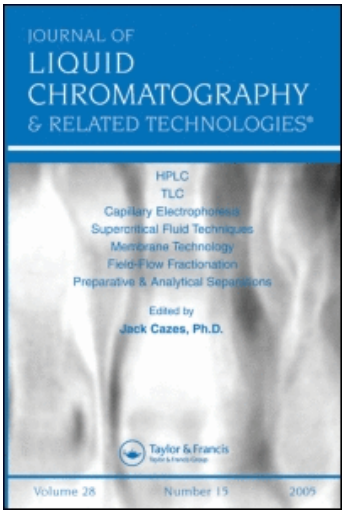


**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# A Comparison and Clinical Applications of Ammonium Acetate and Phosphate Based Mobile Phases for the Separation of Catecholamines on Reversed Phase Columns

W. A. Bartlett[a]
[a] Department of Biochemical, Medicine Ninewells Hospital and Medical School, Dundee

**To cite this Article** Bartlett, W. A.(1985) 'A Comparison and Clinical Applications of Ammonium Acetate and Phosphate Based Mobile Phases for the Separation of Catecholamines on Reversed Phase Columns', Journal of Liquid Chromatography & Related Technologies, 8: 4, 719 — 751
**To link to this Article: DOI:** 10.1080/01483918508067115
**URL:** http://dx.doi.org/10.1080/01483918508067115

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(4), 719-751 (1985)

# A COMPARISON AND CLINICAL APPLICATIONS OF AMMONIUM ACETATE AND PHOSPHATE BASED MOBILE PHASES FOR THE SEPARATION OF CATECHOLAMINES ON REVERSED PHASE COLUMNS

W. A. Bartlett
*Department of Biochemical Medicine*
*Ninewells Hospital and Medical School*
*Dundee*



ABSTRACT

A novel ion paired high performance liquid chromatographic system on reversed phase columns with ammonium acetate buffer as eluent is described for the separation of catecholamines. The advantages of using ammonium acetate buffer have been systematically studied and compared with the more widely employed phosphate buffer. The applicability of the method was demonstrated by analysis of catecholamines in clinical specimens.

## INTRODUCTION

The catecholamines, particularly noradrenaline, adrenaline and dopamine, function physiologically in a wide variety of systems, with homeostatic functions including the maintenance of blood pressure. Catecholamine secreting tumours, such as phaechromo-



 0148-3919/85/0804-0719$3.50/0

cytoma, may lead to characteristic clinical syndromes and this has lead to a demand for catecholamine estimation as a diagnostic tool in clinical chemistry laboratories. A great number of high performance liquid chromatographic methods have been developed for the assay of catecholamines in body fluids [1] and the use of reversed phase ion paired chromatography with phosphate buffered mobile phases has been investigated in detail [2] and applied with some success.

It was thought that the use of ammonium acetate as an alternative mobile phase solute might prove advantageous for this particular application. Ammonium ions are an effective masking agent for residual silanol groups on packing materials [3,4] and are also added to mobile phases to facilitate peak sharpening, while acetate buffers have found wide spread general use. The use of ammonium acetate mobile phases combines both of these advantages, with the further additional advantages of high salt solubility and good miscibility with organic solvents (e.g., methanol and acetonitrile). The salt is also compatible with most HPLC detection systems and is volatile enabling easier coupling to mass spectrometers [3]. The parameters affecting separation of catecholamines in reversed phase HPLC, with ion pairing, using ammonium acetate as the buffering solute have therefore been investigated and compared with the more widely phosphate system.



## EQUIPMENT AND REAGENTS

### HPLC Apparatus

The high performance liquid chromatography system consisted of a Pye Unicam Model PU4010

pump (Pye Unicam Ltd., Cambridge, England) with a Bioanalytical Systems LC4A amperometric detector utilising a LC17 TL5 cube glassy carbon electrode, and silver/silver chloride reference electrode (Bioanlaytical Systems, Purdue Research Park, West Lafayette, IN 47906, USA). The injection port was a Rheodyne model 7120 syringe loading loop sample injector fitted with 20 µl or 100 µl loops where appropriate (Rheodyne Incorporated, Berkley, California, USA).



Columns

Columns of 10, 16 and 25 cm in length and 4.5 mm internal diameter and column packings (Hypersil 5 µ particle size O.D.S., M.O.S. and S.A.S.) were purchased from Shandon Southern Ltd., (Cheshire, England).

Reagents

Noradrenaline, adrenaline, dopamine, normet-adrenaline, metadrenaline, 3-methoxy tyramine, 3-methoxy-4-hydroxymandelic acid, α-methyl-dopa, dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenol ethylene glycol and homovanillic acid were all purchased from Sigma Chemicals Ltd. (Poole, Dorset, England). Stock solutions were prepared at approximately 100 mgm/l in o.05 mol/l perchloric acid with 0.005 mol/l sodium metabisulphite and stored at $4^{o}C$ in dark bottles. Fresh working dilutions were made daily in the appropriate media.

Heptanesulphonic acid sodium salt monohydrate and 3,4-dihydroxy-benzylamine (DHBA) were obtained from Aldrich Chemicals (Gillingham, Dorset, England).

Bond Elut S.C.X. columns were obtained from Jones Chromatography Ltd (Llanbradach, Wales, U.K.).

All other reagents were of analar grade and purchased from B.D.H. (Poole, Dorset, England).

## METHODS

### Phosphate Buffer System

Stock standards were diluted in distilled water to give solutions of noradrenaline (0.50 mol/l), adrenaline (0.55 µmol/l), dopamine (0.65 µmol/l) and dihydroxy-benzylamine (0.45 µmol/l). A sample injection volume of 20 µl was applied to a 10 cm octadecyl silica (O.D.S.) column with a mobile phase consisting of 0.1 mol/l sodium dihydrogen phosphate adjusted to the desired pH with 1 mol/l sodium hydroxide, 0.5 mmol/l disodium E.D.T.A. and containing varying concentrations of methanol and heptane sulphonic acid. A mobile phase flow rate of 1 ml per minute was maintained with a continuous helium degas of the mobile phase reservoir.

### Ammonium Acetate Buffer System

Investigation of the effects of varying the components of the ammonium acetate mobile phase were carried out on a 10 cm O.D.S. column with a solvent flow rate of 1 ml/min unless otherwise stated. The mobile phase included ammonium acetate buffered to required pH with glacial acetic acid, 0.5 mmol/l disodium E.D.T.A. and heptane sulphonic acid. Standards of noradrenaline, adrenaline, DHBA and dopamine were diluted from stock daily and an injection volume of 100 µl employed.

## RESULTS AND DISCUSSION

### High performance liquid chromatography of catecholamine standards.

Chromatographs of catecholamine standard utilising a phosphate based mobile phase (Fig. 1) and an ammonium acetate system (Fig. 2) are shown. Baseline separation of all compounds was achieved with both systems.

### The effect of mobile phase pH upon catecholamine separation.

The capacity factors (K') of the catecholamines increased as the hydrogen ion content of the phosphate mobile phase (Fig. 3) and the ammonium acetate mobile phase (Fig. 4) decreased. If phosphate is used, then the change in K' was in the region of 10% for a 1.2 pH unit (from pH 3.8-5.0) increase for adrenaline, DHBA and dopamine whereas there was a 15% increase in that of noradrenaline. Increasing the pH of the acetate mobile phase produced a much greater effect on K' values observed. An increase of 1 pH unit (pH 4.00 to pH 5.00) produced K' values for noradrenaline, adrenaline, DHBA and dopamine of 230%, 327% 469% and 480% respectively.

Although it is difficult to compare the effects of the two mobile phases directly, because of their different heptane sulphonic acid content, the evidence suggests that some factor other than pH is influencing the separation in the ammonium acetate system. On the basis of results obtained with a phosphate mobile phase, Molnor and Horvath (5) suggested that, as pH increases, ionisation of

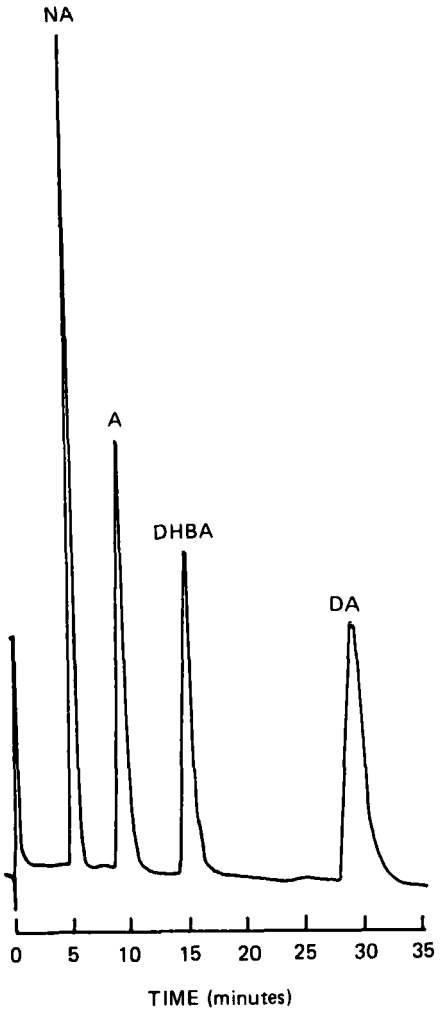


Fig 1 Chromatography of noradrenaline (NA), adrenaline (A), dihydroxy-benzylamine (DHBA) and dopamine (DA). A 10 cm O.D.S. column was used with 0.1M phosphate buffer pH 5.0, 0.5 mmol/l EDTA, 5 mmol/l heptane sulphonic acid, 4% (v/v) methanol. Flow rate 1ml/min.

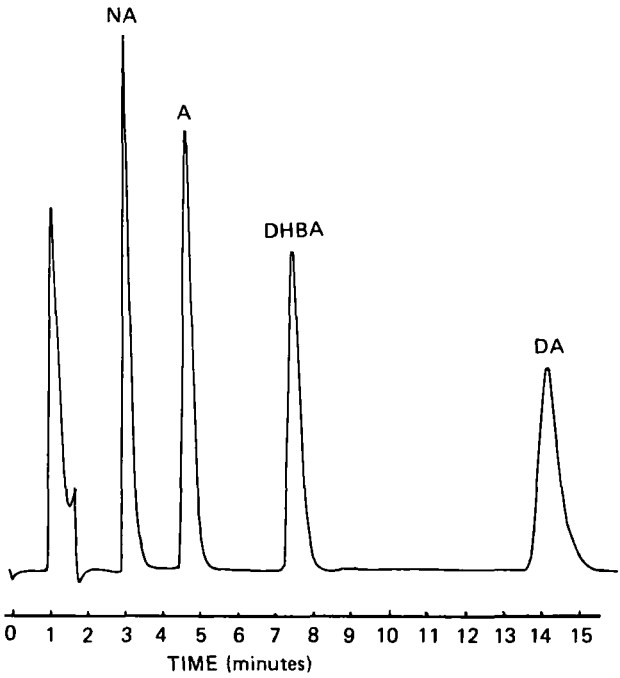


Fig.2 Chromatography of catecholamines by reversed phase ion paired high performance liquid chromatography on an ODS column with an ammonium acetate mobile phase. 20 µl of a standard solution containing noradrenaline 0.618 µmol/l (NA), adrenaline 0.548 µmol/l (A), dihydroxybenzylamine 0.473 µmol/l (DHB A), and dopamine 0.525 µmol/l, chromatographed on a 10 cm ODS column in 0.5M ammonium acetate, 0.5 mmol/l EDTA, 10 mmol/l heptane sulphonic acid, pH 5.16. Flow rate 1 ml/minute.

catecholamines is suppressed thereby increasing hydrophobocity and therefore retention. They did not, however, observe an effect upon retention of the magnitude seen with the ammonium acetate system until the pH of the mobile phase exceeded 6.50. The acetate pH effect allows, therefore, much more control over retention than the phosphate system.

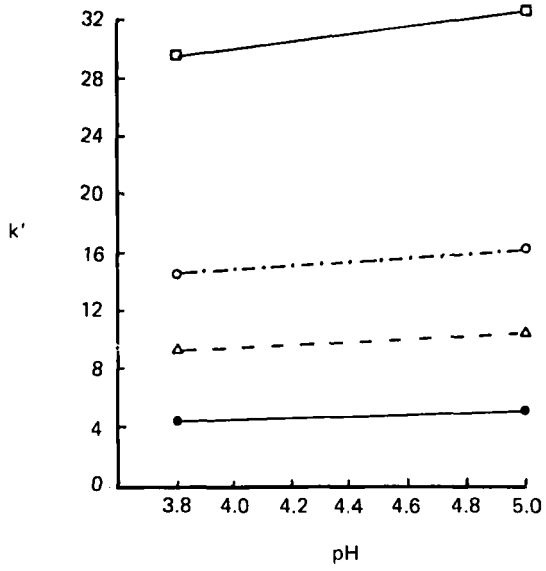


Fig. 3 The effect of increasing pH upon the capacity factors (K') of noradrenaline (●——●), adrenaline (△---△), dihydroxybenylamine (○·····○), and dopamine (□——□). Chromatography performed on a 10cm O.D.S. column with 0.1M phosphate buffer, 5 mmol/l heptane sulphonic acid, 0.5 mmol/l EDTA, 2% methanol (v/v). Flow rate 1 ml/min.



Influence of increasing methanol content of the phosphate mobile phase upon catecholamine separation.

Increasing the methanol content of the phosphate mobile phase caused a significant decrease in the K' values of all components of the standard mix (Fig. 5) with the greatest effect being upon dopamine.

It should be noted that the ammonium acetate system does not require the use of small amounts

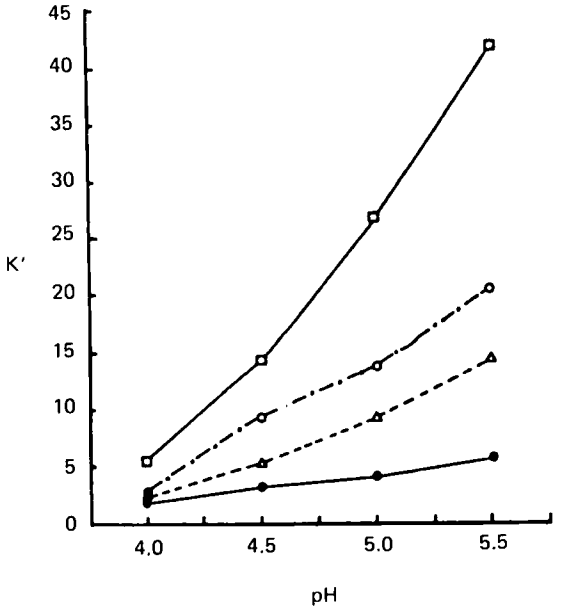


Fig. 4 Effect of ammonium acetate mobile phase pH on the capacity factors (K') of noradrenaline (●——●), adrenaline (△- - -△), DHBA (○·····○) and dopamine (□——□). Chromatography performed on 10 cm O.D.S. column with 0.25 mol/l ammonium acetate, 0.5 mmol/l EDTA, 10 mmol/l heptane sulphonic acid. Flow rate 1 ml/min.



of organic modifyer, thus acoiding gradual changes in retention time that result as the methanol content of the mobile phase is depleted by evaporation during the course of continuous helium degas. This is of importance since increased retention leads to peak broadening thus reducing maximum concentrations presented to the working electrode of the amperometric detector which in turn leads to errors in quantitation as peak detector response is reduced as retention increases.

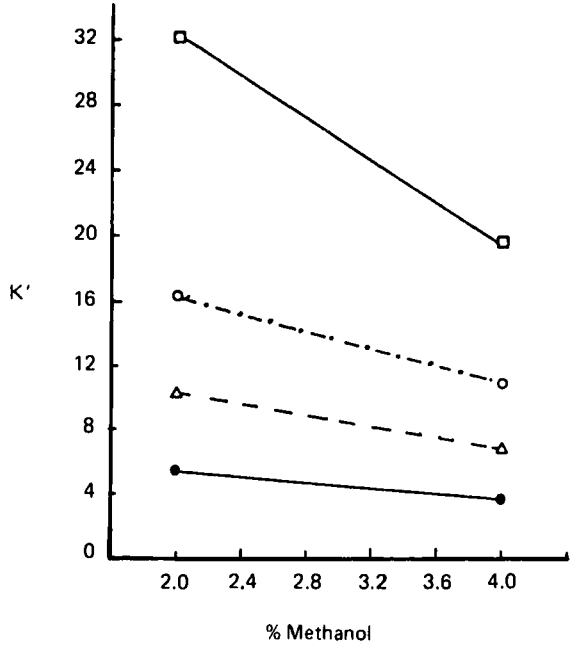


Fig. 5 Effect of increasing methanol content of the mobile phase upon the capacity factors (K') of noradrenaline (●——●), adrenaline (△---△), dihydroxy-benzylamine (○·····○) and dopamine (□——□). Chromatography performed on a 10 cm O.D.S. column with 0.1M phosphate pH 5.0, 5 mmol heptane sulphonic acid 0.5 mmol/l EDTA. Flow rate 1 ml/min.



Influence of molar strength of ammonium acetate upon separation.

Increasing the molar concentration of ammonium acetate concentration at a pH of 5.16 leads to a significant decrease in the capacity factors (K') for all four standard constituents (Fig. 6). At a concentration of 0.5 mol/l, baseline separation of all four components of the standard injection mix was achieved (Fig. 2). An increase in concentration

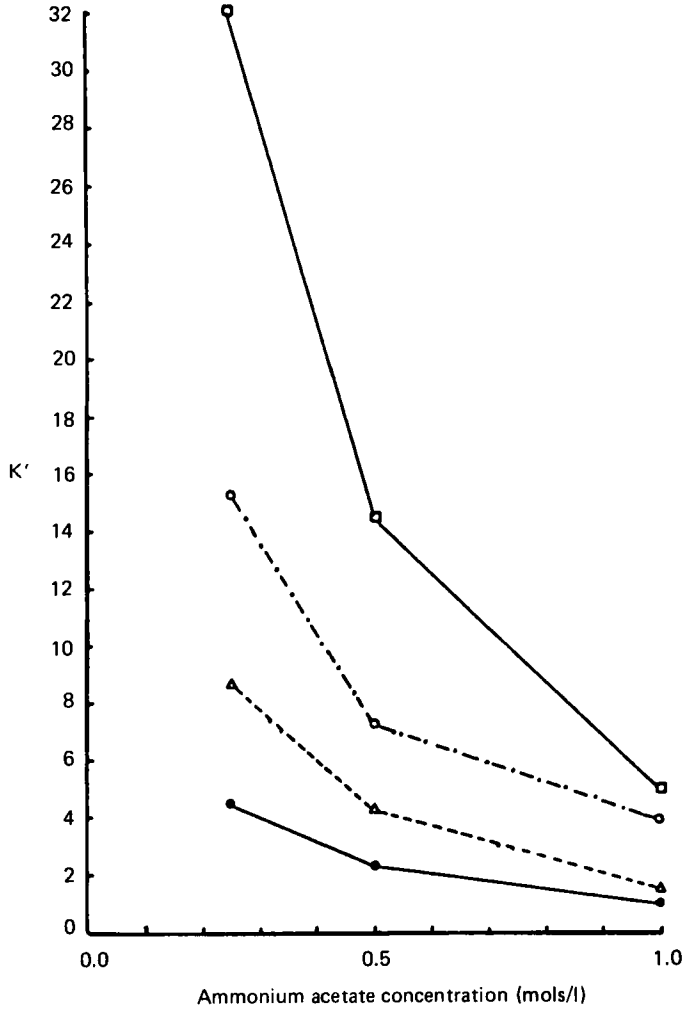


Fig. 6 Effect of increasing molar strength of ammonium acetate of HPLC mobile phase upon the capacity factors of noradrenaline (●——●), adrenaline (△---△), dihydroxybenzylamine (○·-·-○), and dopamine (□——□). Chromatography was performed on a 10 cm O.D.S. column with ammonium acetate pH 5.16, 0.5 mmol/l EDTA, 10 mmol/l heptane sulphonic acid. Flow rate 1 ml/min.

to 1.0 mol/l resulted in elution of noradrenaline very close to the void volume of the column with incomplete separation of DHBA from adrenaline. Decreasing ammonium acetate concentrations to 0.25 mol/l resulted in an excessive capacity factor for dopamine with complete separation occuring at 32 minutes.

This observation contrasts with that of Moyer and Jiang [6] who demonstrated that an increase in the molar strength of phosphate buffer had very little effect on retention times and that any effect was in a positive rather than a negative direction. This finding may be explained by the fact that increasing concentration of ammonium ions will lead to a more effective masking of residual silanol groups, thus reducing any adsorbtion component within the chromatographic process.



Influence of heptane sulphonic acid content of the mobile phase.

Increasing the concentration of heptane sulphonic acid in the phosphate mobile phase (Fig. 7) and the acetate mobile phase (Fig. 8 ) produced an increase in the K' values of all four components of the standard mix. The effect is maximum upon dopamine in both cases but of greater magnitude in the phosphate buffer system.

Hydrodynamic voltamograms of catecholamines and dihydroxy benzylamine.

A hydrodynamic voltamogram, constructed by measuring detector response to injection of the standard mix at increasing cell voltage reveals

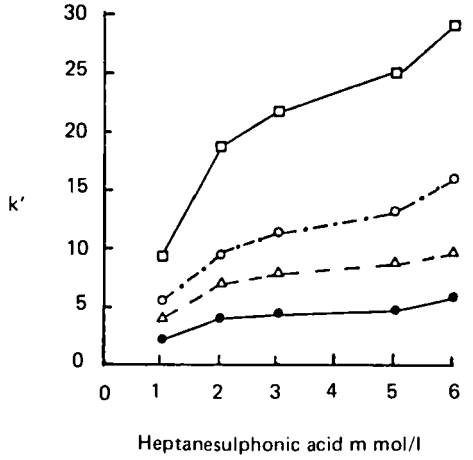


Fig. 7 Effect of increasing heptane sulphonic acid concentration of the mobile phase upon capacity factors (K') of noradrenaline (●——●), adrenaline (△----△), dihydroxy-benzylamine (○----○) and dopamine (□——□). Chromatography performed on 10 cm O.D.S. column with 0.1M phosphate buffer pH 5.0, o.5 mmol/l EDTA, 4% (v/v) methanol. Flow rate 1 ml/min.



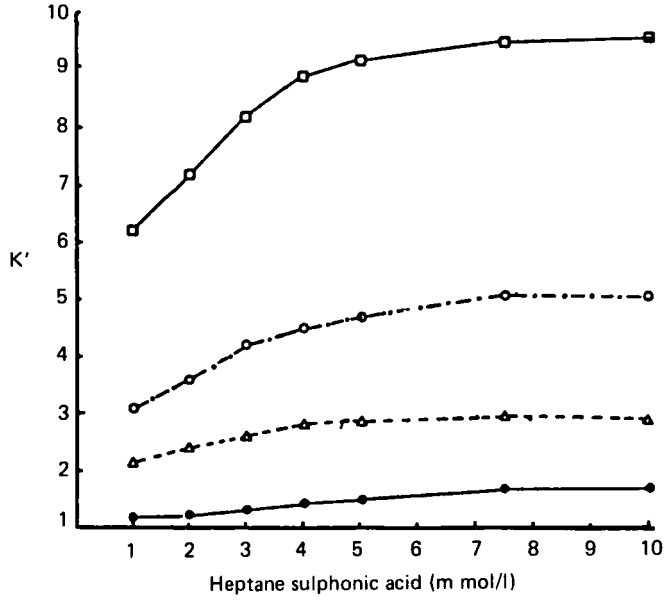


Fig. 8 Effect of increasing heptane sulphonic acid content of ammonium acetate mobile phase on capacity factors of noradrenaline (●——●), adrenaline (△----△), DHBA (○----○) and dopamine (□——□). Chromatography performed on 10 cm O.D.S. column with 0.5 mol/l ammonium acetate buffer pH 5.0, 0.5 mmol/l EDTA, 10 mmol/l heptane sulphonic acid. Flow rate 1 ml/min.

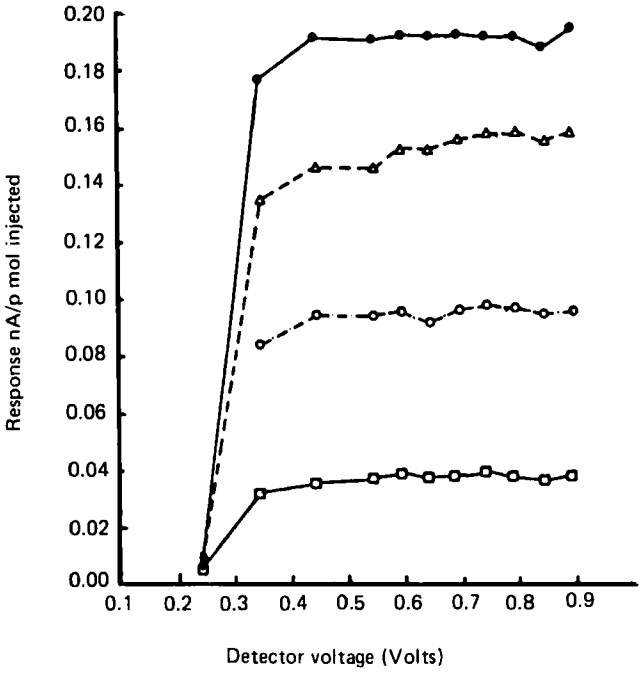


Fig. 9 Hydrodynamic voltamograms of noradrenaline (●——●), adrenaline (△----△), dihydroxy-benzylamine (○----○), and dopamine (□——□). A 10 cm O.D.S. column was used with 0.1M phosphate buffer pH 5.0, 0.5 mmol/l EDTA, 5.0 mmol/l heptanesulphonic acid, 4% (v/v) methanol. Flow rate 1 ml/min.

a plateau, when using the phosphate system, from + 0.45 V to + 0.90 V. (Fig. 9).

Voltamograms were similarly produced at three concentrations of ammonium acetate in response to 100 µl injection of a standard mixture containing neoadrenaline, adrenaline, DHBA and dopamine at concentrations of 0.309 µmol/l, 0.274 µmol/l, 0.237 µmol/l and 0.236 µmol/l respectively (Fig. 10). They were observed to be significantly different from those seen in the phosphate system (Fig. 9);

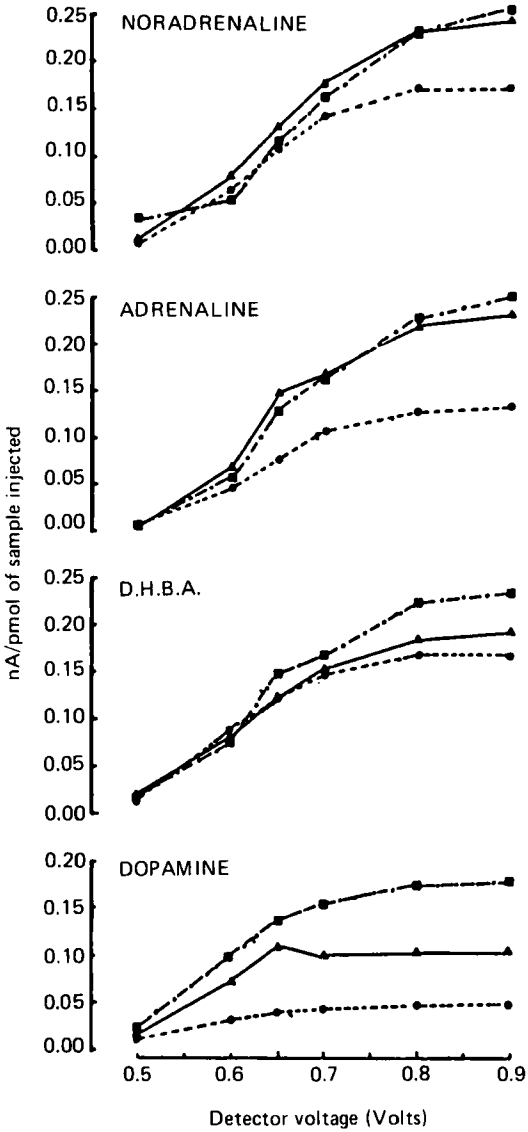


Fig. 10 Hydrodynamic voltamograms of noradrenaline, adrenaline dihydroxy-benzylamine and dopamine at three concentrations of ammonium acetate 0.25 M (●----●), 0.5 M (▲——▲), and 1.0 M (■---■). Chromatographic conditions were a 10 cm O.D.S. column with ammonium acetate buffer pH 5.10, 0.5 mmol/l EDTA, 10 mmol/l heptane sulphonic acid. Flow 1 ml/min.

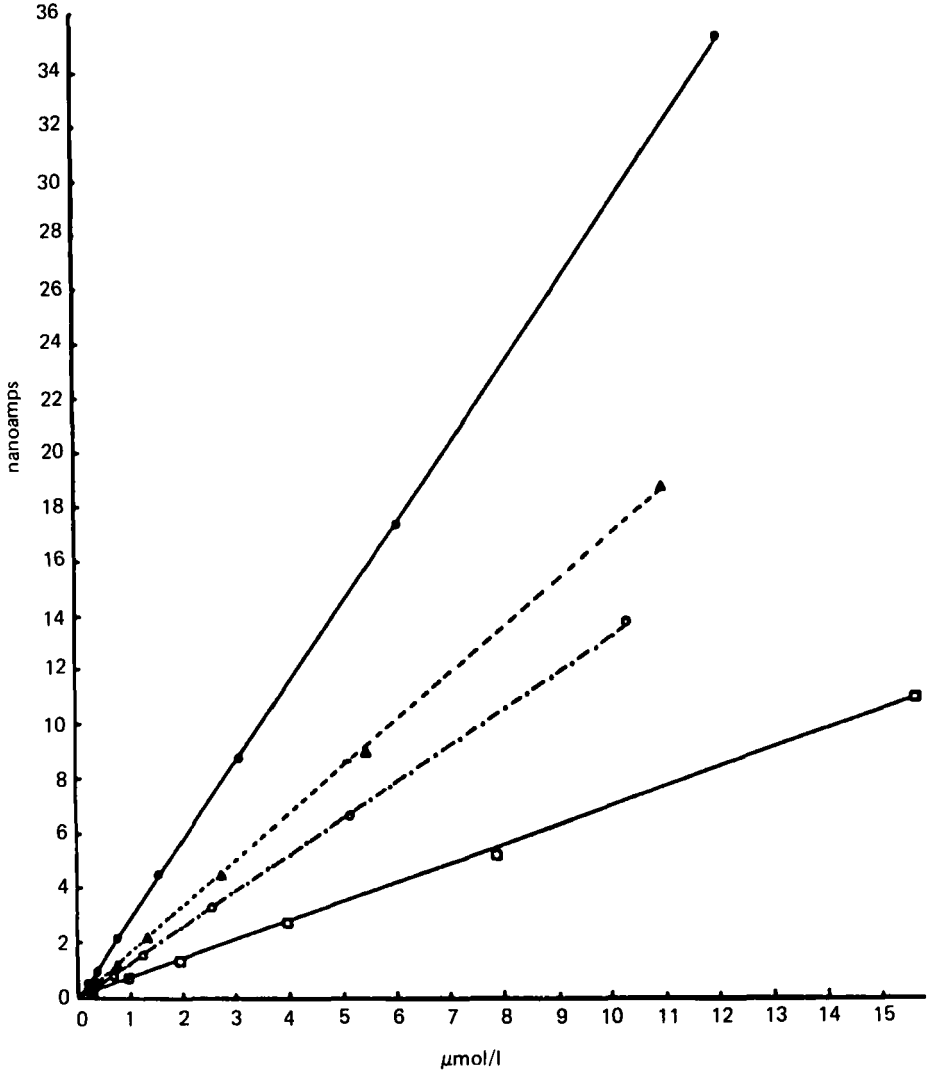


Fig. 11 Response of electrochemical detector to increasing concentrations of noradrenaline (●——●), adrenaline (△---△), dihydroxy-benzylamine (○·-·-○), and dopamine (□——□).
Chromatography performed on a 10 cm O.D.S. column with 0.1 M phosphate buffer pH 5.0, 0.5 mmol/l EDTA, 5.0 mmol/l heptane sulphonic acid, 4% (v/v) methanol. Flow rate 1 ml/min sample injection volume 20 ul.

the rise to the plateau is a gentle progression when ammonium acetate is used, with maximum responses being approached at an applied voltage of + 0.80 V. In contrast, the rise in the case of the phosphate system was rapid with a maximum response reached at + 0.45 V. Once achieved the plateau response tended to be greater in the presence of ammonium acetate. These results suggest that at low voltages ammonium acetate may either reduce detector efficiency or protect the catecholamines from oxidation.

The ability to work at lower detector voltages is advantageous in that detector selectivity may be improved. Use of voltages of less than + 0.80 V with the ammonium acetate system would result in the use of the intermediate region of the voltamogram, a region which is vulnerable to small fluctuations in applied potential and hence change in cell conditions may lead to imprecision of measurement.

The voltamograms show that decreasing the molar strength of the acetate reduces detector response to injected standards. This effect is probably related to column retention times for the catecholamines, which increases as the concentration of acetate decreases (Fig. 6). As the retention time increases, then the volume in which the eluted substances is distributed is likely to increase and therefore its concentration at the detector will be reduced.

This would explain why the effect is more pronounced with the longer retained components dopamine and DHBA, and why there is little difference in the 1.0 mol/l voltamograms of noradrenaline,

(where there is a smaller effect on retention than that seen for DHBA). Measurement of the area under the chromatograph peaks, to give an integrated measure of response instead of measurement of peak response, may remove or reduce the observed effect.

Detector sensitivity and linearity

The detector response to increasing concentrations of catecholamines introduced into the phosphate chromatographic system as a 20 µl sample volume was seen to be linear to levels in excess of those likely to be found in non-pathological and many pathological specimens (Fig. 11).

The sensitivity of the detector for the catecholamines and internal standrads (DHBA) as determined from the slope of the response lines (Fig. 11) were for noradrenaline 0.145 nA/pmol injected, adrenaline 0.085 nA/pmol injected, DHBA 0.66 nA/pmol injected and dopamine 0.035 nA/pmol injected. The detector sensitivity and linearity was checked at three concentrations of ammonium acetate (0.25 mol/l, 0.50 mol/l and 1.00 mol/l) and at two detector voltages (0.65 V and 0.80 V). Increased sensitivity follows an increase in ammonium acetate concentration and an increased applied oxidation voltage (Fig. 12). The relationship between sensitivity and ammonium acetate concentration is most likely to be due to the same differences in retention of catecholamines producing the effect on the voltamograms.

Linearity was checked at two voltage levels at each of the three concentrations of ammonium acetate, the first voltage of + 0.65 V was chosen

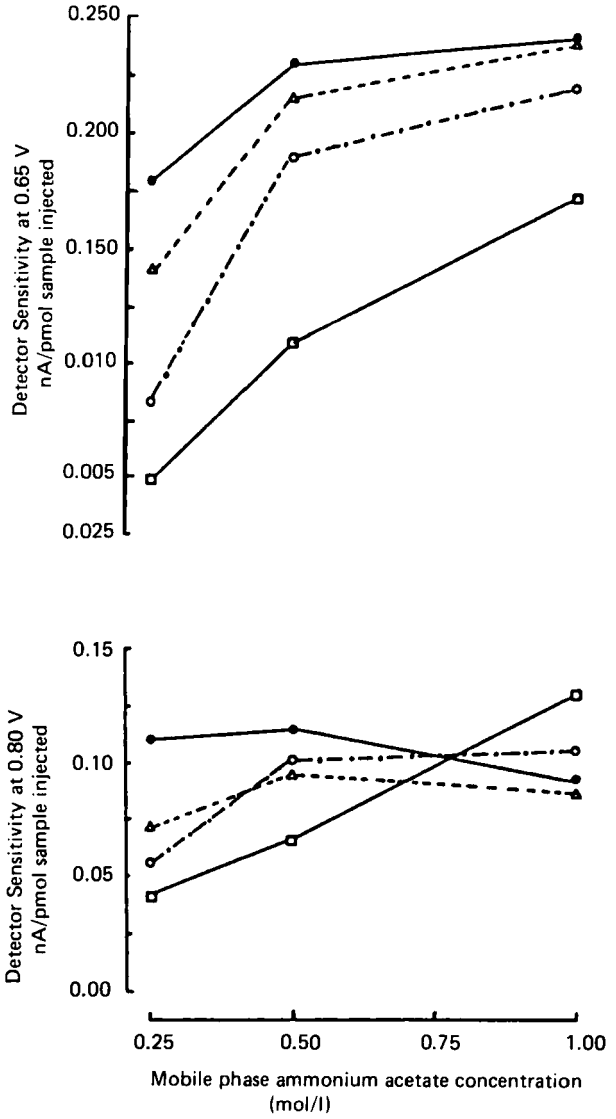


Fig. 12 Detector sensitivity for noradrenaline (●——●), arenaline (△- - -△), dihydroxy-benzylamine (○-·-·-○), and dopamine (□——□) at two applied detector voltages (0.65V and 0.80V) and three different molarity ammonium acetate buffers.
Chromatography was performed on a 10 cm O.D.S. column with ammonium acetate buffer pH 5.16, 0.5 mmol/l EDTA, 10 mmol/l heptanesulphonic acid.
Flow rate 1 ml/min.

as a non-plateau voltage and + 0.80 V as a plateau voltage (Fig. 13). In all cases the response lines were linear over the concentration ranges employed. The linearity of the non-plateau voltage was checked since it may prove necessary under particular circumstances to use non-plateau values to enable an increase in selectivity of the detection system.

Effect of carbon chain length on catecholamine separation and the influence of sample matrix identity.

The effect of increasing the carbon chain length of the reversed phase packing material on the separation of the catecholamines was investigated using 10 cm by 4.5 mm internal diameter columns, packed with Hypersil ODS (C18), MOS (C8) and SAS (C1). A mobile phase of low molarity and high pH was chosen to enable adequate retention on the shorter chain length columns; this phase consisted of 0.2 mol/l ammonium acetate, adjusted to pH 5.5 with glacial acetic acid, 10 mmol/l heptane sulphonic acid and 0.5 mol/l disodium EDTA.

Standards were initially diluted to working dilutions in distilled water and later in such a manner as to maintain matrix identity with the mobile phase.

Inspection of the C18 chromatograph of aqueous standard solution revealed peaks of good symmetry for noradrenaline, adrenaline, DHBA and dopamine, while that of the C8 system revealed a shorter retention time for all compounds with peaks showing

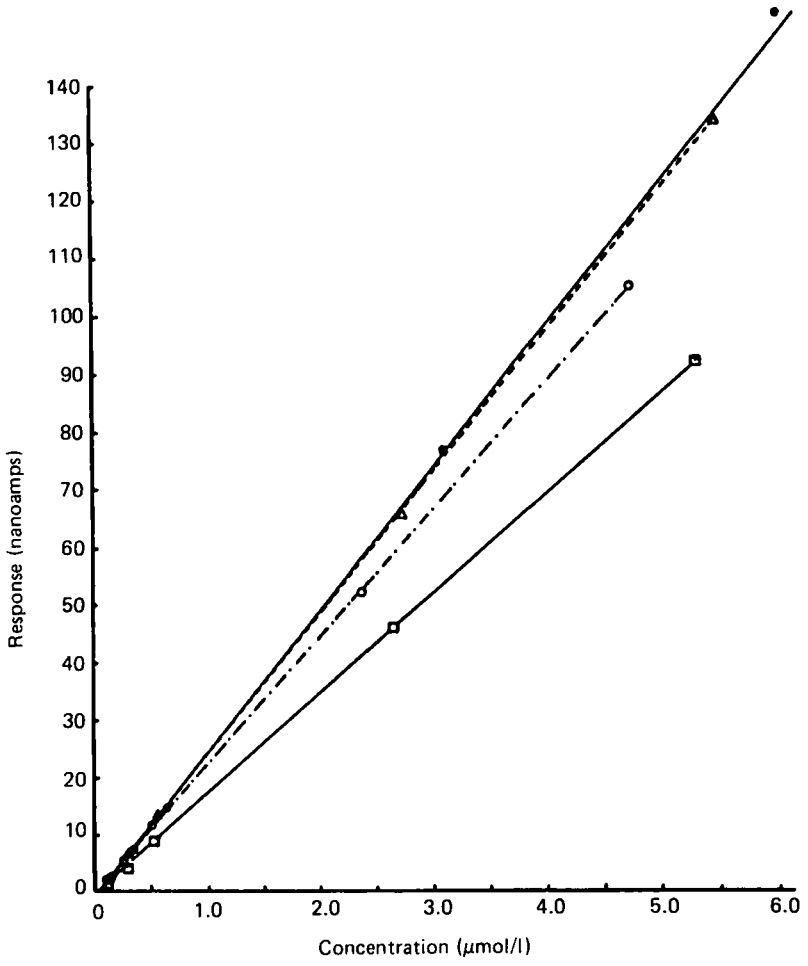


Fig. 13 Electrochemical detector linearity and sensitivity at an applied voltage of 0.80 V for the detection of catecholamines.
Noradrenaline (●——●), adrenaline (△----△), dihydroxybenzylamine (○-·-·-○), and dopamine (□——□). Chromatography performed on a 10 cm O.D.S. column with 1.0 M ammonium acetate buffer pH 5.16, 0.5 mmol/l EDTA, 10 mmol/l heptanesulphonic acid.
Flow rate 1 ml/min.

very obvious tailing and a suggestion of splitting of the DHBA peak. The C1 chromatograph produced a more complex picture, with the retention times of noradrenaline, adrenaline and DHBA being shorter than those observed on C8 (with failure of base line separation between adrenaline and DHBA), but now the dopamine standard had slpit into two peaks with very different retentions, the first peak (peak 1) having a retention of 7 minutes and the second peak (peak 2) with a retention time of 9 minutes (Fig. 14). This effect was not due to impurities in the standard mix since a different batch of dopaline produced the same effect. A second C8 column also produced the same elution pattern. However, alteration of the standard matrix markedly influenced the elution profiles. For example, a stock solution of dopamine, produced by dissolving the solid directly in the mobile phase, chromatographed as a single peak with a retention similar to peak 2, but a 1:5 dilution of this solution in distilled water eluted as two peaks. The dopamine standard therefore appeared homogenous, but, to confirm this, the two peaks were collected separately after injection of 100 µl of aqueous dopamine (0.65 mmol/l) onto the HPLC with a UV detection system (354 nm, electrochemical detection is destructive). When aliquots of the fraction containing the separate peaks were reinjected onto the same column under the same conditions, with electrochemical detection it was found that both peaks 1 and 2 eluted as single peaks with identical retention of 8.5 minutes. Since the

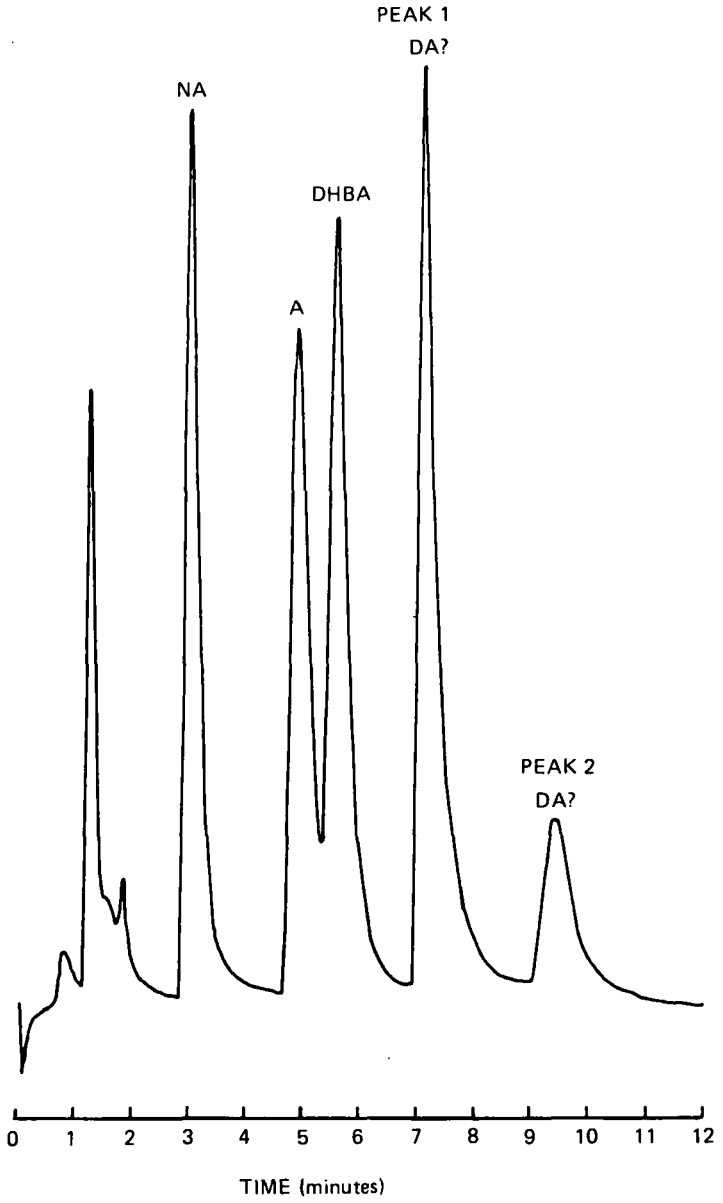


Fig. 14 Seperation of catecholamines on a 10 cm S.A.S. column.
Noradrenaline (NA), adrenaline (A), dihydroxy-benzylamine (DHBA) dopamine (DA). Mobile phase consisted of 0.2 M ammonium acetate buffer pH 5.5, 0.5 mmol/l EDTA, 10 mmol/l heptanesulphonic acid.
Flow rate 1 ml/min.

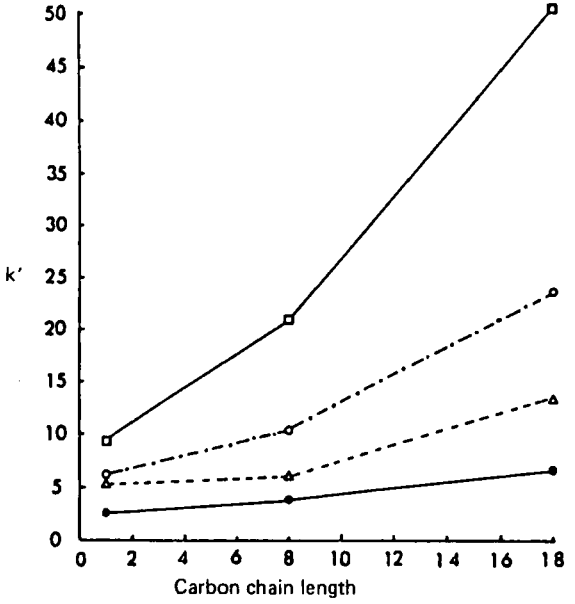


Fig. 15 Effect of column packing chain length on catecholamine capacity factors (K'). Noradrenaline (●——●), adrenaline (△---△), DHBA (○-·-·-○) and dopamine (□——□). Chromatography was on 10 cm columns with 0.2 M ammonium acetate pH 5.5, 0.5 mmol/l EDTA, 10 mmol/l heptanesulphonic acid. Flow rate 1 ml/min.



collected peaks were effectively dissolved in mobile phase, the effect of diluting them in 1:5 in distilled water was investigated and it was seen that each single isolated peak after dilution again generated two fractions on chromatography.

The dopamine standard is therefore homogenous and it would appear that a combination of short column chain length with low molarity acetate buffer will be extremely sensitive to a non-parity of the sample matrix composition with that of the mobile phase composition, and therefore liable

to generation of artefactual separations. The mechanisms involved in the generation of this phenomana are likely to be multifactorial and complex. It is likely that addition of 100 µl of a non identical matrix to the column results in a pocket of mobile phase with a significantly altered composition, in terms of molarity and ion pair content, all of which affect the separation. C1 columns are unstable under purely aqueous conditions and loss of bonded phase would lead to peak splitting due to mixed mechanisms, particularly when ion pairing agent is diluted. Increase in column packing chain length results in an increase in the capacity factors forthe four compounds of the standard mix diluted in mobile phase (Fig. 15). It should be noted that the matrix effect was minimum on the C18, intermediate upon the C8 and maximum upon the C1 columns, thus suggesting that column packing with longer chain length lend resistance to matrix effects. An explanation for this is that, under totally aqueous conditions C18, chains collapse covering the stationary phase more effectively, thus producing a more truely hydrophobic situation; C8 would produce an intermediate effect while C1 is both unstable and unable to perform this function.



Application of the Separation System to Catecholamine Metabolites.

The separation and detection of a wide range of acidic, basic and neutral catecholamine deratives is possible using the present system (Fig. 16). This point is important in consideration of the various extraction procedures used in the initial isolation of catecholamines.

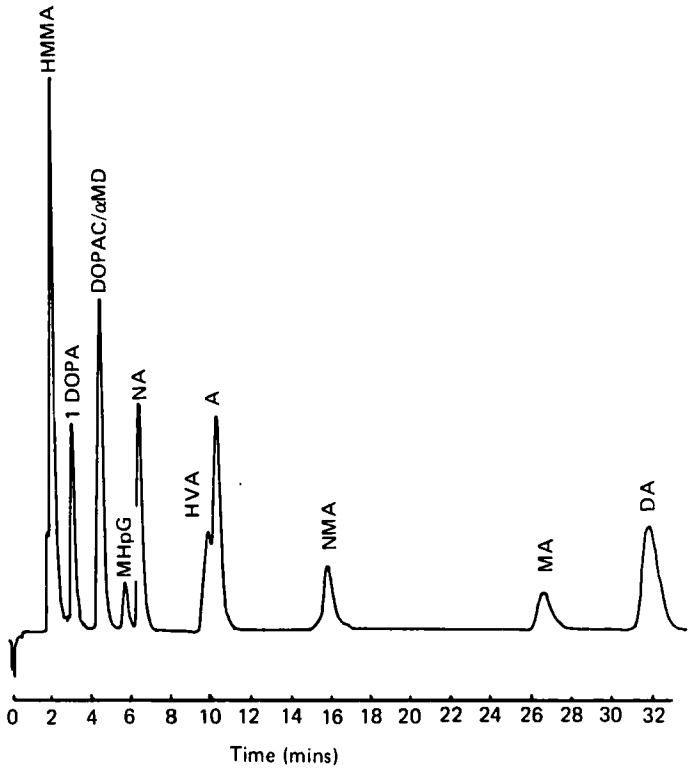


Fig. 16 Seperation of catecholamine metabolites on a 16 cm O.D.S. column with ammonium acetate mobile phase. Hydroxy methyl mandelic acid (HMMA), 1 dihydroxy-phenylalanine (1 DOPA), dihydroxyphenylacetic acid (DOPAC), α methyl dopa (αMD), methoxy-hydroxyphenylethylene glycol (MHPG), noradrenaline (NA), homovanillic acid (HVA) adrenaline (A), normetadrenaline (NMA), metadrenaline (MA), and dopamine (DA). Mobile phase consisted of 0.5 M ammonium acetate buffer pH 5.10, 0.5 mmol/l EDTA, 10 mmol/l heptane sulphonic acid. Flow rate 1 ml/min on a 16 cm O.D.S. column.

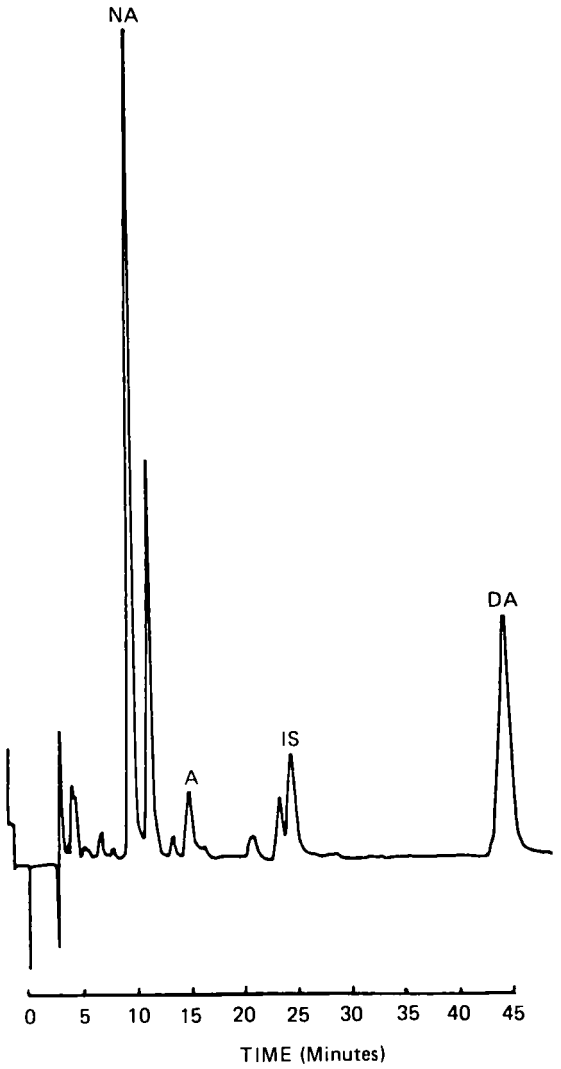


Fig. 17 Chromatograph of a urine from a female aged 33 years with suspected pheaochromocytoma.
Urinary free catecholamine excretion was for noradrenaline (NA) 9.12 µmol/24 hr, adrenaline (A) 0.42 µmol/24 hr, and dopamine (DA) 6.1 µmol/24 hr. Conditions as described in the test with the exception of the column, the length of which was increased to 250 mm to facilatate seperation of the noradrenaline peak from a suspected drug peak. Internal standard (IS).

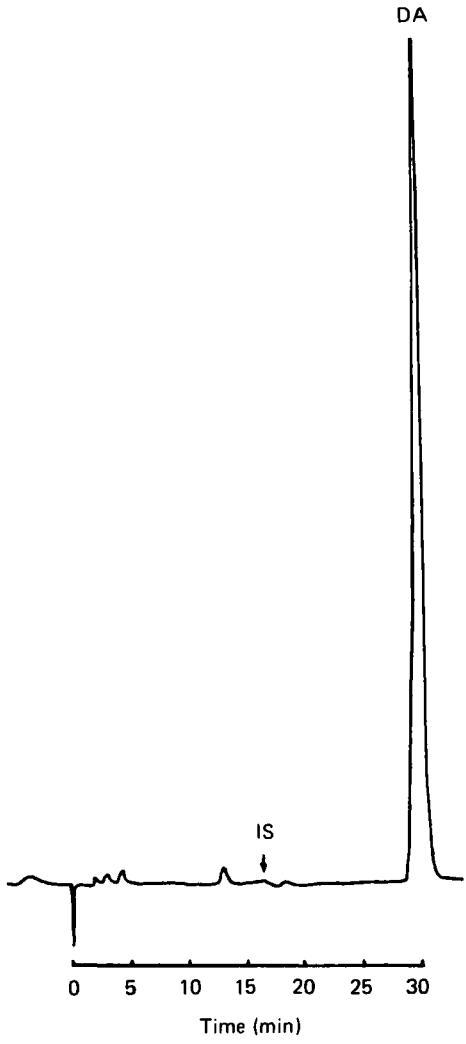


Fig. 18 Chromatograph of a urine from a patient aged 6 years 9 months with Neuroblastoma.
Urinary free catecholamine excretion was for noradrenaline 0.19 µmol/24 hr, adrenaline 0.09 µmol/24 hr, and dopamine (DA) 140.00 µmol/24 hr. Internal standard (IS). For assay conditions see text.

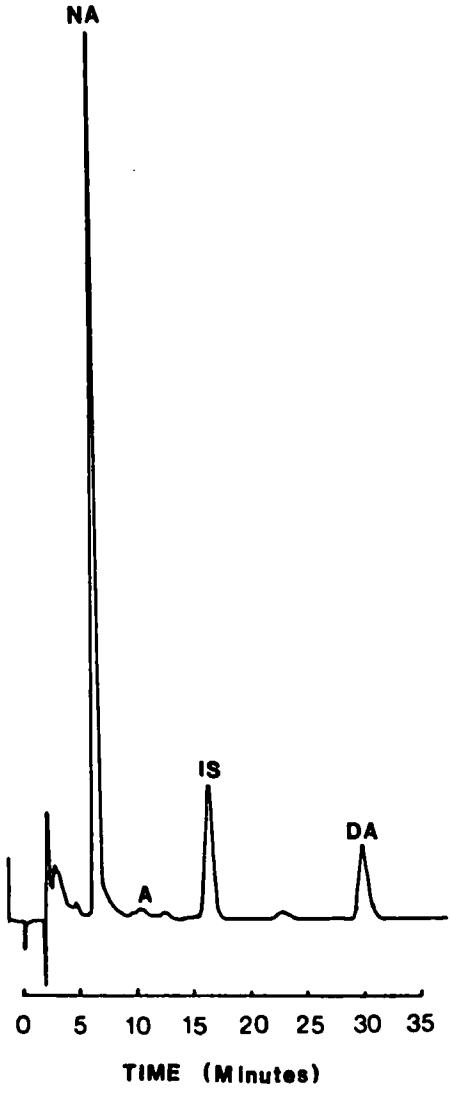


Fig. 19 Chromatograph of a urine from a female aged 7½ years with malignant hypertension and a tumour in the right supra renal area.
Urinary free catecholamine excretion was for noradrenaline (NA) 4.11 µmol/24 hr, adrenaline (A) 0.09 µmol/24hr, and dopamine (DA) 1.29 µmol/24hr. Internal standard (IS). For assay conditions see text.

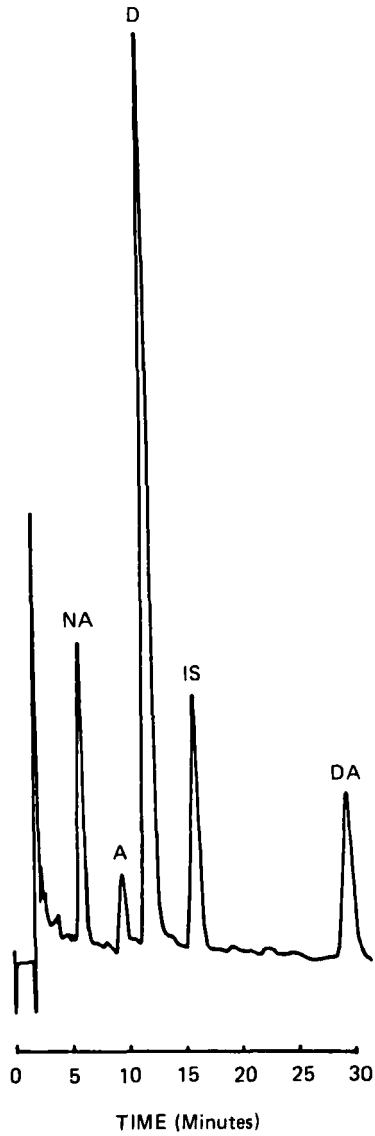


Fig. 20 Chromatograph of a urine from a 57 year old male with iniperable phaeochromocytoma.
For conditions see text. Urinary free catecholamine excretion was for noradrenaline (NA) 1.40 µmol/24 hr, adrenaline (A) 0.99 µmol/24hr, and dopamine (DA) 1.95 µmol/24 hr. Internal standard (IS).
Peak D is seen in patients undergoing treatment with Labetolol.

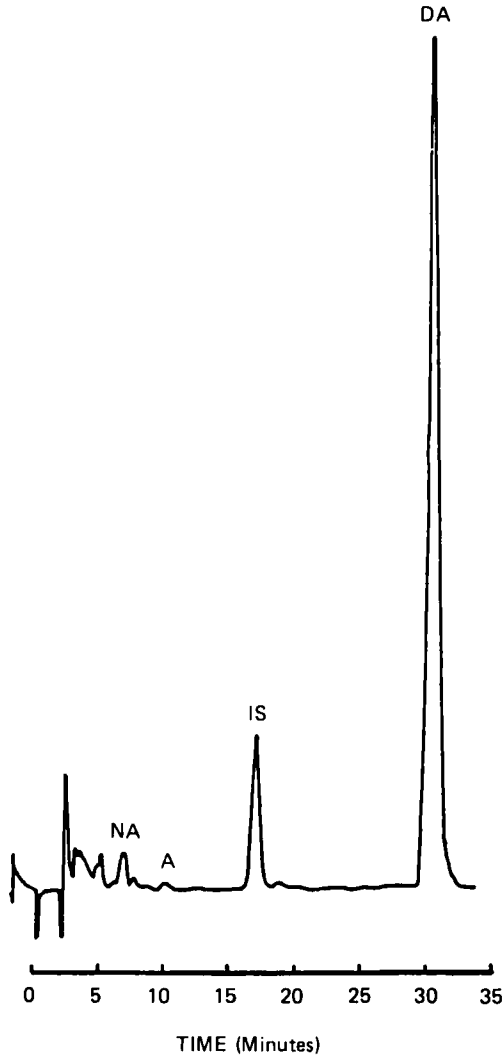


Fig. 21 Chromatograph of a urine from a 19 year old female with cystic teratoma and neuroblastoma.
Urinary free catecholamine excretion was for noradrenaline (NA) 0.31 µmol/24hr, adrenaline (A) 0.09 µmol/24hr, and dopamine (DA) 22.39 µmol/24hr. Internal standard (IS). Conditions as described in the text.

Application to Clinical Samples.

Samples from five patients with catecholamine secreting tumours were assayed for urinary free catecholamines (Figs. 17-21). Sample preparation performed by application to S.C.X. cation exchange columns followed by chromatography upon 16 cm or 25 cm O.D.S. column with a mobile phase consisting of 0.4 M ammonium acetate, 10 mmol/l heptane sulphonic acid, 0.5 mm E.D.T.A. pH 5.10 with a flow rate of 0.9 ml/min and detection voltage of 0.65 V. Results obtained were compatible with the clinical information received.



Conclusion

The ammonium acetate system is superior to the more conventional phosphate based systems used for the separation of catecholamines. Such a system allows the development of more robust and less tedious prechromatographic sample preparation procedures. The use of disposable cation exchange columns, such as those used for sample preparation in this present work, is one possible route to the production of a robust assay system for use in chemical pathology laboratories in order to enable investigation of both normal physiology and autonomic dysfunction.

## ACKNOWLEDGEMENTS

This work was carried out within the department of Clinical Chemistry at Northwich Park Hospital, Harrow, Middlesex. I am grateful to Dr. C.K. Lim and

Mr. D.J. Wright for the helpful suggestions during method development, and to the staff of Prof. M. Sandlers laboratory for supplying urine samples from patients with catecholamine secreting tumours. Thanks are also expressed to Miss M. Gardiner for typing the manuscript.

## REFERENCES

1. Krstoluvic, A.M.. Investigations of Catecholamine Metabolism using High performance Liquid Chromatography. J. Chromatog., 229, 1, 1982.

2. Molnor, I., Horvath, C.. Reverse Phase Chromatography of Polar biological substances: Seperation of Catechol Compounds by High performance Liquid Chromatography. Clin Chem, 22, 1497, 1976.

3. Lim, C.K.. Chromatography Discussion Group Bulletin, 12, 1983.

4. Lim, C.K., Rideout, J.M. and Wright, O.J.. High Performance Liquid Chromatography of Naturally Occurring 8-, 7-, 6-, 5-, and 4-, Carboxylic Porphyrin Isomers. J. Chromatog., 282, 629, 1983.

5. Molnar, I., Horvath, C.. Catecholamines and Related Compounds. Effects of Substituents in Reversed Phase Chromatography. J. Chromatog., 145, 371,1978.

6. Moyer, T.P. and Jiang, N.S.. Optinized Isocratic Conditions for Analyses of Catecholamines by High Performance Reversed Phase Paired-Ion Chromatography with Amperometric Detection. J. Chromatog., 153, 365, 1978.