

ATTACHMENT A.2

METABOLISM STUDY: FINAL REPORT

FINAL REPORT

**Determination of Metaxalone Metabolism in Expressed
Recombinant Human Enzymes CYP1A2, CYP2A6, CYP2C9,
CYP2C19, CYP2D6, CYP2E1, and CYP3A4**

Sponsor: Mutual Pharmaceutical Company
United Research Laboratories, Inc.
1100 Orthodox Street
Philadelphia, PA 19124
Tel.: (215) 807-1076
Fax: (215) 807-1083

Sponsor Representative: Jie Du, Ph.D.
Email: jie_du@URLMutual.com

Testing Facility: In Vitro Technologies, Inc.
1450 South Rolling Road
Baltimore, MD 21227
Tel.: (410) 455-1242
Fax: (410) 455-1245

Study Director: Genfu Chen, Ph.D.
E-mail: cheng@invitrotech.com

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Participation

The following principal staff participated in the conduct of this study:

Study Director:

Genfu Chen, Ph.D.

Scientists:

Paryeen Kaushal, PhD.

Bridget McKenzie-Fogle, M.Sc.

Carrie Crooks, B.Sc.

Drew Fackett, B.Sc.

Jonathan Smith, B.Sc.

Michael Chesebrough, B.Sc.

Wei Zhang, M.Sc.

Technical Writers:

Blaise Considine, B.A.

Steve Sorrow, B.Sc.

Study Dates and Data Retention

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29 September 2005

Date protocol signed by Sponsor:

28 September 2005

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7 October 2005

Experimental end date:

12 October 2005

Study completion date:

15 November 2005

In Vitro Technologies will retain all supporting documentation, including raw data and written records, for a period of up to five years following issuance of the final report. At the end of this period, Mutual Pharmaceutical will be notified to determine whether the data (excluding proprietary information) will be transferred, retained, or destroyed.

Statement of Compliance

This study was conducted using good documentation practices. The study was conducted under my scientific guidance and management.

Genfu Chen, Ph.D.
Study Director


Signature

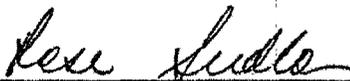
15 NOV 2005
Date

Quality Assurance Statement

This study was inspected in accordance with In Vitro Technologies standard operating procedures. Based on audits conducted, the results reported accurately reflect the methods used and the data collected for this study.

All findings were reported to the Study Director and In Vitro Technologies Management.

Inspection/Audit Dates:	Study Phase Audited:	Date(s) reported to Study Director and Management:
07 October 2005	Incubation of 2C9 & 3A4	07 October 2005
25-27 October 2005	Data & Report	28 October 2005


Quality Assurance

15 Nov. 05.
Date

Glossary of Abbreviations

CYP.....	cytochrome P450
HPLC	high-performance liquid chromatography
HPLC-UV	HPLC with UV detection
LC/MS.....	liquid chromatography/mass spectrometry
NADPH.....	nicotinamide adenosine dinucleotide phosphate
NRS.....	NADPH regenerating system

Summary

The objective of this study was to determine the metabolism of metaxalone in Supersomes™ containing one of the cytochrome P450 (CYP) isoforms CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. Supersomes containing expressed human CYP isoforms were incubated in the presence of metaxalone. The metabolism of metaxalone was evaluated by measuring the disappearance of metaxalone by high-performance liquid chromatography (HPLC). Metaxalone was metabolized by CYP1A2. It was also metabolized by CYP2C19, although to a lesser extent.

Introduction

The liver represents the major organ for drug metabolism and contains the CYP enzymes, the major enzyme systems for xenobiotic metabolism (1). The identification of human CYP enzymes involved in drug metabolism allows the prediction of potential drug-drug interactions such as co-administration with isoform-selective inhibitors or inducers, and interpatient variability in drug exposure. Various approaches, including inhibition studies with specific chemical inhibitors and inhibitory antibodies, correlation studies, and studies using heterologously expressed CYP isoforms, have been applied to identify human enzymes involved in drug metabolism (2, 3).

In this study, individual CYP isoforms expressed from human cDNA and P450 reductase cDNA (Supersomes) were used. Supersomes provide a readily available and well-characterized biological model for use in identifying human CYP enzymes involved in drug metabolism.

The objective of this study was to determine the metabolism of metaxalone in Supersomes™ containing CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. Supersomes containing expressed human CYP isoforms were incubated in the presence of metaxalone, and the metabolism of metaxalone was evaluated by measuring the disappearance of metaxalone by HPLC.

Experimental Methods

Test Article Information & Preparation

The test article was identified in this study as follows:

- Metaxalone: 5-[(3,5-dimethylphenoxy)methyl]-2-oxazolidinone, molecular weight = 221 g/mol, lot MTX-K4013, purity 99.9% by HPLC

Metaxalone stock solutions were prepared in methanol at 100 times (100X) the final concentration. The stock solutions were added to incubation mixtures to obtain the final concentrations of 0.5, 2.5, and 25 μM each containing 1% methanol. (The concentrations in ng/mL were 110.5, 552.5, and 5,525 ng/mL.)

Substrates for the CYP isoforms

Positive controls were incubated with the following CYP450 isoform-selective substrates. Each substrate was prepared as 100X stock solutions in the solvent listed below. The final substrate concentrations and solvents were:

CYP isoform	Isoform-selective substrate	Substrate concentration	Solvent
CYP1A2	Phenacetin	50 μM	Acetonitrile
CYP2A6	Coumarin	8 μM	Acetonitrile
CYP2C9	Tolbutamide	150 μM	Acetonitrile
CYP2C19	S-Mephenytoin	50 μM	Acetonitrile
CYP2D6	Dextromethorphan	5 μM	Water
CYP2E1	Chlorzoxazone	50 μM	Acetonitrile
CYP3A4	Testosterone	100 μM	Acetonitrile

Test Article Incubations

All incubations were conducted at 37 ± 1 °C in a shaking water bath. The sample size was N = 3 replicates for experimental groups.

Incubation mixtures were prepared in 0.1 M Tris buffer and contained Supersomes (10 pmol P450) and metaxalone (at each concentration). After a 5-minute pre-incubation, NADPH regenerating system (NRS) was added to the incubation mixtures to initiate reactions. The final incubation volume was 0.5 mL. Incubations were continued for 30 minutes, except for CYP2C19 incubations, which were incubated for 36 minutes.

Incubations were terminated by adding an equal volume of methanol. Samples were transferred to cryovials. Samples were stored at -70 °C \pm 10 °C prior to analysis.

Control Incubations

All incubations were conducted at 37 ± 1 °C in a shaking water bath. The sample size was $N = 3$ for all control groups.

Matrix Control

Matrix control samples were included to provide a source of background from matrix components. Incubation mixtures were prepared in 0.1 M Tris buffer and contained Supersomes (10 pmol P450) and 1% methanol. After a 5-minute pre-incubation, NRS was added to the incubation mixtures to initiate the reactions. The final incubation volume was 0.5 mL. Incubations were continued for 30 minutes, except for CYP2C19 incubations, which were incubated for 36 minutes.

Metabolic Negative Control

Metabolic negative control samples were included to distinguish potential non-enzymatic metabolism from P450-mediated metabolism of metaxalone. Incubation mixtures were prepared in 0.1 M Tris buffer and contained Supersomes (10 pmol P450) and metaxalone (at each concentration). After a 5-minute pre-incubation, 2% sodium bicarbonate solution was added to the incubation mixtures. The final incubation volume was 0.5 mL. Incubations were continued for 30 minutes.

Positive Control

Incubation mixtures were prepared in 0.1 M Tris buffer and contained Supersomes (10 pmol P450) and an isoform-selective substrate. After a 5-minute pre-incubation, NRS was added to the incubation mixtures to initiate reactions. The final incubation volume was 0.5 mL. CYP3A4 incubations were continued for 10 minutes. All other incubations were continued for 30 minutes.

The test system was considered metabolically active and the incubations acceptable since the measured concentration of the metabolites from the isoform-selective substrate was above the lower limit of quantitation.

Termination of Control Incubations

Matrix controls and metabolic negative controls were terminated by adding an equal volume of methanol. Samples were transferred to cryovials. Samples were stored at -70 °C \pm 10 °C prior to analysis.

Positive controls were terminated by adding an equal volume of methanol. Samples were transferred to cryovials. Samples were stored at -70 °C \pm 10 °C prior to analysis.

Analyses

In Vitro Technologies evaluated the test article-treated, matrix control, and metabolic negative control samples by monitoring metaxalone by HPLC.

In Vitro Technologies evaluated the positive control samples by measuring the formation of metabolites from CYP isoform-selective substrates using the following analytical procedures:

Phenacetin O-Deethylase (CYP1A2)

The activity of CYP1A2 was determined by measuring the formation of acetaminophen. Samples were analyzed using an LC/MS method.

Coumarin 7-Hydroxylase (CYP2A6)

The activity of CYP2A6 was determined by measuring the formation of 7-hydroxycoumarin. Samples were analyzed using an HPLC method.

Tolbutamide 4'-Methyl Hydroxylase (CYP2C9)

The activity of CYP2C9 was determined by measuring the formation of 4'-methylhydroxytolbutamide. Samples were analyzed using an LC/MS method.

S-Mephenytoin 4'-Hydroxylase (CYP2C19)

The activity of CYP2C19 was determined by measuring the formation of 4'-hydroxymephenytoin. Samples were analyzed using an LC/MS method.

Dextromethorphan O-Demethylase (CYP2D6)

The activity of CYP2D6 was determined by measuring the formation of dextrorphan. Samples were analyzed using an LC/MS method.

Chlorzoxazone 6-Hydroxylase (CYP2E1)

The activity of CYP2E1 was determined by measuring the formation of its metabolite, 6-hydroxychlorzoxazone. Samples were analyzed using an LC/MS method.

Testosterone 6 β -Hydroxylase (CYP3A4)

The activity of CYP3A4 was determined by measuring the formation of 6 β -hydroxytestosterone. Samples were analyzed using an HPLC-UV method.

Description of Data Calculations

The disappearance of metaxalone is reported. The data are expressed as mean \pm standard deviation.

Criteria for Data Acceptance

Bioanalytical data were accepted in accordance with the In Vitro Technologies standard operating procedure(s) on bioanalytical data acceptance.

Results

Metabolic Positive Control

Various levels of activity for each CYP isoform tested are presented in Table 1. The amount of metabolite formed from the CYP isoform-selective substrates is above the lower limit of quantitation for the assay (for CYP3A4, the amount of the selective metabolite from one of the three replicates is 0.09772 μ M, which is below the lower limit of quantitation of 0.1 μ M). Therefore, the incubations met the criterion set in the protocol.

Metaxalone Incubation with Supersome™

CYP1A2

Disappearance of metaxalone was detected following incubation with CYP1A2 in the presence of NADPH-regenerating system. Disappearance of metaxalone ranged from 10.1% to 19.6% (Table 2). Results indicate that CYP1A2 is involved in the metabolism of metaxalone.

CYP2A6

No metaxalone disappearance was detected following incubation with metaxalone at the concentrations tested when compared with respective metabolic negative controls (Table 3). Results indicate that CYP2A6 is not involved in the metabolism of metaxalone.

CYP2C9

No metaxalone disappearance was detected following incubation with metaxalone at the concentrations tested when compared with respective metabolic negative controls (Table 4). Results indicate that CYP2C9 is not involved in the metabolism of metaxalone.

CYP2C19

Metaxalone disappearance was evident following incubation with metaxalone at the concentration of 0.5 μ M (Table 5). However, the mean disappearance was only 6.6%. The apparent disappearance of metaxalone at the concentrations of 2.5 and 25 μ M was not statistically significant ($p > 0.05$). Results indicate that CYP2C19, though with less extent, is also involved in the metabolism of metaxalone.

CYP2D6

No metaxalone disappearance was detected following incubation with metaxalone at the concentrations tested when compared with respective metabolic negative controls (Table 6). Results indicate that CYP2D6 is not involved in the metabolism of metaxalone.

CYP2E1

No metaxalone disappearance was detected following incubation with metaxalone at the concentrations tested when compared with respective metabolic negative controls (Table 7). Results indicate that CYP2E1 is not involved in the metabolism of metaxalone.

CYP3A4

No metaxalone disappearance was detected following incubation with metaxalone at the concentrations tested when compared with respective metabolic negative controls (Table 8). Results indicate that CYP3A4 is not involved in the metabolism of metaxalone.

Matrix Control

The analytical method did not detect interference from matrix containing CYP1A2, CYP2A6, CYP2C9, CYP2D6, CYP2E1, or CYP3A4 (Tables 2, 3, 4, 6, 7, and 8). No interference was detected from two of three matrix control samples containing CYP2C19 (Table 5).

Conclusions

Metaxalone was metabolized by CYP1A2. It was also metabolized by CYP2C19, although to a lesser extent.

References

1. Spatzenegger, M.; Jaeger, W. Clinical importance of hepatic cytochrome P450 in drug metabolism. *Drug Metab. Rev.* **1995**, *27*, 397-417.
2. Clarke, S.E. In vitro assessment of human cytochrome P450. *Xenobiotica.* **1998**, *28*, 1167-1202.
3. Rodrigues A. D. Integrated cytochrome P450 reaction phenotyping: Attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. *Biochem. Pharmacol.* **1999**, *57*, 465-480.

Table 1: Metabolic Positive Control in Recombinant Human Cytochrome P450 Enzymes (Supersomes™)

Control Identification	Substrate Identification	Conc. (µM)	Time (Minutes)	Metabolite Formation			Specific Activity (pmol metabolite/min/pmol protein)	
				Raw (µM)	Adjusted (µM)		Individual	Mean ± SD
Human CYP1A2 + P450 Reductase Supersomes™ - Metabolite: Acetaminophen								
PC-1A2	Phenacetin	50	30	3.67330	3.67	3.73 ± 0.0858	12.2	12.4 ± 0.286
				3.68786	3.69		12.3	
				3.82874	3.83		12.8	
Human CYP2A6 + OR + Cytochrome b₅ Supersomes™ - Metabolite: 7-Hydroxycoumarin								
PC-2A6	Coumarin	8	30	1.92343	1.92	1.98 ± 0.0501	6.41	6.59 ± 0.167
				1.98206	1.98		6.61	
				2.02303	2.02		6.74	
Human CYP2C9*1 + P450 Reductase + Cytochrome b₅ Supersomes™ - Metabolite: 4'-OH Methyl Tolbutamide								
PC-2C9	Tolbutamide	150	30	0.70932	0.709	0.735 ± 0.0255	2.36	2.45 ± 0.0849
				0.73401	0.734		2.45	
				0.76023	0.760		2.53	
Human CYP2C19 + P450 Reductase + Cytochrome b₅ Supersomes™ - Metabolite: 4'-OH Mephenytoin								
PC-2C19	S-Mephenytoin	50	30	2.84183	2.84	2.91 ± 0.170	9.47	9.71 ± 0.566
				2.79046	2.79		9.30	
				3.10696	3.11		10.4	
Human CYP2D6*1 + P450 Reductase Supersomes™ - Metabolite: Dextrorphan								
PC-2D6	Dextromethorphan	5	30	1.24397	1.24	1.21 ± 0.0275	4.15	4.04 ± 0.0916
				1.20223	1.20		4.01	
				1.19213	1.19		3.97	

Table 1 (continued): Metabolic Positive Control in Recombinant Human Cytochrome P450 Enzymes (Supersomes™)

Control Identification	Substrate Identification	Conc. (µM)	Time (Minutes)	Metabolite Formation		Specific Activity (pmol metabolite/min/pmol protein)		
				Raw (µM)	Adjusted (µM)	Individual	Mean ± SD	
Human CYP2E1 + P450 Reductase + Cytochrome b₅ Supersomes™ - Metabolite: 6-OH Chlorzoxazone								
PC-2E1	Chlorzoxazone	50	30	0.62863	0.629	0.690 ± 0.107	2.10	2.30 ± 0.358
				0.62674	0.627		2.09	
				0.81366	0.814		2.71	
Human CYP3A4 + P450 Reductase Supersomes™ - Metabolite: 6β-OH Testosterone								
PC-3A4	Testosterone	100	10	0.10537	0.105	<0.102 ± 0.00300	1.05	<1.02 ± 0.0300
				0.10038	0.100		1.00	
				0.09772 ^a	<0.100		<1.00	

Abbreviations: Conc., concentration; SD, standard deviation; PC, positive control; Min, minute

^aThe observed analyzed value (µM) is below the lowest concentration on the standard curve (0.1 µM)

Note: For all calculations above, the resulting values are shown with at least three significant figures for display purposes only.

Table 2: Metabolism of Metaxalone in Expressed Recombinant Human Cytochrome P450 Enzymes (Human CYP1A2 + P450 Reductase SupersomesTM)

Metaxalone Concentration (μM)	Metaxalone Present			Percent of Metabolic Negative Control	
	Raw (μM)	Adjusted (μM)		Individual	Mean \pm SD
		Individual	Mean \pm SD		
MNC (0.5)	0.20195	0.404	0.391 \pm 0.0113	103	100 \pm 2.88
	0.19430	0.389		99.3	
	0.19097	0.382		97.6	
0.5	0.15087	0.302	0.352 \pm 0.0761	77.1	89.9 \pm 19.4
	0.21975	0.440		112	
	0.15734	0.315		80.4	
MNC (2.5)	0.65183	1.30	1.33 \pm 0.0221	98.3	100 \pm 1.67
	0.66350	1.33		100	
	0.67394	1.35		102	
2.5	0.52700	1.05	1.07 \pm 0.0167	79.5	80.4 \pm 1.26
	0.52908	1.06		79.8	
	0.54235	1.08		81.8	
MNC (25)	10.11453	20.2	19.8 \pm 0.360	102	100 \pm 1.82
	9.76568	19.5		98.5	
	9.86156	19.7		99.5	
25	8.20521	16.4	16.6 \pm 0.337	82.8	83.7 \pm 1.70
	8.19232	16.4		82.6	
	8.49030	17.0		85.6	
MXC (0)	0.00000 ^a	N/A	N/A \pm N/A	N/A	N/A \pm N/A
	0.00000 ^a	N/A		N/A	
	0.00000 ^a	N/A		N/A	

Abbreviations: SD, standard deviation; MNC, metabolic negative control; MXC, matrix control; N/A, not applicable

^a The Raw value (μM) was below the lowest concentration on the standard curve (0.05 μM)

Note: For all calculations above, the resulting values are shown with at least three significant figures for display purposes only.

Table 3: Metabolism of Metaxalone in Expressed Recombinant Human Cytochrome P450 Enzymes (Human CYP2A6 + P450 Reductase + Cytochrome b₅ Supersomes™)

Metaxalone Concentration (μM)	Metaxalone Present			Percent of Metabolic Negative Control	
	Raw (μM)	Adjusted (μM)		Individual	Mean \pm SD
		Individual	Mean \pm SD		
MNC (0.5)	0.15455	0.309	0.311 \pm 0.00446	99.4	100 \pm 1.43
	0.15795	0.316		102	
	0.15375	0.308		98.9	
0.5	0.15457	0.309	0.299 \pm 0.0124	99.5	96.1 \pm 3.99
	0.15112	0.302		97.2	
	0.14253	0.285		91.7	
MNC (2.5)	0.74261	1.49	1.52 \pm 0.0353	97.9	100 \pm 2.33
	0.75568	1.51		99.6	
	0.77755	1.56		102	
2.5	0.79130	1.58	1.61 \pm 0.0373	104	106 \pm 2.46
	0.79791	1.60		105	
	0.82642	1.65		109	
MNC (25)	7.74594	15.5	15.3 \pm 0.147	101	100 \pm 0.959
	7.64948	15.3		99.8	
	7.60163	15.2		99.2	
25	7.76399	15.5	15.6 \pm 0.0975	101	102 \pm 0.636
	7.85044	15.7		102	
	7.84628	15.7		102	
MXC (0)	0.00000 ^a	N/A	N/A \pm N/A	N/A	N/A \pm N/A
	0.00000 ^a	N/A		N/A	
	0.00000 ^a	N/A		N/A	

Abbreviations: SD, standard deviation; MNC, metabolic negative control; MXC, matrix control; N/A, not applicable

^a The Raw value (μM) was below the lowest concentration on the standard curve (0.05 μM)

Note: For all calculations above, the resulting values are shown with at least three significant figures for display purposes only.

Table 4: Metabolism of Metaxalone in Expressed Recombinant Human Cytochrome P450 Enzymes (Human CYP2C9*1 (Arg144) + P450 Reductase + Cytochrome b₅ Supersomes™)

Metaxalone Concentration (µM)	Metaxalone Present			Percent of Metabolic Negative Control	
	Raw (µM)	Adjusted (µM)		Individual	Mean ± SD
		Individual	Mean ± SD		
MNC (0.5)	0.17052	0.341	0.348 ± 0.00997	97.9	100 ± 2.86
	0.17229	0.345		98.9	
	0.17990	0.360		103	
0.5	0.18004	0.360	0.355 ± 0.00608	103	102 ± 1.75
	0.17784	0.356		102	
	0.17403	0.348		99.9	
MNC (2.5)	0.93197	1.86	1.93 ± 0.0605	96.8	100 ± 3.14
	0.96526	1.93		100	
	0.99235	1.98		103	
2.5	0.96842	1.94	1.92 ± 0.0246	101	99.7 ± 1.28
	0.96593	1.93		100	
	0.94597	1.89		98.2	
MNC (25)	10.31249	20.6	21.3 ± 0.620	97.1	100 ± 2.92
	10.63201	21.3		100	
	10.93245	21.9		103	
25	10.66111	21.3	21.5 ± 0.144	100	101 ± 0.675
	10.80454	21.6		102	
	10.72836	21.5		101	
MXC (0)	0.00000 *	N/A	N/A ± N/A	N/A	N/A ± N/A
	0.00000 *	N/A		N/A	
	0.00000 *	N/A		N/A	

Abbreviations: SD, standard deviation; MNC, metabolic negative control; MXC, matrix control; N/A, not applicable

* The Raw value (µM) was below the lowest concentration on the standard curve (0.05 µM)

Note: For all calculations above, the resulting values are shown with at least three significant figures for display purposes only.

Table 5: Metabolism of Metaxalone in Expressed Recombinant Human Cytochrome P450 Enzymes (Human CYP2C19 + P450 Reductase + Cytochrome b₅ Supersomes™)

Metaxalone Concentration (µM)	Raw (µM)	Metaxalone Present		Percent of Metabolic Negative Control	
		Adjusted (µM)		Individual	Mean ± SD
		Individual	Mean ± SD		
MNC (0.5)	0.18718	0.374	0.370 ± 0.00898	101	100 ± 2.43
	0.18763	0.375		102	
	0.17964	0.359		97.2	
0.5	0.16773	0.335	0.345 ± 0.0104	90.8	93.4 ± 2.82
	0.17180	0.344		93.0	
	0.17808	0.356		96.4	
MNC (2.5)	0.72720	1.45	1.39 ± 0.0560	105	100 ± 4.03
	0.67562	1.35		97.2	
	0.68261	1.37		98.2	
2.5	0.67218	1.34	1.34 ± 0.00561	96.7	96.5 ± 0.404
	0.67254	1.35		96.7	
	0.66751	1.34		96.0	
MNC (25)	9.84488	19.7	20.1 ± 1.03	97.8	100 ± 5.13
	9.69255	19.4		96.3	
	10.65287	21.3		106	
25	9.34508	18.7	18.6 ± 0.120	92.9	92.6 ± 0.597
	9.35948	18.7		93.0	
	9.24903	18.5		91.9	
MXC (0)	0.00000 ^a	N/A	N/A ± N/A	N/A	N/A ± N/A
	0.00000 ^a	N/A		N/A	
	0.06454	N/A		N/A	

Abbreviations: SD, standard deviation; MNC, metabolic negative control; MXC, matrix control; N/A, not applicable

^a The Raw value (µM) was below the lowest concentration on the standard curve (0.05 µM)

Note: For all calculations above, the resulting values are shown with at least three significant figures for display purposes only.

Table 6: Metabolism of Metaxalone in Expressed Recombinant Human Cytochrome P450 Enzymes (Human CYP2D6*1 + P450 Reductase Supersomes™)

Metaxalone Concentration (μM)	Metaxalone Present			Percent of Metabolic Negative Control	
	Raw (μM)	Adjusted (μM)		Individual	Mean \pm SD
		Individual	Mean \pm SD		
MNC (0.5)	0.14509	0.290	0.292 \pm 0.00220	99.4	100 \pm 0.755
	0.14716	0.294		101	
	0.14547	0.291		99.7	
0.5	0.18683	0.374	0.319 \pm 0.0477	128	109 \pm 16.3
	0.14857	0.297		102	
	0.14305	0.286		98.0	
MNC (2.5)	0.79025	1.58	1.56 \pm 0.0184	101	100 \pm 1.18
	0.78433	1.57		100	
	0.77221	1.54		98.7	
2.5	0.75826	1.52	1.53 \pm 0.0111	96.9	97.7 \pm 0.707
	0.76852	1.54		98.2	
	0.76697	1.53		98.0	
MNC (25)	9.63762	19.3	19.2 \pm 0.0994	100	100 \pm 0.517
	9.54788	19.1		99.4	
	9.62976	19.3		100	
25	9.52577	19.1	19.2 \pm 0.436	99.2	99.9 \pm 2.27
	9.84529	19.7		103	
	9.42917	18.9		98.2	
MXC (0)	0.00000 ^a	N/A	N/A \pm N/A	N/A	N/A \pm N/A
	0.00000 ^a	N/A		N/A	
	0.00000 ^a	N/A		N/A	

Abbreviations: SD, standard deviation; MNC, metabolic negative control; MXC, matrix control; N/A, not applicable

^a The Raw value (μM) was below the lowest concentration on the standard curve (0.05 μM)

Note: For all calculations above, the resulting values are shown with at least three significant figures for display purposes only.

Table 7: Metabolism of Metaxalone in Expressed Recombinant Human Cytochrome P450 Enzymes (Human CYP2E1 + P450 Reductase + Cytochrome b₅ Supersomes™)

Metaxalone Concentration (μM)	Metaxalone Present			Percent of Metabolic Negative Control	
	Raw (μM)	Adjusted (μM)		Individual	Mean \pm SD
		Individual	Mean \pm SD		
MNC (0.5)	0.18358	0.367	0.355 \pm 0.0104	103	100 \pm 2.92
	0.17510	0.350		98.6	
	0.17416	0.348		98.1	
0.5	0.17871	0.357	0.352 \pm 0.00648	101	99.0 \pm 1.83
	0.17235	0.345		97.0	
	0.17662	0.353		99.4	
MNC (2.5)	0.89075	1.78	1.69 \pm 0.117	105	100 \pm 6.89
	0.77998	1.56		92.2	
	0.86695	1.73		102	
2.5	0.88299	1.77	1.76 \pm 0.00318	104	104 \pm 0.188
	0.87990	1.76		104	
	0.88209	1.76		104	
MNC (25)	9.11125	18.2	17.8 \pm 0.410	103	100 \pm 2.30
	8.70811	17.4		98.0	
	8.84728	17.7		99.5	
25	8.73183	17.5	19.2 \pm 2.71	98.2	108 \pm 15.3
	11.15149	22.3		125	
	8.87878	17.8		99.9	
MXC (0)	0.00000 ^a	N/A	N/A \pm N/A	N/A	N/A \pm N/A
	0.00000 ^a	N/A		N/A	
	0.00000 ^a	N/A		N/A	

Abbreviations: SD, standard deviation; MNC, metabolic negative control; MXC, matrix control; N/A, not applicable

^a The Raw value (μM) was below the lowest concentration on the standard curve (0.05 μM)

Note: For all calculations above, the resulting values are shown with at least three significant figures for display purposes only.

Table 8: Metabolism of Metaxalone in Expressed Recombinant Human Cytochrome P450 Enzymes (Human CYP3A4 + P450 Reductase Supersomes™)

Metaxalone Concentration (μM)	Metaxalone Present			Percent of Metabolic Negative Control	
	Raw (μM)	Adjusted (μM)		Individual	Mean \pm SD
		Individual	Mean \pm SD		
MNC (0.5)	0.16014	0.320	0.318 \pm 0.00502	101	100 \pm 1.58
	0.15592	0.312		98.2	
	0.16039	0.321		101	
0.5	0.15978	0.320	0.320 \pm 0.00333	101	101 \pm 1.05
	0.16159	0.323		102	
	0.15826	0.317		99.6	
MNC (2.5)	0.85285	1.71	1.72 \pm 0.0127	99.3	100 \pm 0.741
	0.86553	1.73		101	
	0.85828	1.72		99.9	
2.5	0.85730	1.71	1.68 \pm 0.0289	99.8	98.0 \pm 1.68
	0.82923	1.66		96.5	
	0.83738	1.67		97.5	
MNC (25)	8.65154	17.3	17.4 \pm 0.0906	99.4	100 \pm 0.521
	8.71767	17.4		100	
	8.73830	17.5		100	
25	8.53809	17.1	17.1 \pm 0.192	98.1	98.1 \pm 1.10
	8.44686	16.9		97.1	
	8.63905	17.3		99.3	
MXC (0)	0.00000 ^a	N/A	N/A \pm N/A	N/A	N/A \pm N/A
	0.00000 ^a	N/A		N/A	
	0.00000 ^a	N/A		N/A	

Abbreviations: SD, standard deviation; MNC, metabolic negative control; MXC, matrix control; N/A, not applicable

^a The Raw value (μM) was below the lowest concentration on the standard curve (0.05 μM)

Note: For all calculations above, the resulting values are shown with at least three significant figures for display purposes only.

Appendix 1: In Vitro Technologies Protocol No. 1178

In Vitro Technologies, Inc.
Protocol No. 1178
Version: Final (28 September 2005)

**Determination of Metaxalone Metabolism in Expressed
Recombinant Human Enzymes CYP1A2, CYP2A6, CYP2C9,
CYP2C19, CYP2D6, CYP2E1, and CYP3A4**

Sponsor: Mutual Pharmaceutical Company
United Research Laboratories, Inc.
1100 Orthodox Street
Philadelphia, PA 19124
Tel.: (215) 807-1076
Fax: (215) 807-1083

Sponsor Representative: Jie Du, Ph.D.
Email: jie_du@URLMutual.com

Testing Facility: In Vitro Technologies, Inc.
1450 South Rolling Road
Baltimore, MD 21227
Tel.: (410) 455-1242
Fax: (410) 455-1245

Study Director: Genfu Chen, Ph.D.
E-mail: cheng@invitrotech.com

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INC. AND MUTUAL PHARMACEUTICAL COMPANY

Objective

The objective of this study is to determine the metabolism of metaxalone in Supersomes™ containing one of the cytochrome P450 (CYP) isoforms CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4.

Test Article Information

The test article will be identified in this study as follows:

- Metaxalone (molecular weight = 221 g/mol)

Mutual Pharmaceutical Company will provide metaxalone and will be responsible for the derivation, characterization, retention, and stability testing of metaxalone. Additionally, Mutual Pharmaceutical will be responsible for providing In Vitro Technologies with detailed information regarding handling and storage requirements, diluents or cosolubilizers, and safety hazards and precautions (Material Safety Data Sheet or other documentation) for metaxalone, before or upon initiation of this study.

Test System Identification

The test system that will be used in this study is microsomes from baculovirus-infected insect cells (BD Supersomes™ Enzymes) containing single expressed recombinant human CYP enzymes obtained from BD Biosciences Discovery Labware (Woburn, MA).

Test System Justification

The liver represents the major organ for drug metabolism and contains the CYP enzymes, the major enzyme systems for xenobiotic metabolism (1). The identification of human CYP enzymes involved in drug metabolism allows the prediction of potential drug-drug interactions such as co-administration with isoform-selective inhibitors or inducers, and interpatient variability in drug exposure. Various approaches, including inhibition studies with specific chemical inhibitors and inhibitory antibodies, correlation studies, and studies using heterologously expressed CYP isoforms, have been applied to identify human enzymes involved in drug metabolism (2,3).

In this study, individual CYP isoforms expressed from human cDNA and P450 reductase cDNA (Supersomes) will be used. Supersomes provide a readily available and well-characterized biological model for use in identifying human CYP enzymes involved in drug metabolism.

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Description of Study

Supersomes containing expressed human CYP isoforms will be incubated in the presence of metaxalone. The metabolism of metaxalone will be evaluated by measuring the disappearance of metaxalone by high-performance liquid chromatography (HPLC) or liquid chromatography/mass spectrometry (LC/MS).

Experimental Methods

Test Article Preparation

Metaxalone stock solutions will be prepared in methanol at 100 times (100X) the final concentration. The stock solutions will be added to incubation mixtures to obtain the final concentrations of 0.5, 2.5, and 25 μM ¹, each containing 1% methanol. Modifications in test article preparation, which pertain to changes in solvent used or changes in dosing concentrations, may be made with the approval of the Study Director. These modifications and their rationale will be communicated to Mutual Pharmaceutical and will be described in the study report.

Substrates for the CYP isoforms

Positive controls will be incubated with the following CYP450 isoform-selective substrates. Each substrate will be prepared as 100X stock solutions in the solvent listed below. The final substrate concentrations and solvents will be:

CYP isoform	Isoform-selective substrate	Substrate concentration	Solvent
CYP1A2	Phenacetin	50 μM	ACN
CYP2A6	Coumarin	8 μM	ACN
CYP2C9	Tolbutamide	150 μM	ACN
CYP2C19	S-Mephenytoin	50 μM	ACN
CYP2D6	Dextromethorphan	5 μM	Water
CYP2E1	Chlorzoxazone	50 μM	ACN
CYP3A4	Testosterone	100 μM	ACN

Test Article Incubations

All incubations will be conducted at 37 ± 1 °C in a shaking water bath. The sample size will be N = 3 replicates for experimental groups.

Incubation mixtures will be prepared in 0.1 M Tris buffer and will contain Supersomes (10 pmol P450) and metaxalone (at each concentration). After a 5-minute pre-incubation, NADPH regenerating system (NRS) will be added to the incubation mixtures to initiate reactions. The final incubation volume will be 0.5 mL. Incubations will be continued for 30 minutes.

¹ The concentrations in ng/mL would be 110.5, 552.5, and 5,525 ng/mL.

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Incubations will be terminated by adding an equal volume of methanol. Samples will be transferred to cryovials. If analysis does not occur immediately after incubation, samples will be stored at $-70\text{ }^{\circ}\text{C} \pm 10\text{ }^{\circ}\text{C}$.

Control Incubations

All incubations will be conducted at $37 \pm 1\text{ }^{\circ}\text{C}$ in a shaking water bath. The sample size will be $N = 3$ for all control groups.

Matrix Control

Matrix control samples will be included to provide a source of background from matrix components. Incubation mixtures will be prepared in 0.1 M Tris buffer and will contain Supersomes (10 pmol P450) and 1% methanol. After a 5-minute pre-incubation, NRS will be added to the incubation mixtures to initiate the reactions. The final incubation volume will be 0.5 mL. Incubations will be continued for 30 minutes.

Metabolic Negative Control

Metabolic negative control samples will be included to distinguish potential non-enzymatic metabolism from P450-mediated metabolism of metaxalone. Incubation mixtures will be prepared in 0.1 M Tris buffer and will contain Supersomes (10 pmol P450) and metaxalone (at each concentration). After a 5-minute pre-incubation, 2% sodium bicarbonate solution will be added to the incubation mixtures. The final incubation volume will be 0.5 mL. Incubations will be continued for 30 minutes.

Positive Control

Incubation mixtures will be prepared in 0.1 M Tris buffer and will contain Supersomes (10 pmol P450) and an isoform-selective substrate. After a 5-minute pre-incubation, NRS will be added to the incubation mixtures to initiate reactions. The final incubation volume will be 0.5 mL. CYP3A4 incubations will be continued for 10 minutes. All other incubations will be continued for 30 minutes.

The test system will be considered metabolically active and the incubations acceptable if the measured concentration of the metabolites from the isoform-selective substrate is above the lower limit of quantitation. If this criterion is not met, then the study will be repeated.

Termination of Control Incubations

Matrix controls and metabolic negative controls will be terminated by adding an equal volume of methanol. Samples will be transferred to cryovials. If analysis does not occur immediately after incubation, samples will be stored at $-70\text{ }^{\circ}\text{C} \pm 10\text{ }^{\circ}\text{C}$.

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Positive controls will be terminated by adding an equal volume of methanol. Samples will be transferred to cryovials. If analysis does not occur immediately after incubation, samples will be stored at $-70\text{ }^{\circ}\text{C} \pm 10\text{ }^{\circ}\text{C}$.

Analyses

In Vitro Technologies will evaluate the test article-treated, matrix control, and metabolic negative control samples by monitoring metaxalone by HPLC or LC/MS.

In Vitro Technologies will evaluate the positive control samples by measuring the formation of metabolites from CYP isoform-selective substrates using the following analytical procedures:

Phenacetin O-Deethylase (CYP1A2)

The activity of CYP1A2 will be determined by measuring the formation of acetaminophen. Samples will be analyzed using an LC/MS method.

Coumarin 7-Hydroxylase (CYP2A6)

The activity of CYP2A6 will be determined by measuring the formation of 7-hydroxycoumarin. Samples will be analyzed using an HPLC method.

Tolbutamide 4'-Methyl Hydroxylase (CYP2C9)

The activity of CYP2C9 will be determined by measuring the formation of 4'-methylhydroxytolbutamide. Samples will be analyzed using an LC/MS method.

S-Mephenytoin 4'-Hydroxylase (CYP2C19)

The activity of CYP2C19 will be determined by measuring the formation of 4'-hydroxymephenytoin. Samples will be analyzed using an LC/MS method.

Dextromethorphan O-Demethylase (CYP2D6)

The activity of CYP2D6 will be determined by measuring the formation of dextrophan. Samples will be analyzed using an LC/MS method.

Chlorzoxazone 6-Hydroxylase (CYP2E1)

The activity of CYP2E1 will be determined by measuring the formation of its metabolite, 6-hydroxychlorzoxazone. Samples will be analyzed using an LC/MS method.

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Testosterone 6 β -Hydroxylase (CYP3A4)

The activity of CYP3A4 will be determined by measuring the formation of 6 β -hydroxytestosterone. Samples will be analyzed using an HPLC-UV method.

Description of Data Calculations

The disappearance of metaxalone will be reported. The data will be expressed as mean \pm standard deviation.

Criteria for Data Acceptance

Bioanalytical data will be accepted in accordance with the In Vitro Technologies standard operating procedure(s) on bioanalytical data acceptance.

Study Report

A copy of the final study report will be issued to Mutual Pharmaceutical and will include, but not be limited to, the following information:

- Participating Personnel
- Study Dates and Data Retention
- Statement of Compliance
- Quality Assurance Statement
- Summary
- Introduction
- Experimental Methods
- Results
- Conclusions
- Description of Data Calculations
- Copy of study protocol

Data Retention

In Vitro Technologies will retain all supporting documentation, including raw data and written records, for a period of up to five years following issuance of the final report. At the end of this period, Mutual Pharmaceutical will be notified to determine whether the data (excluding proprietary information) will be transferred, retained, or destroyed.

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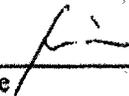
Protocol Approval

Sponsor Approval

This protocol has been reviewed and approved by the following:

Jie Du, Ph.D.

Sponsor Representative
Mutual Pharmaceutical Company

Signature 

Date

9/28/05

Study Director Review

This study will be conducted using good documentation practices and using equipment that is properly maintained and calibrated in accordance with In Vitro Technologies standard operating procedures. The study will be conducted under my scientific guidance and management. I have reviewed the procedures outlined in this protocol.

Genfu Chen, Ph.D.

Study Director
In Vitro Technologies

Signature 

Date

29 Sept 2005

References

1. Spatzenegger, M.; Jaeger, W. Clinical importance of hepatic cytochrome P450 in drug metabolism. *Drug Metab. Rev.* 1995, 27, 397-417.
2. Clarke, S.E. In vitro assessment of human cytochrome P450. *Xenobiotica.* 1998, 28, 1167-1202.
3. Rodrigues A. D. Integrated cytochrome P450 reaction phenotyping: Attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. *Biochem. Pharmacol.* 1999, 57, 465-480.

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Appendix 2: Protocol Deviation

CYP2C19 incubations were incubated for 36 minutes instead of the 30 minute incubation period specified in the protocol. There was no impact since data were expressed as percent of metabolic negative control (MNC), and both MNC and test article metabolism samples were incubated for the same period of time.