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Evaluation of Human Interindividual Variation in Bioactivation of Estragole Using Physiologically Based Biokinetic Modeling

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The present study investigates interindividual variation in liver levels of the proximate carcinogenic metabolite of estragole, 1′-hydroxyestragole, due to variation in two key metabolic reactions involved in the formation and detoxification of this metabolite, namely 1′-hydroxylation of estragole and oxidation of 1′-hydroxyestragole. Formation of 1′-hydroxyestragole is predominantly catalyzed by P450 1A2, 2A6, and 2E1, and results of the present study support that oxidation of 1′-hydroxyestragole is catalyzed by 17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2). In a first approach, the study defines physiologically based biokinetic (PBBK) models for 14 individual human subjects, revealing a 1.8-fold interindividual variation in the area under the liver concentration-time curve (AUC) for 1′-hydroxyestragole within this group of human subjects. Variation in oxidation of 1′-hydroxyestragole by 17β-HSD2 was shown to result in larger effects than those caused by variation in P450 enzyme activity. In a second approach, a Monte Carlo simulation was performed to evaluate the extent of variation in liver levels of 1′-hydroxyestragole that could occur in the population as a whole. This analysis could be used to derive a chemical-specific adjustment factor (CSAF), which is defined as the 99th percentile divided by the 50th percentile of the predicted distribution of the AUC of 1′-hydroxyestragole in the liver. The CSAF was estimated to range between 1.6 and 4.0, depending on the level of variation that was taken into account for oxidation of 1′-hydroxyestragole. Comparison of the CSAF to the default uncertainty factor of 3.16 for human variability in biokinetics reveals that the default uncertainty factor adequately protects 99% of the population.

Key Words: **estragole; PBBK or PBPK; metabolism; *in vitro*; interindividual differences; Monte Carlo simulation.**

The herbal constituent estragole is an alkenylbenzene that has been demonstrated to be hepatocarcinogenic in animal experiments at high dose levels (Drinkwater *et al*., 1976; Miller *et al*., 1983; Wiseman *et al*., 1987). The most important dietary sources of estragole are basil, fennel, and anise and extracts of these herbs used as flavoring agent in, among others, baked goods, nonalcoholic beverages, and candy (Smith *et al*., 2002). Dietary human exposure to estragole has been estimated to range between 0.01 and 0.07 mg/kg body weight (bw)/day, depending on the method used (SCF, 2001; Smith *et al*., 2002).

The observed carcinogenicity of estragole depends on the formation of a genotoxic metabolite, which is formed via a two-step bioactivation pathway catalyzed by cytochromes P450 and sulfotransferases (Drinkwater *et al*., 1976; Miller *et al*., 1983). The first step of the bioactivation is the conversion of estragole into the proximate carcinogenic metabolite 1′-hydroxyestragole (Fig. 1). Sulfonation of 1′-hydroxyestragole leads to formation of the ultimate carcinogenic metabolite of estragole, which is unstable and degrades in aqueous environment to give rise to a reactive carbocation capable of forming DNA adducts (Phillips *et al*., 1981, 1984; Randerath *et al*., 1984; Wiseman *et al*., 1985). Other phase I metabolic reactions of estragole than 1′-hydroxylation are also presented in Figure 1 and include *O*-demethylation, 2′,3′-epoxidation, and 3′-hydroxylation, leading to the formation of, respectively, 4-allylphenol, estragole-2′,3′-oxide, and 3′-hydroxyanethole. Of these metabolic pathways, both *O*-demethylation and 3′-hydroxylation result in detoxification of estragole (Anthony *et al*., 1987; Solheim and Scheline, 1973). The epoxide metabolite of estragole can form DNA adducts *in vitro*, but this metabolite is considered not to contribute to the genotoxic effect of estragole *in vivo*, due to rapid detoxification of estragole-2′,3′-oxide by epoxide hydrolases and via glutathione conjugation (Guenthner and Luo, 2001; Luo et al.,1992; Phillips *et al*., 1981). Detoxification of 1′-hydroxyestragole can occur through glucuronidation and oxidation, the latter resulting in formation of 1′-oxoestragole (Anthony *et al*., 1987; Sangster *et al*., 1987; Solheim and Scheline, 1973). The oxidation of 1′-hydroxyestragole is identified to be an important metabolic route of 1′-hydroxyestragole in humans (Punt *et al*., 2009). 1′-Oxoestragole has been shown to be able to form adducts with 2′-deoxyguanosine in a direct reaction with this nucleoside

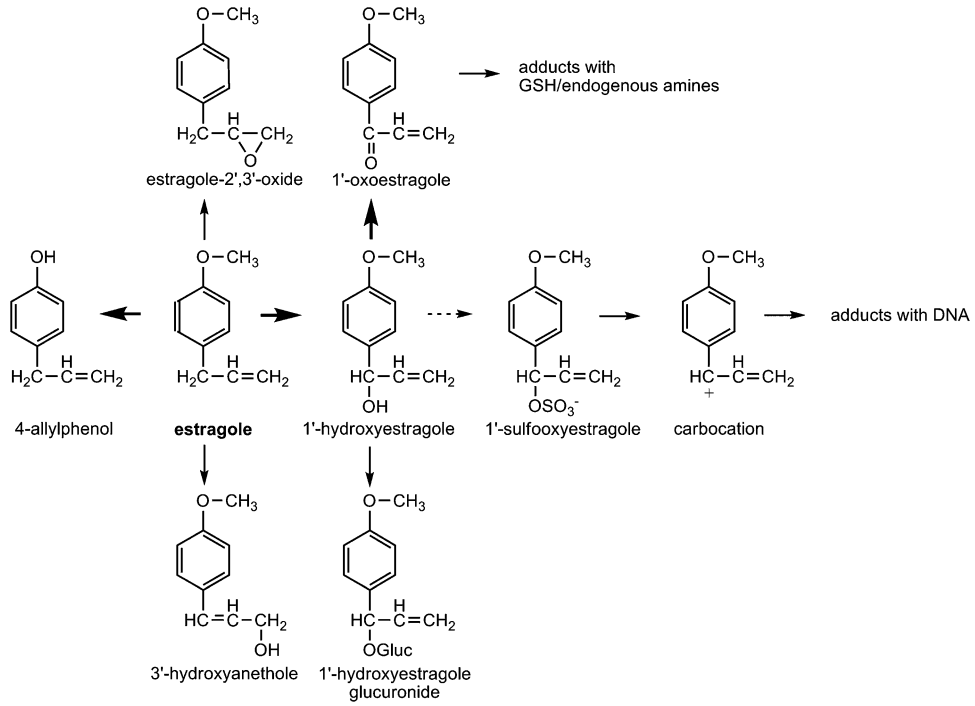

FIG. 1. Metabolism of estragole. Bold arrows represent major metabolic pathways and the dotted arrow represents a minor metabolic pathway in human.

(Phillips *et al*., 1981). In spite of this, 1′-oxoestragole was not carcinogenic *in vivo* in mice (Wiseman *et al*., 1987). This has been ascribed to extensive detoxification of 1′-oxoestragole via conjugation with glutathione or endogenous amines (Fennell *et al*., 1984; Wiseman *et al*., 1987; Wislocki *et al*., 1977).

The relative extent of bioactivation of estragole to its ultimate carcinogenic metabolite 1′-sulfooxyestragole in humans can be affected by genotype- and lifestyle-based factors that influence the activity of enzymes involved in key metabolic reactions. Based on a physiologically based biokinetic (PBBK) model for estragole in human and a sensitivity analysis for this model, it was previously demonstrated that formation of 1′-hydroxyestragole and subsequent oxidation of this metabolite to 1′-oxoestragole are the key metabolic reactions affecting the levels of 1′-hydroxyestragole in the liver (Punt *et al*., 2009). Subsequent formation of 1′-sulfooxyestragole will depend on this level of 1′-hydroxyestragole in the liver and also on the kinetics for its sulfonation (Punt *et al*., 2009). The aim of the present study was to obtain insight in the interindividual variability within the human population in liver 1′-hydroxyestragole levels, caused by variability in the two key reactions that determine these levels, namely 1′-hydroxylation of estragole and oxidation of 1′-hydroxyestragole.

To this end, a PBBK model was developed and parameter values were identified for estragole metabolism in the 14 subjects, allowing evaluation of the contribution of variability in estragole 1′-hydroxylation and 1′-hydroxyestragole oxidation to the predicted interindividual differences in liver levels of 1′-hydroxyestragole among this set of 14 individuals. In a next step, a Monte Carlo simulation was performed to evaluate the level of variation in liver levels of 1′-hydroxyestragole that could occur in the population as a whole.

To understand what level of variation in the activity of enzymes involved in 1′-hydroxylation of estragole and oxidation of 1′-hydroxyestragole is representative for the total population, knowledge about the enzymes involved in these reactions is essential. Jeurissen *et al*. (2007) identified the human cytochrome P450 enzymes involved in 1′-hydroxylation of estragole. It was concluded that especially P450 1A2 and P450 2A6 play a pivotal role in this reaction. To a small extent, P450 2D6, 2C19, and 2E1 are also able to catalyze 1′-hydroxylation of estragole (Jeurissen *et al*., 2007). The enzymes involved in the oxidation of 1′-hydroxyestragole to 1′-oxoestragole have not been identified so far. It has been suggested (Fennell *et al*., 1984) that this reaction could be catalyzed by alcohol dehydrogenases present in liver cytosol. Other enzymes that can catalyze dehydrogenase reactions in the liver not only encompass cytochrome P450 enzymes but also 17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2) (Peltoketo *et al*., 1999; Stupans *et al*., 2000). This latter enzyme plays a role in the regulation of steroid hormones by catalyzing the oxidation of 17β-hydroxysteroids and is expressed in microsomal fractions of tissues such as liver, gastrointestinal and urinary tract, placenta, and uterus (Peltoketo *et al*., 1999). To enable the modeling of interindividual variability in oxidation of 1′-hydroxyestragole, it was essential to elucidate the nature of the major enzyme

TABLE 1
Kinetic Parameters Obtained with Gentest Microsomes Expressing P450 Single Enzymes Involved in Estragole 1′-Hydroxylation as Obtained from Jeurissen *et al.* (2007)

Enzyme	$V_{maxP450,\ Gen}$[a] (pmol/min/mg protein)	K_m (μM)	$V_{maxP450,\ Gen}/K_m$ (μl/min/mg microsomal protein)
P450 1A2	65 ± 3	11 ± 2	5.9
P450 2A6	355 ± 16	8 ± 2	44.4
P450 2C19	167 ± 21	$1.0 \times 10^3 \pm 0.2 \times 10^3$	0.2
P450 2D6	389 ± 104	$1.3 \times 10^3 \pm 0.5 \times 10^3$	0.3
P450 2E1	80 ± 4	49 ± 7	1.6

[a] $V_{maxP450,\ Gen}$ values expressed as nmol/min/(mg microsomal protein) were recalculated from the k_{cat} values expressed as nmol/min/(nmol P450) as reported by Jeurissen *et al.* (2007).

involved in this conversion, and to this end, the present study also reports experiments indentifying the cellular localization and cofactor specificity of the enzyme involved in this reaction. Altogether the experiments and models of the present article allow evaluation of human interindividual variation in liver levels of 1′-hydroxyestragole.

MATERIALS AND METHODS

Chemicals and biological materials. Individual human liver microsomes (eight females and six males), pooled mixed gender human liver microsomes, and pooled mixed gender human liver cytosol were purchased from BD Gentest (Woburn, MA). Nicotinamide adenine dinucleotide phosphate ($NADP^+$), reduced nicotinamide adenine dinucleotide phosphate (NADPH), nicotinamide adenine dinucleotide (NAD^+), and reduced nicotinamide adenine dinucleotide (NADH) were obtained from Roche Diagnostics (Mannheim, Germany). Tris(hydroxymethyl)-aminomethane was obtained from GIBCO BRL Life Technologies (Paisley, Scotland). Hydrochloric acid (37%) was purchased from Merck (Darmstadt, Germany). Estragole (4-allylanisol), estradiol, gallic acid, acetonitrile (chromatography grade), methanol, dimethyl sulfoxide (DMSO), and reduced glutathione (GSH) were purchased from Sigma-Aldrich (Steinheim, Germany). 1′-Hydroxyestragole was synthesized as described previously (Jeurissen *et al.*, 2007; Punt *et al.*, 2007).

1′-Hydroxylation of estragole by individual human liver microsomes. Table 1, derived from Jeurissen *et al.* (2007), presents the kinetic constants for 1′-hydroxylation of estragole by Gentest microsomes expressing single cytochrome P450 enzymes.

When the relative activity levels of different P450 enzymes in liver microsomes of an individual human subject are known, these kinetic constants can be used to estimate the kinetics for 1′-hydroxylation by the individual human liver microsomes. The K_m values for 1′-hydroxylation by the different P450 enzymes in Gentest microsomes and in individual human liver microsomes are assumed to be the same (Bogaards *et al.*, 2000; Lipscomb and Poet, 2008; Rostami-Hodjegan and Tucker, 2007). The $V_{maxP450,\ Gen}$ values are scaled by converting the activity level of a specific P450 enzyme in the Gentest microsomes to the activity level of this enzyme in the liver microsomal sample, all measured using enzyme-selective marker substrates (Bogaards *et al.*, 2000; Lipscomb and Poet, 2008; Rostami-Hodjegan and Tucker, 2007). The relative activity levels of P450 enzymes in individual human liver microsomes were calculated using relative activity factors (RAFs), representing the ratio between (1) the conversion of a P450-selective marker substrate (at saturating substrate conditions) expressed as pmol/min/(mg microsomal protein) by liver microsomes from an individual human subject and (2) the conversion of this marker substrate (at saturating substrate conditions) expressed as pmol/min/(mg microsomal protein), by Gentest microsomes expressing a single P450 enzyme (Bogaards *et al.*, 2000; Lipscomb and Poet, 2008). The RAFs of different P450 enzymes in liver microsomes of 14 different individual human subjects were calculated based on the marker substrate activities for individual human liver microsomes and Gentest microsomes as obtained from the supplier (Lipscomb and Poet, 2008).

Based on this scaling method, the overall 1′-hydroxylation rate by the microsomal fraction obtained from an individual human liver can be described by the sum of the 1′-hydroxylation rates by all P450 enzymes capable of catalyzing the reaction, using the following equation:

$$\begin{aligned} v = &\left(V_{max1A2,\ Gen} \times RAF_{1A2}\right) \times C_E/\left(K_{m1A2} + C_E\right) \\ &+ \left(V_{max2A6,\ Gen} \times RAF_{2A6}\right) \times C_E/\left(K_{m2A6} + C_E\right) \\ &+ \left(V_{max2C19,\ Gen} \times RAF_{2C19}\right) \times C_E/\left(K_{m2C19} + C_E\right) \\ &+ \left(V_{max2D6,\ Gen} \times RAF_{2D6}\right) \times C_E/\left(K_{m2D6} + C_E\right) \\ &+ \left(V_{max2E1,\ Gen} \times RAF_{2E1}\right) \times C_E/\left(K_{m2E1} + C_E\right), \end{aligned} \quad (1)$$

in which v (pmol/min/[mg microsomal protein]) is the overall 1′-hydroxylation rate by microsomes obtained from an individual human liver, $V_{maxP450,\ Gen}$ (pmol/min/[mg microsomal protein]) is the maximum catalytic rate for estragole 1′-hydroxylation by the Gentest microsomes expressing a single cytochrome P450 (P450 = cytochrome P450 1A2, 2A6, 2C19, 2D6, or 2E1), RAF_{P450} corresponds to the RAF of a P450 enzyme in different individual human subjects, and K_{mP450} (μM) is the Michaelis-Menten constant for the formation of 1′-hydroxyestragole by the Gentest microsomes expressing a single P450 enzyme. In the equation, the product $V_{maxP450,\ Gen} \times RAF_E$ corresponds to the maximum 1′-hydroxylation rate by the specific enzyme present in an individual human microsomal sample. C_E (μM) represents the *in vitro* substrate concentration of estragole.

Validation of the outcomes of Equation 1. To validate whether with Equation 1 the kinetics for 1′-hydroxylation can be correctly described, the calculated 1′-hydroxylation rate by individual human liver microsomes at a substrate concentration of 1000μM estragole (representing saturating substrate conditions [Punt *et al.*, 2009]) was compared to the measured 1′-hydroxylation rate by these individual human liver microsomes at the same substrate concentration. Triplicate incubations with individual human liver microsomes were performed in 100-μl incubation mixtures containing (final concentrations) 3mM NADPH and 1 mg/ml microsomal protein in 0.2M Tris-HCl (pH 7.4). After preincubating at 37°C for 1 min, the reactions were started by the addition of the substrate estragole. The reactions were terminated by addition of 25-μl cold acetonitrile. All samples were centrifuged for 5 min at 16,000 × g, and 50 μl of the supernatant of each sample was analyzed by high performance liquid chromatography (HPLC) as described previously (Punt *et al.*, 2008).

Characteristics of the enzymatic conversion of 1′-hydroxyestragole to 1′-oxoestragole. The subcellular distribution and cofactor specificity of the enzymatic oxidation of 1′-hydroxyestragole to 1′-oxoestragole was studied by performing incubations with both pooled human liver microsomes and pooled human liver cytosol in the presence of NAD^+, NADH, $NADP^+$, or NADPH as cofactor. Formation of 1′-oxoestragole was analyzed in these incubations using GSH to trap and, subsequently, quantify the transient 1′-oxoestragole (Punt *et al.*, 2009). The incubation mixtures had a final volume of 100 μl, containing (final concentrations) 3mM of the specific cofactor, 2mM GSH, and 1 mg/ml microsomal or cytosolic protein in 0.2M Tris-HCl (pH 7.4). The reactions were started by addition of the substrate 1′-hydroxyestragole (3mM final concentration added from a 300mM stock solution in DMSO). Incubations were performed for 10 min and were terminated by addition of 25-μl cold acetonitrile. Quantification of the formed 1′-oxoestragole glutathione adduct (GS-1′-oxoestragole), reflecting the formation of 1′-oxoestragole, was performed by HPLC as described previously (Punt *et al.*, 2009).

Additional experiments were performed to investigate the possible involvement of 17β-HSD2. This was done by performing incubations in the presence of estradiol, a competitive inhibitor of this enzyme (Poirier *et al.*,

2001), and in the presence of gallic acid, which is an identified stimulator of 17β-HSD2 (Stupans *et al*., 2000). Incubation conditions were as described above and were performed at a substrate concentration of 25μM 1′-hydroxyestragole (added from a 5mM stock solution in DMSO). The final concentration of estradiol or gallic acid was 500μM (added from a 100mM stock solution in DMSO). Formation of GS-1′-oxoestragole in these incubations was compared to the formation of GS-1′-oxoestragole in control incubations without chemical inhibitor/stimulator but with 1% DMSO as solvent control.

Oxidation of 1′-hydroxyestragole by individual human liver microsomes. Interindividual differences in the maximum oxidation rate of 1′-hydroxyestragole were determined. Because it is assumed that this reaction is catalyzed by one enzyme, namely 17β-HSD2, it was assumed that the K_m values for oxidation will be similar among individual human subjects and also similar to the K_m obtained with pooled human liver microsomes (Punt *et al*., 2009). The V_{max} values for different individual human liver microsomes were determined by measuring the maximum 1′-hydroxylation rate by the microsomal fractions of the same 14 individual human subjects as used to determine the kinetics for 1′-hydroxylation of estragole. Formation of 1′-oxoestragole was analyzed in these incubations using NAD^+ as cofactor and GSH to trap and, subsequently, quantify the transient 1′-oxoestragole (Punt *et al*., 2009). Incubations were performed and analyzed as described above and were performed at a saturating substrate concentration of 3mM 1′-hydroxyestragole (added from a 300mM stock solution in DMSO). Incubations were carried out for 10 min, in which formation of GS-1′-oxoestragole was linear with time and protein concentration.

PBBK modeling. PBBK models for estragole in different individual human subjects were developed using kinetic data obtained with liver microsomal fractions of individual human subjects. The basic structure of the models was obtained from the previously developed PBBK model for estragole in human (Punt *et al*., 2009). A schematic diagram of the model is displayed in Figure 2. The model code in MMD file format is provided in the supplemantary material. Within the models for individual human subjects, the kinetics for 1′-hydroxylation of estragole and oxidation of 1′-hydroxyestragole were described for each individual human subject specifically.

The kinetics for 1′-hydroxylation of estragole were described by Equation 1, in which the RAFs of the P450 enzymes are specific for each individual human subject. The maximum 1′-hydroxylation rate by each specific enzyme in individual human liver microsomes, expressed as pmol/min/(mg microsomal protein), was scaled to the liver using a microsomal protein yield of 32 mg/g liver (Barter *et al*., 2007). In addition, these maximum 1′-hydroxylation rates were multiplied by a correction factor of 1.9 to account for an observed level of underestimation of the 1′-hydroxylation rates by individual human liver microsomes using Equation 1 (see the "Results" section for justification of this correction factor). The *in vivo* K_m values for 1′-hydroxylation by the different P450 enzymes were assumed to be identical to those measured *in vitro* using Gentest microsomes.

The maximum oxidation rate of 1′-hydroxyestragole, as determined in the present study for different individual human liver microsomes, was scaled to the liver using a microsomal protein yield of 32 mg/g liver (Barter *et al*., 2007). The K_m values for oxidation were kept for each individual to the value previously obtained with pooled individual human liver microsomes (i.e., 354μM) (Punt *et al*., 2009).

Interindividual variation in metabolic reactions other than 1′-hydroxylation of estragole and oxidation of 1′-hydroxyestragole were assumed not to influence the liver concentration of 1′-hydroxyestragole in the different individual human subjects significantly (see the sensitivity analysis in the "Result" section for evaluation of this assumption). For this reason, the formation of these metabolites was estimated based on *in vitro* kinetic data obtained with pooled human liver microsomes as described previously (Punt *et al*., 2009). Interindividual differences in physiological parameters were not taken into account in the evaluation of interindividual human variation in liver levels of 1′-hydroxyestragole since variation in these parameters will not affect the balance between bioactivation and detoxification pathways and, therefore, do not affect the overall extent of bioactivation.

Sensitivity analysis. A sensitivity analysis was performed to analyze the relative effect of changes in activity levels of the enzymes involved in 1′-hydroxylation of estragole and oxidation of 1′-hydroxyestragole as well as of other metabolic parameters on the area under the liver concentration-time curve (AUC) of 1′-hydroxyestragole in different individual human subjects. This was done to identify the key metabolic reactions that influence the levels of 1′-hydroxyestragole in the liver. In addition to the metabolic parameters, the sensitivity to different physiological parameters was analyzed as well. Normalized sensitivity coefficients (SCs) were determined according to the following equation: $SC = (C' - C)/(P' - P)(P/C)$, where C is the initial value of the model output, C' is the modified value of the model output resulting from an increase in parameter value, P is the initial parameter value, and P' is the modified parameter value (Evans and Andersen, 2000). A 5% increase in model parameter was chosen to analyze the effect of a change in parameter value on the AUC of 1′-hydroxyestragole after 24 h (Evans and Andersen, 2000). Each parameter was analyzed individually, keeping the other parameters to their initial values.

Evaluation of interindividual variability on population level. The level of variation in the AUC of 1′-hydroxyestragole that could occur in the population as a whole was analyzed with a Monte Carlo simulation. For this analysis, a total of 10,000 simulations were performed, where in each simulation, the V_{max} values for 1′-hydroxylation of estragole by different P450 enzymes and the V_{max} value for oxidation of 1′-hydroxyestragole were randomly taken from a log-normal distribution. The V_{max} values for the different metabolic reactions were assumed to vary independently. For this

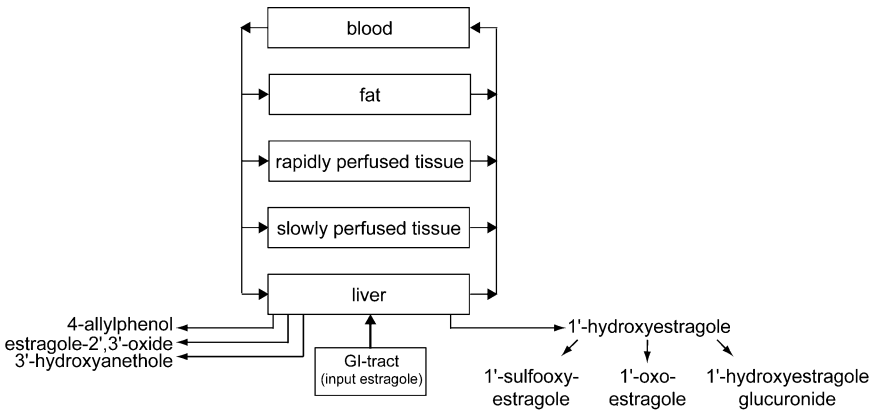

FIG. 2. Schematic diagram of the PBBK model for estragole in human.

TABLE 2
RAFs[a] of Different P450 Enzymes in Different Individual Human Liver Microsomes

Human subject	Gender	RAF_{1A2}	RAF_{2A6}	RAF_{2C19}	RAF_{2D6}	RAF_{2E1}
HH837	F	2.44	1.06	0.22	0.23	1.42
HH37	M	1.94	0.52	0.04	0.63	3.42
HG3	F	0.33	1.17	0.11	0.22	1.83
HH741	M	0.67	0.83	0.04	0.18	2.42
HH18	F	2.25	0.61	0.05	0.07	1.00
HG64	M	0.42	0.41	0.06	0.40	2.25
HH47	F	1.00	0.28	0.01	0.33	2.50
HK37	F	1.97	0.26	0.15	0.33	1.42
HG43	F	1.58	0.26	1.00	0.02	0.80
HG93	F	1.58	0.16	0.15	0.10	1.75
HH13	M	1.44	0.14	0.00	0.30	1.67
HG74	M	1.44	0.20	0.12	0.30	1.17
HG103	M	1.08	0.20	0.08	0.19	1.25
HG95	F	0.81	0.10	0.02	0.33	1.08
Fold variation		7	12	225	36	4

Note. M, male; F, female.

[a]Relative activity factors (RAFs) were calculated as the ratio between (1) the conversion of a P450-selective marker substrate (at saturating substrate conditions) expressed as pmol/min/(mg microsomal protein) by liver microsomes from an individual human subject and (2) the conversion of this marker substrate (at saturating substrate conditions) expressed as pmol/min/(mg microsomal protein) by Gentest microsomes expressing a single P450 enzyme.

simulation, the mean μ_W and SD σ_W describing the log-normal distribution of each V_{max} value were derived from the 14 individual human subjects of the present study using the following equations:

$$\mu_W = \ln\left(\mu_X/\sqrt{(1+CV_X^2)}\right) \text{ and } \sigma_W^2 = \ln\left(1+CV_X^2\right),$$

where μ_x is the mean and CV_x is the coefficient of variation of the non–log-transformed V_{max} values of the different metabolic reactions as observed with the 14 individual human subjects of the present study (Zhang *et al*., 2007).

RESULTS

Relative Activity Factors

Table 2 displays the RAFs of the P450 enzymes involved in 1′-hydroxylation of estragole for the set of 14 different individual human liver microsomes used in the present study. The RAFs of the P450 enzymes vary significantly between the selected individual human subjects and allow evaluation of the effect of these individual differences on the overall 1′-hydroxylation rate.

Validation of the Outcomes of Equation 1

Based on Equation 1 and using the kinetic constants for 1′-hydroxylation by specific P450 enzymes as displayed in Table 1 and the RAFs for the different P450 enzymes as displayed in Table 2, the 1′-hydroxylation rate by different individual human liver microsomes can be described. To validate whether with this equation the rate for 1′-hydroxylation of estragole by individual human liver microsomes can be determined correctly, the 1′-hydroxylation rate at a saturating substrate concentration of 1000μM was calculated using Equation 1 (Table 3) and compared to the 1′-hydroxylation rate actually measured using the different individual human liver microsomes. In Figure 3, the calculated 1′-hydroxylation rates obtained for the 14 individual human liver microsomes are plotted against the measured 1′-hydroxylation rates by these individual human liver microsomes. Although the calculated values are overall ~1.9-fold lower than the ones actually measured in incubations with the respective liver microsomes, a relatively strong correlation ($r^2 = 0.70$) between the calculated and measured 1′-hydroxylation rates is observed.

Contribution of the Different P450 Enzymes to the Overall 1′-Hydroxylation Rate

As shown in Equation 1, the sum of the 1′-hydroxylation rates by the different P450 enzymes in individual human liver microsomes represents the overall 1′-hydroxylation rate for these individual human liver microsomes. Table 3 shows, in addition to the calculated overall 1′-hydroxylation rate by individual human liver microsomes at a saturating substrate concentration of 1000μM, the contribution of the different P450 enzymes to this overall 1′-hydroxylation rate. Of the different P450 enzymes involved in estragole 1′-hydroxylation, P450 1A2, 2A6, and 2E1 are the enzymes predominantly involved in this reaction in the different individual human subjects, contributing with, respectively, 22 ± 10%, 32 ± 16%, and 32 ± 10% to the overall 1′-hydroxylation rate. The highest 1′-hydroxylation rate was calculated and measured for individual HH837, expressing a relatively high activity of both P450 1A2 and 2A6. The lowest 1′-hydroxylation rate was calculated and measured for individual HG95 who has a relatively low 2A6 activity. Overall, a 3.1-fold variation in 1′-hydroxylation rate at saturating substrate conditions by these individual human liver microsomes was observed (Table 3).

Comparing the percentage contribution of the different P450 enzymes to the total calculated 1′-hydroxylation rate by the different individual human liver microsomes reveals that when the contribution of one enzyme is relatively low in an individual human subject, other enzymes are still carrying out the reaction, resulting in an increased relative contribution of these enzymes to the overall hydroxylation rate. This indicates that the impact of variation in one enzyme on the overall hydroxylation rate is limited due to the remaining activity of other enzymes.

Characteristics of the Enzymatic Conversion of 1′-Hydroxyestragole to 1′-Oxoestragole

When analyzing the subcellular distribution and cofactor specificity of the enzymatic oxidation of 1′-hydroxyestragole to 1′-oxoestragole (Fig. 4), it is observed that the highest level of oxidation occurs in incubations with human liver

TABLE 3
Calculated[a] Contribution of Single P450 Enzymes to the Overall Maximum 1′-Hydroxylation Rate by Different Individual Human Liver Microsomes

	Calculated 1′-hydroxylation rate in individual human liver microsomes by specific P450 enzymes					
Human subject	P450 1A2	P450 2A6	P450 2C19	P450 2D6	P450 2E1	Overall 1′-hydroxylation rate
HH837	157 (23)	372 (54)	19 (3)	38 (6)	108 (16)	694
HH37	125 (18)	182 (27)	4 (1)	106 (16)	261 (39)	678
HG3	21 (3)	411 (66)	9 (2)	37 (6)	140 (23)	618
HH741	43 (8)	293 (53)	3 (1)	31 (6)	184 (33)	554
HH18	145 (32)	216 (48)	4 (1)	12 (3)	76 (17)	453
HG64	27 (6)	143 (34)	5 (1)	68 (16)	172 (41)	415
HH47	64 (16)	100 (24)	1 (0)	55 (13)	191 (46)	411
HK37	127 (32)	90 (23)	13 (3)	55 (14)	109 (28)	394
HG43	102 (30)	90 (27)	84 (25)	3 (1)	61 (18)	340
HG93	102 (32)	55 (17)	12 (4)	17 (5)	133 (42)	320
HH13	93 (29)	49 (15)	0 (0)	51 (16)	127 (40)	320
HG74	93 (30)	70 (22)	10 (3)	51 (16)	89 (28)	313
HG103	70 (25)	70 (26)	6 (2)	32 (12)	95 (35)	273
HG95	52 (23)	35 (16)	2 (1)	55 (24)	83 (37)	226

Note. Within parenthesis, the percentage contribution to the calculated overall 1′-hydroxylation rate is given.

[a]The 1′-hydroxylation rate was calculated at a concentration of 1000μM estragole (representing a saturating substrate concentration) using Equation 1, and the RAFs of different enzymes in individual human liver microsomes as displayed in Table 2. The 1′-hydroxylation rate is expressed as pmol/min/(mg microsomal protein).

microsomes in the presence of NAD^+ as cofactor, suggesting an NAD^+-dependent microsomal enzyme to be involved in this reaction. Of all possible options, especially, 17β-HSD2 fulfills these requirements. No significant oxidation rate was observed in incubations with pooled human liver cytosol in the presence of any cofactor, indicating that the reaction is not catalyzed by alcohol dehydrogenases or other enzymes present in the cytosol.

To further investigate a possible role for 17β-HSD2 in the oxidation of 1′-hydroxyestragole, additional incubations were performed in the presence of estradiol, which is a competitive inhibitor of 17β-HSD2 (Poirier *et al*., 2001), and gallic acid, which is an identified stimulator of 17β-HSD2 (Stupans *et al*., 2000). Figure 5 shows that the inhibitor estradiol reduces the oxidation rate significantly. In addition, the stimulator gallic acid increases the oxidation rate significantly. Both results corroborate the involvement of 17β-HSD2 in the oxidation reaction.

Maximum Oxidation Rate of 1′-Hydroxyestragole by Individual Human Liver Microsomes

The maximum oxidation rate of 1′-hydroxyestragole to 1′-oxoestragole by 14 different individual human liver microsomes, at saturating substrate conditions, was analyzed to determine the occurrence of interindividual differences in this reaction. The oxidation rate at a substrate concentration of 3mM 1′-hydroxyestragole by the 14 different individual human liver microsomes is displayed in Figure 6. The highest oxidation rate was observed for individual HG3 for which the rate was twofold higher than for individual HG93.

PBBK Modeling

A PBBK model for estragole metabolism was developed and parameterized for each of the 14 individuals with respect to the kinetics for 1′-hydroxylation of estragole and oxidation of 1′-hydroxyestragole based on the kinetics for these reactions by the individual human liver microsomes. Using the PBBK models obtained for the 14 individuals, the contribution of the observed interindividual differences in kinetics for 1′-hydroxylation of estragole and oxidation of 1′-hydroxyestragole on the predicted liver concentration

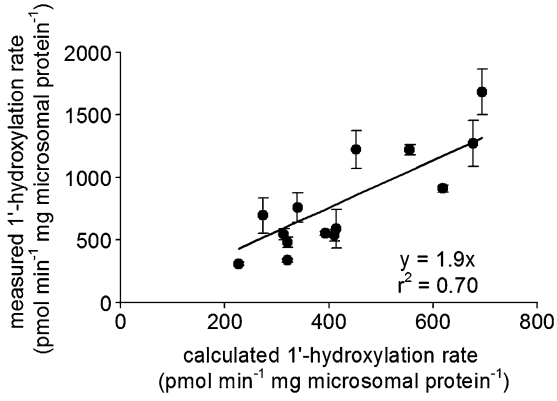

FIG. 3. Calculated 1′-hydroxylation rate for different individual human liver microsomes plotted against the measured values for these human liver microsomes. The calculated values are obtained using Equation 1, and the RAFs of different enzymes in individual human liver microsomes as displayed in Table 2.

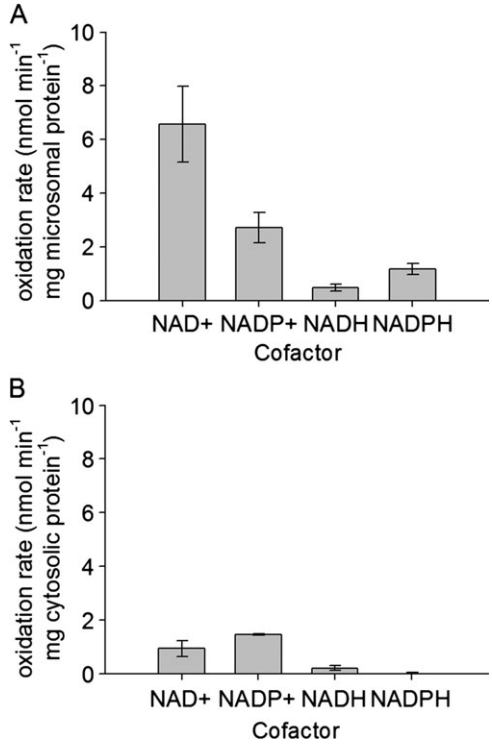

FIG. 4. Oxidation rate of 1′-hydroxyestragole to 1′-oxoestragole in incubations with (A) pooled human liver microsomes in the presence of NAD^+, $NADP^+$, NADH, or NADPH as cofactor and (B) pooled human liver cytosol in the presence of NAD^+, $NADP^+$, NADH, or NADPH as cofactor. Bars indicate average activities of triplicate measurements ± SD ($n = 3$).

of 1′-hydroxyestragole at estragole intake levels relevant for dietary human intake can be analyzed (Fig. 7). Figure 7A shows the predicted time-dependent liver concentration of 1′-hydroxyestragole in 14 different individual human subjects after exposure to 0.07 mg/kg bw estragole. This dose corresponds to the estimated dietary human intake of estragole (SCF, 2001). Simulations were performed with the entire quantity consumed as a single bolus, rather than spread out

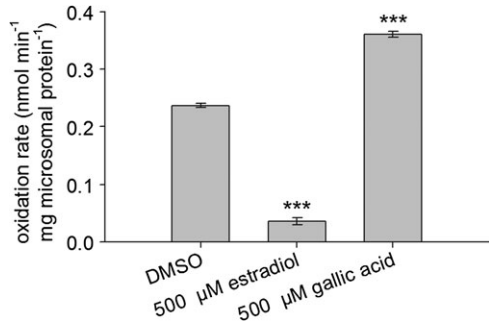

FIG. 5. Oxidation rate of 1′-hydroxyestragole to 1′-oxoestragole in incubations with pooled human liver microsomes with NAD^+ as cofactor at a substrate concentration of 25µM 1′-hydroxyestragole in the presence of 1% DMSO (as solvent blank), estradiol, or gallic acid. Bars indicate average activities of triplicate measurements ± SD ($n = 3$). ***Indicates significant differences from the incubations without inhibitor (Student′s t-test, $p < 0.001$).

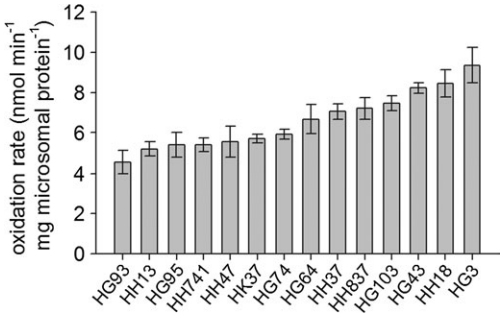

FIG. 6. Oxidation rate of 1′-hydroxyestragole to 1′-oxoestragole by different individual human liver microsomes at a saturating substrate concentration of 3mM. Bars indicate average activities of triplicate measurements ± SD ($n = 3$).

over multiple meals. The AUC obtained for the different individual human subjects is displayed in Figure 7B. Overall, the AUC of 1′-hydroxyestragole is predicted to vary 1.8-fold.

The predicted AUC of 1′-hydroxyestragole in the individual human subjects was negatively correlated with the maximum oxidation rate of 1′-hydroxyestragole by these subjects ($r = -0.71$), with the highest AUC being observed for individuals HG93 and HH741 who displayed a relative low detoxification rate of 1′-hydroxyestragole via oxidation (Fig. 6) and the lowest AUC being observed for individuals HG103 and HG3 who displayed a relative high elimination rate of 1′-hydroxyestragole via oxidation (Fig. 6). No significant correlation between the predicted AUC and maximum 1′-hydroxylation rates of estragole are observed ($r = 0.25$), indicating that individual differences in AUC of 1′-hydroxyestragole mainly depend on the level of variation in oxidation of 1′-hydroxyestragole rather than on the level of variation in the rate for 1′-hydroxylation of estragole.

Sensitivity Analysis

The sensitivity of the AUC of 1′-hydroxyestragole as predicted with the models for individual HG95, HH47, and HH837, which were observed to have a low, median, and high maximum 1′-hydroxylation activity, respectively (Table 3), to changes in all metabolic parameters was analyzed. Normalized SCs were calculated for different metabolic parameters at a dose of 0.07 mg/kg bw estragole by increasing parameter values with 5%. Only metabolic parameters that had a normalized SC higher than 0.1 (in absolute value) in at least one of the three models are displayed in Figure 8. The predicted AUC of 1′-hydroxyestragole was observed to be the most sensitive to changes in the kinetic constants for oxidation of 1′-hydroxyestragole (Fig. 8). Of the different P450 enzymes involved in estragole 1′-hydroxylation, the predicted AUC of 1′-hydroxyestragole is found to be to some extent sensitive to changes in kinetic constants for P450 1A2 and 2A6 but not to changes in kinetic constants for P450 2C19, 2D6, and 2E1 (Fig. 8). Furthermore, the predicted AUC of 1′-hydroxyestragole is observed to be sensitive to changes in

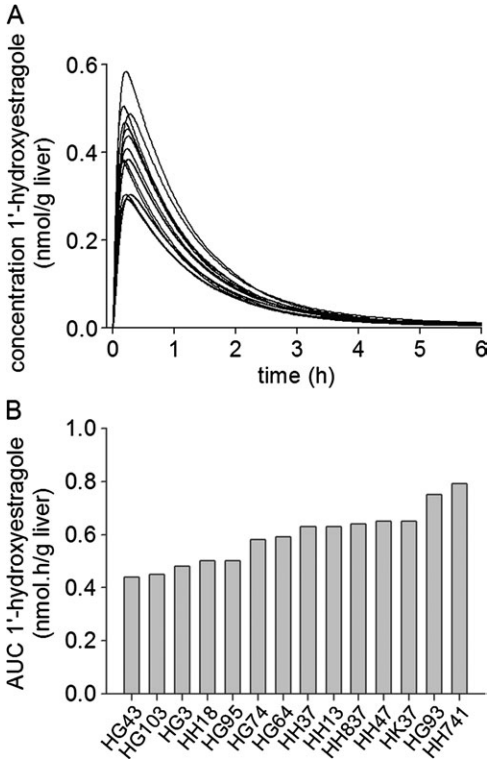

FIG. 7. Predicted time-dependent concentration of 1′-hydroxyestragole in the liver of different human subjects at an oral dose of 0.07 mg/kg bw as predicted by PBBK models for estragole metabolism in these human subjects based on individual enzyme kinetic data for 1′-hydroxylation of estragole and oxidation of 1′-hydroxyestragole (A) and AUC values derived from these curves for the individual human subjects (B).

kinetic constants for epoxidation of estragole, which is the major competing metabolic route to 1′-hydroxylation. The sensitivity toward metabolic parameters for 1′-hydroxylation by P450 1A2 and 2A6 as well as for the metabolic parameters for epoxidation is overall relatively low and when the maximum 1′-hydroxylation activity becomes higher, as in individual HH47 and HH837 (Table 2), the sensitivity to these metabolic parameters becomes even lower.

Of the different physiological parameters (including body weight, tissue volumes, and blood flow rates), the predicted AUC of 1′-hydroxyestragole appears to be only sensitive to changes in the value of the liver volume (SC = 0.9 for all three individuals analyzed).

Evaluation of Interindividual Variability on Population Level

A Monte Carlo simulation was performed to evaluate the extent of variation in liver levels of 1′-hydroxyestragole that could occur in the population as a whole due to variation in 1′-hydroxylation of estragole and oxidation of 1′-hydroxyestragole. In Table 4, the parameters that were varied and the distributions assigned to these parameters are given. The probability distribution for each parameter has been derived from the 14 individual human subjects for whom the V_{max} values for 1′-hydroxylation of estragole by different P450 enzymes and the V_{max} values for oxidation of 1′-hydroxyestragole have been defined in the present study. In case of the different P450 enzymes, the coefficients of variation obtained correspond to those observed in the literature for a larger group of people, which have been reported to be 67, 84, 106, 61, and 61% for P450 1A2, 2A6, 2C19, 2D6, and 2E1, respectively (Rostami-Hodjegan and Tucker, 2007). In case of oxidation of 1′-hydroxyestragole, a coefficient of variation of 22% is derived from the data obtained with the 14 individual human subjects. Current knowledge on the enzyme involved in this reaction, namely 17β-HSD2, is not sufficient to provide an indication of whether this level of variation is representative for the population as a whole. The effect of a possible larger level of variation in activity of this enzyme that could occur in the population was taken into account by performing a Monte Carlo simulation at a threefold higher coefficient of variation for this enzyme as well. A 93-fold difference is then observed between the highest and the lowest V_{max} value for oxidation of 1′-hydroxyestragole.

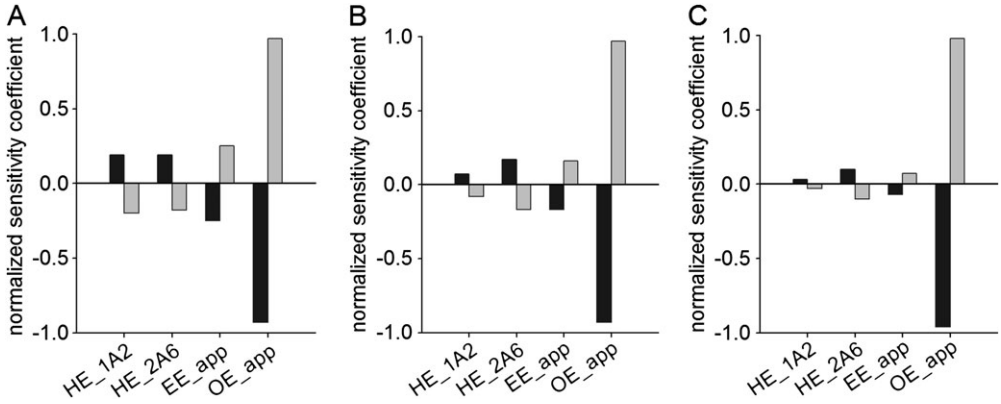

FIG. 8. Sensitivity of the predicted AUC of 1′-hydroxyestragole to changes in V_{max} values (black bars) and K_m values (gray bars) for (1) cytochrome P450 1A2 and 2A6 catalyzed formation of 1′-hydroxyestragole (HE_1A2 and HE_2A6), (2) apparent formation of estragole-2′,3′-oxide (EE_app), and (3) apparent formation of 1′-oxoestragole (OE_app) in the PBBK models for individual HG95 (A), HH47 (B), and HH837 (C).

TABLE 4
Distributions for the Parameters Varied in the Monte Carlo Simulation

Parameter	Mean[a]	CV[b]	Minimum–maximum value in simulation	Fold variation
$V_{max,\ HE\_1A2}$	88	49	12–489	40
$V_{max,\ HE\_2A6}$	156	79	8–1654	207
$V_{max,\ HE\_2C19}$	22	171	0.1–903	7813
$V_{max,\ HE\_2D6}$	100	59	11–507	44
$V_{max,\ HE\_2E1}$	136	42	25–566	23
$V_{max,\ OE}$	6515	22	3206–14953	5

[a]Mean of the non–log-transformed V_{max} values (pmol/min/[mg microsomal protein]).
[b]Coefficient of variation of the non–log-transformed V_{max} values (%).

The outcome of the Monte Carlo simulation, based on the parameters displayed in Table 4, is shown in Figure 9, where the predicted distribution of the AUC of 1′-hydroxyestragole for the total population is given. The difference between the highest and the lowest predicted value of the AUC of 1′-hydroxyestragole is fivefold. The highest value amounts to 1.3 nmol·h/g liver, which was due to a low level of detoxification of 1′-hydroxyestragole via oxidation. This situation occurred once in the 10,000 simulations. The lowest value of the AUC of 1′-hydroxyestragole amounts to 0.2 nmol·h/g liver and was due to a high level of oxidation of 1′-hydroxyestragole as well as a low level of formation of 1′-hydroxyestragole due to a limited activity level of P450 2A6. This situation occurred twice in the 10,000 simulations. The 50th, 95th, and 99th percentiles of the distribution of the AUC of 1′-hydroxyestragole correspond to 0.56, 0.78, and 0.90 nmol·h/g liver, respectively. When a larger level of variation for oxidation of 1′-hydroxyestragole is modeled by assuming that the coefficient of variation is threefold higher than was determined with the sample of the 14 individual human subjects (i.e., 66 instead of 22%), the 95th and 99th percentiles of the distribution of the AUC of 1′-hydroxyestragole are about twofold higher and correspond to 1.50 and 2.26 nmol·h/g liver, respectively, and the 50th percentile stays unchanged. At this level of variation, the difference between the highest and the lowest value of the AUC of 1′-hdyroxyestragole was 100-fold, with the highest value being 6.1 nmol·h/g liver and the lowest value being 0.06 nmol·h/g liver.

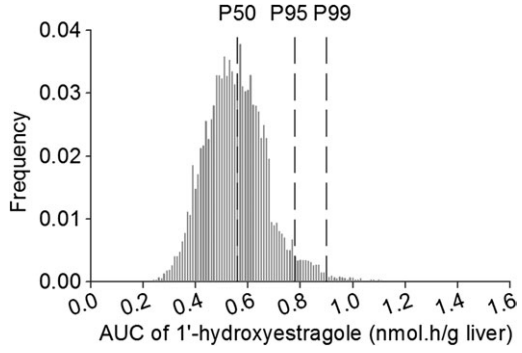

FIG. 9. Predicted distribution of the AUC of 1′-hydroxyestragole.

DISCUSSION

A PBBK model for estragole metabolism was developed and parameterized for individual human subjects with respect to the kinetics for 1′-hydroxylation of estragole and oxidation of 1′-hydroxyestragole. These reactions are key metabolic steps that influence the concentration of 1′-hydroxyestragole in the liver that is available for formation of the ultimate carcinogen 1′-sulfooxyestragole. With the models obtained the effect of interindividual variability in 1′-hydroxylation of estragole, and oxidation of 1′-hydroxyestragole to the predicted liver levels of 1′-hydroxyestragole was evaluated. In addition, the variability in liver levels of 1′-hydroxyestragole to be expected in the human population as a whole was analyzed with a Monte Carlo analysis.

The present study focused on the effects of interindividual variability in 1′-hydroxylation of estragole and oxidation of 1′-hydroxyestragole on the levels of 1′-hydroxyestragole in human liver. Since formation of the ultimate carcinogenic metabolite 1′-sulfooxyestragole depends on the levels of 1′-hydroxyestragole in the liver, the predicted interindividual variability in liver levels of 1′-hydroxyestragole will translate directly to variability in formation of 1′-sulfooxyestragole. However, subsequent formation of 1′-sulfooxyestragole will also be affected by interindividual variability in the kinetic constants of this metabolic reaction itself (Punt *et al*., 2009). To date, the sulfotransferase enzymes involved in sulfonation of 1′-hydroxyestragole have not been identified. The principal sulfotransferase enzymes that are present in the human liver are SULT1A1 (phenol sulfotransferase) and SULT2A1 (hydroxysteroid sulfotransferase), which can, thus, be expected to be involved (Glatt, 2000). For both enzymes, genetic polymorphisms have been described, which were generally observed to lead to reduced enzyme activity (Glatt, 2000; Nagar *et al*., 2006; Thomae *et al*., 2002). In addition, dietary factors might influence the sulfotransferase activity as well. It was, for instance, recently observed by Jeurissen *et al*. (2008) that basil extract is able to efficiently inhibit the sulfotransferase-mediated DNA adduct formation in HepG2 human hepatoma cells exposed to 1′-hydroxyestragole. Whether this inhibition could also occur *in vivo* was, however, not yet established. When evaluating interindividual differences in the overall extent of bioactivation of estragole to 1′-sulfooxyestragole, variability in this reaction should be taken into account as well.

Interindividual differences in glucuronidation of 1′-hydroxyestragole and other phase I metabolic reactions than 1′-hydroxylation of estragole were assumed to not affect interindividual variability in bioactivation of estragole since the

predicted AUC of 1′-hydroxyestragole was not observed to be sensitive to changes in kinetic constants for these reactions. Only in case of epoxidation of estragole, the predicted AUC of 1′-hydroxyestragole appeared to be somewhat sensitive to changes in the kinetic parameters for this reaction. However, when variation in the V_{max} values for epoxidation was taken into account in the Monte Carlo analysis, by deriving the probability distribution for this parameter from the fourteen individual human subjects of the present study, the predicted percentiles for the AUC of 1′-hydroxyestragole were not different from the situation where variation in epoxidation was not considered (data not shown). This supports that it is not necessary to take interindividual differences in epoxidation of estragole into account in the evaluation of interindividual differences in bioactivation of estragole.

In the first approach of the present study, the relative contribution of interindividual variability in 1′-hydroxylation of estragole and oxidation of 1′-hydroxyestragole to the variability in the predicted AUC of 1′-hydroxyestragole was evaluated using PBBK models for estragole metabolism in 14 different individual human subjects and individual kinetic data for these reactions. With the models obtained the AUC of 1′-hydroxyestragole was predicted to vary 1.8-fold, even though the activities of the major enzymes involved in estragole 1′-hydroxylation (i.e., P450 1A2, 2A6, and 2E1) were observed to vary 7-, 12-, and 4-fold, respectively, in the sample set used. The predicted interindividual differences in the AUC of 1′-hydroxyestragole appeared to be mainly dependent on a twofold variation in the oxidation rate of 1′-hydroxyestragole. The observed relatively small effect of variability in activity levels of P450 enzymes on the AUC of 1′-hydroxyestragole can be explained by the fact that different P450 enzymes are involved in the conversion of estragole to 1′-hydroxyestragole and that the impact of variation in one enzyme on the overall hydroxylation rate is limited due to the remaining activity of other enzymes. The outcomes of the sensitivity analysis corroborate that the sensitivity of the predicted AUC of 1′-hydroxyestragole to changes in activity levels of different P450 enzymes is much smaller than the sensitivity of the AUC to changes in the oxidation rate of 1′-hydroxyestragole.

In a second approach, the effect of variation that could occur in the population as a whole was analyzed. Knowledge about the enzymes involved in 1′-hydroxylation of estragole and oxidation of 1′-hydroxyestragole provides information on the theoretical variation in enzyme activity that could occur in the overall human population. Of the major P450 enzymes involved in 1′-hydroxylation of estragole, the variability in activity of both P450 1A2 and 2E1 mainly depends on lifestyle factors. Smoking and consumption of charbroiled meat can influence the activity levels of P450 1A2 (Jiang *et al*., 2006). Consumption of alcohol can influence the activity levels of P450 2E1 (Wenker *et al*., 2001). Overall, the variability in activity of these two enzymes can vary more than 60-fold between individual human subjects (Jiang *et al*., 2006; Wenker *et al*., 2001). Interindividual variability in P450 2A6 activity mainly depends on genetic differences. Polymorphisms occur that lead to poor metabolizer phenotypes or whole deletion genotypes (Ingelman-Sundberg *et al*., 1999). In the Monte Carlo simulation, the activity levels of P450 1A2, 2A6, and 2E1 varied 40-, 207-, and 23-fold, respectively. These levels of variation were assumed to be adequately representative for the population as a whole (Jiang *et al*., 2006; Rostami-Hodjegan and Tucker, 2007; Wenker *et al*., 2001).

To gain insight in the level of variability in oxidation of 1′-hydroxyestragole that could occur in the human population, it is of importance to characterize the enzyme involved in this reaction. Experiments reported in this paper revealed efficient 1′-hydroxyestragole oxidation especially in incubations with liver microsomes and NAD^+ as the cofactor and inhibition of the oxidation reaction by estradiol and activation by gallic acid. This indicates the possible involvement of 17β-HSD2 (Poirier *et al*., 2001; Stupans *et al*., 2000), which is present in the microsomal fraction of the liver and uses NAD^+ as the preferred cofactor (Peltoketo *et al*., 1999).

Current knowledge about 17β-HSD2 does not give a complete insight in the level of variation in enzyme activity that could occur in the human population. This enzyme is widely and abundantly expressed (Peltoketo *et al*., 1999). Recently, however, a specific sequence variant of the gene encoding 17β-HSD2 has been identified, which was observed to result in an enzyme with reduced stability in a transfected cell system (Plourde *et al*., 2008). Whether this protein instability could also lead to a reduced enzyme activity *in vivo* was, however, not established. In case of the related 3β-hydroxysteroid dehydrogenase type 2 enzyme, similar protein instability of several mutant proteins was observed to be involved in the occurrence of deficiencies of this enzyme *in vivo* (Moisan *et al*., 1999; Simard *et al*., 2005). This indicates that protein instability of 17β-HSD type 2 could, in theory, also lead to deficiencies of the corresponding enzyme *in vivo*. In addition to genotype-dependent effects, dietary factors might influence the activity of 17β-HSD2 (Schuster *et al*., 2008). Among different flavonoids, quercetin has, for instance, been observed to be an inhibitor (IC_{50} = 1.5μM) of 17β-HSD2-catalyzed oxidation of estradiol to estrone *in vitro* (Schuster *et al*., 2008). Whether such effects could also occur *in vivo* was, however, not elucidated. In the Monte Carlo simulation, the activity levels of 17β-HSD2 varied fivefold at a coefficient of variation of 22%, the coefficient of variation indicated by the 14 individual human subjects of the present study. When the coefficient of variation was assumed to be threefold higher (i.e., 66%), the activity levels of 17β-HSD2 varied 93-fold. Additional studies will be necessary to provide an indication of what level of variation in 17β-HSD2 will be representative for the human population.

With the results obtained with the Monte Carlo simulation on the variability in human liver levels of 1′-hydroxyestragole,

a so-called chemical-specific adjustment factor (CSAF) can be derived, which can be used to evaluate the appropriateness of the default factor of 3.16 generally assumed to reflect interindividual variation in biokinetics within the human population (Gentry *et al*., 2002; IPCS, 2005). Based on the guidelines of the International Program on Chemical Safety (IPCS, 2005), CSAFs can be calculated as the ratio between given percentiles (such as 95th, 97.5th, or 99th) and the central tendency for the whole population. Using the 99th percentile to represent a sensitive individual and the 50th percentile to represent the average individual, the CSAF might amount to 1.6 and 4.0 depending on the level of variation in oxidation of 1′-hydroxylation that is taken into account (i.e., a coefficient of variation of 22 vs. 66%). This predicted CSAF is comparable to the default factor of 3.16 for kinetic variability. Obviously, definite conclusions on the value of the CSAF have to await further data on the actual variation in the 17β-HSD2 activity to be expected within the human population.

Altogether the results of the present study indicate that in humans the oxidation of 1′-hydroxyestragole to 1′-oxoestragole, and not 1′-hydroxyestragole formation by cytochromes P450, is a major determinant leading to interindividual variability in the liver concentration of 1′-hydroxyestragole. Depending on the level of interindividual differences that might occur in this reaction, the default uncertainty factor of 3.16 is adequately protective for 99% of the population.

SUPPLEMENTARY DATA

Supplementary material is available online at http://toxsci.oxfordjournals.org/.

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