Int Arch Occup Environ Health (1999) 72: 161–168 © Springer-Verlag 1999

ORIGINAL ARTICLE

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Detection of polycyclic aromatic hydrocarbon metabolites by high-pressure liquid chromatography after purification on immunoaffinity columns in urine from occupationally exposed workers

Received: 21 April 1998 / Accepted: 17 January 1999

Abstract ***Objective:*** The objective in our study was to quantitate benzo[*a*]pyrene (B[a]P) metabolites by a combination of immunoaffinity chromatography and high-pressure liquid chromatography (HPLC) with fluorescence detection in urine from workers exposed to high levels of polycyclic aromatic hydrocarbons (PAH). Furthermore, by the simultaneous quantitation of 1-hydroxypyrene, the correlation between the B[a]P-tetrol and 1-hydroxypyrene would provide a means of evaluating the validity of 1-hydroxypyrene as a surrogate biomarker for occupational exposure to the potent carcinogen B[a]P in an electrode paste plant. ***Methods:*** The study was carried out at an electrode paste plant that produces electrode paste for Söderberg electrodes. A total of 34 pre- and post-shift urine samples and 17 personal air samples were collected from 17 workers during a normal work week. The concentration of 1-hydroxypyrene was measured in all urine samples. A recent method of quantitating B[a]P-*r*-7, *t*-8, *t*-9, *c*-10-tetrol in urine of humans exposed to low levels of PAH has been described. A modified version of this method involving purification of urine samples on immunoaffinity columns and HPLC analysis with fluorescence detection was used on urine samples from workers exposed to high levels of PAH. A monoclonal antibody (8*E*11) with binding affinity to B[a]P-tetrols was used. This antibody also binds several PAH-DNA adducts and metabolites, including 1-hydroxypyrene. Gas chromatography/mass spectroscopy (GC/MS) was also used for identification of metabolites isolated by HPLC fractionation. ***Results:*** From personal air sampling the mean exposure to particulate PAHs was 38 $\mu g/m^3$. The mean concentration of urinary 1-hydroxypyrene was 3.9 μmol/mol creatinine in preshift samples and 10.2 μmol/mol creatinine in postshift samples. We could not identify detectable amounts of urinary B[a]P-tetrol by HPLC or fluorescence spectroscopy after purification on immunoaffinity columns. However, in the HPLC analysis we identified several hydroxyphenantrene metabolites that were detected at relatively high concentrations in all of the workers' urine samples. We could not separate 2- and 3-hydroxyphenanthrene (2 + 3-OH-Phe) in peak 1, and peak 2 contained both 1- and 9-hydroxyphenanthrene (1 + 9-OH-Phe). The phenanthrene metabolites were mainly conjugated to glucuronic acid and sulfate. There was a significant correlation between the 1-hydroxypyrene concentration and 2 + 3-OH-Phe (r = 0.73) and 1 + 9-OH-Phe (r = 0.64) in the urine samples. 1-Hydroxypyrene was measured in all post-shift urine samples but was not significantly correlated with workplace pyrene exposure, indicating that skin exposure is an important route of pyrene exposure in this factory. As with 1-hydroxypyrene, dermal PAH uptake may also account for the poor correlation between 2 + 3- and 1 + 9-OH-Phe and ambient phenanthrene. ***Discussion:*** Since dermal uptake is likely to be important in occupational PAH exposure in addition to inhalation, estimation of total PAH exposure is best achieved by quantitation of PAHs excreted into body fluids. However, it remains unclear whether there might be a difference in uptake and urinary excretion of 3-ring, 4-ring, or 5-ring PAHs and in the correlation between these metabolites and ambient-air PAH measurements. In summary, using immunaffinity chromatography, we did not find detectable amounts of B[a]P-tetrol in urine from workers occupationally exposed to PAH. However, by an HPLC/immunoaffinity method, relatively high amounts of 1-hydroxypyrene as well as 2 + 3- and

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1 + 9-OH-Phe were quantitated in the urine samples, both of which are relevant as biomarkers of PAH exposure.

Key words Biomarkers · Immunoaffinity chromatography · 1-Hydroxypyrene · Benzo[*a*]pyrene-tetrol · Hydroxyphenanthrene · Workplace monitoring

Introduction

The mutagenic and carcinogenic effects of individual polycyclic aromatic hydrocarbons (PAHs) and PAH-containing products such as coal tars have been well documented in several animal models and in humans (IARC 1983a, b). PAHs are widespread environmental pollutants, and methods of reliable monitoring of human exposure to PAHs as a preventive health measure are necessary. The most common methods for monitoring of occupational PAH exposure are measurements of the concentration of airborne PAHs (Bjørseth and Becher 1986; Mark 1986; Wilsey et al. 1996). However, monitoring of only the inhalable fraction of PAHs in the work environment may prove to be insufficient since several PAHs have been shown to be absorbed through the skin and to give an important contribution as compared with exposure by inhalation (VanRooij et al. 1993; Quinlan et al. 1995; Malkin et al. 1996).

The concentration of the pyrene metabolite 1-hydroxypyrene in urine has been widely used as a biological indicator of occupational exposure to PAHs. 1-Hydroxypyrene has been a preferential biomarker because pyrene is a dominant compound in the PAH mixture and because it is the main urinary pyrene metabolite and is well suited for quantitation (Jongeneelen et al. 1987; Øvrebø et al. 1995a; Jongeneelen 1997; Bentsen et al. 1998). However, simultaneous determination of several urinary PAH metabolites by a simple and reliable quantitation method would provide a more comprehensive means of achieving a reliable estimation of overall individual exposure to PAH. Because of their carcinogenic potential to humans, PAHs classified as being carcinogenic to experimental animals (IARC 1983b) are of particular interest. By high-pressure liquid chromatography (HPLC) analysis of urine from aluminum plant workers, Øvrebø et al. (1995b) found several peaks originating from the PAH exposure since they were not present in nonexposed, nonsmoking controls. Methods for quantitation of several urinary PAH metabolites have been reported (Becher and Bjørseth 1983; Jacob and Grimmer 1996; Angerer et al. 1997; Grimmer et al. 1997), including monohydroxy derivates and dihydrodiols of phenanthrene, pyrene, chrysene, and benzo[*a*]pyrene. PAH metabolites originating from more-than-3-ring PAHs are mainly excreted in the feces (Jacob and Grimmer 1996) and may therefore be difficult to detect in urine. Recently, Weston et al. (1993) detected and quantitated the hydrolyzed metabolite B[a]P-*r*-7, *t*-8, *t*-9, *c*-10-tetrol of the ultimate carcinogen B[a]P-7,8-diol-9,10-epoxide in the urine of volunteers consuming a diet of char-broiled food by a method using immunoaffinity chromatography (IAC) and reverse-phase HPLC.

The objective in our study was to quantitate urinary B[a]P metabolites by IAC and HPLC (Weston et al. 1993) and to evaluate this method on workers exposed to high levels of PAH. A monoclonal antibody (8*E*11) with proven binding capacity to B[a]P-tetrols was used (Santella et al. 1984; Weston et al. 1993). This antibody has been shown to recognize several PAH-DNA adducts and metabolites, including 1-hydroxypyrene (Strickland et al. 1994). Furthermore, by the simultaneous quantitation of 1-hydroxypyrene, the correlation between the B[a]P-tetrol and 1-hydroxypyrene would provide means of evaluating the validity of 1-hydroxypyrene as a surrogate biomarker for occupational exposure to the potent carcinogen B[a]P in the electrode paste plant.

Materials and methods

Study group

The study was carried out at an electrode paste plant that produces electrode paste for Söderberg electrodes. A total of 34 pre- and postshift urine samples and 17 personal air samples were collected from 17 workers during a normal work week. A personal questionnaire was used to record information about work tasks and smoking habits. The workers performed different work tasks such as the mixing of calcined ground anthracite with coal tar to produce electrode paste, the filling of paste into molds, or truck driving, all of which involved exposure to relatively high levels of PAHs.

Air sampling

A 25-mm closed-face total aerosol sampler (Gelman Sciences, Ann Arbor, Mich., USA) was mounted near the breathing zone of the workers. The sampler was equipped with an acrylic copolymer membrane filter with a pore size of 0.8 μm (Versapore 800, Gelman Sciences). An adsorbent for gaseous PAHs (XAD-2, SKC, Blandford Forum, UK) was placed behind the filters. The sampling was performed with Casella AFC 123 personal pumps (Casella, London, UK) at a flow rate of 2 l min^{-1} over a sampling period of 7–8 h.

PAH determination

The filters were extracted with 2.0 ml dichloromethane containing an internal standard using Soxhlett extraction for 15 h. The extracts were concentrated and injection volumes of 2 μl were analyzed on an HP5890 gas chromatograph (Hewlett-Packard, Wilmington, Del., USA) with a HP 5970 mass spectrometer. The gas chromatograph was equipped with an HP-5 column (Hewlett-Packard, 30 m × 0.25 mm inside diameter; d_f 0.1 μm). The injector temperature was 250 °C. The oven temperature program was set as follows: 80 °C for 5 min, increase to 140 °C at 6 °C/min, and hold there for 15 min before an increase to 220 °C at 4 °C/min and then hold for 35 min. Finally, the injector temperature was increased from 220° to 300 °C at 8 °C/min and held for 15 min. Helium was used as the carrier gas at a constant flow rate of 0.7 ml/min.

PAH collected on XAD was extracted with dichloromethane. One eluate of 5 ml and two eluates of 3 ml were concentrated at

50 °C under a stream of nitrogen (Bjørseth et al. 1978). The eluates were analyzed separately on an HP 5890 Series II gas chromatograph (Hewlett-Packard, Wilmington, Del., USA) equipped with a flame ionization detector (FID) at a temperature of 300 °C. Injections were done in the splitless mode with a 2-min splitless time. A CP-Sil 8 CB fused-silica column (25 m × 0.25 mm inside diameter; d_f 0.25 μm; Chrompack, Raitan, N.J., USA) was used. Helium was used as the carrier gas at a flow rate of 1 ml/min. The oven temperature was held at 35 °C for 2 min, increased from 35° to 150 °C at 6 °C/min and then from 150° to 310 °C at 10 °C/min, and held at 310 °C for 15 min. An internal standard method was used for quantification of the PAH compounds. A total of 17 PAHs were chosen according to the NIOSH manual of analytical methods (NIOSH 1994).

Determination of urinary 1-hydroxypyrene

The determination of 1-hydroxypyrene in urine was carried out essentially according to the method described by Jongeneelen et al. (1987). In brief, 10 ml urine was treated with an enzyme mixture of glucuronidase and sulfatase at 37 °C for 16 h (Boehringer Mannheim, Germany) and was then purified on a C_{18} octadecyl silica cartridge (Sep-Pak, Waters, Milford, Mass.). The sample was eluted from the C_{18} cartridge with 10 ml methanol, evaporated to dryness, and resuspended in 2 ml methanol. Then, 20 μl of this extract was analyzed by HPLC with a methanol-water and column temperature of 40 °C. The flow rate was 0.8 ml min^{-1}. A 150 × 3.9 mm Nova-pak C_{18} column (Waters, Milford, Mass.) was used in a Waters HPLC system equipped with a Perkin-Elmer LC240 fluorescence detector (Beaconsfield, UK; excitation wavelength 242 nm, emission wavelength 388 nm). The detection limit was 0.4 pmol/ml for the injection of a 20-μl sample. The urine samples were corrected for creatinine content (Alessio et al. 1985). Spiked urine samples containing 1-hydroxypyrene at 10, 20, 40, 100, and 250 nmol/l (Janssen Chimica, Geel, Belgium) were treated as unknown and used as standards.

Isolation and quantitation of B[a]P-tetrols in urine by immunoaffinity columns

The isolation and quantitation of B[a]P-metabolites was performed with a method based in principle on that developed by Weston et al. (1993) for the quantitation of B[a]P-7,10/8,9-tetrol in human urine. In brief, 10 ml urine was diluted with 20 ml sodium acetate (pH 5.0). The urine samples were treated with an enzyme mixture of glucuronidase and sulfatase (Boehringer Mannheim, Germany) overnight at 37 °C. In addition, some experiments were carried out with acid hydrolysis (0.1 *M* HCl for 3 h at 90 °C) as described by Weston et al.(1993). The samples were then purified using octadecasilane chromatography columns (Sep-Pak C_{18}, Waters, Milford, Mass.) that had been primed with 100% methanol followed by distilled water. The Sep-Pak columns were washed with distilled water after sample application to remove polar materials, and nonpolar materials were eluted with 10 ml 100% methanol. The eluates were dried at 45 °C under a stream of nitrogen gas and were redissolved in 10 ml phosphate-buffered saline (Sigma Chemical Co., St. Louis, Mo.) for 1 h at 40 °C, which gave a better yield of 1-hydroxypyrene than did dissolution in 1 ml H_2O as described by Weston et al. (1993). The eluates were then subjected to IAC with a monoclonal antibody (8*E*11) directed against BPDE-modified DNA (Santella et al. 1984), which has a proven binding affinity for urinary benzo[*a*]pyrene-tetrol (Weston et al. 1993). The immunoaffinity columns were prepared with the Affinica antibody orientation kit from Schleicher & Schuell (Keene, N.H., USA). Prior to use we tested the columns for recovery by applying 6400 fmol B[a]P-*r*-7, *t*-8, *t*-9, *c*-10-tetrahydrotetrol standard (NCI, Kansas City, Mo.) in 1 ml H_2O. The recovery was $>75\%$.

Materials not bound to the antibodies were removed with 5 ml TRIS-HCl (10 m*M*, pH 7.4). Bound material was eluted with 4 ml 100% methanol. The methanol fractions were dried at 45 °C under nitrogen gas and redissolved in 450 μl 10% methanol, and sample injections of 200 μl were analyzed by HPLC. A 150 × 3.9 mm Nova-pak C_{18} column (Waters, Milford, Mass.) was used in an HPLC system equipped with a Perkin-Elmer LC240 fluorescence detector (Beaconsfield, UK). The extracts were eluted with a methanol-water gradient of 30–100% methanol for 40 min and were held for 10 min at 100%, and the methanol content was then reduced to 30% in 1 min and held for 19 min before the next injection. The column temperature was 40 °C and the flow rate, 1 ml min^{-1}. The fluorescence detection was carried out at an excitation wavelength of 341 nm and an emission wavelength of 381 nm. The detection limit of the assay was 0.02 pmol B[a]P-*r*-7, *t*-8, *t*-9, *c*-10-tetrol/ml for a 10-ml urine sample.

The following phenanthrene standards were obtained from J. Jacob (Jacob et al. 1996): 1-, 2-, 3-, 4-, and 9-OH-Phe; 1,2-diOH-Phe; and 3,4-diOH-Phe. The concentrations of the monohydroxyphenanthrene standards were determined by UV absorption. The concentrations of the phenanthrene metabolites detected in the urine samples were estimated on the basis of the 1-hydroxypyrene standard and were corrected by comparison between the 1-hydroxypyrene standard and the monohydroxyphenanthrene standards. Peak 1 contained 2 + 3-OH-Phe, and peak 2 contained 1 + 9-OH-Phe. We calculated the average fluorescence extinction coefficients for peak 1 and peak 2 on the basis of the distribution of the different monohydroxylated phenanthrene metabolites in other occupational exposure studies (Grimmer et al. 1993; Angerer et al. 1997; Popp et al. 1997). Approximate quantitations were achieved by assuming that peak 1 contained 33% 2-OH-Phe and 67% 3-OH-Phe and that peak 2 contained 6% 9-OH-Phe and 94% 1-OH-Phe.

Fluorescence analysis of HPLC fractions

Samples of the unknown peaks were collected from the HPLC system in fractions of 0.5 ml and were analyzed by fluorescence spectrophotometry on a Shimadzu RF-5000 spectrofluorophotometer (Shimadzu, Kyoto, Japan). The characteristics of the fluorescence spectra were compared with those of synthetic phenanthrene standards by the following method: an excitation scan was run from 200 to 350 nm, with fixed emission wavelengths ranging between 360 and 383 nm, and an emission scan was run from 300 to 450 nm, with fixed excitation wavelengths ranging between 251 and 255 nm.

Capillary gas chromatography analysis and mass spectrometric identification of unknown metabolites in urine

The two HPLC peaks containing the unknown metabolites (Fig. 1) were preparatorily separated. The fractions were silylated (Jacob and Grimmer 1988) and subjected to analysis on a Varian MAT 112S mass spectrometer in conjuction with a Perkin-Elmer PE 8320 gas chromatograph (GC). The mass spectrometer operated at 70 eV. GC separation was achieved by a Nordibond 25-m × 0.32-mm SD54 coated silica capillary (Nordion, Helsinki, Finland). After injection of the sample at 100 °C the following temperature program was applied: (1) from 100° to 120 °C at 30 °C/min followed by 3 min of isothermic heating, (2) from 120° to 150°C at 10 °C/min, and (3) from 150° to 275°C at 3°C/min.

Statistical analysis

The data sets with skewed distributions were log-transformed before analysis using the Pearson correlation test. The analyses were carried out on SPSS 6.1 statistical software (SPSS Inc., Chicago). The comparison between the two quantitative methods used for measurement of urinary 1-hydroxypyrene was carried out according to the method described by Bland and Altman (1986).

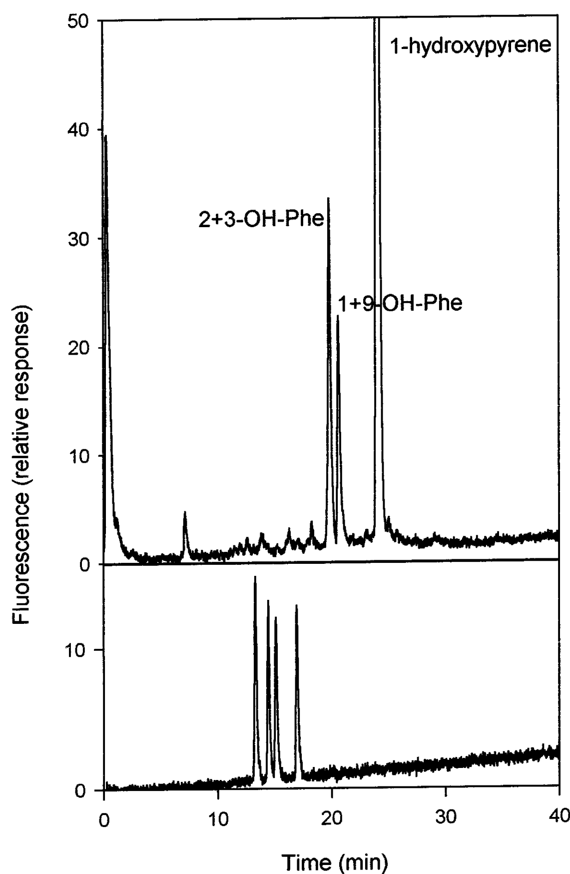

Fig. 1 *Top*: HPLC analysis of an electrode paste-plant worker's urine, showing the hydroxyphenanthrene metabolites and 1-hydroxypyrene levels measured in every worker's urine sample analyzed after immunoaffinity chromatography. *Bottom*: An analysis of the four main isomeric B[a]P-tetrol standards (in the following order: B[a]P-*r*-7, *t*-8, *t*-9, *c*-10-tetrol; B[a]P-*r*-7, *t*-8, *c*-9, *t*-10-tetrol, B[a]P-*r*-7, *t*-8, *t*-9, *t*-10-tetrol; and B[a]P-*r*-7, *t*-8, *c*-9, *c*-10-tetrol), comparing their retention on the chromatogram with that of the hydroxyphenanthrenes. In the postshift urine chromatogram the concentration of 1-hydroxypyrene is 1.73 μmol/mol creatinine as measured with the immunoaffinity method. The concentration of 2 + 3-OH-Phe (*peak 1*) is 4.05 μmol/mol creatinine and the concentration of 1 + 9-OH-Phe (*peak 2*) is 0.96 μmol/mol creatinine. For mean values, see Table 3

Results

The mean concentration of PAH and the distribution of selected PAH compounds in the particulate and gaseous phase that were collected on personal samplers in the electrode paste plant are presented in Table 1. Among the PAHs, high concentrations of the volatile anthracene and phenanthrene and of the semivolatile compounds fluoranthene and pyrene were measured in the work atmosphere.

We used an HPLC method for the quantitation of PAH metabolites in the urine of samples prepurified by C_{18} Sep-pak and immunoaffinity columns with an antibody against B[a]P-tetrol. We could not identify B[a]P-tetrol in the workers' urine after deconjugation either by enzyme hydrolysis (Fig. 1) or by acid hydrolysis prior to IAC. Samples not hydrolyzed by acid or by enzymes (glucuronidase and sulfatase) gave only negligible peaks in the HPLC chromatograms with retention times similar to those of the B[a]P-tetrol isomers. The chemical structure of B[a]P-*r*-7, *t*-8, *t*-9, *c*-10-tetrahydrotetrol is presented in Fig. 2.

Experiments were carried out to test whether 1-hydroxypyrene competed with B[a]P-tetrol for antibody binding on the immunoaffinity columns. At concentrations of up to 5 pmol/ml for B[a]P-*r*-7, *t*-8, *t*-9, *c*-10-tetrahydrotetrol and 200 pmol/ml for 1-hydroxypyrene in H_2O, which approximately equals the average 1-hydroxypyrene concentrations detected in the urine samples, competition for antibody binding sites between 1-hydroxypyrene and B[a]P-tetrol was not shown.

We found two peaks in the HPLC chromatograms obtained from all the urine samples subjected to enzyme hydrolysis and IAC (Fig. 1). Identification of these was attempted by gas chromatography/mass spectroscopy after silylation of isolated HPLC fractions. The results were inconclusive due to insufficient amounts. However, fragments of dihydrodiol oxygen-trimethylsilyl (OTMS) ethers indicating a K-region dihydrodiol configuration and, possibly, another with a non-K-region dihydrodiol configuration were detected, which may be attributable to phenanthrene metabolites. Chromatographic comparison between phenanthrene metabolites showed that peak 1 corresponded to 2-OH-Phe and 3-OH-Phe and peak 2 corresponded to 1-OH-Phe and 9-OH-Phe. There were only small differences in the fluorescence spectra obtained for 2-OH-Phe and 3-OH-Phe, and the two phenanthrene metabolites could not be separated on the HPLC chromatogram. When acid hydrolysis of the urine samples was carried out before IAC, peaks with retention times similar to those of 2- + 3-OH-Phe and 1- + 9-OH-Phe were found, but in considerably lower amounts than after enzyme hydrolysis. We tested the binding of monohydroxylated phenanthrene standards on the immunoaffinity column, and the recovery varied. An average of 58% of 1-OH-Phe and 63% of 2-OH-Phe was recovered, as was 76% of 3-OH-Phe and 43% of 4-OH-Phe. For 9-OH-Phe the average recovery was 41% after IAC. We also tested the binding of 1,2-diOH-Phe and 3,4-diOH-Phe to the immunoaffinity column and found approximately 20% binding of 1,2-diOH-Phe but no binding of 3,4-diOH-Phe.

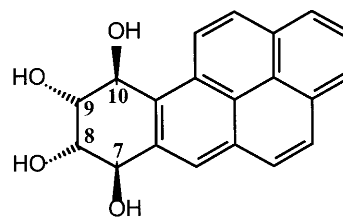

Fig. 2 Chemical structure of B[a]P-*r*-7, *t*-8, *t*-9, *c*-10-tetrol, one hydrolysis product of BPDE-I (*r*-7,*t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene)

Table 1 PAH exposure measurements of samples collected with personal samplers in the electrode paste plant ($n = 17$). Distribution of PAHs in the particulate and gaseous phase (μg/m^3) (*ND* Not detected)

PAH compound	Particulate phase			Gaseous phase			% In gaseous phase	
	Mean	SD	Median	Mean	SD	Median	Mean	Median
Anthracene	3.9	5.2	2.7	16.6	37.1	6.0	81.0	69.0
Phenanthrene	13.2	13.7	9.6	148.0	268.4	44.5	91.8	82.3
Fluoranthene	7.6	8.0	5.4	18.4	32.8	6.3	70.8	53.8
Pyrene	3.5	3.8	2.5	9.0	15.0	3.5	72.0	58.3
Benzo[*a*]pyrene	0.3	0.3	0.2	ND	ND	ND	0	0
PAH[a]	38.0	41.6	23.3					

[a] Sum of 17 PAHs according to NIOSH (1994). Particulate phase only

The pyrene metabolite 1-hydroxypyrene was detected in all samples analyzed. The average urinary concentration of 1-hydroxypyrene was 3.9 (SD 3.2; $n = 17$) μmol/mol creatinine in preshift urine samples and 10.2 (SD 6.6; $n = 17$) μmol/mol creatinine in postshift urine samples.

Urinary 1-hydroxypyrene was measured by both the IAC method and a standard HPLC method (Jongeneelen et al. 1987). The correlation coefficient between 1-hydroxypyrene concentrations quantitated by the two different methods was 0.70 ($P < 0.001$, $n = 34$; Table 2). The agreement between the two methods for the measurement of urinary 1-hydroxypyrene was also assessed according to the method described by Bland and Altman (1986) by calculation of the bias estimated by the mean difference. No consistent bias was found between the two methods. The concentrations of 1-hydroxypyrene recorded in our results are those measured by the standard method according to Jongeneelen et al. (1987).

Table 2 Correlation coefficient (Pearson) between 1-hydroxypyrene, 2 + 3-OH-Phe, and 1 + 9-OH-Phe (all urine samples, $n = 34$)[a]

	1-HP [1]	1-HP[2] IAC	2 + 3-OH-Phe
1-HP[2] IAC	0.70 ($P < 0.001$)		
2 + 3-OH-Phe	0.73 ($P < 0.001$)	0.63 ($P < 0.001$)	
1 + 9-OH-Phe	0.64 ($P < 0.001$)	0.67 ($P = 0.001$)	0.82 ($P < 0.001$)

[a] 1-HP[1] = standard HPLC method (Jongeneelen et al. 1987), 1-HP[2] IAC = immunoaffinity column method; all data are natural log-transformed

The correlation coefficients between the monohydroxylated phenanthrene metabolites and 1-hydroxypyrene are listed in Table 2. Significant correlation coefficients of 0.73 and 0.64 were found. We calculated the average amount of 2 + 3-OH-Phe (peak 1) and 1 + 9-OH-Phe (peak 2; expressed in micromoles per mole of creatinine) to be 31% and 69%, respectively, using an average fluorescence extinction coefficient as described in Materials and methods. The correlation coefficients were very low between the biomarkers 2- + 3-OH-Phe, 1- + 9-OH-Phe, or 1-hydroxypyrene in postshift urine samples and air concentrations of particulate PAH, benzo[*a*]pyrene, or particulate and gaseous anthracene, fluoranthene, phenanthrene, and pyrene. The correlation coefficient between postshift urinary 1-hydroxypyrene and pyrene (sum of the particulate and the gaseous phases) was 0.30 ($P = 0.25$, $n = 17$), and it was 0.39 ($P = 0.12$, $n = 17$) for both 2- + 3-OH-Phe and 1- + 9-OH-Phe as correlated with the sum of particulate and gaseous phenanthrene.

In the HPLC analysis the mean concentration of 2 + 3-OH-Phe detected in both pre- and postshift urine samples was about 25% of that measured for 1-hydroxypyrene with the Jongeneelen method, and the amount of 1 + 9-OH-Phe was about 12% (Table 3). The average ratios between the concentrations of 1-hydroxypyrene, 2 + 3-OH-Phe, and 1 + 9-OH-Phe detected in preshift and postshift urine samples are shown

Table 3 Measurements of 1-hydroxypyrene and phenanthrene metabolites (μmol/mol creatinine) and the ratio between these compounds in urine from electrode-paste plant workers ($n = 34$)[a]

PAH compound	Preshift urine samples			Postshift urine samples			Significance (P)[b]
	Mean	SD	Median	Mean	SD	Median	Pre-/postshift urine samples
1-HP[1]	3.93	3.20	2.57	10.20	6.58	8.94	< 0.001
1-HP[2]IAC	0.95	1.62	0.40	1.38	1.10	1.16	0.01
2 + 3-OH-Phe	0.88	0.47	0.80	2.70	2.73	1.82	0.001
1 + 9-OH-Phe	0.50	0.29	0.55	1.11	1.30	0.85	0.02
1-HP[2]IAC/2 + 3-OH-Phe	0.99	1.29	0.65	0.59	0.40	0.43	0.29
1-HP[2]IAC/1 + 9-OH-Phe	1.75	2.03	1.39	1.49	0.96	1.33	0.76
2 + 3-OH-Phe/1 + 9-OH-Phe	2.06	0.84	1.97	2.82	1.08	2.66	0.002

[a] 1-HP[1] = standard HPLC method (Jongeneelen et al. 1987), 1-HP[2] IAC = immunoaffinity column method

[b] Wilcoxon matched-pair signed-rank test

in Table 3. 1-Hydroxypyrene measured by the IAC method was used for ratio comparisons since the hydroxyphenanthrene metabolites were analyzed by this method. The ratios varied in relation to the time elapsing since the PAH exposure, indicating that the elimination half-life of the metabolites are different. The results indicate that both 2 + 3-OH-Phe and 1 + 9-OH-Phe have a shorter half-life than does 1-hydroxypyrene.

Discussion

In the present study we examined exposure to PAH and the excretion of PAH metabolites in the urine of workers in an electrode paste plant. The workers were exposed to relatively high levels of PAH, which provided the opportunity to study various urinary PAH metabolites. 1-Hydroxypyrene was measured in all postshift urine samples but was not significantly correlated with the workplace pyrene exposure; this observation was in accordance with the results of a larger study conducted at the same electrode paste plant (Bentsen et al. 1998). Recent studies have shown that there is a significant absorption of PAHs through the skin of PAH-exposed workers (VanRooij et al. 1992, 1993; Boogaard and van Sittert 1994; Malkin et al. 1996). Skin exposure may therefore account for the poor correlation observed between the urinary pyrene and phenanthrene metabolites and ambient-air PAHs in this factory.

We attempted to measure urinary benzo[*a*]pyrene (B[a]p) metabolites, but these metabolites were not detected by HPLC or fluorescence spectroscopy after purification on immunoaffinity coloumns. The antibody used has a known affinity for B[a]P-tetrols (Santella et al. 1984). This antibody was also used by Weston et al. (1993), who detected B[a]P-*r*-7, *t*-8, *t*-9, *c*-10-tetrol in the urine (0.24–3.12 pmol/ml) of individuals who had consumed a diet including large quantities of char-broiled meat. Becher and Bjørseth (1983) found B[a]P metabolites at 0–0.58 μmol/mol creatinine in urine from aluminum reduction workers with a typical workplace PAH exposure of 100 $\mu g/m^3$, whereas Jongeneelen et al. (1987) did not detect hydroxylated metabolites of B[a]P in urine samples from creosote-exposed workers. B[a]P metabolites are typically scarce in human urine samples and are preferentially excreted via the bile into the feces (Jacob and Grimmer 1996). The simultaneous determination of 1-hydroxypyrene and 3-hydroxybenzo[*a*]pyrene in the urine of coke-oven workers showed 200- to 2000-fold higher concentrations of 1-hydroxypyrene as reported by Jongeneelen (1997).

In all of the workers' urine samples we detected relatively high concentrations of 2 + 3-OH-Phe (peak 1 in Fig. 1) and 1 + 9-OH-Phe (peak 2 in Fig. 1). The hydroxyphenanthrene metabolites were mainly conjugated to glucuronic acid and sulfate, since only small quantities were found when enzyme treatment of the urine samples was omitted. The proportion of 2 + 3-OH-Phe (peak 1) and 1 + 9-OH-Phe (peak 2) was approximately 31% and 69%, respectively, which is similar to the distribution found in occupational exposure studies by Popp et al. (1997; 38–62%) and Grimmer et al. (1993; 28–72%).

There was a significant correlation between 1-hydroxypyrene and 2 + 3-OH-Phe and 1 + 9-OH-Phe in the workers' urine. This is reasonable since in workplace exposure, individual PAHs have been shown to correlate (Øvrebø et al. 1994). The correlation coefficients observed between different PAH metabolites give information about the usefulness of, for example, 1-hydroxypyrene as a single PAH exposure marker. 1-Hydroxypyrene was excreted at a slower rate than the hydroxyphenanthrenes, probably due to its higher molecular weight.

The recovery of hydroxyphenanthrene standards after IAC was tested and varied from 41% to 76%. Also as experienced by Weston et al. (1993), the recovery of urinary PAH metabolites by IAC was not 100%; therefore, the IAC method is only semiquantitative. The lower recovery most likely explains the lower concentrations of 1-hydroxypyrene measured by the IAC method as compared with the standard method described by Jongeneelen et al. (1987; Table 3).

Because of the high concentrations of several PAHs measured in the work atmosphere, including anthracene, phenanthrene, fluoranthene, and pyrene, these are likely to be absorbed into the body and excreted in relatively large amounts in urine. As with 1-hydroxypyrene, dermal PAH uptake may also account for the poor correlation observed between the phenanthrene metabolites and measured PAHs. In their study of four coke-plant workers, Jacob and Grimmer (1996) found good correlation between the inhaled amounts of phenanthrene and those of its phenol and dihydrodiol metabolites. Although this was not the case in our study, the correlation between the concentration in air and the urinary metabolites was better for phenanthrene than for pyrene among coke-oven workers (Popp et al. 1997).

Metabolites of phenanthrene have been identified in urine from PAH-exposed workers by several authors (Grimmer et al. 1993; Angerer et al. 1997; Popp et al. 1997). In the studies of Grimmer et al. (1993) and Popp et al. (1997) the most prominent metabolite excreted was a phenanthrene dihydrodiol conjugate. The gas chromatographic determination of phenanthrene dihydrodiols described by Grimmer et al. (1993) is complex and laborious, whereas Angerer et al. (1997) enrich the metabolites on a precolumn consisting of copper phthalocyanine-modified silica gel and use an HPLC method for the quantitation of monohydroxylated phenanthrene metabolites (1-, 2 + 9-, 3-, and 4-hydroxyphenanthrene), which accounted for about 10% of the total phenanthrene metabolites.

Because PAH exposure in several industries now appears to be the result of dermal uptake in addition to inhalation exposure (VanRooij et al. 1992; Boogard and van Sittert 1994; Malkin et al. 1996; Bentsen et al. 1998), estimation of total PAH exposure is best achieved by quantitation of PAHs excreted into urine or other body

fluids. Since the introduction of urinary 1-hydroxypyrene as a biomarker for human exposure assessment, it has gained acceptance as a valid and sensitive indicator of occupational PAH exposure (Jongeneelen 1997). However, it remains unclear whether there is a difference in the uptake and excretion of 3-ring, 4-ring, or 5-ring PAHs and in the correlation between these metabolites and ambient-air PAH measurements. As a surrogate marker for carcinogenic PAHs, more knowledge of these relationships is important in the evaluation of 1-hydroxypyrene as a biomarker for total PAH exposure.

Immunoaffinity columns are effective in the purification of urine samples (Strickland et al. 1994). Besides, various immunoassays have been used to quantitate urinary PAH metabolites (Gomes and Santella 1990; Herikstad et al. 1993). Thus, by the acquisition of greater knowledge of compound-antibody specificity it should be feasible to quantitate several PAH metabolites after purification on immunoaffinity columns.

In summary, we isolated two monohydroxyphenanthrene metabolites that, in addition to 1-hydroxypyrene, are relevant as biomarkers of PAH exposure by an HPLC/IAC method, which gives us the opportunity to establish even more reliable means of determining occupational PAH exposure.

Acknowledgements The analyses of PAH on filters were carried out at the research institute SINTEF, Oslo, Norway. We used chemicals from the NCI Chemical Carcinogen Repository (Midwest Research Institute, Kansas City, Mo.) in this study. We are thankful to Dr. R. Santella for providing the monoclonal antibody (8*E*11). This study was supported by the Confederation of Norwegian Industries (NHO), National Fund of Occupational Health, grants 0534 and 0728.

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