Report

Determination of Serum Spermidine by High-Performance Liquid Chromatography after Fluorescence Derivatization with Orthophthalaldehyde

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Abstract

A sensitive method was developed for the determination of serum polyamines, which consisted of (1) deproteinization with perchloric acid, (2) separation of spermidine from amino acids by CM-Sephadex C-25 column chromatography, (3) pre-column derivatization with orthophthalaldehyde, and (4) high-performance liquid chromatography on SP-5PW cation exchanger with fluorescence detection. The detection limit (S/N=3) of the present method was 0.5 pmol per injection, which allowed the assay of spermidine with a small volume of serum. Linearity, recovery and reproducibility of the method were highly satisfatory. The concentrations of spermidine in sera were determined to be 93.2±31.2 and 83.0±27.9 pmol/ml (mean ± SD) for healthy men (n=22) and women (n=18) volunteers, respectively.

Introduction

Polyamine level in biological fluids has recently attracted much attention as one of the tumor markers.[1-16] Thus, a number of methods have been reported for the determination of polyamines such as putrescine, spermidine and spermine in biological samples.[17] They are enzyme assay,[18-22] thin-layer chromatography,[2, 5, 23-24] gas-liquid chromatography (GC),[25] GC-mass spectrometry,[26] and high-performance liquid chromatography (HPLC) including amino acid analyzer.[6, 10, 14, 27-35] Among them, HPLC methods have been widely used for the assay of polyamines in urine, blood and tissues, in combination with pre- or post-column derivatization with fluorogenic reagents such as dansyl chloride[10, 14, 27-30] and orthophthalaldehyde (OPA)-2-mercaptoethanol.[32-35] However, few reports have appeared on the determination of polyamines in serum or plasma[29-32] because of their low contents in the body fluids. A sensitive method which allows the assay of low levels of

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polyamines seems to give useful information on the efficacy of therapies and the recurrence of the diseases of individual patients.[3-6, 8-9, 31, 36]

Spermidine, a major polyamine in serum or plasma,[29, 31] has been known to form a stable fluorescent adduct upon reaction with OPA alone sequentially in alkali and then in acid.[37, 38] This fluorescence reaction is relatively specific and highly sensitive for spermidine. By using this reaction as a pre-column derivatization of spermidine, we developed a simple and sensitive HPLC method for the assay of serum spermidine.

Experimental

Materials

Spermidine triphosphate hexahydrate, diethylenetriamine and OPA were purchased from Nakarai Chemicals, Kyoto, Japan. OPA solution was prepared by dissolving in methanol before use. CM-Sephadex C-25 (Pharmacia Fine Chemicals AB Uppsala, Sweden) was completely swollen with boiling deionized and distilled water for 2 hours. The CM-Sephadex C-25 column was prepared by packing 0.1 ml of the resin in a disposable syringe (1 ml, Terumo, Japan). All other reagents used were of analytical grade. Serum samples were provided by Dr. S. Kinoshita of the Health Administration Center of Hokkaido University.

Instrumentation

The high-performance liquid chromatographic system consisted of a Shimadzu LC 5A pump (Shimadzu, Kyoto, Japan), a Reodyne Model 7125 injector with a 100 μl loop (Reodyne, Calif., USA), a Shimadzu RF-530 spectrofluorometer equipped with a 12 μl flow cell, and a Shimadzu chromatographic date processor Model CR-3A. Fluorescence measurements were made with a Shimadzu Model RF-500 fluorescence spectrophotometer using 10 mm-diameter cuvettes. For centrifugation, Kubota Model 1900 micro centrifuge (Kubota, Tokyo, Japan) was used.

Fluorescence reaction of spermidine with OPA

To a sample solution (1.28 ml) containing spermidine was added 120 μl of 0.1 M NaOH to give final pH of 11.8. The reaction was initiated by addition of 60 μl of 2 mM OPA. After reaction at 40℃ for 15 min, the mixture was acidified to pH 2.5 by addition of 64 μl of 0.3 M sulfuric acid and its fluorescence intensity at 396 nm was measured at an excitation wavelength of 350 nm. For the pre-column derivatization of spermidine, this fluorescence reaction was carried out with half the volumes of reagents and samples as described above.

Determination of free spermidine in serum

To a serum sample (300 μl) was added 300 μl of 1 M perchloric acid. The mixture was shaken and centrifuged at 3000g for 10 min. An aliquot (450 μl) of the supernatant was removed, neutralized with 112 μl of 2 M KOH, placed in an ice bath for about 10 min, and recentrifuged at 3000g for 10 min. The supernatant (480 μl) was applied to a CM-Sephadex C-25 column. Amino acids adsorbed in the column were removed by washing once with 1.0 ml of water and then three times with 1.0 ml of 0.05 M Na_2SO_4. The adsorbed spermidine was then eluted with 600 μl of 0.5 M Na_2SO_4. To the eluate, 40 μl of diethylenetriamine was added as an internal standard. After

the mixure was subjected to pre-column derivatization with OPA as described above, a 100 μl portion of the final reaction mixture was analyzed by HPLC. The amount of free spermidine in serum was calculated from the ratio of the peak area of spermidine to that of the internal standard.

Determination of total spermidine in serum

The deproteinized serum (450 μl) was mixed with an equal volume of concentrated hydrochloric acid and heated at 105℃ for 16 hours in a sealed tube. A 800 μl portion of the hydrolysate was evaporated in vacuo and the residue obtained was dissolved in 1.0 ml of distilled water. A portion (900 μl) of the solution was subjected to the CM-Sephadex C-25 column. Spermidine was eluted from the column, derivatized with OPA and analyzed by HPLC in the same manner as described above.

Chromatographic conditions

An HPLC column (75×7.5 mm) packed with a cation exchanger (TSK gel SP-5PW, 10 μm, Toyo Soda, Tokyo, Japan) was preceded by a column of TSK gurdgel SP-5PW (10×6 mm, Toyo Soda). The column temperature was ambient. The mobile phase was 0.2 M potassium phosphate buffer (pH 7.0) at a flow-rate of 0.9 ml/min. The fluorescence intensity of eluent was monitored with an emission wavelength at 396 nm and an excitation wavelength at 350 nm.

Results and Discussion

Primary amines including polyamines react with OPA in the presence of thiol such as 2-mercaptoethanol to give fluorescent isoindoles.[39] Though this reagent system has been used for post-column fluorescence derivatization of polyamines, its practical application to pre-column derivatization is limited because of the instability of the fluorescent isoindoles.[40]

On the other hand, spermidine produces a stable fluorescent adduct upon reaction with OPA alone in alkaline medium, followed by acidification of the solution.[37, 38] In order to employ this fluorescence reaction to the pre-column derivatization of spermidine, we investigated this reaction in detail to optimize its reaction conditions. The standard procedure was established and shown in Experimental Section. Table 1 shows the specificity of this fluorescence reaction. Spermidine showed a stable fluorescence with an excitation wavelength at 350 nm and an emission wavelength at 396 nm. The fluorescence intensities seen with other polyamines such as spermine, putrescine and cadaverine were less than 0.7 % of that with spermidine. N-Acetylspermidines showed no detectable fluorescence. Though glutathione, histamine or histidine gave fluorescent adduct with an intensity less than 9 % of that with spermidine, other amino acids and biological amines showed no fluorescence. These results indicate that this fluorescence reaction is relatively specific for spermidine. Since the fluorescent adduct formed from spermidine was stable in the final acidic solution for at least 24 hours at 4℃, this fluorescence reaction can be used for pre-column derivatization of spermidine.

Though the derivatization was relatively specific for spermidine, deproteinization and separation of spermidine from amino acids were needed prior to the derivatization for the assay of serum spermidine, since most of the OPA was consumed by reaction with proteins and amino acids

Table 1 Specificity of the fluorescence reaction with OPA.

compound	RFI
Spermidine	100
Putrescine	< 0.01
Cadaverine	< 0.01
Spermine	0.7
N^1-Acetylspermidine	< 0.01
N^8-Acetylspermidine	< 0.01
Histamine	4.1
Serotonin	< 0.01
Glutathione	8.7
Histidine	0.3
Other amino acids and amines	< 0.01

Fluorescence intensities at 396 nm were measured using an excitation wavelength at 350 nm after reaction with OPA as described in Experimental, and corrected on the same molar basis. The intensity due to 0.78 μM spermidine was taken as 100. RFI : Relative fluorescence intensity.

present in serum at considerably higher levels. Both amino acids and spermidine were adsorbed on a mini-column packed with the CM-Sephadex C-25, however, amino acids were first eluted with 0.05 M Na_2SO_4 and then spermidine with 0.5 M Na_2SO_4. The recovery of spermidine was quantitative as shown below.

Glutathione and histidine being contained in serum at higher levels than spermidine, developed fluorescence in the reaction with OPA though their intensities were lower than with spermidine (Table 1). These interfering substances were also adsorbed on the CM-Sephadex C-25 column and were easily separated from spermidine by elution with 0.05M Na_2SO_4. On the other hand, histamine and spermine could not be separated from spermidine by the column chromatography. However, their interference were negligible because of their low fluorescence intensities (Table 1) and low concentrations in serum. According to these basic data, serum spermidine can be measured specifically by the fluorometric method with OPA after separation by CM-Sephadex C-25 column chromatography. In the present study, however, we also employed the HPLC method to attain higher accuracy and sensitivity which allows the assay of serum spermidine with smaller volumes of serum samples.

Figure 1a shows the HPLC chromatogram of standard spermidine after derivatization with OPA. Diethylenetriamine, which is a synthetic triamine and gives a fluorescent adduct under the same derivatization conditions, was used as an internal standard. The OPA adducts of spermidine and diethylenetriamine were completely separated from each other with retention times of 11.0 and 13.8 min, respectively, by an HPLC equipped cation exchanger SP-5PW column with 0.2 M phosphate buffer (pH 7.0) as a mobile phase at a flow rate of 0.9 ml/min. The peak intensities were found to be constant over the range of pH 2-9 of mobile phase. The relative peak areas of spermidine to the internal standard were plotted against the amounts of spermidine and shown in Fig. 2. The linear calibration curve ($r=0.999$) was obtained over the range of 1-50 pmol with

detection limit (S/N=3) of 0.5 pmol spermidine per 100 μl of injection volume.

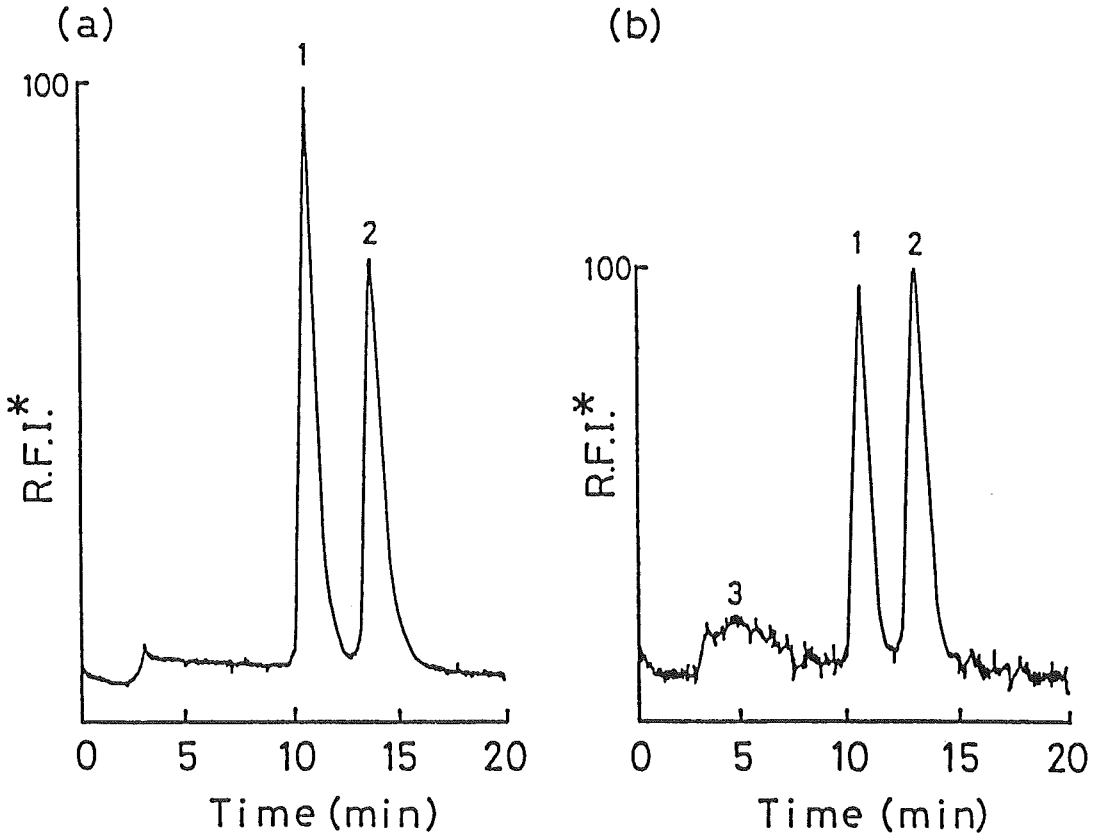

Fig. 1 High-performance liquid chromatograms of standard spermidine(a) and human serum(b) pre-derivatized with OPA.
Chromatographic conditions were as decribed in Experimental. RFI ; Relative fluorescence intensity. Amount of standard spermidine or diethylenetriamine (internal standard) injected in a 100 μl volume was 10 pmol or 100 pmol, respectively. peak 1 : spermidine, peak 2 : diethylenetriamine, peak 3 : unknown.

Figure 1b shows a typical HPLC chromatogram with human serum after the deproteinization, the CM-Sephadex column chromatography and the derivatization with OPA. The OPA adducts of spermidine and the internal standard appeared at 11.0 and 13.8 min, respectively. The peaks at the retention time of 3.0-7.0 min were due to unknown substances. The recovery and reproducibility of this method were tested using 300 μl of pooled serum as a sample. The spermidine content in the serum was estimated to be 95.7±2.1 pmol/ml (mean ± SD, n=5). The recovery of spermidine added to the serum (15 pmol/300 μl) was 101±2.5% (mean ± SD, n=5). The relative standard deviation of within-day and day-to day of this method were 2.1 and 2.7%, respectively (n=5). The contents of spermidine in sera of healthy men (n=22, 20-40 years), and women (n=18, 20-40 years) were determined to be 92.3±31.2 and 83.0±27.9 pmol/ml (mean ± SD),

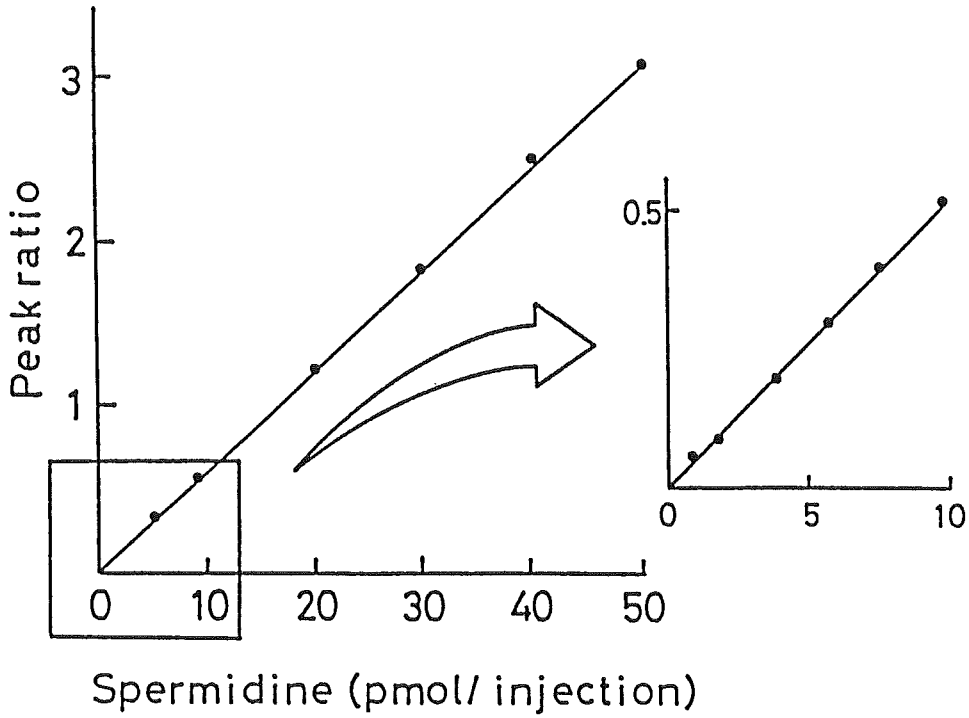

Fig. 2 Calibration curve of the present method for spermidine.
The ratio of peak area with spermidine to that with internal standard (diethylenetriamine) was plotted against the amounts of spermidine. The assay conditions were as described in Experimental.

respectively.

Monoacetylspermidines are also present in serum as conjugated forms of spermidine. Since the acetylspermidines showed no fluorescence in the reaction with OPA (Table 1), the serum spermidine measured as above was free spermidine. Therefore, total spermidine was determined after hydrolysis of serum samples with hydrochloric acid. The concentrations of total and free spermidines were determined using one pooled serum to be 103.3 ± 0.93 and 69.3 ± 1.32 pmol/ml (mean $\pm$ SD, n=5), respectively, indicating that about one-third the amounts of spermidine in serum was present as its acetyl derivatives.

Spermidine is present at higher levels in tissues and urine but at quite lower levels in serum and plasma. Among numerous methods reported so far, only a few methods have been employed for the assay of serum spermidine[29-32]. These methods do not appear to be sensitive enough for clinical use, since more than 2 ml of serum or plasma are necessary to assay spermidine. Therefore, this method offers several advantages such as sensitivity and simplicity over the other methods reported so far ; the present method allows the determination of spermidine with 300 μl of serum.

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