

Determination of 1-Hydroxypyrene as a Biomarker for the Effects of Polycyclic Aromatic Hydrocarbons in Urine by Chromatography–Mass Spectrometry

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Abstract—A simple and sensitive procedure has been developed for the determination of 1-hydroxypyrene in urine as a biological marker of the effects of polycyclic aromatic hydrocarbons. After enzymatic hydrolysis for conjugate cleavage, the analyte was extracted from the matrix by liquid–liquid extraction with *n*-hexane, the extract was evaporated to dryness, and the analyte was derivatized with the silylating reagent *N,O*-bis(trimethylsilyl)trifluoroacetamide into trimethylsilyl ether at room temperature. The trimethylsilyl extract was analyzed by capillary gas chromatography with mass-selective detection. Quantitative high-precision determination was achieved with the use of the isotope-labeled 1-hydroxypyrene-d9 reference standard. No matrix effects were observed. The detection limit was 0.02 ng/mL, and the linearity range was 0.1–100 ng/mL. Relative standard deviations were 0.044 and 0.064 under the conditions of repeatability and intralaboratory precision, respectively. The percentage recovery was 96–102%.

Keywords: 1-hydroxypyrene in urine, *N,O*-bis(trimethylsilyl)trifluoroacetamide, chromatography–mass spectrometry analysis, isotope-labeled standard

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Polycyclic aromatic hydrocarbons (PAHs), which have high carcinogenic and mutagenic activity, are harmful to human health and reproduction. Due to the high danger of PAHs, the most dangerous benzo(*a*)pyrene is monitored in environmental samples and food products in Russia and other countries. An alternative assessment of the effect of PAHs on human beings is based on the determination of their metabolites in biological media, but it is used only in developed countries.

The concentrations of unmetabolized PAH residues both in the blood circulatory system and in human urine are very low and difficult to measure using modern analytical methods. However, the concentration levels of some metabolites are sufficiently high. Urine is the most common and readily available biological fluid for the determination of PAH metabolites in the human body. In the majority of cases, PAHs are present in the form of mixtures, in which pyrene is a major component in terms of concentration. The pyrene metabolite 1-hydroxypyrene is a stable compound, which can be relatively easily and reliably determined in urine. Based on a large number of studies, it is possible with sufficient confidence to recognize 1-hydroxypyrene as an adequate biomarker for the presence of PAHs in the environment [1–3].

To determine 1-hydroxypyrene in urine, HPLC with fluorimetric detection was used [4]. Sample preparation included preliminary enzymatic hydrolysis with β -glucuronidase at 37°C for 16 h, solid phase extraction (SPE) on a C₁₈ cartridge, evaporation of the eluate to dryness, and dissolution of the residue in methanol. The detection limit was 0.1 ng/mL, the limit of determination was 2 ng/mL at a sample volume of 10 mL, the relative standard deviation RSD (reproducibility) was 12.6%, the correlation coefficient of a calibration graph was 0.990, and the percentage recovery was 88 ± 9%.

Gas chromatography with mass-selective detection (GC–MS) in electron ionization (EI) mode is another method suitable for the determination of 1-hydroxypyrene in urine [5, 6]. The main advantage of GC–MS analysis over HPLC is the better separation of components and the possibility of using an isotope-labeled d9-1-hydroxypyrene standard (deuterated standard). Both 1-hydroxypyrene and its deuterated analogue should be derivatized for GC–MS analysis because the presence of an OH group causes low volatility, low sensitivity, and analyte interaction with the stationary phase of the column. Acylation and silylation are the most common derivatization reactions for substances containing hydroxyl groups [7, 8]. In acylation reactions, compounds containing mobile

Table 1. Data for the identification of 1-hydroxypyrene and its isotope-labeled standard in mass chromatograms

Compound	Retention time, min	<i>m/z</i> (main)	<i>m/z</i> (confirmatory)
1-Hydroxypyrene-d9 (internal standard)	17.14	299 (100%)	284 (20 ± 5%)
1-Hydroxypyrene	17.16	290 (100%)	275 (20 ± 5%)

hydrogen atoms are converted into esters by the action of a carboxylic acid or its anhydrides. Due to the presence of residual acid, the products cannot be directly injected into a gas chromatograph, and a step of purification is required. In silylation reactions, mobile hydrogen atoms are replaced by a trimethylsilyl or *tert*-butyldimethylsilyl group [9]. As a rule, the products of this reaction are more volatile and thermally stable. Unlike acylation, silylation usually does not require a step of purification, and the derivatives can be directly injected into a GC instrument. Silylation is the most commonly used derivatization method [9], and trimethylchlorosilane (TMCS), (trimethylsilyl)imidazole, *N*-methyl(trimethylsilyl)trifluoroacetamide, *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide are classic reagents for this method; the two last-named compounds are most often used [10].

The GC–MS determination involved the following steps [5, 6, 10]: enzymatic hydrolysis with β -glucuronidase at 37°C for 17–18 h, SPE, evaporation of the extract in a flow of nitrogen, derivatization of the dry residue with the silylating reagent BSTFA at 60°C for 40 min, and GC–MS analysis. The duration of separation was 40 min. The detection limit was 0.5 ng/mL, the determination limit was 1 ng/mL with a sample volume of 3 mL, RSD was 6.7–13.1%, the correlation coefficient of a calibration graph was 0.995, and the percentage recovery was 88.7%.

The aim of this work was to develop a GC–MS procedure for the determination of 1-hydroxypyrene in urine using an isotope-labeled standard. For this purpose, the following problems were solved: an increase in the determination sensitivity by increasing the degree of extraction from a biological matrix and a significant decrease in sample preparation time due to a decrease in the duration of enzymatic hydrolysis and 1-hydroxypyrene derivatization with the silylating reagent BSTFA.

EXPERIMENTAL

Equipment. An Agilent 7890A gas chromatograph with an Agilent 5975C mass-selective detector (EI), an Agilent 7693 autosampler, and an HP-5MS capillary column (30 m × 0.25 mm, 0.25 μ m); a Stuart block thermostat (30–130°C); a Biosan vibratory mixer; an Eppendorf 5804 centrifuge; and a nitrogen-flow evaporation system were used.

Reagents and standards. 1-Hydroxypyrene (99.8%, Aldrich); 1-hydroxypyrene-d9 (98.8%, Santa

Cruz); acetonitrile (Kriokhrom); type H-2 β -glucuronidase from *Helix Pomatia*, aqueous solution (>85000 units/mL, Aldrich); an acetate buffer solution with pH 5; chemically pure magnesium sulfate; *n*-hexane (Kriokhrom); and BSTFA containing 1% TMCS (Fluka) were used in this study.

Sample preparation. A 20- μ L portion of a solution of 1-hydroxypyrene-d9 in acetonitrile (5 μ g/mL), 1 mL of an acetate buffer solution with pH 5, and 20 μ L of an aqueous solution of β -glucuronidase were added to 2 mL of urine in a 15-mL centrifuge tube, and the mixture was heated at 55°C for 1 h. Then, the solution was cooled for 20 min to room temperature, and 0.5 g of magnesium sulfate and 2 mL of *n*-hexane were added; the contents were vigorously shaken on a vibratory mixer for 2 min, and the phases were separated by centrifugation at 4000 rpm for 10 min. The hexane extract was separated and transferred to a 5-mL vial with a conical bottom; 2 mL of *n*-hexane was added again; the process was repeated, and the extracts were combined. The extract was evaporated to dryness in a small flow of nitrogen at a water-bath temperature of 60°C; then, 100 μ L of BSTFA was added to the dry residue, and the mixture was allowed to stand for 5 min. Thereafter, it was transferred into a glass insert in a 200- μ L microvial and analyzed.

GC–MS analysis. The determination was carried out under the following conditions: injector temperature, 300°C; injected sample volume, 1 μ L; splitless injection mode, 0.7 min; linear velocity in the column, 40 cm/s; column oven temperature programming (an exposure at 60°C for 2 min, heating to 300°C at a rate of 15 K/min, and an exposure for 2 min); interface temperature, 290°C; EI ion source temperature, 230°C; quadrupole temperature, 150°C; and filament turn-on delay time, 16.6 min. GC–MS data acquisition was carried out in the selected ion monitoring mode with *m/z* 290, 275, 299, and 284.

1-Hydroxypyrenes and 1-hydroxypyrenes-d9 as derivatives (trimethylsilanes) were identified in the chromatograms based on retention times and detected ion intensity ratios (Table 1).

Quantitative determination by an internal standard method was performed with the use of 1-hydroxypyrene solutions in urine with concentrations from 0.1 to 100 ng/mL.

RESULTS AND DISCUSSION

Silylation with *N,O*-bis(trimethylsilyl)trifluoroacetamide. The silylation of 1-hydroxypyrene with BSTFA

Table 2. Dependence of the degree of derivatization of 1-hydroxypyrene with *N,O*-bis(trimethylsilyl)trifluoroacetamide on the reaction time and temperature

Retention time, min	S_{abs}^*		S_{rel}^{**}	
	20–25°C	90°C	20–25°C	90°C
5	3150	3492	0.38	0.34
30	3039	3065	0.39	0.36
45	3419	3174	0.37	0.33
s_r	0.061	0.068	0.026	0.044

* S_{abs} is the absolute value of the peak area of a trimethylsilyl ether of 1-hydroxypyrene.

** S_{rel} is a ratio between the peak areas of trimethylsilyl ethers of 1-hydroxypyrene and 1-hydroxypyrene-d9.

at room temperature (20–25°C) and 90°C and at various reaction times was compared (Table 2). It can be seen that the temperature and silylation reaction time did not significantly affect the analytical signal, especially, if this was a relative peak area (height). It was sufficient to perform silylation at room temperature for 5 min with an isotope-labeled standard; in this case, the value of RSD was minimal.

Extraction of 1-hydroxypyrene from a sample of urine. It was found that the degree of extraction of 1-hydroxypyrene from urine was 8 or about 90% with the use of SPE or liquid–liquid extraction (LLE) with *n*-hexane, respectively (Table 3).

Although SPE is a more advanced extraction method than LLE, the degree of analyte recovery by SPE is very low. This fact can be mainly explained by the presence of a large amount of polar compounds containing OH–, COOH–, NH=, and –NH₂ groups in the urine sample, and their concentrations are higher than that of the analyte. These components interfere with the silylation of 1-hydroxypyrene by BSTFA; therefore, a very small amount of 1-hydroxypyrene enters the silylation reaction. In the case of LLE with hexane, nonpolar or low-polarity compounds, which do not interfere with derivatization, are extracted. Double LLE with hexane is an optimal method for extracting 1-hydroxypyrene from a biological matrix.

Selection of conditions for the liquid–liquid extraction of 1-hydroxypyrene using mathematical planning. The main factors affecting the efficiency of LLE are the type of an extractant, the duration of extraction, the number of extractions, and the nature

and amount of a salting-out agent. It was found that *n*-hexane is a more effective extractant than diethyl ether and toluene due to the reduction of interfering effects on derivatization. It is better to use magnesium sulfate rather than sodium sulfate as a salting-out agent because it provides a higher degree of analyte recovery. The following quantitative parameters of the LLE of 1-hydroxypyrene were optimized using mathematical planning [11]: the weight of magnesium sulfate, the duration of extraction, and the number of extraction steps (Table 4).

In a multifactorial experiment, three factors were varied simultaneously. A planning matrix consisted of eight experiments. The optimization parameter y was taken as the degree of analyte extraction. Each experiment in the planning matrix (Table 5) was repeated three times.

The following equation was obtained statistically:

$$y = 84 + 8x_3 + 2.07x_1x_2 - 2x_1x_2x_3. \quad (1)$$

Equation (1) indicates that the number of extraction steps (x_3) makes a larger contribution to the formation of a response (degree of extraction) than that of the weight of magnesium sulfate and the extraction time (shaking in a vibratory mixer). The degree of extraction of the analyte is higher upon double extraction due to an increase in the analyte concentration in an organic phase. Substituting the coded values of the factors into the equation, we calculated the theoretical values of an optimization parameter, which showed that the degree of extraction remained unchanged (Table 6). Therefore, it is unreasonable to increase the weight of magnesium sulfate x_1 and the extraction time x_2 . It is advisable to fix the weight of magnesium sulfate at a zero level and shorten the extraction time to 2 min. Thus, the optimal conditions for the LLE of 1-hydroxypyrene from urine are the following: magnesium sulfate weight, 0.5 g; extraction time, 1–2 min; and double extraction.

Enzymatic hydrolysis. Table 7 summarizes the results of the analysis of real urine samples from aluminum workers obtained using two versions of hydrolysis with β -glucuronidase. It can be seen that the concentrations of 1-hydroxypyrene obtained by the two methods do not differ much. In the second version, the analyte concentration was higher; therefore, it is better to carry out enzymatic hydrolysis with β -glucuronidase at 55°C for 60 min.

Procedure validation. The detection limit, determination limit, linearity range, repeatability, intralabo-

Table 3. Extraction of 1-hydroxypyrene from aqueous and biological matrices using different techniques

Extraction technique	Matrix	Conditions	R , %
SPE	Urine	C_{18} sorbent; eluant, methanol	8
	Water		88
LLE	Urine	Double extraction with hexane	90

Table 4. Conditions for the design of a three-factor experiment

Factor	Zero level, x_0	Range of variation in J	Lower level of the factor	Upper level of the factor
x_1 — weight of $MgSO_4$, g	0.5	0.5	0	1
x_2 — extraction time, min	3	2	1	5
x_3 — number of extraction steps (quality factor)	—	—	1	2

Table 5. Planning matrix for a three-factor experiment and optimization parameters found

Experiment no.	Factors						Optimization parameter y_j ($n = 3$)
	natural			coded			
	x_1	x_2	x_3	x_1	x_2	x_3	
1	0	1	1	—	—	—	80.3
2	1	1	1	+	—	—	73.2
3	0	5	1	—	+	—	71.6
4	1	5	1	+	+	—	80.0
5	0	1	2	—	—	+	93.0
6	1	1	2	+	—	+	93.4
7	0	5	2	—	+	+	89.8
8	1	5	2	+	+	+	90.2

ratory precision, accuracy, matrix effect, and selectivity were evaluated [12].

The detection limit calculated for a signal-to-noise ratio of ≥ 3 was 0.02 ng/mL, and the determination limit (a signal-to-noise ratio of ≥ 10) was 0.1 ng/mL. The linearity range was established for six model urine samples with different concentrations of 1-hydroxy-

pyrene (0.1, 2, 10, 20, 40, and 100 ng/mL); the correlation coefficient was $r > 0.999$. An acceptable value of r was no lower than 0.990. Thus, the linearity range was from 0.1 to 100 ng/mL. The correctness (precision and accuracy) was evaluated using four urine samples with analyte additives of 0.5, 2, 20, and 40 ng/mL, and each sample was analyzed twice for five days. Accord-

Table 6. Experimental conditions and theoretical values of the optimization parameter

Experiment no.	Natural factors			Coded factors			y
	x_1	x_2	x_3	x_1	x_2	x_3	
1	0.5	1	2	0	−1	1	92.0
2	0.5	2	2	0	−0.5	1	92.0
3	0.5	3	2	0	0	1	92.0
4	0.5	4	2	0	0.5	1	92.0
5	0.5	5	2	0	1	1	92.0
6	0.6	3	2	0.20	0	1	92.0
7	0.8	3	2	0.60	0	1	92.0
8	1	3	2	1.00	0	1	92.0

Table 7. Concentrations (ng/mL) of 1-hydroxypyrene in the urine of aluminum workers upon enzymatic hydrolysis with β -glucuronidase under different conditions in production with variants

Urine sample no.	Conditions of enzymatic hydrolysis with β -glucuronidase	
	37°C, 16 h	55°C, 1 h
1	0.5	0.6
2	1.2	1.5
3	2.7	2.9
4	2.4	2.5
5	34.5	39
6	0.83	0.96
7	0.31	0.35
8	22.3	25

ing to the results of the analysis, the values of s_r (repeatability and intralaboratory precision) and the percentage recovery as a ratio between the found and added concentrations were calculated (Table 8). All of the ratios in a range from 96.5 to 102% based on a nominal analyte concentration correspond to an acceptable value ($100 \pm 7\%$). The repeatability and intralaboratory precision satisfy acceptable criteria (no higher than 12%). The concentrations added fall in the confidence intervals of found values.

Table 8. Precision and accuracy of the determination of 1-hydroxypyrene in urine ($P = 0.95$)

Added, ng/mL	Found, ng/mL	Intralaboratory precision RSD ($n = 5$)	Repeatability RSD ($n = 2$)	Recovery, %
0.5	0.51 ± 0.04	6.3	4.4	102
2	1.9 ± 0.1	6.4	1.0	97
20	19 ± 1	6.4	1.7	96.5
40	40 ± 3	6.0	3.9	99.3

Table 9. Matrix effect in the determination of 1-hydroxypyrene

Sample no.	1-Hydroxypyrene amount, ng	$S_{rel, s}$	$S_{rel, u}$	$b_{ME}, \%$
1	0.4	0.00417	0.00423	1.6
2	4	0.0409	0.0415	1.5
3	20	0.219	0.210	3.9
4	40	0.435	0.446	2.66

The matrix effect was evaluated as the relative bias b_{ME} by comparing the relative signals of the analyte introduced into an organic solvent ($S_{rel, s}$) and into a sample of urine ($S_{rel, u}$) (Table 9). The calculation was carried out according to the following formula:

$$b_{ME} = |S_{rel, u} - S_{rel, s}| \times 100 / S_{rel, s} \quad (2)$$

The maximum matrix effect was 3.9%.

Figure 1 shows the mass chromatograms of a blank sample and three samples with different concentrations of 1-hydroxypyrene (0.1, 0.5, and 39 ng/mL) (Fig. 1). The peak of trimethylsilyl-1-hydroxypyrene is narrow (peak half-width, 1.5 s) and symmetrical, and there are no interfering peaks. Therefore, a high selectivity of determination was achieved. The value of RSD for the measured retention times is 2.7%.

The procedure developed for the quantitative determination of 1-hydroxypyrene in urine was tested with the urine samples of aluminum workers (31 persons). The analyte content of urine varied from 0.1 to 263 ng/mL. Urine samples of workers (14 persons) from other industries without PAHs in the workplace air were also analyzed; the concentrations of 1-hydroxypyrene were in a range from 0.08 to 0.9 ng/mL.

* * *

Thus, the use of this procedure allowed us to shorten the duration of analysis due to a decrease in the time of enzymatic hydrolysis and derivatization of 1-hydroxypyrene, as compared with well-known procedures. The determination accuracy was high due to the use of the isotope-labeled standard 1-hydroxypyrene-d9, and the sensitivity of determination was

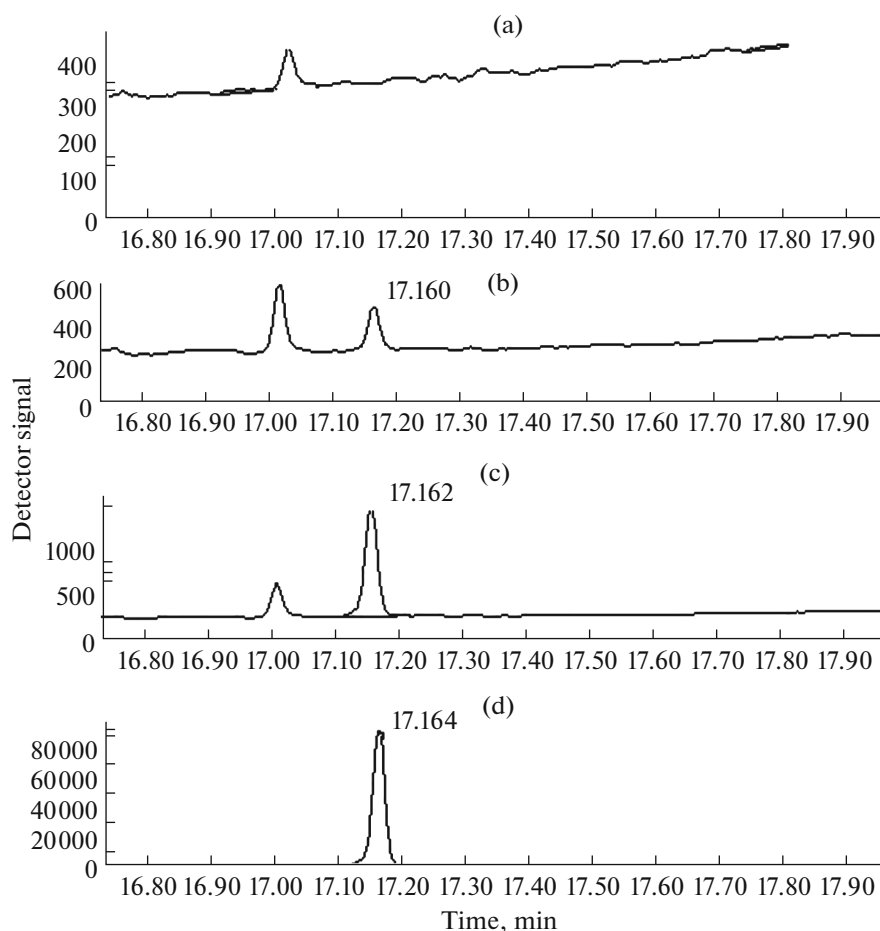


Fig. 1. Mass chromatograms (at m/z 290) of (a) a blank sample; urine samples with 1-hydroxypyrene concentrations of (b) 0.1 and (c) 0.5 ng/mL; and (d) a urine sample of a worker exposed to PAHs (1-hydroxypyrene concentration, 39 ng/mL).

increased by increasing the degree of extraction from the biological matrix.

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