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Coupled-column high-performance liquid chromatographic method for the determination of four metabolites of polycyclic aromatic hydrocarbons, 1-, 4- and 9-hydroxyphenanthrene and 1-hydroxypyrene, in urine

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Abstract

A coupled-column high-performance liquid chromatographic method applying system-integrated sample processing on a copper phthalocyanine modified solid-phase material, gradient elution and fluorescence detection for the quantification of 1-, 4- and 9-hydroxyphenanthrene as well as for 1-hydroxypyrene in urine is described. The automated chromatographic system tolerates direct and repeated injection of urine samples. Validation results presented indicate quantitative and matrix-independent recoveries, low imprecisions in within-series and between-series analysis in combination with broad linear working ranges (2–400 nmol/l) and low detection limits (< 0.1 nmol/l).

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are generated during combustion of natural or synthetic organic materials. In the environment they are present mainly due to their release from motor vehicles, domestic coal- or oil-fired heating systems, and various industrial sources. Remarkable high PAH concentrations can be measured at some workplaces like coke ovens, aluminium reduction plants, steel industry, creosote impregnating plants, gas and petroleum industries. Exposure to PAHs involves a potential health risk because some of these lipophilic compounds are considered to be human carcinogens [1]. Thus a thorough monitoring of subjects exposed to PAHs, especially at workplaces where high PAH concentrations are expected, is necessary. Besides the determination of the external exposure to PAHs by quantifying representative PAHs in the ambient air (ambient monitoring) more and more efforts are made to determine the internal, individual exposure (biological monitoring) of an exposed person.

In the case of PAHs biological monitoring is very difficult because the PAH mixtures found in the environments of interest contain a great number of various PAHs. After absorption in the body every compound is transferred to a lot of metabolites by the mammalian system and it is

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impossible to quantify all these substances. One helpful approach to biological monitoring can be seen in the determination of one or more representative metabolites usable as biological indicators for the internal PAH burden of the organism. During the last years the suitability of the renally excreted pyrene metabolite 1-hydroxypyrene as a biological marker of the internal dose of PAHs was investigated and the quantification of this metabolite was successfully used for the biological monitoring of both, occupational and environmental exposure to PAHs [2–10].

The HPLC method mostly used for the determination of 1-hydroxypyrene [11,12] includes three main steps: (1) enzymatic hydrolysis of the sulphate and glucuronide conjugates as which the analyte is excreted, (2) manually performed solid-phase extraction of the enzymatically treated urine on a conventional C_{18} cartridge and (3) HPLC analysis with fluorescence detection of the extract obtained after the preceding steps.

With the objective to automate principal analysis steps we developed in previous works a novel coupled-column HPLC method for the determination of 1-hydroxypyrene in urine that was distinguished by its high selectivity and practicability [13]. Special feature of the method was the "tailor-made" precolumn packing material that exhibits a high selectivity for the analyte. By integrating the precolumn and thus sample processing into the HPLC system it was possible to reduce the HPLC analysis of 1-hydroxypyrene to the following steps: (1) enzymatic treatment of the urine and (2) centrifugation, injection and HPLC analysis with fluorescence detection of the pre-treated urine. On the basis of this method principle in recent studies a coupled-column HPLC method for the determination of three isomeric hydroxylated phenanthrenes and 1-hydroxypyrene in urine could be developed.

The hydroxylated phenanthrenes are renally excreted metabolites of phenanthrene and they seem to be suitable as additional biological indicators for the internal PAH burden caused by exposure to PAHs [14–17]. Up to now the quantification of phenanthrols in urine is mostly done with gas chromatography–flame ionization detection. Therefore sample preparation normally includes chemical derivatization of the polar metabolites besides usual steps like enzymatic hydrolysis, chromatographic clean-up and enrichment of the analytes [14–18].

In this note the first coupled-column HPLC method with HPLC-integrated sample processing for the quantification of 1-, 4- and 9-hydroxyphenanthrene as well as 1-hydroxypyrene in urine after direct injection of the enzymatically treated biological sample can be presented.

2. Experimental

2.1. Chemicals

β-Glucuronidase/arylsulphatase solution (107 000/1140 units/ml) was from Sigma (Deisenhofen, Germany). HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). HPLC-grade water was generated by double quartz glass distillation. All other chemicals were of the highest purity available.

2.2. Reference substances and standard solutions

1-Hydroxypyrene was obtained from Janssen Chimica (Beerse, Belgium). The five phenanthrene metabolites, 1-, 2-, 3-, 4- and 9-hydroxyphenanthrene, were kindly received from Dr. A. Seidel and Professor Dr. Oesch (Institut für Toxikologie, Universität Mainz, Mainz, Germany) for research use.

Stock solutions in methanol were prepared by weighing in, standard solutions with different concentrations in water–methanol (50:50) were obtained by diluting the stock solutions. All the solutions were stored in the dark at −20°C and checked regularly by HPLC analysis.

2.3. Instrumentation

The HPLC system consisted of a gradient pump (Model 300 CS) with a programmable microprocessor unit (Model M 250 B) from Gynkotek (Germering, Germany), an automatic

six-port switching valve from Besta (Wilhelmsfeld, Germany), a Model 590 pump from Waters (Eschborn, Germany), a fluorescence detector 1050, an autosampler 655A-40 (optional) and an analytical column (Superspher 100, RP-18, 4 μm, 250 × 4 mm I.D.) from Merck Darmstadt, Germany). The precolumn (copper phthalocyanine-modified silica, 30 μm, 5 × 4 mm I.D.) is available as BioPAcK from Walfort & Partner (Reinhardshagen, Germany). For heating the analytical column a unit consisting of a thermostat from Haake (Karlsruhe, Germany) and a self-constructed stainless-steel jacket was used.

2.4. Samples

Urine samples (20–25 ml) used for the evaluation of the matrix-dependent recovery of the analytes were collected in standard polyethylene or polypropylene tubes and immediately frozen at −20°C until sample preparation. Urine samples from exposed subjects were generously provided by Dr. F.J. Jongeneelen and his working group (Department of Toxicology, University of Nijmegen, Nijmegen, Netherlands). After slowly thawing the samples they were enzymatically treated to hydrolyse the sulphate and glucuronide conjugates of the metabolites. The enzymatic hydrolysis was carried out according to the procedure described in detail already elsewhere [13].

2.5. Chromatography

The chromatographic system (Fig. 1) is based on the following main components:

(1) The six-port automatic switching valve, which allows programmed connection and disconnection of pre- and analytical column.

(2) The precolumn, the packing-material of which shows high selectivity for the analytes and that is therefore used for the HPLC-integrated sample processing.

(3) The analytical column on which the analytes are separated after sample processing.

The gradient controller which can be activated by the autosampler controls the gradient pump, the switching valve and the integrator.

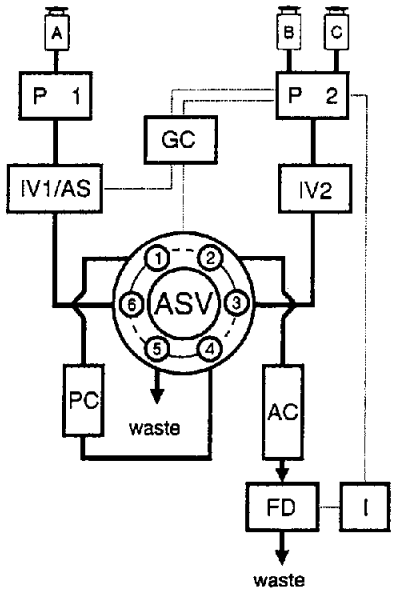

Fig. 1. Module configuration and valve switching positions. A = Methanol–water (10:90, v/v); B = methanol–water (60:40, v/v); C = methanol; P1 = pump 1; P2 = gradient pump; GC = gradient controller; IV = injection valve; AS = autosampler; ASV = automatic switching valve; PC = precolumn; AC = = analytical column; FD = fluorescence detector; I = integrator. Solid line = flow in valve position LOAD; dashed line = flow in valve position INJECT.

A second injection valve in front of the gradient pump (P 2) allows the direct injection and analysis of standards on the analytical column in valve position LOAD. Thus recoveries on the precolumn can be examined.

Eluent compositions, switching-valve positions and procedures during a coupled-column analysis of 1-, 4- and 9-hydroxyphenanthrene and 1-hydroxypyrene are given in Table 1.

3. Results and discussion

Compared with the previously introduced HPLC method [13], the procedures and their principal functions during a coupled-column HPLC analysis of PAH metabolites in urine did not change because the general strategy remained the same. For this reason, the single steps of an analysis cycle will not be explained in detail again, only significant modifications are mentioned.

Table 1
Timetable for the coupled-column HPLC analysis of hydroxyphenanthrenes and 1-hydroxypyrene

Time (min)	B (%)	C (%)	Flow-rate (ml/min)	Switching valve position	Procedure
0	100	0	0.8	LOAD	Sample processing and equilibration of the analytical column
10	100	0	0.8	INJECT	Transfer of the analytes, begin of data acquisition
14	100	0	0.8	LOAD	Analytical separation
35	100	0	0.8	LOAD	
43	38	62	0.8	LOAD	
45	38	62	0.8	LOAD	
45.1	0	100	0.8	INJECT	Clean-up and reconditioning of the columns
48	0	100	0.8	LOAD	
51	0	100	0.8	LOAD	

During the analysis cycle pump 1 operates at a flow-rate of 1 ml/min with eluent A. Eluents: A = methanol–water (10:90, v/v); B = methanol–water (60:40, v/v); C = methanol. Injection volume: 100 μl of the enzymatically treated urine sample or 100 μl of a standard. Fluorescence detection: excitation 242 nm; emission 388 nm. Column temperature: 40°C.

3.1. Sample processing

The injection volume of the urine sample and the duration of the HPLC-integrated sample processing could be reduced from 250 to 100 μl and from 15 to 10 min (water–methanol, 90:10, v/v, at 1 ml/min, pump 1) respectively. The chromatograms represented in Fig. 2 demonstrate that the analytes are selectively separated from the residual biological matrix which is nearly completely eluted into the waste during sample processing.

3.2. Transfer

A fast and quantitative transfer of the analytes from the precolumn to the top of the analytical column is achieved by increasing the methanol content of the eluent. This leads to a desorption and fast elution of the compounds from the precolumn in backflush mode [methanol–water (60:40, v/v) at 0.8 ml/min for 4 min, pump 2].

3.3. Analytical separation and detection

In order to obtain a separation of the isomeric hydroxyphenanthrenes the previously used short reversed-phase column (125 × 4 mm I.D., 5 μm) was replaced by a longer one with smaller particles (250 × 4 mm I.D., 4 μm). Within the limits determined by the conditions of the preceding transfer step and the back-pressure of the analytical column the influence of various parameters like eluent composition (acetonitrile–, tetrahydrofuran–, methanol–water), gradient elution, column temperature and flow-rate on the separation was investigated. The best separation was achieved using a methanol gradient at 40°C with 0.8 ml/min. On these conditions 1-, 4- and 9-hydroxyphenanthrene and 1-hydroxypyrene were separated. It was not possible to prevent co-elution of 2- and 3-hydroxyphenanthrene. For the detection of the analytes their natural fluorescence was used (excitation 240 nm, emission 390 nm).

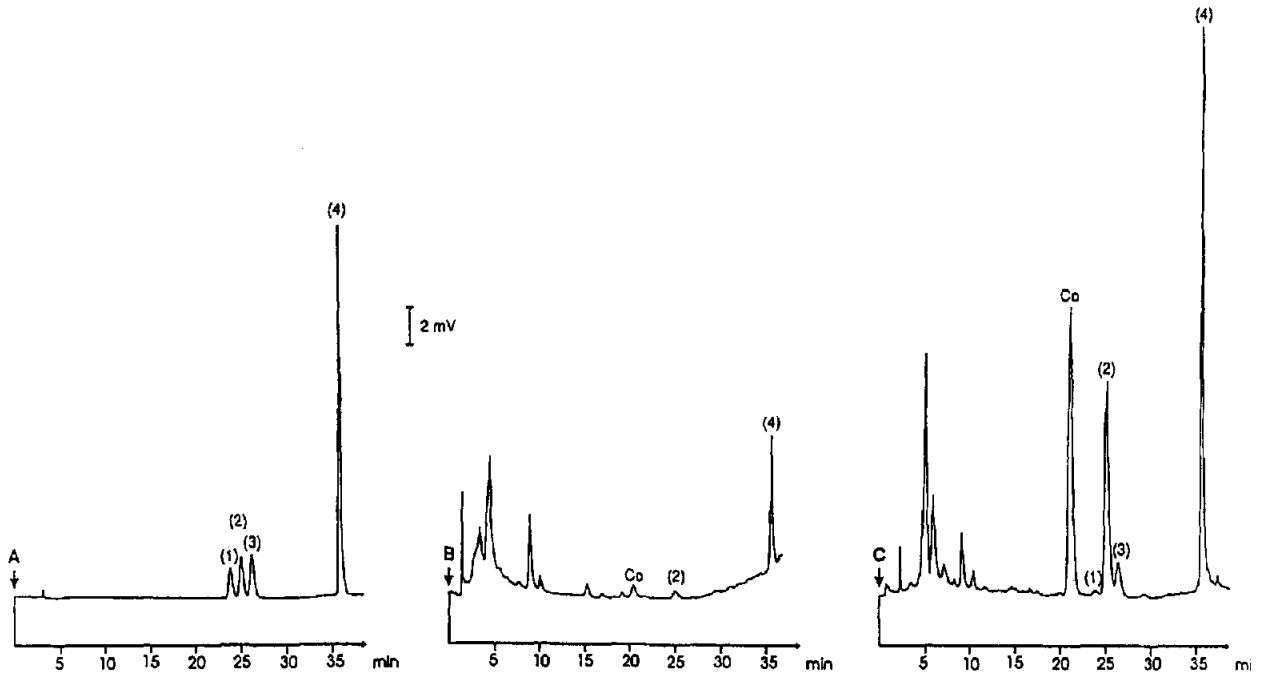

Fig. 2. Coupled-column HPLC analysis of 9-hydroxyphenanthrene (1), 1-hydroxyphenanthrene (2), 4-hydroxyphenanthrene (3) and 1-hydroxypyrene (4). Co = 2- and 3-hydroxyphenanthrene, co-eluting. (A) Calibration standard (1: 24.0 nmol/l; 2: 22.0 nmol/l; 3: 28.0 nmol/l; 4: 28.0 nmol/l). (B) Urine of a non-exposed subject (2: 4.2 nmol/l; 4: 5.4 nmol/l). (C) Urine of an occupationally exposed subject (1: 5.1 nmol/l; 2: 84.7 nmol/l; 3: 19.3 nmol/l; 4: 30.4 nmol/l). Injection volume 100 μl. For chromatographic conditions see Table 1.

A separation of 2- and 3-hydroxyphenanthrene on the conditions investigated could not be managed. The co-eluting analytes exhibit different fluorescence characteristics leading to different signal intensities at identical concentrations. Therefore a calibration and quantification of 2- and 3-hydroxyphenanthrene is not possible with the method presented.

3.4. Cleaning and reconditioning

After analytical separation analytical column and precolumn are purged with methanol to elute very hydrophobic substances. During the last 3 min the precolumn is reconditioned for the next analysis which can be started 51 min after the preceding injection of a urine sample.

3.5. Validation

To obtain data on the reliability of the method recoveries (Table 2), calibration functions, imprecisions (Table 3) and detection limits were determined. For the matrix-independent recovery 100 μl of standard were analysed twice in the single-column and in the coupled-column mode, respectively (concentration range: 1.8–1100 nmol/l). For the matrix-dependent recovery urine samples from non-exposed persons were pooled, enzymatically hydrolysed and the concentrations of the analytes were determined. Aliquots of the thus treated urine were spiked with known amounts of the metabolites and 100 μl of the samples were analysed twice in the coupled-column mode (concentration range: 2.2–1100 nmol/l). The recoveries (Table 2) are

Table 2
Matrix-independent and matrix-dependent recoveries

	1-HPH		4-HPH		9-HPH		1-HP	
	r (%)	s (%)	r (%)	s (%)	r (%)	s (%)	r (%)	s (%)
Standards ($n = 7$)	99	4	98	4	97	3	97	6
Urine samples ($n = 8$)	101	4	99	6	93	6	91	5

HPH = Hydroxyphenanthrene; HP = hydroxypyrene; r = recovery; s = standard deviation of the recovery.

independent of the concentration and of the biological matrix. These results are confirmed by a statistical investigation [19] of the calculated recovery functions: neither sample processing on the precolumn nor the biological matrix led to a significant loss of precision or to significant systematic deviations. For this reason calibration was done by analysing 100 μl of the calibration standards [methanol–water (50:50, v/v)] twice in the coupled-column mode. The calibration graphs were linear at least in a range 2–400 nmol/l for the phenanthrene metabolites and in the range 0.3–100 nmol/l for 1-hydroxypyrene. The correlation coefficients of all calibration graphs were >0.999, the relative standard deviations of the method were low with values for the relative standard deviation of the method V_{x0} between 2.4 and 5.2% [$V_{x0} = s_{x0} \cdot X^{-1} \cdot 100$; $s_{x0} = s_y \cdot (\Delta A/\Delta c)^{-1}$ with s_y: residual standard deviation, $(\Delta A/\Delta x)$: sensitivity, X: mean concentration of the calibration standards]. The sensitivities are increasing from 9-hydroxyphenanthrene over 4-hydroxyphenanthrene and 1-hydroxyphenanthrene to 1-hydroxypyrene. For the determination of the within-run and between-run imprecision (Table 3) two urine samples of occupationally exposed persons were enzymatically treated, aliquoted, frozen and the aliquots (injection volume: 100 μl) were analysed in the coupled-column mode in series and on 8 subsequent days.

The specificity of the method presented in this note is due to the selective fluorescence detection and to the selective sample processing on the precolumn (only compounds owing a planar structure consisting of three or more fused rings are retained quantitatively on the copper phthalocyanine-modified precolumn packing material).

The broad linear working ranges in combination with the low detection limits (DL) (0.05–0.1 nmol/l, according to $DL = 3(\Delta A/\Delta c)^{-1} s_{bl}$ with $(\Delta A/\Delta c)$: sensitivity and s_{bl}: standard deviation of blank values) makes it possible to quantify

Table 3
Within-run and between-run-imprecisions in urine

	1-HPH		4-HPH		9-HPH		1-HP	
	C.V. (%)	c (nmol/l)	C.V. (%)	c (nmol/l)	C.V. (%)	c (nmol/l)	C.V. (%)	c (nmol/l)
Series ($n = 8$)	5.5	41.0	6.9	8.2	7.6	2.8	7.6	11.8
Day/day ($n = 10$)	5.1	44.8	6.1	8.4	3.9	2.2	9.2	13.0

HPH = Hydroxyphenanthrene; HP = hydroxypyrene; C.V. = coefficient of variation; c = concentration.

high metabolite concentrations in urine deriving from e.g. occupational exposure as well as low concentrations caused by e.g. environmental exposure to PAHs.

4. Conclusions

On the basis of a special precolumn used for selective, HPLC-integrated sample processing a coupled-column HPLC method for the determination of 1-, 4- and 9-hydroxyphenanthrene and 1-hydroxypyrene in urine could be developed. Because of the high automation and the selectivity of the HPLC-integrated sample processing the method distinguishes on good practicability and high reliability which make it attractive for routine analysis in biological monitoring of exposure to PAHs. An important objective for future works will be the analytical separation and thus the quantification of 2- and 3-hydroxyphenanthrene. Further applications of the method or rather of their general principle can be seen in the research field of PAH metabolism in mammalian organisms.

References

[1] *Polynuclear Aromatic Compounds, Part 1, Chemical, Environmental and Experimental Data (IARC monograph*, Vol. 32), International Agency for Research on Cancer, Lyon, 1983.
[2] J.G.M. Van Rooij, M.M. Bodelier-Bade and F.J. Jongeneelen, *Brit. J. Ind. Med.*, 50 (1993) 623.
[3] F.J. Jongeneelen, F.E. van Leeuwen, S. Oosterink, R.B.M. Anzion, F. van der Loop, R.P. Bos and H.G. van Veen, *Brit. J. Ind. Med.*, 47 (1990) 454.
[4] J.P. Buchet, J.P. Gennart, J.F. Mercado-Calderon, J.P. Delavignette, L. Cupers and R. Lauwerys, *Brit. J. Ind. Med.*, 49 (1992) 761.
[5] T.J. Buckley and P.J. Lioy, *Brit. J. Ind. Med.*, 49 (1992) 113.
[6] E. Clonfero, M. Zordan, P. Venier, M. Paleologo, A.G. Levis, D. Cottica, L. Pozzoli, F.J. Jongeneelen, R.P. Bos and R.B.M. Anzion, *Int. Arch. Occup. Environ. Health*, 61 (1989) 363.
[7] W.P. Tolos, P.B. Shaw, L.K. Lowry, B.A. MacKenzie, J. Deng and H.L. Markel, *Appl. Occup. Environ. Hyg.*, 5 (1990) 303.
[8] Z.H. Zhab, W.Y. Quan and D.H. Tian, *Sci. Tot. Environ.*, 92 (1990) 145.
[9] T. Kanoh, M. Fukuda, H. Onozuka, T. Kinouchi and Y. Ohnishi, *Environ. Res.*, 62 (1993) 230.
[10] K. Gardiner, K.A. Hale, I.A. Calvert, C. Rice and J.M. Harrington, *Ann. Occup. Hyg.*, 36 (1992) 681.
[11] F.J. Jongeneelen, R.B.M. Anzion and P.Th. Henderson, *J. Chromatogr.*, 413 (1987) 137.
[12] F.J. Jongeneelen and R.B.M. Anzion, in J. Angerer and K.-H. Schaller (Editors), *Analyses of Hazardous Substances in Biological Materials*, Vol. 3, VCH, Weinheim, 1991, p. 151.
[13] K.-S. Boos, J. Lintelmann and A. Kettrup, *J. Chromatogr.*, 600 (1992) 189.
[14] G. Grimmer, G. Dettbarn, K.-W. Naujack and J. Jacob, *Int. J. Environ. Anal. Chem.*, 43 (1991) 177.
[15] G. Grimmer, G. Dettbarn and K.-W. Naujack, in F. Schuckmann and S. Schopper-Jochum (Editors), *Berufskrankheiten, Krebserzeugende Arbeitsstoffe, Biological-Monitoring Arbeitsmedizinisches Kolloquium der Gewerblichen Berufsgenossenschaften*, Gentner Verlag, Stuttgart, 1991, p. 283.
[16] F. Martin, I. Hoepfner, G. Scherer, F. Adlkofer, G. Dettbarn and G. Grimmer, *Environ. Int.*, 15 (1989) 41.
[17] G. Grimmer, G. Dettbarn and J. Jacob, *Int. Arch. Occup. Environ. Health*, 65 (1993) 189.
[18] J. Jacob and G. Grimmer, *Rev. Anal. Chem.*, 9 (1987) 49.
[19] W. Funk, V. Damman and G. Donnevert, *Qualitätssicherung in der Analytischen Chemie*, VCH, Weinheim, 1992.