

# **Metabolic Stability Report**

## **Metabolic Stability Study of SB400868 in Rat Liver Microsomes with and without the Presence of NADPH**

### ***SPONSORED BY:***

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REPORT APPROVAL

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Date

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## 1 Executive Summary

1  $\mu$ M of the test compound and control (propranolol) were incubated with rat 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 life of SB400868 in rat liver microsomes is 17.7 min.

Both propranolol and SB400868 are slightly unstable in microsomes without the presence of NADPH, probably caused by experimental uncertainty.

## 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 <sub>2</sub> PO <sub>4</sub>	99%, Reagent, ACS (BDH-VWR)
K <sub>2</sub> HPO <sub>4</sub>	98%, Reagent, ACS (BDH-VWR)
MgCl <sub>2</sub> •6H <sub>2</sub> O	99.9%, AR ACS grade (Mallinckrodt Chemicals)
NADPH Tetrasodium Salt	97.4% (EMD Biosciences)

### 2.2 Biological Materials

Rat Liver Microsomes	SD, Male, 20 mg protein/mL, Pool of 433, Lot No. 1210376 (XenoTech)
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### 2.3 Experimental Procedures

#### 2.3.1 Liver Microsomal Incubation

The test compound and control (propranolol) were incubated at a concentration of 1  $\mu$ M with rat 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 <sub>2</sub> HPO <sub>4</sub> -KH <sub>2</sub> PO <sub>4</sub> Buffer (pH 7.4)	435	465	90 mM
33 mM MgCl <sub>2</sub>	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<sub>XR</sub> Pumps and SIL-20AC<sub>XR</sub> Autosampler  
 Column: Venusil XBP C18(2), 5 μm, 2.1x50 mm  
 Mobile phase A: 0.1% HOAc 1 mM NH<sub>4</sub>OAc 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  $\ln(\text{peak area})$  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 compound in rat 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:

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

where

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

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

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

$C_{\text{mp}}$  = 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)

$CL_{\text{int}}$  = 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<sup>1-4</sup> are listed in Table 4. No rat 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 rat liver microsomal incubation is 17.7 min, while the value for propranolol is 3.01 min. The intrinsic clearance of SB400868 is 142 mL/min/kg, higher than that of propranolol, which is about 829 mL/min/kg. The hepatic extraction ratio of SB400868 is about 0.669, while that of propranolol is around 0.922.

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. Both propranolol and SB400868 are slightly unstable in microsomes without the presence of NADPH, probably caused by experimental uncertainty.

## 4 Conclusions

The half life of SB400868 in rat liver microsomes is 17.7 min.

Both propranolol and SB400868 are slightly unstable in microsomes without the presence of NADPH, probably caused by experimental uncertainty.

## 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				
		Rat				
		Incubation Time (min.)				
		0	7.5	15	30	60
Propranolol	1	512000	79200	15700	17400	16900
	2	524000	63100	17100	17500	19600
SB400868	1	603000	521000	331000	321000	312000
	2	633000	532000	355000	322000	279000

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

<b>Species</b>	<b>Liver weight per kg body weight (g/kg)</b>	<b>Hepatic blood flow Q (mL/min/kg)</b>	<b>Microsomal protein content in liver (mg/g)</b>	<b>Microsomal protein incubation concentration (mg/mL)</b>
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	Rat					
		Half Life (min)		CL <sub>int</sub> (mL/min/kg)		E	
		Individual	Average	Individual	Average	Individual	Average
Propranolol	1	2.98	3.01	837	829	0.923	0.922
	2	3.04		821		0.921	
SB400868	1	17.3	17.7	144	142	0.673	0.669
	2	18		139		0.665	

Note: 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.



**Table 4: Liver Microsomal Intrinsic Clearance Reference Value for Propranolol**

Reference	CL <sub>int</sub>					Unit	CL <sub>int</sub> (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	
		Rat	
		Incubation Time (min.)	
		0	60
Propranolol	1	466000	329000
	2	428000	329000
SB400868	1	490000	310000
	2	487000	349000

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

Compound	Replicate	% Remaining after 1-hr Incubation	
		Rat	
		Individual	Average
Propranolol	1	70.6	73.8
	2	76.9	
SB400868	1	63.3	67.5
	2	71.7	

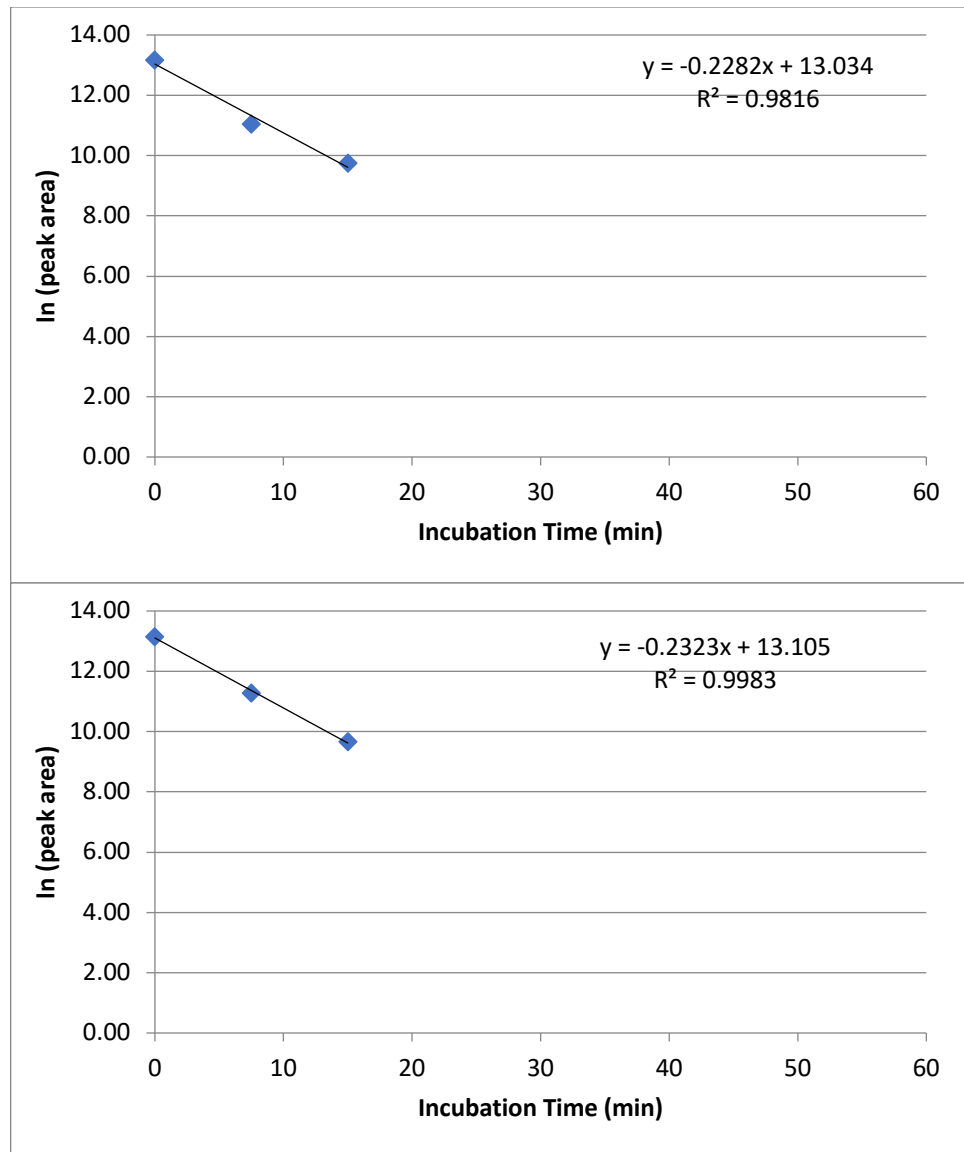


Figure 1. ln (peak area) vs. time plot for propranolol in rat liver microsomes (with NADPH) (top: replicate 1; bottom: replicate 2).

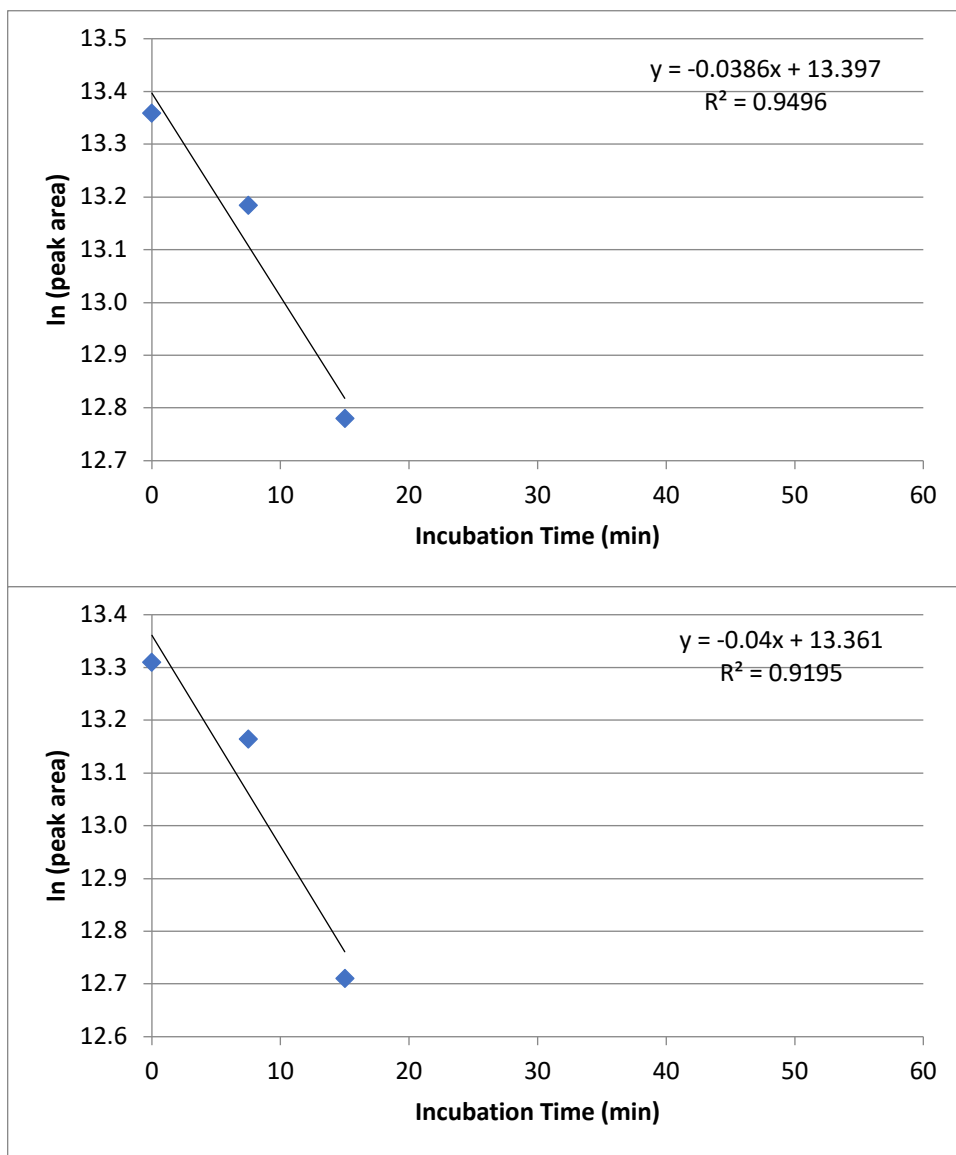


Figure 2. ln (peak area) vs. time plot for SB400868 in rat liver microsomes (with NADPH) (top: replicate 1; bottom: replicate 2).