Metabolic Stability Report

Metabolic Stability Study of SB400868 in Mouse Liver Microsomes with and without the Presence of NADPH

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REPORT APPROVAL

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1 Executive Summary

1 μM of the test compound 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 calculate the half life, intrinsic clearance, hepatic extraction ratio, percent remaining.

SB400868 is not very stable in mouse liver microsomes, with a half life of 8.3 min.

SB400868 is stable in mouse liver microsomes without the presence of NADPH.

2 Experimental

2.1 Chemicals

2.1.1 Reference compound

SB400868	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_2PO_4	99%, Reagent, ACS (BDH-VWR)
K_2HPO_4	98%, Reagent, ACS (BDH-VWR)
$MgCl_2 \cdot 6H_2O$	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)

2.3 Experimental Procedures

2.3.1 Liver Microsomal Incubation

The test compound and control (propranolol) were incubated at a concentration of 1 µM 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_2HPO_4-KH_2PO_4 Buffer (pH 7.4)	435	465	90 mM
33 mM MgCl2	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-20ADXR Pumps and SIL-20ACXR Autosampler
Column: Venusil XBP C18(2), 5 µm, 2.1x50 mm
Mobile phase A: 0.1% HOAc 1 mM NH_4OAc in water
Mobile phase B: Acetonitrile
Injection volume: 2 µL
HPLC flow rate: 0.5 mL/min.
Run time: 2.25 min.
Gradient:

Time (min)	Mobile Phase B (%)
0	20
1	85
1.5	85
1.51	20
2.25	20

2.5 MS Instrument Parameters

Instrument: Applied Biosystems/MDS Sciex API 4000
Gas and temperature settings:

Ionization Source	Turbo Ionspray
Polarity	Positive
Curtain gas	15

CAD	8
Gas1	30
Gas2	50
Interface heater	On
IonSpray voltage	5000
Temperature	450°C

Compound dependent parameters:

	Q1	Q3	Time (msec)	DP	EP	CE (V)	CXP
Propranolol	260.2	116.1	200	66	8	23.5	5
SB400868	306.15	278.1	200	61	10	43	16

3 Results and Discussion

Peak areas of propranolol and the test compound 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 (peak area) vs. time (min) as shown below:

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

where
k = slope of ln(peak area) vs. time line: ln(peak area) = intercept + kt

The plots of ln(peak area) vs. time for propranolol and the test compound in mouse liver microsomes with the presence of NADPH are shown in Figures 1 to 2.

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

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

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:

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

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 references[1-4] 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 compound are very consistent. The half life value of SB400868 in mouse liver microsomal incubation is 8.3 min, while the value for propranolol is 11.9 min. The intrinsic clearance of SB400868 is 677 mL/min/kg, higher than that of propranolol, which is about 472 mL/min/kg. The hepatic extraction ratio of SB400868 is about 0.883, while that of propranolol is around 0.84.

Peak areas of propranolol and the test compound 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. SB400868 is stable in microsomes without the presence of NADPH.

4 Conclusions

SB400868 is not very stable in mouse liver microsomes, with a half life of 8.3 min.

SB400868 is stable in mouse liver microsomes without the presence of NADPH.

5 References

1. Chuang Lu, Ping Li, Richard Gallegos, Vinita Uttamsingh, Cindy Q. Xia, Gerald T. Miwa, Suresh K. Balani, and Liang-Shang Gan, Comparison of Intrinsic Clearance in Liver Microsomes and Hepatocytes from Rats and Humans: Evaluation of Free Fraction and Uptake in Hepatocytes, DRUG METABOLISM AND DISPOSITION 34:1600–1605, 2006
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3. Robert J. Riley, D. F. McGinnity, and R. P. Austin, A Unified Model for Predicting Human Hepatic, Metabolic Clearance from in Vitro Intrinsic Clearance Data in Hepatocytes and Microsomes, DRUG METABOLISM AND DISPOSITION, 33:1304–1311, 2005
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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	538000	291000	224000	167000	102000
	2	540000	300000	226000	165000	107000
SB400868	1	497000	228000	117000	40400	5640
	2	467000	224000	130000	36000	5470

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 Compound (with NADPH)

Compound	Replicate	Mouse					
		Half Life (min)		CL_{int} (mL/min/kg)		E	
		Individual	Average	Individual	Average	Individual	Average
Propranolol	1	11.9	11.9	472	472	0.84	0.84
	2	11.9		472		0.84	
SB400868	1	8.38	8.3	670	677	0.882	0.883
	2	8.22		683		0.884	

Note: For propranolol, only data from 0 to 15 min. was in the linear portion of the Ln (peak area) vs. time plot and was used in linear regression. For AP-39, only data from 0 to 30 min. was in the linear portion of the Ln (peak area) vs. time plot and 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	615000	621000
	2	612000	595000
SB400868	1	524000	548000
	2	491000	507000

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

Compound	Replicate	% Remaining after 1-hr Incubation	
		Mouse	
		Individual	Average
Propranolol	1	101	99.1
	2	97.2	
SB400868	1	105	104
	2	103	

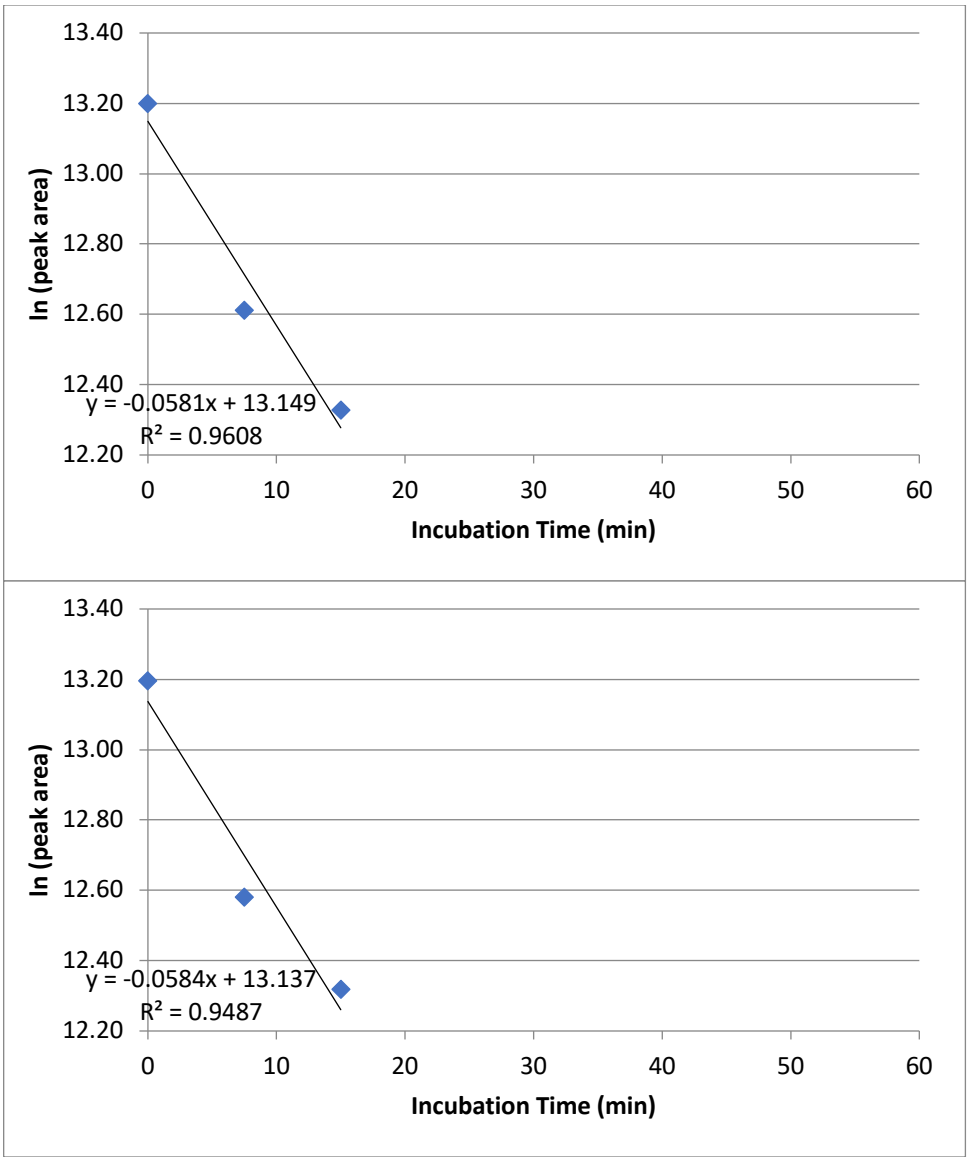

Figure 1. ln (peak area) vs. time plot for propranolol in mouse liver microsomes (with NADPH) (top: replicate 1; bottom: replicate 2).

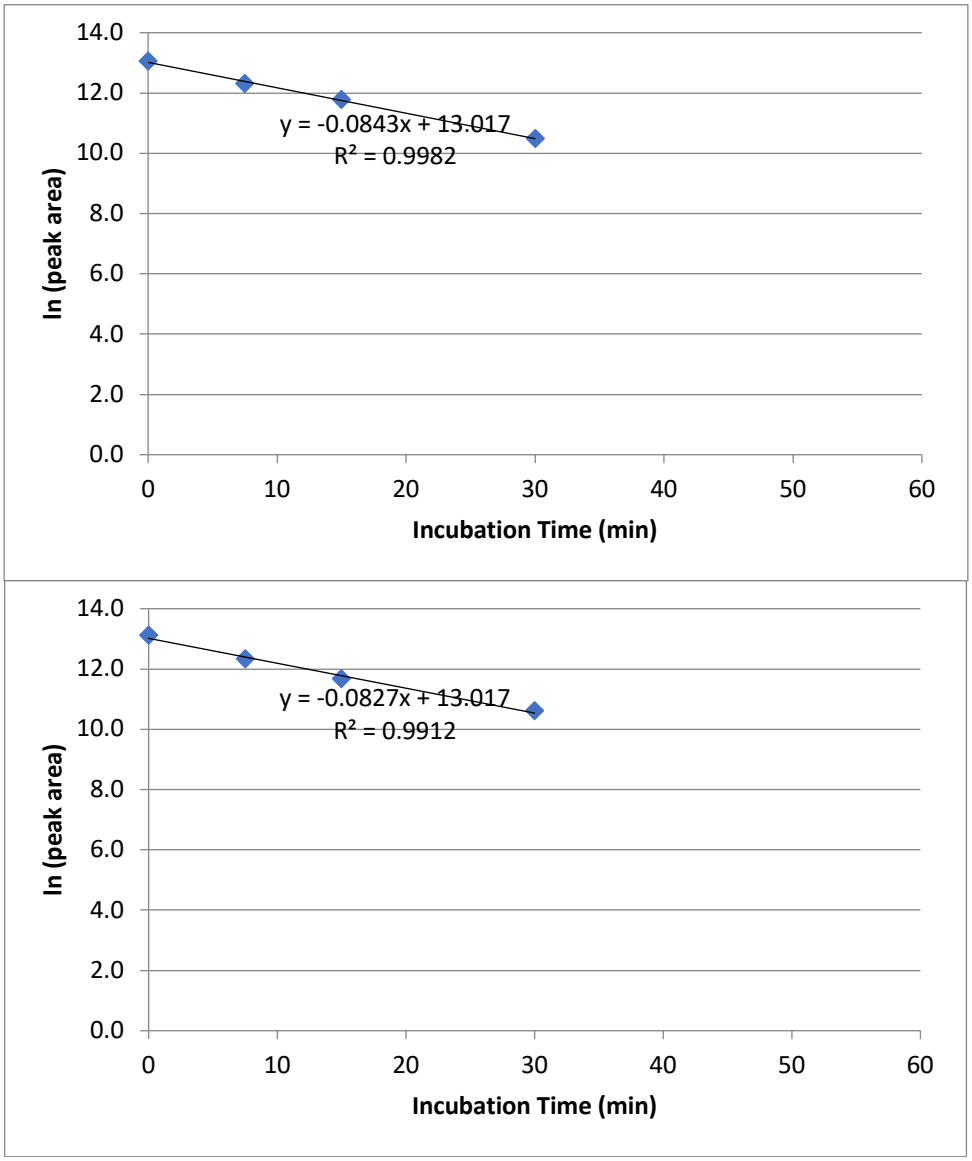

Figure 2. ln (peak area) vs. time plot for SB400868 in mouse liver microsomes (with NADPH) (top: replicate 1; bottom: replicate 2).