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Note

High-speed liquid chromatographic determination of putrescine, spermidine and spermine in human urine

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Interest in aliphatic diamines and polyamines was stimulated by the reports of Russell et al. [1, 2] that these amines are present at elevated concentrations in the urine of patients with cancer. The compounds under consideration are putrescine, spermidine and spermine.

In a previous paper [3], we established a high-speed liquid chromatographic method for the determination of polyamines, based on the formation of the tosylated derivatives. It was found, however, that co-existing compounds in urine interfered in this method. This paper describes a procedure for pretreatment of the urine consisting in the hydrolysis of conjugated polyamines and the purification of the polyamines. A gradient elution technique for the tosylated polyamines is also described.

EXPERIMENTAL

Apparatus

This work was carried out in a Du Pont 840 liquid chromatograph equipped with an ultraviolet absorption detector (254 nm) and a high-pressure pump (Model KWU 32H Minimicro pump, Kyowa Seimitsu). The separation was carried out with a 1 m × 2.1 mm I.D. column of Zipax Permaphase ETH (30—50 μm) purchased from Shimadzu Seisakusho (Kyoto, Japan). Gradient elution was carried out in the apparatus shown in Fig. 1. Other experimental details are given in the legends to the figures.

Reagents

Putrescine dihydrochloride and spermidine phosphate were obtained from Tokyo Organic Chemicals (Tokyo, Japan), and spermine phosphate, 1,10-diaminodecane and *p*-toluenesulfonyl chloride (Ts-Cl) from Wako Chemicals

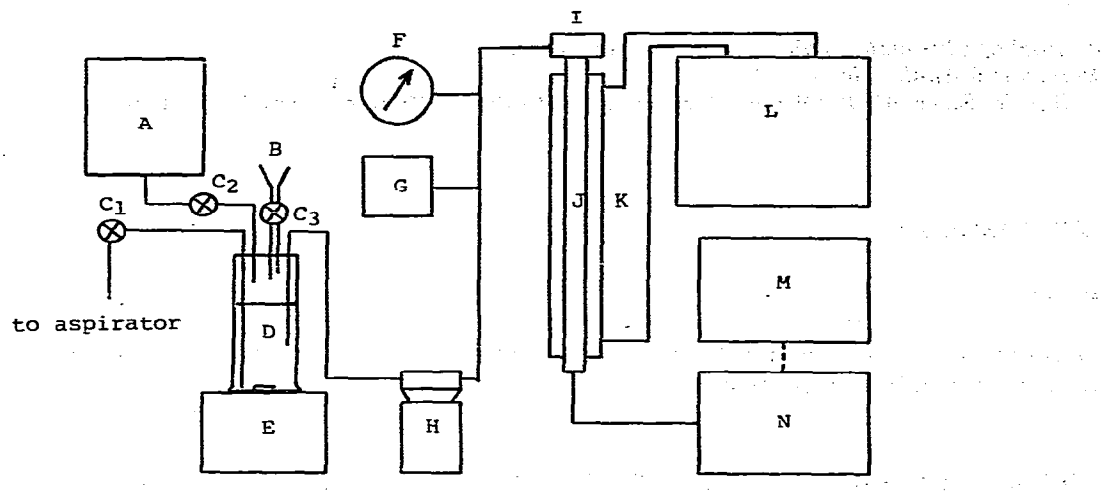

Fig. 1. Schematic diagram of gradient system. A, reservoir (75% acetonitrile); B, funnel; C, cock; D, mixing vessel; E, magnetic stirrer; F, pressure gauge (60—70 kg/cm², flow-rate 0.6 ml/min); G, damper; H, pump; I, injection port; J, column (1-m Zipax Permaphase ETH); K, column jacket; L, constant-temperature circulator (40°); M, recorder; N, UV detector (254nm). The gradient was prepared in the following manner. 1. About 25 ml of 25% aqueous acetonitrile solution is placed in the mixing vessel (D) from the funnel (B). 2. Aqueous acetonitrile, 25% solution, is pumped through the column. 3. When 25% aqueous acetonitrile solution is decreased to 20 ml, the pump is stopped and the sample is injected. 4. The cock C_3 is closed and the cock C_2 is opened so as to connect the mixing vessel with reservoir in which 75% aqueous acetonitrile solution is placed. The pump is then started. 5. When the separation comes to end, the pump is stopped. 6. The cock C_2 is closed and the cocks C_1 and C_3 are opened. The mixing vessel is washed with 25% aqueous acetonitrile solution before next run.

(Osaka, Japan). Acetone, *n*-hexane and methanol were redistilled before use. The other organic solvents and reagents used were of reagent grade.

Procedure

Three milliliters of urine are mixed with 4 ml of hydrochloric acid in a screw-cap tube and hydrolyzed for 3 h at 120° in an autoclave. The hydrolyzate is transferred to a 10-ml centrifuging tube, diluted to 10 ml with water and centrifuged (1000 *g*, 5 min). Then 8.0 ml of the supernatant are taken and evaporated to dryness on a rotary evaporator. The dried residue is dissolved in 10 ml of water, applied on a 10 × 0.5 cm column of 50—100 mesh Amberlite IRA-410 (OH^-), and the column is washed with water. The first 20 ml of eluate are collected. To this eluate, 10 ml of 0.1 *N* hydrochloric acid are added and the solution is applied on to a 5 × 0.5 cm column of 50—100 mesh Dowex 50W-X8 (H^+). After the column has been washed with 30 ml of 1 *N* hydrochloric acid, the polyamines adsorbed on the column are eluted with 6 *N* hydrochloric acid. The first 10 ml of eluate are collected. A 100-μl portion of the aqueous internal standard (1,10-diaminodecane) solution is added to this eluate and the mixture is evaporated to dryness on a rotary evaporator. The residue is dissolved in 1 ml of water. To this solution, 1 ml of 0.5 *M* sodium hydrogen carbonate and 20 mg of Ts-Cl dissolved in 2 ml

of acetone are added. The mixture is then warmed in a water-bath at about 70° for 1 h. The mixture is cooled, then 10 ml of a 1 *N* sodium hydroxide solution are added. The mixture is washed with four 5-ml volumes of *n*-hexane, and after the addition of 15 ml of 1 *N* hydrochloric acid, the tosylated polyamines are extracted with 10 ml carbon tetrachloride. The organic phase is dried on sodium sulfate, and carbon tetrachloride is evaporated to dryness on a rotary evaporator. The residue is redissolved in a few drops of methanol, and 10 μl of the resulting solution are subjected to the high-speed liquid chromatograph.

RESULTS AND DISCUSSION

For the pre-purification of the polyamines from a hydrolyzed urine sample, an Amberlite IRA-410 (OH^-) column (50—100 mesh, 10 × 0.5 cm) and Dowex 50W-X8 (H^+) column (50—100 mesh, 5 × 0.5 cm) were used.

Fig. 2 is an elution graph of the polyamines from the Dowex 50W-X8 column with 6 *N* hydrochloric acid. But 1,10-diaminodecane (I.S.) adsorbed on the column was not eluted with 6 *N* hydrochloric acid. These amines could not be eluted with hydrochloric acid diluted below 1 *N*.

Fig. 3A shows the effect of the pre-purification of a hydrolyzed urine sample from a patient with cancer, with the Dowex 50W-X8 column. The chromatogram shown in Fig. 3B was obtained by passing the hydrolyzed urine sample through the Amberlite IRA-410 column before application to the Dowex 50W-X8 column. Unfortunately, a poor separation of the tosyl-

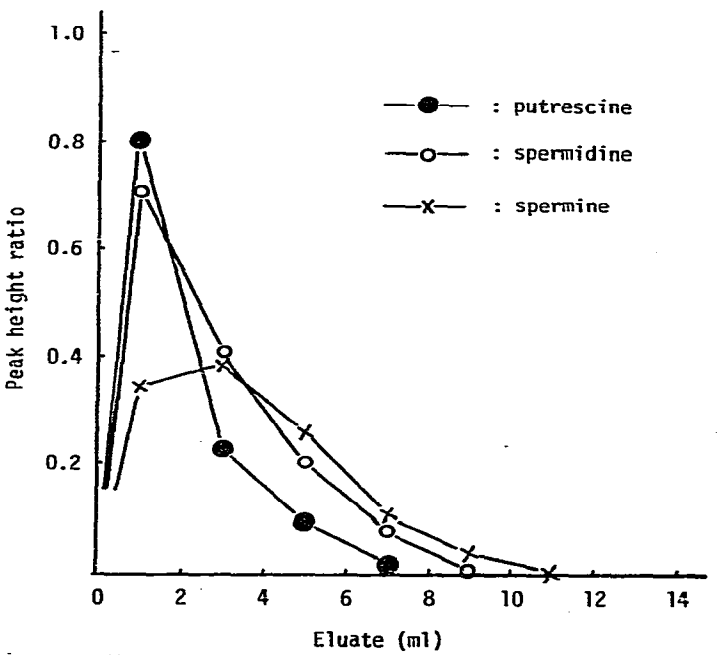

Fig. 2. Elution graph of the polyamines from Dowex 50W-X8 column (5 × 0.5 cm) with 6 *N* HCl.

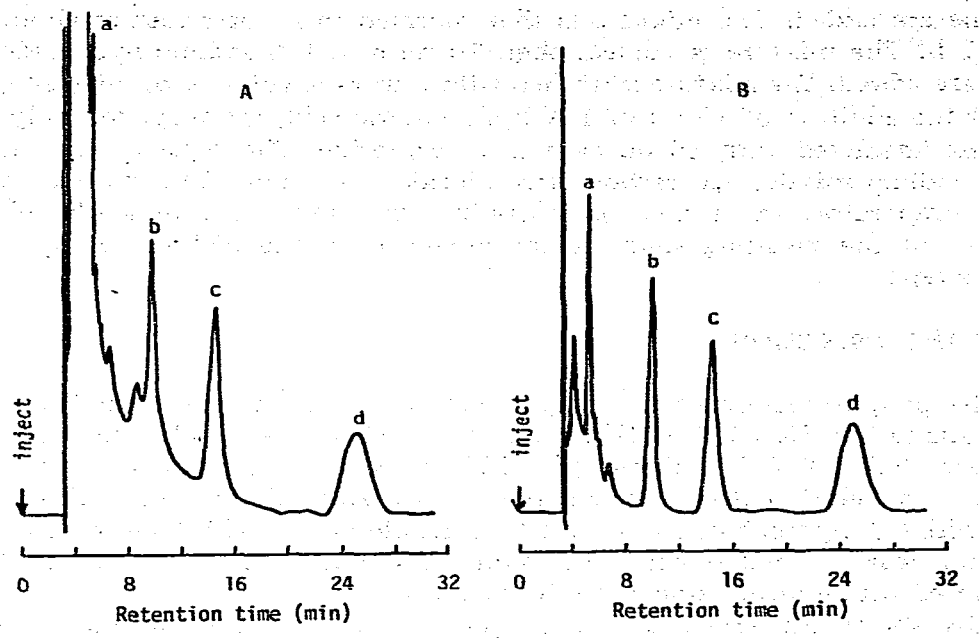

Fig. 3. Effect of pre-purification of a hydrolyzed urine sample on the liquid chromatogram. Operating conditions: column, 1-m Zipax Permaphase ETH (particle size, 30—50 μm); mobile phase, 40% acetonitrile; column temperature, 35°; flow-rate, 0.40 ml/min (pressure, 50 kg/cm²); detector, UV photometer (254 nm). Peaks: a = Ts-putrescine; b = Ts-spermidine; c = Ts-1,10-diaminodecane; d = Ts-spermine.

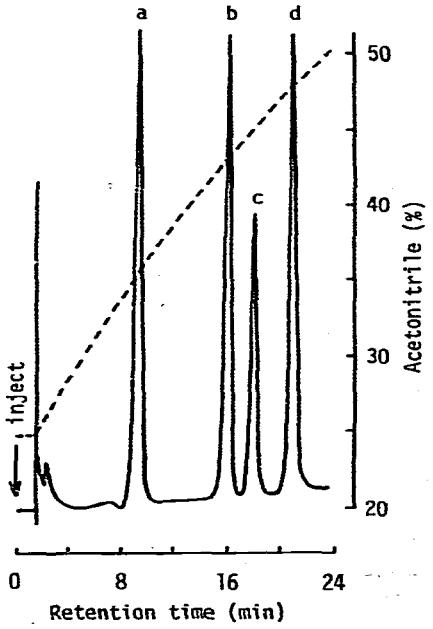

Fig. 4. Gradient elution of Ts-polyamines and Ts-1,10-diaminodecane. For operating conditions, see legend to Fig. 1. Peaks: a = Ts-putrescine; b = Ts-spermidine; c = Ts-1,10-diaminodecane; d = Ts-spermine.

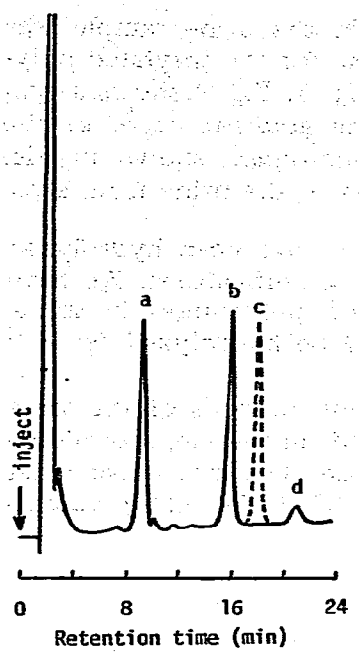

Fig. 5. Chromatogram obtained for the determination of polyamines in the urine from a patient with cancer. For operating conditions, see legend to Fig. 1. Peaks: a = Ts-putrescine; b = Ts-spermidine; c = Ts-1,10-diaminodecane; d = Ts-spermine.

TABLE I

RELATIONSHIP BETWEEN THE EXTENT OF HYDROLYSIS OF CONJUGATED POLYAMINES AND HYDROLYSIS TIME

Results are expressed in peak height ratios. Pu = putrescine; Spd = spermidine, and Sp = spermine.

Sample		Hydrolysis time (h) at 120°					Hydrolysis time (h) at 110°			
		1	2	3	5	15	1	2	15	20
A	Pu	1.73	1.81	1.82	1.82	1.85				
	Spd	0.46	0.51	0.50	0.50	0.51				
	Sp	—	—	—	—	—				
B	Pu	7.96	9.60	9.72	9.72	10.12	6.00	7.88	9.76	9.92
	Spd	18.48	21.24	21.36	21.44	21.40	16.80	19.48	21.64	21.32
	Sp	1.48	1.73	1.74	1.68	1.64	1.26	1.61	1.72	1.73
C	Pu	2.12	2.40	2.85	2.66	2.67				
	Spd	1.29	1.36	1.43	1.51	1.48				
	Sp	0.07	0.09	0.09	0.09	0.08				
D	Pu	1.74	1.77	1.80	1.79	1.82	1.04	1.47	1.80	1.82
	Spd	0.82	0.90	0.94	0.88	0.95	0.54	0.68	0.94	0.93
	Sp	0.11	0.17	0.17	0.17	0.17	0.10	0.13	0.17	0.17

ated polyamines from other compounds present in the urine sample was occasionally observed. The gradient elution technique for the tosylated polyamines was tried by using the apparatus shown in Fig. 1. Fig. 4 shows a typical chromatogram of tosylated polyamines and the gradient curve of the acetonitrile concentration in the eluent. The chromatogram shown in Fig. 5 was obtained for the determination of polyamines in the urine from a patient with cancer.

In most methods reported, conjugated polyamines have been hydrolyzed by the procedures of Marton et al. [4] with some modifications. We have investigated the hydrolysis conditions of conjugated polyamines in urine. Table I shows that the conjugated polyamines could be hydrolyzed for 3 h under our conditions.

To check the precision of this method, seven 3-ml portions of the same urine sample, supplemented each with 10 μg each of putrescine, spermidine and spermine were analyzed by the over-all procedure. The mean recoveries were 90.7% (S.D. 3.5%), 87.4% (S.D. 3.2%) and 67.0% (S.D. 5.2%), respectively.

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