Supporting Information

Deuteration and fluorination of 1,3-bis(2-phenylethyl)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione to improve its pharmacokinetic properties

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Experimental Section

General Procedures

All reactions were carried out in oven- or flame-dried glassware under an atmosphere of air unless otherwise noted. Except as otherwise indicated, all reactions were magnetically stirred and monitored by analytical thin-layer chromatography using Whatman pre-coated silica gel flexible plates (0.25 mm) with F_{254} indicator or Merck pre-coated silica gel plates with F_{254} indicator. Visualization was accomplished by UV light (256 nm) or by potassium permanganate and/or phosphomolybdic acid solution as an indicator. Flash column chromatography was performed using silica gel 60 (mesh 230-400) supplied by E. Merck. Yields refer to chromatographically and spectrographically pure compounds, unless otherwise noted. Commercial grade reagents and solvents were used without further purification except as indicated below. Tetrahydrofuran (THF) was distilled from sodium-benzophenone ketyl under an atmosphere of dry nitrogen.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance III (500 MHz ^{1}H, 125 MHz ^{13}C) with a DCH Cryo-Probe. Chemical shift values (δ) are reported in ppm relative to $CDCl_3$ [δ 7.26 ppm (^{1}H), 77.16 ppm (^{13}C)]. The proton spectra are reported as δ (multiplicity, number of protons). Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), p (pentet), h (heptet), m (multiplet), and br (broad). Elemental analyses were performed by Atlantic Microlab Inc., Norcross, GA. The C, H, and N analyses were performed by combustion using automatic analyzers, and all of the compounds analyzed showed >95% purity.

Typical procedure for the synthesis of PYT analogues with deuterium on the carbon chains

$ZrCl_4$ (20 mmol) was slowly added to anhydrous THF (40 mL) under stirring at room temp under argon. To the mixture was added $NaBH_4$/$NaBD_4$ (5 mmol) portionwise. Immediate gas evolution was observed upon mixing, and a cream colored suspension was obtained. A solution of

RCD_2CN[11]/RCH_2CN (4 mmol) in anhydrous THF (10 mL) was added to the mixture, which was stirred for 5 h at room temp. The reaction was quenched by the addition of water (30 mL) with ice cooling, and the mixture was then extracted with EtOAc (3 x 30 mL). The organic phase was collected and washed with 1 M HCl (3 x 30 mL). The aqueous phase was collected and concentrated *in vacuo* to afford the desired $RCH_2CD_2NH_3^+Cl^-$/$RCD_2CH_2NH_3^+Cl^-$/ $RCD_2CD_2NH_3^+Cl^-$ salts in 70-85% yield.

To the mixture of deuterated $RCH_2CH_2NH_3^+Cl^-$ salt (3 mmol) and anhydrous THF (10 mL) was slowly added triphosgene (0.75 mmol) at -78 ºC. The reaction mixture was stirred for 1 h and then warm up to room temp slowly. After being stirred at room temp overnight, the reaction mixture was partitioned between Et_2O (50 mL) and H_2O (30 mL). The organic layer was washed with water and brine twice and dried over Na_2SO_4. After removal of solvent *in vacuo*, the crude product was purified by silica gel chromatography to afford deuterated urea in 70-80% yield.

To a solution of the deuterated urea (1 mmol) in CH_2Cl_2 (5 mL), was added malonyl dichloride (1 mmol) slowly. The reaction mixture was stirred for 2 h at room temp and then partitioned between EtOAc (20 mL) and H_2O (10 mL). The organic layer was washed with water and brine twice and dried over Na_2SO_4. After removal of solvent *in vacuo*, the crude product was purified by silica gel chromatography to afford the deuterated PYT compound in 85-90% yield.

1,3-Bis(1,1-d$_2$-2-phenylethyl)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.31-7.23 (m, 10 H), 3.58 (s, 2 H), 2.86 (s, 4 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.1, 151.0, 137.7, 128.9, 128.5, 126.7, 39.5, 33.7. Isotopic purity: >98 atom % D. EC_{50} = 1.70 μM.

1,3-Bis(2,2-d$_2$-2-phenylethyl)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (3). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.31-7.23 (m, 10 H), 4.08 (s, 4 H), 3.58 (s, 2 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.1, 151.0, 137.7, 128.9, 128.5, 126.7, 42.9, 39.5. Isotopic purity: >98 atom % D. EC_{50} = 1.57 μM.

1,3-Bis(1,1,2,2-d$_4$-2-phenylethyl)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (4). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.31-7.23 (m, 10 H), 3.58 (s, 2 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.1, 151.0, 137.7, 128.9, 128.5, 126.7, 39.5. Isotopic purity: >98 atom % D. EC_{50} = 1.05 μM.

Typical procedure for the synthesis of the PYT analogs with fluorine on the carbon chains

To a plastic flask containing $RPhCOCH_2Br$ (10 mmol) was added DAST (10.5 mmol) at 0 ºC with stirring, and the mixture was stirred at room temp for 7 days. The reaction mixture was partitioned between EtOAc (300 mL) and saturated $NaHCO_3$ (300 mL). The organic layer was washed with brine twice and dried over Na_2SO_4. After removal of solvent *in vacuo*, the crude $RPhCF_2CH_2Br$ residue was dissolved in anhydrous DMSO (15 mL), and to this solution was added NaN_3 (15 mmol). After being stirred at 110 ºC overnight, the reaction mixture was cooled to room temp and partitioned between EtOAc (300 mL) and H_2O (200 mL). The organic layer was washed with brine twice and dried over Na_2SO_4. After removal of solvent *in vacuo*, the crude $RPhCF_2CH_2N_3$ was dissolved in EtOH (30 mL). To this solution was added 1 N HCl (15 mL) and $Pd(OH)_2/C$ (20%, 200 mg). The reaction mixture was allowed to stir under one atmosphere of H_2 for 2 days. The Pd catalyst was removed by filtration, and the solvent was evaporated. The resulting solid was dried under vacuum overnight to afford $RPhCF_2CH_2NH_3^+Cl^-$ in 50-80% overall yield.

To the mixture of $RPhCF_2CH_2NH_3^+Cl^-$ (6 mmol) and anhydrous THF (20 mL) was slowly added triphosgene (1.5 mmol) at -78 ºC. The reaction mixture was stirred for 1 h and then warmed to room temp slowly. After being stirred at room temp overnight, the reaction mixture was partitioned between Et_2O (100 mL) and H_2O (50 mL). The organic layer was washed with water and brine twice and dried over Na_2SO_4. After removal of solvent *in vacuo*, the crude product was purified by silica gel chromatography to afford $RPhCF_2CH_2$-urea in 60-70% yield.

To the solution of $RPhCF_2CH_2$-urea (2 mmol) in CH_2Cl_2 (10 mL), was added malonyl dichloride (2 mmol) slowly. The reaction mixture was stirred for 2 h at room temp and then partitioned between EtOAc (40 mL) and H_2O (20 mL). The organic layer was washed with water and brine twice and dried over Na_2SO_4. After removal of solvent *in vacuo*, the crude product was purified by silica gel chromatography to afford the fluorinated PYT compound in 85-90% yield.

1,3-Bis(2,2-difluoro-2-phenylethyl)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (5). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.57 (m, 4 H), 7.48 (m, 6 H), 4.57 (t, 4 H), 3.79 (s, 2 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.8, 150.9, 134.7, 134.5, 134.3, 130.8, 128.8, 125.4, 125.3, 122.0, 120.0, 118.0, 46.9, 46.7, 46.5, 39.7. Elemental Analysis: Comb. Anal. ($C_{20}H_{16}F_4N_2O_3$), Calculated: C, 58.83; H, 3.95; N, 6.88; Found: C, 58.67; H, 3.86; N, 6.68; Purity: >95%. EC_{50} = 2.19 μM.

1,3-Bis(2,2-difluoro-2-(4-fluorophenyl)ethyl)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (6). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.56 (m, 4 H), 7.15 (m, 4 H), 4.55 (t, 4 H), 3.81 (s, 2 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.1, 163.7, 163.1, 150.9, 130.7, 130.5, 130.3, 127.6, 121.6, 119.7, 117.7, 116.1, 115.9, 46.8, 46.6, 46.4, 39.6. Elemental Analysis: Comb. Anal. ($C_{20}H_{14}F_6N_2O_3$), Calculated: C, 54.06; H, 3.18; N, 6.30; Found: C, 53.99; H, 3.44; N, 6.10; Purity: >95%. EC_{50} = 3.50 μM.

11,3-Bis(2-(3-chlorophenyl)-2,2-difluoroethyl)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (7). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.57-7.36 (m, 8 H), 4.55 (t, 4 H), 3.83 (s, 2 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.7, 156.5, 150.9, 136.3, 135.0, 131.1, 130.3, 125.7, 123.6, 121.2, 119.2, 117.2, 46.7, 46.5, 46.2, 39.6. Elemental Analysis: Comb. Anal. ($C_{20}H_{14}Cl_2F_4N_2O_3$), Calculated: C, 50.33; H, 2.96; N, 5.87; Found: C, 50.43; H, 3.07; N, 5.95; Purity: >95%. EC_{50} = 2.69 μM.

1,3-Bis(2,2-difluoro-2-(3-fluorophenyl)ethyl)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (8). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.46-7.05 (m, 8 H), 4.60 (t, 4 H), 3.83 (s, 2 H); ^{13}C NMR (125 MHz,

$CDCl_3$) δ 164.6, 163.8, 161.8, 151.0, 140.1, 130.1, 124.5, 115.9, 115.7, 113.8, 113.6, 113.4, 46.8, 46.6, 46.4, 39.7. EC_{50} = 2.54 μM.

Mutant SOD1-induced cytotoxicity protection assay

Cells were seeded at 15,000 cells/well in 96-well plates and incubated 24 h prior to compound addition. Compounds were assayed in twelve-point dose response experiments to determine potency and efficacy. The highest compound concentration tested was 32 μM, which was decreased by one-half with each subsequent dose. After 24 h incubation with the compounds, MG132 was added at a final concentration of 100 nM. MG132 is a well-characterized proteasome inhibitor, which would be expected to enhance the appearance of protein aggregation by blocking the proteosomal clearance of aggregated proteins. Cell viability was measured 48 h later using the fluorescent viability probe, Calcein-AM (Molecular Probes). Briefly, cells were washed twice with PBS, Calcein-AM was added at a final concentration of 1 μM for 20 min at room temperature, and fluorescence intensity was read in a POLARstar fluorescence plate reader (BMG). Fluorescence data were coupled with compound structural data, then stored and analyzed using the CambridgeSoft Chemoffice Enterprise Ultra software package.

In vitro ADME studies

In vitro ADME properties of PYT compounds were tested at Apredica, Inc. (Watertown, MA), a contract research organization.

Supporting Information - SP-1

Compound	Apredica ID
1	CAM-020-05
9	CAM-020-02
10	CAM-020-03
11	CAM-020-04

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1 Objective

The objective of this study was to evaluate the *in vitro* ADME properties of the test agents.

1.1 Regulatory Guidelines

This study was not conducted under US FDA Good Laboratory Practice Regulations (GLPs). Standard operating procedures of Apredica were used throughout the study.

2 Test Articles

Apredica ID	Client ID	Physical Form	Submitted FW	Parent MW	Stock solutions
CAM-020-01	**CMB-087618**	solid		408	10 mM DMSO
CAM-020-02	**CMB-087649**	solid		344	10 mM DMSO
CAM-020-03	**CMB-087647**	solid		340	10 mM DMSO
CAM-020-04	**CMB-087648**	solid		340	10 mM DMSO
CAM-020-05	**CMB-021805**	solid		336	10 mM DMSO

Test agent powders were stored at -20 °C. Stock solutions were stored at -20 °C.

3 Test Methods

Testing was performed at Apredica in Watertown, MA.

3.1 Analytical Methods

3.1.1 Method development

The signal was optimized for each compound by ESI positive or negative ionization mode. A MS2 scan was used to identify the precursor ion and a product ion analysis was used to identify the best fragment for analysis and to optimize the collision energy. An ionization ranking was assigned indicating the compound's ease of ionization.

3.1.2 Analysis

Samples were analyzed by LC/MS/MS using either an Agilent 6410 mass spectrometer coupled with an Agilent 1200 HPLC and a CTC PAL chilled autosampler, all controlled by MassHunter software (Agilent), or an ABI2000 mass spectrometer coupled with an Agilent 1100 HPLC and a CTC PAL chilled autosampler, all controlled by Analyst software (ABI). After separation on a C18 reverse phase HPLC column (Agilent, Waters, or equivalent) using an acetonitrile-water gradient system, peaks were analyzed by mass spectrometry (MS) using ESI ionization in MRM mode.

3.2 In vitro *ADME-Tox Experimental Conditions*

Additional protocol details are given in Appendix A.

3.2.1 PBS solubility experimental conditions

Apredica ID	Client ID	Test conc	Medium	Incub-ation	Ref. comp.	Analytical method
CAM-020-01 **CAM-020-02** **CAM-020-03** **CAM-020-04** **CAM-020-05**	**CMB-087618** **CMB-087649** **CMB-087647** **CMB-087648** **CMB-021805**	500, 250, 125, 62.5, 31.3, 15.6, 7.81, 3.91 μM	PBS	45 min, ON 37 °C	reserpine tamoxifen verapamil	UV/Vis

3.2.2 Microsomal stability experimental conditions

Apredica ID	Client ID	Test conc.	Micro-some source	Protei n conc.	Incub-ation	Ref. comp.	Analytical method
CAM-020-01 **CAM-020-02** **CAM-020-03** **CAM-020-04** **CAM-020-05**	**CMB-087618** **CMB-087649** **CMB-087647** **CMB-087648** **CMB-021805**	1 μM	human, mouse	0.3 mg/mL	0, 10, 20, 40, 60 min 37 °C	verapamil warfarin	LC/MS/MS

3.2.3 Plasma stability experimental conditions

Apredica ID	Client ID	Test conc.	Plasma source	Incub-ation	Reference compounds	Analytical method
CAM-020-01 **CAM-020-02** **CAM-020-03** **CAM-020-04** **CAM-020-05**	**CMB-087618** **CMB-087649** **CMB-087647** **CMB-087648** **CMB-021805**	5 μM	mouse	0, 10, 20, 40, 60 min 37 °C	propantheline	LC/MS/MS

3.2.4 PBS stability experimental conditions

Apredica ID	Client ID	Test conc	Medium	Incub-ation	Ref. comp.	Analytical method
CAM-020-01 **CAM-020-02** **CAM-020-03** **CAM-020-04** **CAM-020-05**	**CMB-087618** **CMB-087649** **CMB-087647** **CMB-087648** **CMB-021805**	5 μM	PBS[a]	0, 10, 20, 40, 60 min 37 °C	warfarin	LC/MS/MS

[a]PBS is Ca^{2+}, Mg^{2+}-free phosphate-buffered saline, pH 7.2.

4 Results

4.1 Analytical

4.1.1 Method development

Client ID	MW	Polari-zation	Precursor m/z	Product m/z	Collision energy (V)	Ionization classification[a]
CMB-087618	408	neg	407.1	136.1	40	1
CMB-087649	344	neg	343.2	236.1	22	1
CMB-087647	340	neg	339.3	233.1	18	1
CMB-087648	340	neg	339.2	234.1	18	1
CMB-021805	336	neg	335	127	26	1

[a]Ionization classification:
1 = Highly ionizable
2 = Intermediately ionizable
3 = Poorly ionizable

The product ion spectrum and a sample chromatogram are shown in Appendix B.

4.2 In vitro *ADME-Tox Summary*

4.2.1 PBS express solubility summary

Client ID	Medium	Solubility limit (μM)[a] 45 min	Solubility limit (μM)[a] 16 hr	comment
Verapamil	PBS	>=500	>=500	negative control
Reserpine	PBS	31.3	31.3	positive control
Tamoxifen	PBS	15.6	31.3	positive control
CMB-021805	PBS	15.6	31.25	
CMB-087618	PBS	125.0	125.0	
CMB-087647	PBS	31.3	31.3	
CMB-087648	PBS	31.3	31.3	
CMB-087649	PBS	62.5	62.5	

[a]Solubility limit is highest concentration with no detectable precipitate.

4.2.2 Microsomal intrinsic clearance summary

Client ID	test conc (μM)	test species	NADPH-dependent CL_{int}[a] (μl min^{-1} mg-^{1})	NADPH-dependent $T_{1/2}$[b] (min)	NADPH-free CL_{int}[a] (μl min^{-1} mg-^{1})	NADPH-free $T_{1/2}$[b] (min)	comment
Verapamil	1.0	Human	135.5	17.0	2.4	>180	highly metabolized control
Verapamil	1.0	Mouse	>1000	5.5	4.8	>180	highly metabolized control
Warfarin	1.0	Human	0.0	>180	0.9	>180	poorly metabolized control
Warfarin	1.0	Mouse	8.1	>180	0.0	>180	poorly metabolized control
CMB-021805	1.0	Human	36	64	9	>180	
CMB-021805	1.0	Mouse	145	16	14	167	
CMB-087618	1.0	Human	49	48	17	132	
CMB-087618	1.0	Mouse	272	9	30	76	
CMB-087647	1.0	Human	31	75	5	>180	
CMB-087647	1.0	Mouse	147	16	9	>180	

CMB-087648	1.0	Human	36	64	6	>180
CMB-087648	1.0	Mouse	153	15	14	171
CMB-087649	1.0	Human	36	64	8	>180
CMB-087649	1.0	Mouse	163	14	21	109

[a]Microsomal Intrinsic Clearance
[b]Half-life

4.2.3 Plasma half-life summary

Client ID	test conc (μM)	test species	Plasma $T_{1/2}$[a] (min)	Fraction remaining, last time point (%)	comment
Propantheline	5.0	Mouse Plasma	11.4	0.6%	metabolized control
Warfarin	5.0	Mouse Plasma	>60	85.9%	nonmetabolized control
CMB-021805	5.0	Mouse Plasma	63.5	52.9%	
CMB-087618	5.0	Mouse Plasma	81.2	60.4%	
CMB-087647	5.0	Mouse Plasma	49.8	39.4%	
CMB-087648	1.0	Mouse Plasma	54.0	46.0%	
CMB-087649	5.0	Mouse Plasma	49.4	39.1%	

[a]Half-life

4.2.4 Buffer half-life summary

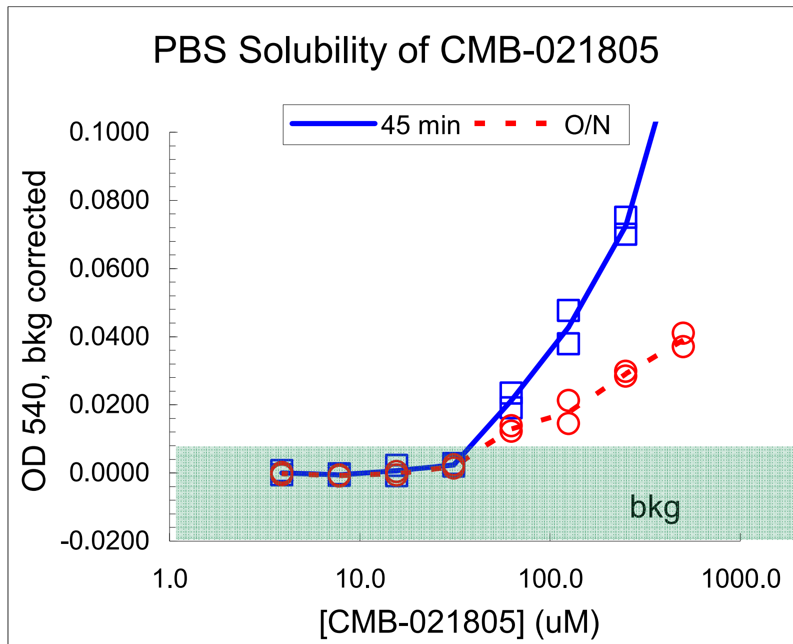
Client ID	Test conc (μM)	Medium	Buffer $T_{1/2}$[a] (min)	Fraction remaining, last time point (%)	comment
Warfarin	5.0	PBS[b]	>60	104.1%	stable control
CMB-021805	5.0	PBS[b]	>60	110.4%	
CMB-087618	5.0	PBS[b]	>60	66.9%	
CMB-087647	5.0	PBS[b]	>60	79.5%	
CMB-087648	1.0	PBS[b]	>60	86.7%	
CMB-087649	5.0	PBS[b]	>60	95.9%	

[a]Half-life
[b]PBS is Ca^{2+}, Mg^{2+}-free phosphate-buffered saline, pH 7.2.

4.3 In vitro *ADME-Tox Individual Data*

4.3.1 PBS express solubility individual data

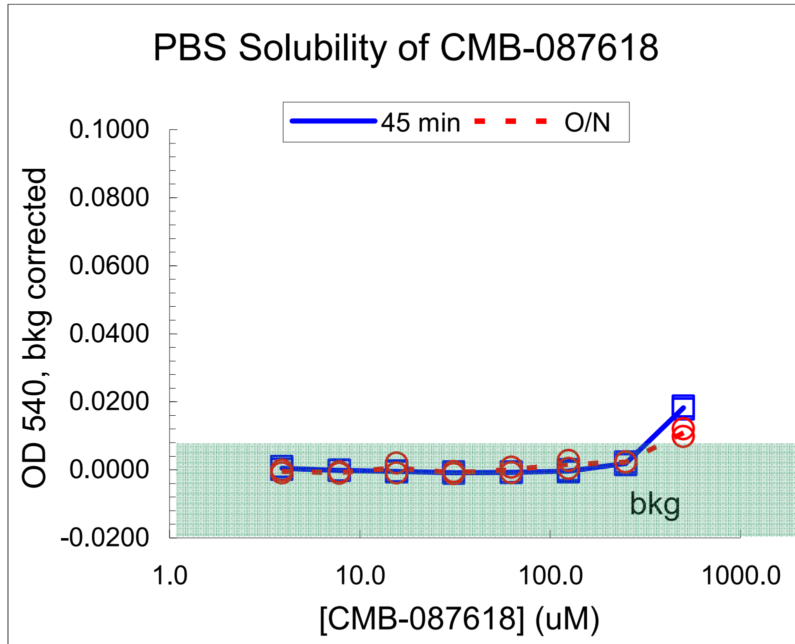
Shaded area represents OD below background.



Test Agent: CMB-021805

Medium PBS

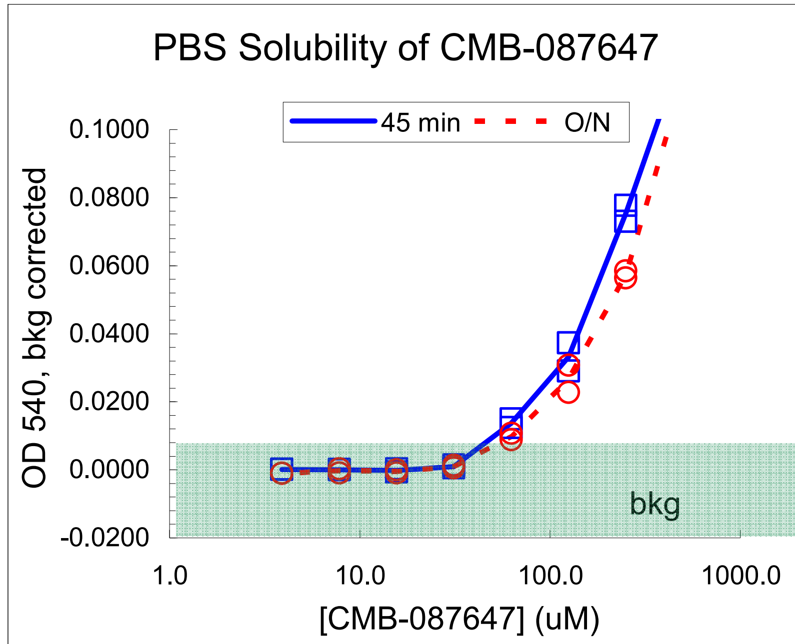
	OD 540 nm, bkg subtracted					
	45 min			O/N		
conc (uM)	**rpt 1**	**rpt2**	**mean**	**rpt 1**	**rpt2**	**mean**
500.0	***0.1369***	***0.1258***	***0.131***	***0.0371***	***0.0410***	***0.0390***
250.0	***0.0700***	***0.0750***	***0.073***	***0.0284***	***0.0298***	***0.0291***
125.0	***0.0476***	***0.0379***	***0.043***	***0.0213***	***0.0145***	***0.0179***
62.5	***0.0234***	***0.0191***	***0.021***	***0.0138***	***0.0122***	***0.0130***
31	0.0028	0.0020	***0.002***	0.0024	0.0014	***0.0019***
16	-0.0008	0.0022	0.001	0.0004	-0.0007	-0.0001
8	-0.0008	-0.0003	-0.001	-0.0006	-0.0009	-0.0008
4	-0.0006	0.0006	0.000	-0.0005	0.0001	-0.0002
vehicle	avg	0.0001		avg	0.0007	
	SD	0.0003		SD	0.0011	
	cutoff	0.0010		cutoff	0.0039	



Test Agent: CMB-087618

Medium PBS

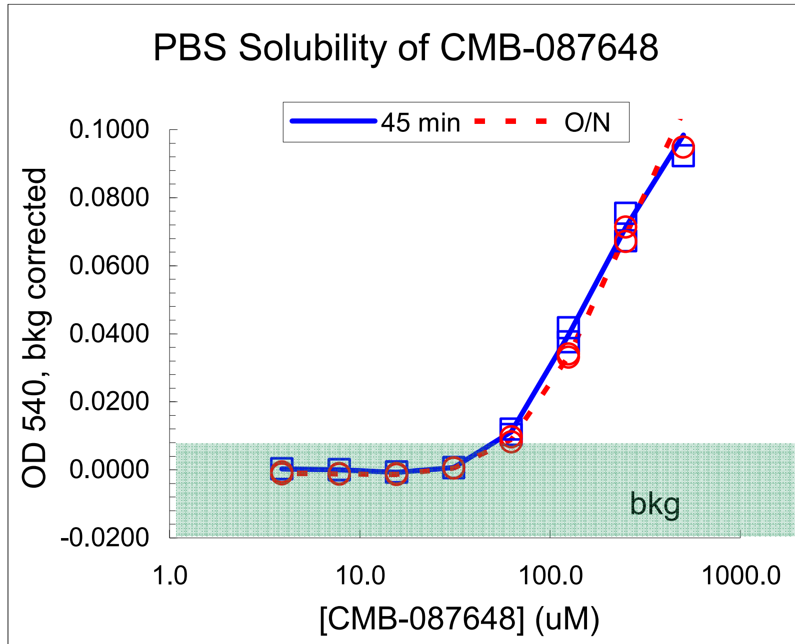
	OD 540 nm, bkg subtracted					
	45 min			O/N		
conc (uM)	**rpt 1**	**rpt2**	**mean**	**rpt 1**	**rpt2**	**mean**
500.0	***0.0178***	***0.0188***	***0.018***	***0.010***	***0.012***	***0.0109***
250.0	0.0022	0.0015	***0.002***	0.002	0.002	***0.0023***
125.0	0.0000	-0.0007	0.000	0.003	0.000	***0.0016***
62.5	-0.0006	-0.0009	-0.001	0.001	-0.001	0.0000
31	-0.0006	-0.0010	-0.001	-0.001	-0.001	-0.0009
16	-0.0006	-0.0004	0.000	-0.001	0.002	0.0005
8	-0.0002	-0.0001	0.000	-0.001	-0.001	-0.0009
4	0.0009	0.0001	0.001	0.000	-0.001	-0.0005
vehicle	avg	0.0001		avg	0.0007	
	SD	0.0003		SD	0.0011	
	cutoff	0.0010		cutoff	0.0039	



Test Agent: CMB-087647

Medium PBS

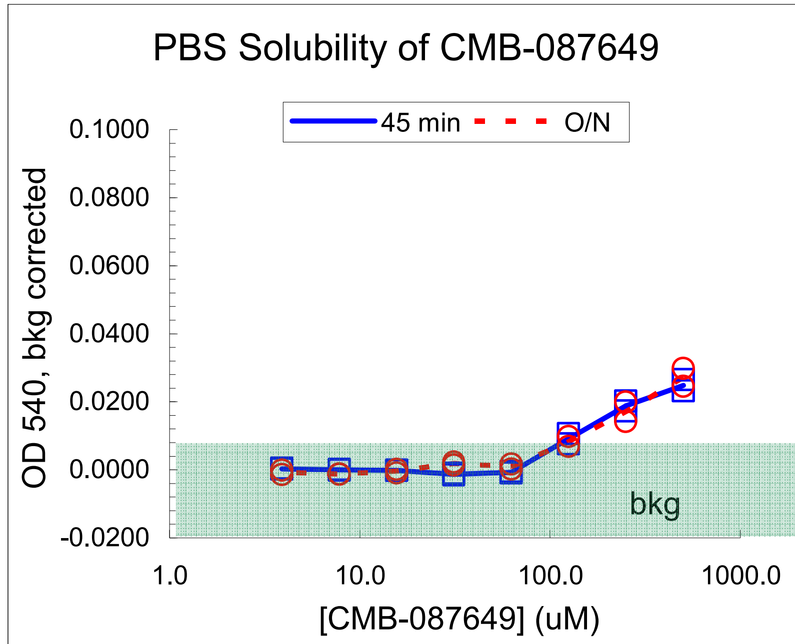
	OD 540 nm, bkg subtracted					
	45 min			O/N		
conc (uM)	**rpt 1**	**rpt2**	**mean**	**rpt 1**	**rpt2**	**mean**
500.0	***0.1233***	***0.1244***	***0.124***	***0.117***	***0.113***	***0.1153***
250.0	***0.0777***	***0.0730***	***0.075***	***0.058***	***0.056***	***0.0574***
125.0	***0.0291***	***0.0373***	***0.033***	***0.023***	***0.031***	***0.0267***
62.5	***0.0150***	***0.0124***	***0.014***	***0.011***	***0.009***	***0.0098***
31	0.0014	0.0006	0.001	0.001	0.001	0.0010
16	0.0003	-0.0007	0.000	0.000	-0.001	-0.0004
8	0.0001	0.0000	0.000	0.000	-0.001	-0.0003
4	0.0002	0.0001	0.000	-0.001	-0.001	-0.0010
vehicle	avg	0.0001		avg	0.0007	
	SD	0.0003		SD	0.0011	
	cutoff	0.0010		cutoff	0.0039	



Test Agent: CMB-087648
Medium PBS

conc (uM)	45 min rpt 1	45 min rpt2	45 min mean	O/N rpt 1	O/N rpt2	O/N mean
500.0	***0.1046***	***0.0923***	***0.098***	***0.116***	***0.095***	***0.1057***
250.0	***0.0754***	***0.0673***	***0.071***	***0.071***	***0.067***	***0.0692***
125.0	***0.0376***	***0.0418***	***0.040***	***0.033***	***0.034***	***0.0336***
62.5	***0.0120***	***0.0104***	***0.011***	***0.010***	***0.008***	***0.0090***
31	0.0007	0.0006	0.001	0.001	0.000	0.0005
16	-0.0006	-0.0008	-0.001	-0.001	-0.001	-0.0013
8	0.0000	0.0000	0.000	-0.001	-0.001	-0.0012
4	0.0003	0.0003	0.000	-0.001	-0.001	-0.0009
vehicle	avg	0.0001		avg	0.0007	
	SD	0.0003		SD	0.0011	
	cutoff	0.0010		cutoff	0.0039	

OD 540 nm, bkg subtracted

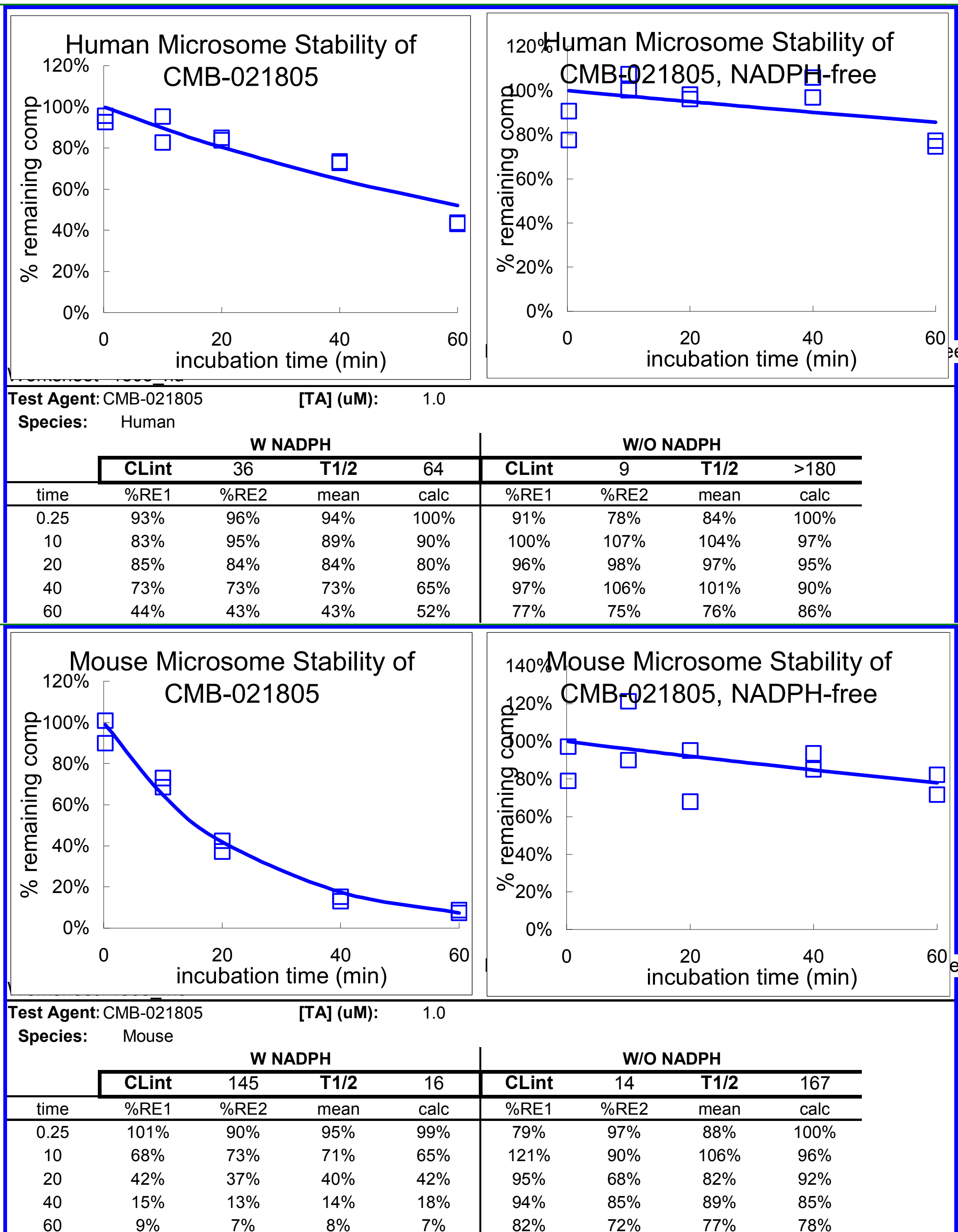


Test Agent: CMB-087649

Medium PBS

	OD 540 nm, bkg subtracted					
	45 min			O/N		
conc (uM)	**rpt 1**	**rpt2**	**mean**	**rpt 1**	**rpt2**	**mean**
500.0	***0.0265***	***0.0232***	***0.025***	***0.025***	***0.030***	***0.0271***
250.0	***0.0173***	***0.0203***	***0.019***	***0.014***	***0.020***	***0.0171***
125.0	***0.0076***	***0.0107***	***0.009***	***0.007***	***0.010***	***0.0083***
62.5	-0.0009	-0.0005	-0.001	0.000	0.002	0.0010
31	-0.0010	-0.0015	-0.001	0.001	0.002	***0.0019***
16	-0.0001	-0.0002	0.000	-0.001	0.000	-0.0004
8	0.0001	0.0000	0.000	-0.001	-0.001	-0.0012
4	0.0002	0.0005	0.000	-0.001	0.000	-0.0008
vehicle	avg	0.0001		avg	0.0007	
	SD	0.0003		SD	0.0011	
	cutoff	0.0010		cutoff	0.0039	

4.3.2 Microsomal intrinsic clearance individual data



Test Agent: CMB-021805 **[TA] (uM):** 1.0

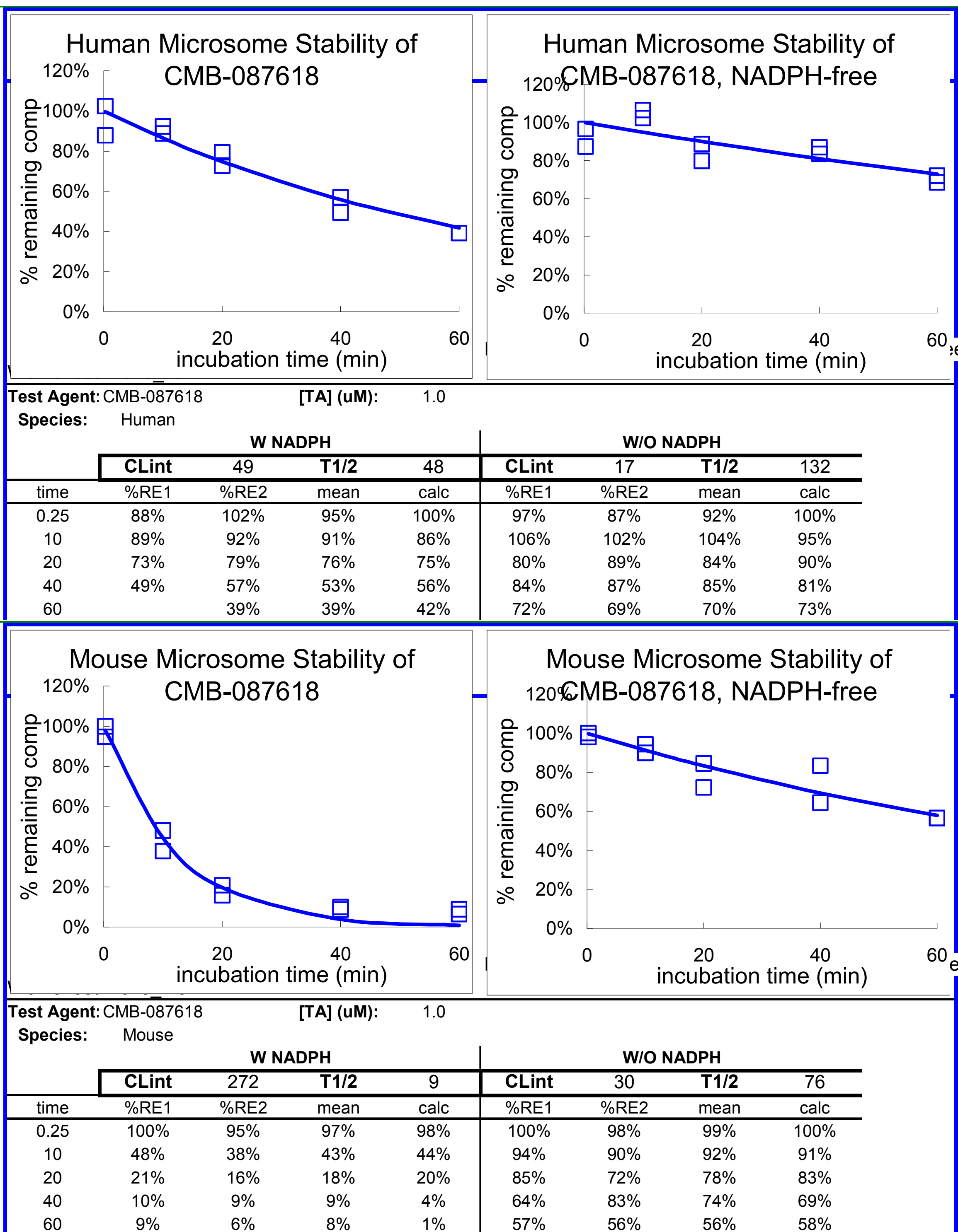
Species: Human

	W NADPH				W/O NADPH			
	CLint	36	**T1/2**	64	**CLint**	9	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	93%	96%	94%	100%	91%	78%	84%	100%
10	83%	95%	89%	90%	100%	107%	104%	97%
20	85%	84%	84%	80%	96%	98%	97%	95%
40	73%	73%	73%	65%	97%	106%	101%	90%
60	44%	43%	43%	52%	77%	75%	76%	86%

Test Agent: CMB-021805 **[TA] (uM):** 1.0

Species: Mouse

	W NADPH				W/O NADPH			
	CLint	145	**T1/2**	16	**CLint**	14	**T1/2**	167
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	101%	90%	95%	99%	79%	97%	88%	100%
10	68%	73%	71%	65%	121%	90%	106%	96%
20	42%	37%	40%	42%	95%	68%	82%	92%
40	15%	13%	14%	18%	94%	85%	89%	85%
60	9%	7%	8%	7%	82%	72%	77%	78%



Test Agent: CMB-087618 **[TA] (uM):** 1.0

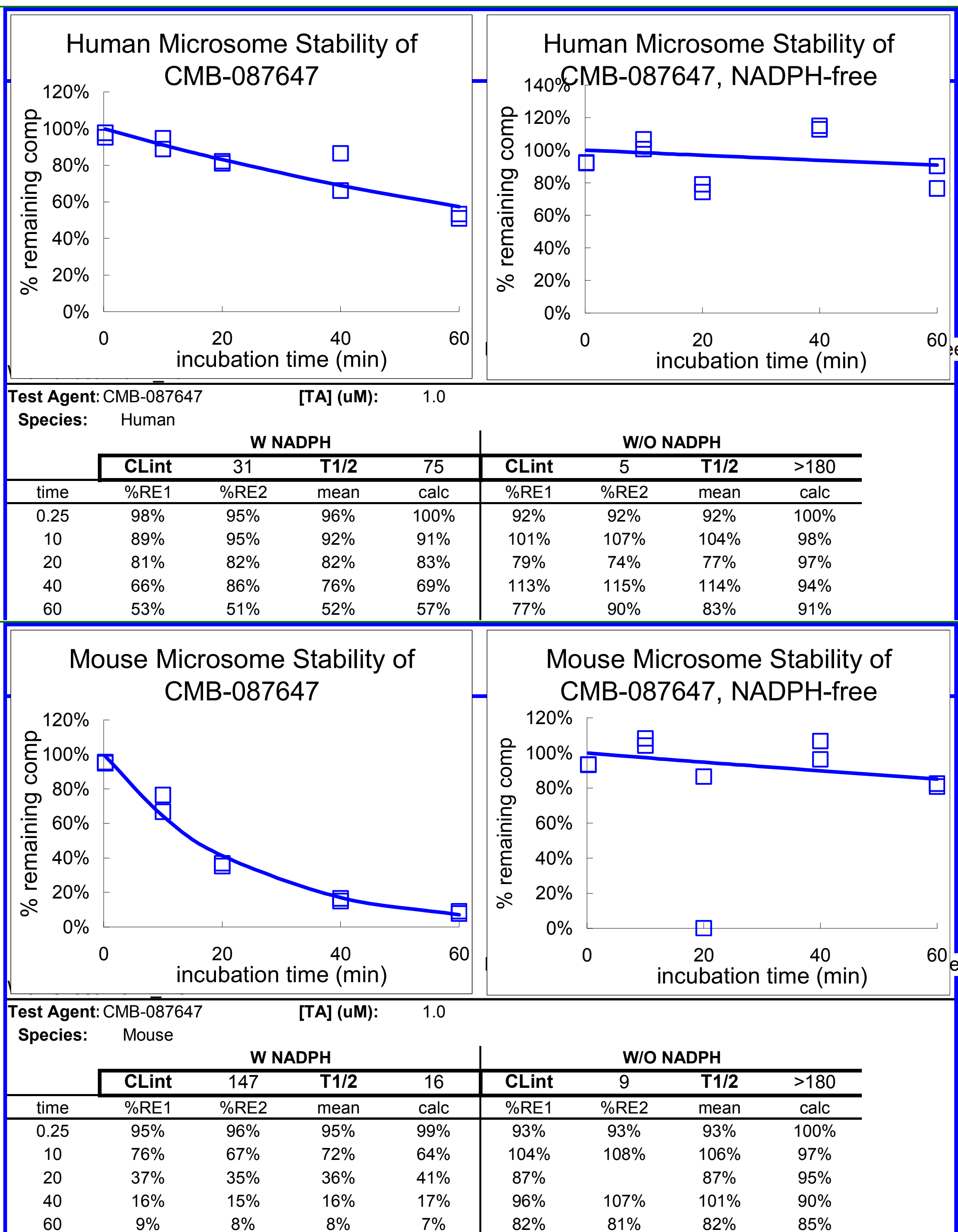
Species: Human

	W NADPH				W/O NADPH			
	CLint	49	**T1/2**	48	**CLint**	17	**T1/2**	132
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	88%	102%	95%	100%	97%	87%	92%	100%
10	89%	92%	91%	86%	106%	102%	104%	95%
20	73%	79%	76%	75%	80%	89%	84%	90%
40	49%	57%	53%	56%	84%	87%	85%	81%
60		39%	39%	42%	72%	69%	70%	73%

Test Agent: CMB-087618 **[TA] (uM):** 1.0

Species: Mouse

	W NADPH				W/O NADPH			
	CLint	272	**T1/2**	9	**CLint**	30	**T1/2**	76
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	100%	95%	97%	98%	100%	98%	99%	100%
10	48%	38%	43%	44%	94%	90%	92%	91%
20	21%	16%	18%	20%	85%	72%	78%	83%
40	10%	9%	9%	4%	64%	83%	74%	69%
60	9%	6%	8%	1%	57%	56%	56%	58%



Test Agent: CMB-087647 **[TA] (uM):** 1.0

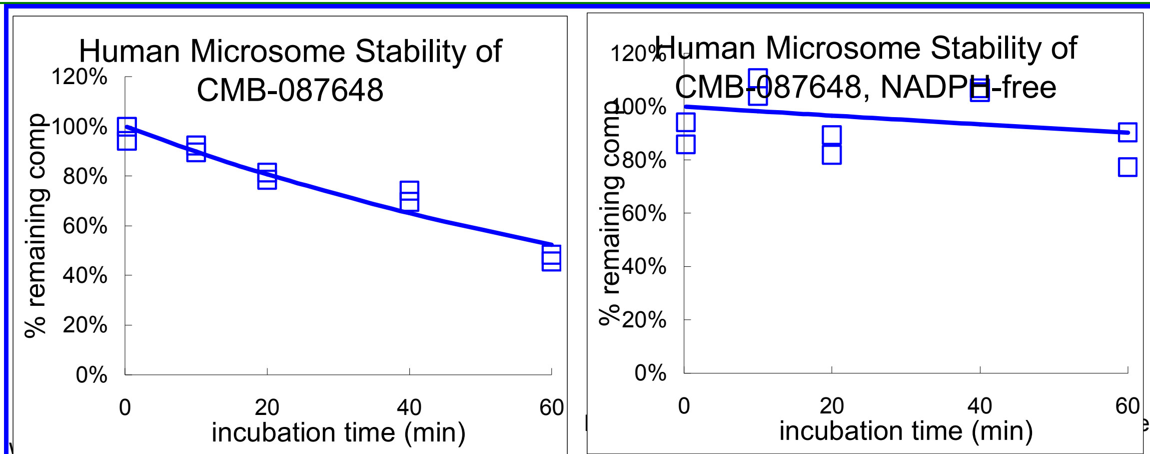
Species: Human

	W NADPH				W/O NADPH			
	CLint	31	**T1/2**	75	**CLint**	5	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	98%	95%	96%	100%	92%	92%	92%	100%
10	89%	95%	92%	91%	101%	107%	104%	98%
20	81%	82%	82%	83%	79%	74%	77%	97%
40	66%	86%	76%	69%	113%	115%	114%	94%
60	53%	51%	52%	57%	77%	90%	83%	91%

Test Agent: CMB-087647 **[TA] (uM):** 1.0

Species: Mouse

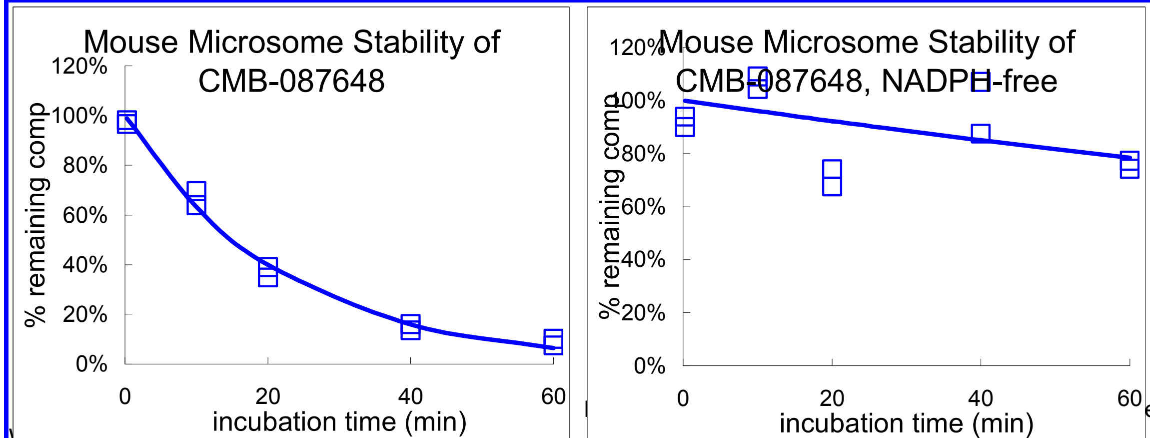
	W NADPH				W/O NADPH			
	CLint	147	**T1/2**	16	**CLint**	9	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	95%	96%	95%	99%	93%	93%	93%	100%
10	76%	67%	72%	64%	104%	108%	106%	97%
20	37%	35%	36%	41%	87%		87%	95%
40	16%	15%	16%	17%	96%	107%	101%	90%
60	9%	8%	8%	7%	82%	81%	82%	85%



Test Agent: CMB-087648 **[TA] (uM):** 1.0

Species: Human

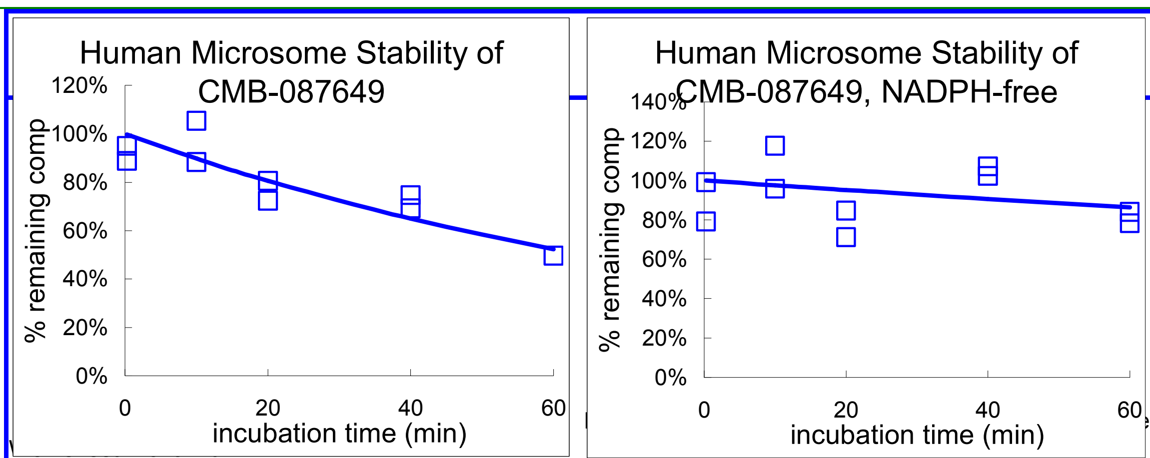
	W NADPH				W/O NADPH			
	CLint	36	**T1/2**	64	**CLint**	6	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	100%	94%	97%	100%	94%	86%	90%	100%
10	89%	92%	91%	90%	111%	104%	107%	98%
20	81%	78%	80%	81%	89%	82%	86%	97%
40	70%	74%	72%	65%	105%	107%	106%	93%
60	48%	46%	47%	52%	77%	90%	84%	90%



Test Agent: CMB-087648 **[TA] (uM):** 1.0

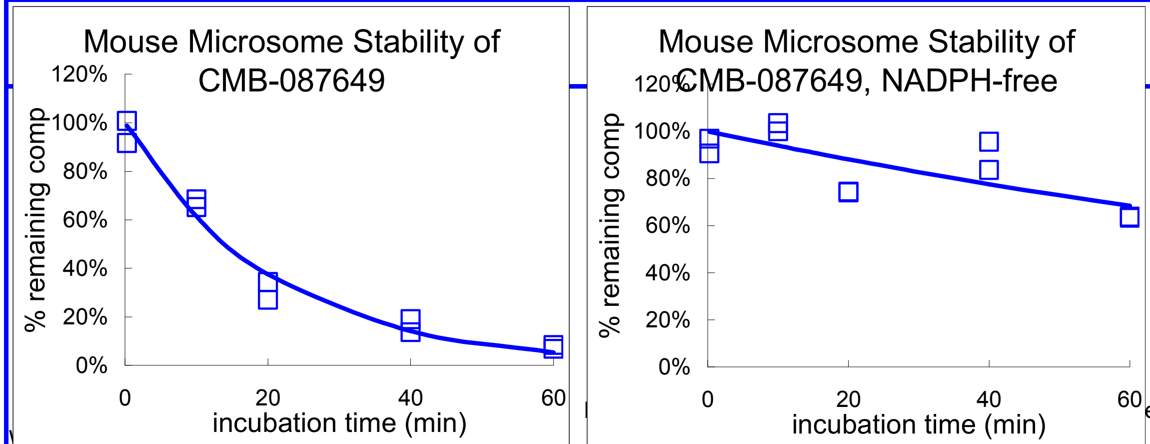
Species: Mouse

	W NADPH				W/O NADPH			
	CLint	153	**T1/2**	15	**CLint**	14	**T1/2**	171
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	98%	97%	97%	99%	90%	94%	92%	100%
10	64%	70%	67%	63%	109%	104%	107%	96%
20	39%	35%	37%	40%	74%	68%	71%	92%
40	16%	14%	15%	16%	88%	107%	97%	85%
60	10%	7%	9%	6%	74%	77%	76%	78%



Test Agent: CMB-087649 **[TA] (uM):** 1.0
Species: Human

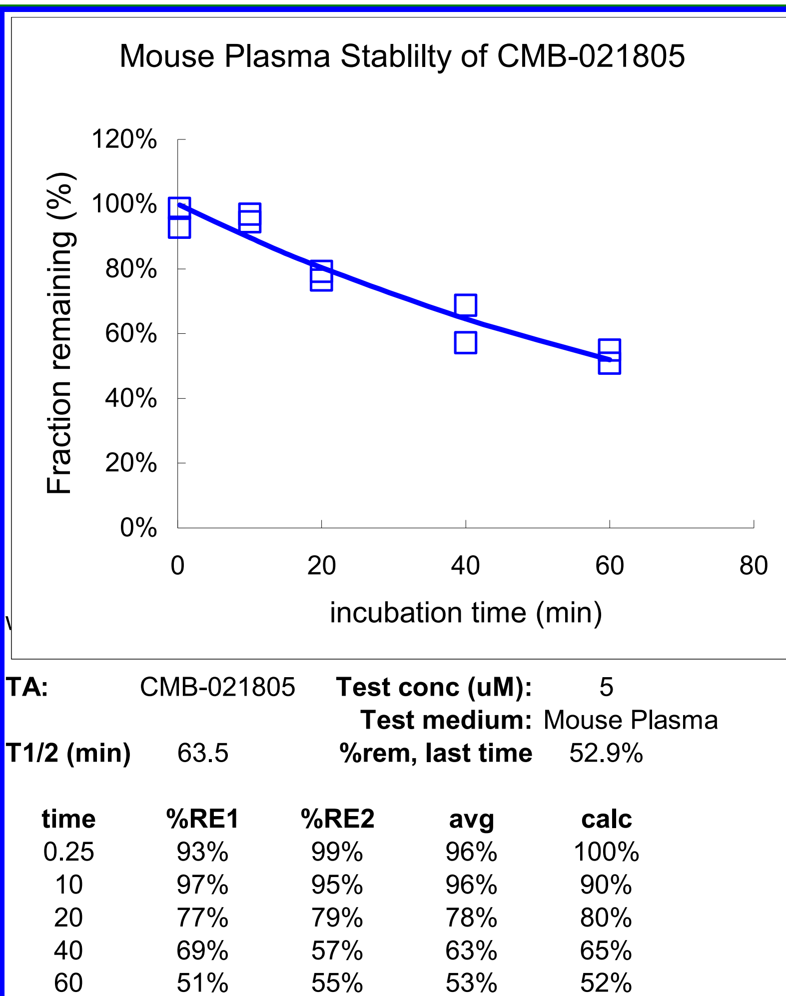
	W NADPH				W/O NADPH			
	CLint	36	**T1/2**	64	**CLint**	8	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	89%	95%	92%	100%	99%	79%	89%	100%
10	88%	105%	97%	90%	96%	118%	107%	98%
20	81%	72%	76%	81%	85%	71%	78%	95%
40	69%	75%	72%	65%	102%	107%	105%	91%
60		50%	50%	52%	84%	78%	81%	86%



Test Agent: CMB-087649 **[TA] (uM):** 1.0
Species: Mouse

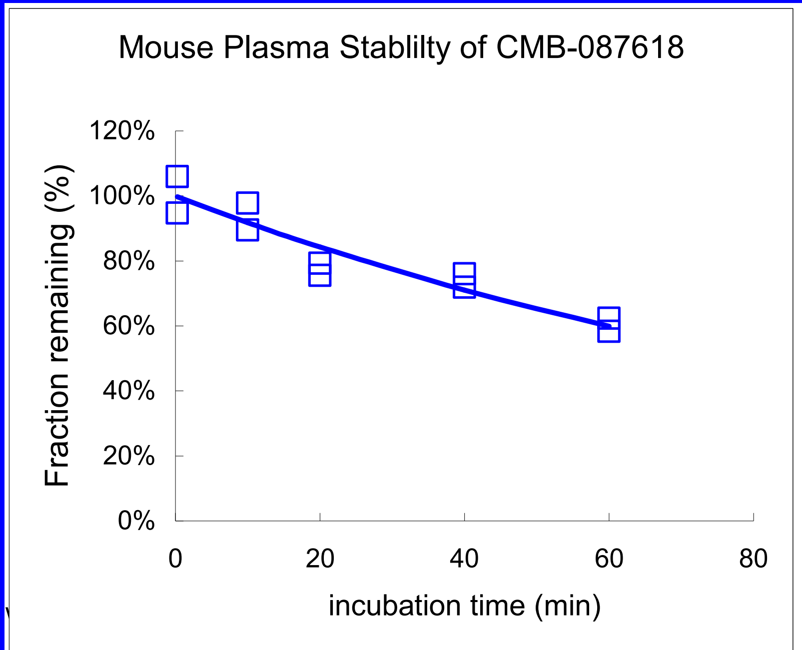
	W NADPH				W/O NADPH			
	CLint	163	**T1/2**	14	**CLint**	21	**T1/2**	109
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	92%	101%	96%	99%	97%	91%	94%	100%
10	68%	65%	67%	61%	103%	100%	102%	94%
20	34%	27%	31%	38%	74%	74%	74%	88%
40	19%	14%	16%	14%	84%	96%	90%	78%
60	8%	7%	8%	5%	64%	63%	63%	68%

4.3.3 Plasma half-life individual data



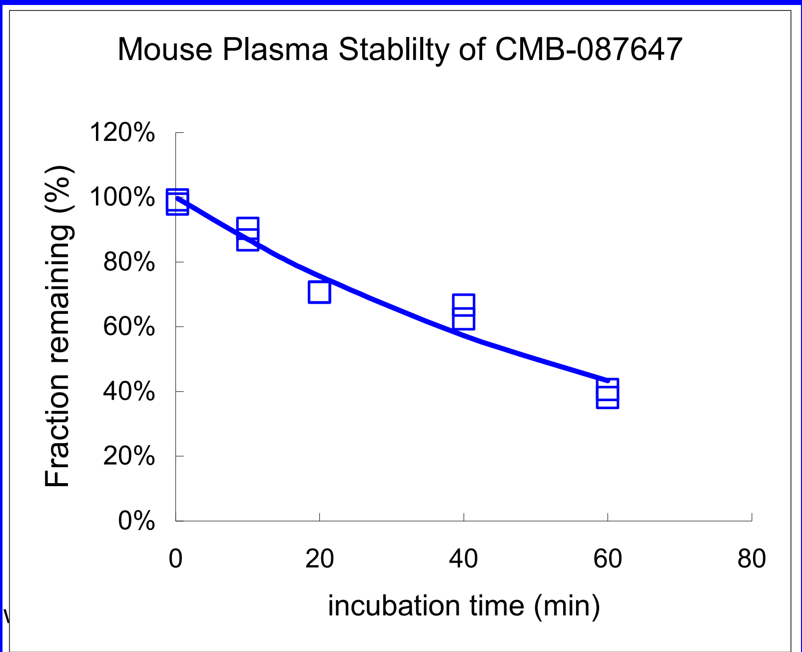
TA: CMB-021805 **Test conc (uM):** 5
Test medium: Mouse Plasma
T1/2 (min) 63.5 **%rem, last time** 52.9%

time	%RE1	%RE2	avg	calc
0.25	93%	99%	96%	100%
10	97%	95%	96%	90%
20	77%	79%	78%	80%
40	69%	57%	63%	65%
60	51%	55%	53%	52%



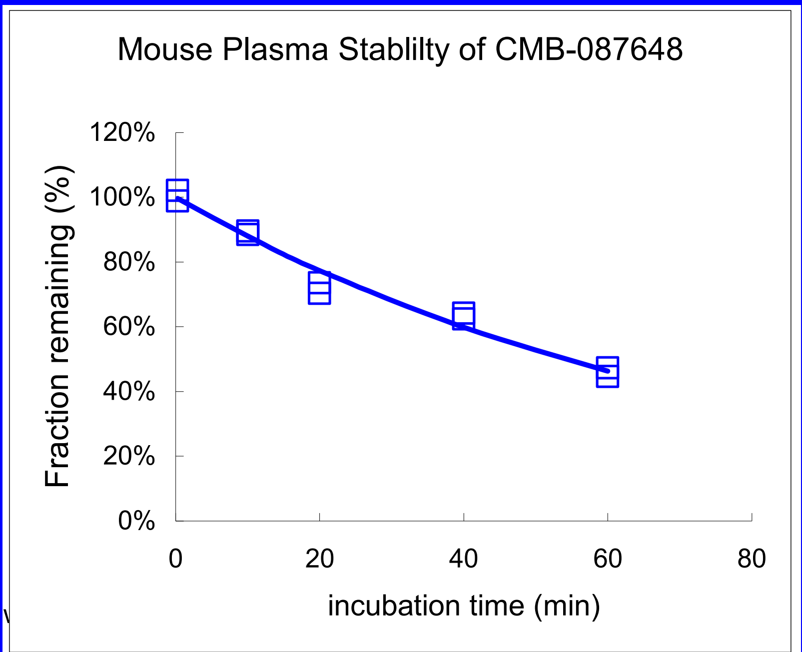
TA: CMB-087618 **Test conc (uM):** 5
Test medium: Mouse Plasma
T1/2 (min) 81.2 **%rem, last time** 60.4%

time	%RE1	%RE2	avg	calc
0.25	95%	106%	100%	100%
10	98%	90%	94%	92%
20	79%	76%	77%	84%
40	76%	72%	74%	71%
60	58%	62%	60%	60%



TA: CMB-087647 **Test conc (uM):** 5
Test medium: Mouse Plasma
T1/2 (min) 49.8 **%rem, last time** 39.4%

time	%RE1	%RE2	avg	calc
0.25	98%	99%	99%	100%
10	90%	87%	89%	87%
20	71%	71%	71%	76%
40	62%	67%	65%	57%
60	41%	38%	39%	43%

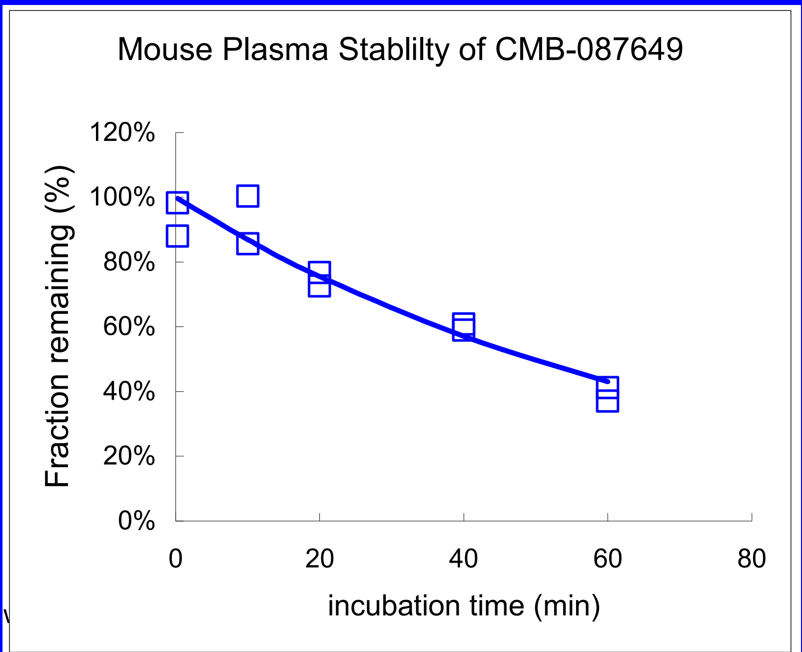


TA: CMB-087648 **Test conc (uM):** 1

Test medium: Mouse Plasma

T1/2 (min) 54.0 **%rem, last time** 46.0%

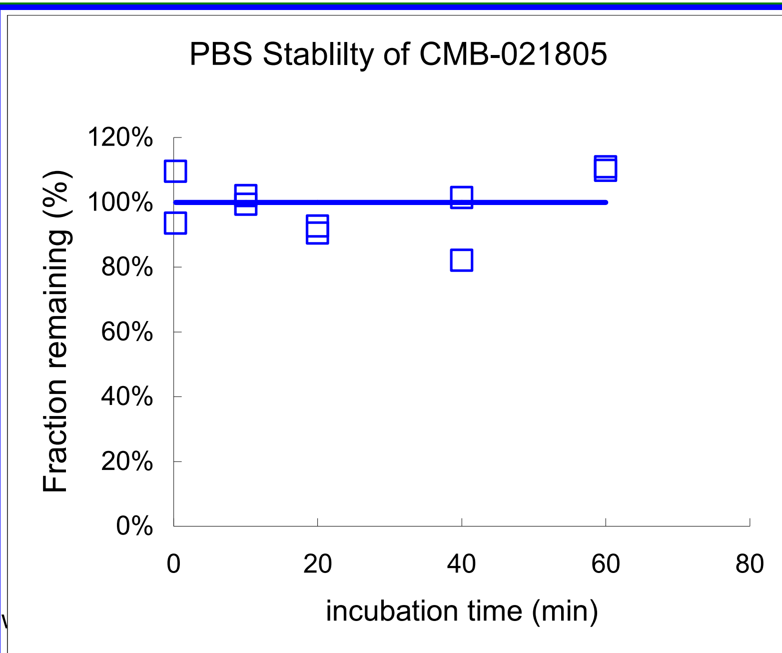
time	%RE1	%RE2	avg	calc
0.25	102%	99%	100%	100%
10	88%	90%	89%	88%
20	70%	74%	72%	77%
40	64%	62%	63%	60%
60	45%	47%	46%	46%



TA: CMB-087649 **Test conc (uM):** 5
Test medium: Mouse Plasma
T1/2 (min) 49.4 **%rem, last time** 39.1%

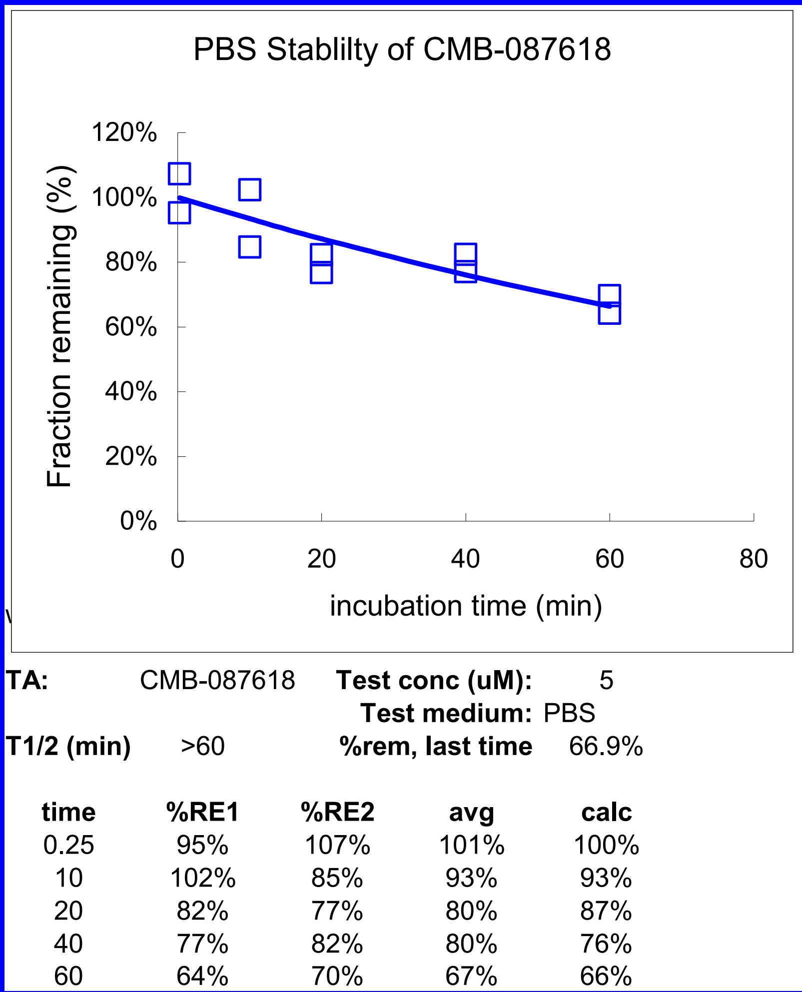
time	%RE1	%RE2	avg	calc
0.25	88%	98%	93%	100%
10	100%	86%	93%	87%
20	73%	77%	75%	76%
40	59%	61%	60%	57%
60	37%	41%	39%	43%

4.3.4 Buffer half-life individual data



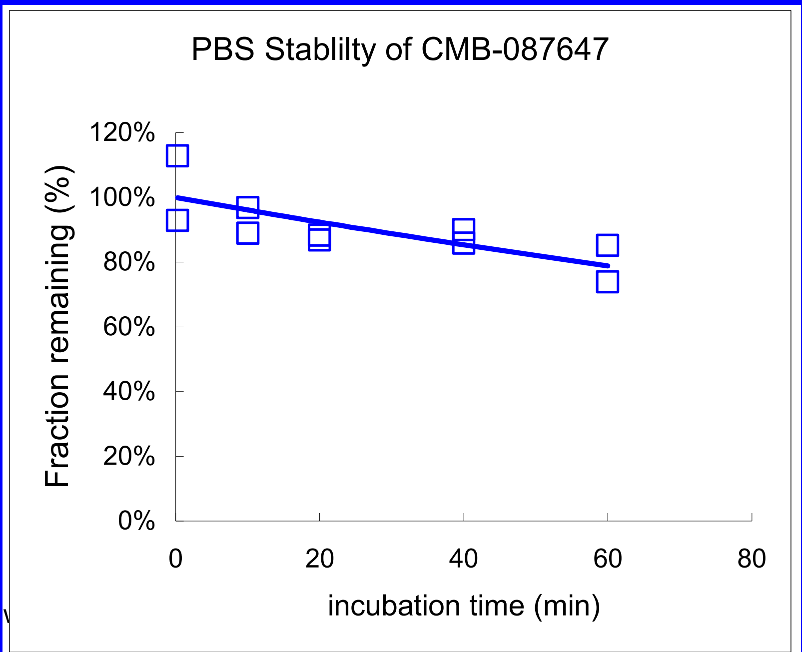
TA: CMB-021805 **Test conc (uM):** 5
Test medium: PBS
T1/2 (min) >60 **%rem, last time** 110.4%

time	%RE1	%RE2	avg	calc
0.25	94%	110%	102%	100%
10	99%	102%	101%	100%
20	93%	91%	92%	100%
40	101%	82%	92%	100%
60	111%	110%	110%	100%



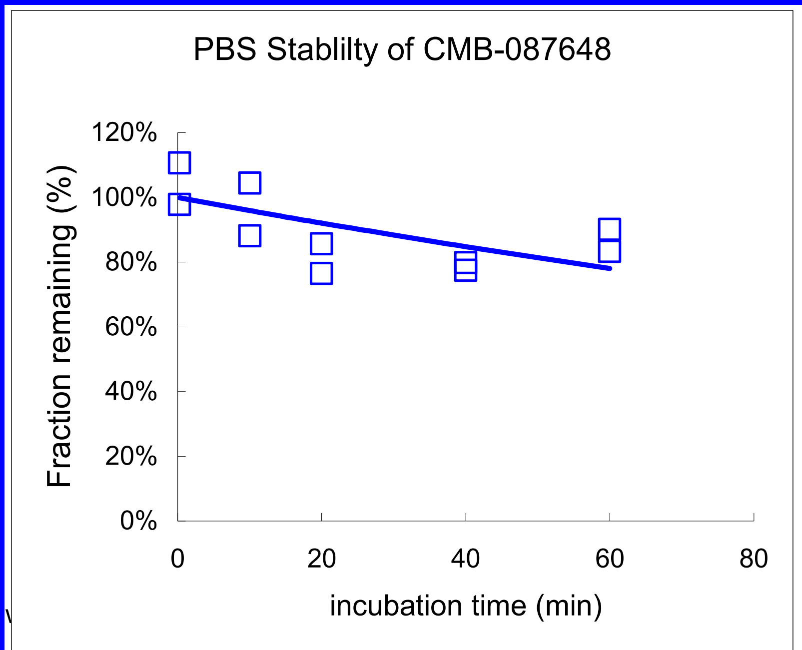
TA: CMB-087618 **Test conc (uM):** 5
Test medium: PBS
T1/2 (min) >60 **%rem, last time** 66.9%

time	%RE1	%RE2	avg	calc
0.25	95%	107%	101%	100%
10	102%	85%	93%	93%
20	82%	77%	80%	87%
40	77%	82%	80%	76%
60	64%	70%	67%	66%



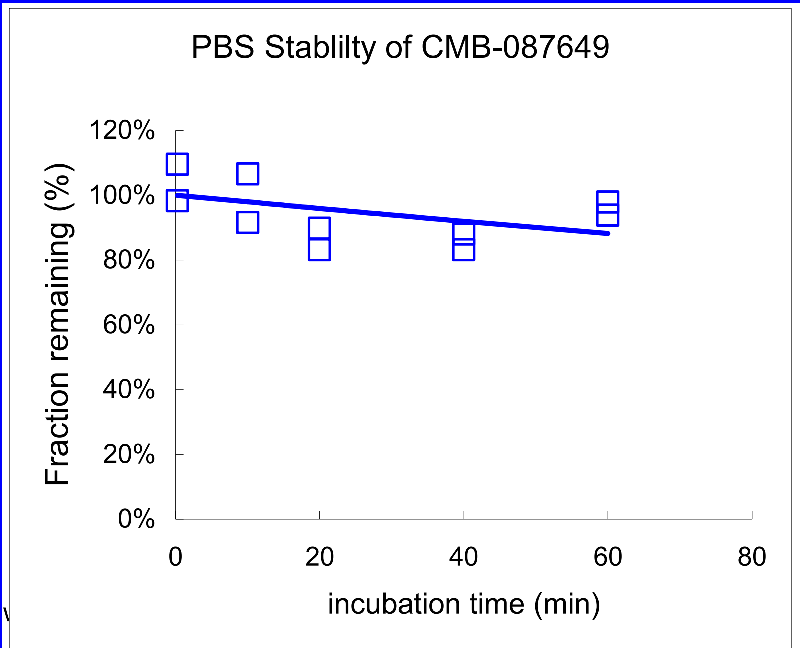
TA:	CMB-087647	**Test conc (uM):**	5
		Test medium:	PBS
T1/2 (min)	>60	**%rem, last time**	79.5%

time	**%RE1**	**%RE2**	**avg**	**calc**
0.25	93%	113%	103%	100%
10	97%	89%	93%	96%
20	88%	87%	88%	92%
40	90%	86%	88%	85%
60	85%	74%	80%	79%



TA:	CMB-087648	**Test conc (uM):**	1
		Test medium:	PBS
T1/2 (min)	>60	**%rem, last time**	86.7%

time	**%RE1**	**%RE2**	**avg**	**calc**
0.25	98%	111%	104%	100%
10	104%	88%	96%	96%
20	86%	76%	81%	92%
40	77%	80%	79%	85%
60	83%	90%	87%	78%



TA: CMB-087649 **Test conc (uM):** 5

Test medium: PBS

T1/2 (min) >60 **%rem, last time** 95.9%

time	%RE1	%RE2	avg	calc
0.25	98%	110%	104%	100%
10	107%	92%	99%	98%
20	90%	83%	86%	96%
40	83%	88%	86%	92%
60	94%	98%	96%	88%

5 References

Stewart, BH, *et al*. (1995) "Comparison of intestinal permeabilities determined in multiple *in vitro* and *in situ* models: Relationship to absorption in humans." Pharm. Res. 12:693.

Houston, JB (1994) "Utility of in vitro drug metabolism data in predicting in vivo metabolic clearance." Biochem. Pharmacol. 47:1469.

Singh, R, *et al*. (1996) "*In vitro* metabolism of a potent HIV-protease inhibitor (141W94) using rat, monkey and human liver S9." Rapid Commun Mass Spectrom 10:1019.

6 Storage and Retention of Records

All documents generated in this study (raw data, the study plan, a copy of this report, etc.) will be stored for three years from the date of this document. Only authorized Apredica employees will have access to the archives.

The original final report will be provided to the sponsor and will be kept by the sponsor under its sole responsibility.

7 Appendices

7.1 Appendix A. Standard Apredica Methods

Buffer half-life

The test agent is incubated in duplicate with test medium at 37 ºC. The reaction contains medium and 2% DMSO. At the indicated times, an aliquot is removed from each experimental reaction and mixed with three volumes of ice-cold Stop Solution (methanol containing propranolol, diclofenac, or other internal standard). Stopped reactions are incubated at least ten minutes at -20 ºC. The samples are centrifuged to remove any precipitate, and the supernatants are analyzed by LC/MS/MS to quantitate the remaining parent. Data are converted to % remaining by dividing by the time zero concentration value. Data are fit to a first-order decay model to determine half-life.

Microsomal intrinsic clearance

The test agent is incubated in duplicate with microsomes at 37 ºC. The reaction contains microsomal protein in 100 mM potassium phosphate, 2 mM NADPH, 3 mM $MgCl_2$, pH 7.4. A control is run for each test agent omitting NADPH to detect NADPH-free degradation. The indicated times, an aliquot is removed from each experimental and control reaction and mixed with an equal volume of ice-cold Stop Solution (0.3% acetic acid in acetonitrile containing haloperidol, diclofenac, or other internal standard). Stopped reactions are incubated at least ten minutes at -20 ºC, and an additional volume of water is added. The samples are centrifuged to remove precipitated protein, and the supernatants are analyzed by LC/MS/MS to quantitate the remaining parent. Data are converted to % remaining by dividing by the time zero concentration value. Data are fit to a first-order decay model to determine half-life. Intrinsic clearance is calculated from the half-life and the protein concentrations: $CL_{int} = \ln(2) /(T_{1/2}\ [\text{microsomal protein}])$.

Plasma half-life

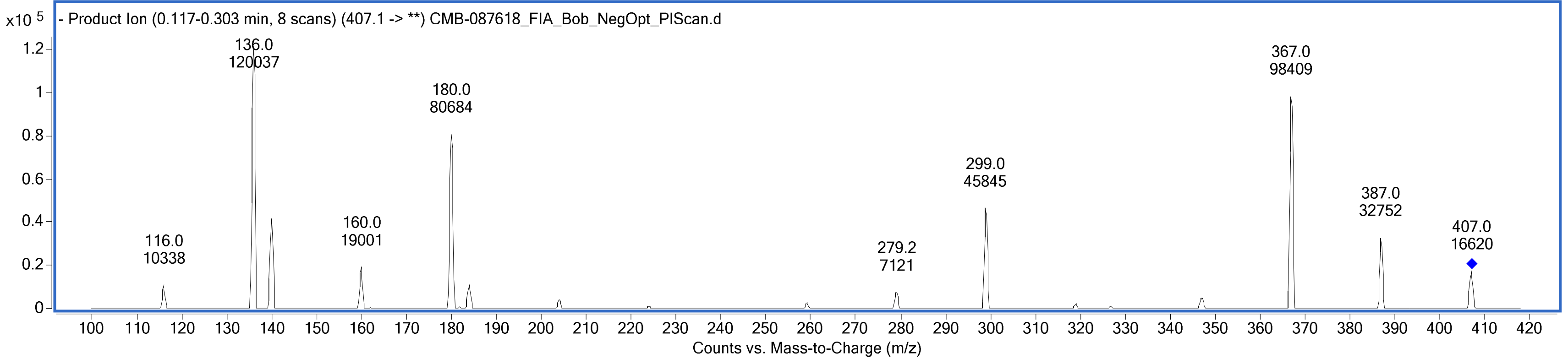
The test agent is incubated in duplicate with plasma at 37 ºC. The reaction contains plasma and 2% DMSO. At the indicated times, an aliquot is removed from each experimental reaction and mixed with three volumes of ice-cold Stop Solution (methanol containing propranolol, diclofenac, or other internal standard). Stopped reactions are incubated at least ten minutes at -20 ºC. The samples are centrifuged to remove precipitated protein, and the supernatants are analyzed by LC/MS/MS to quantitate the remaining parent. Data are converted to % remaining by dividing by the time zero concentration value. Data are fit to a first-order decay model to determine half-life.

PBS express solubility

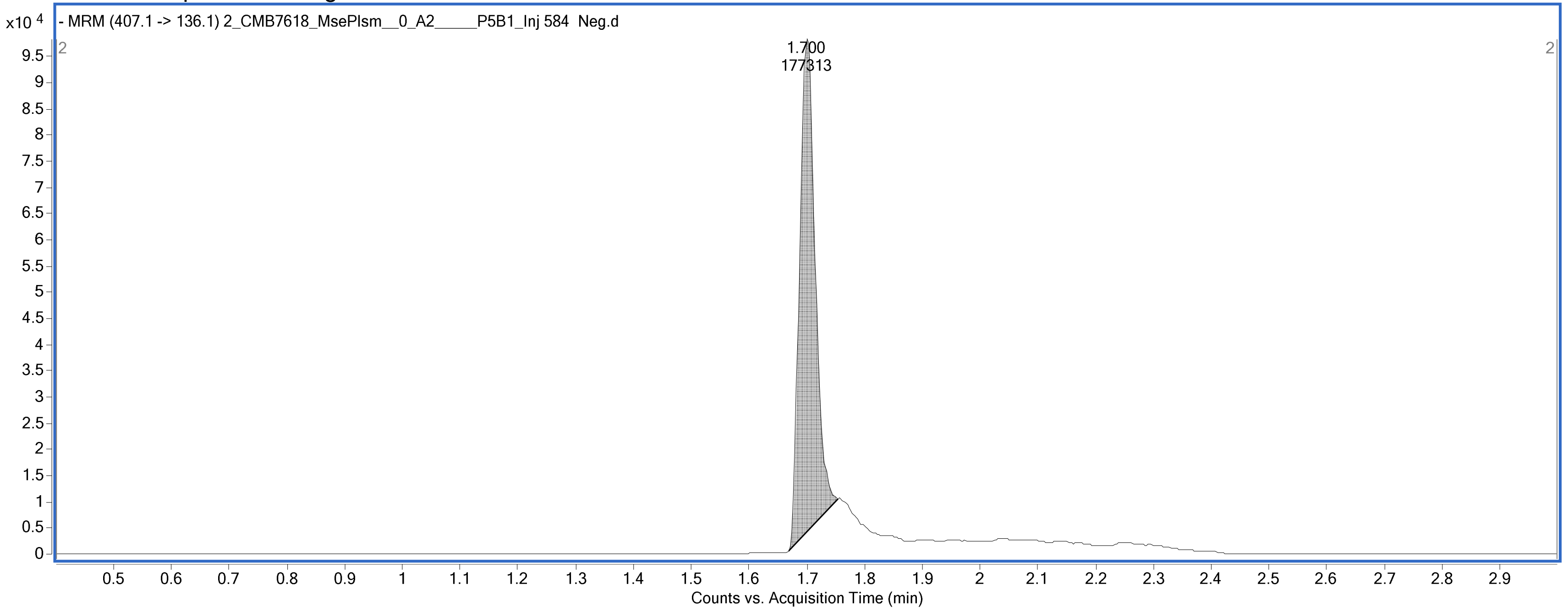
Serial dilutions of test agent are prepared in test agent at 100x the final concentration. Test agent solutions are diluted 100-fold into PBS in a 96-well plate and mixed. The absorbance of the PBS-containing plate is measured prior to adding the test agents to determine the background absorbance. After 45 min and 16 hr, the presence of precipitate is then detected by turbidity (absorbance at 540 nm). An absorbance value of greater than (mean + 3x standard deviation of the blank), after subtracting the pre-experiment background, is indicative of turbidity. For brightly colored compounds, a visual inspection of the plate is performed to verify the solubility limit determined by UV absorbance. The solubility limit is reported as the highest experimental concentration with no evidence of turbidity.

7.2 Appendix B. Sample Spectra and Chromatograms of the Test Agents

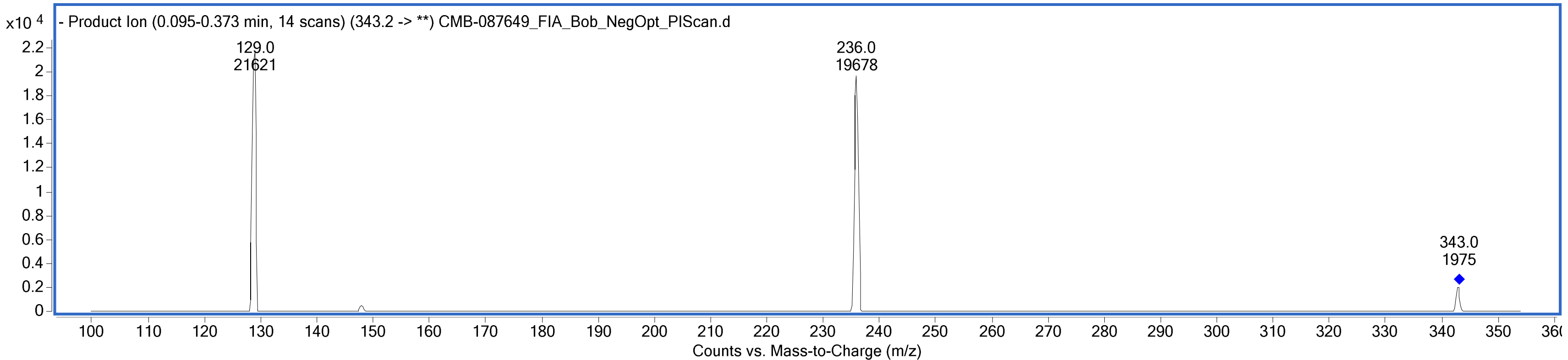
CMB-087618 Product ion Scan



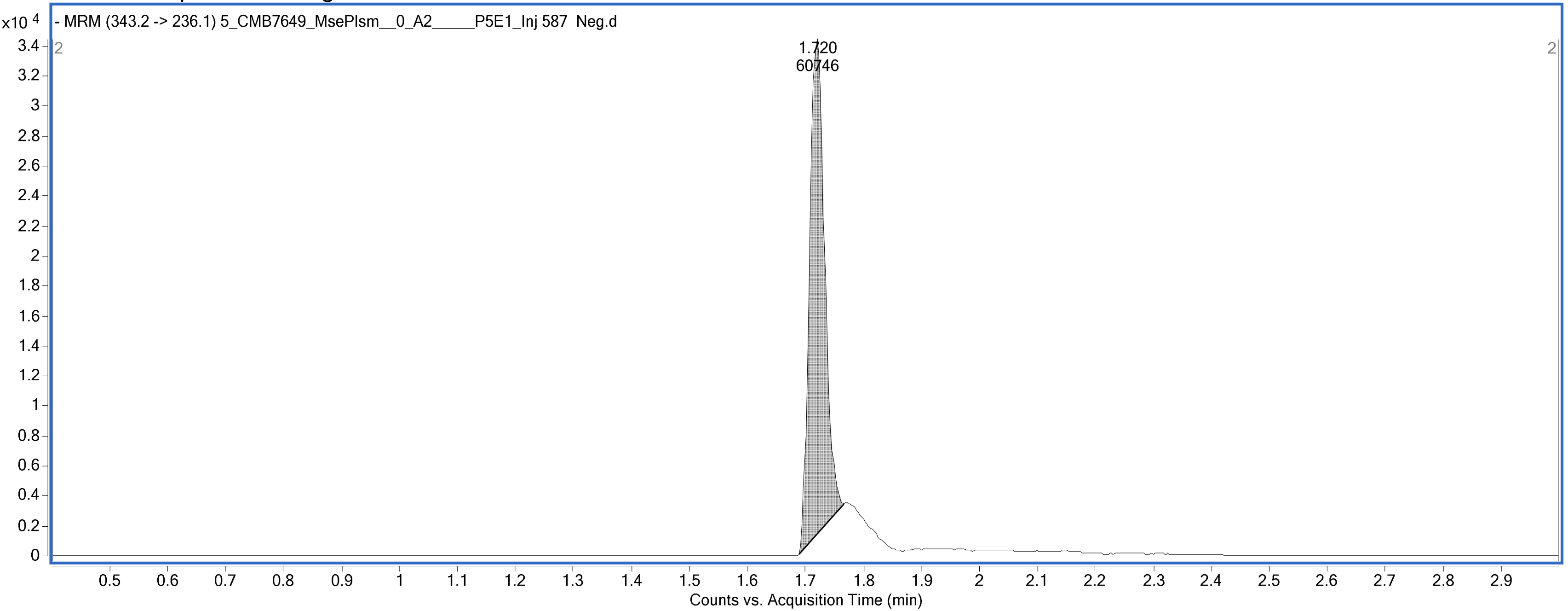
CMB-087618 Sample chromatogram



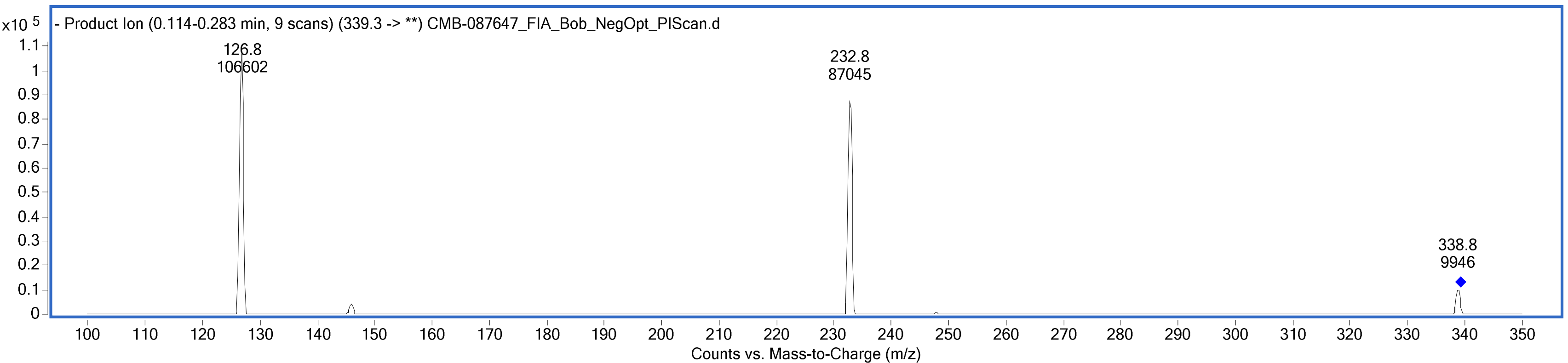
CMB-087649 Product ion Scan



CMB-087649 Sample chromatogram



CMB-087647 Product ion Scan



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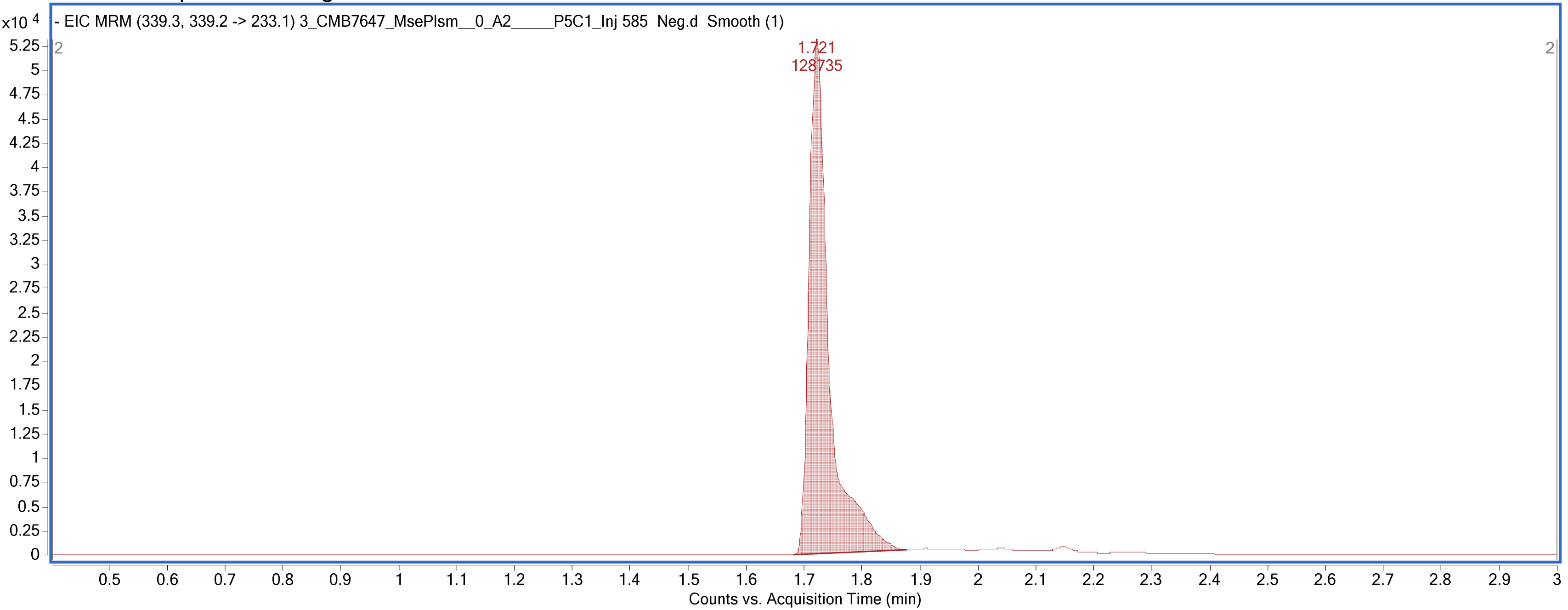
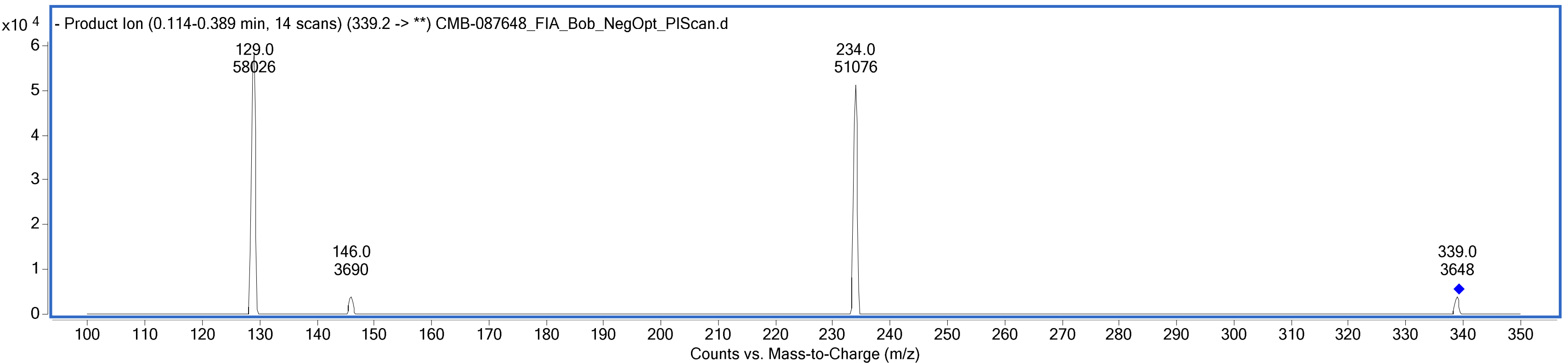
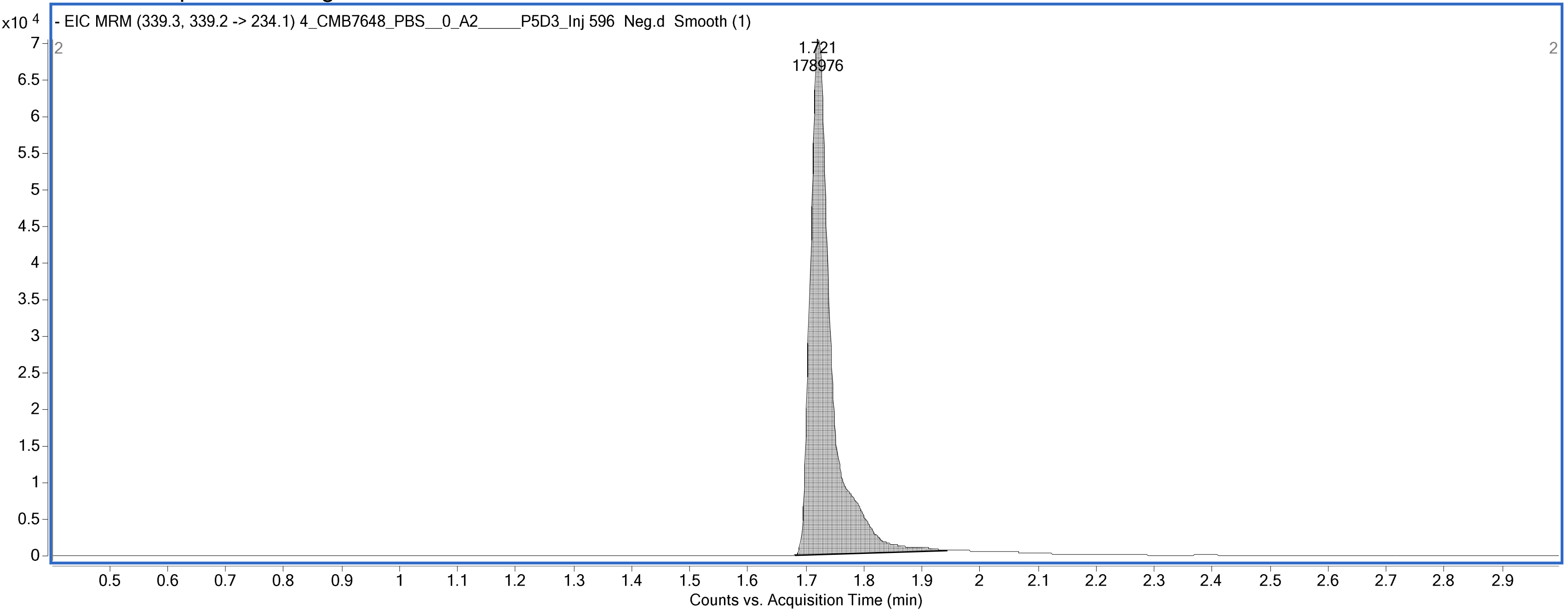


CMB-087648 Product ion Scan



CMB-087648 Sample chromatogram



Supporting Information - SP-2

Compound	Apredica ID
1	CAM-015-01

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1 Objective

The objective of this study was to identify potential metabolites

1.1 Regulatory Guidelines

This study was not conducted under US FDA Good Laboratory Practice Regulations (GLPs). Standard operating procedures of Apredica were used throughout the study.

2 Test Articles

Apredica ID	Client ID	Physical Form	Submitted FW	Parent MW	Stock solutions
CAM-015-01	**CMB-021805**	solid	**336**	**336**	50 mM DMSO

Test agent powders were stored at -20 °C. Stock solutions were stored at -20 °C.

3 Test Methods

Testing was performed at Apredica in Watertown, MA.

3.1 Analytical Methods

3.1.1 Method development

The signal was optimized for each compound by ESI positive or negative ionization mode. A MS2 scan was used to identify the precursor ion and a product ion analysis was used to identify the best fragment for analysis and to optimize the collision energy. An ionization ranking was assigned indicating the compound's ease of ionization.

3.1.2 Analysis

Samples were analyzed by LC/MS/MS using either an Agilent 6410 mass spectrometer coupled with an Agilent 1200 HPLC and a CTC PAL chilled autosampler, all controlled by MassHunter software (Agilent), or an ABI2000 mass spectrometer coupled with an Agilent 1100 HPLC and a CTC PAL chilled autosampler, all controlled by Analyst software (ABI). After separation on a C18 reverse phase HPLC column (Agilent, Waters, or equivalent) using an acetonitrile-water gradient system, peaks were analyzed by mass spectrometry (MS) using ESI ionization in MRM mode.

3.2 In vitro *ADME-Tox Experimental Conditions*

3.2.1 Metabolite ID experimental conditions

Samples: Duplicate samples were prepared by incubating CMB-021805 at 50 μM with 1 mg/mL mouse microsomal protein in 100 mM phosphate buffer containing 2 mM NADPH for 1 hr at 37 °C. The samples were quenched with 1 volume acetonitrile and centrifuged, and the supernatant was transferred to a vial for analysis. An NADPH-free control was prepared by omitting NADPH. A compound-free control was prepared by incubating microsomes and NADPH without compound for 1 hr, then adding the test agent.

LC/MS/MS: A 6 min acetonitrile-water gradient (containing 0.1% formic acid) was used to separate the metabolites. The major contaminants from microsomes and NADPH elute in the first minute, and signals from this background obscure the signals from any metabolites, so data from the first minute of the gradient were not analyzed. For the initial injections, a full-scan mass spectrum was obtained at each point along the gradient. Peaks in the TIC (total ion current) that are present in the NADPH-containing samples, but not in the controls, are indicative of potential metabolites. For the three metabolites identified, product ion scans across the gradient were obtained to look for fragments.

4 Results

4.1 Analytical

4.1.1 Method development

The analytical method used in previous studies (CAM-016)was used for this study.

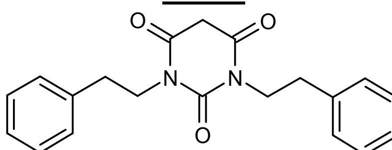
4.2 In vitro *ADME-Tox Summary*

4.2.1 Metabolite ID

Three peaks corresponding to metabolites were identified. Two peaks with m/z 351 in negative mode ionization and m/z 353 in positive mode ionization, corresponding to oxidation products were identified. In addition, a peak with m/z 335 in positive mode ionization was identified. Based on the spectral and chromatographic evidence outlined below, the following metabolites are proposed.

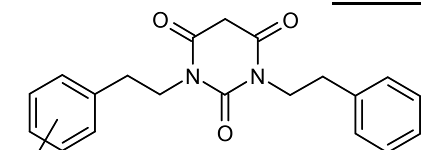
Potential Metabolites Identified

Parent



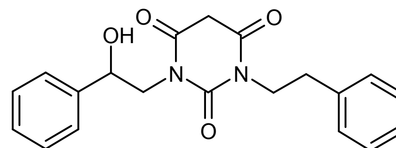
CMB-021805
m/z=337 $(M+H)^+$

Potential Metabolites

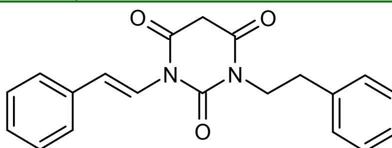


m/z=353 $(M+O+H)^+$
m/z=351 $(M+O-H)^-$

OR



m/z=353 $(M+O+H)^+$
m/z=351 $(M+O-H)^-$



m/z=335 $(M-H_2+H)^+$

No conclusive evidence for other metabolites was observed.

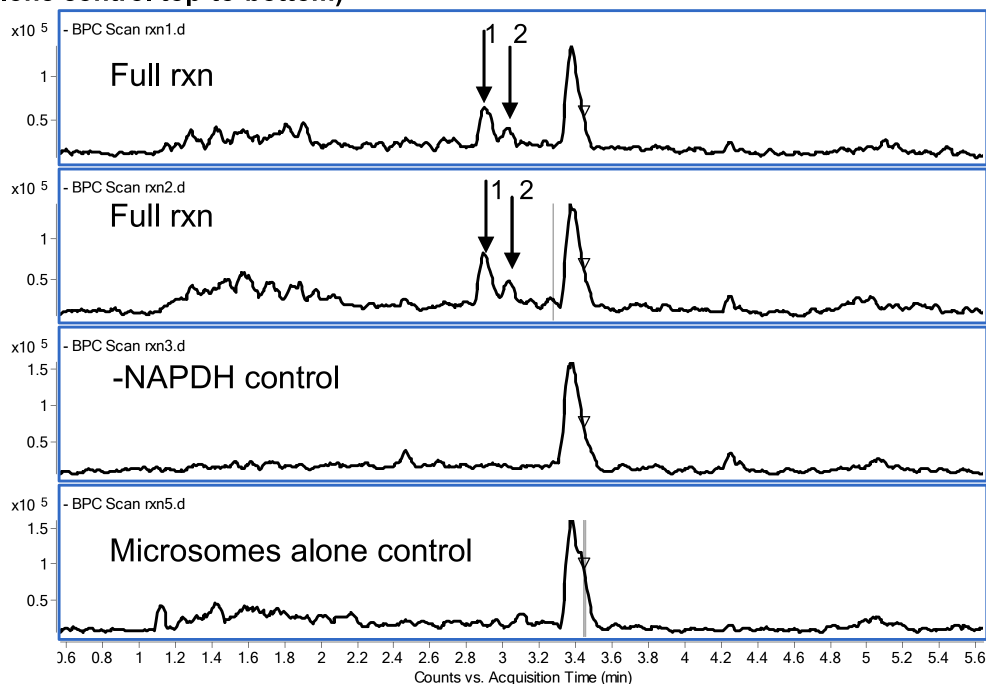
4.3 In vitro *ADME-Tox Individual Data*

4.3.1 Metabolite ID individual data

ESI- Full mass (MS2) Scans

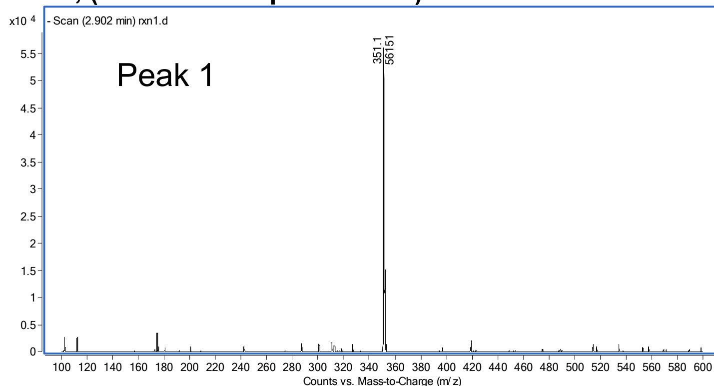
ESI- mode ionization was examined, since the previous study showed that this was more sensitive than ESI+ mode. In the initial ESI- MS2 scan (see figure below), two peaks (labeled 1 and 2) corresponding to potential metabolites were observed (labeled Peak 1). The peak due to unmetabolized CMB-021805 at retention time (RT) 3.4 min is also observed. In this figure, the chromatograms from two samples incubated with NADPH, the NADPH-free control is shown in red, and the microsome alone control are shown. The broad peak at RT=1.2 min, and the shoulder on the CMB-021805 peak at RT = 3.5 min (indicated with the triangle) are due to microsomal degradation of NADPH, and appear in the controls as well (third and fourth scans).

ESI- MS2 Scan BPC (two+NADPH samples in black, -NADPH control, microsomes alone control top-to-bottom)

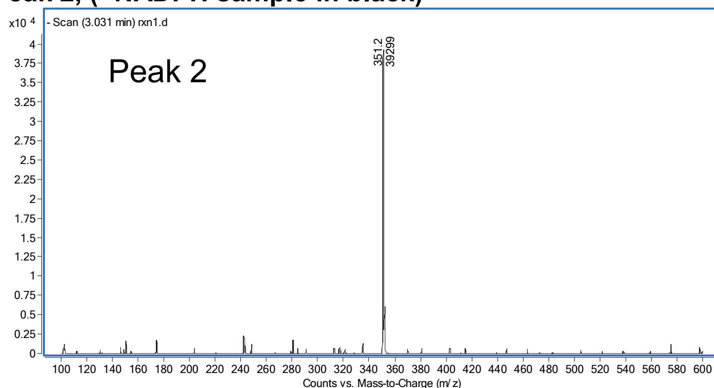


Mass spectra were examined for peaks 1 and 2. The results are shown below. Examination of the mass spectra for peak 1 and 2 shows fragments with m/z. These correspond to the singly oxidized metabolites of CMB-021805.

Peak 1, (+NADPH sample in black)



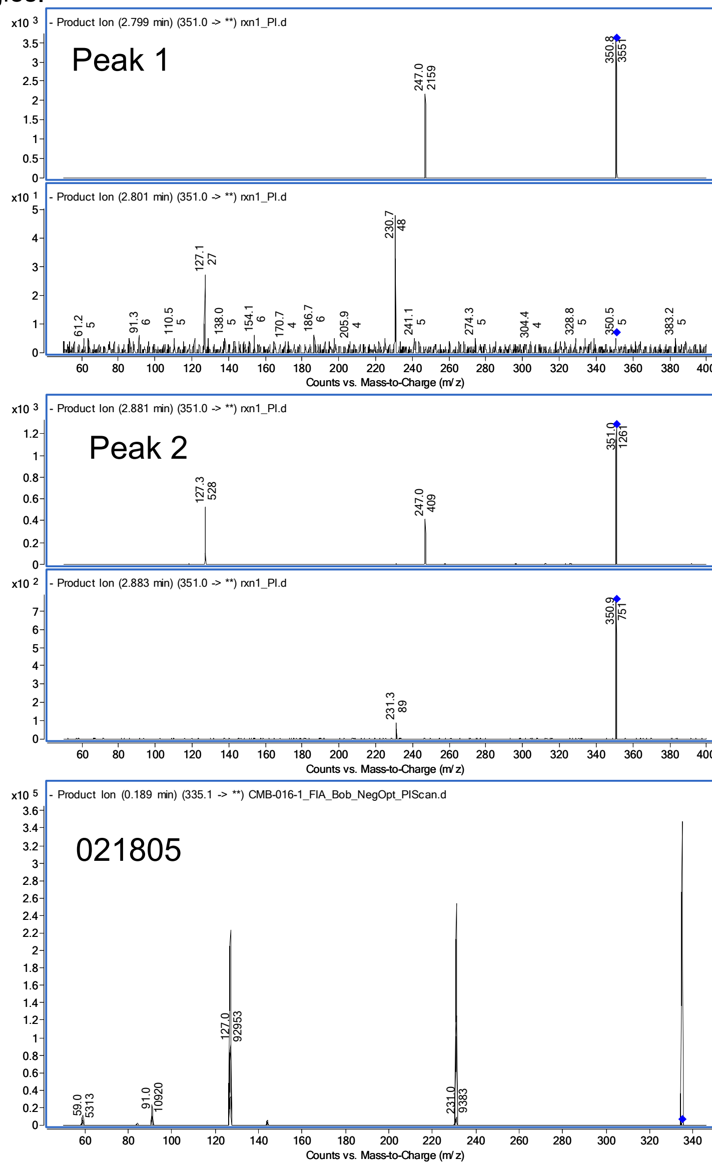
Peak 2, (+NADPH sample in black)



Product Ion (Fragmentation) Scans

Product-ion scans were used to determine the mass spectral fragments of the m/z 351 peaks 1 and 2 (see next figure). Both peaks show peaks of m/z 247 and m/z 231, corresponding to loss of non-oxidized and oxidized phenethyl group from oxidized parent.

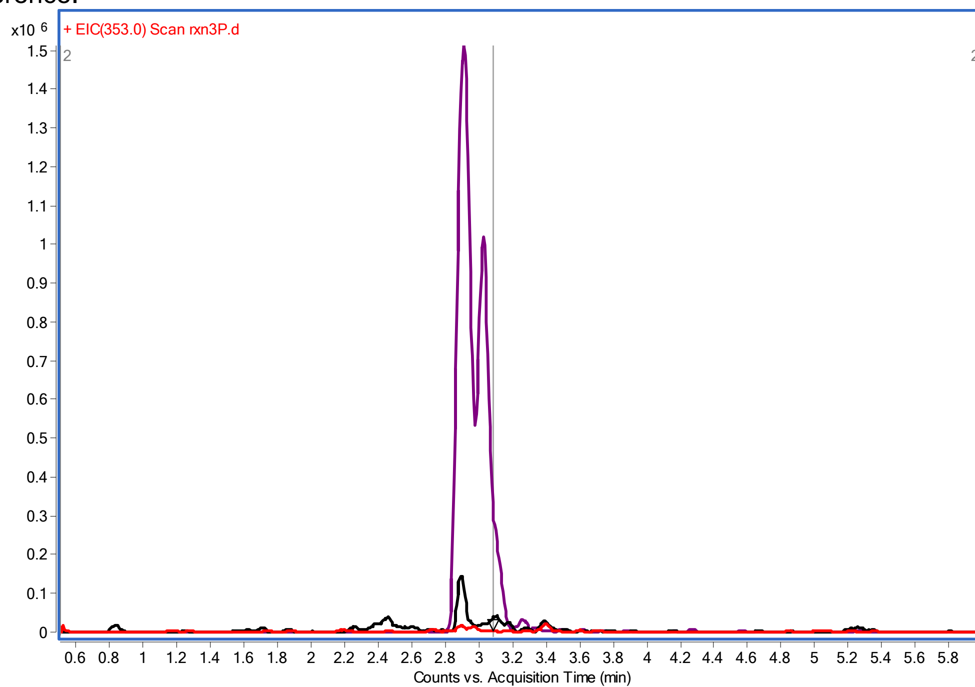
Product ion spectra (ESI- ionization) for Peak 1 (top panel), and Peak 2 (middle panel) and CMB-021805 (bottom panel). Different panes in the panel show spectra at different collision energies.

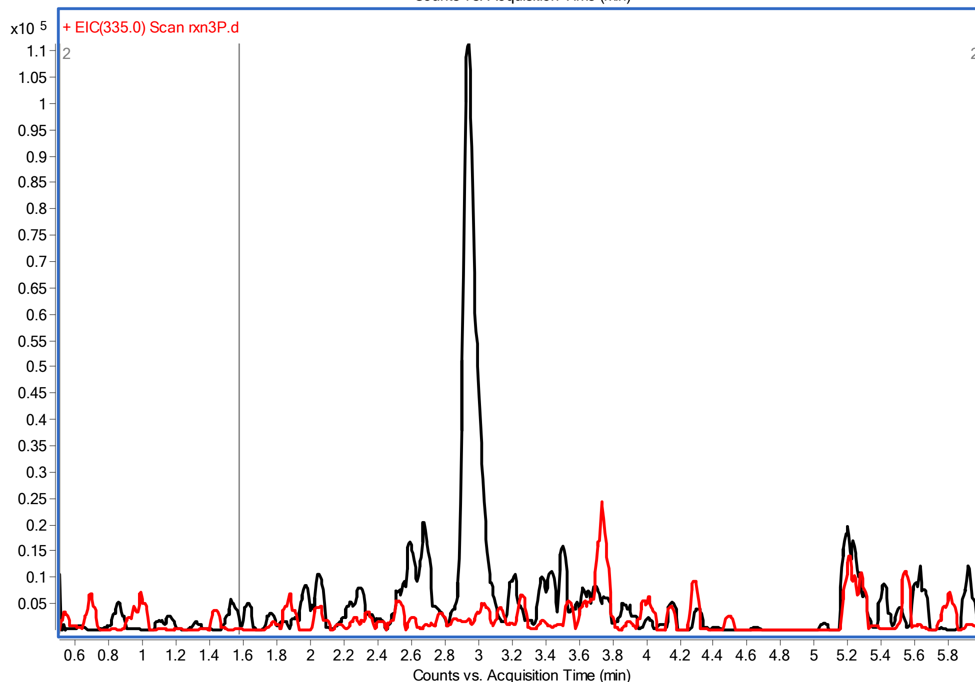


ESI+ Full mass (MS2) Scans

ESI+ mode ionization was examined, since the previous metabolite ID study identified oxidation desaturation products in ESI+ mode. The EIC (extracted ion spectra) of these scans corresponding to the m/z 353 (oxidation product) and m/z 335 (desaturation products) are shown in the following figure. These scans show that the desaturation product is present in mouse microsomes reactions, as well as in human. Both peaks identified in the ESI- scan are also seen in the ESI+ scan as well. The m/z 335 peak was too small to identify fragments in the product ion scan.

EIC for m/z 353 (top panel) and m/z 335 (bottom panel) from ESI+ MS2 scans. The black curve shows the metabolite reaction. The red curve shows the –NADPH control. The purple curve in the top panel shows the position of the negative mode peaks for reference.





5 Storage and Retention of Records

All documents generated in this study (raw data, the study plan, a copy of this report, etc.) will be stored for three years from the date of this document. Only authorized Apredica employees will have access to the archives.

The original final report will be provided to the sponsor and will be kept by the sponsor under its sole responsibility.

Supporting Information - SP-3

Compound	Apredica ID
1	CAM-016-01
6	CAM-016-02
2	CAM-016-03
5	CAM-016-04

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1 Objective

The objective of this study is to evaluate the *in vitro* ADME properties of the test agents.

1.1 Regulatory Guidelines

This study was not conducted under US FDA Good Laboratory Practice Regulations (GLPs). Standard operating procedures of Apredica were used throughout the study.

2 Test Articles

Apredica ID	Client ID	Physical Form	Submitted FW	Parent MW	Stock solutions
CAM-016-01	**21805 (1)**	solid	**336**	**336**	50 mM DMSO, very slightly yellow in color
CAM-016-02	**(2)**	solid	**372.37**	**372**	50 mM DMSO, very slightly yellow in color
CAM-016-03	**(3)**	solid	**408.36**	**408**	50 mM DMSO, pale orange in color
CAM-016-04	**(4)**	solid	**444.34**	**444**	50 mM DMSO, pale yellow in color

Test agent powders were stored at -20 °C. Stock solutions were stored at -20 °C.

3 Test Methods

Testing was performed at Apredica in Watertown, MA.

3.1 *Analytical Methods*

3.1.1 Method development

The signal was optimized for each compound by ESI positive or negative ionization mode. A MS2 scan was used to identify the precursor ion and a product ion analysis was used to identify the best fragment for analysis and to optimize the collision energy. An ionization ranking was assigned indicating the compound's ease of ionization.

3.1.2 Analysis

Samples were analyzed by LC/MS/MS using either an Agilent 6410 mass spectrometer coupled with an Agilent 1200 HPLC and a CTC PAL chilled autosampler, all controlled by MassHunter software (Agilent), or an ABI2000 mass spectrometer coupled with an Agilent 1100 HPLC and a CTC PAL chilled autosampler, all controlled by Analyst software (ABI). After separation on a C18 reverse phase HPLC column (Agilent, Waters, or equivalent) using an acetonitrile-water gradient system, peaks were analyzed by mass spectrometry (MS) using ESI ionization in MRM mode.

3.2 In vitro *ADME-Tox Experimental Conditions*

Additional protocol details are given in Appendix A.

3.2.1 PBS solubility experimental conditions

Apredica ID	Client ID	Test conc	Medium	Incub-ation	Ref. comp.	Analytical method
CAM-016-01 **CAM-016-02** **CAM-016-03** **CAM-016-04**	**21805 (1)** **(2)** **(3)** **(4)**	500, 250. 125, 63, 31, 16, 7.8, 3.9 μM	PBS[a]	45 min, 16 hr RT	tamoxifen reserpine verapamil	UV/Vis

[a]PBS is Ca^{2+}, Mg^{2+}-free phosphate-buffered saline, pH 7.2.

3.2.2 Caco-2 monolayer permeability experimental conditions

Apredica ID	Client ID	Test conc.	Assay Time	Direction	Reference compounds	Analytical method
CAM-016-01 **CAM-016-02** **CAM-016-03** **CAM-016-04**	**21805 (1)** **(2)** **(3)** **(4)**	50 μM	2 hr	A->B B->A	warfarin ranitidine	LC/MS/MS

3.2.3 PBS stability experimental conditions

Apredica ID	Client ID	Test conc	Medium	Incub-ation	Ref. comp.	Analytical method
CAM-016-01 **CAM-016-02** **CAM-016-03** **CAM-016-04**	**21805 (1)** **(2)** **(3)** **(4)**	5 μM	PBS[a]	0, 10, 20, 40, 60 min 37 °C	propantheline	LC/MS/MS

[a]PBS is Ca^{2+}, Mg^{2+}-free phosphate-buffered saline, pH 7.2.

3.2.4 Plasma stability experimental conditions

Apredica ID	Client ID	Test conc.	Plasma source	Incub-ation	Reference compounds	Analytical method
CAM-016-01 **CAM-016-02** **CAM-016-03** **CAM-016-04**	**21805 (1)** **(2)** **(3)** **(4)**	5 μM	PBS[a]	0, 10, 20, 40, 60 min 37 °C	propantheline	LC/MS/MS

3.2.5 Microsomal stability experimental conditions

Apredica ID	Client ID	Test conc.	Micro-some source	Protein conc.	Incub-ation	Ref. comp.	Analytical method
CAM-016-01 **CAM-016-02** **CAM-016-03** **CAM-016-04**	**21805 (1)** **(2)** **(3)** **(4)**	5 μM	human, mouse	0.3 mg/ mL	0, 10, 20, 40, 60 min 37 °C	verapamil warfarin	LC/MS/MS

4 Results

4.1 Analytical

4.1.1 Method development

Client ID	MW	Polari-zation	Precursor m/z	Product m/z	Collision energy (V)	Ionization classification[a]
21805 (1)	336	neg	335.1	231.1	18	1
(2)	372	neg	371.1	127.0	26	1
(3)	408	neg	407.1	136.1	34	1
(4)	444	neg	443.1	154.1	38	1

[a]Ionization classification:
1 = Highly ionizable
2 = Intermediately ionizable
3 = Poorly ionizable

The full scan mass spectrum, the product ion spectrum, and a sample chromatogram are shown in Appendix B.

4.2 In vitro ADME-Tox Summary

4.2.1 PBS express solubility summary

Client ID	Medium	Solubility limit (μM)[a] 45 min	Solubility limit (μM)[a] 16 hr	comment
Tamoxifen	PBS	15.6	31.3	low solubility control
Reserpine	PBS	15.6	15.6	low solubility control
Verapamil	PBS	≥500	≥500	high solubility control
21085 (1)	PBS	62.5	62.5	
(2)	PBS	31.3	31.3	
(3)	PBS	≥500	250	fibrous ppt at highest conc at 16 hr
(4)	PBS	250	125	fibrous ppt at highest two concs at 16 hr

[a]Solubility limit is highest concentration with no detectable precipitate.

Visible inspection of wells at 16 hr time showed cloudiness (fine precipitate) at concentrations that agree with UV determination, except as noted. See individual data below for UV data.

4.2.2 Caco-2 permeability summary

Client ID	test conc (μM)	Assay duration (hr)	mean A->B P_{app} [a] (10^{-6} cm s^{-1})	mean B->A P_{app} [a] (10^{-6} cm s^{-1})	Asymmetry ratio[b]	comment
Ranitidine	50	2	1.2	2.9	2.4	low permeability control
Warfarin	50	2	44.3	16.9	0.4	high permeability control
21085 (1)	50	2	73.4	26.0	0.4	
(2)	50	2	66.3	17.1	0.3	
(3)	50	2	60.7	14.0	0.2	
(4)	50	2	51.7	10.5	0.2	

[a]Apparent permeability
[b]P_{app}(B->A) / P_{app}(A->B)

4.2.3 Buffer half-life summary

Client ID	Test conc (µM)	Medium	Buffer $T_{1/2}$[a] (min)	Fraction remaining, last time point (%)	comment
Propantheline	5.0	PBS[b]	>60	80.9%	stable control
CMB-087250	5.0	PBS[b]	>60	110.4%	
CMB-087251	5.0	PBS[b]	>60	86.5%	
CMB-005304	5.0	PBS[b]	>60	107.8%	
CMB-050384-3	5.0	PBS[b]	>60	98.5%	

[a]Half-life
[b]PBS is Ca^{2+}, Mg^{2+}-free phosphate-buffered saline, pH 7.2.

4.2.4 Plasma half-life summary

Client ID	test conc (µM)	test species	Plasma $T_{1/2}$[a] (min)	Fraction remaining, last time point (%)	comment
Propantheline	5.0	Mouse Plasma	30.3	23.3%	metabolized control
21085 (1)	5.0	Mouse Plasma	60.5	47.1%	Calc T1/2 =60.5 min
(2)	5.0	Mouse Plasma	>60	69.5%	Calc T1/2 =131.2 min
(3)	5.0	Mouse Plasma	>60	95.2%	Calc T1/2 =>1000 min
(4)	5.0	Mouse Plasma	>60	101.3%	Calc T1/2 =>1000 min

[a]Half-life

Calculated half-life from the data is shown in the comment section. Calculated half-lives greater than twice the experimental duration have a limited reliability.

4.2.5 Microsomal intrinsic clearance summary

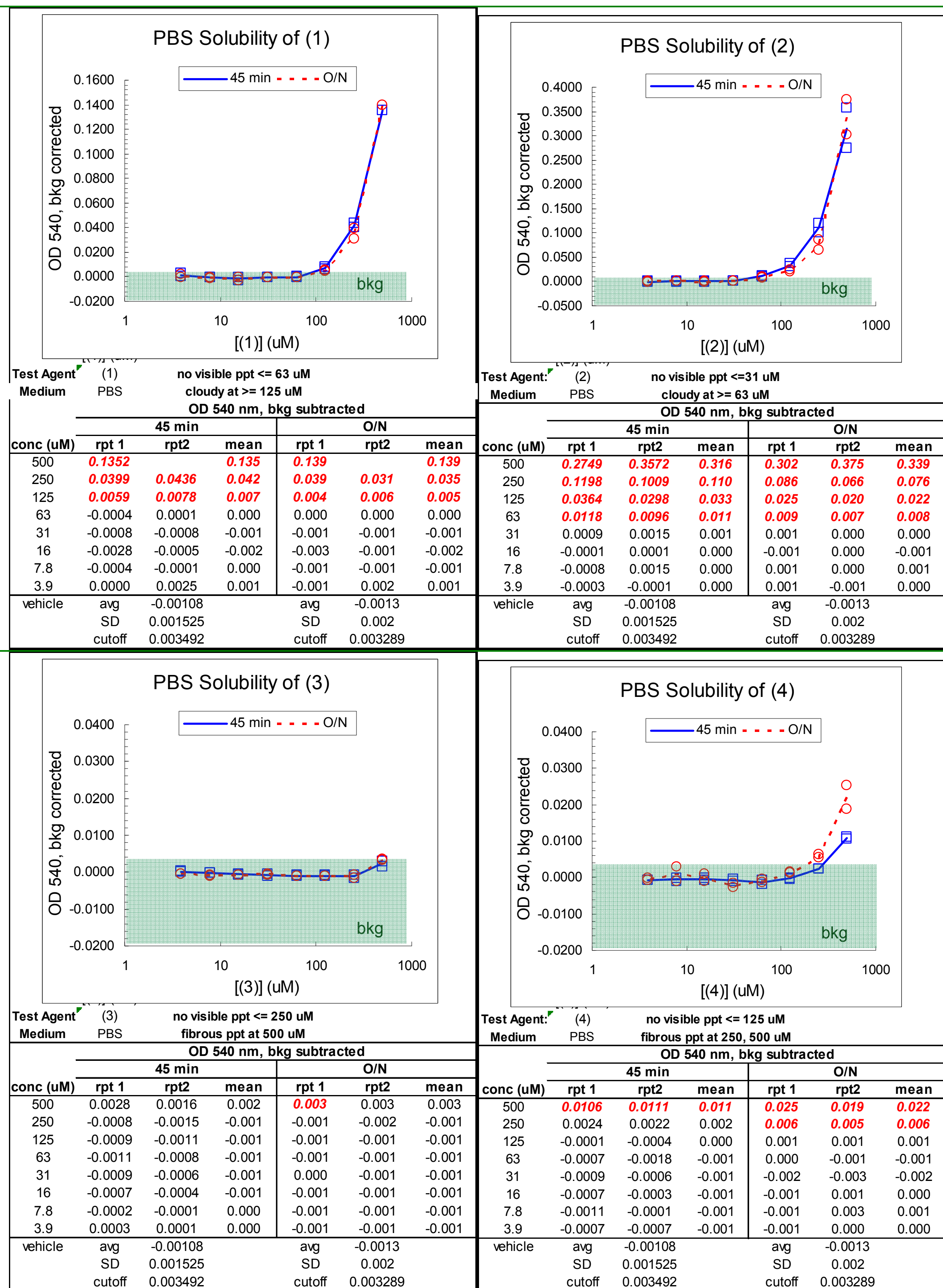
Client ID	test conc (μM)	test species	NADPH-dependent CL_{int}[a] (μl min^{-1} mg-^{1})	NADPH-dependent $T_{1/2}$[b] (min)	NADPH-free CL_{int}[a] (μl min^{-1} mg-^{1})	NADPH-free $T_{1/2}$[b] (min)	comment
Verapamil	1.0	Human	79.9	28.9	0.0	>180	highly metabolized control
Verapamil	1.0	Mouse	260.4	8.9	0.0	>180	highly metabolized control
Warfarin	1.0	Human	0.0	>180	0.0	>180	poorly metabolized control
Warfarin	1.0	Mouse	0.0	>180	0.0	>180	poorly mebabolized control
CAM-016-1	1.0	Human	38	61	0	>180	
CAM-016-1	1.0	Mouse	60	39	1	>180	
CAM-016-2	1.0	Human	74	31	4	>180	
CAM-016-2	1.0	Mouse	103	22	3	>180	
CAM-016-3	1.0	Human	5	>180	0	>180	
CAM-016-3	1.0	Mouse	54	43	0	>180	
CAM-016-4	1.0	Human	12	>180	0	>180	
CAM-016-4	1.0	Mouse	118	20	1	>180	

[a]Microsomal Intrinsic Clearance
[b]Half-life

4.3 In vitro *ADME-Tox Individual Data*

4.3.1 PBS express solubility individual data

Values with OD 540 higher than the limit of significance (mean +3 SD for vehicle-treated samples) are indicated in red. Missing points represent unreliable data that were not included in the analysis. For test agent (3), there was a visible fibrous precipitate at the highest concentration in both wells, so this concentration was scored positive for the presence of precipitate.



Test Agent (1) — **no visible ppt <= 63 uM**
Medium PBS — **cloudy at >= 125 uM**

	OD 540 nm, bkg subtracted					
	45 min			O/N		
conc (uM)	rpt 1	rpt2	mean	rpt 1	rpt2	mean
500	***0.1352***		***0.135***	***0.139***		***0.139***
250	***0.0399***	***0.0436***	***0.042***	***0.039***	***0.031***	***0.035***
125	***0.0059***	***0.0078***	***0.007***	***0.004***	***0.006***	***0.005***
63	-0.0004	0.0001	0.000	0.000	0.000	0.000
31	-0.0008	-0.0008	-0.001	-0.001	-0.001	-0.001
16	-0.0028	-0.0005	-0.002	-0.003	-0.001	-0.002
7.8	-0.0004	-0.0001	0.000	-0.001	-0.001	-0.001
3.9	0.0000	0.0025	0.001	-0.001	0.002	0.001
vehicle	avg	-0.00108		avg	-0.0013	
	SD	0.001525		SD	0.002	
	cutoff	0.003492		cutoff	0.003289	

Test Agent: (2) — **no visible ppt <=31 uM**
Medium PBS — **cloudy at >= 63 uM**

	OD 540 nm, bkg subtracted					
	45 min			O/N		
conc (uM)	rpt 1	rpt2	mean	rpt 1	rpt2	mean
500	***0.2749***	***0.3572***	***0.316***	***0.302***	***0.375***	***0.339***
250	***0.1198***	***0.1009***	***0.110***	***0.086***	***0.066***	***0.076***
125	***0.0364***	***0.0298***	***0.033***	***0.025***	***0.020***	***0.022***
63	***0.0118***	***0.0096***	***0.011***	***0.009***	***0.007***	***0.008***
31	0.0009	0.0015	0.001	0.001	0.000	0.000
16	-0.0001	0.0001	0.000	-0.001	0.000	-0.001
7.8	-0.0008	0.0015	0.000	0.001	0.000	0.001
3.9	-0.0003	-0.0001	0.000	0.001	-0.001	0.000
vehicle	avg	-0.00108		avg	-0.0013	
	SD	0.001525		SD	0.002	
	cutoff	0.003492		cutoff	0.003289	

Test Agent (3) — **no visible ppt <= 250 uM**
Medium PBS — **fibrous ppt at 500 uM**

	OD 540 nm, bkg subtracted					
	45 min			O/N		
conc (uM)	rpt 1	rpt2	mean	rpt 1	rpt2	mean
500	0.0028	0.0016	0.002	***0.003***	0.003	0.003
250	-0.0008	-0.0015	-0.001	-0.001	-0.002	-0.001
125	-0.0009	-0.0011	-0.001	-0.001	-0.001	-0.001
63	-0.0011	-0.0008	-0.001	-0.001	-0.001	-0.001
31	-0.0009	-0.0006	-0.001	0.000	-0.001	-0.001
16	-0.0007	-0.0004	-0.001	-0.001	-0.001	-0.001
7.8	-0.0002	-0.0001	0.000	-0.001	-0.001	-0.001
3.9	0.0003	0.0001	0.000	-0.001	-0.001	-0.001
vehicle	avg	-0.00108		avg	-0.0013	
	SD	0.001525		SD	0.002	
	cutoff	0.003492		cutoff	0.003289	

Test Agent: (4) — **no visible ppt <= 125 uM**
Medium PBS — **fibrous ppt at 250, 500 uM**

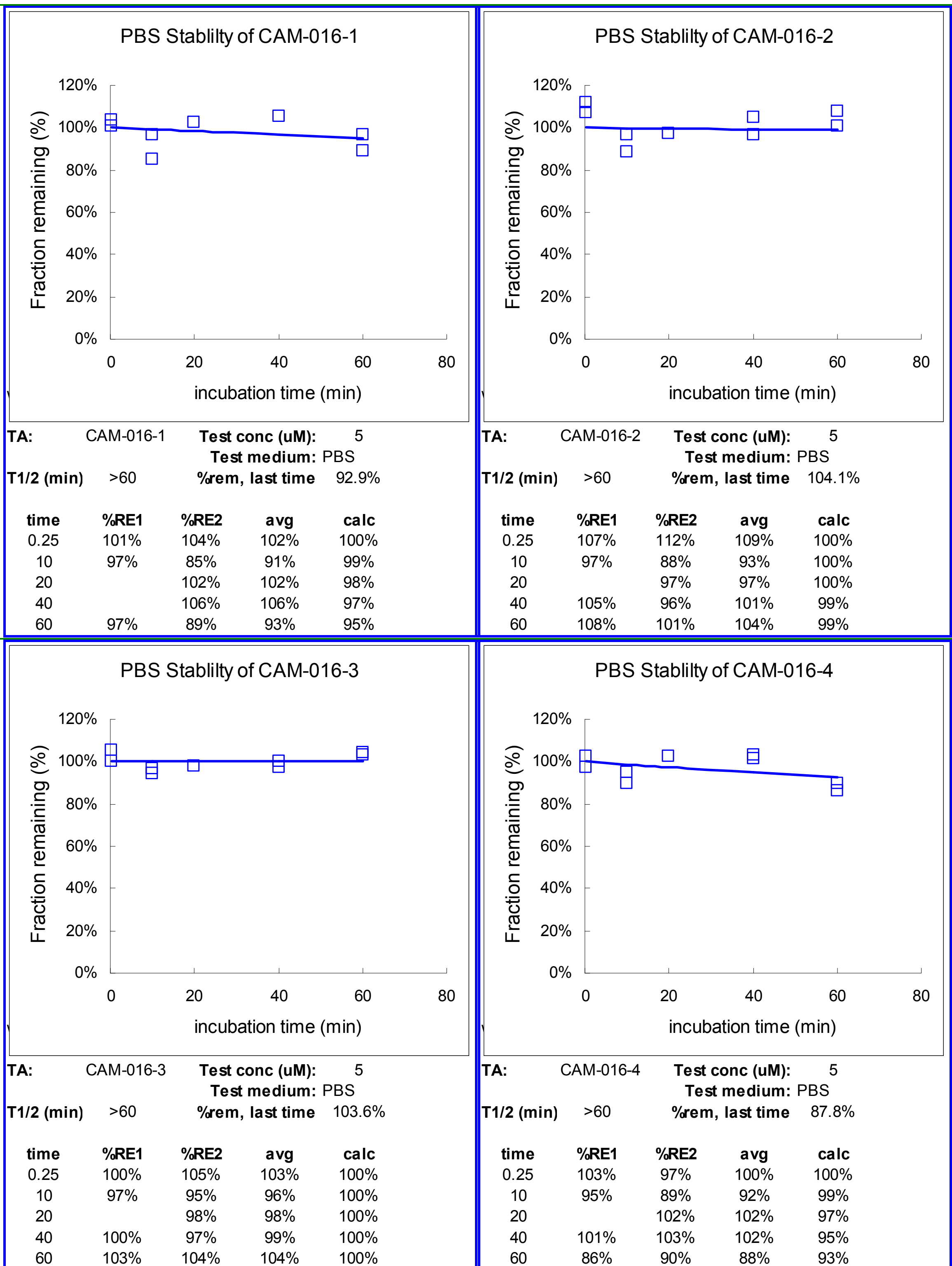
	OD 540 nm, bkg subtracted					
	45 min			O/N		
conc (uM)	rpt 1	rpt2	mean	rpt 1	rpt2	mean
500	***0.0106***	***0.0111***	***0.011***	***0.025***	***0.019***	***0.022***
250	0.0024	0.0022	0.002	***0.006***	***0.005***	***0.006***
125	-0.0001	-0.0004	0.000	0.001	0.001	0.001
63	-0.0007	-0.0018	-0.001	0.000	-0.001	-0.001
31	-0.0009	-0.0006	-0.001	-0.002	-0.003	-0.002
16	-0.0007	-0.0003	-0.001	-0.001	0.001	0.000
7.8	-0.0011	-0.0001	-0.001	-0.001	0.003	0.001
3.9	-0.0007	-0.0007	-0.001	-0.001	0.000	0.000
vehicle	avg	-0.00108		avg	-0.0013	
	SD	0.001525		SD	0.002	
	cutoff	0.003492		cutoff	0.003289	

4.3.2 Caco-2 permeability individual data

Client ID	*test conc (μM)*	*direction*	*value*	*1st*	*2nd*	*mean*	*comment*
1	50	A->B	dQ/dt[a]	1.0E-03	9.0E-04	9.6E-04	
		A->B	C_0[b]	40.3	39.4	39.8	
		B->A	dQ/dt[a]	3.5E-04	3.1E-04	3.3E-04	
		B->A	C_0[b]	38.9	38.1	38.5	
2	50	A->B	dQ/dt[a]	2.4E-03	2.0E-03	2.2E-03	
		A->B	C_0[b]	99.8	102.9	101.4	
		B->A	dQ/dt[a]	5.9E-04	5.6E-04	5.8E-04	
		B->A	C_0[b]	99.9	105.2	102.5	
3	50	A->B	dQ/dt[a]	1.9E-03	1.7E-03	1.8E-03	
		A->B	C_0[b]	87.1	93.6	90.4	
		B->A	dQ/dt[a]	4.1E-04	4.3E-04	4.2E-04	
		B->A	C_0[b]	92.6	87.5	90.0	
4	50	A->B	dQ/dt[a]	3.0E-03	2.6E-03	2.8E-03	
		A->B	C_0[b]	159.5	168.8	164.2	
		B->A	dQ/dt[a]	5.1E-04	6.0E-04	5.6E-04	
		B->A	C_0[b]	164.8	158.4	161.6	

[a]rate of test agent permeation, area units/sec
[b]initial concentration (area units/cm^3)

4.3.3 Buffer half-life individual data



TA: CAM-016-1 **Test conc (uM):** 5
Test medium: PBS
T1/2 (min) >60 **%rem, last time** 92.9%

time	%RE1	%RE2	avg	calc
0.25	101%	104%	102%	100%
10	97%	85%	91%	99%
20		102%	102%	98%
40		106%	106%	97%
60	97%	89%	93%	95%

TA: CAM-016-2 **Test conc (uM):** 5
Test medium: PBS
T1/2 (min) >60 **%rem, last time** 104.1%

time	%RE1	%RE2	avg	calc
0.25	107%	112%	109%	100%
10	97%	88%	93%	100%
20		97%	97%	100%
40	105%	96%	101%	99%
60	108%	101%	104%	99%

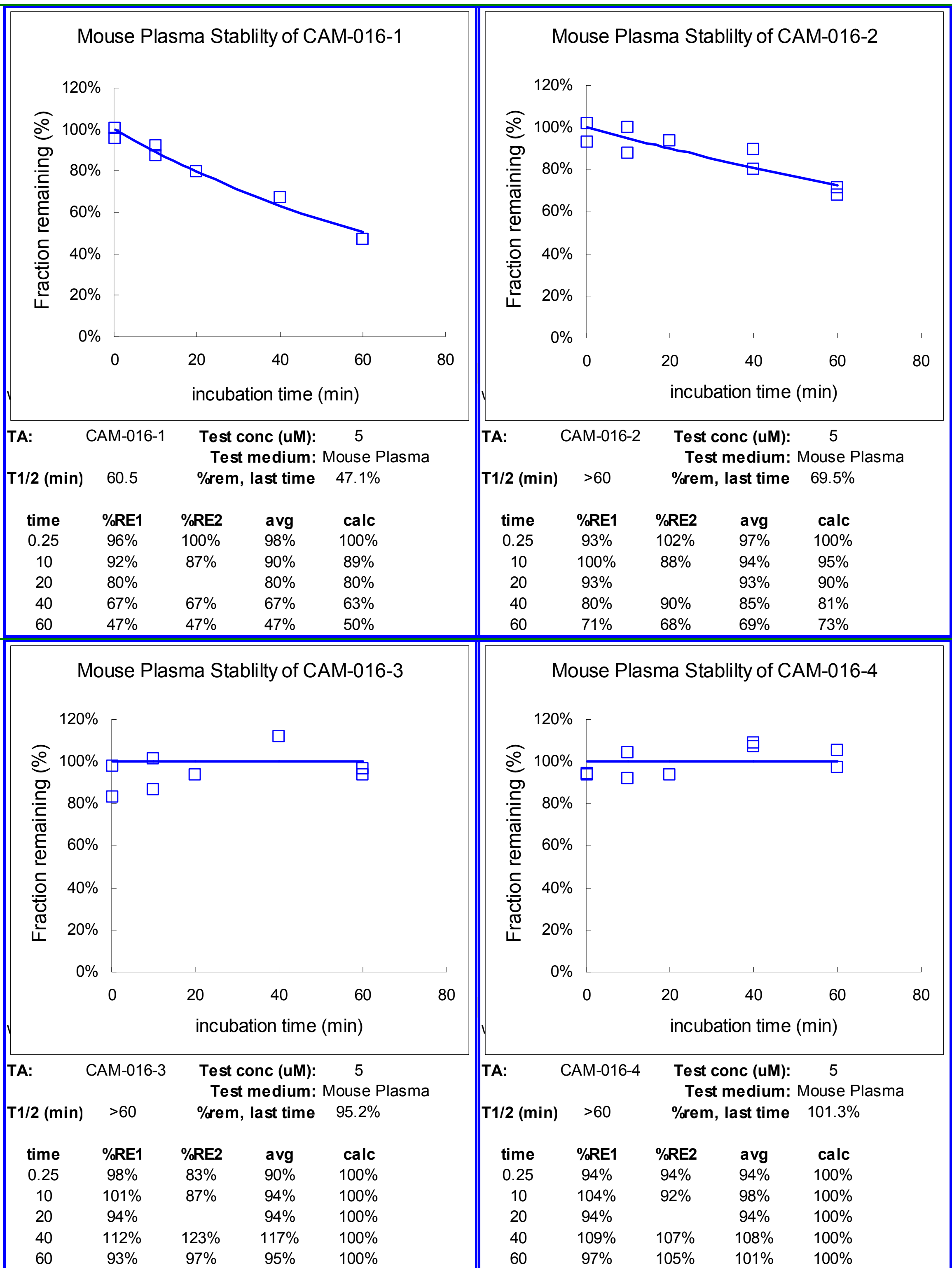
TA: CAM-016-3 **Test conc (uM):** 5
Test medium: PBS
T1/2 (min) >60 **%rem, last time** 103.6%

time	%RE1	%RE2	avg	calc
0.25	100%	105%	103%	100%
10	97%	95%	96%	100%
20		98%	98%	100%
40	100%	97%	99%	100%
60	103%	104%	104%	100%

TA: CAM-016-4 **Test conc (uM):** 5
Test medium: PBS
T1/2 (min) >60 **%rem, last time** 87.8%

time	%RE1	%RE2	avg	calc
0.25	103%	97%	100%	100%
10	95%	89%	92%	99%
20		102%	102%	97%
40	101%	103%	102%	95%
60	86%	90%	88%	93%

4.3.4 Plasma half-life individual data



TA: CAM-016-1 **Test conc (uM):** 5
Test medium: Mouse Plasma
T1/2 (min) 60.5 **%rem, last time** 47.1%

time	%RE1	%RE2	avg	calc
0.25	96%	100%	98%	100%
10	92%	87%	90%	89%
20	80%		80%	80%
40	67%	67%	67%	63%
60	47%	47%	47%	50%

TA: CAM-016-2 **Test conc (uM):** 5
Test medium: Mouse Plasma
T1/2 (min) >60 **%rem, last time** 69.5%

time	%RE1	%RE2	avg	calc
0.25	93%	102%	97%	100%
10	100%	88%	94%	95%
20	93%		93%	90%
40	80%	90%	85%	81%
60	71%	68%	69%	73%

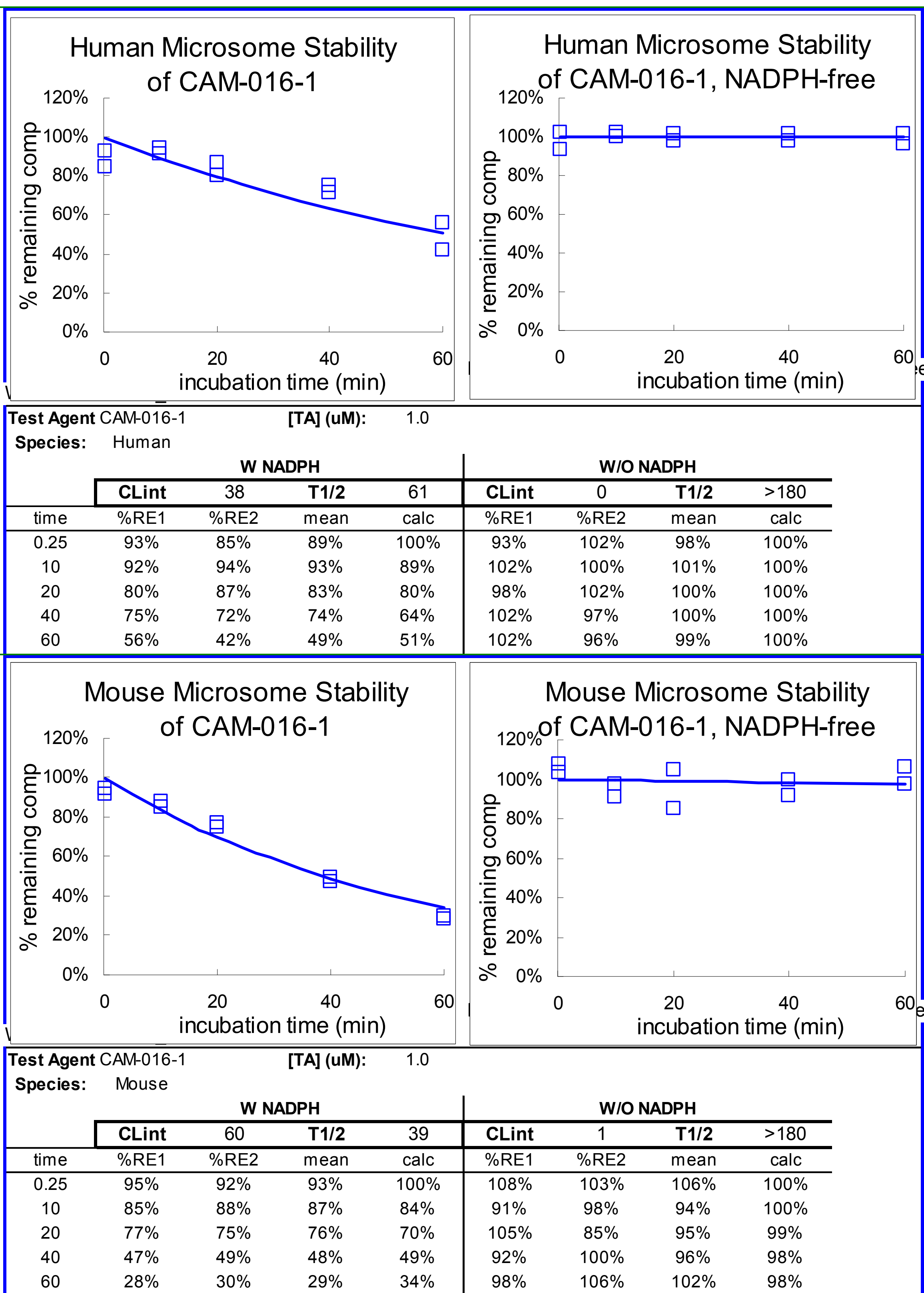
TA: CAM-016-3 **Test conc (uM):** 5
Test medium: Mouse Plasma
T1/2 (min) >60 **%rem, last time** 95.2%

time	%RE1	%RE2	avg	calc
0.25	98%	83%	90%	100%
10	101%	87%	94%	100%
20	94%		94%	100%
40	112%	123%	117%	100%
60	93%	97%	95%	100%

TA: CAM-016-4 **Test conc (uM):** 5
Test medium: Mouse Plasma
T1/2 (min) >60 **%rem, last time** 101.3%

time	%RE1	%RE2	avg	calc
0.25	94%	94%	94%	100%
10	104%	92%	98%	100%
20	94%		94%	100%
40	109%	107%	108%	100%
60	97%	105%	101%	100%

4.3.5 Microsomal intrinsic clearance individual data



Test Agent CAM-016-1 **[TA] (uM):** 1.0

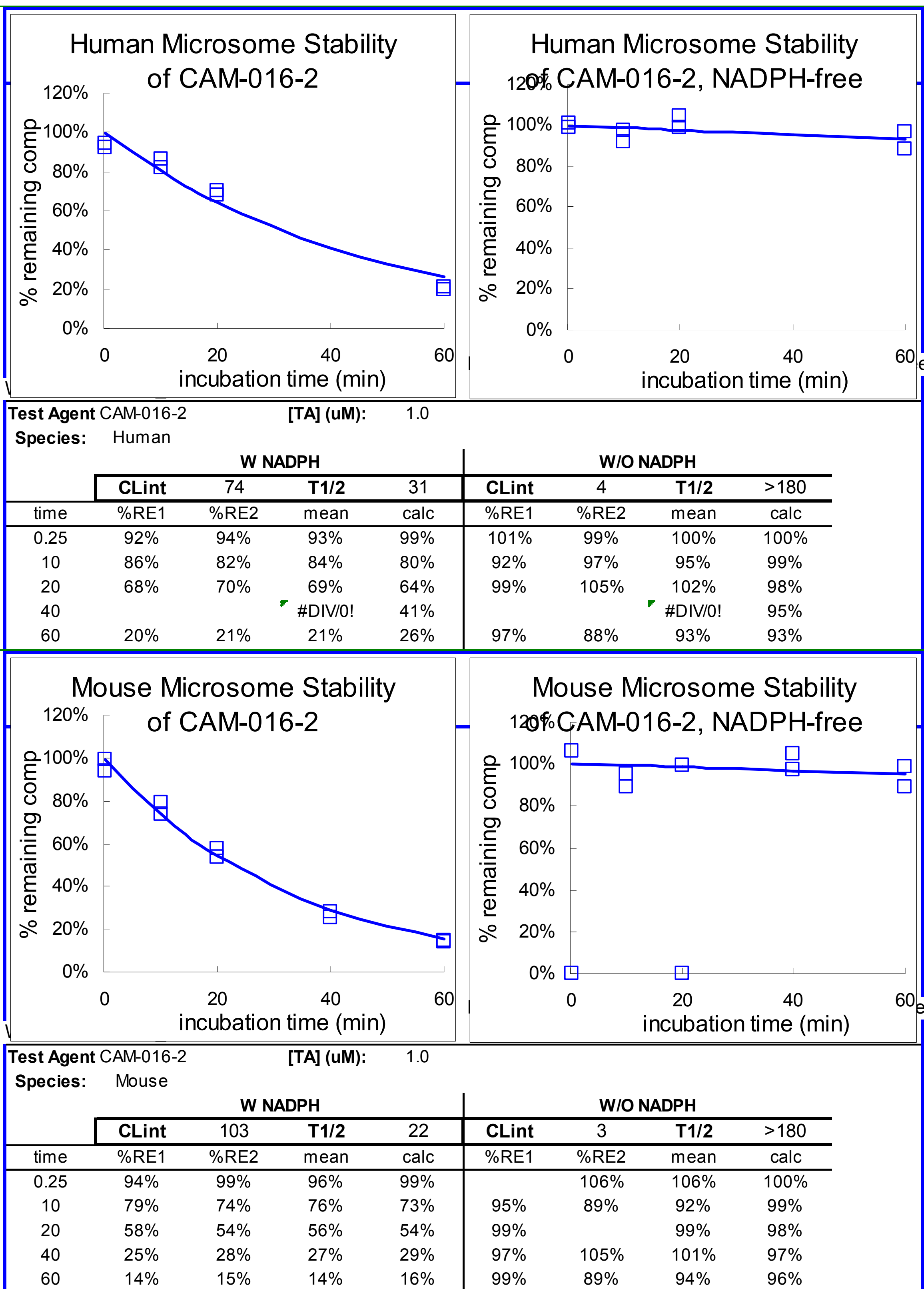
Species: Human

	W NADPH				W/O NADPH			
	CLint	38	**T1/2**	61	**CLint**	0	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	93%	85%	89%	100%	93%	102%	98%	100%
10	92%	94%	93%	89%	102%	100%	101%	100%
20	80%	87%	83%	80%	98%	102%	100%	100%
40	75%	72%	74%	64%	102%	97%	100%	100%
60	56%	42%	49%	51%	102%	96%	99%	100%

Test Agent CAM-016-1 **[TA] (uM):** 1.0

Species: Mouse

	W NADPH				W/O NADPH			
	CLint	60	**T1/2**	39	**CLint**	1	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	95%	92%	93%	100%	108%	103%	106%	100%
10	85%	88%	87%	84%	91%	98%	94%	100%
20	77%	75%	76%	70%	105%	85%	95%	99%
40	47%	49%	48%	49%	92%	100%	96%	98%
60	28%	30%	29%	34%	98%	106%	102%	98%

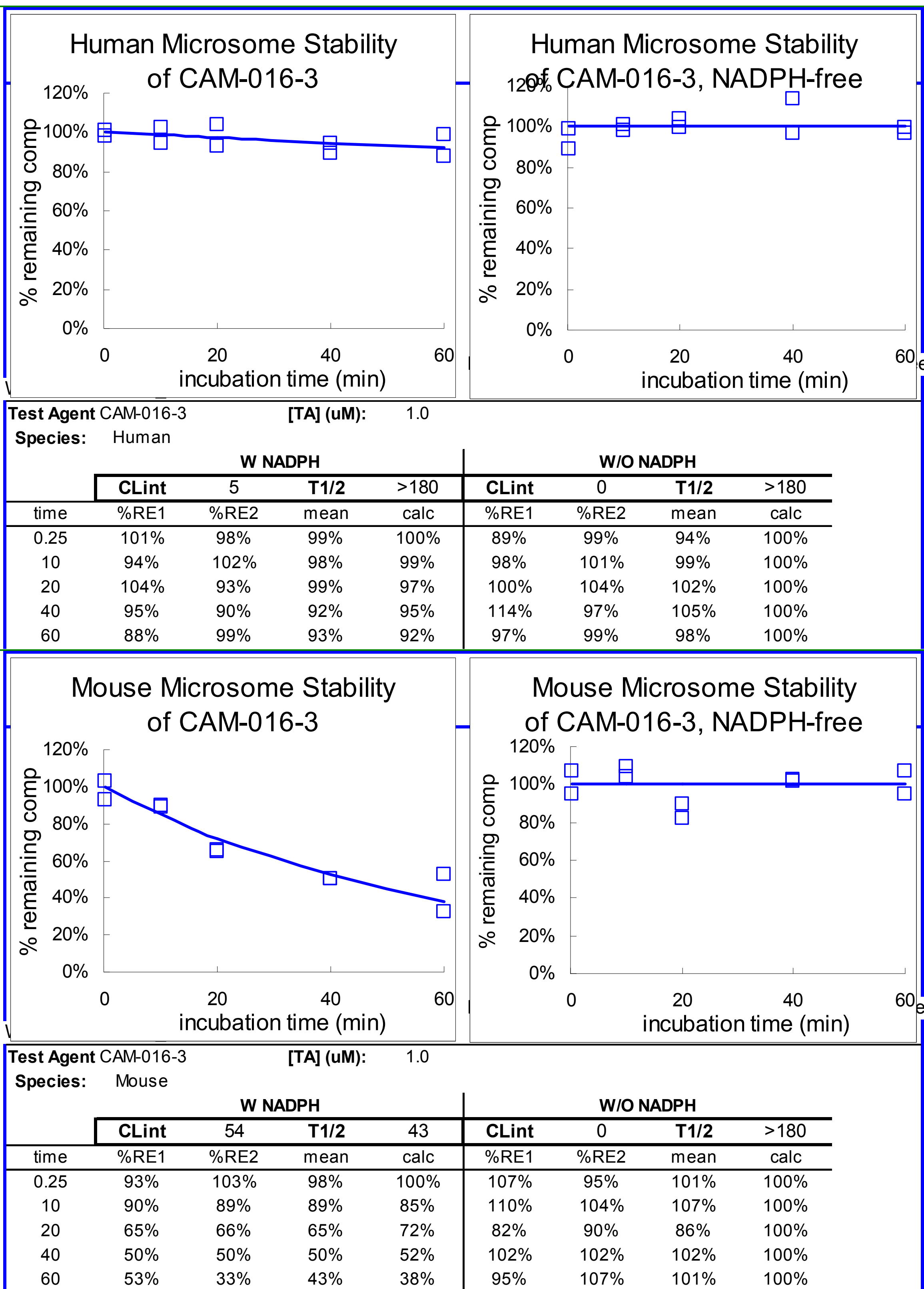


Test Agent CAM-016-2 **[TA] (uM):** 1.0
Species: Human

	W NADPH				W/O NADPH			
	CLint	74	**T1/2**	31	**CLint**	4	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	92%	94%	93%	99%	101%	99%	100%	100%
10	86%	82%	84%	80%	92%	97%	95%	99%
20	68%	70%	69%	64%	99%	105%	102%	98%
40			#DIV/0!	41%			#DIV/0!	95%
60	20%	21%	21%	26%	97%	88%	93%	93%

Test Agent CAM-016-2 **[TA] (uM):** 1.0
Species: Mouse

	W NADPH				W/O NADPH			
	CLint	103	**T1/2**	22	**CLint**	3	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	94%	99%	96%	99%		106%	106%	100%
10	79%	74%	76%	73%	95%	89%	92%	99%
20	58%	54%	56%	54%	99%		99%	98%
40	25%	28%	27%	29%	97%	105%	101%	97%
60	14%	15%	14%	16%	99%	89%	94%	96%

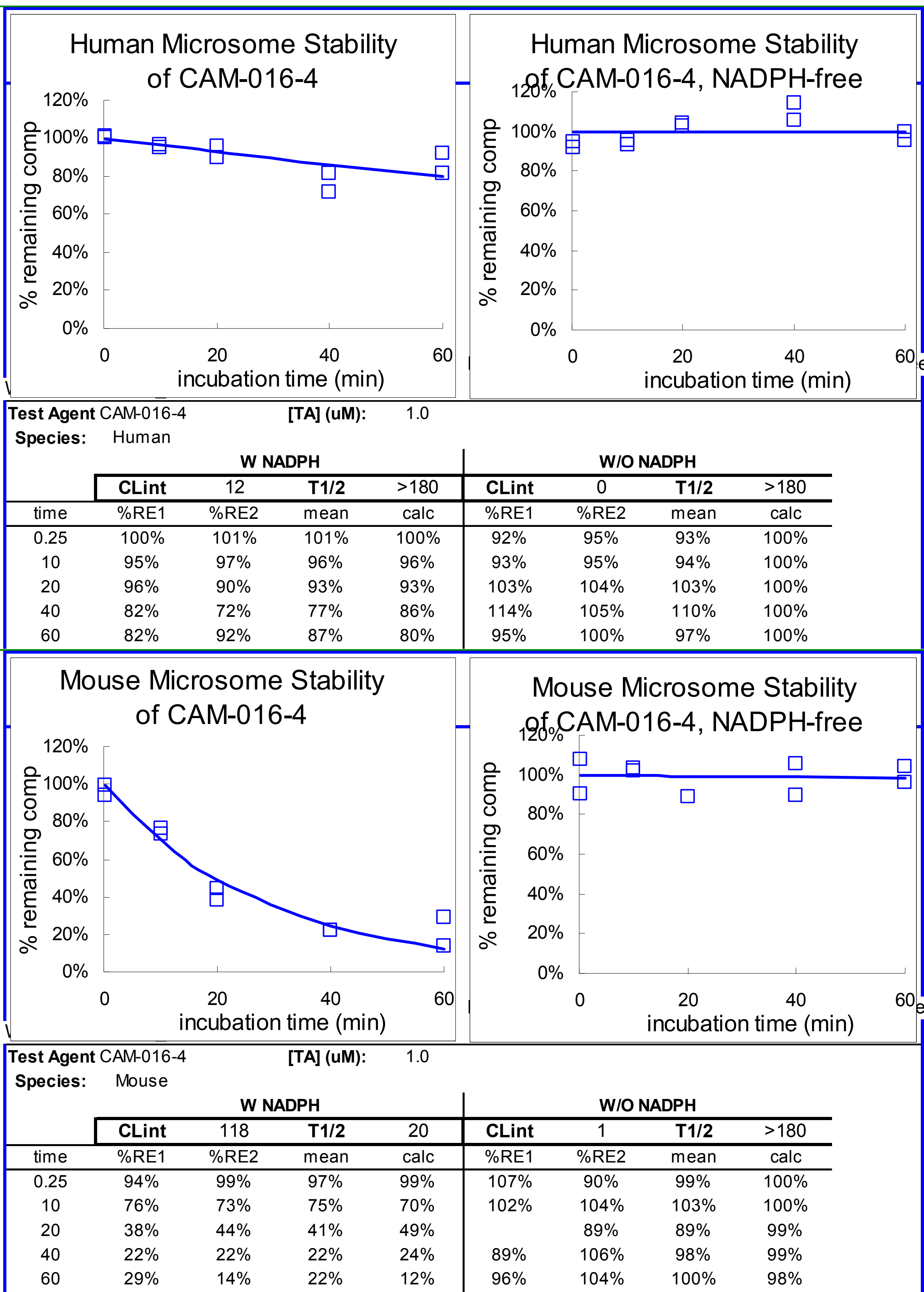


Test Agent CAM-016-3 **[TA] (uM):** 1.0
Species: Human

	W NADPH				W/O NADPH			
	CLint	5	**T1/2**	>180	**CLint**	0	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	101%	98%	99%	100%	89%	99%	94%	100%
10	94%	102%	98%	99%	98%	101%	99%	100%
20	104%	93%	99%	97%	100%	104%	102%	100%
40	95%	90%	92%	95%	114%	97%	105%	100%
60	88%	99%	93%	92%	97%	99%	98%	100%

Test Agent CAM-016-3 **[TA] (uM):** 1.0
Species: Mouse

	W NADPH				W/O NADPH			
	CLint	54	**T1/2**	43	**CLint**	0	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	93%	103%	98%	100%	107%	95%	101%	100%
10	90%	89%	89%	85%	110%	104%	107%	100%
20	65%	66%	65%	72%	82%	90%	86%	100%
40	50%	50%	50%	52%	102%	102%	102%	100%
60	53%	33%	43%	38%	95%	107%	101%	100%



Test Agent CAM-016-4 **[TA] (uM):** 1.0

Species: Human

	W NADPH				W/O NADPH			
	CLint	12	**T1/2**	>180	**CLint**	0	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	100%	101%	101%	100%	92%	95%	93%	100%
10	95%	97%	96%	96%	93%	95%	94%	100%
20	96%	90%	93%	93%	103%	104%	103%	100%
40	82%	72%	77%	86%	114%	105%	110%	100%
60	82%	92%	87%	80%	95%	100%	97%	100%

Test Agent CAM-016-4 **[TA] (uM):** 1.0

Species: Mouse

	W NADPH				W/O NADPH			
	CLint	118	**T1/2**	20	**CLint**	1	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	94%	99%	97%	99%	107%	90%	99%	100%
10	76%	73%	75%	70%	102%	104%	103%	100%
20	38%	44%	41%	49%		89%	89%	99%
40	22%	22%	22%	24%	89%	106%	98%	99%
60	29%	14%	22%	12%	96%	104%	100%	98%

Note: Missing values represent unreliable data that was not used in the calculations.

5 References

Stewart, BH, *et al*. (1995) "Comparison of intestinal permeabilities determined in multiple *in vitro* and *in situ* models: Relationship to absorption in humans." Pharm. Res. 12:693.

Houston, JB (1994) "Utility of in vitro drug metabolism data in predicting in vivo metabolic clearance." Biochem. Pharmacol. 47:1469.

Singh, R, *et al*. (1996) "*In vitro* metabolism of a potent HIV-protease inhibitor (141W94) using rat, monkey and human liver S9." Rapid Commun Mass Spectrom 10:1019.

6 Storage and Retention of Records

All documents generated in this study (raw data, the study plan, a copy of this report, etc.) will be stored for three years from the date of this document. Only authorized Apredica employees will have access to the archives.

The original final report will be provided to the sponsor and will be kept by the sponsor under its sole responsibility.

7 Appendices

7.1 Appendix A. Standard Apredica Methods

PBS express solubility

Serial dilutions of test agent are prepared in test agent at 100x the final concentration. Test agent solutions are diluted 100-fold into PBS in a 96-well plate and mixed. The absorbance of the PBS-containing plate is measured prior to adding the test agents to determine the background absorbance. After 45 min and 16 hr, the presence of precipitate is then detected by turbidity (absorbance at 540 nm). An absorbance value of greater than (mean + 3x standard deviation of the blank), after subtracting the pre-experiment background, is indicative of turbidity. For brightly colored compounds, a visual inspection of the plate is performed to verify the solubility limit determined by UV absorbance. The solubility limit is reported as the highest experimental concentration with no evidence of turbidity.

Caco-2 monolayer permeability

CaCo-2 cells grown in tissue culture flasks are trypsinized, suspended in medium, and the suspensions were applied to wells of a collagen-coated BioCoat Cell Environment in 24-well format (BD Biosciences) at 24,500 cells per well. The cells are allowed to grow and differentiate for three weeks, feeding at 2-day intervals.

For Apical to Basolateral (A->B) permeability, the test agent is added to the apical (A) side and amount of permeation is determined on the basolateral (B) side; for Basolateral to Apical (B>A) permeability, the test agent is added to the B side and the amount of permeation is determine on the A side. The A-side buffer contains 100 μM Lucifer yellow dye, in Transport Buffer (1.98 g/L glucose in 10 mM HEPES, 1x Hank's Balanced Salt Solution) pH 6.5, and the B-side buffer is Transport Buffer, pH 7.4. CaCo-2 cells are incubated with these buffers for 2 h., and the receiver side buffer is removed for analysis by LC/MS/MS.

To verify the CaCo-2 cell monolayers are properly formed, aliquots of the cell buffers are analyzed by fluorescence to determine the transport of the impermeable dye Lucifer Yellow.

Data are expressed as permeability (P_{app}): $P_{app} = \frac{dQ/dt}{C_0 A}$.

where dQ/dt is the rate of permeation, C_0 is the initial concentration of test agent, and A is the area of the monolayer.

In bidirectional permeability studies, the asymmetry index (AI) is also calculated:

$$\mathrm{AI} = \frac{P_{app}(B \rightarrow A)}{P_{app}(A \rightarrow B)}.$$

An AI > 1 indicated a potential substrate for PGP or other active transporters.

Buffer half-life

The test agent is incubated in duplicate with test medium at 37 ºC. The reaction contains medium and 2% DMSO. At the indicated times, an aliquot is removed from each experimental reaction and mixed with three volumes of ice-cold Stop Solution (methanol containing propranolol, diclofenac, or other internal standard). Stopped reactions are incubated at least ten minutes at -20 ºC. The samples are centrifuged to remove any precipitate, and the supernatants are analyzed by LC/MS/MS to quantitate the remaining

parent. Data are converted to % remaining by dividing by the time zero concentration value. Data are fit to a first-order decay model to determine half-life.

Plasma half-life

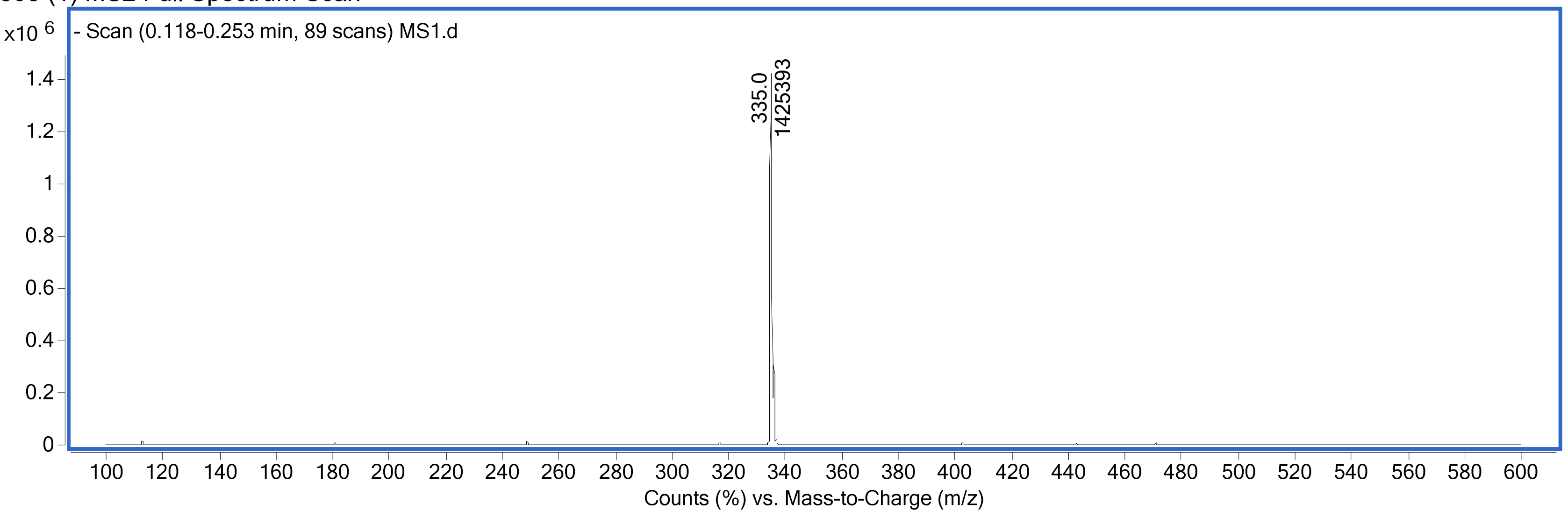
The test agent is incubated in duplicate with plasma at 37 ºC. The reaction contains plasma and 2% DMSO. At the indicated times, an aliquot is removed from each experimental reaction and mixed with three volumes of ice-cold Stop Solution (methanol containing propranolol, diclofenac, or other internal standard). Stopped reactions are incubated at least ten minutes at -20 ºC. The samples are centrifuged to remove precipitated protein, and the supernatants are analyzed by LC/MS/MS to quantitate the remaining parent. Data are converted to % remaining by dividing by the time zero concentration value. Data are fit to a first-order decay model to determine half-life.

Microsomal intrinsic clearance

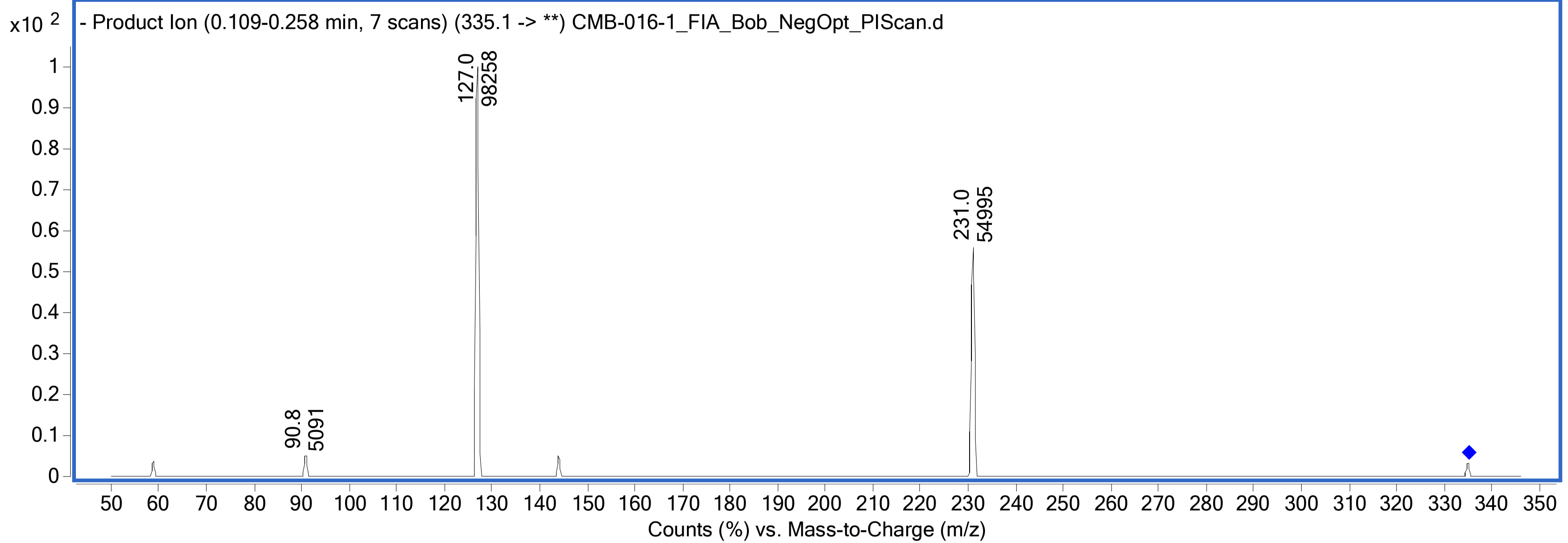
The test agent is incubated in duplicate with microsomes at 37 ºC. The reaction contains microsomal protein in 100 mM potassium phosphate, 2 mM NADPH, 3 mM $MgCl_2$, pH 7.4. A control is run for each test agent omitting NADPH to detect NADPH-free degradation. The indicated times, an aliquot is removed from each experimental and control reaction and mixed with an equal volume of ice-cold Stop Solution (0.3% acetic acid in acetonitrile containing haloperidol, diclofenac, or other internal standard). Stopped reactions are incubated at least ten minutes at -20 ºC, and an additional volume of water is added. The samples are centrifuged to remove precipitated protein, and the supernatants are analyzed by LC/MS/MS to quantitate the remaining parent.
Data are converted to % remaining by dividing by the time zero concentration value. Data are fit to a first-order decay model to determine half-life. Intrinsic clearance is calculated from the half-life and the protein concentrations: $CL_{int} = \ln(2) /(T_{1/2} [\text{microsomal protein}])$.

7.2 Appendix B. Sample Spectra and Chromatograms of the Test Agents

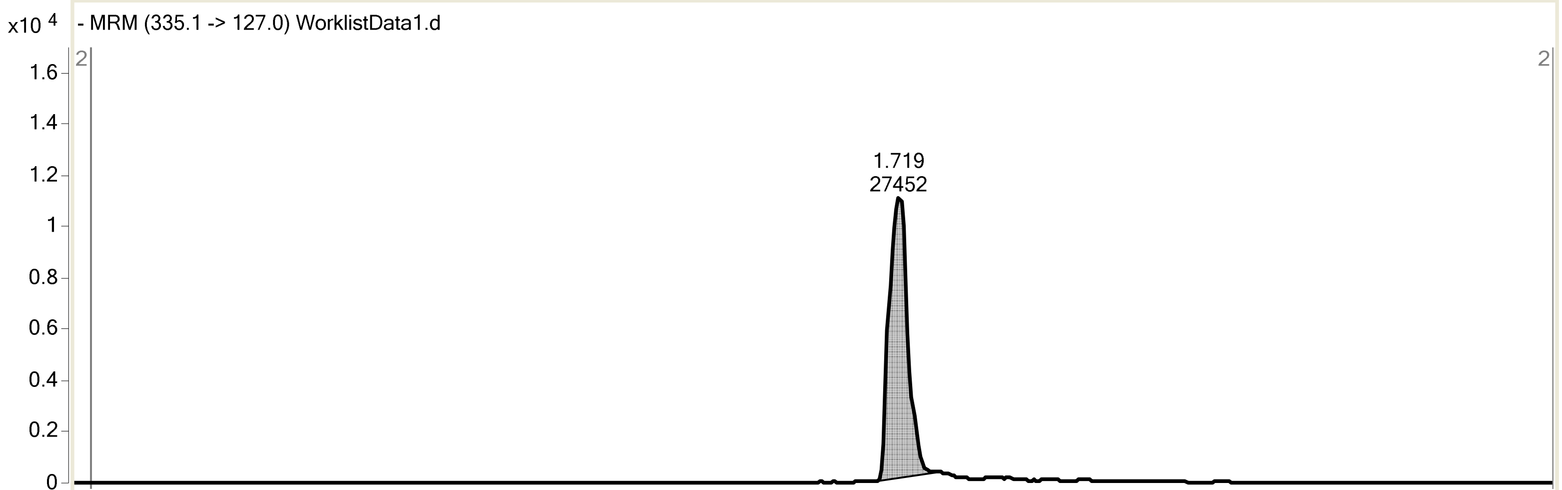
21805 (1) MS2 Full Spectrum Scan

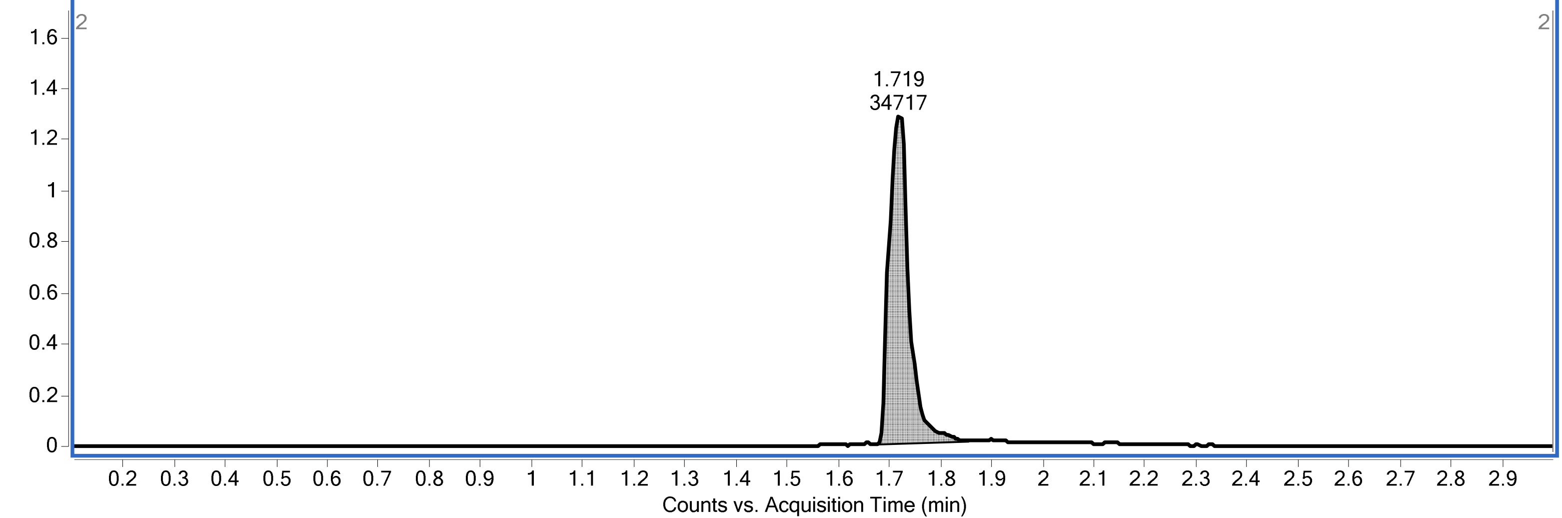


21805 (1) Product Ion Scan

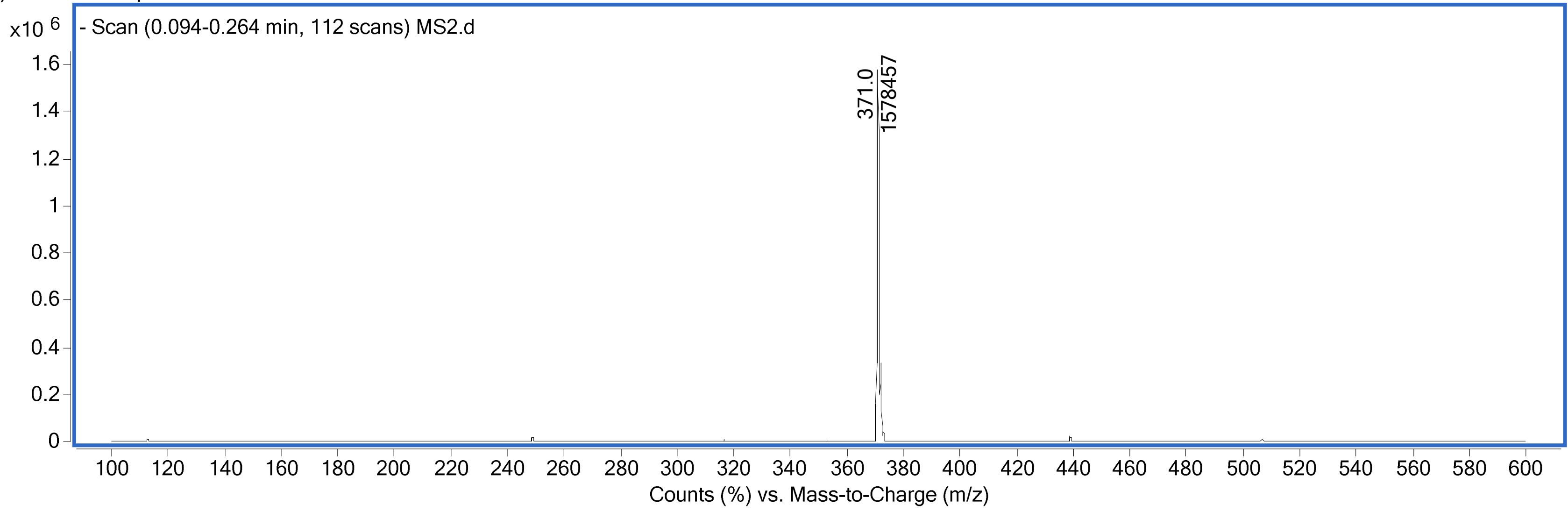


21805 (1) Sample Chromatogram

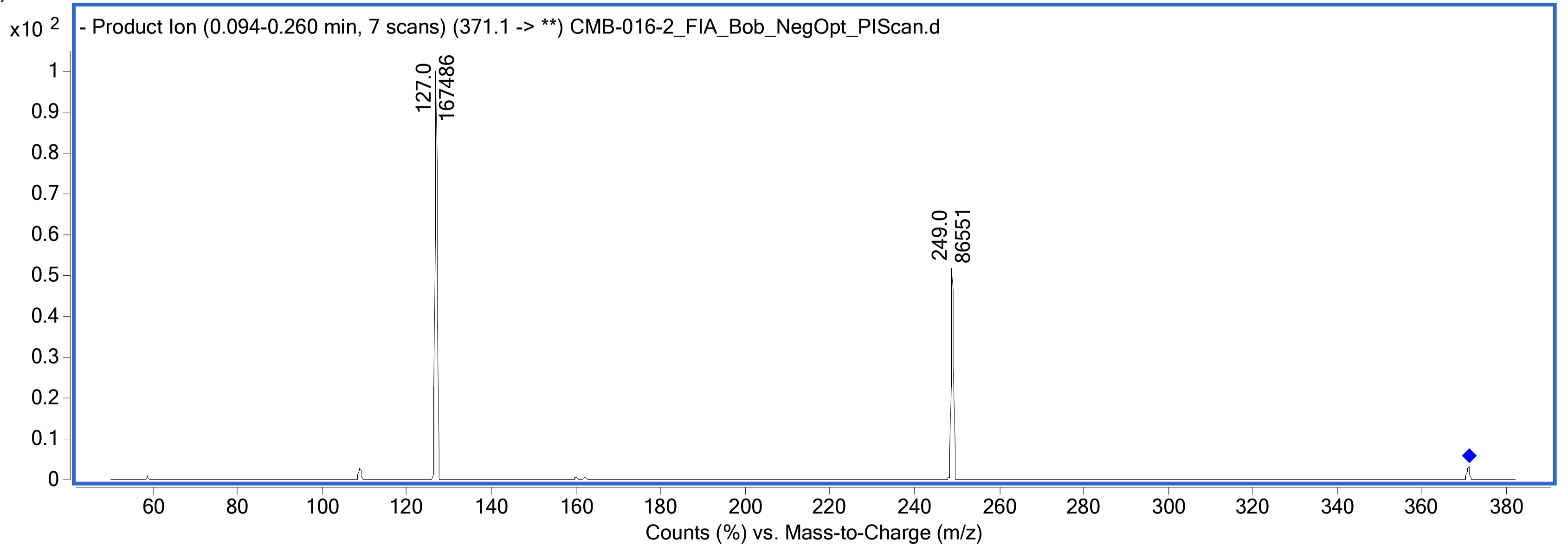




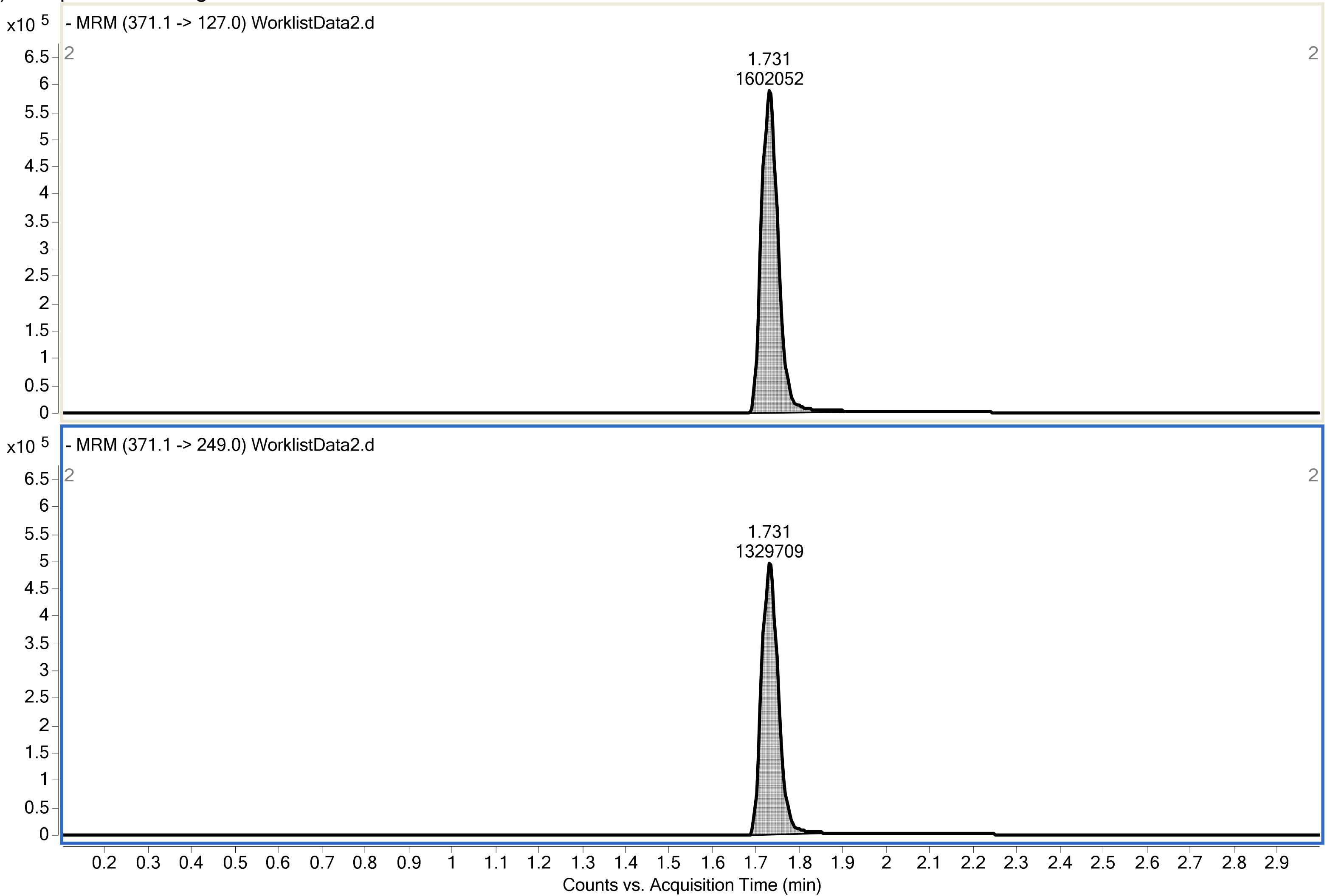
(2) MS2 Full Spectrum Scan



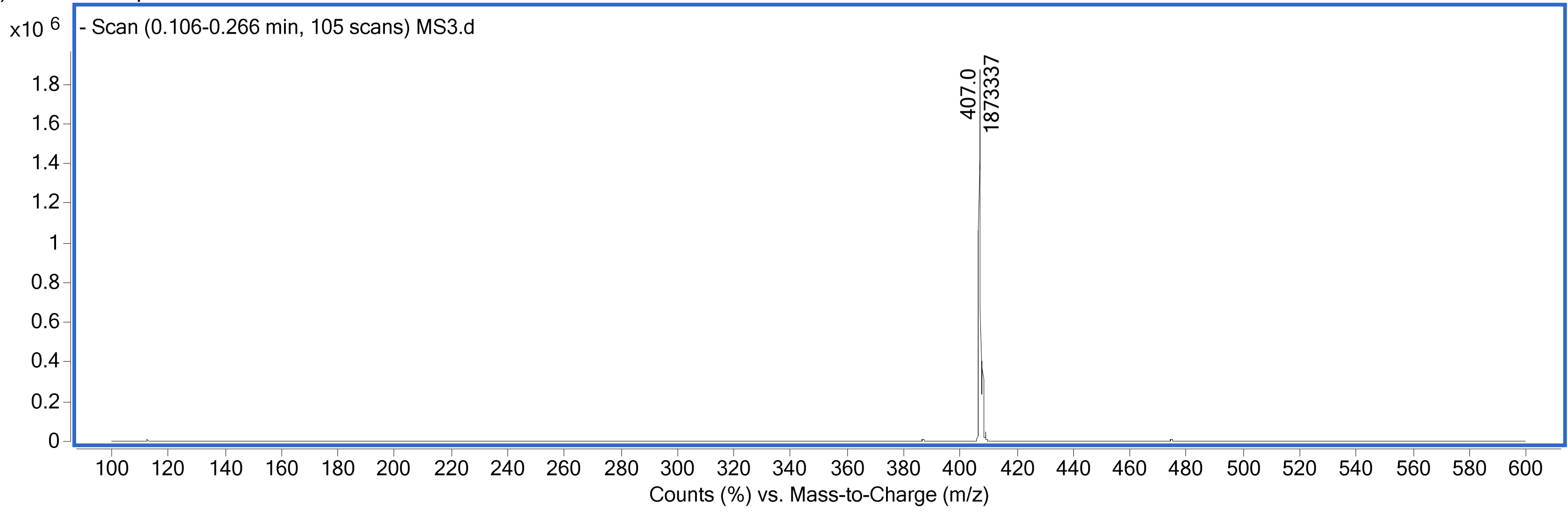
(2) Product Ion Scan



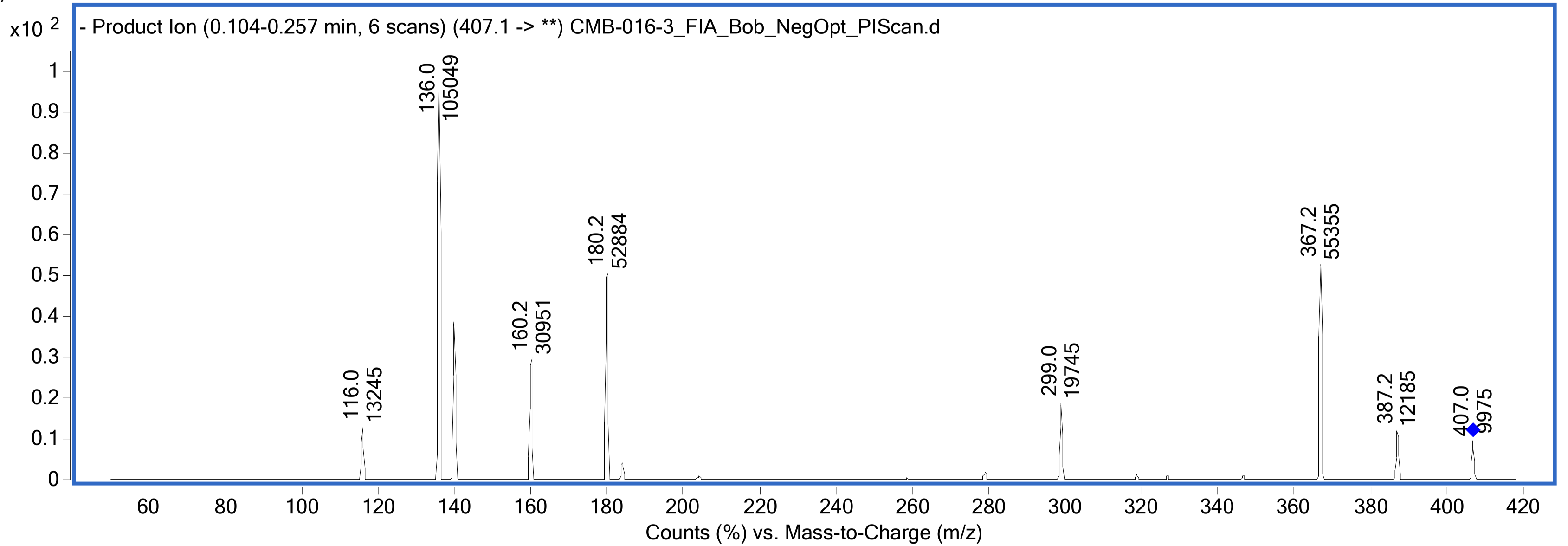
(2) Sample Chromatogram

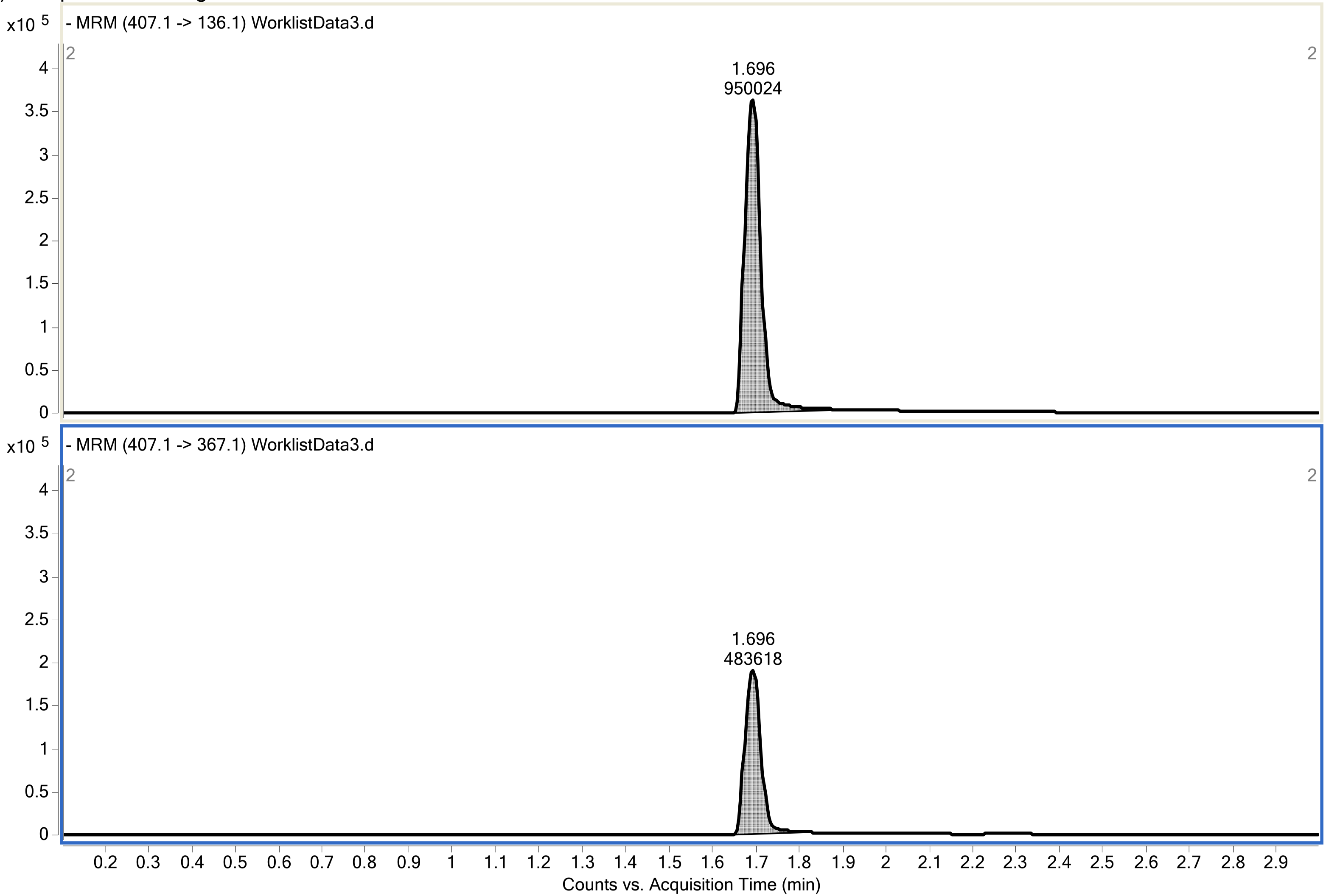


(3) MS2 Full Spectrum Scan

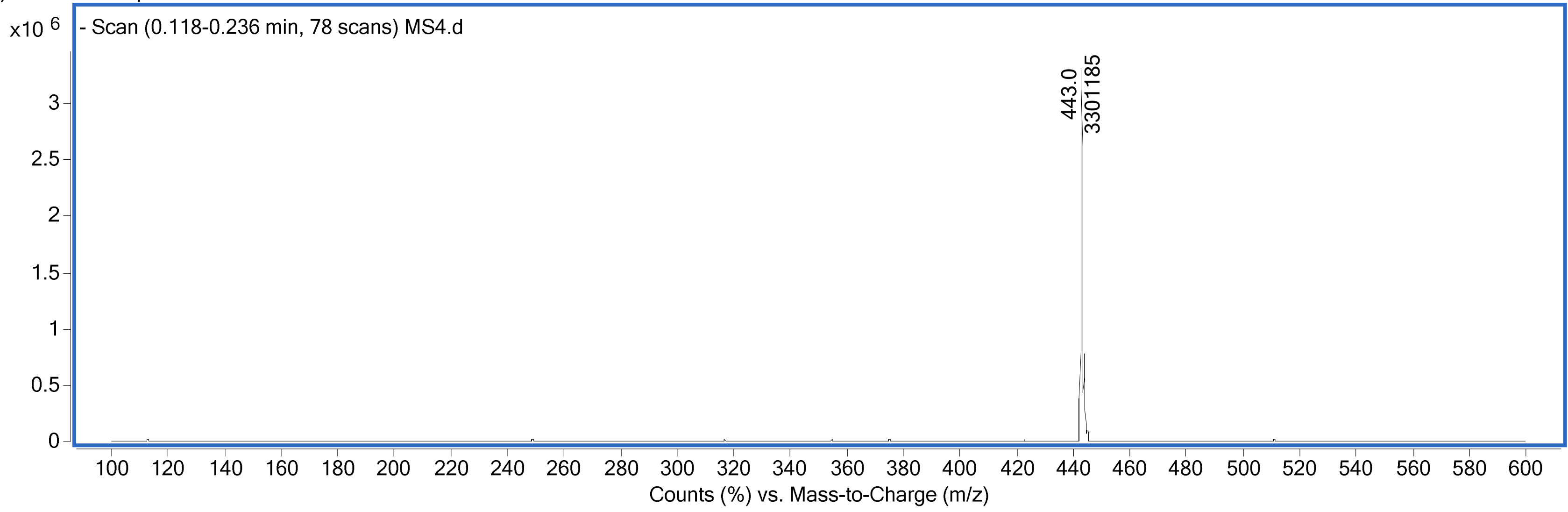


(3) Product Ion Scan

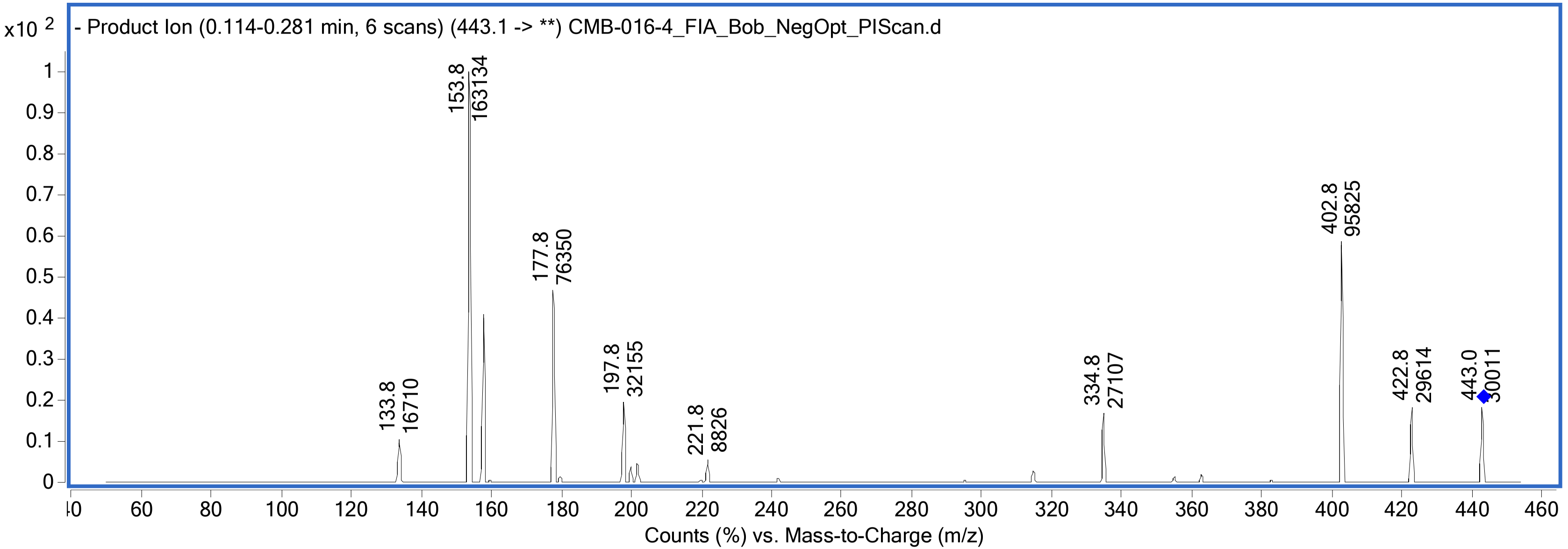



(3) Sample Chromatogram
x10 5
- MRM (407.1 -> 136.1) WorklistData3.d
1.696
950024
x10 5
- MRM (407.1 -> 367.1) WorklistData3.d
1.696
483618
0 0.5 1 1.5 2 2.5 3 3.5 4
0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1 1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8 1.9 2 2.1 2.2 2.3 2.4 2.5 2.6 2.7 2.8 2.9
Counts vs. Acquisition Time (min)

(4) MS2 Full Spectrum Scan



(4) Product Ion Scan



(4) Sample Chromatogram