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

Stephen S. Hecht,* Steven G. Carmella, Peter W. Villalta, and J. Bradley Hochalter

Masonic Cancer Center, University of Minnesota, Mayo Mail Code 806, 420 Delaware Street SE, Minneapolis, Minnesota 55455

Received December 23, 2009

One widely accepted metabolic activation pathway of the prototypic carcinogenic polycyclic aromatic hydrocarbon (PAH) benzo[*a*]pyrene (BaP) proceeds through the "bay region diol epoxide" BaP-(7*R*,8*S*)-diol-(9*S*,10*R*)-epoxide (**2**). However, few studies have addressed the analysis of human urinary metabolites of BaP, which result from this pathway. Phenanthrene (Phe) is structurally related to BaP, but human exposure to Phe is far greater, and its metabolites can be readily detected in urine. Thus, Phe metabolites have been proposed as biomarkers of PAH exposure and metabolic activation. Phe-tetraols in particular could be biomarkers of the diol epoxide pathway. While BaP-tetraols and Phe-tetraols have been previously quantified in human urine, no published studies have determined their enantiomeric composition. This is important because different enantiomers would result from the bay region diol epoxide and "reverse" diol epoxide pathways, the latter being associated with weak mutagenicity and carcinogenicity. We addressed this problem using chiral HPLC to separate the enantiomers of BaP-7,8,9,10-tetraol and Phe-1,2,3,4-tetraol. Urine samples from smokers were subjected to solid-phase extraction, chiral HPLC, and GC-NICI-MS/MS analysis for silylated Phe-1,2,3,4-tetraols. The results demonstrated that >96% of Phe-1,2,3,4-tetraol in smokers' urine was Phe-(1*S*,2*R*,3*S*,4*R*)-tetraol (**12**), resulting from the "reverse" diol epoxide pathway, whereas less than 4% resulted from the "bay region diol epoxide" pathway of Phe metabolism. Urine from creosote workers was similarly analyzed for BaP-7,8,9,10-tetraol enantiomers. In contrast to the results of the Phe-tetraol analyses, 78% of BaP-7,8,9,10-tetraol in these human urine samples was BaP-(7*R*,8*S*,9*R*,10*S*)-tetraol (**3**) resulting from the "bay region diol epoxide" pathway of BaP metabolism. These results provide further support for the bay region diol epoxide pathway of BaP metabolism in humans and demonstrate differences in BaP and Phe metabolism, which may be important when considering Phe-tetraols as biomarkers of PAH metabolic activation.

Introduction

Polycyclic aromatic hydrocarbons (PAHs), which are formed in the incomplete combustion of organic matter, are commonly found in polluted air and water, tobacco products and their smoke, broiled foods, and occupational environments involving coke production from coal or other processes that generate soots and tars (*1–5*). Many PAHs are potent carcinogens and are implicated as causes of cancers of the skin and lung in occupationally exposed individuals (*3, 6–9*). PAHs are also believed to be among the major causative agents for lung cancer in smokers (*10*). The most thoroughly studied of all PAHs, and often considered a prototype, is benzo[*a*]pyrene (BaP, Scheme 1), rated as a human carcinogen by the International Agency for Research on Cancer (*6*). A number of PAHs, including BaP, are "reasonably anticipated to be human carcinogens" according to the U.S. Department of Health and Human Services (*3*).

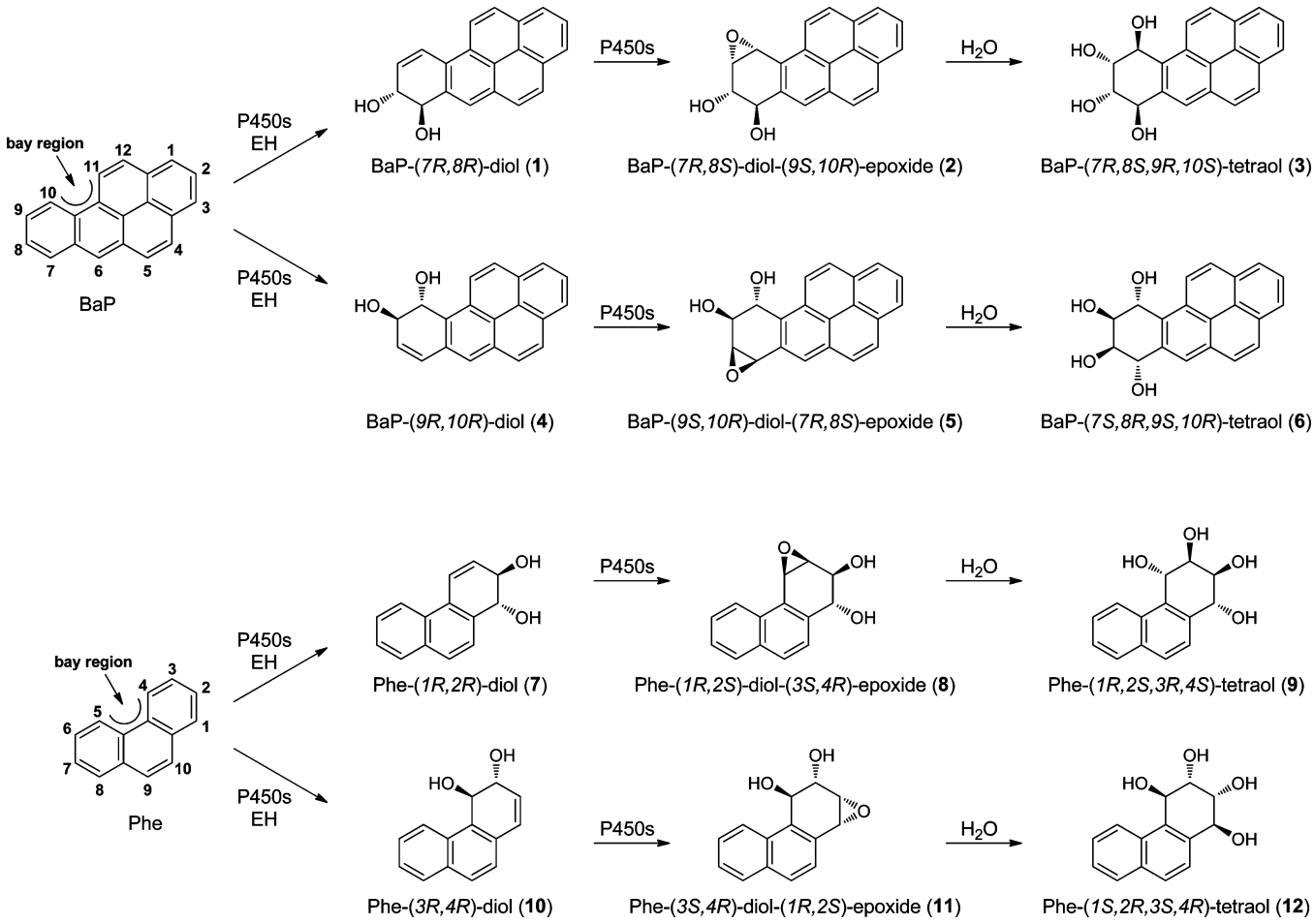
PAHs require metabolic activation to exert their carcinogenic effects (*11*). One widely accepted metabolic activation pathway for BaP proceeds by initial cytochrome P450-catalyzed oxidation of its 7,8-bond followed by epoxide hydrolase-catalyzed hydration yielding the proximate carcinogen BaP-(7*R*,8*R*)-diol (**1**, Scheme 1) (*12–16*). Diol **1**, which is more carcinogenic than BaP on mouse skin, undergoes cytochrome P450-catalyzed oxidation to give BaP-(7*R*,8*S*)-diol-(9*S*,10*R*)-epoxide (**2**), also known in the literature as BPDE. Diol epoxide **2** is highly carcinogenic in newborn mice and reacts easily with DNA, producing adducts that have been extensively characterized in vitro and in laboratory animals treated with BaP (*17, 18*). Diol epoxide **2** is considered to be one major ultimate carcinogen of BaP. The reaction of diol epoxide **2** with H_2O produces predominantly BaP-(7*R*,8*S*,9*R*,10*S*)-tetraol (**3**). An analogous pathway of BaP metabolism gives BaP-(9*R*,10*R*)-diol (**4**), which could be further metabolized to diol epoxide **5** and tetraol **6**. While diol **4** is an established metabolite of BaP in vitro, much less is known about its conversion to diol epoxide **5** and tetraol **6** (*13*).

Many studies demonstrate large interindividual differences in the metabolism of PAH, leading to the logical hypothesis that those people who metabolically activate PAH more extensively should be at higher risk for cancer (*19–27*). A vast literature has investigated this hypothesis, but its validity still remains relatively unsettled (*28–35*). Our approach to this question has been to use phenanthrene (Phe, Scheme 1) as a model compound (*36*). Phe is the simplest PAH with a bay region, a feature often associated with carcinogenicity, although Phe is not generally considered carcinogenic. The metabolism of Phe to diol epoxides follows pathways that are quite analogous to those of BaP, as shown in Scheme 1 (*13, 37*). We have proposed that Phe-tetraol (**9** and/or **12**, Scheme 1) is a practical biomarker of PAH metabolic activation by the diol

* To whom correspondence should be addressed. Tel: 612-624-7604. Fax: 612-626-5135. E-mail: hecht002@umn.edu.

10.1021/tx9004538
Published on Web 04/06/2010

Scheme 1. Tetraol Formation from BaP and Phe Bay Region Diol Epoxides (2 and 8) and Reverse Diol Epoxides (5 and 11)



epoxide pathway and that its levels may be used to evaluate lung cancer susceptibility in smokers (*36*). Phe-tetraol is readily measured in human urine by GC-MS/MS and is present in quantities 10000 times greater than those of BaP-tetraols. All humans have Phe-tetraol in their urine, and levels are higher in smokers than in nonsmokers (*38, 39*). Thus, Phe-tetraol could be a biomarker of PAH exposure plus metabolic activation.

In previous studies in which we quantified Phe-tetraol, no attempt was made to distinguish its enantiomers **9** and **12**. Studies of Phe metabolism in vitro have shown that the formation of diols **7** and **10** is stereoselective, producing mainly the (*R*,*R*)-enantiomers shown in Scheme 1 (*37, 40*). Similar stereoselectivity has been observed in the metabolism of BaP to diols (Scheme 1) (*13*). Metabolic epoxidation of Phe diols **7** and **10** followed by hydrolysis gives mainly the tetraols arising from *trans* ring opening of the *anti*-diol epoxides, as shown in Scheme 1, based on analysis of Phe-tetraol in human urine (*36*). Only minor amounts of tetraols resulting from the *syn* diol epoxides or from *cis* ring opening of the *anti*-diol epoxides were observed. Thus, it becomes evident, as shown in Scheme 1, that the amounts of Phe-tetraol **9** in human urine would reflect the "bay region diol epoxide" pathway of Phe metabolism, proceeding through diol **7** and diol epoxide **8**, whereas the amounts of Phe-tetraol **12** would reflect the "reverse bay region diol epoxide" pathway proceeding through **10** and **11**. In a recent study carried out with human hepatocytes, we obtained data indicating that the latter pathway may predominate (*41*). This could impact the interpretation of our biomarker data. Therefore, in this study, we investigated levels of the tetraol enantiomers **9** and **12** in human urine.

As this study progressed, we realized that there had been no similar investigations of BaP-tetraol enantiomers **3** and **6** in human urine. Other than racemic tetraols (*42, 43*), there are to our knowledge no reports in the literature on human urinary BaP metabolites relevant to the widely accepted bay region diol epoxide pathway. The only in vivo evidence for the existence of this pathway of BaP metabolic activation in humans is based on analyses of diol epoxide adducts to globin, albumin, and DNA, and some of these have produced mixed results with regard to detectability (*44–46*). Therefore, we extended our investigation to determine the levels of BaP-tetraol enantiomers in the urine of creosote workers who were exposed to relatively high amounts of PAH.

Materials and Methods

Chemicals, Enzymes, and Chromatography Supplies. Racemic BaP-7,8,9,10-tetraol (**3** + **6**) and BaP-(7*R*,8*R*)-diol (**1**) were obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository. Racemic Phe-1,2,3,4-tetraol (**9** + **12**) was kindly supplied by Drs. D. Jerina and H. Yagi, National Institutes of Health (Bethesda, MD). Phe-(1*R*,2*R*)-diol (**7**) and Phe-(3*R*,4*R*)-diol (**10**) were prepared by incubation of Phe with cytochrome P450 1A1 and cofactors, followed by HPLC purification (*47*). Phe-(1*R*,2*S*)-diol-(3*S*,4*R*)-epoxide (**8**) was prepared by oxidation of **7** with *m*-chloroperbenzoic acid (*47*). Purities of these standards were >95%, as determined by HPLC analysis. *bis*-Trimethylsilyltrifluoroacetamide (BSTFA) was purchased from Regis Technologies. β-Glucuronidase and arylsulfatase (from *Helix pomatia*) were obtained from Roche Diagnostics Corp. (Indianapolis, IN). Strata-X polymeric SPE cartridges (200 mg/6 mL, #8B-S10-FCH) were obtained from Phenomenex. Bond Elute phenylboronic acid SPE

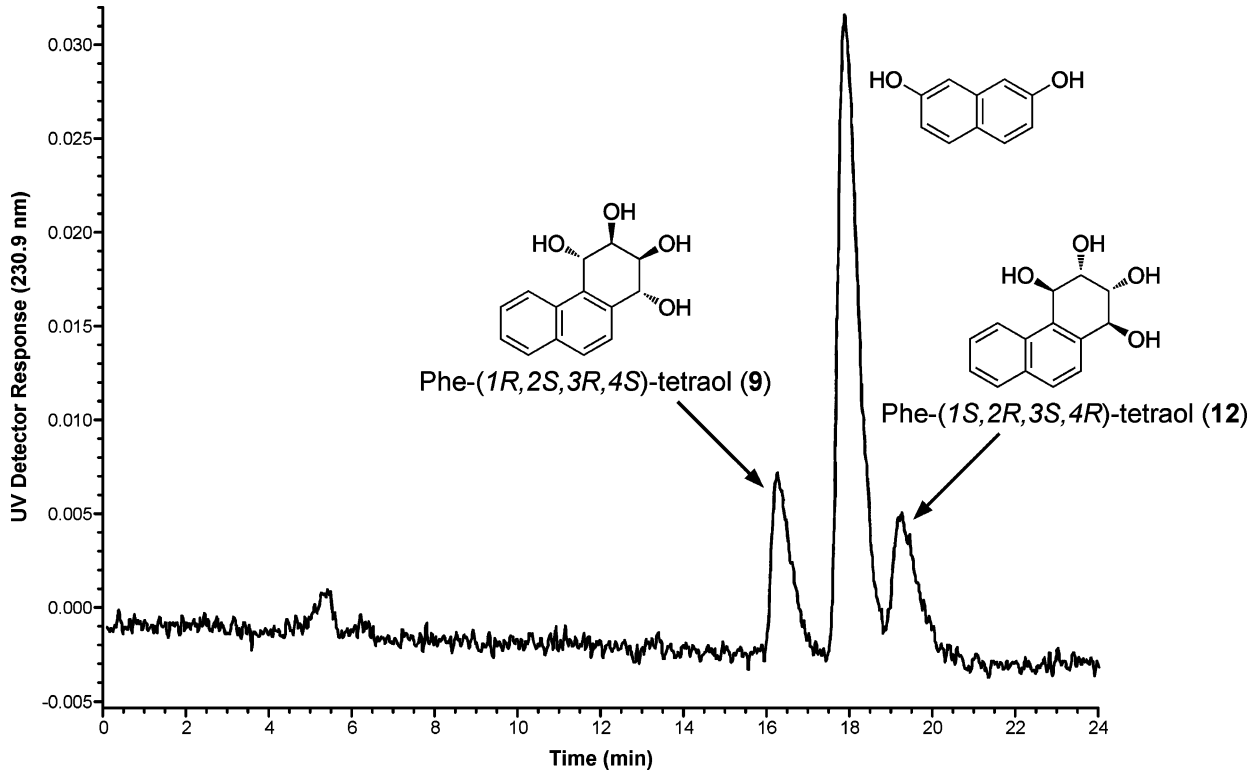


Figure 1. Separation of standard Phe-tetraols **9** and **12** on a Cyclobond II HPLC column. 2,7-Dihydroxynaphthalene was added as a UV marker. For the analysis of urine, the UV peaks corresponding to **9** and **12** cannot be seen; fractions encompassing the correct retention times for Phe-tetraols **9** and **12** were collected using 2,7-dihydroxynaphthalene as a marker (see the Materials and Methods for details).

cartridges (100 mg/1 mL, #12102018) were from Varian, and Oasis MAX SPE cartridges (60 mg/1 mL/ #186000378) were from Waters. An Astec Cyclobond II (250 mm × 4.6 mm, 5 μm) chiral HPLC column was obtained from Sigma-Aldrich and a (*R*,*R*) Whelk-O 5/100 Kromasil #780201 (250 mm × 4.6 mm, 5 μm) Pirkle chiral HPLC column was procured from Regis.

Urine Samples. Urine samples from smokers were obtained from ongoing studies at the University of Minnesota Tobacco Use Research Center. Urine samples from creosote workers were kindly provided by Mary Wolff (Mt. Sinai Medical Center, New York). Analysis of these samples for 1-hydroxypyrene showed levels that were several hundred times higher than in smokers or nonsmokers.

Human Hepatocyte Incubations. These have been previously described (*41*). Incubation mixtures of racemic **7** or **10** from that study were analyzed for enantiomeric tetraols **9** and **12**. Using the same conditions as those described (*41*), Phe-(1*R*,2*R*)-diol (**7**) or Phe-(3*R*,4*R*)-diol (**10**) was also incubated with hepatocytes, and the resulting mixtures were analyzed for tetraols **9** and **12**.

Analysis of Phe-Tetraol Enantiomers 9 and 12 in Smokers' Urine. This was carried out using a modification of a previously described method (*41*). A 0.5 mL aliquot of urine was added to a 1.5 mL polypropylene tube containing 0.5 mL of H_2O, 0.15 mL of 2.5 M NaOAc buffer, pH 5, β-glucuronidase (3500 units), and arylsulfatase (28000 units), and the mixture was incubated overnight with shaking at 37 °C. After incubation, the sample was partially purified by loading on a Strata-X polymeric sorbent cartridge that had been activated with 5 mL of CH_3OH and 5 mL of H_2O. The cartridge was washed twice with 1 mL of H_2O and then 5 mL of 1% NH_4OH in 10% CH_3OH, and the analyte was eluted with 5 mL of 50% CH_3OH, collected in an 8 mL silanized vial, and the solvents were removed on a Speedvac. The residue was dissolved in 500 μL of H_2O with sonication and loaded on a phenylboronic acid cartridge that had been activated with 1 mL of CH_3OH and 1 mL of H_2O. The cartridge was washed with 100 μL of H_2O and placed under vacuum overnight. It was then washed twice with 1 mL of acetone (that had been dried with Na_2SO_4), and Phe-tetraols were eluted with 1 mL of 80% CH_3OH in H_2O. This fraction was collected in a 2 mL silanized vial, and the solvent removed on a Speedvac. The residue was dissolved in 100 μL of CH_3OH with sonication, transferred to an insert vial, and concentrated to dryness. The residue was dissolved using sonication in 50 μL of 10% isopropanol in H_2O containing 500 ng of 2,7-dihydroxynaphthalene as a UV marker, 500 pg of [D_{10}]Phe-tetraol **9** [prepared from metabolically formed [D_{10}]Phe-1,2-diol (**7**) and containing small amounts of [D_{10}]Phe-tetraol **12**] as enantiomer markers, and injected onto the Cyclobond II chiral HPLC column. The column was held at 23 °C, eluted with 0.6 mL/min H_2O, and monitored by UV detection at 230.9 nm. One quarter of a min to 2 min fractions were collected in silanized vials over the entire 10−24 min portion of the chromatogram. Each vial contained 1 pmol of *cis*,*anti*-Phe-tetraol as a derivatization monitor. The UV marker eluted at 18 min, and the Phe-tetraol enantiomers eluted at 16 and 19 min (Figure 1). Fractions were dried on a Speedvac, and the residue was dissolved in 100 μL of CH_3OH with sonication, transferred to an insert vial, concentrated to dryness, and dissolved in 10 μL of BSTFA and 10 μL of acetonitrile. The purpose of the acetonitrile was to break the bonding between residual Cyclobond II stationary phase bleed and Phe-tetraol analyte so that TMS derivatization by BSTFA could proceed. The samples were heated to 60 °C for 60 min with periodic mixing, and 2 μL was analyzed by GC-NICI-MS/MS.

Analysis of BaP-Tetraol Enantiomers in Creosote Workers' Urine. This was performed using a modification of the method described above for Phe-tetraol enantiomers. A 1.0 mL aliquot of urine was added to a 1.5 mL polypropylene tube containing 0.15 mL of 2.5 M NaOAc buffer, pH 5, β-glucuronidase (3500 units), and arylsulfatase (28000 units), and the mixture was incubated overnight with shaking at 37 °C. After incubation, the sample was partially purified by loading onto a Strata-X cartridge as described for the Phe-tetraols. The cartridge was washed twice with 1 mL of H_2O, 5 mL of 1% NH_4OH in 50% CH_3OH, and then 0.5 mL of CH_3OH, and the analyte was eluted using 2 mL of CH_3OH. The analyte fraction was collected in a 4 mL silanized vial, and the solvents were removed on a Speedvac. The sample was dissolved in 750 μL of 30% CH_3OH in H_2O with sonication and loaded onto a phenylboronic acid cartridge as described for Phe-tetraols. The

cartridge was washed with 100 μL of 30% CH_3OH in H_2O and placed under vacuum overnight. The BaP-tetraols were recovered from the cartridge and transferred to an insert vial as described for Phe-tetraols. The residue was dissolved with the aid of sonication in 50 μL of isopropanol containing 500 ng of 2,7-dihydroxynaphthalene as a UV marker and injected onto the Pirkle chiral HPLC column. The column was eluted with 0.5 mL/min isopropanol, and the eluant was monitored by UV at 230.9 nm. The two enantiomers were collected in 4 mL silanized vials, each vial containing 1 pmol of *trans*,*anti*-Phe-tetraol as a derivatization monitor. The UV marker eluted at 7 min, and the two BaP-tetraol enantiomers eluted at 10 and 21 min. Fractions were collected over the entire chromatogram encompassing the two enantiomers. Fractions were dried on a Speedvac, and the residue was dissolved in 100 μL of CH_3OH with sonication, transferred to an insert vial, concentrated to dryness, and dissolved in 10 μL of BSTFA. The samples were heated to 60 °C for 60 min with periodic mixing, and 2 μL was analyzed by GC-NICI-MS/MS.

Analysis of Phe-Diol Enantiomers in Smokers' Urine. The method was similar to that used for the analysis of tetraols. Urine samples were worked up as described above for tetraols, and after the tetraols were eluted from the Strata-X cartridge using 5 mL of 50% CH_3OH, the cartridge was washed with 3 mL of CH_3OH to elute Phe-1,2-diol and Phe-3,4-diol. The solvents were removed on a Speedvac, and the sample was dissolved in 1 mL of H_2O with sonication and loaded onto a Oasis MAX cartridge that had been activated using 1 mL of CH_3OH and 1 mL of 3% NH_4OH in H_2O. The cartridge was washed with 1 mL of 3% NH_4OH in H_2O and 1 mL of H_2O, the Phe-diols were eluted with 1 mL of CH_3OH into a 2 mL silanized vial, and the solvent was removed on the Speedvac. The residue was dissolved in 100 μL of CH_3OH with sonication, transferred to an insert vial, and concentrated to dryness. For Phe-1,2-diols, the residue was dissolved using sonication in 50 μL of 10% isopropanol in hexane containing 500 ng of 2,7-dihydroxynaphthalene as a UV marker, 100 pmol of [$^{13}C_6$]Phe(1*R*,2*R*)-diol as an enantiomer marker, and injected onto the Pirkle chiral HPLC column. The column was eluted with 1 mL/min of 10% isopropanol in hexane, and the eluant was monitored by UV at 230.9 nm. Fractions were collected from 8 to 14 min in 2 mL silanized vials, each vial containing 236 pmol of Phe-9,10-diol as a derivatization monitor. The UV marker eluted at 15 min, and the two Phe-1,2-diol enantiomers eluted at 10 and 11 min. The Phe-3,4-diols were analyzed the same way except that the residue was dissolved in 50% isopropanol in hexane, 94 pmol of [$^{13}C_6$]Phe(3*R*,4*R*)-diol was used as an enantiomer marker, the column was eluted with 50% isopropanol in hexane, fractions were collected from 6 to 11 min, each vial contained 79 pmol of Phe-1,2-diol as derivatization standard, the UV marker eluted at 4 min, and the two Phe-3,4-diol enantiomers eluted at 8 and 9 min. Fractions were dried on a Speedvac, and the residue was dissolved in 100 μL of methanol with sonication, transferred to an insert vial, concentrated to dryness, dissolved in 10 μL of BSTFA, and heated to 60 °C for 60 min with periodic mixing, and 2 μL was analyzed by GC-NICI-MS/MS.

GC-NICI-MS/MS Analysis. This was carried out with a TSQ Quantum instrument (Thermo Electron). All analytes were detected as their trimethylsilyl derivatives. For the detection of tetraols, a 0.25 mm i.d. × 0.15 μm film thickness × 30 m DB17-MS column (Agilent) with a 0.53 mm i.d. × 3 m deactivated fused silica precolumn was used, and for the detection of diols, a 0.18 mm i.d. × 20 m, 0.18 μm film thickness, DB-5 MS UI column (Agilent) was used. The oven temperature program was 80 °C for 1 min (except for Phe-3,4-diol, 3 min was used), then 80−200 at 35 °C/min, then 200−215 at 3 °C/min, then 215−320 at 35 °C/min, and then hold for 3 min. The carrier gas was He at a flow rate of 1.0 mL/min, and the injection port (except for analysis of Phe-3,4-diol) and MS transfer line were kept at 250 and 320 °C, respectively. For the analysis of Phe-3,4-diol, a programmed temperature vaporization injector with baffled inlet liner was used. The injector was operated in the splitless mode, the evaporation temperature was 80 °C for 2 min, then 80−260 at 5 °C/s, and then hold for 1 min. The NICI-MS conditions were as follows: CI gas, methane at 2.0 mL/min except for Phe-3,4-diol, which required argon at 3.3 mL/min; source temperature, 200 °C; and emission current, 100 μA for diols and 500 μA for tetraols. Selected ion monitoring (SIM) with an electron energy of −150 eV for Phe-1,2-diol and −100 eV for Phe-3,4-diol was used to detect the diols at *m*/*z* 193. Selected reaction monitoring (SRM) with a collision energy of 12 eV, electron energy of −100 eV for Phe-tetraols, and −150 eV for BaP-tetraols, and Ar collision gas at 1.0 mTorr was used to detect Phe-tetraols, [D_{10}]Phe-tetraols, and BaP-tetraols at *m*/*z* 372 → 210, *m*/*z* 382 → 220, and *m*/*z* 446 → 255, respectively.

Results

The first step was to separate and characterize Phe-tetraol enantiomers **9** and **12**. Racemic *trans*,*anti*-Phe-1,2,3,4-tetraol was separated into its component enantiomers on a Cyclobond II chiral HPLC column. This is a γ-cyclodextrin bonded to silica, in which glucopyranose units form a truncated cone, and enantiomers are separated by forming inclusion complexes. Baseline separation was achieved as illustrated in Figure 1. The large peak eluting between the two enantiomers is 2,7-dihydroxynaphthalene, which was added as a UV marker to facilitate collection of the appropriate fractions containing each tetraol enantiomer.

The next step was to assign structures, for example, **9** or **12**, to the separated tetraols. This was accomplished by isolating Phe-(1*R*,2*R*)-diol (**7**) and Phe-(3*R*,4*R*)-diol (**10**) from incubation mixtures of human P450 1A1, Phe, and cofactors, conditions that are known to produce these diols stereoselectively (*37, 40, 48*). Oxidation of **7** with *m*-chloroperbenzoic acid selectively produces *anti*-diol epoxide **8** (*48*), which upon hydrolysis yields tetraol **9**, from *trans* ring opening, as the major product (*36*). Similarly, epoxidation and hydrolysis of diol **10** give tetraol **12**. Analysis of each of these tetraols on the Cyclobond II column gave essentially one peak, each of which coeluted with one of the two peaks shown in Figure 1. Thus, we were able to assign the first eluting tetraol peak in Figure 1 as tetraol **9** and the second eluting peak as tetraol **12**. These tetraols were also separable on a Pirkle HPLC column and had the same order of elution.

Fifteen urine samples from smokers were then analyzed for tetraols **9** and **12**. The first step in the analysis was treatment with β-glucuronidase and aryl sulfatase to produce the unconjugated tetraols. The urine was then partially purified by sequential solid-phase extractions using Strata-X reverse phase and phenyl boronic acid cartridges. The phenyl boronic acid cartridge binds tetraols due to their adjacent hydroxyl groups. The appropriate fraction from the phenyl boronic acid cartridge was injected on the Cyclobond II HPLC column, and fractions containing each enantiomer were collected and analyzed for tetraols **9** and **12** by GC-MS/MS. Typical chromatograms are illustrated in Figure 2A−D. Clear peaks were observed for the enantiomeric tetraols, the corresponding internal standard, and for *cis*,*anti*-Phe-tetraol (resulting from *cis* ring opening of *anti*-Phe-1,2-diol-3,4-epoxide), which was used as an injection standard to monitor silylation efficiency.

The results are summarized in Table 1. In each case, 93% or more of Phe-1,2,3,4-tetraol was the second eluting peak from the Cyclobond II column, tetraol **12**, and 7% or less was tetraol **9**. These results indicated that most of Phe-1,2,3,4-tetraol in smokers' urine results from hydrolysis of the reverse diol epoxide **11**, while less than 7% originates from the bay region diol epoxide **8** (Scheme 1). However, this conclusion would only be valid if the formation of Phe-diols **7** and **10** in humans were stereoselective, as shown in Scheme 1 and indicated in

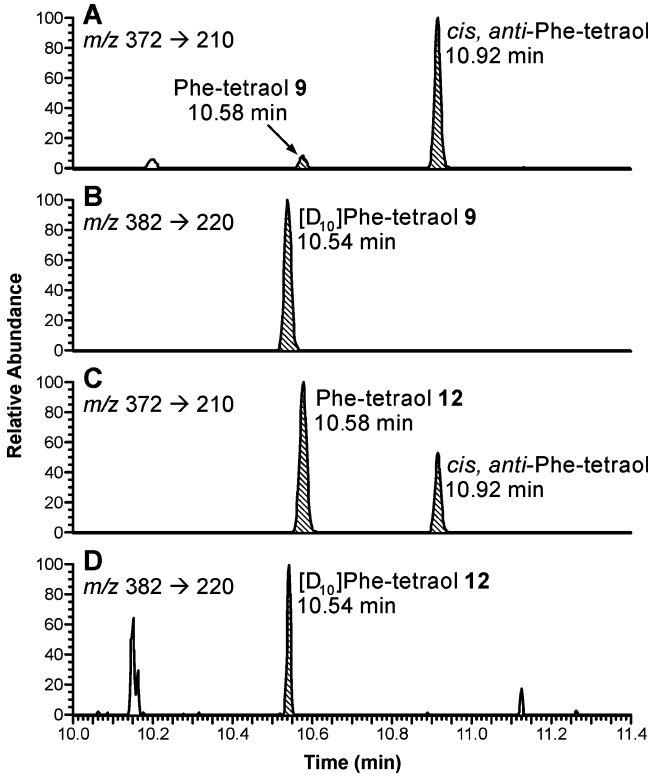


Figure 2. Chromatograms obtained upon GC-MS/MS analysis of tetratrimethylsilyl derivatives of (A) Phe-tetraol **9**, (B) internal standard [D_{10}]Phe-tetraol **9**, (C) Phe-tetraol **12**, and (D) internal standard [D_{10}]Phe-tetraol **12**, which were isolated from the urine of smokers by solid-phase extraction and chiral HPLC as described in the text. A second internal standard, *cis*,*anti*-Phe-tetraol, was added to each sample to monitor silylation efficiency. (Internal standards [D_{10}]Phe-tetraol **9** and [D_{10}]Phe-tetraol **12** were prepared from metabolically formed [D_{10}]Phe-1,2-diol, which was mostly the (*R*,*R*)-enantiomer. Therefore, [D_{10}]Phe-tetraol is predominantly **9** with smaller amounts of **12**, as seen in panels B and D).

Table 1. Enantiomeric Composition of Phe-Tetraols in the Urine of 15 Smokers

	percent composition	
urine sample	Phe-(1*R*,2*S*,3*R*,4*S*)-tetraol (**9**)	Phe-(1*S*,2*R*,3*S*,4*R*)-tetraol (**12**)
1	4.0	96.0
2	7.0	93.0
3	3.8	96.2
4	2.0	98.0
5	4.2	95.8
6	0.2	99.8
7	4.0	96.0
8	2.5	97.5
9	1.8	98.2
10	4.8	95.2
11	5.8	94.2
12	5.7	94.3
13	2.9	97.1
14	0.5	99.5
15	2.0	96.6
mean	3.4 ± 2.0	96.5 ± 1.9

previous in vitro studies (*37, 40*). Because there were no data in the literature on the stereochemistry of Phe-diols **7** and **10** in human urine, we addressed this topic.

Enantiomers of Phe-1,2-diol and Phe-3,4-diol were separated on a Pirkle column, as shown in Figure 3A,B. In each case, the (*R*,*R*)-diol, **7** and **10** (Scheme 1), eluted first, as established by analyzing the diols formed by in vitro metabolism of Phe with human P4501A1 and cofactors. For analysis of the Phe diols in human urine, the samples were treated with β-glucuronidase

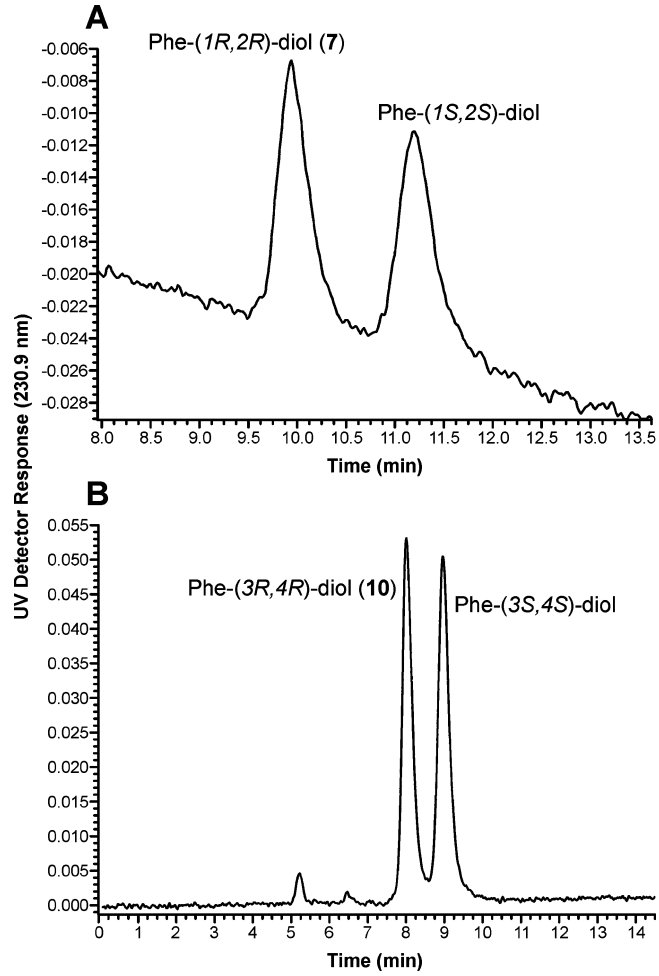


Figure 3. Separation of (A) enantiomers of Phe-1,2-diol and (B) enantiomers of Phe-3,4-diol on a Pirkle HPLC column. For the analysis of urine, the UV peaks corresponding to the diols cannot be seen; they were collected based on retention times.

and aryl sulfatase and then partially purified by solid-phase extraction, followed by injection of the appropriate fraction on the Pirkle column. The fractions containing the diols were collected and analyzed by GC-MS/MS. The results of this study, carried out on urine samples from four smokers, showed that greater than 96% of both Phe-1,2-diol and Phe-3,4-diol were the (*R*,*R*)-enantiomers, for example, compounds **7** and **10** of Scheme 1.

Although these results demonstrated that the Phe-diols were formed stereoselectively in smokers, it was still possible that metabolism of these diols to quinones followed by reduction could lead to biological racemization, possibly confounding the results. We investigated this possibility by analyzing the enantiomeric tetraols formed in incubations of each enantiomeric or racemic diol with human hepatocytes. The results showed that tetraol **9** was formed from diol **7**, and tetraol **12** was formed from diol **10**. Furthermore, the enantiomeric tetraols **9** and **12** were also formed selectively from the racemic diols (data not shown).

In the next phase of this study, we extended this research to BaP. We analyzed urine samples from creosote workers who were exposed to high levels of PAH, thus facilitating the analysis of BaP-tetraols in urine. BaP-tetraols **3** and **6** were separated on the Pirkle column as illustrated in Figure 4. On the basis of the comparison of their CD spectra to published data (*49*), BaP-tetraol **3** eluted first, and BaP-tetraol **6** eluted second. This was confirmed by preparing tetraol **3** from BaP-(7*R*,8*R*)-diol (**1**), by

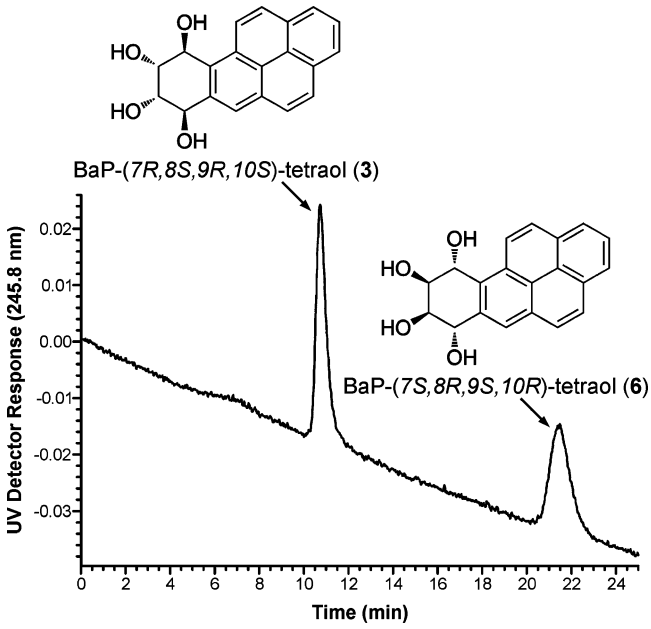


Figure 4. Separation of BaP-tetraols **3** and **6** on a Pirkle HPLC column. For the analysis of urine, the UV peaks corresponding to **3** and **6** cannot be seen; fractions encompassing their retention times were collected.

m-chloroperbenzoic acid oxidation to **2** followed by hydrolysis. Urine samples from four creosote workers were analyzed using a method similar to that employed for the Phe-tetraols. After partial purification and collection from the Pirkle column, the appropriate fractions were silylated, and the tetra-trimethylsilyl derivatives of the BaP-tetraols were analyzed by GC-MS/MS. Fragments and their relative intensities in a daughter ion spectrum of *m/z* 446 are illustrated in Scheme 2; little or no molecular ion, *m/z* 608, was observed. We monitored the transition *m/z* 446 → *m/z* 255. The chromatograms shown in Figure 5A,C have clear peaks eluting at the correct retention times for BaP-tetraols **3** and **6**, respectively. These retention times were confirmed by coinjection with racemic BaP-7,8,9,10-tetraol. The relative intensities of the daughter ion peaks of the isolated BaP-tetraols **3** and **6** were similar to those of standard racemic BaP-7,8,9,10-tetraol (Scheme 2). BaP-tetraol **3** comprised 78 ± 1.7% and BaP-tetraol **6** 22 ± 1.7% of BaP-7,8,9,10-tetraol, indicating that BaP-7,8,9,10-tetraol in the urine of creosote workers results mainly from hydrolysis of the bay region diol epoxide BaP-(7*R*,8*S*)-diol-(9*S*,10*R*) epoxide (**2**).

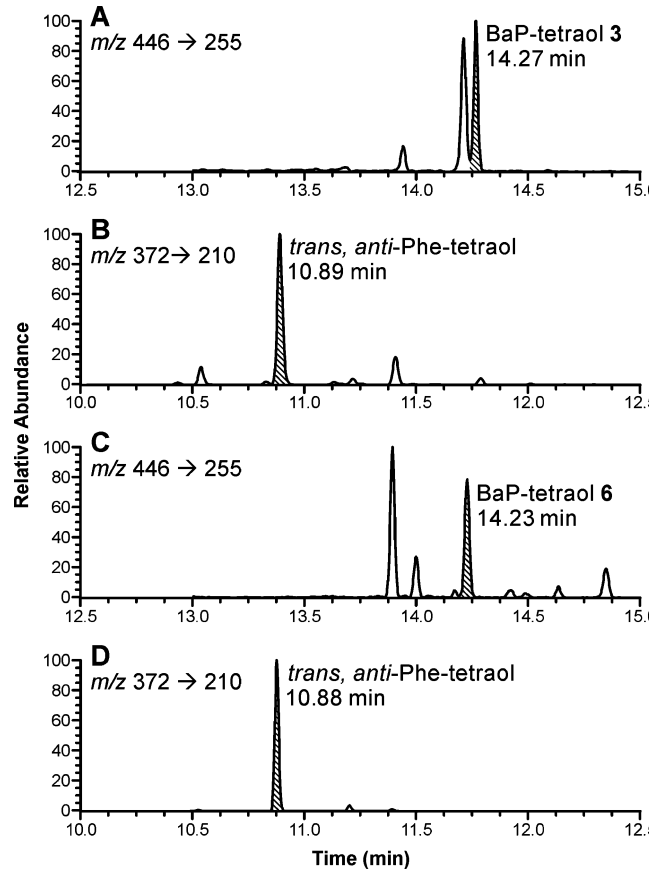
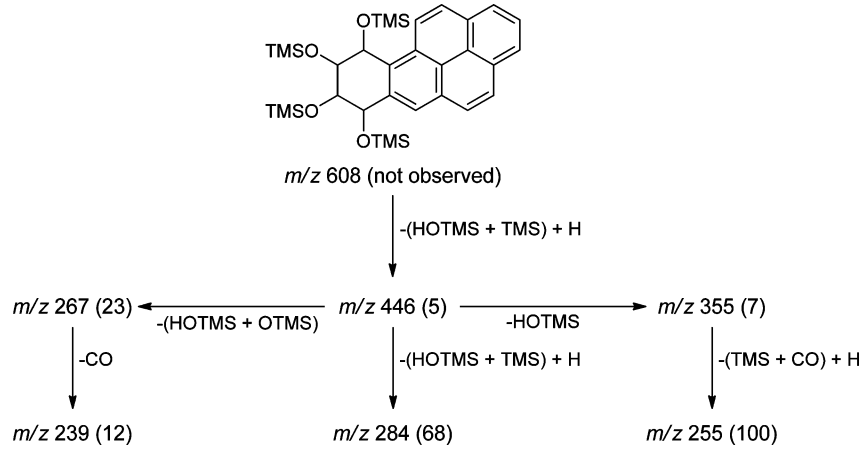


Figure 5. Chromatograms obtained upon GC-MS/MS analysis of urine samples from creosote workers for BaP-7,8,9,10-tetraols. The shaded peaks are tetra-trimethylsilyl derivatives of (A) BaP-tetraol **3**; (B and D) racemic *trans*,*anti*-Phe-tetraol added upon collection of the BaP-tetraol enantiomers to monitor silylation efficiency; and (C) BaP-tetraol **6**.

Discussion

We present the first data on BaP-tetraol and Phe-tetraol enantiomer composition in human urine. The BaP-tetraol results are fully consistent with the view that BaP is metabolically activated in humans to bay region diol epoxide **2** as an ultimate carcinogen. A vast amount of in vitro data using human tissues and P450 enzymes provide strong support for this hypothesis (*12–16, 50–56*), but there are relatively few studies of BaP metabolites in humans. Pertinent to the diol epoxide pathway, only racemic tetraols have been previously analyzed (*42, 43*).

Scheme 2. Fragmentation Pattern and Relative Intensities (in Parentheses) in the Daughter Ion Spectrum of *m/z* 446 of Racemic Tetra-trimethylsilyl-BaP-tetraol; TMS = $(CH_3)_3Si$



The only other studies of BaP metabolites in human urine quantified 3-hydroxyBaP as a biomarker of exposure (*57, 58*). Consistent with our results and the bay region diol epoxide hypothesis, a number of studies using structure specific methods have characterized DNA adducts of BaP resulting from this pathway, although some have reported negative results (*44, 46*). BaP-tetraols have also been identified in hydrolysates of globin and albumin (*44, 45, 59–62*). Our results are consistent with in vitro studies that indicate that BaP-7,8-diol is converted to a diol epoxide more extensively than is BaP-9,10-diol (*63*).

The results reported here leave no doubt that Phe-tetraol in human urine is predominantly comprised of enantiomer **12** originating from metabolism of Phe-(3*R*,4*R*)-diol **10**, with far smaller amounts of enantiomer **9** derived from metabolism of Phe-(1*R*,2*R*)-diol **7**. Thus, in contrast to BaP, angular ring Phe-diol metabolism in humans proceeds mainly through the reverse diol epoxide **11**, as opposed to the bay region diol epoxide **8**. These results are consistent with those of our recent study of human hepatocyte metabolism of Phe diols, which demonstrated 2.5−29-fold more conversion of Phe-3,4-diol than Phe-1,2-diol to tetraols (*41*). That study also demonstrated more extensive glutathione conjugation of the reverse diol epoxide than of the bay region diol epoxide.

A number of investigations have examined the further metabolism in vitro of PAH bay region diols analogous to Phe-3,4-diol (*13*). These studies mainly used rat liver microsomal preparations. As mentioned above, there was limited conversion to tetraols resulting from reverse diol epoxides in the metabolism of the bay region 9,10-diol of BaP, and similar results were obtained with benzo[*e*]pyrene-9,10-diol. However, studies of the metabolism of the bay region diols of benz[*a*]anthracene and chrysene found moderate and high conversion, respectively, to tetraols (*13*). Corresponding investigations of Phe diol metabolism do not seem to have been reported. On the basis of the available literature, it would appear that the extent of metabolism of bay region diols to tetraols via reverse diol epoxides is quite dependent on the structure of the parent PAH.

We also observed enantiomeric preference in the composition of Phe-diols in human urine, with Phe-(1*R*,2*R*)-diol (**7**) and Phe-(3*R*,4*R*)-diol (**10**) predominating. These results are consistent with in vitro metabolism studies of Phe, using both rat and human enzymes (*37, 40*). Phe-diols have been previously analyzed in human urine, but their stereochemistry was not reported. Those studies found higher levels of Phe-1,2-diol than Phe-3,4-diol (*64, 65*). It is not clear whether that observation results from preferential formation of Phe-1,2-diol or preferential metabolism, as indicated here, of Phe-3,4-diol.

Our results raise some questions about the utility of Phe metabolism as a surrogate for BaP metabolism, as we have previously proposed. Although many features of their metabolic transformations are similar in terms of enzyme involvement and stereochemistry, the tetraol end products of the diol epoxide pathway result predominantly from different intermediate diol epoxides. Thus, measurement of racemic Phe-tetraol in urine, as we have reported in previous studies, does not reflect mainly the carcinogenic bay region diol epoxide pathway but rather the reverse diol epoxide pathway, which is generally associated with weak mutagenicity and carcinogenicity (*12, 47, 66*). Nevertheless, the same enzymes are involved in both pathways, so a higher level of racemic Phe-tetraol is still likely to reflect a higher level of bay region diol epoxide formation from carcinogenic PAH such as BaP. This question needs to be addressed in more detail. We are currently developing a method for analysis specifically of Phe-tetraol **9** in human urine. Although it represents only about 3% of racemic Phe-tetraol, the levels of racemic Phe-tetraol are approximately 10000 times as great as those of racemic BaP-tetraol in most samples. Therefore, its concentration should still be 300 times greater than that of racemic BaP-tetraol in samples from smokers and others only moderately exposed to PAHs and may be a more practical biomarker than BaP-tetraol.

In summary, the results of this study provide the first analysis of human urinary PAH tetraol stereochemistry. We confirm the widely held view that BaP is metabolized by the bay region diol epoxide pathway in humans. In contrast, Phe is metabolized predominantly via the reverse diol epoxide pathway. These results provide some important new insights relative to the development of biomarkers to assess PAH metabolism in humans.

Acknowledgment. This study was supported by Grant CA-92025 from the National Cancer Institute. We thank Dr. Silvia Balbo for carrying out the hepatocyte incubations and Bob Carlson for editorial assistance.

References

(1) Brandt, H. C., and Watson, W. P. (2003) Monitoring human occupational and environmental exposures to polycyclic aromatic compounds. *Ann. Occup. Hyg. 47*, 349–378.
(2) Luch, A. (2005) Polycyclic aromatic hydrocarbon-induced carcinogenesis-an introduction. In *The Carcinogenic Effects of Polycyclic Aromatic Hydrocarbons* (Luch, A., Ed.) pp 1−18, Imperial College Press, London.
(3) U.S. Department of Health and Human Services (2004) Report on Carcinogens, 11th ed., pp III-220−III-222, U.S. Department of Health and Human Services, Research Triangle Park, NC.
(4) Ding, Y. S., Ashley, D. L., and Watson, C. H. (2007) Determination of 10 carcinogenic polycyclic aromatic hydrocarbons in mainstream cigarette smoke. *J. Agric. Food Chem. 55*, 5966–5973.
(5) Stepanov, I., Villalta, P. W., Knezevich, A., Jensen, J., Hatsukami, D., and Hecht, S. S. (2010) Analysis of 23 polycyclic aromatic hydrocarbons in smokeless tobacco by gas chromatography-mass spectrometry. *Chem. Res. Toxicol. 23*, 66–73.
(6) Straif, K., Baan, R., Grosse, Y., Secretan, B., El Ghissassi, F., and Cogliano, V. (2005) Carcinogenicity of polycyclic aromatic hydrocarbons. *Lancet Oncol. 6*, 931–932.
(7) International Agency for Research on Cancer (1983) Polynuclear aromatic compounds, Part 1. Chemical, environmental, and experimental data. *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*, Vol. 32, pp 33−91, IARC, Lyon, France.
(8) International Agency for Research on Cancer (1984) Polynuclear aromatic compounds, Part 3. Industrial exposures in aluminum production, coal gasification, coke production, and iron and steel founding. *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*, Vol. 34, pp 65−131, IARC, Lyon, France.
(9) International Agency for Research on Cancer (1985) Polynuclear aromatic compounds, Part 4. Bitumens, coal-tars and derived products, shale oils and soots. *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*, Vol. 35, pp 83−241, IARC, Lyon, France.
(10) Hecht, S. S. (1999) Tobacco smoke carcinogens and lung cancer. *J. Natl. Cancer Inst. 91*, 1194–1210.
(11) Luch, A., and Baird, W. M. (2005) Metabolic activation and detoxification of polycyclic aromatic hydrocarbons. In *The Carcinogenic Effects of Polycyclic Aromatic Hydrocarbons* (Luch, A., Ed.) pp 19−96, Imperial College Press, London.
(12) Conney, A. H. (1982) Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G.H.A. Clowes Memorial Lecture. *Cancer Res. 42*, 4875–4917.
(13) Thakker, D. R., Yagi, H., Levin, W., Wood, A. W., Conney, A. H., and Jerina, D. M. (1985) Polycyclic aromatic hydrocarbons: metabolic activation to ultimate carcinogens. In *Bioactivation of Foreign Compounds* (Anders, M. W., Ed.) pp 177−242, Academic Press, Inc., New York.
(14) Cooper, C. S., Grover, P. L., and Sims, P. (1983) The metabolism and activation of benzo[*a*]pyrene. *Prog. Drug Metab. 7*, 295–396.
(15) Dipple, A., Moschel, R. C., and Bigger, C. A. H. (1984) Polynuclear aromatic hydrocarbons. In *Chemical Carcinogens*, 2nd ed. (Searle,

C. E., Ed.) ACS Monograph 182, Vol. 1, pp 41−163, American Chemical Society, Washington, DC.
(16) Harvey, R. G. (1991) *Polycyclic Aromatic Hydrocarbons: Chemistry and Carcinogenicity*, Cambridge University Press, Cambridge, England.
(17) Geacintov, N. E., Cosman, M., Hingerty, B. E., Amin, S., Broyde, S., and Patel, D. J. (1997) NMR solution structures of stereoisomeric covalent polycyclic aromatic carcinogen-DNA adducts: Principles, patterns, and diversity. *Chem. Res. Toxicol. 10*, 112–146.
(18) Szeliga, J., and Dipple, A. (1998) DNA adduct formation by polycyclic aromatic hydrocarbon dihydrodiol epoxides. *Chem. Res. Toxicol. 11*, 1–11.
(19) Welch, R. M., Harrison, Y. E., Conney, A. H., Poppers, P. J., and Finster, M. (1968) Cigarette smoking: Stimulatory effect on metabolism of 3,4-benzpyrene by enzymes in human placenta. *Science 160*, 541–542.
(20) Kellermann, G., Shaw, C. R., and Luyten-Kellerman, M. (1973) Aryl hydrocarbon hydroxylase inducibility and bronchogenic carcinoma. *N. Engl. J Med. 289*, 934–937.
(21) Harris, C. C., Autrup, H., Connor, R., Barrett, L. A., McDowell, E. M., and Trump, B. F. (1976) Interindividual variation in binding of benzo[*a*]pyrene to DNA in cultured human bronchi. *Science 194*, 1067–1069.
(22) Sabadie, N., Richter-Reichhelm, H. B., Saracci, R., Mohr, U., and Bartsch, H. (1981) Inter-individual differences in oxidative benzo(*a*)pyrene metabolism by normal and tumorous surgical lung specimens from 105 lung cancer patients. *Int. J. Cancer 27*, 417–425.
(23) Nowak, D., Schmidt-Preuss, U., Jorres, R., Liebke, F., and Rudiger, H. W. (1988) Formation of DNA adducts and water-soluble metabolites of benzo[*a*]pyrene in human monocytes is genetically controlled. *Int. J. Cancer 41*, 169–173.
(24) McLemore, T. L., Adelberg, S., Liu, M. C., McMahon, N. A., Yu, S. J., Hubbard, W. C., Czerwinski, M., Wood, T. G., Storeng, R., Lubet, R. A., Eggleston, J. C., Boyd, M. R., and Hines, R. N. (1990) 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. 82*, 1333–1339.
(25) Kiyohara, C., Nakanishi, Y., Inutsuka, S., Takayama, K., Hara, N., Motohiro, A., Tanaka, K., Kono, S., and Hirohata, T. (1998) The relationship between CYP1A1 aryl hydrocarbon hydroxylase activity and lung cancer in a Japanese population. *Pharmacogenetics 8*, 315–323.
(26) Nebert, D. W. (2000) Drug-metabolizing enzymes, polymorphisms and interindividual response to environmental toxicants. *Clin. Chem. Lab. Med. 38*, 857–861.
(27) Alexandrov, K., Cascorbi, I., Rojas, M., Bouvier, G., Kriek, E., and Bartsch, H. (2002) CYP1A1 and GSTM1 genotypes affect benzo[a]pyrene DNA adducts in smokers' lung: Comparison with aromatic/hydrophobic adduct formation. *Carcinogenesis 23*, 1969–1977.
(28) Bartsch, H., Nair, U., Risch, A., Rojas, M., Wikman, H., and Alexandrov, K. (2000) Genetic polymorphism of CYP genes, alone or in combination, as a risk modifier of tobacco-related cancers. *Cancer Epidemiol. Biomarkers Prev. 9*, 3–28.
(29) Smith, G. B., Harper, P. A., Wong, J. M., Lam, M. S., Reid, K. R., Petsikas, D., and Massey, T. E. (2001) 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. 10*, 839–853.
(30) Lee, W. J., Brennan, P., Boffetta, P., London, S. J., Benhamou, S., Rannug, A., To-Figueras, J., Ingelman-Sundberg, M., Shields, P., Gaspari, L., and Taioli, E. (2002) Microsomal epoxide hydrolase polymorphisms and lung cancer risk: A quantitative review. *Biomarkers 7*, 230–241.
(31) Benhamou, S., Lee, W. J., Alexandrie, A. K., Boffetta, P., Bouchardy, C., Butkiewicz, D., Brockmoller, J., Clapper, M. L., 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, S. J., Nazar-Stewart, V., Onon-Kihara, M., Rannug, A., Romkes, M., Ryberg, D., Seidegard, J., Shields, P., Strange, R. C., Stucker, I., To-Figueras, J., Brennan, P., and Taioli, E. (2002) Meta- and pooled analyses of the effects of glutathione *S*-transferase M1 polymorphisms and smoking on lung cancer risk. *Carcinogenesis 23*, 1343–1350.
(32) Hung, R. J., Boffetta, P., Brockmoller, J., Butkiewicz, D., Cascorbi, I., Clapper, M. L., Garte, S., Haugen, A., Hirvonen, A., Anttila, S., Kalina, I., Le Marchand, L., London, S. J., Rannug, A., Romkes, M., Salagovic, J., Schoket, B., Gaspari, L., and Taioli, E. (2003) CYP1A1 and GSTM1 genetic polymorphisms and lung cancer risk in Caucasian non-smokers: A pooled analysis. *Carcinogenesis 24*, 875–882.
(33) Veglia, F., Matullo, G., and Vineis, P. (2003) Bulky DNA adducts and risk of cancer: A meta-analysis. *Cancer Epidemiol. Biomarkers Prev. 12*, 157–160.
(34) Raimondi, S., Paracchini, V., Autrup, H., Barros-Dios, J. M., Benhamou, S., Boffetta, P., Cote, M. L., Dialyna, I. A., Dolzan, V., Filiberti, R., Garte, S., Hirvonen, A., Husgafvel-Pursiainen, K., Imyanitov, E. N., Kalina, I., Kang, D., Kiyohara, C., Kohno, T., Kremers, P., Lan, Q., London, S., Povey, A. C., Rannug, A., Reszka, E., Risch, A., Romkes, M., Schneider, J., Seow, A., Shields, P. G., Sobti, R. C., Sorensen, M., Spinola, M., Spitz, M. R., Strange, R. C., Stucker, I., Sugimura, H., To-Figueras, J., Tokudome, S., Yang, P., Yuan, J. M., Warholm, M., and Taioli, E. (2006) Meta- and pooled analysis of *GSTT1* and lung cancer: A HuGE-GSEC review. *Am. J. Epidemiol. 164*, 1027–1042.
(35) Carlsten, C., Sagoo, G. S., Frodsham, A. J., Burke, W., and Higgins, J. P. (2008) Glutathione S-transferase M1 (GSTM1) polymorphisms and lung cancer: A literature-based systematic HuGE review and meta-analysis. *Am. J. Epidemiol. 167*, 759–774.
(36) Hecht, S. S., Chen, M., Yagi, H., Jerina, D. M., and Carmella, S. G. (2003) 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. 12*, 1501–1508.
(37) Shou, M., Korzekwa, K. R., Krausz, K. W., Crespi, C. L., Gonzalez, F. J., and Gelboin, H. V. (1994) Regio- and stereo-selective metabolism of phenanthrene by twelve cDNA-expressed human, rodent, and rabbit cytochromes P-450. *Cancer Lett. 83*, 305–313.
(38) Hecht, S. S., Chen, M., Yoder, A., Jensen, J., Hatsukami, D., Le, C., and Carmella, S. G. (2005) Longitudinal study of urinary phenanthrene metabolite ratios: Effect of smoking on the diol epoxide pathway. *Cancer Epidemiol. Biomarkers Prev. 14*, 2969–2974.
(39) Hecht, S. S., Carmella, S. G., Yoder, A., Chen, M., Li, Z., Le, C., Jensen, J., and Hatsukami, D. K. (2006) Comparison of polymorphisms in genes involved in polycyclic aromatic hydrocarbon metabolism with urinary phenanthrene metabolite ratios in smokers. *Cancer Epidemiol. Biomarkers Prev. 15*, 1805–1811.
(40) Nordqvist, M., Thakker, D. R., Vyas, K. P., Yagi, H., Levin, W., Ryan, D. E., Thomas, P. E., Conney, A. H., and Jerina, D. M. (1981) Metabolism of chrysene and phenanthrene to bay-region diol epoxides by rat liver enzymes. *Mol. Pharmacol. 19*, 168–178.
(41) Hecht, S. S., Berg, J. Z., and Hochalter, J. B. (2009) Preferential glutathione conjugation of a reverse diol epoxide compared to a bay region diol epoxide of phenanthrene in human hepatocytes: Relevance to molecular epidemiology studies of glutathione-*S*-transferase polymorphisms and cancer. *Chem. Res. Toxicol. 22*, 426–432.
(42) Simpson, C. D., Wu, M. T., Christiani, D. C., Santella, R. M., Carmella, S. G., and Hecht, S. S. (2000) 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. 13*, 271–280.
(43) Weston, A., Bowman, E. D., Carr, P., Rothman, N., and Strickland, P. T. (1993) Detection of metabolites of polycyclic aromatic hydrocarbons in human urine. *Carcinogenesis 14*, 1053–1055.
(44) Boysen, G., and Hecht, S. S. (2003) Analysis of DNA and protein adducts of benzo[*a*]pyrene in human tissues using structure-specific methods. *Mutat. Res. 543*, 17–30.
(45) Ragin, A. D., Crawford, K. E., Etheredge, A. A., Grainger, J., and Patterson, D. G., Jr. (2008) A gas chromatography-isotope dilution high-resolution mass spectrometry method for quantification of isomeric benzo[a]pyrene diol epoxide hemoglobin adducts in humans. *J. Anal. Toxicol. 39*, 728–736.
(46) Beland, F. A., Churchwell, M. I., Von Tungeln, L. S., Chen, S., Fu, P. P., Culp, S. J., Schoket, B., Gyorffy, E., Minarovits, J., Poirier, M. C., Bowman, E. D., Weston, A., and Doerge, D. R. (2005) High-performance liquid chromatography electrospray ionization tandem mass spectrometry for the detection and quantitation of benzo[*a*]pyrene-DNA adducts. *Chem. Res. Toxicol. 18*, 1306–1315.
(47) Hecht, S. S., Villalta, P. W., and Hochalter, J. B. (2008) Analysis of phenanthrene diol epoxide mercapturic acid detoxification products in human urine: Relevance to molecular epidemiology studies of glutathione-*S*-transferase polymorphisms. *Carcinogenesis 29*, 937–943.
(48) Whalen, D. L., Ross, A. M., Yagi, H., Karle, J. M., and Jerina, D. M. (1978) Stereoelectronic factors in the solvolysis of bay region diol epoxides of polycyclic aromatic hydrocarbons. *J. Am. Chem. Soc. 100*, 5218–5221.
(49) Weems, H. B., and Yang, S. K. (1989) Chiral stationary phase high-performance liquid chromatographic resolution and absolute configuration of enantiomeric benzo[a]pyrene diol-epoxides and tetrols. *Chirality 1*, 276–283.
(50) Jiang, H., Shen, Y. M., Quinn, A. M., and Penning, T. M. (2005) Competing roles of cytochrome P450 1A1/1B1 and aldo-keto reductase 1A1 in the metabolic activation of (±)-7,8-dihydroxy-7,8-dihydro-

benzo[*a*]pyrene in human bronchoalveolar cell extracts. *Chem. Res. Toxicol. 18*, 365–374.
(51) Shimada, T., Gillam, E. M. J., Oda, Y., Tsumara, F., Sutter, T. R., Guengerich, F. P., and Inoue, K. (1999) 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. 12*, 623–629.
(52) Kim, J. H., Stansbury, K. H., Walker, N. J., Trush, M. A., Strickland, P. T., and Sutter, T. R. (1998) Metabolism of benzo[*a*]pyrene and benzo[*a*]pyrene-7,8-diol by human cytochrome P450 1B1. *Carcinogenesis 19*, 1847–1853.
(53) Staretz, M. E., Murphy, S. E., Nunes, M. G., Koehl, W., Amin, S., Koenig, L., Guengerich, F. P., and Hecht, S. S. (1997) Comparative metabolism of the tobacco smoke carcinogens benzo[*a*]pyrene, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, and *N*′-nitrosonornicotine in human hepatic microsomes. *Drug Metab. Dispos. 25*, 154–162.
(54) Bauer, E., Guo, Z., Ueng, Y. F., Bell, L. C., Zeldin, D., and Guengerich, F. P. (1995) Oxidation of benzo[*a*]pyrene by recombinant human cytochrome P450 enzymes. *Chem. Res. Toxicol. 8*, 136–142.
(55) Shou, M., Korzekwa, K. R., Crespi, C. L., Gonzalez, F. J., and Gelboin, H. V. (1994) The role of 12 *c*DNA-expressed human, rodent, and rabbit cytochromes P450 in the metabolism of benzo[*a*]pyrene and benzo[*a*]pyrene trans-7,8-dihydrodiol. *Mol. Carcinog. 10*, 159–168.
(56) Jacob, J., Doehmer, J., Grimmer, G., Soballa, V., Raab, G., Seidel, A., and Greim, H. (1996) Metabolism of phenanthrene, benz[a]anthracene, benz[a]pyrene, chrysene and benzo[c]phenanthrene by eight cDNA-expressed human and rate cytochromes P450. *Polycyclic Aromat. Compd. 10*, 1–9.
(57) Gündel, J., and Angerer, J. (2000) 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 738*, 47–55.
(58) Forster, K., Preuss, R., Rossbach, B., Bruning, T., Angerer, J., and Simon, P. (2008) 3-Hydroxybenzo[a]pyrene in the urine of workers with occupational exposure to polycyclic aromatic hydrocarbons in different industries. *Occup. Environ. Med. 65*, 224–229.
(59) Ozbal, C. C., Skipper, P. L., Yu, M. C., London, S. J., Dasari, R. R., and Tannenbaum, S. R. (2000) Quantification of (7*S*,8*R*)-dihydroxy-(9*R*,10*S*)-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene adducts in human serum albumin by laser- induced fluorescence: Implications for the in vivo metabolism of benzo[*a*]pyrene. *Cancer Epidemiol. Biomarkers Prev. 9*, 733–739.
(60) Melikian, A. A., Sun, P., Pierpont, C., Coleman, S., and Hecht, S. S. (1997) Gas chromatography-mass spectrometric determination of benzo[*a*]pyrene and chrysene diol epoxide globin adducts in humans. *Cancer Epidemiol. Biomarkers Prev. 6*, 833–839.
(61) Pastorelli, R., Restano, J., Guanci, M., Maramonte, M., Magagnotti, C., Allevi, R., Lauri, D., Fanelli, R., and Airoldi, L. (1996) Hemoglobin adducts of benzo[*a*]pyrene diolepoxide in newspaper vendors: Association with traffic exhaust. *Carcinogenesis 17*, 2389–2394.
(62) Pastorelli, R., Guanci, M., Cerri, A., Minoia, C., Carrer, P., Negri, E., Fanelli, R., and Airoldi, L. (2000) Benzo(a)pyrene diolepoxide-haemoglobin and albumin adducts at low levels of benzo(a)pyrene exposure. *Biomarkers 5*, 245–251.
(63) Thakker, D. R., Yagi, H., Lehr, R. E., Levin, W., Buening, M., Lu, A. Y., Chang, R. L., Wood, A. W., Conney, A. H., and Jerina, D. M. (1978) Metabolism of *trans*-9,10-dihydroxy-9,10-dihydrobenzo[*a*]pyrene occurs primarily by arylhydroxylation rather than formation of a diol epoxide. *Mol. Pharmacol. 14*, 502–513.
(64) Seidel, A., Spickenheuer, A., Straif, K., Rihs, H. P., Marczynski, B., Scherenberg, M., Dettbarn, G., Angerer, J., Wilhelm, M., Bruning, T., Jacob, J., and Pesch, B. (2008) New biomarkers of occupational exposure to polycyclic aromatic hydrocarbons. *J. Toxicol. Environ. Health A 71*, 734–745.
(65) Jacob, J., Grimmer, G., and Dettbarn, G. (1999) Profile of urinary phenanthrene metabolites in smokers and non-smokers. *Biomarkers 4*, 319–327.
(66) Glatt, H., Wameling, C., Elsberg, S., Thomas, H., Marquardt, H., Hewer, A., Phillips, D. H., Oesch, F., and Seidel, A. (1993) Genotoxicity characteristics of reverse diol-epoxides of chrysene. *Carcinogenesis 14*, 11–19.

TX9004538