HHS Public Access

Author manuscript

Chemosphere. Author manuscript; available in PMC 2018 December 01.

Published in final edited form as:

Chemosphere. 2017 December ; 189: 365–372. doi:10.1016/j.chemosphere.2017.09.077.

Measurement of Urinary Benzo[a]pyrene Tetrols and Their Relationship to Other Polycyclic Aromatic Hydrocarbon Metabolites and Cotinine in Humans

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Abstract

Biomonitoring of exposure to polycyclic aromatic hydrocarbons (PAHs) typically uses measurement of metabolites of PAHs with four or less aromatic rings, such as 1-hydroxypyrene, even though interest may be in exposure to larger and carcinogenic PAHs, such as benzo[*a*]pyrene (B[*a*]P). An improved procedure for measuring two tetrol metabolites of B[*a*]P has been developed. Using 2 mL urine, the method includes enzymatic deconjugation of the tetrol conjugates, liquid-liquid extraction, activated carbon solid phase extraction (SPE) and Strata-X SPE, and gas chromatography–electron capture negative ionization–tandem mass spectrometric determination. Limits of detection were 0.026 pg/mL (benzo[*a*]pyrene-r-7,t-8,t-9,c-10-tetrahydrotetrol, BPT I-1) and 0.090 pg/mL (benzo[*a*]pyrene-r-7,t-8,c-9,c-10-tetrahydrotetrol, BPT II-1). We quantified BPT I-1 and BPT II-1 in urine from a volunteer who consumed one meal containing high levels of PAHs (barbequed chicken). We also measured urinary concentrations of BPT I-1 and BPT II-1 in smokers and nonsmokers, and compared these concentrations with those of monohydroxy PAHs (OH-PAHs) and cotinine. Urinary elimination of BPT I-1 and BPT II-1 as a function of time after dietary exposure was similar to that observed previously for OH-PAHs. While the median BPT I-1 concentration in smokers' urine (0.069 pg/mL) significantly differs from nonsmokers (0.043 pg/mL), BPT I-1 is only weakly correlated with cotinine. The urinary concentration of BPT I-1 shows a weaker relationship to tobacco smoke than metabolites of smaller PAHs, suggesting that other routes of exposure such as for example dietary routes may be of larger quantitative importance.

Keywords

Benzo[*a*]pyrene; PAH; Methylnaphthalene; 1-Hydroxypyrene; Tobacco Smoke; Polycyclic Aromatic Hydrocarbons

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CONFLICT OF INTERST

The authors declare no conflict of interest.

Supplementary Information

Supporting information includes a list of standard materials used, demographic information, and detailed instrumental conditions. This material is available at doi:...

1. INTRODUCTION

Smoking, diet, and combustion processes are common sources of exposure to polycyclic aromatic hydrocarbons (PAHs) in the general population (EFSA 2008). While PAHs, as a class, are recognized as hazardous, individual PAHs possess different degrees of toxicity and carcinogenicity (EPA 2010). For example, benzo[*a*]pyrene (B[*a*]P) is a human carcinogen, while phenanthrene and pyrene are not classified as human carcinogens (IARC, 2002, 2010). Due to the absence of reliable methods for assessing B[a]P exposure in humans, indirect methods have been used, such as urinary 1-hydroxypyrene, a metabolite of pyrene that has been shown to correlate with external exposure to PAHs, including B[*a*]P (Brandt and Watson, 2003). However, the relative proportion of B[*a*]P and pyrene differs from source to source (Dennis et al., 1991; Khalili et al., 1995; Lee et al., 2011), adding to the uncertainty in the estimated exposure of B[*a*]P when only based on 1-hydroxypyrene measurements. While B[*a*]P exposure from tobacco smoke can be estimated based on knowledge of the smoke intake and composition of the smoke (Ding et al., 2012), such estimates are associated with uncertainty because smoke chemistry varies across individual tobacco products and individual smokers' puff frequencies and durations (St Charles et al., 2010). For these reasons, the use of specific B[*a*]P human exposure biomarkers may be preferable for exposure assessment, particularly when evaluating relative sources of exposure.

B[*a*]P is oxidized by cytochrome P450 to yield several epoxides (two are shown in Fig. 1). The epoxides can rearrange to form monohydroxy B[*a*]P, such as 3-hydroxybenzo[*a*]pyrene (3-OH-B[*a*]P). 3-OH-B[*a*]P has been proposed as a biomarker, but shows a low rate of detection (Lafontaine et al., 2006) or requires large (50 mL) sample volume (Yao et al., 2014). Alternatively, the epoxide may be hydrolyzed to a diol by an epoxide hydrolase (EH), as is shown for the 7,8-epoxide in Fig. 1. The 7,8-diol is further oxidized to the diolepoxide (Conney, 1982), which is hydrolyzed to a tetrol. Methods such as ELISA or ^{32}P-postlabeling, intended to measure B[*a*]P adducts, possess adequate sensitivity but lack specificity as they may also respond to compounds other than the B[*a*]P metabolites (Gyorffy et al., 2008). Quantitative methods with structural specificity for determining B[a]P metabolites in blood (Pastorelli et al., 1996; Melikian et al., 1997; Ozbal et al., 2000) and urine (Simpson et al., 2000; Lafontaine et al., 2006) have been reported. Methods to measure adducts of B[a]P to DNA or proteins have shown low levels of detection in nonsmokers (Boysen and Hecht, 2003). Ragin et al. (2008) measured BPT I-1 and BPT II-1 individually in human hemoglobin, with BPT II-1 present at levels between 4% and 63% of the BPT I-1 in the same sample. Methods for determining B[a]P metabolites in urine have also shown low detection rates for nonsmokers until a method was reported (Zhong et al., 2011) for measuring benzo[*a*]pyrene-r-7,t-8,t-9,c-10-tetrahydrotetrol (BPT I-1) in urine. The method uses two solid phase extraction (SPE) steps and gas chromatography – electron capture negative ionization – tandem mass spectrometry (GC-ECNI-MS/MS). This method was reported to give 100% detection in nonsmokers, but depended on a phenylboronate SPE cartridge, which subsequently has not retained BPT I-1 in our hands. This change in performance was also noted in a revised method which provides results for specific B[*a*]P tetrol enantiomers (Hecht and Hochalter, 2014). Both methods detected BPT I-1 in all urine

samples analyzed and showed BPT I-1 to be two times higher in smokers than nonsmokers. Neither of these methods reports values for BPT II-1.

While 1-hydroxypyrene is commonly used to estimate exposure to PAHs, this has largely been because of the relative consistency of the composition of PAHs in industrial settings (Hansen et al., 2008). The reliability of 1-hydroxypyrene as a marker in association with the more carcinogenic PAHs, such as B[*a*]P, has yet to be demonstrated in non-occupationally exposed persons, particularly in any attempt to show relative contributions of B[*a*]P from various sources.

We developed a method for measuring BPT I-1 and benzo[*a*]pyrene-r-7,t-8,c-9,c-10-tetrahydrotetrol (BPT II-1) in urine. We tested the usefulness of BPT I-1 and BPT II-1 as exposure biomarkers by examining their urinary concentrations in samples from a volunteer who consumed one meal containing high levels of PAHs (barbequed chicken). We also measured concentrations of BPT I-1 and BPT II-1 in commercially obtained smokers and nonsmokers urine and compared these concentrations with those of other urinary PAH biomarkers and cotinine.

2. EXPERIMENTAL PROCEDURES

2.1. Materials and Standards

All reagents and solvents were of pesticide or equivalent grade. PAH tetrols were obtained from the MRI Global Chemical Carcinogen Repository (Kansas City, MO, USA). $^{13}C_{12}$ decachlorobiphenyl ($^{13}C_{12}$-PCB209) and $^{13}C_6$ labeled BPT I-1 and II-1 were obtained from Cambridge Isotope Laboratory (Andover, MA). All neat standards had purity at or above 99%. β-Glucuronidase type H-1 with sulfatase activity, from *Helix pomatia,* was obtained from Sigma-Aldrich (St. Louis, MO, USA). ENVI-Carb cartridges (0.5 g, 6 mL) were obtained from Supelco (Bellefonte, PA, USA) and Strata-X cartridges (200 mg, 6 mL) were obtained from Phenomenex (Torrance, CA, USA). Standard reference materials SRM 3672 (smokers urine) and SRM 3673 (nonsmokers urine), used as quality control (QC) materials, were obtained from the U.S. National Institute of Standards and Technology (Gaithersburg, MD, USA).

First morning void urine samples from 30 smokers and 30 nonsmokers were purchased from Bioreclamation (Westbury, NY, USA). Smoking status was self-reported at the time of sample collection and was further confirmed by cotinine measurement at the Centers for Disease Control and Prevention (CDC) using an LC-MS/MS method (Wei, et al., 2014). Nonsmoker samples with a continine urinalry level above 100 ng/mL were excluded (n=4), as this level of cotine is expected only in smokers (Haufroid and Lison, 1998).

The collection of urine specimens used to evaluate the excretion profile of BPT I-1 and BPT II-1 after a controlled dietary exposure was described in detail elsewhere (Li et al., 2012). In brief, non-occupationally exposed nonsmoking participants ate a barbequed chicken meal known to contain high levels of PAHs, and collected urine before and after the meal. We measured the two PAH tetrols in a subset of samples taken from one participant 0.5, 7, 17.5, 20.5, and 21.5 hours after consumption of the meal. (Sufficient urine samples were available

from the previous study only for the one participant.) The samples at 0.5 and at 21.5 hours had shown only background levels of monohydroxylated metabolites of small PAHs (OH-PAHs) in prior work (Li et al., 2012). The CDC Institutional Review Board reviewed and approved the study activities.

2.2. Analytical Methods

Sample preparation included enzymatic hydrolysis, liquid-liquid extraction, purification on activated carbon, fractionation on a Strata-X solid phase extraction cartridge, and derivatization (Fig. 2). Urine (2 mL) was added to sodium acetate buffer containing 10 mg/mL β-glucuronidase/arylsulfatase (1M, pH 5.5, 1 mL) and internal standard solution ($^{13}C_6$-BPT I-1 and $^{13}C_6$-BPT II-1, each 2 ng/mL, in 10% methanol [MeOH]/90% dimethylformamide [DMF], 10 μL). After overnight enzymatic hydrolysis (37 °C), the sample was diluted with water (2 mL) and washed twice with 20% toluene in pentane (5 mL). The aqueous fraction was extracted twice with ethyl acetate (EtOAc, 5 mL). The combined EtOAc fractions were washed with 0.1 M sodium ascorbate (2 mL). The organic fraction was evaporated to dryness, re-dissolved in MeOH (1 mL), diluted with water (4 mL) and applied to an ENVI-Carb cartridge, after the cartridge was conditioned with EtOAc, MeOH, and water (4 mL each). The cartridge was rinsed with EtOAc (4 mL) and 20% MeOH in EtOAc (4 mL), and eluted with 45% toluene in MeOH (26 mL). The eluent, which contained the PAH tetrols, was evaporated to dryness, re-dissolved in water (4 mL), and loaded onto a Strata-X cartridge (200 mg, 6 mL) that was pre-conditioned with 15% acetonitrile in MeOH, MeOH, and water (each 3 mL). The Strata-X cartridge was washed with water (2 mL), 1% ammonium hydroxide and 10% MeOH in water (5 mL), and 60% MeOH in water (4 mL), followed by the elution of the PAH tetrols with 15% acetonitrile in MeOH (10 mL). The eluent was concentrated to 0.5 mL, transferred to a GC vial, and evaporated to dryness. Recovery standard ($^{13}C_{12}$-PCB209, 30 ng/mL in DMF, 5 μL) and *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA, 15 μL) was added and the target analytes in the urine extract were derivatized to trimethylsilyl derivatives at 60 °C for 1 hour.

GC-ECNI-MS/MS was performed on a Thermo Trace GC Ultra and TSQ Quantum tandem mass spectrometer (Thermo Scientific, Waltham, MA, USA) configured with a programmable temperature vaporization inlet. A 10 μL injection was made under large volume injection (solvent vent) conditions. After injection, the solvent was evaporated in the inlet at 70 °C, then the inlet temperature was raised to 310 °C for transfer of analytes to the column. Separations were carried out using an Rxi®-17Sil MS column (30 m × 0.25 mm × 0.25 μ, Restek) using helium as the carrier gas. The GC oven was programmed from 70 °C to 310 °C at 30 °C/min and holding at final temperature for 6 min.

Ammonia was used as the ECNI reagent gas and argon as the collision gas. MS detection used the transition at 446.00 → 255.20 for B[*a*]P tetrols with the corresponding transition of 452.50 → 261.20 for the ^{13}C-labeled B[*a*]P tetrol. Further instrumental conditions are given in Table S2. Peak identities were confirmed by relative retention time within ±0.25%.

Samples were run across 7 batches, each including method blanks (N = 4), SRM 3672 (N = 2), SRM 3673 (N = 2), and 10 study samples. Results for PAH tetrols were blank corrected by subtracting the mean value determined for method blanks in each sample batch. The limit

of detection (LOD) according to Taylor (Taylor, 1987) was less than the standard deviation of method blanks (N = 28), pooled across batches. Hence, the method LOD was computed as three times the pooled standard deviation of the method blanks.

Recovery was determined by adding labeled $^{13}C_6$ BPT I-1 and $^{13}C_6$ BPT II-1 (20 pg each) to unspiked and spiked urine (a smoker, and a nonsmoker, 2 replicates each) after all extraction steps. For spiking, 20 pg each unlabeled BPT I-1 and BPT II-1 were added to 2 mL urine. The area ratio obtained from the GC/MS analysis was compared to the area ratios obtained by combining the same quantity of spike and internal standard into a GC vial with solvent and evaporated derivatized, and chromatographed with the extracts from the urine samples.

Urinary OH-PAHs (monohydroxylated metabolites of naphthalene, fluorene, phenanthrene and pyrene), monohydroxylated methylnaphthols (Me-OH-NAPs), and cotinine were measured on the samples for this study according to published methods (Li, et al., 2014; Wei et al., 2014).

2.3. B[*a*]P Tetrol Peak Identification

Detection of B[*a*]P tetrols in urine was confirmed by comparisons of retention times of BPT I-1 and BPT II-1 to ^{13}C labeled BPT I-1 and BPT II-1 across three chromatographic stationary phases: ZB-5 MS (Phenomenex), Rxi-17 (Restek), and DB-1701 (Agilent). Additionally the ratio of peak areas for unlabeled and labeled compound for each BPTI-1 and BPT II-1 were examined across the three stationary phases for indication of coeluting interferences.

2.4. Data Analysis

Statistical analysis was performed with SAS Enterprise Guide Version 5.1 (SAS Institute, Cary NC, USA). Probability plots (Q-Q) showed the PAH metabolites (OH-PAHs and tetrols) tended to be log normally distributed both within the smokers and the nonsmokers.

Statistical evaluation of concentration data was limited to analytes with a detection frequency over 50% to limit the influence of measurements falling below LOD. Concentrations < LOD were substituted with LOD divided by the square root of 2 for calculating interquartile range, median, geometric means, and correlation (Hornung and Reed, 1990). We compared geometric means by smoking status using t-test on log-transformed data. For BPTI-1, we also used a nonparametric test (Wilcoxon rank sum) to compare medians because of the low (< 60%) detection frequency in nonsmokers. We calculated the correlation of log-transformed PAH metabolites in the combined smokers and nonsmokers with log-transformed urinary cotinine, 1-hydroxypyrene and BPT I-1.

3. RESULTS

3.1. BPT I-1 Method Characteristics

The LODs for BPT I-1 and BPT II-1 in urine were 0.026 pg/mL and 0.090 pg/mL, respectively. Average recovery was 58% (BPT I-1) and 42% (BPT II-1). The concentration

of BPT I-1 in SRM 3672 (smokers urine) was determined to be 0.079 pg/mL (SD = 0.013, N = 14).

Exact matches of retention times were used to confirm presence of BPT I-1 and BPT II-1 in urine from individual smokers on three GC stationary phases (ZB-5 MS, Rxi-17, DB-1701). The peak area ratio between each analyte and the corresponding labeled internal standard was consistent across GC stationary phases suggesting absence of interfering coeluting compounds. Example chromatograms of BPT I-1 and BPT II-1 in a smoker and a nonsmoker are shown in Fig. 3.

3.1.1. Excretion Profile—The urinary concentrations of BPT I-1 and BPT II-1 increased in the volunteer after the consumption of a high PAH-containing barbeque chicken meal, supporting the potential of BPTI-1 as a biomarker of B[*a*]P exposure. The BPT I-1 concentration increased from < LOD at the time of the PAH containing meal to 0.98 pg/mL 7 hours after the meal (Table S3), an increase of at least 40 times the initial value, and then decreased to baseline at approximately 24 hours (Fig. 4). BPT II-1 showed a similar trend, with an increase from < LOD at the time of the meal to 0.97 pg/mL at 7 hours (an increase of at least 11 times). The elimination pattern of the BPT isomers was similar to those reported previously for the OH-PAHs (Li et al., 2012), as exemplified by 1-hydroxypyrene and 2-hydroxynaphthalene in Fig. 4.

3.1.2. Comparison of Smokers and Nonsmokers—The demographics of the 60 smoking and nonsmoking donors are detailed in Table S1. Smokers ranged in age from 18 to 63 years (median = 23 years) while nonsmokers ranged in age from 19 to 75 years (median = 26 years). Four of the 30 self-reported nonsmokers had urinary cotinine values above 100 ng/mL (Haufroid and Lison 1998) and were excluded from further analyses. Urinary cotinine levels for nonsmokers (n = 26) ranged from undetectable (< 5 ng/mL, N = 24) to 83 ng/mL (median < 5 ng/mL) and for smokers (n = 30) from 47 ng/mL to 13,500 ng/mL (median = 2,970 ng/mL).

BPT I-1 was present at a higher concentration in smokers (median: 0.069 pg/mL) than nonsmokers (median: 0.043 pg/mL) [Wilcoxon rank sum test, p = 0.076]. The interquartile ranges of BPT I-1 in nonsmokers and smokers were < LOD – 0.074 pg/mL and 0.031 – 0.10 pg/mL, respectively (Table 1). BPT I-1 was detectable in 54% of nonsmokers compared to 83% in smokers. BPT II-1 was detected in less than 25% of samples for both the nonsmokers and the smokers. Therefore, we did not perform statistical comparison on BPT II-1 by smoking status.

OH-PAHs and Me-OH-NAPs (LOD = 10 pg/mL except hydroxylnaphthalenes at 40 pg/mL) were detected in all smokers, while the detection rates in nonsmokers were 100% for all metabolites, except 5-methyl-2-hydroxynaphthalene (85%), 4-hydroxyphenanthrene (85%), and 1-hydroxypyrene (96%). The concentration of OH-PAHs and Me-OH-NAPs were significantly higher in smokers than in nonsmokers ($p < 0.03$ to $p < 0.0003$), Table 1. The results for OH-PAHs are similar to those obtained for smokers and nonsmokers in the 2011–2012 NHANES (CDC 2015) with results in the current work being about 20% higher for the analytes in nonsmokers and about 5% lower in smokers (Table S4).

Overall, the largest difference in geometric mean concentrations between smokers and nonsmokers observed was for methylnaphthalene metabolites with 8–15 times higher concentration in smokers, followed by naphthalene metabolites (6.0–6.4 times). The smallest difference observed was for phenanthrene metabolites, 1-hydroxypyrene, and BPT I-1, with 1.5 to 2.3 times higher concentration in smokers compared to nonsmokers.

Correlation coefficients between measured PAH metabolites and cotinine, 1-hydroxypyrene and BPT I-1 for smokers and nonsmokers combined are given in Table 2. All correlations were statistically significant ($p < 0.05$). Correlation coefficient with cotinine was the highest (Pearson's $r > 0.89$) for methylnaphthalene metabolites. 1-Hydroxypyrene shows the greatest correlation ($r > 0.8$) with 1-, 2-, 3-, and summed hydroxyphenanthrenes and with 9-hydroxyfluorene. BPT I-1 was most highly correlated ($r > 0.7$) with 1-hydroxyphenanthrene, 1-hydroxypyrene, summed hydroxyphenanthrenes and 9- hydroxyfluorene. The ratio of 1-hydroxypyrene to BPT I-1 in smokers ranged from 602 to 11300 with a mean of 3960. In nonsmokers, this ratio ranged from 698 to 4490, with a mean of 1190. The means of the ratios were significantly different between smokers and nonsmokers ($p < 0.02$).

4. DISCUSSION

4.1. Analytical Method for BPT I-1 and BPT II-1

Our proposed method included deconjugation followed by clean-up through several steps, including liquid-liquid extraction, activated carbon SPE, and Strata-X SPE. A previous method for BPT I-1 used deconjugation followed by Strata-X SPE and phenylboronate SPE (Zhong et al., 2011). The same group of researchers subsequently published a revised method that involves fractionation of samples on an LC column and uses separate GC injections to measure the enantiomers of BPT I-1 (Hecht and Hochalter, 2014). The revised technique no longer used a phenylboronate cartridge, which no longer retains B[a]P-tetrols with *cis*-tetrol groups. We developed and optimized the multiple sample clean-up steps in the present method, and we were able to provide adequate sensitivity and selectivity and obtain results for BPT I-1 and BPT II-1 in a single GC injection.

We found that B[*a*]P tetrols easily absorbed to glass surfaces from aprotic solvents (data not shown), therefore, a small quantity of methanol (10%) is required in the solvent (DMF) used for making standards and for transferring the final extract to the GC-vial. With the transfer solvents completely evaporated from the samples, we used an excess of MSTFA to reach a complete derivatization in the final GC-vial. This results in an increased volume of the final derivatized extract (20 μL). Due to this dilution of the sample, we used a large volume injector and injected 10 μL of the derivatized extract into the GC-ECNI-MS/MS.

4.1.1. Elimination Profile after a Known Dietary Exposure—The elimination profiles for BPT I-1 and BPT II-1 (Fig. 4) show a rise and fall above background over several hours following a known dietary exposure (a barbecued meal), demonstrating the potential utility of these compounds as biomarkers to reflect recent dietary exposure. Tetrol urinary concentrations appeared to remain elevated longer than for OH-PAHs. Further studies are needed to assess the usability of tetrol metabolites as makers for dietary exposure to B[a]P.

4.1.2. PAHs and Smoke Exposure—The geometric mean of BPT I-1 in smokers is about 1.5 times that in nonsmokers, similar to results reported by others (2 times greater in smokers) (Zhong et al., 2011, Hecht and Hochalter, 2014). While tobacco smoke may be an important individual source of exposure to B[*a*]P in non-occupationally exposed persons, diet has been estimated to contribute more B[*a*]P than smoking for the average smoker (Hattemer-Frey and Travis, 1991). In the current study, all donors are from the same commercial source and smokers are not limited to those above a particular number of cigarettes per day. The concentration of BPT I-1 across smokers is only 50% greater than in nonsmokers. This can be contrasted with the concentration of BPT I-1 after consumption of a barbecued meal by a volunteer, which rose to a urinary concentration of 0.98 pg/mL, well above that measured at the 75th percentile in the smokers (0.10 pg/mL), and remained above 0.10 pg/mL until approximately 20 hours after consumption of the meal. For assessment of exposure to B[*a*]P from smoking in the general population, exposure from other sources cannot be assumed to be inconsequential. While the level of BPT II-1 measurably elevated in the volunteer following the barbecued meal, the detection rate of less than 25% in both smokers and nonsmokers for BPT II-1 shows this biomarker to be of interest, but not usable for studies of the general population at the current limit of detection.

Cotinine, 1-hydroxypyrene and BPT I-1, while correlated to varying degrees, are limited in their ability to predict each other. Metabolites of the smaller PAHs, such as naphthalene and fluorene, showed better correlation with tobacco smoke exposure, as indicated by cotinine, than metabolites of the larger PAH such as 1-hydroxypyrene and BPT I-1. Additionally the urinary concentrations of naphthalene and methylnaphthalene metabolites in smokers were 6–14 times higher than in nonsmokers, strongly suggesting that the main source of these compounds in smokers is tobacco smoke. Phenanthrene metabolites, 1-hydroxypyrene and BPT I-1 showed a smaller increase in smokers over nonsmokers (less than 4 times) and were not as well correlated with cotinine, which was most likely due to a larger contribution of other sources than tobacco smoke, such as diet (Khalili et al., 1995; St Charles et al., 2010; Li et al., 2012). These observations generally agree with other work in which a metabolite for a lower molecular weight PAH (1-hydroxyfluorene) was considered to be a good biomarker for tobacco smoke (St Helen et al., 2012) and 1-hydroxypyrene was shown to correlate poorly with cotinine (Murphy et al., 2004). The metabolites of the larger PAHs show variation in concentration which reflect other factors than just exposure to tobacco smoke. While 1-hydroxypyrene and BPT I-1 are correlated with each other, the ratio between the two metabolites differs between the smokers and nonsmokers. This difference may reflect differences in PAH sources, metabolism, or both. Because BPT I-1 is a metabolite of B[*a*]P through the formation of diol-expoxide, and, the diol-expoxide is known to be a carcinogenic metabolite of B[a]P, (Conney, 1982) the use of BPT I-1 as a biomarker merits further investigation.

5. CONCLUSIONS

This work demonstrates an analytical method that is suitable for measurement of BPT I-1 and BPT II-1 in urine, with a LOD that enables the detection of BPT I-1 in non-occupationally exposed smokers as well as in nonsmokers. The BPT I-1 concentration is found to be elevated in smokers compared to nonsmokers by a factor of 1.5, in agreement

with other researchers. The ratio of 1-hydroxypyrene to BPT I-1 differs between the two groups, suggesting the BPT I-1 and 1-hydroxypyrene concentrations provide differing information regarding PAH exposure or metabolism. The elevated concentration of BPT I-1 after consumption of a PAH-rich meal suggests that B[a]P exposure from sources other than tobacco smoke is significant, and that measurement of a biomarker for B[a]P, rather than for other PAHs, may be important in evaluating relative contribution of B[a]P exposure from various sources.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Drs. Benjamin Blount and Jun Feng for providing the urine cotinine measurements, Lee-Yang Wong for advice in statistical data analysis, Johnathan Parker and Alisha Etheridge for assistance in confirmation of analytes, and Hassan Carmichael and Erin (Nikki) Pittman for the measurements of OH-PAHs and Me-OH-NAPs. This work was funded through interagency agreement 224-10-9022 from the US Food and Drug Administration Center for Tobacco Products. The findings and conclusions in this report are those of the authors and do not necessarily represent the Centers for Disease Control and Prevention. Use of trade names is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention, the Public Health Service, or the U.S. Department of Health and Human Services.

ABBREVIATIONS

1-PYR	1-Hydroxypyrene
$^{13}C_{12}$-PCB209	$^{13}C_{12}$ Labeled decachlorobiphenyl
B[*a*]P	Benzo[*a*]pyrene
BPT I-1	benzo[*a*]pyrene-r-7,t-8,t-9,c-10-tetrahydrotetrol
BPT II-1	benzo[*a*]pyrene-r-7,t-8,c-9,c-10-tetrahydrotetrol
DMF	Dimethylformamide
EtOAc	Ethyl Acetate
FLU	Fluorene
GC-ECNI-MS/MS	Gas Chromatography with electron capture/negative ionization tandem mass spectrometry
LOD	Limit of detection
MeOH	Methanol
MSTFA	*N*-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide
NAP	Naphthalene
OH-PAH	Monohydroxylated PAH
PAH	Polycyclic aromatic hydrocarbon

PHE	Phenanthrene
PYR	Pyrene

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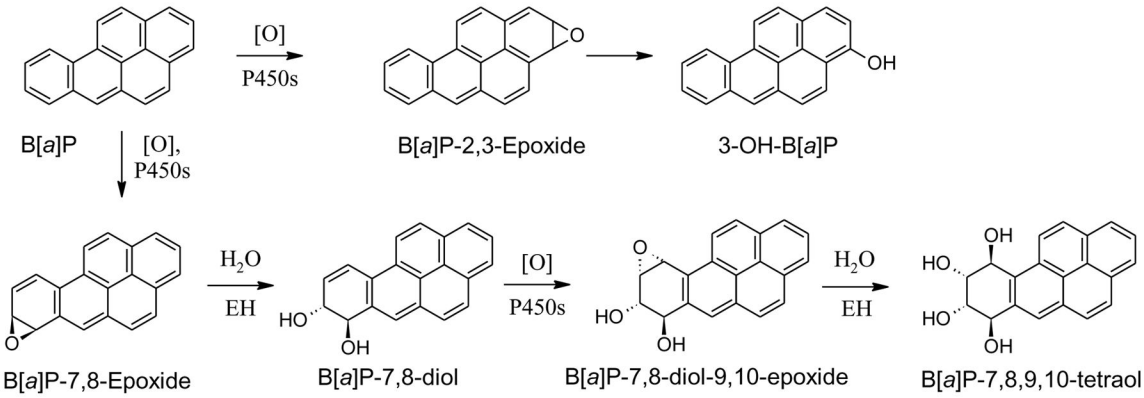

Figure 1.

Pathways to benzo[a]pyrene metabolites. The metabolites shown, 3-OH-B[a]P and BPT I-1, have been used as biomarkers (Lafontaine et al., 2006; Zhong et al., 2011).

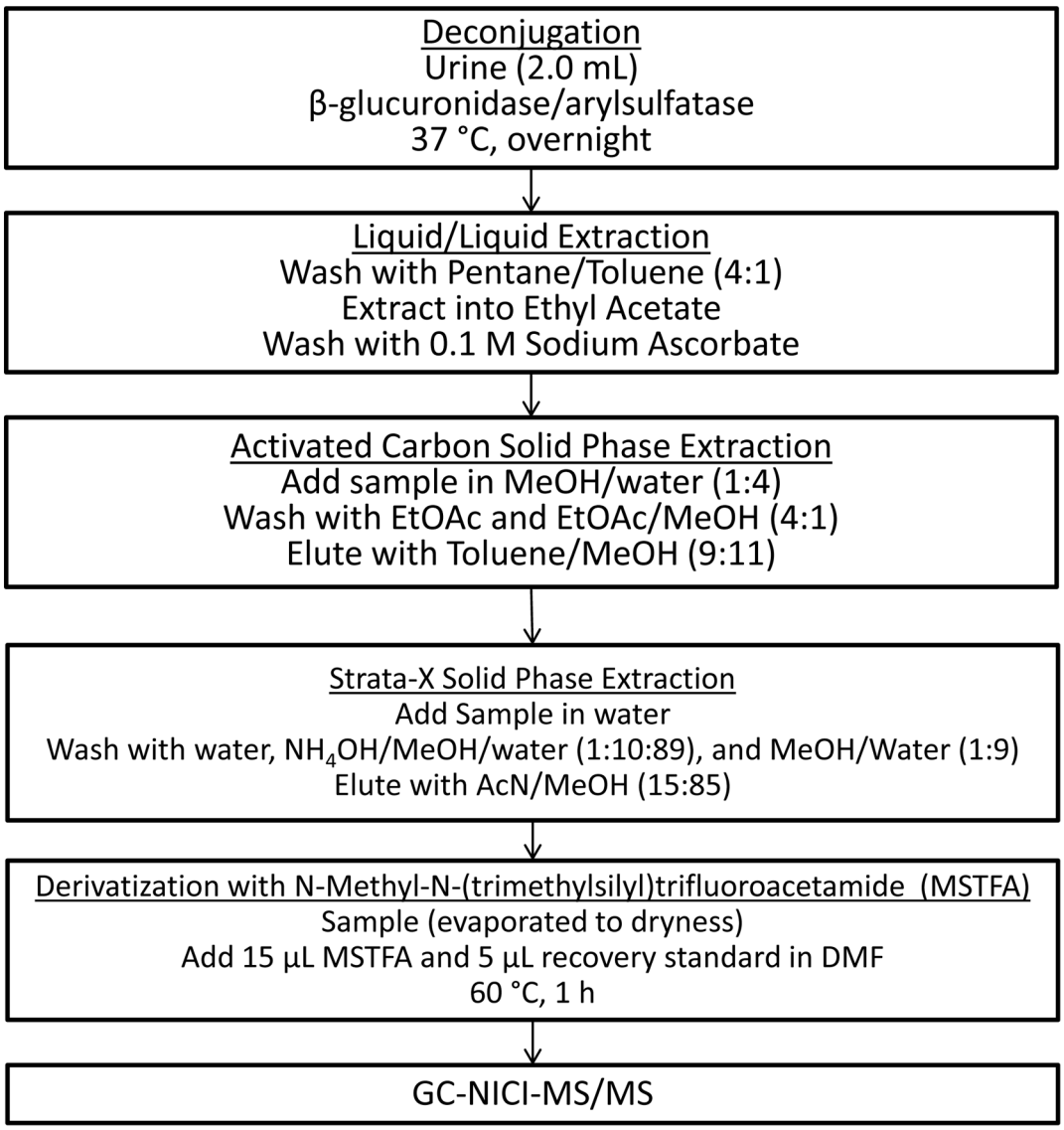

Figure 2.
Sample Preparation for the isolation and quantification of BPT I-1 and BPT II-1 in Urine.

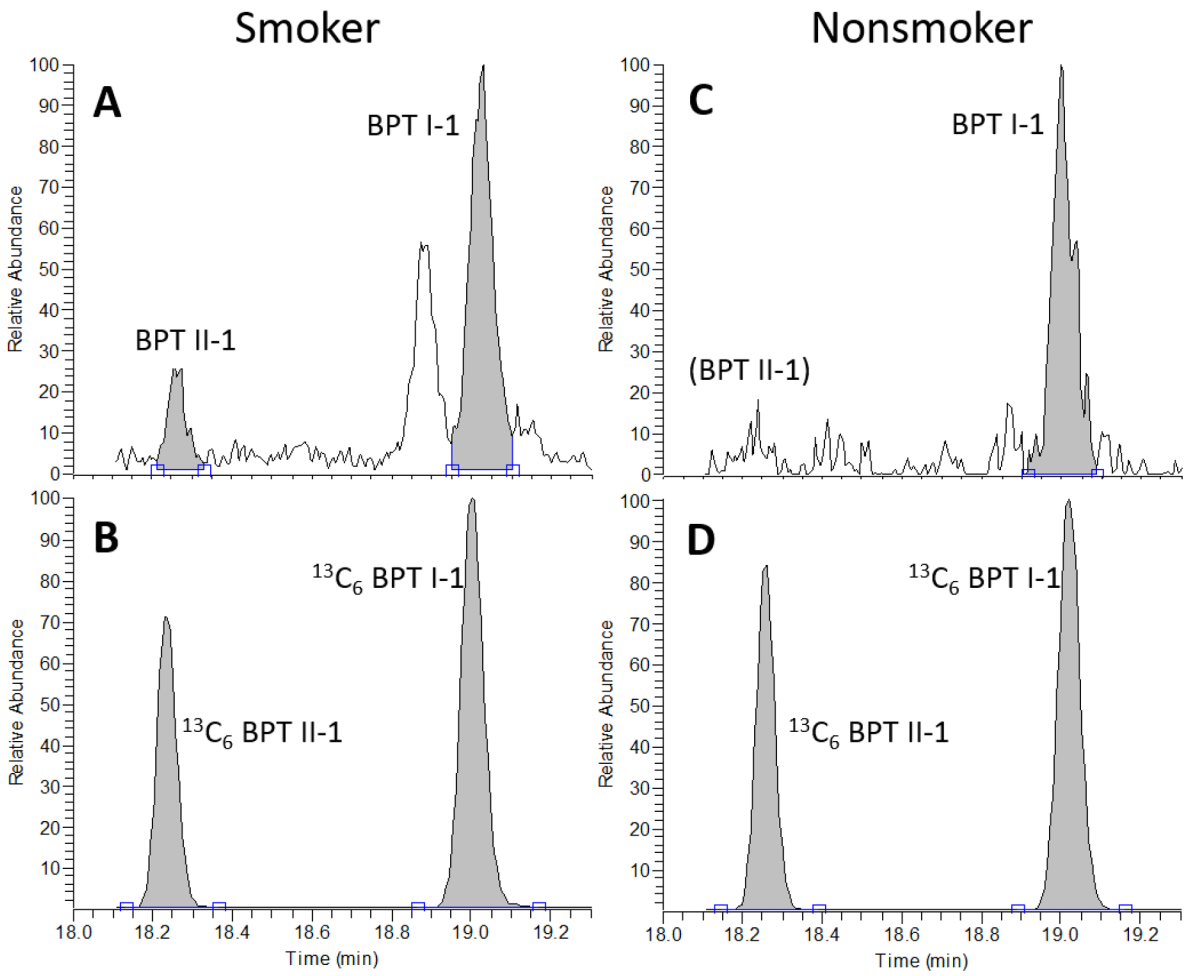

Figure 3.

GC-ECNI-MS/MS Chromatogram of BPT I-1 and BPT II-1 in a smoker (A [native] and B [labeled]) and a nonsmoker (C [native] and D [labeled]). BPT I-1 concentration in smoker is 0.11 pg/mL. BPT I-1 in the nonsmoker and BPT II-1 in both are below the method limit of detection.

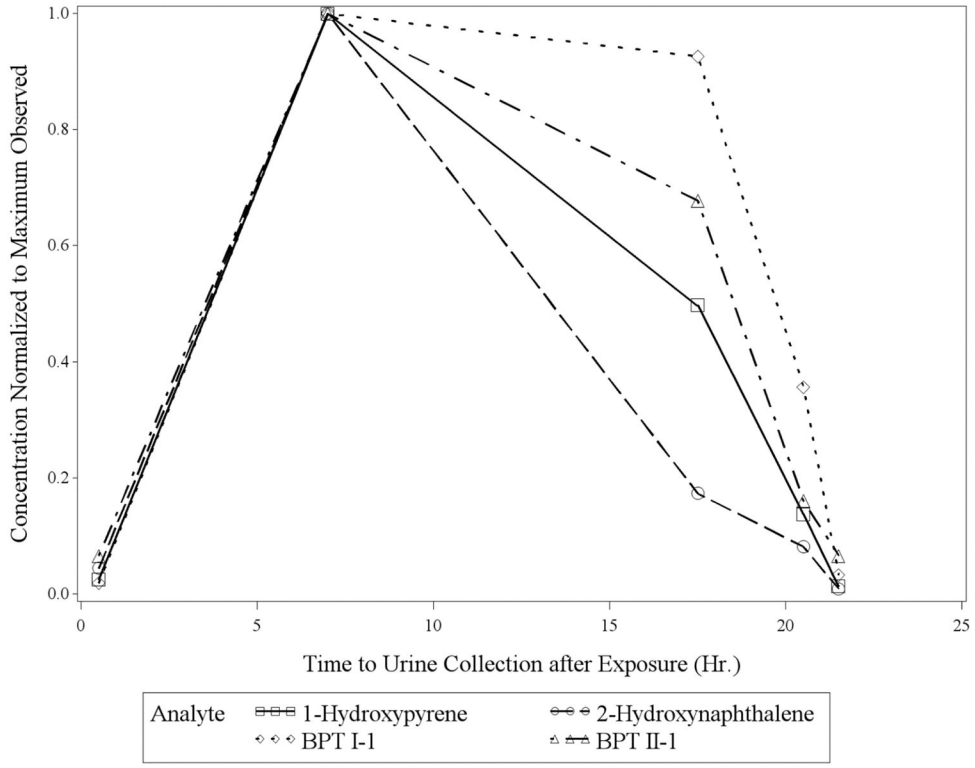

Figure 4.

Urinary concentrations in one subject (normalized to the maximum concentration observed) of BPT I-1 and BPT II-1 after consumption of a barbecued chicken meal (at time 0) compared to profiles for 2- hydroxynaphthalene and 1-hydroxypyrene measured in the same urine samples.

Chemosphere. Author manuscript; available in PMC 2018 December 01.

Table 1

PAH metabolite median, interquartile ranges, geometric mean (GM) concentrations (pg/mL) in nonsmokers (N=26[a]) and smokers (N=30) with the ratio of GMs (smoker/nonsmoker) and significance of the ratio (t-test).

	Nonsmoker		Smoker		Ratio GM Smokers to GM Nonsmokers	
Compound	**Median (IQR)**	**GM**	**Median (IQR)**	**GM**	**Ratio**	**P-value [b]**
Hydroxynaphthalenes (sum)	3620 (2,490 – 5,020)	3,570	23,000 (15,900 – 29,500)	21,700	6.1	<.0001
1-Hydroxynaphthalene	904 (524 – 1,570)	793	6,000 (2,200 – 9,860)	5060	6.4	<.0001
2-Hydroxynaphthalene	2,280 (1400 – 4,470)	2,370	15,800 (8,970 – 25,200)	14,100	6.0	<.0001
Hydroxymethylnaphthalenes (Sum)	875 (588 – 1,650)	922	12,000 (3,250 – 27,600)	10,200	11.0	<.0001
3-Methyl-2-Hydroxynaphthalene/6-Methyl-1-Hydroxynaphthalene	389 (188 – 501)	321	3,120 (966 – 5,870)	2,700	8.4	<.0001
4-Methyl-1-Hydroxynaphthalene	72.8 (56.5 – 130)	80.9	1,480 (288 – 3,260)	1,020	12.6	<.0001
4-Methyl-2-Hydroxynaphthalene/6-Methyl-2-Hydroxynaphthalene/7-Methyl-2-Hydroxynaphthalene	231 (156 – 507)	252	3220 (838 – 9,230)	3,080	12.2	<.0001
5-Methyl-1-Hydroxynaphthalene	97 (55 – 240)	102	1,710 (408 – 3,200)	1,330	13.0	<.0001
5-Methyl-2-Hydroxynaphthalene	21 (14 – 61)	30.1	591 (105 – 1,580)	441	14.7	<.0001
7-Methyl-1-Hydroxynaphthalene/3-Methyl-1-Hydroxynaphthalene/8-Methyl-2-Hydroxynaphthalene	96.4 (50 – 163)	91.8	2,060 (258 – 4,440)	1,300	14.2	<.0001
Hydroxyfluorene (sum)	567 (292 – 696)	511	1610 (1,160 – 2,280)	1,660	3.2	<.0001
2-Hydroxyfluorene	188 (117 – 274)	183	666 (445 – 972)	717	3.9	<.0001
3-Hydroxyfluorene	45.1 (36 – 82)	53.5	321 (160 – 841)	351	6.6	<.0001
9-Hydroxyfluorene	293 (131 – 484)	261	537 (372 – 795)	513	2.0	0.0015
Hydroxyphenanthrene (Sum)	351 (216 – 458)	308	532 (434 – 809)	539	1.7	0.0027
1-Hydroxyphenanthrene	139 (81 – 198)	131	226 (148 – 287)	201	1.5	0.0215
2-Hydroxyphenanthrene	87.3 (53 – 134)	78.5	129 (82 – 191)	126	1.6	0.0093
3-Hydroxyphenanthrene	66.5 (47 – 107)	69.7	160 (105 – 285)	159	2.3	0.0001
4-Hydroxyphenanthrene	25.1 (14.3 – 35.7)	25.1	42.8 (32 – 63)	41.1	1.6	0.0037
1-Hydroxypyrene	118 (71.3 – 177)	100	243 (152 – 365)	223	2.2	0.0004
Benzo[*a*]pyrene-r-7,t-8,t-9,c-10-tetrahydrotetrol (+/−) (BPT I-1)	0.043 (<LOD – 0.074)	0.043	0.069 (0.031 – 0.10)	0.065	1.5[d]	0.091
benzo[*a*]pyrene-r-7,t-8,c-9,c-10-tetrahydrotetrol (BPT II-1) [c]	<LOD		<LOD			

[a] Four nonsmoking subjects were excluded from study because cotinine in urine indicated noncompliance (cotinine > 100 ng/mL).

[b] Probability for the test of the ratio of geometric means = 1.

[c] The detection rate for BPT II-1 is not high enough to give meaningful values for quartiles or geometric means.

[d] There are significant number of non-detects in the computation of the GMs for BPT I-1, the median values may be a better representation, with a ratio for smokers to nonsmokers (1.6) and a significant difference between the two values (Wilcoxon rank sum test, p = 0.076)

Table 2

Correlation coefficients of PAH metabolites with cotinine, 1-PYR, and BPT I-1 (log10-transformed) in 30 smokers and 26 nonsmokers combined. All correlations are significant at the $p<0.05$ level. Correlation coefficients over 0.70 are in bold.

	Cotinine	1-PYR	BPT I-1
Hydroxynaphthalenes (Sum)	**0.780**	0.648	0.482
1-Hydroxynaphthalene	**0.840**	0.649	0.566
2-Hydroxynaphthalene	0.659	0.579	0.413
Hydroxymethylnaphthalenes (Sum)	**0.924**	0.608	0.506
3-Methyl-2-Hydroxynaphthalene/6-Methyl-1-Hydroxynaphthalene	**0.917**	0.633	0.536
4-Methyl-1-Hydroxynaphthalene	**0.912**	0.596	0.483
4-Methyl-2-Hydroxynaphthalene/6-Methyl-2-Hydroxynaphthalene/7-Methyl-2-Hydroxynaphthalene	**0.919**	0.599	0.481
5-Methyl-1-Hydroxynaphthalene	**0.916**	0.602	0.518
5-Methyl-2-Hydroxynaphthalene	**0.895**	0.579	0.504
7-Methyl-1-Hydroxynaphthalene/3-Methyl-1-Hydroxynaphthalene/8-Methyl-2-Hydroxynaphthalene	**0.892**	0.531	0.446
Hydroxyfluorene (sum)	**0.736**	**0.835**	0.684
2-Hydroxyfluorene	**0.780**	**0.784**	0.635
3-Hydroxyfluorene	**0.850**	**0.748**	0.572
9-Hydroxyfluorene	0.489	**0.827**	**0.712**
Hydroxyphenanthrene (Sum)	0.378	**0.922**	**0.719**
1-Hydroxyphenanthrene	0.335	**0.902**	**0.739**
2-Hydroxyphenanthrene	0.301	**0.859**	0.662
3-Hydroxyphenanthrene	0.429	**0.869**	0.627
4-Hydroxyphenanthrene	0.472	**0.780**	0.610
1-Hydroxypyrene	0.467		**0.728**
BPT I-1	0.361	**0.728**	