Sulfation is an important reaction in the biotransformation of steroid hormones, neurotransmitters, drugs, and other xenobiotics, yet little is known about the effects of organic solvents on sulfotransferase (SULT) activities in vitro. Initial experiments found that surprisingly low levels of solvent had dramatic effects on sulfotransferase activity. Consequently, we evaluated the effects of five commonly used solvents (methanol, ethanol, acetonitrile, dimethyl sulfoxide, and dimethyl formamide) on activities of cDNA-expressed sulfotransferase isozymes 1A1 (4-nitrophenol sulfation), 1A3 (dopamine sulfation), 1E1 (ethynylestradiol sulfation), and 2A1 (dehydroepiandrosterone sulfation). In addition, 1-hydroxypyrene was used as a general fluorescent probe for all four sulfotransferase isoforms examined. When substrates were present at their respective isoform-specific $K_m$ values, methanol and ethanol (0.4%, v/v) generally had less effect than acetonitrile, dimethyl sulfoxide, and dimethyl formamide on sulfotransferase activities. Acetonitrile, a commonly used solvent in cytochrome P450 studies, inhibited SULT1A1 activities (−40%) at 0.4% (v/v), but activated SULT1E1-mediated 1-hydroxypyrene sulfation 2.6-fold. Assuming a two-site kinetic model, studies revealed that solvent affected $V_{max}$, $K_{max}$, and the $K_m$ value of 1-hydroxypyrene sulfation mediated by SULT1E1. In contrast, the $K_m$ value was not affected, suggesting that solvent may potentially alter binding interactions of the second substrate molecule, but not the first. Additional experiments with expressed SULT1A1, supplemented with control protein, revealed that the inhibitory effect of solvent (0.4%, v/v) was reduced to <15% for all solvents examined. Thus, it is recommended that ethanol is used as the preferred solvent vehicle and that incubations with expressed enzyme contain >12 μg/ml total protein.

Sulfate conjugation is an important pathway in the detoxication of xenobiotics as well as in the modulation of endogenous compounds such as thyroid hormones, steroids, and neurotransmitters (Coughtrie et al., 1998). This reaction is catalyzed by sulfotransferases (SULTs1), a family of cytosolic enzymes (Nagata and Yamazoe, 2000). Sulfotransferases facilitate the transfer of sulfate from a cosubstrate, 3'-phosphoadenosine-5'-phosphosulfate (PAPS), to an acceptor molecule forming a sulfate conjugate. In human, at least 11 members of SULT have been identified and divided into several gene families based on their amino acid sequence and substrate specificity. Members of SULT1 family (divided into four subfamilies: 1A, 1B, 1C, and 1E) sulfotransferase isozymes isozyme 1A1 (4-nitrophenol sulfation), 1A3 (dopamine sulfation), 1E1 (ethynylestradiol sulfation), and 2A1 (dehydroepiandrosterone sulfation). In addition, 1-hydroxypyrene was used as a general fluorescent probe for all four sulfotransferase isoforms examined. When substrates were present at their respective isoform-specific $K_m$ values, methanol and ethanol (0.4%, v/v) generally had less effect than acetonitrile, dimethyl sulfoxide, and dimethyl formamide on sulfotransferase activities. Acetonitrile, a commonly used solvent in cytochrome P450 studies, inhibited SULT1A1 activities (−40%) at 0.4% (v/v), but activated SULT1E1-mediated 1-hydroxypyrene sulfation 2.6-fold. Assuming a two-site kinetic model, studies revealed that solvent affected $V_{max}$, $K_{max}$, and the $K_m$ value of 1-hydroxypyrene sulfation mediated by SULT1E1. In contrast, the $K_m$ value was not affected, suggesting that solvent may potentially alter binding interactions of the second substrate molecule, but not the first. Additional experiments with expressed SULT1A1, supplemented with control protein, revealed that the inhibitory effect of solvent (0.4%, v/v) was reduced to <15% for all solvents examined. Thus, it is recommended that ethanol is used as the preferred solvent vehicle and that incubations with expressed enzyme contain >12 μg/ml total protein.

### Materials and Methods

**Chemicals.** PAPS, 1-hydroxypyrene, formic acid, ammonium formate, 4-nitrophenol, 4-nitrophenol sulfate, dopamine, dehydroepiandrosterone (DHEA), sodium EDTA, and sodium 1-decanesulfonic acid were purchased from Sigma-Aldrich (St. Louis, MO). [$^3$H]Dopamine, [$^3$H]ethynylestradiol, and [$^3$H]DHEA were obtained from PerkinElmer Life Sciences (Boston, MA). All solvents and other reagents were of analytical grade or higher.

**Human Tissue Cytosol and cDNA-Expressed Sulfotransferases.** Cytosol prepared from human liver and jejunum were purchased from Xenotech, LLC (Lenexa, KS) and Tissue Transformation Technologies (Edison, NJ), respec-
Recombinant human sulfotransferase prepared from insect cells, infected with baculovirus containing human SULT cDNA, was obtained in-house (SULTs 1A1 and 1A3; Merck Research Laboratories, West Point, PA) or from PanVera Corp. (SULTs 1E1 and 2A1; Madison, WI).

Incubations. Incubation mixtures (0.5 ml) contained 100 mM sodium phosphate (pH 7.4), 1 mM magnesium chloride, 0.19 to 80 μg of cytosolic protein, 0 to 10 μl of solvent, 50 μM PAPS, and probe substrates. 4-Nitrophenol (1 μM), dopamine (10 μM), ethynylestradiol (6 nM), and DHEA (2 μM) were used as marker substrates for SULTs 1A1, 1A3, 1E1, and 2A1, respectively. 1-Hydroxypyrene was used at the concentration of 10 nM, 1 μM, 20 nM, and 2 μM for SULTs 1A1, 1A3, 1E1, and 2A1, respectively. Concentration of the marker substrates was chosen based on the \( K_m \) values reported in the literature (Falany et al., 1989; Veronese et al., 1994) or determined in-house (Table 1) (Schrag et al., 2002). For ethynylestradiol and DHEA, stock solutions in organic solvent (by the vendor) were pipetted into clean tubes and then dried under nitrogen. The dried substrate was resuspended in the incubation mixture, followed by sonication for 1 min. The recovery of the substrate in the incubation mixture was determined to be \( >95\% \). The amount of cytosolic protein used for 1-hydroxypyrene sulfation in expressed SULTs 1A1, 1A3, 1E1, and 2A1 was 0.4, 1.38, 0.19, and 0.9 μg, respectively. In addition, the activity of SULTs 1A1 (4-nitrophenol), 1A3 (dopamine), 1E1 (ethynylestradiol), and 2A1 (DHEA) was determined using 1.6, 20, 0.19, and 6.75 αM.

Table 1: Kinetic parameters for 1-hydroxypyrene sulfation by cDNA-expressed sulfotransferases

<table>
<thead>
<tr>
<th>SULT isoform</th>
<th>( K_m^* ) μM</th>
<th>( K_i ) μM</th>
<th>( V_{max1} ) pmol/min/μg protein</th>
<th>( V_{max2} ) pmol/min/μg protein</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1</td>
<td>0.008 ± 0.001</td>
<td>0.151 ± 0.035</td>
<td>378 ± 17</td>
<td>181 ± 4</td>
<td>0.994</td>
</tr>
<tr>
<td>1A3</td>
<td>0.8 ± 0.1</td>
<td>1.7 ± 0.3</td>
<td>2268 ± 227</td>
<td>0 ± 29</td>
<td>0.998</td>
</tr>
<tr>
<td>1E1</td>
<td>0.021 ± 0.006</td>
<td>0.176 ± 0.316</td>
<td>81 ± 7</td>
<td>67 ± 3</td>
<td>0.973</td>
</tr>
<tr>
<td>2A1</td>
<td>2.3 ± 0.1</td>
<td>45 ± 1</td>
<td>378 ± 17</td>
<td>181 ± 4</td>
<td>0.998</td>
</tr>
</tbody>
</table>

* Kinetic analysis was performed using eq. 1 listed under Materials and Methods. Data from SULT2A1 was fitted to the Michaelis-Menten equation. Values are denoted as mean ± standard error.

Fig. 1. Kinetics of 1-hydroxypyrene sulfation in cDNA-expressed SULTs 1A1 (A), 1A3 (B), 1E1 (C), and 2A1 (D).

Incubations were described under Materials and Methods. Each data point represents the mean of three determinations (for SULTs 1A1 and 2A1) or two determinations (for SULTs 1A3 and 1E1).
µg of protein, respectively. Sulfation of 4-nitrophenol and 1-hydroxypyrene was performed using 10 and 2 µg of human liver cytosol, whereas 80 and 6 µg were used in human jejunum preparations, respectively. The reaction was initiated with the addition of PAPS after a 2-min preincubation at 37°C. Acetonitrile (0.1 ml) was added to stop the reaction at 5 to 30 min. The samples were centrifuged at 2000 g for 10 min, and the supernatant fractions were analyzed by HPLC. Standard curves of the sulfation product were run at the beginning and end of each HPLC analytical run to confirm metabolite stability.

For the kinetic studies of 1-hydroxypyrene sulfation, 0.2 to 1.4 µg of cytosolic protein were incubated with 4 nM to 20 µM substrate for 8 min. Substrate consumption was <8% in all incubations performed. The formation rate of the sulfate product was linear with respect to incubation time as well as the amount of cytosolic protein used. To estimate the amount of 1-hydroxypyrene sulfate, a standard curve was prepared by incubating a known concentration of 1-hydroxypyrene in the presence of PAPS and SULT1A1 and letting the reaction go to completion. The resulting yield of 1-hydroxypyrene sulfate was >97% in all cases.

HPLC Analysis. HPLC analyses were conducted with an Agilent 1100 LC system (Agilent Technologies, Palo Alto, CA), equipped with an auto-injector, a fluorescence detector, and a diode array detector. For titrated samples, column eluent was analyzed by a Packard (500TR, PerkinElmer Life Sciences) or IN/US (β-ram 2, IN/US Systems, Inc., Tampa, FL) online radiochemical detector equipped with a liquid flow cell. The flow of scintillation cocktail (Packard Ultima-Flo M) was maintained at 3 ml/min. Separation of parent substrate and its sulfate conjugate was achieved by the following methods.

4-Nitrophenol sulfation. 4-Nitrophenol and its sulfate were separated using a method developed by Duescher and Elfarra (1993) with modifications. Analyte was eluted through a Zorbax XDB-C18 column (4.6 mm × 25 cm, 5 µm; Agilent Technologies) at a constant flow of 1 ml/min with 30% (v/v) acetonitrile in water containing 0.1% trifluoroacetic acid. Column effluent was monitored by UV absorbance at 300 nm.

Dopamine sulfation. An HPLC method using sodium decanesulfonate as the ion-pairing agent was adapted to separate dopamine and its sulfate (Hollenbach et al., 1998). Separation was performed on a Spherisorb ODS-2 column (4.6 mm × 25 cm, 5 µm; Thomson Instrument Company, Oceanside, CA). The mobile phase consisted of 0.15 M sodium phosphate (pH 4.0) with 10 mM sodium decanesulfonate and 0.1 mM sodium EDTA in water (solvent A) and methanol (solvent B) at a constant flow of 1 ml/min. The solvent gradient initiated at 5% B for 5 min and then increased linearly to 80% B in 5 min. After isocratic flow (80% B) for 5 min, the gradient returned to 5% B in 0.5 min. The column was re-equilibrated at initial conditions for 7.5 min before injection of the next sample.

Ethynylestradiol sulfation. Ethynylestradiol and its sulfate (3 position) were separated using a Zorbax SB-C8 column (4.6 mm × 7.5 cm, 3.5 µm). The mobile phase consisted of 25 mM ammonium formate, pH 3.0 (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The solvent gradient started from 30% B at 1 ml/min and increased linearly to 50% B in 8 min. The gradient returned to 30% B in 0.2 min. Followed by re-equilibration for 4.8 min.

DHEA sulfation. An HPLC method similar to that described above for ethynylestradiol was used with minor modifications. A Metachem Inertsil ODS-3 column (2.1 mm × 5 cm, 5 µm; Ansys Technologies Inc., Lake Forrest, CA) was used with a solvent gradient that increased from 10% B to 80% B in 8 min, and then returned back to 10% B in 0.2 min. The column was re-equilibrated for 4.8 min before the next injection.

1-Hydroxypyrene sulfation. The method established for ethynylestradiol was slightly modified for 1-hydroxypyrene sulfation. The solvent gradient

![Fig. 2. Solvent effect (0.4%) on sulfotransferase activities of SULTs 1A1 (A), 1A3 (B), 1E1 (C), and 2A1 (D).](image-url)
increased from 45% B to 80% B in 0.5 min. After holding at 80% B for 4 min, the gradient returned to 45% B in 0.5 min. The column was then re-equili-
brated at initial conditions for 2 min before injection of the next sample. 
Fluorescence detection was performed with excitation and emission wave-
lengt hes set at 340 nm and 390 nm, respectively.

**Determination of Kinetic Parameters.** With the exception of 1-hydroxy-
pyrene sulfation in SULT2A1, data from the kinetic studies were fitted into the 
equation derived from a two-substrate model by Zhang et al. (1998).

\[
V = \frac{V_{\text{max1}}(1 + \frac{V_{\text{max2}}S}{V_{\text{max1}}K_i})}{1 + (K_m/S) + (S/K_i)}
\]

It is important to note that the $K_m$ value for the binding of the second 
substrate is denoted as $K_i$ in this equation. Data from SULT2A1-mediated 
1-hydroxypyrene sulfation were fitted into the Michaelis and Menten equation.
Curve-fitting was performed using SigmaPlot (SPSS Inc., Chicago, IL).

**Results and Discussion**

In the present study, probe substrates were used at concentrations 
corresponding to isoform-specific apparent $K_m$ values. Dopamine, 
ethynylestradiol, and DHEA sulfates were detected using [$^3$H]-labeled 
material, whereas the sulfate of 1-hydroxypyrene was detected with 
fluorescence. 1-Hydroxypyrene was a substrate for all the sulfo-
transferase isoforms tested. Thus, it was developed as a sensitive, conven-
nient, and universal probe to monitor sulfotransferase activity.

Kinetic studies of 1-hydroxypyrene sulfation by different SULT 
isozymes revealed that substrate inhibition, a phenomenon commonly 
observed in sulfate conjugation (Sundaram et al., 1989), was detected 
in reactions catalyzed by SULTs 1A1, 1A3, and 1E1, but not 
SULT2A1 (Fig. 1). Since the consumption of 1-hydroxypyrene was 
<8% in all incubations performed, it is unlikely that the formation and 
accumulation of 3', 5'-diphosphoadenosine caused the observed in-
hibition. Based upon a previous report in the literature by Zhang et al. 
(1998), a two-site model was selected to describe the kinetic data 
observed. The use of this model is further supported by the recent 
observation of two 4-nitrophenol molecules within the active site of 
crystalized SULT1A1 (Gamage et al., 2003). Moreover, it was also 
observed that the corresponding SULT1A1-mediated sulfation of 
4-nitrophenol yielded substrate inhibition kinetics, similar to that 
observed for sulfation of 1-hydroxypyrene. The apparent $K_m$, $K_i$, and 
$V_{\text{max}}$ values for 1-hydroxypyrene sulfation are reported in Table 1. It is 
interesting to note that the $K_m$ value of the first binding site ranged 
from 8 nM to 3 μM, indicating that 1-hydroxypyrene has relatively 
high affinity for all four SULT isoforms tested.

The effect of different solvents on cDNA-expressed SULT activi-
ties was shown in Fig. 2. In general, the alcohols, methanol and 
ethanol, had relatively less inhibitory effects on SULT activities than 
acetonitrile, dimethyl sulfoxide, and dimethyl formamide. Statistical 
analysis (analysis of variance test) revealed that ethanol had the least 
inhibitory effect on SULT1A1-mediated 1-hydroxypyrene sulfation.

![Effect of ethanol on activities of SULTs 1A1 (A), 1A3 (B), 1E1 (C), and 2A1 (D).](image_url)
among the five solvents tested ($p < 0.001$). In contrast, dimethyl formamide inhibition of 1-hydroxypyrene sulfation catalyzed by SULT1A3 ($p < 0.001$) and SULT2A1 ($p < 0.05$) was more significant than the other test solvents. Acetonitrile, the preferred solvent in cytochrome P450-mediated reactions (Chauret et al., 1998; Busby et al., 1999), significantly inhibited SULT1A1 activity by ~40%, but activated SULT1E1-mediated 1-hydroxypyrene sulfation ~2.6-fold ($p < 0.001$).

Ethanol was chosen as a probe solvent to study the concentration-dependent inhibition of sulfotransferase activities (Fig. 3). In all cases, the inhibition of sulfotransferase activity increased with ethanol concentration. Generally, 0.1 and 0.5% (v/v) ethanol inhibited SULT activities up to 10 and 20%, respectively. Ethanol content at 2% (v/v) was sufficient to inhibit ≥40% of the SULT1A1 activity and ≥60% of the SULT1E1 activity, whereas ≤25% activity was inhibited for SULT1A3 and SULT2A1, indicating SULTs 1A1 and 1E1 are more susceptible to ethanol than other SULT isoforms. In addition, 1-hydroxypyrene sulfation appeared to be slightly more sensitive to ethanol, especially at 1 to 2% (v/v), as compared with other known substrates.

The acetonitrile-mediated activation of 1-hydroxypyrene sulfation (SULT1E1) was examined with additional kinetic studies. As acetonitrile content was increased from 0 to 0.4% (v/v), the apparent first and second $V_{\text{max}}$ values, determined by fitting data into a two-substrate binding model, increased 58 and 328%, respectively (Fig. 4). The mean of two determinations.

![A. Acetonitrile](image)

![B. Ethanol](image)

**Fig. 4.** Effect of acetonitrile (A) and ethanol (B) on the kinetics of 1-hydroxypyrene sulfation by SULT1E1.

1-Hydroxypyrene sulfation was performed in the presence of 0% (●), 0.4% (□), and 2% (▲) (v/v) acetonitrile (panel A) or ethanol (panel B). Each data point represents the mean of two determinations.

1-Hydroxypyrene sulfation was also studied. In agreement with the results shown in Fig. 2C, SULT1E1 activity was inhibited by ethanol in a concentration-dependent manner with a resulting ~75% drop in both of the $V_{\text{max}}$ values in the presence of 2% (v/v) ethanol. However, the apparent $K_m$ value was not significantly affected by ethanol at concentration up to 2% (v/v), similar to that observed in acetonitrile. In the context of a two-site model, these results suggest that the impact of solvent lies on binding of the second substrate molecule ($K_i$) and its effects on productive catalysis ($V_{\text{max}}$). It is also noteworthy that effects on $V_{\text{max}}$ could be due to a number of factors such as protein conformational change or altered affinity/binding of PAPS.

In addition to experiments with expressed enzyme, the effect of solvent was investigated in tissue cytosol. Substrates for SULT1A1, 4-nitrophenol and 1-hydroxypyrene, were chosen for this study because expressed SULT1A1 was relatively more sensitive to solvents as compared with other SULT isoforms (Fig. 1). In general, SULT1A1 activity in cytosolic preparation of human liver (Fig. 5A) and jejunum (Fig. 5B) was relatively more resistant to solvents [up to 0.4% (v/v)] than that determined for recombinant SULT1A1. To determine whether sulfotransferase is stabilized by the higher level of protein in the tissue preparations (5- to 50-fold higher, relative to expressed enzyme), the solvent effect on 4-nitrophenol and 1-hydroxypyrene sulfation in expressed SULT1A1 was further examined. Incubations containing expressed SULT1A1 were supplemented with cytosolic protein from control insect cells (12 μg/ml final concentration). The inhibitory effect of solvent [0.4% (v/v) methanol, ethanol, acetonitrile, dimethyl sulfoxide, or dimethyl formamide] on SULT1A1 activity was reduced to <15% of control values in the presence of supplemented protein (data not shown), indicating that the addition of control cytosolic protein can counterbalance the effect of

**TABLE 2**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Percentage (v/v)</th>
<th>$K_m$</th>
<th>$K_i$</th>
<th>$V_{\text{max}1}$</th>
<th>$V_{\text{max}2}$</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>0.4</td>
<td>0.021 ± 0.006</td>
<td>0.176 ± 0.316</td>
<td>81 ± 7</td>
<td>67 ± 3</td>
<td>0.973</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>2</td>
<td>0.022 ± 0.011</td>
<td>0.333 ± 0.246</td>
<td>128 ± 37</td>
<td>220 ± 5</td>
<td>0.994</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.4</td>
<td>0.020 ± 0.016</td>
<td>0.780 ± 0.664</td>
<td>49 ± 17</td>
<td>108 ± 8</td>
<td>0.968</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2</td>
<td>0.029 ± 0.003</td>
<td>0.402 ± 0.165</td>
<td>75 ± 4</td>
<td>48 ± 1</td>
<td>0.993</td>
</tr>
</tbody>
</table>

* Kinetic analysis was performed using eq. 1 listed under Materials and Methods. Values are denoted as mean ± standard error.
The open bar represents 4-nitrophenol, whereas the hatched bar represents 1-hydroxypyrene. Each data point is equivalent to the mean ± standard deviation (n = 3). Buffer was used in the no-solvent controls.

Acknowledgments. We thank Dr. Tom Rushmore for cloning and expressing the human SULTs 1A1 and 1A3 genes.

References


