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Automated column-switching high-performance liquid chromatography method for the determination of 1-hydroxypyrene in human urine

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Abstract

An on-line sample treatment method to determine 1-hydroxypyrene (1-OHP), a metabolite of polycyclic aromatic hydrocarbons (PAHs), in human urine has been developed. The hydrolysed biological fluid was directly injected into the chromatographic system after only centrifugation. A miniature precolumn loop packed with a preparative phase and coupled on-line to a liquid chromatographic (LC) system was used for analyte enrichment. The analytes were non-selectively desorbed with the LC eluent and cleaned by means of a column-switching procedure comprising two purification columns and an analytical column. Pre-treatment and analysis were performed within 2 and 20 min, respectively. Average 1-OHP recovery reached 99% in the 1–25 μg/l range of urine, and the quantitation limit was 20 ng/l for 100 μl of injected sample. A comparison with a more time-consuming off-line method was performed by analysing 120 urine samples of PAH-exposed and expected unexposed workers; the statistical treatment indicated that both methods are in agreement. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Column-switching; 1-Hydroxypyrene

1. Introduction

Many polycyclic hydrocarbons (PAHs) are suspected or known carcinogens, and exposure to these compounds involves a potential health risk [1]. Environmental exposure occurs when smoking, when air pollution is present, or during the consumption of broiled or smoked food products. High PAH exposure levels are encountered more particularly in various industrial sectors, such as coke ovens, aluminium reduction plants, the steel industry, creosote impregnation plants, etc.

In addition to personal air sampling, biological monitoring is also used to assess exposure; urinary 1-hydropyrene (1-OHP; Fig. 1) is often used as an indicator of exposure to PAH mixtures at workplaces [2–6].

Several analytical methods have been described in the literature to determine 1-OHP in urine [7–12]. They mainly employ unautomated and time-consuming sample clean-up procedures with solid-phase C_{18} extraction cartridges [7–10]. These methods require treatment times of up to 30 min, with the exception of the automated sample clean-up method using

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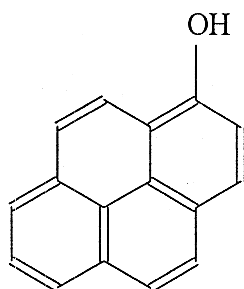

Fig. 1. Structural formula of 1-OHP.

'tailor-made' copper phthalocyanine-modified porous glass precolumns [11,12]. The column-switching technique, which has found many applications in clinical laboratories and in biological monitoring, is very attractive for sample clean-up and routine analysis using liquid chromatography. A new and easily automated column-switching method has been developed to determine urinary 1-OHP.

The method described in this work removes the need for a liquid–liquid extraction step or an off-line purification step using solid-phase extraction mini-columns. It allows the direct injection of an enzymatically treated urine sample after only centrifugation. Purification and analysis by high-performance liquid chromatography (HPLC) were carried out in isocratic mode with a fluorescence detector. To improve the urinary detection limit, a concentration and clean-up procedure can be performed with a re-usable precolumn, which is used as an injection loop and is coupled on-line to the column-switching system. Less than 1 ml of urine is needed to detect 20 ng/l of 1-OHP in urine.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical reagent grade. Acetic acid, ascorbic acid and sodium acetate were obtained from Merck (Darmstadt, Germany). Water was purified by passing it through a Milli-Q treatment system (Millipore, Bedford, MA, USA). Chromatographic-grade acetonitrile and methanol were obtained from S.D.S (Peypin, France) and Merck, respectively. Triton X-100 R and 1-OHP were purchased from Sigma–Aldrich Chimie (St. Quentin Fallavier, France). β-Glucuronidase-arylsulphatase solution (from *Helix pomatia*) was from Merck, and Lyphochek® urine control was provided by Bio-Rad (Ivry/Seine, France).

The methanol (MeOH) used for the preparation of the mobile phases must be freshly distilled in order to eliminate impurities that cause interference and serious disturbance of the baseline.

2.2. Apparatus

2.2.1. On-line method

The HPLC system consisted of the following: two chromatographic pumps, a Model 590 (Waters, Milford, MA, USA) and a Model 5000 (Varian, San Fernando, CA, USA); four Model 7000 automated switching valves (Rheodyne, Berkeley, CA, USA), one of which was equipped with a miniature precolumn loop; and a Model RF-551 fluorescence detector (Shimadzu, Kyoto, Japan) set to 265 nm for excitation and 385 nm for emission. The entire system was controlled by the Waters chromatographic pump. The output signal was recorded with a Chromjet integrator (Thermo Separation Products, Fremont, CA, USA) or a recorder (Kipp and Zonen, Touzart et Matignon, Vitry-sur-Seine, France).

2.2.2. Off-line method

The HPLC system consisted of a Model P 1500 chromatographic pump (Thermo Separation Products) combined with a Model 7410 sample injector (Rheodyne) equipped with a 5-μl loop and a Model F-1050 fluorescence detector (Hitachi, Tokyo, Japan) set to 240 nm for excitation and 390 for emission. The output signal was recorded with a Model D-2500 integrator (Merck) or a recorder (Kipp and Zonen).

2.3. Columns

2.3.1. On-line method

The precolumn loop was a 2 cm×0.21 cm I.D. stainless-steel guard column cartridge (Upchurch Scientific, Oak Harbor, WA, USA) filled with approximately 50 mg of 20–40 μm Sepralyte C_1 Methyl (Analytichem International, Harbor City, CA,

USA). Purification columns C1 and C2 were 3.5 cm×0.32 cm I.D. and 3.5 cm×0.46 cm I.D. stainless-steel tubes packed with 5 μm Kromasil C_4 (The Separation Group, Hesperia, CA, USA), for C1, and 5 μm Spherisorb OD/CN (Phase Separations, Franklin, MA, USA), regardless of whether Nucleosil NO_2 or Polygosil NO_2 (Macherey-Nagel, Düren, Germany) had been applied for C2. Analytical column C3 was a 20 cm×0.32 cm I.D. stainless-steel tube packed with 5 μm Vydac 201 TPB (The Separation Group), Lichrosorb RP Select B (Merck) or Kromasil C_{18} (The Separation Group).

2.3.2. Off-line method

The analytical column was a 12.5×0.4 cm I.D. stainless-steel tube filled with Lichrospher HPA 5 μm (Merck) set to 30°C with a column thermostat (CTO-10ASVP, Shimadzu).

With the exception of the precolumn loop, which was dry-filled, all of the columns were packed in the laboratory at $4 \cdot 10^7$ Pa using a mixture of 95% ethanol–2-propanol–toluene (1:1:1, v/v/v) as the slurry solvent, followed by methanol and then by water as the displacement liquid.

2.4. Analytical procedure

2.4.1. On-line method

A schematic diagram of the switching system is shown in Fig. 2, and the timetable of the analytical procedure is given in Table 1. The mobile phase (E1) used for the purification columns (C1 and C2) was a mixture of water–methanol–acetic acid (60:39.9:0.1, v/v/v). The mobile phase (E2) used for the analytical column (C3) was a mixture of the same solvents in the ratio 75:24.8:0.2 (v/v/v). They were all eluted in isocratic mode at 0.5 ml/min. Prior to analysis, the mobile phases were degassed with helium for 5 min and kept under a helium atmosphere during the analysis.

Prior to each injection, the precolumn loop was manually preconditioned with 200 μl of water, and the variable volume samples (10–200 μl) were then injected into the chromatographic system with a HPLC glass syringe. After the samples had been loaded, the precolumn loop was flushed with 200 μl of $2 \cdot 10^{-1}$ *M* CH_3COOH (in water–MeOH; 8:2, v/v) at about 2 ml/min, to remove undesirable impurities (step 1). This step can be automated using appro-

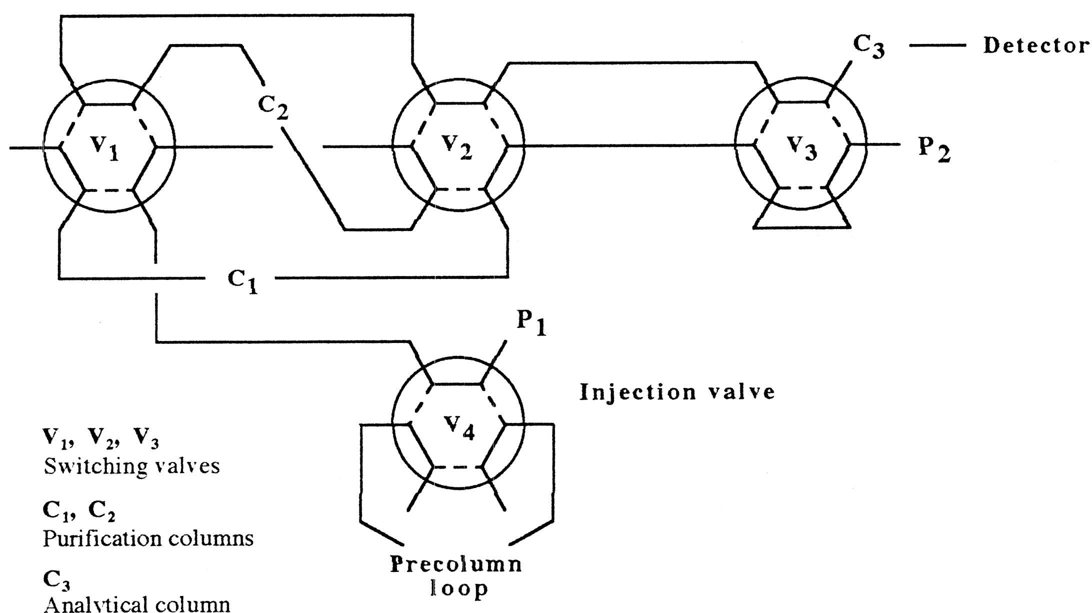

Fig. 2. Layout of the automated sample pre-treatment system for determining 1-OHP: Valves V1–V4 are all shown in position A (cf. step 6, Table 1). The system is described in the Experimental section.

Table 1
Column-switching analytical procedure using the scheme according to Fig. 2

Step event		Valve positions[a]				
		Time (min)	Valve 1	Valve 2	Valve 3	Valve 4
1	Manual[b] precolumn loop flushing with sample and CH_3COOH solution (about 1 min)		A	B	B	A
2	Start of the automated analytical sequence. Flushing precolumn loop with E1 (0.5 min, 0.25 ml) pump P1, analyte transfer to C1 (injection)	0	B	A	B	B
3	Switch injection valve; manual precolumn wash. Purification on C1	0.5	B	A	B	A
4	Analyte transfer from C1 to C2	2.5	B	B	B	A
5	Purification on C2. Clean-up of C1 in back-flush mode	5	A	A	B	A
6	Analyte transfer from C2 to C3. Flushing C1 with E2, pump P2	8	A	A	A	A
7	Analysis on C3. Flushing C1 and C2 in back-flush mode with E1 (reconditioning)	12	A	B	B	A

[a] Position A corresponds to the valve positions shown in Fig. 2.
[b] The flushing precolumn loop procedure could be automated using the appropriate equipment.

priate equipment. The next steps of the column-switching analytical procedure are summarised in Table 1. During the last step, 1-OHP was eluted on C3 with E2, while columns C1 and C2 were cleaned and reconditioned in back-flush mode with E1 to prepare them for the next injection. After each analysis, the precolumn loop was cleaned with 200 µl of CH_3OH–CH_3COOH (99:1, v/v) and reconditioned with water. After every 50 injections, the purification columns and analytical column were cleaned with a mixture of H_2O–MeOH–CH_3COOH (9:90:1, v/v/v). Inexplicably, the addition of ascorbic acid to the mobile phases at concentrations of 5 or 10 mg/l did not significantly improve the reproducibility and the sensitivity of the analysis, contrary to what Bouchard et al. [9] had noted previously.

2.4.2. Off-line method

The mobile phase, a mixture of acetonitrile–water (1:1, v/v), was pumped isocratically at a flow-rate of 1 ml/min. After sample treatment, 5 µl of the extract were injected into the HPLC system. As for the on-line method, the mobile phase was degassed with helium for 5 min before the analysis, then kept under a helium atmosphere during the analysis. In contrast to the on-line method and in accordance with the method of Bouchard et al. [9], ascorbic acid was added to the eluent at a concentration of 5 mg/l to improve reproducibility and sensitivity. The analytical column was cleaned with acetonitrile every day after 30 injections.

2.5. Human urine collection and treatment

'Spot' urine samples from volunteers from three different types of workshops, an artificial shooting target factory, an aluminium reduction plant and a research centre, were collected in polyethylene bottles and refrigerated. If the urine samples were not analysed within one week of being collected, they were fractionated and kept frozen at −20°C. After the urine samples had been thawed, they were pretreated following a previously described procedure [7]. Briefly, the urine (0.5–5 ml) was diluted twofold with sodium acetate buffer ($5\cdot10^{-2}$ *M*, pH 5), vortex-mixed (0.5 min) and incubated in an oven at 37°C overnight with 4 β-glucuronidase-arylsulphatase.

2.5.1. On-line method

After hydrolysis, a methanolic solution of Triton X-100 R (50 g/l) was added to the mixture at a ratio of 1:10 (v/v). Before direct injection of 20 µl of the sample into the precolumn loop, it was briefly vortex-mixed (0.5 min), then centrifuged at 2000 *g* for 3 min. Depending on the sample, the injected volume ranged from 10 to 200 µl; when the injected volumes were higher than 50 µl, a 5-g/l concentration (instead 50 g/l) of Triton X-100 R in methanol was added to the hydrolysed urine sample. Before switchover of the injection valve, the precolumn was flushed with 200 µl of a mixture

containing MeOH–H_2O–CH_3COOH (20:79.8:0.2, v/v/v) at a flow-rate of about 2 ml/min.

2.5.2. Off-line method

Prior to injection into the chromatographic system, a manual extraction procedure was used according to a method modified from Jongeneelen et al. [7]. Briefly, 10 ml of buffered and hydrolysed urine was vacuum drawn through a C_{18} Sep-Pak cartridge (Waters). After being washed with water (5 ml), the cartridge was vacuum dried, then washed with *n*-hexane (3 ml). Finally, the 1-OHP was eluted with CH_2Cl_2 (5 ml). The latter was evaporated at 35°C under a gentle flow of nitrogen and the residue was dissolved in 0.5–5 ml of a mixture of acetonitrile–methanol (1:1, v/v).

2.6. Preparation of standards

The commercial 1-OHP standard was dissolved in acetonitrile (50 mg/l) and stored at −20°C.

2.6.1. On-line method

A reconstituted Lyphochek® quantitative control urine was used to obtain spiked 1-OHP solutions. The urine was diluted 1:2 (v/v) with sodium acetate buffer (10^{-1} *M*, pH 5) and enzymatically hydrolysed. After hydrolysis, a methanolic solution of Triton X-100 R (50 or 5 g/l depending on the injected volume) was added to the hydrolysed sample at a ratio of 1:10 and briefly vortex-mixed (0.5 min). Spiked solutions (25, 5 and 1 μg/l) were prepared by diluting the stock solution with this sample. The resulting samples were directly injected (generally 20 μl) into the precolumn loop after centrifugation at 2000 *g* for 3 min.

After the addition of the methanolic solution of surfactant, the hydrolysed urine standard solutions are stable for at least one month at 4°C, as are the majority of hydrolysed urine samples from subjects. Aqueous standards of 1-OHP are also stable for 24 h at 4°C provided that a methanolic solution containing 50 g/l of Triton X-100 R is added to the water at a ratio of 1:10 (v/v). The aqueous or urinary standards were used to determine the respective switching times, to check the efficiency of the method and to test its reliability.

2.6.2. Off-line method

The starting solution (50 mg/l) was further diluted in acetonitrile–methanol (1:1, v/v) to obtain spiked 1-OHP solutions at 500, 50 and 5 μg/l.

2.6.2.1. 1-OHP analysis. For a given urine sample, the retention time was compared with that of an external standard, and peak-height measurement method was used for quantitative assessment.

3. Results and discussion

An interesting automated method was described by Boos et al. [11], who employed a 'tailor-made' copper phthalocyanine-modified porous glass precolumn for the purification and concentration of urine samples. This precolumn is on-line coupled to a column-switching HPLC system that uses a discontinuous methanol gradient to transfer 1-OHP from the precolumn to the analytical column, and a linear methanol gradient as the elution mode for the analysis.

An isocratic elution mode seems to us to be simpler and more practical than the gradient elution mode and, moreover, no column reequilibration time is necessary. Consequently, we used automated switching multicolumns, which had been employed previously in our laboratory [13] for aflatoxin M_1 (AFM_1) analysis. Besides elution in isocratic mode, the developed method makes use of the difference in selectivity of the chromatographic phases to clean up urine samples. As a result, the direct transfer technique or 'heart cut' technique was retained and the optimum chromatographic conditions sought: best eluents (composition, ionic strength, pH, nature of organic modifiers, etc.), combinations of various bonded phases with various parameters (particle diameter, column dimensions, number of columns, etc.).

The results of our investigations are shown in Fig. 3 where the chromatograms of different urine samples illustrate the high selectivity of the developed method; apart from 1-OHP, very few other residual urine sample components appear in the chromatograms. Two purification columns were used to clean up the urine, and high-volume samples were able to be directly injected into the chromatographic system

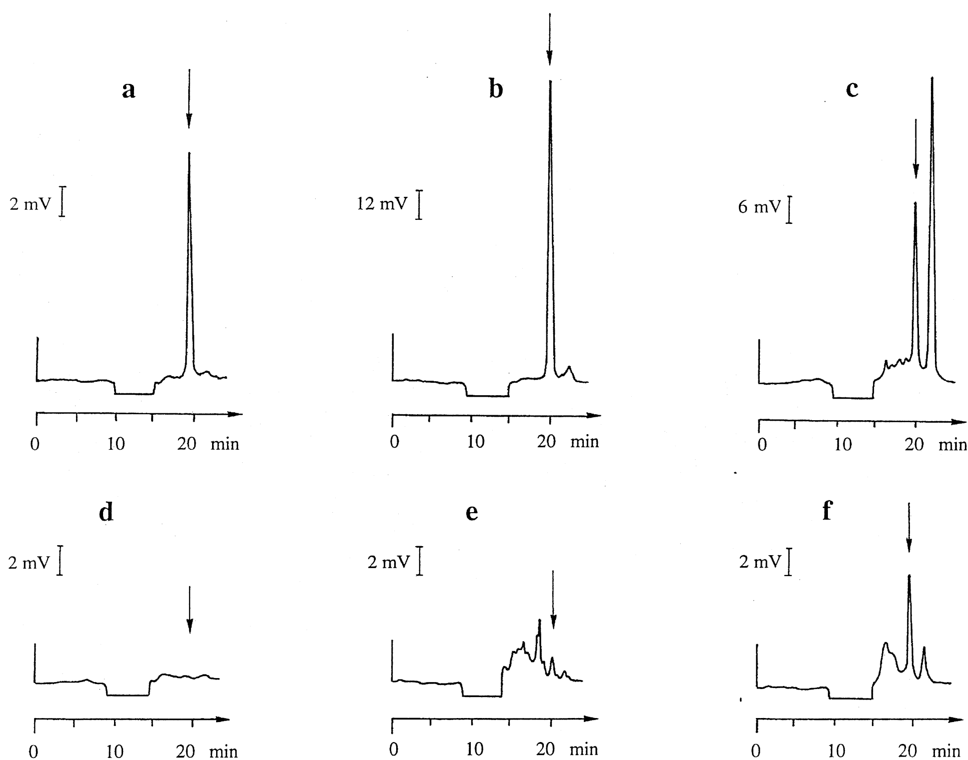

Fig. 3. Chromatograms of various urine samples obtained after clean-up with the switching system: (a) Urinary standard of 5.5 μg/l 1-OHP; 20 μl injected; (b) urine sample 1, 40.4 μg/l 1-OHP; 10 μl injected; (c) urine sample 2, 2.4 μg/l 1-OHP; 100 μl injected; (d) blank, eluant; (e) 'blank' urine sample, 0.040 μg/l 1-OHP; 200 μl injected; (f) urine sample 4, 0.450 μg/l1-OHP; 100 μl injected. The system peak at time 10–15 min corresponds to the transfer and injection of analyte from C2 into the analytical column. The chromatographic conditions are detailed in Section 2.

to improve the quantitation limit. As previously observed by Boos et al. [11], all urine samples contain 1-OHP. However, as systematically low amounts of 1-OHP (less than 50 ng/l) were found in Lyphochek® quantitative urine control samples (Fig. 3e) and as 1-OHP is particularly stable in the urinary matrix, this material was used after enzymatic hydrolysis for the preparation of 1-OHP standards.

The efficiency of the purification and concentration procedures for urine samples on the pre-column loop was checked by comparison with the direct injection (without treatment) of methanolic or aqueous standards. The matrix-dependent recovery was assessed in the same way. No loss of 1-OHP occurs during the pretreatment process, provided that a surfactant is added to the enzymatically hydrolysed urine sample. Also, after addition of Triton X-100 R, the recoveries of 1-OHP from spiked urines at concentrations of 1, 5 and 25 μg/l are almost quantitative (99±2%, $n=6$) and independent of the concentration of 1-OHP and of the volume of the urine sample loaded; conversely, in the absence of surfactant, apparent 1-OHP losses (20 to 40%) are observed.

The calibration curve for 1-OHP using peak-height (mm) measurement vs. analyte concentration (μg/l) was linear over the range investigated (0.25–25 μg/l; i.e. 5–500 pg for a 20-μl injection), and the calibration curve equation was $y=-2.33+96.71x$, with a regression coefficient (r^2) equal to 0.998. The precision of the method was established on a sample of pooled urine (1.45 g/l creatinine) to which 1-OHP had been added at concentrations of 1, 4 and 10 μg/l; for the 1 μg/l concentration, the coefficients of variation (C.V.) were less than 3 and 4% for within-day and between-day precision, respectively,

and for the 4 and 10 μg/l concentrations, they were less than 2 and 3%, respectively.

The between-day variation was related primarily to degassing of the mobile phase, which was necessary to prevent quenching of the fluorescence signal of 1-OHP by the oxygen dissolved in the mobile phase. This problem has been discussed in the literature [14].

When the laboratory temperature is not regulated, excessive retention time variations (C.V.>10%) can occur on the purification columns; thus, as far as possible, purification columns and the analytical column should be thermostated.

Since the quantitation limit varies with the volume of the injected urine sample or standard, this limit was estimated at 20 ng/l ($S/N=10$) for a 100-μl injection, and at 100 ng/l for a 20-μl injection. The detection limit was estimated at 5 ng/l ($S/N=3$) for 100 μl of injected aqueous standard; this value is much lower than those occurring in the urine of controls or exposed subjects. The range of the spiked standard solutions (0.25–25 μg/l) was determined according to the range of 1-OHP values generally found in the urine of workers exposed or unexposed to PAHs [2,4,6].

The reliability of the switching method was confirmed by comparing it with the manual off-line purification method. Urine samples (120) from exposed and supposedly unexposed workers were analysed by both methods. The urinary levels of 1-OHP are plotted on a regression line (Fig. 4). The regression line equation ($y=-73.07+1.025x$) and the product–moment correlation coefficient ($r=0.996$) show that both methods give similar results and that no marked systematic bias exists between them.

These results were confirmed, as shown in Fig. 5 (Bland-Altman plot), which shows the relationship between y-residuals ($X-Y$) and mean $(X+Y)/2$ urinary 1-OHP concentration readings (ng/l) obtained by the switching method (X) and those obtained by the off-line method (Y). It can be seen that the mean difference is not 'significantly' different from zero (mean of $X-Y=-45.66$); the occasional lack of agreement between the two methods can be attributed to random errors.

The result of the Student's t-test indicate that there is no significant difference between the two series of analytical results; $t=1.075$ ($t=1.980$, 95%, $n=100$).

Fig. 6 shows the chromatograms of the same urine

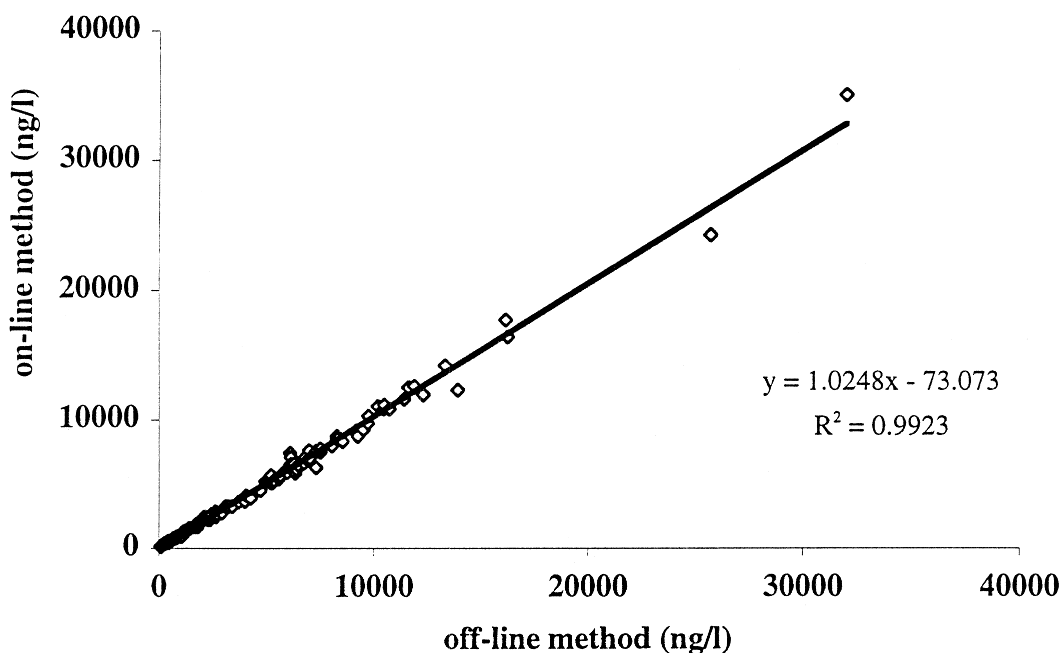

Fig. 4. Comparison of 1-OHP measurements in 120 urine samples using the off-line purification method and the switching method.

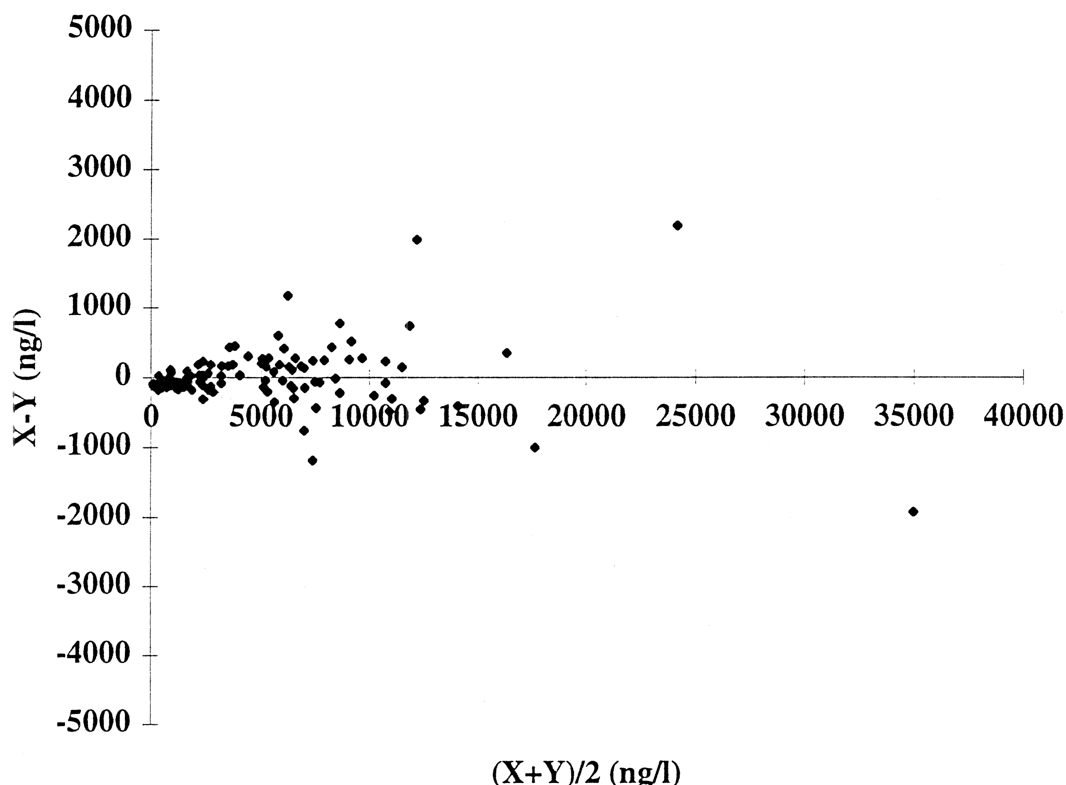

Fig. 5. Relationship between the difference (switching method−off-line method) and mean (switching method+off-line method/2) assays of 1-OHP in 120 urine samples analysed by both methods.

samples treated by both methods. It is clear that the specificity and the selectivity of the on-line method are better. With the off-line method, much higher background from residual components in the sample appear in addition to 1-OHP, which is often eluted in a tailing peak. Moreover, late residual peaks appear after 1-OHP and this contrasts with the on-line method in which no peak appears after a 25-min elution time.

Compared to the manual off-line method, the switching method offers a number of advantages, the most important being that time-consuming handling operations with the associated risk of error are reduced.

3.1. Hydrolysis of OHP glucuronides

The switching method is particularly well adapted to routine analyses and, for example, to studying the kinetics of 1-OHP glucuronide hydrolysis. Thus, the method was used during a study of urinary 1-OHP excretion in exposed workers, to confirm the abnormally low values observed for some samples. These samples were analysed again and particular attention was paid to the efficiency of 1-OHP glucuronide hydrolysis. Fig. 7 shows the hydrolysis kinetic curve obtained every hour for six problematic samples. It can be seen that the 1-OHP glucuronide hydrolysis is complete after less than 3 h and that, beyond this time, an unexplained and sudden reduction in 1-OHP occurs, except in one case. Moreover, in spite of the regular addition of enzyme to compensate for possible degradation or excessive consumption, the decrease in 1-OHP did not reverse or stop. Likewise, when a five- or 20-fold excess of starting enzyme material was added to the same urine samples, greater 1-OHP concentrations than those in the previous experiment were obtained after 1 h of

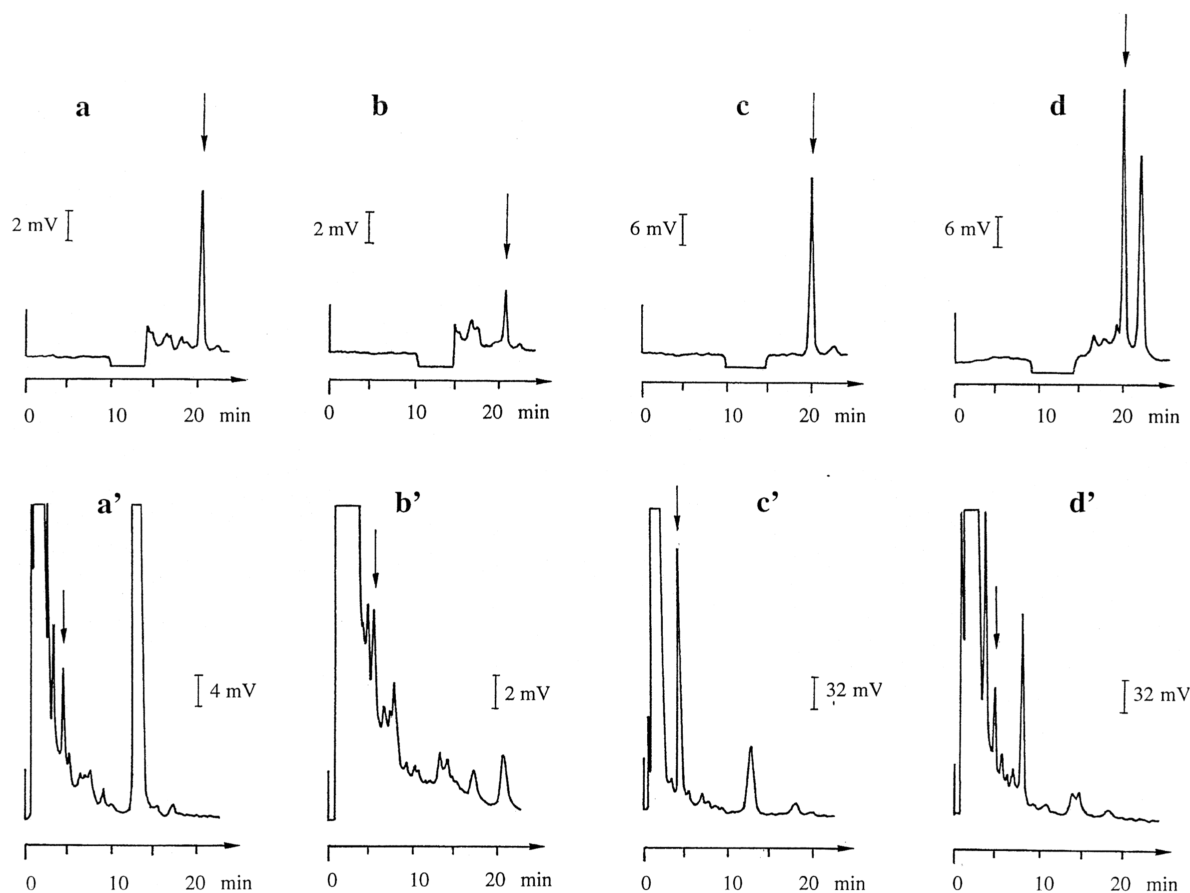

Fig. 6. Chromatograms of the urine samples obtained after purification with the described method (a, b, c, d) and with the off-line purification method (a′, b′, c′, d′): (a) urine sample 5, 0.5 μg/l 1-OHP; 100 μl injected; (a′) same urine sample concentrated tenfold; 5 μl injected; (b) urine sample 6, 0.18 μg/l 1-OHP; 100 μl injected; (b′) same urine sample concentrated tenfold; 5 μl injected; (c) urine sample 7, 27.0 μg/l 1-OHP; 10 μl injected; (c′) same urine sample concentrated 2.5-fold; 5 μl injected; (d) urine sample 8, 3.4 μg/l 1-OHP; 100 μl injected; (d′) same urine sample concentrated tenfold; 5 μl injected. The system peak at time 10–15 min in chromatograms a, b, c and d corresponds to the transfer and injection of analyte from C2 into the analytical column. The chromatographic conditions are detailed in Section 2.

hydrolysis; however, a decrease in the amount of 1-OHP still occurred with the passage of time. Lastly, an aqueous solution of Triton X-100 R (50 g/l) was added to the problematic urine samples at a ratio of 1:10 (v/v) before the addition of enzyme. Under these hydrolysis conditions, no 1-OHP loss was observed, even after 16 h of hydrolysis. The 1-OHP losses, only observed for a few urine samples during the preliminary experiment, cannot be explained clearly given that they are not the result of adsorption on the walls of vials; probably some unknown proteins or substances present in urine are responsible for these losses, which might depend on the sample being studied. These results suggest that it would be better to add a small quantity of surfactant to the urine samples before hydrolysis in order to prevent any loss of 1-OHP.

3.2. Technical remarks

Concerning the HPLC switching system, the pumps, valves, columns and connections were laid out in such a way that a minimum amount of equipment was used and that the two purification

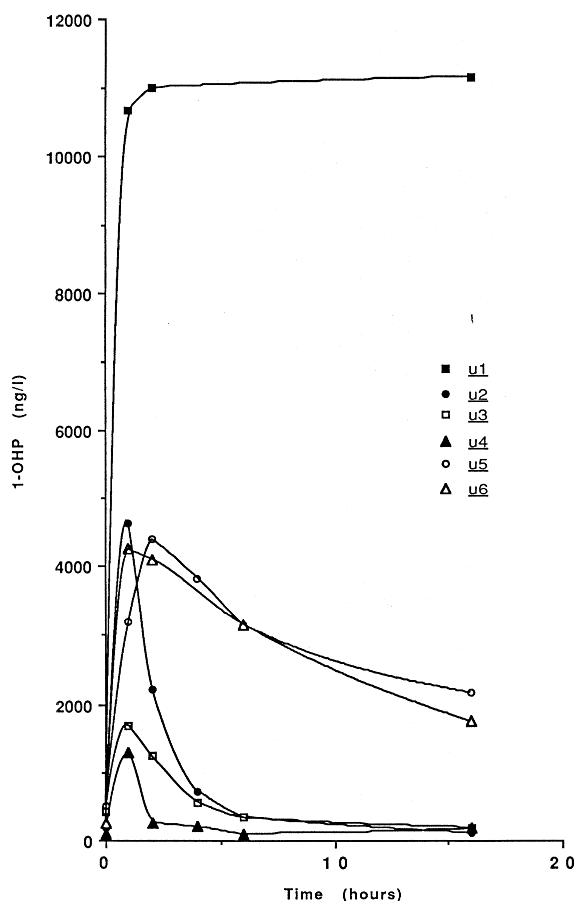

Fig. 7. Hydrolysis kinetics curve obtained for six urine samples.

columns were cleaned and reconditioned in back-flush mode during the 1-OHP elution on the analytical column. The nature of the purification column was chosen by determining the capacity factor of 1-OHP for a series of different packing materials.

The use of ion-exchange bonded phases proved to be disappointing. In addition, uncommon stationary phases, such as CN- and NO_2-bonded phases, were tested, with the latter being known for its chromatographic behaviour with respect to PAHs. Satisfactory results were obtained with the two selected bonded phases and, more particularly, with the NO_2-phase, which formed the best compromise, offering a minimum number of interfering components transferred in a minimum retention time. In order to reconcentrate the 1-OHP on the top of the analytical column, the size of the purification column was also optimised so that the strength of the mobile phase was lower than that of the analytical column. Up to 500 injections of 20 μl, requiring about 400 urine samples, were performed with the same purification and analytical columns without any loss of resolution and performance.

Concerning the analytical column, various alkyl bonded phases (C_8 and C_{18}) were tested under the same elution conditions. As Bouchard et al. [9] noted previously, the fluorescence signal for 1-OHP was slightly different from one phase to another (±10%), except for one that gave an inexplicably weak signal (about tenfold lower than the mean of the other phases). Moreover, mixing ascorbic acid with the mobile phase neither improved the 1-OHP fluorescence temporal signal, nor stopped its progressive deterioration. Under the elution conditions described, the three selected bonded phases gave a similar and stable 1-OHP fluorescence response with time. When a Kromasil C_{18} phase was used, the eluent strength of E2 was increased slightly to elute 1-OHP within 20 min (H_2O–MeOH–CH_3COOH; 19.2:80:0.2, v/v/v).

The reliability of the method was tested regularly by injecting a urinary standard solution every five samples. As for the urine samples, the urinary 1-OHP standard solutions were passed through the entire column system in order to subject them to the same purification treatment. This procedure allows the detection of chromatographic troubleshooting occurring upstream of the analytical column (i.e., changes in 1-OHP retention times on the purification columns, small leaks of eluent, etc.).

With respect to the injection precolumn loop, Sepralyte C_1 Methyl, Chromabond CN or C_1 phase was selected. The precolumn is easy to change, can be filled with cheap bonded phase, and is able to purify up to 50×100 μl urine samples without loss of clean-up or retention characteristics.

The addition of Triton X-100 R to the urine sample or aqueous standards is necessary to avoid a decrease in the fluorescence signal of 1-OHP in comparison with the corresponding methanolic solution. This decrease in signal intensity was not only related to the presence of the precolumn loop, as the same effect was observed when 1-OHP aqueous standard solutions were injected directly into the analytic column with a simple loop. The original

signal intensity is recovered as long as a surfactant is added to the aqueous 1-OHP solution. This apparent loss of 1-OHP was related to the water content of the injected solutions of 1-OHP, as well as to the use of the precolumn loop. These observations are probably due to the low water solubility of 1-OHP, to interactions (i.e., adsorptions on active sites) between 1-OHP and the inner walls of the vials or tubing, and to the bonded support. These interactions are suppressed by the addition of a surfactant to the aqueous 1-OHP solution. The formation of micelles probably promotes 1-OHP solubilisation, and masks the adsorption sites of the chromatographic support or inner walls of the tubes or vials. Other surfactants were tested [Tween 40, Brij 35, sodium dodecyl sulphate (SDS), etc.], with the best results being obtained using Triton X-100 R. During the preparation of standards, a methanolic solution of Triton X-100 R was added to the hydrolysed urines before successive dilutions were performed, in order to improve the dissolution of 1-OHP.

Methanolic standards with a volume greater than 10 μl could not be injected directly, due to the elution effect of the MeOH during the injection and the precleaning of the sample on the precolumn loop.

4. Conclusion

The great advantage of the proposed column-switching method is the reduction of sample handling by excluding the time-consuming solid–liquid extraction step. Moreover, the urine sample volume is low, less than 1 ml, which facilitates biological sampling and transport. The sample pretreatment time is short, not exceeding 2 min, and the analysis time is less than 20 min. The quantitation limit for 1-OHP is 20 ng/l for 100 μl of injected biological sample. Furthermore, the chromatograms are particularly clean at very low 1-OHP levels compared to those obtained using the off-line method. The possibility of reusing the silica support of the precolumn loop to concentrate the 1-OHP with a high degree of extraction repeatability is another interesting aspect of the method. The method will be adapted to look for traces of 3-hydroxybenzo[*a*]pyrene in urine.

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