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Note

Determination of hydroxylated metabolites of polycyclic aromatic hydrocarbons in urine

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Environmental pollution caused by polycyclic aromatic hydrocarbons (PAHs) is widespread [1]. There is sufficient evidence that eleven PAHs are carcinogenic to experimental animals [2]. Workers in industrial operations with high airborne PAH levels, such as gasworks [3,4], cokeworks [5–8] and the aluminium industry [9,10], show excess rates of lung cancers. Estimates of the health risks due to environmental or occupational exposure to these compounds strongly depend on the quality of analytical methods used to assess the intake or uptake of PAHs.

In this respect, an important role is played by biological monitoring, i.e. the systematic registration of the exposure level of individual workers and groups of workers, measured in biological specimens. PAHs are metabolized extensively and the enzymes involved are classified in two broad categories: phase 1 enzymes, which catalyse oxidative reactions, and phase 2 enzymes, which catalyse conjugative reactions of oxidized PAHs with endogenous compounds such as sulphuric acid, glucuronic acid and glutathione. PAH metabolites may be excreted either as free or as conjugated compounds.

The known carcinogenic PAHs are of a large molecular type [2]. For practical reasons we selected three large-molecular PAHs (pyrene, benz(*a*)anthracene and benzo(*a*)pyrene) for use as indicators. Measurement of the hydroxylated metabolites of these compounds in urine may be a useful starting point for the development of a method for biological monitoring of PAHs.

Adequate analysis of low exposure levels requires the use of sophisticated methods. In this note we present a high-performance liquid chromatographic (HPLC) method with enzymic hydrolysis, rapid sample clean-up and metabolite-specific fluorescence detection. This method allowed the determination of

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the sum of free and conjugated hydroxylated PAHs in urine at the nmol/l level. It was shown that people exposed to coal tar excrete 1-hydroxypyrene principally as a conjugate.

EXPERIMENTAL

Chemicals

Authentic benzo(*a*)pyrene and benz(*a*)anthracene metabolite reference standards were obtained from the NCI Chemical Carcinogen Reference Standard Repository (Chicago, IL, U.S.A.). 1-Hydroxypyrene was synthesized by Janssen (Beerse, Belgium), and its identity was confirmed by gas chromatography–mass spectrometry (GC–MS) and NMR spectroscopy. β-Glucuronidase/arylsulphatase (100 000 Fishman U/ml and 800 000 Roy U/ml) was obtained from Boehringer (Mannheim, F.R.G.). HPLC-grade methanol was from Fisons (Loughborough, U.K.). HPLC-grade water (conductivity > 18 Ω/cm) was generated from distilled water by a water purification system (Millipore, Milford, MA, U.S.A.). All other chemicals were of the highest purity obtainable.

Enzymic hydrolysis

An aliquot of urine (10–25 ml) was adjusted to pH 5.0 with 1.0 *M* hydrochloric acid and 0.1 *M* acetate buffer (pH 5.0) was added to a total volume of 30 ml. This mixture was incubated overnight (16 h) with 12.5 μl of β-glucuronidase/arylsulphatase at 37°C in an electronically controlled rotary shaking bath (210 rpm).

Extraction procedure

A sample enrichment and purification cartridge packed with C_{18} reversed-phase liquid chromatographic material (Sep-Pak C_{18} cartridge, Waters, Milford, MA, U.S.A.) was used for the extraction of the metabolites. The cartridge was primed with 5 ml of methanol, followed by 10 ml of distilled water, and the hydrolysed sample was passed through the cartridge at ca. 10 ml/min. Subsequently the cartridge was washed with 8 ml of distilled water. The retained solutes were eluted with 10 ml of methanol. The solvent was evaporated at 60°C under a gentle flow of nitrogen, and the residue was dissolved in 2.0 ml of methanol.

Calibration procedure

Samples of hydrolysed urine of non-exposed persons (blank urines) were spiked with the given analyte. These calibration samples were treated as described in the extraction procedure. At least five different concentrations across the working range were measured in duplo. Calibration curves were calculated by the least-squares method. Reagents blanks were used to monitor for interferences.

Reversed-phase HPLC analysis

The analyses were conducted on a HPLC gradient system controlled by a microprocessor (Kipp Analytica, Kipp and Zonen, Delft, The Netherlands). A fully automatic sample injector (Kipp Analytica) filled a 20-μl sample loop valve under slight pressure from sealed 2-ml vials and injected it subsequently on a

TABLE I

EXCITATION AND EMISSION WAVELENGTHS

Metabolite	λ_{ex} (nm)	λ_{em} (nm)
1-Hydroxypyrene	242	388
3-Hydroxybenz(*a*)anthracene	290	402
3-Hydroxybenzo(*a*)pyrene	265	430

150×4 mm I.D. LiChrosorb RP-18 (5 μm) column (Merck, Darmstadt, F.R.G.). The column temperature was 40°C and the flow-rate 0.8 ml/min. The solvent gradient was as follows: 5 min of methanol–water (46:54); a linear gradient in 35 min to methanol–water (94:6); hold for 10 min. The chromatograph was equipped with a fluorescence spectrophotometer (Perkin-Elmer, Type LS 4). The excitation and emission slits were both set to 5 nm. Throughout the chromatographic analysis, three pairs of programmed excitation and emission wavelengths were set by the microprocessor prior to the peak appearing. Because the pair of wavelengths can be changed during the run, it is possible to measure several HPLC-separated metabolites with optimum sensitivity and selectivity. The wavelengths used in this assay are listed in Table I. Peak heights were used for quantification.

RESULTS AND DISCUSSION

The optimum incubation conditions for the enzymic hydrolysis of glucuronide and sulphate conjugates of PAH metabolites were determined with urine samples of three female dermatological patients undergoing coal tar treatment. These patients excreted 1-hydroxypyrene after topical application of a 10% coal tar ointment [11]. Table II shows that the level of 1-hydroxypyrene increased seven- to nine-fold after enzymic hydrolysis. It was found that an incubation time of 2 h is enough to hydrolyse the 1-hydroxypyrene conjugates. Nevertheless, a much longer incubation time (16 h) was chosen for the determination of urinary PAH metabolites to ensure that the other conjugated metabolites were also hydrolysed. It appeared that 6.25 μl of enzyme solution gave equal results. Again, an excess of enzyme solution was used (12.5 μl) for the hydrolysis of all conjugated metabolites.

The HPLC analysis for the separation of PAH metabolites is essentially based on the reversed-phase separation that is generally applied in studies of the metabolism of PAH [12–16]. Reversed-phase analysis of hydroxylated PAH metabolites in urine is very satisfactory because of a high resolution and a low level of interfering compounds. In the present study it was found that metabolite-specific fluorescence detection maximized the sensitivity and minimized interferences, resulting in a non-laborious extraction procedure. Fig. 1 shows chromatograms of a sample from a non-exposed person (blank urine), of the blank urine spiked with three PAH metabolites and of a urine sample from a PAH-exposed worker at a creosote plant.

The urine of workers exposed to PAHs showed levels of 1-hydroxypyrene up to

TABLE II

ENZYMIC HYDROLYSIS OF 1-HYDROXYPYRENE CONJUGATES IN URINE FROM THREE PATIENTS EXPOSED TO COAL TAR

The experiments were performed with 2-ml aliquots of urine diluted with buffer to a volume of 30 ml in the presence of 12.5 μl of β-glucuronidase/arylsulphatase solution.

Incubation time (h)	1-Hydroxypyrene (μmol/l)		
	Patient 1	Patient 2	Patient 3
0	1.1	0.4	0.6
0.5	8.1	1.4	1.8
1.0	13.3	1.6	2.4
2.0	14.1	1.6	2.4
4.0	13.3	1.7	2.4
6.0	13.3	1.7	2.6
24.0	12.4	1.5	2.5

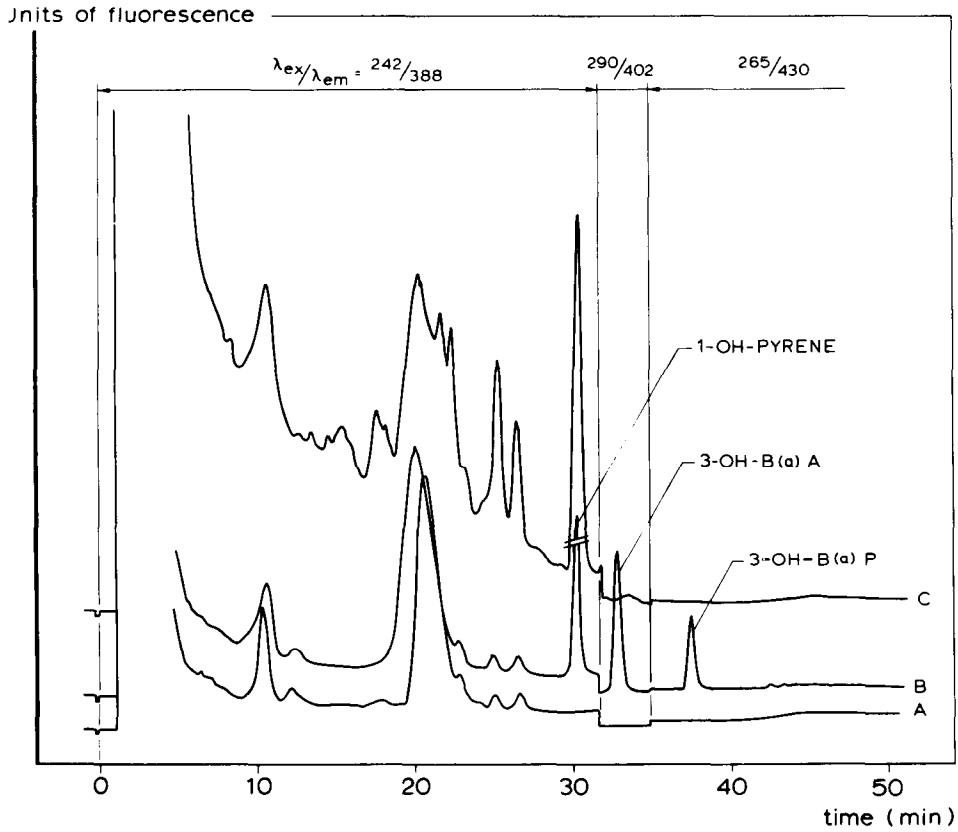

Fig. 1. HPLC profiles of extracts from (A) a blank urine, (B) a blank urine spiked to contain ca. 400 nmol/l of each of the three hydroxylated PAHs and (C) a urine sample from a worker exposed to creosote, a PAH-containing wood preservative. Peaks: 1-OH-pyrene = 1-hydroxypyrene; 3-OH-B(a)A = 3-hydroxybenz(*a*)anthracene; 3-OH-B(a)P = 3-hydroxybenzo(*a*)pyrene.

1500 nmol/l [11,17]. The recovery of the analyte was determined in spiked urine samples at three concentrations (40, 200 and 400 nmol/l). The recovery (mean ± S.D.) from four determinations was 88 ± 9.0%, 83 ± 4.4% and 84 ± 2.9%, respectively. The calibration curves for 1-hydroxypyrene were set up in hydrolysed urine across the working range (ca. 0, 10, 20, 40, 400 and 800 nmol/l in duplo). The standard curves were linear with a correlation coefficient of at least 0.99. The y-intercept of the curve was not significantly different from zero at the 95% confidence level. The detection limit of urinary 1-hydroxypyrene was 0.5 nmol/l (signal-to-noise ratio > 4), assayed with an autosampling and auto-injection system using 2-ml vials. Manual injection allowed the manipulation of more concentrated samples and decreased the detection limit by a factor 10.

The present method offers the possibility of measuring more metabolites in one run by setting the optimum excitation and emission wavelengths at the corresponding retention times. It is probable that PAH-exposed workers excrete other metabolites of PAH. Attempts were made to measure 3-hydroxybenz(a)anthracene and 3-hydroxybenzo(a)pyrene in workers' urine, but so far these metabolites have remained undetectable, just as when other extraction techniques were applied. The detection limits of 3-hydroxybenz(a)anthracene and 3-hydroxybenzo(a)pyrene were 5.0 and 4.0 nmol/l, respectively. Attention has now been focused on the identification of other hydroxylated metabolites.

A bulk urine sample was spiked with 180 nmol/l 1-hydroxypyrene. The urine was divided into aliquots of 20 ml and stored at −18°C. Over the course of 12 months, 24 aliquots were assayed. No loss of 1-hydroxypyrene was recorded during this period. The long-term stability of 1-hydroxypyrene in urine is therefore good. The average concentration of these 24 determinations was 167 nmol/l and the relative standard deviation (coefficient of variation) was 12.6%. The latter may be used as an estimation of the random error of 1-hydroxypyrene analyses.

The analytical potential for 1-hydroxypyrene appeared to work out well across the working range. Mathieu et al. [18] did not apply a hydrolysis step in the analytical method of trace amounts of urinary metabolites of benzo(a)pyrene. Keimig et al. [19] reported that initial treatment of urine with heat and strong acid to hydrolyse 1-hydroxypyrene conjugates did not increase the yield. The results of the present study, however, show that 1-hydroxypyrene is principally excreted as a conjugate. The enzymic hydrolysis appeared to be very effective and necessary in measuring trace amounts of PAH metabolites in urine at the nmol/l level. The urine samples can be stored for at least a year without loss of 1-hydroxypyrene.

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