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A toxicokinetic study to elucidate 3-hydroxybenzo(a)pyrene atypical urinary excretion profile following intravenous injection of benzo(a)pyrene in rats

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ABSTRACT: The toxicokinetics of benzo(a)pyrene (BaP) and 3-hydroxybenzo(a)pyrene (3-OHBaP) were assessed in 36 male Sprague–Dawley rats injected intravenously with 40 μmol kg^{-1} of BaP to explain the reported atypical urinary excretion profile of 3-OHBaP. Blood, liver, kidney, lung, adipose tissue, skin, urine and feces were collected at t = 2, 4, 8, 16, 24, 33, 48, 72 h post-dosing. BaP and 3-OHBaP were measured by high-performance liquid chromatography/fluorescence. A biexponential elimination of BaP was observed in blood, liver, skin and kidney ($t_{½}$ of 4.2–6.1 h and 12.3–14.9 h for initial and terminal phases, respectively), while a monoexponential elimination was found in adipose tissue and lung ($t_{½}$ of 31.2 and 31.5 h, respectively). A biexponential elimination of 3-OHBaP was apparent in blood, liver and skin ($t_{½}$ of 7.3–11.7 h and 15.6–17.8 h for initial and terminal phases, respectively), contrary to adipose tissue, lung and kidney. In adipose tissue and lung, a monophasic elimination of 3-OHBaP was observed ($t_{½}$ of 27.0 h and 24.1 h, respectively). In kidney, 3-OHBaP kinetics showed a distinct pattern with an initial buildup during the first 8 h post-dosing followed by a gradual elimination ($t_{½}$ of 15.6 h). In the 72-h post-treatment, 0.21 ± 0.09% (mean ± SD) of dose was excreted as 3-OHBaP in urine and 12.9 ± 1.0% in feces while total BaP in feces represented 0.40 ± 0.16% of dose. This study allowed the identification of the kidney as a retention compartment governing 3-OHBaP atypical urinary excretion. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: benzo(a)pyrene; 3-hydroxybenzo(a)pyrene; kinetics; rats; biomonitoring

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are gaseous and particulate pollutants emitted during the incomplete combustion of organic matter. PAH are found in some industrial areas and, at lower levels, in the general environment. Some particulate PAH, characterized by a minimum of four aromatic rings, are of major concern since they are carcinogenic in humans (Boffetta *et al*., 1997; Mastrangelo *et al*., 1996). Among these, benzo(a)pyrene (BaP) has been classified in Group 1 by the International Agency for Research on Cancer (IARC) in 2005 (Straif *et al*., 2005).

Exposure assessment of carcinogenic PAH is a key element in the identification of population groups at increased risk of cancer development. It is now commonly accepted that biomonitoring is the best approach to determine individual exposure to toxicants, such as PAH, for risk assessment (Angerer *et al*., 2007; Jacob and Seidel, 2002). The metabolite of pyrene, 1-hydroxypyrene (1-OHP), is extensively used for PAH biomonitoring, but its usefulness is limited by the fact that it is derived from a non-carcinogenic PAH and that the profiles of PAH emissions vary from one source to another (Maître, 2008). Therefore, 3-hydroxybenzo(a)pyrene (3-OHBaP) has been explored as a biomarker of particular interest since it is the metabolite of BaP, a carcinogenic PAH and one of its major metabolites measured in urine (Bouchard and Viau, 1996; Wang *et al*., 2003). This is a non-negligible aspect considering that BaP metabolites are present in human urine in very small amounts, 1000–10 000-fold less than 1-OHP (Gendre *et al*., 2002), and their urinary detection in humans requires highly sensitive analytical procedures (Barbeau *et al*., 2008; Simon *et al*., 2000).

Urinary 3-OHBaP has already been measured in different populations exposed to PAH occupationally (Forster *et al*., 2008;

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Gendre *et al.*, 2002, 2004; Lafontaine *et al.*, 2004) or non-occupationally (Fan *et al.*, 2006; Godschalk *et al.*, 1998; Lafontaine *et al.*, 2006; Wang *et al.*, 2005). However, a good correlation between BaP atmospheric concentrations and 3-OHBaP urinary concentrations is not always found, due in part to the use of individual protective devices (Maître *et al.*, 2008) but also to the sampling time (Forster *et al.*, 2008). Indeed, 3-OHBaP urinary excretion is relatively slow compared with that of 1-OHP, with maximum excretion lagging by about 15 h that of 1-OHP in PAH exposed workers according to Gendre *et al.* (2004). Accordingly, rat studies showed a maximum urinary excretion rate occurring *ca* 10 h following an intravenous injection of 40 μmol kg^{-1} BaP (Bouchard and Viau, 1996). Interestingly, *trans*-4,5-dihydrodiolBaP (BaP-4,5-diol), another BaP metabolite excreted in urine, exhibited a quasi log–linear and rapid elimination, as observed for 1-OHP, whereas 3-OHBaP excretion profile showed an initial build up followed by a gradual decrease (Bouchard and Viau, 1996). Following intravenous, oral or cutaneous administration of BaP, urinary excretion of BaP-4,5-diol was almost complete 24 h post-dosing in contrast with that of 3-OHBaP, which persisted even after 72 h post-dosing (Bouchard and Viau, 1997).

The underlying mechanism responsible for such a delayed urinary excretion of 3-OHBaP is not known. Bouchard and Viau (1996) showed that phase I biotransformation was not the rate-limiting step in the excretion of this metabolite, since 3-OHBaP excretion kinetics was similar following intravenous injection of the metabolite or the parent compound. This suggests a delay related to the excretion of the metabolite itself. Although the tissue kinetics of BaP have been reported in different studies using ^{14}C-BaP or ^{3}H-BaP administration in rats, no information on kinetics of specific metabolites was obtained with this approach. Metabolites were only determined from the difference between total radioactivity and free BaP quantified by high performance liquid chromatography (HPLC) (Moir *et al.*, 1998; Withey *et al.*, 1993), or by water-soluble volatile tritium counts (Mitchell, 1982, 1983; Uziel and Haglund, 1988). Ramesh *et al.* (2001a, b) determined the time courses of the parent compound BaP and total aqueous and organic metabolites in blood, tissues and excreta of rats following a very high oral dose of BaP (400 μmol kg^{-1}), together with the relative distribution of specific BaP metabolites including 3-OHBaP. However, the specific time courses of the metabolites were not reported and distribution in the kidney was not studied, although it is a potential key tissue responsible for the atypical urinary excretion profile of 3-OHBaP.

Therefore, there is a paucity of data on the blood and tissue distribution and elimination of 3-OHBaP specifically following intravenous injection, which is considered a reference route of exposure, and on the mechanistic comprehension of 3-OHBaP delayed urinary excretion. It is, however, of utmost importance to clarify the excretion mechanisms of this biomarker in order to optimize its use as a disease prevention tool. The understanding of the rate-limiting steps governing the overall excretion dynamic is also necessary for the development of mathematical models enabling the establishment of biological reference values protective of occupationally exposed subjects. It has to be pointed out that published toxicokinetic models are often established from blood concentrations data only, without knowledge of the detailed tissue distribution, which remains essential.

The purpose of the present study is therefore to determine the kinetics of BaP and 3-OHBaP specifically in blood, tissues and excreta of rats, in order to identify the compartments responsible for the slow urinary excretion of 3-OHBaP.

METHODS

Chemicals

BaP (99% purity) was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Reference standards of 3-OHBaP and 3-benzo[a]pyrenyl-β-D-glucopyranosiduronic acid (3-OHBaPGlu) (99% purity) were obtained from the National Cancer Institute (NCI) Chemical Reference Standards distributed by Midwest Research Institute (Kansas, MO, USA). β-Glucuronidase/arylsulfatase (100 000 Fishman U ml^{-1} and 800 000 Roy U ml^{-1} from *Helix pomatia*) was obtained from Roche Diagnostics (Laval, Quebec, Canada). Alkamuls EL-620 was kindly provided by Debro Chemicals and Pharmaceuticals (Dorval, Quebec, Canada). HPLC-grade methanol, ethyl acetate, ascorbic and citric acids as well as thymol were obtained from Fisher Scientific Company (Ottawa, Ontario, Canada) and ammonium formate from Sigma-Aldrich (Oakville, Ontario, Canada).

Animals and Treatment

Thirty-six male Sprague–Dawley rats (Charles River Canada Inc., St-Constant, Quebec, Canada) weighing 260–290 g were used. Lighting was maintained on a 12 h light–dark cycle and room temperature was kept at 22 ± 3°C. Prior to intravenous injection, rats were kept in plastic cages in groups of two; following injection, animals were put in individual metabolic cages. The principles and guidelines of the Canadian Council on Animal Care were followed.

Rats were provided with food and tap water *ad libitum* prior to the experiment. Rats were provided with water containing D-glucose (40 g l^{-1}) *ad libitum* 12 h before intravenous injection and throughout the experiment to induce a polydipsic behavior with associated aqueous diuresis, allowing frequent urine collections (Bouchard *et al.*, 1998). Food was removed 2 h prior to intravenous injection, and then provided 1 h per day throughout the experiment.

Rats received a single intravenous dose of 40 μmol kg^{-1} of BaP. The study was limited to a single dose and route given that the emphasis was on the determination of the detailed time course of BaP and 3-OHBaP in several tissues and excreta. A relatively high dose was also administered to allow the detection of low levels of 3-OHBaP in certain tissues, based on a prior study by Bouchard *et al.* (1998) on the tissue distribution and elimination of pyrene and 1-OHP metabolite in rats. The vehicle used for the injection was a 20% alkamuls:80% isotonic glucose solution. Three milliliters of solution containing BaP was injected per kilogram of body weight.

Sampling

Groups of four rats were euthanized by CO_2 inhalation at $t = 2$, 4, 8, 16, 24, 33, 48 and 72 h post-dosing. An additional group of four rats used as controls received an injection of the vehicle and were euthanized 24 h post-dosing. Total blood (about 8 ml) was withdrawn by cardiac puncture, and several tissues (liver, kidney, perirenal adipose tissue, lung, and abdominal skin) were quickly removed, rinsed with saline, blotted dry and weighed. These tissues were selected for their potential to explain the atypical urinary excretion time course of 3-OHBaP. Urine and feces voided between the time of injection and euthanization were also collected. Thymol was added to the urine collection tubes prior to

sampling. After collection, volumes of urine samples were measured, and feces weighed. All samples were kept on ice before storage at −20 °C until analysis.

Analysis of 3-OHBaP in Urine

Urine samples were analyzed using an analytical procedure adapted from Simon *et al.* (2000). After the urine samples had thawed, they were heated for 30 min at 37 °C to dissolve possible precipitate, and well homogenized. One milliliter of each urine void was then sampled as quickly as possible to avoid residue sedimentation, and diluted 1 : 2 (v/v) with a sodium acetate buffer (0.1 M, pH 5.0). Samples were then incubated for 16 h with 10 µL of β-glucuronidase–arylsulfatase at 37 °C in a shaking bath. Afterward, 200 µL of Triton X-100 R (50 g l^{-1} in methanol) was added to the samples; they were then vortex-mixed and transferred to HPLC vials prior to analysis.

The HPLC system consisted of a Model AS-100 automatic injector (Bio-Rad, Richmond, CA, USA), and a 1100 series apparatus equipped with two quaternary pumps, three automated switching valves and a fluorescence detector from Agilent Technologies (Mississauga, Ontario, Canada). The fluorescence detector was set to excitation and emission wavelengths of 382 and 441 nm, respectively, and the detector signal was recorded with ChemStation software.

Three purification columns, C1, C2 and C3, were used for the on-line extraction of 3-OHBaP and corresponded to a C_8 column (50 × 3.0 mm, 5 µm) from Interchim (Montluçon, France), a Spherisorb OD/CN column (50 × 3.2 mm, 5 µm), and a phenyl column (75 × 3.2 mm, 5 µm), respectively. C2 and C3 were kindly prepared by P. Simon from the Institut National de Recherche et de Sécurité (INRS). The analytical column was a C_{18} Grace Vydac column (150 × 3.2 mm, 5 µm). The temperature of the purification columns was set at 45°C. Urine analysis was performed in 40 min using a 60% methanol (citric acid 5 mM, ascorbic acid 10 mg l^{-1})–40% water (citric acid 5 mM, ascorbic acid 10 mg l^{-1}) mobile phase for the purification columns, and a 90% methanol (citric acid 5 mM, ascorbic acid 10 mg l^{-1})–10% water (citric acid 5 mM, ascorbic acid 10 mg l^{-1}) mobile phase for the analytical column. Ascorbic acid was added to the mobile phase in accordance with Bouchard *et al.* (1994). Elution was performed in isocratic mode at a flow rate of 0.5 ml min^{-1}. The injection volume was 100 µL and samples were kept at 10 °C on the injection tray. The limit of detection of the method was 5 fmoles of 3-OHBaP injected onto the column, corresponding to 100 pmol/L of urine using a 1 mL sample. The quantification was performed using an external calibration curve of 3-OHBaPGlu prepared in blank rat urine prior to enzymatic hydrolysis.

Analysis of BaP and 3-OHBaP in Blood, Tissues and Feces

Blood, tissues and feces were analyzed using a method adapted from Bouchard *et al.* (1998). A 12.5% (w/v) homogenate of the liver, a 6.25% (w/v) homogenate of the kidney, of the adipose tissue and of all the feces voided by a given rat, as well as a 5% (w/v) homogenate of the lung, were prepared in a sodium acetate buffer (0.1 M, pH 5.0). The homogenate of adipose tissue was mixed with a 5% (w/v) homogenate of control liver in order to stabilize 3-OHBaP in this tissue. Skin samples required a special homogenization procedure. A circular punch of 17.5 mm i.d. was performed in skin with a cork borer. The skin punch was immediately frozen at −20 °C and then in liquid nitrogen; this was followed by grinding in a stainless steel mortar previously cooled in liquid nitrogen. From the resulting skin powder, a 5% (w/v) homogenate was prepared.

Aliquots of 4 ml of each homogenate were transferred in Pyrex tubes. For blood, samples of 500 µL were buffered with 3.5 ml of sodium acetate buffer (0.1 M, pH 5.0). Liver, lung and kidney homogenates were heated at 90 °C in a water bath for 5 min. Blood, tissue and fecal homogenates were incubated with 30 µL of β-glucuronidase-arylsulfatase at 37 °C in a shaking bath, 16 h for liver and feces, 1 h for lung, kidney and blood, and 30 min for adipose tissue and skin. Samples were then extracted twice with 4 ml of ethyl acetate saturated with water, shaken for 30 min, and centrifuged for 30 min at 3000 rpm (4 °C). The organic extracts were combined and evaporated under a gentle nitrogen stream at 40 °C. The residue was redissolved in 1 ml of methanol and analyzed with the HPLC system described previously, but using only an analytical column from Supelco (250 × 4.6 mm, 5 µm) without purification columns. Elution was performed in 35 min at a flow rate of 1 ml min^{-1} with a 80 : 20 to 100 : 0 methanol (ascorbic acid 1 mg l^{-1})–water (ammonium formate 5 mM, pH 3.5) gradient over 20 min. For calibration, a standard curve in pure methanol was used. The analytical limits of detection ranged from 11 to 22 injected fmol for BaP and 4 to 13 injected fmol for 3-OHBaP.

Efficiency of the extraction of BaP and 3-OHBaP was determined using untreated rat blood, tissue and feces samples spiked with BaP and 3-OHBaPGlu authentic reference standards, before incubation with 30 µL of β-glucuronidase-arylsulfatase at 37 °C. Recovery of BaP from spiked samples was 127 ± 22% in blood, 87 ± 15% in liver, 110 ± 19% in kidney, 125 ± 27% in lung, 99 ± 11% in feces, 93 ± 6% in skin, and 82 ± 8% in adipose tissue (mean ± SD for 24 samples, i.e. four independent series of two levels of spiking in triplicate, within the expected concentration range for blood, tissues and excreta). Recovery of 3-OHBaP from spiked samples was 65 ± 14% in blood, 65 ± 11% in liver, 95 ± 14% in kidney, 80 ± 13% in lung, 78 ± 11% in feces, 70 ± 14 % in skin, and 71 ± 9% in adipose tissue (mean ± SD for 24 samples, i.e. four independent series of two levels of spiking in triplicate, within the expected concentration range for blood, tissues and excreta). Results presented herein were adjusted for recovery.

BaP recoveries in blood, lung and kidney exceeded 100% due to the steeper slope of the relation between peak height and BaP concentration in methanolic extracts of tissues compared with that obtained with pure methanol and used for calibration. Concerning 3-OHBaP, too long an enzymatic hydrolysis period decreased the recovery of 3-OHBaP in some of the tissues. The hydrolysis time was thus reduced from 16 h (as routinely used for urine) to 1 h in lung, kidney and blood. 3-OHBaP recovery was lower in adipose tissue and only a 30 min hydrolysis was thus performed.

Toxicokinetic Analysis

Elimination rate constants of BaP and 3-OHBaP in blood and tissues were determined by least square fit adjustments of the following general functions to the experimental average time course data, using the algorithm *genfit* included in MathCad (MathCad 14.0, Parametric Technology Corporation, Needham, MA, USA); this function uses an optimized version of the Levenberg–Marquardt method for least square curve fitting (Gill and Murray, 1978). Henceforth, monophasic elimination was represented by the equation $C(t) = C_0 e^{-Kt}$, where C_0 is the

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concentration at time zero and K is the elimination rate constant, and the apparent elimination half-life ($t_{½}$) was calculated using the equation $t_{½} = 0.693\ K^{-1}$. Biphasic elimination was represented by the equation $C(t) = Ae^{-\alpha t} + Be^{-\beta t}$ where A and B are the preexponential coefficients and α and β are the hybrid rate coefficients for the initial and terminal phases, respectively. Apparent $t_{½}$ values were calculated for the initial and terminal elimination phases using the equation $t_{½}\alpha = 0.693/\alpha$ and $t_{½}\beta = 0.693/\beta$, respectively.

From the time course curves of BaP and 3-OHBaP in blood, other calculated parameters include the discrete version of the area under the concentration-time curve (AUC), the area under the first moment of concentration–time curve (AUMC), the clearance from blood (CL), the apparent volume of distribution (V_β) and the mean residence time (MRT) calculated as:

$$\mathrm{AUC} = \frac{1}{2}\sum_{\forall i}(t_i - t_{i+1})\left[C(t_i) + C(t_{i+1})\right]$$

$$\mathrm{AUMC} = \frac{1}{2}\sum_{\forall i}(t_i - t_{i+1})\left[t_i C(t_i) + t_{i+1} C(t_{i+1})\right]$$

$$\mathrm{CL} = \frac{\mathrm{Dose}}{\mathrm{AUC}}$$

$$V_\beta = \frac{\mathrm{CL}}{\beta}$$

$$\mathrm{MRT} = \frac{\mathrm{AUMC}}{\mathrm{AUC}}$$

RESULTS

Time Courses of BaP in Blood and Tissues

The time courses of BaP in blood and tissues (expressed as a percentage of administered dose) over the 72 h period following intravenous injection of BaP (40 µmol kg^{-1}) in rats are presented in Fig. 1 and Table 1. Blood, liver, kidney and skin profiles evolved in parallel over the studied period. Following tissue distribution (≤4 h post-injection), two apparent elimination phases were observed in blood and these tissues, with an initial more rapid phase (≤24 h period following injection) followed by a slower phase (≥24 h post-treatment). Mean apparent elimination half-lives of 6.1, 5.0, 4.2 and 5.8 h were calculated for the initial phase in blood, liver, kidney and skin, respectively; corresponding half-life values for the terminal phase were 12.5, 14.9, 13.9 and 12.3 h (Table 2). In adipose tissue and lung, a mono-exponential elimination of BaP was observed and $t_{½}$ values of 31.2 and 31.5 h were calculated for these tissues, respectively (Table 2). Table 3 also shows AUC, AUMC, CL, V_β and MRT of BaP calculated from the average blood time course curve.

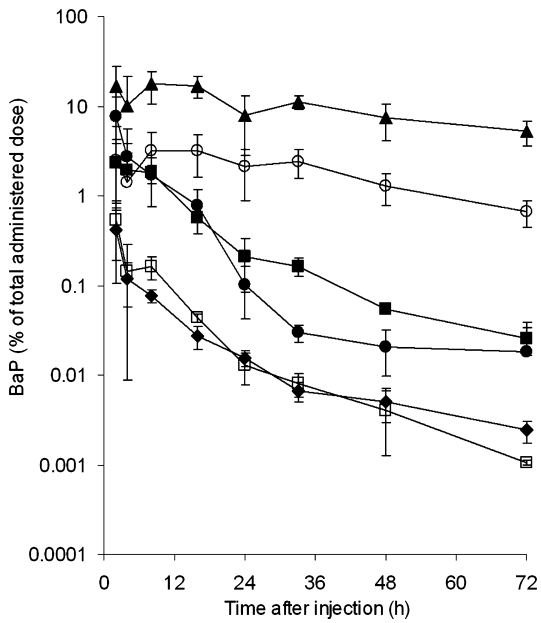

Figure 1. Time courses of BaP in blood and tissues (expressed as a percentage of administered dose) following intravenous injection of 40 µmol kg^{-1} of BaP in male Sprague–Dawley rats. Each point represents mean and vertical bars are standard deviations (n = 4). ▲, Lung; ○, adipose tissue; ■, liver; ●, skin; □ kidney; ◆, blood.

At 2 h post-dosing, BaP was highly predominant in lung, representing 17% of injected dose, and to a lesser extent in skin, adipose tissue and liver. Then, BaP decreased more rapidly in skin and liver than in lung and adipose tissue, resulting in BaP being found in highest percentages in lung and adipose tissue from 8 to 72 h post-dosing. At all time points, the percentage of injected dose recovered as BaP was highest in lung.

Time Courses of 3-OHBaP in Blood and Tissues

Figure 2 and Table 1 depict the time courses of 3-OHBaP in blood and tissues (expressed as a percentage of administered dose) over the 72 h period following intravenous injection of BaP (40 µmol kg^{-1}) in rats. In liver, peak levels of 3-OHBaP were observed between 2 and 4 h post-dosing, reflecting its relatively rapid rate of formation. The time profiles of 3-OHBaP in blood, liver and skin readily evolved in unison within 8 h post-dosing. A biexponential elimination of 3-OHBaP was apparent for blood and these tissues, with a more rapid elimination rate (≤24 h post-dosing) followed by a slower rate (≥24 h post-injection). Apparent $t_{½}$ values of 7.3, 11.0 and 11.7 h were calculated for the initial phase in blood, liver and skin, respectively; corresponding half-life values for the terminal phase were 16.9, 17.8 and 16.1 h (Table 2). Table 3 also shows the AUC, AUMC, CL, V_β and MRT of 3-OHBaP calculated from the average blood time course curve, indicating a quite similar blood disposition and clearance of BaP and 3-OHBaP.

In the kidney, 3-OHBaP showed a distinct pattern with a buildup during the first 8 h post-dosing followed by a gradual decrease. However, the time course of 3-OHBaP in the kidney appeared to reach an equilibrium with blood after 16 h, and showed an apparent elimination half-life of 15.6 h for the 24–72 h period, thus similar to that of blood, liver and skin (Table 2). In the lung, as for BaP, a monophasic elimination of 3-OHBaP was observed with a half-life of 24.1 h (Table 2). In adipose tissue, following a relatively slow uptake of 3-OHBaP (peak levels at 8 h post-dosing), a monophasic elimination was observed with an apparent half-life value of 27.0 h.

Until 8 h post-injection, 3-OHBaP was predominantly found in liver, skin and kidney. From 8 to 72 h post-injection, kidney levels of 3-OHBaP exceeded those of other tissues and accounted for up to 0.65% of the dose at 8 h post-dosing. Contrary to BaP, low percentages of BaP dose were found as 3-OHBaP in the lung at all time points.

Time Courses of BaP and 3-OHBaP in Urine and Feces

The cumulative urinary and fecal excretion time courses of BaP and 3-OHBaP following BaP injection (all voids collected over 2, 4, 8, 16, 24, 33, 48, 72 h post-dosing and expressed as a

Table 1. Mean percentage of administered dose recovered as BaP in blood, tissues and excreta at the different sampling times (2, 4, 8, 16, 24, 33, 48, and 72 h post-dosing) following intravenous administration of 40 μmol kg^{-1} of BaP in male Sprague–Dawley rats

Tissues and biological fluids	Mean percentage of dose in tissues and biological fluids (n = 4)															
	BaP								3-OHBaP							
	Time after injection (h)								Time after injection (h)							
	2	4	8	16	24	33	48	72	2	4	8	16	24	33	48	72
Blood	0.4216	0.1178	0.0760	0.0274	0.0156	0.0066	0.0050	0.0024	0.1051	0.0863	0.0912	0.0388	0.0235	0.0142	0.0084	0.0047
Kidney	0.5300	0.1451	0.1636	0.0435	0.0127	0.0080	0.0040	0.0010	0.1954	0.4290	0.6504	0.5829	0.3466	0.2931	0.0936	0.0512
Liver	2.3071	1.9086	1.7928	0.5739	0.2102	0.1646	0.0546	0.0254	0.4897	0.5879	0.1405	0.0819	0.0528	0.0505	0.0149	0.0096
Lung	17.0251	10.1916	17.5873	16.9215	8.0200	11.1726	7.3491	5.1808	0.0230	0.0236	0.0176	0.0153	0.0102	0.0085	0.0047	0.0045
Adipose tissue	2.4732	1.4033	3.2298	3.1947	2.1066	2.3975	1.2693	0.6633	0.0241	0.0381	0.0582	0.0348	0.0330	0.0322	0.0192	0.0114
Skin	7.5588	2.7401	1.7023	0.7833	0.1023	0.0296	0.0205	0.0180	0.3845	0.2315	0.1346	0.1032	0.0410	0.0215	0.0148	0.0073
Urine	–	–	–	–	–	–	–	–	0.0001	0.0022	0.0119	0.0604	0.0680	0.1897	0.1493	0.2087
Feces	0.0005	0.0020	0.0127	0.0779	0.3507	0.3823	0.3539	0.3966	0.0001	0.0004	0.0035	0.4707	4.7078	7.7161	8.7544	12.8652
Total	30.3164	16.5085	24.5646	21.6222	10.8181	14.1612	9.0565	6.2875	1.2220	1.3990	1.1079	1.3879	5.2831	8.3257	9.0592	13.1625

percentage of administered dose) are shown in Fig. 3 and Table 1. On average, at 72 h, 12.9 ± 1.0% and 0.40 ± 0.16% of the administered dose was recovered as 3-OHBaP and BaP in feces, respectively, and 0.21 ± 0.09% as 3-OHBaP in urine. BaP was therefore mainly excreted in feces in the metabolized form.

BaP was excreted more rapidly than 3-OHBaP since 88% of total BaP excretion in feces occurred in the first 24 h post-dosing, in comparison to 37 and 33% during the same period of time for 3-OHBaP in feces and urine, respectively.

When considering blood, tissues and excreta all together, the molar fraction of injected dose recovered in total as BaP and 3-OHBaP reached a maximum of 31% at 2 h post-dosing and still represented 19% at 72 h post-dosing. The fraction of injected dose recovered as 3-OHBaP until 16 h post-dosing was smaller than that of BaP and did not exceed 1.4%. Between 24 and 72 h post-dosing, that fraction increased from 5.3 to 13.2% of injected dose, mainly due to 3-OHBaP appearance in expelled feces, and eventually exceeded total amounts of BaP found in the analyzed biological samples for the same sampling period (Table 1).

DISCUSSION

This study is the first to report the toxicokinetics of 3-OHBaP specifically in rat blood, excreta and tissues, including kidney, following intravenous injection of BaP. Our study showed that BaP was rapidly distributed throughout the body, peak concentrations occurring in blood and all tissues in the first 2 h post-dosing. This is in accordance with the observations of Kotin *et al.* (1959), who reported that less than 1% of administered dose was recovered as BaP in blood 10 min after an intravenous injection of the parent compound. BaP was excreted mainly in feces, in the metabolized form, with 3-OHBaP excretion in feces being 30 times higher than BaP fecal excretion, as also determined by Van Schooten *et al.* (1997) in rats over a 6-day period following an oral administration of BaP. Although 3-OHBaP was the only metabolite investigated in our study, its urinary and fecal excretion reached on average 13% of injected dose 72 h post-dosing. Similar to our results, Tzekova *et al.* (2004) reported that the mean fraction of BaP dose recovered as 3-OHBaP in feces varied between 3.1 and 13.7% in rats intraperitoneally exposed to a mixture of BaP and pyrene once daily for 10 consecutive days, depending on the dose and the ratio of pyrene and BaP in the mixture.

In urine, the small fraction of injected dose recovered as 3-OHBaP over the 72-h period post-dosing, 0.21 ± 0.09%, is in agreement with previous results from Bouchard and Viau (1996), indicating that 0.1% of dose was excreted in rat urine during the 24-h period following an intravenous injection of 40 μmol kg^{-1} of BaP. The same order of magnitude for 3-OHBaP excretion in urine, 0.4 and 0.6% of total administered dose, was obtained in rats over a 21-day period following an oral and cutaneous exposure to 40 μmol kg^{-1} of BaP, respectively (Godschalk *et al.*, 2000).

These results translate into a ratio of *ca* 60 between fecal and urinary excretion of 3-OHBaP. This ratio was found by other authors to vary depending on the exposure dose of BaP. In particular, Tzekova *et al.* (2004) reported that the ratio of 3-OHBaP in feces compared with urine over a 24-h period post-dosing ranged from 9 to 48 in rats exposed intraperitoneally once a day for 10 consecutive days to a mixture of BaP and pyrene, depending on the dose and the BaP-to-pyrene ratio.

As for the molar fraction of injected dose recovered in total as BaP and 3-OHBaP in blood, tissues and excreta in the current

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Table 2. First-order elimination half-lives of BaP and 3-OHBaP in blood and tissues following intravenous injection of 40 μmol kg^{-1} of BaP in male Sprague–Dawley rats

	Mean first-order elimination half-life (h) (n = 4)							
	BaP				3-OHBaP			
Tissue	Initial phase[a]	R^{2b}	Terminal phase[c]	R^{2b}	Initial phase[a]	R^{2b}	Terminal phase[c]	R^{2b}
Blood	6.1	0.99	12.5	0.91	7.3	0.99	16.9	0.97
Liver	5.0	0.99	14.9	0.97	11.0	0.99	17.8	0.88
Kidney	4.2	0.99	13.9	0.99	–	–	15.6	0.95
Skin	5.8	0.97	12.3	0.89	11.7	0.90	16.1	0.94
Adipose tissue	31.2[d]	0.91	–	–	27.0[d]	–	0.91	–
Lung	31.5[d]	0.81	–	–	24.1[d]	–	0.96	–

[a]BaP and 3-OHBaP elimination kinetics in blood and tissues appeared biphasic and reported $t_{½}$ values for the initial phase (corresponding to the elimination rate α) were calculated over the 8–24 h period post-dosing.

[b]R^2 is the coefficient of determination defined by:

$$R^2 = 1 - \frac{\sum_{\forall i}\left(y_i^{\exp} - y_i^{theo}\right)^2}{\sum_{\forall i}\left(y_i^{\exp} - \overline{y}_i^{\exp}\right)^2}$$

where the superscript 'exp' refers to the experimental data points while 'theo' is related to model prediction of data points at time i.

[c]Terminal phase $t_{½}$ values (corresponding to the elimination rate β) for BaP and 3-OHBaP elimination kinetics were obtained for the 24–72 h period.

[d]Elimination kinetics of BaP and 3-OHBaP appeared monophasic in adipose tissue and lung after the distribution phase, and reported $t_{½}$ values were calculated for the 8–72 h

Table 3. Toxicokinetic parameters for BaP and 3-OHBaP in blood following intravenous injection of 40 μmol kg^{-1} of BaP in male Sprague–Dawley rats

	First-order toxicokinetic values	
Model parameters[a]	BaP	3-OHBaP
AUC[b] [(nmol × h ml^{-1}) kg^{-1}]	48.7	41.9
AUMC[c] [(nmol × h^2 ml^{-1}) kg^{-1}]	498.0	720.1
CL[d] (ml h^{-1})	821.0	954.8
V_β[e] (l)	0.25	0.39
MRT[f] (h)	10.2	17.2

[a]Toxicokinetic parameters were calculated from the average time course curves.

[b]The discrete version of the area under the curve is calculated as: $\mathrm{AUC} = \frac{1}{2}\sum_{\forall i}(t_i - t_{i+1})\left[C(t_i) + C(t_{i+1})\right]$.

[c]The area under the first moment of concentration–time curve is approximated by:

$$\mathrm{AUMC} = \frac{1}{2}\sum_{\forall i}(t_i - t_{i+1})\left[t_i C(t_i) + t_{i+1} C(t_{i+1})\right].$$

[d]With the dose being 40 μmol kg^{-1}, the clearance from blood was determined by: CL = dose/AUC.

[e]The volume of distribution was computed by: $V_\beta = \mathrm{CL}/\beta$.

[f]The mean residence time is defined as: MRT = AUMC/AUC.

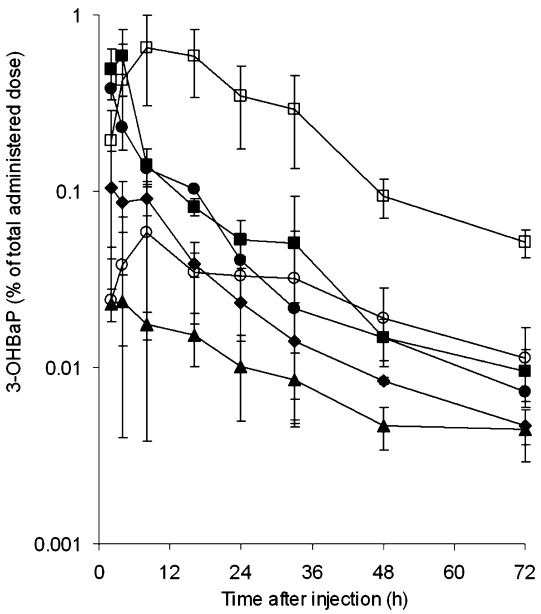

Figure 2. Time courses of 3-OHBaP in blood and tissues (expressed as a percentage of administered dose) following intravenous injection of 40 μmol kg^{-1} of BaP in male Sprague–Dawley rats. Each point represents mean and vertical bars are standard deviations (n = 4). □, Kidney; ■, liver; ●, skin; ▲, lung; ○, adipose tissue; ◆, blood.

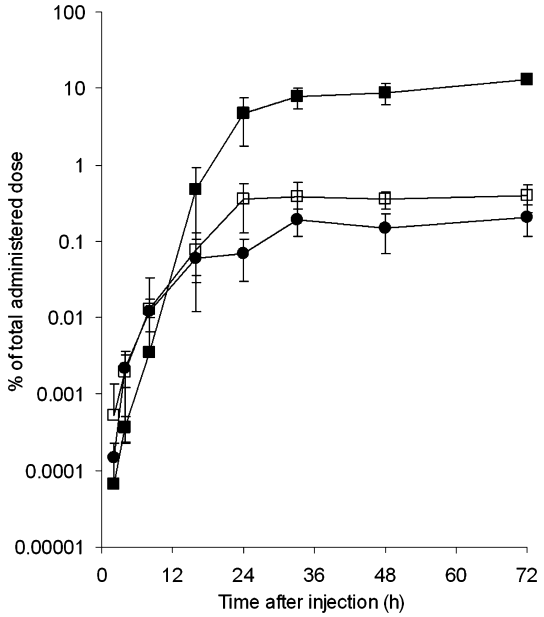

Figure 3. Cumulative excretion time courses of 3-OHBaP in urine and BaP and 3-OHBaP in feces, expressed as percentages of total administered dose (all voids collected over 2, 4, 8, 16, 24, 33, 48, 72 h post-dosing), following intravenous injection of 40 μmol kg^{-1} of BaP in male Sprague–Dawley rats. Each point represents mean value observed in separate groups of rats and vertical bars are standard deviations ($n = 4$). ■, 3-OHBaP in feces; □, BaP in feces; ●, 3-OHBaP in urine.

study, it reached a maximum of 31%, which might be explained by the fact that only the 3-OHBaP metabolite was quantified and not all tissues were monitored. Indeed, the metabolite 3-OHBaP specifically was the focus of the study, whereas other metabolites are known to result from BaP biotransformation (Xue and Warshawsky, 2005). Furthermore, other organs such as reproductive organs, for instance, are known to capture important amounts of BaP because of their lipid contents but they were not included in our study since they were not primarily suspected of being responsible for 3-OHBaP body retention (Ramesh *et al.*, 2001a).

With regard to the distribution of BaP in tissues, at all time points, the highest levels of unmetabolized compound were found in lung. The second tissue most concentrated in BaP was the adipose tissue, except for the two first sampling time points where liver and skin concentrations were either higher or similar. BaP in lung and adipose tissue followed very similar time profiles; its elimination in these tissues was monophasic over the 72 h period post-dosing and the calculated apparent elimination half-lives, 31.5 and 31.2 h respectively, were longer than those found for the initial elimination phase of BaP in blood and other tissues (liver, kidney and skin), that is 4.2–6.1 h over the 24 h period post-dosing. Following an intravenous injection of 24 or 60 μmol kg^{-1} of ^{14}C-BaP in rats, Moir *et al.* (1998) also found that BaP concentrated mostly in lung over a 2–32 h period following intravenous injection and the adipose tissue was the second tissue most concentrated in BaP. Similar to our findings, these authors also found that BaP elimination from the lung was slower than in blood, kidney, liver and adipose tissue. A slow elimination of BaP from the lung was further reported by Mitchell (1982) in rats after inhalation of ^{3}H-BaP. In accordance with our results, an apparent BaP elimination half-life of 50 h was calculated by these authors over the 25–56 h period post-dosing. The fact that BaP concentrated in adipose tissue can be linked directly to its lipophilicity given the aromatic structure of the molecule while BaP concentration in the lung may be explained by the fact that it receives total cardiac output and is therefore the first organ, after the heart, to receive total iv dose injection (Moir *et al.*, 1998). Indeed, after oral administration of BaP in rats, Ramesh *et al.* (2001a) showed no such accumulation in lung; in the latter study, BaP levels were similar in lung and liver while plasma was more concentrated in BaP.

On the other hand, in blood, liver, skin and kidney, BaP elimination curves evolved in parallel, indicating that a quasi-steady state equilibrium was rapidly reached (within 2 h post-dosing) between blood and these tissues. A biexponential elimination of BaP was observed in these matrices with an initial more rapid phase (≤24 h following injection) with an average half-life value of 5.3 followed by a slower phase (≥24 post-dosing) with a corresponding average half-life value of 13.4 h. The more rapid phase appears to reflect the renal clearance and biliary elimination rate of rapidly distributed and metabolized BaP, while the terminal elimination phase is most plausibly driven by the slower elimination of BaP stored in adipose tissue or concentrated in lung. The BaP apparent terminal elimination half-life value of 12.5 h in blood, corresponding to a β value of 0.0092 min^{-1}, is in accordance with the β value of 0.0017 min^{-1} found by Moir *et al.* (1998) in male Wistar rats following intravenous injection of 60 μmol kg^{-1} of BaP. The apparent volume of distribution of BaP obtained in our study (250 ml) was also in the same value range as the one reported by the latter authors (486 ml). It was also compatible with the volume of distribution of 238 ml obtained for pyrene in rats following intravenous injection of the parent compound (Bouchard *et al.*, 1998). This high volume of distribution indicates that BaP is extensively distributed in tissues when compared with the average rat blood volume of 18 ml in our study. Weyand and Bevan (1986) determined a slightly higher volume of distribution of BaP (1.29 l) in canulated male Sprague–Dawley rats intravenously exposed to 1 μg kg^{-1} of BaP, but BaP clearance calculated by these authors (822 ml h^{-1}) was very similar to our value of 821 ml h^{-1}.

The slower elimination of BaP in the lung compared with other tissues, such as the liver, may be explained by a retention of the parent compound in lung components, and/or by a slower monooxygenase (phase I) metabolism of BaP in the lung compared with the liver (Mitchell, 1983; Prough *et al.*, 1979). Nevertheless, in isolated perfused rat lung and liver, although hepatic metabolism was found to be more rapid than lung metabolism, overall pulmonary metabolism (on a unit weight basis) was shown to be more efficient than hepatic metabolism and free metabolites and especially free diols and phenols were found in higher amounts in the lung than in the liver (Mollière *et al.*, 1987). It appears that phase I biotransformation by monooxygenases might be less rapid in the lung than in the liver but eventually as efficient while phase II conjugation in the lung may be limited. This could explain in part the carcinogenicity of BaP in lung due to a high adduction level of BaP metabolites to lung DNA and macromolecules, including BaP diol-epoxide ultimate carcinogen. In fact, Tzekova *et al.* (2004) observed more protein adducts in the lung than in the liver and blood following a repeated intraperitoneal injection of a mixture of BaP and pyrene over 10 days. Similarly, Godschalk *et al.* (2000) found more DNA adducts in the lung compared with white blood cells, whatever the route of exposure to BaP (intratracheal, dermal and oral).

Further characterization of specific protein or DNA adducts of BaP, using a quantification method such as the one developed by Marie *et al.* (2007), would be necessary to elucidate the proportion of BaP bound to macromolecules in lung in comparison with other organs.

As for the specific disposition of 3-OHBaP, biphasic eliminations were obtained in blood, liver and skin, with the mean terminal elimination rate for these tissues ($t_{½} \approx 17$ h) appearing to be driven by the elimination rate of BaP stored in adipose tissue or concentrated in the lung ($t_{½} \approx 31$ h). The toxicokinetic parameters for 3-OHBaP in blood were similar to those obtained for BaP.

In the lung, the elimination rate of BaP also appears to govern the overall elimination rate of 3-OHBaP in that tissue ($t_{½} \approx 24$ h). In adipose tissues, given their slow perfusion, a gradual uptake of 3-OHBaP was observed from 2 to 8 h post-dosing where maximum excretion was reached, and was followed by an elimination phase with a $t_{½}$ of 27.0 h; the accumulation of 3-OHBaP in adipose tissue reflects the lipophilicity of the metabolite itself even after monohydroxylation of the parent compound.

In the particular case of the kidney, 3-OHBaP time course presented a distinct pattern with a significant buildup from 2 to 8 h post-dosing followed by a gradual decrease. This is probably due to a distinct retention phenomenon of 3-OHBaP in this organ. This is the most important aspect of this study, which was primarily designed to provide insights into the phenomenon governing the atypical excretion kinetics of 3-OHBaP in urine reported in the past (Bouchard and Viau, 1995; Gendre *et al.*, 2004). Previous results suggested that this may be due to a retention of 3-OHBaP in the body. In our study, the time course of 3-OHBaP urinary excretion rate, expressed as pmol excreted per hour, could not be obtained because rats were different at each sampling time. However, Bouchard and Viau (1996) documented such profile in the same experimental conditions, their study being designed to obtain the urinary excretion profile with the same rats over a 96-h period following intravenous injection of 40 µmol kg^{-1} of BaP. Interestingly, the profile of 3-OHBaP in the kidney obtained in our study perfectly matched the urinary excretion time course previously documented by Bouchard and Viau (1996) (not shown), confirming the role of the kidney in the retention phenomenon.

Our results are in accordance with previous findings on the toxicokinetics of labelled BaP. Both following intravenous injection of ^{14}C-BaP (Moir *et al.*, 1998) or inhalation of ^{3}H-BaP (Mitchell, 1982; Withey *et al.*, 1993), radioactivity peaked in kidney 4 and 6 h post-dosing, respectively. This increase was not linked to free BaP but to metabolites determined as the difference between total radioactivity and free BaP (Moir *et al.*, 1998; Withey *et al.*, 1993). The retention seems specific to 3-OHBaP since no similar retention was observed in kidney with 1-OHP after intravenous administration of pyrene in rats (Bouchard *et al.*, 1998). Moreover, Bouchard and Viau (1996) reported that the urinary excretion kinetics of BaP-4,5-diol was monotonous in contrast to 3-OHBaP urinary excretion time course. This would suggest that no such retention is occurring for other BaP metabolites.

The retention of 3-OHBaP in the kidney may be explained by a delayed active tubular secretion of the conjugated form of this metabolite in proximal tubules (Gosselin *et al.*, 2005). Indeed, 3-OHBaP is excreted in urine in the glucurono- and sulfo-conjugated form and is transferred from blood to urine by organic anion transporters present on both the basolateral and apical membrane of the proximal tubules (Van Aubel *et al.*, 2000; Wright and Dantzler, 2004). These secreted molecules thus have to actively cross the basolateral membrane of proximal tubules to enter the intracellular medium and then again actively cross the apical membrane to be excreted in the tubular lumen. Since organic anion transporters are different between the basolateral and the apical membranes (Van Aubel *et al.*, 2000), the transfer of conjugated 3-OHBaP from blood to the tubules may be more efficient than the secretion from the tubules to the lumen, resulting in a transitory accumulation in the proximal tubules and therefore in the kidney. If urinary excretion of 3-OHBaP occurred mainly by glomerular filtration, the toxicokinetic profiles in blood and urine would be similar, which is not the case.

Overall, the current study will serve as a basis for the development of a toxicokinetic model for BaP and 3-OHBaP, which is a key tool for the determination of biological reference values protective of PAH exposed workers.

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