

Short Communication

Effect of Commonly Used Organic Solvents on the Kinetics of Cytochrome P450 2B6- and 2C8-Dependent Activity in Human Liver Microsomes

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ABSTRACT:

The effect of common organic solvents on the activities of various human cytochromes P450 has been reported. However, very little is known about their influence on CYP2B6 and CYP2C8 enzymes. The purpose of this study was to investigate the effect of solvents on the kinetics of representative CYP2B6 (bupropion hydroxylase) and CYP2C8 (paclitaxel hydroxylase) reactions in human liver microsomes. Methanol, ethanol, dimethyl sulfoxide (DMSO), and acetonitrile were studied at increasing volumes (v/v). Acetonitrile, DMSO, and ethanol were shown to increase the K_m and decrease the intrinsic clearance (CL_{int}) of CYP2B6-mediated bupropion hydroxylation in a concentration-dependent manner. These solvents did not noticeably alter the V_{max} at concentrations of $\leq 1\%$ (v/v). Unlike the other solvents studied, the effect of methanol ($\leq 0.5\%$, v/v) on CYP2B6 kinetics was negligible. Both DMSO and ethanol increased the K_m and de-

creased the CL_{int} of CYP2C8-mediated paclitaxel hydroxylation in a concentration-dependent manner. Acetonitrile had minimal influence on CYP2C8 enzyme kinetics at concentrations of $\leq 1\%$ (v/v). Methanol decreased the K_m of paclitaxel at low concentrations followed by an increase at concentrations of $\geq 2\%$ (v/v). This differential influence on K_m resulted in an increased CL_{int} at low concentrations followed by a decrease at high concentrations. The studied solvents had minimal influence on V_{max} of paclitaxel. Collectively, DMSO and ethanol were not suitable for characterizing CYP2B6- and CYP2C8-mediated reactions because they showed concentration-dependent inhibition. Methanol and acetonitrile at concentrations of $\leq 0.5\%$ and $\leq 1\%$ (v/v) appeared to be suitable for the measurement of CYP2B6- and CYP2C8-mediated activities, respectively.

Cytochrome P450 (P450) enzymes are a major class of heme-containing proteins critical for the metabolism of a large number of xenobiotics and endogenous compounds. Human liver microsomes (HLM) are routinely used for identifying P450 enzymes responsible for metabolism (reaction phenotyping) and/or for investigating the enzyme kinetics and inhibitory effects of discovery and development compounds (Walsky and Obach, 2004; Obach et al., 2005).

The primary function of P450 enzymes is to convert lipophilic drug molecules into hydrophilic forms that can be readily excreted from the body. Because *in vitro* metabolism studies are typically conducted in physiological buffers, problems related to the solubilization of substrates or inhibitors may occur. Organic solvents like methanol, ethanol, acetonitrile, and dimethyl sulfoxide (DMSO) are commonly used to facilitate solubilization of highly lipophilic compounds of interest. However, owing to their lipophilic nature, organic solvents may alter the activities of the P450 enzymes directly by either modifying the native environment surrounding these enzymes or by disrupting the integrity of the enzymes, leading to erroneous interpretation of data and misleading conclusions regarding enzyme kinetics and inhibition.

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CYP2C8 constitutes about 7% of the total microsomal P450 content in human liver (Rendic and Di Carlo, 1997). Despite the high expression level, this polymorphic enzyme has been a relatively less explored member of the P450 family, and it is only recently that its role in metabolism has been considered. Noteworthy drug-drug interactions involving inhibition of CYP2C8 have done much to raise awareness of its importance (Backman et al., 2002; Niemi et al., 2003). Likewise, CYP2B6 is polymorphic, and large interindividual differences in enzyme expression have generated significant interest in substrates of CYP2B6 and their associated drug-drug interactions (Shimada et al., 1994; Faucette et al., 2000). Fortunately, the availability of selective probes in recent times has enabled the *in vitro* assessment of CYP2B6 and CYP2C8 inhibition and P450 reaction-phenotyping (isozyme-mapping) studies. However, with any *in vitro* study one has to consider the effects of solvents that are used to solubilize substrates and inhibitors. Toward this end, numerous efforts have been made to evaluate the impact of organic solvents on recombinant P450s or P450 activities in HLM (Chauret et al., 1998; Hickman et al., 1998; Busby et al., 1999; Tang et al., 2000). Because the effects of organic solvents on CYP2B6- and CYP2C8-mediated enzyme activities in HLM have not been thoroughly assessed, we undertook the task of evaluating the effects of organic solvents on these relatively less-studied enzymes in the present report.

In the current communication, an attempt was made to system-

ABBREVIATIONS: P450, cytochrome P450; HLM, human liver microsome(s); DMSO, dimethyl sulfoxide; LC/MS/MS, liquid chromatography/tandem mass spectrometry; CL_{int} , intrinsic clearance.

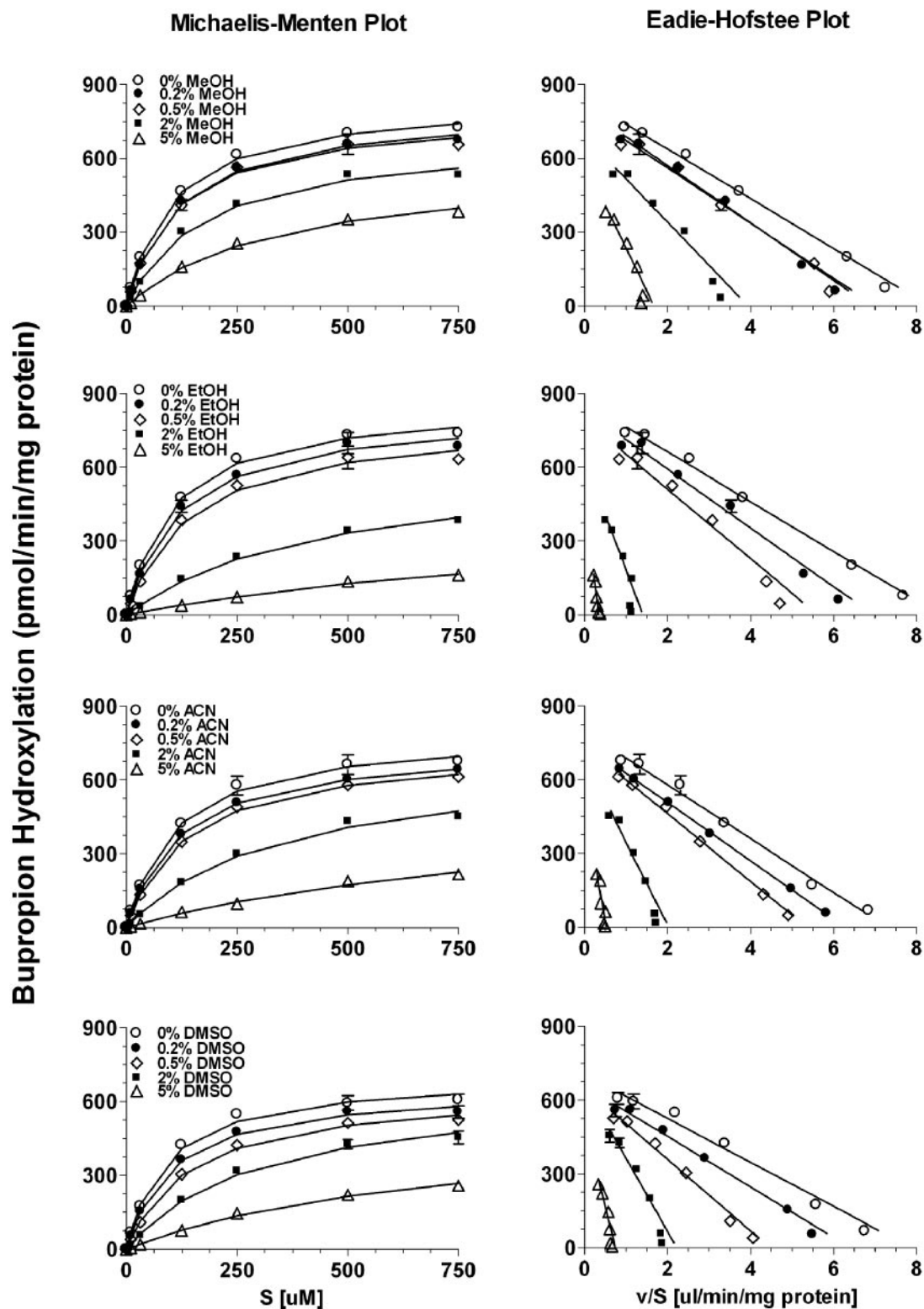


FIG. 1. Effects of methanol, ethanol, acetonitrile, and DMSO on the kinetics of hydroxybupropion formation in the presence of HLM. Michaelis-Menten (left) and Eadie-Hofstee (right) plots of hydroxybupropion formation from bupropion. Effects of different incubation concentrations [0 (control), 0.2, 0.5, 2, and 5%, v/v] of methanol, ethanol, acetonitrile, and DMSO on the rates of hydroxybupropion formation were assessed using HLM ($n = 3$). The symbols and solid lines represent the observed (mean \pm S.D.) and model-predicted values, respectively.

atically examine the influence of four commonly used organic solvents (methanol, ethanol, DMSO, and acetonitrile) on the kinetics of a representative, but selective, CYP2B6 and CYP2C8

substrate in HLM. The intent was to identify suitable solvents that enable in vitro studies with minimal impact on CYP2C8 and CYP2B6 enzymes.

Materials and Methods

Chemicals, Supplies, and Enzyme Sources. All the solvents and chemicals were obtained from commercial sources. Pooled HLM (9 males and 17 females) were obtained from BD Biosciences (Woburn, MA).

Assay Linearity. For the CYP2B6 and CYP2C8 assays ($n = 3$), the time linearity was evaluated by incubating the reaction mixture (200 μl) containing bupropion (50 μM) or paclitaxel (20 μM), KH_2PO_4 buffer, pH 7.4 (100 mM), containing EDTA (1 mM), microsomal protein (0.05 mg/ml), and NADPH (1 mM) for 5, 10, and 20 min. The protein linearity was evaluated by incubating the above reaction mixture (200 μl) at HLM protein concentrations ranging from 0.025 to 0.2 mg/ml for 10 min (CYP2B6 assay) or at protein concentrations ranging from 0.025 to 0.1 mg/ml for 5 min (CYP2C8 assay). The reactions of CYP2B6 and CYP2C8 assays were terminated by adding acetonitrile (400 μl) containing trazodone (50 nM) (internal standard) or methanol (400 μl) containing 10-deacetylpaclitaxel (internal standard) (100 nM), respectively. Incubation mixtures were subsequently vortexed and centrifuged at 2000g for 5 min, and the supernatant was monitored for hydroxybupropion or 6 α -hydroxypaclitaxel by liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis. Both reactions were linear with respect to time of incubation (up to 20 min) and HLM protein concentration (0.025 to 0.1 mg/ml).

Determination of Enzyme Kinetic Parameters. The incubation procedure used for these studies was similar to the one described above. The substrate concentrations used for the determination of enzyme kinetics were 0.5, 1.56, 6.25, 12.5, 25, 37.5, and 50 μM (paclitaxel) and 10, 31.25, 125, 250, 500, 750, and 1000 μM (bupropion). Based on the results of protein and time linearity studies, a protein concentration of 0.05 mg/ml and an incubation time of 5 min were chosen for these studies. Whereas the stock solutions of bupropion were prepared in water, those of paclitaxel were prepared in methanol. The final concentration of methanol in the CYP2C8 incubation mixture was 0.025% (v/v).

Effect of Solvents on the Enzyme Kinetic Parameters of Paclitaxel (CYP2C8) and Bupropion (CYP2B6). The microsomal incubations for evaluating the effects of organic solvents (methanol, ethanol, DMSO, and acetonitrile) on the enzyme kinetic parameters of paclitaxel and bupropion were conducted as above, except 10 μl of either 4, 10, 40, or 100% organic solvent (v/v) in water was added to each incubation to yield final solvent concentrations of 0.2, 0.5, 2, or 5% (v/v) in the incubates, respectively. The effects of methanol and acetonitrile on the enzyme kinetic parameters of bupropion and paclitaxel, respectively, were also evaluated at 1% concentration (v/v) in an attempt to identify the threshold concentrations of organic solvents that these enzymes could readily withstand without being subjected to any alteration. In addition to the above solvent concentrations (because paclitaxel was dissolved in methanol), all the CYP2C8 incubates consisted of an additional 0.025% of methanol.

LC/MS/MS Analysis. For quantification of metabolites (6 α -hydroxy paclitaxel and hydroxybupropion), the LC/MS/MS system comprised a Sciex (Concord, ON, Canada) API 4000 tandem mass spectrometer equipped with an electrospray ionization source (operated in electrospray ionization negative and positive modes for CYP2C8 and CYP2B6 assays, respectively), two LC-10ADvp pumps with an SCL-10ADvp controller and DGU-14 solvent degasser (Shimadzu, Columbia, MD), and a CTC PAL autosampler (Leap Technology, Zwingen, Switzerland). XBridge, 5 μm , 50 \times 2 mm (Waters Corp., Milford, MA) (CYP2B6) and Luna Phenyl-Hexyl, 5 μm , 150 \times 2.0 mm (Phenomenex, Torrance, CA) (CYP2C8) columns were used to achieve high-performance liquid chromatography separation. Gradient elution at a flow rate of 0.3 ml/min was achieved using the following mobile phase: A = 95:5 0.1% formic acid/acetonitrile and B = 95:5 acetonitrile/0.1% formic acid. Detection of each analyte was achieved through selected reaction monitoring (914.5 > 541.3 and 256.2 > 238.1 for 6 α -hydroxy paclitaxel and hydroxybupropion, respectively).

Data Analysis. Kinetic parameters were estimated based on least-squares nonlinear regression analysis using the WinNonlin program (Pharsight, Mountain View, CA). The appropriate model was selected after visual inspection of the Eadie-Hofstee-transformed plots (rate versus rate/substrate concentration). Hydroxybupropion and 6 α -hydroxypaclitaxel formations from bupropion and

paclitaxel, respectively, were best described by a simple Michaelis-Menten equation: $V = V_{\text{max}} \cdot [S]/K_m + [S]$.

The intrinsic clearance (CL_{int}) values of bupropion and paclitaxel in the absence (control) and presence of solvent were calculated by dividing the corresponding V_{max} (maximum velocity) by their respective K_m (apparent affinity constant). The linearity of metabolite formation with respect to the incubation time and protein concentration was evaluated by linear regression analysis using Microsoft Excel (Microsoft, Redmond, WA). Significant differences ($P < 0.05$) between control and solvent-treated groups were established using Student's t test. All the data are reported as mean \pm S.D. ($n = 3$ determinations). The data in the tables were expressed as percentage of the control.

Results and Discussion

CYP2B6-Dependent Hydroxylation of Bupropion. Metabolism of bupropion to hydroxybupropion, catalyzed primarily by the CYP2B6 in HLM, was best described by simple hyperbolic Michaelis-Menten kinetics in the absence and presence of solvent (Fig. 1). The kinetic parameters (expressed as percentage of control) derived by nonlinear regression analysis are presented in Table 1. The K_m and V_{max} of bupropion obtained by fitting the kinetic data from control liver microsomes (no solvent) were $101.4 \pm 4.2 \mu\text{M}$ and $842.4 \pm 4.7 \text{ pmol/min/mg protein}$, respectively. These values are similar to literature estimates of K_m (81–130 μM) and V_{max} (82–7675 pmol/min/mg protein) (Faucette et al., 2000; Walsky and Obach, 2004). Although the K_m values reported by different laboratories were quite consistent, significant variability was observed in the V_{max} for bupropion hydroxylation. This variability was mainly attributed to differential expression of CYP2B6 in various HLM preparations (Faucette et al., 2004).

Methanol did not significantly ($P > 0.05$) alter any of the enzyme kinetic parameters (K_m , V_{max} , and CL_{int}) when incubated at concentrations of $\leq 0.5\%$ (v/v). However, increases in the K_m (2.1-, 1.7-, and 3.4-fold) and decreases in CL_{int} (49, 53, and 80%) were observed when methanol was added at concentrations of 1, 2, and 5% (v/v), respectively ($P < 0.05$). Ethanol, acetonitrile, and DMSO showed concentration-dependent effects on K_m and CL_{int} of bupropion hydroxylation; an increase in the concentration of ethanol, acetonitrile, and DMSO from 0.2% to 5% (v/v) resulted in 1.2- to 13.3-fold, 1.1- to 9.0-fold, and 1.1- to 7.6-fold increases in K_m and 19 to 96%, 14 to 93%, and 19 to 90% decreases in the CL_{int} values, respectively (Table 1). At the lowest concentration of ethanol, acetonitrile, and DMSO (0.2%, v/v), the effects on K_m and CL_{int} were not statistically significant ($P > 0.05$). Despite these concentration-dependent effects on K_m and CL_{int} , ethanol, acetonitrile, and DMSO did not significantly alter the V_{max} values at concentrations of $\leq 0.5\%$, $\leq 2\%$, and $\leq 2\%$ (v/v), respectively ($P > 0.05$).

The effects of organic solvents on the kinetic parameters of CYP2B6 enzyme using HLM were generally similar to the effects reported previously using human recombinant CYP2B6 enzyme (Busby et al., 1999). The previous study used 7-ethoxy-4-trifluoromethyl coumarin as a probe substrate for assessing the activity of CYP2B6 enzyme. Similar to the results described herein, methanol was found to be least inhibitory at concentrations of $\leq 1\%$ (v/v), whereas acetonitrile, DMSO, and ethanol exhibited concentration-dependent effects on CYP2B6 activity.

CYP2C8-Dependent Hydroxylation of Paclitaxel. The formation of 6 α -hydroxypaclitaxel from paclitaxel in the presence of HLM conformed to simple Michaelis-Menten kinetics (Fig. 2), and the kinetic parameters are presented in Table 2. The K_m and V_{max} were $8.9 \pm 0.1 \mu\text{M}$ and $404.2 \pm 12.9 \text{ pmol/min/mg protein}$, respectively, and were consistent with the values reported in the literature (Rahman et al., 1994; Vaclavikova et al., 2004).

TABLE 1

*Effect of organic solvents on the kinetics of CYP2B6-mediated bupropion hydroxylation in the presence of HLM*Kinetic parameters are expressed as % Control (mean \pm S.D.).

	Solvent (% v/v)			
	Methanol	Ethanol	Acetonitrile	DMSO
K_m				
0 (Control)	100.0 \pm 4.2	100.0 \pm 8.5	100.0 \pm 3.9	100.0 \pm 2.3
0.2	115.1 \pm 0.5	118.1 \pm 1.1	108.2 \pm 2.5	114.7 \pm 1.5
0.5	110.9 \pm 2.0	139.5 \pm 1.6 ^a	125.4 \pm 1.2 ^a	162.3 \pm 0.6 ^b
1	209.8 \pm 45.9 ^a	N.E.	N.E.	N.E.
2	174.1 \pm 3.0 ^c	444.7 \pm 14.5 ^c	311.0 \pm 6.7 ^b	332.3 \pm 40.4 ^a
5	341.2 \pm 22.5 ^c	1327.8 \pm 293.0 ^a	901.4 \pm 18.4 ^b	763.2 \pm 126.6 ^a
V_{max}				
0 (Control)	100.0 \pm 3.2	100.0 \pm 2.0	100.0 \pm 3.6	100.0 \pm 3.4
0.2	95.6 \pm 3.0	96.1 \pm 1.2	93.2 \pm 1.4	93.3 \pm 3.0
0.5	93.4 \pm 1.9	91.7 \pm 2.4	92.2 \pm 2.2	92.2 \pm 1.0
1	105.0 \pm 5.6	N.E.	N.E.	N.E.
2	82.4 \pm 0.9 ^c	73.3 \pm 1.5 ^c	86.1 \pm 0.7 ^a	93.9 \pm 8.1
5	69.0 \pm 0.4 ^c	54.1 \pm 9.1 ^a	65.2 \pm 1.9 ^c	72.3 \pm 7.8 ^a
CL_{int}				
0 (Control)	100.0 \pm 4.6	100.0 \pm 6.5	100.0 \pm 7.5	100.0 \pm 5.7
0.2	82.9 \pm 3.0	81.1 \pm 0.2	86.0 \pm 3.3	81.4 \pm 3.6
0.5	84.2 \pm 3.2	65.5 \pm 0.9 ^a	73.4 \pm 1.1 ^a	56.8 \pm 0.8 ^a
1	50.5 \pm 8.4 ^a	N.E.	N.E.	N.E.
2	47.3 \pm 1.4 ^c	16.4 \pm 0.2 ^c	27.7 \pm 0.4 ^c	28.3 \pm 1.0 ^c
5	20.3 \pm 1.2 ^c	4.1 \pm 0.2 ^c	7.2 \pm 0.1 ^c	9.5 \pm 0.6 ^c

N.E., not evaluated.

^a Significantly different from control: $P < 0.05$, Student's t test.^b Significantly different from control: $P < 0.001$, Student's t test.^c Significantly different from control: $P < 0.01$, Student's t test.

As opposed to the effects on the CYP2B6 enzyme, methanol showed mixed effects on the kinetic parameters of paclitaxel (Table 2). At low concentrations (0.2 and 0.5%, v/v) methanol decreased the K_m (32 and 35%), whereas at high concentrations (2 and 5%, v/v) it increased the K_m by 1.3- and 1.7-fold ($P < 0.05$). This differential influence on K_m resulted in an increase in CL_{int} (1.4- and 1.5-fold) at low concentrations (0.2 and 0.5%, v/v) followed by a decrease (13 and 25%) at high concentrations (2 and 5%, v/v) ($P < 0.05$). Ethanol had the most deleterious effect of all the solvents on CYP2C8-mediated paclitaxel hydroxylation. Relative to ethanol, the effects of DMSO were minor. In general, both ethanol and DMSO showed a concentration-dependent increase of K_m and a corresponding decrease in the CL_{int} of paclitaxel: increase in the concentration of ethanol and DMSO from 0.2% to 5% (v/v) resulted in 1.6- to 8.9-fold and 1.2- to 2.0-fold increases in K_m and 36 to 89% and 17 to 52% decreases in the CL_{int} values, respectively. Acetonitrile did not significantly alter any of the enzyme kinetic parameters when incubated at concentrations of $\leq 1\%$ (v/v) ($P > 0.05$). However, 1.3- and 1.8-fold increases in the K_m and 33 and 58% decreases in CL_{int} were observed when acetonitrile was incubated at concentrations of 2 and 5% (v/v), respectively ($P < 0.05$). In contrast to the effects on K_m and CL_{int} , none of the solvents investigated had a major influence on the V_{max} of paclitaxel at concentrations of $\leq 2\%$ (v/v) ($P > 0.05$).

The results of the current study using HLM were not in agreement with the results published previously using recombinant CYP2C8 enzyme with paclitaxel as substrate (Busby et al., 1999). In the earlier study, CYP2C8 was found to be resistant to the inhibitory effects of methanol, ethanol, acetonitrile, and DMSO at concentrations of $< 3\%$ (v/v) (Busby et al., 1999). Although ethanol, acetonitrile, and DMSO showed minor inhibition ($< 20\%$) of enzyme activity at 3% (v/v), these inhibitory effects on CYP2C8 enzyme can be considered negligible when compared with the inhibition on other P450 enzymes in their study (Busby et al., 1999). These conflicting observations suggest that the same enzyme could potentially exhibit different degrees of sensitivity to organic solvents depending on the environment (HLM

versus insect cell membranes) in which the enzymes are expressed. Although speculative, the structural and chemical differences between insect cell membranes and microsomal membranes may have contributed to these differences.

The concentration-dependent effects of organic solvents (with the exception of the effect of methanol on CYP2C8 enzyme) on K_m of both the enzymes studied and the inhibitory effects of organic solvents on the V_{max} [observed only at high concentrations ($\geq 2\%$, v/v)] of these enzymes can be explained by two different mechanisms of action. Whereas the concentration-dependent effects on K_m may be caused by competitive interaction of the solvents with the substrates, their effects on V_{max} observed only at high concentrations may be the result of disruption of the integrity of the enzyme at high concentrations, leading to a decrease in the overall activity. These competitive effects observed in the present study were not without any precedence. Although we are not aware of any reports investigating these solvents as substrates of CYP2C8 and CYP2B6 enzymes per se, DMSO has long been recognized as a substrate for NADPH-dependent drug-metabolizing enzymes (Gerhards et al., 1965), and ethanol and methanol have also been identified as substrates for alcohol-metabolizing enzymes (Teschke et al., 1975). Our current results combined with the previous findings suggest that organic solvents have a potential to behave as competitive inhibitors of P450s.

In conclusion, results of the current evaluation suggest that methanol and acetonitrile at concentrations of $\leq 0.5\%$ and $\leq 1\%$ (v/v) are suitable solvents for the measurement of CYP2B6 and CYP2C8 activities in HLM, respectively. DMSO and ethanol are not suitable for characterizing CYP2B6- and CYP2C8-mediated reactions because they showed concentration-dependent effects on enzyme kinetics. To avoid misinterpretation of the data, the effects of these organic solvents should be considered before initiating in vitro P450 inhibition and reaction-phenotyping studies with new chemical entities.

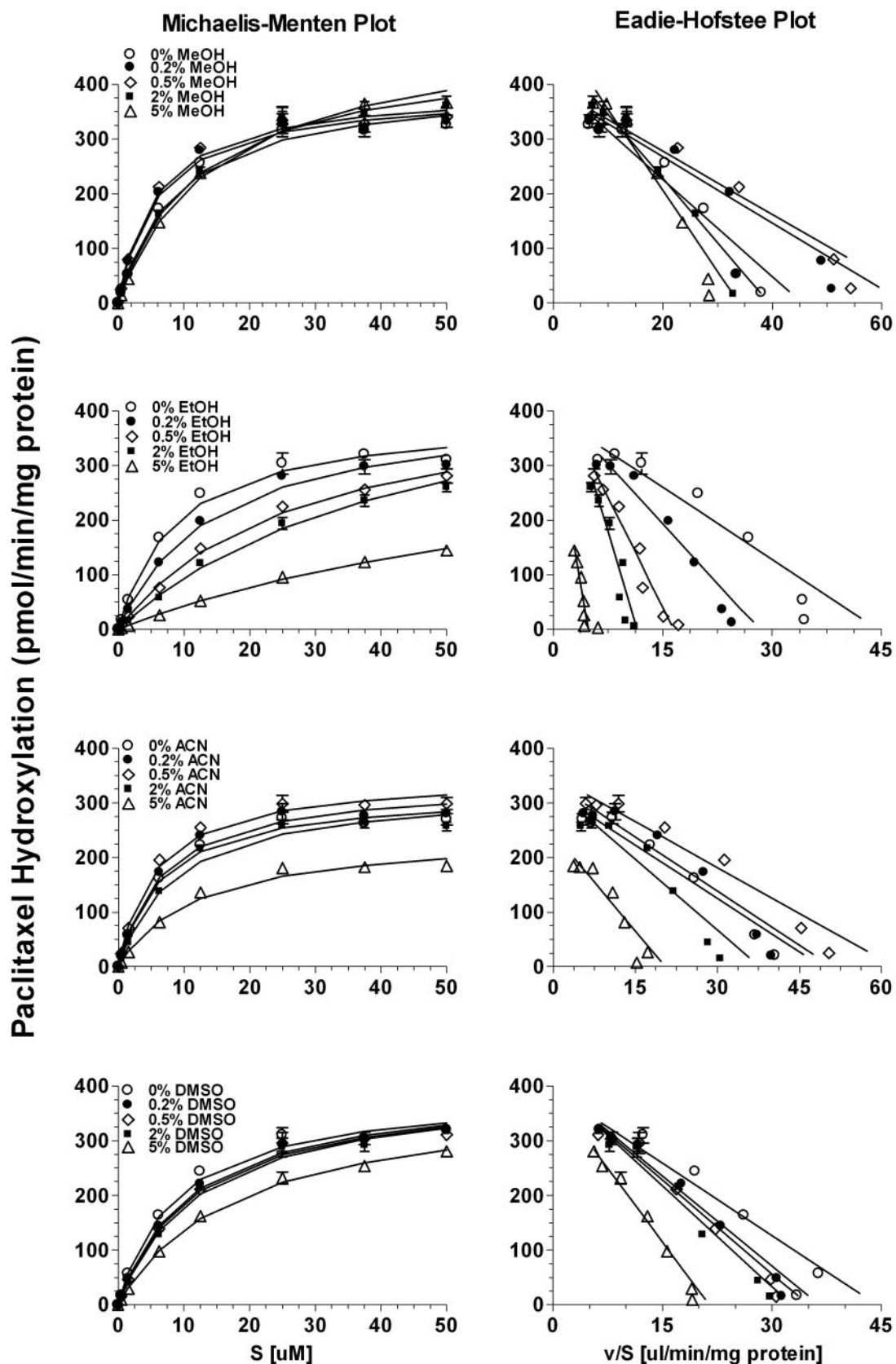


FIG. 2. Effects of methanol, ethanol, acetonitrile, and DMSO on the kinetics of 6α -hydroxypaclitaxel formation in the presence of using HLM. Michaelis-Menten (left) and Eadie-Hofstee (right) plots of 6α -hydroxypaclitaxel formation from paclitaxel. Effects of different incubation concentrations [0 (control), 0.2, 0.5, 2, and 5%, v/v] of methanol, ethanol, acetonitrile, and DMSO on the rates of 6α -hydroxypaclitaxel formation were assessed using HLM ($n = 3$). The symbols and solid lines represent the observed (mean \pm S.D.) and model-predicted values, respectively.

TABLE 2

Effect of organic solvents on the kinetics of CYP2C8-mediated paclitaxel hydroxylation in the presence of HLM

Kinetic parameters are expressed as % Control (mean \pm S.D.)

	Solvent (% v/v)			
	Methanol	Ethanol	Acetonitrile	DMSO
K_m				
0 (Control)	100.0 \pm 1.4	100.0 \pm 3.2	100.0 \pm 2.2	100.0 \pm 4.0
0.2	67.8 \pm 1.7 ^a	165.3 \pm 12.1 ^b	101.9 \pm 0.5	123.7 \pm 2.4 ^b
0.5	65.1 \pm 2.4 ^a	303.4 \pm 14.5 ^a	101.5 \pm 0.1	128.8 \pm 8.0 ^b
1	N.E.	N.E.	92.9 \pm 16.5	N.E.
2	132.0 \pm 7.6 ^b	507.4 \pm 26.5 ^a	132.3 \pm 2.2 ^a	139.0 \pm 1.6 ^a
5	166.6 \pm 6.4 ^a	893.4 \pm 53.4 ^a	183.8 \pm 12.0 ^b	202.8 \pm 7.2 ^a
V_{max}				
0 (Control)	100.0 \pm 3.2	100.0 \pm 3.4	100.0 \pm 2.1	100.0 \pm 2.0
0.2	96.1 \pm 2.9	105.0 \pm 5.4	105.3 \pm 2.5	102.4 \pm 3.3
0.5	97.4 \pm 2.6	112.6 \pm 6.5	109.2 \pm 2.2	102.2 \pm 1.2
1	N.E.	N.E.	96.6 \pm 6.5	N.E.
2	114.6 \pm 6.3	131.4 \pm 9.4	101.9 \pm 2.7	102.9 \pm 3.1
5	124.5 \pm 3.8 ^b	96.9 \pm 0.8	76.3 \pm 3.3 ^b	98.2 \pm 3.3
CL_{int}				
0 (Control)	100.0 \pm 4.6	100.0 \pm 0.2	100.0 \pm 4.3	100.0 \pm 6.0
0.2	141.7 \pm 7.7 ^b	63.5 \pm 1.4 ^c	103.2 \pm 1.9	82.6 \pm 1.0
0.5	149.8 \pm 9.5 ^b	37.1 \pm 0.4 ^c	107.5 \pm 2.2	79.4 \pm 5.8
1	N.E.	N.E.	103.4 \pm 11.5	N.E.
2	86.8 \pm 0.2 ^b	25.9 \pm 0.5 ^c	77.0 \pm 3.3 ^b	74.0 \pm 3.1 ^b
5	74.7 \pm 0.5 ^b	10.9 \pm 0.6 ^c	41.5 \pm 0.9 ^a	48.4 \pm 0.1 ^a

N.E. = Not evaluated.

^a Significantly different from control: $P < 0.01$, Student's t test.^b Significantly different from control: $P < 0.05$, Student's t test.^c Significantly different from control: $P < 0.001$, Student's t test.

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