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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

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Abstract

The described high-performance liquid chromatographic method with fluorescence detection (HPLC–FD) permits the simultaneous determination of 3-hydroxybenzo[*a*]pyrene and 3-hydroxybenz[*a*]anthracene as the most important metabolites of the carcinogenic polycyclic aromatic hydrocarbons (PAHs) benzo[*a*]pyrene and benz[*a*]anthracene in human urine. After enzymatic hydrolysis, to release the conjugated metabolites, the analytes are separated from the matrix by means of a liquid–solid extraction step which is followed by a coupled column HPLC procedure using an enriching precolumn consisting of silica modified with copper phthalocyanine. This special precolumn selectively adsorbs PAHs with at least three condensed rings and thus separates them from the urine matrix. The quantitative analysis was carried out using a switchable fluorescence detector. The detection limits were 6 ng/l urine (3-hydroxybenzo[*a*]pyrene) and 8 ng/l urine (3-hydroxybenz[*a*]anthracene). The relative standard deviations of the within-series imprecision ranged between 4.0% and 9.0%. The between-day imprecision was 7.7% (3-hydroxybenz[*a*]anthracene) and 12.9% (3-hydroxybenzo[*a*]pyrene). The recovery rates ranged between 102% and 124%. Using this analytical method we determined PAH metabolites in post shift urine samples from 19 workers engaged in the production of fire-proof materials. The urinary concentrations ranged from 3 to 198 ng 3-hydroxybenzo[*a*]pyrene per g creatinine and from 15 to 1871 ng 3-hydroxybenz[*a*]anthracene per g creatinine.

Keywords: 3-Hydroxybenzo[*a*]pyrene; 3-Hydroxybenz[*a*]anthracene

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) represent a large group of organic compounds widely distributed in the environment as pollutants of air,

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water and soil [1]. PAHs are formed during various processes that involve incomplete combustion of organic material.

The International Agency for Research on Cancer (IARC) has characterized different PAHs as carcinogens in animal experiments [1]. Some mixtures containing PAHs, e.g., tar, pitch or exhaust fumes from coke plants, are considered carcinogenic to humans [2]. To minimize the carcinogenic risk safe production methods and working conditions are necessary as well as occupational health surveillance: these are controlled by measuring air concentrations of PAHs. In Germany a threshold limit value for carcinogenic substances (technical guidance concentration: TRK value) of 2 $\mu g/m^3$ benzo[*a*]pyrene must be adhered to [2]. Because the dermal absorption of PAHs is an important route of uptake [3–5] biological monitoring is very important for the prevention of diseases.

In 1986 Jongeneelen et al. [6] developed a high-performance liquid chromatographic method for the determination of 1-hydroxypyrene, the main metabolite of pyrene. Since its introduction this method has been used in various studies for the biological monitoring of exposure to PAHs. As pyrene shows less carcinogenic properties the need exists for a sensitive and practical method for the routine analysis of PAH metabolites in urine which result from carcinogenic PAHs as for example benzo[*a*]pyrene and benz[*a*]anthracene. Grimmer et al. [7] developed a procedure for the simultaneous quantification of a variety of PAH metabolites eliminated in urine such as phenols and dihydrodiols of pyrene, phenanthrene, chrysene, fluoranthene, benz[*a*]anthracene and benzo[*a*]pyrene using capillary gas chromatography with mass spectrometry (GC–MS). In this method a number of preparation steps such as derivatization of the phenols and dihydrodioles followed by multiple-column chromatographic purification make the procedure difficult to carry out routinely.

This paper describes a new, simple and sensitive high-performance liquid chromatography (HPLC) method for the quantitative determination of 3-hydroxybenzo[*a*]pyrene and 3-hydroxybenz[*a*]anthracene as the major metabolites of benzo[*a*]-pyrene and benz[*a*]anthracene in urine using a coupled column HPLC system with a special enriching precolumn and fluorescence detection (FD). The method was used in the analysis of urine samples from 19 workers engaged in the production of fire-proof materials.

2. Materials and methods

2.1. Chemicals and materials

3-Hydroxybenzo[*a*]pyrene, 3-hydroxybenz[*a*]-anthracene, 1-, 2- and 3-hydroxychrysene were received from Dr. A. Seidel from the Institute of Toxicology (Dir.: Professor Dr. F. Oesch), University of Mainz, Germany.

Acetic acid glacial, sodium hydroxide pellets, hydrochloric acid (37%), ascorbic acid, diethyl ether, methanol (LiChrosolv) and deionized water were obtained from Merck, Germany. All chemicals and solvents used were of analytical grade.

β-Glucuronidase/arylsulfatase and Micr-O-protect to stabilize the enzyme were supplied by Boehringer Mannheim, Germany.

Polyethylene reservoirs (70 ml) and adapters were from ICT, Germany. Glass cartridges for solid-phase extraction (SPE) (empty reservoirs, 8 ml), PTFE frits and 40 μm reversed-phase (RP) octadecyl (C_{18}) material 60 Å were obtained from Baker, Germany. For vacuum SPE a Varian Vacelut SPS 24 workstation (Varian, Palo Alto, CA, USA) was used.

In each SPE cartridge a frit was inserted and the cartridges were then filled with 0.5 g RP-C_{18} adsorbent.

Conditioning of the cartridges was performed successively with 12 ml methanol and 30 ml deionized water. During the whole conditioning procedure the cartridges were not permitted to run dry.

2.2. Solutions

0.3 *M* Sodium acetate buffer (pH 5.7) was prepared as follows: 893 ml 0.3 *M* sodium acetate solution was adjusted to pH 5.7 with approximately 107 ml 0.3 *M* acetic acid solution.

0.3 *M* Sodium acetate buffer with ascorbic acid (0.75 g/l, pH 5.7) was prepared as follows: 75 ml ascorbic acid (10 g/l) were diluted to the mark with 0.3 *M* sodium acetate buffer (pH 5.7) in a 1000-ml

glass volumetric flask. The pH was adjusted to 5.7 with 1 *M* sodium hydroxide solution.

Solvents used for HPLC were: solvent A (methanol), solvent B (methanol–water, 6:4, v/v) and solvent C (methanol–water, 3:7, v/v).

2.3. Sample collection and preparation

The post-shift urine from the workers was collected in polypropylene tubes and immediately frozen at −20°C until sample preparation.

A 20-ml volume of urine was buffered with 40 ml 0.3 *M* sodium acetate buffer with ascorbic acid (pH 5.7; 0.75 g/l ascorbic acid). The pH was controlled and corrected if necessary with 1 *M* hydrochloric acid or 1 *M* sodium hydroxide solution. For hydrolysis of conjugated PAH metabolites 80 μl β-glucuronidase/arylsulfatase was added to the urine samples. This solution was incubated for 16 h at 37°C in a waterbath. Afterwards the hydrolyzed urine samples were applied to the preconditioned cartridges. The cartridges were washed with 20 ml deionized water and dried in a vacuum (0.5 bar). The analytes were eluted with 15 ml diethyl ether. After adding 0.5 ml sodium acetate buffer (pH 5.7; 0.75 g/l ascorbic acid) the diethyl ether was removed by a gentle stream of nitrogen. A 6-ml volume of methanol and 5.5 ml sodium acetate buffer (pH 5.7; 0.75 g/l ascorbic acid) were added to the residue. Fig. 1 shows an overview of the whole clean-up procedure.

A reagent blank is included in each analytical series. Ultrapure water instead of urine is subjected to the sample processing described above.

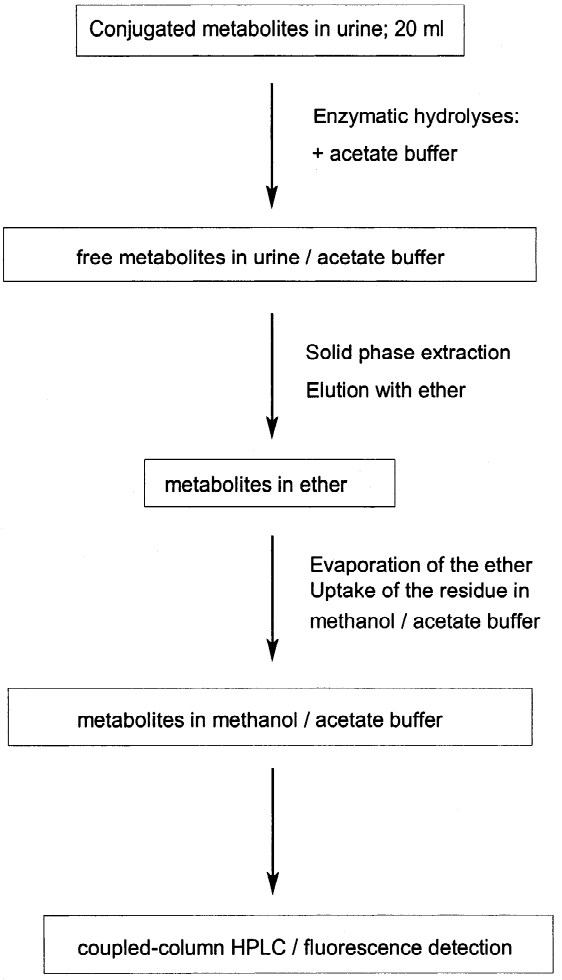

Fig. 1. Sample processing.

2.4. High-performance liquid chromatographic analysis

Analysis was carried out using a HPLC system which consisted of an autosampler (AS-4000, Merck, Darmstadt, Germany), an automatic six-port switching valve (Vici, Schenkon, Switzerland), a pump (L-6000, Merck), a gradient pump (L-6200, Merck), a enriching precolumn (copper phthalocyanine modified silica, 5×4 mm I.D.; Dr. Andreas Walfort and Partner, Reinhardshagen, Germany), a column inlet filter (7335, Rheodyne, Cotati, CA, USA), an analytical column (LiChrospher PAH, 250×4 mm I.D., Merck), a column thermostat (T-6300, Merck), a fluorescence detector (F-1080, Merck) and of a computer (D-7000 HPLC System Manager, Merck).

The HPLC system (Fig. 2) allows the simultaneous determination of 3-hydroxybenzo[*a*]pyrene and 3-hydroxybenz[*a*]anthracene in urine. The enriching precolumn selectively adsorbs PAHs with at least three condensed rings as a result of π,π interaction and thus separates them from the urine matrix.

A 4-ml volume of the solution containing the analytes was automatically injected into the HPLC system and transported by solvent C to the enriching precolumn. The PAH metabolites were adsorbed on

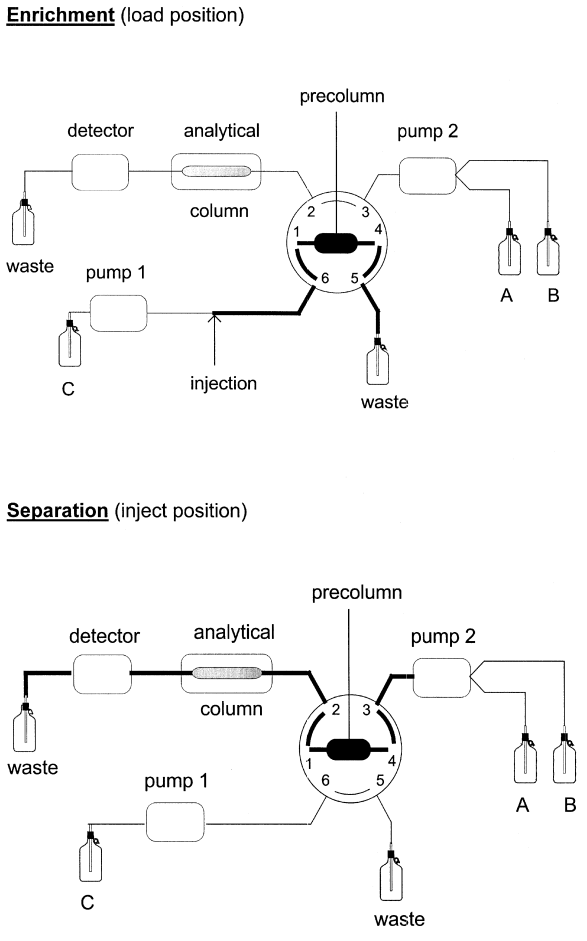

Fig. 2. Coupled-column HPLC system.

the precolumn while the matrix compounds were discarded. By switching the automatic valve in the separation position, solvent A was pumped through the precolumn. This way the PAH metabolites were desorbed in the back-flush procedure and were online transferred to the analytical column. By switching the automatic valve in the enrichment position, again, the analytes were separated on the analytical column by a methanol gradient (solvent A and B) and quantified using fluorescence detection.

After the analytical separation both, the precolumn and the analytical column were washed and then conditioned again.

During the entire analytical period solvent C flowed at a rate of 1 ml/min.

In Table 1 the timetable for the coupled-column HPLC analysis of 3-hydroxybenzo[*a*]pyrene and 3-hydroxybenz[*a*]anthracene and for the fluorescence detector is described.

2.5. Calibration

A starting solution was prepared as follows: 2.6 mg 3-hydroxybenzo[*a*]pyrene and 8 mg 3-hydroxybenz[*a*]anthracene were dissolved with methanol in a 100-ml glass volumetric flasks.

Stock solution A was prepared as follows: 1000 μl of the starting solution was diluted with methanol in a 100-ml glass volumetric flask (3-hydroxybenzo[*a*]pyrene: 0.26 mg/l, 3-hydroxybenz[*a*]anthracene: 0.80 mg/l).

Stock solution B was prepared as follows: 1000 μl of stock solution A was diluted with methanol in a 25-ml glass volumetric flask (3-hydroxybenzo[*a*]pyrene: 10.4 μg/l, 3-hydroxybenz[*a*]anthracene: 32 μg/l).

Calibration standards were prepared in a urine–buffer mixture. For this purpose urine of a non-smoking person, occupationally not exposed to PAHs was mixed with 0.3 *M* sodium acetate buffer (pH 5.7) in the ratio 1:2. To avoid analyte losses due to oxidation ascorbic acid was added to the urine–buffer mixture (0.75 g/l).

Six calibration standards with concentrations ranging from 8 to 156 ng/l 3-hydroxybenzo[*a*]pyrene and from 24 to 480 ng/l 3-hydroxybenz[*a*]anthracene were received by diluting the stock solutions with the urine–buffer mixture.

The calibration standards were stable for at least 5 months at −20°C.

The calibration standards were processed in the same manner as the urine samples (Section 2.3).

Linear calibration curves were obtained by plotting the peak areas as a function of the concentrations used. These graphs were taken to ascertain the unknown concentrations of the metabolites in urine samples from exposed persons.

2.6. Human studies

We investigated a group of 19 workers employed in a factory producing fire-proof materials on basis of graphite and tar. Post-shift urine of the workers were collected in the middle of the working week in

Table 1
Timetable of the HPLC and the FD program[a]

Time (min)	A (%)	B (%)	Flow-rate (ml/min)	Position	Flow-rate C (ml/min)	λ_{Ex} (nm)	λ_{Em} (nm)	Procedure
0	0	100	0.8	Load	1	290	402	Enrichment of the analytes and conditioning of the analytical column
14.4	0	100	0.8	Load	1	290	402	
14.5	100	0	0.8	Load	1	290	402	
15.0	100	0	0.8	Inject	1	290	402	Transfer of the analytes to the analytical column
16.0	100	0	0.8	Inject	1	290	402	
16.1	0	100	0.8	Inject	1	290	402	
25.0	0	100	0.8	Load	1	290	402	Separation of the analytes
25.5	0	100	0.6	Load	1	290	402	
53.0	65	35	0.6	Load	1	265	391	
53.5	65	35	0.8	Load	1	265	391	
59.5	65	35	0.8	Load	1	262	439	
65.0	100	0	0.8	Load	1	262	439	
75.5	100	0	0.8	Inject	1	262	439	Rinsing of the enrichment precolumn and of the analytical column
90.0	100	0	0.8	Load	1	262	439	
110.0	100	0	0.8	Load	1	262	439	
110.5	0	100	0.8	Load	1	262	439	

[a] Eluent A: methanol (contains 1 mg/l ascorbic acid); eluent B: deionized water–methanol (60:40, v/v); eluent C: deionized water–methanol (70:30, v/v). λ_{Ex}: Excitation wavelength. λ_{Em}: Emission wavelength.

sealable plastic bottles and stored in the deep freezer until it was processed. Urinary creatinine was determined photometrically as picrate according to the Jaffé method [8]. To compensate fluctuations due to diuresis the urinary concentration of 3-hydroxybenz[*a*]anthracene and 3-hydroxybenzo[*a*]pyrene was related to creatinine.

3. Results and discussion

The principle of the method described here is based on studies by Lintelmann et al. [9], Hackl et al. [10], Rinno et al. [11] and our own investigations [12]. The method makes use of the selective adsorption of PAH metabolites on a silica phase loaded with copper phthalocyanine. This enables PAH metabolites to be enriched and at the same time separated from other interfering urinary components. The method is particularly practical as this clean-up/enrichment step can be combined online with analytical separation. This procedure, successfully used for the determination of 1-hydroxypyrene [12], could not, however, be adapted unchanged for the determination of 3-hydroxybenzo[*a*]pyrene and 3-hydroxybenz[*a*]anthracene. In the case of occupational exposure to PAHs these metabolites occur in urine in the lower ng/l concentration range. To be able to determine such concentrations, larger volumes of urine must be used. Pilot tests have shown, however, that under these conditions – despite the use of a selective enriching column – there is great analytical interference. We have therefore included an additional clean-up step. In this step 20 ml urine is passed through a reversed-phase column. The PAH metabolites are enriched on the reversed-phase column and at the same time separated from many accompanying polar substances. Unlike Jongeneelen et al. [13] we used diethyl ether for elution of the analytes which is in comparison to methanol less polar. This produced an eluate of higher purity. After evaporation in a stream of nitrogen the residue is injected into the HPLC system, and transferred online to the enriching precolumn and finally the separating column. In this way it is possible to reduce interference considerably and to achieve detection limits for the PAH metabolites in the lower ng/l range. However, despite these detection limits, the determination of 3-hydroxy-

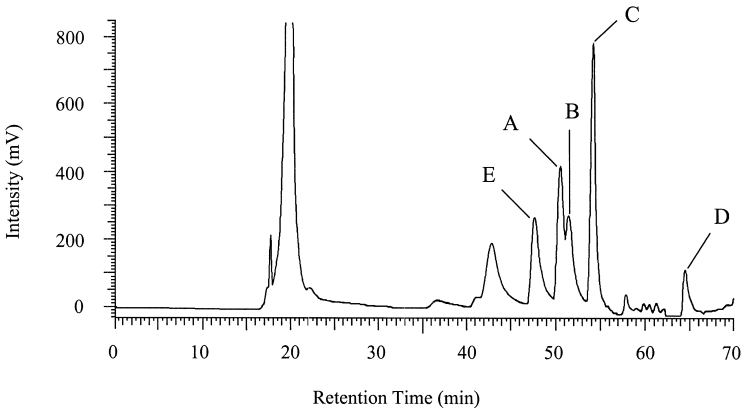

Fig. 3. Chromatogram of a standard solution; (A) 3-hydroxybenz[*a*]anthracene (480 ng/l); (B) 2-hydroxychrysene (528 ng/l); (C) 1-hydroxychrysene (2205 ng/l); (D) 3-hydroxybenzo[*a*]pyrene (156 ng/l); (E) 3-hydroxychrysene (720 ng/l).

benz[*a*]anthracene and 3-hydroxybenzo[*a*]pyrene in urine of persons occupationally not exposed to PAHs is not possible.

Due to a lack of reference substances, we used data from Grimmer and Jacob for the hydrolysis of the PAH conjugates in urine [14]. We discovered, that under these conditions – even with strict observance of the pH value – analyte losses occur as a result of oxidation. Therefore we carried out hydrolysis under reductive conditions. The addition of ascorbic acid has proved highly suitable, and losses were no longer observed.

For elution of the analytes from the enrichment precolumn we used pure methanol. In this way we were able to achieve complete desorption from the enriching precolumn. Warming of the elution agent, as necessary in the method of Hackl et al. [10], was not necessary.

With our optimized method we could not only determine 3-hydroxybenzo[*a*]pyrene and 3-hydroxybenz[*a*]anthracene but also 1-, 2- and 3-hydroxychrysene (as Fig. 3 shows). Investigation of workers exposed to PAHs showed, however, that the chrysene metabolites are excreted in only very small amounts and therefore cannot be separated from other components sufficiently (Fig. 4). We have therefore

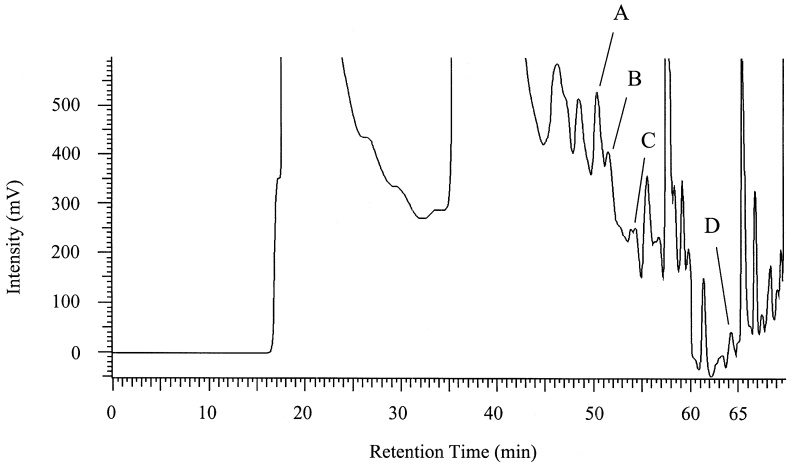

Fig. 4. Processed urine sample of a person exposed to PAHs; (A) 3-hydroxybenz[*a*]anthracene (518 ng/l); (B) 2-hydroxychrysene (not evaluable); (C) 1-hydroxychrysene (not evaluable); (D) 3-hydroxybenzo[*a*]pyrene (24 ng/l).

Table 2
Imprecision and detection limits of the method

	n	3-Hydroxybenzo[*a*]pyrene		3-Hydroxybenz[*a*]anthracene	
		Concentration (ng/l)	RSD (%)	Concentration (ng/l)	RSD (%)
Within-series imprecision	7	38	7.0	113	4.0
		137	9.0	394	4.4
Between-day imprecision	6	94	12.9	270	7.7
Imprecision of the method tested by using different urine matrices	10	208	3.8	600	11.5
Detection limit		6		8	

restricted our method to the determination of 3-hydroxybenzo[*a*]pyrene and 3-hydroxybenz[*a*]-anthracene, particularly as the parent PAHs of these metabolites have greater carcinogenic potentials than chrysene.

3.1. Reliability of the method

3.1.1. Precision

Within-series imprecision was determined by seven-fold analysis of a urine–acetate buffer mixture which was spiked with different amounts of the analytes. The relative standard deviations (RSDs) ranged between 4.0% (3-hydroxybenz[*a*]anthracene) and 9.0% (3-hydroxybenzo[*a*]pyrene) (Table 2).

Between-day imprecision was determined using spiked urine samples. These were prepared by adding defined amounts of the analytes to a urine sample of a non-smoking person, occupationally not exposed to PAHs. Afterwards the samples were analyzed on six different days twice. The RSDs were 7.7% (3-hydroxybenz[*a*]anthracene) and 12.9% (3-hydroxybenzo[*a*]pyrene) at concentrations of 270 and 94 ng/l, respectively (Table 2).

3.1.2. Accuracy

The accuracy of the method was checked by carrying out recovery experiments, by determining the losses during the whole procedure and by checking the influence of different urine matrices on the analytical results.

In order to determine the recovery, a urine–acetate buffer mixture was spiked with different amounts of the analytes. These samples were analyzed seven times. Recovery was found to be between 102% and 124% (Table 3).

In order to determine the losses which occur during sample preparation a urine–acetate buffer mixture was spiked with the analytes and subjected to the sample preparation, the enrichment procedure and the HPLC separation, described. In addition, the same amounts of analytes were pipetted into a solution made up of equal amounts of methanol and acetate buffer. This solution was analyzed by HPLC–FD, avoiding the enriching precolumn. The

Table 3
Accuracy of the method

	Concentration (ng/l)	Recovery (%)	Losses during sample preparation (%)
3-Hydroxybenzo[*a*]pyrene	38	124	–
	137	121	–
	104	–	52.5
3-Hydroxybenz[*a*]anthracene	113	102	–
	394	115	–
	300	–	33.5

losses which occur during sample preparation can be calculated from the quotients of the area units which are achieved with and without the enriching pre-column. The preparation losses are 52.5% for 3-hydroxybenzo[*a*]pyrene and 33.5% for 3-hydroxybenz[*a*]anthracene (Table 3). These losses, however, are compensated by the calibration procedure used. The good precision of the described analytical method – even at low concentration ranges – indicates that the losses during sample preparation are reproducible and therefore do not affect the reliability of the method. High preparation losses for the determination of 3-hydroxybenzo[*a*]pyrene (58%) were also found by Ariese et al. [15] using solid-phase extraction.

The influence of the urine matrix on the analytical results was additionally investigated. For this purpose 10 individual urine samples of different persons were spiked with the analytes and subjected to the sample preparation, the enrichment procedure and the HPLC separation, described. RSDs of 3.8% (3-hydroxybenz[*a*]anthracene) and 11.5% (3-hydroxybenzo[*a*]pyrene) were found (Table 2). These relative standard deviations, however, lie in the range of the within series imprecision (Table 2). It can be concluded that different urine matrices have no influence on the analytical results.

3.1.3. Detection limits

Under the given conditions of sample preparation and the conditions for HPLC–FD, taking into consideration a three-fold signal-to-noise ratio a detection limit of 6 ng/l 3-hydroxybenzo[*a*]pyrene and 8 ng/l 3-hydroxybenz[*a*]anthracene was determined.

3.1.4. Sources of error

Impurities in the HPLC solvents used lead to unspecific background interference. For this reason the solvents, A (pure methanol), B (methanol–water, 60:40) and C (methanol–water, 30:70) must be of highest purity. Before each analysis series a solvent blank is recorded to determine possible impurities. In addition, in particular with the retention time of 3-hydroxybenzo[*a*]pyrene an unspecific reagent blank can occur which is usually due to impurities in the ether used. For this reason care must be taken that this solvent is also of highest purity. A reagent blank must be included when a new batch of ether is used.

In aqueous solutions it cannot be excluded that the unconjugated analytes are adsorbed on plastic surfaces. In the preparation of native urine samples, to avoid losses glass vessels should be used for sample preparation after enzymatic hydrolysis. An exception are polyethylene reservoirs which are used during solid-phase extraction, as such vessels are not available made of glass. With reference standards and calibration solutions the analytes are already present in unconjugated form so that these solutions must be stored in glass vessels [16].

3.2. Examination of exposed persons

In all the urine samples we investigated we could determine 3-hydroxybenz[*a*]anthracene. The concentrations were between 15 and 1871 ng/g creatinine. 3-Hydroxybenzo[*a*]pyrene, however, could not be detected in two urine samples. The highest level of 3-hydroxybenzo[*a*]pyrene excreted (198 ng/g creatinine) was lower than the highest value for 3-hydroxybenz[*a*]anthracene by a factor of 10. The median values of both PAH metabolites differ by about a factor of 10, also (Table 4). The comparatively small amount of 3-hydroxybenzo[*a*]pyrene excreted is due to the fact that the five-ring PAH, benzo[*a*]pyrene, unlike the four-ring PAH, benz[*a*]anthracene, is excreted to a large extent via the faeces. Fig. 4 shows the HPLC–FD chromatogram of

Table 4
Results of the biological monitoring[a]

	50% (ng/l)	$\bar{x} \pm s$ (ng/l)	90% (ng/l)	Range (ng/l)	Persons investigated	Results below detection limit
3-Hydroxybenzo[*a*]pyrene	14	37±56	171	3–198	19	2
3-Hydroxybenz[*a*]anthracene	174	376±466	1162	15–1871	19	–

[a] 50%: Median; 90%: 90%-percentile; $\bar{x}$: mean; *s*: standard deviation.

a urine sample from a person engaged in the production of fire-proof materials.

4. Conclusions

The analytical method for the determination of 3-hydroxybenzo[*a*]pyrene and 3-hydroxybenz[*a*]-anthracene in human urine samples presented here is reproducible, sensitive and specific. The reliability data of the method like within-series imprecision, between-day imprecision, the detection limit or the recovery can be regarded as good. The urinary 3-hydroxybenz[*a*]anthracene concentration is a diagnostically sensitive parameter for the biological monitoring of persons occupationally exposed to PAHs. Compared to 1-hydroxypyrene, 3-hydroxybenz[*a*]anthracene has the advantage that it is a metabolite of a PAH which has greater carcinogenic potential than pyrene. 3-Hydroxybenzo[*a*]pyrene can only be detected in urine samples from persons with occupationally high PAH exposure, such as in the production of graphite electrodes or in coking plants. With low external exposures such as found in road construction or in the environment, the excretion of 3-hydroxybenzo[*a*]pyrene is too low to be detected with the analytical methods available to date.

Acknowledgements

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