Chapter 2

Based on:

A physiologically based biodynamic (PBBD) model for estragole DNA binding in rat liver based on *in vitro* kinetic data and estragole DNA adduct formation in primary hepatocytes

By:

Paini A, Punt A, Viton F, Scholz G, Delatour T, Marin-Kuan M, Schilter B, van Bladeren PJ, Rietjens IMCM.

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Abstract

Estragole has been shown to be hepatocarcinogenic in rodent species at high dose levels. Translation of these results into the likelihood of formation of DNA adducts, mutation, and ultimately cancer upon more realistic low dose exposures remains a challenge. Recently we have developed physiologically based biokinetic (PBBK) models for rat and human predicting bioactivation of estragole. These PBBK models, however, predict only kinetic characteristics. The present study describes the extension of the rat PBBK model to a so-called physiologically based biodynamic (PBBD) model predicting *in vivo* DNA adduct formation of estragole in rat liver. This PBBD model was developed using *in vitro* data on DNA adduct formation in rat primary hepatocytes exposed to 1′-hydroxyestragole. To define the PBBD model, the PBBK model was extended by linking the area under the curve for 1′-hydroxyestragole formation predicted by the PBBK model to the area under the curve for 1′-hydroxyestragole in the *in vitro* experiments. The outcome of the PBBD model revealed a linear increase in DNA adduct formation with increasing estragole doses up to 100 mg/kg bw. Although DNA adduct formation of genotoxic carcinogens is generally seen as a biomarker of exposure rather than a biomarker of response, the PBBD model now developed is one step closer to the ultimate toxic effect of estragole than the PBBK model described previously. Comparison of the rat PBBD model outcome to available data on *in vivo* DNA adduct formation showed that the model predicts the dose dependent level of DNA adduct formation for low levels of exposure up to a dose level shown to cause cancer in rodent bioassays providing a proof of principle for modeling a toxicodynamic *in vivo* endpoint on the basis of solely *in vitro* experimental data.

2.1 Introduction

The regular human diet is known to contain several compounds that have been shown to be genotoxic and carcinogenic when tested at high doses in rodents. Estragole is such a food-borne natural compound, which is known to be a genotoxic hepatocarcinogen in rodent studies at high dose levels (Miller *et al*., 1983). Estragole itself is not reactive but upon its rapid absorption in the gastrointestinal track it is transported to the liver, where the compound is bioactivated (Smith *et al*., 2002). Metabolism of estragole can follow different pathways (Figure 2.1). It is detoxified via *O*-demethylation to 4-allylphenol and epoxidation to estragole 2,3-oxide (Phillips *et al*., 1981; Smith *et al*., 2002). The main pathway for bioactivation of estragole proceeds by hydroxylation on the allyl side chain by P 450 enzymes resulting in the formation of 3′-hydroxyanethole and 1′-hydroxyestragole. 1′-Hydroxyestragole can be detoxified by glucuronidation or via oxidation to 1′-oxoestragole (Bock *et al*., 1978; Iyer *et al*., 2003; Phillips *et al.*, 1981). Alternatively, sulfonation of 1′-hydroxyestragole by sulfotransferases gives rise to formation of the unstable ultimate carcinogenic metabolite 1′-sulfooxyestragole which decomposes to generate the reactive carbocation which covalently binds to DNA and proteins (Gardner *et al*., 1997). Several adducts are formed upon reaction of 1′-sulfooxyestragole with DNA: N^2-(*trans*-isoestragol-3′-yl)-2′-deoxyguanosine, N^2-(estragol-1′-yl)-2′-deoxyguanosine, 7-(trans-isoestragol-3′-yl)-2′-guanine, 8-(trans-isoestragol-3′-yl)-2′-deoxyguanosine (Phillips *et al*., 1981; Punt *et al*., 2007).

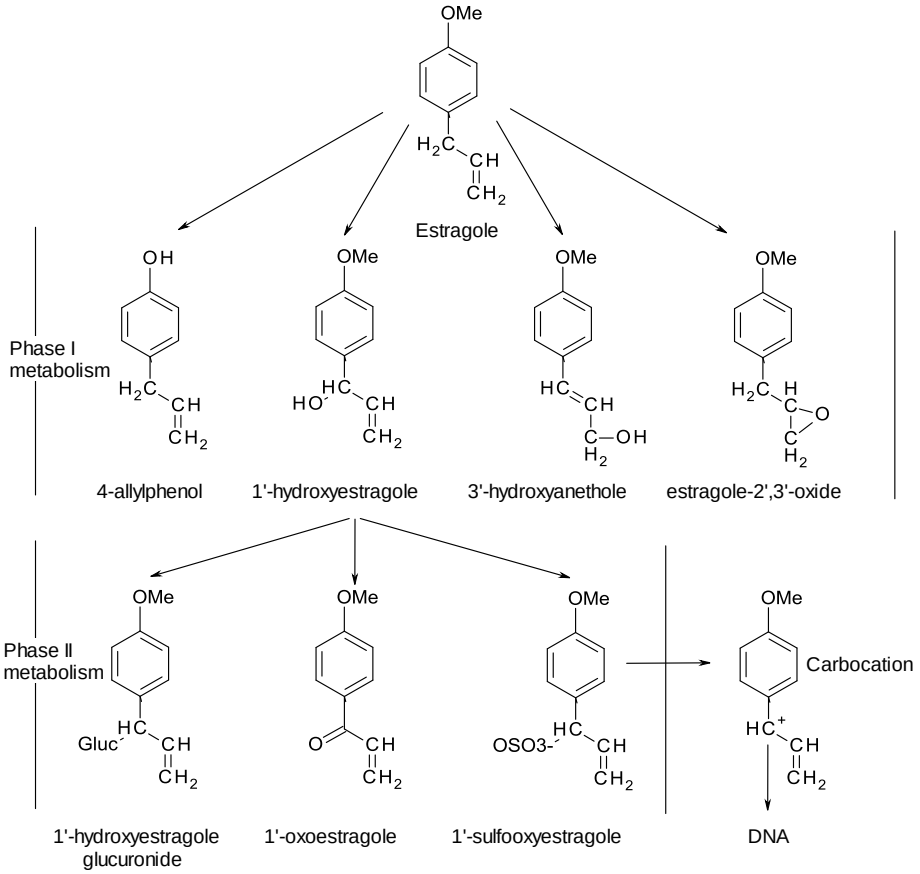

Figure 2.1. Metabolism of estragole (adapted from Punt et al., 2009).

The major adduct formed is N^2-(*trans*-isoestragol-3′-yl)-2′-deoxyguanosine (E-3′-N^2-dG) (Figure 2.1) which is considered to play a role in the genotoxic and carcinogenic effects induced by estragole (Philips *et al*., 1981; Smith *et al*, 2002). A physiologically based biokinetic (PBBK) model is represented as a set of mathematical equations that together describe the absorption, distribution, metabolism and excretion of a compound of interest within an organism (Yang *et al*., 2004). However, a toxic response *in vivo* will be determined by kinetic and dynamic characteristics. Therefore, integration of additional information on the subsequent reactions with target molecules or organs causing toxicity, i.e. toxicodynamic parameters, will give a more complete picture of the consequences of chemical exposure. It has been discussed that PBBK and physiologically based biodynamic (PBBD) models will give more insight from animal systems to reliably predict the likelihood and magnitude of adverse health effect in human (Coleman *et al*., 2008; Mager *et al.*, 2009). Recently, a PBBK model was developed to simulate estragole bioactivation and detoxification in both rat (Punt *et al*., 2008) and human (Punt *et al*., 2009) based on *in vitro* metabolic parameters only, providing possibilities to model metabolism of estragole at different oral doses in both species. These models provide more insight in relative dose- and species-dependent differences in bioactivation and detoxification of estragole, and are able to provide dose dependent predictions on the level of formation of the proximate and ultimate carcinogenic metabolites 1′-hydroxyestragole and 1′-sulfooxyestragole, respectively, in the target organ, the liver. As a result these PBBK models allow improved extrapolation of kinetic characteristics for both detoxification as well as bioactivation from high to low dose levels as well as from one species to another including human (Rietjens *et al*., 2009). However, formation of a DNA reactive metabolite cannot be directly extrapolated to the actual level of DNA binding since in addition to the level of 1′-sulfooxyestragole formed also other factors, including the rates of adduct formation and DNA repair will influence the ultimate DNA adduct formation. Although not all DNA adducts have a mutagenic effect (Bignold, 2004), the formation of DNA adducts is considered as a marker of exposure (Shuker and Farmer, 1992; Phillips, 2005) and has also been accepted as a hazard indication pointing at a potential cancer risk (Renwick and Walton, 2001), whereas the reduction of DNA adduct levels by for example chemoprevention can result in reduction of tumors (Preston and Williams, 2005; Sander *et al*., 2005). The processes determining the level of DNA binding as possible key event in tumor induction are part of what is called the toxicodynamic phase.

The objective of the present study was to extend the previously developed PBBK model that allowed prediction of the levels of 1′-hydroxyestragole and 1′-sulfooxyestragole in the liver of rats with a description of pharmacodynamic interactions enabling prediction of the dose dependent levels of DNA binding in the liver of rat at varying oral dose levels of estragole. It is well recognized that the level of DNA adduct formation as such cannot predict a cancer risk since several steps in the process of cancer induction are not yet taken into account including the relationship of the DNA adducts formed with mutagenicity and carcinogenicity. However, the PBBD model can take into account the processes of DNA binding and DNA repair, thereby allowing evaluation of dose- and species-dependent differences in bioactivation of estragole at a level one step beyond the biokinetic phase.

2.2 Materials and Methods

2.2.1 Chemicals and reagents

Estragole was obtained from Acros Organics (Geel, Belgium). 2′-Deoxyguanosine was purchased from Sigma (Basel, Switzerland). 1,2,3,7,9-$^{15}N_5$-2′-Deoxyguanosine ($^{15}N_5$-dG) was obtained from Cambridge Isotope Laboratories (Cambridge, MA). ITS+ (Insulin, Transferrin, and Selenious acid), l-glutamine and matrigel were obtained from collaborative Biomedical Products (Bedford, MA). Penicillin/streptomycin was acquired from Gibco (Paisley, UK). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, Utah). Earle's balanced salts solution (EBSS), William's E, William's E without phenol red, dexamethasone, ethylene glycol tetra acetic acid (EGTA), collagenase from Clostridium hystoliticum type IV-S, dimethyl sulfoxide (DMSO), Hepes, $CaCl_2$*$2H_2O$, $MgCl_2$*$6H_2O$, trypan blue, calf thymus DNA (ctDNA), methanol, zinc sulphate (heptahydrate), phosphodiesterase I from Crotalus adamanteus (venom phosphodiesterase), phosphodiesterase II from bovine spleen (spleen phosphodiesterase), and alkaline phosphatase were purchased from Sigma (Schnelldorf, Germany). Acetonitrile, formic acid, dichloromethane, HCl (1N), sodium carbonate (1M), ammonium bicarbonate, sodium sulphate, petroleum ether, ethyl acetate, sodium acetate, Tris-HCl, ethylenediamine tetra acetic acid (EDTA), ammonia 30%, and ethanol were purchased from Merck (Darmstadt, Germany). Nuclease P1 was obtained from MP Biochemicals (Aurora, OH). Triethylacetate ammonium buffer, acetic anhydride, and pyridine were purchased from Fluka (Buchs, Switzerland) and β-glucuronidase was purchased from Roche (Mannheim, Germany).

Both 1'-hydroxyestragole and 1'-acetoxyestragole are carcinogenic to rodents and should be handled accordingly.

2.2.2 Preparation and cultivation of primary rat hepatocytes

White male Sprague-Dawley rats, 8 weeks old, with a weight of 200-250 g were used to isolate fresh primary hepatocytes by perfusion. Rat primary hepatocytes were isolated and cultured according to Cavin *et al*., (2007), based on a protocol first described by Sidhu *et al*. (1993). Briefly, the rat was anesthetized and the liver perfused with medium containing calcium free EBSS, 0.75 mM EGTA, and 100 U/mL penicilin/streptomycin, for 10 min at a flow rate of 20 mL/min. After this a switch was made to perfusion with medium containing EBSS, 100 mg collagenase, 10mM Hepes pH 7.4, 100 U/mL penicillin/streptomycin, 1.8 mM $CaCl_2$, and 0.8 mM $MgCl_2$; the flow rate was kept at 25 mL/min for 8 to 10 min depending on the liver size. The organ was rapidly excised and transferred to William’s E medium containing phenol red, supplemented with 2 mM L-glutamine, 10 mM Hepes pH 7.4, 1% ITS+, 100U/mL penicillin/streptomycin, and 100 nM dexamethasone. After gentle shaking the cells were passed through a sterilized filter nylon membrane of 200 µm cutoff and centrifuged using an Allegra X-15R Centrifuge Beckman coulter (Nyon, Switzerland) for 2 min at 37 g (400 rpm). Cells were washed with William's E containing 2 mM l-glutamine, 10 mM Hepes pH 7.4, 100 U/mL penicillin/streptomycin, and 100 nM dexamethasone. After which cells were filtered with a cutoff membrane of 60 µm to obtain single cells. Viability of the isolated hepatocytes was determined by a trypan blue test, and was at least 90%. Cells were seeded at a density of $3{,}5*10^6$ cells/plate on a 60 mm culture dish in 3.5 mL William's E medium containing phenol red, supplemented with 2mM L-

glutamine, 10 mM Hepes pH 7.4, 1% ITS+, 100 U/ml penicillin/streptomycin, 25 nM dexamethasone and 5% FBS. Hepatocytes were allowed to attach (at 37°C with 5% CO_2) for two to three hrs and then washed with EBSS to remove unattached cells. Fresh serum-free medium containing 25 nM dexamethasone was added and matrigel (233 µg/mL) was then applied. Cells were kept overnight at 37°C with 5% CO_2 before treatment with test compounds.

2.2.3 Exposure of primary rat hepatocytes to 1′-hydroxyestragole

Rat primary hepatocytes were exposed to 1′-hydroxyestragole. The test compound was dissolved in DMSO to 100 mM stock solution. Further dilution was made first in DMSO and then in exposure media with final concentrations in exposure media of 0 (only DMSO), 0.5, 1, 2.5, 5, 10, 25, 50, 100, 150 µM of 1′-hydroxyestragole, with the final concentration of DMSO not exceeding 1%, a concentration that did not result in cytotoxicity for the rat hepatocytes. Exposure to the test compound was for 2 hrs at 37°C with 5% CO_2. It is known that DNA adduct formation is a fast reaction but that also repair pathways in rat primary hepatocytes are active as self defense mechanism; assumption was made that the two pathway were active in parallel. A 2 hr incubation was selected, during which DNA adduct formation appeared to be higher than repair of the DNA adducts since DNA adduct formation could be detected (see result section). To obtain a sufficient amount of DNA all concentrations of 1′-hydroxyestragole were tested in quadruplicates using hepatocytes from the same liver, and pooled together before the digestion step. For biological replicates, hepatocytes isolated from four individual perfusions were used. The supernatants of the incubations were used to measure cytotoxicity by detecting lactate dehydrogenase (LDH) activity and for the measurement of residual 1′-hydroxyestragole as described below.

2.2.4 DNA extraction from primary hepatocytes and enzymatic digestion

DNA was extracted from primary rat hepatocytes exposed to the test compound using the Get pure DNA Kit-Cell protocol (Dojindo Molecular Technology Inc., Kumamoto, Japan) for $3*10^6$ to $1*10^7$ cells (following the manufacturer's instructions); the protocol was modified by doubling the quantity of all reagents. The final DNA pellet was dissolved in a volume of 100 µL MilliQ water. The yield and purity of the extracted DNA was determined using Nanodrop technique by measuring the absorbance ratio A260 nm / A280 nm. DNA samples with an absorbance ratio of 1.8-2 were considered sufficiently pure. The quantity of DNA per sample was calculated from the Nanodrop output in ng/mL using a molar extinction coefficient for double stranded DNA of 50 ($L*mol^{-1}cm^{-1}$). The method of digestion was adjusted based on Delatour *et al.,* (2008) for the release of the nucleosides E-3′-N^2-dG. Per 50 µg DNA in 100 µL water, 20 µL buffer P1 (300 mM sodium acetate, 1 mM $ZnSO_4$, pH 5.3), 12 µL SPDE (spleen phosphodiesterase) solution (0.0004 U/µL in water), and 10 µL nuclease P1 (0.5 µg/µL in water) were added and the resulting solution was incubated for 4 hrs at 37°C. Following incubation, 20 µL PA buffer (500 mM Tris-HCl, 1 mM EDTA, pH 8.0), 13 µL VPDE (venom phosphodiesterase) solution (0.00026 U/µL in water), and 5 µL alkaline phosphatase (0.764 U/µL in water) were added and the mixture was incubated for 3 hrs at 37°C (Delatour *et al.,* 2008). Samples were filtered using eppendorf tubes with a cutoff membrane of 5.000 NMWL (Millipore). The hydrolyzed sample was evaporated to dryness and reconstituted in 50 µL water. The completeness of the digestion was confirmed by agarose gel electrophoresis (data not shown).

2.2.5 Synthesis of 1′-hydroxyestragole

1′-Hydroxyestragole was synthesized based on a reaction described by Drinkwater *et al*. (1976) adapted from Borchert *et al*. (1973). 1′-Hydroxyestragole was formed by a Grignard reaction: Vinyl Grignard (34.52 mmol) was added drop wise to a solution of 4-methoxybenzaldehyde (28.77 mmol) in 20.0 mL of anhydrous tetrahydrofuran cooled down to 0°C and purged under argon. The reaction mixture was stirred in an ice/water cold bath for 3 hrs (temperature maintained below 10°C) and quenched by the addition of 10.0 mL of saturated NH_4Cl. The reaction medium was then diluted with 30 mL of ethyl acetate and 10 mL of water. The two phases were separated and the aqueous phase was extracted with 2*40 mL of ethyl acetate. The combined organic extracts were dried over Na_2SO_4, filtered and evaporated under vacuum to afford 5.90 g of yellow oily residue. This oily residue was purified by column chromatography over silica gel using a Biotage SP-1 equipment with a gradient of hexane/ethyl acetate (from 5% to 40% ethyl acetate). 3.75 g (22.85 mmoL, 80% isolated yield) of (±)-1′-hydroxyestragole were isolated as a yellow oil. Satisfactory purity was demonstrated by NMR (> 96%), HPLC (> 96%) and GC-Tof analysis (pure). NMR analysis of 1′-hydroxyestragole:

^{1}H-NMR ($CDCl_3$, 360 MHz): δ (ppm) 1.86 (d, 1H, J = 3.9 Hz, OH); 3.81 (s, 3H, OMe); 5.17 (bm, 1H, CH); 5.19 (dt, 1H, J = 10.3 Hz; J = 1.4 Hz, =CH); 5.34 (dt, 1H, J = 17.2 Hz, J = 1.4 Hz, =CH); 6.05 (ddd, 1H, J = 17.2 Hz, J = 10.3 Hz, J = 5.9 Hz, =CH); 6.89 (m, 2H, ArH); 7.30 (m, 2H, ArH).

^{13}C-NMR ($CDCl_3$, 90 MHz): δ (ppm) 58.12 (1C, OMe); 77.72 (1C, CH); 116.76 (2C, ArH); 117.61 (1C, $=CH_2$); 130.50 (2C, ArH); 137.64 (1C; C^{IV} Ar); 143.17 (1C, =CH); 162.05 (1C, C^{IV} OAr).

GC-Tof analysis was performed on a DB-5 GC column (Length 30 m; Int. Diameter 250 µm; Film thickness 0.25 µm) in split-less mode using helium as the carrier gas. Inlet purge time: 120 s; Inlet purge flow: 30 mL/min; Inlet total flow: 31.27 mL/min; Inlet temperature: 250°C. (±)-1′-Hydroxyestragole eluted in these conditions with a retention time of 20.2 min and the mass spectrum was in agreement with the structure of (±)-1′-hydroxyestragole. GC-Tof mass spectrum: m/z (rel. int. %): 55(100), 77(97), 109(79), 39 (63), 121(53), 94(45), 135(43), 65(40) 164(36), 137(31), 115(17), 147 (14).

2.2.6 Synthesis of 1′-acetoxyestragole

1′-Hydroxyestragole (46 mg, 0.280 mmol) was dissolved in 1 mL of dichloromethane and this solution was cooled down to 0-5°C in an ice/water bath. Subsequently 35 µL of acetic anhydride (0.375 mmol) and 200 µL pyridine (2.48 mmol) were added and the reaction mixture was stirred for 10-15 min at 0-5°C. The reaction mixture was further stirred for 24 hrs at room temperature. After 24 hr, 10 mL dichloromethane were added to the reaction mixture and this solution was extracted with 2 mL MilliQ water. The pH of the aqueous extract was controlled (pH 8). The dichloromethane solution was then extracted with 2 mL HCl 1N. Control of the aqueous extract pH showed a pH of 2-3. The dichloromethane solution was further extracted with 2 mL sodium carbonate (1M), then dried with sodium sulphate, filtered, and evaporated to dryness under vacuum to obtain 60 mg of 1'acetoxyestragole (0.242 mmol, 86% isolated yield). Purity of 1′-acetoxyestragole was verified by HPLC UV, NMR and GC-ToF. NMR analysis of 1′-acetoxyestragole:

^{1}H-NMR ($CDCl_3$, 360 MHz): δ (ppm) 2.23 (s, 3H, OAc); 3.81 (s, 3H, OMe); 5.26 (bm, 1H, =CH); 5.24-5.31 (dt, 1H, J = 28.2 Hz; J = 1.0 Hz; =CH); 6.05 (ddd, 1H, J = 17.2 Hz; J = 10.1 Hz; J = 6.0 Hz; =CH); 6.20 (bd, 1H, J = 5 Hz, CH); 6.91 (m, 2H, ArH); 7.30 (m, 2H, ArH).
^{13}C-NMR ($CDCl_3$, 90 MHz): δ (ppm) 24.80 (1C, CH_3, OAc); 58.12 (1C, OMe); 78.51 (1C, CH); 116.57 (2C, ArH); 118.80 (1C, $=CH_2$); 131.35 (2C, ArH); 134.01 (1C; C^{IV} Ar); 139.43 (1C, =CH); 162.34 (1C, C^{IV} OAr); 169.31 (1C, CO, OAc).
GC-Tof Mass spectrum: m/z (rel. int. %): 103(100), 164(67) 146(67), 131(67), 147(56), 135(44), 115(44), 163(22), 206(17).

2.2.7 Synthesis of E-3′-N^2-deoxyguanosine (E-3′-N^2-dG) and ($^{15}N_5$) E-3′-N^2-deoxyguanosine (($^{15}N_5$) E-3′-N^2-dG)

E-3′-N^2-dG and ($^{15}N_5$) E-3′-N^2-dG were prepared as described by Punt *et al*., (2007). Briefly, 250 µl of a 0.01g/mL solution of 1′-acetoxyestragole in DMSO were added to 2250 µL of 2.5 mM 2'-deoxyguanosine (non-labeled or labeled, respectively) dissolved in 2.5 mM ammonium bicarbonate at pH 7.6. The incubation was stirred for 60 hrs at 37°C. In order to obtain the adduct E-3'-N^2-dG, the reaction mixture was purified using HPLC UV (HP1090 with a UV detector HP 1090) according to Punt *et al*. (2007). The synthesized nucleotides E-3′-N^2-dG and ($^{15}N_5$) E-3′-N^2-dG were further purified using a Waters semi preparative column: C18 7.8 cm * 150 mm, 7 µm. The gradient was made with ultrapure water and 100% acetonitrile, with a flow rate of 2 mL/min. 200 µL of the crude nucleoside preparation were injected. For elution, a linear gradient was applied over 40 min from 20% to 30% acetonitrile, followed by an increase to 100% acetonitrile in 2 min, and keeping the gradient at 100% acetonitrile for one minute. Then acetonitrile was lowered to 20% in 2 min and the column was equilibrated with 20% acetonitrile for 10 min. Batches of 200 µL of the crude mixture were injected and each individual peak was collected. Under these conditions, peaks corresponding to E-3′-N^2-dG and ($^{15}N_5$) E-3′-N^2-dG eluted at a retention time of 7.5 min.

2.2.8 LC-ESI-MS/MS method for detection and quantification of E-3'-N^2-dG

The LC-ESI-MS/MS method was adapted from Punt *et al*. (2007). LC-ESI-MS/MS analysis was performed on an Agilent 1100 series (Agilent Technologies, Waldbronn, Germany) coupled to a TSQ Quantum triple quadruple mass spectrometer from Thermo Finnigan (San Jose, CA, USA). Samples were injected on an Agilent Zorbax Extend-C18 column, 2.1*50 mm, 3.5 Micron 80 Å (Basel, Switzerland), with a Zorbax guard column. The gradient was made with ultrapure water containing 0.1% (v/v) formic acid and 100% acetonitrile. The flow rate was set at 0.3 mL/min. A linear gradient was applied from 20% to 70% acetonitrile over 8 min, after which the percentage of acetonitrile was brought to 100% in 1 min, and kept at 100% acetonitrile over 2 min. The amount of acetonitrile was lowered to 20% over 1 min, and the column was equilibrated at these initial conditions for 8 min. E-3'-N^2-dG eluted at 4.94 min. The mass spectrometric analysis was done in positive ion mode. The electro-spray capillary voltage was set at 4kV and the capillary temperature at 250°C. The cone voltage was set to 20V; extractor voltage 3V; and the RF lens to 0.3V. Nitrogen was used as sheath gas with a pressure of 7 bar (100 psi). The collision gas pressure was set at 350 mbar (5 psi) in the collision cell. The dwell time per transition was 0.02s. A divert valve was used in order to discard the first 2 min and the gradient after elution of the peak. Sample analysis was carried out using selected reaction

monitoring (SRM) mode, and characteristic transitions were recorded. The most intense transition was used as quantifier and the second transition as qualifier: 414 → 298 m/z (qualifier), 414 → 164 m/z (quantifier), and 414 → 147 (qualifier) for E-3'-N^2-dG; 419 → 303 m/z (qualifier), and 419 → 169 m/z (quantifier) for ($^{15}N_5$) E-3'-N^2-dG (table 2.1). The limit of detection (LOD) and limit of quantification (LOQ) were measured by adding to digested DNA from untreated rat hepatocytes a constant amount of the internal standard (0.012 nmol) and increasing amount of E-3'-N^2-dG (0-0.0157-0.04-0.0785-0.12-0.14-0.16 nmol). The LOD was defined as three times over the background level and the LOQ was defined as 10 times above the background level.

Table 2.1. Transition reaction (m/z) and the collision energy used to obtain the daughter fragments by LC-ESI-MS/MS.

	Transition (m/z) (used for quantification)	Collision energy (eV)	Transition (m/z) (used for conformation)	Collision energy (eV)
E-3′-N^2-dG	414→164	34	414→298 414→147	26 45
($^{15}N^5$)E-3′-N^2-dG	419→169	34	419→303	26

2.2.9 Quantification of nucleoside E-3′-N^2-dG by isotope dilution

Samples of 50 µL of digested rat hepatocyte DNA were spiked with 10 µL of the internal standard ($^{15}N_5$) E-3'-N^2-dG to achieve 5 ng/µL (0.012 nmol) injection of the internal standard into the instrument using an injection volume of 10µL. In order to quantify the amount of adduct formed in the samples, a calibration curve was prepared using increasing concentrations of E-3'-N^2-dG (0, 0.0157, 0.04, 0.0785, 0.12, 0.14, and 0.16 nmol) with a constant concentration of ($^{15}N_5$) E-3'-N^2-dG (0.012 nmol). Calibration curves were prepared plotting the ratio of the peak areas against the ratio of the concentrations of E-3'-N^2-dG and the internal standard, ($^{15}N_5$) E-3'-N^2-dG. Furthermore, these calibration curves were injected before and after each sample sequence run to ensure that the instrument response did not vary during the run of the sample sequence. As reported by Delatour *et al*., (2009) the response was considered linear when the response factor was lower than 15%. Data analysis of the calibration series and sample sequence was performed using Xcalibur software (Thermo Electron Corporation) and integrated using an excel spreadsheet developed internally (Nestlé Research Center), to comply with EU guidelines 2002/657/EC concerning the performance of analytical methods and the interpretation of results (EU guidelines: 2002/657/EC).

The amount of E-3′-N^2-dG detected in the samples was related to the total amount of digested DNA detected in each sample and adjusted for the average Mw of DNA monophosporylated nucleotides (nt) of 327g/mol, in order to quantify the number of E-3′-N^2-dG adducts per 1000 nt.

2.2.10 Determination of 1′-hydroxyestragole in primary rat hepatocytes supernatant

Primary rat hepatocytes were exposed to 50 µM and 100 µM of 1′-hydroxyestragole and incubated at 37°C at increasing time intervals up to 2 hrs. To quantify possible protein binding of 1′-hydroxyestragole, samples containing 50 µM of 1′-hydroxyestragole were incubated for the same time intervals without cells with bovine serum albumin at a concentration of either 1.25 mg/mL (physiological level) or 50 mg/mL (high level). Furthermore, chemical stability of 1′-hydroxyestragole was quantified by incubating 1′-hydroxyestragole without cells for increasing time intervals also up to 2 hrs. The supernatants and samples thus obtained were analyzed by HPLC-UV (HP1050 with a UV detector HP 1050) with detection at wavelengths of 220-260-275 nm in order to quantify the 1′-hydroxyestragole in the incubation medium. The gradient was made with ultra-pure water and acetonitrile, with a flow rate of 1 mL/min. A linear gradient was applied from 20% to 30% acetonitrile in 20 min, followed by an increase in acetonitrile in 2 min to 100%, and keeping acetonitrile at 100% for one minute. Acetonitrile was lowered to 20% in 2 min and the HPLC instrument was equilibrated to 80% water and 20% acetonitrile for 10 min. 20 µL of sample was injected onto a Supelcosil LC 18 DB 25 cm * 4,6 mm, 5 µm column with a Supelco guard column Sulpelco. The elution time of 1′-hydroxyestragole was at 9.9 min. Furthermore, 90 µL of each sample were incubated for 2 hrs at 37°C with 2.5 µL of β-glucuronidase (0.35U, 140 U/mL) and 10 µL of 1M potassium phosphate buffer (pH 6.2) followed by analysis described above by HPLC-UV to detect 1′-hydroxyestragole. and investigate to what extent the reduction in 1′-hydroxyestagole in the incubation media could be ascribed to formation of 1’-hydroxyestagole glucuronide, representing a detoxification pathway.

2.2.11 Cytotoxicity assay

Cytotoxicity was determined by measuring the activity of cytoplasmic lactate dehydrogenase (LDH) in cell culture supernatants reflecting leakage from the cytoplasm of damaged cells, using ENZYLINE™ LDH Optimise 10 (BioMerieux® SA, Lyon, France) solution measured with the Roche COBAS MIRA plus analyzer (Minnesota, USA). The LDH was assayed by following the consumption of NADH to NAD^+, during the conversion of pyruvate to lactate. Absorbance of NADH was recorded at a wavelength of 340 nm at a temperature of 37°C, with an extinction coefficient of 6.22 mM^{-1} cm^{-1} (at 340 nm and pH 7.1). In order to validate the measurements, commercially available quality controls were freshly prepared and run in parallel. The quality controls used were: zymotrol™ (BioMerieux® SA, Lyon, France), duotrol™, and duotrol abnormal™ (Biomed, Germany).

2.2.12 Physiologically based biodynamic modeling

The PBBD model for rats was developed based on the PBBK model developed by Punt *et al*., (2008). The equation for DNA adduct formation was introduced into the PBBK rat model for estragole, and parameters were kept similar to those reported and used before (Punt *et al*., 2008). The time of simulation by the model was shortened from 24 hrs to 2 hrs in order to match the incubation time of the treated hepatocytes with 1′-hydroxyestragole. In order to calculate the amount of DNA adducts formed at each external dose of estragole given the model was extended as follows. The mass balance equation for the level of 1′-

hydroxyestragole in the liver reported in Punt *et al.,* 2008 in the PBBK model was used to derive the AUC for 1′-hydroxyestragole in the liver within the first two hours of exposure.

Liver: $dAL_{HE}/dt = + V_{max,L\_HE} * CL_E/PL_E/(K_{m,L\_HE} + CL_E/PL_E) - V_{max,L\_OE} * CL_{HE}/PL_{HE}/(K_{m,L\_OE} + CL_{HE}/PL_{HE}) - V_{max,L\_HEG} * CL_{HE}/PL_{HE}/(K_{m,L\_HEG} + CL_{HE}/PL_{HE}) - V_{max,L\_HES} * CL_{HE}/PL_{HE}/(K_{m,L\_HES} + CL_{HE}/PL_{HE})$

$CL_{HE} = AL_{HE}/VL$

$dAUC_{HE}/dt = CL_{HE}$

where CL_{HE} is the 1'-hydroxyestragole concentration in the liver (µmol/L); CL_E is the concentration of estragole in the liver; PL_E is estragole liver/blood partition coefficient; VL volume of the liver (in Liters); AL_{HE} is the amount of 1'-hydroxyestragole in the liver (L) in µmol; PL_{HE} is the liver/blood partition coefficient of 1'-hydroxestragole; V_{max,L_M} and K_{m,L_M} are the maximum rate of formation and the Michaelis–Menten constant for the formation of 1'-oxoestragole (OE), 1'-hydroxyestragole (HE) and for the phase II metabolites 1'-hydroxyestragole glucuronide (HEG) and 1'-sulfooxyestragole (HES) in the liver (Punt *et al.,* 2008). AUC_{HE} (hr*µmol/L) is the area under the concentration-time curve of 1'-hydroxyestragole in the liver. Differential equations were integrated using the Berkley Madonna 8.0.1 software (Macey and Oster, UC Bekeley, CA, USA).

The following equation was added to the model to describe the formation of E-3′-N^2-dG DNA adducts as a function of the AUC for 1′-hydroxyestragole:

$$DNA_{dG} = A + B * AUC_{HE}$$

The DNA_{dG} (#adducts/1000nt) is the amount of DNA adduct formed due to the specific binding of 1′-hydroxyestragole to deoxyguanosine nucleoside, A and B are the intercept and the slope calculated based on the data from the *in vitro* experiments.

2.2.13 Sensitivity Analysis

A sensitivity analysis was performed by analyzing all model parameters to identify key parameters that influence the outcome of the PBBD model, the DNA adducts formation. Individual parameters were modified without changing the other model parameters, which were left at initial values. Normalized sensitivity coefficients (SC) for each parameter of the model were determined according to the following equation:

$$SC = (C'-C)/(P'-P)x(P/C),$$

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). A 5 % increase in parameter values was chosen, to analyze the effect of a change in parameter on formation of DNA adducts in the rat hepatocytes.

2.3 Results

2.3.1 Cytotoxicity of 1′-hydroxyestragole in primary rat hepatocytes

Concentrations of 1′-hydroxyestragole tested for cytotoxicity ranged from 0 µM to 1000 µM (Figure 2.2). When primary rat hepatocytes were exposed to 1′-hydroxyestragole an increase in the medium of the LDH levels was observed above 200 µM (Figure 2.2), which is in line to what was reported by Chan and Caldwell, (1992) who measured cytotoxicity by LDH measurement in medium of hepatocytes exposed for 17 hrs to 1′-hydroxyestragole and observed cytotoxicity at concentrations higher than 100 µM. Based on these results 1′-hydroxyestragole concentrations selected for measuring DNA adduct formation in primary rat hepatocytes exposed to 1′-hydroxyestragole were chosen to be below 150 µM. Below 150 µM and at 2 hrs exposure time of the primary rat hepatocytes to 1′-hydroxyestragole no cytotoxicity was observed (data not shown).

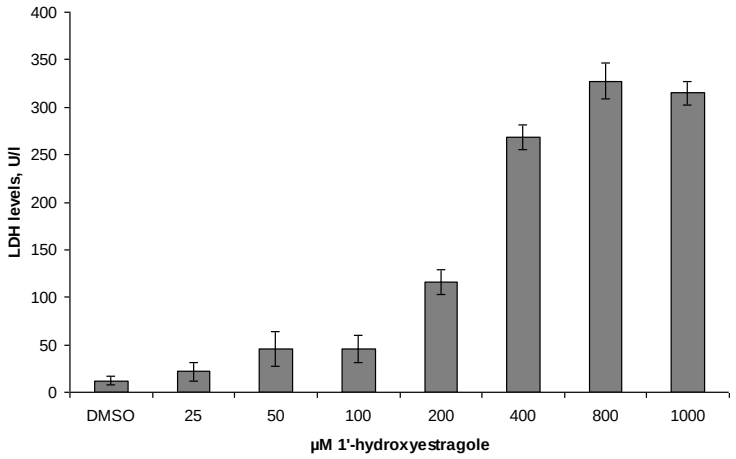

Figure 2.2. Cytotoxicity of 1′-hydroxyestragole towards isolated rat primary hepatocytes as detected by LDH leakage upon 2 hours exposure (extinction coefficient of NADH of 6.22 mM^{-1} cm^{-1}).

2.3.2 Quantification of E-3′-N^2-dG in primary rat hepatocytes exposed to 1′-hydroxyestragole by isotope dilution and LC-ESI-MS/MS

The reaction of the sulphate metabolite of 1′-hydroxyestragole with double strand DNA has been reported to produce four different adducts with the major one being E-3′-N^2-dG (Phillips *et al*., 1981; Wiseman *et al*., 1985; Wiseman *et al*., 1987; Punt *et al*., 2007). Via isotope dilution and LC-ESI-MS/MS analysis the amount of E-3′-N^2-dG DNA adducts formed in primary rat hepatocytes exposed to 1′-hydroxyestragole was quantified. Detection and quantification limits of the LC-ESI-MS/MS were 0.033 pmol and 0.08 pmol of E-3′-N^2-dG respectively. At concentrations above 0.5 µM 1′-hydroxyestragole formation of E-3′-N^2-dG DNA adducts was detected in the primary rat hepatocytes. Figure 2.3 presents the amount of E-3′-N^2-dG DNA adducts formed (expressed as # adducts/ 1000 nt) with increasing concentrations of 1′-hydroxyestragole (µM). These experimental data show a dose dependent increase in E-3′-N^2-dG DNA adduct formation at increasing concentrations of 1′-hydroxyestragole up to 150 µM, the highest non-cytotoxic dose level tested. At 150 µM 1′-hydroxyestragole the level of E-3′-N^2-dG DNA adducts formed amounted to 4.82

adducts/1000nt (average of four individual experiments). The results obtained reveal a linear relationship that passes through the origin between the concentration of 1′-hydroxyestragole and the level of E-3′-N^2-dG DNA adduct formation in the exposed rat primary hepatocytes.

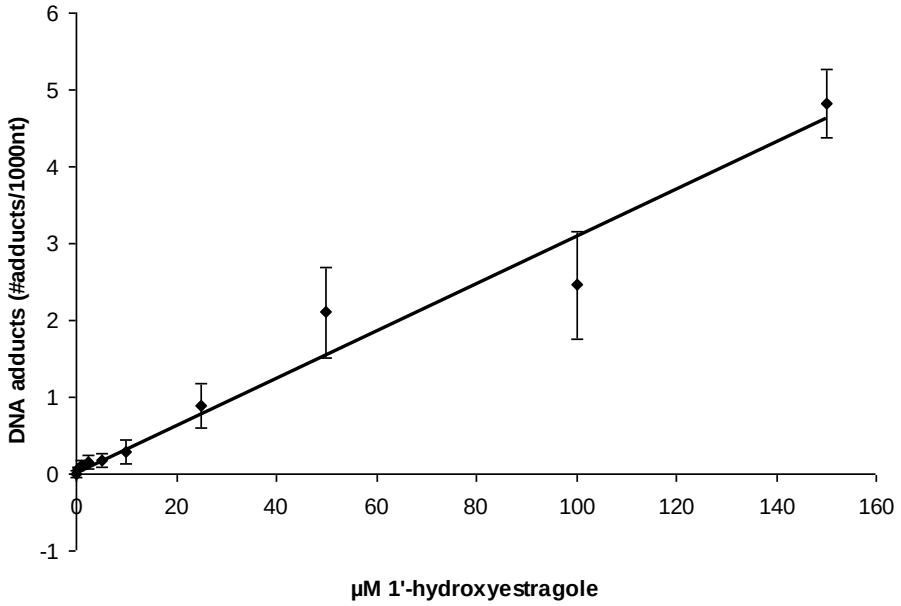

Figure 2.3. E-3′-N^2-dG adduct formation after 2 hours treatment in primary rat hepatocytes expressed in # adducts/1000nt as a function of different concentrations of 1′-hydroxyestragole, and quantified by LC-ESI-MS/MS using isotope dilution technique (average of four individual experiments).

2.3.3 Determination of 1′-hydroxyestragole in cell culture supernatants

To be able to define the AUC in order to link the PBBK model to the results from the *in vitro* studies on formation of DNA adducts in rat primary hepatocytes exposed to 1′-hydroxyestragole, the PBBK predicted AUC values for 1′-hydroxyestragole during the first two hours after *in vivo* exposure (AUC_{HE}) were linked to an equation defining the dependence of DNA adduct formation as a function of the AUC for 1′-hydroxyestragole as determined in the *in vitro* incubations ($AUC_{HE(in\ vitro)}$). To be able to define the AUC for 1′-hydroxyestragole in the *in vitro* model the time dependent changes in the level of 1′-hydroxyestragole in the medium of rat hepatocytes exposed to 1′-hydroxyestragole at different concentrations were quantified. The $AUC_{HE(in\ vitro)}$ was calculated using the generic formula of the area of the trapezoid: X = ba+b(c-a)/2 or X = b(c+a)/c. Figure 2.4A presents the time-dependent decrease in 1′-hydroxyestragole in the cell culture supernatants of hepatocytes exposed to 50 µM and to 100 µM 1′-hydroxyestragole up to 2 hrs. Control incubations in the absence of hepatocytes reveal no time dependent loss of the test compound (data not shown). In addition, control incubations in the absence of hepatocytes but in the presence of bovine serum albumin (BSA) resulted in about 10-20% loss of the test compound (data not shown), indicating a low level of protein binding. These results indicate that the compound is rapidly and effectively taken up and potentially metabolized by the cells. The majority of 1′-hydroxyestragole detoxification is known to occur through glucuronidation. In order to determine if the primary hepatocytes in our model are competent to metabolize

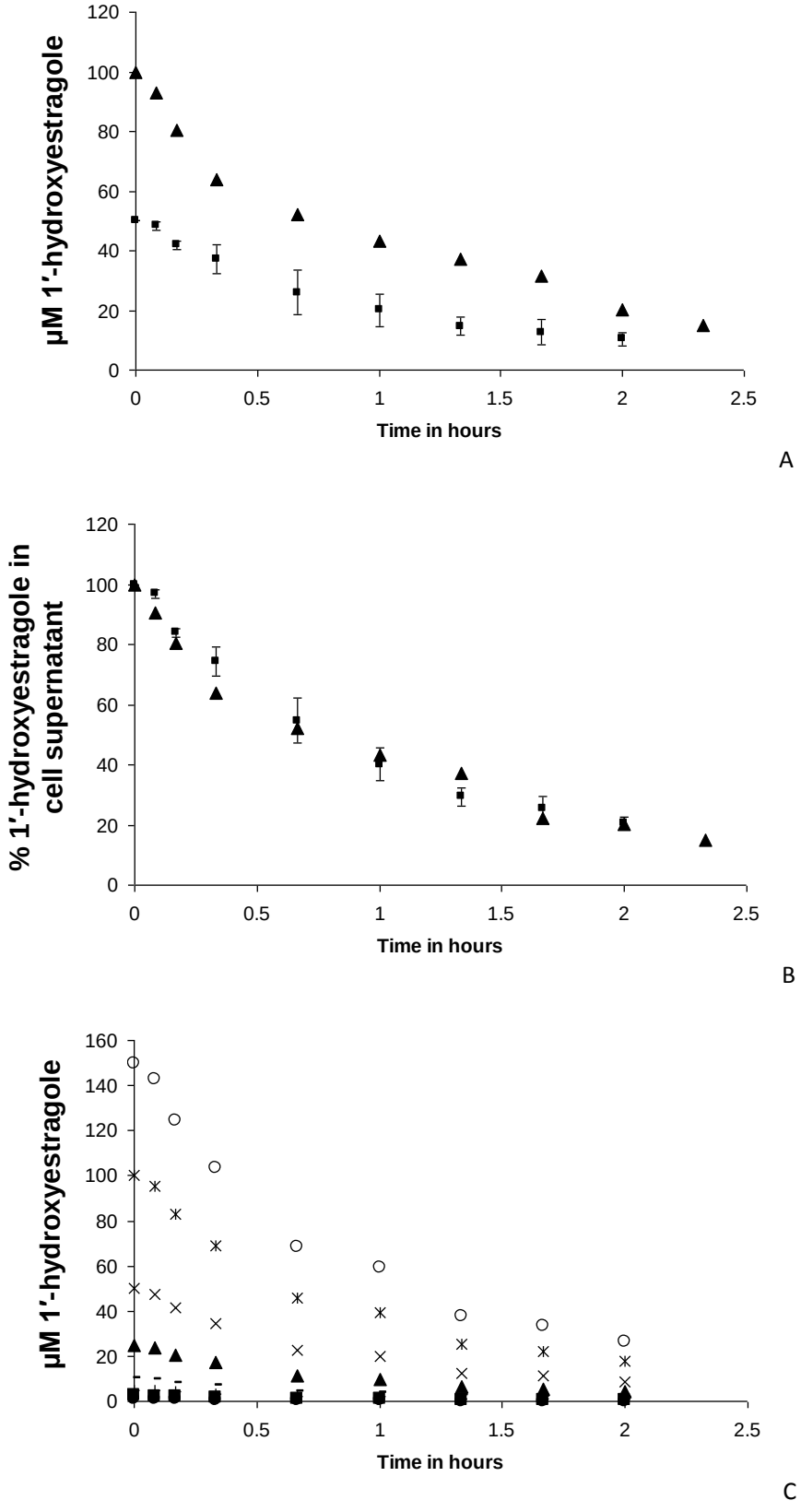

Figure 2.4. Time dependent decrease of 1'-hydroxyestragole in the media of primary rat hepatocytes exposed to either 50 (■) or 100 (▲) µM 1′-hydroxyestragole expressed in absolute concentrations (A) or as percentage of the starting concentration (B), and representation of the calculated time-dependent 1'-hydroxyestragole decrease in cell culture supernatant at each concentration tested 150µM (○), 100µM (∗), 50µM (x), 25µM (▲), 10µM(–), 5µM (+), 2.5µM (■), 1µM (•) from which the respective $AUC_{HE(in\ vitro)}$ (hr*µmol/L) values were calculated (C).

1′-hydroxyestragole comparable to the *in vivo* situation, supernatants of the treated primary rat hepatocytes were treated with β-glucuronidase to release free 1′-hydroxyestragole from the glucuronic acid conjugates and reanalyzed for their 1′-hydroxyestragole levels. The results obtained revealed a recovery of more than 60-80% of 1′-hydroxyestragole upon treatment with β-glucuronidase (data not shown). This confirms that glucuronidation is a major pathway for conversion of 1′-hydroxyestragole in rat primary hepatocytes. Furthermore, taking into account that 10-20% of the originally added 1′-hydroxyestragole is lost due to possible protein binding it can be concluded that glucuronidation of 1′-hydroxyestragole accounts for most of the decrease in 1′-hydroxyestragole in the *in vitro* incubations, which would be in line with its metabolic fate in the *in vivo* situation. This confirms that the primary rat hepatocytes under these conditions are metabolically competent to form glucuronic acid conjugates as the major pathway for phase II metabolism of 1′-hydroxyestragole.

2.3.4 Definition of the PBBD model by linking *in vitro* data on DNA adduct formation to the PBBK model

From the experimental data on the decrease of 1′-hydroxyestragole as a function of time in the incubations with rat primary hepatocytes (Figure 2.4A) the $AUC_{HE(in\ vitro)}$ values were calculated. Experimental data obtained at 50 and 100 µM 1′-hydroxyestragole were also presented as the decrease in the percentage of the original concentration of the test compound (Figure 2.4B). This reveals that the time dependent decrease follows a similar pattern at both concentrations. From these experimental findings the time dependent decrease in the concentration of 1′-hydroxyestragole with other starting concentrations was modeled in line with these curves (Figure 2.4C) and this allowed calculation of the area under time curve ($AUC_{HE(in\ vitro)}$) values at all concentrations of 1′-hydroxyestragole tested.

In a next step these $AUC_{HE(in\ vitro)}$ values were used to replace the x-axis values in Figure 2.3 resulting in Figure 2.5. Figure 2.5 represent the level of E-3′-N^2-dG DNA adduct formation in the rat primary hepatocytes as a function of the AUC for 1′-hydroxyestragole ($AUC_{HE(in\ vitro)}$). In line with Figure 2.3 also Figure 2.5 reveals a linear relationship with an intercept at the origin. Thus, the curve presented in Figure 2.5 can be fitted by a linear equation passing through zero according to:

$$DNA_{dG} = 0.032 * AUC_{HE}$$

with $r^2 = 0.9635$, and DNA_{dG} representing the amount of E-3′-N^2-dG DNA adducts (#adducts/1000nt) formed in the hepatocytes at a certain $AUC_{HE(in\ vitro)}$ (hr*µmol/L) of 1′-hydroxyestragole.

By incorporating this equation in the PBBK model and defining that $AUC_{HE(in\ vitro)}$ should equal AUC_{HE} (hr*µmol/L) in the *in vivo* PBBK model defined by Punt *et al.,* (2008) a link between the AUC predicted in the PBBK model (AUC_{HE}) and the AUC from the hepatocyte incubations ($AUC_{HE(in\ vitro)}$) was established; ultimately providing a link between the PBBK model and the formula for DNA binding *in vitro* and defining a PBBD model that can predict DNA binding as a function of the estragole dose.

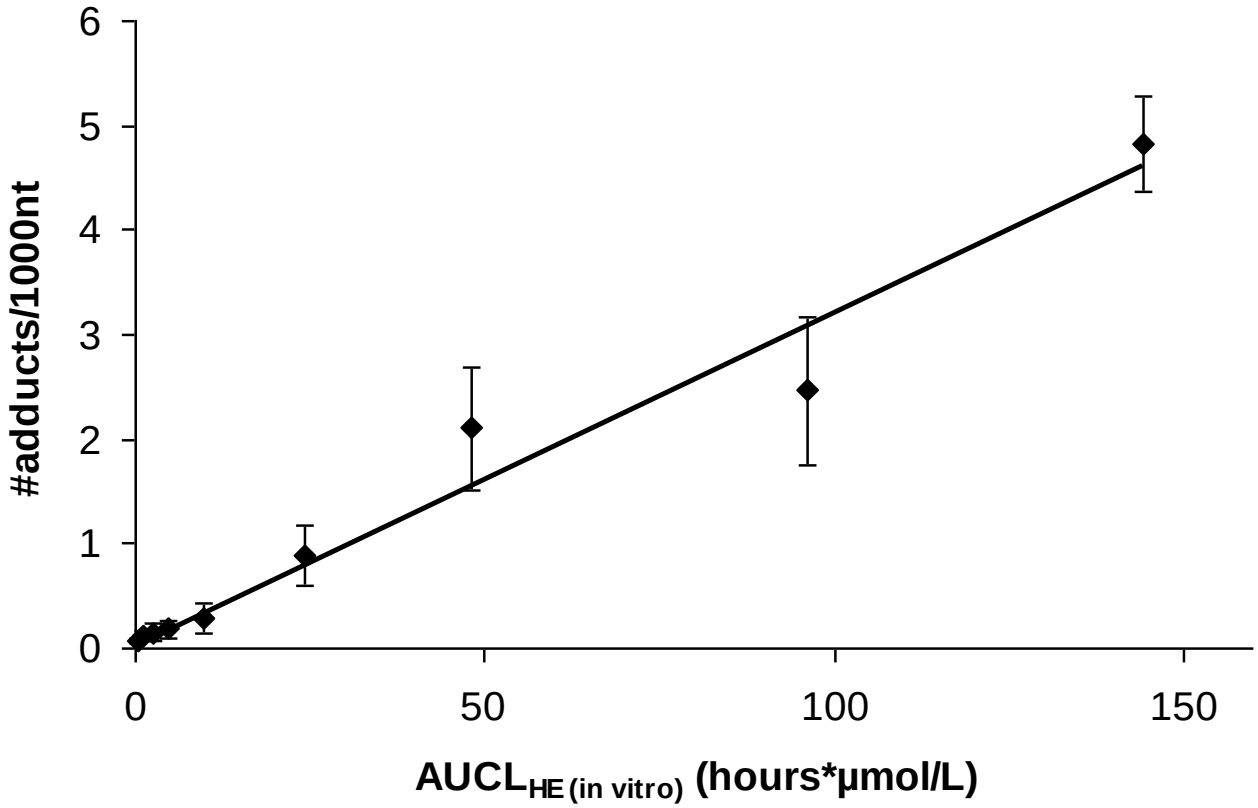

Figure 2.5. E-3′-N^2-dG adduct formation in primary rat hepatocytes expressed in #adducts/1000nt as a function of the $AUC_{HE(in\ vitro)}$ (hr*µmol/L) of 1′-hydroxyestragole, and quantified by LC-ESI-MS/MS using isotope dilution technique (average of four individual experiments).

2.3.5 Rat PBBD model predictions

The newly defined PBBD model was used to simulate the dose dependent DNA adduct formation in the liver of rat exposed to estragole. The results of this simulations are displayed in Figure 2.6 and reveals that up to dose levels of 100 mg/kg bw the E-3′-N^2-dG DNA adduct formation is predicted to be linear with increasing dose of estragole.

At a dose level of 0.01 mg/kg bw (Smith *et al.,* 2002), and 0.07 mg/kg bw estragole, (estimated daily human exposure for a 60 kg person reported by the Scientific Committee on food of the European Union) (SCF, 2001), the PBBK model predicted amounts of E-3′-N^2-dG DNA adduct formed of respectively 2, and 12.8 in 10^8 nt.

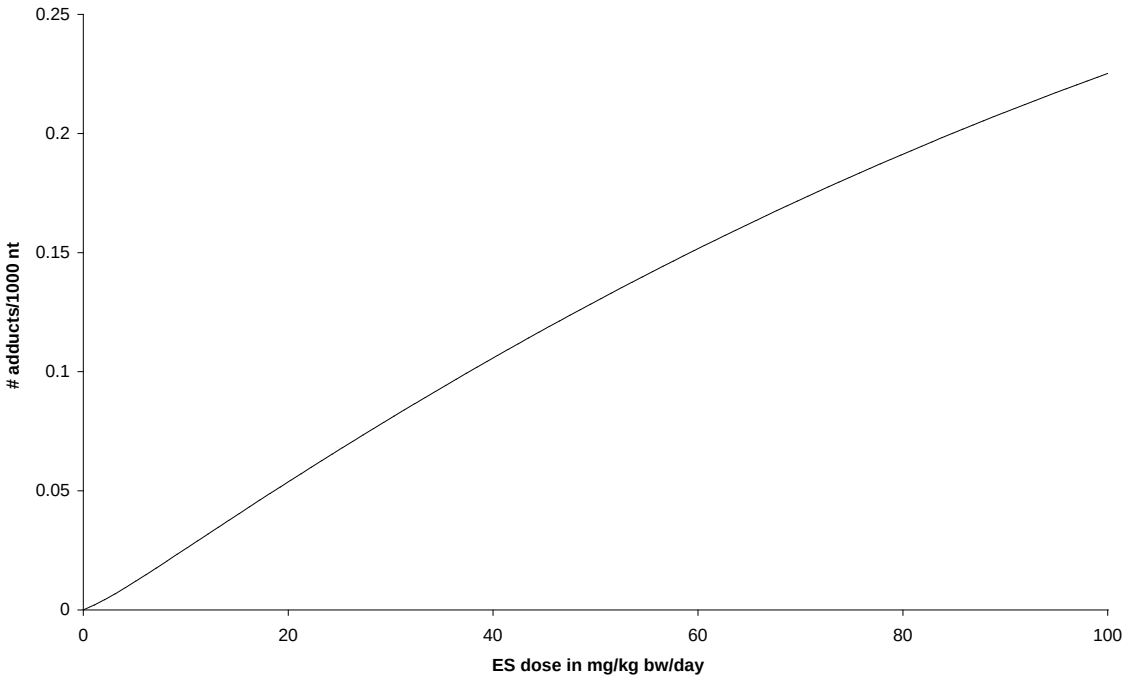

Figure 2.6. Dose dependent, PBBD model predicted DNA adduct formation (#adducts/1000nt) in the liver of rats exposed orally to estragole.

2.3.6 Sensitivity Analysis

Sensitivity analysis was performed at different estragole doses to identify the key parameters that can influence the DNA adduct formation in the PBBD model outcome. The estragole doses set for sensitivity analysis were: 0.07 mg/kg bw (corresponding to the average daily intake for humans, SCF 2001), and 400 mg/kg bw (the dose used in the *in vivo* study by Randerath *et al*., 1984). All parameters of the model were changed and only the ones which resulted in a normalized sensitivity coefficient higher than a 0.1 (in absolute value) were kept and displayed in Figure 2.7.

Figure 2.7 shows that the parameters with a major impact on the model prediction of the levels of DNA adducts were the slope of the DNA_{dG} versus $AUC_{HE(in\ vitro)}$ curve obtained from the *in vitro* hepatocyte data. Further, the kinetic parameters V_{max} and K_m for the glucuronidation of 1′-hydroxyestragole have a major impact. High sensitivity was found also when changing the liver/blood partition coefficient for 1′-hydroxyestragole (PLHE). Additionally, the dose played a role; at 0.07 mg/kg bw parameters such as body weight; volume Liver; blood through the liver; the uptake constant Ka; and the kinetic parameters V_{max} and K_m of the 1′-hydroxy metabolite had a higher impact than at 400 mg/kg bw. On the contrary at 400 mg/kg bw the blood through fat and the V_{max} of 1′-hydroxyestragole in the liver had a higher impact.

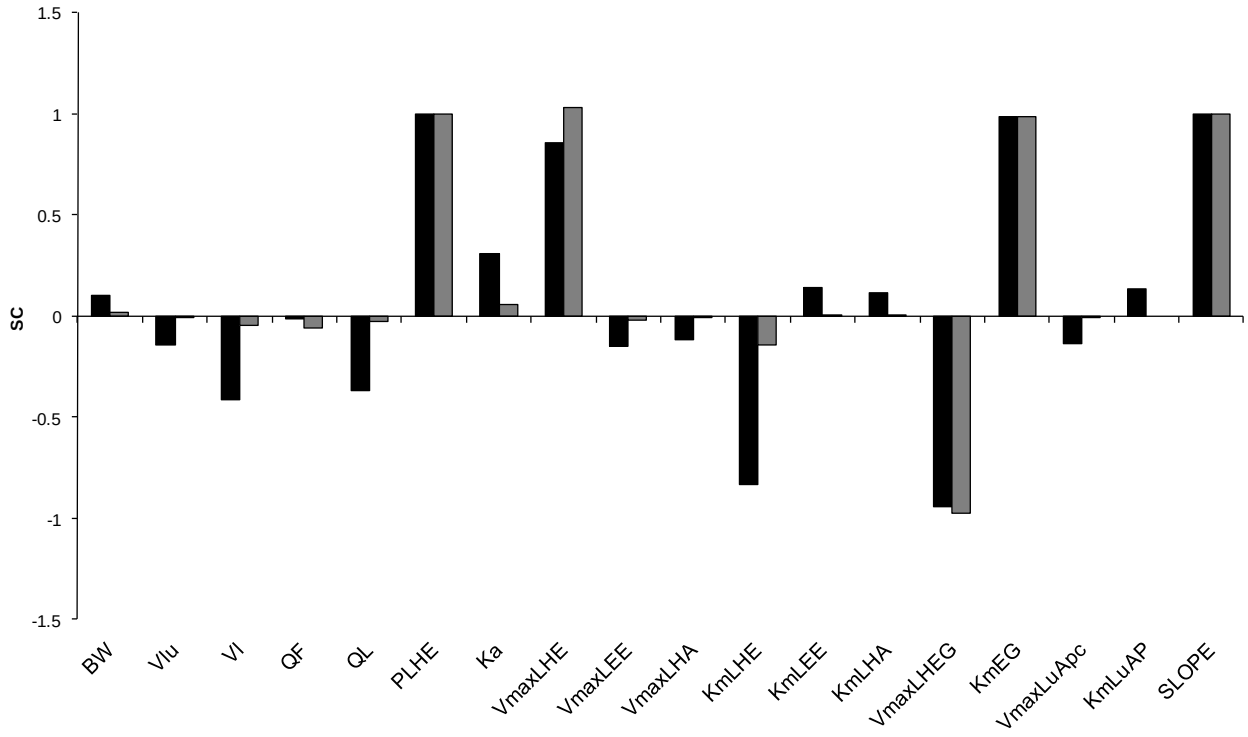

Figure 2.7. Sensitivity analysis of the PBBD model predictions at a dose of estragole of 0.07 mg/kg bw/day (black bars) and 400 mg/kg bw/day (grey bars). Model parameters evaluated include: the V_{max} and K_m of the different metabolites in the liver (L) and lung (Lu): 1′-hydroxyestragole (HE), estragole-2′,3′-oxide (EE), 3′-hydroxyanethole (HA), 4-allylphenol (AP). 1′-hydroxyestragole glucuronide (HEG). Bw, body weight; VK, Volume kidney; VL, Volume Liver; QF, blood flow through fat; QL, blood flow through the liver; PLHE, liver/blood partition coefficient 1′-hydroxyestragole; and slope of the DNA_{dG} versus $AUC_{HE(in\ vitro)}$ curve.

2.4 Discussion

The present study illustrates the development of a physiologically based biodynamic (PBBD) model describing the dose dependent DNA adduct formation in liver of rats exposed to estragole. The PBBD model was developed based on combining our previously developed PBBK model for predicting *in vivo* formation of 1′-hydroxyestragole in the liver of rats exposed to estragole (Punt *et al*., 2008) with *in vitro* data from experiments in which the formation of the major estragole DNA adduct, E-3′-N^2-dG, in isolated rat hepatocytes exposed to 1′-hydroxyestragole was quantified. In parallel, measurement of the time dependent decrease in 1′-hydroxyestragole in the medium of the exposed rat hepatocytes defined the concentration versus time area under the curve of each concentration of the test compound, $AUC_{HE(in\ vitro)}$. By defining the equation describing the E-3′-N^2-dG DNA adduct formation in rat primary hepatocytes as a function the $AUC_{HE(in\ vitro)}$, and by adding this equation to the PBBK model and defining the $AUC_{HE(in\ vitro)}$ to be equal to the AUC_{HE} already defined in the rat model it became possible to couple biokinetic with dynamic data and to extend the PBBK model to a PBBD model. The PBBD model allows estimation of the level of DNA adduct formation in rat liver upon different doses of estragole. This AUC approach was used previously in a paper on dynamical coupling of PBPK/PD and AUC-based toxicity models for arsenic *in vivo* in tilapia (Liao *et al*., 2005). The PBBD model outcome showed a linear relationship between the level of E-3′-N^2-dG DNA adduct formation and the dose of estragole up to at least 100 mg/kg bw/day. At a dose level of 0.01 mg/kg bw/day (Smith *et al*., 2002) and 0.07 mg/kg bw/day estragole (SCF, 2001), the PBBK model predicted amounts of E-3′-N^2-dG DNA adduct formed equal to respectively 2 and 12.8 in 10^8nt. These levels of adducts were 2 to 3 order of magnitude below the background level of DNA damage of 1 adduct in 10^6nt, which are of no known consequence (Williams, 2008). Furthermore levels are 1 to 2 order of magnitude lower when compared to the background levels of DNA adducts formed by low molecular weight alkylating agents, like for example N-nitrosamines, that are in a range of 1–10 adducts in 10^7 nucleotide (Farmer, 2005; Farmer, 2008).

To validate the newly developed PBBD model, *in vivo* experimental data on DNA adduct formation in rats exposed to estragole would be required but they are not available in the literature. Since generation of such *in vivo* data was beyond the objective of the present study the predicted PBBD model based outcomes were compared to data available in the literature on DNA adduct formation in the liver of mice exposed to estragole. Randerath *et al*. (1984) reported in female CD-1 mice the formation of 1 adduct in 10.000 – 15.000 DNA nucleotides after a single i.p. injection of 10 mg estragole per mouse. Assuming a mouse weight of 0.025 kg, the average intake dose was estimated to be 400 mg/kg bw/day. At this dose the PBBD model predicts the formation of E-3′-N^2-dG in the liver of rat at a level amounting to 4 adducts in 10.000nt. Taking into account that we quantified by LC-ESI-MS/MS the formation of only the E-3′-N^2-dG DNA adduct representing a major but not the only DNA adduct formed, whereas in the Randerath *et al*. (1984) DNA adducts were determined using ^{32}P-postlabelling detecting 1 adduct in 10.000nt, levels of DNA adduct formation in these two studies are within the same order of magnitude. The slight difference can be explained by the difference in the setup analysis of the two studies. In the present paper we used primary rat hepatocytes form male Sprague Dawley rats and the adducts were analyzed with the sensitive LC-ESI-MS/MS 2 hrs after exposure while Randerath *et al*. (1984) used female CD1 mice and analysis of samples was done by ^{32}P-postlabelling 24 hrs after treatment.

In risk assessment different mathematical models (ex. Linear model, Multistage model, Gamma model, Probit model, and Weibull model) can be used to extrapolate cancer risks from high dose animal exposure to cancer risks connected to low dose exposure relevant for the human situation. However, this approach to use mathematical models for high to low dose extrapolation of cancer risks is still a topic for considerable debate, since is not known whether or not these mathematical models take into account all biological process of relevant genotoxic carcinogenic compounds interacting with the body. PBBK models appear to provide a useful tool in risk assessment when modeling interspecies and high to low dose extrapolation of the ADME characteristics including bioactivation of genotoxic carcinogenic compound which are present in the human diet at low doses (Punt *et al*., 2008; Punt *et al*., 2009). Extension of the rat PBBK model to a rat PBBD model, by linking the area under the curve for 1′-hydroxyestragole formation predicted by the rat PBBK model to the area under the curve for 1′-hydroxyestragole in the culture medium of 1′-hydroxyestragole exposed rat primary hepatocytes, as reported in the present study, will give more insight on how the body reacts to the chemical at various dose levels. Clearly a limitation is that the present PBBD model predicts DNA adduct formation as the endpoint and does not yet allow prediction of estragole mutagenicity or carcinogencity, since for DNA adducts to induce mutations the cells must survive, proliferate, and replicate (Bailey *et al*., 2009), which still is a long process before achieving the endpoint of actual tumor formation. However, although DNA adduct formation of genotoxic carcinogens is generally seen as a biomarker of exposure rather than a biomarker of response, the PBBD model now developed is one step closer to the ultimate toxic effect of estragole than the PBBK model for bioactivation and detoxification of estragole described previously. The PBBD model predicts the dose dependent level of E-3′-N^2-dG DNA adduct formation for low levels of exposure up to a dose level shown to cause cancer in the rodent bioassays and provides a proof of principle for modeling a toxicodynamic endpoint. Up to now only a few papers have described PBBD type models for genotoxic compounds. Conolly *et al*. (2004) reported a PBBD model for formaldehyde and Young *et al*. (2007) for acrylamide. However, both papers describe kinetic and dynamic modeling based on *in vivo* data, whereas the PBBD model of the present study is based solely on parameters from *in vitro* experimental data. Therefore, the present paper demonstrates for the first time that relatively reliable PBBD model linking doses to DNA adduct formation *in vivo* can be developed based solely on *in vitro* data. This provides a promising complement to the current risk assessment procedure, and may even have a substantial potential in the refinement, reduction or replacement of animals experimentation.

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