



***In Vitro* Metabolic Stability of ALC-0159 in CD-1/ICR Mouse, Sprague Dawley Rat, Wistar Han Rat, Cynomolgus Monkey, and Human Hepatocytes**

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Medicilon Preclinical Research (Shanghai) LLC  
Test Article: ALC-0159  
Study No.: 01049-20022

## **SUMMARY**

This study evaluated the *in vitro* metabolic stability of ALC-0159 in hepatocytes of CD-1/ICR mouse, Sprague Dawley rat, Wistar Han rat, cynomolgus monkey, and human. ALC-0159 was stable after an approximately 4-hour incubation with hepatocytes from all these species.

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### 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.

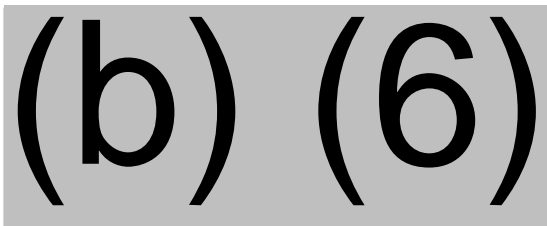
Study Director Approval:

(b) (6)  


Study Director

2020/08/10  
Date

Sponsor Approval:

(b) (6)  


Study Monitor

August 10, 2020  
Date

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## 1. OBJECTIVE

To evaluate the *in vitro* metabolic stability of ALC-0159 in hepatocytes from different species.

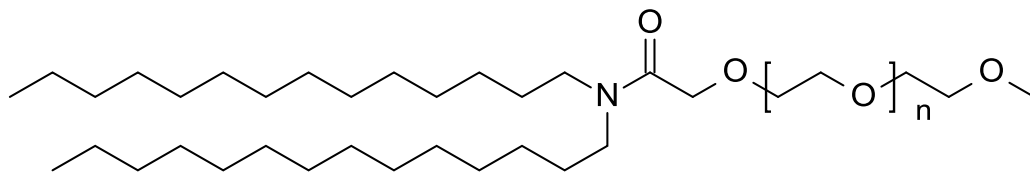
## 2. MATERIALS

### 2.1 Test Article

Name: ALC-0159

Molecular Formula: C<sub>30</sub>H<sub>60</sub>NO (C<sub>2</sub>H<sub>4</sub>O)<sub>n</sub> (n = 45~50)

MW (g/mol): ~2400-2600



### 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 <sup>6</sup>
Sprague Dawley rat (male)	XenoTech	RPCH1000	1810189	5.0×10 <sup>6</sup>
Wistar Han rat	BioIVT	M00065	YMV	5.0×10 <sup>6</sup>
Cynomolgus monkey (male)	RILD Shanghai	HP-SXH-02M	CJJC	5.0×10 <sup>6</sup>
Human (mixed gender)	XenoTech	HPCH10	1810156	5.0×10 <sup>6</sup>

### 3. EXPERIMENTAL PROCEDURES

#### 3.1 Stock solution:

4.24 mg of ALC-0159 was weighed and dissolved in 169.60 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-0159	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 ( $2 \times 10^6$  viable cells/mL) and then pre-warmed at 37 °C for 10 min.

- 3.5** 40  $\mu$ L 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  $\mu$ L of internal standard solution (IS solution, 10 ng/mL verapamil in ethanol) was added, followed by 40  $\mu$ L of pre-warmed 2 $\times$  dosing solution. The final concentration of test article or positive control in the incubation mixture was 1  $\mu$ M.
- 3.7** For the 30, 60, 90, 120, 180, and 240 min samples, 40  $\mu$ L of pre-warmed 2 $\times$  dosing solution was added to initiate the reaction. The final concentration of test article or positive control in the incubation mixture was 1  $\mu$ M.
- 3.8** Samples were incubated at 37 °C. At 30, 60, 90, 120, 180, and 240 min time points, the reaction was stopped by adding 480  $\mu$ L 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  $\mu$ L of the supernatants were transferred from each well into a 96-well sample plate for LC-MS/MS.

## 4. BIOANALYSIS

### 4.1 Instruments

SHIMADZU: UPLC system

Sciex Triple Quad 6500+ with ESI ion source

### 4.2 LC/MS/MS Conditions

Column: Agilent Zorbax SB-CN 3.5  $\mu$ m (100 mm\*2.1 mm)

Gradient for ALC-0159:

Time (min)	Solvent A (%)	Solvent B (%)
0.00	80	20
0.40	30	70
1.60	10	90
2.70	10	90
2.71	80	20
3.00	80	20



Solvent A: 0.1% formic acid in water  
Solvent B: 0.1% formic acid in acetonitrile  
Flow rate: 600  $\mu$ L/min  
Column temperature: 40  $^{\circ}$ C  
Autosampler temperature: 4 $^{\circ}$ C  
MS Conditions: MRM detection

Compound	Q1(m/z)	Q3(m/z)	DP	CE	Retention Time
ALC-0159	1164.00	494.70	45	71	~1.33
tolbutamide (IS)	271.10	172.00	70	18	~1.03

### 4.3 Detection of ALC-0159

Representative chromatograms of ALC-0159 in each matrix are shown in [Appendix 1](#).

## 5. DATA ANALYSIS

The % remaining parent compound (ALC-0159 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 (T<sub>1/2</sub>) (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  $\times$  V (1 mL incubation/ $10^6$  cells)  $\times$  Scaling Factor ( $10^6$  cells/kg),  
Scaling Factor ( $10^6$  cells/kg) = Hepatocellularity ( $10^6$  cells/g liver)  $\times$  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 <sup>6</sup> cells/g liver)	Liver Weight (g/kg BW)	Scaling Factor (10 <sup>6</sup> 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-0159 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-0159 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-0159. 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-0159 in hepatocytes of CD-1/ICR mouse, Sprague Dawley rat, Wistar Han rat, cynomolgus monkey, and human. ALC-0159 was stable after an approximately 4-hour incubation with hepatocytes from all these species.



**Table 2. Summary of Hepatocyte Stability of ALC-0159, Testosterone and 7-Hydroxycoumarin**

Test Article	Species		Percent Remaining (%)							T <sub>1/2</sub> (minute)	CL'int (mL/min/kg)
			0 min	30 min	60 min	90 min	120 min	180 min	240 min		
ALC-0159	CD-1/ICR mouse	Mean	100.00	100.85	94.92	94.28	87.08	94.92	102.75	>240	<34.1
		RSD of Area Ratio	0.60	4.16	1.89	0.95	3.10	0.63	3.21		
	Sprague Dawley rat	Mean	100.00	93.37	91.81	90.25	89.47	93.96	94.93	>240	<13.5
		RSD of Area Ratio	7.44	1.48	5.70	3.36	2.16	4.11	2.61		
	Wistar Han rat	Mean	100.00	113.04	105.07	112.80	104.11	102.90	98.79	>240	<13.5
		RSD of Area Ratio	3.42	2.42	4.23	3.94	5.58	0.00	3.11		
	Cynomolgus monkey	Mean	100.00	90.23	92.93	94.59	97.51	89.81	92.93	>240	<11.3
		RSD of Area Ratio	3.82	8.47	7.28	7.77	2.11	3.93	3.48		
	Human	Mean	100.00	106.34	101.58	92.67	96.04	93.66	102.57	>240	<7.35
		RSD of Area Ratio	1.96	0.79	1.93	5.44	0.87	3.89	6.55		
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		

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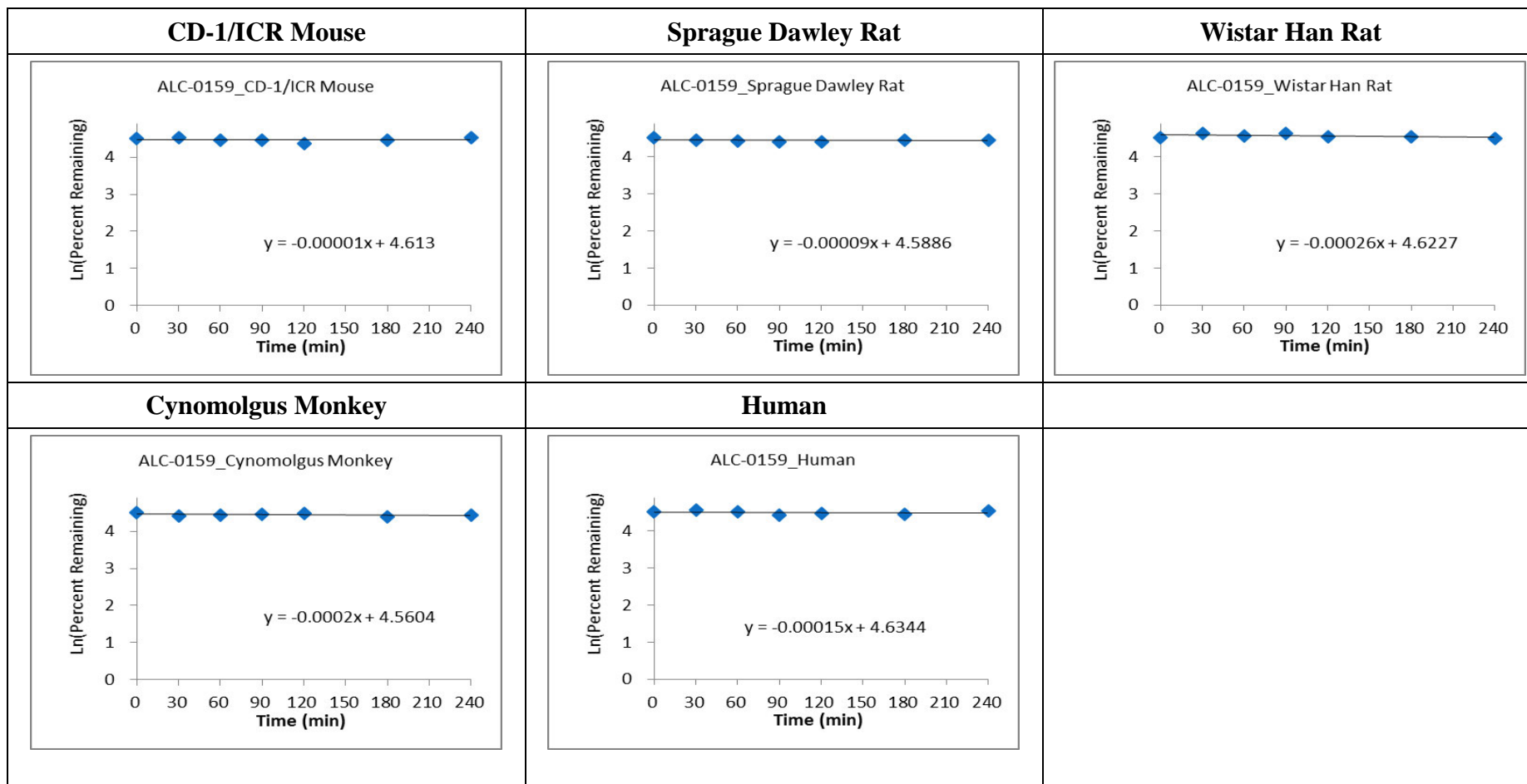
7- Hydroxycou marin	CD-1/ICR mouse	Mean	100.00	35.05	3.20	BQL	BQL	BQL	BQL	12.1	677
		RSD of Area Ratio	1.22	15.06	8.46	N/A	N/A	N/A	N/A		
	Sprague Dawley rat	Mean	100.00	20.97	BQL	BQL	BQL	BQL	BQL	13.3	244
		RSD of Area Ratio	2.99	10.49	N/A	N/A	N/A	N/A	N/A		
	Wistar Han rat	Mean	100.00	19.11	BQL	BQL	BQL	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	100.00	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	100.00	40.70	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-0159 in Mouse, Rat, Monkey and Human Hepatocytes



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

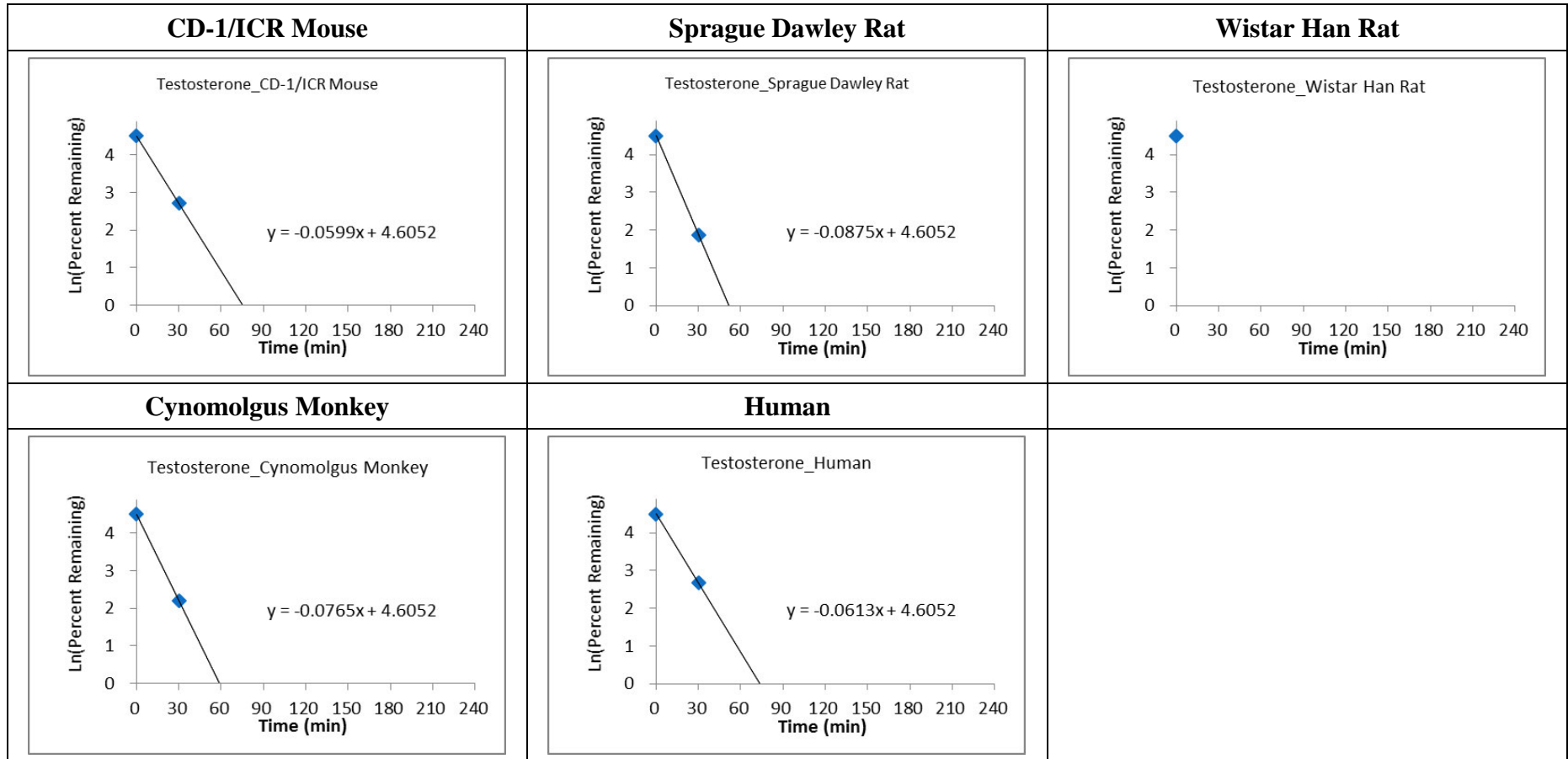
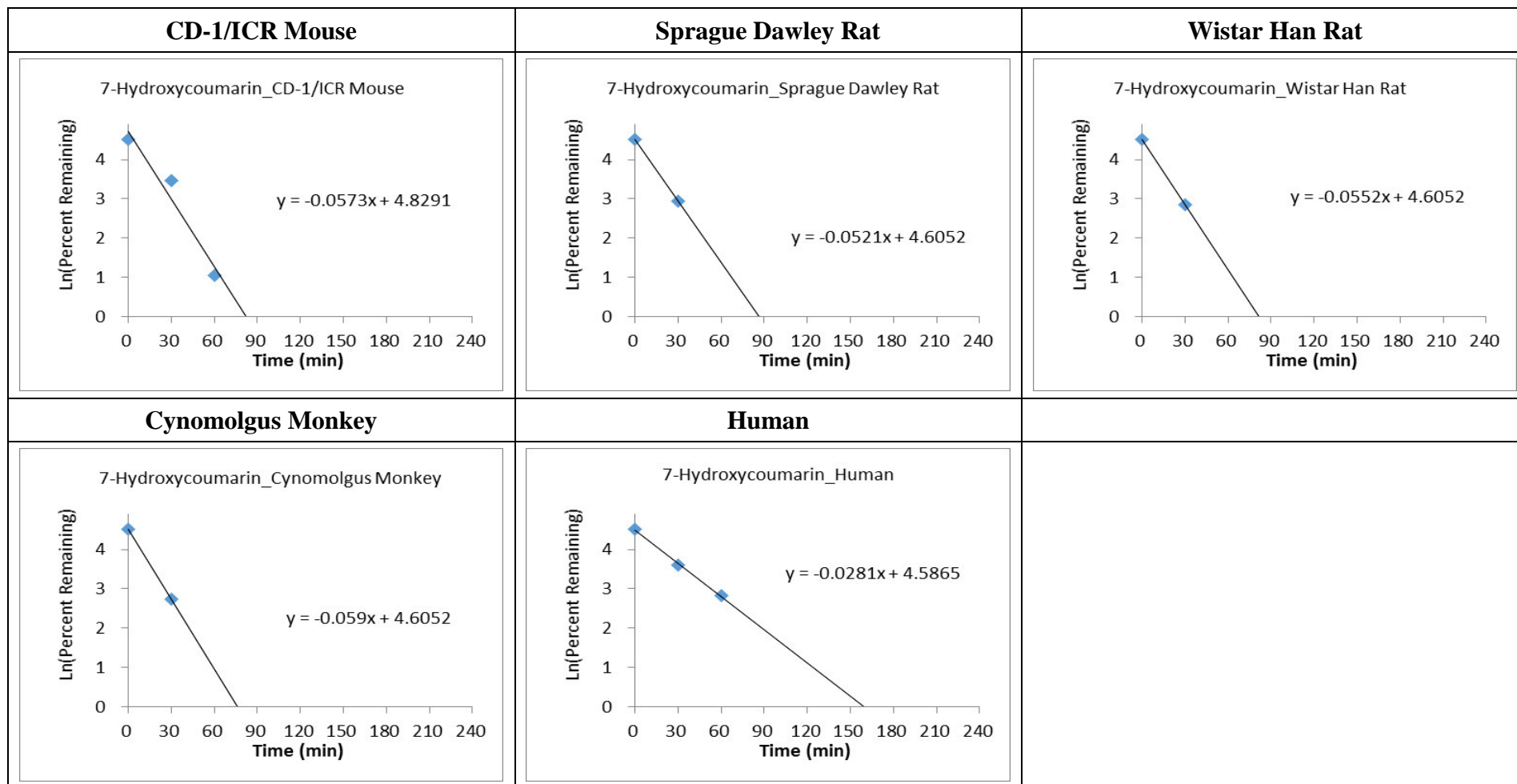




Figure 3. Stability of 7-Hydroxycoumarin in Mouse, Rat, Monkey and Human Hepatocytes



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## 8. APPENDICES

**Appendix 1** – Representative Chromatograms of ALC-0159 in Mouse, Rat, Monkey and Human Hepatocytes

**Appendix 2** – Stability of ALC-0159 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** – 01049-20022-ALC-0159-Hepatocytes Stability\_Protocol



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Test Article: ALC-0159  
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## **APPENDIX 1**

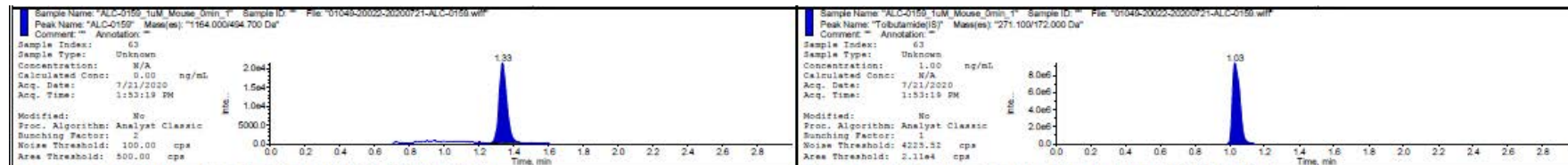
Representative Chromatograms of ALC-0159 in Mouse, Rat, Monkey and Human Hepatocytes

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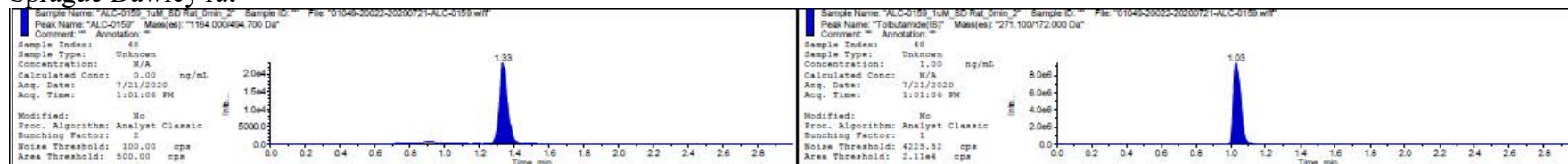




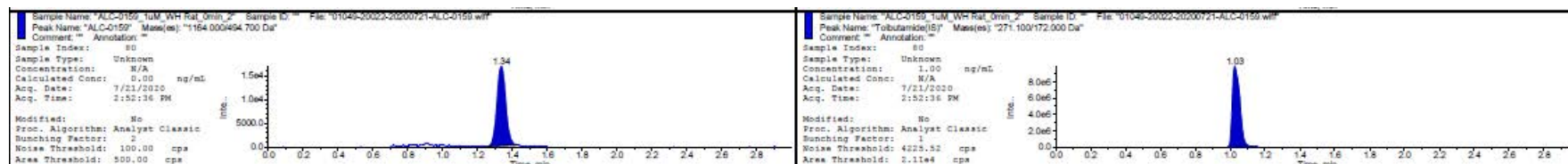
### CD 1/ICR mouse



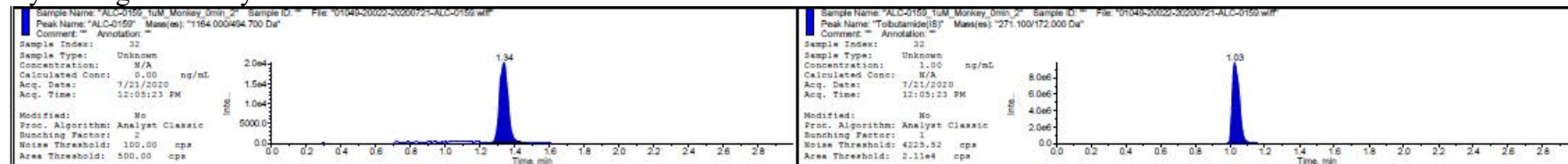
### Sprague Dawley rat



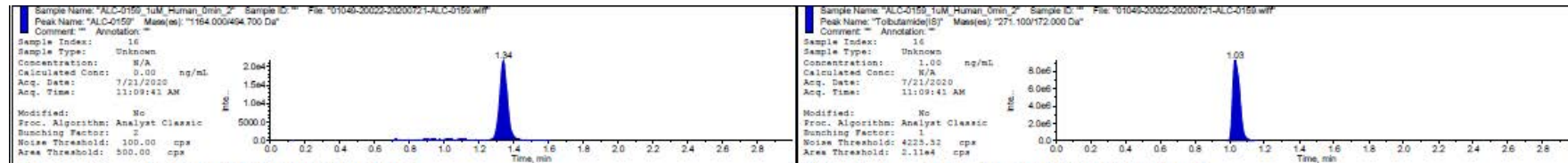
### Wistar Han rat



### Cynomolgus monkey



### Human



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## APPENDIX 2

Stability of ALC-0159 in Mouse, Rat, Monkey and Human Hepatocyte – Raw Data

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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-0159	CD-1/ICR mouse	240	6.41E+04	6.72E+04	2.70E+07	2.71E+07	0.002	0.002
		180	6.18E+04	6.20E+04	2.74E+07	2.78E+07	0.002	0.002
		120	5.40E+04	5.71E+04	2.69E+07	2.72E+07	0.002	0.002
		90	6.03E+04	5.93E+04	2.69E+07	2.69E+07	0.002	0.002
		60	6.06E+04	5.99E+04	2.67E+07	2.70E+07	0.002	0.002
		30	6.52E+04	6.18E+04	2.66E+07	2.68E+07	0.002	0.002
		0	6.30E+04	6.21E+04	2.66E+07	2.64E+07	0.002	0.002
ALC-0159	Sprague Dawley rat	240	6.44E+04	6.55E+04	2.69E+07	2.65E+07	0.002	0.002
		180	6.55E+04	6.23E+04	2.64E+07	2.66E+07	0.002	0.002
		120	5.92E+04	6.09E+04	2.61E+07	2.61E+07	0.002	0.002
		90	6.22E+04	5.93E+04	2.63E+07	2.63E+07	0.002	0.002
		60	6.40E+04	5.93E+04	2.61E+07	2.63E+07	0.002	0.002
		30	6.43E+04	6.22E+04	2.65E+07	2.62E+07	0.002	0.002
		0	6.39E+04	7.03E+04	2.63E+07	2.61E+07	0.002	0.003
ALC-0159	Wistar Han rat	240	5.74E+04	6.00E+04	2.86E+07	2.88E+07	0.002	0.002
		180	6.11E+04	6.01E+04	2.87E+07	2.82E+07	0.002	0.002
		120	5.87E+04	6.32E+04	2.83E+07	2.82E+07	0.002	0.002
		90	6.30E+04	6.59E+04	2.78E+07	2.75E+07	0.002	0.002
		60	6.12E+04	5.78E+04	2.73E+07	2.74E+07	0.002	0.002
		30	6.40E+04	6.59E+04	2.79E+07	2.77E+07	0.002	0.002
		0	5.89E+04	5.70E+04	2.78E+07	2.83E+07	0.002	0.002
ALC-0159	Cynomolgus monkey	240	6.16E+04	5.89E+04	2.69E+07	2.70E+07	0.002	0.002
		180	5.89E+04	5.65E+04	2.66E+07	2.69E+07	0.002	0.002
		120	6.19E+04	6.31E+04	2.68E+07	2.66E+07	0.002	0.002
		90	5.73E+04	6.50E+04	2.66E+07	2.71E+07	0.002	0.002
		60	5.73E+04	6.32E+04	2.70E+07	2.69E+07	0.002	0.002
		30	6.31E+04	5.59E+04	2.75E+07	2.74E+07	0.002	0.002
		0	6.32E+04	6.69E+04	2.70E+07	2.71E+07	0.002	0.002
ALC-0159	Human	240	6.22E+04	6.86E+04	2.52E+07	2.53E+07	0.002	0.003
		180	5.84E+04	6.26E+04	2.53E+07	2.58E+07	0.002	0.002
		120	6.12E+04	6.19E+04	2.51E+07	2.57E+07	0.002	0.002
		90	5.70E+04	6.08E+04	2.54E+07	2.50E+07	0.002	0.002
		60	6.56E+04	6.26E+04	2.52E+07	2.48E+07	0.003	0.003
		30	6.75E+04	7.09E+04	2.53E+07	2.63E+07	0.003	0.003
		0	6.53E+04	6.35E+04	2.55E+07	2.55E+07	0.003	0.002

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### APPENDIX 3

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

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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

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Medicilon Preclinical Research (Shanghai) LLC  
Test Article: ALC-0159  
Study No.: 01049-20022

## APPENDIX 4

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

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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

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Medicilon Preclinical Research (Shanghai) LLC  
Test Article: ALC-0159  
Study No.: 01049-20022

## APPENDIX 5

01049-20022-ALC-0159-Hepatocytes Stability\_Protocol

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***In Vitro* Metabolic Stability of ALC-0159 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**

01049-20022

**Study Director**

(b) (6)

**Sponsor**

Acuitas Therapeutics Inc.

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**1. INTRODUCTION**

**1.1. Study Number**

01049-20022

**1.2. Study Title**

*In Vitro* Metabolic Stability of ALC-0159 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-0159 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)

(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

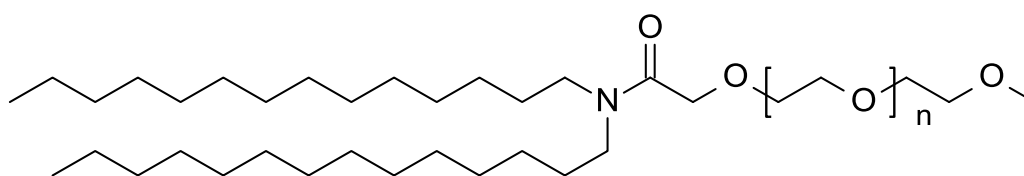
## 2. MATERIALS

### 2.1. Test Article

Name: ALC-0159

Molecular Formula:  $C_{30}H_{60}NO (C_2H_4O)_n$  ( $n = 45\sim 50$ )

MW (g/mol): ~2400-2600



### 2.2. Positive Control and Internal Standard

Testosterone and 7-hydroxycoumarin will be used as positive controls. Tolbutamide 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°C 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<sup>6</sup> 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 °C . 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 °C 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

SHIMADZU: UPLC system

Sciex Triple Quad 6500+ with ESI ion source

##### 4.2. LC/MS/MS Conditions

Column: Agilent Zorbax SB-CN 3.5um (100mm\*2.1mm)

Gradient for ALC-0159

Time (min)	Solvent A (%)	Solvent B (%)
0.00	80	20
0.40	30	70
1.60	10	90
2.70	10	90
2.71	80	20
3.00	80	20

A: 0.1%Formic acid in water

B: 0.1%Formic acid in acetonitrile

Flow rate: 600 µL/min

Column temperature: 40 °C

Autosampler temperature: 4°C

Compound	Q1(m/z)	Q3(m/z)	Retention Time (min)
ALC-0159	1164.00	494.70	~1.30
Tolbutamide (IS)	271.10	172.00	~1.02

#### 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:

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

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

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

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

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

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	Liver Weight	Scaling Factor
	(10 <sup>6</sup> cells/g liver)	(g/kg BW)	(10 <sup>6</sup> 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**

(b) (6)

Sponsor Representative

July 15, 2020

Date

**Study Director Approval**

(b) (6)

Study Director

2020 / 07 / 15  
Date