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DETERMINATION OF POLYAMINES AND RELATED COMPOUNDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: IMPROVED SEPARATION SYSTEMS

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SUMMARY

By employing a column with a high capacity factor and high resolution, separation of the ion pairs with octane sulphonic acid of the natural di- and polyamines, and related compounds, was considerably improved. The new separation systems were applied to the analysis of urine samples and tissue extracts, and allowed the determination not only of the usual polyamines, and the monoacetylspermidines, but also of monoacetylputrescine, putreanine and isoputreanine.

INTRODUCTION

Several years ago we published a method for the determination of polyamines, which was based on the separation of their ion pairs with octane sulphonic acid on a reversed-phase column, post-column derivatization with the *o*-phthalaldehyde—2-mercaptoethanol reagent of Roth, and recording of fluorescence intensity [1]. This method has found numerous applications. Its major drawback was that it did not allow the determination of monoacetylputrescine in tissue extracts or urine samples. Since monoacetylputrescine is the major urinary excretion form of putrescine in humans [2], either procedures based on the dansylation reaction [3, 4] or ion-exchange chromatographic procedures [5—7] had to be used to obtain a complete picture of the polyamine pattern of human urine. Another limitation was, for example, that it was not possible to determine putreanine with this method, although it allowed the detection of isoputreanine [8]. Putreanine is a natural amino acid deriving from spermidine, which among vertebrate tissues occurs uniquely in brain [9, 10], although it can be found in urine [11]. None of the ver-

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sions of our original method that have been published in the meantime [12—14] extended the applicability of the method.

With the advent of reversed-phase columns with an increased capacity factor and improved resolution, it was possible to work out gradient elution systems which allow the separation of compounds of greatly differing polarity in one run.

In this paper we describe several solvent systems that are useful in the establishment of complete polyamine patterns.

MATERIALS AND METHODS

Chemicals

With the exception of *o*-phthalaldehyde (Roth, Karlsruhe, F.R.G.) and octane sulphonic acid (sodium salt) (Eastman Kodak, Rochester, NJ, U.S.A.), all commercial reagents (A-grade or for chromatographic use) were from E. Merck (Darmstadt, F.R.G.). Polyamines were from Fluka (Buchs, Switzerland), and monoacetyl derivatives of the polyamines were prepared in the form of hydrochlorides in our laboratory according to published procedures [15, 16].

Instruments

A Varian Vista 5500 and a Varian 8500 liquid chromatograph were used. Both instruments were equipped with loop injector valves with 200-μl loops. In the case of the Vista 5500, the column eluate first passed a UV detector. The eluate of the other instrument was immediately mixed in a 1:1 ratio with the *o*-phthalaldehyde—2-mercaptoethanol reagent, and after passing through a PTFE coil (1 m × 0.5 mm I.D.), fluorescence was continuously recorded using a Varian Fluorichrome or a Perkin-Elmer fluorescence spectrophotometer (Model 204A). The fluorescence detectors were equipped with a 12.5-μl and a 10-μl flow cell, respectively. Signals were usually recorded at two sensitivities (Omniscribe two-channel pen recorder, Houston Instruments, Gistel, Belgium). Separations were performed using Beckman Ultrasphere^{TM}-IP columns. These columns (25 cm × 4.6 mm I.D.) are filled with 5-μm pellicular material with chemically bonded C_{18} groups. The separation columns were protected by guard columns (7 cm × 2.1 mm I.D.) filled with Pellicular ODS (C_{18} groups bonded to 37—53 μm particles; Whatman, Clifton, NJ, U.S.A.). Pre-column, column, mixing T-piece and PTFE coil were kept at ambient temperature (22°C).

Solvents

All solvents were prepared using water distilled over phosphoric acid. Gradients were usually prepared by mixing three solvents.

Solvent A: 0.1 *M* sodium acetate (pH adjusted to 4.50 with acetic acid) with 2.16 g of sodium octane sulphonate per litre (10 m*M*).

Solvent B: 0.2 *M* sodium acetate (pH 4.50)—acetonitrile (10:3) with 2.16 g of sodium octane sulphonate per litre (10 m*M*).

Solvent C: methanol.

Solvents A and B are identical with those previously suggested for polyamine

separations using μBondapak C_{18} columns (Waters, Paris, France) [1].

From the experience with the ternary systems, the following binary system could be developed.

Solvent a: A + C (9:1).

Solvent b: B + C (9:1).

Gradients

For the separation of polyamines, their monoacetyl derivatives and related compounds, the gradients given in Table I were found to be appropriate (flow-rate 1 ml/min).

TABLE I

COMPOSITION OF GRADIENTS I—III

Gradient I				Gradient II				Gradient III		
Elution time (min)	Percentage of solvent			Elution time (min)	Percentage of solvent			Elution time (min)	Percentage of solvent	
	A	B	C		A	B	C		a	b
0	90	10	0	0	90	0	10	0	100	0
40	0	100	0	17	70	20	10	12	100	0
45	0	90	10	17.1	50	40	10	16	60	40
				40	50	40	10	36	60	40
				50	0	90	10	48	0	100
End time (min)	55			65				55		
Equilibration time (min)	30			30				30		

Sample preparation

Tissues were homogenized with 10 vols. of 0.2 *M* perchloric acid. The supernatants were appropriately diluted with 0.2 *M* perchloric acid and applied on the column. Separations were not significantly influenced by sample volumes of 200 μl.

Urines were diluted with 10 vols. of 0.2 *M* perchloric acid and 200-μl aliquots were directly separated, after centrifugation.

For the preparation of hydrolysates, 1-ml samples were mixed with the same volume of 12 *M* hydrochloric acid, sealed in glass tubes, and heated for 18 h at 120°C. After evaporation to dryness the residues were dissolved in 11 ml of 0.2 *M* perchloric acid, and centrifuged. Aliquots were submitted to chromatographic separation.

o-Phthalaldehyde—2-mercaptoethanol reagent

The reagent was almost the same as that used previously [1]. It is prepared by dissolving 50 g of boric acid and 44 g of potassium hydroxide per litre water; 3 ml of Brij-35 solution and 3 ml of 2-mercaptoethanol and a solution of 400 mg of *o*-phthalaldehyde, dissolved in 5 ml of methanol, are added to

the borate buffer. The reagent was stored in dark bottles, and was used without any further precautions, not, however, for longer than two days.

RESULTS AND DISCUSSION

Compounds which appear in the following chromatograms are shown in Table II. They were numbered according to their order of elution by gradient I. The same numbers are used in all figures.

Separations with gradient I

Fig. 1 shows the separation of a mixture of polyamines, monoacetylpolyamines and related amino acids, as well as amino acids and dipeptides, with chromatographic properties similar to those compounds of our specific interest. There is only one interference of importance which should be pointed out: 1,3-diaminopropane is very close to cadaverine (not shown). 1,3-Diaminopropane normally does not occur in the vertebrate organism, but its determination has some interest in plants. The separation of agmatine from N^8-acetylspermidine has previously been demonstrated [1]. It was not included in the standard mixture. Similarly it has been shown [1] that in practice there is little interference from certain biogenic amines, due to their usually low concentrations.

Fig. 2A demonstrates the direct separation of a sample of human urine, which was diluted prior to chromatography with 10 vols. of 0.2 *M* perchloric acid; Fig. 2B shows the separation of an appropriately diluted hydrolysate of

TABLE II

COMPOUNDS APPEARING IN THE CHROMATOGRAMS OF FIGS. 1—6

Number*	Compound	Number	Compound
1	Tyrosine	14	Isoputreanine [N-(3-aminopropyl)-4-aminobutyric acid]
2	Anserine (N^1-methylcarnosine)	15	Putrescine (1,4-diaminobutane)
3	Phenylalanine	16	Cadaverine (1,5-diaminopentane)
4	Carnosine (β-alanyl-L-histidine)	17	Decarboxylated S-adenosylmethionine (S-adenosyl-5′-deoxy-(5′)-3-methylthiopropylamine)
5	Homocarnosine (γ-aminobutyryl-L-histidine)	18	Histamine
6	Acetylputrescine	19	N^1-Acetylspermidine
7	Putreanine [N-(4-aminobutyl)-3-aminopropionic acid]	20	N^8-Acetylspermidine
8	S-Adenosylmethionine	21	1,7-Diaminoheptane (internal standard)
9	Arginine	22	Spermidine [N^1-(3-aminopropyl)-putrescine]
10	Acetylcadaverine	23	N^1-Acetylspermine
11	Tryptophan	24	Spermine [N^1,N^4-bis(3-aminopropyl)-putrescine]
12	5′-Methylthioadenosine		
13	Isoputreanine lactam [N-(3-aminopropyl)pyrrolidin-2-one]		

*These numbers are used in Figs. 1—6 for the identification of compounds.

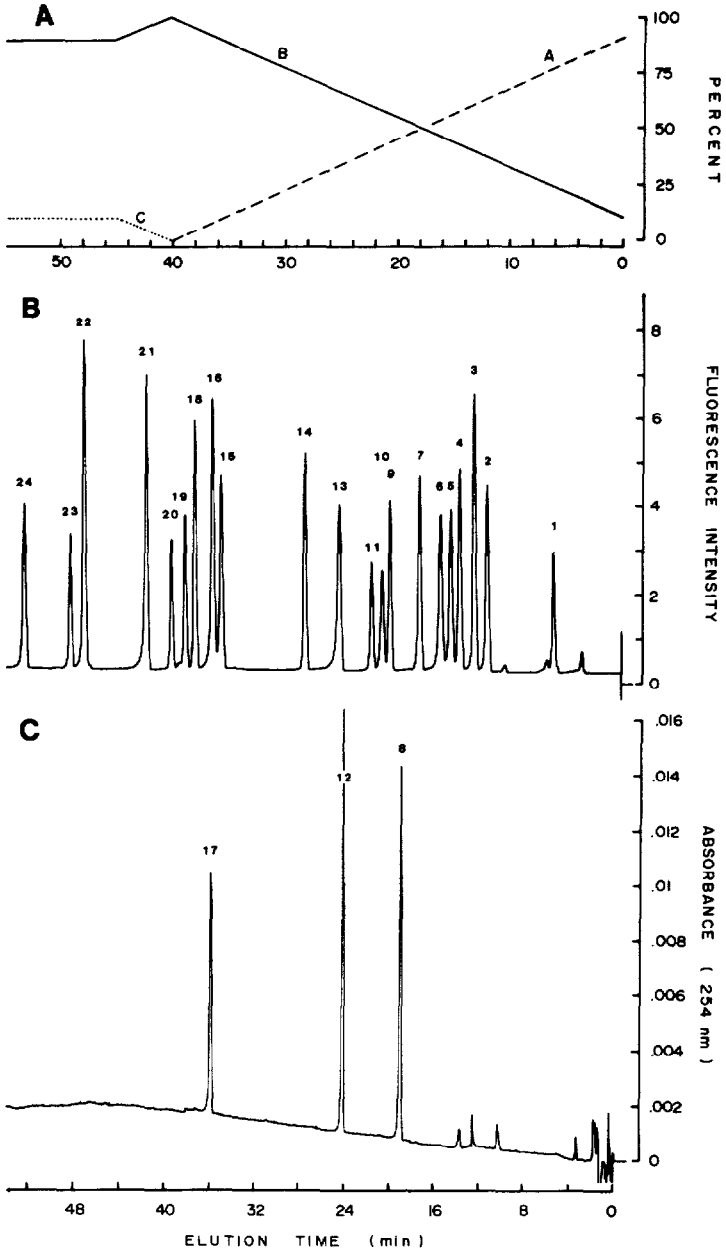

Fig. 1. Separation of polyamines and related compounds using gradient I. (A) Composition of gradient. See text for compositions of solvents A—C. (B) Fluorescence intensity after reaction with *o*-phthalaldehyde—2-mercaptoethanol. Amount of each compound is 1 nmol per 0.1 ml. (C) Absorbance at 254 nm. Amount of adenosine derivatives is 100 pmol per 0.1 ml. For details see Materials and methods. Tissue components do not interfere with the determination of the adenosine derivatives. For peak identification, see Table II.

the same urine sample. Acetylputrescine is well separated from other urinary constituents. Putrescine, cadaverine and spermidine concentrations are low, in agreement with previous findings [2—4]. After hydrolysis (Fig. 2B) acetyl-

putrescine and the peaks corresponding to N^1- and N^8-acetylspermidine disappeared, and the putrescine and spermidine peaks increased proportionately. The peak corresponding in its chromatographic behaviour to acetylcadaverine is not uniform. However, the appearance of large amounts of cadaverine in

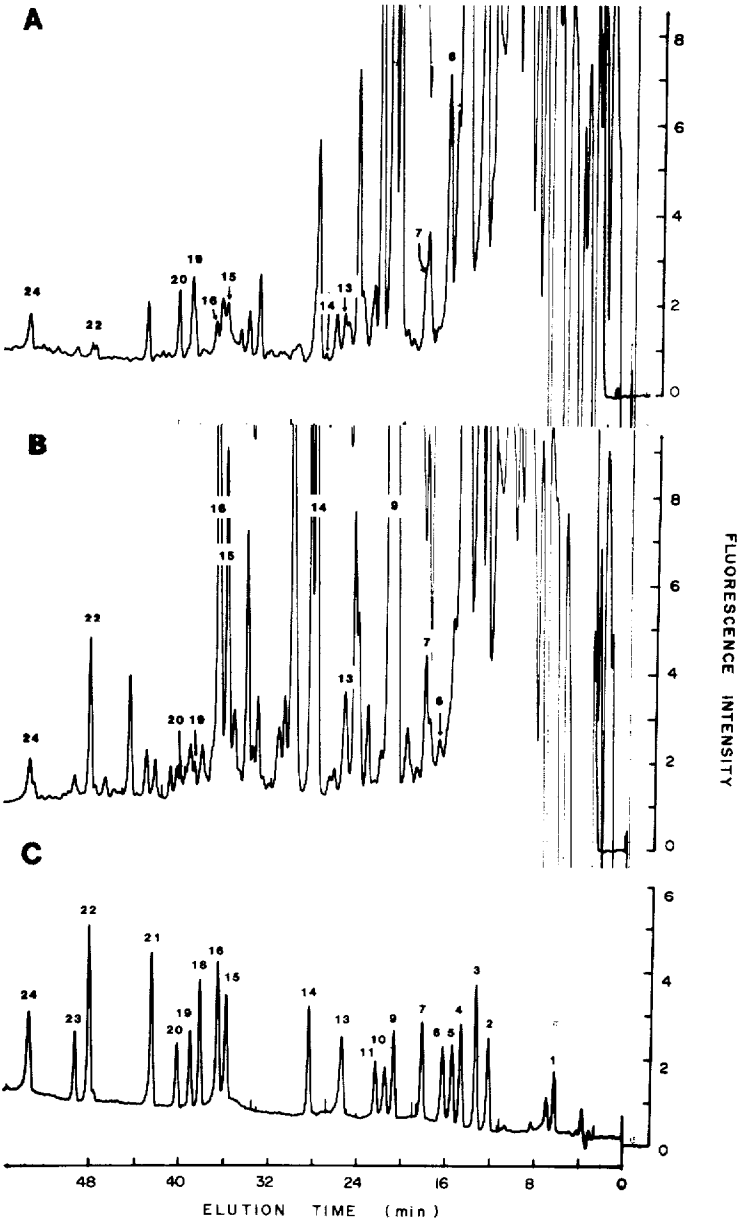

Fig. 2. Separation of human urine, using the conditions described in the legend of Fig. 1. (A) Urine sample diluted with 10 vols. of 0.2 *M* perchloric acid. (B) Hydrolysate (6 *M* hydrochloric acid, 120°C, 18 h) of the same urine sample after 1:10 dilution with 0.2 *M* perchloric acid. (C) Standard mixture (50 pmol per 0.1 ml). For peak identification see Table II.

the hydrolysate suggests that not only putrescine, but also cadaverine, is excreted in a conjugated form, most probably in the form of acetylcadaverine.

While only small peaks are observed in the positions of isoputreanine lactam and isoputreanine in normal human urine, a large peak corresponding in its chromatographic behaviour to isoputreanine is seen in the chromatogram of the urine hydrolysate. This finding is in agreement with the notion that isoputreanine is not excreted as such, but in the form of conjugates of its lactam [8]. Further evidence for the identity of the "isoputreanine peak" will be given below.

The putreanine peak in urine separations is not free of interferences, but its separation is improved by hydrolysis.

If determination of acetylputrescine and related compounds is not required, the short version of the elution programme given in Table III is suitable.

The high proportion of solvent B in the initial eluent allows the time for column equilibration to be reduced considerably.

Under these conditions acetylputrescine is eluted close to the solvent front. This elution mode was routinely used in the past for numerous polyamine determinations in tissue extracts and rat urine.

TABLE III

ELUTION PROGRAMME

	Elution time (min)	Percentage of solvent		
		A	B	C
	0	60	40	0
	30	0	100	0
	35	0	90	10
End time (min)	40			
Equilibration time (min)	8			

Separations with gradients II and III

The main advantage of gradient II is the constant proportion of methanol. This allowed us to establish an elution mode which is suitable for equipment with only two pumps for gradient making. Fig. 3 shows the separation of a standard mixture, using gradient III. In Figs. 4—6 separations using gradient II are shown. These latter separations are very similar to those obtained with gradient III.

A comparison between the chromatograms obtained with gradients I and III can be made by comparing Figs. 1 and 4. Elution by the somewhat complex gradient III improved the separations considerably, especially those of acetylputrescine, and of N^1- and N^8-acetylspermidine. Putrescine is well separated from the background, so that its determination is more precise than after elution by gradient I. Thus it appears from Fig. 4A that the concentration of free putrescine can be negligibly low in human urine. The chromatogram of the hydrolysate (Fig. 4B) demonstrates the quantitative transformation of the acetyl derivatives into the non-conjugated polyamines.

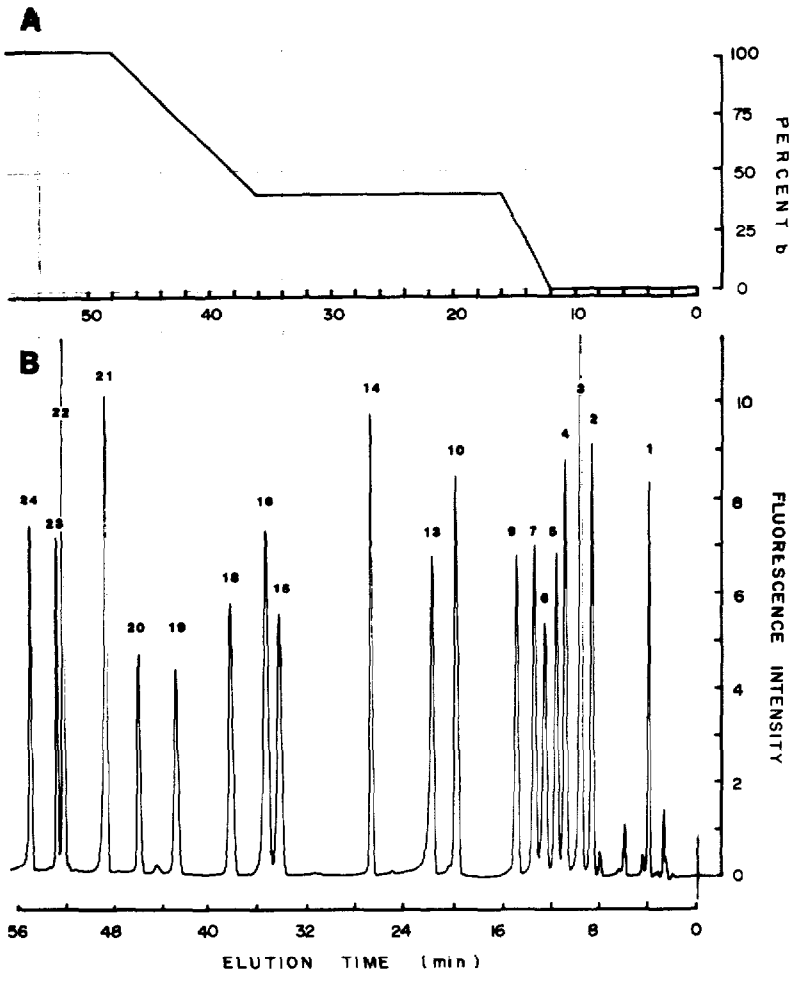

Fig. 3. Separation of polyamines and related compounds using gradient III. (A) Gradient composition. See text for compositions of solvents a and b. (B) Fluorescence intensity after reaction with o-phthalaldehyde—2-mercaptoethanol. Amount of each compound is 1 nmol per 0.1 ml. For details see Materials and methods. For peak identification, see Table II.

In contrast with humans, rats excrete most of the putrescine as such, not in the form of acetylputrescine, as can be seen in Fig. 5A. Only a small acetylputrescine peak is observed. Although N^1-acetylspermidine and N^8-acetylspermidine can be found in rat urine, a major proportion of spermidine is excreted as such [17].

Some rats were treated with 50 mg of aminoguanidine sulphate per kg body weight on three consecutive days, and 24-h urine samples were collected. The chromatogram in Fig. 5B shows the urinary polyamines pattern of a rat treated with aminoguanidine sulphate. The reason for this experiment was as follows. Aminoguanidine is an inhibitor of diamine oxidase [18]. Inhibition of this enzyme is expected to prevent putrescine and cadaverine catabolism, and to increase their urinary excretion. This effect is clearly seen in Fig. 5B, and in the chromatogram of the hydrolysate of this urine (Fig. 6B). One can also

expect that under these conditions the excretion of monoacetylputrescine will be enhanced. This effect, however, is not great, as appears from the comparison of Fig. 5A and B.

It has previously been shown that treatment with aminoguanidine prevents the formation of isoputreanine lactam, both from exogenously administered, and from endogenous, spermidine [8, 19]. In agreement with this finding, isoputreanine, the product of acid hydrolysis of isoputreanine lactam, was absent in the urine hydrolysates of aminoguanidine-treated rats, and the spermidine peak was correspondingly higher (Fig. 6B). This demonstrates the usefulness of the method for the determination of spermidine catabolism to isoputreanine. A capillary gas–liquid chromatographic method suitable for the determination of polyamines and isoputreanine in urine hydrolysates has recently been reported [20].

Since the size of the putreanine peak was not changed by treatment with aminoguanidine, it will be necessary to establish the identity of this peak

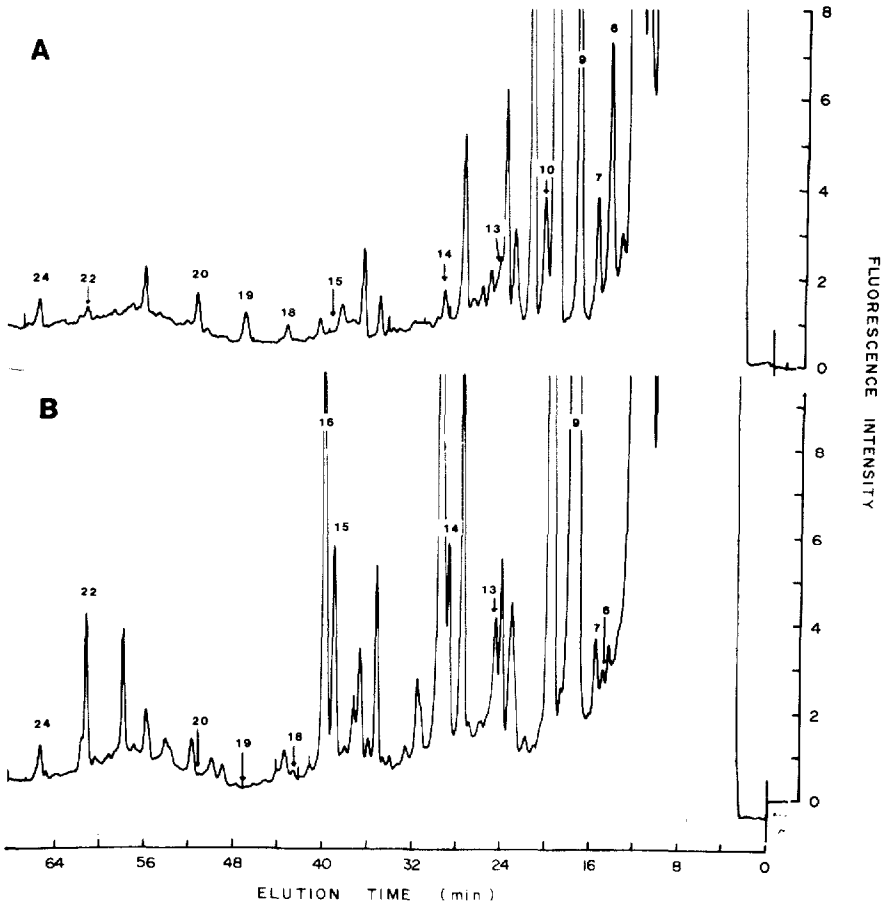

Fig. 4. Separation of human urine, using gradient II. (A) Urine sample diluted with 10 vols. of 0.2 *M* perchloric acid. (B) Hydrolysate (6 *M* hydrochloric acid, 120°C, 18 h) of the same urine sample after 1:10 dilution with 0.2 *M* perchloric acid. For peak identification, see Table II.

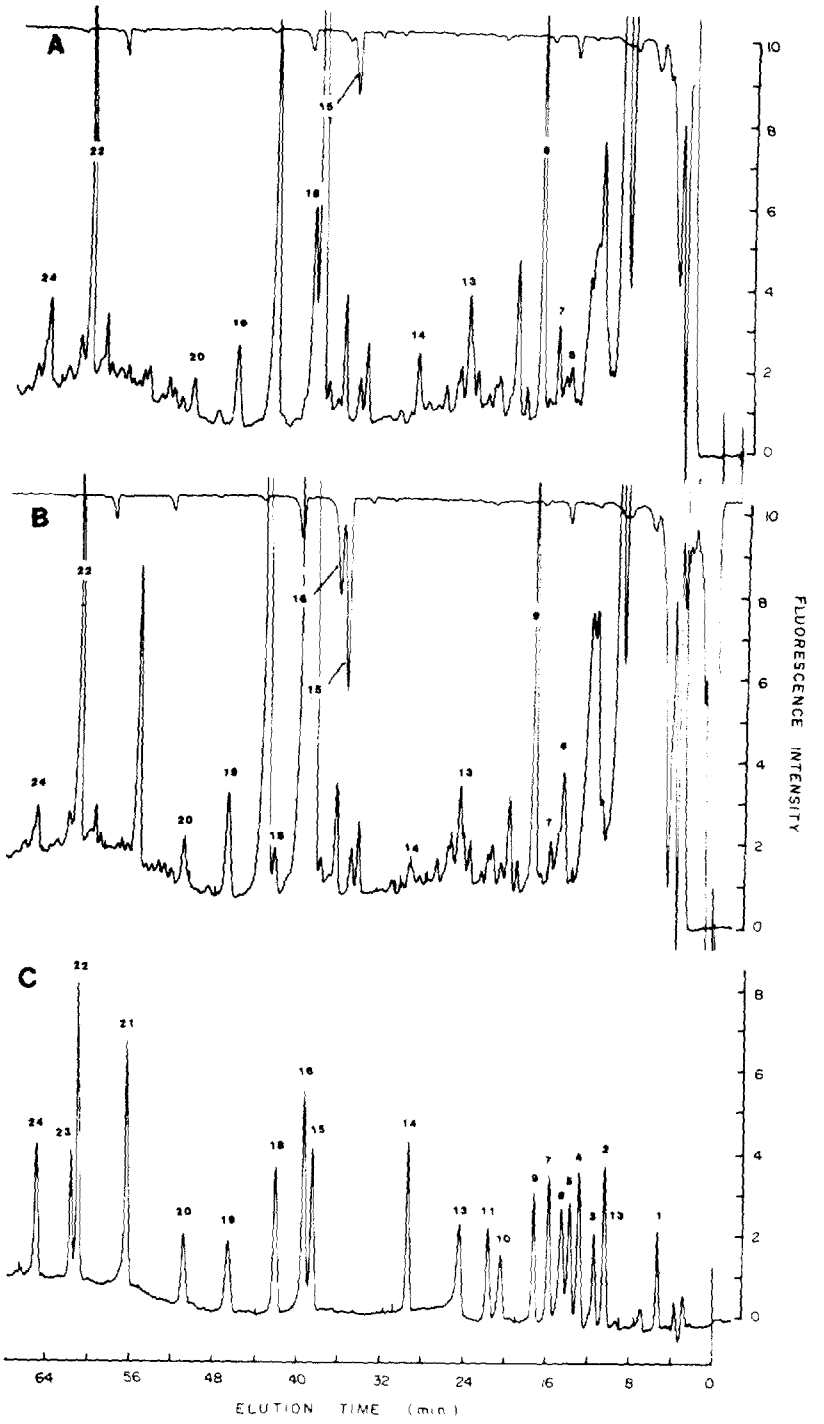

Fig. 5. Separation of rat urine, using gradient II. (A) Normal rat urine, diluted with 10 vols. of 0.2 *M* perchloric acid. (B) Urine of a rat after three days of daily intraperitoneal administration of 50 mg aminoguanidine sulphate per kg body weight (1:10 dilution with 0.2 *M* perchloric acid). (C) Standard mixture of polyamines and related compounds (50 pmol per 0.1 ml). For details see Materials and methods. For peak identification, see Table II.

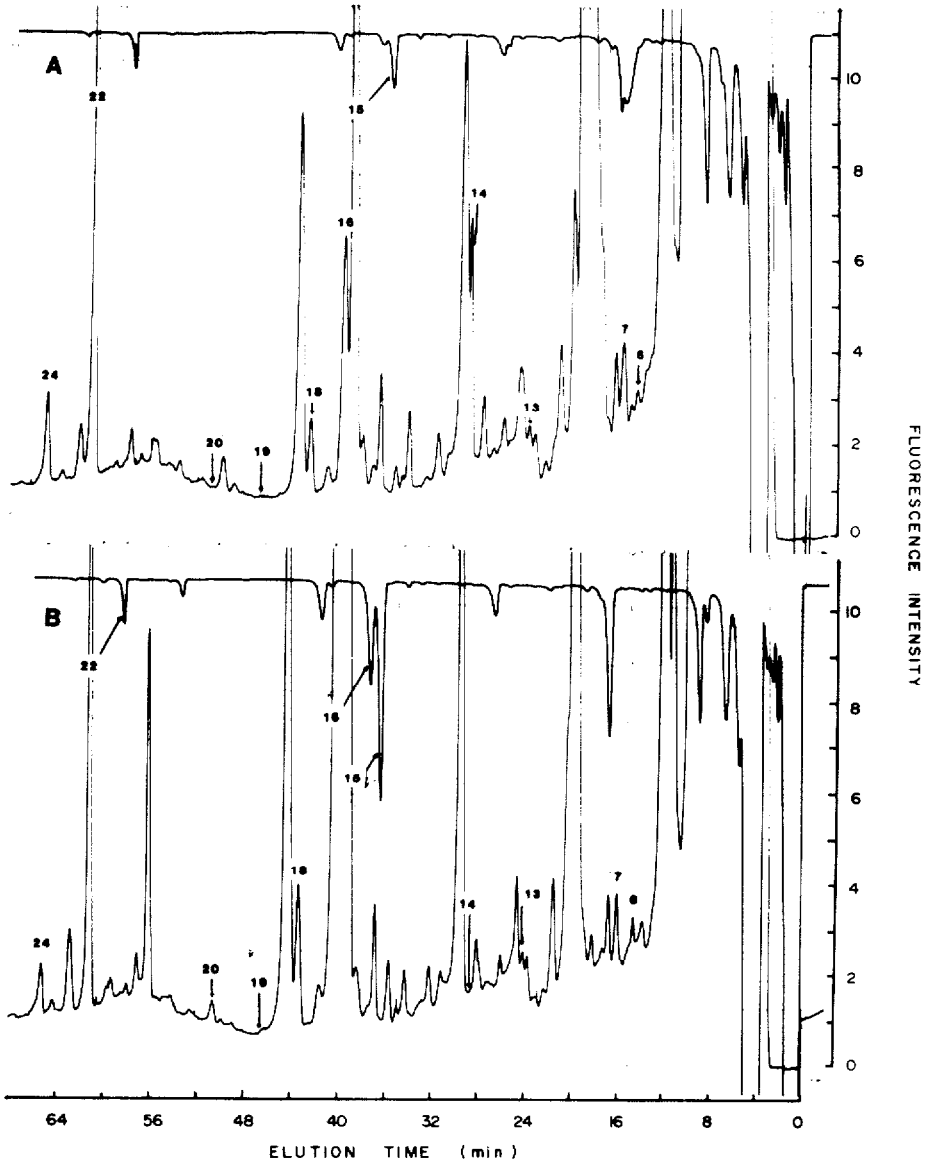

Fig. 6. Separation of rat urine hydrolysates (6 *M* hydrochloric acid, 120°C, 18 h), using gradient II. The urine are the same as those mentioned in Fig. 5. (A) Hydrolysate of a normal rat urine. (B) Hydrolysate of the urine of a rat treated with aminoguanidine sulphate. For details see Materials and methods. For peak identification, see Table II.

with putreanine, before its determination in urine by the present method can be recommended. However, the usefulness of the present method for the determination for putreanine in brain has firmly been established. If only determinations of putreanine, acetylputrescine or of the compounds that are eluting around these is required, it is most advantageous to use the isocratic part of gradient III (Fig. 3). Elution with a mixture of 90% A and 10% C is a most suitable procedure for the separation of these compounds. Up to

60 chicken brain extracts have been consecutively applied on a column without a washing step. Nevertheless, it is advised to remove the polar components of tissue extracts from the pre-column and the separation column by washing with a mixture of 70% A and 30% C after each series, or whenever interfering compounds appear in the eluate. Equilibration of the column for 30 min is required to restore starting conditions.

Determination of S-adenosylmethionine, 5'-methylthioadenosine and decarboxylated S-adenosylmethionine

S-Adenosylmethionine is decarboxylated to decarboxylated S-adenosylmethionine. This serves as donor of the aminopropyl group which is necessary for the formation of spermidine from putrescine and spermine from spermidine. 5'-Methylthioadenosine is the other product of the aminopropyl transfer reaction [21]. The determination of these adenosine derivatives is, therefore, of importance in certain studies of polyamine metabolism. Wagner et al. [12] have previously determined these compounds by absorbance at 254 nm in the eluate of a similar ion-pair reversed-phase method. As shown in Fig. 1, these adenosine derivatives can be determined as well, using the new elution programmes. Moreover, our original chromatographic procedure [1] is also suitable for this purpose. There is no interference by aromatic acids and related naturally occurring compounds.

CONCLUSIONS

One of the major difficulties in the establishment of separation procedures for a series of compounds of greatly differing polarity is the construction of gradients in such a way that resolution is not impaired by peak broadening or tailing.

This was not achieved in most of the previously published procedures of polyamine separations. It is demonstrated in the present work that even columns with a high capacity factor allow one to produce separations with peaks of nearly identical width over the entire range, if the gradient is constructed appropriately. Liquid chromatographs with the possibility of forming gradients from three solvents simplify the task enormously. In our case, for example, we were only able to work out a suitable binary system after separations with ternary systems had been established.

The separations presented in this paper do not improve the reproducibility or sensitivity of polyamine determination significantly; these are the same as were previously reported [1]. However, the methods allow one to establish a more complete pattern of the polyamines and their metabolites in one run than was previously possible.

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