



**ATTACHMENT A.1**  
**METABOLISM STUDY: PROTOCOL**

**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**

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## 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  $\mu\text{M}$ <sup>1</sup>, 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 $\mu\text{M}$	ACN
CYP2A6	Coumarin	8 $\mu\text{M}$	ACN
CYP2C9	Tolbutamide	150 $\mu\text{M}$	ACN
CYP2C19	S-Mephenytoin	50 $\mu\text{M}$	ACN
CYP2D6	Dextromethorphan	5 $\mu\text{M}$	Water
CYP2E1	Chlorzoxazone	50 $\mu\text{M}$	ACN
CYP3A4	Testosterone	100 $\mu\text{M}$	ACN

### Test Article Incubations

All incubations will be conducted at  $37 \pm 1$  °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.

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<sup>1</sup> 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 $\beta$ -Hydroxylase (CYP3A4)**

The activity of CYP3A4 will be determined by measuring the formation of 6 $\beta$ -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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