Mass Spectrometric Separation and Determination of \(N^1,N^{12}\)-Diacetylspermine in the Urine of Cancer Patients

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An ionspray ionization mass-spectrometric method for the determination of \(N^1,N^{12}\)-diacetylspermine (Ac\(_2\)Spm) was developed using \(^{15}\)N-labeled Ac\(_2\)Spm as the internal standard. Concentrations of Ac\(_2\)Spm in the urine obtained from 17 cancer patients measured by the present method correlated well with those measured by ELISA, showing the usefulness of the two methods.

Key words  diacetylspermine; IS-MS; \(^{15}\)N-polyamine; biomarker; cancer; analysis of urine

\(N^1,N^{12}\)-Diacetylspermine (Ac\(_2\)Spm) was found in the urine of a patient with non-Hodgkin’s lymphoma and of pregnant women using gas chromatography by Van den Berg et al.\(^{1,2}\) It has been drawn attention as a probable marker of malignancy, after mostly disappointing results obtained by measuring polyamines (putrescine, spermidine, spermine) and their monoacetyl derivatives in various biological samples for the purpose of cancer diagnosis or prognosis.\(^{3,4}\) Ac\(_2\)Spm has no primary amino group, so that pre-column derivatization or an alternative device is required for detection. Most recently, Shimpo et al.\(^{3,4}\) reported a reversed-phase HPLC method for the determination of benzoyl derivative of Ac\(_2\)Spm, and the significance of urinary Ac\(_2\)Spm as a biomarker of cancer and pregnancy. Hiramatsu et al. also developed a post-column HPLC method with an enzyme reactor and electrochemical detection system\(^{5}\) and a ELISA,\(^{6}\) showing the usefulness of measuring urinary Ac\(_2\)Spm.

The present short investigation was conducted to include Ac\(_2\)Spm in the method for simultaneous determination of polyamines by ionspray ionization-mass spectrometry (IS-MS)\(^{6}\) without the need for coupling with other separation techniques, and to measure Ac\(_2\)Spm in the urine of cancer patients to compare the data with those obtained by the ELISA.

MATERIALS AND METHODS

Chemicals  Heptafluorobutyric anhydride (GC grade), acetonitrile (HPLC grade), and \([p-\text{benzoyloxy-carbonyloxy}]\text{phenyl}\)dimethylsulfoxonium methylslufate (Z-DSP) were purchased from Wako Pure Chemical Ind., Ltd. (Tokyo). Hydrazine hydrate (98%) and Silica Gel 60N (spherical, neutral, 40—50 \(\mu m\)) used for column chromatography were obtained from Kanto Chemical Co., Inc. (Tokyo), and ammonium acetate (99% +) from Aldrich (Japan).

Standard polyamines (putrescine·2HCl, spermidine·3HCl, spermine·4HCl) were purchased from Sigma (Japan). Potassium \([\text{\(^{15}\)}\text{N}]\text{phthalalimide}, [\text{\(^{15}\)}\text{N}]\text{-}(3\text{-bromopropyl})\text{phthalalimide}, [1,4,8,15\text{-\(^{15}\)}\text{N}]\text{putrescine} \text{\((\text{\(^{15}\)}\text{N}\text{-Put})\text{·2HCl}, [1,4,8,15\text{-\(^{15}\)}\text{N}]\text{spermidine} \text{\((\text{\(^{15}\)}\text{N}\text{-Spd})\text{·3HCl}, and [1,4,9,12,15\text{-\(^{15}\)}\text{N}]\text{spermine} \text{\((\text{\(^{15}\)}\text{N}\text{-Spm})\text{·4HCl were prepared in this laboratory.\(^{5,6}\) All other chemicals and solvents used in this study were of the purest grade available.}

Synthesis of \([1,4,9,12,15\text{-\(^{15}\)}\text{N}]\text{-}N^1\text{-N}^{12}\text{-Diacetylspermine (\text{\(^{15}\)}\text{N}-\text{Ac}_2\text{Spm)}\) According to the synthetic method for \(^{15}\)N-Spm,\(^{8}\) an intermediate compound, \([1,4,9,12,15\text{-\(^{15}\)}\text{N}]\text{-}N^1\text{-dibenzylnspermine} (1) was prepared using \(^{15}\)N-Put·2HCl (100 mg, 0.6 mmol) and \([\text{\(^{15}\)}\text{N}]\text{-}(3\text{-bromopropyl})\text{phthalalimide} (480 mg, 1.8 mmol). 1 (0.5 mmol) was reacted with acetic anhydride (0.17 ml, 1.8 mmol) in pyridine (5 ml) at room temperature for 1 h to obtain \([1,4,9,12,15\text{-\(^{15}\)}\text{N}]\text{-N}^1\text{-N}^{12}\text{-dibenzylnspermine-N}^1\text{-dibenzylnspermine (2). After evaporation in vacuo, the residue was purified by silica gel (4 g) column chromatography, initially equilibrated with a solvent system of CHCl\(_3\)–MeOH–AcOH (7 : 1 : 0.1) and eluted with CHCl\(_3\)–MeOH–AcOH (1 : 3 : 0.1). The purified 2 dissolved in AcOH (2 ml) was then hydrolyzed at 60°C in the presence of 10% Palladium Carbon (Kojima Chemical Co., Ltd., Tokyo) (20 mg). The mixture was stirred until hydrogen uptake ceased, then filtered through a Milllex-FG syringe driven filter unit (Millipore). Hydrochloric acid (1.8 mmol) was added to the filtrate, and the mixture was evaporated to dryness. The white crystal was recrystallized from EtOH. Pure \(^{15}\)N-Ac\(_2\)Spm·2HCl was obtained (104 mg, 0.3 mmol). \textit{Anal.} Calcd for C\(_{48}\)H\(_{32}\)\text{\(^{15}\)}\text{N}_4\text{O}_2\text{Cl}_2·1/3\text{H}_2\text{O}: C, 45.49; H, 8.84; \textit{\(^{15}\)}\text{N}, 16.25. Found: C, 45.50; H, 8.80; \textit{\(^{15}\)}\text{N}, 16.38.

Synthesis of \([1,4,8\text{-\(^{15}\)}\text{N}]\text{-}N1\text{-N}^{8}\text{-Monoacetylspermidine (\text{\(^{15}\)}\text{N-AcSpd)}\) According to the synthetic method for \(^{15}\)C,\(^{15}\)N-Spd,\(^{9}\) a protected precursor of labeled spermidine, \([1,4,8,15\text{-\(^{15}\)}\text{N}]\text{-}N^1\text{-phthaloxy-N}^{8}\text{-benzyl-N}^8\text{-benzoxycarbonylspermidine (3) was prepared using \([\text{\(^{15}\)}\text{N}](3\text{-bromopropyl})\text{phthalalimide and monobenzyloxycarbonyl-15N-Put prepared by an improved method using Z-DSP.\(^{9}\) For N\(_1\)-AcSpd, the phthaloxy group of 3 (0.2 g, 0.4 mmol) was removed by refluxing it in MeOH (5 ml) and hydrate hydrate (0.1 ml, 2 mmol), and the residue after evaporation was extracted with 4\text{\(M\) NH}_3\text{-CHCl}_3. The resulting deprotected 3 having an N\(_1\)-primary amino group after evaporation of CHCl\(_3\) was reacted with acetic anhydride (0.08 ml, 0.8 mmol) in pyridine (5 ml) at room temperature for 1 h to obtain \([1,4,8,15\text{-\(^{15}\)}\text{N}]\text{-N}^1\text{-acetyl-N}^4\text{-benzyl-N}^8\text{-benzoxycarbonylspermidine (4). After evaporation in vacuo, the residue dissolved in AcOH (2 ml) was hydrogenolyzed at 60°C in the presence of 10% Palladium Carbon (20 mg), and deprotected \([\text{\(^{15}\)}\text{N}]\)-
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RESULTS AND DISCUSSION

The reported method for the simultaneous determination of Put, Spd, and Spm by IS-MS\(^7\)) was the first example of a series of compounds that were determined with only a mass spectrometer. In the method, biological samples were subjected to pretreatment using a small column of CM-cellulose with the elution buffer of pyridine-acetic acid to obtain a polyamine fraction before HFB derivatization procedure. In the fraction, compounds having at least two basic amino groups were collected, such as Ac\(_2\)Spm and monoacetyl-Spd (AcSpd), which were suggested previously to be determined by IS-MS under the same conditions as those reported for polyamines.\(^7\)

To determine Ac\(_2\)Spm or AcSpd by IS-MS, 15\(^N\)-labeled Ac\(_2\)Spm or 15\(^N\)-labeled AcSpd was used as internal standards were synthesized. High yield syntheses of these compounds are described in Materials and Methods, in which two isomers of AcSpd (N\(^1\)-acetyl and N\(^8\)-acetyl) were prepared from the common intermediate, N\(^1\)-phthaloyl, N\(^8\)-benzyl, N\(^8\)-benzoxycarbonyl-protected spermidine, and AcSpm by the reductive removal of two benzyl groups of the acetylated precursor, N\(^4\)/N\(^8\)-dibenzyloxyacetyl-1\(^N\)-diaminopimelic acid. The acetamide group of Ac\(_2\)Spm and AcSpd was shown to be stable under the conditions of HFB derivatization with no detection of HFB-Spm and HFB-Spd by IS-MS, respectively. Under the conditions of mass spectrometry for HFB-polyamines, a major peak of each HFB-polyamine carried ammonium ion with a minor peak attached proton. On the other hand, a major peak of HFB-Ac\(_2\)Spm or HFB-AcSpd carried a proton with a minor peak attached ammonium ion. The difference was significant as shown in Fig. 1, in which a mass spectrum of HFB-derivatives of five authentic samples with their 15\(^N\)-labeled counterparts is exemplified with the magnification of ion peaks for HFB-Ac\(_2\)Spm. AcSpd was not determined in this study due to the difference in ion intensities of two isomers, though it was possible to determine either N\(^1\)-acetyl Spd or N\(^8\)-acetyl Spd alone.

The determination of Ac\(_2\)Spm based on the ratio of major ion intensity of Ac\(_2\)Spm to that of 15\(^N\)-labeled Ac\(_2\)Spm was then examined by the standard addition method using the urine of a cancer patient (Table 1). A linear relationship between the ratios and added amounts of Ac\(_2\)Spm was shown, and the average coefficient of variation was 5.7%. These results suggested that the present method was sufficiently reliable.

Urine of 17 cancer patients, given concentrations of Ac\(_2\)Spm by ELISA,\(^6\) were then examined by the present method. The results were compared with those of ELISA, and the correlation graph is shown in Fig. 2. The value of the correlation coefficient, 0.94, showed that the two methods were reliable.

At present, it is unknown whether or not Ac\(_2\)Spm in the urine of cancer patients is formed at the site of malignant cells. The present method will be useful in the future for elucidating the origin of Ac\(_2\)Spm.

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REFERENCES


Table 1. Reliability of the Method Examined by Addition of Various Amounts of Ac$_2$Spm to Urine of a Cancer Patient

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<th>Ac$_2$Spm added (nmol)</th>
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<td>Ratio$^b$</td>
<td>0.37±0.02$^f$</td>
<td>0.42±0.02</td>
<td>0.49±0.03</td>
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<td>0.61±0.04</td>
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<td>cv (%)</td>
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<td>4.8</td>
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$^a$) To 0.1 ml of urine were added various amounts of Ac$_2$Spm and 0.6 nmol of $^{15}$N-Ac$_2$Spm as internal standard. See the experimental details in Materials and Methods.
$^b$) Ac$_2$Spm/$^{15}$N-Ac$_2$Spm. $^c$) Mean±S.D. ($n=9$: 3 samples, 3 determinations/sample).

Fig. 1. Simultaneous Mass Analysis of a Mixture of HFB-Put, HFB-AcSpd, HFB-Ac$_2$Spm, HFB-Spd, HFB-Spm, and Their $^{15}$N-Labeled Counterparts 

$m/z$ 483.7 corresponds to [HFB-1$^{15}$N-Put+H$^+$], $m/z$ 500.7 to [HFB-1$^{15}$N-Put+NH$_4$], $m/z$ 583.5 to [HFB-2$^{15}$N-AcSpd+H$^+$], $m/z$ 600.5 to [HFB-2$^{15}$N-AcSpd+NH$_4$], $m/z$ 683.6 to [HFB-Ac$_2$Spm+H$^+$], $m/z$ 700.5 to [HFB-Ac$_2$Spm+NH$_4$], $m/z$ 737.5 to [HFB-1$^{15}$N-Spd+H$^+$], $m/z$ 754.5 to [HFB-1$^{15}$N-Spd+NH$_4$], $m/z$ 1008.6 to [HFB-1$^{15}$N-Spm+NH$_4$]$^+$. Arrow shows the enlargement of HFB-diacetylspermine fraction. See the experimental details in Materials and Methods.

Fig. 2. Correlation Graph of Ac$_2$Spm Concentrations in the Urine of 17 Cancer Patients Measured by ELISA and IS-MS

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