Novel Method to Determine Bioequivalence of Nanomedicines

summary of work conducted for

U.S. Food & Drug Administration
(Inter-Agency Award 224-16-3001S)

Nanotechnology Characterization Laboratory
Frederick National Laboratory for Cancer Research
Leidos Biomedical Research, Inc.
Frederick, MD 21702
(301) 846-6939 • ncl@mail.nih.gov
http://ncl.cancer.gov

TABLE OF CONTENTS

The complexity of nanomedicine drug formulations poses unique scientific challenges. In contrast to conventional small molecule formulations, the active pharmaceutical ingredient (API) in a systemically administered nanomedicine formulation exists in several forms: (a) nanomedicine-encapsulated, (b) unencapsulated, free/unbound, and (c) unencapsulated, protein-bound (**Figure 1**). While the free, unbound form is considered the only biologically active form of the API, all three fractions are important in characterizing a nanomedicine's pharmacokinetics–especially in evaluation of bioequivalence (BE) (pharmacokinetic similarity). Existing methods to measure these various nanomedicine fractions (e.g. solid phase extraction, conventional ultrafiltration, etc.) are not ideal due to a variety of shortcomings as described in the literature [1].

The primary objective of this project was to evaluate a novel bioanalytical technique developed at the NCL to fractionate the various subpopulations of a nanomedicine in plasma, in an effort to fulfill this unmet need and facilitate regulatory review of new classes of generic nanomedicines [2, 3]. This novel method is an ultrafiltration technique that utilizes a stable isotope tracer of the API to determine protein binding, which allows for calculation of the remaining drug fractions (the bioanalytical method is described in Appendix A). In order to evaluate the performance of this fractionation method, it was used to determine the BE of two controlled release nanomedicine products currently on the market, Janssen's Doxil® and Sun Pharma's doxorubicin HCl liposome formulations, and two fast-releasing nanomedicine products, Celgene's Abraxane® and Samyang's Genexol-PM (approved in South Korea), as well as the legacy paclitaxel formulation, Taxol (generic).

Nanomedicine Encapsulated

Free/Unbound

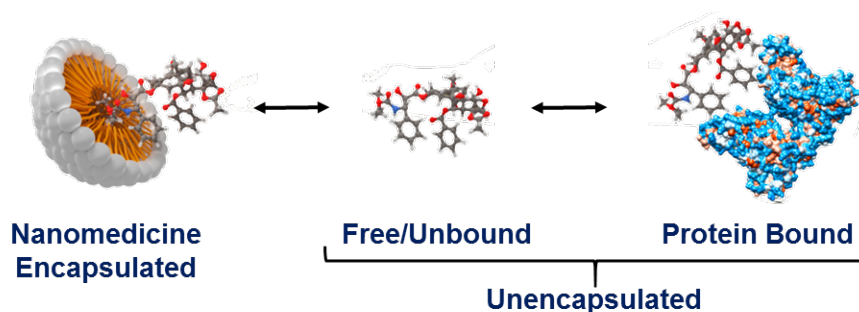Protein Bound

Unencapsulated

Figure 1. Nanomedicine Drug Fractions. The active pharmaceutical ingredient (API), or drug, in a systemically administered nanomedicine formulation exists in several forms: (a) nanomedicine-encapsulated, (b) unencapsulated, free/unbound, and (c) unencapsulated, protein-bound.

The stable isotope tracer ultrafiltration assay (SITUA; see Appendix A) was used to evaluate the bioequivalence of two controlled release nanomedicines–Janssen's Doxil® and Sun Pharma's doxorubicin HCl liposome formulations, and two fast-releasing nanomedicine products–Celgene's Abraxane® and Samyang's Genexol-PM (approved in South Korea). The legacy paclitaxel formulation, Taxol (generic) was also included as a compartor in the latter studies.

In brief, the bioanalytical method for measurement of doxorubicin (DXR) and paclitaxel (PTX) analytes, as well as their stable isotopically labelled versions, were successfully validated in both human and rat plasma and protein-free plasma in accordance with FDA bioanalytical guidance [4]. Furthermore, the assay was validated with regard to process induced drug release, spike recovery, organic spike stability, and plasma volume requirements. Analysis by the validated SITUA method revealed the unencapsulated and encapsulated drug concentration-time profiles for both Doxil and Sun Pharma formulations, as well as the estimated pharmacokinetic (PK) parameters, were very comparable. Similarly, the total, unencapsulated and unbound drug PK parameters were similar for the Abraxane and Genexol-PM formulations. However, a statistical analysis of bioequivalence by two one-sided t-tests determined that neither drug comparators (i.e., Sun Pharma to Doxil or Genexol-PM to Abraxane) were bioequivalent. The lack of bioequivalence is most likely attributed to low study power, due to the low number of animals utilized per group (N=8), and the parallel design that includes both intra- and intersubject variability.

Importantly, though, the SITUA method was able to evaluate nanomedicine bioequivalence, as well as differentiate between formulations in which drug is stably encapsulated (i.e., Doxil and Sun Pharma's doxorubicin HCl liposome), bound in equilibrium (i.e., Taxol), or simply solubilized (i.e., Genexol-PM and Abraxane). The SITUA method should facilitate both characterization and generic development of complex drugs. The resulting higher quality pharmacokinetic data may also decrease patient sample size and facilitate regulatory determination of bioequivalence.

Doxil vs. SunPharma
Three independent lots of both Doxil and Sun Pharma formulations were evaluated for in vitro drug release using the validated method. Doxil and Sun Pharma liposome drug release were similar over the 6 hr incubation period, at both 37°C and 42°C, in both human and rat plasma, averaging ~1.5% total drug release and not following a temporal trend. Although there were instances of statistically significant differences at various time points and concentrations, these differences were not consistent between lots or nanomedicine products over the study period.

Single lots of each formulation were also evaluated in a bioequivalence study using Sprague Dawley rats. The Doxil and Sun Pharma unencapsulated and encapsulated drug concentration-time profiles were very comparable, as were the estimated pharmacokinetic parameters. However, a statistical analysis of bioequivalence by two one-sided t-tests determined that all PK parameters, except for encapsulated C_{max}, were not equivalent. Therfore, the two formulations were not bioequivalent in this study. This lack of bioequivalence is most likely the result of low study power, due to the low number of animals utilized per group (N=8), and the parallel design that includes both intra- and intersubject variability.

Important differences were observed for the unencapsulated drug profile in this stable isotope tracer method study compared to a previous conventional solid phase extraction (SPE) method study in rats [5], including 18-fold lower unencapsulated drug concentrations, an 8-fold later

unencapsulated T_{max} (33 vs. 4 hr), and a much longer unencapsulated terminal half-life (145 vs. 30 hr), resulting in data that are more scientifically sound from a pharmacokinetic perspective.

Abraxane vs. Genexol-PM vs. Taxol (generic)

Three independent lots of both Genexol-PM and Abraxane formulations, as well as a single lot of generic Taxol comparator, were evaluated for in vitro drug release using the validated method. Drug release for all three formulations was similar over the 2 hr incubation period, in both human and rat plasma, with all formulations releasing all drug immediately and not following a concentration-dependent or temporal trend. Although there were instances of statistically significant differences at various time points and concentrations, these differences were not consistent between lots or nanomedicine products over the study period. Notably, however, there were differences in the relationship between formulation concentration and free drug fraction. In rat plasma, paclitaxel, Abraxane, and Genexol-PM displayed similar saturated binding, suggesting the Abraxane-albumin nanoparticle and Genexol-PM micelle do not contribute to protein binding. In human plasma, Genexol-PM displayed linear binding, suggesting a contribution of the micelle to drug binding at high concentrations, while paclitaxel and Abraxane displayed similar saturated binding. In both rat and human plasma, Taxol (generic) displayed concentration-dependent binding, suggesting the cremophor micelle strongly binds to free drug.

Separate lots of each formulation were also evaluated in a bioequivalence study using Sprague Dawley rats. Total, unencapsulated and unbound drug PK parameters were similar for Abraxane and Genexol-PM, but notably different for Taxol. Although Abraxane and Genexol-PM had similar PK, a statistical analysis of bioequivalence by two one-sided t-tests determined that Abraxane and Genexol-PM were not bioequivalent. This lack of bioequivalence is most likely the result of high variability and insufficient power, due to the low number of animals used.

Taxol demonstrated lower total, unencapsulated and unbound CL, Vd and Vss than Genexol-PM and Abraxane, consistent with strong equilibrium binding of drug to stable cremophor micelles. This equilibrium binding was reflected in non-linear/reduced unbound drug fraction at high drug concentrations for the Taxol formulation. Surprisingly, and inconsistent with pharmacological theory regarding the effect of protein binding on active, unbound drug PK for a low extraction drug administered parenterally, unbound PK was altered by this equilibrium formulation-binding. This effect on active, unbound drug PK could potentially influence drug therapy, and help explain differences between Abraxane and Taxol pharmacology [6]. Unbound drug PK appears to be a discriminating criteria for drug formulations that influence drug PK by equilibrium binding. The SITUA assay was a very predictive and accurate method for identifying formulation effects on unbound drug fraction resulting from equilibrium binding.

DOXIL vs. SUN PHARMA

Design and Methods

The bioequivalence of Doxil and Sun Pharma products was evaluated in a Sprague Dawlay rat model. A parallel design bioequivalence study was conducted in double jugular catheterized 15-week-old male Sprague Dawley rats (approx. weight of 400 grams, Charles River Laboratories, Raleigh, N.C.). Test articles, Janssen's Doxil® (Lot 600520P1) or Sun Pharma's Doxorubicin HCl Liposome (Lot JKR0865A) were diluted to a concentration of 1 mg DXR/mL in saline. Rats were treated intravenously by left catheter with 5 mg DXR/5 mL/kg of Janssen's Doxil® or Sun Pharma's Doxorubicin HCl Liposome generic (8/treatment group). Blood samples (400 μL) were collected in K_2EDTA tubes by the right jugular catheter at 0.25, 0.5, 1, 4, 8, 12, 24, 48 and 96 hr. Blood was spun at 2500x*g* for 10 min and plasma (~200 μL) collected in a glass vial.

Husbandry

Animals arrived the day prior to study initiation. In order to keep the catheters patent, catheters were stored and flushed with 500 IU/mL heparin in PBS. Animal rooms were kept at 50% relative humidity, 68-72°F with 12 hr light/dark cycles. Rats were housed two animals/cage (rat polycarbonate cage type with ¼" corncob bedding). Animals were allowed *ad libitum* access to Purina 5L79 and reverse osmosis water.

The Frederick National Laboratory for Cancer Research is accredited by AAALAC International and follows the Public Health Service *Policy for the Care and Use of Laboratory Animals* (Health Research Extension Act of 1985, Public Law 99-158, 1986). Animal care was provided in accordance with the procedures outlined in the *Guide for Care and Use of Laboratory Animals* (National Research Council, 1996; National Academy Press, Washington, D.C.). All animal protocols were approved by the NCI at Fredrick institutional Animal Care and Use Committee. The experiments outlined herein are scientifically justified and do not represent an unnecessary duplication of previous work by the sponsor.

Noncompartmental Pharmacokinetic Analysis

Noncompartmental pharmacokinetic parameters were determined using Phoenix WinNonlin version 6.3 software (Pharsight Corporation, Mountain View, CA): the area under the time concentration curve including all time points (AUC_{all}) was calculated using the linear trapezoidal rule without extrapolation; the area under the time concentration curve to time infinity (AUC_{inf}) was calculated using the linear trapezoidal rule with extrapolation to time infinity; the C_{max} term is the maximum concentration; the T_{max} term is the time of maximum concentration.

Statistics

In vivo PK parameters were evaluated by two one-sided t-tests, with α=0.05 and Ө=0.2, to determine the 90% CI of the geometric mean of log transformed T/R ratio [7]. The FDA bioequivalence criteria is a 90% CI between 80 and 125% [8]. Outliers were identified as data points two standard deviations away from the mean of all data points in the group. Only outliers that did not fit the overall drug concentration profile of the treatment group were excluded from the bioequivalence analysis.

Results and Discussion

The Doxil and Sun Pharma unencapsulated and encapsulated drug concentration-time profiles in rats were very comparable, as were the estimated pharmacokinetic parameters (**Figure 2, Table 1**). However, a statistical analysis of bioequivalence by two one sided t-tests determined that all PK parameters, except for encapsulated C_{max}, were not equivalent (**Figure 3**). Therefore, the two formulations were not bioequivalent in this study. This lack of bioequivalence is most likely the result of low study power, due to the low number of animals utilized per group (*N*=8), and the parallel design that includes both intra- and intersubject variability.

Importantly, several differences were observed for the unencapsulated drug profile in this stable isotope tracer method study compared to a previous conventional solid phase extraction (SPE) method study in rats [5], including 18-fold lower unencapsulated drug concentrations, an 8-fold later unencapsulated T_{max} (33 vs. 4 hr), and a much longer unencapsulated terminal half-life (145 vs. 30 hr).

Figure 2. Unencapsulated and Encapsulated DXR Comparison. Presented is the encapsulated and unencapsulated DXR time-concentration comparison for Doxil and Sun Pharma (*N*=8, Mean).

Table 1. PK Comparison of Sun Pharma and Doxil. Displayed are the individual animal pharmacokinetic parameters for the Doxil and Sun Pharma, encapsulated and unencapsulated plasma analysis: area under the time concentration curve to time infinity (AUC_{inf}); maximum concentration (C_{max}); area under the time concentration curve all time points (AUC_{all}); time of maximum concentration (T_{max}).

		Unencapsulated			Encapsulated			
		T_{max}	C_{max}	AUC_{all}	T_{max}	C_{max}	AUC_{all}	AUC_{inf}
	Animal #	h	ng/mL	ng*h/mL	h	ng/mL	ng*h/mL	ng*h/mL
Sun Pharma	1	48	171	12522	1	166940	4341721	4541653
	3	24	117	8871	0.25	185303	7569868	9067173
	5	24	224	9827	1	173156	6894633	8199833
	7	48	157	11167	0.5	191985	8056557	10190358
	9	24	104	8011	0.25	202248	7194422	8911107
	11	24	115	7863	0.5	191702	7198496	8403677
	13	48	125	8427	0.25	195679	5739068	6339516
	15	24	241	14367	1	180606	6758153	7585164
	AVG	33	157	10132	0.59	185952	6719115	7904810
	SD	12	52	2359	0.35	11855	1171877	1764188
Doxil	17	48	155	11556	0.25	156683	5883992	7031063
	19	48	139	10770	4	145434	5641037	6337460
	21	48	139	10770	4	172599	6080370	7044728
	23	96	273	16159	4	145738	6324194	7617542
	25	24	201	12397	0.5	163420	5645537	6109138
	27	12	198	13022	0.25	153541	5015026	5083284
	29	12	163	12182	0.5	180756	5643580	6575046
	31	0.25	218	13410	0.25	169214	6626650	8017609
	AVG	36	186	12533	1.72	160923	5857548	6726984
	SD	31	46	1751	1.89	12804	493830	918891

90% CI of geometric mean of log transformed T/R ratio

Figure 3. TOST bioequivalence analysis. Presented are the results of two one-sided t-tests (TOST) of PK parameters for Doxil (reference) in comparison to Sun Pharma (test). All PK parameters, except encapsulated C_{max}, were found to be not equivalent, with the 90%CI of the test (Sun Pharma)/reference (Doxil) ratio outside the 80-125% criteria by TOST.

Design and Methods

Abraxane, Genexol-PM, and Taxol (generic) were evaluated in a bioequivalence study using Sprague Dawley rats. A parallel design bioequivalence study was conducted in double jugular catheterized 15-week-old male Sprague Dawley rats (approx. weight of 400 grams, Charles River Laboratories, Raleigh, N.C.). Test articles, Abraxane (Lot 6115194), Genexol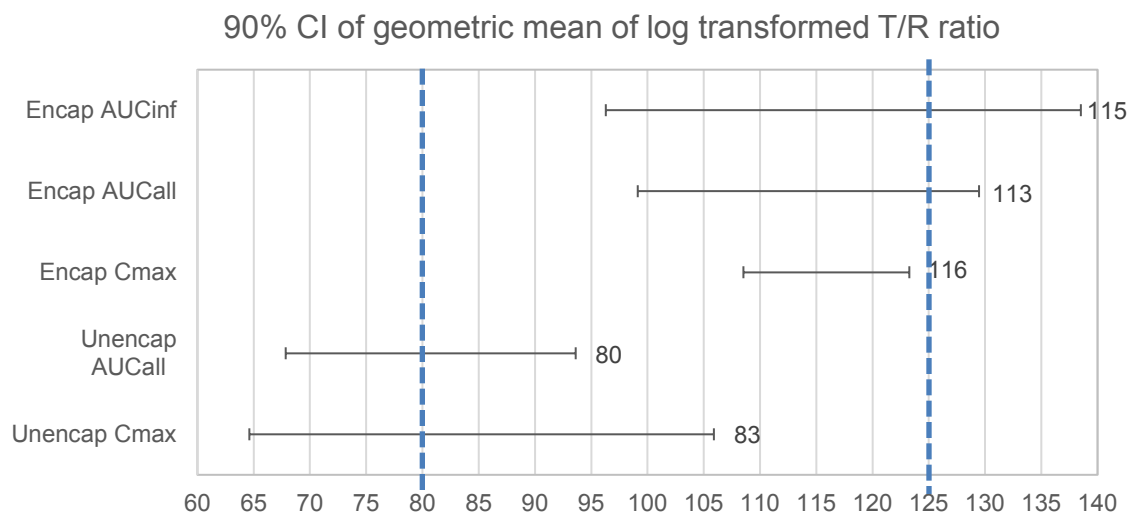-PM (Lot GP31771), or Taxol generic (Lot PACCA1018) were diluted to a concentration of 2 mg PTX/mL in saline. Rats were treated intravenously by left catheter with 6 mg PTX/3 mL/kg of Abraxane, Genexol-PM, or Taxol generic (8/treatment group). Blood samples (400 µL) were collected in K_2EDTA tubes by the right jugular catheter at 0.25, 0.5, 2, 4, 6, 8, 24, 48 and 72 hr. Blood was spun at 2500x*g* for 10 min and plasma (~200 µL) collected in a glass vial.

Husbandry

Animals arrived the day prior to study initiation. In order to keep the catheters patent, catheters were stored and flushed with 500 IU/mL heparin in PBS. Animal rooms were kept at 50% relative humidity, 68-72°F with 12 hr light/dark cycles. Rats were housed two animals/cage (rat polycarbonate cage type with ¼" corncob bedding). Animals were allowed *ad libitum* access to Purina 5L79 and reverse osmosis water.

Noncompartmental Pharmacokinetic Analysis

Noncompartmental pharmacokinetic parameters were determined using Phoenix WinNonlin version 6.3 software (Pharsight Corporation, Mountain View, CA): the area under the time concentration curve including all time points (AUC_{all}), was calculated using the linear trapezoidal rule without extrapolation; the area under the time concentration curve to time infinity (AUC_{inf}), was calculated using the linear trapezoidal rule with extrapolation to time infinity; the C_{max} term is the maximum measured concentration; the T_{max} term is the time of maximum concentration; clearance (CL) was determined by the equation CL=dose/AUC; the AUMC term is the area under the first moment curve; volume of distribution steady state (Vss), is determined by the equation Vss=AUC/AUMC; Mean residence time (MRT_{inf}), is determined by the equation MRT_{inf}=(dose/AUCinf)*(AUMC/AUC); the λ_z term is the slope of the terminal elimination phase; half-life ($T_{1/2}$), is determined by $T_{1/2}$=0.693/ λ_z; Concentration time zero (C_0), is the extrapolated concentration of the intial slope to the y-intercept; Volume of distribution apparent (Vd), is determined by the equation Vd=dose/C_0.

Statistics

In vivo PK parameters were evaluated by two one-sided t-tests, with α=0.05 and Ө=0.2, to determine the 90% CI of the geometric mean of log transformed T/R ratio [7] as well as ANOVA, with Duncan's Multiple Range test post hoc comparisons. The FDA bioequivalence criteria is a 90% CI between 80 and 125% [8]. Outliers were identified as data points two standard deviations away from the mean of all data points in the group. Only outliers that did not fit the overall drug concentration profile of the treatment group were excluded from the statistical analysis.

Results and Discussion

Encapsulated drug, as measured by the SITUA method, was only observed at the earliest time point, 15 min, for the Taxol and Genexol-PM formulations, but not the Abraxane formulation. This is consistent with stable cremophore and PEG-polylactic acid micelles at this early time point for the Taxol and Genexol-PM formulations, respectively, corresponding with peak excipient concentrations. While the Abraxane, Genexol-PM and Taxol concentration-time profiles for total, unencapsulated and unbound drug, were all identical, displaying a triphasic curve as described previously in the literature (**Figure 4**) [9]. The PK parameters calculated from these profiles were significantly different between Taxol and the Abraxane and Genexol-PM formulations (**Tables 2-4**).

Total, unencapsulated and unbound drug PK parameters were similar for Abraxane and Genexol-PM, but notably different for Taxol. Although Abraxane and Genexol-PM PK parameters were similar, a statistical analysis of bioequivalence by two one-sided t-tests determined that Abraxane and Genexol-PM were not bioequivalent (**Figure 5**). This lack of bioequivalence is probably the result of high variability and insufficient power, due to the low number of animals used. Taxol demonstrated lower total, unencapsulated and unbound CL, Vd and Vss than Genexol-PM and Abraxane, consistent with strong equilibrium binding to stable cremophor micelles. This equilibrium binding was reflected in non-linear/reduced unbound drug fraction at high drug concentrations for the Taxol formulation, as determined for both the normoisotopic drug and stable isotope tracer (**Figure 6**). Importantly, the stable isotope tracer allowed discrimination between drug stably bound to cremophore micelles and dynamic drug binding to micelles in equilibrium with unbound drug. Surprisingly, and inconsistent with pharmacological theory regarding the effect of protein binding on active, unbound drug PK for a low extraction drug like paclitaxel administered parenterally, unbound drug PK was apparently altered by this equilibrium formulation binding [6].

Although cremophore has been shown to inhibit paclitaxel metabolism at high concentrations in vitro [10], the differences in unbound drug PK for the Taxol formulation does not appear to be the result of changes in drug metabolism, since changes in metabolic clearance would have resulted in altered drug half-life ($T_{1/2}=0.693/(Vss/CL)$), which was not the case. By contrast, alterations in free drug fraction result in compensatory changes in both volume of distribution and clearance resulting in no change in drug half-life, as observed [6]. This is an important finding, as it would suggest that binding of paclitaxel to the Taxol cremophore micelle affects unbound drug PK differently than changes in binding to protein, and can influence hepatic drug extraction and tissue distribution of unbound drug, even for drugs that are not perfusion limited. This effect of cremophore on unbound drug disposition is consistent with previous findings in the isolated liver perfusion and liver slice models [10, 11], in which researchers observed a decrease in liver uptake of drug which in turn decreased drug metabolism. By decreasing the volume of distribution of active, unbound drug, the Taxol cremophore micelle could potentially influence drug therapy, and help explain differences between Abraxane and Taxol pharmacology [6]. While Abraxane has been approved for treatment of pancreatic cancer, Taxol has not, and the reasons for this lack of Taxol efficacy may be due to the underlying pharmacology of the formulaiton, not just the fact that Abraxane can be administered at higher doses [12].

Unbound drug PK apears to be a discriminating criteria for drug formulations that influence drug PK by equilibrium binding. The SITUA assay is a very predictive and accurate method for identifying formulation effects on unbound drug fraction resulting from equilibrium binding.
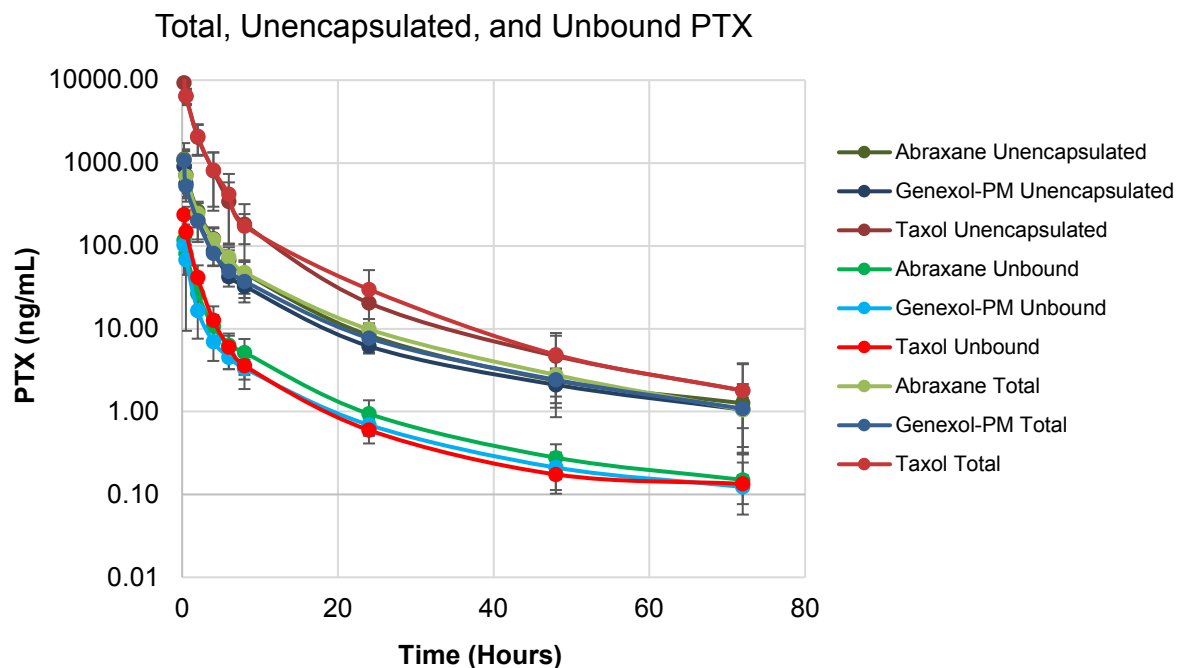
Figure 4. Total, Unencapsulated and Unbound PTX PK Comparison. Presented are the total, unencapsulated, and unbound PTX time-concentration comparisons for Abraxane, Genexol-PM and Taxol (*N*=8, Mean±SD).

Table 2. Total Drug PK Comparison. Displayed are the individual animal pharmacokinetic parameters: concentration time zero (C_0); slope of the terminal eliminiation phase (λ_z); Half-life ($T_{1/2}$); area under the time concentration curve to time infinity (AUC_{inf}); maximum concentration (C_{max}); area under the time concentration curve all time points (AUC_{all}); Volume of distribution (Vd); clearance (CL); Mean residence time (MRT_{inf}); Volume of distribution steady state (Vss).

	Animal	C_o ng/mL	C_{max} ng/mL	λ_z h^{-1}	$T_{1/2}$ h	AUC_{all} ng*h/mL	AUC_{inf} ng*h/mL	Vd mL/kg	CL mL/h*kg	MRT_{inf} h	Vss mL/kg
Abraxane Total											
6 mg/mL	1	1860	1218	0.051964	13	2529	2541	3226	2362	6	13333
	3	2664	1690	0.011352	61	4316	4962	2253	1209	19	26121
	5	1351	811	0.02031	34	2111	2235	4441	2685	11	32066
	7	1748	1036	0.050009	14	2338	2352	3433	2551	6	15740
	9	1495	916	0.041708	17	2106	2132	4014	2814	7	20309
	11	1358	971	0.066454	10	2810	2820	4417	2128	7	14548
	13	1162	800	0.053085	13	1850	1863	5161	3221	7	21464
	15	2167	1285	0.006819	102	2863	4014	2768	1495	42	84448
	AVG	1726	1091	0.047	17	2291	2324	4115	2627	7	20512
	SD	498	298	0.015	9	343	332	715	379	2	6780
Genexol-PM Total											
6 mg/mL	17	4314	2312	0.038093	18	4090	4131	1391	1452	5	7863
	19	2274	1210	0.050525	14	2455	2469	2638	2431	6	14109
	21	1113	746	0.015923	44	1828	2003	5391	2996	14	46535
	23	1126	686	0.053317	13	1620	1630	5328	3681	6	23615
	25	1319	938	0.055108	13	2341	2354	4549	2549	7	17059
	27	805	425	0.041627	17	1187	1210	7456	4960	11	53181
	29	1674	868	0.055104	13	1536	1545	3584	3884	6	22741
	31	1580	1101	0.042293	16	2352	2375	3798	2527	6	14509
	AVG	1413	853	0.044	15	2176	2214	4267	3060	8	16649
	SD	481	264	0.013	2	896	897	1861	1083	3	5900
Taxol Total											
6 mg/mL	33	19515	11979	0.057647	12	20691	20719	307	290	3	852
	35	17414	9797	0.059938	12	13747	13758	345	436	2	965
	37	16406	9292	0.083809	8	16532	16549	366	363	3	1080
	39	18047	8987	0.08945	8	14429	14440	332	416	4	1652
	41	17067	10735	0.052564	13	23464	23495	352	255	3	798
	43	21541	11051	0.070788	10	16116	16124	279	372	2	857
	45	14204	10454	0.045474	15	30110	30258	422	198	5	995
	47	30155	16237	0.061243	11	22805	22818	199	263	2	516
	AVG	19294*	11066*	0.065*,ABX	11	19737*	19770*	325*	324*	3*	964*
	SD	4890	2303	0.015	2	5597	5635	66	85	1	325

*p<0.05, significantly different from other formulations; *,ABXp<0.05 significantly different from abraxane; ANOVA, with Duncan's Multiple range post hoc test comparison; Outliers highlighted in red text were excluded from statistical analysis.

Table 3. Unencapsulated Drug PK Comparison. Displayed are the individual animal pharmacokinetic parameters: concentration time zero (C_0); slope of the terminal eliminiation phase (λ_z); Half-life ($T_{1/2}$); area under the time concentration curve to time infinity (AUC_{inf}); maximum concentration (C_{max}); area under the time concentration curve all time points (AUC_{all}); Volume of distribution (Vd); clearance (CL); Mean residence time (MRT_{inf}); Volume of distribution steady state (Vss).

	Animal	C_o	C_{max}	λ_z	$T_{1/2}$	AUC_{all}	AUC_{inf}	Vd	CL	MRT_{inf}	Vss
Abraxane Unencapsulated											
		ng/mL	ng/mL	h⁻¹	h	ng*h/mL	ng*h/mL	mL/kg	mL/h*kg	h	mL/kg
6 mg/mL	1	2357	1433	0.034	20	2636	2672	2545	2246	6	12778
	3	2858	1776	0.009	78	4362	5183	2099	1158	19	30698
	5	1080	769	0.014	49	2051	2267	53554	2647	11	44684
	7	2181	1067	0.042	17	2261	2282	2751	2629	6	15942
	9	1541	860	0.037	19	1982	2009	3894	2987	7	20588
	11	1272	970	0.062	11	3098	3110	4719	1929	7	12940
	13	1221	828	0.050	14	1885	1898	4914	3161	7	19647
	15	1668	1231	0.003	204	3455	5534	3598	1084	42	108521
	AVG	1772	1117	0.040	22	2319	2373	4063*	2600*	7	18766*
	SD	631	347	0.016	14	466	450	1219	457	2	6697
Genexol-PM Unencapsulated											
	Animal	C_o	C_{max}	λ_z	$T_{1/2}$	AUC_{all}	AUC_{inf}	Vd	CL	MRT_{inf}	Vss
		ng/mL	ng/mL	h⁻¹	h	ng*h/mL	ng*h/mL	mL/kg	mL/h*kg	h	mL/kg
6 mg/mL	17	7299	2092	0.0319	22	3701	3754	822	1598	5	7918
	19	1609	1077	0.0294	24	2316	2366	3730	2536	6	17766
	21	733	630	0.0268	26	1714	1764	8189	3401	14	27778
	23	827	618	0.0378	18	1690	1713	7251	3504	6	23512
	25	939	818	0.0416	17	2223	2247	6390	2670	7	18959
	27	529	362	0.0385	18	1104	1128	11350	5317	11	56273
	29	1181	748	0.0570	12	1444	1451	5080	4134	6	24971
	31	1150	956	0.0426	16	2087	2104	5218	2852	6	16138
	AVG	995	744	0.038	18	2035	2066	5976*	3251*	8	19577*
	SD	354	237	0.010	4	786	797	1620	1126	3	6624
Taxol Unencapsulated											
	Animal	C_o	C_{max}	λ_z	$T_{1/2}$	AUC_{all}	AUC_{inf}	Vd	CL	MRT_{inf}	Vss
		ng/mL	ng/mL	h⁻¹	h	ng*h/mL	ng*h/mL	mL/kg	mL/h*kg	h	mL/kg
6 mg/mL	33	13216	9949	0.064	11	18784	18801	454	319	3	893
	35	11421	7980	0.104	7	12017	12035	525	499	2	967
	37	14158	8569	0.062	11	15457	15470	424	388	3	1107
	39	12183	7624	0.088	8	12170	12168	492	493	4	1232
	41	10660	8493	0.045	15	20754	20799	563	288	3	929
	43	14336	9191	0.050	14	15432	15459	419	388	2	1057
	45	9691	8444	0.042	16	27054	27195	619	221	5	1062
	47	23740	14018	0.052	13	19929	19953	253	301	2	594
	AVG	12238*	9283*	0.063*,GXL	12*,GXL	17699*	17735*	469*	362*	3*	980*
	SD	1765	2041	0.022	3	5014	5053	111	99	1	189

p<0.05, significantly different from other formulations;,GXLp<0.05 significantly different from genexol; ANOVA, with Duncan's Multiple range post hoc test comparison; Outliers highlighted in red text were excluded from statistical analysis.

Table 4. Unbound Drug PK Comparison. Displayed are the individual animal pharmacokinetic parameters: concentration time zero (C_0); slope of the terminal eliminiation phase (λ_z); Half-life ($T_{1/2}$); area under the time concentration curve to time infinity (AUC_{inf}); maximum concentration (C_{max}); area under the time concentration curve all time points (AUC_{all}); Volume of distribution (Vd); clearance (CL); Mean residence time (MRT_{inf}); Volume of distribution steady state (Vss).

	Animal	C_0	C_{max}	λ_z	$T_{1/2}$	AUC_{all}	AUC_{inf}	Vd	CL	MRT_{inf}	Vss
Abraxane Unbound											
		ng/mL	ng/mL	h^{-1}	h	ng*h/mL	ng*h/mL	mL/kg	mL/h*kg	h	mL/kg
6 mg/mL	1	193	127	0.031	22	309	314	31143	19131	6	124681
	3	225	165	0.007	102	408	518	26658	11586	30	426431
	5	161	96	0.018	38	218	236	37171	25420	13	359746
	7	184	104	0.041	17	220	223	32686	26932	6	175102
	9	94	96	0.034	21	232	236	64020	25423	7	184146
	11	184	119	0.062	11	327	328	32658	18281	7	129303
	13	168	104	0.044	16	211	214	35755	28100	6	177124
	15	187	128	0.005	153	341	496	32057	12102	51	854809
	AVG	174	117	0.038	21	283	320	36518	20872*	8	303918*
	SD	38	23	0.015	9	73	123	11545	6582	3	248205
Genexol-PM Unbound											
		ng/mL	ng/mL	h^{-1}	h	ng*h/mL	ng*h/mL	mL/kg	mL/h*kg	h	mL/kg
6 mg/mL	17	970	214	0.033	21	371	376	6184	15937	5	77019.0
	19	151	91	0.034	20	191	195	39676	30802	7	220001.8
	21	109	69	0.026	27	159	164	55052	36504	9	323738.9
	23	118	72	0.037	19	180	183	50667	32820	7	242908.7
	25	202	112	0.039	18	229	232	29748	25897	7	191305.8
	27	110	51	0.034	20	126	130	54634	46149	11	534880.3
	29	160	79	0.057	12	141	141	37545	42477	6	271593.2
	31	164	102	0.036	20	195	198	36678	30239	7	216701.7
	AVG	145	99	0.037	19	199	202	38773	32603*	8	259769*
	SD	34	50	0.009	5	77	78	16073	9470	2	131868
Taxol Unbound											
		ng/mL	ng/mL	h^{-1}	h	ng*h/mL	ng*h/mL	mL/kg	mL/h*kg	h	mL/kg
6 mg/mL	33	272	214	0.050	14	406	408	22060	14723	4	54623
	35	328	230	0.088	8	327	328	18308	18319	2	35158
	37	329	221	0.052	13	396	397	18211	15111	4	56979
	39	324	201	0.071	10	309	310	18512	19372	3	60471
	41	360	246	0.035	20	514	517	16660	11596	4	47131
	43	480	244	0.034	20	354	357	12496	16803	4	62008
	45	249	183	0.028	25	689	708	24120	8478	8	72254
	47	610	370	0.040	17	507	509	9834	11783	3	30242
	AVG	446*	239*	0.050	16	438*	442*	17525*	14523*	4*	52359*
	SD	183	57	0.021	6	127	132	4649	3706	2	14110

*$p < 0.05$, significantly different from other formulations, <u>ANOVA</u>, with Duncan's Multiple range post hoc test comparisons. Outliers highlighted in red text were excluded from statistical analysis.

90% CI of geometric mean of log transformed T/R ratio
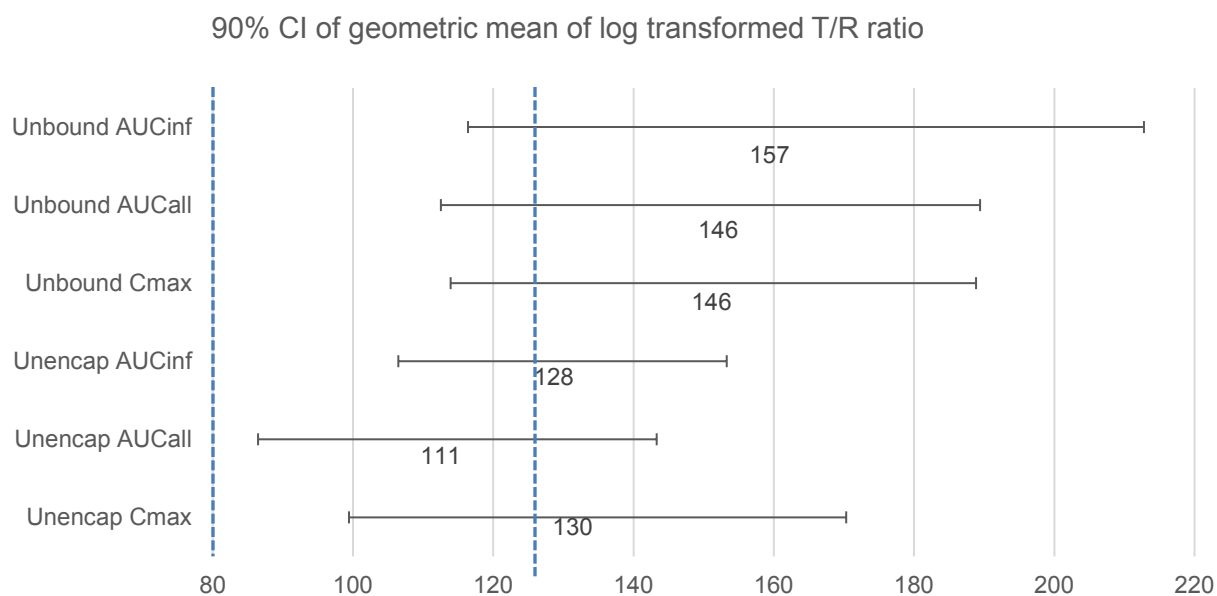
Figure 5. TOST bioequivalence analysis. Presented are the results of two-sided t-tests (TOST) of PK parameters for Abraxane (reference) in comparison to Genexol-PM (test). None of the PK parameters were found to be equivalent, with 90%CI of the test (Abraxane)/reference (Genexol-PM) ratio falling outside the 80-125% range by TOST.

A. Normoisotope %Fu vs. Total Drug Concentration
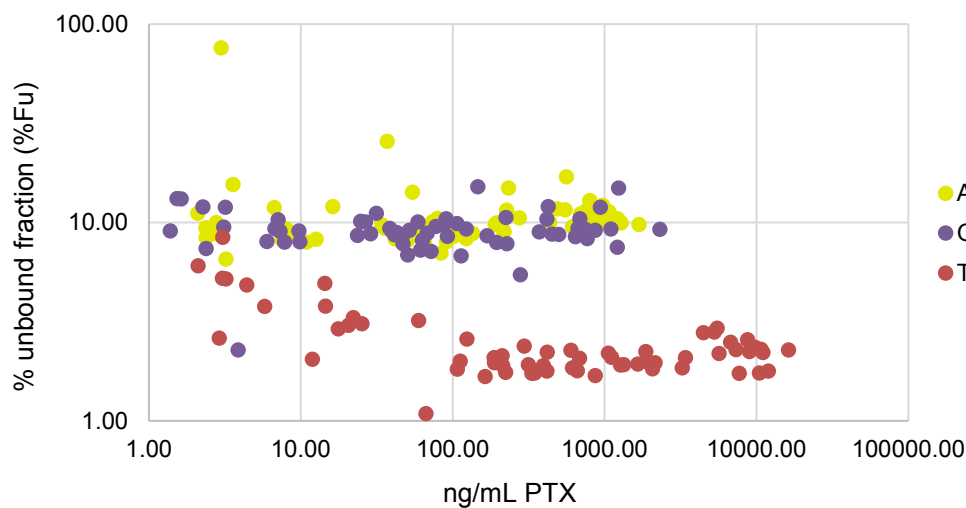
B. Stable isotope %Fu vs. Total Drug Concentration
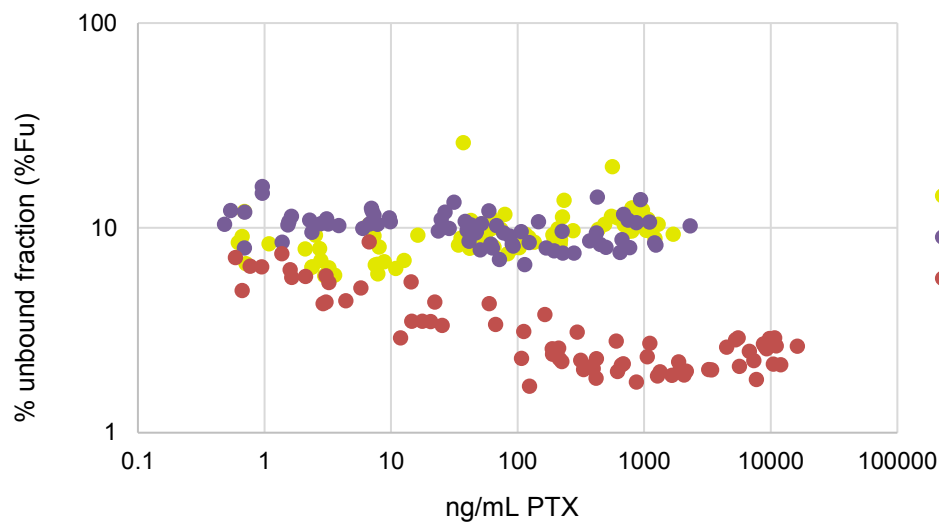
Figure 6. %Fu vs. Total PTX Concentration. Presented are the individual %Fu vs. total PTX concentration based on (**A**) normoisotopic filtrate/reservoir concentrations and (**B**) stable isotope tracer filtrate/reservoir concentrations for Abraxane, Genexol-PM and Taxol (*N*=8).

Appendix A. Stable Isotope Tracer Ultrafiltration Assay (SITUA)

The stable isotope tracer ultrafiltration assay (SITUA) method [2, 3] detailed below was utilized for all in vitro drug release and in vivo bioequivalence (BE) studies.

Design and Methods

Our laboratory has developed a novel ultrafiltration method to measure nanomedicine encapsulated and unencapsulated AP) in plasma, and assess nanomedicine API release [2, 3]. This method utilizes a stable isotope of the API to account for formulation-induced changes in protein-binding, as well as binding of the API to formulation components.

In this method, stable, isotopically labeled drug is spiked into a plasma sample containing the nanomedicine formulation (**Figure A-1**). This can be a plasma sample from an in vitro incubation or pharmacokinetic study. The sample is incubated for a predefined time to allow isotope equilibration, an aliquot of the sample is taken, and the sample is then filtered using an ultrafiltration apparatus. Both the sample aliquot and the ultrafiltrate are analyzed by liquid chromatography-mass spectrometry to determine concentrations of the normoisotopic and isotopically labeled drug.

Since the stable isotopically labeled drug (**D***) equilibrates with protein and formulation components identical to the unlabeled, normoisotopic drug (**D**) released from the nanomedicine formulation, the ultrafilterable fraction of the isotopically labeled drug represents a reliable measurement of free unbound fraction. Bound fraction can be calculated from equation (i):

$$(i) \qquad \%Bound\ D^* = \frac{([Total\ D^*] - [Ultrafilterable\ D^*]) * 100}{[Total\ D^*]}$$

The encapsulated and unencapsulated nanomedicine fractions can then be easily calculated using equations (ii) and (iii):

$$(ii) \qquad [Unencapsulated\ D] = \frac{[Ultrafilterable\ D]}{(1-(\%Bound\ D^*/100))}$$

$$(iii) \qquad [Encapsulated] = [Total\ D] - [Unencapsulated\ D]$$

Figure A-1. Drug Release Assay Using SITUA. The stable isotopically labeled drug (D*) is spiked into nanomedicine (NM-D) in plasma. D* behaves identically to normoisotopic drug (D) with regard to protein binding (Pro-D/D*). After protein binding equilibrium is reached, the plasma sample is transferred to an ultrafiltration device and the filtrate is separated by centrifugation. The stable isotope tracer free fraction, represented as the ultrafilterable fraction, can be used to calculate protein bound, unencapsulated and encapsulated drug fractions, according to equations (i), (ii) and (iii) above.

In Vitro Drug Release in Human Plasma

Human blood was collected in K_2-EDTA tubes and pooled from 6 donors. Blood was spun at 2500x*g* for 10 min to collect plasma. HEPES buffer (pH 7.4) was added at 50 µL/2 mL plasma to maintain a pH of 7.4. Plasma in glass vials was spiked with samples (free drug or nanomedicine) at final drug concentrations of 0.5 µg/mL, 1 µg/mL, and 5 µg/mL doxorubicin (DXR) or paclitaxel (PTX) in triplicate. All samples were spiked with 0.1 µg/mL stable isotope tracer.

Samples were then incubated at 37°C with agitation. At time points 10 min, 30 min, 1 hr and 2 hr, 50 µL of plasma was taken for protein precipitation as described below; this sample was used to determine total drug concentration. Additionally, 400 µL (see Note 1) of sample was transferred to a prewarmed ultrafiltration tube (10 kDa Microcon for doxorubicin and 10 kDa Vivacon for paclitaxel) and spun at 6000x*g* for 10 min. 50 µL of this filtrate was also taken for protein precipitation; this sample was used to determine free/unbound drug concentration.

Notes, for in vitro studies 400 µL of plasma was used. For analysis of the samples from the in vivo study, 150 µL of plasma was used. No change in assay performance was noted for the reduced sample volume (confirmed by control studies comparing reduced sample volumes).

Protein Precipitation Method

The two 50 µL samples from above (non-centrifuged and centrifuged) were added to 200 µL ice cold acetonitrile (ACN) with 0.1% formic acid (FA) containing 25 ng/mL internal standard (ISTD) (see Appendices B and C for materials). The samples were frozen at -80°C for 10 min, thawed at room temperature, then spun at 4°C, 18,000x*g* for 20 min. The supernatant was transferred to a clean Eppendorf tube and dried using a centrifuge speed vacuum for 25 min at 50°C and 5 Torr. Following protein precipitation, samples were reconstituted in 150 µL 40% ACN with 0.1% FA. The reconstituted sample was then transferred to clean a Eppendorf tube and spun at

18,000xg at 4°C for 5 min, then transferred to an HPLC vial. Samples were analyzed on the Q-Orbitrap as described in Appendices B and C.

In Vitro Drug Release in Rat Plasma
In vitro drug release in rat plasma was conducted similarly. Sprague Dawley rat blood was collected in K_2-EDTA tubes. Blood was spun at 2500xg for 10 min to collect plasma. HEPES buffer (pH 7.4) was added at 50 µL/2 mL plasma to maintain a pH of 7.4. Plasma in glass vials was spiked with samples (free drug or nanomedicine) at final drug concentrations of 0.5 µg/mL, 1 µg/mL, and 5 µg/mL DXR or PTX in triplicate. All samples were spiked with 0.1 µg/mL stable isotope tracer and processed as described above.

Appendix B. LC-Orbitrap Method for Doxorubicin

1. LC-Orbitrap Set Up

1. The LC-Orbitrap system consisted of a Q Exactive basic quadrupole Orbitrap mass spectrometer, Vanquish UHPLC system, binary pump and autosampler (Thermo Fisher Scientific).

2. The LC conditions were, 20 µL injection volume, 40°C column oven, 10°C autosampler and flow rate of 0.4 µL /min. Mobile phase A consisted of water with 0.1% formic acid, and mobile phase B consisted of acetonitrile with 0.1% formic acid. The following gradient was used; hold 25% B for 3 min, linear increase to 80% B from 3-7 min, linear increase to 95% B from 7.0-7.5 min, hold at 95% B for 0.5 min, and linear decrease to 25% B from 8.0-8.1 min, with a column regeneration time between injections of 4 min. The column was a Zorbax-SB-C18 RRHD 1.8 µm particle, 2.1x100 mm (Agilent technologies, Inc) with a Zorbax-SB-C18 1.8 µm particle, 2.1x5 mm guard column (Agilent Technologies, Inc).

3. Doxorubicin (DXR) and $^{13}C^{2}H_{3}$-Doxorubicin (DXR_C13) elution times were 1.63 min, respectively, and aclarubicin internal standard (ISTD) elution time was 5.56 min (**Figures B-1 and B-2**). The Orbitrap mass spectrometer was run in ESI positive mode, spray voltage was 3.5 kV, and the capillary and auxiliary gas temperatures were 270°C and 290°C, respectively. The collision energy was set at 13 AU for doxorubicin and $^{13}C^{2}H_{3}$-doxorubicin, and 15 AU for aclarubicin. Parallel reaction monitoring (PRM) of the following transitions were used: DXR 544.18 → 130.08, 379.07, 397.08; DXR_C13 548.20 → 130.08, 383.09, 401.11; and Aclarubicin 812.34 → 570.23.

2. Materials

2.1 Doxorubicin hydrochloride (DXR) – Sigma-Aldrich, Catalog # D1515

2.2 $^{13}C,^{2}H_{3}$-Doxorubicin trifluoroacetate salt (DXR_C13) – Alsa Chim, Catalog # C2451

2.3 Aclarubicin hydrochloride – Alfa Aesar, Catalog # J66842

2.4 Acetonitrile – VWR, Catalog # BJLC015-1

2.5 Formic acid – Thermo Scientific, Catalog # 28905

2.6 Zorbax-SB-C18 RRHD 1.8 μm particle, 2.1 x 100 mm – Agilent Technologies, Inc., Catalog # 858700-902

2.7 Zorbax-SB-C18 1.8 µm particle, 2.1 x 5 mm – Agilent Technologies, Inc., Catalog # 821725-902

2.8 Amicon Ultra-4 centrifugal filter unit Ultracel 30 membrane – Millipore, Catalog # UFC803024

2.9 Microcon 10 kDa MWCO centrifugal filter unit with Ultracel 10 membrane – Millipore, Catalog # MRCPRT010

2.10 Rat plasma (Sprague Dawley) – collected fresh in K_2EDTA tubes

2.11 Human plasma (pooled) – collected fresh from six human donors in K_2EDTA tubes

2.12   HEPES Buffer (1 M) – Gibco, catalog # 15630080

2.13 K_2EDTA vacutainer tubes – Moore Medical, catalog # 87770

3.   Preparation of Human and Rat Plasma and Protein-Free Plasma

3.1   Blood was collected from six human donors or Sprague Dawley rats in K_2EDTA tubes

3.2   Blood was centrifuged at 2,500x*g* for 10 min and plasma was pooled

3.3   50 µL of HEPES buffer was added for every 2 mL of plasma and pH adjusted to 7.4

3.4   To prepare protein-free plasma, plasma was transferred to 4 mL centrifugal filter units with a 30 kDa MWCO, centrifuged at 5000x*g* for 1 hr, and filtrate was collected

4.   Calibration and Quality Control Standards Preparation

4.1   Stock solutions of DXR and DXR_C13 were prepared in 25% ACN. These stocks were used to prepare calibration and quality control standards.

4.2   DXR and DXR_C13 calibration standards were prepared in human protein-free plasma, human plasma, rat protein-free plasma, and rat plasma with concentrations ranging from 0.1 ng/mL to 300 µg/mL depending on the matrix and range needed for unknown samples. Aclarubicin was used as an internal standard at a concentration of 25 ng/mL.

4.3   DXR and DXR_C13 high, medium and low QCs were also prepared in appropriate matrix at concentrations dependent on the range of the calibration curve.

5.   Sample Preparation

5.1   50 µL of standard and QC in plasma or protein-free plasma were added to 1.5 mL Eppendorf tube, followed by addition of 200 µL ice cold ACN with 0.1% formic acid containing 25 ng/mL internal standard and vortexed.

5.2   The sample was placed at -80°C for 10 min, then thawed at room temperature.

5.3   The thawed sample was then spun at 18,000x*g* for 20 min at 4°C to pellet precipitated proteins.

5.4   The supernatant was transferred to a 1.5 mL Eppendorf tube and dried in a speed vac at 50°C.

5.5   The dried residue was resuspended in 150 µL 25% ACN with 0.1% formic acid.

5.6   The extracted sample was spun again at 18,000x*g* for 10 min at room temperature.

5.7   The supernatant was transferred to a 1.5 mL amber glass screw top HPLC vial with fixed Teflon insert and cap, and placed in an HPLC auto sampler vial rack.

5.8   Plasma blank, ISTD spiked plasma blank and quality control samples were run with each calibration curve.

A. Analytes

Doxorubicin $^{13}C,\ ^2H_3$-Doxorubicin

B. Internal Standard

Aclarubicin

Figure B-1. Analytes and Internal Standard Used in the Drug Release Assay. Structures of analytes (**A**) doxorubicin (DXR) and (**B**) $^{13}C,^2H_3$-doxorubicin (DXR_C13), and (**C**) ISTD aclarubicin are presented.
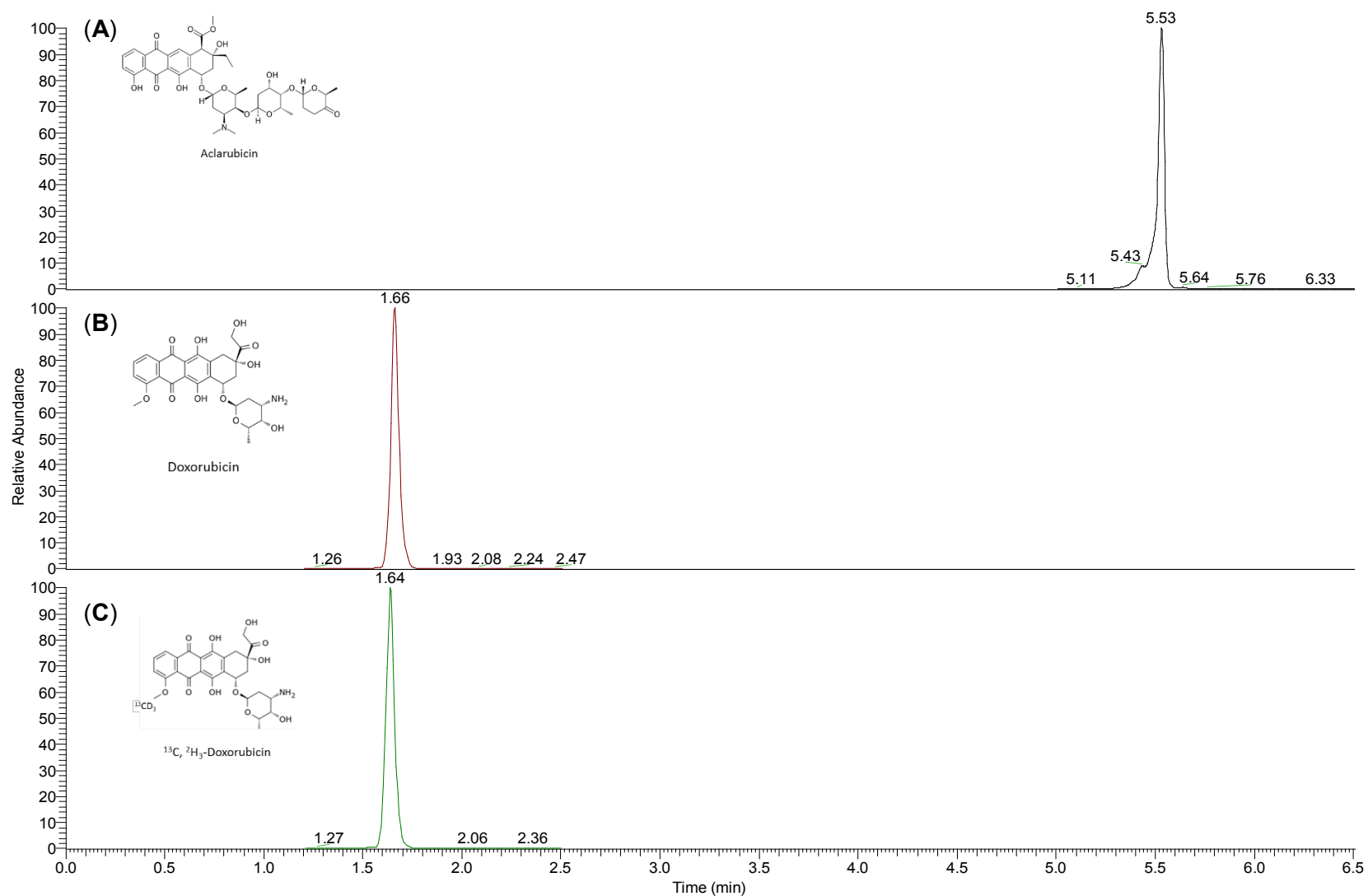
Figure B-2. LC-Q Orbitrap Parallel Reaction Monitoring of Analytes and Internal Standard. Displayed are chromatograms of (**A**) ISTD, (**B**) DXR and (**C**) DXR_C13. The transitions used were: DXR 544.18 → 130.08, 379.07, 397.08; DXR_C13 548.20 → 130.08, 383.09, 401.11; and Aclarubicin 812.34 → 570.23

Appendix C. LC-Orbitrap Method for Paclitaxel

1. LC-Orbitrap Set Up

1.1 The LC-Orbitrap system consisted of a Q Exactive basic quadrupole Orbitrap mass spectrometer, Vanquish UHPLC system, binary pump and autosampler (Thermo Fisher Scientific).

1.2 The LC conditions were: 20 µL injection volume for protein-free plasma samples and 10 µL injection volume for plasma samples, 40°C column oven, 10°C autosampler, and flow rate of 0.4 µL/min. Mobile phase A consisted of water with 0.1% formic acid, and mobile phase B consisted of acetonitrile with 0.1% formic acid. The following gradient was used: linear increase from 40% B to 100% B from 0 to 2 min, hold at 100% B for 2 min, and linear decrease from 100% B to 40% B in 0.5 min, with a column regeneration time between injections of 3.5 min. The column was a Zorbax-SB-C18 RRHD 1.8 µm particle, 2.1x100 mm (Agilent technologies, Inc) with a Zorbax-SB-C18 1.8 µm particle, 2.1x5 mm guard column (Agilent Technologies, Inc).

1.3 Paclitaxel (PTX), $^{13}C_6$-Paclitaxel (PTX_C13) tracer, and $^2H_5$-Paclitaxel (PTX_d5) internal standard (ISTD) elution times were all 2.03 min (**Figures C-1 and C-2**). The Orbitrap mass spectrometer was run in ESI positive mode, spray voltage was 3.5 kV, and the capillary and auxiliary gas temperatures were 150°C and 400°C, respectively. The collision energy was set at 13 AU for all three analytes. Parallel reaction monitoring (PRM) of the following transitions were used: PTX: 854.34 → 286.10, 509.21; PTX_C13: 860.36 → 286.10, 515.23, and; PTX_d5: 859.37 → 291.13, 509.21.

2. Materials

2.1 Paclitaxel (PTX) – LC Laboratories, catalog #P-9600

2.2 $^{13}C_6$-Paclitaxel (PTX_C13) – Santa Cruz Biotechnology, catalog # sc-477982

2.3 $^2H_5$-Paclitaxel (PTX_d5) – Santa Cruz Biotechnology, catalog # sc-219546

2.4 Acetonitrile – VWR, catalog # BJLC015-1

2.5 Formic acid – Thermo Scientific, catalog # 28905

2.6 Zorbax-SB-C18 RRHD 1.8 µm particle, 2.1 x 100 mm – Agilent Technologies, Inc., catalog # 858700-902

2.7 Zorbax-SB-C18 1.8 µm particle, 2.1 x 5 mm – Agilent Technologies, Inc., catalog # 821725-902

2.8 Amicon Ultra-4 centrifugal filter unit Ultracel 30 membrane – Millipore, catalog # UFC803024

2.9 Vivacon 10 kDa MWCO centrifugal filter unit with Hydrosart regenerated cellulose membrane – Satorius, catalog # VN01H02

2.10 Rat plasma (Sprague Dawley) – collected fresh in K_2EDTA tubes

2.11 Human plasma (pooled) – collected fresh from six human donors in K_2EDTA tubes, collected under NCI at Frederick Protocol OH99-C-N046

2.12 HEPES Buffer (1 M) – Gibco, catalog # 15630080

2.13 K$_2$EDTA vacutainer tubes – Moore Medical, catalog # 87770

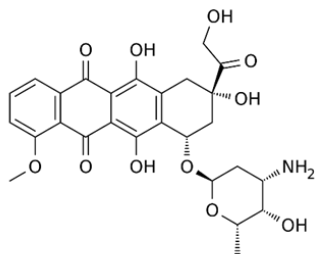## 3. Preparation of Human and Rat, Plasma and Protein-Free Plasma

3.1 Blood collected from six human donors or Sprague Dawley rats in K$_2$EDTA tubes.

3.2 Blood was centrifuged at 2,500x*g* for 10 min and plasma was pooled together.

3.3 50 µL of HEPES buffer (pH 7.4) for every 2 mL of plasma was added and pH adjusted to 7.4.

3.4 To prepare protein-free plasma, plasma was transferred to 4 mL centrifugal filter units with a 30 kDa MWCO, centrifuged at 5000x*g* for 1 hr, and filtrate was collected.

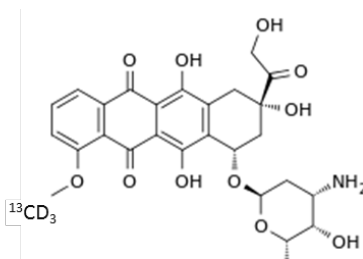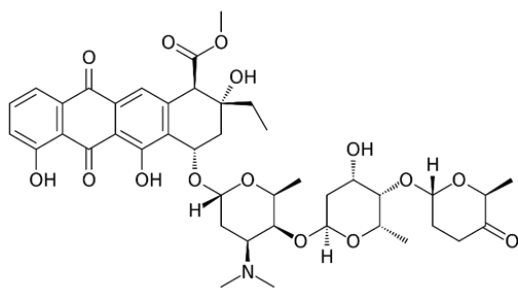## 4. Calibration and Quality Control Standards Preparation

4.1 Stock solutions of PTX and PTX_C13 were prepared in acetonitrile. These stocks were used to prepare calibration and quality control standards.

4.2 PTX and PTX_C13 calibration standards were prepared in human protein-free plasma, human plasma, rat protein-free plasma, and rat plasma with concentrations ranging from 0.5 ng/mL to 10 µg/mL depending on the matrix and range needed for unknown samples. PTX_d5 was used as an internal standard at a concentration of 25 ng/mL.

4.3 PTX and PTX_C13 low, medium and high QCs were also prepared in appropriate matrix at concentrations dependent on the range of the calibration curve.

5. Sample Preparation

5.1 50 µL of plasma or protein-free plasma containing standards, QCs, or unknowns were added to 1.5 mL Eppendorf tubes, followed by addition of 200 µL ice cold ACN with 0.1% formic acid containing 25 ng/mL internal standard and vortexed.

5.2 The sample was placed at -80°C for 10 min and then thawed at room temperature.

5.3 The thawed sample was then spun at 18,000x*g* for 20 min at 4°C to pellet precipitated proteins.

5.4 The supernatant was transferred to a 1.5 mL Eppendorf tube and dried in a speed vac for 25 min at 50°C and 5 Torr.

5.5 The dried residue was resuspended in 150 µL 40% ACN with 0.1% formic acid.

5.6 The extracted sample was spun again at 18,000x*g* for 10 min at room temperature.

5.7 The supernatant was transferred to a 1.5 mL amber glass screw top HPLC vial with fixed Teflon insert, capped, and placed in an HPLC auto sampler vial rack.

5.8 Plasma blank, ISTD spiked plasma blank, and quality control samples were run with each calibration curve.

A. Analytes

Paclitaxel

* = ^{13}C

^{13}C$_6$-Paclitaxel

B. Internal Standard

^{2}H$_5$-Paclitaxel

Figure C-1. Analytes and Internal Standard Used in the Drug Release Assay. Structures of analytes, (**A**) paclitaxel (PTX) and (**B**) ^{13}C$_6$-paclitaxel (PTX_C13), and (**C**) ISTD ^{2}H$_5$-paclitaxel (PTX_d5) are shown.

RT: 0.00 - 2.50
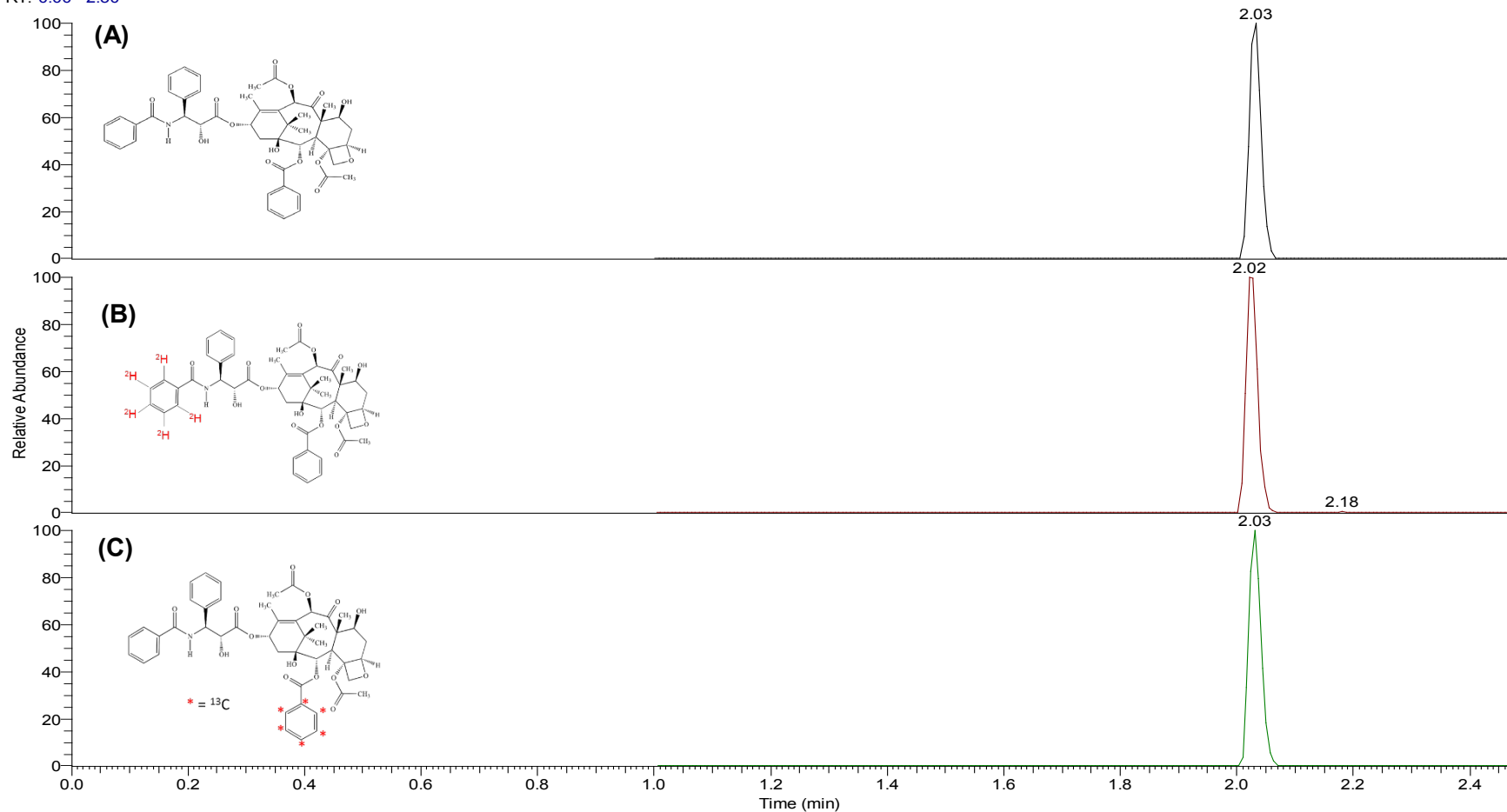
Figure C-2. LC-Q Orbitrap Parallel Reaction Monitoring Chromatograms of Analytes and Internal Standard. Displayed are chromatograms of (**A**) PTX, (**B**) PTX_d5 and (**C**) PTX_C13. The transitions used were: PTX: 854.34 → 286.10, 509.21; PTX_C13: 860.36 → 286.10, 515.23, and; PTX_d5: 859.37 → 291.13, 509.21.

1. Ambardekar, V.V. and Stern, S.T. *NBCD Pharmacokinetics and Drug Release Methods* in Non-Biological Complex Drugs; The Science and the Regulatory Landscape, D.J.A. Crommelin and J.S.B. de Vlieger, eds. **2015**, p. 261-287, Springer International Publishing.

2. Skoczen, S., McNeil, S.E., and Stern, S.T., *Stable isotope method to measure drug release from nanomedicines.* J Control Release. **2015**, 220(Pt A), 169-174.

3. Skoczen, S.L. and Stern, S.T., *Improved Ultrafiltration Method to Measure Drug Release from Nanomedicines Utilizing a Stable Isotope Tracer.* Methods Mol Biol. **2018**, 1682, 223-239.

4. Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), *Guidance for Industry: Bioanalytical Method Validation,* **2013**. Available from: https://www.fda.gov/downloads/drugs/guidances/ucm368107.pdf.

5. Kousba, A., Underbrink, R., and Kruppa, D., *Comparison of Encapsulated and Non-encapsulated Doxorubicin Pharmacokinetics Following Single Intravenous Administration of ATI-0918, an Encapsulated Liposomal Formulation, and DOXIL in Female Rats.* AAPS Annual meeting, San Antonio, TX. **2013**.

6. Stern, S.T., Martinez, M.N., and Stevens, D.M., *When Is It Important to Measure Unbound Drug in Evaluating Nanomedicine Pharmacokinetics?* Drug Metab Dispos. **2016**, 44(12), 1934-1939.

7. Limentani, G.B., Ringo, M.C., Ye, F., Berquist, M.L., and McSorley, E.O., *Beyond the t-test: statistical equivalence testing.* Anal Chem. **2005**, 77(11), 221A-226A.

8. Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), *Guidance for Industry: Statistical approaches to establishing bioequivalence,* **2001**. Available from: http://www.fda.gov/downloads/Drugs/Guidances/ucm070244.pdf.

9. Sparreboom, A., Scripture, C.D., Trieu, V., Williams, P.J., De, T., Yang, A., Beals, B., Figg, W.D., Hawkins, M., and Desai, N., *Comparative preclinical and clinical pharmacokinetics of a cremophor-free, nanoparticle albumin-bound paclitaxel (ABI-007) and paclitaxel formulated in Cremophor (Taxol).* Clin Cancer Res. **2005**, 11(11), 4136-4143.

10. Jamis-Dow, C.A., Klecker, R.W., Katki, A.G., and Collins, J.M., *Metabolism of taxol by human and rat liver in vitro: a screen for drug interactions and interspecies differences.* Cancer Chemother Pharmacol. **1995**, 36(2), 107-114.

11. Ellis, A.G. and Webster, L.K., *Inhibition of paclitaxel elimination in the isolated perfused rat liver by Cremophor EL.* Cancer Chemother Pharmacol. **1999**, 43(1), 13-18.

12. Goldstein, D., El-Maraghi, R.H., Hammel, P., Heinemann, V., Kunzmann, V., Sastre, J., Scheithauer, W., Siena, S., Tabernero, J., Teixeira, L., Tortora, G., Van Laethem, J.L., Young, R., Penenberg, D.N., Lu, B., Romano, A., and Von Hoff, D.D., *nab-Paclitaxel plus gemcitabine for metastatic pancreatic cancer: long-term survival from a phase III trial.* J Natl Cancer Inst. **2015**, 107(2).

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ABX	Abraxane
ACN	acetonitrile
ANOVA	analysis of variance
API	active pharmaceutical ingredient
AU	absorbance units
AUC_{all}	area under the time concentration curve including all time points
AUC_{inf}	area under the time-concentration curve extrapolated to time infinity
AUMC	area under the first moment curve
BE	bioequivalence
C_{max}	concentration maximum
CI	confidence interval
CL	clearance
DXR	doxorubicin
ESI	electrospray ionization
FA	formic acid
FDA	Food and Drug Administration
GXL	Genexol-PM
HPLC	high performance liquid chromatography
ISTD	internal standard
λ_z	slope of the terminal elimination phase
MRT_{inf}	mean residence time
MWCO	molecular weight cut off
NCL	Nanotechnology Characterization Laboratory
PBS	phosphate buffered saline
PRM	parallel reaction monitoring
PK	pharmacokinetic
PTX	paclitaxel
QC	quality control
SD	standard deviation
SITUA	stable isotope tracer ultrafiltration assay
SPE	solid phase extraction
T_{max}	time to maximum concentration
TOST	two one-sided t-tests
T/R	test to reference ratio
Vd	volume of distribution apparent
Vss	volume of distribution steady state

Nanotechnology Characterization Laboratory

Stephan T. Stern
Scott E. McNeil
Rachael M. Crist
Sarah Skoczen
Kelsie Snapp

**Novel Method to Determine Bioequivalence of Nanomedicines:
Doxil vs. Sun Pharma**

prepared for

**U.S. Food & Drug Administration
(Inter-Agency Award 224-16-3001S)**

The complexity of nanomedicine drug formulations poses unique scientific challenges. In contrast to conventional small molecule formulations, the active pharmaceutical ingredient (API) in a systemically administered nanomedicine formulation exists in several forms: (a) nanomedicine-encapsulated, (b) unencapsulated, free/unbound, and (c) unencapsulated, protein-bound. While the free, unbound form is considered the only biologically active form of the API, all three fractions are important in characterizing a nanomedicine's pharmacokinetics– especially in evaluation of bioequivalence (BE) (pharmacokinetic similarity). Existing methods to measure these various nanomedicine fractions (e.g. solid phase extraction, conventional ultrafiltration, etc.) are not ideal due to a variety of shortcomings as described in the literature [1].

The primary objective of this project was to evaluate a novel bioanalytical technique developed at the NCL to fractionate the various subpopulations of a nanomedicine in plasma, in an effort to fulfill this unmet need and facilitate regulatory review of new classes of generic nanomedicines [2, 3]. This novel method is an ultrafiltration technique that utilizes a stable isotope tracer of the API to determine protein binding, which allows for calculation of the remaining drug fractions (the bioanalytical method is described in more detail in Section I). In order to evaluate the performance of this fractionation method, it was used to determine the BE of two controlled release nanomedicine products currently on the market, Janssen's Doxil® and Sun Pharma's doxorubicin HCl liposome formulations. Drug release from these nanoformulations was assessed in vitro, in both human and rat plasma, and the bioequivalence of the products determined in rats.

Nanomedicine Encapsulated   **Free/Unbound**   **Protein Bound**

Unencapsulated
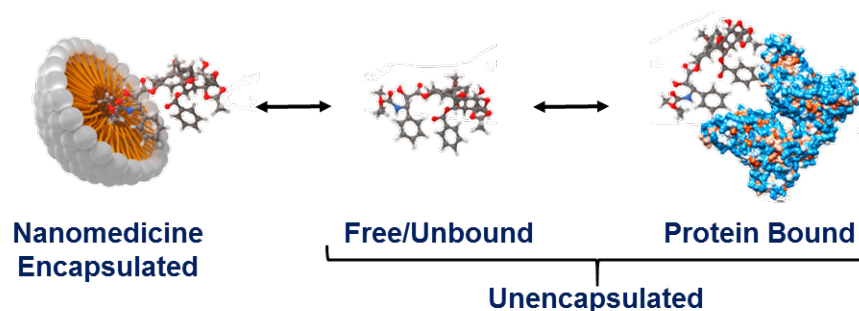
In brief, the bioanalytical methods for measurement of the doxorubicin (DXR) and doxorubicin stable isotope tracer (DXR_C13) analytes were successfully validated in both human and rat plasma and protein-free plasma, in accordance with FDA bioanalytical guidance [4]. Furthermore, the stable isotope tracer ultrafiltration assay (SITUA) was validated with regard to process induced drug release, spike recovery, organic spike stability, and plasma volume requirements. The validation and control studies are outlined in Section II.

Three independent lots of both Doxil and Sun Pharma formulations were evaluated for in vitro drug release using the validated method, as noted in the Table below. The in vitro drug release lot comparisons of Doxil are presented in Section III, Sun Pharma's lot comparison in Section IV, and a comparison of Doxil vs. Sun Pharma is presented in Section V. Doxil and Sun Pharma liposome drug release were similar over the 6 hr incubation period, at both 37°C and 42°C, in both human and rat plasma, averaging ~1.5% total drug release and not following a temporal trend. Although there were instances of statistically significant differences at various time points and concentrations, these differences were not consistent between lots or nanomedicine products over the study period.

Doxil Lots	Sun Pharma Lots	Lot Abbreviation	Color Coding
600220P1	JKR0494A	A	Green
600120P1	JKR0154A	B	Yellow
600520P1	JKR0491A	C	Red
	JKR0865A	D	Purple
Free Doxorubicin Control			Blue

Single lots of each formulation (bolded in the table above) were also evaluated in a bioequivalence study using Sprague Dawley rats (see Section VI). The Doxil and Sun Pharma unencapsulated and encapsulated drug concentration-time profiles were very comparable (**Figure VI-10**), as were the estimated pharmacokinetic parameters (**Table VI-15**). However, a statistical analysis of bioequivalence by two one-sided t-tests determined that all PK parameters, except for encapsulated C_{max}, were not equivalent (**Figure VI-12**). Therfore, the two formulations were not bioequivalent in this study. This lack of bioequivalence is most likely the result of low study power, due to the low number of animals utilized per group (*N*=8), and the parallel design that includes both intra- and intersubject variability.

Important differences were observed for the unencapsulated drug profile in this stable isotope tracer method study compared to a previous conventional solid phase extraction (SPE) method study in rats [5], including 18-fold lower unencapsulated drug concentrations, an 8-fold later unencapsulated T_{max} (33 vs. 4 hr), and a much longer unencapsulated terminal half-life (145 vs. 30 hr), resulting in data that are more scientifically sound from a pharmacokinetic perspective.

I. Bioanalytical Method

Stable Isotope Tracer Ultrafiltration Assay (SITUA)

The stable isotope tracer ultrafiltration assay (SITUA) method [2, 3] detailed below was utilized for all in vitro drug release and in vivo bioequivalence (BE) studies in this report.

Design and Methods

Our laboratory has developed a novel ultrafiltration method to measure nanomedicine encapsulated and unencapsulated active pharmaceutical ingredient (API) in plasma, and assess nanomedicine API release [2, 3]. This method utilizes a stable isotope of the API to account for formulation-induced changes in protein-binding, as well as binding of the API to formulation components.

In this method, stable, isotopically labeled drug is spiked into a plasma sample containing the nanomedicine formulation (**Figure I-1**). This can be a plasma sample from an in vitro incubation or pharmacokinetic study. The sample is incubated for a predefined time to allow isotope equilibration, an aliquot of the sample is taken, and the sample is then filtered using an ultrafiltration apparatus. Both the sample aliquot and the ultrafiltrate are analyzed by liquid chromatography-mass spectrometry to determine concentrations of the normoisotopic and isotopically labeled drug.

Since the stable isotopically labeled drug (**D***) equilibrates with protein and formulation components identical to the unlabeled, normoisotopic drug (**D**) released from the nanomedicine formulation, the ultrafilterable fraction of the isotopically labeled drug represents a reliable measurement of free unbound fraction. Bound fraction can be calculated from equation (i):

$$(i) \qquad \%\text{Bound D*} = \frac{([\text{Total D*}] - [\text{Ultrafilterable D*}]) * 100}{[\text{Total D*}]}$$

The encapsulated and unencapsulated nanomedicine fractions can then be easily calculated using equations (ii) and (iii):

$$(ii) \qquad [\text{Unencapsulated D}] = \frac{[\text{Ultrafilterable D}]}{(1-(\%\text{Bound D*}/100))}$$

$$(iii) \qquad [\text{Encapsulated}] = [\text{Total D}] - [\text{Unencapsulated D}]$$

D* =

^{13}C, $^2H_3$-Doxorubicin

Figure I-1. Drug Release Using SITUA. The stable isotopically labeled drug (D*) is spiked into nanomedicine (NM-D) in plasma. D* behaves identically to normoisotopic drug (D) with regard to protein binding (Pro-D/D*). After protein binding equilibrium is reached, the plasma sample is transferred to an ultrafiltration device and the filtrate is separated by centrifugation. The stable isotope tracer free fraction, represented as the ultrafilterable fraction, can be used to calculate protein bound, unencapsulated and encapsulated drug fractions, according to equations (i), (ii) and (iii) on the previous page.

In Vitro Drug Release in Human Plasma
Human blood was collected in K_2EDTA tubes and pooled from 6 donors. Blood was spun at 2500x*g* for 10 min to collect plasma. HEPES buffer was added at 50 µL/2 mL plasma to maintain a pH of 7.4. Plasma in glass vials was spiked with samples (free drug or nanoparticle) at final drug concentrations of 0.5 µg/mL, 1 µg/mL, and 5 µg/mL doxorubicin (DXR) in triplicate. All samples were spiked with 0.1 µg/mL $^{13}C^2H_3$-DXR (stable isotope tracer).

Samples were then incubated at 37°C or 42°C with agitation. At time points 10 min, 2 hr, 6 hr, and 24 hr (see Note 1), 50 µL of plasma was taken for protein precipitation as described below; this sample was used to determine total drug concentration. Additionally, 400 µL (see Note 2) of sample was transferred to a prewarmed ultrafiltration tube (10 kDa Microcon) and spun at 6000x*g* for 10 min. 50 µL of this filtrate was also taken for protein precipitation; this sample was used to determine free/unbound drug concentration.

Protein Precipitation Method
The two 50 µL samples from above (non-centrifuged and centrifuged) were added to 200 µL ice cold acetonitrile (ACN) with 0.1% formic acid (FA) containing 25 ng/mL aclarubicin as an internal standard (ISTD). The samples were frozen at -80°C for 10 min, thawed at room temperature, then spun at 4°C, 18,000x*g* for 20 min. The supernatant was transferred to a clean Eppendorf tube and dried using either a nitrogen turbo evaporator or a centrifuge speed vacuum for 25 min at 50°C and 5 Torr (see Note 3). Following protein precipitation, samples were reconstituted in 150 µL 25% ACN with 0.1% FA. The reconstituted sample was then transferred to a clean Eppendorf tube, spun at 18,000x*g* at 4°C for 5 min, then transferred to an HPLC vial. Samples were analyzed on the Q-Orbitrap as described on the following page.

Notes:

1. The 24 hr timepoint was included only in the first samples analyzed (Sun Pharma lot comparison at 37°C), because significant degradation of the drug was noted at this timepoint. It was eliminated from subsequent studies.
2. For in vitro studies, 400 µL of plasma was used. For analysis of samples from the in vivo study, 150 µL of plasma was used. No change in assay performance was noted for the reduced sample volume. See control study comparing reduced sample volumes in Section II.
3. Initial studies showed low levels of cross-contamination using the turbo evaporator. This was only apparent because of the extreme sensitivity of the Q-Orbitrap system. Nevertheless, the turbo evaporator was replaced with a centrifugal speed vacuum to reduce cross-contamination. There was no noted difference in the assay results from either apparatus. See control study in Section II.

In Vitro Drug Release in Rat Plasma
In vitro drug release in rat plasma was conducted similarly. Sprague Dawley rat blood was collected in K_2EDTA tubes. Blood was spun at 2500x*g* for 10 min to collect plasma. HEPES buffer was added at 50 µL/2 mL plasma to maintain a pH of 7.4. Plasma in glass vials was spiked with samples (free drug or nanoparticle) at final drug concentrations of 0.5 µg/mL, 1 µg/mL, and 5 µg/mL DXR in triplicate. All samples were spiked with 0.1 µg/mL $^{13}C^2H_3$-DXR (stable isotope tracer) and processed as described above.

LC-Orbitrap Method

The LC-Orbitrap bioanalytical method detailed below was utilized for all validation studies performed in this section, as well as all in vitro drug release and in vivo BE studies in this report.

1. LC-Orbitrap Set Up

1. The LC-Orbitrap system consisted of a Q Exactive basic quadrupole Orbitrap mass spectrometer, Vanquish UHPLC system, binary pump and autosampler (Thermo Fisher Scientific).

2. The LC conditions were, 20 µL injection volume, 40°C column oven, 10°C autosampler and flow rate of 0.4 µL /min. Mobile phase A consisted of water with 0.1% formic acid, and mobile phase B consisted of acetonitrile with 0.1% formic acid. The following gradient was used; hold 25% B for 3 min, linear increase to 80% B from 3-7 min, linear increase to 95% B from 7.0-7.5 min, hold at 95% B for 0.5 min, and linear decrease to 25% B from 8.0-8.1 min, with a column regeneration time between injections of 4 min. The column was a Zorbax-SB-C18 RRHD 1.8 µm particle, 2.1x100 mm (Agilent technologies, Inc) with a Zorbax-SB-C18 1.8 µm particle, 2.1x5 mm guard column (Agilent Technologies, Inc).

3. Doxorubicin (DXR) and $^{13}C^2H_3$-Doxorubicin (DXR_C13) elution times were 1.63 min, respectively, and aclarubicin internal standard (ISTD) elution time was 5.56 min (**Figures I-2 and I-3**). The Orbitrap mass spectrometer was run in ESI positive mode, spray voltage was 3.5 kV, and the capillary and auxiliary gas temperatures were 270°C and 290°C, respectively. The collision energy was set at 13 AU for doxorubicin and $^{13}C^2H_3$-doxorubicin, and 15 AU for aclarubicin. Parallel Reaction Monitoring (PRM) of the following transitions were used: DXR 544.18 → 130.08, 379.07, 397.08; DXR_C13 548.20 → 130.08, 383.09, 401.11; and Aclarubicin 812.34 → 570.23 (**Figure I-4**).

2. Materials

2.1 Doxorubicin hydrochloride (DXR) – Sigma-Aldrich, Catalog # D1515

2.2 $^{13}C,^2H_3$-Doxorubicin trifluoroacetate salt (DXR_C13) – Alsa Chim, Catalog # C2451

2.3 Aclarubicin hydrochloride – Alfa Aesar, Catalog # J66842

2.4 Acetonitrile – VWR, Catalog # BJLC015-1

2.5 Formic acid – Thermo Scientific, Catalog # 28905

2.6 Zorbax-SB-C18 RRHD 1.8 µm particle, 2.1 x 100 mm – Agilent Technologies, Inc., Catalog # 858700-902

2.7 Zorbax-SB-C18 1.8 µm particle, 2.1 x 5 mm – Agilent Technologies, Inc., Catalog # 821725-902

2.8 Amicon Ultra-4 centrifugal filter unit Ultracel 30 membrane – Millipore, Catalog # UFC803024

2.9 Microcon 10 kDa MWCO centrifugal filter unit with Ultracel 10 membrane – Millipore, Catalog # MRCPRT010

2.10   Rat plasma (Sprague Dawley) – collected fresh in K$_2$EDTA tubes

2.11   Human plasma (pooled) – collected fresh from six human donors in K$_2$EDTA tubes

2.12   HEPES Buffer (1 M) – Gibco, catalog # 15630080

2.13   K$_2$EDTA vacutainer tubes – Moore Medical, catalog # 87770

3.   Preparation of Human and Rat Plasma and Protein-Free Plasma

3.1   Blood was collected from six human donors or Sprague Dawley rats in K$_2$EDTA tubes

3.2   Blood was centrifuged at 2,500xg for 10 min and plasma was pooled

3.3   50 µL of HEPES buffer was added for every 2 mL of plasma and pH adjusted to 7.4

3.4   To prepare protein-free plasma, plasma was transferred to 4 mL centrifugal filter units with a 30 kDa MWCO, centrifuged at 5000xg for 1 hr, and filtrate was collected

4.   Calibration and Quality Control Standards Preparation

4.1   Stock solutions of DXR and DXR_C13 were prepared in 25% ACN. These stocks were used to prepare calibration and quality control standards.

4.2   DXR and DXR_C13 calibration standards were prepared in human protein-free plasma, human plasma, rat protein-free plasma, and rat plasma with concentrations ranging from 0.1 ng/mL to 300 µg/mL depending on the matrix and range needed for unknown samples. Aclarubicin was used as an internal standard at a concentration of 25 ng/mL.

4.3   DXR and DXR_C13 high, medium and low QC were also prepared in appropriate matrix at concentrations dependent on the range of the calibration curve.

5.   Sample Preparation

5.1   50 µL of standard and QC in plasma or protein-free plasma were added to 1.5 mL Eppendorf tube, followed by addition of 200 µL ice cold ACN with 0.1% formic acid containing 25 ng/mL internal standard and vortexed.

5.2   The sample was placed at -80°C for 10 min, then thawed at room temperature.

5.3   The thawed sample was then spun at 18,000xg for 20 min at 4°C to pellet precipitated proteins.

5.4   The supernatant was transferred to a 1.5 mL Eppendorf tube and dried in a speed vac at 50°C.

5.5   The dried residue was resuspended in 150 µL 25% ACN with 0.1% formic acid.

5.6   The extracted sample was spun again at 18,000xg for 10 min at room temperature.

5.7   The supernatant was transferred to a 1.5 mL amber glass screw top HPLC vial with fixed Teflon insert and cap, and placed in an HPLC auto sampler vial rack.

5.8   Plasma blank, ISTD spiked plasma blank and quality control samples were run with each calibration curve.

A. Analytes

Doxorubicin ^{13}C, ^{2}H$_3$-Doxorubicin

B. Internal Standard

Aclarubicin

Figure I-2. Analytes and Internal Standard Used in the Drug Release Assay. Structures of analytes (**A**) doxorubicin (DXR) and (**B**) ^{13}C,^{2}H$_3$-doxorubicin (DXR_C13), and (**C**) ISTD aclarubicin are presented.
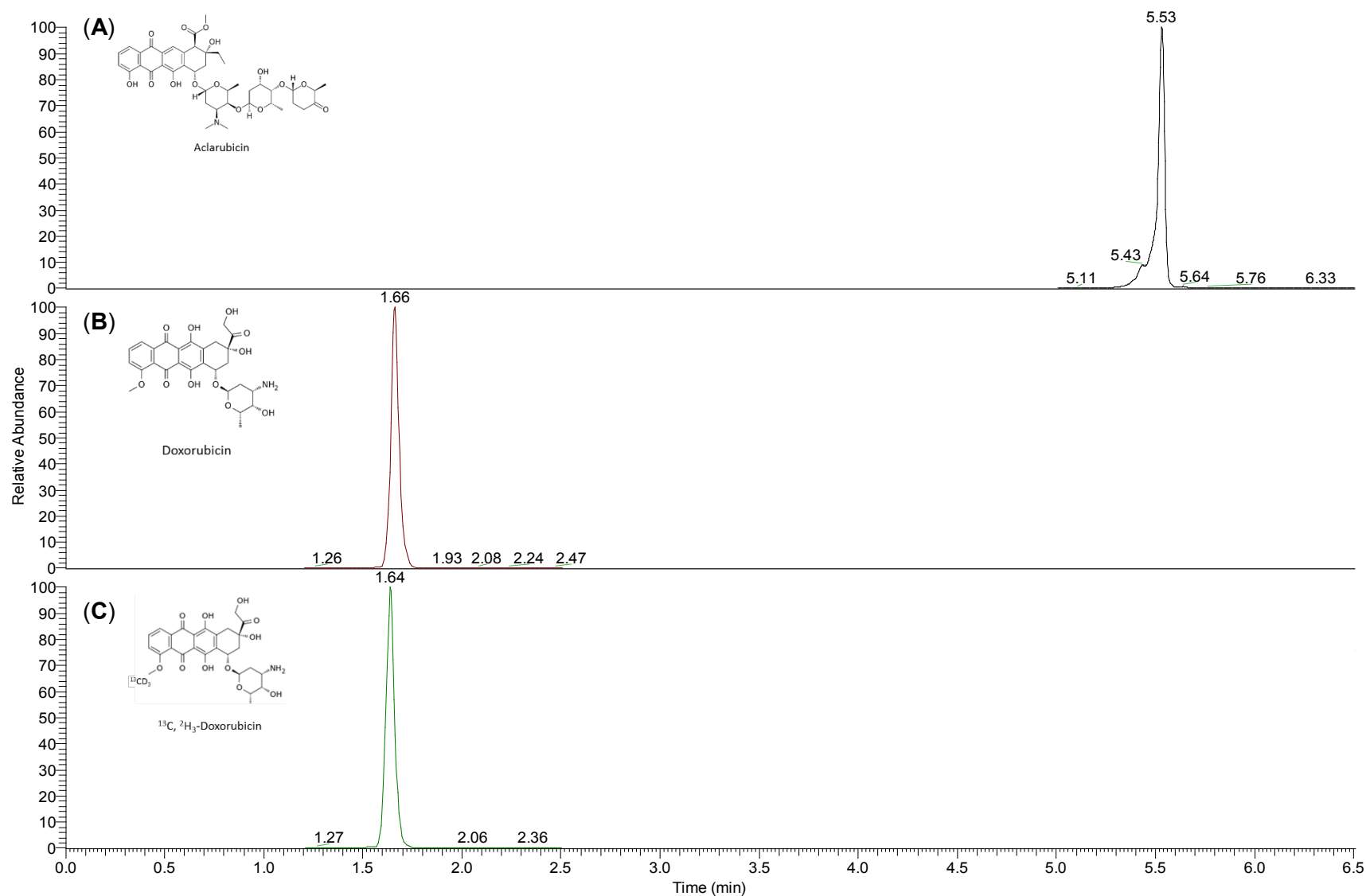
Figure I-3. LC Chromatograms of Analytes and Internal Standard. Displayed are chromatograms of (**A**) ISTD, (**B**) DXR and (**C**) DXR_C13.
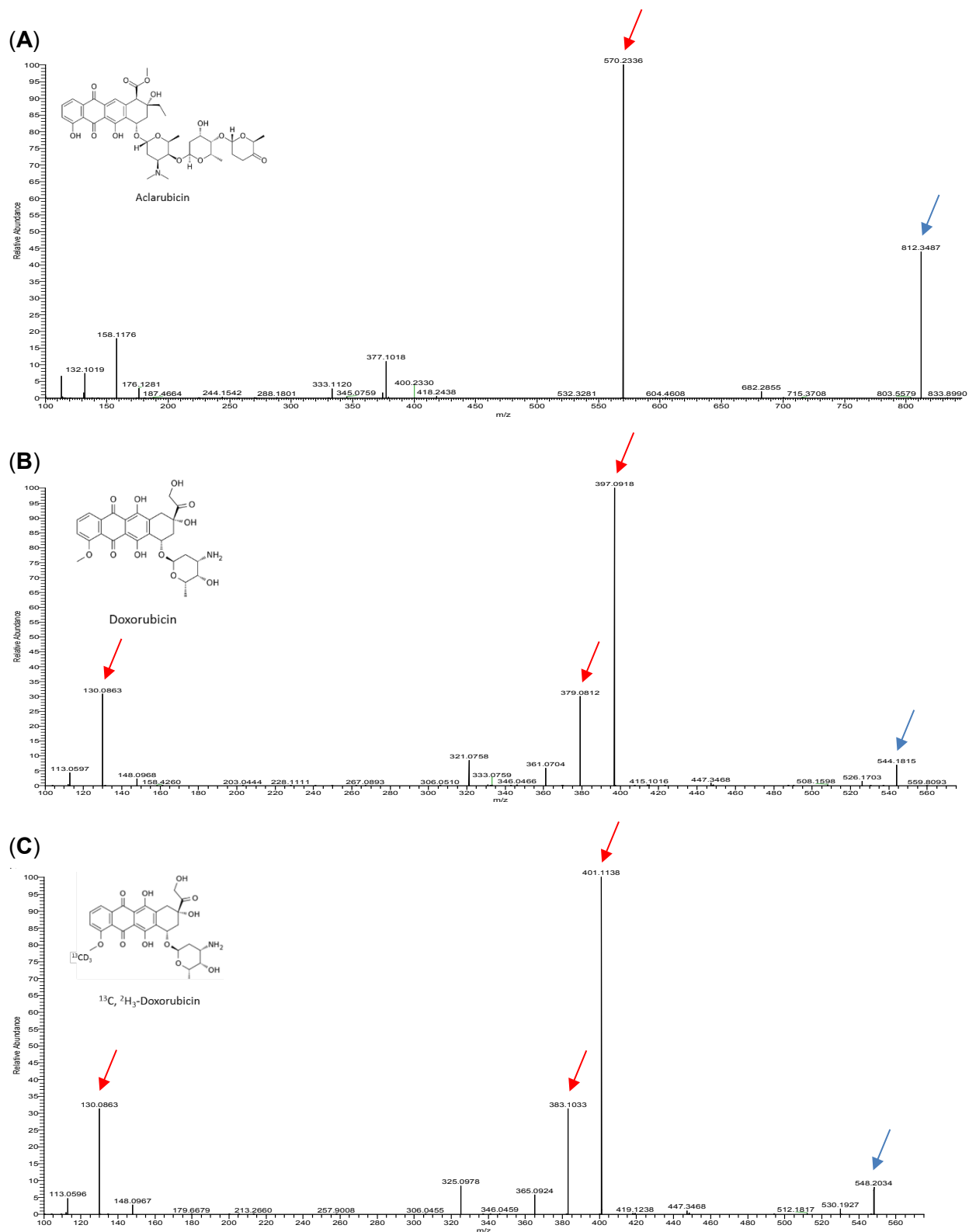
Figure I-4. MS Spectrograms of Analytes and Internal Standard. Displayed are sample MS Spectrograms of (**A**) ISTD, (**B**) DXR and (**C**) DXR_C13. The m/z peaks of the parent molecules are denoted with blue arrows, and major fragments with red arrows.

II. Validation and Control Studies

Validation Criteria and Study Design

The LC-Orbitrap method for DXR and DXR_C13 in human plasma and protein-free plasma matrices was validated according to the FDA guidelines for bioanalytical methods [4]. As per the FDA guidance, three concentrations representing the entire range of the standard curve should be studied: 1) the low QC within 3x of the lower limit of quantitation (LLOQ); 2) the mid QC at the middle of the calibration curve; and 3) the high QC near the upper boundary of the standard curve. Our calibration curve contained six calibrants, ranges dependent on matrix. The QC standards were selected within the concentration range of the calibration curve as described. The following criteria were used for validation of the LC-Orbitrap method.

Figure II-1. Graphical representation of validation criteria terms.

Precision defines random errors, bias defines systematic errors, and accuracy contains both errors (**Figure II-1**). Accuracy deviation is a measure of the distance between the estimated and actual values (**Accuracy deviation** = [(actual-estimated)/ref]*100%). The mean value of the estimate at each QC level should be within 15% of the actual value. Precision is a measure of assay repeatability, and is defined as **Precision** = percent relative standard deviation (% RSD) = [standard deviation/mean]*100%. The precision of the assay at each QC level should be equal to or less than 15% except at LLOQ, where it should be equal or less than 20%. For intra-day validation, six individual QC samples were run at each QC level. These samples were distributed throughout the beginning, middle and end of the run. For the inter-day validation, six individual QC samples were run at each QC level on three separate days. System reproducibility was determined by injecting mid QC standards in six replicates on three separate days, and evaluating retention time and peak area precision.

Interpolation from Calibration Curve
Unknowns and QC sample concentrations were interpolated from the calibration curve, using the linear formula area ratio = slope*concentration ratio + y-intercept.

Full Validation in Human Plasma

Summary

The purpose of this study was to validate the LC-Orbitrap quantitation method for DXR and DXR_C13 in human plasma matrix at a standard curve range of 10-5000 ng/mL. For specifics of the LC-Orbitrap method please see Section I. This analytical method was validated for extraction efficiency in human plasma and quality control precision and accuracy based on general FDA guidelines for bioanalytical methods [4].

Calibration standards were prepared by adding DXR and DXR_ C13 analytical standards to blank human plasma in the range from 10-5000 ng/mL, to arrive at six calibration levels. Matrix interference was not observed for the analytes or internal standard. Complete sets of the combined DXR and DXR_C13 calibration standards were run on three different days.

The DXR and DXR_C13 calibration curves, analyte area/internal standard area (area ratio) vs. standard concentration, were suitable for linear regression with 1/x weighting, resulting in correlation coefficients of 0.99 or better. The accuracy deviation of the regression-calculated standard value from the true value was 15% or less for all calibration levels. The lower limit of quantitation (LLOQ) was established at 10 ng/mL. The method precision at the LLOQ was 2% RSD for DXR and 2% RSD for DXR_C13.

No carry over from the high calibration standard was seen for any of the three analytes. Analytical system reproducibility, evaluated by determining the peak area and retention time precision of six replicate samples at the 100 ng/mL concentration level for the plasma extracted DXR and DXR_C13 analytes, met the acceptance criterion established for retention time and peak area at 10% RSD for all analytes. The estimated LOD and LOQ for DXR were 2 and 6 ng/mL, respectively. The estimated LOD and LOQ for DXR_C13 were 2 and 5 ng/mL, respectively. For intra-day validation, six individual QC samples were run at each QC level, 10 (low), 100 (mid) and 1000 (high) ng/mL for both analytes. These samples were distributed throughout the beginning, middle and end of the run. For the inter-day validation, six individual QC samples were run at each level on three separate days. The low, mid and high QC standards had intra-day and inter-day accuracy deviation and precision of ≤11% for both DXR and DXR_C13 (see **Table II-6**). This meets the acceptance criteria of accuracy deviation not exceeding 15% from the true value, and precision not exceeding 15% RSD, at all QC levels. The calculated absolute recovery for plasma extraction was between 72-103% for all analytes, at all calibration levels. This validation did not attempt to assess either short-term room temperature stability or freeze-thaw stability of samples, or accuracy and precision of diluted samples.

In conclusion, this LC-Orbitrap analytical method for determination of DXR and DXR_C13 in human plasma was successfully validated.

Results & Conclusions

Calibration Curves

Calibration curves, peak area ratio vs. concentration, were linear over the concentration range, from 10-5000 ng/mL, for DXR and DXR_C13 (**Figures II-1 and II-2**). Complete sets of six combined DXR and DXR_C13 calibration levels were run on three different days. The DXR and DXR_C13 curves, calibration area/internal standard area (ratio) vs. standard concentration, were suitable for linear regression with 1/x weighting, resulting in correlation coefficients of 0.99 or better. The percent accuracy deviation of the regression-calculated value from the true value and precision was 15% or less, for all calibration levels (**Tables II-1 and II-2**). The lower limit of quantitation (LLOQ) was established at 10 ng/mL for both analytes.
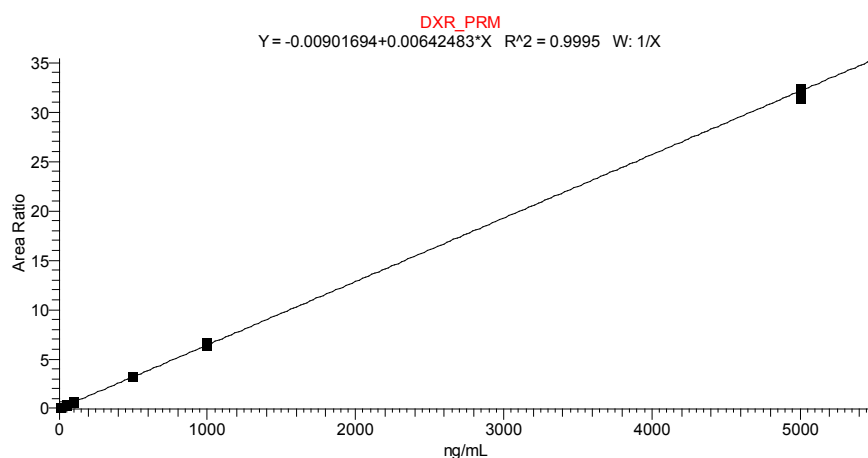
Figure II-1. Example of DXR Calibration Curve in Human Plasma.
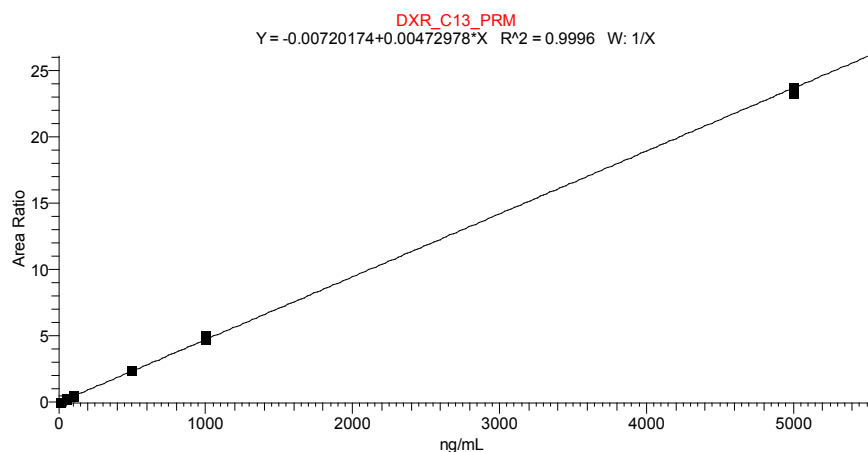
Figure II-2. Example of DXR_C13 Calibration Curve in Human Plasma.

Table II-1. DXR Calibration Curves in Human Plasma.

Standard Conc (ng/mL)	DXR Calibration 1			DXR Calibration 2			DXR Calibration 3		
	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision
10.0	9.1	-9%	2%	10.3	3%	5%	10.2	2%	2%
10.0	9.3	-7%		9.6	-4%		9.9	-1%	
50	48	-3%	6%	51	2%	8%	49	-3%	1%
50	53	5%		46	-8%		49	-2%	
100	97	-3%	7%	97	-3%	3%	98	-2%	1%
100	107	7%		101	1%		96	-4%	
500	496	-1%	5%	512	2%	0.1%	534	7%	5%
500	536	7%		512	2%		497	-1%	
1000	1045	5%	2%	1000	0.03%	3%	989	-1%	5%
1000	1018	2%		1049	5%		1061	6%	
5000	4718	-6%	7%	5042	1%	2%	4620	-8%	10%
5000	5183	4%		4890	-2%		5307	6%	
Linear Regression (ax+b) with 1/x Weighting									
Linear coefficient	0.005			0.006			0.004		
Intercept	0.006			-0.009			-0.007		
R^2	0.99			0.99			0.99		

Table II-2. DXR_C13 Calibration Curves in Human Plasma.

Standard Conc (ng/mL)	DXR_C13 Calibration 1			DXR_C13 Calibration 2			DXR_C13 Calibration 3		
	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision
10.0	9.1	-9%	1%	10.4	4%	6%	10.2	2%	2%
10.0	9.2	-8%		9.6	-4%		10.0	-0.2%	
50	48	-4%	7%	51	3%	7%	48	-4%	1%
50	53	6%		46	-7%		49	-2%	
100	98	-2%	6%	97	-3%	3%	97	-3%	2%
100	108	8%		101	1%		95	-5%	
500	486	-3%	6%	509	2%	1%	535	7%	5%
500	531	6%		506	1%		496	-1%	
1000	1035	4%	1%	1002	0.2%	4%	998	-0.2%	5%
1000	1044	4%		1057	6%		1072	7%	
5000	4706	-6%	7%	5001	0.02%	1%	4666	-7%	8%
5000	5192	4%		4931	-1%		5243	5%	
Linear Regression (ax+b) with 1/x Weighting									
Linear coefficient	0.004			0.005			0.003		
Intercept	0.005			-0.007			-0.005		
R²	0.99			0.99			0.99		

Carry Over

Carry over was examined by injecting triplicates of the high and low (LLOQ) standards, and a water blank following the high standard run (**Table II-3**). There was no carry over seen for any of the analytes.

Table II-3. Carry Over.

Analyte	PRM Transitions	Retention Time	High STD Area	LLOQ Area	High STD-Blank Carry Over Area	% LLOQ of Blank
DXR	130.086 + 379.080 +397.085	1.7	30554362	1484206	0	0
DXR_C13	130.086 + 383.100 + 401.115	1.7	256608915	1235729	0	0
Aclarubicin	570.228	5.7	58769997	56890098	0	0

Intra-Day Precision and Accuracy

Over the intra-day period, the six replicate QC samples maintained a high degree of accuracy and precision (**Tables II-4 and II-6**). The low QC DXR standard, at 10 ng/mL, had an average accuracy deviation of 6% and precision of 1%. The mid QC DXR standard, at 100 ng/mL, had an average accuracy deviation of 7% and precision of 2%. The high QC DXR standard, at 1000 ng/mL, had an average accuracy deviation of 8% and precision of 3%.

The low DXR_C13 QC standard, at 10ng/mL, had an average accuracy deviation of 8% and precision of 3%. The mid QC DXR_C13 standard, at 100 ng/mL, had an average accuracy deviation of 11% and precision of 3%. The high QC DXR_C13 standard at, 1000 ng/mL, had an average accuracy deviation of 7% and precision of 5%.

Table II-4. Intra-Day Precision and Accuracy. Precision and accuracy deviation data are presented for the intra-day six replicate samples of the DXR and DXR_C13 analytes by QC levels, 10 ng/mL (low), 100 ng/mL (mid), 1000 ng/mL (high). **(A)** QC Low DXR, **(B)** QC Mid DXR, **(C)** QC High DXR, **(D)** QC Low DXR_C13, **(E)** QC Mid DXR_C13, **(F)** QC High DXR_C13.

A. QC Low DXR

QC Low DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	10.0	10.5	5	
Replicate 2	10.0	10.5	5	
Replicate 3	10.0	10.7	7	
Replicate 4	10.0	10.5	5	
Replicate 5	10.0	10.8	8	
Replicate 6	10.0	10.4	4	
AVG		**10.6**	**6**	**1**

B. QC Mid DXR

QC Mid DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	100	109	9	
Replicate 2	100	105	5	
Replicate 3	100	104	4	
Replicate 4	100	105	5	
Replicate 5	100	109	10	
Replicate 6	100	106	6	
AVG		**106**	**7**	**2**

C. QC High DXR

QC High DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	1000	1119	12	
Replicate 2	1000	1083	8	
Replicate 3	1000	1113	11	
Replicate 4	1000	1073	7	
Replicate 5	1000	1044	4	
Replicate 6	1000	1043	4	
AVG		**1079**	**8**	**3**

D. QC Low DXR_C13

QC Low DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	10.0	10.7	7	
Replicate 2	10.0	10.7	7	
Replicate 3	10.0	10.9	9	
Replicate 4	10.0	10.9	9	
Replicate 5	10.0	11.3	13	
Replicate 6	10.0	10.5	5	
AVG		**10.8**	**8**	**3**

E. QC Mid DXR_C13

QC Mid DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	100	111	11	
Replicate 2	100	115	15	
Replicate 3	100	107	7	
Replicate 4	100	108	8	
Replicate 5	100	113	13	
Replicate 6	100	109	9	
AVG		**111**	**11**	**3**

F. QC High DXR_C13

QC High DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	1000	1133	13	
Replicate 2	1000	1036	4	
Replicate 3	1000	1112	11	
Replicate 4	1000	1101	10	
Replicate 5	1000	1010	1	
Replicate 6	1000	1054	5	
AVG		**1074**	**7**	**5**

Inter-Day Precision and Accuracy

Over the inter-day period, QC samples maintained a high degree of accuracy and precision (**Tables II-5 and II-6**). The low QC DXR standard, at 10 ng/mL, had an average accuracy deviation of 5% and precision of 6%. The mid QC DXR standard, at 100 ng/mL, had an average accuracy deviation of 7% and precision of 1%. The high QC DXR standard at, 1000 ng/mL, had an average accuracy deviation of 9% and precision of 1%.

The low DXR_C13 QC standard, at 10 ng/mL, had an average accuracy deviation of 7% and precision of 5%. The mid QC DXR_C13 standard, at 100 ng/mL, had an average accuracy deviation of 8% and precision of 3%. The high QC DXR_C13 standard, at 1000 ng/mL, had an average accuracy deviation of 9% and precision of 2%.

Table II-5. Inter-Day Precision and Accuracy. Precision and accuracy deviation data are presented for the inter-day DXR and DXR_C13 analysis by QC levels, 10 ng/mL (low), 100 ng/mL (mid), and 1000 ng/mL (high), on three separate days. Data for each study day represents an average of six replicate estimations of each QC level on that study day. **(A)** QC Low DXR, **(B)** QC Mid DXR, **(C)** QC High DXR, **(D)** QC Low DXR_C13, **(E)** QC Mid DXR_C13, **(F)** QC High DXR_C13. (See Appendix A for inter-day individual data).

A. QC Low DXR

QC Low DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	10.0	9.8	-2	
Day 2	10.0	11.1	11	
Day 3	10.0	10.6	6	
AVG	**10.0**	**10.5**	**5**	**6**

B. QC Mid DXR

QC Mid DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	100	108	8	
Day 2	100	106	6	
Day 3	100	106	6	
AVG	**100**	**107**	**7**	**1**

C. QC High DXR

QC High DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	1000	1090	9	
Day 2	1000	1109	11	
Day 3	1000	1079	8	
AVG	**1000**	**1093**	**9**	**1**

D. QC Low DXR_C13

QC Low DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	10.0	10.1	1	
Day 2	10.0	11.3	13	
Day 3	10.0	10.8	8	
AVG	**10.0**	**10.7**	**7**	**5**

E. QC Mid DXR_C13

QC Mid DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	100	109	9	
Day 2	100	105	5	
Day 3	100	111	11	
AVG	**100**	**108**	**8**	**3**

F. QC High DXR_C13

QC High DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	1000	1106	11	
Day 2	1000	1100	10	
Day 3	1000	1074	7	
AVG	**1000**	**1094**	**9**	**2**

Table II-6. Intra- & Inter-Day Precision and Accuracy Summary. The summarized precision and accuracy deviation data are presented for the intra- and inter-day validation study of DXR and DXR_C13 QC levels, 10 ng/mL (low), 100 ng/mL (mid), and 1000 ng/mL (high). Data for each QC level represents an average of all validation data presented previously in the report.

QC Standard	Intra-Day Summary		Inter-Day Summary	
	Accuracy Deviation (%)	Precision (%)	Accuracy Deviation (%)	Precision (%)
QC Low DXR	6	1	5	6
QC Mid DXR	6	2	7	1
QC High DXR	8	3	9	1
QC Low DXR_C13	8	3	7	5
QC Mid DXR_C13	11	3	8	3
QC High DXR_C13	7	5	9	2

Limit of Detection (LOD) and Limit of Quantitation (LOQ) Analysis

The LOQ and LOD was assessed by running six replicates of the LLOQ on three different days, for both DXR and DXR_C13, and determining the standard deviation of the concentration interpolated from the standard curve run on the corresponding day. The LOD was defined as 3 x SD, while LOQ was defined as limit of 10 x SD. The estimated LOD and LOQ for DXR were 2 and 6 ng/mL, respectively (**Table II-7A**). The estimated LOD and LOQ for DXR_C13 were 2 and 5 ng/mL, respectively (**Table II-7B**).

Table II-7. LOD and LOQ Determination.

A. DXR

Human Plasma Samples Spiked at the LLOQ[a]	Day 1	Day 2	Day 3
	Concentration (ng/mL)		
Replicate 1	10.1	11.1	10.5
Replicate 2	9.9	11.2	10.5
Replicate 3	9.3	10.8	10.7
Replicate 4	9.8	11.4	10.5
Replicate 5	10.2	11.1	10.8
Replicate 6	9.6	11.3	10.4
Mean	10.5		
SD[b]	0.6		
%RSD[c]	5.6%		
LOD[d]	1.8		
LOQ[e]	5.9		

B. DXR_C13

Human Plasma Samples Spiked at the LLOQ[a]	Day 1	Day 2	Day 3
	Concentration (ng/mL)		
Replicate 1	10.0	11.4	10.7
Replicate 2	10.0	10.8	10.7
Replicate 3	10.1	11.1	10.9
Replicate 4	10.1	11.7	10.9
Replicate 5	10.4	11.2	11.3
Replicate 6	10.1	11.3	10.5
Mean	10.7		
SD[b]	0.5		
%RSD[c]	5.0%		
LOD[d]	1.6		
LOQ[e]	5.3		

[a] Lower limit of quantitation (LLOQ) = 10.0 ng/mL
[b] SD = standard deviation
[c] %RSD = percent relative standard deviation
[d] LOD = limit of detection (3 x SD)
[e] LOQ = limit of quantitation (10 x SD)

Analytical System Reproducibility

Analytical system reproducibility was explored by determining the peak area and retention time precision of six replicate samples of the three plasma extracted analyte standards, at the 100 ng/mL QC level on three separate days (**Table II-8**). The runs met the acceptance criterion established for retention time and peak area at <10% RSD for all analytes.

Table II-8. Analytical System Suitability, 100 ng/mL.

A. Day 1

	DXR		DXR_C13		Aclarubicin	
	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area
Replicate 1	1.69	30047093	1.66	26113365	5.68	61910113
Replicate 2	1.69	31726486	1.67	26736413	5.69	64320511
Replicate 3	1.69	32291425	1.67	27435353	5.68	64717053
Replicate 4	1.69	29072017	1.67	24681245	5.68	54026736
Replicate 5	1.69	30155801	1.67	25320874	5.68	56750717
Replicate 6	1.69	30033350	1.67	24458367	5.68	55346846
AVG	1.69	30554362	1.67	25790936	5.68	59511996
SD	0.003	1206336	0.003	1177294	0.004	4712253
%RSD	0.2	4	0.2	5	0.1	8

B. Day 2

	DXR		DXR_C13		Aclarubicin	
	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area
Replicate 1	1.72	27302405	1.70	19629475	5.71	57249561
Replicate 2	1.71	24489666	1.69	19053816	5.71	53489419
Replicate 3	1.72	25409028	1.70	18354432	5.71	55664294
Replicate 4	1.74	30078538	1.72	21727477	5.71	65414158
Replicate 5	1.72	27008320	1.70	19650162	5.71	56344165
Replicate 6	1.72	26162113	1.69	18888901	5.71	56190028
AVG	1.72	26741678	1.70	19550710	5.71	57391937
SD	0.01	1934092	0.01	1172210	0	4126333
%RSD	0.6	7	0.5	6	0	7

C. Day 3

	DXR		DXR_C13		Aclarubicin	
	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area
Replicate 1	1.73	31724623	1.71	24081356	5.70	46134009
Replicate 2	1.73	32193297	1.71	23483941	5.70	47168884
Replicate 3	1.72	31542227	1.71	23225431	5.70	46953791
Replicate 4	1.73	30731532	1.71	22568010	5.70	51051170
Replicate 5	1.73	31652084	1.71	22563151	5.70	44793046
Replicate 6	1.73	32332533	1.70	23933928	5.70	47835578
AVG	1.73	31696049	1.71	23309303	5.70	47322746
SD	0.004	567287	0.001	652689	0	2104111
%RSD	0.2	2	0.1	3	0	4

D. Overall

	DXR		DXR_C13		Aclarubicin	
	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area
Overall Mean	1.71	29664030	1.69	22883650	5.70	54742227
Overall SD	0.02	2524707	0.02	2811556	0.01	6540455
Overall %RSD	**1**	**9**	**1**	**12**	**0.2**	**12**

Absolute Recovery

Absolute recovery was evaluated by comparing the spectrogram areas of the six calibration standards of the plasma extracted analytes DXR and DXR_C13 to their solvent standards, at each calibration level (**Table II-9**). The calculated absolute recovery was between 72-103% for both analytes.

Table II-9. Absolute Recovery in Plasma. Data for each calibration level represents an average peak area of two replicates ± standard deviation.

A. DXR

	Mobile Phase Area	Plasma Area	% Extraction Efficiency
10 ng/mL	2873206 ± 120829	2361991 ± 171547	82
50 ng/mL	14537814 ± 602021	13082506 ± 467127	90
100 ng/mL	29082548 ± 891451	27758647 ± 869171	85
500 ng/mL	139042479 ± 9681903	143283933 ± 1089240	103
1000 ng/mL	300534549 ± 7758572	296737255 ± 13273944	99
5000 ng/mL	1445432257 ± 70190338	14689364443 ± 55206944	102

B. DXR_C13

	Mobile Phase Area	Plasma Area	% Extraction Efficiency
10 ng/mL	2423672 ± 91922	1749991 ± 113528	72
50 ng/mL	12109472 ± 355880	9584698 ± 377221	79
100 ng/mL	24165875 ± 580080	20188869 ± 495952	84
500 ng/mL	117886110 ± 6853469	100795836 ± 5078736	86
1000 ng/mL	252681535 ± 3762276	212447440 ± 16447317	84
5000 ng/mL	1225897856 ± 49556201	1065317820 ± 47456168	87

Full Validation in Human Protein-Free Plasma

Summary

The purpose of this study was to validate the LC-Orbitrap quantitation method for DXR and DXR_C13 in human protein-free plasma matrix at a standard curve range of 0.1-1000 ng/mL. For specifics of the LC-Orbitrap method please refer to Section I. This analytical method was validated for extraction efficiency in human protein-free plasma and quality control precision and accuracy based on general FDA guidelines for bioanalytical methods [4].

Calibration standards were prepared by adding DXR and DXR_C13 analytical standards to blank human protein-free plasma in the range from 0.1-1000 ng/mL, to arrive at six calibration levels. Matrix interference was not observed for the analytes or internal standard. Complete sets of the combined DXR and DXR_C13 calibration standards were run on three different days.

The DXR and DXR_C13 calibration curves, analyte area/internal standard area vs. standard concentration, were suitable for linear regression with $1/x$ weighting, resulting in correlation coefficients of 0.99 or better. The accuracy deviation of the regression-calculated standard value from the true value was 15% or less for all calibration levels. The lower limit of quantitation (LLOQ) was established at 0.1 ng/mL. The method precision at the LLOQ was 8% relative standard deviation (% RSD) for DXR and 6% RSD for DXR_C13.

No carry over from the high calibration standard was seen for any of the three analytes. Analytical system suitability, evaluated by determining the peak area and retention time precision of six replicate samples at the 5 ng/mL concentration level for the protein-free plasma extracted DXR and DXR_C13 analytes, met the acceptance criterion established for retention time and peak area at $\pm10\%$ RSD for all analytes. The estimated LOD and LOQ for DXR were 0.03 and 0.08 ng/mL, respectively. The estimated LOD and LOQ for DXR_C13 were 0.01 and 0.03 ng/mL, respectively. For intra-day validation, six individual QC samples were run at each QC level, 0.1 (low), 5 (mid) and 100 (high) ng/mL for both analytes. These samples were distributed throughout the beginning, middle and end of the run. For the inter-day validation, six individual QC samples were run at each level on three separate days. The low, mid and high QC standards had intra-day and inter-day accuracy deviation and precision of ≤8% for both DXR and (see **Table II-15**). This meets the acceptance criteria of accuracy deviation not exceeding 15% from the true value, and precision not exceeding 15%, at all QC levels. The calculated absolute recovery for protein-free plasma extraction was between 53-114% for all analytes, at all calibration levels.

This validation did not attempt to assess either short-term room temperature stability or freeze-thaw stability of samples, or accuracy and precision of diluted samples.

In conclusion, this LC-Orbitrap analytical method for determination of DXR and DXR_C13 in human protein-free plasma was successfully developed and validated.

Results & Conclusions

Analytical System Suitability

Calibration curves, peak area ratio vs. concentration, were linear over the concentration range, from 0.1-1000 ng/mL, for DXR and DXR_C13 (**Figures II-4 and II-5**). Complete sets of six combined DXR and DXR_C13 calibration levels were run on three different days. The DXR and DXR_C13 curves, calibration area/internal standard area vs. standard concentration, were suitable for linear regression with $1/x^2$ weighting, resulting in correlation coefficients of 0.99 or better. The percent accuracy deviation of the regression-calculated value from the true value was 15% or less, for all calibration levels (**Tables II-10 and II-11**). The lower limit of quantitation (LLOQ) was established at 0.1 ng/mL.

DXR_PRM
$Y = -3.9316e\text{-}005 + 0.00625724 \cdot X \quad R^2 = 0.9948 \quad W: 1/X^2$
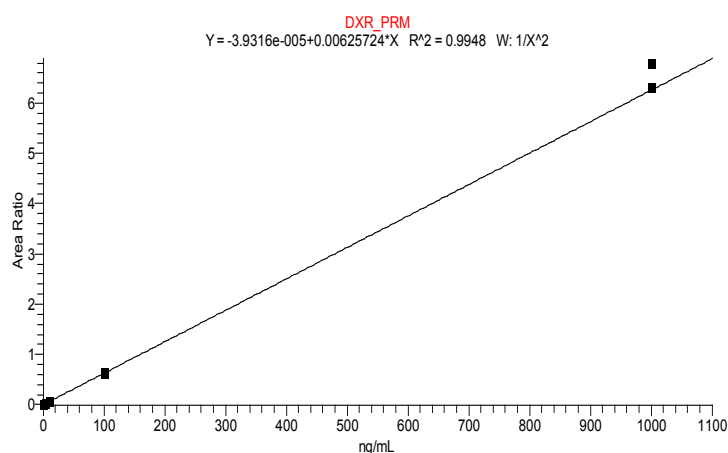
Figure II-4. Example of DXR Calibration Curve in Human Protein-Free Plasma.

DXR_C13_PRM
$Y = -8.47631e\text{-}005 + 0.00506509 \cdot X \quad R^2 = 0.9985 \quad W: 1/X^2$
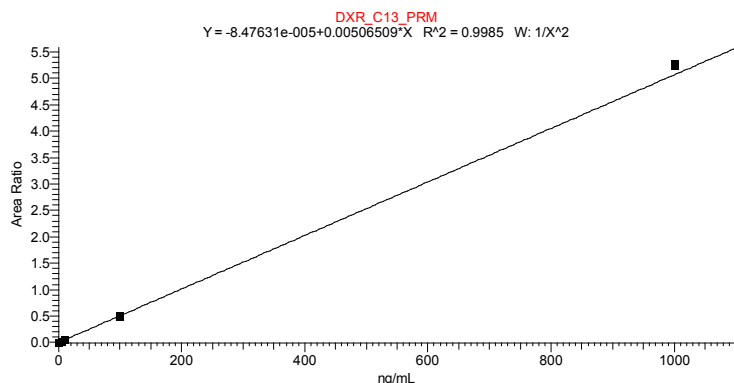
Figure II-5. Example of DXR_C13 Calibration Curve in Human Protein-Free Plasma.

Table II-10. DXR Calibration Runs.

Standard Conc (ng/mL)	DXR Calibration 1			DXR Calibration 2			DXR Calibration 3		
	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision
0.100	0.090	-10%	15%	0.096	-4%	7%	0.095	-5%	8%
0.100	0.110	10%		0.105	5%		0.106	6%	
1.00	0.99	-1%	1%	0.97	-3%	3%	0.96	-4%	2%
1.00	1.01	1%		0.93	-7%		0.98	-2%	
5.00	4.61	-8%	1%	4.82	-4%	3%	4.99	-0.2%	5%
5.00	4.54	-9%		5.04	1%		4.68	-6%	
10.0	9.9	-1%	6%	9.8	-2%	3%	9.7	-3%	1%
10.0	10.7	7%		10.3	3%		9.8	-2%	
100.0	99.6	-0.4%	2%	95.9	-4%	9%	98.8	-1%	2%
100.0	102.1	2%		108.4	8%		100.9	1%	
1000.0	1007.3	1%	5%	1020.1	2%	2%	1087.9	9%	0.4%
1000.0	1084.2	8%		1047.5	5%		1082.1	8%	
Linear Regression (ax+b) with $1/x^2$ Weighting									
Linear coefficient	0.006			0.006			0.005		
Intercept	-4E-05			5E-05			5E-05		
R^2	0.99			0.99			0.99		

Table II-11. DXR_C13 Calibration Runs.

Standard Conc (ng/mL)	DXR_C13 Calibration 1			DXR_C13 Calibration 2			DXR_C13 Calibration 3		
	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision
0.100	0.101	1%	2%	0.100	1%	0.1%	0.096	-4%	6%
0.100	0.099	-1%		0.101	1%		0.104	4%	
1.00	1.02	2%	0.2%	0.94	-6%	1%	0.96	-4%	2%
1.00	1.01	1%		0.95	-5%		0.99	-1%	
5.00	4.59	-8%	2%	4.81	-4%	4%	4.98	-0.4%	5%
5.00	4.72	-6%		5.05	1%		4.66	-7%	
10.0	10.2	2%	1%	10.2	2%	0.05%	9.8	-2%	1%
10.0	10.0	0.4%		10.2	2%		9.9	-1%	
100.0	99.4	-0.6%	2%	94.7	-5%	9%	100.4	0.4%	0.1%
100.0	101.7	2%		106.9	7%		100.4	0.4%	
1000.0	1034.0	3%	1%	1037.8	4%	0.05%	1067.0	7%	0.02%
1000.0	1044.1	4%		1038.6	4%		1066.6	7%	
Linear Regression (ax+b) with $1/x^2$ Weighting									
Linear coefficient	0.0050			0.0043			0.0033		
Intercept	-8.48E-05			-4.71E-05			-4.15E-05		
R^2	0.99			0.99			0.99		

Carry Over

Carry over was examined by injecting triplicates of the high and low (LLOQ) standards, and a water blank following the high standard run (**Table II-12**). There was no carry over seen for any of the analytes.

Table II-12. Carry Over.

	PRM	Retention Time	High STD Area	LLOQ Area	High STD-Blank Carry Over Area	% LLOQ of Blank
DXR	130.086 + 379.080 +397.085	1.7	39454345	1955	0	0
DXR_C13	130.086 + 383.100 + 401.115	1.7	32497795	1207	0	0
Aclarubicin	570.228	5.7	62911835	65991249	0	0

Intra-Day Precision and Accuracy
Over the intra-day period, the six replicate QC samples maintained a high degree of accuracy and precision (**Tables II-13 and II-15**). The low QC DXR standard, at 0.1 ng/mL, had an average accuracy deviation of 6% and precision of 6%. The mid QC DXR standard, at 5 ng/mL, had an average accuracy deviation of -8% and precision of 2%. The high QC DXR standard, at 100 ng/mL, had an average accuracy deviation of 6% and precision of 2%.

The low DXR_C13 QC standard, at 0.1ng/mL, had an average accuracy deviation of -6% and precision of 2%. The mid QC DXR_C13 standard, at 5 ng/mL, had an average accuracy deviation of -7% and precision of 3%. The high QC DXR_C13 standard at, 100 ng/mL, had an average accuracy deviation of 8% and precision of 2%.

Table II-13. Intra-Day Precision and Accuracy. Precision and accuracy deviation data are presented for the intra-day six replicate samples of the DXR and DXR_C13 analytes by QC levels, 0.1 ng/mL (low), 5 ng/mL (mid), 100 ng/mL (high). **(A)** QC Low DXR, **(B)** QC Mid DXR, **(C)** QC High DXR, **(D)** QC Low DXR_C13, **(E)** QC Mid DXR_C13, **(F)** QC High DXR_C13.

A. QC Low DXR

QC Low DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	0.100	0.102	2	
Replicate 2	0.100	0.110	10	
Replicate 3	0.100	0.097	-3	
Replicate 4	0.100	0.106	6	
Replicate 5	0.100	0.102	2	
Replicate 6	0.100	0.115	15	
AVG		**0.105**	**6**	**6**

B. QC Mid DXR

QC Mid DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	5.00	4.56	-9	
Replicate 2	5.00	4.53	-9	
Replicate 3	5.00	4.63	-7	
Replicate 4	5.00	4.73	-6	
Replicate 5	5.00	4.54	-9	
Replicate 6	5.00	4.61	-8	
AVG		**4.60**	**-8**	**2**

C. QC High DXR

QC High DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	100.0	108.8	9	
Replicate 2	100.0	108.3	8	
Replicate 3	100.0	105.0	5	
Replicate 4	100.0	104.7	5	
Replicate 5	100.0	105.7	6	
Replicate 6	100.0	102.3	2	
AVG		**105.8**	**6**	**2**

D. QC Low DXR_C13

QC Low DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	0.100	0.093	-7	
Replicate 2	0.100	0.094	-6	
Replicate 3	0.100	0.098	-2	
Replicate 4	0.100	0.092	-8	
Replicate 5	0.100	0.097	-3	
Replicate 6	0.100	0.093	-7	
AVG		**0.094**	**-6**	**2**

E. QC Mid DXR_C13

QC Mid DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	5.00	4.60	-8	
Replicate 2	5.00	4.48	-11	
Replicate 3	5.00	4.55	-9	
Replicate 4	5.00	4.69	-6	
Replicate 5	5.00	4.59	-8	
Replicate 6	5.00	4.85	-3	
AVG		**4.63**	**-7**	**3**

F. QC High DXR_C13

QC High DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	100.0	110.1	10	
Replicate 2	100.0	106.8	7	
Replicate 3	100.0	108.9	9	
Replicate 4	100.0	109.2	9	
Replicate 5	100.0	105.7	6	
Replicate 6	100.0	104.8	5	
AVG		**107.6**	**8**	**2**

Inter-Day Precision and Accuracy

Over the inter-day period, QC samples maintained a high degree of accuracy and precision (**Tables II-14 and II-15**). The low QC DXR standard, at 0.1 ng/mL, had an average accuracy deviation of 8% and precision of 3%. The mid QC DXR standard, at 5 ng/mL, had an average accuracy deviation of -0.3% and precision of 7%. The high QC DXR standard at, 100 ng/mL, had an average accuracy deviation of 5% and precision of 4%.

The low DXR_C13 QC standard, at 0.1 ng/mL, had an average accuracy deviation of 4% and precision of 8%. The mid QC DXR_C13 standard, at 5 ng/mL, had an average accuracy deviation of 0.2% and precision of 7%. The high QC DXR_C13 standard, at 100 ng/mL, had an average accuracy deviation of 6% and precision of 4%.

Table II-14. Inter-Day Precision and Accuracy. Precision and accuracy deviation data are presented for the inter-day DXR and DXR_C13 analysis by QC levels, 0.1 ng/mL (low), 5 ng/mL (mid), and 100 ng/mL (high), on three separate days. Data for each study day represents an average of six replicate estimations of each QC level on that study day. **(A)** QC Low DXR, **(B)** QC Mid DXR, **(C)** QC High DXR, **(D)** QC Low DXR_C13, **(E)** QC Mid DXR_C13, **(F)** QC High DXR_C13. (See Appendix B for inter-day individual data).

A. QC Low DXR

QC Low DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	0.100	0.106	6	
Day 2	0.100	0.111	11	
Day 3	0.100	0.105	6	
AVG	**0.100**	**0.108**	**8**	**3**

B. QC Mid DXR

QC Mid DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	5.00	5.05	1	
Day 2	5.00	5.30	6	
Day 3	5.00	4.60	-8	
AVG	**5.00**	**4.98**	**-0.3**	**7**

C. QC High DXR

QC High DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	100.0	100.3	0.3	
Day 2	100.0	109.2	9	
Day 3	100.0	105.8	6	
AVG	**100.0**	**105.1**	**5**	**4**

Validation Studies

D. QC Low DXR_C13

QC Low DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	0.100	0.107	7	
Day 2	0.100	0.110	10	
Day 3	0.100	0.094	-6	
AVG	**0.100**	**0.104**	**4**	**8**

E. QC Mid DXR_C13

QC Mid DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	5.00	5.10	2	
Day 2	5.00	5.31	6	
Day 3	5.00	4.63	-7	
AVG	**5.00**	**5.01**	**0.2**	**7**

F. QC High DXR_C13

QC High DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	100.0	102.1	2	
Day 2	100.0	109.3	9	
Day 3	100.0	107.6	8	
AVG	**100.0**	**106.3**	**6**	**4**

Table II-15. Intra- & Inter-Day Precision and Accuracy Summary. The summarized precision and accuracy deviation data are presented for the intra- and inter-day validation study of DXR and DXR_C13 QC levels, 0.1 ng/mL (low), 5 ng/mL (mid), and 100 ng/mL (high). Data for each QC level represents and average of all validation data presented previously in the report.

QC Standard	Intra-Day Summary		Inter-Day Summary	
	Accuracy Deviation (%)	Precision (%)	Accuracy Deviation (%)	Precision (%)
QC Low DXR	6	6	8	3
QC Mid DXR	-8	2	-0.3	7
QC High DXR	6	2	5	4
QC Low DXR_C13	-6	2	4	8
QC Mid DXR_C13	-7	3	0.2	7
QC High DXR_C13	8	2	6	4

Limit of Detection (LOD) and Limit of Quantitation (LOQ) Analysis
The LOQ and LOD was assessed by running six replicates of the LLOQ on three different days, for both DXR and DXR_C13, and determining the standard deviation of the concentration interpolated from the standard curve run on the corresponding day. The LOD was defined as 3 x SD, while LOQ was defined as limit of 10 x SD. The estimated LOD and LOQ for DXR were 0.02 and 0.1 ng/mL, respectively (**Table II-16A**). The estimated LOD and LOQ for DXR_C13 were 0.03 and 0.1 ng/mL, respectively (**Table II-16B**).

Table II-16. LOD and LOQ Determination.

A. DXR

Human Protein-Free Plasma Samples Spiked at the LLOQ[a]	Day 1	Day 2	Day 3
	Concentration (ng/mL)		
Replicate 1	0.117	0.117	0.102
Replicate 2	0.100	0.103	0.110
Replicate 3	0.105	0.112	0.097
Replicate 4	0.114	0.121	0.106
Replicate 5	0.095	0.099	0.102
Replicate 6	0.108	0.114	0.115
Mean	**0.108**		
SD[b]	**0.01**		
%RSD[c]	**7.2**		
LOD[d]	**0.023**		
LOQ[e]	**0.078**		

B. DXR_C13

Human Protein-Free Plasma Samples Spiked at the LLOQ[a]	Day 1	Day 2	Day 3
	Concentration (ng/mL)		
Replicate 1	0.102	0.108	0.093
Replicate 2	0.116	0.100	0.094
Replicate 3	0.118	0.113	0.098
Replicate 4	0.108	0.112	0.092
Replicate 5	0.099	0.117	0.097
Replicate 6	0.096	0.108	0.093
Mean	**0.104**		
SD[b]	**0.01**		
%RSD[c]	**8.7**		
LOD[d]	**0.027**		
LOQ[e]	**0.091**		

[a] Lower limit of quantitation (LLOQ) = 0.1 ng/mL
[b] SD = standard deviation
[c] %RSD = percent relative standard deviation
[d] LOD = limit of detection (3 x SD)
[e] LOQ = limit of quantitation (10 x SD)

Analytical System Reproducibility
Analytical system reproducibility was evaluated by determining the peak area and retention time precision of six replicate samples of the three plasma extracted analyte standards, at the 5 ng/mL QC level on three separate days (**Table II-17**). The runs met the acceptance criterion established for retention time and peak area at ≤10% RSD for all analytes.

Table II-17. Analytical System Suitability.

A. Day 1

	DXR		DXR_C13		Aclarubicin	
	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area
Replicate 1	1.69	1850652	1.67	1504709	5.68	60877162
Replicate 2	1.69	1597583	1.67	1397213	5.68	51674492
Replicate 3	1.70	1858762	1.67	1437102	5.68	54220815
Replicate 4	1.70	1832314	1.67	1456986	5.69	59165433
Replicate 5	1.69	1638938	1.67	1326525	5.68	50831938
Replicate 6	1.70	1748878	1.68	1450837	5.68	57161786
AVG	1.69	1754521	1.67	1428896	5.68	55655271
SD	0.01	113296	0.005	60941	0.004	4074991
%RSD	0.4	6	0.3	4	0.1	7

B. Day 2

	DXR		DXR_C13		Aclarubicin	
	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area
Replicate 1	1.70	1729652	1.68	1150509	5.70	75305433
Replicate 2	1.71	1738632	1.68	1132865	5.70	76221228
Replicate 3	1.71	1666423	1.68	1079852	5.71	71498819
Replicate 4	1.71	1704834	1.68	1116656	5.71	71623928
Replicate 5	1.71	1818820	1.69	1214634	5.70	79610816
Replicate 6	1.72	1797956	1.69	1246068	5.70	77419702
AVG	1.71	1742719	1.68	1156764	5.70	75279988
SD	0.01	57073	0.01	62383	0.01	3221336
%RSD	0.4	3	0.3	5	0.1	4

C. Day 3

	DXR		DXR_C13		Aclarubicin	
	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area
Replicate 1	1.72	1597725	1.70	1099639	5.69	46283895
Replicate 2	1.72	1689140	1.69	1149009	5.69	47509289
Replicate 3	1.73	1690496	1.70	1153892	5.69	49734172
Replicate 4	1.73	1549877	1.70	1061280	5.70	44780562
Replicate 5	1.73	1722217	1.70	1169401	5.70	49325413
Replicate 6	1.73	1713957	1.70	1164809	5.70	55159106
AVG	1.73	1660568	1.70	1133005	5.70	48798739
SD	0.01	70093	0.005	43096	0.01	3626005
%RSD	0.3	4	0.3	4	0.1	7

D. Overall

	DXR		DXR_C13		Aclarubicin	
	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area
Overall Mean	1.70	1719270	1.68	1239555	5.69	59911333
Overall SD	0.02	89594	0.01	147859	0.01	12047573
Overall %RSD	**1**	**5**	**1**	**12**	**0.2**	**20**

Absolute Recovery

Absolute recovery was explored by comparing the spectrogram areas of the six calibration standards of the plasma extracted analytes DXR and DXR_C13 to their solvent standards, at each calibration level (**Table II-18**). The calculated absolute recovery was between 53-114% for both analytes.

Table II-18. Absolute Recovery in Protein-Free Plasma. Data for each calibration level represents an average peak area of two replicates $\pm$ standard deviation.

A. DXR

	Mobile Phase Area	PFP Area	% Extraction Efficiency
0.1 ng/mL	49220 $\pm$ 7251	27178 $\pm$ 232	55
1 ng/mL	371716 $\pm$ 17939	289954 $\pm$ 10694	78
5 ng/mL	1835203 $\pm$ 27855	1468186 $\pm$ 41753	80
10 ng/mL	3728638 $\pm$ 28209	3076410 $\pm$ 38104	83
100 ng/mL	36045394 $\pm$ 319118	35142439 $\pm$ 1439237	98
1000 ng/mL	337695084 $\pm$ 18363559	386458177 $\pm$ 4259945	114

B. DXR_C13

	Mobile Phase Area	PFP Area	% Extraction Efficiency
0.1 ng/mL	24126 $\pm$ 2352	12823 $\pm$ 56	53
1 ng/mL	227221 $\pm$ 10575	166906 $\pm$ 1998	74
5 ng/mL	1119173 $\pm$ 3877	870816 $\pm$ 32872	78
10 ng/mL	2274530 $\pm$ 10043	1838816 $\pm$ 10786	81
100 ng/mL	21830034 $\pm$ 229126	20667723 $\pm$ 1526527	95
1000 ng/mL	207902349 $\pm$ 19968717	221853377 $\pm$ 6789967	107

Partial Validation in Rat Plasma and Protein-Free Plasma

Summary

The purpose of this study was to run a partial validation on the LC-Orbitrap quantitation method for DXR and DXR_C13 in rat plasma and rat protein-free matrices at standard curve ranges equivalent to those used for human plasma and human protein-free plasma. For specifics of the LC orbitrap method please refer to Section I. This partial validation consisted of a determination of intra-day precision and accuracy at three different QC levels, 10 ng/mL (low), 100 ng/mL (mid), and 1000 ng/mL (high) for plasma and QC levels 0.1 ng/mL (low), 5 ng/mL (mid), and 100 ng/mL (high) for protein-free plasma, and assessment of analytical reproducibility.

Calibration standards were prepared by adding DXR and DXR_C13 analytical standards to blank rat plasma in the range from 10-5000 ng/mL and to blank rat protein-free plasma in a range from 0.1-1000 ng/mL, to arrive at six calibration levels for each matrix. Matrix interference was not observed for the analytes or internal standard, and carry over from the high calibration standard was less than 5% of the low standard, 10 ng/mL in plasma and 0.1 ng/mL in protein-free plasma.

The DXR and DXR_C13 calibration curves, analyte area/internal standard area vs. standard concentration, were suitable for linear regression of $1/x$ weighting for plasma and $1/x^2$ weighting for protein-free plasma, resulting in correlation coefficients of 0.99 or better. The accuracy deviation of the regression-calculated standard value from the true value was 15% or less for all calibration levels.

For intra-day validation, six individual QC samples were run at each QC level, 10 (low), 100 (mid), and 1000 (high) ng/mL for plasma and QC levels 0.1 (low), 5 (mid), and 100 (high) ng/mL for protein-free plasma. These samples were distributed throughout the beginning, middle, and end of the run. The low, mid, and high QC standards for DXR in plasma had intra-day accuracy deviation/precision of -16%/3.2%, -8%/5%, and 5%/4%. The low, mid, and high QC standards for DXR_C13 in plasma had intra-day accuracy deviation/precision of -11%/4%, -10%/5%, and 4%/3%. The low, mid and high QC standards for DXR in protein-free plasma had intra-day accuracy deviation/precision of -9%/7%, -1%/4%, and 5%/3%. The low, mid and high QC standards for DXR_C13 in protein-free plasma had intra-day accuracy deviation/precision of 1%/5%, -1%/4%, and 3%/4%. This meets the acceptance criteria of accuracy deviation not exceeding 15% from the true value except at LLOQ, where it does not exceed 20% of the true value, and precision not exceeding 15% for all QC levels. The runs met the acceptance criterion established for retention time and peak area at <10% RSD for both analytes and internal standard.

This validation did not attempt to assess inter-day precision and accuracy, either short-term room temperature stability or freeze-thaw stability of samples, or accuracy and precision of diluted samples.

In conclusion, this LC-Orbitrap analytical method for determination of DXR and DXR_C13 in rat plasma and protein-free plasma was successfully validated.

Results and Conclusions

Calibration Curves

Calibration curves, area ratio vs. concentration, were linear over the concentration range from 10-5000 ng/mL for DXR and DXR_C13 in plasma (**Figures II-6 and II-7**). Calibration curves, area ratio vs. concentration, were linear over the concentration range from 0.1-1000 ng/mL for DXR and DXR_C13 in protein-free plasma (**Figures II-8 and II-9**). Complete sets of six calibration levels were run in duplicate. The DXR and DXR_C13 curves, calibration area/internal standard area vs. standard concentration, were suitable for linear regression with 1/x weighting for plasma and $1/x^2$ weighting for protein-free plasma, resulting in correlation coefficients of 0.99 or better. The percent accuracy deviation of the regression-calculated value from the true value, and precision (%RSD) was 15% or less for all calibration levels except at LLOQ, where it was 20% or less (**Tables II-19 to II-22**).

Table II-19. DXR Calibration Standard Curve in Rat Plasma.

| Standard Conc (ng/mL) | DXR Calibration 1 | | |
	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision
10.0	8.5	-15%	2%
10.0	8.8	-12%	
50	52	4%	5%
50	48	-3%	
100	107	7%	3%
100	103	3%	
500	542	8%	2%
500	528	6%	
1000	1029	3%	0.04%
1000	1029	3%	
5000	4767	-5%	5%
5000	5097	2%	
Linear Regression (ax+b) with 1/x Weighting			
Linear coefficient	0.0170		
Intercept	2.80E-02		
R^2	0.99		

DXR_PRM
$Y = 0.0281177 + 0.0176518 \cdot X$ $R^2 = 0.9984$ W: 1/X
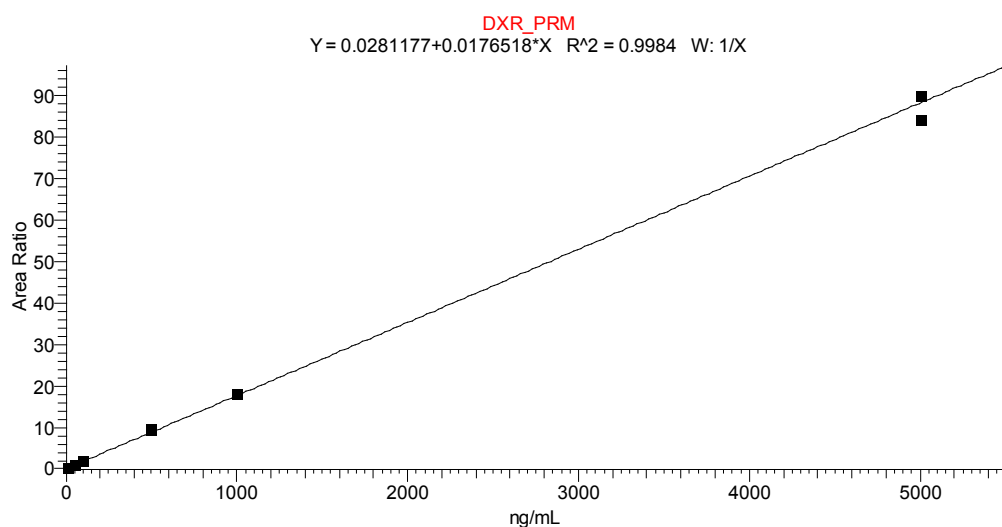
Figure II-6. DXR Calibration Curve in Rat Plasma.

Table II-20. DXR_C13 Calibration Curve in Rat Plasma.

| Standard Conc (ng/mL) | DXR_C13 Calibration 1 | | |
	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision
10.0	9.3	-7%	1%
10.0	9.4	-6%	
50	52	4%	5%
50	48	-3%	
100	104	4%	1%
100	106	6%	
500	544	9%	3%
500	522	4%	
1000	1022	2%	1%
1000	1035	4%	
5000	4795	-4%	4%
5000	5074	1%	
Linear Regression (ax+b) with 1/x Weighting			
Linear coefficient	0.0150		
Intercept	1.90E-02		
R^2	0.99		

DXR_C13_PRM
Y = 0.0185855+0.0149376*X   R^2 = 0.9987   W: 1/X
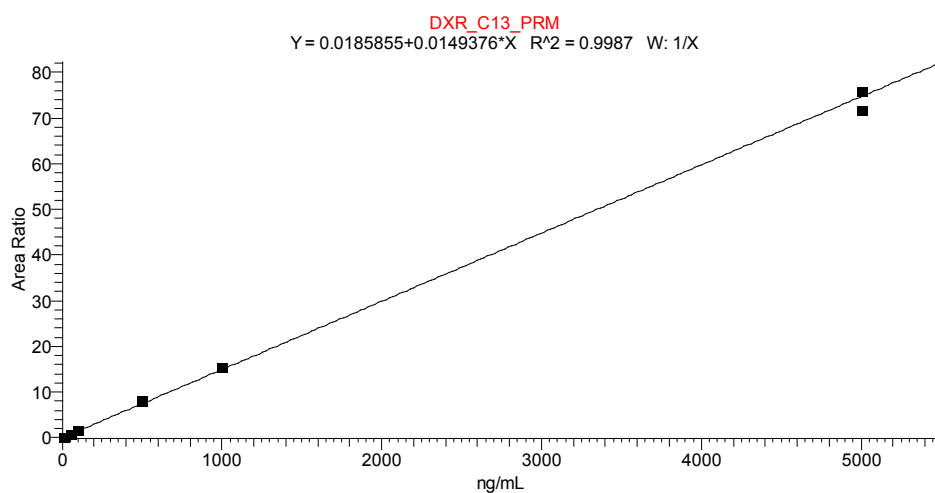
Figure II-7. DXR_C13 Calibration Curve in Rat Plasma.

Table II-21. DXR Calibration Standard Curve in Rat Protein-Free Plasma.

| Standard Conc (ng/mL) | DXR Calibration 1 | | |
	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision
0.100	0.097	-3%	4%
0.100	0.104	4%	
1.00	0.97	-3%	0.3%
1.00	0.97	-3%	
5.00	4.95	-1%	4%
5.00	4.66	-7%	
10.0	10.4	4%	1%
10.0	10.3	3%	
100.0	102.8	3%	0.2%
100.0	102.6	3%	
1000.0	1011.2	1%	0.3%
1000.0	1006.6	1%	
Linear Regression (ax+b) with $1/x^2$ Weighting			
Linear coefficient	0.0069		
Intercept	2.30E-05		
R^2	0.99		

DXR_PRM
$Y = 2.34343e{-}005 + 0.00689963{*}X$ $R^2 = 0.9987$ W: $1/X^2$
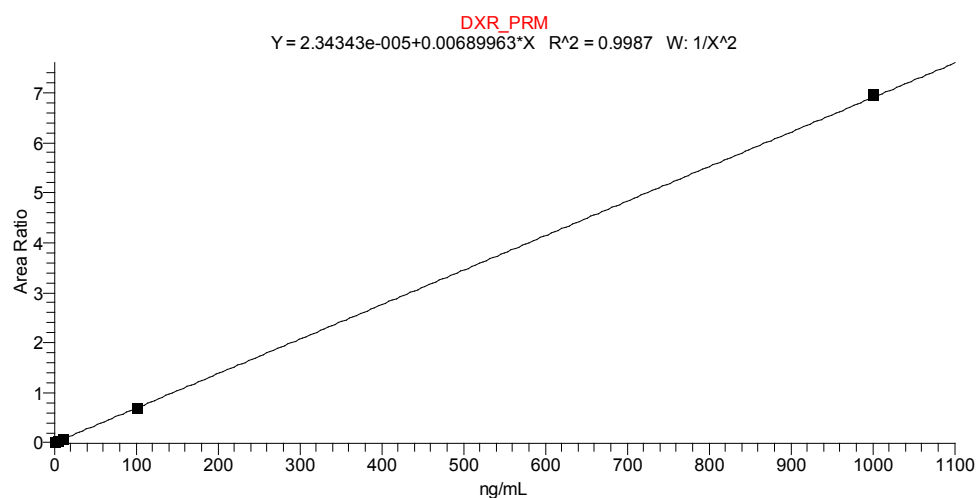
Figure II-8. DXR Calibration Curve in Rat Protein-Free Plasma.

Table II-22. DXR_C13 Calibration Curve in Rat Protein-Free Plasma.

| Standard Conc (ng/mL) | DXR_C13 Calibration 1 | | |
	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision
0.100	0.098	-2%	3%
0.100	0.103	3%	
1.00	0.93	-7%	5%
1.00	1.00	0.3%	
5.00	5.03	1%	5%
5.00	4.71	-6%	
10.0	10.0	-0.2%	2%
10.0	10.3	3%	
100.0	101.9	2%	2%
100.0	105.3	5%	
1000.0	959.2	-4%	6%
1000.0	1048.8	5%	
Linear Regression (ax+b) with $1/x^2$ Weighting			
Linear coefficient	0.0044		
Intercept	-8.70E-05		
R^2	0.99		

DXR_C13_PRM
Y = -8.67687e-005+0.00439295*X   R^2 = 0.9982   W: 1/X^2
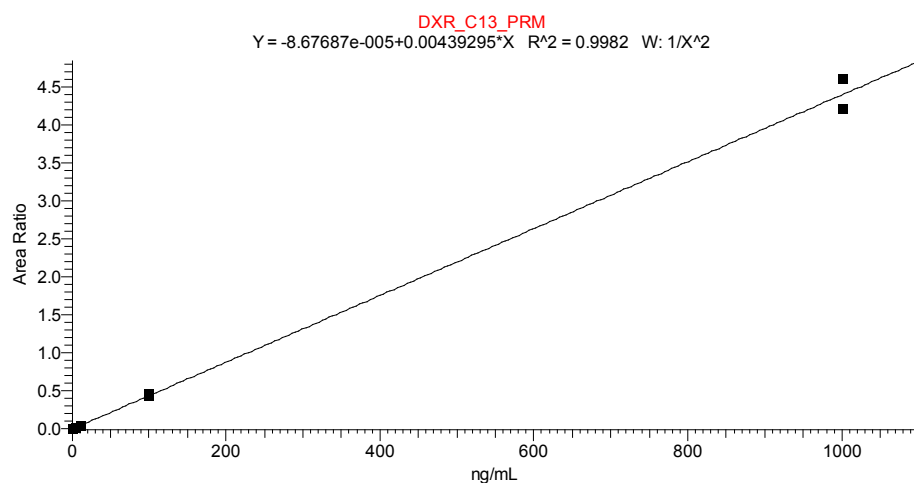
Figure II-9. DXR_C13 Calibration Curve in Rat Protein-Free Plasma.

Intra-Day Precision and Accuracy

Over the intra-day study period, the six replicate QC samples maintained a high degree of accuracy and precision in plasma (**Table II-23**). The low QC DXR standard, at 10 ng/mL, had an average accuracy deviation of 16% and precision of 3%. The mid QC DXR standard, at 100 ng/mL, had an average accuracy deviation of -8% and precision of 5%. The high QC DXR standard, at 1000 ng/mL, had an average accuracy deviation of 5% and precision of 4%. The low QC DXR_C13 standard, at 10 ng/mL, had an average accuracy deviation of -11% and precision of 4%. The mid QC DXR_C13 standard at 100 ng/mL, had an average accuracy deviation of -10% and precision of 5%. The high QC DXR_C13 standard, at 1000 ng/mL, had an average accuracy deviation of 4% and precision of 3%.

Over the intra-day study period, the six replicate QC samples also maintained a high degree of accuracy and precision in protein-free plasma (**Table II-24**). The low QC DXR standard, at 0.1 ng/mL, had an average accuracy deviation of -9% and precision of 7%. The mid QC DXR standard, at 5 ng/mL, had an average accuracy deviation of -1% and precision of 4%. The high QC DXR standard, at 100 ng/mL, had an average accuracy deviation of 5% and precision of 3%. The low QC DXR_C13 standard, at 0.1 ng/mL, had an average accuracy deviation of 1% and precision of 5%. The mid QC DXR_C13 standard, at 5 ng/mL, had an average accuracy of -1% and precision of 4%. The high QC DXR_C13 standard, at 100 ng/mL, had an average accuracy of 3% and precision of 4%.

Table II-23. Intra-Day Precision and Accuracy in Rat Plasma. Precision and accuracy deviation data are presented for the intra-day six replicate samples, QC levels,10 ng/mL (low), 100 ng/mL (mid), and 1000 ng/mL (high), for DXR and DXR_C13. **(A)** QC Low DXR, **(B)** QC Mid DXR, **(C)** QC High DXR, **(D)** QC Low DXR_C13, **(E)** QC Mid DXR_C13, **(F)** QC High DXR_C13.

A. QC Low DXR

QC Low DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	10.0	8.8	-12	
Replicate 2	10.0	8.0	-20	
Replicate 3	10.0	8.5	-16	
Replicate 4	10.0	8.6	-14	
Replicate 5	10.0	8.4	-16	
Replicate 6	10.0	8.2	-18	
AVG		**8.4**	**-16**	**3**

B. QC Mid DXR

QC Mid DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	100	92	-8	
Replicate 2	100	89	-11	
Replicate 3	100	88	-12	
Replicate 4	100	101	1	
Replicate 5	100	92	-8	
Replicate 6	100	89	-12	
AVG		**92**	**-8**	**5**

C. QC High DXR

QC High DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	1000	1032	3	
Replicate 2	1000	1049	5	
Replicate 3	1000	1019	2	
Replicate 4	1000	1128	13	
Replicate 5	1000	1015	2	
Replicate 6	1000	1041	4	
AVG		**1047**	**5**	**4**

D. QC Low DXR_C13

QC Low DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	10.0	9.5	-5	
Replicate 2	10.0	8.6	-15	
Replicate 3	10.0	8.6	-14	
Replicate 4	10.0	9.0	-10	
Replicate 5	10.0	9.1	-9	
Replicate 6	10.0	8.7	-13	
AVG		**8.9**	**-11**	**4**

E. QC Mid DXR_C13

QC Mid DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	100	91	-9	
Replicate 2	100	88	-12	
Replicate 3	100	87	-13	
Replicate 4	100	100	-0.4	
Replicate 5	100	90	-10	
Replicate 6	100	86	-14	
AVG		**90**	**-10**	**5**

F. QC High DXR_C13

QC High DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	1000	1028	3	
Replicate 2	1000	1049	5	
Replicate 3	1000	1003	0.1	
Replicate 4	1000	1100	10	
Replicate 5	1000	1020	2	
Replicate 6	1000	1013	1	
AVG		**1036**	**4**	**3**

Table II-24. Intra-Day Precision and Accuracy in Rat Protein-Free Plasma. Precision and accuracy deviation data are presented for the intra-day six replicate samples QC levels,0.1 ng/mL (low), 5 ng/mL (mid), and 100 ng/mL (high), for DXR and DXR_C13. **(A)** QC Low DXR, **(B)** QC Mid DXR, **(C)** QC High DXR, **(D)** QC Low DXR_C13, **(E)** QC Mid DXR_C13, **(F)** QC High DXR_C13.

A. QC low DXR

QC Low DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	0.100	0.082	-18	
Replicate 2	0.100	0.099	-1	
Replicate 3	0.100	0.089	-11	
Replicate 4	0.100	0.093	-7	
Replicate 5	0.100	0.096	-4	
Replicate 6	0.100	0.089	-11	
AVG		**0.091**	**-9**	**7**

B. QC Mid DXR

QC Mid DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	5.00	5.14	3	
Replicate 2	5.00	5.14	3	
Replicate 3	5.00	5.10	2	
Replicate 4	5.00	4.69	-6	
Replicate 5	5.00	4.83	-4	
Replicate 6	5.00	4.77	-5	
AVG		**4.95**	**-1**	**4**

C. QC High DXR

QC High DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	100.0	107.5	8	
Replicate 2	100.0	100.4	0.4	
Replicate 3	100.0	106.6	7	
Replicate 4	100.0	106.9	7	
Replicate 5	100.0	104.1	4	
Replicate 6	100.0	101.6	2	
AVG		**104.5**	**5**	**3**

D. QC Low DXR_C13

QC Low DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	0.100	0.097	-3	
Replicate 2	0.100	0.104	4	
Replicate 3	0.100	0.099	-1	
Replicate 4	0.100	0.096	-4	
Replicate 5	0.100	0.101	1	
Replicate 6	0.100	0.110	10	
AVG		**0.101**	**1**	**5**

E. QC Mid DXR_C13

QC Mid DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	5.00	5.06	1	
Replicate 2	5.00	5.11	2	
Replicate 3	5.00	5.19	4	
Replicate 4	5.00	4.76	-5	
Replicate 5	5.00	4.72	-6	
Replicate 6	5.00	5.01	0.1	
AVG		**4.98**	**-1**	**4**

F. QC High DXR_C13

QC High DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	100.0	100.2	0.2	
Replicate 2	100.0	97.9	-2	
Replicate 3	100.0	107.1	7	
Replicate 4	100.0	106.2	6	
Replicate 5	100.0	106.6	7	
Replicate 6	100.0	102.1	2	
AVG		**103.4**	**3**	**4**

Analytical System Reproducibility

Analytical system reproducibility was evaluated by determining the peak area and retention time precision of six replicate samples at the 100 ng/mL QC level (**Table II-25**) for plasma and 5 ng/mL QC level (**Table II-26**) for protein-free plasma. The runs met the acceptance criterion established for retention time and peak area at <10% RSD for both the analytes, DXR and DXR_C13, and internal standard, aclarubicin, in both plasma and protein-free plasma.

Table II-25. Analytical System Suitability in Rat Plasma.

	DXR		DXR_C13		Aclarubicin	
	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area
Replicate 1	1.68	37228187	1.66	30765169	5.70	22431674
Replicate 2	1.68	35992516	1.66	29805965	5.70	22427947
Replicate 3	1.68	34681660	1.66	28972112	5.70	22011125
Replicate 4	1.68	35377026	1.65	29373086	5.70	19605652
Replicate 5	1.68	34231763	1.65	28200059	5.70	20790938
Replicate 6	1.67	33747782	1.65	27506262	5.70	21209465
AVG	1.68	35209822	1.66	29103775	5.70	21412800
SD	0.005	1271808	0.005	1158612	0.003	1106387
%RSD	**0.17**	**4**	**0.3**	**4**	**0.03**	**5**

Table II-26. Analytical System Suitability in Rat Protein-Free Plasma.

	DXR		DXR_C13		Aclarubicin	
	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area
Replicate 1	1.70	1437838	1.67	897917	5.70	40518546
Replicate 2	1.69	1375340	1.67	867560	5.69	38784562
Replicate 3	1.70	1422543	1.67	917454	5.70	40371113
Replicate 4	1.70	1396646	1.67	897930	5.70	43114966
Replicate 5	1.70	1174149	1.67	727069	5.70	35227544
Replicate 6	1.70	1327793	1.68	882462	5.70	40293599
AVG	1.70	1355718	1.67	865065	5.70	39718388
SD	0.003	96971	0.005	69650	0.002	2606669
%RSD	**0.3**	**7**	**0.3**	**8**	**0.05**	**7**

Control Study – Process and Spike Controls

Summary
A series of control studies were performed to evaluate assay accuracy and identify possible processing artifacts. A 5 ng/mL DXR spike into 1 μg/mL liposomal DXR in plasma was recovered within 20% of theoretical (an encapsulated:unencapsulated ratio of 200). Double processing and organic solvent stable isotope spike of liposomal plasma samples did not alter formulation stability.

Design and Methods
A double spin control was performed to identify the potential for processing-induced drug release, which could inflate unencapsulated drug release estimates. A free doxorubicin spike control was performed to assess the accuracy of the unencapsulated drug estimation. Lastly, an organic spike control was included to ensure the organic solvent used to solubilize the $^{13}C^2H_3$-DXR stable isotope tracer did not disrupt the formulation.

Double Spin Control
A plasma sample containing liposomal doxorubicin underwent two successive filtrations, in order to exclude artificial sample processing induced drug release. A Sun Pharma sample (Lot JKR0494A) was spiked into human plasma at a concentration of 1 μg/mL, and 0.1 μg/mL $^{13}C^2H_3$-DXR stable isotope tracer was added. The sample was incubated at 37°C for 2 hours. Following incubation, the sample was spun at 6000x*g* for 10 min, and 50 μL of the filtrate was collected for analysis. The retentate was transferred to a new filter tube and spun again for 10 min at 6000x*g*. 50 μL of the filtrate was again collected for analysis. A 50 μL unspun plasma sample was also collected for sample concentration measurement.

Spike Control
A plasma sample containing the liposomal formulation was spiked with free doxorubicin, and spike recovery was calculated. Accurate spike recovery supports the accuracy of the unencapsulated drug measurement. A Sun Pharma sample (Lot JKR0494A) was spiked into human plasma at a concentration of 1 μg/mL, and 0.1 μg/mL $^{13}C^2H_3$-DXR stable isotope tracer was added. Free DXR at 5 ng/mL was also spiked into the sample. After incubating at 37°C for 2 hours, samples were spun at 6000x*g* for 10 min. 50 μL of the filtrate was collected for analysis. A 50 μL unspun plasma sample was also collected for sample concentration measurement.

No Organic Control
A control for the organic spike was performed by preparing two identical formulation-containing plasma samples. One sample received the stable isotope tracer spike, the other did not. If the organic spike has no effect on the formulation stability, the concentration of doxorubicin in the filtrate of both samples should be identical, ideally within 15% of each other.

The Sun Pharma formulation (Lot JKR0494A) was spiked into two sets of human plasma at a concentration of 1 μg/mL in triplicate. To one set, no $^{13}C^2H_3$-DXR stable isotope tracer was added, while to the other set, the usual 0.1 μg/mL $^{13}C^2H_3$-DXR stable isotope tracer was added. The sample was incubated at 37°C for 2 hours. Following incubation, the sample was spun at 6000x*g* for 10 min, and 50 μL of the filtrate was collected for analysis. A 50 μL unspun plasma sample was also collected for sample concentration measurement.

Results and Discussion

Double processing of the Sun Pharma liposome plasma samples did not alter the unencapsulated DXR estimate, supporting the fact that the centrifugation/filtration step does not alter formulation stability (**Tables II-27 and II-28, Figure II-10**). The 5 ng/mL spike recovery was within 20% of theoretical (**Tables II-27 and II-28, Figure II-10**). The organic stable isotope spike did not change the protein binding estimate (within 2%), confirming that the organic stable isotope spike does not alter formulation stability in this example (**Tables II-27 and II-28**).

Table II-27. Double Spin, Spike Recovery and No-Organic Spike Controls Analytical Data. Presented are the analytical data for the double spin, spike recovery and no organic controls. The % protein binding, encapsulated and unencapsulated measurements were calculated as described in the SITUA Design and Methods in Section I. The concentration of Sun Pharma liposome was 1 µg DXR/mL in plasma, the DXR spike was 5 ng/mL, and the incubation parameters were 37°C for 2 hours.

	Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR	% Bound	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13	% Bound DXR_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG	SD
			= Filtrate DXR / Reservoir DXR *100	= 100 – %Unbound DXR			= Filtrate DXR_C13 / Reservoir DXR_C13 * 100	= 100 – %Unbound C13	= Fil DXR/ (1-(%Bound DXR_C13/100))	= Reservoir DXR- Unencapsulated	= (Unencapsulated/ Reservoir DXR) *100		
Sun Pharma Spin 1	0.8	953	0.1	99.9	10.0	71	14.0	86.0	5.5	947	0.6	0.7	0.1
	1.6	1068	0.2	99.8	14.9	71	20.8	79.2	7.8	1060	0.7		
	0.9	1119	0.1	99.9	9.4	76	12.5	87.5	7.3	1111	0.6		
Sun Pharma Spin 2	1.5	953	0.2	99.8	16.2	71	22.8	77.2	6.7	946	0.7	0.6	0.1
	1.6	1068	0.1	99.9	15.2	71	21.2	78.8	7.5	1061	0.7		
	1.3	1119	0.1	99.9	18.8	76	24.9	75.1	5.1	1114	0.5		
Sun Pharma + 5 ng DXR spike	3.1	1084	0.3	99.7	15.7	73	21.4	78.6	14.3	1069	1.3	1.2	0.2
	2.6	927	0.3	99.7	14.5	71	20.3	79.7	12.6	914	1.4		
	2.5	1172	0.2	99.8	16.2	76	21.5	78.5	11.8	1160	1.0		
Sun Pharma No Organic	1.2	1232	0.1	99.9									
	1.3	1092	0.1	99.9									
	1.0	1099	0.1	99.9									

Table II-28. Double Spin, Spike Recovery and No-Organic Controls. Presented are the unencapsulated DXR concentrations and percent protein binding values of the normoisotopic drug, for the double spin and spike recovery controls, and the no organic controls, respectively. The unencapsulated DXR measurements and percent protein binding were calculated as described in the SITUA Design and Methods in Section I. The concentration of Sun Pharma liposome was 1 μg DXR/mL in plasma, the DXR spike was 5 ng/mL, and the incubation parameters were 37°C for 2 hours (N = 3, mean ± SD).

	AVG Unencapsulated DXR (ng/mL)	SD
Spin 1	6.9	1.2
Spin 2	6.4	1.2
5 ng/mL Spike DXR	12.9	1.3
Spiked Difference	6.0	
	% Protein Binding	**SD**
With Organic SI	99.7	0.06
No Organic SI	99.9	0.02
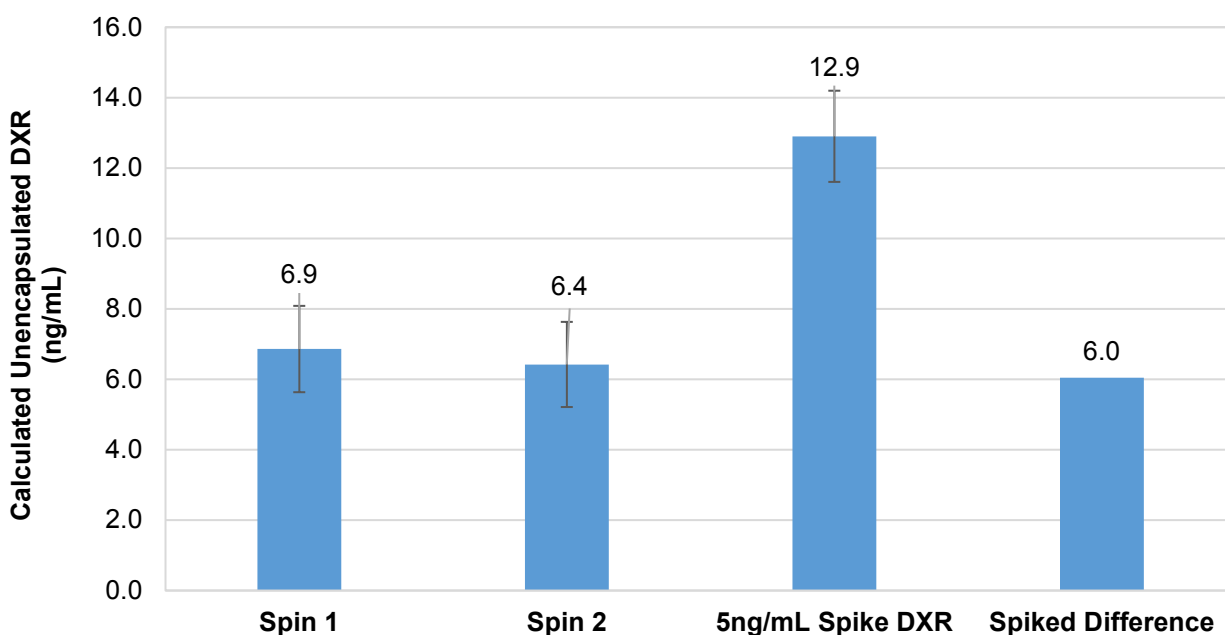
Figure II-10. Double Spin and Spike Recovery Controls. Displayed are the unencapsulated DXR concentrations, calculated from Eq. ii, for the first (Spin 1) and second spin (Spin 2) of the double processing controls and the 5 ng/mL spike DXR control. The "Spiked Difference" is the difference between the 5 ng/mL spike and Spin 1 controls. The concentration of liposome was 1 μg DXR/mL in plasma, and the incubation parameters were 37°C for 2 hours (N = 3, mean ± SD).

Control Study – Reduced Sample Volumes

Summary

The in vitro drug release studies used 400 µL of plasma in the filter apparatus. However, plasma from the in vivo studies will be limited due to restrictions on the blood volumes permitted to be collected over the 4-day study period. For the in vivo studies, only 150 µL of plasma will be used in the filter apparatus instead of 400 µL. This control study confirmed that the reduced sample volume did not influence the assay performance.

Design and Methods

The following study compared results using 150 µL vs. 400 µL of plasma sample in the filter apparatus. The study utilized free DXR and a single lot of Sun Pharma liposome (Lot JKR0494A). Human blood was collected in K_2EDTA tubes and pooled from 6 donors. Blood was spun at 2500x*g* for 10 min to collect plasma. HEPES buffer was added at 50 µL/2 mL plasma to maintain a pH of 7.4. Plasma in glass vials was spiked with samples (free DXR or Sun Pharma liposome) at final drug concentrations of 0.5 µg/mL, 1 µg/mL, and 5 µg/mL DXR in triplicate. All samples were then spiked with 0.1 µg/mL $^{13}C^2H_3$-DXR (stable isotope tracer). The incubation period was for 2 hours at 37ºC. All other experimental procedures were the same as described previously in Section I.

Results and Discussion

The results of the drug release study for the free DXR controls and Sun Pharma liposome (Lot JKR0494A) were not significantly different at any of the drug concentration levels between the 150 or 400 µL plasma volume groups (**Tables II-29 and II-30, Figures II-11 and II-12**).

Table II-29. Free Doxorubicin HCl. Presented are the % release results for the plasma volume comparison study at 150 and 400 µL (Mean ± SD, *N=3*).

Sample Volume	Conc.	Avg % Release	SD	% CV
400 µL	0.5 µg/mL	103.4	1.9	1.9
	1 µg/mL	104.5	1.1	1.0
	5 µg/mL	107.0	1.8	1.7
150 µL	0.5 µg/mL	104.5	1.9	1.8
	1 µg/mL	105.8	4.1	3.9
	5 µg/mL	106.5	1.2	1.1
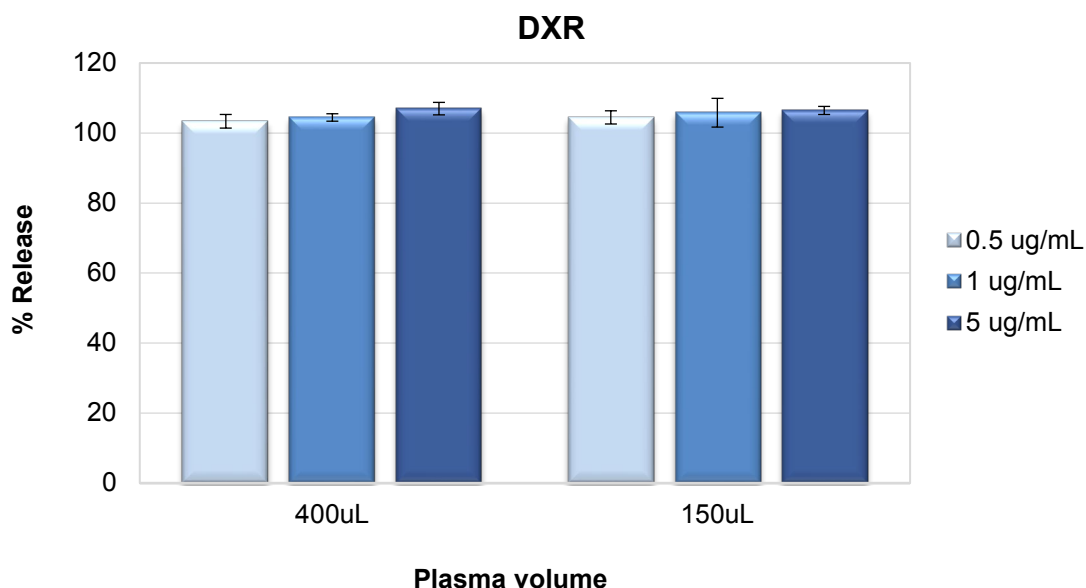
Figure II-11. Free Doxorubicin HCl. Presented are the % release results for the plasma volume comparison study (Mean ± SD, *N=3*).

Table II-30. Sun Pharma, Lot A (JKR0494A). Presented are the % release results of the plasma volume comparison study (Mean ± SD, *N=3*).

Sample Volume	Conc.	Avg % Release	SD	% CV
400 µL	0.5 µg/mL	0.51	0.05	10.5
	1 µg/mL	0.65	0.08	12.1
	5 µg/mL	0.45	0.10	21.5
150 µL	0.5 µg/mL	0.53	0.002	0.4
	1 µg/mL	0.59	0.03	5.1
	5 µg/mL	0.46	0.10	21.7

Sun Pharma (JKR0494A)
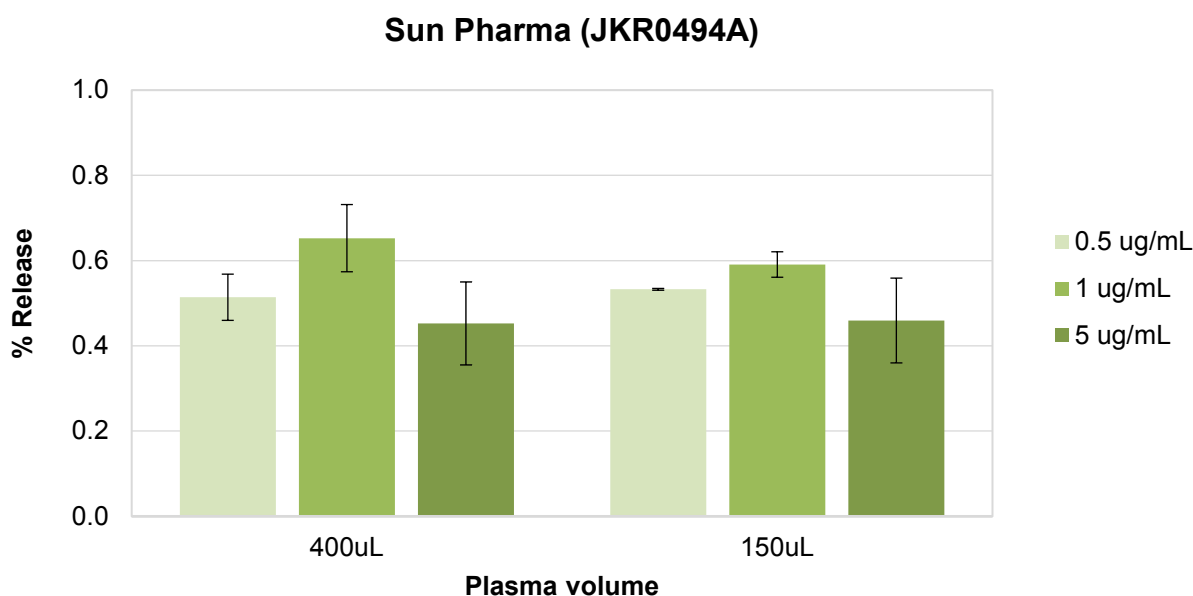
Table II-12. Sun Pharma, Lot A (JKR0494A). Presented are the % release results of the plasma volume comparison study (Mean ± SD, *N=3*).

Control Study – Speed Vac vs. Turbo Vap

Summary
Low levels of cross contamination were initially seen in blank plasma and protein-free plasma (PFP) samples which interfered with the low end of the standard curves and low QC. The cross contamination was traced to the turbo evaporator used to concentrate the samples following the protein precipitation method. The turbo evaporator was replaced with a speed vacuum for the sample concentration step. A comparison of standard curves using the two sample concentrating methods was performed in order to demonstrate that the change in apparatus minimized the cross-contamination problem.

Design and Methods
Two sets of DXR/^{13}C^{2}H$_3$-DXR combined standard curves were prepared in human plasma and human PFP in duplicate. One set of calibrations was dried in the turbo evaporator while the other set was dried in the speed vacuum.

Results and Discussion
Although human plasma and PFP standard curve accuracy was similar using the two concentration methods, the samples which were dried in the speed vacuum showed decreased cross contamination in both blank matrices compared to the turbo evaporator, supporting the use of the speed vacuum method (**Tables II-31 and II-32**).

Table II-31. Speed Vac. vs. Turbo Vap. Plasma Standard Curves. Presented below are the (**A**) area units and (**B**) calculated accuracy for the DXR and DXR_C13 standard curves prepared using either the turbo evaporator or speed vacuum sample concentrating methods.

A. Area Units for the DXR and DXR_C13 Standard Curves.

Sample	DXR Area Units		% Reference	DXR_C13 Area Units		% Reference
	Turbo Vap	Speed Vac	= (Turbo Vap / Speed Vac)*100	Turbo Vap	Speed Vac	= (Turbo Vap / Speed Vac)*100
Blank	4264	0	--	18528	5915	313
Blank	4661	9252	50	88632	0	--
Blank + ISTD	1561	0	--	24672	10704	230
Blank + ISTD	3221	487	661	2845	3784	75
10.0 ng/mL	1827364	1806550	101	1580237	1415196	112
10.0 ng/mL	1862153	1899205	98	1462425	1523934	96
50 ng/mL	9132323	9782509	93	7484117	7835102	96
50 ng/mL	10176223	9883760	103	8038872	7938823	101
100 ng/mL	19895954	20287352	98	15587255	16236242	96
100 ng/mL	20892269	20813350	100	16393962	16867286	97
500 ng/mL	103972158	105602418	98	84270883	84689484	100
500 ng/mL	116029278	105131102	110	92279654	81099597	114
1000 ng/mL	217950786	21193010	1028	174341019	167760019	104
1000 ng/mL	226084808	201104409	112	177079231	161156675	110
5000 ng/mL	1036559229	1003458246	103	825455668	811141988	102
5000 ng/mL	1096706952	982188376	112	859304577	769383845	112
10000 ng/mL	1950344240	1913300422	102	1545841639	1518949218	102
10000 ng/mL	1913206776	1873846576	102	1537070969	1547543136	99

B. Calculated Accuracy for the DXR and DXR_C13 Standard Curves.

Conc.	DXR Turbo Vap		DXR Speed Vac		DXR_C13 Turbo Vap		DXR_C13 Speed Vac	
	ng/mL	% Accuracy Deviation	ng/mL	% Accuracy Deviation	ng/mL	% Accuracy Deviation	ng/mL	% Accuracy Deviation
10.0 ng/mL	8.8	-12	8.5	-15	9.4	-6	8.4	-16
10.0 ng/mL	9.0	-10	8.7	-13	8.6	-14	8.9	-11
50 ng/mL	47	-7	49	-3	48	-4	49	-2
50 ng/mL	49	-2	45	-9	48	-4	46	-10
100 ng/mL	105	5	105	5	103	3	105	5
100 ng/mL	101	1	104	4	99	-1	105	5
500 ng/mL	475	-5	530	6	484	-3	529	6
500 ng/mL	557	11	534	7	557	11	533	7
1000 ng/mL	1095	9	1081	8	1101	10	1068	7
1000 ng/mL	1082	8	1084	8	1066	7	1081	8
5000 ng/mL	5227	5	4879	-2	5236	5	4908	-2
5000 ng/mL	5089	2	5487	10	5015	0.3	5321	6
10000 ng/mL	9929	-1	9569	-4	9898	-1	9452	-6
10000 ng/mL	9546	-5	9834	-2	9646	-4	10105	1

Table II-32. Speed Vac. vs. Turbo Vap. Protein-Free Plasma Standard Curves. Presented below are the (**A**) area units and (**B**) calculated accuracy for the DXR and DXR_C13 standard curves prepared using either the turbo evaporator or speed vacuum sample concentrating methods.

A. Area Units for the DXR and DXR_C13 Standard Curves.

Sample	DXR		% Reference = (Turbo Vap / Speed Vac)*100	DXR_C13		% Reference = (Turbo Vap / Speed Vac)*100
	Turbo Vap	Speed Vac		Turbo Vap	Speed Vac	
Blank	2102	0	--	12623	1226	1030
Blank	1726	616	280	26458	8327	318
Blank + ISTD	0	0	--	1159	0	--
Blank + ISTD	410	519	79	16579	1273	1302
0.100 ng/mL	10892	10952	99	9823	9715	101
0.100 ng/mL	13894	12638	110	10310	8047	128
0.50 ng/mL	79673	85004	94	66727	56319	118
0.50 ng/mL	73190	65808	111	64317	58435	110
1.00 ng/mL	185918	183717	101	149688	126056	119
1.00 ng/mL	176653	190976	93	138744	132644	105
5.0 ng/mL	985191	1000651	98	767353	722454	106
5.0 ng/mL	969904	1032475	94	770293	709149	109
10.0 ng/mL	1950679	2162321	90	1534465	1530821	100
10.0 ng/mL	1720746	2029236	85	1348068	1457455	92
100 ng/mL	21887731	22402686	98	17133525	16492170	104
100 ng/mL	20236630	21286870	95	15888430	16557243	96
1000 ng/mL	207206335	227812725	91	164815666	180291867	91
1000 ng/mL	219224551	237735307	92	174505883	181566267	96

B. Calculated Accuracy for the DXR and DXR_C13 Standard Curves.

Conc.	DXR Turbo Vap		DXR Speed Vac		DXR_C13 Turbo Vap		DXR_C13 Speed Vac	
	ng/mL	% Accuracy Deviation	ng/mL	% Accuracy Deviation	ng/mL	% Accuracy Deviation	ng/mL	% Accuracy Deviation
0.100 ng/mL	0.094	-6	0.098	-2	0.098	-2	0.105	5
0.100 ng/mL	0.109	9	0.105	5	0.104	4	0.097	-3
0.50 ng/mL	0.41	-17	0.499	-0.1	0.43	-15	0.52	5
0.50 ng/mL	0.46	-8	0.462	-8	0.50	-0.2	0.45	-10
1.00 ng/mL	1.10	9	0.95	-5	1.10	10	1.00	-0.5
1.00 ng/mL	1.10	9	0.95	-5	1.07	7	0.93	-7
5.0 ng/mL	4.8	-3	5.1	1	4.7	-6	5.2	4
5.0 ng/mL	4.5	-10	4.7	-6	4.5	-10	4.8	-5
10.0 ng/mL	10.3	3	10.3	3	10.2	2	9.7	-3
10.0 ng/mL	10.9	9	9.5	-5	10.7	7	8.7	-13
100 ng/mL	97	-3	106	6	96	-4	108	8
100 ng/mL	103	3	99.5	-0.5	101	1	103	3
1000 ng/mL	1030	3	1087	9	1030	3	1095	10
1000 ng/mL	1030	3	1081	8	1031	3	1058	6

III. In Vitro Doxil Lot Comparison

Intra-Day Three Lot Comparison in Human Plasma at 37°C

Summary

The objective of this study was to compare the in vitro drug release of three lots of Doxil in human plasma at 37°C over a 6 hr period. All lots had similar drug release profiles over the 6 hr period, although some instances of statistically significant differences were observed between lots.

Design and Methods

Three lots of Doxil, 600220P1, 600120P1, and 600520P1, also denoted as lots A, B, and C, respectively, were evaluated for drug release in human plasma at 37°C over a 6 hr period, according to the SITUA method described in Section I. All three lots were run on a single day, and free doxorubicin HCl at identical concentrations and time points was included as a control. The human plasma and protein-free plasma DXR and DXR_C13 standard calibration curves used for this study are also provided.

Results and Discussion

The DXR and DXR_C13 standard calibration curves used for this study are presented in **Tables III-1 to III-4 and Figures III-1 to III-4**. The free doxorubicin HCl controls averaged between 98-107% of theoretical for all concentrations and time points (**Tables III-5 and III-9, and Figure III-5**). The Doxil drug release was similar for Lot's A-C, at approximately 1% release over the 6 hr period, without a clear temporal trend (**Tables III-6 to III-8 and III-10 to III-12, and Figures III-6 to III-9**).

Table III-1. DXR Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	1214791	10.6	106
10.0 ng/mL	990272	11.0	110
50 ng /mL	5898490	45	90
50 ng /mL	6077639	53	105
100 ng /mL	12180354	101	101
100 ng /mL	10709406	92	92
500 ng /mL	59486746	537	107
500 ng /mL	58113007	477	95
1000 ng /mL	125338586	1030	103
1000 ng /mL	122701815	927	93
5000 ng /mL	647450098	5051	101
5000 ng /mL	585539573	4696	94
10000 ng /mL	1210552114	10041	100
10000 ng /mL	1325663970	10249	102
QC			
QCL	991142	11.0	110
QCL	941145	10.9	109
QCM	11320401	94	94
QCM	9261144	93	93
QCH	119298643	972	97
QCH	111115726	1034	103

DXR_PRM
Y = -0.00900378+0.00293757*X   R^2 = 0.9989   W: 1/X
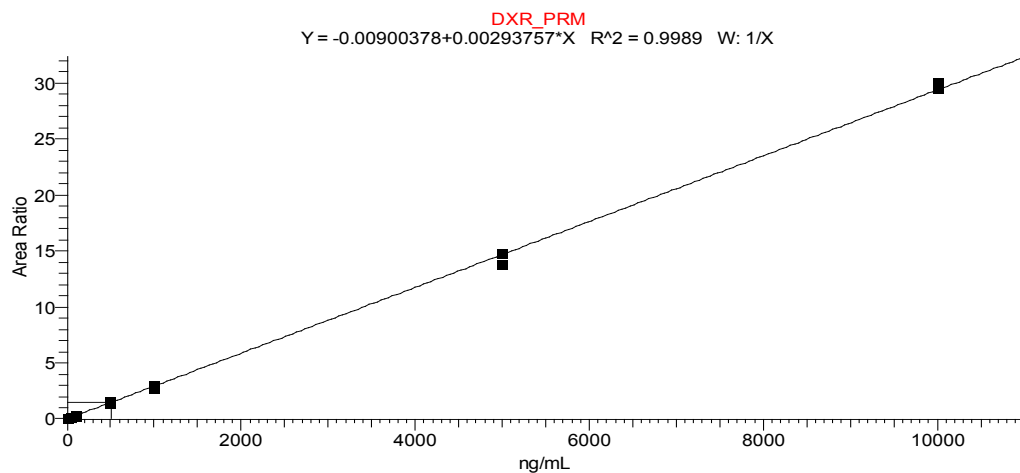
Figure III-1. DXR Plasma Standard Curve.

Table III-2. DXR_C13 Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	953683	9.6	96
10.0 ng/mL	867327	11.0	110
50 ng /mL	5080267	49	99
50 ng /mL	4656664	52	103
100 ng /mL	9555737	102	102
100 ng /mL	8140534	90	90
500 ng /mL	44942822	531	106
500 ng /mL	45557932	489	98
1000 ng /mL	97811351	1052	105
1000 ng /mL	94832429	937	94
5000 ng /mL	490307432	5012	100
5000 ng /mL	450513605	4734	95
10000 ng /mL	920971766	10011	100
10000 ng /mL	1010610327	10240	102
QC			
QCL	758415	9.8	98
QCL	740809	10.0	100
QCM	9124959	98	98
QCM	7244110	94	94
QCH	91491206	976	98
QCH	85832639	1046	105

DXR_C13_PRM
$Y = -0.00415256+0.00224131*X$ $R^2 = 0.9991$ W: 1/X
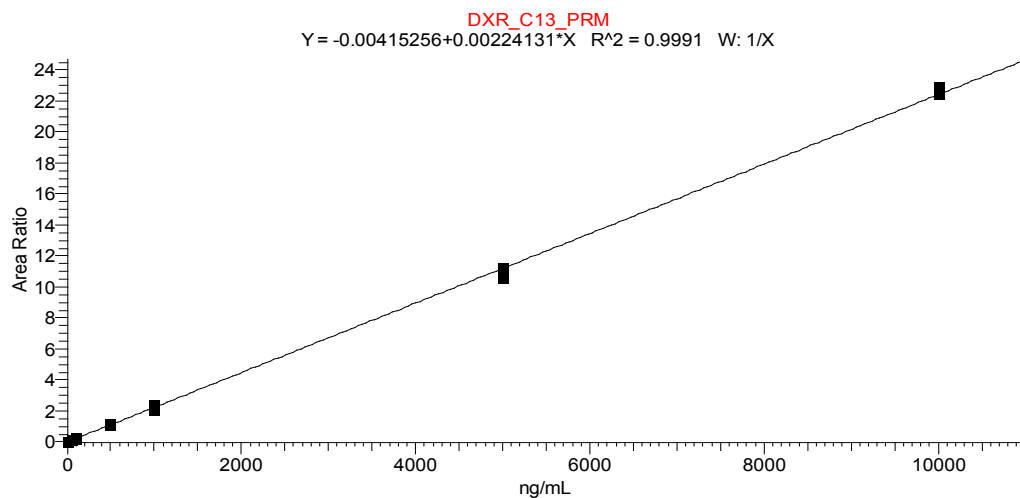
Figure III-2. DXR_C13 Plasma Standard Curve.

Table III-3. DXR Protein-Free Plasma Standard Curve.

	Area	**Conc. (ng/mL)**	**% Accuracy**
0.100 ng/mL	9668	0.103	103
0.100 ng/mL	10089	0.102	102
0.50 ng/mL	68274	0.46	91
0.50 ng/mL	73048	0.46	92
1.00 ng/mL	162792	0.91	91
1.00 ng/mL	164230	0.93	93
5.00 ng/mL	923452	4.83	97
5.00 ng/mL	953707	4.79	96
10.0 ng/mL	1986107	10.2	102
10.0 ng/mL	1886216	10.4	104
100.0 ng/mL	21430704	108.2	108
100.0 ng/mL	21137612	108.0	108
1000.0 ng/mL	229930412	1088.4	109
1000.0 ng/mL	233263053	1055.5	106
QC			
QCL	11982	0.110	110
QCL	10485	0.108	108
QCM	160661	0.96	96
QCM	160703	0.97	97
QCH	1960467	9.4	94
QCH	1907367	10.0	100

DXR_PRM
$Y = -0.000263962 + 0.00501164 \cdot X$ $R^2 = 0.9945$ W: $1/X^2$
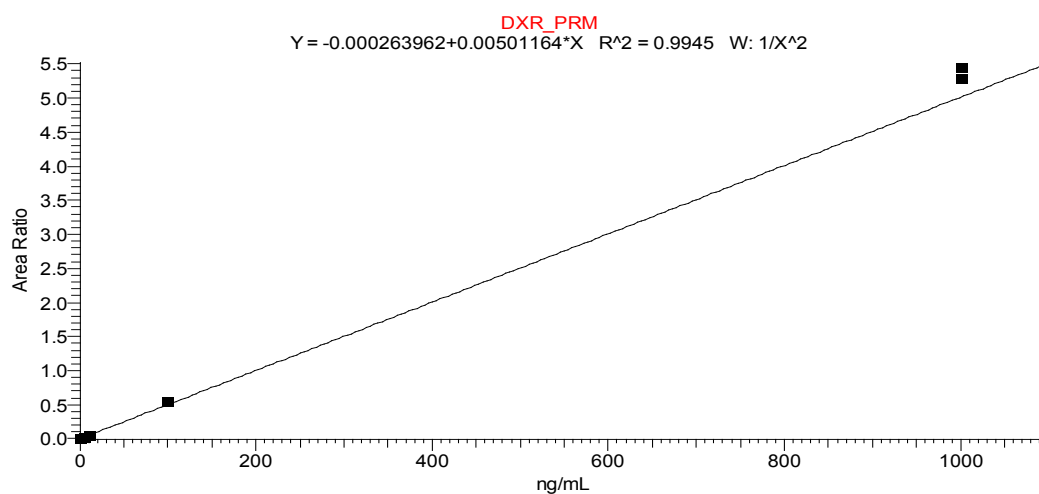
Figure III-3. DXR Protein-Free Plasma Standard Curve.

Table III-4. DXR_C13 Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	9551	0.108	108
0.100 ng/mL	8047	0.956	96
0.50 ng/mL	56489	0.48	95
0.50 ng/mL	58689	0.47	93
1.00 ng/mL	126056	0.90	90
1.00 ng/mL	132644	0.96	96
5.00 ng/mL	720213	4.87	97
5.00 ng/mL	709149	4.61	92
10.0 ng/mL	1530821	10.1	101
10.0 ng/mL	1457455	10.4	104
100.0 ng/mL	16346351	106.9	107
100.0 ng/mL	16149844	106.9	107
1000.0 ng/mL	180201568	1104.9	111
1000.0 ng/mL	174833974	1024.7	103
QC			
QCL	8736	0.098	98
QCL	8474	0.103	103
QCM	122249	0.93	93
QCM	124655	0.97	97
QCH	1526369	9.5	95
QCH	1474958	10.0	100

DXR_C13_PRM
Y = -0.000170892+0.00386905*X   R^2 = 0.9947   W: 1/X^2
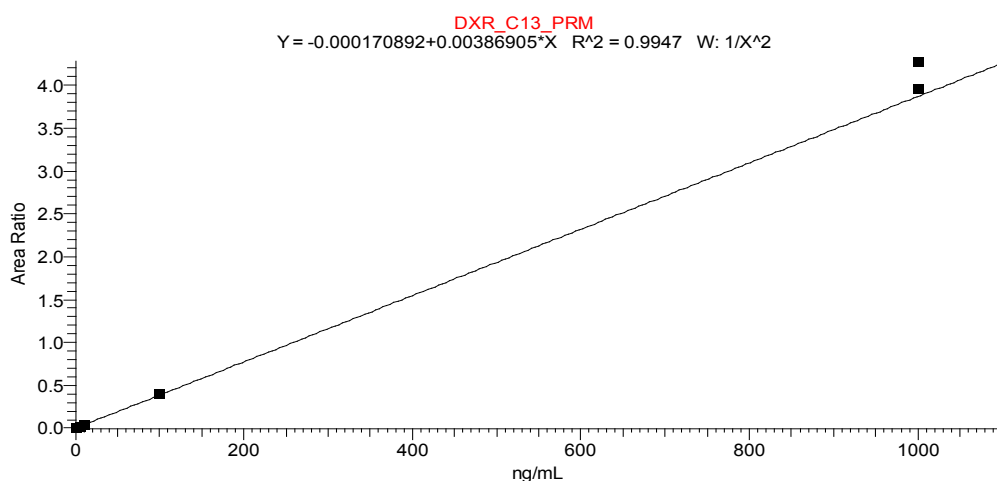
Table III-4. DXR_C13 Protein-Free Plasma Standard Curve.

Table III-5. Free Doxorubicin HCl Control Analytical Data. Presented are the analytical data for the free doxorubicin HCl controls. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 – %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/(1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	60.9	511	11.9	88.1	11.7	99	11.9	88.1	513.7	-3	100.5	99.7	2.0
		50.4	423	11.9	88.1	12.3	101	12.2	87.8	412.4	11	97.4		
		56.1	506	11.1	88.9	10.7	97	11.0	89.0	511.4	-6	101.2		
	1 µg/mL	154.1	1024	15.0	85.0	14.4	95	15.2	84.8	1012.2	12	98.9	101.1	2.4
		136.5	1031	13.2	86.8	12.4	94	13.1	86.9	1040.4	-9	100.9		
		148.6	1021	14.6	85.4	13.5	96	14.0	86.0	1057.7	-37	103.6		
	5 µg/mL	706.2	5142	13.7	86.3	12.7	92	13.8	86.2	5120.2	22	99.6	102.8	3.0
		816.3	5251	15.5	84.5	14.4	97	14.7	85.3	5545.0	-294	105.6		
		783.9	5354	14.6	85.4	14.3	101	14.2	85.8	5530.3	-176	103.3		
2 hr	0.5 µg/mL	53.7	503	10.7	89.3	10.5	97	10.8	89.2	496.6	7	98.7	97.8	0.9
		46.1	400	11.5	88.5	11.2	95	11.9	88.1	387.6	13	96.9		
		49.9	440	11.3	88.7	9.6	83	11.6	88.4	430.1	10	97.7		
	1 µg/mL	124.1	1007	12.3	87.7	11.4	94	12.1	87.9	1026.4	-19	101.9	102.0	1.2
		116.3	924	12.6	87.4	10.4	86	12.2	87.8	954.4	-30	103.3		
		131.8	1015	13.0	87.0	12.1	94	12.9	87.1	1023.9	-9	100.8		
	5 µg/mL	642.7	4844	13.3	86.7	11.4	89	12.9	87.1	4997.0	-153	103.2	99.7	3.2
		724.6	5013	14.5	85.5	13.5	91	14.9	85.1	4851.0	162	96.8		
		724.6	4942	14.7	85.3	13.5	91	14.8	85.2	4894.4	48	99.0		
6 hr	0.5 µg/mL	23.2	293	7.9	92.1	4.5	63	7.2	92.8	320.3	-28	109.5	107.2	2.1
		23.2	272	8.5	91.5	5.6	69	8.1	91.9	286.1	-14	105.3		
		32.2	343	9.4	90.6	6.2	70	8.8	91.2	367.2	-24	106.9		
	1 µg/mL	71.9	822	8.8	91.2	6.7	77	8.6	91.4	833.0	-11	101.3	105.3	3.5
		65.0	797	8.2	91.8	6.1	79	7.6	92.4	849.6	-53	106.6		
		104.0	993	10.5	89.5	9.5	98	9.7	90.3	1072.7	-80	108.0		
	5 µg/mL	315.0	3604	8.7	91.3	5.7	70	8.1	91.9	3910.8	-307	108.5	105.6	3.8
		444.2	3923	11.3	88.7	7.9	75	10.6	89.4	4197.1	-274	107.0		
		577.0	5362	10.8	89.2	10.6	100	10.6	89.4	5435.7	-74	101.4		

Table III-6. Doxil, Lot A (600220P1) Drug Release Analytical Data. Presented are the analytical data for Doxil Lot A (600220P1). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 – %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	0.7	681	0.1	99.9	10.7	97	11.0	89.0	6.7	674	1.0	1.3	0.3
		0.7	645	0.1	99.9	9.0	128	7.1	92.9	9.5	636	1.5		
		1.0	639	0.2	99.8	10.1	93	10.8	89.2	9.3	630	1.5		
	1 µg/mL	1.7	1372	0.1	99.9	10.1	87	11.5	88.5	14.9	1357	1.1	1.1	0.2
		1.8	1221	0.1	99.9	9.3	84	11.0	89.0	16.3	1205	1.3		
		1.3	1356	0.1	99.9	9.2	94	9.8	90.2	13.2	1343	1.0		
	5 µg/mL	4.3	5815	0.1	99.9	9.0	86	10.5	89.5	41.1	5774	0.7	0.7	0.02
		4.2	5696	0.1	99.9	8.7	77	11.3	88.7	37.6	5659	0.7		
		5.3	6149	0.1	99.9	10.2	81	12.6	87.4	42.5	6106	0.7		
2 hr	0.5 µg/mL	0.9	695	0.1	99.9	10.3	82	12.5	87.5	7.2	688	1.0	1.2	0.2
		0.9	664	0.1	99.9	10.1	75	13.5	86.5	6.9	657	1.0		
		1.0	633	0.2	99.8	9.0	76	11.8	88.2	8.6	624	1.4		
	1 µg/mL	2.2	1325	0.2	99.8	10.5	79	13.3	86.7	16.7	1309	1.3	1.2	0.1
		2.1	1201	0.2	99.8	9.7	72	13.5	86.5	15.9	1186	1.3		
		1.6	1306	0.1	99.9	9.1	77	11.8	88.2	13.7	1292	1.1		
	5 µg/mL	7.3	5652	0.1	99.9	10.7	68	15.6	84.4	46.7	5605	0.8	0.9	0.1
		7.1	5625	0.1	99.9	10.3	70	14.6	85.4	48.7	5576	0.9		
		7.7	4485	0.2	99.8	10.1	56	17.9	82.1	43.0	4442	1.0		
6 hr	0.5 µg/mL	0.9	792	0.1	99.9	8.1	79	10.3	89.7	8.3	783	1.1	1.1	0.2
		0.9	766	0.1	99.9	8.3	72	11.6	88.4	7.9	758	1.0		
		1.1	717	0.2	99.8	8.2	71	11.6	88.4	9.4	708	1.3		
	1 µg/mL	2.3	1501	0.2	99.8	8.9	73	12.1	87.9	18.9	1482	1.3	1.2	0.05
		1.9	1306	0.1	99.9	7.4	66	11.2	88.8	16.7	1289	1.3		
		1.9	1293	0.1	99.9	8.1	66	12.3	87.7	15.3	1278	1.2		
	5 µg/mL	7.4	5821	0.1	99.9	7.9	60	13.2	86.8	55.9	5765	1.0	0.9	0.05
		7.1	5931	0.1	99.9	8.1	61	13.3	86.7	53.0	5878	0.9		
		7.3	6286	0.1	99.9	7.8	65	11.9	88.1	61.6	6224	1.0		

Table III-7. Doxil, Lot B (600120P1) Drug Release Analytical Data. Presented are the analytical data for Doxil Lot B (600120P1). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 – %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/(1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	0.9	684	0.1	99.9	10.2	85	12.0	88.0	7.6	677	1.1	1.4	0.3
		1.3	691	0.2	99.8	10.3	83	12.3	87.7	10.3	680	1.5		
		1.6	697	0.2	99.8	11.8	84	14.0	86.0	11.3	686	1.6		
	1 µg/mL	1.2	1331	0.1	99.9	5.5	88	6.3	93.7	19.8	1312	1.5	1.3	0.2
		2.5	1344	0.2	99.8	13.2	93	14.2	85.8	18.0	1326	1.3		
		1.6	1251	0.1	99.9	9.9	80	12.3	87.7	13.2	1238	1.1		
	5 µg/mL	5.5	5264	0.1	99.9	9.9	73	13.6	86.4	40.4	5224	0.8	0.8	0.02
		5.7	5523	0.1	99.9	10.2	73	13.9	86.1	41.2	5481	0.7		
		7.1	6566	0.1	99.9	13.1	87	15.0	85.0	47.5	6518	0.7		
2 hr	0.5 µg/mL	1.2	538	0.2	99.8	9.5	66	14.5	85.5	8.5	529	1.6	1.9	0.3
		1.6	499	0.3	99.7	10.4	64	16.2	83.8	9.7	490	1.9		
		1.5	586	0.3	99.7	9.2	79	11.6	88.4	12.7	573	2.2		
	1 µg/mL	1.5	1159	0.1	99.9	8.8	76	11.6	88.4	13.0	1146	1.1	1.5	0.3
		2.2	1164	0.2	99.8	9.3	77	11.9	88.1	18.6	1146	1.6		
		2.2	1014	0.2	99.8	10.1	78	13.0	87.0	16.9	997	1.7		
	5 µg/mL	7.3	6049	0.1	99.9	9.9	77	12.7	87.3	57.4	5991	0.9	1.1	0.1
		7.8	5559	0.1	99.9	9.5	82	11.6	88.4	66.7	5492	1.2		
		7.8	5334	0.1	99.9	10.3	71	14.4	85.6	53.7	5280	1.0		
6 hr	0.5 µg/mL	1.4	746	0.2	99.8	9.2	70	13.2	86.8	10.2	736	1.4	1.5	0.2
		1.3	701	0.2	99.8	8.1	66	12.3	87.7	10.3	691	1.5		
		1.5	700	0.2	99.8	8.2	67	12.3	87.7	11.7	689	1.7		
	1 µg/mL	1.8	1422	0.1	99.9	8.4	72	11.6	88.4	15.1	1406	1.1	1.2	0.1
		2.2	1491	0.1	99.9	8.2	72	11.5	88.5	19.1	1472	1.3		
		2.2	1473	0.1	99.9	8.9	71	12.5	87.5	17.5	1455	1.2		
	5 µg/mL	7.1	6240	0.1	99.9	7.7	65	11.9	88.1	59.4	6180	1.0	1.0	0.1
		8.5	5863	0.1	99.9	8.4	66	12.7	87.3	66.8	5796	1.1		
		7.4	6327	0.1	99.9	7.9	65	12.3	87.7	60.7	6267	1.0		

Table III-8. Doxil, Lot C (600520P1) Drug Release Analytical Data. Presented are the analytical data for Doxil Lot C (600520P1). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 – %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	0.7	701	0.1	99.9	10.0	104	9.6	90.4	7.5	693	1.1	1.2	0.2
		0.9	674	0.1	99.9	10.0	105	9.5	90.5	9.0	665	1.3		
		0.9	621	0.1	99.9	10.4	96	10.8	89.2	8.2	613	1.3		
	1 µg/mL	1.2	1261	0.1	99.9	11.3	95	11.8	88.2	10.3	1250	0.8	0.9	0.2
		1.6	1407	0.1	99.9	10.5	107	9.8	90.2	16.3	1391	1.2		
		1.1	1448	0.1	99.9	9.8	106	9.3	90.7	12.3	1435	0.9		
	5 µg/mL	4.1	6398	0.1	99.9	9.8	103	9.5	90.5	42.8	6355	0.7	0.7	0.04
		4.7	6083	0.1	99.9	10.9	104	10.4	89.6	44.7	6038	0.7		
		4.3	6433	0.1	99.9	10.4	105	9.8	90.2	43.6	6389	0.7		
2 hr	0.5 µg/mL	0.8	630	0.1	99.9	9.7	90	10.7	89.3	7.2	623	1.1	1.3	0.1
		0.9	638	0.1	99.9	10.0	98	10.2	89.8	8.6	629	1.4		
		0.9	612	0.2	99.8	10.3	91	11.3	88.7	8.4	603	1.4		
	1 µg/mL	1.2	1237	0.1	99.9	10.2	92	11.1	88.9	11.0	1226	0.9	1.1	0.2
		1.6	1241	0.1	99.9	10.1	94	10.8	89.2	15.2	1226	1.2		
		1.6	1188	0.1	99.9	11.5	89	12.9	87.1	12.3	1176	1.0		
	5 µg/mL	6.1	5910	0.1	99.9	12.2	92	13.3	86.7	45.5	5865	0.8	0.8	0.02
		5.3	5749	0.1	99.9	10.6	92	11.5	88.5	46.6	5702	0.8		
		5.3	6158	0.1	99.9	10.5	94	11.1	88.9	47.9	6110	0.8		
6 hr	0.5 µg/mL	0.8	635	0.1	99.9	9.0	87	10.3	89.7	7.9	627	1.2	1.4	0.1
		0.9	654	0.1	99.9	8.5	93	9.1	90.9	9.9	644	1.5		
		1.0	696	0.1	99.9	9.1	97	9.4	90.6	10.1	686	1.4		
	1 µg/mL	1.3	1817	0.1	99.9	8.7	113	7.7	92.3	16.4	1800	0.9	1.1	0.2
		1.6	1550	0.1	99.9	8.8	103	8.6	91.4	18.5	1532	1.2		
		1.3	1568	0.1	99.9	8.2	108	7.6	92.4	16.7	1552	1.1		
	5 µg/mL	5.5	7576	0.1	99.9	8.7	108	8.0	92.0	68.8	7507	0.9	0.9	0.04
		5.4	8143	0.1	99.9	8.5	107	7.9	92.1	68.5	8075	0.8		
		5.9	7868	0.1	99.9	9.0	108	8.3	91.7	70.9	7797	0.9		

Table III-9. Free Doxorubicin HCl Control Data. Displayed is the calculated % release for the doxorubicin HCl control data. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	99.7	2.0	2.0
	1 µg/mL	101.1	2.4	2.4
	5 µg/mL	102.8	3.0	3.0
2 hr	0.5 µg/mL	97.8	0.9	0.9
	1 µg/mL	102.0	1.2	1.2
	5 µg/mL	99.7	3.2	3.3
6 hr	0.5 µg/mL	107.2	2.1	2.0
	1 µg/mL	105.3	3.5	3.3
	5 µg/mL	105.6	3.8	3.6
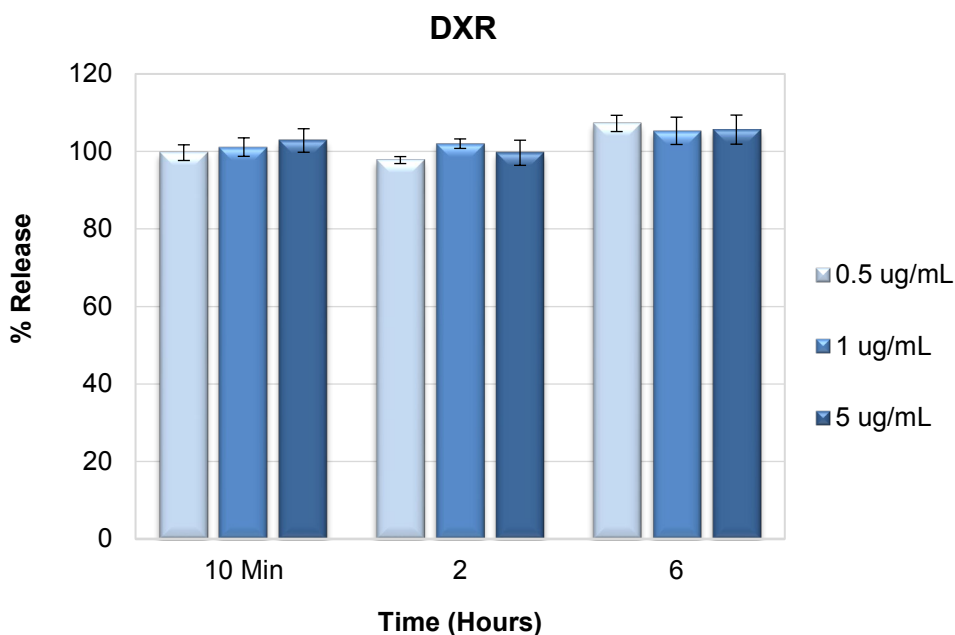
Figure III-5. Free Doxorubicin HCl Control Data. Displayed is the calculated % release for the doxorubicin HCl control data. (Mean $\pm$ SD, *N=3*)

Table III-10. Doxil, Lot A (600220P1) Drug Release. Displayed is the calculated % release for Doxil, Lot A (600220P1). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	1.3	0.3	21.3
	1 µg/mL	1.1	0.2	16.1
	5 µg/mL	0.7	0.02	3.5
2 hr	0.5 µg/mL	1.2	0.2	16.4
	1 µg/mL	1.2	0.1	11.5
	5 µg/mL	0.9	0.1	7.6
6 hr	0.5 µg/mL	1.1	0.2	13.6
	1 µg/mL	1.2	0.05	4.0
	5 µg/mL	0.9	0.05	4.8
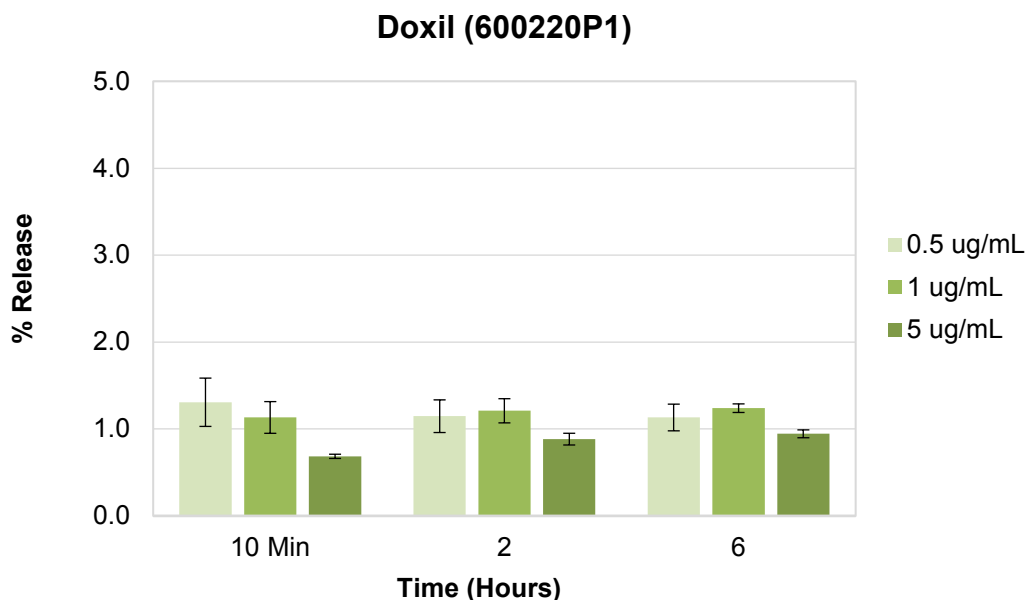
Figure III-6. Doxil, Lot A (600220P1) Drug Release. Displayed is the calculated % release for Doxil, Lot A (600220P1). (Mean $\pm$ SD, *N=3*)

Table III-11. Doxil, Lot B (600120P1) Drug Release. Displayed is the calculated % release for Doxil, Lot B (600120P1). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	1.4	0.3	19.0
	1 µg/mL	1.3	0.2	17.0
	5 µg/mL	0.8	0.02	3.0
2 hr	0.5 µg/mL	1.9	0.3	15.6
	1 µg/mL	1.5	0.3	20.2
	5 µg/mL	1.1	0.1	12.4
6 hr	0.5 µg/mL	1.5	0.2	10.5
	1 µg/mL	1.2	0.1	9.3
	5 µg/mL	1.0	0.1	10.5
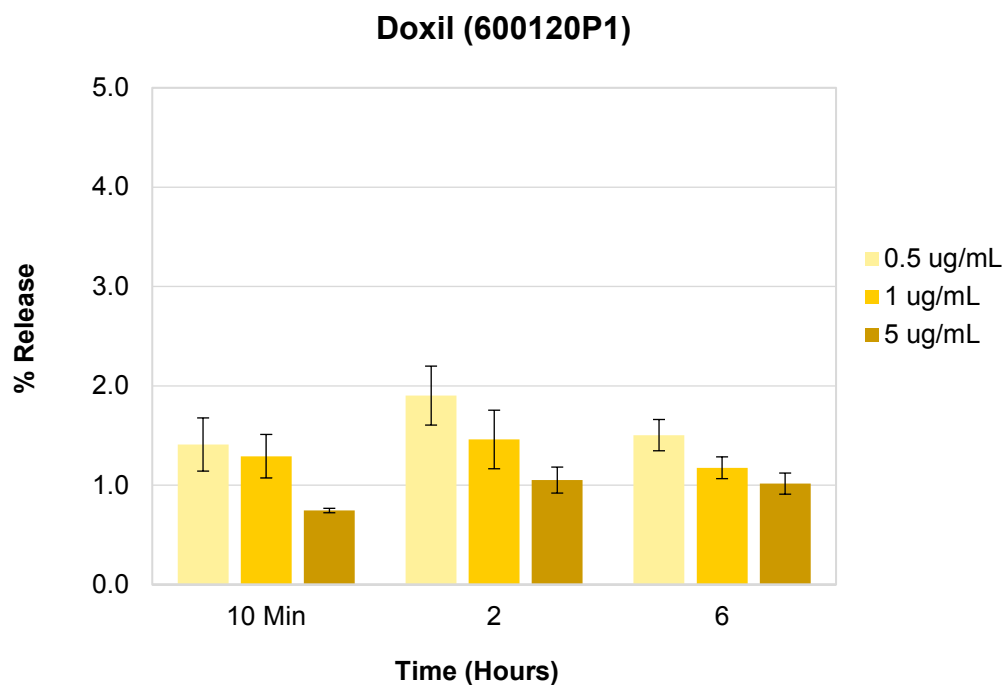
Figure III-7. Doxil, Lot B (600120P1) Drug Release. Displayed is the calculated % release for the Doxil, Lot B (600120P1). (Mean $\pm$ SD, *N=3*)

Table III-12. Doxil, Lot C (600520P1) Drug Release. Displayed is the calculated % release for Doxil, Lot C (600520P1). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	1.2	0.2	11.9
	1 µg/mL	0.9	0.2	20.1
	5 µg/mL	0.7	0.04	5.1
2 hr	0.5 µg/mL	1.3	0.1	9.6
	1 µg/mL	1.1	0.2	15.9
	5 µg/mL	0.8	0.02	2.7
6 hr	0.5 µg/mL	1.4	0.1	10.0
	1 µg/mL	1.1	0.2	13.8
	5 µg/mL	0.9	0.04	4.2
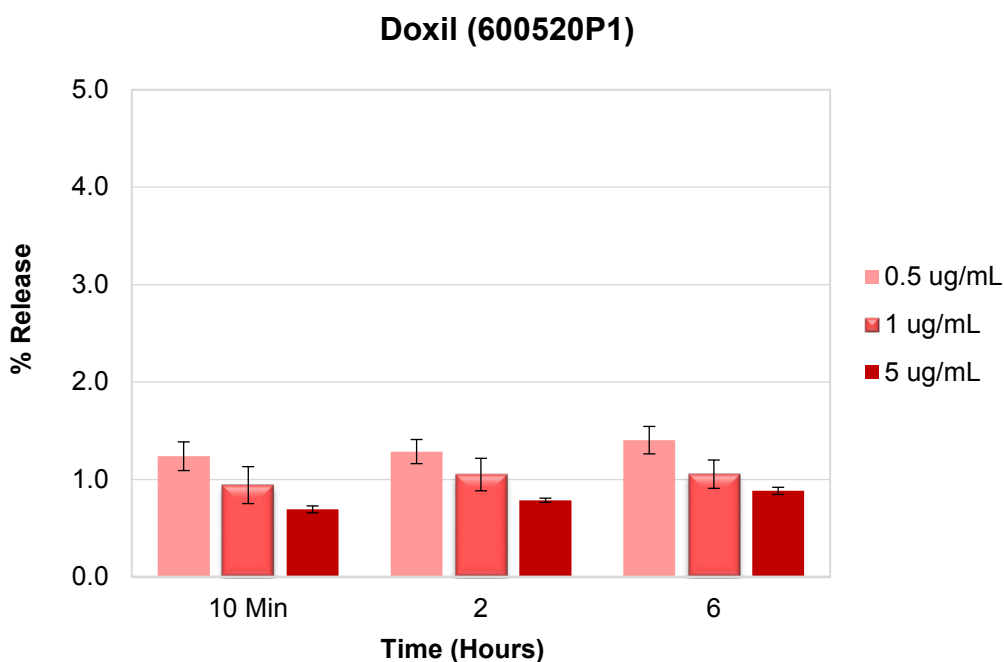
Figure III-8. Doxil, Lot C (600520P1) Drug Release. Displayed is the calculated % release for the Doxil, Lot C (600520P1). (Mean $\pm$ SD, *N=3*)
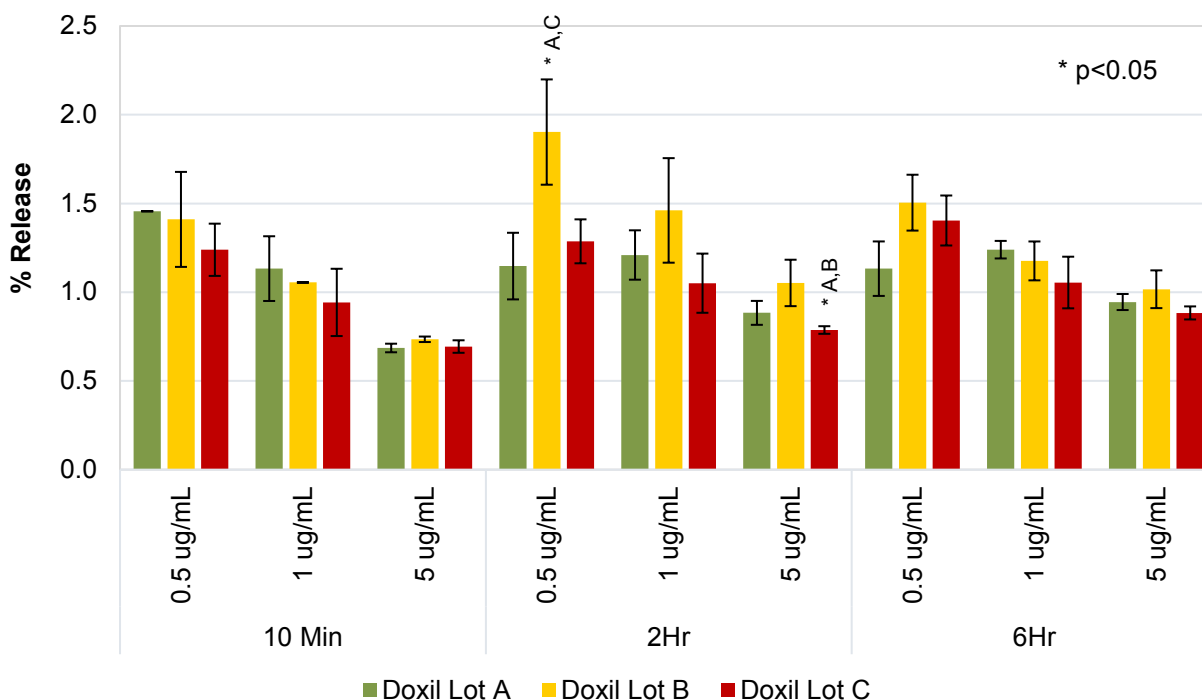
Figure III-9. Doxil Drug Release Lot Comparison. Displayed are the 10 min-6 hr calculated % release data for the Doxil Lots A, B, C. (Mean + SD, *N*=3), *p<0.05, ANOVA with Duncan's Multiple Range posthoc test.

Intra-Day Three Lot Comparison in Human Plasma at 42°C

Summary

The objective of this study was to compare the in vitro drug release of three lots of Doxil in human plasma at 42°C over a 6 hr period. All lots had similar drug release profiles over the 6 hr period, although some instances of statistically significant differences were observed between lots.

Design and Methods

Three lots of Doxil, 600220P1, 600120P1, and 600520P1, also denoted as lots A, B, and C, respectively, were evaluated for drug release in human plasma at 42°C over a 6 hr period, according to the SITUA method described in Section I. All three lots were run on a single day, and free doxorubicin HCl at identical concentrations and time points was included as a control. The human plasma and protein-free plasma DXR and DXR_C13 standard calibration curves used for this study are also provided.

Results and Discussion

The DXR and DXR_C13 standard calibration curves used for this study are presented in **Tables III-13 to III-16 and Figures III-10 to III-13**. The free doxorubicin HCl controls averaged between 86-93% of theoretical for all concentrations and time points (**Tables III-17 and III-21, and Figure III-14**). The Doxil drug release was similar for Lot's A-C, at approximately 1% release over the 6 hr period, without a clear temporal trend (**Tables III-18 to III-20 and III-22 to III-24, and Figures III-15 to III-18**).

Table III-13. DXR Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	1266071	10.0	100
10.0 ng/mL	1359355	10.3	103
50 ng /mL	5417557	47	94
50 ng /mL	6619267	49	98
100 ng /mL	14424204	94	94
100 ng /mL	13813824	94	94
500 ng /mL	72685967	493	99
500 ng /mL	76740561	525	105
1000 ng /mL	150424560	1063	106
1000 ng /mL	147718123	1091	109
5000 ng /mL	680128417	4663	93
5000 ng /mL	721054668	5183	104
10000 ng /mL	1389569509	9944	99
10000 ng /mL	1409088763	10053	101
QC			
QCL	1104803	9.2	92
QCL	1205026	9.8	98
QCM	12682730	96	96
QCM	12961696	96	96
QCH	151266113	1075	107
QCH	149588031	1014	101

DXR_PRM
Y = 0.00211304+0.00446164*X   R^2 = 0.9990   W: 1/X
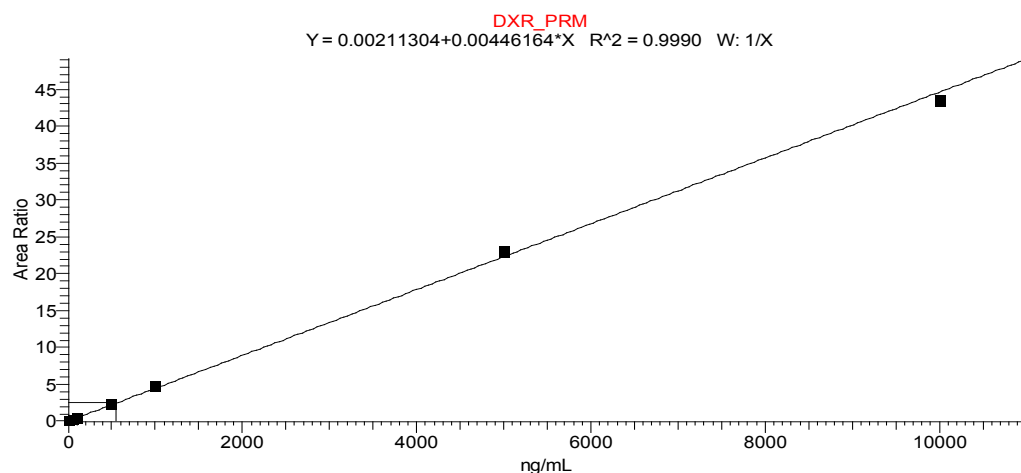
Figure III-10. DXR Plasma Standard Curve.

Table III-14. DXR_C13 Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	1031414	10.6	106
10.0 ng/mL	1064941	10.6	106
50 ng /mL	4547580	50	99
50 ng /mL	5291186	49	99
100 ng /mL	11642961	95	95
100 ng /mL	10876768	93	93
500 ng /mL	53259828	452	90
500 ng /mL	60045961	514	103
1000 ng /mL	117871099	1041	104
1000 ng /mL	117731287	1087	109
5000 ng /mL	538515919	4613	92
5000 ng /mL	562471464	5051	101
10000 ng /mL	1123165891	10042	100
10000 ng /mL	1145522388	10211	102
QC			
QCL	1003223	10.7	107
QCL	971306	10.3	103
QCM	10158673	96	96
QCM	10197896	94	94
QCH	120744590	1072	107
QCH	119944727	1017	102

DXR_C13_PRM
$Y = 0.00256778 + 0.00306105 \cdot X$ $R^2 = 0.9987$ W: 1/X
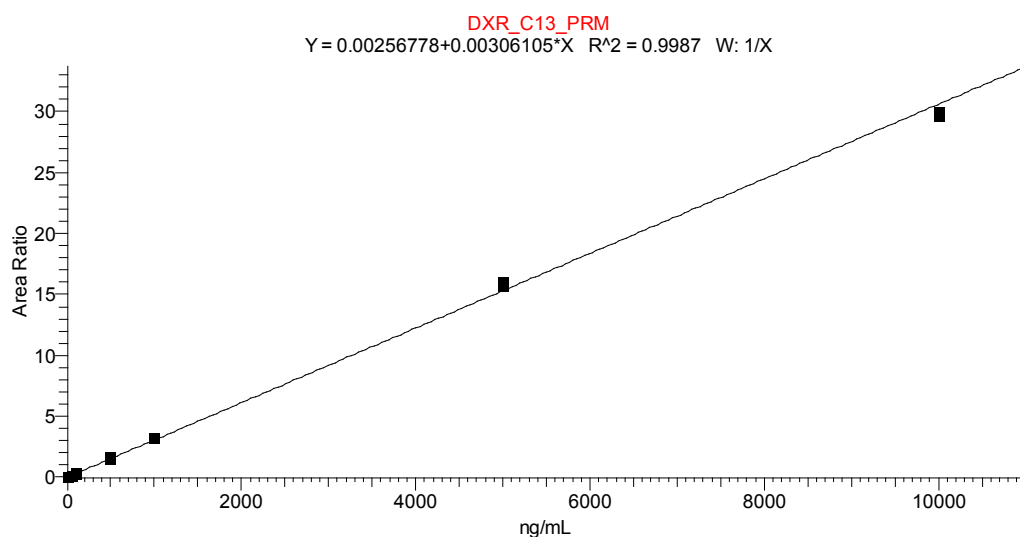
Figure III-11. DXR_C13 Plasma Standard Curve.

Table III-15. DXR Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	27541	0.109	109
0.100 ng/mL	24028	0.092	92
0.50 ng/mL	99423	0.52	105
0.50 ng/mL	100919	0.49	99
1.00 ng/mL	199167	0.92	92
1.00 ng/mL	180930	0.93	93
5.00 ng/mL	942201	4.64	93
5.00 ng/mL	922083	4.67	93
10.0 ng/mL	1987576	10.1	101
10.0 ng/mL	1968702	9.7	97
100.0 ng/mL	21523279	104.4	104
100.0 ng/mL	21573748	109.1	109
1000.0 ng/mL	225023557	1095	109
1000.0 ng/mL	208969009	1026	103
QC			
QCL	11982	0.103	103
QCL	10485	0.102	102
QCM	170861	0.901	90
QCM	176334	0.944	94
QCH	1905849	9.7	97
QCH	1906268	9.9	99

DXR_PRM
$Y = 9.72645e\text{-}005 + 0.00467628 \cdot X \quad R^2 = 0.9946 \quad W: 1/X^2$
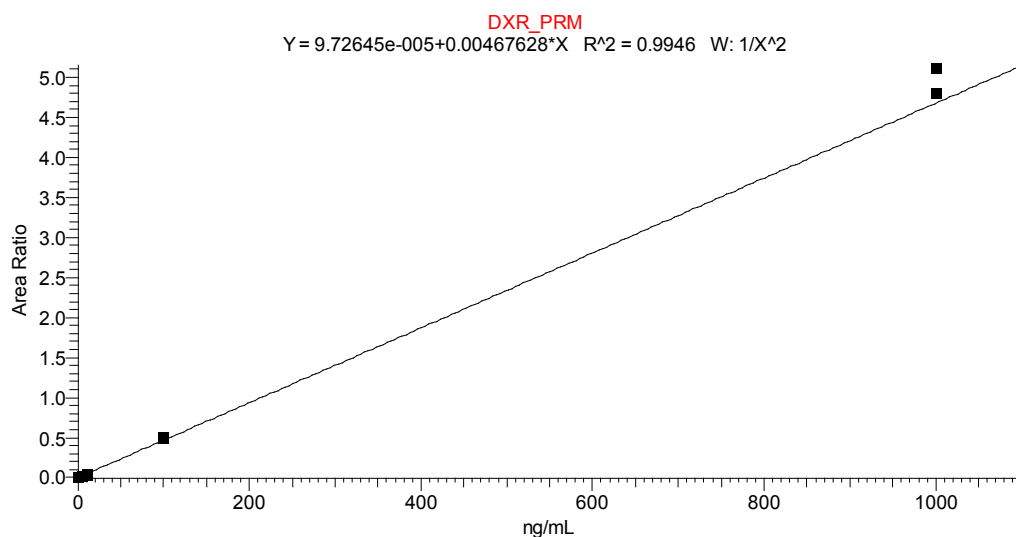
Figure III-12. DXR Protein-Free Plasma Standard Curve.

Table III-16. DXR_C13 Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	9840	0.111	111
0.100 ng/mL	6951	0.092	92
0.50 ng/mL	52822	0.46	92
0.50 ng/mL	59985	0.48	97
1.00 ng/mL	135846	0.96	96
1.00 ng/mL	124409	0.98	98
5.00 ng/mL	666591	4.75	95
5.00 ng/mL	648229	4.76	95
10.0 ng/mL	1407456	10.3	103
10.0 ng/mL	1354350	9.6	96
100.0 ng/mL	15136830	105.0	105
100.0 ng/mL	15206192	109.9	110
1000.0 ng/mL	149453679	1039	104
1000.0 ng/mL	151240495	1061	106
QC			
QCL	9551	0.108	108
QCL	8047	0.096	96
QCM	132675	1.067	107
QCM	126642	1.035	104
QCH	1396810	10.2	102
QCH	1330958	9.9	99

DXR_C13_PRM
$Y = -0.000147586+0.0032729*X$ $R^2 = 0.9949$ W: $1/X^2$
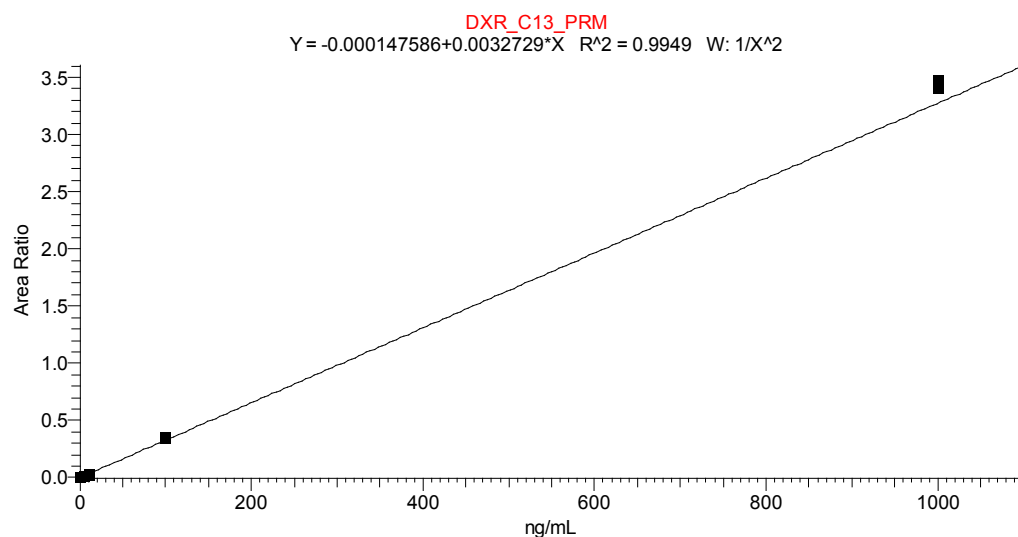
Figure III-13. DXR_C13 Protein-Free Plasma Standard Curve.

Table III-17. Free Doxorubicin HCl Control Analytical Data. Presented are the analytical data for the free doxorubicin HCl controls. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 − %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	50.3	535	9.4	90.6	7.4	70	10.5	89.5	476.8	58	89.1	89.1	0.5
		60.3	637	9.5	90.5	8.8	82	10.7	89.3	564.2	73	88.6		
		38.0	592	6.4	93.6	6.1	85	7.2	92.8	529.8	62	89.5		
	1 µg/mL	107.0	1217	8.8	91.2	7.9	82	9.7	90.3	1101.5	116	90.5	92.7	2.0
		112.1	1392	8.1	91.9	8.0	94	8.5	91.5	1314.6	77	94.5		
		113.9	1336	8.5	91.5	8.2	89	9.2	90.8	1243.5	92	93.1		
	5 µg/mL	633.3	6420	9.9	90.1	9.0	86	10.5	89.5	6031.1	389	93.9	92.4	7.2
		657.0	5461	12.0	88.0	10.2	84	12.2	87.8	5392.0	69	98.7		
		680.9	6382	10.7	89.3	10.1	80	12.6	87.4	5397.8	985	84.6		
2 hr	0.5 µg/mL	49.2	464	10.6	89.4	7.3	62	11.8	88.2	417.6	47	89.9	89.6	0.5
		56.4	474	11.9	88.1	8.3	63	13.2	86.8	426.2	48	89.9		
		41.9	471	8.9	91.1	6.8	68	10.0	90.0	419.8	52	89.1		
	1 µg/mL	104.3	963	10.8	89.2	7.9	65	12.1	87.9	865.1	98	89.8	88.1	3.2
		102.2	959	10.6	89.4	7.6	64	11.8	88.2	863.6	96	90.0		
		110.7	929	11.9	88.1	8.3	59	14.1	85.9	784.3	145	84.4		
	5 µg/mL	701.2	4109	17.1	82.9	10.5	55	19.2	80.8	3649.8	459	88.8	88.6	0.2
		604.9	5357	11.3	88.7	9.0	70	12.8	87.2	4736.6	620	88.4		
		613.0	5073	12.1	87.9	9.0	66	13.6	86.4	4492.0	581	88.5		
6 hr	0.5 µg/mL	31.5	352	8.9	91.1	4.9	47	10.5	89.5	300.6	52	85.4	86.0	2.4
		32.6	361	9.0	91.0	4.9	48	10.2	89.8	320.1	41	88.6		
		27.6	321	8.6	91.4	4.8	47	10.3	89.7	269.2	52	83.9		
	1 µg/mL	75.7	671	11.3	88.7	6.0	47	12.6	87.4	602.3	69	89.7	87.8	1.7
		40.2	710	5.7	94.3	3.1	48	6.6	93.4	613.2	97	86.3		
		65.1	753	8.6	91.4	5.1	52	9.9	90.1	657.7	96	87.3		
	5 µg/mL	390.2	4564	8.5	91.5	5.7	61	9.3	90.7	4203.6	361	92.1	90.4	1.6
		426.2	4373	9.7	90.3	6.4	58	11.0	89.0	3885.8	487	88.9		
		436.8	4126	10.6	89.4	6.5	55	11.7	88.3	3727.9	398	90.3		

Table III-18. Doxil, Lot A (600220P1) Drug Release Analytical Data. Presented are the analytical data for Doxil Lot A (600220P1). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 – %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	0.7	637	0.1	99.9	10.0	90	11.1	88.9	6.5	630	1.0	1.2	0.2
		1.0	582	0.2	99.8	11.6	92	12.6	87.4	7.7	574	1.3		
		0.7	641	0.1	99.9	9.5	94	10.2	89.8	7.3	634	1.1		
	1 µg/mL	1.2	1142	0.1	99.9	9.1	84	10.8	89.2	10.9	1132	1.0	1.0	0.1
		1.2	1118	0.1	99.9	9.3	89	10.4	89.6	11.9	1106	1.1		
		1.2	1351	0.1	99.9	10.0	100	10.0	90.0	11.9	1339	0.9		
	5 µg/mL	4.9	5922	0.1	99.9	9.0	88	10.2	89.8	48.0	5874	0.8	0.8	0.1
		4.3	5576	0.1	99.9	8.7	87	10.0	90.0	43.5	5533	0.8		
		5.3	5920	0.1	99.9	11.1	84	13.2	86.8	40.4	5880	0.7		
2 hr	0.5 µg/mL	1.0	716	0.1	99.9	8.1	80	10.1	89.9	10.2	706	1.4	1.6	0.2
		1.3	694	0.2	99.8	8.1	80	10.2	89.8	12.7	682	1.8		
		1.0	720	0.1	99.9	8.3	84	9.9	90.1	10.5	710	1.5		
	1 µg/mL	2.6	1370	0.2	99.8	10.5	81	12.9	87.1	20.6	1350	1.5	1.5	0.2
		1.9	1136	0.2	99.8	7.9	79	10.0	90.0	18.6	1117	1.6		
		1.9	1259	0.1	99.9	8.6	77	11.2	88.8	16.6	1242	1.3		
	5 µg/mL	8.2	6120	0.1	99.9	8.0	80	10.1	89.9	80.8	6039	1.3	1.4	0.1
		7.5	5500	0.1	99.9	7.4	82	9.0	91.0	83.8	5416	1.5		
		9.5	5992	0.2	99.8	9.6	80	12.0	88.0	79.1	5913	1.3		
6 hr	0.5 µg/mL	1.2	741	0.2	99.8	5.3	55	9.7	90.3	12.8	728	1.7	2.0	0.3
		1.4	588	0.2	99.8	5.5	53	10.4	89.6	13.3	575	2.3		
		1.1	672	0.2	99.8	4.4	55	8.1	91.9	13.5	659	2.0		
	1 µg/mL	2.6	1410	0.2	99.8	5.4	52	10.4	89.6	24.7	1385	1.8	2.0	0.2
		2.8	1181	0.2	99.8	5.8	53	10.9	89.1	25.7	1155	2.2		
		2.4	1054	0.2	99.8	5.1	48	10.6	89.4	22.8	1032	2.2		
	5 µg/mL	9.0	6165	0.1	99.9	3.8	56	6.8	93.2	133.2	6032	2.2	2.5	0.5
		10.2	5630	0.2	99.8	2.6	45	5.8	94.2	174.3	5456	3.1		
		10.1	5639	0.2	99.8	4.1	53	7.7	92.3	131.0	5508	2.3		

Table III-19. Doxil, Lot B (600120P1) Drug Release Analytical Data. Presented are the analytical data for Doxil Lot B (600120P1). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*) †These replicates were removed from final calculations as outliers.

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR	% Bound	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13	% Bound DXR_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG	SD
				= Filtrate DXR / Reservoir DXR *100	= 100 – %Unbound DXR			= Filtrate DXR_C13 / Reservoir DXR_C13 * 100	= 100 – %Unbound C13	= Fil DXR/ (1-(%Bound DXR_C13/100))	= Reservoir DXR-Unencapsulated	= (Unencapsulated/ Reservoir DXR) *100		
10 min	0.5 µg/mL	0.7	742	0.1	99.9	7.9	96	8.2	91.8	7.9	734	1.1	1.3	0.2
		0.7	575	0.1	99.9	8.5	94	9.0	91.0	7.9	567	1.4		
		1.0	687	0.1	99.9	10.1	94	10.8	89.2	9.4	678	1.4		
	1 µg/mL	0.9	1413	0.1	99.9	7.4	90	8.2	91.8	11.3	1402	0.8	0.9	0.1
		1.5†	1242	0.1	99.9	6.6	92	7.1	92.9	20.8	1221	1.7		
		1.0	1372	0.1	99.9	7.5	92	8.2	91.8	12.7	1359	0.9		
	5 µg/mL	18.8†	5778	0.3	99.7	13.7	82	16.6	83.4	113.1	5665	2.0	0.8	0.1
		4.9	6017	0.1	99.9	9.0	79	11.4	88.6	42.7	5974	0.7		
		5.7	5982	0.1	99.9	9.8	86	11.4	88.6	49.6	5933	0.8		
2 hr	0.5 µg/mL	1.5	744	0.2	99.8	9.0	85	10.6	89.4	14.3	730	1.9	1.9	0.1
		1.2	663	0.2	99.8	9.0	88	10.2	89.8	12.1	651	1.8		
		1.5	675	0.2	99.8	9.0	83	10.8	89.2	13.6	661	2.0		
	1 µg/mL	2.1	1405	0.2	99.8	7.4	78	9.6	90.4	22.3	1382	1.6	1.6	0.04
		1.8	1292	0.1	99.9	7.5	83	9.0	91.0	20.3	1271	1.6		
		1.8	1525	0.1	99.9	6.9	87	7.9	92.1	22.9	1503	1.5		
	5 µg/mL	9.1	6076	0.1	99.9	7.8	78	10.0	90.0	90.9	5985	1.5	1.5	0.01
		7.4	5900	0.1	99.9	6.5	78	8.4	91.6	87.8	5812	1.5		
		9.3	5862	0.2	99.8	8.1	77	10.5	89.5	88.2	5774	1.5		
6 hr	0.5 µg/mL	1.6	708	0.2	99.8	6.1	53	11.3	88.7	13.7	695	1.9	2.2	0.2
		1.4	671	0.2	99.8	5.2	57	9.0	91.0	15.2	656	2.3		
		1.4	675	0.2	99.8	4.6	51	9.1	90.9	15.6	659	2.3		
	1 µg/mL	2.2	1410	0.2	99.8	4.3	50	8.7	91.3	25.8	1384	1.8	1.9	0.1
		2.4	1568	0.2	99.8	4.8	64	7.5	92.5	31.7	1536	2.0		
		2.2	1538	0.1	99.9	3.9	55	7.1	92.9	30.5	1508	2.0		
	5 µg/mL	9.3	7407	0.1	99.9	3.6	60	6.0	94.0	154.0	7253	2.1	2.2	0.1
		8.5	7259	0.1	99.9	3.1	60	5.1	94.9	166.1	7093	2.3		
		9.8	6911	0.1	99.9	3.7	58	6.4	93.6	153.7	6757	2.2		

Table III-20. Doxil, Lot C (600520P1) Drug Release Analytical Data. Presented are the analytical data for Doxil Lot C (600520P1). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 – %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	0.6	665	0.1	99.9	5.2	75	6.9	93.1	9.0	656	1.4	1.5	0.2
		0.6	788	0.1	99.9	4.7	79	5.9	94.1	10.9	777	1.4		
		0.9	684	0.1	99.9	5.8	78	7.5	92.5	11.6	672	1.7		
	1 µg/mL	1.0	1168	0.1	99.9	6.6	74	9.0	91.0	11.4	1156	1.0	0.9	0.1
		0.9	1477	0.1	99.9	6.0	79	7.6	92.4	11.9	1465	0.8		
		1.2	1385	0.1	99.9	5.9	71	8.3	91.7	14.5	1371	1.0		
	5 µg/mL	5.0	5412	0.1	99.9	7.1	59	12.0	88.0	41.8	5371	0.8	0.8	0.1
		4.1	5217	0.1	99.9	6.4	73	8.8	91.2	47.1	5170	0.9		
		4.1	6945	0.1	99.9	6.0	76	7.8	92.2	52.1	6893	0.8		
2 hr	0.5 µg/mL	1.1	667	0.2	99.8	7.3	64	11.3	88.7	9.8	657	1.5	1.6	0.3
		0.7	559	0.1	99.9	3.8	41	9.2	90.8	7.6	552	1.4		
		1.4	615	0.2	99.8	7.0	61	11.4	88.6	12.0	603	2.0		
	1 µg/mL	1.8	1329	0.1	99.9	7.3	62	11.9	88.1	14.9	1314	1.1	1.3	0.1
		1.9	1125	0.2	99.8	8.1	64	12.8	87.2	14.7	1110	1.3		
		2.0	1361	0.1	99.9	7.0	64	11.0	89.0	18.6	1343	1.4		
	5 µg/mL	9.6	6299	0.2	99.8	8.2	63	12.9	87.1	74.3	6225	1.2	1.3	0.1
		8.7	5419	0.2	99.8	6.8	61	11.3	88.7	77.1	5342	1.4		
		8.8	5531	0.2	99.8	7.4	62	11.8	88.2	73.9	5457	1.3		
6 hr	0.5 µg/mL	1.3	900	0.1	99.9	5.4	61	8.8	91.2	15.1	885	1.7	1.7	0.1
		1.0	738	0.1	99.9	1.3	18	7.5	92.5	13.3	725	1.8		
		1.2	880	0.1	99.9	4.7	57	8.3	91.7	14.9	865	1.7		
	1 µg/mL	2.3	1753	0.1	99.9	5.1	59	8.5	91.5	27.4	1726	1.6	1.6	0.1
		2.0	1645	0.1	99.9	4.4	57	7.8	92.2	25.7	1619	1.6		
		2.3	1742	0.1	99.9	4.3	54	7.9	92.1	29.5	1713	1.7		
	5 µg/mL	9.5	7569	0.1	99.9	4.3	64	6.7	93.3	141.8	7427	1.9	2.3	0.6
		10.6	6441	0.2	99.8	2.5	45	5.5	94.5	191.7	6249	3.0		
		9.9	7368	0.1	99.9	4.1	60	6.9	93.1	143.8	7224	2.0		

Table III-21. Free Doxorubicin HCl Control Data. Displayed is the calculated % release for the doxorubicin HCl control data. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	89.1	0.5	0.5
	1 µg/mL	92.7	2.0	2.2
	5 µg/mL	92.4	7.2	7.8
2 hr	0.5 µg/mL	89.6	0.5	0.5
	1 µg/mL	88.1	3.2	3.6
	5 µg/mL	88.6	0.2	0.2
6 hr	0.5 µg/mL	86.0	2.4	2.8
	1 µg/mL	87.8	1.7	2.0
	5 µg/mL	90.4	1.6	1.8
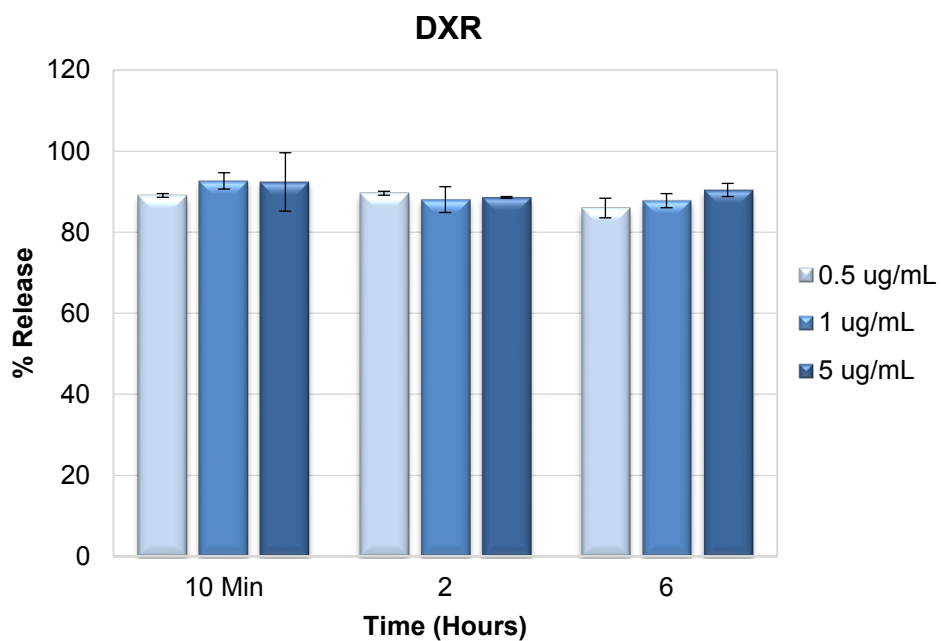
Figure III-14. Free Doxorubicin HCl Control Data. Displayed is the calculated % release for the doxorubicin HCl control data. (Mean $\pm$ SD, *N=3*)

Table III-22. Doxil, Lot A (600220P1) Drug Release. Displayed is the calculated % release for Doxil, Lot A (600220P1). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	1.2	0.2	13.7
	1 µg/mL	1.0	0.1	9.5
	5 µg/mL	0.8	0.1	8.9
2 hr	0.5 µg/mL	1.6	0.2	14.5
	1 µg/mL	1.5	0.2	10.7
	5 µg/mL	1.4	0.1	8.5
6 hr	0.5 µg/mL	2.0	0.3	13.2
	1 µg/mL	2.0	0.2	11.8
	5 µg/mL	2.5	0.5	19.8
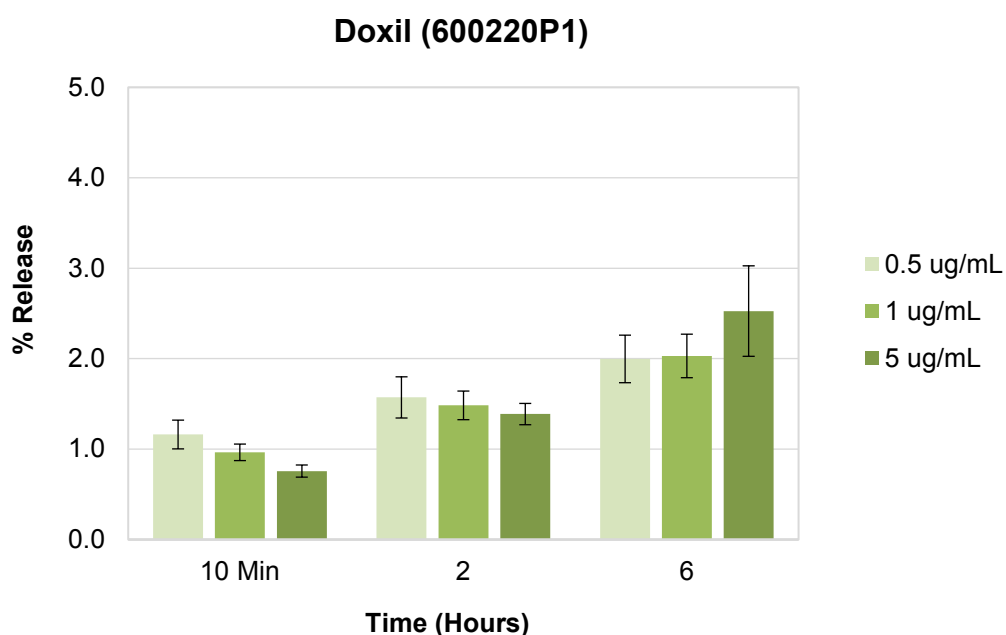
Figure III-15. Doxil, Lot A (600220P1) Drug Release. Displayed is the calculated % release for Doxil, Lot A (600220P1). (Mean $\pm$ SD, *N=3*)

Table III-23. Doxil, Lot B (600120P1) Drug Release. Displayed is the calculated % release for Doxil, Lot B (600120P1). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	1.3	0.2	14.1
	1 µg/mL	0.9	0.1	10.2
	5 µg/mL	0.8	0.1	10.9
2 hr	0.5 µg/mL	1.9	0.1	5.1
	1 µg/mL	1.6	0.04	2.8
	5 µg/mL	1.5	0.01	0.6
6 hr	0.5 µg/mL	2.2	0.2	9.5
	1 µg/mL	1.9	0.1	5.2
	5 µg/mL	2.2	0.1	4.9
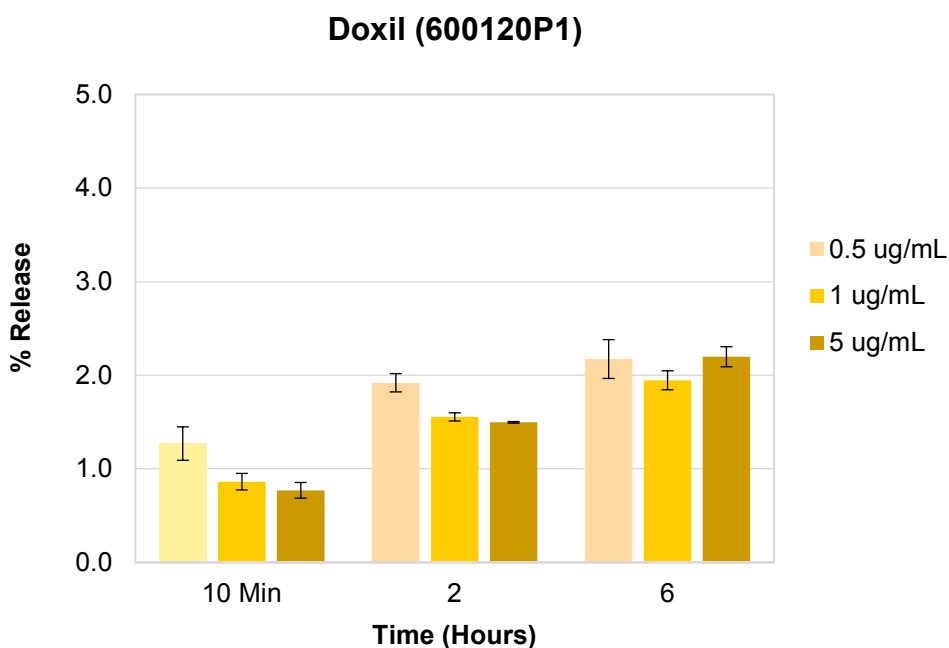
Figure III-16. Doxil, Lot B (600120P1) Drug Release. Displayed is the calculated % release for the Doxil, Lot B (600120P1). (Mean $\pm$ SD, *N=3*)

Table III-24. Doxil, Lot C (600520P1) Drug Release. Displayed is the calculated % release for Doxil, Lot C (600520P1). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	1.5	0.2	13.1
	1 µg/mL	0.9	0.1	13.3
	5 µg/mL	0.8	0.1	10.1
2 hr	0.5 µg/mL	1.6	0.3	20.1
	1 µg/mL	1.3	0.1	10.1
	5 µg/mL	1.3	0.1	9.4
6 hr	0.5 µg/mL	1.7	0.1	3.9
	1 µg/mL	1.6	0.1	4.8
	5 µg/mL	2.3	0.6	27.1
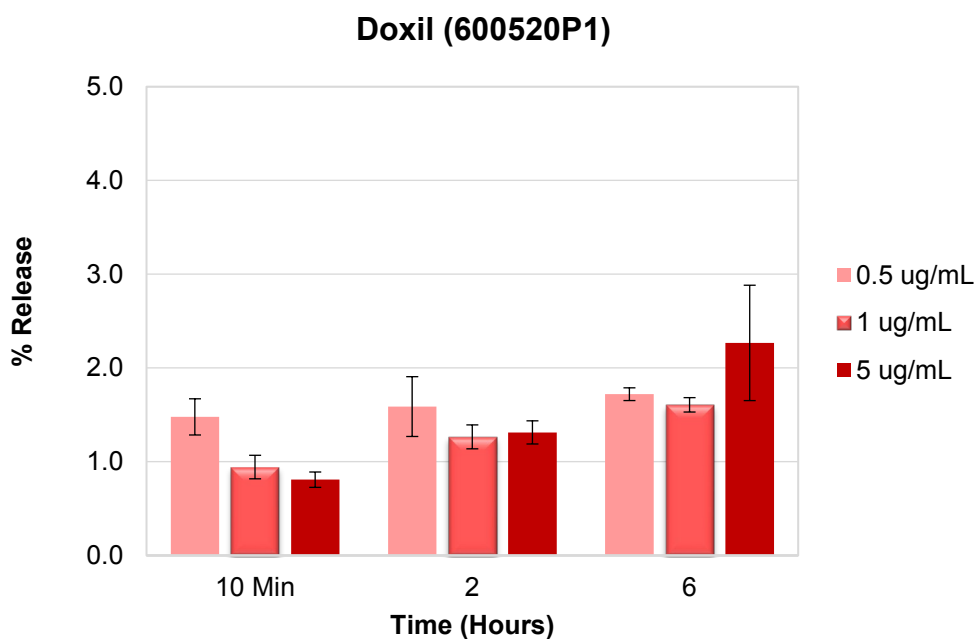
Doxil (600520P1)

Figure III-17. Doxil, Lot C (600520P1) Drug Release. Displayed is the calculated % release for the Doxil, Lot C (600520P1). (Mean $\pm$ SD, *N=3*)
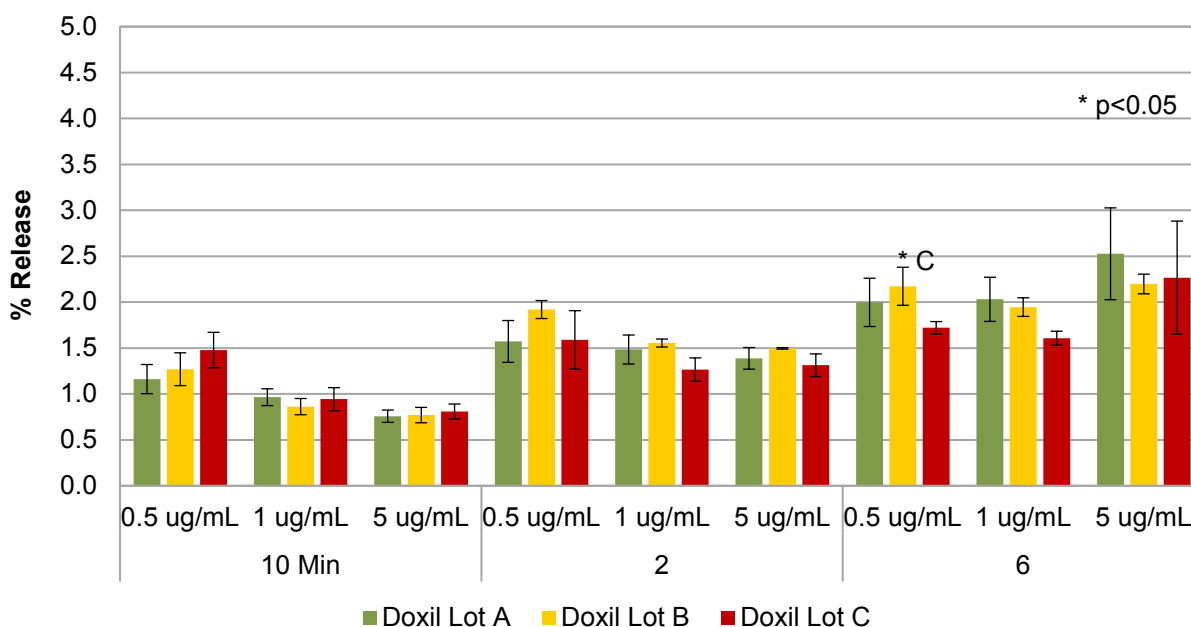
Figure III-18. Doxil Drug Release Lot Comparison. Displayed are the 10 min-6 hr calculated % release data for the Doxil Lots A, B, C. (Mean $\pm$ SD, *N=3*), *$p \leq 0.05$, ANOVA with Duncan's Multiple Range posthoc test.

IV. In Vitro Sun Pharma Lot Comparison

Intra-Day Three Lot Comparison in Human Plasma at 37°C

Summary

The objective of this study was to compare the in vitro drug release of three lots of Sun Pharma's liposomal doxorubicin HCl in human plasma at 37°C over a 6 hr period. All lots had similar drug release profiles over the 6 hr period, although some instances of statistically significant differences were observed between lots.

Design and Methods

Three lots of Sun Pharma's liposomal doxorubicin HCl, JKR0494A, JKR0154A, and JKR0491A, also denoted as lots A, B, and C, respectively, were evaluated for drug release in human plasma at 42°C over a 6 hr period, according to the SITUA method described in Section I. All three lots were run on a single day, and free doxorubicin HCl at identical concentrations and time points was included as a control. The human plasma and protein-free plasma DXR and DXR_C13 standard calibration curves used for this study are also provided.

Results & Conclusions

The DXR and DXR_C13 standard calibration curves used for this study are presented in **Tables IV-1 to IV-4 and Figures IV-1 to IV-4**. The free doxorubicin HCl controls averaged between 90-126% of theoretical for all concentrations and time points (**Tables IV-5 and IV-9, and Figure IV-5**). The Sun Pharma drug release was similar for Lot's A-C, at approximately 1% release over the 6 hr period, without a clear temporal trend (**Tables IV-6 to IV-8 and IV-10 to IV-12, and Figures IV-6 to IV-8**). Due to high variability (%CV>50) and deviation of free doxorubicin from theoretical (>122%) at the 24 hr time point, this time point was not included in later studies.

Table IV-1. DXR Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	2692086	11.3	113
10.0 ng/mL	2488860	11.3	113
50 ng/mL	13581502	45	89
50 ng/mL	13669389	47	94
100 ng/mL	27880471	97	97
100 ng/mL	27897602	92	92
500 ng/mL	153482889	498	100
500 ng/mL	149200919	502	100
1000 ng/mL	304496982	1035	104
1000 ng/mL	305213298	978	98
5000 ng/mL	1574425873	5045	101
5000 ng/mL	1568176390	4957	99
QC			
QCL	2623114	11.1	111
QCL	2707798	11.3	113
QCM	29102859	94	94
QCM	3128445	100	100
QCH	301868936	1069	107
QCH	336310237	1051	105

DXR_PRM
Y = -0.0228911+0.00828529*X   R^2 = 0.9997   W: 1/X
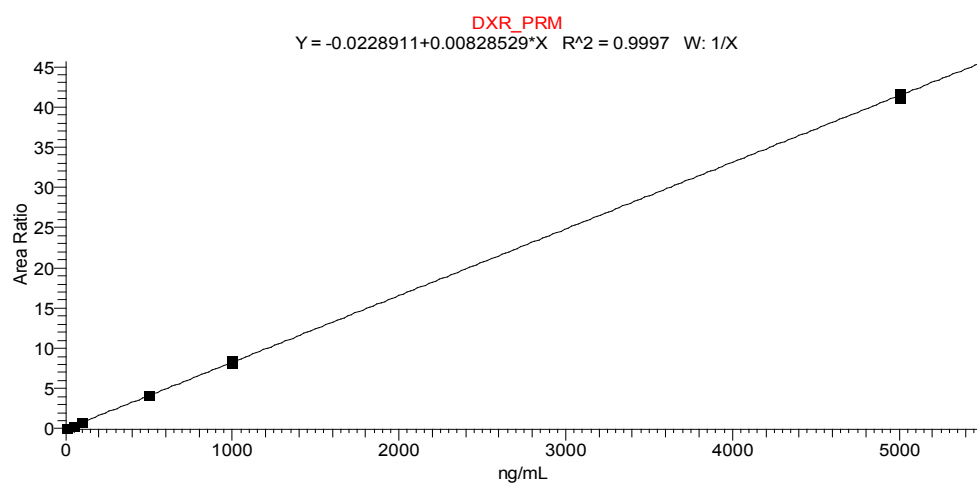
Figure IV-1. DXR Plasma Standard Curve.

Table IV-2. DXR_C13 Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	1423401	11.2	112
10.0 ng/mL	1310823	11.1	111
50 ng/mL	7207239	45	90
50 ng/mL	7115259	47	93
100 ng/mL	14469240	96	96
100 ng/mL	14431941	91	91
500 ng/mL	77637801	496	99
500 ng/mL	81366330	510	102
1000 ng/mL	162735651	1060	106
1000 ng/mL	161758808	993	99
5000 ng/mL	817959967	5021	100
5000 ng/mL	815569916	4939	99
QC			
QCL	1369878	10.9	109
QCL	1377554	10.8	108
QCM	15172469	93	94
QCM	16259170	99	99
QCH	150052526	1018	102
QCH	172945313	1035	104

DXR_C13_PRM
Y = -0.0109779+0.00432503*X   R^2 = 0.9995   W: 1/X
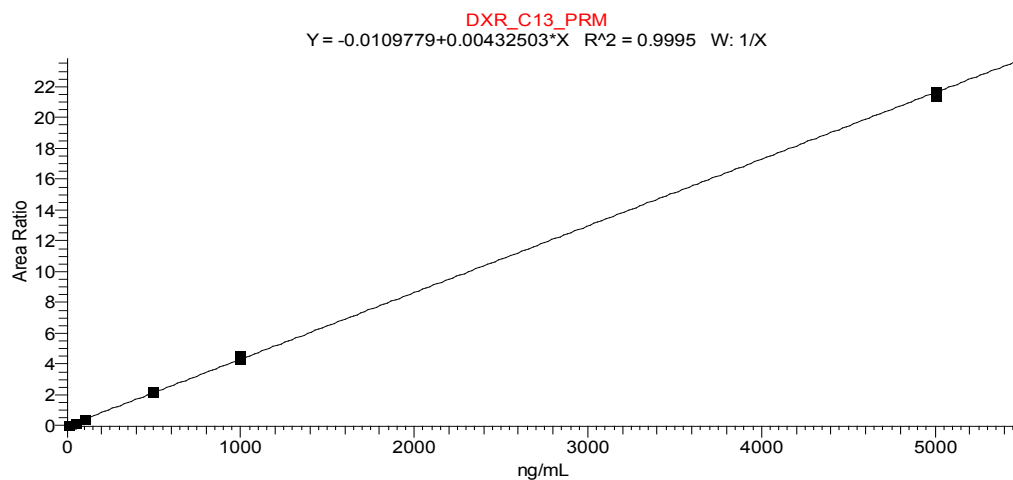
Figure IV-2. DXR_C13 Plasma Standard Curve.

Table IV-3. DXR Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	23387	0.106	106
0.100 ng/mL	18189	0.095	95
1.00 ng/mL	251805	0.92	92
1.00 ng/mL	266179	0.97	97
5.00 ng/mL	1671179	5.57	111
5.00 ng/mL	1408102	4.86	97
10.0 ng/mL	2985762	9.8	98
10.0 ng/mL	2911653	9.2	92
100.0 ng/mL	32929084	105.8	106
100.0 ng/mL	33790011	103.6	104
1000.0 ng/mL	358392962	1058.2	106
1000.0 ng/mL	353819312	962.7	96
QC			
QCL	20565	0.105	105
QCL	21464	0.107	107
QCM	1387863	4.55	91
QCM	1409503	5.00	100
QCH	34575903	102.6	103
QCH	33541632	101.1	101

DXR_PRM
Y = -0.000407639+0.0093624*X   R^2 = 0.9955   W: 1/X^2
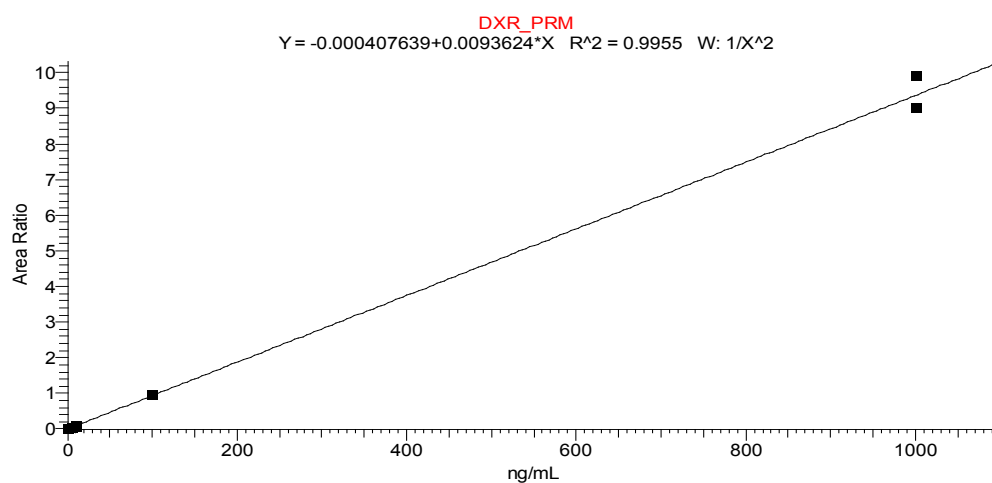
Figure IV-3. DXR Protein-Free Plasma Standard Curve.

Table IV-4. DXR_C13 Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	6225	0.094	94
0.100 ng/mL	7882	0.106	107
1.00 ng/mL	118890	0.95	95
1.00 ng/mL	126108	1.00	100
5.00 ng/mL	694960	5.00	100
5.00 ng/mL	680972	5.07	101
10.0 ng/mL	1373923	9.8	98
10.0 ng/mL	1407900	9.5	95
100.0 ng/mL	15219895	105.2	105
100.0 ng/mL	15839525	104.5	105
1000.0 ng/mL	164360113	1044.7	105
1000.0 ng/mL	162901404	954.1	95
QC			
QCL	7737	0.108	108
QCL	7855	0.109	109
QCM	666188	4.72	94
QCM	671863	5.15	103
QCH	17115308	109.4	109
QCH	16417097	106.6	107

DXR_C13_PRM
$Y = -0.000253653 + 0.00434929 \cdot X$ $R^2 = 0.9977$ W: $1/X^2$
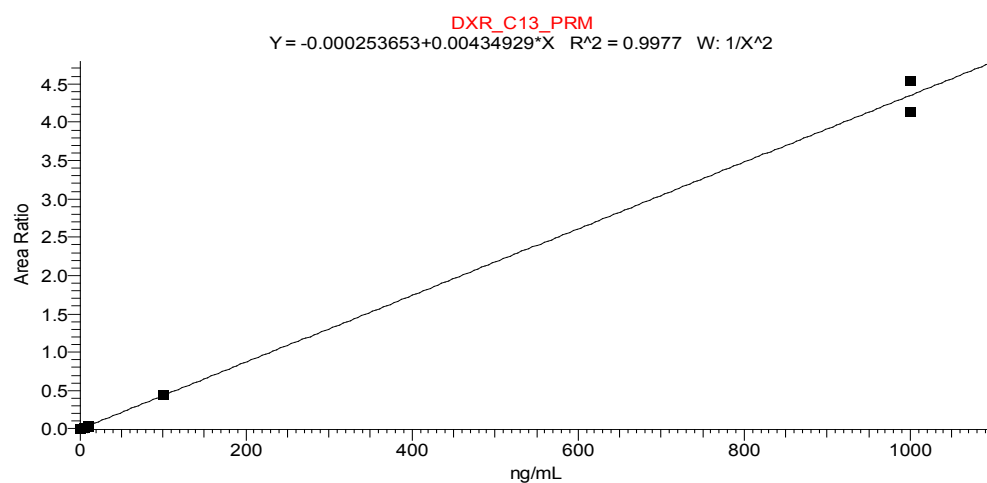
Table IV-4. DXR_C13 Protein-Free Plasma Standard Curve.

Table IV-5. Free Doxorubicin HCl Control Analytical Data. Presented are the analytical data for the free doxorubicin HCl controls. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 − %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	80.3	475	16.9	83.1	22.2	119	18.7	81.3	429.1	46	90.2	90.4	0.3
		74.8	449	16.7	83.3	20.9	113	18.5	81.5	405.5	43	90.3		
		81.5	464	17.6	82.4	21.5	111	19.4	80.6	420.7	43	90.8		
	1 µg/mL	172.9	902	19.2	80.8	23.0	109	21.0	79.0	821.5	81	91.1	91.3	0.6
		177.5	1036	17.1	82.9	23.5	126	18.6	81.4	953.6	83	92.0		
		163.0	888	18.4	81.6	20.9	104	20.2	79.8	807.5	81	90.9		
	5 µg/mL	911.5	5148	17.7	82.3	22.9	119	19.2	80.8	4756.7	392	92.4	91.6	0.7
		961.7	5050	19.0	81.0	24.5	117	20.9	79.1	4599.1	451	91.1		
		815.1	4284	19.0	81.0	21.6	104	20.8	79.2	3917.9	366	91.5		
2 hr	0.5 µg/mL	69.6	425	16.4	83.6	19.1	105	18.1	81.9	384.1	41	90.3	90.0	0.6
		67.5	407	16.6	83.4	18.7	102	18.3	81.7	368.1	39	90.4		
		61.4	393	15.6	84.4	16.6	95	17.5	82.5	351.1	42	89.4		
	1 µg/mL	125.4	793	15.8	84.2	16.7	96	17.4	82.6	722.1	71	91.1	91.2	0.8
		139.9	838	16.7	83.3	19.0	103	18.4	81.6	758.6	80	90.5		
		139.8	851	16.4	83.6	18.1	102	17.8	82.2	784.0	67	92.1		
	5 µg/mL	602.7	4041	14.9	85.1	15.3	93	16.4	83.6	3669.0	372	90.8	91.7	0.8
		747.7	3944	19.0	81.0	19.6	95	20.5	79.5	3640.8	304	92.3		
		716.6	4099	17.5	82.5	19.1	100	19.0	81.0	3765.4	333	91.9		
6 hr	0.5 µg/mL	56.8	239	23.7	76.3	15.6	48	32.5	67.5	174.7	65	73.0	99.1	28.1
		56.3	263	21.4	78.6	15.5	69	22.5	77.5	250.4	12	95.4		
		42.3	264	16.0	84.0	11.2	90	12.4	87.6	340.3	-76	128.9		
	1 µg/mL	78.4	568	13.8	86.2	10.5	70	15.0	85.0	524.3	43	92.4	97.8	8.4
		126.2	584	21.6	78.4	16.9	73	23.1	76.9	545.9	38	93.6		
		100.7	624	16.1	83.9	13.3	89	15.0	85.0	670.9	-47	107.5		
	5 µg/mL	363.8	2957	12.3	87.7	9.3	87	10.7	89.3	3387.6	-430	114.5	100.5	14.0
		638.6	3226	19.8	80.2	16.4	72	22.9	77.1	2790.8	435	86.5		
		651.0	3602	18.1	81.9	17.1	95	18.0	82.0	3613.1	-11	100.3		
24 hr	0.5 µg/mL	17.4	134	13.0	87.0	4.9	34	14.5	85.5	120.2	13	90.0	98.2	13.3
		21.5	125	17.2	82.8	6.1	32	18.9	81.1	113.7	11	91.1		
		0.9	15	6.1	93.9	0.3	5	5.4	94.6	16.5	-2	113.5		
	1 µg/mL	1.3	15	8.6	91.4	0.2	4	5.1	94.9	24.9	-10	169.6	126.3	39.4
		47.1	278	16.9	83.1	6.5	36	18.3	81.7	257.8	21	92.6		
		4.4	44	10.0	90.0	0.6	7	8.6	91.4	51.2	-7	116.6		
	5 µg/mL	8.9	80	11.2	88.8	0.3	4	6.2	93.8	144.8	-65	181.4	122.8	50.8
		238.5	1752	13.6	86.4	6.2	42	14.6	85.4	1638.6	113	93.6		
		224.2	1550	14.5	85.5	6.1	39	15.5	84.5	1446.3	104	93.3		

Table IV-6. Sun Pharma, Lot A (JKR0494A) Drug Release Analytical Data. Presented are the analytical data for Sun Pharma, Lot A (JKR0494A). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 – %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	0.8	339	0.2	99.8	30.3	137	22.1	77.9	3.8	335	1.1	1.1	0.2
		0.6	333	0.2	99.8	28.6	131	21.9	78.1	2.9	331	0.9		
		1.3	369	0.4	99.6	34.8	130	26.9	73.1	4.9	364	1.3		
	1 µg/mL	1.2	702	0.2	99.8	30.3	129	23.5	76.5	5.0	697	0.7	0.8	0.1
		1.5	683	0.2	99.8	35.7	126	28.2	71.8	5.2	678	0.8		
		1.4	759	0.2	99.8	31.1	136	22.8	77.2	6.3	752	0.8		
	5 µg/mL	4.9	3497	0.1	99.9	35.6	127	28.0	72.0	17.6	3479	0.5	0.4	0.1
		3.3	3568	0.1	99.9	31.1	125	24.9	75.1	13.2	3555	0.4		
		2.8	3481	0.1	99.9	30.4	129	23.5	76.5	11.8	3469	0.3		
2 hr	0.5 µg/mL	0.8	357	0.2	99.8	20.3	109	18.7	81.3	4.0	353	1.1	1.3	0.2
		0.8	290	0.3	99.7	20.2	98	20.6	79.4	4.1	286	1.4		
		1.1	354	0.3	99.7	26.6	119	22.3	77.7	4.9	349	1.4		
	1 µg/mL	1.4	843	0.2	99.8	24.5	113	21.7	78.3	6.4	836	0.8	0.9	0.1
		1.9	690	0.3	99.7	27.9	108	25.7	74.3	7.2	683	1.0		
		1.4	749	0.2	99.8	25.6	115	22.2	77.8	6.2	743	0.8		
	5 µg/mL	5.4	3766	0.1	99.9	31.1	120	26.0	74.0	20.9	3745	0.6	0.5	0.1
		3.9	3299	0.1	99.9	20.5	107	19.1	80.9	20.4	3278	0.6		
		2.7	3408	0.1	99.9	25.4	116	21.8	78.2	12.3	3396	0.4		
6 hr	0.5 µg/mL	0.5	312	0.2	99.8	6.4	97	6.6	93.4	7.8	304	2.5	1.9	0.6
		0.8	302	0.3	99.7	12.7	86	14.7	85.3	5.4	296	1.8		
		1.0	419	0.2	99.8	18.4	106	17.3	82.7	5.5	414	1.3		
	1 µg/mL	1.3	698	0.2	99.8	13.1	86	15.4	84.6	8.2	690	1.2	1.1	0.3
		1.3	715	0.2	99.8	14.0	99	14.2	85.8	9.0	706	1.3		
		1.2	838	0.1	99.9	19.1	100	19.2	80.8	6.4	832	0.8		
	5 µg/mL	4.4	3495	0.1	99.9	18.3	95	19.3	80.7	22.7	3472	0.6	0.6	0.3
		4.0	3686	0.1	99.9	11.2	92	12.2	87.8	33.0	3653	0.9		
		3.2	4013	0.1	99.9	20.1	95	21.1	78.9	15.0	3998	0.4		
24 hr	0.5 µg/mL	1.2	395	0.3	99.7	0.1	4	3.2	96.8	35.7	360	9.0	4.0	4.4
		0.9	521	0.2	99.8	1.6	15	10.7	89.3	8.5	513	1.6		
		0.9	441	0.2	99.8	5.5	33	16.7	83.3	5.4	436	1.2		
	1 µg/mL	2.0	750	0.3	99.7	1.0	10	9.7	90.3	20.7	729	2.8	2.5	1.6
		2.0	1208	0.2	99.8	0.4	9	4.0	96.0	49.1	1159	4.1		
		1.5	1137	0.1	99.9	6.2	38	16.2	83.8	9.3	1128	0.8		
	5 µg/mL	7.6	4417	0.2	99.8	5.4	44	12.4	87.6	61.3	4356	1.4	5.7	7.7
		10.6	4805	0.2	99.8	0.3	22	1.5	98.5	700.0	4105	14.6		
		6.2	4865	0.1	99.9	5.6	49	11.4	88.6	54.5	4811	1.1		

Table IV-7. Sun Pharma, Lot B (JKR0154A) Drug Release Analytical Data. Presented are the analytical data for Sun Pharma, Lot B (JKR0154A). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR	% Bound	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13	% Bound DXR_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG	SD
				= Filtrate DXR / Reservoir DXR *100	= 100 − %Unbound DXR			= Filtrate DXR_C13 / Reservoir DXR_C13 * 100	= 100 − %Unbound C13	= Fil DXR/ (1-(%Bound DXR_C13/100))	= Reservoir DXR- Unencapsulated	= (Unencapsulated/ Reservoir DXR) *100		
10 min	0.5 µg/mL	0.9	348	0.2	99.8	34.1	125	27.2	72.8	3.2	345	0.9	1.0	0.2
		0.9	362	0.3	99.7	34.8	127	27.3	72.7	3.4	358	1.0		
		1.5	387	0.4	99.6	38.4	127	30.3	69.7	4.8	382	1.3		
	1 µg/mL	1.6	743	0.2	99.8	35.6	125	28.4	71.6	5.4	738	0.7	0.7	0.04
		1.3	753	0.2	99.8	27.5	123	22.4	77.6	5.6	747	0.7		
		1.3	779	0.2	99.8	30.7	127	24.1	75.9	5.3	774	0.7		
	5 µg/mL	4.6	3323	0.1	99.9	32.9	120	27.3	72.7	16.7	3306	0.5	0.9	0.5
		5.4	3362	0.2	99.8	28.0	124	22.5	77.5	23.9	3338	0.7		
		11.6	3473	0.3	99.7	27.4	126	21.7	78.3	53.2	3420	1.5		
2 hr	0.5 µg/mL	0.8	344	0.2	99.8	25.9	119	21.8	78.2	3.4	340	1.0	1.1	0.1
		0.8	320	0.2	99.8	24.4	109	22.4	77.6	3.4	317	1.1		
		1.0	390	0.3	99.7	25.8	118	21.8	78.2	4.7	386	1.2		
	1 µg/mL	1.4	840	0.2	99.8	22.8	122	18.7	81.3	7.4	832	0.9	0.8	0.1
		1.4	801	0.2	99.8	26.9	118	22.8	77.2	5.9	795	0.7		
		1.4	808	0.2	99.8	29.8	113	26.4	73.6	5.4	802	0.7		
	5 µg/mL	4.1	3758	0.1	99.9	24.7	120	20.5	79.5	19.8	3738	0.5	0.9	0.5
		5.5	3490	0.2	99.8	24.2	114	21.2	78.8	25.7	3464	0.7		
		12.1	3491	0.3	99.7	25.5	109	23.3	76.7	52.0	3439	1.5		
6 hr	0.5 µg/mL	0.6	516	0.1	99.9	17.3	84	20.7	79.3	2.9	513	0.6	0.7	0.2
		0.6	498	0.1	99.9	15.9	68	23.2	76.8	2.7	495	0.5		
		0.8	434	0.2	99.8	17.2	77	22.3	77.7	3.7	430	0.9		
	1 µg/mL	1.6	1001	0.2	99.8	14.9	50	30.0	70.0	5.4	995	0.5	0.5	0.04
		1.1	1043	0.1	99.9	17.5	82	21.5	78.5	5.2	1038	0.5		
		1.0	1079	0.1	99.9	17.3	83	21.0	79.0	4.9	1074	0.5		
	5 µg/mL	4.2	4510	0.1	99.9	16.2	86	18.9	81.1	22.1	4487	0.5	0.6	0.3
		4.7	4688	0.1	99.9	16.1	63	25.4	74.6	18.3	4670	0.4		
		9.3	4783	0.2	99.8	16.9	78	21.6	78.4	43.1	4740	0.9		
24 hr	0.5 µg/mL	0.8	575	0.1	99.9	6.1	40	15.1	84.9	5.1	570	0.9	0.8	0.1
		0.8	772	0.1	99.9	4.7	33	14.2	85.8	5.7	766	0.7		
		0.9	654	0.1	99.9	6.4	40	15.9	84.1	5.8	648	0.9		
	1 µg/mL	1.5	1254	0.1	99.9	0.4	10	4.1	95.9	35.7	1219	2.8	1.6	1.1
		1.6	970	0.2	99.8	6.4	43	14.9	85.1	10.5	960	1.1		
		1.6	1176	0.1	99.9	6.4	43	14.9	85.1	10.7	1166	0.9		
	5 µg/mL	7.1	4899	0.1	99.9	4.7	45	10.4	89.6	67.5	4832	1.4	1.6	0.3
		8.5	5298	0.2	99.8	5.3	50	10.6	89.4	80.1	5218	1.5		
		10.1	4896	0.2	99.8	5.0	47	10.6	89.4	94.9	4801	1.9		

Table IV-8. Sun Pharma, Lot C (JKR0491A) Drug Release Analytical Data. Presented are the analytical data for Sun Pharma, Lot C (JKR0491A). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR	% Bound	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13	% Bound DXR_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG	SD
				= Filtrate DXR / Reservoir DXR *100	= 100 − %Unbound DXR			= Filtrate DXR_C13 / Reservoir DXR_C13 * 100	= 100 − %Unbound C13	= Fil DXR/ (1-(%Bound DXR_C13/100))	= Reservoir DXR- Unencapsulated	= (Unencapsulated/ Reservoir DXR) *100		
10 min	0.5 µg/mL	0.9	540	0.2	99.8	19.2	121	15.9	84.1	5.5	534	1.0	1.2	0.4
		0.7	512	0.1	99.9	19.3	118	16.3	83.7	4.5	507	0.9		
		1.4	502	0.3	99.7	20.0	123	16.2	83.8	8.7	494	1.7		
	1 µg/mL	5.9	950	0.6	99.4	23.5	107	22.0	78.0	27.0	922	2.8	1.5	1.1
		1.7	916	0.2	99.8	21.8	104	21.0	79.0	7.9	908	0.9		
		1.5	991	0.2	99.8	19.5	110	17.8	82.2	8.5	982	0.9		
	5 µg/mL	5.6	4634	0.1	99.9	23.3	115	20.2	79.8	27.6	4607	0.6	0.6	0.02
		5.5	5075	0.1	99.9	19.7	108	18.3	81.7	30.1	5045	0.6		
		6.2	5070	0.1	99.9	20.7	107	19.4	80.6	31.8	5038	0.6		
2 hr	0.5 µg/mL	0.7	399	0.2	99.8	17.2	99	17.3	82.7	4.1	395	1.0	1.0	0.1
		0.7	408	0.2	99.8	17.0	93	18.4	81.6	3.9	404	1.0		
		0.8	420	0.2	99.8	17.2	103	16.8	83.2	4.6	415	1.1		
	1 µg/mL	1.3	950	0.1	99.9	12.9	89	14.5	85.5	9.0	941	0.9	0.9	0.1
		1.4	971	0.1	99.9	16.5	94	17.5	82.5	8.1	963	0.8		
		1.4	824	0.2	99.8	15.7	82	19.2	80.8	7.4	817	0.9		
	5 µg/mL	5.0	4413	0.1	99.9	18.0	99	18.1	81.9	27.4	4385	0.6	0.6	0.03
		4.5	4201	0.1	99.9	13.6	85	16.0	84.0	27.9	4173	0.7		
		5.3	4553	0.1	99.9	16.4	88	18.6	81.4	28.2	4524	0.6		
6 hr	0.5 µg/mL	0.8	397	0.2	99.8	13.4	96	14.0	86.0	5.5	391	1.4	1.4	0.1
		0.6	369	0.2	99.8	11.0	92	12.0	88.0	5.3	364	1.4		
		0.8	316	0.2	99.8	14.4	75	19.3	80.7	4.1	312	1.3		
	1 µg/mL	1.6	540	0.3	99.7	6.8	65	10.5	89.5	15.3	525	2.8	1.8	0.9
		1.4	763	0.2	99.8	13.4	92	14.6	85.4	9.3	753	1.2		
		1.4	642	0.2	99.8	12.0	76	15.9	84.1	8.7	634	1.4		
	5 µg/mL	5.7	2791	0.2	99.8	14.0	65	21.5	78.5	26.6	2764	0.95	1.1	0.2
		4.6	4204	0.1	99.9	8.0	97	8.3	91.7	55.2	4149	1.31		
		5.5	4185	0.1	99.9	13.0	101	12.8	87.2	42.9	4142	1.02		
24 hr	0.5 µg/mL	6.2	181	3.4	96.6	4.2	32	13.2	86.8	47.3	134	26.1	11.3	12.9
		1.1	148	0.8	99.2	2.2	15	14.4	85.6	7.9	140	5.3		
		1.0	206	0.5	99.5	6.1	32	18.9	81.1	5.3	200	2.6		
	1 µg/mL	2.4	638	0.4	99.6	0.2	6	3.2	96.8	76.4	562	12.0	9.3	6.3
		2.0	598	0.3	99.7	3.9	25	15.6	84.4	12.5	585	2.1		
		2.8	784	0.4	99.6	0.2	8	2.6	97.4	108.7	675	13.9		
	5 µg/mL	8.2	3583	0.2	99.8	4.6	38	12.1	87.9	67.7	3515	1.9	5.1	5.5
		12.4	3619	0.3	99.7	0.5	18	3.0	97.0	413.2	3206	11.4		
		9.2	3788	0.2	99.8	4.2	34	12.4	87.6	74.7	3713	2.0		

Table IV-9. Free Doxorubicin HCl Control Data. Displayed is the calculated % release for the doxorubicin HCl control data. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	90.4	0.3	0.3
	1 µg/mL	91.3	0.6	0.6
	5 µg/mL	91.6	0.7	0.7
2 hr	0.5 µg/mL	90.0	0.6	0.6
	1 µg/mL	91.2	0.8	0.9
	5 µg/mL	91.7	0.8	0.8
6 hr	0.5 µg/mL	99.1	28.1	28.4
	1 µg/mL	97.8	8.4	8.6
	5 µg/mL	100.5	14.0	14.0
24 hr	0.5 µg/mL	98.2	13.3	13.5
	1 µg/mL	126.3	39.4	31.2
	5 µg/mL	122.8	50.8	41.4
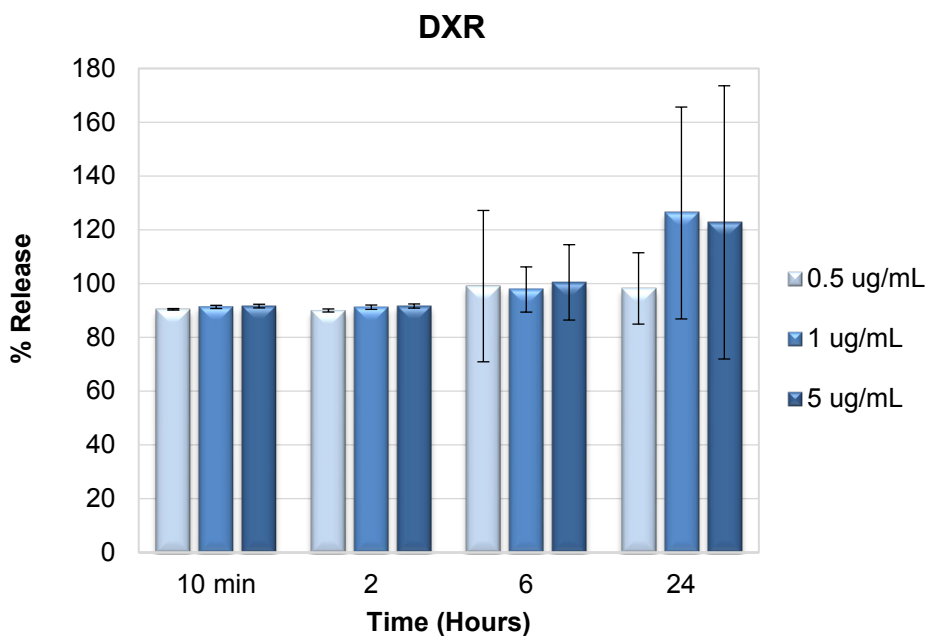
Figure IV-5. Free Doxorubicin HCl Control Data. Displayed is the calculated % release for the doxorubicin HCl control data. (Mean $\pm$ SD, *N=3*)

Table IV-10. Sun Pharma, Lot A (JKR0494A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot A (JKR0494A). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	1.1	0.2	21.3
	1 µg/mL	0.8	0.1	7.7
	5 µg/mL	0.4	0.1	21.7
2 hr	0.5 µg/mL	1.3	0.2	11.6
	1 µg/mL	0.9	0.1	16.5
	5 µg/mL	0.5	0.1	26.2
6 hr	0.5 µg/mL	1.9	0.6	32.3
	1 µg/mL	1.1	0.3	24.7
	5 µg/mL	0.6	0.3	40.8
24 hr	0.5 µg/mL	4.0	4.4	110.9
	1 µg/mL	2.5	1.6	64.1
	5 µg/mL	5.7	7.7	135.1
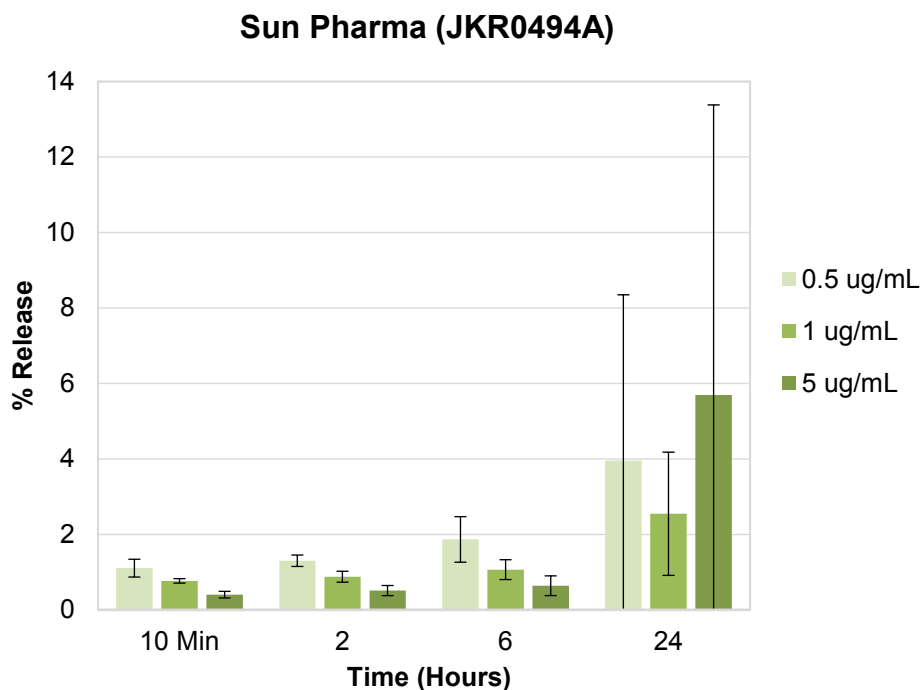
Figure IV-6. Sun Pharma, Lot A (JKR0494A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot A (JKR0494A). (Mean $\pm$ SD, *N=3*)

Table IV-11. Sun Pharma, Lot B (JKR0154A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot B (JKR0154A). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	1.0	0.2	17.9
	1 µg/mL	0.7	0.04	5.1
	5 µg/mL	0.9	0.5	59.4
2 hr	0.5 µg/mL	1.1	0.1	9.3
	1 µg/mL	0.8	0.1	14.5
	5 µg/mL	0.9	0.5	55.1
6 hr	0.5 µg/mL	0.7	0.2	27.2
	1 µg/mL	0.5	0.04	8.3
	5 µg/mL	0.6	0.3	45.5
24 hr	0.5 µg/mL	0.8	0.1	10.0
	1 µg/mL	1.6	1.1	66.6
	5 µg/mL	1.6	0.3	18.2
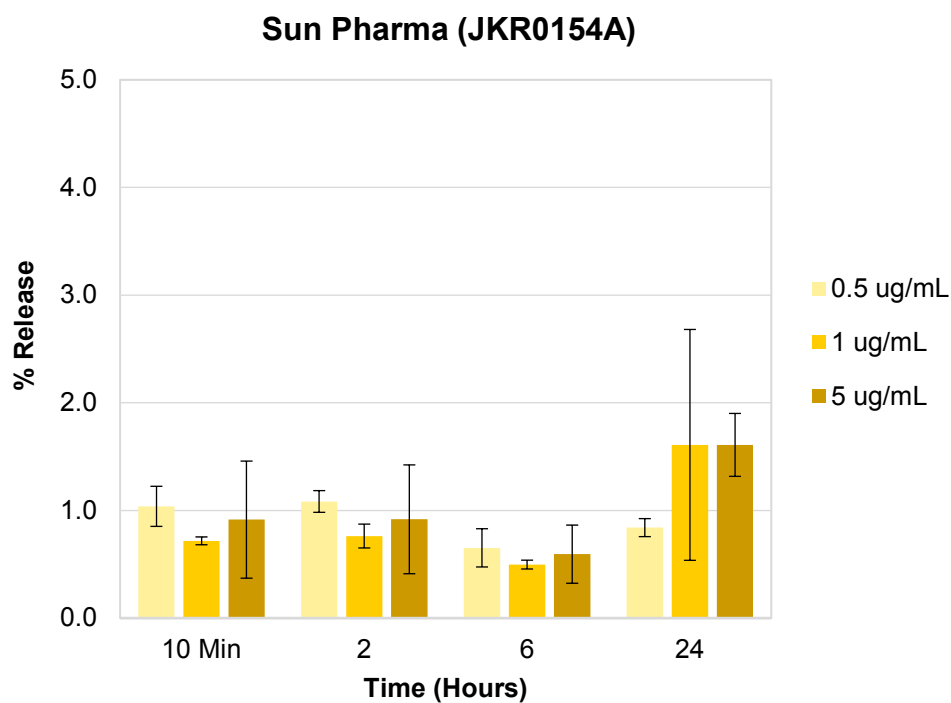
Figure IV-7. Sun Pharma, Lot B (JKR0154A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot B (JKR0154A). (Mean $\pm$ SD, *N=3*)

Table IV-12. Sun Pharma, Lot C (JKR0491A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot C (JKR0491A). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	1.2	0.4	37.0
	1 µg/mL	1.5	1.1	75.1
	5 µg/mL	0.6	0.02	3.1
2 hr	0.5 µg/mL	1.0	0.1	7.0
	1 µg/mL	0.9	0.1	6.5
	5 µg/mL	0.6	0.02	4.0
6 hr	0.5 µg/mL	1.4	0.1	5.6
	1 µg/mL	1.8	0.9	49.6
	5 µg/mL	1.1	0.2	17.4
24 hr	0.5 µg/mL	11.3	12.9	113.6
	1 µg/mL	9.3	6.3	67.9
	5 µg/mL	5.1	5.5	107.5
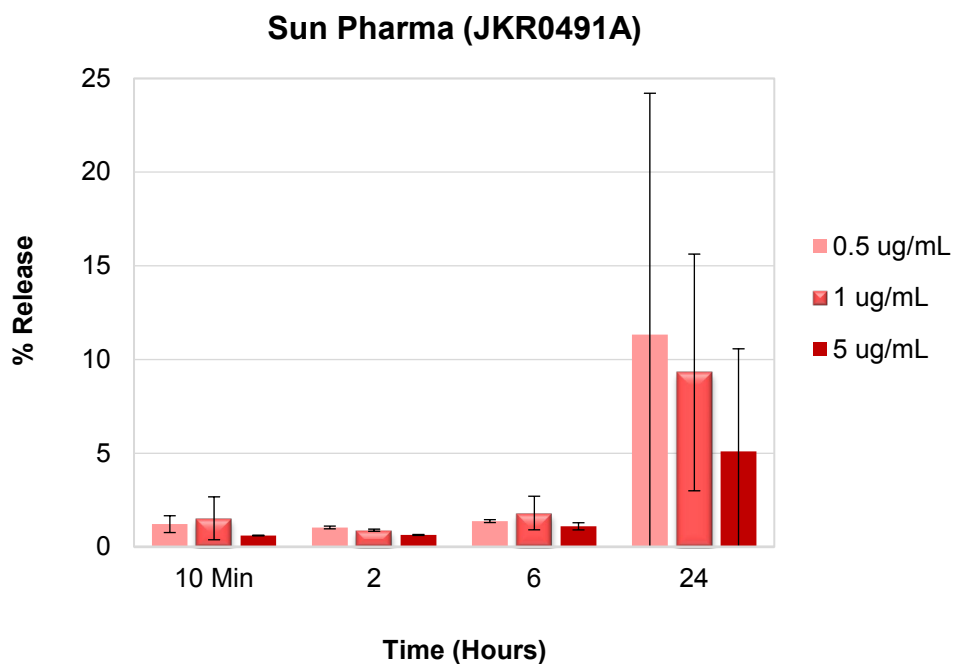
Figure IV-8. Sun Pharma, Lot C (JKR0491A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot C (JKR0491A). (Mean $\pm$ SD, *N=3*)

A.
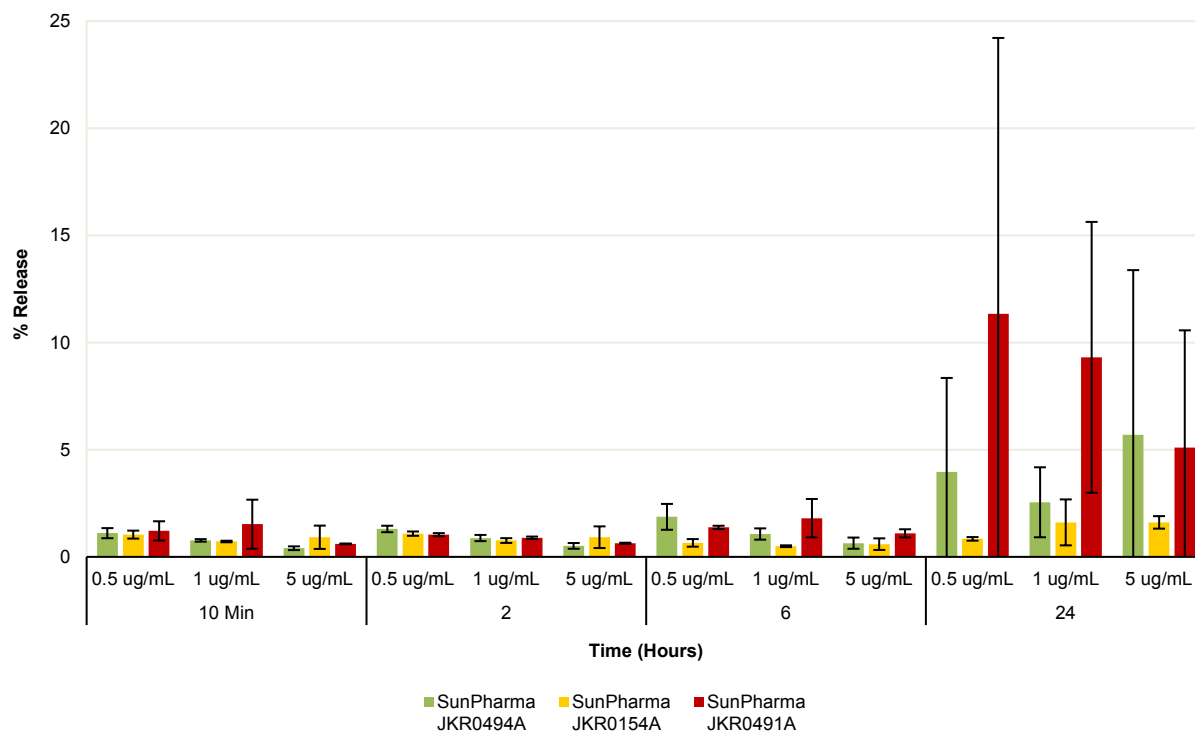
B.
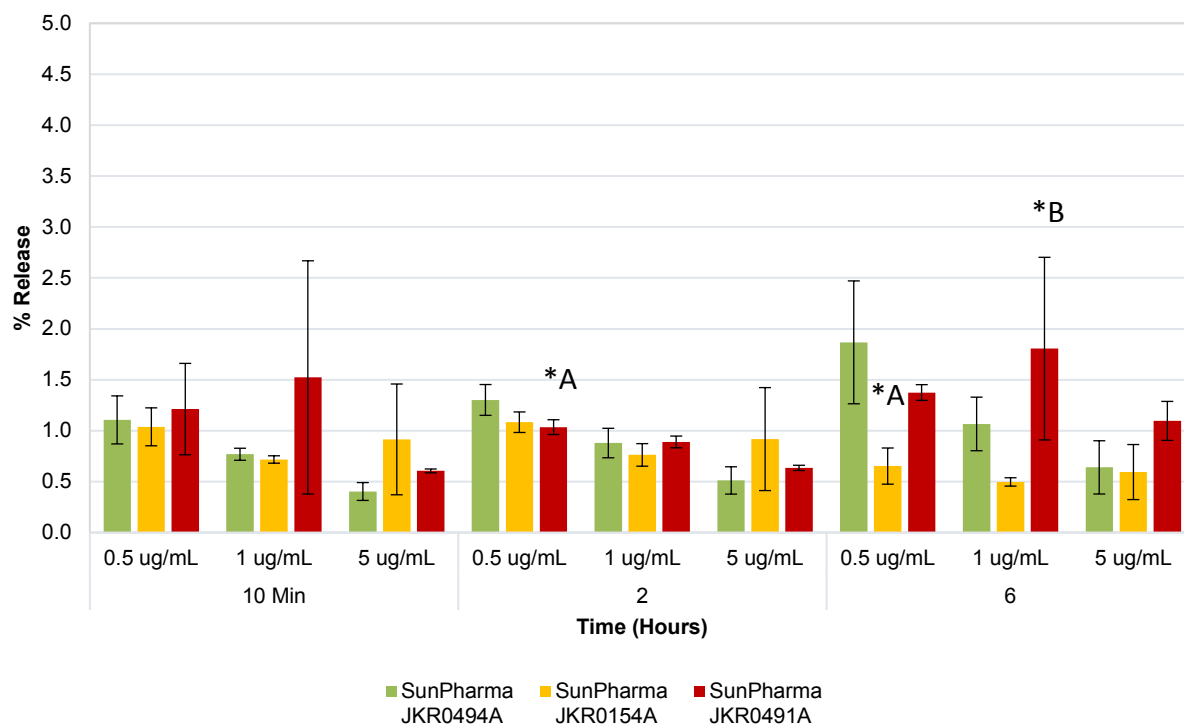
Figure IV-9. Sun Pharma Drug Release Lot Comparison. Displayed are the calculated % release for the Sun Pharma Lots JKR0494A, JKR0154A, and JKR0491A, also denoted as lots A, B, and C, time points 10 min-24 hr (**A**) and 0-6 hr (**B**). (Mean $\pm$ SD, *N=3*), *$p \leq 0.05$, ANOVA with Duncan's Multiple Range posthoc test.

Bridging Study for In Vivo BE Sun Pharma Lot

Summary
The objective of this study was to compare the in vitro drug release of a new lot of Sun Pharma's liposomal doxorubicin, to be used in the rat BE studies, to two previous lots in human plasma at 37°C over a 6 hr period. All lots had similar drug release profiles over the 6 hr period, although some instances of statistically significant differences were observed between lots.

Design and Methods
The new lot of Sun Pharma used in this study is JKR0865A (also denoted as lot D), and it is this lot that was used in the in vivo BE study. This lot was compared to lots JKR0494A and JKR0154A (also denoted as lots A and B). All three lots were evaluated on the same day for drug release in human plasma at 37°C over a 6 hr period, according to the SITUA method described in Section I. Free doxorubicin HCl at identical concentrations and time points was included as a control. The human plasma and protein-free plasma DXR and DXR_C13 standard calibration curves used for this study are also provided.

Results & Conclusions
The DXR and DXR_C13 standard calibration curves used for this study are presented in **Tables IV-13 to IV-16 and Figures IV-10 to IV-13**. The free doxorubicin HCl controls averaged between 105-117% of theoretical for all concentrations and time points (**Tables IV-17 and 21, and Figure IV-14**). The Sun Pharma drug release was similar for Lot's A, B and D, at approximately 1% release over the 6 hr period, without a clear temporal trend (**Tables IV-18 to IV-20 and IV-22 to IV-24, and Figures IV-15 to IV-19**).

Table IV-13. DXR Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	2663714	10.7	107
10.0 ng/mL	2700617	10.8	108
50 ng/mL	14620688	49	98
50 ng/mL	13950939	47	94
100 ng/mL	30118691	99	99
100 ng/mL	29704494	97	97
500 ng/mL	150046299	483	97
500 ng/mL	153843256	495	99
1000 ng/mL	310382104	997	100
1000 ng/mL	319924934	1027	103
5000 ng/mL	1549728762	4968	99
5000 ng/mL	1562274672	5008	100
10000 ng/mL	3185434389	10209	102
10000 ng/mL	3064124028	9820	98
QC			
QCL	2936939	11.6	115
QCL	2771288	11.0	110
QCM	30263394	99	99
QCM	27993056	92	92
QCH	279788398	899	90
QCH	313203415	1006	101

DXR_PRM
$Y = 0.00211304 + 0.00446164 \cdot X$ $R^2 = 0.9990$ W: 1/X
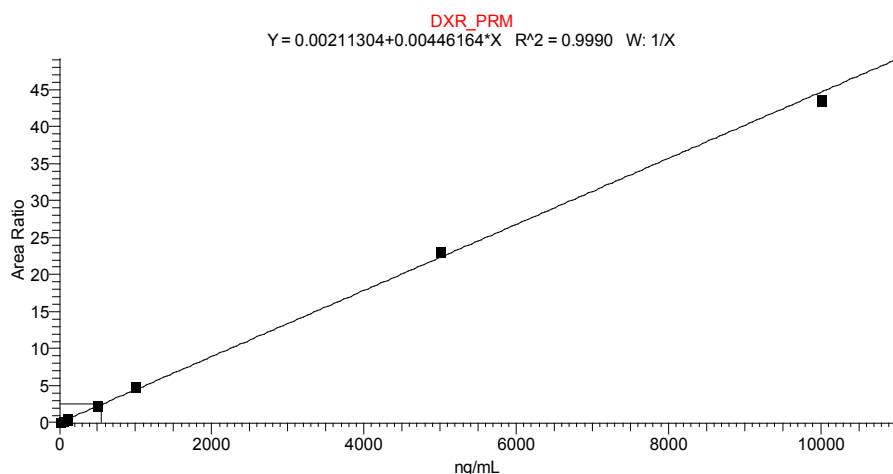
Figure IV-10. DXR Plasma Standard Curve.

Table IV-14. DXR_C13 Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	1330186	9.9	98
10.0 ng/mL	1440011	10.6	106
50 ng/mL	7330871	49	98
50 ng/mL	7073394	48	95
100 ng/mL	14951541	99	99
100 ng/mL	14708668	98	98
500 ng/mL	73707531	484	97
500 ng/mL	76761511	504	101
1000 ng/mL	157927327	1037	104
1000 ng/mL	157946059	1037	104
5000 ng/mL	756776674	4963	99
5000 ng/mL	785580835	5152	103
10000 ng/mL	1516932787	9948	99
10000 ng/mL	1506659334	9880	99
QC			
QCL	1431310	10.5	105
QCL	1392581	10.3	103
QCM	14923818	99	99
QCM	13875848	92	92
QCH	141328969	928	93
QCH	160286068	1052	105

DXR_C13_PRM
$Y = 0.00256778 + 0.00306105 * X$ $R^2 = 0.9987$ W: 1/X
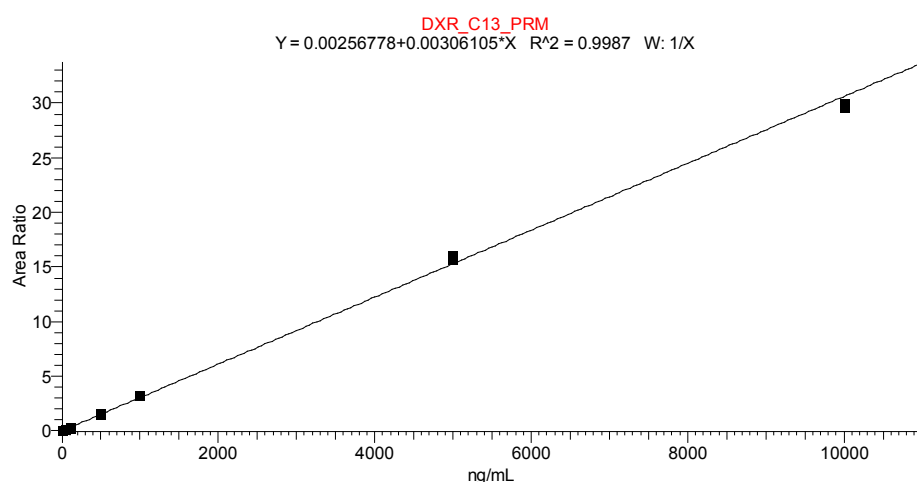
Figure IV-11. DXR_C13 Plasma Standard Curve.

Table IV-15. DXR Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	23913	0.094	94
0.100 ng/mL	37136	0.109	109
0.50 ng/mL	138728	0.49	98
0.50 ng/mL	132092	0.49	98
1.00 ng/mL	291707	0.96	96
1.00 ng/mL	304713	0.87	87
5.00 ng/mL	1630476	4.62	92
5.00 ng/mL	1602109	4.65	93
10.0 ng/mL	3470234	9.8	98
10.0 ng/mL	3537159	10.5	105
100.0 ng/mL	38153356	107.9	108
100.0 ng/mL	36770406	107.1	107
1000.0 ng/mL	395923611	1101.7	110
1000.0 ng/mL	398185092	1049.7	105
QC			
QCL	30486	0.084	84
QCL	32021	0.120	120
QCM	302121	1.01	101
QCM	289110	0.94	94
QCH	3524591	10.1	101
QCH	3423363	9.8	98

DXR_PRM
$Y = -2.06484e\text{-}005 + 0.00404479 * X$ $R^2 = 0.9937$ W: $1/X^2$
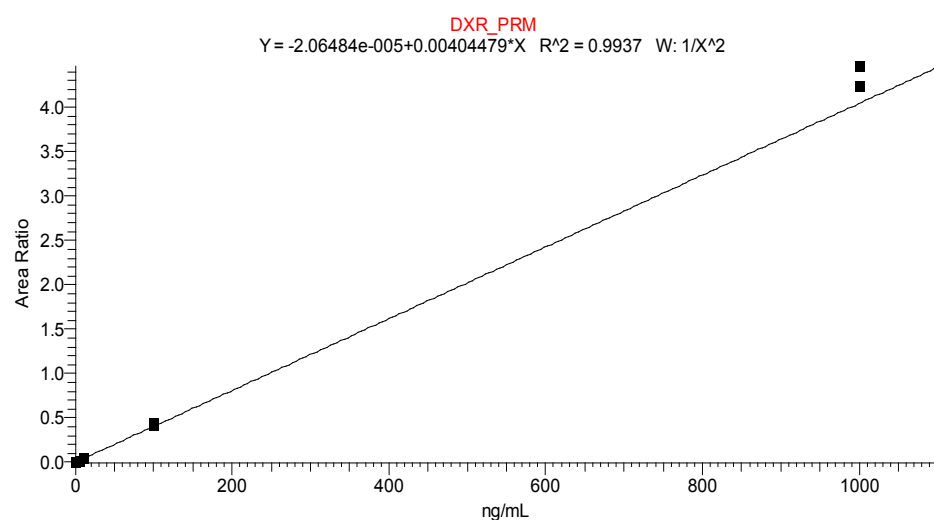
Figure IV-12. DXR Protein-Free Plasma Standard Curve.

Table IV-16. DXR_C13 Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	21312	0.091	91
0.100 ng/mL	32227	0.110	110
0.50 ng/mL	93943	0.53	106
0.50 ng/mL	77710	0.45	91
1.00 ng/mL	168006	0.92	92
1.00 ng/mL	220865	1.06	106
5.00 ng/mL	909023	4.48	90
5.00 ng/mL	912069	4.61	92
10.0 ng/mL	1945089	9.6	96
10.0 ng/mL	1982298	10.3	103
100.0 ng/mL	21725346	108.1	108
100.0 ng/mL	20275903	103.9	104
1000.0 ng/mL	222029294	1087.5	109
1000.0 ng/mL	222008228	1030.1	103
QC			
QCL	34274	0.108	108
QCL	18641	0.069	69
QCM	174944	0.98	98
QCM	181787	0.99	99
QCH	1968010	9.8	98
QCH	1951655	9.8	98

DXR_C13_PRM
$Y = 0.000110937 + 0.00229778 \cdot X \quad R^2 = 0.9929 \quad W: 1/X^2$
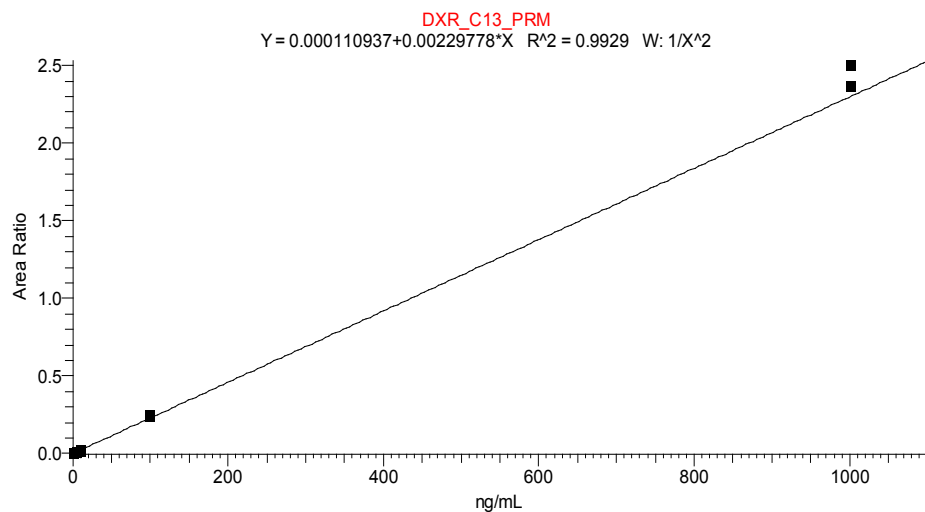
Figure IV-13. DXR_C13 Protein-Free Plasma Standard Curve.

Table IV-17. Free Doxorubicin HCl Control Analytical Data. Presented are the analytical data for the free doxorubicin HCl controls. The %protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 − %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	66.8	563	11.9	88.1	14.4	125	11.5	88.5	581.2	-18	103.2	104.8	1.7
		63.2	573	11.0	89.0	13.0	123	10.5	89.5	599.6	-26	104.6		
		70.7	629	11.2	88.8	14.3	136	10.5	89.5	670.9	-42	106.7		
	1 µg/mL	139.0	1096	12.7	87.3	14.2	119	11.9	88.1	1163.3	-67	106.2	107.8	1.5
		144.7	1308	11.1	88.9	14.2	139	10.2	89.8	1412.3	-104	108.0		
		135.4	1265	10.7	89.3	13.3	136	9.8	90.2	1380.7	-116	109.2		
	5 µg/mL	852.3	6528	13.1	86.9	15.9	131	12.2	87.8	6985.7	-457	107.0	108.1	3.3
		929.8	6326	14.7	85.3	17.3	124	13.9	86.1	6673.8	-348	105.5		
		925.5	7042	13.1	86.9	17.3	147	11.8	88.2	7872.9	-831	111.8		
2 hr	0.5 µg/mL	81.2	544	14.9	85.1	16.3	117	13.9	86.1	583.3	-39	107.2	106.2	1.1
		70.2	582	12.1	87.9	14.2	125	11.3	88.7	618.8	-37	106.4		
		77.9	479	16.3	83.7	16.3	105	15.5	84.5	503.2	-24	105.0		
	1 µg/mL	156.8	967	16.2	83.8	15.2	101	15.0	85.0	1042.3	-76	107.8	108.5	0.6
		176.3	1009	17.5	82.5	17.8	111	16.1	83.9	1096.4	-88	108.7		
		163.4	893	18.3	81.7	16.0	95	16.8	83.2	972.3	-80	108.9		
	5 µg/mL	1015.4	5464	18.6	81.4	19.0	111	17.0	83.0	5959.4	-496	109.1	107.7	2.1
		1014.8	5692	17.8	82.2	18.7	114	16.4	83.6	6192.9	-501	108.8		
		1006.7	5537	18.2	81.8	19.1	111	17.3	82.7	5827.3	-291	105.2		
6 hr	0.5 µg/mL	55.8	446	12.5	87.5	10.4	92	11.3	88.7	494.7	-49	111.0	114.4	3.1
		50.4	394	12.8	87.2	9.3	85	10.9	89.1	461.5	-67	117.0		
		60.8	426	14.3	85.7	11.2	90	12.4	87.6	490.3	-64	115.1		
	1 µg/mL	69.8	602	11.6	88.4	6.3	63	10.0	90.0	699.6	-97	116.2	116.8	1.4
		123.4	753	16.4	83.6	11.4	81	14.1	85.9	872.4	-119	115.8		
		127.8	880	14.5	85.5	11.3	92	12.3	87.7	1040.9	-161	118.3		
	5 µg/mL	786.8	5946	13.2	86.8	13.2	115	11.5	88.5	6860.0	-915	115.4	117.1	3.9
		799.8	4887	16.4	83.6	13.2	98	13.5	86.5	5942.6	-1056	121.6		
		753.6	5023	15.0	85.0	12.4	94	13.1	86.9	5742.0	-719	114.3		

Table IV-18. Sun Pharma, Lot D (JKR0865A) Drug Release Analytical Data. Presented are the analytical data for Sun Pharma, Lot D (JKR0865A). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 − %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	0.7	632	0.1	99.9	10.5	150	7.0	93.0	10.2	622	1.6	1.6	0.3
		0.7	611	0.1	99.9	12.2	145	8.4	91.6	8.4	603	1.4		
		0.9	616	0.2	99.8	11.2	142	7.8	92.2	12.0	604	1.9		
	1 µg/mL	1.2	1210	0.1	99.9	11.7	138	8.5	91.5	14.7	1195	1.2	1.2	0.01
		1.2	1180	0.1	99.9	11.6	134	8.7	91.3	14.3	1165	1.2		
		1.0	1105	0.1	99.9	10.2	131	7.8	92.2	13.1	1092	1.2		
	5 µg/mL	4.1	4953	0.1	99.9	10.6	129	8.3	91.7	49.7	4903	1.0	1.0	0.01
		4.6	5094	0.1	99.9	11.5	128	9.0	91.0	50.6	5044	1.0		
		4.7	4910	0.1	99.9	11.9	124	9.7	90.3	48.3	4862	1.0		
2 hr	0.5 µg/mL	0.8	536	0.2	99.8	8.6	119	7.2	92.8	11.4	525	2.1	2.0	0.3
		0.7	491	0.2	99.8	10.9	119	9.2	90.8	8.0	483	1.6		
		0.9	515	0.2	99.8	9.1	115	7.9	92.1	11.5	503	2.2		
	1 µg/mL	1.4	1111	0.1	99.9	11.0	126	8.7	91.3	15.9	1095	1.4	1.4	0.1
		1.2	972	0.1	99.9	10.3	114	9.1	90.9	13.1	958	1.4		
		1.2	914	0.1	99.9	10.7	109	9.8	90.2	11.9	902	1.3		
	5 µg/mL	5.0	4747	0.1	99.9	11.8	121	9.8	90.2	50.9	4696	1.1	1.1	0.04
		4.9	4500	0.1	99.9	11.0	116	9.5	90.5	51.8	4448	1.2		
		4.8	4820	0.1	99.9	10.2	116	8.8	91.2	54.3	4765	1.1		
6 hr	0.5 µg/mL	0.6	507	0.1	99.9	4.6	70	6.6	93.4	8.7	498	1.7	1.7	0.3
		0.6	533	0.1	99.9	7.5	96	7.8	92.2	7.7	525	1.4		
		0.7	490	0.1	99.9	5.5	78	7.1	92.9	9.9	480	2.0		
	1 µg/mL	1.0	959	0.1	99.9	5.6	73	7.7	92.3	12.4	946	1.3	1.3	0.03
		0.9	907	0.1	99.9	6.5	88	7.3	92.7	11.9	895	1.3		
		1.0	887	0.1	99.9	6.0	70	8.5	91.5	11.1	876	1.3		
	5 µg/mL	4.7	4640	0.1	99.9	7.4	88	8.4	91.6	55.4	4585	1.2	1.3	0.1
		4.7	4207	0.1	99.9	8.3	93	8.9	91.1	53.3	4153	1.3		
		3.7	3860	0.1	99.9	4.9	69	7.2	92.8	51.5	3808	1.3		

Table IV-19. Sun Pharma, Lot A (JKR0494A) Drug Release Analytical Data. Presented are the analytical data for Sun Pharma, Lot A (JKR0494A). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 – %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	0.4	556	0.1	99.9	12.1	135	9.0	91.0	4.4	552	0.8	0.7	0.1
		0.3	594	0.1	99.9	11.0	144	7.7	92.3	4.5	590	0.8		
		0.3	601	0.1	99.9	12.4	149	8.3	91.7	3.9	597	0.7		
	1 µg/mL	0.5	1241	0.04	100.0	11.5	147	7.8	92.2	6.6	1234	0.5	0.6	0.1
		0.4	1212	0.03	100.0	9.8	138	7.1	92.9	5.6	1206	0.5		
		0.6	1347	0.04	100.0	10.2	159	6.4	93.6	9.2	1338	0.7		
	5 µg/mL	0.8	6199	0.01	100.0	9.7	154	6.3	93.7	13.0	6186	0.2	0.2	0.02
		1.0	6122	0.02	100.0	9.7	150	6.5	93.5	15.1	6107	0.2		
		0.9	6008	0.02	100.0	10.1	143	7.1	92.9	12.9	5995	0.2		
2 hr	0.5 µg/mL	0.4	502	0.1	99.9	10.1	114	8.9	91.1	4.2	498	0.8	0.9	0.2
		0.4	510	0.1	99.9	9.2	124	7.4	92.6	5.5	505	1.1		
		0.3	461	0.1	99.9	9.0	112	8.0	92.0	3.7	458	0.8		
	1 µg/mL	0.6	1087	0.1	99.9	10.0	124	8.1	91.9	7.4	1079	0.7	0.7	0.1
		0.5	1078	0.05	100.0	8.5	119	7.1	92.9	7.3	1071	0.7		
		0.6	968	0.1	99.9	8.8	109	8.0	92.0	7.9	960	0.8		
	5 µg/mL	1.3	4748	0.03	100.0	10.1	110	9.2	90.8	14.3	4734	0.3	0.3	0.01
		1.6	4516	0.04	100.0	11.5	105	10.9	89.1	14.9	4501	0.3		
		1.3	4297	0.03	100.0	9.3	99	9.4	90.6	13.6	4284	0.3		
6 hr	0.5 µg/mL	0.3	504	0.1	99.9	5.4	73	7.4	92.6	4.4	500	0.9	1.1	0.3
		0.4	335	0.1	99.9	7.2	84	8.5	91.5	4.7	330	1.4		
		0.4	333	0.1	99.9	8.0	83	9.6	90.4	3.7	329	1.1		
	1 µg/mL	0.6	975	0.1	99.9	5.7	70	8.2	91.8	7.7	967	0.8	0.9	0.1
		0.5	860	0.1	99.9	3.9	56	7.0	93.0	7.4	853	0.9		
		0.7	915	0.1	99.9	5.4	67	8.1	91.9	8.5	906	0.9		
	5 µg/mL	1.8	4895	0.04	100.0	5.2	81	6.4	93.6	28.2	4866	0.6	0.7	0.2
		2.9	4768	0.1	99.9	4.5	66	6.7	93.3	42.9	4725	0.9		
		1.8	4944	0.04	100.0	6.2	91	6.7	93.3	26.1	4918	0.5		

Table IV-20. Sun Pharma, Lot B (JKR0154A) Drug Release Analytical Data. Presented are the analytical data for **Sun Pharma, Lot B (JKR0154A)**. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 − %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/(1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR-Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	**0.5 µg/mL**	0.9	692	0.1	99.9	11.2	141	8.0	92.0	11.6	680	1.7	1.4	0.2
		0.9	899	0.1	99.9	12.4	168	7.4	92.6	11.7	888	1.3		
		0.7	729	0.1	99.9	10.4	135	7.8	92.2	8.9	721	1.2		
	1 µg/mL	1.1	1370	0.1	99.9	12.5	135	9.2	90.8	12.0	1358	0.9	0.9	0.1
		0.9	1340	0.1	99.9	10.8	136	7.9	92.1	11.4	1329	0.9		
		1.1	1410	0.1	99.9	11.4	143	7.9	92.1	13.9	1396	1.0		
	5 µg/mL	1.6	6374	0.02	100.0	11.7	135	8.6	91.4	18.4	6356	0.3	0.3	0.04
		1.8	5126	0.04	100.0	11.6	107	10.8	89.2	16.7	5109	0.3		
		2.0	6643	0.03	100.0	12.5	147	8.5	91.5	23.9	6620	0.4		
2 hr	**0.5 µg/mL**	1.2	604	0.2	99.8	12.6	115	10.9	89.1	10.9	594	1.8	1.5	0.3
		1.1	612	0.2	99.8	14.7	113	13.0	87.0	8.3	604	1.4		
		0.9	567	0.2	99.8	14.2	104	13.7	86.3	6.8	561	1.2		
	1 µg/mL	1.3	1111	0.1	99.9	13.9	105	13.3	86.7	9.7	1101	0.9	0.8	0.05
		1.0	1070	0.1	99.9	12.1	98	12.3	87.7	8.3	1062	0.8		
		1.3	1067	0.1	99.9	14.7	102	14.4	85.6	8.9	1058	0.8		
	5 µg/mL	2.3	4855	0.05	100.0	15.1	102	14.8	85.2	15.6	4839	0.3	0.4	0.03
		2.5	5372	0.05	100.0	13.8	104	13.3	86.7	19.1	5353	0.4		
		3.1	5130	0.1	99.9	16.2	103	15.8	84.2	19.9	5110	0.4		
6 hr	**0.5 µg/mL**	1.0	645	0.1	99.9	9.1	97	9.3	90.7	10.2	635	1.6	1.3	0.2
		0.8	648	0.1	99.9	9.8	101	9.7	90.3	8.1	640	1.2		
		0.9	699	0.1	99.9	11.8	102	11.6	88.4	7.9	692	1.1		
	1 µg/mL	1.1	1186	0.1	99.9	10.0	91	10.9	89.1	10.3	1175	0.9	0.9	0.01
		1.0	1144	0.1	99.9	8.6	90	9.6	90.4	9.9	1134	0.9		
		1.0	1260	0.1	99.9	9.3	102	9.1	90.9	11.0	1249	0.9		
	5 µg/mL	2.4	5832	0.04	100.0	9.7	102	9.5	90.5	25.7	5806	0.4	0.5	0.02
		2.6	5788	0.04	100.0	9.3	101	9.3	90.7	27.8	5760	0.5		
		2.5	6038	0.04	100.0	8.8	100	8.8	91.2	28.3	6010	0.5		

Table IV-21. Free Doxorubicin HCl Control Data. Displayed is the calculated % release for the doxorubicin HCl control data. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	104.8	1.7	1.7
	1 µg/mL	107.8	1.5	1.4
	5 µg/mL	108.1	3.3	3.0
2 hr	0.5 µg/mL	106.2	1.1	1.0
	1 µg/mL	108.5	0.6	0.5
	5 µg/mL	107.7	2.1	2.0
6 hr	0.5 µg/mL	114.4	3.1	2.7
	1 µg/mL	116.8	1.4	1.2
	5 µg/mL	117.1	3.9	3.4
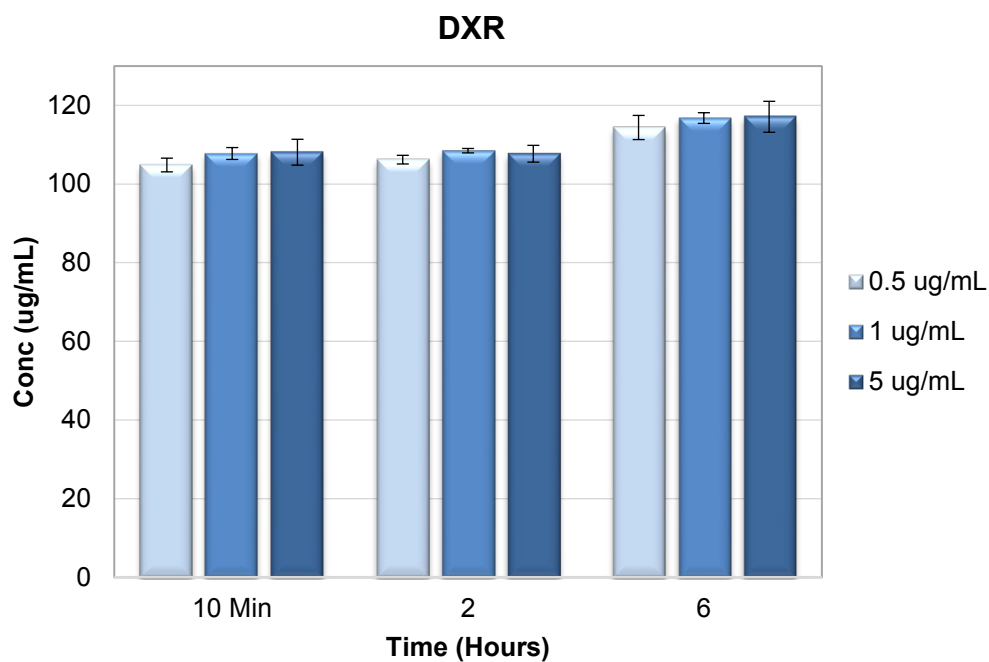
Figure IV-14. Free Doxorubicin HCl Control Data. Displayed is the calculated % release for the doxorubicin HCl control data. (Mean $\pm$ SD, *N=3*)

Table IV-22. Sun Pharma, Lot D (JKR0865A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot D (JKR0865A). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	1.6	0.3	17.5
	1 µg/mL	1.2	0.01	1.1
	5 µg/mL	1.0	0.01	1.0
2 hr	0.5 µg/mL	2.0	0.3	16.1
	1 µg/mL	1.4	0.1	4.6
	5 µg/mL	1.1	0.04	3.6
6 hr	0.5 µg/mL	1.7	0.3	16.6
	1 µg/mL	1.3	0.03	2.4
	5 µg/mL	1.3	0.1	5.6

Sun Pharma (JKR0865A)
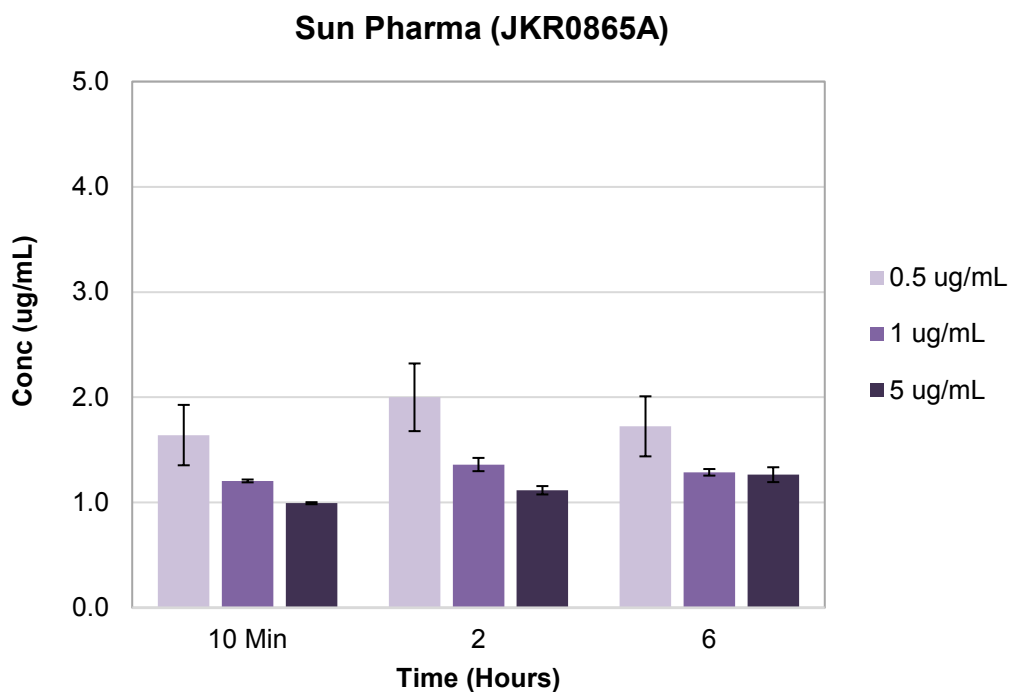
Figure IV-15. Sun Pharma, Lot D (JKR0865A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot D (JKR0865A). (Mean $\pm$ SD, *N=3*)

Table IV-23. Sun Pharma, Lot A (JKR0494A). Displayed is the calculated % release for Sun Pharma, Lot A (JKR0494A). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	0.7	0.1	9.7
	1 µg/mL	0.6	0.1	20.3
	5 µg/mL	0.2	0.02	8.6
2 hr	0.5 µg/mL	0.9	0.2	16.6
	1 µg/mL	0.7	0.1	10.7
	5 µg/mL	0.3	0.01	4.7
6 hr	0.5 µg/mL	1.1	0.3	24.1
	1 µg/mL	0.9	0.1	8.1
	5 µg/mL	0.7	0.2	30.3

Sun Pharma (JKR0494A)
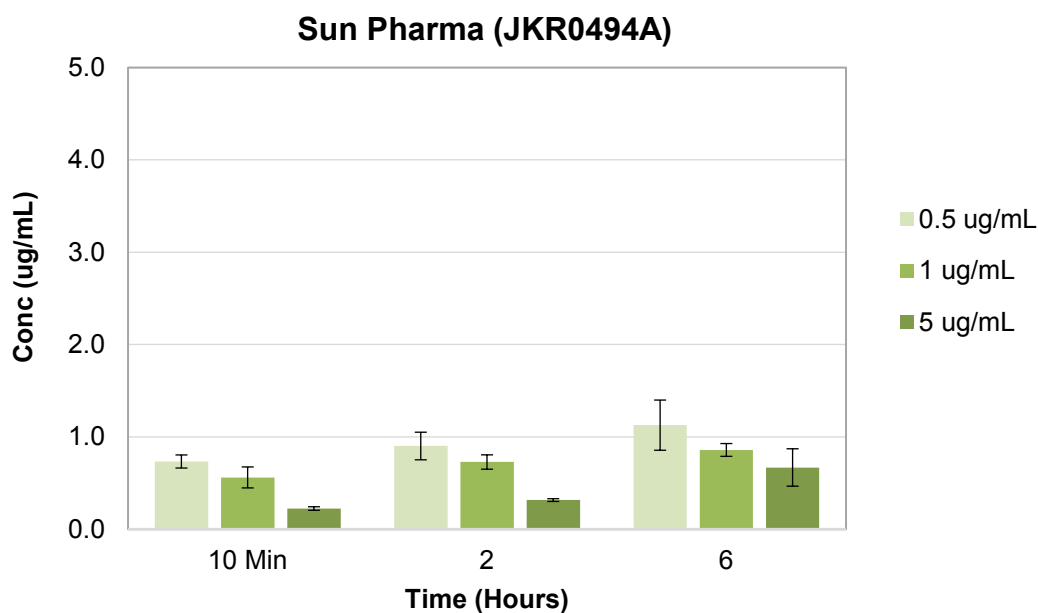
Figure IV-16. Sun Pharma, Lot A (JKR0494A) Drug Release. Displayed is the calculated % release for the Sun Pharma, Lot A (JKR0494A). (Mean ± SD, *N=3*)

Table IV-24. Sun Pharma, Lot B (JKR0154A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot B (JKR0154A). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	1.4	0.2	17.5
	1 µg/mL	0.9	0.1	8.1
	5 µg/mL	0.3	0.04	10.9
2 hr	0.5 µg/mL	1.5	0.3	21.8
	1 µg/mL	0.8	0.05	5.9
	5 µg/mL	0.4	0.03	9.1
6 hr	0.5 µg/mL	1.3	0.2	18.0
	1 µg/mL	0.9	0.01	0.6
	5 µg/mL	0.5	0.02	4.5
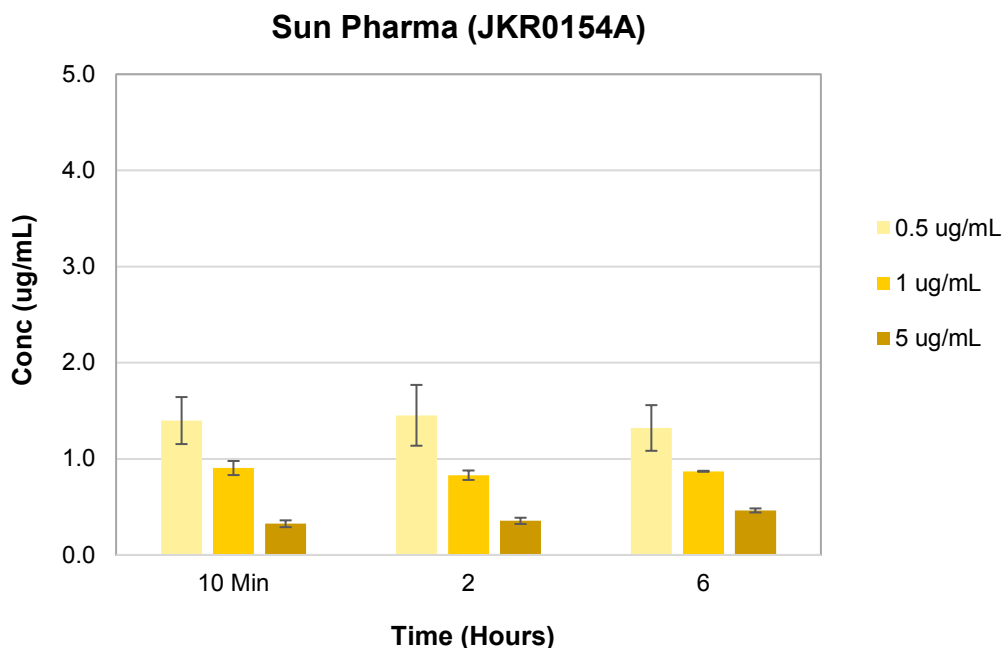
Figure IV-17. Sun Pharma, Lot B (JKR0154A) Drug Release. Displayed is the calculated % release for the Sun Pharma, Lot B (JKR0154A). (Mean + SD, *N=3*)
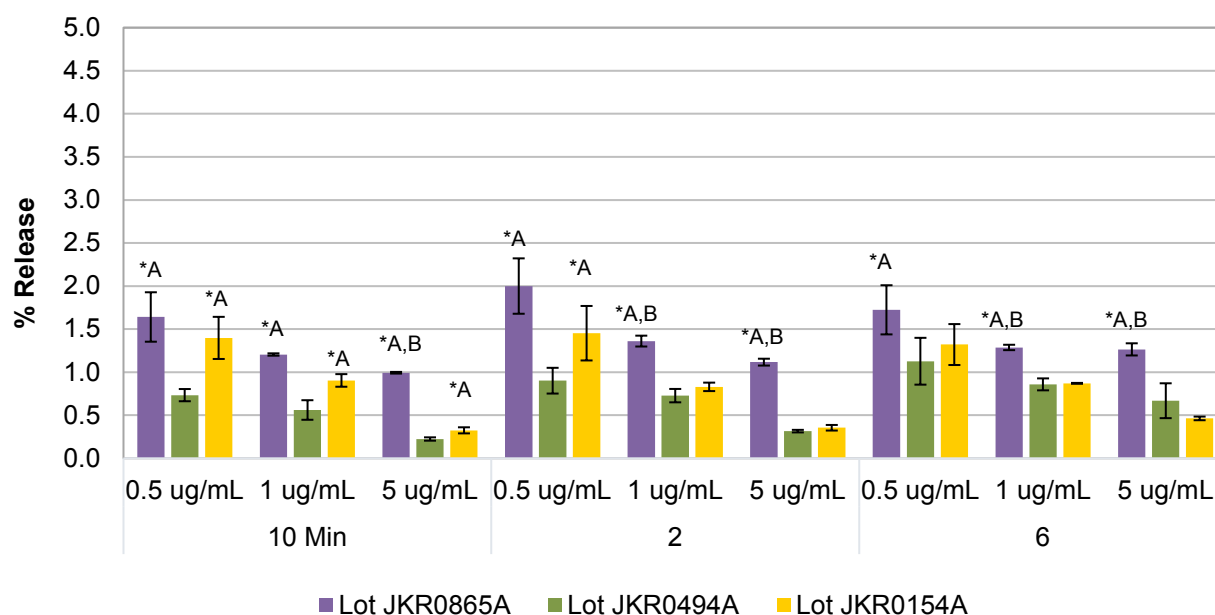
Figure IV-18. Sun Pharma Drug Release Compared. Displayed are the 10 min-6 hr calculated % release data for the Sun Pharma lots JKR0865A, JKR0494A and JKR0154A, also denoted as lots D, A and B, respectively. (Mean $\pm$ SD, *N=3*), *$p \leq 0.05$, ANOVA with Duncan's Multiple Range posthoc test.
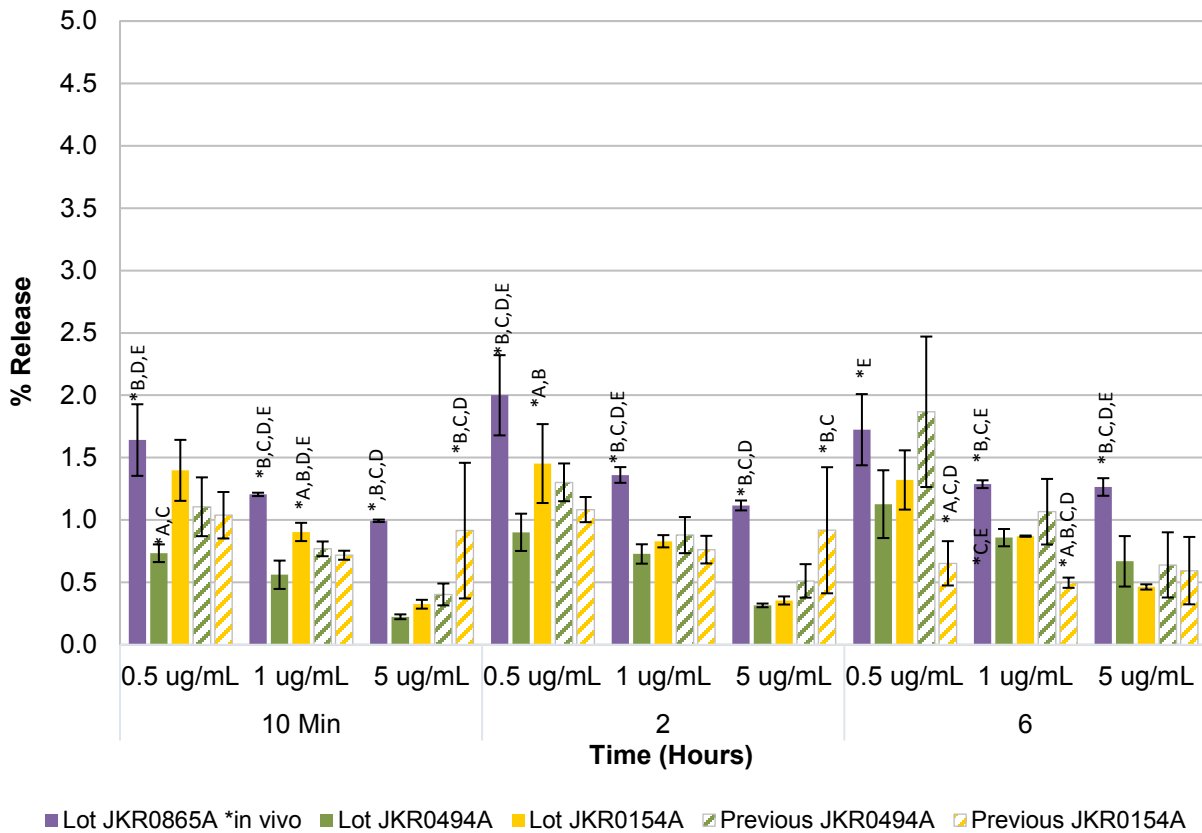
Figure IV-19. Sun Pharma Drug Release Compared to Previous Data. Displayed are the calculated 10 min-6 hr % release data for the Sun Pharma lots JKR0865A, JKR0494A and JKR0154A, and previous data for lots JKR0494A and JKR0154A, denoted in this figure as groups1-5, respectively, for purposes of statistical labeling. (Mean $\pm$ SD, *N=3*), *$p \leq 0.05$, ANOVA with Duncan's Multiple Range posthoc test.

Intra-Day Three Lot Comparison in Human Plasma at 42°C

Summary

The objective of this study was to compare the in vitro drug release of three lots of Sun Pharma in human plasma at 42°C over a 6 hr period. All lots had similar drug release profiles over the 6 hr period, although some instances of statistically significant differences were observed between lots.

Design and Methods

Three lots of Sun Pharma were JKR0494A, JKR0154A, and JKR0491A, also denoted as lots A, B, and C, respectively, were evaluated for drug release in human plasma at 42°C over a 6 hr period, according to the SITUA method described in Section I. All three lots were run on a single day, and free doxorubicin HCl at identical concentrations and time points was included as a control. The human plasma and protein-free plasma DXR and DXR_C13 standard calibration curves used for this study are also provided.

Results & Conclusions

The DXR and DXR_C13 standard calibration curves used for this study are presented in **Tables IV-25 to IV-28 and Figures IV-20 to IV-23**. The free doxorubicin HCl controls averaged between 102-103% of theoretical for all concentrations and time points (**Tables IV-29 and IV-33, and Figure IV-24**). The Sun Pharma drug release was similar for Lot's A- C, at approximately 1.5% release over the 6 hr period, without a clear temporal trend (**Tables IV-30 to IV-32 and IV-34 to IV-36, and Figures IV-25 to IV-28**).

Table IV-25. DXR Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	1844503	9.5	95
10.0 ng/mL	1790797	9.1	91
50 ng/mL	9892383	51	102
50 ng/mL	9550898	48	97
100 ng/mL	18893351	95	95
100 ng/mL	19414905	100	100
500 ng/mL	101711793	524	105
500 ng/mL	101599282	511	102
1000 ng/mL	184015970	1039	104
1000 ng/mL	210938052	1091	109
5000 ng/mL	1054503036	5188	104
5000 ng/mL	1024997258	5127	103
10000 ng/mL	1984165538	9796	98
10000 ng/mL	1974331982	9732	97
QC			
QCL	1851836	9.3	93
QCL	1889366	9.6	96
QCM	19872473	101	101
QCM	19369479	96	96
QCH	206163832	1003	100
QCH	204092352	1001	100

DXR_PRM
Y = 0.00211304+0.00446164*X   R^2 = 0.9990   W: 1/X
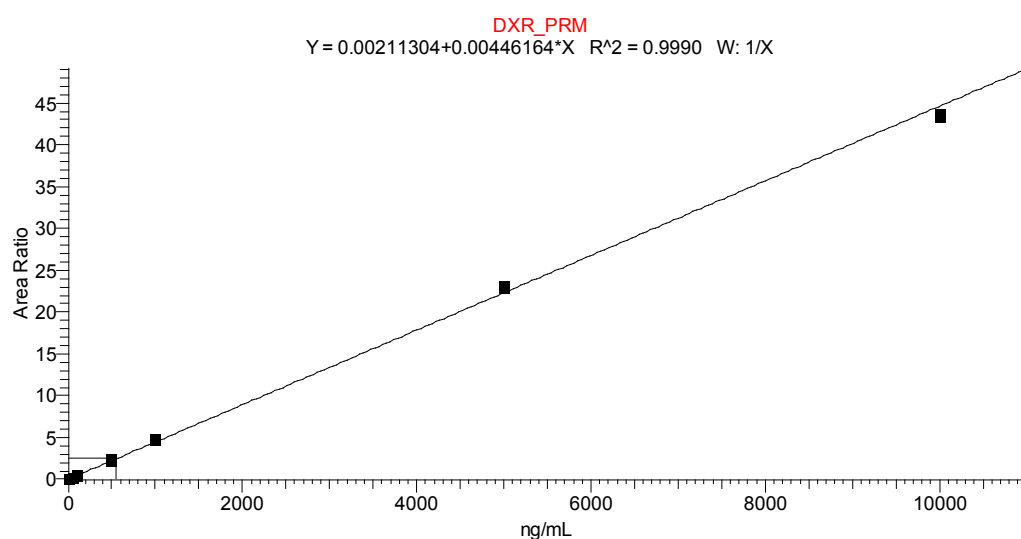
Figure IV-20. DXR Plasma Standard Curve.

Table IV-26. DXR_C13 Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	1319202	9.5	95
10.0 ng/mL	1257929	9.0	90
50 ng/mL	6794564	50	101
50 ng/mL	6549385	48	96
100 ng/mL	13125267	95	95
100 ng/mL	13432015	100	100
500 ng/mL	70308697	528	106
500 ng/mL	69754896	511	102
1000 ng/mL	128723506	1059	106
1000 ng/mL	141397079	1066	107
5000 ng/mL	732290619	5251	105
5000 ng/mL	704240265	5134	103
10000 ng/mL	1359707818	9784	98
10000 ng/mL	1346712656	9676	97
QC			
QCL	1326483	9.4	94
QCL	1358544	9.7	97
QCM	13853031	103	103
QCM	13516723	97	97
QCH	144330129	1023	102
QCH	141991829	1015	101

DXR_C13_PRM
Y = 0.00256778+0.00306105*X   R^2 = 0.9987   W: 1/X
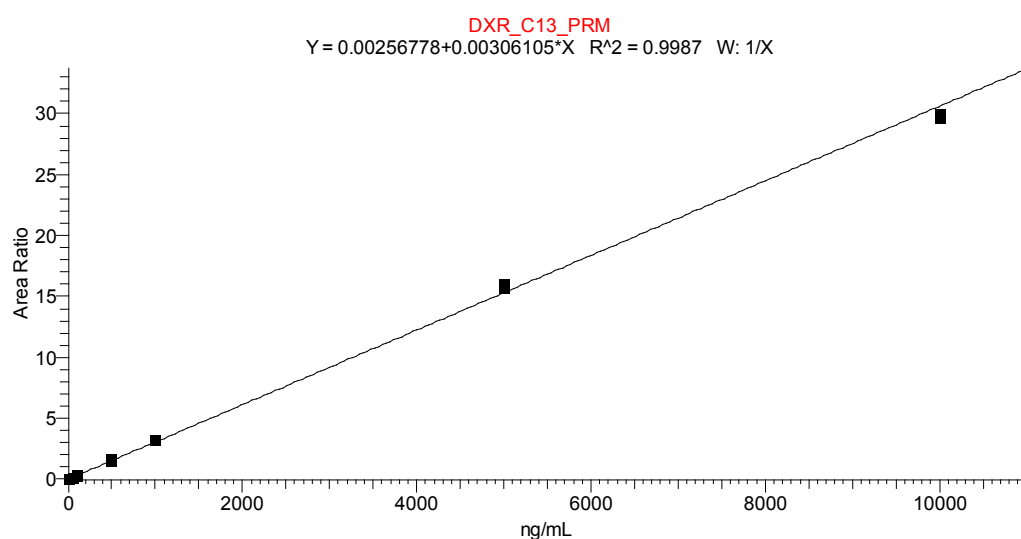
Figure IV-21. DXR_C13 Plasma Standard Curve.

Table IV-27. DXR Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	10358	0.097	97
0.100 ng/mL	11902	0.108	108
0.50 ng/mL	77002	0.451	90
0.50 ng/mL	76980	0.455	91
1.00 ng/mL	168973	0.92	92
1.00 ng/mL	169547	0.91	91
5.00 ng/mL	977778	4.67	93
5.00 ng/mL	995034	5.31	106
10.0 ng/mL	2151755	10.1	101
10.0 ng/mL	2030014	10.4	104
100.0 ng/mL	22310549	103.3	103
100.0 ng/mL	20893558	106.4	106
1000.0 ng/mL	232738355	1046	105
1000.0 ng/mL	208693546	1110	111
QC			
QCL	11428	0.096	96
QCL	13980	0.116	116
QCM	175482	0.93	93
QCM	180208	0.91	91
QCH	2111559	9.5	95
QCH	2120806	9.6	96

DXR_PRM
$Y = -0.000276772+0.00557506*X$ $R^2 = 0.9935$ W: $1/X^2$
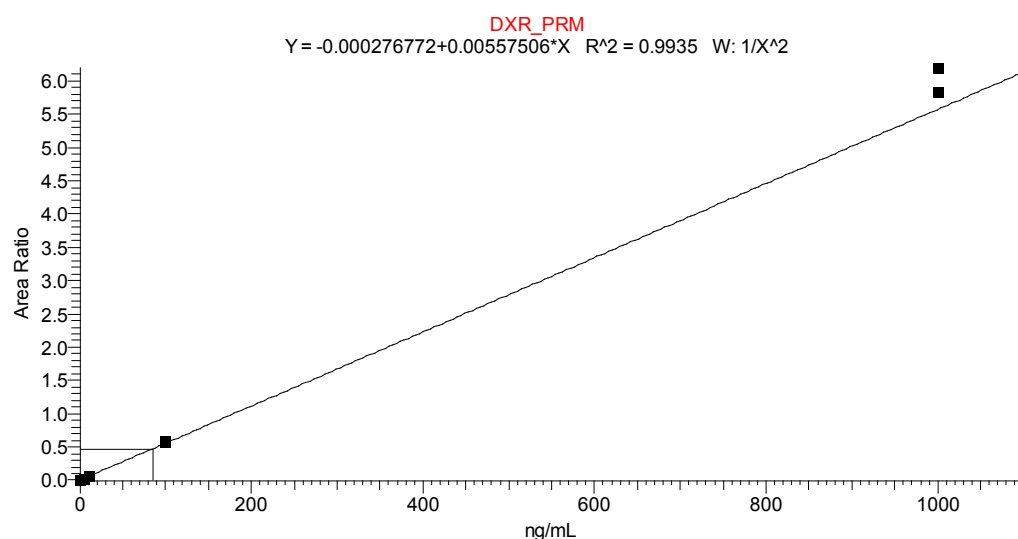
Figure IV-22. DXR Protein-Free Plasma Standard Curve.

Table IV-28. DXR_C13 Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	9823	0.099	99
0.100 ng/mL	10310	0.106	106
0.50 ng/mL	63898	0.45	90
0.50 ng/mL	64317	0.46	92
1.00 ng/mL	138089	0.92	92
1.00 ng/mL	141539	0.92	92
5.00 ng/mL	780667	4.56	91
5.00 ng/mL	793426	5.18	104
10.0 ng/mL	1702631	9.8	98
10.0 ng/mL	1686955	10.6	106
100.0 ng/mL	18256631	103.5	104
100.0 ng/mL	17706522	110.4	110
1000.0 ng/mL	192850895	1061.8	106
1000.0 ng/mL	168662908	1098.9	110
QC			
QCL	10144	0.095	95
QCL	10218	0.103	103
QCM	140518	0.91	91
QCM	147964	0.91	91
QCH	1761798	9.7	97
QCH	1779952	9.8	98

DXR_C13_PRM
Y = -0.000200041+0.00455269*X   R^2 = 0.9933   W: 1/X^2
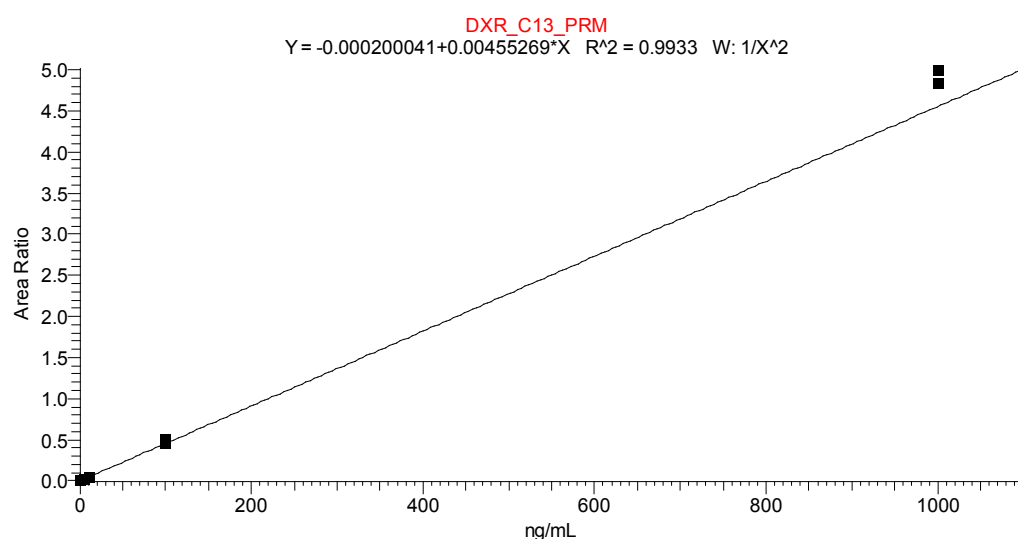
Figure IV-23. DXR_C13 Protein-Free Plasma Standard Curve.

Table IV-28. Free Doxorubicin HCl Control Analytical Data. Presented are the analytical data for the free doxorubicin HCl controls. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 − %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	79.0	554	14.3	85.7	17.2	124	13.8	86.2	572.8	-19	103.4	102.3	1.2
		50.9	508	10.0	90.0	11.3	114	9.9	90.1	513.1	-5	101.0		
		66.9	531	12.6	87.4	13.6	111	12.3	87.7	544.2	-13	102.5		
	1 µg/mL	146.0	1073	13.6	86.4	14.7	116	12.7	87.3	1147.5	-74	106.9	105.2	3.3
		105.3	1070	9.8	90.2	11.2	116	9.7	90.3	1084.7	-15	101.4		
		122.4	1064	11.5	88.5	12.4	115	10.7	89.3	1142.2	-79	107.4		
	5 µg/mL	787.5	4916	16.0	84.0	15.9	100	15.9	84.1	4962.8	-47	100.9	104.6	3.4
		795.7	5028	15.8	84.2	14.4	95	15.1	84.9	5278.0	-250	105.0		
		824.3	5304	15.5	84.5	15.7	109	14.4	85.6	5714.0	-410	107.7		
2 hr	0.5 µg/mL	42.3	412	10.3	89.7	8.6	93	9.2	90.8	457.6	-45	110.9	109.7	1.5
		40.6	392	10.4	89.6	8.2	86	9.6	90.4	423.1	-31	108.0		
		50.3	437	11.5	88.5	9.7	93	10.4	89.6	481.6	-45	110.2		
	1 µg/mL	101.2	827	12.2	87.8	9.9	87	11.3	88.7	893.9	-67	108.1	110.3	2.4
		110.4	804	13.7	86.3	10.9	87	12.5	87.5	883.8	-80	110.0		
		110.7	796	13.9	86.1	10.5	85	12.3	87.7	898.2	-102	112.8		
	5 µg/mL	523.2	3964	13.2	86.8	10.7	87	12.3	87.7	4241.8	-278	107.0	108.1	2.3
		675.8	4149	16.3	83.7	12.6	85	14.7	85.3	4593.5	-444	110.7		
		600.1	3942	15.2	84.8	12.1	85	14.3	85.7	4200.1	-258	106.5		
6 hr	0.5 µg/mL	29.8	205	14.6	85.4	6.1	47	13.0	87.0	229.6	-25	112.1	110.0	2.2
		21.4	151	14.1	85.9	4.4	34	13.1	86.9	162.9	-12	107.6		
		31.6	184	17.2	82.8	6.4	41	15.6	84.4	202.6	-19	110.2		
	1 µg/mL	57.4	371	15.5	84.5	5.6	42	13.4	86.6	428.7	-58	115.5	113.3	2.5
		98.5	512	19.2	80.8	9.5	55	17.4	82.6	566.3	-55	110.6		
		96.7	528	18.3	81.7	9.2	57	16.1	83.9	600.5	-73	113.7		
	5 µg/mL	470.4	2406	19.5	80.5	9.5	55	17.3	82.7	2717.8	-312	113.0	110.8	3.3
		593.4	2807	21.1	78.9	10.7	57	18.8	81.2	3157.0	-351	112.5		
		392.6	2475	15.9	84.1	7.5	50	14.8	85.2	2648.1	-173	107.0		

Table IV-29. Sun Pharma, Lot A (JKR0494A) Drug Release Analytical Data. Presented are the analytical data for Sun Pharma, Lot A (JKR0494A). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 − %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	0.6	612	0.1	99.9	9.7	100	9.7	90.3	6.1	606	1.0	1.0	0.03
		0.5	479	0.1	99.9	10.1	94	10.7	89.3	4.9	474	1.0		
		0.4	519	0.1	99.9	7.4	99	7.5	92.5	5.5	514	1.1		
	1 µg/mL	0.9	1162	0.1	99.9	9.5	110	8.6	91.4	10.2	1152	0.9	1.0	0.1
		0.9	840	0.1	99.9	10.3	100	10.2	89.8	8.8	831	1.0		
		0.9	920	0.1	99.9	9.4	106	8.9	91.1	9.6	910	1.0		
	5 µg/mL	3.3	5103	0.1	99.9	8.8	93	9.5	90.5	35.2	5068	0.7	0.7	0.02
		5.0	4287	0.1	99.9	14.5	91	15.9	84.1	31.4	4256	0.7		
		2.7	4028	0.1	99.9	8.6	94	9.2	90.8	28.9	3999	0.7		
2 hr	0.5 µg/mL	0.9	459	0.2	99.8	10.2	87	11.8	88.2	7.6	451	1.7	1.7	0.1
		0.8	369	0.2	99.8	10.4	81	12.8	87.2	6.3	363	1.7		
		0.7	360	0.2	99.8	9.1	78	11.6	88.4	6.4	354	1.8		
	1 µg/mL	1.3	888	0.1	99.9	9.0	88	10.2	89.8	12.3	876	1.4	1.5	0.3
		1.4	790	0.2	99.8	10.0	78	12.9	87.1	10.7	780	1.4		
		1.7	818	0.2	99.8	9.2	83	11.2	88.8	15.1	802	1.9		
	5 µg/mL	6.4	4257	0.2	99.8	10.2	86	11.9	88.1	54.0	4203	1.3	1.2	0.1
		5.1	3293	0.2	99.8	9.1	72	12.7	87.3	40.1	3253	1.2		
		5.7	3960	0.1	99.9	10.0	81	12.3	87.7	46.3	3914	1.2		
6 hr	0.5 µg/mL	1.1	505	0.2	99.8	6.8	63	10.7	89.3	10.0	495	2.0	2.1	0.3
		1.1	438	0.2	99.8	6.9	57	12.1	87.9	8.8	429	2.0		
		1.1	371	0.3	99.7	6.8	56	12.1	87.9	9.1	362	2.5		
	1 µg/mL	2.2	956	0.2	99.8	7.2	59	12.3	87.7	18.2	938	1.9	2.3	0.3
		2.2	746	0.3	99.7	6.6	54	12.3	87.7	18.2	728	2.4		
		2.3	702	0.3	99.7	7.2	54	13.3	86.7	17.3	684	2.5		
	5 µg/mL	8.3	4343	0.2	99.8	3.8	49	7.9	92.1	105.6	4238	2.4	2.2	0.2
		7.5	3373	0.2	99.8	5.6	51	10.9	89.1	69.2	3304	2.1		
		7.9	3474	0.2	99.8	5.5	51	10.8	89.2	72.6	3401	2.1		

Table IV-30. Sun Pharma, Lot B (JKR0154A) Drug Release Analytical Data. Presented are the analytical data for Sun Pharma, Lot B (JKR0154A). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 – %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	1.0	578	0.2	99.8	12.2	104	11.8	88.2	8.1	570	1.4	1.6	0.1
		0.9	493	0.2	99.8	10.4	93	11.2	88.8	8.1	485	1.6		
		0.9	530	0.2	99.8	9.9	93	10.6	89.4	8.6	522	1.6		
	1 µg/mL	1.1	1290	0.1	99.9	12.5	110	11.3	88.7	10.0	1280	0.8	1.1	0.3
		1.4	1096	0.1	99.9	10.1	104	9.7	90.3	13.9	1082	1.3		
		1.3	994	0.1	99.9	11.9	98	12.1	87.9	10.9	983	1.1		
	5 µg/mL	4.0	5127	0.1	99.9	9.2	87	10.6	89.4	37.5	5090	0.7	0.7	0.1
		3.5	5768	0.1	99.9	8.7	97	8.9	91.1	39.5	5729	0.7		
		3.5	5528	0.1	99.9	10.7	97	11.0	89.0	31.9	5496	0.6		
2 hr	0.5 µg/mL	1.1	600	0.2	99.8	10.3	95	10.8	89.2	9.8	590	1.6	2.0	0.4
		1.3	588	0.2	99.8	8.6	82	10.5	89.5	12.0	576	2.0		
		1.4	539	0.3	99.7	9.8	90	10.9	89.1	12.6	526	2.3		
	1 µg/mL	1.4	1111	0.1	99.9	9.2	82	11.2	88.8	12.3	1099	1.1	1.3	0.1
		1.6	951	0.2	99.8	10.2	80	12.7	87.3	12.3	939	1.3		
		1.7	964	0.2	99.8	10.8	83	12.9	87.1	13.3	950	1.4		
	5 µg/mL	6.8	5402	0.1	99.9	9.5	78	12.1	87.9	56.4	5346	1.0	1.0	0.1
		5.6	5136	0.1	99.9	6.8	72	9.5	90.5	59.0	5077	1.1		
		5.4	5105	0.1	99.9	9.0	79	11.3	88.7	47.8	5057	0.9		
6 hr	0.5 µg/mL	1.9	523	0.4	99.6	10.7	64	16.7	83.3	11.2	512	2.1	2.6	0.4
		2.2	488	0.5	99.5	9.1	58	15.8	84.2	13.9	474	2.9		
		2.0	438	0.5	99.5	9.0	56	16.2	83.8	12.6	426	2.9		
	1 µg/mL	2.3	900	0.2	99.8	7.6	52	14.8	85.2	15.2	885	1.7	2.1	0.5
		3.0	936	0.3	99.7	4.3	35	12.0	88.0	24.9	911	2.7		
		2.1	946	0.2	99.8	6.8	56	12.1	87.9	17.5	929	1.9		
	5 µg/mL	11.1	4991	0.2	99.8	6.9	56	12.2	87.8	90.6	4900	1.8	2.4	1.1
		16.3	5129	0.3	99.7	3.4	39	8.8	91.2	185.3	4944	3.6		
		10.2	4610	0.2	99.8	7.3	54	13.3	86.7	76.4	4534	1.7		

Table IV-31. Sun Pharma, Lot C (JKR0491A) Drug Release Analytical Data. Presented are the analytical data for Sun Pharma, Lot C (JKR0491A). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 − %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	0.9	594	0.1	99.9	10.4	105	10.0	90.0	8.6	585	1.4	1.6	0.1
		0.8	547	0.2	99.8	10.3	107	9.6	90.4	8.7	538	1.6		
		0.9	445	0.2	99.8	10.7	94	11.4	88.6	7.7	438	1.7		
	1 µg/mL	1.2	1233	0.1	99.9	9.6	106	9.1	90.9	13.5	1219	1.1	1.3	0.1
		1.1	834	0.1	99.9	9.2	98	9.4	90.6	11.5	822	1.4		
		1.0	926	0.1	99.9	9.4	109	8.6	91.4	12.0	914	1.3		
	5 µg/mL	4.1	4923	0.1	99.9	9.0	94	9.6	90.4	43.0	4880	0.9	1.0	0.1
		4.3	4310	0.1	99.9	9.5	90	10.6	89.4	40.4	4269	0.9		
		4.9	4058	0.1	99.9	10.6	94	11.3	88.7	43.0	4015	1.1		
2 hr	0.5 µg/mL	1.0	472	0.2	99.8	8.5	92	9.3	90.7	10.9	461	2.3	2.3	0.04
		1.0	389	0.3	99.7	9.4	86	10.9	89.1	9.2	380	2.4		
		0.8	395	0.2	99.8	7.9	86	9.2	90.8	9.0	386	2.3		
	1 µg/mL	1.7	870	0.2	99.8	8.5	87	9.8	90.2	17.1	853	2.0	2.4	0.6
		1.7	823	0.2	99.8	8.1	82	9.9	90.1	17.2	805	2.1		
		2.4	858	0.3	99.7	7.1	75	9.4	90.6	25.8	832	3.0		
	5 µg/mL	4.8	4220	0.1	99.9	6.6	82	8.1	91.9	59.9	4160	1.4	1.6	0.2
		6.2	3622	0.2	99.8	7.8	79	9.9	90.1	62.6	3560	1.7		
		6.9	3423	0.2	99.8	8.7	72	12.1	87.9	57.0	3366	1.7		
6 hr	0.5 µg/mL	1.5	526	0.3	99.7	7.6	63	12.1	87.9	12.0	514	2.3	2.6	0.4
		1.6	369	0.4	99.6	8.0	57	14.1	85.9	11.2	358	3.0		
		1.5	446	0.3	99.7	7.3	54	13.4	86.6	11.0	435	2.5		
	1 µg/mL	3.1	978	0.3	99.7	7.7	57	13.5	86.5	23.1	955	2.4	2.9	0.5
		3.1	812	0.4	99.6	4.0	34	11.8	88.2	26.6	786	3.3		
		3.1	704	0.4	99.6	4.3	31	13.8	86.2	22.3	682	3.2		
	5 µg/mL	12.2	4252	0.3	99.7	6.9	53	13.1	86.9	93.2	4159	2.2	3.0	0.8
		12.1	3407	0.4	99.6	4.6	43	10.7	89.3	112.8	3294	3.3		
		11.3	2995	0.4	99.6	2.8	27	10.4	89.6	108.2	2886	3.6		

Table IV-32. Free Doxorubicin HCl Control Data. Displayed is the calculated % release for the doxorubicin HCl control data. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	102.3	1.2	1.2
	1 µg/mL	105.2	3.3	3.2
	5 µg/mL	104.6	3.4	3.3
2 hr	0.5 µg/mL	109.7	1.5	1.4
	1 µg/mL	110.3	2.4	2.1
	5 µg/mL	108.1	2.3	2.1
6 hr	0.5 µg/mL	110.0	2.2	2.0
	1 µg/mL	113.3	2.5	2.2
	5 µg/mL	110.8	3.3	3.0
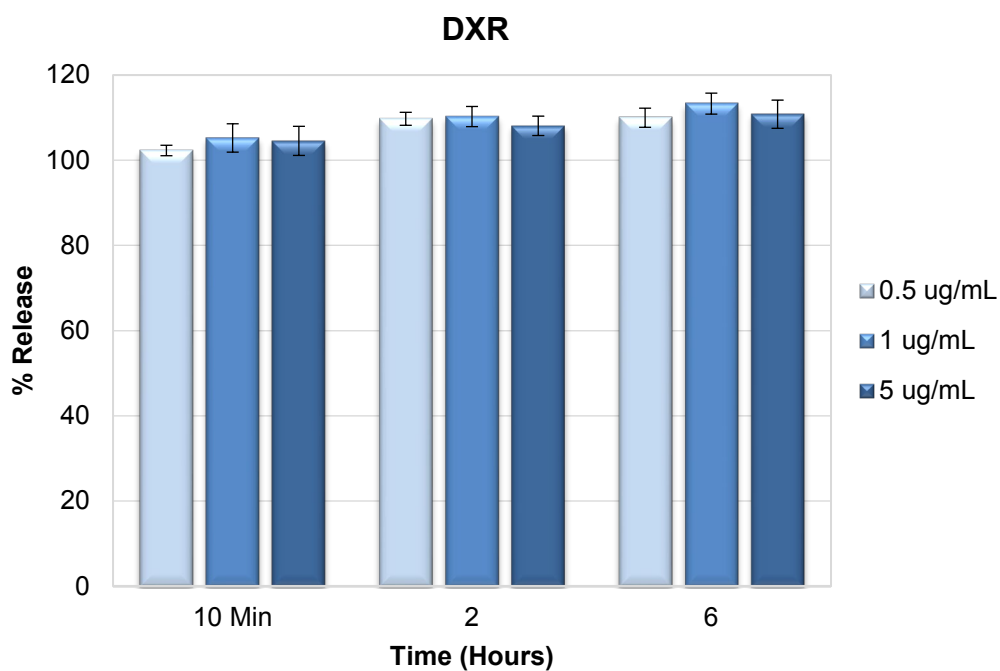
Figure IV-24. Free Doxorubicin HCl Control Data. Displayed is the calculated % release for the doxorubicin HCl control data. (Mean $\pm$ SD, *N=3*)

Table IV-33. Sun Pharma, Lot A (JKR0494A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot A (JKR0494A). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	1.0	0.03	2.7
	1 µg/mL	1.0	0.1	9.5
	5 µg/mL	0.7	0.02	3.1
2 hr	0.5 µg/mL	1.7	0.1	3.4
	1 µg/mL	1.5	0.3	18.1
	5 µg/mL	1.2	0.1	4.1
6 hr	0.5 µg/mL	2.1	0.3	12.6
	1 µg/mL	2.3	0.3	13.9
	5 µg/mL	2.2	0.2	9.5
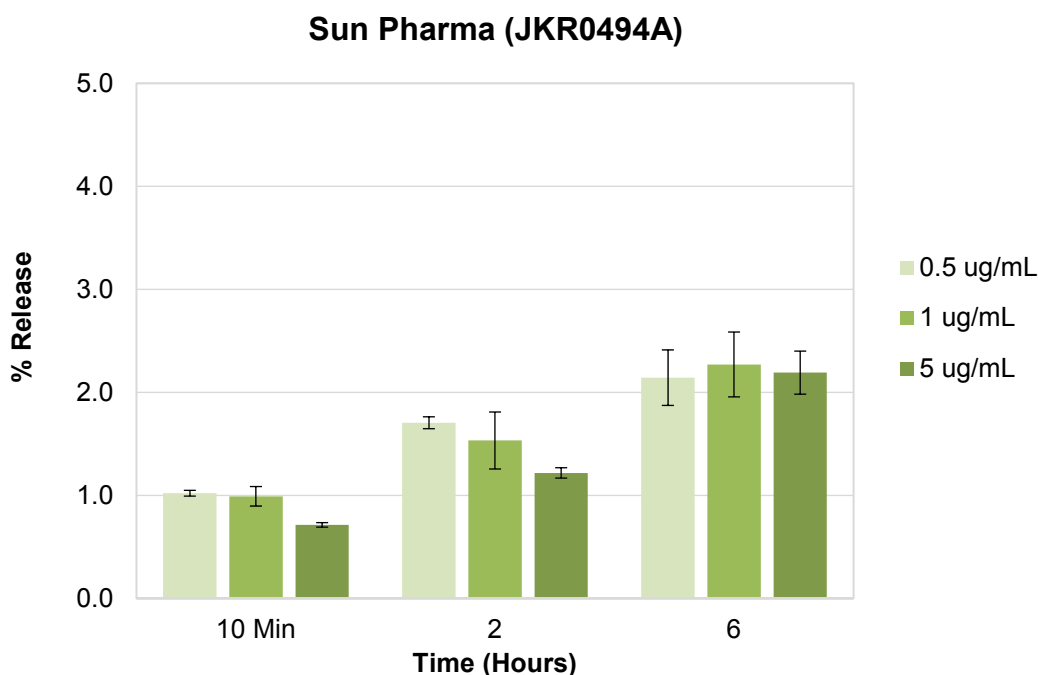
Figure IV-25. Sun Pharma, Lot A (JKR0494A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot A (JKR0494A). (Mean $\pm$ SD, *N=3*)

Table IV-34. Sun Pharma, Lot B (JKR0154A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot B (JKR0154A). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	1.6	0.1	8.4
	1 µg/mL	1.1	0.3	24.0
	5 µg/mL	0.7	0.1	12.0
2 hr	0.5 µg/mL	2.0	0.3	18.0
	1 µg/mL	1.3	0.1	11.0
	5 µg/mL	1.0	0.1	10.1
6 hr	0.5 µg/mL	2.6	0.4	16.0
	1 µg/mL	2.1	0.5	25.0
	5 µg/mL	2.4	1.1	46.0
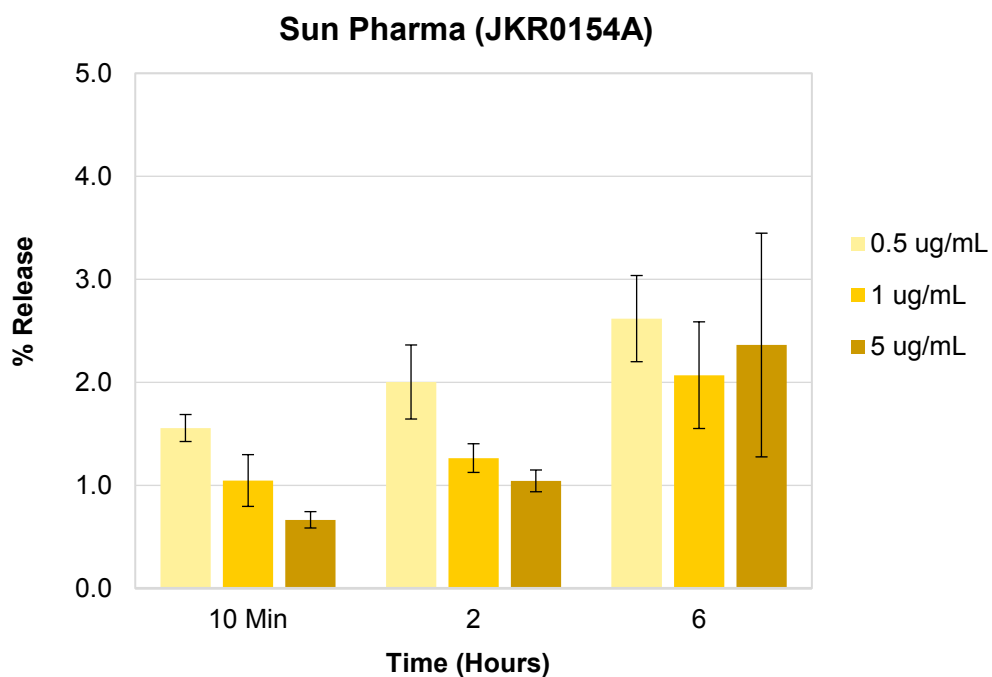
Figure IV-26. Sun Pharma, Lot B (JKR0154A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot B (JKR0154A). (Mean $\pm$ SD, *N=3*)

Table IV-35. Sun Pharma, Lot C (JKR0491A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot C (JKR0491A). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	1.6	0.1	8.5
	1 µg/mL	1.3	0.1	11.5
	5 µg/mL	1.0	0.1	9.9
2 hr	0.5 µg/mL	2.3	0.04	1.8
	1 µg/mL	2.4	0.6	24.1
	5 µg/mL	1.6	0.2	10.2
6 hr	0.5 µg/mL	2.6	0.4	15.4
	1 µg/mL	2.9	0.5	16.9
	5 µg/mL	3.0	0.8	24.6
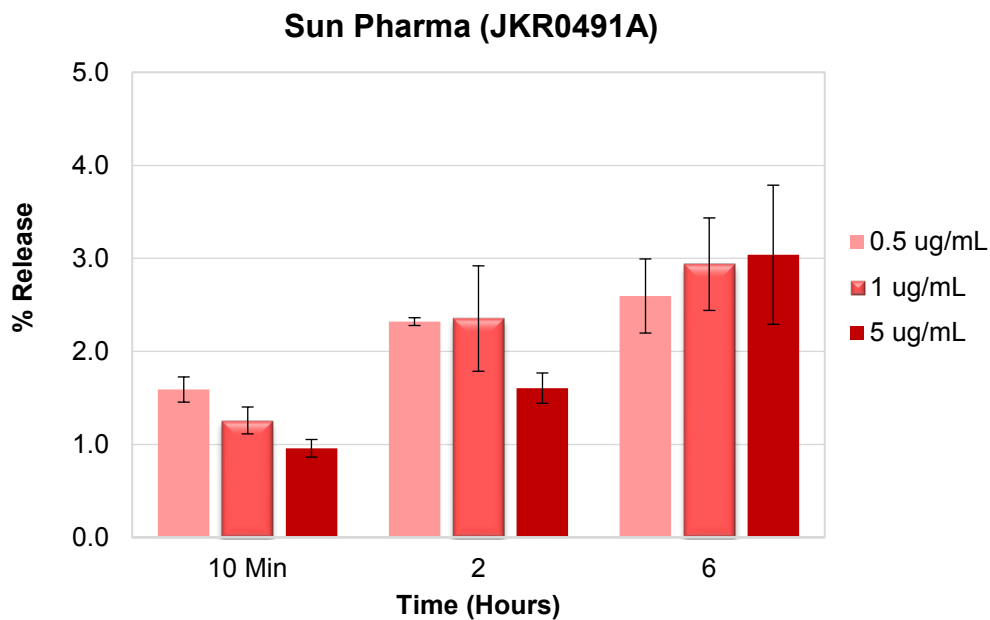
Figure IV-27. Sun Pharma, Lot C (JKR0491A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot C (JKR0491A). (Mean $\pm$ SD, *N=3*)
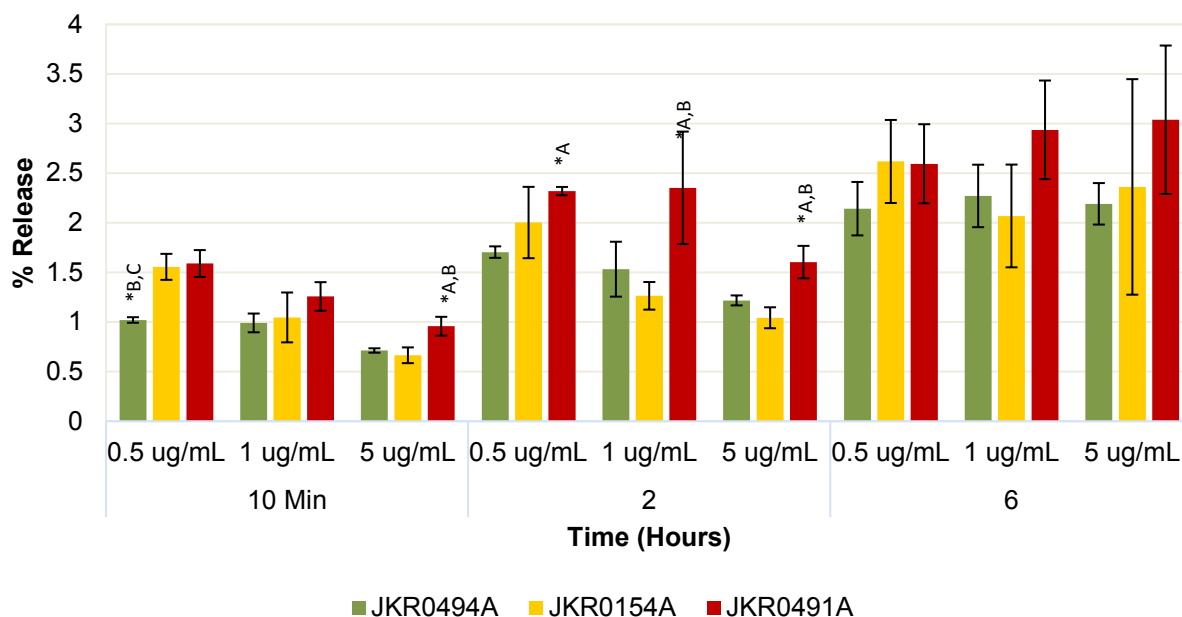
Figure IV-28. Sun Pharma Drug Release Lot Comparison. Displayed are the 10 min-6 hr calculated % release data for the Sun Pharma lots JKR0494A, JKR0154A, and JKR0491A, also denoted as lots A, B, and C, respectively. (Mean $\pm$ SD, *N=3*), *$p \leq 0.05$, ANOVA with Duncan's Multiple Range posthoc test.

V. In Vitro Doxil vs. Sun Pharma Comparisons

Intra-day Comparison in Human Plasma at 37°C

Summary

The objective of this study was to compare the in vitro drug release of a single lot of Doxil to that of a single lot of Sun Pharma in human plasma at 37°C over a 6 hr period. All lots had similar drug release profiles over the 6 hr period, although some instances of statistically significant differences were observed between lots.

Design and Methods

A single lot of Doxil, 600120P1 (lot B), and a single lot of Sun Pharma, JKR0494A (lot A), were evaluated for drug release in human plasma at 37°C over a 6 hr period, according to the SITUA method described in Section I. Both lots were run on a single day, and free doxorubicin HCl at identical concentrations and time points were included as a control. The human plasma and protein-free plasma DXR and DXR_C13 standard calibration curves used for this study are also provided.

Results & Conclusions

The DXR and DXR_C13 standard calibration curves used for this study are presented in **Tables V-1 to V-4 and Figures V-1 to V-4**. The free doxorubicin HCl controls averaged between 98-110% of theoretical for all concentrations and time points (**Tables V-5 and V-8, and Figure V-5**). The Doxil and Sun Pharma drug release was similar, at approximately 1% release over the 6 hr period, without a clear temporal trend (**Table V-6, V-7, V-9 and V-10, and Figures V-6 to V-8**).

Table V-1. DXR Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	2727016	9.2	92
10.0 ng/mL	2509530	8.4	84
50 ng/mL	12790648	48	96
50 ng/mL	13466309	50	100
100 ng/mL	26287361	103	103
100 ng/mL	26040963	101	101
500 ng/mL	133555739	522	104
500 ng/mL	138136872	522	104
1000 ng/mL	269738547	1067	107
1000 ng/mL	279192640	1075	108
5000 ng/mL	1344226914	5141	103
5000 ng/mL	1289923907	5175	104
10000 ng/mL	2598995261	9899	99
10000 ng/mL	2459435560	9600	96
QC			
QCL	2323215	11.0	110
QCL	2371844	10.1	101
QCM	27042102	99	99
QCM	26487439	98	98
QCH	274696464	1051	105
QCH	257274107	1039	104

DXR_PRM
Y = 0.00685308+0.00797759*X   R^2 = 0.9988   W: 1/X
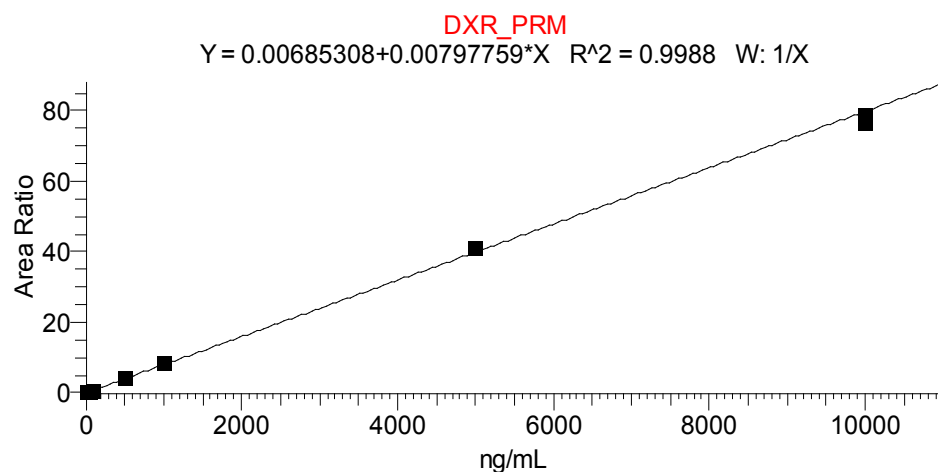
Figure V-1. DXR Plasma Standard Curve.

Table V-2. DXR_C13 Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	3877115	8.6	86
10.0 ng/mL	4360447	11.3	113
50 ng/mL	10925270	49	98
50 ng/mL	10936922	48	96
100 ng/mL	18586630	95	95
100 ng/mL	18125411	92	92
500 ng/mL	93483128	526	105
500 ng/mL	93988991	511	102
1000 ng/mL	182898828	1053	105
1000 ng/mL	190112008	1065	107
5000 ng/mL	903136802	5069	101
5000 ng/mL	878975795	5176	104
10000 ng/mL	1775829845	9938	99
10000 ng/mL	1687583791	9678	97
QC			
QCL	2644676	7.3	73
QCL	3410623	10.7	107
QCM	18554195	88	88
QCM	21853660	108	108
QCH	198905658	1107	111
QCH	177535032	1043	104

DXR_C13_PRM

$Y = 0.067624 + 0.00542312 \cdot X \quad R^2 = 0.9991 \quad W: 1/X$
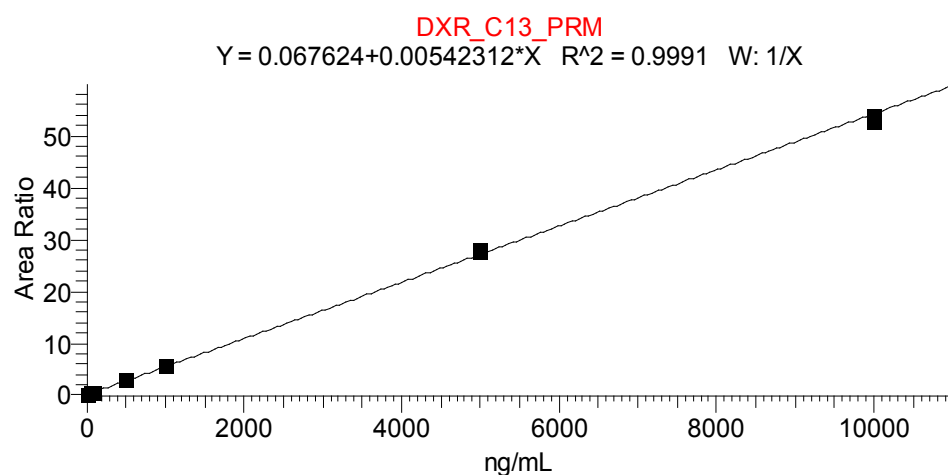
Figure V-2. DXR_C13 Plasma Standard Curve.

Table V-3. DXR Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	21768	0.110	111
0.100 ng/mL	11391	0.091	91
0.50 ng/mL	114869	0.47	95
0.50 ng/mL	107784	0.51	101
1.00 ng/mL	225721	0.93	93
1.00 ng/mL	227953	1.00	100
5.00 ng/mL	1248798	4.87	98
5.00 ng/mL	1189081	4.50	90
10.0 ng/mL	2415519	9.8	98
10.0 ng/mL	2566798	10.2	102
100.0 ng/mL	27685002	106.5	107
100.0 ng/mL	27015250	107.8	108
1000.0 ng/mL	309299063	1027.0	103
1000.0 ng/mL	226617146	1051.1	105
QC			
QCL	18929	0.101	101
QCL	18260	0.101	101
QCM	270334	0.96	96
QCM	268081	0.92	92
QCH	3066429	10.2	102
QCH	2441537	10.2	102

DXR_PRM
$Y = -0.000491086 + 0.012035 \cdot X \quad R^2 = 0.9952 \quad W: 1/X^2$
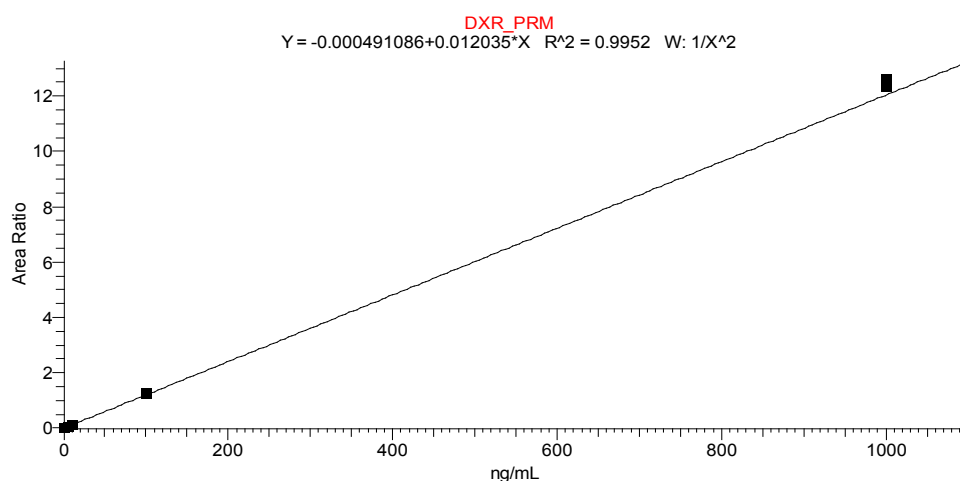
Figure V-3. DXR Protein-Free Plasma Standard Curve.

Table V-4. DXR_C13 Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	19469	0.110	110
0.100 ng/mL	10918	0.094	94
0.50 ng/mL	95266	0.47	93
0.50 ng/mL	80848	0.45	91
1.00 ng/mL	197324	0.97	97
1.00 ng/mL	184245	0.96	96
5.00 ng/mL	1061422	4.95	99
5.00 ng/mL	1019450	4.61	92
10.0 ng/mL	2157629	10.5	105
10.0 ng/mL	2084612	9.9	99
100.0 ng/mL	23182483	106.8	107
100.0 ng/mL	22569828	107.8	108
1000.0 ng/mL	258941627	1029.5	103
1000.0 ng/mL	191650916	1064.4	106
QC			
QCL	16309	0.109	109
QCL	17848	0.112	112
QCM	113399	0.93	93
QCM	117582	0.93	93
QCH	1312560	9.7	97
QCH	1037888	9.6	96

DXR_C13_PRM
$Y = -0.000359353 + 0.0100507 \cdot X \quad R^2 = 0.9948 \quad W: 1/X^2$
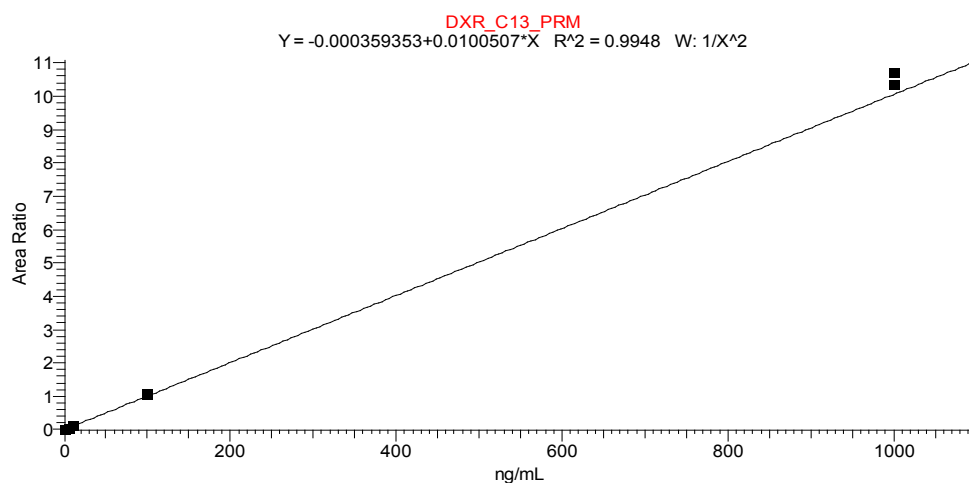
Figure V-4. DXR_C13 Protein-Free Plasma Standard Curve.

Table V-5. Free Doxorubicin HCl Control Analytical Data. Presented are the analytical data for the free doxorubicin HCl controls. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 – %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	60.3	518	11.6	88.4	10.8	102	10.6	89.4	569.6	-52	110.0	108.2	1.6
		52.2	510	10.2	89.8	9.4	98	9.6	90.4	545.9	-36	107.1		
		54.3	520	10.4	89.6	9.8	101	9.7	90.3	559.2	-39	107.6		
	1 µg/mL	117.3	1030	11.4	88.6	10.3	98	10.5	89.5	1115.2	-85	108.2	108.7	2.0
		133.7	1022	13.1	86.9	11.6	95	12.2	87.8	1093.1	-71	106.9		
		114.1	974	11.7	88.3	9.7	92	10.6	89.4	1080.3	-106	110.9		
	5 µg/mL	-	4981	-	-	-	96	-	-	-	-	-	110.2	0.4
		774.0	4902	15.8	84.2	13.5	94	14.3	85.7	5416.1	-514	110.5		
		707.0	4882	14.5	85.5	12.1	92	13.2	86.8	5364.7	-483	109.9		
2 hr	0.5 µg/mL	66.1	458	14.4	85.6	11.5	92	12.5	87.5	530.7	-72	115.8	112.4	3.0
		80.2	477	16.8	83.2	14.3	93	15.3	84.7	524.6	-48	110.0		
		68.4	447	15.3	84.7	11.8	86	13.7	86.3	498.6	-52	111.5		
	1 µg/mL	138.3	906	15.3	84.7	11.8	85	13.8	86.2	1000.6	-95	110.5	109.7	2.0
		140.1	907	15.5	84.6	11.9	86	13.9	86.1	1008.5	-102	111.2		
		143.2	902	15.9	84.1	12.7	86	14.8	85.2	969.4	-67	107.4		
	5 µg/mL	849.7	4453	19.1	80.9	14.9	87	17.1	82.9	4974.9	-522	111.7	110.4	1.4
		852.8	4543	18.8	81.2	15.2	90	17.0	83.0	5023.7	-481	110.6		
		876.3	4495	19.5	80.5	15.2	85	17.9	82.1	4900.4	-405	109.0		
6 hr	0.5 µg/mL	47.3	346	13.7	86.3	8.7	63	13.8	86.2	342.8	4	99.0	97.9	1.2
		44.3	311	14.3	85.7	8.1	56	14.6	85.4	304.3	7	97.9		
		42.2	317	13.3	86.7	7.6	55	13.8	86.2	306.2	11	96.7		
	1 µg/mL	100.1	677	14.8	85.2	8.9	59	15.0	85.0	669.5	7	98.9	98.6	0.5
		108.4	733	14.8	85.2	9.6	64	14.9	85.1	725.1	8	98.9		
		103.8	696	14.9	85.1	9.4	62	15.2	84.8	682.1	14	98.0		
	5 µg/mL	557.5	3431	16.3	83.8	10.0	61	16.5	83.5	3370.8	60	98.3	99.1	1.1
		532.3	3534	15.1	84.9	9.6	64	15.0	85.0	3548.7	-15	100.4		
		541.1	3701	14.6	85.4	9.8	66	14.8	85.2	3653.8	47	98.7		

Table V-6. Doxil, Lot B (600120P1) Drug Release Analytical Data. Presented are the analytical data for Doxil, Lot B (600120P1). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 − %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	1.5	466	0.3	99.7	13.7	102	13.4	86.6	11.1	455	2.4	3.6	2.1
		2.0	455	0.4	99.6	18.4	102	18.0	82.0	10.9	444	2.4		
		4.7	489	1.0	99.0	16.4	103	16.0	84.0	29.5	460	6.0		
	1 µg/mL	3.0	1079	0.3	99.7	14.9	100	14.8	85.2	20.4	1059	1.9	2.2	0.3
		3.6	872	0.4	99.6	16.6	97	17.2	82.8	21.0	852	2.4		
		3.1	890	0.4	99.6	15.0	98	15.3	84.7	20.5	870	2.3		
	5 µg/mL	6.8	4736	0.1	99.9	16.2	92	17.6	82.4	38.6	4698	0.8	1.1	0.4
		9.9	4528	0.2	99.8	12.7	94	13.5	86.5	73.2	4455	1.6		
		5.4	4482	0.1	99.9	12.6	91	13.7	86.3	39.5	4443	0.9		
2 hr	0.5 µg/mL	1.6	413	0.4	99.6	11.9	91	13.0	87.0	12.0	401	2.9	2.9	0.2
		1.7	444	0.4	99.6	13.4	95	14.1	85.9	11.9	432	2.7		
		2.2	478	0.5	99.5	13.3	91	14.6	85.4	14.8	463	3.1		
	1 µg/mL	3.0	931	0.3	99.7	12.9	97	13.3	86.7	22.6	908	2.4	2.4	0.1
		3.2	856	0.4	99.6	12.3	82	15.0	85.0	21.0	835	2.5		
		3.1	895	0.4	99.6	13.3	88	15.2	84.8	20.7	874	2.3		
	5 µg/mL	7.2	4224	0.2	99.8	12.0	90	13.3	86.7	54.4	4170	1.3	1.2	0.1
		7.1	4327	0.2	99.8	11.5	87	13.3	86.7	53.2	4274	1.2		
		7.8	4343	0.2	99.8	12.6	82	15.3	84.7	50.9	4292	1.2		
6 hr	0.5 µg/mL	1.3	381	0.3	99.7	8.7	65	13.5	86.5	9.3	372	2.4	2.4	0.04
		1.3	364	0.4	99.6	9.8	64	15.3	84.7	8.6	356	2.4		
		1.5	420	0.4	99.6	9.7	63	15.3	84.7	9.9	410	2.4		
	1 µg/mL	2.4	769	0.3	99.7	9.4	61	15.4	84.6	15.7	753	2.0	2.1	0.1
		2.2	737	0.3	99.7	7.6	54	14.0	86.0	15.8	722	2.1		
		2.2	727	0.3	99.7	8.9	59	15.1	84.9	14.4	713	2.0		
	5 µg/mL	6.6	3245	0.2	99.8	7.9	53	15.1	84.9	43.7	3201	1.3	1.4	0.1
		6.1	2983	0.2	99.8	5.4	38	14.2	85.8	42.7	2940	1.4		
		6.1	2943	0.2	99.8	6.4	41	15.6	84.4	39.0	2905	1.3		

Table V-7. Sun Pharma, Lot A (JKR0494A) Drug Release Analytical Data. Presented are the analytical data for Sun Pharma, Lot A (JKR0494A). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 − %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	1.9	349	0.6	99.5	17.1	112	15.2	84.8	12.5	336	3.6	2.6	1.5
		0.5	351	0.2	99.8	9.9	100	9.9	90.1	5.3	345	1.5		
		28.7	384	7.5	92.5	18.4	108	17.1	82.9	167.8	216	43.7		
	1 µg/mL	0.6	792	0.1	99.9	6.0	107	5.6	94.4	10.3	781	1.3	1.1	0.2
		0.4	698	0.1	99.9	6.4	97	6.6	93.4	5.9	692	0.8		
		0.9	634	0.1	99.9	11.1	90	12.3	87.7	7.2	627	1.1		
	5 µg/mL	1.5	3668	0.04	100.0	7.0	107	6.6	93.4	22.1	3646	0.6	1.0	0.8
		8.1	3542	0.2	99.8	11.8	100	11.7	88.3	69.3	3472	2.0		
		1.0	3574	0.03	100.0	6.6	96	6.9	93.1	14.4	3560	0.4		
2 hr	0.5 µg/mL	0.4	326	0.1	99.9	8.3	84	9.9	90.1	4.1	322	1.3	1.3	0.2
		0.4	386	0.1	99.9	7.0	86	8.1	91.9	4.6	381	1.2		
		0.5	383	0.1	99.9	8.1	90	8.9	91.1	5.8	377	1.5		
	1 µg/mL	1.0	812	0.1	99.9	10.3	85	12.1	87.9	8.4	804	1.0	0.9	0.1
		0.7	818	0.1	99.9	9.1	91	10.0	90.0	6.7	812	0.8		
		0.7	854	0.1	99.9	8.4	88	9.5	90.5	7.3	847	0.9		
	5 µg/mL	2.2	3323	0.1	99.9	8.4	94	9.0	91.0	24.2	3299	0.7	0.5	0.2
		1.6	3687	0.04	100.0	9.4	94	10.0	90.0	15.8	3671	0.4		
		1.7	4138	0.04	100.0	8.4	98	8.5	91.5	20.3	4118	0.5		
6 hr	0.5 µg/mL	0.5	498	0.1	99.9	8.7	69	12.6	87.4	3.7	495	0.7	0.8	0.1
		0.4	478	0.1	99.9	6.8	60	11.3	88.7	3.5	474	0.7		
		0.5	506	0.1	99.9	7.9	71	11.2	88.8	4.7	501	0.9		
	1 µg/mL	2.2	1007	0.2	99.8	5.1	41	12.5	87.5	17.2	990	1.7	1.0	0.6
		0.6	871	0.1	99.9	7.3	62	11.7	88.3	5.4	866	0.6		
		0.7	883	0.1	99.9	4.5	40	11.2	88.8	6.5	877	0.7		
	5 µg/mL	2.4	4117	0.1	99.9	6.6	56	11.9	88.1	20.0	4097	0.5	0.5	0.05
		3.4	3811	0.1	99.9	9.1	56	16.3	83.7	21.1	3790	0.6		
		2.3	3727	0.1	99.9	6.2	47	13.1	86.9	17.4	3710	0.5		

142

Table V-8. Free Doxorubicin HCl Control Data. Displayed is the calculated % release for the doxorubicin HCl control data. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	108.2	1.6	1.4
	1 µg/mL	108.7	2.0	1.8
	5 µg/mL	110.2	0.4	0.4
2 hr	0.5 µg/mL	112.4	3.0	2.7
	1 µg/mL	109.7	2.0	1.8
	5 µg/mL	110.4	1.4	1.2
6 hr	0.5 µg/mL	97.9	1.2	1.2
	1 µg/mL	98.6	0.5	0.5
	5 µg/mL	99.1	1.1	1.1
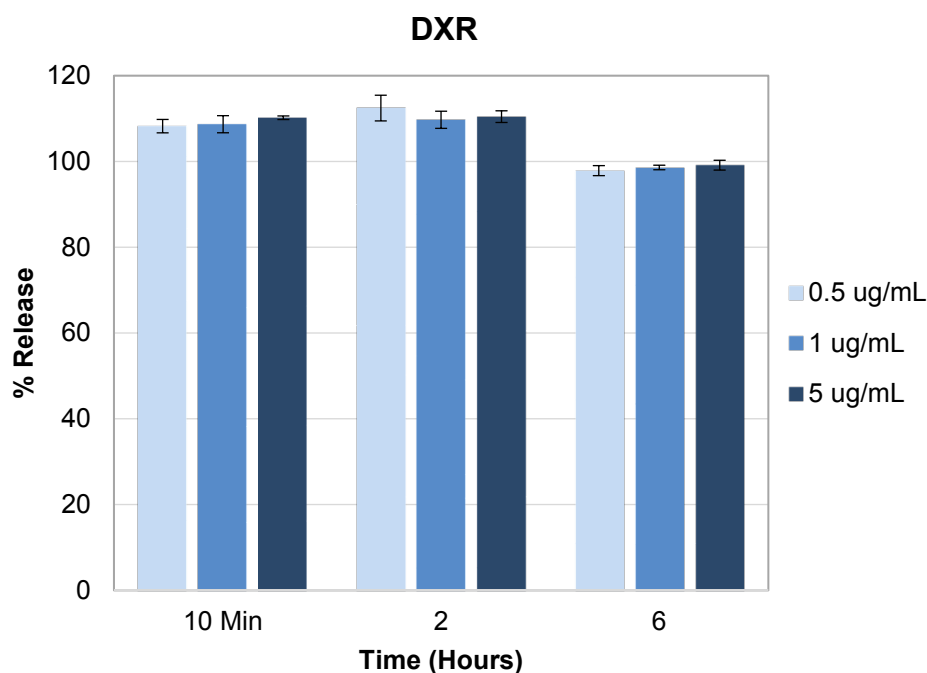
Figure V-5. Free Doxorubicin HCl Control Data. Displayed is the calculated % release for the doxorubicin HCl control data. (Mean $\pm$ SD, *N=3*)

Table V-9. Doxil, Lot B (600120P1) Drug Release. Displayed is the calculated % release for Doxil, Lot B (600120P1). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	3.6	2.1	58.2
	1 µg/mL	2.2	0.3	12.3
	5 µg/mL	1.1	0.4	40.4
2 hr	0.5 µg/mL	2.9	0.2	7.2
	1 µg/mL	2.4	0.1	3.2
	5 µg/mL	1.2	0.1	4.7
6 hr	0.5 µg/mL	2.4	0.04	1.6
	1 µg/mL	2.1	0.1	3.9
	5 µg/mL	1.4	0.1	4.0
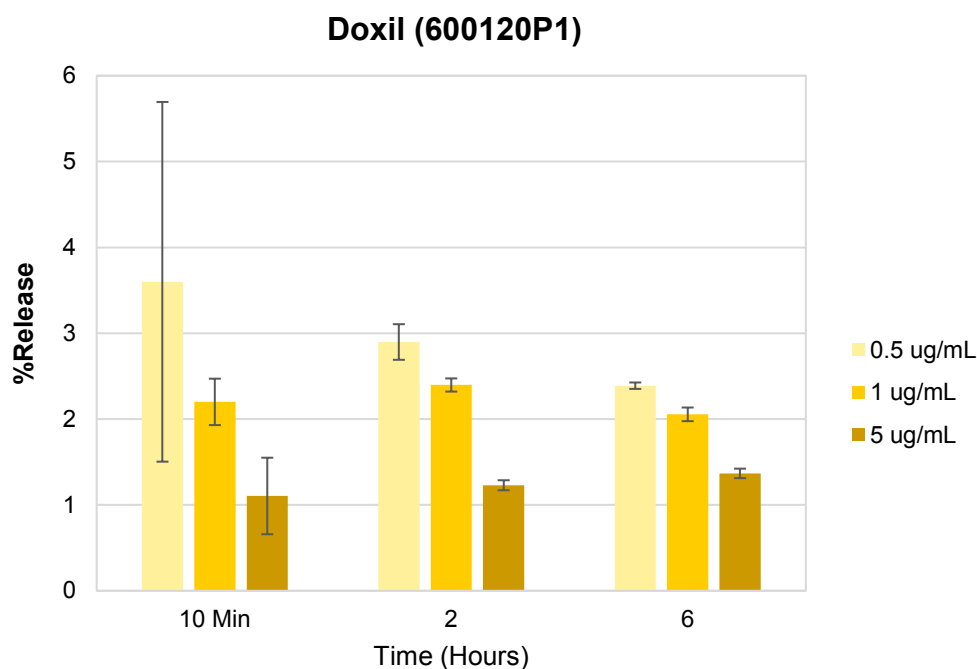
Figure V-6. Doxil, Lot B (600120P1) Drug Release. Displayed is the calculated % release for Doxil, Lot B (600120P1). (Mean $\pm$ SD, *N=3*)

Table IV-10. Sun Pharma, Lot A (JKR0494A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot A (JKR0494A). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	2.6	1.5	57.1
	1 µg/mL	1.1	0.2	21.4
	5 µg/mL	1.0	0.8	85.8
2 hr	0.5 µg/mL	1.3	0.2	12.3
	1 µg/mL	0.9	0.1	12.9
	5 µg/mL	0.5	0.2	28.6
6 hr	0.5 µg/mL	0.8	0.1	15.0
	1 µg/mL	1.0	0.6	58.7
	5 µg/mL	0.5	0.05	9.2

Sun Pharma (JKR0494A)
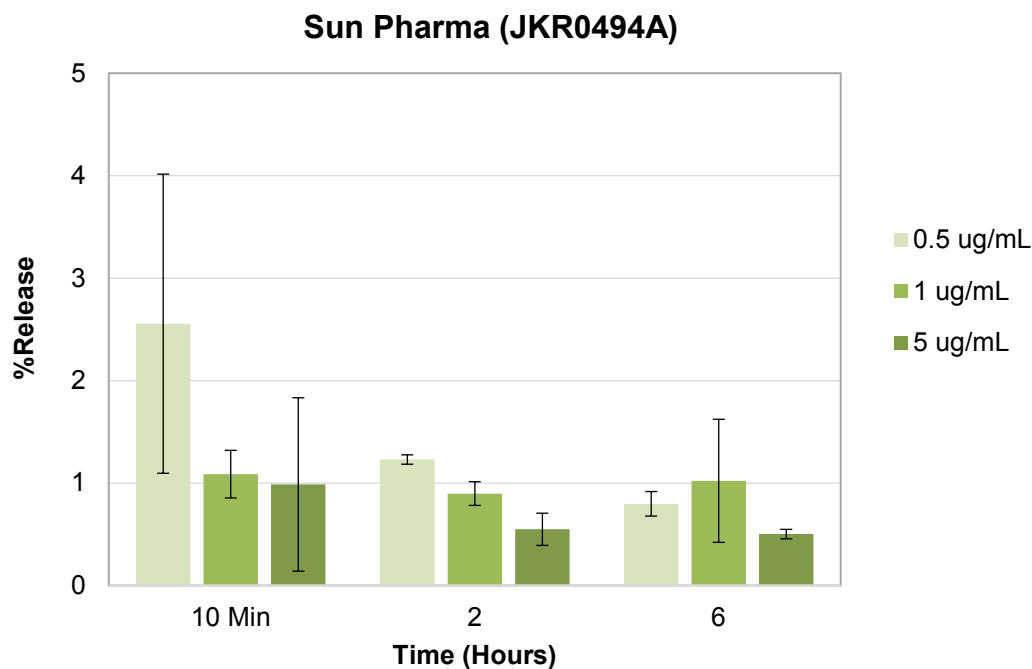
Figure IV-7. Sun Pharma, Lot A (JKR0494A) Drug Release. Displayed is the calculated % release for the Sun Pharma, Lot A (JKR0494A). (Mean $\pm$ SD, *N=3*)
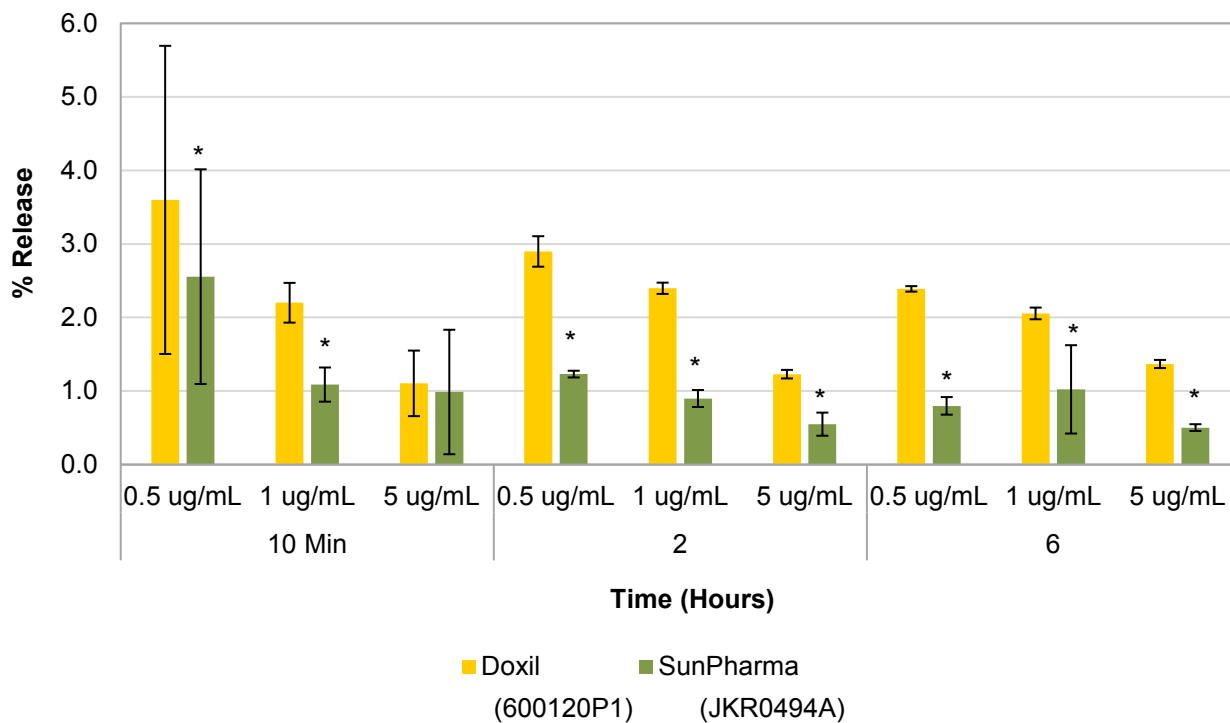
Figure V-8. Doxil and Sun Pharma Drug Release Lot Comparison. Displayed are the 10 min-6 hr calculated % release data for the Doxil and Sun Pharma Lots 600120P1 and JKR0494A, respectively. (Mean $\pm$ SD, *N=3*), *$p \leq 0.05$, Student's t-test.

Intra-day Comparison in Human Plasma at 42°C

Summary

The objective of this study was to compare the in vitro drug release of a single lot of Doxil to that of a single lot of Sun Pharma in human plasma at 42°C over a 6 hr period. All lots had similar drug release profiles over the 6 hr period, although some instances of statistically significant differences were observed between lots.

Design and Methods

A single lot of Doxil, 600120P1 (lot B), and a single lot of Sun Pharma, JKR0494A (lot A), were evaluated for drug release in human plasma at 42°C over a 6 hr period, according to the SITUA method described in Section I. Both lots were run on a single day, and free doxorubicin HCl at identical concentrations and time points were included as a control. The human plasma and protein-free plasma DXR and DXR_C13 standard calibration curves used for this study are also provided.

Results & Conclusions

The DXR and DXR_C13 standard calibration curves used for this study are presented in **Tables V-11 to V-14 and Figures V-9 to V-12**. The free doxorubicin HCl controls averaged between 100-106% of theoretical for all concentrations and time points (**Tables V-15 and V-18, and Figure V-13**). The Doxil and Sun Pharma drug release was similar, at approximately 1-3% release over the 6 hr period, without a clear temporal trend (**Tables V-16 to V-17 and V-19 to V-20, and Figures V-14 to V-16**).

Table V-11. DXR Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	1789312	11.1	111
10.0 ng/mL	1815761	11.1	111
50 ng/mL	9514348	48	97
50 ng/mL	9793362	50	100
100 ng/mL	19196632	94	94
100 ng/mL	18660777	93	93
500 ng/mL	103015421	508	102
500 ng/mL	96989141	472	95
1000 ng/mL	206334148	1019	102
1000 ng/mL	206811431	1008	101
5000 ng/mL	984766969	4579	92
5000 ng/mL	1010461312	5005	100
10000 ng/mL	2092009859	10380	104
10000 ng/mL	1960769409	10041	100
QC			
QCL	1826364	10.4	104
QCL	1812140	10.2	102
QCM	19295370	91	91
QCM	19612097	92	92
QCH	209717245	974	97
QCH	198936578	969	97

DXR_PRM
$Y = -0.0091865 + 0.00539319 \cdot X \quad R^2 = 0.9984 \quad W: 1/X$
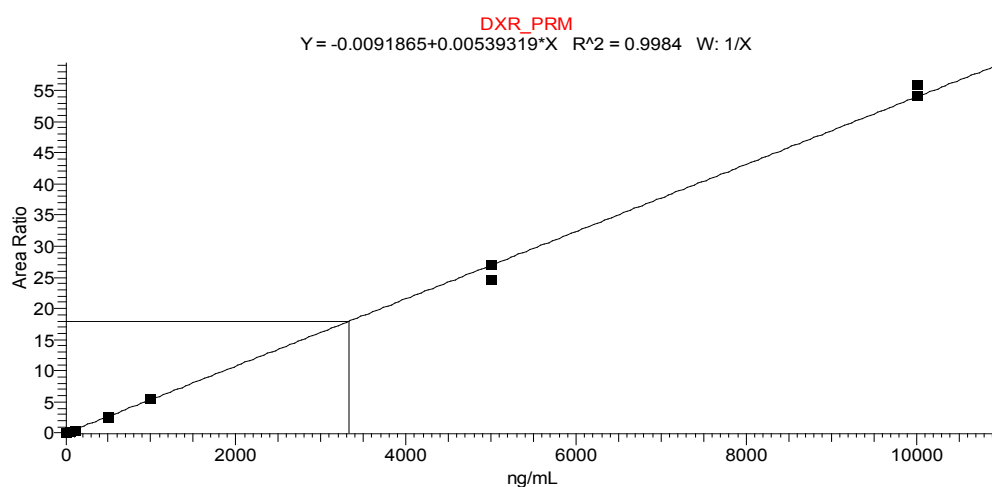
Figure V-9. DXR Plasma Standard Curve.

Table V-12. DXR_C13 Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	1430079	11.7	117
10.0 ng/mL	1414527	11.5	115
50 ng/mL	7468464	46	93
50 ng/mL	8055193	50	101
100 ng/mL	15736642	94	94
100 ng/mL	15158863	91	91
500 ng/mL	84622427	500	100
500 ng/mL	80205631	468	94
1000 ng/mL	167693427	991	99
1000 ng/mL	172531120	1007	101
5000 ng/mL	836870812	4656	93
5000 ng/mL	834817797	4948	99
10000 ng/mL	1743062991	10348	104
10000 ng/mL	1647742620	10096	101
QC			
QCL	1485489	11.2	112
QCL	1469516	10.9	109
QCM	15803544	90	90
QCM	16014174	91	91
QCH	170604700	949	95
QCH	161243644	940	94

DXR_C13_PRM
Y = -0.0122881+0.00450807*X   R^2 = 0.9987   W: 1/X
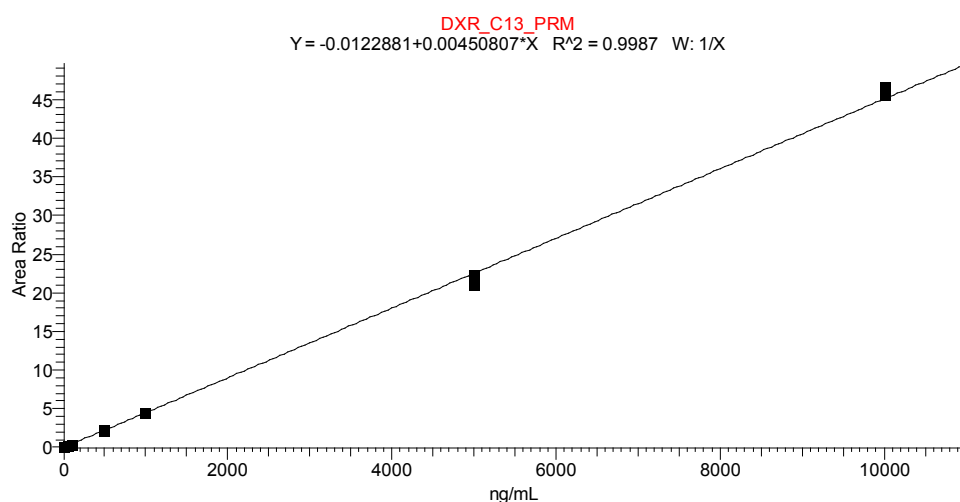
Figure V-10. DXR_C13 Plasma Standard Curve.

Table V-13. DXR Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	18603	0.104	104
0.100 ng/mL	17269	0.100	100
0.50 ng/mL	80285	0.46	92
0.50 ng/mL	83950	0.47	94
1.00 ng/mL	172814	0.95	95
1.00 ng/mL	176635	0.92	93
5.00 ng/mL	967543	4.67	94
5.00 ng/mL	982507	5.19	104
10.0 ng/mL	2065895	9.7	97
10.0 ng/mL	2020253	10.2	102
100.0 ng/mL	22426286	103.4	103
100.0 ng/mL	20843477	108.2	108
1000.0 ng/mL	231657867	1061.3	106
1000.0 ng/mL	204850302	1080.9	108
QC			
QCL	11780	0.084	84
QCL	13429	0.089	89
QCM	172443	0.98	98
QCM	174019	0.94	94
QCH	2079508	9.6	96
QCH	2110600	9.5	95

DXR_PRM

$Y = -0.0002135 + 0.00527703*X \quad R^2 = 0.9961 \quad W: 1/X^2$
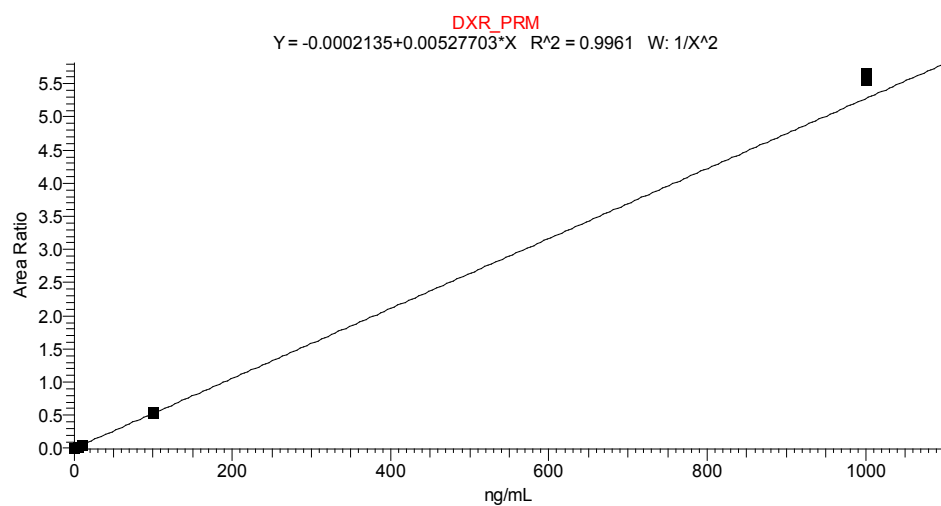
Figure V-11. DXR Protein-Free Plasma Standard Curve.

Table V-14. DXR_C13 Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	9265	0.098	98
0.100 ng/mL	10023	0.104	104
0.50 ng/mL	66638	0.49	98
0.50 ng/mL	64987	0.47	94
1.00 ng/mL	143068	0.98	98
1.00 ng/mL	143193	0.93	93
5.00 ng/mL	796665	4.66	93
5.00 ng/mL	810971	5.19	104
10.0 ng/mL	1694219	9.6	96
10.0 ng/mL	1685605	10.3	103
100.0 ng/mL	18332776	101.8	102
100.0 ng/mL	17400549	108.9	109
1000.0 ng/mL	180169346	994.3	99
1000.0 ng/mL	169783177	1079.2	108
QC			
QCL	6073	0.088	88
QCL	9575	0.109	109
QCM	153158	1.07	107
QCM	146948	0.99	99
QCH	1724615	9.6	96
QCH	1765369	9.6	96

DXR_C13_PRM
$Y = -0.000216167 + 0.00486666 \cdot X$ $R^2 = 0.9968$ W: $1/X^2$
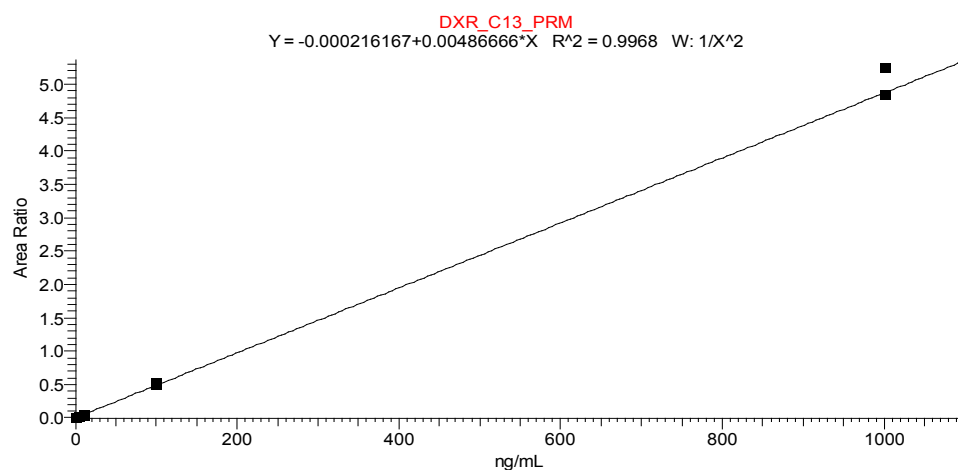
Figure V-12. DXR_C13 Protein-Free Plasma Standard Curve.

Table V-15. Free Doxorubicin HCl Control Analytical Data. Presented are the analytical data for the free doxorubicin HCl controls. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 – %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/(1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	56.6	487	11.6	88.4	10.5	91	11.5	88.5	492.5	-6	101.2	102.8	2.2
		47.9	460	10.4	89.6	9.0	91	9.9	90.1	484.9	-25	105.3		
		51.9	478	10.9	89.1	9.8	92	10.7	89.3	485.7	-8	101.7		
	1 µg/mL	119.0	937	12.7	87.3	11.3	90	12.5	87.5	954.5	-18	101.9	101.3	0.7
		106.6	882	12.1	87.9	10.6	89	11.9	88.1	895.6	-14	101.5		
		122.9	892	13.8	86.2	12.2	89	13.7	86.3	896.8	-5	100.5		
	5 µg/mL	800.0	4567	17.5	82.5	15.0	86	17.4	82.6	4598.2	-31	100.7	103.5	2.6
		793.5	4573	17.4	82.6	14.4	88	16.4	83.6	4834.1	-261	105.7		
		740.5	4490	16.5	83.5	13.6	86	15.8	84.2	4681.2	-191	104.3		
2 hr	0.5 µg/mL	73.6	449	16.4	83.6	13.8	84	16.4	83.6	448.6	0	99.9	100.7	0.7
		69.1	456	15.2	84.8	13.4	89	15.0	85.0	460.5	-5	101.1		
		75.6	455	16.6	83.4	14.3	87	16.4	83.6	460.2	-5	101.1		
	1 µg/mL	148.8	823	18.1	81.9	14.4	80	18.0	82.0	824.6	-2	100.2	100.1	0.7
		163.7	815	20.1	79.9	16.6	83	19.9	80.1	821.5	-6	100.7		
		155.2	799	19.4	80.6	15.5	79	19.5	80.5	793.9	5	99.3		
	5 µg/mL	827.5	4192	19.7	80.3	15.6	80	19.5	80.5	4243.5	-52	101.2	102.8	1.5
		883.0	4113	21.5	78.5	16.4	79	20.9	79.1	4232.8	-120	102.9		
		934.5	3901	24.0	76.0	17.2	75	23.0	77.0	4068.1	-167	104.3		
6 hr	0.5 µg/mL	44.2	296	14.9	85.1	8.3	57	14.5	85.5	304.2	-8	102.6	102.6	0.8
		39.1	288	13.6	86.4	7.7	58	13.3	86.7	293.6	-5	101.8		
		38.3	301	12.7	87.3	7.2	59	12.3	87.7	311.5	-10	103.4		
	1 µg/mL	92.5	605	15.3	84.7	8.9	60	14.7	85.3	630.6	-26	104.3	102.9	2.6
		97.7	570	17.1	82.9	9.7	59	16.4	83.6	594.7	-25	104.4		
		79.1	559	14.1	85.9	8.4	59	14.2	85.8	558.8	1	99.9		
	5 µg/mL	509.8	3112	16.4	83.6	9.4	60	15.6	84.4	3267.9	-156	105.0	105.7	0.6
		443.7	2936	15.1	84.9	8.2	57	14.2	85.8	3118.7	-183	106.2		
		509.9	3066	16.6	83.4	9.3	59	15.7	84.3	3249.5	-184	106.0		

Table V-16. Doxil, Lot B (600120P1) Drug Release Analytical Data. Presented are the analytical data for Doxil, Lot B (600120P1). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 – %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	0.8	530	0.1	99.9	9.7	96	10.1	89.9	7.5	523	1.4	1.4	0.04
		0.9	522	0.2	99.8	10.9	91	12.0	88.0	7.8	514	1.5		
		0.8	540	0.2	99.8	9.8	91	10.9	89.1	7.7	532	1.4		
	1 µg/mL	1.5	1080	0.1	99.9	10.0	91	11.0	89.0	14.1	1066	1.3	1.3	0.1
		1.5	1085	0.1	99.9	9.3	91	10.2	89.8	15.0	1070	1.4		
		1.4	1102	0.1	99.9	9.7	92	10.5	89.5	13.7	1088	1.2		
	5 µg/mL	6.9	4468	0.2	99.8	10.5	84	12.5	87.5	54.7	4413	1.2	1.2	0.1
		5.8	4467	0.1	99.9	8.9	82	10.8	89.2	53.5	4413	1.2		
		6.7	5122	0.1	99.9	10.5	89	11.7	88.3	56.8	5066	1.1		
2 hr	0.5 µg/mL	1.3	543	0.2	99.8	9.0	74	12.1	87.9	10.3	532	1.9	2.1	0.2
		1.2	510	0.2	99.8	6.9	68	10.2	89.8	11.6	499	2.3		
		1.1	501	0.2	99.8	7.2	69	10.5	89.5	10.4	491	2.1		
	1 µg/mL	2.7	1071	0.2	99.8	8.5	70	12.1	87.9	22.0	1049	2.1	1.9	0.1
		2.0	1038	0.2	99.8	7.1	73	9.8	90.2	20.2	1018	1.9		
		2.0	871	0.2	99.8	7.2	55	13.1	86.9	15.6	855	1.8		
	5 µg/mL	9.8	4988	0.2	99.8	7.6	65	11.7	88.3	84.0	4904	1.7	1.9	0.2
		8.7	4692	0.2	99.8	6.3	64	9.8	90.2	88.8	4603	1.9		
		8.9	4542	0.2	99.8	6.5	66	9.9	90.1	90.2	4452	2.0		
6 hr	0.5 µg/mL	1.6	413	0.4	99.6	3.3	30	11.1	88.9	14.0	399	3.4	3.4	0.1
		1.6	414	0.4	99.6	2.5	23	10.9	89.1	14.5	400	3.5		
		1.5	416	0.4	99.6	2.5	24	10.4	89.6	14.1	401	3.4		
	1 µg/mL	3.0	893	0.3	99.7	2.9	29	9.8	90.2	30.2	863	3.4	3.6	0.3
		3.3	766	0.4	99.6	3.8	30	12.4	87.6	26.4	740	3.4		
		4.1	823	0.5	99.5	3.3	26	12.5	87.5	33.0	790	4.0		
	5 µg/mL	12.8	4260	0.3	99.7	2.0	30	6.7	93.3	190.3	4070	4.5	4.6	0.6
		12.0	3693	0.3	99.7	1.5	25	6.1	93.9	195.8	3498	5.3		
		11.2	3674	0.3	99.7	2.5	34	7.3	92.7	153.2	3520	4.2		

Table V-17. Sun Pharma, Lot A (JKR0494A) Drug Release Analytical Data. Presented are the analytical data for Sun Pharma, Lot A (JKR0494A). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR	% Bound	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13	% Bound DXR_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG	SD
				= Filtrate DXR / Reservoir DXR *100	= 100 – %Unbound DXR			= Filtrate DXR_C13 / Reservoir DXR_C13 * 100	= 100 – %Unbound C13	= Fil DXR/ (1-(%Bound DXR_C13/100))	= Reservoir DXR- Unencapsulated	= (Unencapsulated/ Reservoir DXR) *100		
10 min	0.5 µg/mL	0.4	500	0.1	99.9	10.4	91	11.5	88.5	3.7	496	0.7	0.8	0.1
		0.4	511	0.1	99.9	11.5	95	12.1	87.9	3.3	508	0.6		
		0.4	490	0.1	99.9	9.8	93	10.6	89.4	4.2	486	0.9		
	1 µg/mL	0.6	999	0.1	99.9	9.7	91	10.6	89.4	6.0	993	0.6	0.5	0.1
		0.6	985	0.1	99.9	10.0	87	11.5	88.5	5.1	980	0.5		
		0.6	1060	0.1	99.9	10.3	90	11.4	88.6	5.2	1055	0.5		
	5 µg/mL	3.1	4763	0.1	99.9	10.6	85	12.5	87.5	24.4	4739	0.5	0.5	0.02
		2.9	4421	0.1	99.9	10.0	84	12.0	88.0	23.9	4397	0.5		
		2.8	4630	0.1	99.9	9.8	81	12.1	87.9	23.4	4607	0.5		
2 hr	0.5 µg/mL	0.7	517	0.1	99.9	7.8	73	10.7	89.3	6.3	511	1.2	1.2	0.1
		0.6	480	0.1	99.9	8.5	77	11.1	88.9	5.1	475	1.1		
		0.6	495	0.1	99.9	7.6	72	10.5	89.5	6.1	489	1.2		
	1 µg/mL	1.2	1074	0.1	99.9	8.8	76	11.6	88.4	10.4	1063	1.0	1.0	0.1
		1.0	985	0.1	99.9	6.6	67	9.9	90.1	10.4	974	1.1		
		0.9	1024	0.1	99.9	7.8	73	10.7	89.3	8.8	1015	0.9		
	5 µg/mL	4.5	4863	0.1	99.9	7.6	72	10.6	89.4	42.7	4821	0.9	1.0	0.2
		5.2	4359	0.1	99.9	6.0	63	9.5	90.5	54.2	4304	1.2		
		5.0	4614	0.1	99.9	8.0	71	11.2	88.8	44.9	4570	1.0		
6 hr	0.5 µg/mL	1.1	392	0.3	99.7	3.3	26	12.6	87.4	8.5	383	2.2	2.5	0.6
		0.9	342	0.3	99.7	4.4	36	12.2	87.8	7.5	334	2.2		
		1.3	323	0.4	99.6	2.7	21	12.5	87.5	10.4	312	3.2		
	1 µg/mL	2.1	807	0.3	99.7	4.9	41	12.0	88.0	17.6	790	2.2	2.7	0.6
		2.4	759	0.3	99.7	1.7	19	9.2	90.8	25.6	733	3.4		
		2.4	727	0.3	99.7	3.5	28	12.8	87.2	18.9	708	2.6		
	5 µg/mL	8.7	3303	0.3	99.7	3.4	35	9.5	90.5	91.4	3211	2.8	4.2	1.5
		11.6	3647	0.3	99.7	1.3	24	5.4	94.6	212.1	3435	5.8		
		10.6	3335	0.3	99.7	2.4	29	8.0	92.0	132.3	3203	4.0		

Table V-18. Free Doxorubicin HCl Control Data. Displayed is the calculated % release for the doxorubicin HCl control data. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	102.8	2.2	2.2
	1 µg/mL	101.3	0.7	0.7
	5 µg/mL	103.5	2.6	2.5
2 hr	0.5 µg/mL	100.7	0.7	0.7
	1 µg/mL	100.1	0.7	0.7
	5 µg/mL	102.8	1.5	1.5
6 hr	0.5 µg/mL	102.6	0.8	0.8
	1 µg/mL	102.9	2.6	2.5
	5 µg/mL	105.7	0.6	0.6
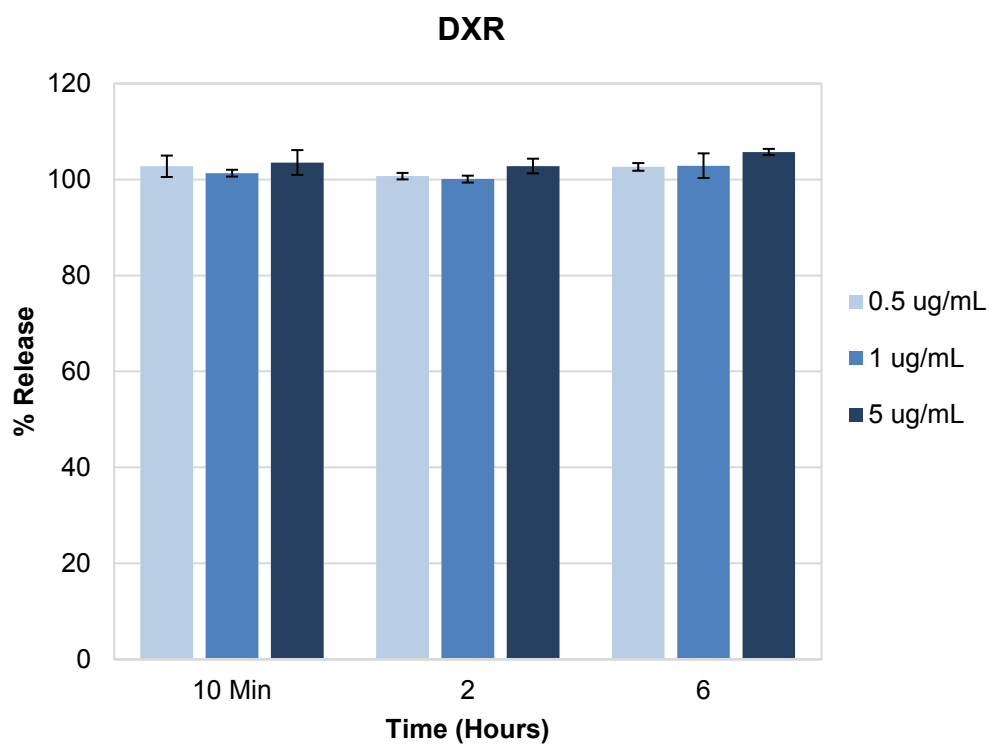
Figure V-13. Free Doxorubicin HCl Control Data. Displayed is the calculated % release for the doxorubicin HCl control data. (Mean $\pm$ SD, *N=3*)

Table V-19. Doxil, Lot B (600120P1) Drug Release. Displayed is the calculated % release for Doxil, Lot B (600120P1). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	1.5	0.04	2.9
	1 µg/mL	1.3	0.1	5.1
	5 µg/mL	1.2	0.1	5.2
2 hr	0.5 µg/mL	2.1	0.2	9.3
	1 µg/mL	1.9	0.1	6.8
	5 µg/mL	1.9	0.2	8.3
6 hr	0.5 µg/mL	3.4	0.1	1.9
	1 µg/mL	3.6	0.4	9.6
	5 µg/mL	4.7	0.6	12.6
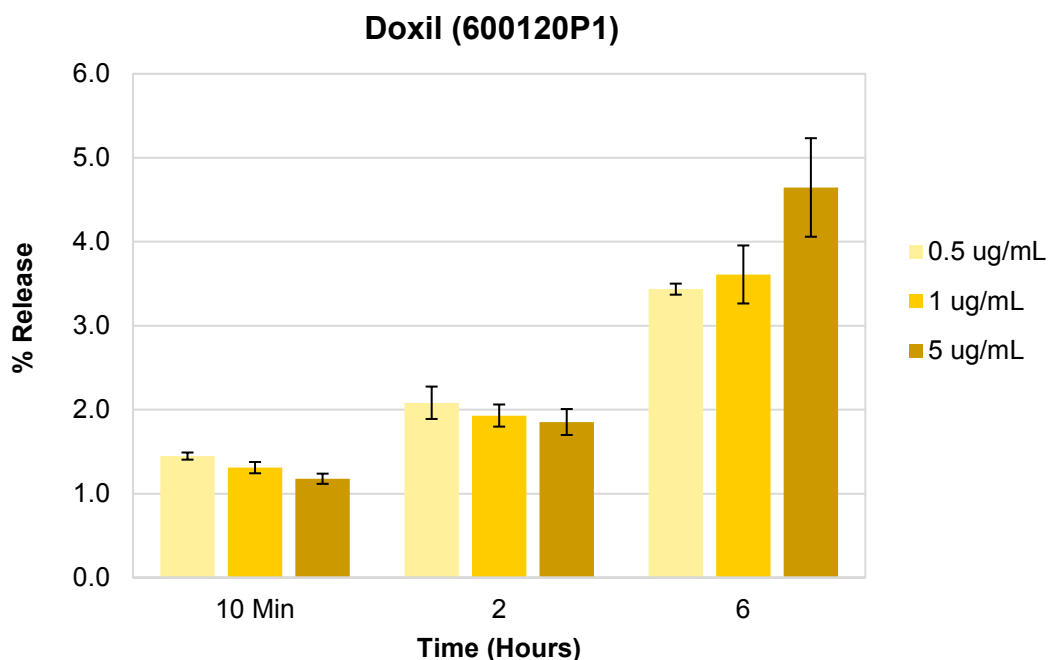
Figure V-14. Doxil, Lot B (600120P1) Drug Release. Displayed is the calculated % release for Doxil, Lot B (600120P1). (Mean $\pm$ SD, *N=3*)

Table V-20. Sun Pharma, Lot A (JKR0494A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot A (JKR0494A). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	0.8	0.1	14.0
	1 µg/mL	0.5	0.1	10.6
	5 µg/mL	0.5	0.02	3.6
2 hr	0.5 µg/mL	1.2	0.1	7.7
	1 µg/mL	1.0	0.1	10.2
	5 µg/mL	1.0	0.2	18.4
6 hr	0.5 µg/mL	2.5	0.6	24.2
	1 µg/mL	2.7	0.6	22.3
	5 µg/mL	4.2	1.5	36.7

Sun Pharma (JKR0494A)
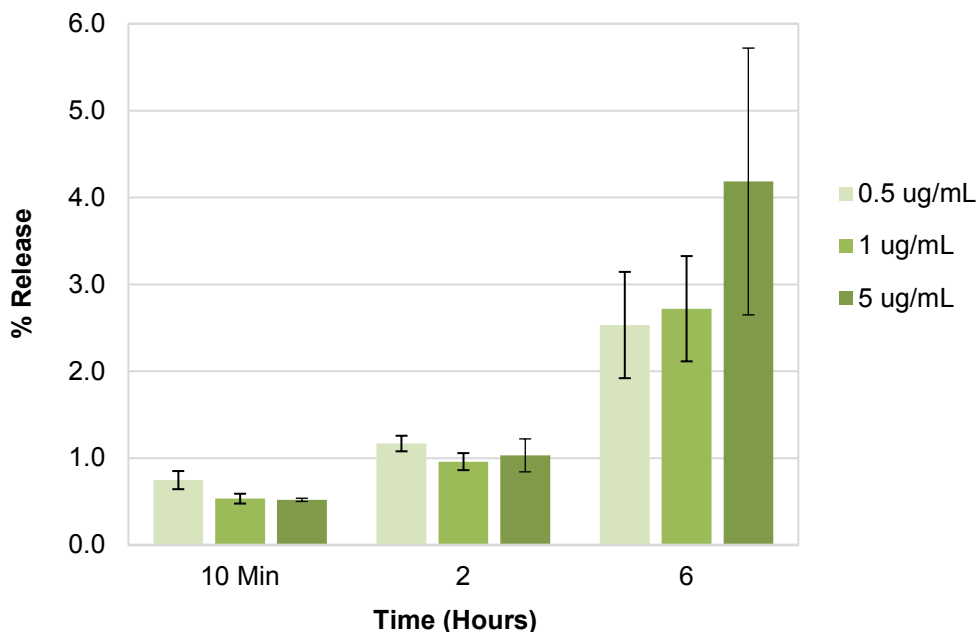
Figure V-15. Sun Pharma, Lot A (JKR0494A) Drug Release. Displayed is the calculated % release for the Sun Pharma, Lot A (JKR0494A). (Mean + SD, *N=3*)
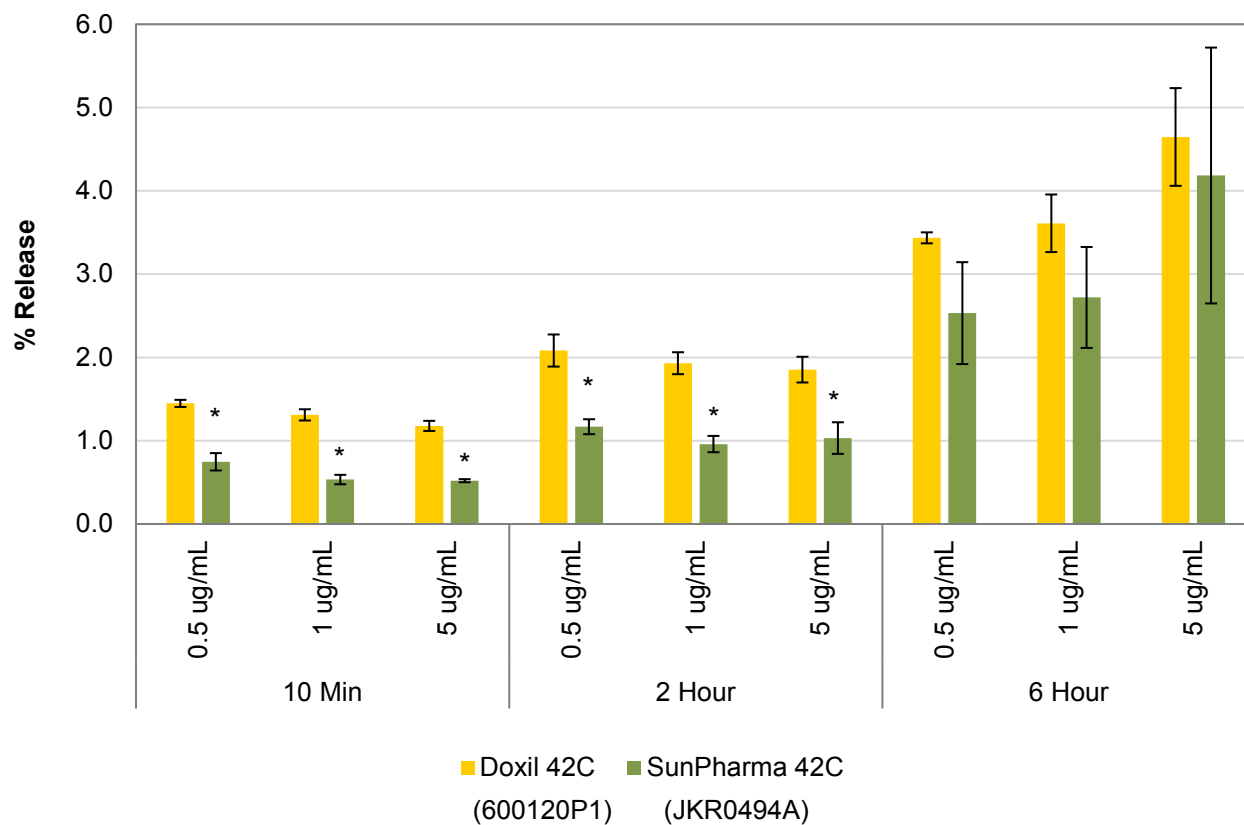
Figure V-16. Doxil and Sun Pharma Drug Release Lot Comparison. Displayed are the 10 min-6 hr calculated % release data for the Doxil and Sun Pharma lots 600120P1 and JKR0494A, respectively. (Mean $\pm$ SD, *N=3*), *p$\leq$0.05, Student's t-test.

Intra-day Comparison in Rat Plasma at 37°C

Summary
The objective of this study was to compare the in vitro drug release of a single lot of Doxil to that of a single lot of Sun Pharma in rat plasma at 37°C over a 6 hr period. All lots had similar drug release profiles over the 6 hr period, although some instances of statistically significant differences were observed between lots.

Design and Methods
A single lot of Doxil, 600520P1 (lot C), and a single lot of Sun Pharma, JKR0865A (lot D), were evaluated for drug release in rat plasma at 37°C over a 6 hr period, according to the SITUA method described in Section I. Both lots were run on a single day, and free doxorubicin HCl at identical concentrations and time points were included as a control. The human plasma and protein-free plasma, DXR and DXR_C13 standard calibration curves used for this study are also provided.

Results & Conclusions
The DXR and DXR_C13 standard calibration curves used for this study are presented in **Tables V-21 to V-24 and Figures V-17 to V-20**. The free doxorubicin HCl controls averaged between 93-114% of theoretical for all concentrations and time points (**Tables V-25 and V-28, and Figure V-21**). The Doxil and Sun Pharma drug release was similar, at approximately 1-3% release over the 6 hr period, without a clear temporal trend (**Tables V-26, V-27, V-29 and V-30, and Figures V-22 to V-24**).

Table V-21. DXR Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	814971	11.3	113
10.0 ng/mL	920480	12.1	121
50 ng/mL	5194650	49	99
50 ng/mL	4903013	48	96
100 ng/mL	11059892	95	95
100 ng/mL	10435735	94	94
500 ng/mL	63367759	540	108
500 ng/mL	61944736	522	104
1000 ng/mL	114933198	1043	104
1000 ng/mL	121350899	1028	103
5000 ng/mL	606671052	4914	98
5000 ng/mL	595262758	4971	99
10000 ng/mL	1195908335	9706	97
10000 ng/mL	1226502457	10291	103
QC			
QCL	1367320	12.5	125
QCL	1065930	12.9	129
QCM	9689224	95	95
QCM	11702020	101	101
QCH	123873690	995	99
QCH	121401818	1039	104

DXR_PRM
Y = -0.0218868+0.00126815*X   R^2 = 0.9991   W: 1/X
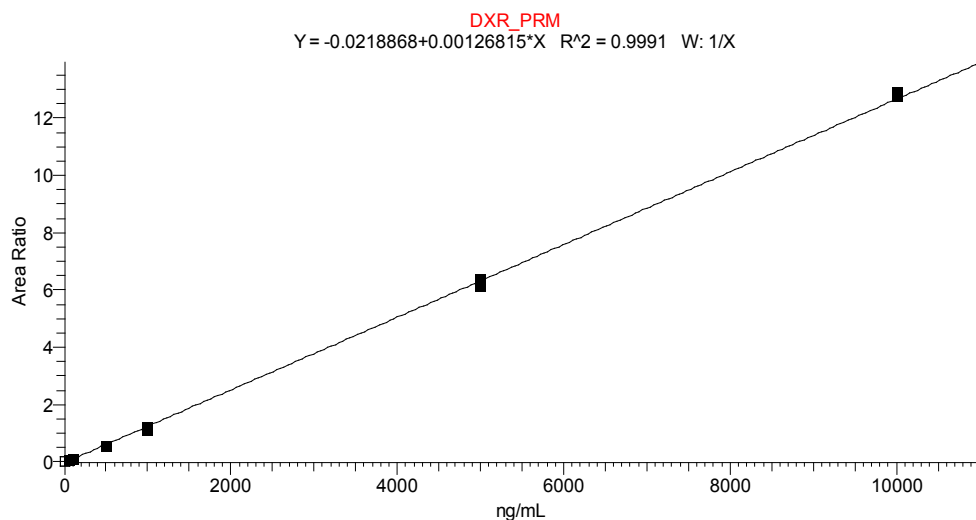
Figure V-17. DXR Plasma Standard Curve.

Table V-22. DXR_C13 Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	951429	10.9	109
10.0 ng/mL	917335	10.4	104
50 ng/mL	3884734	47	93
50 ng/mL	3807232	47	93
100 ng/mL	8111685	92	92
100 ng/mL	7770482	92	92
500 ng/mL	43259603	509	102
500 ng/mL	45263598	527	105
1000 ng/mL	84765508	1067	107
1000 ng/mL	88431743	1039	104
5000 ng/mL	439059812	4950	99
5000 ng/mL	432767306	5032	101
10000 ng/mL	843652377	9536	95
10000 ng/mL	886694551	10362	104
QC			
QCL	1138929	9.1	91
QCL	1045124	11.3	113
QCM	7169817	93	93
QCM	8722706	100	100
QCH	90412581	1007	101
QCH	89018737	1057	106

DXR_C13_PRM
Y = -0.0199869+0.00105685*X   R^2 = 0.9990   W: 1/X
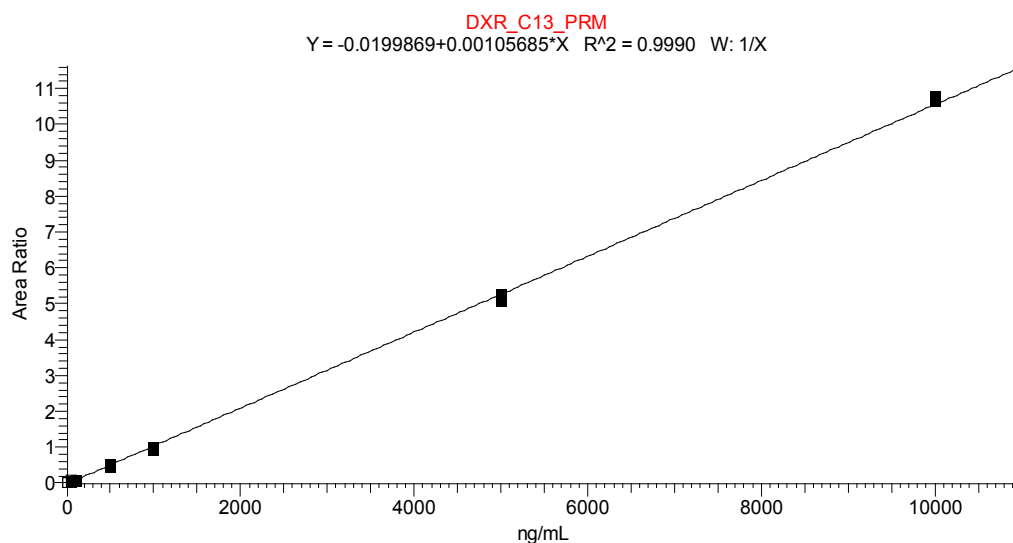
Figure V-18. DXR_C13 Plasma Standard Curve.

Table V-23. DXR Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	13319	0.101	101
0.100 ng/mL	12661	0.102	102
0.50 ng/mL	71838	0.47	94
0.50 ng/mL	72319	0.47	95
1.00 ng/mL	136960	0.93	93
1.00 ng/mL	158222	0.99	99
5.00 ng/mL	796700	4.89	98
5.00 ng/mL	797447	4.94	99
10.0 ng/mL	1703628	9.7	97
10.0 ng/mL	1618471	9.7	97
100.0 ng/mL	18566189	105.1	105
100.0 ng/mL	18061977	104.2	104
1000.0 ng/mL	198410833	1084.5	109
1000.0 ng/mL	203716575	1073.6	107
QC			
QCL	9449	0.095	95
QCL	14190	0.113	113
QCM	147538	1.01	101
QCM	151413	1.01	101
QCH	1615623	9.8	98
QCH	1711895	9.8	98

DXR_PRM
$Y = -0.000156087 + 0.00385002 \cdot X \quad R^2 = 0.9970 \quad W: 1/X^2$
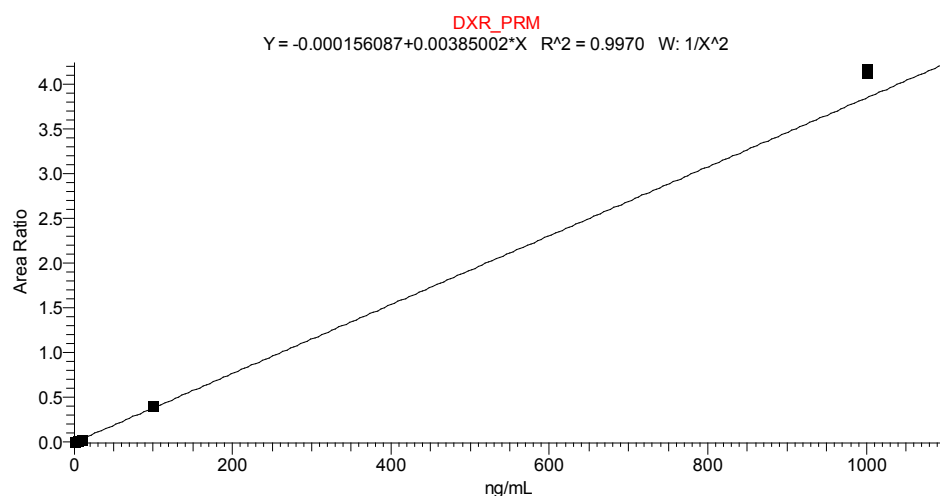
Figure V-19. DXR Protein-Free Plasma Standard Curve.

Table V-24. DXR_C13 Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	3604	0.100	100
0.100 ng/mL	4127	0.106	106
0.50 ng/mL	40159	0.43	86
0.50 ng/mL	42987	0.45	91
1.00 ng/mL	90196	0.94	94
1.00 ng/mL	96351	0.93	93
5.00 ng/mL	523552	4.78	96
5.00 ng/mL	543895	5.01	100
10.0 ng/mL	1178125	10.0	100
10.0 ng/mL	1105863	9.9	99
100.0 ng/mL	12953773	108.3	108
100.0 ng/mL	12816072	109.2	109
1000.0 ng/mL	136001881	1097.3	110
1000.0 ng/mL	139933840	1088.6	109
QC			
QCL	3885	0.109	109
QCL	4532	0.110	110
QCM	93510	0.99	99
QCM	93040	0.96	96
QCH	1099089	9.9	99
QCH	1205690	10.3	103

DXR_C13_PRM
$Y = -0.000198474 + 0.00260832 \cdot X$ $R^2 = 0.9931$ W: $1/X^2$
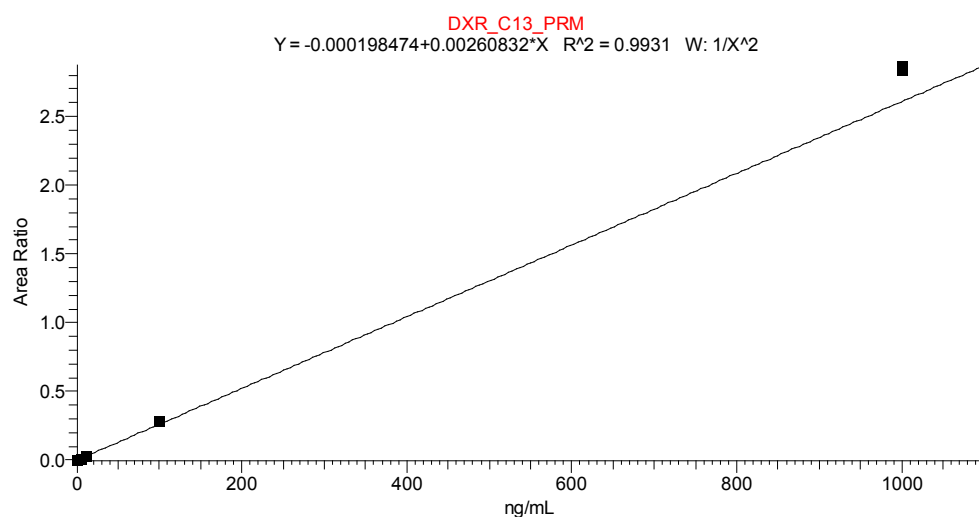
Figure V-20. DXR_C13 Protein-Free Plasma Standard Curve.

Table V-25. Free Doxorubicin HCl Control Analytical Data. Presented are the analytical data for the free doxorubicin HCl controls. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 – %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	83.4	592	14.1	85.9	12.2	84	14.5	85.5	576.0	16	97.4	92.6	6.8
		76.3	574	13.3	86.7	216.8	76	286.6	-186.6	26.6	548	4.6		
		78.2	599	13.1	86.9	12.2	82	14.9	85.1	525.5	73	87.8.		
	1 µg/mL	142.2	1016	14.0	86.0	10.1	76	13.4	86.6	1063.8	-48	104.7	100.0	6.7
		150.6	1104	13.6	86.4	16.5	79	20.9	79.1	721.4	383	65.3		
		141.8	1048	13.5	86.5	10.9	77	14.2	85.8	998.5	50	95.2		
	5 µg/mL	838.3	5072	16.5	83.5	12.1	77	15.8	84.2	5301.6	-229	104.5	98.9	7.2
		874.2	4951	17.7	82.3	14.4	74	19.4	80.6	4496.4	455	90.8		
		874.5	4893	17.9	82.1	12.5	71	17.6	82.4	4965.3	-73	101.5		
2 hr	0.5 µg/mL	78.4	542	14.5	85.5	11.3	76	14.8	85.2	528.4	14	97.5	100.1	2.6
		75.8	571	13.3	86.7	9.9	75	13.3	86.7	571.9	-1	100.2		
		78.7	453	17.4	82.6	10.9	64	16.9	83.1	465.8	-13	102.8		
	1 µg/mL	136.3	828	16.5	83.5	10.2	64	15.8	84.2	863.6	-35	104.3	104.6	3.2
		130.5	918	14.2	85.8	9.5	68	14.0	86.0	932.6	-14	101.6		
		147.4	746	19.8	80.2	10.5	57	18.3	81.7	805.5	-59	107.9		
	5 µg/mL	787.4	4678	16.8	83.2	11.7	71	16.5	83.5	4769.2	-91	101.9	107.5	8.7
		842.6	3992	21.1	78.9	11.2	63	18.0	82.0	4692.4	-700	117.5		
		837.9	4999	16.8	83.2	11.7	72	16.3	83.7	5144.9	-146	102.9		
6 hr	0.5 µg/mL	30.3	338	9.0	91.0	4.5	53	8.5	91.5	356.2	-19	105.5	110.4	6.4
		35.2	362	9.7	90.3	4.3	52	8.3	91.7	425.7	-64	117.7		
		43.3	372	11.6	88.4	6.0	55	10.8	89.2	401.9	-30	108.0		
	1 µg/mL	81.5	626	13.0	87.0	6.1	51	11.8	88.2	689.8	-64	110.2	113.0	2.8
		83.2	647	12.9	87.1	5.9	52	11.4	88.6	729.9	-83	112.9		
		93.9	620	15.1	84.9	6.6	50	13.1	86.9	718.4	-98	115.8		
	5 µg/mL	522.8	3021	17.3	82.7	7.6	51	14.9	85.1	3516.0	-495	116.4	113.6	2.4
		544.2	3736	14.6	85.4	7.6	59	13.0	87.0	4196.6	-461	112.3		
		641.4	3782	17.0	83.0	8.8	58	15.1	84.9	4243.2	-461	112.2		

Table V-26. Doxil, Lot C (600520P1) Drug Release Analytical Data. Presented are the analytical data for Doxil, Lot C (600520P1). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 − %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	2.6	494	0.5	99.5	12.4	93	13.3	86.7	19.8	474	4.0	4.1	0.2
		2.1	335	0.6	99.4	12.5	84	14.8	85.2	14.4	320	4.3		
		2.6	473	0.6	99.4	13.4	94	14.2	85.8	18.5	455	3.9		
	1 µg/mL	2.8	919	0.3	99.7	12.2	89	13.6	86.4	20.8	898	2.3	2.5	0.2
		3.2	786	0.4	99.6	13.4	86	15.6	84.4	20.8	765	2.6		
		3.0	882	0.3	99.7	11.7	90	13.0	87.0	23.1	859	2.6		
	5 µg/mL	9.2	4575	0.2	99.8	14.3	91	15.7	84.3	58.4	4517	1.3	1.4	0.1
		7.6	3701	0.2	99.8	11.4	87	13.2	86.8	57.5	3644	1.6		
		8.3	4439	0.2	99.8	12.2	96	12.7	87.3	65.0	4374	1.5		
2 hr	0.5 µg/mL	2.1	792	0.3	99.7	7.9	83	9.5	90.5	21.5	770	2.7	2.6	0.2
		2.1	529	0.4	99.6	10.9	65	16.8	83.2	12.5	517	2.4		
		2.6	702	0.4	99.6	10.7	80	13.5	86.5	19.0	683	2.7		
	1 µg/mL	3.0	1200	0.2	99.8	10.7	76	14.2	85.8	21.1	1179	1.8	1.7	0.05
		2.7	1357	0.2	99.8	9.7	81	11.9	88.1	23.0	1334	1.7		
		3.0	1480	0.2	99.8	10.6	92	11.5	88.5	26.5	1453	1.8		
	5 µg/mL	7.8	6972	0.1	99.9	8.3	88	9.4	90.6	82.8	6889	1.2	1.3	0.3
		12.7	6290	0.2	99.8	10.4	85	12.3	87.7	103.3	6186	1.6		
		8.3	5721	0.1	99.9	9.8	80	12.2	87.8	68.3	5652	1.2		
6 hr	0.5 µg/mL	3.0	538	0.6	99.4	6.6	56	11.6	88.4	26.0	512	4.8	3.8	0.9
		2.1	590	0.4	99.6	7.9	70	11.3	88.7	18.7	571	3.2		
		2.2	598	0.4	99.6	7.4	68	10.9	89.1	19.8	578	3.3		
	1 µg/mL	2.2	977	0.2	99.8	5.3	69	7.7	92.3	28.0	949	2.9	2.8	0.04
		2.6	979	0.3	99.7	5.7	62	9.2	90.8	28.2	951	2.9		
		3.1	964	0.3	99.7	7.4	64	11.6	88.4	27.0	937	2.8		
	5 µg/mL	12.4	5076	0.2	99.8	4.6	55	8.4	91.6	147.8	4928	2.9	2.2	0.6
		8.1	4280	0.2	99.8	6.7	63	10.6	89.4	76.1	4204	1.8		
		5.9	3868	0.2	99.8	4.8	57	8.3	91.7	70.5	3798	1.8		

Table V-27. Sun Pharma, Lot D (JKR0865A) Drug Release Analytical Data. Presented are the analytical data for Sun Pharma, Lot D (JKR0865A). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 − %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated	% Release = (Unencapsulated/ Reservoir DXR) *100	AVG	SD
10 min	0.5 µg/mL	1.3	415	0.3	99.7	11.3	83	13.6	86.4	9.2	406	2.2	2.4	0.4
		1.5	360	0.4	99.6	11.8	81	14.5	85.5	10.5	350	2.9		
		1.5	507	0.3	99.7	12.3	90	13.7	86.3	10.6	497	2.1		
	1 µg/mL	2.5	794	0.3	99.7	11.4	85	13.5	86.5	18.3	776	2.3	2.5	0.3
		2.3	645	0.3	99.7	10.9	90	12.1	87.9	18.6	626	2.9		
		2.1	732	0.3	99.7	10.1	82	12.3	87.7	17.1	714	2.3		
	5 µg/mL	8.9	3668	0.2	99.8	9.7	67	14.4	85.6	61.6	3607	1.7	1.9	0.2
		9.2	3021	0.3	99.7	10.5	70	15.0	85.0	61.2	2960	2.0		
		10.8	3507	0.3	99.7	11.2	70	16.1	83.9	67.5	3440	1.9		
2 hr	0.5 µg/mL	1.2	653	0.2	99.8	9.8	74	13.2	86.8	9.2	643	1.4	1.5	0.2
		1.3	580	0.2	99.8	8.5	62	13.6	86.4	9.8	571	1.7		
		1.2	643	0.2	99.8	9.1	64	14.1	85.9	8.3	634	1.3		
	1 µg/mL	2.1	1316	0.2	99.8	8.8	74	11.9	88.1	17.8	1299	1.4	1.5	0.2
		2.4	1117	0.2	99.8	7.9	62	12.6	87.4	19.3	1098	1.7		
		2.0	1179	0.2	99.8	8.3	67	12.4	87.6	16.3	1163	1.4		
	5 µg/mL	8.6	6388	0.1	99.9	8.0	71	11.3	88.7	76.0	6312	1.2	1.2	0.1
		9.5	5371	0.2	99.8	10.1	63	16.0	84.0	59.7	5311	1.1		
		10.5	5314	0.2	99.8	9.5	63	15.1	84.9	69.8	5244	1.3		
6 hr	0.5 µg/mL	1.1	516	0.2	99.8	5.8	58	10.1	89.9	10.5	505	2.0	2.5	0.7
		1.6	522	0.3	99.7	4.1	45	9.1	90.9	17.5	505	3.4		
		1.2	555	0.2	99.8	5.8	56	10.4	89.6	11.8	543	2.1		
	1 µg/mL	2.7	1096	0.2	99.8	6.1	59	10.3	89.7	25.9	1070	2.4	2.8	0.6
		3.6	989	0.4	99.6	4.5	43	10.4	89.6	34.2	955	3.5		
		2.9	1017	0.3	99.7	6.0	52	11.7	88.3	24.6	992	2.4		
	5 µg/mL	8.3	4876	0.2	99.8	4.7	53	8.9	91.1	93.7	4782	1.9	1.9	0.2
		8.7	5196	0.2	99.8	5.5	57	9.5	90.5	91.5	5105	1.8		
		10.1	4850	0.2	99.8	5.4	56	9.7	90.3	104.0	4746	2.1		

Table V-28. Free Doxorubicin HCl Control Data. Displayed is the calculated % release for the doxorubicin HCl control data. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	92.6	6.8	7.3
	1 µg/mL	100.0	6.7	6.7
	5 µg/mL	98.9	7.2	7.3
2 hr	0.5 µg/mL	100.1	2.6	2.6
	1 µg/mL	104.6	3.2	3.1
	5 µg/mL	107.5	8.7	8.1
6 hr	0.5 µg/mL	110.4	6.4	5.8
	1 µg/mL	113.0	2.8	2.5
	5 µg/mL	113.6	2.4	2.1
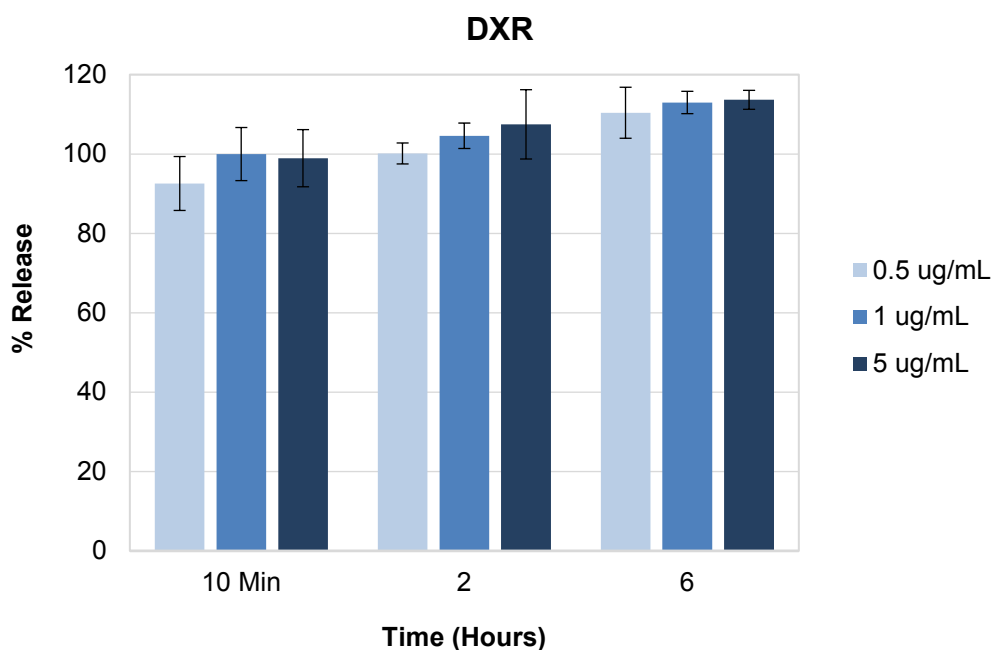
Figure V-21. Free Doxorubicin HCl Control Data. Displayed is the calculated % release for the doxorubicin HCl control data. (Mean + SD, *N=3*)

Table V-29. Doxil, Lot C (600520P1) Drug Release. Displayed is the calculated % release for Doxil, Lot C (600520P1). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	4.1	0.2	4.7
	1 µg/mL	2.5	0.2	8.7
	5 µg/mL	1.4	0.1	9.9
2 hr	0.5 µg/mL	2.6	0.2	7.9
	1 µg/mL	1.7	0.05	2.8
	5 µg/mL	1.3	0.3	19.5
6 hr	0.5 µg/mL	3.8	0.9	24.5
	1 µg/mL	2.9	0.04	1.6
	5 µg/mL	2.2	0.6	29.6
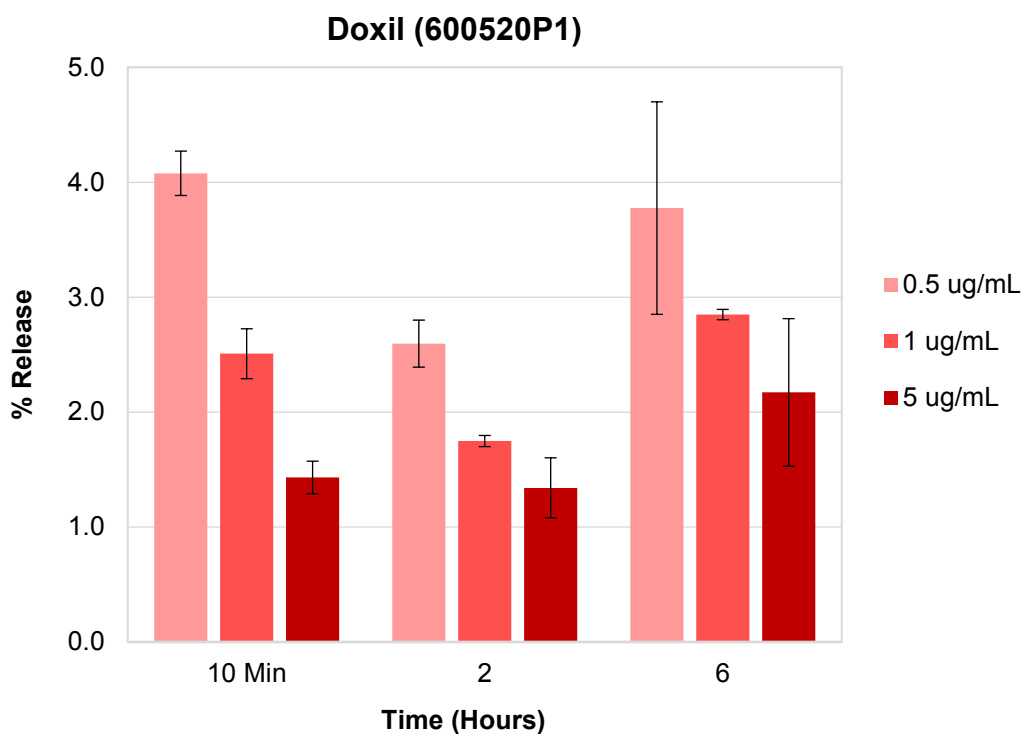
Figure V-22. Doxil, Lot C (600520P1) Drug Release. Displayed is the calculated % release for Doxil, Lot C (600520P1). (Mean $\pm$ SD, *N=3*)

Table V-30. Sun Pharma, Lot D (JKR0865A) Drug Release. Displayed is the calculated % release for Sun Pharma, Lot D (JKR0865A). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	2.4	0.4	18.4
	1 µg/mL	2.5	0.3	12.8
	5 µg/mL	1.9	0.2	9.5
2 hr	0.5 µg/mL	1.5	0.2	13.8
	1 µg/mL	1.5	0.2	13.9
	5 µg/mL	1.2	0.1	8.4
6 hr	0.5 µg/mL	2.5	0.7	29.4
	1 µg/mL	2.8	0.6	22.5
	5 µg/mL	1.9	0.2	9.9
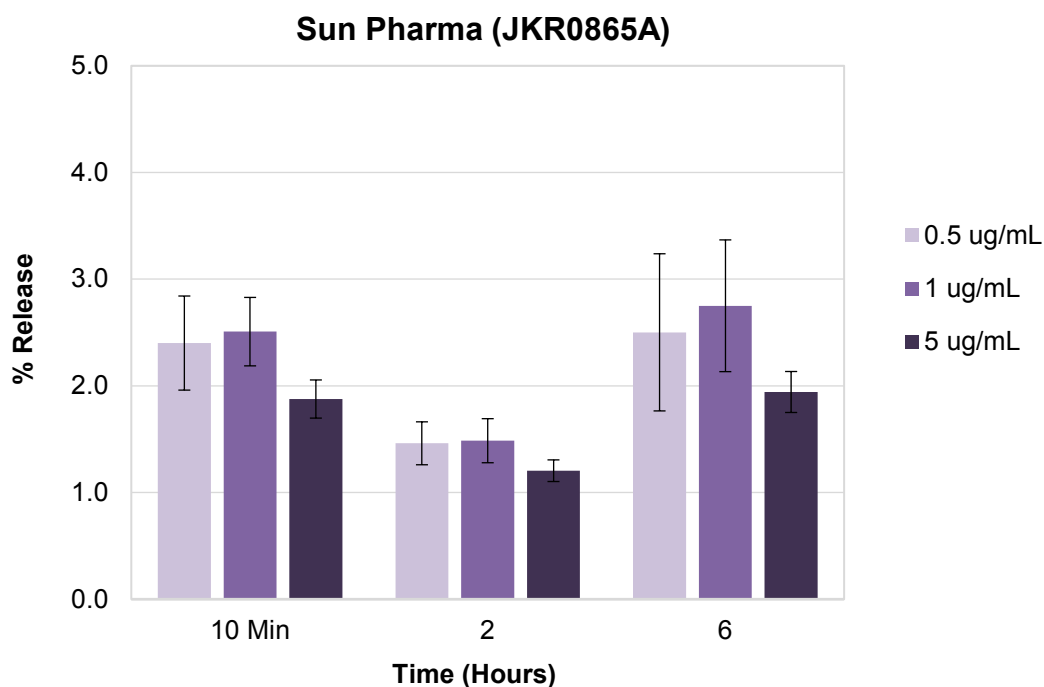
Figure V-23. Sun Pharma, Lot D (JKR0865A) Drug Release. Displayed is the calculated % release for the Sun Pharma, Lot D (JKR0865A). (Mean $\pm$ SD, *N=3*)
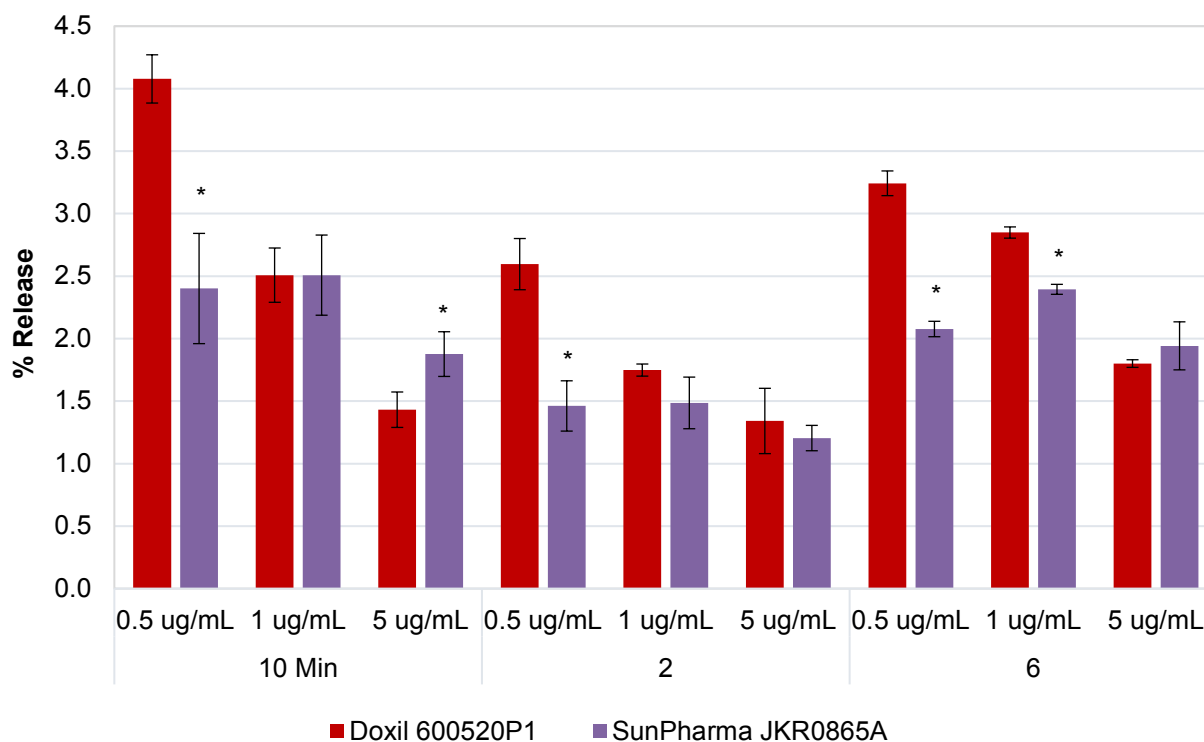
Figure V-24. Doxil and Sun Pharma Drug Release Lot Comparison. Displayed are the 10 min-6 hr calculated % release data for the Doxil and Sun Pharma Lot C (600520P1) and Lot D (JKR0865A), respectively. (Mean $\pm$ SD, *N=3*), *$p \leq 0.05$, Student's t-test.

VI. Doxil vs. Sun Pharma Bioequivalency Study in Rats

Bioequivalency Study

Summary
The objective of this study was to evaluate the bioequivalence of Doxil and Sun Pharma products in a Sprague Dawlay rat in vivo study. The Doxil and Sun Pharma unencapsulated and encapsulated drug concentration-time profiles in rats were very comparable, as were the estimated pharmacokinetic parameters. However, a statistical analysis of bioequivalence by two one sided t-tests determined that all PK parameters, except for encapsulated C_{max}, were not equivalent. Therefore, the two formulations were not bioequivalent in this study. This lack of bioequivalence is most likely the result of low study power, due to the low number of animals utilized per group (N=8), and the parallel design that includes both intra- and intersubject variability. Importantly, several differences were observed for the unencapsulated drug profile in this stable isotope tracer method study compared to a previous conventional solid phase extraction (SPE) method study in rats [5], including 18-fold lower unencapsulated drug concentrations, an 8-fold later unencapsulated T_{max} (33 vs. 4 hr), and a much longer unencapsulated terminal half-life (145 vs. 30 hr).

Design and Methods

Test Article Preparation
Test articles, Janssen's Doxil® (**Lot C (600520P1)**) or Sun Pharma's Doxorubicin HCl Liposome (**Lot D (JKR0865A)**) were diluted to a concentration of 1 mg DXR/mL in saline.

Animal Study Design
A parallel design bioequivalence study was conducted in double jugular catheterized 15-week-old male Sprague Dawley rats (approx. weight of 400 grams, Charles River Laboratories, Raleigh, N.C.). Rats were treated intravenously by left catheter with 5 mg DXR/5 mL/kg of Janssen's Doxil® or Sun Pharma's Doxorubicin HCl Liposome generic (8/treatment group). Blood samples (400 µL) were collected in K_2EDTA tubes by the right jugular catheter at 0.25, 0.5, 1, 4, 8, 12, 24, 48 and 96 hr. Blood was spun at 2500xg for 10 min and plasma (~200 µL) collected in a glass vial.

The analysis of the plasma samples was conducted as described in Section I, using the stable isotope tracer method. However, for the in vivo studies, approximately 200 µL of plasma was the maximum obtainable volume for each time point. Therefore, the volumes were adjusted from those used in the in vitro studies to 200 µL. This volume change was validated in Section II. Briefly, 50 ng/mL of the $^{13}C^2H_3$-DXR stable isotope was added to 200 µL of plasma and incubated at 37°C for 10 min with agitation. After incubation, 25 µL plasma was removed to Eppendorf tubes containing 200 µL ACN/0.1% formic acid with 25 ng/mL aclarubicin (ISTD) for total drug concentration analysis. The remainder of the plasma was transferred to a filter tube and spun at 12,000xg for 10 min at 37°C. 50 µL of the filtrate was added to an Eppendorf tube containing 200 µL ACN with 0.1% FA and 25 ng/mL aclarubicin internal standard for analysis. In some instances, we were unable to obtain 50 µL of the filtrate. In these cases, the total volume available was noted and added to the ACN. The dilution factor was adjusted accordingly during data analysis. Samples were frozen at -80°C until analysis. Samples were thawed, centrifuged, and supernatants dried in a vacuum centrifuge and the resulting residue reconstituted in 150 µL 25% ACN/0.1% formic acid. Samples were then analyzed on a Thermo Fisher Q Exactive OrbiTrap, using matrix matched standard curves and controls as described in Section I (**Tables VI-1 to V-4, Figures VI-1 to VI-4**). The standard curve range used in this study was higher than was initially validated for the rat plasma validation study in Section II. For this reason, a partial analytical validation was run and is presented in **Appendix C**.

Husbandry

Animals arrived the day prior to study initiation. In order to keep the catheters patent, catheters were stored and flushed with 500 IU/mL heparin in PBS. Animal rooms were kept at 50% relative humidity, 68-72°F with 12 hr light/dark cycles. Rats were housed two animals/cage (rat polycarbonate cage type with ¼" corncob bedding). Animals were allowed *ad libitum* access to Purina 5L79 and reverse osmosis water*.*

The Frederick National Laboratory for Cancer Research is accredited by AAALAC International and follows the Public Health Service *Policy for the Care and Use of Laboratory Animals* (Health Research Extension Act of 1985, Public Law 99-158, 1986). Animal care was provided in accordance with the procedures outlined in the *Guide for Care and Use of Laboratory Animals* (National Research Council, 1996; National Academy Press, Washington, D.C.). All animal protocols were approved by the NCI at Fredrick institutional Animal Care and Use Committee. The experiments outlined herein are scientifically justified and do not represent an unnecessary duplication of previous work by the sponsor.

Noncompartmental Pharmacokinetic Analysis

Noncompartmental pharmacokinetic parameters were determined using Phoenix WinNonlin version 6.3 software (Pharsight Corporation, Mountain View, CA): the area under the time concentration curve including all time points (AUC_{all}) was calculated using the linear trapezoidal rule without extrapolation; the area under the time concentration curve to time infinity (AUC_{inf}) was calculated using the linear trapezoidal rule with extrapolation to time infinity; the C_{max} term is the maximum concentration; the T_{max} term is the time of maximum concentration.

Statistics

In vivo PK parameters were evaluated by two one-sided t-tests, with α=0.05 and Ө=0.2, to determine the 90% CI of the geometric mean of log transformed T/R ratio [6]. The FDA bioequivalence criteria is a 90% CI between 80 and 125% [7]. Outliers were identified as data points two standard deviations away from the mean of all data points in the group. Only outliers that did not fit the overall drug concentration profile of the treatment group were excluded from the bioequivalence analysis.

Table VI-1. DXR Standard Curve in Rat Plasma.

	Area	Conc. (ng/mL)	% Accuracy
10000 ng/mL	118801482	8861	89
10000 ng/mL	104089651	8664	87
25000 ng/mL	263709537	24088	96
25000 ng/mL	241286990	27481	110
50000 ng/mL	488416482	52317	105
50000 ng/mL	470235386	51592	103
75000 ng/mL	784304300	74401	99
75000 ng/mL	777110874	80684	108
100000 ng/mL	951632693	107861	108
100000 ng/mL	977069434	102491	103
200000 ng/mL	1727446885	204738	102
200000 ng/mL	1633451875	193312	97
300000 ng/mL	2438036785	294555	98
300000 ng/mL	2370054229	288955	96
QC			
QCL	103967235	10493	105
QCL	97749040	9639	96
QCM	461998144	52653	105
QCM	508563348	53321	107
QCH	926838817	99943	100
QCH	976731424	107195	107

DXR_PRM
$Y = 5.82844 + 1.56017 \cdot X$ $R^2 = 0.9973$ W: 1/X
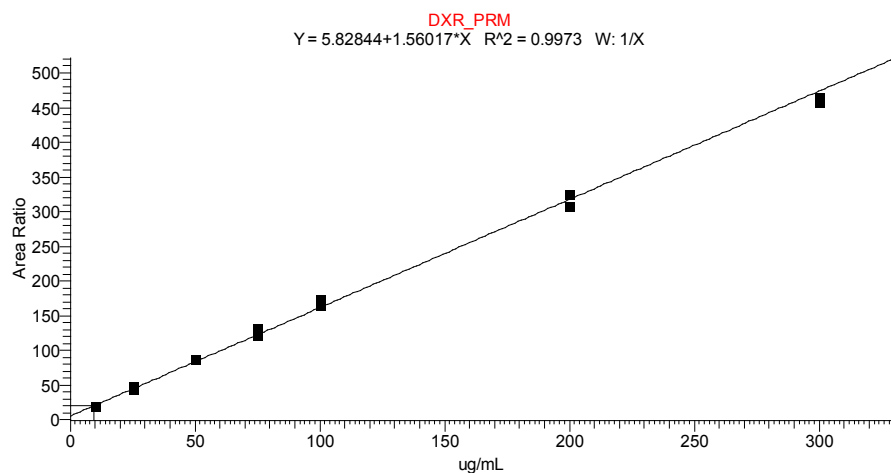
Figure VI-1. DXR Standard Curve in Rat Plasma.

Table VI-2. DXR_C13 Standard Curve in Rat Plasma.

Table VI-2 ^{13}C Doxorubicin Standard Curve in Rat Plasma			
	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	216821	9.9	99
10.0 ng/mL	234596	10.1	101
50 ng/mL	920985	52	105
50 ng/mL	841933	45	90
100 ng/mL	2102157	104	104
100 ng/mL	1589844	108	108
500 ng/mL	7374293	541	108
500 ng/mL	8130444	530	106
1000 ng/mL	21412463	944	95
1000 ng/mL	25460486	922	92
5000 ng/mL	112928524	4792	96
5000 ng/mL	93953145	10913	218
10000 ng/mL	239159794	10998	110
10000 ng/mL	265762415	8632	86
QC			
QCL	261701	10	102
QCL	256769	9	87
QCM	2825409	100	100
QCM	2747580	101	101
QCH	28552474	907	91
QCH	28604793	876	88

DXR_C13_PRM
Y = -0.0023803+0.000923126*X   R^2 = 0.9927   W: 1/X^2
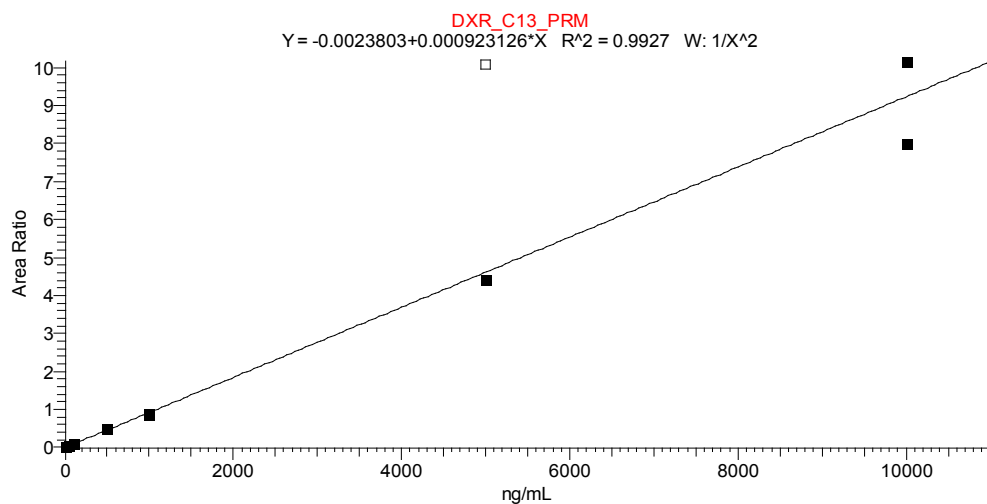
Figure VI-2. DXR_C13 Standard Curve in Rat Plasma.

Table VI-3. DXR Standard Curve in Protein-Free Rat Plasma.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	3951	0.103	103
0.100 ng/mL	4548	0.094	94
0.50 ng/mL	60329	0.76	152
0.50 ng/mL	46689	0.56	113
1.00 ng/mL	84522	1.11	111
1.00 ng/mL	73583	1.01	101
5.00 ng/mL	406422	4.66	93
5.00 ng/mL	360916	4.40	88
10.0 ng/mL	850769	10.3	103
10.0 ng/mL	815928	10.1	101
100.0 ng/mL	8321535	97.0	97
100.0 ng/mL	8407682	96.2	96
1000.0 ng/mL	85946365	985.3	99
1000.0 ng/mL	89309973	1027.4	103
QC			
QCL	6132	0.113	113
QCL	6672	0.116	116
QCM	77981	1.01	101
QCM	95661	1.09	109
QCH	794430	10.0	100
QCH	852518	9.6	96

DXR_PRM
$Y = -0.000240636 + 0.00509929 \cdot X$ $R^2 = 0.9945$ W: $1/X^2$
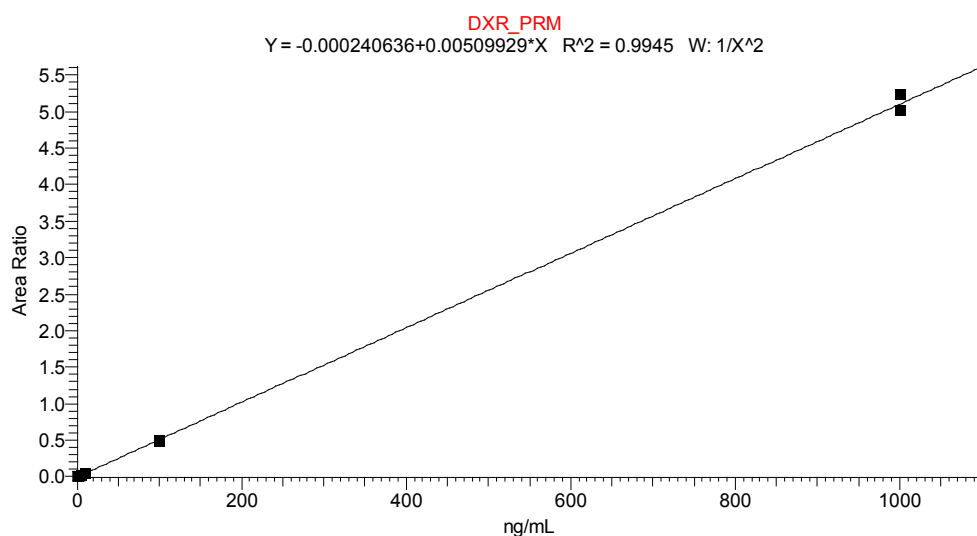
Figure VI-3. DXR Standard Curve in Protein-Free Rat Plasma.

Table VI-4. DXR_C13 Standard Curve in Protein-Free Rat Plasma.

	Area	Conc. (ng/mL)	% Accuracy
0.100 ng/mL	778	0.104	104
0.100 ng/mL	7702	0.220	220
0.50 ng/mL	18126	0.45	90
0.50 ng/mL	19002	0.44	89
1.00 ng/mL	44701	1.04	104
1.00 ng/mL	39975	0.97	97
5.00 ng/mL	243986	4.80	96
5.00 ng/mL	227322	4.76	95
10.0 ng/mL	531751	11.0	110
10.0 ng/mL	520338	11.0	110
100.0 ng/mL	5138461	102.0	102
100.0 ng/mL	5146906	100.4	100
1000.0 ng/mL	50394217	984.2	98
1000.0 ng/mL	53102815	1040.7	104
QC			
QCL	689	0.098	98
QCL	796	0.099	99
QCM	48378	1.10	110
QCM	44634	0.91	91
QCH	517406	11.1	111
QCH	526521	10.2	102

DXR_C13_PRM
$Y = -0.000255524 + 0.00299333*X \quad R^2 = 0.9950 \quad W: 1/X^2$
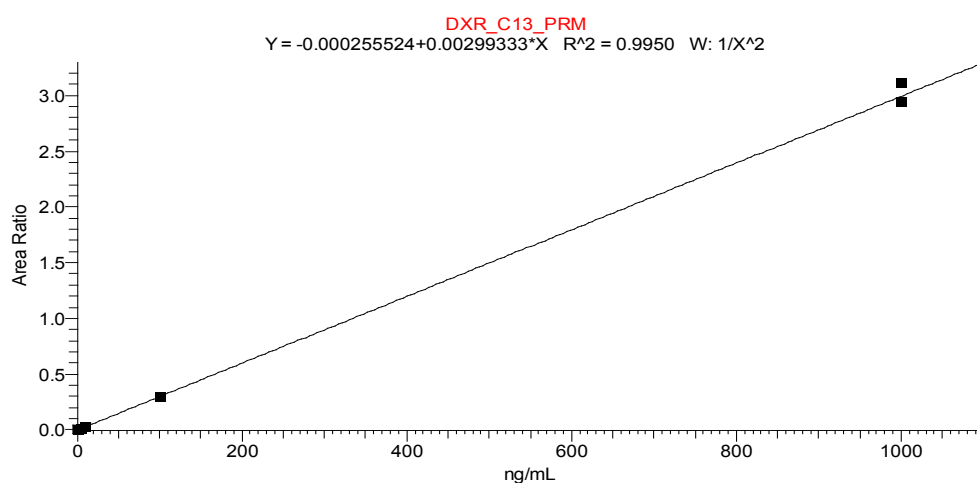
Figure VI-4. DXR_C13 Standard Curve in Protein-Free Rat Plasma.

Results and Discussion

The encapsulated and unencapsulated drug concentration measurement results of the stable isotope tracer analysis of the rat plasma are displayed in **Tables VI-5, VI-6, VI-9 and VI-10**, and **Figures VI-5 to VI-8**. The analytical data for these analyses are displayed in **Tables VI-7, IV-8, VI-11 and IV-12**. The Doxil and Sun Pharma unencapsulated and encapsulated drug concentration-time profiles were very similar (**Tables VI-13 and VI-14 and Figures VI-9 to VI-11**), as were the calculated pharmacokinetic parameters (**Table VI-15**). An analysis of bioequivalence by two one sided t-tests determined that all PK parameters, except for encapsulated C_{max}, were not equivalent (**Figure VI-12, Table VI-16**). The lack of bioequivalence is most likely the result of low study power, due to the low number of animals utilized per group (*N*=8), and the parallel design that includes both intra- and intersubject variability. Important differences were observed for this stable isotope tracer method study compared to a previous literature SPE method study in rats [5]:

1) Unencapsulated drug concentrations were much lower for the stable isotope tracer study, resulting in encapsulated/unencapsulated drug ratios of approx. 800 compared to approx. 60 for the previous SPE method study;

2) The terminal slope of the unencapsulated profile did not parallel the encapsulated profile, as it did for the previous SPE method study; and

3) T_{max} is much later for the stable isotope study at approx. 33 hr compared to approx. 4 hr for the SPE study.

Table VI-5. Doxil, Lot C (600520P1), Unencapsulated DXR.

Hours	0.25	0.5	1	4	8	12	24	48	96
	Unencapsulated DXR (ng/mL)								
Animal 17	53	76	53	43	99	127	124	155	85
Animal 19	56	42	101	35	93	122	108	139	89
Animal 21	54	63	34	21	68	92	83	95	50
Animal 23	81	231	23	48	100	91	181	136	273
Animal 25	46	48	21	67	116	140	201	114	116
Animal 27	47	64	38	93	159	198	144	552	120
Animal 29	52	49	62	50	104	163	146	144	97
Animal 31	218	47	61	35	96	146	177	655	111
Average	76	77	49	49	104	135	146	130	118
STD	58	63	26	22	26	36	40	22	67
% CV	77	81	53	45	25	27	27	17	57

Values were excluded

Values were calculated to be outliers, but were not excluded

A.

Doxil Unencapsulated DXR
(*including all data points)

B.

Doxil Unencapsulated DXR
(*excluding outliers)
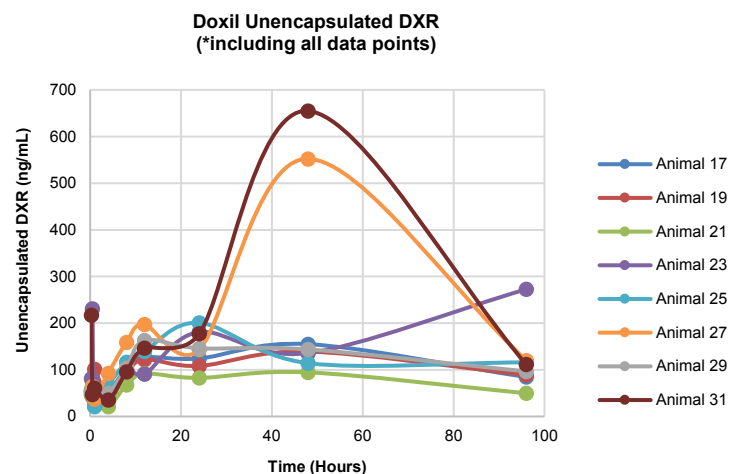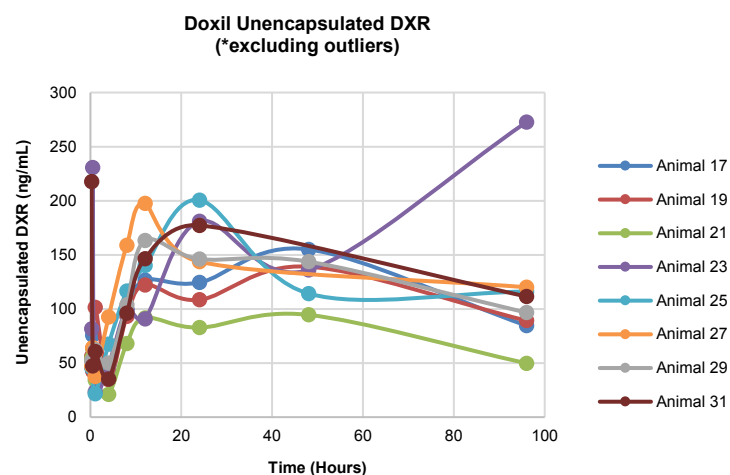
Figure VI-5. Doxil concentration-time profiles for unencapsulated drug. Displayed are concentration-time profiles for unencapsulated drug including (**A**) or excluding (**B**) outliers.

Table VI-6. Doxil, Lot C (600520P1), Encapsulated DXR.

	Encapsulated DXR (ng/mL)								
Hours	**0.25**	**0.5**	**1**	**4**	**8**	**12**	**24**	**48**	**96**
Animal 17	156683	123426	128748	118047	121990	120909	71088	51934	22115
Animal 19	141304	127262	121621	145434	121649	100329	80672	47167	15716
Animal 21	127830	144445	139080	172599	124495	126364	76068	48972	20403
Animal 23	137902	115680	135119	145738	116581	113047	84916	55897	23657
Animal 25	158607	163420	147532	158204	124150	106961	78295	45989	12320
Animal 27	153541	144090	152463	137277	120844	114071	83120	33215	2793
Animal 29	170977	180756	154527	145828	112403	91093	78214	46105	20203
Animal 31	169214	165041	143297	126669	128973	124261	100859	51470	26179
Average	152007	145515	140298	143725	121386	112129	81654	47594	17923
STD	15221	22860	11479	17099	5062	12189	8852	6723	7513
% CV	10	16	8	12	4	11	11	14	42

(red)	Values were excluded
(orange)	Values were calculated to be outliers, but were not excluded

Figure VI-6. Doxil concentration-time profiles for encapsulated drug. Displayed are concentration-time profiles for encapsulated drug.

Table VI-7. Analytical data for Doxil, Lot C (600520P1). Presented are the analytical data for Doxil, Lot C (600520P1). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I.

Time point	Rat #	Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 − %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR-Unencapsulated
15 min	R17	10.1	156736	0.01	99.99	7.2	38	19	81	53.3	156683
	R19	17.7	141360	0.01	99.99	10.7	34	32	69	56.1	141304
	R21	15.1	127884	0.01	99.99	11.1	40	28	72	54.3	127830
	R23	17.9	137983	0.01	99.99	8.7	40	22	78	81.5	137902
	R25	9.7	158653	0.01	99.99	9.0	42	21	79	45.6	158607
	R27	17.2	153589	0.01	99.99	14.8	41	36	64	47.4	153541
	R29	10.4	171029	0.01	99.99	9.0	45	20	80	52.0	170977
	R31	61.2	169432	0.04	99.96	11.7	42	28	72	217.5	169214
30 min	R17	21.4	123502	0.02	99.98	9.1	32	28	72	75.5	123426
	R19	9.4	127305	0.01	99.99	6.4	29	22	78	42.4	127262
	R21	12.6	144509	0.01	99.99	6.9	35	20	80	63.4	144445
	R23	57.6	115911	0.05	99.95	7.4	30	25	75	230.7	115680
	R25	8.4	163467	0.01	99.99	6.8	39	18	82	47.6	163420
	R27	17.2	144154	0.01	99.99	8.7	32	27	73	64.1	144090
	R29	7.5	180805	0.004	100.00	7.3	48	15	85	48.8	180757
	R31	12.1	165088	0.01	99.99	13.3	52	26	74	47.2	165041
1 hr	R17	13.7	128801	0.01	99.99	10.2	40	26	74	53.0	128748
	R19	26.1	121722	0.02	99.98	8.8	34	26	74	101.4	121621
	R21	8.4	139115	0.01	99.99	8.9	37	24	76	34.3	139080
	R23	5.1	135143	0.004	100.00	7.1	33	22	78	23.4	135119
	R25	5.1	147554	0.003	100.00	9.5	40	24	76	21.4	147532
	R27	8.9	152500	0.01	99.99	9.1	38	24	76	37.6	152463
	R29	15.7	154589	0.01	99.99	13.0	51	19	81	62.1	154527
	R31	18.3	143358	0.01	99.99	12.1	40	32	69	60.7	143297
4 hr	R17	8.9	118091	0.01	99.99	9.1	44	28	72	43.5	118047
	R19	5.3	145468	0.004	100.00	6.1	40	22	78	34.6	145434
	R21	2.9	172620	0.002	100.00	6.7	49	21	79	21.0	172599
	R23	6.9	145786	0.005	100.00	6.5	45	36	64	48.2	145738
	R25	12.8	158272	0.01	99.99	8.5	45	20	80	67.3	158205
	R27	17.4	137369	0.01	99.99	6.9	37	28	72	92.6	137277
	R29	9.7	145878	0.01	99.99	7.3	38	28	72	50.0	145828
	R31	7.4	126704	0.01	99.99	7.5	35	22	78	35.2	126669
8 hr	R17	22.8	122089	0.02	99.98	9.6	42	20	80	98.8	121990
	R19	23.0	121742	0.02	99.98	8.6	35	25	75	93.1	121649
	R21	10.6	124563	0.01	99.99	5.3	34	18	82	67.8	124495
	R23	22.1	116681	0.02	99.98	19.2	87	27	73	99.8	116581
	R25	16.3	124267	0.01	99.99	4.3	31	15	85	116.5	124150
	R27	29.1	121002	0.02	99.98	8.0	44	26	74	158.9	120844
	R29	26.6	112507	0.02	99.98	9.6	37	26	74	103.9	112403
	R31	18.4	129069	0.01	99.99	7.2	37	19	81	96.0	128973

Values were excluded

Values were calculated to be outliers, but were not excluded

Table VI-8. Analytical data for Doxil, Lot C (600520P1), continued. Presented are the analytical data for Doxil, Lot C (600520P1). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I.

Time point	Rat #	Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 − %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated
12 hr	R17	16.6	121035	0.01	99.99	6.6	50	13	87	126.5	120909
	R19	24.5	100452	0.02	99.98	9.1	45	20	80	122.2	100329
	R21	11.5	126456	0.01	99.99	5.8	47	13	88	91.7	126364
	R23	13.4	113138	0.01	99.99	6.6	44	15	85	90.7	113047
	R25	21.5	107101	0.02	99.98	9.0	58	15	85	140.0	106961
	R27	33.1	114269	0.03	99.97	8.6	51	17	83	197.5	114071
	R29	41.3	91256	0.05	99.95	9.6	38	25	75	163.1	91093
	R31	23.2	124407	0.02	99.98	7.3	46	16	84	146.5	124261
24 hr	R17	23.1	71213	0.03	99.97	7.3	39	19	81	124.4	71088
	R19	21.2	80781	0.03	99.97	6.5	33	20	80	108.5	80672
	R21	13.4	76151	0.02	99.98	5.9	36	16	84	82.7	76068
	R23	28.9	85097	0.03	99.97	7.9	49	16	84	181.0	84916
	R25	30.6	78496	0.04	99.96	10.3	68	15	85	200.6	78295
	R27	22.5	83263	0.03	99.97	9.9	63	16	84	143.6	83120
	R29	24.9	78360	0.03	99.97	7.7	45	17	83	146.1	78214
	R31	39.3	101037	0.04	99.96	9.2	42	22	78	177.2	100860
48 hr	R17	21.5	52089	0.04	99.96	6.2	45	14	86	155.0	51934
	R19	21.8	47307	0.05	99.95	6.5	41	16	84	139.1	47168
	R21	14.6	49067	0.03	99.97	6.1	40	15	85	94.5	48973
	R23	21.3	56033	0.04	99.96	6.1	39	16	84	136.0	55897
	R25	9.1	46104	0.02	99.98	6.5	81	8	92	114.2	45989
	R27	59.6	33767	0.18	99.82	29.1	269	11	89	552.3	33215
	R29	10.6	46249	0.02	99.98	6.9	93	7	93	143.7	46105
	R31	115.5	52125	0.22	99.78	13.0	74	18	82	654.8	51470
96 hr	R17	7.6	22200	0.03	99.97	4.9	55	9	91	84.6	22115
	R19	8.0	15805	0.05	99.95	4.0	45	9	91	89.2	15716
	R21	6.3	20452	0.03	99.97	6.9	54	13	87	49.8	20403
	R23	33.6	23929	0.14	99.86	6.8	55	12	88	272.7	23657
	R25	9.5	12436	0.08	99.92	6.6	81	8	92	116.1	12320
	R27	31.6	2913	1.08	98.92	122.9	467	26	74	120.0	2793
	R29	9.4	20299	0.05	99.95	7.7	79	10	90	96.6	20203
	R31	7.3	26290	0.03	99.97	6.8	104	7	94	111.5	26179

■ Values were excluded

■ Values were calculated to be outliers, but were not excluded

Table VI-9. Sun Pharma, Lot D (JKR0865A), Unencapsulated DXR.

Sun Pharma Unencapsulated DXR (ng/mL)								
Hours	0.25	0.5	1	4	8	12	24	48	96
Animal 1	20.6	13.0	26.9	30.7	no sample	106.9	84.0	170.9	148.5
Animal 3	23.3	27.0	29.3	17.0	37.5	66.9	116.6	104.4	92.3
Animal 5	27.1	26.1	19.9	25.1	76.2	99.8	224.5	88.7	56.4
Animal 7	3.2	66.9	18.6	33.0	58.1	112.1	114.4	156.6	90.4
Animal 9	51.8	34.2	22.0	23.4	46.5	78.1	104.1	74.6	102.5
Animal 11	30.8	19.1	31.1	29.2	49.5	69.9	115.3	99.2	53.6
Animal 13	46.1	29.4	21.8	24.9	33.7	65.0	108.7	125.1	48.4
Animal 15	36.1	25.2	23.4	32.6	356.7	101.4	241.2	188.8	82.3
Average	29.9	30.1	24.1	27.0	50.2	87.5	138.6	126.0	84.3
STD	15.3	16.2	4.5	5.4	15.4	19.5	59.3	41.6	32.9
% CV	51.1	53.7	18.7	20.1	30.7	22.2	42.8	33.0	39.0

Values were excluded

Values were calculated to be outliers, but were not excluded

A.

Sun Pharma Unencapsulated DXR
(*exluding outlier)

B.

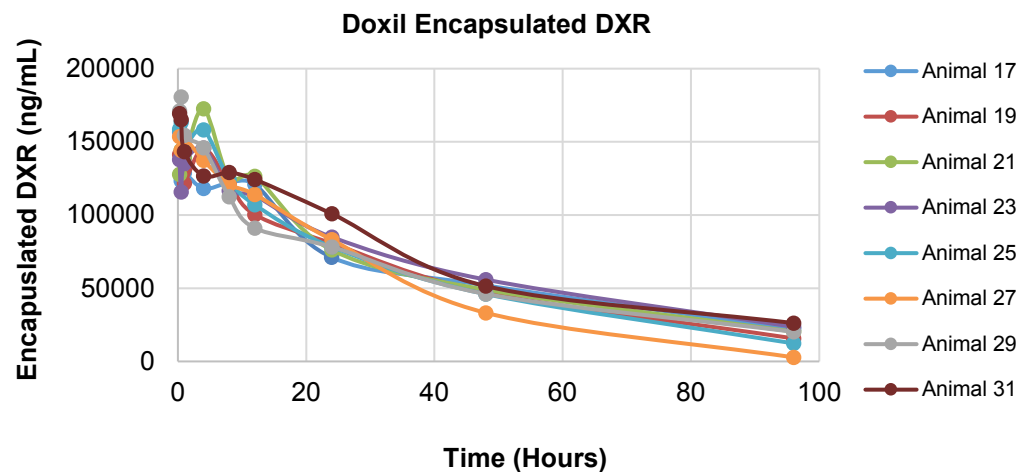Sun Pharma Unencapsulated DXR
(*including all data points)
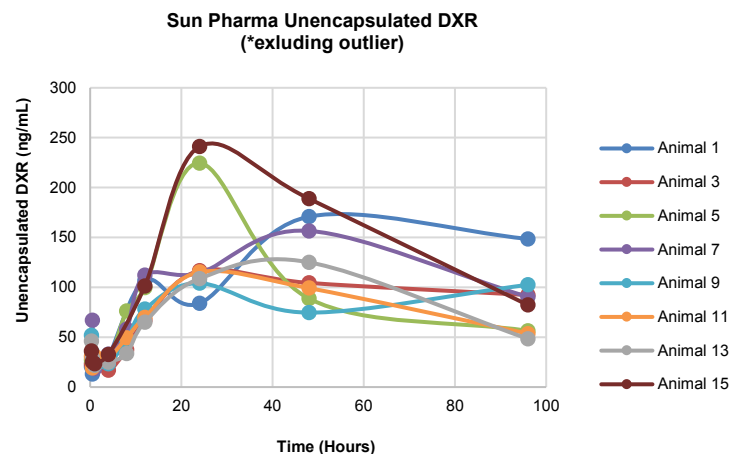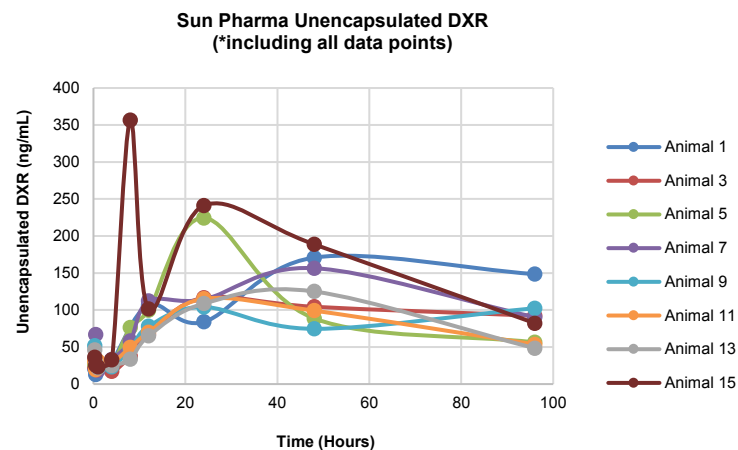
Figure VI-7. Sun Pharma concentration-time profiles for unencapsulated drug. Displayed are concentration-time profiles for unencapsulated drug including (**A**) or excluding (**B**) outliers.

Table VI-10. Sun Pharma, Lot D (JKR0865A), Encapsulated DXR.

Hours	0.25	0.5	1	4	8	12	24	48	96
Sun Pharma Encapsulated DXR (ng/mL)									
Animal 1	163159	161223	166940	132308	no sample	96674	62449	26369	6662
Animal 3	185303	176113	179029	169865	153348	125058	97682	68368	28830
Animal 5	161920	149303	173156	146947	133380	122223	92164	60533	25393
Animal 7	-3733	191985	176474	106012	145900	141616	116051	71979	34658
Animal 9	202248	198638	170325	115306	123897	126319	104753	61736	30325
Animal 11	179167	191702	170503	149792	143434	127688	97762	63631	24629
Animal 13	195679	161526	152726	145125	100824	102851	75618	52315	14757
Animal 15	175093	177268	180606	175154	106635	111727	97087	60645	18574
Average	180367	175970	171220	142564	133464	119270	92946	58197	22979
STD	15293	17553	8800	24112	19007	14634	16763	14114	9153
% CV	8	10	5	17	14	12	18	24	40

Values were excluded

Values were calculated to be outliers, but were not excluded

Sun Pharma Encapuslated DXR
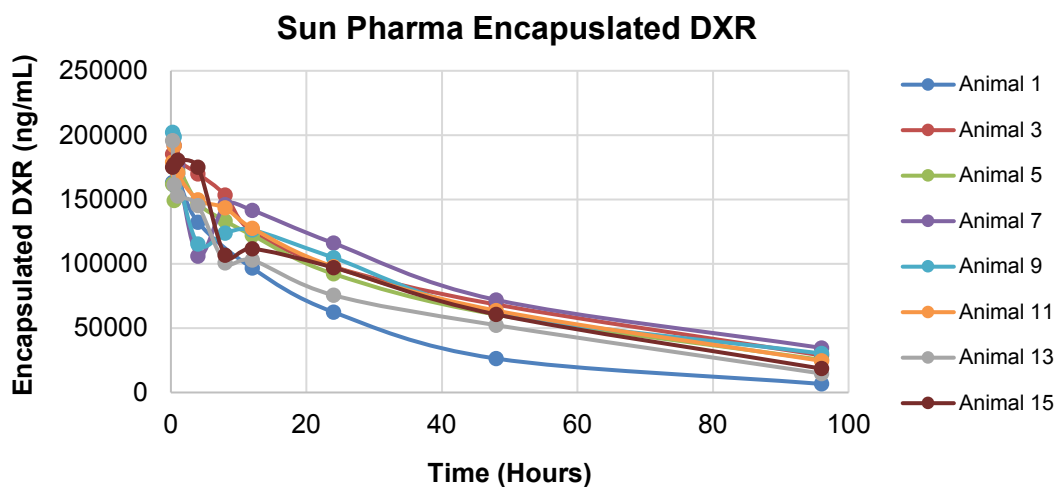
Figure VI-8. Sun Pharma concentration-time profiles for encapsulated drug. Displayed are concentration-time profiles for encapsulated drug.

Table VI-11. Analytical data for Sun Pharma, Lot D (JKR0865A). Presented are the analytical data for Sun Pharma, Lot D (JKR0865A). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I.

Time point	Rat #	Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 – %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/ (1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated
15 min	R1	2.9	163179	0.002	100.00	7.8	55	14	86	20.6	163159
	R3	4.2	185326	0.002	100.00	8.3	46	18	82	23.3	185303
	R5	5.4	161947	0.003	100.00	8.6	44	20	80	27.1	161920
	R7	0.2	-3730	-0.01	100.01	5.6	80	7	93	3.2	-3733
	R9	8.1	202300	0.004	100.00	7.6	48	16	84	51.8	202248
	R11	6.3	179198	0.004	100.00	6.9	34	21	80	30.8	179167
	R13	11.8	195725	0.01	99.99	12.3	48	26	74	46.1	195679
	R15	6.8	175129	0.004	100.00	9.1	48	19	81	36.1	175093
30 min	R1	1.5	161236	0.001	100.00	7.0	61	12	89	13.0	161223
	R3	5.3	176140	0.003	100.00	9.0	46	20	80	27.0	176113
	R5	6.2	149329	0.004	100.00	9.7	41	24	76	26.1	149303
	R7	13.9	192052	0.01	99.99	8.0	38	21	79	66.9	191985
	R9	5.9	198672	0.003	100.00	8.5	49	17	83	34.2	198638
	R11	3.5	191721	0.002	100.00	8.1	45	18	82	19.1	191702
	R13	5.5	161556	0.003	100.00	7.5	40	19	81	29.4	161526
	R15	4.7	177293	0.003	100.00	8.0	43	19	81	25.2	177268
1 hr	R1	4.5	166967	0.003	100.00	9.4	56	17	83	26.9	166940
	R3	7.0	179059	0.004	100.00	10.2	43	24	76	29.3	179029
	R5	7.3	173176	0.004	100.00	9.5	26	37	63	19.9	173156
	R7	4.8	176493	0.003	100.00	11.2	43	26	74	18.6	176474
	R9	5.1	170347	0.003	100.00	10.7	46	23	77	22.0	170325
	R11	7.4	170534	0.004	100.00	8.4	35	24	76	31.1	170503
	R13	6.2	152748	0.004	100.00	9.0	32	28	72	21.8	152726
	R15	3.4	180629	0.002	100.00	6.0	41	14	86	23.4	180606
4 hr	R1	6.4	132339	0.005	100.00	11.4	54	21	79	30.7	132308
	R3	2.3	169882	0.001	100.00	5.4	40	14	86	17.0	169865
	R5	4.9	146972	0.003	100.00	7.1	36	19	81	25.1	146947
	R7	7.3	106045	0.01	99.99	10.5	47	22	78	33.0	106012
	R9	6.8	115329	0.01	99.99	8.3	28	29	71	23.4	115306
	R11	5.5	149821	0.004	100.00	6.4	34	19	81	29.2	149792
	R13	5.3	145150	0.004	100.00	7.4	35	21	79	24.9	145125
	R15	8.6	175186	0.005	100.00	11.7	44	27	74	32.6	175154
8 hr	R1	-	-	-	-	-	-	-	-	-	-
	R3	4.7	153386	0.003	100.00	6.7	54	13	88	37.5	153348
	R5	15.0	133457	0.01	99.99	9.8	50	20	80	76.2	133380
	R7	9.9	145958	0.01	99.99	8.0	47	17	83	58.1	145900
	R9	13.5	123944	0.01	99.99	9.9	34	29	71	46.5	123897
	R11	8.0	143484	0.01	99.99	6.2	38	16	84	49.5	143434
	R13	8.3	100857	0.01	99.99	8.5	34	25	75	33.7	100824
	R15	53.4	106991.75	0.05	99.95	7.79	52	15	85	356.7	106635

Values were excluded

Values were calculated to be outliers, but were not excluded

Table VI-12. Analytical data for Sun Pharma, Lot D (JKR0865A), continued. Presented are the analytical data for Sun Pharma, Lot D (JKR0865A). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I.

Time point	Rat #	Filtrate DXR (ng/mL)	Reservoir DXR (ng/mL)	%Unbound DXR = Filtrate DXR / Reservoir DXR *100	% Bound = 100 – %Unbound DXR	Filtrate DXR_C13 (ng/mL)	Reservoir DXR_C13 (ng/mL)	%Unbound DXR_C13 = Filtrate DXR_C13 / Reservoir DXR_C13 * 100	% Bound DXR_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil DXR/(1-(%Bound DXR_C13/100))	Encapsulated (ng/mL) = Reservoir DXR- Unencapsulated
12 hr	R1	12.2	96781	0.01	99.99	6.4	56	11	89	106.9	96674
	R3	9.9	125125	0.01	99.99	5.6	38	15	85	66.9	125058
	R5	17.2	122323	0.01	99.99	7.8	45	17	83	99.8	122224
	R7	17.7	141728	0.01	99.99	6.9	44	16	84	112.1	141616
	R9	10.6	126397	0.01	99.99	5.2	38	14	86	78.1	126319
	R11	9.0	127758	0.01	99.99	5.8	45	13	87	69.9	127689
	R13	12.6	102916	0.01	99.99	7.9	41	19	81	65.0	102851
	R15	14.3	111829	0.01	99.99	7.3	52	14	86	101.4	111727
24 hr	R1	11.9	62533	0.02	99.98	10.2	72	14	86	84.0	62449
	R3	13.6	97799	0.01	99.99	6.1	52	12	88	116.6	97682
	R5	45.5	92389	0.05	99.95	19.0	94	20	80	224.5	92164
	R7	14.4	116166	0.01	99.99	6.1	49	13	87	114.4	116051
	R9	15.3	104857	0.01	99.99	7.7	53	15	85	104.1	104753
	R11	15.5	97877	0.02	99.98	6.9	51	14	87	115.3	97762
	R13	18.5	75726	0.02	99.98	6.9	40	17	83	108.7	75618
	R15	42.5	97328	0.04	99.96	8.6	49	18	82	241.2	97087
48 hr	R1	14.5	26540	0.05	99.95	7.0	82	9	92	170.9	26369
	R3	13.8	68473	0.02	99.98	6.1	46	13	87	104.4	68368
	R5	14.3	60622	0.02	99.98	6.5	40	16	84	88.7	60533
	R7	24.2	72136	0.03	99.97	6.2	40	15	85	156.6	71979
	R9	11.4	61811	0.02	99.98	7.2	47	15	85	74.6	61736
	R11	16.1	63731	0.03	99.97	6.1	38	16	84	99.2	63631
	R13	12.6	52440	0.02	99.98	7.0	69	10	90	125.1	52315
	R15	29.8	60834	0.05	99.95	7.5	47	16	84	188.8	60645
96 hr	R1	9.9	6811	0.15	99.85	5.7	85	7	93	148.5	6662
	R3	7.6	28922	0.03	99.97	4.4	53	8	92	92.3	28830
	R5	7.0	25449	0.03	99.97	7.5	60	13	88	56.4	25393
	R7	8.9	34748	0.03	99.97	6.0	61	10	90	90.4	34658
	R9	9.6	30428	0.03	99.97	7.3	78	9	91	102.5	30325
	R11	6.2	24683	0.02	99.98	7.7	67	12	89	53.6	24630
	R13	5.4	14805	0.04	99.96	9.1	82	11	89	48.4	14757
	R15	8.3	18657	0.04	99.96	6.9	69	10	90	82.2	18575

■	Values were excluded
■	Values were calculated to be outliers, but were not excluded

Table VI-13. Unencapsulated DXR Comparison

Hours	Sun Pharma Unencapsulated DXR (ng/mL)			Doxil Unencapsulated DXR (ng/mL)		
	Average	STD	% CV	Average	STD	% CV
0.25	30	15	51	76	58	77
0.5	30	16	54	77	63	81
1	24	5	19	49	26	53
4	27	5	20	49	22	45
8	50	15	31	104	26	25
12	88	19	22	135	36	27
24	139	59	43	146	40	27
48	126	42	33	130	22	17
96	84	33	39	118	67	57
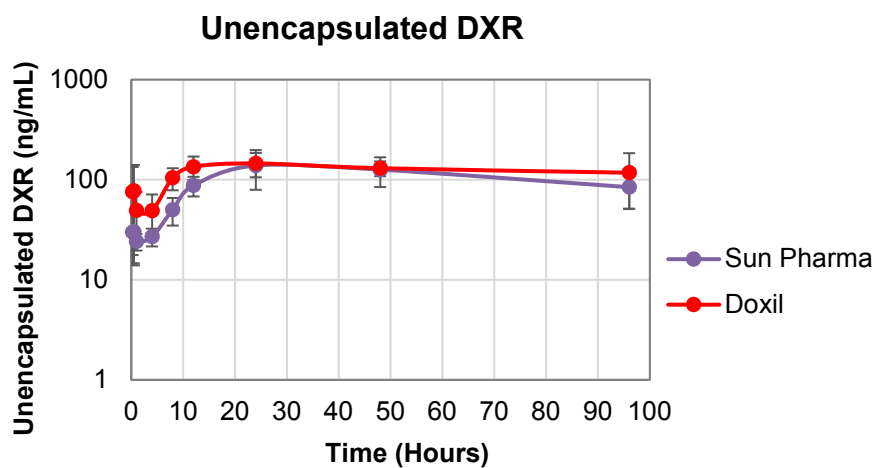
Figure VI-9. Unencapsulated DXR Comparison. Presented is the encapsulated DXR time-concentration comparison for Doxil and Sun Pharma (*N*=8, Mean $\pm$ SD).

Table 14. Encapsulated DXR Comparison

Hours	Sun Pharma Encapsulated DXR (ng/mL)			Doxil Encapsulated DXR (ng/mL)		
	Average	STD	% CV	Average	STD	% CV
0.25	180367	15293	8	152007	15221	10
0.5	175970	17553	10	145515	22860	16
1	171220	8800	5	140298	11479	8
4	142564	24112	17	143725	17099	12
8	133464	19007	14	121386	5062	4
12	119270	14634	12	112129	12189	11
24	92946	16763	18	81654	8852	11
48	58197	14114	24	47594	6723	14
96	22979	9153	40	17923	7513	42
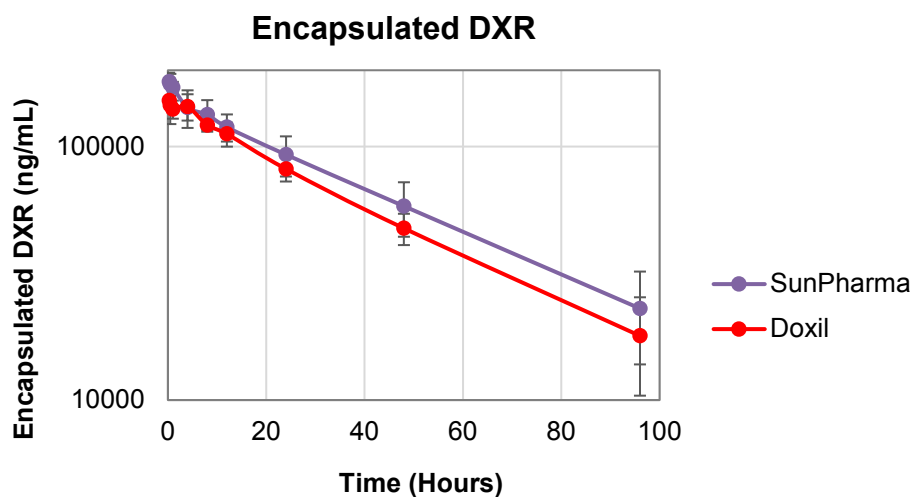
Figure VI-10. Encapsulated DXR Comparison. Presented is the encapsulated DXR time-concentration comparison for Doxil and Sun Pharma (*N*=8, Mean $\pm$ SD).
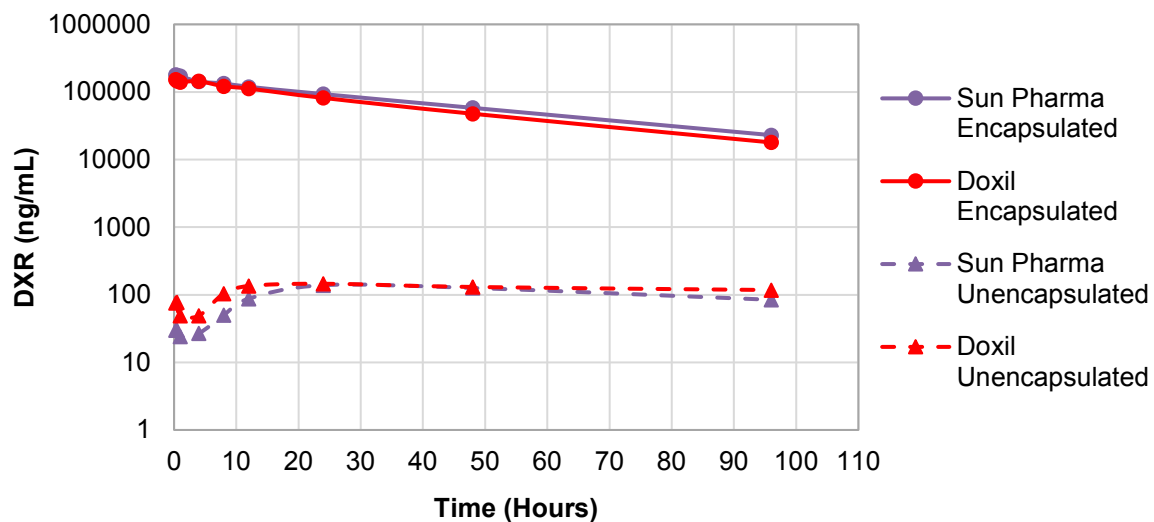
Figure VI-10. Unencapsulated and Encapsulated DXR Comparison. Presented is the encapsulated and unencapsulated DXR time-concentration comparison for Doxil and Sun Pharma (*N*=8, Mean).

Table VI-15. PK Comparison of Sun Pharma and Doxil. Displayed are the individual animal pharmacokinetic parameters for the Doxil and Sun Pharma, encapsulated and unencapsulated plasma analysis: area under the time concentration curve to time infinity (AUC_{inf}); maximum concentration (C_{max}); area under the time concentration curve all time points (AUC_{all}); time of maximum concentration (T_{max}).

		Unencapsulated			Encapsulated			
		T_{max}	C_{max}	AUC_{all}	T_{max}	C_{max}	AUC_{all}	AUC_{inf}
	Animal #	h	ng/mL	ng*h/mL	h	ng/mL	ng*h/mL	ng*h/mL
Sun Pharma	1	48	171	12522	1	166940	4341721	4541653
	3	24	117	8871	0.25	185303	7569868	9067173
	5	24	224	9827	1	173156	6894633	8199833
	7	48	157	11167	0.5	191985	8056557	10190358
	9	24	104	8011	0.25	202248	7194422	8911107
	11	24	115	7863	0.5	191702	7198496	8403677
	13	48	125	8427	0.25	195679	5739068	6339516
	15	24	241	14367	1	180606	6758153	7585164
	AVG	33	157	10132	0.59	185952	6719115	7904810
	SD	12	52	2359	0.35	11855	1171877	1764188
Doxil	17	48	155	11556	0.25	156683	5883992	7031063
	19	48	139	10770	4	145434	5641037	6337460
	21	48	139	10770	4	172599	6080370	7044728
	23	96	273	16159	4	145738	6324194	7617542
	25	24	201	12397	0.5	163420	5645537	6109138
	27	12	198	13022	0.25	153541	5015026	5083284
	29	12	163	12182	0.5	180756	5643580	6575046
	31	0.25	218	13410	0.25	169214	6626650	8017609
	AVG	36	186	12533	1.72	160923	5857548	6726984
	SD	31	46	1751	1.89	12804	493830	918891

Figure VI-12. TOST bioequivalence analysis. Presented are the results of two one-sided t-tests (TOST) of PK parameters for Doxil (reference) in comparison to Sun Pharma (test). All PK parameters, except encapsulated Cmax, were found not to be equivalent, with 90%CI of the test (Sun Pharma)/reference (Doxil) ratio outside the 80-125% criteria by TOST. See **Table VI-16** for TOST analysis data.

Table VI-16. TOST Bioequivalence Analysis. In vivo PK parameters were evaluated by two one-sided t-tests, with α=0.05 and Ө=0.2, to determine the 90% CI of the geometric mean of log transformed T/R ratio.

		Unencapsulated		Encapsulated		
		C_{max}	AUC_{all}	C_{max}	AUC_{all}	AUC_{inf}
		ng/mL	ng*h/mL	ng/mL	ng*h/mL	ng*hr/mL
Sun Pharma	Ln mean, Y1	5.01	9.20	12.13	15.70	15.86
	Ln SD	0.32	0.22	0.06	0.20	0.25
Doxil	Ln mean, Y2	5.20	9.43	11.99	15.58	15.71
	Ln SD	0.24	0.13	0.08	0.09	0.14
	LN (Y1-Y2)	-0.19	-0.23	0.15	0.12	0.14
90%CI of LN (Y1-Y2)	(U-mean-L)	(0.06)-(-0.19)-(-0.44)	(-0.07)-(-0.23)-(-0.39)	0.209-0.150-(0.081)	0.258-0.125-(-0.009)	0.33-0.14-(-0.04)
90%CI of (Y1/Y2)	(U-mean-L)	1.06-0.82-0.65	0.94-0.80-068	1.233-1.157-1.085	1.29-1.13-0.99	1.39-1.15-0.96

Appendix A. Inter-Day Individual Data for Human Plasma Validation

A. QC Low DXR in Human Plasma.

QC Low DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	10	10	1	
Study Day 1	10	10	-1	
Study Day 1	10	9	-7	
Study Day 1	10	10	-2	
Study Day 1	10	10	2	
Study Day 1	10	10	-4	
AVG		**10**	**-2**	**3**
Study Day 2	10	11	11	
Study Day 2	10	11	12	
Study Day 2	10	11	8	
Study Day 2	10	11	14	
Study Day 2	10	11	11	
Study Day 2	10	11	13	
AVG		**11**	**11**	**2**
Study Day 3	10	11	5	
Study Day 3	10	10	5	
Study Day 3	10	11	7	
Study Day 3	10	11	5	
Study Day 3	10	11	8	
Study Day 3	10	10	4	
AVG		**11**	**6**	**1**

B. QC Mid DXR in Human Plasma.

QC Mid DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	100	102	2	
Study Day 1	100	103	4	
Study Day 1	100	105	5	
Study Day 1	100	113	13	
Study Day 1	100	112	12	
Study Day 1	100	114	14	
AVG		**108**	**8**	**4**
Study Day 2	100	108	8	
Study Day 2	100	108	8	
Study Day 2	100	106	6	
Study Day 2	100	95	-5	
Study Day 2	100	111	11	
Study Day 2	100	107	7	
AVG		**106**	**6**	**5**
Study Day 3	100	109	9	
Study Day 3	100	105	5	
Study Day 3	100	104	4	
Study Day 3	100	105	5	
Study Day 3	100	109	10	
Study Day 3	100	106	6	
AVG		**106**	**6**	**2**

C. QC High DXR in Human Plasma.

QC High DXR	Theoretical Concentration	Experimental Concentration	Accuracy Deviation	Precision (%)
Study Day 1	1000	1057	6	
Study Day 1	1000	1148	15	
Study Day 1	1000	1145	15	
Study Day 1	1000	1059	6	
Study Day 1	1000	1067	7	
Study Day 1	1000	1063	6	
AVG		**1090**	**9**	**4**
Study Day 2	1000	1091	9	
Study Day 2	1000	1096	10	
Study Day 2	1000	1125	13	
Study Day 2	1000	1117	12	
Study Day 2	1000	1094	9	
Study Day 2	1000	1132	13	
AVG		**1109**	**11**	**2**
Study Day 3	1000	1119	12	
Study Day 3	1000	1083	8	
Study Day 3	1000	1113	11	
Study Day 3	1000	1073	7	
Study Day 3	1000	1044	4	
Study Day 3	1000	1043	4	
AVG		**1079**	**8**	**3**

D. QC Low DXR_C13 in Human Plasma.

QC Low DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	10	10	-0.1	
Study Day 1	10	10	-0.1	
Study Day 1	10	10	1	
Study Day 1	10	10	1	
Study Day 1	10	10	4	
Study Day 1	10	10	1	
AVG		**10**	**1**	**1**
Study Day 2	10	11	14	
Study Day 2	10	11	8	
Study Day 2	10	11	11	
Study Day 2	10	12	17	
Study Day 2	10	11	12	
Study Day 2	10	11	13	
AVG		**11**	**13**	**3**
Study Day 3	10	11	7	
Study Day 3	10	11	7	
Study Day 3	10	11	9	
Study Day 3	10	11	9	
Study Day 3	10	11	13	
Study Day 3	10	10	5	
AVG		**11**	**8**	**3**

E. QC Mid DXR_C13 in Human Plasma.

QC Mid DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	100	106	6	
Study Day 1	100	104	4	
Study Day 1	100	106	6	
Study Day 1	100	114	14	
Study Day 1	100	112	12	
Study Day 1	100	111	11	
AVG		**109**	**9**	**4**
Study Day 2	100	107	7	
Study Day 2	100	107	7	
Study Day 2	100	106	6	
Study Day 2	100	95	-5	
Study Day 2	100	108	8	
Study Day 2	100	107	7	
AVG		**105**	**5**	**5**
Study Day 3	100	111	11	
Study Day 3	100	115	15	
Study Day 3	100	107	7	
Study Day 3	100	108	8	
Study Day 3	100	113	13	
Study Day 3	100	109	9	
AVG		**111**	**11**	**3**

F. QC High DXR_C13 in Human Plasma.

QC High DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	1000	1067	7	
Study Day 1	1000	1138	14	
Study Day 1	1000	1146	15	
Study Day 1	1000	1107	11	
Study Day 1	1000	1084	8	
Study Day 1	1000	1092	9	
AVG		**1106**	**11**	**3**
Study Day 2	1000	1106	11	
Study Day 2	1000	1063	6	
Study Day 2	1000	1119	12	
Study Day 2	1000	1078	8	
Study Day 2	1000	1117	12	
Study Day 2	1000	1119	12	
AVG		**1100**	**10**	**2**
Study Day 3	1000	1133	13	
Study Day 3	1000	1036	4	
Study Day 3	1000	1112	11	
Study Day 3	1000	1101	10	
Study Day 3	1000	1010	1	
Study Day 3	1000	1054	5	
AVG		**1074**	**7**	**5**

Appendix B. Inter-Day Individual Data for Human Protein-Free Plasma Validation

A. QC Low DXR in Human Protein-Free Plasma.

QC Low DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	0.1	0.1	17	
Study Day 1	0.1	0.1	-0.2	
Study Day 1	0.1	0.1	5	
Study Day 1	0.1	0.1	14	
Study Day 1	0.1	0.1	-5	
Study Day 1	0.1	0.1	8	
AVG		**0.1**	**6**	**3**
Study Day 2	0.1	0.1	17	
Study Day 2	0.1	0.1	3	
Study Day 2	0.1	0.1	12	
Study Day 2	0.1	0.1	21	
Study Day 2	0.1	0.1	-1	
Study Day 2	0.1	0.1	14	
AVG		**0.1**	**11**	**2**
Study Day 3	0.1	0.1	5	
Study Day 3	0.1	0.1	5	
Study Day 3	0.1	0.1	7	
Study Day 3	0.1	0.1	5	
Study Day 3	0.1	0.1	8	
Study Day 3	0.1	0.1	4	
AVG		**0.1**	**6**	**1**

B. QC Mid DXR in Human Protein-Free Plasma.

QC Mid DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	5.0	4.9	-3	
Study Day 1	5.0	4.9	-1	
Study Day 1	5.0	5.5	10	
Study Day 1	5.0	5.0	-1	
Study Day 1	5.0	5.2	3	
Study Day 1	5.0	4.9	-2	
AVG		**5.1**	**1**	**3**
Study Day 2	5.0	5.4	7	
Study Day 2	5.0	5.5	11	
Study Day 2	5.0	5.3	6	
Study Day 2	5.0	5.4	8	
Study Day 2	5.0	5.4	9	
Study Day 2	5.0	4.8	-4	
AVG		**5.3**	**6**	**2**
Study Day 3	5.0	4.6	-9	
Study Day 3	5.0	4.5	-9	
Study Day 3	5.0	4.6	-7	
Study Day 3	5.0	4.7	-6	
Study Day 3	5.0	4.5	-9	
Study Day 3	5.0	4.6	-8	
AVG		**4.6**	**-8**	**1**

C. QC High DXR in Human Protein-Free Plasma.

QC High DXR	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	100.0	99.6	-0.4	
Study Day 1	100.0	98.4	-2	
Study Day 1	100.0	101.5	2	
Study Day 1	100.0	92.8	-7	
Study Day 1	100.0	104.7	5	
Study Day 1	100.0	104.9	5	
AVG		**100.3**	**0.3**	**3**
Study Day 2	100.0	109.4	9	
Study Day 2	100.0	113.5	14	
Study Day 2	100.0	110.2	10	
Study Day 2	100.0	111.1	11	
Study Day 2	100.0	104.2	4	
Study Day 2	100.0	106.8	7	
AVG		**109.2**	**9**	**2**
Study Day 3	100.0	108.8	9	
Study Day 3	100.0	108.3	8	
Study Day 3	100.0	105.0	5	
Study Day 3	100.0	104.7	5	
Study Day 3	100.0	105.7	6	
Study Day 3	100.0	102.3	2	
AVG		**105.8**	**6**	**1**

D. QC Low DXR_C13 in Human Protein-Free Plasma.

QC Low DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	0.1	0.1	2	
Study Day 1	0.1	0.1	16	
Study Day 1	0.1	0.1	18	
Study Day 1	0.1	0.1	9	
Study Day 1	0.1	0.1	-1	
Study Day 1	0.1	0.1	-4	
AVG		**0.1**	**7**	**3**
Study Day 2	0.1	0.1	8	
Study Day 2	0.1	0.1	0.1	
Study Day 2	0.1	0.1	13	
Study Day 2	0.1	0.1	12	
Study Day 2	0.1	0.1	17	
Study Day 2	0.1	0.1	8	
AVG		**0.1**	**10**	**2**
Study Day 3	0.1	0.1	-7	
Study Day 3	0.1	0.1	-6	
Study Day 3	0.1	0.1	-2	
Study Day 3	0.1	0.1	-8	
Study Day 3	0.1	0.1	-3	
Study Day 3	0.1	0.1	-7	
AVG		**0.1**	**-6**	**1**

E. QC Mid DXR_C13 in Human Protein-Free Plasma.

QC Mid DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	5.0	4.9	-2	
Study Day 1	5.0	5.4	7	
Study Day 1	5.0	5.2	5	
Study Day 1	5.0	4.9	-2	
Study Day 1	5.0	5.2	3	
Study Day 1	5.0	5.0	1	
AVG		**5.1**	**2**	**3**
Study Day 2	5.0	5.4	9	
Study Day 2	5.0	5.5	9	
Study Day 2	5.0	5.3	6	
Study Day 2	5.0	5.4	8	
Study Day 2	5.0	5.4	8	
Study Day 2	5.0	4.8	-3	
AVG		**5.3**	**6**	**2**
Study Day 3	5.0	4.6	-8	
Study Day 3	5.0	4.5	-11	
Study Day 3	5.0	4.5	-9	
Study Day 3	5.0	4.7	-6	
Study Day 3	5.0	4.6	-8	
Study Day 3	5.0	4.8	-3	
AVG		**4.6**	**-7**	**1**

F. QC High DXR_C13 in Human Protein-Free Plasma.

QC High DXR_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	100.0	99.6	-0.4	
Study Day 1	100.0	98.4	-2	
Study Day 1	100.0	101.5	2	
Study Day 1	100.0	92.8	-7	
Study Day 1	100.0	104.7	5	
Study Day 1	100.0	104.9	5	
AVG		**100.3**	**0.3**	**3**
Study Day 2	100.0	109.4	9	
Study Day 2	100.0	113.5	14	
Study Day 2	100.0	110.2	10	
Study Day 2	100.0	111.1	11	
Study Day 2	100.0	104.2	4	
Study Day 2	100.0	106.8	7	
AVG		**109.2**	**9**	**2**
Study Day 3	100.0	108.8	9	
Study Day 3	100.0	108.3	8	
Study Day 3	100.0	105.0	5	
Study Day 3	100.0	104.7	5	
Study Day 3	100.0	105.7	6	
Study Day 3	100.0	102.3	2	
AVG		**105.8**	**6**	**1**

Appendix C. Rat Plasma Partial Validation for BE Study

Summary

The purpose of this study was to run a partial validation of the LC-Orbitrap quantitation method for DXR in rat plasma matrix at a higher standard curve range (10-300 µg/mL) than the previous partial validation in Section II, for use in the in vivo BE study. For specifics of the LC orbitrap method please refer to Section I. This partial validation consisted of a determination of intra-day precision and accuracy at three different QC levels, 10 (low), 50 (mid) and 100 (high) µg/mL, and analytical reproducibility.

Calibration standards were prepared by adding DXR analytical standards to blank rat plasma in the range from 10-300 µg/mL, to arrive at six calibration levels. Matrix interference was not observed for the analytes or internal standard, and carry over from the high calibration standard was less than 5% of the low standard, 10 µg/mL.

The DXR calibration curves, analyte area/internal standard area vs. standard concentration, were suitable for linear regression with 1/x weighting, resulting in correlation coefficients of 0.999 or better. The accuracy deviation of the regression-calculated standard value from the true value was 15% or less for all calibration levels. The lower limit of quantitation (LLOQ) was not established in this partial validation.

For intra-day validation, six individual QC samples were run at each QC level, 10 (low), 50 (mid) and 100 (high) µg/mL. These samples were distributed throughout the beginning, middle and end of the run. The low, mid and high QC standards had intra-day accuracy deviation/precision of -1.6%/6.1%, 1.6%/2.5% and 3.8%/1.5%. This meets the acceptance criteria of accuracy deviation not exceeding 15% from the true value, and precision not exceeding 15%, at all QC levels. The runs met the acceptance criterion established for retention time and peak area at <10% RSD for both the analyte, DXR, and internal standard, aclarubicin.

Materials and Methods

See Section I for specifics of the LC-Orbitrap analytical method.

Validation Criteria and Study Design

The calibration curve contained six calibrants 10, 25, 75, 100, 200, and 300 μg/mL. The QC standards selected within this concentration range were, 10, 50 and 100 μg/mL, representing the low, mid and high QC standards, respectively.

The following criteria were used for this partial validation of the LC-Orbitrap method (**Figure C-1**).
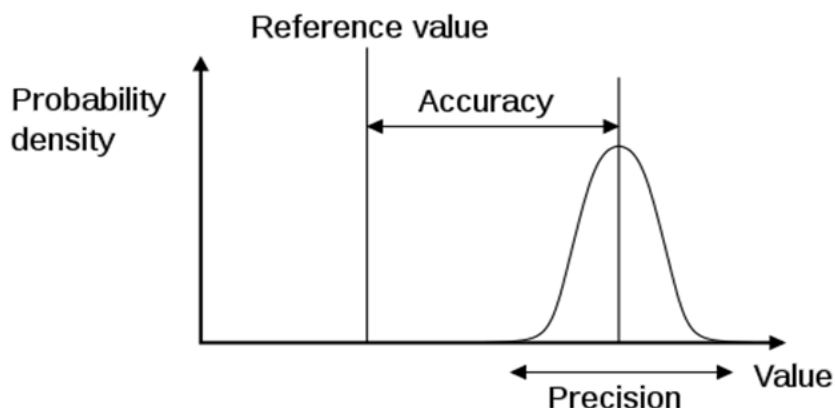
Figure C-1. Graphical Representation of Validation Criteria Terms.

Precision defines random errors, bias defines systematic errors and accuracy contains both errors. Accuracy deviation is a measure of the distance between the estimated and actual values (**Accuracy deviation** = [(actual-estimated)/reference value]*100%). The mean value of the estimate at each QC level should be within 15% of the actual value. Precision is a measure of assay repeatability, and is defined as **Precision** = % RSD = [standard deviation/Mean]*100%. The precision of the assay at each QC level should be equal to or less than 15% for all QC levels. For this intra-day validation, six individual QC samples were run at each QC level. These samples were distributed throughout the beginning, middle and end of the run. Analytical system reproducibility was also assessed with regard to linear regression fit of the three-separate standard curve runs, and the peak area and retention time precision of six replicate samples at the 50 μg/mL QC level, with acceptance criteria of <10% RSD.

Interpolation from Calibration Curve

Standard and QC sample concentrations were interpolated from the calibration curve, using the linear formula area ratio = slope*concentration ratio + y-intercept.

Results

Analytical System Suitability

Calibration curves, area ratio vs. concentration, were linear for DXR over the concentration range, from 10-300 µg/mL (**Figures C-2 to C-4**). Complete sets of six calibration curves were run three times. The DXR curves, calibration area/internal standard area vs. standard concentration, were suitable for linear regression with 1/x weighting, resulting in correlation coefficients of 0.998 or better. The percent accuracy deviation of the regression-calculated value from the true value, and precision (%RSD) was 15% or less for all calibration levels, except at the LLOQ were it was less than 20% (**Tables C-1 to C-3**).

Table C-1. DXR Calibration Curve 1 in Rat Plasma.

Standard	Peak Area	ISTD Area	Area Ratio	Calculated Concentration (µg/mL)	% Diff From Theoretical	% RSD	RT
10 µg/mL	131672302	9102805	14.47	8.34	-16.6%		1.67
10 µg/mL	142572799	9293398	15.34	9.16	-8.4%	6.7%	1.67
25 µg/mL	332042946	10346075	32.09	24.97	-0.1%		1.67
25 µg/mL	323944094	9694355	33.42	26.21	4.9%	3.4%	1.66
50 µg/mL	575093852	9489819	60.60	51.86	3.7%		1.64
50 µg/mL	578192522	9292721	62.22	53.39	6.8%	2.1%	1.66
75 µg/mL	855366093	9356162	91.42	80.94	7.9%		1.64
75 µg/mL	818617175	9268372	88.32	78.02	4.0%	2.6%	1.64
100 µg/mL	1041739211	9035587	115.29	103.46	3.5%		1.62
100 µg/mL	1037663107	9203495	112.75	101.06	1.1%	1.7%	1.62
200 µg/mL	1934364838	8937706	216.43	198.88	-0.6%		1.57
200 µg/mL	1871841564	8779168	213.21	195.85	-2.1%	1.1%	1.58
300 µg/mL	2690120878	8497842	316.57	293.35	-2.2%		1.58
300 µg/mL	2664422491	8384395	317.78	294.50	-1.8%	0.3%	1.53
QCL 1	114142446	6910336	16.52	10.27	2.7%		1.65
QCL 2	123506769	7795092	15.84	9.64	-3.6%	4.5%	1.65
QCM 1	533134400	8749413	60.93	52.18	4.4%		1.63
QCM 2	549004621	9460147	58.03	49.44	-1.1%	3.8%	1.62
QCH 1	999160975	8643690	115.59	103.75	3.7%		1.60
QCH 2	1003032652	8526181	117.64	105.68	5.7%	1.3%	1.61
Blank Plasma	0	0					
Blank Plasma	0	0					
Blank Plasma + ISTD	0	12291759					
Blank Plasma + ISTD	0	12131964					
Linear Regression (ax+b) with 1/x Weighting							
Linear coefficient	1.0599						
Intercept	5.6299						
R^2	0.9982						

RT=retention time; ISTD=internal standard

DXR_PRM
Y = 5.62987+1.05993*X R^2 = 0.9982 W: 1/X

Figure C-2. DXR Calibration Curve 1.

Table C-2. DXR Calibration Curve 2 in Rat Plasma.

Standard	Peak Area	ISTD Area	Area Ratio	Calculated Concentration (µg/mL)	% Diff From Theoretical	% RSD	RT
10 µg/mL	115791921	8902807	13.01	8.57	-14.3%		1.65
10 µg/mL	114335862	8211903	13.92	9.36	-6.4%	6.2%	1.66
25 µg/mL	277046163	8487420	32.64	25.51	2.0%		1.65
25 µg/mL	273813646	8572570	31.94	24.90	-0.4%	1.7%	1.65
50 µg/mL	538916415	8252755	65.30	53.68	7.4%		1.63
50 µg/mL	519618243	8230235	63.14	51.81	3.6%	2.5%	1.62
75 µg/mL	752483878	8127159	92.59	77.21	2.9%		1.61
75 µg/mL	736435530	7933590	92.82	77.42	3.2%	0.2%	1.61
100 µg/mL	934395744	7362945	126.91	106.81	6.8%		1.63
100 µg/mL	945516974	7877260	120.03	100.88	0.9%	4.0%	1.61
200 µg/mL	1859520625	7690088	241.81	205.91	3.0%		1.59
200 µg/mL	1761356417	7828957	224.98	191.40	-4.3%	5.2%	1.58
300 µg/mL	2679293027	7684214	348.67	298.09	-0.6%		1.54
300 µg/mL	2528902096	7492928	337.51	288.45	-3.8%	2.3%	1.54
QCL 3	98598794	7068608	13.95	9.39	-6.1%		1.66
QCL 4	98574330	7288483	13.52	9.02	-9.8%	2.8%	1.66
QCM 3	477742858	7704907	62.01	50.83	1.7%		1.63
QCM 4	475056927	7902817	60.11	49.20	-1.6%	2.3%	1.64
QCH 3	928066985	7644808	121.40	102.06	2.1%		1.61
QCH 4	926099337	7537031	122.87	103.33	3.3%	0.9%	1.61
Blank Plasma	0	0					
Blank Plasma	0	0					
Blank Plasma + ISTD	0	11397794					
Blank Plasma + ISTD	10016	11369003					
Linear Regression (ax+b) with 1/x Weighting							
Linear coefficient	1.1594						
Intercept	3.0673						
R^2	0.9978						

RT=retention time; ISTD=internal standard

DXR_PRM
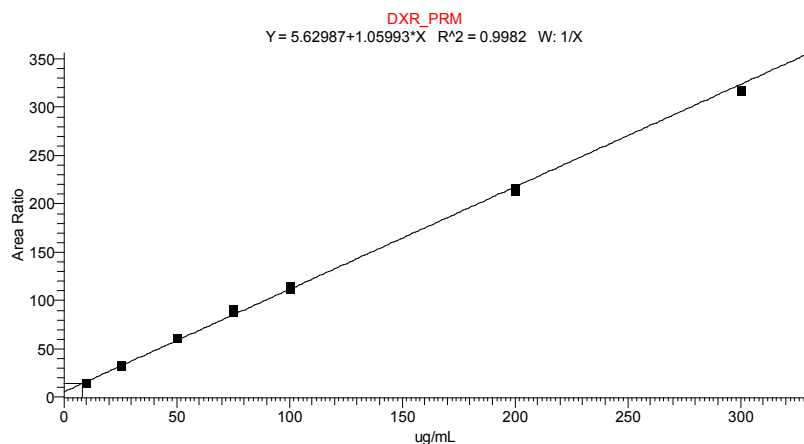Y = 3.06731+1.15942*X   R^2 = 0.9978   W: 1/X
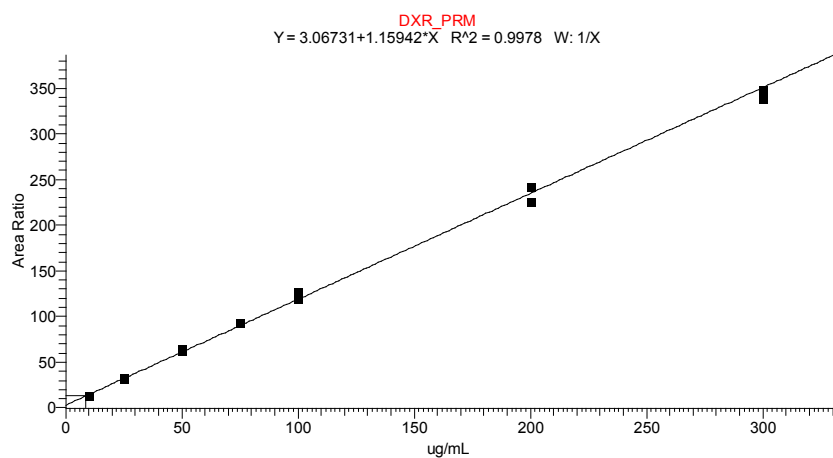
Figure C-3. DXR Calibration Curve 2.

Table C-3. DXR Calibration Curve 3 in Rat Plasma.

Standards	Peak Area	ISTD Area	Area Ratio	Calculated Concentration (µg/mL)	Average Concentration (µg/mL)	% Diff From Theoretical	% RSD	RT
10 µg/mL	112903922	6705897	16.84	8.68		-13.2%		1.65
10 µg/mL	123878589	7357519	16.84	8.68	8.7	-13.2%	0.004%	1.65
25 µg/mL	293832532	8081338	36.36	25.94		3.8%		1.64
25 µg/mL	280640237	7640887	36.73	26.27	26.1	5.1%	0.9%	1.64
50 µg/mL	532195492	7824586	68.02	53.93		7.9%		1.63
50 µg/mL	482080059	7314864	65.90	52.06	53.0	4.1%	2.5%	1.61
75 µg/mL	708672422	7526972	94.15	77.03		2.7%		1.62
75 µg/mL	700066103	7508535	93.24	76.23	76.6	1.6%	0.7%	1.62
100 µg/mL	950926636	7571024	125.60	104.84		4.8%		1.62
100 µg/mL	828044390	6686496	123.84	103.28	104.1	3.3%	1.1%	1.61
200 µg/mL	1714706555	7465534	229.68	196.85		-1.6%		1.56
200 µg/mL	1590979458	6985108	227.77	195.16	196.0	-2.4%	0.6%	1.57
300 µg/mL	2316565187	6643633	348.69	302.06		0.7%		1.53
300 µg/mL	2305505567	6904927	333.89	288.98	295.5	-3.7%	3.1%	1.53
QCL 5	105010025	5530899	18.99	10.58		5.8%		1.64
QCL 6	106390328	5761623	18.47	10.12	10.4	1.2%	3.1%	1.64
QCM 5	475027124	7253011	65.49	51.70		3.4%		1.63
QCM 6	481842261	7379730	65.29	51.52	51.6	3.0%	0.2%	1.63
QCH 5	876586001	7139952	122.77	102.34		2.3%		1.60
QCH 6	923088155	7302129	126.41	105.56	104.0	5.6%	2.2%	1.60
Blank Plasma	0	0						
Blank Plasma	0	0						
Blank Plasma + ISTD	0	11817640						
Blank Plasma + ISTD	0	10609872						
Linear Regression (ax+b) with 1/x Weighting								
Linear coefficient	1.1312							
Intercept	7.0137							
R^2	0.9982							

RT=retention time; ISTD=internal standard

DXR_PRM
Y = 7.01369+1.13115*X   R^2 = 0.9982   W: 1/X
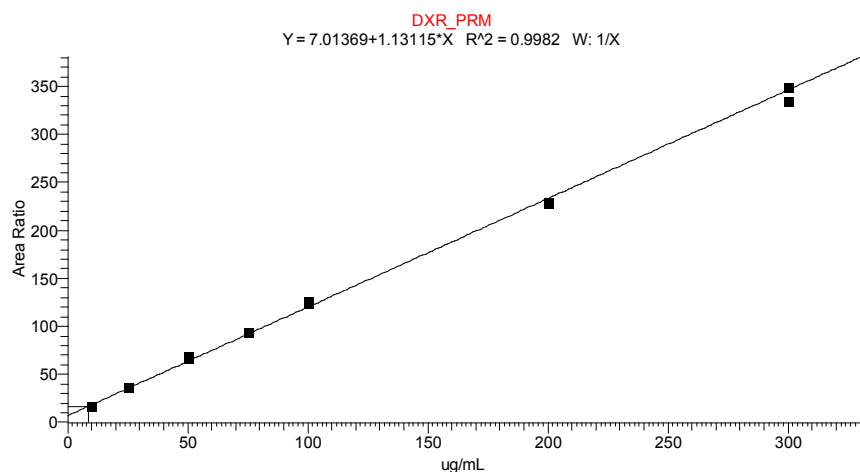
Figure C-4. DXR Calibration Curve 3.

Intra-Day Precision and Accuracy

Over the intra-day study period, the six replicate QC samples maintained a high degree of accuracy and precision (**Table C-4**). The low QC standard, at 10 µg/mL, had an average accuracy deviation of -1.6% and precision of 6.1%. The mid QC standard, at 50 µg/mL, had an average accuracy deviation of 1.6% and precision of 2.5%. The high QC standard, at 100 µg/mL, had an average accuracy deviation of 3.8% and precision of 1.5%.

Table C-4. Intra-Day Precision and Accuracy. Precision and accuracy deviation data are presented for six replicates at each QC level, 10 µg/mL (QC Low **(A)**), 50 µg/mL (QC Mid **(B)**), and 100 µg/mL (QC High **(C)**).

A. QC Low DXR

QC Low DXR	Theoretical (µg/mL)	Concentration (µg/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	10	10.3	2.7%	
Replicate 2	10	9.6	-3.6%	
Replicate 3	10	9.4	-6.1%	
Replicate 4	10	9.0	-9.8%	
Replicate 5	10	10.6	5.8%	
Replicate 6	10	10.1	1.2%	
AVG		**9.8**	**-1.6%**	**6.1%**

B. QC Mid DXR

QC Mid DXR	Theoretical (µg/mL)	Concentration (µg/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	50	52.2	4.4%	
Replicate 2	50	49.4	-1.1%	
Replicate 3	50	50.8	1.7%	
Replicate 4	50	49.2	-1.6%	
Replicate 5	50	51.7	3.4%	
Replicate 6	50	51.5	3.0%	
AVG		**50.8**	**1.6%**	**2.5%**

C. QC High DXR

QC High DXR	Theoretical (µg/mL)	Concentration (µg/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	100	103.7	3.7%	
Replicate 2	100	105.7	5.7%	
Replicate 3	100	102.1	2.1%	
Replicate 4	100	103.3	3.3%	
Replicate 5	100	102.3	2.3%	
Replicate 6	100	105.6	5.6%	
AVG		**103.8**	**3.8%**	**1.5%**

Analytical System Reproducibility

Analytical system reproducibility was explored by determining the peak area and retention time precision of six replicate samples at the 50 µg/mL QC level (**Table C-3**). The runs met the acceptance criterion established for retention time and peak area at <10% RSD for both the analyte, DXR, and internal standard, aclarubicin.

Table C-3. Analytical System Reproducibility. Presented are the RT and peak area results for the 6 replicates at the 50 µg/mL QC level.

Samples	DXR		Aclarubicin, ISTD	
	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area
Replicate 1	1.63	533134400	5.57	8749413
Replicate 2	1.62	549004621	5.57	9460147
Replicate 3	1.63	477742858	5.57	7704907
Replicate 4	1.64	475056927	5.56	7902817
Replicate 5	1.63	475027124	5.56	7253011
Replicate 6	1.63	481842261	5.56	7379730
AVG	1.63	498634698	5.57	8075004
SD	0.01	33343792	0.00	860133
%RSD	0.37	6.7	0.05	10.7

ISTD=internal standard

1. Ambardekar, V.V. and Stern, S.T. *NBCD Pharmacokinetics and Drug Release Methods* in Non-Biological Complex Drugs; The Science and the Regulatory Landscape, D.J.A. Crommelin and J.S.B. de Vlieger, eds. **2015**, p. 261-287, Springer International Publishing.

2. Skoczen, S.L. and Stern, S.T., *Improved Ultrafiltration Method to Measure Drug Release from Nanomedicines Utilizing a Stable Isotope Tracer.* Methods Mol Biol. **2018**, 1682, 223-239.

3. Skoczen, S., McNeil, S.E., and Stern, S.T., *Stable isotope method to measure drug release from nanomedicines.* J Control Release. **2015**, 220(Pt A), 169-174.

4. Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), *Guidance for Industry: Bioanalytical Method Validation,* **2013**. Available from: https://www.fda.gov/downloads/drugs/guidances/ucm368107.pdf.

5. Kousba, A., Underbrink, R., and Kruppa, D., *Comparison of Encapsulated and Non-encapsulated Doxorubicin Pharmacokinetics Following Single Intravenous Administration of ATI-0918, an Encapsulated Liposomal Formulation, and DOXIL in Female Rats.* AAPS Annual meeting, San Antonio, TX. **2013**.

6. Limentani, G.B., Ringo, M.C., Ye, F., Berquist, M.L., and McSorley, E.O., *Beyond the t-test: statistical equivalence testing.* Anal Chem. **2005**, 77(11), 221A-226A.

7. Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), *Guidance for Industry: Statistical approaches to establishing bioequivalence,* **2001**. Available from: http://www.fda.gov/downloads/Drugs/Guidances/ucm070244.pdf.

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ACN	acetonitrile
API	active pharmaceutical ingredient
AU	absorbance units
AUC_{all}	area under the time concentration curve including all time points
AUC_{inf}	area under the time-concentration curve extrapolated to time infinity
BE	bioequivalence
C_{max}	concentration maximum
CDER	Center for Drug Evaluation and Research
CI	confidence interval
Conc	concentration
CV	coefficient of variation
CVM	Center for Veterinary Medicine
DXR	doxorubicin hydrochloride
DXR_C13	$^{13}C,^{2}H_3$-doxorubicin trifluoroacetate salt
ESI	electrospray ionization
FA	formic acid
FDA	Food and Drug Administration
HPLC	high performance liquid chromatography
ISTD	internal standard
LLOQ	lower limit of quantitation
LOD	limit of detection
LOQ	limit of quantitation
MS	mass spectrometry
MWCO	molecular weight cut off
NCL	Nanotechnology Characterization Laboratory
PBS	phosphate buffered saline
PFP	protein-free plasma
PRM	parallel reaction monitoring
PK	pharmacokinetic
QC	quality control
QCH	quality control, high
QCL	quality control, low
QCM	quality control, mid
RSD	relative standard deviation
SD	standard deviation
SPE	solid phase extraction
SITUA	stable isotope tracer ultrafiltration assay
T_{max}	time to maximum concentration
TOST	two one-sided t-tests

Nanotechnology Characterization Laboratory

Stephan T. Stern
Scott E. McNeil
Jennifer Grossman
Rachael M. Crist
Sarah Skoczen
Kelsie Snapp

NCL201902B-DRAFT

Novel Method to Determine Bioequivalence of Nanomedicines: Abraxane vs. Genexol-PM vs. Taxol (Generic)

prepared for

U.S. Food & Drug Administration
(Inter-Agency Award 224-16-3001S)

The complexity of nanomedicine drug formulations poses unique scientific challenges. In contrast to conventional small molecule formulations, the active pharmaceutical ingredient (API) in a systemically administered nanomedicine formulation exists in several forms: (a) nanomedicine-encapsulated, (b) unencapsulated, free/unbound, and (c) unencapsulated, protein-bound. While the free, unbound form is considered the only biologically active form of the API, all three fractions are important in characterizing a nanomedicine's pharmacokinetics– especially in evaluation of bioequivalence (BE) (pharmacokinetic similarity). Existing methods to measure these various nanomedicine fractions (e.g. solid phase extraction, conventional ultrafiltration, etc.) are not ideal due to a variety of shortcomings as described in the literature [1].

The primary objective of this project was to evaluate a novel bioanalytical technique developed at the NCL to fractionate the various subpopulations of a nanomedicine in plasma, in an effort to fulfill this unmet need and facilitate regulatory review of new classes of generic nanomedicines [2, 3]. This novel method is an ultrafiltration technique that utilizes a stable isotope tracer of the API to determine protein binding, which allows for calculation of the remaining drug fractions (the bioanalytical method is described in more detail in Section I). In order to evaluate the performance of this fractionation method, it was used to determine the BE of two controlled release nanomedicine products currently on the market, Janssen's Doxil® and Sun Pharma's doxorubicin HCl liposome formulations. This work was detailed in an earlier report to the FDA, "Novel Method to Determine Bioequivalence of Nanomedicines: Doxil vs. Sun Pharma." Herein, a follow-on assessment is detailed, utilizing two fast-releasing nanomedicine products, Celgene's Abraxane® and Samyang's Genexol-PM (approved in South Korea), as well as the legacy paclitaxel formulation, Taxol (generic).

Nanomedicine Encapsulated   **Free/Unbound**   **Protein Bound**

Unencapsulated
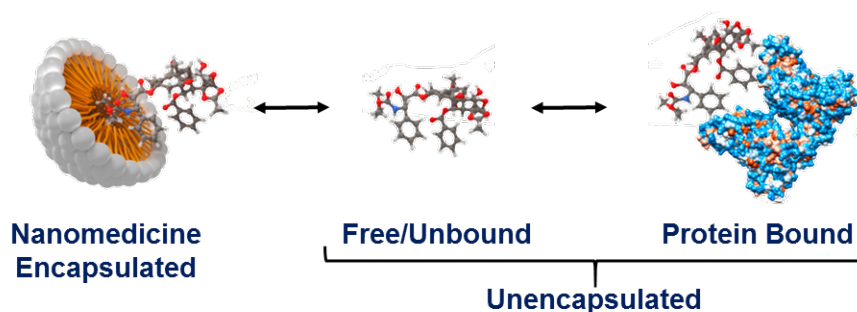
In brief, the bioanalytical methods for measurement of the paclitaxel (PTX) and paclitaxel stable isotope tracer (PTX_C13) analytes were successfully validated in both human and rat plasma and protein-free plasma, in accordance with FDA bioanalytical guidance [4]. Furthermore, the stable isotope tracer ultrafiltration assay (SITUA) was validated with regard to process induced drug release, spike recovery, organic spike stability, and plasma volume requirements. The validation and control studies are outlined in Section II.

Three independent lots of both Genexol-PM and Abraxane formulations, as well as a single lot of generic Taxol comparator, were evaluated for in vitro drug release using the validated method, as noted in the Table below. The in vitro drug release lot comparisons of Abraxane are presented in Section III, Genexol-PM lot comparisons in Section IV, and a comparison of

Abraxane vs. Genexol-PM is presented in Section V. A comparison of Abraxane vs. Genexol-PM vs. Taxol (generic) is presented in Section VI. Drug release for all three formulations was similar over the 2 hr incubation period, in both human and rat plasma, with all formulation releasing all drug immediately and not following a concentration-dependent or temporal trend. Although there were instances of statistically significant differences at various time points and concentrations, these differences were not consistent between lots or nanomedicine products over the study period. Notably, however, there were differences in the relationship between formulation concentration and free drug fraction. In rat plasma, paclitaxel, Abraxane, and Genexol-PM displayed similar saturated binding, suggesting the Abraxane-albumin nanoparticle and Genexol-PM micelle do not contribute to protein binding. In human plasma, Genexol-PM displayed linear binding, suggesting a contribution of the micelle to drug binding at high concentrations, while paclitaxel and Abraxane displayed similar saturated binding. In both rat and human plasma, Taxol (generic) displayed concentration-dependent binding, suggesting the cremophor micelle strongly binds to free drug.

Abraxane Lots	Genexol-PM Lots	Generic Taxol Lots	Lot Abbreviation	Color Coding
6113658	GP31681	PACCA1018	A	Green
6111839	GP316A1	-	B	Orange
6111355	GP31741	-	C	Red
6115664	GP31771	-	D	Purple
6115194	-	-	E	Yellow
6115306	-	-	F	Grey
Free Paclitaxel Control				Blue

Separate lots of each formulation (Abraxane (Lot E, 6115194); Genexol-PM (Lot D, GP31771); or Taxol generic (Lot A, PACCA1018)) were also evaluated in a bioequivalence study using Sprague Dawley rats (see Section VII). Total, unencapsulated and unbound drug pharmacokinetic (PK) parameters were similar for Abraxane and Genexol-PM, but notably different for Taxol. Although Abraxane and Genexol-PM had similar PK, a statistical analysis of bioequivalence by two one-sided t-tests determined that Abraxane and Genexol-PM were not bioequivalent. This lack of bioequivalence is probably the result of high variability and insufficient power, due to the low number of animals used. Taxol demonstrated lower total, unencapsulated and unbound CL, Vd and Vss than Genexol-PM and Abraxane, consistent with strong equilibrium binding of drug to stable cremophor micelles. This equilibrium binding was reflected in non-linear/reduced unbound drug fraction at high drug concentrations for the Taxol formulation. Surprisingly, and inconsistent with pharmacological theory regarding the effect of protein binding on active, unbound drug PK for a low extraction drug administered parenterally, unbound PK was altered by this equilibrium formulation-binding. This effect on active, unbound drug PK could potentially influence drug therapy, and help explain differences between Abraxane and Taxol pharmacology [5]. Unbound drug PK appears to be adiscriminating criteria for drug formulations that influence drug PK by equilibrium binding. The SITUA assay is a very predictive and accurate method for identifying formulation effects on unbound drug fraction resulting from equilibrium binding.

Importantly, the SITUA method can be used to evaluate nanomedicine bioequivalence, as well as differentiate between formulations in which drug is stably encapsulated (i.e., Doxil and Sun Pharma's doxorubicin HCl liposome), bound in equilibrium (i.e., Taxol), or simply solubilized (i.e., Genexol-PM and Abraxane). The SITUA method should facilitate both characterization and generic development of complex drugs. The resulting higher quality pharmacokinetic data may also decrease patient sample size and facilitate regulatory determination of bioequivalence.

I. Bioanalytical Method

Stable Isotope Tracer Ultrafiltration Assay (SITUA)

The stable isotope tracer ultrafiltration assay (SITUA) method [2, 3] detailed below was utilized for all in vitro drug release and in vivo bioequivalence (BE) studies in this report.

Design and Methods

Our laboratory has developed a novel ultrafiltration method to measure nanomedicine encapsulated and unencapsulated active pharmaceutical ingredient (API) in plasma, and assess nanomedicine API release [2, 3]. This method utilizes a stable isotope of the API to account for formulation-induced changes in protein-binding, as well as binding of the API to formulation components.

In this method, stable, isotopically labeled drug is spiked into a plasma sample containing the nanomedicine formulation (**Figure I-1**). This can be a plasma sample from an in vitro incubation or pharmacokinetic study. The sample is incubated for a predefined time to allow isotope equilibration, an aliquot of the sample is taken, and the sample is then filtered using an ultrafiltration apparatus. Both the sample aliquot and the ultrafiltrate are analyzed by liquid chromatography-mass spectrometry to determine concentrations of the normoisotopic and isotopically labeled drug.

Since the stable isotopically labeled drug (**D***) equilibrates with protein and formulation components identical to the unlabeled, normoisotopic drug (**D**) released from the nanomedicine formulation, the ultrafilterable fraction of the isotopically labeled drug represents a reliable measurement of free unbound fraction. Bound fraction can be calculated from equation (i):

(i)   %Bound D* = ([Total D*] - [Ultrafilterable D*]) * 100
                              [Total D*]

The encapsulated and unencapsulated nanomedicine fractions can then be easily calculated using equations (ii) and (iii):

(ii)   [Unencapsulated D]   =   [Ultrafilterable D]
                                          (1-(%Bound D*/100))

(iii)   [Encapsulated] = [Total D] – [Unencapsulated D]

Figure II-1. Drug release assay using SITUA. The stable isotopically labeled drug (D*), $^{13}C_6$-paclitaxel in the studies detailed in this report, is spiked into nanomedicine (NM-D) in plasma. D* behaves identically to normoisotopic drug (D) with regard to protein binding (Pro-D/D*). After protein binding equilibrium is reached, the plasma sample is transferred to an ultrafiltration device and the filtrate is separated by centrifugation. The stable isotope tracer free fraction, represented as the ultrafilterable fraction, can be used to calculate protein bound, unencapsulated and encapsulated drug fractions, according to equations (i), (ii) and (iii) above.

In Vitro Drug Release in Human Plasma

Human blood was collected in K_2-EDTA tubes and pooled from 6 donors. Blood was spun at 2500x*g* for 10 min to collect plasma. HEPES buffer (pH 7.4) was added at 50 µL/2 mL plasma to maintain a pH of 7.4. Plasma in glass vials was spiked with samples (free drug or nanomedicine) at final drug concentrations of 0.5 µg/mL, 1 µg/mL, and 5 µg/mL paclitaxel (PTX) in triplicate. All samples were spiked with 0.1 µg/mL $^{13}C_6$-PTX (stable isotope tracer) (PTX_C13).

Samples were then incubated at 37°C with agitation. At time points 10 min, 30 min, 1 hr and 2 hr, 50 µL of plasma was taken for protein precipitation as described below; this sample was used to determine total drug concentration. Additionally, 400 µL (see Note 1) of sample was transferred to a prewarmed ultrafiltration tube (10 kDa Vivacon) and spun at 6000x*g* for 10 min. 50 µL of this filtrate was also taken for protein precipitation; this sample was used to determine free/unbound drug concentration.

Notes:

1. For in vitro studies, 400 µL of plasma was used. For analysis of the samples from the in vivo study, 150 µL of plasma was used. No change in assay performance was noted for the reduced sample volume. See control study comparing reduced sample volumes in Section II.

Protein Precipitation Method

The two 50 µL samples from above (non-centrifuged and centrifuged) were added to 200 µL ice cold acetonitrile (ACN) with 0.1% formic acid (FA) containing 25 ng/mL $^2H_5$-PTX as an internal standard (ISTD). The samples were frozen at -80°C for 10 min, thawed at room temperature, then spun at 4°C, 18,000x*g* for 20 min. The supernatant was transferred to a clean Eppendorf tube and dried using a centrifuge speed vacuum for 25 min at 50°C and 5 Torr. Following protein precipitation, samples were reconstituted in 150 µL 40% ACN with 0.1% FA. The reconstituted sample was then transferred to clean a Eppendorf tube and spun at 18,000xg at 4°C for 5 min, then transferred to an HPLC vial. Samples were analyzed on the Q-Orbitrap as described on the next page.

In Vitro Drug Release in Rat Plasma

In vitro drug release in rat plasma was conducted similarly. Sprague Dawley rat blood was collected in K_2-EDTA tubes. Blood was spun at 2500x*g* for 10 min to collect plasma. HEPES buffer (pH 7.4) was added at 50 µL/2 mL plasma to maintain a pH of 7.4. Plasma in glass vials was spiked with samples (free drug or nanomedicine) at final drug concentrations of 0.5 µg/mL, 1 µg/mL, and 5 µg/mL PTX in triplicate. All samples were spiked with 0.1 µg/mL $^{13}C_6$-PTX (stable isotope tracer) and processed as described above.

LC-Orbitrap Method

The LC-Orbitrap bioanalytical method detailed below was utilized for all studies detailed in this report.

1. LC-Orbitrap Set Up

1.1 The LC-Orbitrap system consisted of a Q Exactive basic quadrupole Orbitrap mass spectrometer, Vanquish UHPLC system, binary pump and autosampler (Thermo Fisher Scientific).

1.2 The LC conditions were: 20 μL injection volume for protein-free plasma samples and 10 μL injection volume for plasma samples, 40°C column oven, 10°C autosampler, and flow rate of 0.4 μL/min. Mobile phase A consisted of water with 0.1% formic acid, and mobile phase B consisted of acetonitrile with 0.1% formic acid. The following gradient was used: linear increase from 40% B to 100% B from 0 to 2 min, hold at 100% B for 2 min, and linear decrease from 100% B to 40% B in 0.5 min, with a column regeneration time between injections of 3.5 min. The column was a Zorbax-SB-C18 RRHD 1.8 μm particle, 2.1x100 mm (Agilent technologies, Inc) with a Zorbax-SB-C18 1.8 μm particle, 2.1x5 mm guard column (Agilent Technologies, Inc).

1.3 Paclitaxel (PTX), $^{13}C_6$-Paclitaxel (PTX_C13) tracer, and $^2H_5$-Paclitaxel (PTX_d5) internal standard (ISTD) elution times were all 2.03 min (**Figures I-2 and I-3**). The Orbitrap mass spectrometer was run in ESI positive mode, spray voltage was 3.5 kV, and the capillary and auxiliary gas temperatures were 150°C and 400°C, respectively. The collision energy was set at 13 AU for all three analytes. Parallel reaction monitoring (PRM) of the following transitions were used: PTX: 854.34 → 286.10, 509.21; PTX_C13: 860.36 → 286.10, 515.23, and; PTX_d5: 859.37 → 291.13, 509.21 (**Figure I-3**).

2. Materials

2.1 Paclitaxel (PTX) – LC Laboratories, catalog #P-9600

2.2 $^{13}C_6$-Paclitaxel (PTX_C13) – Santa Cruz Biotechnology, catalog # sc-477982

2.3 $^2H_5$-Paclitaxel (PTX_d5) – Santa Cruz Biotechnology, catalog # sc-219546

2.4 Acetonitrile – VWR, catalog # BJLC015-1

2.5 Formic acid – Thermo Scientific, catalog # 28905

2.6 Zorbax-SB-C18 RRHD 1.8 μm particle, 2.1 x 100 mm – Agilent Technologies, Inc., catalog # 858700-902

2.7 Zorbax-SB-C18 1.8 μm particle, 2.1 x 5 mm – Agilent Technologies, Inc., catalog # 821725-902

2.8 Amicon Ultra-4 centrifugal filter unit Ultracel 30 membrane – Millipore, catalog # UFC803024

2.9 Vivacon 10 kDa MWCO centrifugal filter unit with Hydrosart regenerated cellulose membrane – Satorius, catalog # VN01H02

2.10 Rat plasma (Sprague Dawley) – collected fresh in K_2EDTA tubes

2.11 Human plasma (pooled) – collected fresh from six human donors in K_2EDTA tubes, collected under NCI at Frederick Protocol OH99-C-N046

2.12 HEPES Buffer (1 M) – Gibco, catalog # 15630080

2.13 K_2EDTA vacutainer tubes – Moore Medical, catalog # 87770

3. Preparation of Human and Rat, Plasma and Protein-Free Plasma

3.1 Blood collected from six human donors or Sprague Dawley rats in K_2EDTA tubes.

3.2 Blood was centrifuged at 2,500x*g* for 10 min and plasma was pooled together.

3.3 50 µL of HEPES buffer (pH 7.4) for every 2 mL of plasma was added and pH adjusted to 7.4.

3.4 To prepare protein-free plasma, plasma was transferred to 4 mL centrifugal filter units with a 30 kDa MWCO, centrifuged at 5000x*g* for 1 hr, and filtrate was collected.

4. Calibration and Quality Control Standards Preparation

4.1 Stock solutions of PTX and PTX_C13 were prepared in acetonitrile. These stocks were used to prepare calibration and quality control standards.

4.2 PTX and PTX_C13 calibration standards were prepared in human protein-free plasma, human plasma, rat protein-free plasma, and rat plasma with concentrations ranging from 0.5 ng/mL to 10 µg/mL depending on the matrix and range needed for unknown samples. PTX_d5 was used as an internal standard at a concentration of 25 ng/mL.

4.3 PTX and PTX_C13 low, medium and high QCs were also prepared in appropriate matrix at concentrations dependent on the range of the calibration curve.

5. Sample Preparation

5.1 50 µL of plasma or protein-free plasma containing standards, QCs, or unknowns were added to 1.5 mL Eppendorf tubes, followed by addition of 200 µL ice cold ACN with 0.1% formic acid containing 25 ng/mL internal standard and vortexed.

5.2 The sample was placed at -80°C for 10 min and then thawed at room temperature.

5.3 The thawed sample was then spun at 18,000x*g* for 20 min at 4°C to pellet precipitated proteins.

5.4 The supernatant was transferred to a 1.5 mL Eppendorf tube and dried in a speed vac for 25 min at 50°C and 5 Torr.

5.5   The dried residue was resuspended in 150 µL 40% ACN with 0.1% formic acid.

5.6   The extracted sample was spun again at 18,000x*g* for 10 min at room temperature.

5.7   The supernatant was transferred to a 1.5 mL amber glass screw top HPLC vial with fixed Teflon insert, capped, and placed in an HPLC auto sampler vial rack.

5.8   Plasma blank, ISTD spiked plasma blank, and quality control samples were run with each calibration curve.

A. Analytes

Paclitaxel

* = ^{13}C

^{13}C$_6$-Paclitaxel

B. Internal Standard

^{2}H$_5$-Paclitaxel

Figure I-2. Analytes and internal standard used in the drug release assay. Structures of analytes, (**A**) paclitaxel (PTX) and (**B**) ^{13}C$_6$-paclitaxel (PTX_C13), and (**C**) ISTD ^{2}H$_5$-paclitaxel (PTX_d5) are shown.

Figure I-3. LC-Q Orbitrap Parallel Reaction Monitoring Chromatograms of Analytes and Internal Standard. Displayed are chromatograms of (**A**) PTX, (**B**) PTX_d5 and (**C**) PTX_C13. The transitions used were: PTX: 854.34 → 286.10, 509.21; PTX_C13: 860.36 → 286.10, 515.23, and; PTX_d5: 859.37 → 291.13, 509.21.

II. Validation and Control Studies

Validation Criteria and Study Design

The LC-Orbitrap method for PTX and PTX_C13 in human plasma and protein-free plasma matrices was validated according to the FDA Guidelines for bioanalytical methods [4]. As per the FDA guidance, three concentrations representing the entire range of the standard curve should be studied: 1) the low QC within 3x of the lower limit of quantification (LLOQ), 2) the mid QC at the middle of the calibration curve, and 3) the high QC near the upper boundary of the standard curve. Our calibration curve contained seven calibrants, ranges dependent on matrix. The QC standards were selected within the concentration range of the calibration curve as described. The following criteria were used for validation of the LC-Orbitrap method.

Figure II-1. Graphical representation of validation criteria terms

Precision defines random errors, bias defines systematic errors, and accuracy contains both errors (**Figure II-1**). Accuracy deviation is a measure of the distance between the estimated and actual values (Accuracy deviation = [(actual-estimated)/ref]*100%). The mean value of the estimate at each QC level should be within 15% of the actual value. Precision is a measure of assay repeatability, and is defined as Precision = percent relative standard deviation (% RSD) = [standard deviation/mean]*100%. The precision of the assay at each QC level should be equal to or less than 15% except at LLOQ, where it should be equal or less than 20%. For intra-day validation, six individual QC samples were run at each QC level. These samples were distributed throughout the beginning, middle and end of the run. For the inter-day validation, six individual QC samples were run at each QC level on three separate days. System reproducibility was determined by injecting mid QC standards in six replicates on three separate days, and evaluating retention time and peak area precision.

Interpolation from Calibration Curve
Unknowns and QC sample concentrations were interpolated from the calibration curve, using the linear formula, area ratio = slope*concentration ratio + y-intercept.

Full Validation in Human Plasma

Summary

The purpose of this study was to validate the LC-Orbitrap quantitation method for PTX and PTX_C13 in human plasma matrix at a standard curve range of 10-10,000 ng/mL. For specifics of the LC-Orbitrap method please see Section I. This analytical method was validated for extraction efficiency in human plasma and quality control precision and accuracy based on general FDA guidelines for bioanalytical methods [5].

Calibration standards were prepared by adding PTX and PTX_ C13 analytical standards to blank human plasma in the range from 10-10,000 ng/mL, to arrive at seven calibration levels. Matrix interference was not observed for the analytes or internal standard. Complete sets of the combined PTX and PTX_C13 calibration standards were run on three different days.

The PTX and PTX_13C calibration curves, analyte area/internal standard area (area ratio) vs. standard concentration, were suitable for linear regression with $1/x^2$ weighting, resulting in correlation coefficients of 0.99 or better. The accuracy deviation of the regression-calculated standard value from the true value was 15% or less for all calibration levels. The lower limit of quantitation (LLOQ) was established at 10 ng/mL. The method precision at the LLOQ was 2% RSD for PTX and 2% RSD for PTX_C13.

No carry over from the high calibration standard was seen for analytes or internal standard. Analytical system reproducibility, evaluated by determining the peak area and retention time precision of six replicate samples at the 100 ng/mL concentration level for the plasma extracted PTX and PTX_C13 analytes, met the acceptance criterion established for retention time and peak area at 15% RSD for all analytes. The estimated LOD and LOQ for PTX were 2 and 6 ng/mL, respectively. The estimated LOD and LOQ for PTX_C13 were 1 and 5 ng/mL, respectively. For intra-day validation, six individual QC samples were run at each QC level, 10 (low), 100 (mid) and 1000 (high) ng/mL for both analytes. These samples were distributed throughout the beginning, middle and end of the run. For the inter-day validation, six individual QC samples were run at each level on three separate days. The low, mid and high QC standard's intra- and inter-day accuracy deviation and precision, for both PTX and PTX_C13, met the acceptance criteria of accuracy deviation not exceeding 15% from the true value and precision not exceeding 15% RSD. The calculated absolute recovery for plasma extraction was between 45-91% for all analytes, at all calibration levels.

This validation did not attempt to assess either short-term room temperature stability, or freeze-thaw stability of samples, or accuracy and precision of diluted samples.

In conclusion, this LC-Orbitrap analytical method for determination of PTX and PTX_C13 in human plasma was successfully validated.

Results & Conclusions

Calibration Curves

Calibration curves, peak area ratio vs. concentration, were linear over the concentration range, from 10-10000 ng/mL, for PTX and PTX_C13 (**Figures II-2 and II-3**). Complete sets of seven combined PTX and PTX_C13 calibration levels were run on three different days. The PTX and PTX_C13 curves, calibration area/internal standard area (ratio) vs. standard concentration, were suitable for linear regression with $1/x^2$ weighting, resulting in correlation coefficients of 0.99 or better. The percent accuracy deviation of the regression-calculated value from the true value and precision was 15% or less, for all calibration levels (**Tables II-1 and II-2**). The lower limit of quantitation (LLOQ) was established at 10 ng/mL for both analytes.

PTX
Y = 0.00547456+0.00890258*X   R^2 = 0.9983   W: 1/X^2
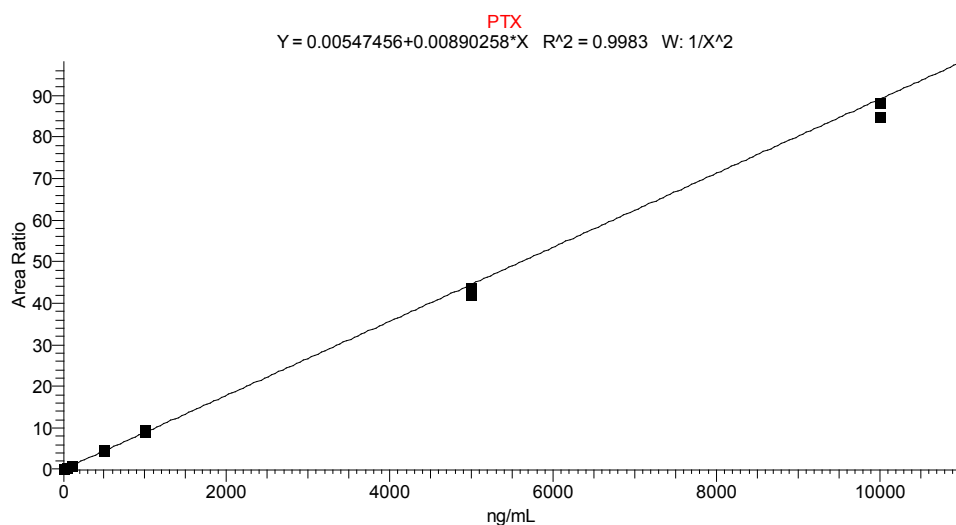
Figure II-2. Example of PTX Calibration Curve in Human Plasma.

PTX_C13
Y = -0.00276628+0.00828887*X   R^2 = 0.9964   W: 1/X^2
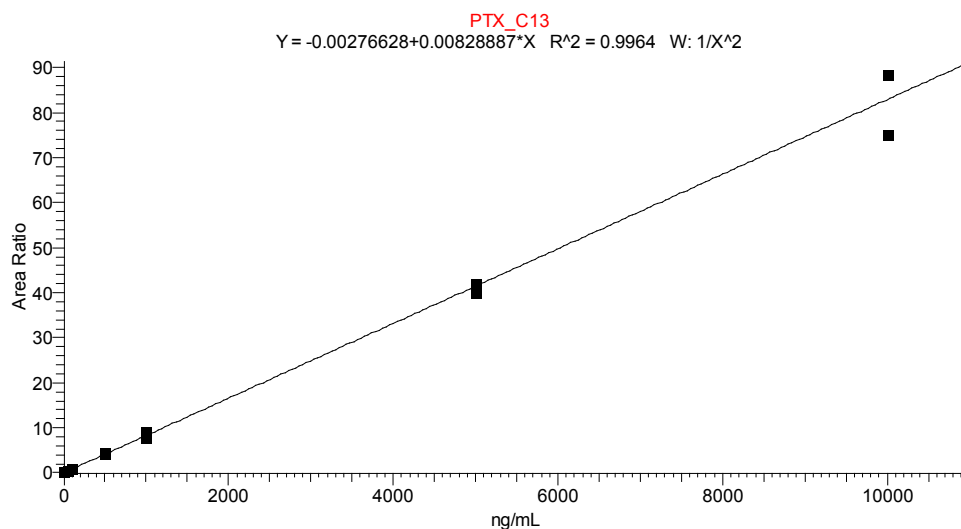
Figure II-3. Example of PTX_C13 Calibration Curve in Human Plasma.

Table II-1. PTX Calibration Runs in Human Plasma.

Standard Conc (ng/mL)	PTX Calibration 1			PTX Calibration 2			PTX Calibration 3		
	Experimental Conc (ng/mL) *	% Accuracy Deviation	% Precision	Experimental Conc (ng/mL) *	% Accuracy Deviation	% Precision	Experimental Conc (ng/mL) *	% Accuracy Deviation	% Precision
10.0	10.0	-1%		9.9	-1%		10.0	-0.2%	
10.0	9.9	-1%		9.9	-1%		9.9	-1%	
10.0	9.9	-1%	9%	9.9	-1%	2%	10.0	0.1%	2%
50	51	3%		53	6%		49	1%	
50	52	3%		52	3%		51	2%	
50	51	2%	5%	51	2%	3%	50	0.1%	5%
100	99	-1%		102	2%		95	-0.1%	
100	99	-1%		101	2%		103	3%	
100	103	3%	3%	103	3%	3%	97	-3%	5%
500	504	1%		507	1%		472	-2%	
500	512	2%		504	1%		502	0.4%	
500	511	2%	3%	523	5%	3%	522	4%	3%
1000	984	-2%		1001	0.1%		990	4%	
1000	1039	4%		1035	4%		1035	4%	
1000	991	-1%	3%	1027	3%	2%	1045	5%	5%
5000	4881	-2%		4922	-2%		4942	3%	
5000	4829	-3%		4970	-1%		5046	1%	
5000	5068	1%	8%	4884	-2%	4%	4819	-4%	5%
10000	10151	2%		9320	-7%		9034	-6%	
10000	9531	-5%		9243	-8%		9091	-9%	
10000	9312	-7%	6%	9118	-9%	1%	9710	-3%	3%
Linear Regression (ax+b) with $1/x^2$ Weighting									
	Curve 1	Curve 2	Curve 3	Curve 1	Curve 2	Curve 3	Curve 1	Curve 2	Curve 3
Linear coefficient	0.007	0.007	0.007	0.007	0.008	0.008	0.008	0.008	0.009
Intercept	0.004	0.003	0.013	0.015	0.005	0.011	0.005	0.002	0.005
R^2	0.997	0.997	0.996	0.997	0.998	0.997	0.997	0.996	0.998

*Data based on average of duplicate replicates.

Table II-2. PTX_C13 Calibration Runs in Human Plasma.

Standard Conc (ng/mL)	PTX_C13 Calibration 1			PTX_C13 Calibration 2			PTX_C13 Calibration 3		
	Experimental Conc (ng/mL) *	% Accuracy Deviation	% Precision	Experimental Conc (ng/mL) *	% Accuracy Deviation	% Precision	Experimental Conc (ng/mL) *	% Accuracy Deviation	% Precision
10.0	10.0	-0.1%		9.9	-1%		9.7	-1%	
10.0	10.0	-0.1%		10.0	-1%		9.9	-1%	
10.0	10.1	1%	7%	9.9	-1%	3%	10.0	0.3%	3%
50	49	-1%		52	4%		49	2%	
50	51	1%		51	3%		51	2%	
50	49	-2%	8%	50	1%	2%	50	-0.4%	5%
100	103	3%		98	-2%		95	1%	
100	99	-1%		98	-2%		100	0.4%	
100	98	-2%	3%	103	3%	4%	97	-3%	7%
500	509	2%		510	2%		475	0.3%	
500	498	-0.3%		505	1%		512	2%	
500	516	3%	3%	528	6%	6%	519	4%	5%
1000	995	-1%		1045	5%		1022	5%	
1000	1016	2%		1037	4%		1015	2%	
1000	1035	4%	6%	1048	5%	3%	1017	2%	6%
5000	5187	4%		4883	-2%		4823	1%	
5000	5244	5%		4942	-1%		4948	-1%	
5000	5067	1%	5%	4753	-5%	5%	4953	-1%	5%
10000	9187	-8%		9382	-6%		9254	-8%	
10000	9381	-6%		9565	-4%		9512	-5%	
10000	9539	-5%	2%	9112	-9%	2%	9849	-2%	5%
Linear Regression (ax+b) with $1/x^2$ Weighting									
	Curve 1	Curve 2	Curve 3	Curve 1	Curve 2	Curve 3	Curve 1	Curve 2	Curve 3
Linear coefficient	0.008	0.008	0.008	0.008	0.008	0.008	0.009	0.009	0.008
Intercept	-0.0002	-0.005	-0.002	0.001	-0.008	-0.002	-0.003	-0.008	-0.003
R^2	0.996	0.997	0.997	0.996	0.998	0.996	0.997	0.998	0.996

*Data based on average of duplicate replicates.

Carry Over

Carry over was examined by injecting triplicates of the high and low (LLOQ) standards, and a water blank following the high standard run (**Table II-3**). There was no carry over seen for any of the analytes.

Table II-3. Carry Over

Analyte	PRM Transitions	Retention Time	High STD Area	LLOQ Area	High STD-Blank Carry Over Area	% LLOQ of Blank
PTX	854.34 → 286.10 + 509.21	1.98	28663782	344864	0	0
PTX_C13	860.36 → 286.10 + 515.23	1.98	31484542	330949	0	0
PTX_d5	859.37 → 291.13 + 509.21	1.98	3886462	4397744	0	0

Intra-Day Precision and Accuracy

Over the intra-day period, the six replicate QC samples maintained a high degree of accuracy and precision (**Tables II-4 and II-6**). The low QC PTX standard at 10.0 ng/mL, had an average accuracy deviation of 2% and precision of 2%. The mid QC PTX standard, at 100 ng/mL, had an average accuracy deviation of 3% and precision of 4%. The high QC PTX standard, at 1000 ng/mL, had an average accuracy deviation of 6% and precision of 3%.

The low PTX_C13 QC standard, at 10.0 ng/mL had an average accuracy deviation of 1% and precision of 2%. The mid QC PTX_C13 standard, at 100 ng/mL, had an average accuracy deviation of 1% and precision of 4%. The high QC PTX_C13 standard at, 1000 ng/mL, had an average accuracy deviation of 7% and precision of 2%.

Table II-4. Intra-Day Precision and Accuracy. Precision and accuracy deviation data are presented for the intra-day six replicate samples of the PTX and PTX_C13 analytes by QC levels, 10.0 ng/mL (low), 100 ng/mL (mid), 1000 ng/mL (high). **(A)** Low QC PTX, **(B)** Mid QC PTX, **(C)** High QC PTX, **(D)** Low QC PTX_C13, **(E)** Mid QC PTX_C13, **(F)** High QC PTX_C13.

A. QC Low PTX

QC Low PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	10.0	10.1	1%	
Replicate 2	10.0	9.9	-1%	
Replicate 3	10.0	10.2	2%	
Replicate 4	10.0	10.3	3%	
Replicate 5	10.0	10.5	5%	
Replicate 6	10.0	10.2	2%	
AVG		**10.2**	**2%**	**2%**

B. QC Mid PTX

QC Mid PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	100	107	7%	
Replicate 2	100	100	0.04%	
Replicate 3	100	108	8%	
Replicate 4	100	102	2%	
Replicate 5	100	100	-0.3%	
Replicate 6	100	100	-0.02%	
AVG		**103**	**3%**	**4%**

C. QC High PTX

QC High PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	1000	1053	5%	
Replicate 2	1000	1056	6%	
Replicate 3	1000	1007	1%	
Replicate 4	1000	1041	4%	
Replicate 5	1000	1083	8%	
Replicate 6	1000	1094	9%	
AVG		**1055**	**6%**	**3%**

D. QC Low PTX_C13

QC Low PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	10.0	9.9	-1%	
Replicate 2	10.0	10.2	2%	
Replicate 3	10.0	10.4	4%	
Replicate 4	10.0	10.1	1%	
Replicate 5	10.0	9.7	-3%	
Replicate 6	10.0	10.1	1%	
AVG		**10.1**	**1%**	**2%**

E. QC Mid PTX_C13

QC Mid PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	100	105	5%	
Replicate 2	100	101	1%	
Replicate 3	100	106	6%	
Replicate 4	100	101	1%	
Replicate 5	100	96	-4%	
Replicate 6	100	97	-3%	
AVG		**101**	**1%**	**4%**

F. QC High PTX_C13

QC High PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	1000	1044	4%	
Replicate 2	1000	1053	5%	
Replicate 3	1000	1068	7%	
Replicate 4	1000	1095	9%	
Replicate 5	1000	1072	7%	
Replicate 6	1000	1074	7%	
AVG		**1067**	**7%**	**2%**

Inter-Day Precision and Accuracy

Over the inter-day period, QC samples maintained a high degree of accuracy and precision (**Tables II-5 and II-6**). The low QC PTX standard, at 10.0 ng/mL, had an average accuracy deviation of 1% and precision of 3%. The mid QC PTX standard, at 100 ng/mL, had an average accuracy deviation of 3% and precision of 2%. The high QC PTX standard at, 1000 ng/mL, had an average accuracy deviation of 5% and precision of 1%.

The low PTX_C13 QC standard, at 10.0 ng/mL, had an average accuracy deviation of 2% and precision of 2%. The mid QC PTX_C13 standard, at 100 ng/mL, had an average accuracy deviation of 1% and precision of 2%. The high QC PTX_C13 standard, at 1000 ng/mL, had an average accuracy deviation of 5% and precision of 2%.

Table II-5. Inter-Day Precision and Accuracy. Precision and accuracy deviation data are presented for the inter-day PTX and PTX_C13 analysis by QC levels, 10.0 ng/mL (low), 100 ng/mL (mid), and 1000 ng/mL (high), on three separate days. Data for each study day represents an average of six replicate estimations of each QC level on that study day. **(A)** QC Low PTX, **(B)** QC Mid PTX, **(C)** QC High PTX, **(D)** QC Low PTX_C13, **(E)** QC Mid PTX_C13, **(F)** QC High PTX_C13. (See Appendix A for inter-day individual data).

A. QC Low PTX

QC Low PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	10.0	10.3	3%	
Day 2	10.0	9.8	-2%	
Day 3	10.0	10.2	2%	
AVG		**10.1**	**1%**	**3%**

B. QC Low PTX

QC Mid PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	100	101	1%	
Day 2	100	105	5%	
Day 3	100	103	3%	
AVG		**103**	**3%**	**2%**

C. QC High PTX

QC High PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	1000	1054	5%	
Day 2	1000	1036	4%	
Day 3	1000	1055	6%	
AVG		**1048**	**5%**	**1%**

D. QC Low PTX_C13

QC Low PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	10.0	10.1	1%	
Day 2	10.0	10.5	5%	
Day 3	10.0	10.1	1%	
AVG		**10.2**	**2%**	**2%**

E. QC Mid PTX_C13

QC Mid PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	100	99	-1%	
Day 2	100	103	3%	
Day 3	100	101	1%	
AVG		**101**	**1%**	**2%**

F. QC High PTX_C13

QC High PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	1000	1051	5%	
Day 2	1000	1036	4%	
Day 3	1000	1067	7%	
AVG		**1052**	**5%**	**2%**

Table II-6. Intra- & Inter-Day Precision and Accuracy Summary. The summarized precision and accuracy deviation data are presented for the intra and inter-day validation study of PTX and PTX_C13 QC levels, 10.0 ng/mL (low), 100 ng/mL (mid), and 1000 ng/mL (high). Data for each QC level represents an average of all validation data presented previously in the report.

QC Standard	Intra-Day Summary		Inter-Day Summary	
	Accuracy Deviation (%)	Precision (%)	Accuracy Deviation (%)	Precision (%)
QC Low PTX	2%	2%	1%	3%
QC Mid PTX	3%	4%	3%	2%
QC High PTX	6%	3%	5%	1%
QC Low PTX_C13	1%	2%	2%	2%
QC Mid PTX_C13	1%	4%	1%	2%
QC High PTX_C13	7%	2%	5%	2%

Limit of Detection (LOD) and Limit of Quantitation (LOQ) Analysis

The LOQ and LOD was assessed by running six replicates of the LLOQ on three different days, for both PTX and PTX_C13, and determining the standard deviation of the concentration interpolated from the standard curve run on the corresponding day. The LOD was defined as 3 x SD, while LOQ was defined as limit of 10 x SD. The estimated LOD and LOQ for PTX were 2 and 6 ng/mL, respectively (**Table II-7A**). The estimated LOD and LOQ for PTX_C13 were 2 and 5 ng/mL, respectively (**Table II-7B**).

Table II-7. LOD and LOQ Determination.

A. PTX

Human Plasma Samples Spiked at the LLOQ	Day 1	Day 2	Day 3
	Concentration (ng/mL)		
Replicate 1	10.2	10.5	10.1
Replicate 2	10.7	8.4	9.9
Replicate 3	11.0	10.4	10.2
Replicate 4	10.7	10.4	10.3
Replicate 5	9.1	9.6	10.5
Replicate 6	9.9	9.5	10.2
Mean[a]	10.1		
SD[b]	0.6		
% RSD[c]	6.4		
LOD[d]	1.9		
LOQ[e]	6.4		

B. PTX_C13

Human Plasma Samples Spiked at the LLOQ	Day 1	Day 2	Day 3
	Concentration (ng/mL)		
Replicate 1	9.7	10.5	9.9
Replicate 2	10.7	11.4	10.2
Replicate 3	10.6	10.5	10.4
Replicate 4	10.3	10.6	10.1
Replicate 5	9.6	10.0	9.7
Replicate 6	9.6	9.8	10.1
Mean[a]	10.2		
SD[b]	0.5		
% RSD[c]	4.5		
LOD[d]	1.4		
LOQ[e]	4.6		

[a] Lower limit of quantitation (LLOQ) = 10.0 ng/mL
[b] SD - standard deviation
[c] %RSD = percent relative standard deviation
[d] LOD = limit of detection (3 x SD)
[e] LOQ = limit of quantitation (10 x SD)

Analytical System Reproducibility

Analytical system reproducibility was explored by determining the peak area and retention time precision of six replicate samples of the three plasma extracted analyte standards, at the 100 ng/mL QC level on three separate days (**Table II-8**). The runs met the acceptance criterion established for retention time and peak area at <15% RSD for all analytes.

Table II-8. Analytical System Suitability.

A. Day 1

	PTX		PTX_C13		PTX_d5	
	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)
Replicate 1	3048048	2.03	3476562	2.03	4498178	2.02
Replicate 2	2937137	2.03	3343261	2.02	4264656	2.02
Replicate 3	3650739	2.03	4062415	2.03	5216861	2.02
Replicate 4	2556281	2.03	2838301	2.02	3679329	2.02
Replicate 5	3472130	2.03	3531001	2.02	4886397	2.02
Replicate 6	3525003	2.03	3713776	2.03	4808307	2.02
AVG	3198223	2.03	3494219	2.03	4558955	2.02
SD	421756	0.003	406382	0.003	541532	0.00
%RSD	13	0.1	12	0.1	12	0.00

B. Day 2

	PTX		PTX_C13		PTX_d5	
	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)
Replicate 1	4421102	2.02	4831830	2.03	5345143	2.02
Replicate 2	4112428	2.02	4492774	2.02	5384641	2.02
Replicate 3	4671012	2.02	4444244	2.03	5643645	2.02
Replicate 4	4914648	2.03	4530479	2.03	5667398	2.02
Replicate 5	4667063	2.03	5177737	2.03	5699630	2.03
Replicate 6	4856105	2.03	5230955	2.03	6102799	2.03
AVG	4607060	2.03	4784670	2.03	5640543	2.02
SD	297847	0.003	352591	0.002	272268	0.003
%RSD	6	0.2	7	0.1	5	0.2

C. Day 3

	PTX		PTX_C13		PTX_d5	
	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)
Replicate 1	4376071	1.98	4539978	1.98	5061232	1.98
Replicate 2	4694649	1.98	4990985	1.98	5816126	1.97
Replicate 3	4189039	1.98	4173370	1.98	4660401	1.97
Replicate 4	4286676	1.98	4305113	1.98	5011412	1.98
Replicate 5	4153190	1.98	3734438	1.98	4637615	1.98
Replicate 6	3785837	1.98	3405370	1.98	4416274	1.98
AVG	4247577	1.98	4191542	1.98	4933843	1.98
SD	297809	0.002	565819	0.002	496199	0.01
%RSD	7	0.1	13	0.1	10	0.3

D. Overall

	PTX		PTX_C13		PTX_d5	
	Peak Area	**Retention Time**	**Peak Area**	**Retention Time**	**Peak Area**	**Retention Time**
Overall Mean	4017620	2.01	4156811	2.01	5044447	2.01
Overall SD	339137	0.003	441597	0.002	436666	0.003
Overall %RSD	**8**	**0.1**	**11**	**0.1**	**9**	**0.1**

Absolute Recovery

Absolute recovery was evaluated by comparing the spectrogram areas of the seven calibration standards of the plasma extracted analytes PTX and PTX_C13 to their solvent standards, at each calibration level (**Table II-9**). The calculated absolute recovery was between 45-91% for both analytes.

Table II-9. Absolute Recovery. Data for each calibration level represents an average peak area of two replicates ± standard deviation.

A. PTX

	Extracted Mobile Phase Area	**Plasma Area**	**% Extraction Efficiency**
10.0 ng/mL	451935 ± 84805	237697 ± 5753	53
50 ng/mL	2337974 ± 841921	1041188 ± 24981	45
100 ng/mL	3892671 ± 121048	2050706 ± 7176	53
500 ng/mL	17529886 ± 2340659	10953979 ± 949277	62
1000 ng/mL	33543551 ± 3477658	22573040 ± 2064197	67
5000 ng/mL	132488852 ± 8465264	92549929 ± 4057676	70
10000 ng/mL	191228289 ± 15489279	158467369 ± 1369746	83

B. PTX_C13

	Mobile Phase Area	**Plasma Area**	**% Extraction Efficiency**
10.0 ng/mL	416951 ± 62378	251751 ± 3152	60
50 ng/mL	2396149 ± 842445	1149918 ± 323	48
100 ng/mL	4043680 ± 34836	2270806 ± 16504	56
500 ng/mL	18426392 ± 3190873	12697196 ± 1189211	69
1000 ng/mL	35707808 ± 6754811	26670826 ± 3431185	75
5000 ng/mL	140045580 ± 10162565	109624409 ± 1896140	78
10000 ng/mL	201049365 ± 13284806	183211517 ± 10634734	91

Full Validation in Human Protein-Free Plasma

Summary

The purpose of this study was to validate the LC-Orbitrap quantitation method for PTX and PTX_C13 in human protein-free plasma matrix at a standard curve range of 0.5-500 ng/mL. For specifics of the LC-Orbitrap method please refer to Section I. This analytical method was validated for extraction efficiency in human protein-free plasma, and quality control precision and accuracy based on general FDA guidelines for bioanalytical methods [4].

Calibration standards were prepared by adding PTX and PTX_ C13 analytical standards to blank human protein-free plasma in the range from 0.5-500 ng/mL, to arrive at seven calibration levels. Matrix interference was not observed for the analytes or internal standard. Complete sets of the combined PTX and PTX_C13 calibration standards were run on three different days.

The PTX and PTX_13C calibration curves, analyte area/internal standard area vs. standard concentration, were suitable for linear regression with $1/x^2$ weighting, resulting in correlation coefficients of 0.99 or better. The accuracy deviation of the regression-calculated standard value from the true value was 15% or less for all calibration levels. The lower limit of quantitation (LLOQ) was established at 0.5 ng/mL. The method precision at the LLOQ was 3% relative standard deviation (% RSD) for PTX and 8% RSD for PTX_C13.

No carry over from the high calibration standard was seen for any of the analytes or internal standard. Analytical system suitability, evaluated by determining the peak area and retention time precision of six replicate samples at the 5 ng/mL concentration level for the protein-free plasma extracted PTX and PTX_C13 analytes, met the acceptance criterion established for retention time and peak area at ±15% RSD for all analytes. The estimated LOD and LOQ for PTX were 0.10 and 0.32 ng/mL, respectively. The estimated LOD and LOQ for PTX_C13 were 0.16 and 0.53 ng/mL, respectively. For intra-day validation, six individual QC samples were run at each QC level, 0.5 (low), 5 (mid) and 50 (high) ng/mL for both analytes. These samples were distributed throughout the beginning, middle and end of the run. For the inter-day validation, six individual QC samples were run at each level on three separate days. The low, mid and high QC standard's intra- and inter-day accuracy deviation and precision, for both PTX and PTX_C13, met the acceptance criteria of accuracy deviation not exceeding 15% from the true value, except at LLOQ which does not exceed 20%, and precision not exceeding 15%. The calculated absolute recovery for protein-free plasma extraction was between 89-131% for all analytes, at all calibration levels.

This validation did not attempt to assess either short-term room temperature stability, freeze-thaw stability of samples, or accuracy and precision of diluted samples.

In conclusion, this LC-Orbitrap analytical method for determination of PTX and PTX_C13 in human protein-free plasma was successfully developed and validated.

Results & Conclusions

Analytical System Suitability

Calibration curves, peak area ratio vs. concentration, were linear over the concentration range, from 0.5-500 ng/mL, for PTX and PTX_C13 (**Figures II-4 and II-5**). Complete sets of seven combined PTX and PTX_C13 calibration levels were run on three different days. The PTX and PTX_C13 curves, calibration area/internal standard area vs. standard concentration, were suitable for linear regression with $1/x^2$ weighting, resulting in correlation coefficients of 0.99 or better. The percent accuracy deviation of the regression-calculated value from the true value was 15% or less, for all calibration levels, except LLOQ which was 20% or less (**Tables II-10 and II-11**). The lower limit of quantitation (LLOQ) was established at 0.5 ng/mL.

PTX
Y = -0.000724704+0.00937717*X   R^2 = 0.9916   W: 1/X^2
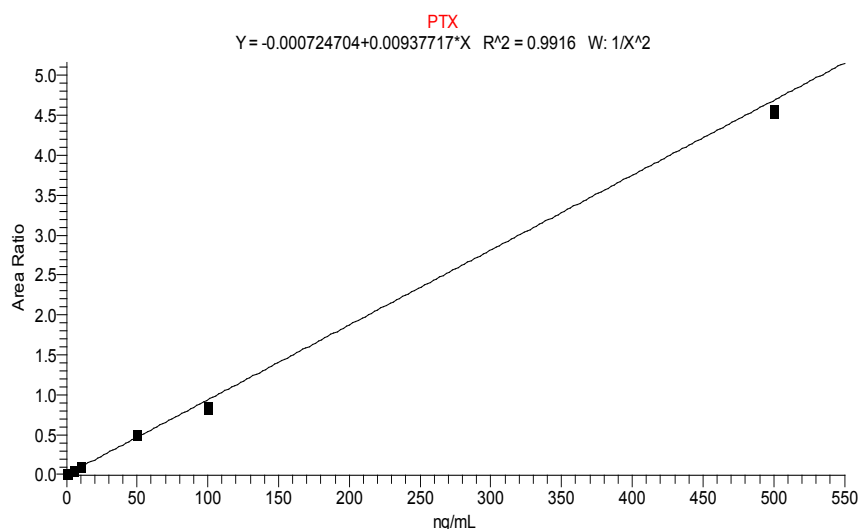
Figure II-4. Example of PTX Calibration Curve in Human Protein-Free Plasma.

PTX_C13
Y = -0.00241342+0.00911564*X   R^2 = 0.9942   W: 1/X^2
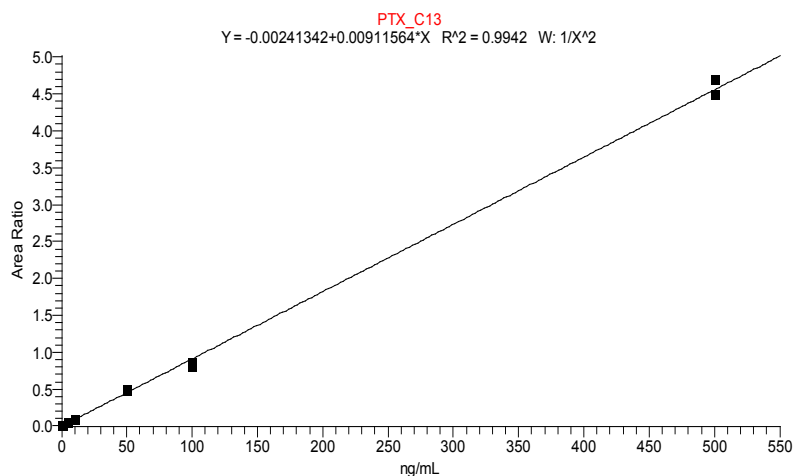
Figure II-5. Example of PTX_C13 Calibration Curve in Human Protein-Free Plasma.

Table II-10. PTX Calibration Runs in Human Protein-Free Plasma.

Standard Conc (ng/mL)	PTX Calibration 1			PTX Calibration 2			PTX Calibration 3		
	Experimental Conc (ng/mL) *	% Accuracy Deviation	% Precision	Experimental Conc (ng/mL) *	% Accuracy Deviation	% Precision	Experimental Conc (ng/mL) *	% Accuracy Deviation	% Precision
0.50	0.48	-5%		0.51	2%		0.51	3%	
0.50	0.51	1%		0.50	-1%		0.49	-1%	
0.50	0.48	-3%	12%	0.48	-5%	3%	0.48	-4%	6%
1.00	1.10	10%		0.94	-6%		0.92	-8%	
1.00	0.96	-4%		1.00	0.3%		1.01	1%	
1.00	1.05	5%	4%	1.08	8%	-0.2%	1.06	6%	4%
5.00	5.03	1%		5.36	7%		5.60	12%	
5.00	5.35	7%		5.47	10%		5.38	8%	
5.00	5.42	8%	1%	5.41	8%	3%	5.55	11%	2%
10.0	9.7	-3%		10.5	5%		10.1	1%	
10.0	10.1	1%		10.0	-0.1%		10.1	1%	
10.0	9.7	-3%	4%	9.9	-1%	3%	10.4	4%	3%
50.0	49.2	-2%		49.8	-1%		52.6	5%	
50.0	50.0	1%		50.2	0.4%		50.2	1%	
50.0	51.2	3%	1%	49.7	-1%	1%	49.0	-2%	2%
100.0	104.7	5%		98.0	-2%		89.7	-10%	
100.0	94.2	-6%		90.4	-10%		91.6	-8%	
100.0	93.5	-7%	4%	92.7	-7%	-0.2%	90.1	-10%	1%
500.0	471.9	-6%		470.1	-6%		486.3	-3%	
500.0	499.0	-0.2%		502.8	1%		498.7	-0.3%	
500.0	485.1	-3%	2%	486.4	-3%	-0.1%	475.4	-5%	2%
Linear Regression (ax+b) with $1/x^2$ Weighting									
	Curve 1	Curve 2	Curve 3	Curve 1	Curve 2	Curve 3	Curve 1	Curve 2	Curve 3
Linear coefficient	0.010	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.010
Intercept	-0.0009	0.001	-0.0006	-0.0001	-0.0005	-0.0005	-0.0007	0.0005	-0.0008
R^2	0.995	0.995	0.994	0.996	0.996	0.995	0.992	0.996	0.993

*Data based on average of duplicate replicates.

Table II-11. PTX_C13 Calibration Runs in Human Protein-Free Plasma.

Standard Conc (ng/mL)	PTX_C13 Calibration 1			PTX_C13 Calibration 2			PTX_C13 Calibration 3		
	Experimental Conc (ng/mL) *	% Accuracy Deviation	% Precision	Experimental Conc (ng/mL) *	% Accuracy Deviation	% Precision	Experimental Conc (ng/mL) *	% Accuracy Deviation	% Precision
0.50	0.50	-1%		0.51	2%		0.51	1%	
0.50	0.50	-0.4%		0.52	4%		0.52	4%	
0.50	0.50	-0.4%	14%	0.51	2%	6%	0.50	-1%	7%
1.00	1.00	-0.1%		0.98	-2%		0.97	-3%	
1.00	1.00	0.04%		0.92	-8%		0.91	-9%	
1.00	1.00	-1%	13%	0.96	-4%	6%	1.02	2%	5%
5.00	5.40	8%		4.73	-5%		5.22	4%	
5.00	5.26	5%		5.09	2%		5.21	4%	
5.00	5.51	10%	2%	5.13	3%	4%	5.04	1%	2%
10.0	9.4	-6%		9.4	-6%		9.8	-2%	
10.0	9.7	-3%		9.7	-3%		9.4	-6%	
10.0	9.2	-8%	1%	9.6	-4%	3%	9.8	-2%	3%
50.0	52.1	4%		51.8	4%		53.5	7%	
50.0	50.3	1%		51.4	3%		53.2	6%	
50.0	50.9	2%	2%	52.9	6%	2%	52.2	4%	2%
100.0	91.2	-9%		106.6	7%		91.4	-10%	
100.0	96.0	-4%		101.5	2%		101.7	2%	
100.0	94.3	-6%	2%	95.4	-5%	2%	95.2	-5%	2%
500.0	515.9	3%		505.6	1%		504.2	1%	
500.0	507.2	1%		504.5	1%		493.8	-1%	
500.0	512.1	2%	4%	510.4	2%	3%	505.6	1%	3%
Linear Regression (ax+b) with $1/x^2$ Weighting									
	Curve 1	Curve 2	Curve 3						
Linear coefficient	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.010
Intercept	-0.003	-0.001	-0.002	-0.002	-0.003	-0.002	-0.002	-0.002	-0.003
R^2	0.991	0.994	0.992	0.993	0.997	0.997	0.994	0.996	0.998

*Data based on average of duplicate replicates.

Carry Over

Carry over was examined by injecting triplicates of the high and low (LLOQ) standards and a water blank following the high standard run (**Table II-12**). There was no carry over seen for any of the analytes.

Table II-12. Carry Over.

	PRM	Retention Time	High STD Area	LLOQ Area	High STD-Blank Carry Over Area	% LLOQ of Blank
PTX	854.34 → 286.10 + 509.21	1.98	4460028	40148	0	0
PTX_C13	860.36 → 286.10 + 515.23	1.98	4211740	22543	0	0
PTX_d5	859.37 → 291.13 + 509.21	1.98	9352384	8432526	0	0

Intra-Day Precision and Accuracy

Over the intra-day period, the six replicate QC samples maintained a high degree of accuracy and precision (**Tables II-13 and II-15**). The low QC PTX standard at 0.5 ng/mL, had an average accuracy deviation of 8% and precision of 3%. The mid QC PTX standard, at 5 ng/mL, had an average accuracy deviation of 8% and precision of 2%. The high QC PTX standard, at 50 ng/mL, had an average accuracy deviation of 5% and precision of 3%.

The low PTX_C13 QC standard, at 0.5g/mL had an average accuracy deviation of 5% and precision of 8%. The mid QC PTX_C13 standard, at 5 ng/mL, had an average accuracy deviation of 4% and precision of 5%. The high QC PTX_C13 standard at, 50 ng/mL, had an average accuracy deviation of 3% and precision of 2%.

Table II-13. Intra-Day Precision and Accuracy. Precision and accuracy deviation data are presented for the intra-day six replicate samples of the PTX and PTX_C13 analytes by QC levels, 0.5 ng/mL (low), 5 ng/mL (mid), 50 ng/mL (high). **(A)** QC Low PTX, **(B)** QC Mid PTX, **(C)** QC High PTX, **(D)** QC Low PTX_C13, **(E)** QC Mid PTX_C13, **(F)** QC High PTX_C13.

A. QC Low PTX

QC Low PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	0.50	0.55	9%	
Replicate 2	0.50	0.55	9%	
Replicate 3	0.50	0.52	3%	
Replicate 4	0.50	0.55	11%	
Replicate 5	0.50	0.54	9%	
Replicate 6	0.50	0.55	10%	
AVG		**0.54**	**8%**	**3%**

B. QC Mid PTX

QC Mid PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	5.00	5.34	7%	
Replicate 2	5.00	5.37	7%	
Replicate 3	5.00	5.41	8%	
Replicate 4	5.00	5.23	5%	
Replicate 5	5.00	5.38	8%	
Replicate 6	5.00	5.63	13%	
AVG		**5.39**	**8%**	**2%**

C. QC High PTX

QC High PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	50.0	52.8	6%	
Replicate 2	50.0	51.1	2%	
Replicate 3	50.0	53.8	8%	
Replicate 4	50.0	54.7	9%	
Replicate 5	50.0	50.1	0.2%	
Replicate 6	50.0	51.4	3%	
AVG		**52.3**	**5%**	**3%**

D. QC Low PTX_C13

QC Low PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	0.50	0.46	-9%	
Replicate 2	0.50	0.57	14%	
Replicate 3	0.50	0.56	12%	
Replicate 4	0.50	0.55	9%	
Replicate 5	0.50	0.49	-1%	
Replicate 6	0.50	0.51	3%	
AVG		**0.52**	**5%**	**8%**

E. QC Mid PTX_C13

QC Mid PTX_C13	Theoretical (ng/mL)	Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	5.00	5.30	6%	
Replicate 2	5.00	5.42	8%	
Replicate 3	5.00	5.09	2%	
Replicate 4	5.00	5.48	10%	
Replicate 5	5.00	4.97	-1%	
Replicate 6	5.00	4.88	-2%	
AVG		**5.19**	**4%**	**5%**

F. QC High PTX_C13

QC High PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	50.0	51.4	3%	
Replicate 2	50.0	53.8	8%	
Replicate 3	50.0	51.9	4%	
Replicate 4	50.0	51.0	2%	
Replicate 5	50.0	51.0	2%	
Replicate 6	50.0	50.2	0.3%	
AVG		**51.5**	**3%**	**2%**

Inter-Day Precision and Accuracy

Over the inter-day period, QC samples maintained a high degree of accuracy and precision (**Tables II-14 and II-15**). The low QC PTX standard, at 0.5 ng/mL, had an average accuracy deviation of 5% and precision of 3%. The mid QC PTX standard, at 5 ng/mL, had an average accuracy deviation of 7% and precision of 1%. The high QC PTX standard at, 50 ng/mL, had an average accuracy deviation of 2% and precision of 2%.

The low PTX_C13 QC standard, at 0.5 ng/mL, had an average accuracy deviation of 3% and precision of 3%. The mid QC PTX_C13 standard, at 5 ng/mL, had an average accuracy deviation of 4% and precision of 1%. The high QC PTX_C13 standard, at 50 ng/mL, had an average accuracy deviation of 3% and precision of 1%.

Table II-14. Inter-Day Precision and Accuracy. Precision and accuracy deviation data are presented for the inter-day PTX and PTX_C13 analysis by QC levels, 0.5 ng/mL (low), 5 ng/mL (mid), and 50 ng/mL (high), on three separate days. Data for each study day represents an average of six replicate estimations of each QC level on that study day. **(A)** QC Low PTX, **(B)** QC Mid PTX, **(C)** QC High PTX, **(D)** QC Low PTX_C13, **(E)** QC Mid PTX_C13, **(F)** QC High PTX_C13. (See Appendix B for inter-day individual data).

A. QC Low PTX

QC Low PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	0.50	0.51	2%	
Day 2	0.50	0.52	4%	
Day 3	0.50	0.54	8%	
AVG		**0.52**	**5%**	**3%**

B. QC Mid PTX

QC Mid PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	5.00	5.33	7%	
Day 2	5.00	5.30	6%	
Day 3	5.00	5.39	8%	
AVG		**5.34**	**7%**	**1%**

C. QC High PTX

QC High PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	50	49.9	-0.3%	
Day 2	50	50.7	1%	
Day 3	50	52.3	5%	
AVG		**51.0**	**2%**	**2%**

D. QC Low PTX_C13

QC Low PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	0.50	0.52	5%	
Day 2	0.50	0.50	0%	
Day 3	0.50	0.52	5%	
AVG		**0.52**	**3%**	**3%**

E. QC Mid PTX_C13

QC Mid PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	5.00	5.29	6%	
Day 2	5.00	5.18	4%	
Day 3	5.00	5.19	4%	
AVG		**5.22**	**4%**	**1%**

F. QC High PTX_C13

QC High PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Day 1	50	51.0	2%	
Day 2	50	51.5	3%	
Day 3	50	51.5	3%	
AVG		**51.3**	**3%**	**1%**

Table II-15. Intra- & Inter-Day Precision and Accuracy Summary. The summarized precision and accuracy deviation data are presented for the intra and inter-day validation study of PTX and PTX_C13 QC levels, 0.5 ng/mL (low), 5 ng/mL (mid), and 50 ng/mL (high). Data for each QC level represents an average of all validation data presented previously in the report.

PFP QC Standard	Intra-Day		Inter-Day	
	Accuracy Deviation (%)	Precision (%)	Accuracy Deviation (%)	Precision (%)
QC Low PTX	8%	3%	5%	3%
QC Mid PTX	8%	2%	7%	1%
QC High PTX	5%	3%	2%	2%
QC Low PTX_C13	5%	8%	3%	3%
QC Mid PTX_C13	4%	5%	4%	1%
QC High PTX_C13	3%	2%	3%	1%

Limit of Detection (LOD) and Limit of Quantitation (LOQ) Analysis

The LOQ and LOD was assessed by running six replicates of the LLOQ on three different days, for both PTX and PTX_C13, and determining the standard deviation of the concentration interpolated from the standard curve run on the corresponding day. The LOD was defined as 3 x SD, while LOQ was defined as limit of 10 x SD. The estimated LOD and LOQ for PTX were 0.10 and 0.32 ng/mL, respectively (**Table II-16A**). The estimated LOD and LOQ for PTX_C13 were 0.16 and 0.53 ng/mL, respectively (**Table II-16B**).

Table II-16. LOD and LOQ Determination.

A. PTX

Human Protein-Free Plasma Samples Spiked at the LLOQ	Day 1	Day 2	Day 3
	Concentration ng/mL		
Replicate 1	0.51	0.50	0.55
Replicate 2	0.55	0.54	0.55
Replicate 3	0.45	0.49	0.52
Replicate 4	0.56	0.55	0.55
Replicate 5	0.52	0.51	0.54
Replicate 6	0.47	0.53	0.55
Mean[a]	0.52		
SD[b]	0.03		
% RSD[c]	6.14		
LOD[d]	0.10		
LOQ[e]	0.32		

B. PTX_C13

Human Plasma Samples Spiked at the LLOQ	Day 1	Day 2	Day 3
	Concentration ng/mL		
Replicate 1	0.54	0.57	0.46
Replicate 2	0.58	0.43	0.57
Replicate 3	0.50	0.49	0.56
Replicate 4	0.40	0.52	0.55
Replicate 5	0.57	0.45	0.49
Replicate 6	0.54	0.53	0.51
Mean[a]	0.52		
SD[b]	0.05		
% RSD[c]	10.22		
LOD[d]	0.16		
LOQ[e]	0.53		

[a] Lower limit of quantitation (LLOQ) = 0.5 ng/mL
[b] SD - standard deviation
[c] %RSD = percent relative standard deviation
[d] LOD = limit of detection (3 x SD)
[e] LOQ = limit of quantitation (10 x SD)

Analytical System Reproducibility

Analytical system reproducibility was evaluated by determining the peak area and retention time precision of six replicate samples of the three plasma extracted analyte standards at the 5 ng/mL QC level on three separate days (**Table II-17**). The runs met the acceptance criterion established for retention time and peak area at ≤15% RSD for all analytes.

Table II-17. Analytical System Suitability

A. Day 1

	PTX		PTX_C13		PTX_d5	
	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)
Replicate 1	487602	2.03	439335	2.03	9909342	2.02
Replicate 2	535666	2.03	486616	2.03	10877424	2.02
Replicate 3	497128	2.03	433727	2.03	9571327	2.02
Replicate 4	535521	2.03	487114	2.03	10567174	2.03
Replicate 5	443971	2.03	443971	2.03	9462576	2.03
Replicate 6	447075	2.03	447075	2.03	10052433	2.02
AVG	491160	2.03	456307	2.03	10073379	2.02
SD	40416	0.002	24096	0.002	555483	0.01
%RSD	8	0.1	5	0.1	6	0.3

B. Day 2

	PTX		PTX_C13		PTX_d5	
	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)
Replicate 1	464330	1.98	376119	1.98	8959828	1.97
Replicate 2	457548	1.98	396785	1.98	9095200	1.97
Replicate 3	430683	1.98	385917	1.98	8873954	1.98
Replicate 4	432546	1.98	423892	1.98	8903149	1.98
Replicate 5	391622	1.98	410704	1.98	8284973	1.97
Replicate 6	424626	1.98	436678	1.98	8884169	1.97
AVG	433559	1.98	405016	1.98	8833545	1.97
SD	25962	0.003	23059	0.002	280849	0.01
%RSD	6	0.1	6	0.1	3	0.3

C. Day 3

	PTX		PTX_C13		PTX_d5	
	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)
Replicate 1	447642	1.98	416765	1.98	9074524	1.97
Replicate 2	468349	1.98	443684	1.98	9444741	1.97
Replicate 3	429898	1.98	397316	1.98	8659032	1.97
Replicate 4	409134	1.98	421437	1.98	8509412	1.97
Replicate 5	444881	1.98	387847	1.98	8558074	1.97
Replicate 6	429922	1.98	350907	1.98	7903858	1.97
AVG	438304	1.98	402993	1.98	8691607	1.97
SD	20124	0.002	32125	0.002	526588	0.00
%RSD	5	0.1	8	0.1	6	0.00

D. Overall

	PTX		PTX_C13		PTX_d5	
	Peak Area	Retention Time	Peak Area	Retention Time	Peak Area	Retention Time
Overall Mean	454341	2.00	421438	2.00	9199510	1.99
Overall SD	28834	0.003	26427	0.002	454307	0.003
Overall %RSD	6	0.1	6	0.1	5	0.2

Absolute Recovery

Absolute recovery was explored by comparing the chromatogram areas of the seven calibration standards of the plasma extracted analytes PTX and PTX_C13 to their solvent standards, at each calibration level (**Table II-18**). The calculated absolute recovery was between 89-131% for both analytes.

Table II-18. Absolute Recovery. Data for each calibration level represents an average peak area of two replicates + standard deviation.

A. PTX

	Extracted Area	PFP Area	% Extraction Efficiency
0.50 ng/mL	27244 ± 3699	35530 ± 1701	130
1.00 ng/mL	69863 ± 3327	91534 ± 1933	131
5.00 ng/mL	396047 ± 44550	394863 ± 51645	100
10.0 ng/mL	779134 ± 133892	834195 ± 99359	107
50.0 ng/mL	4181065 ± 135415	4262130 ± 552211	102
100.0 ng/mL	7088938 ± 383139	6310855 ± 73472	89
500.0 ng/mL	36246489 ± 5616325	38801475 ± 5615136	107

B. PTX_C13

	Extracted Area	PFP Area	% Extraction Efficiency
0.50 ng/mL	13768 ± 1057	13030 ± 125	95
1.00 ng/mL	47832 ± 2850	62639 ± 256	131
5.00 ng/mL	366883 ± 51733	362926 ± 36280	99
10.0 ng/mL	723491 ± 140836	788289 ± 89133	109
50.0 ng/mL	4342255 ± 192937	4257807 ± 751320	98
100.0 ng/mL	4014640 ± 412787	6570744 ± 98303	94
500.0 ng/mL	37083237 ± 5225312	39180492 ± 5737863	106

Partial Validation in Rat Plasma and Protein-Free Plasma

Summary

The purpose of this study was to run a partial validation on the LC-Orbitrap quantitation method for PTX and PTX_C13 in rat plasma and rat protein-free matrices at standard curve ranges equivalent to those used for human plasma and human protein-free plasma. For specifics of the LC orbitrap method please refer to Section I. This partial validation consisted of a determination of intra-day precision and accuracy at three different QC levels, 10 (QCL), 100 (QCM), and 1000 (QCH) ng/mL for plasma and QC levels 0.5 (QCL), 5 (QCM), and 50 (QCH) ng/mL for protein-free plasma, and analytical reproducibility.

Calibration standards were prepared by adding PTX and PTX_C13 analytical standards to rat plasma in the range from 10-10000 ng/mL and to rat protein-free plasma in a range from 0.5-500 ng/mL, to arrive at seven calibration levels for each matrix. Matrix interference was not observed for the analytes or internal standard, and carry over from the high calibration standard was less than 5% of the low standard, 10ng/mL in plasma and 0.1 ng/mL in protein-free plasma.

The PTX and PTX_C13 calibration curves, analyte area/internal standard area vs. standard concentration, were suitable for linear regression with a $1/x^2$ weighting for both plasma and protein-free plasma, resulting in correlation coefficients of 0.99 or better. The accuracy deviation of the regression-calculated standard value from the true value was 15% or less for all calibration levels, exept the LLOQ at which it was 20% or less.

For intra-day validation, six individual QC samples were run at each QC level, 10 (QCL), 100 (QCM), and 1000 (QCH) ng/mL for plasma and QC levels 0.5 (QCL), 5 (QCM), and 50 (QCH) ng/mL for protein-free plasma. These samples were distributed throughout the beginning, middle, and end of the run. The low, mid and high QC standard's intra- and inter-day accuracy deviation/precision met the acceptance criteria of accuracy deviation not exceeding 15% from the true value, except at LLOQ where it does not exceed 20%, and precision not exceeding 15%, in both plasma and protein-free plasma. The runs met the acceptance criterion established for retention time and peak area at <15% RSD for both analytes and internal standard.

This validation did not attempt to assess inter-day precision and accuracy, either short-term room temperature stability or freeze-thaw stability of samples, or accuracy and precision of diluted samples.

In conclusion, this LC-Orbitrap analytical method for determination of PTX and PTX_C13 in rat plasma and protein-free plasma was successfully validated.

Results and Conclusions

Calibration Curves

Calibration curves, area ratio vs. concentration, were linear over the concentration range from 10-10,000 ng/mL for PTX and PTX_C13 in plasma (**Figures II-6 and II-7**). Calibration curves, area ratio vs. concentration, were linear over the concentration range from 0.5-500 ng/mL for PTX and PTX_C13 in protein-free plasma (**Figures II-8 and II-9**). Complete sets of seven calibration levels were run in duplicate. The PTX and PTX_C13 curves, calibration area/internal standard area vs. standard concentration, were suitable for linear regression with $1/x^2$ weighting for both plasma and protein-free plasma, resulting in correlation coefficients of 0.99 or better. The percent accuracy deviation of the regression-calculated value from the true value, and precision (%RSD) was 15% or less for all calibration levels, except at LLOQ where it was 20% or less (**Tables II-19 to II-22**).

Table II-19. PTX Calibration Standard Curve in Rat Plasma.

Standard Conc (ng/mL)	PTX Calibration		
	Experimental Conc (ng/mL) *	% Accuracy Deviation	% Precision
10.0	10.1	1%	
10.0	10.1	1%	
10.0	10.0	0.4%	10%
50	48	-3%	
50	49	-3%	
50	50	0.1%	2%
100	95	-5%	
100	98	-3%	
100	96	-4%	3%
500	497	-1%	
500	518	4%	
500	482	-4%	5%
1000	1025	3%	
1000	1010	1%	
1000	1040	4%	3%
5000	5116	2%	
5000	5064	1%	
5000	5138	3%	6%
10000	10269	3%	
10000	9855	-2%	
10000	10042	0.4%	8%
Linear Regression (ax+b) with $1/x^2$ Weighting			
Linear coefficient	0.008	0.010	0.008
Intercept	-0.02	-0.01	-0.01
R^2	0.996	0.996	0.997

*Data based on average of duplicate replicates.

PTX
Y = -0.0164351+0.00808277*X   R^2 = 0.9960   W: 1/X^2
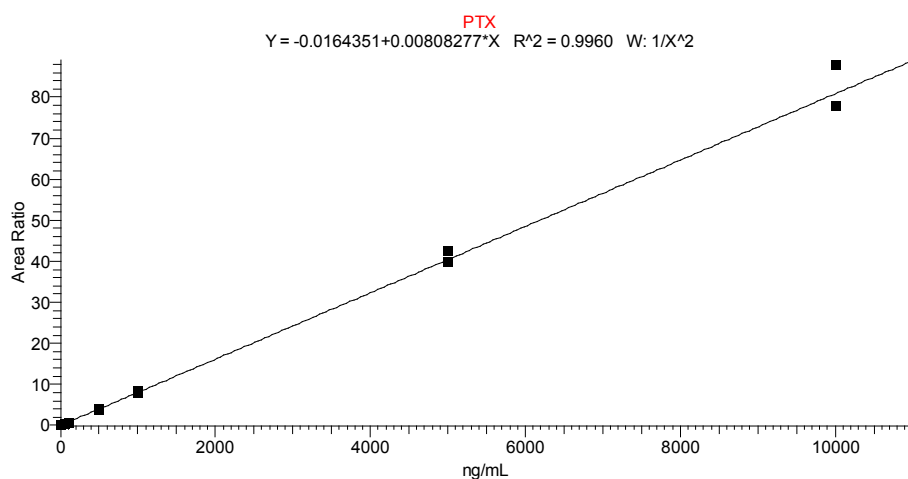
Figure II-6. PTX Calibration Curve in Rat Plasma.

Table II-20. PTX_C13 Calibration Standard Curve in Rat Plasma.

Standard Conc (ng/mL)	PTX_C13 Calibration		
	Experimental Conc (ng/mL) *	% Accuracy Deviation	% Precision
10.0	10.2	2%	
10.0	10.0	0.4%	
10.0	10.0	0.2%	5%
50	46	-8%	
50	50	-0.01%	
50	50	0.4%	2%
100	99	-1%	
100	96	-4%	
100	97	-3%	3%
500	491	-2%	
500	519	4%	
500	495	-1%	5%
1000	1038	4%	
1000	999	-0.1%	
1000	1051	5%	4%
5000	5155	3%	
5000	5051	1%	
5000	5086	2%	7%
10000	10207	2%	
10000	9930	-1%	
10000	9692	-3%	9%
Linear Regression (ax+b) with $1/x^2$ Weighting			
Linear coefficient	0.008	0.009	0.009
Intercept	-0.01	-0.01	-0.01
R^2	0.996	0.997	0.997

*Data based on average of duplicate replicates.

PTX_C13
Y = -0.0144817+0.00790549*X   R^2 = 0.9959   W: 1/X^2
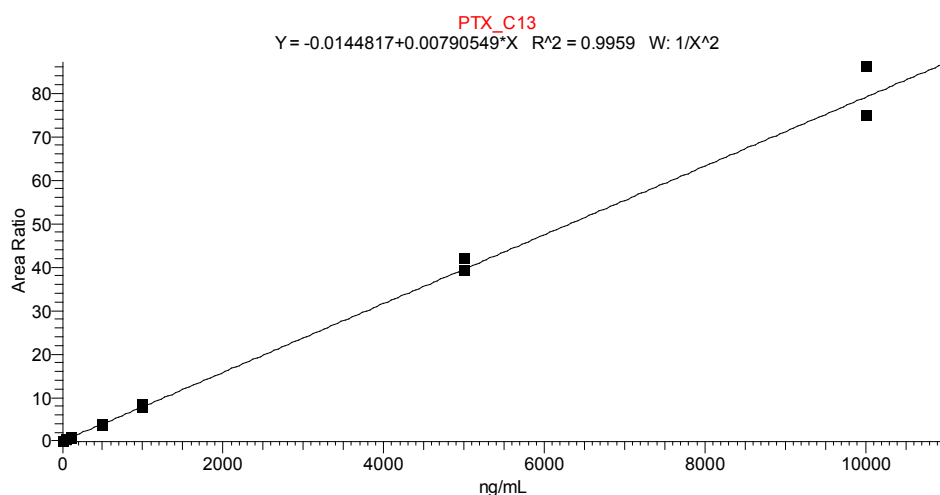
Figure II-7. PTX_C13 Calibration Curve in Rat Plasma.

Table II-21. PTX Calibration Standard Curve in Rat Protein-Free Plasma.

Standard Conc (ng/mL)	PTX Calibration		
	Experimental Conc (ng/mL) *	% Accuracy Deviation	% Precision
0.50	0.50	-0.02%	
0.50	0.50	0.3%	
0.50	0.48	-5%	8%
1.00	0.99	-1%	
1.00	0.98	-2%	
1.00	1.08	8%	5%
5.00	5.38	8%	
5.00	5.48	10%	
5.00	5.45	9%	3%
10.0	9.8	-3%	
10.0	10.0	-0.3%	
10.0	9.9	-1%	2%
50.0	50.7	1%	
50.0	51.2	2%	
50.0	49.8	-1%	2%
100.0	96.4	-4%	
100.0	92.5	-8%	
100.0	90.8	-9%	1%
500.0	491.5	-2%	
500.0	489.8	-2%	
500.0	493.9	-1%	1%
Linear Regression (ax+b) with $1/x^2$ Weighting			
Linear coefficient	0.010	0.010	0.008
Intercept	0.00001	0.0005	-0.01
R^2	0.997	0.994	0.997

*Data based on average of duplicate replicates.

PTX
$Y = 1.28182e\text{-}005 + 0.00974719 \cdot X$ $R^2 = 0.9972$ W: $1/X^2$
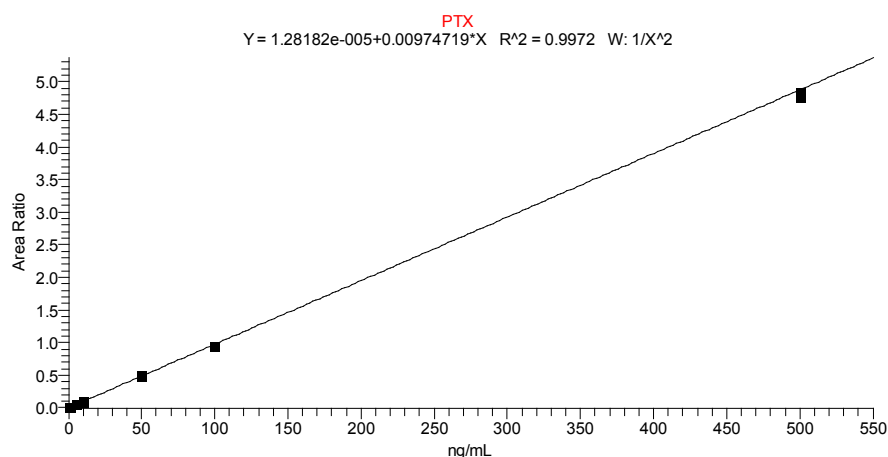
Figure II-8. PTX Calibration Curve in Rat Protein-Free Plasma.

Table II-22. PTX_C13 Calibration Curve in Rat Protein-Free-Plasma.

Standard Conc (ng/mL)	PTX_C13 Calibration		
	Experimental Conc (ng/mL)	% Accuracy Deviation	% Precision
0.50	0.48	-5%	
0.50	0.51	2%	
0.50	0.48	-4%	13%
1.00	1.10	10%	
1.00	0.97	-4%	
1.00	1.09	9%	2%
5.00	5.31	6%	
5.00	5.31	6%	
5.00	5.11	2%	3%
10.0	9.2	-8%	
10.0	9.0	-10%	
10.0	9.5	-5%	1%
50.0	50.4	1%	
50.0	51.9	4%	
50.0	49.1	-2%	3%
100.0	96.7	-3%	
100.0	98.8	-1%	
100.0	100.5	1%	3%
500.0	499.6	-0.1%	
500.0	516.3	3%	
500.0	497.9	-0.4%	1%
Linear Regression (ax+b) with $1/x^2$ Weighting			
Linear coefficient	0.010	0.009	0.010
Intercept	-0.003	-0.002	-0.003
R^2	0.993	0.991	0.996

*Data based on average of duplicate replicates.

PTX_C13
$Y = -0.00267788 + 0.0100695*X$ $R^2 = 0.9934$ W: $1/X^2$
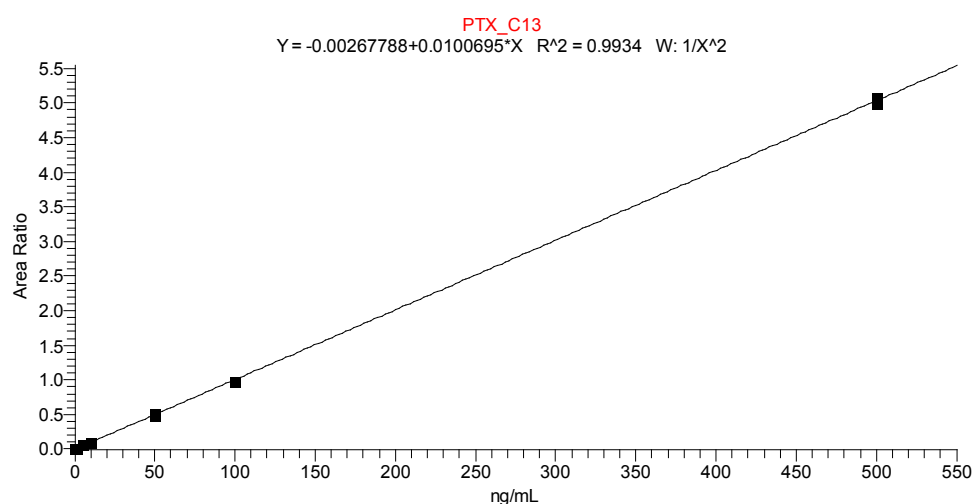
Figure II-9. PTX_C13 Calibration Curve in Rat Protein-Free Plasma.

Intra-Day Precision and Accuracy

Over the intra-day study period, the six replicate QC samples maintained a high degree of accuracy and precision in plasma (**Table II-23**). The low QC PTX standard, at 10 ng/mL, had an average accuracy deviation of 9% and precision of 3%. The mid QC PTX standard, at 100 ng/mL, had an average accuracy deviation of 4% and precision of 4%. The high QC PTX standard, at 1000 ng/mL, had an average accuracy deviation of 1% and precision of 6%. The low QC PTX_C13 standard, at 10 ng/mL, had an average accuracy deviation of 5% and precision of 5%. The mid QC PTX_C13 standard at 100 ng/mL, had an average accuracy deviation of 4% and precision of 4%. The high QC PTX_C13 standard, at 1000 ng/mL, had an average accuracy deviation of 3% and precision of 7%.

Over the intra-day study period, the six replicate QC samples also maintained a high degree of accuracy and precision in protein-free plasma (**Table II-24**). The low QC PTX standard, at 0.5 ng/mL, had an average accuracy deviation of 2% and precision of 8%. The mid QC PTX standard, at 5 ng/mL, had an average accuracy deviation of 6% and precision of 2%. The high QC PTX standard, at 50 ng/mL, had an average accuracy deviation of 2% and precision of 3%. The low QC PTX_C13 standard, at 0.5 ng/mL, had an average accuracy deviation of 8% and precision of 5%. The mid QC PTX_C13 standard, at 5 ng/mL, had an average accuracy of 5% and precision of 1%. The high QC PTX_C13 standard, at 50 ng/mL, had an average accuracy of 0.3% and precision of 4%.

Table II-23. Intra-Day Precision and Accuracy for PTX in Rat Plasma. Precision and accuracy deviation data are presented for the intra-day six replicate samples, QC levels,10 ng/mL (low), 100 ng/mL (mid), and 1000 ng/mL (high), for PTX and PTX_C13. **(A)** QC Low PTX, **(B)** QC Mid PTX, **(C)** QC High PTX, **(D)** QC Low PTX_C13, **(E)** QC Mid PTX_C13, **(F)** QC High PTX_C13.

A. QC Low PTX

QC Low PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	10.0	11.3	13%	
Replicate 2	10.0	10.7	7%	
Replicate 3	10.0	10.6	6%	
Replicate 4	10.0	11.0	10%	
Replicate 5	10.0	11.0	10%	
Replicate 6	10.0	10.5	5%	
AVG		10.9	9%	3%

B. QC Mid PTX

QC Mid PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	100	108	8%	
Replicate 2	100	99	-1%	
Replicate 3	100	107	7%	
Replicate 4	100	109	9%	
Replicate 5	100	100	-0.1%	
Replicate 6	100	102	2%	
AVG		104	4%	4%

C. QC High PTX

QC High PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	1000	1050	5%	
Replicate 2	1000	1038	4%	
Replicate 3	1000	996	-0.4%	
Replicate 4	1000	894	-11%	
Replicate 5	1000	1023	2%	
Replicate 6	1000	1048	5%	
AVG		1008	1%	6%

D. QC Low PTX_C13

QC Low PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	10.0	10.8	8%	
Replicate 2	10.0	10.2	2%	
Replicate 3	10.0	10.0	0.2%	
Replicate 4	10.0	10.2	2%	
Replicate 5	10.0	11.3	13%	
Replicate 6	10.0	10.2	2%	
AVG		10.5	5%	5%

E. QC Mid PTX_C13

QC Mid PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	100	105	5%	
Replicate 2	100	99	-1%	
Replicate 3	100	104	4%	
Replicate 4	100	112	12%	
Replicate 5	100	101	1%	
Replicate 6	100	102	2%	
AVG		104	4%	4%

F. QC High PTX_C13

QC High PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	1000	1081	8%	
Replicate 2	1000	1041	4%	
Replicate 3	1000	1024	2%	
Replicate 4	1000	883	-12%	
Replicate 5	1000	1099	10%	
Replicate 6	1000	1039	4%	
AVG		1028	3%	7%

Table II-24. Intra-Day Precision and Accuracy for PTX in Rat ProteinFree Plasma.
Precision and accuracy deviation data are presented for the intra-day six replicate samples QC levels, 0.5 ng/mL (low), 5 ng/mL (mid), and 50 ng/mL (high), for PTX and PTX_C13. **(A)** QC Low PTX, **(B)** QC Mid PTX, **(C)** QC High PTX, **(D)** QC Low PTX_C13, **(E)** QC Mid PTX_C13, **(F)** QC High PTX_C13.

A. QC Low PTX

QC Low PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	0.50	0.53	6%	
Replicate 2	0.50	0.53	7%	
Replicate 3	0.50	0.46	-8%	
Replicate 4	0.50	0.46	-8%	
Replicate 5	0.50	0.54	8%	
Replicate 6	0.50	0.55	10%	
AVG		0.51	2%	8%

B. QC Mid PTX

QC Mid PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	5.00	5.49	10%	
Replicate 2	5.00	5.15	3%	
Replicate 3	5.00	5.41	8%	
Replicate 4	5.00	5.26	5%	
Replicate 5	5.00	5.19	4%	
Replicate 6	5.00	5.27	5%	
AVG		5.29	6%	2%

C. QC High PTX

QC High PTX	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	50.0	50.8	2%	
Replicate 2	50.0	49.6	-1%	
Replicate 3	50.0	51.7	3%	
Replicate 4	50.0	53.0	6%	
Replicate 5	50.0	49.7	-1%	
Replicate 6	50.0	51.8	4%	
AVG		51.1	2%	3%

D. QC Low PTX_C13

QC Low PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	0.50	0.52	4%	
Replicate 2	0.50	0.51	3%	
Replicate 3	0.50	0.55	10%	
Replicate 4	0.50	0.53	5%	
Replicate 5	0.50	0.55	10%	
Replicate 6	0.50	0.58	17%	
AVG		0.54	8%	5%

E. QC Mid PTX_C13

QC Mid PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	5.00	5.23	5%	
Replicate 2	5.00	5.24	5%	
Replicate 3	5.00	5.30	6%	
Replicate 4	5.00	5.33	7%	
Replicate 5	5.00	5.34	7%	
Replicate 6	5.00	5.15	3%	
AVG		5.26	5%	1%

F. QC High PTX_C13

QC High PTX_C13	Theoretical Concentration (ng/mL)	Experimental Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Replicate 1	50.0	50.2	0.5%	
Replicate 2	50.0	47.7	-5%	
Replicate 3	50.0	50.0	-0.02%	
Replicate 4	50.0	53.7	7%	
Replicate 5	50.0	48.9	-2%	
Replicate 6	50.0	50.5	1%	
AVG		50.2	0.3%	4%

Analytical System Reproducibility

Analytical system reproducibility was evaluated by determining the peak area and retention time precision of six replicate samples at the 100 ng/mL QC level (**Table II-25**) for plasma and 5 ng/mL QC level (**Table II-26**) for protein-free plasma. The runs met the acceptance criterion established for retention time and peak area at <15% RSD for both the analytes, PTX and PTX_C13, and internal standard, PTX_d5 in both plasma and protein-free plasma.

Table II-25. Analytical System Suitability in Rat Plasma.

	PTX		PTX_C13 (Tracer)		PTX_d5 (ISTD)	
	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)
Replicate 1	6251545	1.98	7218211	1.98	8721635	1.98
Replicate 2	5704261	1.98	6446902	1.97	7979968	1.98
Replicate 3	6072776	1.98	6863400	1.98	7949763	1.98
Replicate 4	6504722	1.98	7701697	1.97	8769651	1.98
Replicate 5	6188345	1.98	7111003	1.97	8321324	1.98
Replicate 6	5965064	1.98	6791403	1.97	8017718	1.98
AVG	6114452	1.98	7022103	1.97	8293343	1.98
SD	271616	0.002	428363	0.002	375049	0.002
%RSD	4	0.1	6	0.1	5	0.1

Table II-26. Analytical System Suitability in Rat Protein-Free Plasma.

	PTX		PTX_C13 (Tracer)		PTX_d5 (ISTD)	
	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)	Peak Area	Retention Time (minutes)
Replicate 1	503345	1.98	469991	1.98	9410927	1.97
Replicate 2	477105	1.98	476459	1.99	9510791	1.98
Replicate 3	498104	1.98	446100	1.98	9398500	1.97
Replicate 4	473155	1.98	438555	1.98	9194349	1.98
Replicate 5	455237	1.98	452133	1.98	8868475	1.97
Replicate 6	476817	1.98	449821	1.99	9158052	1.98
AVG	480627	1.98	455510	1.98	9256849	1.98
SD	17601	0.002	14617	0.003	233578	0.003
%RSD	4	0.1	3	0.2	3	0.2

Control Study – Process and Spike Controls

Summary

A series of control studies were performed to evaluate assay accuracy and identify possible processing artifacts. A 200 ng/mL PTX spike into 1 µg/mL Abraxane or Genexol-PM containing plasma was recovered within 20% of theoretical (an encapsulated:unencapsulated ratio of 5). A 200 ng/mL PTX spike into 1, 5, o 20 µg/mL Taxol generic containing plasma was recovered within 20% of theoretical (an encapsulated:unencapsulated ratio of 5). Double processing and organic solvent stable isotope spike of Abraxane, Genexol-PM, or Taxol generic containing plasma samples did not alter formulation stability.

Design and Methods

A double spin control was performed to identify the potential for processing-induced drug release, which could inflate unencapsulated drug release estimates. A free paclitaxel spike control was performed to assess the accuracy of the unencapsulated drug estimation. Lastly, an organic spike control was included to ensure the organic solvent used to solubilize the PTX_C13 stable isotope tracer did not disrupt the formulation.

Double Spin Control

A plasma sample containing Abraxane, Genexol-PM, or Taxol generic underwent two successive filtrations, in order to exclude artificial sample processing induced drug release. Abraxane and Genexol-PM samples were spiked into human plasma at a concentration of 1 µg/mL; Taxol generic samples were spiked into human plasma at a concentration of 1,5, or 20 µg/mL, and PTX_C13 stable isotope tracer was added at a final concentration of 100 ng/mL. The sample was incubated at 37°C for 10 minutes. Following incubation, the sample was spun at 6000x*g* for 10 min, and 50 µL of the filtrate was collected for analysis. The retentate was transferred to a new filter tube and spun again for 10 min at 6000x*g*. 50 µL of the filtrate was again collected for analysis. A 50 µL unspun plasma sample was also collected for sample concentration measurement.

Spike Control

A plasma sample containing Abraxane, Genexol-PM, or Taxol generic was spiked with free paclitaxel, and spike recovery was calculated. Accurate spike recovery supports the accuracy of the unencapsulated drug measurement. Abraxane and Genexol-PM samples were spiked into human plasma at a concentration of 1 µg/mL; Taxol generic samples were spiked into human plasma at a concentration of 1,5, or 20 µg/mL, and PTX_C13 stable isotope tracer was added at a final concentration of 100 ng/mL. Free PTX at 200 ng/mL was also spiked into the sample. After incubating at 37°C for 10 minutes, samples were spun at 6000x*g* for 10 min. 50 µL of the filtrate was collected for analysis. A 50 µL unspun plasma sample was also collected for sample concentration measurement.

No Organic Control

A control for the organic spike was performed by preparing two identical Abraxane, Genexol-PM, or Taxol generic formulation-containing plasma samples. One sample received the stable isotope tracer spike, the other did not. If the organic spike has no effect on the formulation stability, the concentration of paclitaxel in the filtrate of both samples should be identical, ideally within 15% of each other.

The Abraxane and Genexol-PM formulations were spiked into two sets of human plasma at a concentration of 1 µg/mL in triplicate; Taxol generic formulation was spiked into two sets of human plasma at a concentration of 1, 5, or 20 µg/mL in triplicate. To one set no stable isotope tracer was added, while to the other set the normal PTX_C13 stable isotope tracer was added at a final concentration of 100 ng/mL. The sample was incubated at 37°C for 10 minutes. Following incubation, the sample was spun at 6000x*g* for 10 min, and 50 µL of the filtrate was collected for analysis. A 50 µL unspun plasma sample was also collected for sample concentration measurement.

Results and Discussion

Double processing of the Abraxane plasma samples did not alter the unencapsulated PTX estimate, supporting the fact that the centrifugation/filtration step does not alter formulation stability (**Tables II-27 and II-28, Figure II-10**). The 200 ng/mL spike recovery was within 20% of theoretical (**Tables II-27 and II-28, Figure II-10**). The organic stable isotope spike did not change the protein binding estimate (within 2%), confirming that the organic stable isotope spike does not alter formulation stability in this example (**Tables II-27 and II-28**).

Double processing of the Genexol-PM liposome plasma samples did not alter the unencapsulated PTX estimate, supporting the fact that the centrifugation/filtration step does not alter formulation stability (**Tables II-29 to II-32, and Figures II-11 and II-12**). The 200 ng/mL spike recovery was within 20% of theoretical (**Tables II-29 to II-32, and Figures II-11 and II-12**). The organic stable isotope spike did not change the protein binding estimate (within 2%), confirming that the organic stable isotope spike does not alter formulation stability in this example (**Tables II-29 to II-32**).

Double processing of the Taxol generic plasma samples did not alter the unencapsulated PTX estimate, supporting the fact that the centrifugation/filtration step does not alter formulation stability (**Tables II-33 to II-36, and Figures II-13 to II-15**). The 200 ng/mL spike recovery was within 20% of theoretical (**Tables II-33 to II-36, and Figures II-13 to II-15**). The organic stable isotope spike did not change the protein binding estimate (within 2%), confirming that the organic stable isotope spike does not alter formulation stability in this example (**Tables II-33 to II-36**).

Table II-27. Abraxane double spin, spike recovery, and organic spike controls analytical data. Presented are the analytical data for the double spin, spike recovery and no organic controls for Abraxane, lot 6113658. The % protein binding, encapsulated and unencapsulated measurements were calculated as described in the SITUA methods section above (Section I). The concentration of Abraxane was 1 µg PTX/mL in plasma, the PTX spike was 200 ng/mL, and the incubation parameters were 37°C for 10 minutes.

	Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX	% Bound	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13	% Bound PTX_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG	SD
			= Filtrate PTX / Reservoir PTX *100	= 100 – %Unbound PTX			= Filtrate PTX_C13 / Reservoir PTX_C13 * 100	= 100 – %Unbound C13	= Fil PTX/ (1-(%Bound PTX_C13/100))	= Reservoir PTX- Unencapsulated	= (Unencapsulated/ Reservoir PTX) *100		
Abraxane Spin 1	39.8	816	4.9	95.1	5.3	113	4.7	95.3	852.2	-36	104.4	105.2	1.1
	48.2	803	6.0	94.0	6.4	114	5.6	94.4	855.0	-52	106.4		
	42.0	841	5.0	95.0	5.8	121	4.8	95.2	881.6	-41	104.9		
Abraxane Spin 2	51.6	816	6.3	93.7	7.2	113	6.4	93.6	801.9	15	98.2	100.5	2.7
	50.1	803	6.2	93.8	6.9	114	6.0	94.0	831.0	-28	103.5		
	51.5	841	6.1	93.9	7.4	121	6.1	93.9	840.3	0	100.0		
Abraxane + 200 ng PTX spike	56.9	1082	5.3	94.7	5.9	116	5.1	94.9	1117.2	-36	103.3	100.1	2.9
	52.6	1041	5.1	94.9	5.8	115	5.1	94.9	1032.5	8	99.2		
	53.5	1102	4.9	95.1	5.9	118	5.0	95.0	1076.7	25	97.7		
Abraxane No Organic	36.7	852	4.3	95.7									
	35.6	841	4.2	95.8									
	32.2	840	3.8	96.2									

Table II-28. Abraxane double spin, spike recovery, and no organic controls. Presented are the unencapsulated PTX concentrations and percent protein binding values of the normoisotopic drug, for the double spin and spike recovery controls, and the no organic controls, respectively. The unencapsulated PTX measurements and percent protein binding were calculated as described in the SITUA methods section above (Section I). The concentration of Abraxane, lot 6113658, was 1 µg PTX/mL in plasma, the PTX spike was 200 ng/mL, and the incubation parameters were 37°C for 10 minutes (*N* = 3, mean ± SD).

	AVG Unencapsulated PTX (ng/mL)	SD
Spin 1	863	16.2
Spin 2	824	20.0
200 ng/mL free PTX spike	1075	42.4
Spiked Difference	213	42.4
	% Protein Binding	SD
With Organic SI	94.7	0.6
No Organic SI	95.9	0.3

Abraxane, Lot A (6113658)

Figure II-10. Abraxane double spin and spike recovery controls. Displayed are the unencapsulated PTX concentrations, calculated from Eq. ii, for the first (Spin 1) and second spin (Spin 2) of the double processing controls and the 200 ng/mL spike PTX control. The "Spiked Difference" is the difference between the 200 ng/mL spike and Spin 1 controls. The concentration of Abraxane, lot 6113658, was 1 µg PTX/mL in plasma, and the incubation parameters were 37°C for 10 minutes (*N* = 3, mean ± SD).

Table II-29. Genexol-PM double spin, spike recovery, and organic spike controls analytical data. Presented are the analytical data for the double spin, and no organic controls for Genexol-PM, lot GP31681. The % protein binding, encapsulated and unencapsulated measurements were calculated as described in the SITUA methods section above (Section I). The concentration of Genexol-PM was 1 μg PTX/mL in plasma, and the incubation parameters were 37°C for 10 minutes.

	Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 – %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated	% Release = (Unencapsulated/ Reservoir PTX) *100	AVG	SD
Genexol-PM Spin 1	37.2	1007	3.7	96.3	5.2	128	4.0	96.0	927.5	80	92.1	96.3	4.6
	45.2	870	5.2	94.8	6.3	117	5.4	94.6	832.9	37	95.7		
	38.3	842	4.6	95.4	5.3	117	4.5	95.5	851.8	-10	101.2		
Genexol-PM Spin 2	52.3	1007	5.2	94.8	7.3	128	5.7	94.3	922.0	85	91.5	97.5	5.2
	73.0	870	8.4	91.6	9.8	117	8.4	91.6	867.0	3	99.6		
	49.7	842	5.9	94.1	6.8	117	5.8	94.2	852.8	-11	101.3		
Genexol-PM No Organic	101.6	852	11.9	88.1									
	35.1	866	4.1	95.9									
	39.4	871	4.5	95.5									

Table II-30. Genexol-PM spike recovery analytical data. Presented is the analytical data for the spike recovery controls for Genexol-PM, lot GP31681. The % protein binding, encapsulated and unencapsulated measurements were calculated as described in the SITUA methods section above (Section I). The concentration of Genexol-PM was 1 μg PTX/mL in plasma, the PTX spike was 200 ng/mL, and the incubation parameters were 37°C for 10 minutes.

	Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 – %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated	% Release = (Unencapsulated/ Reservoir PTX) *100	AVG	SD
Genexol-PM Spin	63.1	1367	4.6	95.4	5.5	130	4.3	95.7	1482.4	-115	108.4	107.7	5.3
	57.3	1319	4.3	95.7	4.9	126	3.9	96.1	1485.0	-166	112.6		
	55.6	1371	4.1	95.9	4.7	117	4.0	96.0	1399.1	-28	102.1		
Genexol PM + 200 ng/mL Spike	87.3	1607	5.4	94.6	6.2	120	5.2	94.8	1678.4	-72	104.5	102.9	3.0
	87.9	1673	5.3	94.7	6.2	117	5.3	94.7	1663.0	10	99.4		
	80.5	1562	5.2	94.8	5.6	114	4.9	95.1	1637.9	-76	104.9		

Table II-31. Genexol-PM double spin, and no organic controls. Presented are the unencapsulated PTX concentrations and percent protein binding values of the normoisotopic drug, for the double spin controls, and the no organic controls, respectively. The unencapsulated PTX measurements and percent protein binding were calculated as described in SITUA methods section above (Section I). The concentration of Genexol-PM, lot GP31681, was 1 µg PTX/mL in plasma, and the incubation parameters were 37°C for 10 minutes (*N* = 3, mean ± SD).

	AVG Unencapsulated PTX (ng/mL)	SD
Spin 1	871	50.1
Spin 2	881	36.6
	% Protein Binding	**SD**
With Organic SI	95.5	0.8
No Organic SI	95.7	0.3

Genexol-PM, Lot A (GP31681)
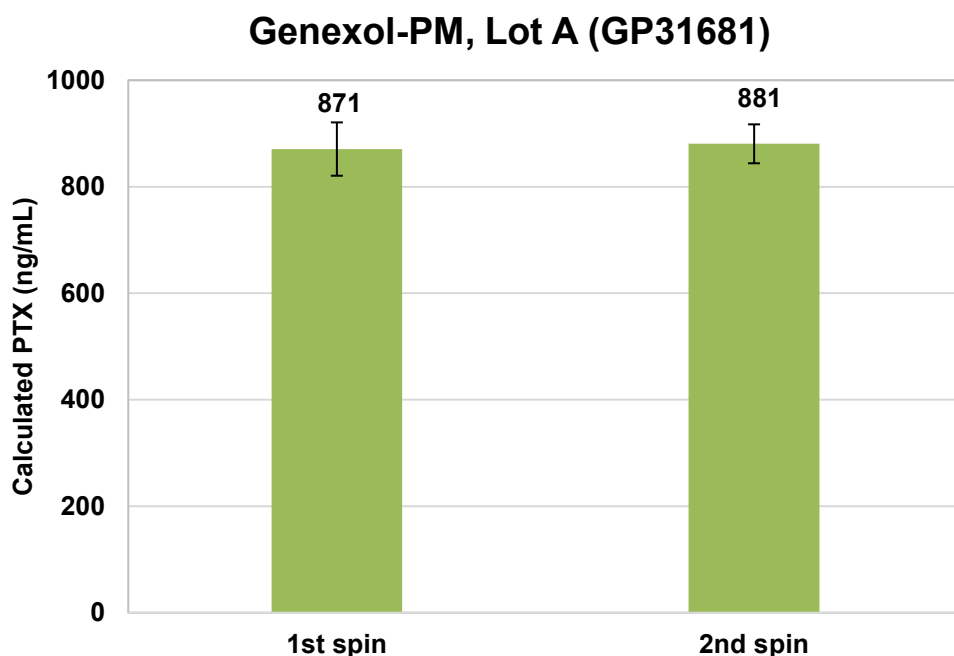
Figure II-11. Genexol-PM double spin and spike recovery controls. Displayed are the unencapsulated PTX concentrations, calculated from Eq. ii, for the first (Spin 1) and second spin (Spin 2) of the double processing controls. The concentration of Genexol-PM, lot GP31681, was 1 µg PTX/mL in plasma, and the incubation parameters were 37°C for 10 minutes (*N* = 3, mean ± SD).

Table II-32. Genexol-PM spike recovery controls. Presented are the unencapsulated PTX concentrations and percent protein binding values of the normoisotopic drug, for the spike recovery controls. The unencapsulated PTX measurements was calculated as described in SITUA methods section above (Section I). The concentration of Genexol-PM, lot GP31681, was 1 µg PTX/mL in plasma, the PTX spike was 200 ng/mL, and the incubation parameters were 37°C for 10 minutes (N = 3, mean ± SD).

	AVG Unencapsulated PTX (ng/mL)	SD
Spin	1456	48.9
200 ng/mL Spike PTX	1660	20.5
Spiked Difference	204	20.5
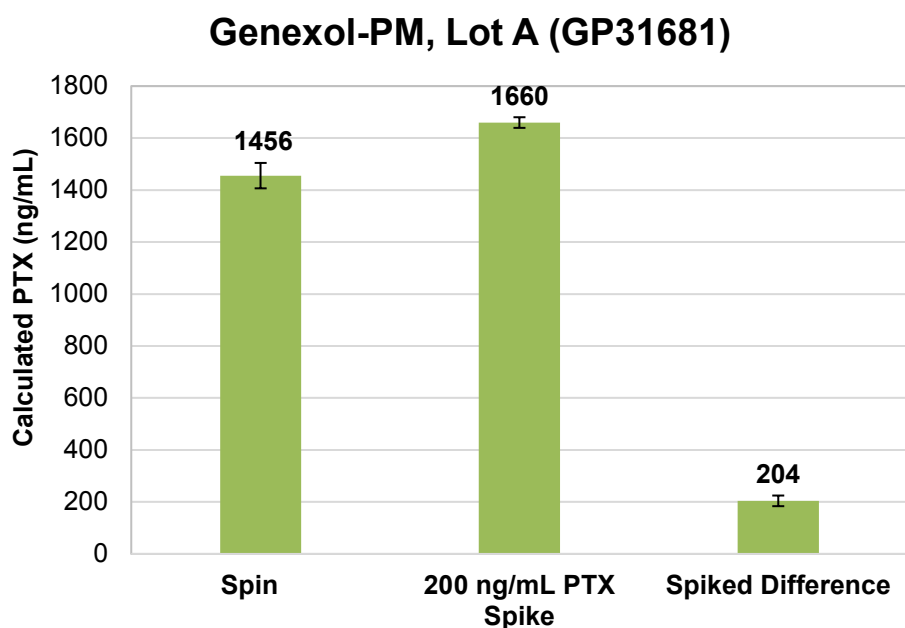
Figure II-12. Genexol-PM spike recovery control. Displayed are the unencapsulated PTX concentrations, calculated from Eq. ii, for the first spin (Spin) and the 200 ng/mL spike PTX control. The "Spiked Difference" is the difference between the 200 ng/mL spike and Spin 1 controls. The concentration of Genexol-PM, lot GP31681, was 1 µg PTX/mL in plasma, and the incubation parameters were 37°C for 10 minutes (N = 3, mean ± SD).

Table II-33. Taxol double spin, spike recovery, and organic spike controls analytical data. Presented are the analytical data for the double spin, spike recovery and no organic controls for Taxol generic, lot PACCA1018. The % protein binding, encapsulated and unencapsulated measurements were calculated as described in the SITUA methods section above (Section I). The concentration of Taxol was 1, 5, and 20 µg PTX/mL in plasma, the PTX spike was 200 ng/mL, and the incubation parameters were 37°C for 10 minutes.

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX	% Bound	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13	% Bound PTX_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG	SD
				= Filtrate PTX / Reservoir PTX *100	= 100 − %Unbound PTX			= Filtrate PTX_C13 / Reservoir PTX_C13 * 100	= 100 − %Unbound C13	= Fil PTX/ (1-(%Bound PTX_C13/100))	= Reservoir PTX- Unencapsulated	= (Unencapsulated/ Reservoir PTX) *100		
1 µg/mL	Taxol Spin 1	46.3	1066	4.3	95.7	4.4	106	4.1	95.9	1118.2	-53	104.9	96.2	9.4
		41.5	1033	4.0	96.0	3.8	93	4.1	95.9	1006.2	26	97.4		
		44.8	1009	4.4	95.6	4.4	86	5.1	94.9	870.5	139	86.2		
	Taxol Spin 2	54.5	1066	5.1	94.9	5.3	106	5.0	95.0	1090.2	-25	102.3	97.8	7.0
		50.9	1033	4.9	95.1	4.5	93	4.9	95.1	1047.3	-15	101.4		
		60.4	1009	6.0	94.0	5.7	86	6.7	93.3	906.1	103	89.8		
	Taxol + 200 ng PTX spike	69.4	1316	5.3	94.7	5.3	97	5.5	94.5	1259.8	56	95.7	93.2	4.0
		66.3	1235	5.4	94.6	4.9	88	5.6	94.4	1177.5	58	95.3		
		58.1	1213	4.8	95.2	4.2	77	5.4	94.6	1074.0	139	88.5		
	Taxol No Organic	47.8	1095	4.4	95.6									
		42.5	1037	4.1	95.9									
		48.5	1038	4.7	95.3									
5 µg/mL	Taxol Spin 1	222.1	5568	4.0	96.0	4.1	85	4.8	95.2	4618.4	950	82.9	83.0	0.6
		196.6	4834	4.1	95.9	4.0	81	4.9	95.1	4046.5	787	83.7		
		242.8	5446	4.5	95.5	4.7	87	5.4	94.6	4489.7	956	82.4		
	Taxol Spin 2	315.8	5568	5.7	94.3	5.7	85	6.8	93.2	4662.9	905	83.7	82.5	3.3
		261.5	4834	5.4	94.6	5.6	81	6.9	93.1	3803.7	1030	78.7		
		306.4	5446	5.6	94.4	5.7	87	6.6	93.4	4629.4	817	85.0		
	Taxol + 200 ng PTX spike	241.7	5540	4.4	95.6	4.1	82	5.0	95.0	4801.9	738	86.7	86.0	1.7
		272.4	5319	5.1	94.9	5.2	86	6.1	93.9	4468.4	850	84.0		
		240.5	5180	4.6	95.4	4.5	85	5.3	94.7	4520.5	660	87.3		
	Taxol No Organic	228.0	4950	4.6	95.4									
		254.2	4513	5.6	94.4									
		239.5	4790	5.0	95.0									
20 µg/mL	Taxol Spin 1	710.6	19724	3.6	96.4	3.4	81	4.2	95.8	16736.9	2987	84.9	83.6	1.1
		692.8	19874	3.5	96.5	3.4	81	4.2	95.8	16507.4	3366	83.1		
		733.3	20186	3.6	96.4	3.6	81	4.4	95.6	16748.3	3437	83.0		
	Taxol Spin 2	1035.1	19724	5.2	94.8	5.2	81	6.4	93.6	16183.9	3540	82.1	84.0	2.3
		981.1	19874	4.9	95.1	4.8	81	5.9	94.1	16592.6	3281	83.5		
		1013.0	20186	5.0	95.0	4.7	81	5.8	94.2	17476.9	2709	86.6		
	Taxol + 200 ng PTX spike	751.4	20550	3.7	96.3	3.5	80	4.4	95.6	17254.3	3296	84.0	82.3	2.5
		787.1	20504	3.8	96.2	3.8	79	4.8	95.2	16291.5	4212	79.5		
		775.1	20431	3.8	96.2	3.9	86	4.5	95.5	17041.1	3389	83.4		
	Taxol No Organic	802.8	21048	3.8	96.2									
		781.6	20923	3.7	96.3									
		778.6	20250	3.8	96.2									

Table II-34. 1 µg/mL Taxol double spin, spike recovery, and no organic controls.
Presented are the unencapsulated PTX concentrations and percent protein binding values of the normoisotopic drug, for the double spin and spike recovery controls, and the no organic controls, respectively. The unencapsulated PTX measurements and percent protein binding were calculated as described in the SITUA methods section above (Section I). The concentration of Taxol generic, lot PACCA1018, was 1 µg PTX/mL in plasma, the PTX spike was 200 ng/mL, and the incubation parameters were 37°C for 10 minutes (*N* = 3, mean ± SD).

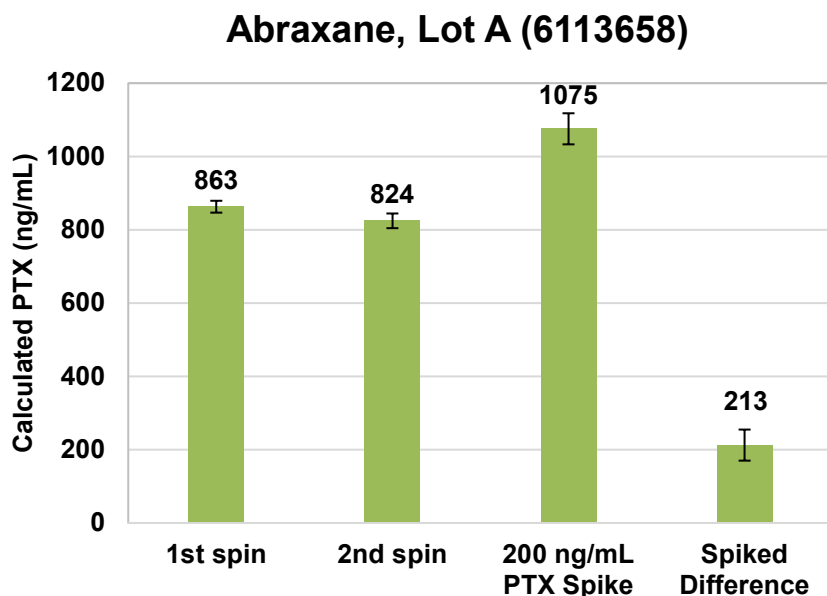|	AVG Unencapsulated PTX (ng/mL)	SD
Spin 1	998	124.0
Spin 2	1015	96.3
200 ng/mL free PTX spike	1170	93.1
Spiked Difference	172	93.1
	% Protein Binding	SD
With Organic SI	94.7	0.6
No Organic SI	95.6	0.3

1 µg/mL Taxol generic, Lot PACCA1018
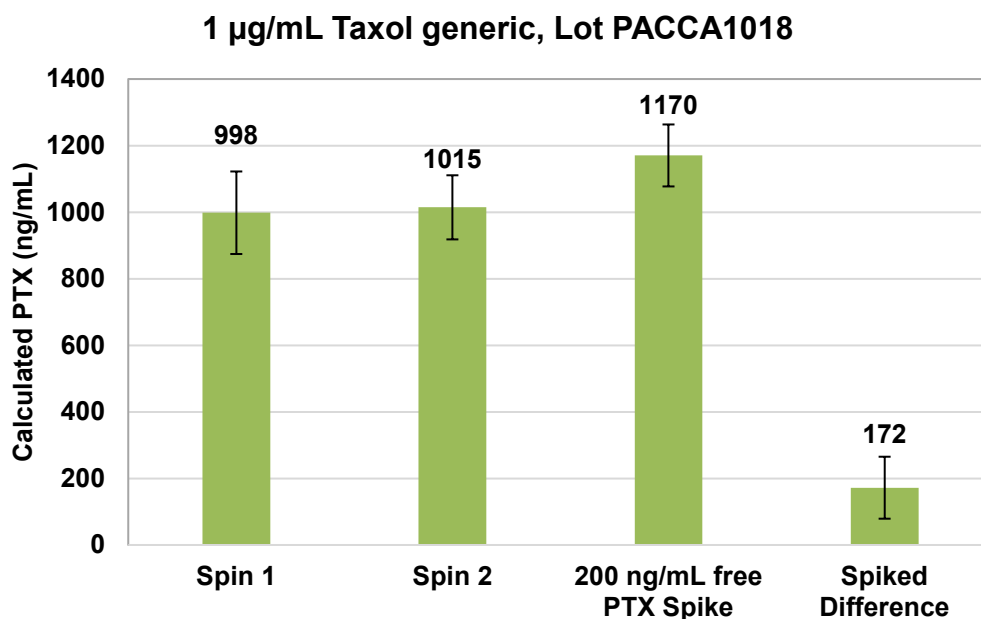
Figure II-13. 1 µg/mL Taxol double spin and spike recovery controls. Displayed are the unencapsulated PTX concentrations, calculated from Eq. ii, for the first (Spin 1) and second spin (Spin 2) of the double processing controls and the 200 ng/mL spike PTX control. The "Spiked Difference" is the difference between the 200 ng/mL spike and Spin 1 controls. The concentration of Taxol generic, lot PACCA1018, was 1 µg PTX/mL in plasma, and the incubation parameters were 37°C for 10 minutes (*N* = 3, mean ± SD).

Table II-35. 5 µg/mL Taxol double spin, spike recovery, and no organic controls.
Presented are the unencapsulated PTX concentrations and percent protein binding values of
the normoisotopic drug, for the double spin and spike recovery controls, and the no organic
controls, respectively. The unencapsulated PTX measurements and percent protein binding
were calculated as described in the SITUA methods section above (Section I). The
concentration of Taxol generic, lot PACCA1018, was 5 µg PTX/mL in plasma, the PTX spike
was 200 ng/mL, and the incubation parameters were 37°C for 10 minutes (N = 3, mean ± SD).

	AVG Unencapsulated PTX (ng/mL)	SD
Spin 1	4385	300.0
Spin 2	4365	486.7
200 ng/mL free PTX spike	4597	179.4
Spiked Difference	212	179.4
% Protein Binding		
With Organic SI	95.3	0.4
No Organic SI	94.9	0.5
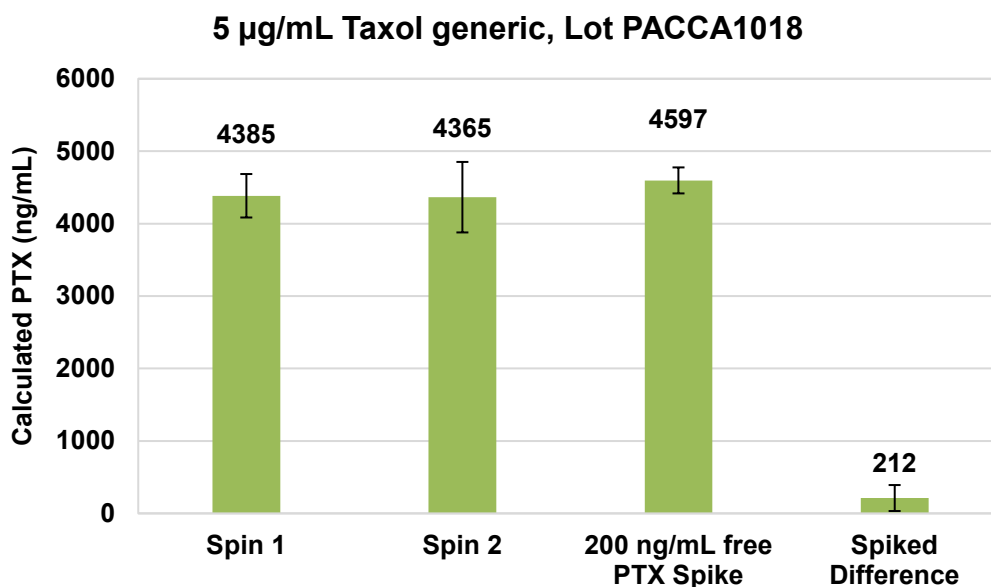
Figure II-14. 5 µg/mL Taxol double spin and spike recovery controls. Displayed are the
unencapsulated PTX concentrations, calculated from Eq. ii, for the first (Spin 1) and second
spin (Spin 2) of the double processing controls and the 200 ng/mL spike PTX control. The
"Spiked Difference" is the difference between the 200 ng/mL spike and Spin 1 controls. The
concentration of Taxol generic, lot PACCA1018, was 5 µg PTX/mL in plasma, and the
incubation parameters were 37°C for 10 minutes (N = 3, mean ± SD).

Table II-36. 20 μg/mL Taxol double spin, spike recovery, and no organic controls.
Presented are the unencapsulated PTX concentrations and percent protein binding values of the normoisotopic drug, for the double spin and spike recovery controls, and the no organic controls, respectively. The unencapsulated PTX measurements and percent protein binding were calculated as described in the SITUA methods section above (Section I). The concentration of Taxol generic, lot PACCA1018, was 20 μg PTX/mL in plasma, the PTX spike was 200 ng/mL, and the incubation parameters were 37°C for 10 minutes (N = 3, mean ± SD).

	AVG Unencapsulated PTX (ng/mL)	SD
Spin 1	16664	135.9
Spin 2	16751	660.9
200 ng/mL free PTX spike	16862	505.7
Spiked Difference	198	505.7
	% Protein Binding	SD
With Organic SI	96.2	0.1
No Organic SI	96.2	0.1

20 μg/mL Taxol generic, Lot PACCA1018
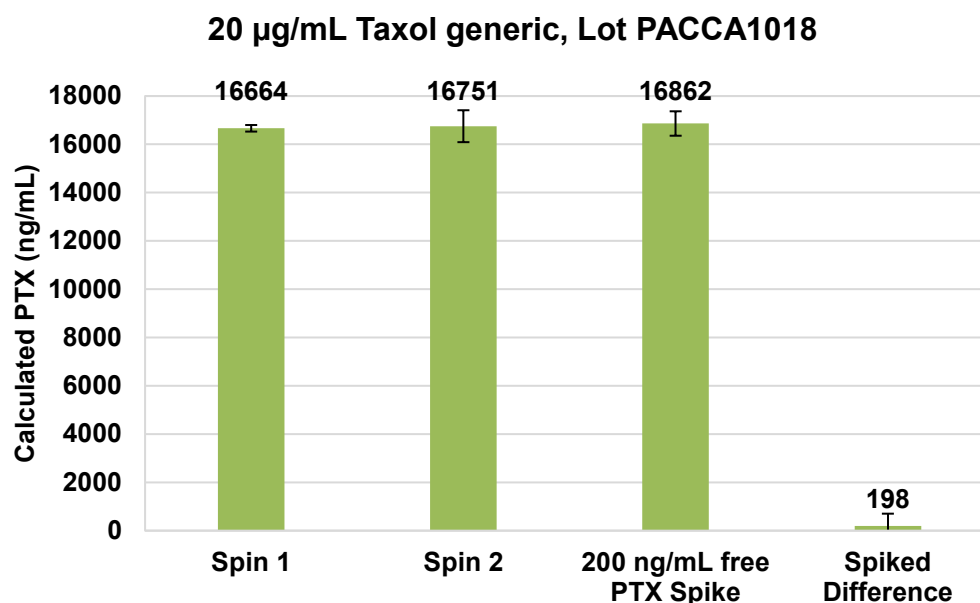
Figure II-15. 20 μg/mL Taxol double spin and spike recovery controls. Displayed are the unencapsulated PTX concentrations, calculated from Eq. ii, for the first (Spin 1) and second spin (Spin 2) of the double processing controls and the 200 ng/mL spike PTX control. The "Spiked Difference" is the difference between the 200 ng/mL spike and Spin 1 controls. The concentration of Taxol generic, lot PACCA1018, was 20 μg PTX/mL in plasma, and the incubation parameters were 37°C for 10 minutes (N = 3, mean ± SD).

Control Study – Reduced Sample Volumes

Summary

The in vitro drug release studies used 400 µL of plasma in the filter apparatus. However, plasma from the in vivo studies will be limited due to restrictions on the blood volumes permitted to be collected over the 4-day study period. For the in vivo studies, only 150-175 µL of plasma will be used in the filter apparatus instead of 400 µL. This control study confirmed that the reduced sample volume did not influence the assay performance.

Design and Methods

The following study compared results using 150 µL vs 400 µL of plasma sample in the filter apparatus. The study utilized free PTX, Abraxane (lot 6113658), and Genexol-PM (lot GP31681). Human blood was collected in K_2-EDTA tubes and pooled from 6 donors. Blood was spun at 2500x*g* for 10 min to collect plasma. HEPES buffer (pH 7.4) was added at 50 µL/2 mL plasma to maintain a pH of 7.4. Plasma in glass vials was spiked with samples (free PTX, Abraxane, or Genexol-PM) at final drug concentrations of 0.5 µg/mL, 1 µg/mL, and 5 µg/mL PTX, in triplicate. All samples were then spiked with ^{13}C-PTX stable isotope tracer at a final concentration of 100 ng/mL. The incubation period was for 10 minutes at 37ºC. All other experimental procedures were the same as described previously.

Results and Discussion

The results of the drug release study for the free PTX controls, Abraxane (lot 6113658), and Genexol-PM (lot GP31681) were not significantly different at any of the drug concentration levels between the 150 or 400 µL plasma volume groups (**Tables II-37 to II-39, Figures II-16 to II-18**).

Table II-37. Free Paclitaxel. Presented are the % release results for the plasma volume comparison study at 150 and 400 µL (Mean $\pm$ SD, *N=3*).

Sample Volume	Conc.	Avg % Release	SD	% CV
400 µL	0.5 µg/mL	105	1.1	1.0
	1 µg/mL	97	0.7	0.7
	5 µg/mL	100	2.4	2.4
150 µL	0.5 µg/mL	110	10.0	9.1
	1 µg/mL	108	7.2	6.7
	5 µg/mL	108	10.3	9.6

Figure II-16. Free Paclitaxel. Presented are the % release results for the plasma volume comparison study (Mean $\pm$ SD, *N=3*).

Table II-38. Abraxane, Lot 6113658. Presented are the % release results of the plasma volume comparison study (Mean $\pm$ SD, *N=3*).

Sample Volume	Conc.	Avg % Release	SD	% CV
400 µL	0.5 µg/mL	99	1.8	1.8
	1 µg/mL	105	1.1	1.0
	5 µg/mL	100	0.9	0.9
150 µL	0.5 µg/mL	97	6.5	6.7
	1 µg/mL	102	0.1	0.1
	5 µg/mL	102	1.5	1.5
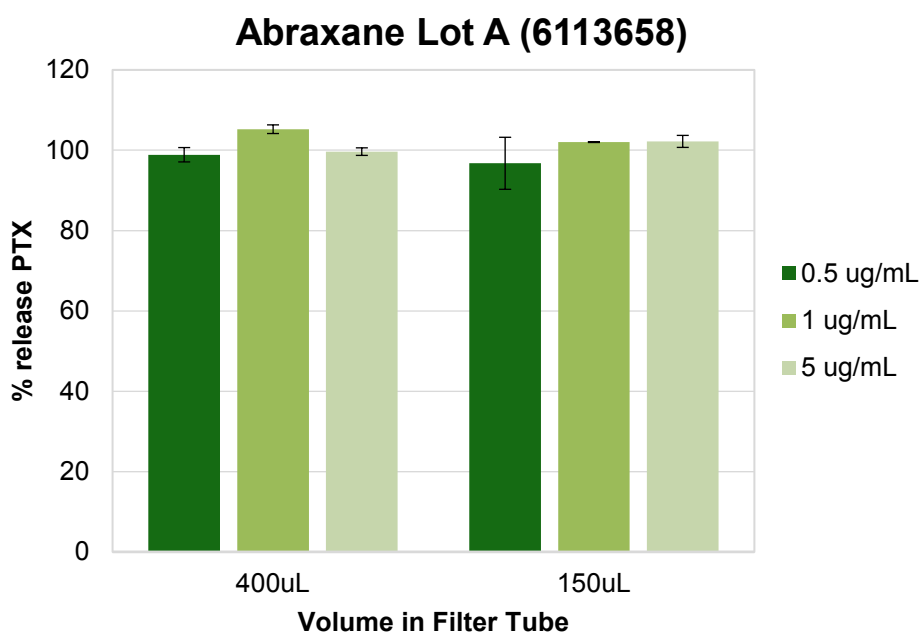
Figure II-17. Abraxane, Lot 6113658. Presented are the % released results of the plasma volume comparison study (Mean $\pm$ SD, *N=3*).

Table II-39. Genexol-PM, Lot GP31681. Presented are the % release results of the plasma volume comparison study (Mean $\pm$ SD, *N=3*).

Sample Volume	Conc.	Avg % Release	SD	% CV
400 µL	0.5 µg/mL	101	4.5	4.5
	1 µg/mL	96	4.6	4.7
	5 µg/mL	99	0.3	0.3
150 µL	0.5 µg/mL	101	1.1	1.1
	1 µg/mL	98	4.9	5.0
	5 µg/mL	96	2.6	2.7
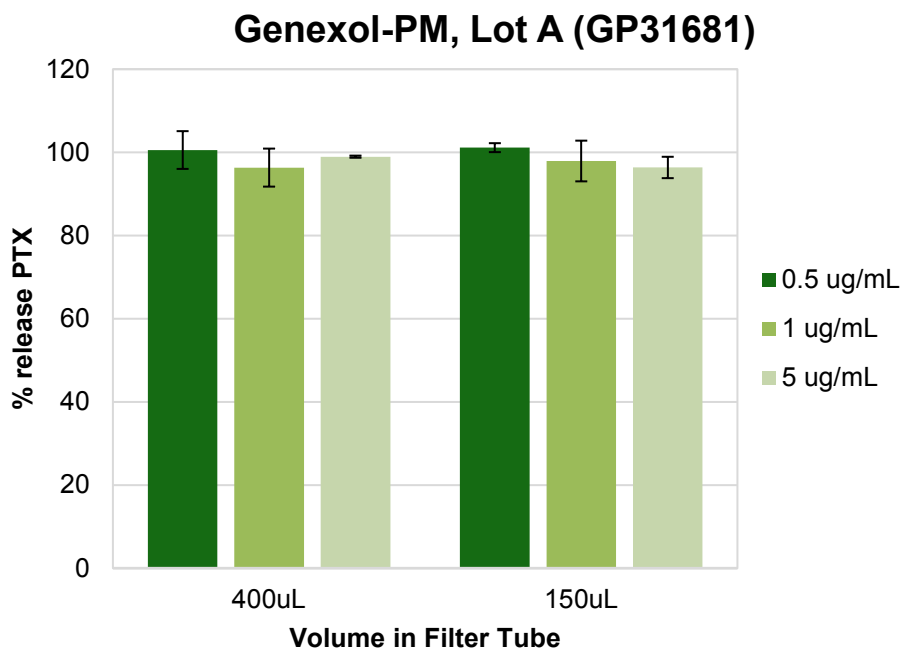
Figure II-18. Genexol-PM, Lot GP31681. Presented are the % released results of the plasma volume comparison study (Mean $\pm$ SD, *N=3*).

III. In Vitro Abraxane Lot Comparison

Intra-Day Three Lot Comparison in Human Plasma at 37°C

Summary

The objective of this study was to compare the in vitro drug release of three lots of Abraxane in human plasma at 37°C over a 2 hr period. All lots had similar drug release profiles over the 2 hr period, although some instances of statistically significant differences were observed between lots.

Design and Methods

Three lots of Abraxane, 6113658, 6111839, and 6111355, also denoted as lots A, B, and C, respectively, were evaluated for drug release at 0.5, 1 and 5 µg/mL PTX equivalents in human plasma at 37°C over a 2 hr period, according to the SITUA method described in Section I. All three lots were run on a single day, and free paclitaxel at identical concentrations and time points was included as a control. The human plasma and protein-free plasma, PTX and PTX_C13 standard calibration curves used for this study are also provided.

Results & Conclusions

The PTX and PTX_C13 standard calibration curves used for this study are presented in **Tables III-1 to III-4 and Figures III-1 to III-4**. The free paclitaxel controls averaged between 94-112% release for all concentrations and time points (**Tables III-5 and III-9, and Figure III-5**). The Abraxane drug release was similar for lots A-C, at approximately 100% release at the earliest 10 min time point for all concentrations, without a clear concentration-dependent or temporal trend (**Tables III-6 to III-8 and III-10 to III-12, and Figures III-6 to III-9**).

Table III-1. PTX Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	530648	10.4	104
10.0 ng/mL	478488	9.4	94
50 ng/mL	1664833	51	102
50 ng/mL	2359610	53	105
100 ng/mL	3683684	101	101
100 ng/mL	3432923	98	98
500 ng/mL	16545844	525	105
500 ng/mL	30310037	535	107
1000 ng/mL	54214250	1055	105
1000 ng/mL	56659949	1053	105
5000 ng/mL	274505789	4421	88
5000 ng/mL	304417403	5015	100
QC			
QCL	558989	10.4	104
QCL	481147	10.5	105
QCM	5143552	104	104
QCM	6240014	107	107
QCH	57099282	1046	105
QCH	56988139	1124	112

PTX_C13
$Y = 0.0205132 + 0.0214021 \ast X$ $R^2 = 0.9932$ W: $1/X^2$
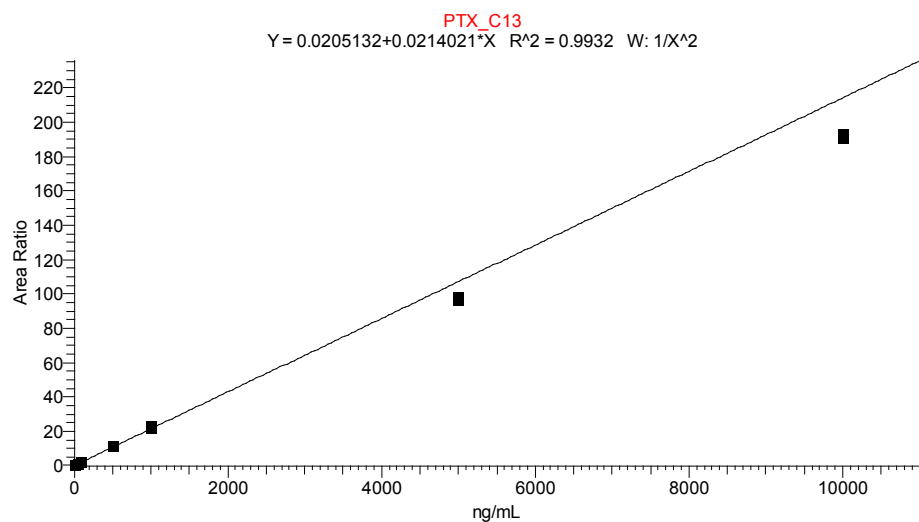
Figure III-1. PTX Plasma Standard Curve.

Table III-2. PTX_C13 Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	1039800	10.2	102
10.0 ng/mL	970084	9.6	96
50 ng/mL	2980219	48	97
50 ng/mL	4527440	53	107
100 ng/mL	7313859	107	107
100 ng/mL	6864541	105	105
500 ng/mL	31556211	537	107
500 ng/mL	57452663	544	109
1000 ng/mL	102551111	1071	107
1000 ng/mL	103003812	1027	103
5000 ng/mL	529963391	4584	92
5000 ng/mL	507281860	4488	90
QC			
QCL	1094717	10.2	102
QCL	909185	9.9	99
QCM	10080805	109	109
QCM	11023535	101	101
QCH	111913102	1101	110
QCH	102642245	1086	109

PTX_C13
$Y = 0.0205132 + 0.0214021 * X$ $R^2 = 0.9932$ W: 1/X^2
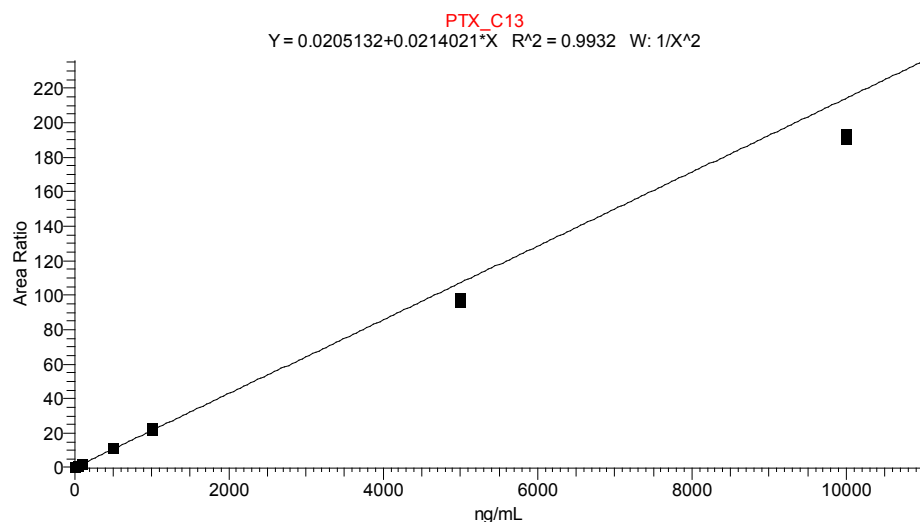
Figure III-2. PTX_C13 Plasma Standard Curve.

Table III-3. PTX Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.50 ng/mL	212740	0.50	100
0.50 ng/mL	437666	0.49	98
1.00 ng/mL	654186	1.01	101
1.00 ng/mL	832773	1.04	104
5.00 ng/mL	2067334	5.1	101
5.00 ng/mL	4172093	5.1	101
10.0 ng/mL	7482627	9.5	95
10.0 ng/mL	7170079	9.2	92
50.0 ng/mL	35757389	50.1	100
50.0 ng/mL	36028483	53.0	106
100.0 ng/mL	62204068	105.6	106
100.0 ng/mL	59110158	88.7	89
500.0 ng/mL	284738402	514.4	103
500.0 ng/mL	257962572	520.0	104
QC			
QCL	575432	3.03	607*
QCL	443464	0.54	108
QCM	3510744	4.79	96
QCM	3455488	4.79	96
QCH	34193460	53.80	108
QCH	33410605	51.85	104

*Out of specification

PTX
$Y = 0.00171011 + 0.0125915 \cdot X$ $R^2 = 0.9966$ W: $1/X^2$
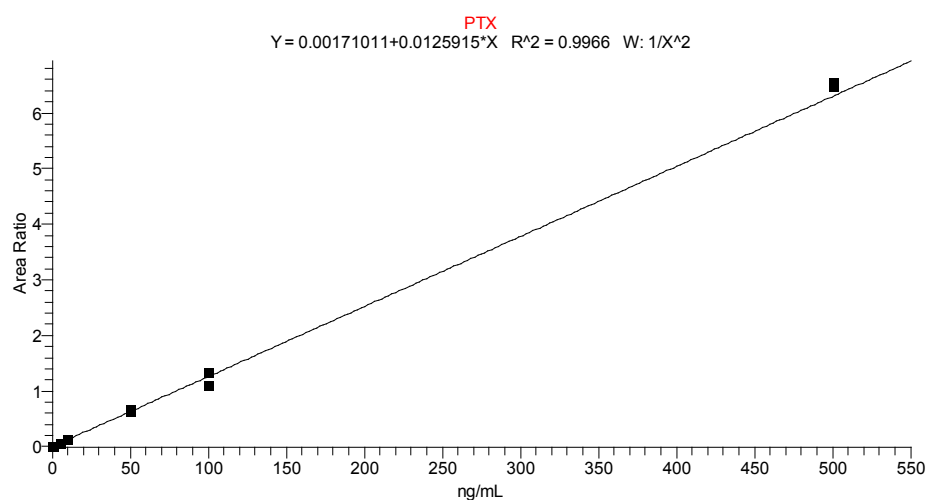
Figure III-3. PTX Protein-Free Plasma Standard Curve.

Table III-4. PTX_C13 Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.50 ng/mL	368750	0.50	100
0.50 ng/mL	214736	0.54	108
1.00 ng/mL	1102237	0.90	90
1.00 ng/mL	1483421	0.98	98
5.00 ng/mL	4441335	5.4	107
5.00 ng/mL	8935484	5.4	107
10.0 ng/mL	14803907	9.2	92
10.0 ng/mL	14956017	9.4	94
50.0 ng/mL	74511825	50.6	101
50.0 ng/mL	73621134	52.4	105
100.0 ng/mL	119592150	98.3	98
100.0 ng/mL	124432280	90.4	90
500.0 ng/mL	605471411	529.0	106
500.0 ng/mL	541175634	527.6	106
QC			
QCL	802559	0.52	105
QCL	653447	0.45	90
QCM	7393276	5.0	100
QCM	7009793	4.8	96
QCH	69799015	53.2	106
QCH	71776846	54.0	108

PTX_C13
Y = 0.000808855+0.0260389*X   R^2 = 0.9948   W: 1/X^2

Table III-4. PTX_C13 Protein-Free Plasma Standard Curve.

Table III-5. Free Paclitaxel Control Analytical Data. Presented are the analytical data for the free paclitaxel controls. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX	% Bound	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13	% Bound PTX_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG	SD
				= Filtrate PTX / Reservoir PTX *100	= 100 – %Unbound PTX			= Filtrate PTX_C13 / Reservoir PTX_C13 * 100	= 100 – %Unbound C13	= Fil PTX/ (1-(%Bound PTX_C13/100))	= Reservoir PTX- Unencapsulated	= (Unencapsulated/ Reservoir PTX) *100		
10 min	0.5 µg/mL	25.7	599	4.3	95.7	5.0	119	4.2	95.8	613	-13	102.2	100.6	2.9
		26.2	516	5.1	94.9	5.5	106	5.2	94.8	501	14	97.2		
		24.1	579	4.2	95.8	5.0	123	4.1	95.9	592	-14	102.4		
	1 µg/mL	56.3	1294	4.3	95.7	5.6	118	4.7	95.3	1198	96	92.6	101.2	7.7
		62.1	1163	5.3	94.7	6.2	120	5.2	94.8	1205	-42	103.6		
		65.9	1257	5.2	94.8	6.4	130	4.9	95.1	1349	-92	107.3		
	5 µg/mL	268.4	5780	4.6	95.4	5.0	110	4.6	95.4	5878	-98	101.7	98.8	3.6
		272.1	6110	4.5	95.5	5.2	118	4.5	95.5	6104	6	99.9		
		209.7	6564	3.2	96.8	4.4	131	3.4	96.6	6226	338	94.8		
30 min	0.5 µg/mL	23.0	525	4.4	95.6	4.8	111	4.3	95.7	536	-12	102.2	105.4	3.9
		25.4	479	5.3	94.7	5.8	113	5.1	94.9	499	-20	104.2		
		21.1	482	4.4	95.6	4.8	119	4.0	96.0	528	-47	109.7		
	1 µg/mL	55.1	905	6.1	93.9	5.9	101	5.8	94.2	948	-43	104.7	106.7	3.2
		48.6	852	5.7	94.3	5.0	97	5.2	94.8	941	-89	110.4		
		55.4	1027	5.4	94.6	5.8	112	5.1	94.9	1078	-50	104.9		
	5 µg/mL	239.6	4855	4.9	95.1	4.6	105	4.4	95.6	5447	-592	112.2	103.9	7.2
		228.5	5451	4.2	95.8	4.9	117	4.2	95.8	5443	8	99.8		
		218.3	4950	4.4	95.6	4.8	108	4.4	95.6	4934	15	99.7		
1 hr	0.5 µg/mL	20.8	561	3.7	96.3	4.0	114	3.5	96.5	600	-38	106.8	106.5	0.4
		24.1	525	4.6	95.4	5.2	120	4.3	95.7	560	-35	106.6		
		19.0	612	3.1	96.9	4.1	139	2.9	97.1	649	-38	106.2		
	1 µg/mL	50.8	1123	4.5	95.5	5.0	116	4.3	95.7	1170	-47	104.2	106.3	2.7
		45.5	1050	4.3	95.7	4.4	108	4.1	95.9	1107	-57	105.4		
		53.4	1084	4.9	95.1	5.2	116	4.5	95.5	1186	-101	109.4		
	5 µg/mL	215.7	5921	3.6	96.4	4.0	115	3.5	96.5	6174	-252	104.3	106.5	2.2
		205.4	4995	4.1	95.9	4.2	112	3.8	96.2	5433	-437	108.8		
		208.2	5539	3.8	96.2	4.1	115	3.5	96.5	5903	-364	106.6		
2 hr	0.5 µg/mL	22.7	559	4.1	95.9	4.6	122	3.8	96.2	602	-43	107.8	105.4	4.6
		19.1	516	3.7	96.3	4.0	116	3.4	96.6	560	-44	108.5		
		20.4	530	3.8	96.2	4.5	116	3.8	96.2	531	-1	100.1		
	1 µg/mL	38.6	1021	3.8	96.2	3.8	107	3.6	96.4	1081	-60	105.9	107.1	2.1
		47.8	860	5.6	94.4	4.8	95	5.1	94.9	942	-82	109.5		
		47.1	1088	4.3	95.7	4.9	120	4.1	95.9	1153	-65	106.0		
	5 µg/mL	204.5	4604	4.4	95.6	4.1	103	4.0	96.0	5176	-572	112.4	105.3	6.4
		233.8	5399	4.3	95.7	4.5	109	4.2	95.8	5587	-188	103.5		
		216.1	5730	3.8	96.2	4.3	115	3.8	96.2	5738	-7	100.1		

Table III-6. Abraxane, Lot A (6113658) Drug Release Analytical Data. Presented are the analytical data for Abraxane, lot A (6113658). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section II. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX	% Bound	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13	% Bound PTX_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG	SD
				= Filtrate PTX / Reservoir PTX *100	= 100 − %Unbound PTX			= Filtrate PTX_C13 / Reservoir PTX_C13 * 100	= 100 − %Unbound C13	= Fil PTX/ (1-(%Bound PTX_C13/100))	= Reservoir PTX- Unencapsulated	= (Unencapsulated/ Reservoir PTX) *100		
10 min	0.5 µg/mL	18.1	464	3.9	96.1	5.5	138	4.0	96.0	451	12	97.3	102.0	8.3
		15.8	360	4.4	95.6	4.5	115	3.9	96.1	401	-42	111.6		
		17.0	454	3.7	96.3	5.0	130	3.9	96.1	440	14	97.0		
	1 µg/mL	34.8	953	3.6	96.4	4.6	123	3.8	96.2	921	31	96.7	101.6	4.3
		34.9	893	3.9	96.1	4.8	130	3.7	96.3	936	-43	104.8		
		32.3	894	3.6	96.4	4.2	121	3.5	96.5	924	-29	103.3		
	5 µg/mL	196.6	4311	4.6	95.4	5.3	122	4.4	95.6	4513	-202	104.7	99.7	4.4
		185.1	4290	4.3	95.7	5.3	120	4.4	95.6	4196	94	97.8		
		176.8	4329	4.1	95.9	5.0	118	4.2	95.8	4183	146	96.6		
30 min	0.5 µg/mL	18.7	390	4.8	95.2	5.6	122	4.6	95.4	408	-18	104.5	105.6	1.5
		16.5	372	4.4	95.6	4.8	115	4.1	95.9	399	-27	107.2		
		16.2	383	4.2	95.8	4.6	115	4.0	96.0	402	-19	104.9		
	1 µg/mL	35.9	741	4.8	95.2	5.2	114	4.6	95.4	777	-36	104.9	106.0	1.9
		30.1	711	4.2	95.8	4.7	119	3.9	96.1	770	-58	108.2		
		33.8	767	4.4	95.6	4.8	115	4.2	95.8	806	-38	105.0		
	5 µg/mL	164.0	3446	4.8	95.2	5.0	114	4.4	95.6	3750	-304	108.8	110.6	3.9
		168.7	3379	5.0	95.0	5.2	112	4.6	95.4	3643	-265	107.8		
		149.7	3285	4.6	95.4	4.1	105	4.0	96.0	3780	-495	115.1		
1 hr	0.5 µg/mL	18.1	443	4.1	95.9	4.9	133	3.7	96.3	490	-46	110.5	107.0	3.1
		16.8	362	4.6	95.4	5.2	117	4.4	95.6	379	-17	104.7		
		15.6	350	4.5	95.5	4.7	111	4.2	95.8	370	-20	105.8		
	1 µg/mL	34.6	797	4.3	95.7	4.5	115	3.9	96.1	885	-88	111.0	109.9	2.8
		47.3	715	6.6	93.4	6.7	108	6.2	93.8	763	-48	106.7		
		33.3	751	4.4	95.6	4.5	113	4.0	96.0	841	-90	112.0		
	5 µg/mL	196.5	3286	6.0	94.0	5.9	104	5.6	94.4	3493	-207	106.3	107.0	2.3
		174.0	3587	4.9	95.1	4.9	107	4.6	95.4	3775	-187	105.2		
		168.6	3591	4.7	95.3	4.8	113	4.3	95.7	3935	-344	109.6		
2 hr	0.5 µg/mL	16.4	374	4.4	95.6	4.4	111	4.0	96.0	412	-38	110.2	109.3	2.3
		16.7	380	4.4	95.6	4.8	117	4.1	95.9	406	-26	106.8		
		12.6	333	3.8	96.2	3.6	106	3.4	96.6	369	-37	111.1		
	1 µg/mL	32.8	840	3.9	96.1	4.4	116	3.8	96.2	864	-24	102.8	104.2	4.1
		29.0	789	3.7	96.3	4.2	116	3.6	96.4	797	-8	101.0		
		26.4	643	4.1	95.9	3.7	97	3.8	96.2	700	-57	108.8		
	5 µg/mL	153.9	3740	4.1	95.9	4.4	116	3.8	96.2	4033	-293	107.8	105.5	3.1
		156.0	2421	6.4	93.6	4.4	69	6.3	93.7	2468	-46	101.9		
		154.0	3156	4.9	95.1	4.4	96	4.6	95.4	3369	-212	106.7		

Table III-7. Abraxane, Lot B (6111839) Drug Release Analytical Data. Presented are the analytical data for Abraxane, lot B (6111839). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section II. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX	% Bound	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13	% Bound PTX_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG	SD
				= Filtrate PTX / Reservoir PTX *100	= 100 − %Unbound PTX			= Filtrate PTX_C13 / Reservoir PTX_C13 * 100	= 100 − %Unbound C13	= Fil PTX/ (1-(%Bound PTX_C13/100))	= Reservoir PTX- Unencapsulated	= (Unencapsulated/ Reservoir PTX) *100		
10 min	0.5 µg/mL	16.6	435	3.8	96.2	4.6	121	3.8	96.2	432	3	99.3	103.8	5.3
		17.9	461	3.9	96.1	4.8	135	3.5	96.5	505	-44	109.6		
		15.5	508	3.0	97.0	4.3	145	3.0	97.0	520	-12	102.4		
	1 µg/mL	38.3	864	4.4	95.6	5.4	123	4.4	95.6	881	-17	101.9	98.8	5.6
		34.8	873	4.0	96.0	4.7	122	3.9	96.1	892	-18	102.1		
		31.3	888	3.5	96.5	4.5	117	3.8	96.2	820	68	92.4		
	5 µg/mL	186.9	4398	4.3	95.7	5.2	121	4.3	95.7	4365	33	99.3	101.7	2.2
		203.2	4364	4.7	95.3	5.2	114	4.5	95.5	4503	-139	103.2		
		191.9	4346	4.4	95.6	5.0	117	4.3	95.7	4466	-120	102.8		
30 min	0.5 µg/mL	15.2	367	4.2	95.8	4.6	116	3.9	96.1	388	-21	105.9	106.4	1.6
		15.1	386	3.9	96.1	4.3	117	3.7	96.3	406	-20	105.2		
		16.4	368	4.5	95.5	4.8	117	4.1	95.9	398	-30	108.2		
	1 µg/mL	32.4	692	4.7	95.3	4.9	110	4.5	95.5	728	-35	105.1	103.8	2.4
		29.8	744	4.0	96.0	4.2	105	4.0	96.0	751	-7	101.0		
		31.8	752	4.2	95.8	4.9	121	4.0	96.0	791	-39	105.2		
	5 µg/mL	152.5	3525	4.3	95.7	4.7	115	4.1	95.9	3690	-165	104.7	107.3	3.2
		161.7	3668	4.4	95.6	4.4	109	4.0	96.0	4067	-399	110.9		
		155.1	3686	4.2	95.8	4.4	112	4.0	96.0	3919	-233	106.3		
1 hr	0.5 µg/mL	17.0	350	4.9	95.1	4.5	111	4.1	95.9	414	-64	118.4	111.6	5.9
		17.4	381	4.6	95.4	4.9	117	4.2	95.8	414	-33	108.6		
		18.1	381	4.8	95.2	5.1	116	4.4	95.6	411	-30	107.9		
	1 µg/mL	35.1	719	4.9	95.1	4.8	115	4.1	95.9	847	-128	117.8	108.8	8.0
		33.5	803	4.2	95.8	4.6	117	3.9	96.1	851	-48	106.0		
		41.3	790	5.2	94.8	5.8	114	5.1	94.9	810	-20	102.5		
	5 µg/mL	190.2	3539	5.4	94.6	5.4	113	4.7	95.3	4009	-470	113.3	112.2	1.2
		179.8	3711	4.8	95.2	4.8	110	4.4	95.6	4113	-403	110.9		
		202.0	3554	5.7	94.3	5.3	104	5.1	94.9	3992	-439	112.3		
2 hr	0.5 µg/mL	13.6	320	4.3	95.7	4.1	104	4.0	96.0	342	-22	106.8	106.1	2.5
		16.4	434	3.8	96.2	4.5	129	3.5	96.5	470	-35	108.1		
		12.9	340	3.8	96.2	3.9	106	3.7	96.3	351	-11	103.3		
	1 µg/mL	34.3	720	4.8	95.2	4.7	107	4.4	95.6	783	-63	108.8	108.3	1.7
		33.1	796	4.2	95.8	4.5	116	3.9	96.1	846	-51	106.4		
		33.6	631	5.3	94.7	4.6	96	4.9	95.1	692	-61	109.6		
	5 µg/mL	162.3	4048	4.0	96.0	4.4	114	3.9	96.1	4206	-158	103.9	104.9	0.9
		173.5	4478	3.9	96.1	4.5	122	3.7	96.3	4707	-229	105.1		
		171.5	3739	4.6	95.4	4.5	103	4.3	95.7	3953	-214	105.7		

Table III-8. Abraxane, Lot C (6111355) Drug Release Analytical Data. Presented are the analytical data for Abraxane, lot C (6111355). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section II. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX	% Bound	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13	% Bound PTX_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG	SD
				= Filtrate PTX / Reservoir PTX *100	= 100 − %Unbound PTX			= Filtrate PTX_C13 / Reservoir PTX_C13 * 100	= 100 − %Unbound C13	= Fil PTX/ (1-(%Bound PTX_C13/100))	= Reservoir PTX- Unencapsulated	= (Unencapsulated/ Reservoir PTX) *100		
10 min	0.5 µg/mL	19.0	447	4.3	95.7	5.4	130	4.2	95.8	456	-9	102.0	101.7	0.2
		16.3	445	3.7	96.3	4.7	129	3.6	96.4	452	-7	101.6		
		16.5	338	4.9	95.1	5.2	109	4.8	95.2	344	-5	101.6		
	1 µg/mL	32.9	915	3.6	96.4	5.0	135	3.7	96.3	891	24	97.4	101.0	3.3
		35.6	819	4.3	95.7	5.0	120	4.2	95.8	851	-32	103.9		
		33.2	929	3.6	96.4	4.9	139	3.5	96.5	946	-17	101.8		
	5 µg/mL	202.8	4838	4.2	95.8	4.8	127	3.8	96.2	5331	-493	110.2	107.4	5.9
		163.7	4128	4.0	96.0	4.4	113	3.9	96.1	4155	-27	100.7		
		188.1	4288	4.4	95.6	4.7	120	3.9	96.1	4773	-485	111.3		
30 min	0.5 µg/mL	16.9	384	4.4	95.6	4.8	117	4.1	95.9	412	-28	107.4	109.6	2.0
		17.0	349	4.9	95.1	4.9	113	4.4	95.6	389	-40	111.4		
		15.8	339	4.7	95.3	4.8	113	4.3	95.7	373	-34	110.0		
	1 µg/mL	29.4	641	4.6	95.4	4.4	108	4.1	95.9	721	-80	112.5	110.2	2.1
		29.3	737	4.0	96.0	4.5	123	3.7	96.3	798	-61	108.2		
		29.3	588	5.0	95.0	4.3	96	4.5	95.5	647	-59	110.0		
	5 µg/mL	175.1	3544	4.9	95.1	4.8	107	4.5	95.5	3928	-383	110.8	108.1	4.2
		178.4	3727	4.8	95.2	4.7	108	4.3	95.7	4105	-379	110.2		
		175.4	4073	4.3	95.7	4.9	117	4.2	95.8	4205	-132	103.3		
1 hr	0.5 µg/mL	14.7	394	3.7	96.3	4.1	121	3.4	96.6	431	-38	109.6	103.6	5.9
		17.3	381	4.5	95.5	5.4	116	4.6	95.4	373	8	97.9		
		14.3	370	3.9	96.1	4.5	119	3.7	96.3	383	-13	103.4		
	1 µg/mL	29.4	717	4.1	95.9	4.2	112	3.8	96.2	782	-64	109.0	107.1	3.4
		29.8	786	3.8	96.2	4.3	116	3.7	96.3	811	-25	103.2		
		28.7	601	4.8	95.2	4.2	95	4.4	95.6	656	-55	109.1		
	5 µg/mL	164.2	4031	4.1	95.9	4.8	125	3.8	96.2	4297	-266	106.6	112.0	10.4
		140.6	3682	3.8	96.2	4.0	110	3.6	96.4	3881	-199	105.4		
		152.5	3159	4.8	95.2	4.3	111	3.9	96.1	3918	-759	124.0		
2 hr	0.5 µg/mL	12.4	368	3.4	96.6	3.5	109	3.2	96.8	391	-23	106.2	105.5	1.3
		13.7	480	2.9	97.1	4.0	145	2.7	97.3	499	-19	103.9		
		13.2	322	4.1	95.9	4.1	107	3.9	96.1	343	-20	106.2		
	1 µg/mL	27.8	698	4.0	96.0	4.2	109	3.8	96.2	729	-31	104.4	107.3	2.6
		23.9	700	3.4	96.6	3.6	115	3.1	96.9	766	-66	109.4		
		27.4	740	3.7	96.3	4.1	119	3.4	96.6	800	-60	108.1		
	5 µg/mL	155.3	3745	4.1	95.9	4.2	111	3.8	96.2	4103	-358	109.6	107.6	1.9
		142.6	3796	3.8	96.2	4.0	113	3.5	96.5	4018	-222	105.8		
		143.3	4318	3.3	96.7	3.7	120	3.1	96.9	4636	-318	107.4		

Table III-9. Free Paclitaxel Control Data. Displayed is the calculated % release for the paclitaxel control data. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	100.6	2.9	2.9
	1 µg/mL	101.2	7.7	7.6
	5 µg/mL	98.8	3.6	3.6
30 min	0.5 µg/mL	105.4	3.9	3.7
	1 µg/mL	106.7	3.2	3.0
	5 µg/mL	103.9	7.2	6.9
1 hr	0.5 µg/mL	106.5	0.4	0.3
	1 µg/mL	106.3	2.7	2.5
	5 µg/mL	106.5	2.2	2.1
2 hr	0.5 µg/mL	105.4	4.6	4.4
	1 µg/mL	107.1	2.1	1.9
	5 µg/mL	105.3	6.4	6.0
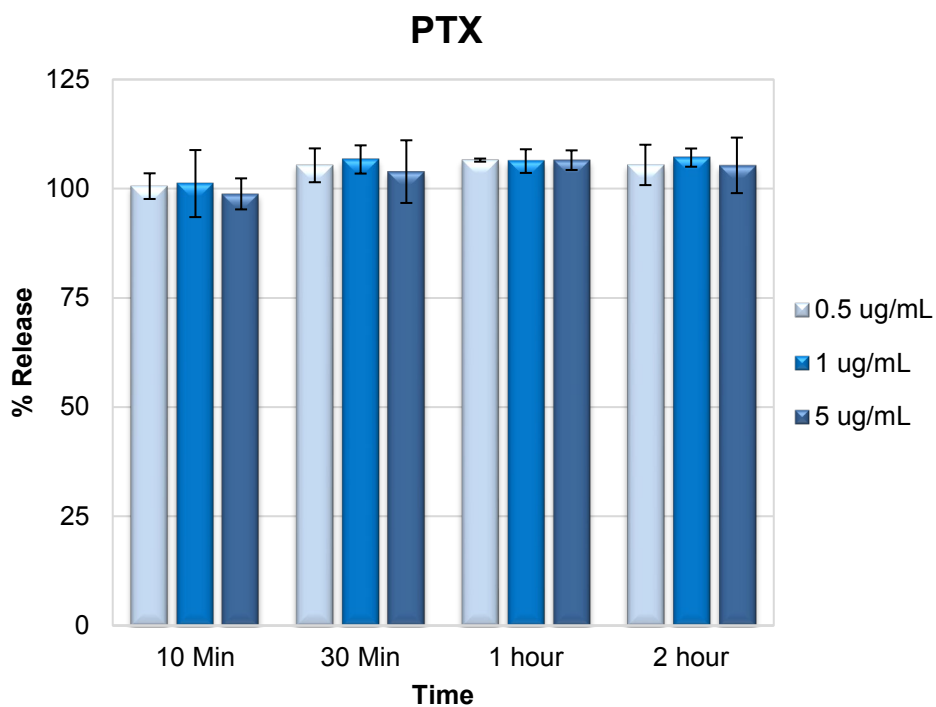
Figure III-5. Free Paclitaxel Control Data. Displayed is the calculated % release for the paclitaxel control data. (Mean $\pm$ SD, *N=3*)

Table III-10. Abraxane, Lot A (6113658) Drug Release. Displayed is the calculated % release for Abraxane, lot A (6113658). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	102.0	8.3	8.1
	1 µg/mL	101.6	4.3	4.2
	5 µg/mL	99.7	4.4	4.4
30 min	0.5 µg/mL	105.6	1.5	1.4
	1 µg/mL	106.0	1.9	1.8
	5 µg/mL	110.6	3.9	3.6
1 hrh	0.5 µg/mL	107.0	3.1	2.9
	1 µg/mL	109.9	2.8	2.6
	5 µg/mL	107.0	2.3	2.1
2 hr	0.5 µg/mL	109.3	2.3	2.1
	1 µg/mL	104.2	4.1	3.9
	5 µg/mL	105.5	3.1	3.0
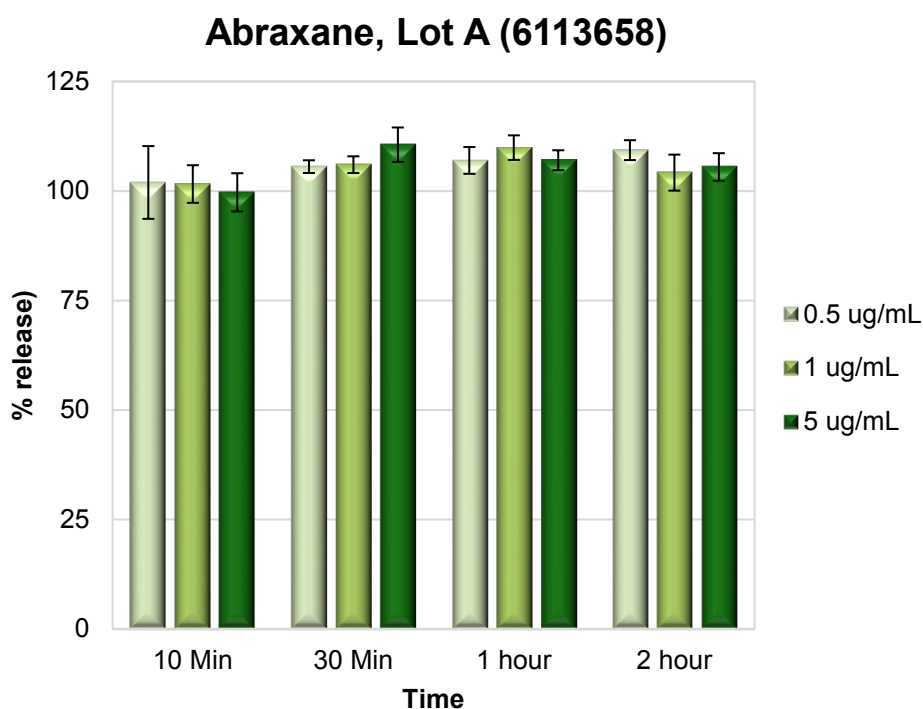
Figure III-6. Abraxane, Lot A (6113658) Drug Release. Displayed is the calculated % release for Abraxane, lot A (6113658). (Mean $\pm$ SD, *N=3*)

Table III-11. Abraxane, Lot B (6111839) Drug Release. Displayed is the calculated % release for Abraxane, lot B (6111839). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	103.8	5.3	5.1
	1 µg/mL	98.8	5.6	5.6
	5 µg/mL	101.7	2.2	2.1
30 min	0.5 µg/mL	106.4	1.6	1.5
	1 µg/mL	103.8	2.4	2.3
	5 µg/mL	107.3	3.2	3.0
1 hr	0.5 µg/mL	111.6	5.9	5.3
	1 µg/mL	108.8	8.0	7.4
	5 µg/mL	112.2	1.2	1.1
2 hr	0.5 µg/mL	106.1	2.5	2.4
	1 µg/mL	108.3	1.7	1.5
	5 µg/mL	104.9	0.9	0.9
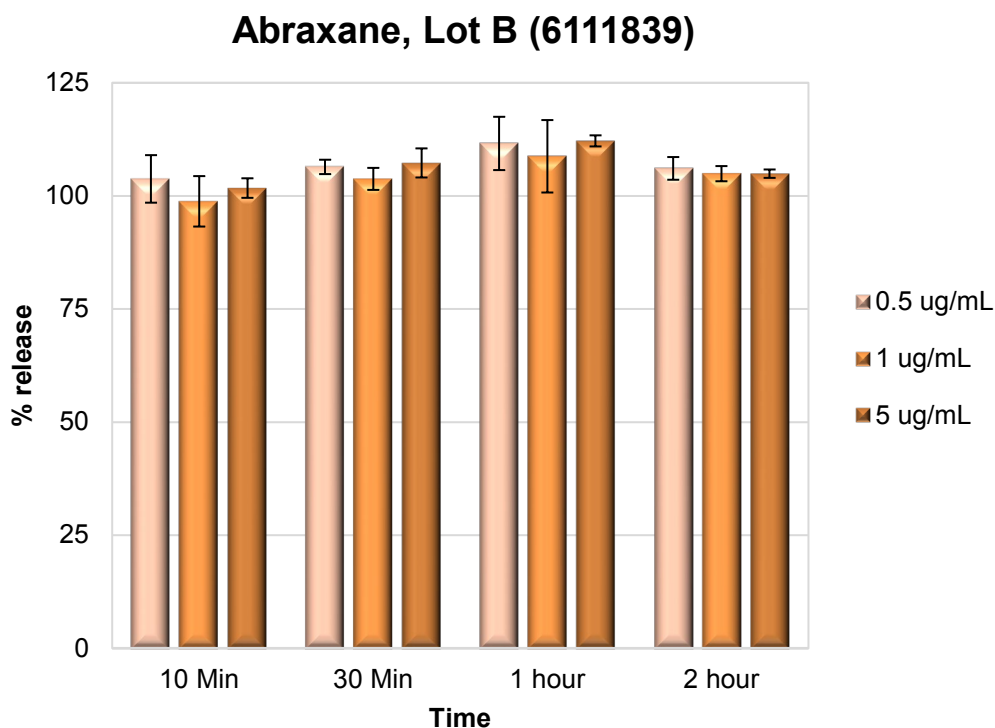
Figure III-7. Abraxane, Lot B (6111839) Drug Release. Displayed is the calculated % release for the Abraxane, lot B (6111839). (Mean $\pm$ SD, *N=3*)

Table III-12. Abraxane, Lot C (6111355) Drug Release. Displayed is the calculated % release for Abraxane, lot C (6111355). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	101.7	0.2	0.2
	1 µg/mL	101.0	3.3	3.3
	5 µg/mL	107.4	5.9	5.5
30 min	0.5 µg/mL	109.6	2.0	1.8
	1 µg/mL	110.2	2.1	1.9
	5 µg/mL	108.1	4.2	3.9
1 hr	0.5 µg/mL	103.6	5.9	5.6
	1 µg/mL	107.1	3.4	3.2
	5 µg/mL	112.0	10.4	9.3
2 hr	0.5 µg/mL	105.5	1.3	1.3
	1 µg/mL	107.3	2.6	2.4
	5 µg/mL	107.6	1.9	1.7
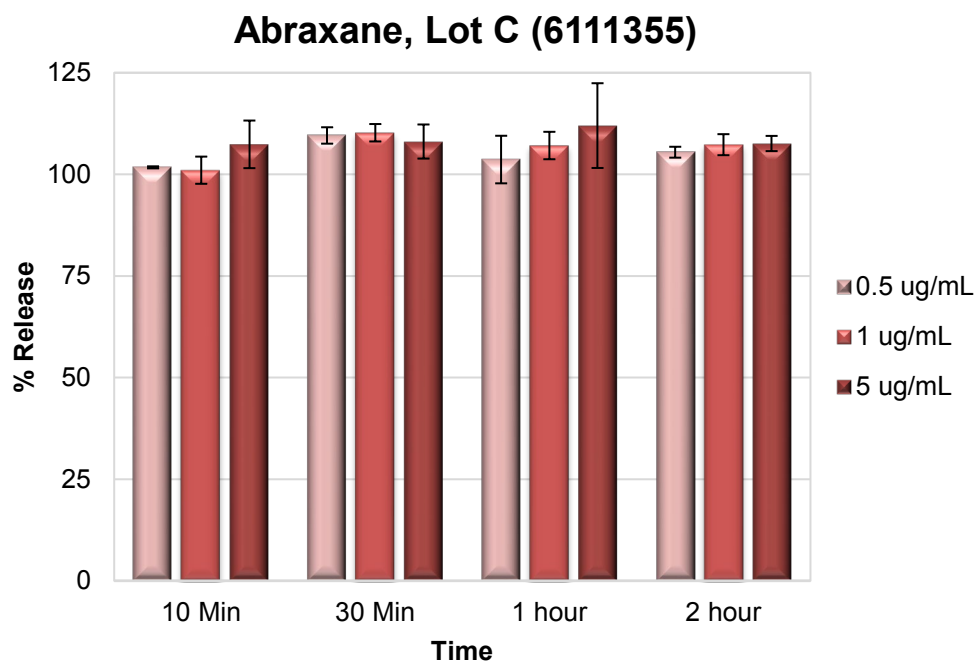
Figure III-8. Abraxane, Lot C (6111355) Drug Release. Displayed is the calculated % release for the Abraxane, lot C (6111355). (Mean $\pm$ SD, *N=3*)

Abraxane 3 Lot Comparison, % Release
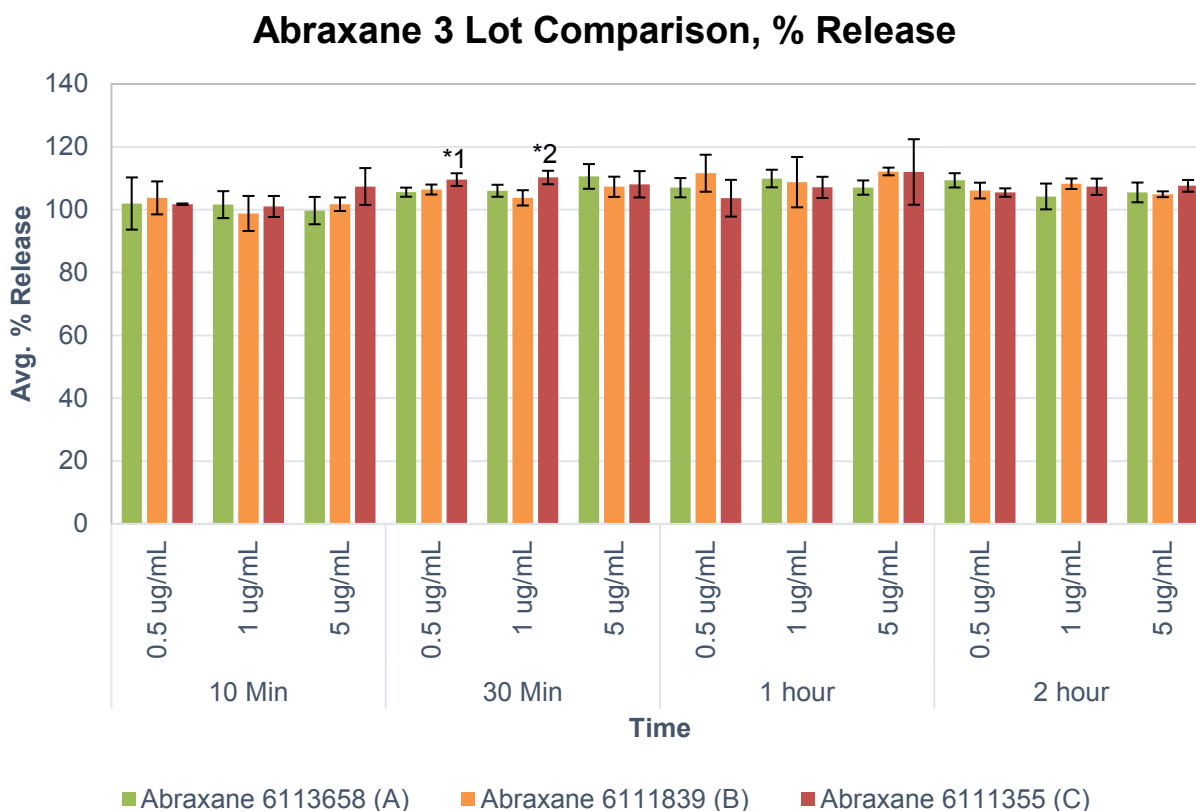
Figure III-9. Abraxane Drug Release Lot Comparison. Displayed are the 10 min-2 hr calculated % release data for the Abraxane lots 6113658, 6111839, and 6111355, also denoted as lots A, B, and C. (Mean $\pm$ SD, *N=3*), *p$\leq$0.05, ANOVA with Duncan's Multiple Range test posthoc comparisons. (*1 is statistically signficant compared to lot A. *2 is statistically signiicant compared to lot B.)

IV. In Vitro Genexol-PM Lot Comparison

Intra-Day Three Lot Comparison in Human Plasma at 37°C

Summary

The objective of this study was to compare the in vitro drug release of three lots of Genexol-PM in human plasma at 37°C over a 2 hr period. All lots had similar drug release profiles over the 2 hr period, although some instances of statistically significant differences were observed between lots.

Design and Methods

Three lots of Genexol-PM, GP31681 (lot A), GP316A1 (lot B), and GP31741 (lot C), also denoted as lots A, B, and C, respectively, were evaluated for drug release at 0.5, 1 and 5 µg/mL PTX equivalents in human plasma at 37°C over a 2 hr period, according to the SITUA method described in Section I. All three lots were run on a single day, and free paclitaxel at identical concentrations and time points was included as a control. The human plasma and protein-free plasma, PTX and PTX_C13 standard calibration curves used for this study are also provided.

Results and Discussion

The PTX and PTX_C13 standard calibration curves used for this study are presented in **Tables IV-1 to IV-4 and Figures IV-1 to IV-4**. The free paclitaxel drug release controls averaged between 104-109% release for all concentrations and time points (**Table IV-5 and IV-9, Figure IV-5**). The Genexol-PM drug release was similar for lots A-C, at approximately 100% release at the earliest 10 min time point for all concentrations, without a clear concentration-dependent or temporal trend (**Tables IV-6 to IV-8 and IV-10 to IV-12, and Figures IV-6 to IV-9**).

Table IV-1. PTX Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	891588	9.9	99
10.0 ng/mL	1861866	10.1	101
50 ng/mL	10807210	51	101
50 ng/mL	4318981	50	101
100 ng/mL	7245881	89	89
100 ng/mL	13078065	103	103
500 ng/mL	93339195	532	106
500 ng/mL	31477163	548	110
1000 ng/mL	94069689	965	96
1000 ng/mL	91479188	1120	112
5000 ng/mL	545487167	4631	93
5000 ng/mL	315697973	4398	88
10000 ng/mL	329642738	11294	113
10000 ng/mL	572768368	8825	88
QC			
QCL	1505267	11.4	114
QCL	1570620	10.6	106
QCM	14958871	100	100
QCM	13483310	103	103
QCH	88827708	1148	115
QCH	159979936	1038	104

PTX
$Y = -0.0155433 + 0.0123394 \cdot X$ $R^2 = 0.9913$ W: $1/X^2$
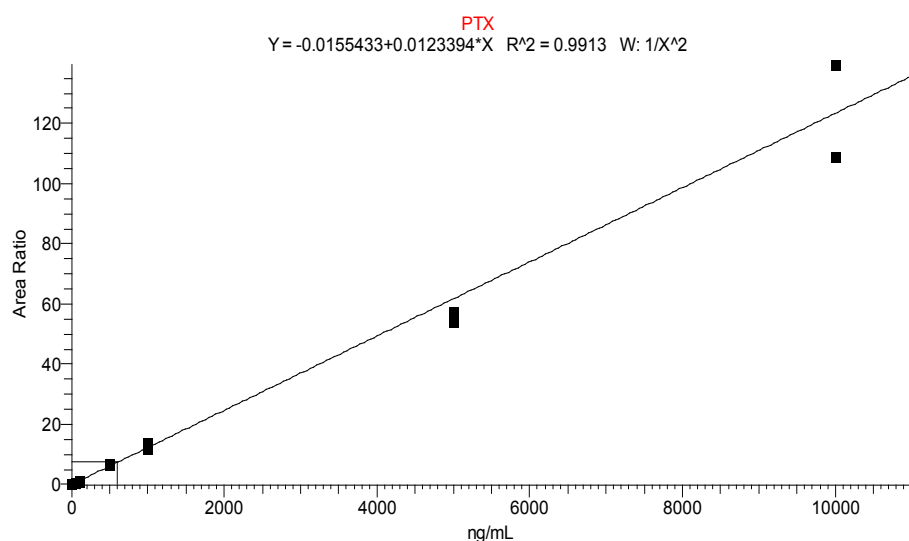
Figure IV-1. PTX Plasma Standard Curve.

Table IV-2. PTX_C13 Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	1929426	9.9	99
10.0 ng/mL	3939814	9.9	99
50 ng /mL	21703079	51	101
50 ng /mL	8940947	52	104
100 ng /mL	15430602	95	95
100 ng /mL	26826797	106	106
500 ng /mL	186629230	538	108
500 ng /mL	65266054	574	115
1000 ng /mL	194499707	1009	101
1000 ng /mL	182734752	1132	113
5000 ng /mL	1064879825	4577	92
5000 ng /mL	657950549	4641	93
10000 ng /mL	498940819	8656	87
10000 ng /mL	1130238815	8817	88
QC			
QCL	3138370	11.1	111
QCL	3515552	11.0	110
QCM	29781718	100	100
QCM	28079309	108	108
QCH	175480233	1148	115
QCH	323860812	1063	106

PTX_C13
$Y = -0.0108231 + 0.0243685 \cdot X \quad R^2 = 0.9909 \quad W: 1/X^2$
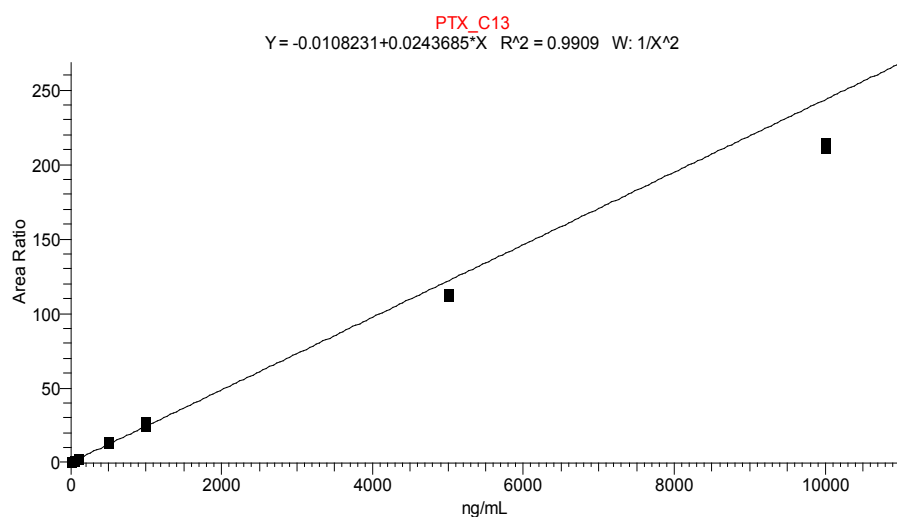
Figure IV-2. PTX_C13 Plasma Standard Curve.

Table IV-3. PTX Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.50 ng/mL	272145	0.49	99
0.50 ng/mL	321633	0.51	103
1.00 ng/mL	734658	1.06	106
1.00 ng/mL	692989	0.92	92
5.0 ng/mL	3813347	5.3	106
5.0 ng/mL	3616142	4.9	97
10.0 ng/mL	6248632	9.3	93
10.0 ng/mL	6256724	9.4	94
50.0 ng/mL	32679644	52.2	104
50.0 ng/mL	31811435	50.0	100
100.0 ng/mL	57375650	96.5	96
100.0 ng/mL	56135929	97.3	97
500.0 ng/mL	255629419	539.3	108
500.0 ng/mL	248312459	524.1	105
QC			
QCL	330510	0.55	109
QCL	290425	0.54	109
QCM	3314058	5.14	103
QCM	3531922	5.45	109
QCH	33357765	53.44	107
QCH	33807730	54.49	109

PTX
$Y = -0.000433235 + 0.0132404 \cdot X$ $R^2 = 0.9960$ W: $1/X^2$
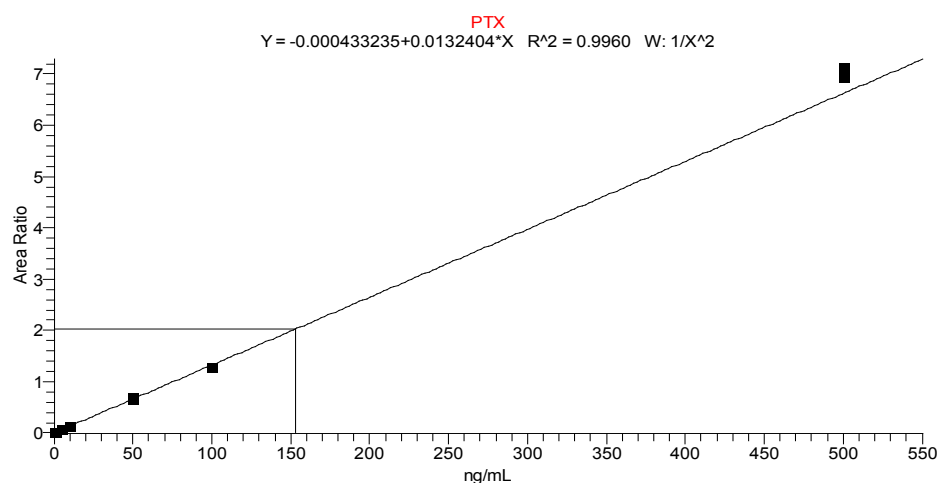
Figure IV-3. PTX Protein-Free Plasma Standard Curve.

Table IV-4. PTX_C13 Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.50 ng/mL	555410	0.48	95
0.50 ng/mL	696986	0.53	105
1.00 ng/mL	1400820	0.97	97
1.00 ng/mL	1607918	1.01	101
5.0 ng/mL	8336679	5.6	112
5.0 ng/mL	7703681	5.0	99
10.0 ng/mL	13842264	9.9	99
10.0 ng/mL	12697723	9.2	92
50.0 ng/mL	66570982	51.1	102
50.0 ng/mL	67148133	50.7	101
100.0 ng/mL	111574129	90.3	90
100.0 ng/mL	114867234	95.8	96
500.0 ng/mL	510754115	518.5	104
500.0 ng/mL	525294804	533.5	107
QC			
QCL	644965	0.51	101
QCL	631385	0.56	112
QCM	7018322	5.2	105
QCM	7324849	5.4	109
QCH	66890952	51.6	103
QCH	69926110	54.2	108

PTX_C13
$Y = -0.000665521 + 0.027514 \cdot X \quad R^2 = 0.9954 \quad W: 1/X^2$

Figure IV-4. PTX-C13 Protein-Free Plasma Standard Curve.

Table IV-5. Free Paclitaxel Control Analytical Data. Presented are the analytical data for the free paclitaxel controls. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX	% Bound	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 STD (ng/mL)	%Unbound PTX_C13	% Bound PTX_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG % Release	SD
				=Filtrate PTX /Reservoir PTX *100	=100-%Unbound PTX			=Filtrate PTX C13/Reservoir PTX C13 * 100	=100 - %Unbound PTX C13	=Filtrate PTX/ (1-(%Bound PTX C13/100))	=Reservoir PTX-Unencapsulated	=(Unencapsulated/ Reservoir PTX) *100		
10 min	0.5 µg/mL	27.8	557	5.0	95.0	5.4	112	4.8	95.2	578	-21	103.8	104.0	0.5
		26.1	550	4.7	95.3	5.1	111	4.6	95.4	569	-19	103.5		
		26.2	608	4.3	95.7	4.9	120	4.1	95.9	636	-27	104.5		
	1 µg/mL	59.2	1100	5.4	94.6	5.6	114	4.9	95.1	1203	-103	109.3	105.0	5.8
		57.4	1293	4.4	95.6	5.4	119	4.5	95.5	1272	21	98.3		
		57.7	1120	5.2	94.8	5.3	110	4.8	95.2	1201	-81	107.2		
	5 µg/mL	285.0	5569	5.1	94.9	5.5	109	5.1	94.9	5618	-49	100.9	107.4	7.4
		253.3	4258	5.9	94.1	5.2	101	5.2	94.8	4913	-655	115.4		
		218.9	4220	5.2	94.8	4.5	91	4.9	95.1	4476	-256	106.1		
30 min	0.5 µg/mL	25.0	552	4.5	95.5	5.0	115	4.3	95.7	579	-27	105.0	108.0	3.4
		24.4	477	5.1	94.9	4.7	102	4.6	95.4	533	-55	111.6		
		28.2	541	5.2	94.8	5.1	106	4.9	95.1	581	-40	107.4		
	1 µg/mL	66.4	1097	6.1	93.9	6.4	109	5.9	94.1	1133	-36	103.3	104.5	1.1
		54.7	1008	5.4	94.6	5.5	107	5.2	94.8	1063	-55	105.4		
		59.6	1599	3.7	96.3	5.7	161	3.6	96.4	1674	-76	104.7		
	5 µg/mL	278.0	5042	5.5	94.5	5.4	104	5.1	94.9	5420	-378	107.5	108.7	1.1
		257.1	4880	5.3	94.7	5.2	107	4.8	95.2	5356	-476	109.8		
		278.7	5224	5.3	94.7	5.1	105	4.9	95.1	5693	-469	109.0		
1 hr	0.5 µg/mL	24.2	517	4.7	95.3	4.8	108	4.4	95.6	548	-31	106.0	107.1	2.6
		29.3	505	5.8	94.2	5.9	111	5.3	94.7	556	-51	110.1		
		25.6	599	4.3	95.7	5.1	126	4.1	95.9	631	-32	105.3		
	1 µg/mL	64.8	1089	5.9	94.1	6.1	111	5.5	94.5	1174	-85	107.8	107.3	0.7
		61.1	1050	5.8	94.2	5.7	106	5.4	94.6	1129	-79	107.6		
		61.8	1045	5.9	94.1	5.9	107	5.6	94.4	1113	-68	106.5		
	5 µg/mL	282.1	5371	5.3	94.7	5.4	109	4.9	95.1	5722	-351	106.5	104.4	5.5
		277.2	5299	5.2	94.8	5.3	110	4.8	95.2	5748	-449	108.5		
		253.9	5224	4.9	95.1	5.1	104	5.0	95.0	5123	101	98.1		
2 hr	0.5 µg/mL	24.2	447	5.4	94.6	4.7	99	4.7	95.3	513	-66	114.7	108.4	6.9
		24.0	495	4.8	95.2	5.0	105	4.8	95.2	500	-5	101.0		
		24.5	489	5.0	95.0	4.6	100	4.6	95.4	535	-46	109.4		
	1 µg/mL	53.6	1099	4.9	95.1	5.3	105	5.0	95.0	1070	29	97.4	105.7	7.2
		45.0	844	5.3	94.7	4.3	88	4.9	95.1	923	-79	109.3		
		54.6	1035	5.3	94.7	5.1	106	4.8	95.2	1143	-108	110.4		
	5 µg/mL	200.4	5101	3.9	96.1	3.9	101	3.9	96.1	5155	-55	101.1	107.3	5.9
		231.7	4279	5.4	94.6	4.6	97	4.8	95.2	4829	-550	112.9		
		224.0	4593	4.9	95.1	4.4	96	4.5	95.5	4952	-359	107.8		

Table IV-6. Genexol-PM, Lot A (GP31681) Drug Release Analytical Data. Presented are the analytical data for Genexol-PM lot A (GP31681). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX =Filtrate PTX /Reservoir PTX *100	% Bound =100-%Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 STD (ng/mL)	%Unbound PTX_C13 =Filtrate PTX C13/Reservoir PTX C13 * 100	% Bound PTX_C13 =100 - %Unbound PTX C13	Unencapsulated (ng/mL) =Filtrate PTX/ (1-(%Bound PTX C13/100))	Encapsulated (ng/mL) =Reservoir PTX-Unencapsulated	% Release = (Unencapsulated/ Reservoir PTX) *100	AVG % Release	SD
10 min	0.5 µg/mL	14.5	369	3.9	96.1	3.9	108	3.6	96.4	402	-34	109.1	106.9	2.5
		15.4	374	4.1	95.9	4.4	114	3.8	96.2	401	-27	107.3		
		15.6	378	4.1	95.9	4.3	108	4.0	96.0	394	-16	104.2		
	1 µg/mL	32.5	734	4.4	95.6	4.6	112	4.1	95.9	788	-53	107.3	104.4	3.6
		34.0	865	3.9	96.1	4.8	122	3.9	96.1	868	-3	100.4		
		29.9	828	3.6	96.4	4.1	121	3.4	96.6	873	-45	105.4		
	5 µg/mL	178.4	3754	4.8	95.2	5.1	114	4.4	95.6	4021	-268	107.1	105.7	2.0
		153.2	3456	4.4	95.6	4.5	107	4.2	95.8	3605	-149	104.3		
		154.2	5088	3.0	97.0	4.1	111	3.7	96.3	4138	950	81.3*		
30 min	0.5 µg/mL	15.9	369	4.3	95.7	4.3	106	4.1	95.9	387	-19	105.1	105.4	0.7
		15.1	336	4.5	95.5	4.4	105	4.2	95.8	357	-21	106.1		
		14.7	357	4.1	95.9	4.2	107	3.9	96.1	375	-17	104.9		
	1 µg/mL	31.2	738	4.2	95.8	4.7	116	4.0	96.0	774	-36	104.9	101.8	3.7
		36.2	766	4.7	95.3	5.4	111	4.8	95.2	748	18	97.7		
		36.0	754	4.8	95.2	4.9	107	4.6	95.4	776	-22	102.9		
	5 µg/mL	144.1	3491	4.1	95.9	4.2	111	3.8	96.2	3819	-328	109.4	107.8	2.2
		155.4	3389	4.6	95.4	4.7	112	4.2	95.8	3685	-296	108.7		
		168.3	3592	4.7	95.3	4.9	110	4.4	95.6	3785	-193	105.4		
1 hr	0.5 µg/mL	17.0	359	4.7	95.3	4.4	103	4.3	95.7	395	-36	110.0	108.9	2.5
		14.5	298	4.9	95.1	4.4	101	4.4	95.6	330	-31	110.5		
		16.4	376	4.4	95.6	4.6	113	4.1	95.9	398	-23	106.0		
	1 µg/mL	34.2	784	4.4	95.6	5.0	112	4.4	95.6	771	12	98.4	105.2	5.9
		31.7	732	4.3	95.7	4.5	112	4.0	96.0	793	-61	108.3		
		33.0	744	4.4	95.6	4.4	109	4.1	95.9	810	-65	108.8		
	5 µg/mL	168.3	3606	4.7	95.3	4.9	112	4.4	95.6	3849	-243	106.7	109.0	2.1
		168.2	3502	4.8	95.2	4.9	111	4.4	95.6	3835	-333	109.5		
		168.8	3309	5.1	94.9	4.9	106	4.6	95.4	3665	-356	110.8		
2 hr	0.5 µg/mL	12.7	361	3.5	96.5	3.8	110	3.5	96.5	365	-4	101.2	101.5	0.3
		14.7	362	4.1	95.9	4.4	111	4.0	96.0	369	-6	101.7		
		13.2	348	3.8	96.2	3.8	102	3.7	96.3	354	-6	101.7		
	1 µg/mL	27.9	770	3.6	96.4	4.2	110	3.9	96.1	724	46	94.0	99.6	5.4
		30.0	730	4.1	95.9	4.5	109	4.1	95.9	730	0	99.9		
		26.9	727	3.7	96.3	3.8	107	3.5	96.5	762	-34	104.7		
	5 µg/mL	147.7	3186	4.6	95.4	4.5	104	4.3	95.7	3454	-269	108.4	107.7	4.6
		149.2	3195	4.7	95.3	4.6	100	4.5	95.5	3283	-88	102.7		
		143.6	3315	4.3	95.7	4.0	104	3.9	96.1	3708	-393	111.8		

*Data not used for calculating average and standard deviation.

Table IV-7. Genexol-PM, Lot B (GP316A1) Drug Release Analytical Data. Presented are the analytical data for Genexol-PM lot B (GP316A1). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

Time	Conc	Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX =Filtrate PTX /Reservoir PTX *100	% Bound =100-%Unbound PTX	Filtrate PTX_C13 ng/mL	Reservoir PTX_C13 STD ng/mL	%Unbound PTX_C13 =Filtrate PTX C13/Reservoir PTX C13 * 100	% Bound PTX_C13 =100 - %Unbound PTX C13	Unencapsulated ng/mL =Filtrate PTX/ (1-(%Bound PTX C13/100))	Encapsulated ng/mL =Reservoir PTX- Unencapsulated	% Release =(Unencapsulated/ Reservoir PTX) *100	AVG % Release	SD
10 min	0.5 µg/mL	16.0	408	3.9	96.1	4.6	122	3.8	96.2	424	-15	103.8	104.6	2.9
		16.7	340	4.9	95.1	5.3	116	4.5	95.5	367	-27	107.9		
		16.0	405	3.9	96.1	4.8	123	3.9	96.1	414	-9	102.2		
	1 µg/mL	30.6	787	3.9	96.1	4.3	115	3.8	96.2	809	-22	102.9	104.3	1.5
		32.5	729	4.5	95.5	4.5	107	4.2	95.8	772	-43	105.9		
		33.2	753	4.4	95.6	4.5	105	4.2	95.8	784	-31	104.1		
	5 µg/mL	159.3	2840	5.6	94.4	4.4	91	4.8	95.2	3315	-475	116.7	109.7	6.3
		164.3	3602	4.6	95.4	4.6	108	4.2	95.8	3877	-275	107.6		
		162.3	3733	4.3	95.7	4.7	114	4.2	95.8	3909	-177	104.7		
30 min	0.5 µg/mL	16.5	374	4.4	95.6	4.8	113	4.3	95.7	384	-10	102.8	101.3	3.9
		16.1	382	4.2	95.8	5.2	120	4.4	95.6	370	12	96.9		
		17.6	380	4.6	95.4	5.3	118	4.5	95.5	396	-16	104.3		
	1 µg/mL	33.5	730	4.6	95.4	4.5	107	4.2	95.8	797	-66	109.1	105.2	3.9
		34.4	693	5.0	95.0	5.1	104	4.9	95.1	702	-9	101.3		
		32.1	772	4.2	95.8	4.4	110	3.9	96.1	813	-41	105.3		
	5 µg/mL	173.3	3636	4.8	95.2	4.6	104	4.4	95.6	3917	-281	107.7	100.9	6.2
		170.9	3577	4.8	95.2	5.4	107	5.0	95.0	3415	162	95.5		
		161.0	3448	4.7	95.3	4.3	92	4.7	95.3	3431	16	99.5		
1 hr	0.5 µg/mL	15.8	400	3.9	96.1	4.9	114	4.3	95.7	367	32	91.9	102.7	9.4
		13.6	344	4.0	96.0	4.2	114	3.7	96.3	370	-26	107.6		
		15.3	363	4.2	95.8	4.6	117	3.9	96.1	394	-31	108.6		
	1 µg/mL	29.9	766	3.9	96.1	4.3	106	4.1	95.9	730	36	95.2	104.4	7.9
		34.0	737	4.6	95.4	4.6	109	4.2	95.8	802	-66	108.9		
		30.8	704	4.4	95.6	4.2	105	4.0	96.0	767	-63	109.0		
	5 µg/mL	162.9	3203	5.1	94.9	4.7	98	4.8	95.2	3419	-217	106.8	107.1	2.7
		159.2	3472	4.6	95.4	4.8	111	4.4	95.6	3636	-163	104.7		
		147.3	3214	4.6	95.4	4.5	108	4.2	95.8	3534	-321	110.0		
2 hr	0.5 µg/mL	15.1	355	4.2	95.8	4.4	111	3.9	96.1	386	-31	108.7	108.9	1.9
		13.8	332	4.2	95.8	4.2	107	3.9	96.1	355	-23	107.1		
		14.0	362	3.9	96.1	4.0	115	3.5	96.5	402	-40	110.9		
	1 µg/mL	29.3	671	4.4	95.6	3.9	94	4.2	95.8	702	-31	104.6	101.7	2.7
		27.2	673	4.0	96.0	3.9	97	4.0	96.0	681	-7	101.1		
		29.4	709	4.1	95.9	4.1	98	4.2	95.8	704	5	99.3		
	5 µg/mL	147.2	3321	4.4	95.6	4.2	100	4.2	95.8	3485	-164	104.9	107.7	2.9
		142.4	3482	4.1	95.9	3.9	102	3.8	96.2	3739	-257	107.4		
		135.6	3194	4.2	95.8	3.7	96	3.8	96.2	3535	-341	110.7		

Table IV-8. Genexol-PM, Lot C (GP31741) Drug Release Analytical Data. Presented are the analytical data for Genexol-PM lot C (GP31741). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX =Filtrate PTX /Reservoir PTX *100	% Bound =100- %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 STD (ng/mL)	%Unbound PTX_C13 =Filtrate PTX C13/Reservoir PTX C13 * 100	% Bound PTX_C13 =100 - %Unbound PTX C13	Unencapsulated (ng/mL) "=Filtrate PTX/(1-(%Bound PTX C13/100))	Encapsulated (ng/mL) =Resevoir PTX-Unencapsulated	% Release =(Unencapsulated/ Reservoir PTX)*100	AVG % Release	SD
10 min	0.5 µg/mL	17.4	375	4.6	95.4	4.8	107	4.4	95.6	392	-17	104.4	104.0	3.1
		15.8	387	4.1	95.9	4.6	113	4.0	96.0	390	-3	100.8		
		17.2	387	4.4	95.6	4.5	108	4.2	95.8	414	-27	106.9		
	1 µg/mL	29.9	751	4.0	96.0	4.3	110	3.9	96.1	768	-17	102.2	101.8	6.4
		30.8	627	4.9	95.1	4.6	101	4.5	95.5	677	-50	108.0		
		33.9	866	3.9	96.1	4.6	113	4.1	95.9	825	41	95.2		
	5 µg/mL	191.8	3684	5.2	94.8	5.6	112	5.0	95.0	3860	-175	104.8	103.2	2.2
		176.9	3626	4.9	95.1	5.0	103	4.8	95.2	3652	-26	100.7		
		170.2	3669	4.6	95.4	4.9	109	4.5	95.5	3822	-152	104.2		
30 min	0.5 µg/mL	15.0	368	4.1	95.9	4.1	110	3.7	96.3	405	-37	110.2	109.3	0.8
		9.3	335	2.8	97.2	2.6	102	2.6	97.4	363	-29	108.7		
		16.3	327	5.0	95.0	4.4	97	4.6	95.4	356	-29	109.0		
	1 µg/mL	34.5	662	5.2	94.8	4.9	106	4.6	95.4	745	-82	112.4	105.3	7.1
		38.2	704	5.4	94.6	5.5	107	5.2	94.8	740	-36	105.2		
		33.6	774	4.3	95.7	4.8	109	4.4	95.6	760	14	98.2		
	5 µg/mL	185.4	3475	5.3	94.7	5.3	105	5.0	95.0	3698	-222	106.4	106.4	1.4
		171.3	3599	4.8	95.2	4.8	106	4.5	95.5	3777	-178	105.0		
		173.4	3643	4.8	95.2	4.7	107	4.4	95.6	3927	-284	107.8		
1 hr	0.5 µg/mL	15.4	339	4.5	95.5	4.6	109	4.2	95.8	364	-25	107.5	99.8	6.8
		16.9	388	4.4	95.6	4.9	106	4.6	95.4	366	22	94.3		
		16.1	412	3.9	96.1	4.5	112	4.0	96.0	403	9	97.8		
	1 µg/mL	28.7	760	3.8	96.2	4.1	107	3.9	96.1	746	14	98.2	101.2	2.9
		30.5	723	4.2	95.8	4.5	112	4.1	95.9	752	-29	104.1		
		32.6	730	4.5	95.5	4.9	110	4.4	95.6	740	-10	101.4		
	5 µg/mL	170.5	4259	4.0	96.0	4.7	129	3.7	96.3	4621	-362	108.5	104.2	4.4
		182.6	3629	5.0	95.0	5.5	113	4.8	95.2	3782	-153	104.2		
		161.5	3398	4.8	95.2	5.0	105	4.8	95.2	3390	8	99.8		
2 hr	0.5 µg/mL	15.3	342	4.5	95.5	4.1	101	4.1	95.9	376	-34	110.0	108.1	1.8
		14.1	354	4.0	96.0	3.8	104	3.7	96.3	381	-27	107.6		
		14.6	363	4.0	96.0	3.9	104	3.8	96.2	386	-24	106.5		
	1 µg/mL	26.3	688	3.8	96.2	3.7	105	3.5	96.5	742	-54	107.8	107.9	3.6
		26.3	643	4.1	95.9	3.6	99	3.7	96.3	717	-74	111.5		
		28.3	676	4.2	95.8	3.7	93	4.0	96.0	705	-30	104.4		
	5 µg/mL	145.5	3297	4.4	95.6	4.4	102	4.4	95.6	3343	-45	101.4	105.3	3.8
		135.0	3389	4.0	96.0	4.0	106	3.8	96.2	3577	-188	105.5		
		153.0	3133	4.9	95.1	4.7	104	4.5	95.5	3414	-281	109.0		

Table IV-9. Free Paclitaxel Control Data. Displayed is the calculated % release for the paclitaxel control data. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 Min	0.5 µg/mL	104.0	0.5	0.5
	1 µg/mL	105.0	5.8	5.6
	5 µg/mL	107.4	7.4	6.8
30 Min	0.5 µg/mL	108.0	3.4	3.1
	1 µg/mL	104.5	1.1	1.0
	5 µg/mL	108.7	1.1	1.1
1 hour	0.5 µg/mL	107.1	2.6	2.4
	1 µg/mL	107.3	0.7	0.7
	5 µg/mL	104.4	5.5	5.3
2 hour	0.5 µg/mL	108.4	6.9	6.4
	1 µg/mL	105.7	7.2	6.8
	5 µg/mL	107.3	5.9	5.5
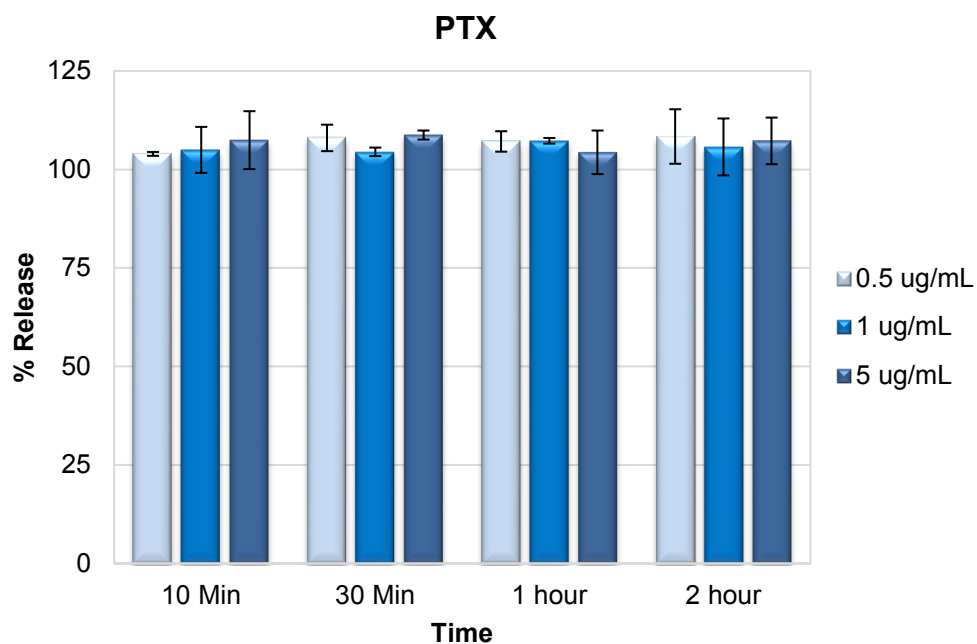
Figure IV-5. Free Paclitaxel Control Data. Displayed is the calculated % release for the paclitaxel control data. (Mean $\pm$ SD, *N=3*)

Table IV-10. Genexol-PM, Lot A (GP31681) Drug Release. Displayed is the calculated % release for Genexol-PM, lot A (GP31681). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 Min	0.5 µg/mL	106.9	2.5	2.3
	1 µg/mL	104.4	3.6	3.4
	5 µg/mL	105.7	2.0	1.9
30 Min	0.5 µg/mL	105.4	0.7	0.6
	1 µg/mL	101.8	3.7	3.7
	5 µg/mL	107.8	2.2	2.0
1 hour	0.5 µg/mL	108.9	2.5	2.3
	1 µg/mL	105.2	5.9	5.6
	5 µg/mL	109.0	2.1	1.9
2 hour	0.5 µg/mL	101.5	0.3	0.3
	1 µg/mL	99.6	5.4	5.4
	5 µg/mL	107.7	4.6	4.3
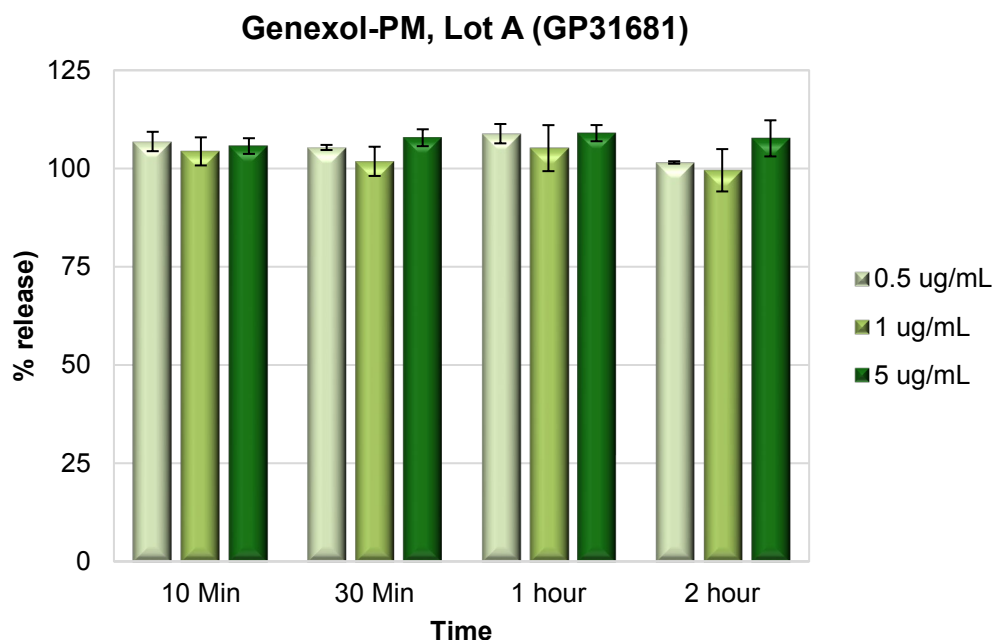
Figure IV-6. Genexol-PM, Lot A (GP31681) Drug Release. Displayed is the calculated % release for Genexol-PM, lot A (GP31681). (Mean ± SD, *N=3*)

Table IV-11. Genexol-PM, Lot B (GP316A1) Drug Release. Displayed is the calculated % release for Genexol-PM, lot B (GP316A1). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 Min	0.5 µg/mL	104.6	2.9	2.8
	1 µg/mL	104.3	1.5	1.5
	5 µg/mL	109.7	6.3	5.7
30 Min	0.5 µg/mL	101.3	3.9	3.9
	1 µg/mL	105.2	3.9	3.7
	5 µg/mL	100.9	6.2	6.2
1 hour	0.5 µg/mL	102.7	9.4	9.1
	1 µg/mL	104.4	7.9	7.6
	5 µg/mL	107.1	2.7	2.5
2 hour	0.5 µg/mL	108.9	1.9	1.8
	1 µg/mL	101.7	2.7	2.6
	5 µg/mL	107.7	2.9	2.7
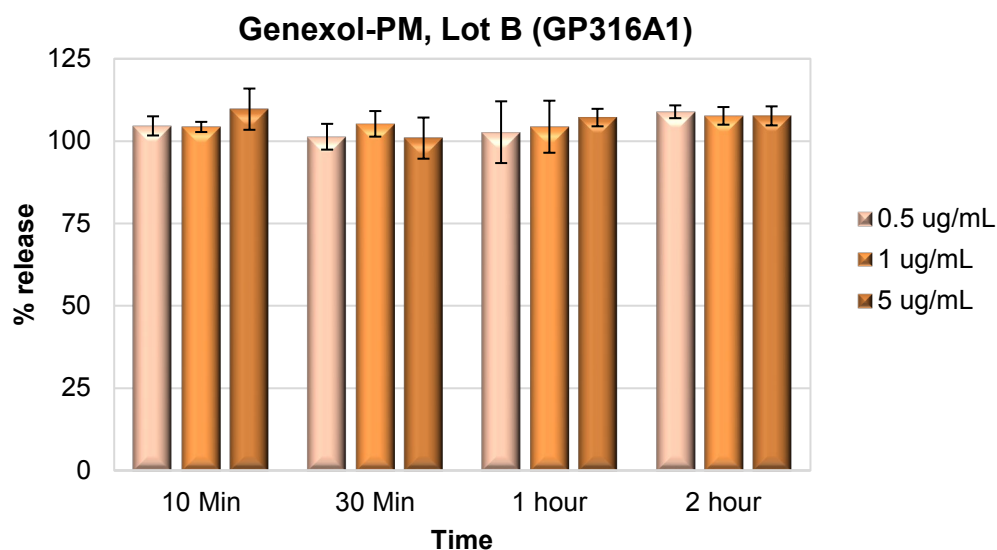
Figure IV-7. Genexol-PM, Lot B (GP316A1) Drug Release. Displayed is the calculated % release for the Genexol-PM, lot B (GP316A1). (Mean $\pm$ SD, *N=3*)

Table IV-12. Genexol-PM, Lot C (GP31741) Drug Release. Displayed is the calculated % release for Genexol-PM, lot C (GP31741). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 Min	0.5 µg/mL	104.0	3.1	3.0
	1 µg/mL	101.8	6.4	6.3
	5 µg/mL	103.2	2.2	2.1
30 Min	0.5 µg/mL	109.3	0.8	0.7
	1 µg/mL	105.3	7.1	6.8
	5 µg/mL	106.4	1.4	1.3
1 hour	0.5 µg/mL	99.8	6.8	6.8
	1 µg/mL	101.2	2.9	2.9
	5 µg/mL	104.2	4.4	4.2
2 hour	0.5 µg/mL	108.1	1.8	1.6
	1 µg/mL	107.9	3.6	3.3
	5 µg/mL	105.3	3.8	3.6
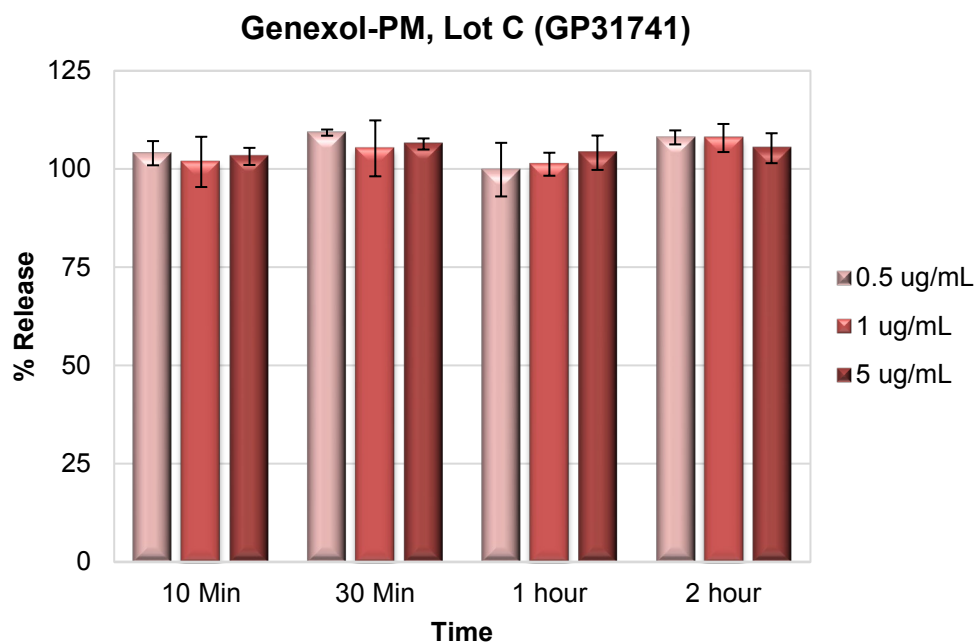
Figure IV-8. Genexol-PM, Lot C (GP31741) Drug Release. Displayed is the calculated % release for the Genexol-PM, lot C (GP31741). (Mean $\pm$ SD, *N=3*)

Genexol 3 Lot Comparison, % Release
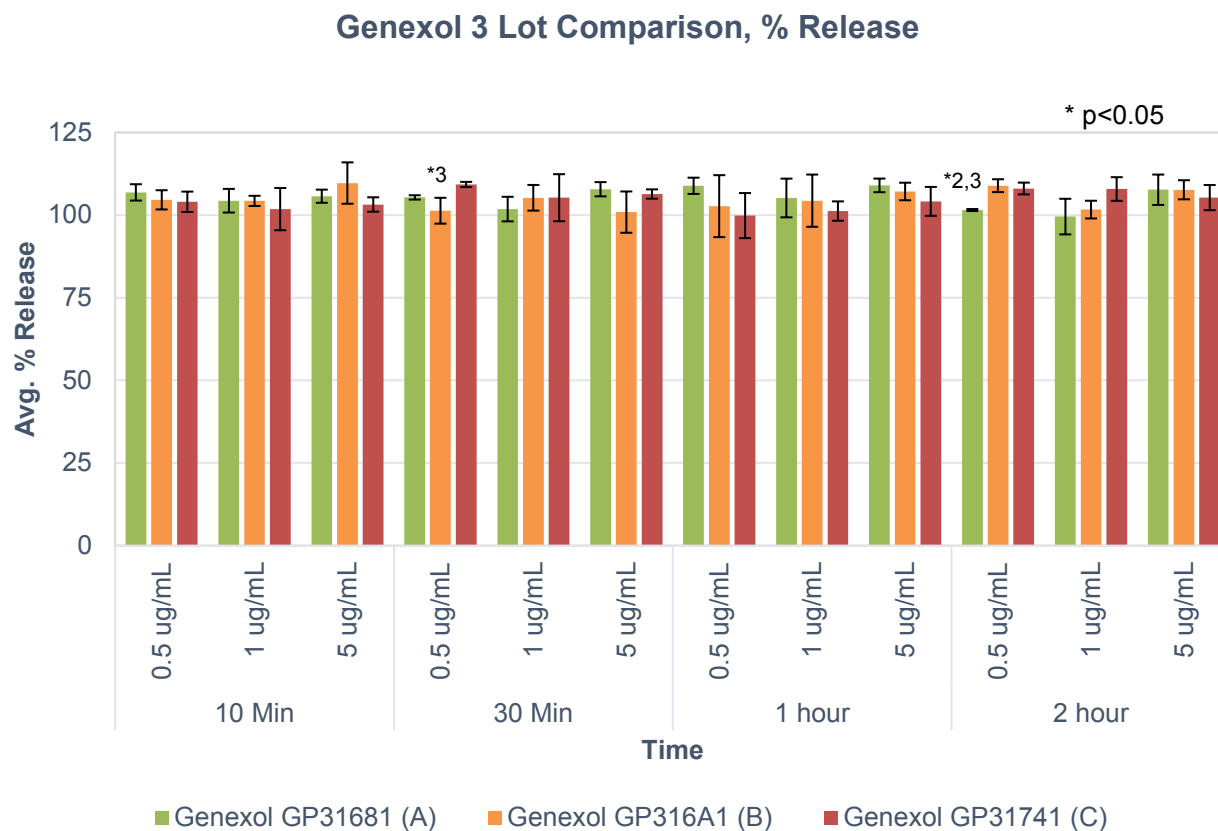
Figure IV-9. Genexol-PM Drug Release Lot Comparison. Displayed are the 10 min-2 hr calculated % release data for the Genexol-PM lots A, B, C. (Mean $\pm$ SD, *N=3*), *p$\leq$0.05, ANOVA with Duncan's Multiple Range test posthoc comparisons. (*2,3 is statistically signficant compared to lots B and C. *3 is statistically signiicant compared to lot C.)

V. In Vitro Abraxane vs. Genexol-PM Comparisons

Intra-day Comparison in Human Plasma at 37°C

Summary
The objective of this study was to compare the in vitro drug release of a single lot of Genexol-PM to that of a single lot of Abraxane in human plasma at 37°C over a 1 hr period. All lots had similar drug release profiles over the 1 hr period, although some instances of statistically significant differences were observed between lots.

Design and Methods
A single lot of Abraxane, 6113658 (lot A), and a single lot of Genexol-PM, GP31681 (lot A), were evaluated for drug release at 0.5, 1 and 5 μg/mL PTX equivalents in human plasma at 37°C over a 1 hr period, according to the SITUA method described in Section I. Both lots were run on a single day, and free paclitaxel at identical concentrations and time points was included as a control. The human plasma and protein-free plasma, PTX and PTX_C13 standard calibration curves used for this study are also provided.

Results & Conclusions
The PTX and PTX_C13 standard calibration curves used for this study are presented in **Tables V-1 to V-4 and Figures V-1 to V-4**. The free paclitaxel controls averaged between 97-105% release for all concentrations and time points (**Tables V-5 andV-8, and Figure V-5**). The Abraxane and Genexol-PM drug release was similar, at approximately 100% release at the earliest 10 min time point, without a clear concentration-dependent or temporal trend (**Tables V-6, V-7, V-9 and V-10, and Figures V-6 to V-8**).

Table V-1. PTX Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	1074029	10.7	107
10.0 ng/mL	130452	9.5	95
50 ng/mL	774671	46	91
50 ng/mL	789650	50	99
100 ng/mL	2947084	105	105
100 ng/mL	2356452	101	101
500 ng/mL	31483661	511	102
500 ng/mL	40788379	523	105
1000 ng/mL	70053213	1004	100
1000 ng/mL	65808206	1074	107
5000 ng/mL	139742605	5136	103
5000 ng/mL	320705576	4777	96
10000 ng/mL	283553328	9789	98
10000 ng/mL	291831454	9247	92
QC			
QCL	411589	10.8	108
QCL	358391	10.1	101
QCM	6732188	105	105
QCM	5079270	102	102
QCH	69878960	1028	103
QCH	15108358	1081	108

PTX
$Y = -0.00666809 + 0.0115253 * X$ $R^2 = 0.9968$ W: $1/X^2$
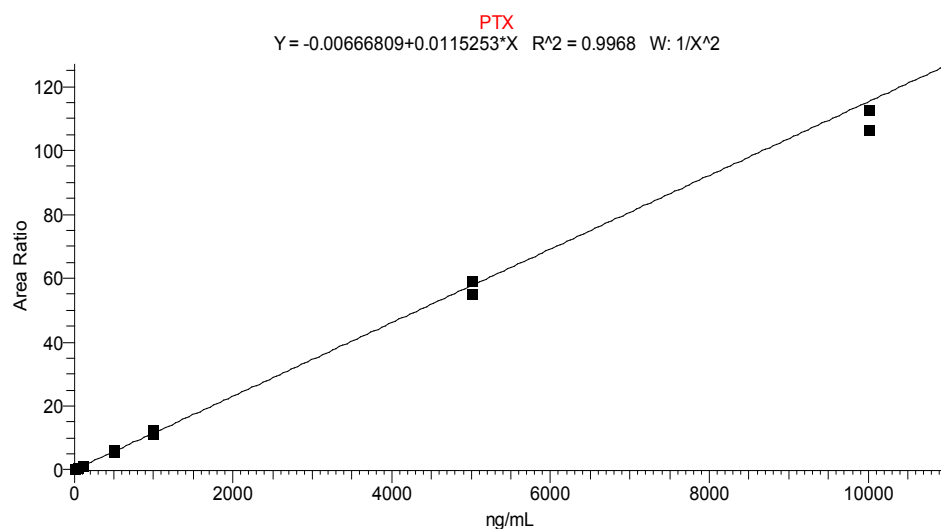
Figure V-1. PTX Plasma Standard Curve.

Table V-2. PTX_C13 Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	2223137	9.8	98
10.0 ng/mL	308830	9.9	99
50 ng/mL	1876718	51	102
50 ng/mL	1831945	53	107
100 ng/mL	6162758	102	102
100 ng/mL	5294894	105	105
500 ng/mL	66811598	508	102
500 ng/mL	86561746	520	104
1000 ng/mL	151891792	1020	102
1000 ng/mL	141675790	1083	108
5000 ng/mL	288219771	4967	99
5000 ng/mL	641952341	4483	90
10000 ng/mL	552249426	8940	89
10000 ng/mL	617973958	9182	92
QC			
QCL	905975	10.6	106
QCL	812837	10.1	101
QCM	14431124	105	105
QCM	11306292	106	106
QCH	143901293	992	99
QCH	31928062	1070	107

PTX_C13
$Y = -0.00100548 + 0.0245781*X$ $R^2 = 0.9956$ W: $1/X^2$
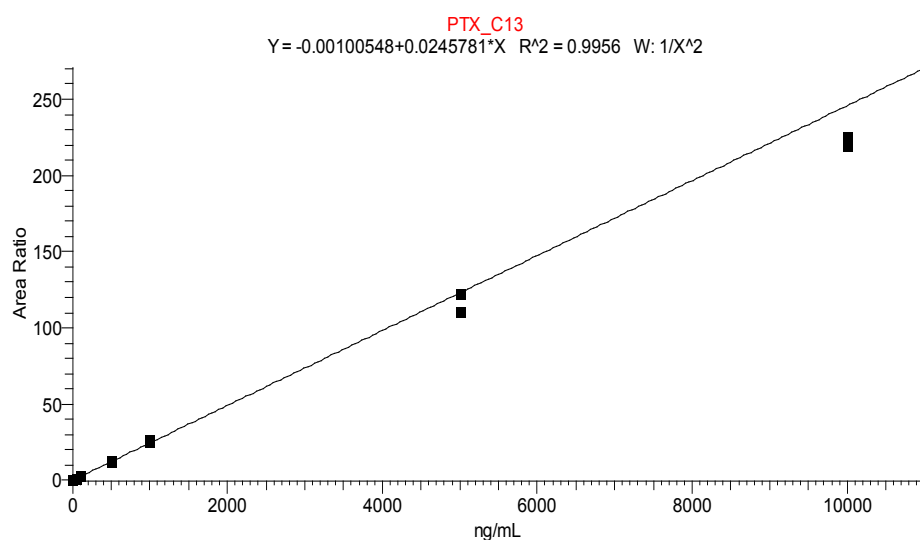
Figure V-2. PTX_C13 Plasma Standard Curve.

Table V-3. PTX Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.50 ng/mL	227061	0.50	100
0.50 ng/mL	265408	0.50	101
1.00 ng/mL	308557	0.92	92
1.00 ng/mL	444262	1.05	105
5.00 ng/mL	2463592	5.17	103
5.00 ng/mL	1504167	5.03	101
10.0 ng/mL	3125567	10.6	106
10.0 ng/mL	3929754	10.1	101
50.0 ng/mL	15347027	51.8	104
50.0 ng/mL	20348672	55.1	110
100.0 ng/mL	27329627	90.5	90
100.0 ng/mL	38632869	88.8	89
500.0 ng/mL	202520371	508.3	102
500.0 ng/mL	184253572	487.4	97
QC			
QCL	207407	0.50	100
QCL	208881	0.51	101
QCM	2090276	5.35	107
QCM	2090633	5.35	107
QCH	21883930	54.2	108
QCH	20802768	50.9	102

PTX
$Y = -0.000444785 + 0.0131622 \cdot X$ $R^2 = 0.9952$ W: $1/X^2$
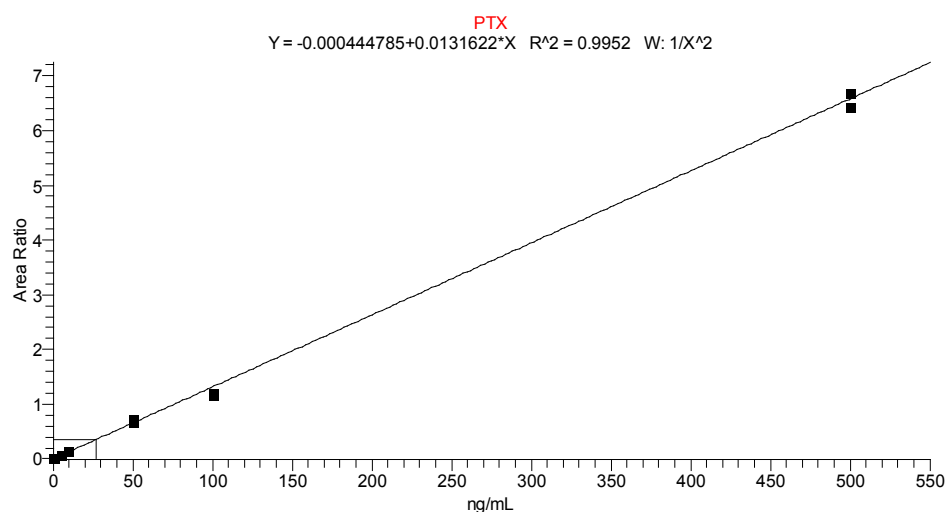
Figure V-3. PTX Protein-Free Plasma Standard Curve.

Table V-4. PTX_C13 Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.50 ng/mL	530229	0.53	106
0.50 ng/mL	569981	0.49	98
1.00 ng/mL	672049	0.93	93
1.00 ng/mL	891044	0.98	98
5.00 ng/mL	5331034	5.30	106
5.00 ng/mL	3281241	5.20	104
10.0 ng/mL	6318292	10.1	101
10.0 ng/mL	8128641	9.9	99
50.0 ng/mL	33888077	54.5	109
50.0 ng/mL	42960443	55.4	111
100.0 ng/mL	56787660	89.6	90
100.0 ng/mL	83081564	91.0	91
500.0 ng/mL	409050837	489.3	98
500.0 ng/mL	384696664	485.0	97
QC			
QCL	456221	0.50	100
QCL	493665	0.54	108
QCM	4457220	5.41	108
QCM	4363514	5.30	106
QCH	44800945	52.9	106
QCH	44674761	52.0	104

PTX_C13

$Y = -0.000235644 + 0.0276154 \cdot X$ $R^2 = 0.9943$ W: $1/X^2$
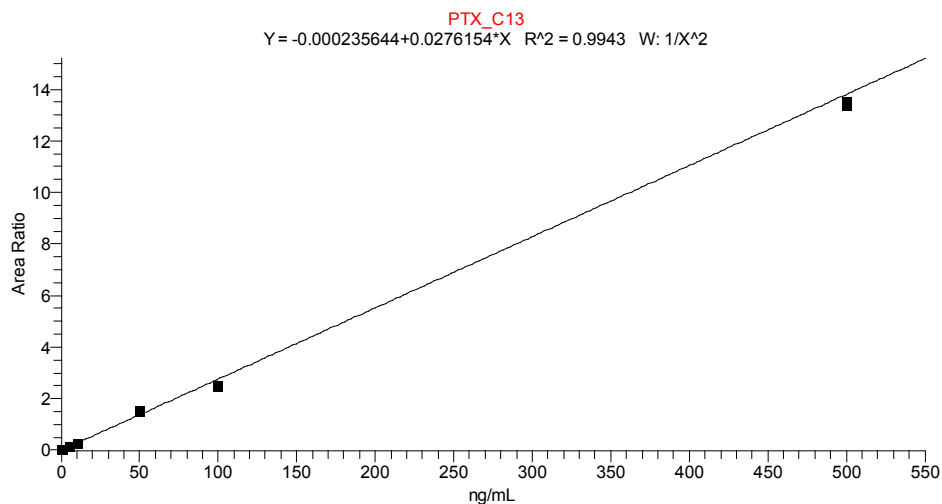
Figure V-4. PTX_C13 Protein-Free Plasma Standard Curve.

Table V-5. Free Paclitaxel Control Analytical Data. Presented are the analytical data for the free paclitaxel controls. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX	% Bound	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13	% Bound PTX_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG	SD
				= Filtrate PTX / Reservoir PTX *100	= 100 − %Unbound PTX			= Filtrate PTX_C13 / Reservoir PTX_C13 * 100	= 100 − %Unbound C13	= Fil PTX/ (1-(%Bound PTX_C13/100))	= Reservoir PTX- Unencapsulated	= (Unencapsulated/ Reservoir PTX) *100		
10 min	0.5 µg/mL	27.0	521	5.2	94.8	5.5	109	5.0	95.0	540.0	-19	103.7	104.7	1.1
		30.9	546	5.7	94.3	5.9	110	5.4	94.6	577.1	-32	105.8		
		30.0	564	5.3	94.7	5.6	111	5.1	94.9	590.1	-26	104.6		
	1 µg/mL	62.2	1148	5.4	94.6	5.8	104	5.6	94.4	1114.5	33	97.1	97.3	0.7
		61.1	1138	5.4	94.6	6.1	111	5.6	94.4	1100.7	38	96.7		
		64.2	1099	5.8	94.2	6.5	110	6.0	94.0	1078.1	21	98.1		
	5 µg/mL	318.4	5813	5.5	94.5	6.2	112	5.5	94.5	5781.4	32	99.4	100.4	3.3
		437.5	5381	8.1	91.9	8.3	101	8.3	91.7	5278.2	103	98.1		
		312.8	5559	5.6	94.4	5.8	106	5.5	94.5	5715.0	-156	102.8		
30 min	0.5 µg/mL	29.8	548	5.4	94.6	5.7	109	5.2	94.8	571.4	-24	104.3	101.0	4.0
		58.7	572	10.3	89.7	11.6	110	10.6	89.4	552.6	20	96.6		
		31.9	564	5.7	94.3	6.1	109	5.6	94.4	575.0	-11	102.0		
	1 µg/mL	74.7	1092	6.8	93.2	7.3	103	7.1	92.9	1047.7	45	95.9	96.4	2.9
		76.4	1134	6.7	93.3	7.2	107	6.8	93.2	1129.2	5	99.6		
		71.9	1102	6.5	93.5	7.1	103	7.0	93.0	1033.6	68	93.8		
	5 µg/mL	314.8	5398	5.8	94.2	6.3	106	6.0	94.0	5267.1	131	97.6	98.9	1.1
		309.3	5101	6.1	93.9	5.9	97	6.1	93.9	5067.8	34	99.3		
		347.1	5687	6.1	93.9	6.4	104	6.1	93.9	5667.4	20	99.6		
60 min	0.5 µg/mL	26.2	574	4.6	95.4	5.3	123	4.3	95.7	610.3	-37	106.4	103.0	4.0
		29.9	542	5.5	94.5	6.0	112	5.3	94.7	563.7	-21	103.9		
		24.0	562	4.3	95.7	4.8	110	4.3	95.7	553.8	8	98.5		
	1 µg/mL	68.0	1169	5.8	94.2	6.5	109	5.9	94.1	1145.8	23	98.0	102.0	3.5
		61.2	1167	5.2	94.8	5.9	119	5.0	95.0	1223.7	-57	104.9		
		62.3	1098	5.7	94.3	6.2	112	5.5	94.5	1131.2	-33	103.0		
	5 µg/mL	333.1	5002	6.7	93.3	6.2	104	5.9	94.1	5642.6	-641	112.8	101.6	9.7
		276.1	5306	5.2	94.8	5.5	103	5.4	94.6	5121.7	184	96.5		
		287.7	5559	5.2	94.8	5.6	103	5.4	94.6	5314.1	245	95.6		

Table V-6. Abraxane, Lot A (6113658) Drug Release Analytical Data. Presented are the analytical data for Abraxane, lot A (6113658). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 − %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated	% Release = (Unencapsulated/ Reservoir PTX) *100	AVG	SD
10 min	0.5 µg/mL	22.0	476	4.6	95.4	5.8	122	4.8	95.2	463.3	12	97.4	98.9	1.8
		19.0	431	4.4	95.6	5.4	121	4.5	95.5	424.4	7	98.4		
		21.9	416	5.3	94.7	6.0	114	5.2	94.8	419.4	-4	100.9		
	1 µg/mL	39.8	816	4.9	95.1	5.3	113	4.7	95.3	852.2	-36	104.4	105.2	1.1
		48.2	803	6.0	94.0	6.4	114	5.6	94.4	855.0	-52	106.4		
		42.0	841	5.0	95.0	5.8	121	4.8	95.2	881.6	-41	104.9		
	5 µg/mL	212.9	4057	5.25	94.8	5.6	107	5.2	94.8	4064.2	-7	100.2	99.7	0.9
		200.7	4122	4.9	95.1	5.6	114	4.9	95.1	4063.1	59	98.6		
		173.0	4209	4.11	95.9	4.7	116	4.1	95.9	4218.4	-9	100.2		
30 min	0.5 µg/mL	18.2	363	5.0	95.0	5.2	111	4.7	95.3	390.0	-27	107.3	103.9	4.8
		19.1	403	4.7	95.3	5.4	113	4.8	95.2	396.6	6	98.4		
		20.8	414	5.0	95.0	5.5	115	4.7	95.3	439.7	-25	106.1		
	1 µg/mL	46.1	851	5.4	94.6	6.2	117	5.3	94.7	867.8	-17	102.0	100.4	1.4
		41.6	798	5.2	94.8	5.9	113	5.3	94.7	792.3	5	99.3		
		44.5	880	5.1	94.9	6.2	123	5.1	94.9	879.6	1	99.9		
	5 µg/mL	239.4	4042	5.9	94.1	6.8	108	6.3	93.7	3783.1	259	93.6	100.5	6.1
		228.7	3748	6.10	93.9	6.2	106	5.8	94.2	3943.9	-196	105.2		
		212.2	3959	5.36	94.6	5.9	113	5.2	94.8	4066.8	-107	102.7		
60 min	0.5 µg/mL	19.1	416	4.6	95.4	5.6	123	4.5	95.5	423.0	-7	101.7	100.6	1.7
		19.1	386	4.9	95.1	5.5	113	4.9	95.1	390.9	-5	101.4		
		20.3	440	4.6	95.4	5.6	120	4.7	95.3	433.6	6	98.6		
	1 µg/mL	40.0	840	4.8	95.2	5.4	120	4.5	95.5	884.8	-45	105.4	102.3	6.2
		37.2	866	4.3	95.7	5.2	114	4.5	95.5	823.4	42	95.1		
		39.1	858	4.6	95.4	5.5	129	4.3	95.7	912.0	-54	106.3		
	5 µg/mL	184.5	4438	4.2	95.8	4.9	116	4.2	95.8	4368.1	70	98.4	95.6	2.98
		173.6	4048	4.3	95.7	4.8	108	4.5	95.5	3878.9	169	95.8		
		181.1	4139	4.4	95.6	5.2	110	4.7	95.3	3827.6	312	92.5		

Table V-7. Genexol-PM, Lot A (GP31681) Drug Release Analytical Data. Presented are the analytical data for Genexol-PM, lot A (GP31681). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 − %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated	% Release = (Unencapsulated/ Reservoir PTX) *100	AVG	SD
10 min	0.5 µg/mL	20.6	439	4.7	95.3	5.1	107	4.8	95.2	432.9	6	98.7	100.6	4.5
		17.7	410	4.3	95.7	4.5	109	4.1	95.9	433.5	-24	105.7		
		17.8	446	4.0	96.0	5.0	123	4.1	95.9	433.8	12	97.3		
	1 µg/mL	37.2	1007	3.7	96.3	5.2	128	4.0	96.0	927.5	80	92.1	96.3	4.6
		45.2	870	5.2	94.8	6.3	117	5.4	94.6	832.9	37	95.7		
		38.3	842	4.6	95.4	5.3	117	4.5	95.5	851.8	-10	101.2		
	5 µg/mL	210.9	4350	4.8	95.2	6.1	123	4.9	95.1	4294.5	56	98.7	99.0	0.3
		214.8	4236	5.1	94.9	5.7	111	5.1	94.9	4203.6	32	99.2		
		216.7	4289	5.1	94.9	5.7	112	5.1	94.9	4242.8	47	98.9		
30 min	0.5 µg/mL	20.3	456	4.4	95.6	4.9	111	4.4	95.6	457.6	-1	100.3	97.8	4.4
		20.6	427	4.8	95.2	5.6	108	5.2	94.8	396.1	31	92.7		
		21.3	428	5.0	95.0	5.8	118	5.0	95.0	429.4	-2	100.4		
	1 µg/mL	40.3	845	4.8	95.2	5.7	114	5.0	95.0	807.0	38	95.5	97.1	2.8
		43.7	861	5.1	94.9	6.0	112	5.3	94.7	821.4	39	95.4		
		38.5	868	4.4	95.6	5.5	124	4.4	95.6	870.7	-3	100.3		
	5 µg/mL	203.4	4659	4.4	95.6	5.7	143	4.0	96.0	5092.7	-433	109.3	103.2	5.3
		239.2	4181	5.7	94.3	6.4	113	5.7	94.3	4209.3	-28	100.7		
		206.5	4164	5.0	95.0	5.7	114	5.0	95.0	4143.7	20	99.5		
60 min	0.5 µg/mL	17.9	439	4.1	95.9	4.6	109	4.2	95.8	426.5	13	97.1	101.2	3.67
		17.5	405	4.3	95.7	4.7	112	4.2	95.8	412.9	-8	102.0		
		19.2	409	4.7	95.3	5.4	120	4.5	95.5	427.2	-18	104.4		
	1 µg/mL	37.0	844	4.4	95.6	5.2	113	4.6	95.4	806.4	38	95.6	101.3	6.1
		37.4	826	4.5	95.5	5.2	117	4.5	95.5	832.1	-6	100.7		
		32.5	836	3.9	96.1	4.6	127	3.6	96.4	901.1	-65	107.8		
	5 µg/mL	205.9	3172	6.5	93.5	6.1	102	6.0	94.0	3453.0	-281	108.9	100.8	7.1
		176.4	4033	4.4	95.6	4.8	106	4.6	95.4	3846.3	187	95.4		
		180.8	4382	4.1	95.9	4.7	112	4.2	95.8	4298.9	83	98.1		

Table V-8. Free Paclitaxel Control Data. Displayed is the calculated % release for the paclitaxel control data. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	104.7	1.1	1.0
	1 µg/mL	97.3	0.7	0.7
	5 µg/mL	100.4	3.3	3.3
30 min	0.5 µg/mL	101.0	4.0	3.9
	1 µg/mL	96.4	2.9	3.0
	5 µg/mL	98.9	1.1	1.1
60 min	0.5 µg/mL	103.0	4.0	3.9
	1 µg/mL	102.0	3.5	3.5
	5 µg/mL	101.6	9.7	9.5
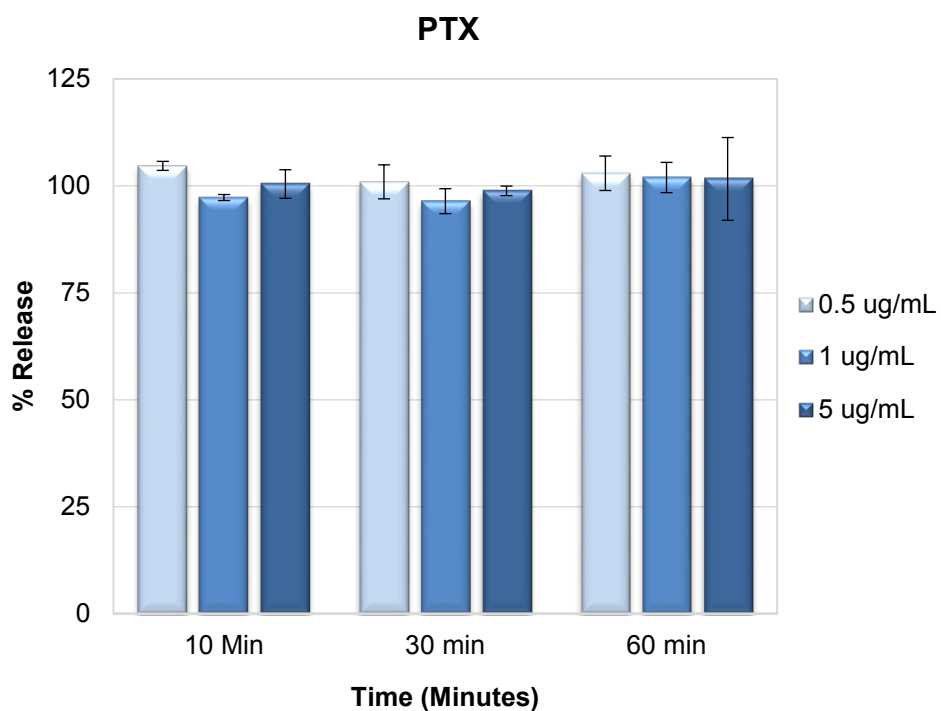
Figure V-5. Free Paclitaxel Control Data. Displayed is the calculated % release for the paclitaxel control data. (Mean $\pm$ SD, *N=3*)

Table V-9. Abraxane, Lot A (6113658) Drug Release. Displayed is the calculated % release for Abraxane, lot A (6113658). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	98.9	1.8	1.8
	1 µg/mL	105.2	1.1	1.0
	5 µg/mL	99.7	0.9	0.9
30 min	0.5 µg/mL	103.9	4.8	4.6
	1 µg/mL	100.4	1.4	1.4
	5 µg/mL	100.5	6.1	6.1
60 min	0.5 µg/mL	100.6	1.7	1.7
	1 µg/mL	102.3	6.2	6.1
	5 µg/mL	95.6	3.0	3.1
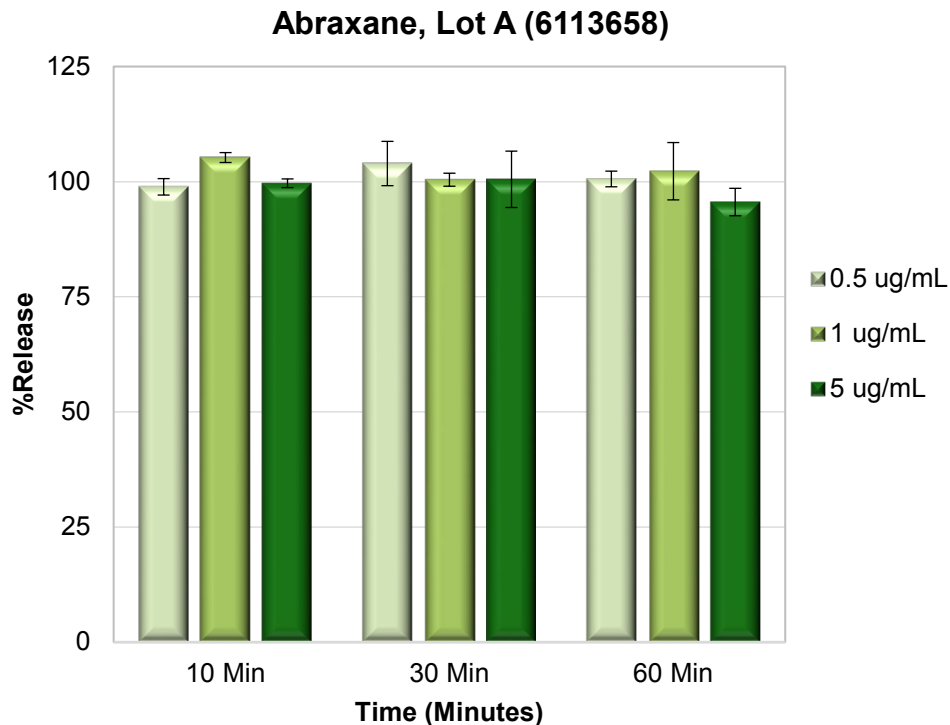
Figure V-6. Abraxane, Lot A (6113658) Drug Release. Displayed is the calculated % release for the Abraxane, lot A (6113658). (Mean $\pm$ SD, *N=3*)

Table V-10. Genexol-PM, Lot A (GP31681) Drug Release. Displayed is the calculated % release for Genexol-PM, lot A (GP31681). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	100.6	4.5	4.5
	1 µg/mL	96.3	4.6	4.7
	5 µg/mL	99.0	0.3	0.3
30 min	0.5 µg/mL	97.8	4.4	4.5
	1 µg/mL	97.1	2.8	2.9
	5 µg/mL	103.2	5.3	5.2
60 min	0.5 µg/mL	101.2	3.7	3.6
	1 µg/mL	101.3	6.1	6.0
	5 µg/mL	100.8	7.1	7.1
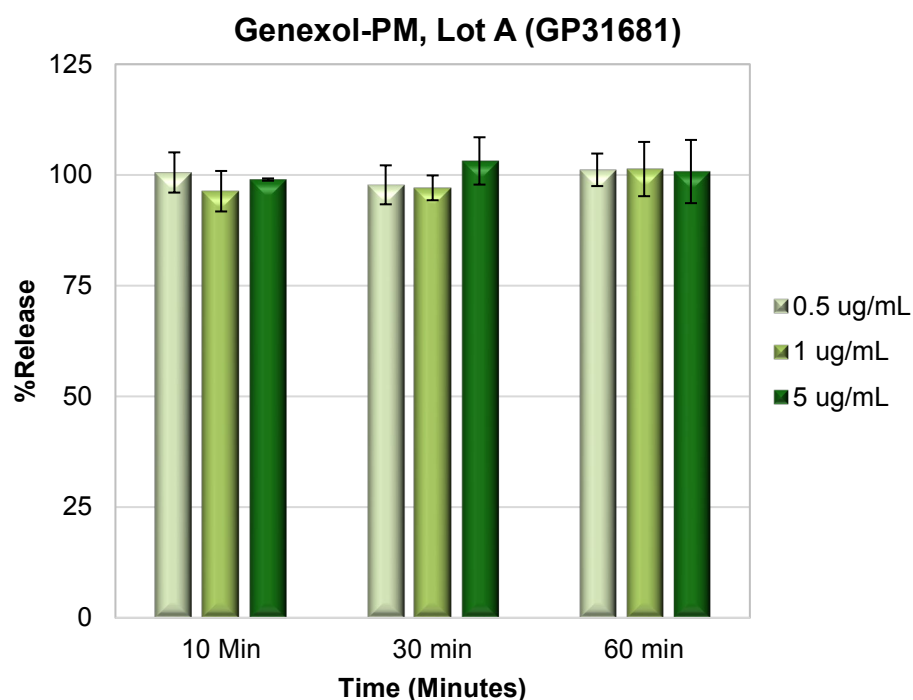
Figure V-7. Genexol-PM, Lot A (GP31681) Drug Release. Displayed is the calculated % release for Genexol-PM, lot A (GP31681). (Mean $\pm$ SD, *N=3*)

Abraxane vs. Genexol-PM, % Release
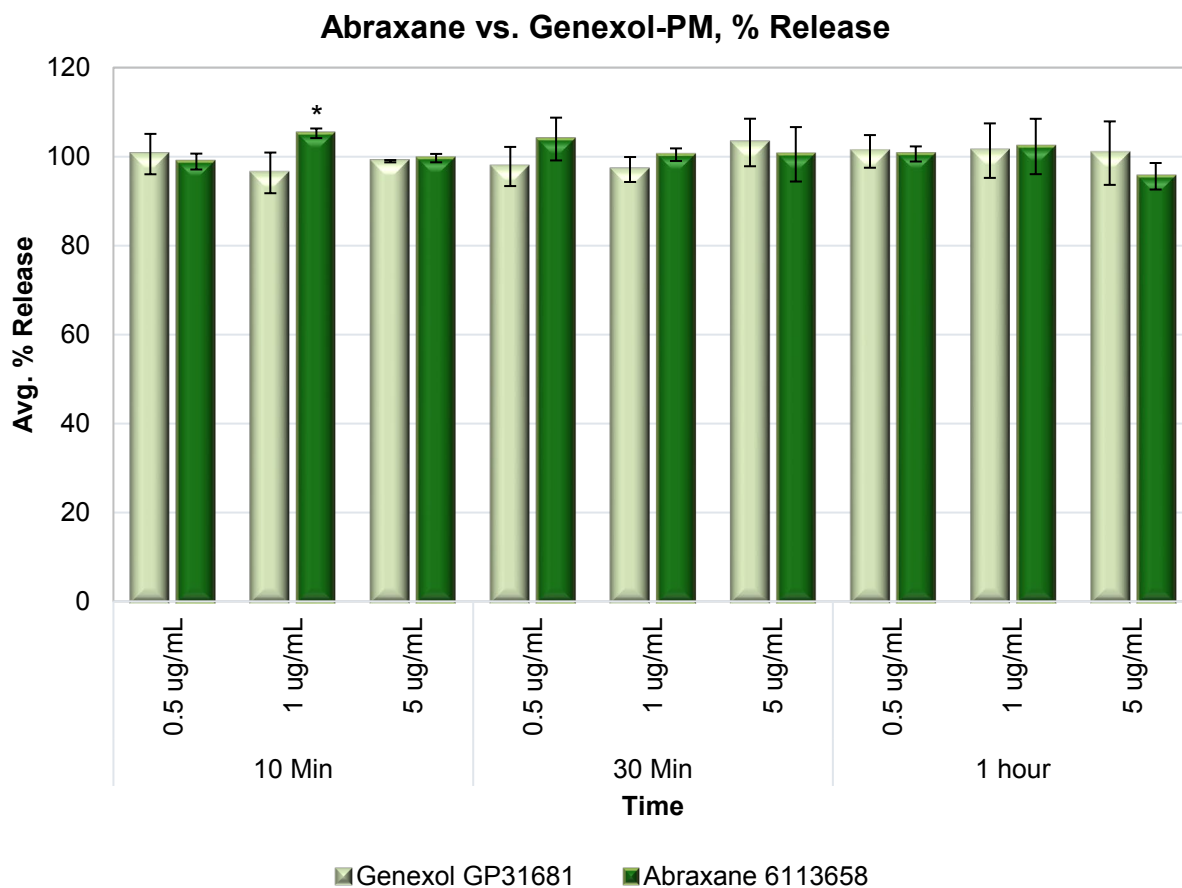
Figure V-8. Abraxane vs. Genexol-PM Drug Release Comparison. Displayed are the 10–60 min calculated % release data for the Abraxane and Genexol-PM lots 6113658 and GP31681, respectively. (Mean $\pm$ SD, *N=3*), *p$\leq$0.05, Student's t-test

Intra-day Comparison in Human Plasma at 37°C, 5 and 25 µg/mL

Summary
The objective of this study was to compare the in vitro drug release of a single lot of Genexol-PM to that of a single lot of Abraxane in human plasma at 37°C over a 4 hr period at a higher concentration than the previous intra-day study, at 5 and 25 µg/mL PTX equivalents. All lots had similar drug release profiles over the 4 hr period, although some instances of statistically significant differences were observed between lots.

Design and Methods
A single lot of Abraxane, 6113658 (lot A), and a single lot of Genexol-PM, GP31681 (lot A), were evaluated for drug release at 5 and 25 µg/mL PTX equivalents in human plasma at 37°C over a 4 hr period, according to the SITUA method described in Section I. Both lots were run on a single day, and free paclitaxel at identical concentrations and time points was included as a control. The human plasma and protein-free plasma, PTX and PTX_C13 standard calibration curves used for this study are also provided.

Results & Conclusions
The PTX and PTX_C13 standard calibration curves used for this study are presented in **Tables V-11 to V-14 and Figures V-9 to V-12**. The free paclitaxel controls averaged between 96-107% release for all concentrations and time points (**Tables V-15 and V-18, and Figure V-13**). The Abraxane and Genexol-PM drug release was similar, at approximately 100% release at the earliest 10 min time point, without a clear concentration-dependent ot temporal trend (**Tables V-16, V-17, V-19 and V-20, and Figures V-14 to V-16**).

Table V-11. PTX Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
50.0 ng/mL	1374559	48.2	96
50.0 ng/mL	1536702	46.8	94
100 ng/mL	2626435	106.7	107
100 ng/mL	2028220	109.2	109
500 ng/mL	11538207	537.3	107
500 ng/mL	11947842	525.5	105
1000 ng/mL	24117219	1058	106
1000 ng/mL	21147770	1094	109
5000 ng/mL	105914593	5110	102
5000 ng/mL	99985232	5239	105
10000 ng/mL	218992163	9292	93
10000 ng/mL	170265481	10930	109
25000 ng/mL	312572602	20205	81
25000 ng/mL	342959681	19071	76
QC			
QCL	5400611	271	542*
QCL	1598838	52	105
QCM	13255592	579	116
QCM	12385303	570	114
QCH	124995986	5202	104
QCH	108362256	5550	111

*Out of specification

PTX
$Y = 0.0506671 + 0.00665124*X$ $R^2 = 0.9848$ W: $1/X^2$
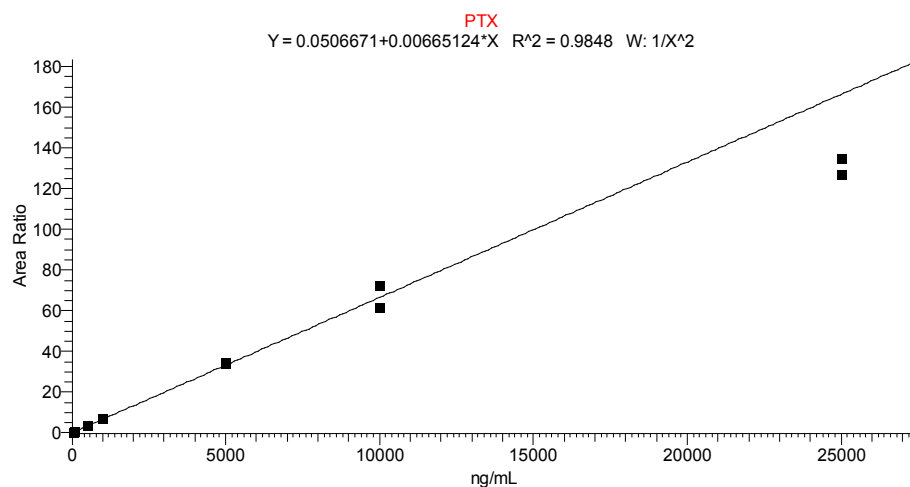
Figure V-9. PTX Plasma Standard Curve.

Table V-12. PTX_C13 Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
50.0 ng/mL	4067081	47.2	94
50.0 ng/mL	4878951	49.6	99
100.0 ng/mL	7986404	102.8	103
100.0 ng/mL	6371123	108.7	109
500.0 ng/mL	36737022	525.3	105
500.0 ng/mL	38172902	515.6	103
1000 ng/mL	74216263	996	100
1000 ng/mL	62367525	986	99
5000 ng/mL	324185786	4768	95
5000 ng/mL	293060124	4680	94
10000 ng/mL	675074948	8727	87
10000 ng/mL	574124643	11228	112
QC			
QCL	3600218	54	107
QCL	4829320	52	104
QCM	41643363	559	112
QCM	37960917	537	107
QCH	414665607	5260	105
QCH	338909209	5290	106

PTX_C13
Y = 0.0667252+0.0218411*X   R^2 = 0.9931   W: 1/X^2
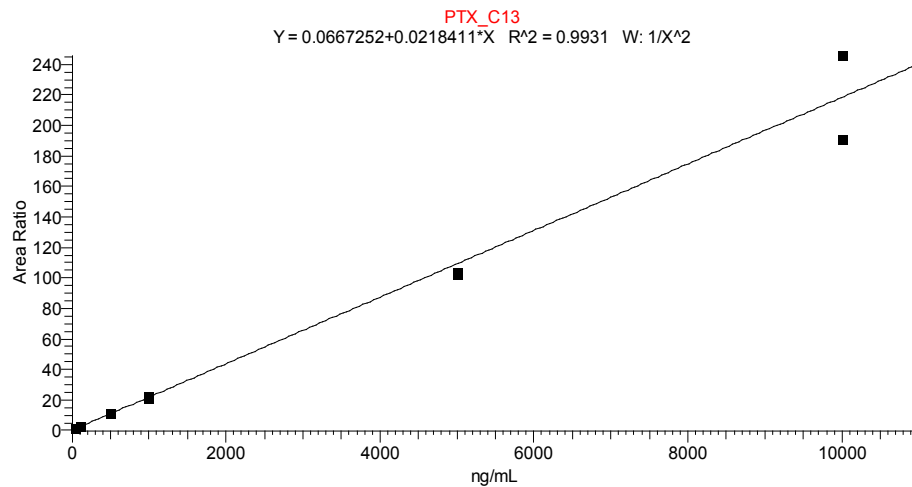
Figure V-10. PTX_C13 Plasma Standard Curve.

Table V-13. PTX Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
5.00 ng/mL	507416	4.71	94
5.00 ng/mL	518411	5.58	112
10.0 ng/mL	838575	9.0	90
10.0 ng/mL	882569	9.4	94
50.0 ng/mL	5270251	54.8	110
50.0 ng/mL	4933282	54.2	108
100.0 ng/mL	8800740	92.6	93
100.0 ng/mL	10448398	108.1	108
500 ng/mL	53795700	536	107
500 ng/mL	49686558	554	111
1000 ng/mL	80622621	907	91
1000 ng/mL	79540101	932	93
5000 ng/mL	333128063	4737	95
5000 ng/mL	323351948	4712	94
QC			
QCL	565917	5.64	113
QCL	560926	5.58	112
QCM	5105450	54.1	108
QCM	4940191	54.0	108
QCH	46636887	532	106
QCH	50467202	524	105

PTX
$Y = 0.00260967 + 0.00755108 \cdot X \quad R^2 = 0.9904 \quad W: 1/X^2$
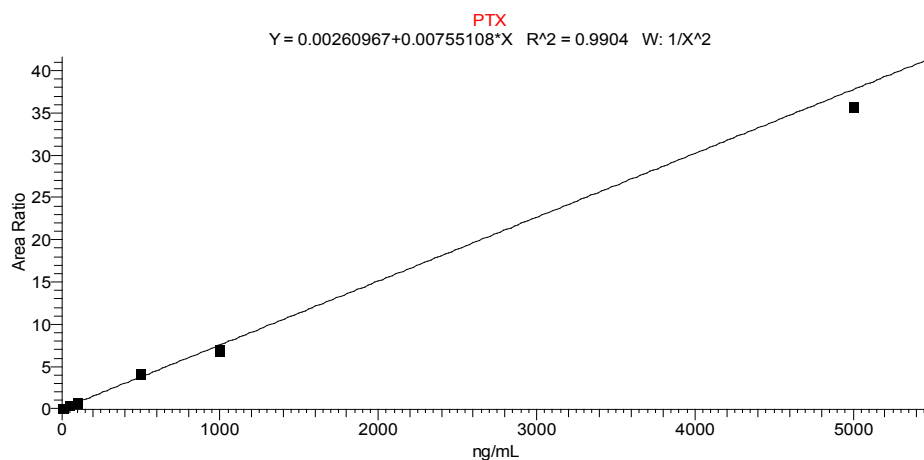
Figure V-11. PTX Protein-Free Plasma Standard Curve.

Table V-14. PTX_C13 Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
5.00 ng/mL	1674133	4.76	95
5.00 ng/mL	1651822	5.44	109
10.0 ng/mL	2750726	9.2	92
10.0 ng/mL	2900637	9.6	96
50.0 ng/mL	17115702	55.3	111
50.0 ng/mL	16140532	55.1	110
100.0 ng/mL	29381821	96.2	96
100.0 ng/mL	33236358	106.9	107
500 ng/mL	170674693	529	106
500 ng/mL	164320195	570	114
1000 ng/mL	260141108	911	91
1000 ng/mL	255835941	933	93
5000 ng/mL	1015662221	4495	90
5000 ng/mL	999422237	4533	91
QC			
QCL	1865514	5.71	114
QCL	1776906	5.41	108
QCM	16149992	53.2	106
QCM	15671510	53.2	106
QCH	161812449	575	115
QCH	165808348	536	107

PTX_C13
$Y = 0.0104529 + 0.0242609 \cdot X$ $R^2 = 0.9895$ W: $1/X^2$
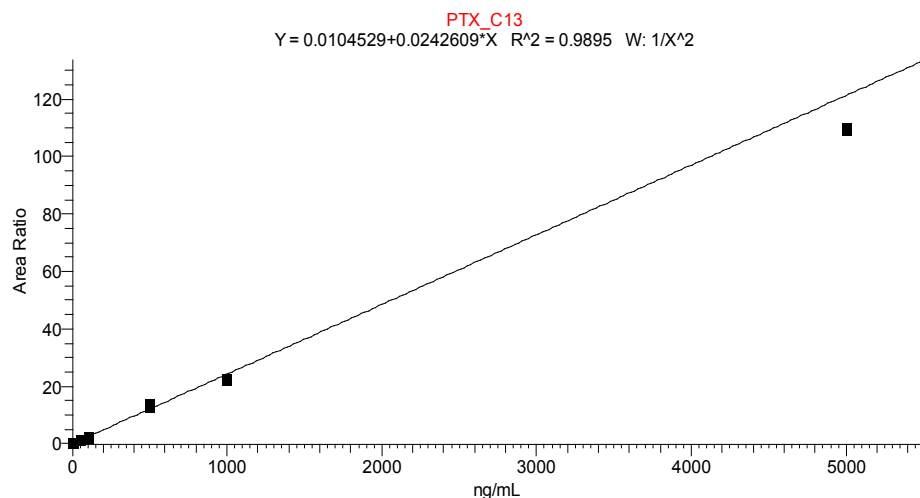
Figure V-12. PTX_C13 Protein-Free Plasma Standard Curve.

Table V-15. Free Paclitaxel Control Analytical Data. Presented are the analytical data for the free paclitaxel controls. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX	% Bound	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13	% Bound PTX_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG	SD
				= Filtrate PTX / Reservoir PTX *100	= 100 − %Unbound PTX			= Filtrate PTX_C13 / Reservoir PTX_C13 * 100	= 100 − %Unbound C13	= Fil PTX/ (1-(%Bound PTX_C13/100))	= Reservoir PTX- Unencapsulated	= (Unencapsulated/ Reservoir PTX) *100		
10 min	5 µg/mL	338	5811	5.8	94.2	6.1	114	5.3	94.7	6338	-527	109	101	8
		350	6137	5.7	94.3	6.4	106	6.1	93.9	5772	365	94		
		354	6432	5.5	94.5	6.2	112	5.6	94.4	6359	74	99		
	25 µg/mL	2550	28850	8.8	91.2	9.5	106	9.0	91.0	28452	398	99	100	1
		2454	28324	8.7	91.3	9.1	106	8.6	91.4	28543	-219	101		
		2833	29944	9.5	90.5	10.2	108	9.5	90.5	29790	154	99		
30 min	5 µg/mL	364	9006	4.0	96.0	6.6	171	3.9	96.1	9335	-329	104	98	5
		339	6459	5.2	94.8	6.2	113	5.5	94.5	6123	335	95		
		340	6409	5.3	94.7	6.0	114	5.2	94.8	6499	-89	101		
	25 µg/mL	2800	33329	8.4	91.6	10.4	111	9.4	90.6	29791	3537	89	96	7
		2559	31753	8.1	91.9	8.9	108	8.3	91.7	30964	789	98		
		2388	30220	7.9	92.1	8.5	110	7.7	92.3	30986	-766	103		
2 hr	5 µg/mL	343	5660	6.1	93.9	6.5	120	5.4	94.6	6308	-648	111	110	3
		352	6049	5.8	94.2	6.2	114	5.4	94.6	6458	-409	107		
		367	5913	6.2	93.8	6.6	118	5.5	94.5	6615	-702	112		
	25 µg/mL	2602	25687	10.1	89.9	9.3	103	9.0	91.0	28926	-3239	113	106	10
		2613	29135	9.0	91.0	9.9	105	9.5	90.5	27624	1511	95		
		2627	25371	10.4	89.6	9.8	105	9.4	90.6	27981	-2610	110		
4 hr	5 µg/mL	306	5526	5.5	94.5	5.6	108	5.2	94.8	5933	-406	107	107	1
		296	5740	5.2	94.8	5.1	105	4.8	95.2	6119	-380	107		
		298	5912	5.0	95.0	5.0	107	4.7	95.3	6370	-458	108		
	25 µg/mL	2382	30098	7.9	92.1	8.1	108	7.5	92.5	31932	-1834	106	103	3
		2387	28039	8.5	91.5	8.4	100	8.4	91.6	28561	-522	102		
		2298	32450	7.1	92.9	8.1	115	7.0	93.0	32846	-397	101		

Table V-16. Abraxane, Lot A (6113658) Drug Release Analytical Data. Presented are the analytical data for Abraxane, lot A (6113658). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX $= \text{Filtrate PTX} / \text{Reservoir PTX} *100$	% Bound $= 100 - \%\text{Unbound PTX}$	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 $= \text{Filtrate PTX\_C13} / \text{Reservoir PTX\_C13} * 100$	% Bound PTX_C13 $= 100 - \%\text{Unbound C13}$	Unencapsulated (ng/mL) $= \text{Fil PTX} / (1-(\%\text{Bound PTX\_C13}/100))$	Encapsulated (ng/mL) $= \text{Reservoir PTX} - \text{Unencapsulated}$	% Release $= (\text{Unencapsulated}/ \text{Reservoir PTX}) *100$	AVG	SD
10 min	5 µg/mL	292	6304	4.6	95.4	4.8	118	4.1	95.9	7177	-873	114	105	7.8
		323	6330	5.1	94.9	5.7	110	5.1	94.9	6298	32	99		
		265	6244	4.2	95.8	4.4	105	4.2	95.8	6340	-96	102		
	25 µg/mL	1674	28647	5.8	94.2	5.5	104	5.3	94.7	31507	-2860	110	108	1.8
		1650	30289	5.4	94.6	5.6	110	5.1	94.9	32388	-2100	107		
		1724	30093	5.7	94.3	5.7	106	5.4	94.6	32124	-2031	107		
30 min	5 µg/mL	408	6411	6.4	93.6	5.9	107	5.5	94.5	7363	-953	115	105	9.8
		332	6640	5.0	95.0	5.8	109	5.3	94.7	6323	316	95		
		313	6318	5.0	95.0	5.1	108	4.7	95.3	6653	-335	105		
	25 µg/mL	1641	29105	5.6	94.4	5.7	101	5.6	94.4	29244	-139	100	104	3.5
		1816	28143	6.5	93.5	6.6	107	6.1	93.9	29718	-1575	106		
		1854	29466	6.3	93.7	6.4	109	5.9	94.1	31548	-2081	107		
2 hr	5 µg/mL	309	5755	5.4	94.6	5.2	109	4.7	95.3	6506	-751	113	108	4.7
		304	5851	5.2	94.8	5.2	108	4.9	95.1	6268	-417	107		
		301	6075	4.9	95.1	5.0	105	4.8	95.2	6311	-236	104		
	25 µg/mL	1660	29708	5.6	94.4	5.7	109	5.2	94.8	31791	-2083	107	109	1.8
		1643	28108	5.8	94.2	5.4	102	5.3	94.7	31082	-2974	111		
		1557	27824	5.6	94.4	5.2	101	5.1	94.9	30246	-2422	109		
4 hr	5 µg/mL	220	5609	3.9	96.1	4.0	105	3.8	96.2	5825	-216	104	99	7.7
		202	5926	3.4	96.6	3.3	100	3.3	96.7	6163	-236	104		
		214	6482	3.3	96.7	3.6	98	3.6	96.4	5874	607	91		
	25 µg/mL	1171	31342	3.7	96.3	3.8	99	3.9	96.1	30340	1002	97	96	2.8
		1272	31612	4.0	96.0	4.7	109	4.3	95.7	29463	2150	93		
		1205	29535	4.1	95.9	4.2	102	4.1	95.9	29154	380	99		

Table V-17. Genexol-PM, Lot A (GP31681) Drug Release Analytical Data. Presented are the analytical data for Genexol-PM, lot A (GP31681). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 – %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated	% Release = (Unencapsulated/ Reservoir PTX) *100	AVG	SD
10 min	**5 µg/mL**	320	5737	5.6	94.4	5.5	105	5.2	94.8	6114	-377	107	105	2.6
		320	5754	5.6	94.4	5.7	108	5.3	94.7	6068	-315	105		
		346	5794	6.0	94.0	6.5	111	5.9	94.1	5885	-91	102		
	25 µg/mL	1868	28710	6.5	93.5	12.8	214	6.0	94.0	31188	-2477	109	102	7.8
		1720	32369	5.3	94.7	6.4	113	5.7	94.3	30152	2218	93		
		1521	29093	5.2	94.8	5.5	108	5.1	94.9	29966	-873	103		
30 min	**5 µg/mL**	338	5963	5.7	94.3	5.8	103	5.6	94.4	6039	-76	101	104	6.2
		347	6077	5.7	94.3	5.7	110	5.2	94.8	6721	-644	111		
		318	6549	4.9	95.1	5.5	111	4.9	95.1	6467	82	99		
	25 µg/mL	1661	29859	5.6	94.4	12.3	210	5.9	94.1	28292	1567	95	96	4.8
		2211	32190	6.9	93.1	8.5	113	7.5	92.5	29404	2786	91		
		1560	33128	4.7	95.3	5.0	106	4.7	95.3	33389	-261	101		
2 hr	**5 µg/mL**	278	5487	5.1	94.9	4.7	98	4.8	95.2	5781	-294	105	107	3.1
		306	5003	6.1	93.9	5.7	103	5.5	94.5	5522	-520	110		
		293	5634	5.2	94.8	5.1	103	5.0	95.0	5897	-263	105		
	25 µg/mL	1577	27697	5.7	94.3	11.2	202	5.6	94.4	28407	-711	103	102	3.1
		1541	28307	5.4	94.6	5.6	108	5.2	94.8	29466	-1158	104		
		1514	28980	5.2	94.8	5.6	105	5.3	94.7	28448	532	98		
4 hr	**5 µg/mL**	266	5206	5.1	94.9	4.3	98	4.3	95.7	6131	-925	118	112	5.0
		267	5536	4.8	95.2	4.6	103	4.5	95.5	6005	-469	108		
		258	5458	4.7	95.3	4.7	109	4.3	95.7	5995	-537	110		
	25 µg/mL	1350	29458	4.6	95.4	9.7	210	4.6	95.4	29186	272	99	105	5.6
		1236	28222	4.4	95.6	4.3	105	4.1	95.9	30015	-1792	106		
		1052	27592	3.8	96.2	3.5	102	3.5	96.5	30363	-2771	110		

Table V-18. Free Paclitaxel Control Data. Displayed is the calculated % release for the paclitaxel control data. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	5 µg/mL	101	7.7	7.6
	25 µg/mL	100	1.1	1.1
30 min	5 µg/mL	98	4.7	4.7
	25 µg/mL	96	6.6	6.9
2 hr	5 µg/mL	110	2.8	2.6
	25 µg/mL	106	9.7	9.1
4 hr	5 µg/mL	107	0.6	0.5
	25 µg/mL	103	2.6	2.6
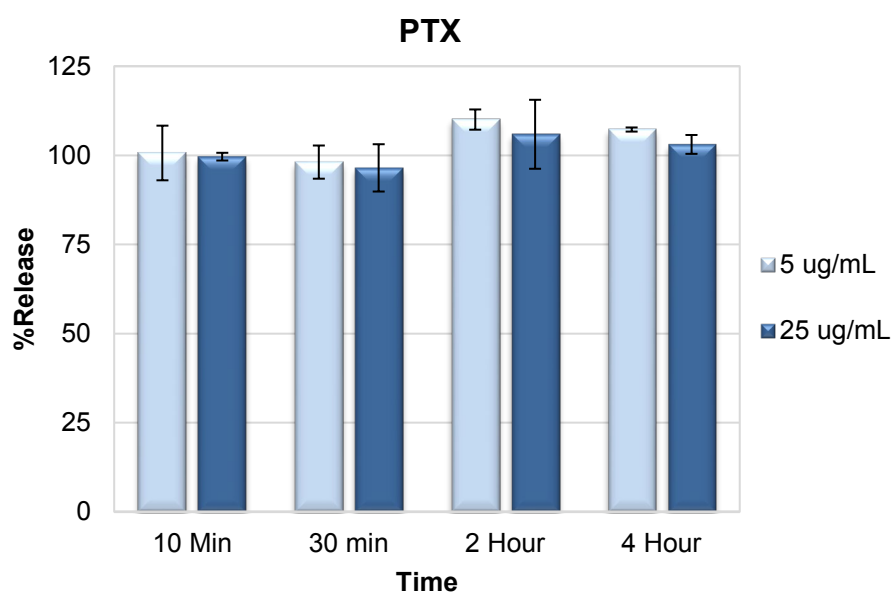
Figure V-13. Free Paclitaxel Control Data. Displayed is the calculated % release for the paclitaxel control data. (Mean $\pm$ SD, *N=3*)

Table V-19. Abraxane, Lot A (6113658) Drug Release. Displayed is the calculated % release for Abraxane, lot A (6113658). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	5 µg/mL	105	7.8	7.4
	25 µg/mL	108	1.8	1.7
30 min	5 µg/mL	105	9.8	9.3
	25 µg/mL	104	3.5	3.3
2 hr	5 µg/mL	108	4.7	4.3
	25 µg/mL	109	1.8	1.6
4 hr	5 µg/mL	99	7.7	7.7
	25 µg/mL	96	2.8	2.9
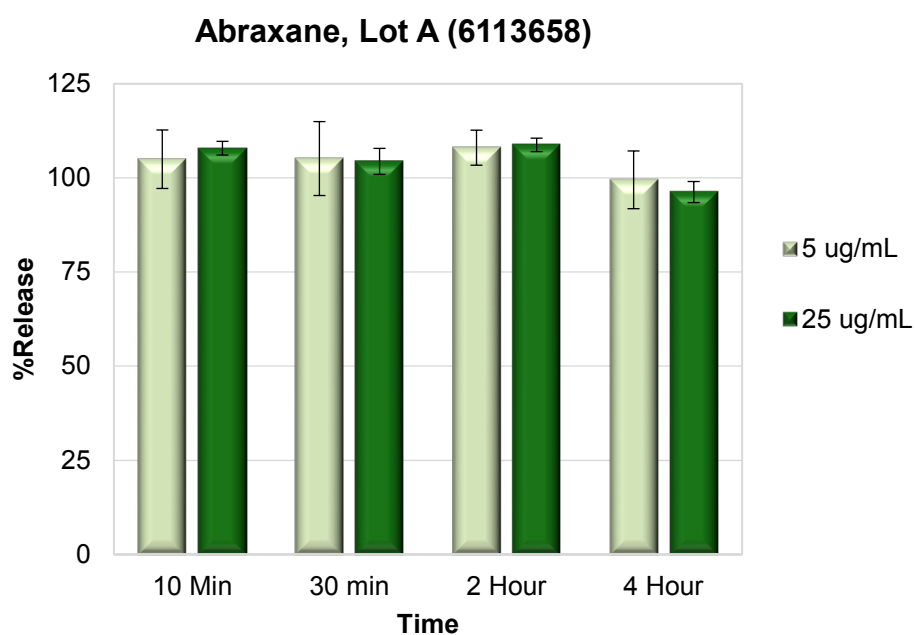
Figure V-14. Abraxane, Lot A (6113658) Drug Release. Displayed is the calculated % release for the Abraxane, lot A (6113658). (Mean $\pm$ SD, *N=3*)

Table V-20. Genexol-PM, Lot A (GP31681) Drug Release. Displayed is the calculated % release for Genexol-PM, lot A (GP31681). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	5 µg/mL	105	7.8	7.4
	25 µg/mL	108	1.8	1.7
30 min	5 µg/mL	105	9.8	9.3
	25 µg/mL	104	3.5	3.3
2 hr	5 µg/mL	108	4.7	4.3
	25 µg/mL	109	1.8	1.6
4 hr	5 µg/mL	99	7.7	7.7
	25 µg/mL	96	2.8	2.9
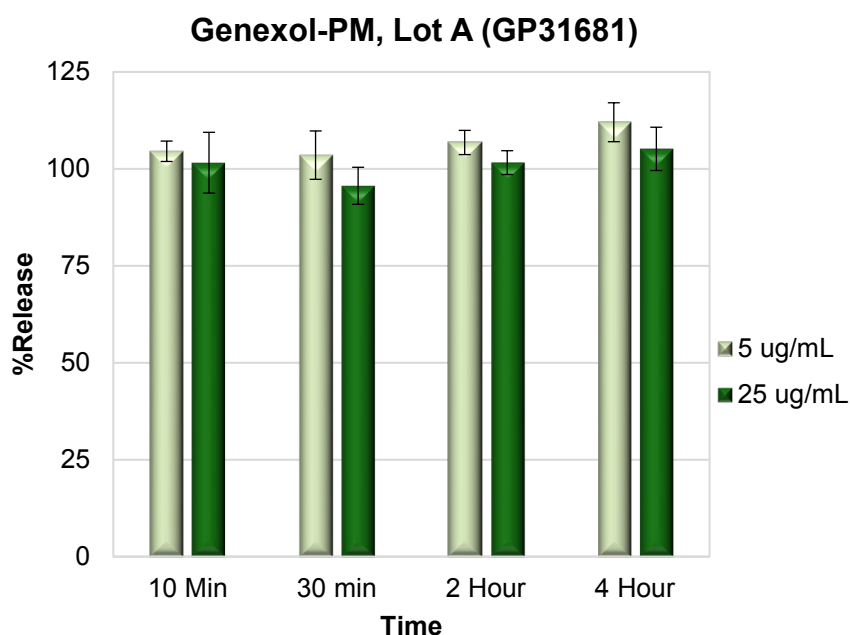
Figure V-15. Genexol-PM, Lot A (GP31681) Drug Release. Displayed is the calculated % release for Genexol-PM,lot A (GP31681). (Mean $\pm$ SD, *N=3*)
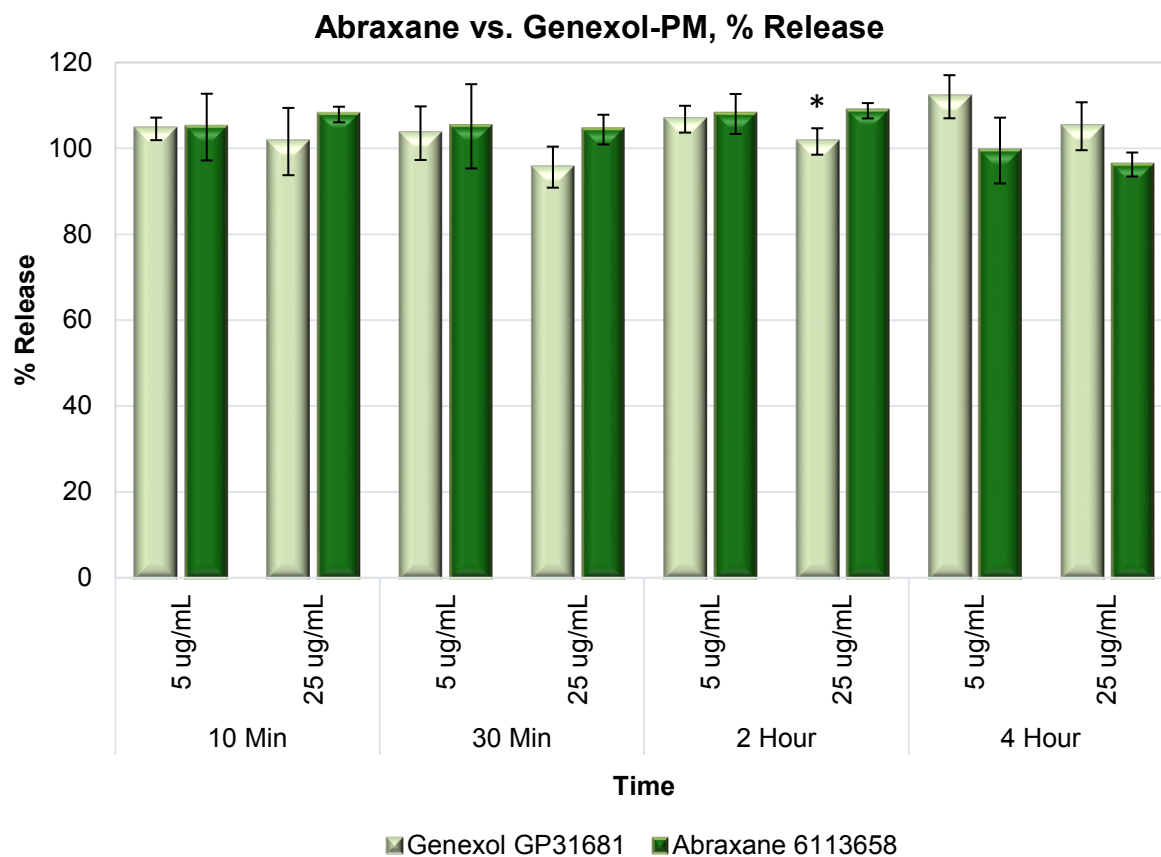
Figure V-16. Abraxane and Genexol-PM Drug Release Lot Comparison. Displayed are the 10 min – 4 hr calculated % release data for the Abraxane and Genexol-PM lots 6113658 and GP31681, respectively. (Mean $\pm$ SD, *N=3*), *$p \leq 0.05$, Student's t-test

Abraxane in Human Plasma at 37°C, 25 and 100 µg/mL

Summary
The objective of this study was to detect the in vitro drug release of a single lot of Abraxane in human plasma at 37°C over a 4 hr period at a higher concentration than the previous intra-day study, 25 and 100 µg/mL.

Design and Methods
A single lot of Abraxane, 6113658 (lot A), was evaluated for drug release at 25 and 100 µg/mL PTX equivalents in human plasma at 37°C over a 4 hr period, according to the SITUA method described in Section II. The lot was run on a single day, and free paclitaxel at identical concentrations and time points was included as a control. The human plasma and protein-free plasma, PTX and PTX_C13 standard calibration curves used for this study are also provided.

Results & Conclusions
The PTX and PTX_C13 standard calibration curves used for this study are presented in **Tables V-21 to V-24 and Figures V-17 to V-20**. The free paclitaxel controls averaged between 90-108% release for all concentrations and time points (**Table V-25 and V-27, and Figure V-21**). The Abraxane drug release was at approximately 100% release at the earliest 10 min time point (**Tables V-26 and V-28, and Figure V-22)**.

Table V-21. PTX Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
1.00 µg/mL	20955927	1.03	103
1.00 µg/mL	18852671	0.99	99
5.00 µg/mL	54960603	4.82	96
5.00 µg/mL	72577805	4.70	94
10.0 µg/mL	163167274	9.9	99
10.0 µg/mL	188577367	9.9	99
25.0 µg/mL	399025687	25.0	100
25.0 µg/mL	331057734	21.7	87
50.0 µg/mL	480793938	55.4	111
50.0 µ/mL	584835980	54.5	109
75.0 µg/mL	921051966	80.1	107
75.0 µg/mL	853442888	71.9	96
100.0 µg/mL	868172618	93.0	93
100.0 µg/mL	1090064814	107.1	107
QC			
QCL	20452163	1.09	109
QCL	20387444	1.03	103
QCM	207824595	10.3	103
QCM	166133566	9.2	92
QCH	692068369	46.2	92
QCH	673650894	49.8	100

PTX
Y = -1.27561+0.00831277*X   R^2 = 0.9943   W: 1/X^2
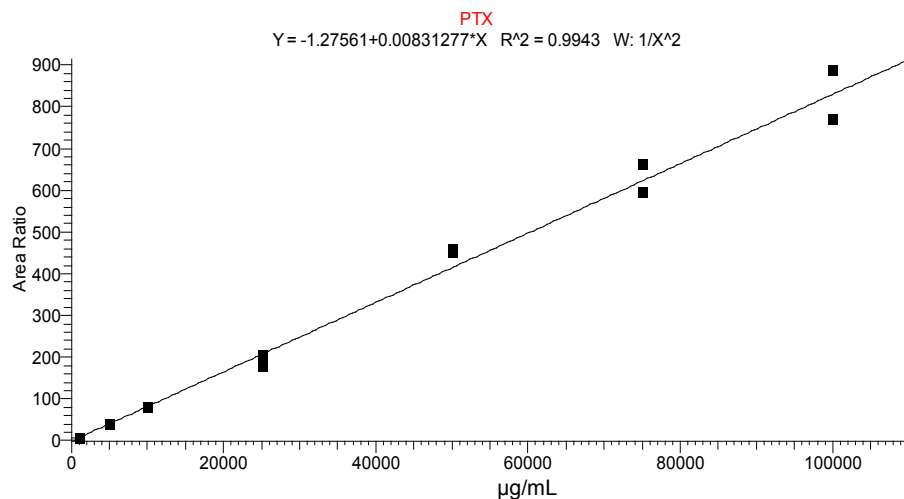
Figure V-17. PTX Plasma Standard Curve.

Table V-22. PTX_C13 Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	368369	10.6	106
10.0 ng/mL	319213	9.7	97
50.0 ng/mL	782702	45.3	91
50.0 ng/mL	1085446	46.3	93
100 ng/mL	2552741	104	104
100 ng/mL	2850063	100	100
500 ng/mL	5710318	242	97
500 ng/mL	5380606	239	95
750 ng/mL	6084864	476	95
750 ng/mL	7375619	466	93
1000 ng/mL	14020071	829	110
1000 ng/mL	14096847	807	108
QC			
QCL	296356	9.4	94
QCL	286499	8.5	85
QCM	3287861	109	109
QCM	2963288	110	110
QCH	11672703	529	106
QCH	10758361	539	108

PTX_C13
$Y = -0.000961937 + 0.0122137*X$ $R^2 = 0.9950$ $W: 1/X^2$
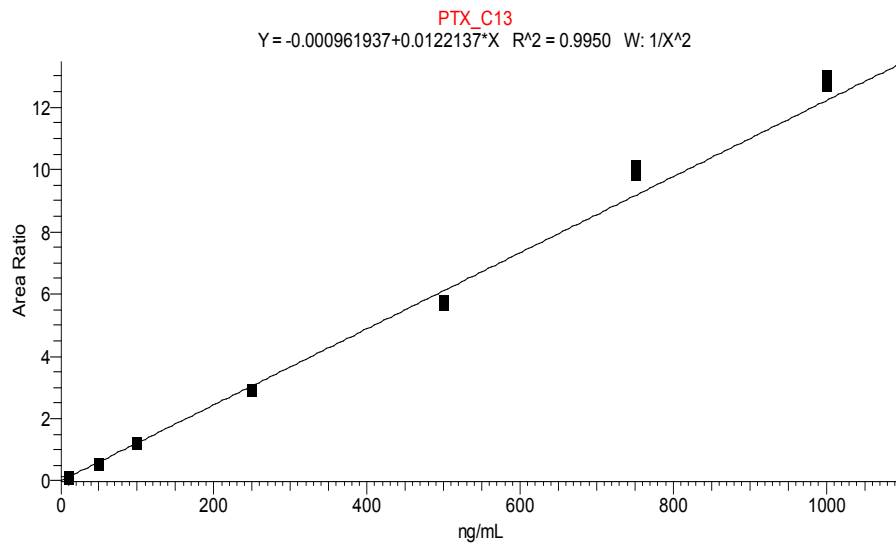
Figure V-18. PTX_C13 Plasma Standard Curve

Table V-23. PTX Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
1.00 ng/mL	165713	1.07	107
1.00 ng/mL	164222	0.94	94
5.00 ng/mL	1087869	4.85	97
5.00 ng/mL	772879	4.82	96
10.0 ng/mL	1349287	10.1	101
10.0 ng/mL	1180144	10.0	100
50.0 ng/mL	5642281	51.8	104
50.0 ng/mL	5697605	54.4	109
100.0 ng/mL	9854318	103.3	103
100.0 ng/mL	9308833	100.4	100
500 ng/mL	47318825	512	102
500 ng/mL	48918364	531	106
1000 ng/mL	82989673	929	93
1000 ng/mL	90816087	927	93
5000 ng/mL	386465587	5179	104
5000 ng/mL	364498248	4558	91
QC			
QCL	24158	0.57	57*
QCL	49927	0.84	84
QCM	723546	9.2	92
QCM	782956	10.5	105
QCH	7893066	96.0	96
QCH	8005853	100.3	100

*Out of specification

PTX
$Y = -0.000879426 + 0.00886241*X$ $R^2 = 0.9966$ W: $1/X^2$
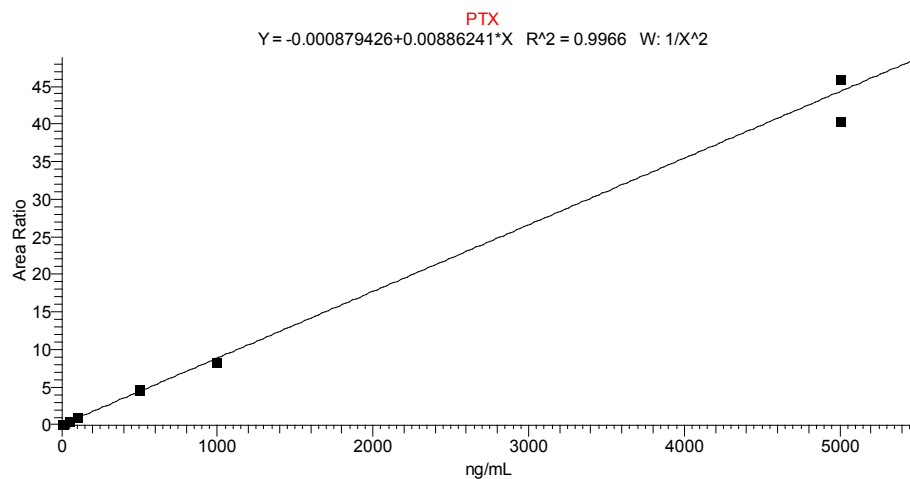
Figure V-19. PTX Protein-Free Plasma Standard Curve.

Table V-24. PTX_C13 Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
1.00 ng/mL	180847	0.92	92
1.00 ng/mL	254200	1.08	108
5.00 ng/mL	1685482	5.03	101
5.00 ng/mL	1205353	5.03	101
10.0 ng/mL	1921079	9.5	95
10.0 ng/mL	1800771	10.1	101
50.0 ng/mL	8944926	53.7	107
50.0 ng/mL	8438519	52.7	105
100.0 ng/mL	15021793	102.8	103
100.0 ng/mL	13822466	97.3	97
500 ng/mL	76344887	539	108
500 ng/mL	74475001	527	105
1000 ng/mL	127321973	930	93
1000 ng/mL	147633655	983	98
5000 ng/mL	575191980	5028	101
5000 ng/mL	523020627	4266	85
QC			
QCL	55544	0.94	94
QCL	84317	1.05	105
QCM	1051013	8.9	89
QCM	1094781	9.7	97
QCH	12227682	97.1	97
QCH	11983233	98.1	98

PTX_C13
Y = -0.00311542+0.013589*X   R^2 = 0.9951   W: 1/X^2
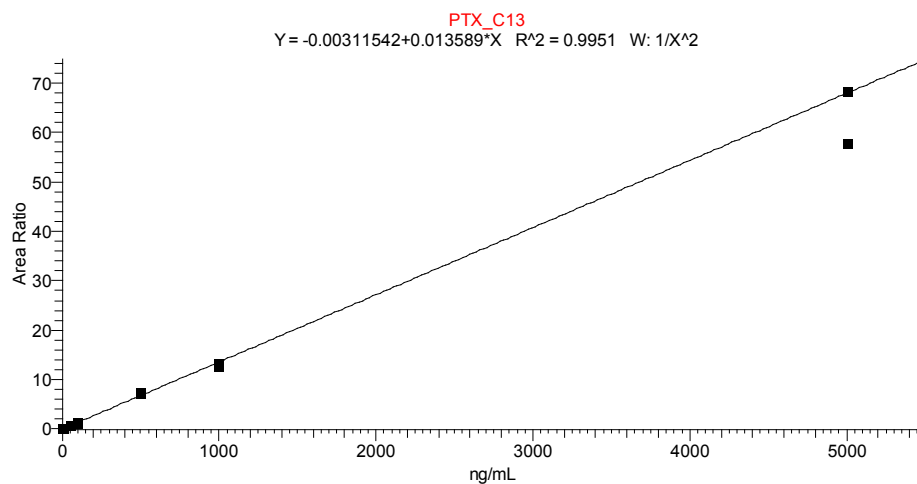
Figure V-20. PTX_C13 Protein-Free Plasma Standard Curve.

Table V-25. Free Paclitaxel Control Analytical Data. Presented are the analytical data for the free paclitaxel controls. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 – %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated	% Release = (Unencapsulated/ Reservoir PTX) *100	AVG	SD
10 min	25 µg/mL	1210	20363	5.9	94.1	5.7	107	5.4	94.6	22445	-2082	110	104	10.3
		1252	25431	4.9	95.1	5.3	118	4.5	95.5	27908	-2477	110		
		1095	22429	4.9	95.1	5.2	98	5.3	94.7	20676	1754	92		
	100 µg/mL	5856	83564	7.0	93.0	6.7	105	6.4	93.6	91465	-7901	109	108	3.7
		5590	82529	6.8	93.2	6.1	99	6.1	93.9	91202	-8673	111		
		6141	96948	6.3	93.7	5.6	91	6.1	93.9	100538	-3590	104		
30 min	25 µg/mL	1411	23602	6.0	94.0	6.4	107	6.0	94.0	23476	126	99	95	5.5
		1297	25241	5.1	94.9	5.8	103	5.7	94.3	22946	2295	91		
		1300	22723	5.7	94.3	6.2	107	5.8	94.2	22411	313	99		
	100 µg/mL	6650	88242	7.5	92.5	6.9	98	7.1	92.9	94194	-5952	107	98	10.6
		5527	93101	5.9	94.1	6.3	91	6.9	93.1	80341	12760	86		
		5964	87899	6.8	93.2	6.2	93	6.7	93.3	89162	-1262	101		
2 hr	25 µg/mL	1224	23487	5.2	94.8	5.3	96	5.6	94.4	22028	1460	94	94	2.8
		1045	23078	4.5	95.5	5.1	102	5.0	95.0	21073	2005	91		
		1242	24554	5.1	94.9	5.4	103	5.2	94.8	23798	756	97		
	100 µg/mL	5015	82063	6.1	93.9	5.8	92	6.3	93.7	79693	2370	97	90	8.4
		5489	94702	5.8	94.2	5.7	90	6.3	93.7	87027	7675	92		
		5033	104297	4.8	95.2	5.3	89	6.0	94.0	84192	20105	81		
4 hr	25 µg/mL	1174	26432	4.4	95.6	4.5	101	4.5	95.5	26084	348	99	96	4.4
		965	20741	4.7	95.3	4.4	94	4.7	95.3	20601	140	99		
		1113	26179	4.3	95.7	4.6	100	4.6	95.4	23942	2236	91		
	100 µg/mL	5232	78010	6.7	93.3	5.5	90	6.1	93.9	85673	-7663	110	106	9.9
		4979	91088	5.5	94.5	5.4	94	5.7	94.3	86692	4396	95		
		4985	82748	6.0	94.0	4.8	91	5.3	94.7	94381	-11632	114		

Table V-26. Abraxane, Lot A (6113658) Drug Release Analytical Data. Presented are the analytical data for Abraxane, lot A (6113658). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 − %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated	% Release = (Unencapsulated/ Reservoir PTX) *100	AVG	SD
10 Min	25 µg/mL	1466	25184	5.8	94.2	5.6	102	5.5	94.5	26451	-1267	105	96	8.0
		1382	28003	4.9	95.1	5.7	105	5.5	94.5	25205	2797	90		
		1310	30540	4.3	95.7	5.1	111	4.6	95.4	28330	2209	93		
	100 µg/mL	5693	99565	5.7	94.3	5.6	101	5.6	94.4	102326	-2761	103	96	5.9
		5994	115294	5.2	94.8	5.4	99	5.5	94.5	108767	6527	94		
		4991	105494	4.7	95.3	5.0	96	5.2	94.8	96431	9063	91		
30 Min	25 µg/mL	1479	27583	5.4	94.6	5.9	104	5.7	94.3	26137	1447	95	93	4.9
		1426	27163	5.3	94.7	6.0	110	5.5	94.5	26139	1024	96		
		1365	24599	5.5	94.5	5.9	93	6.4	93.6	21410	3189	87		
	100 µg/mL	6152	99303	6.2	93.8	6.6	97	6.8	93.2	89975	9328	91	87	3.1
		6133	99562	6.2	93.8	6.8	93	7.3	92.7	84168	15394	85		
		6490	98004	6.6	93.4	6.8	90	7.6	92.4	85280	12724	87		
2 Hour	25 µg/mL	1189	27011	4.4	95.6	5.2	108	4.8	95.2	24663	2349	91	94	5.2
		1163	26191	4.4	95.6	5.1	104	4.9	95.1	23688	2502	90		
		1239	24690	5.0	95.0	5.1	101	5.0	95.0	24651	40	100		
	100 µg/mL	6297	93371	6.7	93.3	6.0	94	6.4	93.6	98911	-5539	106	106	5.2
		6111	94187	6.5	93.5	5.8	99	5.8	94.2	104667	-10481	111		
		5151	100432	5.1	94.9	5.0	99	5.1	94.9	101132	-700	101		
4 Hour	25 µg/mL	1502	28927	5.2	94.8	5.7	107	5.3	94.7	28305	622	98	93	4.5
		1047	24707	4.2	95.8	4.7	99	4.7	95.3	22255	2453	90		
		1083	25912	4.2	95.8	4.6	98	4.6	95.4	23302	2610	90		
	100 µg/mL	5714	98428	5.8	94.2	5.6	95	5.9	94.1	96466	1962	98	96	1.8
		5714	96022	6.0	94.0	5.6	90	6.2	93.8	91710	4312	96		
		4743	88121	5.4	94.6	5.1	90	5.7	94.3	83383	4739	95		

Table V-27. Free Paclitaxel Control Data. Displayed is the calculated % release for the paclitaxel control data. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	25 µg/mL	104	10.3	9.9
	100 µg/mL	108	3.7	3.4
30 min	25 µg/mL	95	5.5	5.8
	100 µg/mL	98	10.6	10.8
2 hr	25 µg/mL	94	2.8	3.0
	100 µg/mL	90	8.4	9.3
4 hr	25 µg/mL	96	4.4	4.5
	100 µg/mL	106	9.9	9.3
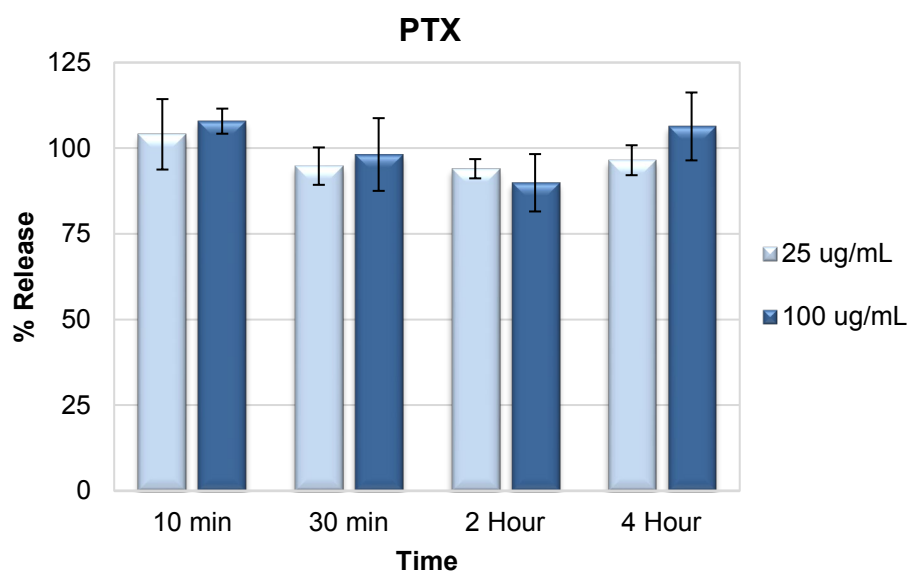
Figure V-21. Free Paclitaxel Control Data. Displayed is the calculated % release for the paclitaxel control data. (Mean $\pm$ SD, *N=3*)

Table IV-28. Abraxane, Lot A (6113658) Drug Release. Displayed is the calculated % release for Abraxane, lot A (6113658). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	25 µg/mL	96	8.0	8.3
	100 µg/mL	96	5.9	6.1
30 min	25 µg/mL	93	4.9	5.3
	100 µg/mL	87	3.1	3.5
2 Hour	25 µg/mL	94	5.2	5.5
	100 µg/mL	106	5.2	4.9
4 Hour	25 µg/mL	93	4.5	4.9
	100 µg/mL	96	1.8	1.8
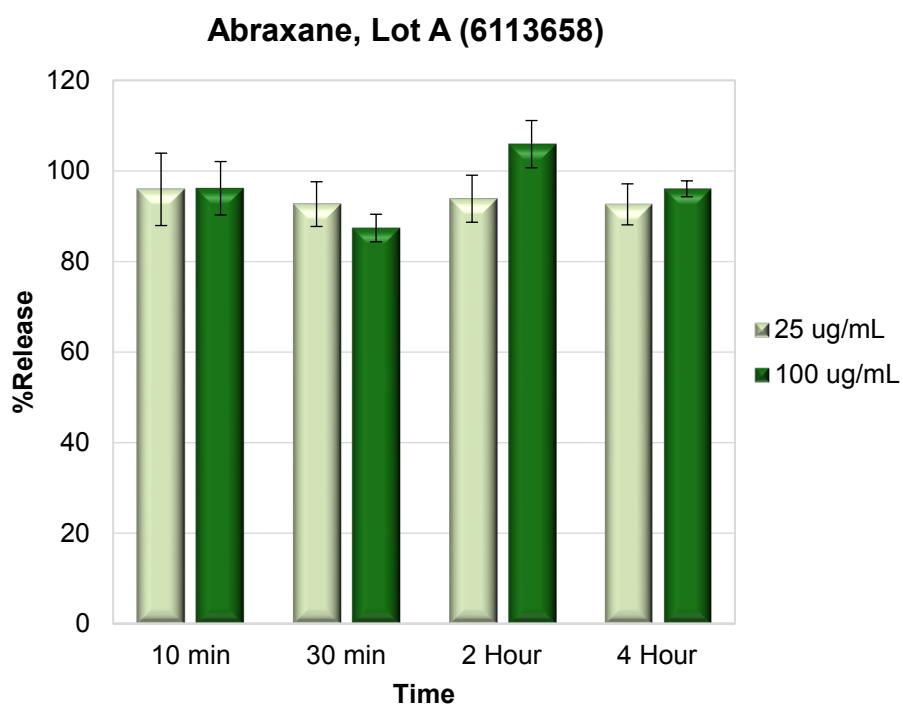
Figure IV-22. Abraxane, Lot A (6113658) Drug Release. Displayed is the calculated % release for the Abraxane, lot A (6113658). (Mean $\pm$ SD, *N=3*)

Intra-day Comparison in Rat Plasma at 37°C

Summary
The objective of this study was to compare the in vitro drug release of a single lot of Abraxane to that of a single lot of Genexol-PM in rat plasma at 37°C over a 1 hr period. All lots had similar drug release profiles over the 1 hr period, although some instances of statistically significant differences were observed between lots.

Design and Methods
A single lot of Abraxane, 6113658 (lot A), and a single lot of Genexol-PM, GP31681 (lot A), were evaluated for drug release at 0.5, 5 and 10 μg/mL PTX equivalents in rat plasma at 37°C over a 1 hr period, according to the SITUA method described in Section I. Both lots were run on a single day, and free paclitaxel at identical concentrations and time points were included as a control. The rat plasma and protein-free plasma, PTX and PTX_C13 standard calibration curves used for this study are also provided.

Results & Conclusions
The PTX and PTX_C13 standard calibration curves used for this study are presented in **Tables V-29 to V-32 and Figures V-23 to V-26**. The free paclitaxel controls averaged between 96-111% release for all concentrations and time points (**Tables V-33 and V-36, and Figure V-27**). The Abraxane and Genexol-PM drug release was similar, at approximately 100% release at the earliest 10 min time point, without a clear concentration-dependent and temporal trend (**Tables V-34, V-35, V-37 and V-38, and Figures V-28 to V-30**).

Table V-29. PTX Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
50.0 ng/mL	147960	47.4	95
50.0 ng/mL	214040	47.4	104
100.0 ng/mL	273806	90.3	90
100.0 ng/mL	164768	90.3	110
500 ng/mL	1623343	478	96
500 ng/mL	1024803	478	106
1000 ng/mL	1653011	1062	106
1000 ng/mL	5211529	1062	108
5000 ng/mL	25818640	5045	101
5000 ng/mL	16226161	5045	98
10000 ng/mL	71649512	8910	89
10000 ng/mL	38883909	8910	96
QC			
QCL	44881	41.7	83
QCL	139440	55.6	83
QCM	758394	524	105
QCM	1834029	542	108
QCH	23624247	5075	101
QCH	12866570	5505	110

PTX
$Y = -0.0681929 + 0.00753035 \cdot X \quad R^2 = 0.9930 \quad W: 1/X^2$
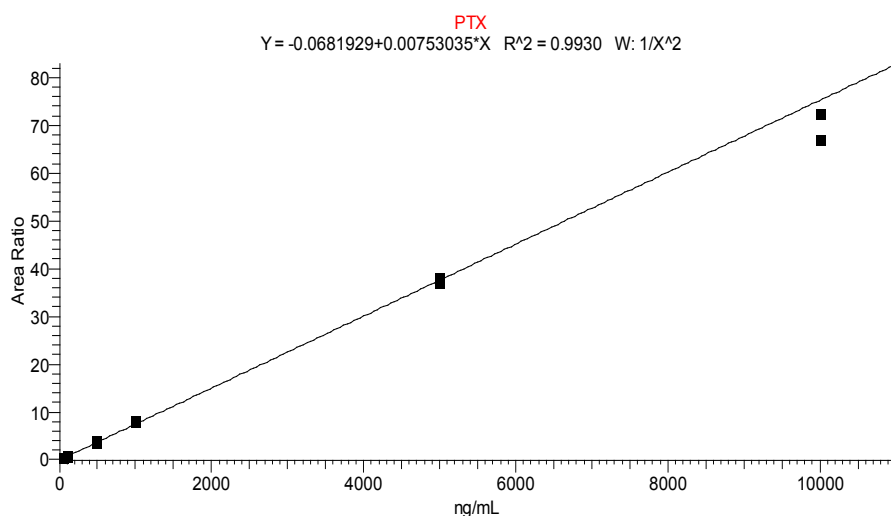
Figure V-23. PTX Plasma Standard Curve.

Table V-30. PTX_C13 Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
50.0 ng/mL	531898	45.8	92
50.0 ng/mL	753519	50.3	101
100.0 ng/mL	999975	96.9	97
100.0 ng/mL	567348	113.2	113
500 ng/mL	5933009	552	110
500 ng/mL	3332972	544	109
1000 ng/mL	5392400	1104	110
1000 ng/mL	16572068	1099	110
5000 ng/mL	77995742	4885	98
5000 ng/mL	46722731	4538	91
10000 ng/mL	198074892	7900	79
10000 ng/mL	114076830	9053	91
QC			
QCL	217631	52.4	105
QCL	459391	51.0	102
QCM	2546731	556	111
QCM	5516407	516	103
QCH	70110523	4827	97
QCH	37078050	5086	102

PTX_C13
$Y = -0.0378605 + 0.023459 \cdot X$ $R^2 = 0.9836$ W: $1/X^2$
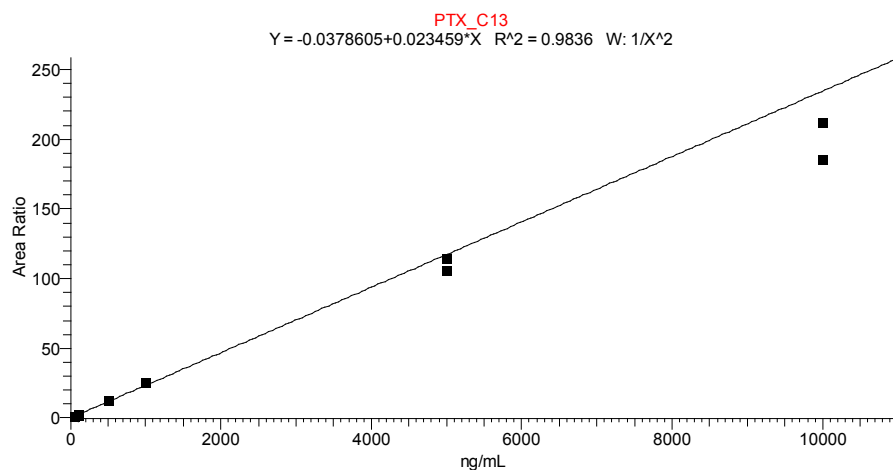
Figure V-24. PTX_C13 Plasma Standard Curve.

Table V-31. PTX Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
1.00 ng/mL	184095	0.95	95
1.00 ng/mL	179008	1.05	105
5.00 ng/mL	1020633	5.23	105
5.00 ng/mL	949829	4.91	98
10.0 ng/mL	1877427	9.9	99
10.0 ng/mL	1832767	9.7	97
50.0 ng/mL	9747801	53.0	106
50.0 ng/mL	9808536	53.8	108
100.0 ng/mL	17086147	95.8	96
100.0 ng/mL	16206008	90.3	90
500.0 ng/mL	81380409	534	107
500.0 ng/mL	83808115	529	106
1000.0 ng/mL	134758431	938	94
1000.0 ng/mL	151415531	955	95
QC			
QCL	215437	1.05	105
QCL	246478	1.17	117
QCM	2176187	9.7	97
QCM	2273842	9.8	98
QCH	20853616	97.4	97
QCH	21538299	99.0	99

PTX
$Y = -0.000492961 + 0.00845329 * X \quad R^2 = 0.9960 \quad W: 1/X^2$
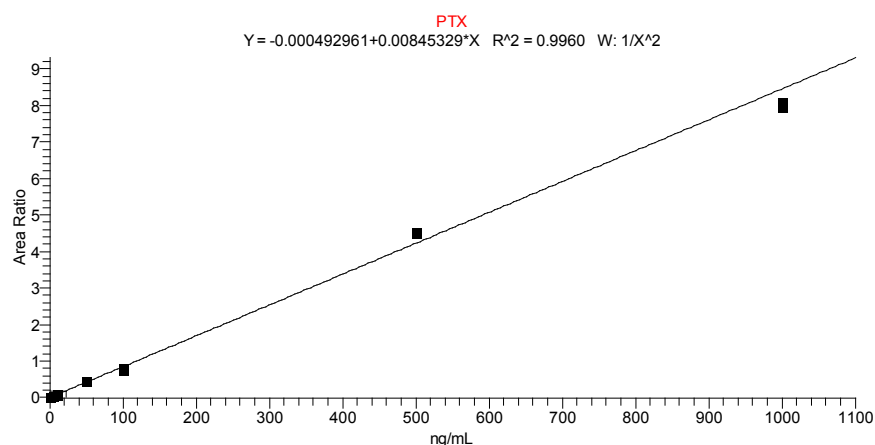
Figure V-25. PTX Protein-Free Plasma Standard Curve.

Table V-32. PTX_C13 Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
1.00 ng/mL	494156	0.91	91
1.00 ng/mL	532604	1.08	108
5.00 ng/mL	3041020	5.00	100
5.00 ng/mL	3330568	5.50	110
10.0 ng/mL	5539700	9.3	93
10.0 ng/mL	5703833	9.5	95
50.0 ng/mL	31318391	53.6	107
50.0 ng/mL	31641316	54.7	109
100.0 ng/mL	53081979	93.6	94
100.0 ng/mL	55516496	97.3	97
500 ng/mL	248788491	513	103
500 ng/mL	268203869	532	106
1000 ng/mL	413340481	904	90
1000 ng/mL	482481435	956	96
QC			
QCL	702298	1.17	117
QCL	671082	1.11	111
QCM	6512915	9.2	92
QCM	7048458	9.6	96
QCH	66650566	97.9	98
QCH	69362491	100.2	100

PTX_C13
Y = -0.00420185+0.0268997*X   R^2 = 0.9938   W: 1/X^2
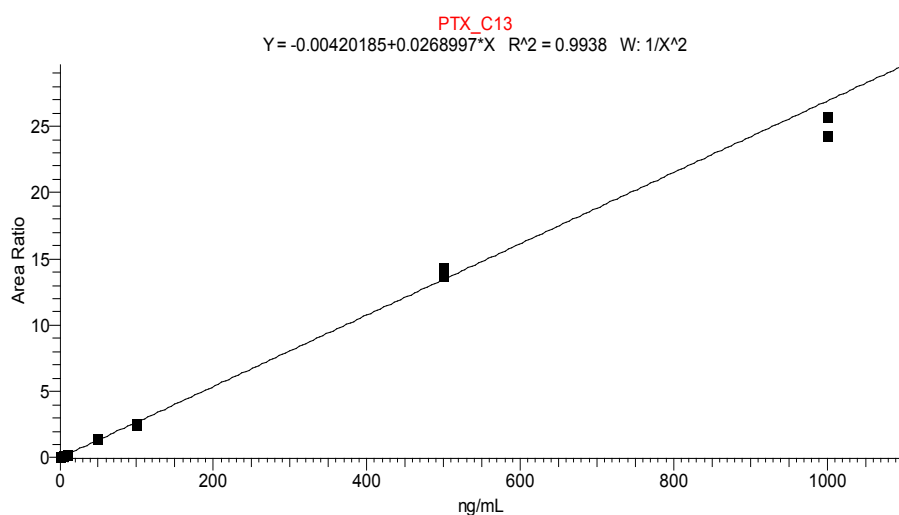
Figure V-26. PTX_C13 Protein-Free Plasma Standard Curve.

Table V-33. Free Paclitaxel Control Analytical Data. Presented are the analytical data for the free paclitaxel controls. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 − %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated	% Release = (Unencapsulated/ Reservoir PTX) *100	AVG	SD
10 min	0.5 µg/mL	22.4	509	4.4	95.6	4.5	110	4.1	95.9	547.6	-38.7	108	109.0	2.0
		21.8	545	4.0	96.0	4.6	116	4.0	96.0	542.8	2.0	100		
		28.5	547	5.2	94.8	5.7	121	4.7	95.3	604.7	-57.3	110		
	5 µg/mL	283.2	6022	4.7	95.3	5.4	112	4.8	95.2	5863.4	159.0	97	99.6	3.2
		333.0	5879	5.7	94.3	5.7	112	5.1	94.9	6553.1	-674.5	111		
		243.8	5632	4.3	95.7	4.7	112	4.2	95.8	5736.4	-104.0	102		
	10 µg/mL	577.0	11699	4.9	95.1	5.6	114	4.9	95.1	11757.1	-58.3	100	96.4	3.7
		600.6	11308	5.3	94.7	5.9	103	5.7	94.3	10543.5	764.4	93		
		506.0	12142	4.2	95.8	5.0	115	4.4	95.6	11592.5	549.9	95		
30 min	0.5 µg/mL	23.5	596	3.9	96.1	4.8	118	4.1	95.9	573.7	22.4	96	99.8	5.3
		24.6	572	4.3	95.7	5.1	116	4.4	95.6	555.8	16.4	97		
		25.2	537	4.7	95.3	5.2	116	4.4	95.6	568.3	-31.7	106		
	5 µg/mL	276.8	5720	4.8	95.2	5.5	112	4.9	95.1	5634.0	86.5	98	104.0	4.9
		279.9	5512	5.1	94.9	5.5	115	4.8	95.2	5819.8	-308.0	106		
		301.1	5432	5.5	94.5	6.0	116	5.1	94.9	5860.3	-428.1	108		
	10 µg/mL	711.9	12098	5.9	94.1	6.8	115	5.9	94.1	12103.0	-4.7	100	101.8	1.9
		698.6	11390	6.1	93.9	6.5	110	5.9	94.1	11813.6	-424.0	104		
		663.1	11166	5.9	94.1	6.6	113	5.8	94.2	11338.2	-172.6	102		
1 hr	0.5 µg/mL	18.7	449	4.2	95.8	4.0	108	3.7	96.3	508.3	-59.4	113	111.4	1.9
		23.5	459	5.1	94.9	5.0	106	4.7	95.3	502.4	-43.3	109		
		21.6	470	4.6	95.4	4.6	111	4.1	95.9	524.5	-54.3	112		
	5 µg/mL	243.5	5779	4.2	95.8	4.8	122	3.9	96.1	6186.2	-407.0	107	108.8	2.9
		282.7	4848	5.8	94.2	5.7	111	5.2	94.8	5435.5	-587.5	112		
		268.0	5112	5.2	94.8	5.4	111	4.9	95.1	5476.9	-364.5	107		
	10 µg/mL	628.0	11129	5.6	94.4	6.1	106	5.8	94.2	10871.5	257.2	98	101.7	5.2
		608.9	11576	5.3	94.7	5.8	110	5.3	94.7	11558.1	17.5	100		
		662.7	10830	6.1	93.9	6.3	111	5.7	94.3	11650.5	-820.0	108		

Table V-34. Abraxane, Lot A (6113658) Drug Release Analytical Data. Presented are the analytical data for Abraxane, lot A (6113658). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 − %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated	% Release = (Unencapsulated/ Reservoir PTX) *100	AVG	SD
10 min	0.5 µg/mL	22.7	603	3.8	96.2	4.0	112	3.6	96.4	629.2	-25.7	104	108	4.7
		23.5	541	4.3	95.7	4.6	119	3.8	96.2	612.3	-70.9	113		
		22.1	573	3.9	96.1	4.1	113	3.6	96.4	606.8	-34.1	106		
	1 µg/mL	262.1	5501	4.8	95.2	4.6	114	4.0	96.0	6511.5	-1010.3	118	107	10.9
		271.4	6415	4.2	95.8	5.2	131	4.0	96.0	6842.2	-426.9	107		
		260.5	6602	3.9	96.1	4.8	117	4.1	95.9	6381.8	220.0	97		
	5 µg/mL	555.8	11943	4.7	95.3	5.0	121	4.2	95.8	13351.7	-1408.4	112	103	7.2
		458.2	12812	3.6	96.4	4.2	115	3.6	96.4	12711.6	100.3	99		
		539.2	12328	4.4	95.6	4.8	108	4.4	95.6	12262.0	66.4	99		
30 min	0.5 µg/mL	21.8	518	4.2	95.8	4.2	111	3.8	96.2	575.3	-57.8	111	114	3.8
		20.2	539	3.8	96.2	4.0	119	3.4	96.6	602.0	-62.7	112		
		26.5	497	5.3	94.7	5.0	110	4.5	95.5	586.6	-89.6	118		
	1 µg/mL	275.5	5545	5.0	95.0	5.1	122	4.2	95.8	6538.0	-993.4	118	115	3.6
		278.3	5534	5.0	95.0	5.2	115	4.5	95.5	6133.0	-599.2	111		
		265.0	5475	4.8	95.2	5.0	119	4.2	95.8	6326.3	-851.2	116		
	5 µg/mL	549.1	11098	4.9	95.1	5.3	115	4.6	95.4	11981.5	-883.5	108	109	1.4
		509.2	10992	4.6	95.4	4.7	110	4.2	95.8	12044.3	-1051.9	110		
		509.4	10965	4.6	95.4	4.6	111	4.2	95.8	12146.8	-1181.6	111		
1 hr	0.5 µg/mL	29.7	573	5.2	94.8	5.5	122	4.5	95.5	662.8	-89.5	116	110	7.8
		23.0	541	4.2	95.8	4.9	115	4.2	95.8	546.1	-4.9	101		
		21.0	525	4.0	96.0	4.1	115	3.5	96.5	593.3	-67.9	113		
	1 µg/mL	288.0	5927	4.9	95.1	5.4	120	4.5	95.5	6351.9	-424.6	107	103	8.3
		281.4	6347	4.4	95.6	5.1	126	4.1	95.9	6897.4	-550.4	109		
		266.4	6784	3.9	96.1	4.9	116	4.2	95.8	6349.6	434.2	94		
	5 µg/mL	548.8	13419	4.1	95.9	5.0	117	4.3	95.7	12723.9	695.4	95	102	7.7
		524.2	10357	5.1	94.9	4.8	97	5.0	95.0	10552.5	-195.4	102		
		541.8	10761	5.0	95.0	4.8	106	4.6	95.4	11859.0	-1097.9	110		

Table V-35. Genexol-PM, Lot A (GP31681) Drug Release Analytical Data. Presented are the analytical data for Genexol-PM, lot A (GP31681). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 − %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/(1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated	% Release = (Unencapsulated/ Reservoir PTX) *100	AVG	SD
10 min	0.5 µg/mL	22.7	518	4.4	95.6	4.7	109	4.3	95.7	524.7	-6.7	101	108	6.0
		23.9	544	4.4	95.6	4.8	120	4.0	96.0	597.5	-53.3	110		
		23.0	526	4.4	95.6	4.5	117	3.9	96.1	594.2	-67.7	113		
	1 µg/mL	275.1	5145	5.3	94.7	5.6	116	4.8	95.2	5686.3	-540.8	111	110	2.6
		263.2	5538	4.8	95.2	5.1	120	4.2	95.8	6231.0	-693.4	113		
		267.9	5718	4.7	95.3	5.4	124	4.4	95.6	6143.6	-425.4	107		
	5 µg/mL	573.9	11772	4.9	95.1	4.8	102	4.8	95.2	12058.8	-287.0	102	103	3.7
		564.4	11255	5.0	95.0	5.4	115	4.7	95.3	12009.4	-754.2	107		
		608.0	11263	5.4	94.6	5.9	108	5.4	94.6	11192.8	70.4	99		
30 min	0.5 µg/mL	26.0	565	4.6	95.4	4.8	119	4.0	96.0	643.5	-79.0	114	112	4.1
		23.7	527	4.5	95.5	4.7	119	3.9	96.1	603.3	-76.3	114		
		23.7	522	4.5	95.5	4.8	113	4.2	95.8	559.4	-37.1	107		
	1 µg/mL	313.1	5429	5.8	94.2	6.1	122	5.0	95.0	6259.1	-830.2	115	112	2.9
		299.9	4886	6.1	93.9	5.9	105	5.6	94.4	5383.3	-497.8	110		
		277.7	5096	5.4	94.6	5.3	108	4.9	95.1	5622.2	-526.0	110		
	5 µg/mL	532.2	10459	5.1	94.9	4.6	99	4.7	95.3	11402.7	-943.8	109	105	4.6
		527.7	10846	4.9	95.1	5.3	108	4.9	95.1	10843.6	2.6	100		
		545.2	10186	5.4	94.6	5.5	108	5.1	94.9	10795.4	-609.2	106		
1 hr	0.5 µg/mL	23.1	573	4.0	96.0	4.7	128	3.7	96.3	632.7	-59.8	110	115	4.0
		22.7	512	4.4	95.6	4.6	123	3.7	96.3	606.2	-93.9	118		
		27.6	499	5.5	94.5	5.5	114	4.8	95.2	576.7	-78.1	116		
	1 µg/mL	256.0	5561	4.6	95.4	5.2	123	4.2	95.8	6064.7	-503.8	109	110	0.66
		300.8	5374	5.6	94.4	5.7	112	5.1	94.9	5877.1	-503.3	109		
		251.1	5279	4.8	95.2	4.8	111	4.3	95.7	5824.5	-545.3	110		
	5 µg/mL	502.7	13086	3.8	96.2	4.5	114	4.0	96.0	12657.9	427.9	97	103	6.5
		568.9	11163	5.1	94.9	5.6	114	4.9	95.1	11624.4	-461.0	104		
		708.5	13141	5.4	94.6	6.4	129	4.9	95.1	14402.0	-1260.9	110		

Table V-36. Free Paclitaxel Control Data. Displayed is the calculated % release for the paclitaxel control data. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	109	2.0	1.9
	5 µg/mL	100	3.2	3.2
	10 µg/mL	96	3.7	3.9
30 min	0.5 µg/mL	100	5.3	5.4
	5 µg/mL	104	4.9	4.7
	10 µg/mL	102	1.9	1.8
1 hr	0.5 µg/mL	111	1.9	1.7
	5 µg/mL	109	2.9	2.7
	10 µg/mL	102	5.2	5.1
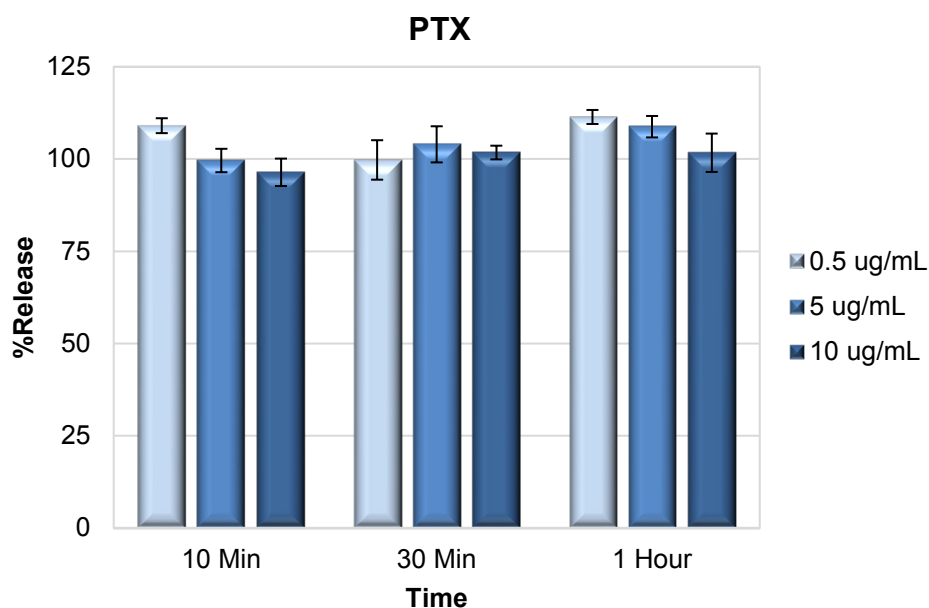
Figure V-27. Free Paclitaxel Control Data. Displayed is the calculated % release for the paclitaxel control data. (Mean $\pm$ SD, *N=3*)

Table V-37. Abraxane, Lot A (6113658) Drug Release. Displayed is the calculated % release for Abraxane, lot A (6113658). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	107.8	4.7	4.4
	5 µg/mL	107.2	10.9	10.1
	10 µg/mL	103.5	7.2	6.9
30 min	0.5 µg/mL	113.6	3.8	3.4
	5 µg/mL	114.8	3.6	3.1
	10 µg/mL	109.4	1.4	1.3
1 hr	0.5 µg/mL	109.8	7.8	7.1
	5 µg/mL	103.1	8.3	8.0
	10 µg/mL	102.3	7.7	7.5
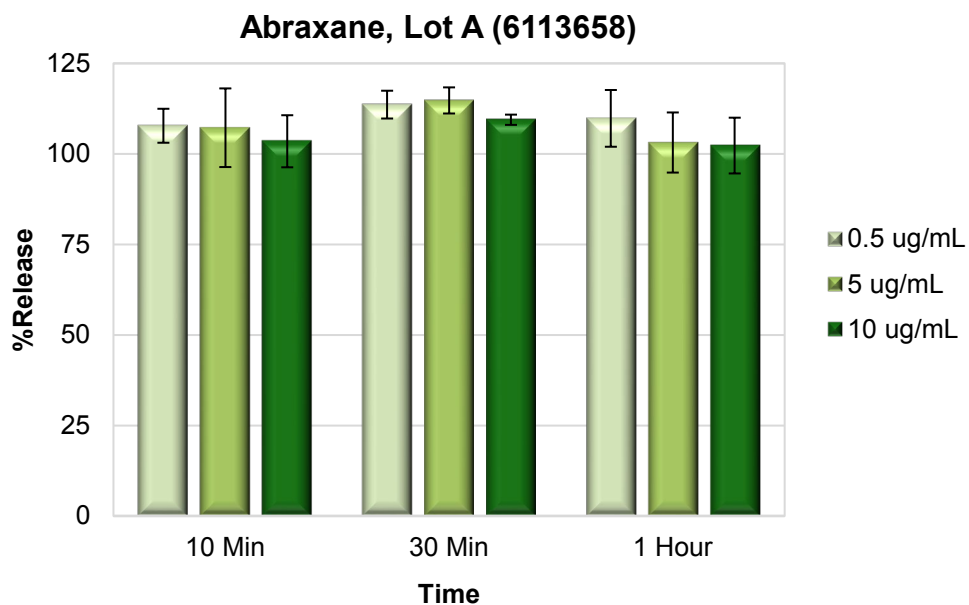
Figure V-28 Abraxane, Lot A (6113658) Drug Release. Displayed is the calculated % release for Abraxane, lot A (6113658). (Mean $\pm$ SD, *N=3*)

Table V-38. Genexol-PM, Lot A (GP31681) Drug Release. Displayed is the calculated % release for Genexol-PM, lot A (GP31681). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	108.0	6.0	5.5
	5 µg/mL	110.2	2.6	2.3
	10 µg/mL	102.8	3.7	3.6
30 min	0.5 µg/mL	111.9	4.1	3.7
	5 µg/mL	111.9	2.9	2.6
	10 µg/mL	105.0	4.6	4.4
1 hr	0.5 µg/mL	114.8	4.0	3.5
	5 µg/mL	109.6	0.7	0.6
	10 µg/mL	103.5	6.5	6.2
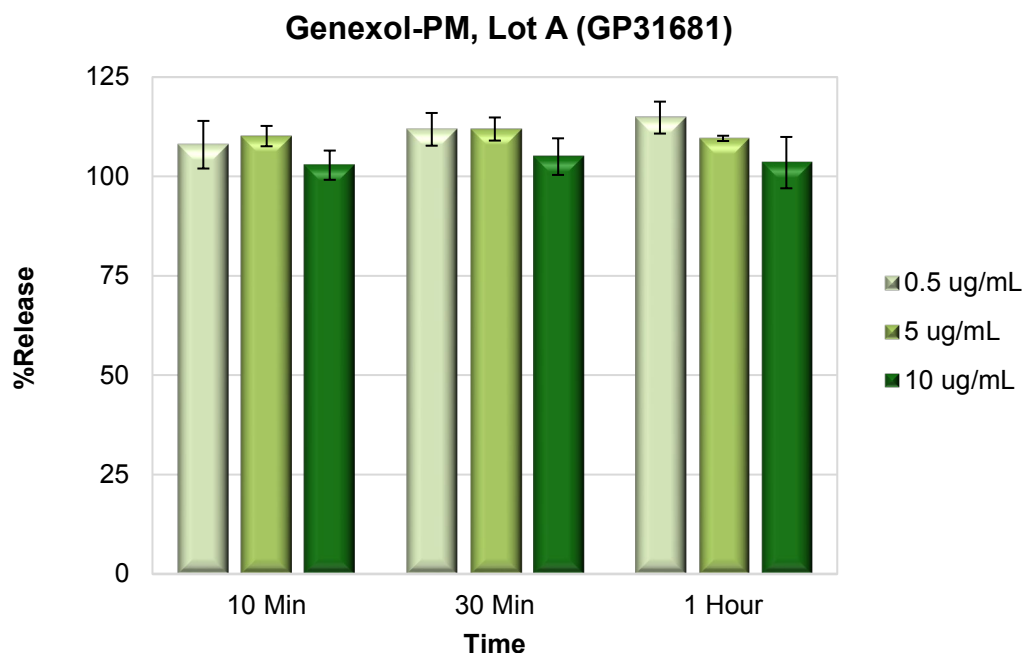
Figure V-29. Genexol-PM, Lot A (GP31681) Drug Release. Displayed is the calculated % release for Genexol-PM, lot A (GP31681). (Mean $\pm$ SD, *N=3*)

Abraxane vs. Genexol-PM, % Release in Rat Plasma

Legend: Genexol GP31681, Abraxane 6113658
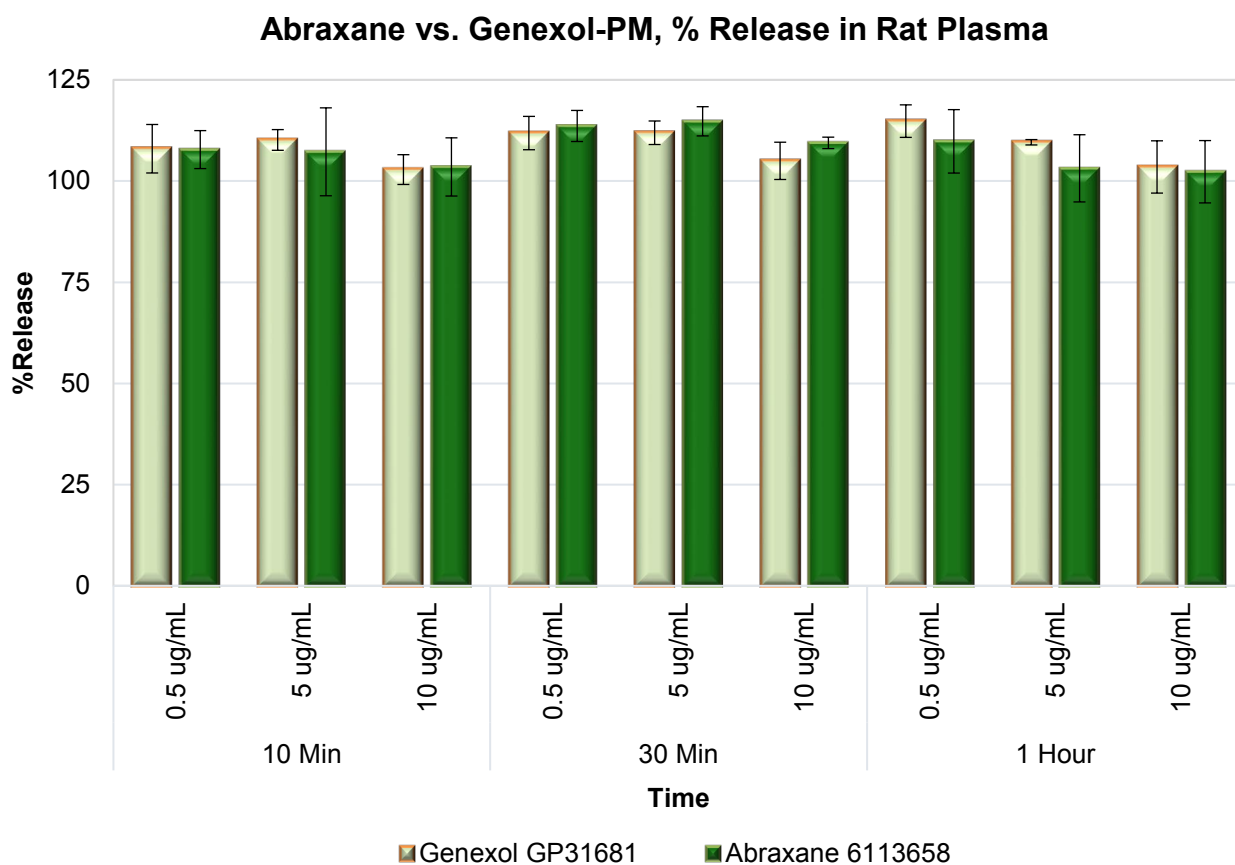
Figure V-30. Abraxane vs. Genexol-PM Drug Release Comparison in Rat Plasma.
Displayed are the 10 min – 1 hr calculated % release data for the Abraxane and Genexol-PM lots 6113658 and GP31681, respectively. (Mean $\pm$ SD, *N=3*), *p$\leq$0.05, Student's t-test

VI. In Vitro Abraxane, Genexol-PM, and Taxol Generic Comparisons

Intra-day Comparison in Human Plasma at 37°C

Summary
The objective of this study was to compare the in vitro drug release of a single lot of Abraxane, a single lot of Genexol-PM, and a single lot of Taxol in human plasma at 37°C over a 2 hr period. All lots had similar drug release profiles over the 2 hr period, although some instances of statistically significant differences were observed between lots. Notably, there were significant differences in drug binding between the formulations over the concentration range.

Design and Methods
A single lot of Abraxane, 6115664 (lot D), a single lot of Genexol-PM, GP31771 (lot D), and a single lot of Taxol generic PACCA1018 (lot A) were evaluated for drug release at 0.5, 5, 25 and 100 μg/mL PTX equivalents in human plasma at 37°C over a 2 hr period, according to the SITUA method described in Section I. All lots were run on a single day, and free paclitaxel at identical concentrations and time points was included as a control. The human plasma and protein-free plasma, PTX and PTX_C13 standard calibration curves used for this study are also provided.

Results & Conclusions
The PTX and PTX_C13 standard calibration curves used for this study are presented in **Tables VI-1 to VI-4 and Figures VI-1 to VI-4**. The free paclitaxel controls averaged between 88-109% of theoretical for all concentrations and time points (**Table VI-5 and VI-9, and Figure VI-5**). The Abraxane, Genexol-PM, and Taxol generic drug release was similar, at approximately 100% release at the earliest 10 min time point, without a clear concentration-dependent or temporal trend (**Tables VI-6 to VI-8 and VI-10 to VI-12, and Figures VI-6 to VI-9**). However, there were significant differences in drug binding between the formulations over the concentration range (**Figure V-10**). Abraxane displayed a saturated binding, suggesting that the Abraxane-albumin nanoparticle does not contribute to the protein binding. Genexol-PM displayed linear binding, suggesting a contribution of the PEG-PLA micelle to drug binding at high concentrations. Taxol generic displayed concentration dependent binding, suggesting the cremophor micelle strongly binds to free drug.

Table VI-1. PTX Plasma Standard Curve.

	Peak Area Ratio	Conc. (ng/mL)	% Accuracy
0.10 µg/mL	0.7	0.10	96
0.10 µg/mL	0.8	0.11	110
0.50 µg/mL	4.0	0.46	91
0.50 µg/mL	3.9	0.45	90
1.00 µg/mL	8.3	0.93	93
1.00 µg/mL	8.2	0.93	93
5.00 µg/mL	42	4.66)	93
5.00 µg/mL	46	5.02	100
10.0 µg/mL	93	10.2	102
10.0 µg/mL	99	10.9	109
50.0 µg/mL	453	49.6	99
50.0 µg/mL	511	55.8	112
100.0 µg/mL	966	106	106
100.0 µg/mL	1039	114	114
250.0 µg/mL	2060	225	90
250.0 µg/mL	2364	259	103
QC			
QCL	4.2	0.48	96
QCL	4.0	0.46	91
QCM	99	10.9	109
QCM	96	10.5	105
QCH	885	96.7	97
QCH	1045	114.3	114

PTX

$Y = -0.216812 + 9.14518 \cdot X$ $R^2 = 0.9922$ W: $1/X^2$
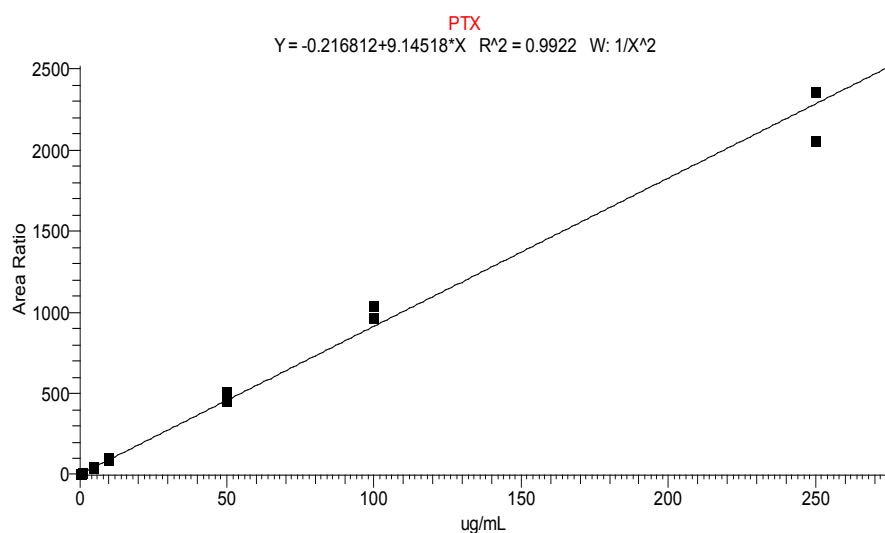
Figure VI-1. PTX Plasma Standard Curve.

Table VI-2. PTX_C13 Plasma Standard Curve.

	Peak Area Ratio	Conc. (ng/mL)	% Accuracy
5.00 ng/mL	0.04	5.30	106
5.00 ng/mL	0.03	4.97	99
10.0 ng/mL*	0.03	5.2	52
10.0 ng/mL	0.07	8.8	88
50.0 ng/mL	0.5	50.2	100
50.0 ng/mL	0.5	51.7	103
100.0 ng/mL	1.0	101.4	101
100.0 ng/mL	1.0	101.8	102
500 ng/mL	5.0	475	95
500 ng/mL	5.6	539	108
1000 ng/mL	10.4	997	100
1000 ng/mL	11.3	1081	108
2500 ng/mL	24	2252	90
2500 ng/mL	26	2473	99
QC			
QCL	0.04	5.83	117
QCL	0.02	4.21	84
QCM	1.1	110.9	111
QCM	1.1	109.4	109
QCH	10.5	1005	100
QCH	10.8	1029	103

*Point excluded from curve

PTX_C13
$Y = -0.0198383 + 0.0104683*X$ $R^2 = 0.9953$ W: 1/X^2
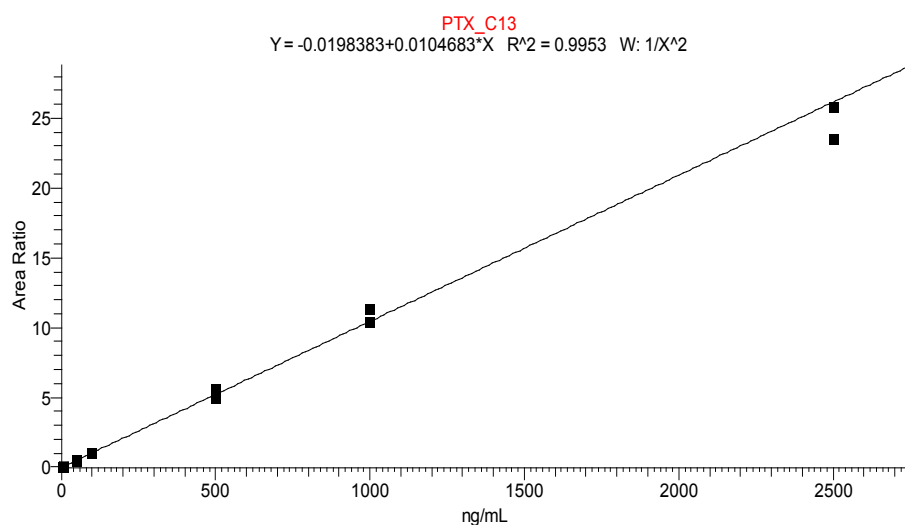
Figure VI-2. PTX_C13 Plasma Standard Curve.

Table VI-3. PTX Protein-Free Plasma Standard Curve.

	Peak Area Ratio	Conc. (ng/mL)	% Accuracy
1.00 ng/mL	0.009	1.05	105
1.00 ng/mL	0.008	0.93	93
5.00 ng/mL	0.04	5.26	105
5.00 ng/mL	0.04	5.17	103
10.0 ng/mL	0.08	10.3	103
10.0 ng/mL	0.08	9.6	96
50.0 ng/mL	0.4	53.5	107
50.0 ng/mL	0.4	53.1	106
100.0 ng/mL	0.8	100.6	101
100.0 ng/mL	0.8	101.3	101
500 ng/mL	4.4	543	109
500 ng/mL	4.2	524	105
1000 ng/mL	7.9	978	98
1000 ng/mL	7.7	955	96
5000 ng/mL	38	4724	94
5000 ng/mL	36	4478	90
10000 ng/mL	78	9580	96
10000 ng/mL	75	9247	92
QC			
QCL	0.05	5.53	111
QCL	0.05	5.72	114
QCM	0.8	103.1	103
QCM	0.8	100.6	101
QCH	40	4938	99
QCH	38	4633	93

PTX
Y = 0.000488322+0.00802169*X   R^2 = 0.9948   W: 1/X^2
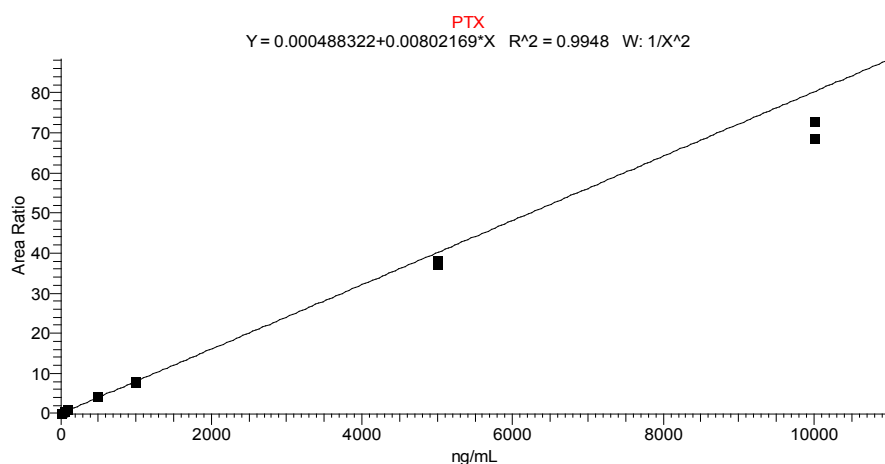
Figure VI-3. PTX Protein-Free Plasma Standard Curve.

Table VI-4. PTX_C13 Protein-Free Plasma Standard Curve.

	Peak Area Ratio	Conc. (ng/mL)	% Accuracy
1.00 ng/mL	0.008	1.10	110
1.00 ng/mL	0.006	0.89	89
5.00 ng/mL	0.05	5.17	103
5.00 ng/mL	0.05	5.06	101
10.0 ng/mL	0.09	10.0	100
10.0 ng/mL	0.09	10.0	100
50.0 ng/mL	0.5	52.0	104
50.0 ng/mL	0.5	53.3	107
100.0 ng/mL	1.0	104.3	104
100.0 ng/mL	0.9	95.9	96
500 ng/mL	5.0	524	105
500 ng/mL	5.2	548	110
1000 ng/mL	9.9	1049	105
1000 ng/mL	8.6	911	91
5000 ng/mL	46	4894	98
5000 ng/mL	43	4530	91
10000 ng/mL	94	9975	100
10000 ng/mL	83	8758	88
QC			
QCL	0.05	5.5	110
QCL	0.05	5.4	108
QCM	1.0	105.6	106
QCM	1.0	102.7	103
QCH	46	4862	97
QCH	42	4468	89

PTX_C13
Y = -0.00179759+0.00934686*X   R^2 = 0.9933   W: 1/X^2
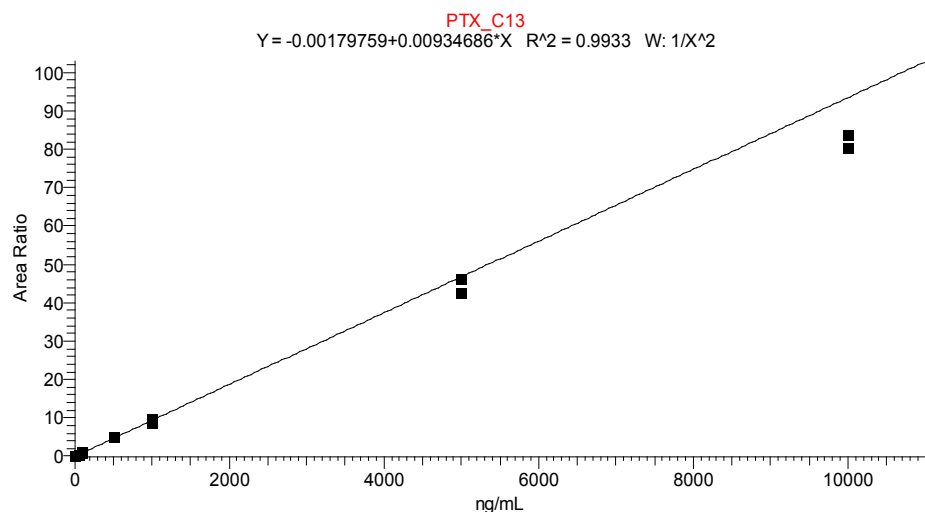
Figure VI-4. PTX_C13 Protein-Free Plasma Standard Curve.

Table VI-5. Free Paclitaxel Control Analytical Data. Presented are the analytical data for the free paclitaxel controls. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 − %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated	% Release = (Unencapsulated/ Reservoir PTX) *100	AVG	SD
10 min	0.5 µg/mL	32	483	6.7	93.3	5.2	87	6.0	94.0	544	-60.6	113	106	9.0
		27	482	5.7	94.3	5.5	93	5.9	94.1	466	16.9	96		
		30	517	5.8	94.2	5.1	88	5.8	94.2	516	0.7	100		
	5 µg/mL	272	4369	6.2	93.8	5.7	94	6.0	94.0	4519	-149.9	103	102	1.7
		269	4559	5.9	94.1	5.7	92	6.1	93.9	4374	184.6	96		
		274	4658	5.9	94.1	5.4	93	5.8	94.2	4707	-48.8	101		
	25 µg/mL	1373	20466	6.7	93.3	6.4	87	7.4	92.6	18548	1918.7	91	93	4.2
		1402	20337	6.9	93.1	6.4	84	7.5	92.5	18603	1733.8	91		
		1532	21722	7.1	92.9	5.8	81	7.2	92.8	21356	365.7	98		
	100 µg/mL	6620	70123	9.4	90.6	8.8	88	10.0	90.0	66150	3972.9	94	93	4.4
		5480	71725	7.6	92.4	7.5	95	7.9	92.1	69112	2612.4	96		
		5748	80307	7.2	92.8	7.4	90	8.1	91.9	70585	9722.4	88		
30 min	0.5 µg/mL	28	490	5.8	94.2	4.9	90	5.5	94.5	519	-28.8	106	109	4.3
		30	478	6.3	93.7	5.2	88	5.9	94.1	511	-32.6	107		
		32	492	6.5	93.5	5.1	89	5.7	94.3	560	-68.0	114		
	5 µg/mL	285	4547	6.3	93.7	5.9	91	6.4	93.6	4439	108.5	98	100	9.1
		278	4817	5.8	94.2	6.0	95	6.3	93.7	4417	400.0	92		
		288	4010	7.2	92.8	5.5	84	6.6	93.4	4397	-386.6	110		
	25 µg/mL	1423	22572	6.3	93.7	6.0	90	6.7	93.3	21383	1188.2	95	94	1.2
		1422	21439	6.6	93.4	6.1	87	7.0	93.0	20301	1137.9	95		
		1415	22494	6.3	93.7	5.7	84	6.8	93.2	20823	1670.9	93		
	100 µg/mL	4471	71169	6.3	93.7	6.9	95	7.3	92.7	61447	9722.3	86	88	3.8
		5595	70241	8.0	92.0	7.9	92	8.6	91.4	65217	5023.8	93		
		5930	77553	7.6	92.4	8.3	94	8.9	91.1	66913	10640.4	86		
2 hr	0.5 µg/mL	26	471	5.4	94.6	5.3	88	6.0	94.0	424	46.8	90	90	3.3
		26	467	5.5	94.5	5.3	89	6.0	94.0	432	35.2	92		
		26	464	5.6	94.4	5.5	85	6.5	93.5	399	64.9	86		
	5 µg/mL	260	4115	6.3	93.7	5.7	87	6.6	93.4	3960	154.7	96	95	3.0
		248	4384	5.7	94.3	5.4	87	6.2	93.8	4012	372.5	92		
		248	4355	5.7	94.3	5.3	91	5.9	94.1	4230	124.7	97		
	25 µg/mL	1359	21259	6.4	93.6	6.0	92	6.5	93.5	21016	242.4	99	96	3.0
		1237	22668	5.5	94.5	5.3	90	5.9	94.1	21102	1565.7	93		
		1345	21730	6.2	93.8	5.4	86	6.3	93.7	21180	549.5	97		
	100 µg/mL	4783	64715	7.4	92.6	6.3	83	7.6	92.4	63089	1626.6	97	97	8.1
		5201	72042	7.2	92.8	7.3	89	8.1	91.9	63972	8069.9	89		
		6257	49696	12.6	87.4	7.8	65	12.0	88.0	52140	-2444.3	105		

Table VI-6. Abraxane, Lot D (6115664) Drug Release Analytical Data. Presented are the analytical data for Abraxane, lot D (6115664). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX	% Bound	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13	% Bound PTX_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG	SD
				= Filtrate PTX / Reservoir PTX *100	= 100 − %Unbound PTX			= Filtrate PTX_C13 / Reservoir PTX_C13 * 100	= 100 − %Unbound C13	= Fil PTX/ (1-(%Bound PTX_C13/100))	= Reservoir PTX- Unencapsulated	= (Unencapsulated/ Reservoir PTX) *100		
10 min	0.5 µg/mL	18	403	4.4	95.6	4.0	91	4.4	95.6	406	-2.8	101	109	9.8
		25	482	5.2	94.8	4.5	103	4.3	95.7	576	-94.0	120		
		20	569	3.5	96.5	4.1	124	3.3	96.7	601	-31.1	105		
	5 µg/mL	215	4493	4.8	95.2	4.4	99	4.4	95.6	4851	-357.9	108	103	4.8
		222	4464	5.0	95.0	5.0	99	5.0	95.0	4411	52.8	99		
		218	4223	5.2	94.8	4.8	94	5.1	94.9	4264	-41.3	101		
	25 µg/mL	1138	23078	4.9	95.1	4.9	94	5.2	94.8	22048	1030.4	96	100	5.3
		1216	21858	5.6	94.4	5.0	88	5.6	94.4	21645	213.1	99		
		1232	20599	6.0	94.0	5.1	91	5.7	94.3	21807	-1208.7	106		
	100 µg/mL	5727	87432	6.5	93.5	6.2	91	6.8	93.2	83679	3753.2	96	94	1.8
		5326	76269	7.0	93.0	6.1	81	7.6	92.4	70293	5976.2	92		
		4891	83870	5.8	94.2	5.7	92	6.3	93.7	78241	5629.1	93		
30 min	0.5 µg/mL	22	452	4.8	95.2	5.1	100	5.1	94.9	428	23.8	95	97	4.5
		23	464	5.0	95.0	5.0	96	5.2	94.8	439	25.7	94		
		22	440	5.1	94.9	4.7	94	5.0	95.0	451	-10.8	102		
	5 µg/mL	257	4307	6.0	94.0	5.0	92	5.4	94.6	4740	-432.9	110	104	5.4
		234	4390	5.3	94.7	4.9	93	5.3	94.7	4422	-31.8	101		
		239	4483	5.3	94.7	5.1	97	5.3	94.7	4517	-34.4	101		
	25 µg/mL	1247	21656	5.8	94.2	5.4	92	5.8	94.2	21355	300.0	99	95	3.7
		1264	23236	5.4	94.6	5.7	97	5.8	94.2	21726	1509.7	94		
		1441	23775	6.1	93.9	6.0	90	6.6	93.4	21758	2017.0	92		
	100 µg/mL	6093	90580	6.7	93.3	6.5	84	7.8	92.2	78508	12072.3	87	94	7.9
		6230	88118	7.1	92.9	6.8	99	6.9	93.1	90346	-2228.7	103		
		6649	82292	8.1	91.9	7.4	86	8.6	91.4	77254	5037.8	94		
2 hr	0.5 µg/mL	22	420	5.2	94.8	4.9	95	5.2	94.8	421	-1.0	100	102	2.2
		25	430	5.9	94.1	5.3	91	5.8	94.2	438	-7.3	102		
		22	432	5.2	94.8	4.6	93	4.9	95.1	452	-19.6	105		
	5 µg/mL	236	4518	5.2	94.8	4.7	94	4.9	95.1	4768	-249.4	106	102	3.0
		244	4426	5.5	94.5	5.1	92	5.5	94.5	4410	16.0	100		
		267	4431	6.0	94.0	5.6	94	5.9	94.1	4502	-70.8	102		
	25 µg/mL	1373	21744	6.3	93.7	5.6	91	6.2	93.8	22207	-463.6	102	101	2.3
		1297	21905	5.9	94.1	5.3	93	5.7	94.3	22628	-722.6	103		
		1408	20777	6.8	93.2	5.8	85	6.9	93.1	20553	224.0	99		
	100 µg/mL	6854	85417	8.0	92.0	6.8	81	8.4	91.6	81969	3447.8	96	97	10.6
		5838	76394	7.6	92.4	6.2	87	7.1	92.9	82246	-5851.8	108		
		6683	94088	7.1	92.9	6.6	80	8.2	91.8	81366	12722.6	86		

Table VI-7. Genexol-PM, Lot D (GP31771) Drug Release Analytical Data. Presented are the analytical data for Genexol-PM, lot D (GP31771). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 − %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated	% Release = (Unencapsulated/ Reservoir PTX) *100	AVG	SD
10 min	0.5 µg/mL	21	440	4.8	95.2	4.2	92	4.5	95.5	473	-33.2	108	108	1.3
		25	448	5.5	94.5	4.7	93	5.1	94.9	489	-40.4	109		
		23	426	5.3	94.7	4.4	89	5.0	95.0	452	-26.9	106		
	5 µg/mL	220	4535	4.9	95.1	5.2	101	5.1	94.9	4327	207.6	95	102	5.8
		227	4455	5.1	94.9	4.8	99	4.9	95.1	4614	-158.9	104		
		234	4418	5.3	94.7	4.8	96	5.0	95.0	4708	-290.8	107		
	25 µg/mL	1233	22821	5.4	94.6	5.2	87	6.0	94.0	20590	2231.5	90	90	2.3
		1061	22880	4.6	95.4	5.0	95	5.3	94.7	20098	2781.9	88		
		1205	22833	5.3	94.7	5.2	91	5.7	94.3	21092	1741.2	92		
	100 µg/mL	4480	78475	5.7	94.3	5.1	83	6.1	93.9	73018	5457.9	93	91	3.9
		4666	86483	5.4	94.6	5.3	93	5.7	94.3	81433	5049.9	94		
		4481	84723	5.3	94.7	5.5	91	6.1	93.9	73593	11130.6	87		
30 min	0.5 µg/mL	24	516	4.6	95.4	4.9	105	4.6	95.4	519	-3.3	101	105	4.3
		24	441	5.4	94.6	4.7	95	4.9	95.1	481	-40.5	109		
		24	466	5.2	94.8	4.5	92	4.9	95.1	492	-25.2	105		
	5 µg/mL	251	4689	5.4	94.6	5.2	94	5.6	94.4	4504	184.7	96	99	2.8
		236	4321	5.5	94.5	5.1	95	5.4	94.6	4387	-66.6	102		
		255	4609	5.5	94.5	5.3	93	5.6	94.4	4525	83.5	98		
	25 µg/mL	1238	23192	5.3	94.7	4.7	91	5.2	94.8	23982	-790.7	103	95	7.6
		1192	22785	5.2	94.8	5.3	90	5.9	94.1	20317	2468.3	89		
		1159	24623	4.7	95.3	4.9	95	5.1	94.9	22608	2015.0	92		
	100 µg/mL	4547	85778	5.3	94.7	5.5	86	6.4	93.6	71325	14453.6	83	85	4.9
		4698	101844	4.6	95.4	5.0	97	5.1	94.9	91651	10193.3	90		
		4935	93600	5.3	94.7	5.3	81	6.6	93.4	75327	18273.5	80		
2 hr	0.5 µg/mL	21	440	4.7	95.3	4.0	89	4.5	95.5	464	-24.2	105	98	6.5
		23	424	5.4	94.6	5.0	85	5.8	94.2	395	29.2	93		
		20	456	4.4	95.6	4.0	88	4.6	95.4	437	19.1	96		
	5 µg/mL	235	4594	5.1	94.9	5.0	87	5.8	94.2	4045	549.3	88	87	2.9
		246	4652	5.3	94.7	5.1	86	5.9	94.1	4180	471.7	90		
		237	4567	5.2	94.8	5.0	82	6.2	93.8	3844	723.2	84		
	25 µg/mL	1130	22870	4.9	95.1	4.8	88	5.5	94.5	20429	2440.7	89	92	4.3
		1248	23688	5.3	94.7	5.5	93	5.9	94.1	21197	2490.8	89		
		1321	24842	5.3	94.7	5.5	100	5.5	94.5	24045	797.2	97		
	100 µg/mL	4693	76709	6.1	93.9	5.3	83	6.4	93.6	73554	3154.1	96	92	6.1
		4911	86786	5.7	94.3	5.1	87	5.9	94.1	82950	3836.1	96		
		5079	90628	5.6	94.4	5.4	83	6.6	93.4	77147	13481.2	85		

Table VI-8. Taxol Generic (PACCA1018) Drug Release Analytical Data. Presented are the analytical data for Taxol generic (PACCA1018). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 – %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/(1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated	% Release = (Unencapsulated/ Reservoir PTX) *100	AVG	SD
10 min	0.5 µg/mL	25	559	4.5	95.5	4.4	94	4.7	95.3	537	21.9	96	103	6.7
		30	560	5.3	94.7	4.5	92	4.8	95.2	613	-53.2	109		
		25	521	4.9	95.1	4.5	94	4.8	95.2	533	-12.5	102		
	5 µg/mL	279	5629	4.9	95.1	4.8	89	5.4	94.6	5167	461.6	92	90	5.4
		255	5267	4.8	95.2	4.6	90	5.2	94.8	4928	339.0	94		
		269	6104	4.4	95.6	4.9	92	5.3	94.7	5097	1007.0	84		
	25 µg/mL	858	24944	3.4	96.6	3.6	89	4.1	95.9	21000	3943.9	84	84	1.2
		831	25955	3.2	96.8	3.3	84	3.9	96.1	21536	4418.6	83		
		705	19941	3.5	96.5	3.4	82	4.1	95.9	17017	2924.0	85		
	100 µg/mL	1408	72388	1.9	98.1	1.9	81	2.4	97.6	59349	13039.0	82	81	1.8
		1391	73599	1.9	98.1	2.0	85	2.3	97.7	60400	13199.5	82		
		1369	84030	1.6	98.4	1.7	85	2.1	97.9	66379	17650.1	79		
30 min	0.5 µg/mL	30	569	5.2	94.8	5.4	90	6.0	94.0	495	73.4	87	96	8.0
		30	531	5.7	94.3	5.1	91	5.6	94.4	542	-11.8	102		
		28	509	5.5	94.5	4.9	88	5.5	94.5	504	4.6	99		
	5 µg/mL	293	5658	5.2	94.8	5.7	89	6.4	93.6	4552	1105.7	80	85	6.3
		289	5338	5.4	94.6	5.5	91	6.1	93.9	4774	563.8	89		
		*2265	*5440	*41.6	*58.4	*33.8	*83	*40.5	*59.5	*5590	*-150.3	*103		
	25 µg/mL	972	27148	3.6	96.4	3.6	91	3.9	96.1	24625	2522.5	91	90	0.6
		963	23210	4.2	95.8	3.8	83	4.6	95.4	20849	2360.8	90		
		823	19362	4.2	95.8	4.1	85	4.7	95.3	17334	2027.6	90		
	100 µg/mL	1901	77564	2.5	97.5	2.2	82	2.7	97.3	70484	7080.7	91	89	2.9
		1774	68475	2.6	97.4	2.5	87	2.9	97.1	61263	7212.3	89		
		1797	76784	2.3	97.7	2.4	89	2.7	97.3	65543	11241.5	85		
2 hr	0.5 µg/mL	27	534	5.0	95.0	4.8	89	5.4	94.6	491	43.2	92	100	8.0
		27	545	5.0	95.0	4.3	86	5.0	95.0	540	4.8	99		
		29	534	5.3	94.7	4.7	94	4.9	95.1	577	-42.4	108		
	5 µg/mL	288	5738	5.0	95.0	4.8	90	5.3	94.7	5387	351.2	94	93	11.4
		257	5367	4.8	95.2	4.5	77	5.9	94.1	4373	993.9	81		
		275	4958	5.6	94.4	4.8	90	5.3	94.7	5164	-206.0	104		
	25 µg/mL	921	21404	4.3	95.7	4.4	89	5.0	95.0	18506	2898.1	86	98	11.4
		1103	22043	5.0	95.0	4.0	86	4.6	95.4	24065	-2022.0	109		
		953	21015	4.5	95.5	3.9	83	4.7	95.3	20467	548.0	97		
	100 µg/mL	1916	71424	2.7	97.3	2.4	79	3.0	97.0	64389	7035.2	90	90	2.5
		2179	73650	3.0	97.0	2.8	82	3.4	96.6	64504	9145.7	88		
		1960	76504	2.6	97.4	2.5	89	2.8	97.2	70864	5639.6	93		

*Excluded from calculated average and standard deviation

Table VI-9. Free Paclitaxel Control Data. Displayed is the calculated % release for the paclitaxel control data. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	106	9.0	8.4
	5 µg/mL	102	1.7	1.6
	25 µg/mL	93	4.2	4.5
	100 µg/mL	93	4.4	4.8
30 min	0.5 µg/mL	109	4.3	4.0
	5 µg/mL	100	9.1	9.2
	25 µg/mL	94	1.2	1.3
	100 µg/mL	88	3.8	4.3
2 hr	0.5 µg/mL	90	3.3	3.7
	5 µg/mL	95	3.0	3.2
	25 µg/mL	96	3.0	3.1
	100 µg/mL	97	8.1	8.3
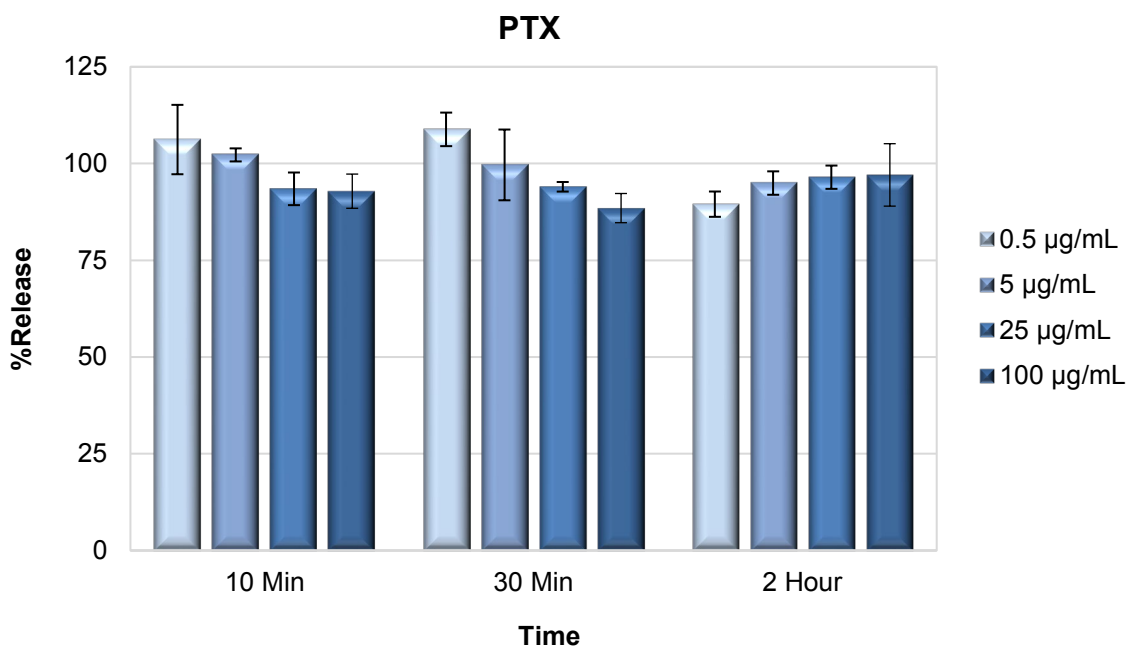
Figure VI-5. Free Paclitaxel Control Data. Displayed is the calculated % release for the paclitaxel control data. (Mean $\pm$ SD, *N=3*)

Table VI-10. Abraxane, Lot D (6115664) Drug Release. Displayed is the calculated % release for Abraxane, lot D (6115664). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	109	9.8	9.0
	5 µg/mL	103	4.8	4.7
	25 µg/mL	100	5.3	5.2
	100 µg/mL	94	1.8	1.9
30 min	0.5 µg/mL	97	4.5	4.7
	5 µg/mL	104	5.4	5.2
	25 µg/mL	95	3.7	3.9
	100 µg/mL	94	7.9	8.4
2 hr	0.5 µg/mL	102	2.2	2.1
	5 µg/mL	102	3.0	2.9
	25 µg/mL	101	2.3	2.2
	100 µg/mL	97	10.6	11.0
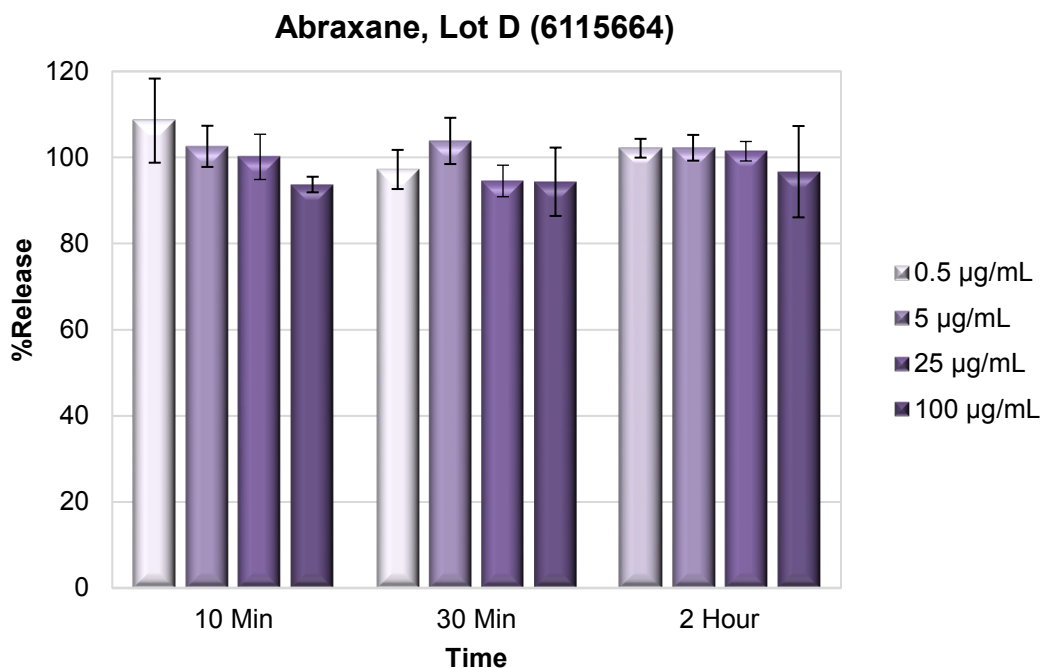
Figure VI-6. Abraxane, Lot D (6115664) Drug Release. Displayed is the calculated % release for the Abraxane, lot D (6115664). (Mean $\pm$ SD, *N=3*)

Table VI-11. Genexol-PM, Lot D (GP31771) Drug Release. Displayed is the calculated % release for Genexol-PM, lot D (GP31771). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	108	1.3	1.2
	5 µg/mL	102	5.8	5.7
	25 µg/mL	90	2.3	2.5
	100 µg/mL	91	3.9	4.3
30 min	0.5 µg/mL	105	4.3	4.1
	5 µg/mL	99	2.8	2.8
	25 µg/mL	95	7.6	8.0
	100 µg/mL	85	4.9	5.8
2 hr	0.5 µg/mL	98	6.5	6.6
	5 µg/mL	87	2.9	3.3
	25 µg/mL	92	4.3	4.6
	100 µg/mL	92	6.1	6.6
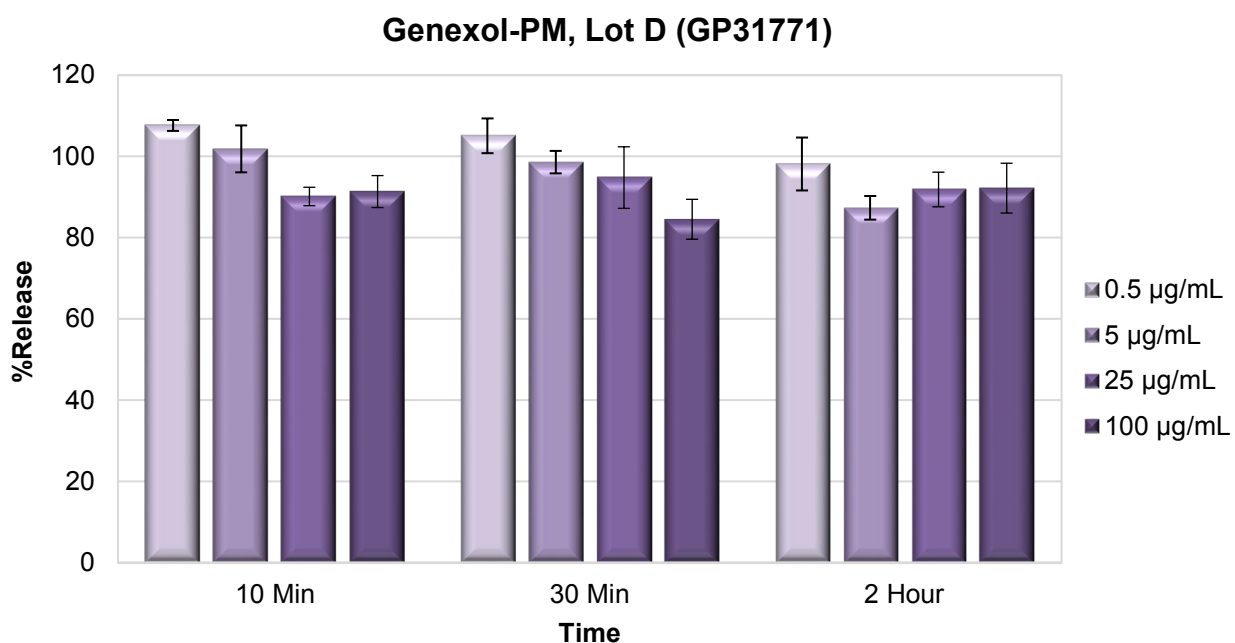
Figure VI-7. Genexol-PM, Lot D (GP31771) Drug Release. Displayed is the calculated % release for Genexol-PM, lot D (GP31771). (Mean $\pm$ SD, *N=3*)

Table VI-12. Taxol Generic (PACCA1018) Drug Release. Displayed is the calculated % release for Taxol generic (PACCA1018). (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	103	6.7	6.5
	5 µg/mL	90	5.4	6.0
	25 µg/mL	84	1.2	1.4
	100 µg/mL	81	1.8	2.2
30 min	0.5 µg/mL	96	8.0	8.3
	5 µg/mL	85	6.3	7.5
	25 µg/mL	90	0.6	0.7
	100 µg/mL	89	2.9	3.2
2 hr	0.5 µg/mL	100	8.0	8.0
	5 µg/mL	93	11.4	12.2
	25 µg/mL	98	11.4	11.6
	100 µg/mL	90	2.5	2.8
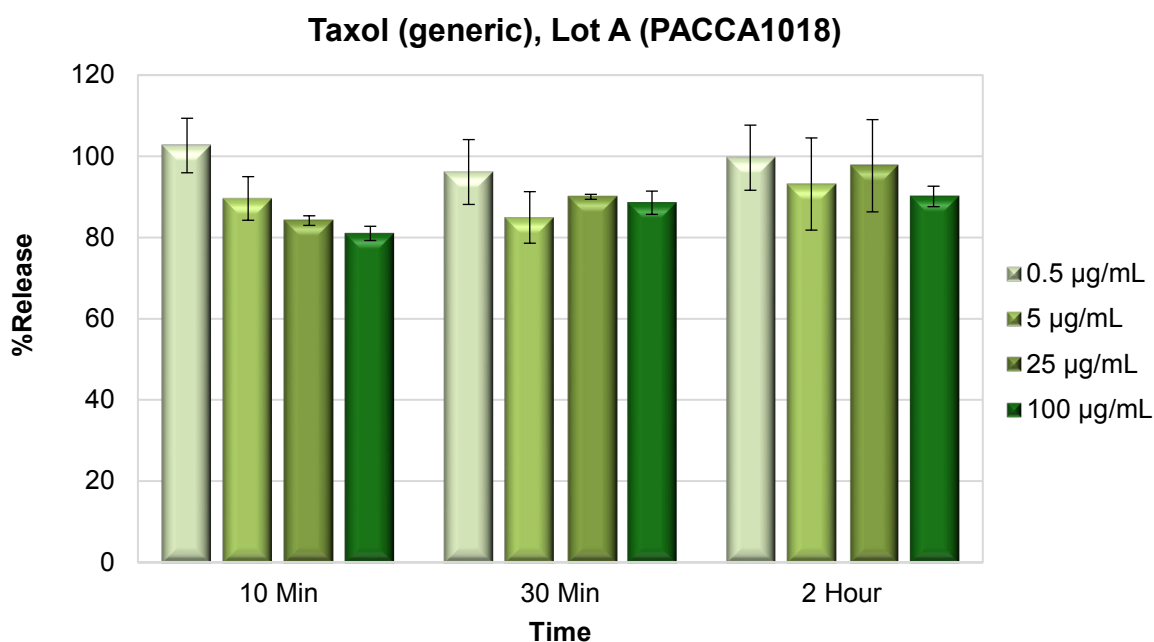
Figure VI-8. Taxol Generic (PACCA1018) Drug Release. Displayed is the calculated % release for the Taxol generic (PACCA1018). (Mean $\pm$ SD, *N=3*)
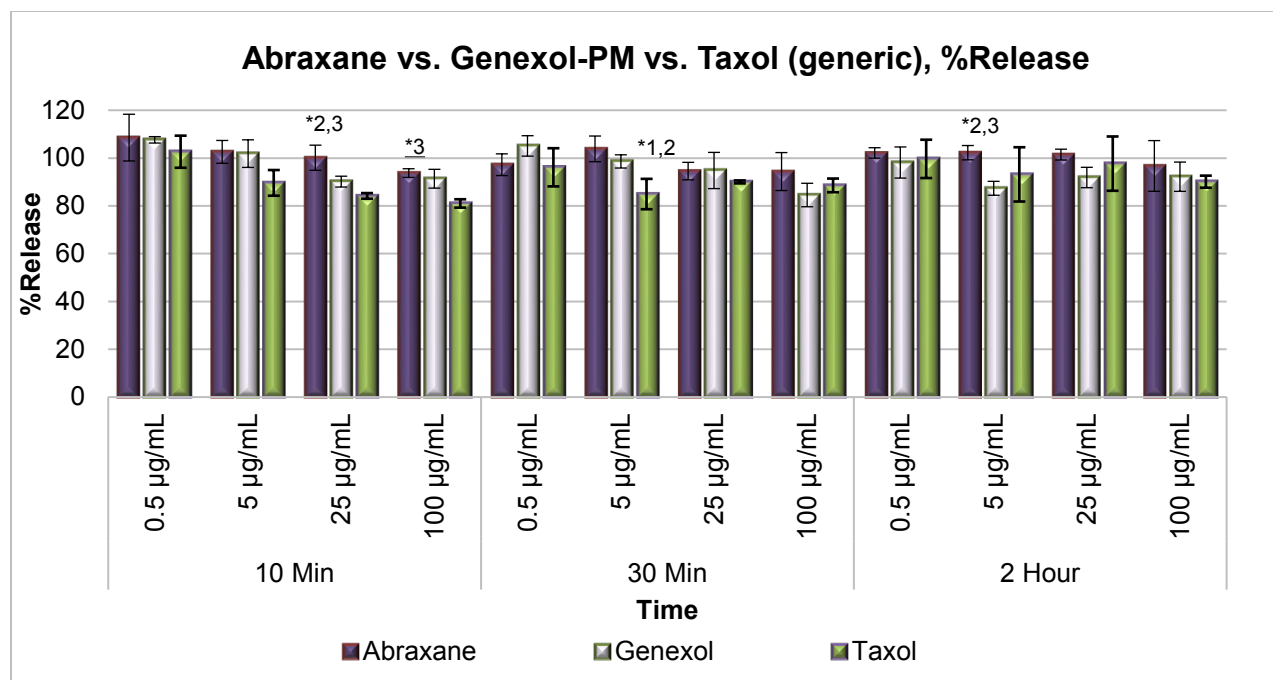
Figure VI-9. Abraxane, Genexol-PM, and Taxol Generic Drug Release Comparison.
Displayed are the 10 min – 2 hr calculated % release data for the Abraxane, Genexol-PM, and Taxol generic lots 6115664, GP31771, and PACCA1018, respectively. (Mean $\pm$ SD, *N=3*); *$p\leq0.05$, ANOVA, with Duncan's Multiple Range test posthoc comparisons; 1,2,3 designates significantly different from Abraxane, Genexol-PM, and Taxol generic, respectively.
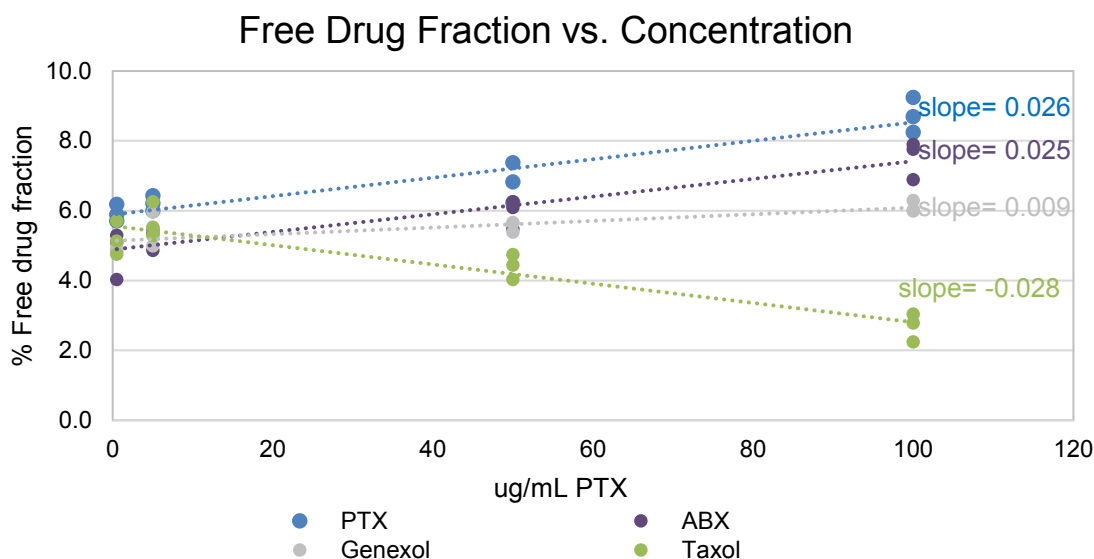
Figure VI-10. Abraxane, Genexol-PM, and Taxol Generic % Free Drug Fraction vs. Concentration Comparison. Displayed are the calculated % free drug fraction vs. concentration for the Abraxane, Genexol-PM, and Taxol generic lots 6115664, GP31771, and PACCA1018, respectively.

Intra-day Comparison in Rat Plasma at 37°C

Summary
The objective of this study was to compare the in vitro drug release of a single lot of Abraxane, a single lot of Genexol-PM, and a single lot of Taxol generic in rat plasma at 37°C over a 2 hr period. All lots had similar drug release profiles over the 2 hr period, although some instances of statistically significant differences were observed between lots. Notably, there were significant differences in drug binding between the formulations over the concentration range

Design and Methods
A single lot of Abraxane, 6115194 (lot E), a single lot of Genexol-PM, GP31771 (lot D), and a single lot of Taxol generic PACCA1018 (lot A) were evaluated for drug release at 0.5, 5, 25 and 100 µg/mL PTX equivalents in rat plasma at 37°C over a 2 hr period, according to the SITUA method described in Section I. All lots were run on a single day, and free paclitaxel at identical concentrations and time points was included as a control. The rat plasma and protein-free plasma, PTX and PTX_C13 standard calibration curves used for this study are also provided.

Results & Conclusions
The PTX and PTX_C13 standard calibration curves used for this study are presented in **Tables VI-13 to V-16 and Figures VI-11 to V-14**. The free paclitaxel controls averaged between 95-109% of theoretical for all concentrations and time points (**Table VI-17 and V-21, and Figure VI-15**). The Abraxane, Genexol-PM, and Taxol generic drug release was similar, at approximately 100% release at the earliest 10 min time point, without a clear concentration-dependent or temporal trend (**Tables VI-18 to V-20 and VI-22 to V-24, and Figures VI-16 to V-22**). However, there were significant differences in drug binding between the formulations over the concentration range (**Figure V-20**). Abraxane and Genexol-PM displayed saturated binding, similar to free paclitaxel, suggesting that the Abraxane-human albumin nanoparticle and Genexol PLA micelle do not contribute to the protein binding. Taxol generic displayed concentration dependent binding, suggesting the cremophor micelle strongly binds to free drug.

Table VI-13. PTX Plasma Standard Curve.

	Peak Area Ratio	Conc. (ng/mL)	% Accuracy
0.10 µg/mL	0.5	0.11	108
0.10 µg/mL	0.4	0.10	99
0.50 µg/mL	2.7	0.43	87
0.50 µg/mL	2.8	0.45	90
1.00 µg/mL	6.2	0.92	92
1.00 µg/mL	5.8	0.87	87
5.00 µg/mL	36	5.16	103
5.00 µg/mL	31	4.39	88
10.0 µg/mL	75	10.4	104
10.0 µg/mL	74	10.2	102
50.0 µg/mL	446	52.5	105
50.0 µg/mL	462	54.1	108
100.0 µg/mL	1146	111.9	112
100.0 µg/mL	1122	110.1	110
250.0 µg/mL	3094	227.1	91
250.0 µg/mL	3702	255.8	102
QC			
QCL	2.8	0.44	88
QCL	2.6	0.41	82
QCM	77.2	10.7	107
QCM	77.4	10.7	107
QCH	1125	110.4	110
QCH	1048	104.5	105

PTX

$Y = -0.295375 + 6.95665*X + 0.0293829*X^2$ $R^2 = 0.9882$ W: $1/X^2$
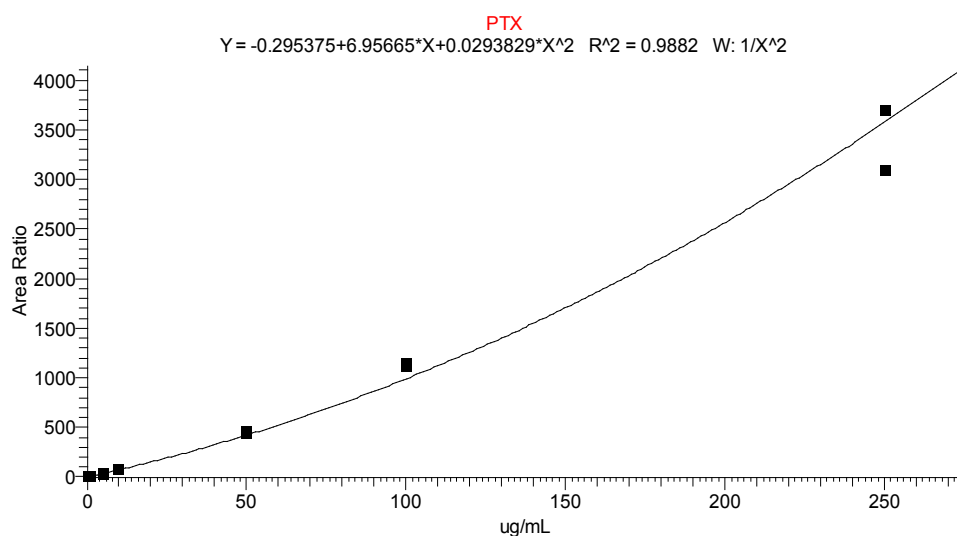
Figure VI-11. PTX Plasma Standard Curve.

Table VI-14. PTX_C13 Plasma Standard Curve.

	Peak Area Ratio	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	0.02	10.0	100
10.0 ng/mL	0.01	9.8	98
50.0 ng/mL	0.6	55.0	110
50.0 ng/mL	0.5	46.7	93
100.0 ng/mL	1.2	106.8	107
100.0 ng/mL	1.2	106.3	106
500 ng/mL	5.5	459	92
500 ng/mL	5.7	471	94
1000 ng/mL	11.9	977	98
1000 ng/mL	12.7	1042	104
2500 ng/mL	27	2218	89
2500 ng/mL	33	2718	109
QC			
QCL	0.02	10.3	103
QCL	0.01	9.6	96
QCM	1.1	99.4	99
QCM	1.3	111.2	111
QCH	12.0	980	98
QCH	12.3	1003	100

PTX_C13
$Y = -0.108805 + 0.0123252 \cdot X$ $R^2 = 0.9935$ W: $1/X^2$
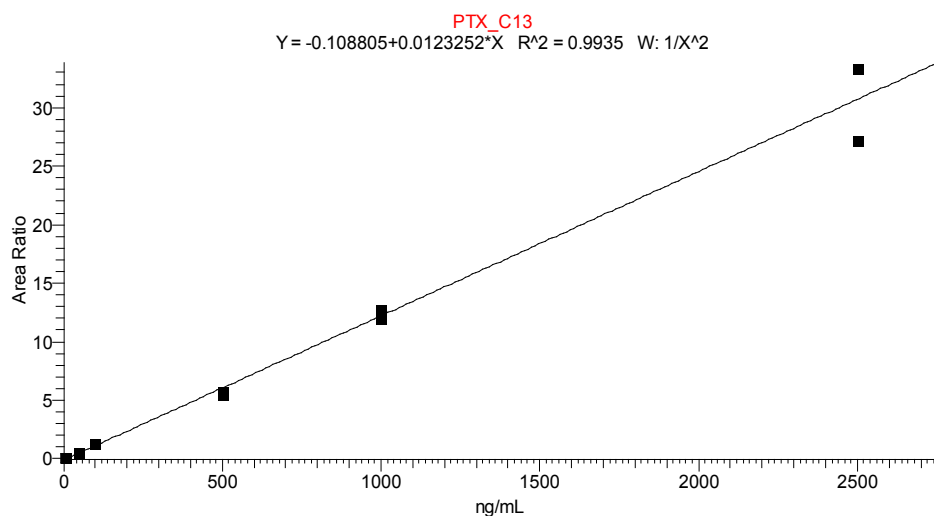
Figure VI-12. PTX_C13 Plasma Standard Curve.

Table VI-15. PTX Protein-Free Plasma Standard Curve.

	Peak Area Ratio	Conc. (ng/mL)	% Accuracy
1.00 ng/mL	0.005	1.03	103
1.00 ng/mL	0.005	0.98	98
5.00 ng/mL	0.03	4.74	95
5.00 ng/mL	0.03	4.76	95
10.0 ng/mL	0.06	10.3	103
10.0 ng/mL	0.07	10.6	106
50.0 ng/mL	0.3	51.9	104
50.0 ng/mL	0.3	52.0	104
100.0 ng/mL	0.6	102.6	103
100.0 ng/mL	0.6	99.0	99
500 ng/mL	3.2	524	105
500 ng/mL	3.0	495	99
1000 ng/mL	5.5	902	90
1000 ng/mL	5.6	918	92
5000 ng/mL	28	5141	103
5000 ng/mL	28	5157	103
10000 ng/mL	47	9969	100
10000 ng/mL	46	9881	99
QC			
QCL	0.03	5.05	101
QCL	0.03	4.89	98
QCM	0.6	98.0	98
QCM	0.6	100.6	101
QCH	29	5295	106
QCH	29	5407	108

PTX
$Y = -0.000982108 + 0.00621969 \cdot X - 1.53962e\text{-}007 \cdot X^2$ $R^2 = 0.9975$ W: $1/X^2$
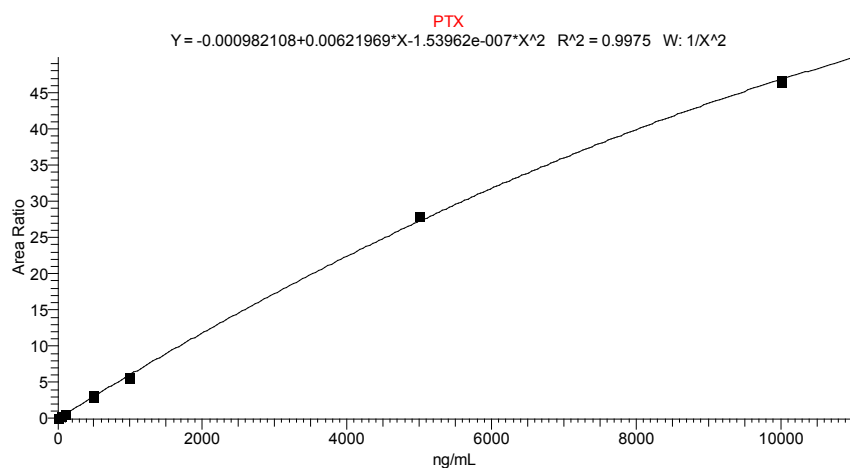
Figure VI-13. PTX Protein-Free Plasma Standard Curve.

Table VI-16. PTX_C13 Protein-Free Plasma Standard Curve.

	Peak Area Ratio	Conc. (ng/mL)	% Accuracy
1.00 ng/mL	0.006	0.96	96
1.00 ng/mL	0.007	1.04	104
5.00 ng/mL	0.05	5.24	105
5.00 ng/mL	0.05	4.90	98
10.0 ng/mL	0.11	9.8	98
10.0 ng/mL	0.10	9.6	96
50.0 ng/mL	0.6	52.0	104
50.0 ng/mL	0.6	54.1	108
100.0 ng/mL	1.2	102.2	102
100.0 ng/mL	1.1	97.7	98
500 ng/mL	5.8	510	102
500 ng/mL	5.7	501	100
1000 ng/mL	11.3	990	99
1000 ng/mL	11.4	1001	100
5000 ng/mL	55	4850	97
5000 ng/mL	53	4646	93
QC			
QCL	0.06	5.45	109
QCL	0.06	5.47	109
QCM	1.1	94.5	95
QCM	1.1	96.9	97
QCH	52	4557	91
QCH	59	5205	104

PTX_C13
Y = -0.0049116+0.0114278*X   R^2 = 0.9982   W: 1/X^2

Figure VI-14. PTX_C13 Protein-Free Plasma Standard Curve.

Table VI-17. Free Paclitaxel Control Analytical Data. Presented are the analytical data for the free paclitaxel controls. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

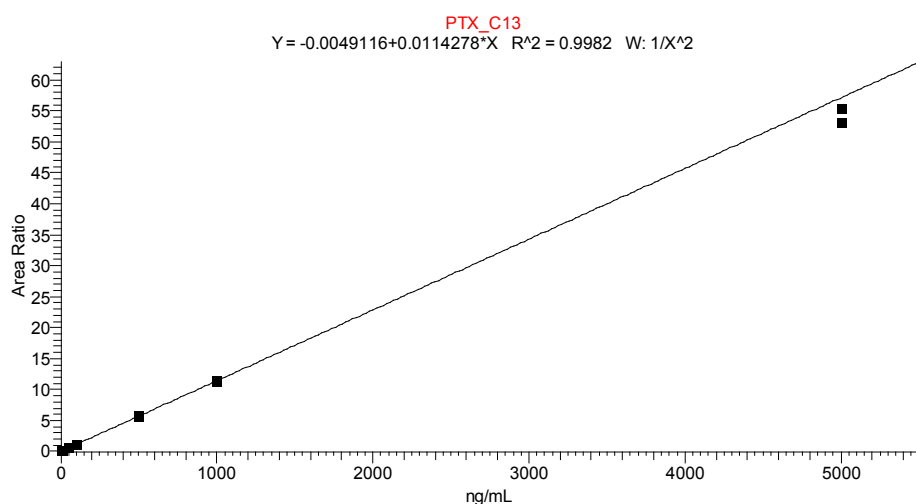|		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX	% Bound	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13	% Bound PTX_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG	SD
				= Filtrate PTX / Reservoir PTX *100	= 100 – %Unbound PTX			= Filtrate PTX_C13 / Reservoir PTX_C13 * 100	= 100 – %Unbound C13	= Fil PTX/ (1-(%Bound PTX_C13/100))	= Reservoir PTX- Unencapsulated	= (Unencapsulated/ Reservoir PTX) *100		
10 min	0.5 µg/mL	20	476	4.2	95.8	3.6	90	4.1	95.9	488	-11.7	102	98	4.5
		18	453	4.1	95.9	3.7	88	4.2	95.8	438	15.0	97		
		21	464	4.6	95.4	4.5	92	4.9	95.1	435	29.4	94		
	5 µg/mL	246	4560	5.4	94.6	5.5	93	5.9	94.1	4199	360.5	92	95	4.0
		237	4473	5.3	94.7	5.2	92	5.7	94.3	4174	299.1	93		
		250	4371	5.7	94.3	5.5	96	5.8	94.2	4352	19.1	100		
	25 µg/mL	1395	22765	6.1	93.9	5.8	97	5.9	94.1	23572	-807.5	104	95	7.7
		1349	27369	4.9	95.1	5.7	103	5.5	94.5	24390	2979.1	89		
		1379	24645	5.6	94.4	5.5	90	6.1	93.9	22587	2057.9	92		
	100 µg/mL	6202	79352	7.8	92.2	6.6	96	6.9	93.1	89884	-10531.9	113	109	6.8
		6314	88352	7.1	92.9	6.4	90	7.1	92.9	89434	-1082.3	101		
		5972	75145	7.9	92.1	6.9	98	7.1	92.9	84653	-9507.9	113		
30 min	0.5 µg/mL	23	499	4.5	95.5	3.9	87	4.5	95.5	503	-3.7	101	102	6.0
		21	429	4.9	95.1	3.9	87	4.5	95.5	464	-35.0	108		
		22	589	3.8	96.2	4.8	121	3.9	96.1	567	22.2	96		
	5 µg/mL	264	4400	6.0	94.0	5.8	90	6.4	93.6	4127	273.4	94	93	8.6
		301	6048	5.0	95.0	6.3	106	5.9	94.1	5073	975.1	84		
		277	4518	6.1	93.9	6.2	102	6.1	93.9	4559	-41.2	101		
	25 µg/mL	1807	26103	6.9	93.1	7.3	92	7.9	92.1	22730	3372.7	87	96	7.7
		1506	30760	4.9	95.1	6.3	129	4.9	95.1	30737	23.4	100		
		1527	30260	5.0	95.0	5.9	119	5.0	95.0	30487	-226.6	101		
	100 µg/mL	9707	106991	9.1	90.9	8.2	96	8.6	91.4	112920	-5929.3	106	97	8.8
		8955	119455	7.5	92.5	7.0	92	7.6	92.4	117089	2365.3	98		
		7937	104501	7.6	92.4	7.8	91	8.6	91.4	92026	12475.3	88		
2 hr	0.5 µg/mL	19	414	4.7	95.3	3.5	82	4.3	95.7	449	-34.1	108	107	1.3
		18	507	3.6	96.4	3.9	114	3.4	96.6	537	-29.6	106		
		19	437	4.3	95.7	4.1	105	3.9	96.1	473	-35.1	108		
	5 µg/mL	245	4641	5.3	94.7	5.2	95	5.5	94.5	4438	203.0	96	91	5.0
		251	5807	4.3	95.7	5.3	112	4.7	95.3	5289	517.7	91		
		251	5355	4.7	95.3	4.9	89	5.5	94.5	4582	773.2	86		
	25 µg/mL	1460	24839	5.9	94.1	6.1	96	6.3	93.7	23008	1831.3	93	90	3.0
		1444	27510	5.2	94.8	6.1	101	6.0	94.0	23865	3645.0	87		
		1360	27476	5.0	95.0	5.2	96	5.4	94.6	25026	2449.8	91		
	100 µg/mL	7798	118401	6.6	93.4	6.9	99	6.9	93.1	112440	5961.5	95	96	16.0
		9615	120222	8.0	92.0	7.6	76	10.0	90.0	96146	24076.1	80		
		9788	110027	8.9	91.1	8.9	112	7.9	92.1	123199	-13172.4	112		

Table VI-18. Abraxane, Lot E (6115194) Drug Release Analytical Data. Presented are the analytical data for Abraxane, lot E (6115194). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 − %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated	% Release = (Unencapsulated/ Reservoir PTX) *100	AVG	SD
10 min	0.5 µg/mL	20	496	3.9	96.1	4.2	107	3.9	96.1	504	-7.9	102	97	7.2
		19	535	3.5	96.5	4.1	102	4.0	96.0	475	60.0	89		
		20	505	3.9	96.1	4.1	107	3.9	96.1	510	-4.4	101		
	5 µg/mL	244	5975	4.1	95.9	4.9	119	4.1	95.9	5903	71.6	99	96	3.7
		271	7484	3.6	96.4	5.3	141	3.8	96.2	7210	274.3	96		
		277	5672	4.9	95.1	5.2	97	5.3	94.7	5192	480.3	92		
	25 µg/mL	1564	30461	5.1	94.9	5.6	100	5.7	94.3	27575	2886.1	91	93	3.4
		1340	25689	5.2	94.8	4.9	86	5.7	94.3	23686	2003.5	92		
		1458	23881	6.1	93.9	5.7	90	6.3	93.7	23161	719.5	97		
	100 µg/mL	6988	90816	7.7	92.3	6.4	98	6.5	93.5	107390	-16574.5	118	104	13.4
		7428	99172	7.5	92.5	5.7	78	7.3	92.7	101592	-2420.5	102		
		7025	103235	6.8	93.2	6.3	85	7.4	92.6	94629	8605.9	92		
30 min	0.5 µg/mL	24	577	4.1	95.9	4.6	115	4.0	96.0	591	-14.0	102	100	16.2
		22	460	4.8	95.2	4.3	102	4.2	95.8	526	-66.5	114		
		22	542	4.1	95.9	4.5	89	5.0	95.0	447	95.5	82		
	5 µg/mL	272	5265	5.2	94.8	5.5	111	5.0	95.0	5473	-207.7	104	109	4.8
		269	4566	5.9	94.1	5.2	100	5.2	94.8	5176	-610.1	113		
		257	6090	4.2	95.8	5.2	135	3.8	96.2	6697	-606.7	110		
	25 µg/mL	1627	30358	5.4	94.6	6.5	99	6.6	93.4	24799	5559.4	82	90	8.2
		1491	27633	5.4	94.6	5.9	98	6.0	94.0	24721	2912.3	89		
		1552	25493	6.1	93.9	6.0	97	6.2	93.8	25004	489.1	98		
	100 µg/mL	8134	118975	6.8	93.2	6.9	97	7.1	92.9	113885	5089.6	96	95	7.2
		8418	154201	5.5	94.5	6.6	122	5.4	94.6	157242	-3041.4	102		
		7351	132969	5.5	94.5	6.3	99	6.3	93.7	116410	16558.8	88		
2 hr	0.5 µg/mL	22	492	4.4	95.6	4.8	97	5.0	95.0	435	56.5	89	97	9.6
		19	470	4.1	95.9	4.3	99	4.3	95.7	442	28.2	94		
		20	483	4.2	95.8	4.6	116	3.9	96.1	517	-34.6	107		
	5 µg/mL	279	5945	4.7	95.3	5.6	115	4.9	95.1	5730	215.1	96	101	5.6
		255	6390	4.0	96.0	5.5	138	4.0	96.0	6418	-27.8	100		
		294	5560	5.3	94.7	5.7	116	4.9	95.1	5970	-409.7	107		
	25 µg/mL	1665	32784	5.1	94.9	6.0	110	5.5	94.5	30389	2395.5	93	101	9.1
		1404	25939	5.4	94.6	5.9	121	4.9	95.1	28761	-2822.4	111		
		1334	25276	5.3	94.7	5.8	111	5.3	94.7	25352	-75.9	100		
	100 µg/mL	7508	114594	6.6	93.4	6.3	80	7.9	92.1	95065	19528.9	83	97	12.4
		6438	105006	6.1	93.9	6.5	112	5.8	94.2	111521	-6514.7	106		
		6549	125277	5.2	94.8	6.4	125	5.1	94.9	127874	-2597.3	102		

Table VI-19. Genexol-PM, Lot D (GP31771) Drug Release Analytical Data. Presented are the analytical data for Genexol-PM, lot D (GP31771). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX	% Bound	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13	% Bound PTX_C13	Unencapsulated (ng/mL)	Encapsulated (ng/mL)	% Release	AVG	SD
				= Filtrate PTX / Reservoir PTX *100	= 100 − %Unbound PTX			= Filtrate PTX_C13 / Reservoir PTX_C13 * 100	= 100 − %Unbound C13	= Fil PTX/ (1-(%Bound PTX_C13/100))	= Reservoir PTX- Unencapsulated	= (Unencapsulated/ Reservoir PTX) *100		
10 min	0.5 µg/mL	24	476	5.0	95.0	4.5	103	4.4	95.6	536	-59.3	112	99	11.4
		19	463	4.0	96.0	4.4	101	4.3	95.7	431	32.3	93		
		20	456	4.4	95.6	4.7	99	4.7	95.3	423	33.9	93		
	5 µg/mL	236	4526	5.2	94.8	5.1	101	5.0	95.0	4716	-189.9	104	101	3.5
		256	4615	5.5	94.5	5.4	100	5.5	94.5	4673	-57.8	101		
		259	4759	5.4	94.6	5.7	102	5.6	94.4	4626	132.6	97		
	25 µg/mL	1398	24332	5.7	94.3	6.0	103	5.8	94.2	23930	402.7	98	97	9.6
		1559	27123	5.7	94.3	6.5	98	6.6	93.4	23540	3582.8	87		
		1459	25145	5.8	94.2	5.5	101	5.5	94.5	26633	-1488.3	106		
	100 µg/mL	7251	98959	7.3	92.7	6.5	101	6.4	93.6	112517	-13557.1	114	108	6.7
		6575	93436	7.0	93.0	6.3	98	6.4	93.6	101950	-8513.3	109		
		6951	110671	6.3	93.7	6.1	98	6.2	93.8	111271	-599.4	101		
30 min	0.5 µg/mL	23	586	3.9	96.1	4.7	124	3.8	96.2	608	-21.3	104	98	12.7
		22	621	3.6	96.4	4.7	142	3.3	96.7	665	-44.1	107		
		23	540	4.2	95.8	4.9	97	5.0	95.0	451	89.1	83		
	5 µg/mL	317	5622	5.6	94.4	5.7	86	6.7	93.3	4751	870.6	85	92	14.1
		273	4728	5.8	94.2	5.8	109	5.3	94.7	5137	-409.4	109		
		281	4888	5.7	94.3	6.0	87	6.9	93.1	4099	788.7	84		
	25 µg/mL	1661	22500	7.4	92.6	6.7	90	7.4	92.6	22432	68.3	100	105	7.8
		1549	25700	6.0	94.0	6.4	121	5.3	94.7	29261	-3561.3	114		
		1725	26000	6.6	93.4	6.7	103	6.6	93.4	26242	-242.4	101		
	100 µg/mL	7267	93345	7.8	92.2	7.1	83	8.6	91.4	84751	8594.0	91	91	3.4
		7285	91860	7.9	92.1	6.6	79	8.4	91.6	86621	5238.7	94		
		7320	92347	7.9	92.1	6.8	76	9.1	90.9	80856	11491.3	88		
2 hr	0.5 µg/mL	19	445	4.3	95.7	4.5	104	4.3	95.7	444	1.1	100	97	9.5
		22	480	4.5	95.5	4.6	108	4.3	95.7	504	-24.1	105		
		19	513	3.7	96.3	3.4	78	4.3	95.7	444	68.7	87		
	5 µg/mL	255	5016	5.1	94.9	5.1	115	4.5	95.5	5716	-700.0	114	101	11.8
		259	5461	4.8	95.2	5.0	103	4.9	95.1	5308	153.2	97		
		259	5315	4.9	95.1	4.9	91	5.4	94.6	4841	473.6	91		
	25 µg/mL	1422	26951	5.3	94.7	5.3	80	6.6	93.4	21407	5544.3	79	85	4.8
		1327	25143	5.3	94.7	4.9	83	6.0	94.0	22214	2929.3	88		
		1638	25320	6.5	93.5	6.5	87	7.5	92.5	21980	3339.9	87		
	100 µg/mL	6326	115368	5.5	94.5	6.0	112	5.3	94.7	118407	-3038.9	103	90	11.2
		6952	135368	5.1	94.9	5.8	93	6.3	93.7	110864	24503.9	82		
		7168	126519	5.7	94.3	6.0	90	6.7	93.3	107516	19003.0	85		

Table VI-20. Taxol Generic, Lot A (PACCA1018) Drug Release Analytical Data. Presented are the analytical data for Taxol generic, lot A (PACCA1018). The % protein binding, encapsulated and unencapsulated values were calculated as described in Section II. (*N=3*)

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 – %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated	% Release = (Unencapsulated/ Reservoir PTX) *100	AVG	SD
10 min	0.5 µg/mL	25	517	4.9	95.1	4.9	110	4.5	95.5	569	-52.2	110	88	18.8
		23	749	3.1	96.9	4.6	113	4.0	96.0	574	174.8	77		
		21	565	3.8	96.2	4.1	86	4.8	95.2	444	120.5	79		
	5 µg/mL	241	5984	4.0	96.0	5.3	143	3.7	96.3	6514	-529.4	109	101	7.4
		255	5952	4.3	95.7	5.0	116	4.3	95.7	5870	81.9	99		
		238	4704	5.1	94.9	5.3	100	5.4	94.6	4447	256.6	95		
	25 µg/mL	847	32876	2.6	97.4	4.3	176	2.5	97.5	34422	-1546.0	105	99	14.2
		864	25778	3.4	96.6	4.0	132	3.0	97.0	28319	-2541.0	110		
		747	27861	2.7	97.3	4.0	124	3.2	96.8	23151	4709.6	83		
	100 µg/mL	1907	127586	1.5	98.5	2.9	148	2.0	98.0	96181	31404.8	75	76	6.6
		2030	117621	1.7	98.3	3.1	128	2.5	97.5	82821	34799.7	70		
		1828	112188	1.6	98.4	2.8	143	2.0	98.0	93577	18610.7	83		
30 min	0.5 µg/mL	37	645	5.8	94.2	4.5	87	5.2	94.8	713	-68.0	111	103	8.7
		22	468	4.7	95.3	4.4	98	4.4	95.6	497	-28.6	106		
		22	554	4.0	96.0	4.0	95	4.2	95.8	519	34.8	94		
	5 µg/mL	250	5486	4.6	95.4	5.1	91	5.6	94.4	4442	1044.2	81	91	10.0
		244	5328	4.6	95.4	5.2	114	4.5	95.5	5376	-47.5	101		
		226	5660	4.0	96.0	5.0	113	4.4	95.6	5086	573.6	90		
	25 µg/mL	861	22531	3.8	96.2	4.6	85	5.4	94.6	15919	6611.9	71	85	14.3
		952	24648	3.9	96.1	4.5	100	4.5	95.5	21263	3384.5	86		
		896	31548	2.8	97.2	4.9	172	2.9	97.1	31289	259.4	99		
	100 µg/mL	1878	90839	2.1	97.9	3.1	133	2.3	97.7	80945	9893.9	89	86	4.2
		2106	82951	2.5	97.5	3.1	107	2.9	97.1	72024	10927.2	87		
		1871	89391	2.1	97.9	2.7	105	2.6	97.4	72308	17082.5	81		
2 hr	0.5 µg/mL	21	477	4.4	95.6	3.9	97	4.1	95.9	513	-36.6	108	97	10.6
		21	453	4.5	95.5	4.1	86	4.7	95.3	434	18.6	96		
		20	441	4.6	95.4	3.8	71	5.3	94.7	382	59.0	87		
	5 µg/mL	253	4501	5.6	94.4	5.3	105	5.0	95.0	5043	-542.2	112	110	3.3
		243	4898	5.0	95.0	5.1	110	4.7	95.3	5193	-294.6	106		
		255	4211	6.1	93.9	5.5	101	5.4	94.6	4681	-469.9	111		
	25 µg/mL	872	18034	4.8	95.2	4.8	95	5.1	94.9	17173	861.2	95	95	0.3
		916	21366	4.3	95.7	4.9	110	4.5	95.5	20382	984.5	95		
		880	21131	4.2	95.8	4.6	104	4.4	95.6	20023	1107.8	95		
	100 µg/mL	2001	74408	2.7	97.3	3.2	98	3.3	96.7	60690	13718.1	82	86	5.5
		2225	75757	2.9	97.1	3.4	105	3.2	96.8	69728	6028.9	92		
		2298	75822	3.0	97.0	3.2	89	3.6	96.4	63817	12004.5	84		

Table VI-21. Free Paclitaxel Control Data. Displayed is the calculated % release for the paclitaxel control data. (*N=3*)

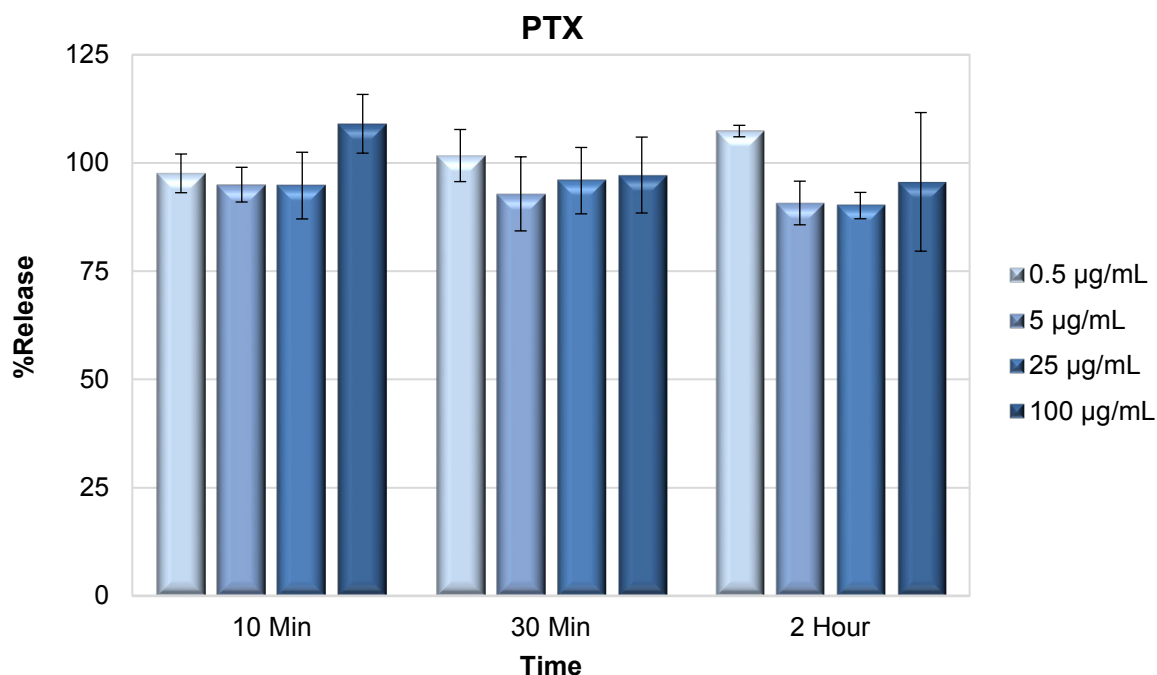| Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	98	4.5	4.6
	5 µg/mL	95	4.0	4.2
	25 µg/mL	95	7.7	8.1
	100 µg/mL	109	6.8	6.2
30 min	0.5 µg/mL	102	6.0	5.9
	5 µg/mL	93	8.6	9.2
	25 µg/mL	96	7.7	8.0
	100 µg/mL	97	8.8	9.0
2 hr	0.5 µg/mL	107	1.3	1.2
	5 µg/mL	91	5.0	5.6
	25 µg/mL	90	3.0	3.4
	100 µg/mL	96	16.0	16.7

Figure VI-15. Free Paclitaxel Control Data. Displayed is the calculated % release for the paclitaxel control data. (Mean $\pm$ SD, *N=3*)

Table VI-22. Abraxane, Lot E (6115194) Drug Release. Displayed is the calculated % release for Abraxane, lot E (6115194). (*N=3*)

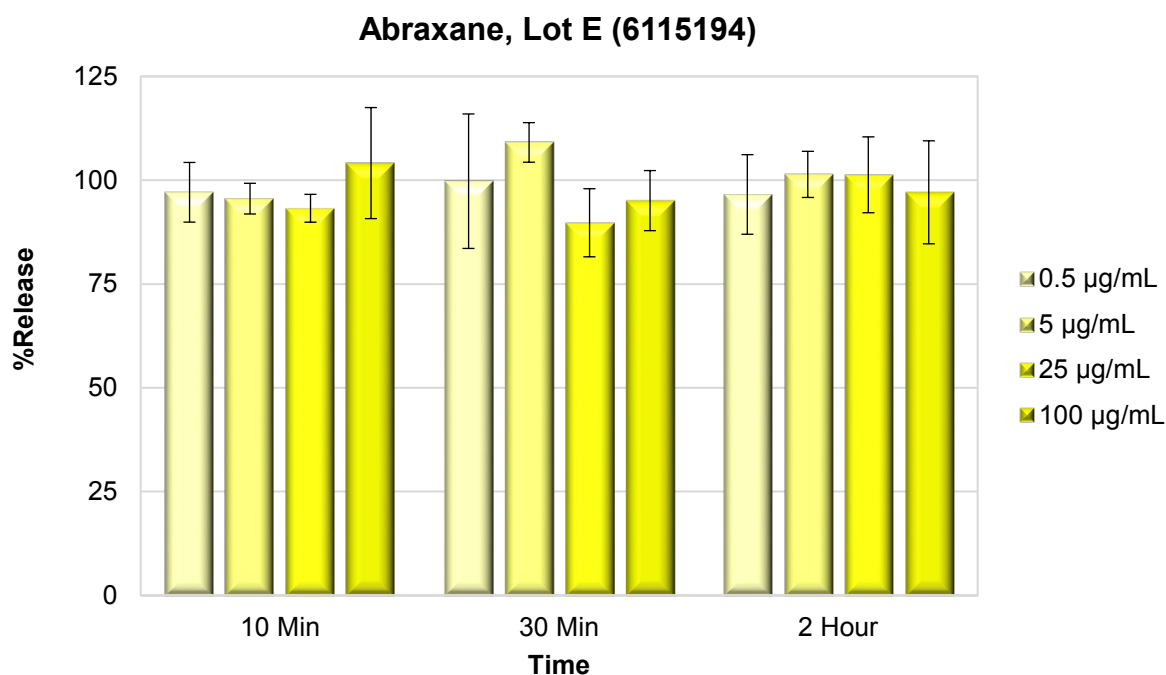| Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	97	7.2	7.4
	5 µg/mL	96	3.7	3.9
	25 µg/mL	93	3.4	3.6
	100 µg/mL	104	13.4	12.8
30 min	0.5 µg/mL	100	16.2	16.2
	5 µg/mL	109	4.8	4.4
	25 µg/mL	90	8.2	9.1
	100 µg/mL	95	7.2	7.6
2 hr	0.5 µg/mL	97	9.6	9.9
	5 µg/mL	101	5.6	5.5
	25 µg/mL	101	9.1	9.0
	100 µg/mL	97	12.4	12.8

Figure VI-16. Abraxane, Lot E (6115194) Drug Release. Displayed is the calculated % release for the Abraxane, lot E (6115194). (Mean $\pm$ SD, *N=3*)

Table VI-23. Genexol-PM, Lot D (GP31771) Drug Release. Displayed is the calculated % release for Genexol-PM, lot D (GP31771). (*N=3*)

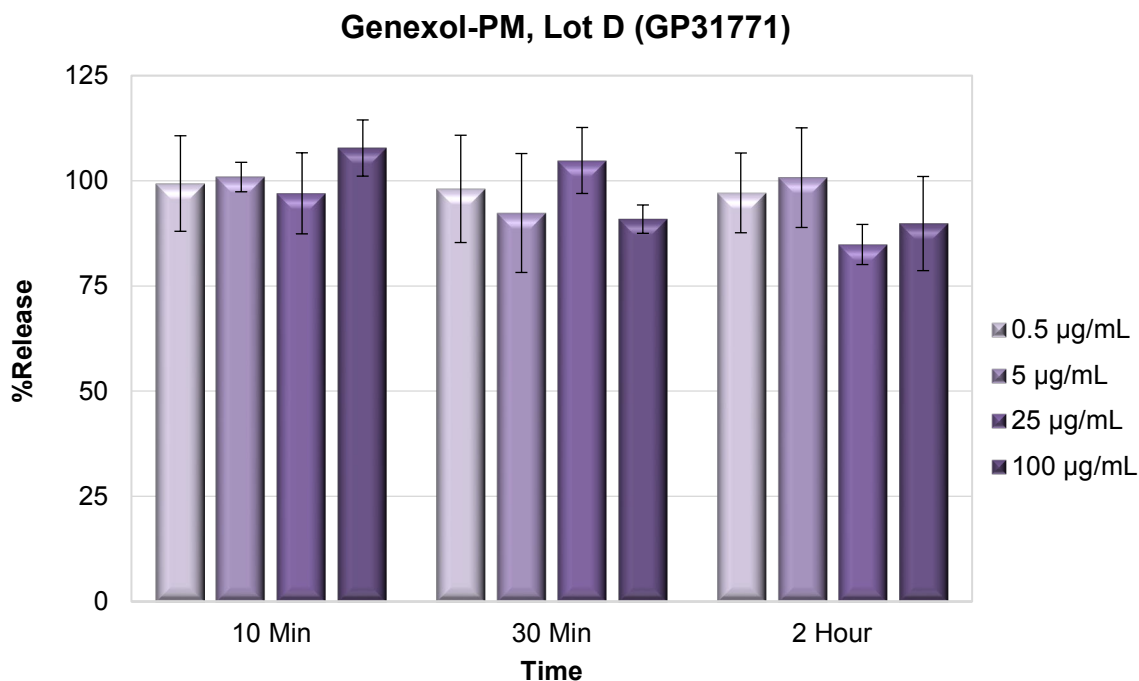| Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	99	11.4	11.4
	5 µg/mL	101	3.5	3.5
	25 µg/mL	97	9.6	9.9
	100 µg/mL	108	6.7	6.2
30 min	0.5 µg/mL	98	12.7	13.0
	5 µg/mL	92	14.1	15.3
	25 µg/mL	105	7.8	7.5
	100 µg/mL	91	3.4	3.7
2 hr	0.5 µg/mL	97	9.5	9.8
	5 µg/mL	101	11.8	11.8
	25 µg/mL	85	4.8	5.6
	100 µg/mL	90	11.2	12.5

Figure VI-17. Genexol-PM, Lot D (GP31771) Drug Release. Displayed is the calculated % release for Genexol-PM, lot D (GP31771). (Mean $\pm$ SD, *N=3*)

Table VI-24. Taxol Generic (PACCA1018) Drug Release. Displayed is the calculated % release for Taxol generic (PACCA1018). (*N=3*)

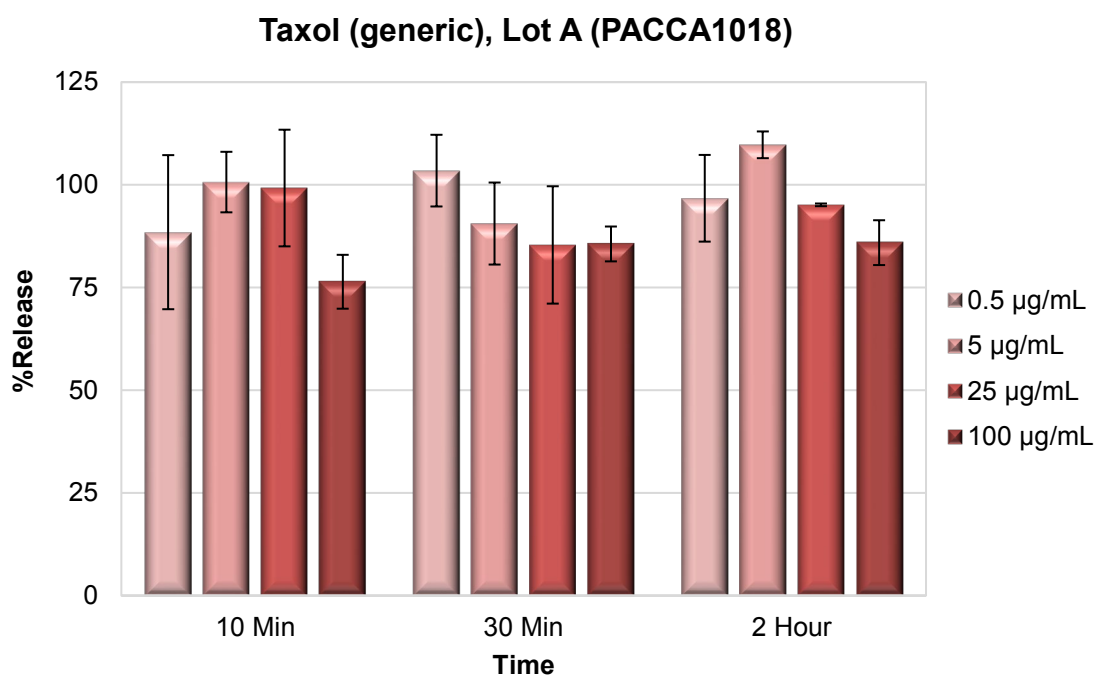| Time	Conc.	Avg % Release	SD	% CV
10 min	0.5 µg/mL	88	18.8	21.2
	5 µg/mL	101	7.4	7.3
	25 µg/mL	99	14.2	14.3
	100 µg/mL	76	6.6	8.6
30 min	0.5 µg/mL	103	8.7	8.4
	5 µg/mL	91	10.0	11.0
	25 µg/mL	85	14.3	16.7
	100 µg/mL	86	4.2	5.0
2 hr	0.5 µg/mL	97	10.6	10.9
	5 µg/mL	110	3.3	3.0
	25 µg/mL	95	0.3	0.3
	100 µg/mL	86	5.5	6.3

Taxol (generic), Lot A (PACCA1018)

Figure VI-18. Taxol Generic (PACCA1018) Drug Release. Displayed is the calculated % release for the Taxol generic (PACCA1018). (Mean $\pm$ SD, *N=3*)
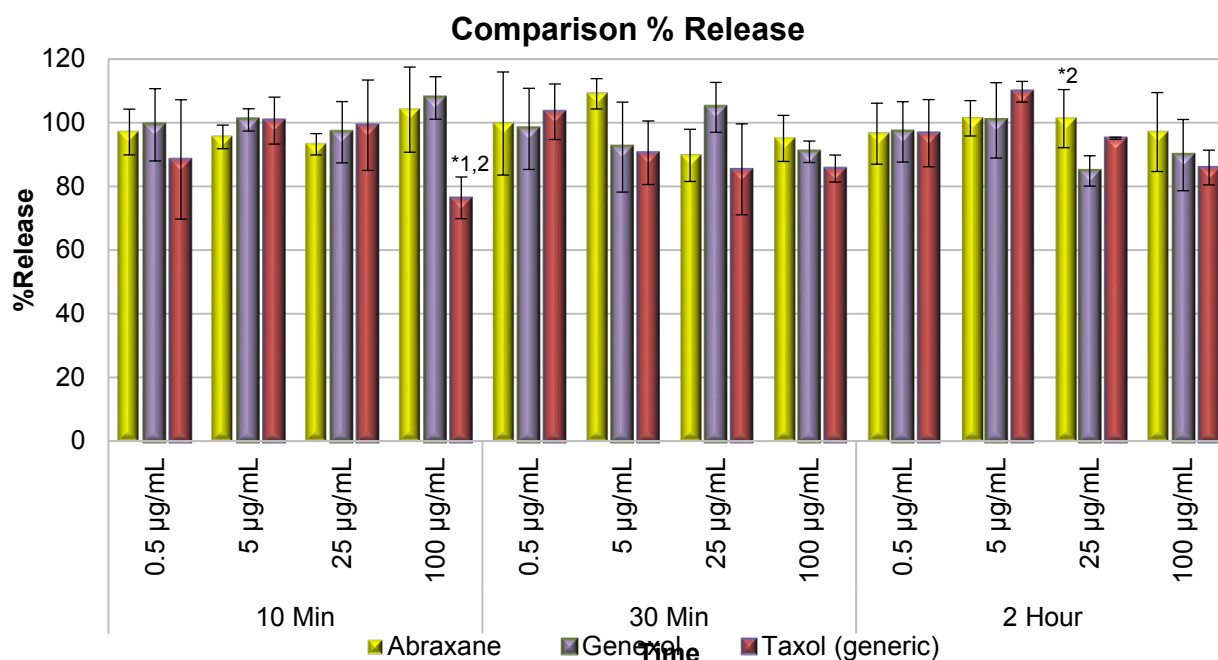
Comparison % Release

Figure VI-19. Abraxane, Genexol-PM, and Taxol Generic Drug Release Comparison.
Displayed are the 10 min – 2 hr calculated % release data for the Abraxane, Genexol-PM, and Taxol generic lots 6115194, GP31771, and PACCA1018, respectively. (Mean $\pm$ SD, *N=3*); *p$\leq$0.05, ANOVA, with Duncan's Multiple range test posthoc comparisons; 1 and 2 designate significantly different from Abraxane and Genexol-PM, respectively.
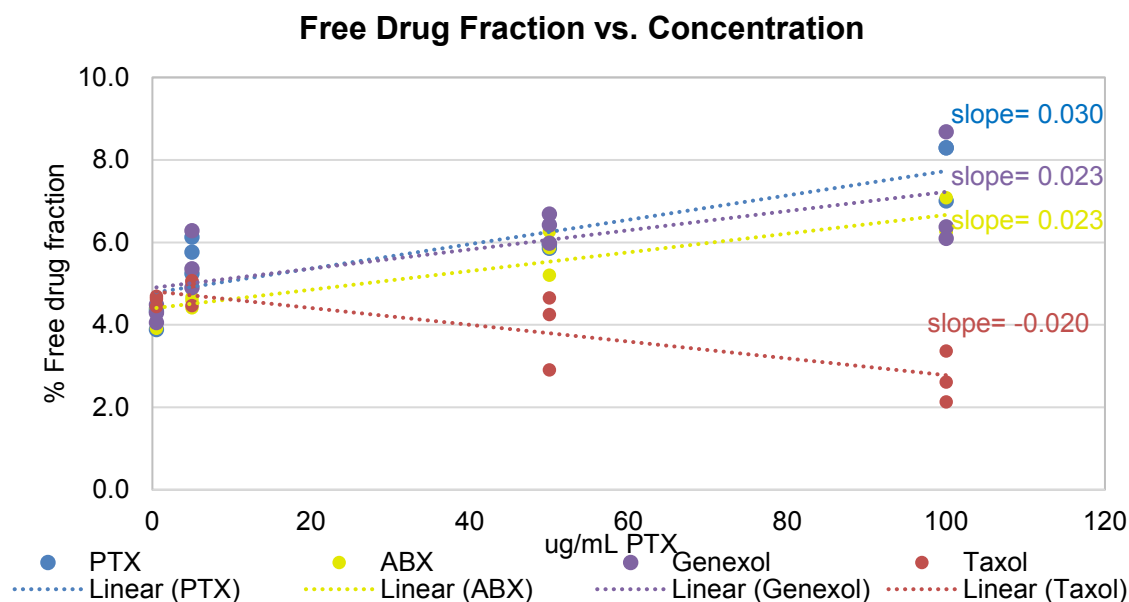
Free Drug Fraction vs. Concentration

Figure VI-20. Abraxane, Genexol-PM, and Taxol Generic % Free drug Fraction vs. Concentration Comparison. Displayed are the calculated % free fraction vs. concentration for the Abraxane, Genexol-PM, and Taxol generic lots 6115194, GP31771, and PACCA1018, respectively.

VII. Abraxane, Genexol-PM, and Taxol (generic) Bioequivalency Study in Rats

Bioequivalency Study

Summary

Seperate lots of each formulation (Abraxane (Lot E, 6115194), Genexol-PM (Lot D, GP31771), and Taxol generic (Lot A, PACCA1018)) were evaluated in a bioequivalence study using Sprague Dawley rats. Total, unencapsulated and unbound drug PK parameters were similar for Abraxane and Genexol-PM, but notably different for Taxol. Although Abraxane and Genexol-PM PK parameters were similar, a statistical analysis of bioequivalence by two one-sided t-tests determined that Abraxane and Genexol-PM were not bioequivalent. This lack of bioequivalence is most likely the result of high variability and insufficient power, due to the low number of animals used. Taxol demonstrated lower total, unencapsulated and unbound CL, Vd and Vss than Genexol-PM and Abraxane, consistent with strong equilibrium binding to stable cremophor micelles. This equilibrium binding was reflected in non-linear/reduced unbound drug fraction at high drug concentrations for the Taxol formulation. Surprisingly, and inconsistent with pharmacological theory regarding the effect of protein binding on active, unbound drug PK for a low extraction drug administered parenterally, unbound PK was apparently altered by this equilibrium formulation binding. This effect on active, unbound drug PK could potentially influence drug therapy and help explain differences between Abraxane and Taxol pharmacology [5]. Unbound drug PK apears to be a discriminating criteria for drug formulations that influence drug PK by equilibrium binding. The SITUA assay is a very predictive and accurate method for identifying formulation effects on unbound drug fraction resulting from equilibrium binding.

Design and Methods

Test Article Preparation
Test articles, Abraxane (Lot E, 6115194), Genexol-PM (Lot D, GP31771), or Taxol generic (Lot A, PACCA1018) were diluted to a concentration of 2 mg PTX/mL in saline.

Animal Study Design
A parallel design bioequivalence study was conducted in double jugular catheterized 15-week-old male Sprague Dawley rats (approx. weight of 400 grams, Charles River Laboratories, Raleigh, N.C.). Rats were treated intravenously by left catheter with 6 mg PTX/3 mL/kg of Abraxane, Genexol-PM, or Taxol generic (8/treatment group). Blood samples (400 µL) were collected in K_2EDTA tubes by the right jugular catheter at 0.25, 0.5, 2, 4, 6, 8, 24, 48 and 72 hr. Blood was spun at 2500x*g* for 10 min and plasma (~200 µL) collected in a glass vial.

The analysis of the plasma samples was conducted as described in Section I, using the stable isotope tracer method. However, for the in vivo studies, approximately 200 µL of plasma was the maximum obtainable volume for each time point. Therefore, the volumes were adjusted from those used in the in vitro studies to 200 µL. This volume change was validated in Section II. Briefly, 100 ng/mL of the PTX_C13 stable isotope was added to 200 µL of plasma and incubated at 37°C for 10 min with agitation. After incubation, 25 µL plasma was removed to Eppendorf tubes containing 200 µL ACN with 0.1% formic acid with 25 ng/mL PTX_d5 internal standard for total drug concentration analysis. The remainder of the plasma was transferred to a filter tube and spun at 12,000x*g* for 10 min at 37°C. 50 µL of the filtrate was added to an Eppendorf tube containing 200 µL ACN with 0.1% FA and 25 ng/mL PTX_d5 internal standard for analysis. In some instances, we were unable to obtain 50 µL of the filtrate. In these cases,

the total volume available was noted and added to the ACN. The dilution factor was adjusted accordingly during data analysis. Samples were frozen at -80°C until analysis.

Prior to analysis, samples were thawed, centrifuged, supernatants dried in a vacuum centrifuge, and the resulting residue reconstituted in 150 µL 40% ACN with 0.1% formic acid. Samples were then analyzed on a Thermo Fisher Q Exactive OrbiTrap, using matrix matched standard curves and controls as described in Section I. The standard curve range used in this study (**Tables VII-1 to VII-4 and Figures VII-1 to VII-4**) was higher than was initially validated for the rat plasma validation study in Section II.

Husbandry
Animals arrived the day prior to study initiation. In order to keep the catheters patent, catheters were stored and flushed with 500 IU/mL heparin in PBS. Animal rooms were kept at 50% relative humidity, 68-72°F with 12 hr light/dark cycles. Rats were housed two animals/cage (rat polycarbonate cage type with ¼" corncob bedding). Animals were allowed *ad libitum* access to Purina 5L79 and reverse osmosis water.

The Frederick National Laboratory for Cancer Research is accredited by AAALAC International and follows the Public Health Service *Policy for the Care and Use of Laboratory Animals* (Health Research Extension Act of 1985, Public Law 99-158, 1986). Animal care was provided in accordance with the procedures outlined in the *Guide for Care and Use of Laboratory Animals* (National Research Council, 1996; National Academy Press, Washington, D.C.). All animal protocols were approved by the NCI at Fredrick institutional Animal Care and Use Committee. The experiments outlined herein are scientifically justified and do not represent an unnecessary duplication of previous work by the sponsor.

Noncompartmental Pharmacokinetic Analysis
Noncompartmental pharmacokinetic parameters were determined using Phoenix WinNonlin version 6.3 software (Pharsight Corporation, Mountain View, CA): the area under the time concentration curve including all time points (AUC_{all}), was calculated using the linear trapezoidal rule without extrapolation; the area under the time concentration curve to time infinity (AUC_{inf}), was calculated using the linear trapezoidal rule with extrapolation to time infinity; the C_{max} term is the maximum measured concentration; the T_{max} term is the time of maximum concentration; clearance (CL) was determined by the equation CL=dose/AUC; the AUMC term is the area under the first moment curve; volume of distribution steady state (Vss), is determined by the equation Vss=AUC/AUMC; Mean residence time (MRT_{inf}), is determined by the equation MRT_{inf}=(dose/AUCinf)*(AUMC/AUC); the λ_z term is the slope of the terminal elimination phase; half-life ($T_{1/2}$), is determined by $T_{1/2}$=0.693/ λ_z; Concentration time zero (C_0), is the extrapolated concentration of the intial slope to the y-intercept; Volume of distribution apparent (Vd), is determined by the equation Vd=dose/C_0.

Statistics
In vivo PK parameters were evaluated by two one-sided t-tests, with α=0.05 and Ө=0.2, to determine the 90% CI of the geometric mean of log transformed T/R ratio [6] as well as ANOVA, with Duncan's Multiple Range test post hoc comparisons. The FDA bioequivalence criteria is a 90% CI between 80 and 125% [6]. Outliers were identified as data points two standard deviations away from the mean of all data points in the group. Only outliers that did not fit the overall drug concentration profile of the treatment group were excluded from the statistical analysis.

Table VII-1. PTX Plasma Standard Curve.

	Peak Area Ratio	Conc. (ng/mL)	% Accuracy
0.50 ng/mL	0.0009	0.57	113
0.50 ng/mL	0.0005	0.44	87
1.00 ng/mL	0.0023	1.01	101
1.00 ng/mL	0.0045	1.71	171
10.0 ng/mL	0.029	9.6	96
10.0 ng/mL	0.028	9.3	93
100 ng/mL	0.31	101	101
100 ng/mL	0.31	98	98
1,000 ng/mL	3.2	1,009	101
1,000 ng/mL	3.1	990	99
10,000 ng/mL	31	10,044	100
10,000 ng/mL	30	9,525	95
50,000 ng/mL	170	54,265	109
50,000 ng/mL	167	53,368	107
QC			
QCL	0.0027	1.12	112
QCL	0.0034	1.37	137
QCM	0.32	101	101
QCM	0.31	101	101
QCH	163	52,127	104
QCH	171	54,485	109

PTX
$Y = -0.000838722 + 0.00313193 \cdot X$ $R^2 = 0.9946$ W: $1/X^2$
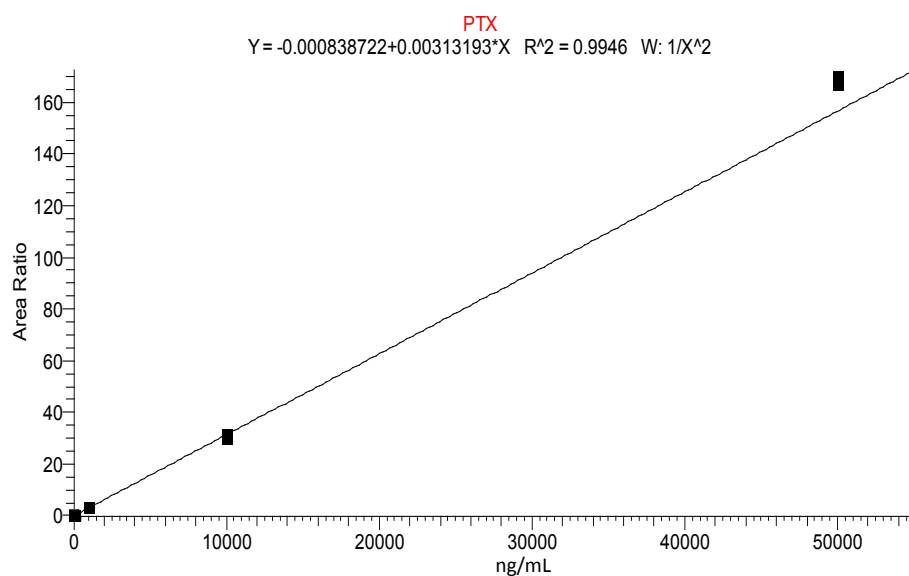
Figure VII-1. PTX Plasma Standard Curve.

Table VII-2. PTX_C13 Plasma Standard Curve.

	Peak Area Ratio	Conc. (ng/mL)	% Accuracy
0.50 ng/mL	0.0008	0.46	92
0.50 ng/mL	0.0013	0.55	111
1.00 ng/mL	0.0127	2.58	258
1.00 ng/mL	0.0036	0.96	96
10.0 ng/mL	0.058	10.7	97
10.0 ng/mL	0.046	8.5	94
100 ng/mL	0.54	97	99
100 ng/mL	0.53	94	102
1,000 ng/mL	5.6	993	96
1,000 ng/mL	5.7	1,020	95
10,000 ng/mL	54	9,577	113
10,000 ng/mL	53	9,488	114
50,000 ng/mL	316	51,944	104
50,000 ng/mL	320	57,160	114
QC			
QCL	0.0047	1.15	115
QCL	0.0044	1.10	110
QCM	0.59	106	106
QCM	0.58	103	103
QCH	291	51,944	104
QCH	321	57,160	114

PTX_C13
$Y = -0.00177199 + 0.00560944*X$ $R^2 = 0.9910$ W: $1/X^2$
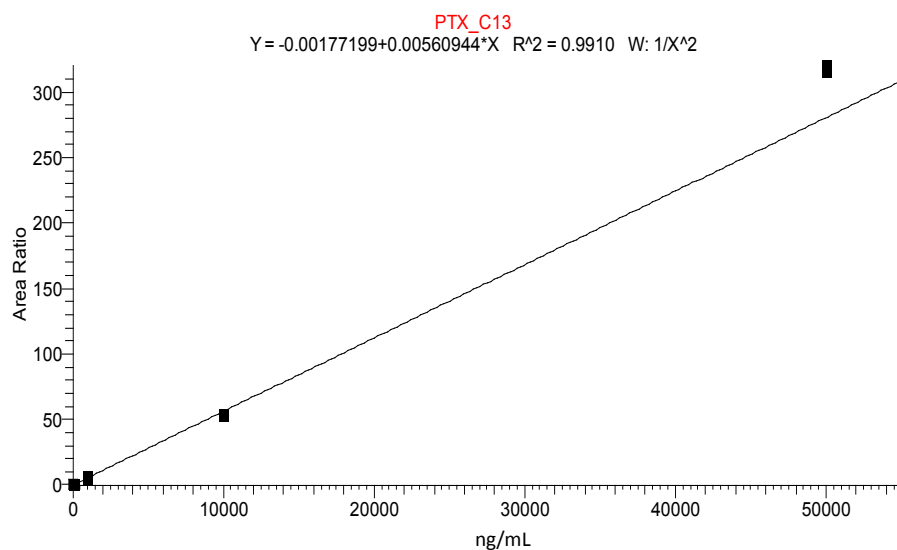
Figure VII-2. PTX_C13 Plasma Standard Curve.

Table VII-3. PTX Protein-Free Plasma Standard Curve.

	Peak Area Ratio	**Conc. (ng/mL)**	**% Accuracy**
0.10 ng/mL	0.0004	0.09	85
0.10 ng/mL	0.0006	0.11	114
0.50 ng/mL	0.0034	0.56	111
0.50 ng/mL	0.0029	0.47	93
1.00 ng/mL	0.0062	0.98	98
1.00 ng/mL	0.0063	1.01	101
5.0 ng/mL*	0.160	25.1	501
5.0 ng/mL	0.031	4.9	98
10.0 ng/mL	0.064	10.1	101
10.0 ng/mL	0.065	10.1	101
50.0 ng/mL	0.32	50.4	101
50.0 ng/mL	0.32	49.5	99
100.0 ng/mL	0.59	92.0	92
100.0 ng/mL	0.58	91.5	92
500.0 ng/mL	3.578	560.4	112
500.0 ng/mL	3.218	504.0	101
QC			
QCL	0.0031	0.50	100
QCL	0.0034	0.55	110
QCM	0.066	10.3	103
QCM	0.068	10.7	107
QCH	3.14	492.3	98
QCH	3.17	496.5	99

*Point excluded from standard curve

PTX
$Y = -0.000105057 + 0.00638551 \cdot X \quad R^2 = 0.9925 \quad W: 1/X^2$
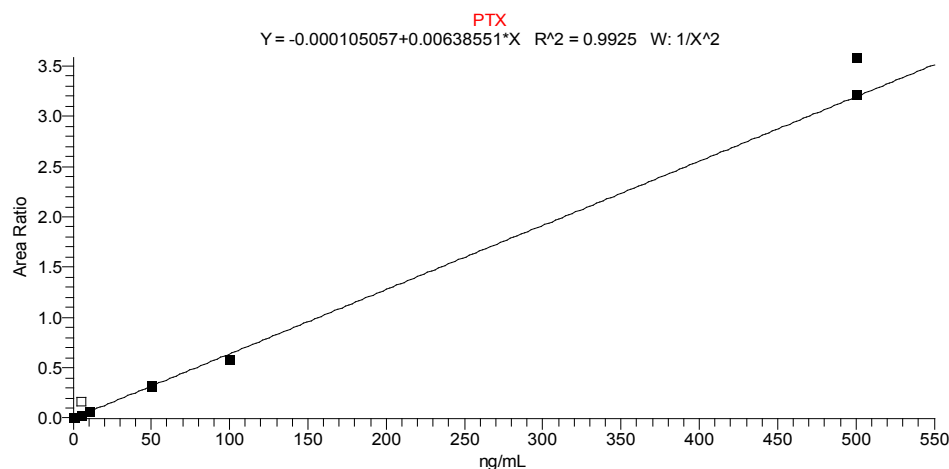
Figure VII-3. PTX Protein-Free Plasma Standard Curve.

Table VII-4. PTX_C13 Protein-Free Plasma Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
0.50 ng/mL	0.0043	0.54	108
0.50 ng/mL	0.0030	0.43	86
1.00 ng/mL	0.011	1.07	107
1.00 ng/mL	0.010	1.06	106
5.0 ng/mL	0.057	5.0	100
5.0 ng/mL	0.059	5.2	104
10.0 ng/mL	0.11	9.4	94
10.0 ng/mL	0.11	9.3	93
50.0 ng/mL	0.59	50.8	102
50.0 ng/mL	0.57	49.0	98
100.0 ng/mL	1.1	93.4	93
100.0 ng/mL	1.1	92.7	93
500.0 ng/mL	6.7	574.3	115
500.0 ng/mL	5.9	504.9	101
QC			
QCL	0.0046	0.57	113
QCL	0.0037	0.49	98
QCM	0.11	9.5	95
QCM	0.11	9.4	94
QCH	5.5	466.7	93
QCH	5.4	465.6	93

PTX_C13
Y = -0.00199933+0.0117006*X   R^2 = 0.9920   W: 1/X^2
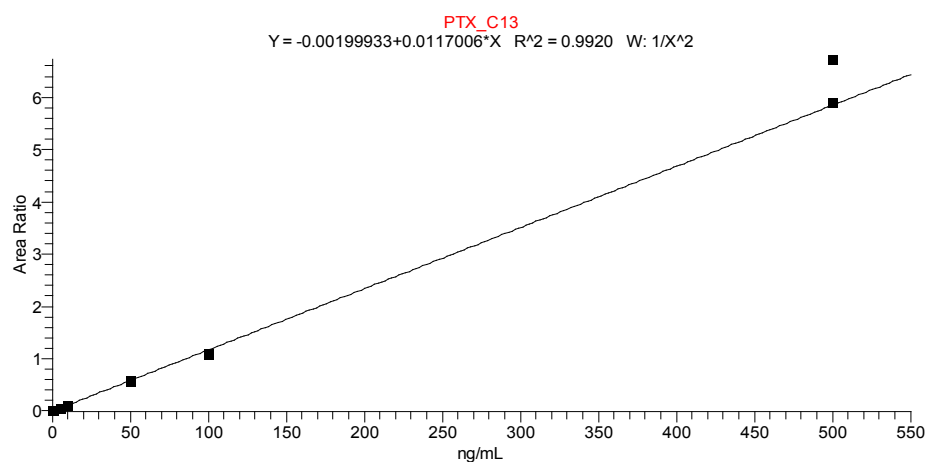
Figure VII-4. PTX_C13 Protein-Free Plasma Standard Curve.

Results and Discussion

Encapsulated drug, as measured by the SITUA method, was only observed at the earliest time point, 15 min, for the Taxol and Genexol-PM formulations, but not the Abraxane formulation (**Tables VII-8, VII-9, VII-13, VII-14, VII-18 and VII-19**). This is consistent with stable cremophore and PEG-polylactic acid micelles at this early time point for the Taxol and Genexol-PM formulations, respectively, corresponding with peak excipient concentrations. While the Abraxane, Genexol-PM and Taxol concentration-time profiles for total, unencapsulated and unbound drug, were all identical (**Tables VII-5 to VII-7, VII-10 to VII-12 ans VII-15 to VII-17, Figures VII-5 to VII-13**), displaying a triphasic curve as described previously in the literature (**Figures VII-14 to VII-16**) [7]. The PK parameters calculated from these profiles were significantly different between Taxol and the Abraxane and Genexol-PM formulations (**Tables VII-20 toVII-22**).

Total, unencapsulated and unbound drug PK parameters were similar for Abraxane and Genexol-PM, but notably different for Taxol. Although Abraxane and Genexol-PM PK parameters were similar, a statistical analysis of bioequivalence by two one-sided t-tests determined that Abraxane and Genexol-PM were not bioequivalent (**Figure VII-19**). This lack of bioequivalence is probably the result of high variability and insufficient power, due to the low number of animals used. Taxol demonstrated lower total, unencapsulated and unbound CL, Vd and Vss than Genexol-PM and Abraxane, consistent with strong equilibrium binding to stable cremophor micelles. This equilibrium binding was reflected in non-linear/reduced unbound drug fraction at high drug concentrations for the Taxol formulation, as determined for both the normoisotopic drug and stable isotope tracer (**Figures VII-17 and VII-18**). Importantly, the stable isotope tracer allowed discrimination between drug stably bound to cremophore micelles and dynamic drug binding to micelles in equilibrium with unbound drug. Surprisingly, and inconsistent with pharmacological theory regarding the effect of protein binding on active, unbound drug PK for a low extraction drug like paclitaxel administered parenterally, unbound drug PK was apparently altered by this equilibrium formulation binding [5].

Although cremophore has been shown to inhibit paclitaxel metabolism at high concentrations in vitro [8], the differences in unbound drug PK for the Taxol formulation does not appear to be the result of changes in drug metabolism,since changes in metabolic clearance would have resulted in altered drug half-life ($T_{1/2}=0.693/(Vss/CL)$), which was not the case. By contrast, alterations in free drug fraction result in compensatory changes in both volume of distribution and clearance resulting in no change in drug half-life, as observed [5]. This is an important finding, as it would suggest that binding of paclitaxel to the Taxol cremophore micelle affects unbound drug PK differently than changes in binding to protein, and can influence hepatic drug extraction and tissue distribution of unbound drug, even for drugs that are not perfusion limited. This effect of cremophore on unbound drug disposition is consistent with previous findings in the isolated liver perfusion and liver slice models [8, 9], in which researchers observed a decrease in liver uptake of drug which in turn decreased drug metabolism. By decreasing the volume of distribution of active, unbound drug, the Taxol cremophore micelle could potentially influence drug therapy, and help explain differences between Abraxane and Taxol pharmacology [5]. While Abraxane has been approved for treatment of pancreatic cancer, Taxol has not, and the reasons for this lack of Taxol efficacy may be due to the underlying pharmacology of the formulaiton, not just the fact that Abraxane can be administered at higher doses [10].

Unbound drug PK apears to be a discriminating criteria for drug formulations that influence drug PK by equilibrium binding. The SITUA assay is a very predictive and accurate method for identifying formulation effects on unbound drug fraction resulting from equilibrium binding.

Table VII-5. Abraxane, Lot E (6115194) Total PTX

Hours	Total PTX (ng/mL)								
	0.25	0.5	2	4	6	8	24	48	72
Animal 1	1218.0	797.7	226.5	102.9	61.1	37.1	7.5	3.2	0.6
Animal 3	1689.5	1071.6	434.8	217.7	123.0	83.8	12.6	3.6	7.3
Animal 5	810.9	486.7	207.8	115.1	61.1	41.8	6.7	2.4	2.5
Animal 7	1036.2	614.3	216.7	100.0	64.2	42.8	7.8	2.7	0.7
Animal 9	915.7	561.0	194.6	91.0	56.8	35.8	8.1	2.8	1.1
Animal 11	970.6	693.6	274.7	135.1	79.4	54.5	16.3	2.4	0.7
Animal 13	800.0	550.6	190.1	73.6	NA	34.2	8.8	2.1	0.7
Animal 15	1285.0	761.9	232.7	115.0	65.0	50.4	10.9	3.0	7.8
Average	1090.7	692.2	247.3	118.8	72.9	47.5	9.8	2.8	1.0
STD	298.4	187.3	80.3	43.9	23.2	16.3	3.2	0.5	0.7
% CV	27	27	32	37	32	34	33	18	70

Values were excluded

No sample for this time point

Abraxane Total PTX
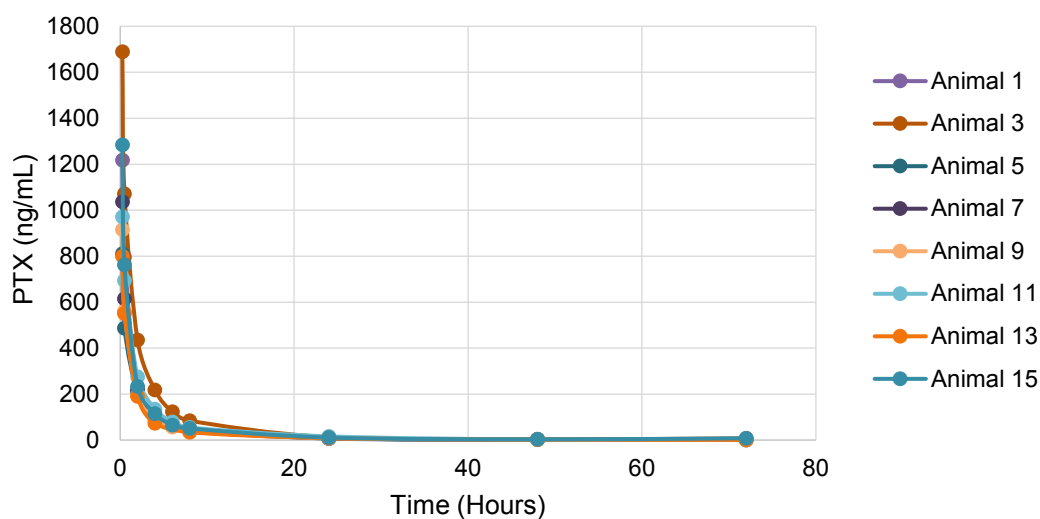
Figure VII-5. Abraxane concentration-time profiles for total drug. Displayed are concentration-time profiles for total drug including outliers.

Table VII-6. Abraxane, Lot E (6115194) Unencapsulated PTX

Hours	Unencapsulated PTX (ng/mL)								
	0.25	0.5	2	4	6	8	24	48	72
Animal 1	1432.5	870.5	232.1	110.3	55.7	32.0	6.2	2.4	1.2
Animal 3	1776.0	1103.5	442.9	223.1	113.4	76.1	11.1	6.3	7.3
Animal 5	769.4	547.9	207.4	118.7	50.5	36.0	5.9	2.7	3.0
Animal 7	1066.7	521.7	235.2	111.5	54.8	35.7	6.6	2.3	0.9
Animal 9	859.6	479.5	222.1	91.5	55.3	31.1	6.0	2.5	1.0
Animal 11	970.1	740.1	299.3	140.3	71.7	81.6	15.3	2.2	0.8
Animal 13	828.4	562.1	200.8	75.1	NA	37.0	7.0	2.2	0.6
Animal 15	1231.4	909.3	254.8	110.8	64.0	43.9	8.3	24.1	7.1
Average	1116.8	716.8	261.8	122.6	66.5	46.7	8.3	2.9	2.7
STD	346.7	226.0	79.4	44.8	21.9	20.3	3.3	1.5	2.8
% CV	31	32	30	37	33	43	40	52	104

Values were excluded

No sample for this time point

Abraxane Unencapsulated PTX

Figure VII-6. Abraxane concentration-time profiles for unencapsulated drug. Displayed are concentration-time profiles for unencapsulated drug including outliers.

Table VII-7. Abraxane, Lot E (6115194) Unbound PTX

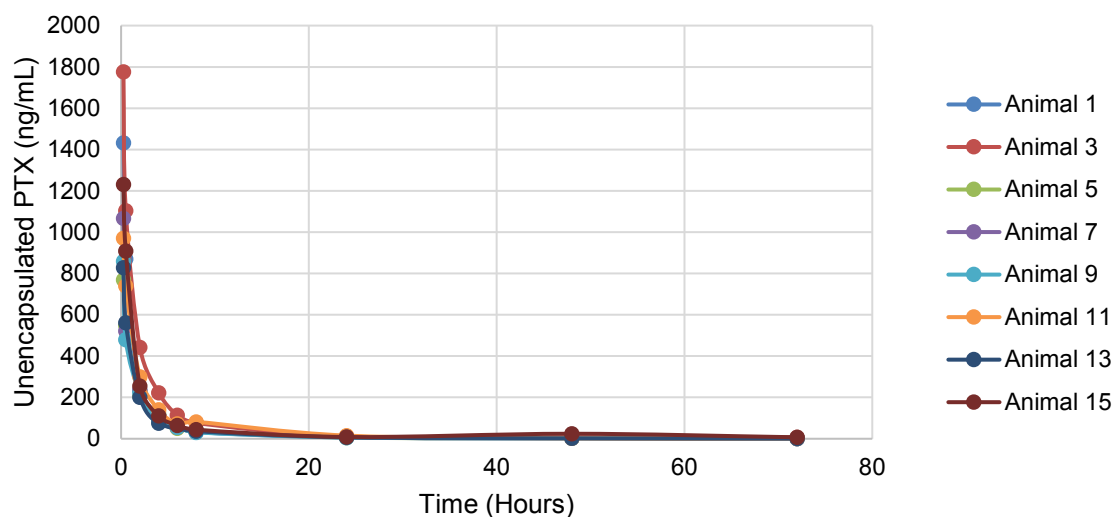| Hours	Unbound PTX (ng/mL)								
	0.25	0.5	2	4	6	8	24	48	72
Animal 1	127.3	84.1	26.2	8.8	5.5	9.5	0.6	0.2	0.1
Animal 3	165.5	121.6	43.5	19.5	10.2	5.9	1.0	0.6	0.7
Animal 5	96.0	57.1	20.6	10.4	5.7	3.5	0.8	0.2	0.3
Animal 7	103.6	58.5	19.7	9.8	5.6	3.7	0.7	0.2	0.1
Animal 9	93.7	95.6	19.4	7.3	5.3	3.3	0.7	0.3	0.2
Animal 11	118.6	76.6	29.1	11.9	8.3	7.8	2.0	0.2	0.1
Animal 13	103.6	64.0	18.6	7.4	4.8	3.3	0.8	0.2	0.1
Animal 15	128.3	87.9	34.7	10.3	5.2	4.1	0.9	2.3	0.7
Average	117.1	80.7	26.5	10.7	6.3	5.1	0.9	0.5	0.1
STD	23.7	21.7	8.9	3.9	1.9	2.4	0.4	0.7	0.1
% CV	20	27	34	37	30	46	46	136	95

Values were excluded
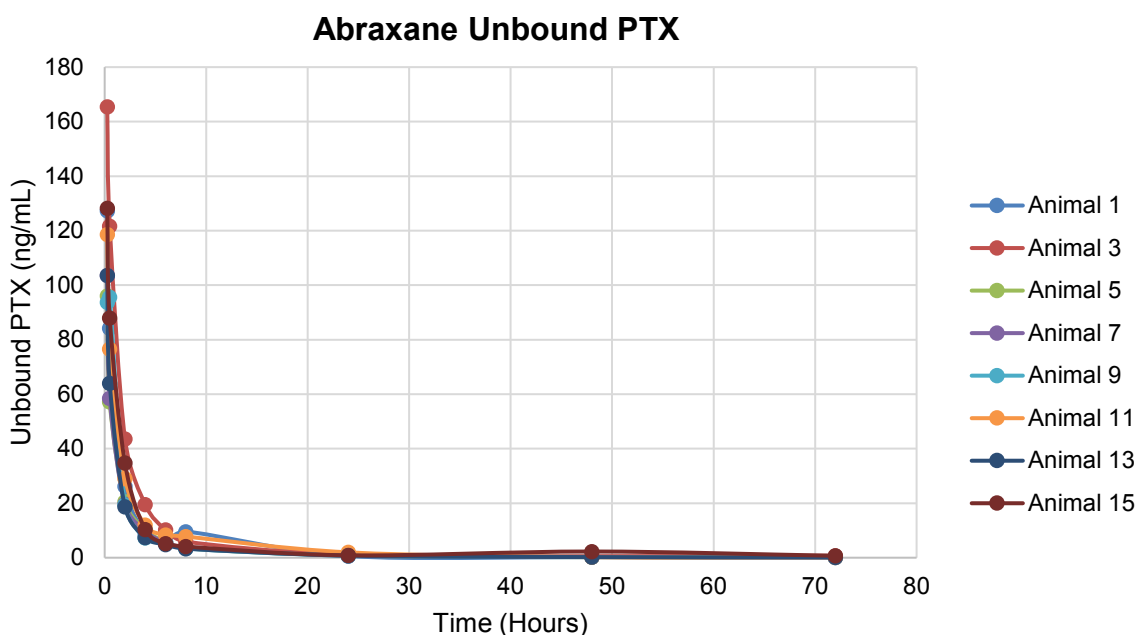
Figure VII-7. Abraxane concentration-time profiles for unbound drug. Displayed are concentration-time profiles for unbound drug including outliers.

Table VII-8 Analytical data for Abraxane, Lot E (6115194). Presented are the analytical data for Abraxane, Lot E (6115194). The Total, Unencapsulated, and Unbound values were calculated as described in Section I. * No sample for this time point.

Time point	Rat #	Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 − %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated
15 min	R1	127.3	1218.0	10.5	89.5	10.4	117.0	8.9	91.1	1432.5	-214.5
	R3	165.5	1689.5	9.8	90.2	10.2	109.9	9.3	90.7	1776.0	-86.5
	R5	96.0	810.9	11.8	88.2	11.3	90.8	12.5	87.5	769.4	41.5
	R7	103.6	1036.2	10.0	90.0	7.3	75.5	9.7	90.3	1066.7	-30.6
	R9	93.7	915.7	10.2	89.8	9.1	83.5	10.9	89.1	859.6	56.2
	R11	118.6	970.6	12.2	87.8	10.6	86.6	12.2	87.8	970.1	0.5
	R13	103.6	800.0	12.9	87.1	15.5	124.0	12.5	87.5	828.4	-28.4
	R15	128.3	1285.0	10.0	90.0	9.6	92.4	10.4	89.6	1231.4	53.6
30 min	R1	84.1	797.7	10.5	89.5	9.2	95.0	9.7	90.3	870.5	-72.8
	R3	121.6	1071.6	11.3	88.7	11.6	105.4	11.0	89.0	1103.5	-31.9
	R5	57.1	486.7	11.7	88.3	10.5	101.1	10.4	89.6	547.9	-61.3
	R7	58.5	614.3	9.5	90.5	8.3	74.0	11.2	88.8	521.7	92.6
	R9	95.6	561.0	17.0	83.0	12.5	62.6	19.9	80.1	479.5	81.4
	R11	76.6	693.6	11.0	89.0	9.5	91.7	10.3	89.7	740.1	-46.5
	R13	64.0	550.6	11.6	88.4	10.9	95.8	11.4	88.6	562.1	-11.5
	R15	87.9	761.9	11.5	88.5	9.8	101.8	9.7	90.3	909.3	-147.4
2 hr	R1	26.2	226.5	11.6	88.4	12.4	109.8	11.3	88.7	232.1	-5.5
	R3	43.5	434.8	10.0	90.0	8.8	89.6	9.8	90.2	442.9	-8.1
	R5	20.6	207.8	9.9	90.1	9.4	94.7	9.9	90.1	207.4	0.5
	R7	19.7	216.7	9.1	90.9	8.4	100.6	8.4	91.6	235.2	-18.5
	R9	19.4	194.6	10.0	90.0	9.7	111.5	8.7	91.3	222.1	-27.5
	R11	29.1	274.7	10.6	89.4	11.4	117.4	9.7	90.3	299.3	-24.6
	R13	18.6	190.1	9.8	90.2	9.3	100.4	9.3	90.7	200.8	-10.7
	R15	34.7	232.7	14.9	85.1	13.1	95.7	13.6	86.4	254.8	-22.1
4 hr	R1	8.8	102.9	8.6	91.4	9.1	113.8	8.0	92.0	110.3	-7.3
	R3	19.5	217.7	9.0	91.0	10.0	114.4	8.8	91.2	223.1	-5.3
	R5	10.4	115.1	9.0	91.0	9.3	106.1	8.7	91.3	118.7	-3.5
	R7	9.8	100.0	9.8	90.2	7.9	90.3	8.8	91.2	111.5	-11.6
	R9	7.3	91.0	8.0	92.0	8.3	104.7	8.0	92.0	91.5	-0.5
	R11	11.9	135.1	8.8	91.2	7.8	91.3	8.5	91.5	140.3	-5.1
	R13	7.4	73.6	10.1	89.9	10.5	106.4	9.9	90.1	75.1	-1.5
	R15	10.3	115.0	9.0	91.0	11.9	127.5	9.3	90.7	110.8	4.2
6 hr	R1	5.5	61.1	9.0	91.0	9.9	99.9	9.9	90.1	55.7	5.4
	R3	10.2	123.0	8.3	91.7	9.0	100.5	9.0	91.0	113.4	9.6
	R5	5.7	61.1	9.3	90.7	10.7	95.0	11.2	88.8	50.5	10.6
	R7	5.6	64.2	8.7	91.3	9.7	95.3	10.2	89.8	54.8	9.4
	R9	5.3	56.8	9.3	90.7	9.0	94.2	9.5	90.5	55.3	1.6
	R11	8.3	79.4	10.5	89.5	13.1	112.4	11.6	88.4	71.7	7.6
	R13*	4.8				9.5					
	R15	5.2	65.0	7.9	92.1	5.8	71.9	8.1	91.9	64.0	1.0

Table VII-9 Analytical data for Abraxane, Lot E (6115194), continued. Presented are the analytical data for Abraxane, Lot E (6115194). The Total, Unencapsulated, and Unbound values were calculated as described in Section I. * No sample for this time point.

Time point	Rat #	Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 – %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated
8 hr	R1	9.55	37.1	25.7	74.3	26.0	87.3	29.8	70.2	32.0	5.1
	R3	5.88	83.8	7.0	93.0	7.5	97.0	7.7	92.3	76.1	7.6
	R5	3.46	41.8	8.3	91.7	7.9	82.6	9.6	90.4	36.0	5.8
	R7	3.74	42.8	8.7	91.3	10.86	103.7	10.5	89.5	35.7	7.1
	R9	3.35	35.8	9.4	90.6	8.9	82.8	10.8	89.2	31.1	4.7
	R11	7.77	54.5	14.3	85.7	8.6	90.7	9.5	90.5	81.6	-27.1
	R13	3.33	34.2	9.7	90.3	8.2	91.7	9.0	91.0	37.0	-2.8
	R15	4.09	50.4	8.1	91.9	9.0	96.5	9.3	90.7	43.9	6.4
24 hr	R1	0.62	7.5	8.2	91.8	6.6	66.3	10.0	90.0	6.2	1.3
	R3	1.04	12.6	8.2	91.8	6.9	73.9	9.4	90.6	11.1	1.5
	R5	0.80	6.7	11.9	88.1	10.4	77.4	13.4	86.6	5.9	0.7
	R7	0.73	7.8	9.3	90.7	6.6	60.2	11.0	89.0	6.6	1.2
	R9	0.75	8.1	9.2	90.8	8.1	65.5	12.3	87.7	6.0	2.0
	R11	1.96	16.27	12.0	88.0	9.2	72.0	12.8	87.2	15.3	1.0
	R13	0.76	8.8	8.6	91.4	6.8	62.6	10.9	89.1	7.0	1.9
	R15	0.87	10.9	8.0	92.0	6.3	60.6	10.5	89.5	8.3	2.6
48 hr	R1	0.21	3.2	6.6	93.4	6.4	71.9	8.9	91.1	2.4	0.8
	R3	0.56	3.6	15.5	84.5	5.9	67.0	8.8	91.2	6.3	-2.8
	R5	0.20	2.4	8.4	91.6	6.4	85.5	7.5	92.5	2.7	-0.3
	R7	0.24	2.7	8.8	91.2	7.9	76.5	10.3	89.7	2.3	0.4
	R9	0.28	2.8	10.0	90.0	6.9	61.7	11.2	88.8	2.5	0.3
	R11	0.22	2.4	9.4	90.6	6.4	63.4	10.1	89.9	2.2	0.2
	R13	0.23	2.1	11.2	88.8	7.9	72.8	10.8	89.2	2.2	-0.1
	R15	2.27	3.0	76.0	24.0	5.8	62.0	9.4	90.6	24.1	-21.1
72 hr	R1	0.14	0.6	22.3	77.7	8.5	74.8	11.4	88.6	1.2	-0.6
	R3	0.75	7.3	10.2	89.8	9.1	89.1	10.3	89.7	7.3	0.1
	R5	0.33	2.5	13.2	86.8	9.2	84.1	11.0	89.0	3.0	-0.5
	R7	0.10	0.7	13.6	86.4	6.7	61.9	10.8	89.2	0.9	-0.2
	R9	0.15	1.1	13.8	86.2	8.4	57.0	14.7	85.3	1.0	0.1
	R11	0.10	0.7	14.4	85.6	9.1	72.2	12.6	87.4	0.8	-0.1
	R13	0.09	0.7	12.5	87.5	12.0	89.6	13.4	86.6	0.6	0.05
	R15	0.70	7.8	9.0	91.0	6.0	59.7	10.0	90.0	7.1	0.8

🟥 Values were excluded

Table VII-10. Genexol-PM Lot D (GP31771) Total PTX

Hours	Total PTX (ng/mL)								
	0.25	**0.5**	**2**	**4**	**6**	**8**	**24**	**48**	**72**
Animal 17	2311.9	1239.1	370.5	123.1	68.1	61.4	9.7	3.9	1.6
Animal 19	1210.0	643.7	194.4	92.2	51.8	50.6	7.8	2.4	0.7
Animal 21	745.7	499.6	168.0	71.9	47.1	38.6	6.0	1.4	2.8
Animal 23	686.0	417.9	146.1	90.4	40.0	26.7	7.0	1.6	0.5
Animal 25	938.1	667.3	227.7	87.1	59.1	42.8	9.9	3.2	0.7
Animal 27	425.2	224.7	106.5	41.4	31.5	24.8	7.1	3.1	1.0
Animal 29	868.0	450.0	113.8	63.3	NA	23.6	6.8	1.5	0.5
Animal 31	1101.2	767.7	278.5	77.4	46.6	28.9	7.3	2.3	1.0
Average	1083.2	524.4	200.7	80.9	49.2	37.2	7.7	2.4	1.1
STD	654.2	183.1	89.5	23.9	12.1	13.7	1.4	0.9	0.8
% CV	60	35	45	30	25	37	18	37	71

Values were excluded

No sample for this time point

Genexol-PM Total PTX
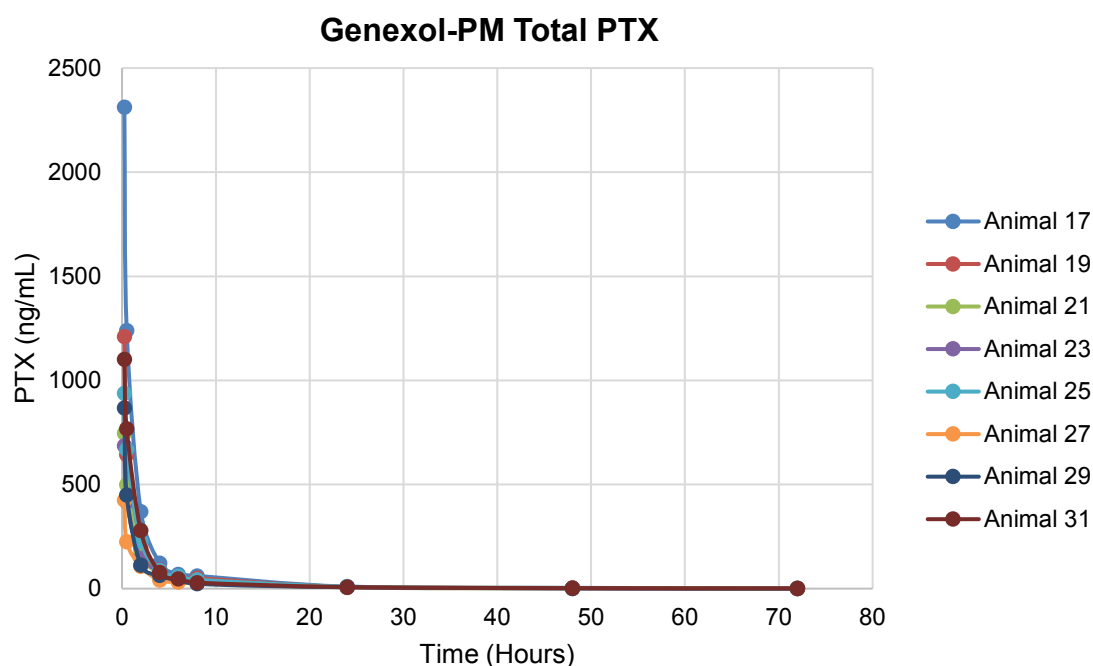
Figure VII-8. Genexol-PM concentration-time profiles for total drug. Displayed are concentration-time profiles for total drug including outliers.

Table VII-11. Genexol-PM Lot D (GP31771) Unencapsulated PTX

Hours	Unencapsulated PTX (ng/mL)								
	0.25	**0.5**	**2**	**4**	**6**	**8**	**24**	**48**	**72**
Animal 17	2091.5	2245.4	385.1	134.9	58.6	53.4	7.9	0.9	1.7
Animal 19	1076.6	720.5	199.9	96.6	45.1	44.6	6.0	1.8	1.5
Animal 21	630.4	542.3	180.4	73.6	38.4	33.6	4.8	1.5	1.3
Animal 23	617.6	461.0	207.4	113.7	37.1	22.5	5.3	1.9	0.9
Animal 25	818.3	713.2	236.8	93.0	49.1	36.9	7.4	3.6	1.0
Animal 27	362.2	248.2	109.7	41.9	26.3	22.9	6.0	2.7	0.9
Animal 29	748.1	473.9	116.8	64.6	NA	21.1	6.0	2.0	0.4
Animal 31	956.5	795.6	202.2	78.3	42.1	25.5	5.7	2.5	0.7
Average	912.7	564.9	204.8	87.1	42.4	32.6	6.1	2.1	1.1
STD	524.3	191.2	85.3	29.1	10.1	11.8	1.0	0.8	0.4
% CV	57	34	42	33	24	36	16	40	40

Values were excluded
No sample for this time point

Figure VII-9. Genexol-PM concentration-time profiles for unencapsulated drug. Displayed are concentration-time profiles for unencapsulated drug including outliers.

Table VII-12. Genexol-PM Lot D (GP31771) Unbound PTX

Hours	0.25	0.5	2	4	6	8	24	48	72
				Unbound PTX (ng/mL)					
Animal 17	213.6	184.9	33.3	11.4	6.0	4.5	0.9	0.1	0.2
Animal 19	91.0	54.8	15.4	7.9	4.7	3.5	0.6	0.2	0.1
Animal 21	68.9	43.6	14.4	5.2	3.7	3.6	0.5	0.1	0.1
Animal 23	71.8	43.6	22.2	9.5	3.6	2.7	0.7	0.2	0.1
Animal 25	112.2	62.5	17.8	8.5	6.0	3.8	0.8	0.4	0.1
Animal 27	51.2	23.9	10.5	3.6	3.5	2.5	0.7	0.3	0.1
Animal 29	79.3	39.3	7.7	5.2	3.1	2.0	0.6	0.2	0.04
Animal 31	102.1	63.8	15.2	7.4	4.0	2.5	0.7	0.3	0.1
Average	102.7	68.1	16.5	6.9	4.5	3.3	0.7	0.2	0.1
STD	58.1	58.7	9.0	2.9	1.3	0.9	0.1	0.1	0.05
% CV	57	86	54	41	28	27	21	52	38

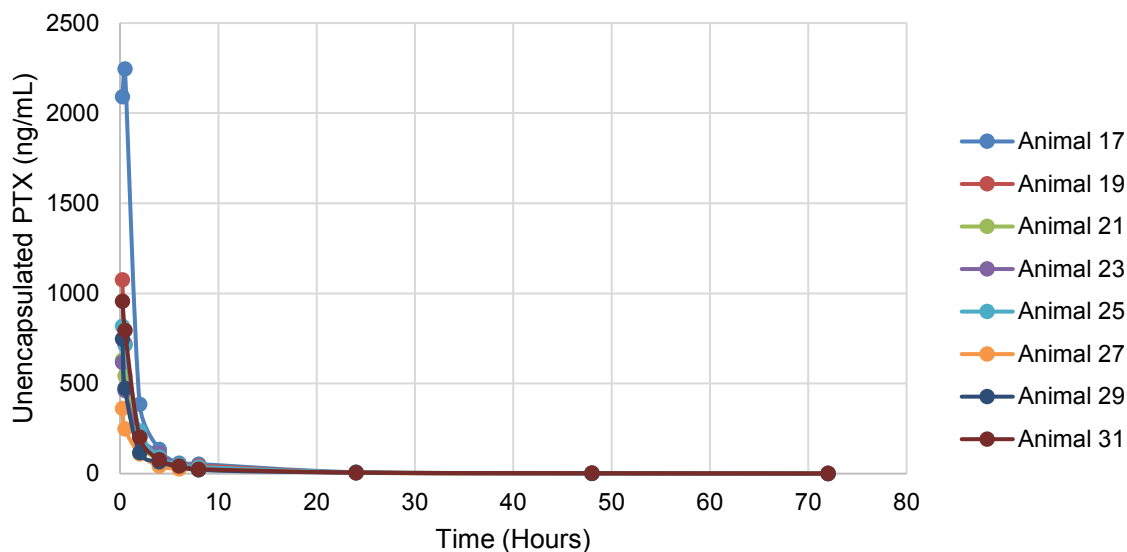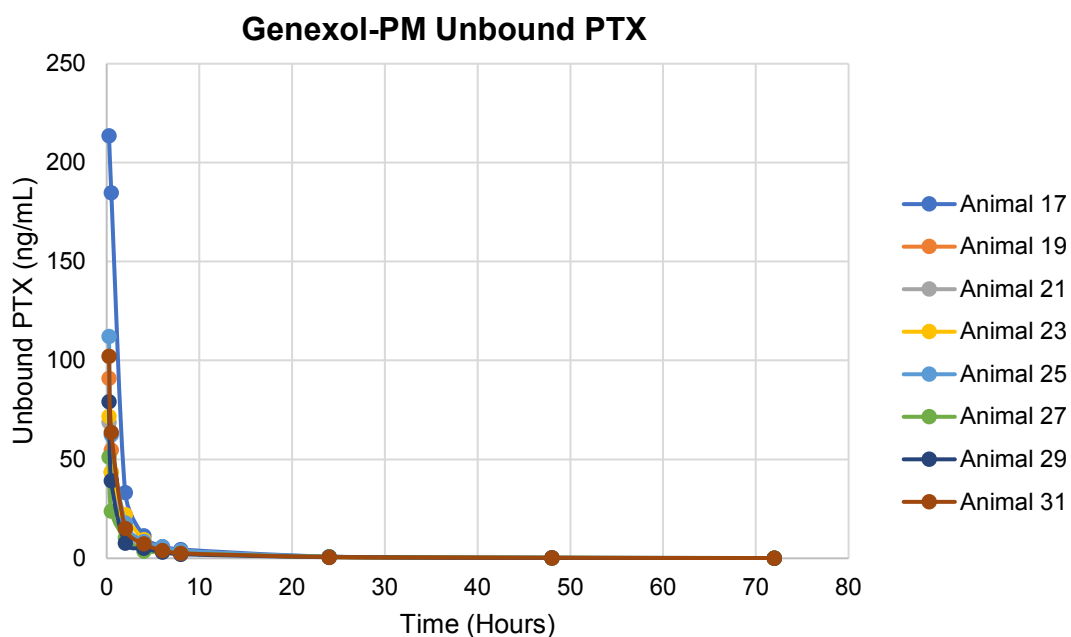**Figure VII-10. Genexol-PM concentration-time profiles for unbound drug.** Displayed are concentration-time profiles for unbound drug.

Table VII-13 Analytical data for Genexol-PM Lot D (GP31771). Presented are the analytical data for Genexol-PM Lot D (GP31771). The Total, Unencapsulated, and Unbound values were calculated as described in Section I. *No sample for this time point.

Time point	Rat #	Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 − %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX-Unencapsulated
15 min	R17	213.6	2311.9	9.2	90.8	9.4	91.7	10.2	89.8	2091.5	220.4
	R19	91.0	1210.0	7.5	92.5	7.0	82.7	8.5	91.5	1076.6	133.4
	R21	68.9	745.7	9.2	90.8	10.1	92.6	10.9	89.1	630.4	115.3
	R23	71.8	686.0	10.5	89.5	10.6	90.8	11.6	88.4	617.6	68.4
	R25	112.2	938.1	12.0	88.0	11.7	85.5	13.7	86.3	818.3	119.8
	R27	51.2	425.2	12.0	88.0	12.9	91.2	14.1	85.9	362.2	63.0
	R29	79.3	868.0	9.1	90.9	7.5	70.5	10.6	89.4	748.1	119.9
	R31	102.1	1101.2	9.3	90.7	11.0	103.1	10.7	89.3	956.5	144.7
30 min	R17	184.9	1239.1	14.9	85.1	9.6	116.8	8.2	91.8	2245.4	-1006.3
	R19	54.8	643.7	8.5	91.5	7.9	103.3	7.6	92.4	720.5	-76.8
	R21	43.6	499.6	8.7	91.3	9.5	118.1	8.0	92.0	542.3	-42.7
	R23	43.6	417.9	10.4	89.6	9.5	100.3	9.5	90.5	461.0	-43.1
	R25	62.5	667.3	9.4	90.6	8.1	92.9	8.8	91.2	713.2	-45.9
	R27	23.9	224.7	10.6	89.4	7.8	81.0	9.6	90.4	248.2	-23.5
	R29	39.3	450.0	8.7	91.3	8.3	100.0	8.3	91.7	473.9	-23.8
	R31	63.8	767.7	8.3	91.7	8.9	110.7	8.0	92.0	795.6	-27.8
2 hr	R17	33.3	370.5	9.0	91.0	10.2	118.2	8.6	91.4	385.1	-14.7
	R19	15.4	194.4	7.9	92.1	7.9	102.2	7.7	92.3	199.9	-5.5
	R21	14.4	168.0	8.6	91.4	10.4	130.7	8.0	92.0	180.4	-12.4
	R23	22.2	146.1	15.2	84.8	18.4	172.0	10.7	89.3	207.4	-61.2
	R25	17.8	227.7	7.8	92.2	9.4	124.3	7.5	92.5	236.8	-9.2
	R27	10.5	106.5	9.9	90.1	11.5	119.5	9.6	90.4	109.7	-3.2
	R29	7.7	113.8	6.8	93.2	4.6	68.9	6.6	93.4	116.8	-3.0
	R31	15.2	278.5	5.5	94.5	8.5	112.9	7.5	92.5	202.2	76.3
4 hr	R17	11.4	123.1	9.3	90.7	9.8	114.9	8.5	91.5	134.9	-11.8
	R19	7.9	92.2	8.5	91.5	8.0	98.4	8.2	91.8	96.6	-4.4
	R21	5.2	71.9	7.2	92.8	7.1	100.7	7.0	93.0	73.6	-1.7
	R23	9.5	90.4	10.5	89.5	6.3	75.2	8.3	91.7	113.7	-23.2
	R25	8.5	87.1	9.7	90.3	8.5	92.9	9.1	90.9	93.0	-5.9
	R27	3.6	41.4	8.7	91.3	8.7	101.0	8.6	91.4	41.9	-0.5
	R29	5.2	63.3	8.1	91.9	9.9	124.0	8.0	92.0	64.6	-1.4
	R31	7.4	77.4	9.6	90.4	9.8	103.3	9.5	90.5	78.3	-0.8
6 hr	R17	6.0	68.1	8.8	91.2	8.8	85.5	10.3	89.7	58.6	9.5
	R19	4.7	51.8	9.1	90.9	9.8	93.5	10.5	89.5	45.1	6.7
	R21	3.7	47.1	7.8	92.2	9.0	94.1	9.6	90.4	38.4	8.7
	R23	3.6	40.0	9.0	91.0	7.5	76.9	9.7	90.3	37.1	2.9
	R25	6.0	59.1	10.1	89.9	8.6	71.3	12.1	87.9	49.1	10.1
	R27	3.5	31.5	11.1	88.9	11.6	87.3	13.3	86.7	26.3	5.2
	R29*	3.1				7.5					
	R31	213.6	2311.9	9.2	90.8	9.4	91.7	10.2	89.8	2091.5	220.4

Values were excluded

Table VII-14 Analytical data for Genexol-PM Lot D (GP31771), continued. Presented are the analytical data for Genexol-PM lot D (GP31771). The Total, Unencapsulated, and Unbound values were calculated as described in Section I.

Time point	Rat #	Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 – %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 – %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated
8 hr	R17	4.5	61.4	7.3	92.7	9.5	114.0	8.3	91.7	53.4	8.0
	R19	3.5	50.6	6.9	93.1	7.8	100.0	7.8	92.2	44.6	6.0
	R21	3.6	38.6	9.4	90.6	9.6	89.3	10.8	89.2	33.6	5.0
	R23	2.7	26.7	10.1	89.9	9.0	75.2	12.0	88.0	22.5	4.1
	R25	3.8	42.8	8.9	91.1	8.4	80.9	10.3	89.7	36.9	5.9
	R27	2.5	24.8	10.1	89.9	8.9	80.7	11.0	89.0	22.9	1.9
	R29	2.0	23.6	8.6	91.4	8.2	84.9	9.7	90.3	21.1	2.6
	R31	2.5	28.9	8.8	91.2	9.0	90.2	9.9	90.1	25.5	3.3
24 hr	R17	0.9	9.7	9.1	90.9	8.9	80.0	11.2	88.8	7.9	1.8
	R19	0.6	7.8	8.0	92.0	6.9	65.8	10.4	89.6	6.0	1.8
	R21	0.5	6.0	8.0	92.0	6.5	65.6	9.9	90.1	4.8	1.1
	R23	0.7	7.0	9.4	90.6	10.1	81.1	12.5	87.5	5.3	1.7
	R25	0.8	9.9	8.0	92.0	7.5	69.8	10.7	89.3	7.4	2.5
	R27	0.7	7.1	10.3	89.7	7.4	60.7	12.1	87.9	6.0	1.0
	R29	0.6	6.8	9.3	90.7	7.9	75.3	10.4	89.6	6.0	0.7
	R31	0.7	7.3	9.1	90.9	12.0	103.6	11.6	88.4	5.7	1.6
48 hr	R17	0.1	3.9	2.3	97.7	6.7	65.1	10.3	89.7	0.9	3.0
	R19	0.2	2.4	7.4	92.6	6.3	66.2	9.5	90.5	1.8	0.5
	R21	0.1	1.4	9.1	90.9	4.7	55.5	8.5	91.5	1.5	-0.1
	R23	0.2	1.6	13.2	86.8	6.5	57.5	11.4	88.6	1.9	-0.3
	R25	0.4	3.2	12.0	88.0	6.7	64.7	10.4	89.6	3.6	-0.5
	R27	0.3	3.1	9.5	90.5	7.6	68.3	11.1	88.9	2.7	0.4
	R29	0.2	1.5	13.2	86.8	6.7	64.5	10.3	89.7	2.0	-0.4
	R31	0.3	2.3	12.0	88.0	7.8	71.8	10.9	89.1	2.5	-0.2
72 hr	R17	0.2	1.6	11.7	88.3	6.0	56.1	10.7	89.3	1.7	-0.1
	R19	0.1	0.7	16.8	83.2	2.9	36.3	8.0	92.0	1.5	-0.8
	R21	0.1	2.8	5.0	95.0	8.9	85.2	10.5	89.5	1.3	1.4
	R23	0.1	0.5	19.4	80.6	6.9	56.4	12.2	87.8	0.9	-0.3
	R25	0.1	0.7	17.0	83.0	7.5	62.5	11.9	88.1	1.0	-0.3
	R27	0.1	1.0	14.5	85.5	8.6	58.6	14.7	85.3	0.9	0.01
	R29	0.04	0.5	8.5	91.5	4.8	45.9	10.4	89.6	0.4	0.1
	R31	0.1	1.0	12.2	87.8	13.6	85.5	15.9	84.1	0.7	0.2

Table VII-15. Taxol generic Lot A (PACCA1018) Total PTX

| Hours	Total PTX (ng/mL)								
	0.25	0.5	2	4	6	8	24	48	72
Animal 33	11979.0	7353.4	2145.4	867.1	331.5	223.0	25.3	5.8	1.6
Animal 35	9796.6	5511.2	1057.0	394.8	188.6	107.4	11.9	2.9	0.7
Animal 37	9291.7	5262.5	1868.4	685.2	347.8	187.2	22.2	3.1	1.4
Animal 39	8986.5	4474.9	1336.0	419.7	213.6	112.0	66.8	2.1	1.0
Animal 41	10734.9	6751.9	3241.8	1274.4	661.7	295.2	20.6	4.4	1.6
Animal 43	11051.3	5669.6	1651.7	416.7	211.3	124.0	17.6	3.2	0.6
Animal 45	10453.7	7693.4	3411.1	1890.4	1109.0	600.1	59.7	14.4	6.7
Animal 47	16236.6	8742.3	2068.6	614.2	314.8	163.9	14.6	3.1	0.8
Average	11066.3	6432.4	2097.5	820.3	422.3	173.2	29.8	4.9	1.8
STD	2303.3	1439.6	841.1	523.5	315.5	68.5	21.1	4.0	2.0
% CV	21	22	40	64	75	40	71	82	114

▨ Values were excluded
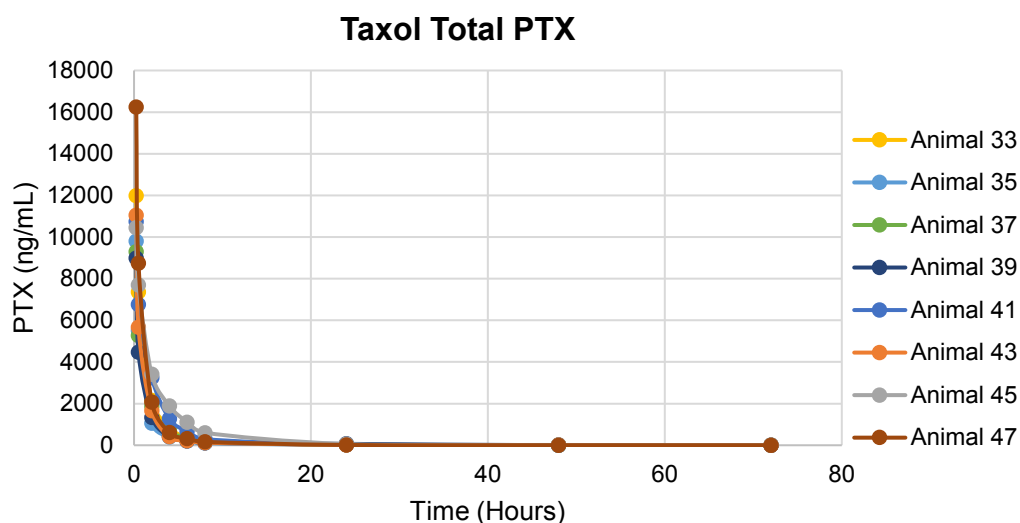
Figure VII-11. Taxol generic concentration-time profiles for total drug. Displayed are concentration-time profiles for total drug including outliers.

Table VII-16. Taxol generic Lot A (PACCA1018) Unencapsulated PTX

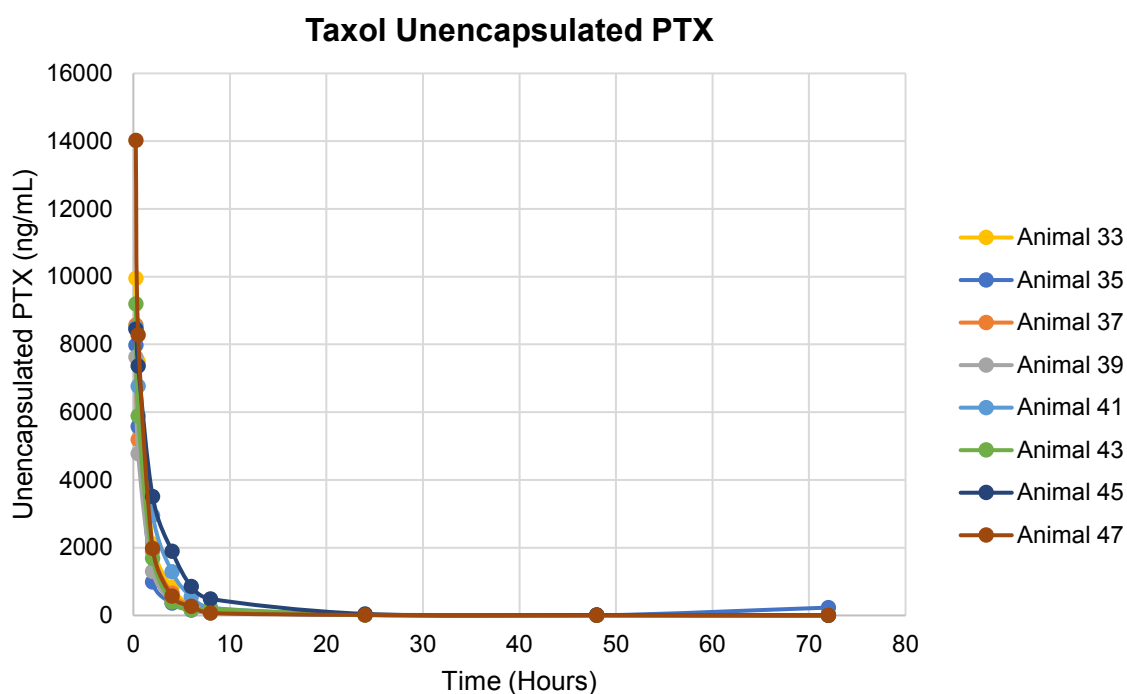| Hours	Unencapsulated PTX (ng/mL)								
	0.25	0.5	2	4	6	8	24	48	72
Animal 33	9949.2	7489.7	2110.6	830.5	283.9	176.0	23.4	4.3	1.1
Animal 35	7980.2	5576.1	986.0	364.7	153.4	84.9	8.4	1.8	231.2
Animal 37	8568.8	5186.2	1884.6	655.1	288.8	151.7	16.9	4.4	0.9
Animal 39	7623.9	4770.8	1297.7	405.8	176.6	71.9	21.5	2.2	0.000
Animal 41	8493.0	6766.3	2946.9	1288.0	552.4	226.7	17.9	4.8	2.0
Animal 43	9190.5	5891.7	1680.4	403.1	174.3	189.8	14.7	3.1	1.3
Animal 45	8443.8	7357.4	3505.7	1895.8	850.1	487.3	44.8	13.1	6.0
Animal 47	14018.1	8277.5	1977.4	572.0	268.5	72.7	15.7	3.7	1.3
Average	9283.4	6414.5	2048.7	801.9	343.5	182.6	20.4	4.7	1.8
STD	2041.1	1243.7	827.6	536.6	240.6	136.1	10.8	3.6	1.9
% CV	22	19	40	67	70	75	53	76	108

Values were excluded

Figure VII-12. Taxol generic concentration-time profiles for unencapsulated drug.
Displayed are concentration-time profiles for unencapsulated drug including outliers.

Table VII-17. Taxol generic Lot A (PACCA1018) Unbound PTX

| Hours	Unbound PTX (ng/mL)								
	0.25	**0.5**	**2**	**4**	**6**	**8**	**24**	**48**	**72**
Animal 33	213.9	168.2	42.1	14.7	5.8	3.9	0.8	0.2	0.1
Animal 35	230.4	161.9	23.2	7.5	3.7	2.0	0.2	0.1	11.5
Animal 37	220.5	147.6	41.8	14.2	6.1	3.9	0.7	0.3	0.1
Animal 39	201.0	124.7	25.7	9.3	4.1	2.2	0.7	0.1	0.00
Animal 41	246.4	168.6	60.0	24.4	11.8	7.0	0.6	0.2	0.1
Animal 43	244.3	124.3	32.0	7.4	4.5	3.2	0.5	0.2	0.1
Animal 45	182.6	134.0	71.0	37.8	23.2	13.6	1.9	0.7	0.5
Animal 47	370.4	224.8	37.9	11.4	6.1	2.7	0.6	0.2	0.1
Average	238.7	156.8	41.7	15.8	8.2	4.8	0.8	0.2	0.1
STD	57.3	33.0	16.5	10.4	6.6	3.9	0.5	0.2	0.2
% CV	24	21	40	66	81	81	65	82	127

Values were excluded
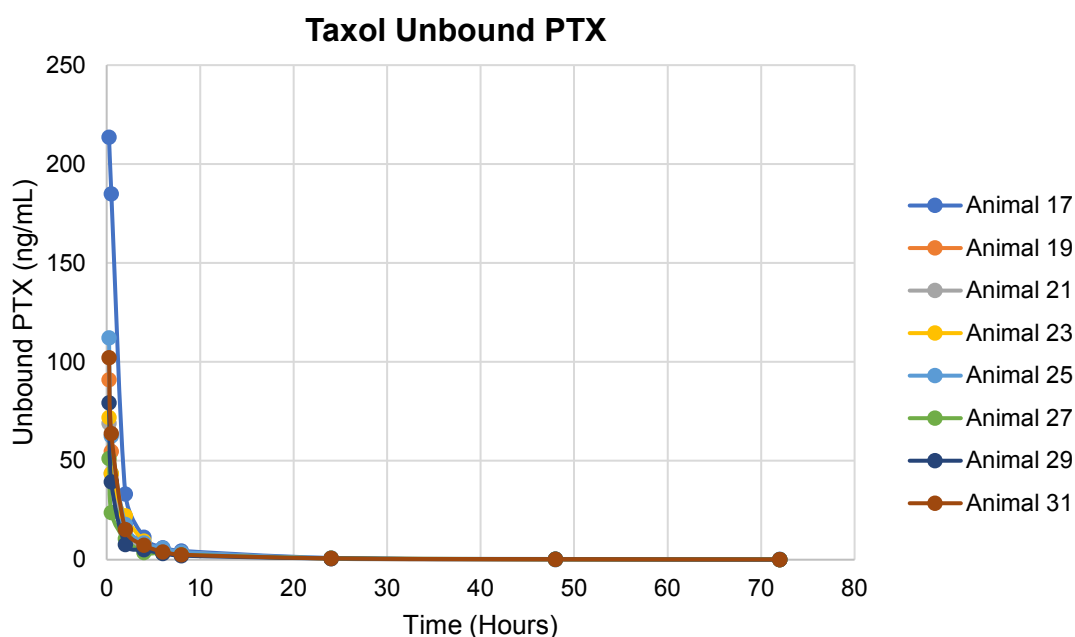
Figure VII-13. Taxol generic concentration-time profiles for unbound drug. Displayed are concentration-time profiles for unbound drug including outliers.

Table VII-18. Analytical data for Taxol generic Lot A (PACCA1018). Presented are the analytical data for Taxol generic lot A (PACCA1018). The Total, Unencapsulated, and Unbound values were calculated as described in Section I.

Time point	Rat #	Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 − %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX-Unencapsulated
15 min	R33	213.9	11979.0	1.8	98.2	2.0	93.6	2.1	97.9	9949.2	2029.8
	R35	230.4	9796.6	2.4	97.6	2.4	82.7	2.9	97.1	7980.2	1816.4
	R37	220.5	9291.7	2.4	97.6	2.3	91.2	2.6	97.4	8568.8	722.9
	R39	201.0	8986.5	2.2	97.8	4.7	180.0	2.6	97.4	7623.9	1362.6
	R41	246.4	10734.9	2.3	97.7	2.0	67.5	2.9	97.1	8493.0	2241.9
	R43	244.3	11051.3	2.2	97.8	2.1	79.6	2.7	97.3	9190.5	1860.8
	R45	182.6	10453.7	1.7	98.3	2.6	118.7	2.2	97.8	8443.8	2009.9
	R47	370.4	16236.6	2.3	97.7	2.0	77.1	2.6	97.4	14018.1	2218.6
30 min	R33	168.2	7353.4	2.3	97.7	2.5	110.9	2.2	97.8	7489.7	-136.4
	R35	161.9	5511.2	2.9	97.1	2.9	100.6	2.9	97.1	5576.1	-64.9
	R37	147.6	5262.5	2.8	97.2	2.7	96.2	2.8	97.2	5186.2	76.3
	R39	124.7	4474.9	2.8	97.2	3.1	118.7	2.6	97.4	4770.8	-295.9
	R41	168.6	6751.9	2.5	97.5	2.0	82.0	2.5	97.5	6766.3	-14.4
	R43	124.3	5669.6	2.2	97.8	2.1	97.8	2.1	97.9	5891.7	-222.1
	R45	134.0	7693.4	1.7	98.3	1.8	100.8	1.8	98.2	7357.4	336.0
	R47	224.8	8742.3	2.6	97.4	3.0	109.2	2.7	97.3	8277.5	464.7
2 hr	R33	42.1	2145.4	2.0	98.0	1.9	95.8	2.0	98.0	2110.6	34.8
	R35	23.2	1057.0	2.2	97.8	2.1	87.6	2.4	97.6	986.0	71.0
	R37	41.8	1868.4	2.2	97.8	2.1	96.1	2.2	97.8	1884.6	-16.2
	R39	25.7	1336.0	1.9	98.1	1.9	95.1	2.0	98.0	1297.7	38.3
	R41	60.0	3241.8	1.9	98.1	1.9	95.6	2.0	98.0	2946.9	294.8
	R43	32.0	1651.7	1.9	98.1	2.1	112.5	1.9	98.1	1680.4	-28.7
	R45	71.0	3411.1	2.1	97.9	1.8	88.9	2.0	98.0	3505.7	-94.6
	R47	37.9	2068.6	1.8	98.2	1.7	90.3	1.9	98.1	1977.4	91.1
4 hr	R33	14.7	867.1	1.7	98.3	1.6	90.8	1.8	98.2	830.5	36.6
	R35	7.5	394.8	1.9	98.1	2.3	113.8	2.1	97.9	364.7	30.1
	R37	14.2	685.2	2.1	97.9	1.7	77.0	2.2	97.8	655.1	30.0
	R39	9.3	419.7	2.2	97.8	2.4	105.4	2.3	97.7	405.8	13.9
	R41	24.4	1274.4	1.9	98.1	1.7	90.4	1.9	98.1	1288.0	-13.7
	R43	7.4	416.7	1.8	98.2	1.4	76.7	1.8	98.2	403.1	13.6
	R45	37.8	1890.4	2.0	98.0	2.0	100.3	2.0	98.0	1895.8	-5.4
	R47	11.4	614.2	1.9	98.1	1.7	83.3	2.0	98.0	572.0	42.2
6 hr	R33	5.8	331.5	1.7	98.3	1.7	83.5	2.0	98.0	283.9	47.6
	R35	3.7	188.6	2.0	98.0	1.9	77.3	2.4	97.6	153.4	35.2
	R37	6.1	347.8	1.7	98.3	1.3	61.9	2.1	97.9	288.8	58.9
	R39	4.1	213.6	1.9	98.1	1.8	79.3	2.3	97.7	176.6	37.1
	R41	11.8	661.7	1.8	98.2	1.6	75.0	2.1	97.9	552.4	109.3
	R43	4.5	211.3	2.1	97.9	1.9	73.8	2.6	97.4	174.3	37.0
	R45	23.2	1109.0	2.1	97.9	2.1	76.7	2.7	97.3	850.1	258.9
	R47	6.1	314.8	1.9	98.1	2.0	88.8	2.3	97.7	268.5	46.3

Table VII-19. Analytical data for Taxol generic Lot A (PACCA1018), continued. Presented are the analytical data for Taxol generic lot A (PACCA1018). The Total, Unencapsulated, and Unbound values were calculated as described in Section I.

Time point	Rat #	Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX = Filtrate PTX / Reservoir PTX *100	% Bound = 100 − %Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 (ng/mL)	%Unbound PTX_C13 = Filtrate PTX_C13 / Reservoir PTX_C13 * 100	% Bound PTX_C13 = 100 − %Unbound C13	Unencapsulated (ng/mL) = Fil PTX/ (1-(%Bound PTX_C13/100))	Encapsulated (ng/mL) = Reservoir PTX- Unencapsulated
8 hr	R33	3.9	223.0	1.8	98.2	1.7	76.6	2.2	97.8	176.0	47.0
	R35	2.0	107.4	1.8	98.2	1.7	74.2	2.3	97.7	84.9	22.4
	R37	3.9	187.2	2.1	97.9	2.0	79.4	2.6	97.4	151.7	35.5
	R39	2.2	112.0	2.0	98.0	2.8	89.4	3.1	96.9	71.9	40.1
	R41	7.0	295.2	2.4	97.6	2.2	71.3	3.1	96.9	226.7	68.5
	R43	3.2	124.0	2.6	97.4	1.7	101.6	1.7	98.3	189.8	-65.8
	R45	13.6	600.1	2.3	97.7	2.0	72.1	2.8	97.2	487.3	112.8
	R47	2.7	163.9	1.7	98.3	2.6	68.9	3.8	96.2	72.7	91.3
24 hr	R33	0.8	25.3	3.1	96.9	2.2	65.8	3.3	96.7	23.4	1.9
	R35	0.2	11.9	2.1	97.9	2.1	71.0	2.9	97.1	8.4	3.5
	R37	0.7	22.2	3.3	96.7	3.0	69.9	4.3	95.7	16.9	5.2
	R39	0.7	66.8	1.1	98.9	2.5	72.7	3.4	96.6	21.5	45.3
	R41	0.6	20.6	3.0	97.0	2.6	73.1	3.5	96.5	17.9	2.7
	R43	0.5	17.6	2.9	97.1	2.4	69.6	3.5	96.5	14.7	3.0
	R45	1.9	59.7	3.2	96.8	2.7	64.1	4.3	95.7	44.8	14.9
	R47	0.6	14.6	3.8	96.2	2.3	64.4	3.5	96.5	15.7	-1.2
48 hr	R33	0.2	5.8	3.8	96.2	3.2	62.9	5.1	94.9	4.3	1.5
	R35	0.1	2.9	2.6	97.4	2.6	60.7	4.3	95.7	1.8	1.1
	R37	0.3	3.1	8.4	91.6	3.7	63.6	5.8	94.2	4.4	-1.4
	R39	0.1	2.1	6.1	93.9	3.7	63.3	5.8	94.2	2.2	-0.1
	R41	0.2	4.4	4.9	95.1	2.4	55.5	4.4	95.6	4.8	-0.4
	R43	0.2	3.2	5.2	94.8	3.3	61.7	5.4	94.6	3.1	0.1
	R45	0.7	14.4	4.9	95.1	3.1	57.6	5.4	94.6	13.1	1.3
	R47	0.2	3.1	5.2	94.8	2.2	51.1	4.3	95.7	3.7	-0.6
72 hr	R33	0.1	1.6	4.3	95.7	3.6	57.3	6.2	93.8	1.1	0.5
	R35	11.5	0.7	1714.9	-1614.9	3.4	68.8	5.0	95.0	231.2	-230.6
	R37	0.1	1.4	4.7	95.3	5.6	74.8	7.5	92.5	0.9	0.5
	R39	0.00	1.0	0.00	100.0	3.6	56.5	6.4	93.6	0.00	1.0
	R41	0.1	1.6	7.1	92.9	4.1	71.0	5.7	94.3	2.0	-0.4
	R43	0.1	0.6	16.3	83.7	3.4	47.4	7.2	92.8	1.3	-0.7
	R45	0.5	6.7	7.6	92.4	5.5	64.6	8.6	91.4	6.0	0.8
	R47	0.1	0.8	10.9	89.1	4.2	64.3	6.5	93.5	1.3	-0.5

Values were excluded

Table VII-20. Total drug PK comparison. Displayed are the individual animal pharmacokinetic parameters: concentration time zero (C_0); slope of the terminal eliminiation phase (λ_z); Half-life ($T_{1/2}$); area under the time concentration curve to time infinity (AUC_{inf}); maximum concentration (C_{max}); area under the time concentration curve all time points (AUC_{all}); Volume of distribution (Vd); clearance (CL); Mean residence time (MRT_{inf}); Volume of distribution steady state (Vss).

	Animal	C_0 ng/mL	C_{max} ng/mL	λ_z h^{-1}	$T_{1/2}$ h	AUC_{all} ng*h/mL	AUC_{inf} ng*h/mL	Vd mL/kg	CL mL/h*kg	MRT_{inf} h	Vss mL/kg
Abraxane Total											
6 mg/mL	1	1860	1218	0.051964	13	2529	2541	3226	2362	6	13333
	3	2664	1690	0.011352	61	4316	4962	2253	1209	19	26121
	5	1351	811	0.02031	34	2111	2235	4441	2685	11	32066
	7	1748	1036	0.050009	14	2338	2352	3433	2551	6	15740
	9	1495	916	0.041708	17	2106	2132	4014	2814	7	20309
	11	1358	971	0.066454	10	2810	2820	4417	2128	7	14548
	13	1162	800	0.053085	13	1850	1863	5161	3221	7	21464
	15	2167	1285	0.006819	102	2863	4014	2768	1495	42	84448
	AVG	1726	1091	0.047	17	2291	2324	4115	2627	7	20512
	SD	498	298	0.015	9	343	332	715	379	2	6780
Genexol-PM Total											
6 mg/mL	17	4314	2312	0.038093	18	4090	4131	1391	1452	5	7863
	19	2274	1210	0.050525	14	2455	2469	2638	2431	6	14109
	21	1113	746	0.015923	44	1828	2003	5391	2996	14	46535
	23	1126	686	0.053317	13	1620	1630	5328	3681	6	23615
	25	1319	938	0.055108	13	2341	2354	4549	2549	7	17059
	27	805	425	0.041627	17	1187	1210	7456	4960	11	53181
	29	1674	868	0.055104	13	1536	1545	3584	3884	6	22741
	31	1580	1101	0.042293	16	2352	2375	3798	2527	6	14509
	AVG	1413	853	0.044	15	2176	2214	4267	3060	8	16649
	SD	481	264	0.013	2	896	897	1861	1083	3	5900
Taxol Total											
6 mg/mL	33	19515	11979	0.057647	12	20691	20719	307	290	3	852
	35	17414	9797	0.059938	12	13747	13758	345	436	2	965
	37	16406	9292	0.083809	8	16532	16549	366	363	3	1080
	39	18047	8987	0.08945	8	14429	14440	332	416	4	1652
	41	17067	10735	0.052564	13	23464	23495	352	255	3	798
	43	21541	11051	0.070788	10	16116	16124	279	372	2	857
	45	14204	10454	0.045474	15	30110	30258	422	198	5	995
	47	30155	16237	0.061243	11	22805	22818	199	263	2	516
	AVG	19294*	11066*	0.065*,ABX	11	19737*	19770*	325*	324*	3*	964*
	SD	4890	2303	0.015	2	5597	5635	66	85	1	325

*$p \leq 0.05$, significantly different from other formulations; *,ABX$p \leq 0.05$ significantly different from abraxane; <u>ANOVA</u>, with Duncan's Multiple range post hoc test comparison; Outliers highlighted in red text were excluded from statistical analysis.
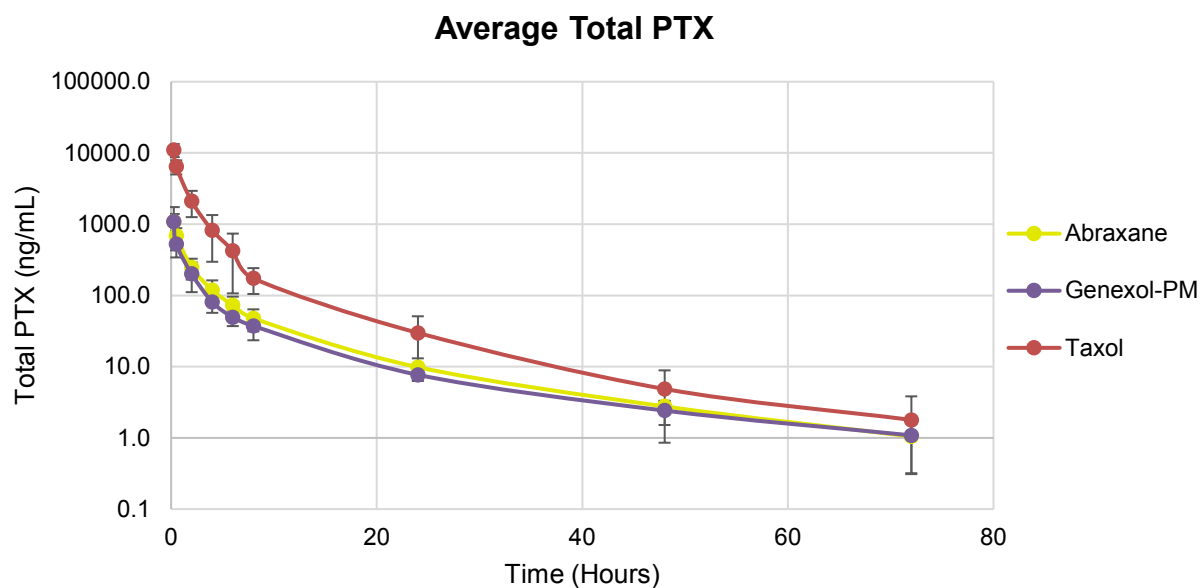
Average Total PTX

Figure VII-14. Total drug PK comparison. Presented are the total PTX time-concentration comparisons for Abraxane, Genexol-PM and Taxol (*N*=8, Mean$\pm$SD).

Table VII-21. Unencapsulated drug PK comparison. Displayed are the individual animal pharmacokinetic parameters: concentration time zero (C_0); slope of the terminal eliminiation phase (λ_z); Half-life ($T_{1/2}$); area under the time concentration curve to time infinity (AUC_{inf}); maximum concentration (C_{max}); area under the time concentration curve all time points (AUC_{all}); Volume of distribution (Vd); clearance (CL); Mean residence time (MRT_{inf}); Volume of distribution steady state (Vss).

	Animal	C_o	C_{max}	λ_z	$T_{1/2}$	AUC_{all}	AUC_{inf}	Vd	CL	MRT_{inf}	Vss
Abraxane Unencapsulated											
		ng/mL	ng/mL	h⁻¹	h	ng*h/mL	ng*h/mL	mL/kg	mL/h*kg	h	mL/kg
6 mg/mL	1	2357	1433	0.034	20	2636	2672	2545	2246	6	12778
	3	2858	1776	0.009	78	4362	5183	2099	1158	19	30698
	5	1080	769	0.014	49	2051	2267	53554	2647	11	44684
	7	2181	1067	0.042	17	2261	2282	2751	2629	6	15942
	9	1541	860	0.037	19	1982	2009	3894	2987	7	20588
	11	1272	970	0.062	11	3098	3110	4719	1929	7	12940
	13	1221	828	0.050	14	1885	1898	4914	3161	7	19647
	15	1668	1231	0.003	204	3455	5534	3598	1084	42	108521
	AVG	1772	1117	0.040	22	2319	2373	4063*	2600*	7	18766*
	SD	631	347	0.016	14	466	450	1219	457	2	6697
Genexol-PM Unencapsulated											
		ng/mL	ng/mL	h⁻¹	h	ng*h/mL	ng*h/mL	mL/kg	mL/h*kg	h	mL/kg
6 mg/mL	17	7299	2092	0.0319	22	3701	3754	822	1598	5	7918
	19	1609	1077	0.0294	24	2316	2366	3730	2536	6	17766
	21	733	630	0.0268	26	1714	1764	8189	3401	14	27778
	23	827	618	0.0378	18	1690	1713	7251	3504	6	23512
	25	939	818	0.0416	17	2223	2247	6390	2670	7	18959
	27	529	362	0.0385	18	1104	1128	11350	5317	11	56273
	29	1181	748	0.0570	12	1444	1451	5080	4134	6	24971
	31	1150	956	0.0426	16	2087	2104	5218	2852	6	16138
	AVG	995	744	0.038	18	2035	2066	5976*	3251*	8	19577*
	SD	354	237	0.010	4	786	797	1620	1126	3	6624
Taxol Unencapsulated											
		ng/mL	ng/mL	h⁻¹	h	ng*h/mL	ng*h/mL	mL/kg	mL/h*kg	h	mL/kg
6 mg/mL	33	13216	9949	0.064	11	18784	18801	454	319	3	893
	35	11421	7980	0.104	7	12017	12035	525	499	2	967
	37	14158	8569	0.062	11	15457	15470	424	388	3	1107
	39	12183	7624	0.088	8	12170	12168	492	493	4	1232
	41	10660	8493	0.045	15	20754	20799	563	288	3	929
	43	14336	9191	0.050	14	15432	15459	419	388	2	1057
	45	9691	8444	0.042	16	27054	27195	619	221	5	1062
	47	23740	14018	0.052	13	19929	19953	253	301	2	594
	AVG	12238*	9283*	0.063*,GXL	12*,GXL	17699*	17735*	469*	362*	3*	980*
	SD	1765	2041	0.022	3	5014	5053	111	99	1	189

*p≤0.05, significantly different from other formulations; *,GXLp≤0.05 significantly different from genexol; <u>ANOVA</u>, with Duncan's Multiple range post hoc test comparison; Outliers highlighted in red text were excluded from statistical analysis.
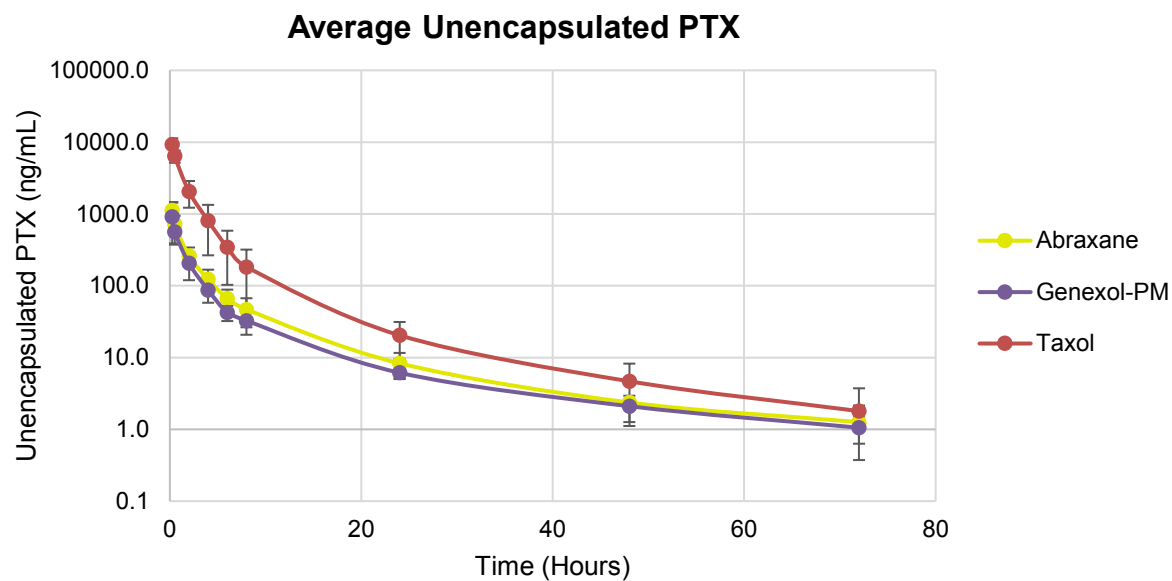
Figure VII-15. Unencapsulated drug PK comparison. Presented are the uncapsulated PTX time-concentration comparisons for Abraxane, Genexol-PM and Taxol (*N*=8, Mean+SD).

Table VII-22. Unbound drug PK comparison. Displayed are the individual animal pharmacokinetic parameters: concentration time zero (C_0); slope of the terminal eliminiation phase (λ_z); Half-life ($T_{1/2}$); area under the time concentration curve to time infinity (AUC_{inf}); maximum concentration (C_{max}); area under the time concentration curve all time points (AUC_{all}); Volume of distribution (Vd); clearance (CL); Mean residence time (MRT_{inf}); Volume of distribution steady state (Vss).

	Abraxane Unbound									
Animal	C_o	C_{max}	λ_z	$T_{1/2}$	AUC_{all}	AUC_{inf}	Vd	CL	MRT_{inf}	Vss
	ng/mL	ng/mL	h^{-1}	h	ng*h/mL	ng*h/mL	mL/kg	mL/h*kg	h	mL/kg
6 mg/mL 1	193	127	0.031	22	309	314	31143	19131	6	124681
3	225	165	0.007	102	408	518	26658	11586	30	426431
5	161	96	0.018	38	218	236	37171	25420	13	359746
7	184	104	0.041	17	220	223	32686	26932	6	175102
9	94	96	0.034	21	232	236	64020	25423	7	184146
11	184	119	0.062	11	327	328	32658	18281	7	129303
13	168	104	0.044	16	211	214	35755	28100	6	177124
15	187	128	0.005	153	341	496	32057	12102	51	854809
AVG	174	117	0.038	21	283	320	36518	20872*	8	303918*
SD	38	23	0.015	9	73	123	11545	6582	3	248205
	Genexol-PM Unbound									
Animal	C_o	C_{max}	λ_z	$T_{1/2}$	AUC_{all}	AUC_{inf}	Vd	CL	MRT_{inf}	Vss
	ng/mL	ng/mL	h^{-1}	h	ng*h/mL	ng*h/mL	mL/kg	mL/h*kg	h	mL/kg
6 mg/mL 17	970	214	0.033	21	371	376	6184	15937	5	77019.0
19	151	91	0.034	20	191	195	39676	30802	7	220001.8
21	109	69	0.026	27	159	164	55052	36504	9	323738.9
23	118	72	0.037	19	180	183	50667	32820	7	242908.7
25	202	112	0.039	18	229	232	29748	25897	7	191305.8
27	110	51	0.034	20	126	130	54634	46149	11	534880.3
29	160	79	0.057	12	141	141	37545	42477	6	271593.2
31	164	102	0.036	20	195	198	36678	30239	7	216701.7
AVG	145	99	0.037	19	199	202	38773	32603*	8	259769*
SD	34	50	0.009	5	77	78	16073	9470	2	131868
	Taxol Unbound									
Animal	C_o	C_{max}	λ_z	$T_{1/2}$	AUC_{all}	AUC_{inf}	Vd	CL	MRT_{inf}	Vss
	ng/mL	ng/mL	h^{-1}	h	ng*h/mL	ng*h/mL	mL/kg	mL/h*kg	h	mL/kg
6 mg/mL 33	272	214	0.050	14	406	408	22060	14723	4	54623
35	328	230	0.088	8	327	328	18308	18319	2	35158
37	329	221	0.052	13	396	397	18211	15111	4	56979
39	324	201	0.071	10	309	310	18512	19372	3	60471
41	360	246	0.035	20	514	517	16660	11596	4	47131
43	480	244	0.034	20	354	357	12496	16803	4	62008
45	249	183	0.028	25	689	708	24120	8478	8	72254
47	610	370	0.040	17	507	509	9834	11783	3	30242
AVG	446*	239*	0.050	16	438*	442*	17525*	14523*	4*	52359*
SD	183	57	0.021	6	127	132	4649	3706	2	14110

*p≤0.05, significantly different from other formulations; <u>ANOVA</u>, with Duncan's Multiple range post hoc test comparison; Outliers highlighted in red text were excluded from statistical analysis.
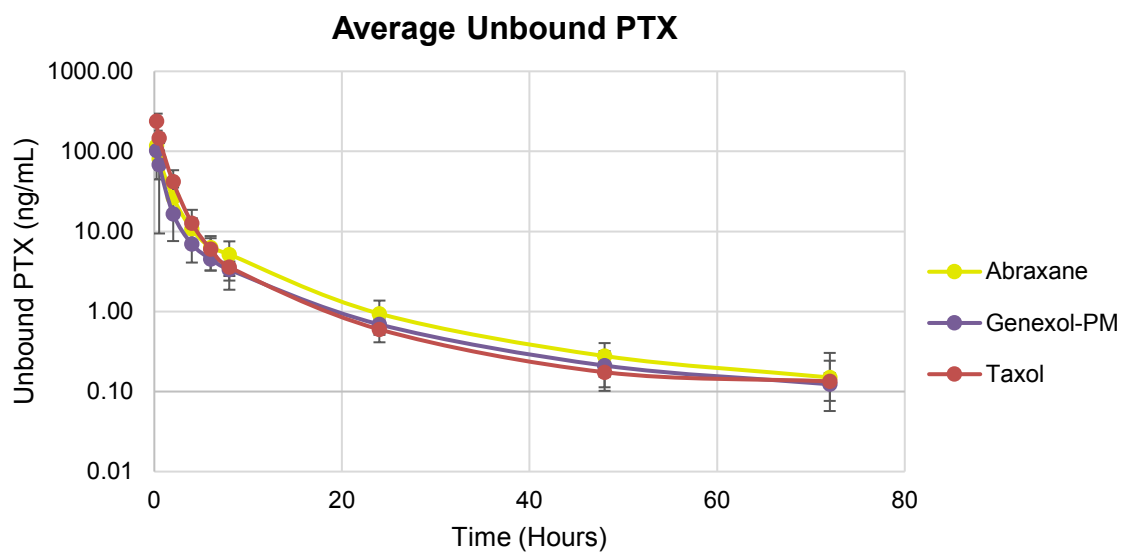
Average Unbound PTX

Figure VII-16. Unbound drug PK comparison. Presented are the unbound PTX time-concentration comparisons for Abraxane, Genexol-PM and Taxol (*N*=8, Mean+SD).

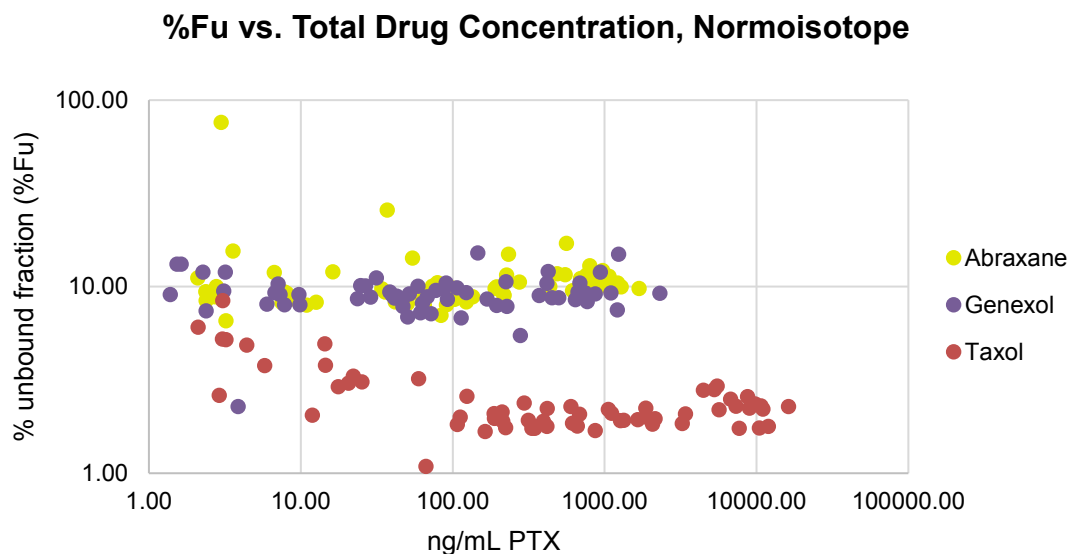**%Fu vs. Total Drug Concentration, Normoisotope**

Figure VII-17. Normoisotopic %Fu vs. Total PTX concentration. Presented are the individual %Fu vs. Total PTX concentration based on normoisotopic filtrate/reservoir concentrations for Abraxane, Genexol-PM and Taxol (*N*=8).
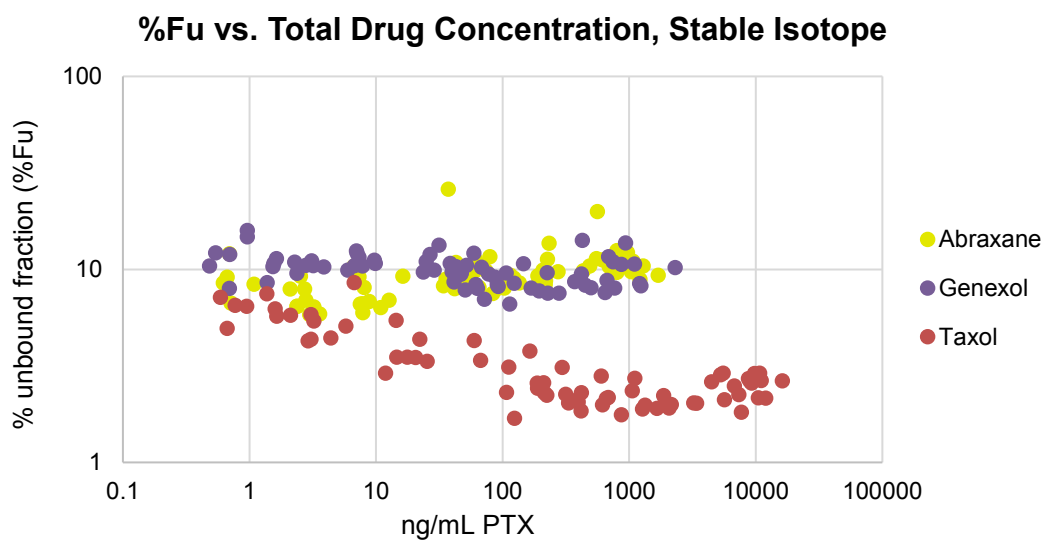
%Fu vs. Total Drug Concentration, Stable Isotope

Figure VII-18. Stable isotope tracer %Fu vs. Total PTX concentration. Presented are the individual %Fu vs. Total PTX concentration based on stable isotope tracer filtrate/reservoir concentrations for Abraxane, Genexol-PM and Taxol (*N*=8).

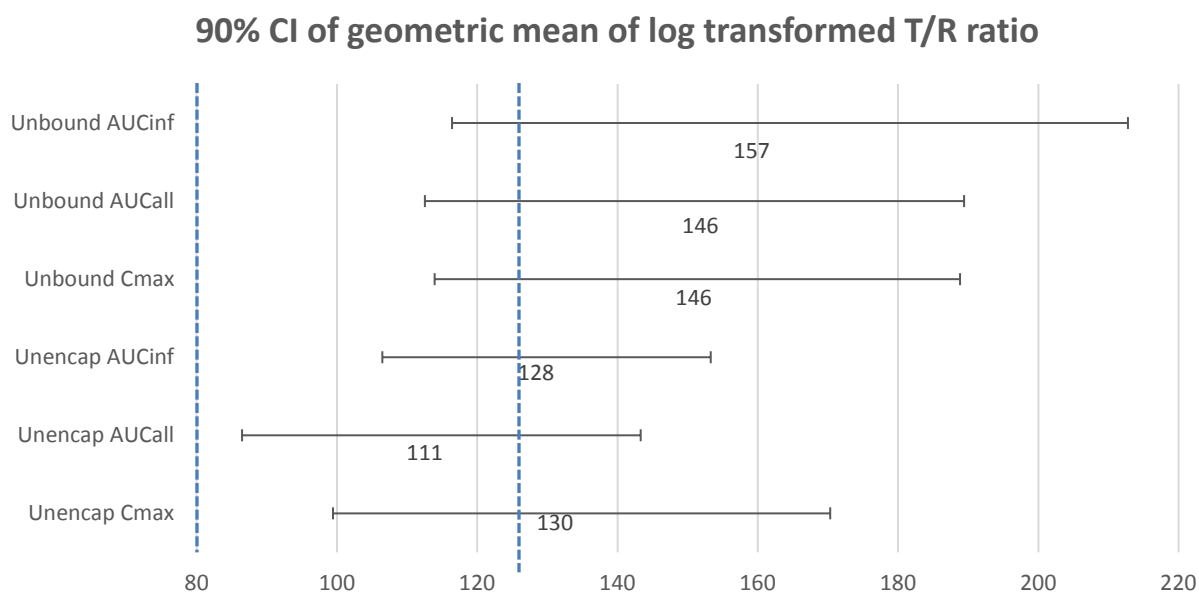**90% CI of geometric mean of log transformed T/R ratio**

Figure VII-19. TOST bioequivalence analysis. Presented are the results of two-sided t-tests (TOST) of PK parameters for Abraxane (reference) in comparison to Genexol-PM (test). None of the PK parameters were found to be equivalent, with 90%CI of the test (Abraxane)/reference (Genexol-PM) ratio falling outside the 80-125% range by TOST.

Appendix A. Inter-Day Individual Data for Human Plasma Validation

A. QC Low PTX in Human Plasma

QC Low PTX	Theoretical (ng/mL)	Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	10.0	10.2	2%	
Study Day 1	10.0	10.7	7%	
Study Day 1	10.0	11.0	10%	
Study Day 1	10.0	10.7	7%	
Study Day 1	10.0	9.1	-9%	
Study Day 1	10.0	9.9	-1%	
AVG		**10.3**	**3%**	**7%**
Study Day 2	10.0	10.5	5%	
Study Day 2	10.0	8.4	-16%	
Study Day 2	10.0	10.4	4%	
Study Day 2	10.0	10.4	4%	
Study Day 2	10.0	9.6	-4%	
Study Day 2	10.0	9.5	-5%	
AVG		**9.8**	**-2%**	**8%**
Study Day 3	10.0	10.1	1%	
Study Day 3	10.0	9.9	-1%	
Study Day 3	10.0	10.2	2%	
Study Day 3	10.0	10.3	3%	
Study Day 3	10.0	10.5	5%	
Study Day 3	10.0	10.2	2%	
AVG		**10.2**	**2%**	**2%**

B. QC Mid PTX in Human Plasma

QC Mid PTX	Theoretical (ng/mL)	Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	100	101	1%	
Study Day 1	100	98	-2%	
Study Day 1	100	102	2%	
Study Day 1	100	101	1%	
Study Day 1	100	102	2%	
Study Day 1	100	105	5%	
AVG		**101**	**1%**	**2%**
Study Day 2	100	109	9%	
Study Day 2	100	100	0.2%	
Study Day 2	100	102	2%	
Study Day 2	100	106	6%	
Study Day 2	100	109	9%	
Study Day 2	100	106	6%	
AVG		**105**	**5%**	**3%**
Study Day 3	100	107	7%	
Study Day 3	100	100	0.04%	
Study Day 3	100	108	8%	
Study Day 3	100	102	2%	
Study Day 3	100	100	-0.3%	
Study Day 3	100	100	-0.02%	
AVG		**103**	**3%**	**4%**

C. QC High PTX in Human Plasma

QC High PTX	Theoretical (ng/mL)	Concentration (ng/mL)	Accuracy Deviation	Precision (%)
Study Day 1	1000	1055	6%	
Study Day 1	1000	1042	4%	
Study Day 1	1000	1009	1%	
Study Day 1	1000	1081	8%	
Study Day 1	1000	1052	5%	
Study Day 1	1000	1087	9%	
AVG		**1054**	**5%**	**3%**
Study Day 2	1000	1037	4%	
Study Day 2	1000	1014	1%	
Study Day 2	1000	1073	7%	
Study Day 2	1000	1020	2%	
Study Day 2	1000	1018	2%	
Study Day 2	1000	1050	5%	
AVG		**1036**	**4%**	**2%**
Study Day 3	1000	1053	5%	
Study Day 3	1000	1056	6%	
Study Day 3	1000	1007	1%	
Study Day 3	1000	1041	4%	
Study Day 3	1000	1083	8%	
Study Day 3	1000	1094	9%	
AVG		**1055**	**6%**	**3%**

D. QC Low PTX_C13 in Human Plasma

QC Low PTX_C13	Theoretical (ng/mL)	Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	10.0	9.7	-3%	
Study Day 1	10.0	10.7	7%	
Study Day 1	10.0	10.6	6%	
Study Day 1	10.0	10.3	3%	
Study Day 1	10.0	9.6	-4%	
Study Day 1	10.0	9.6	-4%	
AVG		**10.1**	**1%**	**5%**
Study Day 2	10.0	10.5	5%	
Study Day 2	10.0	11.4	14%	
Study Day 2	10.0	10.5	5%	
Study Day 2	10.0	10.6	6%	
Study Day 2	10.0	10.0	-0.5%	
Study Day 2	10.0	9.8	-2%	
AVG		**10.5**	**5%**	**5%**
Study Day 3	10.0	9.9	-1%	
Study Day 3	10.0	10.2	2%	
Study Day 3	10.0	10.4	4%	
Study Day 3	10.0	10.1	1%	
Study Day 3	10.0	9.7	-3%	
Study Day 3	10.0	10.1	1%	
AVG		**10.1**	**1%**	**2%**

E. QC Mid PTX_C13 in Human Plasma

QC Mid PTX_C13	Theoretical (ng/mL)	Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	100	100	0%	
Study Day 1	100	100	0%	
Study Day 1	100	99	-1%	
Study Day 1	100	98	-2%	
Study Day 1	100	99	-1%	
Study Day 1	100	100	0%	
AVG		**99**	**-1%**	**1%**
Study Day 2	100	108	8%	
Study Day 2	100	99	-1%	
Study Day 2	100	99	-1%	
Study Day 2	100	101	1%	
Study Day 2	100	107	7%	
Study Day 2	100	101	1%	
AVG		**103**	**3%**	**4%**
Study Day 3	100	105	5%	
Study Day 3	100	101	1%	
Study Day 3	100	106	6%	
Study Day 3	100	101	1%	
Study Day 3	100	96	-4%	
Study Day 3	100	97	-3%	
AVG		**101**	**1%**	**4%**

F. QC High PTX_C13 in Human Plasma

QC High PTX_C13	Theoretical (ng/mL)	Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	1000	1090	9%	
Study Day 1	1000	1052	5%	
Study Day 1	1000	998	0%	
Study Day 1	1000	1075	8%	
Study Day 1	1000	1034	3%	
Study Day 1	1000	1061	6%	
AVG		**1051**	**5%**	**3%**
Study Day 2	1000	1019	2%	
Study Day 2	1000	1040	4%	
Study Day 2	1000	1016	2%	
Study Day 2	1000	1035	4%	
Study Day 2	1000	1041	4%	
Study Day 2	1000	1063	6%	
AVG		**1036**	**4%**	**2%**
Study Day 3	1000	1044	4%	
Study Day 3	1000	1053	5%	
Study Day 3	1000	1068	7%	
Study Day 3	1000	1095	9%	
Study Day 3	1000	1072	7%	
Study Day 3	1000	1074	7%	
AVG		**1067**	**7%**	**2%**

Appendix B. Inter-Day Individual Data for Human Protein-Free Plasma Validation

A. QC Low PTX in Human Protein-Free Plasma

QC Low PTX	Theoretical (ng/mL)	Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	0.50	0.51	1%	
Study Day 1	0.50	0.55	10%	
Study Day 1	0.50	0.45	-10%	
Study Day 1	0.50	0.56	12%	
Study Day 1	0.50	0.52	4%	
Study Day 1	0.50	0.47	-6%	
AVG		**0.51**	**2%**	**9%**
Study Day 2	0.50	0.50	-1%	
Study Day 2	0.50	0.54	9%	
Study Day 2	0.50	0.49	-2%	
Study Day 2	0.50	0.55	11%	
Study Day 2	0.50	0.51	2%	
Study Day 2	0.50	0.53	7%	
AVG		**0.52**	**4%**	**5%**
Study Day 3	0.50	0.55	9%	
Study Day 3	0.50	0.55	9%	
Study Day 3	0.50	0.52	3%	
Study Day 3	0.50	0.55	11%	
Study Day 3	0.50	0.54	9%	
Study Day 3	0.50	0.55	10%	
AVG		**0.54**	**8%**	**3%**

B. QC Mid PTX in Human Protein-Free Plasma

QC Mid PTX	Theoretical (ng/mL)	Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	5.00	5.16	3%	
Study Day 1	5.00	5.38	8%	
Study Day 1	5.00	5.40	8%	
Study Day 1	5.00	5.36	7%	
Study Day 1	5.00	5.52	10%	
Study Day 1	5.00	5.14	3%	
AVG		**5.33**	**7%**	**3%**
Study Day 2	5.00	5.50	10%	
Study Day 2	5.00	5.34	7%	
Study Day 2	5.00	5.33	7%	
Study Day 2	5.00	5.34	7%	
Study Day 2	5.00	5.13	3%	
Study Day 2	5.00	5.18	4%	
AVG		**5.30**	**6%**	**3%**
Study Day 3	5.00	5.34	7%	
Study Day 3	5.00	5.37	7%	
Study Day 3	5.00	5.41	8%	
Study Day 3	5.00	5.23	5%	
Study Day 3	5.00	5.38	8%	
Study Day 3	5.00	5.63	13%	
AVG		**5.39**	**8%**	**2%**

C. QC High PTX in Human Protein-Free Plasma

QC High PTX	Theoretical (ng/mL)	Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	50.0	47.0	-6%	
Study Day 1	50.0	49.5	-1%	
Study Day 1	50.0	50.8	2%	
Study Day 1	50.0	49.5	-1%	
Study Day 1	50.0	50.6	1%	
Study Day 1	50.0	51.7	3%	
AVG		**49.9**	**-0.3%**	**3%**
Study Day 2	50.0	48.8	-2%	
Study Day 2	50.0	50.5	1%	
Study Day 2	50.0	50.7	1%	
Study Day 2	50.0	49.7	-1%	
Study Day 2	50.0	51.7	3%	
Study Day 2	50.0	52.8	6%	
AVG		**50.7**	**1%**	**3%**
Study Day 3	50.0	52.8	6%	
Study Day 3	50.0	51.1	2%	
Study Day 3	50.0	53.8	8%	
Study Day 3	50.0	54.7	9%	
Study Day 3	50.0	50.1	0.2%	
Study Day 3	50.0	51.4	3%	
AVG		**52.3**	**5%**	**3%**

D. QC Low PTX_C13 in Human Protein-Free Plasma

QC Low PTX_C13	Theoretical (ng/mL)	Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	0.50	0.54	9%	
Study Day 1	0.50	0.58	15%	
Study Day 1	0.50	0.50	0%	
Study Day 1	0.50	0.40	-19%	
Study Day 1	0.50	0.57	15%	
Study Day 1	0.50	0.54	9%	
AVG		**0.52**	**5%**	**12%**
Study Day 2	0.50	0.57	14%	
Study Day 2	0.50	0.43	-15%	
Study Day 2	0.50	0.49	-1%	
Study Day 2	0.50	0.52	4%	
Study Day 2	0.50	0.45	-10%	
Study Day 2	0.50	0.53	7%	
AVG		**0.50**	**0%**	**11%**
Study Day 3	0.50	0.46	-9%	
Study Day 3	0.50	0.57	14%	
Study Day 3	0.50	0.56	12%	
Study Day 3	0.50	0.55	9%	
Study Day 3	0.50	0.49	-1%	
Study Day 3	0.50	0.51	3%	
AVG		**0.52**	**5%**	**8%**

E. QC Mid PTX_C13 in Human Protein-Free Plasma

QC Mid PTX_C13	Theoretical (ng/mL)	Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	5.00	5.49	10%	
Study Day 1	5.00	5.39	8%	
Study Day 1	5.00	5.04	1%	
Study Day 1	5.00	5.17	3%	
Study Day 1	5.00	5.46	9%	
Study Day 1	5.00	5.19	4%	
AVG		**5.29**	**6%**	**3%**
Study Day 2	5.00	4.75	-5%	
Study Day 2	5.00	4.93	-1%	
Study Day 2	5.00	4.93	-1%	
Study Day 2	5.00	5.37	7%	
Study Day 2	5.00	5.56	11%	
Study Day 2	5.00	5.52	10%	
AVG		**5.18**	**4%**	**7%**
Study Day 3	5.00	5.30	6%	
Study Day 3	5.00	5.42	8%	
Study Day 3	5.00	5.09	2%	
Study Day 3	5.00	5.48	10%	
Study Day 3	5.00	4.97	-1%	
Study Day 3	5.00	4.88	-2%	
AVG		**5.19**	**4%**	**5%**

F. QC High PTX_C13 in Human Protein-Free Plasma

QC High PTX_C13	Theoretical (ng/mL)	Concentration (ng/mL)	Accuracy Deviation (%)	Precision (%)
Study Day 1	50.0	48.0	-4%	
Study Day 1	50.0	51.8	4%	
Study Day 1	50.0	51.4	3%	
Study Day 1	50.0	50.2	0.4%	
Study Day 1	50.0	51.7	3%	
Study Day 1	50.0	52.6	5%	
AVG		**51.0**	**2%**	**3%**
Study Day 2	50.0	51.6	3%	
Study Day 2	50.0	53.4	7%	
Study Day 2	50.0	48.2	-4%	
Study Day 2	50.0	50.2	0.3%	
Study Day 2	50.0	52.5	5%	
Study Day 2	50.0	52.9	6%	
AVG		**51.5**	**3%**	**4%**
Study Day 3	50.0	51.4	3%	
Study Day 3	50.0	53.8	8%	
Study Day 3	50.0	51.9	4%	
Study Day 3	50.0	51.0	2%	
Study Day 3	50.0	51.0	2%	
Study Day 3	50.0	50.2	0.3%	
AVG		**51.5**	**3%**	**2%**

Appendix C. Abraxane Release in 4.5% Human Serum Albumin

Summary

Consistent with the visual and DLS analysis of the free paclitaxel and Abraxane 4.5% HSA solutions, precipitated and stable Abraxane nanoparticle fractions were identified as encapsulated fractions by the SITUA method. The 100 and 500 µg/mL concentrations of Abraxane in 4.5% HSA contained stable Abraxane nanoparticles, as determined by DLS and visual inspection, and these fractions were identified as encapsulated fractions by the stable isotope method (**Table C-13 and 16; Figure C-15**). Similarly, concentrations of Abraxane below 100 µg/mL, at which the stable Abraxane nanoparticle was not observed, were determined to be 100% released with no encapsulated drug fraction (**Table C-13 and 16; Figure C-15**). Free PTX at 100 and 500 µg/mL concentrations in 4.5% HSA, which were found by visual inspection and DLS analysis to be precipitated, were also determined to have an "encapsulated" drug fraction, consistent with SITUA assay theory (**Table C-11 and 15; Figure C-14**). Stable isotope tracer addition in acetonitrile did not change normoisotopic PTX % protein binding, supporting the lack of effect of solvent addition on PTX solubility and Abraxane stability (**Table C-11-14, 17; Figure C-16**). However, Abraxane at high concentrations in 4.5% HSA increases stable isotope tracer protein binding, either due to tracer binding to Abraxane albumin or Abraxane nanoparticle (**Table C-18; Figure C-17**). These data strongly support the validity of the stable isotope method to distinguish stable Abraxane nanoparticles/precipitated drug from free drug in equilibrium with protein.

Design and Methods

Drug Release in 4.5% Human Serum Albumin

Albutein human serum albumin (HSA) 25% solution was diluted to 4.5% in 0.9% saline. In glass vials, 4.5% HSA was spiked with samples (free drug or Abraxane) at final drug concentrations of 10 µg/mL, 50 µg/mL, 100 µg/mL, and 500 µg/mL PTX in triplicate. Set 1 of samples were spiked with 0.1 µg/mL PTX_C13 (stable Isotope tracer) in acetonitrile and set 2 of samples was not spiked with PTX_C13.

Samples were then incubated at 26°C with agitation. At time points 10 min, 30 min, and 2 hr, 50 µL of 4.5% HSA sample was taken for protein precipitation as described below; this sample was used to determine total drug concentration. Additionally, 400 µL of 4.5% HSA sample was transferred to a prewarmed ultrafiltration tube (10 kDa Vivacon) and spun at 6000x*g* for 10 min at 26°C. 50 µL of this filtrate sample was taken for protein precipitation; this sample was used to determine free/unbound drug concentration.

Protein Precipitation Method

The two 50 µL samples from above (non-centrifuged and centrifuged) were added to 200 µL ice cold acetonitrile (ACN) with 0.1% formic acid (FA) containing 25 ng/mL PTX_d5 as an internal standard (ISTD). The samples were frozen at -80°C for 10 min, thawed at room temperature, then spun at 4°C, 18,000x*g* for 20 min. The supernatant was transferred to a clean Eppendorf tube and dried using a centrifuge speed vacuum for 25 min at 50°C and 5 Torr. Following protein precipitation, samples were reconstituted in 150 µL 40% ACN with 0.1% FA. The reconstituted sample was then transferred to a clean Eppendorf tube and spun at 18,000xg at 4°C for 5 min, then transferred to an HPLC vial. Samples were analyzed by the Q-Orbitrap as described in the LC-Orbitrap Method section, using matrix matched 4.5% HSA and filtered 4.5% HSA standard curves.

Dynamic Light Scattering, DLS
Samples prepared from the above drug release in 4.5% HSA section were analyzed by DLS. Abraxane samples were analyzed at time points, time zero, 30 min, and 2 hr. Paclitaxel samples were analyzed at time zero. Abraxane and paclitaxel were also prepared in 0.9% saline and analyzed at time zero.

Results and Discussion

Visual inspection and DLS analysis of the 100 and 500 µg/mL concentrations of free PTX in 4.5% HSA, with and without PTX_C13 tracer addition, determined that these solutions had precipitated immediately (**Table C-6; Figure C-1, C-9**); the 100 and 500 µg/mL concentrations of free PTX in 4.5% HSA appear opaque, and the DLS analysis shows micron size particle populations typical of precipitated drug. By contrast, although the 500 µg/mL concentrations of Abraxane in 4.5% HSA, appear opaque, the DLS analysis of these shows a uniform particle size of ~150 nm over the 2 hr period, similar to the appearance and DLS analysis of 0.5-5 µg/mL Abraxane in saline (**Table C-1-2, Figure C-2-3,4-5**). At concentrations below 100 µg/mL Abraxane in 4.5 % HSA, the solutions were clear, and the Abraxane nanoparticle dissociated immediately into a DLS population of 7 nm (**Table C-3-5, Figure C-2, 6-8**). These data suggest that the Abraxane nanoparticle is stable in HSA at 100 and 500 µg/mL, but not at concentrations below 100 µg/mL.

Consistent with visual and DLS analysis of the free paclitaxel and Abraxane 4.5% HSA solutions, precipitated stable Abraxane nanoparticle fractions were identified as "encapsulated" fractions by the stable isotope method. The PTX and PTX_C13, 4.5% HSA and filtered 4.5% HSA standard curves used for SITUA analysis are displayed in **Tables C-7-10 and Figures C-10-14**. The 100 and 500 µg/mL concentrations of Abraxane in 4.5% HSA contained stable Abraxane nanoparticles, as determined by DLS and visual inspection, and these fractions were identified as encapsulated fractions by the stable isotope method (**Table C-13 and 16; Figure C-15**). Similarly, concentrations of Abraxane below 100 µg/mL, at which the stable Abraxane nanoparticle was not observed, were determined to be 100% released with no encapsulated drug fraction (**Table C-13 and 16; Figure C-15**). Free PTX at 100 and 500 µg/mL concentrations in 4.5% HSA, which were found by visual inspection and DLS analysis to be precipitated, were also determined to have an "encapsulated" drug fraction, consistent with SITUA assay theory (**Table C-11 and 15; Figure C-14**). Stable isotope tracer addition in acetonitrile did not change normoisotopic PTX % protein binding, supporting the lack of effect of solvent addition on PTX solubility and Abraxane stability (**Table C-11-14, 17; Figure C-16**). However, Abraxane at high concentrations in 4.5% HSA increases stable isotope tracer protein binding, either due to tracer binding to Abraxane albumin or Abraxane nanoparticle (**Table C-18; Figure C-17**). These data strongly support the validity of the stable isotope method to distinguish stable Abraxane nanoparticles/precipitated drug from free drug in equilibrium with protein.
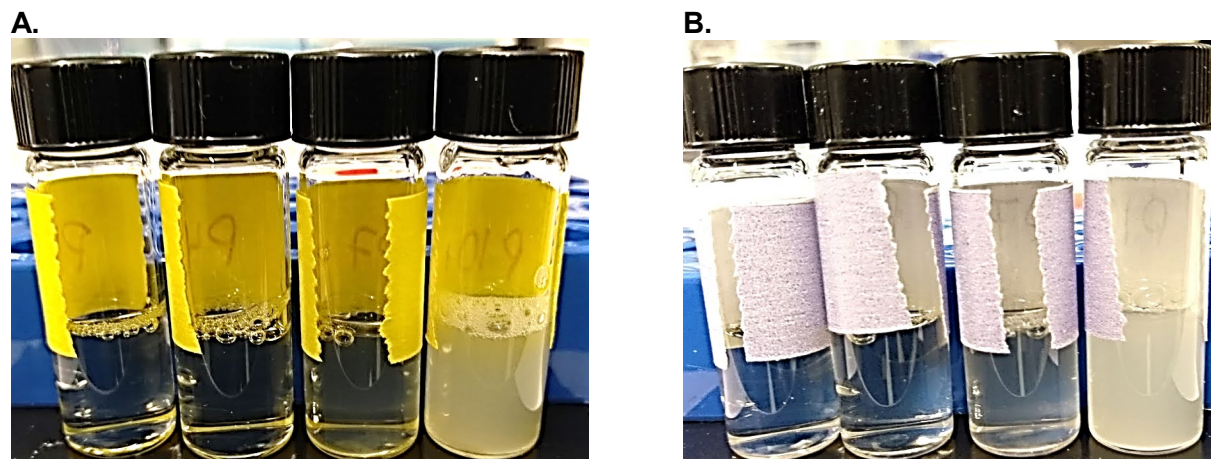
A.

B.

Figure C-1. Comparison of Turbidity of Paclitaxel in 4.5% HSA with and without PTX_C13 Tracer. Displayed are the paclitaxel free controls in 4.5% HSA at 10 µg/mL, 50 µg/mL, 100 µg/mL, and 500 µg/mL (left to right) with PTX_C13 tracer **(A)** and 10 µg/mL, 50 µg/mL, 100 µg/mL, and 500 µg/mL (left to right) without tracer **(B)**.
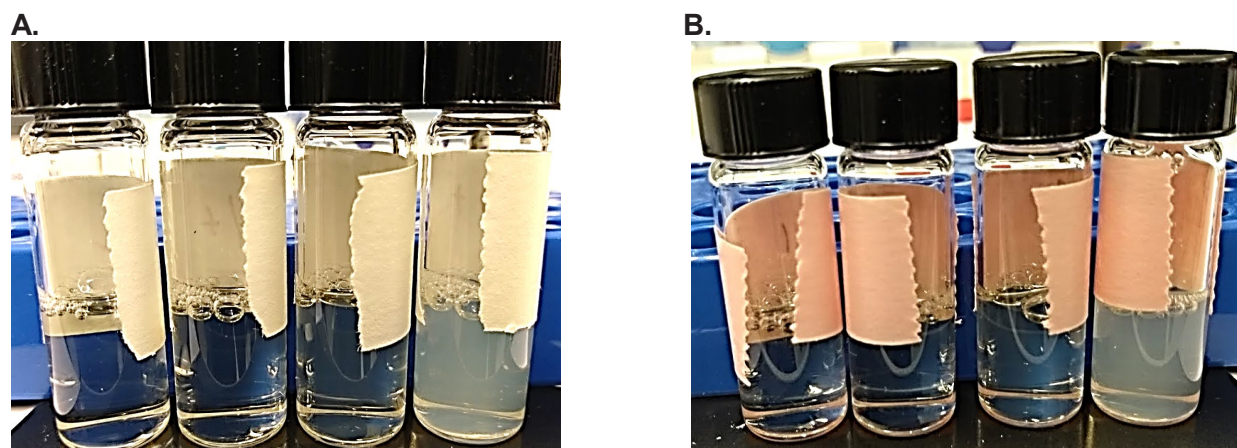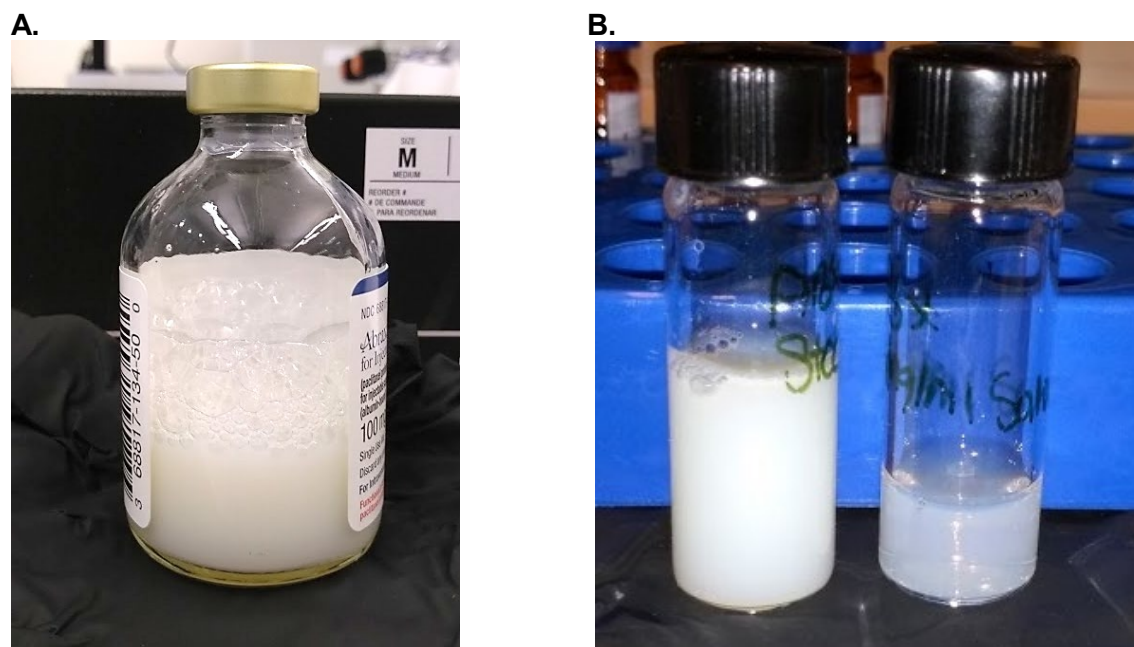
A.

B.

Figure C-2. Comparison of Turbidity of Abraxane Lot 6115306 in 4.5% HSA with and without PTX_C13 Tracer. Displayed are the Abraxane Lot 6115306 in 4.5% HSA at 10 µg/mL, 50 µg/mL, 100 µg/mL, and 500 µg/mL (left to right) with PTX_C13 tracer **(A)** and 10 µg/mL, 50 µg/mL, 100 µg/mL, and 500 µg/mL (left to right) without tracer **(B)**.

A.

B.

Figure C-3. Abraxane Lot 6115306 stock reconstitute in 0.9% Saline Comparison.
Displayed are the Abraxane Lot 6115306 stock reconstitute in 0.9% saline at 5 mg/mL **(A)**, and
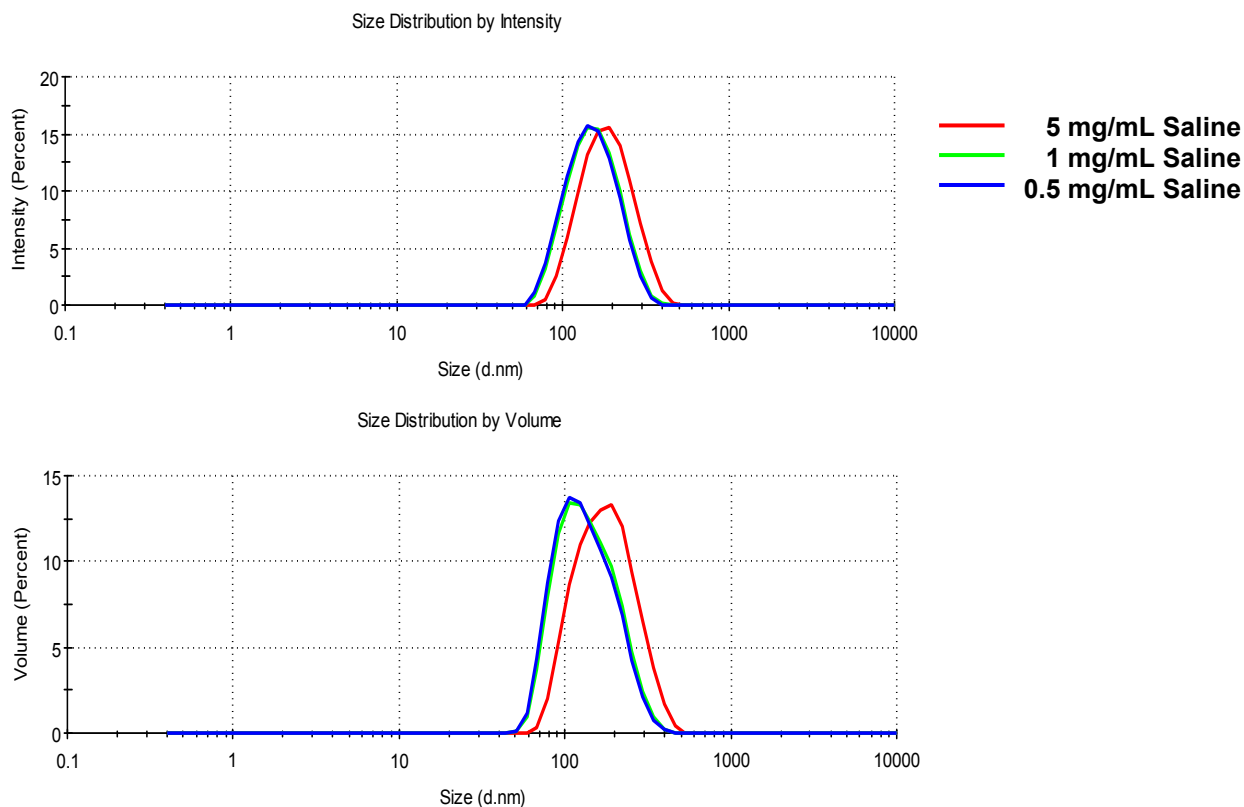5 mg/mL and 1 mg/mL (left to right) **(B)**.

Figure C-4. Hydrodynamic Size of Abraxane Lot 6115306 in Saline. Displayed are the size distribution by intensity and volume of Abraxane lot 6115306 in 0.9% saline at 0.5 mg/mL, 1 mg/mL, and 5 mg/mL. (*N=10*)

Table C-1. Hydrodynamic Size of Abraxane Lot 6115306 in Saline. Displayed are the are the calculated values for hydrodynamic size of Abraxane lot 6115306 in 0.9% saline at 0.5 mg/mL, 1 mg/mL, and 5 mg/mL. (*N=10*)

Abraxane	Z-Avg, nm	PdI	Int-Peak, nm	%Int	Vol-Peak, nm	%Vol
5 mg/mL Saline	170 (1)	0.116 (0.009)	193 (4)	100 (0)	185 (2)	100 (0)
1 mg/mL Saline	142 (1)	0.110 (002)	161 (3)	100 (0)	145 (2)	100 (0)
0.5 mg/mL Saline	139 (1)	0.11 (0.01)	157 (2)	100 (0)	140 (2)	100 (0)

Note: Results are the average of at least 10 measurements. Sizes are shown as diameter in nm (d.nm). Standard deviations are shown in parentheses. Z-Avg is the intensity-weighted average. PdI is the polydispersity index. Int-Peak is the intensity-weighted average over the primary peak. % Int is the percentage of the intensity spectra occupied by the primary peak. Vol-Peak is the volume-weighted average over the primary peak. % Vol is the percentage of the volume spectra occupied by the primary peak.
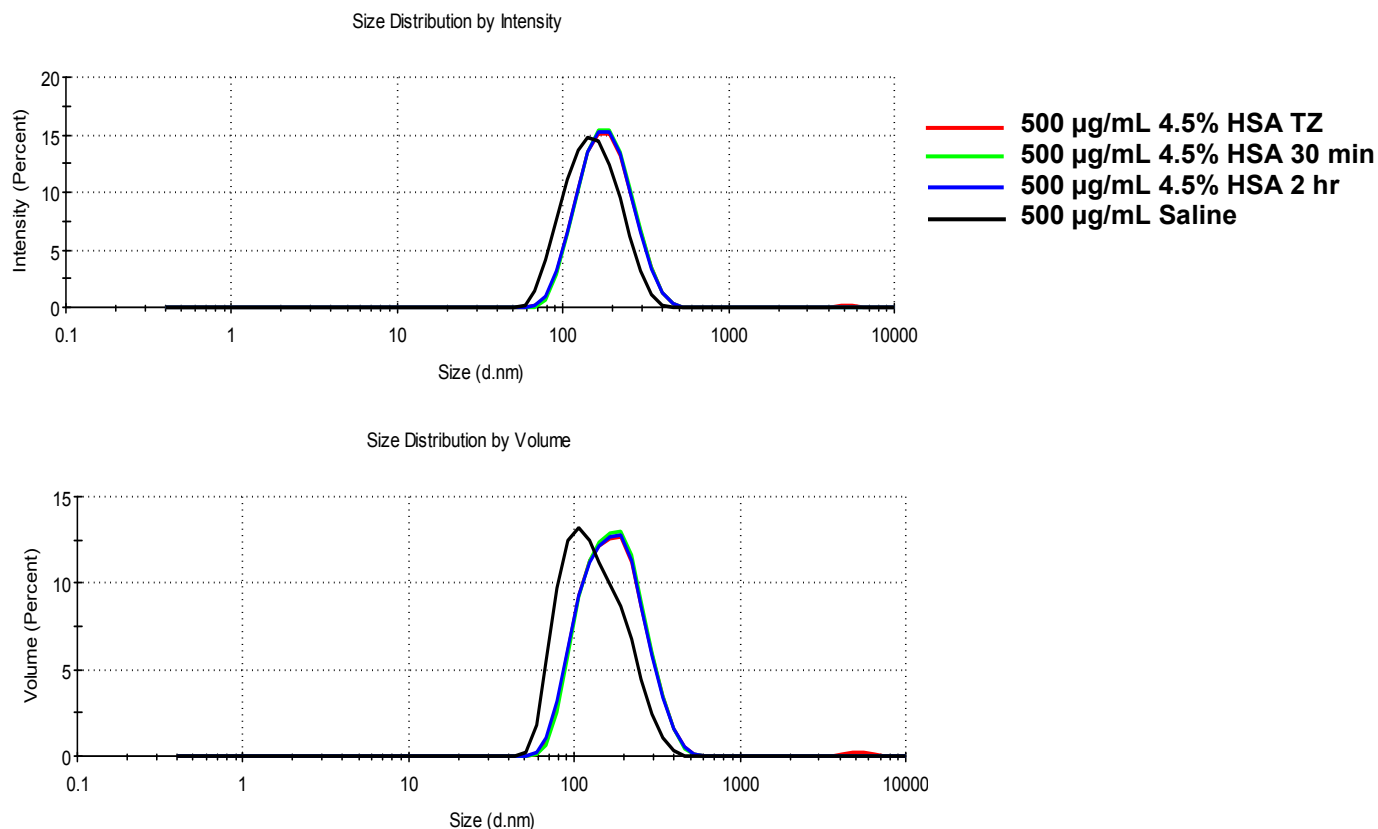
Size Distribution by Intensity

Size Distribution by Volume

Figure C-5. Hydrodynamic Size of Abraxane Lot 6115306 at 500 µg/mL. Displayed are the size distribution by intensity and volume of Abraxane lot 6115306 at 500 µg/mL in 4.5% HSA at time zero, 30 min, and 2 hr and in saline at time zero. (*N=5 for HSA samples and N=3 for saline samples*).

Table C-2. Hydrodynamic Size of Abraxane Lot 6115306 at 500 µg/mL. Displayed are the calculated values for hydrodynamic size of Abraxane lot 6115306 at 500 µg/mL in 4.5% HSA at time zero, 30 min, and 2 hr and in 0.9% saline at time zero. (*N=5 for HSA samples and N=3 for saline samples*).

500 µg/mL Abraxane	Z-Avg, nm	PdI	Int-Peak, nm	%Int	Vol-Peak, nm	%Vol
4.5% HSA TZ	159.5 (0.8)	0.19 (0.02)	190 (4)	100 (1)	181 (4)	99 (2)
4.5% HSA 30 min	158.8 (0.9)	0.19 (0.01)	190 (4)	100 (0)	182 (2)	100 (0)
4.5% HSA 2 Hour	158.2 (0.5)	0.18 (0.02)	189 (5)	100 (0)	180 (2)	100 (0)
Saline	139.2 (0.5)	0.12 (0.01)	159 (3)	100 (0)	139.7 (0.6)	100 (0)

Size Distribution by Intensity
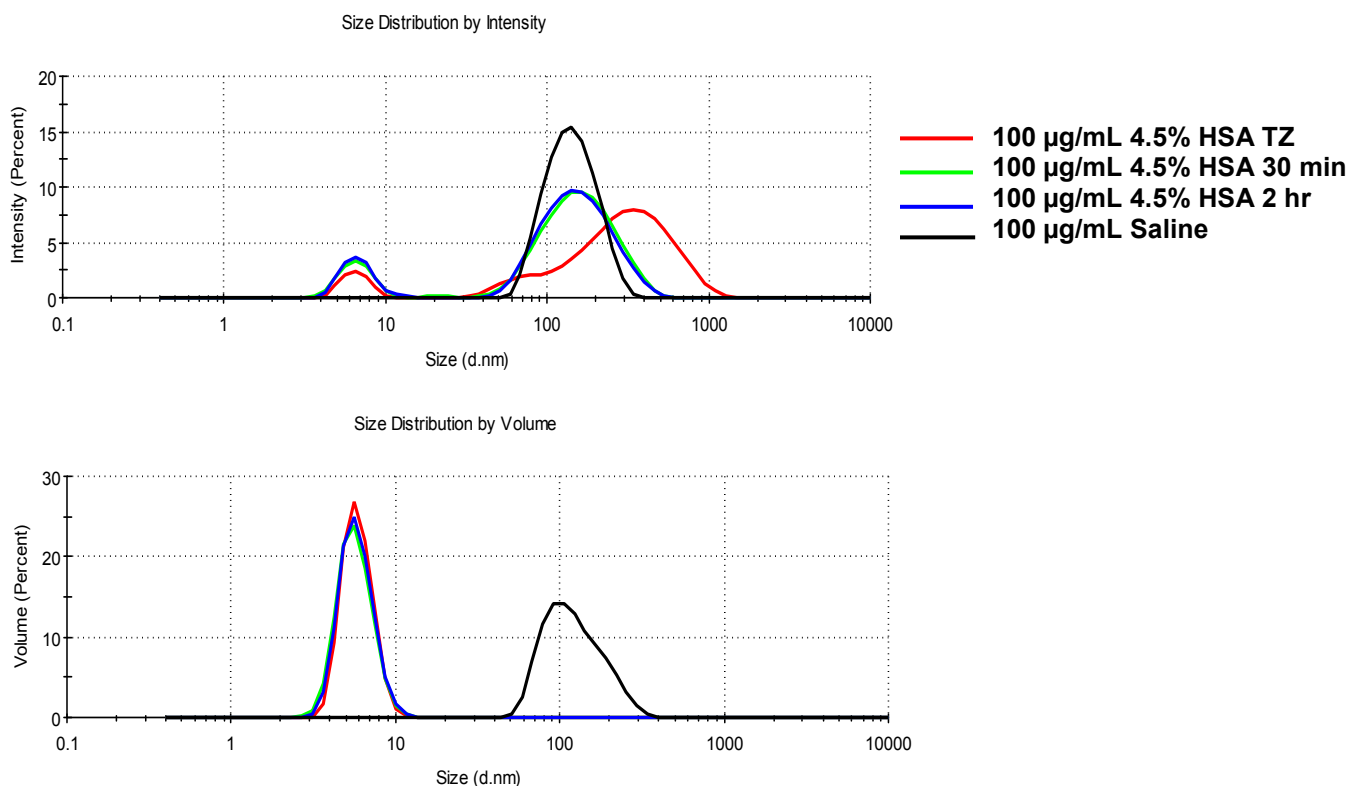

Size Distribution by Volume

Figure C-6. Hydrodynamic Size of Abraxane Lot 6115306 at 100 µg/mL. Displayed are the size distribution by intensity and volume of Abraxane lot 6115306 at 100 µg/mL in 4.5% HSA at time zero, 30 min, and 2 hr and in saline at time zero. (*N=5 for HSA samples and N=3 for saline samples*).

Table C-3. Hydrodynamic Size of Abraxane Lot 6115306 at 100 µg/mL. Displayed are the are the calculated values for hydrodynamic size of Abraxane lot 6115306 at 100 µg/mL in 4.5% HSA at time zero, 30 min, and 2 hr and in 0.9% saline at time zero. (*N=5 for HSA samples and N=3 for saline samples*).

100 µg/mL Abraxane	Z-Avg, nm	PdI	Peak 1 Int-Peak, nm	Peak 1 %Int	Peak 2 Int-Peak, nm	Peak 2 %Int	Vol-Peak, nm	%Vol
4.5% HSA TZ	144 (4)	0.68 (0.05)	353 (12)	87 (6)	6.6 (0.1)	8.8 (0.3)	5.9 (0.1)	100 (0)
4.5% HSA 30 min	68.7 (0.6)	0.744 (0.004)	172 (3)	85 (1)	6.7 (0.2)	14.2 (0.5)	5.8 (0.3)	100 (0)
4.5% HSA 2 Hour	66.2 (0.2)	0.733 (0.003)	168 (7)	84.6 (0.4)	7.0 (0.5)	15.4 (0.4)	5.9 (0.2)	100 (0)
Saline	129.8 (0.8)	0.12 (0.01)	148 (2)	100 (0)	--	--	129 (2)	100 (0)

Size Distribution by Intensity
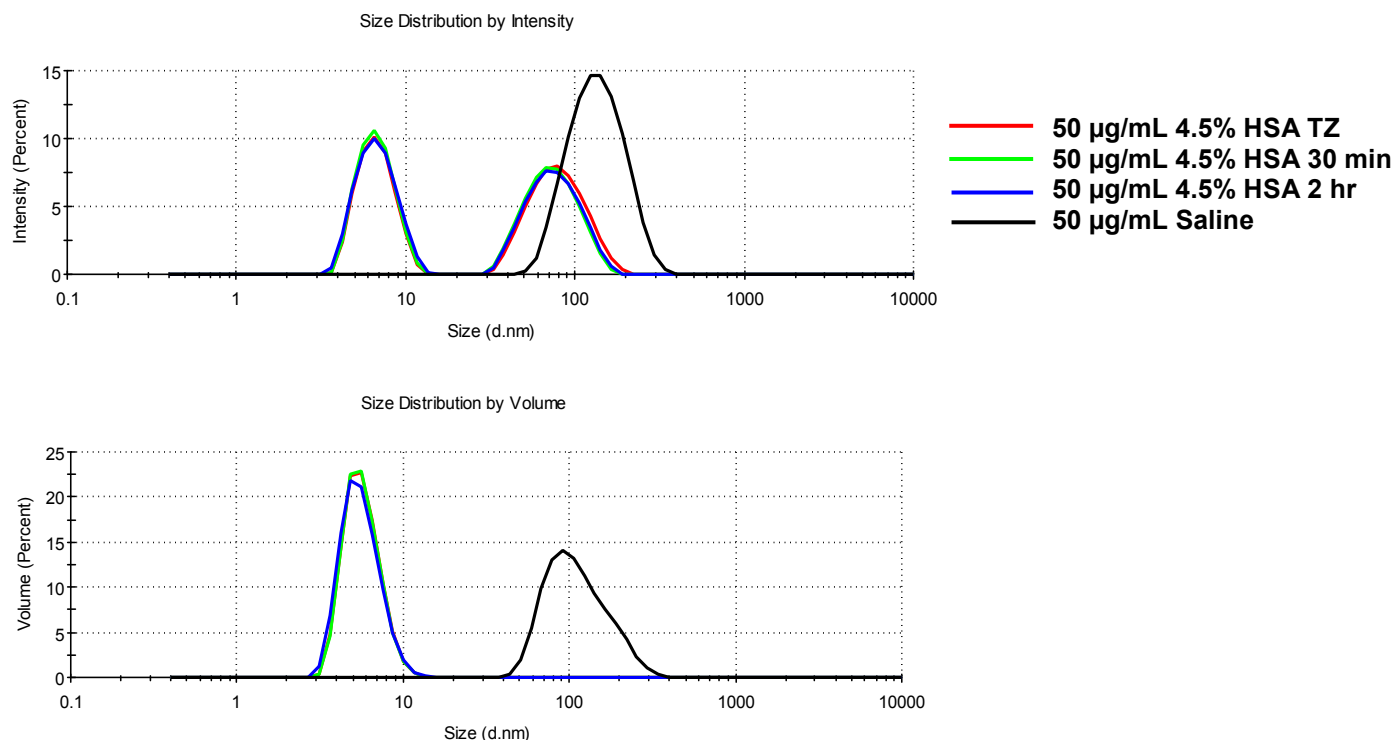
Size Distribution by Volume

Figure C-7. Hydrodynamic Size of Abraxane Lot 6115306 at 50 µg/mL. Displayed are the size distribution by intensity and volume of Abraxane lot 6115306 at 50 µg/mL in 4.5% HSA at time zero, 30 min, and 2 hr and in saline at time zero. (*N=5 for HSA samples and N=3 for saline samples*).

Table C-4. Hydrodynamic Size of Abraxane Lot 6115306 at 50 µg/mL. Displayed are the are the calculated values for hydrodynamic size of Abraxane lot 6115306 at 50 µg/mL in 4.5% HSA at time zero, 30 min, and 2 hr and in 0.9% saline at time zero. (*N=5 for HSA samples and N=3 for saline samples*).

50 µg/mL Abraxane	Z-Avg, nm	PdI	Peak 1 Int-Peak, nm	Peak 1 %Int	Peak 2 Int-Peak, nm	Peak 2 %Int	Vol-Peak, nm	%Vol
4.5% HSA TZ	14.5 (0.6)	0.56 (0.03)	83 (5)	54 (2)	6.8 (0.1)	46 (2)	5.8 (0.1)	100 (0)
4.5% HSA 30 min	13.5 (0.04)	0.519 (0.002)	76 (1)	51.2 (0.4)	6.8 (0.1)	48.8 (0.4)	5.8 (0.1)	100 (0)
4.5% HSA 2 Hour	13.5 (0.04)	0.520 (0.001)	78 (1)	50.8 (0.4)	6.9 (0.1)	49.2 (0.4)	5.7 (0.1)	100 (0)
Saline	121.6 (0.6)	0.14 (0.02)	142 (3)	100 (0)	--	--	119 (3)	100 (0)

Size Distribution by Intensity
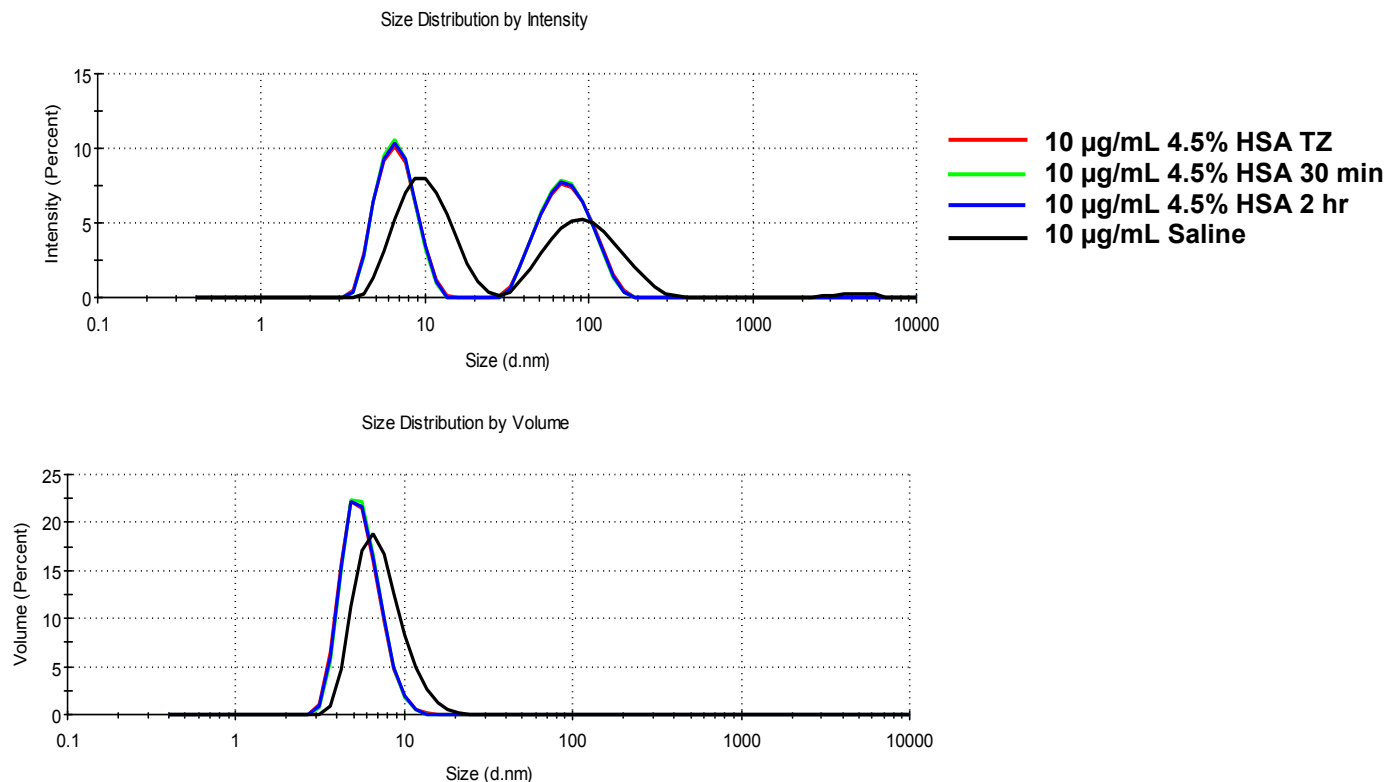

Size Distribution by Volume

Figure C-8. Hydrodynamic Size of Abraxane Lot 6115306 at 10 µg/mL. Displayed are the size distribution by intensity and volume of Abraxane lot 6115306 at 10 µg/mL in 4.5% HSA at time zero, 30 min, and 2 hr and in saline at time zero. (*N=5 for HSA samples and N=3 for saline samples*).

Table C-5. Hydrodynamic Size of Abraxane Lot 6115306 at 10 µg/mL. Displayed are the are the calculated values for hydrodynamic size of Abraxane lot 6115306 at 10 µg/mL in 4.5% HSA at time zero, 30 min, and 2 hr and in 0.9% saline at time zero. (*N=5 for HSA samples and N=3 for saline samples*).

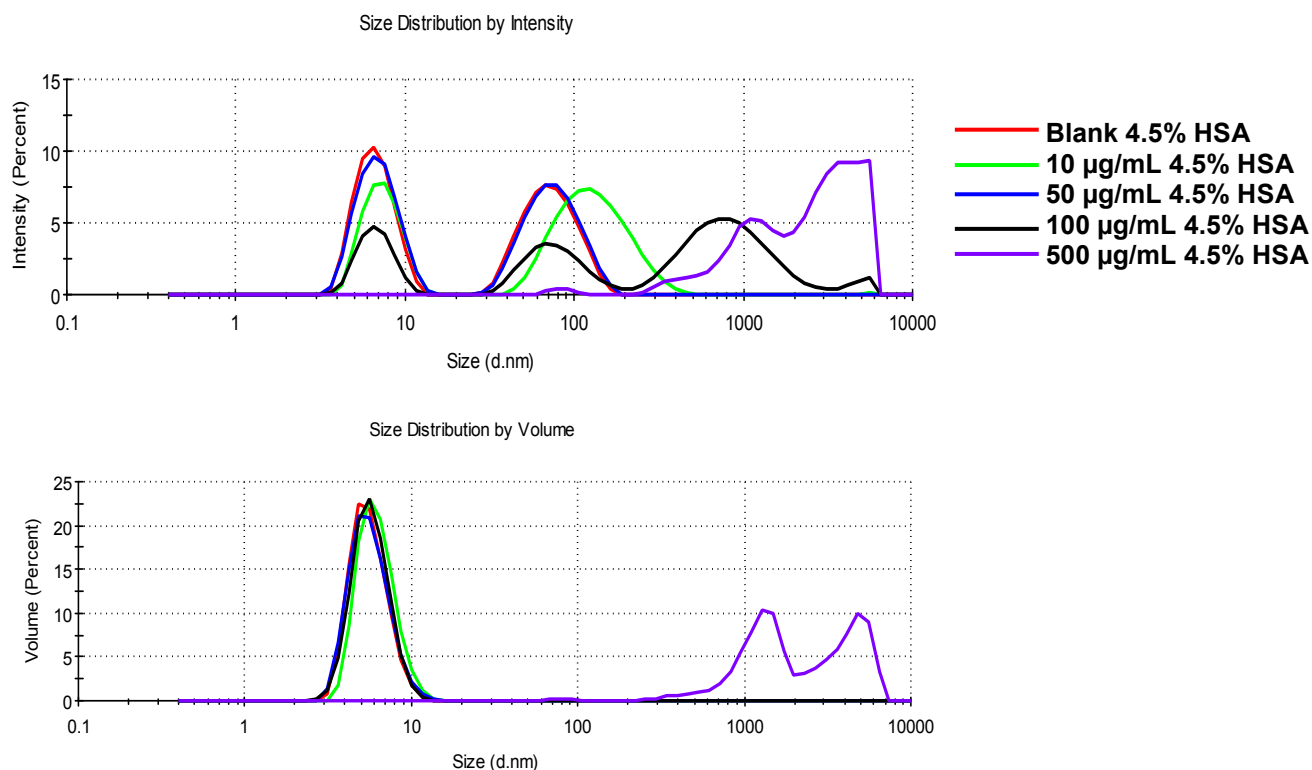| 10 µg/mL Abraxane	Z-Avg, nm	PdI	Peak 1 Int-Peak, nm	Peak 1 %Int	Peak 2 Int-Peak, nm	Peak 2 %Int	Vol-Peak, nm	%Vol
4.5% HSA TZ	14.5 (0.6)	0.558 (0.03)	83 (5)	54 (2)	6.8 (0.1)	46 (2)	5.8 (0.1)	100 (0)
4.5% HSA 30 min	13.5 (0.04)	0.519 (0.002)	76 (1)	51.2 (0.4)	6.8 (0.1)	48.8 (0.4)	5.8 (0.1)	100 (0)
4.5% HSA 2 Hour	13.5 (0.04)	0.520 (0.001)	78 (1)	50.8 (0.4)	6.9 (0.1)	49.2 (0.4)	5.7 (0.1)	100 (0)
Saline	121.6 (0.6)	0.14 (0.02)	142 (3)	100 (0)	--	--	119 (3)	100 (0)

Figure C-9. Hydrodynamic Size of Paclitaxel in 4.5% HSA. Displayed are the size distribution by intensity and volume of paclitaxel at 10 μg/mL, 50 μg/mL, 100 μg/mL, and 500 μg/mL in 4.5% HSA at time zero and blank 4.5% HSA. (*N=5*).

Table C-6. Hydrodynamic Size of Paclitaxel in 4.5% HSA. Displayed are the are the calculated values for hydrodynamic size of paclitaxel at 10 μg/mL, 50 μg/mL, 100 μg/mL, and 500 μg/mL in 4.5% HSA at time zero and blank 4.5% HSA. (*N=5*).

Paclitaxel	Z-Avg, nm	PdI	Peak 1 Int-Peak, nm	Peak 1 %Int	Peak 2 Int-Peak, nm	Peak 2 %Int	Vol-Peak, nm	%Vol
Blank 4.5% HSA	13.4 (0.1)	0.516 (0.003)	75 (1)	51.2 (0.4)	6.7 (0.04)	48.8 (0.4)	5.7 (0.1)	100 (0)
10 μg/mL PTX 4.5% HSA	21.1 (0.3)	0.80 (0.01)	139 (4)	62.9 (0.5)	7.4 (0.1)	36.9 (0.5)	6.3 (0.1)	100 (0)
50 μg/mL PTX 4.5% HSA	13.9 (0.1)	0.525 (0.002)	78 (2)	51.4 (0.6)	7.0 (0.1)	48.6 (0.6)	5.7 (0.2)	100 (0)
100 μg/mL PTX 4.5% HSA	66 (6)	0.99 (0.01)	899. (187)	52 (2)	80 (7)	24 (2)	5.8 (0.3)	100 (0)
500 μg/mL PTX 4.5% HSA	1695 (85)	0.6 (0.1)	3146. (1083)	77 (11)	1659 (2098)	22 (12)	687 (518)	29 (31)

Table C-7. PTX 4.5% HSA Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
1.00 µg/mL	15713278	1.05	105
1.00 µg/mL	30203495	0.92	92
5.00 µg/mL	127928848	5.06	101
5.00 µg/mL	107414064	5.56	111
10.0 µg/mL*	132056812	19.2	192
10.0 µg/mL	144305288	10.3	103
50.0 µg/mL	936207749	56.7	113
50.0 µg/mL	942555260	55.1	110
100.0 µg/mL	1847809224	106.4	106
100.0 µg/mL	1857814792	103.1	103
250.0 µg/mL	3696498967	222.0	89
250.0 µg/mL	2794144981	219.7	88
500.0 µg/mL	3405208716	439.4	88
500.0 µg/mL	3672290239	450.8	90
QC			
QCL	38835217	0.98	98
QCL	29550708	0.93	93
QCM	932317683	54.7	109
QCM	1145471406	53.2	106
QCH	3788334518	429.6	86
QCH	3624048578	481.1	96

*Point not included in Standard Curve

PTX
Y = 0.667254+7.64381*X   R^2 = 0.9893   W: 1/X^2
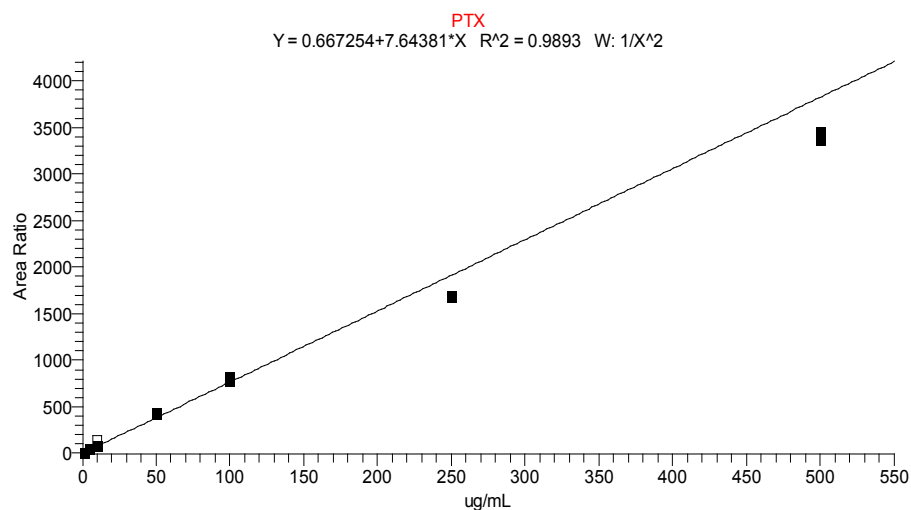
Figure C-10. PTX 4.5% HSA Standard Curve.

Table C-8. PTX_C13 4.5% HSA Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
10.0 ng/mL	139585	9.6	96
10.0 ng/mL	334528	10.5	105
50.0 ng/mL	1434554	50.0	100
50.0 ng/mL	1102286	50.2	100
100.0 ng/mL*	1313383	164.3	164
100.0 ng/mL	1541625	95.3	95
500.0 ng/mL	9349699	483.3	97
500.0 ng/mL	9766234	486.5	97
1000 ng/mL	21794236	1068	107
1000 ng/mL	21932546	1036	104
2500 ng/mL	46260972	2362	94
2500 ng/mL	38022291	2543	102
5000 ng/mL	47996567	5265	105
5000 ng/mL	46912405	4896	98
QC			
QCL	433166	11.1	111
QCL	320972	10.4	104
QCM	11098589	555.5	111
QCM	13437896	532.6	107
QCH	49958367	4816	96
QCH	41462291	4679	94

*Point not included in Standard Curve

PTX_C13
Y = -0.00893839+8.99579*X   R^2 = 0.9979   W: 1/X^2

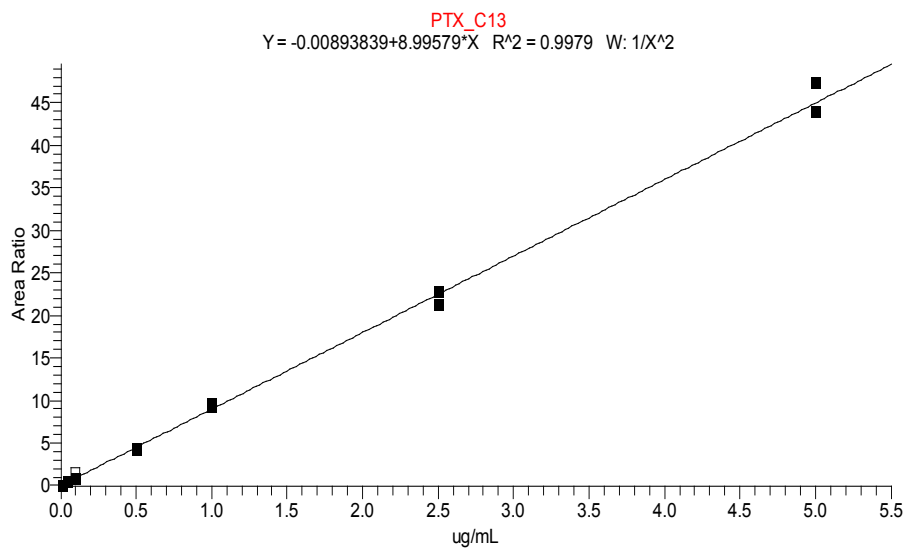**Figure C-11. PTX_C13 4.5% HSA Standard Curve.**

Table C-9. PTX 4.5% HSA Filtered Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
100.0 ng/mL	26875050	94.1	94
100.0 ng/mL	23583686	96.2	96
250.0 ng/mL	60321602	275.5	110
250.0 ng/mL	47541261	270.9	108
500.0 ng/mL	85653538	540.0	108
500.0 ng/mL	77606407	525.4	105
1000 ng/mL	144133356	996	100
1000 ng/mL	115657793	968	97
5000 ng/mL	628603910	5197	104
5000 ng/mL	571200443	4959	99
10000 ng/mL	1002800942	10098	101
10000 ng/mL	952918116	9603	96
25000 ng/mL	1634630841	23456	94
25000 ng/mL	1684737571	21929	88
QC			
QCL	10263580	96.0	96
QCL	9905094	97.6	98
QCM	95331981	927	93
QCM	95464415	917	92
QCH	1707033839	23040	92
QCH	1617233372	22926	92

PTX
$Y = 0.0056099 + 0.00798951 \times X$ $R^2 = 0.9940$ W: $1/X^2$
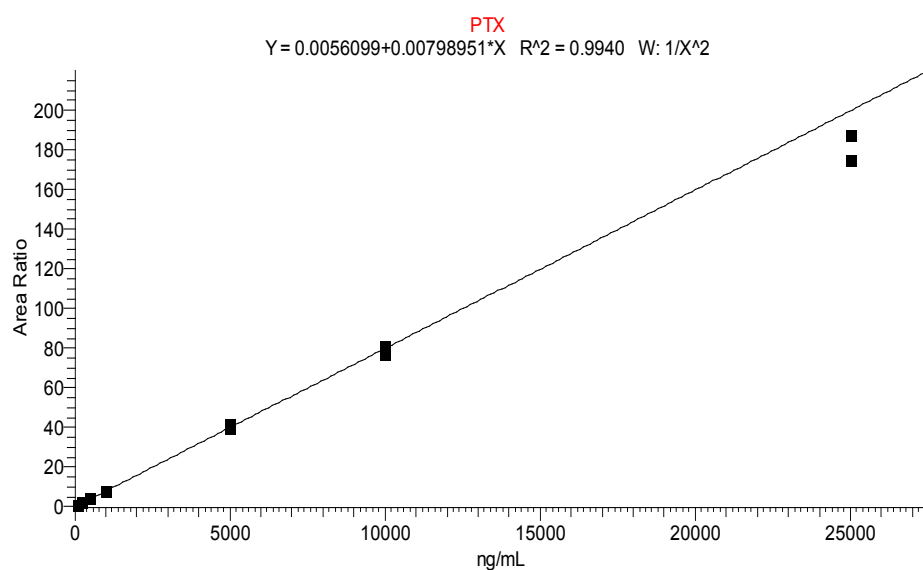
Figure C-12. PTX 4.5% HSA Filtered Standard Curve.

Table C-10. PTX_C13 4.5% HSA Filtered Standard Curve.

	Area	Conc. (ng/mL)	% Accuracy
1.00 ng/mL	259984	0.95	95
1.00 ng/mL	249818	1.04	104
2.50 ng/mL	622340	2.54	102
2.50 ng/mL	515078	2.62	105
5.00 ng/mL	886139	4.81	96
5.00 ng/mL	874372	5.09	102
10.0 ng/mL	1699501	9.9	99
10.0 ng/mL	1337787	9.5	95
50.0 ng/mL	7930946	54.4	109
50.0 ng/mL	6965049	50.2	100
100.0 ng/mL	11265716	94.0	94
100.0 ng/mL	11191021	93.4	93
250.0 ng/mL	23819367	282.8	113
250.0 ng/mL	21791888	234.7	94
QC			
QCL	89287	0.88	88
QCL*	72938	0.79	79
QCM	1173990	9.6	96
QCM	1186310	9.6	96
QCH	20289544	226.6	91
QCH	19766508	231.9	93

*Point not included in Standard Curve

PTX_C13
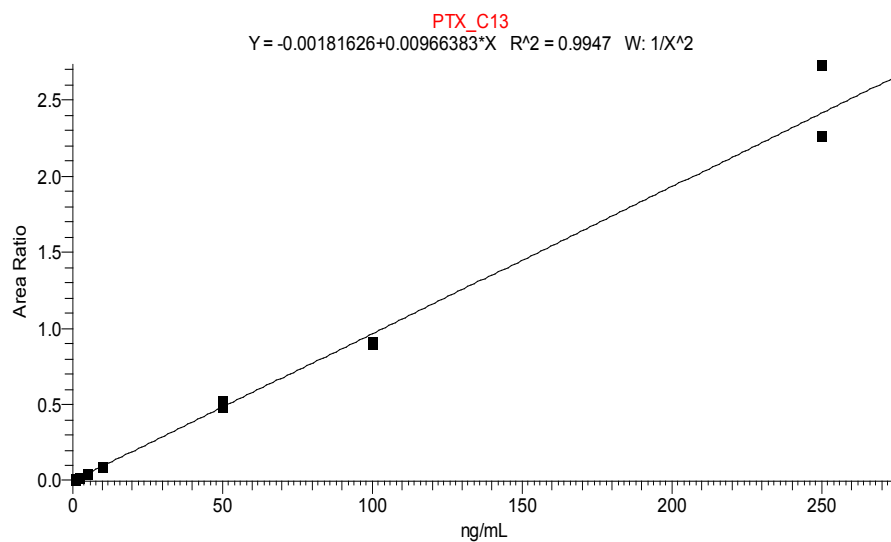$Y = -0.00181626+0.00966383*X$ $R^2 = 0.9947$ W: $1/X^2$

Figure C-13. PTX_C13 4.5% HSA Filtered Standard Curve.

Table C-11. Free Paclitaxel Control Analytical Data. Presented are the analytical data for the free paclitaxel controls. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX =Filtrate PTX/Reservoir PTX *100	% Bound =100-%Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 STD (ng/mL)	%Unbound PTX_C13 =Filtrate PTX C13/Reservoir PTX C13 * 100	% Bound PTX_C13 =100 - %Unbound PTX C13	Unencapsulated (ng/mL) =Filtrate PTX/ (1-(%Bound PTX C13/100))	Encapsulated (ng/mL) =Reservoir PTX- Unencapsulated	% Release =(Unencapsulated/ Reservoir PTX) *100	AVG % Release	SD
10 min	10 µg/mL	3408	9905	34.4	65.6	36.0	109	32.9	67.1	10359	-454	104.6	102.8	1.7
		3398	10328	32.9	67.1	34.9	109	32.1	67.9	10590	-262	102.5		
		3296	9255	35.6	64.4	37.2	106	35.2	64.8	9375	-119	101.3		
	50 µg/mL	14886	43193	34.5	65.5	33.7	97	34.9	65.1	42679	514	98.8	95.7	3.5
		15680	46191	33.9	66.1	37.6	102	36.9	63.1	42446	3745	91.9		
		15060	46863	32.1	67.9	33.9	102	33.4	66.6	45149	1714	96.3		
	100 µg/mL	23687	99398	23.8	76.2	30.8	99	31.2	68.8	76035	23363	76.5	79.6	9.2
		22553	100719	22.4	77.6	33.0	107	31.0	69.0	72869	27850	72.3		
		23251	85583	27.2	72.8	32.2	106	30.2	69.8	76952	8631	89.9		
	500 µg/mL	20774	432009	4.8	95.2	20.0	99	20.2	79.8	102765	329244	23.8	30.0	6.0
		23243	364176	6.4	93.6	17.9	100	17.9	82.1	129994	234181	35.7		
		23642	409335	5.8	94.2	19.3	102	19.0	81.0	124623	284712	30.4		
30 min	10 µg/mL	3140	10297	30.5	69.5	32.0	113	28.4	71.6	11055	-758	107.4	101.6	7.3
		3974	10534	37.7	62.3	39.4	109	36.3	63.7	10953	-420	104.0		
		3397	10806	31.4	68.6	35.8	106	33.7	66.3	10092	715	93.4		
	50 µg/mL	15720	52993	29.7	70.3	34.0	104	32.9	67.1	47814	5178	90.2	89.6	6.7
		14730	54387	27.1	72.9	34.9	107	32.8	67.2	44923	9464	82.6		
		16154	52534	30.8	69.2	34.2	107	32.0	68.0	50405	2129	95.9		
	100 µg/mL	23716	93451	25.4	74.6	33.8	103	32.9	67.1	72076	21375	77.1	80.2	6.1
		23028	90342	25.5	74.5	31.1	106	29.3	70.7	78728	11614	87.1		
		23219	92873	25.0	75.0	31.6	96	32.8	67.2	70789	22084	76.2		
	500 µg/mL	22719	460456	4.9	95.1	19.7	95	20.7	79.3	109842	350614	23.9	23.3	0.6
		22440	479222	4.7	95.3	18.5	92	20.1	79.9	111493	367730	23.3		
		23049	468529	4.9	95.1	20.5	94	21.7	78.3	106275	362254	22.7		
2 hr	10 µg/mL	3086	10218	30.2	69.8	34.1	111	30.8	69.2	10030	188	98.2	95.8	3.6
		3281	11368	28.9	71.1	32.9	104	31.5	68.5	10418	950	91.6		
		3095	9845	31.4	68.6	33.8	105	32.2	67.8	9601	244	97.5		
	50 µg/mL	14336	49083	29.2	70.8	33.4	105	31.9	68.1	44902	4181	91.5	100.1	15.3
		16585	40638	40.8	59.2	35.5	102	34.7	65.3	47863	-7225	117.8		
		15858	52130	30.4	69.6	34.9	104	33.4	66.6	47489	4641	91.1		
	100 µg/mL	20472	82575	24.8	75.2	28.6	99	28.9	71.1	70956	11619	85.9	79.7	5.5
		22058	91232	24.2	75.8	30.6	98	31.3	68.7	70507	20725	77.3		
		22767	94387	24.1	75.9	32.0	101	31.8	68.2	71595	22792	75.9		
	500 µg/mL	21126	356672	5.9	94.1	17.0	84	20.2	79.8	104742	251930	29.4	30.7	1.3
		19994	296958	6.7	93.3	16.6	78	21.2	78.8	94497	202461	31.8		
		21274	332348	6.4	93.6	17.7	86	20.6	79.4	103091	229257	31.0		

Table C-12. Free Paclitaxel Control without Tracer Analytical Data. Presented are the analytical data for the free paclitaxel controls without Tracer. The % protein binding values were calculated as described in Section I. (*N=3*).

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX =Filtrate PTX /Reservoir PTX *100	% Bound =100- %Unbound PTX	AVG % Protein Binding	SD
10 min	**10 µg/mL**	3630	10831	33.5	66.5	66.1	0.6
		3537	10232	34.6	65.4		
		3645	10881	33.5	66.5		
	50 µg/mL	14602	48398	30.2	69.8	65.3	5.3
		14477	43284	33.4	66.6		
		20254	50007	40.5	59.5		
	100 µg/mL	23493	100139	23.5	76.5	73.2	6.8
		25881	74873	34.6	65.4		
		20374	91095	22.4	77.6		
	500 µg/mL	21619	438082	4.9	95.1	94.5	0.6
		23772	440948	5.4	94.6		
		25555	417665	6.1	93.9		
30 min	**10 µg/mL**	3324	11276	29.5	70.5	67.8	2.8
		3576	10221	35.0	65.0		
		3411	10645	32.0	68.0		
	50 µg/mL	12277	47240	26.0	74.0	72.5	1.6
		13930	50916	27.4	72.6		
		14314	49121	29.1	70.9		
	100 µg/mL	21957	107980	20.3	79.7	77.6	1.9
		23361	101244	23.1	76.9		
		22434	93870	23.9	76.1		
	500 µg/mL	24797	472469	5.2	94.8	94.7	0.1
		23335	441878	5.3	94.7		
		23697	436569	5.4	94.6		
2 hr	**10 µg/mL**	3535	10631	33.3	66.7	67.8	1.5
		3518	10691	32.9	67.1		
		3328	10914	30.5	69.5		
	50 µg/mL	13417	47947	28.0	72.0	70.9	1.1
		13870	47608	29.1	70.9		
		15258	50538	30.2	69.8		
	100 µg/mL	20764	83255	24.9	75.1	72.8	7.6
		21089	100047	21.1	78.9		
		32700	91638	35.7	64.3		
	500 µg/mL	26152	368195	7.1	92.9	93.3	0.4
		21667	325764	6.7	93.3		
		25326	396951	6.4	93.6		

Table C-13. Abraxane, Lot 6115306 Analytical Data. Presented are the analytical data for Abraxane lot 6115306. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX =Filtrate PTX /Reservoir PTX *100	% Bound =100-%Unbound PTX	Filtrate PTX_C13 (ng/mL)	Reservoir PTX_C13 STD (ng/mL)	%Unbound PTX_C13 =Filtrate PTX C13/Reservoir PTX C13 * 100	% Bound PTX_C13 =100 - %Unbound PTX C13	Unencapsulated (ng/mL) =Filtrate PTX/ (1-(%Bound PTX C13/100))	Encapsulated (ng/mL) =Reservoir PTX- Unencapsulated	% Release =(Unencapsulated/ Reservoir PTX) *100	AVG % Release	SD
10 min	10 µg/mL	3521	11069	31.8	68.2	34.2	106	32.1	67.9	10963	106	99.0	95.8	3.2
		3045	11208	27.2	72.8	31.9	109	29.3	70.7	10388	820	92.7		
		2951	11150	26.5	73.5	29.9	108	27.7	72.3	10656	494	95.6		
	50 µg/mL	15050	54587	27.6	72.4	29.3	113	26.1	73.9	57702	-3114	105.7	109.9	5.2
		13707	41389	33.1	66.9	29.2	102	28.6	71.4	47867	-6478	115.7		
		16203	49564	32.7	67.3	31.2	103	30.2	69.8	53694	-4130	108.3		
	100 µg/mL	18551	110351	16.8	83.2	27.3	111	24.7	75.3	75130	35221	68.1	67.0	1.1
		19234	105989	18.1	81.9	27.6	102	27.2	72.8	70815	35174	66.8		
		18961	101524	18.7	81.3	27.3	96	28.3	71.7	66966	34558	66.0		
	500 µg/mL	20068	548512	3.7	96.3	10.2	108	9.5	90.5	210986	337527	38.5	36.2	8.4
		16982	529849	3.2	96.8	11.8	99	11.9	88.1	142280	387569	26.9		
		21804	476998	4.6	95.4	10.3	97	10.6	89.4	206499	270499	43.3		
30 min	10 µg/mL	2940	12695	23.2	76.8	30.4	108	28.1	71.9	10463	2232	82.4	95.0	11.6
		3031	11702	25.9	74.1	28.3	115	24.6	75.4	12335	-633	105.4		
		3074	11414	26.9	73.1	27.5	99	27.7	72.3	11085	328	97.1		
	50 µg/mL	15861	57297	27.7	72.3	30.9	112	27.7	72.3	57216	81	99.9	97.5	8.1
		14800	53496	27.7	72.3	31.5	101	31.3	68.7	47339	6157	88.5		
		17301	54614	31.7	68.3	30.9	102	30.4	69.6	56870	-2256	104.1		
	100 µg/mL	19064	120159	15.9	84.1	23.8	102	23.3	76.7	81891	38268	68.2	65.4	3.9
		17260	108391	15.9	84.1	26.4	101	26.1	73.9	66097	42293	61.0		
		21449	123289	17.4	82.6	25.6	99	25.9	74.1	82714	40574	67.1		
	500 µg/mL	25226	470174	5.4	94.6	10.2	97	10.4	89.6	241543	228632	51.4	47.1	3.9
		20648	404476	5.1	94.9	11.0	94	11.7	88.3	177164	227312	43.8		
		22473	499607	4.5	95.5	9.0	92	9.7	90.3	230771	268836	46.2		
2 hr	10 µg/mL	2897	12019	24.1	75.9	29.3	105	28.0	72.0	10340	1678	86.0	91.5	5.8
		3399	11179	30.4	69.6	34.4	110	31.2	68.8	10909	270	97.6		
		3121	11569	27.0	73.0	32.4	109	29.6	70.4	10527	1042	91.0		
	50 µg/mL	15922	57115	27.9	72.1	31.6	109	29.0	71.0	54851	2264	96.0	95.6	11.6
		13818	60238	22.9	77.1	29.7	109	27.4	72.6	50466	9772	83.8		
		16306	54292	30.0	70.0	29.5	105	28.1	71.9	58120	-3828	107.0		
	100 µg/mL	16993	108826	15.6	84.4	24.0	100	23.9	76.1	71030	37796	65.3	69.3	9.6
		17577	100620	17.5	82.5	27.0	96	28.0	72.0	62742	37878	62.4		
		19691	107596	18.3	81.7	26.3	115	22.8	77.2	86308	21288	80.2		
	500 µg/mL	18751	485102	3.9	96.1	9.6	98	9.8	90.2	190519	294584	39.3	38.9	2.4
		19466	614005	3.2	96.8	12.1	139	8.7	91.3	223061	390943	36.3		
		19476	433629	4.5	95.5	9.3	85	10.9	89.1	178121	255507	41.1		

Table C-14. Abraxane, Lot 6115306 without Tracer Analytical Data. Presented are the analytical data for Abraxane lot 6115306 without Tracer. The % protein binding, encapsulated and unencapsulated values were calculated as described in Section I. (*N=3*).

|		Filtrate PTX (ng/mL)	Reservoir PTX (ng/mL)	%Unbound PTX =Filtrate PTX /Reservoir PTX *100	% Bound =100-%Unbound PTX	AVG % Protein Binding	SD
10 min	10 µg/mL	3420 3210 3530	11725 10294 11785	29.2 31.2 30.0	70.8 68.8 70.0	69.9	1.0
	50 µg/mL	16220 16469 15496	67016 58456 52797	24.2 28.2 29.3	75.8 71.8 70.7	72.8	2.7
	100 µg/mL	18045 17806 21971	101172 94948 101078	17.8 18.8 21.7	82.2 81.2 78.3	80.6	2.0
	500 µg/mL	20216 19591 19433	463748 477842 490605	4.4 4.1 4.0	95.6 95.9 96.0	95.9	0.2
30 min	10 µg/mL	3095 3747 3902	12076 12234 13065	25.6 30.6 29.9	74.4 69.4 70.1	71.3	2.7
	50 µg/mL	20462 25135 16641	62250 60838 51231	32.9 41.3 32.5	67.1 58.7 67.5	64.4	5.0
	100 µg/mL	20932 19969 19739	94286 112193 116023	22.2 17.8 17.0	77.8 82.2 83.0	81.0	2.8
	500 µg/mL	22240 21419 20134	519729 485772 423745	4.3 4.4 4.8	95.7 95.6 95.2	95.5	0.2
2 hr	10 µg/mL	3675 3260 2919	12653 11218 11462	29.0 29.1 25.5	71.0 70.9 74.5	72.1	2.1
	50 µg/mL	16647 16777 16302	56474 61169 58092	29.5 27.4 28.1	70.5 72.6 71.9	71.7	1.0
	100 µg/mL	19668 19605 20572	106251 108007 111252	18.5 18.2 18.5	81.5 81.8 81.5	81.6	0.2
	500 µg/mL	20779 18850 22154	486185 509106 477723	4.3 3.7 4.6	95.7 96.3 95.4	95.8	0.5

Table C-15. Free Paclitaxel Control Data. Displayed are the calculated % release for the paclitaxel control data. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	10 µg/mL	102.8	1.7	1.6
	50 µg/mL	95.7	3.5	3.7
	100 µg/mL	79.6	9.2	11.5
	500 µg/mL	30.0	6.0	19.9
30 min	10 µg/mL	101.6	7.3	7.2
	50 µg/mL	89.6	6.7	7.5
	100 µg/mL	80.2	6.1	7.6
	500 µg/mL	23.3	0.6	2.5
2 hr	10 µg/mL	95.8	3.6	3.8
	50 µg/mL	100.1	15.3	15.3
	100 µg/mL	79.7	5.5	6.8
	500 µg/mL	30.7	1.3	4.1
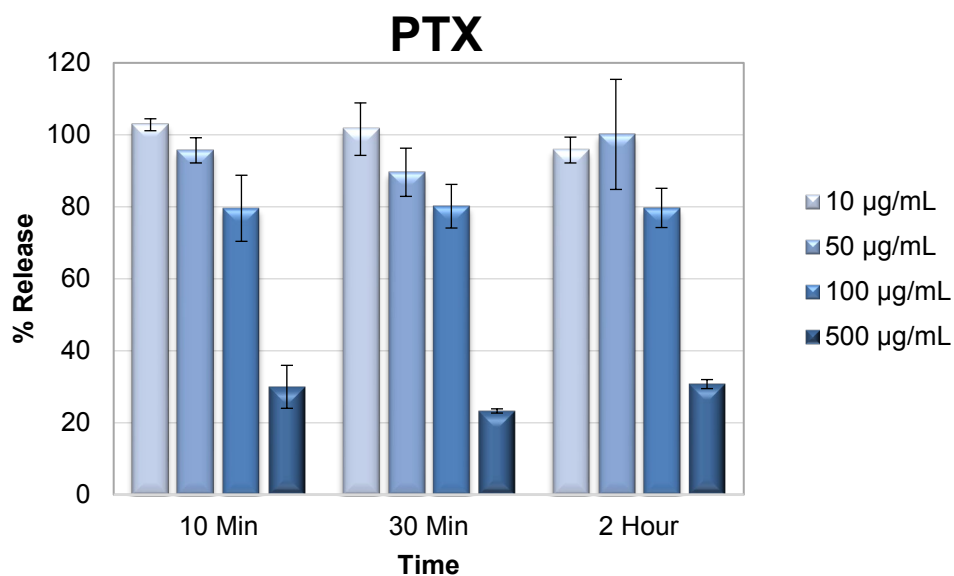
Figure C-14. Free Paclitaxel Control Data. Displayed are the calculated % release for the paclitaxel control data. (Mean $\pm$ SD, *N=3*)
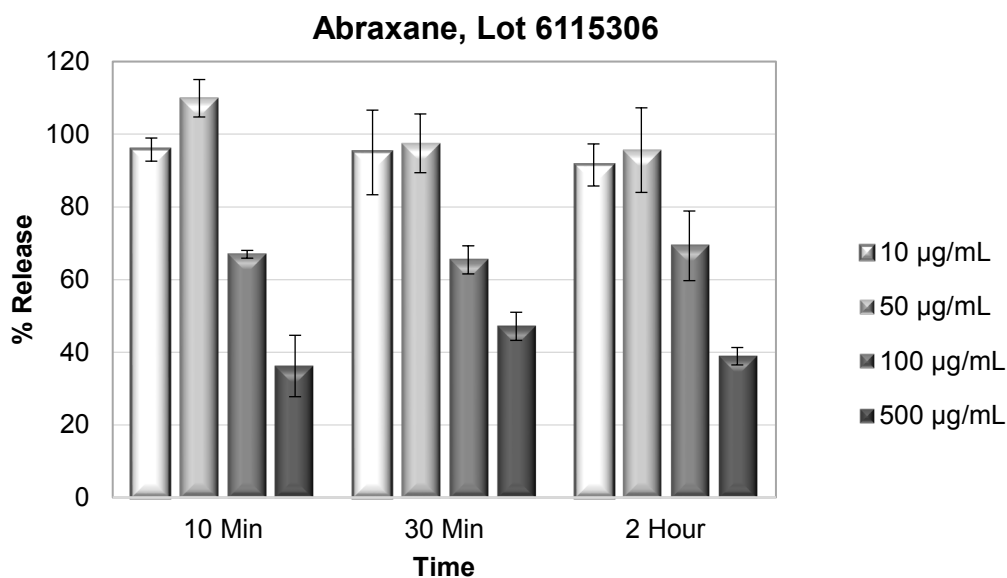
Table C-16. Abraxane, Lot 6115306 Drug Release. Displayed are the calculated % release for Abraxane lot 6115306. (*N=3*)

Time	Conc.	Avg % Release	SD	% CV
10 min	10 µg/mL	95.8	3.2	3.3
	50 µg/mL	109.9	5.2	4.7
	100 µg/mL	67.0	1.1	1.6
	500 µg/mL	36.2	8.4	23.3
30 min	10 µg/mL	95.0	11.6	12.3
	50 µg/mL	97.5	8.1	8.3
	100 µg/mL	65.4	3.9	5.9
	500 µg/mL	47.1	3.9	8.2
2 hr	10 µg/mL	91.5	5.8	6.3
	50 µg/mL	95.6	11.6	12.2
	100 µg/mL	69.3	9.6	13.8
	500 µg/mL	38.9	2.4	6.2

Figure C-15. Abraxane, Lot 6115306 Drug Release. Displayed are the calculated % release for Abraxane lot 6115306. (Mean $\pm$ SD, *N=3*)

Table C-17. % Protein Binding Comparison. Displayed are the calculated normoisotopic paclitaxel % protein binding for paclitaxel and Abraxane lot 6115306, with and without tracer. (*N=3*)

		PTX	SD	PTX no tracer	SD	Abraxane 6115306	SD	Abraxane 6115306 no tracer	SD
10 min	10 µg/mL	65.7	1.4	66.1	0.6	71.5	2.9	69.9	1.0
	50 µg/mL	66.5	1.2	65.3	5.3	69.9	3.6	72.8	2.7
	100 µg/mL	75.5	2.5	73.2	6.8	82.1	1.0	80.6	2.0
	500 µg/mL	94.3	0.8	94.5	0.6	96.2	0.7	95.9	0.2
30 min	10 µg/mL	65.4	4.5	67.8	2.8	74.7	1.9	71.3	2.7
	50 µg/mL	70.8	1.9	72.5	1.6	71.0	2.3	64.4	5.0
	100 µg/mL	74.7	0.3	77.6	1.9	83.6	0.9	81.0	2.8
	500 µg/mL	95.2	0.1	94.7	0.1	95.0	0.4	95.5	0.2
2 hr	10 µg/mL	69.8	1.3	67.8	1.5	72.8	3.2	72.1	2.1
	50 µg/mL	66.5	6.4	70.9	1.1	73.1	3.6	71.7	1.0
	100 µg/mL	75.6	0.4	72.8	7.6	82.9	1.4	81.6	0.2
	500 µg/mL	93.6	0.4	93.3	0.4	96.2	0.7	95.8	0.5
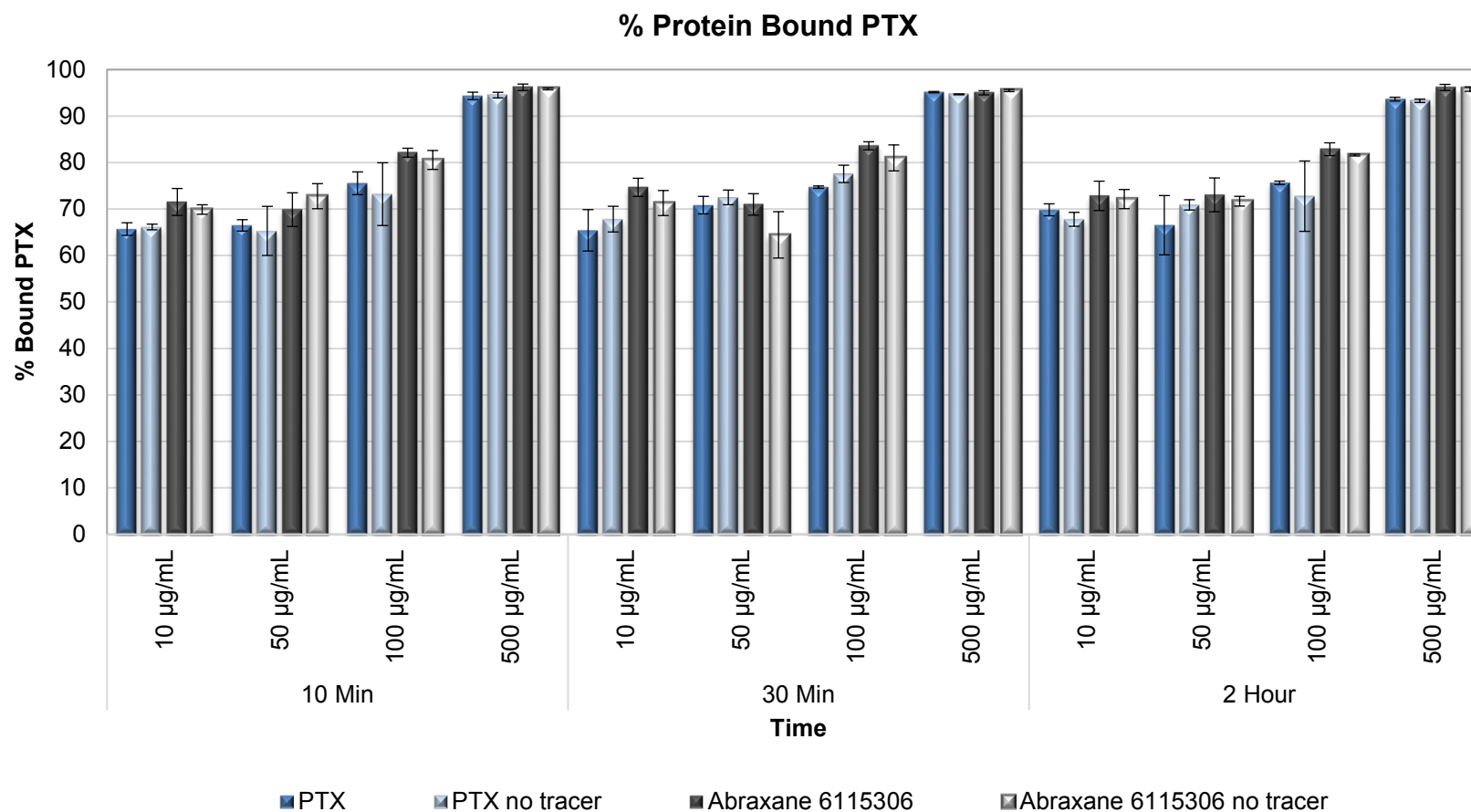
Figure C-16. % Protein Binding Comparison. Displayed are the calculated % protein binding for paclitaxel and Abraxane lot 6115306, with and without tracer. (Mean $\pm$ SD, *N=3*)
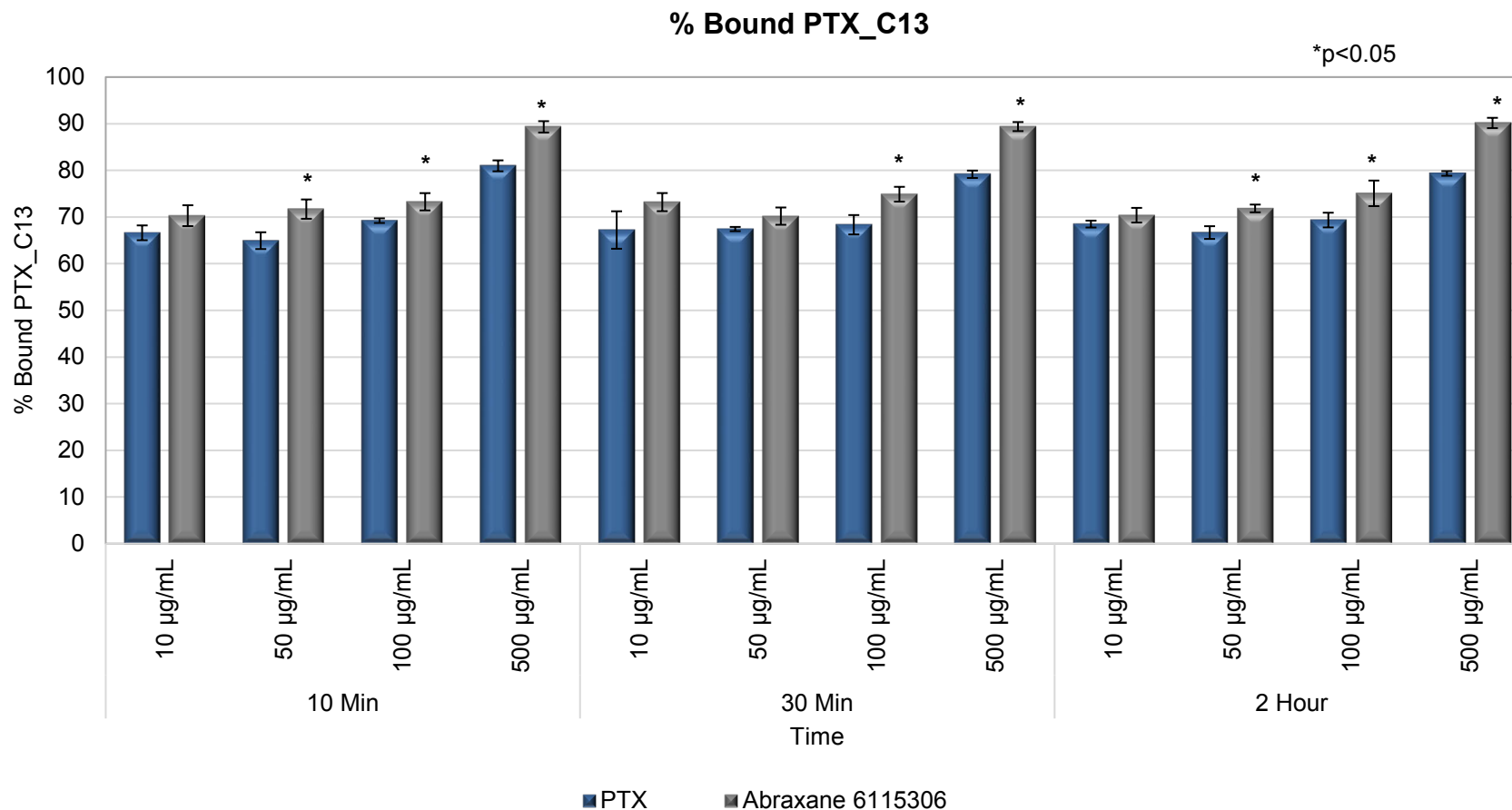
Table C-18. % Protein Binding Tracer PTX_C13 Comparison. Displayed are the calculated tracer PTX_C13 % protein binding for paclitaxel and Abraxane lot 6115306. (*N=3*)

		PTX	SD	Abraxane 6115306	SD
10 min	10 µg/mL	66.6	1.6	70.3	2.2
	50 µg/mL	64.9	1.8	71.7	2.1
	100 µg/mL	69.2	0.5	73.3	1.9
	500 µg/mL	81.0	1.2	89.3	1.2
30 min	10 µg/mL	67.2	4.0	73.2	1.9
	50 µg/mL	67.4	0.5	70.2	1.9
	100 µg/mL	68.3	2.1	74.9	1.6
	500 µg/mL	79.2	0.8	89.4	1.0
2 hr	10 µg/mL	68.5	0.7	70.4	1.6
	50 µg/mL	66.7	1.4	71.8	0.8
	100 µg/mL	69.4	1.6	75.1	2.7
	500 µg/mL	79.3	0.5	90.2	1.1

Figure C-17. % Protein Binding Tracer PTX_C13 Comparison. Displayed are the calculated tracer PTX_C13 % protein Binding for paclitaxel and Abraxane Lot 6115306. (Mean $\pm$ SD, *N=3*), *p$\leq$0.05, ANOVA with Duncan's Multiple Range test posthoc comparisons.

[1]   V.V. Ambardekar, S.T. Stern, NBCD Pharmacokinetics and Drug Release Methods, in: D.J.A. Crommelin, J.S.B. de Vlieger (Eds.), Non-Biological Complex Drugs; The Science and the Regulatory Landscape, Springer International Publishing, 2015, pp. 261-287.

[2]   S.L. Skoczen, S.T. Stern, Improved Ultrafiltration Method to Measure Drug Release from Nanomedicines Utilizing a Stable Isotope Tracer, Methods Mol Biol, 1682 (2018) 223-239.

[3]   S. Skoczen, S.E. McNeil, S.T. Stern, Stable isotope method to measure drug release from nanomedicines, Journal of controlled release : official journal of the Controlled Release Society, 220(Pt A) (2015) 169-174.

[4]   Guidance for Industry: Bioanalytical Method Validation, Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), 2013.

[5]   S.T. Stern, M.N. Martinez, D.M. Stevens, When Is It Important to Measure Unbound Drug in Evaluating Nanomedicine Pharmacokinetics?, Drug Metab Dispos, 44(12) (2016) 1934-1939.

[6]   Guidance for Industry: Statistical approaches to establishing bioequivalence, Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), 2001.

[7]   A. Sparreboom, C.D. Scripture, V. Trieu, P.J. Williams, T. De, A. Yang, B. Beals, W.D. Figg, M. Hawkins, N. Desai, Comparative preclinical and clinical pharmacokinetics of a cremophor-free, nanoparticle albumin-bound paclitaxel (ABI-007) and paclitaxel formulated in Cremophor (Taxol), Clin Cancer Res, 11(11) (2005) 4136-43.

[8]   C.A. Jamis-Dow, R.W. Klecker, A.G. Katki, J.M. Collins, Metabolism of taxol by human and rat liver in vitro: a screen for drug interactions and interspecies differences, Cancer Chemother Pharmacol, 36(2) (1995) 107-14.

[9]   A.G. Ellis, L.K. Webster, Inhibition of paclitaxel elimination in the isolated perfused rat liver by Cremophor EL, Cancer Chemother Pharmacol, 43(1) (1999) 13-8.

[10]   D. Goldstein, R.H. El-Maraghi, P. Hammel, V. Heinemann, V. Kunzmann, J. Sastre, W. Scheithauer, S. Siena, J. Tabernero, L. Teixeira, G. Tortora, J.L. Van Laethem, R. Young, D.N. Penenberg, B. Lu, A. Romano, D.D. Von Hoff, nab-Paclitaxel plus gemcitabine for metastatic pancreatic cancer: long-term survival from a phase III trial, J Natl Cancer Inst, 107(2) (2015).

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ACN	acetonitrile
ANOVA	analysis of variance
API	active pharmaceutical ingredient
AU	absorbance units
AUC_{all}	area under the time concentration curve including all time points
AUC_{inf}	area under the time-concentration curve extrapolated to time infinity
AUMC	area under the first moment curve
BE	bioequivalence
C_{max}	concentration maximum
CI	confidence interval
CL	clearance
Conc	concentration
CV	coefficient of variation
ESI	electrospray ionization
FA	formic acid
FDA	Food and Drug Administration
HPLC	high performance liquid chromatography
HSA	human serum albumin
ISTD	internal standard
λ_z	slope of the terminal elimination phase
LLOQ	lower limit of quantitation
LOD	limit of detection
LOQ	limit of quantitation
MRT_{inf}	mean residence time
MS	mass spectrometry
MWCO	molecular weight cut off
NCL	Nanotechnology Characterization Laboratory
PBS	phosphate buffered saline
PFP	protein-free plasma
PRM	parallel reaction monitoring
PK	pharmacokinetic
PTX	paclitaxel
PTX_13C	$^{13}C_6$-paclitaxel (stable isotope tracer)
PTX_d5	$^2H_5$-paclitaxel (internal standard)
QC	quality control
QCH	quality control, high
QCL	quality control, low
QCM	quality control, mid
RSD	relative standard deviation
SD	standard deviation
SITUA	stable isotope tracer ultrafiltration assay
T_{max}	time to maximum concentration
TOST	two one-sided t-tests
T/R	test to reference ratio
UHPLC	ultra high performance liquid chromatography
Vd	volume of distribution apparent
Vss	volume of distribution steady state

CONTRIBUTORS & ACKNOWLEDGEMENTS

Nanotechnology Characterization Laboratory

Stephan T. Stern
Scott E. McNeil
Rachael M. Crist
Sarah Skoczen
Kelsie Snapp

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products or organizations imply endorsement by the US Government.