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ADVANTAGES OF A NEW ION-EXCHANGE RESIN FOR THE LIQUID CHROMATOGRAPHY OF BIOGENIC AMINES: A METHACRYLIC ACID POLYMER CROSS-LINKED WITH BUTANEDIOLDIACRYLATE

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SUMMARY

The synthesis of a methacrylic acid polymer, cross-linked with 11% 1,4-butanedioldiacrylate is described. This was shown to be much more efficient than conventional carboxylic resins for the liquid chromatography of biogenic amines, at least in the pH range 7–9. The height equivalent to a theoretical plate was from 2–3 particle diameters for most of the amines tested. The peaks given by the remainder could often be sharpened by adding imidazole to the eluting buffer. A low-pressure system based on the new resin, giving a mean peak width of only 1.1 ml will separate 18 or more mono-, di- and polyamines simultaneously.

INTRODUCTION

Synthetic resins bearing carboxyl groups have been widely used for the liquid chromatography (LC) of biogenic amines[1–13]. The pH range 7.5–11.5 is particularly suitable for such separations because within it the ionic states of all amines vary and therefore their elution orders will be very sensitive to pH; versatile and flexible chromatographic systems are thus obtained. However, commercial carboxylic resins tend to chromatograph aromatic amines inefficiently especially at neutral[14] and alkaline pH values[15]. One reason for the broad tailed peaks observed is probably aromatic–aromatic interaction between the divinylbenzene (DVB) residues used to cross-link commercial resins and the benzene and indole rings found in so many physiologically active amines.

Thus, as a preliminary experiment, the chromatographic performance of a completely aliphatic resin, cross-linked with 1,4-butanedioldiacrylate (BDDA) residues, was compared with that of two conventional resins. One of these, formed by polymerising a mixture of methacrylic acid and DVB, corresponded to the old form of Zeo-Karb 226; the other, prepared from ethylacrylate, methacrylic acid and DVB, with subsequent hydrolysis of the ester groups, corresponded to the current commercial resin Zerolit 236. The standard recipes were adopted except that the Zeo-Karb 226 was prepared from unusually pure DVB containing 80% of the required

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TABLE I

SPECIFICATION OF THE THREE SYNTHETIC RESINS USED IN THE PRELIMINARY EXPERIMENTS

Industrial chemicals were used throughout except as mentioned in the text. The DVB used for preparing Zerolit 236 was the least pure of these and a typical analysis would be: 1,4-divinylbenzene, 19%; 1,3-divinylbenzene, 43%; 1,3- and 1,4-ethylvinylbenzene, 34%; diethylbenzene, 2.4%. Where the monomers do not add up to 100% the discrepancy is due to the impurities involuntarily added with the DVB.

	Zeo-Karb 226	*Zerolit 236**	*"Aliphatic" resin*
Composition of the monomer mixtures (molar percentages)	Methacrylic acid, 95% Divinylbenzene, 4%	Methacrylic acid, 30% Ethylacrylate, 63% Divinylbenzene, 4%	Methacrylic acid, 94.5% 1,4-Butanedioldiacrylate, 5.5%
Mean particle diameter, measured in pH 7.0, 0.2 *M* sodium phosphate buffer (μm)	41 (100% between 33 and 50)	44 (93% between 34 and 54)	47 (95% between 37 and 57)

* Now marketed as Duolite C436.

monomer. BDDA tends to give less rigid resins than DVB, so relatively more of it was used. The resins, whose detailed specification is given in Table I, were all made from the same batch of commercial methacrylic acid.

Fig. 1 and Table II illustrate the results obtained when the three resins were used for the chromatography of representative aromatic amines at a pH of 8.85. All substrates gave similar elution volumes and plate numbers for dopamine, a compound that scarcely interacts at this pH because its phenolic hydroxy groups are partially ionised. However, the "aliphatic" resin was clearly the most efficient for chromatographing *p*-tyramine and tryptamine, whether or not allowance was made

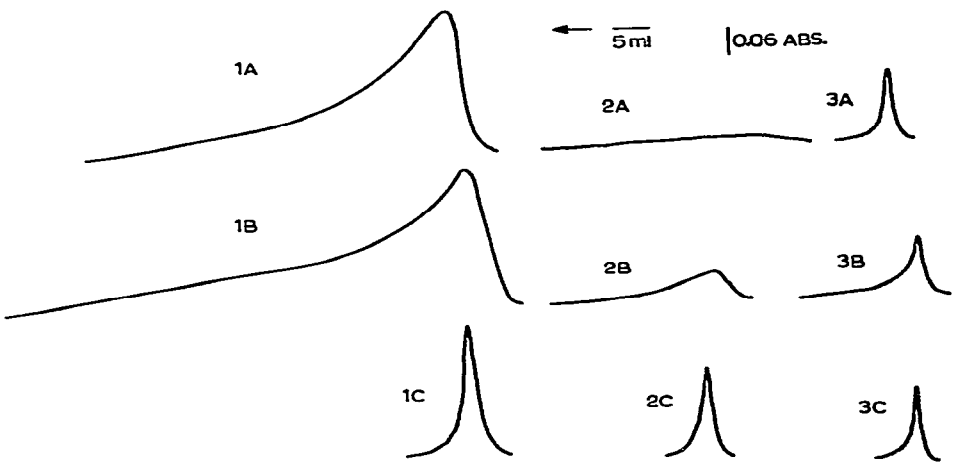

Fig. 1. Shape of peaks given by aromatic amines on three different ion-exchange resins. A = Zeo-Karb 226; B = Zerolit 236; C = "Aliphatic" resin; 1 = tryptamine; 2 = *p*-tyramine; 3 = dopamine.

for particle diameter; it also gave very much lower elution volumes and near-gaussian peaks (Fig. 1). In contrast, the resins containing DVB gave late, tailed peaks, suggesting the presence of secondary adsorption sites. Here elution volumes were often dependent on the history of the column. Thus the elution volume of tryptamine on Zerolit 236 was initially 166 ml, declining to 65 ml after the fourth 25-μmole sample had been applied. Table II shows that factors other than the nature of the cross-linking agent affect chromatographic performance but the "aliphatic" resin was so promising that the rest of this paper is devoted to describing its preparation and performance in more detail.

TABLE II

THE CHROMATOGRAPHY OF AROMATIC AMINES ON THREE SYNTHETIC RESINS IN pH 8.85 BORATE BUFFER

Columns of resin, 30 × 0.6 cm I.D., were eluted with pH 8.85, 0.01 *M* borax buffer containing 0.4 *M* sodium nitrate at a flow-rate of 7.2–8.4 ml h^{-1}. Typical loadings were: dopamine, 1 μmole; *p*-tyramine 5 μmole; tryptamine 50 μmole. However, only 5 μmole samples of tryptamine were chromatographed on the "aliphatic" resin. The temperature was 18–23°C and the column effluents were analysed by an automatic device that reacted 0.83-ml fractions with Folin's reagent (sodium salt of 1,2-naphthaquinone-4-sulphonic acid)[19]. The chromatographic peaks were reconstructed from these results. The figures in the table are averages of duplicate or triplicate measurements.

Amine	*Zeo-Karb 226*	*Zerolit 236*	*"Aliphatic" resin*
	Adjusted elution volumes (ml)		
Dopamine	6.4	6.7	6.2
p-Tyramine	54.1	65.4	18.5
Tryptamine	27.1	65.1	16.8
	Chromatographic efficiencies (plates/cm)		
Dopamine	4.4	5.9	5.1
p-Tyramine	2.5	16.0	28.4
Tryptamine	0.2	4.5	16.7

EXPERIMENTAL

Preparation of "aliphatic" resin

The usual technique was adopted, that of polymerising a monomer mixture in suspension in an aqueous medium. The cross-linking agent, BDDA is susceptible to acid hydrolysis so it had to be copolymerised with a free acid, sparingly soluble in the aqueous suspending medium. This was the reason why methacrylic acid rather than acrylic acid wasadopted as the major monomer. A mixture of BDDA, methacrylic acid and appropriate catalyst will not polymerise at all as a suspension except under an oxygen-free atmosphere. It then does so giving distorted spheres mixed with irregular lumps; the latter are probably formed from monomers dissolved in the aqueous phase. Certainly the physical uniformity of the product was greatly improved by adding the polymerisation inhibitor, ammonium thiocyanate to the suspending medium. Azo-bis-isobutyronitrile (AZDN) was adopted as the polymerisation catalyst because it was non-aromatic. The following recipe gives a resin nominally containing

11% of BDDA, calculated on a molar basis; this was used for all the chromatographic studies described here. However, resins containing 2.7% and 5.5% of BDDA were also successfully prepared.

Monomer mixture	
Commercial methacrylic acid	100 ml
1,4-Butanedioldiacrylate	26 ml
AZDN	0.25 g
Suspending medium	
Commercial sodium chloride	210 g
Sodium carboxymethylcellulose (cellofas B, grade B50)	6 g
Ammonium thiocyanate	600 μg
Water	600 ml

The monomers were distributed in the suspending medium by rapid stirring to give a suspension containing droplets of organic phase averaging 30 μm in diameter. These were polymerised in an atmosphere of nitrogen by heating the suspension to 70°C for 2.5 h and, subsequently, to 85°C for 16 h. The resulting resin was precipitated with 12 ml 12 *M*-hydrochloric acid and washed with 6 × 2.5 l water.

The product was hydraulically graded in distilled water, using a modification of Hamilton's arrangement[16]. The fraction used for this work had particles 29 ± 10 μm in diameter; 95% of the particles fell within this range when diameters were measured in pH 7.0, 0.2 *M* sodium phosphate buffer.

Most of our results were obtained using this resin without further pre-treatment. However, as explained later, we recommend that each bed volume of resin should be extracted before use with 9 bed volumes of 50% (v/v) aqueous acetone for 3 days at 25°C. The aqueous acetone probably extracts residual monomers and low molecular weight material; it certainly hardens the resin, so improving its physical as well as its chromatographic properties. Fig. 2 shows a photomicrograph of the resin after acetone extraction.

Chromatographic techniques

The column pressure was comparatively low, never exceeding 4 bars (0.4 MPa). Samples of amine hydrochlorides (0.1–0.3 μmole at 100 μmole/ml in 1 *M* sucrose) were normally layered onto the surface of the resin and washed directly into the column with eluting buffer.

Amines were usually detected by reacting them with 2,4,6-trinitrobenzene sulphonic acid (TNBS) as described by Snyder and Sobocinski[17]. In our modification, a segmented stream continuous analyser was used to mix aqueous TNBS (0.13 g/l) and pH 9.7, 0.1 *M* borax buffer, each reagent being supplied at a flow-rate of 9.6 ml h^{-1}. The buffered TNBS was allowed to react with the column effluent for 35 min at 30°C and the resulting optical density was recorded at 420 nm.

Where appropriate, the results were used to calculate plate numbers by Glueckauff's method[18]. Adjusted elution volumes (*i.e.* adjusted retention volumes, V'_R) were measured relative to those of glutamate or phthalate, depending on whether

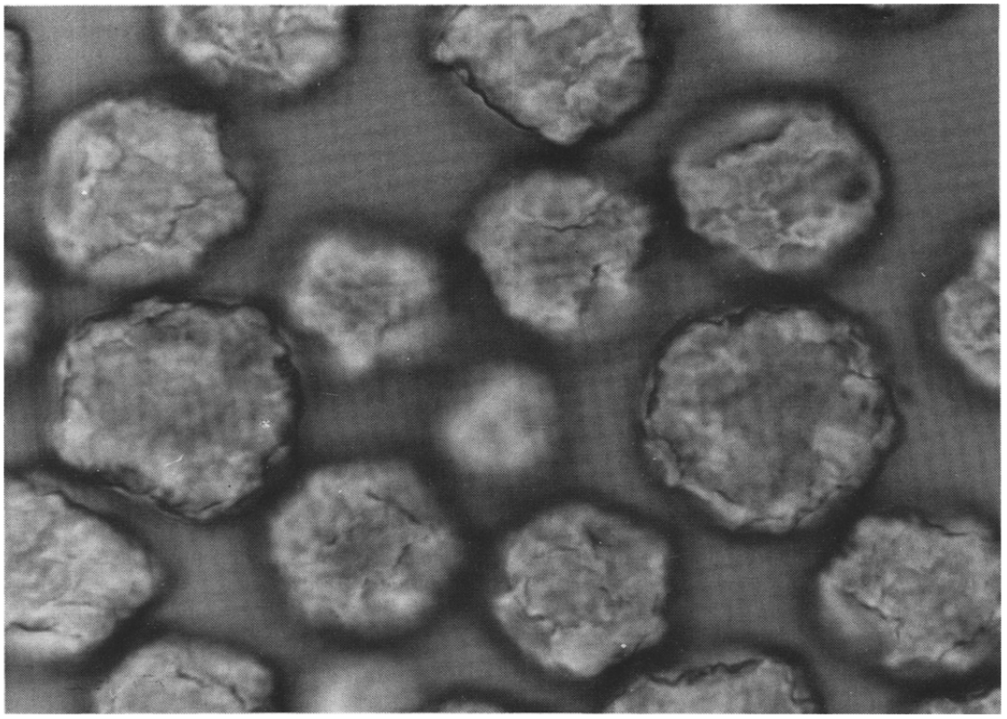

Fig. 2. Photomicrograph of methacrylate/1,4-butanedioldiacrylate resin after extraction with aqueous acetone.

detection was by TNBS or UV absorbance. Both these compounds were negatively charged in our eluting buffers and as they had the same elution volume within experimental error (± 0.1 ml), were assumed not to interact with the resin.

Unless otherwise stated, all the figures in the tables are averages of duplicate measurements. The relative standard error of the elution volume measurements is 3% and that of the plate number estimates is approximately 7%.

Influence of variables on chromatographic performance

Anions. The borate ion buffers from pH 8.1–10.7 and will not support the growth of micro-organisms so it forms a particularly suitable basis for an alkaline eluting buffer. Table III shows that 0.02 *M* borax, used in the presence of chloride and orthophosphate, reduced the average adjusted elution volume of the three catecholamines from 54.8 to 3.7 ml. This dramatic effect was probably due to the formation of catecholamine–borate complexes. It was not shown by β-phenylethylamine, *p*-tyramine or by five aliphatic monoamines; here the addition of borax only reduced elution volumes by an average of 5% and this is adequately explained by the higher sodium concentration of the modified buffer.

Nevertheless, the nature of the anion can have a general effect on amine chromatography. Table IV shows the results obtained when borate buffers having the same pH and sodium ion concentration but containing high concentrations of different major anions, are used to elute representative di- and polyamines. Compared

TABLE III

EFFECT OF BORATE ON THE ELUTION VOLUMES OF THE CATECHOLAMINES

The standard eluant was pH 7.0, 0.02 *M* sodium phosphate buffer containing 0.37 *M* sodium chloride; where appropriate, the pH was readjusted to 7.0 after adding borax. Catecholamine samples (0.4 μmole) were eluted, at 25°C, from a column of resin, 26 × 0.6 cm I.D. at a flow-rate of 6.4 ± 0.3 ml h^{-1} and were detected directly by their absorbance at 272 nm.

	Adjusted elution volumes (ml)	
Amine	*Phosphate eluting buffer*	*Phosphate eluting buffer containing 0.02 M borax*
Epinephrine	35.6	2.35
Dopamine	58.1	4.35
Norepinephrine	70.8	4.55

with chloride, citrate substantially increases and iodide decreases the elution volumes of all the compounds studied.

The anions giving the lowest elution volumes give the lowest chromatographic efficiencies; under conditions corresponding to Table IV, citrate doubles and iodide halves plate numbers relative to chloride and nitrate. In this case, excessively short elution times could be limiting efficiency but this can only be a partial explanation.

These anion effects provide an unexpected, additional way of modifying the elution pattern. Table IV shows that the elution order in citrate is quite different from that in iodide while the catecholamines separate much better in phosphate–chloride than they do when borate is also present (Table III).

Cations. Table V shows that the potassium ion will displace amines from the resin quite effectively, though it gives higher elution volumes than the sodium ion. Chromatographic efficiency is approximately the same whichever cation is used, while the elution order is clearly sensitive to the K^+/Na^+ ratio.

Temperature. Tables VI and VII show that temperature adjustments are also a

TABLE IV

EFFECT OF FOUR ANIONS ON THE ELUTION VOLUMES OF DI- AND POLYAMINES

The eluting buffer was 0.05 *M* borax containing 1 g/l imidazole, adjusted to pH 9.3 after the addition of the appropriate salt. The amines were eluted from a column of resin, 28 × 0.6 cm I.D. (when measured in chloride containing buffer) at 20°C and at a flow-rate of 7.9 ± 1.6 ml h^{-1}.

Amine	*Adjusted elution volumes (ml)*			
	Eluting buffer + 0.67 M sodium citrate	*Eluting buffer + 2.0 M sodium nitrate*	*Eluting buffer + 2.0 M sodium chloride*	*Eluting buffer + 2.0 M sodium iodide*
1,4-Diaminobutane	35.6	21.3	18.1	8.3
1,6-Diaminohexane	23.5	10.5	9.7	3.7
Spermidine	83.2	27.4	23.2	6.2
Spermine	63.0	35.0	31.4	4.2

TABLE V

EFFECT OF TWO CATIONS ON THE ELUTION VOLUMES OF DI- AND POLYAMINES

The eluting buffer was 0.05 *M* borax containing 10 g/l imidazole, adjusted to pH 9.3 after the addition of the appropriate salt. The amines were eluted from a column of resin 27.5 × 0.6 cm I.D. at 50°C and at a flow-rate of 7.9 ± 0.1 ml h^{-1}.

Amine	*Adjusted elution volumes (ml)*	
	Eluting buffer + 2.1 M sodium iodide	*Eluting buffer + 2.1 M potassium iodide*
Histamine	3.05	3.75
1,4-Diaminobutane	6.6	17.8
1,6-Diaminohexane	5.55	10.3
Spermidine	2.45	13.0
Spermine	1.6	14.7
Agmatine	10.4	26.1

very effective way of manipulating elution order. In most cases chromatographic efficiency increases with temperature and this is particularly pronounced for compounds showing anomalously low efficiencies like the polyamines and aromatic amines. High temperatures are particularly useful when chromatographing spermine and spermidine because, here, the increase in efficiency is associated with a considerable reduction in elution volumes. Thus the resin is best used at above-ambient temperatures.

pH. High pH values suppress the ionisation of amino groups and should pre-

TABLE VI

EFFECT OF TEMPERATURE ON THE CHROMATOGRAPHY OF DI- AND POLYAMINES

The eluting buffer was 0.05 *M* borax, containing 2.0 *M* sodium iodide and 1 g/l imidazole, adjusted to pH 9.3 immediately before use. The amines were eluted from a column of resin 27.5 × 0.6 cm I.D. at a flow-rate of 8.5 ± 1.0 ml h^{-1}.

Amine	*20°C*	*40°C*	*60°C*	*80°C*
	Adjusted elution volumes (ml)			
Histamine	4.5	4.45	3.5	2.5
1,4-Diaminobutane	8.3	8.0	5.85	4.15
1,6-Diaminohexane	3.7	4.9	4.95	4.2
Spermidine	6.2	4.65	3.0	2.0
Spermine	4.2	2.9	1.55	0.75
Agmatine	12.7	13.1	11.0	8.8
	Chromatographic efficiencies (plates/cm)			
Histamine	3.9	5.0	8.0	13.7
1,4-Diaminobutane	12.9	18.4	21.4	25.1
1,6-Diaminohexane	11.2	16.3	23.1	25.2
Spermidine	4.7	9.5	12.8	20.8
Spermine	1.8	4.4	6.3	9.3
Agmatine	11.6	15.8	19.9	28.6

TABLE VII

EFFECT OF TEMPERATURE ON THE CHROMATOGRAPHY OF THE MONOAMINES

The eluting buffer was 0.02 *M* borax containing 0.4 *M* sodium nitrate and 2 g/l imidazole, adjusted to pH 8.85 immediately before use. The amines were eluted from a column of resin 30 × 0.6 cm I.D. at a flow-rate of 7.5 ± 0.6 ml h^{-1}.

Amine	*20°C*	*40°C*	*55°C*
	Adjusted elution volumes (ml)		
Methylamine	34.0	39.5	42.6
Ethylamine	17.8	20.8	22.8
n-Butylamine	8.3	10.5	12.3
β-Phenylethylamine	9.7	11.0	10.9
p-Tyramine	19.6	19.8	18.2
	Chromatographic efficiencies (plates/cm)		
Methylamine	86	133	112
Ethylamine	58	131	120
n-Butylamine	54	102	93
β-Phenylethylamine	29	39	45
p-Tyramine	43	56	82

TABLE VIII

EFFECT OF pH ON THE CHROMATOGRAPHY OF MONOAMINES

The eluting buffer was 0.02 *M* borax, containing 0.4 *M* sodium nitrate, adjusted to the required pH before use. The amines were eluted from a column of resin 30 × 0.6 cm I.D. at 55°C and at a flow-rate of 6.9 ± 0.5 ml h^{-1}.

Amine	*pH 7.5*	*8.0*	*8.5*	*9.0*	*9.5*	*10.0*	*10.5*
	Adjusted elution volumes (ml)						
Methylamine	33.7	33.8	35.9	35.7	31.0	21.9	16.9
Ethylamine	21.0	22.4	22.2	20.2	18.0	14.3	10.7
n-Propylamine	16.1	15.3	15.1	14.5	12.7	8.3	6.3
n-Butylamine	13.1	12.8	12.3	11.0	9.7	7.3	4.9
n-Hexylamine	11.6	9.5	9.5	8.2	7.5	5.1	4.0
β-Phenylethylamine	14.9	12.3	12.0	12.1	5.5	3.1	1.8
p-Tyramine	25.7	22.1	20.2	12.2	6.9	2.5	1.3
Norepinephrine	5.3	2.5	1.6	0.9	*	*	*
	Chromatographic efficiencies (plates/cm)						
Methylamine	93	93	86	83	85	92	75
Ethylamine	104	109	111	110	115	88	16.
n-Propylamine	108	116	111	107	87	65	40
n-Butylamine	110	109	117	113	99	87	66
n-Hexylamine	84	85	83	88	67	63	41
β-Phenylethylamine	65	67	69	76	55	49	40
p-Tyramine	108	104	87	56	50	46	50
Norepinephrine	104	50	62	62	*	*	*

* No peak observed: decomposition.

vent them from binding to the resin. Table VIII shows that, as expected, increasing pH does accelerate amine elution.

It also decreases chromatographic efficiency. The elution volume at which efficiency begins to fall is different for each compound so the diminished plate numbers at high pH are not just due to the reduced elution times and probably imply that the free base forms of the amines are beginning to partition into the resin.

From the experimental standpoint, it is impractical to elute a strongly bound component with a low molarity buffer of extreme pH. Thus spermine can be readily displaced by 0.3 *M* Na^+ at pH 11.4 but with an efficiency of less than 0.1 plates/cm. The pH of the eluting buffer should not exceed 9.0 for general purpose amine chromatography.

TABLE IX

PRACTICAL LC SYSTEM FOR THE SEPARATION OF MONOAMINES: TNBS DETECTION

The eluting buffer was 0.02 *M* borax, containing 0.4 *M* sodium nitrate, adjusted to pH 8.5 immediately before use. The resin had been pre-extracted with aqueous acetone and a column of it, 100 × 0.34 cm I.D., was eluted at 55 ± 1°C and at a flow-rate of 1.65 ± 0.1 ml h^{-1}.

Amine	*Adjusted elution volumes (ml)*	*Peak width at base measured after triangulation (ml)*	*Total plate number*
Histidine	0.90	1.54	140
Dopamine	1.62	*	*
Glucosamine	3.0	0.45	3400
Galactosamine	3.65	0.68	1850
3,4-Dimethoxyphenylethylamine	7.8	0.49	8500
β-Hydroxyphenylethylamine	9.1	0.51	10300
tert.-Butylamine	9.5	0.44	14400
Isoamylamine	9.65	0.49	11600
n-Hexylamine	9.7	0.54	9900
n-Heptylamine	9.85	0.70	5900
2-Methylbutylamine	10.2	0.49	12600
n-Octylamine	10.45	0.66	7300
sec.-Butylamine	10.65	0.44	17000
n-Amylamine	10.8	0.44	17400
Normetanephrine	11.0	0.64	8500
Benzylamine	11.65	0.59	10600
β-Phenylethylamine	11.95	0.64	9600
n-Butylamine	12.65	0.51	16100
Isopropylamine	14.35	0.61	14000
3-Methoxytyramine	14.7	0.72	10400
Lysine	15.1	0.66	12700
n-Propylamine	15.8	0.66	14000
Octopamine	16.3	0.90	7700
Arginine	19.45	4.3	460
p-Tyramine	20.95	1.02	9300
Ethylamine	22.2	0.95	11800
Histamine	31.45	2.72	2700
Ethanolamine	34.8	1.29	14300
Methylamine	39.4	1.60	11590

* Dopamine gives a sharp, discrete peak when detected by TNBS but absorbance measurements at 272 nm show that it decomposes into several components, like other catecholamines.

Flow-rate. When measured under the conditions of Table IX, except that the pH was 8.9, typical aliphatic monoamines were chromatographed at their optimum flow-rate when moving down the column at approximately 10 cm h^{-1}. For methylamine, *n*-propylamine and *n*-hexylamine this corresponded to actual flow-rates of 45, 27 and 18 ml cm^{-2} h^{-1}, respectively. When the flow-rate was increased or decreased by a factor of 2, the plate numbers declined by an average of 25%. Every compound under each set of elution conditions will have its own optimum flow-rate but these figures suggest that the flow-rate range used in this work has been a reasonable one.

Method of packing column. Columns were prepared from the slurry formed by suspending wet resin in the minimum volume of eluting buffer. Columns packed in short sections gave better chromatographic efficiencies than those which were not. Thus a 0.6 cm diameter column, eluted as described in Table IX gave the following average values for β-phenylethylamine, *p*-tyramine and 5 aliphatic monoamines: bed packed in 8 × 3 cm sections, 81 plates/cm; bed packed as 1 × 25 cm section, 52 plates/cm. Multiple section colums were used for all the later experiments (Tables IX, X, XI; Figs. 3 and 4) though the plate numbers recorded in Tables VII and VIII suggest that single section columns do not invariably give bad results.

Column diameter. Varying the column diameter from 0.6–0.34 cm made only a

TABLE X

PRACTICAL LC SYSTEM FOR THE SEPARATION OF MONOAMINES: UV DETECTION

Conditions were identical to those for Table IX except that amine samples (0.1–0.03 μmole) were chromatographed in a buffer containing 0.4 *M* sodium chloride instead of sodium nitrate and were detected directly by their absorbance at 272 nm. Column back pressure was 0.5 bars (0.05 MPa).

Amine	*Adjusted elution volumes (ml)*	*Peak width at base after triangulation (ml)*	*Total plate number*
Macromerine	1.97	0.76	870
N-Methyl-3,4-dimethoxyphenyl-ethylamine	2.6*	0.56	1960
Hordenine	6.25	0.54	5400
N-Methyl-4-methoxyphenyl-ethylamine	6.35	0.58	4800
3,4-Dimethoxyphenylethylamine	8.3	0.63	5700
Gramine	8.85	0.65	5900
Normetanephrine	10.9	0.72	6500
N-Methyltryptamine	11.2	0.81	5400
Synephrine	11.65	0.72	7200
3-Phenyl-1-propylamine	12.95	0.86	6000
3-Methoxytyramine	15.2	0.86	7700
Octopamine	17.05	1.04	6300
o-Tyramine	17.7	0.92	8600
Tryptamine	20.7	1.10	7800
p-Tyramine	21.6	1.22	6900
5-Hydroxytryptamine	33.8	1.51	9800

* Our sample of N-Methyl-3,4-dimethoxyphenylethylamine also gave a major peak at 8.84 ml. The 2.6-ml peak is probably the authentic one because N-methylation would be expected to accelerate the elution of 3,4-dimethoxyphenylethylamine.

TABLE XI

PRACTICAL LC SYSTEM FOR THE SEPARATION OF DI- AND POLYAMINES

The eluting buffer was 0.019 *M* borax containing 1.1 *M* sodium iodide and 0.5 *M* potassium iodide, adjusted to pH 8.5 immediately before use. It was stored protected from light to prevent the release of iodine. The resin had been pre-extracted with aqueous acetone and a column of it, 100 × 0.34 cm I.D., was eluted at 55 ± 1°C and at a flow-rate of 1.9 ± 0.1 ml h^{-1}.

Amine	*Adjusted elution volume (ml)*	*Peak width at base measured after triangulation (ml)*	*Total plate number*
1,6-Diaminohexane	9.35	0.51	9940
1,2-Diaminopropane	9.45	7.2	50
1,5-Diaminopentane	13.2	0.65	10300
1,4-Diaminobutane	18.4	0.79	12100
Spermine	22.1	1.02	9950
Spermidine	29.4	1.50	7630
Agmatine	30.3	3.85	1230
1,3-Diaminopropane	41.2	4.61	1490

marginal difference to efficiency, but the optimum diameter was close to 0.5 cm at the flow-rates employed relative to cross sectional area (25 ml cm^{-2} h^{-1}).

Change in efficiency of column packing with time. The chromatographic efficiency of a 0.6 cm diameter column declines by 2–3% each run under the conditions of Table IX, when tested with β-phenylethylamine, *p*-tyramine and 3 aliphatic monoamines. The effect is a genuine one: when such a column is used for 1000 h (*ca.* 100 runs) and then repacked, plate numbers increase by a factor of 3.0, which is certainly statistically significant. Narrower columns almost certainly lose their efficiency more slowly if indeed they lose it at all. Nevertheless all the results given here have been obtained from columns repacked every 6–10 runs.

Long term changes in the properties of the resin itself. The cross-linking agent, BDDA, is susceptible to hydrolysis but appears to be stable indefinitely under all our

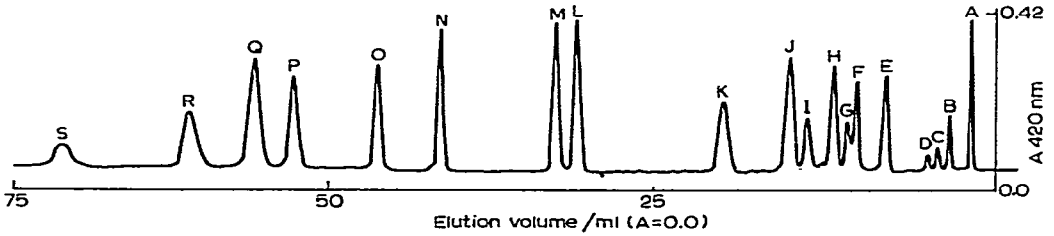

Fig. 3. Two-buffer general purpose LC system for the separation of biogenic amines. A column of resin 100 × 0.34 cm I.D., pre-extracted with aqueous acetone, was eluted at 55 ± 1°C. Initially the buffer was 0.02 *M* borax (AR) containing 0.4 *M* sodium nitrate (AR) adjusted to pH 8.5. At 28 ml this was replaced with 0.019 *M* borax (AR) containing 1.1 *M* sodium iodide and 0.5 *M* potassium iodide also adjusted to pH 8.5. The flow-rate, initially 2.85 ml h^{-1}, fell to 2.4 ml h^{-1} after the introduction of the second buffer. Elution volumes have been calculated from the mean flow-rate so are not exact throughout the range. A = Glutamate; B = dopamine; C = glucosamine; D = galactosamine; E = 3,4-dimethoxyphenylethylamine; F = isoamylamine; G = normetanephrine; H = β-phenylethylamine; I = 3-methoxytyramine; J = octopamine; K = *p*-tyramine; L = ethanolamine; M = methylamine; N = 1,6-diaminohexane; O = 1,5-diaminopentane; P = 1,4-diaminobutane; Q = spermine; R = spermidine; S = agmatine.

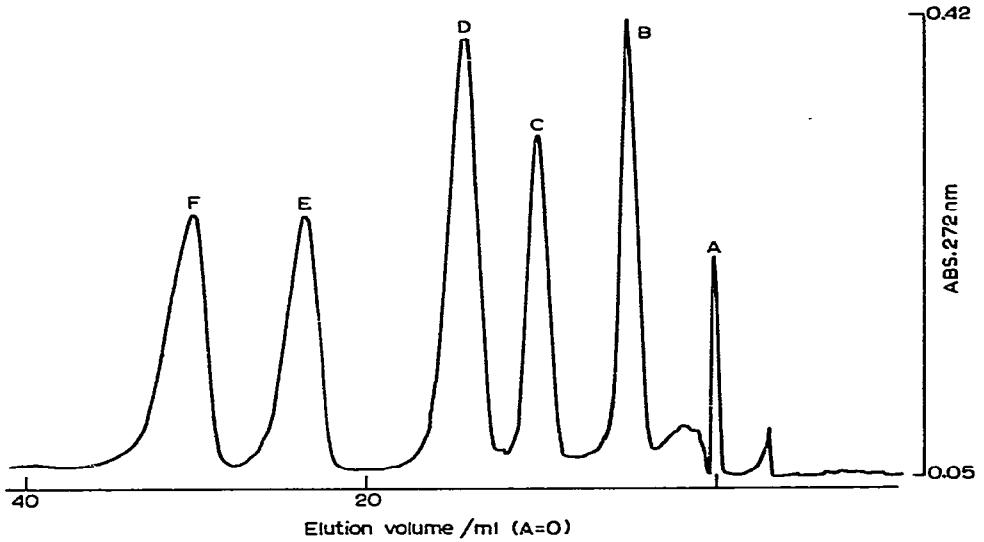

Fig. 4. LC system for the separation of catecholamines and metanephrines. A column of resin, pre-extracted with acetone, 24 × 0.6 cm I.D., was eluted with 0.02 *M* sodium phosphate containing 1.2 *M* sodium chloride (AR) adjusted to pH 7.0. The temperature was 25°C, the flow-rate 6.9 ml h^{-1} and the amines were detected directly by their absorbance at 272 nm. A = Phthalate; B = metanephrine; C = normetanephrine; D = epinephrine; E = dopamine; F = norepinephrine.

experimental conditions. Thus exposure to a pH 9 buffer for 2 years, often at elevated temperatures, has not caused the resin to swell or soften detectably.

Its chromatographic properties are also stable for at least 4 years when stored in the dark at 25°C. Yet after prolonged treatment with flowing eluting buffers it gives substantially better plate numbers and lower elution volumes (Table XII). These effects are particularly noticeable for the aromatic amines which are initially chromatographed rather inefficiently.

The "ageing" effect will only increase plate numbers by an insignificant 5% during a single investigation, but it does prevent valid comparisons between the results given in different tables. For example, the "used" resin on which Table VIII is based had slightly different properties from the "used" resin of Table XII.

Fortunately, a highly efficient resin, giving a reproducible performance, can be obtained by extracting the newly-synthesised material with aqueous acetone as described earlier. The acetone treated resin has a marginally higher chromatographic efficiency than that of the best resin modified by prolonged elution.

Practical chromatographic systems

Amine fractions from biological sources always contain basic amino acids. Thus we have chromatographed arginine, lysine and histidine as well as all the biogenic amines we have been able to obtain and detect: these are listed in order of elution in Tables IX–XI. All systems are based on acetone extracted resin, usually in the form of a 100 × 0.34 cm I.D. column.

A good general purpose buffer for the separation of monoamines is pH 8.5, 0.02 *M* borax containing 0.4 *M* sodium nitrate, used at 55°C (Table IX). If detection

TABLE XII

LONG TERM CHANGES IN CHROMATOGRAPHIC PROPERTIES OF RESIN

The eluting buffer was pH 8.85, 0.01 *M* borax containing 0.4 *M* sodium nitrate. The amines were eluted from a column of resin, 30 × 0.6 cm I.D. at 20°C and at a flow-rate of 9.2 ± 2.7 ml h^{-1}. A freshly synthesised sample of resin ("fresh" resin) was exposed to a variety of, mainly, high molarity eluting buffers at 20–50°C for approximately 1200 h. It then gave the results listed under "used" resin.

	"Fresh" resin	*"Used" resin*
	Adjusted elution volumes (ml)	
Methylamine	39.1	34.0
Ethylamine	18.7	17.8
n-Butylamine	8.5	8.2
β-Phenylethylamine	12.0	9.7
p-Tyramine	25.3	19.6
Dopamine	1.55	1.45
Tryptamine	28.0	22.4
	Chromatographic efficiency (plates/cm)	
Methylamine	62	86
Ethylamine	55	58
n-Butylamine	30	54
β-Phenylethylamine	19	29
p-Tyramine	26	43
Dopamine	4	27
Tryptamine	14	44
Average for all compounds	30	49

of amines is to be by UV absorbance it is better to replace the nitrate with chloride (Table X).

A high molarity borax–iodide buffer containing both sodium and potassium is suitable for the separation of di- and polyamines, again at 55°C (Table XI). The monoamine and polyamine buffers are compatible with each other and Fig. 3 illustrates the results given by such a combination.

Finally, catecholamines separate badly in borate and decompose at the comparatively high pH and temperature of the general monoamine systems. However, these and the related metanephrines can be easily separated in a pH 7 high-molarity phosphate–chloride buffer (Fig. 4).

DISCUSSION AND CONCLUSIONS

Something of the interactions between the resin and the amines can be deduced from these results. Aliphatic monoamines, like aliphatic diamines, are generally eluted more rapidly the higher their molecular weights (Tables IX, XI) so molecular sieving must be an important factor. The lower monoamines probably interact with the resin predominantly by ion exchange; the higher monoamines, being less polar, probably also enter the resin by partition and so penetrate more deeply. The onset of partition would explain why the relationship between molecular weight and elution

volume reverses for the C_6–C_8 monoamines and why these are chromatographed less efficiently than their lower homologues.

Tables IX and X suggest that efficiency is not limited by elution time providing the net elution volume is greater than 6 ml. Thus the diaminopropanes, imidazoles and guanidine derivatives must all be showing anomalously low plate numbers. Histamine is typical of this group, giving a tailed peak, which shows a higher plate number if either a larger sample is applied or if several identical samples are run successively. This behaviour implies that histamine is strongly bound to a very small number of secondary adsorption sites: a molecule so bound will either not be displaced at all or will be displaced so slowly that it will tail behind the main band.

Imidazole at 1 g/l, added to pH 9.7 2 *M* nitrate–borax buffer had a dramatic effect on the behaviour of histamine. There was a significant reduction in elution volume and a 3.7X increase in efficiency as measured in plates/cm. Thus imidazole appears to be competing with histamine for these postulated secondary adsorption sites. Moreover, a pH 8.9 buffer containing 2 g/l imidazole but otherwise identical to that used for Table IX, gave the following plates/cm values: histidine, 13; histamine, 70; arginine, 65. The improvement in efficiency in the presence of imidazole, ranging from 2.6X for histamine to 14X for arginine, cannot be explained by differences in the resin or pH. Agmatine, too, gives relatively sharper peaks in the presence of imidazole. Thus imidazole improves the chromatography of at least 4 "difficult" compounds suggesting that they all interact with the same type of secondary site. The diaminopropanes may well behave in the same way since all substances chromatographed with anomalously low efficiency have two polar nitrogens, close together.

Imidazole does not react with TNBS and, even at 10 g/l, does not affect the chromatography of any other amino compound tested so it was once routinely incorporated into eluting buffers. Its use was abandoned because it will support the growth of micro-organisms and ultimately led to infected columns. Clearly there is a strong case for its re-introduction.

However, even using buffers having no organic supplements, acetone extracted resin chromatographs most of the biogenic amines tested very efficiently. Aromatic compounds probably interact slightly by partition and so still give marginally worse plate numbers than the lower aliphatic amines. Nevertheless, relative peak sharpness for aromatics is very much better than when DVB cross-linked resins are used. This is a most important gain, because 50% of the biogenic amines so far reported contain either a benzene or indole nucleus.

All plate numbers have been based on the peak widths actually measured. However, there was substantial band broadening in the A_{272} direct detection system. Thus, for the 5 compounds that appear both in Tables IX and X, UV absorbance detection gave peaks an average of 0.14 ml wider than did TNBS detection. This difference was not due to the nitrate–chloride substitution because the phthalate reference peak was also 0.17 ml wider than its glutamate counterpart. If the results in Table X are recalculated to allow for a band broadening of 0.14 ml in the detection system, the mean plate number for all aromatics, from hordenine onwards, becomes 9900.

Peak bases must also have been spread slightly by the automatic analyser used for the TNBS reaction. Allowing for this and for an average sample volume of 0.02 ml, the resin must chromatograph most amines with an efficiency of 100–200 plates/

cm, the height corresponding to 1 theoretical plate being from 2–3 particle diameters. This is an excellent performance for a substrate that is not even composed of perfect spheres (Fig. 2). The physical inhomogeneity of the resin is, however, the probable reason why column performance tends to decline with time and is dependent on packing technique.

From the experimental standpoint, the resin can easily separate members of a homologous series such as the *n*-aliphatic monoamines from C_1 to C_6 (Table IX) and the α,ω-diamines from C_3 to C_6 (Table XI). It will also resolve structural isomers of the aliphatic monoamines including *tert.*-, *sec.*- and *n*-butylamine, together with 2-methylbutylamine, iso- and *n*-amylamine (Table IX). Aromatic amines, too, are well spread out through the elution pattern. Here the elution volume is sensitive to the addition of a hydroxy group and to its position, as shown by the data for β-phenylethylamine, β-hydroxyphenylethylamine, *o*-tyramine and *p*-tyramine (Tables IX and X). Methylation of a hydroxy group as when 3-methoxytyramine is converted to 3,4-dimethoxyphenylethylamine increases mobility dramatically (Table IX). Methylation of the nitrogen atom has a similar effect: compare the elution volumes of tryptamine and *p*-tyramine with those of N-methyltryptamine and N,N-dimethyltyramine (hordenine) in Table X.

Fig. 3 shows that a combination of buffers will separate at least 18 biogenic amines in one run. The di-/polyamine buffer was optimised as part of this combined system so it gives better spaced peaks here than when used alone.

A good LC system should generate sharp peaks and be able to separate a large number of components simultaneously and rapidly. Table XIII uses these criteria to compare our combined system (Fig. 3) with the best that has been achieved by previous workers. Clearly the BDDA-cross-linked resin gives much better resolution and peak sharpness than any previous carboxylic resin. It also enables workers with very modest equipment, who have no facilities for high-pressure chromatography, to achieve results as good as the best so far obtained in this field.

TABLE XIII

COMPARISON OF OUR RESULTS WITH THE BEST ONES REPORTED IN THE LITERATURE

The literature is extensive but has been reviewed[9,12,21–27]. The systems chosen for comparison were those recording the separation of the greatest number of amines, whether free or derivatised, in a single run.

Reference and comments	*Resolving power: maximum number of biogenic amines separable in one run*	*Mean peak width at base after triangulation (ml)*	*Time taken for each run (h)*
Our combined system, Fig. 3	18	1.1	28
Ref. 5. Best published system based on carboxylic resin	12	6.8	4
Ref. 20. Best LC system for amine separation	18	1.3	3

The resolving power of the new resin is quite impressive but there is potential for further improvement. A 2 or 3 m column is a practical proposition at the flow-rates employed here. Moreover, Fig. 3 shows that there are some quite large spaces

between certain peaks. If these could be filled either by modifying the elution order or by devising a more universal detection system, it should be possible to separate appreciably more biogenic amines in one run.

The one disadvantage of our system, its lack of speed, could be minimised by using resin of smaller particle diameter. However, ideally, the methacrylate–BDDA polymer would be best employed as a surface phase bonded to 5-μm silica spheres. In this form it could well out-perform any substrate currently available for the LC of amines.

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