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Trace determination of urinary 3-hydroxybenzo[*a*]pyrene by automated column-switching high-performance liquid chromatography

P. Simon*, M. Lafontaine, P. Delsaut, Y. Morele, T. Nicot

Institut National de Recherche et de Sécurité, Avenue de Bourgogne, 54501 Vandoeuvre, France

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Abstract

3-Hydroxybenzo[*a*]pyrene (3-OHB[*a*]P), one of the metabolites of benzo[*a*]pyrene (B[*a*]P), has been determined in human urine using an automated column-switching procedure. The hydrolysed biological sample is centrifuged just prior to being injected into a reusable precolumn loop, which is packed with a preparative phase and coupled on-line to a liquid chromatographic (LC) system. A rapid pre-treatment of the hydrolysed sample, consisting of a concentration and a crude clean-up, is performed on the precolumn. The analytes are then non-selectively desorbed with the LC eluent and the sample is cleaned again in three successive purification columns using the direct transfer or ''heart-cut'' technique. The pre-treatment does not exceed 3 min. and the entire analytical purification and separation procedure takes less than 30 min. Average 3-OHB[*a*]P recovery reaches 95% in the 1–50 ng/l range of urine, and the detection limit is 0.1 ng/l urine for a 3 ml injection of hydrolysed urine. The developed method was compared with a more time-consuming off-line method to analyse urines of B[*a*]P gavaged rats; the statistical treatment indicates that both methods are in agreement. The method was applied to purify and concentrate the urine samples of workers exposed and apparently unexposed to polycyclic aromatic hydrocarbons (PAHs).

Keywords: Column switching; 3-hydroxybenzo[*a*]pyrene

1. Introduction

Many PAHs are suspected or known carcinogens, and exposure to these compounds involves a potential health risk [1]. B[*a*]P is probably the most thoroughly studied and the most representative of this class of contaminants. Environmental human exposure is related to smoking, air pollution, and the consumption of broiled or smoked food products. Particularly high exposure to PAHs can be encountered in certain industrial areas including coke ovens, aluminium reduction plants, the steel industry, and creosote impregnation plants.

In addition to personal air sampling, biological monitoring is also used to assess pollutant exposure, and urinary 1-hydroxypyrene (1-OHP) is often used as an indicator of the total exposure to PAHs at workplaces [2–5]. However, when a poor correlation

*Corresponding author. Tel.: +33-83-50-2000; fax: +33-83-50-8787.

E-mail address: simonp@inrs.fr (P. Simon).

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is observed between atmospheric pyrene and B[*a*]P, urinary 1-OHP does not provide an accurate assessment of the potential risk [6], and 3-OHB[*a*]P (Fig. 1) seems to be more suitable as a biological indicator of exposure to carcinogenic PAHs. The experimental demonstration of a correlation between the urinary excretion of 1-OHP and other PAH metabolites such as 3-OHB[*a*]P would support this assumption. To verify if such a correlation is available, an extremely sensitive method is necessary to detect urinary 3-OHB[*a*]P, because its excreted fraction in urine is much smaller than that of other metabolites such as 1-OHP and hydroxy phenanthrenes [7]. As reported by Jongeneelen et al. [8] and Ariese et al. [9], the 3-OHB[*a*]P concentrations were 200 to 2500 lower than the 1-OHP concentrations in the urine samples of dermatological patients after treatment with a coal tar ointment (very high exposure to PAHs) and in the urine samples of coke-oven workers.

In order to examine if such correlation can be found for occupationally exposed workers or control subjects, the analytical method must be able to detect and quantify 3-OHB[*a*]P in urine at concentrations lower than 1 ng/l. Few analytical methods that are selective and sensitive enough to achieve this have been described in the literature [9–14]. To clean the samples, these methods employ the simple but tedious and time-consuming liquid–liquid extraction procedure [10,11], the more practical but still complex solid-phase chromatography [12,13], or an automated sample cleanup method using ''tailor made'' copper phthalocyanine modified porous glass precolumns [14–16]. To separate, detect and quantify, these methods use gas chromatography coupled with mass spectrometry [11,12] or HPLC associated with fluorimetric or amperometric detection [9,12–17] for trace determination. However, these methods require treatment times of up to 30 min, with the exception of the automated sample clean-up method using ''tailor made'' copper phthalocyanine precolumns (20 min).

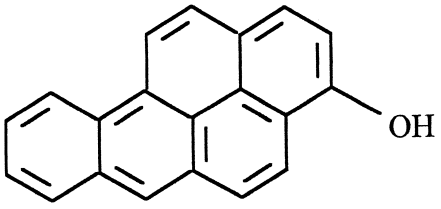

Fig. 1. Structural formula of 3-OHB[*a*]P.

To determine urinary 1-OHP, we have recently developed an easy automated column-switching method [18], which allows direct injection of the hydrolysed urine sample into the liquid chromatographic system after a rapid on-line sample treatment. This method has been modified and adapted to detect and quantify urinary 3-OHB[*a*]P at low ng/l concentrations, purification and analysis being still carried out in isocratic mode with fluorescence detection.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical reagent grade. Citric 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 3-OHB[*a*]P (purity 99.4%) were purchased from Sigma–Aldrich Chimie (St. Quentin Fallavier, France) and NCI Chemical Carcinogen Repository (MRI, Kansas City, MO) respectively. β-Glucuronidase-aryl sulphatase solution (from *Helix pomatia*) was from Merck, and Lyphochek® urine control was provided by Bio-Rad (Ivry/Seine, France).

The MeOH used to prepare the mobile phases must be freshly distilled to eliminate impurities that cause interferences and a serious disturbance of the baseline.

2.2. Apparatus

2.2.1. On-line method

The HPLC system consisted of two chromatographic pumps (Model LC-10 AT, Shimadzu, Kyoto, Japan), four automated switching valves (Model 7000, Rheodyne, Berkeley, CA, USA), one being equipped with a miniature precolumn loop, and a fluorescence detector (Model RF-10AXL, Shimadzu)

set to an excitation and emission wavelength of 425–460 nm respectively, and equipped with a Xe lamp. The entire system was controlled by a programmable microprocessor unit. 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 comprised a chromatographic pump (Model P 1500, Thermo Separation Products), a sample injector (Model 7410, Rheodyne) equipped with a 10 μl loop, and a fluorescence detector (Model F-1050, Hitachi, Tokyo, Japan) set to 425 and 460 nm for excitation and emission respectively. 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×0.32 cm I.D. stainless-steel guard column cartridge (Upchurch Scientific, Oak Harbor, WA, USA) filled with approximately 100 mg of 20–40 μm Sepralyte C1 (Analytichem International, Harbor city, USA). The purification columns, designated C1, C2 and C3 in the remainder of the paper were 5 cm×0.32 cm I.D., 5 cm×0.32 cm I.D., and 7.5 cm×0.46 cm I.D. stainless-steel tubes respectively, packed with: 5 μm Spherisorb C_8 (Phase Separations, Franklin, MA, USA) for C1, 5 μm Spherisorb OD/CN (Phase Separations) for C2, and 5 μm Nucleosil Phenyl (Macherey-Nagel, Düren, Germany) for C3. The analytical column, designated C4, was a 20 cm×0.32 cm I.D. stainless-steel tube packed with 5 μm Vydac 201 TPB (The Separation Group, Hesperia, CA, USA) in preference, otherwise with Lichrosorb RP Select B (Merck) or Kromasil C_{18} (The Separation Group).

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

2.3.2. Off-line method

The analytical column was a Supelcosil LC_{18} 5 μm, 25×0.46 cm I.D. (Supelco, France) thermostated at 30°C with a column thermostat (CTO-10ASVP, Shimadzu).

2.4. Urine collection and treatment

Rat urine samples and ''spot'' urine samples from human volunteers were collected in polyethylene bottles. After sampling, one part (about 100 ml) was refrigerated while the other was divided into three 10 ml samples and kept frozen at −20°C until the analysis. After the urine samples had been thawed, they were heated to 37–38°C to dissolve the maximum of sediment, then well shaken to homogenise the sample thoroughly. The required volume was then sampled as quickly as possible to avoid sedimentation of the deposit, and pre-treated following a previously described procedure [19]. Briefly, the urine (0.5–5 ml) was diluted 1:2 (v/v) with sodium acetate buffer (5×10^{-2} *M*, pH 5), vortex-mixed (0.5 min) and incubated for 1 h with 4 β-glucuronidase-arylsulphatase and Triton X-100 R (approximately 0.5 mg/ml of hydrolysed urine, i.e. 1 mg/ml of urine) at 37°C in an oven.

2.4.1. On-line method

After hydrolysis and before direct injection of the hydrolysed urine sample into the precolumn loop, it was briefly vortex-mixed (0.5 min), then centrifuged at 2000 *g* for 5 min. Depending on the sample, the injected volume ranged from 0.5 to 3 ml. Before the switchover of the injection valve, the precolumn was flushed with 0.5 ml of water, then with 0.5 ml NH_4OH 5×10^{-1} *M* (in water–MeOH, 9.5:0.5, v/v), and finally with 0.5 ml citric acid 10^{-2} *M* (in water–MeOH, 8:2, v/v) at about 2 ml/min to remove impurities. When the injected sample volumes are lower than 0.250 ml the quantity of added surfactant must be increased from 0.5 to 5 mg/ml of hydrolysed urine to avoid solute losses.

2.4.2. Off-line method

Prior to injection into the chromatographic system, a manual extraction procedure was used according to a modified method from Jongeneleen et al. [19]. 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 3-OHB[*a*]P was eluted with CH_2Cl_2 (5 ml). The latter was evaporated at 35°C under a gentle flow of nitrogen and the residue dissolved in 0.5–5 ml of a mixture of acetonitrile–methanol (8:2, v/v).

2.5. Preparation of standards

The commercial 3-OHB[*a*]P standard was dissolved in acetonitrile (50 μg/ml) to constitute the starting solution and stored at −20°C.

2.5.1. On-line method

A reconstituted Lyphochek® quantitative control urine was used, after hydrolysis, to obtain a stock urine solution destined to perform 3-OHB[*a*]P standards. Briefly, the reconstituted urine was diluted 1:2 (v/v) with sodium acetate buffer (10^{-1} *M*, pH 5), and β-glucuronidase-aryl sulphatase was added to the solution. After addition of an aqueous (or methanolic) solution of TRITON X 100 R (5 g/l) at a ratio of 1 to 10, the hydrolysed urine was vortex-mixed briefly (0.5 min) and refrigerated. Standards of 42, 4.2 and 0.84 ng/l were prepared by diluting the 3-OHB[*a*]P starting solution with this mixture. The resulting standard samples were directly injected (generally 1 ml) into the precolumn loop after centrifugation at 2000 *g* for 5 min. As for 1-OHP, after addition of the surfactant, the hydrolysed urine standard solutions of 3-OHB[*a*]P and the majority of hydrolysed urine samples from subjects are stable for at least 2 weeks at 4°C. Aqueous standards of 3-OHB[*a*]P are stable for 24 h at 4°C provided that a methanolic solution of 5 g/l of Triton X 100 R is added to the water at a ratio of 1 to 10 (v/v), but not more.

2.5.2. Off-line method

In contrast to the on-line method, the 3-OHB[*a*]P starting solution (50 μg/ml) was further diluted in acetonitrile–methanol (1:1, v/v) instead of hydrolysed urine, to obtain spiked 3-OHB[*a*]P solutions at 500, 100 and 50 ng/l and to the establish the calibration curve by HPLC. However for the recovery experiments, other calibration standards were prepared in a urine-buffer mixture as for the on-line method.

2.6. Analytical procedure

2.6.1. 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, C2 and C3) was a mixture of water and methanol (35:65, v/v) containing citric acid (5×10^{-3} *M*) and ascorbic

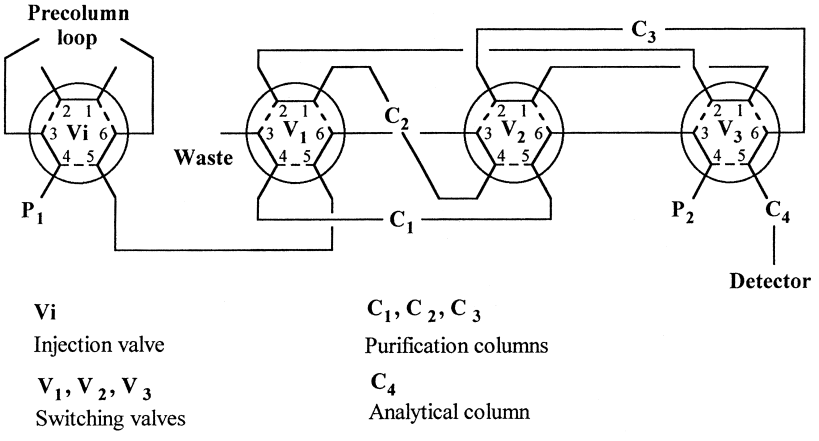

Fig. 2. Layout of the automated sample pre-treatment system to determine 3-OHB[*a*]P: Valves V1–V4 are all shown in position A (cf. steps 4 and 6, Table 1). The system is described in the Experimental section.

Table 1
Column switching analytical procedure using the setup shown in Fig. 1

Step event		Time (min)	Valve positions[a]			
			Valve i	Valve 1	Valve 2	Valve 3
1	Manual[b] precolumn loop flushing with sample and washing solutions (about 2 min)		B	B	A	B
2	Start of the automated analytical sequence. Precolumn loop flushing with E1 (1 min, 0.5 ml) pump P1, analyte transfer to C1 (injection)	0	A	B	A	B
3	Switch injection valve; manual precolumn wash. C1 purification	1	B	B	A	B
4	Analyte transfer from C1 to C2	4	B	B	B	B
5	C2 purification. C1 Clean-up in back-flush mode	8.5	B	A	A	B
6	Analyte transfer from C2 to C3.		B	B	B	B
7	C3 purification. C1 and C2 cleanup in back-flush mode	14	B	A	B	B
8	Analyte transfer from C3 to C4. Flushing C1 and C2 with eluent E2, pump P2	16	B	A	B	A
9	Analysis on C4. Flushing C1, C2 and C3 with eluent E1, pump P1 (reconditioning). C1 and C2 cleanup in back-flush mode	21	B	A	B	B

[a] Position A corresponds to the valve positions shown in Fig. 1.
[b] The precolumn loop flushing procedure could be automated with appropriate equipment.

acid (5 mg/l). The mobile phase (E2) used for the analytical column (C4) was a mixture of the same solvents in the ratio 15:85 (v/v), also containing citric acid (5×10^{-3} *M*) and ascorbic acid (5 mg/l). They were all eluted in isocratic mode at 0.5 ml/min. Prior to the analysis, the mobiles phases were degassed with helium for 5 min and kept under a reduced pressure (5×10^{4} Pa) helium atmosphere during the analysis.

Prior to each injection, the precolumn loop was manually preconditioned with 0.5 ml water, and the variable volumes of hydrolysed urine (0.5–3 ml) were then injected into the chromatographic system with a HPLC glass syringe. After the sample had been loaded, the precolumn loop was flushed with 0.5 ml of water, then with 0.5 ml NH_4OH 0.5 *M* (in water–MeOH, 9.5:0.5, v/v), and finally with 0.5 ml citric acid 10^{-2} *M* (in water–MeOH, 8:2, v/v) at about 2 ml/min to remove undesirable impurities (step 1). This step can be automated using appropriate equipment. The subsequent steps of the column-switching analytical procedure are summarised in Table 1. During the last step, 3-OHB[*a*]P was eluted on C4 with E2, while columns C1 and C2 were cleaned and reconditioned in back-flush mode with E1 in preparation for the next injection. After each analysis, the precolumn loop was cleaned with 0.5 ml of MeOH. In accordance with the method of

Bouchard et al. [19] and in contrast to the 1-OHP method, ascorbic acid was added to the eluent at a concentration of 5 mg/l to improve the reproducibility and the sensitivity of the 3-OHB[*a*]P fluorescence signal and to prevent gradual deterioration

2.6.2. Off-line method

The eluent, a mixture of acetonitrile and water (8:2, v/v), was pumped isocratically at a flow rate of 1 ml/min. After sample treatment, 10 μl of the extract was injected into the HPLC system. In the same way as in the on-line method, the mobile phase was degassed with helium for 5 min before analysis, then kept in a helium atmosphere during the analysis. In accordance with the method of Bouchard et al. [19], ascorbic acid was added to the eluent at a concentration of 5 mg/l. Every day, after about thirty injections, the analytical column was cleaned with acetonitrile.

2.6.3. 3-OHB[a]P analysis

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

2.7. Rat experimental protocol

Six-week-old male Sprague–Dawley rats were purchased from Charles River Breeding Labs. (Lyon, France) and housed individually in glass metabolic cages throughout the study period in a temperature and humidity controlled environment with a 12 h light, 12 h dark photoperiod. Food and water (free from B[*a*]P) were available ad libitum. Ten male rats were gavaged with a single dose of B[*a*]P, five with 2.8 μg (0.5 ml of a solution of 5.67 mg/l B[*a*]P in EtOH–H_2O; 1:1, v/v) and the other five with 28 μg (0.5 ml of a solution of 56.7 mg/l B[*a*]P in EtOH–H_2O; 1:1, v/v). The urines were then collected on ice 0, 8, 16, 24, 32, 40 and 48 h after gavage, and stored at −20°C until analysis. The urine volume ranged from 0.21 to 16 ml.

3. Results and discussion

Among the analytical methods described to detect low-level of PAH metabolites, an interesting method was developed by Boos et al. [14] and improved by Lintelmann et al. [15]. They employ a ''tailor-made'' copper phthalocyanine-modified porous glass precolumn to purify the urine sample and enrich the analyte. This precolumn is included in a fully automated column-switching LC system that uses a methanol gradient as elution mode for the clean-up procedure and the analysis. Gündel and Angerer [16] have recently applied this method to determine 3-OHB[*a*]P in urine, but the detection limit (6 ng/l urine) is not sufficient for the biological monitoring of persons environmentally exposed to low levels of PAHs. Moreover, the method has a number of drawbacks: an additional off-line liquid–solid extraction step is required and the ''tailor-made'' precolumn employed is both expensive.

An isocratic elution mode seems to us simple and practical, and it would be better to use a precolumn filled with an inexpensive support. Consequently, we used a ordinary preparative bonded phase as column packing, and automated switching multicolumns combined with the isocratic elution mode for the purification and analysis, a method that has previously been employed in our laboratory [18]. As a result, there is no analytical column reequilibration time involved and no apparatus solvent programmer is used.

The direct transfer or ''heart cut'' technique was again used in conjunction with chromatographic phase selectivity difference to clean-up the urine samples. The direct transfer or ''heart-cut'' technique consists in discarding the uninteresting parts of a sample initially eluted on a primary column, and in transferring the fraction of interest onto a secondary column with a minimum of overlapping interferences. However, as the 3-OHB[*a*]P urinary concentrations were 2500 times lower than the 1-OHP urinary concentrations, a detection limit improvement was necessary.

An improvement can be made by injecting a higher volume of urine sample, which require better sample clean-up and consequently an increase in both the number of ''heart-cuts'' and the number of the purification columns. Moreover, changing the bonded phase-type of each purification column to improve the sample cleanup would be also necessary. Thus, 0.5–3 ml of hydrolysed urine sample instead of 0.02–0.1 ml were injected, and three purification columns filled with a different packing

were used compared to the two for the 1-OHP method.

A study of the best chromatographic conditions was then carried out: best eluents, combinations of various bonded phases with various parameters, etc. Moreover, as previously described by Ariese et al. [10], the excitation and emission wavelengths selected, 425 and 460 nm respectively, were the best compromise, offering a minimum of background level and a sufficiently high fluorescence 3-OHB[*a*]P response allowing detection at low 1 ng/l concentrations.

The results of our investigations are shown in Figs. 3 and 6a,b,c,d: the chromatograms of the different urine samples, selected as being among the most representative, illustrate the selectivity of the developed method. Apart from 3-OHB[*a*]P, very few residual urine sample components appear in the chromatograms. Fig. 3 shows the human urine chromatograms obtained after cleanup with the switching system, using the analytical conditions required to quantify 3-OHB[*a*]P at the limit of quantitation. The method is potentially sensitive enough to detect 3-OHB[*a*]P in urine samples from persons occupationally exposed to PAHs (Figs. 3d and 6d) and even environmentally exposed to PAHs (Fig. 3b,c).

3.1. Validation of the method

In order to check the efficiency of the purification and concentration procedure of urine samples on the precolumn loop, it was compared with the direct injection (without treatment) of 3-OHB[*a*]P standards. Likewise, the matrix-dependent recovery was assessed. As for 1-OHP, no loss of 3-OHB[*a*]P occurs during the pretreatment provided that a surfactant is added to the aqueous or enzymatically hydrolysed urine sample. Therefore, after addition of Triton X100 R, the recoveries of 3-OHB[*a*]P from spiked urines at concentrations of 0.84, 4.2, and 42 ng/l rise to 96% independently of the concentration of 3-OHB[*a*]P and of the volume of hydrolysed urine sample loaded (range: 0.5–4 ml); conversely, in the absence of surfactant, apparent 3-OHB[*a*]P losses (20–40%) are observed, as for 1-OHP.

The calibration curve of 3-OHB[*a*]P response using peak-height measurement (mm) vs. analyte concentration (ng/l) was linear over the range investigated (0.42–42 ng/l, i.e. 0.42–42 pg for a 1 ml

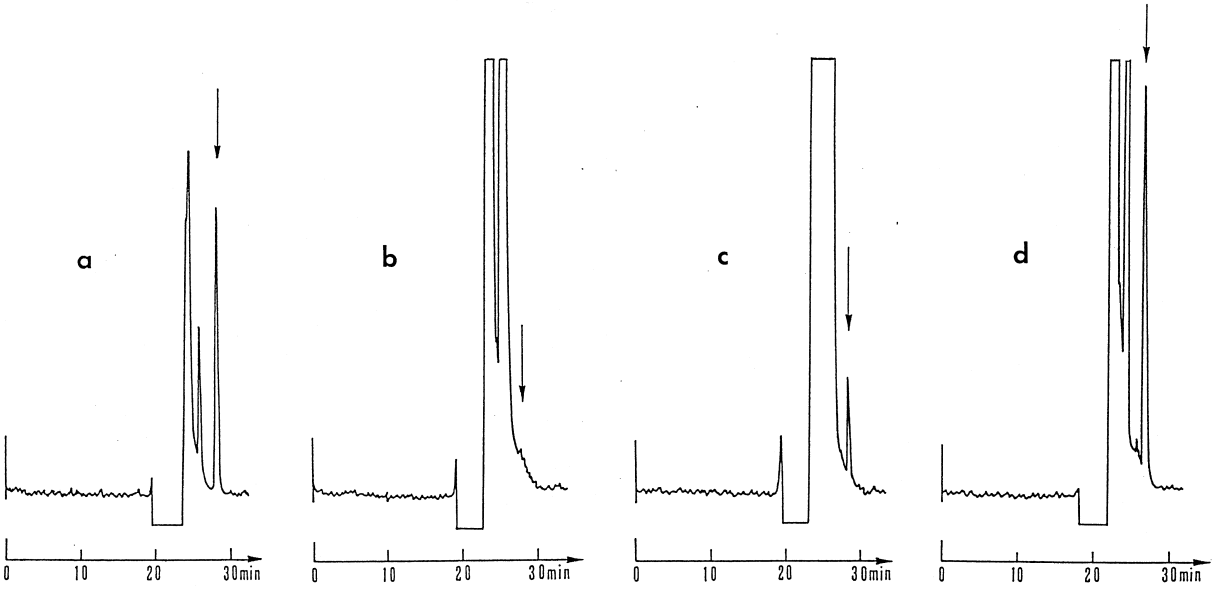

Fig. 3. Chromatograms of various urine samples obtained after cleanup with the switching system: (a) Reconstituted Lyphochek® urine standard 2.1 ng/l 3-OHB[*a*]P, 2 ml injected; (b) human urine sample 1 (unexposed, non smoker), <0.1 ng/l 3-OHB[*a*]P, 3 ml injected; (c) human urine sample 2 (unexposed, smoker), 0.8 ng/l 3-OHB[*a*]P, 3 ml injected; (d) human urine sample 3 (exposed worker), 6.2 ng/l 3-OHB[*a*]P, 1 ml injected. The system peak at time 19–24 min corresponds to the transfer and injection of analyte from C3 into the analytical column. The chromatographic conditions are detailed in Section 2.

injection, i.e. 0.5 ml urine), and the equation of the calibration curve was $y = -1.57 + 20.29\ x$, with a correlation coefficient greater than 0.999. The precision of the method was established on a sample of pooled urine (1.45 g/l creatinine) to which 3-OHB[*a*]P was added in concentrations of 0.84, 4.2 and 42 ng/l; for the 0.84 ng/l concentration, the coefficients of variation (C.V.) are less than 3% and 6% for within-day and between-day precision respectively, and for the 4.2 and 42 ng/l concentration, less than 2 and 5%.

The between-day variation was mainly related to the mobile phase degassing necessary to prevent quenching of the 3-OHB[*a*]P fluorescence signal by the oxygen dissolved in the mobile phase [21].

The purification columns and analytical column should be thermostated to avoid excessive retention time variations (C.V.>10%).

Since the quantitation limit varies with the injected hydrolysed urine sample or standard volume, this limit was estimated at 0.4 ng/l urine ($S/N=10$) for a 3 ml injection of hydrolysed urine sample (corresponding to 1.5 ml of undiluted urine). The detection limit was estimated at 0.1 ng/l urine ($S/N=3$) for a 3 ml injection of hydrolysed urine sample (i.e. 1.5 ml of undiluted urine).

3.2. Comparison of the methods

The reliability of the switching method was confirmed by comparing it with a manual off-line purification method. Urines samples ($n=33$) of rats gavaged with small doses of B[*a*]P were analysed by both methods. The results of urinary 3-OHB[*a*]P are plotted on a regression line (Fig. 4).

The equation of the regression line ($y = -0.585 + 0.950\ x$) and the product–moment correlation coefficient ($r = 0.993$) shows that both methods give similar results and that no marked systematic bias exists between them. The difference between the two mean readings is not statistically significant, and the result of the Student's *t*-test indicates that there is no significant difference between the two series of analytical results; $t = 1.55$ ($t = 1.69$, 95%, $n = 30$).

The agreement of the two methods is confirmed by Fig. 5 (Bland–Altman plot), which shows the relationship between the difference (A−B) and the mean (A+B)/2. The Bland–Altman plot shows that the

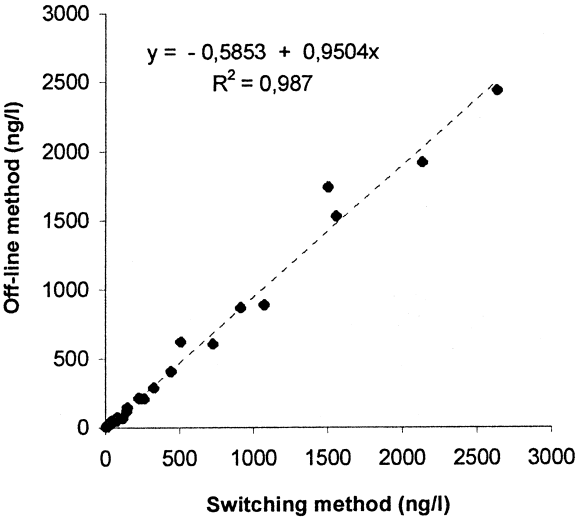

Fig. 4. Comparison of 3-OHB[*a*]P measurements of 33 rat urine samples by the off-line purification method and switching method.

urinary 3-OHB[*a*]P concentration (ng/l) values obtained with the off-line method (A) are slightly higher than those obtained with the on-line method (B), but the mean of differences is not ''significantly'' different from 0 (mean of A−B=21). The occasional lack of agreement between the methods could be attributed to random errors, as well as to the weaker selectivity of the off-line method, which leads to an overestimation of the metabolite values by integrating interferences.

Such a method comparison was performed, because the off-line method chromatograms were sufficiently clean to determine the 3-OHB[*a*]P at relative low levels. Relatively clean chromatograms were obtained by exposing the rats only to B[*a*]P and, as a result, the number of excreted metabolites able to interfere were significantly reduced. Moreover, the B[*a*]P doses chosen were low enough to check the on-line method performances and to determine the 3-OHB[*a*]P within the quantitation limit of the off-line method, the latter becoming imprecise, indeed impossible, below concentrations of 20 ng/l (in rat urine).

Fig. 6 shows the chromatograms of three rat urine samples and of a human urine sample treated respectively following both methods; clearly, the specificity and the selectivity of the on-line method are better.

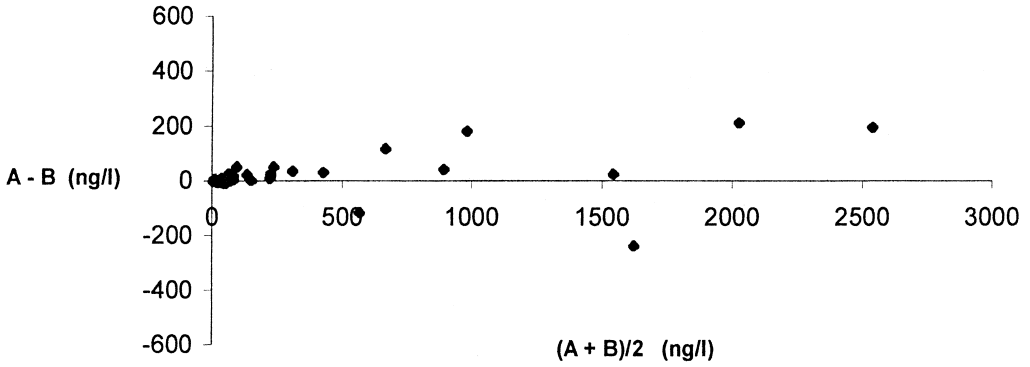

Fig. 5. Relationship between difference (switching method-off-line method) and mean (switching method+off-line method/2) assays of 3-OHB[*a*]P in 33 urines samples analysed by both methods.

With the off-line method, much greater background noise from residual components of the samples appears in addition to 3-OHB[*a*]P, which is often eluted in a tailing peak making quantitation impossible. This can be seen clearly in Fig. 6c′ and d′. The use of a gradient elution mode would probably improve the separation of 3-OHB[*a*]P from the interference but not the quantitation limit sufficiently. Analytical times of the off-line method seem to be shorter than those of the on-line method, but late residual peaks appear after 40 min elution time (not shown on the chromatograms). As regards the rat urine chromatogram 6c′ and the human urine chromatogram 6d′, it is obvious that 3-OHB[*a*]P was eluted in a tailing peak which is particularly large in the chromatogram 6d′. The main reason is that the human worker has been exposed to the entire PAH family, whereas the rats have been exposed only to B[*a*]P. Consequently, many corresponding PAH metabolites have been excreted in the human urine, which were not well separated and eluted in the tailing peak, whereas only B[*a*]P metabolites have been excreted in the rat urine, explaining the relative simplicity of the associated chromatogram. Obviously, the separation of the on-line method (cf. chromatograms 6c, 6d) is more efficient and sensitive. In addition, compared to the manual off-line method, it offers a number of advantages including a reduction in time-consuming handling operations and the associated errors.

To check the ability of the proposed method to determine 3-OHB[*a*]P in human urine at low concentrations, it was applied to a number of urine samples from supposedly non exposed people and workers exposed to PAHs. The results of the analyses (Table 2) and urine chromatograms of each volunteer category (Fig. 3) demonstrate that the developed method can be used in various circumstances. The high specificity of the method could be considered as a disadvantage because, to look for traces of an other PAH metabolite (i.e. 3-hydroxybenzo[*a*]anthracene), the switching times should be modified and consequently some parameters of the method. However, this high specificity due to the successive switches, leads to a high sensitivity.

3.3. Hydrolysis of 3-OHB[a]P glucuronide

The switching method is particularly well adapted to routine analyses, such as the study of the kinetics of conjugated PAH metabolites hydrolysis: it was also used to determine hydrolysis time of 3-OHB[*a*]P glucuronides during development of the method, because a large range of hydrolysis times (3 to 16 h) is mentioned in the literature [10,11,14,16,20]. To determine the hydrolysis end point, ten samples were analysed again after 0.5, 1, 2, 4, 8 and 16 h of incubation at 37°C. For all samples the 3-OHB[*a*]P glucuronides hydrolysis was complete after less than 1 h, and about 80% after a half-hour. As for the 1-OHP glucuronides hydrolysis, an aqueous solution of Triton X 100 R (5 g/l) was added to the diluted urine (urine-buffer mixture)

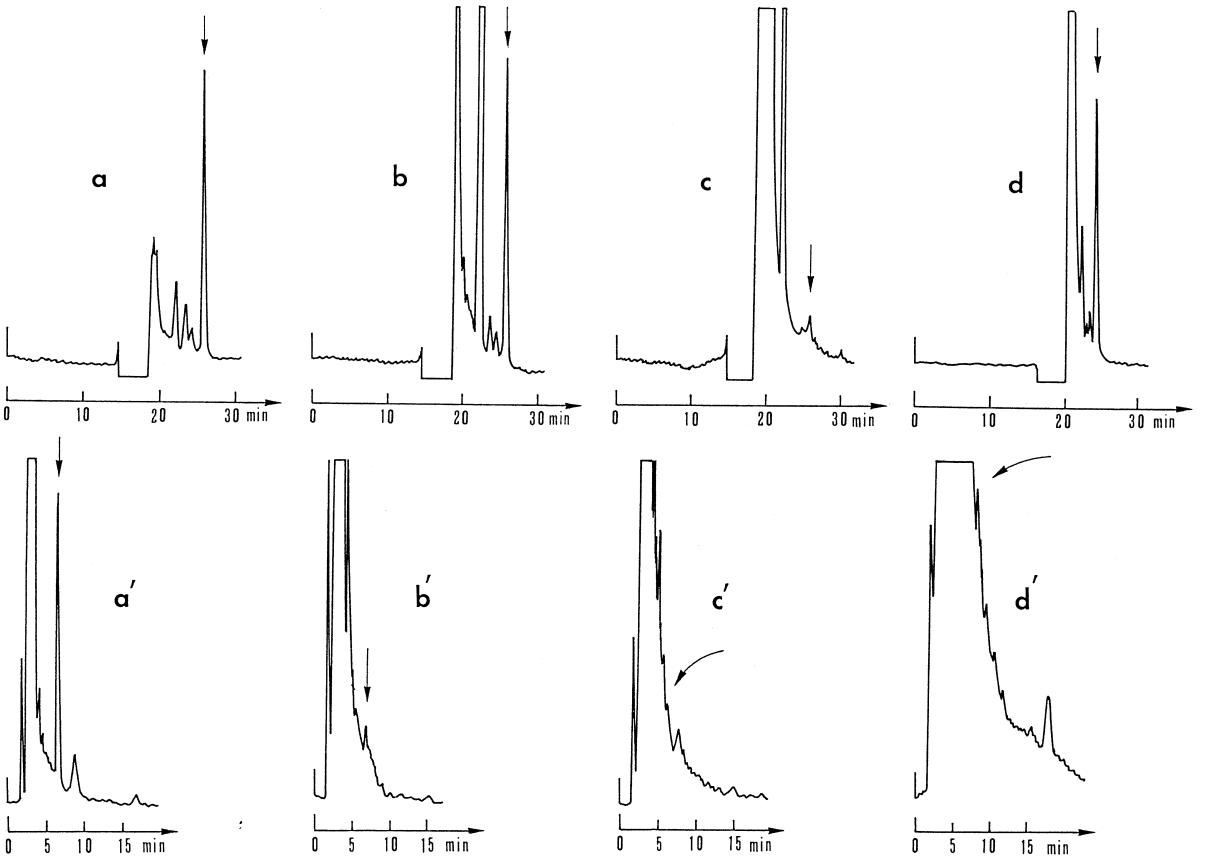

Fig. 6. Chromatograms of the urine samples obtained after purification with the described method (a,b,c,d) and with the off line purification method (a′,b′,c′,d′): (a) rat urine sample 1, 2300 ng/l 3-OHB[*a*]P, 20 μl injected; (a′) same urine sample concentrated tenfold, 2150 ng/l 3-OHB[*a*]P, 10 μl injected; (b) rat urine sample 2, 125 ng/l 3-OHB[*a*]P, 20 μl injected; (b′) same urine sample concentrated tenfold, 150 ng/l 3-OHB[*a*]P,10 μl injected; (c) rat urine sample 3, <1ng/l 3-OHB[*a*]P, 100 μl injected; (c′) same urine sample concentrated tenfold, 3-OHB[*a*]P not quantifiable, 10 μl injected; (d) human urine sample 4, 34 ng/l 3-OHB[*a*]P, 500 μl injected; (d′) same urine sample concentrated tenfold, 3-OHB[*a*]P not quantifiable, 10 μl injected. The system peak at time 15–21 min in chromatograms a,b,c,d corresponds to the transfer and injection of analyte from C3 into the analytical column. The chromatographic conditions are detailed in Section 2.

Table 2
3-OHB[*a*]P concentrations in urine samples from human volunteers[a]

Sample		ng/l	ng/g creatinine
ClS	NE–NS	<0.1	<0.15
MDN	NE–NS	<0.1	<0.05
JCP	NE–NS	0.2	0.05
JMG	NE–S	0.8	0.55
MCH	NE–S	0.1	0.15
MM2	NE–S	0.6	0.2
P16	E	1.7	2.4
P34	E	4.2	1
P311	E	9.5	5.2

[a] NE: non exposed; E: exposed; NS: non smoker; S: smoker.

samples in the ratio 1/10 before the addition of enzyme to prevent any possible loss of 3-OHB[*a*]P.

3.4. Technical remarks

Among the working wavelengths described in the literature [10,12,16,19,20], various possibility were tested. Like Ariese et al., the couple 425 nm for excitation and 460 nm for emission were employed; these long selected wavelenghts, offer a minimum background level due to the interfering compounds present in blank urine and a maximum sensitivity. In addition, preliminary tests have shown that a Xe

lamp rather than Xe-Hg lamp should be used in conjunction with the couple 425–460 nm to improve the detection limit (a better signal-to-noise ratio was obtained with the Xe lamp).

As for the 1-OHP analytical method, the pumps, valves, columns and connections were laid out in such a way as to minimise the number of devices. The column switching arrangement was studied in detail so that two of the three purification columns were cleaned and reconditioned in back-flush mode during elution of the 3-OHB[*a*]P on the analytical column, which contributed to reducing the analysis time.

The purification columns were chosen by determining the capacity factor of 3-OHB[*a*]P for a series of different packing materials. Satisfactory results were obtained with the combination of C_8, OD/CN and Phenyl (whether NO_2) bonded phases, which formed the best compromise, offering a minimum number of interfering components transferred in a minimum retention time. The dimensions of the purification columns were also optimised so that the strength of the mobile phase was lower than that of the analytical column in order to reconcentrate the 3-OHB[*a*]P at the top of the analytical column. Up to 1000 injections of 1 ml of hydrolysed urine sample (i.e. 0.5 ml of urine) were performed with the same purification and analytical columns without loss of resolution and damage to the purification characteristics.

Concerning the analytical column, various alkyl bonded phases (C_8 and C_{18}) were tested in the same elution conditions. Lichrospher RP Select B, Uptisphere ODS 1 and Spherisorb ODS 1 gave satisfactory selectivity, but the best results were obtained with Vydac 201 TPB phase.

As for the urine samples, the standard solution of 3-OHB[*a*]P is passed through the entire column assembly regularly (about every five samples) to test the reliability of the method. By so doing, the standard solution is subject to the same purification treatment as the urine samples. This procedure locates chromatographic malfunctions occurring upstream of the analytical column (i.e., changes of 3-OHB[*a*]P retention times on the purification columns, small eluent leaks, etc . . .).

As for the 1-OHP method, Chromabond C1 or CN phase was used as the chromatographic support in the injection precolumn loop, but the quantity of phase was increased from 50 to 150 mg. The precolumn is easy to change and to fill with inexpensive bonded phase, and about 30 injections of 2 ml of hydrolysed urine samples can be performed with the same packing without solute losses. Upwards about thirty times 2 ml of hydrolysed urine samples injections, a progressive decrease of 3-OHB[*a*]P intensity signal arises.

Finally, when studying the storage and hydrolysis capacities pattern of urine samples taken from exposed and non exposed volunteers, a few samples displayed an abnormal and unexplainable variation in 3-OHB[*a*]P after being thawed repeatedly. These abnormal results were related to the deposit quantity present in the urine samples after they had been thawed, and partly solved by heating (37–38°C) the urine samples to dissolve the maximum of deposit, and by well-shaking the flasks to homogenize the mixtures correctly prior to sampling the required volume. In our view, adsorptions of 3-OHB[*a*]P glucuronides on sediment particles are the main cause of these occasional, abnormal and variable results. These adsorbtions occur when the sample is being frozen. This assumption was confirmed by analysing the filtration residues from thawed urines. After filtration of the urine, the filtration residues were again suspended in acetate buffer and hydrolysed. The analyses have shown that up to 80% of 3-OHB[*a*]P glucuronides could be adsorbed on the sediment particles whereas the remainder of the 3-OHB[*a*]P glucuronides stayed in the filtrate. These observations show just how important urine homogeneity is during sampling. To obtain the most homogeneous sample possible before hydrolysis, the urine must be well mixed and sampled quickly after thawing the urine.

4. Conclusion

The proposed column-switching method offers both the sensitivity and selectivity required to detect 3-OHB[*a*]P, even at 0.1 ng/l urine for a 3 ml injection of hydrolysed urine sample. Furthermore, although the proposed switching system seems complicated, no gradient elution mode is used and sample handling is reduced considerably by exclud-

ing the trying off-line solid–liquid extraction step. Also, the pre-treatment of the hydrolysed sample does not exceed 3 min, and the analysis time is less than 30 min. The chromatograms are particularly clean at very low 3-OHB[*a*]P levels compared to those obtained using the off-line method. As for 1-OHP, the possibility of reusing the silica support of the mini-column loop to concentrate the 3-OHB[*a*]P with good extraction repeatability is another interesting aspect of the method. The proposed system, both practicable and reliable, is particularly attractive for routine analysis and potentially sensitive enough to determine occupational PAH exposure and even environmental PAH exposure.

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