Effect of Regular Organic Solvents on cytochrome P450 Mediated Metabolic Activities in Rat Liver Microsomes

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Abbreviations:

P450 or CYP, cytochrome P450; DMSO, dimethyl sulfoxide; RLM, rat liver microsomes; HLM, human liver microsomes; HPLC, high performance liquid chromatography.
Abstract

The effects of regular organic solvents on the metabolic activities of various human cytochrome P450s have been reported. However, very little is known about their influence on metabolic activities mediated by P450s in the rat liver microsomes (RLM). The purpose of this study was to investigate the effects of organic solvents such as methanol, acetonitrile, dimethyl sulfoxide (DMSO), acetone and ethanol on CYP1A, CYP2C, CYP2D, CYP2E and CYP3A-mediated metabolism using RLM. The results showed that the activities of most rat P450 enzymes appeared to be organic solvent-dependent and, the metabolism of the tested probes were remarkably reduced when the concentration of organic solvents was up to 5% v/v while most organic solvents demonstrated no significant interference when the concentration was below 1% except DMSO. In addition, organic solvents exhibited different inhibitory effects, for example, CYP2D and CYP2E showed significant activities reduction at lower concentration of organic solvents. Hence, this phenomenon should be taken into consideration when designing in vitro metabolism studies of new chemical entities. As a recommendation, acetonitrile was the most suitable solvent and the content of organic solvent should be kept lower than 1% v/v in rat liver microsomal incubations.
Introduction

Cytochrome P450s (CYP, P450) are the principal enzymes for the oxidation of drugs, environment pollutants and a large number of xenobiotics, which present in many mammalian organs such as lungs, kidneys, intestines, and livers. Organic solvents are routinely used to dissolve substrates or other chemicals in the microsomal incubations for drug metabolism, P450 inhibiton and induction studies. For example, ethoxyresorufin and pentoxyresorufin are both hydrophobic substrates as the biomarkers for CYP1A1 and CYP2B1. DMSO or ethanol/bovine serum albumin (BSA) was added to increase the solubility in RLM incubation system (Rutten et al., 1992). Various studies have reported the effects of common organic solvents on the in vitro CYP-mediated metabolic activities in HLM, human hepatocytes, or cDNA expressed human microsomes (Cotreau-Bibbo et al., 1996; Draper et al., 1997; Chauret et al., 1998; Hickman et al., 1998; Busby et al., 1999; Coller et al., 1999 and Palamanda et al., 2000). RLM was quite commonly used during in vitro studies (Bae et al., 2009; Deroussent et al., 2010; Pelkonen et al., 2009 and Yao et al., 2008), however, fewer systemic studies about the effects of organic solvents on the activities of rat P450 enzymes have been reported, while those tested in rats were limited to an old system in which the S9 and older marker substrates were used (Kawalek and Andrews, 1980). Previous study (Martignoni et al., 2006) indicated that the species-specific P450 enzymes of CYP1A, CYP2C, CYP2D and CYP3A showed appreciable interspecies differences in terms of catalytic activity between rat and human. Hence, the sensitivity of the P450 to organic solvents between RLM and HLM might be different. Therefore, we evaluated the effects of 5 common organic solvents (methanol,
acetonitrile, DMSO, acetone and ethanol) on 5 different rat P450 enzyme activities with escalating concentration from 0.1 to 10% v/v. Although species differences in substrate selectivity have been established, it has become common practice in drug discovery/toxicity to use marker substrates for human P450s in animal models such as RLM. The five commonly used reactions comprised of phenacetin O-deethylation for CYP1A, tolbutamide methyl-hydroxylation for CYP2C, dextromethorphan O-demethylation for CYP2D, chlorzoxazone 6-hydroxylation for CYP2E, and testosterone 6β-hydroxylation for CYP3A.
Materials and Methods

Chemicals. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, β-Nicotinamide adenine dinucleotide phosphate, phenacetin and acetamidophenol were purchased from Sigma (St. Louis, MO). Tolbutamide, 4-hydroxytolbutamide, dextromethorphan, dextorphan D-tartrate, chlorzoxazone, 6-hydroxychlorzoxazone, testosterone and 6β-hydroxytestosterone were obtained from Amersham Life Science (Arlington Heights, IL). Bovine serum albumin, Folin & Ciocalteu’s Phenol Reagent, and the modified Lowry protein assay reagents were purchased from Pierce (Rockford, IL, USA). Deionized water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). All other solvents were of highest purity commercially available or HPLC grade.

RLM Preparation. Male SD rats (8 weeks old, weight 200±20g) were purchased from the Department of Laboratory Animal Sciences, Fudan University of Shanghai in China. The rats were deprived of food for 20h prior to sacrificed, and liver was removed and pooled. RLM were prepared by different centrifugation as described previously (van Der Hoeven and Coon, 1974). All processes were performed at 0-4°C. Fractions were immediately frozen at -80°C. Protein was determined by the modified Lowry protein assay reagents, using bovine serum albumin as the standard.

P450 Incubation Assays. Microsomal incubations for the determination of P450 activities were carried out in a total volum of 200 μl. Briefly, each incubation was performed with microsomal protein in 50 mM potassium phosphate buffer at PH 7.4 containing 3.0 mM magnesium chloride, 1.0 mM EDTA, NADPH generating system
(5 mM glucose 6-phosphate, 1.0 mM NADP and 1.0 unit/ml glucose 6-phosphate dehydrogenase), various substrates and different volume of organic solvents. There was a 5-min preincubation step at 37°C. The reaction was started by adding NADPH generation system. At the end of the incubation, the reaction was quenched by adding ice cold methanol. The incubation mixtures were subsequently vortexed and then centrifugated for 15 min at 16,000×g. 50 μl of the supernatant was analyzed by HPLC. All microsomal incubations were conducted in duplicate.

**Assay Linearity.** The reaction time and protein concentration linearity for each enzyme in RLM were evaluated through a series of incubations. At the end, the linear reaction conditions used were as follows: the incubation time and protein concentration were 40 min and 1 mg/ml for phenacetin O-deethylation; 60 min and 1 mg/ml for tolbutamide methyl-hydroxylation; 60 min and 1 mg/ml for dextromethorphan O-demethylation; 30 min and 1 mg/ml for chlorzoxazone 6-hydroxylation and, 40 min and 0.5 mg/ml for testosterone 6β-hydroxylation.

**Determination of Enzyme Kinetic Parameters.** Based on the linearity studies, the proper concentration of substrates were chosen according to the kinetic studies. The phenacetin concentrations were 10, 25, 50, 100, 150 and 200 μM; tolbutamide concentrations were 125, 250, 500, 1000, 2000, and 3000 μM; dextromethorphan concentrations were 2.5, 5, 10, 25, 50 and 75 μM; chlorzoxazone concentrations were 10, 25, 50, 100, 150 and 200 μM, and testosterone concentrations were 20, 40, 80, 120, 160 and 200 μM.

**Reduction of Specific P450 Activities by Selected Solvents.** To investigate the
inhibitory capacity of the solvents methanol, acetonitrile, DMSO, acetone and ethanol on the P450-mediated activities of several substrates in RLM, the solvents final concentration were 0.1, 0.25, 0.5, 1, 2.5, 5, 10% v/v and the control group was just adding water instead. The relative enzyme activity of the control group without organic solvent was defined as 100%.

**Data and Statistical Analysis.** The parameters $K_m$ and $V_{max}$ were processed by nonlinear regression analysis by using GraphPad Prism v5.0 software. Data are expressed as mean ± S.D. of two replicated experiments. Comparisons between each concentration and control group were performed using a two tailed Student’s t test. $P < 0.05$ was considered statically significant. Some solvents at 10, 5 or even at 2.5% v/v showed strong inhibition on the enzyme activities so that the corresponding metabolites were below the limit of quantification which could not be measured by HPLC.
Results and Discussion

As commonly employed by numerous laboratories studying RLM (Alcorn et al., 2007; Hasegawa et al., 2002; Brown et al., 2007; Yao et al., 2008), we used human P450 substrates to investigate the effects of organic solvents on their activities which has become a common practice. Each enzyme activity was characterized in terms of linearity with respect to time and protein concentration.

The Michaelis-Menten parameters of $K_m$ determined for each enzyme (1A, 2C, 2D, 2E and 3A) activities in RLM were $54.35 \pm 11.2$, $751.0 \pm 89.76$, $41.63 \pm 4.67$, $48.12 \pm 2.79$ and $94.19 \pm 17.4 \mu M$. The $K_m$ values of chlorzoxazone was approximate while phenacetin, tolbutamide and testosterone were relative lower than the previous report conducted in uninduced RLM (Easterbrook's et al., 2001 a). Hence, the concentrations for phenacetin, tolbutamide, dextromethorphan, chlorzoxazone and testosterone in the incubations were 100, 750, 40, 50 and 100 $\mu M$, respectively, close to their $K_m$ values except phenacetin because of the lower limit of quantification.

The effects of regular organic solvents (methanol, acetonitrile, DMSO, acetone and ethanol) at different concentrations on the P450-mediated metabolism in RLM were presented in Fig. 1. The sensitivity of the various P450s in RLM was not same to a particular organic solvent. As shown, all of the tested solvents exhibited a significant inhibition on the activities of P450s at concentration of 10%. The rat CYP2D and CYP2E was much more sensitive to all the solvents except acetonitrile. All organic solvents at 1% v/v exhibited $> 10\%$ inhibition on the activity of CYP2D and all but acetonitrile showed $> 40\%$ inhibition on CYP2E. In general, the
inhibitory effect showed organic solvent concentration-dependent increases. DMSO and ethanol demonstrated a stronger inhibition which was consistent with the previous report that the RLM was sensitive to even lower percentage of DMSO and also the inhibitory effect of DMSO was different for each compound (Di et al., 2003). Acetonitrile was relative mild to most P450 enzymes.

**Phenacetin O-Deethylation (1A).** Methanol, ethanol and DMSO at 1% all exhibited > 20% inhibition of phenacetin O-deethylation activities in RLM. Acetonitrile showed a mild effect, however, it demonstrated the strongest inhibition than any other solvents at 10% so that the metabolite of phenacetin was not detectable. Hence, < 1% of acetonitrile should be used for assaying phenacetin O-deethylation in RLM.

**Tolbutamide Methyl-Hydroxylation (2C).** The tolbutamide methyl-hydroxylation activity was not noticeably affected in the presence of 0.25% of methanol, acetonitrile, DMSO and acetone. However, the activity was strongly inhibited in the presence of ethanol by 27% at 0.25% v/v. With the escalating concentrations up to 1%, acetonitrile showed no effects on the activity of CYP2C. 30, 16, 6 and 65% of the enzyme activity was inhibited by methanol, DMSO, acetone and ethanol at 1%, respectively. All solvents at 5% exhibited > 60% inhibition. However, all the study results suggested that the CYP2C was much more resistant to acetonitrile in HLM (Busby et al., 1999) or in RLM as demonstrated in the present study.

**Dextromethorphan O-Demethylation (2D).** CYP2D appeared to be much more sensitive to these organic solvents. Even at 1% v/v, acetonitrile, DMSO, methanol,
ethanol and acetone inhibited the CYP2D activities nearly by 12, 12, 19, 36 and 40%, respectively. Acetone appeared to have the strongest effect on CYP2D activity. Comparing the results between this study and the literature (Busby et al., 1999), < 1% v/v organic solvent is suggested when determining the CYP2D activity in RLM or in HLM.

**Chlorzoxazone 6-Hydroxylation (2E).** For chlorzoxazone 6-hydroxylation, methanol, DMSO and ethanol all exhibited > 50% inhibition at 1% v/v and acetone showed 37% inhibition at 1% v/v. DMSO strongly inhibited the CYP2E activity by 91% even at the concentration of 2.5%. This is consistent with the report that DMSO was a CYP2E inhibitor (Yoo et al., 1987). Acetonitrile had no apparent inhibition on CYP2E activity until a concentration of 2.5% was reached. The results observed in our study was basically consistent with the report that the CYP2E activity in RLM was strongly inhibited by DMSO and ethanol at lower concentration and there was a concentration dependent decrease in activities by methanol and acetonitrile (Nishimura et al., 1999). Similar to other P450 enzyme studies, higher concentration of the organic solvents showed stronger effects on CYP2E. However, acetone demonstrated activation trend at concentration of 1-5% v/v, which is consistent with the previous study that acute acetone treatments increased chlorzoxazone activity by 3.1-fold (González-Jasso et al., 2003).

**Testosterone 6β-Hydroxylation (3A).** All five common organic solvents exhibited minimal inhibition (< 10%) at less than 1% concentration. The inhibition effect on CYP3A activity by methanol, acetonitrile, ethanol and acetone was observed at 5%
v/v and DMSO at 2.5% v/v in RLM. In our study, DMSO at 1% v/v exhibited 8% inhibition on testosterone 6β-hydroxylation activity, which was different from up to 30% inhibition on midazolam 1’-hydroxylation activity observed previously (Di et al., 2003). This apparent substrate-dependent effect of organic solvents on P450 enzyme activities is consistent with previous observations with HLM as well as RLM (Chauret et al., 1998; Hickman et al., 1998; Kawalek and Andrews, 1980).

Generally, the trend of the effects of organic solvents on the P450 activities in HLM and RLM was similar. However, the difference may exist for a specific enzyme and organic solvent. The active site as well as incubation binding site may be different among different species (Boobis et al., 1990) as highlighted in the previous study (Eagling et al., 1998). The detailed difference for the organic solvent effects on P450 enzyme activities between rat and human needs further studies.

Several studies on the effects of organic solvents on a number of P450 activities in HLM or hepatocytes (Chauret et al., 1998; Hickman et al., 1998; Easterbrook et al., 2001 b) have indicated that acetonitrile or methanol appeared to be a better choice with HLM. Our studies with RLM also showed that acetonitrile among several organic solvents tested represents a good choice as long as the concentration is kept below 1%.

Conclusion

The effects of organic solvents on the P450 mediated metabolic activities in RLM should be considered when designing drug metabolism studies in vitro. Most organic solvents should be controlled below 1%. Acetonitrile is the most suitable solvent for
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RLM incubations.
References


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Legends for Figures

Figure 1. The effects of organic solvents at increasing concentrations on P450-mediated metabolic activities in RLM. The effects of methanol, acetonitrile, DMSO, acetone and ethanol on CYP1A (A), CYP2C (B), CYP2D (C), CYP2E (D), and CYP3A (E) activities were determined by coincubation of each solvent (0.1, 0.25, 0.5, 1, 2.5, 5 and 10% v/v) and its corresponding substrate with RLM. Results represented the mean ± S.D. of two replicated experiments. *, P < 0.05; **, P < 0.01, compared with the control group. Some solvents at 10, 5 or even at 2.5% v/v showed strong inhibition on the enzyme activities so that the corresponding metabolites were below the limit of quantification and these results were not shown in the figure.