ELSEVIER

Journal of Chromatography B, 739 (2000) 225–229

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Biomonitoring of environmental polycyclic aromatic hydrocarbon exposure by simultaneous measurement of urinary phenanthrene, pyrene and benzo[*a*]pyrene hydroxides

Juliane Hollender*, Burkhard Koch, Wolfgang Dott

Institute of Hygiene and Environmental Medicine, RWTH Aachen, Pauwelsstrasse 30, 52057 Aachen, Germany

Abstract

A high-performance liquid chromatographic method with fluorescence detection was developed which enables the simultaneous determination of the urinary polycyclic aromatic hydrocarbon metabolites 3-hydroxyphenanthrene, 1-hydroxypyrene and 3-hydroxybenzo[*a*]pyrene. The method has small solvent consumption because of the use of a microbore RP C_{18} column and a relatively short run time. Low detection limits of 0.02 nmol/l for 3-hydroxypyrene to 0.19 nmol/l for 3-hydroxybenzo[*a*]pyrene were attained. In contrast, the detection limits of α-naphthol and 9,10-dihydroxy-9,10-dihydrophenanthrene were not adequate for the determination of environmental exposure. The developed method was successfully used for the analysis of urine samples from children.

Keywords: Biological monitoring; Polynuclear aromatic compounds; Phenanthrene; Pyrene; Benzo[*a*]pyrene

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the environment, produced mainly by incomplete combustion of organic matter such as wood, oil or coal. Since some PAHs such as anthracene, benzo[*a*]pyrene and benzo[*k*]fluoranthene are carcinogenic the investigation of human exposure to these contaminants is very important. Biological monitoring provides a very useful method for the determination of exposure, because the bioavailability of pollutants is also taken into account. Biological monitoring of PAH exposure is usually performed by the measurement of PAH hydroxides in urine samples [1]. In 1987, Jongeneelen et al. [2] developed a high-performance liquid chromatography (HPLC) method for the determination of 1-hydroxypyrene, the main metabolite of pyrene. Since its introduction this method has been used in various studies to determine occupational and environmental PAH exposure. In Germany, the method was published in 1991 by the DFG as a standard method [3]. Intra- and inter-individual variation of 1-hydroxypyrene as well as reference values are described [4,5]. However, pyrene is only one PAH out of several hundred and its use as a biomarker has been called into question [6]. Therefore, in the last few years, methods have been proposed whereby other PAH hydroxides can be determined. Grimmer et al. [7] developed a laborious procedure for the simultaneous analysis of phenols and dihydrodiols of phenanthrene and pyrene in urine by gas chromatog-

*Corresponding author. Tel.: +49-241-808-8282; fax: +49-241-888-8477.

E-mail address: juliane.hollender@post.rwth-aachen.de (J. Hollender)

0378-4347/00/$ – see front matter
PII: S0378-4347(99)00470-3

raphy–mass spectrometry (GC–MS). Lintelmann et al. [8] described a HPLC method with fluorescence detection (FLD) for the quantification of 1-hydroxy-, 4-hydroxy-, and 9-hydroxyphenanthrene in addition to 1-hydroxypyrene which was used for occupational studies [9]. The method was improved in sensitivity to determine environmental exposure also [10]. Because naphthalene is a predominant PAH compound in occupational exposure the determination of 1-naphthol [4,11,12] and napthylamine [13] has been performed in other studies. 3-Hydroxybenzo-[*a*]pyrene, the major urinary benzo[*a*]pyrene metabolite, was only detected in the urine of exposed workers [14] and in animals studies [15,16]. Biomonitoring of benzo[*a*]pyrene is of great interest because of its carcinogenic effect. However, because of its hydrophobicity and molecule size, benzo[*a*]pyrene is mainly eliminated via the gastrointestinal tract.

The aim of our study was the development of a HPLC–FLD method to enable the simultaneous detection of the hydroxides of the highly volatile PAHs such as naphthalene, which occur in air in the highest concentrations, and low volatile carcinogenic PAHs such as benzo[*a*]pyrene. Thereby the environmental PAH exposure from different sources could be better estimated. The method should be sufficiently sensitive to measure the environmental exposure of children because it will be used as a biomarker in a study of the respiratory diseases of children.

2. Experimental

2.1. Chemicals

3-Hydroxyphenanthrene, 1-hydroxypyrene, 3-hydroxybenzo[*a*]pyrene and 9,10-dihydroxy-9,10-dihydrophenanthrene were obtained from Promochem (Wesel, Germany), and α-naphthol and β-glucuronidase/arylsulfatase from Merck (Darmstadt, Germany). The methanol used was gradient grade (Merck) and water was obtained from a Milli-Q water purification system (Millipore, Eschborn, Germany).

2.2. Sample preparation

The method for determining PAH hydroxides in urine consisted of enzymatic hydrolysis, solid-phase extraction on SPE-C-18, reversed-phase HPLC and fluorescence detection according to Ref. [3]. To 20 ml urine, 30 ml 0.1 *M* sodium acetate buffer, pH 5.0, and 25 μl β-glucuronidase/aryl sulfatase (30 and 20 U/ml, respectively) were added. The mixture was incubated for 16 h at 210 rpm at 37°C. The sample was applied to a SPE column packed with 0.5 g RP-C-18 (Baker, Deventer, The Netherlands), which was conditioned with 5 ml methanol and 10 ml water. After washing with 10 ml methanol–water (40:60, v/v%) the PAH metabolites were eluted with 10 ml methanol. The methanol was evaporated to dryness under a stream of nitrogen at 40°C and the residue was dissolved in 2 ml methanol in an ultrasonification bath. After centrifugation at 600 rpm for 5 min the supernatant was stored in a vial with a PTFE-lined septum cap at −20°C until HPLC analysis.

Urine samples were stored at −20°C until analysis. For calibration, pooled urine samples were used which were extracted with RP-C-18 to eliminate traces of PAH metabolites and spiked with standards ranging from 0.19 to 41.24 nmol/l according to sample concentrations of 0.019 to 4.124 nmol/l.

2.3. Column liquid chromatography

A HPLC system consisting of pumps Model 126 (Beckmann, München, Germany), a peltier thermostat BFO-04 (Optilab, Berlin, Germany), an autosampler 1050 and a fluorescence detector 1046A (Hewlett-Packard, Waldbronn, Germany) was used.

Separation was achieved on a 125 mm×2 mm I.D. RP-C_{18} ODS column (particle size 3 μm) with a 10 mm×2 mm I.D. RP-C_{18} guard column (particle size 5 μm) (Hewlett-Packard). Elution was performed by means of a water–methanol gradient: 0–20 min a linear gradient from 40 to 95% methanol, 20–25 min 95% methanol, 25–28 min a linear gradient to 40% methanol and 28–38 min 40% methanol. The flow-rate was 0.30 ml/min and the column temperature was stabilized at 40°C. The injection volume was 10 μl. A time program was used to detect the different

PAH hydroxides at optimal absorption and emission wavelengths: 0–21 min excitation and emission wavelength 242 and 388 nm (3-hydroxyphenanthrene, 1-hydroxypyrene), and 21–38 min excitation and emission wavelength 248 and 439 nm (3-hydroxybenzo[*a*]pyrene). Peak heights were used for quantification.

3. Results and discussion

Initially, the described water–methanol gradient was developed to enable the separation of 9,10-dihydroxy-9,10-dihydrophenanthrene [retention time (t_R)=8.1], α-naphthol ($t_R = 9.5$ min), 3-hydroxyphenanthrene ($t_R = 15.7$ min), 1-hydroxypyrene ($t_R = 18.5$ min) and 3-hydroxybenzo[*a*]pyrene ($t_R = 22.2$ min). The HPLC method has a low methanol consumption (7.1 ml per sample) because of the use of a microbore RP-C-18 column and a relatively short run time (38 min). The excitation and emission wavelengths for each compound were varied to obtain optimal detection, but the natural fluorescence of α-naphthol and 9,10-dihydroxy-9,10-dihydrophenanthrene was not high enough to attain sensitive detection. The detection limits with approximately 70 nmol/l were not adequate for the determination of environmental PAH exposure. For α-naphthol, similar detection limits were determined by HPLC–FLD elsewhere [12,13]. 9,10-Dihydroxy-9,10-dihydrophenanthrene has previously only been measured by GC–MS [7]. Because of the high detection limits the study of these compounds was not pursued further.

Fig. 1A shows a chromatogram of an extract of pooled urine spiked with 3-hydroxyphenanthrene, 1-hydroxypyrene and 3-hydroxybenzo[*a*]pyrene. The PAH hydroxides are well separated from each other and from the matrix compounds, which mainly elute within the first 10 min. The 3-hydroxybenzo-[*a*]pyrene standard as well as the other standards were stable for more than 1 month at −20°C, in contrast to other reports [17].

We attempted to improve the sensitivity of the method by raising the detector signal amplification, increasing the injection volume and increasing the urine sample volume. Singly or in combination this

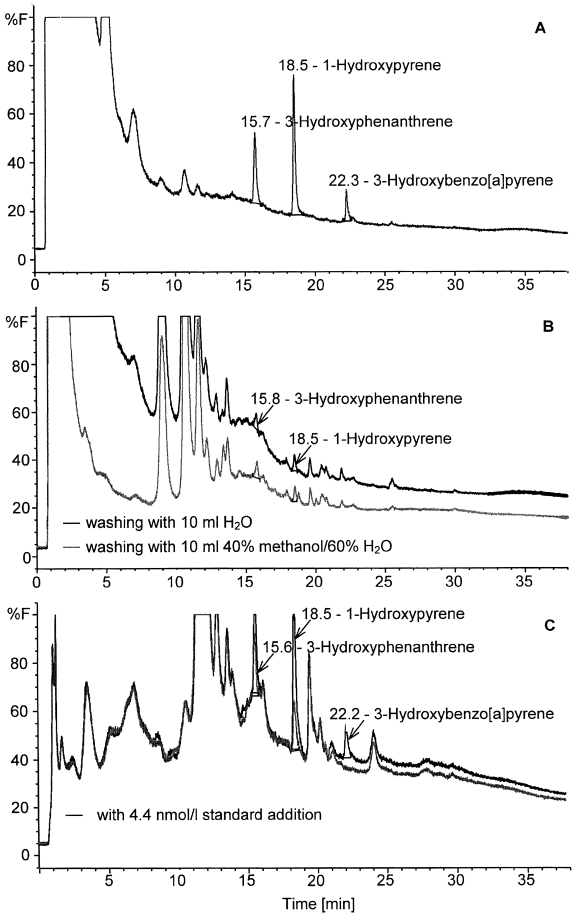

Fig. 1. Chromatograms of extracts of (A) pooled urine spiked with 3-hydroxyphenanthrene (3.3 nmol/l), 1-hydroxypyrene (2.9 nmol/l) and 3-hydroxybenzo[*a*]pyrene (2.4 nmol/l) (signal amplification of the detector: pmt gain 16), (B) native urine sample, with different washing of the SPE column during sample preparation (signal amplification of the detector: pmt gain 16), (C) native urine sample of a child with and without 4.4 nmol/l standard addition (signal amplification of the detector: pmt gain 17).

resulted in interference of the chromatogram by matrix compounds or in peak tailing. Further cleanup of the samples prior to HPLC–FLD analysis was necessary. The best results were achieved by washing the SPE column with 40% methanol (Fig. 1B) without affecting the good recoveries (Table 1). By this method it was possible to raise the signal amplification of the detector to pmt gain 17, which led to larger peaks relative to the background peaks (Fig. 1C). In addition, the urine volume could be

Table 1
Recoveries, detection and quantitation limits of the PAH hydroxides

	3-Hydroxy-phenanthrene	1-Hydroxy-pyrene	3-Hydroxy-benzo[*a*]pyrene
Recovery (%)	77±7	93±8	84±12
Detection limit (nmol/l) (DIN 32645)	0.33	0.26	0.34
Detection limit (nmol/l) ($S/N > 3$)	0.05	0.02	0.19
Quantitation limit[a] (nmol/l) (DIN 32645)	1.08	0.86	1.12
Residue standard deviation (counts)	1.20	0.90	0.40
Standard deviation of the method[b] (nmol/l)	0.10	0.08	0.11
Correlation coefficient	0.997	0.998	0.995

[a] Calculated from the suggestion that the maximal relative uncertainty of the results is 33% at significance level $\alpha = 0.01$.
[b] The standard deviation of the method is defined as residue standard divided by the slope of the calibration function and is a parameter for the quality of the method.

doubled to 20 ml in comparison to the sample preparation described in the standard method [3]. Increasing the injection volume led to peak tailing, therefore it was kept at 10 μl.

To demonstrate the reliability of the method, recoveries, correlation coefficients, detection and quantitation limits were determined using spiked urine samples as described in the Experimental section (Table 1). The recoveries were determined over the whole concentration range and resemble the values reported elsewhere [3,5,8,12]. The *F*-test demonstrated that the calibration function was linear. The correlation coefficients were better and the detection limits lower if the quantification was carried out by peak heights instead of peak area. The sensitivity increased from 3-hydroxybenzo[*a*]pyrene to 3-hydroxyphenanthrene to 1-hydroxypyrene. The detection limits were calculated according to two methods. The values determined by the signal-to-noise ratio are significantly lower than the values determined according to DIN 32645, because the confidence interval is used by the latter method. As proposed in DIN 32645 the parameters for the statistic calculations are set so that the compounds are detected in a sample with a probability of 1%, although they are not in reality in the sample (significance level $\alpha = 0.01$), and also with a 1% probability that the compounds are not detected, although they are in the sample (significance level $\beta = 0.01$). The detection limit and quantitation limit were determined from the calibration curve. In comparison, for the signal-to-noise ratio method it is not evident that the detection limit is, with a defined probability, always statistically different from zero. Therefore, the limits calculated according to DIN 32645 are more reliable. The detection limits of 1-hydroxypyrene and 3-hydroxyphenanthrene are similar to or lower than other values published to date using the same determination method [3,5,8,10,17]. The standard deviation of the method is a parameter of the quality of the method and is valid over the whole concentration range. The calculated values are similar for all hydroxylated PAHs (Table 1).

The method was applied to 10 urine samples from non-exposed children (Fig. 1B,C) because the method will be used in a study of the respiratory diseases of children. 1-Hydroxypyrene and 3-hydroxyphenanthrene were detected in all samples, indicating that the method is very sensitive for these compounds and suitable for the detection of environmental exposure of children. Concentrations of 0.15–2.13 nmol/l 3-hydroxyphenanthrene and 0.44–1.79 nmol/l 1-hydroxypyrene were determined. These values are significantly lower than the 1-hydroxypyrene values for children in a highly industrialized region of Poland (median 3.2 nmol/l for children of smoking mothers and 2.5 nmol/l for children of

non-smoking mothers) [5] and the reference value of 1-hydroxypyrene for non-exposed adults with a geometric mean of 3.2 nmol/l [3]. Therefore, the exposure of adults is also likely to be determined by the method developed here. The standard addition method was used to improve the reliability of peak identification (Fig. 1C), as also performed by Whiton et al. [17]. Only in one sample was 3-hydroxybenzo[*a*]pyrene identified, but the concentration was below the detection limit. Because benzo[*a*]pyrene is eliminated mainly via the gastro-intestinal tract the concentration of metabolites in urine is obviously too low for detection by HPLC with standard fluorescence detection. Therefore, other more sensitive methods such as HPLC with laser-induced fluorescence detection [14,18] are necessary for the measurement of benzo[*a*]pyrene in urine from non-exposed persons, but these methods require laborious sample preparation [14].

4. Conclusions

The sample preparation and the developed HPLC method allowed the simultaneous determination of 3-hydroxyphenanthrene, 1-hydroxypyrene and 3-hydroxybenzo[*a*]pyrene in urine with high sensitivity, small solvent consumption and short run time. The natural fluorescence of α-naphthol and 9,10-dihydroxy-9,10-dihydrophenanthrene was not high enough for sensitive detection. Biomonitoring of environmental phenanthrene and pyrene exposure of non-exposed children can be performed by the described method. The release of benzo[*a*]pyrene metabolites is mostly too low for detection.

Acknowledgements

We thank M. Weishoff-Houben and M. Möller for their kind help.

References

[1] J. Angerer, C. Mannschreck, J. Gündel, Int. Arch. Occup. Environ. Health 70 (1997) 365–377.
[2] F.J. Jongeneelen, R.B.M. Anzion, P.Th. Henderson, J. Chromatogr. 413 (1987) 227–232.
[3] F.J. Jongeneelen, R.B.M. Anzion, in: J. Angerer, K.H. Schaller (Eds.), Analyses of Hazardous Substances in Biological Materials, Vol. 3, VCH, Weinheim, 1991.
[4] A.M. Hansen, J.M. Christensen, D. Sherson, Sci. Total Environ. 163 (1995) 211–219.
[5] E. Siwińska, D. Mielzyńska, E. Smolik, A. Babak, J. Kwapuliński, Sci. Total Environ. 217 (1998) 175–183.
[6] P. Gerde, B.A. Muggenburg, G.G. Scott, J.L. Lewis, K.H. Pyon, A.R. Dahl, Carcinogenesis 19 (1998) 493–500.
[7] G. Grimmer, G. Dettbarn, J. Jacob, Int. Arch. Environ. Health 65 (1993) 189–199.
[8] J. Lintelman, C. Helleman, A. Kettrup, J. Chromatogr. B 660 (1994) 67–73.
[9] J. Angerer, C. Mannschreck, J. Gündel, Int. Arch. Occup. Environ. Health 69 (1997) 323–331.
[10] J. Gündel, C. Mannschreck, K. Büttner, U. Ewers, J. Angerer, Arch. Environ. Contam. Toxicol. 31 (1996) 585–590.
[11] A.M. Hansen, O.M. Poulsen, J.M. Christensen, S.H. Hansen, J. Liq. Chromatogr. 15 (1992) 479–499.
[12] P. Heikkila, M. Luotamo, V. Riihimäki, Int. Arch. Occup. Environ. Health 67 (1995) 211–217.
[13] A.M. Hansen, X. Omland, O.M. Poulsen, D. Sherson, T. Sigsgaard et al., Int. Arch. Occup. Environ. Health 65 (1994) 385–394.
[14] F. Ariese, M. Verkaik, G.P. Hoornweg, R.J. van de Nesse, S.R. Jukema-Leenstra et al., J. Anal. Toxicol. 18 (1994) 195–204.
[15] M. Bouchard, C. Dodd, C. Viau, J. Anal. Toxicol. 18 (1994) 261–264.
[16] M. Bouchard, C. Viau, Toxicol. Appl. Pharmacol. 139 (1996) 301–309.
[17] R.S. Whiton, C.L. Witherspoon, T.J. Buckley, J. Chromatogr. B 665 (1995) 390–394.
[18] C.J. Smith, J. Grainger, D.G. Patterson Jr., J. Chromatogr. A 803 (1998) 241–247.