Physiologically based biokinetic (PBBK) models to characterize dose-dependent effects, species differences, and interindividual human variation in bioactivation and detoxification of estragole

Ans Punt

Promotoren:	Prof. Dr. Ir. I.M.C.M. Rietjens Hoogleraar in de Toxicologie Wageningen Universiteit
	Prof. Dr. P.J. van Bladeren Hoogleraar in de Toxicokinetiek en Biotransformatie Wageningen Universiteit
Co-promotor:	Dr. B. Schilter Head of the Chemical Food Safety Group Nestlé Research Center, Switzerland
Promotiecommissie:	Prof. Dr. B.J. Blaauboer Universiteit Utrecht
	Prof. Dr. F.G.M. Russel Radboud Universiteit Nijmegen
	Prof. Dr. M. van den Berg Universiteit Utrecht
	Prof. Dr. R.F. Witkamp Wageningen Universiteit

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Abstract

Estragole is a natural constituent of several herbs and spices and their essential oils. This compound has been found to be hepatocarcinogenic in different rodents when administered at high doses. Determining the cancer risk in humans at low dose dietary intake levels requires extrapolation of the animal carcinogenicity data with respect to species, dose and interindividual human variation.

The aim of the present thesis was to build physiologically based biokinetic (PBBK) models for both rats and (individual) human subjects to obtain quantitative insight into dose-dependent effects, species differences, and interindividual human variation in bioactivation and detoxification of estragole. 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. For the development of the PBBK models, metabolic parameters were determined for all detoxification and bioactivation steps of estragole *in vitro* using relevant tissue fractions. The physiological parameters, needed for the PBBK models, were obtained from the literature and partition coefficients were estimated from the octanol-water partition coefficient based on an in silico method.

With the PBBK model defined for estragole in rat, the factors underlying the dose-dependent differences in estragole bioactivation could be revealed. Due to saturation of the *O*-demethylation pathway in the lung and kidney at high doses of estragole a relative increase in formation of the proximate carcinogenic metabolite 1'-hydroxyestragole in the liver occurs, leading to a relative increase in bioactivation of estragole to 1'-sulfooxyestragole at higher doses of estragole. In contrast to rat, the PBBK model defined for estragole in human predicted a relative decrease in formation of 1'-sulfooxyestragole in human liver with increasing dose levels. This was due to the fact that in humans efficient *O*-demethylation in lung and kidney was absent, whereas in the rat these conversions reduced the level of bioactivation at low doses.

Species-differences in formation of the ultimate carcinogenic metabolite

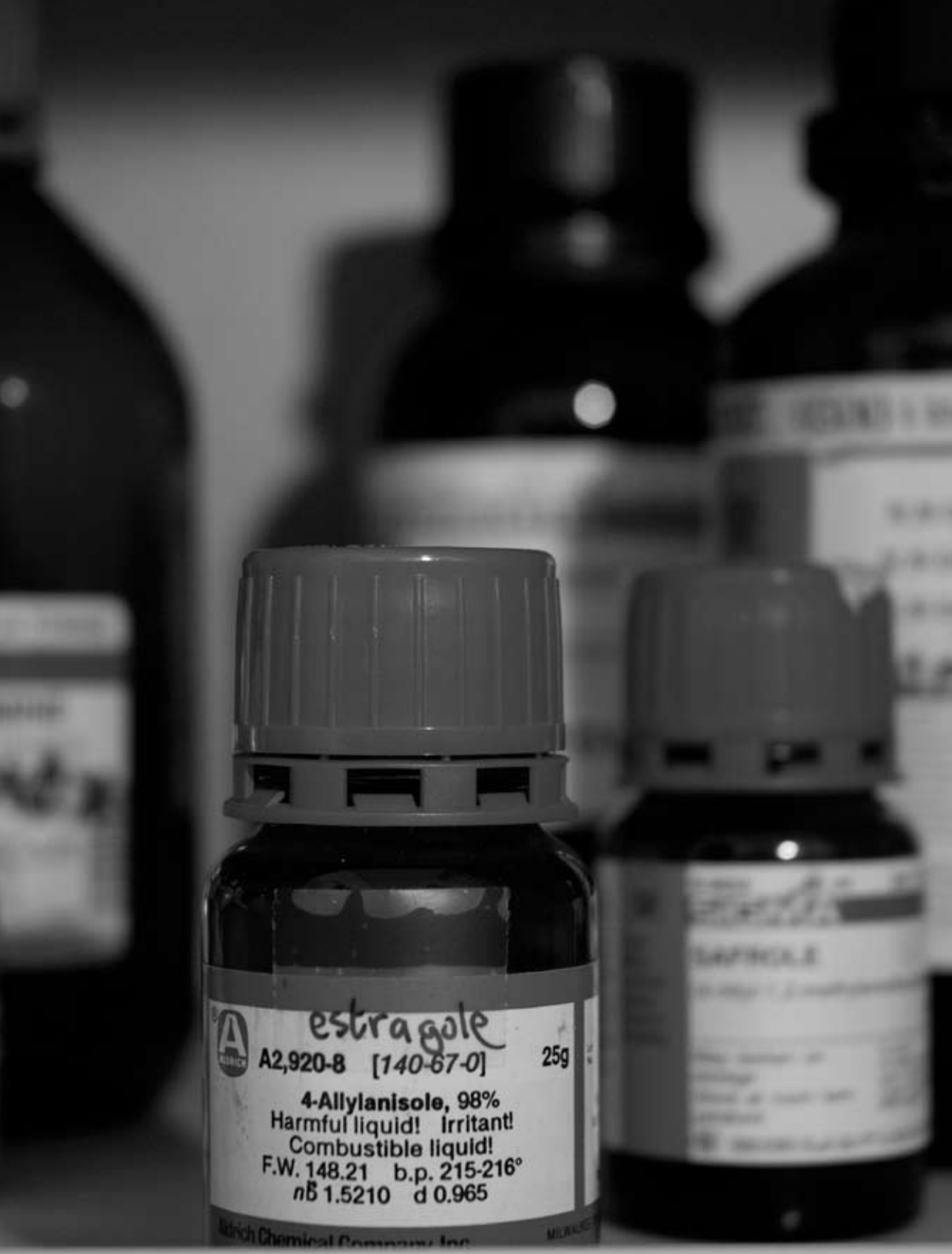
1'-sulfooxyestragole between human and rat were predicted to be relatively low. Even though the formation of the proximate carcinogen 1'-hydroxyestragole was predicted to be 4-fold higher in human, formation of 1'-sulfooxyestragole was predicted to be only 2-fold higher in human due to a high level of detoxification of 1'-hydroxyestragole in human liver by oxidation to 1'-oxoestragole as compared to rat liver. It was concluded that in spite of significant differences in the relative extent of different metabolic pathways between human and male rat, there is a minor influence of species differences on the ultimate overall bioactivation of estragole to 1'-sulfooxyestragole.

The level of interindividual variation in liver levels of the proximate carcinogenic metabolite 1'-hydroxyestragole, due to variation in 1'-hydroxylation of estragole and oxidation of 1'-hydroxyestragole, was investigated. These are two key metabolic reactions involved in formation and detoxification of this metabolite. Formation of 1'-hydroxyestragole was shown to be predominantly catalyzed by P450 1A2, 2A6, and 2E1. Oxidation of 1'-hydroxyestragole was shown to be catalyzed by type 2 17β-hydroxysteroiddehydrogenase (17β-HSD). It was observed that the oxidation of 1'-hydroxyestragole to 1'-oxoestragole, and not 1'-hydroxyestragole formation by cytochromes P450, was the major determinant leading to interindividual variability in the liver levels of 1'-hydroxyestragole. Both dietary and genotype-based influences on type 2 17β-HSD activity might lead to reduced or diminished oxidation activity. Based on a worst case estimate of the level of interindividual variability that might occur in this reaction it was concluded that liver levels of 1'-hydroxyestragole may vary one to two orders of magnitude within the human population and might thus be larger than the default factor of 3.16 generally assumed to reflect interindividual variation in biokinetics within the human population.

Altogether, the results presented in this thesis show that integrating *in vitro* metabolic parameters using a PBBK model as a framework, provides a good method to evaluate the occurrence of dose-dependent effects, species

differences, and human variability in bioactivation of estragole. The model predictions obtained can be used to provide a more mechanistic basis for the assessment of the cancer risk in humans at low dose dietary intake levels based on carcinogenicity data obtained in experiments with rodents at high dose levels. Overall, the PBBK results of the present study showed that linear extrapolation of the cancer risk in rats at high dose levels to dose levels is feasible. In addition, estimates of the cancer risk obtained can be considered relevant for the human situation.

Contents


estragole
A2,920-8 [140-67-0]
25g
4-Allylanisole, 98%
Harmful liquid! Irritant!
Combustible liquid!
F.W. 148.21 b.p. 215-216°
nD 1.5210 d 0.965

1

General introduction

Background

The research of this PhD project focuses on the compound estragole (Figure 1), which is an alkenylbenzene that occurs naturally in different herbs such as tarragon, basil, and fennel and is present in products derived from these herbs such as pesto and essential oils (Smith *et al.*, 2002; Siano *et al.*, 2003). There is interest in the risk of estragole as a food constituent, since estragole has been identified to be genotoxic *in vitro* and carcinogenic in rodent studies performed at high dose levels (Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Wiseman *et al.*, 1987).

Several evaluations have been performed to assess the safety of human exposure to estragole at low dietary intake levels. In an evaluation performed by the Scientific Committee on Food of the European Committee (SCF) in 2001 it was concluded that estragole is genotoxic and carcinogenic, and restrictions in use levels were indicated (SCF, 2001). The Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) classified estragole in 1965 as GRAS (Generally Recognized as Safe) under conditions of intended use as flavoring substance in food (Hall and Oser, 1965). In 2002 the FEMA re-evaluated the data available for estragole and concluded again that exposure to estragole from food, mainly as spices or added as such, does not pose a significant cancer risk to humans (Smith *et al.*, 2002). In this conclusion, it was taken into account that there are experimental data suggesting a non-linear relationship between dose and profiles of metabolism and metabolic

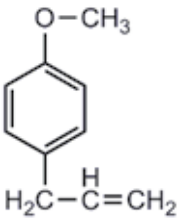


Figure 1. Structure of estragole

activation. In a more recent evaluation performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2008, it was indicated that although evidence of carcinogenicity to rodents given high doses of estragole exists, further research is needed to assess the potential risk to human health from low-level dietary exposure to estragole present in foods and essential oils and used as flavoring agents (JECFA 2008).

Overall these different expert judgments denote a general problem in cancer risk assessment studies, which is a lack in scientific consensus to translate carcinogenicity data obtained in experiments with rodents at high levels of exposure to the situation for humans with low levels of exposure. Determining the cancer risk in humans at low dose dietary intake levels requires extrapolation of the animal carcinogenicity data with respect to species, dose and interindividual variation, which is larger in the human population than in the inbred strains used for rodent experiments. Uncertainties exist in the shape of the dose-response curve below the range of the animal experimental data, and in addition, species and interindividual differences in metabolism and metabolic activation can occur. The aim of the present PhD project was to obtain quantitative insight into the consequences of dose- and species-dependent effects and of interindividual differences for the bioactivation and detoxification of estragole, using physiologically based biokinetic (PBBK) modeling.

Exposure to estragole

Estragole occurs naturally in a number of herbs such as tarragon, basil, fennel, anise, and star anise (Smith *et al.*, 2002). Estragole can also be added as such to food products, which only occurs in the US on a small scale (Smith *et al.*, 2002). Overall, consumption of estragole mainly occurs due to consumption of products containing herbs or extracts of these herbs added as flavoring to products (Smith *et al.*, 2002). The concentrations of estragole in herbs can vary considerably, depending on the plant maturity at harvest, harvesting

techniques, storage conditions, processing (e.g. drying), and geographical origin of the plant (Smith *et al.*, 2002). Of different products, especially pesto sauce was observed to contain relative high amounts of estragole amounting to 5.26 (0.05-19.30) mg per kg product (Siano *et al.*, 2003). Consumption of a portion of 10 g pesto can therefore result in intake levels of estragole ranging from 0.5 to 193 µg, which can contribute significantly to the dietary human exposure levels of estragole.

The average daily per capita intake of estragole was estimated by the Scientific Committee on Food of the European Union (SCF) to amount to about 4.3 mg per day (with a 95th percentile of 8.7 mg per day) (SCF, 2001). This estimation is based on a conservative method using theoretical maximum use levels of estragole in 28 food categories and consumption data for these food categories based on seven days dietary records of adult individuals (SCF, 2001). Using a different method, a lower average daily per capita intake of estragole was estimated by the FEMA (Smith *et al.,* 2002). This estimation was performed using production volume data of herbs, essential oils, and flavoring substances containing estragole in the US (Smith *et al.*, 2002). Based on the average concentrations of estragole in herbs, essential oils, and flavoring substances, and assuming that only 10% of the population consumes all of the food containing estragole (‘eaters only'), the FEMA estimated the daily per capita intake for the US population to be less than 10 µg/kg bw/day (corresponding to 0.6 mg/day for 60 kg person) (Smith *et al.*, 2002). The majority (63%) of this intake was derived from the herbs and spices that naturally contain estragole, 27% was derived from essential oils containing estragole, and 10% from estragole added as such (Smith *et al.*, 2002).

Genotoxicity of estragole

Estragole as such has generally not been observed to be mutagenic in the bacterial Ames test with or without exogenous activation (Dorange *et al.*, 1977; Swanson *et al.,* 1979; Sekizawa and Shibamoto 1982; To *et al,.* 1982;

Zeiger *et al*., 1987). Of the different estragole metabolites (for a detailed description of estragole metabolism see below), however, 1'-hydroxyestragole and estragole-2',3'-oxide could induce bacterial mutagenicity (Swanson *et al.,* 1979). Estragole was not observed to induce chromosomal aberrations in V79 cells (Müller *et al*., 1994). In cultured rat hepatocytes both estragole and its 1'-hydroxy metabolite were observed to induce unscheduled DNA synthesis (Howes *et al.,* 1990; Chan and Caldwell 1992). In these experiments, the cell system that was used retains metabolic activity allowing bioactivation. The 1'-hydroxy metabolite of estragole was observed to be more potent than the parent compound in inducing unscheduled DNA synthesis, suggesting the involvement of this metabolite in the metabolic activation of estragole (Howes *et al.,* 1990).

In several *in vivo* studies both estragole and 1'-hydroxyestragole have been shown to be able to induce DNA adduct formation. In female CD-1 mice that were exposed to 10 mg estragole by intraperitoneal injection the formation of DNA adducts was observed at a level of 1 adduct in 10,000 – 15,000 DNA nucleotides as analyzed by ^{32}P-post-labelling of DNA obtained from the livers (Randerath *et al.*, 1984). Phillips *et al*. (1981) and Wiseman *et al.* (1985) characterized the different DNA adducts formed in the liver of female CD-1 mice after exposure to ^{3}H-labelled 1'-hydroxyestragole. In these experiment four different adducts with 2'-deoxyguanosine and one adduct with 2'-deoxyadenosine were identified, which were postulated to result from a reaction of the bases with an electrophilic reaction product derived from 1'-hydroxyestragole. Figure 2 presents the characterized adducts with 2'-deoxyguanosine. The major adduct formed was N^2-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine (E-3'-N^2-dGuo), which can arise after an allylic shift of a carbocation (see Figure 3 for its structure) derived from 1'-hydroxestragole prior to the reaction with the exocyclic amino group of 2'-deoxyguanosine. No adducts with other electrophilic metabolites of 1'-hydroxyestragole, including 1'-hydroxyestragole-2',3'-oxide and 1'-oxoestragole, were detected in these mice exposed to 1'-hydroxyestragole (Phillips *et al*., 1981).

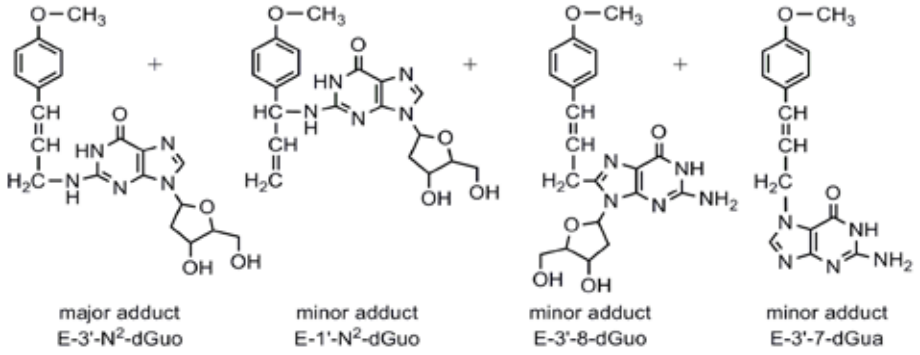


Figure 2. Structure of estragole DNA adducts with 2'-deoxyganosine as identified by Phillips *et al*. (1981) and Wiseman *et al.* (1985). E-3'-N^2-dGuo = N^2-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine, E-3'-8-dGuo = 8-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine, E-1'-N^2-dGuo = N^2-(estragol-1'-yl)-2'-deoxyguanosine, E-3'-7-dGua = 7-(*trans*-isoestragol-3'-yl)-2'-deoxyguanine.

Carcinogenicity of estragole

Both estragole and 1'-hydroxyestragole have been shown to induce hepatic tumors in several bioassays performed with different mouse strains at high dose levels. Upon exposure of female CD-1 mice to estragole at dose levels corresponding to 150-300 or 300-600 mg/kg bw/day over a period of 12 months, hepatic tumor incidences were found to amount to 58% and 71%, respectively, while no tumors were found in the control group (Miller *et al.,* 1983). In different bioassays the 1'-hydroxy metabolite of estragole was observed to be a more potent carcinogen than estragole (Miller *et al.*, 1983). Formation of hepatocellular carcinomas in B6C3F1 mice given an intraperitoneal injection of estragole was significantly inhibited by the sulfotransferases inhibitor pentachlorophenol (Wiseman *et al.*, 1987), indicating the involvement of a sulfate metabolite in the estragole induced carcinogenesis. Other estragole metabolites including estragole-2',3'-oxide and 1'-oxoestragole did not result in a significant increase in tumor incidences in bioassays with mice (Miller *et al.,* 1983; Wiseman *et al.,* 1987).

Absorption, distribution, metabolism, and excretion of estragole

General

In vivo studies with both rodents and humans revealed that following oral intake estragole is rapidly absorbed, metabolized, distributed, and excreted (Anthony *et al.*, 1987; Sangster *et al.*, 1987). The overall disposition of ^{14}C-methoxy-labelled estragole in female Wistar rats and male CD-1 mice was observed to be dose-dependent (Anthony *et al.*, 1987). In female Wistar rats orally exposed to estragole at dose levels of 0.05 – 50 mg/kg bw, the major part of the radioactivity was excreted in the 0-48 h expired air (average of 58%), whereas at a higher dose range of 500 and 1000 mg/kg bw, the major part (average of 59%) was excreted in the 0-48 h urine (Anthony *et al.*, 1987). These dose-dependent effects in excretion of the radioactivity were ascribed to dose-dependent differences in the biotransformation of estragole (Anthony *et al*., 1987). The disposition of estragole has also been studied in two volunteers who were exposed to an oral dose of ~1 µg/kg bw of ^{14}C-methoxy-labelled estragole (Sangster *et al.*, 1987). After 48 h the major part of this oral dose, corresponding to ~58%, was observed to be excreted in the urine. More than 11% of the radioactivity was excreted in the expired air after 8 h (Sangster *et al.*, 1987).

Principal metabolic reactions

Based on the disposition studies of ^{14}C-methoxy-labelled estragole in rats, mice and humans, and identification of the metabolites excreted, the principal metabolic pathways of estragole have been established (Figure 3) (Anthony *et al*., 1987; Sangster *et al*., 1987). Figure 3 presents an overview of estragole metabolism. The main phase I metabolic pathways include 1'-hydroxylation, *O*-demethylation, epoxidation and 3'-hydroxylation of estragole, which are catalyzed by cytochrome P450 enzymes. The main further metabolic pathways of the proximate carcinogen 1'-hydroxyestragole are sulfonation to 1'-sulfooxyestragole, glucuronidation to 1'-hydroxyestragole glucuronide,

and oxidation to 1'-oxoestragole. In the next sections the principal metabolic routes are described in more detail.

Hydroxylation to 1'-hydroxyestragole

Of the different phase I metabolites, 1'-hydroxyestragole has been recognized as the proximate carcinogenic metabolite of estragole, based on a considerably higher carcinogenicity of the 1'-hydroxy metabolite in male CD-1 mice compared to estragole (Drinkwater *et al*., 1976). Recently, 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 estragole 1'-hydroxylation. To a small extent also P450 2D6, 2C19, and 2E1 are able to catalyze the 1'-hydroxylation of estragole (Jeurissen *et al*., 2007).

Based on characterization of the hepatic DNA adducts formed after administration of 1'-hydroxyestragole, it was postulated that 1'-sulfooxyestragole is the major electrophilic and carcinogenic metabolite of estragole (Phillips *et al*., 1981; Wiseman *et al*., 1985). This was corroborated by the observation that the sulfotransferases inhibitor pentachlorophenol could significantly inhibit hepatocellular carcinoma formation in B6C3F1 mice given an intraperitoneal injection of estragole (Wiseman *et al*., 1987). The carbocation that is formed due to the instability of 1'-sulfooxyestragole in aqueous environment can bind to protein and DNA (Phillips *et al*., 1981; Wiseman *et al*., 1985).

In addition to sulfonation of 1'-hydroxyestragole, proposed metabolic pathways of 1'-hydroxyestragole include glucuronidation to 1'-hydroxyestragole glucuronide and oxidation to 1'-oxoestragole (Solheim and Scheline, 1973; Fennell *et al*., 1984; Anthony *et al*., 1987). The glucuronosyl conjugate of 1'-hydroxyestragole is a stable metabolite, which is excreted in the urine, leading to detoxification of 1'-hydroxyestragole (Solheim and Scheline, 1973; Fennell *et al*., 1984; Anthony *et al*., 1987). In both rats and mice, the extent of excretion of 1'-hydroxyestragole glucuronide after oral administration of estragole was observed to be dose-dependent (Anthony

et al., 1987). In case of rats, the proportion excreted as 1'-hydroxyestragole glucuronide increased from 1.3-5.4% of the dose in the range of 0.05-50 mg/kg bw to 11.4-13.7% in the dose range of 500-1000 mg/kg bw (after a single dose). Along with the relative increase in excretion of 1'-hydroxyestragole glucuronide, a relative decrease in the proportion of *O*-demethylation was observed (as determined by the percentage of exhalation as $^{14}CO_2$). An explanation for these changes in metabolism with the dose could be a shift in phase I metabolism from *O*-demethylation at low doses to 1'-hydroxylation at higher doses (Anthony *et al*., 1987). Such a shift could lead to a relative increase in the formation of 1'-hydroxyestragole with increasing doses, which can result in a concomitant relative increase in formation of its metabolites

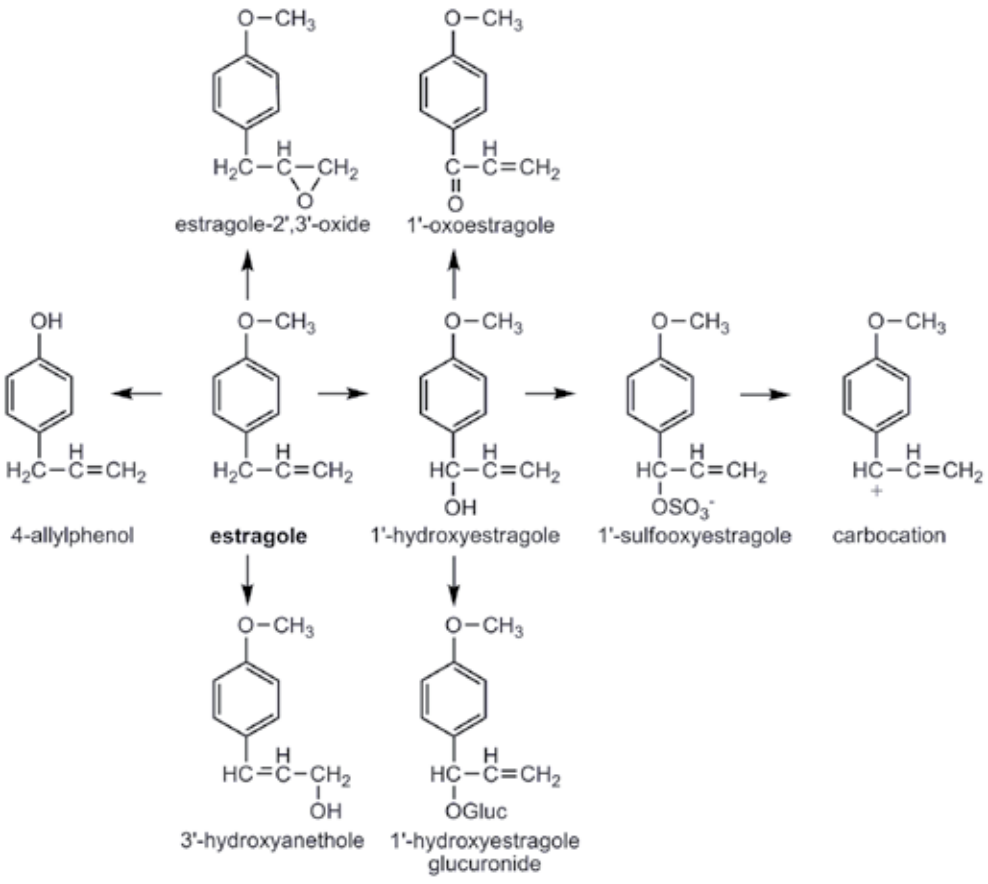


Figure 3. Metabolism of estragole.

1'-hydroxyestragole glucuronide, 1'-oxoxestragole and 1'-sulfooxyestragole, with the latter being the ultimate carcinogenic metabolite of estragole. This indicates that the relative extent of bioactivation can be dependent on the dose of estragole.

The 1'-oxoestragole metabolite of 1'-hydroxyestragole is a reactive metabolite, that has been shown to be able to form adducts with 2'-deoxyguanosine in a direct reaction with this nucleoside (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 (Phillips *et al*., 1981; Fennell *et al*., 1984). To date, the formation of 1'-oxoestragole has been considered to be of minor importance in rats since only relatively small amounts of derivatives of this metabolite have been detected in the urine of rats after exposure to estragole (Solheim and Scheline, 1973). The relative importance of this metabolic route in humans was not established so far (Sangster *et al*., 1987), but was a topic of the present thesis (Chapter 6).

_O_-demethylation to 4-allylphenol

O-demethylation of estragole results in the formation of 4-allylphenol, which is excreted as sulfate and glucuronic acid conjugates in the urine, leading to detoxification of estragole (Solheim and Scheline, 1973; Anthony *et al*., 1987). In both rats and mice the proportion of *O*-demethylation has been observed to decrease with increasing dose of estragole. In case of rats this metabolic pathway decreased from 52.5-58.1% of the dose in the range of 0.05-50 mg/kg bw to 28.6-29.1% in the dose range of 500-1000 mg/kg bw (Anthony *et al*., 1987). In humans this metabolic route has been observed to be relatively less important, amounting to about 10% of the oral dose at a dose of 0.001 mg/kg bw (Sangster *et al*., 1987).

Epoxidation to estragole-2',3'-oxide

Epoxidation of estragole results in the formation of estragole-2',3'-oxide.

Although estragole-2',3'-oxide has been observed to be genotoxic *in vitro* (Swanson, 1979), this metabolite did not result in a significant increase in tumor formation in male CD-1 mice exposed to this metabolite (Guenthner and Luo, 2001). Furthermore, no DNA adducts have been identified *in vivo* for which an epoxide metabolite precursor can be rationalized (Guenthner and Luo, 2001). This absence of carcinogenicity and genotoxicity of this epoxide *in vivo* has been ascribed to the rapid detoxification of estragole-2',3'-oxide by epoxide hydrolases and/or glutathione-S-transferases (Luo *et al*., 1992; Guenthner and Luo, 2001). For this reason it was concluded previously that this pathway does not contribute to the genotoxic effects of estragole *in vivo* (Guenthner and Luo, 2001).

3'-Hydroxylation to 3'-hydroxyanethole

Finally, the formation of 3'-hydroxyanethole is an important phase I metabolic route, although it is not clear whether this metabolite is formed via isomerisation of 1'-hydroxyestragole or as a result of 3'-hydroxylation of estragole (Solheim and Scheline, 1973; Anthony *et al*., 1987). 3'-Hydroxyanethole can be oxidized to 4-methoxycinnamic acid, which in turn is further oxidized to 4-methoxybenzoic acid, which is excreted as such. Furthermore, glucuronosyl conjugates of 3'-hydroxanethole have been observed in the urine of rats (Anthony *et al.,* 1987), indicating glucuronidation of 3'-hydroxyanethole.

Altogether data available on metabolism of estragole so far indicate the formation of various metabolites and provide proof for the nature of the bioactivation route proceeding via formation of 1'-hydroxyestragole, which is subsequently converted to 1'-sulfooxyestragole. The latter metabolite is unstable and degrades to generate a carbocation that binds covalently to DNA resulting in genotoxicity which is suggested to be the cause of the hepatocarcinogenicity induced by estragole.

Cancer risk assessment strategies

Since the occurrence of estragole in food can not readily be eliminated or avoided, estimation of the cancer risk at low dose levels will be necessary to indicate whether the presence of low concentrations of estragole is of concern to public health. No international consensus exists on how to evaluate the potential risk of genotoxic carcinogens that naturally occur in food. When the carcinogenicity is caused by a genotoxic mode of action, risk assessors generally advice that exposure should be as low as reasonable achievable (ALARA). This approach, however, only identifies the hazard and does not give a quantitative estimate of the cancer risk at intake levels relevant for dietary human exposure (Dybing *et al*., 2008).

Numerical estimates of the risk associated with human exposure can be derived by extrapolation of carcinogenicity data obtained in animals at high dose levels to low dose levels relevant for the human situation. Many mathematical models have been proposed by which such an extrapolation below the available experimental data could be performed (reviewed by COC, 2004). These mathematical models are either based on fitting a curve to the experimental data points providing possibilities for extrapolation to low doses, or extrapolate from a specific point of departure to lower doses. Such a point of departure can be a point in the range of observations such as the T25, which is the dose that increases the tumor incidence by 25% (Barlow *et al*., 2006; Dybing *et al*., 2008). The point of departure can also be derived by fitting a curve to the dose-response data from an animal bioassay and using this curve fit to estimate the intake level that corresponds to a fixed tumor incidence. This can for instance be done using the so-called Benchmark Dose (BMD) approach in which the Benchmark Dose can be defined as the dose that gives a 10% extra cancer incidence (BMD_{10}) and in which either the BMD_{10} or the lower confidence bound of this BMD_{10}, the $BMDL_{10}$, can be used as the point of departure (Barlow *et al*., 2006; Dybing *et al*., 2008). The simplest way to estimate the risk associated with the average dietary human intake is to apply linear extrapolation from the BMD_{10} or T25 as the point of departure to zero

dose at zero risk. Based on this extrapolation a so-called virtual safe dose (VSD) might be derived, corresponding to the dose that results in an additional cancer risk of one in a million upon lifetime exposure, which is generally considered acceptable by risk managers. Extrapolating from animal tumor data at high doses using mathematical modeling in order to obtain estimates of the risk to humans at low exposures has been much debated, since it is not known whether or not the model chosen actually reflects the underlying biological processes. In addition, numerical estimates may be misused or misinterpreted in further risk management and risk communication, if the uncertainties and inaccuracy connected to the model may not be communicated (EFSA, 2005).

Recently, the margin of exposure (MOE) approach was developed by the European Food Safety Authority (EFSA) as a harmonized approach for risk assessment of substances which are both genotoxic and carcinogenic (EFSA, 2005). The MOE represents the margin between a reference point derived from the dose–response curve ($BMDL_{10}$) and the estimated dietary human exposure (EDI) ($BMDL_{10}$/EDI). The magnitude of the MOE reflects the possible magnitude of the cancer risk, but does not create implicit assumptions about a ‘safe' dose (Dybing *et al*., 2008). The opinion of the EFSA indicates that, when this margin is higher than 10,000, the compound is considered to be of low priority for risk management actions (EFSA, 2005). This margin of 10,000 is applied to adequately allow for various uncertainties in the MOE approach, such as interspecies differences and human variability in biokinetics, the shape of the dose-response curve below the experimental data, and human interindividual variability in cell cycle control and DNA repair (EFSA, 2005; Barlow *et al*., 2006; Dybing *et al*., 2008).

PBBK models

As outlined above, an overall problem in current risk assessment strategies is the need to extrapolate the cancer risk orders of magnitude below the experimental data obtained in animal experiments at high dose levels to a low dose human situation. Uncertainties about the i) shape of the dose-response curve at dose levels relevant for dietary human intake, about ii) species

differences in metabolism and metabolic activation and about iii) the influence of the larger interindividual variability in the human population, as compared to the variability in the inbred strains used for the rodent bioassays, make it difficult to perform such extrapolations. Physiologically based biokinetic (PBBK) modeling can provide a method to obtain a better mechanistic basis for extrapolations from data obtained in experimental animal studies to the human situation (Andersen and Krishnan 1994; Clewell *et al*., 2001; Clewell *et al*., 2002).

A PBBK model consists of a set of mathematical equations that together describe the absorption, distribution, metabolism and excretion (ADME) characteristics of a compound within an organism on the basis of three types of parameters (Krishnan and Andersen, 2001). These parameters include physiological parameters (e.g. cardiac output, tissue volumes, and tissue blood flows), physico-chemical parameters (e.g. blood/tissue partition coefficients), and kinetic parameters (e.g. kinetic constants for metabolic reactions) (Krishnan and Andersen, 2001). Solution of the PBBK equations results in outcomes that are an indication of, for example, the tissue concentration of a compound or its metabolite in any tissue over time at a certain dose, allowing analyses of effects at both high but also more realistic low dose levels. Furthermore, such PBBK models can be developed for different species, which facilitates interspecies extrapolation. In addition, by incorporating equations and kinetic constants for metabolic conversions by individual human samples and/or specific isoenzymes, modeling of interindividual variations and genetic polymorphisms becomes feasible (Bogaards *et al*., 2000).

In cancer risk assessment strategies, PBBK models have been used to convert external exposure values to an internal dose metric (e.g. the predicted plasma or tissue levels of a parent compound or its reactive metabolites) in animal bioassays, as well as in human exposure scenarios (Andersen and Krishnan, 1994). Such an internal dose metric is considered to be more closely related to the toxic response than the applied external doses. Extrapolation of the cancer risk from high to low doses and from animals to humans can be performed based on the internal dose metrics rather than the applied external

doses (Andersen and Krishnan, 1994). The value of applying PBBK models in cancer risk assessment was first demonstrated with the chemical agent dichloromethane (Andersen and Krishnan, 1994). Based on the model for this compound in mice it could be revealed that conjugation of dichloromethane with glutathione is a principal bioactivation pathway of this compound, since tumor incidences in mice were observed to correlate well with the predicted formation of this glutathione conjugate, and not with the formation of other metabolites. With the developed models for dichloromethane in both mice and humans, the occurrence of dose-dependent effects and species differences in formation of the glutathione conjugate was evaluated. For mice a non-linear increase in formation of the glutathione conjugate with increasing doses was predicted. Comparison of the predicted formation of the glutathione conjugate between mice and humans revealed a higher formation of this metabolite in mice. By taking these dose-dependent effects and interspecies differences into account in the extrapolation of the cancer risk from high doses in mice to a low dose human situation, the cancer risk in humans was predicted to be 57-fold lower than would have been estimated if a default linear extrapolation would have been be applied (Andersen and Krishnan, 1994).

For the development of a PBBK model for a specific compound, model parameters need to be obtained. The physiological parameters (e.g. blood flow rates and tissue volumes) of a specific species can be obtained from the literature (Brown *et al*., 1997). Tissue-blood partition coefficients might be obtained experimentally *in vitro* using microdialysis techniques (Krishnan and Andersen, 2001; Lipscomb and Poet, 2008), but can also be obtained using *in silico* methods. Several *in silico* models have been published by which tissue-blood partition coefficients of a compound can be calculated based on their octanol-water partition coefficient (Payne and Kenny, 2002 and references therein). Biochemical parameters for PBBK models, including metabolic parameters, are most often obtained by making preliminary assumptions about metabolic routes and optimizing the kinetic constants by fitting the model to available *in vivo* data (Krishnan and Andersen, 2001). Alternatively, metabolic parameters might also be derived from *in vitro* experiments with

tissue fractions, primary cell cultures, or tissue slices of organs involved in the metabolism of the compound. Lipscomb and Poet (2008) have pointed out some advantages of using *in vitro* metabolic parameters to define PBBK models, which include the ability to separately define and analyze individual metabolic processes, such as phase I metabolism and phase II metabolism, or bioactivation and detoxification, and to compare contributions from individual conversions to the overall metabolism across species and between individuals, when limited *in vivo* data are available as is often the case for humans (Lipscomb and Poet, 2008).

Objective and outline of this thesis

The aim of the present PhD project was to build physiologically based biokinetic (PBBK) models for both rats and (individual) human subjects to obtain a quantitative insight into dose-dependent effects, species differences, and interindividual differences in bioactivation and detoxification of estragole. For the development of these models metabolic parameters were determined *in vitro* using relevant tissue fractions. The physiological parameters were obtained from the literature (Brown *et al*., 1997) and partition coefficients were estimated from the octanol-water partition coefficient based on an *in silico* method (DeJongh *et al.,* 1997).

In **Chapter 1,** the present chapter of this thesis, an introduction to the bioactivation, detoxification, genotoxicty and carcinogenicity of estragole as well as a short introduction to PBBK modeling and the aim and content of the present thesis are given. In **Chapter 2** the kinetic constants for sulfonation of 1'-hydroxyestragole by liver S9 homogenates of male rat, male mouse, and humans were described to identify the kinetic constants and possible species differences for this bioactivation step. **Chapter 3** defines a PBBK model for estragole metabolism in male rat, elucidating dose-dependent effects in bioactivation and detoxification of estragole. In **Chapter 4** a PBBK model

for estragole bioactivation and detoxification in human is defined of which the outcomes were compared to those predicted by the PBBK model for estragole in male rat, described in **Chapter 3,** to evaluate the occurrence of species differences in metabolic activation and detoxification of estragole. Whereas the PBBK model for estragole metabolism in human describes the relative extent of bioactivation in an average situation, interindividual differences can occur within the whole population. The activity levels of the enzymes involved in these reactions can vary in the human population due to genotype- and lifestyle-based factors, which could influence the level of bioactivation of estragole between different human subjects to a level that is higher than what would be observed in animals studies with inbred rodent strains. To gain insight in the level of variability in 1'-hydroxylation of estragole and oxidation of 1'-hydroxyestragole that could occur in the human population it is of importance to characterize the enzymes involved in these reactions. Therefore, in **Chapter 5** the human cytochrome P450 enzymes involved in the bioactivation of estragole to its proximate carcinogen 1'-hydroxyestragole were identified. In **Chapter 6** 17β-hydroxysteroid dehydrogenase type 2 is identified as the major enzyme involved in 1'-hydroxyestragole oxidation, shown to be a major reaction affecting the ultimate level of estragole bioactivation and detoxification. **Chapter 6** also defines a PBBK models for estragole metabolism in individual human subjects using individual kinetic data for 1'-hydroxylation, determined based on the kinetic constants for individual P450 enzymes as obtained in **Chapter 5**, and for oxidation of 1'-hydroxyestragole. Based on the models obtained the interindividual variation in the liver levels of 1'-hydroxyestragole due to variation in these two metabolic reactions were evaluated. In a second step, the individual models were combined with literature data on interindividual variation in activity levels of the key enzymes involved in formation and detoxification of 1'-hydroxyestragole, to model the maximum level of variability in the general population. Finally, **Chapter 7** presents a summary of the results obtained in this thesis and provides a discussion on how these results can be used in the risk assessment of estragole.

References

Andersen, M. E. and Krishnan, K. (1994). Physiologically-based pharmacokinetics cancer risk assessment. Environ Health Persp 102, 103-108.

Anthony, A., Caldwell, J., Hutt, A. J., and Smith, R. L. (1987). Metabolism of estragole in rat and mouse and influence of dose size on excretion of the proximate carcinogen 1'-hydroxyestragole. Food Chem Toxicol 25, 799-806.

Barlow, S., Renwick, A. G., Kleiner, J., Bridges, J. W., Busk, L., Dybing, E., Edler, L., Eisenbrand, G., Fink-Gremmels, J., Knaap, A., Kroes, R., Liem, D., Muller, D. J., Page, S., Rolland, V., Schlatter, J., Tritscher, A., Tueting, W., and Wurtzen, G. (2006). Risk assessment of substances that are both genotoxic and carcinogenic report of an International Conference organized by EFSA and WHO with support of ILSI Europe. Food Chem Toxicol 44, 1636-1650.

Bogaards, J. J., Hissink, E. M., Briggs, M., Weaver, R., Jochemsen, R., Jackson, P., Bertrand, M. and van Bladeren, P. J. (2000). Prediction of interindividual variation in drug plasma levels *in vivo* from individual enzyme kinetic data and physiologically based pharmacokinetic modeling. Eur J Pharm Sci 12, 117-124.

Brown, R. P., Delp, M. D., Lindstedt, S. L., Rhomberg, L. R. and Beliles, R. P. (1997). Physiological parameter values for physiologically based pharmacokinetic models. Toxicol Ind Health 13, 407-484.

Chan, V. S. and Caldwell, J. (1992). Comparative induction of unscheduled DNA synthesis in cultured rat hepatocytes by allylbenzenes and their 1'-hydroxy metabolites. Food Chem Toxicol 30, 831-836.

Clewell, H. J., Gentry, P. R., Gearhart, J. M., Allen, B. C., and Andersen, M. E. (2001). Comparison of cancer risk estimates for vinyl chloride using animal and human data with a PBPK model. Sci Total Environ 274, 37-66.

Clewell, H. J., 3rd, Andersen, M. E., and Barton, H. A. (2002). A consistent

approach for the application of pharmacokinetic modeling in cancer and noncancer risk assessment. Environ Health Perspect 110, 85-93.

Committee on Carcinogenicity of chemicals in food, consumer products and the environment (COC) (2004). Guidance on a strategy for the risk assessment of chemical carcinogens. http://www.advisorybodies.doh.gov.uk/coc/guideline04.pdf.

DeJongh, J., Verhaar, H. J. and Hermens, J. L. (1997). A quantitative property-property relationship (QPPR) approach to estimate in vitro tissue-blood partition coefficients of organic chemicals in rats and humans. Arch Toxicol 72, 17-25.

Dorange, J. L., Delaforge, M., Janiaud, P. and Padieu, P. (1977). Mutagenicity of the metabolites of the epoxide-diol pathway of safrole and its analogs. Study on Salmonella typhimurium. C R Seances Soc Biol Fil 171, 1041-1048. First published on Pouvoir mutagene de metabolites de la voie epoxyde-diol du safrol et d'analogues. Etude sur Salmonella typhimurium.

Drinkwater, N. R., Miller, E. C., Miller, J. A., and Pitot, H. C. (1976). Hepatocarcinogenicity of estragole (1-allyl-4-methoxybenzene) and 1'-hydroxyestragole in the mouse and mutagenicity of 1'-acetoxyestragole in bacteria. J Natl Cancer Inst 57, 1323-1331.

Dybing, E., O'Brien, J., Renwick, A. G., and Sanner, T. (2008). Risk assessment of dietary exposures to compounds that are genotoxic and carcinogenic--an overview. Toxicol Lett 180, 110-117.

EFSA (2005). Opinion of the scientific committee on a request from EFSA related to a harmonised approach for risk assessment of substances which are both genotoxic and carcinogenic. http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/sc_op_ej282_gentox_en3.pdf?ssbinary=true

Fennell, T. R., Miller, J. A., and Miller, E. C. (1984). Characterization of the biliary and urinary glutathione and N-acetylcysteine metabolites of the hepatic carcinogen 1'-hydroxysafrole and its 1'-oxo metabolite in rats and mice. Cancer Res 44, 3231-3240.

Guenthner, T. M. and Luo, G. (2001). Investigation of the role of the 2',3'-epoxidation pathway in the bioactivation and genotoxicity of dietary

allylbenzene analogs. Toxicology 160, 47-58.

Hall, R.L. and Oser, B.L. (1965). Recent progress in the consideration of flavoring ingredients under the food additives amendment III. GRAS substances. Food Technology 253, 151–197.

Howes, A. J., Chan, V. S., and Caldwell, J. (1990). Structure-specificity of the genotoxicity of some naturally occurring alkenylbenzenes determined by the unscheduled DNA synthesis assay in rat hepatocytes. Food Chem Toxicol 28, 537-542.

JECFA (2008). Sixty-ninth meeting Rome, Italy, 17-26 June 2008. http://www.who.int/entity/ipcs/food/jecfa/summaries/summary69.pdf.

Jeurissen, S. M. F., Punt, A., Boersma, M. G., Bogaards, J. J., Fiamegos, Y. C., Schilter, B., van Bladeren, P. J., Cnubben, N. H. P. and Rietjens, I. M. C. M. (2007). Human cytochrome P450 enzyme specificity for the bioactivation of estragole and related alkenylbenzenes. Chem Res Toxicol 20, 798-806.

Krishnan, K. and Andersen, M. E. (2001). Physiologically-based pharmacokinetic modeling and toxicology. In principles and methods of toxicology (A. W. Hayes, Ed.), pp. 193-241. Raven Press, N.Y.

Lipscomb, J. C. and Poet, T. S. (2008). In vitro measurements of metabolism for application in pharmacokinetic modeling. Pharmacol Ther 118, 82-103.

Luo, G., Qato, M. K., and Guenthner, T. M. (1992). Hydrolysis of the 2',3'-allylic epoxides of allylbenzene, estragole, eugenol, and safrole by both microsomal and cytosolic epoxide hydrolases. Drug Metab Dispos 20, 440-445.

Miller, E. C. and Miller, J. A. (1986). Carcinogens and mutagens that may occur in foods. Cancer 58, 1795-1803.

Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A., and Miller, J. A. (1983). Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. Cancer Res 43, 1124-1134.

Müller, L., Kasper, P., Muller-Tegethoff, K. and Petr, T. (1994). The genotoxic potential in vitro and *in vivo* of the allyl benzene etheric oils estragole,

basil oil and trans-anethole. Mutat Res 325, 129-36.

Payne, M.P., Kenny, L.C. (2002) Comparison of models for the estimation of biological partition coefficients. J. Toxicol. Environ. Health A 65, 897–931.

Phillips, D. H., Miller, J. A., Miller, E. C., and Adams, B. (1981). Structures of the DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1'-hydroxyestragole. Cancer Res 41, 176-186.

Randerath, K., Haglund, R. E., Phillips, D. H., and Reddy, M. V. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. Carcinogenesis 5, 1613-1622.

Sangster, S. A., Caldwell, J., Hutt, A. J., Anthony, A., and Smith, R. L. (1987). The metabolic disposition of [methoxy-^{14}C]-labelled trans-anethole, estragole and *p*-propylanisole in human volunteers. Xenobiotica 17, 1223-1232.

SCF (2001). Opinion of the scientific committee on food on estragole (1-allyl-4-methyxybenzene). http://ec.europa.eu/food/fs/sc/scf/out104_en.pdf.

Sekizawa J. and Shibamoto T. (1982). Genotoxicity of safrole-related chemicals in microbial test systems. Mutat Res 101, 127-140.

Siano, F., Ghizzoni, C., Gionfriddo, F., Colombo, E., Servillo, L., and Castaldo, D. (2003). Determination of estragole, safrole and eugenol methyl ether in food products. Food Chemistry 81, 469-475.

Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., Rogers, A. E., Caldwell, J., and Sipes, I. G. (2002). Safety assessment of allylalkoxybenzene derivatives used as flavouring substances - methyl eugenol and estragole. Food Chem Toxicol 40, 851-870.

Solheim, E. and Scheline, R. R. (1973). Metabolism of alkenebenzene derivatives in the rat. I. *p*-Methoxyallylbenzene (Estragole) and *p*-methoxypropenylbenzene (Anethole). Xenobiotica 3, 493-510.

Swanson, A. B., Chambliss, D. D., Blomquist, J. C., Miller, E. C., and Miller,

J. A. (1979). The mutagenicities of safrole, estragole, eugenol, trans-anethole, and some of their known or possible metabolites for Salmonella typhimurium mutants. Mutat Res 60, 143-153.

To, L. P., Hunt, T. P., and Andersen, M. E. (1982). Mutagenicity of trans-anethole, estragole, eugenol, and safrole in the Ames Salmonella typhimurium assay. Bull Environ Contam Toxicol 28, 647-654.

US-EPA (2005). (U.S. Environmental Protection Agency) Guidelines for carcinogen risk assessment. http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=116283

Wiseman, R. W., Fennell, T. R., Miller, J. A., and Miller, E. C. (1985). Further characterization of the DNA adducts formed by electrophilic esters of the hepatocarcinogens 1'-hydroxysafrole and 1'-hydroxyestragole in vitro and in mouse liver *in vivo*, including new adducts at C-8 and N-7 of guanine residues. Cancer Res 45, 3096-3105.

Wiseman, R. W., Miller, E. C., Miller, J. A., and Liem, A. (1987). Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J x C3H/HeJ F1mice. Cancer Res 47, 2275-2283.

Zeiger E., Anderson B., Haworth S., Lawlor T., Mortelmans K., Speck W. (1987). Salmonella mutagenicity tests: III. Results from the testing of 255 chemicals. Environ Mutagen 9 (Suppl. 9), 1-109.

2

Tandem mass spectrometry analysis of N^2-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine as a strategy to study species differences in sulfotransferase conversion of the proximate carcinogen 1'-hydroxyestragole

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Abstract

To obtain insight into possible species differences in the bioactivation of estragole, the kinetics for sulfonation of the proximate carcinogen 1'-hydroxyestragole were compared for male rat, male mouse, and mixed gender human liver S9 homogenates. In order to quantify sulfonation, 2'-deoxyguanosine was added to the incubation mixtures, in which sulfonation of 1'-hydroxyestragole was catalyzed, to trap the reactive 1'-sulfooxyestragole. A method was developed, with which the formation of the most abundant adduct with 2'-deoxyguanosine could be quantified using isotope dilution LC-ESI-MS/MS. Comparing the kinetics for sulfonation by liver S9 homogenates of male rat, male mouse, and human revealed that sulfonation was about 30 times more efficient by male rat liver S9 than by human liver S9, whereas the catalytic efficiency by male mouse and human liver S9 was about the same. This indicates, as far as the bioactivation by sulfotransferase is concerned, that when extrapolating the cancer risk from laboratory animals to humans, using data from male rats may overestimate the cancer risk in humans, whereas using data from male mice may provide a better estimate of the cancer risk in humans.

Introduction

The alkenylbenzene estragole is a natural constituent of several herbs and spices, such as basil, fennel, anise, and tarragon. Estragole is also present in food as a result of the addition of essential oils of these herbs and spices as flavoring agents or by addition of the compound itself as flavoring agent. In 2001, the Scientific Committee on Food (SCF) of the European Union launched a scientific evaluation on estragole and concluded that estragole is genotoxic and carcinogenic and that reductions in exposure and restrictions in use levels are indicated (SCF, 2001). This conclusion was based on the findings of several studies demonstrating that estragole and its metabolite 1'-hydroxyestragole can induce hepatic tumors in mice and, in the case of the 1'-hydroxy metabolite, also in rats (Drinkwater *et al*., 1976; Miller *et al*., 1983; Wiseman *et al.,* 1987). This was further supported by the detection of DNA adducts in mice exposed to estragole (Phillips *et al.*, 1981; Phillips *et al.*, 1984; Randerath *et al.*, 1984; Wiseman *et al.*, 1985). The Flavor and Extract Manufacturers Association (FEMA) concluded in their evaluation of estragole that exposure to estragole from food does not pose a significant cancer risk to consumers (Smith *et al*., 2002). In this evaluation, experimental data that suggested a non-linear relationship between dose and profiles of metabolism and metabolic activation were taken into account (Anthony *et al.*, 1987; Smith *et al*., 2002). The average daily intake of estragole was estimated by the SCF to be 4.3 mg per day (SCF, 2001). The expert panel of the FEMA estimated the average daily intake of estragole to be 0.06-0.60 mg per day (Smith *et al*., 2002). These intake estimates of the SCF and FEMA are based on different methodologies. The estimation of the SCF is based on theoretical maximum use levels of estragole in various food categories and consumption data for these food categories, whereas the intake estimation of the FEMA is based on production volume data (SCF, 2001; Smith *et al*., 2002).

The carcinogenicity of estragole is linked to its metabolic conversion to genotoxic metabolites (Drinkwater *et al*., 1976; Miller *et al*., 1983; Wiseman *et al.,* 1987). The first step of the bioactivation is the conversion of estragole

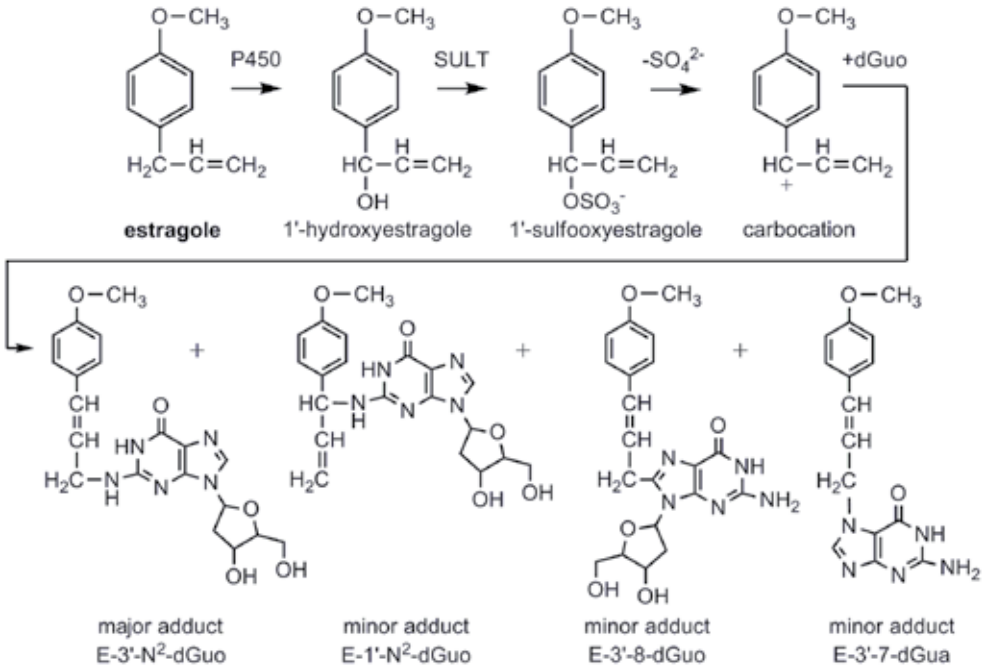

Figure 1. Bioactivation of estragole and the structure of adducts of its reactive metabolite with 2'-deoxyganosine as identified by Phillips *et al*. (1981) and Wiseman *et al*. (1985). P450 = cytochrome P450, SULT = sulfotransferase enzymes, dGuo = 2'-deoxyguanosine, E-3'-N^2-dGuo = N^2-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine, E-3'-8-dGuo = 8-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine, E-1'-N^2-dGuo = N^2-(estragol-1'-yl)-2'-deoxyguanosine, and E-3'-7-Gua = 7-(*trans*-isoestragol-3'-yl)-2'-guanine.

to the proximate carcinogenic metabolite 1'-hydroxyestragole, which is catalyzed by cytochrome P450 enzymes in the liver (Figure 1). Sulfonation of 1'-hydroxyestragole leads to formation of the ultimate carcinogenic metabolite, which is unstable and degrades in aqueous environment to give rise to a reactive carbocation capable of forming DNA adducts possibly leading to the formation of liver tumors (Drinkwater *et al*., 1976; Boberg *et al.*, 1983; Miller *et al*., 1983; Wiseman *et al.,* 1987). So far, four different adducts with 2'-deoxyguanosine and one adduct with 2'-deoxyadenosine were identified when the proximate carcinogenic metabolite 1'-hydroxyestragole was administered to mice. The major adduct formed was N^2-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine (E-3'-N^2-dGuo), which can arise after an allylic shift

of the carbocation prior to the reaction with the exocyclic amino group of 2'-deoxyguanosine (Phillips *et al.*, 1981; Wiseman *et al.*, 1985).

When determining the risk of estragole at low dose human intake, it is important to take into account both nonlinear effects and species differences in biokinetics and biodynamics when extrapolating the cancer risk from laboratory animals to humans. The formation of DNA adducts from the proximate carcinogenic metabolite 1'-hydroxyestragole requires sulfonation to form the ultimate carcinogenic 1'-sulfooxyestragole. So far, no data are available regarding the kinetics for sulfonation of 1'-hydroxyestragole in any species. This is partly due to the instability of 1'-sulfooxyestragole in aqueous environment, which is reflected by the fact that it cannot be synthesized (Drinkwater *et al*., 1976; Phillips *et al.*, 1981; Wiseman *et al.*, 1985; Wiseman *et al.*, 1987). Because of this instability, it is difficult to detect 1'-sulfooxyestragole; however, by addition of a nucleophilic compound to the incubation mixture, such as rRNA, the reactive carbocation that arises from 1'-sulfooxyestragole can be trapped (Wislocki *et al.*, 1976; Fennell *et al.*, 1986). The objective of the present study was to compare the kinetics for the formation of 1'-sulfooxyestragole in male rats, male mice, and humans, using the formation of E-3'-N^2-dGuo as trapping method. To this end, 2'-deoxyguanosine was added as trapping agent to the incubation mixtures, in which the sulfonation of 1'-hydroxyestragole was catalyzed, resulting in the formation of several adducts with 2'-deoxyguanosine. An isotope dilution LC-ESI-MS/MS method was developed to quantify the formation of the major adduct, namely, E-3'-N^2-dGuo. With this approach, the kinetics for the sulfotransferase dependent formation of E-3'-N^2-dGuo by male rat, male mouse, and human S9 homogenates were studied. The results obtained are discussed with emphasis on the implications of interspecies differences in the bioactivation of estragole for the extrapolation of cancer risk from male rats and male mice to humans.

Materials and Methods

Chemicals and biological materials

Pooled male rat liver S9 (Sprague-Dawley), pooled male mouse liver S9 (CD-1), and pooled mixed gender human liver S9 were purchased from BD Gentest (Woburn, MA). Stable isotope labeled 1,2,3,7,9-$^{15}N_5$-2'-deoxyguanosine ($^{15}N_5$-dGuo) was obtained from Cambridge Isotope Laboratories (Cambridge, MA). Diethyl ether, tris(hydroxymethylamino methane), formic acid, acetonitrile (chromatography grade), ammonium carbonate, acetic anhydride, sodium acetate, acetic acid, and sodium carbonate were purchased from Merck (Darmstadt, Germany). Hydrochloric acid (37%) was from Roche Diagnostics (Mannheim, Germany). Pyridine was obtained from Acros Organics (Geel, Belgium) and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) from Fluka (Buchs, Switzerland). NADPH was obtained from Boehringer (Mannheim, Germany). Estragole (4-allylanisol), vinylmagnesium bromide, *p*-anisaldehyde, tetrahydrofuran (THF), dimethylsulfoxide (DMSO), magnesium sulfate, dichloromethane, bovine serum albumin (BSA), pentachlorophenol, 2'-deoxyguanosine, ascorbic acid, isopropyl alcohol, 7-hydroxycoumarin, and 7-hydroxycoumarin sulfate were purchased from Sigma-Aldrich (Steinheim, Germany)

Synthesis of 1'-hydroxyestragole

1'-Hydroxyestragole was synthesized as described by Drinkwater *et al*. (1976) as an adaption of a method of Borchert *et al*. (1973) for the synthesis of 1'-hydroxysafrole. The synthesis of 1'-hydroxyestragole encompassed a Grignard reaction, using *p*-anisaldehyde and vinylmagnesium bromide as the Grignard reagent (1 M solution in THF). In short, *p*-anisaldehyde (0.0165 mol) was dissolved in 10 mL of dry THF, and this solution was added dropwise over a period of 30 min to the Grignard reagent (0.035 mol) while stirring at 50 °C under anhydrous conditions in a nitrogen atmosphere. The reaction mixture was further incubated for 90 min, and the resulting solution was added to a solution of 4.5 g of ammonium chloride in 200 mL of ice cold water.

The emulsion was stirred for several minutes, and 1'-hydroxyestragole was extracted with diethyl ether. The organic solution was dried with magnesium sulfate, and the yield was 97%.

Structural confirmation was obtained with HP6890 GC-MS from Hewlett-Packard (Palo Alto, CA) using a DB-5 0.25 μm film column, 30 m×0.25 mm from J&W Scientific (Folsom, CA). The temperature gradient was programmed from 50 to 250 °C at 5 °C min^{-1}, the inlet temperature was 260 °C, and the pressure of helium carrier gas was 12.0 psi. The mass spectrum (EI (70 eV): m/z (rel. int. %): 109 (100), 164 (84), 135 (70), 77 (66), 121 (65), 163 (63), 137 (61), 133 (43), 94 (42), 108 (38)) was comparable to that described in the literature (Drinkwater *et al.*, 1976). The purity of 1'-hydroxyestragole was more than 98% according to GC-MS analysis.

1'-Hydroxyestragole is a chiral molecule. To determine whether a racemic mixture of 1'-hydroxyestragole was obtained, the synthesized product was analyzed by HPLC on a Waters M600 liquid chromatography system (Waters, Milford, MA) coupled to a Chiral-AGP 5 μm column, 150 × 4 mm (ChromTech, Congleton, U.K.), with an isocratic elution using 10 mM sodium acetate (pH 4.5) containing 0.5% isopropyl alcohol at a flow rate of 0.9 mL min^{-1}. Detection was carried out using a Waters 966 photodiode array detector (Waters, Milford, MA) at 280 nm. The ratio between the peak areas of the two enantiomers was calculated, but the stereochemistry of the enantiomers was not established. To determine whether cytochrome P450 enzymes also produce a racemic mixture of 1'-hydroxyestragole, incubations were performed with male rat, male mouse, and human liver S9 in a 500 μL incubation mixture, containing (final concentrations of) 3 mM NADPH, 1 mM ascorbic acid, and 1 mg S9 protein per mL in 0.2 M Tris-HCl (pH 7.4). The reaction was started by the addition of the substrate estragole (1000 μM final concentration, added from a 100 mM stock solution in DMSO). Incubations were performed at 37 °C, and the reaction was terminated after 60 min by adding 25 μL of ice-cold acetonitrile. 1'-Hydroxyestragole was extracted from the samples with diethyl ether (3 × 2 mL), and the combined extract was evaporated in a nitrogen atmosphere. The samples were reconstituted in 150 μL of Tris-HCl, and

aliquots of 50 µL were analyzed by HPLC as described above.

Synthesis of E-3'-N^2-dGuo and ($^{15}N_5$)E-3'-N^2-dGuo

E-3'-N^2-dGuo was synthesized from a reaction between 1'-acetoxyestragole and 2'-deoxyguanosine based on the protocol of Phillips *et al*. (1981). For this reaction, 1'-acetoxyestragole was synthesized from 1'-hydroxyestragole as described for 1'-acetoxysafrole by Borchert *et al*. (1973) and adapted for 1'-acetoxyestragole by Drinkwater *et al*. (1976). In brief, 1'-hydroxyestragole (50 mg) was dissolved in 200 µL of pyridine. Acetic anhydride (33 µL) was added dropwise to this solution, and the reaction mixture was stirred for 5 h at room temperature, after which 400 µL of dichloromethane was added. The reaction mixture was extracted several times with aliquots of 200 µL of 1 N HCl. When the aqueous phase reached pH 2-3, the organic layer was immediately extracted with 400 µL of 1 M sodium carbonate solution. The organic solution was dried with magnesium sulfate, and the solvent was evaporated in a nitrogen atmosphere. Structural conformation of the synthesized product was acquired by GC-MS as described for 1'-hydroxyestragole. The mass spectrum (EI (70 eV): m/z (rel. int. %): 164 (100), 146 (89), 147 (55), 131 (48), 103 (46), 135 (36), 115 (30), 163 (28), 206 (25)) was comparable to that described in the literature (Drinkwater *et al*., 1976).

For the synthesis of E-3'-N^2-dGuo, the reaction product containing 1'-acetoxyestragole was diluted 100-fold in DMSO, from which 250 µL was added to 2250 µL of 2.5 mM 2'-deoxyguanosine solution in 2.5 mM ammonium bicarbonate (pH 7). The same reaction was performed for the synthesis of $^{15}N_5$-labeled E-3'-N^2-dGuo, in which 1'-acetoxyestragole was allowed to react with $^{15}N_5$-labeled 2'-deoxyguanosine. The incubations were stirred for 48 h at 37 °C. Both E-3'-N^2-dGuo and ($^{15}N_5$)E-3'-N^2-dGuo were purified by HPLC on a Waters M600 liquid chromatography system (Waters, Milford, MA) equipped with an Alltima C18 5 µm column, 150 × 4.6 mm (Alltech, Breda, The Netherlands). The gradient was made with ultrapure water and acetonitrile. The flow rate was 1 mL min^{-1}. A linear gradient was applied from 20% to 30% acetonitrile over 40 min, after which the percentage

of acetonitrile was increased to 100% over 2 min. Detection was carried out using a Waters 966 photodiode array detector (Waters, Milford, MA) at 260 nm. Both E-3'-N^2-dGuo and ($^{15}N_5$)E-3'-N^2-dGuo were collected at a retention time of 19 min. The purity of both E-3'-N^2-dGuo and ($^{15}N_5$)E-3'-N^2-dGuo was more than 98%, according to LC-ESI-MS/MS and HPLC analyses.

Incubations with male rat, male mouse, and human liver S9 homogenates.

Incubations with male rat, male mouse, and human liver S9 homogenates were performed in 100 µL incubation mixtures, containing (final concentrations of) 1 mM PAPS, 1 mM 2'-deoxyguanosine, 1 mg S9 protein per mL, and a substrate concentration that ranged from 50 to 2000 µM 1'-hydroxyestragole in 0.1 M Tris-HCl (pH 7.4) (Wislocki *et al*., 1976; Fennell *et al*., 1986). After preincubating at 37 °C for 1 min, the reaction was started by the addition of PAPS. All incubations with S9 homogenates were carried out for 30 min. Under these conditions, sulfonation was linear with time and protein concentration for each type of S9 incubation. The reactions were terminated by the addition of 25 µL of cold acetonitrile, after which the incubations were centrifuged for 5 min at 16,000g to precipitate the S9 proteins, and the supernatant was analyzed by LC-ESI-MS/MS as described below. In the blank incubations performed without cofactor, low amounts of E-3'-N^2-dGuo were formed, indicating that 1'-hydroxyestragole itself can also react with 2'-deoxyguanosine. Therefore, all data had to be corrected for the amount of E-3'-N^2-dGuo formed in the blank incubations. In the present study, the substrate was added prior to the cofactor, and the reaction was initiated by the addition of the cofactor PAPS. To determine whether the order of addition of substrate and PAPS influences the results obtained, the order of pipetting was reversed. Experiments, in which the substrate was added after PAPS revealed, however, that the order of cofactor and substrate addition did not significantly influence the rate of formation of E-3'-N^2-dGuo (data not shown).

The kinetic values (K_m and V_{max}) for the PAPS dependent formation of E-3'-N^2-dGuo were determined by fitting the data to the standard Michaelis-Menten equation:

$$v = V_{max}/(1 + (K_m/[S])$$

with [S] being the concentration of 1'-hydroxyestragole, using the LSW data analysis toolbox (version 1.1.1, MDL Information Systems, Inc.).

To allow for the comparison of the V_{max} values obtained to the activity of sulfotransferases present in each preparation, incubations were performed to determine the sulfotransferase activity in the S9 homogenates of the three species with the standard substrate 7-hydroxycoumarin. Incubations with male rat, male mouse, and human liver S9 homogenates were performed in a 100 µL incubation mixture, containing (final concentrations of) 1 mM PAPS, 0.4 mg S9 protein per mL, and a substrate concentration of 25 µM 7-hydroxycoumarin (representing saturating substrate conditions (Wang *et al.*, 2006) in 0.1 M Tris-HCl (pH 7.4). Incubations were carried out for 10 min, and the reactions were terminated by the addition of 25 µL of cold acetonitrile, after which the incubations were centrifuged for 5 min at 16,000g. The supernatant obtained was analyzed by HPLC on a Waters M600 liquid chromatography system coupled to an Alltima C18 5 µm column, 150 mm × 4.6 mm (Alltech, Breda, The Netherlands). The flow rate was 1 mL min^{-1}, starting at 10% acetonitrile in nanopure water containing 0.1% acetic acid for 3 min. A linear gradient was then applied from 10% to 30% acetonitrile over 10 min, after which the percentage of acetonitrile was increased to 100% over 2 min. Under these conditions, 7-hydroxycoumarin sulfate had a retention time of 9 min. Detection was carried out using a Waters 966 photodiode array detector (Waters, Milford, MA) at 280 nm. Quantification was obtained by comparison to a standard curve of 7-hydroxycoumarin sulfate. Because the rates of formation of both 1'-sulfooxyestragole and 7-hydroxycoumarin sulfate are expressed as pmol min^{-1} (mg S9 protein)$^{-1}$, the V_{max} values obtained for

the sulfonation of 1'-hydroxyestragole can also be expressed as a percentage of the sulfotransferase activity of the standard substrate 7-hydroxycoumarin to allow for the comparison of the V_{max} values obtained to the activity of sulfotransferases present in each preparation with this standard substrate.

In the present study, liver S9 homogenates were used for the incubations, in which the sulfonation of 1'-hydroxyestragole was catalyzed. Liver S9 homogenates contain cytosol as a source of sulfotransferase enzymes, but they also contain microsomal protein, in which some sulfatase enzymes are localized (Parenti *et al*., 1997; Diez-Roux and Ballabio, 2005). To determine whether the microsomal fraction present in the liver S9 homogenate can influence the relative difference in the rate of formation of 1'-sulfooxyestragole by the three species, incubations were performed with the cytosol obtained from the S9 homogenates of the three species. The liver S9 homogenates were split into microsomes and cytosol by centrifugation at 100,000g for 1 h. Incubations with the cytosol obtained were performed as described for the incubations with liver S9 homogenates, using 5 μL of cytosol instead of 5 μL of S9 and substrate concentrations of 2000 and 3000 μM 1'-hydroxyestragole. The incubations were analyzed by LC-ESI-MS/MS as described below.

Additional experiments were performed to analyze the effect of the sulfotransferase inhibitor pentachlorophenol on the formation of E-3'-N^2-dGuo. To this end experiments in the presence of 25 μM (final concentration) pentachlorophenol were performed for all S9 incubations at 100 μM (final concentration) substrate. Furthermore, the influence of the presence of protein in the incubations on the scavenging capacity of 2'-deoxyguanosine was analyzed as well, for which incubations with male rat liver S9 were performed in the presence of BSA at a concentration range of 2-10 mg mL^{-1} and the decrease in E-3'-N^2-dGuo formation was compared to its formation in the absence of additionally added BSA.

Quantification of E-3'-N^2-dGuo by isotope dilution LC-ESIMS/MS

LC-ESI-MS/MS analysis was performed on an HP 1100 series liquid chromatography system (Hewlett-Packard, Palo Alto, CA) coupled to a

Finnigan MAT TSQ 7000 triple quadrupole mass spectrometer (San Jose, CA). Aliquots of 15 µL of the incubation mixture were directly injected on a Gemini C18 5 µm 110 Å column, 150 × 2 mm (Phenomenex, Torrance, CA). The gradient was made with ultrapure water containing 0.1% (v/v) formic acid and acetonitrile. The in-column flow rate was 50 µL min^{-1}. A linear gradient was applied from 20% to 30% acetonitrile over 30 min, after which the percentage of acetonitrile was increased to 100% over 2 min, kept at 100% for 1 min, lowered to 20% over 2 min, and equilibrated at these initial conditions for 15 min. Mass spectrometrical analysis was done in positive ion mode. The electospray capillary voltage was set at 4.5 kV and the capillary temperature at 250 °C. Nitrogen was used as sheath gas with a pressure of 40 psi. The pressure was set at 3.2 mTorr in the collision cell with argon as collision gas. The quantitative determination of E-3'-N^2-dGuo in samples was achieved by isotope dilution to ensure high trueness and precision. Typically, 20 µL of the incubations with male rat, male mouse, and human liver S9 homogenates was spiked with 10 µL of 0.1 pmol ($^{15}N_5$)E-3'-N^2-dGuo per µL. Sample analysis was carried out by the selected reaction monitoring (SRM) mode, and three characteristic transitions were recorded for E-3'-N^2-dGuo, whereas two were chosen for ($^{15}N_5$)E-3'-N^2-dGuo. The dwell time per transition was 0.6 s. For each molecule, the most intense transition was used for the quantification (quantifier), whereas the others (two for E-3'-N^2-dGuo and one for ($^{15}N_5$)E-3'-

Table 1. Transition reactions and their corresponding collision energies monitored by LC-ESI-MS/MS

	Transition (m/z) (used for quantification)	Collision energy (eV)	Transition (m/z) (used for conformation)	Collision energy (eV)
N^2-(*trans*-isoestragol-3'-yl)- 2'-deoxyguanosine	414 → 298	16	414 → 164	26
			414 → 147	36
Internal standard	419 → 303	16	419 → 169	26

N^2-dGuo) were used to confirm the presence of the compound (qualifier). The ratios quantifier:qualifier were controlled in samples and compared to standards (calibration points) to improve the certainty of the detection. The various transitions and collision energies are shown in Table 1.

Results

Stereochemistry of 1'-hydroxyestragole

Analysis of the synthesized 1'-hydroxyestragole by HPLC on a Chiral-AGP column revealed that 1'-hydroxyestragole consists of a racemic mixture of about 50:50%. Incubations with male rat, male mouse, and human liver S9 homogenates in the presence of estragole as substrate and NADPH as cofactor were performed to determine whether cytochrome P450 enzymes produce a racemic mixture of 1'-hydroxyestragole. Analysis of these incubations revealed that both enantiomers are formed by cytochrome P450 enzymes present in male rat, male mouse, and human liver S9 homogenates in a ratio of about 75:25%. In the present study, it is assumed that a racemic mixture of 1'-hydroxyestragole can be used as substrate to determine the kinetics for the sulfotransferase dependent formation of E-3'-N^2-dGuo by male rat, male mouse, and human liver S9 homogenates because the actual 1'-hydroxyestragole metabolite formed by the liver cytochromes P450 was shown to be a racemic mixture as well. A different ratio of enantiomers might affect the kinetics for the sulfonation of 1'-hydroxyestragole to some extent, but it is assumed that this will not change the relative efficiency in the sulfonation of 1'-hydroxyestragole by the three species studied.

Synthesis and MS/MS-based characterization of E-3'-N^2-dGuo and ($^{15}N_5$)E-3'-N^2-dGuo

Incubation of 1'-acetoxyestragole with 2'-deoxyguanosine resulted in the formation of several adducts with 2'-deoxyguanosine as detected by HPLC (Figure 2). Our chromatographic profile was comparable to that reported in the

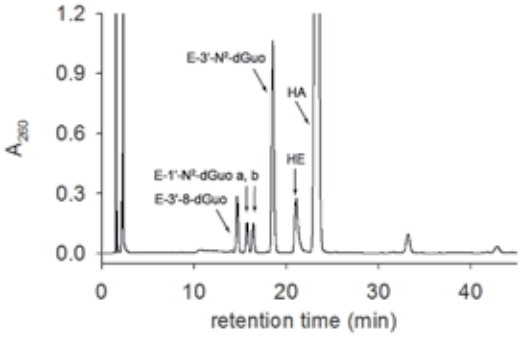

Figure 2. HPLC chromatogram of estragole adducts with 2'-deoxyguanosine formed in a reaction between 1'-acetoxyestragole and 2'-deoxyguanosine. On the basis of previous identification by Phillips *et al*. (1981) and Wiseman *et al*. (1985) peaks were characterized as 8-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine (E-3'-8-dGuo), two diastereomers of N^2-(estragol-1'-yl)-2'-deoxyguanosine (E-1'-N^2-dGuo), and N^2-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine (E-3'-N^2-dGuo). HE and HA correspond to 1'-hydroxyestragole and 3'-hydroxyanethole, respectively.

literature for the same reaction, and without further identification, we assigned the peaks in our chromatogram according to the data of the literature (Phillips *et al*., 1981, Wiseman *et al*., 1985). This assumption was supported by the structural identification of the peak at 19 min in our chromatogram, which was identified, on the basis of its collision-induced dissociation (CID) spectrum, as E-3'-N^2-dGuo (see below). Indeed, E-3'-N^2-dGuo was shown to be the most abundant compound (on a UV-detection basis) generated during the reaction, not only in the current study but also in the works reported by Phillips *et al.* (1981) and Wiseman *et al.* (1985). Therefore, E-3'-N^2-dGuo was collected at 19 min to be used as a standard to develop the quantitative LC-ESI-MS/MS method that formed the basis for the 2'-deoxyguanosine scavenging method aimed at detecting 1'-sulfooxyestragole. A similar chromatographic profile was obtained for ($^{15}N_5$)E-3'-N^2-dGuo, and the isolation of the compound was undertaken under the same conditions.

The characterizations of E-3'-N^2-dGuo and ($^{15}N_5$)E-3'-N^2-dGuo were carried out on the basis of the CID spectra depicted in Figure 3. The MS spectrum of E-3'-N^2-dGuo provided a quasi-molecular ion at m/z 414 corresponding

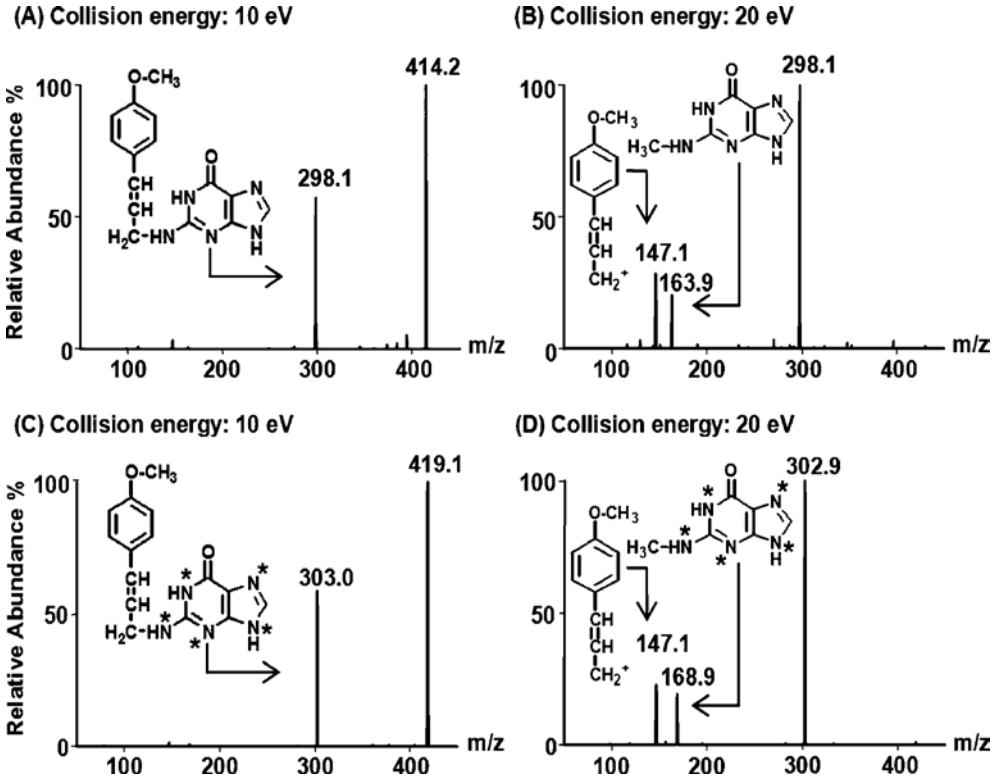

Figure 3. Positive-ion electrospray MS/MS spectra and proposed stuctures of the fragment ions of E-3'-N^2-dGuo (A and B) and ($^{15}N_5$)E-3'-N^2-dGuo (C and D). The asterisks indicate the ^{15}N-labeled sites in the internal standard.

to the protonated molecule $[M + H]^+$. The 5 Th-upmass shift observed in the MS spectrum of ($^{15}N_5$)E-3'-N^2-dGuo (m/z 419) was consistent with the penta-^{15}N labeling of the guanine moiety. When fragmented by collision, the ion at m/z 414 gave rise to three major fragment ions at m/z 298, 164, and 147. The fragment ion at m/z 298 (ΔM = -116 Da) was rationalized in terms of a loss of the 2-deoxy-β-D-erythro-pentofuranose moiety, as commonly observed in nucleosides (Apruzzese and Vouros, 1998; Wang *et al*., 2003; Singh and Farmer, 2006). This was further supported by the observation of the same mass loss in the transition m/z 419 → 303 obtained in the case of ($^{15}N_5$)E-3'-N^2-dGuo. Similarly, the fragment ion at m/z 164 observed for E-3'-N^2-dGuo was also 5 Th-upshifted to m/z 169 when the CID spectrum was recorded with ($^{15}N_5$)E-3'-N^2-dGuo instead of the non-labeled adduct. This

demonstrated unambiguously that the five nitrogen atoms of guanine were involved in the structure of the fragment. Thus, it is very likely that the loss of the 2-deoxy-β-D-erythro-pentofuranose and the cleavage of the C-C bond in the β-position of the exocyclic amine of guanine led to the formation of the protonated *N*^{2}-methylguanine, observed at 164 Th for E-3'-N^2-dGuo and at 169 Th for ($^{15}N_5$)E-3'-N^2-dGuo. In contrast, no mass shift was observed for the ion at m/z 147 when ($^{15}N_5$)E-3'-N^2-dGuo was analyzed instead of E-3'-N^2-dGuo, suggesting the exclusive contribution of atoms from the estragole moiety in this fragment. Therefore, the fragment at m/z 147 was assigned to be the carbocation radical [estragole-H$^{\bullet}$]$^+$.

Performance of the LC-ESI-MS/MS method

On the basis of the fragmentation pattern of E-3'-N^2-dGuo and ($^{15}N_5$)E-3'-N^2-dGuo, an isotope dilution LC-ESI-MS/MS method was developed using multiple-transitions SRM to ensure a high selectivity of the detection. The control of the quantifier:qualifier ratios did not reveal any contamination in the signals of interest. For E-3'-N^2-dGuo, the limit of detection (S/N = 3) was 0.05 pmol on column, whereas the limit of quantification was found at 0.1 pmol on column. For quantitative analysis of E-3'-N^2-dGuo, a calibration curve was constructed in the presence of a 0.5 pmol internal standard. This calibration curve was at least linear from the limit of quantification to 4 pmol on column ($r^2 > 0.991$), using six different concentrations of E-3'-N^2-dGuo.

2'-Deoxyguanosine-mediated scavenging of 1'-sulfooxyestragole

Incubations with male rat, male mouse, and human liver S9 homogenates in the presence of 1'-hydroxyestragole as substrate, PAPS as cofactor, and 2'-deoxyguanosine as trapping agent were performed to determine the possibility of detecting the formation of 1'-sulfooxyestragole as E-3'-N^2-dGuo. The LC-ESI-MS/MS analysis of an incubation in the presence of male rat liver S9 with 1'-hydroxyestragole at a concentration of 50 μM (1 mM PAPS and 1 mM 2'-deoxyguanosine) revealed the formation of E-3'-N^2-dGuo (peak eluting at ca. 30 min) as well as two unidentified minor adducts that were also observed

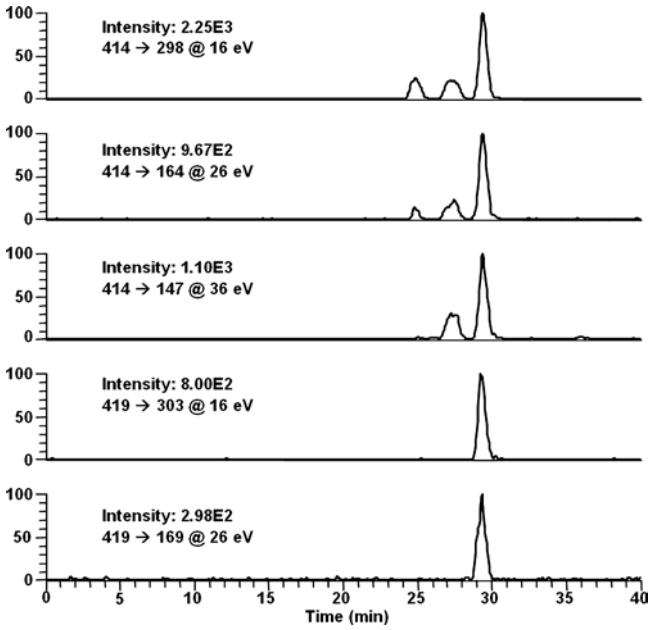

Figure 4. LC-ESI-MS/MS analysis of an incubation with male rat liver S9 homogenate at a substrate concentration of 50 µM 1'-hydroxyestragole with PAPS as cofactor and 2'-deoxyguanosine as trapping agent. The chromatograms correspond to the recorded transitions: m/z 414 → 298, m/z 414 → 164 and m/z 414 → 147 for E-3'-N^2-dGuo, while m/z 419 → 303 and m/z 419 → 169 for ($^{15}N_5$)E-3'-N^2-dGuo.

in vivo by Phillips *et al*. (1981) and Wiseman *et al*. (1985) (Figure 4). When 25 µM of the sulfotransferase inhibitor pentachlorophenol was added to the incubation mixture, levels of E-3'-N^2-dGuo were equal to the levels in the blank incubation (no cofactor), indicating that the formation of E-3'-N^2-dGuo required, at least in our model, the transient 1'-sulfooxyestragole.

Because of the formation of three 2'-deoxyguanosine-mediated adducts, the quantification of E-3'-N^2-dGuo does not represent the total amount of 1'-sulfooxyestragole formed by the S9. With our data (Figure 4) and those from the literature (Phillips *et al*., 1981; Wiseman *et al*., 1985), it can roughly be estimated that E-3'-N^2-dGuo represents about 60-70% of the total amount of adducts formed when the 1'-sulfooxyestragole is trapped by 2'-deoxyguanosine. Furthermore, it was found that the amount of E-3'-N^2-dGuo formed at a defined level of S9 protein, PAPS, and 1'-hydroxyestragole was

increasing linearly with the amount of 2'-deoxyguanosine added up to the highest soluble amount of 2'-deoxyguanosine of 1 mM (data not shown). This indicates that the amount of 2'-deoxyguanosine added is not providing a complete scavenging of 1'-sulfooxyestragole, leading to an underestimation of the generated amount of 1'-sulfooxyestragole. Because of the low solubility of 2'-deoxyguanosine in water, the optimal 2'-deoxyguanosine concentration for maximal scavenging could not be determined, and therefore, the extent of the underestimation could not be quantified. The protein content in the incubations could also influence the scavenging efficiency by providing an alternative reaction possibility for the reactive 1'-sulfooxyestragole. When increasing the protein content in the incubations by the addition of BSA, a small but insignificant decrease in the established rate of formation of 1 pmol E-3'-N^2-dGuo min^{-1} mg $protein^{-1}$ was observed for each 1 mg mL^{-1} increase in BSA concentration. On the basis of this result, it can be estimated that at a level of 1 mg S9 protein per mL, the protein content does not significantly decrease the apparent V_{max} (data not shown).

Michaelis-Menten kinetics

Using the newly developed 2'-deoxyguanosine scavenging method, the kinetics for the sulfotransferase dependent formation of E-3'-N^2-dGuo for male rat, male mouse, and human liver S9 homogenates was investigated. Figure 5 shows the plots of the sulfotransferase dependent formation of E-3'-N^2-dGuo versus the substrate concentration for liver S9 homogenates of the three species. Table 2 summarizes the apparent K_m and V_{max} values obtained from these plots. A comparison of the kinetics for sulfotransferase dependent formation of E-3'-N^2-dGuo in liver S9 homogenates of the three species reveals that the rate of formation of E-3'-N^2-dGuo by male rat liver S9 is higher than the rate of formation by human liver S9, whereas the rate of formation of E-3'-N^2-dGuo by male mouse liver S9 was about the same as that by human liver S9. The apparent V_{max} for male rat liver S9 was found to be 2.6-fold higher than for human liver S9, and the apparent K_m was found to be 12-fold lower. In the case of male mouse liver S9, the apparent V_{max} was found to be 2.3-

fold lower than for human liver S9, and the apparent K_m was found to be 2.5-fold lower. Overall, the catalytic efficiency for the sulfotransferase dependent formation of E-3'-N^2-dGuo, calculated as the apparent V_{max}/K_m, was about 30 times higher for male rat liver S9 than for human liver S9 homogenates, whereas the catalytic efficiency for male mouse and human liver S9 was about the same.

A comparison of the apparent V_{max} for sulfotransferase dependent

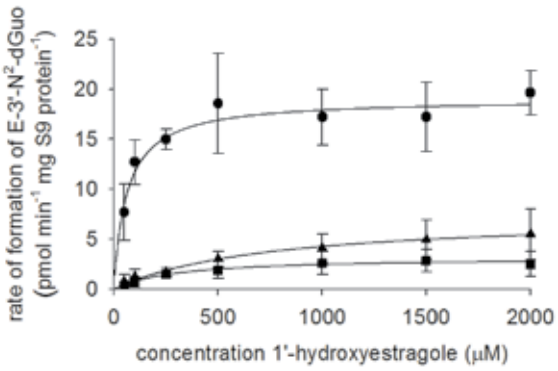

Figure 5. Plots of sulfotransferase dependent formation of E-3'-N^2-dGuo vs the substrate concentration by male rat (●), male mouse (■), and human (▲) liver S9 homogenates. In the plots each point represents the mean (±SD) of three replicates.

Table 2. Kinetic parameters for sulfotransferase dependent formation of E-3'-N^2-dGuo for male rat, male mouse, and human liver S9 homogenates

Species	Apparent V_{max} [a]	Apparent K_m [b]	Catalytic efficiency V_{max}/K_m [c]	7-Hydroxycoumarin sulfotransferase activity[a]	Relative V_{max} [d]
Male rat	19.0 ± 0.8	63 ± 13	0.30	2387 ± 94	0.8
Male mouse	3.2 ± 0.2	296 ± 64	0.01	277 ± 12	1.2
Human	7.4 ± 0.8	727 ± 185	0.01	1111 ± 93	0.7

[a] pmol min^{-1} (mg S9 protein)$^{-1}$
[b] μM
[c] μL min^{-1} (mg S9 protein)$^{-1}$
[d] V_{max} for sulfotransferase dependent formation of E-3'-N^2-dGuo calculated as percentage of the 7-hydroxycoumarin sulfotransferase activity at 25 μM 7-hydroxycoumarin (apparent V_{max}/7-hyroxycoumarin sulfotransferase activity × 100).

formation of E-3'-N^2-dGuo by the S9 homogenates of the three species with the sulfotransferase activity for sulfonation of the standard substrate 7-hydroxycoumarin by these preparations reveals that the differences between the apparent V_{max} of the S9 homogenates of the three species are comparable to the differences for the sulfonation of 7-hydroxycoumarin for the same samples. The apparent V_{max} for the sulfotransferase dependent formation of E-3'-N^2-dGuo by liver S9 homogenates of the three species was about 0.7-1.2% of the sulfotransferase activity for the sulfonation of 7-hydroxycoumarin.

Liver S9 homogenates contain sulfotransferase enzymes, but they also contain sulfatases in the microsomal fraction, which in theory may catalyze the deconjugation of 1'-sulfooxyestragole (Parenti *et al*., 1997; Diez-Roux and Ballabio, 2005). However, because the half-life of 1'-sulfooxyestragole in aqueous environment is estimated to be very short, it is not likely that 1'-sulfooxyestragole will be a substrate for sulfatase enzymes. Incubations with the cytosolic fraction obtained from the used S9 homogenates, which does not contain sulfatases (Parenti *et al*., 1997; Diez-Roux and Ballabio, 2005), revealed the same relative differences among male rats, male mice, and humans in the sulfonation of 1'-hydroxyestragole as observed with S9 fractions (data not shown). This indicates that the microsomal fraction present in liver S9 homogenates does not influence the relative difference in the rate of formation of 1'-sulfooxyestragole between the three species.

Discussion

To obtain insight into possible species differences in the bioactivation of estragole, the kinetics for the sulfonation of the proximate carcinogen 1'-hydroxyestragole were compared for male rat, male mouse, and human liver S9 homogenates. A method was developed, with which the 1'-sulfooxyestragole formed in incubations with male rat, male mouse, and human liver S9 homogenates was scavenged by 2'-deoxyguanosine, and the most abundant adduct (E-3'-N^2-dGuo) could be quantified by isotope dilution

LC-ESI-MS/MS. With this method, only a certain percentage of the formed 1'-sulfooxyestragole is trapped, and the absolute V_{max} and consequently the absolute catalytic efficiency for sulfonation could not be determined. However, it should be pointed out that this will not change the relative efficiency in the sulfonation of 1'- hydroxyestragole by the three species studied. High selectivity of our MS-based method was achieved through the monitoring of three characteristic transitions for E-3'-N^2-dGuo as well as two for the internal standard, ($^{15}N_5$)E-3'-N^2-dGuo. The accuracy of the quantification was censured by a low limit of detection at 0.05 pmol on column for E-3'-N^2-dGuo. This method can be further adapted for the analysis of E-3'-N^2-dGuo in genomic DNA from *in vitro* treatments and *in vivo* samples.

The catalytic efficiency (V_{max}/K_m) for the sulfotransferase dependent formation of E-3'-N^2-dGuo by male rat liver S9 was observed to be higher than by male mouse and human liver S9 because a higher apparent V_{max} and a lower apparent K_m were found. A higher V_{max} can be the result of a general relatively higher expression of sulfotransferase enzymes in rat liver compared to mouse and human liver (Glatt, 2002). The ratio between the apparent V_{max} values for the sulfonation of 1'-hydroxyestragole by male rat, male mouse, and human liver was found to be comparable to the ratio of the catalytic activities for the sulfonation of the standard substrate 7-hydroxycoumarin by these preparations. This supports the conclusion that the differences observed reflect the generally observed higher expression of sulfotransferases in male rat liver compared to human and male mouse livers. Extrahepatic expression of sulfotransferases might be higher than the hepatic expression of sulfotransferases in humans and mice. However, given the fact that the liver is the main target organ, in which tumors develop after exposure to estragole, it is assumed that the sulfonation of 1'-hydroxyestragole in the liver is most important for its bioactivation (Drinkwater *et al*., 1976; Miller *et al*., 1983; Wiseman *et al.,* 1987).

Differences in the sulfonation between species can furthermore be a result of differences in the substrate specificity of orthologous sulfotransferase enzymes. This has for instance been demonstrated with the compound

tamoxifen, which is bioactivated by the sulfonation of its hydroxylated metabolite α-hydroxytamoxifen (Boocock *et al.*, 2000; Glatt, 2000). A higher efficiency in the sulfonation of α-hydroxytamoxifen for rats compared to that for humans was ascribed to a greater specific activity of rat hydroxysteroids SULTa versus its orthologous human hSULT2A1 (Glatt, 2000). Another possible explanation for species differences in sulfonation is that different sulfotransferase enzyme subfamilies are responsible for sulfonation. It has for instance been shown that the sulfotransferase mediated bioactivation of the hydroxyl metabolite of acetoaminofluorene in rats occurs by sulfotransferases of the 1C family, whereas in humans, sulfotransferases of the 1A family are involved (Glatt, 2000).

So far, no data are available that reveal which iso-enzymes are involved in the sulfonation of 1'-hydroxyestragole in male rats, male mice, and humans. In the present study, the sulfotransferase dependent formation of E-3'-N^2-dGuo was completely blocked by 25 μM pentachlorophenol in incubations with liver S9 homogenates from all three species. These findings are in agreement with studies, in which pentachlorophenol reduced the sulfotransferase dependent binding to rRNA of the analogous alkenylbenzene 1'-hydroxy-2'-3'-dehydroestragole in hepatic cytosols of mice and 1'-hydroxysafrole in the hepatic cytosols of rats (Wislocki *et al.*, 1976; Fennell *et al.*, 1986). Because pentachlorophenol is a specific inhibitor of phenol sulfotransferase enzymes (Glatt, 2000), the results of the present study indicate that phenol sulfotransferases are involved in the sulfonation of 1'-hydroxyestragole. In order to explain the differences in the sulfonation of 1'-hydroxyestragole between species and to determine inter-individual variation in sulfonation, it is interesting to know, which sulfotransferase subfamilies are involved in the sulfonation of 1'-hydroxyestragole in male rats, male mice, and humans. This was, however, beyond the scope of the present study.

The induction of tumors by estragole has been demonstrated in mice exposed to high concentrations of estragole (Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Wiseman *et al.,* 1987). Some risk assessment strategies imply linear extrapolation from these animal experiments to a low dose human situation.

Such a linear extrapolation does not take into account species differences and nonlinear effects in the biokinetics and biodynamics of estragole. The present study demonstrates that male rats are more efficient in the sulfonation of 1'-hydroxyestragole than humans, whereas male mice were equally efficient in the sulfonation of 1'-hydroxyestragole as humans. This indicates, as far as the bioactivation by sulfotransferase is concerned, that when extrapolating the cancer risk from laboratory animals to humans, using data from male rats may overestimate the cancer risk in humans, whereas using data from male mice may provide a better estimate of the cancer risk in humans. At present, quantitative data on possible species differences in susceptibility toward carcinogenic effects of estragole *in vivo* are not available. Long-term carcinogenicity studies for estragole have so far only been conducted in mice (Drinkwater *et al*., 1976; Miller *et al*., 1983; Wiseman *et al.,* 1987). To provide the whole picture of species dependent differences in estragole bioactivation more insight is needed in species dependent differences in sensitivity to estragole carcinogenicity, species differences in P450-mediated bioactivation of estragole, and species differences in DNA adduct formation by estragole. This latter aspect can now be studied using the newly developed isotope dilution LC-ESIMS/MS method developed and reported in the present study.

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References

Anthony, A., Caldwell, J., Hutt, A. J. and Smith, R. L. (1987). Metabolism of estragole in rat and mouse and influence of dose size on excretion of the proximate carcinogen 1'-hydroxyestragole. Food Chem Toxicol 25, 799-

806.

Apruzzese, W. A. and Vouros, P. (1998). Analysis of DNA adducts by capillary methods coupled to mass spectrometry: a perspective. J Chromatogr A 794, 97-108.

Boberg, E. W., Miller, E. C., Miller, J. A., Poland, A. and Liem, A. (1983). Strong evidence from studies with brachymorphic mice and pentachlorophenol that 1'-sulfooxysafrole is the major ultimate electrophilic and carcinogenic metabolite of 1'-hydroxysafrole in mouse liver. Cancer Res 43, 5163-5173.

Boocock, D. J., Maggs, J. L., Brown, K., White, I. N. and Park, B. K. (2000). Major inter-species differences in the rates of O-sulphonation and O-glucuronylation of alpha-hydroxytamoxifen in vitro: a metabolic disparity protecting human liver from the formation of tamoxifen-DNA adducts. Carcinogenesis 21, 1851-1858.

Borchert, P., Wislocki, P. G., Miller, J. A. and Miller, E. C. (1973). The metabolism of the naturally occurring hepatocarcinogen safrole to 1'-hydroxysafrole and the electrophilic reactivity of 1'-acetoxysafrole. Cancer Res 33, 575-89.

Diez-Roux, G. and Ballabio, A. (2005). Sulfatases and human disease. Annu Rev Genomics Hum Genet 6, 355-79.

Drinkwater, N. R., Miller, E.C., Miller, J.A and Pitot H.C. (1976). Hepatocarcinogenicity of estragole (1-allyl-4-methoxybenzene) and 1'-hydroxyestragole in the mouse and mutagenicity of 1'-acetoxyestragole in bacteria. J Natl Cancer Inst 57, 1323-1331.

Fennell, T. R., Juhl, U., Miller, E. C. and Miller, J. A. (1986). Identification and quantitation of hepatic DNA adducts formed in B6C3F1 mice from 1'-hydroxy-2',3'-dehydroestragole: comparison of the adducts detected with the 1'-3H-labelled carcinogen and by ^{32}P-postlabelling. Carcinogenesis 7, 1881-1887.

Glatt, H. (2000). Sulfotransferases in the bioactivation of xenobiotics. Chem Biol Interact 129, 141-170.

Glatt, H. (2002). Sulphotransferases. In Enzyme Systems that Metabolise

Drugs and Other Xenobiotics (Ioannides, C., Ed.) pp 353–439, John Wiley & Sons Ltd., New York.

Miller, E. C., Swanson, A. B., Phillips, D. H., Fetcher, T. L., Liem, A. and Miller, J. A. (1983). Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occuring and synthetic alkenylbenzene derivatives related to safrole and estragole. . Cancer Res 43, 1124-1134.

Parenti, G., Meroni, G. and Ballabio, A. (1997). The sulfatase gene family. Curr Opin Genet Dev 7, 386-391.

Phillips, D. H., Miller, J. A., Miller, E. C. and Adams, B. (1981). Structures of the DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1'-hydroxyestragole. Cancer Res 41, 176-186.

Phillips, D. H., Reddy, M. V. and Randerath, K. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. II. Newborn male B6C3F1 mice. Carcinogenesis 5, 1623-1628.

Randerath, K., Haglund, R. E., Phillips, D. H. and Reddy, M. V. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. Carcinogenesis 5, 1613-1622.

SCF (2001). Opinion of the scientific committee on food on estragole (1-allyl-4-methyxybenzene). http://ec.europa.eu/food/fs/sc/scf/out104_en.pdf

Singh, R. and Farmer, P. B. (2006). Liquid chromatography-electrospray ionization-mass spectrometry: the future of DNA adduct detection. Carcinogenesis 27, 178-196.

Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., Rogers, A. E., Caldwell, J. and Sipes, I. G. (2002). Safety assessment of allylalkoxybenzene derivatives used as flavouring substances - methyl eugenol and estragole. Food Chem Toxicol 40, 851-870.

Wang, J. J., Marshall, W. D., Frazer, D. G., Law, B. and Lewis, D. M. (2003). Characterization of DNA adducts from lung tissue of asphalt fume-exposed

mice by nanoflow liquid chromatography quadrupole time-of-flight mass spectrometry. Anal Biochem 322, 79-88.

Wang, Q., Ye, C., Jia, R., Owen, A. J., Hidalgo, I. J. and Li, J. (2006). Inter-species comparison of 7-hydroxycoumarin glucuronidation and sulfation in liver S9 fractions. In Vitro Cell Dev Biol Anim 42, 8-12.

Wiseman, R. W., Fennell, T. R., Miller, J. A. and Miller, E. C. (1985). Further characterization of the DNA adducts formed by electrophilic esters of the hepatocarcinogens 1'-hydroxysafrole and 1'-hydroxyestragole in vitro and in mouse liver in vivo, including new adducts at C-8 and N-7 of guanine residues. Cancer Res 45, 3096-3105.

Wiseman, R. W., Miller, E. C., Miller, J. A. and Liem, A. (1987). Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J x C3H/HeJ F_1 mice. Cancer Res 47, 2275-2283.

Wislocki, P. G., Borchert, P., Miller, J. A. and Miller, E. C. (1976). The metabolic activation of the carcinogen 1'-hydroxysafrole in vivo and in vitro and the electrophilic reactivities of possible ultimate carcinogens. Cancer Res 36, 1686-1695.


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A physiologically based biokinetic (PBBK) model for estragole bioactivation and detoxification in rat

Based on: Punt A, Freidig AP, Delatour T, Scholz G, Boersma MG, Schilter B, Van Bladeren PJ, and Rietjens IMCM
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Abstract

The present study defines a physiologically based biokinetic (PBBK) model for the alkenylbenzene estragole in rat based on i) *in vitro* metabolic parameters determined using relevant tissue fractions, ii) *in silico* derived partition coefficients, and iii) physiological parameters derived from the literature. The model consists of eight compartments including liver, lung and kidney as metabolizing compartments, and additional compartments for fat, arterial blood, venous blood, rapidly perfused tissue and slowly perfused tissue. Evaluation of the model was performed by comparing the PBBK model-predicted dose-dependent formation of the estragole metabolites 4-allylphenol and 1'-hydroxyestragole glucuronide to literature reported levels of these metabolites, which were demonstrated to be in the same order of magnitude. With the model obtained the relative extent of bioactivation and detoxification of estragole at different oral doses was examined. At low doses formation of 4-allylphenol, leading to detoxification, is observed to be the major metabolic pathway, occurring mainly in the lung and kidney due to formation of this metabolite with high affinity in these organs. Saturation of this metabolic pathway in the lung and kidney leads to a relative increase in formation of the proximate carcinogenic metabolite 1'-hydroxyestragole, occurring mainly in the liver. This relative increase in formation of 1'-hydroxyestragole leads to a relative increase in formation of 1'-hydroxyestragole glucuronide and 1'-sulfooxyestragole as well, with the latter being the ultimate carcinogenic metabolite of estragole. These results indicate that the relative importance of different metabolic pathways of estragole may vary in a dose-dependent way, leading to a relative increase in bioactivation of estragole at higher doses.

Introduction

The alkenylbenzene estragole is a natural constituent of several herbs including basil, fennel, anise, and tarragon. Estragole is also present in food as a result of the addition of essential oils of these herbs as flavoring agents. Because estragole is demonstrated to be hepatocarcinogenic in different rodents when administered at high doses, there is interest in its safety as a food constituent. In 2001, the Scientific Committee on Food (SCF) of the European Union published a scientific opinion on estragole, in which it was concluded that estragole is genotoxic and carcinogenic and that reductions in exposure and restrictions in use levels are indicated (SCF, 2001). The expert panel of the Flavor and Extract Manufacturers association (FEMA) concluded that exposure to estragole, resulting from spice consumption, does not pose a significant cancer risk to humans. In this conclusion, experimental data that suggested a non-linear relationship between dose and profiles of metabolism and metabolic activation were taken into account (Smith *et al*., 2002). Based on different methodologies the average daily intake of estragole was estimated by the SCF to be 0.07 mg/kg bw/day for a 60 kg person (SCF, 2001), while the FEMA estimated the average daily intake of estragole to be less than 0.01 mg/kg bw/day (Smith *et al*., 2002). The estimation of the SCF is based on theoretical maximum use levels of estragole in various food categories and consumption data for these food categories, whereas the intake estimation of the FEMA is based on production volume data for flavor use (SCF, 2001; Smith *et al*., 2002).

Rodent carcinogenicity of estragole has been linked to its metabolic conversion to a genotoxic metabolite (Drinkwater *et al*., 1976; Miller *et al*., 1983; Wiseman *et al*., 1987). The first step of the bioactivation of estragole to the proximate carcinogenic metabolite is 1'-hydroxylation by cytochrome P450 enzymes (Figure 1). Further bioactivation of 1'-hydroxyestragole requires the involvement of sulfotransferases converting 1'-hydroxyestragole to 1'-sulfooxyestragole, which is unstable and degrades in aqueous environment to a reactive carbocation that is capable of binding covalently to different

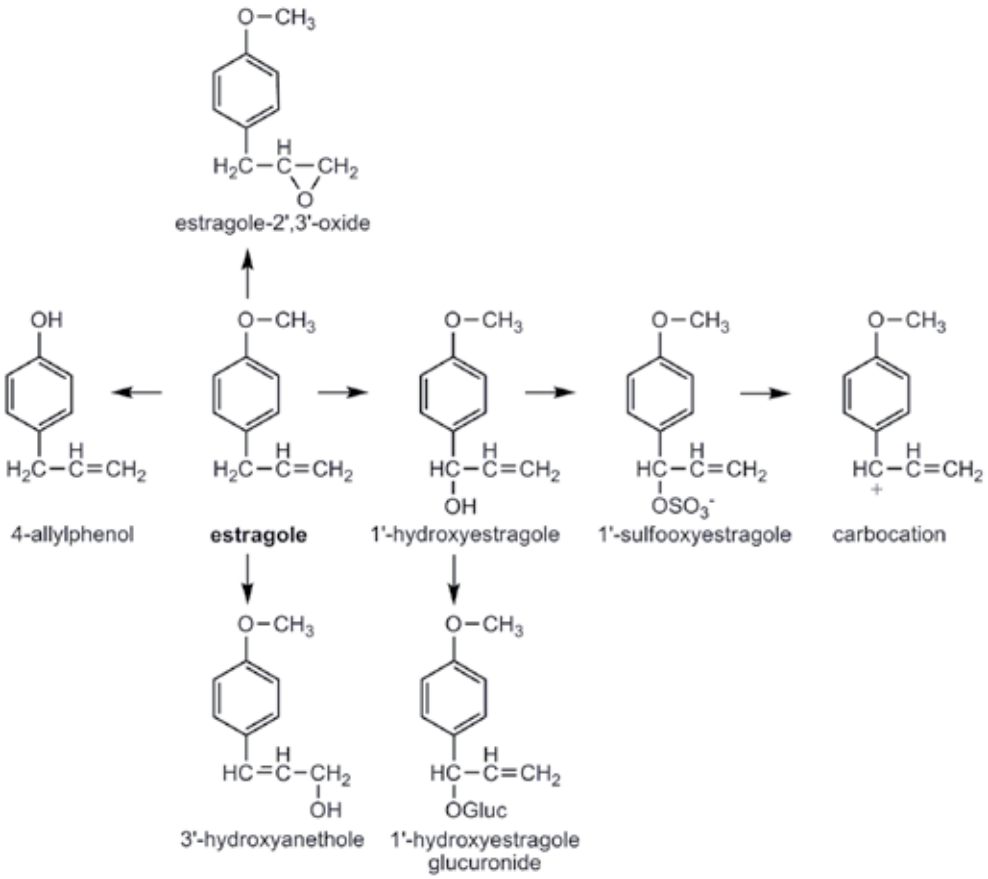

Figure 3. Metabolism of estragole.

nucleophilic molecules including glutathione, RNA, and DNA (Phillips *et al*., 1981; Phillips *et al*.,1984; Randerath *et al*.,1984;Wiseman *et al*.,1985). Conjugation of 1'-hydroxyestragole with glucuronic acid leads to the formation of a stable metabolite that is excreted in urine and is therefore an important detoxification route (Solheim and Scheline, 1973; Anthony *et al*., 1987). In addition to 1'-hydroxylation of estragole, three other major phase I metabolic pathways have been defined. *O*-demethylation of estragole results in the formation of 4-allylphenol, which is excreted as sulfo-, or glucuronosyl conjugate in the urine and leads to detoxification of estragole (Solheim and Scheline, 1973; Anthony *et al*., 1987). Furthermore, estragole can be epoxidated, leading to the formation of estragole-2',3'-oxide. This epoxide has been shown

to be able to form DNA adducts *in vitro*, but these adducts are not found *in vivo*. This has been ascribed to the rapid detoxification of estragole-2',3'-oxide by epoxide hydrolases and/or glutathione-S-transferases *in vivo* (Luo *et al*., 1992; Guenthner and Luo, 2001). Finally formation of 3'-hydroxyanethole is an important phase I metabolic route, however, it is not known whether this metabolite is formed by isomerisation of 1'-hydroxyestragole or from conversion of estragole (Solheim and Scheline, 1973; Anthony *et al*., 1987). 3'-Hydroxyanethole can be oxidized to 4-methoxycinnamic acid, which in turn can further be oxidized to 4-methoxybenzoic acid. Glucuronosyl conjugates of 3'-hydroxanethole have also been observed in the urine of rats (Anthony *et al*., 1987), which indicates that glucuronidation of 3'-hydroxyanethole can occur as well.

A change in metabolism of estragole with increasing dose is seen in both rats and mice (Anthony *et al*., 1987). In the case of rats the proportion of *O*-demethylation decreases at higher doses (as determined by the percentage of exhalation as $^{14}CO_2$) and excretion of 1'-hydroxyestragole glucuronide in the urine increases from 1.3–5.4% of the dose in the range of 0.05–50 mg/kg bw to 11.4–13.7% in the dose range of 500–1000 mg/kg bw (after a single dose). An explanation for these changes in metabolism with the dose could be a shift in phase I metabolism, from *O*-demethylation at low doses to 1'-hydroxylation at higher doses (Anthony *et al*., 1987). Such a shift could lead to a relative increase in the formation of 1'-hydroxyestragole and consequently 1'-hydroxyestragole glucuronide at higher doses. Alternatively, the observed relative increase in urinary excretion of 1'-hydroxyestragole glucuronide could also be explained by a shift in conjugation of 1'-hydroxyestragole from sulfonation at low doses to glucuronidation at higher doses, with only the glucuronide being excreted in urine because of the labile character of 1'-sulfooxyestragole. Such a shift in conjugation has been reported before for other phenolic compounds and is generally related to the fact that sulfonation is a high affinity, low capacity reaction (low K_m, low V_{max}) whereas glucuronidation shows high K_m and high V_{max} values (Koster *et al*., 1981).

To predict the extent of bioactivation and detoxification of estragole at low dose intake, it is important to take into account non-linear effects in biokinetics when extrapolating from high to low doses. The objective of the present study was to build a physiologically based biokinetic (PBBK) model providing insight in possible dose-dependent changes in estragole metabolism with emphasis on detoxification and bioactivation pathways. Kinetics for relevant phase I and phase II reactions were determined *in vitro* using tissue fractions of organs that were found to be capable of metabolizing estragole. Based on these *in vitro* data a PBBK model was constructed and the performance of the model was evaluated by comparing the predicted *O*-demethylation and formation of 1'-hydroxyestragole glucuronide to the reported levels of these metabolic routes in rats exposed to different concentrations of estragole as described by Anthony *et al*. (1987).

Material and Methods

Chemicals and biological materials

Pooled liver, lung, kidney, and small intestine microsomes from male Sprague Dawley rats and pooled liver microsomes from female Sprague Dawley, male Wistar, and female Wistar rats were purchased from BioPredic International (Rennes, France). Pooled liver S9 from male Sprague Dawley rat was obtained from BD Gentest (Woburn, MA) Hydrochloric acid (37%), potassium dihydrogen phosphate, di-potassiumhydrogenphosphate trihydrate, magnesiumchloride hexahydrate, and acetic acid were purchased from Merck (Darmstadt, Germany). β-Glucuronidasewas obtained from Roche Diagnostics (Mannheim, Germany). Tris(hydroxymethyl) aminomethane was obtained from Gibco BRL Life Technologies (Paisley, Scotland) and reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) was from Boehringer (Mannheim, Germany). CD_3OD was purchased from Eurisotop (Gif-sur-Yvette, France). Estragole (4-allylanisol), *p*-anisaldehyde, acetonitrile (chromatography grade), methanol, dimethylsulfoxide (DMSO), ascorbic

acid, alamethicin, and uridine 5'-diphosphoglucuronic acid (UDPGA) were purchased from Sigma-Aldrich (Steinheim, Germany).

Synthesis of phase I metabolites

1'-Hydroxyestragole was synthesized starting from *p*-anisaldehyde as described previously (Punt *et al*., 2007), 4-allylphenol (4-hydroxylallylbenzene) was synthesized from estragole as described by Agharahimi and LeBel (1995) and estragole-2',3'-oxide was synthesized from estragole as described by Luo *et al*. (1992). 3'-Hydroxyanethole (4-methoxycinnamyl alcohol) was synthesized from estragole based on a method described by Iyer *et al*. (2003) for the synthesis of 1'-hydroxyestragole. NMR, GC-MS, and HPLC-DAD analysis of the product revealed that the method described resulted in formation of 3'-hydroxyanethole (Passreiter *et al*., 2004; Paraskar and Sudalai, 2006). The ^{1}H-NMR spectrum of 3'-hydroxyanethole was measured using a Bruker AMX 500 MHz spectrometer (Bruker, Karlsruhe, Germany). The spectrum was acquired at 25 °C with 3.5 μs pulses (50° flip angle), a sweep width of 7353 Hz and 32 k data points. The spectrum was processed using Perch NMR Software (PERCH solutions, Kuopio, Finland). ^{1}H NMR (500 MHz, CD_3OD): δ 3.78 (3H, s, $-OCH_3$), δ4.19 (2H, m, J=5.9 Hz, J=−1.5 Hz, $-CH_2$), δ 6.21 (1H, m, J=15.9 Hz, J=5.9 Hz,=CH), δ 6.53 (1H, m, J=15.9 Hz, J=−1.5 Hz, J=-0.5 Hz,=CH), δ 6.86 (2H, m, J=2.7 Hz, J=0.4 Hz,J=8.6 Hz, arom), δ 7.33 (2H, m, J=2.4 Hz, J=8.6 Hz, J=−0.5 Hz, J=0.4 Hz, arom). GC-MS analysis was performed on a HP6890 GC-MS from Hewlett–Packard (Palo Alto, CA) using a AT-5ms 0.25 μm film column, 30 m×0.25 mm from Alltech (Breda, The Netherlands). The temperature gradient was programmed from 50–250 °C at 5 °C min^{-1}, the inlet temperature was 260 °C and the pressure of helium carrier gas was 12.0 psi. EI (70 eV): m/z (rel. int. %): 121 (100),108 (38), 164 (33), 77 (18), 32 (15),103 (11). HPLC-DAD analysis was performed as described below. UV (λ_{max} 206 nm and λ_{max} 262 nm).

Phase I metabolism

To determine which organs are involved in the phase I metabolism of

estragole, microsomal preparations of the liver, lung, kidney, and small intestine from male Sprague Dawley rats were incubated with estragole in the presence of NADPH and the formation of metabolites was monitored in time. The incubation mixtures had a final volume of 100 µL, containing (final concentrations) 3 mM NADPH, 1 mM ascorbic acid and 1 mg mL^{-1} microsomal protein in 0.2 M Tris–HCl (pH 7.4). After pre-incubating at 37 °C for 1 min, the reactions were started by the addition of the substrate estragole. Initial incubations were performed at a substrate concentration of 100 µM (final concentration) at several incubation times. The reactions were terminated by addition of 25 µL cold acetonitrile. Blank incubations were performed without the cofactor NADPH.

The kinetic constants for the formation of different phase I metabolites of estragole were determined in liver, lung, and kidney microsomes, which were found to be capable of metabolizing estragole (see Results section). The incubation mixtures were as described above. Incubations with male rat liver microsomes were carried out for 10 min at a substrate concentration that ranged from 50 to 1000 µM estragole. Incubations with male rat lung and kidney microsomes were carried out for 2 min at a substrate concentration that ranged from 1 to 400 µM estragole. All incubations were started by addition of substrate from a 100 times concentrated stock solution in DMSO and terminated by addition of 25 µL cold acetonitrile. Under these conditions the formation of the different estragole metabolites was linear with time and microsomal protein concentration for all the microsomal fractions. In the blank incubations performed without NADPH low amounts of 3'-hydroxyanethole were also formed, corresponding to ~0.6% of the applied concentration of estragole. Therefore the formation of 3'-hydroxyanethole was corrected for the amount of 3'-hydroxyanethole formed in the blank incubations. To determine whether 3'-hydroxyanethole is formed from estragole or 1'-hydroxyestragole, additional incubations were performed, in which 1'-hydroxyestragole was added as substrate instead of estragole. These incubations had a final volume of 100 µL, containing (final concentrations) 3 mM NADPH, 1 mM ascorbic acid and 1 mg microsomal protein per mL in 0.2 M Tris-HCl (pH 7.4). After

pre-incubating at 37 °C for 1 min, the reactions were started by the addition of 5 μM 1'-hydroxyestragole (final concentration) from a 100 times concentrated stock solution in DMSO. The reactions were terminated after 10 min by addition of 25 μL cold acetonitrile.

Kinetic constants for formation of phase I metabolites were also determined in incubations with liver microsomes obtained from female Sprague Dawley rats, and male and female Wistar rats. The incubation mixtures were as described above and were carried out for 10 min at a substrate concentration that ranged from 50 to 1000 μM estragole. As for the incubations with liver microsomes from male Sprague Dawley rats, the formation of 3'-hydroxyanethole was corrected for the amount of 3'-hydroxyanethole formed in the blank incubations without cofactor.

Glucuronidation of 1'-hydroxyestragole

Incubations with male Sprague Dawley rat liver S9 were performed to determine the kinetic constants for glucuronidation of 1'-hydroxyestragole. The incubation mixtures had a final volume of 200 μL, containing (final concentrations) 10 mM UDPGA and 0.2 mg mL^{-1} S9 protein in 0.2 M Tris-HCl (pH 7.4) containing 10 mM $MgCl_2$. Enzyme latency was observed due to the luminal localization of the uridine diphosphate glucuronosyltransferase active site. For this reason the incubation mixture was pre-treated with 0.025 mg mL^{-1} of the poreforming peptide alamethicin from a 200 times concentrated stock in methanol on ice for 15 min, to overcome enzyme latency and obtain maximal glucuronidation activity (Fisher *et al*., 2000; Lin and Wong, 2002). The reactions were started by the addition of the substrate 1'-hydroxyestragole from a 200 times concentrated stock solution in DMSO, after pre-incubating at 37 °C for 1 min. The reactions were carried out for 10 min and terminated by addition of 50 μL cold acetonitrile. Blank incubations were performed without the cofactor UDPGA.

Additional incubations were performed, in which the samples obtained were treated with β-glucuronidase to verify whether 1'-hydroxyestragole glucuronide had indeed been formed. For these incubations, 90 μL of sample

was added to 10 µL of 1 M potassium phosphate (pH 6.2). After the addition of 2 µL of β-glucuronidase solution (0.3 U), the mixture was incubated for 1 h at 37 °C. The nature of 1'-hydroxyestragole glucuronide was furthermore verified by LC-MS, which was performed on a Thermo Finnigan HPLC system coupled to an LCQ mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Aliquots of 20 µL (injected volume) were separated on an Alltima C18 5 U column, 150×2.1 mm (Alltech, Breda, The Netherlands). The gradient was made with ultra pure water containing 0.1% (v/v) acetic acid and acetonitrile and the flow rate was set to 0.2 mL min^{-1}. A linear gradient was applied from 10% to 25% acetonitrile over 30 min, after which the percentage of acetonitrile was increased to 100% over 2 min, and kept at 100% for 1 min. Mass spectrometric analysis was in the negative electrospray mode using a spray voltage of 4.5 kV, a capillary temperature of 200 °C, and nitrogen as sheath gas (60 arbitrary units).

Quantification of phase I metabolites and 1′-hydroxyestragole glucuronide by HPLC

Before HPLC analysis of the samples, all incubations were centrifuged for 5 min at 16,000 g. Aliquots of 50 µL of the supernatant of each sample were analyzed on a Waters 2695 HPLC system (Waters, Milford, MA) equipped with an Alltima C18 5u column, 150×4.6 mm (Alltech, Breda, The Netherlands). Detection was performed between 200 and 350 nm using a Waters 2996 photodiode array detector (Waters, Milford, MA).

For the analysis of phase I metabolites the gradient was made with ultrapure water and acetonitrile. The flow rate was 1 mL min^{-1} and a gradient was applied from 0% to 25% acetonitrile over 5 min and was kept at 25% for 20 min, after which the percentage of acetonitrile was increased to 35% over 15 min and then increased to 100% over 2 min, and kept at 100% for 1 min. Quantification of 4-allylphenol, 1'-hydroxyestragole, and estragole-2',3'-diol reflecting formation of estragole-2',3'-oxide (see Results section) was achieved by comparison of the peak areas of the different metabolites in the chromatograms obtained at a wavelength of 225 nm to the calibration

curve of the corresponding synthesized metabolites. Quantification of 3'-hydroxyanetholewas achieved by comparison of the peak areas of the metabolite in the chromatograms obtained at a wavelength of 206 nm to the calibration curve of the corresponding synthesized metabolite.

For quantification of 1'-hydroxyestragole glucuronide the gradient was made with ultrapure water containing 0.1% (v/v) acetic acid and acetonitrile. The flow rate was 1 mL min^{-1} and a gradient was applied from 10% to 25% acetonitrile over 30 min, after which the percentage of acetonitrile was increased to 100% over 2 min, and kept at 100% for 1 min. No synthetic standard of 1'-hydroxyestragole glucuronide was available for quantification of this conjugate. Because 1'-hydroxyestragole glucuronide has the same UV spectrum as 1'-hydroxyestragole (data not shown) it was assumed that it has the same molar extinction coefficient as 1'-hydroxyestragole, allowing quantification of this metabolite by comparison of the peak area of 1'-hydroxyestragole glucuronide in the chromatograms obtained at a wavelength of 225 nm to the calibration curve of 1'-hydroxyestragole. This calibration curve was prepared with the synthesized 1'-hydroxyestragole, which was analyzed by HPLC on an Alltima C18 5 µm column, 150 mm x 4.6 mm (Alltech, Breda, The Netherlands) coupled to a Waters 2695 alliance HPLC system (Waters, Etten-Leur, the Netherlands). An isocratic mobile phase gradient was applied consisting of 25% acetonitrile and 75% ultrapure water containing 0.1% (v/v) acetic acid.

The apparent maximum velocity (V_{max}(app)) and apparent Michaelis–Menten constant ($K_{m(app}$) for the formation of the different phase I metabolites of estragole and glucuronidation of 1'-hydroxyestragole were determined by fitting the data to the standard Michaelis–Menten equation:

$$v = V_{max}/(1+(K_m/ [S]))$$

with [S] being the substrate concentration, using the LSW data analysis toolbox (version 1.1.1, MDL Information Systems, Inc.).

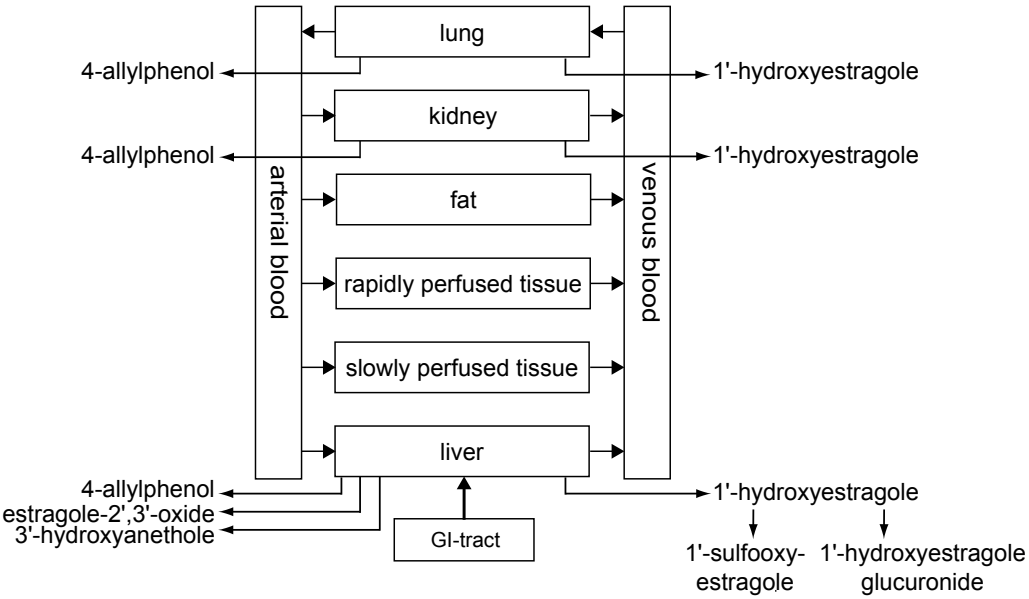

Figure 2. Diagram of the proposed PBBK model for estragole in rat.

Estragole PBBK model

A PBBK model was developed in order to estimate the formation of different estragole metabolites in male rat at different oral dose levels of estragole. A schematic diagram of the PBBK model developed is shown in Figure 2. The final model includes separate compartments for liver, lung, and kidney tissue, which were found to be involved in the metabolism of estragole (see Results section). Furthermore, a separate compartment for fat tissue was included in order to take into account the relatively higher partition coefficient of estragole in fat tissue. All other tissues were lumped into a rapidly perfused tissue group, comprising tissues such as adrenals, brain, and heart and a slowly perfused tissue group, comprising tissues such as muscle, and skin (Ramsey and Andersen, 1984).

The uptake of estragole from the gastrointestinal tract was described by a first-order process, assuming direct entry from the intestine to the liver compartment. The absorption rate constant (Ka) was 1.0 h^{-1}, resulting in a rapid absorption of estragole from the gastrointestinal tract with an absorption half-life of 0.7 h (Anthony *et al.*, 1987). Based on *in vitro* conversion data (see Results

section), conversion of estragole to 4-allylphenol and 1'-hydroxyestragole was described in the liver, lung, and kidney compartment, whereas conversion of estragole to estragole-2',3'-oxide and 3'-hydroxyanethole was described in the liver compartment only. Mass balance equations for estragole in metabolizing tissues are as follows:

Liver:
$$dAL_E/dt = dUptake_E/dt + QL*(CA_E - CL_E/PL_E) - V_{max,\,L\_AP} * CL_E/PL_E/ (K_{m,\,L\_AP} + CL_E/PL_E) - V_{max,\,L\_HE} * CL_E/PL_E/ (K_{m,\,L\_HE} + CL_E/PL_E) - V_{max,\,L\_EE} * CL_E/PL_E/ (K_{m,\,L\_EE} + CL_E/PL_E) - V_{max,\,L\_HA} * CL_E/PL_E/ (K_{m,\,L\_HA} + CL_E/PL_E)$$
$$dUptake_E/dt = - dAGI_E/dt = K_a*AGI_E,\ AGI_E\,(0) = \text{oral dose}$$
$$CL_E = AL_E/VL$$

Kidney:
$$dAK_E/dt = QK*(CA_E - CK_E/PK_E) - V_{max,\,K\_AP} * CK_E/PK_E/ (K_{m,\,K\_AP} + CK_E/PK_E) - V_{max,\,K\_HE} * CK_E/PK_E/ (K_{m,\,K\_HE} + CK_E/PK_E)$$
$$CK_E = AK_E/VK$$

Lung:
$$dALu_E/dt = QC*(CV_E - CLu_E/PLu_E) - V_{max,\,Lu\_AP} * CLu_E/PLu_E/ (K_{m,\,Lu\_AP} + CLu_E/PLu_E) - V_{max,\,Lu\_HE} * CLu_E/PLu_E/ (K_{m,\,Lu\_HE} + CLu_E/PLu_E)$$
$$CLu_E = ALu_E/VLu$$

where $Uptake_E$ is the amount of estragole taken up from the gastrointestinal tract (µmol), AGI_E (µmol) is the amount of estragole remaining in the gastrointestinal tract, ATi_E is the amount of estragole in a tissue (Ti=L (liver), K (kidney) or Lu (lung)) (µmol), CTi_E is the estragole concentration in a tissue (µmol L^{-1}), CA_E is the estragole concentration in the arterial blood (µmol L^{-1}), CV_E is the estragole concentration in the venous blood (µmol L^{-1}), QTi is the blood flow rate to a tissue (L h^{-1}), QC is the cardiac output (L h^{-1}),

VTi is the volume of a tissue, PTi_E is the tissue/blood partition coefficient of estragole, and V_{max,Ti_M} and $K_{m,\ Ti\_M}$ are the maximum rate of formation and the Michaelis–Menten constant for the formation of the different phase I metabolites: 4-allylphenol (AP), 1'-hydroxyestragole (HE), estragole-2',3'-oxide (EE), and 3'-hydroxyanethole (HA) in a tissue.

In case of 4-allylphenol, estragole-2',3'-oxide, and 3'-hydroxanethole only formation of these metabolites is taken into account and no further reactions with these metabolites were modeled. Formation of 1'-hydroxyestragole in the liver, but not in the lung and kidney was followed up by phase II reactions, since 1'-hydroxylation of estragole was found to occur predominantly in the liver (see Results section). Furthermore, distribution of these metabolites in the body was not taken into account. The quantities of the phase II metabolites that were estimated to be formed in the liver were also used as an estimate of the total formation of these metabolites. To describe the disposition of 1'-hydroxyestragole and its phase II metabolites a simplified structure was applied to the model. Thereby, a near quantitative intrahepatic conversion of 1'-hydroxyestragole by phase II enzymes was assumed. This assumption was based on the findings that the overall catalytic efficiency for the phase II reaction was higher than the catalytic efficiency for the formation of 1'-hydroxyestragole. For this reason only intraorgan distribution of 1'-hydroxyestragole was modeled and no distribution of this metabolite over the body was taken into account. This simplification of the model reduced the number of parameters considerably, but might lead to an overestimation of the rates of the phase II reactions (Filser *et al.*, 2001). The mass balance equation for 1'-hydroxyestragole in the liver is as follows:

$$\begin{aligned} \text{Liver}: dAL_{HE}/dt = &+ V_{max,\ L\_HE} * CL_E/PL_E/(K_{m,\ L\_HE} + CL_E/PL_E) \\ &- V_{max,\ L\_HEG} * CL_{HE}/PL_{HE}/(K_{m,\ L\_HEG} + CL_{HE}/PL_{HE}) \\ &- V_{max,\ L\_HES} * CL_{HE}/PL_{HE}/(K_{m,\ L\_HES} + CL_{HE}/PL_{HE}) \\ CL_{HE} = &\ AL_{HE}/VL \end{aligned}$$

where AL_{HE} is the amount of 1'-hydroxyestragole in the liver (µmol), CL_{HE}

is the 1'-hydroxyestragole concentration in the liver (μmol L^{-1}), PL_{HE} is the liver/blood partition coefficient of 1'-hydroxestragole, and V_{max,L_M} and K_{m,L_M} are the maximum rate of formation and the Michaelis–Menten constant for the formation of the phase II metabolites: 1'-hydroxyestragole glucuronide (HEG) and 1'-sulfooxyestragole (HES) in the liver.

The kinetic constants for the formation of phase I metabolites and for glucuronidation of 1'-hydroxyestragole were determined *in vitro* in the present study, whereas the kinetic constants for sulfonation of 1'-hydroxyestragole were obtained from Punt *et al*. (2007). The V_{max} values for the different phase I metabolic pathways in the liver, expressed as nmol min^{-1} (mg microsomal protein)$^{-1}$, were scaled to the liver using a microsomal protein yield of 35 mg per g liver (Medinsky *et al*., 1994). The V_{max} values for the different phase I metabolic pathways in the lung and kidney were scaled accordingly using a microsomal protein yield of 20 mg per g lung, and 7 mg per g kidney (Medinsky *et al*., 1994; Beierschmitt and Weiner, 1986). The V_{max} values for sulfonation and glucuronidation of 1'-hydroxyestragole, expressed as nmol min^{-1} (mg S9 protein)$^{-1}$, were scaled to the liver using a S9 protein yield of 143 mg per g liver, which corresponds to the sum of the literature reported cytosolic protein yield of 108 mg per g liver and the micrososomal protein yield of 35 mg per g liver (Medinsky *et al*., 1994). Furthermore, this scaling factor was verified by comparing the rate of glucuronidation of 1'-hydroxyestragole by male rat liver S9 to the rate of glucuronidation by male rat liver microsomes at a substrate concentration of 2000 μM (representing saturating substrate conditions), 10 mM UDPGA as cofactor, and 0.2 mg per mL S9 or microsomal protein, which were pre-treated with 0.025 mg per mL alamethicin. The incubations were performed and analyzed by HPLC as described above. The rate of glucuronidation of 1'-hydroxyestragole was found to be about 4 times lower in the incubations with male rat liver S9 than with male rat liver microsomes (data not shown). This indicates that the scaling factor from liver S9 to the liver needs to be 4 times higher than the scaling factor from liver microsomes to the liver.

Table 1. Parameters used in the physiologically based biokinetic model for estragole in rat.

Physiological parameters[a]		Tissue: blood partition coefficients[b]	
Body weight (kg)	0.25		
Percentage of body weight		Estragole	
Liver	3.4	Liver	2.4
Lung	0.5	Lung	2.4
Kidney	0.7	Kidney	2.4
Fat	7.0	Fat	76.9
Rapidly perfused	4.4	Rapidly perfused	2.4
Slowly perfused	67.6	Slowly perfused	0.8
Arterial blood	1.85	1' -Hydroxyestragole	
Venous blood	5.55	Liver	1.1
Cardiac output (L/ hr/kg $bw^{0.74}$)	15.0		
Percentage of cardiac output			
Liver	25.0		
Kidney	14.1		
Fat	7.0		
Rapidly perfused	36.9		
Slowly perfused	17.0		

[a] Brown *et al*. (1997)

[b] DeJongh *et al*.(1997)

The physiological parameters used in the estragole model were obtained from Brown *et al*. (1997). Partition coefficients were estimated from the octanol-water partition coefficient based on a method of DeJongh *et al*. (1997) (Table 1). Octanol-water partition coefficients were estimated with the software package ClogP version 4.0 (Biobyte, Claremont, CA), and was 3.1 for estragole and 1.6 for 1'-hydroxyestragole. Model equations were coded and numerically integrated in Berkeley Madonna 8.0.1 (Macey and Oster, UC Berkeley, CA, USA), using the Rosenbrock's algorithm for stiff systems.

Sensitivity analysis

To identify the key parameters, to which the model output is most sensitive, a sensitivity analysis was performed. Normalized sensitivity coefficients (SC) were determined according to the following equation: $SC=(C'-C)/(P'-P)\times(P/C)$, where C is the initial value of 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; Tardif *et al*., 2002; Garg and Balthasar, 2007). Based on the literature a 5% increase in parameter values was chosen, to analyze the effect of a change in parameter on the formation of 4-allylphenol, 1'-hydroxyestragole, 1'-hydroxyestragole glucuronide, and 1'-sulfooxyestragole expressed as nmol (kg bw)$^{-1}$ (Evans and Andersen, 2000). Each parameter was analyzed individually, keeping the other parameters to their initial values.

Results

Formation of phase I metabolites

Incubations with male rat liver, lung, kidney, and small intestine microsomes in the presence of estragole as substrate and NADPH as cofactor revealed that microsomal preparations of liver, lung, and kidney were capable of metabolizing estragole (Figures 3A–C), whereas no conversion of estragole was found in incubations with male rat small intestinal microsomes (data not shown).

In the chromatograms of incubations with liver microsomes four metabolites of estragole were identified: 1'-hydroxyestragole, 3'-hydroxyanethole, 4-allylphenol and estragole-2',3'-diol (Figure 3A). 1'-Hydroxyestragole, 3'-hydroxyanethole, and 4-allylphenol were identified based on the similarities of their retention time and UV spectrum to those of the chemically synthesized reference compounds and had a retention time of 19, 21 and 39 min, respectively. 3'-Hydroxyanethole was found to be formed from estragole directly and not from 1'-hydroxyestragole, since incubations of 1'-hydroxyestragole with liver microsomes and NADPH as cofactor did not result in formation of 3'-hydroxyanethole (data not shown). Estragole-2',3'-oxide could not be identified directly in the chromatogram, because no peak corresponded to the retention time and UV spectrum of the chemically synthesized reference compound. The synthesized estragole-2',3'-oxide had a retention time of 34 min when analyzed by HPLC under the same conditions used to analyze the formation

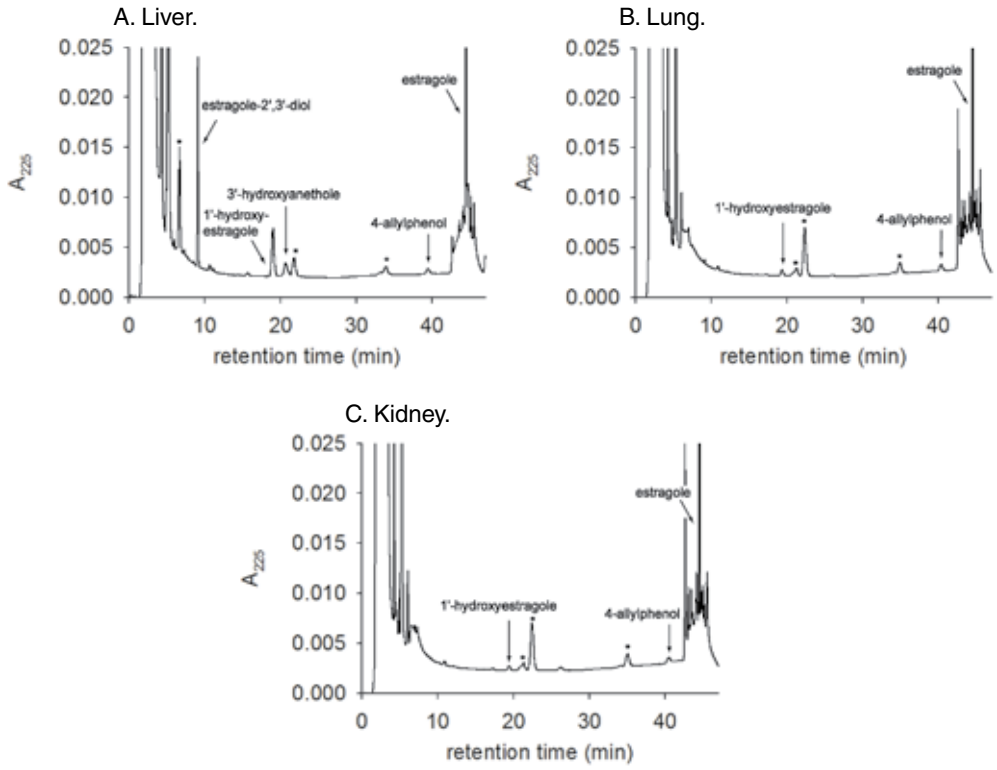

Figure 3. Chromatographic profiles of incubations with liver (A), lung (B), and kidney (C) microsomes obtained at a substrate concentration of 100 µM estragole, NADPH as cofactor, and an incubation time of 10 min for the incubation with liver microsomes, and 2 min for the incubations with lung and kidney microsomes. Peaks marked with an asterisk were also present in the blank incubations performed without cofactor NADPH, in which they had the same area under the curve as in the incubation. UV-VIS analysis revealed these peaks to be different from the phase I metabolites that were identified.

of phase I metabolites of estragole in the incubations. However, incubating estragole-2',3'-oxide with male rat liver microsomes in the absence of any cofactor revealed that in these incubations estragole-2',3'-oxide is completely hydrolyzed to estragole-2',3'-diol within the timeframe of the incubation. Hydrolysis is not observed in blank incubations without microsomes, which indicates that the conversion is most likely catalyzed by epoxide hydrolases present in the microsomal preparation (Luo *et al.*, 1992; Luo and Guenthner, 1996; Guenthner and Luo, 2001). Estragole-2',3'-diol had a retention time of 9 min and quantification of this metabolite was achieved by comparison of the peak area of estragole-2',3'-diol in the chromatograms to the calibration curve of estragole-2',3'-oxide. This could be done because the peak area of estragole-

2',3'-diol after hydrolyzing estragole-2',3'-oxide with liver microsomes was equal to the peak area of estragole-2',3'-oxide in the blank incubation without microsomes.

In the chromatograms of incubations with male rat lung and kidney microsomes two metabolites of estragole were identified, corresponding to 4-allylphenol and 1'-hydroxyestragole (Figures 3B, and C). Formation of estragole-2',3'-oxide or its corresponding estragole-2',3'-diol was not observed in these incubations. A peak corresponding to 3'-hydroxyanethole was observed in the incubations with both lung and kidney microsomes. However, because the area of this peak was equal to the area of the same peak in the blank incubation without NADPH, it was concluded that 3'-hydroxyanethole is not formed in incubations with both lung and kidney microsomes.

Kinetics of phase I metabolism

Male rat liver, lung, and kidney microsomes were incubated with increasing estragole concentrations to determine the enzyme kinetics for the formation of the various phase I metabolites of estragole. Figure 4A shows the rate of formation of estragole-2',3'-oxide, 1'-hydroxyestragole, 3'-hydroxyanethole, and 4-allylphenol in incubations with male rat liver microsomes with increasing estragole concentrations. Table 2 summarizes the apparent K_m and V_{max} values obtained from these plots. The results obtained with male rat liver microsomes reveal that estragole-2',3'-oxide is the most abundant metabolite formed, followed by 1'-hydroxyestragole and 3'-hydroxyanethole. *O*-demethylation was shown to be the least important metabolic route in these incubations and no shift in metabolism from *O*-demethylation at low concentrations to 1'-hydroxylation at higher concentrations is observed. The apparent V_{max} for *O*-demethylation was 1.7-fold lower than the apparent V_{max} for formation of 1'-hydroxyestragole, whereas the apparent K_m was 3.9-fold higher. Overall, the catalytic efficiency for the formation of the *O*-demethylated metabolite 4-allylphenol by male rat liver microsomes, calculated as the apparent V_{max}/K_m, was 7-fold lower than the catalytic efficiency for the formation of 1'-hydroxyestragole.

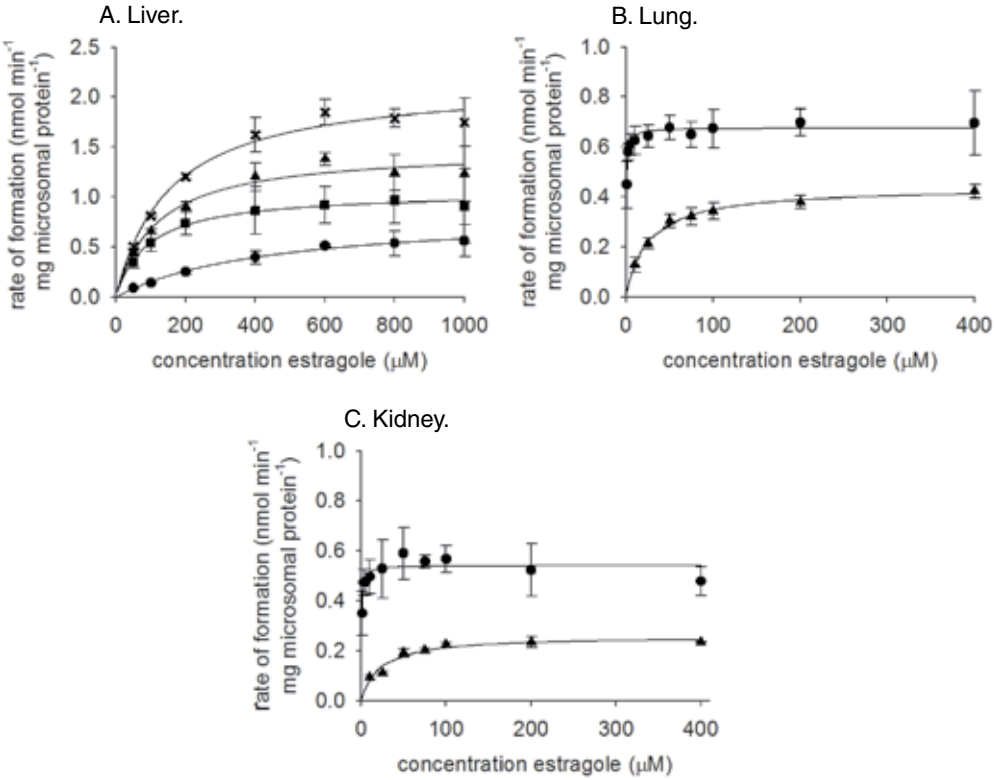

Figure 4. Estragole concentration dependent rate of formation of phase I metabolites by liver (A), lung (B), and kidney (C) microsomal preparations. In the plots each point represents the mean (± SD) of three replicates corresponding to the formation of respectively estragole-2' ,3' -oxide (x), 1' -hydroxyestragole (▲), 3' -hydroxyanethole (■) and 4-allylphenol (●). In incubations with lung and kidney microsomes formation of estragole-2' ,3' -oxide or 3' -hydroxyanethole was not observed.

The results obtained with male rat lung and kidney microsomes reveal that 4-allylphenol, the metabolite resulting from *O*-demethylation, is the most abundant metabolite formed in both the lung and kidney (Figures 4B and C). 4-Allylphenol was formed in microsomal preparations of these organs with a high affinity, reflected by a low apparent K_m of 0.5 μM for lung and 0.5 μM for kidney microsomes. The apparent V_{max} for *O*-demethylation was 1.5-fold higher than for 1'-hydroxylation in incubations with microsomal preparations of lungs and 2.0-fold higher in incubations with microsomal preparations of kidney. The apparent K_m for *O*-demethylation was 50-fold lower than for 1'-hydroxylation in incubations with microsomal preparations of lungs and 44-fold lower in incubations with microsomal preparations of kidney. Overall,

Table 2. Kinetic parameters for phase I metabolism of estragole

Organ	Metabolite	$K_{m(app)}$ [a,b]	$V_{max\ (app)}$ [a,c]	*In vitro* catalytic efficiency ($V_{max\ (app)}$/ $K_{m(app)}$)[d]	Scaled $V_{max,\ in\ vivo}$ [e]	*In vivo* catalytic efficiency scaled $V_{max,\ in\ vivo}$/ $K_{m(app)}$)[i]
Liver	4-allylphenol	458 ± 65	0.85 ± 0.05	1.9	15[f]	0.03
	1' -hydroxyestragole	116 ± 25	1.48 ± 0.08	13	26[f]	0.2
	3' -hydroxyanethole	93 ± 10	1.05 ± 0.03	11	19[f]	0.2
	estragole-2' ,3' -oxide	154 ± 25	2.16 ± 0.10	14	39[f]	0.3
Lung	4-allylphenol	0.5 ± 0.1	0.68 ± 0.01	1360	1.0[g]	2.0
	1' -hydroxyestragole	25 ± 2.0	0.44 ± 0.01	18	0.6[g]	0.03
Kidney	4-allylphenol	0.5 ± 0.1	0.54 ± 0.01	1080	0.4[h]	0.8
	1' -hydroxyestragole	22 ± 5.0	0.26 ± 0.01	12	0.2[h]	0.01

[a] mean ± SD
[b] µM
[c] nmol min^{-1} (mg microsomal protein)$^{-1}$
[d] µL min^{-1} (mg microsomal rotein)$^{-1}$
[e] µmol hr^{-1}
[f] $V_{max\ (app)}$ /(1000 nmol/ µmol) * (60 min/hr) * (35 mg microsomal protein/ g liver) * (34 g liver/kg bw)* (0.25 kg bw)
[g] $V_{max\ (app)}$/(1000 nmol/ µmol) * (60 min/hr) * (20 mg microsomal protein/ g lung) * (5 g lung/kg bw)* (0.25 kg bw)
[h] $V_{max\ (app)}$ /(1000 nmol/ µmol) * (60 min/hr) * (7 mg microsomal protein/ g kidney) * (7 g kidney/kg bw)* (0.25 kg bw)
[f] L hr^{-1}

the catalytic efficiency for the formation of 4-allylphenol in lungs and kidney was respectively 76 and 90-fold higher than the catalytic efficiency for 1'-hydroxylation in these organs.

Comparing the catalytic efficiencies for the formation of phase I metabolites in the different organs that were scaled to the *in vivo* situation reveals that in male rat conversion of estragole to 4-allylphenol is most efficiently catalyzed in the lung and kidney. Formation of 1'-hydroxyestragole, estragole-2',3'-oxide, and 3'-hydroxyanethole on the other hand are most efficiently catalyzed in the liver.

Formation of phase II metabolites: 1'-hydroxyestragole glucuronide

Figure 5 shows a chromatogram of an incubation with male rat liver S9 performed with 1'-hydroxyestragole as substrate and UDPGA as cofactor, which revealed the formation of a metabolite with a retention time of 21 min. Treatment of samples with β-glucuronidase resulted in complete elimination of this metabolite, revealing that the metabolite corresponds to the glucuronosyl conjugate of 1'-hydroxyestragole. Furthermore, the spectrum of the metabolite, obtained by LC-MS, reveals a deprotonated molecule at m/z 339, which corresponds to the theoretically expected mass and confirms that

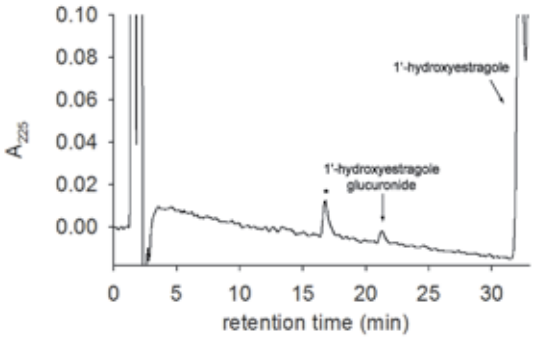

Figure 5. Chromatographic profile of an incubation with male rat liver S9 at a substrate concentration of 500 µM 1' -hydroxyestragole and UDPGA as cofactor. The peak marked with an asterisk was also present in the blank incubation performed without cofactor.

the metabolite corresponds to 1'-hydroxyestragole glucuronide.

Kinetics of phase II metabolism: glucuronidation of 1'-hydroxyestragole

Figure 6 shows the rate of formation of 1'-hydroxyestragole glucuronide with increasing 1'-hydroxyestragole concentration. The kinetic constants were obtained from the graph by fitting the data to the standard Michaelis–Menten equation, resulting in an apparent K_m of 137±27 μM and an apparent V_{max} of 7.0±0.32 nmol min^{-1} (mg S9 protein)$^{-1}$. The catalytic efficiency for glucuronidation of 1'-hydroxyestragole (V_{max}/K_m) is 51 μL min^{-1} (mg S9 protein)$^{-1}$. Liver S9 homogenates contain uridine diphosphate glucuronosyltransferases in the microsomal fraction but also contain sulfotransferase enzymes in the cytosolic fraction. Using liver S9 homogenates to determine the kinetic constants for glucuronidation of 1'-hydroxyestragole therefore allows comparison of the kinetic constants obtained to those for sulfonation of 1'-hydroxyestragole that were previously determined by Punt *et al.* (2007) with the same liver S9 homogenates. Comparing the kinetic constants for glucuronidation to those for sulfonation of 1'-hydroxyestragole reveals that the K_m for glucuronidation is about the same as the K_m for sulfonation, which was determined to be 63 μM (Punt *et al.*, 2007). Sulfotransferase enzymes

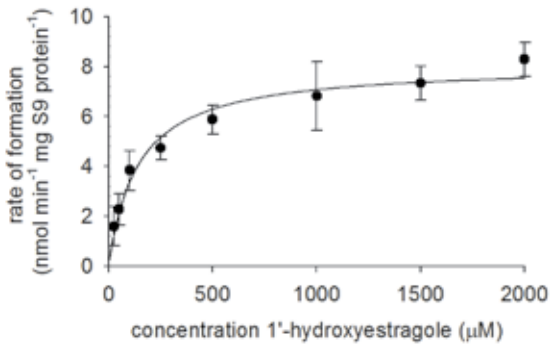

Figure 6. 1' -Hydroxyestragole dependent rate of formation of 1' -hydroxyestragole glucuronide by male rat liver S9 homogenate.

in the liver therefore have about the same affinity for 1'-hydroxyestragole as uridine diphosphate glucuronosyltransferases in this organ. For this reason, no shift can occur from sulfonation at low concentrations to glucuronidation at higher concentrations. The V_{max} for glucuronidation is 368-fold higher than for sulfonation of 1'-hydroxyestragole, which was determined to be 0.019 nmol min^{-1} (mg S9 protein)$^{-1}$ (Punt *et al*., 2007). However, it should be pointed out that the V_{max} for sulfonation is likely underestimated, since formation of the transient 1'-sulfooxyestragole was measured indirectly by trapping with 2'-deoxyguanosine and only the major adduct of the three observed adducts with 2'-deoxyguanosine was quantified (Punt *et al*., 2007).

Performance of the PBBK model

To evaluate the performance of the PBBK model developed, the predicted extent of glucuronidation of 1'-hydroxyestragole and *O*-demethylation were compared to the reported levels of these metabolic routes in female Wistar rats exposed to different doses of estragole (Anthony *et al*., 1987). With the constructed PBBK model it was found that the liver is the organ that predominantly contributes to the formation of 1'-hydroxyestragole, since over the whole dose range of 0.05–1000 mg/kg bw about 95–97% of the total formation of 1'-hydroxyestragole occurred in the liver. For this reason only glucuronidation of 1'-hydroxyestragole in the liver was taken into account to contribute to the total formation of 1'-hydroxyestragole glucuronide. The amount of 1'-hydroxyestragole glucuronide formed in the liver was assumed to equal the amount excreted in urine. The PBBK model predicted the levels of 1'-hydroxyestragole glucuronide more accurately at high doses than at lower doses (Figure 7A). For example at a dose of 1000 mg/kg bw the predicted excretion of 1'-hydroxyestragole glucuronide was 1.8-fold higher than the reported level of this metabolite, whereas at a low dose of 0.05 mg/kg bw the predicted excretion of 1'-hydroxyestragole glucuronide was about 11-fold higher than the reported urinary level of this metabolite. *O*-demethylation, on the other hand, was predicted especially well at the low dose range where there was no difference between predicted and reported extent of *O*-demethylation

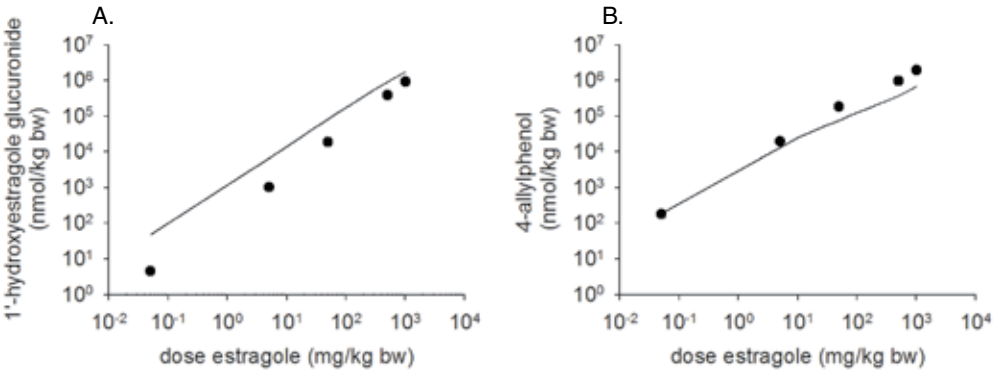

Figure 7. PBBK model-predicted formation of 1' -hydroxyestragole glucuronide (A) and 4-allylphenol (B) at different oral doses of estragole after 24 h, compared to the reported levels of these metabolic routes by Anthony *et al*., (1987). Reported urinary excretion of the glucuronide conjugate of 1' -hydroxyestragole was assumed to correspond to the PBBK model-predicted formation of 1' -hydroxyestragole glucuronide in the liver, whereas the reported exhalation of $^{14}CO_2$ was assumed to correspond to the formation of 4-allylphenol in liver, lung and kidney. The lines represent the model estimation, whereas the points represent the data obtained from Anthony *et al*. (1987).

(Figure 7B). At the high dose range *O*-demethylation was predicted to be 2.7-fold lower than the reported level of this metabolic route.

Due to the high affinity, with which estragole is converted to 4-allylphenol in the lung and kidney, formation of 4-allylphenol in these organs was estimated to reach saturation at 300 mg/kg bw estragole. Saturation of this metabolic route can also be observed in Figure 7B, in which the *O*-demethylation becomes relatively less profound between 10 and 300 mg/kg bw. However, at higher doses the extent of *O*-demethylation increases again due to a relative increase in the contribution of the liver to the total extent of *O*-demethylation. At a dose of 0.05 mg/kg bw estragole, the lung contributes with 80% to the total formation of 4-allylphenol, whereas the kidney contributes with 16%, and the liver with 4%. At a dose of 1000 mg/kg bw estragole, the lung contributes with 14% to the total formation of 4-allylphenol, whereas the kidney with 5%, and the liver with 81%.

Sensitivity analysis

A sensitivity analysis was performed to identify the key parameters

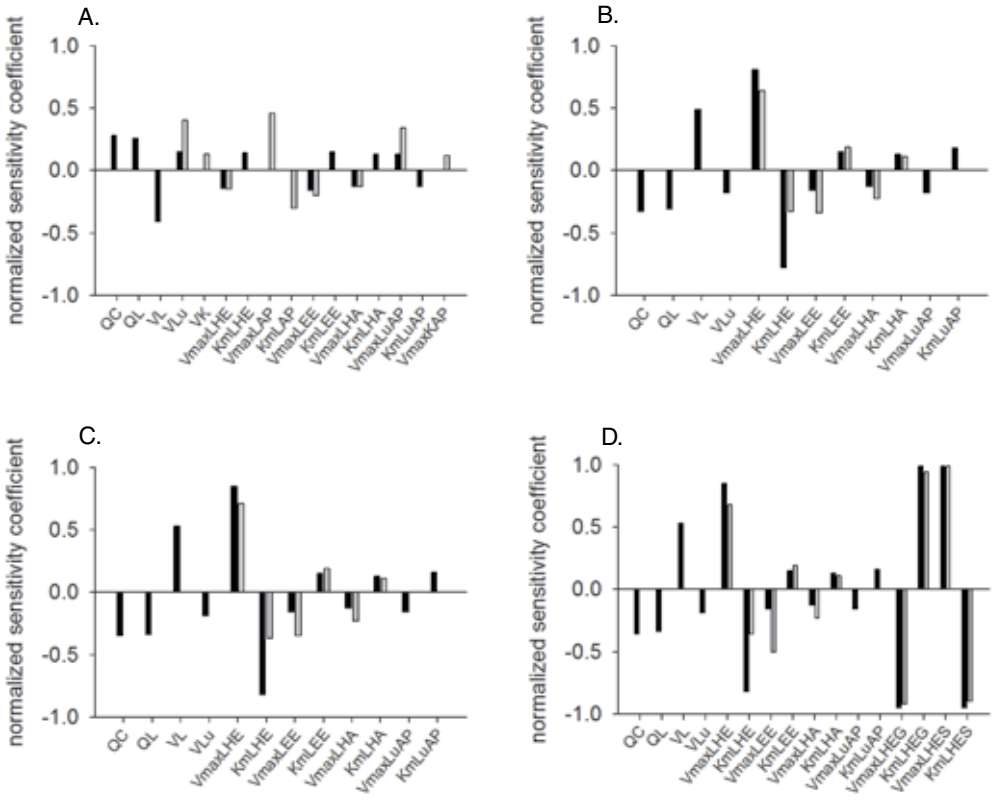

Figure 8. Sensitivity of the predicted formation of 4-allylphenol (A), 1' -hydroxyestragole (B), 1' -hydroxyestragole glucuronide (C), and 1' -sulfooxyestragole (D) to different model parameters. Black bars correspond to the normalized sensitivity coefficients at a dose of 0.05 mg/kg bw and gray bars at a dose of 300 mg/kg bw. QC = cardiac output, QL = blood flow through liver, VTi = volume of a tissue (Ti = L (liver), K (kidney) or Lu (lung)), Vmax,Ti_M and Km, Ti_M are the maximum rate of formation and the Michaelis–Menten constant for the formation of the different metabolites: 4-allylphenol (AP), 1' -hydroxyestragole (HE), estragole-2' ,3' -oxide (EE), 3' -hydroxyanethole (HA), 1' -hydroxyestragole glucuronide (HEG), and 1' -sulfooxyestragole (HES) in a tissue.

that influence the formation of 4-allylphenol, 1'-hydroxyestragole, 1'-hydroxyestragole glucuronide, and 1'-sulfooxyestragole (after 24 h) as model outputs. Normalized sensitivity coefficients were calculated for all parameters at doses of 0.05 and 300 mg/kg bw estragole, but only parameters that had a normalized sensitivity coefficient higher than 0.1 are displayed in Figure 8. Figure 8A reveals the parameters that influence formation of 4-allylphenol. Formation of this metabolite can occur in both the lung and kidney with high efficiency, however, only estragole that is not metabolized in the liver can

reach these tissues. For this reason changes in parameters, which influence the metabolism in the liver, will influence the extent of *O*-demethylation in the lung and kidney. Examples of these parameters are the kinetic constants for formation of the major metabolites in the liver and the liver volume. The extent of *O*-demethylation of estragole is also influenced by cardiac output and the blood flow through the liver, which both influence the transport of estragole from the liver over the body. At a dose of 0.05 mg/kg bw of estragole, *O*-demethylation mainly occurs in the lung, and therefore the kinetic constants for this metabolic route in the lung also influence the extent of *O*-demethylation. At a dose of 300 mg/kg bw *O*-demethylation reaches saturation in the lung and kidney, and the formation of this metabolite in the liver will increase. For this reason parameters that influence the kinetic constants for *O*-demethylation in the liver affect the extent of *O*-demethylation at this oral dose, but also parameters, which influence the capacity of *O*-demethylation in the lung and kidney.

Figure 8B reveals the parameters that influence formation of 1'-hydroxyestragole. Both at a dose of 0.05 and 300 mg/kg bw formation of 1'-hydroxyestragole mainly depends on the kinetic constants for formation of this metabolite in the liver. To a smaller extent formation of 1'-hydroxyestragole can, however, also be influenced by the kinetic constants for competing metabolic conversions that occur in the liver and at a dose of 0.05 mg/kg bw by the parameters that influence the extent of *O*-demethylation of estragole in the lung.

Figures 8C and D reveal that the parameters that influence the formation of 1'-hydroxyestragole also influence the formation of both 1'-hydroxyestragole glucuronide and 1'-sulfooxyestragole, which indicates that 1'-hydroxylation of estragole is the rate limiting step in formation of these metabolites. Formation of 1'-sulfooxyestragole also strongly depends on the kinetic constants for glucuronidation of 1'-hydroxyestragole and the kinetic constants for formation of this metabolite itself. This indicates that glucuronidation of 1'-hydroxyestragole is an important competing metabolic pathway to sulfonation due to its higher catalytic efficiency.

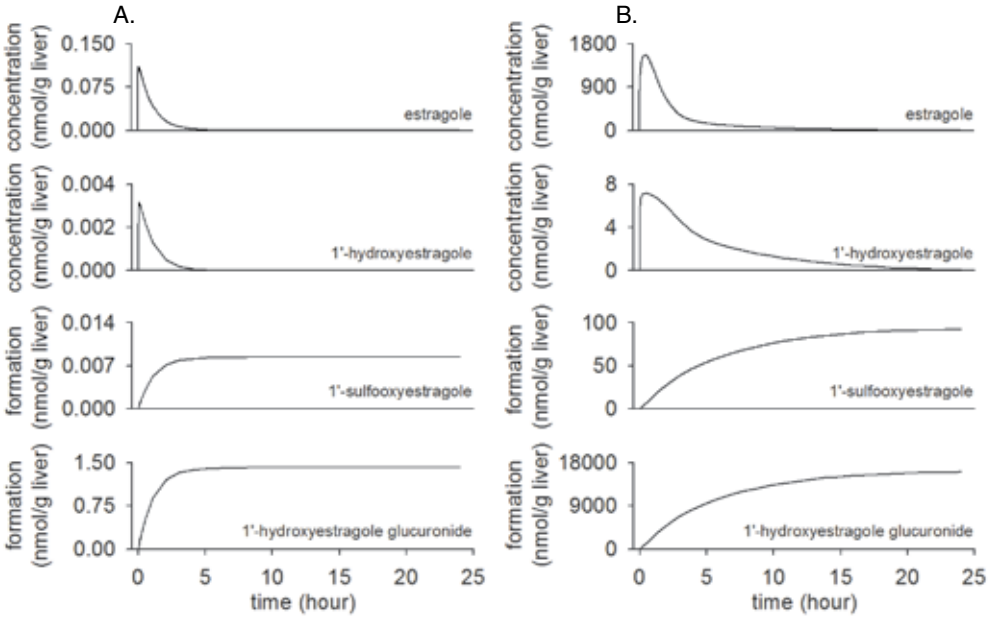

Figure 9. Time dependent PBBK model-predicted concentrations of estragole and 1' -hydroxyestragole, and formation of 1'-sulfooxyestragole and 1' -hydroxyestragole glucuronide in male rat liver following exposure to 0.05 mg/kg bw estragole (A) and 300 mg/kg bw

Model predictions

Figure 9A shows the time-dependent predicted liver concentrations of estragole and 1'-hydroxyestragole, and the formation of 1'-sulfooxyestragole and 1'-hydroxyestragole glucuronide in male rat following exposure to 0.05 mg/kg bw estragole. After 5 h, estragole and 1'-hydroxyestragole are completely metabolized and a maximum formation of 0.01 nmol (g liver)$^{-1}$ of 1'-sulfooxyestragole and 1.4 nmol (g liver)$^{-1}$ of 1'-hydroxyestragole glucuronide are reached, corresponding to 0.08% and 14% of the dose respectively. The formation of 1'-sulfooxyestragole and 1'-hydroxyestragole glucuronide are accumulating, because no further reactions with these metabolites are included in the model to allow estimation of the maximum possible formation of these metabolites in the liver. For this reason the estimated formation of 1'-sulfooxyestragole and 1'-hydroxyestragole glucuronide do not represent the concentrations of these metabolites that would occur in liver, but the total quantities that would theoretically be formed in the liver over time. Figure

9B shows the time-dependent PBBK model-predicted liver concentrations of estragole, 1'-hydroxyestragole, and formation of 1'-sulfooxyestragole and 1'-hydroxyestragole glucuronide in male rat following exposure to 300 mg/kg bw estragole. This dose corresponds to the dose, at which *O*-demethylation in the lung and kidney was found to reach saturation. At this oral dose the total conversion of estragole and 1'-hydroxyestragole was predicted to take longer than at a low dose of 0.05 mg/kg bw, with estragole and 1'-hydroxyestragole being almost completely metabolized after 15 h. This indicates that in the dose range of 0.05–300 mg/kg bw of estragole, both estragole and 1'-hydroxyestragole are readily metabolized within the 24 h time frame. It is, therefore, not expected that accumulation of estragole or its reactive metabolites will occur if a repeated dosing would by applied after 24 h. At a dose of 300 mg/kg bw the percentage of the dose that is ultimately converted into 1'-sulfooxyestragole and 1'-hydroxyestragole glucuronide is relatively higher. At this dose 93 nmol (g liver)$^{-1}$ of 1'-sulfooxyestragole is formed, corresponding to 0.16% of the dose. In the case of 1'-hydroxyestragole glucuronide 16128 nmol (g liver)$^{-1}$ is formed, which corresponds 27% of the dose.

The observed relative increase in percentage of the dose that is ultimately converted into 1'-sulfooxyestragole and 1'-hydroxyestragole glucuronide can be explained by a change in phase I metabolism of estragole. In Figure 10A the percentage of estragole metabolized to the different phase I metabolites is plotted at different oral doses of estragole. At low doses *O*-demethylation is the major metabolic route. A relative decrease in formation of 4-allylphenol is observed from 56% of the dose at a dose of 0.05 mg/kg bw to approximately 14% of the dose at a dose of 300 mg/kg bw. This relative decrease in formation is a result of saturation of the *O*-demethylation pathways in the lung and the kidney. Along with the relative decrease in *O*-demethylation a relative increase in formation of the other phase I metabolites occurs, which are mainly formed in the liver. Of these metabolites the formation of 1'-hydroxyestragole increased from 15% of the dose at a dose of 0.05 mg/kg bw to 29% of the dose at a dose of 300 mg/kg bw. Due to the relative increase in the formation of 1'-hydroxyestragole with increasing dose of estragole, a relative increase

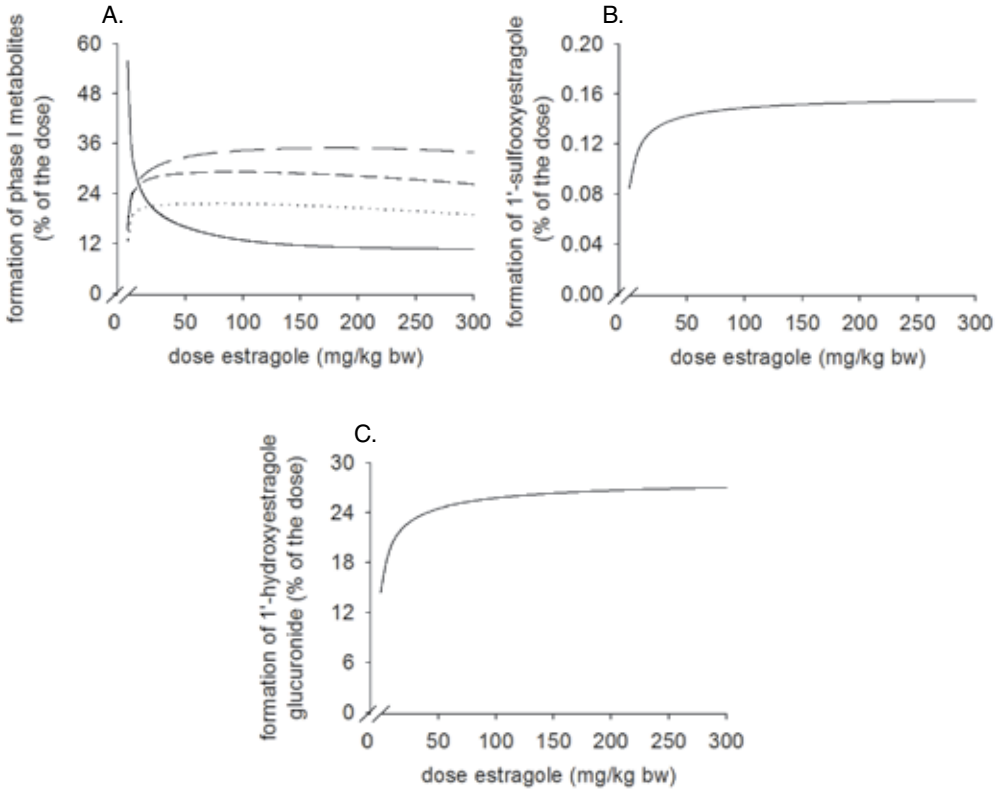

Figure 10. PBBK model-predicted dose-dependent changes in overall formation of phase I metabolites in all organs (A) and dose-dependent changes in formation of 1' -sulfooxyestragole (B), and 1' -hydroxyestragole glucuronide (C) in the liver. The lines in Figure 10A correspond to 4-allylphenol (-), estragole-2' ,3' -oxide (– –), 1' -hydroxyestragole (-----), and 3' -hydroxyanethole (·····).

in formation of phase II metabolites of 1'-hydroxyestragole also occurs. This can be seen Figures 10B, and C, in which the percentage of the dose that is ultimately converted into 1'-sulfooxyestragole and 1'-hydroxyestragole glucuronide is plotted at different oral doses.

Effect of strain and gender differences in kinetics and scaling of *in vitro* metabolic parameters on the model outcome was examined. The present model was developed based on *in vitro* metabolic parameters obtained from male Sprague Dawley rats. Because the performance of the model was evaluated against literature data obtained from female Wistar rats, the possible effect of the strain and gender differences on the model outcome was examined. Based on the sensitivity analysis it can be concluded that metabolism in the liver is

Table 3. Kinetic parameters for phase I metabolism of estragole by liver microsomes of rats of different strain and gender.

Metabolite		$K_{m(app)}$ [a]	$V_{max\ (app)}$ [b]	catalytic efficiency ($V_{max\ (app)}/K_{m(app)}$)[c]
4-allylphenol	Male SD	458 ± 65	0.85 ± 0.05	1.9
	Female SD	260 ± 85	0.34 ± 0.04	1.3
	Male Wistar	364 ± 26	0.25 ± 0.01	0.7
	Female Wistar	277 ± 61	0.34 ± 0.03	1.2
1' -hydroxyestragole	Male SD	116 ± 25	1.48 ± 0.08	13
	Female SD	65 ± 6.9	0.77 ± 0.02	12
	Male Wistar	80 ± 20	0.51 ± 0.03	6.4
	Female Wistar	79 ± 18	0.67 ± 0.03	8.5
3' -hydroxyanethole	Male SD	93 ± 10	1.05 ± 0.03	11
	Female SD	113 ± 43	0.95 ± 0.09	8.4
	Male Wistar	210 ± 25	1.13 ± 0.04	5.4
	Female Wistar	142 ± 34	0.83 ± 0.05	5.8
estragole-2' ,3' -oxide	Male SD	154 ± 25	2.16 ± 0.10	14
	Female SD	1406 ± 300	0.90 ± 0.13	0.6
	Male Wistar	315 ± 76	0.76 ± 0.07	2.4
	Female Wistar	2196 ± 1091	1.4 0± 0.52	0.6

[a] μM

[b] nmol min^{-1} (mg microsomal protein)$^{-1}$

[c] μL min^{-1} (mg microsomal protein)$^{-1}$

the key factor influencing the extent of formation of 1'-hydroxyestragole and its phase II metabolites. For this reason the kinetic constants for formation of different phase I metabolites by liver microsomes from female Sprague Dawley and female and male Wistar rats were determined, in addition to those already determined for male Sprague Dawley rats, and the kinetic constants obtained are displayed in Table 3. Comparison of these kinetic constants reveal no strain and sex differences in formation of 1'-hydroxyestragole, 4-allylphenol, and 3'-hydroxyanethole. Although some variation in K_m and V_{max} values for these metabolic routes occur, the overall catalytic efficiencies are about the same for

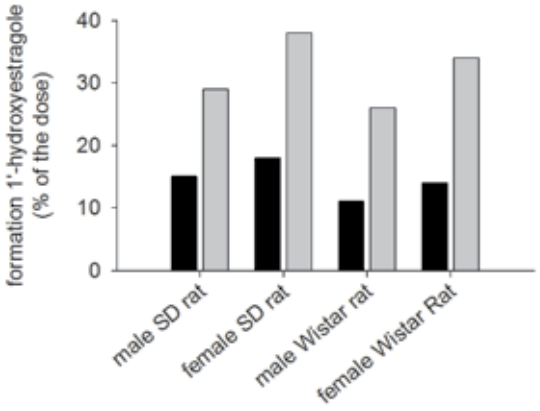

Figure 11. PBBK model-predicted relative formation of 1' -hydroxyestragole when using kinetic constants for phase I metabolism by liver microsomes of male or female Sprague Dawley (SD) rats, or male or female Wistar rats were applied, and all other parameters were kept the same. Black bars correspond to the predicted formation of 1' -hydroxyestragole as % of the dose at a dose of 0.05 mg/kg bw and gray bars at a dose of 300 mg/kg bw.

the different strains and sexes. Strain and sex dependent differences do occur in formation of estragole-2',3'-oxide. Comparison of the kinetic constants for formation of this metabolite reveals that epoxidation is less efficient in female rats compared to male rats. In addition, this metabolic route was to some extent also less efficient in male Wistar rats compared to male Sprague Dawley rats. To examine the influence of these differences in metabolic constants on the outcome of the PBBK model, the effect of applying these kinetic constants in the model on the PBBK model-predicted formation of 1'-hydroxyestragole was determined. Figure 11 presents the PBBK model-predicted formation of 1'-hydroxyestragole as a percentage of the dose when kinetic constants for phase I metabolism by liver microsomes of male or female Sprague Dawley, or male or female Wistar rats were applied, and all other parameters were kept the same. The lower observed catalytic efficiency for epoxidation of estragole in female rats mainly influences the extent of 1'-hydroxylation at a dose of 300 mg/kg bw, which was observed to be 1.2-fold higher than in male Sprague Dawley rats. These results indicate that strain and sex dependent differences in phase I kinetics influence the model outcome only to a minor extent, and

they do not influence the observed non-linear increase in formation of 1'-hydroxyestragole and its phase II metabolites.

In addition to strain and gender dependent differences in kinetics, scaling of *in vitro* metabolic parameters to the *in vivo* situation can influence the model outcome. The microsomal protein yield from different tissues used to scale the V_{max} values to the *in vivo* situation were based on values taken from the literature (Medinsky *et al*., 1994; Beierschmitt and Weiner, 1986). Up to 4-fold differences in scaling factors have been used throughout different PBBK models to scale up *in vitro* V_{max} values (Reitz *et al*., 1996; Quick and Shuler, 1999; Bogaards *et al*., 2001). These differences in scaling factors are due to differences in the method of preparation of microsomes from a tissue, as well as the strain and sex of the rat. Since the extent of 1'-hydroxyestragole formation strongly depends on the V_{max} values for formation of this metabolite and its competing metabolic routes, the effect of a 4-fold decrease in microsomal protein yield of the liver, lung, and kidney on the formation of 1'-hydroxyestragole was examined. A fourfold decrease in liver microsomal protein per gram liver decreased the PBBK model-predicted formation of 1'-hydroxyestragole 2.3-fold at a dose of 0.05 mg/kg bw, whereas at a dose of 300 mg/kg bw a 1.3-fold decrease in formation of 1'-hydroxyestragole was observed. A 4-fold decrease in lung microsomal protein yield increased the formation of 1'-hydroxyestragole 1.2-fold at a dose of 0.05 mg/kg bw and did not influence formation of 1'-hydroxyestragole at a dose of 300 mg/kg bw estragole. In case of the kidney microsomal protein yield, changes in this parameter did not influence the predicted formation of 1'-hydroxyestragole at different oral doses. Some uncertainty also exists in the *in vitro* derived apparent K_m values, which have been used for *in vivo* apparent K_m values. No indication can be given on the level of uncertainty in these K_m values. A 4-fold increase in overall K_m values for phase I metabolism in the different organs has, however, the same effects on the estimated formation of 1'-hydroxyestragole at different oral doses as a 4-fold decrease in scaling of V_{max} values.

Discussion

To further elucidate dose-dependent differences in bioactivation and detoxification of estragole, a PBBK model for estragole in male rat was built, based on *in vitro* metabolic parameters, providing possibilities to model the metabolism of estragole in rats at different oral doses. The advantage of using *in vitro* metabolic parameters and PBBK modeling as compared to *in vivo* experiments is that this PBBK based approach gives insight, in which organs are involved in the metabolism of estragole and in the consequences of saturation of different metabolic pathways. Also the model allows prediction of the amounts of the proximate and ultimate carcinogenic metabolites, 1'-hydroxyestragole and 1'-sulfooxyestagole, formed in the target organ, namely liver.

To evaluate the performance of the newly developed PBBK model, the predicted formation of 1'-hydroxyestragole glucuronide and 4-allylphenol were compared to literature data on the levels of these metabolic routes in rats exposed to different concentrations of estragole (Anthony *et al*., 1987). Overall, the PBBK model-predicted formation of 1'-hydroxyestragole glucuronide and the predicted level of *O*-demethylation were in the same order of magnitude as reported by Anthony *et al*. (1987), except for 1'-hydroxyestragole glucuronide formation at the low dose levels. At these low dose levels the PBBK model-predicted formation of 1'-hydroxyestragole glucuronide was somewhat overestimated (the levels at 0.05 mg/kg bw amounted to 11-times that observed in urine). It has to be kept in mind that the *in vitro* data of the present study were obtained with tissue samples from male Sprague Dawley rats whereas the *in vivo* data from Anthony *et al*. (1987) were obtained with female Wistar rats. This implies in theory that part of the differences between the PBBK model-predicted levels and the experimental data may arise from strain and gender dependent differences in kinetics or scaling of *in vitro* metabolic data. However, sensitivity analysis revealed that uncertainties in both metabolic parameters and scaling of *in vitro* metabolic parameters were found to influence the model outcome to only a minor extent.

Overall it is important to stress that deviations within one order of magnitude are generally considered acceptable within the present state-of-the-art of integrating *in vitro* data and PBBK modeling (Hissink *et al*., 1997; Quick and Shuler, 1999; Lupfert and Reichel, 2005). This implies that conclusions derived from the present model should preferably not be based on absolute outcomes but rather on comparison of different conditions in a relative way as done in the present study. The PBBK model developed in the present study can be used to study dose-dependent bioactivation.

It was demonstrated that a shift from *O*-demethylation at lower doses to 1'-hydroxylation at higher doses occurs, as previously suggested on the basis of dose-dependent *in vivo* kinetic studies in rats (Anthony *et al*., 1987). *O*-demethylation of estragole is the major metabolic route at low doses of estragole, occurring mainly in the lung and kidney of male rat. In these organs 4-allylphenol is formed with high affinity, reflected by a relative low K_m. The fact that metabolism in the lung and kidney can be of importance in rat when the K_m for formation of metabolites in these organs is relatively low has also been demonstrated for other compounds such as 1,3-butadiene, vinyl chloride, trichloroethylene, and carbon tetrachloride (Evelo *et al*., 1993; Yoon *et al*., 2007). In spite of the low K_m, saturation of *O*-demethylation was shown to occur at relatively high doses of estragole (>300 mg/kg bw). Due to saturation of the *O*-demethylation pathway in the lung and kidney, formation of the proximate carcinogenic metabolite 1'-hydroxyestragole, which was shown to occur mainly in the liver of male rat, becomes relatively more important at higher doses of estragole. A relative increase in formation of 1'-hydroxyestragole in the liver at high doses of estragole results in a relative increase in formation of 1'-sulfooxyestragole and 1'-hydroxyestragole glucuronide as well, which could explain the observed relative increase in excretion of 1'-hydroxyestragole glucuronide with increasing dose, found by Anthony *et al*. (1987) in rats exposed to estragole. The ratio between formation of 1'-hydroxyestragole glucuronide and 1'-sulfooxyestragole was found to be the same at different oral doses of estragole ranging from 0.05–300 mg/kg bw. This indicates that in an *in vivo* situation the level of excretion

of 1'-hydroxyestragole glucuronide in male rat is proportional to the level of bioactivation of estragole.

By integrating *in vitro* metabolic parameters using a PBBK model as a framework the factors underlying the dose-dependent differences in estragole bioactivation could be revealed. Due to saturation of the *O*-demethylation pathway in the lung and kidney at high doses of estragole a relative increase in formation of the proximate carcinogenic metabolite 1'-hydroxyestragole in the liver occurs, leading to a relative increase in bioactivation of estragole at higher doses of estragole. This result indicates that when extrapolating the cancer risk for estragole from high dose animal experiments to low doses relevant for dietary human intake, it is important to take into account non-linear effects in biokinetics. In general PBBK models provide a tool, with which the extent of bioactivation can be estimated at different oral doses. In addition to dose-dependent differences, species differences in metabolism and metabolic activation can also occur, which could affect the cancer risk assessment of estragole. We have for instance previously demonstrated that male rats are more efficient in sulfonation of 1'-hydroxyestragole than humans (Punt *et al*., 2007). In order to determine the overall differences between male rats and humans in formation of 1'-sulfooxyestragole, a similar PBBK model is presently being constructed for humans based on the PBBK model developed for male rat. Comparison of the results predicted by such a PBBK model for the human to those predicted by the rat model of the present study can provide further insight in species differences in metabolism and metabolic activation of this alkenylbenzene.

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References

Agharahimi, M. R. and LeBel, N. A. (1995). Synthesis of (-)-monoterpenylmagnolol and magnolol. J Org Chem 60, 1856-1863.

Anthony, A., Caldwell, J., Hutt, A. J., and Smith, R. L. (1987). Metabolism of estragole in rat and mouse and influence of dose size on excretion of the proximate carcinogen 1'-hydroxyestragole. Food Chem Toxicol 25, 799-806.

Beierschmitt, W. P. and Weiner, M. (1986). Age-related changes in renal metabolism of acetaminophen in male fischer 344 rats. Age 9, 7-13.

Bogaards, J. J., Freidig, A. P., and van Bladeren, P. J. (2001). Prediction of isoprene diepoxide levels in vivo in mouse, rat and man using enzyme kinetic data in vitro and physiologically-based pharmacokinetic modelling. Chem Biol Interact 138, 247-265.

Brown, R. P., Delp, M. D., Lindstedt, S. L., Rhomberg, L. R., and Beliles, R. P. (1997). Physiological parameter values for physiologically based pharmacokinetic models. Toxicol Ind Health 13, 407-484.

DeJongh, J., Verhaar, H. J., and Hermens, J. L., (1997). A quantitative property-property relationship (QPPR) approach to estimate in vitro tissue-blood partition coefficients of organic chemicals in rats and humans. Arch Toxicol 72, 17-25.

Drinkwater, N. R., Miller, E. C., Miller, J. A., and Pitot, H. C. (1976). Hepatocarcinogenicity of estragole (1-allyl-4-methoxybenzene) and 1'-hydroxyestragole in the mouse and mutagenicity of 1'-acetoxyestragole in bacteria. J Natl Cancer Inst 57, 1323-1331.

Evans, M. V. and Andersen, M. E. (2000). Sensitivity analysis of a physiological model for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD): assessing the impact of specific model parameters on sequestration in liver and fat in the

rat. Toxicol Sci 54, 71-80.

Evelo, C. T., Oostendorp, J. G., ten Berge, W. F., and Borm, P. J. (1993). Physiologically based toxicokinetic modeling of 1,3-butadiene lung metabolism in mice becomes more important at low doses. Environ Health Perspect 101, 496-502.

Filser, J. G., Faller, T. H., Bhowmik, S., Schuster, A., Kessler, W., Putz, C. and Csanady, G. A. (2001). First-pass metabolism of 1,3-butadiene in once-through perfused livers of rats and mice. Chem Biol Interact 135-136, 249-265.

Fisher, M. B., Campanale, K., Ackermann, B. L., Van Den Branden, M., and Wrighton, S. A. (2000). In vitro glucuronidation using human liver microsomes and the pore-forming peptide alamethicin. Drug Metab Dispos 28, 560-566.

Garg, A. and Balthasar, J. P. (2007). Physiologically-based pharmacokinetic (PBPK) model to predict IgG tissue kinetics in wild-type and FcRn-knockout mice. J Pharmacokinet Pharmacodyn 34, 687-709.

Guenthner, T. M. and Luo, G. (2001). Investigation of the role of the 2',3'-epoxidation pathway in the bioactivation and genotoxicity of dietary allylbenzene analogs. Toxicology 160, 47-58.

Hissink, A. M., Van Ommen, B., Kruse, J., and Van Bladeren, P. J. (1997). A physiologically based pharmacokinetic (PB-PK) model for 1,2-dichlorobenzene linked to two possible parameters of toxicity. Toxicol Appl Pharmacol 145, 301-310.

Iyer, L. V., Ho, M. N., Shinn, W. M., Bradford, W. W., Tanga, M. J., Nath, S. S., and Green, C. E. (2003). Glucuronidation of 1'-hydroxyestragole (1'-HE) by human UDP-glucuronosyltransferases UGT2B7 and UGT1A9. Toxicol Sci 73, 36-43.

Koster, H., Halsema, I., Scholtens, E., Knippers, M., and Mulder, G. J. (1981). Dose-dependent shifts in the sulfation and glucuronidation of phenolic compounds in the rat in vivo and in isolated hepatocytes. The role of saturation of phenolsulfotransferase. Biochem Pharmacol 30, 2569-2575.

Lin, J. H. and Wong, B. K. (2002). Complexities of glucuronidation affecting in vitro in vivo extrapolation. Curr Drug Metab 3, 623-646.

Luo, G. and Guenthner, T. M. (1996). Covalent binding to DNA in vitro of 2',3'-oxides derived from allylbenzene analogs. Drug Metab Dispos 24, 1020-1027.

Luo, G., Qato, M. K., and Guenthner, T. M. (1992). Hydrolysis of the 2',3'-allylic epoxides of allylbenzene, estragole, eugenol, and safrole by both microsomal and cytosolic epoxide hydrolases. Drug Metab Dispos 20, 440-445.

Lupfert, C. and Reichel, A. (2005). Development and application of physiologically based pharmacokinetic-modeling tools to support drug discovery. Chem Biodivers 2, 1462-1486.

Medinsky, M. A., Leavens, T. L., Csanady, G. A., Gargas, M. L., and Bond, J. A. (1994). In vivo metabolism of butadiene by mice and rats: a comparison of physiological model predictions and experimental data. Carcinogenesis 15, 1329-1340.

Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A., and Miller, J. A. (1983). Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. Cancer Res 43, 1124-1134.

Paraskar, A. S. and Sudalai, A. (2006). Enantioselective synthesis of (-)-cytoxazone and (+)-epi-cytoxazone, novel cytokine modulators via Sharpless asymmetric epoxidation and -proline catalyzed Mannich reaction. Tetrahedron 62, 5756-5762.

Passreiter, C. M., Wilson, J., Andersen, R., and Isman, M. B. (2004). Metabolism of thymol and trans-anethole in larvae of Spodoptera litura and Trichoplusia ni (Lepidoptera: Noctuidae). J Agric Food Chem 52, 2549-2551.

Phillips, D. H., Miller, J. A., Miller, E. C., and Adams, B. (1981). Structures of the DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1'-hydroxyestragole. Cancer Res 41, 176-186.

Phillips, D. H., Reddy, M. V., and Randerath, K. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. II. Newborn male B6C3F1 mice. Carcinogenesis 5, 1623-1628.

Punt, A., Delatour, T., Scholz, G., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2007). Tandem mass spectrometry analysis of N^2-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine as a strategy to study species differences in sulfotransferase conversion of the proximate carcinogen 1'-hydroxyestragole. Chem Res Toxicol 20, 991-998.

Quick, D. J. and Shuler, M. L. (1999). Use of in vitro data for construction of a physiologically based pharmacokinetic model for naphthalene in rats and mice to probe species differences. Biotechnol Prog 15, 540-555.

Ramsey, J. C. and Andersen, M. E. (1984). A physiologically based description of the inhalation pharmacokinetics of styrene in rats and humans. Toxicol Appl Pharmacol 73, 159-175.

Randerath, K., Haglund, R. E., Phillips, D. H., and Reddy, M. V. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. Carcinogenesis 5, 1613-1622.

Reitz, R. H., Gargas, M. L., Mendrala, A. L., and Schumann, A. M. (1996). In vivo and in vitro studies of perchloroethylene metabolism for physiologically based pharmacokinetic modeling in rats, mice, and humans. Toxicol Appl Pharmacol 136, 289-306.

SCF (2001). Opinion of the scientific committee on food on estragole (1-allyl-4-methyxybenzene). http://ec.europa.eu/food/fs/sc/scf/out104_en.pdf.

Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., Rogers, A. E., Caldwell, J., and Sipes, I. G. (2002). Safety assessment of allylalkoxybenzene derivatives used as flavouring substances - methyl eugenol and estragole. Food Chem Toxicol 40, 851-870.

Solheim, E. and Scheline, R. (1973). Metabolism of alkenebenzene derivatives in the rat I. *p*-Methoxyallylbenzene (estragole) and

p-Methoxypropenylbenzene (Anethole). Xenobiotica 3, 493-510.

Tardif, R., Droz, P. O., Charest-Tardif, G., Pierrehumbert, G., and Truchon, G. (2002). Impact of human variability on the biological monitoring of exposure to toluene: I. Physiologically based toxicokinetic modelling. Toxicol Lett 134, 155-163.

Wiseman, R. W., Fennell, T. R., Miller, J. A., and Miller, E. C. (1985). Further characterization of the DNA adducts formed by electrophilic esters of the hepatocarcinogens 1'-hydroxysafrole and 1'-hydroxyestragole in vitro and in mouse liver in vivo, including new adducts at C-8 and N-7 of guanine residues. Cancer Res 45, 3096-3105.

Wiseman, R. W., Miller, E. C., Miller, J. A., and Liem, A. (1987). Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J x C3H/HeJ F_1 mice. Cancer Res 47, 2275-2283.

Yoon, M., Madden, M. C., and Barton, H. A. (2007). Extrahepatic metabolism by CYP2E1 in PBPK modeling of lipophilic volatile organic chemicals: impacts on metabolic parameter estimation and prediction of dose metrics. J Toxicol Environ Health A 70, 1527-1541.


1000
Rat
250

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Use of physiologically based biokinetic (PBBK) modeling to study estragole bioactivation and detoxification in humans as compared to rats

Based on: Punt A, Paini A, Boersma MG, Freidig AP, Delatour T, Scholz G, Schilter B, Van Bladeren PJ, and Rietjens IMCM
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Abstract

The extent of bioactivation of the herbal constituent estragole to its ultimate carcinogenic metabolite 1'-sulfooxyestragole depends on the relative levels of both bioactivation and detoxification pathways. The present study investigated the kinetics of the metabolic reactions of both estragole and its proximate carcinogenic metabolite 1'-hydroxyestragole in humans in incubations with relevant tissue fractions. Based on the kinetic data obtained a PBBK model for estragole in human was defined to predict the relative extent of bioactivation and detoxification at dose levels relevant for dietary human intake. The outcomes of the model were subsequently compared to those previously predicted by a PBBK model for estragole in male rat to evaluate the occurrence of species differences in metabolic activation. The results obtained reveal that formation of 1'-oxoestragole, which represents a minor metabolic route for 1'-hydroxyestragole in rat, is the main detoxification pathway of 1'-hydroxyestragole in humans. Due to a high level of this 1'-hydroxyestragole oxidation pathway in human liver, the predicted species differences in formation of 1'-sulfooxyestragole remain relatively low, with the predicted formation of 1'-sulfooxyestragole being 2-fold higher in human compared to male rat, even though the formation of its precursor 1'-hydroxyestragole was predicted to be 4–fold higher in human. Overall it is concluded that in spite of significant differences in the relative extent of different metabolic pathways between human and male rat, there is a minor influence of species differences on the ultimate overall bioactivation of estragole to 1'-sulfooxyestragole.

Introduction

Estragole is an alkenylbenzene that occurs naturally in certain herbs such as tarragon, basil and fennel, and in essential oils of these herbs used as flavoring agents (Smith *et al*., 2002). At high dose levels this compound is found to be hepatocarcinogenic in different rodents (Drinkwater *et al*., 1976; Miller *et al*., 1983; Wiseman *et al*., 1987). Uncertainties about the shape of the dose-response curve at dose levels relevant for dietary human intake and about species differences in metabolism and metabolic activation make it difficult to extrapolate the cancer risk from high dose animal experiments to the low dose human situation. In an evaluation performed by the expert panel of the Flavor and Extract Manufacturers Association (FEMA) it was concluded that exposure to estragole from herbs, essential oils, and flavor substances does not pose a significant cancer risk to humans (Smith *et al*., 2002). In this conclusion, experimental data that suggested a non-linear relationship between dose and profiles of metabolism and metabolic activation were taken into account. The FEMA expert panel estimated the average daily intake of estragole to be less than 0.01 mg/kg bw/day, which was determined based on production volume data for flavor use (Smith *et al*., 2002). The Scientific Committee on Food (SCF) of the European Union concluded in their evaluation of estragole, that estragole is genotoxic and carcinogenic, and restrictions in use levels were indicated (SCF, 2001). The SCF estimated the average daily intake of estragole from all food sources to be 4.3 mg/day, corresponding to ~0.07 mg/kg bw/day for a 60 kg person (SCF, 2001). This estimation is based on theoretical maximum use levels of estragole in various food categories and consumption data for these food categories.

Rodent carcinogenicity of estragole has been linked to its metabolic conversion to a genotoxic metabolite (Drinkwater *et al*., 1976; Miller *et al*., 1983; Wiseman *et al*., 1987). Bioactivation of estragole starts with the conversion of estragole into the proximate carcinogenic metabolite 1'-hydroxyestragole (Figure 1), which is mainly catalyzed by cytochrome P450 1A2 and P450 2A6 (Jeurissen *et al*., 2007). Other phase I metabolic pathways represented

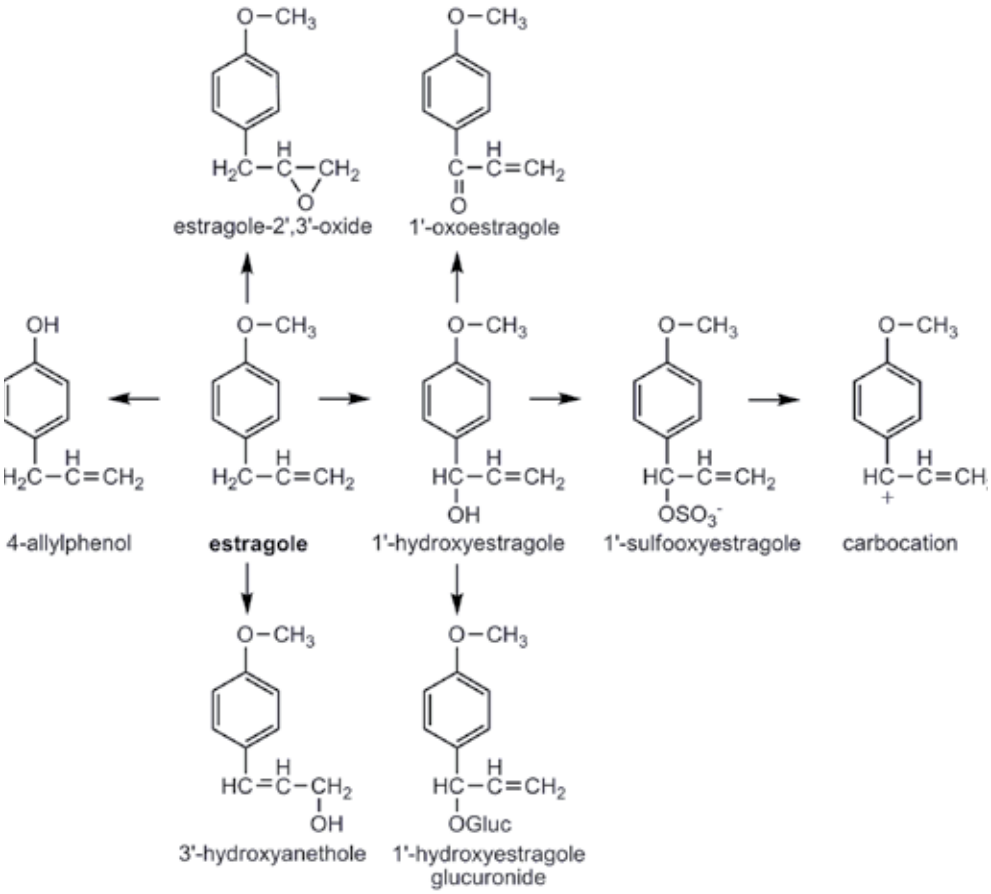

Figure 1. Metabolism of estragole

in Figure 1 include *O*-demethylation, epoxidation and 3'-hydroxylation of estragole, leading to the formation of respectively 4-allylphenol, estragole-2',3'-oxide, and 3'-hydroxyanethole. These metabolic pathways lead to detoxification of estragole (Solheim and Scheline, 1973; Phillips *et al*., 1981; Fennell *et al*., 1984; Anthony *et al*., 1987; Sangster *et al*., 1987; Luo *et al*., 1992; Guenthner and Luo, 2001). Sulfonation of 1'-hydroxyestragole results in the formation of the ultimate carcinogenic metabolite, which is unstable and degrades in aqueous environment to a reactive carbocation that is capable of forming DNA adducts (Phillips *et al*., 1981; Phillips *et al*., 1984; Randerath *et al*., 1984; Wiseman *et al*., 1985). Conjugation of 1'-hydroxyestragole with glucuronic acid results in detoxification of 1'-hydroxyestragole, because it

results in formation of a stable metabolite that has been detected in the urine of both humans and rats exposed to estragole (Sangster *et al*., 1987, Anthony *et al*., 1987). In addition to glucuronidation and sulfonation, oxidation of 1'-hydroxyestragole to 1'-oxoestragole has been reported as a possible metabolic route of 1'-hydroxyestragole (Solheim and Scheline, 1973). The 1'-oxoestragole metabolite of 1'-hydroxyestragole is a reactive metabolite, that has been shown to be able to form adducts with 2'-deoxyguanosine in a direct reaction with this nucleoside (Phillips *et al*., 1981). In spite of this, 1'-oxoestragole was not carcinogenic *in vivo* in mice (Wiseman et al., 1987). To date, formation of 1'-oxoestragole has been considered to be of minor importance, since only relatively small amounts of derivatives of this metabolite have been detected in the urine of rats exposed orally to [methoxy-^{14}C]-labeled estragole (Solheim and Scheline, 1973).

Dose-dependent effects in metabolism of estragole have been revealed in both rats and mice exposed to different doses of estragole. In these species the relative extent of *O*-demethylation decreased with increasing dose accompanied by a relative increase in excretion of 1'-hydroxyestragole glucuronide in the urine (Anthony *et al*., 1987). Based on a previously constructed PBBK model for estragole in male rat it was demonstrated that these dose-dependent effects can be explained by a shift in metabolism of estragole (Punt *et al*., 2008). At low doses, *O*-demethylation is the major metabolic pathway, occurring mainly in the lung and kidney due to formation of this metabolite with high affinity in these organs. Saturation of this metabolic pathway in the lung and kidney at increasing dose levels, leads to a relative increase in formation 1'-hydroxyestragole in the liver and a concomitant increase in formation of 1'-hydroxyestragole glucuronide and the ultimate carcinogenic metabolite 1'-sulfooxyestragole. Such dose-dependent effects can have consequences for the extrapolation of the cancer risk from high dose animal experiments to humans at relevant dietary intake levels.

In addition to dose-dependent effects, species differences in metabolism and metabolic activation can also occur. We have, for instance, previously demonstrated that male rats are more efficient in sulfonation of 1'-

hydroxyestragole than humans (Punt *et al*., 2007). To determine the overall differences between humans and male rats in bioactivation of estragole, it is of importance to compare the relative extent of different metabolic pathways between these species. The present study investigated the kinetics of possible metabolic reactions of estragole and 1'-hydroxyestragole in humans in incubations with relevant human tissue fractions. The kinetic data obtained were used to build a physiologically based biokinetic (PBBK) model for estragole metabolism in human to predict the relative extent of bioactivation and detoxification of estragole at dose levels relevant for dietary human intake. An evaluation of the model defined was performed by comparing the predicted formation of 4-allylphenol and 1'-hydroxyestragole glucuronide to literature reported levels of these metabolites in humans exposed to estragole (Sangster *et al*., 1987). The outcomes of the model were subsequently compared to those of the previously defined PBBK model for estragole in male rat to evaluate the occurrence of species differences in metabolic activation and detoxification of estragole.

Material and Methods

Chemicals and biological materials

Pooled, mixed-gender human lung, kidney and small intestine microsomes were purchased from BioPredic International (Rennes, France). Pooled, mixed-gender human liver S9 and microsomes were purchased from BD Gentest (Woburn, USA). Hydrochloric acid (37%) was purchased from Merck (Darmstadt, Germany). β-Glucuronidase, NADPH and NAD^+ were obtained from Roche Diagnostics (Mannheim, Germany). Tris(hydroxymethyl) aminomethane was obtained from Gibco BRL Life Technologies (Paisley, Scotland). Estragole (4-allylanisol), acetonitrile (chromatography grade), methanol, dimethylsulfoxide (DMSO), ascorbic acid, alamethicin, glutathione (GSH), bovine serum albumin (BSA), uridine 5'-diphosphoglucuronic acid (UDPGA), and a GSH assay kit were purchased from Sigma-Aldrich

(Steinheim, Germany). 1'-Hydroxyestragole, 4-allylphenol, estragole-2',3'-oxide, and 3'-hydroxyanethole were synthesized as described previously (Punt *et al*., 2008).

Phase I Metabolism

Mixed gender human liver, lung, kidney, and small intestine microsomal preparations were incubated with estragole in the presence of NADPH. The incubation mixtures had a final volume of 100 µL, containing (final concentrations) 3 mM NADPH, 1 mM ascorbic acid and 1 mg mL^{-1} microsomal protein in 0.2 M Tris-HCl (pH 7.4). After pre-incubating at 37ºC for 1 min, the reactions were started by adding the substrate estragole. Initial incubations with the different microsomal fractions were performed at a substrate concentration of 100 µM (final concentration) at several incubation times. The reactions were terminated by addition of 25 µL cold acetonitrile. Additional incubations to determine the kinetic constants for the formation of phase I metabolites of estragole were performed only with human liver microsomes, because these were the microsomal samples tested that resulted in metabolic conversion of estragole (see Results section). These incubations were carried out for 10 min at a substrate concentration that ranged from 5 to 400 µM estragole. Under these conditions the formation of the different estragole metabolites was linear with time and microsomal protein concentration. All samples were centrifuged for 5 min at 16,000g and 50 µL of the supernatant of each sample was analyzed by HPLC as described previously (Punt *et al*., 2008). In the blank incubations performed without NADPH low amounts of 3'-hydroxyanethole were formed, corresponding to approximately 0.6% of the applied concentration of estragole. For this reason the formation of 3'-hydroxyanethole was corrected for the amount of 3'-hydroxyanethole formed in the blank incubations. 3'-Hydroxyanethole was quantified by comparison of the peak areas of the metabolite in the chromatograms obtained at a wavelength of 206 nm to the calibration curve of the synthesized metabolite. Formation of 4-allylphenol and 1'-hydroxyestragole were quantified by comparison of the peak areas of the different metabolites in the chromatograms obtained

at a wavelength of 225 nm to the calibration curve of the corresponding synthesized metabolites. Quantification of estragole-2',3'-diol, which reflects formation of estragole-2',3'-oxide (see Results section) was achieved by comparison of the peak area of estragole-2',3'-diol in the chromatograms obtained at a wavelength of 225 nm to the calibration curve of estragole-2',3'-oxide, since estragole-2',3'-diol has a similar molar extinction coefficient as estragole-2',3'-oxide (Punt *et al*., 2008).

Glucuronidation of 1'-hydroxyestragole

Mixed gender human liver microsomal preparations were incubated with 1'-hydroxyestragole in the presence of UDPGA. The incubation mixtures had a final volume of 200 µL, containing (final concentrations) 10 mM UDPGA, and 1 mg mL^{-1} microsomal protein in 0.2 M Tris-HCl (pH 7.4) containing 10 mM $MgCl_2$. The incubation mixtures were pre-treated on ice with 0.025 mg mL^{-1} of the poreforming peptide alamethicin from a 200-times concentrated stock in methanol for 15 min to overcome enzyme latency and obtain maximal glucuronidation activity (Fisher *et al*., 2000; Lin and Wong, 2002). The reactions were started by the addition of the substrate 1'-hydroxyestragole from a 200-times concentrated stock solution in DMSO, after pre-incubating at 37ºC for 1 min. The kinetic constants for glucuronidation of 1'-hydroxyestragole were determined at substrate concentrations that ranged from 50 to 2000 µM 1'-hydroxyestragole. The reactions were terminated after 6 h by addition of 50 µL cold acetonitrile. Under these conditions glucuronidation of 1'-hydroxyestragole was linear with time and microsomal protein concentration. All samples were centrifuged for 5 min at 16,000g and 50 µL of the supernatant of each sample was analyzed by HPLC as described previously (Punt *et al*., 2008). Formation of 1'-hydroxyestragole glucuronide in these incubations was verified by treatment of samples with β-glucuronidase and analysis of samples by LC-MS as described previously (Punt *et al*., 2008). Because 1'-hydroxyestragole glucuronide has the same UV spectrum as 1'-hydroxyestragole (data not shown) it was assumed that it has the same molar extinction coefficient as 1'-hydroxyestragole. Quantification of the 1'-

hydroxyestragole glucuronide was therefore achieved by comparison of the peak area of 1'-hydroxyestragole glucuronide in the chromatograms obtained at a wavelength of 225 nm to the calibration curve of 1'-hydroxyestragole (Chapter 3).

Oxidation of 1'-hydroxyestragole

Formation of 1'-oxoestragole was analyzed in incubations with both human and male rat liver microsomes, using NAD^+ as cofactor and GSH to trap the transient 1'-oxoestragole. The kinetic constants for formation of the 1'-oxoestragole adduct with glutathione, reflecting the formation of 1'-oxoestragole were determined with human but also with male rat liver microsomes at substrate concentrations that ranged from 10 to 1000 μM 1'-hydroxyestragole. These incubation mixtures had a final volume of 100 μL, containing (final concentrations) 3 mM NAD^+, 2 mM GSH and 1 mg mL^{-1} microsomal protein in 0.2 M Tris-HCl (pH 7.4). The reactions were terminated after 10 min by addition of 25 μL cold acetonitrile. Under these conditions the formation of the glutathione adduct, 3'-(glutathion-S-yl)-1'-oxo-2'-3'-dihydroestragole (GS-1'-oxoestragole), was linear with time and microsomal protein concentration. The level of GSH in the incubations was optimized to obtain maximum scavenging of 1'-oxoestragole. To this end incubations were performed in the presence of increasing concentrations of GSH, ranging from 2 mM to 10 mM. At a concentration of 2 mM GSH maximum formation of GS-1'-oxoestragole was reached in the incubations, pointing at maximum scavenging of 1'-oxoestragole at this concentration.

Due to the presence of microsomal protein in the incubations the maximum formation of GS-1'-oxoestragole could be affected by providing an alternative reaction possibility for the reactive 1'-oxoestragole. Experiments, in which the protein content was increased by addition of BSA revealed, however, no decrease in formation of GS-1'-oxoestragole, indicating that at a level of 1 mg mL^{-1} microsomal protein the protein content does not significantly decrease GS-1'-oxoestragole formation (data not shown).

For HPLC analysis the samples were centrifuged for 5 min at 16,000g and

50 µL of the supernatant of each sample was analyzed on Alltima C18 5 µm column, 150 mm x 4.6 mm (Alltech, Breda, The Netherlands) coupled to a Waters 2695 alliance HPLC system (Waters, Etten-Leur, the Netherlands). The gradient was made with acetonitrile and ultrapure water containing 0.1% (v/v) acetic acid. The flow rate was 1 mL min^{-1} and a gradient was applied from 0% to 25% acetonitrile over 5 min, after which the percentage of acetonitrile was kept at 25% for 5 min and then increased to 100% over 2 min. Detection was performed at 280 nm using a Waters 2996 photodiode array detector (Waters, Etten-Leur, the Netherlands).

Formation of GS-1'-oxoestragole in the incubations was verified by LC-MS, which was performed on a Finnigan Surveyor HPLC system coupled to an LXQ mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Aliquots of 20 µL (injected volume) were separated on an Alltima C18 5u column, 150 x 2.1 mm (Alltech, Breda, The Netherlands). The gradient was made with acetonitrile and ultra pure water containing 0.1% (v/v) acetic acid and the flow rate was set to 0.1 mL min^{-1}. A linear gradient was applied from 0% to 25% acetonitrile over 5 min and was kept at 25% for 5 min, after which the percentage of acetonitrile was increased to 100% over 2 min. Mass spectrometric analysis was performed in positive electrospray mode using a spray voltage of 5 kV, a capillary temperature of 275 °C, and nitrogen as sheath gas (60 arbitrary units).

Quantification of GS-1'-oxoestragole was performed by means of a calibration curve. To this end GS-1'-oxoestragole was synthesized as a reference compound from 1'-oxoestragole and GSH based on the protocol of Phillips *et al*. (1981). 1'-Oxoestragole was synthesized from 1'-hydroxyestragole as described for 1'-oxosafrole by Fennel *et al*. (1984). A calibration curve of GS-1'-oxoestragole was prepared by incubating 40 µM 1'-oxoestragole with different concentrations of GSH, ranging from 2 to 20 µM in 0.2 M Tris-HCl (pH 7.4). The reactions were incubated for 4 h at 37°C resulting in maximum formation of GS-1'-oxoestragole. The peak area of GS-1'-oxoestragole in the chromatograms of these reactions were related to the quantity of GSH used in the reactions. This could be done since, according to both LC-MS analysis as

well as HPLC analysis of the reaction mixture of GSH with 1'-oxoestragole, only one product is formed and there is no residual GSH (determined using a GSH assay kit), indicating that all GSH has reacted and that, thus, the concentration of the adduct formed equals the original GSH concentration.

Kinetic analysis

The estragole concentration dependent rate of formation of different phase I metabolites and the 1'-hydroxyestragole concentration dependent glucuronidation and formation of 1'-oxoestragole were fitted to the standard Michaelis-Menten equation:

$$v=V_{max}/(1+(K_m/[S])$$

with [S] being the substrate concentration. The apparent maximum velocity ($V_{max(app)}$), the apparent Michaelis-Menten constant ($K_{m(app)}$), and the catalytic efficiency $V_{max(app)}/ K_{m(app)}$ were determined, using the LSW data analysis toolbox (version 1.1.1, MDL Information Systems, Inc.)

PBBK model

A PBBK model for estragole in human was developed based on the *in vitro* metabolic data. The model defined consists of five compartments including blood, liver, fat, richly perfused tissue and slowly perfused tissue (Ramsey and Andersen, 1984). A schematic diagram of the human PBBK model is shown in Figure 2. The physiological parameters and partition coefficients used in the model are given in table 1. The physiological parameters were obtained from (Brown *et al*., 1997). The partition coefficients were estimated from the log K_{ow} based on a method of DeJongh *et al*. (1997). Log K_{ow} values were estimated with the software package ClogP version 4.0 (Biobyte, Claremont, CA) and amounted to 3.1 for estragole and 1.6 for 1'-hydroxyestragole. Model equations were coded and numerically integrated in Berkeley Madonna 8.0.1 (Macey and Oster, UC Berkeley, CA, USA), using the Rosenbrock's algorithm for stiff systems.

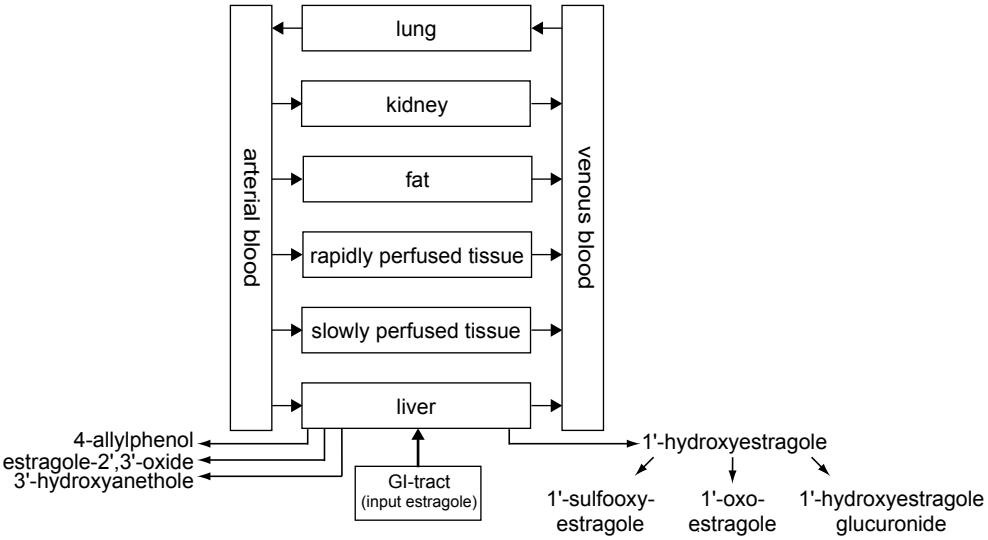

Figure 2. Schematic diagram of the proposed PBBK model for estragole in human.

Based on *in vitr*o conversion data (see Results section), conversion of estragole to 4-allylphenol, 1'-hydroxyestragole, estragole-2',3'-oxide and 3'-hydroxyanethole was described in the liver compartment. The apparent *in vitro* V_{max} values for these reactions, expressed as nmol min^{-1} (mg microsomal protein)$^{-1}$, were scaled to the liver using a microsomal protein yield of 35 mg per g liver (Medinsky *et al*., 1994). The apparent *in vitro* K_m values were assumed to correspond to the apparent *in vivo* K_m values. The uptake of estragole from the gastrointestinal tract was described by a first-order process, assuming direct entry from the intestine to the liver compartment. The absorption rate constant (K_a) was 1.0 h^{-1}. Mass balance equations for estragole in the liver were similar to those described previously in the PBBK model for estragole in male rat (Punt *et al*., 2008)

In the case of 4-allylphenol, estragole-2',3'-oxide, and 3'-hydroxanethole only formation of these metabolites are taken into account, and no further reactions with these metabolites were modeled. Formation of 1'-hydroxyestragole was followed by additional metabolic reactions. To this end a near quantitative intrahepatic conversion of 1'-hydroxyestragole was assumed. This assumption was based on the findings that the overall catalytic

Table 1. Parameters used in the physiologically based biokinetic model for estragole in human.

Physiological parameters[a]		Tissue: blood partition coefficients[b]	
Body weight (kg)	60		
Percentage of body weight:		Estragole	
Liver	2.6	Liver	6.5
Fat	21.4	Fat	105
Rapidly perfused	5	Rapidly perfused	6.5
Slowly perfused	51.7	Slowly perfused	4.1
Blood	7.9	1'-Hydroxyestragole	
		Liver	1.6
Cardiac output (L/ hr/kg $bw^{0.74}$)	15		
Percentage of cardiac output:			
Liver	22.7		
Fat	5.2		
Rapidly perfused	47.3		
Slowly perfused	24.8		

[a] Brown *et al*. (1997)
[b] DeJongh *et al*. (1997)

efficiency for the reactions converting 1'-hydroxyestragole was higher than the catalytic efficiency for the formation of 1'-hydroxyestragole. No distribution of this metabolite throughout the body was therefore modeled. The kinetic constants for glucuronidation of 1'-hydroxyestragole and formation of 1'-oxoestragole were determined in the present study, whereas the kinetic constants for sulfonation of 1'-hydroxyestragole were obtained from Punt *et al*. (2007). The apparent *in vitro* V_{max} values for glucuronidation and formation of 1'-oxoestragole, expressed as nmol min^{-1} (mg microsomal protein)$^{-1}$, were scaled to the liver using a microsomal protein yield of 35 mg per g liver (Medinsky *et al*., 1994). The apparent *in vitro* V_{max} value for sulfonation of 1'-hydroxyestragole, expressed as nmol min^{-1} (mg S9 protein)$^{-1}$, were scaled to the liver using a S9 protein yield of 143 mg per g liver (Medinsky *et al*., 1994). Mass balance equations for 1'-hydroxyestragole in the liver were similar to thosedescribed previously in the PBBK model for estragole in male rat (Punt *et al*., 2008), with the addition of oxidation of 1'-hydroxyestragole as additional metabolic route by adding the following term to the equation

describing the rate of change in amount of 1'-hydroxyestragole in the liver compartment (dAL_{HE}/dt):

$$-V_{max,\ L\_OE} * CL_{HE}/PL_{HE}/ (K_{m,\ L\_OE} + CL_{HE}/PL_{HE})$$

Where AL_{HE} is the amount of 1'-hydroxyestragole in the liver (µmol), CL_{HE} is the 1'-hydroxyestragole concentration in the liver (µmol/L), PL_{HE} is the liver/ blood partition coefficient of 1'-hydroxestragole, and V_{max,L_OE} and K_{m,L_OE} are the maximum rate of formation and the Michaelis-Menten constant for the formation 1'-oxoestragole in the liver.

Sensitivity analysis

A sensitivity analysis was performed to evaluate the relative effect of model parameters on the model output. Normalized sensitivity coefficients (SC) were determined for all model parameters according to the following equation:

$$SC = (C'-C)/(P'-P)*(P/C)$$

where C is the initial value of 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). Based on the literature a 5% increase in parameter value was chosen to analyze the effect of a change in parameter value on the formation of 1'-hydroxyestragole and 1'-sulfooxyestragole (over 24 h), expressed as nmol (kg bw)$^{-1}$ (Evans and Andersen, 2000). Each parameter was analyzed individually, keeping the other parameters to their initial values. Sensitivity coefficients were calculated for all physiological parameters (bw, tissue volumes, and blood flow rates), partition coefficients, and biochemical parameters (K_m and V_{max} values for the different metabolitic reactions and k_a).

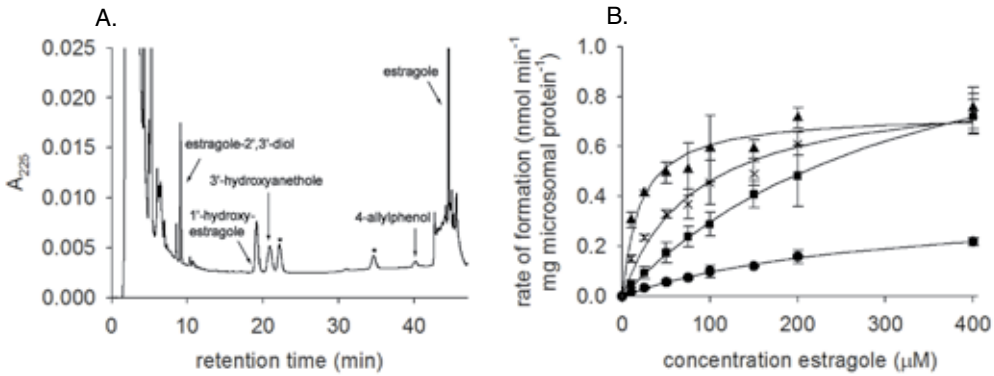

Figure 3. (A) Chromatographic profile of an incubation with human liver microsomes at a substrate concentration of 400 μM estragole, NADPH as cofactor and an incubation time of 10 min. Peaks marked with an asterisk were also present in the blank incubations performed without cofactor NADPH, in which they had the same area under the curve as in this incubation. (B) Estragole concentration dependent rate of formation of phase I metabolites by human liver microsomes. In the plots each point represents the mean (±SD) of three replicates corresponding to the formation of respectively 1'-hydroxyestragole (▲), estragole-2',3'-oxide (x), 3'-hydroxyanethole (■) and 4-allylphenol (●).

Results

Conversion of estragole: phase I metabolism

HPLC analysis of incubations with human liver, lung, kidney, and small intestine microsomes in the presence of estragole as substrate and NADPH as cofactor reveals that conversion of estragole to phase I metabolites only occurs in incubations with human liver microsomes (Figure 3A). No conversion of estragole is observed in incubations with microsomes from the other organs (data not shown). In the chromatograms of the incubations with human liver microsomes the peaks at 18.9, 20.6 and 39.6 min were identified as 1'-hydroxyestragole, 3'-hydroxyanethole, and 4-allylphenol on the basis of comparison of the retention times and UV spectra to the chemically synthesized reference compounds. The peak at 9.1 min was previously identified as estragole-2',3'-diol (Punt et al., 2008), which is formed upon hydrolysis of estragole-2',3'-oxide due to the presence of epoxide hydrolases in human liver microsomes (Luo et al., 1992; Guenthner and Luo, 2001).

Figure 3B shows the rate of formation of 1'-hydroxyestragole, estragole-2',3'-oxide, 3'-hydroxyanethole, and 4-allylphenol in incubations with

Table 2. Kinetic parameters for phase I metabolism of estragole with human liver microsomes

Metabolite	$K_{m(app)}$ [a,b]	$V_{max(app)}$ [a,c]	*In vitro* catalytic efficiency ($V_{max(app)}/K_{m(app}$ * 1000 µL/mL)[d]
4-allylphenol	290 ± 28	0.38 ± 0.02	1.3
1'-hydroxyestragole	21 ± 6	0.73 ± 0.04	35.8
3'-hydroxyanethole	350 ± 20	1.35 ± 0.05	3.9
estragole-2',3'-oxide	83 ± 17	0.85 ± 0.07	10.2

[a] average value ± SEM
[b] µM
[c] nmol min^{-1} (mg microsomal protein)$^{-1}$
[d] µL min^{-1} (mg microsomal protein)$^{-1}$

human liver microsomes with increasing estragole concentrations. Table 2 summarizes the apparent K_m and V_{max} values obtained from these plots as well as the catalytic efficiencies, calculated as V_{max}/K_m. In the incubations with human liver microsomes, the catalytic efficiency for formation of 1'-hydroxyestragole was the highest, followed by that for formation of estragole-2',3'-oxide and 3'-hydroxyanethole. Formation of 4-allylphenol was the least efficient catalytic phase I conversion in these incubations. The high catalytic efficiency for 1'-hydroxylation of estragole is mainly due to a relative low K_m for this reaction, which was respectively 4-, 14- and 17-fold lower than the K_m for epoxidation, *O*-demethylation and 3'-hydroxylation.

Conversion of 1'-hydroxyestragole: formation of 1'-hydroxyestragole glucuronide

Formation of the glucuronosyl conjugate of 1'-hydroxyestragole was analyzed in incubations with pooled human liver microsomes. Figure 4A shows a HPLC-chromatogram of such incubation in the presence of 1'-hydroxyestragole as substrate and UDPGA as cofactor and revealed the formation of a metabolite of 1'-hydroxyestragole with a retention time of 18.9 min. Treatment of samples with β-glucuronidase resulted in complete elimination of this metabolite and formation of a corresponding amount of 1'-hydroxyestragole (data not shown). Based on this enzymatic deconjugation with β-glucuronidase together with LC/MS analysis of the metabolite, revealing a deprotonated molecule at *m/z* 339, which corresponds to the

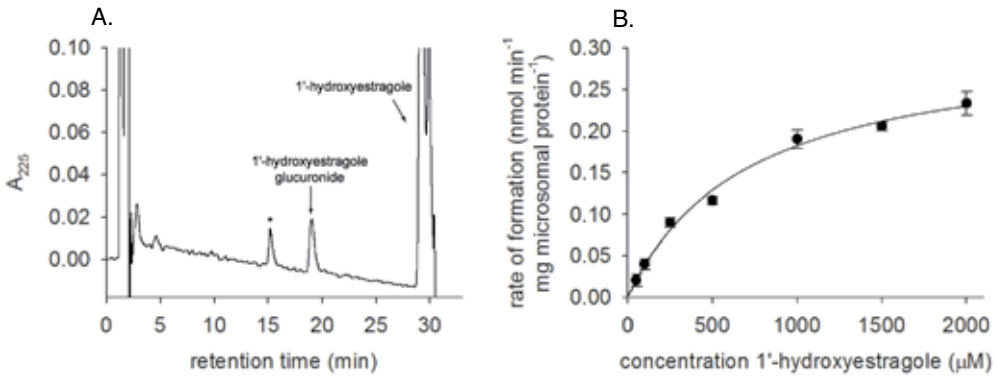

Figure 4. (A) Chromatographic profile of an incubation with human liver microsomes at a substrate concentration of 500 μM 1'-hydroxyestragole, UDPGA as cofactor and an incubation time of 360 min. The peak marked with an asterisk was also present in the blank incubation performed without cofactor. (B) 1'-Hydroxyestragole concentration dependent rate of formation of 1'-hydroxyestragole glucuronide by human liver microsomes. In the plot each point represents the mean (±SD) of three replicates.

theoretically expected mass of 1'-hydroxyestragole glucuronide, confirms the formation of 1'-hydroxyestragole glucuronide. The 1'-hydroxyestragole concentration dependent rate of formation of 1'-hydroxyestragole glucuronide by human liver microsomes is displayed in Figure 4B. The kinetic constants were obtained from the graph by fitting the data to the standard Michaelis-Menten equation, resulting in an apparent K_m of 708 μM and an apparent V_{max} of 0.3 nmol min^{-1} (mg microsomal protein)$^{-1}$.

Conversion of 1'-hydroxyestragole: formation of 1'-oxoestragole

In addition to conjugation reactions of 1'-hydroxyestragole the occurrence of oxidation of 1'-hydroxyestragole to 1'-oxoestragole was examined. Formation of this metabolite was observed in incubations with human liver microsomes using NAD^+ as cofactor and GSH to trap the transient 1'-oxoestragole. Figure 5A shows a HPLC-chromatogram of such incubation. The peak at 7.9 min was identified as GS-1'-oxoestragole on the basis of comparison of the retention time and UV spectrum to the chemically synthesized GS-1'-oxoestragole. Furthermore the mass spectrum of the metabolite, obtained by LC-MS, reveals a protonated molecule at *m/z* 470, which corresponds to the theoretically expected mass and this confirms formation of GS-1'-

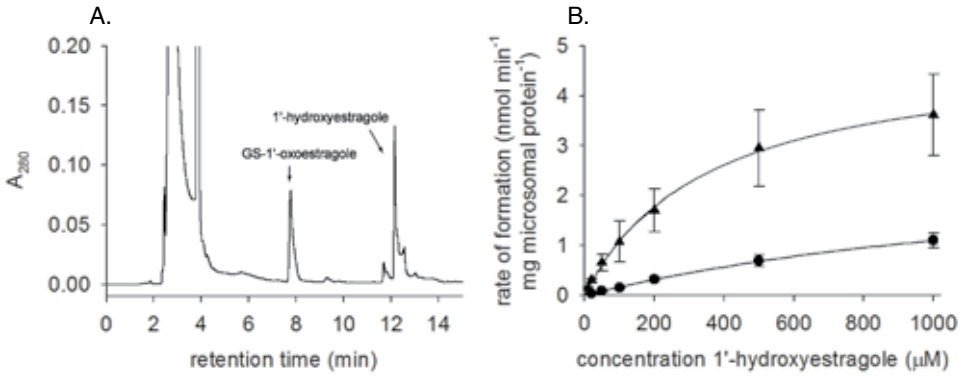

Figure 5. (A) Chromatographic profile of an incubation with human liver microsomes at a substrate concentration of 500 μM 1'-hydroxyestragole, NAD^+ as cofactor and GSH to trap the transient 1'-oxoestragole at an incubation time of 10 min. (B) 1'-Hydroxyestragole concentration dependent rate of formation of 1'-oxoestragole by human (▲) and male rat (●) liver microsomes as determined by quantification of the GS-1'-oxoestragole, which is assumed to represent the formation of 1'-oxoestragole. In the plots each point represents the mean (±SD) of three replicates.

oxoestragole in the incubation. The peak was not present in the incubations without GSH or blank incubations without microsomes or co-factor (data not shown). Since the occurrence of this reaction was previously not analyzed for male rat (Punt *et al*., 2008), additional incubations were performed to determine formation of 1'-oxoestragole by male rat liver microsomes as well. Figure 5B reveals the 1'-hydroxyestragole concentration dependent formation of 1'-oxoestragole by human and male rat liver microsomes as determined by quantification of the GS-1'-oxoestragole, which is assumed to represent the formation of 1'-oxoestragole. The K_m for formation of 1'-oxoestragole by human liver microsomes was determined to be 354 μM and the V_{max} 4.9 nmol min^{-1} (mg microsomal protein)$^{-1}$. For formation of 1'-oxoestragole by rat liver microsomes the K_m was determined to be 1609 μM and the V_{max} 2.9 nmol min^{-1} (mg microsomal protein)$^{-1}$.

Comparison of the kinetic data for estragole bioactivation and detoxification by human and male rat tissue fractions

Table 3 presents a summary of the kinetic parameters for estragole bioactivation and detoxification by human tissue fractions combined with the kinetic parameters previously reported for male rat tissue fractions (Punt *et*

Table 3. Kinetic constants for estragole metabolism by human and male rat tissue fractions.

		Human[a]			Male rat[b]		
Metabolite	Organ	Scaled $V_{max(app)}$[c]	$K_{m(app)}$[d]	Scaled $V_{max(app)}/K_{m(app)}$[e]	Scaled $V_{max(app)}$[c]	$K_{m(app)}$[d]	Scaled $V_{max(app)}/K_{m(app)}$[e]
estragole metabolites							
4-allylphenol	Liver	0.8	290	3	1.8	458	4
	Lung	n.d.[f]	n.d.	-	0.8	0.5	1600
	Kidney	n.d.	n.d.	-	0.2	0.5	400
1'-hydroxyestragole	Liver	1.5	21	73	3.1	116	27
	Lung	n.d.	n.d.	-	0.5	26	19
	Kidney	n.d.	n.d.	-	0.1	22	5
3'-hydroxyanethole	Liver	2.8	350	8	2.2	93	24
estragole-2',3'-oxide	Liver	1.8	83	22	4.5	154	29
1'-hydroxyestragole metabolites							
1'-sulfooxyestragole	liver	0.06	727	0.1	0.2	63	3
1'-hydroxyestragole glucuronide	liver	0.7	708	1	60	137	438
1'-oxoestragole	liver	10.3	354	29	6.1	1609	4

[a] Data obtained from present study and Punt *et al*. (2007)
[b] Data obtained from Punt *et al*. (2007), Punt *et al*. (2008) and in the case of 1'-oxoestragole from the present study.
[c] Scaled $V_{max(app)}$ expressed as μmol h^{-1} (g liver)$^{-1}$, calculated from the *in vitro* $V_{max (app)}$ based on a microsomal protein yield of 35, 20 and 7 mg (g tissue)$^{-1}$ for liver, lung, and kidney respectively, and a S9 protein yield of 143 mg (g liver)$^{-1}$
[d] $K_{m(app)}$ expressed as μM
[e] Catalytic efficiency (scaled $V_{max (app)}/ K_{m(app)}$) expressed as mL h^{-1} (g liver)$^{-1}$
[f] n.d.= not detectable

al., 2007; Punt *et al*., 2008). In this table the V_{max} values expressed as µmol h^{-1} (g liver)$^{-1}$ were obtained by scaling of the *in vitro* V_{max} values to V_{max} values expressed per g liver using microsomal and S9 protein yields from the different organs that were obtained from the literature (Medinsky *et al*., 1994). This conversion allowed comparison of the kinetic constants obtained with different tissue fractions. In the case of phase I metabolism of estragole, comparison of the kinetic constants between humans and male rats reveals that the catalytic efficiency (scaled V_{max}/K_m) for formation of the proximate carcinogenic metabolite 1'-hydroxyestragole is 2.7-fold higher in human liver. Although the V_{max} for 1'-hydroxylation is 2-fold lower in human liver, the K_m is 5.5-fold lower resulting in a higher overall catalytic efficiency. The catalytic efficiencies for *O*-demethylation and epoxidation of estragole are similar for human and male rat liver, but the catalytic efficiency for 3'-hydroxylation of estragole is 3-fold lower in human liver. This lower observed catalytic efficiency is mainly due to a 3.8-fold higher K_m for 3'-hydroxylation in human liver. Another important difference between human and male rat is that no detoxifying *O*-demethylation occurs in human lung and kidney, whereas this metabolic route occurs with high affinity in male rat lung and kidney.

With respect to the metabolic reactions with 1'-hydroxyestragole, the catalytic efficiency for formation of the ultimate carcinogenic metabolite 1'-sulfooxyestragole was previously found to be 30-fold lower in human liver compared to male rat liver (table 3) (Punt *et al*., 2007). Comparison of the catalytic efficiency for glucuronidation of 1'-hydroxyestragole by human and male rat liver samples reveals that this metabolic route is more than 400-times less efficient in the human liver, which is mainly due to an 86-fold lower V_{max} for glucuronidation in human liver. Oxidation of 1'-hydroxyestragole is found to be a major metabolic route in human liver. Comparison of the catalytic efficiency for formation of 1'-oxoestragole by human and male rat liver samples reveals that this metabolic route is a 7.3-fold more efficient in the human liver, which can be explained by both a 1.7-fold higher V_{max} value and a 4.5-fold lower K_m value for 1'-hydroxyestragole oxidation in human liver.

Human PBBK model

Based on the *in vitro* kinetic data for bioactivation and detoxification by human tissue fractions a PBBK model for estragole metabolism in human was developed. With the model obtained predictions can be made on formation of different metabolites in human liver in time and at different oral doses. The model performance was evaluated by comparing the predicted formation of 1'-hydroxyestragole glucuronide and 4-allylphenol to the reported levels of these metabolic routes in humans exposed 0.001 mg/kg bw estragole (Sangster *et al*., 1987). To this end it was assumed that formation of 1'-hydroxyestragole glucuronide equals excretion of this metabolite in the urine, whereas formation of 4-allylphenol is assumed to correspond to the reported level of exhalation of $^{14}CO_2$. In the case of 1'-hydroxyestragole glucuronide the predicted formation of this metabolite, corresponding to 2.0% of the dose, is comparable to the reported *in vivo* level of 0.5% of the dose. The predicted formation of 4-allylphenol, corresponding to 2.5% of the dose, is 4-fold lower than the reported *in vivo* level of ~10% of the dose (Sangster *et al*., 1987). These results indicate that the PBBK model predicts the formation of these metabolites within the same order of magnitude as the reported levels. Formation of the major metabolite, 1'-oxoestragole, could not be evaluated against human *in vivo* data, since excretion of 1'-oxoestragole as GSH or protein adducts was not analyzed in humans exposed to estragole (Sangster *et al*., 1987).

Figure 6 displays the time-dependent predicted concentrations of estragole and 1'-hydroxyestragole, and the formation of 1'-sulfooxyestragole, 1'-hydroxyestragole glucuronide and 1'-oxoestragole in human liver following exposure to 0.07 mg/kg bw estragole, which corresponds to the average dietary human intake as estimated by the SCF (2001). The PBBK model predicts that both estragole and 1'-hydroxyestragole are metabolized within the 24 h timeframe. Accumulation of estragole or its reactive metabolites is therefore not expected to occur when a repeated dosing would be applied after 24 h. After 24 hours a total formation of 0.03 nmol (g liver)$^{-1}$ of 1'-sulfooxyestragole, 0.36 nmol (g liver)$^{-1}$ of 1'-hydroxyestragole glucuronide,

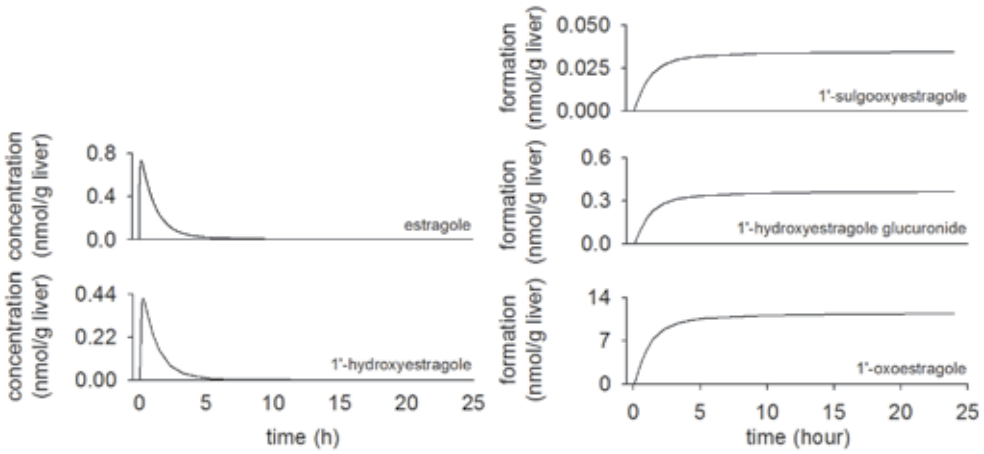

Figure 6. PBBK model-predicted time-dependent concentrations of estragole and 1'-hydroxyestragole and formation of 1'-sulfooxyestragole, 1'-hydroxyestragole glucuronide and 1'-oxoestragole in human liver following exposure to 0.07 mg/kg bw estragole.

and 11.39 nmol (g liver)$^{-1}$ of 1'-oxoestragole is reached, which corresponds to 0.19%, 2.0%, and 62.7% of the dose respectively.

Estimation of the formation of the proximate carcinogenic metabolite 1'-hydroxyestragole at different oral doses revealed that the relative formation of this metabolite decreases with increasing dose due to saturation of this metabolic route. However, the observed relative decrease in formation of 1'-hydroxyestragole was not substantial at doses relevant for dietary human intake, given that at a dose of 0.7 mg/kg bw (i.e. ten-fold higher than the estimated average dietary human intake), the percentage of the dose that is ultimately converted to 1'-hydroxyestragole is similar (64.9%) as at a dose of 0.07 mg/kg bw (64.4%). Only when a dose of 10 mg/kg bw is applied (i.e. 150-fold higher than the average dietary human intake) a decrease of approximately 10% of the percentage of the dose converted to 1'-hydroxyestragole was observed leading to a value of 57.6%. The relative decrease in formation of 1'-hydroxyestagole with increasing doses resulted in a concomitant decrease in formation 1'-sulfooxyestragole, 1'-hyroxyestragole glucuronide, and 1'-oxoestragole amounting to respectively 0.19%, 1.9%, and 62.2% of the dose at 0.7 mg/kg bw and to 0.17%, 1.7%, and 55.6% of the dose at 10 mg/kg bw.

Sensitivity analysis

The sensitivity of the predicted formation of 1'-hydroxyestragole and 1'-sulfooxyestragole to changes in all model parameters was analyzed to identify the key parameters in the human model that influence bioactivation of estragole. Normalized sensitivity coefficients were calculated at a dose of 0.07 mg/kg bw estragole, but only parameters that had a normalized sensitivity coefficient higher than 0.1 (in absolute value) are displayed in Figure 7. Formation of 1'-hydroxyestragole is influenced by the kinetic constants for 1'-hydroxylation of estragole in the liver and to a minor extent also by the kinetic constants for epoxidation, which is the most important competing metabolic pathway to 1'-hydroxylation in the liver. In the case of 1'-sulfooxyestragole, the predicted formation of this metabolite depends on parameters that determine the formation of 1'-hydroxyestragole, but formation of this metabolite is predominantly influenced by the kinetic constants for the formation of 1'-oxoestragole and the kinetic constants for formation of 1'-sulfooxyestragole itself. This indicates that oxidation of 1'-hydroxyestragole is an important competing metabolic pathway to sulfonation due to its higher catalytic efficiency. Physiological parameters (bw, tissue volumes, and blood

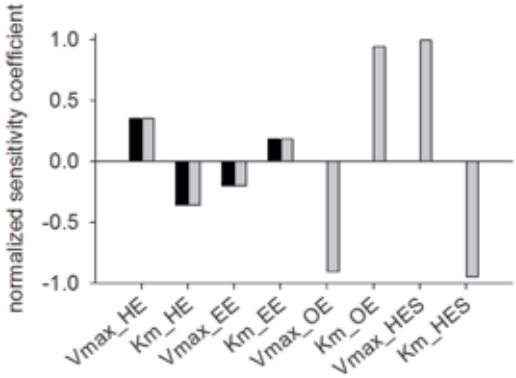

Figure 7. Sensitivity of the predicted formation of 1'-hydroxyestragole (black bars) and 1'-sulfooxyestragole (grey bars) to different model parameters. The Vmax and Km correspond to the maximum rate of formation and the Michaelis-Menten constant for the formation of the different metabolites in the liver: 1'-hydroxyestragole (HE), estragole-2',3'-oxide (EE), 1'-oxoestragole (OE) and 1'-sulfooxyestragole (HES).

flow rates) and partition coefficients do not significantly affect the relative extent of formation of 1'-hydroxyestragole and 1'-sulfooxyestragole in human liver. Differences in, for instance, estimated tissue:blood partition coefficients between human and rat will therefore not affect the comparison between these species in formation of 1'-hydroxyestragole and 1'-sulfooxyestragole.

Predicted species differences bioactivation and detoxification of estragole

Comparison of the *in vitro* metabolic data for rat and human gave some insight into whether species differences are to be expected, but do not reveal the integrated effect of these difference in the overall formation of 1'-hydroxyestragole and 1'-sulfooxyestragole. Using PBBK modeling as a platform for integrating *in vitro* metabolic data, a comparison can be made between humans and male rats in the relative extent of bioactivation and detoxification of estragole. Because the formation of 1'-oxoestragole from 1'-hydroxyestragole was previously not described in the PBBK model for male rat, the rat model was adjusted by the addition of this metabolic route using the kinetic data obtained in the present study to allow equal comparison between the

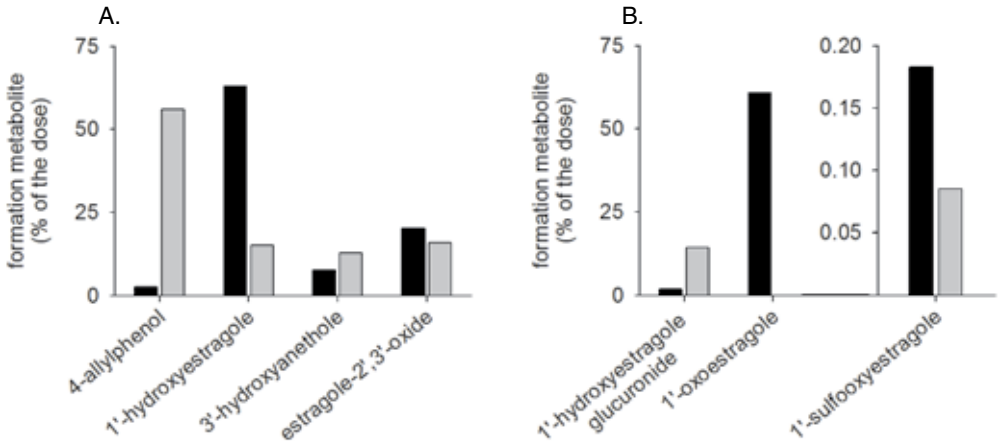

Figure 8. PBBK model-predicted formation of phase I metabolites in human (black bar) and male rat (grey bar) following an oral dose of 0.07 mg/kg bw estragole (A) and the metabolites formed from 1'-hydroxyestragole in human and male rat at this oral dose (B).

rat and human model. The evaluation of species difference presented hereafter is focused on dose levels relevant for dietary human intake. In Figure 8A the predicted formation of phase I metabolites in human and male rat following an oral dose of 0.07 mg/kg bw estragole is displayed. Major differences between human and male rat in formation of 4-allylphenol and 1'-hydroxyestragole are observed, whereas the formation of 3'-hydroxyanethole and estragole-2',3'-oxide are similar in the two species. In the case of 4-allylphenol, the formation of this metabolite is 20-fold lower in human compared to male rat, which is due to the absence of this metabolic route in human lung and kidney, whereas formation of 4-allylphenol occurs with high affinity in male rat lung and kidney. Formation of 1'-hydroxyestragole is 4-fold higher in human, which is due to the lower extent of *O*-demethylation, but also to the higher efficiency for 1'-hydroxylation in human. Figure 8B displays the metabolites formed from 1'-hydroxyestragole in human and male rat. The greater part of the 1'-hydroxyestragole that is formed in human is oxidized to 1'-oxoestragole, which is due to the relative high efficiency for oxidation of 1'-hydroxyestragole in comparison to glucuronidation and sulfonation. When the oxidation of 1'-hydroxyestragole is added to the rat PBBK model, the model predicts low formation of this metabolite at a level corresponding of 0.1% of the dose. This is due to the fact that the catalytic efficiency for the formation of 1'-oxoestragole in male rat is much lower in comparison to glucuronidation of 1'-hydroxyestragole. In male rat 1'-hydroxyestragole is almost completely metabolized to 1'-hydroxyestragole glucuronide. Comparison of the predicted formation of the ultimate carcinogenic metabolite between human and male rat reveals that the predicted overall formation of 1'-sulfooxyestragole is approximately 2-fold higher in human liver.

Discussion

A PBBK model for estragole metabolism in human was defined based on *in vitro* kinetic data, providing possibilities to evaluate the relative extent of

different metabolic routes at dose levels relevant for dietary human intake. With the model obtained, the occurrence of species differences in metabolic activation of estragole was evaluated by comparison of the outcomes of the defined model with those of the previously defined PBBK model for estragole in male rat (Punt *et al.*, 2008).

The performance of the human PBBK model was evaluated by comparing the predicted formation of 4-allylphenol and 1'-hydroxyestragole glucuronide to the reported levels of these metabolic routes in humans exposed to a single oral dose of estragole (Sangster *et al*., 1987). Formation of both 4-allylphenol and 1'-hydroxyestragole glucuronide were predicted within one order of magnitude of the reported levels of these metabolites, which can be considered acceptable within the present state-of-the-art of integrating *in vitro* data and PBBK modeling (Hissink *et al*.,1997; Quick and Shuler, 1999; Lupfert and Reichel, 2005), and provides an indication that the critical metabolic pathways have been captured in the model.

Based on the approach of identifying principal metabolic pathways of estragole in incubations with human tissue fractions, the present study reveals that oxidation of 1'-hydroxyestragole is the main detoxification route of 1'-hydroxyestragole in humans. Validation of the formation of this metabolite against human *in vivo* data was, however, not possible, since excretion of this metabolite or its conjugates was not analyzed in humans exposed to estragole (Sangster *et al*., 1987). In this study on humans exposed to estragole (Sangster et al., 1987), the greater part of the urinary metabolites (34-42% of the oral dose) could not be identified due to insufficient knowledge of the nature of the excreted metabolites. In addition, a significant proportion of ~30% of the radioactivity was not recovered within 48 h (Sangster et al., 1987). It was suggested by the authors that this incomplete recovery of radioactivity could be due to premature termination of the collection of exhaled $^{14}CO_2$ after 8 h, suggesting that metabolic conversion of estragole is not complete within this time-frame (Sangster et al., 1987). The outcomes of the PBBK model of the present study indicate, however, that metabolic conversion of estragole is essentially complete within 8 h and might suggest

another reason for incomplete recovery of radioactivity, which could, for instance, be due to enterohepatic circulation of metabolites. Overall, the large proportion of unidentified metabolites in humans might reflect the formation of 1'-oxoestragole, which was predicted to amount 62.7 % of the dose, based on the PBBK model for estragole in human developed in the present study. Obviously, the relevance of the oxidation pathway of 1'-hydroxyestragole in humans needs to be further confirmed *in vivo*.

Although the extent of oxidation of 1'-hydroxyeestragole to 1'-oxoestragole could not be evaluated against human *in vivo* data, evidence for the presence *in vivo* of the oxidation route of 1'-hydroxyestragole can be found in experiments with rats exposed to estragole or other alkenylbenzenes. Solheim and Scheline (1973) described the excretion of small amounts of amino conjugates in rats exposed to estragole, which were assumed to originate from 1'-oxoestragole. In the case of the related alkenylbenzene safrole, Fennell *et al*. (1984) described the excretion of GS-1'-oxosafrole and *N*-acetylcystein-1'-oxosafrole in rats exposed to the 1'-hydroxy metabolite of this compound, indicating formation of 1'-oxo metabolites. The excretion of 1'-oxosafrole conjugates was however relatively low in comparison to the excretion of glucuronosyl conjugates of 1'-hydroxysafrole, which is in agreement with results of the present study revealing that the predicted formation of 1'-oxoestragole is relatively low and of minor importance in male rat in comparison to the predicted glucuronidation of 1'-hydroxyestragole.

Parameter sensitivity analysis of the predicted formation of 1'-sulfooxyestragole in humans showed that uncertainty and variability in the kinetic constants of the formation of 1'-oxoestragole and 1'-sulfooxyestragole influence the model predicted formation of 1'-sulfooxyestragole to a high extent. Variability in these kinetic constants can for instance arise from individual differences in the expression of the enzymes involved in these metabolic routes in humans. Identification of these enzymes and determination of the individual enzyme kinetic data for the formation of these metabolites could give insight in the level of variability of these parameters (Bogaards *et al.*, 2001). Identifying the kinetics for individual isoenzymes was,

however, beyond the scope of the present study. In addition to variability, uncertainty in the kinetic constants for the formation of 1'-oxoestragole and 1'-sulfooxyestragole can come from scaling of *in vitro* metabolic data, but also from the level of precision of the determined *in vitro* kinetic constants. Both 1'-oxoestragole and 1'-sulfooxyestragole were for instance quantified indirectly by trapping the formed transient metabolites with respectively glutathione and 2'-deoxyguanosine. The maximum rate of formation of these metabolites could therefore be underestimated to some extent. Furthermore in the case of 1'-sulfooxyestragole, only the major adduct of the three observed adducts with 2'-deoxyguanosine was quantified, leading to underestimation of the maximum rate of formation of this metabolite. Overall it should be stated that the present model derived from integrating *in vitro* metabolic data using PBBK modeling as a platform, does not allow conclusions based on absolute outcomes. However, the relative importance of different metabolic pathways in humans can be predicted and moreover a relative comparison of species differences in the extent of bioactivation and detoxification can be made, since the *in vitro* metabolic data for human and male rat are obtained with similar methods.

The present study mainly focused on the extent of species differences at dose levels relevant for dietary human intake, which was considered most relevant. It can be expected that at higher doses species differences between human and male rat will be different from those identified at low doses of estragole. It has for instance previously been demonstrated that formation of 1'-sulfooxyestragole in male rat can relatively increase with increasing doses (Punt *et al.*, 2008), whereas formation of this metabolite in humans is observed to relatively decrease with increasing doses. Based on the developed models it is predicted that at higher doses the relative extent of bioactivation can become 1.5 to 2-fold lower in humans compared to male rats. This was, however, only observed at doses of 100 to 300 mg/kg bw, which are orders of magnitude higher than the dose levels relevant for dietary human intake. At a dose-range within one order of magnitude of the estimated average dietary human intake of 0.07 mg/kg bw, the differences between human and male rat

were as described in the present study.

Comparison of the predicted extent of bioactivation of estragole between humans and male rats at a dose of 0.07 mg/kg bw showed that the formation of the proximate carcinogenic metabolite 1'-hydroxyestragole is higher in human liver compared to male rat liver, which is mainly due to the relative lower extent of detoxification of estragole through *O*-demethylation in human. This observation that *O*-demethylation of estragole is a less important metabolic route in humans than in rats is supported by *in vivo* data showing that only 10-15% of the oral dose was excreted through this metabolic route in humans at a dose of 0.001 mg/kg bw (Sangster *et al*., 1987), whereas in rats this metabolic route accounted for 53% of the oral dose at a similar dose level of 0.05 mg/kg bw (Anthony *et al*., 1987). The relatively high formation of the *O*-demethylated metabolite 4-allylphenol in male rats at low dose levels has previously been demonstrated to be due to formation of 4-allylphenol with high affinity in lung and kidney tissue of male rat (Punt *et al*., 2008). Saturation of this metabolic pathway in the lung and kidney at higher doses resulted in a relative decrease in formation of this metabolite, leading to relative increase formation of 1'-hydroxyestragole and consequently 1'-sulfooxyestragole with increasing doses of estragole (Punt *et al*., 2008). Since *O*-demethylation was not observed to occur in incubations with human lung and kidney fractions, such a relative increase in bioactivation of estragole with increasing dose is not predicted to occur in the human. Formation of 1'-hydroxyestragole, was even predicted to decrease to some extent with increasing dose, due to saturation of this and not of other metabolic routes for estragole at higher dose levels.

The absence of phase I metabolism in human lung and kidney can be rationalized when the P450 enzymes involved are not expressed in these tissues. Jeurissen *et al*., (2007) recently identified that P450 1A2 and P450 2A6 play a pivotal role in estragole 1'-hydroxylation. These enzymes are absent or only poorly expressed in human lung and kidney (Raunio *et al.*, 1995a, Shimada *et al.*, 1996), which is in line with the observation that no significant 1'-hydroxylation activity occurs in the corresponding tissue fractions. The P450 enzymes involved in *O*-demethylation have not been identified so far,

but assuming that similar P450 enzymes as for 1'-hydroxylation are involved, this would explain that overall no phase I metabolism is detected in human lung and kidney tissue.

Even though formation of the proximate carcinogenic metabolite 1'-hydroxyestragole is predicted to be 4–fold higher in human, the formation of the ultimate carcinogenic metabolite 1'-sulfooxyestragole is predicted to be only 2–fold higher in human. This is due to the fact that the major part of 1'-hydroxyestragole is oxidized to 1'-oxoestragole in human, due to the much higher catalytic efficiency for oxidation compared sulfonation and glucuronidation of 1'-hydroxyestragole. Although 1'-oxoestragole can potentially react with DNA (Phillips *et al*., 1981), this metabolite, which is a soft acid, is not expected to contribute to the genotoxicity of estragole *in vivo*, since it can readily react with glutathione (soft base) rather than with DNA molecules (hard bases) (Fennell *et al*., 1984). This is supported by literature data revealing that 1'-oxoestragole is not carcinogenic *in vivo* in mice (Wiseman et al., 1987). For this reason formation of 1'-oxoestragole in the human can be considered as the major detoxification pathway of 1'-hydroxyestragole. Detoxification of 1'-hydroxyestragole through glucuronidation was predicted to hardly occur in human due to a very low catalytic efficiency of this metabolic route in comparison to oxidation of 1'-hydroxyestragole. This observation is supported by *in vivo* data showing that only 0.3% of the oral dose was excreted through this metabolic route in humans at a dose of 0.001 mg/kg bw (Sangster *et al*., 1987). In contrast, in male rat glucuronidation of 1'-hydroxyestragole is predicted to be the major detoxification pathway of 1'-hydroxyestragole, due to the higher efficiency of this metabolic route compared to oxidation of 1'-hydroxyestragole.

When extrapolating cancer data from high dose animal experiments to the low dose human situation it is important to take species differences in metabolism and metabolic activation into account. Comparison of *in vitro* metabolic data for estragole bioactivation and detoxification between human and male rat, as done in the present study using PBBK modeling to integrate these metabolic data, provides an approach that allows evaluation of whether

species differences in bioactivation are to be expected. Overall it is shown that, even though significant species differences in the relative extent of different metabolic pathways occur between human and male rat, these differences ultimately only result in a minor difference in the PBBK model based predicted formation of 1'-sulfooxyestragole at dose levels relevant for dietary human intake It is, therefore, concluded that in spite of significant differences in the catalytic efficiency of specific individual estragole biotransformation reactions there is a minor influence of species differences between human and male rats on the ultimate overall bioactivation of estragole to its ultimate carcinogenic DNA reactive metabolite 1'-sulfooxyestragole.

Acknowledgments

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References

Anthony, A., Caldwell, J., Hutt, A. J., and Smith, R. L. (1987). Metabolism of estragole in rat and mouse and influence of dose size on excretion of the proximate carcinogen 1'-hydroxyestragole. Food Chem Toxicol 25, 799-806.

Bogaards, J. J.P., Freidig, A. P., and Van Bladeren, P. J. (2001). Prediction of isoprene diepoxide levels in vivo in mouse, rat and man using enzyme kinetic data in vitro and physiologically-based pharmacokinetic modelling. Chem Biol Interact 138, 247-265.

Brown, R. P., Delp, M. D., Lindstedt, S. L., Rhomberg, L. R., and Beliles, R. P. (1997). Physiological parameter values for physiologically based pharmacokinetic models. Toxicol Ind Health 13, 407-484.

DeJongh, J., Verhaar, H. J.M., and Hermens, J. L.M. (1997). A quantitative property-property relationship (QPPR) approach to estimate in vitro tissue-

blood partition coefficients of organic chemicals in rats and humans. Arch. Toxicol 72, 17-25.

Drinkwater, N. R., Miller, E. C., Miller, J. A., and Pitot, H. C. (1976). Hepatocarcinogenicity of estragole (1-allyl-4-methoxybenzene) and 1'-hydroxyestragole in the mouse and mutagenicity of 1'-acetoxyestragole in bacteria. J Natl Cancer Inst 57, 1323-1331.

Evans, M. V. and Andersen, M. E. (2000). Sensitivity analysis of a physiological model for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD): assessing the impact of specific model parameters on sequestration in liver and fat in the rat. Toxicol Sci 54, 71-80.

Fennell, T. R., Miller, J. A., and Miller, E. C. (1984). Characterization of the biliary and urinary glutathione and N-acetylcysteine metabolites of the hepatic carcinogen 1'-hydroxysafrole and its 1'-oxo metabolite in rats and mice. Cancer Res 44, 3231-3240.

Fisher, M. B., Campanale, K., Ackermann, B. L., VandenBranden, M., and Wrighton, S. A. (2000). In vitro glucuronidation using human liver microsomes and the pore-forming peptide alamethicin. Drug Metab Dispos 28, 560-566.

Guenthner, T. M. and Luo, G. (2001). Investigation of the role of the 2',3'-epoxidation pathway in the bioactivation and genotoxicity of dietary allylbenzene analogs. Toxicology 160, 47-58.

Hissink, A. M., Van Ommen, B., Kruse, J., and Van Bladeren, P. J. (1997). A physiologically based pharmacokinetic (PB-PK) model for 1,2-dichlorobenzene linked to two possible parameters of toxicity. Toxicol. Appl Pharmacol 145, 301-310.

Jeurissen, S. M. F., Punt, A., Boersma, M. G., Bogaards, J. J., Fiamegos, Y. C., Schilter, B., Van Bladeren, P. J., Cnubben, N.H.P., and Rietjens, I. M. C. M. (2007). Human cytochrome P450 enzyme specificity for the bioactivation of estragole and related alkenylbenzenes. Chem Res Toxicol 20, 798-806.

Lin, J. H. and Wong, B. K. (2002). Complexities of glucuronidation affecting in vitro in vivo extrapolation. Curr Drug Metab 3, 623-646.

Luo, G., Qato, M. K., and Guenthner, T. M. (1992). Hydrolysis of the 2',3'-

allylic epoxides of allylbenzene, estragole, eugenol, and safrole by both microsomal and cytosolic epoxide hydrolases. Drug Metab Dispos 20, 440-445.

Lupfert, C. and Reichel, A. (2005). Development and application of physiologically based pharmacokinetic-modeling tools to support drug discovery. Chem Biodivers 2, 1462-1486.

Medinsky, M. A., Leavens, T. L., Csanady, G. A., Gargas, M. L., and Bond, J. A. (1994). In vivo metabolism of butadiene by mice and rats: a comparison of physiological model predictions and experimental data. Carcinogenesis 15, 1329-1340.

Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A., and Miller, J. A. (1983). Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. Cancer Res 43, 1124-1134.

Phillips, D. H., Miller, J. A., Miller, E. C., and Adams, B. (1981). Structures of the DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1'-hydroxyestragole. Cancer Res 41, 176-186.

Phillips, D. H., Reddy, M. V., and Randerath, K. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. II. Newborn male B6C3F1 mice. Carcinogenesis 5, 1623-1628.

Punt, A., Delatour, T., Scholz, G., Schilter, B., Van Bladeren, P. J., and Rietjens, I. M. C. M. (2007). Tandem Mass Spectrometry Analysis of N^2-(trans-Isoestragol-3'-yl)-2'-deoxyguanosine as a Strategy to Study Species Differences in Sulfotransferase Conversion of the Proximate Carcinogen 1'-Hydroxyestragole. Chem Res Toxicol 20, 991-998.

Punt, A., Freidig, A. P., Delatour, T., Scholz, G., Boersma, M. G., Schilter, B., Van Bladeren, P. J., and Rietjens, I. M. C. M. (2008). A physiologically based biokinetic (PBBK) model for estragole bioactivation and detoxification in rat. Toxicol Appl Pharmacol 231, 248-259.

Quick, D. J. and Shuler, M. L. (1999). Use of in vitro data for construction of

a physiologically based pharmacokinetic model for naphthalene in rats and mice to probe species differences. Biotechnol Prog 15, 540-555.

Ramsey, J. C. and Andersen, M. E. (1984). A physiologically based description of the inhalation pharmacokinetics of styrene in rats and humans. Toxicol Appl Pharmacol 73, 159-75.

Randerath, K., Haglund, R. E., Phillips, D. H., and Reddy, M. V. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. Carcinogenesis 5, 1613-1622.

Raunio H., Pasanen M., Mäenpää J., Hakkola J & Pelkonen O. (1995). Expression of extrahepatic cytochrome P450 in humans. In Advances in Drug Metabolism in Man (G.M. Pacifici, and G.N. Fracchia, Eds.), pp 234-287. European Commission, Office for Official Publications of the European Communities, Luxembourg.

Sangster, S. A., Caldwell, J., Hutt, A. J., Anthony, A., and Smith, R. L. (1987). The metabolic disposition of [methoxy-^{14}C]-labelled trans-anethole, estragole and *p*-propylanisole in human volunteers. Xenobiotica 17, 1223-1232.

SCF (2001). Opinion of the scientific committee on food on estragole (1-allyl-4-methyxybenzene). http://ec.europa.eu/food/fs/sc/scf/out104_en.pdf.

Shimada, T., Yamazaki, H., Mimura, M., Wakamiya, N., Ueng, Y., Genguerich, F.P., and Inui, Y. (1996). Characterization of microsomal cytochrome P450 enzymes involved in the oxidation of xenobiotic chemicals in human fetal liver and adult lungs. Drug Metab Dispos 24, 515-22.

Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., Rogers, A. E., Caldwell, J., and Sipes, I. G. (2002). Safety assessment of allylalkoxybenzene derivatives used as flavouring substances - methyl eugenol and estragole. Food Chem Toxicol 40, 851-870.

Solheim, E. and Scheline, R. R. (1973). Metabolism of alkenebenzene derivatives in the rat. I. *p*-Methoxyallylbenzene (Estragole) and *p*-methoxypropenylbenzene (Anethole). Xenobiotica 3, 493-510.

Wiseman, R. W., Fennell, T. R., Miller, J. A., and Miller, E. C. (1985). Further characterization of the DNA adducts formed by electrophilic esters of the hepatocarcinogens 1'-hydroxysafrole and 1'-hydroxyestragole in vitro and in mouse liver in vivo, including new adducts at C-8 and N-7 of guanine residues. Cancer Res 45, 3096-3105.

Wiseman, R. W., Miller, E. C., Miller, J. A., and Liem, A. (1987). Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J x C3H/HeJ F_1 mice. Cancer Res 47, 2275-2283.


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Human cytochrome P450 enzyme specificity for bioactivation of estragole to the proximate carcinogen 1'-hydroxyestragole

Based on: Jeurissen SMF, Punt A, Boersma G, Bogaards JJP, Fiamegos YC, Schilter B, Van Bladeren PJ, Cnubben NHP, Rietjens IMCM
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Abstract

In the present study human cytochrome P450 enzymes involved in the bioactivation of estragole to its proximate carcinogen 1'-hydroxyestragole were identified. Incubations with Supersomes revealed that all enzymes tested, except P450 2C8, are intrinsically able to catalyse estragole 1'-hydroxylation. Experiments with Gentest microsomes, expressing P450 enzymes to roughly average liver levels, indicated that P450 1A2, 2A6, 2C19, 2D6, and 2E1 might contribute to estragole 1'-hydroxylation in the human liver. Especially P450 1A2 is an important enzyme based on the correlation between P450 1A2 activity and estragole 1'-hydroxylation in fifteen human liver microsomal samples and inhibition of estragole 1'-hydroxylation by the P450 1A2 inhibitor R-naphthoflavone. Kinetic studies revealed that, at physiologically relevant concentrations of estragole, P450 1A2 and 2A6 are the most important enzymes for bioactivation in the human liver showing enzyme efficiencies (k_{cat}/K_m) of, respectively, 59 and 341 min^{-1} mM^{-1}. Only at relatively high estragole concentrations, P450 2C19, 2D6, and 2E1 might contribute to some extent to the bioactivation of estragole in the human liver. A 2.7-fold difference in activities was found between the fifteen human liver microsomes of the correlation study (range: 0.60 – 1.63 nmol min^{-1} nmol $P450^{-1}$). Therefore, interindividual differences might cause variation in sensitivity towards estragole. Polymorphisms in P450 2A6 leading to poor metabolizer phenotypes might diminish the chances on bioactivation of estragole, whereas life-style factors that increase the activity of P450 1A2 such as cigarette smoking and consumption of charbroiled food might increase these chances.

Introduction

The alkenylbenzene estragole (4-allyl-1-methoxybenzene) is a natural ingredient of herbs such as tarragon, basil, fennel, and anise (SCF, 2001a). Estragole is also used as a flavoring substance in, among others, baked goods, nonalcoholic beverages, and hard and soft candy (SCF, 2001a; Hall and Oser, 1965). The Scientific Committee on Food (SCF) of the European Union concluded that estragole is genotoxic and carcinogenic, and this will result in restrictions in the use of this pure compound as a food additive in the European Union (SCF, 2001a; CEC, 2002). The estimated average intake (for consumers only) was estimated by the SCF to amount to 4.3 mg/day, which is equal to 72 µg/kg bw/day assuming 60 kg body weight. On the other hand, the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) of the United States concluded that the present exposure to estragole from food, mainly spices and added as such, does not pose a significant cancer risk (Smith *et al*., 2002). This conclusion was based on evidence of a nonlinear relationship between dose and profiles of metabolism, metabolic activation, and covalent binding of estragole to proteins and DNA (Smith *et al*., 2002). The FEMA Expert Panel estimated the mean daily per capita intake ("eaters only") for estragole to be less than 10 µg/kg bw/day, which is equal to less than 0.6 mg/day for a 60 kg person.

Figure 1 shows the most important bioactivation pathway for estragole. The bioactivation pathway starts with the conversion of estragole into its proximate carcinogen 1'-hydroxyestragole by P450 enzymes (Drinkwater *et al*., 1976; Chan and Caldwell, 1992). Other phase I metabolic reactions of

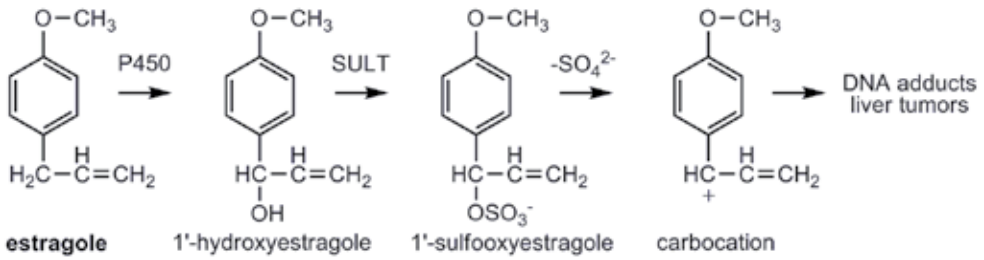

Figure 1. Bioactivation pathway of estragole

estragole include *O*-demethylation, 2',3'epoxidation and 3'-hydroxylation, and represent detoxification pathways (Solheim and Scheline, 1973; Phillips *et al.*, 1981; Fennell *et al.*, 1984; Anthony *et al.*, 1987; Sangster *et al.*, 1987; Luo *et al.*, 1992; Guenthner and Luo, 2001). Sulfonation of 1'-hydroxymetabolite by sulfotransferases leads to the formation of the ultimate carcinogenic species 1'-sulfooxyestragole. 1'-Sulfooxyestragole is unstable in an aqueous environment, and upon loss of the sulfate group, a carbocation remains. This carbocation may bind to DNA and proteins and may cause DNA adducts and liver tumors (Boberg *et al.*, 1983). Detoxification of 1'-hydroxyestragole can occur through glucuronidation and oxidation, the latter resulting in formation of 1'-oxoestragole (Solheim and Scheline, 1973; Anthony *et al.*, 1987; Sangster *et al.*, 1987). The oxidation of 1'-hydroxyestragole is identified to be an important metabolic route of 1'-hydroxyestragole in humans (Chapter 4).

To better estimate the risks associated with the consumption of estragole, it is important to know which enzymes catalyze the different biotransformation steps and to which extent. The P450 enzymes catalyzing the bioactivation of estragole into 1'-hydroxyestragole have not yet been identified. Knowledge of the P450 enzymes involved may identify groups of people at increased or reduced risk for the possible adverse effects of estragole, due to differences in the activities of the enzymes involved in bioactivation caused by genetic polymorphisms or lifestyle factors. Recently, the human P450 enzymes responsible for the first step in the main bioactivation pathway, 1'-hydroxylation, were identified for the related alkenylbenzenes safrole and methyleugenol (Jeurissen *et al.*, 2004; Ueng *et al.*, 2004; Jeurissen *et al.*, 2006a). In this paper, we describe the identification of the P450 enzymes involved in 1'-hydroxylation of estragole in human liver and the kinetics for those P450 enzymes. To investigate which P450 enzymes are able to 1'-hydroxylate estragole in the human liver, incubations were performed with Supersomes en Gentest microsomes expressing single human P450 enzymes, the latter to roughly average liver levels. In addition, a correlation study was performed in which estragole was incubated with a series of fifteen human liver microsomes. The 1'-hydroxylation rates obtained were correlated with

the activities of these microsomes toward specific substrates for nine different P450 enzymes. Furthermore, the effect of specific inhibitors on the extent of 1'-hydroxylation of estragole by pooled human liver microsomes was investigated. Finally, the kinetics for estragole 1'-hydroxylation of the P450 enzymes involved were determined.

Materials and Methods

Chemicals

Ascorbic acid, acetone, and DMSO were purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid was from Acros (Geel, Belgium). NADPH was obtained from Boehringer (Mannheim, Germany). Hydrochloric acid (37%) was purchased from Roche Diagnostics (Mannheim, Germany). Tris(hydroxymethylaminomethane) was obtained from Gibco BRL Life Technologies (Paisley, Scotland). Acetonitrile and methanol were HPLC grade from Lab-Scan, Analytical Sciences (Dublin, Ireland). Estragole (4-allylanisole), *p*-anisaldehyde, α-naphthoflavone, coumarin, quinidine, and ketoconazole, were purchased from Sigma-Aldrich (Steinheim, Germany). (*S*)-*N*-3-Benzylnirvanol, monoclonal antibody for human P450 2B6 (MAB 2B6), and monoclonal antibody for human P450 2C8 (MAB 2C8) were obtained from Gentest (Woburn, MA). Sulfaphenazole was purchased from Ultrafine Chemicals (Manchester, United Kingdom).

Microsomal preparations

Supersomes, prepared from baculovirus-infected insect cells expressing the human individual P450 enzymes 1A2, 2A6, 2B6, 2C8, 2C9*1, 2C19, 2D6*1, 2E1, and 3A4, were obtained from BD Gentest. Also, in all cells, human P450 reductase and (except for P450 1A2) cytochrome b5 were coexpressed. Gentest microsomes, prepared from lymphoblastoid cell lines expressing the same human individual P450 enzymes (described in Crespi *et al*., 1991 and Crespi *et al*., 1993), were obtained from BD Gentest. For P450 2A6, 2C8,

2C9*1, 2D6*1, 2E1, and 3A4, human P450 reductase was coexpressed. For the other enzymes, the catalytic activity was supported by reductase activity endogenous to the cell line. In general, in Gentest microsomes, the activities toward enzyme selective substrates, expressed as nmol min^{-1} mg $protein^{-1}$, were roughly at the same level as the mean activities found in human liver microsomes (the ratio between the activity of the Gentest microsomes and the human liver microsomes toward typical substrates was reported by the producer to be approximately 1 for P450 2A6, 2B6, 2C19, 2C9, and 2E1; 0.5 for P450 1A2; 0.3 for P450 2C8; 3.3 for P450 2D6; and 0.25 for P450 3A4). In Supersomes, the enzyme levels were much higher than those in the human liver (the ratio between the activity of the Supersomes and the human liver microsomes varied from 1.7 to 100 according to the data provided by the producer). Microsomes from fifteen individual human livers were obtained from Human Biologics (Phoenix, AZ). Pooled human liver microsomes (mixed gender pool) were obtained from BD Gentest.

Synthesis of 1′-hydroxyestragole

1'-Hydroxyestragole was synthesized as described previously for 1'-hydroxysafrole (Jeurissen *et al*., 2004), starting from *p*-anisaldehyde instead of piperonal, based on the method developed by Tamayo and Ossorio (1948), which was adapted by Suga *et al*. (1966) and Borchert *et al*. 1973). GC-MS analysis was performed as previously described for safrole (Jeurissen *et al*., 2004). The purity of 1'-hydroxyestragole was estimated to be more than 98% according to GC-MS and HPLC analyses. Structural confirmation was obtained from the UV (λ_{max} 229 nm and λ_{max} 272 nm) and MS data of the compound [*m/z* (rel. int. %) 109 (100), 164 (84), 135 (70), 77 (66), 121 (65), 163 (63), 137 (61), 133 (43), 94 (42), 108 (38)] that were comparable to those reported in the literature (Drinkwater *et al*., 1976).

Incubations with recombinant enzymes

Microsomal incubations with estragole, using Supersomes or Gentest microsomes expressing one single P450 enzyme, were performed in 100 µL

of incubation mixture containing (final concentrations) 3 mM NADPH, 1 mM ascorbic acid, and Supersomes at 0.3 nmol P450 mL^{-1} or microsomes at 1 mg protein mL^{-1} in 0.2 M Tris-HCl, pH 7.4. The reaction was started by adding the substrate estragole (500 μM final concentration, added from a 50 mM stock solution in DMSO). Incubations were performed at 37 °C, and the reaction was terminated after 20 min by adding 25 μL of ice-cold acetonitrile. Product formation was linear in time under these conditions. Incubations were performed in triplicate, and all samples were centrifuged for 5 min at 16,000*g*, and the supernatant was stored at −20 °C until HPLC analysis for quantification of 1'-hydroxyestragole.

Correlation study

The human liver microsomes from Human Biologics were characterized with respect to 7-ethoxyresorufin *O*-dealkylase, coumarin 7-hydroxylase, 7-ethoxy-4-trifluoromethylcoumarin *O*-dealkylase, diclofenac 4'-hydroxylase, *S*-mephenytoin 4'-hydroxylase, bufuralol 1'-hydroxylase, chlorzoxazone 6-hydroxylase, and testosterone 6β-hydroxylase activities, as described previously (Bogaards *et al*., 1995; Bogaards *et al*., 1996; Bogaards *et al*., 2000). Paclitaxel 6α-hydroxylation was determined by incubating 1 mg mL^{-1} human liver microsomes at 37 °C for 15 min in a 200 μL incubation mixture containing 0.1 M potassium phosphate, pH 7.4, 3 mM NADPH, and 50 μM paclitaxel. The reaction was terminated by the addition of 100 μL of ice-cold acetonitrile. After centrifugation for 5 min at 2750*g*, the supernatant was analyzed by HPLC using UV detection (230 nm). HPLC analysis was performed using a 250 mm × 4.6 mm Inertsil ODS-3 column, a gradient of water and acetonitrile, and a flow rate of 1.0 mL min^{-1}. The formation of the product was quantified using a calibration curve of 6α-hydroxypaclitaxel. Data on protein and P450 content were provided by the supplier. Incubations with human liver microsomes from Human Biologics were performed identically to the incubations with Gentest microsomes described above, using an estragole concentration of 500 μM and microsomes in a concentration of 1 mg protein mL^{-1} (range, 0.18–0.82 nmol P450 per mL). Incubations were

performed in duplicate. Samples were centrifuged for 5 min at 16,000*g*, and the supernatant was stored at −20 °C until HPLC analysis for quantification of 1'-hydroxyestragole.

Inhibition study

Microsomal incubations, using pooled human liver microsomes (1 mg protein per mL; 0.36 nmol P450 per mL), were performed in 200 µL incubations containing (final concentrations) 3 mM NADPH, 1 mM ascorbic acid, and 0.2 M Tris-HCl, pH 7.4. To these incubations, 2 µL of a 100× concentrated stock solution of one of the chemical inhibitors in methanol was added as follows: α-naphthoflavone for P450 1A2 (final concentration, 1 µM), coumarin for P450 2A6 (final concentration, 10 µM), sulfaphenazole for P450 2C9 (final concentration, 10 µM), (S)-N-3-benzylnirvanol for P450 2C19 (final concentration, 5 µM), quinidine for P450 2D6 (final concentration, 5 µM), acetone for P450 2E1 (final concentration, 1% v/v), and ketoconazole for P450 3A4 (final concentration, 1 µM). For P450 2B6 and P450 2C8, 5 µL of their respective antibody was added (5 µL per 100 µg microsomal protein). The selection of the specific chemical inhibitors and their concentrations was based on either literature data (Bogaards *et al*., 1995; Bogaards *et al*., 2000; Li *et al*., 1997) or data of the manufacturer [for (S)-N-3-benzylnirvanol, MAB 2B6, and MAB 2C8, see the Gentest catalog]. After 5 min of preincubation, 2 µL of 10 mM estragole (final concentration, 100 µM) was added. The reactions were terminated after 20 min of incubation by adding 50 µL of acetonitrile. All incubations were performed in triplicate, and control incubations without NAPDH and without chemical inhibitor/antibody were performed. Samples were centrifuged for 5 min at 16,000g, and the supernatant was stored at −20 °C until HPLC analysis for quantification of 1'-hydroxyestragole.

K_{cat} and K_m determination for estragole

For Gentest microsomes expressing P450 1A2, 2A6, 2C19, 2D6, and 2E1, the k_{cat} and K_m values for estragole 1'-hydroxylation were determined by incubating these microsomes with estragole concentrations ranging from

0 to 500 μM (for P450 1A2, 2A6, and 2E1) or from 0 to 1000 μM (for P450 2C19 and P450 2D6 in triplicate and for P450 1A2 in quadruplicate). The incubation conditions were similar to the incubation conditions with Gentest microsomes described above. The data from the kinetic studies with Gentest microsomes were fitted to the standard Michaelis–Menten equation:

$$v = V_{max}/(1 + (K_m/[S])$$

in which [S] = substrate concentration, using the LSW data analysis toolbox (version 1.1.1, MDL Information Systems, Inc.). The parameters k_{cat}, K_m, and k_{cat}/K_m were determined.

HPLC analysis

Aliquots (50 μL) of each sample were analyzed on an Alltima C18 5 μm column, 150 mm × 4.6 mm (Alltech, Breda, The Netherlands) using an HPLC (Waters Alliance 2695 Separations Module) coupled to a Waters 2996 photodiode array detector. The gradient was made with ultrapure water containing 0.1% (v/v) trifluoroacetic acid and acetonitrile. The flow rate used was 1.0 mL min^{-1}. HPLC analysis started for 20 min in isocratic mode with 25% (v/v) acetonitrile, followed by a linear increase from 25 to 50% (v/v) acetonitrile over 20 min. The percentage of acetonitrile was increased to 100% over 2 min and kept at 100% for 2 min. The retention time of 1'-hydroxyestragole under these conditions was approximately 16 min. Quantification of the amounts of 1'-hydroxyestragole was performed with a calibration curve measured at 280 nm, made using synthesized 1'-hydroxyestragole. The activities were calculated in nmol 1'-hydroxyestragole min^{-1} nmol $P450^{-1}$ and/or nmol 1'-hydroxyestragole min^{-1} mg $protein^{-1}$.

Statistical analysis

For correlations between the 1'-hydroxylation of estragole and the metabolism of P450 marker substrates, enzyme activities expressed as nmol min^{-1} nmol $P450^{-1}$ were used, because in this way, correlation analysis was

independent of the amount of P450 present in the various samples. Pearson correlation tests were performed to investigate correlations between the metabolism of individual P450 marker substrates and the 1'-hydroxylation of estragole. These statistical analyses were performed with SPSS 10.1 for Windows (SPSS Inc, Chicago, IL). To test whether the inhibition by enzyme specific inhibitors/antibodies was significant, two-sample *t* tests (one-sided, equal variances) were performed, after *F* tests for equal variances were done, using Excel (Microsoft Office 2000).

Results

Formation of 1′-hydroxyestragole by recombinant P450 enzymes

Incubations with Supersomes were performed to define which human P450 enzymes are able to 1'-hydroxylate estragole. The results obtained with the Supersomes (Figure 2) show that many P450 enzymes are intrinsically able to catalyze the bioactivation of estragole. P450 1A2, 2A6, 2C9, 2C19, 2D6, and 2E1 were able to 1'-hydroxylate estragole, whereas for P450 2B6 and P450 3A4, almost no activity was observed, and for P450 2C8, no 1'-hydroxylation activity was observed at all. Data from the incubations with Gentest microsomes

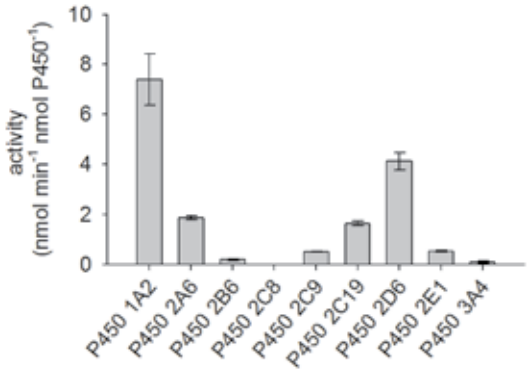

Figure 2. Estragole 1'-hydroxylation activity by Supersomes at a substrate concentration of 500 µM. Bars indicate average activities of triplicate measurements ± SEM (*n* = 3).

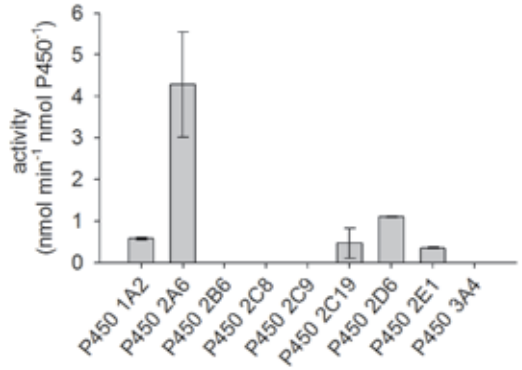

Figure 3. Estragole 1'-hydroxylation activity by Gentest microsomes at a substrate concentration of 500 µM. Bars indicate average activities of triplicate measurements ± SEM (*n* = 3).

are shown in Figure 3. In Figure 3, activities are expressed in nmol min^{-1} nmol $P450^{-1}$, and when expressing the results per mg protein, a comparable pattern of activities is obtained (data not shown). Because in Gentest microsomes, the activities toward enzyme-selective substrates (expressed per mg protein) are in the same order as the mean activities found in human liver microsomes, contrary to Supersomes in which activities are generally much higher than in human liver microsomes, the data in Figure 3 give an impression of the contribution of each enzyme in the human liver. From these data, especially P450 2A6 appears to be active in estragole 1'-hydroxylation. Furthermore, also P450 1A2, 2C19, 2D6, and P450 2E1 showed a moderate activity. Although some activity was observed for Supersomes expressing P450 2B6, 2C9, and 3A4, for Gentest microsomes expressing these enzymes, this was not the case, so it is concluded that these enzymes will not contribute to estragole 1'-hydroxylation in the human liver.

Formation of 1'-hydroxyestragole by human liver microsomes

Table 1 shows the average activities of fifteen human liver microsomes toward nine different P450 substrates. The average rate of 1'-hydroxylation of estragole was 1.20 ± 0.30 nmol 1'-hydroxyestragole min^{-1} nmol $P450^{-1}$. A 2.7-fold variation between different human liver samples was found (range,

Table 1. Correlations between the activities toward P450 enzyme-selective substrates and the formation of 1'-hydroxyestragole by fifteen human liver microsomal samples.

Marker activity[a]	Mean activity ± SD (nmol min^{-1} nmol $P450^{-1}$)	P450 enzyme	Correlation coefficient
EROD	0.15 ± 0.08 (range, 0.02–0.30)	1A	0.71[c]
COUM	3.19 ± 2.64 (range, 0.01–10.4)	2A6	0.41
7-ETC	0.70 ± 0.23 (range, 0.27–1.12)	nonspecific[b]	0.78[c]
PACL	0.55 ± 0.22 (range, 0.18–0.91)	2C8	0.01
DICLF	5.79 ± 2.82 (range, 2.50–12.1)	2C9	0.07
MEPH	0.03 ± 0.06 (range, 0.00–0.23)	2C19	0.43
BUFU	0.38 ± 0.33 (range, 0.05–1.22)	2D6	0.18
CLZOX	6.41 ± 4.13 (range, 1.74–15.0)	2E1	0.06
TEST	10.5 ± 5.16 (range, 2.85–20.7)	3A	0.28

[a] EROD, 7-ethoxyresorufin *O*-dealkylation; COUM, coumarin 7-hydroxylation; 7-ETC, 7-ethoxy-4-trifluoromethylcoumarin *O*-dealkylation; PACL, paclitaxel 6α-hydroxylation; DICLF, diclofenac 4'-hydroxylation; MEPH, *S*-mephenytoin 4'-hydroxylation; BUFU, bufuralol 1'-hydroxylation; CLZOX, chlorzoxazone 6-hydroxylation; and TEST, testosterone 6β-hydroxylation.
[b] 7-ETC is mainly catalyzed by P450 2B6 and P450 1A2 (Bogaards *et al*., 1996).
[c] Statistical significance, $P < 0.05$.

0.60–1.63 nmol min^{-1} nmol $P450^{-1}$). The calculated correlation coefficients between 1'-hydroxylation of estragole and the activities toward all nine substrates are also shown in Table 1. A significant ($P < 0.01$) correlation was found between 7-ethoxyresorufin *O*-dealkylation activity and 1'-hydroxylation of estragole ($r = 0.71$), indicating that P450 1A enzymes are involved in estragole 1'-hydroxylation. Furthermore, a significant correlation between 7-ethoxy-4-trifluoromethylcoumarin *O*-dealkylation (7-ETC) and 1'-hydroxylation of estragole was found ($r = 0.78$). 7-Ethoxy-4-trifluoromethylcoumarin *O*-dealkylation is mainly catalyzed by P450 2B6 and P450 1A2 (Boogaards *et al*., 1996), and because (almost) no intrinsic estragole 1'-hydroxylation was found in the incubations with both Supersomes and Gentest microsomes expressing P450 2B6, it is concluded that P450 2B6 is not involved in estragole 1'-hydroxylation in the human liver and that the observed correlation with 7-ETC is most likely due to the involvement of P450 1A2 in estragole 1'-hydroxylation. The *P* values for the correlation between

the activities of P450 2C19 (r = 0.43) and P450 2A6 (r = 0.41) toward their specific substrates and the 1'-hydroxylation of estragole were 0.1, indicating that these enzymes might also play a role in the bioactivation of estragole.

Inhibition experiment

Estragole 1'-hydroxylation in human liver microsomes was significantly (P < 0.01) inhibited with 39% (remaining activity 61%) by the P450 1A2 inhibitor α-naphthoflavone. For the other eight P450 enzyme-specific inhibitors, the inhibition was less than 10% and not significant (data not shown).

Estragole 1'-hydroxylation

Figure 4 shows the plots of estragole 1'-hydroxylation versus estragole concentration for Gentest microsomes containing, respectively, P450 1A2, 2A6, 2C19, 2D6, and 2E1, and Table 2 presents the parameters k_{cat}, K_m, and k_{cat}/K_m (enzyme efficiency) derived from these studies. P450 2A6 had both a high k_{cat} value (2.73 ± 0.12 nmol min^{-1} nmol $P450^{-1}$) and the lowest K_m (8 ± 2 μM) of all enzymes tested, resulting in the highest enzyme efficiency (k_{cat}/K_m = 341 min^{-1} mM^{-1}). Although the k_{cat} for P450 1A2 was - after the k_{cat} for P450 2E1 - the lowest k_{cat} observed among the five enzymes, the K_m value of P450 1A2 was much lower than the K_m values of the other enzymes (except P450

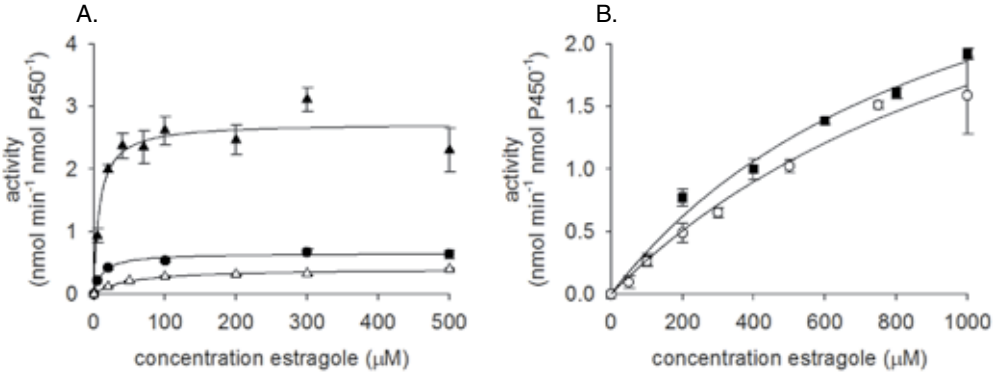

Figure 4. Plots of estragole 1'-hydroxylation versus estragole concentration for Gentest microsomes containing (A) P450 2A6 (▲), P450 1A2 (●), and P450 2E1 (Δ) and (B) P450 2C19 (■) and P450 2D6 (○). Data points represent average activities ± SEM (n = 4 for P450 1A2 and n = 3 for all other enzymes).

Table 2. Kinetic parameters for Gentest microsomes expressing the enzymes involved in estragole 1'-hydroxylation.

Enzyme	k_{cat} (nmol min^{-1} nmol P450^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
P450 1A2	0.65 ± 0.03	11 × 10^{-3} ± 2 × 10^{-3}	59
P450 2A6	2.73 ± 0.12	8 × 10^{-3} ± 2 × 10^{-3}	341
P450 2C19	3.72 ± 0.47	1.0 ± 0.2	4
P450 2D6	3.89 ± 1.04	1.3 ± 0.5	3
P450 2E1	0.40 ± 0.02	49 × 10^{-3} ± 7 × 10^{-3}	8

2A6). Therefore, P450 1A2 had the second highest enzyme efficiency of 59 min^{-1} mM^{-1}. The enzyme efficiencies for P450 2C19, 2D6, and 2E1 were an order of magnitude lower, respectively, 4, 3, and 8 min^{-1} mM^{-1}.

Discussion

The aim of the present study was to characterize the human hepatic P450 enzymes that catalyze the 1'-hydroxylation of estragole and to determine the kinetics for the P450 enzymes involved. Several *in vitro* experiments were performed to elucidate the enzymes catalyzing the 1'-hydroxylation of estragole in the human liver. A pivotal role for P450 1A2 in estragole 1'-hydroxylation was elucidated by the significant correlation ($r = 0.71$, $P < 0.01$) between 7-ethoxyresorufin *O*-dealkylation activities and estragole 1'-hydroxylation activities in the correlation study and the significant 39% inhibition ($P < 0.01$) of estragole 1'-hydroxylation in incubations with human liver microsomes in the presence of the P450 1A2 inhibitor α-naphthoflavone. This was confirmed by the kinetic parameters obtained for P450 1A2 (enzyme efficiency k_{cat}/K_m, 59 min^{-1} mM^{-1}). Because the correlation coefficient for P450 1A2 does not approach 1 and estragole 1'-hydroxylation is only partly inhibited by α-naphthoflavone, this indicates that more enzymes contribute to this bioactivation step. However, no other significant correlations between estragole 1'-hydroxylation and enzyme activities were observed in the correlation study with human liver microsomes and in addition no significant inhibition in estragole 1'-hydroxylation was observed with any other enzyme

specific inhibitor/antibody. Most likely, also P450 2A6 is involved in estragole 1'-hydroxylation since for P450 2A6 the highest enzyme efficiency (k_{cat}/K_m, 341 min^{-1} mM^{-1}) was obtained. In addition, for P450 2A6, the *P* value for the correlation ($r = 0.41$) between the coumarin-7-hydroxylation activities and the estragole 1'-hydroxylation activities was 0.1. Although this correlation is not significant, it is likely that P450 2A6 contributes to estragole 1'-hydroxylation in the human liver. Broad overlap of substrate specificity among P450 enzymes and their relative abundances in the human liver may reduce the reliability of correlation analyses (Lu *et al*., 2003). This could be an explanation for the absence of a significant correlation for P450 2A6 in the current study. P450 2A6 has a low abundance (6% of the total immunoquantified amount of P450) in the human liver (Shimada *et al*., 1994) and P450 2A6 is not the only enzyme that is important in the bioactivation of estragole.

According to the studies with recombinant enzymes, P450 2D6, 2C19, and 2E1 might also be involved; therefore, kinetic studies were performed with these enzymes as well. However, the enzyme efficiencies of P450 2C19, 2D6, and 2E1 appeared to be an order of magnitude lower (k_{cat}/K_m, respectively, 4, 3, and 8 min^{-1} mM^{-1}) than those of P450 1A2 and 2A6 (k_{cat}/K_m, respectively, 59 and 341 min^{-1} mM^{-1}). Moreover, their relative abundances in the liver [based on total immunoquantified (Shimada *et al*., 1994) or spectroscopically quantified (Rodrigues, 1999) P450 levels] are 2% for P450 2D6, 9% for P450 2E1 (Shimada *et al*., 1994), and 4% for P450 2C19 (Rodrigues, 1999) and are lower than or comparable to the abundances of P450 1A2 (18%) and P450 2A6 (6%) (Shimada *et al*., 1994). This indicates that in the human liver, P450 1A2 and P450 2A6 are the most important enzymes in estragole 1'-hydroxylation at physiologically relevant concentrations of estragole and only at relatively higher estragole concentrations P450 2C19, P450 2D6, and P450 2E1 might contribute to some extent.

For the related alkenylbenzenes safrole and methyleugenol, we recently reported on the P450 enzyme specificities (Jeurissen *et al*., 2004; Jeurissen *et al*., 2006a). In case of safrole, P450 2A6 appears to be the most important enzyme involved in safrole 1'-hydroxylation (Jeurissen *et al*., 2004). To some

extent this reaction could also be catalyzed by P450 2C9, 2C19, 2D6, and 2E1 (Jeurissen *et al*., 2004). For methyleugenol, P450 1A2 was previously identified as the most important enzyme in the human liver at physiologically relevant concentrations, whereas P450 2C9 and P450 2C19 might contribute as well at higher substrate concentrations (Jeurissen *et al*., 2006a). The observed differences in enzyme specificity between estragole, methyleugenol, and safrole 1'-hydroxylation illustrate that it is possible that compounds that are structurally similar differ in the pattern of P450 enzymes that convert them and indicate the need for characterization of the P450 enzymes involved in a certain metabolic conversion for each compound of interest.

For a better risk assessment for the alkenylbenzenes, identification of groups of people that might be at increased or decreased risk for the bioactivation of alkenylbenzenes is important. The activities of the two main enzymes in the bioactivation of the herb-based alkenylbenzenes, P450 1A2 and P450 2A6, may vary in the human population due to genotype- and lifestyle-based influences; therefore, interindividual differences in 1'-hydroxymetabolite formation may occur. People bearing polymorphisms in P450 2A6 that lead to poor metabolizer phenotypes or bearing whole deletion genotypes (Ingelman-Sundberg *et al*., 1999) might be at lower risk for adverse effects following 1'-hydroxylation of estragole and safrole. For P450 1A2, three mutations have been described that are associated with decreases in enzyme activity and one mutation has been described that is associated with enhanced inducibility. So far, no allelic variant that is associated with increased enzyme activity has been identified (http://www.cypalleles.ki.se/). Jiang *et al*. (2006) concluded that the P450 1A2 genotype cannot be unequivocally linked to a metabolic phenotype, and this indicates that for interindividual differences in P450 1A2 activity, lifestyle factors are more important than genetic differences. Cigarette smoking (George *et al*., 1995) and the consumption of charbroiled food and cruciferous vegetables can increase the activity of P450 1A2 (reviewed in Pelkonen *et al*., 1998) and might increase the chances on bioactivation of both estragole and methyleugenol.

The addition of estragole and methyleugenol as such to food is presently

being restricted in Europe due to the conclusions drawn by the Scientific Committee on Food of the European Union that these compounds are carcinogenic and genotoxic and that restrictions in their use are necessary (SCF, 2001a; SCF, 2001b; SCF, 2001c). Safrole is already banned from use as a flavor and fragrance substance (Federal Register of December 3, 1960, 25 FR 12412). The question remains whether the use of herbs that contain these alkenylbenzenes, herbal supplements, and foodstuffs in which these herbs are used should be restricted. For a risk assessment for the herb-based exposure to an alkenylbenzene, possible interaction at the level of the P450-catalyzed bioactivation with other compounds present in the herbs should be taken into account. The presence of P450 1A2 and/or P450 2A6 inhibitors in herbs, might decrease the bioactivation and thus the genotoxicity of the alkenylbenzenes. Such P450 1A2 and/or P450 2A6 inhibitors might decrease the chances on 1'-hydroxylation-mediated adverse effects of the alkenylbenzenes and might thus act as anticarcinogens. Recently, we showed that in basil, a herb that contains both estragole (SCF, 2001a) and methyleugenol (SCF, 2001b), inhibitors of P450 1A2 are present (Jeurissen *et al*., 2006b).

The P450 enzymes that are involved in other phase I metabolic reactions than 1'-hydroxylation of estragole are not yet elucidated, but for these metabolic routes, the same issues raised above for P450-catalyzed 1'-hydroxylation are also relevant. The same holds for the second step of the bioactivation pathway, the sulfonation of the 1'-hydroxymetabolites by sulfotransferases (SULT). Also, for the SULT enzymes involved, interindividual differences in bioactivationand possible interactions with other herbal compounds are to be expected. 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.

Altogether, the data in the present paper show that P450 1A2 and P450 2A6 are the main enzymes involved in the bioactivation of herb-based alkenylbenzenes and that competitive interactions between the

alkenylbenzenes may occur at the active site of the P450 enzymes involved in their 1'-hydroxylation. The knowledge on the P450 enzymes involved in 1'-hydroxylation of the alkenylbenzenes can be used to study the presence of possible anticarcinogens in herbs acting through inhibition of P450 1A2 or 2A6 and to implement the possible consequences of genetic and phenotype polymorphisms in the risk assessment for these alkenylbenzenes.

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References

Anthony, A., Caldwell, J., Hutt, A. J., and Smith, R. L. (1987). Metabolism of estragole in rat and mouse and influence of dose size on excretion of the proximate carcinogen 1'-hydroxyestragole. Food Chem Toxicol 25, 799-806.

Boberg, E. W., Miller, E. C., Miller, J. A., Poland, A., and Liem, A. (1983). Strong evidence from studies with brachymorphic mice and pentachlorophenol that 1‘-sulfooxysafrole is the major ultimate electrophilic and carcinogenic metabolite of 1‘-hydroxysafrole in mouse liver. Cancer Res 43, 5163–5173.

Bogaards, J. J. P., van Ommen, B., Wolf, C. R., and van Bladeren, P. J. (1995). Human cytochrome P450 enzyme selectivities in the oxidation of chlorinated benzenes. Toxicol Appl Pharmacol 132, 44–52.

Bogaards, J. J. P., Venekamp, J. C., and van Bladeren, P. J. (1996). The biotransformation of isoprene and the two isoprene monoepoxides by human cytochrome P450 enzymes, compared to mouse and rat liver microsomes. Chem Biol Interact 102, 169–182.

Bogaards, J. J. P., Bertrand, M., Jackson, P., Oudshoorn, M. J., Weaver, R. J., van Bladeren, P. J., and Walther, B. (2000). Determining the best animal model for human cytochrome P450 activities: a comparison of mouse, rat, rabbit, dog, micropig, monkey and man. Xenobiotica 30, 1131–1152.

Borchert, P., Wislocki, P. G., Miller, J. A., and Miller, E. C. (1973). The metabolism of the naturally occurring hepatocarcinogen safrole to 1‘-hydroxysafrole and the electrophilic reactivity of 1‘-acetoxysafrole. Cancer Res 33, 575–589.

Chan, V. S. and Caldwell, J. (1992). Comparative induction of unscheduled DNA synthesis in cultured rat hepatocytes by allylbenzenes and their 1'-hydroxy metabolites. Food Chem Toxicol 30, 831-836.

Commission of the European Communities (CEC) (2002). Commission Decision of 23 January 2002 amending Commission Decision 1999/217/EC as regards the register of flavouring substances used in or on foodstuffs (Text with EEA relevance) (notified under document number C). Off. J. Eur. Commun. L049/1–2.

Crespi, C., Gonzalez, F. J., Steimel, D. T., Turner, T. R., Gelboin, H. V., Penman, B. W., and Langenbach, R. (1991). A metabolically competent cell line expressing five cDNAs encoding procarcinogen-activating enzymes: Applications to mutagenicity testing. Chem Res Toxicol 4, 566–572.

Crespi, C. L., Langenbach, R., and Penman, B. W. (1993). Human cell lines, derived from AHH-1 TK +/- human lymphoblasts, genetically engineered for expression of cytochromes P450. Toxicology 82, 89–104.

Drinkwater, N. R., Miller, E. C., Miller, J. A., and Pitot, H. C. (1976). Hepatocarcinogenicity of estragole (1-allyl-4-methoxybenzene) and 1'-hydroxyestragole in the mouse and mutagenicity of 1'-acetoxyestragole in bacteria. J Natl Cancer Inst 57, 1323-1331.

Fennell, T. R., Miller, J. A., and Miller, E. C. (1984). Characterization of the biliary and urinary glutathione and N-acetylcysteine metabolites of the hepatic carcinogen 1'-hydroxysafrole and its 1'-oxo metabolite in rats and mice. Cancer Res 44, 3231-3240.

George, J., Byth, K., and Farrell, G. C. (1995). Age but not gender selectively

affects expression of individual cytochrome P450 proteins in human liver. Biochem Pharmacol 50, 727–730.

Guenthner, T. M. and Luo, G. (2001). Investigation of the role of the 2',3'-epoxidation pathway in the bioactivation and genotoxicity of dietary allylbenzene analogs. Toxicology 160, 47-58.

Hall, R.L. and Oser, B.L. (1965). Recent progress in the consideration of flavoring ingredients under the food additives amendment III. GRAS substances. Food Technology 253, 151–197.

Ingelman-Sundberg, M., Oscarson, M., and McLellan, R. A. (1999). Polymorphic human cytochrome P450 enzymes: An opportunity for individualized drug treatment. Trends Pharmacol Sci 20, 342–349.

Iyer, L. V., Ho, M. L., Shinn, W. M., Bradford, W. W., Tanga, M. J., Nath, S. S., and Green, C. E. (2003). Glucuronidation of 1‘-hydroxyestragole (1‘-HE) by human UDP-glucuronosyltransferases UGT2B7 and UGT1A9. Toxicol Sci 73, 36–43.

Jeurissen, S. M. F., Bogaards, J. J. P., Awad, H. M., Boersma, M. G., Brand, W., Fiamegos, Y. C., van Beek, T. A., Alink, G. M., Sudholter, E. J. R., Cnubben, N. H. P., and Rietjens, I. M. C. M. (2004). Human cytochrome P450 enzyme specificity for bioactivation of safrole to the proximate carcinogen 1‘-hydroxysafrole. Chem Res Toxicol 17, 1245–1250.

Jeurissen, S. M. F., Bogaards, J. J. P., Boersma, M. G., Ter Horst, J. P. F., Awad, H. M., Fiamegos, Y. C., Van Beek, T. A., Alink, G. M., Sudholter, E. J. R., Cnubben, N. H. P., and Rietjens, I. M. C. M. (2006a). Human cytochrome P450 enzymes of importance for the bioactivation of methyleugenol to the proximate carcinogen 1‘-hydroxymethyleugenol. Chem Res Toxicol 19, 111–116.

Jeurissen, S. M. F., Claassen, F. W., Havlik, J., Bouwmans, E. E., Cnubben, N. H. P., Sudholter, E. J. R., Rietjens, I. M. C. M., and Van Beek, T. A. (2006b). Development of an on-line high performance liquid chromatography detection system for human cytochrome P450 1A2 inhibitors in extracts of natural products. J Chromatogr A 1141, 81–89.

Jeurissen, S. M. F., Punt, A., Delatour, T., and Rietjens, I. M. C. M. (2008).

Basil extract inhibits the sulfotransferase mediated formation of DNA adducts of the procarcinogen 1'-hydroxyestragole by rat and human liver S9 homogenates and in HepG2 human hepatoma cells. Food and Chem Toxicol 46, 2296-2302.

Jiang, Z., Dragin, N., Jorge-Nebert, L. F., Martin, M. V., Guengerich, F. P., Aklillu, E., Ingelman-Sundberg, M., Hammons, G. J., Lyn-Cook, B. D., Kadlubar, F. F. S., S. N., Sorter, M., Vinks, A. A., Nassr, N., Von Richter, O., Jin, L., and Nebert, D. W. (2006). Search for an association between the human CYP1A2 genotype and CYP1A2 metabolic phenotype. Pharmacogenet Genomics 16, 359–367.

Li, Y., Li, N. Y., and Sellers, E. M. (1997). Comparison of CYP2A6 catalytic activity on coumarin 7-hydroxylation in human and monkey liver microsomes. Eur J Drug Metab Pharmacokinet 22, 295–304.

Lu, A. Y., Wang, R. W., and Lin, J. H. (2003). Cytochrome P450 in vitro reaction phenotyping: A re-evaluation of approaches used for P450 isoform identification. Drug Metab Dispos 31, 345–350.

Luo, G., Qato, M. K., and Guenthner, T. M. (1992). Hydrolysis of the 2',3'-allylic epoxides of allylbenzene, estragole, eugenol, and safrole by both microsomal and cytosolic epoxide hydrolases. Drug Metab Dispos 20, 440-445.

Pelkonen, O., Mäenpää, J., Taavitsainen, P., Rautio, A., and Raunio, H. (1998). Inhibition and induction of human cytochrome P450 (CYP) enzymes. Xenobiotica 28, 1203–1253.

Phillips, D. H., Miller, J. A., Miller, E. C., and Adams, B. (1981). Structures of the DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1'-hydroxyestragole. Cancer Res 41, 176-186.

Rodrigues, A. D. (1999). Integrated cytochrome P450 reaction phenotyping. Biochem Pharmacol 57, 465–480.

Sangster, S. A., Caldwell, J., Hutt, A. J., Anthony, A., and Smith, R. L. (1987). The metabolic disposition of [methoxy-^{14}C]-labelled trans-anethole, estragole and p-propylanisole in human volunteers. Xenobiotica 17, 1223-

1232.

SCF (2001a). Opinion of the scientific committee on food on estragole (1-allyl-4-methyxybenzene). http://ec.europa.eu/food/fs/sc/scf/out104_en.pdf.

SCF (2001b) Opinion of the Scientific Committee on Food on methyleugenol (4-allyl-1,2-dimethoxybenzene), http://ec.europa.eu/food/fs/sc/scf/out102_en.pdf.

SCF (2001c) Opinion of the Scientific Committee on Food on the safety of the presence of safrole (1-allyl-3,4-methylene dioxy benzene) in flavourings and other food ingredients with flavouring properties, http://ec.europa.eu/food/fs/sc/scf/out116_en.pdf.

Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., and Guengerich, F. P. (1994). Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: Studies with liver microsomes of 30 Japanese and 30 Caucasians. J Pharmacol Exp Ther 270, 414–423.

Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., Rogers, A. E., Caldwell, J., and Sipes, I. G. (2002). Safety assessment of allylalkoxybenzene derivatives used as flavouring substances - methyl eugenol and estragole. Food Chem Toxicol 40, 851-870.

Solheim, E. and Scheline, R. R. (1973). Metabolism of alkenebenzene derivatives in the rat. I. *p*-Methoxyallylbenzene (Estragole) and *p*-methoxypropenylbenzene (Anethole). Xenobiotica 3, 493-510.

Suga, K., Watanabe, S., and Suematsu, M. (1966). Grignard reaction of vinylchloride with α,β-unsaturated carbonyl compounds. Yuki Gosei Kagaku Kyokai Shi 24, 213–215.

Tamayo, M. L., and Ossorio, R. (1948). La sintesis de acidos fenilalilsuccinicos. An R Soc Espan Fis Quim 44B, 981–998.

Ueng, Y. F., Hsieh, C. H., Don, M. J., Chi, C. W., and Ho, L. K. (2004). Identification of the main human cytochrome P450 enzymes involved in safrole 1‘-hydroxylation. Chem Res Toxicol 17, 1151–1156.


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Evaluation of human interindividual variation in bioactivation of estragole using physiologically based biokinetic (PBBK) modeling

Based on: Punt A, Jeurissen SMF, Boersma MG, Delatour T, Scholz G, Schilter B, Van Bladeren PJ, Rietjens IMCM
In preparation

Abstract

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 formation and detoxification of this metabolite, namely 1'-hydroxylation of estragole and oxidation of 1'-hydroxyestragole. First the study defines physiologically-based biokinetic (PBBK) models for fourteen individual human subjects, revealing a 1.7-fold interindividual variation in the area under the concentration-time curve (AUC) for 1'-hydroxyestragole in the liver within this group of human subjects. In a second step the individual models were combined with literature data on interindividual variation in activity levels of key enzymes involved in formation and detoxification of 1'-hydroxyestragole to model the maximum level of variability in the general population. Formation of 1'-hydroxyestragole is predominantly catalyzed by P450 1A2, 2A6, and 2E1. Interindividual differences in activity levels of the P450 enzymes involved and varying orders of magnitude due to lifestyle factors or polymorphism, were predicted to affect the liver AUC of 1'-hydroxyestragole less than 10-fold. Results of the present study support that oxidation of 1'-hydroxyestragole is catalyzed by 17β-hydroxysteroiddehydrogenase (17β-HSD) type 2. Possible variation in oxidation of 1'-hydroxyestragole by 17β-HSD type 2 was shown to result in larger effects than those caused by variation in P450 enzyme activity. Both dietary and genotype-based influences on 17β-HSD type 2 activity might lead to reduced oxidation activity that could, in theory, lead to 30- to 60-fold variation within the human population in a worst case scenario. It is concluded that the liver AUC values for 1'-hydroxyestragole may vary one to two orders of magnitude within the human population. This interindividual variation in bioactivation of estragole might be larger than the default factor of 3.16 generally assumed to reflect interindividual variation in biokinetics within the human population.

Introduction

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 - 0.07 mg/kg bw, 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.*, 1982). The first step of the bioactivation is the conversion of estragole into the proximate carcinogenic metabolite, 1'-hydroxyestragole (Figure 1). Other phase I metabolic reactions of estragole, also presented in Figure 1, include *O*-demethylation, 2',3'epoxidation and 3'-hydroxylation, and represent detoxification pathways (Solheim and Scheline, 1973; Phillips *et al.*, 1981; Fennell *et al.*, 1984; Anthony *et al.*, 1987; Sangster *et al.*, 1987; Luo *et al.*, 1992; Guenthner and Luo, 2001). Sulfonation of 1'-hydroxyestragole leads to formation of the ultimate carcinogenic metabolite which is unstable and degrades in aqueous environment to give rise to a reactive carbocation capable of forming DNA adducts (Phillips *et al.*, 1981; Phillips *et al.*, 1984; Randerath *et al.*, 1984; Wiseman *et al.*, 1985). Detoxification of 1'-hydroxyestragole can occur through glucuronidation and oxidation, the latter resulting in formation of 1'-oxoestragole (Solheim and Scheline, 1973; Anthony *et al.*, 1987; Sangster *et al.*, 1987). The oxidation of 1'-hydroxyestragole is identified to be an important metabolic route of 1'-hydroxyestragole in humans (Chapter 4). In spite of the fact that 1'-oxoestragole has been shown to be able to form adducts with 2'-deoxyguanosine in a direct reaction with this nucleoside, these adducts were not observed to be formed *in vivo* (Phillips *et al.*, 1981). This has been ascribed to extensive detoxification of 1'-oxoestragole via conjugation

with glutathione or endogenous amines (Phillips *et al.*, 1981; Fennell *et al.*, 1984).

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 for the key metabolic reactions involved. 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 (Chapter 4). Subsequent formation of 1'-sulfooxyestragole will depend

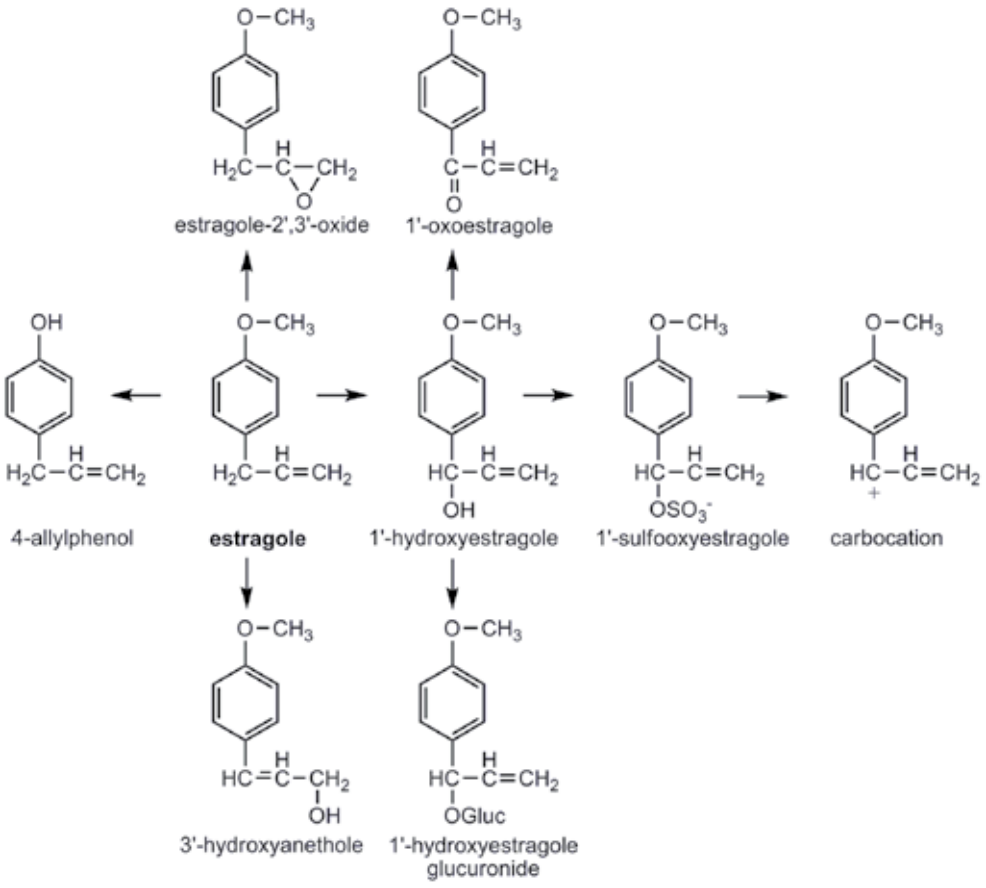

Figure 3. Metabolism of estragole.

on this level of 1'-hydroxyestragole formed and also on the kinetics for its sulfonation (Chapter 4). 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 in a first step PBBK models for estragole metabolism in fourteen different human subjects were developed, allowing evaluation of the contribution of variability in estragole 1'-hydroxylation and 1'-hydroxestragole oxidation to the predicted interindividual differences in liver levels of 1'-hydroxyestragole within this limited set of fourteen individuals. In a next step, the extreme and average individual human PBBK models of the series were used to model the extent of variability in levels of 1'-hydroxyestragole to be expected in the overall human population by introducing into the models variation in enzyme kinetics of these reactions that could occur in the population as a whole.

To allow this modeling of the theoretical variation expected in the human population as a whole, knowledge about the enzymes involved in 1'-hydroxylation of estragole and oxidation of 1'-hydroxyestragole is essential. Recently, 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 enzyme(s) 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 encompass cytochrome P450 enzymes but also 17β-hydroxysteroiddehydrogenase (17β-HSD) type 2 (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 tracts, 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 involved in this conversion and to this end the present study also reports experiments indentifying the cellular localization and co-factor specificity of the enzyme involved in this reaction, thereby elucidating the nature of the major enzyme involved. Altogether the experiments and models of the present paper allow evaluation of human interindividual variation in liver levels of 1'-hydroxyestragole.

Materials and Methods

Chemicals and biological materials

Individual human liver microsomes (8 females and 6 males), mixed gender pooled human liver microsomes, and mixed gender pooled human liver cytosol were purchased from BD Gentest (Woburn, USA). NADPH, $NADP^+$, NADH and NAD^+ 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, dimethylsulfoxide (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

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^{-1} mg $protein^{-1}$)	K_m (μM)	$V_{maxP450, Gen}/K_m$ (μL min^{-1} mg $protein^{-1}$)
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^{-1} (mg microsomal protein)$^{-1}$ were recalculated from the k_{cat} values expressed as nmol min^{-1} nmol $P450^{-1}$ as reported by Jeurissen *et al*. (2007).

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). 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). The relative activity levels of P450 enzymes in individual human liver microsomes were calculated using relative activity factors (RAFs), representing the ratio between i) the conversion of a P450-selective marker substrate (at saturating substrate conditions) expressed as pmol min^{-1} (mg microsomal protein)$^{-1}$ by liver microsomes from an individual human subject, and ii) the conversion of this marker substrate (at saturating substrate conditions) expressed as pmol min^{-1} (mg microsomal protein)$^{-1}$, 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 fourteen 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:

Equation 1: $$v = (V_{max1A2, Gen} * RAF_{1A2}) * C_E/(K_{m1A2} + C_E) + (V_{max2A6, Gen} * RAF_{2A6} * C_E/(K_{m2A6} + C_E) + (V_{max2C19, Gen} * RAF_{2C19}) * C_E/(K_{m2C19} + C_E) + (V_{max2D6, Gen} * RAF_{2D6}) * C_E/(K_{m2D6} + C_E) + (V_{max2E1, Gen} * RAF_{2E1}) * C_E/(K_{m2E1} + C_E)$$

in which v (nmol min^{-1} (mg microsomal protein)$^{-1}$) is the overall 1'-hydroxylation rate by microsomes obtained from an individual human liver, $V_{maxP450, Gen}$ (nmol min^{-1} (mg microsomal protein)$^{-1}$) 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 relative activity factors of the P450 enzymes 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 $V_{maxP450, Gen} * RAF_E$, 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 1000 μM estragole (representing saturating substrate conditions (Chapter 4)) 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) 3 mM NADPH and 1 mg/mL microsomal

protein in 0.2 M Tris-HCl (pH 7.4). After pre-incubating at 37ºC 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 16,000g and 50 µL of the supernatant of each sample was analyzed by 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 (Chapter 4). The incubation mixtures had a final volume of 100 µL, containing (final concentrations) 3 mM of the specific cofactor, 2 mM GSH and 1 mg/mL microsomal or cytosolic protein in 0.2 M Tris-HCl (pH 7.4). The reactions were started by addition of the substrate 1'-hydroxyestragole (3 mM final concentration from a 300 mM 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 (Chapter 4).

Additional experiments were performed to investigate the possible involvement of 17β-hydroxysteroiddehydrogenase (17β-HSD) type 2. 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-HSD type 2 (Stupans *et al.*, 2000). Incubation conditions were as described above and were performed at a substrate concentration of 25 µM 1'-hydroxyestragole (from a 5 mM stock solution in DMSO). The final concentration of estradiol or gallic acid was 500 µM (added from a 100 mM 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-HSD type 2 (see Results section)), 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 (Chapter 4). 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 fourteen 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 (Chapter 4). Incubations were performed and analyzed as described above and were performed at a saturating substrate concentration of 3 mM 1'-hydroxyestragole (added from a 300 mM 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.

Physiologically based biokinetic (PBBK) modeling

PBBK models for estragole in different individual human subjects were developed using kinetic data for 1'-hydroxylation of estragole and oxidation of 1'-hydroxyestragole 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 (Chapter 4), whereas in the newly developed models the kinetics for 1'-hydroxylation of estragole and oxidation of 1'-hydroxyestragole were described for individual human subjects specifically.

In the models for individual human subjects the kinetics for 1'-

Table 2. Relative activity factors (RAFs)[a] of different P450 enzymes in different individual human liver microsomes.

Human subject	Gender	RAF1A2	RAF2A6	RAF2C19	RAF2D6	RAF2E1
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

hydroxylation of estragole were described by equation 1, in which the RAFs of the P450 enzymes are specific for each individual human subject (Table 2). The maximum 1'-hydroxylation rate by each specific enzyme in individual human liver microsomes, expressed as nmol min^{-1} (mg microsomal protein)$^{-1}$, was scaled to the liver using a microsomal protein yield of 35 mg per g liver (Medinsky *et al.*, 1994). 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'-hydrxylation rates by individual human liver microsomes using equation 1 (see Results section for justification of this correction factor). The K_m values for 1'-hydroxylation by the different P450 enzymes in Gentest microsomes were assumed to correspond to the apparent *in vivo* K_m values.

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 35 mg per g liver (Medinsky *et al.*, 1994). The K_m values for oxidation were kept for each individual to the value previously obtained with pooled individual human liver microsomes

(i.e. 354 μM) (Chapter 4).

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 Results 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 (Chapter 4).

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. Normalized sensitivity coefficients (SC) were determined according to the following equation:

$$SC = (C'-C)/(P'-P)*(P/C)$$

where C is the initial value of 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). Based on the literature, the effect of a five percent increase in V_{max} and K_m values for the different metabolic reactions on the predicted area under the concentration-time curve (AUC) of 1'-hydroxyestragole in an individual human subject was analyzed (Evans and Andersen, 2000). Each parameter was analyzed individually, keeping the other parameters to their initial values.

Evaluation of interindividual variability on population level

Based on the identified key metabolic reactions that influenced the levels

of 1'-hydroxyestragole in the liver, the effect of a wider variation in the kinetic constants for these metabolic reactions that could occur within the general population was analyzed. This could be done by modeling theoretical scenarios that might occur in the general population. Starting from the models obtained for the fourteen individuals, the metabolic reactions were set to a theoretically low or high activity level, representative for the variation that could occur in the population as a whole, and the absolute effect on the AUC for 1'-hydroxyestragole in the liver was determined.

Results

Relative activity factors

Table 2 displays the relative activity factors (RAFs) of the P450 enzymes involved in 1'-hydroxylation of estragole for the set of fourteen 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 estragole by individual human liver microsomes can be determined correctly, the 1'-hydroxylation rate at a saturating substrate concentration of 1000 μM 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 2 the calculated 1'-hydroxylation rates thus obtained for the fourteen individual human liver microsomes are plotted against the measured 1'-hydroxylation rates by these

individual human liver microsomes. Although the calculated values are overall about 1.9-fold lower than the ones actually measured in incubations with the respective liver microsomes, a good 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 was 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 1000 μM, 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

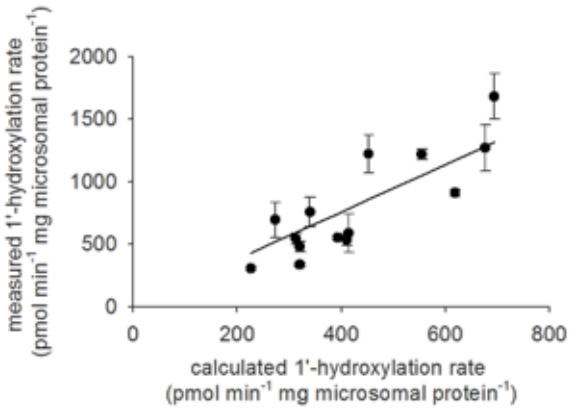

Figure 2. Calculated 1'-hydroxylation rate for different individual human liver microsomes plotted against the measured values for these human liver microsomes. The calculated values are obtained using equation 1, and the relative activity factors (RAFs) of different enzymes in individual human liver microsomes as displayed in Table 2.

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 the contribution of other enzymes increases. Especially in case of P450

Table 3. Calculateda 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. Between brackets the percentage contribution to the calculated overall 1'-hydroxylation rate is given.					
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

[a] The 1'-hydroxylation rate was calculated at a concentration of 1000 μM estragole (representing a saturating substrate concentration) using equation 1, and the relative activity factors (RAFs) of different enzymes in individual human liver microsomes as displayed in Table 2. The 1'-hydroxylation rate is expressed as pmol min^{-1} (mg microsomal protein)$^{-1}$.

2A6 the relative contribution of this enzyme to the overall 1'-hydroxylation rate was observed to be negatively correlated to a significant extent with the relative contribution of other enzymes including P450 1A2, 2D6, and 2E1 (p <0.05 using a Pearson correlation coefficient), indicating that the contribution of these enzymes increases when the P450 2A6 activity is relatively low.

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 (Figure 3), it is observed that the highest level of oxidation occurs in incubations with human liver 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-HSD type 2 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-HSD type 2 in the oxidation of 1'-hydroxestragole additional incubations were performed in the presence

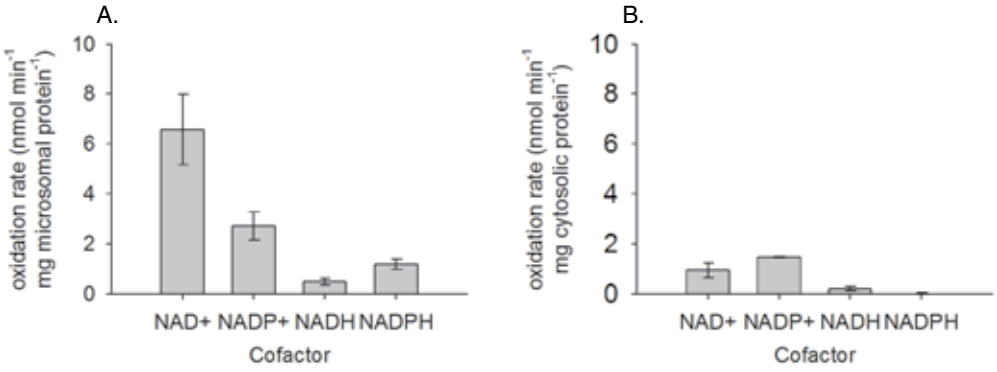

Figure 3. Oxidation rate of 1'-hydroxyestragole to 1'-oxoestragole in incubations with (A) pooled human liver microsomes in the presence of NAD^+, $NADP^+$, NADH, or NADPH as cofactor and (B) pooled human liver cytosol in the presence of NAD^+, $NADP^+$, NADH, or NADPH as cofactor. Bars indicate average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

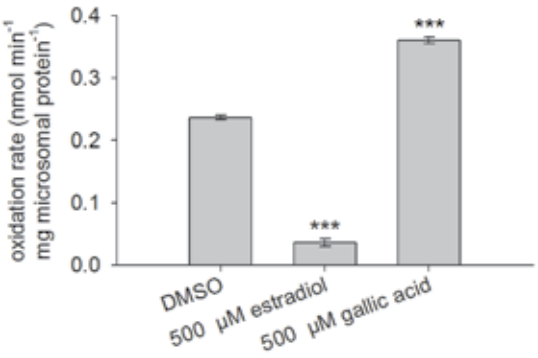

Figure 4. Oxidation rate of 1'-hydroxyestragole to 1'-oxoestragole in incubations with pooled human liver microsomes with NAD^+ as cofactor at a substrate concentration of 25 µM 1'-hydroxyestragole in the presence of 1% DMSO (as solvent blank), estradiol or gallic acid. Bars indicate average activities of triplicate measurements ± SD (n = 3). *** Indicates significant differences from the incubations without inhibitor (Student's t-test, $p < 0.001$).

of estradiol, which is a competitive inhibitor of 17β-HSD type 2 (Poirier *et al.*, 2001), and gallic acid, which is an identified stimulator of 17β-HSD type 2 (Stupans *et al.*, 2000). Figure 4 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-HSD type 2 in the oxidation reaction.

Maximum oxidation of 1'-hydroxyestragole rate by individual human liver microsomes

The maximum oxidation rate of 1'-hydroxyestragole to 1'-oxoestragole by fourteen 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 3 mM 1'-hydroxyestragole by the fourteen different individual human liver microsomes is displayed in Figure 5. The highest oxidation rate was observed for individual HG3 for which the rate was 2-fold higher than for individual HG93.

Physiologically based biokinetic (PBBK) modeling

PBBK models for estragole metabolism in different individual human

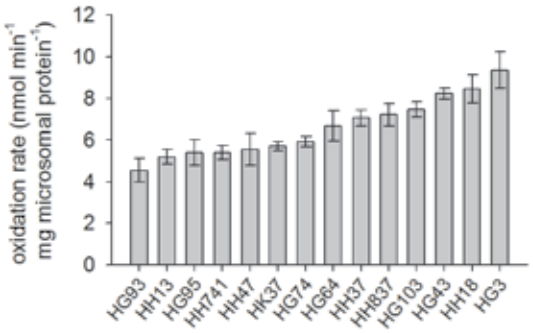

Figure 5. Oxidation rate of 1'-hydroxyestragole to 1'-oxoestragole by different individual human liver microsomes at a saturating substrate concentration of 3 mM. Bars indicate average activities of triplicate measurements ± SD (*n* = 3).

subjects were developed in which the kinetics for 1'-hydroxylation of estragole and oxidation of 1'-hydroxyestragole were described for each human subject specifically, based on the kinetics for these reactions by the individual human liver microsomes. Based on the PBBK models obtained for the fourteen 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 of 1'-hydroxyestragole at estragole intake levels relevant for dietary human intake can be analyzed (Figure 6). Figure 6A shows the predicted time-dependent liver concentration of 1'-hydroxyestragole in fourteen different individual human subjects after exposure to 0.07 mg/kg bw estragole, corresponding to the estimated dietary human intake (SCF, 2001). The area under the liver concentration-time curve (AUC) obtained for the different individual human subjects is displayed in Figure 6B. Overall the AUC of 1'-hydroxyestragole is predicted to vary 1.7-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=-0.74), with the highest AUC being observed for individuals H93 and HH741 who displayed a relative low

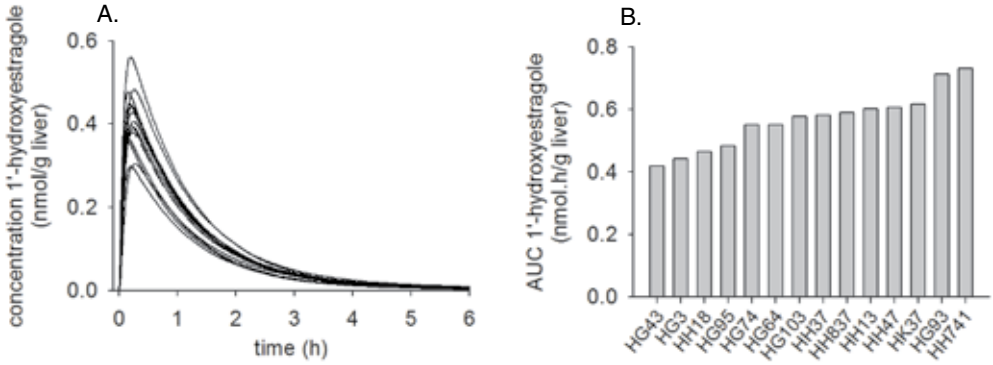

Figure 6. Predicted time-dependent concentration of 1'-hydroxyestragole in the liver of different human subjects at an oral dose of 0.07 mg/kg bw as predicted by PBBK models for estragole metabolism in these human subjects based on individual enzyme kinetic data for 1'-hydroxylation of estragole and oxidation of 1'-hydroxyestragole (A) and AUC values derived from these curves for the individual human subjects (B).

detoxification rate of 1'-hydroxyestragole via oxidation (Figure 5), and the lowest AUC being observed for individuals HH43 and HG3 who displayed a relative high elimination rate of 1'-hydroxyestragole via oxidation (Figure 5). No significant correlation between the predicted AUC and maximum 1'-hydroxylation rates of estragole are observed (r=0.019), 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 sensitivity coefficients were calculated for different metabolic parameters at a dose of 0.07 mg/kg bw estragole by increasing parameter values with five percent. Only parameters that had a normalized sensitivity coefficient higher than 0.1 (in absolute value) in at least one of the three models are displayed in Figure 7. The predicted AUC of 1'-hydroxyestragole was

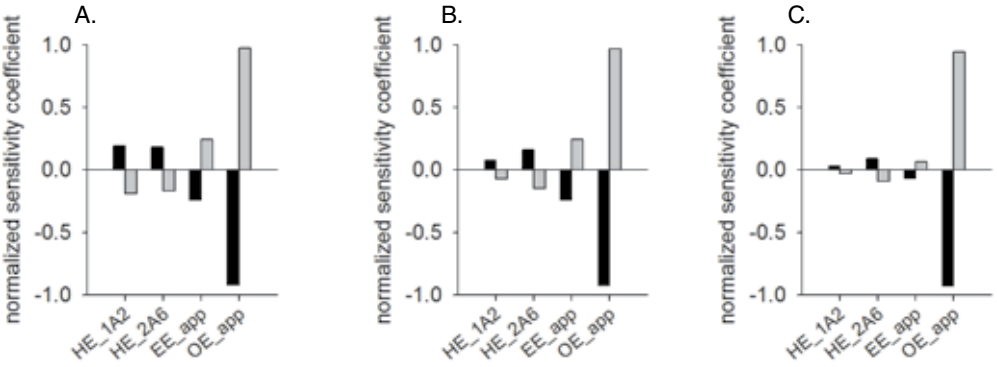

Figure 7. Sensitivity of the predicted AUC of 1'-hydroxyestragole to changes V_{max} values (black bars) and K_m values (grey bars) for i) cytochrome P450 1A2 and 2A6 catalyzed formation of 1'-hydroxyestragole (HE_1A2 and HE_2A6) ii) apparent formation of estragole-2',3'-oxide (EE_app) and iii) apparent formation of 1'-oxoestragole (OE_app) in the PBBK models for individual HG95 (A), HH47 (B), and HH837 (C).

observed to be most sensitive to changes in the kinetic constants for oxidation of 1'-hydroxyestragole (Figure 7). 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 (Figure 7). Furthermore the predicted AUC of 1'-hydroxyestragole is observed to be sensitive to changes in kinetic constants for epoxidation of estragole, which is the major competing metabolic route to 1'-hydroxylation. The sensitivity towards metabolic parameters for 1'-hydroxylation by P450 1A2 and 2A6 as well as for the metabolic parameters for epoxidation is 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.

Evaluation of interindividual variability on population level

The predicted interindividual variation based on the kinetics for 1'-hydroxylation of estragole and oxidation of 1'-hydroxyestragole by fourteen different individual human liver microsomal fractions gives some insight in the level of variation between individuals but does not represent the absolute

variation in the general population, which might be larger. To allow modeling of the variation expected in the human population as a whole knowledge about the variation in the key enzymes involved in 1'-hydroxylation of estragole and oxidation of 1'-hydroxyestragole in the population as a whole is essential.

1'-Hydroxylation of estragole is found to mainly depend on P450 1A2, 2A6 and to some extent on P450 2E1 activity in an individual human subject. Both P450 1A2 and 2E1 activity can vary about 60-fold within the population (Wenker *et al.*, 2001; Jiang *et al.*, 2006a). For P450 2A6 polymorphisms occur that lead to poor metabolizer phenotypes or whole deletion genotypes. Starting from the model for individual HH47, representing median 1'-hydroxylation activity, the effect of these variations in activity levels of P450 1A2, 2A6, and 2E1 on the predicted AUC of 1'-hydroxyestragole was analyzed. To this end the V_{max} for P450 1A2, 2A6, and 2E1 catalyzed 1'-hydroxylation are set to a theoretically low or high level. In case of P450 1A2 and 2E1 the lowest activity level was modeled by decreasing the V_{max} values for these enzymes

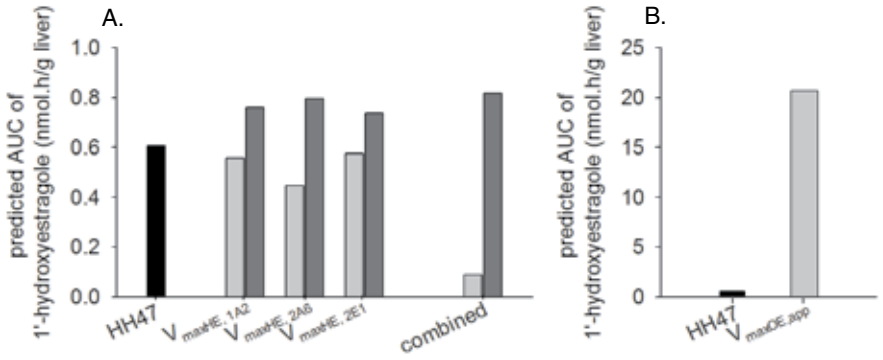

Figure 8. (A) Predicted AUC of 1'-hydroxyestragole in the liver at an oral dose of 0.07 mg/kg bw based on the PBBK model for human HH47 (black bar) in which the maximum 1'-hydroxylation activity of P450 1A2, 2A6, and 2E1 are set to a theoretically low (light gray bars) or high (dark gray bars) activity level by decreasing or increasing the V_{max} for cytochrome P450 1A2, 2A6, and 2E1 catalyzed 1'-hyroxylation ($V_{maxHE, 1A2}$, $V_{maxHE, 2A6}$, and $V_{maxHE, 2E1}$) 10-fold, representing an overall 100-fold variation. Only in case of P450 2A6 the lowest level is set to zero, representing whole deletion genotypes. The bars labelled 'combined' represent the effect of combined changes in the V_{max} for cytochrome P450 1A2, 2A6, and 2E1 catalyzed 1'-hydroxylation on the predicted AUC of 1'-hydroxyestragole. (B) Predicted AUC of 1'-hydroxyestragole in the liver at an oral dose of 0.07 mg/kg bw based on the PBBK model for human HH47 (black bar) in comparison to the theoretical worst case situation in which the maximum oxidation rate of 1'-hydroxyestragole (V_{maxOE}, app) is set to zero (light gray bar) in this individual.

in the HH47 model 10-fold and the highest activity level was modeled by increasing the V_{maxc} values for these enzymes 10-fold, together representing an overall 100-fold variation within the human population. In case of P450 2A6 the lowest level is set to zero, representing whole deletion genotypes, whereas a theoretical high activity level of this enzymes is reached by setting the $V_{max2A6,\ mic}$ 10-fold higher than that in the HH47 model. Figure 8A shows the predicted AUC of 1'-hydroxyestragole in these theoretical cases and also shows the combined effect of changes in P450 1A2, 2A6, and 2E1 activity on the predicted AUC of 1'-hydroxyestragole. It is observed that due to the complementary enzyme activity the effect of decreases and increases in activity levels of single enzymes is relatively small and do not result in more than 1.8-fold variation in the predicted AUC of 1'-hydroxyestragole. When a combined 10-fold increase in expression levels of P450 1A2, 2A6, and 2E1 is applied, the predicted AUC of 1'-hydroxyestragole is not more than 1.3-fold higher than predicted for individual HH47. Only when the expression levels of these three enzymes are set at a theoretical low level the effect on the predicted AUC of 1'-hydroxyestagole is larger, corresponding to a 6.6-fold decrease. In this theoretical scenario where the major P450 enzymes involved in estragole 1'-hydroxylation are set at a theoretical low or high expression level, the overall variation in the AUC of 1'-hydroxyestragole is predicted to be at most 9-fold.

Results of the present study revealed that 17β-HSD type 2 is involved in the oxidation reaction of 1'-hydroxyestragole. The activity of this enzyme might be affected by genotype-based factors (Plourde *et al*., 2008) or dietary flavonoids, such as quercetine and naringenin, which have been observed to reduce the activity of this enzyme in an *in vitro* situation (Schuster *et al*., 2008). No indication can be given on the level of reduction that could occur in an *in vivo* situation due to these factors. Theoretically worst case scenarios can be modeled by reducing the maximum oxidation rate in the different PBBK models for individual human subjects. Reducing the oxidation rate of 1'-hydroxyestragole 10-fold results in about 8-fold increase in AUC of 1'-hydroxyestragole for all fourteen individual human subjects (data not shown).

Modeling of a diminished oxidation rate by setting the maximum oxidation rate to zero results in a 30-fold higher AUC of 1'-hydroxyestragole in case of individual HH47 (Figure 8B). The highest effect of setting the maximum oxidation rate to zero was observed for individual HG3 and resulted in a 60-fold increase in AUC of 1'-hydroxyestragole for this individual. The lowest effect was observed for individual HG93 and resulted in a 28-fold increase in AUC of 1'-hydroxyestragole.

Discussion

The present study defines PBBK models for estragole metabolism in individual human subjects using individual kinetic data 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 could be analyzed by modeling theoretical extreme variations in enzyme kinetics of these reactions.

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 (Chapter 4). To date the sulfotransferase enzymes involved in sulfonation of 1'-hydroxyestragole have

not be 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; Thomae *et al.*, 2002; Nagar *et al.*, 2006). 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 previously demonstrated not to affect interindividual variability in bioactivation of estragole to a significant extent, since the predicted formation of 1'-sulfooxyestragole was previously observed not to be sensitive to changes in kinetic constants of these reactions (Chapter 4).

In a first step the relative contribution of interindividual variability in 1'-hydroxylation of estragole and oxidation of 1'-hydroxyestragole to the variability in predicted AUC of 1'-hydroxyestragole was evaluated using PBBK models for estragole metabolism in fourteen different individual human subjects and individual kinetic data for these reactions. With the models obtained the AUC of 1'-hydroxyestragole was predicted to vary 1.7–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 2-fold variation in the oxidation rate of 1'-hydroxyestragole. The observed relative 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 the relative contribution of one, for example P450 1A2, was found to increase when that of another one, e.g. P450 2A6, decreases. 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.

Although the relative effect of interindividual differences in P450 activity on the predicted liver AUC of 1'-hydroxyestragole is relatively small, the occurrence of large differences in P450 enzyme activities in the human population might still result in significant interindividual differences. 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.,* 2006a). 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.*, 2006a; 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 a second step the effect of such levels of variation, which could occur in the human population, was analyzed. Due to the complementary enzyme activity of different enzymes, the effect of a 100-fold variation in activity levels of single cytochromes P450 appeared to be relatively small and was predicted not to result in more than 1.8-fold variation in the liver AUC of 1'-hydroxyestragole. Only when a combined 10-fold change in the activity level of the major P450 enzymes involved in estragole 1'-hydroxylation was modeled the predicted AUC of 1'-hydroxyestragole varied 9-fold.

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-hydroxysteroid dehydrogenase (17β-HSD) type 2 (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-HSD type 2 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-HSD type 2 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-HSD type 2 (Schuster *et al.*, 2008). Among different flavonoids, quercetin has for instance been observed to be an inhibitor (IC_{50} = 1.5 μM) of 17β-HSD type 2 catalyzed oxidation of estradiol to estrone *in vitro* (Schuster *et al.*, 2008). Whether such effects could also occur *in vivo* was, however, not elucidated. Genotype- and lifestyle-based factors that could influence oxidation of 1'-hydroxyestragole were evaluated by modeling theoretical scenarios in which the oxidation rate was decreased or diminished. This could be done by reducing the maximum oxidation rate of 1'-hydroxyestragole in the PBBK models for the fourteen individual human subjects. A 10-fold reduced oxidation activity results in an about 8-fold increase in 1'-hydroxyestragole levels, whereas homozygous deletion genotypes could, in theory, lead to 30- to 60-fold variation within the population. Obviously, definite conclusions on the effects of variation in 17β-HSD phenotypes on interindividual differences

in estragole bioactivation and detoxification have to await further data on the actual variation in the 17β-HSD activity to be expected within the human population.

Altogether the results of the present study indicate that 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. Due to the level of interindividual differences that might occur in this reaction it can be concluded that liver AUC values for 1'-hydroxyestragole may vary one to two orders of magnitude within the human population. This interindividual variation in bioactivation of estragole may be larger than the default factor of 3.16 generally assumed to reflect interindividual variation in biokinetics within the human population (assuming that the default factor of 10 can be divided into a factor of 3.16 for kinetics and 3.16 for dynamics) (WHO, 1999).

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References

Anthony, A., Caldwell, J., Hutt, A. J., and Smith, R. L. (1987). Metabolism of estragole in rat and mouse and influence of dose size on excretion of the proximate carcinogen 1'-hydroxyestragole. Food Chem Toxicol 25, 799-806.

Bogaards, J. J. P., Hissink, E. M., Briggs, M., Weaver, R., Jochemsen, R., Jackson, P., Bertrand, M., and van Bladeren, P. J. (2000). Prediction of interindividual variation in drug plasma levels in vivo from individual enzyme kinetic data and physiologically based pharmacokinetic modeling. Eur J Pharm Sci 12, 117-124.

Drinkwater, N. R., Miller, E. C., Miller, J. A., and Pitot, H. C. (1976). Hepatocarcinogenicity of estragole (1-allyl-4-methoxybenzene) and 1'-hydroxyestragole in the mouse and mutagenicity of 1'-acetoxyestragole in bacteria. J Natl Cancer Inst 57, 1323-1331.

Evans, M. V. and Andersen, M. E. (2000). Sensitivity analysis of a physiological model for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): assessing the impact of specific model parameters on sequestration in liver and fat in the rat. Toxicol Sci 54, 71-80.

Fennell, T. R., Miller, J. A., and Miller, E. C. (1984). Characterization of the biliary and urinary glutathione and N-acetylcysteine metabolites of the hepatic carcinogen 1'-hydroxysafrole and its 1'-oxo metabolite in rats and mice. Cancer Res 44, 3231-3240.

George, J., Byth, K., and Farrell, G. C. (1995). Age but not gender selectively affects expression of individual cytochrome P450 proteins in human liver. Biochem Pharmacol 50, 727-730.

Glatt, H. (2000). Sulfotransferases in the bioactivation of xenobiotics. Chem Biol Interact 129, 141-170.

Guenthner, T. M. and Luo, G. (2001). Investigation of the role of the 2',3'-epoxidation pathway in the bioactivation and genotoxicity of dietary allylbenzene analogs. Toxicology 160, 47-58.

Ingelman-Sundberg M, Oscarson M, McLellan RA. Polymorphic human cytochrome P450 enzymes: an opportunity for individualized drug treatment. (1999). Trends Pharmacol Sci 20 ,342–9.

Jeurissen, S. M. F., Punt, A., Boersma, M. G., Bogaards, J. J., Fiamegos, Y. C., Schilter, B., van Bladeren, P. J., Cnubben, N. H.P., and Rietjens, I. M. C. M. (2007). Human cytochrome p450 enzyme specificity for the bioactivation of estragole and related alkenylbenzenes. Chem Res Toxicol 20, 798-806.

Jeurissen, S. M. F., Punt, A., Delatour, T., and Rietjens, I. M. C. M. (2008). Basil extract inhibits the sulfotransferase mediated formation of DNA adducts of the procarcinogen 1'-hydroxyestragole by rat and human liver S9 homogenates and in HepG2 human hepatoma cells. Food and Chem

Toxicol 46, 2296-2302.

Jiang, Z., Dragin, N., Jorge-Nebert, L. F., Martin, M. V., Guengerich, F. P., Aklillu, E., Ingelman-Sundberg, M., Hammons, G. J., Lyn-Cook, B. D., Kadlubar, F. F., Saldana, S. N., Sorter, M., Vinks, A. A., Nassr, N., von Richter, O., Jin, L., and Nebert, D. W. (2006a). Search for an association between the human CYP1A2 genotype and CYP1A2 metabolic phenotype. Pharmacogenet Genomics 16, 359-367.

Jiang, Z., Dragin, N., Jorge-Nebert, L. F., Martin, M. V., Guengerich, F. P., Aklillu, E., Ingelman-Sundberg, M., Hammons, G. J., Lyn-Cook, B. D., Kadlubar, F. F. S., S.N., Sorter, M., Vinks, A. A., Nassr, N., Von Richter, O., Jin, L., and Nebert, D. W. (2006b). Search for an association between the human CYP1A2 genotype and CYP1A2 metabolic phenotype. Pharmacogenetics and Genomics 16, 359-367.

Lipscomb, J. C., and Poet, T. S. (2008). In vitro measurements of metabolism for application in pharmacokinetic modeling. Pharmacol Ther 118, 82-103.

Luo, G., Qato, M. K., and Guenthner, T. M. (1992). Hydrolysis of the 2',3'-allylic epoxides of allylbenzene, estragole, eugenol, and safrole by both microsomal and cytosolic epoxide hydrolases. Drug Metab Dispos 20, 440-445.

Medinsky, M. A., Leavens, T. L., Csanady, G. A., Gargas, M. L., and Bond, J. A. (1994). In vivo metabolism of butadiene by mice and rats: a comparison of physiological model predictions and experimental data. Carcinogenesis 15, 1329-1340.

Miller, J. A., Miller, E. C., and Phillips, D. H. (1982). The metabolic activation and carcinogenicity of alkenylbenzenes that occur naturally in many spices. in: H.F> Stich (Ed.), Carcinogens and mutagens in the environment, vol. I, CRC Press, Boca Raton, 83-96.

Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A., and Miller, J. A. (1983). Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. Cancer Res 43, 1124-1134.

Moisan, A. M., Ricketts, M. L., Tardy, V., Desrochers, M., Mebarki, F., Chaussain, J. L., Cabrol, S., Raux-Demay, M. C., Forest, M. G., Sippell, W. G., Peter, M., Morel, Y. and Simard, J. (1999). New insight into the molecular basis of 3beta-hydroxysteroid dehydrogenase deficiency: identification of eight mutations in the HSD3B2 gene eleven patients from seven new families and comparison of the functional properties of twenty-five mutant enzymes. J Clin Endocrinol Metab 84, 4410-4425.

Nagar, S., Walther, S. and Blanchard, R. L. (2006). Sulfotransferase (SULT) 1A1 polymorphic variants *1, *2, and *3 are associated with altered enzymatic activity, cellular phenotype, and protein degradation. Mol Pharmacol 69, 2084-2092.

Peltoketo, H., Luu-The, V., Simard, J., and Adamski, J. (1999). 17beta-hydroxysteroid dehydrogenase (HSD)/17-ketosteroid reductase (KSR) family; nomenclature and main characteristics of the 17HSD/KSR enzymes. J Mol Endocrinol 23, 1-11.

Phillips, D. H., Miller, J. A., Miller, E. C., and Adams, B. (1981). Structures of the DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1'-hydroxyestragole. Cancer Res 41, 176-186.

Phillips, D. H., Reddy, M. V., and Randerath, K. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. II. Newborn male B6C3F1 mice. Carcinogenesis 5, 1623-1628.

Plourde, M., Manhes, C., Leblanc, G., Durocher, F., Dumont, M., Sinilnikova, O. and Simard, J. (2008). Mutation analysis and characterization of HSD17B2 sequence variants in breast cancer cases from French Canadian families with high risk of breast and ovarian cancer. J Mol Endocrinol 40, 161-172.

Poirier, D., Bydal, P., Tremblay, M. R., Sam, K. M. and Luu-The, V. (2001). Inhibitors of type II 17beta-hydroxysteroid dehydrogenase. Mol Cell Endocrinol 171, 119-128.

Punt, A., Delatour, T., Scholz, G., Schilter, B., Bladeren, P. J., and Rietjens,

I. M. C. M. (2007). Tandem mass spectrometry analysis of N^2-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine as a strategy to study species differences in sulfotransferase conversion of the proximate carcinogen 1'-hydroxyestragole. Chem Res Toxicol 20, 991-998.

Punt A., Freidig A.P., Delatour T., Scholz G., Schilter B., Van Bladeren P.J., Rietjens I.M.C.M. (2008). A physiologically based biokinetic (PBBK) model for estragole bioactivation and detoxification in rat. Toxicology and Applied Pharmacology, 231: 248-259.

Randerath, K., Haglund, R. E., Phillips, D. H., and Reddy, M. V. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. Carcinogenesis 5, 1613-1622.

Sangster, S. A., Caldwell, J., Hutt, A. J., Anthony, A., and Smith, R. L. (1987). The metabolic disposition of [methoxy-^{14}C]-labelled trans-anethole, estragole and p-propylanisole in human volunteers. Xenobiotica 17, 1223-1232.

SCF (2001). Opinion of the scientific committee on food on estragole (1-allyl-4-methyxybenzene). http://ec.europa.eu/food/fs/sc/scf/out104_en.pdf.

Schuster, D., Nashev, L. G., Kirchmair, J., Laggner, C., Wolber, G., Langer, T., and Odermatt, A. (2008). Discovery of nonsteroidal 17beta-hydroxysteroid dehydrogenase 1 inhibitors by pharmacophore-based screening of virtual compound libraries. J Med Chem 51, 4188-4199.

Simard, J., Ricketts, M. L., Gingras, S., Soucy, P., Feltus, F. A. and Melner, M. H. (2005). Molecular biology of the 3beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase gene family. Endocr Rev 26, 525-582.

Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., Rogers, A. E., Caldwell, J., and Sipes, I. G. (2002). Safety assessment of allylalkoxybenzene derivatives used as flavouring substances - methyl eugenol and estragole. Food Chem Toxicol 40, 851-870.

Solheim, E. and Scheline, R. R. (1973). Metabolism of alkenebenzene derivatives in the rat. I. *p*-Methoxyallylbenzene (Estragole) and

p-methoxypropenylbenzene (Anethole). Xenobiotica 3, 493-510.

Stupans, I., Stretch, G. and Hayball, P. (2000). Olive oil phenols inhibit human hepatic microsomal activity. J Nutr 130, 2367-70.

Thomae, B. A., Eckloff, B. W., Freimuth, R. R., Wieben, E. D. and Weinshilboum, R. M. (2002). Human sulfotransferase SULT2A1 pharmacogenetics: genotype-to-phenotype studies. Pharmacogenomics J 2, 48-56. Wenker, M. A., Kezic, S., Monster, A. C., and De Wolff, F. A. (2001). Metabolism of styrene in the human liver in vitro: interindividual variation and enantioselectivity. Xenobiotica 31, 61-72.

WHO (1999) International Programme on Chemical Safety (IPCS): Assessing human health risks of chemicals: Principles for the assessment of risk to human health from exposure to chemicals. Environmental Health Criteria 210, World Health Organisation, Geneva. http://www.inchem.org/documents/ehc/ehc/ehc210.htm.

Wiseman, R. W., Fennell, T. R., Miller, J. A., and Miller, E. C. (1985). Further characterization of the DNA adducts formed by electrophilic esters of the hepatocarcinogens 1'-hydroxysafrole and 1'-hydroxyestragole in vitro and in mouse liver in vivo, including new adducts at C-8 and N-7 of guanine residues. Cancer Res 45, 3096-3105.

Wiseman, R. W., Miller, E. C., Miller, J. A., and Liem, A. (1987). Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J x C3H/HeJ F_1 mice. Cancer Res 47, 2275-2283.

7

Summary and general discussion

Summary of the results

Estragole is an alkenylbenzene that is naturally present in different herbs such as tarragon, basil and fennel. Dietary human intake of estragole mainly results from the consumption of these herbs as well as the consumption of their essential oils used as flavoring agents in different products (Smith *et al.*, 2002). There is interest in the evaluation of the safety of estragole as a food constituent since at high dose levels this compound has been observed to be hepatocarcinogenic in rodent studies (Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Wiseman *et al.*, 1987). Induction of the carcinogenicity of estragole proceeds via the formation of a genotoxic metabolite which is formed through a two step bioactivation pathway catalyzed by cytochrome P450 enzymes followed by sulfotransferases (Drinkwater *et al.*, 1976; Wiseman *et al.*, 1987).

In 2001, the Scientific Committee on Food (SCF) of the European Union concluded that estragole is genotoxic and carcinogenic, and restrictions in use were indicated (SCF, 2001). In an evaluation performed by the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) in 2002, it was concluded that exposure to estragole from food does not pose a significant cancer risk to humans (Smith *et al.*, 2002). In this conclusion it was taken into account that there are experimental data suggesting a non-linear relationship between dose and profiles of metabolism and metabolic activation. In a more recent evaluation performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2008, it was indicated that, although evidence of carcinogenicity to rodents given high doses of estragole exists, further research is needed to assess the potential risk to human health from low-level dietary exposure to estragole present in foods and essential oils and used as flavoring agents (JECFA, 2008).

The differences in expert judgments on the safety of estragole result in part from a general problem in cancer risk assessment studies, which is a lack of scientific consensus on how to translate carcinogenicity data obtained in experiments with rodents at high levels of exposure to the situation for

humans exposed at low levels. Such a translation requires extrapolation of the cancer risk orders of magnitude below the levels where tumor incidences are observed in animal experiments. Uncertainties about the i) shape of the dose-response curve at dose levels relevant for dietary human intake, about ii) species differences in metabolism and metabolic activation, and about iii) the influence of the larger interindividual variability in the human population as compared to the variability in the inbred strains used for the rodent bioassays, make it difficult to perform such extrapolations. To facilitate the risk assessment of estragole, additional knowledge on these uncertainties that can influence the extrapolation is needed. The aim of the present PhD project was to build physiologically based biokinetic (PBBK) models for both rats and (individual) human subjects to obtain quantitative insight into dose-dependent effects, species differences, and interindividual differences in bioactivation and detoxification of estragole.

The next section summarizes the results obtained in the present thesis. First, the observed dose-dependent effects in bioactivation and detoxification of estragole in both rat and human will be discussed based on the results obtained in Chapters 3 and 4. Second, the species differences in bioactivation and detoxification of estragole between human and male rat will be discussed based on the results obtained in Chapters 2, 3, and 4. Third, the human interindividual variation in metabolic activation of estragole will be discussed based on the results obtained in Chapters 5 and 6. Finally, the possible implications of the findings for the hazard and risk assessment of estragole will be discussed and some additional considerations will be presented.

Dose dependent differences in biokinetics

In Chapter 3 a PBBK model for estragole metabolism in male rat was developed to gain insight in dose-dependent effects in metabolism and metabolic activation of estragole. It was demonstrated that a shift from *O*-demethylation to 1'-hydroxylation occurs with increasing doses of estragole. *O*-demethylation of estragole is the major metabolic route at low doses of estragole, occurring mainly in the lung and kidney of male rat. In these organs

4-allylphenol is formed with high affinity, reflected by a relative low K_m. Due to saturation of the *O*-demethylation pathway in lung and kidney at higher doses of estragole, formation of the proximate carcinogenic metabolite 1'-hydroxyestragole, which was shown to occur mainly in the liver of male rat, becomes relatively more important. The PBBK model predicted that formation of this metabolite increased from 16% of the dose at a dose of 0.05 mg/kg bw to 29% of the dose at a dose of 300 mg/kg bw. This relative increase in formation of 1'-hydroxyestragole leads to a relative increase in formation of 1'-hydroxyestragole glucuronide, 1'-oxoestragole, and 1'-sulfooxyestragole as well, the latter being the ultimate carcinogenic metabolite of estragole. The formation of 1'-sulfooxyestragole predicted by the PBBK model increased from 0.08% of the dose at a dose of 0.05 mg/kg bw to 0.16% of the dose at a dose of 300 mg/kg bw. Overall, these results indicate that the relative importance of different metabolic pathways of estragole may vary in a dose-dependent way, leading to a relative increase in bioactivation of estragole at higher doses.

The findings of the PBBK model for male rat were in good agreement with observations in the literature, revealing dose-dependent effects on the biokinetics for estragole in female Wistar rats *in vivo.* In these *in vivo* studies the proportion of *O*-demethylation was observed to decrease with increasing doses (as determined by the percentage of exhalation as $^{14}CO_2$), whereas the proportion of the dose excreted as 1'-hydroxyestragole glucuronide in the urine increased from 1.3-5.4% of the dose in the range of 0.05-50 mg/kg bw to 11.4-13.7% in the dose range of 500-1000 mg/kg bw (Anthony *et al.*, 1987). The PBBK model defined in the present thesis provided insight in the mechanism underlying this dose-dependent effect observed *in vivo*, which was identified to be a result of saturation of the *O*-demethylation pathway in the lung and kidney.

Based on the PBBK model for estragole in human that was defined in Chapter 4, dose-dependent effects in bioactivation and detoxification of estragole in humans could be studied as well. In humans no relative increase in formation of 1'-sulfooxyestragole was identified to occur with increasing

dose levels. The PBBK model even predicted that the relative formation of this metabolite decreased from 0.19% of the dose at a dose of 0.07 mg/kg bw to 0.08% of the dose at a dose of 300 mg/kg bw, due to saturation of the 1'-hydroxylation pathway in the liver. Further analysis revealed that this difference between the rat and human model, showing respectively an increase versus a decrease in the relative formation of 1'-sulfooxyestragole with increasing dose, was due to the fact that in the human model efficient *O*-demethylation in lung and kidney was absent, whereas in the rat these conversions reduced the relative formation of the 1'-sulfooxyestragole at low dose levels. The human PBBK model also revealed that at a dose-range within one order of magnitude of the estimated average dietary human intake of 0.07 mg/kg bw/day, these dose-dependent effects on the relative percentage of the dose converted to 1'-sulfooxyestragole were not significant.

Species-dependent differences in biokinetics.

In addition to dose-dependent effects, species differences in metabolism and metabolic activation should also be taken into account when extrapolating the cancer risk from animal experimental data to the human situation. In Chapter 2 the kinetic constants for sulfonation of 1'-hydroxyestragole by liver S9 homogenates of male rat, male mouse, and human were described to identify possible species differences in this bioactivation step. Because 1'-sulfooxyestragole is unstable in aqueous environment and therefore cannot be measured directly, 2'-deoxyguanosine was added to the incubation mixtures in which sulfonation of 1'-hydroxyestragole was catalyzed, in order to trap the transient 1'-sulfooxyestragole. A method was developed with which the formation of the most abundant adduct with 2'-deoxyguanosine could be quantified using isotope dilution LC-ESI-MS/MS. Comparison of the kinetics for sulfonation of 1'-hydroxyestragole by liver S9 samples from different species revealed that this reaction was about 30 times more efficiently catalyzed by male rat liver S9 than by human liver S9, whereas the catalytic efficiency by male mouse and human liver S9 was about the same.

The *in vitro* kinetic data for sulfonation of 1'-hydroxyestragole as

obtained in Chapter 2, as well as the *in vitro* kinetic data for other metabolic reactions obtained in Chapter 3 and 4, gave some insight into whether species differences are to be expected between humans and male rats, but did not reveal the integrated effect of these difference in the overall formation of 1'-hydroxyestragole and 1'-sulfooxyestragole. Using PBBK modeling as a platform for integrating these *in vitro* metabolic data, a comparison was made between humans and male rats in overall differences in bioactivation and detoxification at dose levels of estragole relevant for dietary human intake. The results of this comparison were outlined in Chapter 4 and revealed that the predicted formation of 1'-hydroxyestragole was 4-fold higher in human compared to male rat, due to a relative low level of detoxification of estragole by *O*-demethylation in human compared to male rat. This lower predicted level of *O*-demethylation in human was a result of the absence of this metabolic pathway in human lung and kidney, whereas this metabolic pathway occurred with high affinity in male rat lung and kidney. Even though the formation of 1'-hydroxyestragole was predicted to be 4-fold higher in human, formation of 1'-sulfooxyestragole was predicted to be only 2-fold higher in human due to a high level of detoxification of 1'-hydroxyestragole in human liver by oxidation to 1'-oxoestragole as compared to rat liver.

Formation of this 1'-oxoestragole metabolite has not been considered to be an important metabolic route of 1'-hydroxyestragole before, mainly because in rats only relatively small amounts of derivatives of this metabolite have been detected in the urine after exposure to estragole (Solheim and Scheline, 1973). Based on the approach in the present thesis of identifying principal metabolic pathways of estragole in incubations with tissue fractions of relevant organs, it could be revealed that in humans oxidation of 1'-hydroxyestragole is a major metabolic pathway, which was predicted to account for even 62.7 % of the dose. Validation of the formation of this metabolite against human *in vivo* data was, however, not possible, since excretion of this metabolite, or its conjugates, was not analyzed in humans exposed to estragole (Sangster *et al.*, 1987). In the study by Sangster *et al.* (1987) on humans exposed to estragole, the major part of the metabolites (~70% of the dose) was not identified and

could therefore reflect the formation of 1'-oxoestragole, which was predicted to amount to 62.7 % of the dose, based on the PBBK model for estragole in human. Altogether, it is concluded that the species dependent variation in bioactivation of estragole is smaller than the default factor of 4 generally assumed to reflect interspecies variation in kinetics (assuming that the default factor of 10 can be divided into a factor of 4 for kinetics and 2.5 for dynamics) (WHO, 1999).

Human interindividual variability in biokinetics

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 for the key metabolic reactions involved. Based on the PBBK model for estragole in human as defined in Chapter 4, and a sensitivity analysis for this model, it was demonstrated that formation of 1'-hydroxyestragole and subsequent oxidation of this metabolite to 1'-oxoestragole are key metabolic reactions affecting the amount of 1'-hydroxyestragole in the liver, that is available for formation of the ultimate carcinogen 1'-sulfooxyestragole. It can thus be expected that interindividual differences in these metabolic reactions cause differences in the relative level of bioactivation of estragole between different human subjects. To gain insight in the level of variability in 1'-hydroxylation of estragole and oxidation of 1'-hydroxyestragole that could occur in the human population it is of importance to characterize the enzymes involved in these reactions.

In Chapter 5 the enzymes involved in estragole 1'-hydroxylation were identified. Experiments with Gentest microsomes, expressing P450 enzymes at about average levels of the liver, revealed that 1'-hydroxylation of estragole was most efficiently catalyzed by P450 1A2 and 2A6. To a smaller extent P450 2D6, 2C19, and 2E1 were also able to catalyze 1'-hydroxylation of estragole (Jeurissen *et al*., 2007). Based on these results it was concluded that P450 1A2 and 2A6 play a pivotal role in 1'-hydroxylation of estragole. Activity levels of P450 1A2 can be influenced by smoking and consumption of charbroiled meat (Jiang *et al*., 2006). The activity of P450 1A2 can vary

about 60-fold within the population (Wenker *et al*., 2001; Jiang *et al*., 2006). Interindividual variability in P450 2A6 activity mainly depends on genetic differences (Ingelman-Sundberg *et al*., 1999). Polymorphisms occur that lead to poor metabolizer phenotypes or whole deletion genotypes.

Characterization of the enzyme(s) involved in oxidation of 1'-hydroxyestragole was performed in Chapter 6. It was revealed that type 2 17β-hydroxysteroid dehydrogenase (17β-HSD) is involved in this reaction, which is an enzyme that is present in the microsomal fraction of the liver and uses NAD^+ as the preferred cofactor (Peltoketo *et al*., 1999). Current knowledge about 17β-HSD type 2 does not give a complete insight in the level of variation in enzyme activity that could occur in the human population. It has been reported that 17β-HSD type 2 is widely and abundantly expressed (Peltoketo *et al*., 1999). Recently, however, a specific sequence variant of the gene encoding 17β-HSD type 2 has been identified that might lead to a reduced activity of this enzyme (Plourde *et al*., 2008).

The effect of interindividual variation in 1'-hydroxylation of estragole and oxidation of 1'-hydroxyestragole on the liver levels of the proximate carcinogenic metabolite 1'-hydroxyestragole was investigated in Chapter 6. In this study at first PBBK models were defined for fourteen individual human subjects using the individual kinetic data for these reactions. These models revealed a 1.7-fold interindividual variation in the area under the concentration-time curve (AUC) for 1'-hydroxyestragole in the liver within this group of human subjects. In a second step, the individual models were combined with literature data on interindividual variation in activity levels of key enzymes involved in formation and detoxification of 1'-hydroxyestragole to model the maximum level of variability in the general population. Interindividual differences in activity levels of the P450 enzymes involved, varying orders of magnitude due to lifestyle factors or polymorphism, were predicted to affect the liver AUC of 1'-hydroxyestragole less than 10-fold. Variation in oxidation of 1'-hydroxyestragole was shown to result in larger effects. A 10-fold reduced oxidation activity would result in an about 8-fold increase in 1'-hydroxyestragole formation, whereas homozygous deletion genotypes could,

in theory, lead to 30- to 60-fold variation within the population. Obviously definite conclusions on the effects of variation in 17β-HSD phenotypes on interindividual differences in estragole bioactivation and detoxification have to await further data on the actual variation in the 17β-HSD activity to be expected within the human population. It was concluded that the liver AUC values for 1'-hydroxyestragole may vary about one to, at most, two orders of magnitude within the human population. This interindividual variation in bioactivation of estragole may be larger than the default factor of 3.16 generally assumed to reflect interindividual variation in biokinetics within the human population (assuming that the default factor of 10 can be divided into a factor of 3.16 for kinetics and 3.16 for dynamics) (WHO, 1999).

Summary

Altogether, the results presented in this thesis show that integrating *in vitro* metabolic parameters using a PBBK model as a framework, provides a good method to evaluate the occurrence of dose-dependent effects, species differences, and human variability in bioactivation of estragole. Using this approach, mechanisms underlying dose-dependent effects in bioactivation of estragole in rat were revealed. Furthermore, insight was obtained in the occurrence of species differences and human interindividual variability in metabolism and metabolic activation of estragole. The results obtained in the present study may contribute to a more refined extrapolation of animal carcinogenicity data obtained at high doses to a low dose human situation.

Implications for risk assessment

Worldwide different approaches exist to assess the risk of compounds that are both genotoxic and carcinogenic. Numerical estimates of the risk associated with human exposure might be derived by extrapolation of carcinogenicity data obtained in animals at high dose levels to low dose levels relevant for the human situation. Many mathematical models have been proposed by which such an extrapolation below the available experimental

data can be performed, of which linear extrapolation is the simplest form (reviewed in COC, 2004). Extrapolating from animal tumor data at high doses using mathematical modeling in order to obtain estimates of the risk to humans at low dose exposure levels has been much debated, since it is not known whether or not the model chosen actually reflects the underlying biological processes. It is also argued that by extrapolating linear from animal data to the human situation, species differences are not taken into account. In addition, obtained numerical estimates may be misused or misinterpreted in further risk management and risk communication, in case the uncertainties and inaccuracies connected to the model are not being communicated.

Considering these disadvantages, the Scientific Committee of the European Food safety Authority (EFSA) recommend the use of a different approach, known as the margin of exposure (MOE) approach (EFSA, 2005). The MOE approach uses a reference point, usually derived from data from an animal experiment that represent a dose causing a low but measurable cancer response. It can, for example, be the $BMDL_{10}$, which is the lower confidence bound of the Benchmark Dose that gives 10 % (extra) cancer incidence (BMD_{10}). In that case, the MOE is defined as the ratio between this reference point, the $BMDL_{10}$, and the estimated dietary intake (EDI) in humans. When this margin is higher than 10,000, the carcinogenic potency of the compound is considered to be of low priority for risk management actions (EFSA, 2005; Barlow *et al.*, 2006; Dybing *et al.*, 2008). This safety margin of 10,000 is applied to adequately allow for various uncertainties in the MOE approach, including (EFSA, 2005):

i) a factor of 100 for species differences and human variability in biokinetics and biodynamics. This safety factor is subdivided in a factor of 10 for species differences (consisting of a default value of 4 for kinetics and 2.5 for dynamics) and a factor 10 for interindividual differences (consisting of a default value of 3.16 for kinetics and 3.16 for dynamics) (WHO, 1999).
ii) a factor of 10 for interindividual human variability in cell cycle

control and DNA repair

iii) a factor of 10 for uncertainties in the shape of the dose-response curve outside the observed dose range.

To date, carcinogenicity data for estragole from which a $BMDL_{10}$, and thus an MOE can be derived from results from a long-term carcinogenicity study conducted in mice (Miller *et al*., 1983). Table 1 presents the carcinogenicity data obtained for estragole in female mice in this study. A BMD analysis of these data using BMDS version 1.4.1c and/or PROAST software was performed of which the results are given in table 2. From this table it is concluded that the $BMDL_{10}$ value varies between 9-33 mg/kg bw/day.

Table 1. Overview of the data from Miller *et al*. (1983) on the incidence of hematomas in female mice exposed to estragole for 12 months via the diet.

dose	Estimated dose mg/kg bw/ day	Number of animals	Number of mice with hematomas	Incidence (%)
0	0	43	0	0
0.23% in diet	150-300	48	27	56
0.46% in diet	300-600	49	35	71

Table 2. Results of a BMD analysis of the data from Miller *et al*. (1983) on the incidence of hepatomas in female mice exposed to estragole for 12 months via the diet (Table 1), using BMDS version 1.4.1c and the default settings of extra risk, a Benchmark Response (BMR) of 10% and a 95% confidence limit. To make a worst case estimate the lowest dose levels of the range were used (i.e. 150 and 300 mg/kg bw/day).

mice gender	model	number of parameters	log likelihood	accepted	BMD_{10} mg/kg bw/ day	$BMDL_{10}$ mg/kg bw/ day
female	null	1	-96.1243			
female	full	3	-62.2103			
female	two-stage	1	-62.7403	yes	22.4	18.1
female	gamma	1	-62.7403	yes	22.4	18.1
female	log-logistic	1	-62.2124	yes	13.1	9.2
female	log-probit	1	-62.7928	yes	40.7	32.7
female	Weibull	1	-62.7403	yes	22.4	18.1

The average daily intake of estragole per capita was estimated by the Scientific Committee on Food of the European Union (SCF) to amount to about 4.3 mg per day (corresponding to 0.07 mg/kg bw/day for a 60 kg person) (SCF, 2001). This estimation is based on a relative conservative method using theoretical maximum use levels of estragole in 28 food categories and consumption data for these food categories based on seven days dietary records of adult individuals (SCF, 2001). Using a different method, a lower average per intake of estragole capita daily was estimated by the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) (Smith *et al.,* 2002). This estimation was performed using production volume data of herbs, essential oils, and flavor substances containing estragole in the US (Smith *et al*., 2002). The FEMA estimated the daily intake per capita to be less than 10 µg/kg bw/day (Smith *et al.*, 2002). The majority (63%) of this intake was derived from the herbs and spices that naturally contain estragole, 27% was derived from essential oils containing estragole, and 10% from estragole added as such (Smith *et al.*, 2002). This intake estimate might present the intake of estragole from consumption of spices and their essential oils alone.

Using the exposure assessment provided by the SCF (SCF, 2001) of 0.07 mg/kg bw/day and the $BMDL_{10}$ of 9-33 mg/kg bw/day, the MOE value would amount to 127 up to 569, which is lower than 10,000, indicating that the consumption of estragole at these intake levels might be a high priority for risk management. Using the exposure assessment provided by Smith *et al.* (2001) of 0.01 mg/kg bw/day and the $BMDL_{10}$ of 9-33 mg/kg bw/day, the MOE value would amount to 900 up to 4100. Comparison of this MOE value to the value of 10,000 indicates that at these intake levels the use of estragole containing spices and their essential oils might also be considered a priority for risk management.

In the opinion of the EFSA it has been stated that the default margin of exposure of 10,000 can be reduced or increased when appropriate specific chemical data are available (EFSA, 2005). The results of this thesis can provide insight in especially the applicability of the default safety factors for species differences and interindividual differences in biokinetics used to define the

value of 10,000. The outcomes of the PBBK models of the present thesis reveal that species differences in bioactivation of estragole were observed to be smaller than the default factor of 4 generally assumed to reflect interspecies variation in kinetics, but that human interindividual variation in bioactivation of estragole is likely to be larger than the default factor of 3.16 generally assumed to reflect interindividual variation in biokinetics. Taking these two observations together it becomes clear that these specific chemical data would not support a reduction of the default value of 10,000 and thus a different conclusion on the priority for risk management.

A similar conclusion emerges from the approach in which linear extrapolation from a defined point of departure is used to derive a so-called virtual safe dose (VSD) at which the additional cancer risk upon life time exposure would be one in a million and considered acceptable (EPA 2005). Using the data and BMD analysis of the study of Miller *et al*. (1983) (Table 1 and 2) it can be concluded that in mice, a BMR of 10% extra tumor risk is observed at a BMD_{10} value of 13 to 41 mg/kg bw/day. By linear extrapolation from this point of departure, the VSD that results in an additional cancer risk of 1 in a million is calculated to amount to 0.13 to 0.41 µg/kg bw/day. Comparison of this estimated VSD to the estimated dietary human intake of 10-70 µg/kg bw/day (SCF, 2001; Smith *et al*., 2002) indicates that dietary intake levels are about two orders of magnitude above the VSD, indicating a priority for risk management.

The PBBK models developed in the present thesis for male rat and human indicate that kinetic data do not provide reasons to argue against such a linear extrapolation. This is illustrated in Figure 1, in which the PBBK model-predicted dose-dependent formation of 1'-sulfooxyestragole in the liver of rat and human is displayed. Both curves are observed to be quite linear from doses as high as the BMD_{10} down to as low as the VSD, when plotted on a log-log scale as done in Figure 1 as well as on a linear scale (figure not shown). Deviations from linearity in formation of 1'-sulfooxyestragole are only observed for both rats and humans at dose levels above doses of 70 mg/kg bw/day, which is higher than the BMD_{10}. Since the BMD_{10} appears

to be approximately within the linear part of the curve and since the rat and human curves do not differ substantially, the PBBK results of the present study support that possible nonlinear kinetics and species differences in kinetics should not be used as an argument against using this linear low dose extrapolation from high dose animal data to the low dose human situation. The observed linear formation of 1'-sulfooxyestragole with increasing dose in male rat liver indicates that it is reasonable to extrapolate the cancer risk linear from high doses to low dose levels. In addition, since no significant species differences have been observed to occur at low doses, the VSD thus obtained for estragole in rodent studies can be considered to be relevant for the human situation as well.

Additional considerations

Whereas the risk assessment outlined above takes into account the predicted data on dose-dependent effects, species differences and interindividual differences in bioactivation of estragole, it should be noted that other factors might affect the risk assessment as well. The carcinogenic effects of estragole

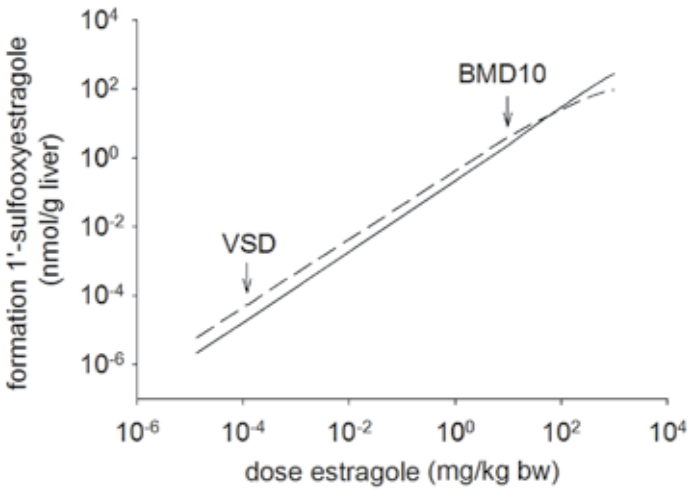


Figure 1. PBBK model-predicted dose-dependent formation of 1'-sulfooxyestragole in the liver of rat (—) and human (- -).

will, for instance, also depend on toxicodynamic processes (i.e. the formation of DNA adducts or induction of mutations and the ultimate formation and development of tumors). Non-linear dose-dependent increases in DNA adduct formation could occur when DNA repair becomes saturated at relatively high doses, resulting in a relative increase in DNA adduct formation with increasing dose. Saturation of DNA repair at increasing dose levels may be a reason why dose dependent increases in cancer incidences should be described by non-linear rather than linear models. This could be investigated in further detail by extending the PBBK models of the present thesis into so-called physiologically based biodynamic (PBBD) models in which dose levels and 1'-sulfooxyestragole formation should be coupled to DNA adduct formation, and, ultimately, cancer incidence. Using the LC-MS method for quantification of estragole DNA adduct formation as developed in the present thesis, research is currently being performed to extend the PBBK models of the present thesis into PBBD models able to quantify 1'-hydroxyestragole dependent DNA adduct formation in the liver *in vivo* based on data obtained in primary hepatocytes exposed to 1'-hydroxyestragole *in vitro*. With these PBBD models the level of DNA adduct formation can be predicted at different oral doses, including doses relevant for dietary human intake. This approach allows taking into account possible effects of DNA repair in the extrapolation from high to low dose estragole for rat and human. Since DNA adduct formation is presently considered a marker of exposure rather than a biomarker for tumor incidence (Phillips 2005; Sander *et al*., 2005), a full PBBD model should ultimately also find a way to link the predicted DNA adduct formation to tumor incidences.

In addition, it should be noted that whereas animal carcinogenicity experiments are conducted with a pure compound, human dietary exposure to estragole occurs in a complex of other herbal ingredients. In a complex food matrix, interactions can occur that can affect the bioavailability of estragole (Schilter *et al*., 2003; Rietjens *et al*., 2008). For example, a slow or incomplete release of estragole from the matrix could result in a reduced bioavailability of estragole as compared to the bioavailability when dosed

as a pure compound by oral gavage. In addition to the effect of the food matrix on the bioavailability, interactions with other herbal ingredients might occur at the level of metabolic activation and/or detoxification of estragole (Schilter *et al*., 2003; Rietjens *et al*., 2008). 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. These results suggest that the bioactivation of estragole and subsequent adverse effects of estragole are probably lower when consumed in a matrix of other basil ingredients than would be expected on the basis of experiments using estragole as a single compound. Whether this inhibition could also occur *in vivo* is, however, not yet established and should be further explored.

In conclusion, the data in the present thesis show that PBBK modeling provides a good method to evaluate the occurrence of dose-dependent effects, species differences, and human variability in bioactivation of estragole. The model predictions obtained can be used to provide a more mechanistic basis for the assessment of the cancer risk in humans at low dose dietary intake levels based on carcinogenicity data obtained in experiments with rodents at high dose levels. However, for a complete assessment of the cancer risk at low dose human intake scenarios additional information is still needed. First of all, additional carcinogenicity studies for estragole might be needed to provide a better definition of the BMD_{10} and $BMDL_{10}$, on which a quantitative cancer risk assessment or a MOE calculation can be based. Second, more information will be needed on the occurrence of polymorphisms in the 17β-hydroxysteroiddehydrogenase enzyme and the effects of such polymorphisms on the extent of detoxification of 1'-hydroxyestragole by oxidation of this metabolite. Third, more insight will be needed in toxicodynamic processes that can affect the risk assessment. This includes increased insight in the relation between the level of 1'-sulfooxyestragole formation, the level of DNA adduct formation and the tumor incidences. Finally, more insight is needed in the modulating effects of other herbal ingredients on the carcinogenic process of estragole. The methods developed in the present thesis can provide a basis

to study these processes affecting the carcinogenicity and the risk assessment of estragole to a further extent.

References

Anthony, A., Caldwell, J., Hutt, A. J., and Smith, R. L. (1987). Metabolism of estragole in rat and mouse and influence of dose size on excretion of the proximate carcinogen 1'-hydroxyestragole. Food Chem Toxicol 25, 799-806.

Barlow, S., Renwick, A. G., Kleiner, J., Bridges, J. W., Busk, L., Dybing, E., Edler, L., Eisenbrand, G., Fink-Gremmels, J., Knaap, A., Kroes, R., Liem, D., Muller, D. J., Page, S., Rolland, V., Schlatter, J., Tritscher, A., Tueting, W., and Wurtzen, G. (2006). Risk assessment of substances that are both genotoxic and carcinogenic report of an International Conference organized by EFSA and WHO with support of ILSI Europe. Food Chem Toxicol 44, 1636-1650.

Committee on Carcinogenicity of chemicals in food, consumer products and the environment (COC) (2004). Guidance on a strategy for the risk assessment of chemical carcinogens. http://www.advisorybodies.doh.gov.uk/coc/guideline04.pdf.

Drinkwater, N. R., Miller, E. C., Miller, J. A., and Pitot, H. C. (1976). Hepatocarcinogenicity of estragole (1-allyl-4-methoxybenzene) and 1'-hydroxyestragole in the mouse and mutagenicity of 1'-acetoxyestragole in bacteria. J Natl Cancer Inst 57, 1323-1331.

Dybing, E., Doe, J., Groten, J., Kleiner, J., O'Brien, J., Renwick, A. G., Schlatter, J., Steinberg, P., Tritscher, A., Walker, R., and Younes, M. (2002). Hazard characterisation of chemicals in food and diet. dose response, mechanisms and extrapolation issues. Food Chem Toxicol 40, 237-282.

Dybing, E., O'Brien, J., Renwick, A. G., and Sanner, T. (2008). Risk assessment of dietary exposures to compounds that are genotoxic and carcinogenic--an overview. Toxicol Lett 180, 110-117.

EFSA (2005). Opinion of the scientific committee on a request from EFSA related to a harmonised approach for risk assessment of substances which are both genotoxic and carcinogenic. http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/sc_op_ej282_gentox_en3.pdf?ssbinary=true

EPA (2005). Guidelines for Carcinogen Risk Assessment and Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens. Washington, DC:U.S. Environmental Protection Agency. http://cfpub.epa.gov/ncea/raf/recordisplay.cfm?deid=116283

Ingelman-Sundberg M, Oscarson M, McLellan RA. (199). Polymorphic human cytochrome P450 enzymes: an opportunity for individualized drug treatment. Trends Pharmacol Sci 20 ,342–349.

JECFA (2008). Sixty-ninth meeting Rome, Italy, 17-26 June 2008. http://www.who.int/entity/ipcs/food/jecfa/summaries/summary69.pdf.

Jeurissen, S. M. F., Punt, A., Boersma, M. G., Bogaards, J. J., Fiamegos, Y. C., Schilter, B., van Bladeren, P. J., Cnubben, N. H. and Rietjens, I. M. C. M. (2007). Human cytochrome p450 enzyme specificity for the bioactivation of estragole and related alkenylbenzenes. Chem Res Toxicol 20, 798-806.

Jeurissen S.M.F, Punt A., Delatour T., Rietjens I.M.C.M. (2008). Basil extract inhibits the sulfotransferase mediated formation of DNA adducts of the procarcinogen 1'-hydroxyestragole by rat and human liver S9 homogenates and in HepG2 Human Hepatoma Cells. Food and Chemical Toxicology, 46: 2296-2302.

Jiang, Z., Dragin, N., Jorge-Nebert, L. F., Martin, M. V., Guengerich, F. P., Aklillu, E., Ingelman-Sundberg, M., Hammons, G. J., Lyn-Cook, B. D., Kadlubar, F. F., Saldana, S. N., Sorter, M., Vinks, A. A., Nassr, N., von Richter, O., Jin, L. and Nebert, D. W. (2006). Search for an association between the human CYP1A2 genotype and CYP1A2 metabolic phenotype. Pharmacogenet Genomics 16, 359-67.

Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A., and Miller, J. A. (1983). Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. Cancer Res 43, 1124-1134.

Peltoketo, H., Luu-The, V., Simard, J., and Adamski, J. (1999). 17beta-hydroxysteroid dehydrogenase (HSD)/17-ketosteroid reductase (KSR) family; nomenclature and main characteristics of the 17HSD/KSR enzymes. J Mol Endocrinol 23, 1-11.

Phillips, D. H. (2005). DNA adducts as markers of exposure and risk. Mutat Res 577, 284-92.

Plourde, M., Manhes, C., Leblanc, G., Durocher, F., Dumont, M., Sinilnikova, O. and Simard, J. (2008). Mutation analysis and characterization of HSD17B2 sequence variants in breast cancer cases from French Canadian families with high risk of breast and ovarian cancer. J Mol Endocrinol 40, 161-172.

Rietjens, I. M. C. M., Slob, W., Galli, C. and Silano, V. (2008). Risk assessment of botanicals and botanical preparations intended for use in food and food supplements: emerging issues. Toxicol Lett 180, 131-136.

Sander, M., Cadet, J., Casciano, D. A., Galloway, S. M., Marnett, L. J., Novak, R. F., Pettit, S. D., Preston, R. J., Skare, J. A., Williams, G. M., Van Houten, B. and Gollapudi, B. B. (2005). Proceedings of a workshop on DNA adducts: biological significance and applications to risk assessment Washington, DC, April 13-14, 2004. Toxicol Appl Pharmacol 208, 1-20.

Sangster, S. A., Caldwell, J., Hutt, A. J., Anthony, A., and Smith, R. L. (1987). The metabolic disposition of [methoxy-^{14}C]-labelled trans-anethole, estragole and p-propylanisole in human volunteers. Xenobiotica 17, 1223-1232.

SCF (2001). Opinion of the scientific committee on food on estragole (1-allyl-4-methyxybenzene). http://ec.europa.eu/food/fs/sc/scf/out104_en.pdf.

Schilter, B., Andersson, C., Anton, R., Constable, A., Kleiner, J., O'Brien, J., Renwick, A.G., Korver, O., Smit, F., and Walker, R., (2003). Guidance for the safety assessment of botanicals and botanical preparation for use in food and food supplements. Food Chem Toxicol 41, 1625-1649.

Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., Rogers, A. E., Caldwell, J., and Sipes, I. G. (2002). Safety assessment of allylalkoxybenzene

derivatives used as flavouring substances - methyl eugenol and estragole. Food Chem Toxicol 40, 851-870.

Solheim, E. and Scheline, R. R. (1973). Metabolism of alkenebenzene derivatives in the rat. I. p-Methoxyallylbenzene (Estragole) and p-methoxypropenylbenzene (Anethole). Xenobiotica 3, 493-510.

Wenker, M. A., Kezic, S., Monster, A. C. and De Wolff, F. A. (2001). Metabolism of styrene in the human liver in vitro: interindividual variation and enantioselectivity. Xenobiotica 31, 61-72.

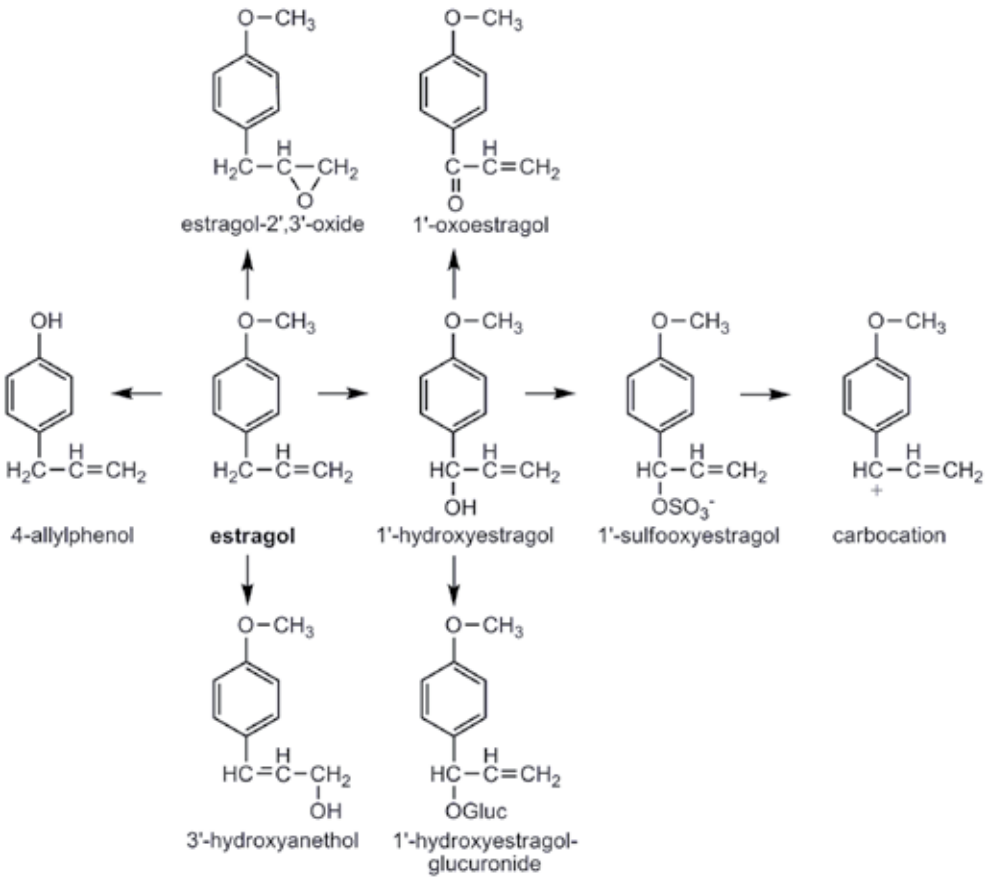
WHO (1999) International Programme on Chemical Safety (IPCS): Assessing human health risks of chemicals: Principles for the assessment of risk to human health from exposure to chemicals. Environmental Health Criteria 210, World Health Organisation, Geneva. http://www.inchem.org/documents/ehc/ehc/ehc210.htm.

Wiseman, R. W., Miller, E. C., Miller, J. A., and Liem, A. (1987). Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J x C3H/HeJ F_1mice. Cancer Res 47, 2275-2283.

8

Samenvatting

Dit proefschrift beschrijft onderzoek naar de alkenylbenzeen estragol. Estragol is een stof die van nature voorkomt in verschillende kruiden zoals dragon, basilicum, anijs en venkel. In studies waarin muizen zijn blootgesteld aan hoge doses estragol, blijken levertumoren te kunnen ontstaan (Miller *et al*., 1983). Verschillende *in vitro* en *in vivo* studies hebben aangetoond dat deze tumoren veroorzaakt worden doordat estragol in de lever door enzymen wordt omgezet in een genotoxische metaboliet, dat wil zeggen; een omzettingsproduct dat met DNA kan reageren (Drinkwater *et al*., 1976; Wiseman *et al*., 1987). Deze omzetting van estragol naar de uiteindelijke genotoxische metaboliet verloopt in meerdere stappen. De eerste stap is de omzetting van estragol naar 1'-hydroxyestragol (Figuur 1). Sulfatering van 1'-hydroxyestragol leidt



Figuur 1. Metabolisme van estragol.

uiteindelijk tot de vorming van de carcinogene metaboliet, welke instabiel is in water en resulteert in vorming van een reactief carbocation dat kan reageren met DNA (Phillips *et al*., 1981; Phillips *et al*., 1984; Randerath *et al*., 1984; Wiseman *et al*., 1985). Andere metabole reacties van estragol zijn *O*-demethylering, 2',3'-epoxidering en 3'-hydroxylering (Figuur 1), welke respectievelijk resulteren in de vorming van 4-allylphenol, estragol-2',3'-oxide, en 3'-hydroxyanethol en leiden tot ontgifting (detoxificering) van estragol (Solheim and Scheline, 1973; Phillips *et al*., 1981; Anthony *et al*., 1987; Sangster *et al*., 1987; Luo *et al*., 1992; Guenthner en Luo, 2001). Detoxificering van 1'-hydroxyestragol kan lopen via glucuronidering en oxidering van deze metaboliet, waarbij deze laatste route resulteert in de vorming van 1'-oxoestragol (Solheim and Scheline, 1973; Anthony *et al*., 1987; Sangster *et al*., 1987).

Omdat estragol een genotoxisch carcinogeen is, is er aandacht voor de risico's van deze stof voor mensen bij de inname van lage hoeveelheden via de voeding. Inname van estragol door de mens vindt vooral plaats via consumptie van kruiden en via de consumptie van de vluchtige oliën verkregen uit kruiden, die gebruikt worden als smaakstoffen in verscheidene producten (Smith *et al*., 2002). Er zijn verschillende evaluaties uitgevoerd om de risico's in te schatten voor de mens van lage blootstelling aan estragol via het dieet. De wetenschappelijke commissie die de Europese Commissie adviseert over voeding (voorheen de Scientific Committee on Food (SCF) en tegenwoordig de European Food Safety Authorithy (EFSA)) heeft bijvoorbeeld geconcludeerd dat estragol genotoxisch en carcinogeen is en dat daarom beperkingen in het gebruik van deze stof wenselijk zijn (SCF, 2001). Bij een andere evaluatie, uitgevoerd door het expert panel van de Amerikaanse smaakstoffen- en extractenindustrie (Flavor and Extract Manufacturers Organisation (FEMA)), is geconcludeerd dat de huidige blootstelling aan estragol via voedselinname geen significant risico met zich meebrengt (Smith *et al*., 2002). In deze conclusie is experimenteel bewijs meegenomen voor een niet-lineair verband tussen de hoogte van de dosis en de vorming van verschillende metabolieten. Een meer recente evaluatie is uitgevoerd door een internationale commissie

van deskundigen op het gebied van voedingsadditieven (Joint FAO/WHO Expert Committee on Food Additives (JECFA)) in 2008. In deze evaluatie werd geconcludeerd dat, hoewel bewijs bestaat dat estragol carcinogeen is in proefdieren bij hoge doses, meer onderzoek nodig is om het potentiële risico voor mensen bij lage blootstelling aan estragol vast te stellen (JECFA, 2008).

De verschillen in de bovengenoemde evaluaties over de veiligheid van humane blootstelling aan estragol zijn deels te verklaren door een gebrek aan wetenschappelijke consensus over hoe de carcinogeniteitsgegevens verkregen in dierproeven moeten worden vertaald naar een humane situatie. Voor een dergelijke vertaling is extrapolatie nodig van de tumorincidentie verkregen in dierproeven bij hoge doses, naar doses die voor de mens relevant zijn, en die over het algemeen vele malen lager liggen. Onzekerheden over i) de vorm van de dosis-responscurve bij lage doses die relevant zijn voor humane inname, over ii) de speciesverschillen in metabolisme en metabole activering, en over iii) het effect van grotere inter-individuele verschillen in de humane populatie in vergelijking met zogenaamde inteeltknaagdieren die gebruikt worden voor dierproeven, maken het complex om zulke extrapolaties uit te voeren. Om de risico-evaluatie van estragol te faciliteren, is meer kennis nodig over deze onzekerheden die het risico op kanker kunnen beïnvloeden. Het doel van dit promotieonderzoek is om zogenaamde physiologically based biokinetic (PBBK) modellen te construeren waarmee kwantitatief inzicht verkregen kan worden in dosisafhankelijke effecten, speciesverschillen en inter-individuele verschillen in bioactivering en detoxificering van estragol.

In de volgende secties wordt een overzicht gegeven van de resultaten die weergegeven zijn in dit proefschrift. Allereerst worden de waargenomen dosisafhankelijke effecten in bioactivering en detoxificering van estragol voor zowel de rat als de mens besproken, naar aanleiding van de resultaten die verkregen zijn in hoofdstuk 3 en 4. Als tweede worden de speciesverschillen in bioactivering en detoxificering van estragol tussen mens en rat besproken naar aanleiding van de resultaten die zijn verkregen in hoofdstuk 2, 3 en 4. Als derde worden de inter-individuele verschillen

tussen mensen in metabole activering van estragol besproken, naar aanleiding van de resultaten die verkregen zijn in hoofdstuk 5 en 6. Als laatste worden implicaties van de verkregen resultaten besproken voor de risicobeoordeling van estragol en nog een aantal extra overwegingen, zoals die weergegeven zijn in hoofdstuk 7.

Dosisafhankelijke effecten in biokinetiek

In hoofdstuk 3 is een PBBK model voor estragol in de rat ontwikkeld, om meer inzicht te krijgen in dosisafhankelijke effecten in metabolisme en metabole activering van estragol. Met dit model is aangetoond dat bij toename van de estragoldosis een verschuiving in metabolisme plaatsvindt van *O*-demethylering naar 1'-hydroxylering. *O*-Demethylering is de belangrijkste metabole route bij lage doses van estragol en wordt vooral gekatalyseerd in de long en nier van de rat. In deze organen wordt 4-allylphenol gevormd met een hoge affiniteit, welke resulteert in een relatief lage K_m voor deze reactie in deze organen. Door verzadiging van deze *O*-demethyleringsroute in de long en nier bij hogere doses van estragol, wordt de vorming van 1'-hydroxyestragol in de lever relatief belangrijker. De door het PBBK model voorspelde vorming van deze metaboliet neemt toe van 16% van de dosis bij een orale dosis van 0.05 mg/kg lichaamsgewicht (lg) tot 29% van de dosis bij een orale dosis van 300 mg/kg lg. Deze relatieve toename in vorming van 1'-hydroxyestragol bij hogere doses resulteert eveneens in een relatieve toename in vorming van 1'-hydroxyestragol glucuronide, 1'-oxoestragol en 1'-sulfooxyestragol. Deze laatste metaboliet is de uiteindelijke carcinogene metaboliet van estragol. De door het PBBK model voorspelde vorming van 1'-sulfooxyestragol neemt toe van 0.08% van de dosis bij een orale dosis van 0.05 mg/kg lg tot 0.16% van de dosis bij een dosis van 300 mg/kg lg. Over het algemeen laten deze resultaten zien dat de relatieve bijdrage van verschillende metabole routes van estragol op een dosisafhankelijke manier varieert, wat leidt to tot een relatieve toename in bioactivering van estragol bij hogere doses.

De met het PBBK model verkregen bevindingen voor estragol in de rat komen overeen met observaties in de literatuur, waar dosisafhankelijke

effecten in biokinetiek van estragol zijn gevonden in *in vivo* experimenten met vrouwelijke Wistar ratten die blootgesteld zijn aan [methoxy-^{14}C]-gelabeld estragol (Anthony *et al*., 1987). In deze *in vivo*-studie bleek dat het aandeel van *O*-demethylering relatief afnam bij hogere doses (vastgesteld als het percentage dat werd uitgescheiden als $^{14}CO_2$), terwijl het aandeel dat werd uitgescheiden als 1'-hydroxyestragolglucuronide in de urine relatief toenam van 1.3-5.4% van de dosis bij een orale dosis van 0.05-50 mg/kg lg tot 11.4-13.7% in de dose-range van 500-1000 mg /kg lg (Anthony *et al*., 1987). Met het PBBK model voor estragol in de rat, dat opgesteld is in dit proefschrift, is inzicht verkregen in het onderliggende mechanisme van deze dosisafhankelijke effecten. Deze effecten blijken een resultaat te zijn van verzadiging van de *O*-demethyleringsroute in de long en nier van de rat.

Met het PBBK model voor estragol in de mens, dat gedefinieerd is in hoofdstuk 4, zijn ook de dosisafhankelijke effecten in bioactivering en detoxificering in de mens bestudeerd. In de mens blijkt geen relatieve toename in vorming van 1'-sulfooxyestragol voor te komen met toenemende doses. Het PBBK model voorspelt zelfs dat de relatieve vorming van deze metaboliet afneemt van 0.19% van de dosis bij een dosis van 0.07 mg/kg lg tot 0.08% van de dosis bij een dosis van 300 mg/kg lg. Dit wordt veroorzaakt door verzadiging van de 1'-hydroxyleringsroute in de lever. Verdere analyses laten zien dat de verschillen tussen de rat en mens, met respectievelijk een toename versus afname in relatieve vorming van 1'-sulfooxyestragol met hogere doses, wordt veroorzaakt doordat in het humane model geen efficiënte *O*-demethylering in long en nier aanwezig is, terwijl in de rat bij lage doses deze omzettingen de relatieve vorming van 1'-sulfooxyestragol kunnen reduceren. Het humane PBBK model laat ook zien dat bij doses binnen een orde van grootte van de geschatte gemiddelde humane inname van 0.07 mg/kg lg, de dosisafhankelijke verschillen in de relatieve vorming van 1'-sulfooxyestragol niet significant zijn.

Speciesafhankelijke verschillen in biokinetiek.

Naast dosisafhankelijke effecten, zouden speciesverschillen in metabolisme

en metabole activering bij extrapolatie van carcinogeniteitsgegevens, verkregen in proefdierstudies, ook meegenomen moeten worden naar de humane situatie. In hoofdstuk 2 zijn de kinetische constanten voor sulfatering van 1'-hydroxyestragol door lever-S9-homogenaten van de rat, muis en mens beschreven om mogelijke speciesverschillen in deze bioactiveringsroute te kunnen identificeren. 1'-Sulfooxyestragol is instabiel in water en kan daarom niet direct worden gemeten. Om de vorming van 1'-sulfooxyestragol toch te kunnen meten, werd 2'-deoxyguanosine toegevoegd aan de incubaties waarin sulfatering van 1'-hydroxyestragol werd gekatalyseerd om het instabiele 1'-sulfo-oxyestragol weg te vangen. Daarnaast is een methode ontwikkeld waarmee de vorming van het meest voorkomende adduct met 2'-deoxyguanosine gekwantificeerd kon worden met behulp van isotopenverdunning LC-ESI-MS/MS. Wanneer de kinetiek voor sulfatering van 1'-hydroxyestragol door de verschillende lever-S9-fracties met elkaar vergeleken wordt, blijkt dat de vorming van de reactive metaboliet ongeveer 30 keer efficiënter gekatalyseerd wordt door ratlever-S9 dan door humaan lever-S9, terwijl de katalytische efficiënties voor muis en humaan lever-S9 ongeveer gelijk zijn.

Met de *in vitro*-kinetische gegevens voor sulfatering van 1'-hydroxyestragol, die zijn verzameld in hoofdstuk 2 en voor andere metabole reacties, die zijn verzameld in de hoofdstukken 3 en 4, is inzicht verkregen in welke speciesverschillen te verwachten zijn tussen de rat en de mens. Deze gegevens laten echter niet het geïntegreerde effect zien van deze verschillen op de totale vorming van 1'-hydroxyestragol en 1'-sulfooxyestragol in deze species. Door deze *in vitro* gegevens te integreren met behulp van PBBK modellen, kon een vergelijking gemaakt worden van de algemene verschillen in bioactivering en detoxificering tussen mensen en ratten, bij doses van estragol die representatief zijn voor humane inname. De resultaten van deze vergelijking zijn beschreven in hoofdstuk 4 en tonen aan dat de voorspelde vorming van 1'-hydroxyestragol bij de mens vier maal hoger ligt dan bij de rat. Dit wordt veroorzaakt door een lagere mate van detoxificering van estragol via *O*-demethylering bij mensen ten opzichte van ratten. Dit is een gevolg van de afwezigheid van deze metabole route in menselijke long en nier, terwijl *O*-demethylering met

hoge affiniteit gekatalyseerd wordt in long en nier van de rat. Hoewel de voorspelde vorming van 1'-hydroxyestragol vier maal hoger is voor de mens, is de voorspelde vorming van de uiteindelijke kankerverwekkende metaboliet 1'-sulfooxyestragol slechts twee maal hoger voor de mens, wat veroorzaakt wordt door een hoge mate van detoxificering van 1'-hydroxyestragol in de menselijke lever via oxidatie naar 1'-oxoestragol.

Vorming van deze 1'-oxoestragolmetaboliet werd niet eerder beschouwd als een belangrijke metabole route van 1'-hydroxyestragol, voornamelijk omdat bij ratten, die blootgesteld zijn aan estragol, slechts een relatief kleine hoeveelheid van deze metaboliet of van zijn conjugaten werd gevonden in de urine (Solheim and Scheline, 1973). Met de benadering in dit proefschrift, waarbij de belangrijkste metabole routes van estragol geïdentificeerd zijn in incubaties met microsomen en/of S9-homogenaten, kon aangetoond worden dat in mensen oxidering van 1'-hydroxyestragol wel een belangrijke metabole route kan zijn. Het humane PBBK model voorspeld dat 62.7% van de dosis bij een dosis van 0.07 mg/kg lg uiteindelijk wordt omgezet in 1'-oxoestragol. Validatie van de mate van vorming van deze metaboliet aan de hand van menselijke *in vivo* data is nog niet mogelijk, aangezien de uitscheiding van deze metaboliet of zijn conjugaten in proeven waarin mensen blootgesteld zijn aan [methoxy-^{14}C]-gelabeled estragol niet is bepaald (Sangster *et al*., 1987). In deze studie van Sangster *et al*. (1987), uitgevoerd met twee proefpersonen, kon het overgrote deel van de uitgescheiden metabolieten (~70% van de dosis) niet worden geïdentificeerd. Deze ~70% zou in potentie overeen kunnen komen met de gevormde 1'-oxoestragol. Al met al kan geconcludeerd worden dat speciesverschillen in de bioactivering van estragol kleiner zijn dan de gebruikelijk gehanteerde factor 4. Deze waarde van 4 is gebaseerd op de interindividuele variatie in kinetiek (waarbij wordt aangenomen dat de normfactor van 10 is opgebouwd uit een factor 4 voor kinetiek en een factor 2.5 voor dynamiek) (WHO, 1999).

Individuele variatie in biokinetiek in de mens

In de humane populatie kan variatie bestaan tussen mensen in bioactivering

van estragol tot de uiteindelijke carcinogene metaboliet 1'-sulfooxyestragol door genetische verschillen en verschillen die veroorzaakt worden door leefstijlfactoren. Dergelijke genetische factoren en leefstijlfactoren zouden de activiteit van enzymen die betrokken zijn bij bioactivering en detoxificering van estragol kunnen beïnvloeden. Aan de hand van het in hoofdstuk 4 geconstrueerde model voor estragol in de mens en een gevoeligheidsanalyse van dit model, is aangetoond dat de hoeveelheid 1'-hydroxyestragol in de lever die beschikbaar is voor de vorming van de uiteindelijke carginogene metaboliet 1'-sulfooxyestragol, vooral beïnvloed wordt door de mate van vorming van 1'-hydroxyestragol en de daarop volgende oxidatie van deze metaboliet tot 1'-oxoestragol. Het is dus te verwachten dat inter-individuele verschillen in deze metabole reacties verschillen in bioactivering van estragol tussen mensen kunnen veroorzaken. Om meer inzicht te krijgen in de mate van variatie in 1'-hydroxylering van estragol en in oxidering van 1'-hydroxyestragol tussen mensen, is het belangrijk dat de enzymen die betrokken zijn bij deze reacties geïdentificeerd worden.

In hoofdstuk 5 zijn de enzymen die betrokken zijn bij 1'-hydroxylering van estragol geïdentificeerd. Experimenten met recombinante enzymen (Gentest microsomen) toonden aan dat 1'-hydroxylering het meest efficiënt gekatalyseerd wordt door P450 1A2 en 2A6. In mindere mate waren ook P450 2D6, 2C19, en 2E1 in staat om 1'-hydroxylering van estragol te katalyseren (Jeurissen *et al*., 2007). Op basis van deze resultaten is geconcludeerd dat P450 1A2 en 2A6 een hoofdrol spelen in de 1'-hydroxylering van estragol. De activiteit van P450 1A2 kan beïnvloed worden door roken en consumptie van vlees bereid op de barbecue (Jiang *et al*., 2006). De activiteit van P450 1A2 kan een factor 60 verschillen binnen de humane populatie (Wenker *et al*., 2001; Jiang *et al*., 2006). Variatie in P450 2A6-activiteit tussen individuen hangt vooral af van genetische verschillen (Ingelman-Sundberg *et al*., 1999). Dergelijke genetische verschillen kunnen leiden tot vermindering of het gehele afwezigheid zijn van de P450 2A6-enzymactiviteit.

Identificatie van het enzym dat betrokken is bij de oxidatie van 1'-hydroxyestragol is beschreven in hoofdstuk 6. Het blijkt dat 17β-

hydroxysteroiddehydrogenase (17β-HSD)-type-2 betrokken is bij deze reactie. Dit is een enzym dat aanwezig is in de microsomale fractie van de lever, en bij voorkeur NAD^+ gebruikt als cofactor (Peltoketo *et al*., 1999). De huidige kennis van 17β-HSD type 2 geeft onvoldoende inzicht in de mate van variatie in de activiteit van dit enzym die op populatieniveau aanwezig zou kunnen zijn. 17β-HSD type 2 is in verschillende organen een veel voorkomend enzym (Peltoketo *et al*., 1999). Recent is een variant geïdentificeerd van het gen dat voor type-2 17β-HSD codeert, welke mogelijk leidt tot een variant van dit enzym met verminderde activiteit (Plourde *et al*., 2008).

In hoofdstuk 6 is het effect van inter-individuele variatie in de vorming van 1'-hydroxyestragol en de detoxificering 1'-hydroxyestragol via oxidering op de concentraties van 1'-hydroxyestragol in de lever onderzocht. In dat hoofdstuk zijn eerst PBBK modellen opgesteld voor veertien individuele mensen met behulp van *in vitro* kinetiekgegevens voor deze twee reacties verkregen voor deze veertien individuen. Binnen deze groep individuen is aan de hand van de verkregen PBBK modellen een inter-individuele variatie van een factor 1.7 in de oppervlakte onder de concentratie-tijdscurve (area under the concentration-time curve (AUC)) voor 1'-hydroxyestragol in de lever voorspeld. In een volgende stap zijn de individuele modellen gecombineerd met literatuurgegevens over inter-individuele variatie in activiteitsniveaus van de betrokken P450 enzymen en 17β-HSD. Hoewel de activiteit van de bij 1'-hydroxylering van estragol betrokken P450-enzymen vele malen kan variëren in de populatie, is het effect van deze variatie op de AUC van 1'-hydroxyestragol kleiner dan een factor 10. Variatie in de oxidering van 1'-hydroxyestragol door 17β-HSD heeft een groter effect. Een reductie in oxidering met een factor 10 veroorzaakt een toename in de AUC van 1'-hydroxyestragol met een factor 8, terwijl een homozygoot deletie-genotype in theorie een variatie in de populatie kan veroorzaken van een factor 30 tot 60. Definitieve conclusies over de effecten van variatie in 17β-HSD geno- en fenotypes op inter-individuele verschillen in de bioactivering en detoxificering van estragol kunnen pas getrokken worden als er meer gegevens over de daadwerkelijke variatie in de 17β-HSD-activiteit in de populatie beschikbaar zijn. Er kan geconcludeerd

worden dat de AUC-waarden voor 1'-hydroxyestragol in de lever in een orde van grootte van 1 tot maximaal 2 kan variëren in de mens. Deze inter-individuele variatie in bioactivering van estragol kan dus groter zijn dan de factor 3.16, waarvan werd aangenomen dat deze de inter-individuele variatie in biokinetiek van de mens verdisconteert (aannemende dat de standaardwaarde van 10 opgedeeld kan worden in een factor 3.16 voor kinetiek, en een factor 3.16 voor dynamiek) (WHO, 1999).

Samenvattend

Het werk in dit proefschrift toont aan dat het integreren van *in vitro* metabole parameters met behulp van PBBK modellen een goede methode is voor het evalueren van het al dan niet voorkomen van dosisafhankelijke effecten, speciesverschillen en inter-individuele verschillen tussen mensen in de bioactivering van estragol. Aan de hand van deze methode is inzicht verkregen in het mechanisme dat ten grondslag ligt aan dosisafhankelijke effecten in bioactivering en detoxificering van estragol in de rat. Daarnaast geeft de methode inzicht in het voorkomen van speciesverschillen en inter-individuele variatie in metabolisme en metabole activering van estragol. De resultaten uit deze studie dragen bij aan een meer verfijnde extrapolatie van carcinogeniteitsgegevens, verkregen in dierexperimenten bij hoge doses, naar de humane situatie bij lage doses.

Implicaties voor de risicobeoordeling

Wereldwijd bestaan er veel verschillende benaderingen bij het beoordelen van risico's van genotoxische carginogenen. Getalsmatige inschattingen van het risico op kanker voor de mens zijn mogelijk af te leiden door extrapolatie van carginogeniteitsgegevens, verkregen in dierstudies bij hoge doses naar doses die relevant zijn voor de humane situatie. Er zijn een groot aantal wiskundige modellen beschikbaar waarmee dergelijke extrapolaties van dierexperimentele gegevens van hoge doses naar lage doses uitgevoerd zouden kunnen worden (COC, 2004). Hiervan is lineaire extrapolatie het

eenvoudigst. Het verkrijgen van een schatting van het risico op kanker voor de mens, waarbij wiskundige modellen gebruikt worden om de tumorincidentie bij hoge doses in proefdieren te extrapoleren naar lage doses bij de mens, wordt veel bediscussieerd aangezien het onbekend is of de gebruikte modellen daadwerkelijk de betrokken biologische processen correct weerspiegelen. Daarnaast wordt betoogd dat bij dergelijke extrapolaties speciesverschillen niet worden meegenomen. Ten slotte speelt ook nog mee dat de verkregen numerieke schattingen verkeerd gebruikt, of verkeerd geïnterpreteerd kunnen worden in toekomstige risicobeoordelingen en in communicatie rond het risico, wanneer onzekerheden en foutenmarges die inherent zijn aan dergelijke modellen niet (kunnen) worden overgebracht.

Vanwege deze nadelen adviseert het Scientific Committee of the European Food Safety Authority (EFSA) een andere benadering te volgen: de margin of exposure (MOE)-benadering (EFSA 2005). De MOE-benadering maakt gebruik van een referentiewaarde. Die waarde wordt normaliter afgeleid van gegevens die verkregen zijn uit dierexperimenten en staat voor de dosis waarbij een klein maar meetbaar aantal dieren kanker ontwikkelt. Deze waarde kan bijvoorbeeld de $BMDL_{10}$ zijn, de lage betrouwbaarheidsintervalwaarde van de Benchmark-dosis (BMD) die een extra toename in ontwikkeling van kanker (Benchmark respons, BMR) van 10% geeft (BMD_{10}). Hierbij wordt de MOE dan gedefinieerd als de ratio tussen de $BMDL_{10}$ en de geschatte inname via het dieet (estimated daily intake, EDI) in de mens. Wanneer deze ratio hoger is dan 10.000, wordt de carcinogeniteit van de stof beschouwd als zijnde van lage prioriteit voor risicomanagement (EFSA, 2005; Barlow *et al*., 2006; Dybing *et al*., 2008). Deze MOE van 10.000 wordt toegepast om rekening te houden met diverse onzekerheden in de MOE-benadering, en is opgebouwd uit de volgende factoren (EFSA, 2005):

i) een factor 100 voor speciesverschillen en inter-individuele verschillen tussen mensen in biokinetiek en biodynamiek. Deze factor is opgebouwd uit een factor 10 voor speciesverschillen en een factor 10 voor inter-individuele verschillen. De factor voor speciesverschillen is op haar beurt weer opgebouwd uit een factor 4 voor kinetiek en

een factor 2.5 voor dynamiek, en de factor voor inter-individuele verschillen is opgebouwd uit een factor 3.16 voor kinetiek en een factor 3.16 voor dynamiek (WHO, 1999),

ii) een factor 10 voor inter-individuele verschillen tussen mensen in celcycluscontrole en reparatie van beschadigd DNA,

iii) een factor 10 voor onzekerheden over de vorm van de dosis-respons-curve buiten het gebied van de in de dierproeven toegepaste dosis.

Tot op heden kunnen carcinogeniteitsdata voor estragol, waarvan een $BMDL_{10}$ en dus en MOE kunnen worden afgeleid, alleen verkregen worden uit langetermijnproeven met muizen (Tabel 1; Miller *et al*., 1983). Met behulp van BMDS versie 1.4.1c en/of PROAST-software is een BMD-analyse van estragol uitgevoerd (tabel 2). Hieruit valt te concluderen dat de $BMDL_{10}$-waarde varieert tussen 9 en 33 mg/kg lg/dag.

Tabel 1. Overzicht van een studie van Miller *et al*. (1983) waarin de mate van hepatomas die gevormd worden in vrouwelijke muizen, die gedurende 12 maanden via het dieet zijn blootgesteld aan estragol, zijn beschreven.

dosis	Geschatte dosis mg/kg lg/dag	Aantal dieren	Aantal muizen met hepatomas	Incidentie (%)
0	0	43	0	0
0.23% in dieet	150-300	48	27	56
0.46% in dieet	300-600	49	35	71

Tabel 2. Resultaten van een Benchmark Dose (BMD) analyse van de data van Miller *et al*. (1983), waarin de mate van hepatomas die gevormd worden in vrouwelijke muizen, die gedurende 12 maanden via het dieet zijn blootgesteld aan estragol, zijn beschreven (Tabel 1). BMD analysis is uitgevoerd met BMDS versie 1.4.1c. Om een 'worst case' schatting te maken zijn de laagste doses in de dose-response gebruikt voor analyse (i.e. 150 en 300 mg/kg lg/dag).

Model	Aantal para-meters	*log* likelihood	Geaccep-teerd	BMD_{10} mg/kg lg/dag	$BMDL_{10}$ mg/kg lg/dag
null	1	-96.1243			
full	3	-62.2103			
two-stage	1	-62.7403	ja	22.4	18.1
gamma	1	-62.7403	ja	22.4	18.1
log-logistic	1	-62.2124	ja	13.1	9.2
log-probit	1	-62.7928	ja	40.7	32.7
Weibull	1	-62.7403	ja	22.4	18.1

De gemiddelde dagelijkse inname van estragol per hoofd van de bevolking is door de SCF geschat op ongeveer 4.3 mg per dag (oftewel 0.07 mg/kg lg/dag, uitgaande van een gemiddeld lichaamsgewicht van 60 kg) (SCF, 2001). Deze schatting is gebaseerd op een relatief conservatieve methodiek, die gebruik maakt van de theoretisch maximale gebruiksniveaus van estragol in 28 voedselcategorieën en consumptiegegevens voor deze voedselcategorieën. Deze consumptiegegevens zijn gebaseerd op zevendaagse dieetbepalingen van volwassenen (SCF, 2001). Een lagere gemiddelde dagelijkse inname van estragol per hoofd van de bevolking is geschat door het expertpanel van de Flavor and Extract Manufacturers Association (FEMA), die een andere methode gebruikte (Smith *et al*., 2002). De FEMA heeft de dagelijkse inname per hoofd van de bevolking op minder dan 10 µg/kg lg/dag geschat (Smith *et al*., 2002). Het merendeel (63%) van deze inname komt voort uit kruiden en specerijen die van nature estragol bevatten, 27% komt voort uit essentiële oliën die estragol bevatten, en 10% komt voort uit estragol die als pure stof wordt toegevoegd in voedsel (Smith *et al*., 2002). Deze schatting van inname weerspiegelt mogelijk alleen de inname van estragol door consumptie van kruiden en hun essentiële oliën.

Bij blootstelling aan 0.07 mg/kg lg/dag, welke overeenkomt met de door de SCF geschatte dagelijkse inname van estragol, en de $BMDL_{10}$ van 9 tot 33 mg/kg lg/dag, zal de MOE-waarde 127 tot 569 zijn, wat beduidend lager is dan 10.000. Dit geeft aan dat de consumptie van estragol bij deze inname een prioriteit zou kunnen zijn voor risicomangement. Op basis van de inschatting van de blootstelling van Smith *et al*., (2001) van 0.01 mg/kg lg/dagen de $BMDL_{10}$ van 9 tot 33 mg/kg lg/dag, zou de MOE-waarde 900 tot 4100 zijn. Vergelijking van deze waarde met de grenswaarde van 10.000 geeft aan dat ook deze inname als gevolg van het gebruik van kruiden die estragol bevatten, en van de daaruit gewonnen essentiële oliën als prioriteit voor risicomanagement moet worden beschouwd.

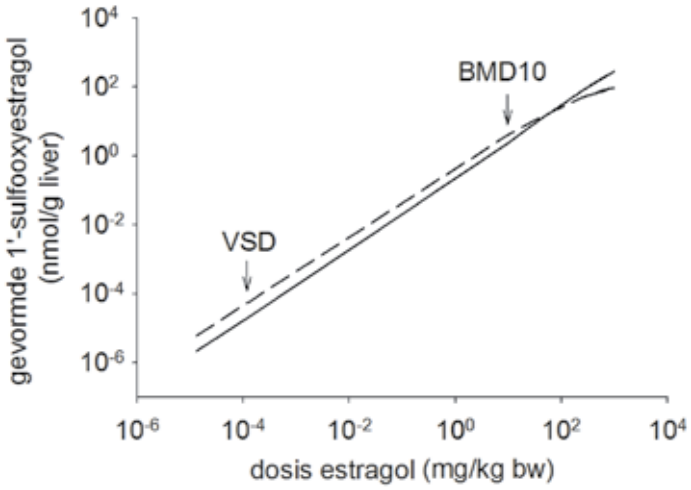
De EFSA stelt dat de waarde voor de MOE voor het maken van de inschattingen van 10.000 verkleind of vergroot kan worden wanneer daarvoor specifieke gegevens van een stof beschikbaar zijn (EFSA, 2005). De data

in dit proefschrift kunnen vooral inzicht verschaffen in de toepasbaarheid van de standaardwaarden voor speciesverschillen en inter-individuele verschillen in biokinetiek die werden gebruikt om de waarde van 10.000 vast te stellen. De resultaten van het PBBK model in dit proefschrift laten zien dat speciesverschillen in de bioactivering van estragol kleiner zijn dan de standaardfactor van 4 die gebruikelijk wordt gehanteerd voor de speciesverschillen in biokinetiek. Daarentegen is de inter-individuele variatie in bioactivering van estragol bij de mens waarschijnlijk groter dan de standaardfactor van 3.16 die gebruikelijk wordt gehanteerd. Wanneer deze twee waarnemingen worden gecombineerd, is het duidelijk dat deze specifieke data voor estragol een reductie van de standaardmarge van 10.000 en daarmee een andere conclusie voor de prioriteitstelling voor risk management niet ondersteunen.

Een vergelijkbare conclusie komt voort uit een tweede benadering die gebruikt wordt voor de risicobeoordeling van genotoxische carcinogenen. Hierbij wordt een zogenaamde ‘virtual safe dose' (VSD) bepaald: een dosis waarbij bijvoorbeeld een toename van het risico op kanker van 1:1.000.000 bij levenslange blootstelling optreedt (EPA 2005). Het vaststellen van die VSD gebeurt door middel van lineaire extrapolatie vanuit een vastgelegd uitgangspunt op de dosisresponse-curve. Op basis van de gegevens die verkregen zijn in de studie van Miller *et al*. (1983) en een BMD-analyse van deze gegevens (tabel 1 en 2) kan geconcludeerd worden dat bij muizen een BMR van 10% extra risico op tumoren wordt waargenomen bij een BMD_{10} van 13 tot 41 mg/kg lg/dag. Door middel van lineaire extrapolatie vanaf dit uitgangspunt wordt de VSD, die resulteert in verhoogde kans op kanker van 1:1.000.000, geschat op 0.13 tot 0.41 µg/kg lg/dag. Wanneer deze geschatte VSD vergeleken wordt met de geschatte menselijke inname van 10-70 µg/kg lg/dag (SCF, 2001; Smith *et al*., 2002), wijst dit erop dat de humane inname bij benadering 2 ordesvan grootte hoger is dan de VSD, wat erop wijst dat de inname van estragol als een prioriteit voor risicomanagement is te beschouwen.

De PBBK modellen voor rat en mens die in dit proefschrift zijn opgesteld,

tonen aan dat kinetische gegevens geen argumenten leveren tegen een dergelijke lineaire extrapolatie. Dit wordt gevisualiseerd in figuur 2, waarin de dosisafhankelijke vorming van 1'-sulfooxyestragol in de lever van rat en mens, zoals voorspeld door de PBBK modellen, worden afgebeeld,. Beide curven zijn lineair tussen de BMD_{10} en de VSD, zowel op *log-log* schaal, (figuur 2), als wanneer ze geplot zijn op een lineaire schaal (niet afgebeeld). Afwijkingen van lineariteit in de vorming van 1'-sulfooxyestragol bij de rat en mens worden pas waargenomen bij doses hoger dan 70 mg/kg lg/dag, wat hoger is dan de BMD_{10}. Aangezien de BMD_{10} binnen het lineaire deel van de curve valt, en aangezien de curven voor rat en mens niet substantieel verschillen, steunen deze resultaten niet dat mogelijke non-lineaire kinetiek en speciesverschillen in kinetiek als argument gebruikt kunnen worden tegen het gebruik van lineaire extrapolatie van dierexperimentele gegevens, verkregen bij hoge doses, naar effecten van lage doses bij mensen. De waargenomen lineaire vorming van 1'-sulfooxyestragol bij een toenemende dosis in de lever van de rat toont aan dat het redelijk is om risico op kanker lineair te extrapoleren van hoge naar lage doses. Daarbij is de VSD die zo bepaald wordt voor estragol met behulp van de studies in knaagdieren relevant voor



Figuur 2. PBBK model-voorspelde dosisafhankelijke vorming van 1'-sulfooxyestragol in de lever van de rat (—) en mens (- -).

de humane situatie, aangezien geen significante speciesverschillen werden waargenomen bij lage doses.

Aanvullende overwegingen

Hoewel in de risicobeoordeling die hierboven beschreven is, de voorspelde data van dosisafhankelijke effecten, speciesverschillen en inter-individuele verschillen in de bioactivering van estragol meegenomen zijn, bestaan er ook nog andere factoren die meegenomen zouden moeten worden in de risicobeoordeling. De carcinogene effecten van estragol zullen bijvoorbeeld ook afhangen van toxicodynamische processen, zoals de vorming van DNA-adducten of de inductie van mutaties en uiteindelijke vorming en ontwikkeling van tumoren. Niet-lineaire dosisafhankelijke toename in de vorming van DNA-adducten kan optreden wanneer bij hoge doses DNA-reparatieprocessen verzadigd raken. Dit zal resulteren in een relatieve toename van DNA-adductvorming bij hogere doses. Verzadiging van DNA-reparatie bij hogere doses kan een reden zijn om de dosisafhankelijke toename in het voorkomen van kanker te beschrijven met non-lineaire modellen, in plaats van met lineaire modellen. Dit zou meer in detail onderzocht kunnen worden door de PBBK modellen uit dit proefschrift verder uit te breiden tot zogenaamde physiologically based biodynamic (PBBD) modellen, waarin de hoogte van de dosis en de vorming van 1'-sulfooxyestragol worden gekoppeld aan de vorming van DNA-adducten, en uiteindelijk aan de incidentie van kanker. Op dit moment loopt een studie waarbij de PBBK modellen, die zijn gedefinieerd in dit proefschrift, worden uitgebreid tot PBBD-modellen die het mogelijk maken de 1'-hydroxyestragol-afhankelijke vorming van DNA-adducten in de lever *in vivo* te kwantificeren. Dit gebeurt op basis van verkregen *in vitro*-gegevens betreffende de vorming van DNA-adducten in primaire hepatocyten die blootgesteld zijn aan 1'-hydroxyestragol, gekwantificeerd aan de hand van de LC-MS methode zoals ontwikkeld in dit proefschrift. Met deze PBBD-modellen kan de vorming van DNA-adducten voorspeld worden

bij verschillende orale doses, inclusief de innameniveaus die relevant zijn voor de humane inname. Deze benadering maakt het mogelijk effecten van DNA-reparatie te betrekken in de extrapolatie van hoge naar lage doses van estragol voor rat en mens. Aangezien vorming van DNA-adducten momenteel beschouwd wordt als een indicator van blootsteling en niet als bioindicator voor de kans op het voorkomen van tumoren (Phillips 2005; Sander *et al*., 2005), zou een dergelijke PBBD-model nog verder uitgebreid moeten worden om het mogelijk te maken om voorspelde vorming van DNA-adducten te koppelen aan het voorkomen van tumoren.

Bovendien is het belangrijk op te merken dat de bepaling van de mate van carcinogeniteit met behulp van dierexperimenten wordt uitgevoerd met een pure stof, terwijl de menselijke blootstelling aan estragol verloopt in een matrix van andere voedingsbestanddelen. In een matrix van andere voedingsbestanddelen kunnen interacties voorkomen die de biobeschikbaarheid van estragol kunnen beïnvloeden (Schilter *et al*., 2003; Rietjens *et al*., 2008). Zo kan bijvoorbeeld langzaam of incompleet vrijkomen van estragol uit de voedingsmatrix een verminderde biobeschikbaarheid tot gevolg hebben in vergelijking met de biobeschikbaarheid van dezelfde orale blootstelling aan de pure stof. Naast het effect van de voedingsmatrix op de biobeschikbaarheid kunnen interacties van andere componenten, aanwezig in bijvoorbeeld de kruiden, op het niveau van metabole activiteit en/of detoxificering van estragol voorkomen (Schilter *et al*., 2003; Rietjens *et al*., 2008). Zo toonden Jeurissen *et al*. (2008) bijvoorbeeld aan dat basilicumextract effectief de door sulfotransferase gemedieerde vorming van DNA-adducten kan remmen in aan 1'-hydroxyestragol blootgestelde menselijke HepG2 hepatomacellen. Deze resultaten suggereren dat de bioactivering van estragol, en de daaropvolgende negatieve effecten van estragol wanneer estragol ingenomen wordt in een matrix van andere basilicumingrediënten, waarschijnlijk lager zullen zijn dan men zou verwachten op basis van experimenten waarbij estragol als pure en enige component wordt toegediend. Of deze inhibitie ook werkelijk *in vivo* plaatsvindt, is echter nog niet vastgesteld, en zou verder onderzocht moeten worden.

Tot conclusie toont dit proefschrift aan dat het gebruik van PBBK modellen een goede methode vormt om de consequenties van dosisafhankelijke effecten, speciesverschillen en menselijke variatie in bioactivering van estragol te evalueren. De verkregen voorspellingen uit de modellen kunnen gebruikt worden voor een meer mechanistische basis voor de evaluatie van risico op kanker bij mensen bij een lage inname via voedsel, op basis van data over de mate van carcinogeniteit uit experimenten met knaagdieren, waarbij met hoge doses wordt gewerkt. Voor een complete beoordeling van het risico op kanker voor de mens bij lage doses is echter meer informatie nodig. Ten eerste zijn er mogelijk aanvullende studies nodig naar de mate van carcinogeniteit van estragol om zo een betere definitie van de BMD_{10} en $BMDL_{10}$ mogelijk te maken, waarop een kwantitatieve risicobeoordeling, een VSD-berekening of een MOE-berekening gebaseerd kan worden. Ten tweede zal er meer informatie nodig zijn over de aanwezigheid van polymorfismen in het 17β-hydroxysteroiddehydrogenase-enzym in de humane populatie en de effecten van dergelijke polymorfismen op de mate van detoxificering van 1'-hydroxyestragol door oxidatie van deze metaboliet. Ten derde is meer inzicht nodig in de toxicodynamische processen die het risico op kanker van estragol kunnen beïnvloeden. Dit zijn onder andere de relaties tussen de vorming van 1'-sulfooxyestragol, de mate van vorming van DNA-adducten en het voorkomen van tumoren. Ten vierde is er meer inzicht nodig in modulerende effecten van andere stoffen, aanwezig in kruiden, op de mate van carcinogeniteit van estragol. De methoden die in dit proefschrift zijn ontwikkeld, vormen een goede basis voor verdere bestudering van deze processen, die de mate van carcinogeniteit en risicobeoordeling van estragol kunnen beïnvloeden.

Referenties

Anthony, A., Caldwell, J., Hutt, A. J., and Smith, R. L. (1987). Metabolism of estragole in rat and mouse and influence of dose size on excretion of the proximate carcinogen 1'-hydroxyestragole. Food Chem Toxicol 25, 799-806.

Barlow, S., Renwick, A. G., Kleiner, J., Bridges, J. W., Busk, L., Dybing, E., Edler, L., Eisenbrand, G., Fink-Gremmels, J., Knaap, A., Kroes, R., Liem, D., Muller, D. J., Page, S., Rolland, V., Schlatter, J., Tritscher, A., Tueting, W., and Wurtzen, G. (2006). Risk assessment of substances that are both genotoxic and carcinogenic report of an International Conference organized by EFSA and WHO with support of ILSI Europe. Food Chem Toxicol 44, 1636-1650.

Committee on Carcinogenicity of chemicals in food, consumer products and the environment (COC) (2004). Guidance on a strategy for the risk assessment of chemical carcinogens. http://www.advisorybodies.doh.gov.uk/coc/guideline04.pdf.

Drinkwater, N. R., Miller, E. C., Miller, J. A., and Pitot, H. C. (1976). Hepatocarcinogenicity of estragole (1-allyl-4-methoxybenzene) and 1'-hydroxyestragole in the mouse and mutagenicity of 1'-acetoxyestragole in bacteria. J Natl Cancer Inst 57, 1323-1331.

Dybing, E., Doe, J., Groten, J., Kleiner, J., O'Brien, J., Renwick, A. G., Schlatter, J., Steinberg, P., Tritscher, A., Walker, R., and Younes, M. (2002). Hazard characterisation of chemicals in food and diet. dose response, mechanisms and extrapolation issues. Food Chem Toxicol 40, 237-282.

Dybing, E., O'Brien, J., Renwick, A. G., and Sanner, T. (2008). Risk assessment of dietary exposures to compounds that are genotoxic and carcinogenic--an overview. Toxicol Lett 180, 110-117.

EFSA (2005). Opinion of the scientific committee on a request from EFSA related to a harmonised approach for risk assessment of substances which are both genotoxic and carcinogenic. http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/sc_op_ej282_gentox_en3.pdf?ssbinary=true

EPA (2005). Guidelines for Carcinogen Risk Assessment and Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens. Washington, DC:U.S. Environmental Protection Agency. http://cfpub.epa.gov/ncea/raf/recordisplay.cfm?deid=116283

Guenthner, T. M. and Luo, G. (2001). Investigation of the role of the 2',3'-epoxidation pathway in the bioactivation and genotoxicity of dietary

allylbenzene analogs. Toxicology 160, 47-58.

Ingelman-Sundberg M, Oscarson M, McLellan RA. (199). Polymorphic human cytochrome P450 enzymes: an opportunity for individualized drug treatment. Trends Pharmacol Sci 20 ,342–349.

JECFA (2008). Sixty-ninth meeting Rome, Italy, 17-26 June 2008. http://www.who.int/entity/ipcs/food/jecfa/summaries/summary69.pdf.

Jeurissen, S. M. F., Punt, A., Boersma, M. G., Bogaards, J. J., Fiamegos, Y. C., Schilter, B., van Bladeren, P. J., Cnubben, N. H. and Rietjens, I. M. C. M. (2007). Human cytochrome p450 enzyme specificity for the bioactivation of estragole and related alkenylbenzenes. Chem Res Toxicol 20, 798-806.

Jeurissen S.M.F, Punt A., Delatour T., Rietjens I.M.C.M. (2008). Basil extract inhibits the sulfotransferase mediated formation of DNA adducts of the procarcinogen 1'-hydroxyestragole by rat and human liver S9 homogenates and in HepG2 Human Hepatoma Cells. Food and Chemical Toxicology, 46: 2296-2302.

Jiang, Z., Dragin, N., Jorge-Nebert, L. F., Martin, M. V., Guengerich, F. P., Aklillu, E., Ingelman-Sundberg, M., Hammons, G. J., Lyn-Cook, B. D., Kadlubar, F. F., Saldana, S. N., Sorter, M., Vinks, A. A., Nassr, N., von Richter, O., Jin, L. and Nebert, D. W. (2006). Search for an association between the human CYP1A2 genotype and CYP1A2 metabolic phenotype. Pharmacogenet Genomics 16, 359-67.

Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A., and Miller, J. A. (1983). Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. Cancer Res 43, 1124-1134.

Peltoketo, H., Luu-The, V., Simard, J., and Adamski, J. (1999). 17beta-hydroxysteroid dehydrogenase (HSD)/17-ketosteroid reductase (KSR) family; nomenclature and main characteristics of the 17HSD/KSR enzymes. J Mol Endocrinol 23, 1-11.

Phillips, D. H., Miller, J. A., Miller, E. C. and Adams, B. (1981). Structures of the DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1'-hydroxyestragole. Cancer Res 41, 176-

186.

Phillips, D. H., Reddy, M. V. and Randerath, K. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. II. Newborn male B6C3F1 mice. Carcinogenesis 5, 1623-1628.

Phillips, D. H. (2005). DNA adducts as markers of exposure and risk. Mutat Res 577, 284-92.

Plourde, M., Manhes, C., Leblanc, G., Durocher, F., Dumont, M., Sinilnikova, O. and Simard, J. (2008). Mutation analysis and characterization of HSD17B2 sequence variants in breast cancer cases from French Canadian families with high risk of breast and ovarian cancer. J Mol Endocrinol 40, 161-172.

Randerath, K., Haglund, R. E., Phillips, D. H. and Reddy, M. V. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. Carcinogenesis 5, 1613-1622.

Rietjens, I. M. C. M., Slob, W., Galli, C. and Silano, V. (2008). Risk assessment of botanicals and botanical preparations intended for use in food and food supplements: emerging issues. Toxicol Lett 180, 131-136.

Sander, M., Cadet, J., Casciano, D. A., Galloway, S. M., Marnett, L. J., Novak, R. F., Pettit, S. D., Preston, R. J., Skare, J. A., Williams, G. M., Van Houten, B. and Gollapudi, B. B. (2005). Proceedings of a workshop on DNA adducts: biological significance and applications to risk assessment Washington, DC, April 13-14, 2004. Toxicol Appl Pharmacol 208, 1-20.

Sangster, S. A., Caldwell, J., Hutt, A. J., Anthony, A., and Smith, R. L. (1987). The metabolic disposition of [methoxy-^{14}C]-labelled trans-anethole, estragole and p-propylanisole in human volunteers. Xenobiotica 17, 1223-1232.

SCF (2001). Opinion of the scientific committee on food on estragole (1-allyl-4-methyxybenzene). http://ec.europa.eu/food/fs/sc/scf/out104_en.pdf.

Schilter, B., Andersson, C., Anton, R., Constable, A., Kleiner, J., O'Brien, J., Renwick, A.G., Korver, O., Smit, F., and Walker, R., (2003). Guidance for

the safety assessment of botanicals and botanical preparation for use in food and food supplements. Food Chem Toxicol 41, 1625-1649.

Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., Rogers, A. E., Caldwell, J., and Sipes, I. G. (2002). Safety assessment of allylalkoxybenzene derivatives used as flavouring substances - methyl eugenol and estragole. Food Chem Toxicol 40, 851-870.

Solheim, E. and Scheline, R. R. (1973). Metabolism of alkenebenzene derivatives in the rat. I. p-Methoxyallylbenzene (Estragole) and p-methoxypropenylbenzene (Anethole). Xenobiotica 3, 493-510.

Wenker, M. A., Kezic, S., Monster, A. C. and De Wolff, F. A. (2001). Metabolism of styrene in the human liver in vitro: interindividual variation and enantioselectivity. Xenobiotica 31, 61-72.

WHO (1999) International Programme on Chemical Safety (IPCS): Assessing human health risks of chemicals: Principles for the assessment of risk to human health from exposure to chemicals. Environmental Health Criteria 210, World Health Organisation, Geneva. http://www.inchem.org/documents/ehc/ehc/ehc210.htm.

Wiseman, R. W., Fennell, T. R., Miller, J. A., and Miller, E. C. (1985). Further characterization of the DNA adducts formed by electrophilic esters of the hepatocarcinogens 1'-hydroxysafrole and 1'-hydroxyestragole in vitro and in mouse liver *in vivo*, including new adducts at C-8 and N-7 of guanine residues. Cancer Res 45, 3096-3105.

Wiseman, R. W., Miller, E. C., Miller, J. A., and Liem, A. (1987). Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J x C3H/HeJ F_1 mice. Cancer Res 47, 2275-2283.

Abbreviations

Acknowledgements

Curriculum Vitae and Publications

Abbreviations

17β-HSD	17β-hydroxysteroid dehydrogenase
ALARA	As low as reasonable achievable
AP	4-allylphenol
BMD	Benchmark Dose
BMR	Benchmark Response
BSA	Bovine Serum Albumin
DAD	Diode Array Detection
dGuo	2'-deoxyguanosine
DMSO	Dimethylsulfoxide
E-1'-N^2-dGuo	N^2-(estragol-1'-yl)-2'-deoxyguanosine
E-3'-7-dGua	7-(*trans*-isoestragol-3'-yl)-2'-deoxyguanine
E-3'-8-dGuo	8-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine
E-3'-N^2-dGuo	N^2-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosin
EDI	Estimated Daily Intake
EE	estragole-2',3'-oxide
EFSA	European Food Safety Authority
ESI	Electrospray ionization
FEMA	Flavor and Extract Manufacturers Association
GS-1'-oxoestragole	3'-(glutathion-S-yl)-1'-oxo-2'-3'-dihydroestragole
GSH	Glutathione
GS-MS	Gas chromatography – mass spectrometry
HA	3'-hydroxyanethole
HE	1'-hydroxyestragole
HEG	1'-hydroxyestragole glucuronide

HPLC	High Performance Liquid Chromatography
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC-MS	Liquid chromatography – mass spectrometry
MOE	Margin of Exposure
NAD^+	Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance
OE	1′-oxoestragole
P450	Cytochrome P450 enzyme
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PBBK	physiologically based biokinetic
PCP	Pentachlorophenol
RAF	Relative Activity Factor
rRNA	ribosomal RNA
SCF	Scientific Committee on Food of the European Union
SD	Standard Deviation
SEM	Standard Error of the Mean
SULT	Sulfotransferase
THF	Tetrahydrofuran
UDPGA	Uridine 5'-diphosphoglucuronic acid
UV	Ultra-violet
VSD	Virtual Safe Dose

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Curriculum vitae

Ans Punt was born in Hoogeveen on 9 December 1978. After graduating from the OSG De Groene Driehoek in Hoogeveen in 1997, she moved to Wageningen to study Nutrition and Health at Wageningen University.

In the academic year 2001-2002 she joined the board of the student club and pop venue Unitas in Wageningen as treasurer.

She obtained a Master Degree in 2004, after doing a MSc thesis and an internship. The subject of her MSc thesis, at the department of Toxicology of Wageningen University, was the quantitative structure activity relationship on the flavonoid mediated inhibition of multidrug resistance protein 1 (Van Zanden *et al*., 2005). At the department of Community and Environmental Toxicology of the University of California, USA, she worked as an intern on the direct effects of particulate air pollution on myocardial cells. After receiving her degree, she worked as a volunteer at the department of Toxicology of Wageningen University on gene expression patterns by natural Ah receptor agonists in citrus fruits and cabbage-like vegetables (De Waard *et al*., 2008).

From October 2004 to October 2008, she worked as a PhD and teaching assistant at the department of Toxicology of Wageningen University and the Chemical Food Safety Group of Nestlé Research Center, Switzerland on a project entitled 'New PBPK concepts for low-dose cancer risk extrapolation', which resulted in this thesis. During her PhD, she was awarded the discussion prize at the PhD days of the Netherlands Society of Toxicology in 2007, and the best presentation at the PhD days of the Netherlands Society of Toxicology in 2008.

Since November 2008 she works as a postdoc at the Institute for Risk Assessment Sciences of Utrecht University, on a project that aims to obtain an overview of the possibilities and bottlenecks of implementing new strategies in toxicological risk assessment of chemicals that are much less relying on animal studies.

As a consultant, she was lecturer of an introductory course on PBBK modeling at the department of Toxicology of Wageningen University

in 2009, and she was consultant in a project for J.S. Polak Koninklijke Specerijenmaalderij B.V. in 2007, developing a PBBK model for the herbal constituent coumarin.

List of publications

Rietjens I.M.C.M., Zaleska M., Boersma M.G., Punt A. (2008) Differences in simulated liver concentrations of toxic coumarin metabolites in rats and different human populations evaluated through physiologically based biokinetic (PBBK) modeling. Toxicology in vitro, 22: 1890-901.

Punt A., Freidig A.P., Delatour T., Scholz G., Schilter B., Van Bladeren P.J., Rietjens I.M.C.M. (2008) A physiologically based biokinetic (PBBK) model for estragole bioactivation and detoxification in rat. Toxicology and Applied Pharmacology, 231: 248-259.

Jeurissen S.M.F, Punt A., Delatour T., Rietjens I.M.C.M. (2008) Basil extract inhibits the sulfotransferase mediated formation of DNA adducts of the procarcinogen 1'-hydroxyestragole by rat and human liver S9 homogenates and in HepG2 Human Hepatoma Cells. Food and Chemical Toxicology, 46: 2296-2302.

de Waard W.J., Aarts J.M., Peijnenburg A.A., Baykus H., Talsma E., Punt A., de Kok T.M., Van Schooten F.J., Hoogenboom L.A. (2008) Gene expression profiling in Caco-2 human colon cells exposed to TCDD, benzo[a]pyrene, and natural Ah receptor agonists from cruciferous vegetables and citrus fruits. Toxicology In Vitro, 22: 396 - 410.

Punt A., Delatour T., Scholz G., Schilter B., Van Bladeren P.J., Rietjens I.M.C.M. (2007) Tandem mass spectrometry analysis of N^2-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine as a strategy to study species differences in sulfotransferase conversion of the proximate carcinogen 1'-hydroxyestragole. Chemical Research in Toxicology, 20: 991 - 998.

Jeurissen S.M.F., Punt A., Boersma M.G., Bogaards, J.J., Fiamegos Y.C.,

Schilter,B. Van Bladeren P.J., Cnubben N.H.P., Rietjens I.M.C.M. (2007) Human cytochrome P450 enzyme specificity for the bioactivation of estragole and related alkenylbenzenes. Chemical Research in Toxicology, 20: 798 -806.

Van Zanden J.J., Bijlsma S., Wortelboer H. M., Punt A., Usta M., Van Bladeren P.J., Rietjens I.M.C.M., Cnubben N.H.P. (2005) Quantitative structure activity relationship on the flavonoid mediated inhibition of multidrug resistance proteins 1 and 2. Biochemical Pharmacology, 69: 699 - 708.

Manuscripts submitted or in preparation

Punt A., Paini A., Freidig A.P., Delatour T., Scholz G., Schilter B., Van Bladeren P.J., Rietjens I.M.C.M. Use of physiologically based biokinetic (PBBK) modeling to study estragole bioactivation and detoxification in humans as compared to rats. Submitted.

Punt A., Jeurissen S.M.F., Delatour T., Scholz G., Schilter B., Rietjens I.M.C.M., Van Bladeren P.J. Evaluation of human interindividual variation in bioactivation of estragole using individual specific kinetic data and physiologically based biokinetic (PBBK) modeling. In preparation.

Rietjens I.M.C.M., Punt A., Schilter B., Scholz G., Delatour T., Van Bladeren P.J. In-silico methods for physiologically based biokinetics. Submitted.

Paini A., Punt A., Scholz G., Delatour T., Viton F., Guignard G., Schilter B., Van Bladeren P.J., Rietjens I.M.C.M. A physiologically based biodynamic (PBBK) model to assess 1'-hydroxyestragole metabolite-induced DNA adduct formation of estragole in rat liver. In preparation.

Alhusainy W., Louisse J., Punt A., Paini A., Vervoort J., Delatour T., Scholz G., Schilter B., Boersma M.G., Van Bladeren P.J., and Rietjens I.M.C.M. Matrix effect on the bioactivation of estragole to its proximate carcinogen. In preparation.

Reports

Rietjens, I.M.C.M., Boersma, M.G., Punt, A. (2007) Matrix effects of cinnamon on coumarin metabolism and PBBK modelling of oHPA formation in the

liver. Report for J.S. Polak koninklijke specerijenmaalderij B.V.

Rietjens, I.M.C.M., Zaleska, M., Boersma, M.G., Punt, A. (2007) Species differences in coumarin metabolism. Physiology based biokinetic (PBBK) study on the bioactivation of coumarin to oHPA in the liver of CYP2A6 deficient and CYP2A6 wild-type human subjects as compared to oHPA formation in rat liver. Report for J.S. Polak koninklijke specerijenmaalderij B.V.

Abstracts

Punt A., Freidig A.P., Delatour T., Scholz G., Schilter B., Van Bladeren P.J., Rietjens I.M.C.M. (2007) A physiologically based biokinetic (PBBK) model for estragole in rats providing more detailed insight in dose dependent bioactivation and detoxification. Toxicology Letters, 172S: S107 - S108.

Punt A., Freidig A.P., Delatour T., Scholz G., Schilter B., Van Bladeren P.J., Rietjens I.M.C.M. (2007) A physiologically based biokinetic (PBBK) model for estragole in rats providing more detailed insight in dose dependent bioactivation and detoxification. Chemico-Biological Interactions, 169: 139-139.

Punt, A., Freidig, A., Delatour, T., Scholz, G., Schilter, B., Van Bladeren, P.J., Rietjens, I.M.C.M. (2006). A PBTK model for estragole metabolism in rat and human to improve the carcinogenic risk assessment of doses relevant to dietary human exposures. Chemico-Biological Interactions 161: 172.

Overview of completed training activities

Courses

Radiation expert 5B (WU)	2005
PBPK beginners workshop (CSU)	2005
Training in LC-MS techniques (NRC)	2005
Method validation (NRC)	2005
Course on Laboratory Animal Science (WU)	2006
Organizing and supervising MSc thesis projects (WU)	2006
Techniques for writing and presenting a scientific paper (WU)	2006
Ecotoxicology (PET)	2007
Toxicological risk assessment (PET)	2008
Medical, forensic and regulatory toxicology (PET)	2008
Organ toxicology (PET)	2008
Immunotoxicology (PET)	2008

Meetings

PhD symposium (Netherlands Society of Toxicology)	2005
PhD symposium (Netherlands Society of Toxicology)	2006
International workshop on uncertainty and variability in PBBK models (U.S. EPA)	2006
PhD symposium (Netherlands Society of Toxicology)	2007
Eurotox 2007 (European Society of Toxicology)	2007
PhD symposium (Netherlands Society of Toxicology)	2008

WU:	Wageningen University
CSU:	Colorado State University
NRC:	Nestlé Research Center
PET:	Postdoctoral Education in Toxicology
US EPA:	U.S. Environmental Protection Agency

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