In Vitro Metabolic Stability of ALC-0315 in CD-1/ICR Mouse, Sprague Dawley Rat, Cynomolgus Monkey, and Human Liver S9 Fractions

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TABLE OF CONTENTS

SUMMARY

This study evaluated the *in vitro* metabolic stability of ALC-0315 in liver S9 fractions of CD-1/ICR mouse, Sprague Dawley rat, cynomolgus monkey, and human. ALC-0315 was stable after an approximately 2-hour incubation with liver S9 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

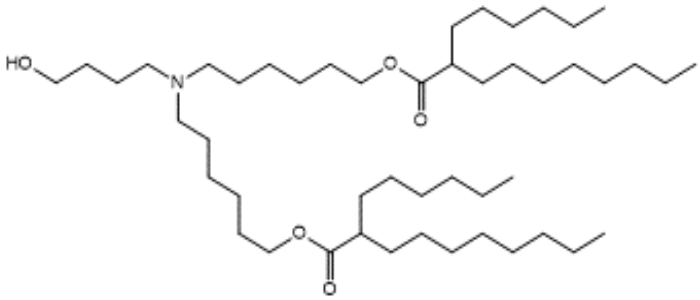
1. OBJECTIVE

To evaluate the *in vitro* metabolic stability of ALC-0315 in liver S9 fractions 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 Controls

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 Liver S9 Fractions

The following pooled liver S9 fractions of CD-1/ICR mouse, Sprague Dawley rat, cynomolgus monkey, and human were stored in a -70°C ultra low temperature freezer prior to use.

Species	Manufacturer	Cat. No.	Lot No.	Protein Concentration (mg/mL)
CD-1/ICR mouse (male)	XenoTech	M1000.S9	1310210	20
Sprague Dawley rat (male)	XenoTech	R1000.S9	1310212	20
Cynomolgus monkey (male)	XenoTech	P2000.S9	0910273	20
Human (mixed gender)	BioreclamationIVT	X008023	BKY	20

2.5 Coenzymes and Pore-forming Agent

NADPH (reduced β-nicotinamide adenine dinucleotide 2′-phosphate) tetrasodium salt was stored at 2~8°C in a refrigerator prior to use. UDPGA (uridine-diphosphate-glucuronic acid trisodium salt) and alamethicin were stored in a -20°C freezer prior to use.

Compound Name	Manufacturer	Cat. No.	Molecular Weight	Purity
NADPH	Roche Diagnostic	10621706001	833.35	97%
UDPGA	Sigma	U6751	646.23	≥98
Alamethicin	Aladdin	A132913	1964.3078	99%

3. EXPERIMENTAL PROCEDURES

3.1 Stock solutions preparation:

2.54 mg of ALC-0315 was weighed and dissolved in 331.48 µL of DMSO to obtain a 10 mM stock solution. 2.547 mg of testosterone was weighed and dissolved in 551.93 µL of DMSO to obtain a 16 mM solution. A 10 mM testosterone stock solution was then prepared by adding 60 µL DMSO to 100 µL of the 16 mM testosterone solution. 3.97 mg of 7-hydroxycoumarin was weighed and dissolved in 1224.25 µL of DMSO to obtain a 20 mM solution. A 10 mM 7-hydroxycoumarin stock solution was then prepared by adding 100 µL DMSO to 100 µL of the 20 mM 7-hydroxycoumarin solution. 5 mg of alamethicin was weighed and dissolved in 495 µL of 100 mM potassium phosphate buffer to obtain a 10 mg/mL alamethicin solution.

3.2 0.5 mM spiking solutions preparation:

Spiking Solution of Test Article or Positive Control			
Conc. of Stock Solution (mM)	Volume of Stock Solution (µL)	Volume of MeOH (µL)	Final Concentration (mM)
10	10	190	0.5

3.3 Preparation of 1.5× liver S9 suspensions (with alamethicin) containing test article or positive control:

1.5× Liver S9 Suspension (with Alamethicin) Containing Test Article or Positive Control						
Livers S9		0.5 mM Spiking Solution (µL)	10 mg/ml Alamethicin Solution	100 mM potassium phosphate buffer containing 5 mM of $MgCl_2$ (pH 7.4) (µL)	Final Concentration	
Conc. of Stock Solution (mg/mL)	Volume of Stock Solution (µL)				Liver S9 Protein (mg/mL)	Compound (µM)
20	37.5	1.5	1.9	459.1	1.5	1.5

3.4 1.5× liver S9 suspensions (with alamethicin) containing test article or positive control were kept on ice for 15 minutes.

3.5 28.51 mg of NADPH was weighed and dissolved in 2.851 mL of 100 mM potassium phosphate buffer to obtain a 12 mM NADPH working solution. 25 mg of UDPGA was weighed and dissolved in 6.448 mL of 100 mM potassium phosphate buffer to obtain a 6 mM UDPGA working solution. 2.8 mL of 12 mM NADPH working solution was added into 2.8 mL of 6 mM UDPGA working solution to obtain a 6 mM NADPH and 3 mM UDPGA mixed working solution. This mixed working solution was then pre-warmed at 37ºC.

3.6 30µL of liver S9 suspension (with alamethicin) containing 1.5 µM test article or positive control was added to 96-well plates in duplicate for each time point (0, 15, 30, 60, 90, and 120 min).

3.7 96-well incubation plates were pre-warmed at 37ºC for 5 min.

3.8 For 0 min samples: 450 µL of ethanol containing internal standard (IS solution) was added before 15 µL of pre-warmed mixed working solution (6 mM NADPH and 3 mM UDPGA) was added.

3.9 For other samples (15, 30, 60, 90, and 120 min): 15 µL of pre-warmed mixed working solution (6 mM NADPH and 3 mM UDPGA) was added to initiate the reaction.

Volume (µL)			Final Concentration in Incubation Mixture		
1.5× Liver S9 Suspension Containing Test Article or Positive Control	3×NADPH Working Solution	Total	Liver S9 Protein (mg/mL)	Test Article or Positive Control (µM)	NADPH (mM)
30	15	45	0.5	1	2

The samples were incubated at 37ºC and 450 µL of IS solution was added to stop the reaction at the corresponding time points (15, 30, 60, 90, and 120 min).

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

3.11 Then 200 µL of the supernatant from each well was transferred 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 1.7 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： 40ºC
Autosampler temperature: 4ºC
MS Conditions: MRM detection

Compound	Q1(m/z)	Q3(m/z)	DP	CE	Retention Time (min)
ALC-0315	766.90	510.60	100	66	~1.08
Verapamil (IS)	455.30	165.20	49	28	~1.21

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 were calculated, when possible, as indicated below.

Elimination rate constant (k) = - slope
Half-life ($t_{1/2}$) = 0.693/k

6. RESULTS

A summary of the % remaining parent compound and half-life of ALC-0315 obtained from a 2-hour incubation with liver S9 from CD-1/ICR mouse, Sprague Dawley rat, cynomolgus monkey, and human is presented in Table 1. The stability of ALC-0315 over time in each matrix is shown in Figure 1. Raw data is presented in Appendix 2.

The liver S9 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 2-hour incubation period, hence the test systems were considered to have yielded valid results. A summary of the % remaining parent compound and half-life of testosterone and 7-hydroxycoumarin is provided in Table 1. 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 liver S9 fractions of CD-1/ICR mouse, Sprague Dawley rat, cynomolgus monkey, and human. ALC-0315 was stable after an approximately 2-hour incubation with liver S9 from all these species.

Table 1. Summary of Liver S9 Stability of ALC-0315 , Testosterone and 7-Hydroxycoumarin

Compounds	Species		Percent Remaining (%)						$T_{1/2}$ (minute)
			0 min	15 min	30 min	60 min	90 min	120 min	
ALC-0315	CD-1/ICR Mouse	Mean	100.00	97.69	97.22	98.61	98.15	96.76	**>120**
		RSD of Area Ratio	0.03	0.03	0.01	0.02	0.00	0.02	
	Sprague Dawley Rat	Mean	100.00	98.85	99.62	99.62	98.85	98.46	**>120**
		RSD of Area Ratio	0.03	0.03	0.06	0.06	0.05	0.03	
	Cynomolgus Monkey	Mean	100.00	99.57	96.96	99.13	98.70	99.57	**>120**
		RSD of Area Ratio	0.04	0.02	0.01	0.01	0.01	0.01	
	Human	Mean	100.00	95.99	97.32	94.98	98.33	99.33	**>120**
		RSD of Area Ratio	0.06	0.03	0.04	0.00	0.04	0.05	
Testosterone	CD-1/ICR Mouse	Mean	100.00	59.38	35.71	6.89	BQL	BQL	**15.5**
		RSD of Area Ratio	0.05	0.11	0.01	0.16	N/A	N/A	
	Sprague Dawley Rat	Mean	100.00	BQL	BQL	BQL	BQL	BQL	**N/A**
		RSD of Area Ratio	0.05	N/A	N/A	N/A	N/A	N/A	
	Cynomolgus Monkey	Mean	100.00	81.12	68.58	45.76	27.19	16.74	**46.6**
		RSD of Area Ratio	0.11	0.11	0.02	0.06	0.02	0.08	
	Human	Mean	100.00	63.69	17.40	BQL	BQL	BQL	**11.9**
		RSD of Area Ratio	0.00	0.02	0.09	N/A	N/A	N/A	
7-Hydroxycoumarin	CD-1/ICR Mouse	Mean	100.00	40.06	16.68	3.57	1.89*	1.54*	**12.5**
		RSD of Area Ratio	0.00	0.11	0.02	0.17	0.18	0.20	
	Sprague Dawley Rat	Mean	100.00	71.60	48.00	26.17*	13.71*	11.92*	**28.3**
		RSD of Area Ratio	0.08	0.04	0.07	0.06	0.00	0.04	
	Cynomolgus Monkey	Mean	100.00	80.70	64.55	38.33	23.76	16.34	**44.8**
		RSD of Area Ratio	0.04	0.04	0.05	0.00	0.01	0.04	
	Human	Mean	100.00	69.01	43.17	19.16	8.29	3.68	**25.0**
		RSD of Area Ratio	0.02	0.00	0.04	0.03	0.03	0.12	

* The compound showed biphasic metabolic kinetics, i.e., an initial fast disappearance phase was followed by a slow disappearance phase. The data points marked with * 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 Liver S9

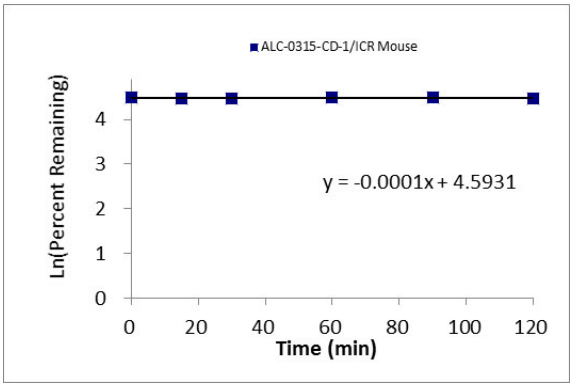
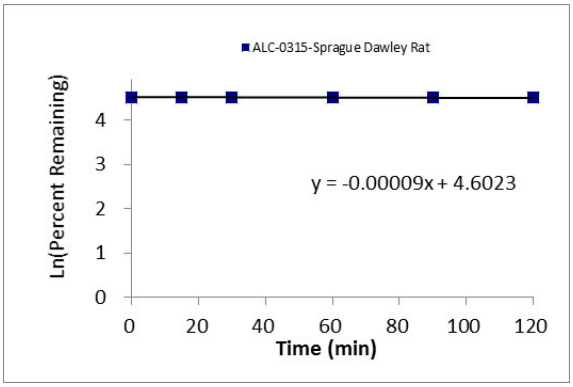
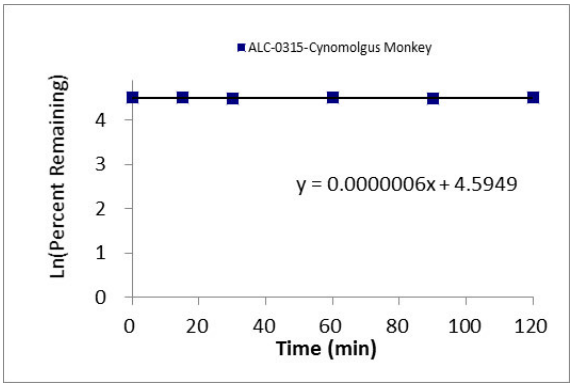
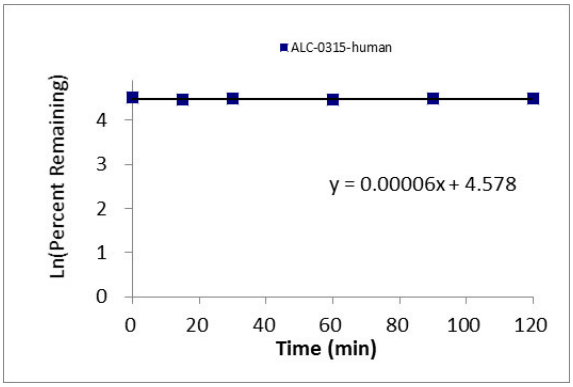
CD-1/ICR Mouse	Sprague Dawley Rat
	
Cynomolgus Monkey	**Human**
	

Figure 2. Stability of Testosterone in Mouse, Rat, Monkey and Human Liver S9

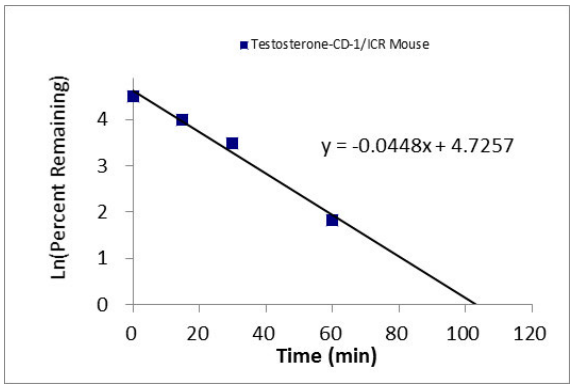
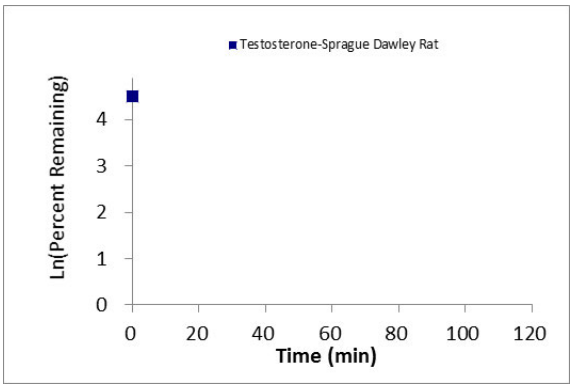
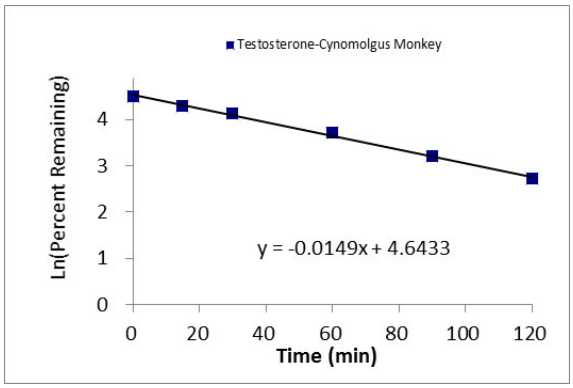
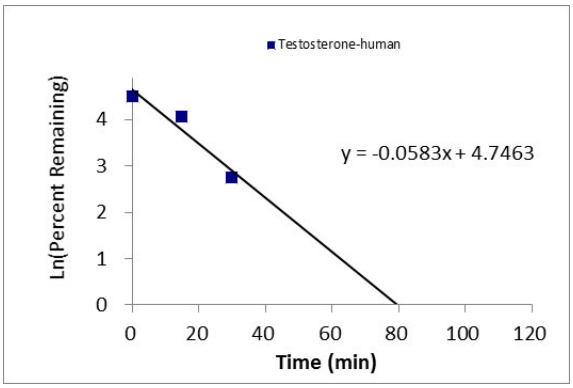
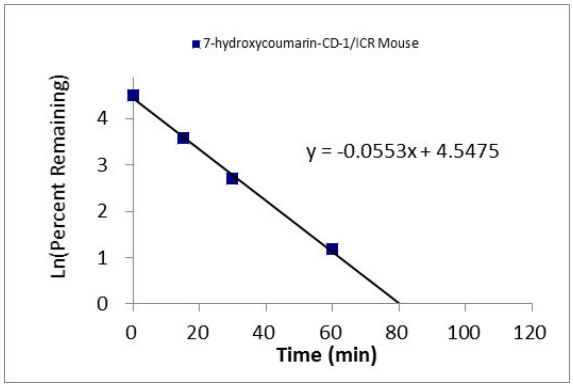
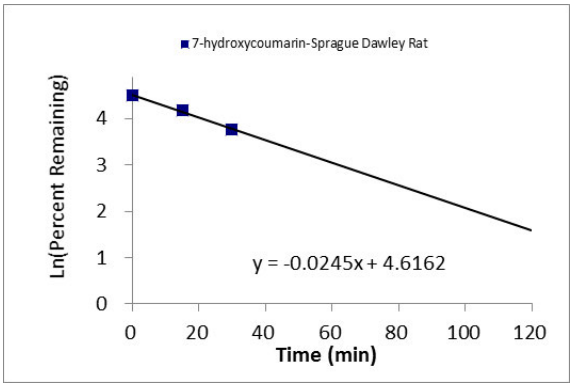
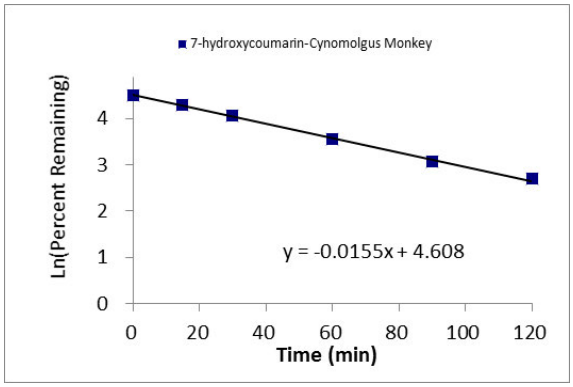
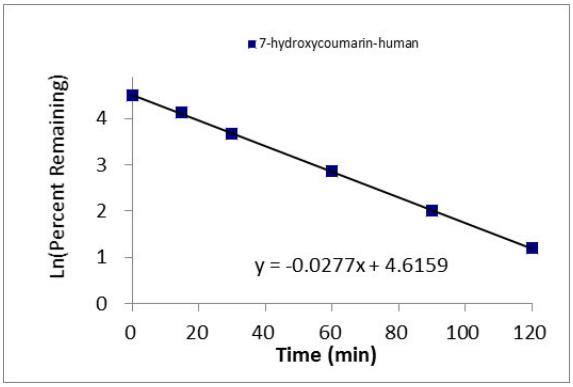
CD-1/ICR Mouse	Sprague Dawley Rat
	
Cynomolgus Monkey	**Human**
	

Figure 3. Stability of 7-Hydroxycoumarin in Mouse, Rat, Monkey and Human Liver S9

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

8. APPENDICES

Appendix 1 – Representative Chromatograms of ALC-0315 in Mouse, Rat, Monkey and Human Liver S9

Appendix 2 – Stability of ALC-0315 in Mouse, Rat, Monkey and Human Liver S9 – Raw Data

Appendix 3 – Stability of Testosterone in Mouse, Rat, Monkey and Human Liver S9 – Raw Data

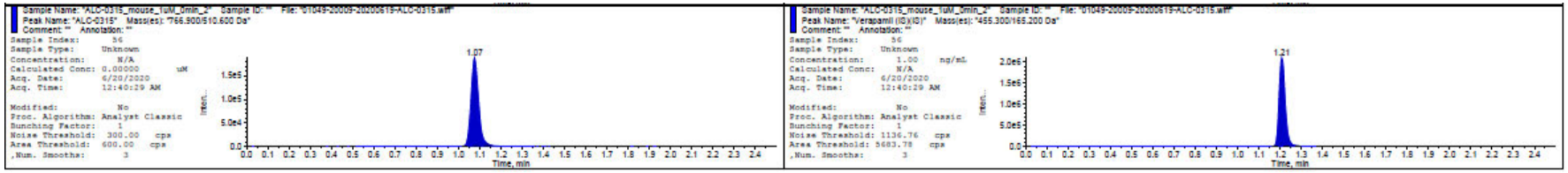
Appendix 4 – Stability of 7-Hydroxycoumarin in Mouse, Rat, Monkey and Human Liver S9 – Raw Data

Appendix 5 – 01049-20009-S9 stability_protocol

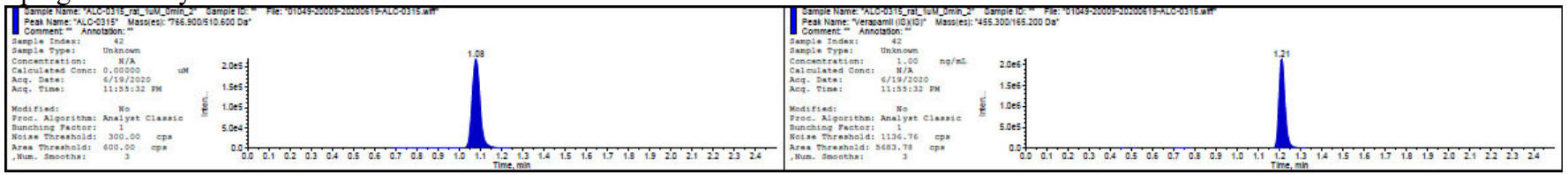
APPENDIX 1

Representative Chromatograms of ALC-0315 in Mouse, Rat, Monkey and Human Liver S9

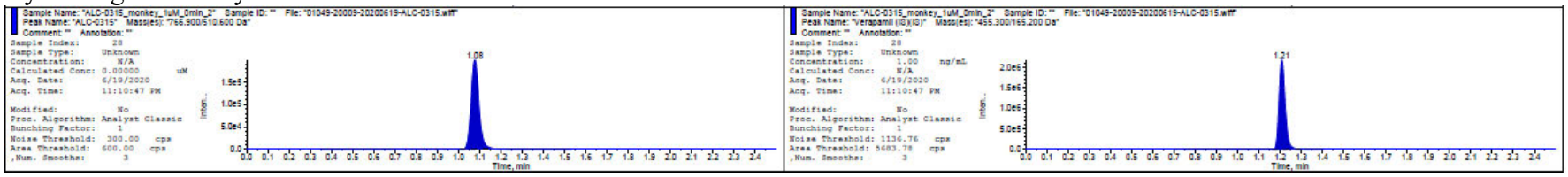
CD 1/ICR mouse



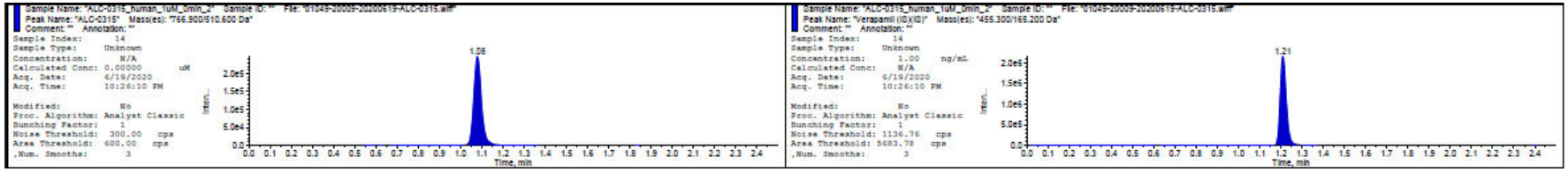
Sprague Dawley rat



Cynomolgus monkey



Human



APPENDIX 2

Stability of ALC-0315 in Mouse, Rat, Monkey and Human Liver S9 – Raw Data

Compounds	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	0	4.33E+05	4.46E+05	4.08E+06	4.06E+06	0.106	0.110
		15	4.15E+05	4.49E+05	4.02E+06	4.15E+06	0.103	0.108
		30	4.21E+05	4.47E+05	4.06E+06	4.23E+06	0.104	0.106
		60	4.27E+05	4.48E+05	4.07E+06	4.15E+06	0.105	0.108
		90	4.47E+05	4.43E+05	4.23E+06	4.16E+06	0.106	0.106
		120	4.24E+05	4.44E+05	4.13E+06	4.17E+06	0.103	0.106
ALC-0315	Sprague Dawley Rat	0	5.23E+05	5.47E+05	4.12E+06	4.13E+06	0.127	0.133
		15	5.16E+05	5.37E+05	4.10E+06	4.11E+06	0.126	0.131
		30	5.10E+05	5.63E+05	4.12E+06	4.17E+06	0.124	0.135
		60	5.14E+05	5.59E+05	4.14E+06	4.15E+06	0.124	0.135
		90	5.22E+05	5.58E+05	4.20E+06	4.19E+06	0.124	0.133
		120	5.30E+05	5.50E+05	4.23E+06	4.22E+06	0.125	0.131
ALC-0315	Cynomolgus Monkey	0	4.57E+05	4.88E+05	4.07E+06	4.15E+06	0.112	0.118
		15	4.69E+05	4.90E+05	4.15E+06	4.21E+06	0.113	0.116
		30	4.60E+05	4.69E+05	4.13E+06	4.18E+06	0.111	0.112
		60	4.66E+05	4.81E+05	4.13E+06	4.19E+06	0.113	0.115
		90	4.73E+05	4.82E+05	4.18E+06	4.23E+06	0.113	0.114
		120	4.86E+05	4.83E+05	4.22E+06	4.23E+06	0.115	0.114
ALC-0315	Human	0	6.76E+05	6.00E+05	4.34E+06	4.20E+06	0.156	0.143
		15	6.28E+05	5.97E+05	4.27E+06	4.27E+06	0.147	0.140
		30	6.60E+05	6.02E+05	4.41E+06	4.26E+06	0.150	0.141
		60	6.17E+05	6.07E+05	4.34E+06	4.27E+06	0.142	0.142
		90	6.44E+05	6.03E+05	4.27E+06	4.21E+06	0.151	0.143
		120	6.44E+05	6.14E+05	4.17E+06	4.28E+06	0.154	0.143

APPENDIX 3

Stability of Testosterone in Mouse, Rat, Monkey and Human Liver S9 – Raw Data

Compounds	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	0	1.74E+04	1.71E+04	5.35E+05	5.68E+05	0.032	0.030
		15	1.06E+04	1.16E+04	6.22E+05	5.77E+05	0.017	0.020
		30	6.44E+03	6.29E+03	5.72E+05	5.69E+05	0.011	0.011
		60	1.10E+03	1.37E+03	5.79E+05	5.69E+05	0.002	0.002
		90	LOD	LOD	5.84E+05	5.74E+05	LOD	LOD
		120	LOD	LOD	5.70E+05	5.75E+05	LOD	LOD
Testosterone	Sprague Dawley Rat	0	1.52E+04	1.62E+04	6.17E+05	6.14E+05	0.025	0.026
		15	LOD	LOD	6.05E+05	5.74E+05	LOD	LOD
		30	LOD	LOD	6.53E+05	5.96E+05	LOD	LOD
		60	LOD	LOD	5.75E+05	5.65E+05	LOD	LOD
		90	LOD	LOD	6.06E+05	5.80E+05	LOD	LOD
		120	LOD	LOD	5.98E+05	6.00E+05	LOD	LOD
Testosterone	Cynomolgus Monkey	0	1.68E+04	1.43E+04	5.88E+05	5.86E+05	0.029	0.024
		15	1.22E+04	1.32E+04	6.16E+05	5.72E+05	0.020	0.023
		30	1.08E+04	1.08E+04	6.04E+05	5.85E+05	0.018	0.018
		60	6.82E+03	7.12E+03	5.88E+05	5.64E+05	0.012	0.013
		90	4.29E+03	4.11E+03	5.87E+05	5.79E+05	0.007	0.007
		120	2.78E+03	2.38E+03	5.94E+05	5.69E+05	0.005	0.004
Testosterone	Human	0	1.66E+04	1.57E+04	5.95E+05	5.63E+05	0.028	0.028
		15	1.02E+04	1.05E+04	5.81E+05	5.81E+05	0.018	0.018
		30	3.05E+03	2.85E+03	5.88E+05	6.27E+05	0.005	0.005
		60	LOD	LOD	5.92E+05	5.75E+05	LOD	LOD
		90	LOD	LOD	6.07E+05	6.32E+05	LOD	LOD
		120	LOD	LOD	5.50E+05	5.76E+05	LOD	LOD

LOD = Limit of detection

APPENDIX 4

Stability of 7-Hydroxycoumarin in Mouse, Rat, Monkey and Human Liver S9 – Raw Data

Compounds	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	0	2.69E+04	2.63E+04	5.42E+05	5.29E+05	0.050	0.050
		15	1.20E+04	1.01E+04	5.59E+05	5.52E+05	0.021	0.018
		30	4.62E+03	4.53E+03	5.49E+05	5.54E+05	0.008	0.008
		60	1.11E+03	8.20E+02	5.54E+05	5.27E+05	0.002	0.002
		90	5.99E+02	4.34E+02	5.62E+05	5.30E+05	0.001	0.001
		120	3.69E+02	4.87E+02	5.59E+05	5.58E+05	0.001	0.001
7-Hydroxycoumarin	Sprague Dawley Rat	0	2.60E+04	2.51E+04	5.20E+05	5.61E+05	0.050	0.045
		15	1.88E+04	1.82E+04	5.37E+05	5.52E+05	0.035	0.033
		30	1.29E+04	1.20E+04	5.39E+05	5.52E+05	0.024	0.022
		60	6.94E+03	6.43E+03	5.38E+05	5.40E+05	0.013	0.012
		90	3.57E+03	3.60E+03	5.49E+05	5.56E+05	0.007	0.006
		120	2.92E+03	3.11E+03	5.32E+05	5.34E+05	0.005	0.006
7-Hydroxycoumarin	Cynomolgus Monkey	0	2.46E+04	2.60E+04	5.32E+05	5.32E+05	0.046	0.049
		15	2.18E+04	2.06E+04	5.53E+05	5.50E+05	0.039	0.037
		30	1.65E+04	1.74E+04	5.57E+05	5.45E+05	0.030	0.032
		60	9.83E+03	9.77E+03	5.40E+05	5.35E+05	0.018	0.018
		90	6.29E+03	6.06E+03	5.52E+05	5.40E+05	0.011	0.011
		120	4.19E+03	4.03E+03	5.23E+05	5.34E+05	0.008	0.008
7-Hydroxycoumarin	Human	0	2.65E+04	2.65E+04	5.32E+05	5.16E+05	0.050	0.051
		15	1.88E+04	1.83E+04	5.37E+05	5.24E+05	0.035	0.035
		30	1.23E+04	1.15E+04	5.49E+05	5.42E+05	0.022	0.021
		60	5.42E+03	5.06E+03	5.48E+05	5.33E+05	0.010	0.009
		90	2.28E+03	2.37E+03	5.55E+05	5.52E+05	0.004	0.004
		120	9.57E+02	1.11E+03	5.60E+05	5.53E+05	0.002	0.002

APPENDIX 5

01049-20009-S9 stability_protocol

In Vitro Metabolic Stability of ALC-0315 in CD-1/ICR Mouse, Sprague Dawley Rat, Cynomolgus Monkey, and Human Liver S9 Fractions

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

Study Number
01049-20009

Study Director
(b) (6)

Sponsor
Acuitas Therapeutics Inc.

CONTENTS

1. INTRODUCTION

1.1. Study Number

01049-20009

1.2. Study Title

In Vitro Metabolic Stability of ALC-0315 in CD-1/ICR Mouse, Sprague Dawley Rat, Cynomolgus Monkey, and Human Liver S9 Fractions

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 liver S9 from different 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)

[redacted]

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

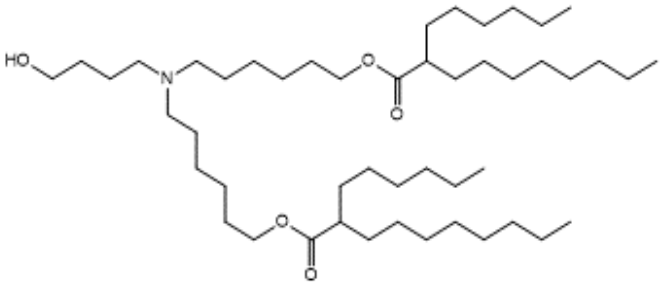
2.1. Test Article

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 experimental records and presented in the report.

2.3. Liver Microsomes and Cofactor

Liver S9 fractions of CD-1/ICR mouse, Sprague Dawley rat, cynomolgus monkey and human were purchased from qualified supplier and stored in a -70ºC ultra low temperature freezer.

NADPH (reduced β-Nicotinamide adenine dinucleotide 2′-phosphate tetrasodium salt) were purchased from qualified supplier and stored at 2-8ºC in a refrigerator.

UDPGA (uridine-diphosphate-glucuronic acid trisodium salt) and alamethicin were purchased from qualified suppliers and stored in a -20ºC freezer.

The source and lot numbers will be documented in the experimental records and presented in the final report.

3. EXPERIMENTAL PROCEDURES

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

(2) Preparation of 0.5 mM spiking solutions:

Spiking Solution of Test Article or Positive Control			
Conc. of stock solution	Volume of stock solution (µL)	Volume of MeOH (µL)	Final Concentration
10 mM	10	190	0.5 mM

(3) Preparation of 1.5× liver S9 suspensions with alamethicin containing test article or positive control:

1.5× Liver S9 Suspension with Alamethicin containing Test article or Positive control						
Livers S9		0.5 mM spiking solution (µL)	10 mg/ml Alamethicin	100 mM potassium phosphate buffer containing 5 mM of $MgCl_2$ (pH 7.4) (µL)	Final Concentration	
Conc. of stock solution (mg/mL)	Volume of stock solution (µL)				Liver S9 protein (mg/mL)	Compound (µM)
20	37.5	1.5	1.9	459.1	1.5	1.5

(4) 1.5× liver S9 suspensions with alamethicin containing test article or positive control are kept on ice for 15 minutes.

(5) 3× master mix of cofactors (6 mM NADPH and 3 mM UDPGA in 100 mM potassium phosphate buffer containing 5 mM of $MgCl_2$, pH7.4) is prepared and then pre-warmed to 37ºC.

(6) 30 µL of liver S9 suspension with alamethicin containing 1.5 µM test article or positive control is added to 96-well plates in duplicate for each time point (0, 15, 30, 60, 90, and 120 min).

(7) 96-well incubation plates (from Step 6) are pre-warmed at 37 ºC for 5 min.

(8) For 0-min samples: 450 µL ethanol containing internal standard (IS solution) is added, followed by 15 µL pre-warmed 3× master mix of cofactors.

(9) For the 15, 30, 60, 90, and 120 min samples, 15 µL pre-warmed 3× master mix of cofactors is added to initiate reaction.

Volume of final incubation system (µL)			Final Concentration			
1.5× Liver S9 Suspension with Alamethicin containing Test article or Positive control	3× Master Mix of Cofactors	Total Volume	Liver S9 protein (mg/mL)	Compound (µM)	UDPGA (mM)	NADPH (mM)
30	15	45	1	1	1	2

The samples are incubated at 37 ºC and 450 µL IS solution (10ng/mL verapamil in ethanol) is added to stop the reaction at the corresponding time points (15, 30, 60, 90, and 120 min).

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

(11) The plates are sealed and stored at -20 ºC in a freezer until bioanalysis.

(12) 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 Chromatography Parameters 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
ALC-0315	766.90	510.60	100	66	~1.07
Verapamil	455.30	165.20	49	28	~1.19

5. DATA ANALYSIS

The % remaining will be 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 will be plotted against time, and the slope of the regression line will be determined. Then elimination constant and half-life will be calculated as below.

Elimination rate constant (k) = - slope

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

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)

June 17, 2020

Sponsor Representative

Date

Study Director Approval

(b) (6)

2020/06/17

Study Director

Date