Comprehensive Physico-chemical Characterization of Liposomal Doxorubicin

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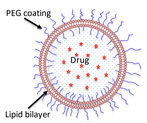
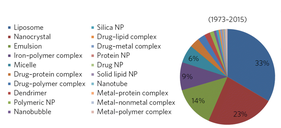
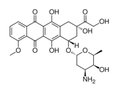
Abstract. Doxil® was approved in 1995 with generic liposomal doxorubicin products approved in recent years by ~~the~~ FDA. Recent publications on potential concerns about the clinical efficacy of generic liposomal doxorubicin prompted this research study to conduct comprehensive physico-chemical characterization of three manufactured lots of Doxil® and two generic products to ascertain differences, if any. The critical quality attributes were evaluated for these nine test articles with various analytical techniques for cross verification of data. Size measurements were conducted with dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), size exclusion chromatography (SEC) and asymmetric flow field flow fractionation (AF4) with multiangle laser light scattering (MALLS) detection; cryo-transmission electron microscopy was utilized for size, morphology and aspect ratio; lipid composition and quantitation were determined by high/ultrahigh performance liquid chromatography with charged aerosol detector (CAD), evaporative light scattering detector (ELSD) or mass spectrometer (MS); Poly(ethylene glycol) layer thickness was determined with fixed aqueous layer thickness (FALT) analysis; quantitation of total, intra-liposomal and extra-liposomal ammonium and sulfate ion content were quantified with ion chromatography; total, encapsulated, and free drug concentration were measured by solid phase extraction (SPE) followed by liquid chromatography and mass spectrometry (UPLC-MS). Additionally, drug release from these liposomes was measured to compare variations between lots as well as between manufacturers. Overall, minor differences in physico-chemical properties were observed among these drug products and further analysis of these minor differences is in progress. This research resulted in the development of three test method standards currently under ballot at ASTM International E56 Sub-Committee on Nanotechnology.

Background



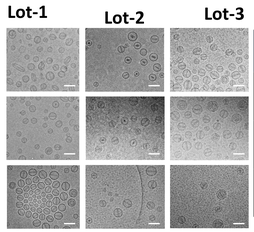


Molecular structure of Doxorubicin



- Liposomes constitute about 1/3 of drug product submissions containing nanomaterial to FDA.
- FDA published general liposome guidance and product-specific guidances to guide liposome product development.
- Doxil® (doxorubicin hydrochloride liposomal injection formulation) is the first FDA approved nano-sized liposomal chemotherapeutic drug.
- Additional generic liposomal doxorubicin products were approved in U.S. and worldwide
- Conflicting reports in public domain led to questions of bioequivalence of generic liposomal doxorubicin formulations despite approval by the FDA after meeting the requirements and standards set forth by the agency.
- A thorough and comprehensive physico-chemical characterization of three manufactured lots of DOXIL and two generic products was conducted to ascertain differences, if any.
- The study resulted in multiple test methods development through ASTM E56-08 via stakeholder engagement.

Cryo-TEM



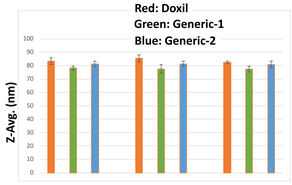
Size and shape distribution

Sample	†Eq. circle dia., ECD (nm)	‡Avg. ECD (nm)	Aspect ratio (AR)	‡Avg. AR
Doxil	61.2 ± 17.1 69.0 ± 16.3 70.4 ± 12.0	66.9 ± 4.9	1.110 1.086 1.087	1.098±0.014
Generic-1	57.1 ± 13.4 69.8 ± 17.5 68.7 ± 17.4	64.2 ± 7.0	1.080 1.115 1.095	1.085±0.018
Generic-2	60.3 ± 13.9 69.6 ± 13.0 71.3 ± 14.0	67.1 ± 5.9	1.090 1.080 1.134	1.089±0.029

† Data are reported as mean ± standard deviation (SD) (N=3 replicates).
‡ Data are reported as mean ± SD (N=3 lots)

- **A minimal difference in circularity and aspect ratio was observed among three products – reference listed drug (RLD) - Doxil, Generic-1 and Generic-2.**

Dynamic light scattering (DLS)



‡ Data are reported as mean ± SD (N=3 lots)

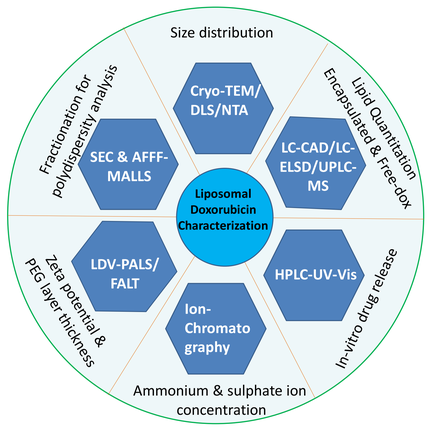
Nanoparticle tracking analysis (NTA)

Hydrodynamic size measured in PBS					
Sample	Mean (nm)	Median (nm)	D50 (nm)	Particle Concentration	‡Average Mean Size (D_h)
Doxil	82.6	69.7	73.5	2.37E-13	82.3±1.0
Doxil	83.2	79.6	80.7	2.36E-13	
Doxil	81.2	79.8	79.6	2.46E-13	
Generic-1	77.2	71.7	73.9	2.96E-13	77.8±0.7
Generic-1	78.6	75.6	76.8	2.62E-13	
Generic-1	77.7	72.0	74.3	3.50E-13	
Generic-2	80.2	76.1	78.0	2.33E-13	79.4±0.9
Generic-2	78.4	75.7	76.8	2.42E-13	
Generic-2	79.5	77.1	77.5	2.97E-13	

‡ Data are reported as mean ± SD (N=3 lots)

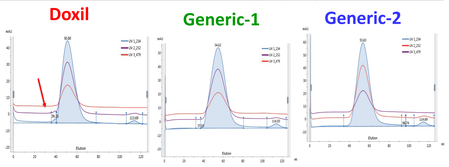
- **No significant differences in average hydrodynamic size were observed from DLS and NTA data analysis.**
- **Particle concentrations for all samples calculated *via* NTA were in the similar range.**

Characterization Techniques

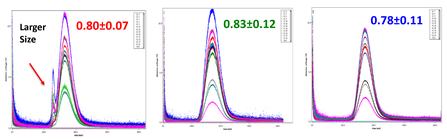


- DLS: Dynamic light scattering
- NTA: Nanoparticle tracking analysis
- TEM: Transmission electron microscopy
- SEC: Size exclusion chromatography
- AFFFF: Asymmetric flow field-flow fractionation
- MALS: Multi-angle light scattering
- HPLC: High performance liquid chromatography
- UPLC: Ultra-high performance liquid chromatography
- CAD: Charged aerosol detector
- ELSD: Evaporative light scattering detector
- MS: Mass spectrometry
- FALT: Fixed aqueous layer thickness analysis

Size exclusion chromatography with MALS



- **Doxil batches showed a distinct early fraction corresponding to a larger size liposomes**



- **No significant differences in Rg/Rh vales were observed among Doxil and generic products.**

pH, charge and PEG distribution

Sample	pH	†Zeta potential (mV)	‡Avg. Zeta potential (mV)	†FALT (nm) 5 points	‡Avg. thickness (nm)
Doxil	6.4	-11.1±0.2		3.5±0.2	
Doxil	6.9	-12.2±0.3	-12.0±0.9	3.4±0.1	3.5±0.1
Doxil	6.5	-12.9±0.3		3.5±0.6	
Generic-1	6.5	-12.2±0.2		3.2±0.2	
Generic-1	6.7	-13.8±0.8	-13.3±0.9	4.1±0.2	3.9±0.6
Generic-1	6.6	-13.8±0.4		4.3±0.8	
Generic-2	6.5	-11.2±0.2		3.2±0.4	
Generic-2	6.5	-12.5±0.5	-11.5±0.9	3.4±0.6	3.4±0.2
Generic-2	6.6	-10.8±0.6		3.5±0.7	

- pH and average zeta potential values of generic drugs do not vary significantly compared to DOXIL.
- No significant difference in PEG thickness-was observed between three drug products.

† Data are reported as mean ± SD (N=3 replicates); ‡ Data are reported as mean ± SD (N=3 lots)

Cholesterol & Lipid Quantitation: UPLC-MS

Sample	†DSPE-PEG 2000 (mg/mL)	†Cholesterol (mg/mL)	†HSPC (mg/mL)	†Total (mg/mL)	Component Ratio
Doxil	3.0 ± 0.2	3.1 ± 0.1	10.3 ± 0.3	16.5 ± 0.4	1.0:1.0:3.4
Generic-1	3.0 ± 0.2	3.2 ± 0.2	10.1 ± 0.2	16.2 ± 0.6	1:1.1.0:3.4
Generic-2	2.9 ± 0.1	3.3 ± 0.1	10.1 ± 0.3	16.2 ± 0.3	1:1.1.0:3.4

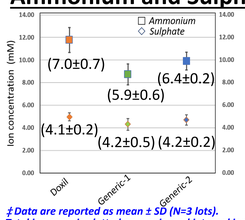
† Data are reported as mean ± SD (N=3 replicates).

Doxorubicin Concentration: UPLC-MS

Sample	†Free Dox (mg/mL)	†Encapsulated Dox (mg/mL)	Total(mg/mL)	‡Avg. Total (mg/mL)	‡Free(%)
Doxil (3-lots)	0.02 ± 0.002	1.876 ± 0.039	1.89	1.98 ± 0.09	2.17 ± 0.75
	0.05 ± 0.002	1.904 ± 0.084	1.95		
	0.03 ± 0.006	2.022 ± 0.055	2.08		
Generic-1 (3-lots)	0.04 ± 0.001	2.110 ± 0.013	2.15	2.11 ± 0.03	2.88 ± 1.12
	0.09 ± 0.046	2.033 ± 0.045	2.12		
	0.05 ± 0.002	2.033 ± 0.017	2.09		
Generic-2 (3-lots)	0.02 ± 0.001	2.087 ± 0.085	2.11	2.04 ± 0.13	2.41 ± 1.42
	0.06 ± 0.002	2.074 ± 0.108	2.13		
	0.07 ± 0.042	1.826 ± 0.119	1.89		

† Data are reported as mean ± SD (N=3 replicates); ‡ Data are reported as mean ± SD (N=3 lots)

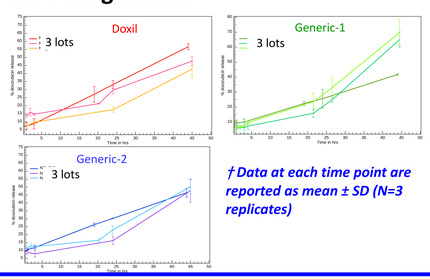
Ammonium and Sulphate Ion



- No significant differences in quantitation of total lipids were observed among 3-producs.
- No significant differences in doxorubicin conc. were observed among 9 samples purchased from 3 manufacturers.
- Ammonium conc. in the generic products were relatively lower than Doxil.

‡ Data are reported as mean ± SD (N=3 lots).
Total ion conc. is plotted on y-axis, and internal ion conc. is reported in parenthesis .

In-vitro drug release



† Data at each time point are reported as mean ± SD (N=3 replicates)

Experimental condition: Drug release experiments were in PBS with 5 mM ammonium chloride and 20 mM histidine.

- **Doxil**: ~ 40-55 %, **Generic-1**: ~ 40-70 % and **Generic-2**: ~ 45-50 % release of drug was observed in 45 hours.
- **Generic-1** showed significant lot-to-lot variations (40-70 %) in the amount of drug released in 45 hours time window.

Conclusions.

- Three manufactured lots of Doxil® and two generic liposomal doxorubicin were thoroughly analyzed through comprehensive physico-chemical characterization with methods development and optimization for each attribute.
- No significant differences were observed in batch mode measurements for size and zeta potential, PEG thickness, and drug concentration.
- A minor difference in total and internal ammonium conc. in generic drug compared to RLD (Doxil) was observed.
- Doxil® contained a small fraction of larger size liposome (through SEC) compared to others.
- In-vitro drug release data for Generic-1 showed variation between three lots.
- This extensive work led to the development of three consensus test method standards, which are in the final balloting through ASTM International E56-08 sub-committee.

Acknowledgement.

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