Electrospray Ionization and Time-of-Flight Mass Spectrometric Method for Simultaneous Determination of Spermidine and Spermine

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A sensitive method for the determination of polyamines in mammalian cells was described using electrospray ionization and time-of-flight mass spectrometer. This method was 50-fold more sensitive than the previous method using ionspray ionization and quadrupole mass spectrometer. The method employed the partial purification and derivatization of polyamines, but allowed a measurement of multiple samples which contained picomol amounts of polyamines. Time required for data acquisition of one sample was approximately 2 min. The method was successfully applied for the determination of reduced spermidine and spermine contents in cultured cells under the inhibition of aminopropyltransferases. In addition, a new proper internal standard was proposed for the tracer experiment using 15N-labeled polyamines.

Key words polyamine; heptfluorobutyl derivative; time-of-flight mass spectrometry; electrospray ionization; stable isotope labeled polyamine; cultured mammalian cell

Spermidine (spd), spermine (spm), and a diamine putrescine are major polyamines in mammalian cells, and they play important roles in cell growth and differentiation.1—4) Although a variety of analytical methods for polyamines have been developed,5—7) there is still a need for a simpler, more sensitive and accurate method. The mass spectrometric method can simultaneously separate and identify polyamines on the basis of the difference of their mass without use of column.8) A quadrupole mass spectrometer with ionspray ionization interface (ISI-Q MS) is the first one used to determine polyamine concentrations in rat tissues.9) Recent progress in mass spectrometer has made it possible to use a time-of-flight mass spectrometer with electrospray ionization interface (ESI-TOF MS), which is more sensitive and provides a higher resolution. The present paper describes a greatly improved ESI-TOF MS method to determine the concentrations of spd and spm in mammalian cells. The data showed that this method had a high sensitivity and reproducibility, and allowed the measurement of multiple samples.

MATERIALS AND METHODS

Materials Dulbecco’s Modified Eagle Media (DMEM), penicillin/streptomycin and trypsin/EDTA purchased were from Invitrogen Corp. (Carlsbad, CA, U.S.A.), and fetal bovine serum (FBS) was from Bio West (Nuaill, France). Cell culture flasks (75 cm²) and 24-well plates were from BD Biosciences (Bedford, MA, U.S.A.) and Corning (NY, U.S.A.), respectively. β-TC6 cells, a mouse pancreatic β-cell line, were purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.).

Standard polyamines (putrescine·2HCl, spermidine·3HCl, spermine·4HCl) were purchased from Sigma (Japan) and recrystallized. 15N-Labeled polyamines, [1,4,15N]-putrescine (15N-cut), [1,4,8,15N]-spd (15N-spd) and [1,4,8,12,15N]spm (15N-spm) were prepared as described previously.10) 1,4-Dibromobutane-1,1,4,4-d₄ (99.2% atom %D) as a starting compound for [1,4,15N₂,1,1,4,4-d₄]putrescine (15N₂D₄-cut) was purchased from CDN ISOTOPES (Quebec, Canada). 15N₂,D₄-Put, N-(3-aminopropyl)-1,4-15N₂-butane-1,1,4,4-d₄ (15N₂D₄-spd) and N,N’-(3-aminopropyl)-1,4-15N₂-butane-1,1,4,4-d₄ (15N₂D₄-spm) were prepared according to the synthetic methods for 15N-labeled polyamines.10) Heptafluorobutyril anhydride, 98% (HFB anhydride) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, U.S.A.). Ammonium acetate (min 97.0%), perchloric acid (60%) (PCA), and CM-cellulose were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). trans-4-Methylcyclohexylamine (MCHA), an inhibitor of spermidine synthase, and N-(3-aminopropyl)cyclohexylamine (APCHA), an inhibitor of spermine synthase, were purified by the respective recrystallization of the hydrochlorides of cis/trans-4-methylcyclohexylamine and N-(3-aminopropyl)-cyclohexylamine (>98%)11) obtained from Tokyo Kasei Kogyo, Ltd. (Tokyo, Japan). All other chemicals and organic solvents were of the purest grade available.

Methods. Cell Culture β-TC6 cells were cultured in DMEM medium containing 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 20 m M glucose in a CO₂ incubator (5% CO₂) at 37°C. When the confluency reached 40—50%, the cells were stripped from the bottom of the flask by adding trypsin/EDTA solution, and cell suspension was transferred into a new flask filled with new medium (10 or 20-fold volume). The cells were subcultured every week. Cells from passage 10—30 were used for all experiments.

Extraction of Polyamines from β-TC6 Cells β-TC6 cells were seeded onto a 24-well plate at 1×10⁵ cells/well. After 1 d culture, the cells were treated with or without 250 µM MCHA or 100 µM APCHA for further 3 d. At the end of the culture, the cells in each well were stripped by 1 ml of trypsin/EDTA solution and transferred into a 1.5 ml centrifuge tube. An aliquot of the cells was stained with a 4% trypan blue solution (Invitrogen) and the number of cells in each tube was counted using a glass hemocytometer. Cells were collected by centrifugation, washed with phosphate-buffered saline (PBS) once and kept in −80°C until the assay of polyamine content. For extraction of polyamines, the cells were homogenized on ice in 100 µl of 0.1 m HCl, and...
then 100 μl of 0.5 M PCA was added to the homogenate. The cell extracts were centrifuged (13000 rpm, 2 min, 4 °C), and the supernatant was used for measurement of the polyamine concentration in β-TC6 cells.

**Preparation of Sample Solutions for Mass Spectrometry** The supernatant was divided into two aliquots for duplicate determination. To each aliquot, 500 pmol each of [15N-labeled spd and 15N-labeled spm were added as internal standards. In addition, 50 pmol each of standard spd and spm were added to one aliquot, and 100 pmol each to the other. The two samples were neutralized with 0.05 M pyridine (1 ml) and then applied on a small CM-cellulose column (bed volume 50 μl) equilibrated with 0.01 M pyridine and acetic acid buffer (Pyr-AcOH) (pH 5.0) prepared by 100-fold dilution of 1 M Pyr-AcOH, a mixture of equal volume of 2 M pyridine and 2 M AcOH. After the columns were washed stepwise with 0.01 M Pyr-AcOH (1 ml) and 0.05 M Pyr-AcOH (2.5 ml), polyamines were eluted with 1 M Pyr-AcOH (0.7 ml). The fractions containing polyamines were evaporated to dryness in vacuo at room temperature, and treated with HFB anhydride (20 μl) at 100 °C for 30 min in acetonitrile (100 μl). The reaction mixtures were then dried under nitrogen stream, and the residues were dissolved in 1 ml of 0.5% ammonium acetate in 50% aqueous acetonitrile (mobile phase). This sample solution was subjected to mass spectrometric analysis.

**Conditions for Mass Spectrometric Analysis** The apparatus used was LCT Premier (TOF MS)-Alliance 2795 (HPLC system) (Waters). The flow rate of the mobile phase was set at 0.2 ml/min, the injection volume of sample solution was 5 μl, and the stop time was set at 2 min. The usual conditions for TOF MS were as follows: mass range was m/z 480—1020 and scan time was 1 s with interscan delay of 500 ms for [HFB-15N-put NH4]+ with 0.01 M Pyr-AcOH (1 ml) and 0.05 M Pyr-AcOH (2.5 ml), polyamines were evaporated to dryness in vacuo for TOF MS were as follows: mass range was m/z 1944 Vol. 30, No. 10. The pretreatment was basically the same as that reported previously using ISI-Q MS. 8,9) The ether extraction step of HFB-polyamines was used previously to obtain a better spectrum in ISI-Q MS, but this step could be omitted in ESI-TOF MS because of its higher sensitivity. This allowed reduction of the amount of injected sample, and simplified the procedure. It was true that the pretreatment is time-consuming, but the demerit can be alleviated by applying many samples at the same time and/or by automation if needed.

**Improved Sensitivity and Analysis Time** A difference in sensitivity between ISI-Q MS and ESI-TOF MS was estimated by the injected amount of internal standard required for each MS. The amount was 2.5 pmol, i.e., 500 pmol divided by a dilution factor of 200 for ESI-TOF MS under the condition described above, whereas it was 150 pmol, i.e., 1.5 nmol divided by a dilution factor of 100 for ISI-Q MS. These results indicated that TOF MS was about 60 fold more sensitive than Q MS in determining the amount of polyamine.

Because the flow rate of the mobile phase, i.e., 0.2 ml/min, in ESI-TOF MS was relatively fast compared to that in ISI-Q MS, i.e., 10 μl/min, it was possible to shorten the stop time to 2 min, while it was 3 min in ISI-Q MS. The length of time in ESI-TOF MS could be shortened as much as 1 min, because total ion chromatogram (TIC) showed the end of peak at around 0.7 min after the injection.

**Quantification by Standard Addition Method** An example of TIC is shown in Fig. 1a. Scans of peak area from 0.3 to 0.7 min were integrated to obtain a mass spectrum (Fig. 1b). The ion intensity ratios of HFB-spd to HFB-15N-spd and HFB-spm to HFB-15N-spm were used for the determination of spd and spm, respectively. Standard addition method was employed to obtain a reliable working curve. A triplicate determination by standard addition method as exemplified in Table 1 was examined using six samples obtained from cultured cells. The average ratios of twelve data for 50 pmol and those of six data for 100 pmol obtained from six samples were 0.090 and 0.180 for 50 and 100 pmol spd, respectively, and 0.096 and 0.192 for 50 and 100 pmol spm, respectively. The results demonstrated the linearity of working curves through the origin. The raw ratios subtracted blank values, which were obtained by the subtraction of the average ratios from apparent ratios for 50 pmol of spd or spm measured as a control, were then converted to pmol amounts based on the average ratios (Table 1). Usually, duplicate determinations were employed with the addition of 50 or 100 pmol of spd and spm as described in Methods, and four sets of data obtained by measuring duplicate samples twice were averaged. Thus, this method allowed the duplicate measurement of many samples at the same time yielding an average ratio for 50 pmol, and has an advantage of excluding a fluctuation of analytical data. In addition, it appeared that the standard addition was useful in obtaining more reliable data especially in dealing with the samples having low contents of spd and spm. The limit of quantification was in the range of pmols in a given sample under the present conditions.

**Application to Cultured Cells** The present method was used to determine the contents of spd and spm in approximately 105 pancreatic β-TC6 cells per a well. Those numbers of cells were too small to determine the polyamine contents...
accurately by fluorescent HPLC method for polyamine determination (unpublished observation). These cells were cultured in the presence or absence of the specific inhibitor of spermidine synthase or spermine synthase, and in some cases, exogenous spd or spm was added. The results are summarized in Tables 2 and 3. The values obtained from the duplicate cultures with the indicated additions gave similar values of spd and spm contents and the spd to spm ratios, showing a good reproducibility. In Table 2, a significant decrease of spd with the concomitant increase of spm was observed when a spermidine synthase inhibitor, MCHA, alone was added. This was expected from the previous observation.\(^{12}\)

The addition of spd alone produced an increase of spd and a decrease of spm. Addition of both MCHA and spd seemed to bring the spd to spm ratio to that of control level when the increasing amounts of spd were added. The results of similar experiments wherein spd and spm contents were examined in the presence of APCHA, an inhibitor of spermine synthase, and/or added exogenous spm (Table 3). A significant increase of spd with a concomitant decrease of spm was expected when APCHA alone was added.\(^{12}\) The addition of spm alone resulted in the undetectable level of spd content, while the spm content being kept at the control level. Because of this effect of exogenous spm, even combination of both APCHA and spm could not increase or restore the levels of spd and spm irrespective of the amount of spm added.

**Internal Standard for Tracer Experiment** A tracer experiment using \(^{15}\)N-labeled polyamines as tracers was re-
ported with ISI-Q MS,\(^{13}\) in which \(^{13}C,^{15}N\)-labeled polyamines were used as internal standards under the negligible presence of sodium ion-binding HFB-polyamines. But the sodium ion-binding HFB-polyamines could not be ignored in ESI-TOF MS. The same mass of 756 for sodium-binding HFB-spd and ammonium-binding HFB-\(^{15}N\)-spd showed that HFB-\(^{13}C,^{15}N\)-spd was unsuitable as an internal standard for spd. A compound, \(^{15}N_2,D_4\)-spd, having a six mass heavier than natural spd was thus prepared as an internal standard for spd. An intermediate, \(^{15}N_2,D_4\)-put, for the preparation of \(^{15}N_2,D_4\)-spd was useful as an alternate internal standard for putrescine. Also, a spm derivative prepared from \(^{15}N_2,D_4\)-put will be useful as an alternate internal standard for spm. A series of authentic HFB-polyamines including \(^{15}N\)-labeled and \(^{15}N_2,D_4\)-labeled polyamines, are shown in the mass spectrum (Fig. 2).

Finally, as can be seen in Fig. 2, the ion intensity of HFB-putrescine was about one fifth of that of HFB-spd or HFB-spm under the measurement of the same amount. In addition to this, cellular putrescine contents were usually very low as compared to those of spd or spm. Therefore, putrescine could not be determined simultaneously under the present ESI-TOF MS conditions. It is possible, however, to determine putrescine after the separation from spd and spm through the step of CM-cellulose fractionation (0.33 M Pyr-AcOH, 0.7 ml).

REFERENCES