The Effects of Single Nucleotide Polymorphisms in CYP2A13 on Metabolism of 5-Methoxypsoralen

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Received June 2, 2010; accepted August 26, 2010

ABSTRACT:

A number of studies have demonstrated that cytochrome P450 (P450) catalyzes monooxygenation at the first step in the metabolism of xenobiotic compounds. In some cases, the catalysis by P450 can generate reactive intermediates that are harmful for human health. Several dietary constituents are converted into toxic reactive metabolites in humans (Zhou et al., 2007). Alkenylbenzenes, such as estragole, safrrole, and methylcyclopentadiene, are genotoxic and carcinogenic through mechanisms initiated by P450 proteins (Jeuringen et al., 2004, 2006, 2007). In addition, certain metabolic products of capsaicin may be harmful when covalently bonded with biomolecules (Reilly and Yost, 2006). Although these studies show potential risks of dietary constituents caused by normal metabolism in human, the risks in foods are not generally investigated as often as the hazards in drugs.

Many beverages and oils derived from fruits and vegetables contain furanocoumarin derivatives, which form covalent bonds to biomolecules. 5-Methoxypsoralen (5-MOP) is a natural furanocoumarin from apiaceous plants. Koenigs and Trager (1998) demonstrated that five furanocoumarins, 5-methoxypsoralen (5-MOP), 8-methoxypsoralen (8-MOP), psoralen, 5-hydroxypsoralen, and 8-hydroxypsoralen, were converted into reactive intermediates. They also showed that efficiency of 8-MOP for the metabolic activation was higher than those of the other derivatives. CYP2A13 is involved in the metabolism of coumarin, nicotine, 4-(methylthio)isocoumarin-1-(3-pyridyl)-1-butanes (NNK), 4-amino-phenyl, phenacetin, theophylline, and aflatoxin B1 (Su et al., 2000; von Weymarn and Murphy, 2003; Bao et al., 2005; He et al., 2006; Nakajima et al., 2006; Fukami et al., 2007). CYP2A13 is involved in the metabolism of coumarin, nicotine, 4-(methylthio)isocoumarin-1-(3-pyridyl)-1-butanes (NNK), 4-amino-phenyl, phenacetin, theophylline, and aflatoxin B1 (Su et al., 2000; von Weymarn and Murphy, 2003; Bao et al., 2005; He et al., 2006; Nakajima et al., 2006; Fukami et al., 2007). Substrate selectivity of CYP2A13 overlaps that of CYP2A6; for example, coumarin, nicotine, and NNK are common substrate of these enzymes. Like CYP2A6, a furanocoumarin derivative 8-MOP was indicated to be converted into metabolic-activated intermediates by CYP2A13 (Koenigs et al., 1997; von Weymarn et al., 2005). Although these studies imply that CYP2A13 is likely to be involved in the metabolism of 5-MOP, there is no article to indentify metabolites or metabolic activity.

Previous studies show that the metabolic activity of the P450 enzyme is influenced by single nucleotide polymorphisms (SNPs).
Nine kinds of SNPs are known in the CYP2A13 gene, and some alleles show regional differences; the CYP2A13*4 allele is more frequent in French whites (3.8%) than in the Japanese population (0.3%), whereas the CYP2A13*8 allele frequency in French whites (1%) is lower than in Japanese (4.9%) and Chinese populations (1.8%) (Wang et al., 2006). Some of the mutations are found in the highly conserved region in P450 enzymes. Arginine 101, which is substituted in the CYP2A13*4, is positioned in putative substrate recognition site 1 and conserved in all human P450 subfamily 1 and 2. Valine 323, which is conserved in most xenobiotic P450s (CYP1A1, CYP2A6, 2B6, 2C8/9/18, 2D6, and 3A4/5), is substituted in the CYP2A13*9 allele. These substitutions can affect substrate specificity or metabolic turnover of the CYP2A13 enzyme. However, functional differences in the SNP variants of CYP2A13 are limited; the studies using recombinant proteins expressed in mammalian cell cultures showed that the CYP2A13*4 protein has decreased metabolic activity on NNK and aflatoxin B1, and that the CYP2A13*2 protein is less efficient in metabolic activation of NNK than wild-type CYP2A13*1 (Zhang et al., 2002; Wang et al., 2003, 2006).

To reveal the effects of SNPs within human CYP2A13 on metabolism of 5-MOP, we used recombinant CYP2A13 enzyme variants overexpressed in Escherichia coli. We demonstrate that the CYP2A13 enzyme is involved in the conversion of 5-MOP into a dihydrodiol form, and that the enzymatic activity of the recombinant CYP2A13*4 enzyme is remarkably decreased. These results show that SNPs within CYP2A13 may affect the pharmacokinetics of 5-MOP.

Materials and Methods

Chemicals. 5-MOP was purchased from Sigma-Aldrich (St. Louis, MO). 7-Hydroxycoumarin (7-HC) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 5-Aminolevulinic acid hydrochloride, isopropyl thioacetamide, and several amino acid residue substitutions (24WRQRKSR30 to24AKKTSSK30) were produced into the fragment using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). Sequences of oligonucleotide primers for mutagenesis were as follows: CYP2A13QC4F, 5'-GAGCTTCCTGCCCTGCTGAGTCGACCT-3'; CYP2A13QC4R, 5'-AGTTCAGCGGGCAAGGCGAGCAGGC-3'; CYP2A13QC5F, 5'-GGAGCTCTTTCTCTTCTACAC-3'; CYP2A13QC5R, 5'-TCTGCATGATGGT-3'; CYP2A13QC6F, 5'-GGAGAGGGCCTCGATGAG-3'; CYP2A13QC6R, 5'-GGGCTTCCTCATCGAG-3'; CYP2A13QC8F, 5'-GAGAGCGCTCGCCTTGCCCGCTGAACT-3'; CYP2A13QC8R, 5'-GAGCTTCCTGCCCTGCTGAGTCGACCT-3'. The substitutions were confirmed by DNA sequence analyses. FIG. 1. A, amino acid sequence alignment of CYP2A13 variants. Modified residues at N termini are in italics, and substituted nucleotides are underlined. B, positions of the amino acid substitutions performed in this study are displayed on wild-type CYP2A13. The basic structure was determined by Smith et al. (2007). Gray sticks, side chains of the residue substituted. Black sticks, heme; black sticks, side chains of the residue substituted.
4°C to sediment the homogenized membrane fraction of the *E. coli* cells. The pellets were resuspended in 6 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 20% glycerol. These fractions were stored at −80°C.

**Validation of P450 Activity and Protein Concentration.** Protein concentration in the membrane fraction was determined by Bradford’s method (Bradford, 1976) using the Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). A standard curve was constructed from diluted bovine serum albumin (0.2–1.0 mg/ml). Reduced CO difference spectra were measured with a Hitachi UV-visible spectrophotometer U-3300 (Hitachi, Tokyo, Japan), according to the protocol published by Imaishi et al. (2000). The P450 hemoprotein contents in the membrane suspensions were determined with an extinction coefficient of 91.1 mM⁻¹ cm⁻¹ (Omura and Sato, 1964).

**Western Blot Analyses.** Production of recombinant CYP2A13 proteins in *E. coli* was detected by immunoblotting with a polyclonal antibody against CYP2A13 (AVIVA Systems Biology LLC, San Diego, CA) diluted 600-fold in Tris-buffered saline Tween 20. The membrane suspensions from *E. coli* containing 0.5 g of total protein were separated by electrophoresis (10% polyacrylamide gel) and transferred to polyvinylidene difluoride membranes by using an iBLOT apparatus (Invitrogen, Carlsbad, CA). A donkey anti-rabbit IgG conjugated to horseradish peroxidase was used as a secondary antibody (1:10,000). The immunoblot was visualized with an ECL Detection System (GE Healthcare UK Ltd., Buckinghamshire, England), according to the manufacturer’s protocol.

**Metabolic Assessment of 5-MOP and 8-MOP.** The reaction mixture was composed of 100 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 20% glycerol, 5 mM glucose 6-phosphate, 1 mM NADPH, and 1 unit/ml glucose 6-phosphate dehydrogenase. In all experiments, the membrane suspensions containing approximately 100 μg of protein were added to 1 ml of the reaction mixture. Concentrations of the CYP2A13 variant enzyme in each reaction mixture were estimated as described above (CYP2A13*1, 65.9 pmol/ml; CYP2A13*4, not determined; CYP2A13*5, 45.1 pmol/ml; CYP2A13*8, 68.6 pmol/ml; CYP2A13*9, 52.7 pmol/ml). The reaction mixtures were incubated at 37°C with shaking at 90 rpm for 0, 5, 15, 30, and 60 min. For measurement of the production of 5-MOP dihydrodiol, 10 μl of 5-MOP solution with dimethyl sulfoxide was added to the mixtures to reach final concentrations of 5 μM 5-MOP. The reaction mixtures were incubated at 37°C with shaking at 90 rpm for 5 min. Enzymatic reactions of CYP2A13 variants were stopped by addition of 250 μl of HCl (1 N) and 3 ml of ethyl acetate to the reaction mixtures, followed by 10 μl of 7-HC (0.5 mM) as an internal control. The organic layers were dried by evaporation, and the residue was dissolved in 150 μl of methanol. Insoluble matter was removed with a Millex-LH filter (Millipore), before proceeding with high-performance liquid chromatography (HPLC) analysis.

**HPLC Analysis.** Twenty microliters of extract was analyzed in an HPLC system (Hitachi High-Technologies Co., Tokyo, Japan) equipped with a TSK-gel C18 column ODS-80Ts (150 × 4.6 mm) (Tosoh Co., Tokyo, Japan). The mobility phase was changed with a linear gradient from 37.5 to 100% methanol over 15 min, and absorbance of metabolites were monitored at 324 nm. The metabolic product was quantified by its ratio to 7-HC.

Fig. 2. Heterologous expression of human CYP2A13 variants representing polymorphisms in *E. coli*. A, SDS-PAGE (left) and Western blot (right) analyses of membrane preparation from transformed *E. coli* cells. The recombinant proteins of CYP2A13 polymorphisms were successfully expressed. The contents of each lane are as follows: lane 1, nonexpressed control using the empty pCW vector; lane 2, CYP2A13*1; lane 3, CYP2A13*4; lane 4, CYP2A13*5; lane 5, CYP2A13*6; lane 6, CYP2A13*8; lane 7, CYP2A13*9. B, reduced CO difference spectra of membrane preparations. An increase in the absorbance near 450 nm was not observed in the spectrum of CYP2A13*4.
Preparation of 5-MOP Dihydrodiol Standard. 5-MOP dihydrodiol standard was isolated by thin-layer chromatography from metabolites of 5-MOP by human CYP2A13 recombinant proteins. The structure of 5-MOP dihydrodiol was determined by liquid chromatography-mass spectrometry and NMR analyses.

Results

Expression of Human CYP2A13 and Its Variants. We constructed bacterial expression vectors encoding CYP2A13*1 (wild type) and five SNP variants [CYP2A13*4 (R101Q), CYP2A13*5 (F453Y), CYP2A13*6 (R494C), CYP2A13*8 (D158E), and CYP2A13*9 (V323L)] (Fig. 1A). The N-terminal region of each construct was modified to yield recombinant proteins from the E. coli expression system (Fig. 1A, italic letters). Substituted residues in each SNP variant are underlined in Fig. 1A and labeled on a structural model of the human CYP2A13 enzyme in Fig. 1B. We prepared membrane fractions from E. coli transformed with each SNP variant of CYP2A13 and analyzed the membrane fractions by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot. Major bands were present at the estimated molecular mass of CYP2A13 (54 kDa), and a specific antibody also recognized these bands (Fig. 2A, arrowhead). Bands with the same mobility as CYP2A13*1 were present in the membrane fractions of E. coli transformed by four variants (CYP2A13*5, CYP2A13*6, CYP2A13*8, and CYP2A13*9). Electrophoretic mobility of the recombinant CYP2A13*4 protein was slightly lower than other recombinant CYP2A13 proteins, but this protein was recognized by the anti-CYP2A13 antibody. These results suggest that protein moieties of the recombinant CYP2A13 enzymes were successfully synthesized in our E. coli expression system.

After establishing the validity of our expression system, we measured reduced CO difference spectra from membrane fractions of CYP2A13 variants (Fig. 2B). The wavelength showing maximum increase of absorption after CO treatment (λ_{max}) for the CYP2A13*1 enzyme was 450.8 nm, indicating the presence of functional cytochrome P450 enzyme. Reduced CO difference spectrum analyses also show that the λ_{max} values for CYP2A13*5, CYP2A13*6, CYP2A13*8, and CYP2A13*9 variants are 449.5, 450.6, 449.9, and 450.2 nm, respectively (Fig. 2B). The expression levels of CYP2A13*1, CYP2A13*5, CYP2A13*6, CYP2A13*8, and CYP2A13*9 were 1.3 ± 0.53, 1.1 ± 0.49, 1.0 ± 0.49, 1.2 ± 0.50, and 0.79 ± 0.34 nmol/mg total proteins (mean ± S.D.), respectively. In contrast, the reduced CO difference spectrum of CYP2A13*4 shows that absorption did not increase at approximately 450 nm but that the peak position is 420 nm (Fig. 2B). We estimate from SDS-PAGE analyses that the expression of CYP2A13*4 was approximately 5% of the total protein, which is similar to the other variants.

Metabolism of 5-MOP by Human CYP2A13. We performed HPLC analyses to validate the creation of 5-MOP metabolites by CYP2A13. Retention times of 7-hydroxycoumarin and of 5-MOP were 6.3 and 11.3 min, respectively (Fig. 3). A peak with a retention time of 5.2 min appeared in the metabolic assay in membrane fractions that included CYP2A13*1 enzymes (Fig. 3A). In the absence of NADPH, the peak disappeared from the HPLC chromatogram (Fig. 3B), and there was no peak at 5.2 min in the HPLC chromatogram using membrane fractions from E. coli transformed with an empty vector (Fig. 3C). Therefore, the peak with a retention time of 5.2 min was a main metabolic product of 5-MOP that is generated by CYP2A13 enzymes. We isolated the peak with a retention time of 5.2 min and identified the peak as 5-MOP dihydrodiol by liquid chromatography-mass spectrometry and NMR structural analyses (data not shown). Koenigs and Trager (1998) proposed that 5-MOP dihydrodiol is a product of 5-MOP oxidation by CYP2A6. We also added CYP2A13*1 enzymes to 8-MOP and analyzed the resulting metabolites of 8-MOP by HPLC. No peaks that signify a metabolite of 8-MOP were detectable, leading us to the hypothesis that the metabolic activity of CYP2A13 on 5-MOP may be higher than that on 8-MOP (Fig. 3D). 5-MOP dihydrodiol was also produced in the experiments with the variants CYP2A13*5, CYP2A13*6, CYP2A13*8, and CYP2A13*9 (Fig. 3, F-I). In metabolic experiments with CYP2A13*4, there were no chromatogram peaks that could be ascribed to 5-MOP dihydrodiol or other metabolites, which demonstrates that the CYP2A13*4 protein has no ability to metabolize 5-MOP (Fig. 3E).

We next determined the kinetic parameters of 5-MOP metabolism by CYP2A13 variant enzymes. The reactions with CYP2A13*1, CYP2A13*5, CYP2A13*6, CYP2A13*8, and CYP2A13*9 enzymes followed Michaelis-Menten kinetics (Fig. 4). As shown in Table 1, K_{m}}
Materials and Methods

TABLE 1

<table>
<thead>
<tr>
<th>Variant</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (nmol/min · nmol P450)</th>
<th>$V_{max}/K_m$</th>
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<tr>
<td>CYP2A13*1</td>
<td>1.44 ± 0.17</td>
<td>4.23 ± 0.36</td>
<td>2.98 ± 0.17</td>
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<tr>
<td>CYP2A13*4a</td>
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<td>N.D.</td>
<td>N.D.</td>
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<td>CYP2A13*5</td>
<td>1.63 ± 0.12</td>
<td>3.20 ± 0.13</td>
<td>1.99 ± 0.13</td>
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<tr>
<td>CYP2A13*6</td>
<td>1.36 ± 0.10</td>
<td>4.69 ± 0.13</td>
<td>3.47 ± 0.17</td>
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<tr>
<td>CYP2A13*8</td>
<td>0.85 ± 0.09</td>
<td>2.34 ± 0.07</td>
<td>2.81 ± 0.21</td>
</tr>
<tr>
<td>CYP2A13*9</td>
<td>0.58 ± 0.06</td>
<td>1.84 ± 0.09</td>
<td>3.22 ± 0.23</td>
</tr>
</tbody>
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N.D., not determined.

*Kinetic parameters are presented as means ± S.E. determined from three (CYP2A13*4, CYP2A13*6) or four (CYP2A13*1, CYP2A13*5, CYP2A13*8, CYP2A13*9) independent experiments.

The CYP2A13*5 variant had a similar $K_m$ value (1.63 ± 0.12 μM) to that of CYP2A13*1 and a $V_{max}$ value [3.20 ± 0.13 nmol/min · nmol P450] that was 25% less than the $V_{max}$ value of CYP2A13*1. $K_m$ values of CYP2A13*8 (0.85 ± 0.09 μM) and CYP2A13*9 (0.58 ± 0.06 μM) were lower than that of CYP2A13*1; these variants also had lower $V_{max}$ values [2.34 ± 0.07 and 1.84 ± 0.09 nmol/min · nmol P450, respectively] than CYP2A13*1. From these data, the catalytic efficiency for 5-MOP metabolism ($V_{max}/K_m$) of CYP2A13*1, CYP2A13*5, CYP2A13*6, CYP2A13*8, and CYP2A13*9 was 2.98 ± 0.17, 1.99 ± 0.13, 3.47 ± 0.17, 2.81 ± 0.21, and 3.22 ± 0.23 ml/(min · nmol P450), respectively. The maximum $V_{max}/K_m$ was less than twice the minimum. These results show that the catalytic efficiency of CYP2A13*5, CYP2A13*6, CYP2A13*8, and CYP2A13*9 do not greatly differ from that of CYP2A13*1; however, CYP2A13*4 was unable to produce 5-MOP dihydrodiol, so the kinetic parameters for this variant cannot be determined (Fig. 4; Table 1). We conclude that the CYP2A13*4 recombinant protein is the product of a loss-of-function allele.

Discussion

We used heterologously expressed enzymes in E. coli to investigate the effects of CYP2A13 polymorphisms on 5-MOP metabolism. There are several advantages to expressing P450 enzymes in E. coli for analysis of their function and structure. In general, bacterial expression is more convenient and the yield is higher than in eukaryotic expression systems. Furthermore, because the E. coli genome contains no P450 genes, the P450 of interest can be analyzed without background activity. Despite these advantages, there is the possibility that the system may not be functional. The characteristics of the recombinant E. coli P450 proteins need to be the same as native P450 proteins, because modification of the membrane-binding region at the N-terminus is essential for expression of the human P450 gene in E. coli (Yun et al., 2006). In our experiments, CYP2A13*4 enzymes were drastically inactivated, whereas other recombinant CYP2A13 enzymes metabolized 5-MOP. Wang et al. (2006) performed the in vitro expression of CYP2A13 variants in mammalian Chinese hamster ovary cells and insect sf9 cells. They proposed that the substitution in CYP2A13*4 led to a nonfunctional and an unstable protein, which were consistent with our study (Figs. 3 and 4). All of the N-terminal-modified CYP2A13 variants, including CYP2A13*4, were useful for analyzing the corresponding native proteins.

We examined expression of the recombinant CYP2A13 enzymes by Western blot assay. The CYP2A13*4 recombinant enzyme was expressed at levels equal to those of wild type and other variants. The amino acid substitution in CYP2A13*4, R101Q, occurs at a distance and $V_{max}$ of CYP2A13*1 enzymes were 1.44 ± 0.17 μM and 4.23 ± 0.36 nmol/min · nmol P450, respectively. The kinetic parameters of CYP2A13*6 [K_m = 1.36 ± 0.10 μM, V_max = 4.69 ± 0.13 nmol/(min · nmol P450)] were almost the same as those for CYP2A13*1.

![Fig. 4. Productions of 5-MOP dihydrodiol mediated by CYP2A13 variants versus reaction time (A) and initial concentration of 5-MOP (B). ○, CYP2A13*1 (65.9 pmol/ml); □, CYP2A13*4 (not determined); △, CYP2A13*5, (89.4 pmol/ml); ●, CYP2A13*6 (45.1 pmol/ml); ■, CYP2A13*8 (68.6 pmol/ml); ●, CYP2A13*9 (52.7 pmol/ml). The concentrations of CYP2A13 enzyme in the reaction mixture, quantified from reduced CO difference spectrum.](image-url)
of less than 3Å from the heme-activation center, and this substitution seems to influence stability and lead to a remarkable decrease in activity (Smith et al., 2007) (Fig. 1B). Surprisingly, though the CYP2A13*4 variant differs from the wild type in only one residue, its electrophoretic mobility was lower. No other examples of a difference in electrophoretic mobility among SNPs of CYP2A13 have been reported in previous studies. Thus, this study clarifies some important characteristics of the CYP2A13*4 enzyme, including its low electrophoretic mobility, nonfunctional reduced CO difference spectrum, and loss of ability to metabolize 5-MOP. The relationship between the low electrophoretic mobility and the functional failure of the CYP2A13*4 enzyme is still unknown, and it remains a question to be examined in future work.

This study demonstrated for the first time that CYP2A13 is involved in conversion of 5-MOP into its dihydrodiol form. Natural furanocoumarins, 5-MOP and 8-MOP, are useful food constituents, which are applied to skin or taken orally in combination with UV irradiation for therapy of some skin diseases (e.g., dermatoses, psoriasis, and vitiligo) (McNeely and Goa, 1998; Wackernagel et al., 2006; Tzaneva et al., 2009). According to the previously postulated metabolic pathway of 5-MOP in humans, formation of 5-MOP dihydrodiol, which was identified as metabolite of 5-MOP in this study, is the result of two reactions: 1) oxidation of 5-MOP to epoxide by CYP2A13 and 2) hydrolysis of epoxide to dihydrodiol either spontaneously or by epoxide hydrolase (John et al., 1992) (Fig. 5). Likewise, Koenigs and Trager (1998) found that generation of the furanoepoxide and γ-ketoal species of 5-MOP, which are capable of forming covalent adducts with proteins and DNA, was mediated by CYP2A6. They also postulated that the binding of these species to CYP2A6 apoprotein caused the inhibition of the CYP2A6 function. Although the furanoepoxide and γ-ketoal species of 5-MOP were not directly detected in this study, these intermediates were probably generated during 5-MOP metabolism by CYP2A13 and were hydrolyzed to dihydrodiol spontaneously. The tissue distribution of CYP2A13 in vivo and its metabolic activity led us to the hypothesis that the human CYP2A13 is a major player in the production of mutagenic intermediates from NNK (Jalas et al., 2003; He et al., 2004; Zhang et al., 2007). Our findings indicate the possibility that reactive intermediates from 5-MOP are produced in respiratory organs by CYP2A13, as well as in liver by other P450s. As 8-MOP is expected to suppress production of carcinogens from NNK (Koenigs et al., 1997; Sellers et al., 2003; von Weymarn et al., 2005), 5-MOP is effective in preventing activation of procarcinogens.

In conclusion, this study shows that one base mutation in the CYP2A13 gene has a role in metabolism of 5-MOP. Most substitutions of amino acid simulating SNPs on CYP2A13 enzyme have a minor effect, but the alteration at position 101 from arginine to glutamine, simulating CYP2A13*4, inactivates the function of CYP2A13 enzyme. This fact suggested that same tendency among CYP2A13 variants will be also observed in metabolisms of other compounds.

Acknowledgments. We thank Takako Yamamoto and Sachiy0 Ito for help in preparing E. coli membrane fractions expressing P450 recombinant proteins.

References


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*Drug Metabolism and Disposition*

Comparison of typical absorption spectra of 5-methoxypsoralen (5-MOP) (dashed line, $\lambda_{\text{max}} = 312\text{nm}$), 5-MOP dihydrodiol (solid line, $\lambda_{\text{max}} = 322\text{nm}$) and 7-hydroxycoumarin (7-HC) (dotted line, $\lambda_{\text{max}} = 322\text{nm}$). The spectra are normalized with $\lambda_{\text{max}}$ values and overlaid. The spectrum of 7-HC was nearly identical to that of 5-MOP dihydrodiol.