Short Communication

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

Received April 11, 2010; accepted August 19, 2010

ABSTRACT:

The effects of regular organic solvents on the metabolic activities of various human cytochromes P450 (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 CYP1A1, 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, whereas most organic solvents demonstrated no significant interference when the concentration was below 1%, with the exception of DMSO. In addition, organic solvents exhibited differential inhibitory effects, for example, CYP2D and CYP2E showed a significant reduction of activities at lower concentrations of organic solvents. Hence, this phenomenon should be taken into consideration when designing in vitro metabolism studies of new chemical entities. Therefore, we recommend acetonitrile as the most suitable solvent for RLM incubations, and the content of organic solvent should be kept lower than 1% v/v.

Introduction

Cytochromes P450 (P450s) are the principal enzymes for the oxidation of drugs, environmental pollutants, and a large number of xenobiotics, which exist 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 inhibition, and induction studies. For example, ethoxyresorufin and pentoxyresorufin are both hydrophobic substrates as the biomarkers for CYP1A1 and CYP2B1, dimethyl sulfoxide (DMSO), or ethanol/bovine serum albumin (BSA) were added to increase the solubility in rat liver microsomes (RLM) incubation system (Rutten et al., 1992). Various studies have reported the effects of common organic solvents on the in vitro P450-mediated metabolic activities in human liver microsomes (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; Palamanda et al., 2000). RLM were quite commonly used during in vitro studies (Yao et al., 2008; Bae et al., 2009; Pelkonen et al., 2009; Deroussent et al., 2010); however, fewer systemic studies about the effects of organic solvents on the activities of rat P450 enzymes have been reported, and those tested in rats were limited to an old system in which the S9 and older marker substrates were used (Kawalek and Andrews, 1980). A previous study (Martignoni et al., 2006) indicated that the species-specific P450 enzymes of CYP1A1, CYP2C, CYP2D, and CYP3A showed appreciable interspecies differences in terms of catalytic activity between rats and humans. Hence, the sensitivity of the P450 enzyme to organic solvents between RLM and HLM might be different. Therefore, we evaluated the effects of five common organic solvents (methanol, acetonitrile, DMSO, acetone, and ethanol) on five different P450 enzyme activities in rats, using escalating concentrations 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 phenacetin O-deethylation for CYP1A1, tolbutamide methylhydroxylation 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-Aldrich (St. Louis, MO). Tolbutamide, 4-hydroxytolbutamide, dextromethorphan, dextrorphan p-tartrate, chlorzoxazone, 6-hydroxychlorzoxazone, testosterona, and 6β-hydroxytestosterone were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). BSA, Folin, Ciocalteu’s Phenol Reagent, and the modified Lowry protein assay reagents were purchased from Pierce (Rockford, IL). Deionized water was purified using a Milli-Q system (Millipore Corporation, Billerica, MA). All other solvents were of the highest purity commercially available or high-performance liquid chromatography (HPLC) grade.

RLM Preparation. Male Sprague-Dawley rats (8 weeks old, weight 200 ± 20 g) were purchased from the Department of Laboratory Animal Sciences (Fudan University of Shanghai, Shanghai, China). The rats were deprived of food for 20 h before being 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 to 4°C. Fractions were immediately frozen at −80°C. Protein was determined by the modified Lowry protein assay reagents, using BSA as the standard.

Abbreviations: P450, cytochrome P450; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; RLM, rat liver microsomes; HLM, human liver microsomes; HPLC, high-performance liquid chromatography.
P450 Incubation Assays. Microsomal incubations for the determination of P450 activities were carried out in a total volume of 200 μl. In brief, each incubation was performed with microsomal protein in 50 mM potassium phosphate buffer, 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 volumes 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 16,000g. Fifty microliters 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, acetoniitrole, DMSO, acetone, and ethanol on the P450-mediated activities of several substrates in RLM, the solvents’ final concentrations were 0.1, 0.25, 0.5, 1, 2.5, 5, and 10%. The relative enzyme activity of the control group without organic solvent was defined as 100%.

Data and Statistical Analysis. The parameters Km and Vmax were processed by nonlinear regression analysis using GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego, CA). 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 to be statistically 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 and could not be measured by HPLC.

Results and Discussion

As commonly used by numerous laboratories studying RLM (Hashigawa et al., 2002; Alcorn et al., 2007; 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 determined Km values in RLM were 54.35 ± 11.2, 751.0 ± 89.76, 41.63 ± 4.67, 48.12 ± 2.79, and 94.19 ± 17.4 μM for CYP1A, CYP2C, CYP2D, CYP2E, and CYP3A, respectively. The Km values of chlorzoxazone were approximate, whereas phenacetin, tolbutamide, and testosterone Km values were relatively lower than those in the previous report conducted in uninduced RLM (Easterbrook et al., 2001a). Hence, the concentrations for phenacetin, tolbutamide, dextromethorphan, chlorzoxazone, and testosterone in the incubations were 100, 750, 40, 50, and 100 μM, respectively. The chosen concentrations were close to their Km values, with the exception of phenacetin due to the lower limit of quantification of its metabolite.

The effects of regular organic solvents (methanol, acetoniitrole, 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 to a particular organic solvent in RLM was not the same. As shown, all of the tested solvents exhibited a significant inhibition on the activities of P450s when the concentration was 10%. The rat CYP2D and CYP2E were much more sensitive to all of the solvents, with the exception of acetoniitrole. All organic solvents at 1% v/v exhibited >10% inhibition on the activity of CYP2D, and all except acetoniitrole showed >40% inhibition on CYP2E. In general, the inhibitory effect showed organic solvent concentration-dependent increases. DMSO and ethanol demonstrated a stronger inhibitory effect, which was consistent with a previous report that the RLM was sensitive to an even lower percentage of DMSO and that the inhibitory effect of DMSO was different for each compound (Di et al., 2003). Acetoniitrole was relatively mild compared with most P450 enzymes.

Phenacetin O-Deethylation (1A). Methanol, ethanol, and DMSO at 1% all exhibited >20% inhibition of phenacetin O-deethylation activities in RLM. Acetoniitrole showed a mild effect; however, it demonstrated the strongest inhibition of all of the other solvents at 10% so that the metabolite of phenacetin was not detectable. Hence, <1% of acetoniitrole 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% methanol, acetoniitrole, 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%, acetoniitrole showed no effects on the activity of CYP2C. Thirty, 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 study results suggested that CYP2C was much more resistant to acetoniitrole 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, acetoniitrole, DMSO, methanol, ethanol, and acetone inhibited the CYP2D activities by nearly 12, 12, 19, 36, and 40%, respectively. Acetone appeared to have the strongest effect on CYP2D activity. In comparing the results between this study and those in the literature (Busby et al., 1999), we suggest that a <1% v/v organic solvent be used when determining the CYP2D activity in RLM or 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 a concentration of 2.5%. This result is consistent with the report that DMSO was a CYP2E inhibitor (Yoo et al., 1987). Acetoniitrole had no apparent inhibition on CYP2E activity until a concentration of 2.5% was reached. The results observed in our study were basically consistent with the reports that the CYP2E activity in RLM was strongly inhibited by DMSO and ethanol at lower concentrations and that there was a concentration-dependent decrease in activities by methanol and acetoniitrole (Nishimura et al., 1999). Similar to other P450 enzyme studies, higher concentrations of the organic solvents showed stronger effects on CYP2E. Similar to other P450 enzyme studies, higher concentrations of the organic solvents showed stronger effects on CYP2E. However, for acetone, the inhibitory effect did not follow the usual organic solvent concentration-dependent inhibition at concentration of 1 to 5% v/v, whereas it was reported previously that acute acetone (5% v/v) treatments increased chlorzoxazone activity by 3.1-fold with Wistar rats (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, acetoniitrole, ethanol, and acetone was observed at 5% v/v and DMSO...
FIG. 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. *, $P < 0.05$ and **, $P < 0.01$ compared with the control group. Some solvents at 10, 5, or even 2.5% v/v showed strong inhibition on the enzyme activities so that the corresponding metabolites were below the limit of quantification. These results are not shown in the figure.
Effect of organic solvents on rat P450 activities

At 2.5% v/v in RLM. In our study, DMSO at 1% v/v exhibited 8% inhibition on testosterone 6β-hydroxylation activity, which differed from the 1 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 (Kawalek and Andrews, 1980; Chauret et al., 1998; Hickman et al., 1998).

In general, 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 a previous study (Eagling et al., 1998). The detailed difference for the organic solvent effects on P450 enzyme activities between rat and human needs further study.

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., 2001b) have indicated that acetaminophen or methanol appeared to be a better choice with HLM. Our studies with RLM also showed that, among several organic solvents tested, acetaminophen 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%. Acetaminophen is the most suitable solvent for RLM incubations.

References


Address correspondence to: Cheng Guo, Ph.D., Professor, Department of Pharmacy, 6th People’s Hospital, Shanghai Jiao Tong University, 600 Yi Shan Road, Shanghai, 200233, China. E-mail: gboss@126.com

American Society for Pharmacology and Experimental Therapeutics