The separation of epinephrine from norepinephrine and dopamine from DOPA on Sephadex G-10

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SYNOPSIS Epinephrine and norepinephrine were separated by acid elution through a Sephadex G-10 column with high recovery (better than 90%), excellent reproducibility, and little overlap (less than 10%). Once packed, the columns could be re-used indefinitely. Total elution time was about six hours and the columns could be left untended since the gravity flow stops automatically once the level of the eluant reaches the gel bed. The resulting dilution was five-fold. 3,4-Dihydroxyphenylalanine (DOPA) was completely separated from 3,4-dihydroxyphenylethylamine (dopamine) by the same procedure.

At present only fluorescence and double isotope derivative techniques (Engelman, Portnoy, and Lovenberg, 1968; Engelman and Portnoy, 1970) are sufficiently sensitive to measure small amounts of catecholamines in biological materials. The most commonly used fluorimetric method is based on the trihydroxyindole reaction, the many modifications of which have been reviewed (Gray and Bacharach, 1967). More recently improved methodology has led to increased sensitivity (Laverty and Taylor, 1968; Weil-Malherbe and Bigelow, 1968) and has allowed a clinical application to be made (Griffiths, Leung, and McDonald, 1970; O'Hanlon, Campuzano, and Horvath, 1970). The differential estimation of epinephrine and norepinephrine in these methods is usually based not on the physical separation of the two compounds but on the combined use of optical methods and the utilization of the different intensities of the fluorophore obtained by oxidizing the epinephrine and norepinephrine at their different optimal pHs. This procedure is tedious, requires the solution of simultaneous equations, and has limited accuracy if one compound is present in much greater amounts than the other. In an analogous fashion normetanephrine has been differentially estimated from metanephrine (Smith and Weil-Malherbe, 1962; Häggendal, 1962a; Brunjes, Wybenga, and Johns, 1964). Hence there is a need for a simple method of physically separating epinephrine from norepinephrine and metanephrine from normetanephrine.

Usually the concentration and isolation of the catecholamines from their 3-O-methylated metabolites is accomplished by selective adsorption onto aluminium oxide from an alkaline solution (Smith and Weil-Malherbe, 1962; Anton and Sayre, 1962; Türlér and Käser, 1971). Drell (1970) has recently re-investigated the use of aluminium oxide and described methods for partially separating catechol acids from the catecholamines. None of the published aluminium oxide methods to date describes the separation of norepinephrine from epinephrine or dopamine (3,4-dihydroxyphenylethylamine) from DOPA (3,4-dihydroxyphenylalanine). Thin-layer chromatography and paper chromatography (Aures, Fleming, and Håkanson, 1968; Vahidi and Sankar, 1969) have resulted in separation of closely related catecholamines and their metabolites but leave these compounds in an inconvenient form for quantitation of small amounts since reliance on staining or fluorescence of the spots for localization limits the sensitivity. In common use are cation and anion exchange resins for the concentration and separation of the catecholamines and their metabolites (Weil-Malherbe and Bigelow, 1968; Häggendal, 1962b; Häggendal, 1963; Bertler, Carlsson, and Rosengren, 1958; Goodall, Harlan, and Alton, 1968; Goodall and Alton, 1969), but these methods have generally resulted in incomplete separation of closely related compounds. Lange, Männl, and Hempel (1970) have described a procedure using sulphonated styrene-divinyl benzene which separated the neutral and acidic catabolites of dopamine, norepinephrine, and

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epinephrine from the parent catecholamines but does not separate the catecholamines from each other. Häggendal (1962b) achieved the separation of norepinephrine, epinephrine, metanephrine, normetanephrine, and dopamine on an Amberlite CG-120 column but noted the disadvantages of slow flow rates, variable amounts of non-specific fluorescent material eluted from the resin and the necessity of re-cycling the columns before again using them. More recently reports of the use of Sephadex column chromatography have been made. Korf, Ottema, and van der Veen (1971) have used Sephadex G-10 to isolate homovanillic acid from biological materials but made no attempt to separate the catecholamines or the other metabolites. Anggård, Sedvall, and Sjöquist (1970) have prepared N-perfluoroacyl derivatives of epinephrine, norepinephrine, dopamine, metanephrine, and normetanephrine (before gas chromatography—mass spectrometry) and chromatographed them on Sephadex LH-20. Demetriou, Macias, McArthur *et al* (1968) in an extensive study of the gel filtration chromatography of phenolic and heterocyclic compounds on Sephadex G-15 noted separation of some of the closely related catecholamines and their metabolites using an acid-salt eluant.

This report outlines a method of separating the following clinically important compounds on Sephadex G-10 using an acid eluant: norepinephrine from epinephrine, and dopamine from DOPA. These compound pairs have similar fluorescent properties and hence their physical separation will facilitate more accurate quantitation.

Materials and Methods

DL-Epinephrine-7-³H-hydrochloride and DL-norepinephrine-7-³H, both with SA 7.1 C/mM, were obtained from the New England Nuclear Corp. They were diluted in 0.1 N acetic acid and stored at 4°C. Just before use they were partially purified by adsorption on alumina (Woelm neutral activity grade I) and elution with 0.2 N acetic acid (Griffiths *et al*, 1970; Anton and Sayre, 1962). Nonradioactive catecholamines and related compounds were obtained from the Sigma Chemical Co, St Louis, Mo, diluted in 0.1 N acetic acid and stored at -10°C. Sephadex G-10 was obtained from the Pharmacia Co, Uppsala, Sweden. All chemicals used were analytical reagent grade.

Counting was carried out using a Phillips liquid scintillation counter with a maximum efficiency of 60% for tritium. The scintillator used contained 400 g naphthalene, 28 g PPO (2,5-diphenyloxazole) and 1.2 g dimethyl-POPOP [1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene] per gallon of dioxane.

After soaking overnight in 0.02 N acetic acid, the Sephadex slurry was packed to a height of 35.5 cm in 10 ml burettes (int diam 0.5 cm) plugged with a small amount of glass wool. Substances under study (10 mμC of tritiated compounds or 10 μg of non-radioactive compounds) were added to the column in 1.0 ml of 0.1 N acetic acid. After the sample had entered the gel, it was eluted with 0.02 N acetic acid in 0.5 ml fractions at a flow rate by gravity of approximately 4 ml per hr. Fractions to be counted were added directly to glass vials containing 10 ml of scintillator, and shaken vigorously for a few seconds. Other fractions were frozen until assayed by the fluorescence method described previously (Lavery and Taylor, 1968; Griffiths *et al*, 1970).

Results

SEPARATION OF EPINEPHRINE AND NOREPINEPHRINE

Both labelled and unlabelled norepinephrine had the same elution pattern on Sephadex G-10. Similar patterns were obtained for labelled and unlabelled epinephrine.

A total of five experiments on three columns were carried out using the unlabelled hormones (added individually to the columns) assayed by fluorescence, and six experiments on two columns using the labelled hormone and scintillation counting. The total recoveries of the added hormones were consistently high: 98.0 ± 8.6% for epinephrine; 93.3 ± 8.3% for norepinephrine (Table I). In all 11 trials

	Average (%) Recovery Epinephrine (range)	Average (%) Recovery Norepinephrine (range)
Unlabelled hormones (5 expts)	99.7 (88.4-113.0)	97.4 (86.5-100)
Labelled hormones (4 expts)	95.9 (86.2-101.0)	90.8 (75.0-98.9)
Overall mean ± SD	98.0 ± 8.6	93.3 ± 8.3

Table I Recovery of catecholamines eluted from columns

the optimum separation point was found between 17 and 18 ml of eluant. The epinephrine fraction is defined as the 5½ ml eluant preceding the optimum separation point and the norepinephrine fraction 5 ml volume following. The percentages of epinephrine and norepinephrine in the elution fractions were calculated according to the total catecholamine eluted and do not represent total recoveries. It should be noted that a small percentage of epinephrine was eluted before the collection for that fraction was begun (Fig. 1).

Using the optimum separation point the epinephrine fraction contains 91.9 ± 2.2% of the eluted

Fig. 1 The elution pattern for epinephrine (E) and norepinephrine (N). The upper graph represents the pattern obtained for a mixture of labelled compounds; the lower that for the unlabelled compounds added individually and determined fluorimetrically.

epinephrine while being contaminated by $6.5 \pm 2.1\%$ of norepinephrine. The norepinephrine fraction contains $92.7 \pm 3.9\%$ of the norepinephrine eluted and is contaminated by $6.9 \pm 3.8\%$ of the eluted epinephrine. The resulting separations are also given for the situation in which the optimum separation points are missed by either + or - 0.5 ml (Table II). The separations are still fair with on the average an 85% separation obtained.

The possibility of the hormones (epinephrine and norepinephrine) together interacting in such a fashion as to change the separation on Sephadex G-10 was ruled out by adding mixed samples in three experiments (Fig. 1).

The effect of serum and urine on the separation procedure was investigated by adding labelled hormones to serum (two experiments) and urine (two experiments). The proteins were precipitated and aliquots of the deproteinized sera and urine were absorbed to and eluted off activated alumina in the exact fashion that would be done in preparation

for fluorescence assay (Griffiths *et al*, 1970). The alumina eluates were then applied to the Sephadex G-10 column and eluted as before. The results of the four experiments in all cases showed identical elution patterns to those obtained with the pure hormones in aqueous solution.

No difference in separation was observed using freshly packed columns as compared with columns prepared nine months earlier and left in 0.02 N acetic acid.

A trial of weaker acids as the solvent for the samples added to the columns resulted in broader peaks and poorer separations. The use of 0.1 N acetic acid to equilibrate with the gel and as the eluant resulted in sharper peaks but poorer separation. An attempt to use 0.2 N acetic acid as the solvent for the samples added to the columns (the strength of acid used to elute the catecholamines off alumina) resulted in a poor separation, with a broad double epinephrine peak but no change in the elution pattern of norepinephrine. A trial of an elution gradient of acetic acid proved of no benefit.

Use of a longer, thinner column for the G-10 gel equilibrated with 0.02 N acetic acid resulted in poorer separation probably due to side effects. The use of a longer column with the same diameter as described might result in more complete separation but would also result in a greater dilution of the compounds which is already 5.5-fold.

SEPARATION OF NORMETANEPHRINE AND METANEPHRINE

The elution patterns of the unlabelled 3-O-methylated catecholamine derivatives were in general analogous to those of epinephrine and norepinephrine with each of the 3-O-methylated compounds being eluted off the columns 2-3 ml before the corresponding catecholamine. There was unfortunately considerably more overlap (Fig. 2). This was due mainly to the broad and double peaked pattern of metanephrine which appeared similar to that obtained for epinephrine using 0.2 N acetic acid as eluant.

Observed (%) Collected		Optimum Separation Point		- 0.5 ml		+ 0.5 ml	
		Epinephrine (%)	Norepinephrine (%)	Epinephrine (%)	Norepinephrine (%)	Epinephrine (%)	Norepinephrine (%)
Unlabelled compounds (average of 5 expts)	Fraction E	92.8	5.5	87.9	2.1	94.6	11.8
	Fraction N	5.4	94.4	11.1	97.4	1.8	88.1
Labelled compounds (average of 6 expts)	Fraction E	90.0	7.3	84.5	2.9	91.2	15.9
	Fraction N	6.8	92.5	14.0	96.9	3.5	84.2
Combined data		Fraction E	91.9 ± 2.2	6.5 ± 2.1	87.6 ± 3.5	2.9 ± 2.1	92.9 ± 2.1
Mean $\pm$ SD		Fraction N	6.9 ± 3.8	92.7 ± 3.9	11.3 ± 3.4	95.7 ± 3.1	2.3 ± 1.2
							85.9 ± 6.0

Table II Effect of choice of separation point on differentiation of catecholamines eluted from columns

Fig. 2 The elution patterns for unlabelled normetanephrine (NM) and metanephrine (M) assayed fluorimetrically.

It also did not prove feasible to separate completely a catecholamine from its 3-O-methylated compound with this system.

THE SEPARATION OF DOPA AND DOPAMINE

The same system worked exceedingly well for the separation of DOPA from dopamine. From Figure 3 it is seen that the DOPA was eluted completely off the G-10 column well before the dopamine started its elution, giving virtually a 100% separation. This was obtained on three experiments on three columns, in one of which the hormones were added as a mixture.

Fig. 3 The elution patterns of unlabelled dopa (D) and dopamine (DA).

Discussion

The results show that using Sephadex G-10 and an acid eluant, a useful physical separation between norepinephrine and epinephrine and between DOPA and dopamine can be achieved before

determination by the double isotope derivative or fluorescent assay techniques. No useful separation was achieved between normetanephrine and metanephrine. There was considerable overlap between members of different pair groups and this paper only investigated the above mentioned compound pairs (chosen due to similar fluorescent properties). For example, the 3-O-methylated catecholamine derivatives can be separated from their parent catecholamine compound by a preliminary step such as adsorption on activated alumina (Brunjes *et al*, 1964).

In separating epinephrine from norepinephrine the optimum separation point may vary by as much as 1 ml. If the midpoint of this range (ie, the epinephrine fraction being collected from 12 ml up to and including 17½ ml) is chosen routinely, then the possible error will be $\pm 1/2$ ml. The resulting decreases in the separation efficiency are shown in Table II and although this is not large it may not be acceptable. For precise work, small amounts of labelled compounds can be added to the sample and then the optimum point and recovery determined individually. If the recovery is estimated individually, the area of possible overlap can be omitted and the loss corrected for.

The five-fold dilution of the sample may present a problem when dealing with the small quantities in plasma and re-concentration may be necessary.

The problem encountered in attempting to separate normetanephrine and metanephrine is not understood. The broad two-peaked elution pattern of metanephrine added to the column in 0.1 N acetic acid was qualitatively similar to that seen with unlabelled epinephrine added to the column in 0.2 N acetic acid and in contrast to the sharp single peak seen with epinephrine added to the column in 0.1 N acetic acid. It was noted that the norepinephrine added to the column in 0.2 N acetic acid showed the same pattern as when added in 0.1 N acetic acid. Further, the same column gives a normal epinephrine pattern if the epinephrine is added in 0.1 N acetic acid after a sample in 0.2 N acetic acid has been eluted with 0.02 N acetic acid; hence any interaction between G-10 gel and the increased acetic acid concentration must be reversible. Both meta- and epi-nephrine have methyl-substituted amine groups and perhaps these interact with the G-10 gel at an increased acetic acid concentration. Possibly a decrease in the acetic acid concentration of the sample added to the column would give a more satisfactory separation of metanephrine and normetanephrine.

The advantages of G-10 Sephadex gel filtration using an acid eluant for separating epinephrine from norepinephrine and DOPA from dopamine are the

following: (1) good reproducibility; (2) ease of column handling; no re-cycling is necessary; the columns are easily and quickly packed; due to surface tension the columns do not run dry if left unattended for short periods; (3) the technician's time required is minimal and the procedure can be completed in several hours; (4) compounds are isolated unchanged chemically and in a form convenient for determination; (5) the compounds are kept in acid throughout the procedure.

The only disadvantage is the five-fold dilution of the compound.

This technique is particularly suitable to differentiate between epinephrine and norepinephrine in laboratories using small filter instruments for fluorescence assays, ie, routine clinical chemistry laboratories doing urinary catecholamines.

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