Metabolic Stability Report

Metabolic Stability Study of 4 Test Compounds 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 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 calculate the half life, intrinsic clearance, hepatic extraction ratio, percent remaining.

The half lives of CA2-209, CA2-220, CA2-005, and ALM-DAI-28 in mouse liver microsomal incubation are 23.5, 5.41, 4.37, and 4.8 min, respectively, while the half life of propranolol is 23.8 min.

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

2 Experimental

2.1 Chemicals

2.1.1 Reference compound

CA2-209	The University of North Carolina at Chapel Hill
CA2-220	The University of North Carolina at Chapel Hill
CA2-005	The University of North Carolina at Chapel Hill
ALM-DAI-28	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 compounds 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: Primesep D, 5 µm, 100 Å, 2.1x100 mm
Mobile phase A: 0.1% Acetic acid 1 mM NH_4OAc in water
Mobile phase B: 50 mM Acetic Acid in Acetonitrile
Injection volume: 2 µL (5 µL for CA2-209; 1 µL for propranolol)
HPLC flow rate: 0.5 mL/min.
Run time: 1.5 min.
Gradient: 50% B (20% B for propranolol) isocratic

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	500°C

Compound dependent parameters:

	Q1	Q3	Time (msec)	DP	EP	CE (V)	CXP
Propranolol	260.2	116.1	200	66	8	23.5	5
CA2-209	278.1	236.1	200	73	10	21	12
CA2-220	289.15	261.15	200	121	10	37	16
CA2-005	253.1	211.1	200	40	10	22	5
ALM-DAI-28	318.15	159.0	200	96	10	55	10

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 (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 compounds in mouse liver microsomes with the presence of NADPH are shown in Figures 1 to 5.

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 compounds are very consistent. The half lives of CA2-209, CA2-220, CA2-005, and ALM-DAI-28 in mouse liver microsomal incubation are 23.5, 5.41, 4.37, and 4.8 min, respectively, while the half life of propranolol is 23.8 min. The intrinsic clearance values of CA2-209, CA2-220, CA2-005, and ALM-DAI-28 are 240, 1040, 1290, and 1170 mL/min/kg, respectively, while the intrinsic clearance of propranolol is 238 mL/min/kg. The hepatic extraction ratios of CA2-209, CA2-220, CA2-005, and ALM-DAI-28 are 0.727, 0.921, 0.935, and 0.929, respectively, while hepatic extraction ratio of propranolol is 0.725.

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 CA2-209, CA2-220, CA2-005, and ALM-DAI-28 in mouse liver microsomal incubation are 23.5, 5.41, 4.37, and 4.8 min, respectively, while the half life of propranolol is 23.8 min.

All test compounds are stable in 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
2. Corning Metabolic Stability in Microsomes Studies, http://www.corning.com/uploadedFiles/Lifesciences/PDFs/ProductInformation/ADME-Tox/Metabolic_Stability_Microsomes_Studies_DS_CLS-DL-GT-028.pdf
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
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	51400	38200	32300	22200	12300
	2	48000	37700	31700	22000	11400
CA2-209	1	53400	38900	35100	15000	880
	2	53500	42100	33400	15600	292
CA2-220	1	44900	16800	6850	2740	664
	2	67400	19000	9440	2340	1480
CA2-005	1	63500	29400	5840	0	0
	2	60100	28400	5600	0	0
ALM-DAI-28	1	362000	101000	41700	6560	3330
	2	361000	102000	41200	7320	3540

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	22.4	23.8	251	238	0.736	0.725
	2	25.1		224		0.713	
CA2-209	1	24.8	23.5	226	240	0.715	0.727
	2	22.1		254		0.738	
CA2-220	1	5.53	5.41	1020	1040	0.919	0.921
	2	5.29		1060		0.922	
CA2-005	1	4.36	4.37	1290	1290	0.935	0.935
	2	4.38		1280		0.934	
ALM-DAI-28	1	4.81	4.8	1170	1170	0.929	0.929
	2	4.79		1170		0.929	

Note: Only data from 0 to 15 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	52600	53600
	2	52300	55400
CA2-209	1	27800	30300
	2	27000	36800
CA2-220	1	54200	53900
	2	54600	52800
CA2-005	1	50700	55200
	2	52400	55000
ALM-DAI-28	1	558000	539000
	2	495000	514000

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	102	104
	2	106	
CA2-209	1	109	123
	2	136	
CA2-220	1	99.4	98.1
	2	96.7	
CA2-005	1	109	107
	2	105	
ALM-DAI-28	1	96.6	100
	2	104	

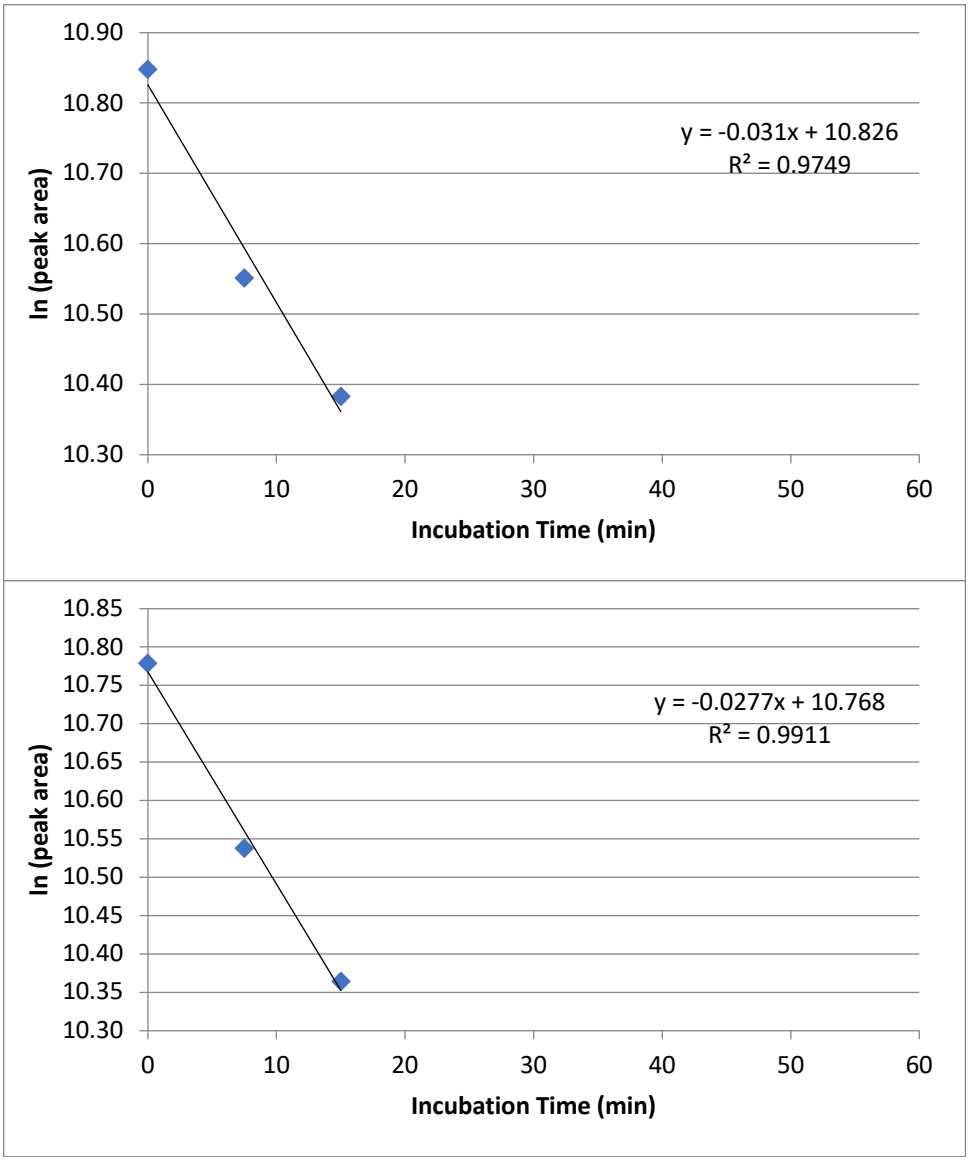

Figure 1. ln (peak area) vs. time plot for propranolol in mouse liver microsomes (with NADPH) (top: replicate 1; bottom: replicate 2)

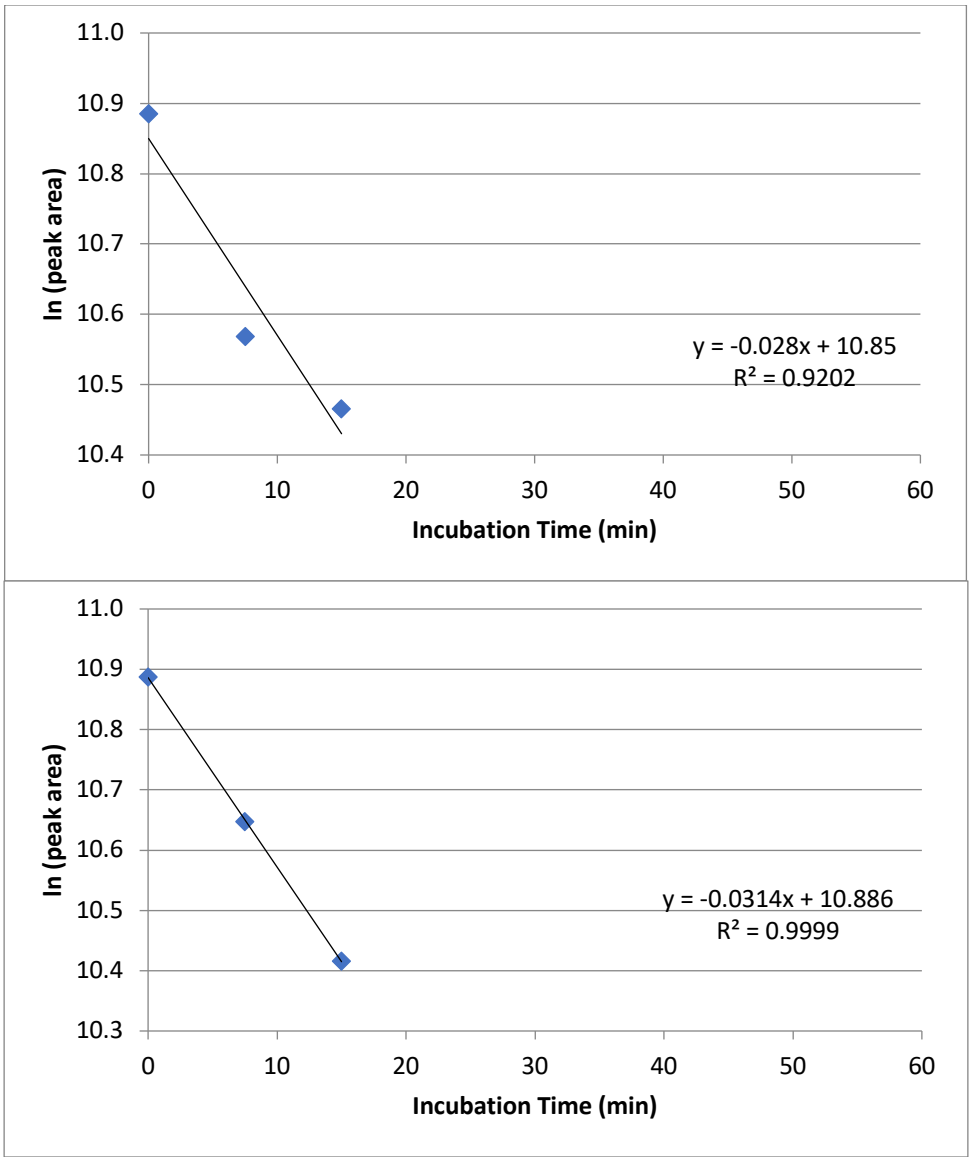

Figure 2. ln (peak area) vs. time plot for CA2-209 in mouse liver microsomes (with NADPH) (top: replicate 1; bottom: replicate 2)

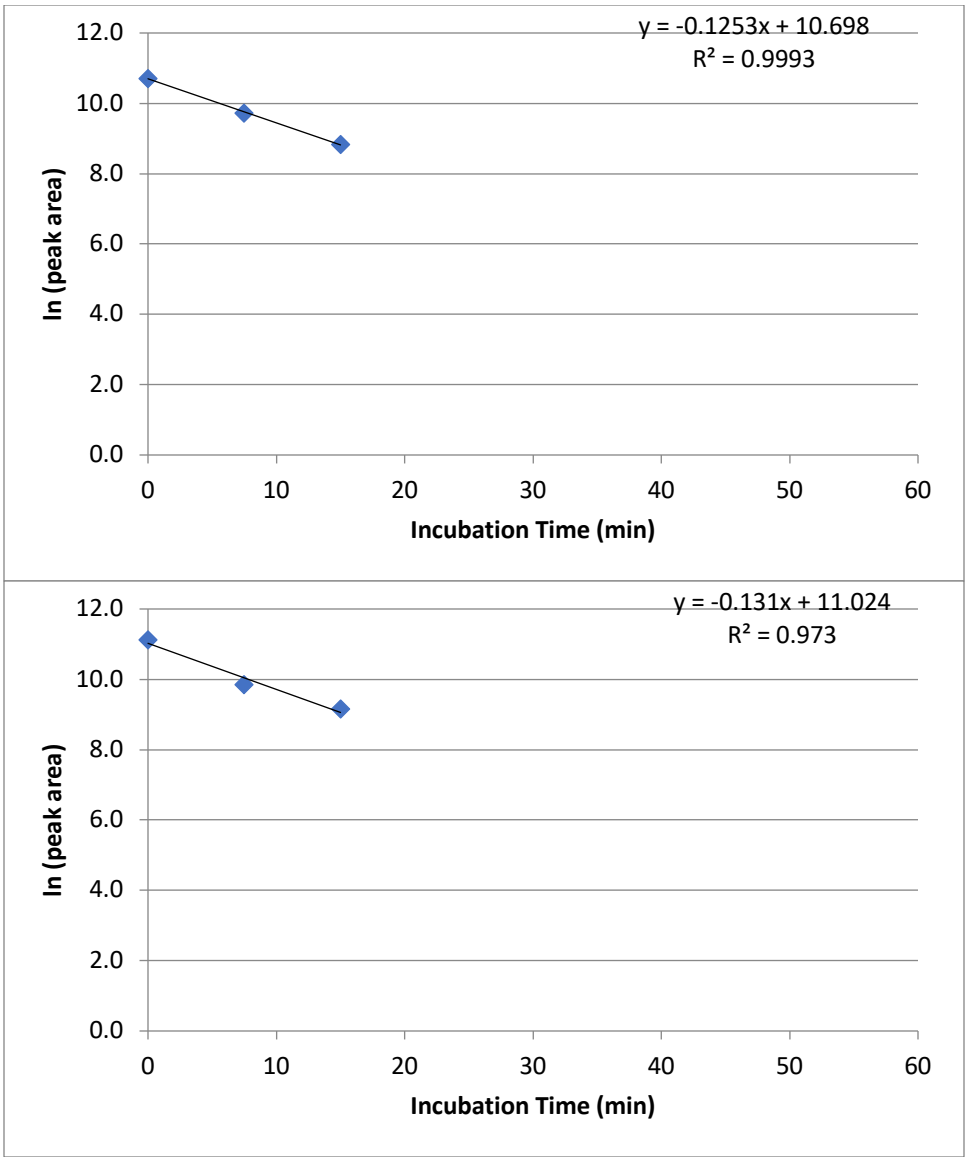

Figure 3. ln (peak area) vs. time plot for CA2-220 in mouse liver microsomes (with NADPH) (top: replicate 1; bottom: replicate 2)

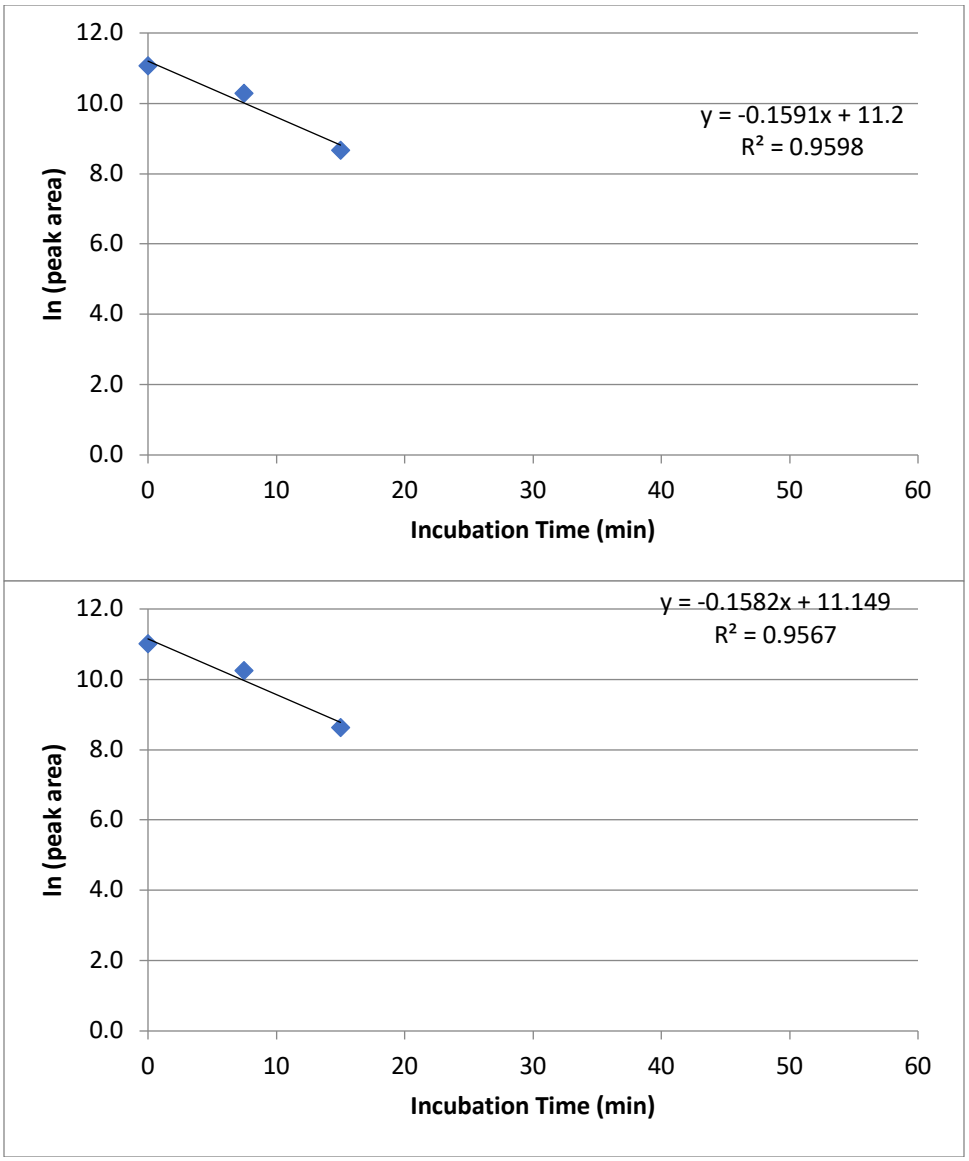

Figure 4. ln (peak area) vs. time plot for CA2-005 in mouse liver microsomes (with NADPH) (top: replicate 1; bottom: replicate 2)

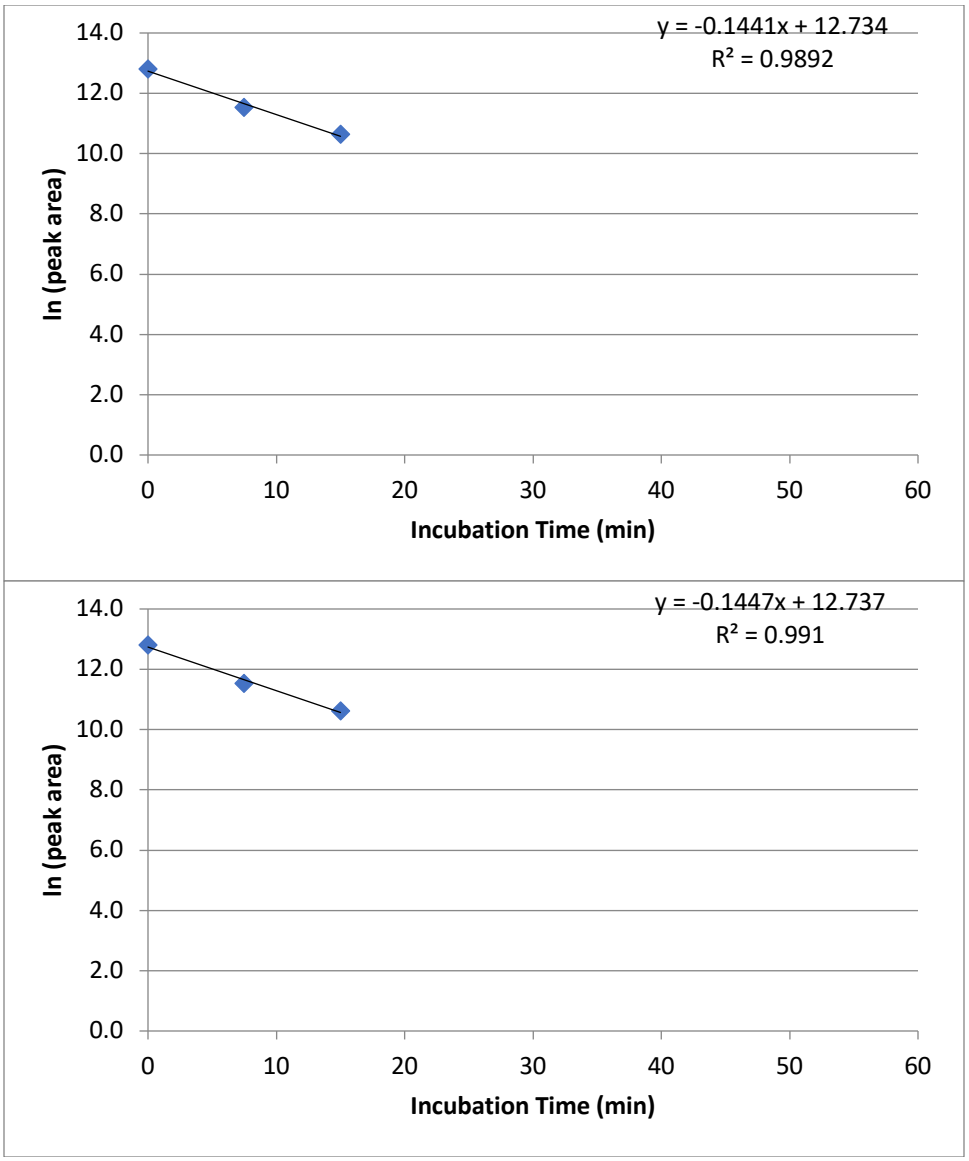

Figure 5. ln (peak area) vs. time plot for ALM-DAI-28 in mouse liver microsomes (with NADPH) (top: replicate 1; bottom: replicate 2)