Arch Toxicol (2004) 78: 351–361
DOI 10.1007/s00204-003-0539-7

GENOTOXICITY

Adela Tzekova · Ross Thuot · Claude Viau

Correlation between biomarkers of polycyclic aromatic hydrocarbon exposure and electrophilic tissue burden in a rat model

Received: 25 June 2003 / Accepted: 16 December 2003 / Published online: 29 January 2004

Abstract This study was aimed at investigating the correlation between biomarkers of exposure to polycyclic aromatic hydrocarbons and, more specifically, at examining the role of urinary 1-hydroxypyrene (1-OHP) as a reliable measure of internal dose linked to the electrophilic tissue burden (ETB), assessed as covalent binding of the ultimate carcinogen benzo(*a*)pyrene diolepoxide (BaPDE) with cellular proteins in target organs. The protocol included experimental verification of a previously proposed algorithm for adjustment of reference values for urinary 1-OHP with exposure to different mixtures of polycyclic aromatic hydrocarbons in a rat model. Hence, the relationships between ETB in liver, lung, and heart as well as the BaPDE–haemoglobin adducts level on the one hand, and urinary/faecal 1-OHP or urinary/faecal 3-hydroxybenzo(*a*)pyrene (3-OHBaP) on the other hand have been examined. Male Sprague-Dawley rats received intraperitoneally, once daily for 10 consecutive days, binary mixtures of benzo(*a*)pyrene (BaP) and pyrene (P) in three different exposure scenarios corresponding to BaP/P ratios of 0.2, 1 and 5, with three doses of BaP (2, 6 and 20 mg/kg) for each scenario. The ETB levels were measured as the ultimate analyte benzo(*a*)pyrene tetrol (BaPTeT) obtained after mild acid hydrolysis of BaPDE adducts with proteins. It was experimentally confirmed that: (1) urinary 1-OHP is a reliable biomarker linked to the ETB in tissues that are targets for carcinogenicity, such as lung, for the BaP/P ratios of 0.2 and 1 (linear regression $p = 0.0099$ and 0.0293, respectively); (2) urinary 3-OHBaP is correlated with the BaPDE–haemoglobin adducts for all three exposure scenarios ($p = 0.0011$ for BaP/P = 0.2, $p < 0.0001$ for BaP/P = 1 and $p = 0.0099$ for BaP/P = 5). The experimental relationship between ETB and urinary 1-OHP was used to interpolate biological limit values for the urinary metabolite assuming three arbitrary critical levels of ETB. These were compared with the values calculated from the algorithm using the BaP/P ratio 1 mixture as a reference. The ratios of calculated to observed values varied from 1.0 to 1.6 for the BaP/P 0.2 mixture, and from 1.9 to 3.0 for the BaP/P 5 mixture. The results obtained in the present study indicate that the algorithm mentioned above applies well for two of the three exposure scenarios corresponding to realistic occupational BaP/P ratios of 0.2 and 1. This suggests that, using ETB as an endpoint, the proposed algorithm will reasonably predict the critical value of urinary 1-OHP for mixtures having different BaP/P ratios. Stronger linear relationships between ETB in all chosen tissues and 1-OHP or 3-OHBaP excretion were obtained with urinary metabolites than with their faecal analogues. Thus urinary 1-OHP and 3-OHBaP are more reliable biomarkers in biological monitoring strategies.

Keywords Benzo(*a*)pyrene · 3-Hydroxybenzo(*a*)pyrene · Benzo(*a*)pyrene diolepoxide · Polycyclic aromatic hydrocarbon · Pyrene · 1-Hydroxypyrene · Electrophilic tissue burden

A. Tzekova · R. Thuot · C. Viau (✉)
Département de santé environnementale et santé au travail, Université de Montréal, PO Box 6128, Station Centre-ville, Montréal, QC H3C 3J7, Canada
E-mail: claude.viau@umontreal.ca
Fax: +1-514-3432200

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants resulting from pyrolysis or incomplete combustion of organic materials and recognized as causative for lung and skin cancer (IARC 1983). They are present as complex mixtures with a prevalence of phenanthrene, benzo(*b*)fluoranthene, fluoranthene, pyrene and benzo(*a*)pyrene among all of the PAHs measured in different work environments. Many PAH metabolites have been studied to assess the "correlation" between external and internal PAH exposure and to investigate their use as probable biomarkers in biological monitoring (Ovrebo et al. 1994; Angerer et al. 1997; Popp et al. 1997; Gündel et al. 2000). Among all

PAHs encountered in the work environments benzo(*a*)pyrene (BaP) and pyrene (P) are the most extensively studied. However, neither pyrene nor its metabolites have been documented as being carcinogenic to humans (IARC 1983, 1987). On the contrary, BaP is classified as a probable human carcinogen (IARC 1983), which is metabolically activated by cytochrome P450 to electrophilic intermediates that bind covalently to cell macromolecules. In this respect, the most important BaP electrophilic metabolite is (+)anti-benzo(*a*)pyrene-7, 8-dihydrodiol-9,10-epoxide (benzo(*a*)pyrene diolepoxide, abbreviated as BaPDE), known as the ultimate BaP-derived human and animal carcinogen, which exerts its genotoxic activity through covalent binding to DNA with the formation of BaPDE–DNA adducts, consequently leading to gene mutations (Poirier and Beland 1992; Peltonen and Dipple 1995).

Recent work suggests that urinary 3-hydroxy BaP could be a useful biomarker of exposure to PAH (Simon et al. 2000; Jacob and Seidel 2002). However, to date the main urinary metabolite used in biomonitoring of exposure to PAH is 1-hydroxypyrene (1-OHP), a major urinary metabolite of pyrene, as proposed by Jongeneelen et al. (1986, 1987). At present no biological exposure limits have been widely adopted for the measured urinary PAH metabolites. Given that PAH profiles vary remarkably in different work environments, a single biological limit value (BLV) for 1-OHP cannot apply to all workplaces (Levin 1995). However, Jongeneelen (1992, 1993) and Ny et al. (1993) have respectively proposed BLVs of 2.3 μmol 1-OHP per mol creatinine for coke-oven workers and 4.3 μmol 1-OHP per mol creatinine for Söderberg potroom workers in aluminium plants, derived from the lower 1-OHP urinary excretion value corresponding to the threshold limit values (TLVs) for coal tar pitch volatiles (CTPVs) and BaP. For other work environments where there are insufficient data to calculate BLVs by such an approach, Bouchard and Viau (1999) have proposed a method to adjust BLVs for urinary 1-OHP from one work environment to another one based on the reported profiles of airborne PAHs and on the fact that 1-OHP excretion remains proportional to the pyrene dose even when administered as a mixture of many PAHs. The algorithm takes into consideration the differences in the ratios of BaP-equivalent concentration of PAHs calculated from the concentration of individual carcinogenic PAHs in the mixtures multiplied by their respective carcinogenic potencies (Krewski et al. 1989) relative to the concentration of pyrene in the mixture.

The equation is as follows:

$$BLV_{(2)} = BLV_{(1)} \times \frac{\left[\frac{[BaPeq]_1}{[P]_1}\right]}{\left[\frac{[BaPeq]_2}{[P]_2}\right]} \quad (1)$$

where $BLV_{(1)}$ and $BLV_{(2)}$ are the biological limit values for work environments 1 and 2, respectively, $[BaPeq]_1$ and $[BaPeq]_2$ are the concentrations of carcinogenic PAHs in work environments 1 and 2 expressed as BaP-equivalent concentration using toxic equivalent factors, and $[P]_1$ and $[P]_2$ are the concentrations of airborne pyrene in work environments 1 and 2. Of course, this equation might not apply where dermal absorption of PAHs is comparable to or exceeds pulmonary absorption.

The general objective of the present study was to experimentally validate this algorithm through determination of urinary 1-OHP levels corresponding to given levels of electrophilic tissue burden (ETB) assessed as covalent binding of the ultimate carcinogen BaPDE with cellular proteins in several selected tissues following repeated exposure to binary mixtures of BaP and pyrene to a rat model. In other words, assuming the BaP-equivalent concentration (or dose) and the ETB are both proportional to the carcinogenic risk, we attempted to verify that:

$$1 - OHP_{(2)} = 1 - OHP_{(1)} \times \frac{\left[\frac{[ETB]_1}{[P]_1}\right]}{\left[\frac{[ETB]_2}{[P]_2}\right]} \quad (2)$$

where $1\text{-}OHP_{(1),(2)}$, $[ETB]_{1,2}$ and $[P]_{1,2}$ correspond to two different exposure scenarios, relating to different BaP/P ratios. Thus, our aim was to examine the role of the urinary 1-hydroxypyrene (1-OHP) as a reliable measure of internal dose linked to the ETB in tissues that are targets for carcinogenicity, such as lung. Correlation between ETB and urinary/faecal 1-OHP as well as urinary/faecal 3-hydroxy benzo(*a*)pyrene (3-OHBaP) has been also studied as related to the different ratios BaP/P.

Materials and methods

Chemicals

Pyrene, scintillation grade >99% purity, was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Benzo(*a*)pyrene, HPLC grade minimum 98% purity, was purchased from Sigma Chemical Co. (St. Louis, MO, USA). A reference standard of 1-OHP was obtained from NCI Chemical Carcinogenic Reference Standards distributed by the Midwest Research Institute (Kansas City, MO, USA). β-Glucuronidase and arylsulfatase (100,000 Fishman U/ml and 800,000 Roy U/ml from *Helix pomatia*), RNase T1 (100,000 U/ml from *Aspergillus oryzae*) and RNase A (100 mg/ml from bovine pancreas) were purchased from Roche Inc. (Laval, QC, Canada). Proteinase (24 U/mg from *Bacillus subtilis*) was purchased from Fluka Co. (Toronto, Canada). Benzo(*a*)pyrene-*r*7,*t*8,9,*c*10-tetrahydrotetrol (BaPTeT), which is liberated upon mild hydrolysis of the adducts, was obtained from the NCI Chemical Carcinogenic Reference Standards distributed by the Midwest Research Institute and was used as standard in high-performance liquid chromatographic (HPLC) analyses. The commercially available corn oil Mazola was used as a vehicle. HPLC-grade methanol was obtained from Fisher Scientific Co. (Witsby, ON, Canada). Water was obtained from a Milli-Q water system (Millipore, Mississauga, ON, Canada).

Animals and treatment

Male Sprague-Dawley rats (Charles River, St. Constant, QC, Canada) weighing 225–250 g were used in this experiment. Rats were

housed in a temperature and humidity controlled room with a 12-h/12-h light/dark cycle and with standard rat chow and water provided ad libitum. The animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care. The animals were housed three per cage during acclimatization for 5 days and were then randomized into four groups of 11 animals each. Individual animals from each group (one per cage) were given, once daily for 10 consecutive days, intraperitoneal (i.p.) doses of one of nine binary mixtures of BaP and P, corresponding to BaP/P ratios of 0.2, 1 and 5 and as three constant doses of BaP equal to 2, 6 and 20 mg/kg for each ratio. The binary mixture doses were dissolved in corn oil as a vehicle and 5 ml of solution was injected per kilogram of body weight. Control animals (two per group) were injected with corn oil alone. After the tenth injection, animals were transferred to individual metabolic cages for urine and faeces collection and food was withheld overnight. Twenty-four hours later, rats were killed by CO_2 inhalation. Livers, lungs and hearts were collected and immediately frozen and stored at −80°C until further processing. Blood was withdrawn from the abdominal aorta and was treated immediately for haemoglobin extraction and quantitation. Urine and faeces collected for a 24-h period following the tenth injection were frozen at −20°C until further processing.

Tissue processing

Tissues were minced individually, washed three times with 0.05 M Tris-KCl buffer pH 7.4 (containing Trisma base, Trisma HCl, KCl), and then homogenized individually in TEM buffer, pH 7.4 (containing Trisma base, Trisma HCl, 10 M Na-EDTA, 1 mM monothioglycerol) at 4 ml/g tissue. The homogenate was further centrifuged at 10,000 *g* for 15 min. The 10,000 *g* pellet was discarded and the 10,000 *g* supernatant fraction (microsomes plus cytosol) was collected and further processed for quantification of the BaPDE–protein adducts. The fact that the nuclear fraction of the homogenate was discarded means that most DNA was eliminated from the analysis. An aliquot of the supernatant was used to determine the protein concentration by the standard kit of Bio-Rad (Richmond, CA, USA), based on the Bradford assay (Bradford 1976).

Blood processing

Blood samples were processed as described by Shugart and Kao (1985) for mouse blood. Briefly, washing of the red blood cells five times with 0.9% solution of heparinized sodium chloride was performed followed by haemoglobin extraction repeated two times by addition of water. Haemoglobin (Hb)content was determined by Total Haemoglobin Kit No.525-A (Sigma Diagnostic Inc., St. Louis, MO, USA). Aliquots of haemoglobin extract were kept for total protein analysis using the standard kit of Bio-Rad to determine the efficiency of haemoglobin extraction. The latter was found to be over 90%.

Analysis of ETB (BaPDE adducts with proteins)

The analytical procedure for the isolation of benzo(a)pyrene tetrol (BaPTeT) obtained after mild acid hydrolysis of the BaPDE–protein adducts (considered as electrophilic tissue burden, ETB) is described by Tzekova et al. (2003) and is mainly based on the one described by Viau et al. (1993). Briefly, enzymatic digestion of the 10,000-*g* supernatant from the primary tissue homogenate with 750 μg Rnase A and 5000 U Rnase T1 for 30 min was performed to facilitate the removal of RNAs as adducted nucleotides together with the "free" BaPTeT. The latter was supposed to result from intracellular hydrolysis of the benzo(*a*)pyrene diolepoxide (BaPDE). Subsequently, the tissue homogenate was submitted to enzymatic digestion with proteinase (65 mU enzyme per mg protein), performed overnight in presence of 10% solution of sodium dodecyl sulfate (SDS, 1/10 v/v of the total homogenate volume for processing), to liberate any free BaPTeT possibly "hidden" in the hydrophobic pockets of proteins. After extraction of the free BaPTeT with 3×3 ml ethyl acetate saturated with water, the tissue homogenate was processed for bound BaPTeT (ETB) quantification. Since acid hydrolysis step (0.1 *N* HCl, 90°C, for 3 h) yielded a white compact precipitate, the ampholytic surfactant diethanolamine (DEA) was introduced at 2.5 ml/sample as a dissolving agent prior to the solid phase extraction step further utilized in the bound BaPTeT analytical procedure. The bound BaPTeT was analysed by an HPLC system composed of a quaternary pump 1100 Series (Agilent Technologies, Palo Alto, CA, USA), an automatic injector AS-100 (Bio-Rad), an LC-8 Zorbax Eclipse 250×4.6 mm column (Agilent Technologies), a fluorescence detector LC-240 (Perkin-Elmer, Foster City, CA, USA) and a PE Nelson 900 interface (Perkin-Elmer). The column temperature was set at 32°C. The solvent system was Milli-Q water (A) and methanol (B) and the elution, at flow rate of 1 ml/min, was performed as follows: 54% B for 11 min, increasing to 80% B for 6 min and decreasing to 54% B for 5 min. Injection volume was 150 μl throughout. Excitation and emission wavelengths were 244 and 398 nm, respectively. A sample of standard BaPTeT was run immediately before the HPLC runs for the tissue extracts in order to verify the expected elution time (8.36 min under the applied conditions). Adduct levels were presented in femtomoles per milligram protein. The results obtained were corrected for the recovery of BaPTeT from control homogenates spiked with authentic BaPTeT. The recovery of BaPTeT was similar for each tissue considered, namely 58% for heart, 66% for lung, 56% for liver and 55% for haemoglobin (Hb). The analytical procedure for quantitation of BaPDE adducts with hemoglobin did not include a proteinase incubation step since around 70–100% of the measured tetrol originated from true adducts (Viau et al. 1993).

Urine analysis

Urine was collected over thymol and frozen at −20°C until analysis. Urine samples were treated using the method of Jongeneelen et al. (1987). Efficiency of the extraction of hydrolysed metabolites was determined using untreated urine samples spiked with authentic reference standards. Recovery was found to be 78 ± 3% (mean ± SD) for 1-OHP, and 57 ± 2% for 3-OHBaP.

Samples were analysed by the same HPLC system as described for BaPTeT analyses.

For 1-OHP analysis, a LC-18 Supelcosil 250×4.6 mm column (Supelco, Oakville, ON, Canada) was used. The solvent system was Milli-Q water (A) and methanol (B) and the elution, at flow rate of 0.8 ml/min, was performed as follows: 50% B for 39 min, increasing to 94% B for 10 min, then to 100% B for 9 min and decreasing to 50% B for 12 min. In order to improve the recovery and reproducibility of the chromatography 1 mg ascorbic acid was added per litre of methanol eluent (Bouchard et al. 1994). Column temperature was set at 32°C. Injection volume was 20 μl throughout. Excitation and emission wavelengths were 242 and 388 nm, respectively. Naphthalene (1 mg/l) was used as an external standard. A sample of standard 1-OHP was run immediately before the HPLC runs for the urine extracts in order to verify the expected retention time (32 min under the applied conditions).

For 3-OHBaP analysis, a LC-18 Supelcosil 250×4.6 mm column (Supelco) was also used. The solvent system was Milli-Q water (A) and methanol (B) and the elution was performed at 80% B. Ascorbic acid (1 mg/l) was added to the eluent to improve analytical conditions (Bouchard et al. 1994). The injection volume was 20 μl throughout. Column temperature was 32°C and flow rate was 0.8 ml/min. Anthracene (1 mg/l) was used as an external standard. Excitation and emission wavelengths were 265 and 430 nm, respectively. The retention time for 3-OHBaP was 14 min under the applied conditions.

Faeces analysis

Faeces were analysed using a method adapted from Chipman et al. (1983) and Gerde et al. (1997). A homogenate of 6.25% (w/v) of all

faeces voided by a given rat was prepared in a sodium acetate buffer (0.1 M, pH 5.0). Aliquots of 4 ml homogenate were transferred into Pyrex tubes and incubated overnight with 10 µl of β-glucuronidase/arylsulfatase in a shaking bath at 37°C. Samples were extracted twice with 4 ml ethyl acetate saturated with water, shaken for 30 min, and centrifuged for 20 min at 3000 rpm at 4°C. The organic phases were combined and evaporated to dryness and the residue was redissolved in 1 ml acetonitrile. Recovery of 1-OHP from spiked faeces samples was 77 ± 3% (mean ± SD) and of 3-OHBaP was 66 ± 7%.

Analyses of faecal 1-OHP and 3-OHBaP were performed using the same HPLC system as for tissue and urine samples and with the same experimental conditions as for urine 1-OHP and 3-OHBaP.

Data analyses

Linear regression analyses (least-squares method) were performed by the JMP IN statistical software (SAS Institute Inc., Cary, NC, USA) and R^2-, β-, and p-values were calculated. Note that in order to avoid confusion between the abbreviation for pyrene (P) and the probability level, a lower case p is used to refer to the latter. A level of $p \leq 0.05$ was considered as the level of significance. Linear regressions were performed to determine the following relationships: BaPDE adduct (ETB) formation and urinary/faecal 1-OHP, or ETB and urinary/faecal 3-OHBaP, as well as to determine the BaP dose-dependent urinary/faecal 3-OHBaP excretion or pyrene dose-dependent urinary/faecal 1-OHPexcretion.

As it is not currently possible to establish either a DNA- or tissue protein-adduct level that entails an unacceptable cancer risk, arbitrary values of ETB had to be set in order to check the validity of Eq. 2. These were established at 30, 40 and 50 fmol/mg protein and were selected as being simply within the span of the observed values in the current study. Thus, assuming for example that 30 fmol/mg protein is a critical endpoint, we calculated the associated critical urinary 1-OHP concentration predicted for the various mixtures using the BaP/P ratio of 1 as the reference point and compared that value with the observed experimental value.

Results

Experimental verification of the relationship between the levels of ETB in liver, lung, heart, blood Hb and urinary/faecal 1-OHP

In general, the relationship between ETB in all tissues studied and urinary 1-OHP was found to be linear for two of the three exposure scenarios corresponding to the BaP/P ratios of 0.2 and 1 (see Table 1). Figure 1 shows linear regressions between the levels of the ETB in liver and urinary 1-OHP for the BaP/P ratios of 0.2 and 1, and a lack of linear regression for the BaP/P ratio 5, where a value of $p = 0.067$ was obtained. In fact, concerning scenario BaP/P 5, a lack of linear relationship between ETB and urinary 1-OHP was found only for liver and lung tissue. For the latter a value of $p = 0.0846$ was obtained.

Regarding the relationship ETB in a given tissue versus faecal 1-OHP levels, linear regressions for all tissues of interest were observed only for the BaP/P ratio 0.2 (see Table 1). Figure 2 shows the relationship between ETB in liver versus faecal 1-OHP, which was found to be linear for all three exposure scenarios. For the BaP/P ratio 1, lack of linear regressions were found only for the relationship ETB in lung versus faecal 1-OHP and ETB of Hb versus faecal 1-OHP. Finally, concerning the scenario BaP/P ratio 5, except for the liver tissue, for all other tissues studied no linear regression was found ($p = 0.5928$ for ETB in lung versus faecal 1-OHP; $p = 0.0796$ for ETB in heart versus faecal 1-OHP; $p = 0.2035$ for ETB of Hb versus faecal 1-OHP).

Experimental verification of the relationship between the levels of ETB in liver, lung, heart, blood Hb and urinary/faecal 3-OHBaP

In general, a remarkable difference in the correlation coefficients obtained for the relationship of ETB versus urinary 3-OHBaP was observed for all tissues of interest as well as for all BaP/P ratios examined. Interestingly, the relationship ETB in liver versus urinary 3-OHBaP was the only one for which no linear relationship was observed for all three exposure scenarios. No linear regression was also found for ETB in lung versus urinary 3-OHBaP for BaP/P ratio1 ($p = 0.0753$). For all other tissues and scenarios studied linear relationships were obtained between ETB and urinary 3-OHBaP (Table 2).

Table 1 Relationships between electrophilic tissue burden (ETB; benzo(*a*)pyrene diolepoxide adducts with proteins) formation in liver, lung, heart and haemoglobin (Hb) and urinary/faecal 1-hydroxypyrene (1-OHP) following intraperitoneal administration to rats of benzo(*a*)pyrene (BaP) in binary mixture with pyrene (P) for three different exposures scenarios corresponding to BaP/P ratios of 0.2, 1, and 5

BaP/P ratio	Tissue	Correlation coefficients					
		ETB vs urinary 1-OHP			ETB vs faecal 1-OHP		
		R^2-value	β-value	p-value	R^2 value	β-value	p-value
0.2	Liver	0.371	0.0093	0.0355	0.371	5.8709	0.0355
	Lung	0.502	0.0164	0.0099	0.898	13.8855	< 0.0001
	Heart	0.793	0.0109	0.0001	0.593	5.9402	0.0034
	Hb	0.880	0.0099	< 0.0001	0.599	5.1958	0.0031
1	Liver	0.787	0.0630	0.0003	0.762	33.423	0.0004
	Lung	0.427	0.0672	0.0293	0.215	25.714	0.1514
	Heart	0.726	0.0573	0.0009	0.556	27.081	0.0084
	Hb	0.575	0.0400	0.0069	0.217	13.283	0.1488
5	Liver	0.297	0.4306	0.0667	0.587	256.016	0.0037
	Lung	0.268	0.7924	0.0846	0.029	111.465	0.5928
	Heart	0.556	0.5243	0.0053	0.276	156.237	0.0796
	Hb	0.592	0.3799	0.0035	0.156	82.649	0.2035

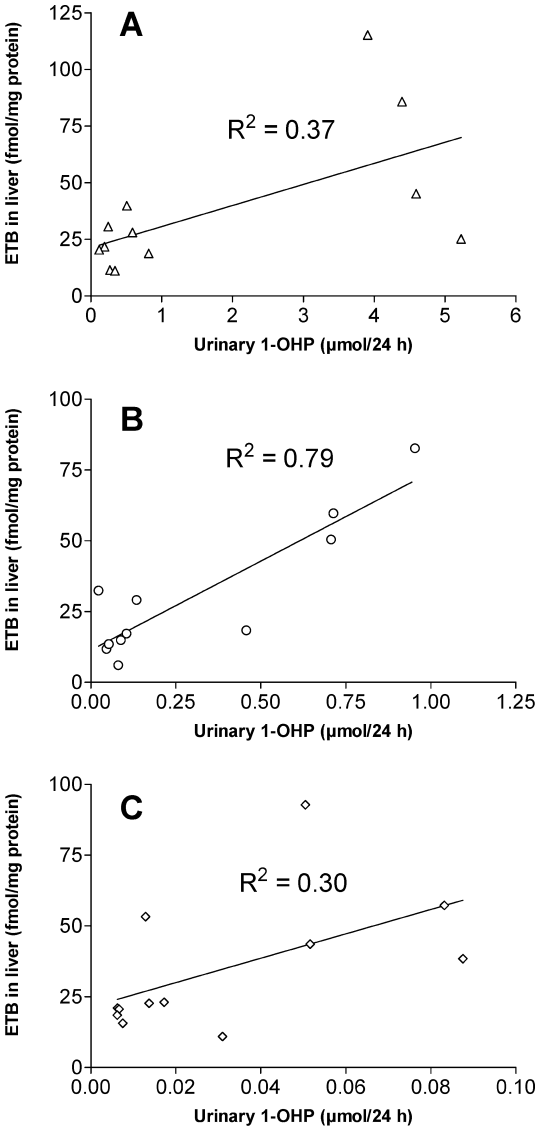

Fig. 1A–C Relationship between the levels of the electrophilic tissue burden (ETB) in liver and urinary 1-hydroxypyrene (1-OHP) following intraperitoneal administration of binary mixtures of benzo(*a*)pyrene (BaP) and pyrene (P) at three constant doses of BaP (2, 6 and 20 mg/kg) for each of the three different exposure scenarios: BaP/P ratio 0.2 (**A**); BaP/P ratio 1 (**B**) and BaP/P ratio 5 (**C**). Each data point represents an individual animal

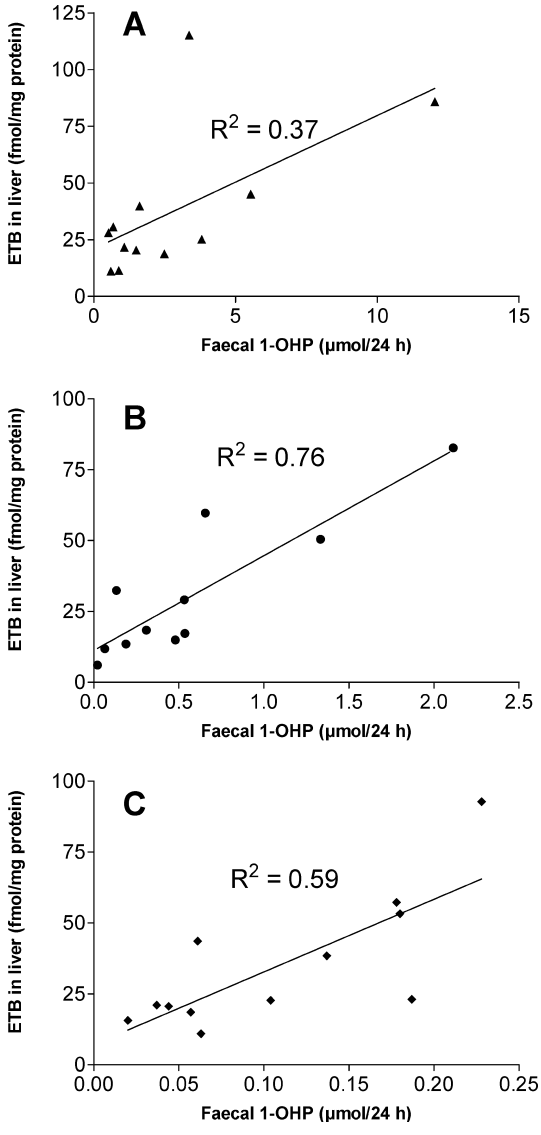

Fig. 2A–C Relationship between the levels of the electrophilic tissue burden (ETB) in liver and faecal 1-hydroxypyrene (1-OHP) following intraperitoneal administration of binary mixtures of benzo(*a*)pyrene (BaP) and pyrene (P) at three constant doses of BaP (2, 6 and 20 mg/kg) for each of the three different exposure scenarios: BaP/P ratio 0.2 (**A**); BaP/P ratio 1 (**B**) and BaP/P ratio 5 (**C**). Each data point represents an individual animal

Figure 3 shows the relationship between Hb adducts and urinary 3-OHBaP for which relatively strong linear regressions were observed for all three exposure scenarios.

Regarding the relationship ETB in a given tissue versus faecal 3-OHBaP, only the scenario BaP/P = 0.2 showed linear regressions for all tissues considered (Table 2). Figure 4 shows the relationship between ETB in liver versus faecal 3-OHBaP, with linear regressions obtained for all scenarios studied. In fact, this is the only one tissue for which linear relationship between ETB and faecal 3-OHBaP was found for all BaP/P ratios examined. Lack of linear regression was observed between ETB in lung and faecal 3-OHBaP and for Hb adducts versus faecal 3-OHBaP for BaP/P ratio1 as well as between ETB in lung or heart or Hb and faecal 3-OHBaP for BaP/P ratio 5.

Dose–response relationship for urinary/faecal 1-OHP and urinary/faecal 3-OHBaP excretion

A linear pyrene dose-dependent urinary 1-OHP excretion ($R^2 = 0.949$, $p < 0.0001$) as well as faecal 1-OHP excretion ($R^2 = 0.694$, $p < 0.0001$) was confirmed (data not shown). A linear BaP dose-dependent urinary

Table 2 Relationships between electrophilic tissue burden (ETB; benzo(*a*)pyrene diolepoxide adducts with proteins) formation in liver, lung, heart and haemoglobin (Hb) and urinary/faecal 3-hydroxybenzo(*a*)pyrene (3-OHBaP) following intraperitoneal administration of binary mixtures of benzo(*a*)pyrene (BaP) and pyrene (P) for three different exposures scenarios corresponding to BaP/P ratios of 0.2, 1, and 5

BaP/P ratio	Tissue	Correlation coefficients					
		ETB vs urinary 3-OHBaP			ETB vs faecal 3-OHBaP		
		R^2-value	β-value	p-value	R^2-value	β-value	p-value
0.2	Liver	0.210	0.3505	0.1344	0.362	0.0267	0.0383
	Lung	0.424	0.7580	0.0217	0.862	0.0628	<0.0001
	Heart	0.848	0.5643	<0.0001	0.528	0.0259	0.0075
	Hb	0.767	0.4668	0.0002	0.547	0.0229	0.0060
1	Liver	0.295	0.3804	0.0844	0.795	0.0361	0.0002
	Lung	0.316	0.5717	0.0717	0.211	0.0270	0.1552
	Heart	0.717	0.5628	0.0010	0.577	0.0292	0.0067
	Hb	0.902	0.4959	<0.0001	0.216	0.0140	0.1496
5	Liver	0.150	0.3553	0.2133	0.534	0.0537	0.0069
	Lung	0.329	1.0197	0.0510	0.035	0.0264	0.5624
	Heart	0.513	0.5884	0.0088	0.244	0.0322	0.1026
	Hb	0.480	0.3974	0.0125	0.211	0.0211	0.1330

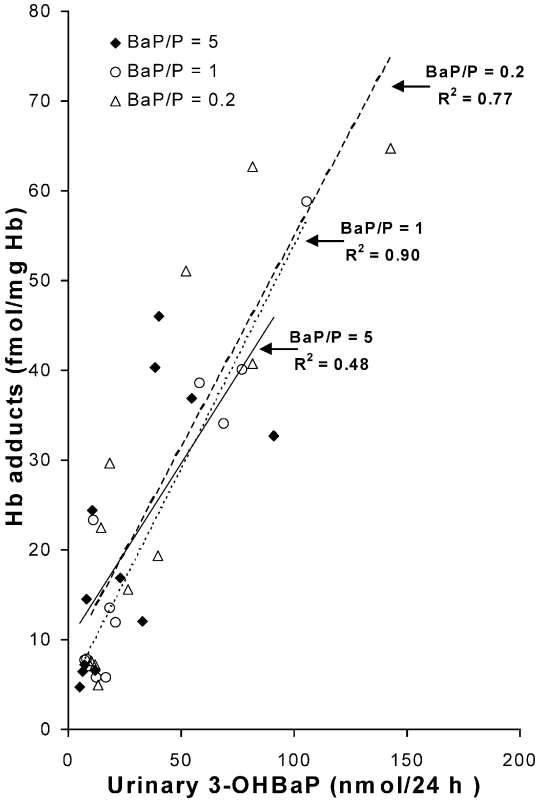

Fig. 3 Correlation between the levels of haemoglobin (Hb) adducts and urinary 3-hydroxybenzo(*a*)pyrene (3-OHBaP) following intraperitoneal administration of binary mixtures of benzo(*a*)pyrene (BaP) and pyrene (P) at three constant doses of BaP (2, 6 and 20 mg/kg) for each of the three different exposure scenarios. Each data point represents an individual animal

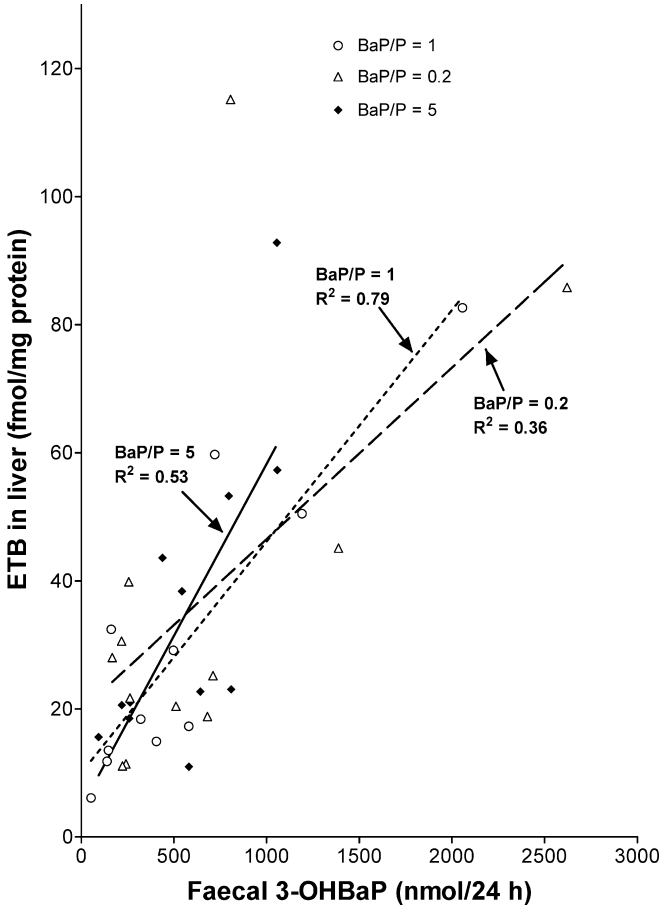

Fig. 4 Correlation between the levels of the electrophilic tissue burden (ETB) in liver and faecal 3-hydroxybenzo(*a*)pyrene (3-OHBaP) following intraperitoneal administration of binary mixtures of benzo(*a*)pyrene (BaP) and pyrene (P) at three constant doses of BaP (2, 6 and 20 mg/kg) for each of the three different exposure scenarios. Each data point represents an individual animal

3-OHBaP excretion ($R^2=0.673$, $p<0.0001$) and faecal 3-OHBaP ($R^2=0.421$, $p<0.0001$) was also observed. As seen, stronger linear regressions were obtained with urinary metabolites than with their faecal analogues.

The mean percentages of the pyrene dose and BaP dose excreted in urine and faeces as 1-OHP and 3-OH-BaP, respectively, during the 24-h collection period were determined.

As seen in Table 3, similar levels of the mean percentage of P dose excreted in urine as 1-OHP was observed for all binary doses examined. The same relationship is confirmed for the mean percentage of

Table 3 Mean percentage of the pyrene (P) dose excreted as 1-hydroxypyrene (1-OHP) and mean percentage of the benzo(*a*)pyrene (BaP) dose excreted as 3-hydroxybenzo(*a*)pyrene (3-OHBaP), in both urine and faeces at 24 h post-dose following the tenth intraperitoneal administration of binary mixtures (BaP + P) (animals received a total of ten injections, one daily). Calculation for the percentage of P and BaP dose excreted as the relevant metabolites in urine and faeces was based on the last (tenth) injection of BaP

BaP/P ratio	Doses (BaP + P, mg/kg)	Mean P dose excreted as 1-OHP (%)		Mean BaP dose excreted as 3-OHBaP (%)	
		Urine	Faeces	Urine	Faeces
0.2	2 + 10	1.7 ± 0.4	5.2 ± 1.3	0.45 ± 0.05	9.5 ± 0.8
	6 + 30	1.1 ± 0.6	3.4 ± 1.8	0.34 ± 0.17	5.6 ± 3.3
	20 + 100	3.0 ± 0.4	4.1 ± 2.7	0.37 ± 0.16	5.7 ± 3.6
1	2 + 2	1.6 ± 0.9	2.3 ± 1.8	0.48 ± 0.18	4.7 ± 2.3
	6 + 6	1.0 ± 0.4	4.6 ± 1.7	0.19 ± 0.08	5.4 ± 2.5
	20 + 20	2.3 ± 0.6	3.6 ± 2.6	0.32 ± 0.10	4.4 ± 3.0
5	2 + 0.4	1.5 ± 0.8	11.4 ± 12.1	0.30 ± 0.12	13.7 ± 12.3
	6 + 1.2	0.9 ± 0.5	5.5 ± 2.3	0.25 ± 0.16	7.7 ± 2.9
	20 + 4	1.1 ± 0.3	2.5 ± 1.2	0.23 ± 0.10	3.1 ± 1.3

P excreted as 1-OHP in faeces. However, large inter-individual difference in urinary and faecal excretion of this P metabolite was observed. The same tendency is confirmed for the mean percentage of BaP dose excreted as 3-OHBaP in urine and faeces (Table 3).

Experimental verification of Eq. 1

A pattern of simulation was performed on the basis of the experimentally established relationships between the levels of ETB in liver versus urinary 1-OHP for the three ratios BaP/P (Fig. 1 and Table 4) when assuming three critical levels of ETB, namely 30, 40 and 50 fmol/mg protein, and determining the corresponding 1-OHP levels. In order to verify Eq. 1 the BaP/P ratio 1 was set as a reference ratio with reference values of urinary 1-OHP corresponding to any given critical level of ETB derived from the relationships of ETB in liver versus urinary 1-OHP (Fig. 1). Thus, while applying Eq. (1) for another BaP/P ratio (from Fig. 1) a calculated value of urinary 1-OHP corresponding to any of the given critical ETB levels was obtained, which was subsequently compared with the experimentally derived value for that BaP/P ratio (from Fig. 1) and named ''observed value'' as shown in Table 4. There is a reasonable agreement between the predicted values and the observed values of urinary 1-OHP for the BaP/P ratios 0.2 and 1 shown in Table 4, which indicates that Eq. 1 is verified for these BaP/P ratios. The same was confirmed when performing similar calculations for the other tissues considered (data not shown).

Table 4 Experimental verification of the algorithm proposed by Bouchard and Viau (1999) for the determination of the threshold urinary 1-hydroxypyrene (1-OHP) based on a reference mixture. Reference was taken as the benzo(*a*)pyrene/pyrene (BaP/P) ratio 1 mixture and calculated values were determined assuming three critical toxic levels of electrophilic tissue burden (ETB). Calculation was subsequently applied to the BaP/P ratios of 0.2 and 5 and results were compared with the experimental value interpolated (observed value) from Fig. 1

BaP/P ratio	Critical ETB level in liver (fmol/mg protein)	Urinary 1-OHP excretion (nmol/24 h)		Ratio of Calculated to Observed values of urinary 1-OHP
		Observed value	Calculated value	
0.2	30	933	1480	1.59
	40	2008	2275	1.13
	50	3083	3070	1.00
1	30	296	296	1.00
	40	455	455	1.00
	50	614	614	1.00
5	30	20	59	2.96
	40	43	91	2.11
	50	66	123	1.85

Discussion

This study was carried out to verify our hypothesis that urinary 1-OHP could serve as a biomarker of the internal dose of PAHs, correlated with a toxicologically relevant biological effect (ETB formation) of PAH mixtures. Given that linear relationship between BaP dose and adduct formation has been observed in experimental studies (Shugart and Kao 1985), and given that linear relationships have been reported between P dose (even as a mixture of PAHs) and urinary 1-OHP (Kuljukka et al. 1997), we expected a linear relationship between 1-OHP and BaPDE adducts with cellular proteins (electrophilic tissue burden, ETB) in target tissues. Importantly, Bouchard and Viau (1999) elaborated a simple algorithm (see Introduction, Eq. 1) that could be further utilized for modulation of biological limit values (BLVs) for 1-OHP according to the actual composition of individual PAHs in typical mixtures encountered in general and occupational environments and that required experimental validation in an animal model. In the present study, we attempted to verify this algorithm in the rat model through verification of Eq. 2 (from the

Introduction) incorporating the ETB derived from exposure to PAHs according to our hypothesis. This would be of great importance in biological monitoring strategies given that a single biological value for 1-OHP can not apply to all work environments due to the variety of PAH profiles encountered (Levin 1995; dell'Omo et al. 1998).

This study was conducted on binary mixtures at doses higher than those encountered in workplaces in order to allow adequate measurements of ETB. Nevertheless, it still represents an interesting tool towards understanding and interpreting the possible additive or antagonistic effects associated with exposure to PAHs. Prior to this study, Bouchard et al. (1998) investigated the effect of exposure to binary and ternary mixtures of PAHs on the urinary excretion kinetics of 1-OHP; no influence of BaP on urinary excretion kinetics of P has been reported, but significant increase in the amount of 1-OHP excreted in urine at doses of 1.3 and 6.5 mg BaP/kg in mixture with 1 mg P/kg (single intravenous administration to rats) was found, probably due to BaP induction of P metabolism. We have chosen higher doses of BaP and P in binary mixtures administered intraperitoneally to ensure sufficient spread in urinary 1-OHP levels for each binary dose tested, making possible the verification of Eq. 2.

In other words, we attempted to determine the urinary 1-OHP levels that correspond to given threshold of ETB in four target tissues of interest: liver, lung, heart and blood Hb. Heart was included in the target group tissues based on the study of Lewtas et al. (1993) reporting highest DNA adduct levels in human heart among several other autopsy tissues examined from individuals previously exposed to tobacco smoke. In this respect high ETB levels in heart was presumed in our case also. In order to verify the correlation between different biomarkers of PAH exposure we have studied, as well, ETB associated with Hb and its relationship with urinary/faecal 1-OHP and 3-OHBaP. Moreover, it has already been reported that BaPDE adducts with Hb could serve as a "surrogate" of ETB in target tissues for carcinogenicity, such as lung (Tzekova et al. 2003). Although BaPDE adducts with Hb and albumin have been considered as important biomarkers of, respectively, repeated and recent human exposure to BaP or PAHs (Lee and Santella 1988; Bechtold et al. 1991; van Welie et al. 1992), such an approach is still distant from genotoxicity endpoints evaluation. Assessing ETB as defined in this report is one step closer towards estimation of the biologically effective PAH dose in target tissues and, consequently, an indirect estimation of PAHs genotoxicity potential in these tissues.

As seen in Table 1 and Fig. 1, statistically significant linear relationships between the levels of ETB in all tissues considered and urinary 1-OHP were observed only for the exposure scenarios of BaP/P ratios 0.2 and 1. Thus, urinary 1-OHP could serve as an indicator of the internal dose linked with the ETB in target tissues for carcinogenicity, such as lung, at low BaP/P ratios (less than or equal to 1) over the range of the exposure doses tested. Taking into consideration the simulation performed in Table 4, this indicates that Eq. 1 is verified for BaP/P ratios equal to 0.2 and 1. However, these two BaP/P ratios correspond to those encountered in work environments (Petry et al. 1996; Bieniek 1998; Gündel et al. 2000), contrary to the BaP/P ratio of 5. In order words, for this last scenario the deviation from the expected linearity in the relationship examined for the liver and lung tissue was not considered an important issue for the typical occupational environments.

As seen in Table 1 and Fig. 2, the relationship between ETB levels in all tissues considered and faecal 1-OHP was found to be linear for all tissues of interest only for the exposure scenario of BaP/P ratio 0.2. However, in the context of biological monitoring, the urinary metabolites examined are those of concern.

Interestingly, while studying the relationship between ETB in all tissues of interest and the urinary/faecal 3-OHBaP excretion, the liver tissue was the only one for which no linear regression was confirmed between ETB and urinary 3-OHBaP for all three BaP/P ratios, and the only one for which linear regressions were obtained with faecal 3-OHBaP for the three exposure scenarios (see Table 2 and Fig. 4), which is an observation that is difficult to explain. Also, relatively strong linear relationships were obtained for BaPDE adducts with Hb versus urinary 3-OHBaP for BaP/P ratios 0.2, 1 and 5 (see Table 2 and Fig. 3), indicating that urinary 3-OHBaP could serve as surrogate for BaPDE–Hb adducts under steady-state exposure conditions. This confirms another previous study reporting high linear correlation between the urinary excretion of 3-OHBaP in Monday 24-h urine samples from rats and the levels of BaPDE–Hb adducts after 4 weeks of treatment with BaP (i.p. administration, once daily, Tuesday to Friday; Bouchard and Viau 1995). However, it is well known that BaP metabolites are mainly excreted in faeces and only a minor part (5–15%) is excreted in urine (Chipman et al. 1981; Mitchell 1982). Also, given that the molecular weight of the glucuronoconjugate of 3-OHBaP (428) is higher than the threshold for biliary excretion in rats (325), it could be a reason for possible competitive inhibition of glucorono-conjugate of 1-OHP (305) biliary transport (Sathirakul et al. 1994). This could result in an increase in the percentage of P excreted as 1-OHP in urine and, consequently, lower proportion of 1-OHP recovered in faeces. However, a significant difference (higher values) in this percentage (ANOVA data) was observed only at the higher exposure binary doses tested (20 mg BaP/kg + 20 mg P/kg and 20 mg BaP/kg + 100 mg P/kg). This could be related to a somewhat more pronounced effect of BaP induction on the P metabolism although a quantitative estimation of such an induction was not performed in the present study. Thus our results for the percentage of P dose excreted as urinary 1-OHP align with those of Bouchard et al. (1998) reporting means of

$1.41 \pm 0.16\%$ and $1.71 \pm 0.27\%$ of the P dose excreted as urinary 1-OHP following single intravenous administration to rat of binary mixtures (1.3 mg BaP/kg + 1 mg P /kg, i.e. BaP/P ratio 1.3, and 6.5 mg BaP/kg + 1 mg P/kg, i.e. BaP/P ratio 6.5, respectively), which compare to our values of $1.5 \pm 0.7\%$ and $0.9 \pm 0.5\%$ of the P dose excreted as urinary 1-OHP at the binary doses (2 mg BaP/kg + 2 mg P/kg, BaP/P ratio 1, and 6 mg BaP/kg + 1.2 mg P/kg, BaP/P ratio 5, respectively). This seems to be in accordance with the study of Viau et al. (1999) reporting that over 90% of the P dose excreted in urine as 1-OHP is excreted in 24 h following a single intravenous, oral and dermal treatment, and suggests that little accumulation of 1-OHP occurs within the 10-day i.p. treatment period in our study. Therefore, urine and faecal collection was made during 24 h following last injection in the present study. The same authors have reported a linear P dose-dependent 1-OHP excretion, as was found in our study following repeated exposure to binary mixture (BaP + P). This is an indication that saturation of the P metabolism has not occurred over the doses examined in the current study.

Recently, Bouchard et al. (2002) have studied the urinary excretion kinetics of 1-OHP in male Sprague-Dawley rats following subchronic oral exposure, on Tuesdays and Fridays for 10 consecutive weeks, to 0.046 mg/kg per day of P or to 0.046, 0.15, and 0.46 mg/kg per day of P in mixture with 12 other PAHs. Those authors found that whatever the administered dose, repeated exposure to both P and PAH mixtures resulted in a progressive time-dependent increase in the urinary excretion of 1-OHP. This was related to slow release of residual P accumulated in a long-term compartment and/or to the enterohepatic recirculation of 1-OHP and other P metabolites. However, our results for the percentage of the P dose excreted as urinary 1-OHP seems to be somewhat lower. Also, relatively high inter-individual difference in 1-OHP urinary excretion in rats was observed in this experiment as has been found in other previous studies following exposure to a given PAH mixture. In addition, it was recently shown that there is a strong correlation in the individual yield of urinary 1-OHP when comparing the excretion of the metabolites in a group of rats with different administrations of P. In other words, animals displaying a low yield of urinary 1-OHP upon a first administration of P will keep this characteristic when injected at a later time (C. Viau et al., report in preparation).

Concerning the ratio between the percentage of the BaP dose excreted in faeces and that in urine for 24-h collection period following last (tenth) i.p. injection of binary mixture (BaP + P), we have obtained values of 9.5 to 43.3 for all exposure mixture doses tested. Hence, it seemed that the importance of the faecal versus urinary excretion of 3-OHBaP depended of the binary dose composition, a fact that was not confirmed statistically due to the high inter-individual difference in urinary 3-OHBaP excretion observed in our study and by Bouchard and Viau (1996). Thus, this ratio is over two times higher in our case than the value of 4 obtained for the same ratio in the study of Tyndyk et al. (1994), in which cumulative excretion for 8 days after a single i.p. administration of 10 mg BaP/kg alone was reported. The same authors have observed that after ten i.p. injections with 10 days cessation of exposure between injections (8-day cumulative excretion) this ratio remains constant, but the quantities of 3-OHBaP excreted in both urine and faeces dropped 1.5–2 times due to age-related changes in BaP-metabolizing enzymes. An average of 0.1% of BaP dose excreted in urine as 3-OHBaP over the 8-day collection period after treatment was reported. Given that around 94% of the total amount of 3-OHBaP excreted in urine over 164 h (7 days) is eliminated during the first 72 h after a single i.p. treatment (Bouchard and Viau 1995), repeated exposure to BaP once daily for 10 days could result in excretion of residual 3-OHBaP from past days suggesting a probable accumulation of BaP and its metabolites upon repeated exposure as well as probable enzyme induction. Thus, our results (0.21–0.49% of BaP dose excreted as urinary 3-OHBaP at 24 h post-dosing, calculated on the basis of the last BaP dose) are two to five times higher than those obtained by Tyndyk et al. (1994) after a single i.p. administration of BaP. In support of this assumption, Bouchard and Viau (1995) have found that values of urinary 3-OHBaP in Mondays urine sample following 1 week of treatment (Monday through Friday, once daily with about 8 mg BaP/kg, i.p. administration) represented, on average, 33% of the previous Thursday sampling values, suggesting that BaP might accumulate in the organism upon repeated dosing and be redistributed, biotransformed and eliminated upon cessation of exposure. However, the amount of BaP in storage compartments, such as fatty tissues in rat, has not been measured in the current study. Moreover, our aim in this study was firstly to verify Eq. (1), and secondly to examine the correlation between different biomarkers of PAH exposure following concomitant exposure to binary mixtures of BaP and P, and thus to better assess their usefulness in biological monitoring. On the other hand, a linear BaP dose-dependant 3-OHBaP excretion in both urine and faeces was observed in our experiment, suggesting that BaP-metabolizing enzymes were not saturated over the range of doses used in the current study.

Again, it is difficult to compare our results for the percentage of BaP dose excreted in urine as 3-OHBaP with other data reported previously because of differences in the administration protocols. However, our results (0.20–0.49% of BaP dose excreted as urinary 3-OHBaP) are somewhat similar to those obtained by Jongeneelen et al. (1985), who reported 0.22–0.35% of BaP dose excreted in urine as 3-OHBaP during 6 days following 3 consecutive days of oral treatment of rats with BaP alone at doses of about 2.4, 5 and 12 mg/kg, calculated on the basis of the last given dose. It has to be kept in mind that the exposure route, duration of exposure, exposure doses and urine collection period, as

well as diet, differed in both studies. Our results did not confirm the findings of Jongeneelen et al. (1985) that the lower the BaP exposure dose the higher is the percentage of BaP dose excreted in urine as 3-OHBaP. However, it is well known that repeated exposure of rats to BaP may trigger induction of the cytochrome P450 enzyme system. There is also possible saturation of BaP metabolism at high BaP doses, and possible metabolic interference in the case of complex PAH mixture exposures, all impacting on the excretion kinetics of urinary 3-OHBaP.

In conclusion, the results obtained in the present report indicate that Eq. 1 is verified for two of three exposure scenarios corresponding to realistic occupational BaP/P ratios of 0.2 and 1. This suggests that using ETB as an endpoint, the proposed algorithm will reasonably predict the critical value of urinary 1-OHP for mixtures having different BaP/P ratios. The predicted value is better for BaP/P ratios ≤ 1. Furthermore, there is a large inter-individual variability in both urinary metabolites and tissue ETB for exposure to a given mixture. Stronger linear relationships between ETB in all chosen tissues and 1-OHP or 3-OHBaP were obtained with urinary metabolites than with their faecal analogues. Thus urinary 1-OHP and 3-OHBaP appear as more reliable biomarkers in biological monitoring strategies, in addition to being more convenient in human studies.

Acknowledgements This study was supported by the Canadian Institutes for Health Research. All experiments were conducted in accordance with the guidelines and principles of the Canadian Council on Animal Care.

References

Angerer J, Mannschreck C, Gundel J (1997) Biological monitoring and biochemical effect monitoring of exposure to polycyclic aromatic hydrocarbons [Review]. Int Arch Occup Environ Health 70:365–377

Bechtold WE, Sun JD, Wolff RK, Griffith WC, Kilmer JW, Bond JA (1991) Globin adducts of benzo(a)pyrene: markers of inhalation exposure as measured in F344/N rats. J Appl Toxicol 11:115–118

Bieniek G (1998) Aromatic and polycyclic hydrocarbons in air and their urinary metabolites in coke plant workers. Am J Ind Med 34:445–454

Bouchard M, Viau C (1995) Benzo(*a*)pyrenediolepoxide-hemoglobin adducts and 3-hydroxybenzo(*a*)pyrene urinary excretion profiles in rats subchronically exposed to benzo(*a*)pyrene. Arch Toxicol 69:540–546

Bouchard M, Viau C (1996) Urinary excretion of benzo[*a*]pyrene metabolites following intravenous, oral, and cutaneous benzo[*a*]pyrene administration. Can J Physiol Pharmacol 75:185–192

Bouchard M, Viau C (1999) Urinary 1-hydroxypyrene as a biomarker of exposure to polycyclic aromatic hydrocarbons: biological monitoring strategies and methodology for determining biological exposure indices for various work environments. Biomarkers 4:159–187

Bouchard M, Dodd C, Viau C (1994) Improved procedure for the high-performance liquid chromatographic determination of monohydroxylated PAH metabolites in urine. J Anal Toxicol 18:261–264

Bouchard M, Krishnan K, Viau C (1998) Urinary excretion kinetics of 1-hydroxypyrene following intravenous administration of binary and ternary mixtures of polycyclic aromatic hydrocarbons in the rat. Arch Toxicol 72:475–482

Bouchard M, Thuot R, Carrier G, Viau C (2002) Urinary excretion kinetics of 1-hydroxypyrene in rats subchronically exposed to pyrene or polycyclic aromatic hydrocarbon mixtures. J Toxicol Environ Health A 65:1195–1209

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chipman JK, Hirom PC, Frost GS, Millburn P (1981) The biliary excretion and enterohepatic circulation of benzo(a)pyrene and its metabolites in the rat. Biochem Pharmacol 30:937–944

Chipman JK, Millburn P, Brooks TM (1983) Mutagenicity and in vivo disposition of biliary metabolites of benzo(*a*)pyrene. Toxicol Lett 17:233–240

dell'Omo M, Muzi G, Marchionna G, Latini L, Carrieri P, Paolemili P, Abbritti G (1998) Preventive measures reduce exposure to polycyclic aromatic hydrocarbons at a graphite electrode plant. Occup Environ Med 55:401–406

Gerde, P, Muggenburg BA, Thornton-Manning JR, Lewis JL, Pyon KH, Dahl AR (1997) Benzo(*a*)pyrene at an environmentally relevant dose is slowly absorbed by, and extensively metabolized in, tracheal epithelium. Carcinogenesis 18:1825–1832

Gündel J, Schaller K-H, Angerer J (2000) Occupational exposure to polycyclic aromatic hydrocarbons in a fireproof stone producing plants: biological monitoring of 1-hydroxypyrene, 1-, 2-, 3- and 4-hydroxyphenanthrene, 3-hydroxybenz(a)anthracene and 3-hydroxybenzo(*a*)pyrene. Int Arch Occup Environ Health 73:270–274

IARC (International Agency for Research on Cancer) (1983) IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans, vol 32. Polynuclear aromatic compounds. Part 1. Chemical, environmental and experimental data. IARC Press, WHO, Lyon

IARC (International Agency for Research on Cancer) (1987) IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans. Supplement 7. Overall evaluations of carcinogenicity: an updating of the IARC monographs vols 1 to 42. IARC Press, Lyon

Jacob J, Seidel A (2002) Biomonitoring of polycyclic aromatic hydrocarbons in human urine. J Chromatogr B Analyt Technol Biomed Life Sci 778:31–47

Jongeneelen FJ (1992) Biological exposure limit for occuptional exposure to coal tar pitch volatiles at cokeovens. Int Arch Occup Environ Health 63:511–516

Jongeneelen FJ (1993) Biological exposure limit for occupational exposure to polycyclic aromatic hydrocarbons in the coke industry. Arch Toxicol 67:55–58

Jongeneelen FJ, Leijdekkers CM, Bos RP, Theuws JLG, Henderson PT (1985) Excretion of 3-hydroxy-benzo(*a*)pyrene and mutagenicity in rat urine after exposure to benzo(*a*)pyrene. J Appl Toxicol 5:277–282

Jongeneelen FJ, Bos RP, Anzion RBM, Theuws JLG, Henderson PT (1986) Biological monitoring of polycyclic aromatic hydrocarbons: metabolites in urine. Scand J Work Environ Health 12:137–143

Jongeneelen FJ, Anzion RBM, Henderson PT (1987) Determination of hydroxylated metabolites of polycyclic aromatic hydrocarbons in urine. J Chromatogr 413:227–232

Krewski D, Thorslund T, Withey J (1989) Carcinogenic risk assessment of complex mixtures. Toxicol Ind Health 5:851–867

Kuljukka T, Vaaranrinta R, Mutanen P, Veidebaum T, Sorsa M, Kalliokoski P, Peltonen K (1997) Assessment of occupational exposure to PAHs in an Estonian coke oven plant: correlation of total external exposure to internal dose measured as 1-hydroxypyrene concentration. Biomarkers 2:87–94

Lee BM, Santella RM (1988) Quantitation of protein adducts as a marker of genotoxic exposure: immunologic detection of benzo(*a*)pyrene–globin adducts in mice. Carcinogenesis 9:1773–1777

Levin JO (1995) First international workshop on hydroxypyrene as a biomarker for PAH exposure in man—summary and conclusions. Sci Total Environ 163:165–168

Lewtas J, Mumford J, Everson RB, Hulka B, Wilcosky T, Kozumbo W, Thompson C, George M, Dobias L, Sram R, Li X, Gallagher J (1993) Comparison of DNA adducts from exposure to complex mixtures in various human tissues and experimental systems. Environ Health Perspect 99:89–97

Mitchell CE (1982) Distribution and retention of benzo(*a*)pyrene in rats after inhalation. Toxicol Lett 11:35–42

Ny ET, Heederik D, Kromhout H, Jongeneelen F (1993) The relationship between polycyclic aromatic hydrocarbons in air and in urine of workers in a Soderberg potroom. Am Ind Hyg Assoc J 54:277–284

Ovrebo S, Haugen A, Fjeldstad PE, Hemminki K, Szyfter K (1994) Biological monitoring of exposure to polycyclic aromatic hydrocarbon in an electrode paste plant. J Occup Med 36:303–310

Peltonen K, Dipple A (1995) Polycyclic aromatic hydrocarbons: chemistry of DNA adduct formation. J Occup Environ Med 37:52–58

Petry T, Schmid P, Schlatter C (1996) Airborne exposure to polycyclic aromatic hydrocarbons (PAHs) and urinary excretion of 1-hydroxypyrene of carbon anode plant workers. Ann Occup Hyg 40:345–357

Poirier MC, Beland FA (1992) DNA adduct measurements and tumor incidence during chronic carcinogen exposure in animal models—implications for DNA adduct-based human cancer risk assessment. Chem Res Toxicol 5:749–755

Popp W, Vahrenholz C, Schell C, Grimmer G, Dettbarn G, Kraus R, Brauksiepe A, Schmeling B, Gutzeit T, von Bülow J, Norpoth K (1997) DNA single strand breakage, DNA adducts, and sister chromatid exchange in lymphocytes and phenanthrene and pyrene metabolites in urine of coke oven workers. Occup Environ Med 54:76–183

Sathirakul K, Suzuki H, Yamada T, Hanano M, Sugiyama Y (1994) Multiple transport systems for organic anions across the bile canalicular membrane. J Pharmacol Exp Ther 268:65–73

Shugart L, Kao J (1985) Examination of adduct formation in vivo in the mouse between benzo(*a*)pyrene and DNA of skin and hemoglobin of red blood cells. Environ Health Perspect 62:223–226

Simon P, Lafontaine M, Delsaut P, Morele Y, Nicot T (2000) Trace determination of urinary 3-hydroxybenzo[*a*]pyrene by automated column-switching high-performance liquid chromatography. J Chromatogr B 748:337–348

Tyndyk ML, Zabezhinski MA, Bykov VJ, Dikun PP, Dymochka LA, Nepomnyaschaya OB, Yatsuk OS, Yermilov VB, Likhachev AJ (1994) Individual values of excretion of benzo[*a*]pyrene metabolites and susceptibility to its carcinogenic effect in rats. Cancer Lett 78:163–170

Tzekova A, Leroux S, Viau C (2003) Electrophilic tissue burden in male Sprague-Dawley rats following repeated exposure to binary mixtures of polycyclic aromatic hydrocarbons. Arch Toxicol (in press) DOI: 10.1007/s00204-003-0518-z

van Welie RTH, van Dijck RGJM, Vermeulen NPE, Vansittert NJ (1992) Mercapturic acids, protein adducts, and DNA adducts as biomarkers of electrophilic chemicals. Crit Rev Toxicol 22:271–306

Viau C, Mercier M, Blondin O (1993) Measurement of hemoglobin and albumin adducts of benzo(*a*)pyrenediolepoxide and their rate of elimination in the female Sprague-Dawley rat. Arch Toxicol 67:468–472

Viau C, Bouchard M, Carrier G, Brunet R, Krishnan K (1999) The toxicokinetics of pyrene and its metabolites in rats. Toxicol Lett 108:201–208