In Vitro Metabolic Stability of ALC-0315 in CD-1/ICR Mouse, Sprague Dawley Rat, Wistar Han Rat, Cynomolgus Monkey, and Human Hepatocytes

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SUMMARY

This study evaluated the *in vitro* metabolic stability of ALC-0315 in hepatocytes of CD-1/ICR mouse, Sprague Dawley rat, Wistar Han rat, cynomolgus monkey, and human. ALC-0315 was stable after an approximately 4-hour incubation with hepatocytes from all these species.

SIGNATURES

Compliance

This was a non-GLP study and was not conducted under full compliance with Good Laboratory Practice (GLP) regulations. Analyses were conducted according to an approved protocol and Standard Operating Procedures (SOPs) of Medicilon Preclinical Research (Shanghai) LLC. All data are documented in analysts' laboratory notebooks and electronic document management systems of Medicilon Preclinical Research (Shanghai) LLC. The content of this report has been reviewed against the raw data listings, summary tables and protocol for accuracy of the report.

Study Director Approval:

(b) (6)

Study Director

2020/08/10
Date

Sponsor Approval:

(b) (6)

Study Monitor

August 10, 2020
Date

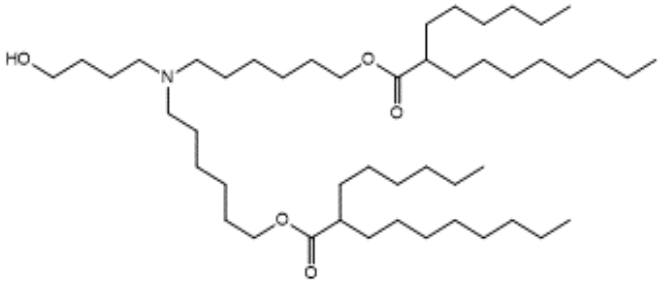
1. OBJECTIVE

To evaluate the *in vitro* metabolic stability of ALC-0315 in hepatocytes from different species.

2. MATERIALS

2.1 Test Article

Name: ALC-0315
Molecular Formula: $C_{48}H_{95}NO_5$
MW (g/mol): 766.27
Exact Mass: 765.72



2.2 Positive Control

Compound Name	Vendor	CAS No.	Cat. No.	Lot No.	Molecular Weight
Testosterone	Aladdin	58-22-0	T102169	K1505051	288.42
7-hydroxycoumarin	J&K Scientific	93-35-6	153384	L630I04	162.14

2.3 Internal Standard

Compound Name	Vendor	CAS No.	Cat. No.	Lot No.	Molecular Weight
Verapamil hydrochloride	TCI	152-11-4	V0118	RFMWJ-RL	491.06
Tolbutamide	Sigma-Aldrich	64-7-7	46968	BCBV8457	270.35

2.4 Hepatocytes

The following cryopreserved hepatocytes of CD-1/ICR mouse, Sprague Dawley rat, Wistar Han rat, cynomolgus monkey, and human were purchased from qualified suppliers and stored in liquid nitrogen until use.

Species	Manufacturer	Cat. No.	Lot No.	Assured Minimum Yield (cells per vial)
CD-1/ICR mouse (male)	XenoTech	MPCH1000	1810242	2.0×10^6
Sprague Dawley rat (male)	XenoTech	RPCH1000	1810189	5.0×10^6
Wistar Han rat	BioIVT	M00065	YMV	5.0×10^6
Cynomolgus monkey (male)	RILD Shanghai	HP-SXH-02M	CJJC	5.0×10^6
Human (mixed gender)	XenoTech	HPCH10	1810156	5.0×10^6

3. EXPERIMENTAL PROCEDURES

3.1 Stock solution:

2.06 mg of ALC-0315 was weighed and dissolved in 268.83 µL of DMSO to obtain a 10 mM stock solution. 3.31 mg of testosterone was weighed and dissolved in 1147.60 µL of DMSO to obtain a 10 mM stock solution. 2.81 mg of 7-hydroxycoumarin was weighed and dissolved in 882.70 µL of DMSO to obtain a 10 mM stock solution.

3.2 4 mM spiking solution:

Spiking Solution of Test Article or Positive Control				
Compound	Conc. of Stock Solution (mM)	Volume of Stock Solution (µL)	Volume of DMSO (µL)	Final Concentration (mM)
ALC-0315	10	20	30	4
Testosterone & 7-Hydroxycoumarin	10	20	10	4

3.3 2 µM dosing solution (2×):

Dosing Solution (2×) of Test Article or Positive Control			
Conc. of Spiking Solution (mM)	Volume of Spiking Solution (µL)	Volume of William's E Medium (µL)	Final Concentration (µM)
4	2	3998	2

3.4 Preparation of hepatocyte suspension:

Cryopreserved hepatocytes were thawed in a 37°C water bath, transferred to hepatocyte thawing medium (William's E Medium with 30% percoll and 5% FBS), and then centrifuged at 100×g for 10 min at room temperature. The cell pellet was resuspended with William's E

Medium, cell viability was determined by trypan blue exclusion analysis, and the density of viable cells was calculated. The hepatocytes were diluted with incubation medium to an appropriate density (2×10^6 viable cells/mL) and then pre-warmed at 37 °C for 10 min.

3.5 40 µL of each hepatocyte suspension was added to 96-well plates in duplicate for each time point (0, 30, 60, 90, 120, 180, and 240 min).

3.6 For 0 min samples: 480 µL of internal standard solution (IS solution, 10 ng/mL verapamil in ethanol) was added, followed by 40 µL of pre-warmed 2× dosing solution. The final concentration of test article or positive control in the incubation mixture was 1 µM.

3.7 For the 30, 60, 90, 120, 180, and 240 min samples, 40 µL of pre-warmed 2× dosing solution was added to initiate the reaction. The final concentration of test article or positive control in the incubation mixture was 1 µM.

3.8 Samples were incubated at 37 ℃ . At 30, 60, 90, 120, 180, and 240 min time points, the reaction was stopped by adding 480 µL ethanol containing internal standard to all of the duplicate wells.

3.9 After quenching, the plates were shaken at 600 rpm for 10 min and then centrifuged at 6,000 rpm for 15 min.

3.10 The plates were sealed and stored at -20 °C until bioanalysis.

3.11 Plates were thawed at room temperature, centrifuged at 6,000 rpm for 15 min, and 200 µL of the supernatants were transferred from each well into a 96-well sample plate for LC-MS/MS.

4. BIOANALYSIS

4.1 Instruments

Waters Acuity UPLC system
Sciex Triple Quad 6500+ with ESI ion source

4.2 LC/MS/MS Conditions

Column: ACQUITY UPLC BEH HILIC 1.7 µm (2.1*100 mm)
Gradient for ALC-0315:

Time (min)	Solvent A (%)	Solvent B (%)
0.01	5	95
1.50	40	60
2.00	40	60
2.01	5	95
2.50	5	95

Solvent A: 10mM ammonium formate, 0.1% formic acid in water
Solvent B: 10mM ammonium formate, 0.1% formic acid in acetonitrile
Flow rate：500 μL/min
Column temperature：40 ºC
Autosampler temperature: 4ºC
MS Conditions: MRM detection

Compound	Q1(m/z)	Q3(m/z)	DP	CE	Retention Time
ALC-0315	766.90	510.60	100	66	~1.21
Verapamil (IS)	455.30	165.20	49	28	~1.32

4.3 Detection of ALC-0315

Representative chromatograms of ALC-0315 in each matrix are shown in Appendix 1.

5. DATA ANALYSIS

The % remaining parent compound (ALC-0315 or positive control, testosterone and 7-hydroxycoumarin) was calculated by dividing the peak area ratio (test article peak area/internal standard peak area) by the time zero peak area ratio. The natural logarithm of % remaining parent compound was plotted against time, and the slope of the regression line was determined. The elimination constant and half-life was calculated, when possible, as indicated below.

Elimination rate constant (k) = - slope
Half-life (T1/2) (minutes) = 0.693/k
Intrinsic clearance, predicted from the *in vitro* hepatocyte stability study, was calculated as shown below:

CL'int (mL/min/kg) = k × V (1 mL incubation/10^6 cells) × Scaling Factor (10^6 cells/kg),
Scaling Factor (10^6 cells/kg) = Hepatocellularity (10^6 cells/g liver) × Normalized Liver Weight (g liver/kg body weight)

The scaling factors are listed in Table 1.

Table 1. Scaling Factors for Intrinsic Clearance Prediction in Mouse, Rat, Monkey, and Human Hepatocytes

Species	Hepatocellularity (10^6 cells/g liver)	Liver Weight (g/kg BW)	Scaling Factor (10^6 cells/kg)
Mouse	135	87.5	11812.5
Rat	117	40	4680
Monkey	120	32	3840
Human	99	25.7	2544.3

6. RESULTS

A summary of the % remaining parent compound, CL'_{int} and half-life of ALC-0315 obtained from a 4-hour incubation with hepatocytes from CD-1/ICR mouse, Sprague Dawley rat, Wistar Han rat, cynomolgus monkey, and human is presented in Table 2. The stability of ALC-0315 over time in each matrix is shown in Figure 1. Raw data is presented in Appendix 2.

The hepatocytes used in this study were tested for activity using metabolism control substrates under incubation conditions identical to those used for ALC-0315. The enzymes were found to exhibit satisfactory activity as determined by significant consumption of the positive control compounds (testosterone and 7-hydroxycoumarin) during the 4-hour incubation period, hence the test systems were considered to have yielded valid results. A summary of the % remaining parent compound, CL'_{int} and half-life of testosterone and 7-hydroxycoumarin is provided in Table 2. The stability of testosterone and 7-hydroxycoumarin over time in each matrix is shown in Figure 2 and Figure 3, respectively. Raw data for controls is presented in Appendix 3 (testosterone) and Appendix 4 (7-hydroxycoumarin).

7. CONCLUSIONS

This study evaluated the *in vitro* metabolic stability of ALC-0315 in hepatocytes of CD-1/ICR mouse, Sprague Dawley rat, Wistar Han rat, cynomolgus monkey, and human. ALC-0315 was stable after an approximately 4-hour incubation with hepatocytes from all these species.

Table 2. Summary of Hepatocyte Stability of ALC-0315, Testosterone and 7-Hydroxycoumarin

Test Article	Species		Percent Remaining (%)							$T_{1/2}$ (minute)	CL'int (mL/min/kg)
			0 min	30 min	60 min	90 min	120 min	180 min	240 min		
ALC-0315	CD-1/ICR mouse	Mean	100.00	101.15	100.77	101.92	98.85	101.15	99.62	**>240**	**<34.1**
		RSD of Area Ratio	1.63	1.07	0.54	0.00	2.19	0.00	1.09		
	Sprague Dawley rat	Mean	100.00	97.75	98.50	99.25	97.38	98.88	101.12	**>240**	**<13.5**
		RSD of Area Ratio	1.59	3.79	3.76	1.60	2.18	4.29	2.10		
	Wistar Han rat	Mean	100.00	102.70	102.32	103.09	99.61	103.47	100.00	**>240**	**<13.5**
		RSD of Area Ratio	0.55	1.06	1.60	0.53	1.10	3.17	7.10		
	Cynomolgus monkey	Mean	100.00	96.36	97.82	100.00	96.36	95.64	93.82	**>240**	**<11.3**
		RSD of Area Ratio	1.54	1.60	2.63	3.60	3.74	1.61	4.39		
	Human	Mean	100.00	100.72	101.44	100.36	100.72	98.92	99.64	**>240**	**<7.35**
		RSD of Area Ratio	2.03	1.01	3.01	2.53	0.00	0.51	0.51		
Testosterone	CD-1/ICR mouse	Mean	100.00	16.60	BQL	BQL	BQL	BQL	BQL	**11.6**	**707**
		RSD of Area Ratio	5.81	11.78	N/A	N/A	N/A	N/A	N/A		
	Sprague Dawley rat	Mean	100.00	7.23	BQL	BQL	BQL	BQL	BQL	**7.92**	**410**
		RSD of Area Ratio	3.17	N/A	N/A	N/A	N/A	N/A	N/A		
	Wistar Han rat	Mean	100.00	BQL	BQL	BQL	BQL	BQL	BQL	**N/A**	**N/A**
		RSD of Area Ratio	8.03	N/A	N/A	N/A	N/A	N/A	N/A		
	Cynomolgus monkey	Mean	100.00	10.07	BQL	BQL	BQL	BQL	BQL	**9.06**	**298**
		RSD of Area Ratio	2.81	41.26	N/A	N/A	N/A	N/A	N/A		
	Human	Mean	100.00	15.92	BQL	BQL	BQL	BQL	BQL	**11.3**	**156**
		RSD of Area Ratio	4.34	7.16	N/A	N/A	N/A	N/A	N/A		

7-Hydroxycoumarin	CD-1/ICR mouse	Mean	100	35.05	3.2	BQL	BQL	BQL	BQL	**12.1**	**677**
		RSD of Area Ratio	1.22	15.06	8.46	N/A	N/A	N/A	N/A		
	Sprague Dawley rat	Mean	100	20.97	BQL	BQL	BQL	BQL	BQL	**13.3**	**244**
		RSD of Area Ratio	2.99	10.49	N/A	N/A	N/A	N/A	N/A		
	Wistar Han rat	Mean	100	19.11	BQL	BQL	BQL	BQL	BQL	**12.6**	**258**
		RSD of Area Ratio	1.97	16.89	N/A	N/A	N/A	N/A	N/A		
	Cynomolgus monkey	Mean	100	17.03	BQL	BQL	BQL	BQL	BQL	**11.7**	**230**
		RSD of Area Ratio	0.85	2.27	N/A	N/A	N/A	N/A	N/A		
	Human	Mean	100	40.7	18.53	3.36	BQL	BQL	BQL	**24.7**	**71.5**
		RSD of Area Ratio	1.52	1.67	8.47	0.73	N/A	N/A	N/A		

* Compound showed biphasic metabolic kinetics, i.e., an initial fast disappearance phase was followed by a slow disappearance phase. The data points marked in * were in the slow disappearance phase and were excluded from half-life calculation.
BQL = Below quantification limit; N/A = not applicable

Figure 1. Stability of ALC-0315 in Mouse, Rat, Monkey and Human Hepatocytes

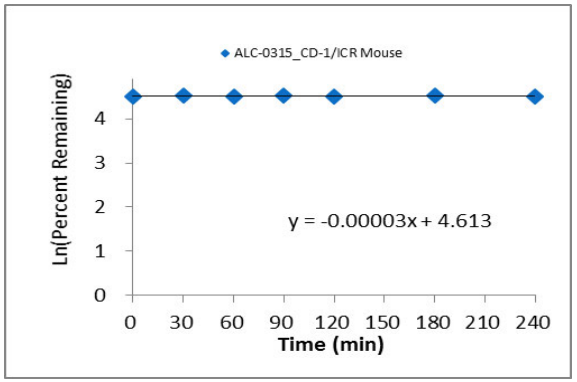
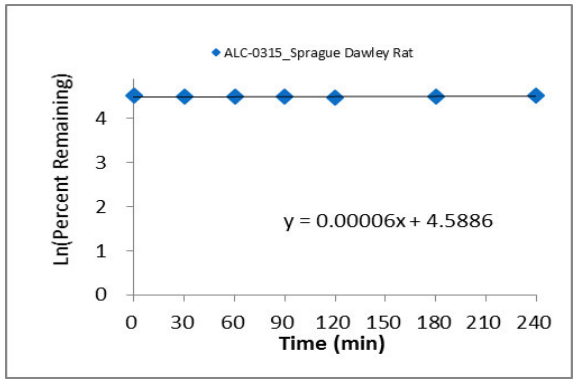
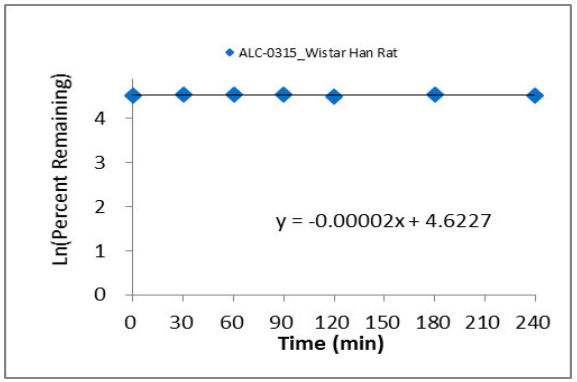
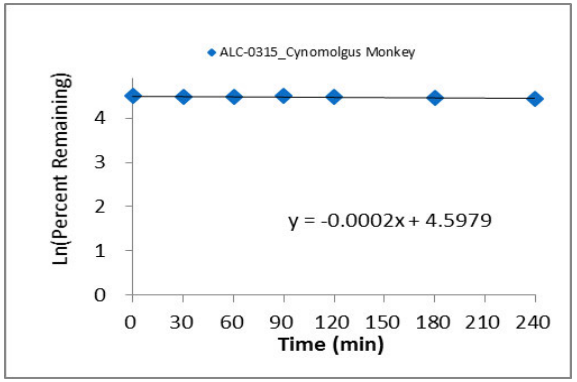
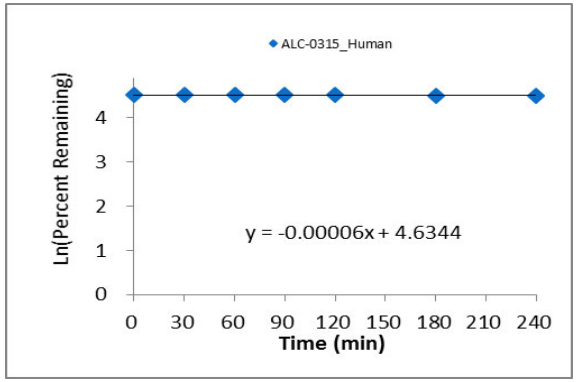
CD-1/ICR Mouse	Sprague Dawley Rat	Wistar Han Rat
		
Cynomolgus Monkey	**Human**	
		

Figure 2. Stability of Testosterone in Mouse, Rat, Monkey and Human Hepatocytes

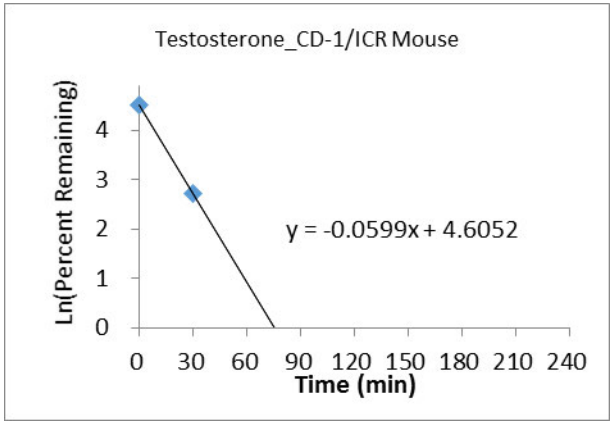
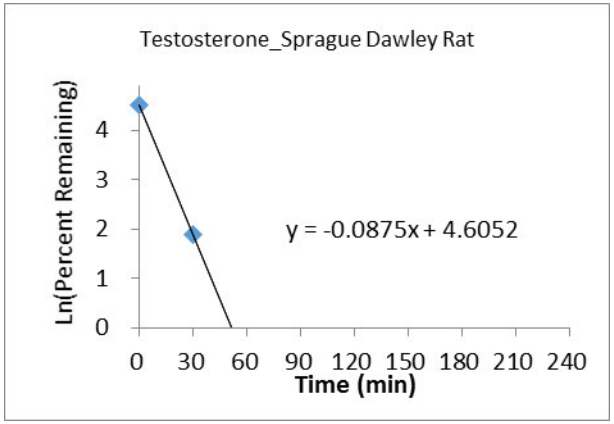
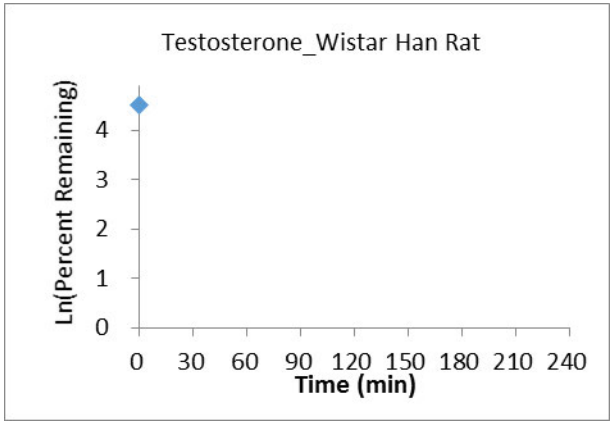
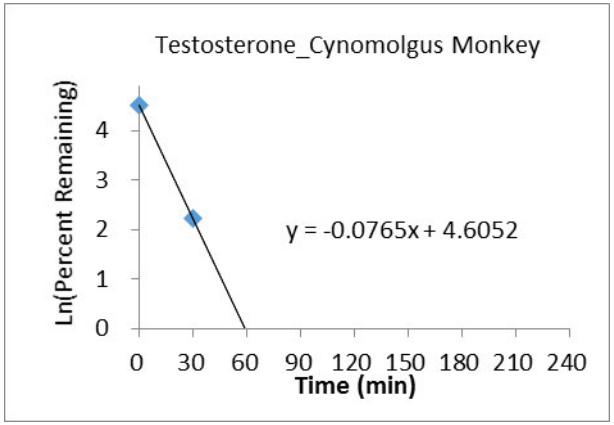
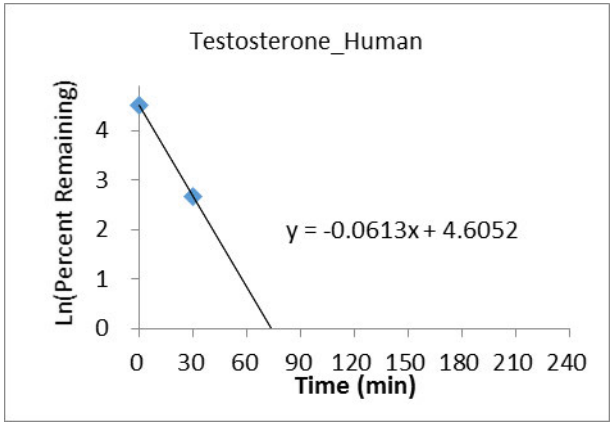
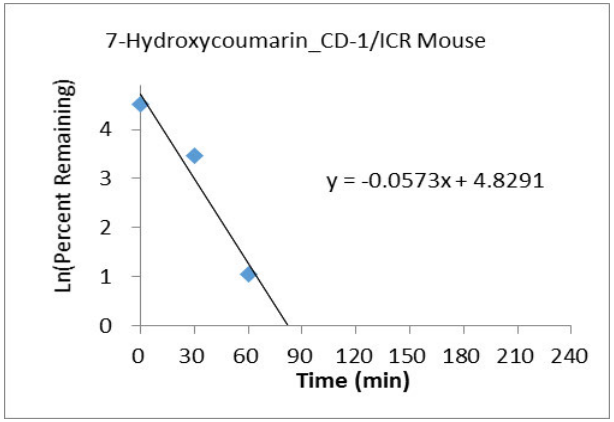
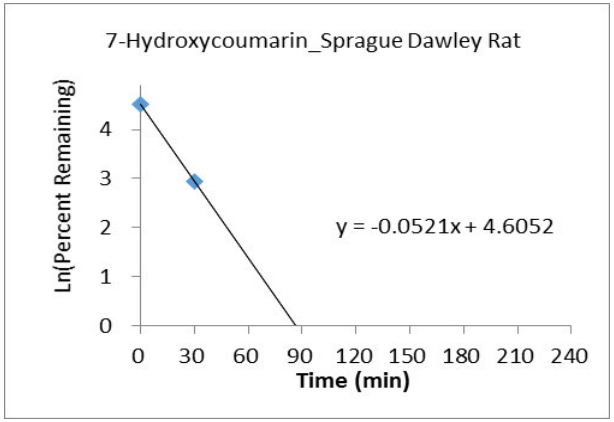
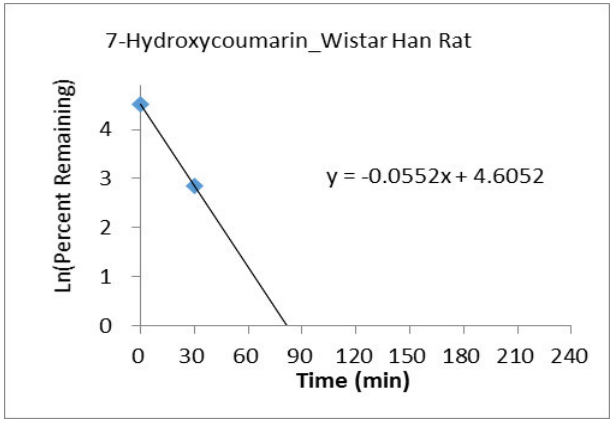
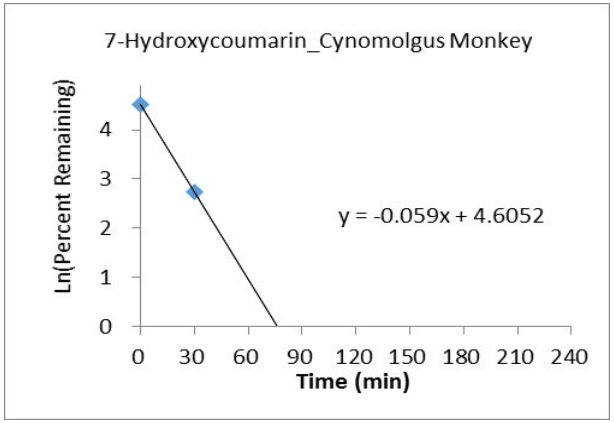
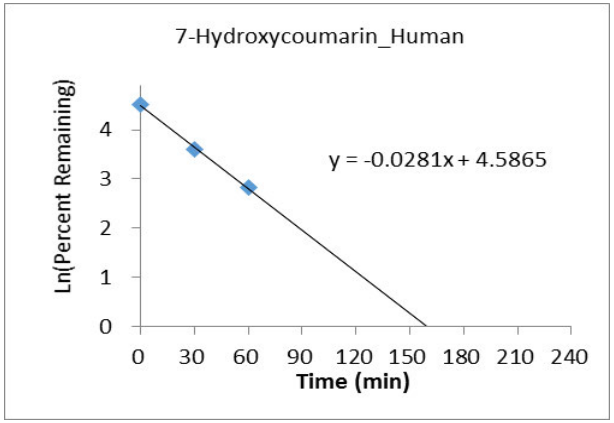
CD-1/ICR Mouse	Sprague Dawley Rat	Wistar Han Rat
		
Cynomolgus Monkey	**Human**	
		

Figure 3. Stability of 7-Hydroxycoumarin in Mouse, Rat, Monkey and Human Hepatocytes

CD-1/ICR Mouse	Sprague Dawley Rat	Wistar Han Rat
		
Cynomolgus Monkey	**Human**	
		

8. APPENDICES

Appendix 1 – Representative Chromatograms of ALC-0315 in Mouse, Rat, Monkey and Human Hepatocytes

Appendix 2 – Stability of ALC-0315 in Mouse, Rat, Monkey and Human Hepatocytes – Raw Data

Appendix 3 – Stability of Testosterone in Mouse, Rat, Monkey and Human Hepatocytes – Raw Data

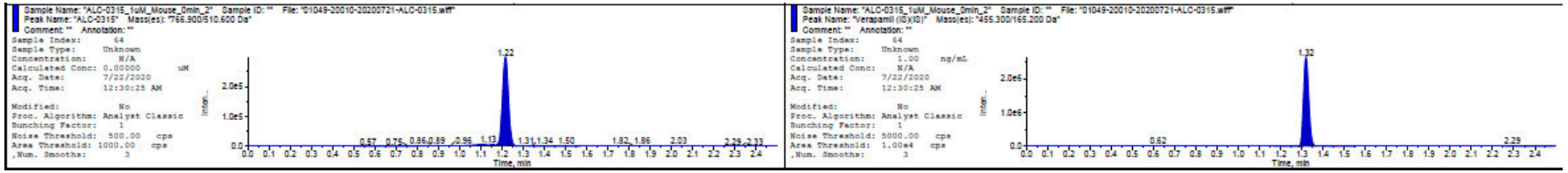
Appendix 4 – Stability of 7-Hydroxycoumarin in Mouse, Rat, Monkey and Human Hepatocytes – Raw Data

Appendix 5 – 01049-20010-ALC-0315-Hepatocytes Stability_Protocol

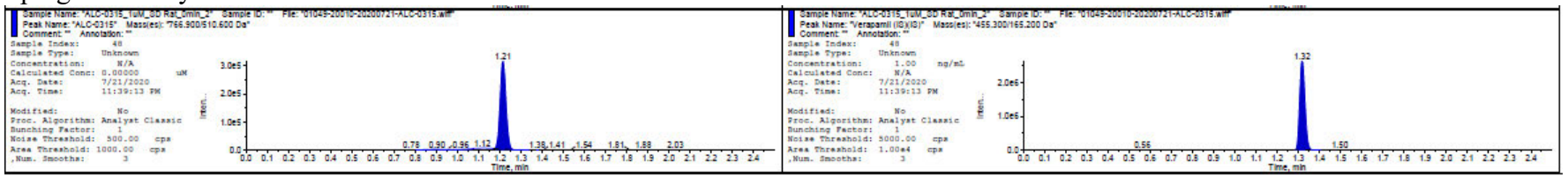
APPENDIX 1

Representative Chromatograms of ALC-0315 in Mouse, Rat, Monkey and Human Hepatocytes

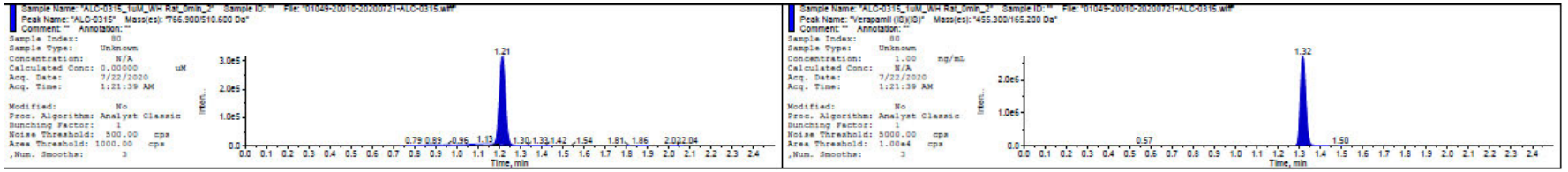
CD 1/ICR mouse



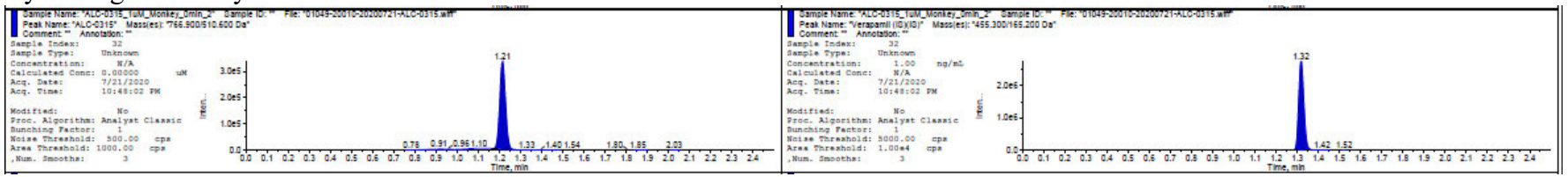
Sprague Dawley rat



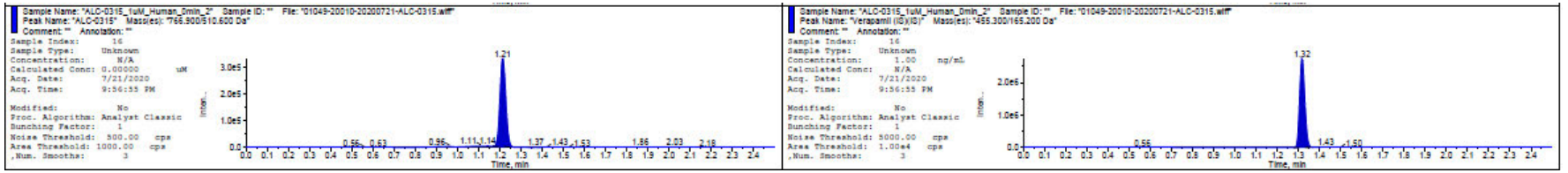
Wistar Han rat



Cynomolgus monkey



Human



APPENDIX 2

Stability of ALC-0315 in Mouse, Rat, Monkey and Human Hepatocyte – Raw Data

Compound	Species	Time(min)	Raw Data					
			Analyte Peak Area (counts)	Analyte Peak Area (counts)	IS Peak Area (counts)	IS Peak Area (counts)	Area Ratio	Area Ratio
ALC-0315	CD-1/ICR mouse	240	6.00E+05	5.90E+05	4.65E+06	4.50E+06	0.129	0.131
		180	5.99E+05	5.96E+05	4.54E+06	4.53E+06	0.132	0.132
		120	5.86E+05	5.84E+05	4.60E+06	4.45E+06	0.127	0.131
		90	5.88E+05	5.83E+05	4.43E+06	4.38E+06	0.133	0.133
		60	5.83E+05	5.92E+05	4.47E+06	4.47E+06	0.131	0.132
		30	5.94E+05	5.89E+05	4.53E+06	4.43E+06	0.131	0.133
		0	5.92E+05	5.65E+05	4.48E+06	4.39E+06	0.132	0.129
ALC-0315	Sprague Dawley rat	240	6.40E+05	5.94E+05	4.69E+06	4.47E+06	0.137	0.133
		180	6.18E+05	5.83E+05	4.53E+06	4.56E+06	0.136	0.128
		120	5.97E+05	5.77E+05	4.53E+06	4.51E+06	0.132	0.128
		90	6.08E+05	5.81E+05	4.53E+06	4.45E+06	0.134	0.131
		60	6.08E+05	5.75E+05	4.51E+06	4.48E+06	0.135	0.128
		30	6.06E+05	5.67E+05	4.53E+06	4.47E+06	0.134	0.127
		0	6.09E+05	5.82E+05	4.50E+06	4.41E+06	0.135	0.132
ALC-0315	Wistar Han rat	240	5.55E+05	6.12E+05	4.50E+06	4.51E+06	0.123	0.136
		180	5.91E+05	6.06E+05	4.52E+06	4.44E+06	0.131	0.137
		120	5.68E+05	5.83E+05	4.45E+06	4.49E+06	0.128	0.13
		90	5.91E+05	5.94E+05	4.40E+06	4.48E+06	0.134	0.133
		60	5.82E+05	5.99E+05	4.46E+06	4.48E+06	0.131	0.134
		30	6.04E+05	5.94E+05	4.51E+06	4.49E+06	0.134	0.132
		0	5.87E+05	5.88E+05	4.55E+06	4.51E+06	0.129	0.13
ALC-0315	Cynomolgus monkey	240	6.17E+05	5.78E+05	4.65E+06	4.64E+06	0.133	0.125
		180	6.09E+05	5.91E+05	4.59E+06	4.54E+06	0.133	0.13
		120	6.28E+05	5.85E+05	4.61E+06	4.55E+06	0.136	0.129
		90	6.42E+05	6.07E+05	4.55E+06	4.55E+06	0.141	0.134
		60	6.38E+05	5.95E+05	4.66E+06	4.50E+06	0.137	0.132
		30	6.03E+05	6.02E+05	4.61E+06	4.49E+06	0.131	0.134
		0	6.32E+05	6.17E+05	4.54E+06	4.55E+06	0.139	0.136
ALC-0315	Human	240	6.30E+05	6.38E+05	4.52E+06	4.64E+06	0.139	0.138
		180	6.44E+05	6.19E+05	4.66E+06	4.52E+06	0.138	0.137
		120	6.49E+05	9.24E+05	4.65E+06	4.54E+06	0.14	0.204
		90	6.51E+05	6.20E+05	4.60E+06	4.52E+06	0.142	0.137
		60	6.53E+05	6.27E+05	4.54E+06	4.54E+06	0.144	0.138
		30	6.42E+05	6.20E+05	4.54E+06	4.46E+06	0.141	0.139
		0	6.42E+05	6.22E+05	4.56E+06	4.55E+06	0.141	0.137

APPENDIX 3

Stability of Testosterone in Mouse, Rat, Monkey and Human Hepatocyte – Raw Data

Compound	Species	Time(min)	Raw Data					
			Analyte Peak Area (counts)	Analyte Peak Area (counts)	IS Peak Area (counts)	IS Peak Area (counts)	Area Ratio	Area Ratio
Testosterone	CD-1/ICR mouse	240	LOD	LOD	7.53E+05	7.48E+05	LOD	LOD
		180	LOD	LOD	7.77E+05	7.83E+05	LOD	LOD
		120	LOD	LOD	7.44E+05	7.99E+05	LOD	LOD
		90	LOD	LOD	7.60E+05	7.89E+05	LOD	LOD
		60	LOD	LOD	7.39E+05	7.46E+05	LOD	LOD
		30	5.29E+03	6.16E+03	7.70E+05	7.58E+05	0.007	0.008
		0	3.64E+04	3.41E+04	7.73E+05	7.88E+05	0.047	0.043
Testosterone	Sprague Dawley rat	240	LOD	LOD	8.19E+05	8.01E+05	LOD	LOD
		180	LOD	LOD	7.97E+05	7.54E+05	LOD	LOD
		120	LOD	LOD	7.48E+05	8.25E+05	LOD	LOD
		90	LOD	LOD	8.12E+05	7.45E+05	LOD	LOD
		60	LOD	LOD	7.59E+05	7.44E+05	LOD	LOD
		30	LOD	2.38E+03	8.25E+05	8.19E+05	LOD	0.003
		0	3.38E+04	3.38E+04	8.23E+05	8.59E+05	0.041	0.039
Testosterone	Wistar Han rat	240	LOD	LOD	7.72E+05	8.57E+05	LOD	LOD
		180	LOD	LOD	7.61E+05	7.44E+05	LOD	LOD
		120	LOD	LOD	7.87E+05	7.53E+05	LOD	LOD
		90	LOD	LOD	7.87E+05	7.71E+05	LOD	LOD
		60	LOD	LOD	7.29E+05	7.93E+05	LOD	LOD
		30	LOD	LOD	7.78E+05	7.87E+05	LOD	LOD
		0	3.34E+04	3.39E+04	8.20E+05	7.44E+05	0.041	0.046
Testosterone	Cynomolgus monkey	240	LOD	LOD	8.17E+05	8.22E+05	LOD	LOD
		180	LOD	LOD	8.26E+05	8.16E+05	LOD	LOD
		120	LOD	LOD	8.22E+05	8.12E+05	LOD	LOD
		90	LOD	LOD	8.44E+05	7.91E+05	LOD	LOD
		60	LOD	LOD	8.47E+05	7.85E+05	LOD	LOD
		30	4.32E+03	2.37E+03	8.24E+05	8.22E+05	0.005	0.003
		0	3.45E+04	3.26E+04	8.72E+05	7.93E+05	0.04	0.041
Testosterone	Human	240	LOD	LOD	8.02E+05	8.22E+05	LOD	LOD
		180	LOD	LOD	8.65E+05	8.75E+05	LOD	LOD
		120	LOD	LOD	8.29E+05	8.22E+05	LOD	LOD
		90	LOD	LOD	8.60E+05	8.16E+05	LOD	LOD
		60	LOD	LOD	8.21E+05	8.47E+05	LOD	LOD
		30	6.13E+03	5.10E+03	8.78E+05	8.09E+05	0.007	0.006
		0	3.25E+04	3.56E+04	8.02E+05	8.26E+05	0.04	0.043

LOD = limit of detection

APPENDIX 4

Stability of 7-Hydroxycoumarin in Mouse, Rat, Monkey and Human Hepatocyte – Raw Data

Compound	Species	Time(min)	Raw Data					
			Analyte Peak Area (counts)	Analyte Peak Area (counts)	IS Peak Area (counts)	IS Peak Area (counts)	Area Ratio	Area Ratio
7-Hydroxycoumarin	CD-1/ICR mouse	240	LOD	LOD	6.12E+05	6.29E+05	LOD	LOD
		180	LOD	LOD	6.12E+05	6.09E+05	LOD	LOD
		120	LOD	LOD	6.11E+05	5.99E+05	LOD	LOD
		90	LOD	LOD	6.29E+05	6.06E+05	LOD	LOD
		60	1.33E+03	1.21E+03	6.10E+05	6.25E+05	0.002	0.002
		30	1.25E+04	1.57E+04	6.23E+05	6.31E+05	0.02	0.025
		0	3.97E+04	4.12E+04	6.25E+05	6.37E+05	0.064	0.065
7-Hydroxycoumarin	Sprague Dawley rat	240	LOD	LOD	6.30E+05	6.18E+05	LOD	LOD
		180	LOD	LOD	6.29E+05	6.25E+05	LOD	LOD
		120	LOD	LOD	6.36E+05	6.49E+05	LOD	LOD
		90	LOD	LOD	6.11E+05	6.30E+05	LOD	LOD
		60	LOD	LOD	6.19E+05	6.07E+05	LOD	LOD
		30	8.21E+03	9.55E+03	6.30E+05	6.32E+05	0.013	0.015
		0	3.98E+04	4.10E+04	6.06E+05	5.99E+05	0.066	0.068
7-Hydroxycoumarin	Wistar Han rat	240	LOD	LOD	6.23E+05	6.17E+05	LOD	LOD
		180	LOD	LOD	6.51E+05	6.11E+05	LOD	LOD
		120	LOD	LOD	6.05E+05	6.24E+05	LOD	LOD
		90	LOD	LOD	6.10E+05	6.15E+05	LOD	LOD
		60	LOD	LOD	6.36E+05	6.05E+05	LOD	LOD
		30	6.78E+03	8.59E+03	6.20E+05	6.18E+05	0.011	0.014
		0	4.01E+04	3.94E+04	6.09E+05	6.14E+05	0.066	0.064
7-Hydroxycoumarin	Cynomolgus monkey	240	LOD	LOD	5.82E+05	6.25E+05	LOD	LOD
		180	LOD	LOD	6.01E+05	6.18E+05	LOD	LOD
		120	LOD	LOD	6.38E+05	6.14E+05	LOD	LOD
		90	LOD	LOD	6.38E+05	6.07E+05	LOD	LOD
		60	LOD	LOD	6.28E+05	6.20E+05	LOD	LOD
		30	7.22E+03	6.96E+03	6.42E+05	6.39E+05	0.011	0.011
		0	4.21E+04	4.15E+04	6.44E+05	6.43E+05	0.065	0.065
7-Hydroxycoumarin	Human	240	LOD	LOD	6.04E+05	6.05E+05	LOD	LOD
		180	LOD	LOD	6.45E+05	6.24E+05	LOD	LOD
		120	LOD	LOD	6.28E+05	6.50E+05	LOD	LOD
		90	1.43E+03	1.40E+03	6.42E+05	6.21E+05	0.002	0.002
		60	7.22E+03	8.24E+03	6.20E+05	6.28E+05	0.012	0.013
		30	1.69E+04	1.68E+04	6.27E+05	6.10E+05	0.027	0.028
		0	4.06E+04	3.99E+04	6.01E+05	6.03E+05	0.068	0.066

LOD = limit of detection

APPENDIX 5

01049-20010-ALC-0315-Hepatocytes Stability_Protocol

In Vitro Metabolic Stability of ALC-0315 in CD-1/ICR Mouse, Sprague Dawley Rat, Wistar Han Rat, Cynomolgus Monkey, and Human Hepatocytes

Testing Facility
Medicilon Preclinical Research (Shanghai) LLC
585 Chuanda Road
Pudong, Shanghai 201299
China

Study Number
01049-20010

Study Director
(b) (6)

Sponsor
Acuitas Therapeutics Inc.

CONTENTS

1. INTRODUCTION

1.1. Study Number

01049-20010

1.2. Study Title

In Vitro Metabolic Stability of ALC-0315 in CD-1/ICR Mouse, Sprague Dawley Rat, Wistar Han Rat, Cynomolgus Monkey, and Human Hepatocytes

1.3. Sponsor Representative

(b) (6)

Acuitas Therapeutics Inc.
6190 Agronomy Road, Suite 402
Vancouver BC V6T 1Z3
Canada

(b) (6)

1.4. Objective

To evaluate the *in vitro* metabolic stability of ALC-0315 in Hepatocytes from different species and to determine intrinsic clearance in each species.

1.5. Compliance

This is a non-GLP study and will be conducted according to the Standard Operating Procedures (SOPs) of Medicilon Preclinical Research (Shanghai) LLC.

1.6. Testing Facility

Medicilon Preclinical Research (Shanghai) LLC
585 Chuanda Road, Pudong, Shanghai 210299, China

1.7. Personnel

1.7.1. Study Director

(b) (6)

1.7.2. Alternate Contact

(b) (6)

1.8. Study Schedule

Study Initiation Date:	Signature date by Study Director
Experiment Start Date:	To be included in the final report
Experiment Termination Date:	To be included in the final report
Draft Report Issue Date:	To be included in the final report

2. MATERIALS

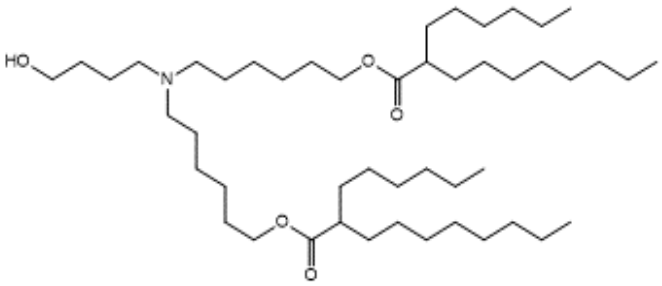
2.1. Test Article

Name: ALC-0315

Molecular Formula: $C_{48}H_{95}NO_5$

MW (g/mol): 766.27

Exact Mass: 765.72



2.2. Positive Control and Internal Standard

Testosterone and 7-hydroxycoumarin will be used as positive controls. Verapamil will be used as internal standard. The sources will be documented in the experimental records and presented in the report.

2.3. Hepatocytes

Cryopreserved hepatocytes of CD-1/ICR mouse, Sprague Dawley rat, Wistar Han rat, cynomolgus monkey, and human were purchased from qualified suppliers and stored in liquid nitrogen until use. The source(s) and lot numbers will be documented in the experimental records and presented in the final report.

3. EXPERIMENTAL PROCEDURES

(1) Preparation of stock solution: Appropriate amount of test article or positive control is weighed and dissolved in dimethyl sulfoxide (DMSO) to obtain a 10 mM stock solution.

(2) Preparation of 4 mM spiking solution:

Spiking Solution of Test Article or Positive Control			
Conc. of Stock Solution (mM)	Volume of Stock Solution (μL)	Volume of DMSO (μL)	Final Concentration (mM)
10	20	30	4

(3) Preparation of 2 μM dosing solution(2×) of test article or positive control:

Dosing Solution (2×) of Test Article or Positive Control			
Conc. of Spiking Solution (mM)	Volume of Spiking Solution (μL)	Volume of William's E Medium (μL)	Final Concentration (μM)
4	2	3998	2

(4) Preparation of hepatocyte suspension: Thaw cryopreserved hepatocytes in a 37℃ water bath. Transfer the hepatocytes to hepatocyte thawing medium (William's E Medium with 30% percoll and 5% FBS) and centrifuge at 100×g for 10 min at room temperature. Resuspend the cell pellet with William's E Medium and determine cell viability by trypan blue exclusion analysis and calculate the viable cell density. Dilute the hepatocytes with incubation medium to an appropriate density (2×10^6 viable cells/mL) and pre-warm at 37 °C for 10 min.

(5) 40 μL of each hepatocyte suspension is added to 96-well plates in duplicate for each time point (0, 30, 60, 90, 120, 180, and 240 min).

(6) For 0 min samples: 480 μL of internal standard solution (IS solution, 10 ng/mL verapamil in ethanol) is added, followed by 40 μL of pre-warmed 2× dosing solution. The final concentration of test article or positive control in the incubation mixture is 1 μM.

(7) For the 30, 60, 90, 120, 180, and 240 min samples, 40 μL of pre-warmed 2× dosing solution is added to initiate reaction. The final concentration of test article or positive control in the incubation mixture is 1 μM.

(8) The samples are incubated at 37℃ . At 30, 60, 90, 120, 180, and 240 min time points, stop the reaction by adding 480 μL ethanol containing internal standard to all of the duplicate wells.

(9) After quenching, shake the plates at 600 rpm for 10 min and then centrifuge them at 6,000 rpm for 15 min.

(10) The plates are sealed and stored at -20℃ freezer until bioanalysis.

(11) Thaw the plates at room temperature, centrifuge them at 6,000 rpm for 15 min, then transfer 200 μL of the supernatant from each well into a 96-well sample plate for LC-MS/MS analysis.

4. BIOANALYSIS

4.1. Instruments

Waters Acquity UPLC system

Sciex Triple Quad 6500+ with ESI ion source

4.2. LC/MS/MS Conditions

Column：ACQUITY UPLC BEH HILIC 1.7μm (2.1*100mm)

Gradient for ALC-0315:

Time (min)	Solvent A (%)	Solvent B (%)
0.01	5	95
1.50	40	60
2.00	40	60
2.01	5	95
2.50	5	95

A: 10mM ammonium formate, 0.1% Formic acid in water

B: 10mM ammonium formate, 0.1% Formic acid in acetonitrile

Flow rate：500 μL/min

Column temperature：40 ºC

Autosampler temperature: 4ºC

Compound	Q1(m/z)	Q3(m/z)	Retention Time (min)
ALC-0315	766.90	510.60	~1.07
Verapamil (IS)	455.30	165.20	~1.19

5. DATA ANALYSIS

The % remaining (parent compound) will be calculated by dividing the peak area ratio (compound peak area/ internal standard peak area) by the 0 min peak area ratio. The natural logarithm of % remaining is plotted against time and the slope of the fitted line will be determined as follows:

Elimination rate constant (k) = - slope

Half-life ($T_{1/2}$) (minutes) = 0.693/k

Intrinsic clearance predicted from the *in vitro* hepatocyte stability study will be calculated as shown below:

CL'_{int} (mL/min/kg) = k * V (1 mL incubation/10^6 cells) * Scaling Factor (10^6 cells/kg),

Scaling Factor (10^6 cells/kg) = Hepatocellularity (10^6 cells/g liver) * Normalized Liver Weight (g liver/kg body weight)

The scaling factors are listed in Table 1.

Table 1. Scaling Factors for Intrinsic Clearance Prediction in Mouse, Rat, Monkey, and Human Hepatocytes

Species	Hepatocellularity (10^6 cells/g liver)	Liver Weight (g/kg BW)	Scaling Factor (10^6 cells/kg)
Mouse	135	87.5	11812.5
Rat	117	40	4680
Monkey	120	32	3840
Human	99	25.7	2544.3

6. FINAL REPORT

After completion of the study, a draft report including the results, analysis and discussion will be sent to the Sponsor in Microsoft Word format.

One month after issuance of the draft report, if no requested revisions or instructions to finalize have been communicated by the Sponsor, the draft report will be issued as a final report, signed by the Study Director, and submitted to the Sponsor in Adobe Acrobat PDF format, containing hyperlinks, as applicable. Any modifications or changes to the draft report requested one month after issuance of the draft will be performed at additional cost to the Sponsor.

7. SIGNATURES

Sponsor Approval

(b) (6)

Sponsor Representative

July 15, 2020

Date

Study Director Approval

(b) (6)

Study Director

2020/07/15

Date