*Journal of Chromatography*, 600 (1992) 189–194
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2508

# Coupled-column high-performance liquid chromatographic method for the determination of 1-hydroxypyrene in urine of subjects exposed to polycyclic aromatic hydrocarbons

Karl-Siegfried Boos*

*Institut für Klinische Chemie, Klinikum Grosshadern der Universität München, Postfach 701260, D-8000 Munich (Germany)*

Jutta Lintelmann and Antonius Kettrup

*GSF-Forschungszentrum für Umwelt und Gesundheit, Institut für Ökologische Chemie, Ingolstädter Landstrasse 1, D-8042 Neuherberg (Germany)*

ABSTRACT

A coupled-column high-performance liquid chromatographic system for integrated, on-line sample processing and the determination of free and conjugated 1-hydroxypyrene in urine has been developed. The method is based on a "tailor-made" copper phthalocyanine-modified porous-glass precolumn packing material, which allows a direct and repeated injection of urine samples and a selective enrichment of trace amounts of particular components. The fully automated method has a low detection limit (0.01 pmol), a quantitative and matrix-independent recovery and a highly reliability, as shown by an interlaboratory comparison of methods.

## INTRODUCTION

The urinary pyrene metabolite 1-hydroxypyrene is a suitable biological indicator for internal exposure to polycyclic aromatic hydrocarbons (PAHs) in several work environments [1]. In the urine of workers occupationally exposed to coal tar and derived products containing PAHs, increased levels of 1-hydroxypyrene, which is mainly excreted as its sulphate and glucuronide conjugate, can be found [1–3].

Until now, urinary 1-hydroxypyrene has been determined by a high-performance liquid chromatographic (HPLC) method developed by Jongeneelen and co-workers [4,5]. This procedure involves a manually performed liquid–solid phase extraction on a cartridge packed with a conventional $C_{18}$ reversed-phase material. Subsequently, the pretreated sample is evaporated and redissolved before injection.

To handle the potentially large number of urine samples to be analysed in the biological monitoring of subjects exposed to PAHs we developed a fully automated coupled-column HPLC method for the urine and direct determination of 1-hydroxypyrene. The method is based on a "tailor-made" precolumn packing material which (1) shows a high selectivity for 1-hydroxypyrene, (2) enriches trace amounts of 1-hydroxypyrene, (3) eliminates the residual sample matrix and (4) allows the direct and repeated injection of native or enzymatically treated urine samples.

## EXPERIMENTAL

### *Chemicals*

1-Hydroxypyrene was obtained from Janssen Chimica (Beerse, Belgium). β-Glucuronidase–arylsulphatase solution (100 000 Fishman U/ml and 800 000 Roy U/ml) was from Boehringer (Mann-

0021-9673/92/$05.00 

heim, Germany). HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). HPLC-grade water was generated by double glass distillation. All other chemicals were of the highest purity obtainable.

*Samples*

Urine samples (20–25 ml) were collected in standard polythylene or polypropylene tubes and immediately frozen at −20°C until sample preparation. After slowly thawing the sample, 2 ml of the urine were placed in a glass vessel and ajusted to pH 5.0 with 1 and 0.1 *M* hydrochloric acid in 10-μl steps using a pH meter. The solution was then diluted with a sodium acetate buffer (0.1 *M*, pH 5.0) to a final volume of 4 ml and incubated for 3 h with 5 μl of 4β-glucuronidase–arylsulphatase at 37°C in a thermostated water-bath. The samples were centrifuged for 5 min at 2000 *g* and 2 ml of the supernatant were transferred into an autosampler glass vial. The enzymatically hydrolysed samples are stable for at least 12 h at 4°C and for at least 6 months at −20°C.

For the interlaboratory method comparison, urine samples from workers of a creosote oil impregnation plant and from non-exposed control subjects were collected and frozen at −20°C at the University of Nijmegen (Netherlands). These samples were analysed in a double-blind study with Dr. Jongeneelen (Department of Toxicology, Faculty of Medicine, University of Nijmegen), who used a manual extraction procedure [4,5].

*Calibration procedure*

From a starting solution (approximately 200 μg/ml methanol), a standard stock solution of 1-hydroxypyrene in HPLC-grade methanol (approximately 10 nmol/ml) was prepared and the concentration photometrically confirmed at 242 nm using the extinction coefficient $\varepsilon = 56.64$ μmol cm$^2$ in methanol. The calibration standards with concentrations ranging from approximately 0.4 to 32 pmol per 100 μl were prepared by diluting the stock solution with an aqueous methanol solution methanol–doubly distilled water (50:50, v/v). The starting solution is stable for 1 month at −20°C, the stock solution for 1 week at −20°C, and the calibration standards for 2 days at 4°C.

The calibration standards were analysed in the coupled-column mode to obtain the calibration graph. Once the linearity of this curve was checked, a single-point calibration with the external standard was applied for day-to-day analysis. Peak areas were used for quantification.

*Instrumentation*

The HPLC system consisted of a Model L-6000 pump (P 1), a Model L-6200 gradient pump with a programmable microprocessor unit (P 2), a Model 655A-40 autosampler (optional) (AS), a Model ELV 7000 automatic switching valve (ASV), a Model F-1050 fluorescence detector (FD), a Model D-2050 integrator, a precolumn (copper phthalocyanine trisulphonic acid-modified porous glass, 30–60 μm, 5 × 4 mm I.D.) (PC) and an analytical column (LiChrospher RP-18, 5μm, 125 × 4 mm I.D.) (AC). The modular units of the HPLC system were obtained from Merck.

*Chromatography*

To perform a coupled-column switching technique, the precolumn and the analytical column were connected via the automatic six-port switching valve, the configurations and positions of which are shown in Fig. 1.

This instrumental set-up allows the use of the HPLC system in the coupled-column (precolumn–analytical column) as well as in the single-column (analytical column) mode. The microprocessor unit of the gradient pump controls the pump itself, the automatic switching valve and the integrator. The timetable with eluent compositions and the switching valve positions for a coupled-column analysis are given in Table I.

For the detection of 1-hydroxypyrene, its natural fluorescence is used (excitation 242 nm, emission 388 nm).

With an injection valve in front of the gradient pump and in the switching valve position LOAD, the instrumental set-up allows the independent use of the single-column (RP-18) HPLC system beside the coupled-column mode. In this mode standard samples can be injected directly onto the analytical column to control the recovery in the coupled-column mode.

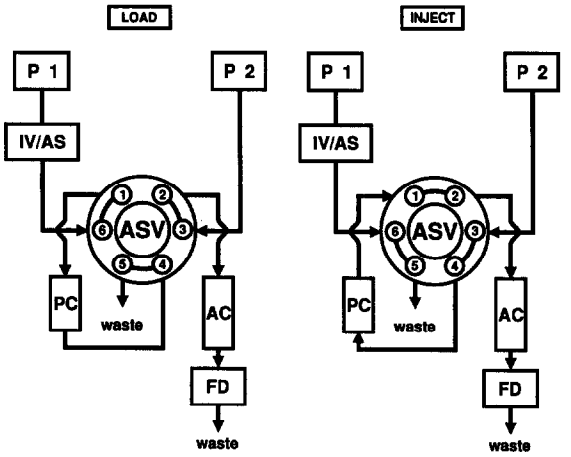


Fig. 1. Valve switching positions. P1 = Pump 1; P 2 = gradient pump; AS = autosampler (optional); IV = injection valve; PC = precolumn; AC = analytical column; FD = fluorescence detector.

TABLE I

TIMETABLE FOR THE COUPLED-COLUMN DETERMINATION OF 1-HYDROXYPYRENE

During the analysis cycle pump 1 operates at a flow-rate of 1 ml/min with eluent A. Eluent A, doubly distilled water–methanol (90:10 v/v); eluent B, HPLC-grade methanol; eluent C, doubly distilled water. Injection volume: 250 µl of the enzymatically treated urine sample, or 100 µl of a calibration standard. Fluorescence detection: excitation 242 nm, emission 388 nm.

| Step | Time (min) | Eluent (%) | | Flow-rate (ml/min) | Switching valve position (Fig. 1) |
|---|---|---|---|---|---|
| | | B | C | | |
| 1 | 0 | 60 | 40 | 1.0 | LOAD |
| 2 | 15 | 60 | 40 | 1.0 | INJECT |
| 3 | 19 | 60 | 40 | 1.0 | LOAD |
| 4 | 33 | 85 | 15 | 1.0 | LOAD |
| 5 | 35 | 100 | 0 | 1.0 | INJECT |
| 6 | 44 | 100 | 0 | 1.0 | LOAD |
| 7 | 50 | 100 | 0 | 1.0 | LOAD |

## RESULTS AND DISCUSSION

The most important part of the HPLC method introduced in this paper is the precolumn packing material, a porous glass support, that shows characteristic properties owing to its composition and modification of the chemical surface.

The hydrophilic surface (glyceryl coating) of the solid-phase material in combination with its size-exclusion properties allows a rapid and almost quantitative elution of the residual biological matrix. The selectivity for 1-hydroxypyrene is introduced by the immobilization of a copper phthalocyanine trisulphonic acid moiety at the surface of the solid phase (Fig. 2) [6].

This surface modification was chosen because water-insoluble salts of copper phthalocyanine tetrasulphonate and solid supports, on which copper trisulphonates are immobilized (Blue Cotton [7–9], Blue pearls [10]), selectivity adsorb compounds with a planar structure consisting of at least three fused rings from aqueous solutions. This selective adsorption is due to hydrophobic and steric interactions, which can easily be destroyed by organic solvents such as methanol. HPLC-integrated sample processing with the new precolumn packing material is thus performed by two different modes of liquid chromatography.

Adsorption chromatography results in a selective retention of the analyte, whereas size-exclusion chromatography allows a simultaneous and quantitative elution of the residual biological matrix into the waste. In the coupled-column mode, an analysis cycle is characterized by the following steps (Table I and Fig. 1).

*Sample processing (step 1)*

Sample application (standard solution, 100 µl; or enzymatically treated urine, 250 µl) is via the autosampler or an injection valve in valve position LOAD, followed by selective sample processing on

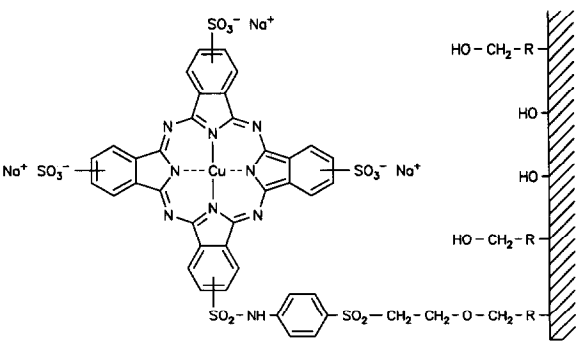


Fig. 2. Structure of the copper phthalocyanine trisulphonic acid derivative bound to the porous-glass support.

the precolumn with eluent A (water–methanol 90:10, v/v; pump 1). The analytical column is simultaneously equilibrated with methanol–water (60:40, v/v) via the gradient pump.

*Transfer (steps 2–3)*

This step involves automatic valve switching into position INJECT and automatic start of the integrator. The analyte is quantitatively eluted from the precolumn in a backflush mode by increasing the methanol content of the eluent (methanol–water, 60:40, v/v; gradient pump) and simultaneously transferred in a single, narrow elution band through valve positions 3–4–1–2 to the top of the series-connected analytical column.

*Separation (steps 3–5)*

The valve is switched into position LOAD and separation on the analytical column is carried out by a linear methanol gradient from 60 to 85% in 14 min and from 85 to 100% in 2 min delivered by the gradient pump. 1-Hydroxypyrene is detected and quantified by its natural fluorescence.

*Reconditioning (steps 5–7)*

In valve position INJECT the analytical column and precolumn are washed with methanol for 9 min to elute highly hydrophobic substances (gradient pump). During the last 6 min of the cycle, the valve is switched into position LOAD and the precolumn is reconditioned for the next analysis (pump 1), which can be started every 50 min. Fig. 3 shows chromatograms obtained by the coupled-column analysis of different urine samples. In addition to 1-hydroxypyrene, which is fully separated, only a few residual sample components appear in the chromatograms. It is interesting to note that no urine sample could be found, even when taken from infants, which contained no 1-hydroxypyrene.

To assess the accuracy of the system recovery ($r$) experiments were carried out. For the matrix-independent recovery, 100 $\mu$l of standard solutions ($n$ =

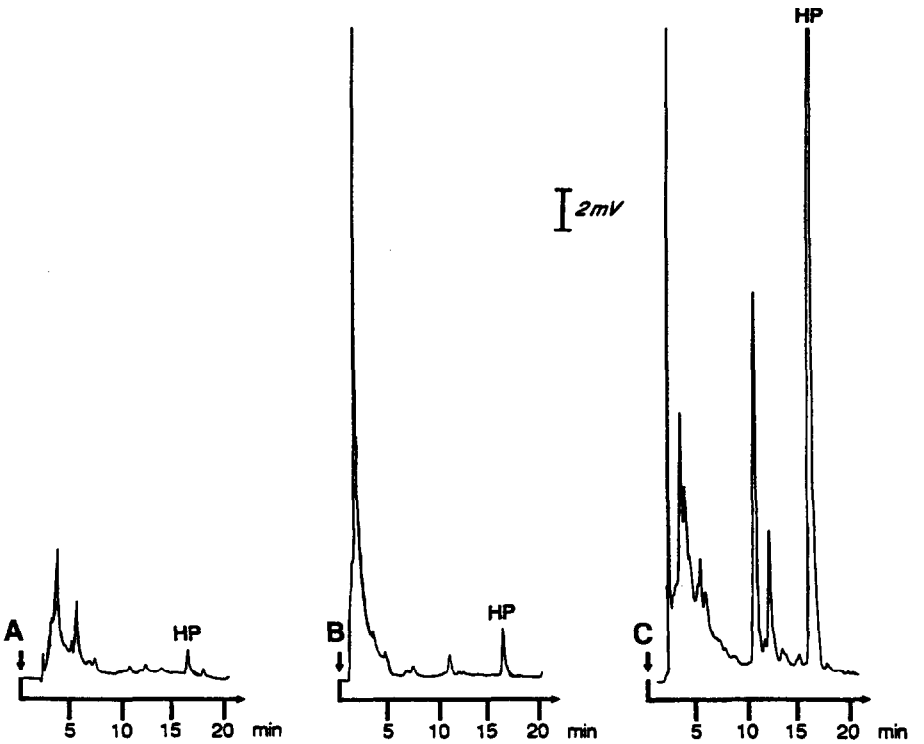


Fig. 3. Coupled-column analysis of 1-hydroxypyrene (HP). (A) Urine of a non-exposed subject after enzymatic hydrolysis (0.27 pmol ≡ 2.16 nmol/l). (B) and (C) Urine sample of an exposed worker of a creosote oil impregnation plant before (B) (0.50 pmol ≡ 4.0 nmol/l) and after (C) enzymatic hydrolysis (12.55 pmol ≡ 100.40 nmol/l). Injection volume 250 $\mu$l.

10) in the concentration range 0.4–32.02 pmol per 100 μl were analysed in the single-column mode and in the coupled-column mode ($r$ = 101.99 ± 3.81%). For the matrix-dependent recovery, a pooled urine sample was enzymatically hydrolysed and the concentration of 1-hydroxypyrene was determined before the addition of a known amount of 1-hydroxypyrene. The spiked urine samples (injection volume 250 μl; $n$ = 10) had concentrations ranging from 0.71 to 28.25 pmol per 250 μl ($r$ = 99.79 ± 3.46%). It showed a quantitative recovery for the coupled-column mode, which is independent of the amount of 1-hydroxypyrene and the biological matrix injected.

To obtain data on the precision of the system, within-series an a between-day precision for the analysis of standards (coefficients of variation: $C.V._{series}$ = 2.49%, $n$ = 15; $C.V._{day/day}$ = 1.83%, $n$ = 7) and urine samples ($C.V._{series}$ = 2.57%, $n$ = 15; $C.V._{day/day}$ = 2.87%, $n$ = 7) were determined. For the determination of the between-day precision of the analysis of urine, including the enzymatic hydrolysis step (C.V. 4.91%), one urine sample from an exposed subject was aliquoted and frozen. The aliquots were enzymatically treated and analysed on 7 subsequent days. The results show that 1-hydroxypyrene can be analysed with high precision, even under routine conditions.

The linearity between peak area and the amount of 1-hydroxypyrene ranges from 0.2 to 35 pmol and covers the concentrations that are of interest in the biological monitoring of exposed subjects.

The detection limit was 0.01 pmol and thus even the very low concentrations which occur in urine of controls or subjects exposed to small amounts of PAHs can be quantified exactly. The specificity of the method relies on the detection of the native fluorescence and on the selective retention mechanism for the analyte on the copper phthalocyanine trisulphonic acid-substituted porous-glass support (only compounds with three or more fused aromatic rings are retained quantitatively).

The good reliability of the HPLC system could be confirmed by an interlaboratory comparison of methods [5]. The results of this double-blind study and its statistical treatment indicate that both methods yield equivalent results with respect to accuracy and precision (Table II).

The principal difference between the two methods is found in the respective sample processing steps. Compared with manually performed off-line method, the coupled-column, *i.e.* HPLC-integrated sample processing, offers many advantages. Time-consuming and error-prone manual working steps such as activation, rinsing and elution of the solid-phase extraction cartridge and evaporation and dissolution of the eluate are greatly reduced, resulting in an increased practicability and high reliability. Finally, the coupled-column system can handle more than 250 urine injections (approximately 32 ml) without any loss of chromatographic performance. Owing to the matrix-independent and quantitative recovery of the analyte, calibration is simplified and needs only an external standard.

TABLE II

RESULTS OF THE INTERLABORATORY COMPARISON OF METHODS

The samples were analysed with the off-line method [4] and the coupled-column method. Spearmans coefficient of rank correlation ($r_s$ = 0.984) and the result for Student's $t$-test [$t$ = 1.60; $t$(95%, 16) = 2.12] indicate that there is no significant difference between the two series of analytical results.

| Sample No. | Concentration of 1-hydroxypyrene (nmol/l) | |
|---|---|---|
| | Off-line method | Coupled-column method |
| 1 | 13.3 | 3.54 |
| 2 | 0.8 | 0.49 |
| 3 | 24.1 | 20.66 |
| 4 | 87.2 | 88.10 |
| 5 | 154.0 | 151.23 |
| 6 | 13.0 | 13.80 |
| 7 | 1.0 | 1.12 |
| 8 | 18.6 | 11.13 |
| 9 | 385.0 | 342.19 |
| 10 | 71.4 | 42.90 |
| 11 | 44.6 | 26.60 |
| 12 | 30.6 | 30.12 |
| 13 | 168.9 | 189.77 |
| 14 | 92.8 | 84.94 |
| 15 | 87.2 | 75.98 |
| 16 | 1.5 | 1.00 |
| 17 | 37.1 | 37.58 |

## CONCLUSIONS

The chemical modification of a glyceryl-coated porous-glass support with a copper phthalocyanine

trisulphonic acid derivative and its integration into a conventional HPLC system via a precolumn and a column switching device has led to an automated coupled-column HPLC system for the determination of 1-hydroxypyrene in urine.

The practicability and high reliability make the system attractive for routine analysis in the biological monitoring of exposure to PAHs.

ACKNOWLEDGEMENTS

The authors thank the "Forschungskommission" of the University of Paderborn for financial support and Dr. Jongeneelen and his working group for their friendly co-operation.

REFERENCES

1 F. J. Jongeneelen, R. B. M. Anzion, P. T. J. Scheepers, R. P. Bos, P. Th. Henderson, E. H. Nijenhuis, S. J. Veenstra, R. M. E. Brouns and A. Winkes, *Ann. Occup. Hyg.*, 32 (1988) 35.
2 F. J. Jongeneelen, R. B. M. Anzion, Ch.-M. Leijdekkers, R. P. Bos and P. Th. Henderson, *Int. Arch. Occup. Environ. Health*, 57 (1985) 47.
3 F. J. Jongeneelen, R. P. Bos, R. B. M. Anzion, J. L. G. Theuws and P. T. Henderson, *Scand. J. Work Environ. Health*, 12 (1986) 137.
4 F. J. Joneneelen, R. B. M. Anzion, P. Th. Henderson, *J. Chromatogr.*, 413 (1987) 227.
5 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.
6 J. Lintelmann, *Ph. D. Thesis*, University of Paderborn, Paderborn, 1990.
7 K. Kusuda, K. Shiraki and T. Miwa, *Anal. Chim. Acta*, 224 (1989) 1.
8 H. Hayatsu, T. Oka, A. Wakata, Y. Ohara, T. Hayatsu, H. Kobayashi and S. Arimoto, *Mutat. Res.*, 119 (1983) 233.
9 H. Hayatsu, H. Kobayashi, A. Michi-ue and S. Arimoto, *Chem. Pharm. Bull.*, 34 (1986) 944.
10 M. Geisert, T. Rose and R. K. Zahn, *Fresenius Z. Anal. Chem.*, 330 (1988) 437.