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REVERSED-PHASE ION-PAIR PARTITION CHROMATOGRAPHY OF PHENYLACETIC, MANDELIC AND PYRIDINECARBOXYLIC ACID DERIVATIVES

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SUMMARY

Phenylacetic, mandelic and pyridinecarboxylic acids of physiological interest have been separated by ion-pair partition chromatography with 1-pentanol as stationary phase and tetrabutylammonium as counter ion in the aqueous mobile phase.

The stability of the chromatographic system is very high and the support is spontaneously re-coated with stationary phase. Untreated plasma samples can be injected in relatively large volumes without serious loss of separating efficiency.

A venting system was found efficiently to reduce disturbing early peaks on injection of biological samples.

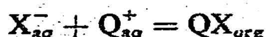
INTRODUCTION

In a previous paper[1], a system for reversed-phase ion-pair chromatography of organic anions was described. The stationary phase, 1-pentanol, was coated on a commercially available hydrophobized silica support and the mobile phase was an aqueous solution of a counter ion, tetrabutylammonium (TBA).

In reversed-phase ion-pair partition chromatography, the capacity factor of an anion X^- is given by

$$k'_X = E_{QX} \cdot [Q^+] \cdot \frac{V_s}{V_m} \qquad (1)$$

where E_{QX} (the extraction constant) is the equilibrium constant of the process



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

Q^+ is the counter ion present in the mobile aqueous phase and V_s/V_m is the phase volume ratio on the column.

Eqn. 1 shows that the capacity factor of the anion is easily regulated by the concentration and the nature of the counter ion. This makes the systems very easy to adapt to different types of samples and it has been shown that the technique presents good possibilities for gradient elution. The systems have also been used for the isola-

tion of hydrophilic acids in human serum by direct injection of the deproteinized samples on the reversed-phase columns.

In the present study, it has been shown that the support (silanized LiChrosorb) is spontaneously coated with stationary phase when equilibrated with 1-pentanol-saturated mobile phase. The aim of this study was to demonstrate the high stability of the reversed-phase liquid–liquid chromatographic system and its ability to separate hydrophilic acidic drugs and metabolites as ion pairs. The stability of the column system on injection of untreated human serum is demonstrated, and also the use of a pre-column venting system for reduction of early peaks obtained on the injection of biological samples.

EXPERIMENTAL

Apparatus

The detector was an LDC Model 1205 UV monitor, wavelength 254 nm, with an 8-μl cell, and an LDC Models 711-26 and 711-47 solvent delivery system (Milton-Roy Minipump with pulse dampener) was used. The columns were made of 316 stainless steel with a polished surface, 3.2 mm I.D. The injector was a high-pressure (3000 p.s.i.) sample injection valve (Altex Scientific).

Chemicals and reagents

1-Pentanol was of Fisher A.C.S. quality. TBA was used as the hydrogen sulphate obtained from Hässle (Mölndal, Sweden) and neutralized with sodium hydroxide prior to use. All other substances were of analytical or reagent grade and used without further purification.

The chromatographic support was LiChrosorb SI 60 silanized (RP 2), mean particle diameter 10 μm, Charge No. YE 374 (E. Merck, Darmstadt, G.F.R.).

Column preparation

The columns were packed by the balanced density slurry technique as described previously[1]. Coating with the stationary phase was performed by an equilibration technique. The column, previously washed with acetone, was equilibrated with the aqueous mobile phase that had been carefully saturated with 1-pentanol at the chromatographic temperature. The column was stable after passage of about 25 column volumes of mobile phase.

The amount of stationary phase was determined by eluting the column with a small volume of water (to remove the salts) and then ethanol. The concentrations of 1-pentanol in this eluate and in the mobile phase were determined by gas chromatography on a Porapak Q column. The amount of stationary phase was calculated after compensation for the concentration of 1-pentanol in the mobile phase.

The interstitial volume was determined by injection of nitrate or dichromate with buffer (pH 7.4) as the mobile phase.

Chromatographic technique

The chromatograph (reservoir, column, detector) was kept in an air-thermostated box at 25.0 $\pm$ 0.1°. Mobile phases were saturated with 1-pentanol and kept in a flask with 1-pentanol present as the upper layer. It was not necessary to use

a pre-column in this system. The detector and the coupling union between the column and the detector were maintained at a temperature below 20°.

A pre-column venting system was used in some of the studies on serum samples. The sample injections were then made on the pre-column, which was connected to the separation column via a low-dead-volume sample valve. The back-pressure in the vent outlet was adjusted to that of the separation column by inserting a packed column.

All samples were dissolved in mobile phase. The mobile phases were made from $TBANaSO_4$ dissolved in phosphate buffer of pH 7.4 (0.04 *M*, ionic strength 0.1).

RESULTS AND DISCUSSION

Coating with pentanol by equilibration

In a previous study[1], 1-pentanol was coated on silanized LiChrosorb by the *in situ* method of Kirkland and Dilks[2] using a 10% solution of the stationary phase in acetone. A higher concentration of stationary phase (75%) gave columns of low efficiency, probably owing to excess coating.

In the present study, all columns were coated by equilibration with mobile phase saturated with 1-pentanol. The stationary phase is then rapidly bound to the support to a loading of *ca.* 0.5 ml/g of support. This coating technique has earlier been used for the coating of hydrophilic silica supports[3-5].

The support used in this study is incompletely silanized and has only moderately hydrophobic character. This is substantiated by its ability to be wetted by water. It is not known if the same coating technique can be used on supports of higher hydrophobicity.

The coating was followed by running test samples of benzoic acids and sulphonates as ion pairs with TBA present in the mobile phase. It was found that the column (15 cm $\times$ 3.2 mm, $V_m \approx 0.65$ ml) was stable after the passage of 17–20 ml of saturated mobile phase. The amount of stationary phase, V_s, was *ca.* 0.30 ml. The solubility of 1-pentanol in the mobile phase is 2.5% (v/v) and theoretically 12 ml of mobile phase (20 column volumes) will be sufficient for the coating.

The results indicate a very rapid and efficient coating as the column reaches the steady state after the passage of 30 column volumes of mobile phase. The equilibration time is 20 min on a 15 cm $\times$ 3.2 mm column when the flow-rate is 1 ml/min.

The spontaneous coating with 1-pentanol has obvious practical advantages. The columns are completely stable provided that a pentanol-saturated mobile phase is used. The rapid coating and high stability is partly due to the high concentration of stationary phase in the mobile phase (2.5%). Unlike most other liquid–liquid chromatographic systems, these columns have been found to work equally well without a pre-column. Furthermore, injection of samples that are not pre-saturated with stationary phase will not damage the column as the stripping of stationary phase will be compensated for by spontaneous re-coating.

The 1-pentanol-coated columns have been used in thousands of runs with mobile phases of different composition without significant changes in their properties. After improper use (*e.g.*, injection of samples that contain non-migrating constituents), the columns can be washed with ethanol and re-coated. However, it has been observed that such procedures can change the properties of the support. On re-coating, the columns do not reach a steady state until 300 ml of mobile phase have passed (Fig. 1).

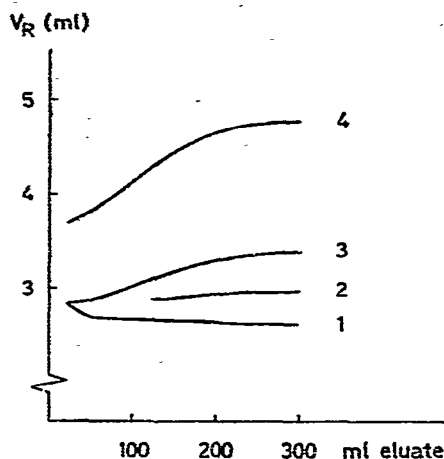


Fig. 1. Change in retention volumes during re-coating of support. Support: LiChrosorb SI 60 silanized, 10 μm (column length, 200 mm). Stationary phase: 1-pentanol. Mobile phase: TBA, 0.03 M, pH 7.4 (0.8 ml/min; 3.4 mm/sec). Samples: 1 = nicotinic acid; 2 = isonicotinic acid; 3 = 5-fluoro-3-hydroxymethylpyridine; 4 = 5-fluoropyridine-3-carboxylic acid.

Ion-pair chromatography can also be performed on this support without a 1-pentanol coating, but the selectivity is lower. Furthermore, the control of retention by the concentration of the counter ion in the mobile phase is limited and a gradual change in the properties of the support has also been observed.

Column efficiency

The height equivalent to a theoretical plate, H, on the 1-pentanol-coated supports is dependent on the flow velocity. Results on 10-μm particles are shown in Fig. 2. $H < 0.08$ mm is obtained at flow velocities below 1.7 mm/sec and capacity factors above 5. On 30-μm particles, $H = 0.65$ mm is obtained under the same conditions[1].

Separation of acidic catecholamine metabolites and related compounds

The reversed-phase ion-pair partition systems are particularly useful for the isolation of hydrophilic, ionizable compounds from complex samples, *e.g.*, biological material. No extraction is needed and the retention time is easily regulated by the concentration and the nature of the counter ion in the mobile aqueous phase.

The systems with 1-pentanol as stationary phase and a quaternary alkylammonium ion as counter ion have a very high selectivity for benzoic, phenylacetic and mandelic acid derivatives. The separation of mandelic acid and two acidic catecholamine metabolites, homovanillic acid and vanilmandelic acid, is demonstrated in Fig. 3. Symmetrical peaks were obtained by elution with 0.10 M TBA in the mobile phase. With a mobile phase containing 0.03 M TBA, mandelic acid and vanilmandelic acid gave tailing peaks with asymmetry factors above 2.5, probably owing to dissociation of the ion pairs in the stationary organic phase. TBA is extracted to some extent into the organic phase as ion pairs with buffer anions (phosphate). The use of 0.10 M TBA in the mobile phase gives rise to a higher concentration of such ion pairs

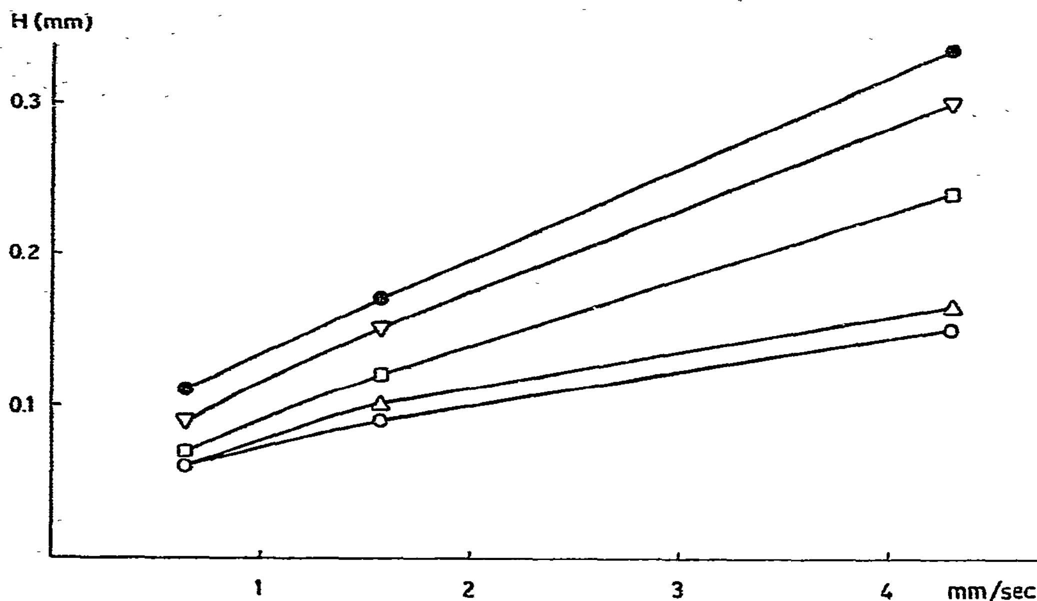


Fig. 2. Effect of capacity factor and flow-rate on column efficiency. Support: LiChrosorb SI 60 silanized, 10 μm (column length, 150 mm). Stationary phase: 1-pentanol (V_s = 0.32 ml). Mobile phase: TBA, 0.03 M, pH 7.4 (V_m = 0.60 ml). Samples: ● = 3-aminobenzoic acid (k' = 0.9); ▽ = 4-hydroxybenzoic acid (k' = 1.9); □ = 3-hydroxybenzoic acid (k' = 3.1); △ = benzoic acid (k' = 6.6); ○ = benzenesulphonic acid (k' = 4.6).

in the organic phase, which leads to suppression of the dissociation of the sample ion pairs[1,6].

The separation of some phenylacetic acid derivatives that differ in the number of hydroxy and methoxy groups is demonstrated in Fig. 4. The same hydrophobic character of the methoxy-substituted derivatives was observed in ion-pair chromatography with an organic mobile phase containing 20% of 1-butanol[7].

Separation of nicotinic acid and related compounds

The separation of some closely related acidic pyridine derivatives is demonstrated in Fig. 5. Nicotinic acid is normally present in biological fluids, isonicotinic acid is one of the metabolites of isoniazide and 5-fluoronicotinic acid is a metabolite of 5-fluoro-3-hydroxymethylpyridine, which has been proposed as an antilipolytic agent[8].

These three carboxylic acids are distributed into the organic phase as ion pairs and the retention can be regulated by the counter-ion concentration. 5-Fluoro-3-hydroxymethylpyridine is a base and the retention can be regulated in the present instance only by pH.

Nicotinic acid and isonicotinic acid are incompletely separated in the present system (separation factor = 1.2), but a complete separation is easily obtained by use of a higher counter-ion concentration. Substitution by a fluoro group in nicotinic acid gives a considerable increase in the hydrophobicity and a separation factor of 2.3 is obtained.

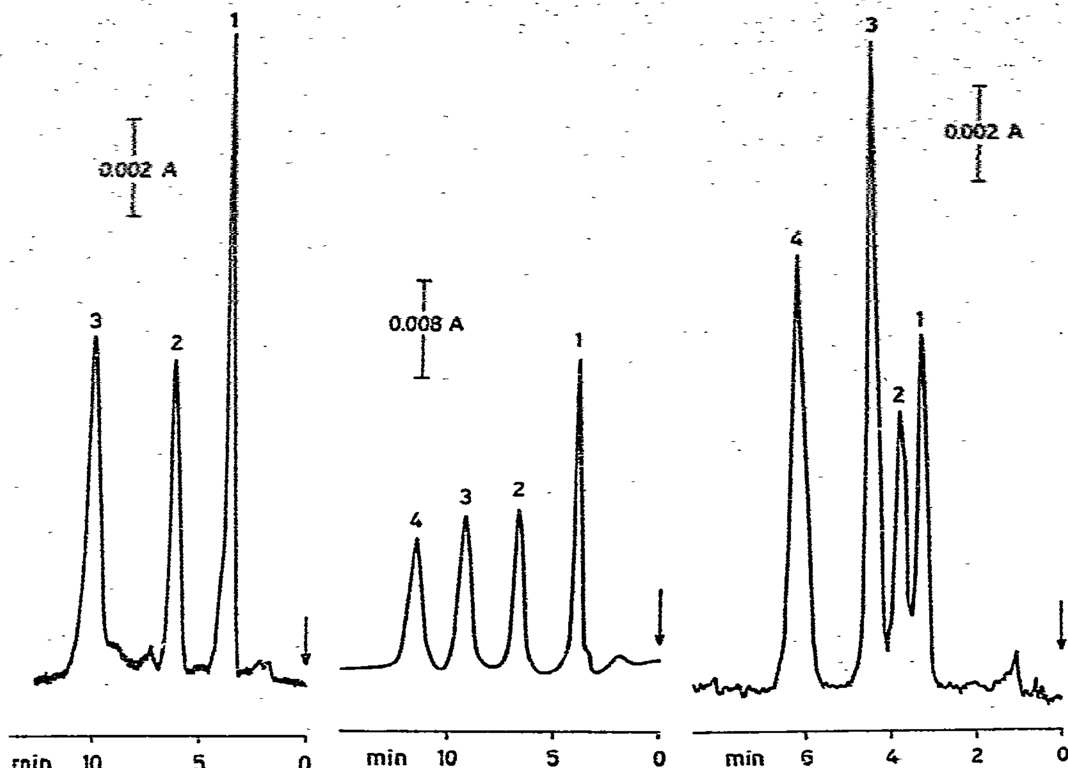


Fig. 3. Separation of vanilmandelic, homovanillic and mandelic acid. Support: LiChrosorb SI 60 silanized, 10 μm (column length, 200 mm). Stationary phase: 1-pentanol. Mobile phase: TBA, 0.1 M, pH 7.4 (0.58 ml/min; 2.3 mm/sec; ΔP = 60 bar). Samples: 1 = vanilmandelic acid (4-hydroxy-3-methoxymandelic acid); 2 = homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid); 3 = mandelic acid. Sample concentration: 4–22$\cdot 10^{-4}$ M (10 μl).

Fig. 4. Separation of phenylacetic acid derivatives. Support: LiChrosorb SI 60 silanized, 10 μm (column length, 150 mm). Stationary phase: 1-pentanol. Mobile phase: TBA, 0.03 M (0.33 ml/min; 1.3 mm/sec; ΔP = 16 bar). Samples: 1 = 4-hydroxy-3-methoxyphenylacetic acid; 2 = 3,4-dimethoxyphenylacetic acid; 3 = 3,4,5-trimethoxyphenylacetic acid; 4 = 4-methoxyphenylacetic acid.

Fig. 5. Separation of acidic and neutral pyridine derivatives. Support: LiChrosorb SI 60 silanized, 10 μm (column length, 200 mm). Stationary phase: 1-pentanol. Mobile phase: TBA, 0.03 M, pH 7.4 (0.80 ml/min; 3.4 mm/sec; ΔP = 70 bar). Samples: 1 = nicotinic acid (88 ng); 2 = isonicotinic acid (100 ng); 3 = 5-fluoro-3-hydroxymethylpyridine hydrochloride (220 ng); 4 = 5-fluoropyridine-3-carboxylic acid (5-fluoronicotinic acid) (220 ng).

Injection of untreated serum

The isolation of nicotinic acid from human serum by reversed-phase ion-pair chromatography after injection of serum on the column was demonstrated previously[1]. The samples were equilibrated with 1-pentanol before injection for two reasons: to prevent stripping of stationary phase and to precipitate proteins. By injection of untreated serum, precipitation of proteins on the column and retention of hydrophobic constituents (*e.g.*, fatty acids) in the stationary phase will be the main disturbing effects, while stripping of stationary phase will be rapidly compensated for by re-coating. The separation column can be protected from the disturbing sample components by use

of a short pre-column, coupled to the separation column via a low-dead-volume union[9]. When the pre-column deteriorates, it is replaced with a new column.

The stability of such a system for untreated serum was tested by repeated injections of filtered human serum. Between the serum injections, the column properties was tested by injection of 5-fluoronicotinic acid dissolved in mobile phase. The results, summarized in Table I, show that no serious damage to the pre-column had occurred up to a total serum volume of 350 μl. The column system was unusable after the injection of 400 μl of serum. Washing with water and re-coating with 1-

TABLE I

INFLUENCE OF UNTREATED SERUM ON COLUMN PROPERTIES

Support: LiChrosorb SI 60 silanized, 10 μm. Stationary phase: 1-pentanol. Mobile phase: TBA, 0.03 M, pH 7.4 (flow velocity, 2.2 mm/sec). Separation column: 150 mm. Pre-column: 50 mm. The same filling was used in the separation column and pre-column. Samples: untreated human serum (55 μl); 5-fluoronicotinic acid (55 μl) (test of column).

Total serum volume applied (µl)	*5-Fluoronicotinic acid*	
	H (mm)	*Capacity factor*
0	0.12	4.9
300	0.12	4.8
350	0.17	4.8

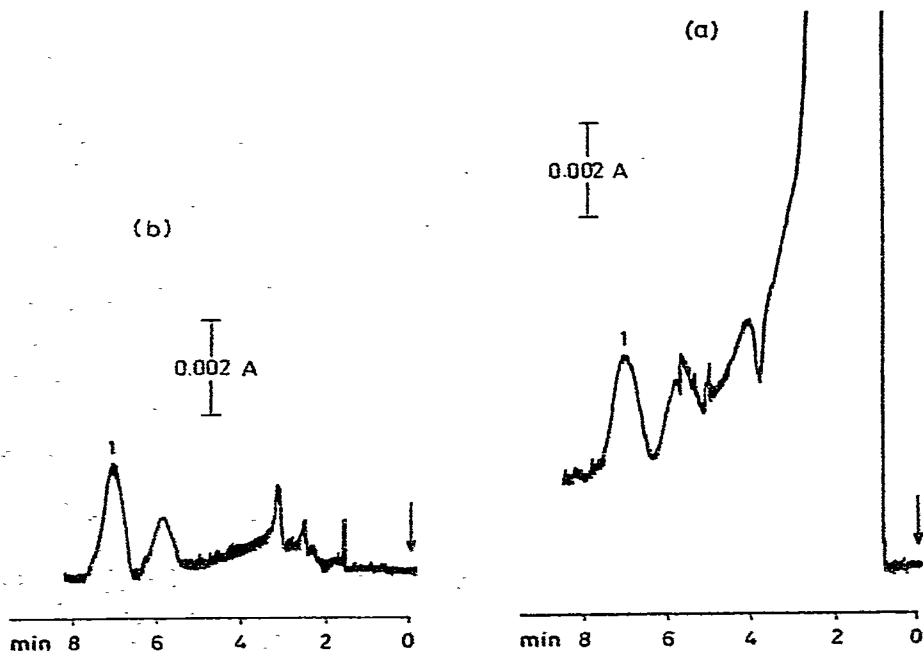


Fig. 6. Pre-column venting: (a) without venting; (b) venting for 1.5 min after injection. Support: LiChrosorb SI 60 silanized, 10 μm (pre-column 50 mm, separation column 150 mm). Stationary phase: 1-pentanol. Mobile phase: TBA, 0.12 M, pH 7.4 (0.80 ml/min; 3.4 mm/sec; ΔP = 70 bar). Sample: 55 μl of serum spiked with 0.82 $\mu g/ml$ of nicotinic acid (I).

pentanol restored its properties: k' was the same as before the application of serum, while H was about 30% higher.

Biological samples directly injected on reversed-phase columns often give rise to broad early peaks, and a pre-column venting system was used to avoid this disturbance[10]. The samples were injected on a short pre-column connected to the separation column via a low-dead-volume sample valve that could direct the flow either to waste or to the separation column.

The advantages of the system were demonstrated by use of serum samples spiked with nicotinic acid. Fig. 6a shows a chromatogram obtained without use of the venting system, while in Fig. 6b, the hydrophilic components, eluted first from the pre-column, were removed to the waste. The flow was then directed to the separation column and nicotinic acid and other more retarded compounds could be isolated without disturbance from the hydrophilic components.

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