Journal of Chromatography, 156 (1978) 340–345
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CHROM. 11,042

Note

Practical gas chromatographic method for the determination of urinary polyamines

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(Received February 27th, 1978)

The polyamines putrescine, spermidine and spermine appear to be associated in many instances with rapid tissue growth[1], and since the initial reports by Russell and co-workers[2,3] indicating that cancer patients excrete increasing amounts of the polyamines, much attention has been focused on the determination of these amines in physiological fluids. Recently it has been also reported that the polyamines are useful indicators of the efficacy of chemotherapy and their potential usefulness may extend to early prediction of relapse[4]. For these reasons, many methods, such as high-voltage paper electrophoresis[3,5], automatic cation-exchange chromatography[6–9], gas chromatography[10,11] and high-speed liquid chromatography[12,13], have been developed for the analysis of urinary polyamines.

We have recently extended the use of isobutyloxycarbonyl (isoBOC) derivatives for the quantitative analyses of a series of amino acids[14,15], simple phenols[16] and phenolic acids[17]. In the present paper we report a further application of these derivatives in the gas chromatographic (GC) analysis of urinary polyamines. The cation-exchange column chromatographic purification stages[18] with some modifications were introduced into the procedure in order to isolate the polyamines from interfering substances in hydrolyzed urine samples. The polyamines thus obtained were converted into their N-isoBOC derivatives by a simple procedure, and then analyzed by temperature-programmed GC.

EXPERIMENTAL

Reagents

Putrescine·2HCl, cadaverine·2HCl, spermidine·3HCl, spermine·4HCl and 1,8-diaminooctane as an internal standard were obtained from Nakarai Chemicals (Kyoto, Japan); 1,8-diaminooctane was purified by recrystallization from water. A standard solution of polyamines (each 250 nmoles/ml) was prepared in water and aliquots were taken for the evaluation of calibration linearity and recovery from urine samples. The solution of the internal standard in water was prepared at a concentration of 300 nmoles/ml. Isobutyl chloroformate was obtained from Sigma (St. Louis, Mo., U.S.A.) and purified by washing twice with two volumes of water and stored over molecular sieve 4 A 1/16 at 4°. Before use, Amberlite CG-120 cation-

exchange resin 100–200 mesh, H^+ was treated as follows: the resin was washed twice with 4 *N* HCl, then covered with 2 *N* NaOH and swirled for 3 h at 70° after successive washing with water until neutral. The resin was regenerated with 4 *N* HCl (three times) and then washed with water until neutral.

All the other reagents were obtained from commercial sources and were reagent-grade purity.

Hydrolysis of urine and isolation of polyamines

Urine samples (24 h) were collected under toluene and the creatinine concentrations were determined by the method of Taussky[19]. A volume of 25 ml of 24-h urine sample was evaporated to dryness in a rotary evaporator at 60° and to the residue was added 25 ml of 6 *N* HCl; then the solution was refluxed for 16 h at 110°. After hydrolysis, the solution was placed in a refrigerator at 4° overnight and the resulting precipitate was filtered off through Whatman No. 30 filter paper. Variable results were obtained if hydrolyzates were not filtered to remove any debris created during hydrolysis. The filtrate was evaporated to dryness in a rotary evaporator at 60° and the residue was dissolved in water, made up to 5 ml. A 1-ml volume of this solution (corresponding to 5 ml of 24-h urine) was diluted to 10 ml with 2% perchloric acid and the whole was loaded on to a column (9 mm I.D.) containing 3-ml bed volume of Amberlite CG-120 cation-exchange resin. The column was washed with 30 ml of 0.1 *M* sodium phosphate buffer (pH 8) containing 0.1 *M* NaCl, and then with 30 ml of 1 *N* HCl. The flow-rate was 1–2 ml/min. Polyamines were eluted with 20 ml of 6 *N* HCl, followed by 10 ml of water, and to the eluate was added 0.5 ml of the internal standard solution (150 nmoles). The eluate containing the internal standard was evaporated to dryness in a rotary evaporator at 60° and the residue was transferred to a 10-ml polyethylene-stoppered vial with 1.5 ml of water.

After use the resin was collected in a flask and treated according to the procedure described above.

Preparation of derivatives

To the sample solution in a vial were added 0.5 ml of 10% NaOH and 0.1 ml of isobutyl chloroformate, and then the mixture was shaken for 10 min at room temperature. The resulting N-isoBOC derivatives of polyamines were extracted three times with 2 ml of diethyl ether, and the combined ether extracts were evaporated to dryness at 50°. The residue was dissolved in 0.1 ml of ethyl acetate and the solution was dried over anhydrous Na_2SO_4. A 4-μl volume of the resulting solution was injected on to the gas chromatograph.

Gas chromatography

A Shimadzu 5A gas chromatograph, equipped with a dual-column oven-bath with dual differential hydrogen-flame detectors, on-column injection ports and a linear temperature programmer, was employed for the analyses. Column packings were prepared from commercial liquid phases and silanized Gas-Chrom P (80–100 mesh) by the solution-coating technique[20]. The liquid phases were dissolved in *n*-BuOH–$CHCl_3$ (1:1). Columns (1.5% SE-30–0.3% SP-1000 and 1% SE-30–0.5% SP-1000) were conditioned with a nitrogen flow-rate of *ca.* 30 ml/min at 275° for 22 h.

RESULTS AND DISCUSSION

In order to evaluate the GC properties and the separation of the N-isoBOC derivatives of polyamines, an aliquot of standard polyamine solution was derivatized and analyzed using variable columns. As a result, symmetrical peaks and good separations could be obtained for all the polyamine derivatives (Fig. 1) when a mixed-phase, 1.5% SE-30–0.3% SP-1000, glass column (0.5 m × 3 mm I.D.) was used. On this column, 1,8-diaminooctane was suitable as an internal standard since it was separated from the other polyamines and showed a symmetrical peak (Fig. 1). This column was used unless otherwise noted.

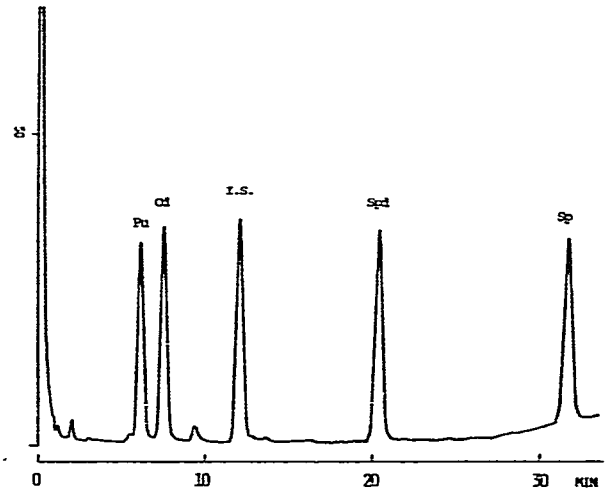

Fig. 1. Chromatogram of the N-isoBOC derivatives obtained from a polyamine standard mixture. Column: 1.5% SE-30–0.3% SP-1000 on silanized Gas-Chrom P (80–100 mesh), 0.5 m × 3 mm I.D., glass. Conditions: isothermal at 150° for 1 min, then programmed at a rate of 4°/min; attenuation, 16×10^2; nitrogen flow-rate, 60 ml/min. Each peak represents 4 nmoles of polyamine. Internal standard: 1,8-diaminooctane, 6 nmoles. Peaks: Pu = putrescine; Cd = cadaverine; Spd = spermidine; Sp = spermine.

In order to test the linearity of the calibration graphs, standard samples at each level in the range 10–250 nmoles were derivatized and analyzed. As shown in Fig. 2, the linearity of the calibration graphs for each polyamine in the range studied and the reproducibility were found to be satisfactory.

The stability of the N-isoBOC derivatives of polyamines with respect to time was investigated. No significant change in the amounts of derivatives present could be observed when compared with a freshly prepared standard 1 week later. Similar reproducibility and stability were demonstrated in the case of urinary extracts. It should be emphasized that all of the derivatives are very stable towards moisture and therefore no precautions to exclude moisture are necessary in their handling and storage.

Based on these results, the application of the proposed method to the determination of urinary polyamines was investigated. Many interfering peaks were observed

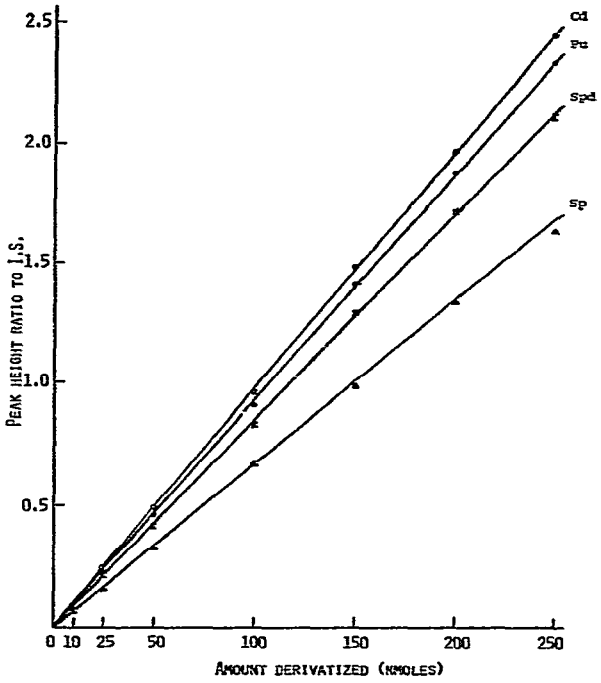

Fig. 2. Calibration graphs for the polyamines. Each point represents the average of three determinations. Internal standard: 1,8-diaminooctane, 150 nmoles.

and quantification was unreliable when urine hydrolyzates were concentrated and immediately derivatized. To overcome this problem, the clean-up procedure originally devised by Inoue and Mizutani[18] was used with some modifications for the simple and rapid isolation of polyamines from urine hydrolyzates. By loading urine hydrolyzates on to the cation-exchange resin column prior to derivatization, the interfering substances could be excluded, and a satisfactory chromatogram could be obtained as shown in Fig. 3A. All the peaks of polyamines obtained from a urine sample were spiked with standards to identify them (Fig. 3B). Separate samples to which the internal standard had not been added were also run to ensure that the internal standard was not being confused with another peak having the same elution time. The results obtained with five normal subjects showed that the internal standard region was free from urinary peaks. In this study, the internal standard was added to the eluate containing the polyamine fraction obtained by column chromatography.

On a 1.5% SE–0.3% SP-1000 column, the determination of cadaverine was hindered by the appearance of another small urinary peak which could not be excluded by cation-exchange column chromatography performed under the present conditions. For the determination of cadaverine, a 1% SE-30–0.5% SP-1000 column (1 m × 3 mm I.D., glass) was suitable. However, the peak for spermine could not be obtained using this column.

Quantification of this method was established from analyses of the hydrolyzed urine samples fortified with 50 or 100 nmoles of each polyamine. The overall recovery

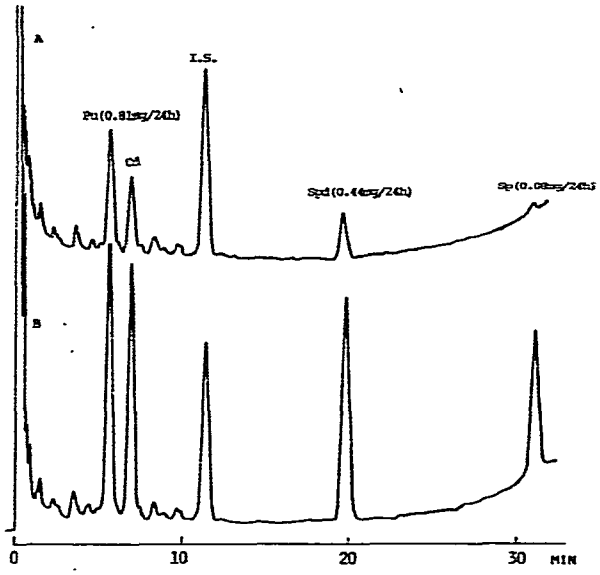

Fig. 3. Chromatograms obtained from (A) a urine sample and (B) the same sample spiked with 100 nmoles of each polyamine. Internal standard: 1,8-diaminooctane. Conditions and peaks as in Fig. 1.

and its relative standard deviation for each polyamine were reasonable, as summarized in Table I. This also indicates that the method is very precise and accurate.

The urinary polyamine concentrations of normal subjects determined by this method are summarized in Table II. The validity of the method is substantiated by the fact that the urinary polyamine levels in normal subjects obtained in this study were in good agreement with those reported in the literature[4,7,8]. The urinary concen-

TABLE I

RECOVERY TEST FOR POLYAMINES ADDED TO URINE SAMPLES

Of each polyamine 50 and 100 nmoles were added to 1 ml of the concentrated hydrolyzed urine (corresponding to 5 ml of 24-h urine) prior to cation-exchange column chromatography. Cadaverine was analyzed using a 1% SE-30–0.5% SP-1000 column.

Sample	*Amount of each polyamine added (nmoles)*	*Recovery (%)*			
		Putrescine	*Cadaverine*	*Spermidine*	*Spermine*
E.Y.	100	96.7	94.1	95.8	91.7
H.Y.	100	94.8	102.5	96.4	107.7
Y.O.	100	93.7	100.9	98.3	99.1
M.M.	100	98.5	—	106.1	96.7
S.Y.	50	94.0	99.4	93.4	100.0
T.M. (♂)	50	92.0	96.3	95.2	97.2
T.M. (♀)	50	94.0	—	101.9	105.1
	Av.	94.8	98.6	98.2	99.6
	R.S.D.*	2.3	3.5	4.5	5.4

* R.S.D. = (S.D./Av.) × 100%.

TABLE II

URINARY EXCRETION OF POLYAMINES IN NORMAL SUBJECTS

Each value is an average from duplicate determinations.

Subject (age)	*Putrescine*		*Spermidine*		*Spermine*	
	mg per 24 h	*mg per g creatinine*	*mg per 24 h*	*mg per g creatinine*	*mg per 24 h*	*mg per g creatinine*
H.Y. (5)	0.88	2.45	0.49	1.31	0.07	0.18
E.Y. (8)	0.81	1.73	0.44	0.93	0.08	0.17
M.M. (22)	1.62	0.96	1.18	0.69	0.23	0.14
Y.O. (23)	2.27	1.52	0.97	0.65	0.31	0.20
M.Y. (31)	1.87	—	0.57	—	0.18	—
S.Y. (36)	1.38	0.84	1.04	0.59	0.16	0.09
T.M. (43)	1.56	1.65	0.96	1.01	0.16	0.17
T.M. (46)	1.79	0.93	1.01	0.52	0.40	0.28

trations of cadaverine obtained in this study are 0.11–1.00 mg per 24 h using a 1% SE-30–0.5% SP-1000 column and these values are within the range reported by Adler *et al.*[8].

In conclusion, we have developed a new method utilizing gas chromatography which permits the complete separation and quantitation of urinary polyamines. Although this method requires the derivatization of polyamines, the preparation of derivatives is simple and rapid, and the derivatives are very stable towards moisture. This method can be less complex and expensive than the automatic cation-exchange and high-speed liquid chromatographic methods.

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