Effect of Dimethyl Sulfoxide on In Vitro Cytochrome P4501A2 Mediated Phenacetin-O-Deethylation in Human Liver Microsomes

Ramakrishna Nirogi*, Vishwottam Kandikere, Gopinadh Bhyrapuneni, Ranjith Kumar Ponnamaneni, Raghava choudary Palacharla and Arunkumar Manoharan
Pharmacokinetics and Drug Metabolism, Discovery Research,
Suven Life Sciences Ltd, Serene Chambers,
Road-5, Avenue-7, Banjara Hills, Hyderabad 500034, India.
Running Title: Effect of dimethyl sulfoxide on Phenacetin-O-deethylation

Address for Correspondence:

Suven Life Sciences Ltd

Serene Chambers, Road -5, Avenue 7,

Banjara Hills, Hyderabad – 500 034, India.

E-mail: ramakrishna_nirogi@yahoo.co.in

Number of

Text Pages: 12

Figures: 2

References: 5

Words in Abstract: 170

Words in Introduction: 277

Words in Results and Discussion: 413

Abbreviations: P450, cytochrome P450; DMSO, Dimethyl sulfoxide;
ABSTRACT

In this study, we report the effect of dimethyl sulfoxide (DMSO), acetonitrile, and methanol on the cytochrome P4501A2 (CYP1A2) mediated metabolism of phenacetin in human liver microsomes (HLM). Phenacetin O-deethylation is the preferred probe reaction for CYP1A2 where the metabolite, acetaminophen, is quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS). DMSO was found to inhibit CYP1A2 mediated phenacetin O-deethylation even at low concentrations (0.1%). Acetonitrile did not significantly change the phenacetin O-deethylation activity at concentrations up to 2%. There was no effect on the phenacetin O-deethylation when methanol was present at levels up to 2%. It was found that the DMSO level should be kept lower than 0.05% because, a concentration of 0.1% strongly affected the metabolism of phenacetin. These findings should be taken into consideration when designing in vitro metabolism studies, especially studies where metabolism of the investigational compound need to be evaluated, which would confound the results. The findings from this study indicate that methanol is the suitable solvent with no significant effects on CYP1A2 mediated phenacetin O-deethylation.
INTRODUCTION
Cytochrome P450 (P450) enzymes play an important role in the metabolism of xenobiotics. The effects of new drugs on probe reactions specific for human drug metabolizing enzymes are examined using in vitro approaches and these approaches use human liver microsomes routinely. Cytochrome P4501A2 (CYP1A2) is one of the major enzymes expressed in liver with significance in metabolism. CYP1A2 constitutes about 15% of the total microsomal P450 content in human liver (Lee et al., 2003). Phenacetin O-deethylation and caffeine N3-demethylation are the two recommended probe reactions for detecting CYP1A2 based drug interaction potential in vitro. However, phenacetin is preferred over caffeine as caffeine N3- demethylation is sensitive to solvent effects (Yuan et al., 2002). Substrates and inhibitors used in metabolism studies need to be dissolved in organic solvents because of solubility problem in physiological buffers employed in these studies.
Organic solvents can inhibit or stimulate the activity of P450 enzymes involved in the biotransformation of xenobiotics. It is always necessary to evaluate the potential effects of organic solvents for in vitro metabolism studies, especially when a new chemical entity is being evaluated. Numerous reports were published on the impact of organic solvents on phenacetin O-deethylation activities in human liver microsomes (Chauret et al., 1998; Hickman et al., 1998; Busby et al., 1999). Chauret et al., 1998 found that phenacetin O-deethylation is not sensitive to DMSO, acetonitrile and methanol. Hickman et al., (1999) found that phenacetin O-deethylation is sensitive to methanol and DMSO at a concentration of 1%.
In this communication, we report new findings on inhibitory effects of DMSO, acetonitrile and methanol on the hepatic activity of phenacetin O-deethylation mediated by CYP1A2 in human liver microsomes.

MATERIALS AND METHODS

Materials

Human liver microsomes were obtained from Xenotech, LLC, USA. β-Nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), acetaminophen, phenacetin and 4-hydroxy butyranilide were purchased from Sigma (St Louis, MO, USA). All other reagents were of the highest purity commercially available or analytical grade.

Effect of organic solvents on Phenacetin O-deethylation assay

All incubations were performed under conditions shown to be linear with respect to time, protein and substrate concentration. Phenacetin stock solution was prepared in water. Incubations were performed with 0.025 mg/mL of microsomal protein in phosphate buffer (100 mM, pH 7.4) containing 50 µM of phenacetin in a final volume of 0.2 mL. All incubations were performed in triplicates.

An appropriate volume of organic solvent (DMSO, acetonitrile and methanol) was added to achieve the desired percentage (0.2, 0.5, 1 & 2%) of organic content and control incubations contained no organic solvent. An aliquot (0.18 mL) of the incubation mixture was transferred to a reaction plate. Reactions were commenced with the addition of NADPH (1 mM) to final incubation volume of 0.2 mL and maintained at 37 °C for 18 min. The reaction mixtures were quenched with 1% formic acid: acetonitrile (70:30 v/v) containing 4-hydroxy butyranilide (1.08 µM) as internal standard (IS). The plates were
then centrifuged at 4000 g for 10 min at 4 °C. An aliquot of the supernatant fraction was mixed with 0.1% formic acid in water, and an aliquot of the mixture was analyzed by LC-MS/MS.

**LC-MS/MS Analysis**

The HPLC system (Shimadzu Corporation, Kyoto, Japan) is equipped with two LC-20AD prominence pumps, a SIL-HTc auto sampler unit, CTO-10AS VP thermo stated column oven and DGU2OA3 degasser unit. Analysis was carried out with API-4000 Qtrap mass spectrometer (Applied Biosystems MDS Sciex, Toronto, Canada) coupled with turbo-spray ionization source. The chromatography was performed using symmetry C8, 3.5 µm, 4.6 x 100 mm ID column. Acetaminophen and internal standard (4-hydroxy butyranilide) were eluted with a mobile phase system consisting of water: acetonitrile: formic acid (80:20:0.1% v/v and 20:80:0.1% v/v for Mobile phase A and B respectively). A constant flow of 0.750 mL/min was employed with a gradient mobile phase system. Mobile phase-B 10% was pumped for 0.8 min, and then increased to 90% in 2 min. Upto 3 min mobile phase-B was pumped constantly and then reversed back to 10% after 3.5 min to restabilize the column for upto 4.5 min. During the run, column oven temperature was maintained at 40 °C.

Analysis was carried out on mass spectrometer in positive MRM (multiple reaction-monitoring) mode with a dwell time of 150 ms for each transition. The mass transitions for acetaminophen and internal standard were as follows: m/z 152.1 → m/z 110.1 and m/z 180.2 → m/z 71.1. Declustering potential (DP) and collision energy (CE) values were set at 70V and 24V for metabolite, and at 60V and 27V for internal standard. Typical source conditions were as follows; the curtain gas was set at 25, ion spray
needle voltage was 5500V, the turbo-gas temperature was set at 400 °C and GS1 as well as GS2 were set at 35 and 40 (arbitrary units). Data was collected and processed using Sciex Analyst 1.4.2 software (MDS-SCIEX, Concord, Ontario, Canada).

Data Analysis

Results are obtained from three different incubations and are represented as a mean ± standard deviation (S.D) except for DMSO concentrations greater than or equal to 0.2% where results are obtained from six determinations (triplicates from two different experiments). The extent of inhibition in the activity is the ratio of the activity in the incubations containing organic solvent over the average activity in the controls containing no organic solvent. Data is expressed as percent of control incubations.

Results and Discussion

The results of the effect of DMSO, acetonitrile and methanol on phenacetin O-deethylation activity are summarized in Fig. 1. Phenacetin-O-deethylase activity is not affected by methanol at concentrations ≤ 2% as was also observed by Chauret et al. (1998) and Busby et al. (1999) with human liver microsomes. This is in contrast to the findings of Hickman et al. (1998) of strong inhibition by methanol of CYP1A2 activity using caffeine as a substrate in liver microsomes. We found that acetonitrile induced the activity of phenacetin O-deethylation at concentrations ≥ 0.5%. The inhibition or inducer effects of acetonitrile on phenacetin O-deethylation are in line with the findings of Chauret et al., (1998), Busby et al., (1999) and Hickman et al., (1998).

We observed strong inhibition effects of DMSO at concentrations ≥ 0.1% (Fig. 2). Initially DMSO was tested at concentrations from 0.2 – 2% and was observed that at concentrations 0.2%, DMSO has inhibitory effects on phenacetin O-deethylation. In
another experiment, to determine the minimum percentage of DMSO that will not affect the phenacetin O-deethylation, DMSO was tested as low as 0.05% and was observed that at these levels DMSO has no inhibitory effects (8% increase, compared to control incubations). This is in contrast to the findings by Chauret et al. (1998) and Busby et al. (1999) where as in line with findings by Hickman et al. (1999) of CYP1A2 activity inhibition by DMSO (1%) where caffeine N3-demethylation was used as probe reaction. The difference in observations on the inhibitory effects of DMSO and methanol between different laboratories is not clear. The difference observed in inhibitory effects of methanol on CYP1A2 activity may be attributed to the different substrate used, caffeine by Hickman et al. (1999) and phenacetin by others.

Our findings show that the choice of solvent may affect the activity of phenacetin O-deethylation in liver microsomes. Use of dimethyl sulfoxide at concentrations ≥ 0.05% in reaction phenotyping studies and enzyme kinetic determinations would lead to wrong interpretation of the data. Although phenacetin is sufficiently soluble in water, the use of inhibitors and test compounds dissolved in dimethylsulfoxide during reaction phenotyping studies may confound the results. The concentration of DMSO should be kept low as indicated in this study or an alternate solvent may be used to solubilize the inhibitors and test compounds. The findings from this study also indicate that methanol apparently is the solvent with no effects on the activity of phenacetin O-deethylation.

Acknowledgements

Authors wish to acknowledge the support received from Mr. Venkateswarlu Jasti, CEO, Suven Life Sciences Ltd, Hyderabad.
Authorship Contribution

Participated in research design: Nirogi, Kandikere, Bhyrapuneni, Ponnamaneni, Palacharla, Manoharan

Conducted experiments: Ponnamaneni, Palacharla, Manoharan

Performed data analysis: Kandikere, Bhyrapuneni, Palacharla, Manoharan

Wrote or contributed to the writing of the manuscript: Nirogi, Kandikere, Bhyrapuneni, Ponnamaneni, Palacharla, and Manoharan.
REFERENCES


FIGURE 1 Effect (mean ± S.D) of organic solvent (0.2 - 2%) on CYP1A2 mediated phenacetin O-deethylation in human liver microsomes. Grey bars, control; black bars, DMSO; white bars, acetonitrile; diamond bars, methanol.

FIGURE 2 Effect (mean ± S.D) of dimethyl sulfoxide (0.05 – 2%) on CYP1A2 mediated phenacetin O-deethylation in human liver microsomes. Grey bars, control; black bars, DMSO;
Figure 1