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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE SEPARATION AND MEASUREMENT OF DI- AND POLYAMINES AND THEIR DERIVATIVES, AND SPECIFIC PREPARATION OF ISOMERS OF THEIR MONOACETYL DERIVATIVES

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SUMMARY

We present a method for separating and quantitating the di- and polyamines and many of their derivatives found in mammalian tissues by high-performance liquid chromatography using a cation-exchange resin with gradient elution. Three different solvent systems are described, each with special advantages. The nitrate anion is used in order to permit increasing the cation concentration without increasing buffering capacity or introducing halide ions, which are corrosive for stainless steel. The specific synthesis of two isomers of N-monoacetylspermidine is described.

INTRODUCTION

Recent observations that N-monoacetylputrescine, two isomeric N-monoacetylated spermidines and the enzymes relevant to their metabolism occur naturally in animal tissues has focused biochemical interest on acetylated derivatives of the polyamines. This paper presents a method suitable for separating these polyamine metabolites in biological samples, and outlines methods for their synthesis.

Several approaches have been used for separating and measuring the polyamines, spermidine and spermine, and their metabolic precursor, putrescine [1]. These include thin-layer chromatography [2], gas—liquid chromatography of derivatives [3—5], radioimmunoassay [3] and high-performance liquid chromatography (HPLC), either by reversed-phase chromatography of fluorescent derivatives such as the dansyl [6, 7] or *o*-phthalaldehyde [8, 9] derivatives or

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by direct chromatography on cation-exchange columns and subsequent derivatization (ninhydrin color [10—12] or fluorescence [13—16]). The most widely used method employs a cationic column with formation of the *o*-phthalaldehyde derivative and measurement by fluorescence. With this approach, amounts as little as 10 pmol can be detected [13].

Polyamines occur naturally in both free and conjugated forms. No method using a chromatographic column with gradient elution without previous derivatization has been proposed for separating and measuring the major simple metabolites such as the monoacetyl derivatives of putrescine, spermidine and spermine, or the partially oxidized derivatives such as γ-aminobutyric acid putreanine, isoputreanine or 2-hydroxyputrescine. Moreover, the methods now in use (with the exception of the derivatized reversed-phase system of Seiler et al. [7]) are based on converted amino acid analyzers and therefore are programmed for stepwise isocratic elution patterns. We have developed a novel system involving a linear gradient which permits separation of most of the major simple metabolic products of the polyamines from one another and from the free amino acids which are usually present in biological samples without precolumn derivatization.

EXPERIMENTAL

Equipment

Solvents are filtered through fritted steel filters and passed through a Gilson Mixograd gradient maker fitted with a C-V General Valve No. 1-17-900 to an Altex 100A double piston pump at 0.8 ml/min and thence through a 2-μm Inline Mobile Phase Filter (Rainin 905-15) to a Rheodyne injection valve (No. 7125) with a 20- or 100-μl injection loop. Samples are applied by the filled loop technique. The flow then passes through a precolumn into a prepacked 250 × 3.5 mm I.D. Partisil PXS 10-25 SCX cation-exchange column (Whatman, Clifton, NJ, U.S.A.) at 50°C. The effluent is led to a T-junction, where it enters at right angles to the flow of the fluorescent reagent. The *o*-phthalaldehyde reagent [17] is pumped at a flow-rate of 0.9 ml/min by an Eldex E-120-S pump through the long limb of the T-connector. The mixed streams then pass through a 10-μl flow cell in a fluorometer (exciting light filtered with a Corning 7-60 filter, peak transmission 330 nm; emitted light filtered through a combination of Corning filters 3289, 5113 and 3-74, peak transmission 455 nm) and the signal from the photomultiplier tube is amplified in a Farand Photometer and recorded on a strip recorder. All connections from the pistons of the pumps to the T-connection are stainless-steel tubing, 1/16 in. O.D. and connections beyond the T-connection are 1/16 in. O.D. PTFE-tubing. It was sometimes advantageous to use isotopic tracers in our biological studies, and on other occasions ^{3}H- or ^{14}C-tracers were added to specimens before analysis in order to verify the location of specific peaks on the chromatogram. When isotopes were used, the effluent from the flow cell could be led directly to a fraction collector, where 1-min samples (1.7 ml fluid volume) were counted after addition of 5 ml of ACS counting fluid (Radiochemical Centre, Amersham, Great Britain).

Reagents

All reagents are analytical grade and are made up in double glass-distilled water, or in redistilled methanol. All aqueous reagents entering the HPLC column are filtered through a 0.3-μm Millipore filter under vacuum to remove dissolved gas and stored at 4°C, when not in use.

The fluorescent reagent is made up from two stock solutions: a potassium borate buffer, 1.0 *M* (pH 10.4), to which has been added 20 ml/l of 5% Brij 35 (Pierce, Rockford, IL, U.S.A.). This reagent can be made up in 4-l lots and stored indefinitely at room temperature. The second stock solution, 2 g *o*-phthalaldehyde in 25 ml methanol (Aldrich No. P 3,940-0, Milwaukee, WI, U.S.A., although the source seems not to affect activity) is stored in a dark bottle in the freezer. Working reagent is prepared by adding to each 100 ml of borate buffer 0.2 ml 14.3 *M* 2-mercaptoethanol and 1.0 ml of the *o*-phthalaldehyde solution. We have found it advantageous to heat this working solution to 55°C for 20 min to remove dissolved gas before using it. The working reagent remains active when kept in a dark bottle at 4°C for several weeks.

The concentrations of the eluting buffers are expressed in terms of their cationic component, i.e., as moles of Na^+. The main system consists of a linear gradient from 0.01 *M* sodium acetate to a mix containing 0.05 *M* sodium acetate in 0.95 *M* sodium nitrate, at a constant pH of 4.60. A second system consisting of a gradient from 0.01 *M* sodium citrate (pH 3.04) to 0.05 *M* sodium citrate in 0.95 *M* sodium nitrate (pH 3.27), is also useful for special separations. When it is necessary to collect fractions for reprocessing under circumstances where removal of the sodium-containing buffer would present a problem, an ammonium formate buffer system may be used, since ammonium formate can be removed by sublimation. In this case, the gradient is from 0.01 to 1.0 *M* ammonium formate at pH 3.40. This system is especially suitable when isotopically labeled compounds are to be detected by their radioactivity, but is not useful for fluorescent measurement since the ammonium ion fluoresces in *o*-phthalaldehyde.

Standards

The following chemical standards were obtained from commercial sources as indicated: putrescine · 2HCl (1,4-diaminobutane · 2HCl; Sigma P-7505, St. Louis, MO, U.S.A.); spermidine · 3HCl (Sigma S-2501); spermine · 4HCl (Aldrich S383-6); γ-glutamylputrescine · HCl (Calbiochem 352491, Los Angeles, CA, U.S.A.); crystalline bovine serum albumin (Sigma A-4503); γ-aminobutyric acid (Calbiochem 1370) and putreanine [N-(4-aminobutyl)-3-aminopropionic acid · 2HCl; Calbiochem 541081]. Monoacetylputrescine, N^1-acetylspermidine and N^8-acetylspermidine were synthesized in our laboratories as described later. A small sample of D,L-2-hydroxyputrescine · 2HCl was kindly supplied by C. Hurwitz, who synthesized it by reduction of 1,4-diaminobutanone [18].

Both ^{14}C- and ^{3}H-radioactive standards were also used, but we found that the purity of the ^{3}H-standards was unreliable. Each batch had to be checked before use, since there was frequently contamination with a front-running radioactive component whose chromatographic mobility remained unchanged after hydrolysis in 8 *N* hydrochloric acid at 110°C for 16 h. It seems likely that some

of this contamination was ^{3}HOH, since part of it could be sublimed away; but the nature of the remainder is uncertain. A possibility is that it represents γ-aminobutyric acid or some other oxidation product. There was negligible contamination with ^{14}C-standards, but their specific radioactivity is so low that they sometimes contributed to the fluorescent measurement when trace amounts were added and the detector set at maximum useful sensitivity. The radioactive materials were obtained from New England Nuclear (Boston, MA, U.S.A.).

Sample preparation

All biological material prepared for application to the column was deproteinized with 5% trichloroacetic acid (TCA). This included samples which had been subjected to proteolysis, since it was essential to remove all traces of protein.

To obtain total polyamines, without concern for the derivatives, samples were hydrolyzed in 8.3 *M* hydrochloric acid at 110°C for 16 h. The hydrochloric acid was then removed in vacuo and the sample reconstituted either in distilled water or in the 0.01 *M* buffer appropriate to the analysis. Acid hydrolysis of biological samples often produced charred residues which were removed by centrifugation prior to lyophilization of the hydrochloric acid. Crystalline bovine serum albumin was prepared in this way to serve as a standard for mixed amino acids.

Chemical syntheses

Monoacetylated diamines. Monoacetyl-1,3-diaminopropane and monoacetyl-1,4-diaminobutane were prepared by the acetic anhydride method described by Tabor et al. [19].

Acetylated spermidines. The nitrile whose reduction yields N^{8}-acetylspermidine was synthesized according to the method of Tabor et al. [19]. After the condensation reaction and subsequent hydrogenation, the catalyst was removed by low-speed centrifugation and washed with water. The pooled sample was concentrated to about 10 ml in vacuo at 40°C. Sulfate ions were removed and the product was converted to the acetate by using the anion-exchange resin Dowex 1 in its acetate form. During concentration of the Dowex 1 eluate, a small amount of white crystalline material appeared, which separated readily, was difficultly soluble in water, had a very high melting point, contained no sulfate and had no demonstrable NMR spectrum. This was filtered off and discarded. The concentrated sample was placed on a 10 × 2.5 cm column of the cation exchanger Dowex 50-X2, 200—400 mesh, in the hydrogen form, and washed with about 50 ml of water. The water eluate was negative to ninhydrin. Elution with 1.5 *N* hydrochloric acid yielded ninhydrin positive material which, after being taken to dryness under reduced pressure at a temperature below 40°C, was repeatedly crystallized from hot absolute ethanol. Low-speed centrifugation was the method of choice for gathering the crystals. A final wash was done with diethyl ether. The yield, based on the nitrile as starting material, was 42%. It was convenient to handle 20 mmol of nitrile.

Tabor and Tabor's method [20] for preparing N^{1}-acetylspermidine was modified as follows: condensation of N-acetyl-1,3-diaminopropane (22 mmol) with 4-bromobutyronitrile (20 mmol) was followed immediately by catalytic hydro-

genation until hydrogen uptake ceased. Sulfate was removed by Dowex 1 as for the N^8-isomer; the preparation was then concentrated and processed with Dowex 50. The elution pattern of the amine was followed by *o*-phthalaldehyde fluorescence. The material eluting with water and with 200 ml of 0.5 *M* hydrochloric acid was discarded. Elution was continued with 1.5 *M* hydrochloric acid. Two hundred ml of eluate were collected, concentrated and recrystallized as for the N^8-isomer. The yield was 19%, based on the nitrile.

Analysis

The identity of the chemicals synthesized was confirmed by elementary analysis, melting point determination, NMR spectrum (Table I) and by hydrolysis to yield the parent compound.

TABLE I

PROOF OF IDENTITY OF SYNTHESIZED COMPOUNDS

Compound*		Elementary analysis (%)**			Melting point corrected (°C)	NMR spectrum*** (1% tetramethylsilane in ^2H_2O)
		C	H	N		
N-Acetyl-1,3-diamino-propane	Observed	39.3	8.7	18.6	160—161	3.27(t,2H,J=6.5 Hz); 3.03(t,2H,J=7.5 Hz); 2.01(s,3H); 1.88(m,2H)
	Theory	39.4	8.6	18.4		
N-Acetyl-1,4-diamino-butane	Observed	43.3	9.3	17.1	138—139	3.22(t,2H,J=6.6Hz); 3.02(t,2H,J=7.5 Hz); 2.00(s,3H); 1.68(m,4H)
	Theory	43.3	9.1	16.8		
N^8-Acetyl-spermidine	Observed	41.0	8.6	17.2	203.5—205	3.42(t,2H); 3.42(t,2H,J=6.6 Hz); 3.13(t,2H,J=7.6 Hz); 3.00(t,2H,J=6.7 Hz); 1.99(s,3H); 1.64(m,6H)
	Theory	41.5	8.9	16.2		
N^1-Acetyl-spermidine	Observed	41.3	9.0	17.3	189—191	3.29(t,2H,J=6.7 Hz); 3.06(m,6H); 2.01(s,3H); 1.90(m,2H); 1.77(m,4H)
	Theory	41.5	8.9	16.2		

*Compounds were prepared as the hydrochloride salts.
**Analysis performed by Baron Consulting Co., Orange, CT, U.S.A.
***NMR spectra were obtained on a Bruker HX-270 instrument.

RESULTS

All of the amino acids are eluted within the first 15 min. During this time, γ-aminobutyric acid and γ-glutamylputrescine also appear. After the gradient has terminated at 40 min, elution is continued isocratically with 1.0 *M* Na^+ if necessary. The retention times and relative fluorescence of the various compounds are shown in Table II. Note that this system does not resolve 2-hydroxyputrescine from N-acetylputrescine, and resolves N^1-acetylspermidine from N^8-acetylspermidine poorly. All other peaks are cleanly resolved.

TABLE II

RETENTION TIMES AND RELATIVE FLUORESCENCE OF POLYAMINES AND RELATED SUBSTANCES

Absolute values of the retention times are individual characteristics of the particular column.

Compound	Acetate buffer			Citrate buffer	
	Retention time (min ± S.D.)	C.V. (%)	Fluorescence vs. putrescine	Retention time (min)	Fluorescence vs. putrescine
γ-Aminobutyric acid	8.1 ± 0.3	3.1	1.48	9.4	1.32
γ-Glutamylputrescine	13.6 ± 1.4	9.9	2.08	15.0 **	1.95
N-Acetylputrescine	16.5 ± 0.3 *	2.0	0.48	15.7	0.49
2-Hydroxyputrescine	16.7 ± 0.1	0.6	0.92	17.7 **	0.76
Putreanine	17.3 ± 0.4	2.0	0.20	17.2 **	0.21
Putrescine	19.2 ± 0.5	2.8	1.00	17.6	1.00
N^8-Acetylspermidine	24.1 ± 0.1	0.4	0.14	21.0 ***	0.09
N^1-Acetylspermidine	25.5 ± 0.8	3.0	0.18	21.4 **	0.10
Spermidine	27.2 ± 0.7	2.6	0.88	21.0	0.61
Spermine	36.4 ± 1.3	3.7	0.38	26.4	0.24
Carnosine	13.3 ± 0.1	0.2	0.36		
Homocarnosine	13.8 ± 0.1	0.1	2.33		
Anserine	16.1 ± 0.7	4.3	0.16		
Cadaverine	21.1 ± 0.1	0.6	1.03		
3,3'-Iminobispropylamine	24.4 ± 1.0	4.0	0.80	18.6	0.76
1,3-Diaminopropane	15.8			16.3	
N-Acetyldiaminopropane	14.1			13.7	
Ammonium ion	10.0			12.7	
Histamine	21.1 ± 1.2	0.8	0.29		
Amino acids	4.5—16			5—13	

*Not resolved by acetate system but resolved by citrate system.
**Not resolved by citrate system, but resolved by acetate system.
***Not resolved by citrate system; partially resolved by acetate system.

The reproducibility of the system is also shown in Table II. Since the peaks are very narrow (Fig. 1) quantitative estimation by measuring peak heights is preferable to attempts to estimate their areas.

The linearity of fluorescence has already been established by others [13] and we could confirm that the fluorescence for any given compound was linear over the range of 20 pmol to 10 nmol. The application of this system to a liver extract is shown in Fig. 2.

A second buffered gradient elution system similar to the sodium acetate system utilized the sodium citrate buffer gradient described previously. This system produced different retention times permitting resolution of N-acetylputrescine from 2-hydroxyputrescine and provided a check on identification of the compounds (Table II).

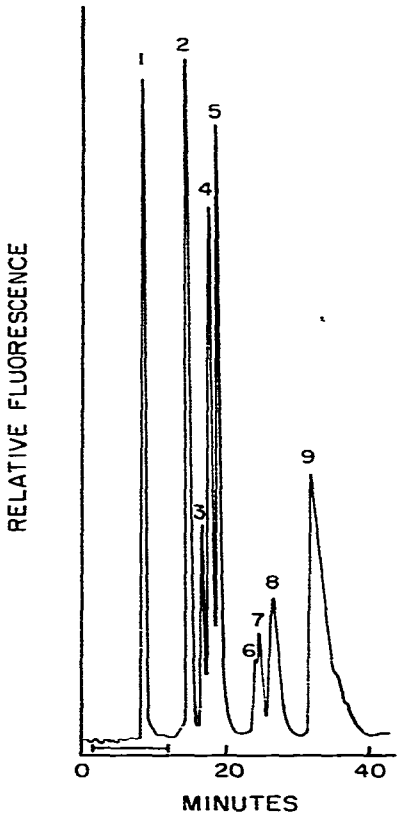

Fig. 1. Chromatogram of mixtures of diamines, polyamines and some of their derivatives, in the acetate system. The compounds are identified as follows: 1, γ-aminobutyric acid; 2, γ-glutamylputrescine; 3, N-acetylputrescine; 4, 2-hydroxyputrescine; 5, putrescine; 6, N^8-acetylspermidine; 7, N^1-acetylspermidine; 8, spermidine; 9, spermine. The location of the amino acids is indicated by the line below the tracing. The vertical line on the left marks the time at which the sample was applied to the column. The total duration of the chromatogram from application of the mixture to the end of the tracing is 39 min. Each peak represents 10 nmol of compound.

DISCUSSION

A linear gradient elution system has advantages over others which have been proposed. It avoids the sudden shifts of baseline which may be seen in making step changes in isocratic systems. The buffers used in this type of system must be fairly acidic in order to maintain complete ionization of the amino groups of the polyamines. The *o*-phthalaldehyde reaction used to generate fluorescence requires a highly basic medium. Sensitivity is lost if the entire increment of cations in the gradient is associated with a buffer anion which results in an altered pH of the reaction mixture. It is therefore advantageous, in the concentrated part of the buffer curve, to provide part of the cations in association with a strong acid such as chloride. However, the use of chloride ion, as recommended by most other workers [9, 11—16], causes corrosion of steel and in-

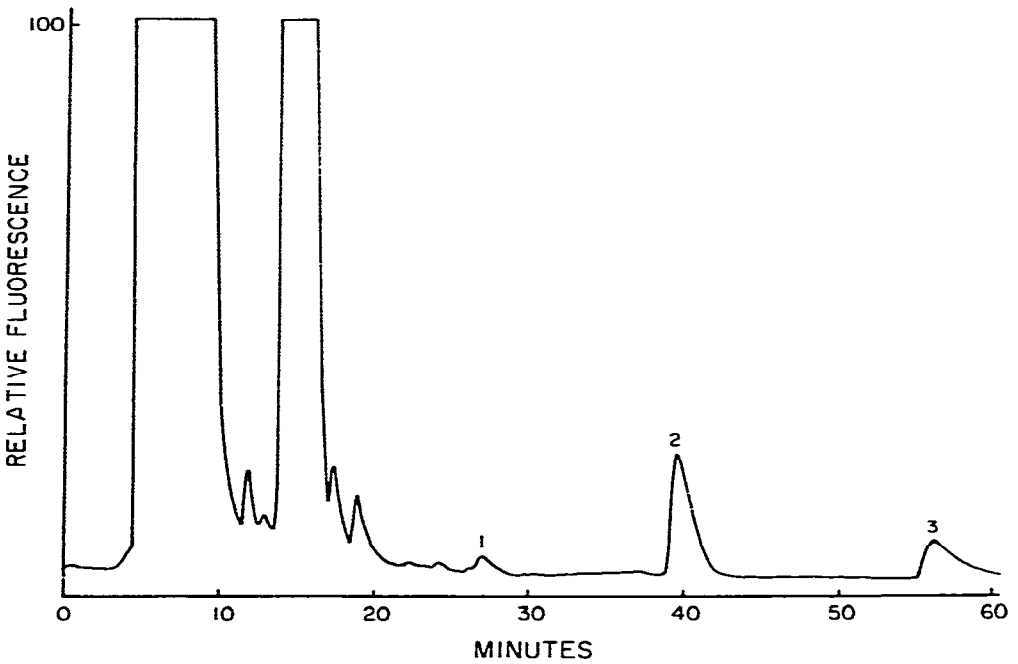

Fig. 2. Chromatogram of an acid extract of the liver of a normal 300-g rat. The tissue was extracted with an equal volume of 0.01 *M* hydrochloric acid. The extract was then precipitated with 10% trichloroacetic acid. The supernatant was hydrolyzed with 8.3 *M* hydrochloric acid at 110°C for 18 h. The hydrochloric acid was then removed in vacuo and the dry residue taken up in distilled water and applied to the column. The peaks were quantitated by standards run before and after the analysis. The tissue levels of putrescine (peak 1), spermidine (peak 2) and spermine (peak 3) (34, 938 and 757 nmol/g wet weight respectively) are in agreement with those recorded in the literature [21]. In addition to the amino acid peaks, several other peaks appear, which have not been identified with certainty.

validates the warranty on such pumps as the Altex which we use. Others have commented on the corrosive effect of chloride-containing buffers, though minimizing the damage it does to the actual valve system [14]. In contrast, the buffers we propose are entirely harmless to the fittings of the HPLC system, since nitrate ion is inert and produces no corrosion. It does not interfere with the assay and is compatible with the compounds under study.

The ability of the system to separate most of the known metabolites of the polyamines from the amino acids present in biological materials as well as from one another without requiring additional steps is a particular advantage in performing metabolic studies. Moreover, because the column can be reconstituted by a wash with the initial buffer for only 20 min, it is suitable for repeated cycling and could easily be automated. In our experience, the columns are suitable for at least 200 cycles, if maintained properly according to the instructions of the manufacturers.

We preferred to use the unique synthesis described here in preparing the monoacetyl derivatives of spermidine because it avoids forming the isomer

which is inevitably present in a synthesis using spermidine as the starting material. Thus, the more cumbersome sequence of synthetic chemical steps was selected in these syntheses because of the level of purity desired in the product.

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