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Rapid and simultaneous determination of hair polyamines as N-heptafluorobutyryl derivatives by gas chromatography–mass spectrometry

Ling Li[a], Kenji Hara[b], Junting Liu[a,*], Yanping Yu[a], Lina Gao[a], Yan Wang[a], Yanfang Wang[a]

[a] College of Pharmacy, China Medical University, Shenyang 110001, PR China
[b] Department of Forensic Medicine, Fukuoka University School of Medicine, Fukuoka 814-0180, Japan

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ABSTRACT

We have developed a simple and sensitive method for the simultaneous determination of putrescine, spermidine and spermine in hair as their N-heptafluorobutyryl derivatives by gas chromatography–mass spectrometry (GC–MS) in selected ion-monitoring (SIM) mode. After base hydrolysis, hair samples were extracted with solid-phase extraction (SPE) with diatomaceous earth columns, followed by derivatization with heptafluorobutyryl chloride (HFB-Cl) and elution with n-hexane simultaneously. This method was linear ($r \geq 0.9989$), reproducible (intra-day R.S.D. = 3.4–15.5%, inter-day R.S.D. = 2.6–14.6%), accurate (recoveries = 67.8–94.6%) and sensitive (LOD = 0.05–1.0 ng). The method was successfully applied to the analysis of 36 hair samples from 14 healthy men and 22 healthy women. Results showed that the levels of hair polyamines were 4.39–12.15 μg/g for putrescine, 3.89–27.91 μg/g for spermidine, and 0.81–15.15 μg/g for spermine. Either in the male or female group, the most abundant hair polyamine was spermidine, followed by putrescine and spermine, while the mean levels of the three polyamines in hair samples were all found to be higher in men than in women.

1. Introduction

Polyamines are found widely in living organisms, which are long-chain aliphatic amines, mainly including putrescine, spermidine and spermine. They have long been closely associated with cell growth, tissue growth and cancer. For example, significantly altered polyamines are related with increased cell proliferation, decreased apoptosis and increased expression of oncogenes [1,2]. It has been reported in a large number of literature that polyamine levels were remarkably increased in the body fluids, tissue and hair of patients with cancer [3–9]. In recent years, Choi et al. [7] observed higher mean levels of polyamines in hair of cervical and ovarian cancer patients compared to healthy subjects. The results of Choi et al. [8] indicated increased hair polyamine levels in patients with Alzheimer's disease. Byun et al. [10] reported markedly altered levels of polyamines in serum of patients with breast cancer. Slavov et al. [11] showed significant differences in urinary polyamines in carcinoma of the bladder and adenocarcinoma of the prostate as compared to healthy subjects. It is thus evident that simultaneously determination of polyamines plays a very important role in early diagnosis and prevention of cancer in patients.

Various techniques have been applied for monitoring polyamine concentrations in human body, including chromatographic [12–18], electrophoretic [19,20], radioimmunoassay [21,22] and enzymatic assays [23–25]. Among these analytical methods, gas chromatography–mass spectrometry (GC–MS) is the most sensitive technique due to the enormous abilities of high-resolution capillary column GC together with selective detection systems [26]. When GC–MS is employed for the polyamine analysis, it is necessary to block active hydrogen atoms in amino groups by derivatization prior to analysis. To date, the determination of hair polyamines was only reported in the literature [7,8,15] by ethoxycarbonyl (EOC) reaction of amino functions combined with subsequent pentafluoropropionyl (PFP) derivatization which was very laborious and time consuming. As a different method, solid-phase extraction (SPE) using diatomaceous earth columns followed by derivatization with HFB-Cl and elution with volatile n-hexane simultaneously proved to be more available [27], which shortened the whole analysis time and simplified the procedure of samples preparation. Till now, the method has not been reported on the analysis of polyamines in hair.

Here we described a new method for the determination of hair polyamines by GC–MS. Taking into account the advantages of hair as the biosample for determination polyamines, such as reflecting long-term chronic biochemical information, convenient collection and stability of endogenous components [15], the method developed in this study was applied to measure polyamines of hair samples from 36 volunteers including 14 healthy men and 22

* Corresponding author. Tel.: +86 24 23256666x5423.
E-mail address: Liujunting8@163.com (J. Liu).

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healthy women by GC–MS with the selected ion-monitoring (SIM) mode.

2. Experimental

2.1. Chemicals and reagents

Hydrochlorides of putrescine, spermidine, spermine, 1,6-diaminohexane (used as an internal standard, I.S.) and diatomaceous earth were obtained from Wako Pure Chemical Ind., Ltd. (Tokyo, Japan). Diatomaceous earth was dried for 3 h at 150 °C prior to use. Heptafluorobutyryl chloride (HFB-Cl) as the derivatization reagent was purchased from Kanto Kasei Chemical Co., Inc. (Tokyo, Japan).

All other chemicals and solvents used in this study were of the purest grade available.

2.2. Hair samples

Tip parts cut from scalp hair strands were collected from 36 volunteers including 14 healthy men (age 21–56) and 22 healthy women (age 18–53). The work has been approved by The Medical Ethics Committee of China Medical University. Hair samples were washed with 0.1% sodium dodecylsulfate (SDS) under ultrasonication, followed by rinsing three times with deionized water and acetone sequentially. After they were dried under a nitrogen stream at room temperature, aliquots of 50 mg hair were precisely weighed into a glass centrifuge tube. Prior to analysis, the hair samples were kept at room temperature.

2.3. Preparation of standard solutions

Stock solutions for standard curves were prepared for each polyamine at 100 ng/μl in 0.1 M HCl. The stock solutions were used to prepare a working solution of varying concentrations (5–1000 ng/ml) in 0.1 M HCl by mixing appropriate aliquots of each stock solution and diluting with 0.1 M HCl. The internal standard (I.S.) working solution was prepared by diluting the stock solution of 1,6-diaminohexane at 10 ng/μl in 0.1 M HCl. All standard solutions prepared were stored at 4 °C.

2.4. Instrumental analysis and data acquisition

GC–MS analyses both in scan and SIM modes were performed with a Shimadzu GC–MS QP2010 (70 eV, electron-impact mode). Chromatographic separation was achieved on a Rtx-5 fused-silica capillary column (10 m × 0.18 mm I.D., 0.2 μm film thickness) using helium (99.999%) as carrier gas at 1.01 ml/min in a constant flow rate mode. A deactivated glass inlet liner (cat. 550733, Restek Co.) was used. The GC–MS was programmed to perform a 1 μl splitless injection at 250 °C and 76.5 kPa during 1 min. The oven temperature was initially at 60 °C for 1 min, programmed at 15 °C/min to 270 °C with a hold at 270 °C for 8 min. The temperatures of interface and ion source were 270 and 200 °C, respectively.

In the scanning mode, the mass range was 40–900 u at a rate of 0.5 s/scan. In the SIM mode, three characteristic ions for each polyamine were used for peak-identification, while one ion underlined which was the most abundant ion was selected for quantification (Table 1). Each polyamine in hair samples was identified by matching the retention time and the relative abundance of three confirming ions with respect to those of polyamine standards. The start time for SIM was programmed from 3.0 to 14.0 min to set up three groups of 5 ions (for putrescine and 1,6-diaminopropane), 3 ions (for spermidine) and 3 ions (for spermine) to be monitored. A dwell time of 0.2 s was chosen for the three groups.

Table 1
SIM parameters for GC–MS analysis of polyamines as N-HFB derivatives.

Polyamine	Retention time (min)	Time window (min)	Ions selected	MW
Putrescine	6.78	3.0–9.0	226 267 480	480
1,6-Diaminohexane	7.98		226 282 508	508
Spermidine	10.38	9.0–11.5	226 536 564	733
Spermine	12.94	11.5–14.0	226 254 576	986

2.5. Solid-phase extraction and N-heptafluorobutyrylation

A mixed standard solution (1 ml) containing three polyamines at varied concentrations together with I.S. (10 ng/μl × 5 μl) was poured onto the solid-phase extraction column filled with 1.7 g of desiccated diatomaceous earth after adjusting to pH 12–13 with 5 M NaOH solution for the reaction of the basic amino functions. After 10 min for equilibration, the analytes were eluted with a mixture of 3 ml n-hexane, 50 μl butyl acetate and 20 μl HFB-Cl. The eluent was collected in a glass tube and evaporated down to ca. 30 μl under a stream of nitrogen followed by GC–MS analysis.

2.6. Method validation

To demonstrate linearity, the calibration samples at six different concentrations (5–1000 ng/ml) together with fixed amount of I.S. (10 ng/μl × 5 μl) were subjected to SPE with a subsequent HFB reaction in triplicate as described above. For the LOD determination, standard solutions at varying concentrations (0.01–1.0 ng/ml) prepared by diluting the working solutions were evaluated and the analyte concentrations of which the signal-to-noise ratios greater than 3 were chosen for each. Likewise, samples for intra- and inter-day assays were prepared individually in triplicate at low (50 ng/ml), medium (200 ng/ml) and high (1000 ng/ml) concentrations using 1 ml of 0.1 M HCl solution and were analyzed in a day except for inter-day assays determined at the same concentrations for 5 days. To determine recoveries, samples at three different concentrations (50, 200 and 1000 ng/ml) were performed and analyzed individually in triplicate as described in Section 2.5. Recoveries were evaluated by comparing the recovered polyamine concentrations with the nominal concentrations. Stability was examined by analyzing a sample at 200 ng/ml. The last derivatives were allowed to stand at 4 °C and analyzed at approximately 0, 2, 4, 6, 8, 12 and 24 h.

2.7. Preparation of hair samples

Each of hair sample (50 mg) was extracted with 1 ml 1 M NaOH solution together with fixed I.S. (10 ng/μl × 5 μl), at 95 °C for 1.5 h in a water bath. After cooling, hair samples were centrifuged at 3000 rpm for 10 min after adjusting to pH < 1 with concentrated hydrochloric acid. The aqueous layer was transferred into a new glass tube and then adjusted to pH 12–13 with 5 M NaOH solution, followed by SPE with a subsequent HFB reaction as described above.

3. Results and discussion

3.1. Mass spectrometry analysis

The derivatization in SPE columns with diatomaceous earth replaced only one active proton in every amino group by a HFB group, which improved the volatility and GC properties of polyamines. Mass spectral patterns of four polyamines as their N-HFB derivatives are displayed in Fig. 1. The molecular ion peaks were very weak in putrescine and 1,6-diaminohexane, cor-

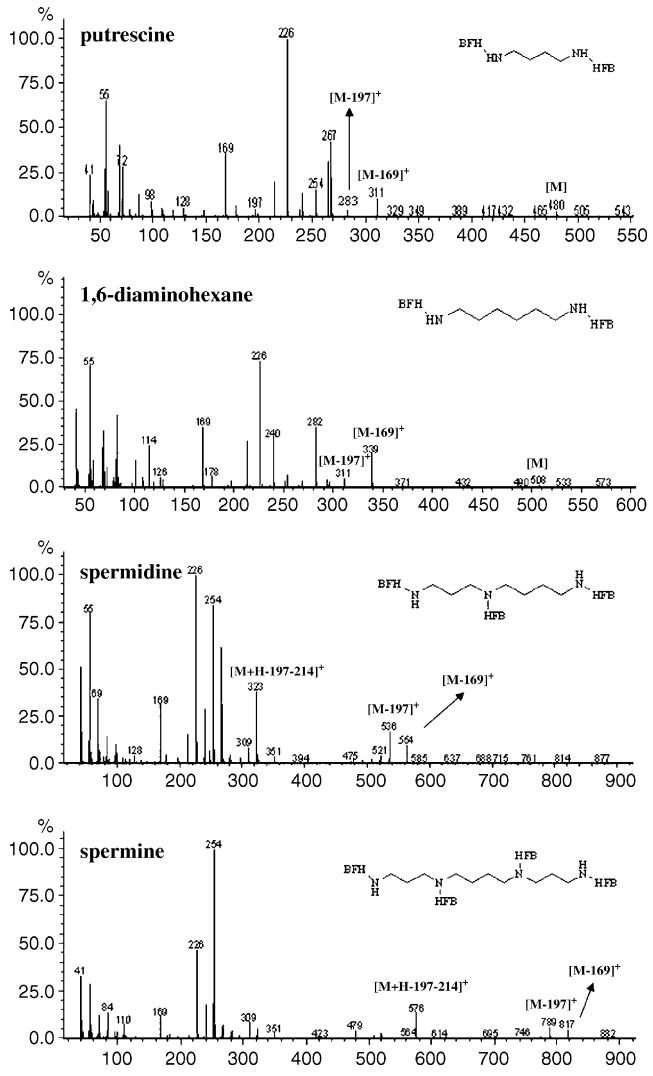

Fig. 1. Electron-impact mass spectra of four polyamines as their N-HFB derivatives obtained in the scannine mode at a rate of 0.5 s/scan with a mass range of *m*/*z* 40–900.

responding to the ions at *m*/*z* 480 and *m*/*z* 508, respectively, while unobservable in spermidine and spermine due to the lability of amides. The base peak ions at *m*/*z* 226 for putrescine, 1,6-diaminohexane and spermidine, but minor for spermine corresponds to $[C_3F_7CONHCH_2]^+$, and *m*/*z* 254 for spermine corresponds to $[C_3F_7CONHC_3H_6]^+$. The ions at *m*/*z* 169, *m*/*z* 197, *m*/*z* 214, *m*/*z* 240 and *m*/*z* 267 observed in all polyamines correspond to $[C_3F_7]^+$, $[C_3F_7CO]^+$, $[C_3F_7C(OH)=NH_2]^+$, $[C_3F_7CONHC_2H_4]^+$, and $[C_3F_7CONHC_4H_7]^+$, respectively. The ion at *m*/*z* 282 was present in 1,6-diaminohexane only, corresponding to $[C_3F_7CONHC_5H_{10}]^+$. The $[M-169]^+$ ions were prominent in all polyamines except for spermine, but $[M-197]^+$ ions were intense in spermidine only. The $[M+H-197-214]^+$ ions and the ions at *m*/*z* 309 corresponding to $[C_3F_7CON(C_3H_6)C_4H_8]^+$ were present in spermidine and spermine only.

3.2. Method validation results

Results of the method validation are summarized in Table 2, including calibration results of three polyamines measured at six different concentrations (5–1000 ng/ml) which were linear (r = 0.9989–0.9998). The overall linearity proved to be acceptable for quantification of the three polyamines in hair samples. No interfering peaks with all analytes were found. The detection limits (LOD) were 0.05 ng for putrescine and spermidine, and 1.0 ng for spermine. Results of intra- and inter-assay precision were all satisfactory at three different concentrations, less than 10% for putrescine and spermidine, and less than 20% for spermine, while recoveries ranged from 67.8% to 94.6%, which were acceptable and feasible for the purpose of this study. Stability of the derivatized polyamines was demonstrated by analysis of the relative standard deviation of measured polyamine concentrations for 24 h. The stability was found to be excellent, with 3.8% for putrescine, 1.1% for spermidine and 5.0% for spermine.

3.3. Quantification of polyamines in human hair

The proposed method was applied to analyze polyamines of hair samples collected from 36 volunteers including 14 healthy men and 22 healthy women. The Fig. 2 showed that separation of four polyamines in the typical SIM chromatogram was achieved with sharp and symmetrical peak shapes, higher responses and less background interferences compared to the total ion chromatogram. The three polyamines were positively detected in aliquots of 50 mg of all hair samples. Results showed that the levels of putrescine detected in hair were between 4.39 and 12.15 μg/g, of spermidine 3.89–27.91 μg/g, and of spermine 0.81–15.15 μg/g (Table 3). Large variations in their levels were demonstrated within each polyamine except for putrescine, mainly due to biological variability. In the male group, the most abundant hair polyamine was spermidine, followed by putrescine and spermine as well as in the female group, while the mean levels of the three polyamines in hair were all found to be higher in men than in women. The normal mean levels of hair

Table 2
Validation data for the analysis of polyamines as their N-HFB derivatives.

Polyamine	Intra-day R.S.D.[a] (%)			Inter-day R.S.D.[a] (%)		
	50 ng/ml	200 ng/ml	1000 ng/ml	50 ng/ml	200 ng/ml	1000 ng/ml
Putrescine	3.4	5.3	3.5	6.0	2.6	4.3
Spermidine	5.2	8.4	4.5	6.1	8.0	6.8
Spermine	11.6	13.2	10.4	15.5	14.6	8.6

	Recovery (%)			Calibration range (ng/ml)	Linearity (r)	LOD[b] (ng)
	50 ng/ml	200 ng/ml	1000 ng/ml			
Putrescine	91.5	83.3	89.5	5–1000	0.9995	0.05
Spermidine	91.0	94.6	94.1	5–1000	0.9998	0.05
Spermine	67.8	69.6	69.9	5–1000	0.9989	1.0

[a] R.S.D.: relative standard deviation.
[b] LOD: limit of detection.

Table 3
Polyamine levels (μg/g) in hair samples from 14 men and 22 women[a].

Polyamine	Male hair (n = 14) mean ± S.D. (median, range)	Female hair (n = 22) mean ± S.D. (median, range)	Total hair (n = 36) mean ± S.D. (median, range)
Putrescine	7.73 ± 1.71 (7.62, 5.55–12.15)	7.13 ± 1.37 (7.28, 4.39–9.78)	7.36 ± 1.52 (7.54, 4.39–12.15)
Spermidine	12.53 ± 7.58 (10.84, 3.89–27.91)	7.48 ± 2.94 (6.68, 4.00–15.67)	9.44 ± 5.72 (7.42, 3.89–27.91)
Spermine	4.90 ± 4.41 (2.95, 0.96–15.15)	1.61 ± 0.82 (1.35, 0.81–4.03)	2.89 ± 3.21 (1.62, 0.81–15.15)

[a] Alkaline extracts of hair (50 mg) collected from 14 healthy men (aged 21–56) and 22 healthy women (aged 18–53) was subjected to SPE with subsequent HFB derivatization for the GC–SIM–MS analysis. GC–SIM–MS conditions are described in Table 1.

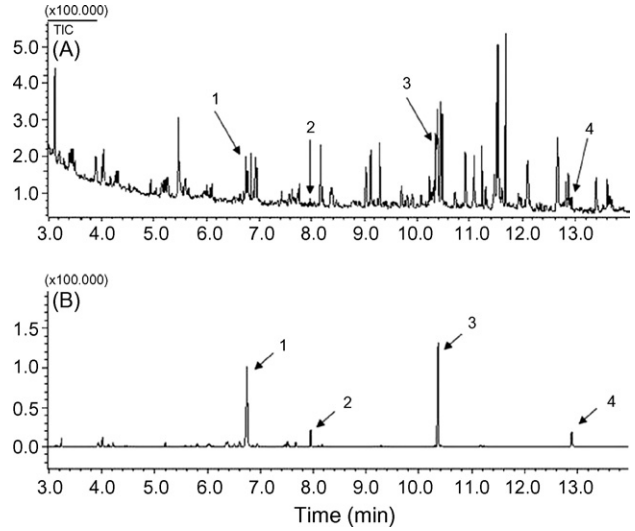

Fig. 2. GC–MS total ion chromatogram (A) and typical SIM chromatogram (B) of hair polyamines as their N-HFB derivatives from a male subject. Chromatographic separation was achieved on a Rtx-5 fused-silica capillary column (10 m × 0.18 mm I.D., 0.2 μm film thickness). The oven temperature was initially at 60 °C (1 min), programmed at 15 °C/min to 270 °C (8 min). The start time for SIM was programmed from 3.0 to 14.0 min to setup three groups of 5 ions (for putrescine and 1,6-diaminopropane), 3 ions (for spermidine) and 3 ions (for spermine) to be monitored with a dwell time of 0.2 s for each group. Peaks: 1 = putrescine (9.12 μg/g); 2 = 1,6-diaminohexane (I.S., 50 ng); 3 = spermidine (17.86 μg/g); 4 = spermine (5.88 μg/g).

polyamines in this study showed to be significantly higher than that reported in the literature [7,8,15] probably due to the difference of hair hydrolysis [28].

4. Conclusion

The GC–SIM–MS method developed in this study has been shown to be suitable with acceptable reproducibility and recoveries for simultaneous determination of putrescine, spermidine and spermine as N-HFB derivatives in hair samples. Advantages offered by the method were simple preparation of hair samples, effective separation, high sensitivity and short-time analysis. The present validated method for measuring hair polyamines may be assistant in early diagnosis and prevention of cancer in patients. Moreover, this study applied for determination polyamines in other biological samples is underway.

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