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ION-PAIR CHROMATOGRAPHY OF ACIDIC DRUG METABOLITES AND ENDOGENIC COMPOUNDS

B. FRANSSON, K.-G. WAHLUND, I. M. JOHANSSON and G. SCHILL

Department of Analytical Pharmaceutical Chemistry, Biomedical Centre, University of Uppsala, Box 574, S-751 23 Uppsala (Sweden)

SUMMARY

Liquid–liquid chromatographic systems based on ion-pair partition with silica microparticles as the support for the stationary phase have been used for the separation of anionic compounds of biochemical and pharmacological interest.

A high separating efficiency can be obtained with both aqueous and organic mobile phases and the retention is easily regulated by the nature and the concentration of the quaternary ammonium counter ion, present in the aqueous phase. The influence of the composition of the liquid phases on the selectivity and separating efficiency has been studied, as well as equilibration methods and the stability of the systems.

Examples are given of separations of sulphonamides, barbiturates, glucuronic and sulphuric acid conjugates of steroidal compounds and phenols, glycine conjugates of carboxylic acids (hippuric, nicotinuric and salicyluric acid) and anionic metabolites of biogenic amines (indoleacetic, benzoic, mandelic and phenylacetic acid derivatives).

INTRODUCTION

Metabolic changes of drugs and related compounds will often give rise to an increase of their hydrophilic character, which increases their tendency to be excreted from the organism, and these processes often lead to the formation of acidic or anionic compounds. Carboxylic acids can be formed by oxidative deamination of aliphatic amines, as in the metabolism of serotonine and catecholamines, and by the hydrolysis of esters. Aromatic hydroxylation is another important pathway in the metabolism and the hydrophilic character can be further increased by conjugation with sulphuric or glucuronic acid. Alcohols can undergo the same processes. Carboxylic acids can be conjugated with amino acids such as aminoacetic acid (glycine) and aminoethanesulphonic acid (taurine).

Acids with such a hydrophilic nature usually cannot be isolated from the biological material by a simple extraction. As they are ionizable, they can, however, be extracted as ion pairs and their hydrophilic character can then be compensated for by a strongly hydrophobic extracting agent (the counter ion)[1–3]. The extract from the biological material is often contaminated and the required compound must be isolated

by a chromatographic procedure, which can be based on the same distribution principle (ion-pair partition chromatography).

Ion-pair chromatography has been used for the isolation of both cationic and anionic compounds[4–7]. Several studies on acidic compounds of biochemical interest have been published during recent years. Persson and co-workers[8–10] studied the isolation of carboxylic acids and sulphonamides in straight-phase systems. Thorough investigations of the separating conditions for acidic metabolites of biogenic amines have been performed by Lagerström[11]. Persson and Lagerström[12] used the methods for analysis of biological fluids. Wahlund developed systems for reversed-phase liquid–liquid ion-pair chromatography of carboxylates and sulphonates and demonstrated their possibilities for gradient elution and direct application of biological fluids[13,14].

This paper describes a study of the separation of acidic compounds of biochemical and pharmacological interest by reversed- and straight-phase ion-pair chromatography. It is demonstrated that the choice of liquid phases, support and technique for column equilibration can have a considerable influence on the possibilities of separating structurally closely related compounds.

EXPERIMENTAL

Apparatus

The detectors were an LDC Model 1205 UV monitor and Chromatronix Model 200-L photometer, both with 8-μl cells; the measuring wavelength was 254 nm. The pump comprised LDC Models 711-26 and 711-47 solvent delivery system (Milton-Roy Minipump with pulse dampener). The injectors were Altex Scientific and Chromatronix high-pressure valves.

The column was made of 316 stainless steel with a polished surface, length 150 and 200 mm, I.D. 3.2 and 4.5 mm, equipped with modified Swagelok connectors and Varian or Altex stainless-steel frits (2 μm).

Chemicals and reagents

1-Pentanol and 1-butanol were of Fisher Scientific (Pittsburgh, Pa., U.S.A.) A.C.S. quality. Chloroform and methylene chloride were zur Analyse products from E. Merck (Darmstadt, G.F.R.). Butyronitrile, puriss p.a. from Fluka (Buchs, Switzerland), was distilled (117–118°) and then extracted five times with phosphate buffer (pH 7.6) to remove light-absorbing impurities. Tetrapropylammonium and tetrabutylammonium hydrogen sulphate from AB Labkemi (Göteborg, Sweden) were neutralized with sodium hydroxide prior to use. Tetraethylammonium bromide and tetrapentylammonium iodide were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.), the latter being converted prior to use into phosphate by shaking with silver oxide and neutralizing the hydroxide with orthophosphoric acid.

All other substances were of analytical or reagent grade and used without further purification.

Chromatographic supports

LiChrosorb RP-2 and RP-18 and LiChrospher SI 100, mean particle diameter 5 and 10 μm (E. Merck), were used.

Column preparation

The columns were packed by a balanced-density slurry technique[15]. The suspending liquids were dioxan–tetrabromoethane–carbon tetrachloride (7:8:9) for LiChrosorb RP-2, tetrachloroethylene for LiChrosorb RP-18 and tetrabromoethane–carbon tetrachloride (1:1) for LiChrospher SI 100. The fillings were washed with *n*-hexane and acetone before coating with stationary phase.

Organic stationary phase

The hydrophobic supports were spontaneously coated with 1-pentanol (RP-2 and RP-18) and butyronitrile (RP-2) by passage of a mobile phase saturated with the organic solvent at a rate of 0.5–1.0 ml/min[14]. The columns were stable after passage of 30–200 column volumes of mobile phase.

The volume of stationary phase was about 0.5 ml/g of support as determined by elution and gas chromatographic (GC) analysis[14].

Aqueous stationary phase

On the hydrophilic support (LiChrospher SI 100) the stationary phase (aqueous solution of quaternary alkylammonium ions) was applied by *in situ* coating. A 50-ml volume of stationary phase + acetone (3:1) followed by 50 ml of undiluted stationary phase were pumped through the column at a rate of about 1 ml/min. The excess of stationary phase was removed by passage of 50–100 ml of mobile phase and the mobile phase was then recycled.

The capacity factors were usually stable after the passage of 500–1000 ml of mobile phase. As the support usually had adsorbed quaternary alkylammonium ions (*i.e.*, counter ions) from the mobile phase during the equilibration[11], a slight change of the composition of the liquid phase in the reservoir had occurred and the final equilibrium was not reached until a change to fresh liquid phases had been made.

The volume of stationary phase was about 0.6 ml/g of support. It was determined by eluting the column with methanol and titrating the water content by the Karl Fischer method. The content of quaternary alkylammonium ions was determined by the picrate or the bromothymol blue method[16,17].

Chromatographic technique

The whole chromatographic system, including the detector but excepting the pump, was kept in an air thermostat (Termaks, Bergen, Norway) at 25.0 ± 0.1°. The mobile and stationary phases were carefully equilibrated with each other in the thermostat before use and an upper layer of stationary phase was always present in the reservoir. No pre-columns were used.

A slow degradation of the butyronitrile was observed. It gave no disturbances if a change to fresh mobile phase was made every third day.

Both types of systems (with either aqueous or organic mobile phase) showed a very high stability and no change of properties has been observed after use for several months.

In systems with an aqueous mobile phase, an LDC detector with cooling channels in the cell holder was used. It permitted thermostatting of the cuvette to 20.0° (ref. 13).

All samples were dissolved in the mobile phase. In systems with an organic

mobile phase the samples were applied as acids or ion pairs with the counter ion used in the chromatographic system, 8–30 μl of sample solution being injected.

RESULTS AND DISCUSSION

Ion-pair partition chromatography is based on a liquid–liquid distribution technique usually called ion-pair extraction, which can be applied to all kinds of ionizable organic substances, both cationic and anionic. The principle is demonstrated by the following example.

An organic anion, X^-, can be transferred from an aqueous to an organic phase by addition to the aqueous phase of an ion Q^+ with the opposite charge and extraction of the ion pair QX into the organic solvent:

$$X^-_{aq} + Q^+_{aq} = QX_{org}$$

A quantitative expression for the extraction is given by the equilibrium constant, E_{QX} (the extraction constant):

$$E_{QX} = [QX]_{org} \cdot [Q^+]^{-1}_{aq} \cdot [X^-]^{-1}_{aq} \quad (1)$$

The distribution of X^- between the two phases is given by the distribution ratio expression:

$$D_X = [QX]_{org}/[X^-]_{aq} = E_{QX} \cdot [Q^+]_{aq} \quad (2)$$

This shows how the distribution can be regulated: by the nature of the counter ion Q^+ and the properties of the organic phase which both affect the magnitude of E_{QX}, and by the concentration of Q^+ (ref. 1).

Control of the retention

The ion-pair extraction principle can be used in partition chromatography with an aqueous solution as the mobile phase and an organic solvent as the stationary phase (reversed-phase system) or *vice versa* (straight-phase system). The counter ion is added to the aqueous solution in both instances.

When the counter ion Q^+ is present in the aqueous stationary phase, the capacity factor, k', of the sample X^- is given by

$$k' = (E_{QX} \cdot [Q^-]_{aq})^{-1} \cdot V_s \cdot V_m^{-1} \quad (3)$$

and in systems with Q^+ in the aqueous mobile phase

$$k' = E_{QX} \cdot [Q^+]_{aq} \cdot V_s \cdot V_m^{-1} \quad (4)$$

where $V_s \cdot V_m^{-1}$ is the phase volume ratio.

The relationship between k', $[Q^+]$ and E_{QX} is illustrated in Fig. 1. In a straight-phase system (Fig. 1A), the retention will increase with decreasing concentration of the counter ion Q^+ and decreasing value of the extraction constant E_{QX}. To obtain a capacity factor of 1–10 at a counter ion concentration between 0.1 and 0.01 M, an

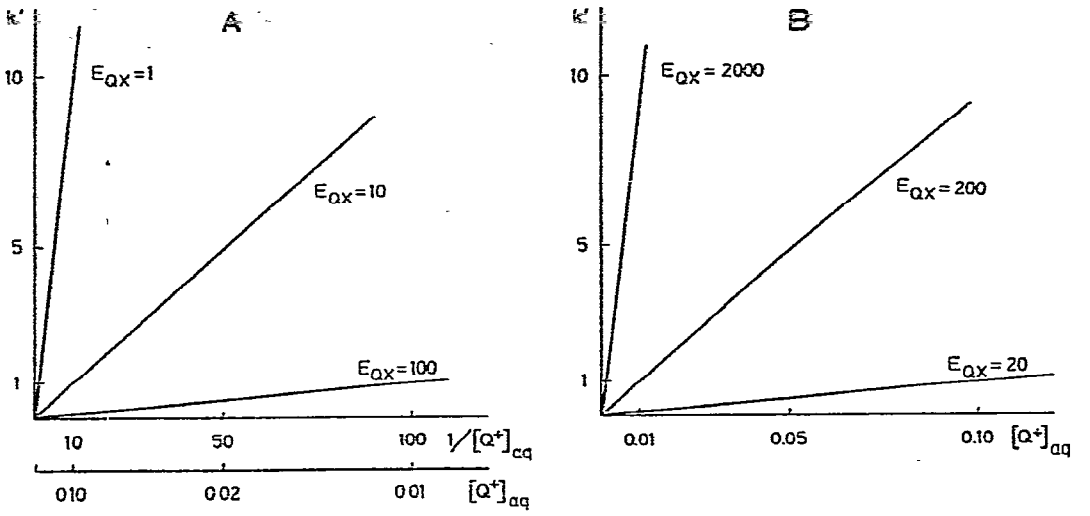

Fig. 1. Regulation of the retention of an anionic compound by the extraction constant (E_{QX}) and the concentration of the counter ion, Q^+, in (A) straight-phase and (B) reversed-phase ion-pair chromatography. V_s/V_m: A, 1.0; B, 0.5.

extraction constant of 1–100 will be required. The reversed-phase system (Fig. 1B) will need an extraction constant of 20–2000 under the same conditions.

The extraction constant can be easily regulated within these limits by the choice of counter ion. Some examples are given in Table I[18], which gives extraction constants of some carboxylic acids, glucuronides and sulphates with chloroform as the organic phase and quaternary alkylammonium ions as counter ions.

Quaternary alkylammonium counter ions have several advantages. They are aprotic and can be used at any pH. The regulation of E_{QX} is easily made by changing

TABLE I

EXTRACTION CONSTANTS[18]

Organic phase: chloroform.

Anionic component	*Log E_{QX}*		
	Tetrapropyl-ammonium	*Tetrabutyl-ammonium*	*Tetrapentyl-ammonium*
Benzoic acid	—	—	2.83
Hippuric acid	—	—	2.65
N-Methylhippuric acid	—	—	2.55
Phenylpropyl 3-sulphate	1.95	4.20	—
2-Naphthyl sulphate	2.64	4.90	—
8-Quinolinyl glucuronide	—	—	1.43
2-Naphthyl glucuronide	—	—	2.33
11-Deoxycorticosterone 21-glucuronide	—	2.08	4.50
11-Deoxycortisol 21-glucuronide	—	1.21	3.66
11-Dehydrocorticosterone 21-glucuronide	—	0.83	3.17
Cortisone 21-glucuronide	—	0.52	2.95

the number of alkyl carbon atoms: removal of one methylene group will decrease log E_{ox} by about 0.5 unit in most organic phases.

Table I also demonstrates that the glucuronic acid conjugates are considerably more hydrophilic than the sulphates. A comparison of the conjugates of 2-naphthol shows that the glucuronide requires a much more hydrophobic (eight alkyl carbons larger) counter ion than the sulphate in order to achieve the same extraction constant.

The extraction of an ion pair can be affected by side-reactions such as protolysis or association processes in the aqueous phase and dissociation or dimerization of the ion pair in the organic phase[2]. The side-reactions will affect the capacity factor, but if the effect is independent of the concentration of the sample it will not have any unfavourable influence on the chromatographic process. If the extent of the side-reaction changes with the concentration of the sample, it will usually give rise to more or less serious peak asymmetry and steps must be taken to avoid or suppress the disturbing process[6,7,13].

Choice of chromatographic conditions

The extraction constant is usually a good basis for the choice of a system for a chromatographic separation. It can often be easily determined by batch extraction between free liquid phases[2,19]. If, however, a rough estimation of the partition properties of the sample can be made from published data, chromatographic test runs may be a more convenient way.

Such tests are most easily made in reversed-phase systems where the counter ion is present in the mobile aqueous phase. A suitable k' value can be obtained in these systems by changing the concentration or/and the nature of the counter ion. An illustration is given in Fig. 2, which shows the relationship between k' and the concentration of the counter ion (tetrabutylammonium) for 4-hydroxybenzoate when 1-pentanol on LiChrosorb RP-2 is used as the stationary phase. For comparison, capacity factors are calculated from distribution ratios obtained by batch extractions. The found

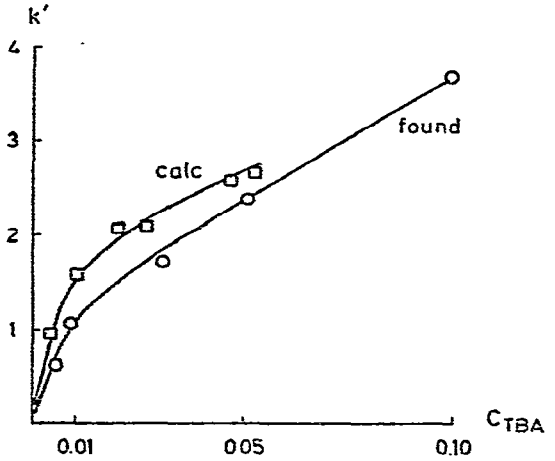

Fig. 2. Regulation of the retention of 4-hydroxybenzoic acid. Mobile phase, tetrabutylammonium (pH 7.4); stationary phase, 1-pentanol; support, LiChrosorb RP-2.

capacity factors deviate only 10–30% from the calculated values, which indicates that the influence of the hydrophobic support is rather small.

The relationship between k' and the counter ion concentration (Fig. 2) deviates from the earlier expressions (eqn. 4) by being non-linear. This result is due to side-reactions that are not taken into consideration in eqn. 4, probably association processes in the aqueous phase between tetrabutylammonium and the sample anion as well as anions of the buffer components.

In a straight-phase system where the counter ion is present in the stationary phase, regulation of the retention by changing the counter ion concentration is more complicated, as it will include a change of the coating of the support. The final regulation of the capacity factor in such systems is usually made by the composition of the mobile organic phase. In such instances it is often favourable to use an organic phase that consists of one weakly and one strongly solvating agent (*e.g.*, a strongly hydrogen-bonding solvent). The interaction between the ion pair and the strongly solvating agent can then be treated as a complexation which will lead to simple means for systematic regulation of the retention[20,21]. It must be emphasized, however, that a change in the composition of the mobile phase can also change the selectivity of the system[22–24]; this is not so when the capacity factor is regulated by changing the counter ion concentration.

Reversed-phase systems

Studies with aqueous mobile phases have so far been performed on two kinds of porous silica microparticles: LiChrosorb RP-2, which is partly hydrophobized with dichlorodimethylsilane, and LiChrosorb RP-18, which is a hydrophobic material of the octadecyl type. Two stationary phases have been tested: 1-pentanol, which is both hydrogen-donating and hydrogen-accepting, and butyronitrile, which is only hydrogen-accepting.

The separating efficiency of the columns was good. An example is given in Fig. 3, which shows the separation of benzoic and benzenesulphonic acid derivatives with tetrabutylammonium as the counter ion. The selectivity is so good that even positional isomers of hydroxy- and aminobenzoic acid are well separated.

A comparison of the selectivity of 1-pentanol and butyronitrile as stationary phases is given in Fig. 4. The differences are rather limited. The weakly hydrogen-accepting nitrile gives a better separation of the unsubstituted benzoic and benzenesulphonic acids while the selectivity for the amino- and hydroxybenzoic acids is lower.

The influence of the support on the separation cannot be disregarded. A comparison of the separation factors on the highly hydrophobic RP-18 and the partly hydrophobized RP-2 (Fig. 4) shows that the benzoates on the latter support are more retarded relative to the sulphonates. The influence of the support was particularly marked for some of the dihydroxylated acids: 3,4-dihydroxyphenylacetic acid on RP-2 gave very strong tailing, which was eliminated on RP-18.

Chromatographic studies have also been performed with a pure aqueous solution of the counter ion as the mobile phase and no organic solvent present in the system. A very low separating efficiency was obtained, as expected, with a highly hydrophobic octadecyl-type support that was not wetted by the mobile phase. The partly hydrophobized RP-2 support gave columns with good efficiency, as demonstrated by the chromatogram in Fig. 5.

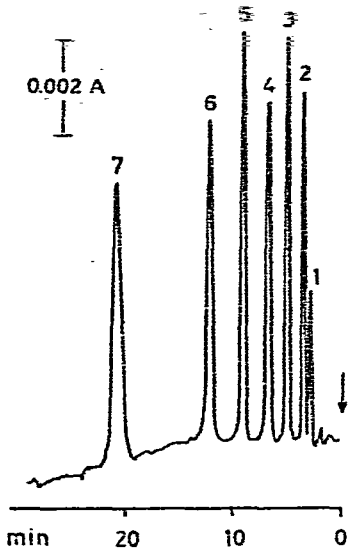

Fig. 3. Reversed-phase ion-pair chromatography. Mobile phase, tetrabutylammonium, 0.03 *M* (pH 7.4), 1.7 mm/sec, 95 bar; stationary phase, 1-pentanol; support, LiChrosorb RP-2 (5 μm). Peaks: 1 = 4-aminobenzoic acid; 2 = 3-aminobenzoic acid; 3 = 4-hydroxybenzoic acid; 4 = 3-hydroxybenzoic acid; 5 = benzenesulphonic acid; 6 = benzoic acid; 7 = toluene-4-sulphonic acid.

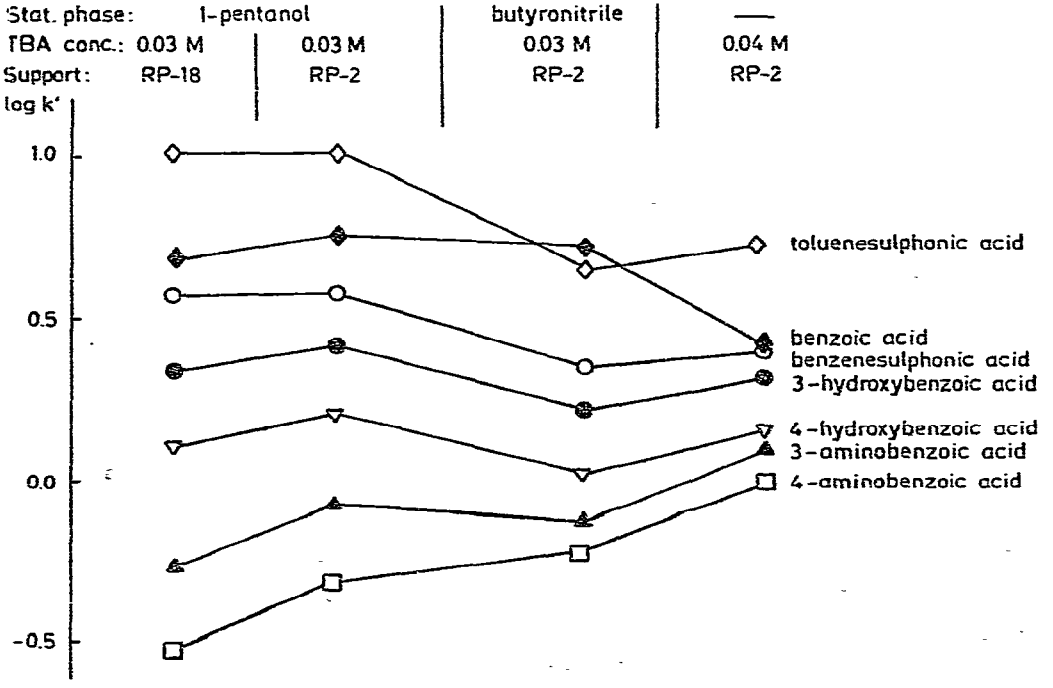

Fig. 4. Influence of support and stationary phase on the selectivity by reversed-phase ion-pair chromatography. Mobile phase, tetrabutylammonium (pH 7.9).

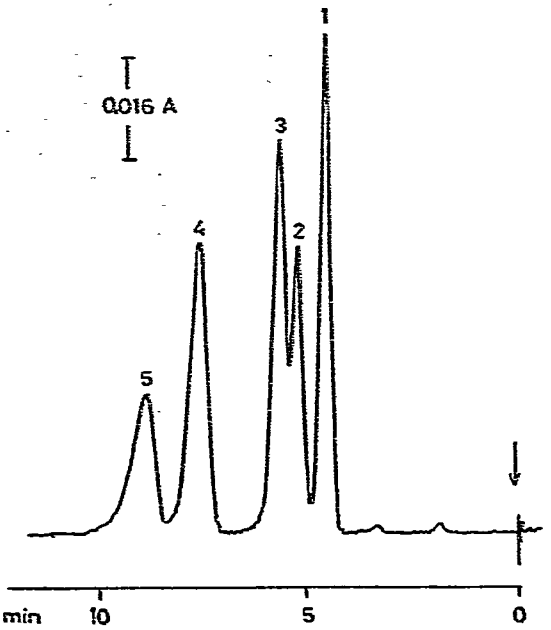

Fig. 5. Reversed-phase ion-pair chromatography without liquid stationary phase. Mobile phase, tetrabutylammonium, 0.03 *M* (pH 7.4), 1.0 mm/sec, 68 bar; support, LiChrosorb RP-2 (10 μm). Peaks: 1 = 4-aminobenzoic acid; 2 = 3-aminobenzoic acid; 3 = 4-hydroxybenzoic acid; 4 = 3-hydroxybenzoic acid; 5 = benzoic acid.

The capacity factor could be controlled by the counter ion concentration up to 0.04 *M* on both supports, but the selectivity was lower than for systems with 1-pentanol or butyronitrile as stationary phase, as demonstrated in Fig. 4. An important drawback of the RP-2 system was its low stability; k' decreased gradually and the column showed an increasing flow resistance. The columns could usually be used only for 2 or 3 weeks.

The high selectivity of the pentanol-containing systems for anions with hydrophilic substituents made them particularly useful for the separation of carboxylic acids of biochemical interest. A separation of homovanillic, indoleacetic and hydroxyindoleacetic acid is demonstrated in Fig. 6, and a survey of the selectivity for derivatives of phenylacetic, mandelic, indoleacetic and benzoic acid is given in Fig. 7.

Figs. 6 and 7 show that not only the type and nature of a substituent but also its position has a considerable influence on the retention. Some of the given k' values are rather low but it must be emphasized that k' can be increased by increasing the counter ion concentration without changing the selectivity.

Hydroxyl substitution will usually give rise to a large decrease in k'. Hydroxylation of IAA in the aromatic ring to HIAA considerably decreases its hydrophobic character and a separation factor of 3.8 is obtained. Hydroxyl substitution in the α-position to a carboxyl group will have a lesser effect: hydroxylation of HVA in the side-chain to VMA gives a separation factor of only 2.2. The effect of the position of the hydroxyl substitution is particularly marked for the dihydroxybenzoic acids, the 2,5-substituted compound being considerably more hydrophobic than the others.

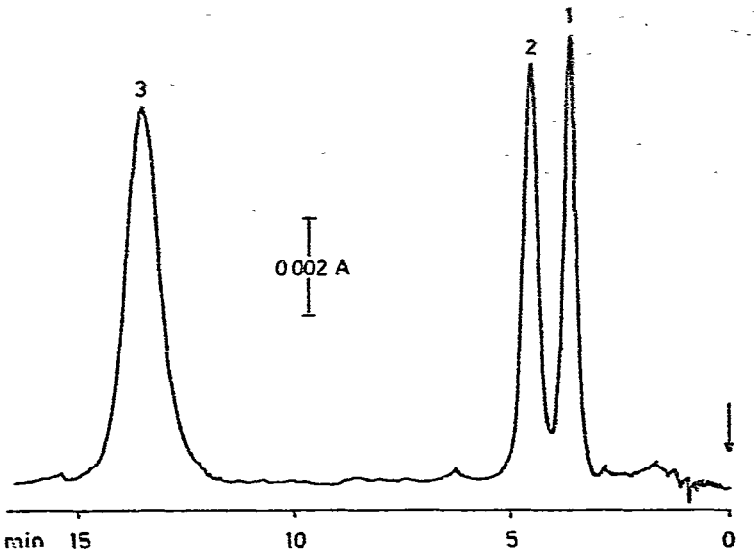

Fig. 6. Reversed-phase ion-pair chromatography. Mobile phase, tetrabutylammonium, 0.03 M (pH 7.4), 2.1 mm/sec, 60 bar; stationary phase, 1-pentanol; support, LiChrosorb RP-2 (10 μm). Peaks: 1 = 4-hydroxy-3-methoxyphenylacetic acid (HVA); 2 = 5-hydroxyindole-3-acetic acid (HIAA); 3 = indole-3-acetic acid (IAA).

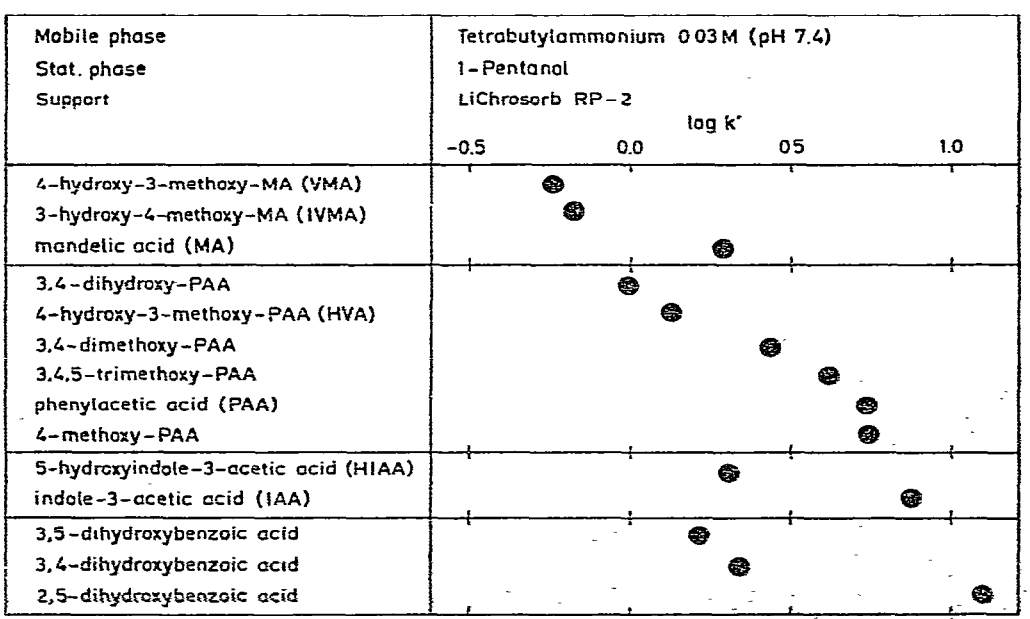

Fig. 7. Selectivity by reversed-phase chromatography of derivatives of mandelic, phenylacetic, indoleacetic and benzoic acid.

The influence of methoxylation of phenylacetic acid is also noteworthy, k' increasing in the order dimethoxy < trimethoxy < monomethoxy.

The pentanol- and butyronitrile-containing systems are also highly suitable for the separation of sulphonamides. A chromatogram obtained with butyronitrile as the stationary phase is given in Fig. 8. The sulphonamides are weak acids with $pK_a = 6–8$ and, under the conditions used, they will migrate as both acids and ion pairs. A comparison of the selectivity by chromatography as acids and ion pairs is given in Fig. 9. There is a drastic change of the elution order and changes in either pH or counter ion concentration can have a considerable influence on the selectivity.

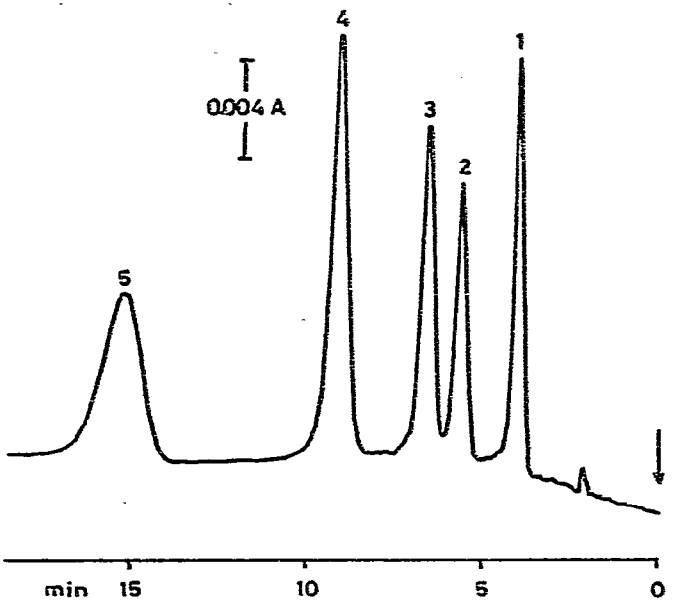

Fig. 8. Reversed-phase ion-pair chromatography of sulphonamides. Mobile phase, tetrabutylammonium, 0.01 *M* (pH 7.9), 1.6 mm/sec, 27 bar; stationary phase, butyronitrile; support, LiChrosorb RP-2 (10 μm). Peaks: 1 = 2-sulphanilamidopyrimidine (sulphadiazine); 2 = 3-methoxy-2-sulphanilamidopyrazine (sulphalene); 3 = 4-methyl-2-sulphanilamidopyrimidine (sulphamerazine); 4 = 5-methyl-3-sulphanilamidoisoxazole (sulphamethoxazole); 5 = 2,4-dimethyl-6-sulphanilamidopyrimidine (sulphaisodimidine).

The N^4-acetyl derivative is the main metabolite of the sulphonamides. It can be easily separated from the parent compound by this chromatographic system, as demonstrated in Fig. 10 for sulphametoxazole and sulphadiazine.

The reversed-phase systems have also been used successfully for the separation of barbiturates. They are weaker acids than most sulphonamides, with $pK_a = 7.5–8.5$, and rather hydrophobic, which means that ion-pair chromatography can be performed only at a high pH. The separation can, however, be effected with the barbiturates partly in acidic form and regulation of the retention mainly by pH. An example is given in Fig. 11. Changing the stationary phase from butyronitrile to pentanol has no drastic influence on the selectivity.

Studies of the separating efficiency for different kinds of substances have shown some differences between 1-pentanol and butyronitrile columns. The 1-pentanol

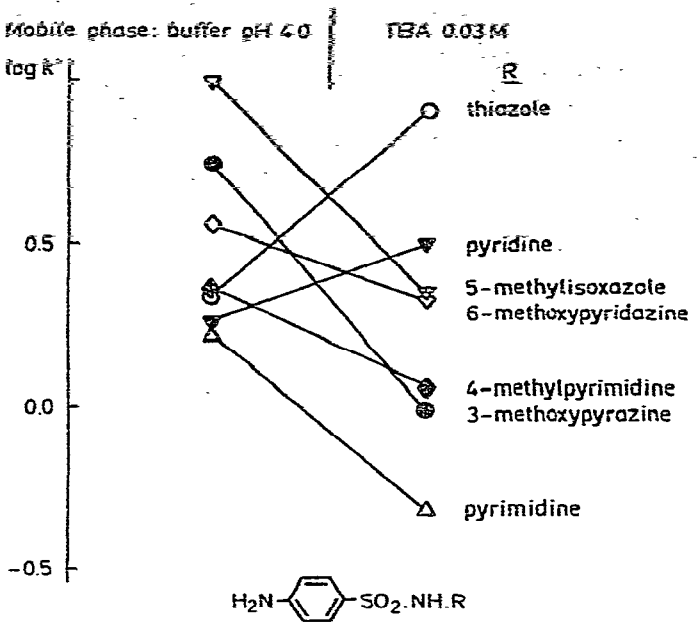

Fig. 9. Selectivity by reversed-phase chromatography of sulphonamides as acids (buffer, pH 4.0) and as ion pairs with tetrabutylammonium (the latter k' are calculated from k' found at pH 7.4 after compensation for distribution as acid and degree of protolysis). Stationary phase, 1-pentanol; support, LiChrosorb RP-2.

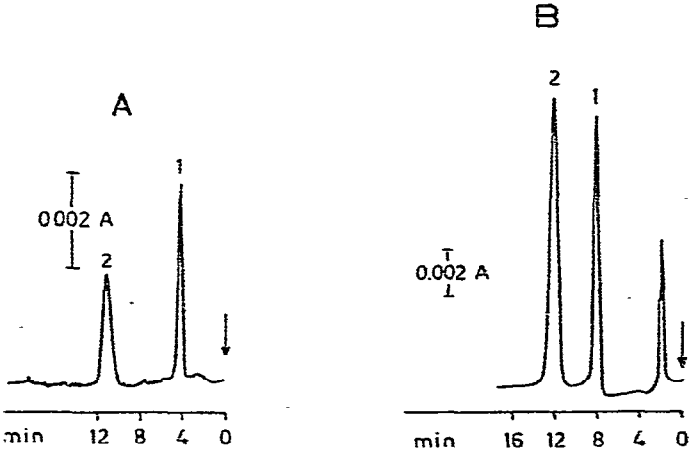

Fig. 10. Separation of parent sulphonamides (1) and N^4-acetyl derivatives (2). Support, LiChrosorb RP-2 (10 μm). (A) Sulphadiazine. Mobile phase, tetrabutylammonium, 0.1 M (pH 7.4), 2.2 mm/sec, 70 bar; stationary phase, 1-pentanol. (B) Sulphamethoxazole. Mobile phase, tetrabutylammonium, 0.03 M (pH 7.9), 1.6 mm/sec, 60 bar; stationary phase, butyronitrile.

columns have a good separating efficiency (with maximum H at $k' = 1$)[13] that is almost independent of the type of substance involved. The butyronitrile columns have the advantage of an equally good and less flow-dependent separating efficiency for sulphonamides and barbiturates, but a significantly higher H has been obtained with the benzoates.

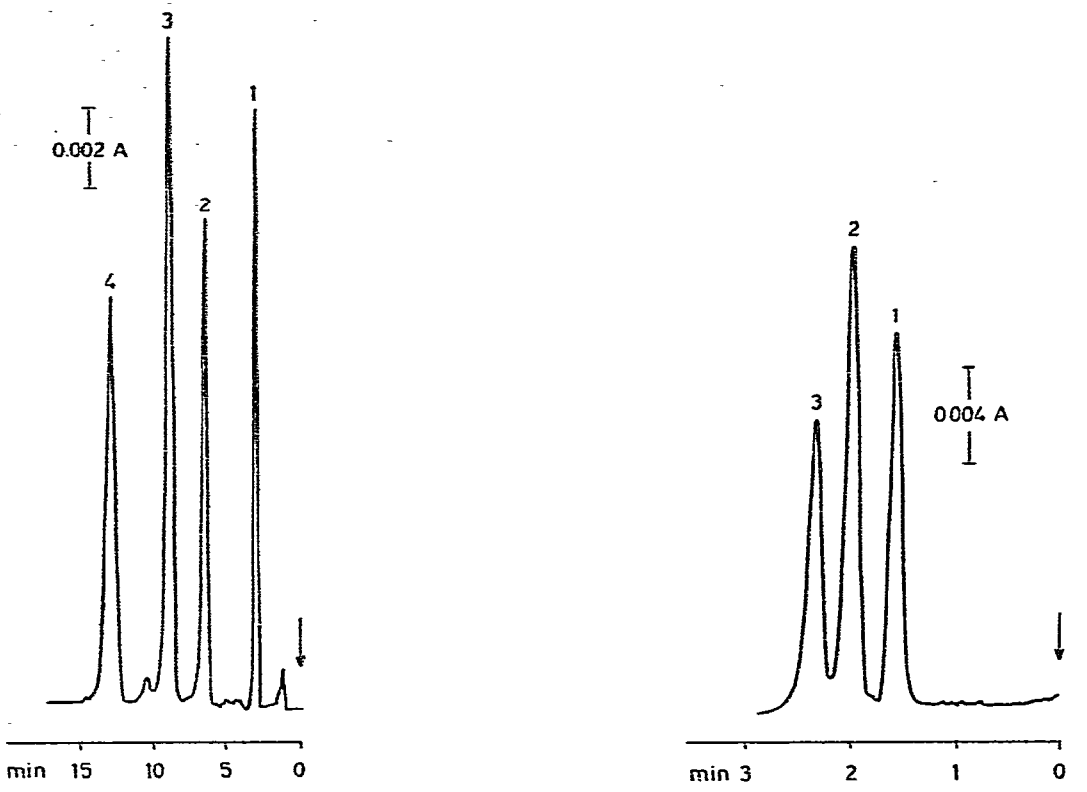

Fig. 11. Reversed-phase chromatography of barbiturates. Mobile phase, tetrabutylammonium, 0.01 *M* (pH 7.7), 1.6 mm/sec, 27 bar; stationary phase, butyronitrile; support, LiChrosorb RP-2 (10 μm). Peaks: 1 = diethylbarbituric acid; 2 = diallylbarbituric acid; 3 = allylisopropylbarbituric acid; 4 = phenylethylbarbituric acid.

Fig. 12. Straight-phase ion-pair chromatography of sulphuric acid conjugates of phenols. Mobile phase, dichloromethane–1-pentanol (9:1), 3.0 mm/sec, 37 bar; stationary phase, tetraethylammonium bromide, 0.1 *M* (applied) pH 7.4, 0.65 ml/g on LiChrospher SI 100 (10 μm). Peaks: 1 = 6-bromo-2-naphthyl sulphate; 2 = 2-naphthyl sulphate; 3 = 4-methylumbelliferyl sulphate.

Straight-phase systems

In straight-phase ion-pair chromatography on porous silica microparticles, the aqueous phase is usually applied by *in situ* coating[8]. The loading, *i.e.* the volume of aqueous phase per gram of support, has usually not been considered as critical. However, by use of quaternary alkylammonium ions as counter ions, it was observed that a change in the loading can have a drastic influence on the properties of the system.

The studies were performed with LiChrospher SI 100 as the support. When it was coated with the maximal amount of stationary phase (about 60% of the support weight), systems with high separating efficiency and selectivity were obtained. An example is given in Fig. 12, which shows the separation of three aryl sulphates. The separating efficiency is demonstrated in Fig. 13. A maximum H is obtained at $k' = 0.3–1.2$, but up to a flow-rate of 3 mm/sec it is still not greater than 0.1 mm.

If the amount of stationary phase on the support is below the maximum, owing

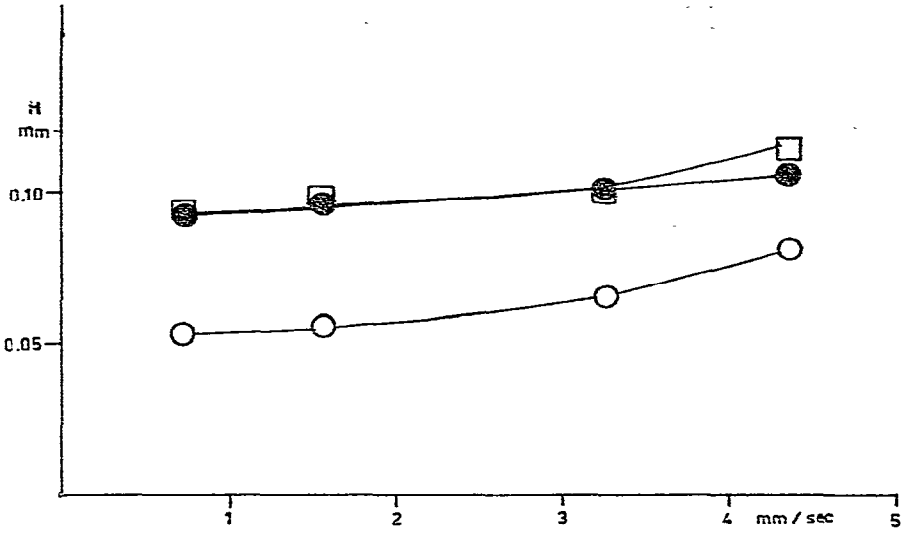

Fig. 13. Effect of capacity factor and flow-rate on the column efficiency by straight-phase ion-pair chromatography. Mobile phase, dichloromethane–1-pentanol (9:1); stationary phase, tetraethylammonium bromide, 0.1 *M* (applied), pH 7.4, 0.65 ml/g on LiChrospher SI 100 (10 μm). Samples: ○, 3-estradiol sulphate ($k' = 0.3$); ●, 17-estradiol sulphate ($k' = 0.9$); □, 3-estriol sulphate ($k' = 5.7$).

either to incomplete coating or equilibration with imperfectly saturated mobile phase, considerable changes in the column properties will occur. The column still has a good separating efficiency, as demonstrated in a separation of glucuronides shown in Fig. 14. The capacity factors found were 10^2–10^5 times higher than those calculated from extraction constants obtained by batch extraction experiments, and the separation factors were considerably lower than expected.

A comparison of the properties of the systems is given in Fig. 15, which demonstrates the separation of estrogen sulphates. With a high loading of stationary phase, 0.6 ml/g of support (Fig. 15A), a suitable retention was obtained with tetraethylammonium as counter ion. By use of a low loading, 0.13 ml/g of support (Fig. 15B), a much more hydrophobic counter ion, tetrapropylammonium, had to be used and the retention of the estradiol and estriol sulphates was considerable. Some changes in the elution order were also obtained.

A survey of the retentions of different types of sulphuric acid conjugates in the two systems is given in Fig. 16. The selectivity is considerably better on the column with high loading: a change of the point of conjugation from 3 to 17 gives a separation factor of 2.8 for the estradiols and 4.2 for the estriols. Bromine substitution in naphthyl sulphate gives a separation factor of 3.5. The agreement between the found and calculated k' values is good.

The difference between columns with high and low loadings was even greater in the systems for the separation of glycine and glucuronic acid conjugates, as demonstrated in Fig. 17. In the system with high loading, tetrapropylammonium was suitable as counter ion. The agreement between found and calculated capacity factors was good. The separation factors between glycine conjugates and parent compounds

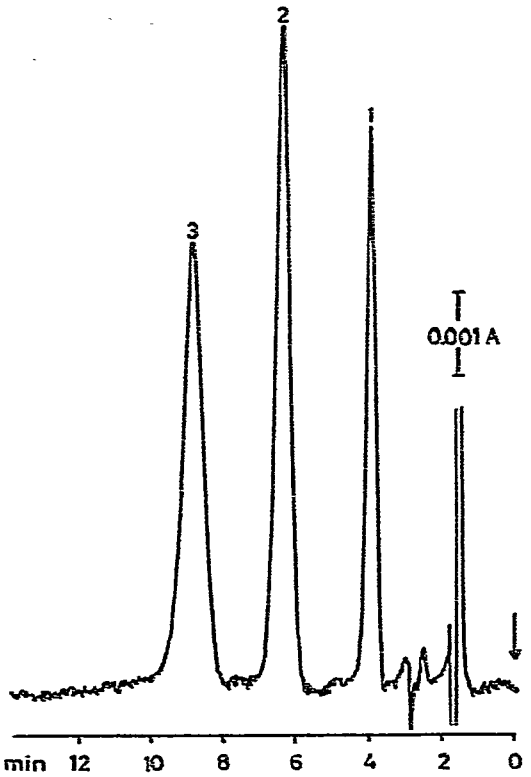

Fig. 14. Straight-phase ion-pair chromatography of glucuronic acid conjugates. Mobile phase, chloroform–1-butanol (19:1), 1.6 mm/sec, 35 bar; stationary phase, tetrapentylammonium, 0.065 *M* (applied) + bromide, 0.001 *M*, pH 7.4, 0.11 ml/g on LiChrospher SI 100 (10 μm). Peaks: 1 = 8-quinolinylglucuronic acid; 2 = 2-naphthylglucuronic acid; 3 = 4-methylumbelliferylglucuronic acid.

is of the order of 2–4. On the column with low loading, the considerably more hydrophobic tetrapentylammonium had to be used as counter ion and the separation factors found were very low in most instances.

It is of particular interest that the order of elution was reversed in numerous instances and the retardation on columns with low loading increased with increasing hydrophobic character of the sample. An investigation of the composition of the stationary phase showed that on columns with low loading, the content of quaternary ammonium ions was 7–9 times higher than that applied. The increase obviously occurred during the equilibration with the mobile phase, which contained 10^{-5}–10^{-4} mol/l of quaternary ammonium ions. It seems likely that the quaternary ammonium ions had been adsorbed on the support, giving the surface a hydrophobic character. The ion-exchange properties of silica gels in the pH range used has earlier been demonstrated[25] as well as the adsorption of quaternary ammonium ions to glass surfaces from aqueous solution[26,27].

An increase in the counter ion content was also observed on the columns with high loading, but it had no significant effect on the ion-pair partition process, as k'_{found} and k'_{calc} showed good agreement when the calculation was based on the applied concentration of counter ion[11].

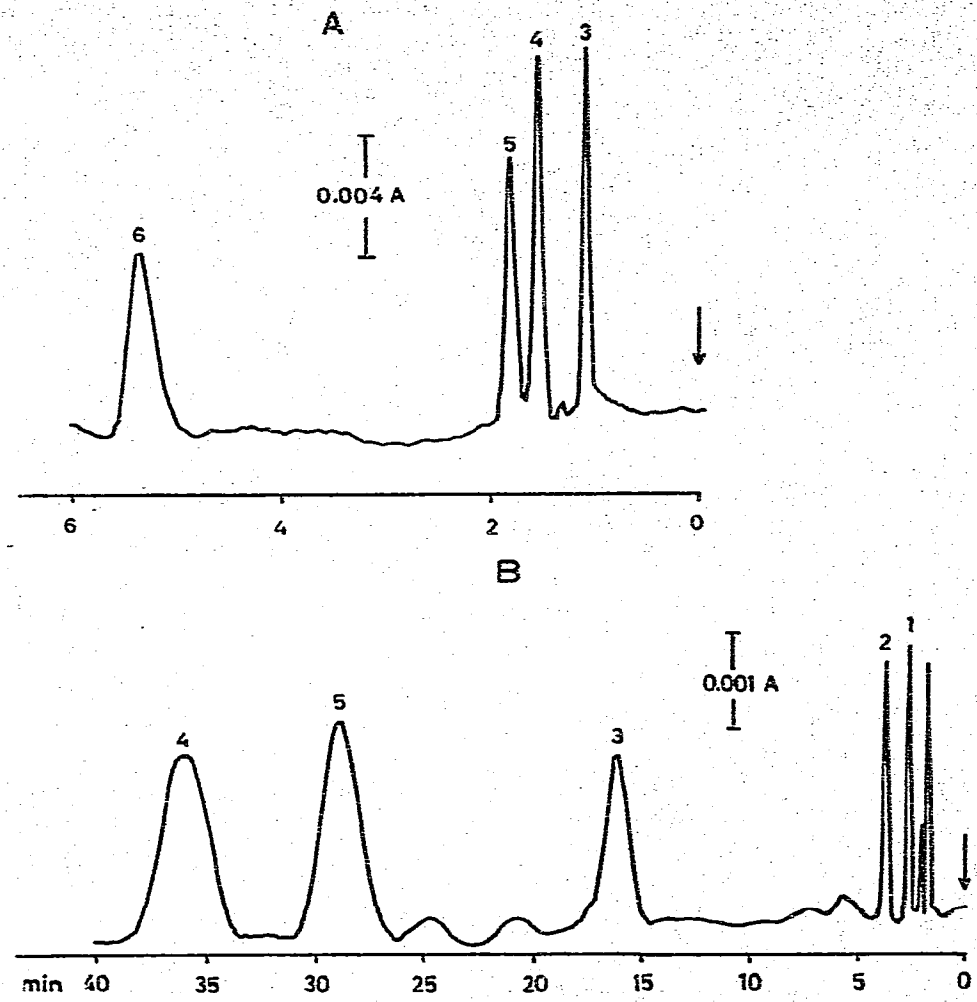

Fig. 15. Straight-phase ion-pair chromatography of steroidal sulphuric acid conjugates. Support, LiChrospher SI 100 (10 μm). (A) Mobile phase, dichloromethane–1-pentanol (9:1), 3.0 mm/sec, 30 bar; stationary phase, tetraethylammonium bromide, 0.1 *M* (applied), pH 7.4, 0.65 ml/g of support. (B) Mobile phase, chloroform–1-butanol (9:1), 1.5 mm/sec, 40 bar; stationary phase, tetrapropylammonium, 0.1 *M* (applied) + bromide, 0.001 *M*, pH 7.4, 0.13 ml/g of support. Peaks: 1 = 3-equilin sulphate; 2 = 17α-dihydro-3-equilin sulphate; 3 = 3-estradiol sulphate; 4 = 17-estradiol sulphate; 5 = 17-estriol sulphate; 6 = 3-estriol sulphate.

These results show that well defined conditions are of fundamental importance for the reproducibility of the chromatographic results. Not only the composition of the stationary and mobile phases, but also the stationary phase loading and the equilibration conditions must be examined thoroughly.

Comparison of reversed- and straight-phase ion-pair chromatography

Both reversed- and straight-phase ion-pair chromatography will give good possibilities for the separation of anionic organic compounds under the conditions given

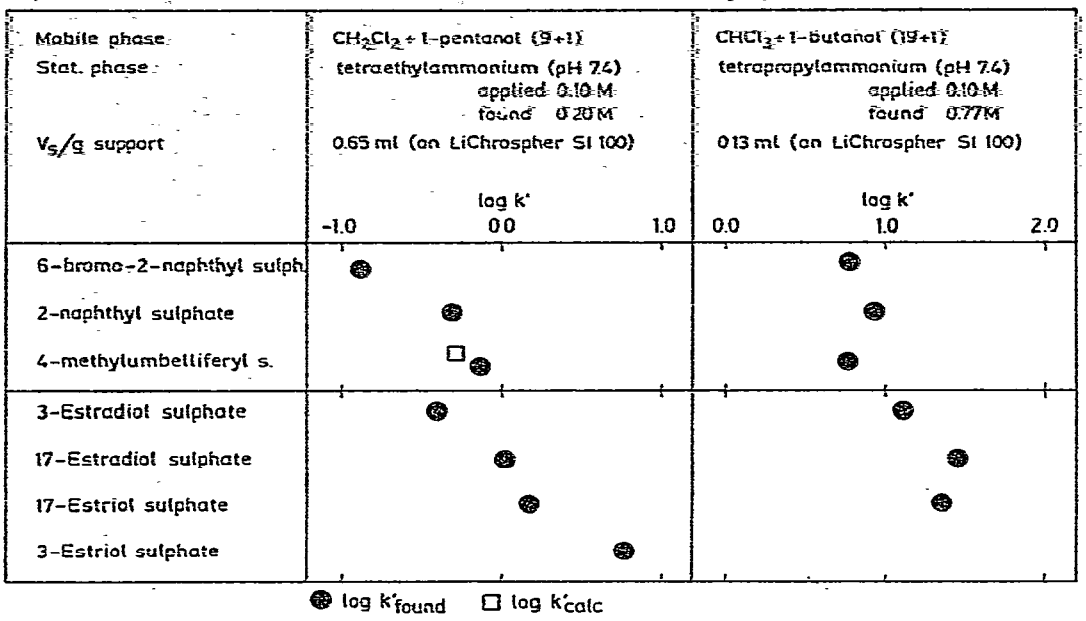

Fig. 16. Selectivity by straight-phase ion-pair chromatography of sulphuric acid conjugates (k'_{calc} for 4-methylumbelliferyl sulphate based on log $E_{ox} = 1.26$).

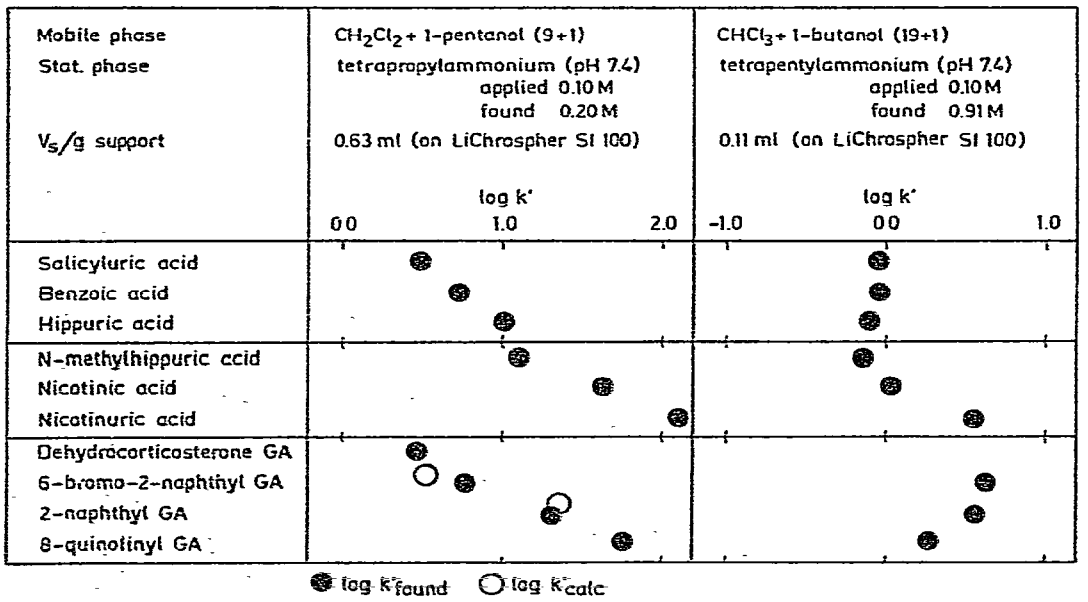

Fig. 17. Selectivity by straight-phase ion-pair chromatography of glucuronic acid (GA) conjugates (k'_{calc} based on log $E_{ox} = -0.38$ for 2-naphthyl-GA and log $E_{ox} = 0.51$ for 6-bromo-2-naphthyl-GA).

above. The reversed-phase technique has the advantage of easy control of the retention by changing the type and concentration of the counter ion in the mobile phase. Gradient elution can be performed in such systems without the risk of stripping off the stationary phase[13]. A further advantage is that hydrophilic samples and samples present in aqueous solution, *i.e.* biological material, can be dissolved directly in the mobile phase[14]. The choice of organic phase is at present limited, as few systems have been thoroughly investigated.

The straight-phase systems have the advantage of a greater choice of organic phase, which may give the possibility of higher selectivity[21]. The retention can be regulated by the composition of the mobile phase, but this can have an unfavourable influence on the stability of the system owing to changes in the mutual solubility of the phases. The sample must be transferred to the organic mobile phase, which might present difficulties for hydrophilic substances. These problems, however, can usually be solved by using a modified extraction technique[12,28].

Both the reversed- and straight-phase techniques seem to be necessary as a means of solving the intricate separation problems within the bioanalytical field.

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REFERENCES

1 G. Schill, in J. A. Marinsky and Y. Marcus (Editors), *Ion Exchange and Solvent Extraction*, Vol. 6, Marcel Dekker, New York, 1974, p. 1.
2 R. Modin and G. Schill, *Acta Pharm. Suec.*, 4 (1967) 301.
3 R. Modin and A. Tilly, *Acta Pharm. Suec.*, 5 (1968) 311.
4 S. Eksborg and B. A. Persson, *Acta Pharm. Suec.*, 8 (1971) 205.
5 K.-G. Wahlund and K. Gröningsson, *Acta Pharm. Suec.*, 7 (1970) 615.
6 S. Eksborg and G. Schill, *Anal. Chem.*, 45 (1973) 2092.
7 S. Eksborg, P.-O. Lagerström, R. Modin and G. Schill, *J. Chromatogr.*, 83 (1973) 99.
8 B. A. Persson and B. L. Karger, *J. Chromatogr. Sci.*, 12 (1974) 521.
9 B. L. Karger, S. C. Su, S. Marchese and B. A. Persson, *J. Chromatogr. Sci.*, 12 (1974) 678.
10 S. C. Su, A. V. Hartkopf and B. L. Karger, *J. Chromatogr.*, 119 (1976) 523.
11 P. O. Lagerström, *Acta Pharm. Suec.*, 13 (1976) 213.
12 B. A. Persson and P.-O. Lagerström, *J. Chromatogr.*, 122 (1976) 305.
13 K.-G. Wahlund, *J. Chromatogr.*, 115 (1975) 411.
14 K.-G. Wahlund and U. Lund, *J. Chromatogr.*, 122 (1976) 269.
15 R. E. Majors, *Anal. Chem.*, 44 (1972) 1722.
16 K. Gustavii and G. Schill, *Acta Pharm. Suec.*, 3 (1966) 241.
17 G. Schill, *Acta Pharm. Suec.*, 2 (1965) 13.
18 B. Fransson and G. Schill, *Acta Pharm. Suec.*, 12 (1975) 107.
19 R. Modin and G. Schill, *Acta Pharm. Suec.*, 7 (1970) 585.
20 B. A. Persson, *Acta Pharm. Suec.*, 5 (1968) 343.
21 R. Modin and G. Schill, *Talanta*, 22 (1975) 1017.
22 K. O. Borg, M. Gabrielsson and T. Jönsson. *Acta Pharm. Suec.*, 11 (1974) 313.
23 M. Schröder-Nielsen, *Acta Pharm. Suec.*, 11 (1974) 541.
24 M. Schröder-Nielsen, *Acta Pharm. Suec.*, 13 (1976) 145.
25 D. N. Strazhesko, V. B. Strelko, V. N. Belyakov and S. C. Rubanik, *J. Chromatogr.*, 102 (1974) 191.
26 L. C. F. Blackman and R. Harrop, *J. Appl. Chem.*, 18 (1968) 37.
27 L. C. F. Blackman and R. Harrop, *J. Appl. Chem.*, 18 (1968) 43.
28 S. Eksborg and G. Schill, *Acta Pharm. Suec.*, 12 (1975) 1.