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# REVERSED-PHASE ION-PAIR PARTITION CHROMATOGRAPHY OF CARBOXYLATES AND SULPHONATES

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## SUMMARY

A reversed-phase partition chromatographic system for separation of organic anions as ion pairs with quaternary ammonium ions has been developed. Commercial, hydrophobized silica supports are used with 1-pentanol as stationary phase and aqueous solutions of tetrabutylammonium (TBA) as mobile phase. The separation of aromatic sulphonates and benzoic acid derivatives is demonstrated. The use of the TBA concentration of the mobile phase to regulate the capacity factor of the anions, as a means of improving separation by gradient elution and direct injection of large sample volumes, is demonstrated. The isolation of nicotinic acid from human serum samples is shown.

## INTRODUCTION

Reversed-phase liquid–liquid partition chromatography, as described by Howard and Martin[1], has found rather limited application, due mainly to the fact that it has previously used columns of low efficiency and stability (*cf.* ref. 2). Recent studies have shown, however, that highly efficient columns can be prepared by application of modern chromatographic techniques. Horwitz and Bloomquist[3] have obtained good columns with bis-(2-ethylhexyl)phosphoric acid as stationary phase, while Kraak and Huber[4] have prepared high-efficiency columns with trioctylamine as stationary phase. In both cases aqueous mobile phases were used.

Reversed-phase, ion-pair, column partition chromatography was first studied by Wahlund and Gröningsson[5] with organic ammonium ions as substrates. Eksborg *et al.*[6] have reported on the reversed-phase chromatography of aminophenols as ion-pair adducts with bis-(2-ethylhexyl)phosphoric acid. In both cases the column efficiency was rather low.

In the present study, the above difficulties have been overcome by the use of a modern technique of column preparation. Systems with high separating efficiency and long-term stability have been obtained by the use of small-diameter supports and an improved packing and coating technique.

The combination of reversed-phase liquid–liquid chromatography and ion-

pair partition has several advantages for the separation of ionized organic compounds. An aqueous sample can be applied directly, which means that a time-consuming, and sometimes complicated, extraction to an organic phase is avoided. This is of particular value for the analysis of hydrophilic compounds. Furthermore, it is simple to regulate the retention of the substrate by varying the type and concentration of the counter ion in the mobile aqueous phase. This opens excellent possibilities for gradient elution and the application of large sample volumes without a loss in separating efficiency.

The aim of the present study is to investigate the properties of a reversed-phase ion-pair system suitable for the separation of hydrophilic organic acids. The influence of the composition of the mobile aqueous phase and side-reactions in the organic phase (dissociation) have been investigated. The selectivity of the system is illustrated by separating a mixture of benzoic and benzenesulphonic acid derivatives. Application of the gradient elution technique is demonstrated, together with the use of the method for isolations from biological material.

## EXPERIMENTAL

### *Apparatus*

Detector: LDC UV-Monitor Model 1205, wavelength 254 nm, cell volume 8 $\mu$l.

Pumps: Chromatronix Cheminert Metering pump CMP-1 L. LDC Solvent Delivery System Model 711-26 (Milton-Roy pump).

Columns: Separation and pre-column of borosilicate glass (silanized), 300 mm × 2.7 mm I.D. 316 stainless-steel columns, polished surface, for small particle supports, 3.1 and 3.2 mm I.D. (pre-column: 150 mm × 4.5 mm I.D.).

Injectors: Septum injector made of Swagelok units. High-pressure (3000 p.s.i.) sample injection valve, Altex Scientific.

Gradient mixer: 11300 Ultrograd gradient mixer (LKB, Bromma, Sweden).

### *Chemicals and reagents*

The 1-pentanol was of Fisher A. C. S. and Fluka *puriss.* quality. Tetrabutylammonium (TBA) hydrogensulphate was obtained from Hässle (Mölndal, Sweden) and the tetrabutylammonium phosphate was prepared from TBA iodide (Eastman-Kodak, Rochester, N.Y., U.S.A.) by shaking with silver oxide and neutralizing the hydroxide with phosphoric acid. All other substances were of analytical or reagent grade and were used without further purification.

Chromatographic supports: LiChrosorb SI 60 silanized, mean particle diameter 30, 10 and 5 $\mu$m (E. Merck, Darmstadt, G.F.R.); Bondapak $C_{18}$/Porasil B (octadecyl derivative), particle size 37–75 $\mu$m (Waters Assoc., Milford, Mass., U.S.A.).

### *Column preparation*

The LiChrosorb particles were packed by a balanced density, slurry technique[7] using equal volumes of dioxane, tetrabromoethane and carbon tetrachloride as the suspending liquid. For the 30 $\mu$m particles, the flow-rate during the packing was maintained at 0.5 ml/min (glass columns); 10 and 5 $\mu$m particles were packed with the highest possible flow-rate (Waters pump, Model M6000).

Coating with the stationary phase, 1-pentanol, was performed using a modifi-

ration of the *in situ* method of Kirkland and Dilks[8]. The column was equilibrated with acetone, followed by 25 ml of a 10% solution of 1-pentanol in acetone at a flow-rate of 1 ml/min. Finally, the mobile phase (aqueous solution) saturated with 1-pentanol was pumped through the column at a flow-rate of 0.2 ml/min until a stable baseline was obtained. The passage of about 100 ml of mobile phase was usually required.

The coating was done with the separation and pre-columns coupled in series and maintained at 25.0 ± 0.1°.

The Bondapak $C_{18}$/Porasil B was coated with stationary phase by evaporation from methylene chloride solution; the slurry was then packed in glass columns with a rod (*cf.* ref. 9).

The amount of stationary phase on the columns was determined by gas chromatography after elution of the stationary phase with ethanol. The loading was 0.4–0.5 ml/g on the LiChrosorb particles, and 0.6 ml/g on the Bondapak support. The interstitial volume was determined by the injection of potassium nitrate or 4-hydroxybenzoate with phosphate buffer pH 7.4 as the mobile phase. None of these compounds was retained on the column.

*Chromatographic technique*

The separation column was always preceded by a pre-column prepared in the same way, with the exception that LiChrosorb 30 μm particles were used in the pre-column to all LiChrosorb packings.

The whole chromatographic system, from the solvent reservoir to the column outlet, was kept in an air-thermostatted box at 25.0 ± 0.1°. In order to prevent pentanol being precipitated in the detector, a cooling tube was coiled around the cell-holder. (The stationary phase dissolves to about 2.5% in the mobile phase and even a small temperature increase can cause precipitation of pentanol.) All mobile phases and sample solvents were also kept at 25.0 ± 0.1°. Columns treated in this way were used continuously for several months without change of properties.

To prevent baseline shifts in gradient elution, it was necessary to use a reference system identical to the separation system. The flow from the gradient-mixer was therefore split into two streams with separate pumps, one for the separation system and one for the reference system. The flow-rate from the latter was passed through the reference channel of the detector.

## RESULTS AND DISCUSSION

*Ion-pair partition and the capacity factor*

An organic anion $X^-$ can be extracted by a counter ion $Q^+$ from an aqueous phase as an ion-pair, according to the reaction

$$Q^+_{aq} + X^-_{aq} = QX_{org} \qquad (1)$$

The distribution ratio of $X^-$ between the organic and aqueous phases is given by

$$D_X = E_{QX} \cdot [Q^+] \qquad (2)$$

where $E_{QX}$, the extraction constant, is the equilibrium constant for reaction (1):

$$E_{QX} = [QX]_{org} \cdot [Q^+]^{-1} \cdot [X^-]^{-1} \quad (3)$$

The magnitude of the extraction constant depends on the properties of the organic phase and the structure of $Q^+$ and $X^-$ (*cf.* ref. 10).

When side-reactions occur (*e.g.* ion-pair dissociation or dimerization in the organic phase, association processes or protolysis in the aqueous phase), it is convenient to exchange the stoichiometric extraction constant $E_{QX}^X$ for a conditional extraction constant $E_{QX}^X$ (*cf.* ref. 9):

$$E_{QX}^X = C_{QX,org} \cdot C_Q^{-1} \cdot C_X^{-1} \quad (4)$$

$C_{QX,org}$ is the total concentration of $X^-$ transferred to the organic phase as ion-pair with $Q^+$. $C_X$ and $C_Q$ are the total concentrations of $X^-$ and $Q^+$ in the aqueous phase. The conditional extraction constant is related to the stoichiometric by the relation

$$E_{QX}^X = E_{QX} \cdot \alpha_{QX} \cdot \alpha_Q^{-1} \cdot \alpha_X^{-1} \quad (5)$$

where the $\alpha$-coefficients express the influence of side-reactions within the phases. The $\alpha$-coefficients are unity when side-reactions are negligible, and increase as the influence of the side-reactions increases. The distribution ratio of $X^-$ is then given by:

$$D_X = E_{QX}^X \cdot C_Q \quad (6)$$

In reversed-phase ion-pair partition chromatography with an organic solvent as stationary phase and an aqueous mobile phase, the capacity factor of the anion $X^-$ is given by:

$$k_X' = V_s \cdot V_m^{-1} \cdot E_{QX}^X \cdot C_Q \quad (7)$$

$V_s$ is the volume of the stationary phase and $V_m$ the interstitial volume. For a given stationary phase the capacity factor can be regulated by the kind and concentration of the counter ion in the aqueous phase. The capacity factor, $k_X'$, can be varied within very wide limits: addition of one methylene group in the quaternary ammonium ions used as counter ions in this study increases $E_{QX}^X$ by about 2.5 times (*cf.* ref. 11).

*Dissociation of the ion pair in the organic phase*

A common side-reaction in the ion-pair partition of low concentrations of organic compounds is the dissociation of the ion-pair in the organic phase[9]:

$$QX_{org} = Q_{org}^+ + X_{org}^- \quad (8)$$

It is especially pronounced in polar solvents, like 1-pentanol, which give dissociation constants of about $10^{-3}$ mol/l[12]. The conditional extraction constant is then given by

$$E_{QX}^X = E_{QX} \cdot \alpha_{QX} = E_{QX}(1 + k_{diss(QX)} \cdot [Q^+]_{org}^{-1}) \quad (9)$$

where the dissociation constant is defined:

$$k_{diss(QX)} = [Q^+]_{org} \cdot [X^-]_{org} \cdot [QX]^{-1}_{org} \quad (10)$$

When $QX$ is the only ion-pair present in the organic phase, $[Q^+]_{org}$ and the capacity factor change with the concentration of the sample $X^-$. This leads to tailing peaks in the chromatogram. Eqn. 9 shows, however, that if $[Q^+]_{org}$ can be kept at a constant level the conditional constant will not change with the sample concentration. This situation can be achieved if $Q^+$ also is extracted by the organic phase as an ion-pair with another anion $Z^-$. If $C_{QZ,org} \gg C_{QX,org}$, and both ion-pairs have dissociation constants of similar magnitude, $[Q^+]_{org}$ will be controlled by $C_{QZ,org}$ and will be independent of $C_{QX,org}$. The conditional extraction constant is then given by:

$$E^X_{QX} = E_{QX} \{1 + k_{diss(QX)} \cdot (k_{diss(QZ)} \cdot E_{QZ} \cdot [Q^+] \cdot [Z^-])^{-\pm}\} \quad (11)$$

Eqn. 11 shows that if the aqueous concentrations of the counter ion $Q^+$ and foreign anion $Z^-$ are kept constant, $E^X_{QX}$ will be independent of the concentration of the sample $X^-$; this is a prerequisite to obtaining symmetrical peaks.

There are several indications that the dissociation of ion-pairs of the organic anions in the present systems is suppressed to a constant level by the extraction of ion-pairs of inorganic anions ($H_2PO_4^-$ or $SO_4^{2-}$) present in the mobile phase: $E^X_{QX}$ for 4-hydroxybenzoate as ion-pair with tetrabutylammonium (TBA) is independent of $C_{QX,org}$ in the range $10^{-4.5}$–$10^{-3.8}$ $M$, when the determination is made at pH 7.4 with 0.04 $M$ phosphate in the aqueous phase (Table I). Without suppressing the dissociation, $E^X_{QX}$ should increase by about four times when $C_{QX,org}$ decreases from $10^{-3.8}$ to $10^{-4.5}$ $M$ if $k_{diss(QX)} = 10^{-5}$. Also, the capacity factor of toluene-4-sulphonate on a column with TBA in 0.04 $M$ phosphate as mobile phase decreases by about 25% if the mobile phase is made 0.1 $M$ with respect to $Br^-$. This is probably due to a decrease in $E^X_{QX}$ (eqn. 11) by $Br^-$ which has a much higher $E_{QZ}$ than phosphate (*cf.* ref. 13).

*Regulation of the capacity factor*

One of the advantages of reversed-phase ion-pair partition chromatography is the presence of the counter ion in the mobile phase: this facilitates regulation of the capacity factor by changing the type or concentration of counter ion. Fig. 1 illustrates the change in the capacity factor of one sulphonate and one carboxylate ion when the concentration of the counter ion TBA is changed from zero to 0.05 $M$. The capacity factor of the hydrophobic ion-pair between toluene sulphonate and TBA rises from

TABLE I

CAPACITY FACTOR OF 4-HYDROXYBENZOATE

Aqueous phase (batch and column): TBA sulphate in 0.04 $M$ phosphate buffer, pH 7.4. Organic phase (batch and column): 1-pentanol. Column support: Bondapak $C_{18}$/Porasil B. $V_s/V_m = 0.79$. Determination of $E^X_{QX}$ made according to ref. 14. $k'_{calc.} = V_s \cdot V_m^{-1} \cdot E^X_{QX} \cdot C_Q$.

| Method | $C_Q$ | $C_{QX,org} \times 10^5$ | $\log E^X_{QX}$ | $k'_{found}$ | $k'_{calc.}$ |
|---|---|---|---|---|---|
| Batch extraction | 0.046 | 3.3–17 | 2.08 | — | — |
| Column chromatography | 0.050 | — | — | 3.6 | 4.8 |

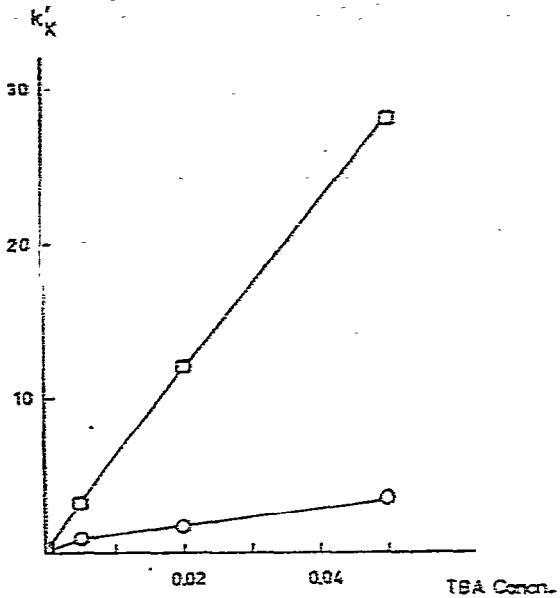


Fig. 1. Regulation of the capacity factor. Support: Bondapak $C_{18}$/Porasil B. Stationary phase: 1-pentanol. Mobile phase: TBA-sulphate in 0.04 *M* phosphate buffer pH 7.4. Samples: □, toluene-4-sulphonic acid; ○, 4-hydroxybenzoic acid.

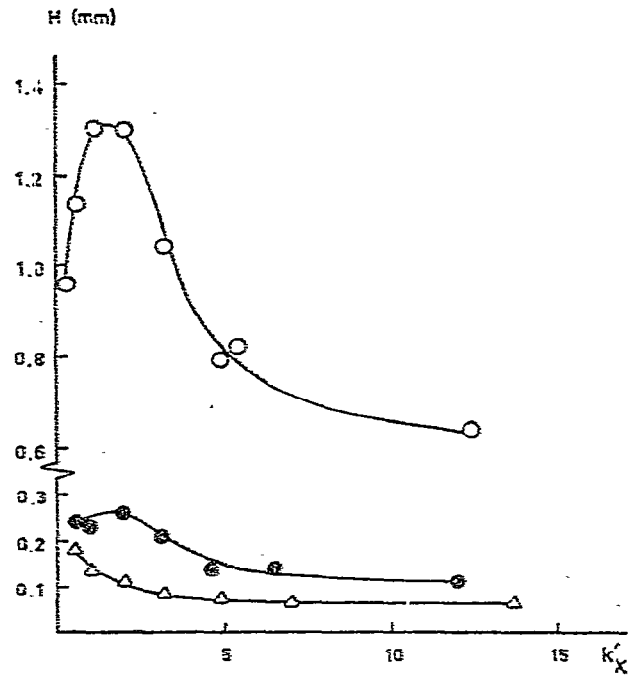


Fig. 2. Column efficiency with different support diameter. Support: LiChrosorb SI 60 silanized. ○, 30 μm (Glass column; flow-rate 2.4 mm/sec); ●, 10 μm (steel column, 250 mm; flow-rate 3.0 mm/sec); △, 5 μm (steel column, 150 mm; flow-rate 1.7 mm/sec). Stationary phase: 1-pentanol. Mobile phase: TBA 0.01, 0.02 or 0.03 *M* in phosphate buffer pH 7.4. Samples: as in Fig. 4.

zero to 28 while the capacity factor of the hydrophilic ion-pair between 4-hydroxybenzoate and TBA reaches only 3.6.

The slopes of the lines should, according to eqn. 7, be proportional to the conditional extraction constant. A test of the validity of eqn. 7 is given in Table I. It gives $E'_{QX}$ for the TBA ion pair of 4-hydroxybenzoate obtained by batch distribution, and the capacity factor found on a column with phases of the same composition. There is fairly good agreement between the capacity factor found and that calculated on the basis of the batch distribution data. Repeated changes of the counter ion concentration did not affect the separating efficiency or stability of the columns.

*Column efficiency*

Column efficiency studies were performed on systems with a LiChrosorb support having mean diameters of 30, 10 and 5 $\mu$m. The relation between the capacity factor and the height $H$ of a theoretical plate is demonstrated in Fig. 2. The variation in capacity factor was obtained by using substances with different partition properties (extraction constants). There is a very large decrease in $H$ when the support diameter is decreased from 30–10 $\mu$m; a further decrease in the particle diameter, however, seems to have little effect.

$H$ is strongly dependent on the flow-rate on 30 $\mu$m particles, as demonstrated in Fig. 3; it reaches a maximum when the capacity factor is about 1.5. This indicates that the dominating band-broadening effect is the mass transfer in the stationary liquid phase (*cf.* refs. 9 and 15) due to the high viscosity of 1-pentanol.

*Separation of organic anions*

An example of the separating power of the present chromatographic method

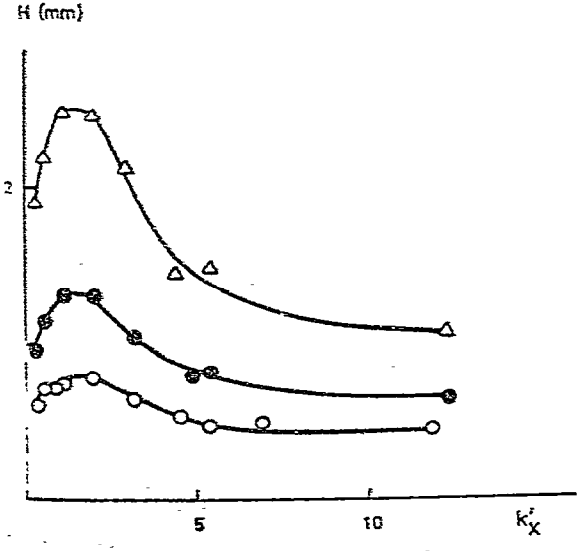


Fig. 3. Effect of capacity factor and flow rate on column efficiency. Support: LiChrosorb SI 60 silanized, 30 $\mu$m (glass column). Stationary phase: 1-pentanol. Mobile phase: TBA 0.01 and 0.02 $M$ in phosphate buffer pH 7.4. Samples: as in Fig. 4. Flow-rates: △, 5.9 mm/sec; ●, 2.4 mm/sec; ○, 1.2 mm/sec.

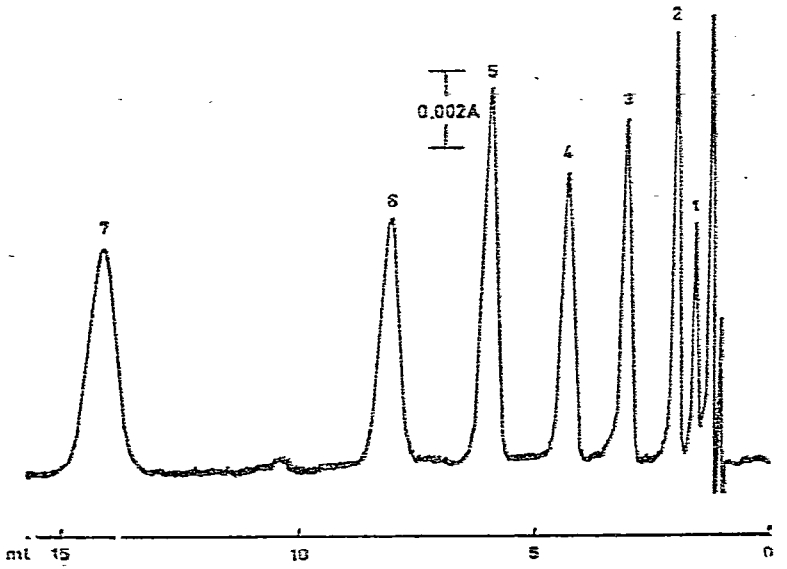


Fig. 4. Separation of sulphonates and benzoates. Support: LiChrosorb SI 60 silanized, 10 μm (steel column, 250 mm). Stationary phase: 1-pentanol. Mobile phase: TBA 0.03 *M* in phosphate buffer pH 7.4. (0.72 ml/min; 3 mm/sec). Sample volume: 22 μl. 1 = 4-aminobenzoic acid (7.6 ng); 2 = 3-aminobenzoic acid (110 ng); 3 = 4-hydroxybenzoic acid (32 ng); 4 = 3-hydroxybenzoic acid (500 ng); 5 = benzenesulphonic acid (3.0 μg); 6 = benzoic acid (580 ng); 7 = toluene-4-sulphonic acid (4.0 μg).

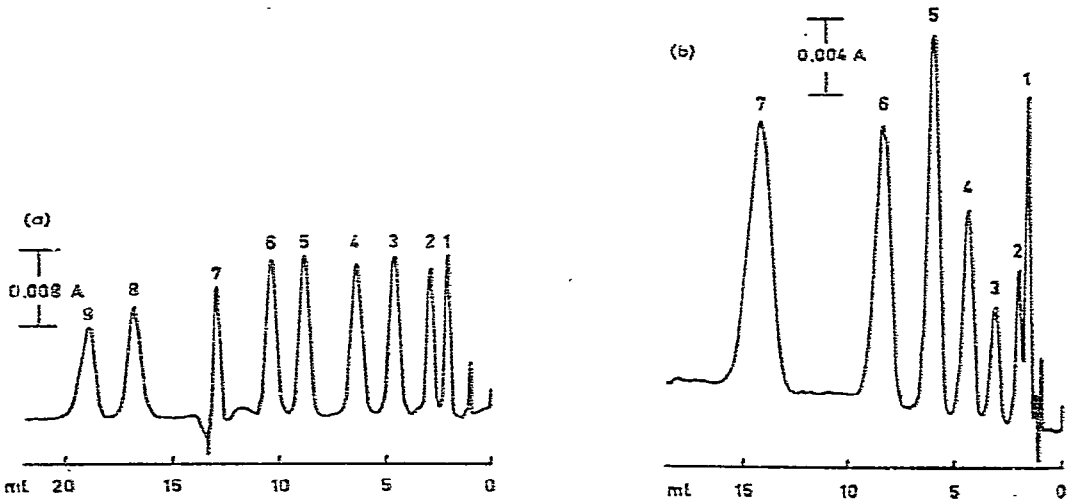


Fig. 5. Separation of sulphonates and benzoates: gradient elution (a) compared to normal elution (b). Support: LiChrosorb SI 60 silanized, 30 μm (glass column, 293 mm). Stationary phase: 1-pentanol. Mobile phase: TBA in phosphate buffer 0.04 *M*, pH 7.4 (0.4 ml/min; 2.0 mm/sec). TBA concentration: (a) 0.10 *M* to zero (linear) in 0–5 ml of mobile phase; (b) 0.04 *M*. Sample volume: 20 μl. 1 = 4-aminobenzoic acid (a, 40 ng; b, 27 ng); 2 = 3-aminobenzoic acid (a, 610 ng; b, 324 ng); 3 = 4-hydroxybenzoic acid (a, 170 ng; b, 63 ng); 4 = 3-hydroxybenzoic acid (a, 2.7 μg; b, 1.8 μg); 5 = benzenesulphonic acid (a, 11 μg; b, 14 μg); 6 = benzoic acid (a, 2.1 μg; b, 3.2 μg); 7 = toluene-4-sulphonic acid (a, 10 μg; b, 19 μg); 8 = sodium 2,4-dimethylbenzenesulphonate (a, 7 μg); 9 = 2-hydroxybenzoic acid (a, 12 μg).

:s shown in Fig. 4, where positional isomers of amino- and hydroxy-benzoates, and :wo homologous aromatic sulphonates, are separated in the same run. The support vas composed of particles with a mean diameter of 10 $\mu$m. pH was chosen so that all ·ample components were present in ionized form and could be transferred to the sta- ionary phase as ion-pairs with TBA. The benzoates with hydrophilic substituents ire eluted first; the toluenesulphonate last. The positional isomers were rather well :eparated: 4-substituted compounds were eluted before 3-substituted ones with sepa- ·ation factors of 1.6–1.7; the separation factor between the homologous sulphonates vas 2.6.

*Gradient elution*

In reversed-phase ion-pair partition chromatography, gradient elution is achieved simply by decreasing the counter ion concentration. This involves no risk of stripping off the stationary phase —one of the main problems when gradient elution is produced by changing the composition of a mobile organic phase.

Gradients were produced by mixing phosphate buffer pH 7.4 with a 0.1 *M* solution of TBA in the same buffer. Fig. 5a shows a gradient run for a mixture of isomeric amino- and hydroxybenzoic acids and three homologous aromatic sulphonic acids. A chromatogram from a normal elution with a constant counter ion concentration, obtained on the same column and with the same flow rate, is shown in Fig. 5b. The gradient gives improved resolution at the beginning of the chromatographic run, smaller peak widths, and the elution of two further components which appear very late in a normal elution. Such effects are normally found in gradient elution[16–18].

The effect of gradient elution on *H* is demonstrated in Fig. 6 which also gives results from a normal elution. The gradient gives improved separating efficiency when the capacity factor is larger than 2. (It should be emphasized that the gradient was not optimized with respect to peak-broadening and resolution in this preliminary study.)

*Injection of large sample volumes*

Large amounts of sample can be injected on a chromatographic column

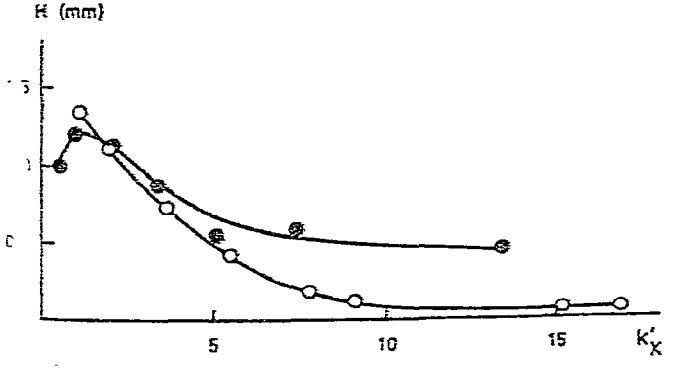


Fig. 6. Column efficiency in gradient and normal elution. Conditions: see Fig. 5. ○, gradient elution; ● normal elution.

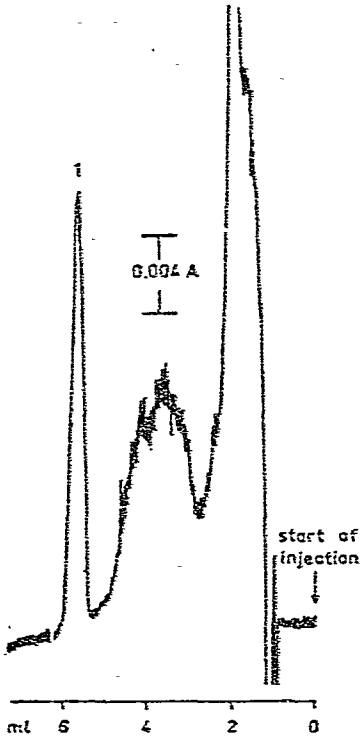


Fig. 7. Injection of large sample volume. Sample: toluene-4-sulphonic acid (I) 5 $\mu$g in 1.075 ml of TBA-sulphate 0.10 $M$ (phosphate buffer pH 7.4). Injected with sample injection valve. Support: LiChrosorb SI 60 silanized, 10 $\mu$m (steel column, 250 mm). Stationary phase: 1-pentanol. Mobile phase: TBA-sulphate 0.0075 $M$ in phosphate buffer 0.04 $M$, pH 7.4 (0.46 ml/min; 1.9 mm/sec).

without significant loss of separating efficiency if the injection can be made so that the sample forms a narrow band at the top of the column. According to the equation

$$w_s = V_0 (1 + k')^{-1}, \qquad (12)$$

where $w_s$ is the volume of the starting zone and $V_0$ is the sample volume, this is best achieved with a gradient giving a high capacity factor during the introduction of the

TABLE II

LARGE SAMPLE VOLUME

Sample: Toluene-4-sulphonic acid in 0.1 $M$ TBA, pH 7.4. $V_m$ = 1.0 ml. Other conditions as in Fig. 7.

| *Sample* | | $k'_X$ | *Peak* | *H* |
|---|---|---|---|---|
| *Volume* ($\mu$l) | *Amount* ($\mu$g) | | *width* ($\mu$l) | *(mm)* |
| 21.6 | 3.5 | 3.6 | 425 | 0.13 |
| 1075 | 5.2 | 4.2* | 630 | 0.23* |

* Calculated from the end of the injection.

ample. The reversed-phase ion-pair partition chromatographic system is ideally uited for this purpose, due to the ease by which the capacity factor can be changed: he simplest way is to dissolve the sample in a solution of a counter ion of such a concentration that a high capacity factor is obtained.

Fig. 7 shows the result of injecting 1.075 ml of sample solution on a column vith an interstitial volume of 1.0 ml. The sample, toluene-4-sulphonic acid, was dissolved in 0.1 *M* TBA to give a capacity factor of 30–40. (The eluent was 0.0075 *M* TBA.) As shown in Table II, the peak-width increased by only 50% as compared vith that obtained on injection of a small sample volume (22 $\mu$l).

*Application to human serum samples*

A preliminary study was made on the isolation of a hydrophilic acid, nicotinic acid, in human serum. 1.0 ml of the serum was initially equilibrated with 0.1 ml of 1-pentanol to prevent "stripping" of the stationary phase. After removal of precipitated proteins by centrifugation, the aqueous phase was injected onto the column. Fig. 8 illustrates the isolation of nicotinic acid (42 ng) from a serum sample of 22 $\mu$l; no losses of nicotinic acid were observed during the analytical procedure. More hydrophobic sample components were easily removed from the column by backflushing with the mobile phase or a phosphate buffer.

The column properties were unchanged even after application of numerous serum samples.

The positive results indicate that reversed-phase ion-pair partition chromatography may open possibilities for the isolation of drugs and related compounds in serum or urine by the direct injection of the biological fluid.

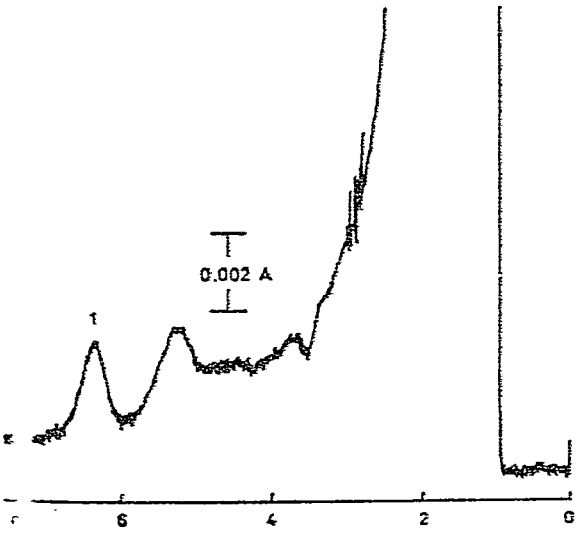


Fig. 8. Isolation of nicotinic acid in human serum. Sample: 42 ng of nicotinic acid in 22 $\mu$l of serum (... Support: LiChrosorb SI 60 silanized, 10 $\mu$m (steel column, 250 mm). Stationary phase: 1-pentanol. Mobile phase: TBA-sulphate 0.12 *M* in phosphate buffer 0.04 *M*, pH 7.4 (0.46 ml/min; 1.1 mm/sec).

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