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SEPARATION OF CATECHOLAMINES AND THEIR METABOLITES BY ADSORPTION, ION-PAIR AND SOAP CHROMATOGRAPHY

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SUMMARY

The separation of catecholamines and their metabolites has been carried out by high-performance liquid chromatography using three systems: liquid–solid adsorption, ion-pair partition, and soap chromatography. In the last newly developed technique a reversed-phase packing is used in conjunction with an aqueous organic eluent containing a detergent. The detergent is chosen so that its ion can form ion-pairs with ions of the solutes. Soap chromatography proved the best technique in terms of column efficiency (giving 3000–5000 plates in 125 mm), resolution and sensitivity of detection. Noradrenaline, adrenaline and dopamine, their 3-O-methyl derivatives, L-3,4-dihydroxyphenylalanine, homovanillic acid and other related compounds could be separated in less than 10 min at the 10–50-ng level on columns whose plate heights were in the range of 20–40 μm. The dependence of retention on the concentration of organic modifier and on detergent concentration for three anionic detergents is reported.

The method is applied to the direct analysis of urine and the potential of the method for such analyses, especially of pathological urines is demonstrated.

Soap chromatography is likely to enlarge the scope of application of high-performance liquid chromatography to biochemical analysis. It is a powerful method for the separation of ionizable compounds which could replace conventional ion-exchange chromatography.

INTRODUCTION

Although many chromatographic techniques have been applied to the separation and quantitation of catecholamines and their metabolites, the major difficulty in dealing with biological material has always been, and still remains, detection of the exceedingly low levels present in bio-fluids such as urine or blood. In such analysis it is generally necessary to carry out a prior extraction and this may have to be followed by appropriate derivatization to enhance the sensitivity of detection. While the most frequently used chromatographic methods for catecholamines are thin-layer (TLC)[1–7] and paper chromatography[8–14], these require quite elaborate pretreatment of samples when applied in biochemical analysis where detection at the nanogram or subnanogram level per millilitre is required. Gas chromatography[15–19] does have the

necessary sensitivity but requires prior derivatization of the catecholamines to render them volatile; since the silylating reagents may react not only with phenolic and alcoholic groups but also with amino groups, errors readily arise from incomplete derivatization. In recent studies using high-performance liquid chromatography (HPLC), Wall[20] has reported separations on a polyacrylic acid cation exchanger, Mori[21–26] has used the Zipax SCX, the DuPont pellicular strong cation exchanger, while Persson and Karger[27] have employed ion pair partition chromatography. The ion-exchange method has been automated by Mori for urine analysis, the procedure involving fluorimetric detection of trihydroxyindole derivatives[22] to give the necessary enhancement of sensitivity over the normal ultraviolet (UV) detection method. Another fluorphore that has been used successfully by Imai[28] for detection of adrenaline and noradrenaline is Fluorescamine® (Hoffmann-La Roche). Very recently Schweat and Bussemas[29] separated Dns derivatives of catecholamines by adsorption chromatography on silica gel and achieved very low levels of detection by using fluorescence. These separations, with the exception of those of Persson and Karger[27], have, however, shown relatively poor resolution, speed and plate height.

There is no doubt that improvements in HPLC performance could reduce and possibly even remove the need for the initial extraction or the formation of special derivatives when examining biological material, for a high plate efficiency might enable the bulk of the endogenous compounds to be separated from the compounds of particular interest. At the same time the elution of sharper peaks from shorter columns would enhance the concentration of eluted solutes and improve sensitivity.

The present study was carried out in an attempt to provide an HPLC method which would give the highest resolution and at the same time be suitable for analysis of urine samples. We have examined adsorption chromatography, ion-pair chromatography and the novel technique of soap chromatography[30]. The last of these has proved to be particularly suitable for catecholamine separation giving chromatographic efficiencies of up to 10,000 theoretical plates, and analysis times of 10–30 min for compounds having capacity ratios (k') of up to 30. The method has the particular advantage, when applied to urine analysis, that the bulk of the endogenous compounds that show UV absorbance elute before the solvent peak ($k' = 0$). These are presumably high-molecular-weight substances that are excluded from the reversed-phase silica gel used as column packing. The catecholamines and their metabolites are well retained and readily separated from other minor UV-absorbing components in urine. While UV spectrophotometry is not sufficiently discriminatory to allow quantitation of many of the catecholamines when normal urine is directly injected into the column, it is sufficiently sensitive to monitor them in the pathological urines of patients being treated for Parkinson's disease using either the 3,4-dihydroxyphenylalanine (L-DOPA) or L-DOPA with benserazide (Madopar®) treatments. Using oneof the fluorescent detection techniques[22,28], it is probable that the sensitivity would be sufficient for the method to be applied to the detection and quantitation of catecholamines in normal urines injected directly on to the column, thus making the extraction stage redundant.

EXPERIMENTAL

The high-performance liquid chromatograph was constructed in the laboratory

and comprised a Haskel high-pressure gas-driven intensifier pump and a variable wavelength UV photometer (Cecil Model 212) as detector. Columns, 5 mm in bore and 125 mm long, were made of internally polished stainless steel. Each column was terminated by an 0.8-μm silver membrane (Selas Flotronics) sandwiched between the 6-μm porosity stainless-steel frits (B.S.A.). The septum injector and column fittings were made to designs similar to those previously described[31]. Columns were operated at ambient temperature. Injections (1–20 μl) were made by microsyringe.

The following column packings were used. (A) For adsorption chromatography: Spherisorb A20, a 20-μm spherical alumina from Phase Separations (Clwyd, Great Britain); (B) for ion-pair partition chromatography: Spherisorb S5, a 5-μm spherical silica gel from Phase Separations; (C) for soap chromatography: ODS/TMS silica 5 μm (Wolfson Liquid Chromatography Unit, Edinburgh, Great Britain). This material[32] is a spherical silica gel (surface area of about 200 m^2/g) whose surface has first been substituted by octadecyl groups and finally treated to replace any residual silanol groups by trimethylsiloxyl groups.

Different packing techniques were employed for the different materials: the 20-μm Spherisorb A was dry-packed by the rotate, bounce and tap method[33]. The Spherisorb S5 and ODS/TMS silica were packed by driving a dense slurry (*ca.* 2 g of packing in 15 cm^3 of methyl iodide) into the column at a pressure of 3000 p.s.i. The methyl iodide was then washed from the column by passage of *n*-hexane. For ion-pair partition chromatography the column (packed with Spherisorb S5) was then dried by passage of air; it was loaded with the aqueous stationary phase (a perchloric acid–sodium perchlorate solution) by drawing 5 cm^3 of the stationary phase through the column using suction. The eluent (a butanol–methylene chloride mixture) was then pumped through the column until the emerging liquid was clear. To preserve the stationary phase a precolumn (5-mm bore, 500 mm long) was placed upstream of the injector: it was packed with 80–100 mesh Chromosorb bearing 20% (w/w) of the stationary phase. For soap chromatography (using ODS/TMS silica) the *n*-hexane was displaced by methanol and the column subsequently conditioned by pumping eluent for several hours before use.

The solutes used in the study are listed in Table I which also shows their formulae and the sources from which they were obtained. The detergents used in soap chromatography were sodium lauryl sulphate (SLS) from BDH (Poole, Great Britain), sodium 1-dodecane sulphonate (SDS) from Cambrian Chemicals (Croydon, Great Britain), and sodium dodecyl benzene sulphonate (SDBS) from BDH.

RESULTS AND DISCUSSION

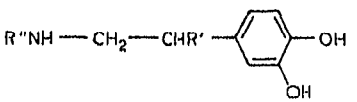
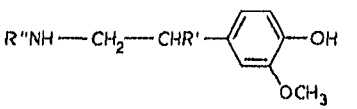
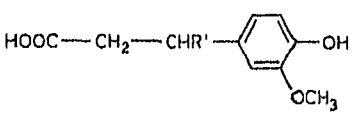
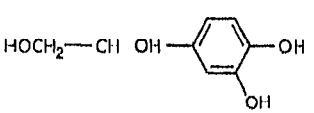
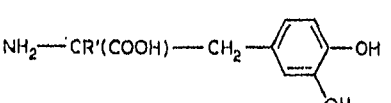
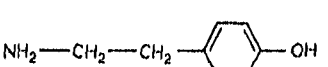
Adsorption chromatography

Mixtures of 1-butanol, acetic acid and water, frequently used as developing solvents in TLC, were employed as eluents. They eluted methyl derivatives before the corresponding unsubstituted catecholamines, and adrenaline before noradrenaline as shown in Fig. 1A. This is essentially the order of increasing polarity.

Addition of small quantities of a less polar solvent such as diethyl ether increased the retention of adrenaline and noradrenaline more than that of metadrenaline and normetadrenaline, and thereby changed the order of elution of adrenaline and normetadrenaline as shown in Fig. 1B.

TABLE I

LIST OF COMPOUNDS STUDIED

Compound	*Symbol*	*Formula*	*R'*	*R''*	*Source*
Catecholamines					
Adrenaline	(A)		OH	CH_3	Hoffmann-La Roche (Basle, Switzerland)
Noradrenaline	(NA)		OH	H	
Dopamine	(DA)		H	H	
3-O-Methylderivatives					
Metadrenaline	(MA)		OH	CH_3	California Corporation for Biochemical Research (Los Angeles, Calif., U.S.A.) Hoffmann-La Roche
Normetadrenaline	(NMA)		OH	H	
3-Methoxytryamine (3-O-Methyl-dopamine)	(MDA)		H	H	
Acidic metabolites					
Homovanillic acid	(HVA)		H		Sigma London (Kingston upon Thames, Great Britain)
Vanilmandelic acid	(VMA)		OH		
Intermediate metabolite					
3,4-Dihydroxyphenyl glycol	(DPG)				
Catecholamine acidic precursors					
L-3,4-Dihydroxyphenylalanine (L-DOPA)	(LD)		H		Hoffmann-La Roche
α-Methyldopa	(MD)		CH_3		Sigma London
Related compound					
Tyramine*					Sigma London

* Formed by decarboxylation of tyrosine, precursor of L-DOPA.

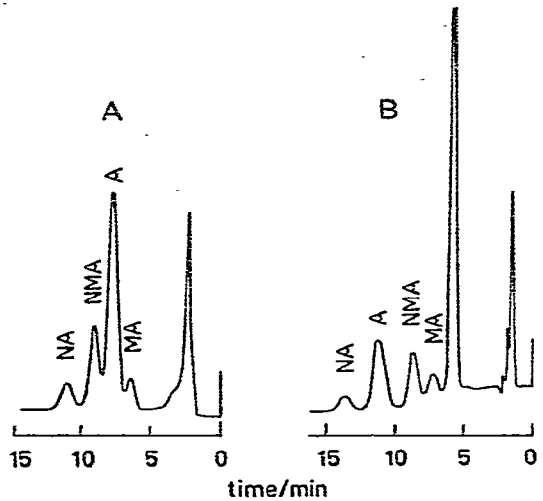


Fig. 1. Liquid–solid adsorption chromatography of catecholamines and derivatives. Packing, Spherisorb A20 (alumina); detection by UV photometry at 254 nm; sensitivity 0.04 absorbance units full scale deflection (a.u.f.s.); pressure drop, 150 p.s.i.; eluent, (A) butanol–acetic acid–water (4:1:1, v/v); (B) butanol–acetic acid–diethyl ether–water (4:1:1:1, v/v). For peak identification see Table I.

The separations in Fig. 1 correspond to plate efficiencies of around 700 giving reduced plate heights ($h = H/d_p$) of about 9 at a reduced velocity ($v = ud_p/D_m$) of about 60. (H = plate height, d_p = particle size, u = linear velocity of eluent calculated as column length/elution time of an unretained solute, D_m = diffusion coefficient of solute in eluent.) Improved efficiencies would undoubtedly be obtained using 5- or 10-μm particles. However, since there was some evidence of irreversible adsorption, and since the components of urine are generally irreversibly adsorbed by alumina and silica gel no further work was carried out on this otherwise promising method of separation.

Ion-pair partition chromatography

The principle of ion-pair partition chromatography has been fully described by Eksborg and Schill[34] who cite references to earlier work by Schill and co-workers on ion-pair partition. It has been used successfully by Kraak and Huber[35] for separation of sulphonic and other acids, by Knox and Jurand[36] for tricyclic antidepressants, and by Persson and Karger[27] for catecholamines. Our technique was similar to that of Persson and Karger. The stationary phase throughout was 0.1 *M* in perchloric acid and 0.9 *M* in sodium perchlorate. Eluents were butanol–methylene chloride mixtures.

A typical separation is shown in Fig. 2. The elution order is similar to that in adsorption chromatography. The plate efficiency is around 1000 for the early peaks and around 2000 for the last peaks giving h from 12–25 at v of about 20. These values are somewhat better than those of Persson and Karger and our separations were obtained at lower pressures (generally below 1000 p.s.i.). Nevertheless the values of h are still much higher than the best values of about 3 obtained in adsorption and reversed-phase bonded liquid chromatography[32]. As in Persson and Karger's work peaks were significantly tailed.

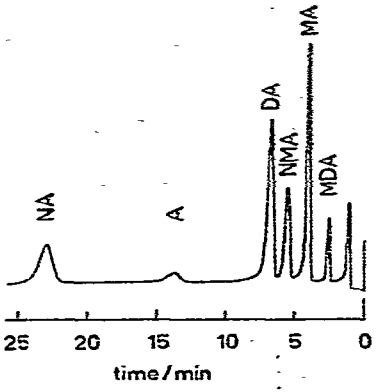


Fig. 2. Ion-pair partition chromatography of catecholamines and derivatives. Packing, Spherisorb S5 (silica gel); detection, 280 nm; sensitivity 0.2 a.u.f.s.; pressure drop, 300 p.s.i.; stationary phase, aqueous 0.1 *M* $HClO_4$ + 0.9 *M* $NaClO_4$; eluent, butanol–methylene chloride (40:60, v/v).

Increase in the proportion of butanol from 40–80% (v/v) reduced k', the column capacity ratio, for all components as shown in Fig. 3. The effects on different solutes were sufficiently different that the elution orders of the pairs adrenaline–noradrenaline and metadrenaline–normetadrenaline could be reversed. These effects are in general accord with the theory of ion-pair partition according to which alcohols are known to solubilize ion-pairs much more effectively than methylene chloride[34,37].

Undoubtedly ion-pair partition chromatography allows considerable flexibility in the degree of retention and elution order even without changing the stationary

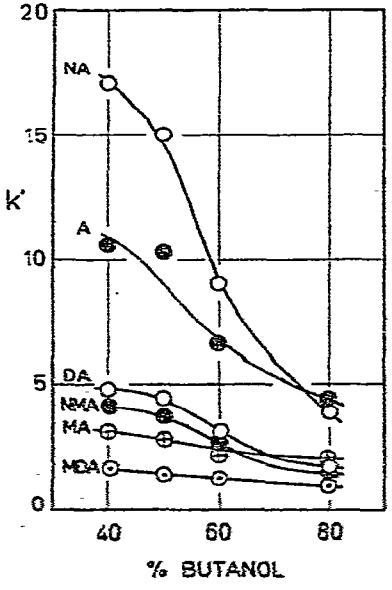


Fig. 3. Dependence of k' upon volume percentage compositions of eluent (butanol–methylene chloride) in ion-pair partition chromatography. Packing and stationary phase as for Fig. 2.

phase and counter ion (ClO_4^- in this work). However, it has several drawbacks. The relatively low plate efficiency and peak asymmetry have been referred to above. A more serious practical problem is the preservation of the stationary phase, which can rather easily be removed from the column and whose quantity is difficult to control unless special precautions are taken. This is a common problem with all liquid–liquid chromatographic systems which employ two physically distinct phases. A way out of the difficulty in simple partition chromatography is to use an adsorbent of large surface area to extract a polar component from a mixed eluent (*e.g.* hexane–acetonitrile, 99:1). It is then possible to form what amounts in practice to a two phase system which is stabilized by the presence of the adsorbent surface. Unfortunately this technique does not appear to be applicable in simple ion-pair partition chromatography where a high concentration of a salt has to be present in the stationary phase.

Soap chromatography described in the next section largely overcomes these problems.

Soap chromatography

Soap chromatography[30] is a technique which uses a reversed-phase packing material in combination with a hydrophilic eluent containing an organic modifier (up to say 30%, v/v) and a small concentration of a detergent or soap (generally 0.1% or less). The detergent is adsorbed by the reversed-phase surface to form a layer which is in some ways akin to an ion exchanger. A detergent is chosen which has the potential to form an ion-pair with an ionized form of the solute. To ensure that the solutes of interest are in the preferred ionic form the pH of the solution may have to be controlled. Thus, for amines, an acidic eluent containing a cationic detergent would generally be used. In this work we have employed SLS, SDS and SDBS as detergents.

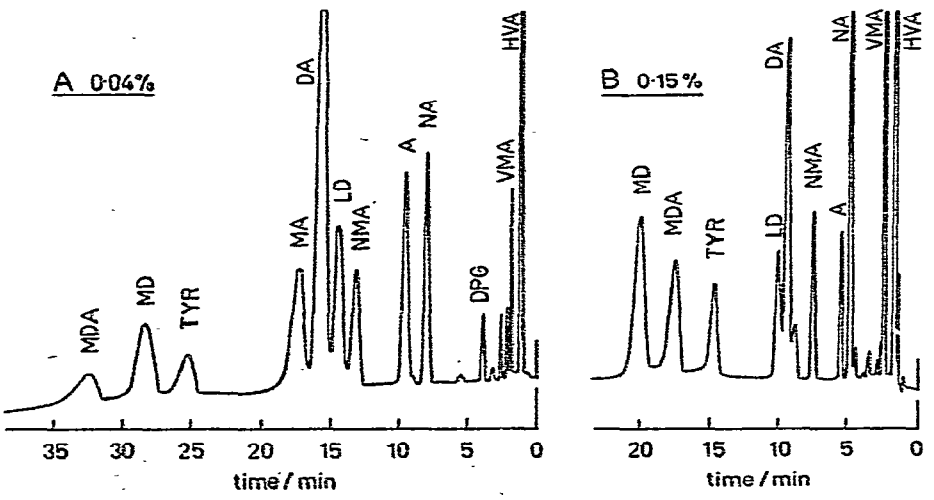


Fig. 4. Soap chromatography of catecholamines and derivatives. Packing, ODS/TMS silica; detection, 280 nm, 0.02 a.u.f.s.; eluent, water–methanol–SDS (72.5:27.5:0.02, v/v/w) with added sulphuric acid: (A) 0.04% (v/v); (B) 0.15% (v/v).

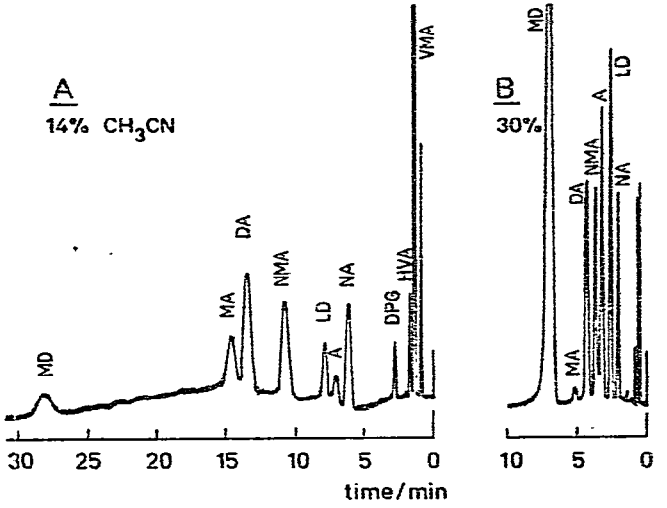


Fig. 5. As for Fig. 4 except: detection, 280 nm, 0.01 a.u.f.s.; eluent, water–acetonitrile–SLS–sulphuric acid. (A) 86:14:0.02:0.04 (v/v/w/v); (B) 70:30:0.02:0.04 (v/v/w/v). The quantity of NA injected for chromatogram B was around 20 ng.

While it was possible to elute the catecholamines and their derivatives from an ODS/TMS silica with a simple acidic eluent, they were either unretained or very slightly retained and then the peaks were generally broad. Resolution of specific compounds was impossible. On addition of a small concentration of any of the above detergents a dramatic improvement occurred in retention, peak sharpness, peak symmetry and resolution. Figs. 4 and 5 illustrate the excellent separations which can be obtained. Catecholamines are eluted in roughly the reverse order to that found in adsorption and ion-pair chromatography. The efficiencies in terms of the number of theoretical plates can be as high as 10,000 for the 125-mm column but 3000–5000 is more usual. The corresponding reduced plate heights are in the range 2–6 at reduced velocities of about 20, and are similar to those obtained in adsorption chromatography. Speed of separation, selectivity and resolution can readily be adjusted by altering any one of a number of parameters such as the nature and concentration of the organic modifier in the eluent, the nature and concentration of the detergent, the nature and concentration of the acid, and by addition of salts, particularly salts with coordinating metal anions.

Amongst the organic modifiers examined, methanol and acetonitrile gave the highest column efficiencies, whereas propanol and dioxane gave poorer performance.

Increase of acid concentration as shown by Fig. 4 increased the speed of analysis by reducing retention and slightly improved the plate efficiency. Unfortunately acid concentrations above about 10^{-2} M (corresponding to 0.1% sulphuric acid) cause slow hydrolysis of bonded alkyl groups and must be avoided for long term work. The addition of acetonitrile or methanol as shown by Fig. 5 reduced retention but with some loss in plate efficiency. A more comprehensive study of the effect of acetonitrile concentration gave the results shown in Fig. 6. In this work, carried out early in the study, an acid concentration of 0.2% and a detergent (SLS) concentration of 0.1% were used. These were substantially higher than the concen-

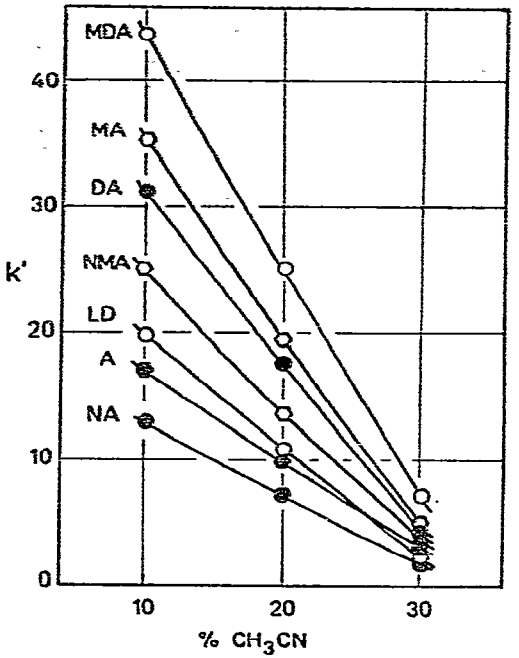


Fig. 6. Dependence of k' upon volume percentage of acetonitrile in soap chromatography. Packing, ODS/TMS silica 5 μm; eluent, water–acetonitrile containing 0.2% (v/v) sulphuric acid and 0.1% (w/v) SLS.

trations used subsequently, for example in the analyses shown in Fig. 5. Nevertheless, the effect of acetonitrile is likely to be similar whatever the acid and detergent concentrations. As the concentration of acetonitrile is increased from 10% to 30%, k' falls more or less linearly, the decrease being from 4 to 7-fold. The order of elution of the catecholamines is unchanged over this range being NA, A, NMA, DA, MA and MDA. However, L-DOPA can be eluted before adrenaline at high acetonitrile concentrations and after it at low concentration. The effect of an organic modifier in reducing the retention is in the sense expected for a reverse phase separation. The addition of detergents increased retention and with minor exceptions the three detergents SLS, SDS and SDBS gave the same order of elution and comparable k' values at around the 0.05% (w/v) concentration level. Detailed results are shown in Fig. 7 for the three detergents. It is noticable that SLS is effective at the lowest concentrations and that addition above about 0.06% has rather little additional effect, especially for the less retained solutes. SDS is less effective at very low concentration but also shows a saturation effect. SDBS in contrast shows the least effect at low concentrations but up to 0.08% k' increases more or less linearly with concentration.

The elution order with SDBS is the same at all concentrations namely NA, A, NMA, LD, DA, MA, MDA, with optimum resolution being obtained at about 0.04%. With SDS the order is the same with SDS concentration above 0.02% but at lower concentrations LD is eluted earlier, and at 0.005% emerges between A and NMA. Best resolution is, however, obtained with an SDS concentration of about 0.02%. With SLS the dependence of retention upon concentration is more complex. At high

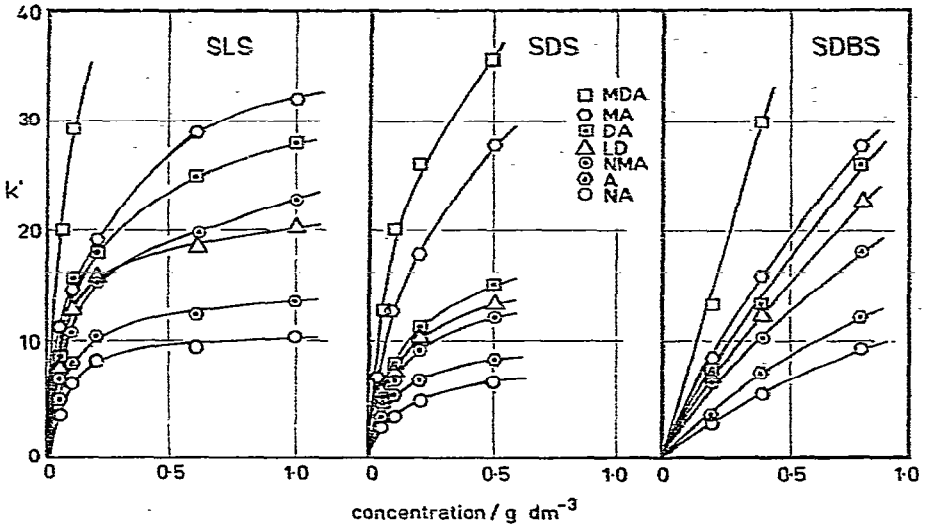


Fig. 7. Dependence of k' upon concentration of detergent. Packing, ODS/TMS silica 5 μm; eluent, water–methanol–sulphuric acid (89:11:0.04, v/v/v).

concentration the elution order is NA, A, LD, NMA, DA, MA, MDA, the order found with low concentrations of SDS. When the SLS concentration is reduced to 0.005% the order of elution becomes NA, A, NMA, LD, DA, MA, MDA, which is the same as with SDBS and at high concentrations of SDS. Excellent resolution can be obtained with SLS at either 0.05 or 0.005%.

The dependence of k' upon detergent concentration found in this study are qualitatively similar to those found by Knox and Laird[30] in their work on sulphonic acids using the cationic detergent cetyltrimethylammonium bromide. They used a short chain bonded silica, SAS silica, and found that k' first rose, then became more or less independent of concentration, and finally fell again. Their concentrations were about ten times higher (up to 2%) and solubilization into the eluent was considered to be the cause of the decline in k' at high concentrations of detergent. The rise in k' at low concentrations was correlated with adsorption of the detergent on to the bonded hydrocarbon surface. Using ODS/TMS silica it would be expected that the detergents would be more strongly adsorbed than on SAS silica and therefore the effects would be observable at lower concentrations. By the same token there would be less solubilization into the eluent.

While the detailed mechanism of soap chromatography is still to be unravelled we believe that it operates rather like ion exchange, where amines (in the present instance) present in the eluent as cations exchange with counter ions that are associated with detergent anions adsorbed onto or into the hydrocarbon surface of the packing. This layer of adsorbed detergent forms an ion exchanger of excellent thermodynamic and kinetic properties so that what amounts to ion-exchange separation can be carried out with high chromatographic efficiency. Furthermore, by changing the concentration and nature of the detergent the degree and order of retention can be simply controlled.

Urine analysis

The determination of catecholamines and their derivatives in urine is of great clinical importance, but, as noted earlier, the problem of detection and quantitation is made difficult by the very low levels present and preliminary extraction coupled with subsequent derivatization is generally required. The method described using soap chromatography gives much better resolution and sensitivity than previous methods yet without one or other of the above procedures it still cannot detect the compounds in normal urine. However, certain pathological conditions are associated with unusually high levels of certain of these compounds in urine. An example is the urine of patients undergoing treatment for Parkinson's disease with either L-DOPA or Madopar.

Fig. 8 shows four chromatograms illustrating the type of results which may be obtained. Chromatogram A shows the elution times of standard compounds. Chromatogram B shows the chromatogram obtained by direct injection of 5 μl of normal urine. The arrows indicate the elution positions of the nine standard compounds. Peaks occur at times close to the elution times of NA, A, NMA and MDS. They are, however, very small and identification is uncertain. Indeed, elution with water–acetonitrile–sulphonic acid–SDS (85:15:0.06:0.01, v/v/v/w) shows that the component eluting at a time corresponding to adrenaline in Fig. 8 is, in fact, some quite different substance. It is, however, worthy of note that the main UV-absorbing components in urine elute very early. Indeed, some elute before the first peak of chromatogram A, indicating that they are probably polymeric. A most important feature of urine analyses carried out on reversed-phase materials is the early elution of the majority of UV absorbing endogenous materials.

Chromatograms C and D are in marked contrast to B in showing substantial

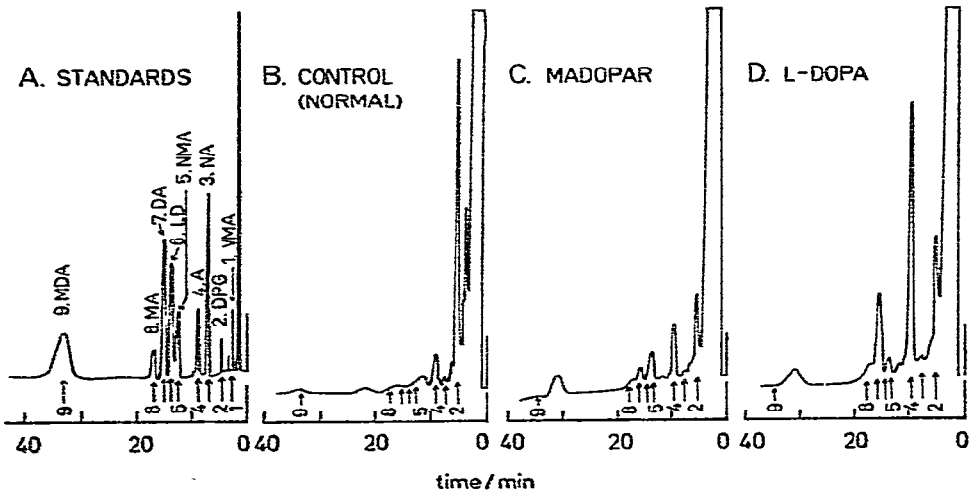


Fig. 8. Soap chromatography of catecholamines and derivatives in urine. Packing, ODS/TMS silica 5 μm; detection 280 nm, 0.04 a.u.f.s.; sample size, 5 μl; eluent, water–methanol–sulphuric acid–SLS (72.5:27.5:0.04:0.005, v/v/v/w). (A) Standards; (B) control normal urine; (C) urine from patient suffering from Parkinson's disease under Madopar treatment; (D) as C but under L-DOPA treatment. Arrows indicate elution times of standard solutes numbered in A.

peaks at the same elution time as L-DOPA and dopamine. The ratio is above 1 in chromatogram C and below 1 in D. Using the second eluent peaks again elute at times corresponding to LD and DA and again the peak area ratio corresponds to those in chromatograms C and D. There is therefore reasonable if not conclusive evidence that these peaks can be identified. The peaks coming at the same time as adrenaline are much larger than in normal urine but again they are not, in fact, due to adrenaline.

These preliminary results show something of the potential for HPLC in clinical analysis, but they also reveal a major pitfall, namely the strong possibility of incorrect identification of peaks if elution time under a single set of conditions is used as the evidence for identification. It is clearly essential to use a number of eluents to confirm identity and preferably independent methods, such as mass spectrometry, to obtain certainty.

CONCLUSIONS

(1) Adsorption chromatography, ion-pair partition chromatography and soap chromatography are all suitable for the separation of the catecholamines. The elution order for the last method (NA, A, NMA, DA, MA, MDA with LD eluting, depending upon conditions, just before or just after NMA) is roughly the reverse of that obtained in the first two methods.

(2) Soap chromatography gives the highest plate efficiency, (up to 10,000 plates in 125 mm). the least peak tailing and the best reproducibility for catecholamines.

(3) The degree of retention in soap chromatography is readily controlled by changes in the nature and concentration of organic modifier, the concentration of acid, the nature and concentration of detergent. Optimum detergent concentrations are 0.02% (SDS), 0.04% (SDBS), 0.005 or 0.05% (SLS).

(4) Soap chromatography is particularly suitable for urine analysis since the major endogenous components are eluted well before the catecholamines. Repeated injections of neat urine cause no deterioration in column performance.

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