Multi-Perfluoroalkyl Derivatization of Polyamines for Selective Liquid Chromatography-Tandem Mass Spectrometric Analysis Utilizing Fluorous Affinity

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Abstract
A novel derivatization method for the selective and sensitive liquid chromatography (LC)-tandem mass spectrometric (MS/MS) analysis of polyamines (putrescine, cadaverine, spermidine, and spermine) was developed. In this study, to utilize the specific affinity between perfluoroalkyl compounds, called ‘fluorous’ affinity, two to four amino groups in each polyamine molecule were transformed with a relatively short perfluoroalkyl reagent, N-succinimidyl 4,4,5,5,6,6,7,7,8,8,9,9,9-tridecafluorononanoate to achieve the corresponding diperfluoroalkyl- to tetrafluoroalkyl-derivatives. Compared with the non-fluorous compounds, the fluorophilicity of the multi-fluorous derivatives was sufficient for realizing strong retention on an LC column with a perfluoroalkyl-modified stationary phase. Furthermore, sensitive analysis of these derivatives could be performed using the multiple reaction monitoring mode in positive electrospray ionization-MS/MS. The limits of detection of the polyamines were in the range of 0.31–1.4 nM. The method was validated using human plasma samples. Although the recoveries from spiked human plasma after ultrafiltration were in the range of 66.4–103%, the derivatives could be determined without interference from matrix effects because of their selective retention on the column, which excluded non-fluorous biological matrix components, such as phospholipids. Therefore, this sensitive and selective analysis method was useful for the determination of trace amounts of polyamines in human plasma.

Keywords: Polyamine; Multi-fluorous derivatization; Liquid chromatography-tandem mass spectrometry; Human plasma

1. Introduction
Polyamines are ubiquitous in living organisms and play important roles in cell growth, division, and differentiation [1,2]. The basal levels of polyamine concentrations in normal cells are highly regulated by biosynthetic enzymes; therefore, determining their endogenous concentrations is well-known method for diagnosing a number of cancer and age-associated diseases because the concentrations in neoplastic cells are higher than those in normal cells [3,4]. Typical analytical approaches for the determination of polyamines have employed liquid chromatography (LC) separation with optical or mass spectrometric (MS) detection [5-14]. Many of these methods require derivatization of the polyamines to be analysed. In the case of fluorescence detection, 9-fluorenylmethyl chloroformate [5], 6-amino-quinolyl-N-hydroxysuccinimidyl carbamate [6], dansyl chloride [7], and o-phthalaldehyde [8] have been often utilized as typical derivatization reagents. For LC-MS analysis, these derivatization methods have been also useful because the high ionization efficiency of the obtained derivatives allows sensitive detection with an electrospray ionization (ESI)-MS source. Moreover, their moderate retention and separation on reversed-phase LC columns were obtained with appropriate mobile phases [9-14].

Previously, we developed an excimer fluorescence derivatization method for selective LC analysis of poly-functional compounds including polyamines [15-17]. The pyrene-derivatized poly-functional compounds could be selectively detected by intramolecular excimer fluorescence (440–540 nm), whereas mono-functional compounds and

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Received: 11 July 2017
Accepted: 9 September 2017
J-STAGE Advance Published: 16 September 2017
DOI: 10.15583/jpchrom.2017.012
reagent blanks, which emitted monomer fluorescence (360–420 nm), did not affect the analysis of the poly-functional compounds. Thus, in general, if poly-functional compounds can be transformed into characteristic derivatives, they can easily be distinguished from complex samples containing mono-functional compounds. To this end, to develop an analysis method for four representative polyamines [putrescine (Put), cadaverine (Cad), spermidine (Spd), and spermine (Spm)] with improved selectivity, sensitivity, and rapidity, all the amino groups in these molecules were derivatized with the perfluoroalkyl (fluorous-tag) reagent N-succinimidyli-4,4,5,5,6,6,7,7,8,8,9,9,9,10,10,11,11,12,12,13,13,14,14,15,15,16,16,17,17,18,18,19,19,20,20,21,21,22,22,23,23,24,24,25,25,26,26,27,27,28,28,29,29,30,30-tridecafluorononanoate (STFN) to achieve the multi-perfluoroalkylated forms (Fig. 1).

Fig. 1. Fluorous derivatization of polyamines with STFN in the present study.

Perfluoroalkyl-containing compounds can be selectively retained and separated on an LC column packed with a perfluoroalkyl-modified stationary phase based on the remarkable affinity between perfluoroalkyl groups, namely ‘fluorous’ affinity (fluorophilicity) [18,19]. Utilizing such fluorous affinity, we reported that accurate and precise LC–tandem mass spectrometric (MS/MS) analysis of biogenic compounds could be performed following derivatization with a fluorous-tag reagent [20-22]. Because the obtained perfluoroalkyl (fluorous) derivatives could be completely separated from non-fluorous species, such as endogenous components in the biological matrix, on the fluorous LC column, this method did not require further sample pretreatment to eliminate matrix-induced effects in MS/MS detection. Although this fluorous derivatization method was useful for the analysis of biological samples with LC-MS/MS, to utilize the fluorous affinity effectively, perfluoroalkyl groups of sufficient length were necessary because the affinity depends on the number or content of perfluoroalkyl groups [23,24]. However, such perfluoroalkyl compounds, especially those containing carboxylate and sulfonate groups, are thought to be environmental pollutants due to their toxicities [25,26], and therefore, the use of shorter perfluoroalkyl reagents is preferable to the conventional derivatization reagents, which have perfluoroalkyl chains of C6F13. For this reason, a shorter fluorous-tag reagent, STFN (which has a perfluoroalkyl chain of C6F13), was used for the derivatization of polyamines in this study. Although the fluorophilicity of mono-fluorous derivatives with STFN may be insufficient, the multi-fluorous polyamine derivatives corresponding to the diperfluoroalkylated to tetraperfluoroalkylated forms could be selectively retained on the fluorous LC column because of their adequate fluorophilicity. Furthermore, the fluorous derivatives could be detected with higher sensitivity using MS/MS owing to an improved ionization response in ESI [20-22]. After optimization of the analytical conditions, including the derivatization, the feasibility of the proposed method was demonstrated by analysing human plasma samples deproteinized with ultrafiltration. Then, the method was applied to the determination of trace amounts of endogenous polyamines in human plasma samples.

2. Experimental

2.1. Reagents and solutions

Polyamines (Put dihydrochloride, Cad dihydrochloride, Spd trihydrochloride, and Spm tetrahydrochloride) were obtained from Sigma-Aldrich (St. Louis, MO, USA). STFN was purchased from Fluorous Technologies (Pittsburgh, PA, USA). Trifluoroacetic acid (TFA) and 2,2,2-trifluoroethanol (TFE) were obtained from Wako Pure Chemicals (Osaka, Japan) and Tokyo Chemical Industry (Tokyo, Japan), respectively. Both of sodium tetraborate decahydrate (borax) and n-nonanoic acid were purchased from Kanto Chemical (Tokyo, Japan). Deionized water was purified using a Millipore EQG system (Billerica, MA, USA) and was used to prepare all aqueous solutions. Unless otherwise noted, all chemicals mentioned above were of the highest purity and were used as received.

Stock solutions of polyamine standards (10 mM) were prepared by dissolving an appropriate amount of each polyamine in deionized water and stored at −20°C. These solutions were diluted further with deionized water to the required concentrations before use. A solution of 20 mM STFN in acetonitrile was stored at 4°C and used within 1 day.

2.2. Derivatization procedure

To 20 µL of sample placed in a 0.5 mL vial, 5 µL of 50 mM borax and 40 µL of 20 mM STFN were added, and the vial was capped and left at room temperature for 30 min. The resulting solution was placed in the autosampler of the LC-MS/MS system.
2.3. Instrumentation and conditions

A Prominence ultrafast liquid chromatography system (Shimadzu, Kyoto, Japan) was used. The system consisted of two LC-20AD pumps, a high-pressure gradient unit, a DGU-20A3 on-line degasser, an SIL-20AC autosampler, and a CTO-20AC column oven. The injection volume was 15 µL. A FluoroSep RP column (100 × 2.1 mm ID, particle size 5 µm; ES Industries, West Berlin, NJ, USA) was used. Solvents A (methanol/water/acidic acid/TFA = 90:10:1:0.1, v/v) and B (TFE/methanol/water/acidic acid/TFA = 50:40:10:1:0.1, v/v) were used as the mobile phases for gradient elution (gradient curve: 0 min, 0% B; 0–3 min, linear change from 0 to 100% B; 3–7 min, 100% B; 7–7.01 min, linear change from 100 to 0% B, and hold at 0% B for 5 min for column re-equilibration; total run time, 12 min). The flow rate and the column oven temperature were set at 0.3 mL/min and 50°C, respectively. The effluent from the LC column was directly introduced into the ion source of the mass spectrometer without splitting.

An API 4000 QTRAP tandem mass spectrometer (AB Sciex, Concord, ON, Canada) was operated in the positive ESI mode. The operating conditions were as follows: ESI capillary voltage, 5500 V; source temperature, 550°C; curtain gas, 10 (arbitrary units); ion source gas 1, 60 (arbitrary units); and ion source gas 2, 80 (arbitrary units). The multiple reaction monitoring (MRM) conditions, i.e., the declustering potentials (DPs), entrance potentials (EPs), collision-induced dissociation energies (CEs), and collision exit potentials (CXP) values, are shown in Table 1, along with the resulting precursor ions and product ions.

Table 1. MRM transitions for fluorous derivatives of polyamines.

|          | Precursor ion (m/z) | Product ion (m/z) | DP (V) | EP (V) | CE (eV) | CXP (V) |
|----------|---------------------|-------------------|--------|--------|---------|---------|
| Put      | 837.1               | 446.1             | 50     | 14     | 50      | 10      |
| Cad      | 851.2               | 460.1             | 70     | 12     | 50      | 40      |
| Spd      | 1268.2              | 432.0             | 100    | 14     | 100     | 30      |
| Spm      | 1699.3              | 432.1             | 100    | 14     | 120     | 20      |

2.4. Comparative study

To evaluate the retention behaviour of the multi-fluorous polyamine derivatives on the fluorous LC column, mono- fluorous and non-fluorous derivatives were prepared for comparison. The mono-fluorous derivatives were obtained by derivatization of monamines [n-butylamine (C4), n-hexylamine (C6), and n-octylamine (C8)] with STFN using the procedure described for polyamines in section 2.2. On the other hand, to prepare the corresponding non-fluorous dialkyl- to tetraalkylated derivatives, the polyamines were derivatized with n-nonanoic acid (which has the same carbon number as STFN) in the presence of the condensation reagent, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) with previously reported procedure [20]. These mono-fluorous and non-fluorous derivatives were analysed by selected ion monitoring mode in the positive ESI mode.

2.5. Human plasma samples

Human plasma samples were obtained from healthy volunteers (n = 5) in our laboratory. A centrifugal blood collection tube (Eiken Chemical, Tokyo, Japan) was used for the collection, and centrifugation was carried out at 2,000 × g for 5 min at room temperature. The obtained plasma was transferred to 1.5 mL screw-cap tubes and stored at −20°C before use. The samples were ultra-filtered by ULTRAFREE®-MC (3000 NMWL, Millipore) with centrifugation at 20,000 × g for 15 min. The filtrate (20 µL) was subjected to derivatization with STFN using the procedure outlined in section 2.2. The present experiments were approved by the Ethics Committee of the Central Research Institute of Fukuoka University, and performed in accordance with established guidelines.

2.6. Method validation

We performed a validation study to assess the precision, the accuracy of the method in terms of the recovery of target analytes from spiked samples, the matrix effect, and the sensitivity of the method. Calibration standards for the calibration curves were prepared in the range of 5–500 nM (5, 10, 50, 100, and 500 nM) for Put, Spd, and Spm, and of 1–100 nM (1, 5, 10, 50, and 100 nM) for Cad. The intra-day precisions of this method were determined using replicate preparations (n = 6) of standard solutions at low (1 nM for Cad, 5 nM for others), medium (10 nM for all analytes), and high (100 nM for all analytes) concentration levels. The limits of detection (LODs) and lower limits of quantification (LLOQs) were defined as the concentrations that gave signal-to-noise (S/N) ratios of 3 and 10, respectively.

The intra-day and inter-day precisions for real samples were also determined using replicate preparations (n = 6) of spiked human plasma samples at low (1 pmol/mL plasma for Cad, 5 pmol/mL plasma for others), medium (10 pmol/mL plasma for all analytes), and high (100 pmol/mL plasma for all analytes) concentration levels. Recovery data were determined by comparing the responses of the standard-spiked human plasma samples with those of the same concentrations of standard solutions using the following equation:

\[
\text{Recovery (\%) = 100 \times \left( \frac{\text{response in spiked plasma} - \text{response in non-spiked plasma}}{\text{response in standard}} \right) }
\]

In addition, matrix effects during LC-MS/MS analysis were estimated using the previously reported approach [21,27]. This was accomplished by comparing the responses from the 10, 50, and 100 nM levels of the derivatized
standard polyamines (non-matrix samples) with those spiked at the same levels into extracts of human plasma (matrix samples) \( (n = 3) \) using the following equation:

Matrix effect (\( \% \)) = \( 100 \times \frac{\text{response in matrix sample}}{\text{response in non-matrix sample}} \)

3. Results and discussion

3.1. MS/MS detection conditions

Structural information about the polyamine derivatives was confirmed by acquisition of their MS and MS/MS spectra. In the MS scan \((m/z \ 400-2000)\), the most intense \( m/z \) value for each multi-fluorous polyamine derivative corresponded to the protonated molecular ions \((\text{[M} + \text{H}\}\text{]}^+)\) in positive ionization mode. Figure 2 shows the MS/MS spectra obtained when the \([\text{M} + \text{H}\]^+) ions of the various multi-fluorous derivatives were set as precursor ions and dissociated under appropriate CE conditions. The MS/MS spectra show that the fragmentation of the derivatives occurred by cleavage of the amide linkage, and specific product ions were observed. These results suggested that the polyamines were successfully transformed into the corresponding multi-fluorous derivatives by the present method, with all amino groups perfluoroalkylated. In this study, the most intense product ions were set as the quantification ions in MRM mode to perform sensitive analysis of the target polyamines (Table 1).

3.2. Derivatization conditions

Succinimidyl ester reagents have been successfully used for the derivatization of amine-containing compounds [15,16]. In this study, the examined polyamines could also be easily derivatized to the corresponding multi-fluorous derivatives with STFN under mild conditions. Optimization of the derivatization conditions was carried out using the polyamine standards \((10 \text{ nM each})\) to obtain the most intense peak areas for the multi-fluorous derivatives. The effects of the solvent for STFN, concentration of STFN, reaction temperature, and reaction time \((10-40 \text{ min})\) were examined. Acetone, acetonitrile, methanol, and tetrahydrofuran were evaluated as solvent. Among these solvents, acetone and methanol could not dissolve STFN, whereas tetrahydrofuran accelerated the derivatization reaction for Put and Cad, but not for Spd and Spm. Consequently, in this study, acetonitrile was found to be the most effective solvent because the multi-fluorous derivatives of each polyamine was successfully obtained, and the following optimization studies were performed using acetonitrile as the solvent for STFN. We tested various STFN concentrations ranging from 0 to 50 mM, and found that the peak intensities of the derivatives of Spd and Spm increased with increasing concentrations of STFN up to 20 mM. Because the peak intensities of the derivatives of Put and Cad were not affected by the concentration of STFN in the examined range, we chose to use 20 mM STFN for the derivatization. On the other hand, the reaction temperature hardly affected this derivatization reaction, when varied from room temperature to \(80^\circ \text{C}\) under a fixed reaction time of 30 min. In addition, a reaction time of 30 min at room temperature was required to obtain constant peak intensities for the polyamine derivatives.

Consequently, 20 mM STFN in acetonitrile and a reaction time of 30 min at room temperature were found to be optimal for derivatization of polyamines in this study. The STFN derivatives of polyamines were stable for at least 1 day in the dark at \(4^\circ \text{C}\).

3.3. Fluorous LC separation

The multi-fluorous polyamine derivatives were retained strongly on the fluorous LC column, and the retention time increased with increasing the number of perfluoroalkyl chains in their structures. The elution of the retained derivatives from the column was easily controlled by the concentration of TFE (fluorophilic solvent) in the mobile phase [21]. Under the present LC conditions, all the polyamine derivatives could be analysed within 7 min. In this study, the retention times of the di-perfluoroalkyl derivatives of Put and Cad were almost the same, which indicates that the retention of the derivatives was based on the number of perfluoroalkyl groups introduced onto amino groups. For comparison, the non-fluorous polyamine derivatives prepared with \(n\)-nonanoic acid, which has same carbon number as STFN, were analysed using the same LC conditions. The non-fluorous derivatives were hardly retained \((t_R \approx \text{ca.} \ 1 \text{ min})\), indicating that the polyamines derivatized with STFN were selectively retained on the
fluorous LC column by means of fluorophilicity and not hydrophobicity (data not shown). Furthermore, we evaluated the retention of mono-fluorous monoamine derivatives (C₄, C₆, and C₈) prepared with STFN. Although the mono-fluorous derivatives showed stronger retention ($t_R = \text{ca.} 2$ min) than the non-fluorous derivatives on the fluorous LC column (data not shown), their retention times were considerable shorter than those of the multi-fluorous polyamine derivatives, even though they contained adequate hydrophobic compounds with long alkyl chains, including a perfluoroalkyl group. This experiment demonstrates the use of fluorous interactions for selective retention and separation. Moreover, selectivity can be achieved by obtaining multi-fluorous derivatives of the analytes using a shorter perfluoroalkyl derivatization reagent.

### 3.4. Method validation

#### 3.4.1. Analysis of standards

As expected, the multi-fluorous polyamine derivatives were detected with high sensitivity in ESI mode. The LODs of Put, Cad, Spd, and Spm were 0.72, 0.31, 0.63, and 1.4 nM (corresponding to 3.3, 1.4, 2.9, and 6.5 fmol on column), respectively. These values were similar to those reported in previous studies employing derivatization with conventional ESI-amenable reagents for sensitive analysis of polyamines [9-14]. The LLOQs of Put, Cad, Spd, and Spm were 2.3, 1.0, 2.1, and 4.6 nM (corresponding to 10, 4.7, 9.6, and 21 fmol on column), respectively. Additionally, the correlation coefficients of the calibration curves for the multi-fluorous polyamine derivatives were greater than 0.9985. The relative standard deviations (RSDs) of the peak areas for intra-day repeated analysis ($n = 6$) were within 13% for all examined concentration levels of the standards. These data are shown in Table 2.

#### 3.4.2. Spike recovery

Prior to application to the determination of polyamines in individual human plasma samples, the developed method was validated by analysing a pooled human plasma sample. The recovery values from human plasma were 66.4–103% for all spiked levels of polyamines (Table 3). Although the recovery values for Spd and Spm from ultrafiltration in this study were relatively low, the RSD values obtained by intra-day and inter-day repeated analysis of spiked human plasma samples ($n = 6$) were within 16%.

#### 3.4.3. Matrix effects

To confirm that this method could overcome matrix effects, human plasma spiked with three different concentration levels of the polyamine derivatives (10, 50, and 100 nM) was analysed ($n = 3$). As shown in Table 4, the matrix effect values obtained for the human plasma samples were in the ranges of 96.5–110% for all concentration levels examined, indicating that excellent separation of the multi-fluorous polyamine derivatives from the biological matrix in human plasma was achieved, leading to the elimination of matrix effects in the LC-MS/MS analysis without further sample pretreatment. Furthermore, to demonstrate the feasibility of

### Table 2. Validation data for the analysis of standard solutions of polyamines.

| Calibration curve | Intra-day RSD$^a$ (%) | LOD (nM)$^b$ | LLOQ (nM)$^b$ |
|-------------------|-----------------------|--------------|--------------|
| Range (nM) | $r$ | Low | Medium | High | Low | Medium | High |
| Put | 5–500 | 0.9993 | 7.5 | 3.4 | 6.1 | 0.72 | 2.3 |
| Cad | 1–100 | 0.9985 | 6.5 | 4.7 | 6.8 | 0.31 | 1.0 |
| Spd | 5–500 | 0.9997 | 5.4 | 7.6 | 9.4 | 0.63 | 2.1 |
| Spm | 5–500 | 0.9999 | 11 | 5.7 | 13 | 1.4 | 4.6 |

$^a$ Relative standard deviations of the peak areas for multi-fluorous polyamine derivatives.

$^b$ Defined as $S/N > 3$.

### Table 3. Validation data for the analysis of polyamines in human plasma samples.

| Intra-day RSD$^a$ (%) | Inter-day RSD$^a$ (%) | Mean recovery (%) |
|-----------------------|-----------------------|------------------|
| Low | Medium | High | Low | Medium | High | Low | Medium | High |
| Put | 7.5 | 7.9 | 5.4 | 13 | 13 | 4.7 | 89.5 | 96.9 | 100 |
| Cad | 4.9 | 3.4 | 6.2 | 11 | 5.6 | 10 | 101 | 91.3 | 103 |
| Spd | 5.7 | 7.7 | 4.8 | 14 | 14 | 6.7 | 75.6 | 66.4 | 82.3 |
| Spm | 8.3 | 10 | 7.3 | 12 | 10 | 16 | 83.4 | 72.4 | 73.4 |

$^a$ Relative standard deviations of the peak areas for multi-fluorous polyamine derivatives.

$^b$ Defined as $S/N > 10$. 

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**Chromatography 2017, 38, 107-113**
this method more clearly, phospholipids in plasma were analysed using the present LC conditions. Phospholipids are known to be the main endogenous components that cause matrix effects in the analysis of plasma, and they can be monitored with the precursor ion scan mode in MS/MS using the product ion m/z 184 (trimethylammonium ethyl phosphate ion) as the typically used common fragment ion of phospholipids [21,28]. The analysis of phospholipids in plasma in this study showed that they were hardly retained on the fluorous LC column (Fig. 3), meaning that the multi-fluorous derivatives of polyamines could be effectively separated from the interfering endogenous components in the biological matrix. These results showed that the present method realized the reliable determination of polyamines in human plasma samples by LC-MS/MS without interference from endogenous components.

Table 4. Evaluation of matrix effects using the present method.

| Matrix effect (%) | 10 nM | 50 nM | 100 nM |
|-------------------|-------|-------|--------|
| Put               | 110 ± 8.2 | 102 ± 6.3 | 100 ± 8.1 |
| Cad               | 106 ± 4.4 | 101 ± 4.1 | 100 ± 8.3 |
| Spd               | 109 ± 7.3 | 96.5 ± 7.7 | 105 ± 13 |
| Spm               | 97.3 ± 5.7 | 97.7 ± 1.9 | 105 ± 11 |

3.5. Analysis of human plasma samples

The determination of polyamines in several native human plasma samples (n = 5) from healthy volunteers was also carried out using this method. As a result, trace amounts of endogenous polyamines were successfully detected in all examined plasma samples (Fig. 3). The concentration ranges of the polyamines in human plasma were as follows: Put 21.8–94.1, Cad 1.9–4.7, Spd 47.8–184.4, and Spm 28.7–75.9 pmol/mL plasma (Table 5). Although the reports concerning the determination of polyamines in human plasma are uncommon, these results are within the expected range according to values taken from the Human Metabolome Database (http://www.hmdb.ca/) [29].

Table 5. Polyamines determined in human plasma samples.

| Sex, age | Determined concentration (pmol/mL plasma) |
|----------|-----------------------------------------|
| Put      | Cad      | Spd   | Spm   |
| Female, 22 | 30.1 | 1.9  | 47.8  | 74.2  |
| Female, 23 | 21.8 | 2.7  | 52.4  | 43.9  |
| Female, 23 | 88.1 | 4.7  | 138.6 | 28.7  |
| Female, 28 | 94.1 | 2.7  | 184.4 | 74.1  |
| Male, 38    | 84.0 | 4.6  | 146.6 | 75.9  |

4. Conclusions

A selective and sensitive LC-MS/MS method was developed for determining polyamines in human plasma samples. In this study, polyamines were easily derivatized with a relatively short fluorous-tag reagent, STFN, to obtain multi-fluorous derivatives. The retention of the obtained derivatives on the fluorous LC column gradually increased as the number of perfluoroalkyl chains increased, whereas the retention of non- and mono-fluorous derivatives was poor. Moreover, positive ESI-MS/MS detection enabled highly sensitive analysis of the multi-fluorous polyaniline derivatives. In addition, this simple derivatization method was successfully applied to the determination of trace amounts of polyamines in human plasma samples without interference from the biological matrix. Accordingly, we believe that this method will be helpful and useful in accurately quantifying polyamines in various biological samples for clinical diagnosis.

Acknowledgements

This work was supported by JSPS KAKENHI (Grant Numbers JP26410166 and JP26460182) and by a fund (No. 147107) from the Central Research Institute of Fukuoka University. We extend thanks to Mr. N. Kaneta and Ms. M. Kuba (Faculty of Pharmaceutical Sciences, Fukuoka University) for excellent technical assistance.

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