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ACCELERATED ION-EXCHANGE CHROMATOGRAPHY OF SOME BIOGENIC AMINES

R. A. WALL*

Department of Chemistry, University of Edinburgh, Edinburgh EH9 3JJ (Great Britain) and Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, Calif. 91109 (U.S.A.)

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SUMMARY

There has been increasing evidence of the importance of biogenic amines, some of which function as transmitting agents for impulses in the central nervous system. This paper attempts to provide procedures for separation and subsequent quantitative analysis of these compounds in physiological fluids. Detection sensitivity and resolving power are sufficient to allow samples of these fluids to be analysed directly after removal of particulate matter and adjustment of acid concentration, thereby avoiding degradation of labile compounds by exposure to aerial oxygen or extremes of heat or pH. Those amines which have primary amino groups may be determined at concentrations of 10^{-3} to 10^{-6} *M* on a standard amino acid analyser with columns of a specific ion-exchange resin and buffers which are completely compatible with reproducible ninhydrin colorimetry. Some of the other biologically important amines which do not have primary amine functions, *e.g.*, catecholamines and indole derivatives, were monitored to even lower concentrations by continuous flow fluorimetry.

INTRODUCTION

The earlier publication[1] from this laboratory on this subject was limited to some of the components of grass ensilage extracts. This study has now been extended to a wider range of biologically important amines, but the basic technique of separation by chromatography on columns of polyacrylic acid cation-exchange resin and continuous monitoring by colorimetry has been retained with the addition of simultaneous detection by continuous fluorimetry.

EXPERIMENTAL

Columns were made from lengths of 0.636 cm precision bore glass tubing, and were connected to the buffer pump and the detection systems with standard Technicon

* National Institute of Mental Health Special Research Fellow (1968–1969), California Institute of Technology.

(Technicon Instruments Co. Ltd., Chertsey, Surrey) column end fittings and 0.16 cm bore thick wall (pressure) and 0.04 cm bore thin wall polyethylene tubing. Little difference in sensitivity was observed when the amines were measured by ninhydrin colorimetry on a Beckman Spinco 120B (Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.) amino acid analyser or a Technicon amino acid analyser fitted with 1.5 cm path length flow cells.

An Aminco-Bowman SPF spectrophotofluorometer was fitted with a micro flow cell made from a U-bend of 1 mm bore quartz capillary tubing which was mounted in a slot machined in the capillary micro cell holder. The column effluent was continuously monitored with this instrument before being mixed with the colour reagent for colorimetry. Sample fluorescence was excited with light of 280 nm wavelength and was followed through an emission monochromator set for light of 340 nm wavelength; standard amplifier settings were: sensitivity 47 and photomultiplier $\times$ 0.03.

TABLE I

ANALYTICAL DATA

	Relative fluorescence	*Relative O.D. (570 nm)*	*O.D. 440 nm/ O.D. 570 nm*
Norleucine	0	1.00	0.23
Ammonia	0	0.85	0.22
Epinephrine	0.47	0.04	0.4
Norepinephrine	0.92	0.18	0.59
Octopamine	0.28	0.90	0.24
Dopamine (oxidised and cyclised)	0.46	0.02	3.8
Tyramine	0.30	0.55	0.25
4-Hydroxy-3-methoxyphenylethylamine	1.38	0.53	0.26
β-Hydroxyphenylethylamine	0	0.90	0.23
Putrescine	0	0.97	0.40
Cadaverine	0	0.83	0.41
Serotonin	2.03	0.25	0.97
Phenylethylamine	0	0.52	0.26
Agmatine	0	0.80	0.27
Histamine	0	0.45	0.44
Tryptamine	3.10	0.47	0.43

Materials

All results reported here were achieved with various samples of the spherical polyacrylic acid cation exchanger Zeo Karb 226 (4½% DVB) (Permutit Co. Ltd., London) which were divided into fractions of narrow ranges of bead diameter by hydraulic fractionation by the method of HAMILTON[2]. Average bead sizes were measured for each fraction by photomicrography, and samples with diameters of 13, 16 and 21 μ were obtained from each of the three resin batches (4, 8 and 17, part 2) examined.

All amines used as standards were commercial products of the highest available purity, except for putrescine and cadaverine, which were purified as their hydrochlorides by recrystallisation from concentrated hydrochloric acid. Propanol was either the standard commercial product purified by distillation from sodium propoxide (1 g sodium/l) or "puriss" grade (Fluka AG, Buchs, Switzerland). Buffers were prepared from reagent grade trisodium citrate and sodium chloride by addition of

reagent grade concentrated hydrochloric acid to the appropriate salt solutions and finally diluted to volume after attainment of the desired pH.

RESULTS AND DISCUSSION

Since the original study[1] was published there have been several other papers[3–5,8] on this problem of biogenic amine analysis with samples as varied as fermented beverages, fish muscle extracts, and physiological fluids. Communications from other interested workers prompted extension of the silage analysis technique to its original range[6] of catecholamines, indoleamines, and decarboxylated amino acids.

One of the objects of the exercise was development of procedures which would permit direct analysis of human physiological fluids of compounds which are known to be sensitive to high pH and oxygen. Samples of urine from juvenile neuroblastoma patients were used as sensitivity monitors and it was possible to determine up to seven compounds of greater basicity than arginine in such fluids with 0.5 cm^3 analytical specimens. Refinement of the detection techniques used in these experiments would readily increase this sensitivity by at least one order of magnitude. That this is possible with colorimetry is demonstrated by the commercial availability of at least three continuous flow photometers with noise levels of 0.002 absorbance units with a 1.0 cm pathlength cell.

Although direct analysis of physiological specimens was the driving force behind the work reported here, this report is mainly concerned with the elution schedules necessary for separation on columns of the exchanger mentioned above.

TABLE II

KEY FOR CHROMATOGRAMS

1	Ethanolamine
2	Ammonia
3	Epinephrine
4	Norepinephrine
5	1-Aminobutane
6	Octopamine
7	Dopamine (oxidised and cyclised)
8	Tyramine
9	β-Hydroxyphenylethylamine
10	4-Hydroxy-,3-methoxyphenylethylamine
11	Putrescine
12	Cadaverine
13	Serotonin
14	Phenylethylamine
15	Agmatine
16	Histamine
17	Tryptamine
18	Not identified

Reproducibility of runs with standard mixtures was certainly as good as the 3% variation usually quoted for amino acid analysis.

The same ion-exchange resin samples employed in the earlier work were used as column packing in the first ventures into the expanded analysis field; batches

4 and 8 were used and appeared to give identical chromatographic results. The pH of the buffer was reduced to 5.30 from the original 6.15, and the temperature, ionic strength, and organic solvent concentration were all increased during chromatographic runs to keep zone widths within acceptable limits. Figs. 1 and 2 are records of elution patterns from a column of Zeo Karb 226 × 4½, batch 8, 21 μ beads, whose packed dimensions were 19 × 0.636 cm. In each case the eluent was pH 5.30, 0.2 *M* trisodium citrate for 70 min, followed by pH 5.30, 0.1 *M* trisodium citrate and 0.3 *M* sodium propionate in 2 *M* propanol; the temperature was increased from 60° to 70° shortly after starting the chromatogram. The eluent pump was set to deliver 40 cm³/h which developed a back pressure of 7.5 atm. The sample in Fig. 1 was a standard mixture of known composition and that in Fig. 2 was 0.5 cm³ of juvenile neuroblastoma urine which was reduced in pH to 5.30 by addition of a small quantity of concentrated hydrochloric acid and clarified by filtration through a cellulose acetate membrane with 0.8 μ pores.

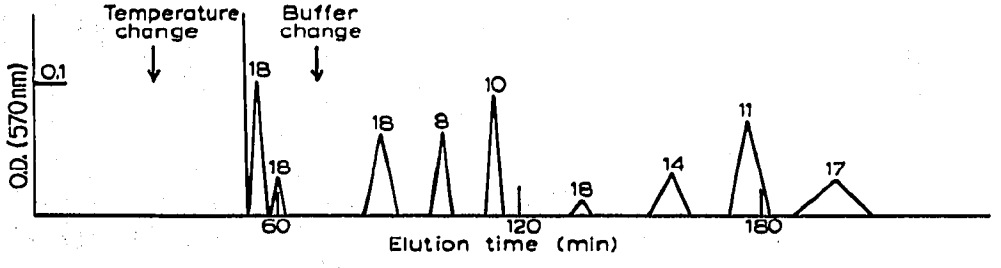

Fig. 1. Urine of juvenile neuroblastoma patient (0.5 cm³ sample volume). See Table II for zone identification.

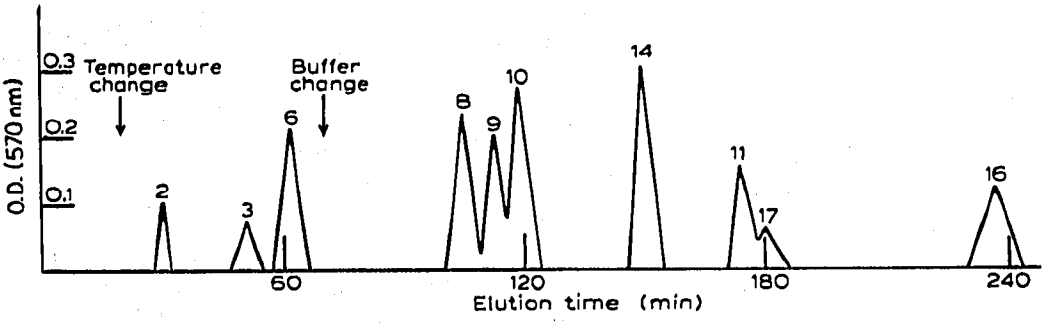

Fig. 2. Amine standard mixture (0.5 cm³ sample volume containing 0.02–0.3 μmoles of individual amines).

When a further batch of the exchanger (batch 17, part 2) was prepared for use, it was soon obvious that its chromatographic characteristics were quite different from those of batches 4 and 8. This was unexpected since the hydraulic separation procedure gave very similar bead size fractions at the same water flow rates, so the densities and hence effective crosslinking must be very nearly identical. Correspondence with the manufacturers failed to produce an adequate reason for these variations, which were apparently well within the production tolerances for this resin.

Fig. 3 is a record of the elution pattern of two columns (of the same volume)

of 21 μ beads from batches 4 and 17, part 2. The samples were identical in content and amount; and the temperature, flow rate, and buffer were also the same.

A chromatogram of the standard mixture using the temperature/buffer elution schedule as in Figs. 1 and 2 on a column packed with the same volume of this new batch of resin gave inadequate resolution of the first compounds to be eluted. Expert advice also suggested that catecholamines were much less susceptible to aerial oxidation at pHs below 4.5, so a new set of experiments was done to develop a buffer/temperature schedule to give adequate separation at pH 4.20. This level of acidity was chosen to preserve a reasonable fluorescence yield from the catecholamines, since this begins to fall off rapidly below pH 4. Another constraint was the preservation of at least a 10 min delay between the emergence of the ammonia zone and the first amine zone, as the colorimetric record is obscured for at least that time by the very high concentrations of ammonia in physiological fluids. Packed resin bed size of 24 × 0.636 cm and a flow rate of 30 cm³/h were adopted as standard for these experiments, which were all done with exchanger from batch 17, part 2.

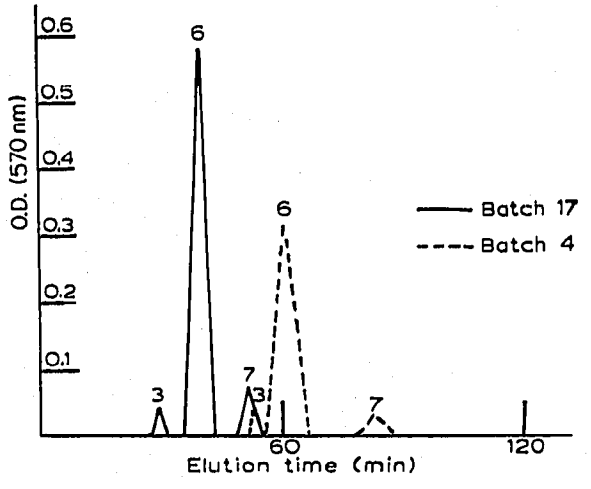

Fig. 3. Amine standard mixture.

Considerable time was wasted before a side reaction between the carboxylic acid resin and the surfactant Brij 35 was confirmed. At acidities below pH 5 a significant proportion of the acid centres of the exchanger are esterified by the surfactant and this drastically changes its chromatographic properties (and in a non-linear manner until equilibrium is reached). This reagent has accordingly to be left out of all buffers used with carboxylic acid ion exchangers.

The separation methods presented in the sequel amount to several systems all based on similar buffers; the elution programmes were varied to improve resolution in certain selected regions of the chromatogram. For maximum efficiency it is necessary to use fluorimetric detection of the effluent before mixing it with the colorimetric reagents in order to analyse compounds like epinephrine, serotonin, and tryptamine (and oxidatively cyclised dopamine) at the 10^{-7} to 10^{-5} M concentration level. Furthermore, some amines which are particularly difficult to separate like the tyramine, β-hydroxyphenylethylamine, and 4-hydroxy-,3-methoxyphenylethylamine trio may be differentiated by their relative fluorescence yields. With this double de-

tection system and gradient elution programmes it is possible to analyse mixtures of some 16–20 biogenic amines in periods of 3–5 h.

The question of what shape of gradient to use depends on the analytical problem, in particular on whether β-hydroxyphenylethylamine and serotonin are likely to be present in a given mixture, since these two amines are most difficult to separate from their immediate neighbours by this chromatographic technique. The best solution to the problem of analysis of the hydroxyphenylethylamine trio seems to be to elute it as two well-resolved zones of tyramine and β-hydroxyphenylethylamine/4-hydroxy-3-methoxyphenylethylamine. The fluorimetry conditions described above give a negligible signal for β-hydroxyphenylethylamine and a strong one for 4-hydroxy-3-methoxyphenylethylamine. Similarly, serotonin can be detected easily in the presence of cadaverine or putrescine by its strong fluorescence, although the diamines can be eluted before or after serotonin and its immediate successor, phenylethylamine, by changing the rate of increase of ionic strength at a cost of loss of resolution in other parts of the chromatogram or addition of another hour to the elution time of the most strongly retained amine

The simplest possible gradient is a linear one; Fig. 4 shows the elution pattern of a standard mixture (amounts of amines vary from 0.02–0.3 μmoles) from a column (24 × 0.636 cm) of ZeoKarb 226 × 4½, batch 17, part 2, 21 μ bead diameter. The column was heated at 60° and was eluted at 30 cm³/h with buffer which varied from pH 4.20, 0.067 *M* trisodium citrate and 0.267 *M* propanol to pH 4.20, 0.211 *M* trisodium citrate, 0.70 *M* sodium chloride, and 2 *M* propanol over 5 h. This elution schedule gives barely adequate separation of the hydroxyphenylethylamines and putrescine is just separated from the cadaverine/serotonin zone. The partial resolution of histamine and agmatine is typical of the best observed at pH 4.20; earlier experiments showed that this pair can be separated easily at pH 4.50 with a similar sodium-ion-concentration gradient.

Combination of a linear gradient in sodium-ion concentration (0.24 *M*/h sodium increase from 0.067 *M* trisodium citrate) and a convex change in propanol concentration (0.267 *M* to 1.33 *M* over 5 h) using a three chamber Varigrad[7] gave results very similar to those of Fig. 4.

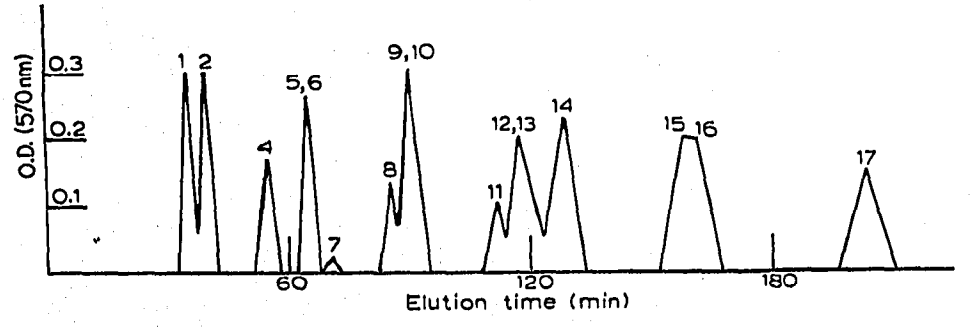

Fig. 4. Amine standards.

A delayed increase in sodium-ion concentration combined with a rapid increase in propanol concentration can be used to improve the separation in the first half of the chromatogram (including the hydroxyphenylethylamines), but this elution procedure adds at least 1 h to total analysis time and worsens the resolution of the diamines.

Fig. 5 is a record of such a chromatogram obtained at the same temperature and flow rate and with the column used for Fig. 4. The propanol concentration was raised exponentially from 0.40 *M* to 0.80 *M* over the first 90 min and was constant thereafter; the salt content was 0.067 *M* trisodium citrate for the initial 90 min and was increased at a rate of 0.24 *M* sodium ion concentration each hour after. This gradient was formed with a 60 cm^3 mixing vessel attached to a sequential four chamber manifold, but similar results were achieved with a convex solvent change and a concave salt change derived from a three chamber Varigrad.

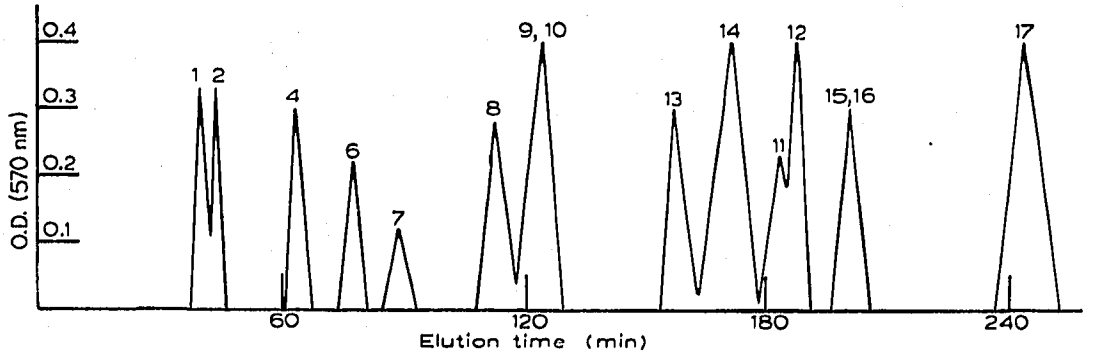

Fig. 5. Amine standards.

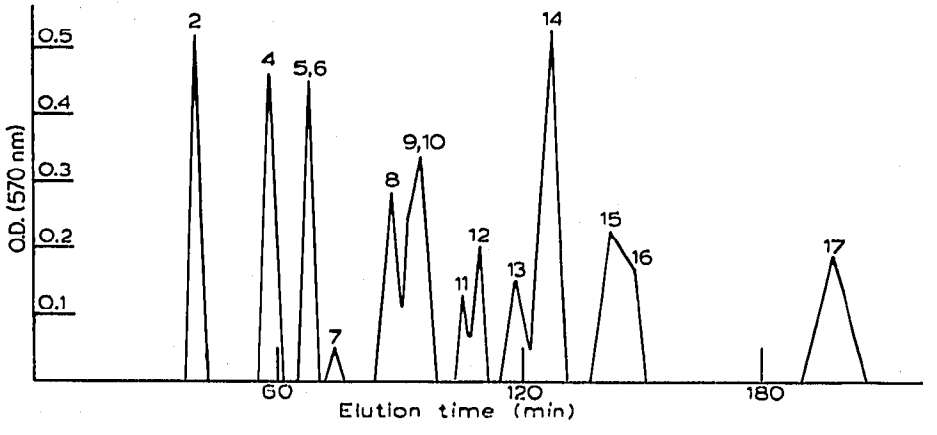

Fig. 6. Amine standards.

Fig. 6 illustrates the consequences of the last elution schedule tested, which had convex increases in both sodium-ion concentration (0.067 *M* trisodium citrate to 0.233 *M* trisodium citrate and 0.60 *M* sodium chloride over 5 h) and propanol concentration (0.267 *M* to 1.33 *M* over 5 h). This system gives the best overall resolution, but still leaves the β-hydroxyphenylethylamine/4-hydroxy-3-methoxyphenylethylamine and histamine/agmatine peaks with a skewed shape that makes accurate quantitation difficult even with differential detection systems.

In all these cases it is possible to decrease the zone width and the elution time of the most strongly retained amine, tryptamine, by an increase in column temperature to 70 or 80° after the phenylethylamine zone has been eluted.

Carboxylic acid ion exchangers undergo large volume changes when the pH

and (to a lesser extent) ionic strength of contacting solutions are altered and this property prevents regeneration of columns of these materials in the manner customary in amino acid analysis. In this series of experiments the general operating procedure has been to wash columns with buffers of sufficient ionic strength to remove the most tightly bound compound (tryptamine at low pH, histamine or agmatine above pH 4.50) likely to be found in the analytical sample and then re-equilibrate with the starting buffer. Experience showed that equilibration is best achieved at the end of a run by a decreasing salt/alcohol gradient to the starting buffer over 1–2 h followed by a further hour or two of that buffer. This technique permitted ten or twelve complete analyses before the column had to be packed again.

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