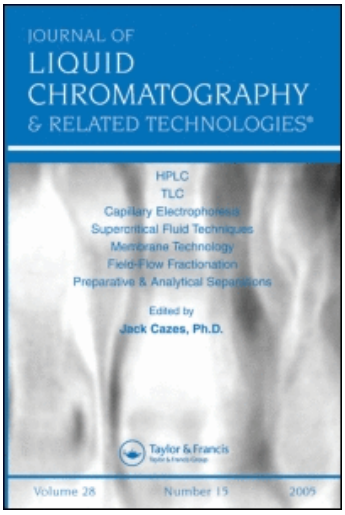

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AUTOMATED QUANTITATION OF POLYAMINES BY IMPROVED CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING A PUMP EQUIPPED WITH A PLUNGER WASHING SYSTEM

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ABSTRACT

An improved method for simple, precise, and sensitive quantitation of polyamines[putrescine, spermidine, spermine, cadaverine(in part) and 1,6-diaminohexane] by cation-exchange high performance liquid chromatography is described. Postcolumn derivatization using the o-phthalaldehyde method was employed to detect them. Analysis took 45 minutes and the minimum detectable amount of each polyamine was 10 - 20 pmol/μl. An isocratic pump equipped with a plunger washing system and a mobile phase of pH 5.3 are the advantages of this improved method. Consequently, an operator can carry out routine and automated polyamine analysis without any hesitation regarding maintenance of the pump or column damage due to crystal precipitation from the mobile phase on or in the pump, which has been a problem previously encountered with cation-exchange resin. Reproducibility of the day to day precision and duplicate determination, and simultaneous reproducibility of the standard mixture were calculated(CV<2.2%).

INTRODUCTION

Quantitation of polyamine is necessary to clarify the precise physiological roles and mechanisms of the regulation of cell proliferation in living organisms(1-3). Many methods, including ion-exchange chromatography(4), high-voltage paper chromatography(5,6), thin-layer chromatography(7), gas chromatography(8-10), HPLC with various columns(11-25), and enzymatic(26) and radioimmunoassays(27) have been employed to achieve this goal. However, most of these methods suffer from various disadvantages, such as reduced sensitivity, insufficient purification steps or time-consuming preparation. Furthermore, they cannot be automated, and thus are inconvenient for routine work. Many HPLC techniques have been used for the determination of polyamine levels(28). Although HPLC with column packed cation ion-exchange has many advantages, there is a problem with crystal precipitation on or in the pump. We modified this method(25) for determination of polyamines in various tissues of the rat and describe here a simple, reproducible, sensitive, automated and improved method involving cation-exchange HPLC and post-column derivatization with OPA.

MATERIALS AND METHODS

Chemicals

All the polyamines and diamine used for the preparation of standard solutions were purchased from Sigma (St. Louis, MO, USA). Potassium hydroxide, 2-mercaptoethanol, boric acid, o-phthalaldehyde, perchloric acid(60%), Brij-35, methanol and trisodium citrate dihydrate were obtained from Nakarai Tesque Inc. (Kyoto, Japan).

HPLC Equipment

Chromatographic analysis was performed with the JASCO analytical chromatographic system [Japan Spectroscopic Co., Ltd, (Tokyo Japan)], composed of an 802-SC system controller, two 880-PU intelligent HPLC pumps equipped with plunger washing system, an 851-AS intelligent sampler, an 860-CO column oven, an 821-FP intelligent spectrofluorometer, an 880-51 degasser and an 805-GI graphic integrator. For the analytical procedure, we used a Polyaminepak column (35 mm x 6mm) protected by a guard-pack column, both made by JASCO. The flow rates were 0.7 ml/min for both the mobile phase solution and the OPA reagent. The temperature of the column oven was kept at 70 ^{o}C throughout the experiment. After post-column derivatization with OPA, fluorescence intensity(excitation at 340 nm, emission at 450nm) was measured with the intelligent spectrofluorometer.

Buffer and OPA Reagent

The buffer solution for the eluation system was prepared by dissolution of 1.0 mol of tri-sodium citrate dihydrate into water in a final volume of 1.0 L. The pH was adjusted to 5.3 with the addition of perchloric acid. This solution was filtrated through a membrane filter [45 μm, from Advantec (Tokyo)] and degassed under a water aspirator at room temperature for 20 min.

The OPA-2-mercaptoethanol for the postcolumn derivatization procedure was prepared according to the method of Seilar and Knoden[23] with minor modifications. Boric acid (24.7 g) and potassium hydroxide (23.0 g) were dissolved in water in a final volume of 1.0 L. After the addition of 2.0 ml of 2-mercaptoethanol to the mixture, the solution was filtrated in the same manner as the buffer solution. This degassed solution was

mixed with 2.0 ml of Brij-35 solution and 1.6 g of OPA dissolv in 10 ml of methanol. The OPA reagent, which was mixed with t solution of the HPLC system behind the Polyaminepak column, w allowed to react with each separated polyamine within the rea tion coil in the column oven at 70 ^{o}C.

Sample Preparation

Male Sprague-Dowley rats (50 day-old, 170 - 180g) wei maintained on 24-h cycles of light/darkness with light from 06. to 18.30. Animal housing conditions were strictly controlled, a food and water were continuously available. Six rats of o group were simultaneously anesthetized with diethylether. A organs and tissues were immediately separated, weighed and th kept in 2.0 ml of 10% trichloroacetic acid (TCA) aqueous soluti in an ice bath. Each organ and tissue in the cold solution w homogenized with a Polytron homogenizer(KINEMATICA,TCU-2-11 Switzerland) and then centrifuged at 2500 rpm for 15 min. T supernatants were washed twice with 5 ml of diethylether eliminate the TCA in the water layer. The water layer was ke in a refrigerator at below -20 ^{o}C until measurement. Ju before measurement, 250 μl of the water layer was diluted with mobile buffer to 500 μl. The solution was filtrated by a mil pore[45 μm, Cosmonice from Nakarai(Kyoto Japan)] and 10 μl of the filtrate was charged with autosamper.

RESULTS AND DISCUSSION

Columns packed with reversed phase resin have been widel used for the determination of polyamines by HPLC, althou prelabeling of such polyamines as benzoyl(12-15), dabsyl(16-1 dansyl(18-19) and tosyl(20) is required. If the column

used in combination with the reagent of ion pairs(21,22,24), it is unnecessary to prepare such polyamine derivatives, but many problems have been encountered in the use of a gradient solvent system requiring two pumps. Therefore, reversed phase HPLC cannot be considered as the best method for the determination of polyamines. A column packed with cation-exchange resin has also been used occasionally for the quantitation of polyamines by HPLC. This method does not require gradient, does not involve pre-derivatization, and exhibits good selectivity and separation. However, there are maintenance problems caused by crystal precipitation on or in the plunger of the pump due to the high salt concentration of the mobile phase. In the present experiment, we used an improved method involving a reduction of the salt concentration and a pump equipped with a plunger washing system.

Effect of pH on the Retention Time of Polyamines

The relationship between the retention times of the polyamines and the change in the pH of the buffer from 5.0 to 6.2 is shown in Fig. 1. The retention times of all of the polyamines were dependent on pH. In other words, the delay in the eluation of the polyamines correlated with the decrease in the hydrogen ion concentration. Lowering of the pH value to 5.0 to shorten the eluation time, however, generally led to crystal precipitation. Therefore, a pH of 5.3 was considered to be the optimum and a buffer of pH 5.3 was used throughout this experiment to prevent crystal precipitation on or in the plunger.

The chromatograph of the standard mixture of polyamines measured by the improved HPLC at this pH indicated an eluation order of putrescine, spermidine, 1,6-diaminohexane and spermine, and good separation as shown in Fig. 2.

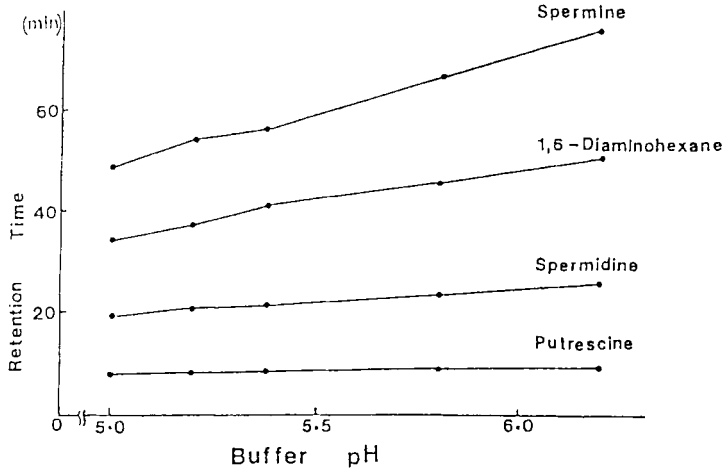

FIGURE 1. Relationship between the retention time of the polyamines and the pH of the mobile phase buffer.

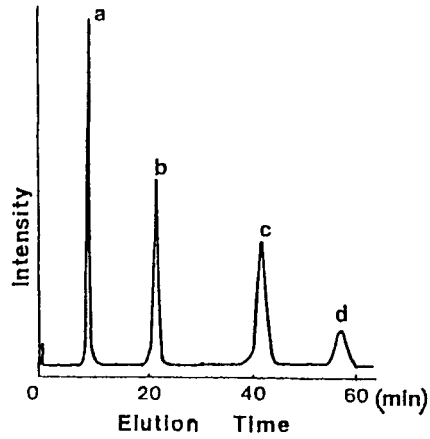

FIGURE 2. Chromatograph of the standard mixture of polyamine determined by the improved HPLC. a:putrescine, b:spermidine c:1,6-diaminohexane and d:spermine.

Simultaneous Reproducibility of the Standard Mixture, Specimens and Separation Time

Simultaneous Reproducibility

The simultaneous reproducibility of 6 charges of 10 μl containing 2.5 nmol of a standard mixture and of 10 charges of 10 μl of rat thymus were measured as shown in Table 1. The CV values ranged from 1.00 to 2.23% for the standard mixture and from 4.99 to 5.08% for the thymus. The CV values of the separation time of 20 charges ranged from 0.08 to 0.42% for the standard mixture. The preciseness of these values was considered acceptable.

Day to day Reproducibility

When charges of the same sample were performed six times per day for six days to determine day-to-day precision, the CV values for reproducibility for putrescine, cadaverine, spermidine, 1,6-diaminohexane and spermine over six days were 6.02, 4.31, 5.19, 5.10 and 6.92%, respectively.

TABLE 1. Simultaneous reproducibility of a standard mixture(St: n=6, nmol/mg) and a sample of the thymus (Thm:n=10, nmol/mg) were calculated from the peak area, and the separation times(ST:n=20, minute) were calculated from the retention times for putrescine (Put), spermidine(Spd), 1,6-diaminohexane(DH) and spermine(Spm). SD is the standard deviation and CV is the coefficient of variation. H, K and T indicate, respectively, the means of each polyamine in the samples of St and Thm and their separation times.

		Put	Spd	DH	Spm
St	H	2.601	2.648	2.603	2.678
	SD	0.026	0.033	0.035	0.060
	CV(%)	1.00	1.22	1.35	2.23
Thm	K	0.204	2.684	–	0.856
	SD	0.010	0.134	–	0.043
	CV(%)	5.08	4.99	–	5.00
ST	T	6.80	16.80	–	43.93
	SD	0.01	0.02	–	0.04
	CV(%)	0.42	0.12	–	0.08

TABLE 2. Reproducibility of duplicate determinations of 60 samples from various tissues. X indicates the content of each polyamines and total polyamine content for first determination and Y for the second determination. PA is total polyamine content. R indicates the correlation coefficient of X and Y. Other abbreviations are the same as in TABLE 1.

n=60		Put	Spd	Spm	PA
X		0.064	1.326	0.843	2.233
SD		0.075	1.912	1.103	3.061
Y		0.066	1.316	0.819	2.201
SD		0.078	1.897	1.044	2.993
R		0.983	1.000	0.998	0.999
Y=AX+B	A	1.02	0.99	0.94	0.98
	B	-0.0001	0.0014	0.0230	0.0180

(nmol/mg)

Reproducibility of Duplicate Determinations

The precision of the assay was tested by duplicate analysis of various tissues in intact rats. The results of duplicate determinations of 60 samples from various tissues are shown in Table 2. An equation of the mathematical linear regression exhibited a good linearity on Y=AX+B with coefficients of A(1.02) and B(-0.0001), A(0.99) and B(0.0014), A(0.94) and B(0.0230), and A(0.98) and B(0.0180) for putrescine, spermidine, spermine and total polyamines, respectively. These values were considered satisfactory for polyamine quantitation.

Calibration Curves for Polyamine Determination

The calibration curves for the determination of polyamines of various concentrations were produced by the improved HPLC method without disturbance of the linearity in their base lines. The linearities of each curve obtained from measurements of standard mixtures of 10, 20, 30, 50, 100, 200, and 250 pmol of polyamines were examined and are shown in Fig. 3. A linear relationship between the polyamine concentration and the peak-

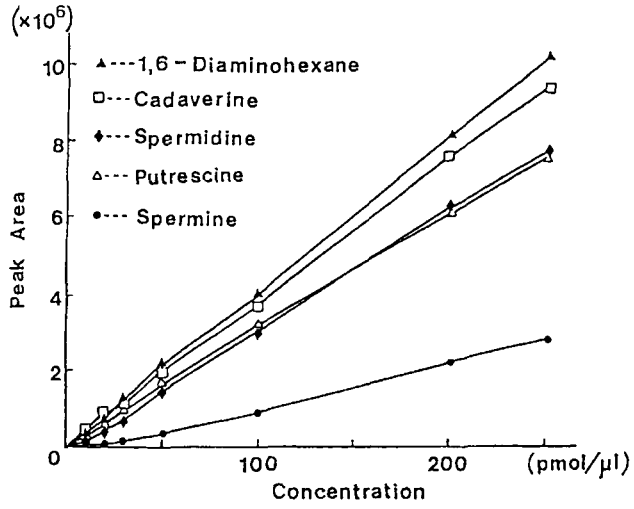

FIGURE 3. Calibration curves for the determination of polyamines.

area ratio relative to the internal standard existed for the entire range up to 250 pmol/μl. The linearities of each curve were quite satisfactory. The detectable concentrations calculated from each peak area were 20 pmol/μl for spermine and 10 pmol/μl for putrescine, cadaverine, spermidine and 1,6-diamnohexane. If allowance is made for the linearity of the base lines, the minimum detectable amount of these polyamines rises to 10 and 1.0 pmol/μl for spermine and putrescine, respectively.

Effect of the Plunger Washing System on the Pump

Before a plunger washing system was attached to the pump, we encountered crystal precipitation about every 20 measurements on or in the plunger at the mobile phase of pH 5.3. However, since addition of the plunger, as shown in Fig 4, crystal precipitation has not been observed at all. In addition, the Polyaminepak could be used for countless measurements.

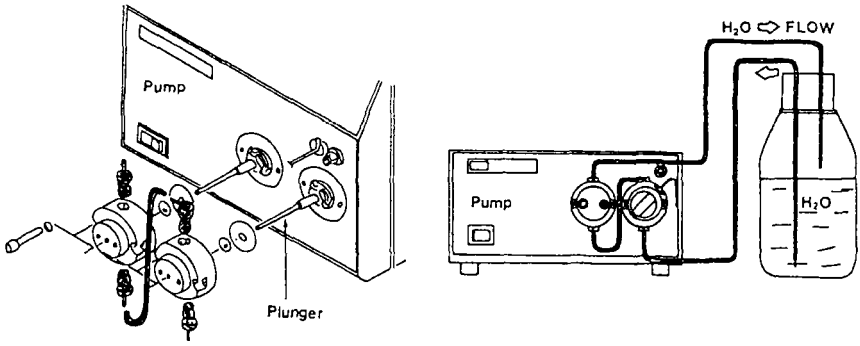

FIGURE 4. Illustrations of the plunger washing system(A) and th pump equipped with it(B).

TABLE 3 Polyamine contents of various tissues and organs i intact rats determined by the improved HPLC. S. intestine, smal intestine;l. intestine, large intestine(rectum); s. vesicle seminal vesicle; s. muscle, skeletal muscle(femoral). Othe abbreviations are the same as in TABLE 1

tissue	g	Put	Spd	Spm	Spd/Spm	tissue	g	Put	Spd	Spm	Spd/Spm
prostate	0.185	0.2598	7.287	4.275	1.72	stomach	0.543	0.0673	0.713	0.660	1.08
SD		0.0254	1.041	0.775	0.14	SD		0.0120	0.046	0.032	0.06
thymus	0.461	0.1538	2.106	0.703	3.02	tongue	0.208	0.0268	0.494	0.432	1.15
SD		0.0256	0.188	0.095	0.22	SD		0.0035	0.108	0.112	0.08
liver	0.889	0.0082	0.687	0.505	1.38	kidney	0.873	0.0164	0.361	0.572	0.63
SD		0.0016	0.080	0.050	0.25	SD		0.0039	0.044	0.093	0.03
s.intes.	0.390	0.1700	1.164	0.561	2.08	s.vesicles	0.463	0.0396	0.504	0.254	1.99
SD		0.0056	0.221	0.072	0.34	SD		0.0054	0.085	0.041	0.14
spleen	0.560	0.0296	1.160	0.654	1.77	lung	0.285	0.0768	0.792	0.402	1.97
SD		0.0040	0.101	0.030	0.15	SD		0.0149	0.126	0.060	0.12
l.intes.	0.232	0.0452	0.700	0.660	1.06	heart	0.701	0.0126	0.281	0.276	1.04
SD		0.0081	0.085	0.044	0.09	SD		0.0035	0.060	0.084	0.11
testis	1.213	0.0091	0.159	0.320	0.50	s.muscle	0.589	0.0095	0.081	0.227	0.36
SD		0.0009	0.013	0.047	0.04	SD		*0.0025*	*0.017*	*0.020*	*0.08*

(nmol/mg)

Polyamine Contents of Various Tissues of the Rat

The polyamine contents of various tissues and organs in intact male rats were measured, as shown in Table 3. The chromatograph of extraction from the tissues and organs showed only four peaks corresponding to polyamines. Therefore, this procedure appears to be quite effective for the determination of polyamines because it does not extract any other compounds. The values obtained from analysis of these tissues and organs seem to be reasonable(29), although it may be difficult to compare these values with those of previous reports because of differences in species, sex and age.

The effectiveness of the column packed with cation-exchange resin for the automated quantitation of polyamines has been significantly improved by addition of the plunger washing system. The combination of these systems, an optimum pH for the mobile phase, and the autosampler make HPLC feasible for long term analysis of these polyamines without the difficulties encountered previously.

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