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

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SUMMARY

This study evaluated the *in vitro* metabolic stability of ALC-0315 in liver microsomes of CD-1/ICR mouse, Sprague Dawley rat, Wistar Han rat, cynomolgus monkey, and human. ALC-0315 was stable after an approximately 2-hour incubation with liver microsomes 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) _____ 2020/08/03

Study Director Date

Sponsor Approval:

(b) (6) _____ August 3, 2020

Study Monitor Date

1. OBJECTIVE

To evaluate the in vitro metabolic stability of ALC-0315 in liver microsomes from different species.

2. MATERIALS

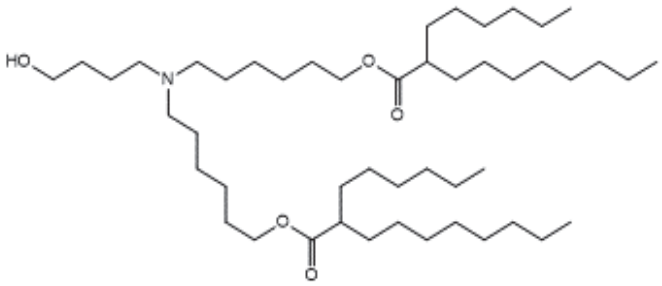
2.1 Test Article

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
Ketanserin	TCI	74050-98-9	K0051	NPGAF-CO	395.43

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

2.4 Liver Microsomes and Cofactor

The following pooled liver microsomes of CD-1/ICR mouse, Sprague Dawley rat, Wistar Han 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	1910002	20
Sprague Dawley rat (male)	XenoTech	R1000	1910100	20
Wistar Han rat	BioIVT	BCF	201801BCF	20
Cynomolgus monkey (male)	RILD Shanghai	LM-SXH-02M	NXNN	20
Human (mixed gender)	XenoTech	H0610	1810003	20

2.5 Coenzyme

NADPH (reduced β-nicotinamide adenine dinucleotide 2′-phosphate) tetrasodium salt was stored at 2-8°C in a refrigerator prior to use.

Compound Name	Manufacturer	Cat. No.	Molecular Weight	Purity
NADPH	Roche Diagnostic	10621706001	833.35	97%

3. EXPERIMENTAL PROCEDURES

3.1 Stock solution: 2.54 mg of ALC-0315 was weighed and dissolved in 331.48 µL of DMSO to obtain a 10 mM stock solution. 3.237 mg of ketanserin was weighed and dissolved in 818.60 µL of DMSO to obtain a 10 mM stock solution.

3.2 0.5 mM spiking solution:

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 1.5× liver microsomes suspension containing test article or positive control:

1.5× Liver Microsomes Suspension Containing Test Article or Positive Control					
Liver Microsomes		0.5 mM spiking solution (μL)	100 mM potassium phosphate buffer (pH 7.4) (μL)	Final Concentration	
Conc. of stock suspension (mg/mL)	Volume of stock suspension (μL)			Liver microsomal protein (mg/mL)	Compound (μM)
20	18.75	1.5	479.75	0.75	1.5

3.4 22.98 mg of NADPH was weighed and dissolved in 4.596 mL of 100 mM potassium phosphate buffer to obtain a 6 mM NADPH working solution. This working solution was then pre-warmed at 37ºC.

3.5 30 μL of 1.5× liver microsomes suspension containing 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).

3.6 96-well incubation plates were pre-warmed at 37 ºC for 5 min.

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

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

Volume (μL)			Final Concentration in Incubation Mixture		
1.5× Liver Microsomes Suspension Containing Test Article or Positive Control	3×NADPH working solution	Total	Liver microsomal protein (mg/mL)	Test Article or Positive Control (μM)	NADPH (mM)
30	15	45	0.5	1	2

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

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

3.10 200 μL of supernatant was transferred 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 1.7 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: 10 mM ammonium formate, 0.1% formic acid in water

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

Flow rate：500 μL/min

Column temperature：40 °C

Autosampler temperature: 4°C

MS Conditions: MRM detection

Compound	Q1(m/z)	Q3(m/z)	DP	CE	Retention Time
ALC-0315	766.90	510.60	100	66	~1.13
Verapamil (IS)	455.30	165.20	49	28	~1.25

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, ketanserin) 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 ($t_{1/2}$) = 0.693/k

The *in vitro* intrinsic clearance, CL'_{int}, was calculated from the $t_{1/2}$ as follows:

CL'_{int} = (0.693/$t_{1/2}$) × (1/ (microsomal protein concentration (0.5 mg/mL))) × Scaling Factor

The scaling factors are listed in Table 1.

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

Species	Microsomal Protein (mg) per Gram of Liver	Liver Weight (g) per kg Body Weight	Scaling Factor (mg/kg)[a]	Hepatic Blood Flow (mL/min/kg)
Mouse	45	87.5	3937.5	90
Rat	44.8	40	1792	55.2
Monkey	45	32.5	1462.5	44
Human	48.8	25.7	1254.2	20.7

[a]Microsomal protein (mg/g liver) × liver weight (g)/kg body weight

6. RESULTS

A summary of the % remaining parent compound, CL'_{int} and half-life of ALC-0315 obtained from a 2-hour incubation of ALC-0315 with liver microsomes 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 liver microsomes used in this study were tested for activity using a metabolism control substrate 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 compound (ketanserin) during the 2-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 ketanserin is provided in Table 2. The stability of ketanserin over time in each matrix is shown in Figure 2. Raw data is presented in Appendix 3.

7. CONCLUSION

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

Table 2. Summary of Liver Microsomal Stability of ALC-0315 and Ketanserin

Test Article	Species		Percent Remaining (%)						$t_{1/2}$ (minute)	CL'int (mL/min/kg)
			0 min	15 min	30 min	60 min	90 min	120 min		
ALC-0315	CD-1/ICR mouse	Mean	100	98.77	97.78	100.49	97.78	96.54	**>120**	<45.5
		RSD of Area Ratio	0.01	0.01	0.01	0	0.04	0.04		
	Sprague Dawley rat	Mean	100	94.39	96.26	99.73	98.66	95.99	**>120**	<20.7
		RSD of Area Ratio	0.05	0.05	0.05	0.05	0.03	0.04		
	Wistar Han rat	Mean	100	96.34	97.32	98.54	94.15	93.66	**>120**	<20.7
		RSD of Area Ratio	0.03	0.03	0.06	0.01	0.01	0.04		
	Cynomolgus monkey	Mean	100	97.96	96.18	100	97.96	97.71	**>120**	<16.9
		RSD of Area Ratio	0.05	0.03	0.01	0.02	0.03	0.03		
	Human	Mean	100	100.24	99.76	101.45	100.48	98.31	**>120**	<14.5
		RSD of Area Ratio	0.03	0.02	0.02	0.02	0.06	0.05		
Ketanserin	CD-1/ICR mouse	Mean	100	61.73	37.16	17.24*	10.16*	6.43*	**21.0**	260
		RSD of Area Ratio	0.04	0.01	0.02	0.05	0.01	0.05		
	Sprague Dawley rat	Mean	100	74.03	51.43	26.11	16.08*	10.01*	**30.7**	80.9
		RSD of Area Ratio	0.04	0.02	0.03	0.05	0.03	0.03		
	Wistar Han rat	Mean	100	54.03	25.10	6.76	2.35	1.18*	**16.4**	151
		RSD of Area Ratio	0.02	0.02	0.01	0.07	0.04	0.06		
	Cynomolgus monkey	Mean	100	71.44	47.42	24.00	13.05*	8.35*	**28.9**	70.1
		RSD of Area Ratio	0.03	0.02	0.01	0.02	0.04	0.02		
	Human	Mean	100	77.74	57.56	38.26	26.22*	24.46*	**43.1**	40.3
		RSD of Area Ratio	0.09	0.01	0.01	0.04	0.12	0.05		

* 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.

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

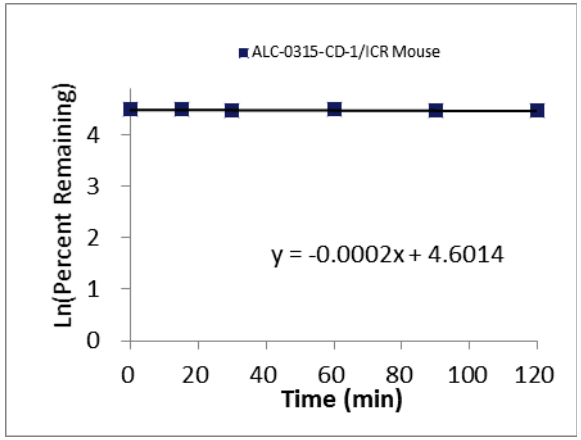
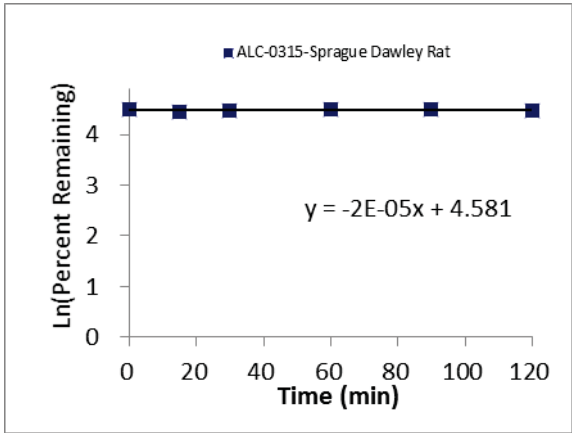
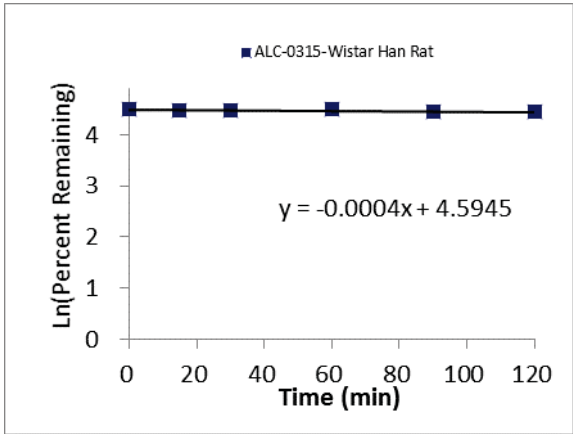
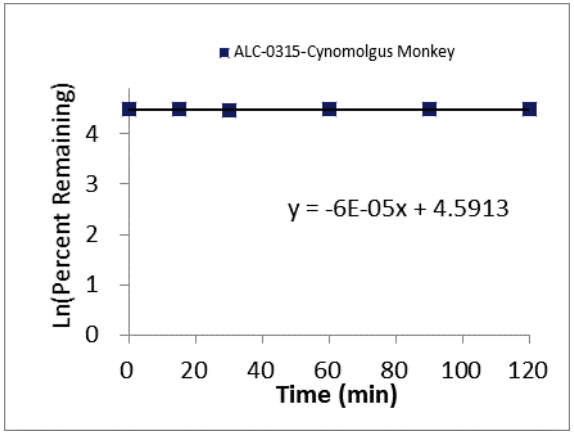
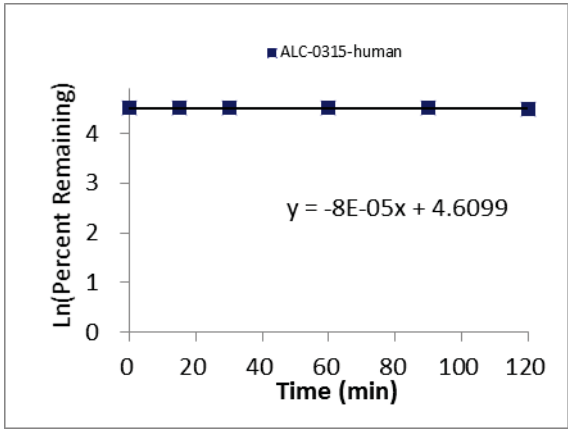
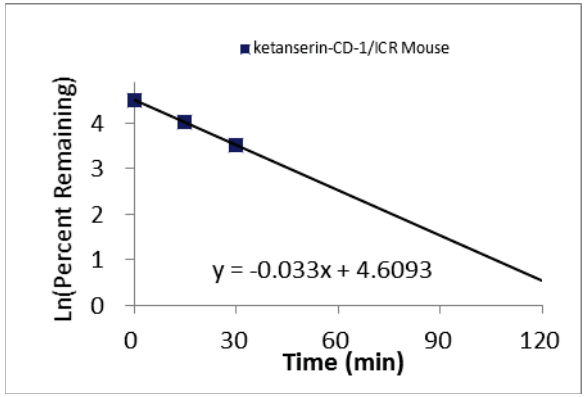
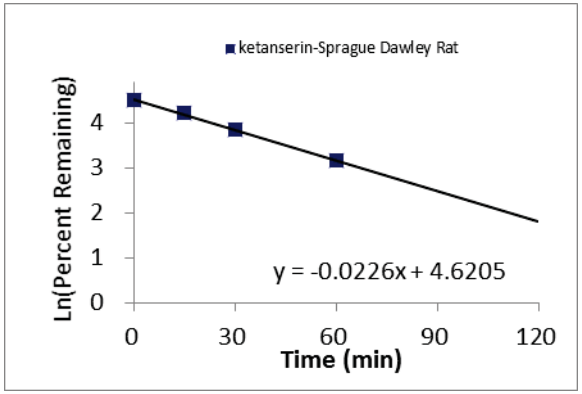
CD-1/ICR Mouse	Sprague Dawley Rat	Wistar Han Rat
		
Cynomolgus Monkey	**Human**	
		

Figure 2. Stability of Ketanserin in Mouse, Rat, Monkey and Human Liver Microsomes

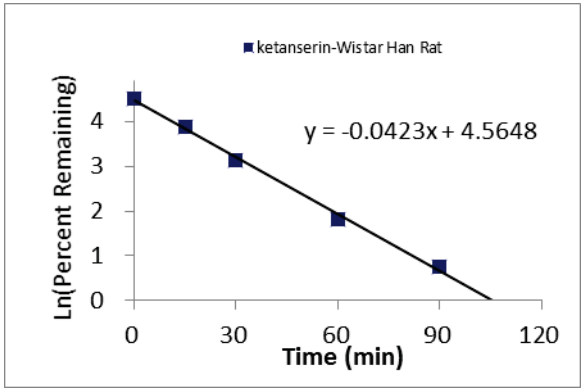
CD-1/ICR Mouse



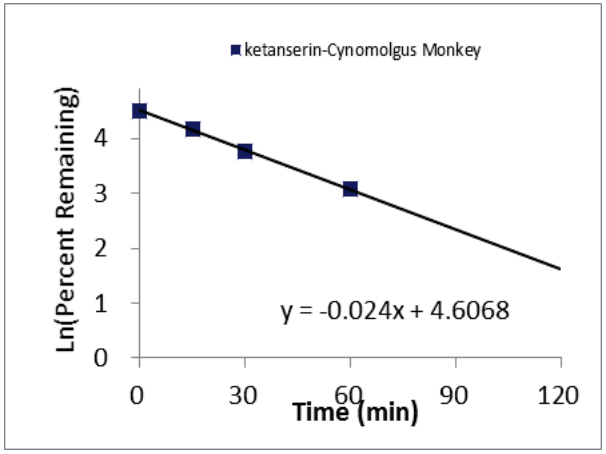
Sprague Dawley Rat



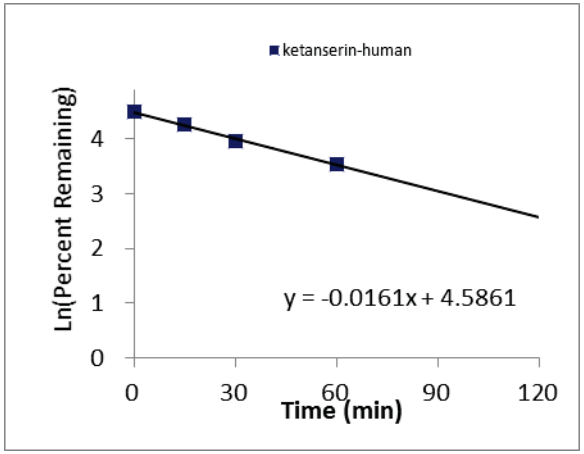
Wistar Han Rat



Cynomolgus Monkey



Human



8. APPENDICES

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

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

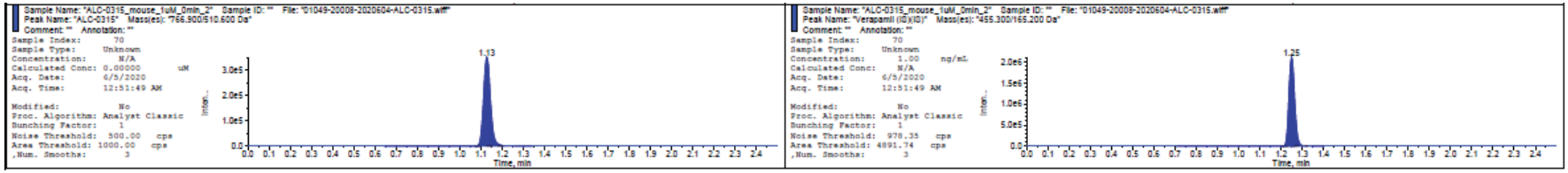
Appendix 3 – Stability of Ketanserin in Mouse, Rat, Monkey and Human Liver Microsomes – Raw Data

Appendix 4 –01049-20008-microsomal stability protocol

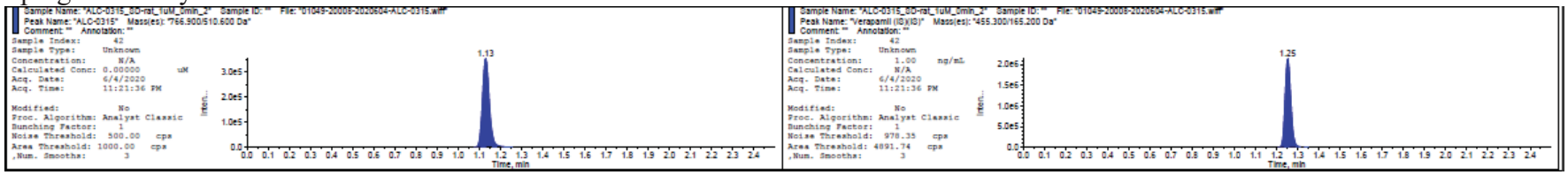
APPENDIX 1

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

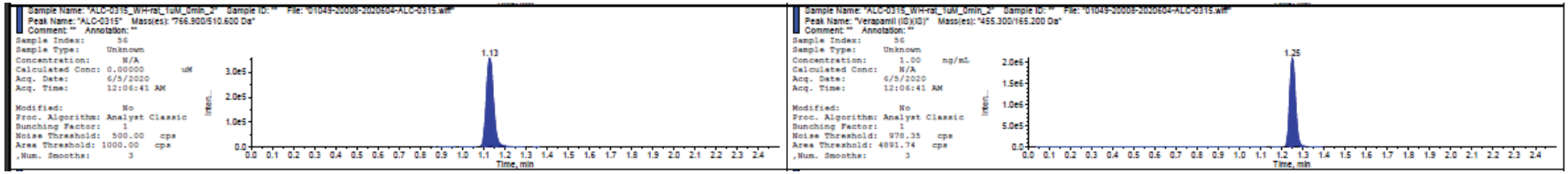
CD-1/ICR mouse



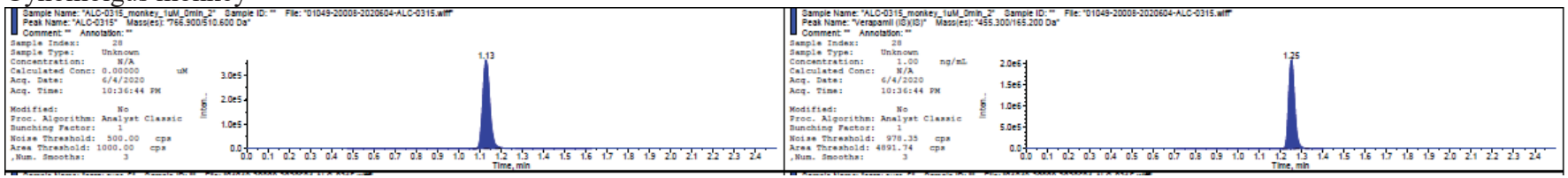
Sprague Dawley rat



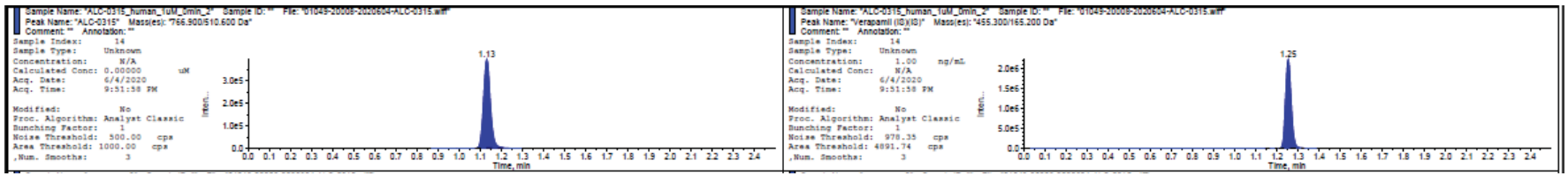
Wistar Han rat



Cynomolgus monkey



Human



APPENDIX 2

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

Stability of ALC-0315 in Mouse, Rat, Monkey and Human Liver Microsomes – 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	0	8.00E+05	7.81E+05	3.91E+06	3.89E+06	0.20	0.20
		15	8.00E+05	7.73E+05	3.96E+06	3.91E+06	0.20	0.20
		30	7.80E+05	7.99E+05	3.99E+06	3.99E+06	0.20	0.20
		60	8.12E+05	8.41E+05	4.01E+06	4.11E+06	0.20	0.20
		90	7.79E+05	7.98E+05	4.04E+06	3.92E+06	0.19	0.20
		120	7.71E+05	7.76E+05	4.05E+06	3.86E+06	0.19	0.20
ALC-0315	Sprague Dawley rat	0	6.97E+05	7.73E+05	3.87E+06	3.98E+06	0.18	0.19
		15	6.87E+05	7.25E+05	4.04E+06	3.96E+06	0.17	0.18
		30	6.94E+05	7.47E+05	3.99E+06	4.01E+06	0.17	0.19
		60	7.16E+05	7.61E+05	3.99E+06	3.93E+06	0.18	0.19
		90	7.19E+05	7.55E+05	4.00E+06	4.00E+06	0.18	0.19
		120	6.82E+05	7.50E+05	3.93E+06	4.06E+06	0.17	0.19
ALC-0315	Wistar Han rat	0	7.65E+05	8.07E+05	3.81E+06	3.87E+06	0.20	0.21
		15	7.76E+05	8.05E+05	4.02E+06	3.98E+06	0.19	0.20
		30	7.59E+05	8.35E+05	3.98E+06	4.02E+06	0.19	0.21
		60	7.95E+05	8.05E+05	3.99E+06	3.95E+06	0.20	0.20
		90	7.80E+05	7.57E+05	4.06E+06	3.90E+06	0.19	0.19
		120	7.22E+05	8.17E+05	3.89E+06	4.12E+06	0.19	0.20
ALC-0315	Cynomolgus monkey	0	7.65E+05	8.06E+05	4.02E+06	3.97E+06	0.19	0.20
		15	7.65E+05	8.11E+05	4.07E+06	4.12E+06	0.19	0.20
		30	7.53E+05	7.62E+05	4.02E+06	3.98E+06	0.19	0.19
		60	7.80E+05	8.28E+05	4.01E+06	4.16E+06	0.19	0.20
		90	7.55E+05	8.13E+05	4.03E+06	4.13E+06	0.19	0.20
		120	7.87E+05	8.03E+05	4.18E+06	4.11E+06	0.19	0.20
ALC-0315	Human	0	7.90E+05	8.60E+05	3.90E+06	4.10E+06	0.20	0.21
		15	8.20E+05	8.50E+05	4.00E+06	4.10E+06	0.21	0.21
		30	8.20E+05	8.50E+05	4.00E+06	4.10E+06	0.20	0.21
		60	8.30E+05	8.50E+05	3.90E+06	4.10E+06	0.21	0.21
		90	8.60E+05	7.80E+05	4.00E+06	3.90E+06	0.22	0.20
		120	8.60E+05	8.00E+05	4.10E+06	4.10E+06	0.21	0.20

APPENDIX 3

Stability of Ketanserin in Mouse, Rat, Monkey and Human Liver Microsomes – Raw Data

Stability of Ketanserin in Mouse, Rat, Monkey and Human Liver Microsomes – 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
Ketanserin	CD-1/ICR mouse	0	1.93E+06	1.99E+06	8.68E+05	8.45E+05	2.22	2.36
		15	1.18E+06	1.17E+06	8.32E+05	8.31E+05	1.42	1.41
		30	7.30E+05	7.08E+05	8.43E+05	8.45E+05	0.87	0.84
		60	3.42E+05	3.24E+05	8.37E+05	8.49E+05	0.41	0.38
		90	1.94E+05	1.94E+05	8.29E+05	8.36E+05	0.23	0.23
		120	1.20E+05	1.28E+05	8.43E+05	8.39E+05	0.14	0.15
Ketanserin	Sprague Dawley rat	0	2.00E+06	1.93E+06	8.58E+05	8.74E+05	2.33	2.21
		15	1.42E+06	1.46E+06	8.57E+05	8.57E+05	1.66	1.70
		30	9.99E+05	1.00E+06	8.34E+05	8.78E+05	1.20	1.14
		60	5.01E+05	5.15E+05	8.76E+05	8.37E+05	0.57	0.61
		90	3.16E+05	3.07E+05	8.43E+05	8.62E+05	0.37	0.36
		120	1.91E+05	1.89E+05	8.55E+05	8.14E+05	0.22	0.23
Ketanserin	Wistar Han rat	0	2.02E+06	2.08E+06	8.55E+05	8.52E+05	2.36	2.44
		15	1.08E+06	1.09E+06	8.41E+05	8.28E+05	1.28	1.31
		30	5.31E+05	5.23E+05	8.76E+05	8.71E+05	0.61	0.60
		60	1.29E+05	1.41E+05	8.41E+05	8.24E+05	0.15	0.17
		90	4.80E+04	4.97E+04	8.74E+05	8.55E+05	0.05	0.06
		120	2.31E+04	2.42E+04	8.56E+05	8.22E+05	0.03	0.03
Ketanserin	Cynomolgus monkey	0	2.07E+06	2.07E+06	8.64E+05	8.34E+05	2.40	2.49
		15	1.43E+06	1.46E+06	8.30E+05	8.23E+05	1.72	1.77
		30	9.68E+05	9.82E+05	8.42E+05	8.42E+05	1.15	1.17
		60	4.84E+05	4.88E+05	8.40E+05	8.18E+05	0.58	0.60
		90	2.68E+05	2.75E+05	8.65E+05	8.40E+05	0.31	0.33
		120	1.69E+05	1.65E+05	8.19E+05	8.19E+05	0.21	0.20
Ketanserin	Human	0	2.11E+06	1.97E+06	8.12E+05	8.57E+05	2.60	2.30
		15	1.57E+06	1.56E+06	8.30E+05	8.13E+05	1.89	1.92
		30	1.09E+06	1.19E+06	7.77E+05	8.37E+05	1.40	1.42
		60	7.23E+05	6.78E+05	7.52E+05	7.42E+05	0.96	0.91
		90	6.14E+05	5.18E+05	8.82E+05	8.80E+05	0.70	0.59
		120	4.40E+05	4.88E+05	7.60E+05	7.88E+05	0.58	0.62

APPENDIX 4

01049-20008-microsomal stability protocol

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

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

Study Number
01049-20008

Study Director
(b) (6)

Sponsor
Acuitas Therapeutics Inc.

CONTENTS

1. INTRODUCTION

1.1. Study Number

01049-20008

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 Liver Microsomes

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 microsomes 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)

(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

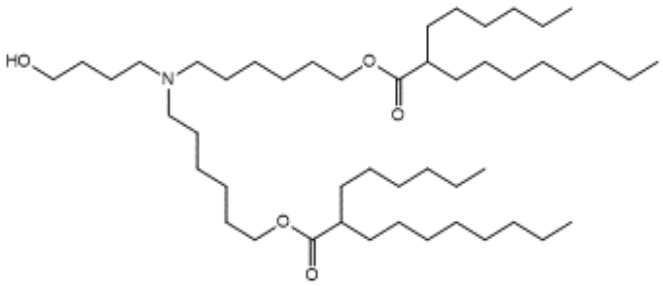
2.1. Test Article

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

Ketanserin and verapamil will be used as positive control and internal standard, respectively. The sources will be documented in experimental records and presented in the report.

2.3. Liver Microsomes and Cofactor

Liver microsomes of CD-1/ICR mouse, Sprague Dawley rat, Wistar Han rat, cynomolgus monkey, and human were purchased from qualified suppliers and stored in a -70°C ultra low temperature freezer. NADPH (reduced β-nicotinamide adenine dinucleotide 2′-phosphate) tetrasodium salt were purchased from a qualified supplier and stored at 2-8°C in a refrigerator. 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 solution: 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 solution:

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) Preparation of 1.5× liver microsomes suspension containing test article or positive control:

1.5× Liver Microsomes Suspension Containing Test Article or Positive Control					
Liver Microsomes		0.5 mM spiking solution (μL)	100 mM potassium phosphate buffer (pH 7.4) (μL)	Final Concentration	
Conc. of stock suspension (mg/mL)	Volume of stock suspension (μL)			Liver microsomal protein (mg/mL)	Compound (μM)
20	18.75	1.5	479.75	0.75	1.5

(4) 3×NADPH working solution (6 mM; 5 mg/mL) will be prepared by dissolving NADPH in 100 mM pH 7.4 potassium phosphate buffer. The working solution is then pre-warmed at 37°C.

(5) 30 μL of 1.5× liver microsomes suspension containing 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).

(6) 96-well incubation plates are pre-warmed at 37 °C for 5 min.

(7) For 0-min samples: 450 μL ethanol containing internal standard (IS solution) is added before 15 μL pre-warmed NADPH working solution (6mM) is added.

(8) For other samples (15, 30, 60, 90, and 120 min): 15 μL pre-warmed NADPH working solution (6 mM) is added to initiate reaction.

Volume (μL)			Final Concentration in incubation mixture		
1.5× Liver Microsomes Suspension Containing Test Article or Positive Control	3×NADPH working solution	Total	Liver microsomal protein (mg/mL)	Test Article or Positive Control (μM)	NADPH (mM)
30	15	45	0.5	1	2

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

(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 ºC in a 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 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.

$$\text{Elimination rate constant (k)} = - \text{ slope}$$

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

The *in vitro* intrinsic clearance, CL'_{int}, will be calculated from the $t_{1/2}$ as follows:

$$CL'_{int} = (0.693/T_{1/2}) \times (1/(\text{microsomal protein concentration (0.5 mg/mL)})) \times \text{Scaling Factor}$$

The scaling factors are listed in Table 1.

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

Species	Microsomal Protein (mg) per Gram of Liver	Liver Weight (g) per kg Body Weight	Scaling Factor (mg/kg)[a]	Hepatic Blood Flow (mL/min/kg)
Mouse	45	87.5	3937.5	90
Rat	44.8	40	1792	55.2
Monkey	45	32.5	1462.5	44
Human	48.8	25.7	1254.2	20.7

[a]Microsomal protein (mg/g liver) × liver weight (g)/kg body weight

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 3, 2020
Date

Sponsor Representative

Study Director Approval

(b) (6)

2020/06/03
Date

Study Director