

Metabolic Stability Report

Metabolic Stability Study of 2 Test Compounds in Mouse Liver Microsomes with and without the Presence of NADPH

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REPORT APPROVAL

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TABLE OF CONTENTS

LIST OF TABLES	iv
LIST OF FIGURES	v
1 Executive Summary	1
2 Experimental.....	2
2.1 Chemicals.....	2
2.1.1 Reference compound.....	2
2.1.2 Other chemicals.....	2
2.2 Biological Materials	2
2.3 Experimental Procedures.....	2
2.3.1 Liver Microsomal Incubation.....	2
2.3.2 Sample Collection and Preparation	3
2.4 HPLC Conditions	3
2.5 MS Instrument Parameters	3
3 Results and Discussion	5
4 Conclusions	7
5 References	8

LIST OF TABLES

Table 1	Propranolol and Test Compound Peak Area in Incubation Samples (with NADPH)	9
Table 2	Standard Values and Parameters Used in Intrinsic Clearance and Hepatic Extraction Ratio Calculation	10
Table 3	Liver Microsomal Half Life, Intrinsic Clearance, and Hepatic Extraction Ratio for Propranolol and Test Compounds (with NADPH)	11
Table 4	Liver Microsomal Intrinsic Clearance Reference Value for Propranolol	12
Table 5	Propranolol and Test Compound Peak Area in Incubation Samples (without NADPH)	13
Table 6	% Remaining for Propranolol and Test Compounds after 1-hr Incubation (without NADPH)	14

LIST OF FIGURES

Figure 1. ln (peak area) vs. time plot for propranolol in mouse liver microsomes (with NADPH) (top: replicate 1; bottom: replicate 2)	15
Figure 2. ln (peak area) vs. time plot for SB-400868 in mouse liver microsomes (with NADPH) (top: replicate 1; bottom: replicate 2)	16
Figure 3. ln (peak area) vs. time plot for ALM-DAI-16 in mouse liver microsomes (with NADPH) (top: replicate 1; bottom: replicate 2)	17

1 Executive Summary

1 μ M of the test compounds and control (propranolol) were incubated with mouse liver microsomes with the presence of NADPH at 37°C for 0, 7.5, 15, 30, and 60 minutes and without the presence of NADPH at 37°C for 0 and 60 minutes. After quenching with acetonitrile, the incubation samples were analyzed on LC-MS/MS. The peak areas of the compounds were used to calculate the half life, intrinsic clearance, hepatic extraction ratio, percent remaining.

The half lives of SB-400868 and ALM-DAI-16 in mouse liver microsomal incubation are 9.87 and 9.08 min, respectively, while the half life of propranolol is 8.33 min.

All test compounds are stable in microsomes without the presence of NADPH.

2 Experimental

2.1 Chemicals

2.1.1 Reference compound

SB-400868	The University of North Carolina at Chapel Hill
ALM-DAI-16	The University of North Carolina at Chapel Hill
Propranolol hydrochloride	Lot No. 07528HH (Sigma-Aldrich)

2.1.2 Other chemicals

Water	Baker Analyzed HPLC Solvent (J.T. Baker)
Acetonitrile	HPLC Solvent (Burdick & Jackson)
Dimethyl Sulfoxide	Baker Analyzed ACS Reagent (J.T. Baker)
Acetic Acid	99.7%, ACS grade (BDH-VWR)
Ammonium Acetate	97%, GR ACS grade (EMD)
KH ₂ PO ₄	99%, Reagent, ACS (BDH-VWR)
K ₂ HPO ₄	98%, Reagent, ACS (BDH-VWR)
MgCl ₂ •6H ₂ O	99.9%, AR ACS grade (Mallinckrodt Chemicals)
NADPH Tetrasodium Salt	97.4% (EMD Biosciences)

2.2 Biological Materials

Mouse Liver Microsomes	CD1, Male, 20 mg protein/mL, Pool of 1396, Lot No. 1710069 (XenoTech)
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2.3 Experimental Procedures

2.3.1 Liver Microsomal Incubation

The test compounds and control (propranolol) were incubated at a concentration of 1 μ M with mouse liver microsomes, with or without the presence of NADPH. The duplicate incubations were conducted in 1-mL 96-well plate in a shaking water bath maintained at 37°C. Ingredients were added as shown below.

Components	Add (μL)		Final Conc.
	With NADPH	Without NADPH	
0.1 M K ₂ HPO ₄ -KH ₂ PO ₄ Buffer (pH 7.4)	435	465	90 mM
33 mM MgCl ₂	60	60	3.3 mM
5 mg/ml Microsomal Protein	60	60	0.5 mg/mL
40 μM Test Compounds or Control in 0.1 M Phosphate Buffer:Acetonitrile 60:40	15	15	1 μM
26 mM NADPH	30	0	1.3 mM
Vortex Vigorously for 5 sec.	Yes	Yes	
Preincubated at 37°C for 5 min.	Yes	Yes	
Pipette 100 μL out as 0 min sample	Yes	Yes	

2.3.2 Sample Collection and Preparation

Samples were collected at 0, 7.5, 15, 30, and 60 min. (0 and 60 min. only for incubation without NADPH) of incubation by pipetting 100 μL of incubation mixture out into a 0.5-mL 96-well plate and quenched by addition of 200 μL of acetonitrile. The plate was capped, vortexed, and centrifuged at 3000 rpm for 10 minutes. The supernatant was injected into LC-MS/MS.

2.4 HPLC Conditions

Instrument: Shimadzu LC-20AD Pumps and SIL-20AHT Autosampler
 Column: Chromolith SpeedRod RP-18e, 4.6x50 mm
 Venusil XBP C18(2), 5 μm, 100 Å, 2.1x50 mm (propranolol)
 Mobile phase A: 150 mM HOAc 50 mM NH₄OAc in acetonitrile:water 1:1 (v/v)
 Mobile phase B: 0.5% Formic acid in methanol
 Injection volume: 5 μL (2 μL for propranolol)
 HPLC flow rate: 0.5 mL min. (0.25 mL/min. for propranolol)
 Run time: 4 min. (2.25 min. for propranolol)
 Gradient:

Propranolol		Other Compounds	
Time (min)	Mobile Phase B (%)	Time (min)	Mobile Phase B (%)
0	0	0	50
0.25	90	4	50
0.75	90		
0.76	0		
2.25	0		

2.5 MS Instrument Parameters

Instrument: Applied Biosystems/MDS Sciex API 3200
 Gas and temperature settings:

Ionization Source	Turbo Ionspray
Polarity	Positive
Curtain gas	10

CAD	6
Gas1	30 (20 for propranolol)
Gas2	50 (40 for propranolol)
Interface heater	On
Ionspray voltage	5500
Temperature	500°C

Compound dependent parameters:

	Q1	Q3	Time (msec)	DP	EP	CE (V)	CXP
Propranolol	260.1	116.1	400	46	11.5	25	4
SB-400868	306.09	278.1	300	71	10.5	47	2
ALM-DAI-16	324.04	238.1	300	81	12	51	2

3 Results and Discussion

Peak areas of propranolol and the test compounds in incubation samples with the presence of NADPH are listed in Table 1. Half life in liver microsomal incubation in min was obtained through linear regression of $\ln(\text{peak area})$ vs. time (min) as shown below:

$$t_{1/2} = -\frac{0.693}{k}$$

where

k = slope of $\ln(\text{peak area})$ vs. time line: $\ln(\text{peak area}) = \text{intercept} + kt$

The plots of $\ln(\text{peak area})$ vs. time for propranolol and the test compounds in mouse liver microsomes with the presence of NADPH are shown in Figures 1 to 3.

Intrinsic clearance in mL/min/kg was obtained as shown below:

$$CL_{\text{int}} = 0.693 * \frac{W_{\text{mp}} * W_{\text{liver}}}{t_{1/2} * C_{\text{mp}}}$$

where

W_{mp} = microsomal protein content in liver (mg/g)

W_{liver} = liver weight per kilogram of body weight (g/kg)

$t_{1/2}$ = test compound half life in liver microsomal incubation (min)

C_{mp} = microsomal protein incubation concentration (mg/mL)

Hepatic extraction ratio was obtained as shown below:

$$E = \frac{CL_{\text{int}}}{Q + CL_{\text{int}}}$$

where

Q = hepatic blood flow (mL/min/kg)

CL_{int} = intrinsic clearance (mL/min/kg)

Standard values and parameters used in the intrinsic clearance and hepatic extraction ratio calculation are listed in Table 2. Half life, intrinsic clearance, and hepatic extraction ratio calculated are listed in Table 3.

The liver microsomal intrinsic clearance values for propranolol from references¹⁻⁴ are listed in Table 4. No mouse and monkey liver microsomal intrinsic clearance values for propranolol are available through search on the internet.

As shown in Table 3, the half life, intrinsic clearance, and hepatic extraction ratio replicate values for propranolol and the test compounds are very consistent. The half lives of SB-400868 and ALM-DAI-16 in mouse liver microsomal incubation are 9.87 and 9.08 min, respectively, while the half life of propranolol is 8.33 min. The intrinsic clearance values of SB-400868 and ALM-DAI-16 are 569 and 619 mL/min/kg, respectively, while the intrinsic clearance of propranolol is 679 mL/min/kg. The hepatic extraction ratios of SB-400868 and ALM-DAI-16 are 0.864 and 0.873, respectively, while hepatic extraction ratio of propranolol is 0.882.

Peak areas of propranolol and the test compounds in incubation samples without the presence of NADPH are listed in Table 5. Percent remaining values after 1-hr incubation without the presence of NADPH are listed in Table 6. All test compounds are stable in microsomes without the presence of NADPH.

4 Conclusions

The half lives of SB-400868 and ALM-DAI-16 in mouse liver microsomal incubation are 9.87 and 9.08 min, respectively, while the half life of propranolol is 8.33 min.

All test compounds are stable in microsomes without the presence of NADPH.

5 References

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4. Robert T. Grbac, Forrest A. Stanley, Tomoko Ambo, Joanna E. Barbara, Lois J. Haupt, Brian D. Smith, David B. Buckley, and Faraz Kazmi, High Content Automated Metabolic Stability and CYP Inhibition Cocktail Screening Assays for Early Drug Development, SLAS 2014 Poster, Jan. 18-22, 2014, San Diego, CA

Table 1: Propranolol and Test Compound Peak Area in Incubation Samples (with NADPH)

Compound	Replicate	Peak Area				
		Mouse				
		Incubation Time (min.)				
		0	7.5	15	30	60
Propranolol	1	14400	4020	3070	786	476
	2	9370	5030	1910	973	508
SB-400868	1	173000	92300	68500	21100	3020
	2	177000	109000	63900	20700	3890
ALM-DAI-16	1	131000	83200	46900	14900	2540
	2	140000	82200	49100	12900	2690
Note: Only data from 0 to 30 min. was used in linear regression.						

Table 2: Standard Values and Parameters Used in Intrinsic Clearance and Hepatic Extraction Ratio Calculation

Species	Liver weight per kg body weight (g/kg)	Hepatic blood flow Q (mL/min/kg)	Microsomal protein content in liver (mg/g)	Microsomal protein incubation concentration (mg/mL)
Mouse	90	90	45	0.5
Rat	40	70	45	0.5
Dog	32	35	45	0.5
Monkey	32	44	45	0.5
Human	21	20	45	0.5

Table 3: Liver Microsomal Half Life, Intrinsic Clearance, and Hepatic Extraction Ratio for Propranolol and Test Compounds (with NADPH)

Compound	Replicate	Mouse					
		Half Life (min)		CL _{int} (mL/min/kg)		E	
		Individual	Average	Individual	Average	Individual	Average
Propranolol	1	7.61	8.33	738	679	0.891	0.882
	2	9.05		620		0.873	
SB-400868	1	10.1	9.87	556	569	0.861	0.864
	2	9.64		582		0.866	
ALM-DAI-16	1	9.46	9.08	593	619	0.868	0.873
	2	8.7		645		0.878	

Note: Only data from 0 to 30 min. was used in linear regression.

Table 4: Liver Microsomal Intrinsic Clearance Reference Value for Propranolol

Reference	CL _{int}					Unit	CL _{int} (mL/min/kg)				
	Human	Monkey	Dog	Rat	Mouse		Human	Monkey	Dog	Rat	Mouse
1	1.72	N/A		79	N/A	L/hr/kg	28.7	N/A		1320	N/A
2	22-33	N/A			N/A	μL/min/mg	20.8-31.2	N/A			N/A
3	13	N/A			N/A	μL/min/mg	12.3	N/A			N/A
4	23.4	N/A	92.8	842	N/A	μL/min/mg	22.1	N/A	134	1516	N/A

Table 5: Propranolol and Test Compound Peak Area in Incubation Samples (without NADPH)

Compound	Replicate	Peak Area	
		Mouse	
		Incubation Time (min.)	
		0	60
Propranolol	1	8660	11400
	2	8880	11500
SB-400868	1	145000	172000
	2	151000	161000
ALM-DAI-16	1	104000	127000
	2	111000	133000

Table 6: % Remaining for Propranolol and Test Compounds after 1-hr Incubation (without NADPH)

Compound	Replicate	% Remaining after 1-hr Incubation	
		Mouse	
		Individual	Average
Propranolol	1	132	131
	2	130	
SB-400868	1	119	113
	2	107	
ALM-DAI-16	1	122	121
	2	120	

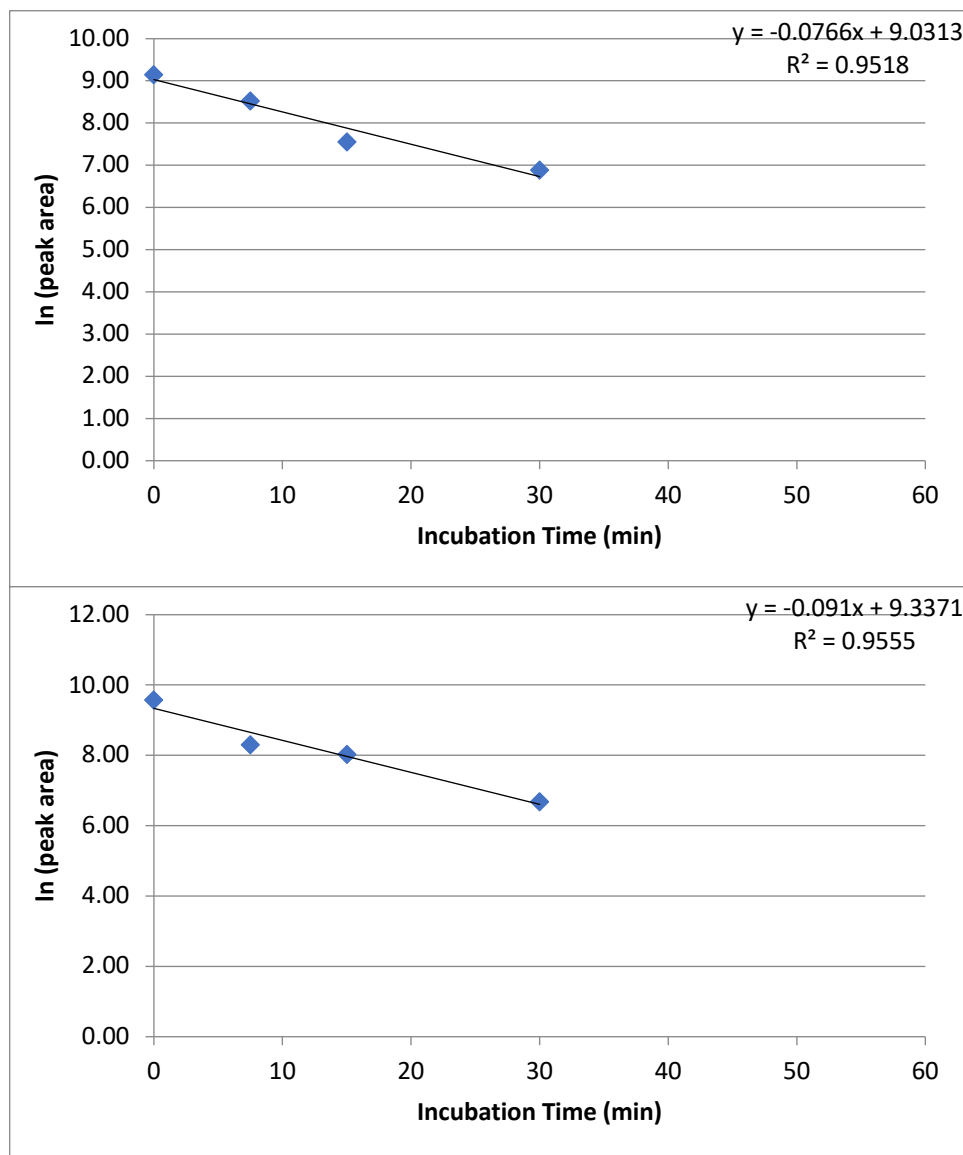


Figure 1. ln (peak area) vs. time plot for propranolol in mouse liver microsomes (with NADPH) (top: replicate 1; bottom: replicate 2)

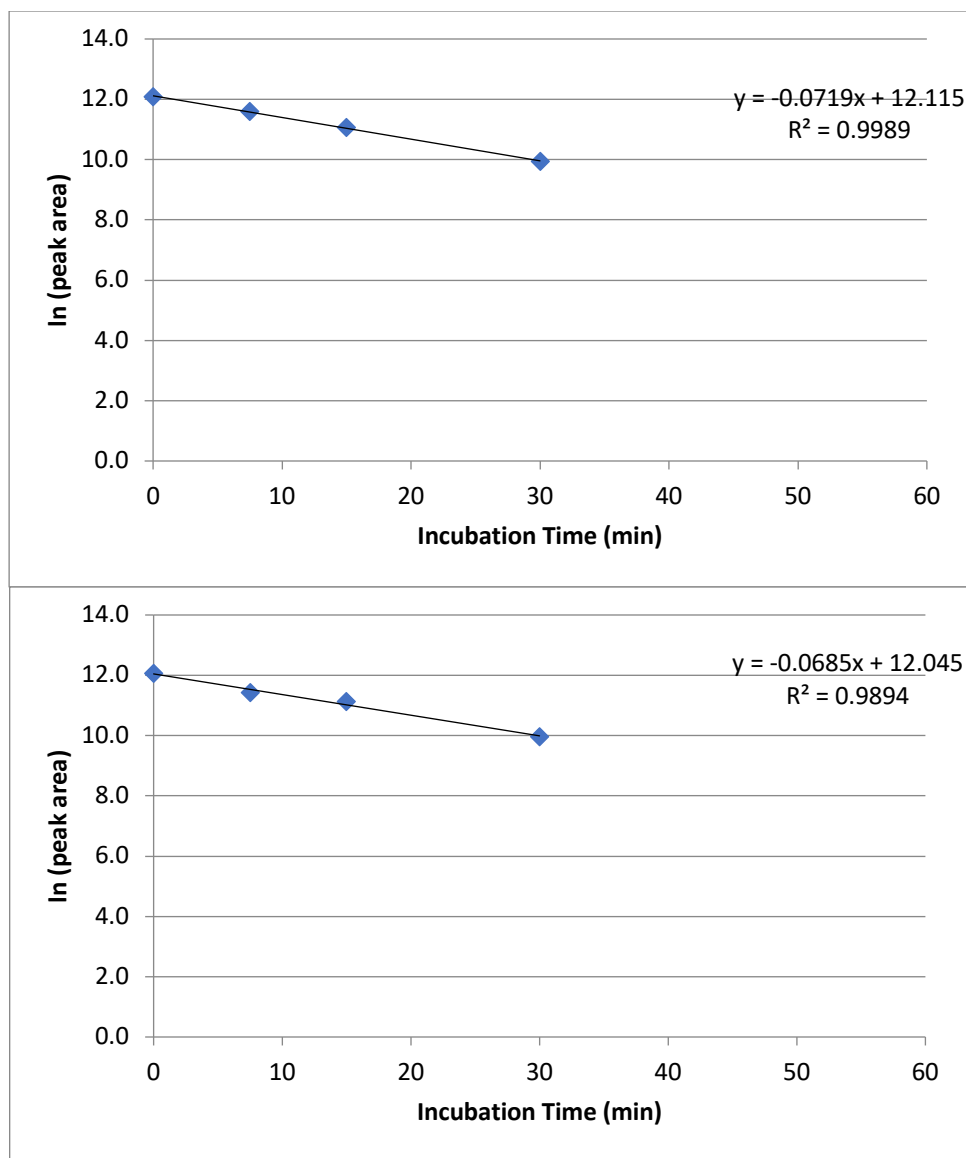


Figure 2. ln (peak area) vs. time plot for SB-400868 in mouse liver microsomes (with NADPH) (top: replicate 1; bottom: replicate 2)

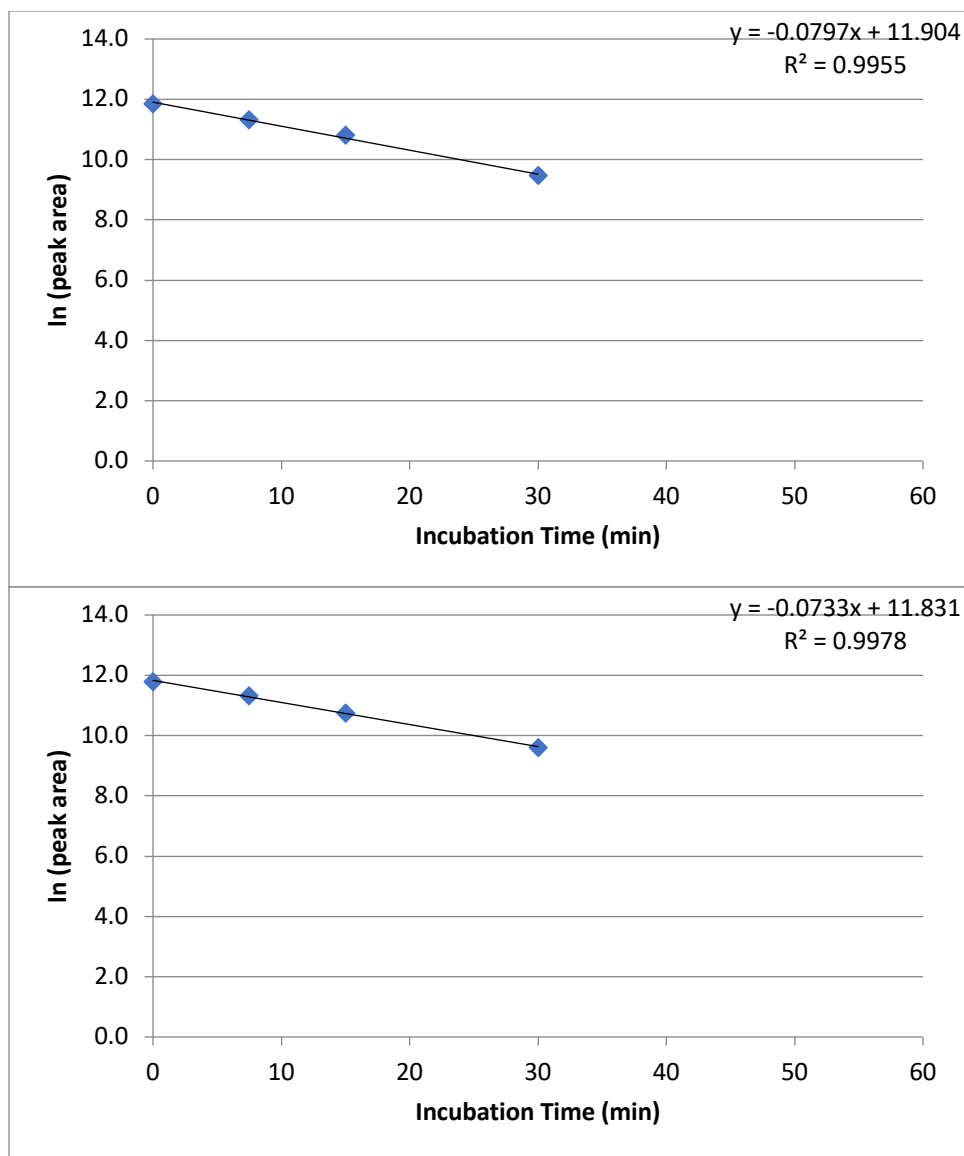


Figure 3. ln (peak area) vs. time plot for ALM-DAI-16 in mouse liver microsomes (with NADPH) (top: replicate 1; bottom: replicate 2)