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DETERMINATION OF POLYAMINES IN HYDROLYSATES OF UREMIC PLASMA BY HIGH-PERFORMANCE CATION-EXCHANGE COLUMN CHROMATOGRAPHY

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SUMMARY

The levels of putrescine, cadaverine, spermidine and spermine in uremic plasma were determined with an automatic polyamine analyzer with a 7.5 × 0.2 cm I.D. cation-exchange column using a stepwise sodium chloride gradient. All four polyamines were higher in ten patients with chronic renal failure than in eight normal subjects. The total polyamine content was also measured in the patients' plasma before and after maintenance dialysis; putrescine and spermidine levels were significantly lowered by the procedure.

INTRODUCTION

Among the methods reported for the separation and quantitation of polyamines [1], various amino acid analyzers coupled with continuous fluorescence monitoring showed good separations [2–6]. A number of papers have been published comparing the polyamine levels in the urine and plasma of cancer patients. To date, however, only a few [7, 8] have reported clinical applications of polyamine determinations in uremic patients. Numerous compounds are known to accumulate in the body fluids of uremic patients, among them polyamines have been proposed as one type of uremic toxin [9]. Recently, elevated polyamine levels in erythrocytes of uremic patients were described [10], and spermine was identified as an inhibitor of erythropoiesis [11]. But the relationship between uremic symptoms and accumulation of uremic toxins is not yet definitely established.

In this paper, a simple and rapid method for the simultaneous determination of putrescine, cadaverine, spermidine and spermine in the plasma of uremic patients is described.

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EXPERIMENTAL

Chemicals

Putrescine, cadaverine, spermidine and spermine were obtained in the form of hydrochlorides from Sigma (St. Louis, MO, U.S.A.). *o*-Phthalaldehyde (OPA) was purchased from Wako Pure Chemicals (Osaka, Japan).

Chromatographic system

Fig. 1 is a flow diagram of the automatic polyamine analyzer which was constructed in our laboratory. Two high-pressure piston pumps (Mitsumi Scientific Industry Co., Tokyo, Japan) served to pump the eluent and OPA solution. The column eluent was pumped through a six-port sample injection valve with a 100-μl sample loop. A jacketed, stainless-steel column, 7.5 × 0.2 cm I.D., was used, slurry-packed with CK-10S cation-exchange resin with a mean particle size of 11.5 μm (Mitsubishi Kasei Co., Tokyo, Japan). The column temperature was kept at 60°C with a Haake constant-temperature circulator (Haake Co., Berlin, G.F.R.). The reservoir of the OPA solution was kept in a refrigerator at 4°C to prevent degradation of the reagent. All buffer solutions were delivered by an automated buffer exchanger (Hijiri Seiko Co., Tokyo, Japan). A JASCO FP-550 fluorescence spectrophotometer (Japan Spectroscopic Co., Tokyo, Japan) was used to detect the fluorescent polyamine derivatives produced by the reaction with OPA—2-mercaptoethanol. The fluorescence setting was activated at 340 nm and emission was measured at 455 nm. The fluorometric

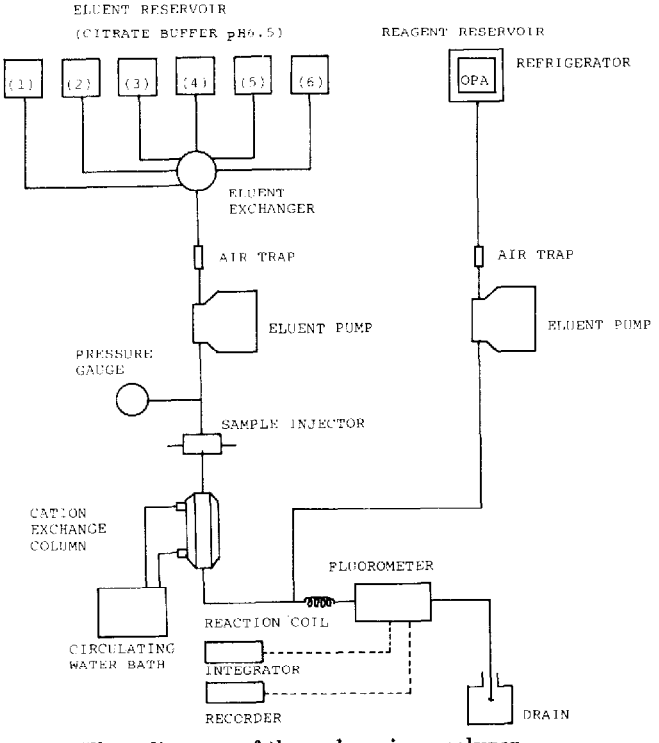

Fig. 1. Flow diagram of the polyamine analyzer.

signal was recorded using a JASCO RC-100 recorder at a chart speed of 12 cm/h. Peak areas were determined with a digital integrator (Chromatopac C-R1A, Shimadzu Seisakusho Co., Kyoto, Japan).

Eluent and reagent solutions

All reagents were of analytical grade, made up in double glass-distilled water. All aqueous reagents were filtered through a 0.45-μm microfilter (Fuji Photo Film Co., Tokyo, Japan), and degassed. Standard polyamine solutions were made in 0.5 *N* hydrochloric acid with putrescine, cadaverine, spermidine and spermine each at 0.5 nmol per 100 μl; these solutions could be kept at 4°C for a month without any degradation. The OPA solution was made with 0.8 g/l OPA dissolved in a 0.4 mol/l potassium borate solution, pH 10.5, and 2 ml/l 2-mercaptoethanol. Six buffers, each prepared with 0.25 *M* sodium citrate (pH 6.5) and sodium chloride, were used to elute polyamines with a stepwise gradient of 0, 0.2, 0.5, 1.5, 2.0 and 3.0 *M* sodium chloride. The first buffer was pumped through the column for 16 min after the introduction of the sample, the second for 2 min, the third for 4 min, the fourth for 7 min, the fifth for 8 min and the sixth for 33 min. The column was operated at a flow-rate of 12 ml/h and a column inlet pressure of 20 kg/cm^2. The OPA solution was pumped into the reaction coil at a flow-rate of 12 ml/h.

Preparation of plasma samples

Whole blood was centrifuged at 1000 *g* for 15 min immediately after removal from the patient. Plasma was mixed thoroughly for 3 min with 50% trichloroacetic acid to a final concentration of 5% trichloroacetic acid [12]. After standing at 4°C for 30 min and centrifugation at 2000 *g* for 20 min, the supernatant was lyophilized. The residue was hydrolysed in 3 ml of 6 *N* hydrochloric acid for 16 h at 110°C. The hydrolysate was evaporated in vacuo. The residue was dissolved in 0.5 ml of 0.5 *N* hydrochloric acid and centrifuged; 100-μl aliquots were separated. Treatment of standard polyamine solutions by this method resulted in recoveries of putrescine, cadaverine, spermidine and spermine of 94%, 88%, 93% and 91%, respectively.

RESULTS

A chromatogram of a standard mixture of polyamines, each at a concentration of 0.5 nmol per 100 μl, is shown in Fig. 2. The reproducibility of the analytical system was evaluated from ten chromatograms of standard samples. The retention times were: putrescine, 43.8 ± 0.2 min; cadaverine, 51.1 ± 0.3 min; spermidine, 55.5 ± 0.3 min; and spermine, 63.7 ± 0.4 min. The retention time of each polyamine compound showed a relative standard deviation of $\leqslant$ 0.6%. The deviation of the peak areas was within 4.4% for all the compounds.

Calibration curves for the polyamines in the range 100 pmol to 20 nmol are shown in Fig. 3. The minimum detectable quantities were 7.9 pmol of putrescine, 7.1 pmol of cadaverine, 6.0 pmol of spermidine and 10.0 pmol of spermine. The limit of detection was defined as the peak height twice that of the noise level. Typical chromatograms of total polyamines in a plasma sample of a normal subject and in a uremic patient are shown in Fig. 4A and B, respective-

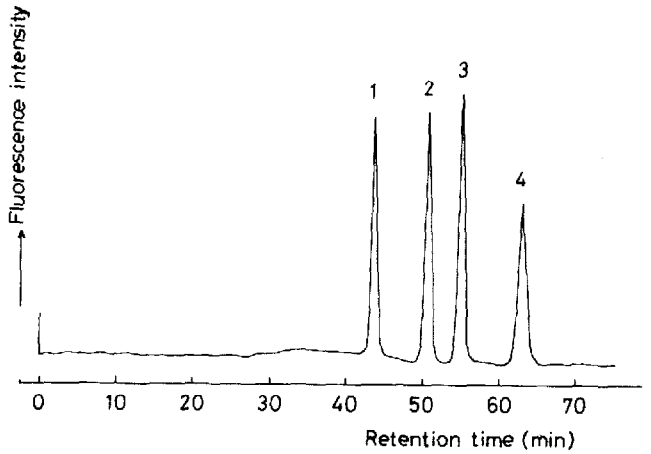

Fig. 2. Chromatogram of a standard mixture of polyamines, each at a concentration of 0.5 nmol per 100 µl. Chromatographic conditions: stationary phase, CK-10S (average particle size 11.5 µm) slurry-packed in a 7.5 × 0.2 cm I.D. stainless-steel column; mobile phase, 0.25 *M* citrate buffer (pH 6.5) with a stepwise sodium chloride gradient; flow-rate, 12 ml/h; detector, fluorescence was activated at 340 nm and emission was at 455 nm; recorder chart speed, 12 cm/h. Peaks: 1 = putrescine; 2 = cadaverine; 3 = spermidine; 4 = spermine.

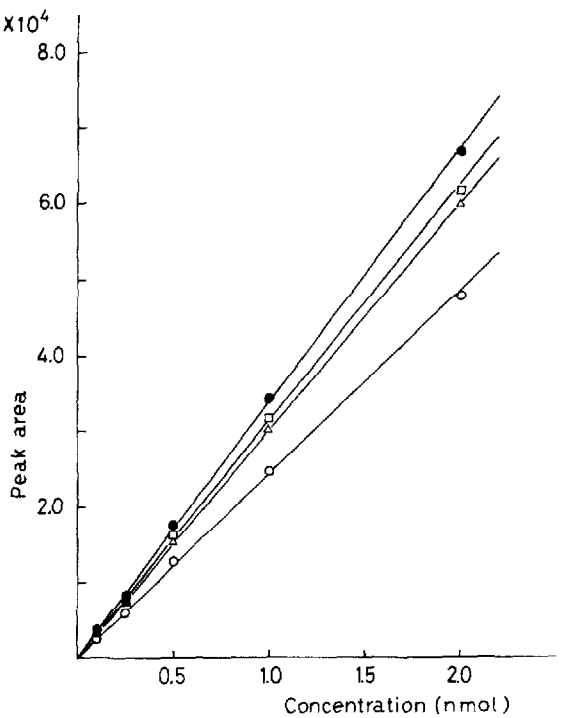

Fig. 3. Calibration curves of polyamines in high-performance liquid chromatography with fluorescence detection. One hundred microliters of a sample containing various amounts (100 pmol to 20 nmol) of polyamines were injected into the column and detected by a fluorescence detector. The conditions are described under Experimental procedures. (○) Putrescine; (△) cadaverine; (●) spermidine; (□) spermine.

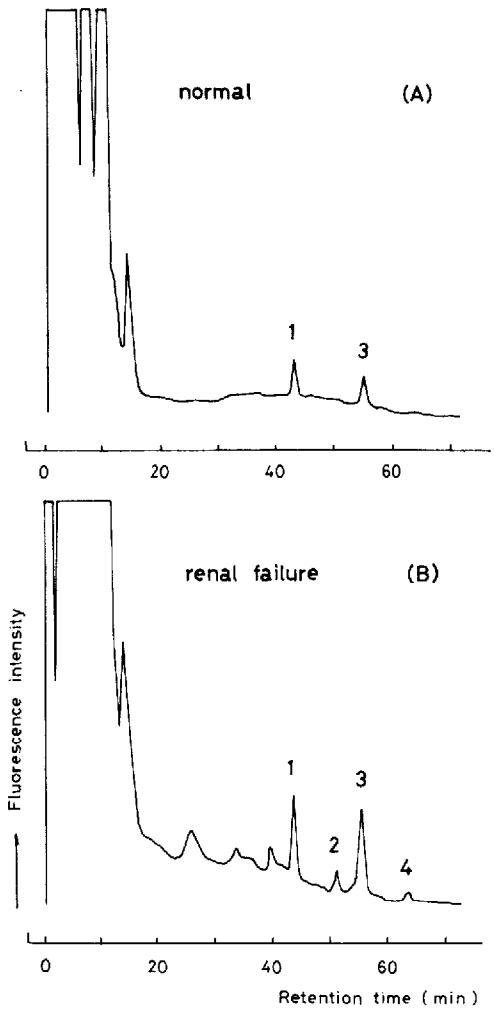

Fig. 4. Chromatograms of the hydrolysates of plasma samples. The conditions are described under Experimental procedures. (A) The hydrolysate of plasma sample from a normal subject. (B) The hydrolysate of plasma sample from a uremic patient. The polyamine peaks are numbered as in Fig. 2.

ly. In normal plasma the concentrations of putrescine and spermidine were 0.24 ± 0.10 (mean ± S.D.) nmol/ml and 0.23 ± 0.06 nmol/ml, respectively. Cadaverine and spermine were below detectable levels.

Table I compares the polyamine levels in patients with chronic renal failure before and after dialysis. The concentrations of all plasma polyamines were lower after the dialysis. In the present experiments we measured the concentration of total polyamines in normal and uremic plasma, and found that all polyamines were significantly elevated in uremic plasma. In ten patients undergoing maintenance hemodialysis, the total polyamine content was measured in plasma before and after dialysis. The mean values before dialysis for both putrescine and spermidine were significantly higher than those in normal subjects, while the mean cadaverine and spermine values were slightly but not

TABLE I

PLASMA LEVELS OF TOTAL POLYAMINES IN NORMAL SUBJECTS AND IN PATIENTS WITH CHRONIC RENAL FAILURE BEFORE AND AFTER DIALYSIS

Compound	Normal plasma (nmol/ml)*	Dialysis plasma (nmol/ml)**	
		Before	After
Putrescine	0.24 ± 0.10 (mean ± S.D.)	0.91 ± 0.43	0.42 ± 0.12
Cadaverine	N.D.***	0.22 ± 0.11	0.16 ± 0.09
Spermidine	0.23 ± 0.06	1.03 ± 0.64	0.45 ± 0.10
Spermine	N.D.	0.07 ± 0.03	0.04 ± 0.04

*n = 8.
**n = 10.
***N.D. = not detected.

significantly elevated. The values after dialysis for both putrescine and spermidine were almost the same as those prior to dialysis.

DISCUSSION

High-performance liquid chromatography with fluorometric detection has proved to be a useful and sensitive method for the determination of polyamines in body fluids [13—16]. The method selected for this study was a high-performance cation-exchange chromatographic method which efficiently separated a series of polyamines with a stepwise sodium chloride gradient. The reaction with OPA—2-mercaptoethanol was utilized for fluorescence detection. Analytical methods so far described using a modified automatic amino-acid analyzer were time-consuming. In our method, automatic quantitative determination was performed within 65 min and the column was regenerated repeatedly by washing with sodium hydroxide.

The method was successfully applied to the simultaneous determination of polyamines in uremic plasma. Quite a number of different amines accumulate in uremic serum [17—19]. We have already reported that the concentrations of aromatic and aliphatic amines were higher in uremic plasma than in normal plasma [20]. Since polyamines are present either free or in conjugated form in plasma [21], urine [22—24], and other body fluids [25], the samples have to be hydrolysed with 6 N hydrochloric acid. Campbell et al. [7] reported that the level of free polyamine in the serum, expressed in spermine equivalent, is elevated in children with uremia. In their report, serum polyamines were determined by a radioimmunoassay method using antispermidine and antispermine antibody. The main disadvantage was the lack of specificity of the method for spermidine and spermine. Swendseid et al. [10] measured free spermidine and spermine in the erythrocytes and putrescine in the urine of patients with renal failure using an amino acid analyzer. In our results, all four polyamines of plasma were higher in patients with chronic renal failure than in normal subjects. After maintenance dialysis, putrescine and spermidine levels were significantly lowered by the procedure.

A large number of samples of plasma and erythrocytes from normal subjects

and uremic patients are currently being analysed by our method, and the results will be the subject of another report.

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