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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FREE AND TOTAL POLYAMINES IN HUMAN SERUM AS FLUORESCAMINE DERIVATIVES

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SUMMARY

A highly sensitive and simple fluorimetric method for the determination of free and total polyamines, spermidine, spermine, putrescine and cadaverine, in human serum by high-performance liquid chromatography is described. The polyamines, obtained after clean-up of deproteinized serum by Cellex P column chromatography, are converted to their fluorescamine derivatives in the presence of nickel ion which inhibits the reaction of interfering amines with fluorescamine, and the derivatives are separated simultaneously by reversed-phase chromatography (LiChrosorb RP-18) with a linear gradient elution. The lower limits of detection are 10 and 5 pmole for spermine and the others in 0.5 ml of serum, respectively.

INTRODUCTION

In recent years, it has been described that the amounts of polyamines, spermidine (Spd), spermine (Spm) and putrescine (Put), are increased in growing tissues [1—3] and cancerous tissues [4—8] and thus the amounts of the

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amines are useful for the evaluation of the efficacy of chemotherapy on cancer [9—11]. Therefore, the quantitation of the polyamines in physiological fluid has become of interest in the possible early diagnosis of malignancy. Several methods have been reported (e.g., enzymatic, ion-exchange chromatographic, gas chromatographic, thin-layer chromatographic and liquid chromatographic methods), which were recently reviewed by Seiler [12]. Among the methods, ion-exchange chromatographic methods using amino acid analysers are popular, but have a limited sensitivity.

The concentrations of the polyamines, Spd, Spm, Put and cadaverine (Cad) in serum are normally very low, and so totals of free and acetylated polyamines have usually been measured after treatment of deproteinized serum with acid-hydrolysis [6, 13, 14]. The quantitation of free polyamines was described in a few reports [15—17].

Samejima [18] described a sensitive fluorimetric method coupled with high-performance liquid chromatography (HPLC) of fluorescamine (FA) derivatives of the polyamines. In the method, the derivatives of Put and Cad and those of Spd and Spm were separately subjected to HPLC because of insufficient resolution of the derivative of Spd from that of Cad. Therefore, the method in practical use required a column chromatographic separation of Put and Cad from Spd and Spm prior to reaction with FA [16].

Recently, we found that FA derivatives of the four polyamines and 1,6-hexanediamine can be separated simultaneously by reversed-phase HPLC, and that the reaction of many biogenic amines other than the polyamines with FA is inhibited considerably by nickel ion, which may serve to minimize the interference from those amines. This paper describes a simple HPLC method with fluorescence detection for the determination of free and total polyamines in human serum utilizing the above findings. 1,6-Hexanediamine is used as an internal standard.

EXPERIMENTAL

Materials and reagents

Sera were obtained from Kyushu University Hospital and healthy volunteers in our laboratory. All chemicals were of reagent grade, unless otherwise noted. Double-distilled water and solvents were used. FA and Cellex P were obtained from Japan Roche (Tokyo, Japan) and Bio-Rad (Richmond, Calif., U.S.A.), respectively.

Cellex P column. Cellex P (H^+ form, 0.9 mequiv./g) should be washed to remove contaminant before use according to the Kremzner and Wilson method [19] with some modifications as follows. Wash 20 g of Cellex P successively with ca. 500 ml of 0.2 *M* sodium hydroxide, ca. 400-ml portions of water, 0.5 *M* hydrochloric acid, water, 1 *M* sodium chloride, water, 1 *M* sodium carbonate, water, 0.1 *M* sodium hydroxide, water, ethanol and water, ca. 800-ml portions of 0.5 *M* sodium chloride and water, and finally ca. 400-ml portions of 0.2 *M* and 0.01 *M* sodium phosphate buffers (pH 6.0). Suspend the washed Cellex P in ca. 500 ml of 0.01 *M* sodium phosphate buffer (pH 6.0) and store in a refrigerator. When required for use, add 0.01 *M* sodium phosphate buffer (pH 6.0) to three times the settling volume of Cellex P after

standing for 1 h, mix and pour onto a glass column (15 × 0.5 cm I.D.) to be 3.0 cm height after settling.

Mobile phase in HPLC. For methanol linear gradient elution, two solutions (A and B) are required. Prepare first a salt solution by dissolving 13.37 g of ammonium chloride, 15.76 g of sodium benzenesulfonate and 1 g of acetic acid in ca. 400 ml of water, adjust the pH to 4.0 at 25° with 1 *M* sodium hydroxide and dilute with water to 500 ml (ammonium chloride, 0.5 *M*; sodium benzenesulfonate, 0.175 *M*). Prepare solution A by mixing 100 ml of the salt solution with 175 ml of water and 225 ml of methanol, and solution B by mixing 100 ml of the salt solution with 400 ml of methanol. Both solutions are de-gassed in the usual manner before use.

Apparatus

The HPLC system consisted of a Mitsumi liquid chromatograph equipped with a 013 high-pressure piston pump and a 7120 syringe loading sample injector, a Hitachi solvent gradient device, a Hitachi 203 spectrofluorimeter equipped with a Hitachi flow-cell unit (cell volume, 20 μl) and a Hitachi 056 recorder (chart speed, 2.5 mm/min). The fluorescence intensity was monitored at the emission wavelength of 490 nm with the excitation wavelength set at 390 nm. A stainless-steel column (15 × 0.4 cm I.D.) was packed with LiChrosorb RP-18, 5 μm (Japan Merck, Tokyo, Japan) as previously described [20]. The column can be used for more than 350 injections with only a small decrease in the theoretical plate number. The column temperature was kept at 30 ± 0.5° by circulation of water through a glass-jacket fitted with the column to obtain certain retention times of peaks in the chromatogram. A Hitachi-Horiba M-7 pH meter was used.

Procedure

Place 0.5 ml of serum in a centrifuge tube, add 0.1 ml of 4 nmole/ml 1,6-hexanediamine as an internal standard and dilute with water to 1.0 ml. Add 0.1 ml of 3 *M* perchloric acid and centrifuge at 1200 *g* for 5 min. Adjust the pH of the supernatant to 7.0 ± 0.5 with 1.5 *M* potassium hydroxide, cool in ice-water and remove potassium perchlorate thus formed by centrifugation. For the sample solution of free polyamines, add 1.0 ml of 0.1 *M* sodium phosphate buffer (pH 7.0) to the supernatant, mix 0.5 ml of chloroform and 0.3 ml of methanol, and centrifuge at 1200 *g* for 5 min. Use the aqueous layer as the sample solution. For the sample solution of total polyamines, place the supernatant obtained after the removal of potassium perchlorate in a screw-cap culture tube, add 1.0 ml of concentrated hydrochloric acid, heat at 115° in an oil-bath for 12 h and dry in vacuo at room temperature to remove excessive acid. Dissolve the residue in 1.0 ml of water and then treat in the same way as in the preparation of the sample solution for free polyamines.

Load the sample solution on the Cellex P column, wash the column by pouring successively 2.0 ml of 0.01 *M* sodium phosphate buffer (pH 6.0), 1.0 ml of water and 1.5 ml of 0.05 *M* sodium chloride and then elute the polyamines and 1,6-hexanediamine with 2.0 ml of 3 *M* sodium chloride. To the eluate, add 0.5 ml of 0.4 *M* borate buffer (pH 9.0) and 0.2 ml of 20 m*M* nickel(II) sulfate, and add 0.5 ml of 1 m*M* FA solution in anhydrous acetone with vigorous mixing.

To the reaction mixture, add 1.0 ml of 0.3 *M* succinic acid, 1 g of sodium chloride and 0.4 ml of ethyl acetate. Shake the mixture vigorously for ca. 30 sec on a Vortex-type mixer and allow to stand for ca. 1 min. Transfer the organic layer to a test-tube, add 3.0 ml of cyclohexane, and extract the FA derivatives with 0.2 ml of 0.4 *M* borate buffer (pH 10.0) with mixing on a Vortex-type mixer for ca. 30 sec. Prepare reagent blanks by treating 0.5 ml of water instead of serum in the same way as for free and total polyamines. Inject 100 μl of each of the extracts of the tests and blanks into the chromatograph, elute by linear gradient elution between solution A and B during 25 min at a constant flow-rate of 1.0 ml/min, and monitor the fluorescence intensities of the eluates.

Calculate the ratios of net peak heights due to the individual polyamines in serum to that due to the internal standard. Read the concentrations of free or total polyamines from working curves prepared as follows. Treat 0.5 ml each of pooled serum added with 0 and 0.05—0.4 ml of the standard solution containing 0.5 nmole/ml each of the polyamines (0 and 25—200 pmole added, respectively) in the same way as for free and total polyamines in serum and plot the ratios of net peak heights due to each polyamine added to that due to the internal standard against the amounts of each polyamine added.
Typical working curves thus obtained are shown in Fig. 1.

RESULTS AND DISCUSSION

HPLC conditions

FA derivatives of the polyamines and 1,6-hexanediamine can be completely separated in 20 min by reversed-phase HPLC with linear gradient elution with a methanol concentration between 45 and 80% (Fig. 2).

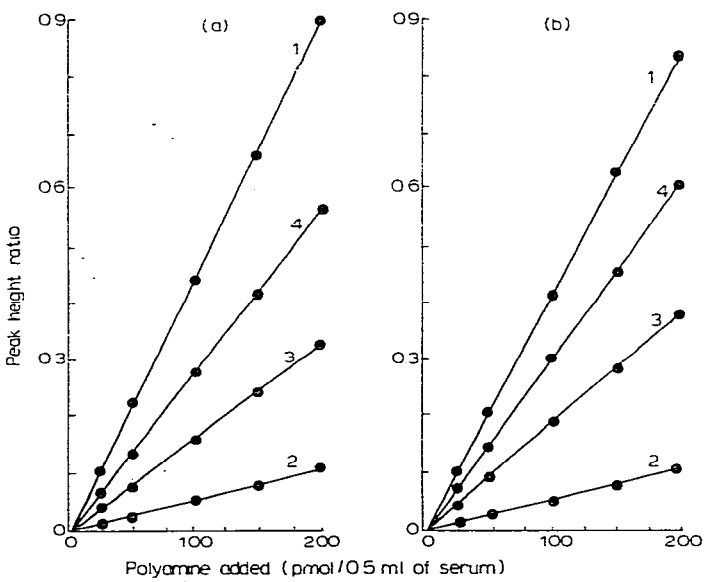

Fig. 1. Working curves for (a) free and (b) total polyamines. Curves: 1, spermidine; 2, spermine; 3, putrescine; 4, cadaverine.

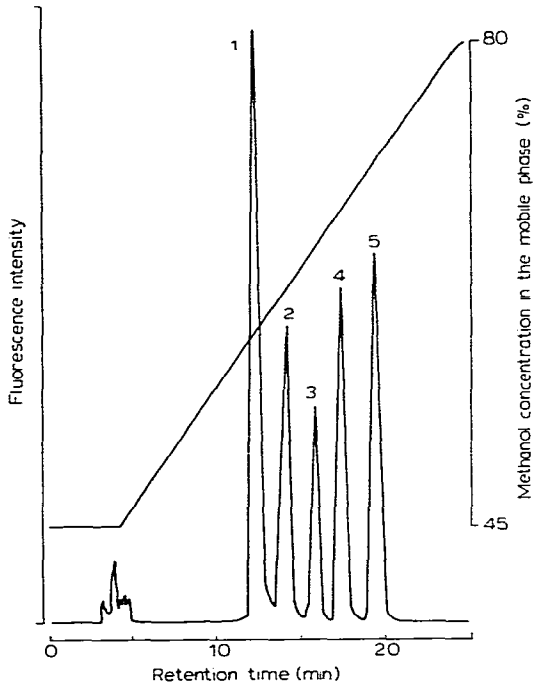

Fig. 2. Chromatogram of fluorescamine derivatives of spermidine, spermine, putrescine, cadaverine and 1,6-hexanediamine. A 0.5-ml aliquot of a standard mixture containing 4 nmole each of the amines was treated as described under *Procedure*. Peaks: 1, spermidine; 2, spermine; 3, putrescine; 4, cadaverine; 5, 1,6-hexanediamine.

The concentrations of ammonium chloride and sodium benzenesulfonate in the mobile phase affect the resolution of the FA derivatives. At the concentration of ammonium chloride lower than the prescribed concentration, 0.1 *M*, the separation of the derivatives of Spd, Spm and Put is incomplete, but the order of their elution remains unchanged, while the higher concentration causes delay of the elution. At the concentration of sodium benzenesulfonate lower or higher than the prescribed concentration, 0.035 *M*, the peaks due to Spd and Spm overlap or those due to Spm and Put nearly overlap, respectively. The pH of the mobile phase affects the elution pattern of the derivatives. The peaks due to Spd and Spm are split into more than two peaks, at pH higher than 8, suggesting that those derivatives exist as more than two fluorescent species in the alkaline medium [16]. At pH higher than 6, the derivatives of Put and Cad elute earlier than that of Spm, and at pH lower than 3, an insufficient resolution of the peaks due to Spd and Spm occurs. At the recommended pH 4, the derivatives of Spd and Spm fluoresce most intensely and the five peaks are well separated.

The relative retention times of FA derivatives of FA-reacting biological amines examined are tabulated in Table I. FA derivatives of many amines eluted earlier than those of the polyamines, but the derivatives of several amines eluted closely to those of the polyamines, which may interfere with the determination of the polyamines if present in the sample solution for

TABLE I

RELATIVE RETENTION TIMES* OF FLUORESCAMINE DERIVATIVES OF THE POLYAMINES AND OTHER BIOLOGICAL AMINES

To a mixture of 2.0 ml of water and 0.5 ml of 0.4 *M* borate buffer (pH 9.0) together with 10 nmole of the amine, 0.5 ml of 1 m*M* fluorescamine solution was added, and 100 µl of the mixture was subjected to HPLC.

Compound	Relative retention time	Compound	Relative retention time
Guanidine	not detectable	Alanine	0.33 0.10**
Agmatine	not detectable	Dopamine	0.35
Histidine	0.05	Tyramine	0.47
Aspartic acid	0.07	Methionine	0.54 0.09**
Glutamic acid	0.07	Tryptophan	0.64
Threonine	0.07	Spermidine	0.71
Tyrosine	0.07	Ornithine	0.76 0.06**
Citrulline	0.07	Spermine	0.77
Glutathione	0.07	1,3-Diaminopropane	0.80 0.59**
Carnosine	0.07	Leucine	0.81
Glycine	0.08	Angiotensin II	0.82 0.54**
Valine	0.08	Lysine	0.82 0.67 0.08**
Histamine	0.08	Isoleucine	0.82 0.35 0.10**
Urea	0.08	Putrescine	0.83
DOPA	0.09	Phenylalanine	0.88 0.37**
Serotonin	0.09	Cadaverine	0.89
Noradrenalin	0.25	1,6-Hexanediamine	1.00

*Retention time of fluorescamine derivative of 1,6-hexanediamine was taken as 1.00.
**Two or three peaks were observed in the chromatograms.

HPLC. Therefore the sample solution should be free from such interfering amines.

Sample solutions for HPLC

The extraction of free and total polyamines, the deproteinization of serum and the acid hydrolysis of acetylated polyamines can be accomplished by the usual methods [6, 13, 14].

Prior to loading of the deproteinized sample solution or the solution obtained after the acid hydrolysis on the Cellex P column for clean-up, lipids in the solutions should be removed to effuse smoothly through the column. This could be done by extraction with chloroform and methanol. The interfering amines held in the column after loading, which may be very large amounts as compared with those of the polyamines, can be removed by successive elution with 0.01 *M* sodium phosphate buffer, water and 0.05 *M* sodium chloride. The polyamines and the internal standard are then eluted with 3 *M* sodium chloride.

Very small amounts of primary amines, which eluted near the FA derivatives of the polyamines in HPLC (see Table I), remained even after the clean-up and occluded as contaminants from the reagents, glass-ware and laboratory environment in the sample solutions and interfered with the determination of

nmole to pmole quantities of the polyamines. Nickel ion inhibits the derivatization of the interfering amines with FA by 40—93%, but not that of the polyamines, under the conditions of the derivatization at pH 9.0 (Table II), which is useful to minimize the interference of those amines in the procedure. No effect of the nickel ion is observed if added after the reaction with FA.

The derivatization of individual polyamines is weakly affected by the pH of the buffer used in the presence of a high concentration of sodium chloride as in the present procedure. Although the most intense fluorescence is obtained in the reaction at pH 8.8—9.5 for Put, Cad and 1,6-hexanediamine, and at pH 7.0—7.5 for Spd and Spm, a small difference between the fluorescence intensities obtained at pH 9.0 and 7.5 is observed. Thus a borate buffer of pH 9.0 was used in the procedure.

FA derivatives of the polyamines and 1,6-hexanediamine in the reaction mixture can be completely extracted with a small amount of ethyl acetate after acidification of the mixture with succinic acid in the presence of sodium chloride. When the extract is subjected to HPLC, an insufficient separation of the derivatives is observed because of broadening of the peaks. A high resolution of the peaks is achieved by back-extraction of the FA derivatives in the extract with a small amount of a borate buffer of pH 10.0. This extraction serves to concentrate the FA derivatives and the peak heights thus observed are ca. 7 times those obtained without the extraction.

TABLE II

INHIBITORY EFFECT OF NICKEL ION ON THE REACTION OF BIOLOGICAL AMINES WITH FLUORESCAMINE

To 10 nmole of the amine in a mixture of 2.0 ml of 3 *M* sodium chloride and 0.5 ml of 0.4 *M* borate buffer (pH 9.0) mixed with 0.2 ml of 20 m*M* nickel sulfate solution or water, 0.5 ml of 1 m*M* fluorescamine solution was added, and the fluorescence intensity was measured with a Hitachi MPF-4 spectrofluorimeter in a cell of 1 × 1 cm at the emission wavelength of 480 nm with the excitation wavelength at 394 nm.

Compound	% Inhibition	Compound	% Inhibition
Histidine	100	Lysine	54
Glutathione	95	Phenylalanine	41
Angiotensin II	93	Isoleucine	40
Histamine	93	Agmatine	0
Tryptophan	91	Spermidine	0
Ornithine	88	Spermine	2
1,3-Diaminopropane	58	Putrescine	0
Leucine	58	Cadaverine	0
Arginine	56	1,6-Hexanediamine	0

Determination of polyamines in serum

Fig. 3 shows typical chromatograms obtained with normal serum and the reagent blank under the conditions of the procedure without acid hydrolysis. The polyamines and 1,6-hexanediamine (internal standard) are completely separated after 11—20 min, in which range of time no peaks ascribable to interfering amines are observed. Three small peaks in the reagent blank (Fig.

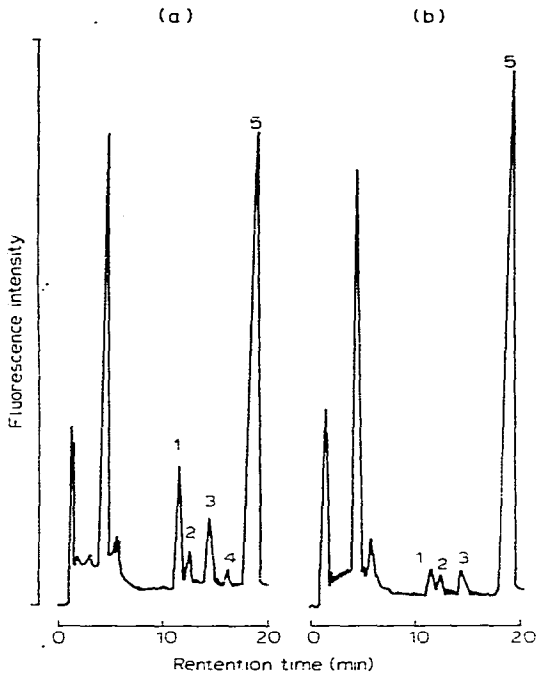

Fig. 3. Chromatograms of fluorescamine derivatives of free polyamines in normal serum (a) and the reagent blank (b), obtained according to the procedure. Peaks: 1, spermidine; 2, spermine; 3, putrescine; 4, cadaverine; 5, 1,6-hexanediamine.

3b, peaks 1—3) have exactly the same retention times as those of Spd, Spm and Put in serum sample (Fig. 3a, peaks 1—3), and increase in their heights when the blank added with Spd, Spm and Put is used, indicating that the peaks are caused by those polyamines occluded as contaminants. When a reagent blank prepared without chromatography on the Cellex P column is subjected to HPLC, only weak peaks ascribable to those amines are observed. This fact suggests that the amines are due mainly from the Cellex P column chromatographic treatment. More intense peaks are observed even if a fresh column packed with commercial Cellex P without washing, as described in the Experimental section, is used, which may interfere with a highly sensitive determination of the polyamines in serum sample. The same is also true when a column used for serum sample or reagent blank is re-used. When the reagent blank is repeatedly applied on the LiChrosorb RP-18 column, constant heights of the peaks due to the polyamines as contaminants are obtained. Thus, in calculation of peak height ratios in the procedure, peak heights of peaks 1—3 in the reagent blank should be subtracted from those in the serum sample.

Linear relationships were observed between the ratios of the peak heights of the polyamines to those of the internal standard and the amounts of the polyamines added in the range of 10—200 pmole to 0.5 ml of serum, both in free and total polyamines (cf. Fig. 1), and no change of the slopes in the graphs of the relationships was observed depending on the serum used. These facts indicate that the present internal standard method permits the deter-

TABLE III

FREE AND TOTAL POLYAMINE CONCENTRATIONS IN NORMAL HUMAN SERUM

Age	Free polyamine* (pmole/ml)				Total polyamine* (pmole/ml)			
	Spd	Spm	Put	Cad	Spd	Spm	Put	Cad
22	150	80	200	0	360	80	260	10
22	90	0	140	10	300	10	260	50
25	80	0	120	0	200	30	160	40
25	10	0	130	20	130	0	290	50
26	60	0	70	10	360	50	300	20
27	10	0	110	10	130	0	190	30
28	50	30	120	10	220	110	270	50
28	180	60	150	0	350	120	400	50
30	10	30	60	10	130	0	180	100
30	80	0	200	0	200	60	350	200
Mean	70	20	130	10	240	50	270	60

*Spd, spermidine; Spm, spermine; Put, putrescine; Cad, cadaverine.

mination of free and total polyamines in serum over wide ranges of their concentrations.

The recoveries of the polyamines added to 0.5 ml of serum in the amounts of 25—200 pmole were 97 ± 3% for free and total Put and Cad, and 75 ± 5% for free and total Spd and Spm. The recoveries were calculated from the determined values obtained with the fortified serum and standard mixtures of the polyamines treated as in the procedure.

The lower limits of detection for Spm and the others are 10 and 5 pmole in 0.5 ml of serum, respectively. The limit was defined as the amount giving a signal-to-noise ratio of 2. The precision of the method was examined by performing 10 assays separately on free and total polyamines in pooled serum containing free Spd, Spm, Put and Cad at 50, 30, 120 and 10 pmole/ml, and total ones at 220, 110, 270 and 50 pmole/ml, respectively. The coefficients of variation were 7, 8, 2 and 3% for free Spd, Spm, Put and Cad, and 4, 6, 2 and 2% for total ones, respectively.

The concentrations of free and total polyamines in the sera of 10 healthy men (22—30 years) determined by this method are shown in Table III. The mean values of individual polyamines are in good agreement with the published data [6, 13—17].

The proposed method is very sensitive and simple to perform, and therefore may be applicable for routine use.

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