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Determination of 1-hydroxypyrene in human urine by high-performance liquid chromatography with fluorescence detection using a deuterated internal standard

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Abstract

A high-performance liquid chromatographic method has been developed for the quantification of 1-hydroxypyrene (1-OHP) in human urine using deuterated 1-hydroxypyrene ([$^2H_9$]1-OHP) as an internal standard with fluorescence detection. [$^2H_9$]1-OHP was prepared enzymatically from deuterated pyrene ([$^2H_{10}$]Pyr) with cytochrome P450 1A1. It eluted immediately prior to non-deuterated 1-OHP on alkylamide-type reversed-phase columns and had nearly the same fluorescence characteristics as non-deuterated 1-OHP. The detection limit was 0.1 μg/L and the calibration range was from 1 to100 nmol/L. Urine sample treatment involved enzymatic hydrolysis followed by solid-phase extraction using Sep-Pak C_{18} cartridges. The proposed method was used to determine urinary 1-OHP in smokers and non-smokers.

Keywords: Hydroxypyrene; Polynuclear aromatic hydrocarbons

1. Introduction

Some polycyclic aromatic hydrocarbons (PAHs) are carcinogenic and/or mutagenic, and some, in particular benzo[*a*]pyrene, have recently been shown to exhibit antiestrogenic and/or antiandrogenic activity [1,2]. Humans are exposed to PAHs from various sources, including work, environmental, medicinal and dietary sources, cigarette smoking and others, through the lung, gastrointestinal tract and skin. The measurement of PAH metabolites in urine can be used to assess recent exposure to these compounds. Pyrene is a dominant compound in environmental PAH mixtures, accounting for 2–10% of PAHs [3], and it is readily excreted in urine. It is metabolized mainly to the 1-hydroxypyrene (1-OHP) intermediate, then 1-OHP is metabolized to the 1-OHP-glucuronide [4] and sulfate [5] conjugates. Elevated levels of 1-OHP have been found in smokers, in patients after coal tar treatment, in road pavers, coke oven and aluminum reduction workers at the end of their shifts and in subjects ingesting charbroiled meat [6]. Thus, 1-OHP is considered a reliable indicator of exposure to PAHs [7] and is

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often used as such an indicator in the workplace [3,4,10,11].

The HPLC method developed by Jongeneelen and co-workers [8,9] has been widely adapted for the analysis of 1-OHP in human urine. At present, an assay for urinary 1-OHP concentration has been established in Europe [10]. Moreover, determination of 1-OHP in urine is also a part of the current German quality assurance program [11]. Therefore, development of a HPLC method for the determination of 1-OHP is still important for routine monitoring of workers in laboratories that carry out toxicological analyses and may also be useful in occupational medicine. Moreover, periodic monitoring of 1-OHP has been shown to be very effective at controlling occupational PAH exposure in several industries [4].

In HPLC, the standard addition method is used widely for the quantification of urinary 1-OHP [8,9]. The main disadvantage of this method is that samples must be run at least three times. On the other hand, with [$^2H_9$]1-OHP (1-OHP-d_9) added to the pretreatment procedure as an internal standard, each sample has to be run only once, an approach more convenient for routine studies. This paper describes the use of 1-OHP-d_9 as an internal standard with fluorescence detection for the determination of 1-OHP in urine samples from smokers and non-smokers.

2. Experimental

2.1. Materials

Glucose 6-phosphate (G6P) and NADPH were obtained from Oriental Koubo (Tokyo, Japan), recombinant human cytochrome (P450) 1A1 expressed in baculovirus-infected insect cells and lymphoblastoid cell microsomes containing epoxide hydrolase (mEH) were purchased from Gentest (Woburn, MA, USA), HPLC-grade acetonitrile was obtained from Nacalai Tesque (Kyoto, Japan) and water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). G6P dehydrogenase (G6PDH) and β-glucuronidase/aryl sulfatase (type H-2: from *Helix pomatia*: β-glucuronidase activity, 100 000 units/mL and sulfatase activity, 5000 units/mL) were from Sigma (St. Louis, MO, USA). Other chemicals were from Wako (Osaka, Japan).

2.2. HPLC conditions

The HPLC system included a DGU-14A degasser, a LC-10AD pump, a CTO-2A column oven, a RF-10AXL fluorescence detector and a C-R7A plus integrator (all from Shimadzu, Kyoto, Japan). Two analytical columns were used for comparison: an RP-Amide column (Discovery RP-Amide C_{16}, 250×4.6 mm I.D., 5 μm Supelco, Bellefonte, PA, USA) and an ODS column (Cosmosil 5C_{18} MS, 250×4.6 mm I.D., 5 μm, Nacalai Tesque) with a guard column (Discovery RP-Amide C_{16}, 2 cm×4.0 mm I.D., 5 μm, Supelco). The mobile phases for the RP-Amide and ODS columns were acetonitrile–phosphate buffers (pH 7.0) (57:43 and 47:53, v/v, respectively) at a flow-rate of 1 mL/min. The column temperature was set at 40 °C. The excitation (λ_{ex}) and emission (λ_{em}) wavelengths were 240 and 387 nm, respectively.

2.3. Preparation and identification of the deuterated internal standard

The reaction mixture contained 100 m*M* potassium phosphate (pH 7.4), 5 m*M* $MgCl_2$, 5 m*M* G6P, 15 μL NADPH, 6 U/mL G6PDH, 40 μ*M* deuterated pyrene ([$^2H_9$]Pyr, Pyr-d_{10}), 5 pmol P450 1A1 and 250 μg/mL epoxide hydrolase in a volume of 300 μL, and was incubated at 37 °C for 2 h. An equal volume (300 μL) of acetone was then added to the reaction mixture and the resultant solution was extracted twice with 500 μL ethyl acetate. The addition of acetone and double extraction with ethyl acetate was repeated four more times. The ethyl acetate solution was evaporated to dryness, and the residue dissolved in methanol (500 μL) for HPLC injection. The eluted fractions containing 1-OHP-d_9 were pooled, evaporated to dryness, and then the residue was redissolved in 1 mL of methanol.

1-OHP-d_9 was identified by comparing its physical and chemical characteristics with those of authentic 1-OHP using LC–MS and fluorescence spectrophotometry. LC–MS was done with a Finnigan

MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) with an electrospray ionization (ESI) system. Spectrophotometry was performed with an F-2500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Excitation wavelength scanning was monitored at an emission wavelength of 387 nm, and emission wavelength scanning was carried out with 240 nm excitation.

2.4. Collection and pretreatment of urine samples

Urine was sampled from five smokers (age range 23–31, mean age 32.7) and nine non-smokers (age range 23–52, mean age 24.8) who lived in Kanazawa City, Japan. The urine specimens were collected in the morning and kept at −20 °C until analysis. Hydroxylated metabolites of PAHs in urine were determined using a slight modification of the method of Jongeneelen et al. [8]. A 10 mL aliquot of the sample was adjusted to pH 5.0 with 0.1 *M* HCl, then buffered with 20 mL of 0.1 *M* acetate buffer (pH 5.0). After the deuterated internal standard was added, the reaction mixture was incubated for 2 h with 15 μL of β-glucuronidase/aryl sulfatase (1655/63 units) at 37 °C. The reaction mixture was then loaded onto a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) that had been primed with 5 mL of methanol and 10 mL of water. The cartridge was sequentially washed with 10 mL of 40% methanol in water. The trapped metabolite was eluted with 10 mL of pure methanol and the eluate was evaporated to dryness. The residue was redissolved in 1 mL of methanol, and sonicated. An aliquot (10 μL) of the solution was injected into the HPLC system.

2.5. Analysis of urinary creatinine

The concentration of urinary creatinine was determined with alkaline picrate using a test kit (Wako) [12].

2.6. Statistical analysis

The means of urinary 1-OHP concentrations in smokers and non-smokers were compared with Student's *t*-test using Stat View 4.0 for Macintosh computers (Nankodo, Tokyo, Japan). A value of $P<0.05$ was considered to be significant.

3. Results and discussion

In this study, 1-OHP-d_9 was selected as an internal standard for the HPLC determination of 1-OHP because its physical and chemical characteristics are similar to those of 1-OHP. The compound collected from the preparative HPLC was identified as 1-OHP-d_9 by MS as follows. 1-OHP-d_9 was observed at m/z 226.4 $[M-H]^-$, while 1-OHP was observed at 217.3 (Fig. 1). On the other hand, the fluorescence spectra of 1-OHP and 1-OHP-d_9 had nearly the same fluorescence characteristics with respect to both the excitation (240 nm) and emission (387 nm) wavelengths (Fig. 2).

To separate the analyte and the internal standard, we selected an RP-Amide column, since this column retains phenols strongly by means of hydrophobic interactions and hydrogen bonding, and has been used successfully for the determination of hydroxylated PAHs such as diethylstilbestrol in calf urine [13]. The best resolution (R_s, 1.45), larger than the R_s on the ODS column (1.03), was obtained on a Discovery RP-Amide C_{16} column. The retention time of 1-OHP was the same on both columns and the retention time of 1-OHP-d_9 was the same on both columns.

A calibration curve was constructed using solutions containing 1-OHP standard and 1-OHP-d_9 as an internal standard. For the linear regression, $r^2 = 0.997$ and the detection limit of 1-OHP was 0.1 μg/L (signal-to-noise ratio >3). The quantification range was from 1 to100 nmol/L. This method was found to be more sensitive than previous methods (0.3 μg/L, signal-to-noise ratio >3) [14].

The urinary 1-OHP concentrations of smokers and non-smokers were determined to demonstrate the application of the proposed method. The chromatogram for a smoker is shown in Fig. 3. 1-OHP-d_9 eluted just before 1-OHP within 30 min. The peaks of both analytes were sharp and symmetrical, and were not obscured by other components of the urine. The recovery of 1-OHP-d_9 was almost 100%, in-

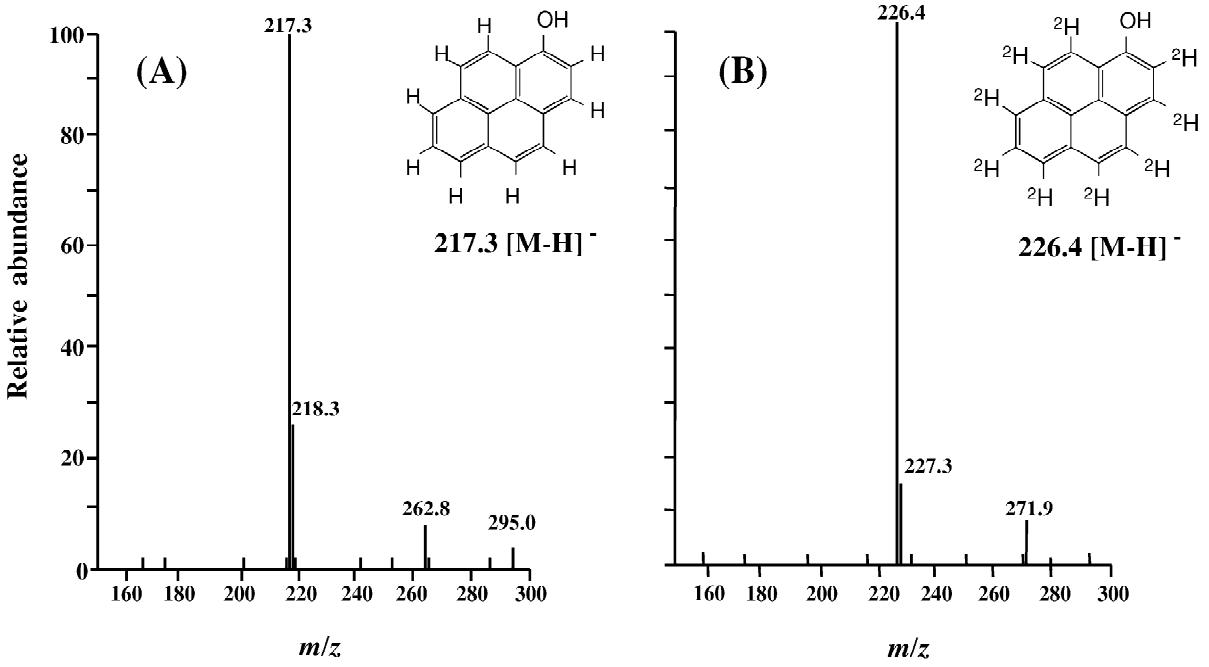

Fig. 1. Mass spectra and chemical structures of 1-OHP (A) and 1-OHP-d_9 (B).

dicating that it was a good choice as an internal standard. The results of the HPLC analyses are shown in Table 1.

Smokers excreted significantly higher concentrations of 1-OHP (62.2 nmol/mol creatinine) than non-smokers (24.1 nmol/mol creatinine). Our value

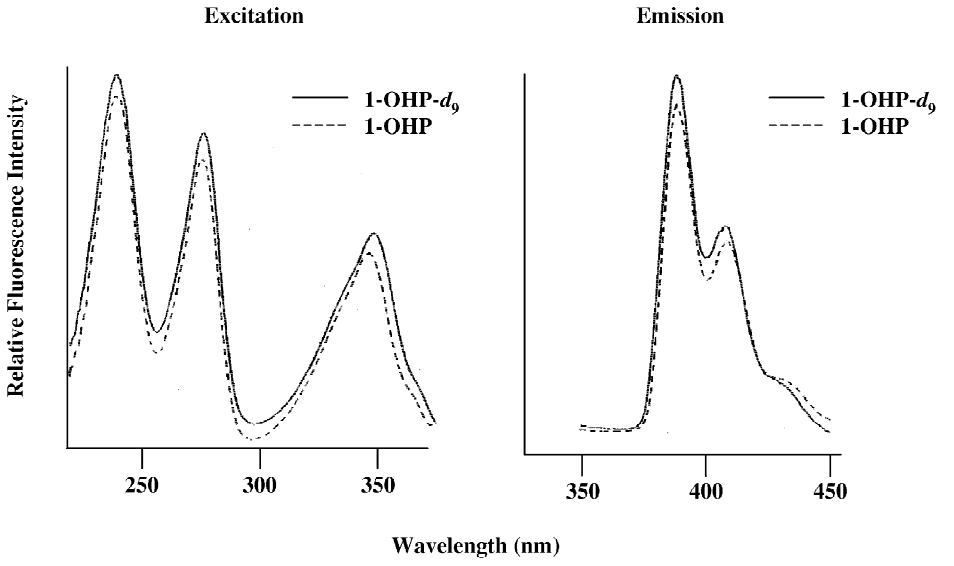

Fig. 2. Fluorescence characteristics of 1-OHP and 1-OHP-d_9. The excitation and emission spectra were obtained using an emission wavelength of 387 nm, and an excitation wavelength of 240 nm, respectively.

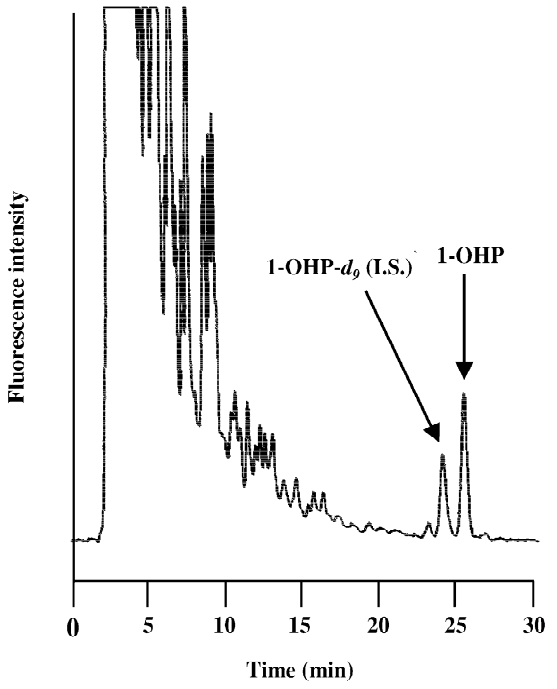

Fig. 3. HPLC chromatogram of a urine sample from a smoker. HPLC conditions: column, Discovery RP-Amide C_{16} (250×4.6 mm I.D., 5 μm, Supelco) at 40 °C; mobile phase, acetonitrile–10 m*M* phosphate buffer (pH 7.0) (57:43, v/v); flow-rate, 1.0 mL/min; fluorescence detection, λ_{ex} 240 nm, λ_{em} 387 nm.

obtained for Japanese subjects is close to that of Korean students [15] and is lower than the levels found in other countries, e.g. China, Netherlands and the USA [6]. Some studies found that 1-OHP levels in smokers who smoked less than 20 cigarettes a day were not significantly different from those of non-smokers [3,16]. It is likely that the difference in the baseline excretion of urinary 1-OHP for our subjects due to environmental PAH levels and/or dietary intake of PAHs is responsible for the significant difference between non-smokers and smokers who smoked 17–20 cigarettes a day.

This study confirms that 1-OHP-d_9 is a suitable internal standard for the determination of 1-OHP in urine and 1-OHP is a potential biomarker of exposure to PAHs from cigarette smoking.

Table 1
Urinary 1-OHP concentrations of smokers and non-smokers

	Smokers	Non-smokers
n	5	9
Mean age (years)	24.8	32.7
Mean±SD (nmol/mol creatine)	62.2±21.8*	22.8±28.8
Range (nmol/mol creatinine)	38.7–92.8	7.8–77.1

*Significantly different from the value of the non-smoker group ($P<0.01$).

4. Conclusions

A new, simple and sensitive HPLC method for determining the concentration of 1-OHP (a metabolite of pyrene) in human urine was developed using a deuterated internal standard with fluorescence detection. The method can be used to evaluate exposure to PAHs to assess the risk of carcinogenesis and/or endocrine disruption, and is suitable for routine analysis.

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