Published in final edited form as:
Chem Res Toxicol. 2011 January 14; 24(1): 73–80. doi:10.1021/tx100287n.

Analysis of *r*-7,*t*-8,9,*c*-10-Tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene in Human Urine: A Biomarker for Directly Assessing Carcinogenic Polycyclic Aromatic Hydrocarbon Exposure plus Metabolic Activation

Yan Zhong[†,‡], **Steven G. Carmella**[†], **J. Bradley Hochalter**[†], **Silvia Balbo**[†], and **Stephen S. Hecht**[*,†]

[†]Masonic Cancer Center, University of Minnesota, Minneapolis, Minnesota 55455, USA

[‡]Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455, USA

Abstract

Polycyclic aromatic hydrocarbons (PAH) are believed to be causative agents for various types of cancers in humans. Benzo[*a*]pyrene (BaP) is a prototypic carcinogenic PAH, which requires metabolic activation to elicit its detrimental effects. The major end product of its diol epoxide metabolic activation pathway is *r*-7,*t*-8,9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*trans, anti*-BaPT). Individual differences in exposure to, and metabolic activation of, carcinogenic PAH may influence cancer risk. Measurement of PAH metabolites in human urine could provide a direct way to assess individual differences in susceptibility to PAH-related cancer. In this paper, we describe a sensitive and reliable method for quantitation of *trans, anti*-BaPT in human urine using gas chromatography-negative ion chemical ionization-tandem mass spectrometry (GC-NICI-MS/MS). [$^{13}C_6$] *trans, anti*-BaPT was used as the internal standard. The urine was treated with β-glucuronidase and sulfatase, and then *trans, anti*-BaPT was enriched by solid-phase extraction with polymeric reversed phase and phenylboronic acid cartridges. The sample was silylated and analyzed by GC-NICI-MS/MS with selected reaction monitoring (SRM) for the trimethylsilyl (TMS) derivatives of *trans, anti*-BaPT (*m/z* 446→ *m/z* 255) and [$^{13}C_6$]*trans, anti*-BaPT (*m/z* 452→ *m/z* 261). The mean assay recovery was 44%. The instrumental on-column detection limit was about 20 amol of *trans, anti*-BaPT (as BaPT-TMS). *trans, anti*-BaPT was readily detected in all urine samples analyzed including 30 smokers (0.71 ± 0.64 fmol/mg creatinine) and 30 non-smokers (0.34 ± 0.2 fmol/mg creatinine) ($P = 0.0018$). The results of this study demonstrate a highly sensitive and selective method for quantitation of *trans, anti*-BaPT in human urine. This is to our knowledge the first study to show that smokers have significantly higher levels of *trans, anti*-BaPT in their urine than do non-smokers. This method may be useful as a direct phenotyping approach to assess individual differences in uptake and metabolic activation of carcinogenic PAH.

Keywords

benzo[*a*]pyrene metabolites; biomarker; metabolic activation; human urine

[*]To whom correspondence should be addressed: Masonic Cancer Center, University of Minnesota, MMC 806, 420 Delaware St SE, Minneapolis, MN 55455, USA. phone: (612) 624-7604, fax: (612) 626-5135, hecht002@umn.edu.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a large class of compounds mainly formed by incomplete combustion of organic matter. They are commonly found in polluted air and water, tobacco smoke, broiled and smoked foods, and in certain occupational environments such as coke production from coal and other processes involving soots and tars (1–7). Humans readily absorb PAH into the body through the lung, gastrointestinal tract, and skin. Many PAHs are carcinogens, and are believed to be causative agents for various types of human cancers, including lung cancer in smokers (6,8–10). Benzo[*a*]pyrene (BaP, Scheme 1), a prototypic PAH, is rated as carcinogenic to humans by the International Agency for Research on Cancer (10). This five-ring PAH is present in virtually all PAH mixtures, and is one of the most carcinogenic of those commonly detected. It has been conclusively demonstrated in laboratory animal studies that BaP is a powerful carcinogen, which readily induces tumors in various tissues such as lung and skin at relatively low doses (2,8,10–12).

PAHs including BaP are procarcinogens that require metabolic activation to elicit their carcinogenic effects (10,13). Considerable evidence supports the view that these carcinogens act as tumor initiators after metabolic oxidation to reactive electrophiles by the bay region diol epoxide pathway (Scheme 1) (13–15). This pathway starts with an initial cytochrome P450-scatalyzed epoxide formation at the 7,8-position followed by epoxide hydrolase - catalyzed hydration (16–18). Subsequent epoxidation at the 9,10-position then predominantly generates BaP-(7*R*,8*S*)-diol-(9*S*,10*R*)-epoxide (BPDE) (Scheme 1) (13–15,19,20). BPDE is considered to be a major ultimate carcinogen of BaP because it is carcinogenic and readily reacts with DNA to produce the same adducts as observed in biological systems exposed to BaP (13–15,19,20). The major reaction of BPDE in biological systems is hydrolysis producing predominantly BaP-(*7R,8S,9R,10S*)-tetraol, which is one enantiomer of *r*-7,*t*-8,9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*trans, anti*-BaPT). Competing with metabolic activation, detoxification pathways of BaP include phenol formation, glutathione conjugation, glucuronidation, and sulfation (8,13,14).

Approximately 11–24% of lifelong smokers develop lung cancer over their lifetimes (6). It would be important to identify individuals in this group with apparently higher cancer susceptibility. Our working hypothesis is that people who metabolically activate tobacco smoke carcinogens more extensively should have higher cancer risk. Large inter-individual differences in the metabolic activation of carcinogenic PAHs have been demonstrated (21–24). Multiple studies have used genotyping methods to examine the relationship between lung cancer risk and polymorphisms in genes such as *CYP1A1* and *GSTM1* which code for enzymes involved in PAH metabolism (25–33). But the results remain varied and inconsistent, likely because of the complexity of PAH metabolism (34). We propose that a phenotyping strategy, with direct measurement of metabolites generated by the metabolic activation pathways, can provide more comprehensive and reliable information on an individual's susceptibility to cancer (9,35). With this goal in mind, we developed and optimized a gas chromatography-negative ion chemical ionization-tandem mass spectrometry (GC-NICI-MS/MS) method to quantify racemic *trans, anti*-BaPT in human urine.

Materials and Methods

Chemicals, Enzymes and Apparatus

All BaP metabolites used in this study were racemic. *r*-7,*t*-8,9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*trans, anti*-BaPT), *r*-7,*t*-8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*cis, anti*-BaPT), *r*-7,*t*-8,,*c*-9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*cis, syn*-BaPT), *r*-7,*t*-8,*c*-9,*t*-10-tetrahydroxy-7,8,9,10-

tetrahydrobenzo[*a*]pyrene (*trans, syn*-BaPT), *trans*-4,5-dihydro-4,5-dihydroxybenzo[*a*]pyrene (BaP-4,5-diol), *trans*-7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene (BaP-7,8-diol) and *trans*-9,10-dihydro-9,10-dihydroxybenzo[*a*]pyrene (BaP-9,10-diol) were obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository, Midwest Research Institute (Kansas City, MO). The internal standard, [$^{13}C_6$]*trans, anti*-BaPT was purchased from Cambridge Isotope Laboratories, Inc (Andover, MA). Purities of these standards were >99%, as determined by HPLC analysis. β-Glucuronidase and arylsulfatase (from *Helix pomatia*) were obtained from Roche Diagnostics Corp.(Indianapolis, IN). Strata-X polymeric reversed phase SPE cartridges (200 mg/6 mL, #8B-S10-FCH) were procured from Phenomenex (Torrance, CA). Bond Elute phenylboronic acid SPE cartridges (100 mg/1 mL, # 12102018) were from Varian, Inc (Palo Alto, CA). *bis*-Trimethylsilyltrifluoroacetamide (BSTFA) was purchased from Regis Technologies (Morton Grove, IL). Removal of solvents was carried out with an SVC-200 Speedvac (Thermo Savant, Holbrook, NY). Gas chromatography-negative ion chemical ionization-tandem mass spectrometry (GC-NICI-MS/MS) was carried out with a TSQ Quantum instrument (Thermo Scientific).

Urine Samples

This study was approved by the University of Minnesota Institutional Review Board. Urine samples from 30 smokers (11 females) were first morning voids obtained from ongoing studies at the University of Minnesota Tobacco Use Research Center. Current smoking status was confirmed by CO and cotinine levels. Urine samples from thirty non-smokers (16 females) were spot samples taken at similar times of day from laboratory personnel. All urine samples were stored in a −20 °C freezer before analysis.

Determination of urinary creatinine

The creatinine content in urine was determined at the University of Minnesota Medical Center, Fairview, Diagnostic Laboratories, using Vitros CREA slides.

Analysis of Urine for *trans, anti*-BaPT

A 1.5 mL aliquot of urine was placed in a 10 mL centrifuge tube containing 780 μL of 0.5M NaOAc buffer, pH 5, β-glucuronidase (3,500 units) and arylsulfatase (28,000 units). [$^{13}C_6$]*trans, anti*-BaPT (20 fmol) in 2 μL CH_3CN was added as internal standard. The mixture was incubated in a water bath overnight with shaking at 37 °C. A Strata-X cartridge was preconditioned with 5 mL of CH_3OH, then with 5 mL of H_2O. The urine sample was applied to the cartridge slowly, along with two 1 mL H_2O washings of the urine sample tubes. The cartridge was washed with 5 mL of 1% NH_4OH in 50% CH_3OH, then with 0.5 mL CH_3OH. *trans, anti*-BaPT was then eluted with 2 mL CH_3OH. This fraction contained the analyte and internal standard. Solvents were removed by overnight concentration on a Speedvac. The residue was dissolved in 1 mL of 30% aq CH_3OH with sonication and loaded onto a 100 mg/1 mL phenylboronic acid cartridge, which had been preconditioned with 1 mL of CH_3OH and then with 1 mL of H_2O. The cartridge was then washed with 100 μL of 30% CH_3OH in H_2O, placed under vacuum overnight to remove residual H_2O, and then washed twice with 1 mL acetone which had been dried with Na_2SO_4. *trans, anti*-BaPT was eluted with 1 mL of 80% CH_3OH in H_2O. The solution was concentrated to dryness. The residue was dissolved in 200 μL of CH_3OH with sonication and vortexing, transferred to an insert vial, dried in a speed vacuum apparatus, and dissolved in 10 μL of BSTFA. The samples were heated at 60 °C for 60 min with periodic mixing by vortexing, and 2.5 μL was injected into the GC-NICI-MS/MS system.

GC-NICI-MS/MS Analysis

This was modified from that described previously (36). The GC was fitted with a 0.25 mm (inside diameter) × 30 m, 0.15-μm film thickness, DB-17MS column (Agilent Technologies, Palo Alto, CA), and a 0.53 mm (inside diameter) × 2 m deactivated fused silica precolumn. The oven temperature program was as follows: 80 °C for 1 min, then 80 to 200 °C at 35 °C/min, then 200 to 215 °C at 3 °C/min, then 215 to 320 °C at 35 °C/min, then hold for 3 min. The injection port temperature was 250 °C, and the MS transfer line temperature was 320 °C. The flow rate was 1 mL/min He. The injector was operated in the splitless mode; the evaporation temperature was 80 °C for 2 min, then 80 to 260 °C at 5 °C/sec, then hold for 1 min. The NICI-MS/MS conditions were as follows: CI gas, methane at 2.0 mL/min; source temperature, 200 °C; emission current, 500 μA. Selected reaction monitoring (SRM) with a collision energy of 18 eV, electron energy of −150 eV, and Ar collision gas pressure of 1.0 mTorr was used to detect *trans, anti*-BaPT-tetratrimethylsilyl ether (*trans, anti*-BaPT-TMS) and [$^{13}C_6$]*trans, anti*-BaPT-TMS at *m/z* 446 → *m/z* 255 and *m/z* 452 → *m/z* 261, respectively.

Hepatocyte Incubations

Primary human hepatocytes were purchased from Cellzdirect (St. Louis, MO), and prepared as described before (37). Hepatocytes (approximately 0.12 mg protein per well) were incubated with 10 μM of BaP-4,5-diol, BaP-7,8-diol or BaP-9,10-diol, or H_2O as a control. Each diol was dissolved in 20 μL DMSO and added to the 2 mL incubation mixtures. One mL of media was collected at 24 hrs. Samples were stored at −80 °C until analysis.

Statistical Analysis

The hypothesis that the level of *trans, anti*-BaPT in the urine of smokers (n=30) was significantly higher than that in non-smokers (n=30) was tested using the two-sided two-sample *t* test.

Results

The analytical method is outlined in Scheme 2. After the addition of [$^{13}C_6$] *trans, anti*-BaPT as the internal standard to a 1.5 mL urine sample, the mixture was incubated with β-glucuronidase and aryl sulfatase to release conjugated BaPT. The samples were purified by solid-phase extraction using polymeric reversed phase cartridges, and then phenylboronic acid cartridges which retain *cis*-hydroxy groups with high specificity (36). *trans, anti*-BaPT was derivatized with BSTFA to produce the corresponding tetra-TMS ethers. A full scan NICI-MS of BaPT-TMS showed a base peak at *m/z* 446 $[M - (OSi(CH_3)_3 + Si(CH_3)_3)]^-$ with little or no molecular ion (*m/z* 608). We used GC-NICI-MS/MS-SRM to monitor the transition *m/z* 446 → *m/z* 255 for *trans, anti*-BaPT-TMS, and *m/z* 452 → *m/z* 261 for the internal standard [$^{13}C_6$]*trans, anti*-BaPT-TMS. The daughter ion peak at *m/z* 255 corresponds to the loss of $[HOSi(CH_3)_3 + (CH_3)_3Si + CO]$ from *m/z* 446 (Scheme 3).

Typical GC-NICI-MS/MS chromatograms obtained upon analysis of *trans, anti*-BaPT in the urine of a smoker and a non-smoker are shown in Figure 1. The internal standard [$^{13}C_6$]*trans, anti*-BaPT-TMS eluted at the same retention time as *trans, anti*-BaPT-TMS. The identity of *trans, anti*-BaPT-TMS was verified by co-injection with a standard. To further confirm the identity of the analyte peak in the urine samples, three different transitions, from *m/z* 446 to daughter ions of *m/z* 255, *m/z* 267, and *m/z* 284 were monitored and compared to standards (Scheme 3). As shown in Table 1, the ratios of the integrated peaks from monitoring the three transitions in a urine sample were very close to those in the standards. The GC-NICI-MS/MS method proved to be highly sensitive. The on-column detection limit was about 20 amol of *trans, anti*-BaPT-TMS. The limit of quantitation of the

assay was less than 0.05 fmol *trans, anti*-BaPT per mL urine. A calibration curve demonstrating linearity when 0.05 – 10 fmol of *trans, anti*-BaPT was derivatized is illustrated in Figure 2.

As multiple tetraols could be formed from BaP, we sought further confirmation of the identity of *trans, anti*-BaPT. Incubation of primary human hepatocytes with BaP-7,8-diol produced four isomers of BaPT, as summarized in Table 2. Each had a retention time identical to those of the TMS derivatives of the corresponding standards - *trans, syn*-BaPT, *trans, anti*-BaPT, *cis, anti*-BaPT, and *cis, syn*-BaPT. *trans, anti*-BaPT was the predominant tetraol formed. Incubation of BaP-9,10-diol with human hepatocytes produced two tetraols which co-eluted with standards – *trans, syn*-BaPT and *trans, anti*-BaPT. There were also two minor products which did not co-elute with *cis, anti*-BaPT and *cis, syn*-BaPT, which was expected since the 9,10-hydroxyl groups in these compounds are *cis*-, not *trans*- as in BaP-9,10-diol. The two minor products probably resulted from *cis*- ring opening of the *syn*- and *anti*-9,10-diol-7,8-epoxides, but this was not pursued. A small peak at the same retention time as *trans, anti*-BaPT was also observed in the incubations of BaP-4,5-diol with human hepatocytes, but the yield of this product was at least 10,000 times lower than those from the two other diols. No tetraol peaks were observed in control incubations.

The intraday precision of the assay was determined by analyzing seven aliquots of a smoker's urine. The results were 1.01 ± 0.05 fmol *trans, anti*-BaPT/mL urine (relative SD, 4.8%). The interday precision based on analyses of a smoker's urine (2 per set as positive controls in four sets of assays) was 15.2% (relative SD). Assay accuracy was assessed by the standard addition method. One mL of the positive control urine, which contained 1.01 fmol/mL *trans, anti*-BaPT, was enriched with 0.1, 0.2, 0.5, 1, and 2.5 fmol *trans, anti*-BaPT. The results of this experiment are presented in Figure 3. The added and measured levels of *trans, anti*-BaPT were highly correlated (r = 0.99) and the y intercept was 0.98 fmol/mL urine, in excellent agreement with the amount of *trans, anti*-BaPT in the untreated sample. Recoveries of internal standard were generally good, averaging 44 ± 19% (n = 80).

The method was applied to the analysis of urine samples from 30 non-smokers and 30 smokers. The results are summarized in Table 3. The mean level of *trans, anti*-BaPT (0.71 ± 0.64 fmol/mg creatinine, n = 30) in the urine of smokers was significantly higher (P = 0.0018) than that in non-smokers (0.34 ± 0.20 fmol/mg creatinine, n = 30). No gender differences in the amounts of this analyte were found among either smokers or non-smokers.

Discussion

We present a highly selective and sensitive GC-NICI-MS/MS-SRM method for quantitation of *trans, anti*-BaPT in human urine. The method is precise, accurate, yet relatively simple with only two SPE purification steps. The GC-NICI-MS/MS-SRM traces illustrated in Figure 1 are quite clean. The sensitivity of the method, with a quantitation limit of 0.05 fmol of *trans, anti*-BaPT/mL urine, was essential for its success. Due to its relative simplicity, this analytical method might be applicable to large numbers of samples in epidemiology studies in the future. A potentially significant advantage of this method is that it measures exposure *plus* metabolic activation of BaP, and therefore may be related to cancer risk.

A major challenge for quantitation of BaP metabolites in human urine is that their levels are extremely low compared to those of smaller PAH such as phenanthrene. The urinary levels of *trans, anti*-BaPT are, for example, approximately 10,000 times lower than those of the corresponding metabolite of phenanthrene, *r*-1,*t*-2,3-*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (*trans, anti*-PheT), which we have previously quantified in the urine of smokers (38,39). The substantially lower levels of *trans, anti*-BaPT in human urine

compared to those of *trans, anti*-PheT results from differences in exposure, metabolism, and excretion. The level of BaP in mainstream cigarette smoke is about 30 times lower than that of phenanthrene (5–10 ng vs. 150–300 ng/cigarette) (4,40). BaP metabolites are mainly excreted in feces while phenanthrene metabolites are excreted mainly in urine, based on studies in laboratory animals (41–44). Metabolism of phenanthrene to *trans, anti*-PheT may exceed metabolism of BaP to *trans, anti*-BaPT, but further studies are required on this point.

Only a few studies have previously described methods for the analysis of BaP metabolites in human urine. 3-Hydroxy BaP was quantified as a biomarker of exposure in industrial workers (45,46). Weston et al. and Bowman et al. analyzed BaPT in human urine using immuno-affinity chromatography for sample purification and synchronous fluorescence spectroscopy for quantitation (47,48). They reported BaPT levels of 240–3120 fmol/mL in the urine of four individuals who were highly exposed to PAHs in their diet, and 150 fmol/mL BaPT in psoriasis patients receiving coal tar therapy. Our laboratory determined urinary *trans, anti*-BaPT by GC-NICI-MS-SIM following three steps of SPE purification, and reported 16 fmol/mL *trans, anti*-BaPT from psoriasis patients and 0.5 fmol/mL from 9 of 21 smokers, with an on-column limit of detection of 1 fmol (39). The lower sensitivity in that study was due to the lower sensitivity of the MS system available at that time. As shown in this paper, with the modified SPE preparation and high performance GC-NICI-MS/MS-SRM, we were able to achieve an ultra low detection limit of 20 amol, at least 50 times greater than our previous method for *trans, anti*-BaPT analysis. Thus, in contrast to our previous study, we were able to detect *trans, anti*-BaPT in 100% of the urine samples from smokers and non-smokers. In addition, the urinary *trans, anti*-BaPT level in smokers we reported here (mean 0.64 fmol/mL urine, or 0.71 fmol/mg creatinine, n = 30) is quite consistent with our previous data for the samples in which quantitation was possible (mean 0.5 fmol/mL urine, n = 9).

Our results clearly demonstrate, apparently for the first time, that cigarette smokers have significantly higher levels of *trans, anti*-BaPT, the end-product of the carcinogenic diol epoxide metabolic activation pathway of BaP, in their urine than do non-smokers. Besides higher BaP exposure in smokers from cigarettes (5–10 ng/cigarette, and mean = 19 cigarettes per day per person, in this study) (4,40), this difference may also result from the induction of P450s 1A1, 1A2, and 1B1, known to occur in smokers (49), as these enzymes are involved in producing *trans, anti*-BaPT. Non-smokers are exposed to BaP probably from polluted air and food. Specifically, our results have shown that smokers excreted approximately twice as much *trans, anti*-BaPT as did non-smokers. This is quite consistent with previous results comparing urinary metabolites from two other PAH components - pyrene and phenanthrene -between smokers and nonsmokers. Levels of 1-hydroxypyrene, a urinary metabolite of pyrene, are about twice as high in smokers' urine as in non-smokers urine, in most studies (35,50,51). Our laboratory has reported that smokers have about three times higher levels of urinary *trans, anti*-PheT than do non-smokers (38).

In this study, we quantified racemic *trans, anti*-BaPT. One enantiomer of this tetraol would be expected to arise from hydrolysis of BPDE, based on known stereoselectivity in the metabolic formation of BPDE, as illustrated in Scheme 1 and summarized previously (36). The opposite enantiomer would be expected from hydrolysis of the reverse diol epoxide, BaP-(*9S,10R*)-diol-(*7R,8S*)-epoxide. Thus, racemic *trans, anti*-BaPT measured here could result from both pathways. We have previously shown that, in the urine of creosote workers exposed to relatively high levels of BaP, 78% of *trans, anti*-BaPT results from hydrolysis of BPDE. Whether that is also the case in smokers and non-smokers, as in this study, requires further investigation.

Measurement of BPDE-DNA adducts in humans may be a more direct approach to examine BaP metabolic activation than that described here. However, methods such as immunoassays and ^{32}P postlabelling for BaP-DNA adduct have limitations, and quantitation can be unreliable (52). Highly sensitive analytical methods such as HPLC-fluorescence and GC-NICI-MS are quantitative, but usually detect BPDE-DNA adducts in only some human tissues and blood samples (53). The urinary metabolite *trans, anti*-BaPT may be a more practical biomarker because it can be reliably quantified and detected in all samples (35,54).

The relationship of *trans, anti*-BaPT levels to lung cancer risk in smokers requires further investigation. Extensive studies of the tumorigenicity of cigarette smoke condensate and its subfractions in mouse skin tumor models indicate that PAH in smoke act mainly as tumor intiators and that expression of their carcinogenicity requires the cocarcinogenic or promoting activity of other smoke constituents (55). Futhermore, cigarette smoke contains multiple carcinogens of other types such as tobacco-specific nitrosamines and volatile organic compounds that most likely contribute to lung cancer in smokers (9). Therefore, it is unlikely that *trans, anti*-BaPT alone could be related to lung cancer risk. *trans, anti*-BaPT however could become part of a biomarker panel that would encompass multiple tobacco smoke constituents and their metabolites or DNA or protein adducts, levels of which collectively could be related to cancer risk (54).

In summary, we have developed a relatively simple and ultra-sensitive method for the analysis of *trans, ant*i-BaPT in human urine. Average levels of this metabolite were 0.34 fmol/mg creatinine in non-smokers, and 0.71 fmol/mg creatinine in smokers. This biomarker can potentially provide a critical component of a carcinogen metabolite phenotyping model that may eventually be used to determine individual susceptibility to PAH-induced human cancer.

Acknowledgments

This study was supported by grant no. CA-92025 from the National Cancer Institute. Biostatistical analysis was carried out by Scott Jackson, Biostatistics and Informatics Shared Resource, Masonic Cancer Center, supported in part by grant no. CA-77598. We thank Bob Carlson for editorial assistance.

References

1. Brandt HC, Watson WP. Monitoring human occupational and environmental exposures to polycyclic aromatic compounds. Ann Occup. Hyg. 2003; 47:349–378. [PubMed: 12855487]
2. U.S. Department of Health and Human Services. Report on Carcinogens. 11th Edition. N.C.: Research Triangle Park; 2004. p. III-220-III-222.
3. Luch, A. Polycyclic aromatic hydrocarbon-induced carcinogenesis-an introduction. In: Luch, A., editor. The carcinogenic effects of polycyclic aromatic hydrocarbons. London: Imperial College Press; 2005. p. 1-18.
4. Ding YS, Ashley DL, Watson CH. Determination of 10 carcinogenic polycyclic aromatic hydrocarbons in mainstream cigarette smoke. J Agric. Food Chem. 2007; 55:5966–5973. [PubMed: 17602652]
5. Stepanov I, Villalta PW, Knezevich A, Jensen J, Hatsukami D, Hecht SS. Analysis of 23 polycyclic aromatic hydrocarbons in smokeless tobacco by gas chromatography-mass spectrometry. Chem. Res. Toxicol. 2010; 23:66–73. [PubMed: 19860436]
6. International Agency for Research on Cancer. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. vol. 83. Lyon, FR: IARC; 2004. Tobacco Smoke and Involuntary Smoking; p. 53-1187.
7. International Agency for Research on Cancer. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. vol. 38. Lyon, FR: IARC; 1986. Tobacco Smoking; p. 37-385.

8. Hecht SS. Tobacco smoke carcinogens and lung cancer. J. Natl. Cancer Inst. 1999; 91:1194–1210. [PubMed: 10413421]
9. Hecht SS. Tobacco carcinogens, their biomarkers, and tobacco-induced cancer. Nature Rev. Cancer. 2003; 3:733–744. [PubMed: 14570033]
10. International Agency for Research on Cancer. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. v. 92. Lyon, FR: IARC; 2010. Some Non-Heterocyclic Polycyclic Aromatic Hydrocarbons and Some Related Exposures; p. 35-818.
11. International Agency for Research on Cancer. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. v.32. Lyon, FR: IARC; 1983. Polynuclear aromatic compounds, part 1, chemical, environmental, and experimental data; p. 33-91.
12. Culp SJ, Gaylor DW, Sheldon WG, Goldstein LS, Beland FA. A comparison of the tumors induced by coal tar and benzo[*a*]pyrene in a two-year bioassay. Carcinogenesis. 1998; 19:117–124. [PubMed: 9472702]
13. Luch, A.; Baird, WM. Metabolic activation and detoxification of polycyclic aromatic hydrocarbons. In: Luch, A., editor. The carcinogenic effects of polycyclic aromatic hydrocarbons. London: Imperial College Press; 2005. p. 19-96.
14. Gelboin HV. Benzo[*a*]pyrene metabolism, activation and carcinogenesis: role and regulation of mixed function oxides and related enzymes. Physiol. Rev. 1980; 60:1107–1166. [PubMed: 7001511]
15. Conney AH, Chang RL, Jerina DM, Wei SJC. Studies on the metabolism of benzo[*a*]pyrene and dose-dependent differences in the mutagenic profile of its ultimate carcinogenic metabolite. Drug Metabol. Rev. 1994; 26:125–163.
16. Shimada T, Martin MV, Pruess-Schwartz D, Marnett LJ, Guengerich FP. Roles of individual human cytochrome P-450 enzymes in the bioactivation of benzo(*a*)pyrene, 7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene, and other dihydrodiol derivatives of polycyclic aromatic hydrocarbons. Cancer Res. 1989; 49:6304–6312. [PubMed: 2509067]
17. Shimada T, Yun CH, Yamazaki H, Gautier JC, Beaune PH, Guengerich FP. Characterization of human lung microsomal cytochrome P-4501A1 and its role in the oxidation of chemical carcinogens. Mol. Pharmacol. 1992; 41:856–864. [PubMed: 1588920]
18. Shimada T, Gillam EMJ, Oda Y, Tsumara F, Sutter TR, Guengerich FP, Inoue K. Metabolism of benzo[*a*]pyrene to *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene by recombinant human cytochrome P450 1B1 and purified liver epoxide hydrolase. Chem. Res. Toxicol. 1999; 12:623–629. [PubMed: 10409402]
19. Conney AH. Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G.H.A. Clowes Memorial Lecture. Cancer Res. 1982; 42:4875–4917. [PubMed: 6814745]
20. Pelkonen O, Nebert DW. Metabolism of polycyclic hydrocarbons: etiologic role in carcinogenesis. Pharmacol. Rev. 1982; 34:189–222. [PubMed: 6287505]
21. Sabadie N, Richter-Reichhelm HB, Saracci R, Mohr U, Bartsch H. Interindividual differences in oxidative benzo(*a*)pyrene metabolism by normal and tumorous surgical lung specimens from 105 lung cancer patients. Int. J Cancer. 1981; 27:417–425. [PubMed: 6268552]
22. Harris CC, Autrup H, Connor R, Barrett LA, McDowell EM, Trump BF. Interindividual variation in binding of benzo[*a*]pyrene to DNA in cultured human bronchi. Science. 1976; 194:1067–1069. [PubMed: 982061]
23. McLemore TL, Adelberg S, Liu MC, McMahon NA, Yu SJ, Hubbard WC, Czerwinski M, Wood TG, Storeng R, Lubet RA, Eggleston JC, Boyd MR, Hines RN. Expression of CYP1A1 gene in patients with lung cancer: evidence for cigarette smoke-induced gene expression in normal lung tissue and for altered gene regulation in primary pulmonary carcinomas. J. Natl. Cancer Inst. 1990; 82:1333–1339. [PubMed: 2380990]
24. Nebert DW. Drug-metabolizing enzymes, polymorphisms and interindividual response to environmental toxicants. Clin. Chem Lab Med. 2000; 38:857–861. [PubMed: 11097340]
25. Bartsch H, Nair U, Risch A, Rojas M, Wikman H, Alexandrov K. Genetic polymorphism of CYP genes, alone or in combination, as a risk modifier of tobacco-related cancers. Cancer Epidemiol Biomarkers & Prev. 2000; 9:3–28.

26. Alexandrov K, Cascorbi I, Rojas M, Bouvier G, Kriek E, Bartsch H. CYP1A1 and GSTM1 genotypes affect benzo[a]pyrene DNA adducts in smokers' lung: comparison with aromatic/hydrophobic adduct formation. Carcinogenesis. 2002; 23:1969–1977. [PubMed: 12507920]
27. Smith GB, Harper PA, Wong JM, Lam MS, Reid KR, Petsikas D, Massey TE. Human lung microsomal cytochrome P4501A1 (CYP1A1) activities: impact of smoking status and CYP1A1, aryl hydrocarbon receptor, and glutathione S-transferase M1 genetic polymorphisms. Cancer Epidemiol Biomarkers & Prev. 2001; 10:839–853.
28. Lee WJ, Brennan P, Boffetta P, London SJ, Benhamou S, Rannug A, To-Figueras J, Ingelman-Sundberg M, Shields P, Gaspari L, Taioli E. Microsomal epoxide hydrolase polymorphisms and lung cancer risk: a quantitative review. Biomarkers. 2002; 7:230–241. [PubMed: 12141066]
29. Benhamou S, Lee WJ, Alexandrie AK, Boffetta P, Bouchardy C, Butkiewicz D, Brockmoller J, Clapper ML, Daly A, Dolzan V, Ford J, Gaspari L, Haugen A, Hirvonen A, Husgafvel-Pursiainen K, Ingelman-Sundberg M, Kalina I, Kihara M, Kremers P, Le Marchand L, London SJ, Nazar-Stewart V, Onon-Kihara M, Rannug A, Romkes M, Ryberg D, Seidegard J, Shields P, Strange RC, Stucker I, To-Figueras J, Brennan P, Taioli E. Meta- and pooled analyses of the effects of glutathione *S*-transferase M1 polymorphisms and smoking on lung cancer risk. Carcinogenesis. 2002; 23:1343–1350. [PubMed: 12151353]
30. Veglia F, Matullo G, Vineis P. Bulky DNA adducts and risk of cancer: a meta-analysis. Cancer Epidemiol. Biomarkers & Prev. 2003; 12:157–160.
31. Raimondi S, Paracchini V, Autrup H, Barros-Dios JM, Benhamou S, Boffetta P, Cote ML, Dialyna IA, Dolzan V, Filiberti R, Garte S, Hirvonen A, Husgafvel-Pursiainen K, Imyanitov EN, Kalina I, Kang D, Kiyohara C, Kohno T, Kremers P, Lan Q, London S, Povey AC, Rannug A, Reszka E, Risch A, Romkes M, Schneider J, Seow A, Shields PG, Sobti RC, Sorensen M, Spinola M, Spitz MR, Strange RC, Stucker I, Sugimura H, To-Figueras J, Tokudome S, Yang P, Yuan JM, Warholm M, Taioli E. Meta- and pooled analysis of *GSTT1* and lung cancer: a HuGE-GSEC review. Am J Epidemiol. 2006; 164:1027–1042. [PubMed: 17000715]
32. Carlsten C, Sagoo GS, Frodsham AJ, Burke W, Higgins JP. Glutathione S-transferase M1 (GSTM1) polymorphisms and lung cancer: a literature-based systematic HuGE review and meta-analysis. Am J Epidemiol. 2008; 167:759–774. [PubMed: 18270371]
33. Hung RJ, Boffetta P, Brockmoller J, Butkiewicz D, Cascorbi I, Clapper ML, Garte S, Haugen A, Hirvonen A, Anttila S, Kalina I, Le Marchand L, London SJ, Rannug A, Romkes M, Salagovic J, Schoket B, Gaspari L, Taioli E. CYP1A1 and GSTM1 genetic polymorphisms and lung cancer risk in Caucasian nonsmokers: a pooled analysis. Carcinogenesis. 2003; 24:875–882. [PubMed: 12771031]
34. Hecht SS, Carmella SG, Yoder A, Chen M, Li Z, Le C, Jensen J, Hatsukami DK. Comparison of polymorphisms in genes involved in polycyclic aromatic hydrocarbon metabolism with urinary phenanthrene metabolite ratios in smokers. Cancer Epidemiol. Biomarkers & Prev. 2006; 15:1805–1811.
35. Hecht SS. Human urinary carcinogen metabolites: biomarkers for investigating tobacco and cancer. Carcinogenesis. 2002; 23:907–922. [PubMed: 12082012]
36. Hecht SS, Carmella SG, Villalta PW, Hochalter JB. Analysis of phenanthrene and benzo[*a*]pyrene tetraol enantiomers in human urine: relevance to the bay region diol epoxide hypothesis of benzo[*a*]pyrene carcinogenesis and to biomarker studies. Chem. Res. Toxicol. 2010; 23:900–908. [PubMed: 20369855]
37. Upadhyaya P, Hochalter JB, Balbo S, McIntee EJ, Hecht SS. Preferential glutathione conjugation of a reverse diol epoxide compared with a bay region diol epoxide of benzo[a]pyrene in human hepatocytes. Drug Metab Dispos. 2010; 38:1397–1402. [PubMed: 20547966]
38. Hecht SS, Chen M, Yagi H, Jerina DM, Carmella SG. *r*-1,*t*-2,3,*c*-4-Tetrahydroxy-1,2,3,4-tetrahydrophenanthrene in human urine: a potential biomarker for assessing polycyclic aromatic hydrocarbon metabolic activation. Cancer Epidemiol. Biomarkers & Prev. 2003; 12:1501–1508.
39. Simpson CD, Wu MT, Christiani DC, Santella RM, Carmella SG, Hecht SS. Determination of *r*-7,*t*-8,9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene in human urine by gas chromatography-negative ion chemical ionization-mass spectrometry. Chem. Res. Toxicol. 2000; 13:271–280. [PubMed: 10775327]

40. Ding YS, Trommel JS, Yan XJ, Ashley D, Watson CH. Determination of 14 polycyclic aromatic hydrocarbons in mainstream smoke from domestic cigarettes. Environ. Sci. Technol. 2005; 39:471–478. [PubMed: 15707046]
41. Kotin P, Falk HL, Busser R. Distribution, retention, and elimination of C14-3,4-benzpyrene after administration to mice and rats. J. Natl. Cancer Inst. 1959; 23:541–555.
42. Hecht SS, Grabowski W, Groth K. Analysis of faeces for benzo[a]pyrene after consumption of charcoal-broiled beef by rats and humans. Food Cosmet. Toxicol. 1979; 17:223–227. [PubMed: 499948]
43. Heidelberger C, Weiss SM. The distribution of radioactivity in mice following administration of 3,4-benzpyrene-5-C14 and 1,2,5,6-dibenzanthracene-9,10-C14. Cancer Res. 1951; 11:885–891. [PubMed: 14886940]
44. Chu I, Ng KM, Benoit FM, Moir D. Comparative metabolism of phenanthrene in the rat and guinea pig. J. Environ. Sci. Health B. 1992; 27:729–749. [PubMed: 1460244]
45. Gundel J, Angerer J. High-performance liquid chromatographic method with fluorescence detection for the determination of 3-hydroxybenzo[a]pyrene and 3-hydroxybenz[a]anthracene in the urine of polycyclic aromatic hydrocarbon-exposed workers. J. Chromatogr. B Biomed. Sci. Appl. 2000; 738:47–55. [PubMed: 10778925]
46. Forster K, Preuss R, Rossbach B, Bruning T, Angerer J, Simon P. 3-Hydroxybenzo[a]pyrene in the urine of workers with occupational exposure to polycyclic aromatic hydrocarbons in different industries. Occup. Environ. Med. 2008; 65:224–229. [PubMed: 17449565]
47. Weston A, Bowman ED, Carr P, Rothman N, Strickland PT. Detection of metabolites of polycyclic aromatic hydrocarbons in human urine. Carcinogenesis. 1993; 14:1053–1055. [PubMed: 8504465]
48. Bowman ED, Rothman N, Hackl C, Santella RM, Weston A. Interindividual variation in the levels of certain urinary polycyclic aromatic hydrocarbon metabolites following medicinal exposure to coal tar ointment. Biomarkers. 1997; 2:321–327.
49. Nebert DW, Dalton TP, Okey AB, Gonzalez FJ. Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. J Biol. Chem. 2004; 279:23847–23850. [PubMed: 15028720]
50. van Delft JH, Steenwinkel MS, van Asten JG, de Vogel N, Bruijntjes-Rozier TC, Schouten T, Cramers P, Maas L, Van Herwijnen MH, van Schooten F, Hopmans PM. Biological monitoring the exposure to polycyclic aromatic hydrocarbons of coke oven workers in relation to smoking and genetic polymorphisms for GSTM1 and GSTT1. Ann. Occup. Hyg. 2001; 45:395–408. [PubMed: 11418090]
51. Nerurkar PV, Okinaka L, Aoki C, Seifried A, Lum-Jones A, Wilkens LR, Le Marchand L. CYP1A1, GSTM1, and GSTP1 genetic polymorphisms and urinary 1-hydroxypyrene excretion in non-occupationally exposed individuals. Cancer Epidemiol. Biomarkers & Prev. 2000; 9:1119–1122.
52. Poirier MC, Santella RM, Weston A. Carcinogen macromolecular adducts and their measurement. Carcinogenesis. 2000; 21:353–359. [PubMed: 10688855]
53. Boysen G, Hecht SS. Analysis of DNA and protein adducts of benzo[*a*]pyrene in human tissues using structure-specific methods. Mutation Res. 2003; 543:17–30. [PubMed: 12510015]
54. Hecht SS, Yuan J-M, Hatsukami DK. Applying tobacco carcinogen and toxicant biomarkers in product regulation and cancer prevention. Chem. Res. Toxicol. 2010; 23:1001–1008. [PubMed: 20408564]
55. Hoffmann, D.; Schmeltz, I.; Hecht, SS.; Wynder, EL. Tobacco carcinogenesis. In: Gelboin, H.; Ts'o, POP., editors. Polycyclic Hydrocarbons and Cancer. New York: Academic Press; 1978. p. 85-117.

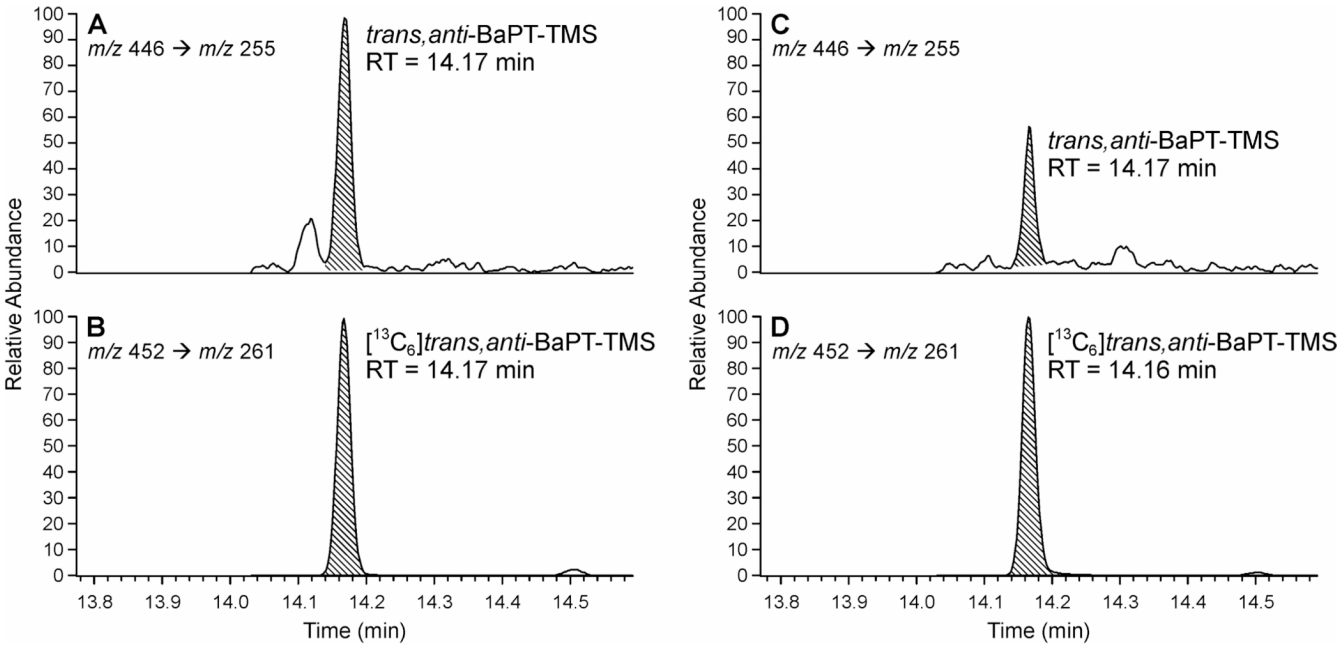


Figure 1.

Chromatograms from GC-NICI-MS/MS SRM analysis of *trans*, *anti*-BaPT in urine of a smoker (A and B) and a non-smoker (C and D). The indicated peaks are TMS derivatives of *trans*, *anti*-BaPT (A and C) and internal standard [$^{13}C_6$]*trans*, *anti*-BaPT (B and D).

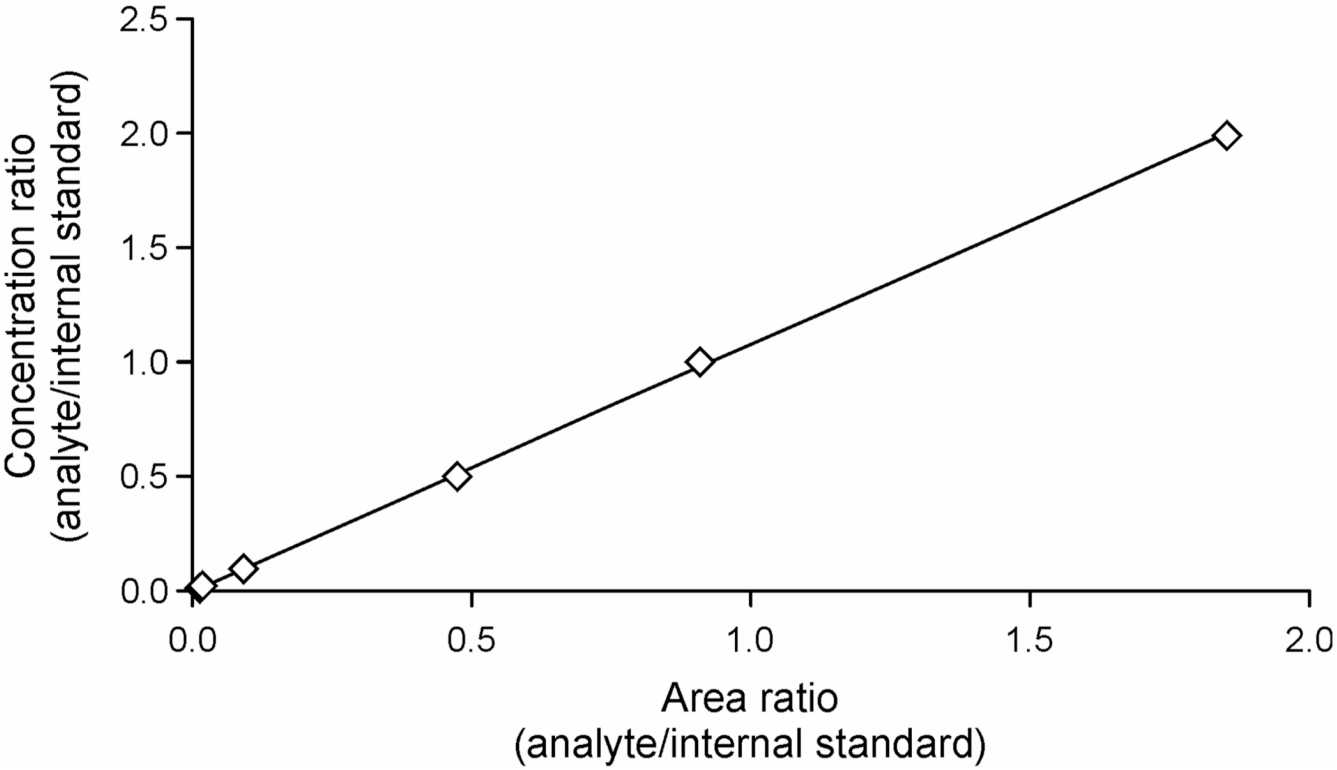


Figure 2.
Calibration curve for *trans, anti*-BaPT-TMS. The amount of standard *trans, anti*-BaPT was increased from 0.05 fmol to 0.1, 0.5, 2.5, 5, and 10 fmol, with a constant amount of internal standard [$^{13}C_6$]*trans, anti*-BaPT (20 fmol). The calibration curve shows the concentration ratio (analyte/internal standard) versus their area ratio.

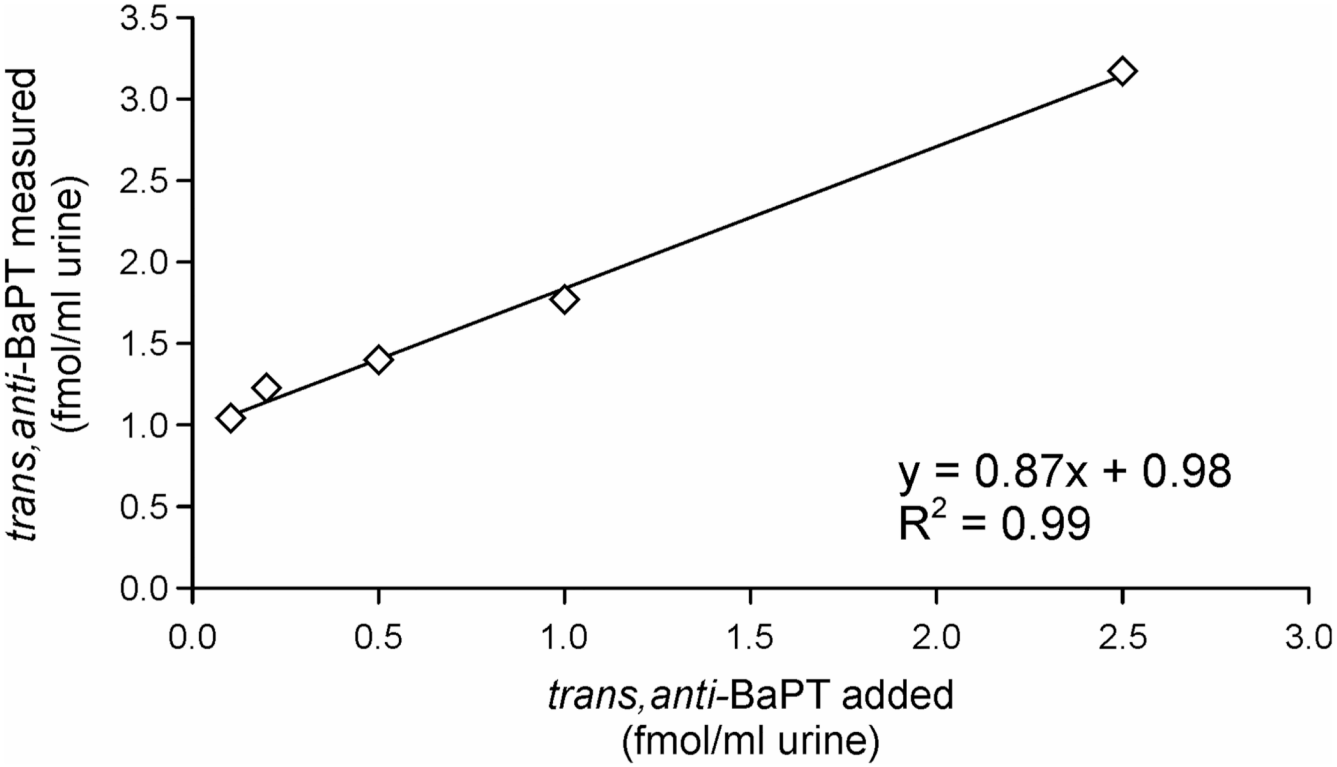
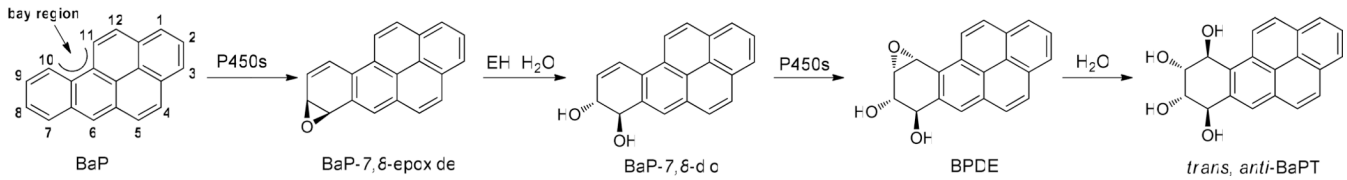
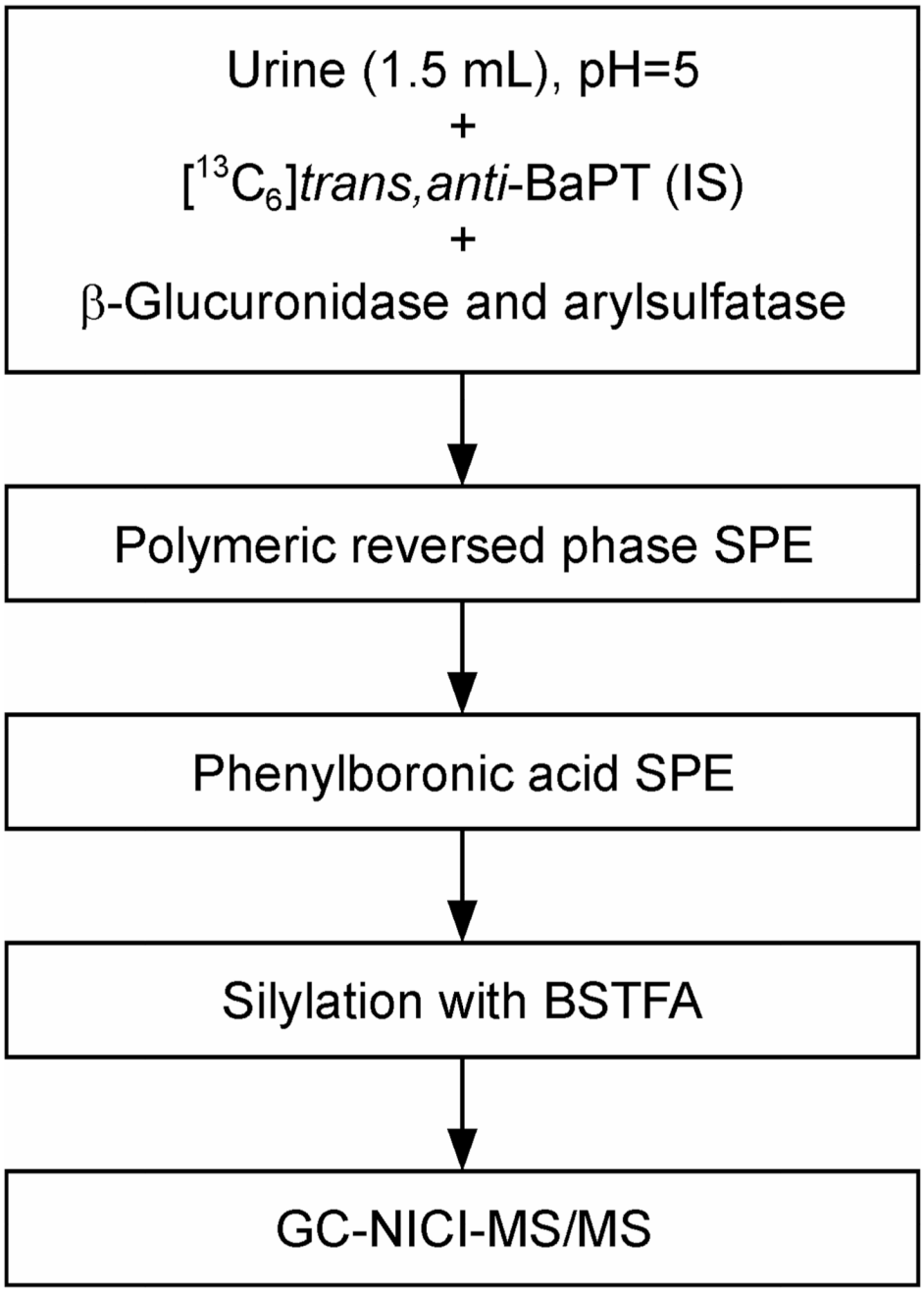


Figure 3.
Relationship between levels of *trans*, *anti*-BaPT added to a urine sample and levels measured (r=0.998). Points, mean of duplicate determinations.

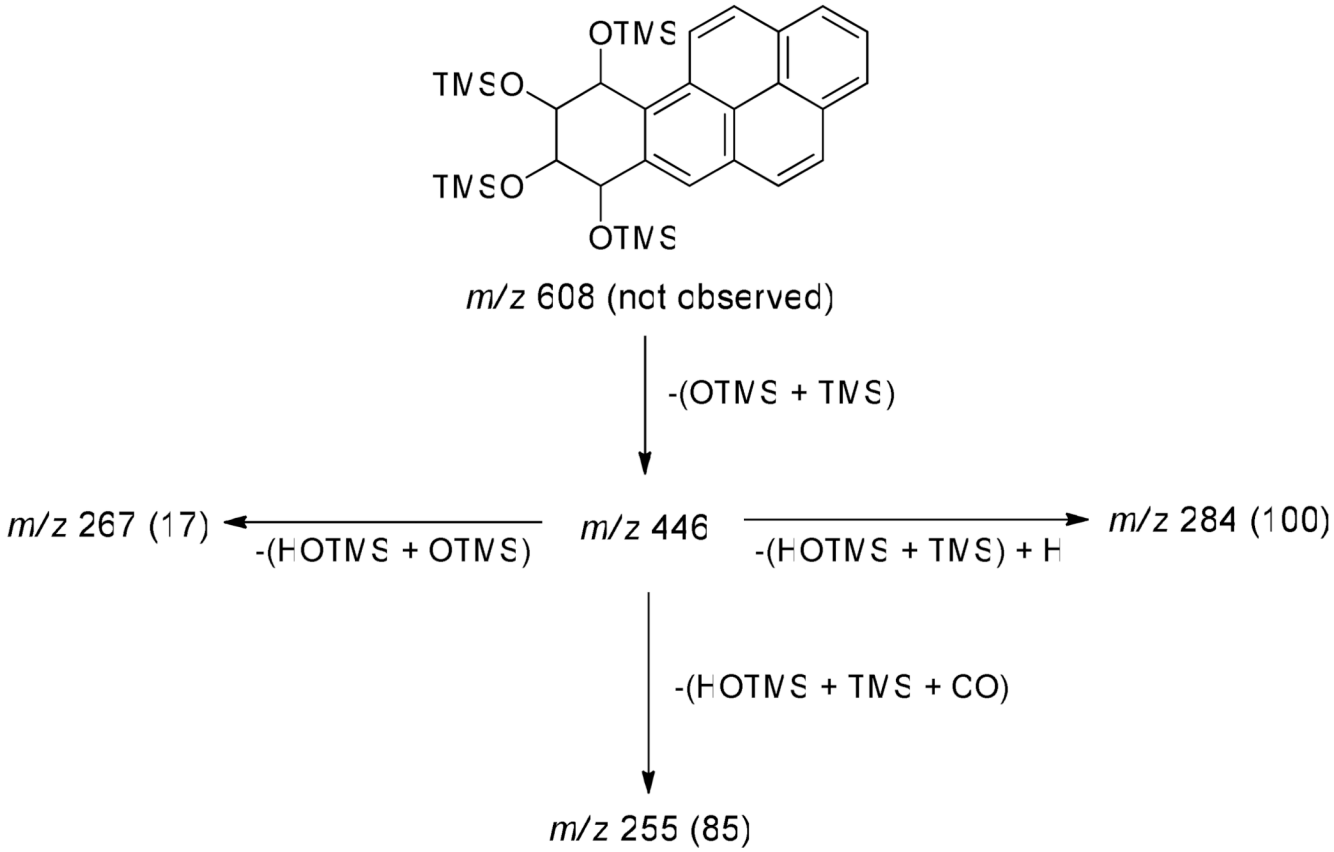


Scheme 1.

Metabolism of benzo[*a*]pyrene (BaP) to *trans, anti*-BaPT via the bay region diol epoxide BPDE. *P450*, cytochrome P450; *EH*, epoxide hydrolase.



Scheme 2.
Analytical scheme for quantitation of *trans*, *anti*-BaPT in human urine. IS, internal standard; SPE, solid phase extraction; BSTFA, *bis*-trimethylsilyltrifluoroacetamide; GC-NICI-MS/MS, gas chromatography-negative ion chemical ionization-tandem mass spectrometry.



Scheme 3.

Fragmentation pattern and relative intensities (in parentheses) in the daughter ion spectrum of *m/z* 446 of *trans*, *anti*-BaPT-TMS, obtained by GC-NICI-MS/MS. TMS = $(CH_3)_3Si$

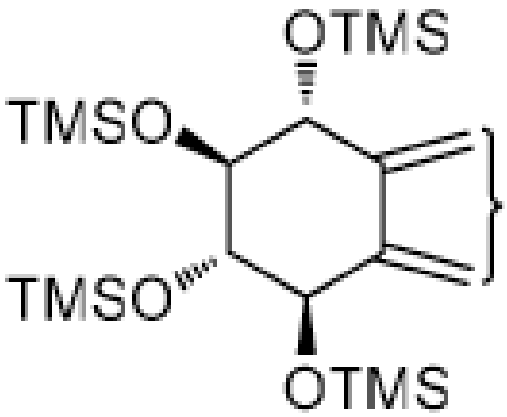
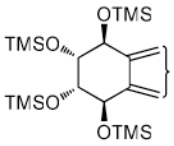
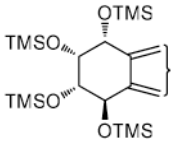
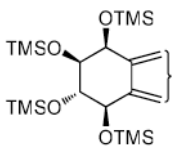
Table 1

Ratios of major *trans, anti*-BaPT-TMS fragment ion intensities from analysis of a human urine sample, compared to *trans, anti*-BaPT-TMS and [$^{13}C_6$]*trans, anti*-BaPT-TMS standards.

	***m/z* 446 → *m/z* 255**	***m/z* 446 → *m/z* 267**	***m/z* 446 → *m/z* 284**
trans, anti-BaPT-TMS from analysis of human urine	1.0	0.2	1.3
trans, anti-BaPT-TMS standard	1.0	0.2	1.1
	m/z 452 → *m/z* 261	*m/z* 452 → *m/z* 273	*m/z* 452 → *m/z* 290
[$^{13}C_6$]*trans, anti*-BaPT-TMS	1.0	0.2	1.2

Table 2

Summary of retention time (min) and yield (pmol/mL media) of BaPT isomers from incubation of three BaP-diols (10 μM) with human hepatocytes, and analyzed as their TMS derivatives by GC-NICI-MS/MS.

	trans, syn- **BaPT-TMS**	***trans, anti-*** **BaPT-TMS**	***cis, anti-*** **BaPT-TMS**	***cis, syn-*** **BaPT-TMS**	**[$^{13}C_6$]*trans,anti-*** **BaPT-TMS**
					
tetraol standards	13.72	13.93	14.25	14.51	13.93
tetraols from BaP-7,8-diol	13.72 (0.4)[a]	13.93 (18.1)	14.25 (1.2)	14.51 (0.8)	13.93
tetraols from BaP-9,10-diol	13.72 (5.6)	13.93 (7.0)	ND[b]	ND	13.93
tetraols from BaP-4,5-diol		13.93 (0.7×10^{-3})			13.93

[a] retention time (yield)

[b] ND, not detected. Two minor peaks from BaP-9,10-diol were not identified.

Table 3

a. Urinary *trans, anti*-BaPT concentrations in non-smokers.

Non-smokers			*trans, anti*-BaPT	
Subject no.	**age**	**gender**	**fmol / ml urine**	**fmol / mg creatinine**
1	28	F	0.37	0.38
2	23	F	0.31	0.25
3	25	F	0.21	0.21
4	22	M	0.23	0.45
5	25	M	0.27	0.99
6	24	M	0.24	0.31
7	28	F	0.21	0.63
8	22	M	0.22	0.26
9	24	M	0.22	0.13
10	25	F	0.22	0.75
11	22	M	0.28	0.20
12	24	F	0.22	0.27
13	60	M	0.28	0.18
14	27	F	0.26	0.35
15	25	F	0.26	0.55
16	57	M	0.24	0.19
17	41	M	0.34	0.51
18	29	F	0.21	0.24
19	24	F	0.26	0.44
20	50	F	0.20	0.26
21	24	F	0.24	0.20
22	52	M	0.28	0.14
23	66	M	0.25	0.39
24	39	M	0.36	0.36
25	28	F	0.26	0.31
26	23	M	0.25	0.10

a. Urinary *trans, anti*-BaPT concentrations in non-smokers.

Non-smokers			*trans, anti*-BaPT	
Subject no.	**age**	**gender**	**fmol / ml urine**	**fmol / mg creatinine**
27	23	M	0.25	0.21
28	28	M	0.25	0.21
29	29	F	0.24	0.31
30	75	M	0.24	0.57
		Mean ± SD Range	0.26 ± 0.04 0.20 – 0.37	0.34 ± 0.20 0.10 – 0.99

3b. Urinary *trans, anti*-BaPT concentrations in smokers.

Smokers (n=30)				*trans, anti*-BaPT	
Subject no.	**age**	**gender**	**cigs/day**	**fmol / ml urine**	**fmol / mg creatinine**
1	24	F	14	0.61	1.38
2	20	F	12	0.60	0.69
3	53	M	15	0.51	0.65
4	24	M	16	0.50	0.74
5	35	F	12	1.20	1.14
6	44	M	20	0.73	0.50
7	66	M	20	0.41	0.35
8	57	M	30	0.62	0.28
9	43	F	15	1.31	0.60
10	56	M	10	0.93	0.33
11	24	F	20	0.56	0.34
12	28	M	24	0.40	0.57
13	20	M	20	0.47	0.47
14	56	M	20	0.62	0.56
15	53	M	25	0.62	0.23
16	49	M	8	0.55	0.33
17	55	M	40	0.36	0.41

3b. Urinary *trans, anti*-BaPT concentrations in smokers.

Smokers (n=30)				*trans, anti*-BaPT	
Subject no.	**age**	**gender**	**cigs/day**	**fmol / ml urine**	**fmol / mg creatinine**
18	50	F	20	0.33	0.33
19	43	M	30	0.27	0.34
20	30	F	20	0.45	0.31
21	42	F	20	0.29	0.23
22	54	F	20	0.29	0.93
23	42	M	10	0.64	0.86
24	27	M	40	0.36	1.07
25	38	M	12	0.38	0.42
26	30	M	20	0.28	0.73
27	43	F	15	1.61	0.86
28	40	F	15	1.38	1.74
29	47	M	23	0.28	0.63
30	38	F	14	0.98	3.62
			Mean ± SD	0.64 ± 0.34	0.71 ± 0.64
			Range	0.27 – 1.61	0.23 – 3.63