Interindividual Variability in 2-Hydroxylation, 3-Sulfation, and 3-Glucuronidation of Ethynylestradiol in Human Liver

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In the current study, we investigated interindividual variability of the 2-hydroxylation, 3-glucuronidation, and 3-sulfation of ethynylestradiol (EE2) using human liver microsomes and cytosol. Km values for the 2-hydroxylation and 3-glucuronidation in pooled liver microsomes and for the 3-sulfation in pooled liver cytosol were 3.34, 23.3, and 2.85 μM, respectively. Vmax/Km (ml/min/g liver) was highest for the 3-sulfation, followed by 2-hydroxylation, suggesting that 3-sulfation is the major metabolic pathway of EE2 in human liver. All further studies were performed at a substrate concentration of 0.1 μM. Microsomal 2-hydroxylation and 3-glucuronidation activities ranged from 0.21 to 5.02 (2.04±1.34, mean±S.D., n=35) and 0.20 to 4.84 (1.20±1.00, n=35) pmol/min/mg protein, respectively. Cytosolic 3-sulfation activity ranged from 4.2 to 24.3 (11.8±4.4, n=21) pmol/min/mg protein. All the measured enzyme activities were neither gender-related nor age-dependent, except that 2-hydroxylation was significantly higher in females than in males (p<0.05). The relative contribution of CYP3A to the 2-hydroxylation in liver microsomes was estimated from the degree of inhibition by 1 μM ketoconazole. The degrees of inhibition were between 17.8 and 78.0% (51.6±16.0%, n=27). These results indicate that there are large interindividual differences in the enzyme activities towards the respective metabolic pathways of EE2 and the relative contribution of CYP3A to the 2-hydroxylation of EE2 in human liver.

Key words ethynylestradiol; sulfotransferase; CYP3A; UDP-glucuronosyltransferase; human liver

Ethynylestradiol (EE2) is commonly used as the major estrogenic component in many oral contraceptive formulations. Orme et al.1) reported that EE2 is metabolized by multiple pathways, and there is a large interindividual variability in the routes and rates of metabolism of EE2 in humans. In addition, marked interindividual differences in the pharmacokinetic parameters of EE2 have been reported.1) The reported systemic bioavailability of orally ingested EE2 ranges from 20 to 65%.1) Factors that could affect oral clearance of a drug include absorption from the gastrointestinal tract, protein binding, intrinsic hepatic clearance, hepatic blood flow, and renal clearance. Urinary excretion of unchanged EE2 was very low;3) suggesting that EE2 is mainly eliminated by metabolism. The 2-hydroxylation of EE2 is mainly catalyzed by CYP3A, CYP2C, and CYP2E enzymes,3,4) and this route is one of the main metabolic pathways in humans.5,6) Additionally, sulfation and glucuronidation also play important roles in the metabolism of EE2. EE2, 3-glucuronide and EE2, 3-sulfate are eliminated into the gastrointestinal tract, hydrolyzed by gut bacteria to EE2, and subsequently reabsorbed. Rifaximin, an inducer of CYP3A, has been reported to significantly increase the oral clearance of EE2.7) This interaction is thought to reflect the significant contribution of CYP3A enzymes to the metabolism of EE2. It is well known that there are large interindividual differences in the expression levels and catalytic activities of CYP3A enzymes in human liver.8) Patki et al.9) indicated that the intrinsic clearance of triazolam, a CYP3A substrate, and CYP3A protein in human liver are reduced in the elderly.

In vitro studies are useful for clarifying the role of respective enzymes on the systemic drug clearance. Plasma concentrations of EE2 achieved during normal therapy are very low (<2 nM).1) However, most previous in vitro studies used substantially high EE2 substrate concentrations (>10 μM). A recent study using human hepatocytes in vitro at a substrate concentration of 1 nM demonstrated that the major EE2 metabolites are direct conjugates, with hydroxylation representing minor pathways, and suggested that the production of EE2, 3-sulfate was increased by pretreatment of hepatocytes with rifampicin.9) The initial rate of conjugate formation was reported to be EE2, 3-sulfate>EE2, 3-glucuronide in human hepatocytes.9)

In the current study, we investigated interindividual variability of the 2-hydroxylation, 3-glucuronidation, and 3-sulfation of EE2 using human liver microsomes and cytosol in the presence of the respective cofactors. Additionally, interindividual variability of the relative contribution of CYP3A to the 2-hydroxylation of EE2 in human liver microsomes was estimated from the degree of inhibition by ketoconazole, a potent inhibitor of CYP3A. All enzyme activities were measured at an EE2 substrate concentration of 0.1 μM, which is under a nearly linear condition (<1/23 of Km values for the respective reactions) for the metabolism of EE2 in human liver, and seems to reflect clinically relevant conditions.

MATERIALS AND METHODS

Chemicals [6,7-3H]-Ethynylestradiol (3H-EE2) was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, U.S.A.), and purified by HPLC before use. The specific radioactivity of the purified 3H-EE2 was 1.517 TBq/mmol, and the radiochemical purity, as checked by HPLC with radiochemical detection, was greater than 99%. Authentic standards of 2-hydroxy-EE2, EE2, 3-sulfate, and EE2, 3-glucuronide were biosynthesized, and their structures were con-

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firmed in our laboratories. EE2, NADP⁺, glucose-6-phosphate (G-6-P), glucose 6-phosphate dehydrogenase (G-6-P DH), and 3’-phosphoadenosine 5’-phosphosulfate (PAPS) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). UDP-glucuronic acid (UDPGA) was obtained from Nacalai Tesque (Kyoto, Japan). Ketocazole was obtained from Ultrafine Chemicals Ltd. (Manchester, U.K.). All other reagents were of the highest purity commercially available.

**Human Liver Microsomes and Cytosol** Pooled human liver microsomes prepared from 46 individuals (2 mg protein/ml; Lot No. 0210171) were purchased from XenoTech, LLC (Lenexa, KS, U.S.A.). Human liver microsomes and cytosol prepared from 12 individuals (coded HG3, HG6, HG23, HG30, HG42, HG43, HG56, HG66, HG70, HG89, HG93, and HG112) were purchased from BD Gentest (Woburn, MA, U.S.A.). Human liver microsomes prepared from 14 individuals (coded HBI 2.0, HBI 5.0, HBI 7.0, HBI 11.0, HBI 12.0, HBI 13.0, HBI 14.0, HBI 15.0, HBI 16.0, HBI 17.0, HBI 18.0, HBI 19.0, HBI 20.0, and HBI 21.0) were purchased from Human Biologies, Inc. (Phoenix, AZ, U.S.A.). Human liver samples of 9 individuals (coded HL-98, HL-109, HL-113, HL-118, HL-127, HL-131, HL-132, HL-133, and HL-136) were obtained as described elsewhere. Each liver specimen was homogenized with 3 volumes of 1.15% KCl using a Teflon-glass homogenizer. The homogenate was centrifuged at 10000×g for 20 min, and the resulting supernatant was centrifuged at 105000×g for 60 min. The supernatant was used as cytosol, and the pellet was suspended in 1.15% KCl and further centrifuged at 105000×g for 60 min. The resulting pellet was resuspended in 1.15% KCl and used as microsomes. Human liver microsomes and cytosol were stored at −80°C until use. Protein concentration of samples prepared in our laboratories was determined by the method of Lowry et al. using bovine serum albumin as the standard.

**Assay for EE2 2-Hydroxylase Activities** The incubation mixture (300 μl) consisted of 3H-EE2, liver microsomes (0.1 mg protein/ml), an NADPH-generating system (2 mM NADP⁺, 10 mM G-6-P, 1 unit/ml G-6-P DH, and 5 mM MgCl₂), and 0.1 M potassium phosphate buffer (pH 7.4). EE2 was dissolved in 50% acetonitrile, and 3 μl of the solution was added to the incubation mixture. The 3H-EE2 substrate concentration was set at 0.1 μM, except for kinetic studies in which substrate concentrations varied from 0.1 to 50 μM. The reaction was started by adding the NADPH-generating system, and the mixture was incubated at 37°C for 15 min. The reaction was terminated by adding 300 μl of methanol and cooling in an ice-bath. After standing for 4 to 5 min, the mixture was centrifuged at 12000×g for 2 min. A 100 μl sample of the supernatant was injected into HPLC and another portion was used to determine radioactivity, in order to calculate the recovery of radioactivity from the incubation mixture. Radioactivity in the supernatant was measured for 2 min using a liquid scintillation counter (Tri-carb 1600CA, PerkinElmer Life Sciences). Control incubations without the NADPH-generating system were concurrently performed to validate enzyme-dependent metabolism. Analytical separation of EE2 and its metabolite was achieved using a Radio-HPLC system equipped with a Waters Alliance 2695 Separations Module, (Milford, MA, U.S.A.). The sample tray was maintained at 4°C. The analytical and guard columns were an Inertsil ODS-3 (5 μm, 150 mm×4.6 mm I.D., GL Sciences Inc., Tokyo, Japan) and a TSKguardgel ODS-80Ts (5 μm, 15 mm×3.2 mm I.D., Tosoh Corporation, Tokyo, Japan), respectively. The mobile phase consisted of solvent A (0.1% acetic acid in water) and solvent B (acetonitrile), and a linear gradient was constructed as follows: 50—70% B, 0—20 min; 70—100% B, 20—20.1 min; 100% B, 20.1—25 min; and 100—50% B, 25—25.1 min. The column temperature and flow rate were set at 40°C and 1 ml/min, respectively. Quantitation of EE2, and its metabolites was performed using a Radiomatic Model 525TR/FLO-ONE flow-through radioactivity detector (PerkinElmer Life Sciences), and peak areas were integrated with Windows-based Radio-HPLC Workstation software. An Ultima-F M liquid scintillator (PerkinElmer Life Sciences) was introduced post-column at a rate of 3 ml/min. Under these conditions, the retention times for 2-hydroxy-EE2 and EE2 were 13.6 and 17.2 min, respectively. The radioactivity detection limit in column eluates using the on-line method described above was set to be twice the background value.

**Assay for EE2 3-Glucuronosyltransferase Activities** The incubation mixture (300 μl) consisted of 3H-EE2, liver microsomes (0.2 mg protein/ml), 5 mM UDPGA, 5 mM MgCl₂, and 0.1 M potassium phosphate buffer (pH 7.4). EE2 was dissolved in 50% acetonitrile, and 3 μl of the solution was added to the incubation mixture. The 3H-EE2 substrate concentration was set at 0.1 μM, except for kinetic studies in which substrate concentrations varied from 0.1 to 100 μM. The reaction was started by adding UDPGA and the mixture was incubated at 37°C for 20 min. The reaction was terminated by adding 300 μl of methanol, followed by cooling in an ice-bath. After standing for 4 to 5 min, the mixture was centrifuged at 12000×g for 2 min. A 100 μl sample of the supernatant was injected into HPLC. The same Radio-HPLC system and columns as described above were used. The mobile phase consisted of solvent A (20 mM ammonium dihydrogenphosphate-phosphoric acid buffer (pH 2.5)) and solvent B (acetonitrile), and a linear gradient was constructed as follows: 35—55% B, 0—20 min; 55—85% B, 20—20.1 min; 85% B, 20.1—25 min; and 85—35% B, 25—25.1 min. The column temperature and flow rate were set at 50°C and 1 ml/min, respectively. Under these conditions, the retention times for EE2-3-glucuronide and EE2 were 4.4 and 15.8 min, respectively.

**Assay for EE2 3-Sulfotransferase Activities** The incubation mixture (300 μl) consisted of 3H-EE2, liver cytosol (0.1 mg protein/ml), 0.2 mM PAPS, 5 mM MgCl₂, 5 mM 2-mercaptoethanol, and 0.1 M potassium phosphate buffer (pH 7.4). EE2 was dissolved in 50% acetonitrile, and 3 μl of the solution was added to the incubation mixture. The 3H-EE2 substrate concentration was set at 0.1 μM, except for kinetic studies in which substrate concentrations varied from 0.1 to 100 μM. The reaction was started by adding PAPS, and the mixture was incubated at 37°C for 10 min. The Radio-HPLC conditions were the same as those described for the analysis of 3-glucuronosyltransferase activities. Under these conditions, the retention times for EE2-3-sulfate and EE2 were 12.4 and 15.4 min, respectively.

**Inhibition of EE2 2-Hydroxylase Activities by Ketoconazole** The effect of ketoconazol, a potent CYP3A inhibitor, on EE2 2-hydroxylation activity was examined in
27 individual human liver microsomes, which showed relatively high EE2 2-hydroxylase activities among 35 individuals. Ketoconazole was dissolved in 50% methanol, and 3 μL of the solution was added to the incubation mixture (a final concentration of 1 μM). EE2 2-hydroxylase activity was measured in the same manner as described above.

Data Analysis The ratio of each metabolite to the total radioactivity in the incubation mixture was calculated by dividing the radioactivity of each metabolite eluted from the HPLC column by the total eluted radioactivity. In calculation of the rate of metabolite formation, the recovery of radioactivity from the incubation mixtures and the HPLC column was assumed to be 100%. All data were analyzed using the average of duplicate determinations. In preliminary experiments, the linearity of the reaction with incubation time and protein concentration was confirmed for each assay condition. Apparent kinetic parameters for the 2-hydroxylation, 3-glucuronidation, and 3-sulfation of EE2 were estimated by a nonlinear least-square program MULTI\(^{16}\) in which raw data were fitted to the following the Michaelis–Menten kinetics (equation 1) or Michaelis-Menten kinetics with noncompetitive substrate inhibition (equation 2):

\[
V = \frac{V_{\text{max}} \cdot S}{K_m + S} \quad \text{(1)}
\]

\[
V = \frac{V_{\text{max}} \cdot S}{(K_m + S)(1 + S/K_{si})} \quad \text{(2)}
\]

where \(V\) is the rate of reaction, \(S\) is the substrate concentration, \(K_m\) is the Michaelis constant, \(V_{\text{max}}\) is the maximum velocity of the reaction, and \(K_{si}\) is the substrate inhibition constant. Intrinsic metabolic clearance (\(CL_{\text{int}}\)) was calculated as a ratio of \(V_{\text{max}}\) to \(K_m\). For the calculation of \(CL_{\text{int}}\) (ml/min/g liver), we used the following parameters: the microsomal and cytosolic protein contents in human liver were assumed to be 21.4 and 59.9 mg/g organ, respectively, as reported by Temellini et al.\(^{17}\) because we could not obtain protein yields for the individual samples included in this study. The coefficient of determination (\(r^2\)) for enzyme activities was determined by linear regression analysis using the SAS (SAS Institute Inc., Cary, NC, U.S.A.). Statistical differences between males and females were determined by Student’s \(t\) test. The significant level was set at \(p<0.05\).

RESULTS

EE2 Metabolism by Human Liver Microsomes and Cytosol The recovery of radioactivity from the incubation mixtures into the supernatant was more than 95% in all cases after incubations with EE2 and human liver microsomes or cytosol in the presence of the respective cofactors. Unchanged EE2 remaining in the incubation mixture following a 15-min incubation was more than 65% for all incubations. The effect of substrate concentrations on the 2-hydroxylation and 3-glucuronidation of EE2 in pooled human liver microsomes, and the 3-sulfation of EE2 in pooled human liver cytosol, are shown in Fig. 1. Apparent kinetic parameters for EE2 metabolism in human liver are summarized in Table 1. In the 3-sulfation of EE2, substrate inhibition was observed at high substrate concentrations (>20 μM); therefore, the data was fitted to Michaelis-Menten kinetics with noncompetitive substrate inhibition (Eq. 2). The \(K_{si}\) value for the 3-sulfation of EE2 was 13.1 μM. \(K_m\) values for the 2-hydroxylation, 3-glucuronidation, and 3-sulfation of EE2 were 3.34, 23.3, and 2.85 μM, respectively, and \(V_{\text{max}}\) values were 201.6, 408.6, and 221.4 pmol/min/mg protein, respectively. The \(CL_{\text{int}}\) (ml/min/g liver) was calculated as a ratio of \(V_{\text{max}}\) to \(K_m\) for different substrates.

Table 1. Apparent Kinetic Parameters for the 2-Hydroxylation and 3-Glucuronidation of Ethynylestradiol in Pooled Human Liver Microsomes and the 3-Sulfation of Ethynylestradiol in Pooled Human Liver Cytosol

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Enzyme source</th>
<th>(K_m) (μM)</th>
<th>(V_{\text{max}}) (pmol/min/mg protein)</th>
<th>(K_{si}) (μM)</th>
<th>(CL_{ rehabilitation}(\text{int})) (V_{\text{max}}/K_m) (ml/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Hydroxylation</td>
<td>Microsomes</td>
<td>3.34</td>
<td>201.6</td>
<td>—</td>
<td>60.4</td>
</tr>
<tr>
<td>3-Glucuronidation</td>
<td>Microsomes</td>
<td>23.3</td>
<td>408.5</td>
<td>—</td>
<td>17.5</td>
</tr>
<tr>
<td>3-Sulfation</td>
<td>Cytosol</td>
<td>2.85</td>
<td>221.4</td>
<td>13.1</td>
<td>77.7</td>
</tr>
</tbody>
</table>

For the 2-hydroxylation and 3-glucuronidation of ethynylestradiol, the data was fitted to Michaelis–Menten kinetics. For the 3-sulfation of ethynylestradiol, the data was fitted to Michaelis–Menten kinetics with noncompetitive substrate inhibition. \(K_m\) is the substrate inhibition constant. \(a) CL_{\text{int}}\) (ml/min/g liver)=(μl/min/mg protein)×(microsomal or cytosolic protein content, mg/g organ). Microsomal and cytosolic protein contents in human liver were assumed to be 21.4 and 59.9 mg/g organ, respectively.
liver) for the 3-sulfation was 3.6- and 12.2-fold higher than that for the 2-hydroxylation and 3-glucuronidation, respectively. All subsequent studies were conducted at a substrate concentration of 0.1 mM ($K_{m}$ values for the respective reactions), which is under a nearly linear condition for the metabolism of EE2 in human liver.

Interindividual variability of the 2-hydroxylation, 3-glucuronidation, and 3-sulfation of EE2 using human liver microsomes and cytosol in the presence of the respective cofactors is shown in Table 2. Microsomal 2-hydroxylation and 3-glucuronidation were determined in 35 liver samples. The 2-hydroxylation activity ranged from 0.21 to 5.02 (2.04 ± 1.34, mean ± S.D.) pmol/min/mg protein. The 3-glucuronidation activity ranged from 0.20 to 4.84 (1.20 ± 0.74) pmol/min/mg protein. On the other hand, cytosolic 3-sulfation of EE2 was determined in 21 liver samples. The 3-sulfation activity ranged from 4.2 to 24.3 (11.8 ± 4.4) pmol/min/mg protein. On average, the rate of 3-sulfation was highest, followed by 2-hydroxylation and 3-glucuronidation. Interindividual variability of the 3-sulfation was relatively small compared with the variability with 2-hydroxylation and 3-glucuronidation. There were no significant gender-related differences in any of the measured enzyme activities, except that the rate of 2-hydroxylation was significantly higher in females than in males ($p<0.05$). The enzyme activities of individual specimens are shown in Fig. 2. No clear age-dependent changes were observed in any of the measured enzyme activities. Additionally, samples from elderly donors (3 donors; 61, 68, and 71 years) did not show clearly low values.

Correlations between the rates of 2-hydroxylation, 3-glucuronidation, and 3-sulfation of Ethynylestradiol by Human Liver Microsomes and Cytosol

The measured enzyme activities were plotted against liver donor age. ●, male; ○, female.

![Fig. 2. Effect of Age and Gender on the 2-Hydroxylation and 3-Glucuronidation of Ethynylestradiol in Human Liver Microsomes and the 3-Sulfation of Ethynylestradiol in Human Liver Cytosol](image)

![Fig. 3. Correlation between 2-Hydroxylation, 3-Glucuronidation, and 3-Sulfation of Ethynylestradiol by Human Liver Microsomes and Cytosol](image)
showed relatively high $EE_2$ 2-hydroxylase activities among 27 individual human liver microsomes, which had a 24-fold difference among individuals (Table 2). Sulfation is catalyzed by sulfo transferase (SULT) enzymes encoded by members of the SULT gene superfamily.15) Humans have at least 11 different SULT enzymes, forming three subfamilies based on their amino acid sequence identity and substrate specificity.21,22) SULT1 family members sulfate phenols and catechols, whereas SULT2 family members sulfate steroids, sterols, and other alcohols. $EE_2$ 3-sulfation appears to be mainly catalyzed by SULT1E1, which is also known as estrogen sulfo transferase.21,22) Recently, Song et al.23) reported that there is large interindividual variation in the expression of SULT1E1 at the protein level. No genetic polymorphisms have been reported for this isoform. To date, little is known about the regulation of SULT expression in humans. SULT1E1 in hormone-dependent tissues is subject to considerable regulatory control by progesterone, and potentially by other compounds as well.24) The induction of $EE_2$ 3-sulfation has been reported in human hepatocytes.10) Genetic and environmental factors seem to contribute to the interindividual variation in the activity of $EE_2$ 3-sulfation, but its regulation mechanism in human liver remains to be determined.

This study showed that there is a 24-fold difference among individuals in the activities of $EE_2$ 2-hydroxylation in human liver microsomes. The activity was significantly higher in females than in males ($p<0.05$). Previous studies reported that 2-hydroxylation of $EE_2$ is mainly catalyzed by CYP3A, CYP2C, and CYP2E enzymes.3,4) Large interindividual differences in the expression levels and the catalytic activities of P450 enzymes have been well documented.5) CYP3A is the major P450 isoform, and the CYP3A content accounted for approximately 30% of the total P450 in adult human liver.8) Therefore, the relative contribution of CYP3A to the 2-hydroxylation of $EE_2$ in 27 individual human liver microsomes was estimated from the degree of inhibition by 1 μM ketoconazole, a potent inhibitor of CYP3A. The degrees of inhibition were between 17.8 and 78.0% (51.6±16.0%, Table 3), indicating that the relative contribution of CYP3A to the 2-hydroxylation of $EE_2$ in liver varied among individuals. There were no significant differences in the extent of inhibition between males and females, and the degree of inhibition by ketoconazole was independent of the rate of 3-sulfation of $EE_2$. Substrate inhibition was observed at high substrate concentrations (>20 μM, Fig. 1), with the $K_i$ value of 13.1 μM. A similar substrate inhibition was commonly observed for phenol sulfo transferases.18,19) Clinically relevant plasma concentrations of $EE_2$ are very low (<2 nm).3) These values indicate that the possibility of substrate inhibition of $EE_2$ 3-sulfation in human liver can be completely ruled out. $K_m$ values for the 2-hydroxylation and 3-glucuronidation of $EE_2$ in pooled liver microsomes were 3.34 and 23.3 μM, respectively, and the $K_m$ value for the 3-sulfation of $EE_2$ in pooled liver cytosol was 2.85 μM (Table 1). The $CL_{int}$ (ml/min/g liver) value for the 3-sulfation was 3.6- and 12.2-fold higher than that for the 2-hydroxylation and 3-glucuronidation, respectively, indicating that the 3-sulfation would be the most important metabolic pathway of $EE_2$ in human liver. This finding is consistent with a previous study,10) which demonstrated that $EE_2$ was rapidly metabolized to $EE_2$-3-sulfate in human hepatocytes. We have shown that there are no clear gender-related or age-dependent differences in the activities of $EE_2$ 3-sulfation in human liver, with a 6-fold difference among individuals (Table 2). Sulfation is catalyzed by sulfo transferase (SULT) enzymes encoded by members of the SULT gene superfamily.15) Humans have at least 11 different SULT enzymes, forming three subfamilies based on their amino acid sequence identity and substrate specificity.21,22) SULT1 family members sulfate phenols and catechols, whereas SULT2 family members sulfate steroids, sterols, and other alcohols. $EE_2$ 3-sulfation appears to be mainly catalyzed by SULT1E1, which is also known as estrogen sulfo transferase.21,22) Inhibition of Ethynylestradiol 2-Hydroxylation by 1 μM Ketoconazole

<table>
<thead>
<tr>
<th>Inhibition rate (%)</th>
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<tbody>
<tr>
<td>Male ($n=13$)</td>
</tr>
<tr>
<td>Female ($n=14$)</td>
</tr>
<tr>
<td>Total ($n=27$)</td>
</tr>
</tbody>
</table>

Values are mean±S.D. (range).
EE₂ 2-hydroxylation (Fig. 4). The inhibitory effect of ketoconazole is relatively CYP3A-specific at low concentrations (≤1 μM). On average, the relative contribution of CYP3A to the 2-hydroxylation of EE₂ human liver microsomes is estimated to be approximately 50%. These findings support previous studies that showed that, in addition to CYP3A, other multiple P450s also play important roles in EE₂ 2-hydroxylation in human liver microsomes. Our study suggests that EE₂ 2-hydroxylation in human liver does not decrease in the elderly. Patki et al. indicated that the intrinsic clearance of triazolam and the content of CYP3A protein in human liver were reduced in the elderly (61—72 years), and suggested that CYP3A gene expression is reduced in this group. Further studies are needed to evaluate the effect of age on the enzyme activities and expression of P450s other than CYP3A4 involved in the metabolism of EE₂.

We have demonstrated that there are no significant gender-related differences in the activities of EE₂ 3-glucuronidation in human liver microsomes, with a 24-fold difference among individuals. Glucuronidation is catalyzed by UDP-glucuronosyltransferases (UGTs), which exist as a gene superfamily. The majority of UGT isoforms show distinct, but overlapping, substrate specificities. Although EE₂ has also been shown to be a substrate for UGT1A1, other isoforms may also catalyze EE₂ 3-glucuronidation. Genetic polymorphism has been reported for UGT1A1 and other isoforms in this study, we could not obtain the genotype information of liver donors. Therefore, we did not evaluate the possible contribution of UGT polymorphisms to the variability in the catalytic activity of EE₂ 3-glucuronidation in this study. The mechanisms that determine EE₂ 3-glucuronidation activities in human liver are largely unknown, but genetic, and possibly dietary, factors may be major contributors. UGTs are membrane-bound enzymes whose active site is located on the luminal side of the endoplasmic reticulum. Incubation conditions are known to modulate UGT activities, particularly disruption of the microsomal membrane structure is required to obtain maximum enzyme activity. Although detergents were traditionally used for UGT activation, a recent study indicated that the pore-forming peptide alamethicin activated human liver microsomal glucuronidation by 2- to 3-fold. In this study, EE₂ 3-glucuronidation in human liver microsomes was determined without activation, therefore further studies using detergents or alamethicin might be required.

The absence of a significant correlation between EE₂ 3-glucuronidation and 3-sulfation in human liver suggests that these activities varied independently. Although the correlations between EE₂ 2-hydroxylation and 3-glucuronidation, as well as between EE₂ 2-hydroxylation and 3-sulfation, were statistically significant, the extent of these correlations was not high (r² < 0.47). Thus, it seems that interindividual variations of EE₂ 2-hydroxylation, 3-glucuronidation and 3-sulfation in human liver are not consequences of tissue isolation and sample preparation, which would be expected to reflect the intrinsic variation of their activities.

The present study was conducted using human liver microsomal and cytosolic preparations. However, it is well known that the intestine plays an important role in the first-pass metabolism of orally ingested drugs in humans. Both phase I and phase II enzymes, including CYP3A4, SULT1E1, and UGT1A1, are reported to be expressed in the intestine. Thus, the intestine could contribute to first-pass metabolism of EE₂, but the relative contributions by hepatic and intestinal enzymes to the first-pass metabolism of EE₂ are still unknown.

In summary, this study suggested that 3-sulfation is the major metabolic pathway of EE₂ in human liver under clinically relevant conditions. There are large interindividual differences in measured enzyme activities towards the respective metabolic pathways of EE₂ and the relative contribution of CYP3A to the 2-hydroxylation of EE₂ in human liver. Genetic regulatory variations and environmental factors may be major contributors to interindividual activity variations.

REFERENCES