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COLUMN LIQUID CHROMATOGRAPHY ON DYNAMICALLY MODIFIED SILICA. I

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SUMMARY

A "reversed-phase" high-performance liquid chromatographic approach using dynamically coated stationary phases has been investigated. The chromatographic separations are performed with silica gel as the solid phase and a mobile phase consisting of a buffer in the pH range 5–9, with a long-chain quaternary ammonium compound added as the reversed-phase-forming agent. The retention and selectivity may be controlled by varying the concentration or nature of the quaternary ammonium ion, the ionic strength or the pH of the buffer or by changing the concentration or nature of the organic modifier in the mobile phase.

It was found that other retention mechanisms including ion exchange and ion-pair formation are involved in the separation of solutes.

INTRODUCTION

In the early 1960s several workers[1–6] coated different supports (*e.g.*, paper and silica) with liquid ion exchangers [*e.g.*, tri-*n*-octylamine, tri-*n*-octylphosphine oxide, di-(2-ethylhexyl)phosphoric acid and methyltricaprylammonium chloride] in order to obtain more efficient separations of cations and anions.

Later, liquid ion exchangers were used as masking agents in reversed-phase high-performance liquid chromatography (*e.g.*, refs. 7–10). The variable amounts of free silanol groups on different brands of chemically bonded reversed-phase materials are covered, giving less tailing of some solutes. In other chromatographic experiments the applicability of liquid ion exchangers has also been demonstrated[11,12].

Recentiy[13], long-chain quaternary ammonium salts have been used in water-rich eluents and with silica as the support, giving chromatographic separations similar to those obtained with chemically bonded reversed-phase materials.

In this work this approach was investigated further in the pH range 5–9.

EXPERIMENTAL

Apparatus

A liquid chromatograph consisting of a Waters 6000 pump, a Rheodyne 7120

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loop injector, a Waters 440 UV absorbance detector (254 nm) and an Omniscribe 5117-5 recorder was used for the experiments.

Chemicals

N-Cetyl-N,N,N-trimethylammonium (CTMA) bromide was obtained from E. Merck (Darmstadt, G.F.R.). N-Tetradecyl-N,N,N-trimethylammonium (TTMA) bromide and N-dodecyl-N,N,N-trimethylammonium (DTMA) bromide were obtained from Sigma (St. Louis, MO, U.S.A.). Tetrabutylammonium hydrogen sulphate (puriss.) (TBA) and tetrahexylammonium hydrogen sulphate (purum) (THA) were obtained from Fluka (Buchs, Switzerland).

All other chemicals were of analytical-reagent grade, and the test substances were of pharmacopoeial quality and were used without further purification.

Chromatography

The chromatographic column (150 × 4.6 mm I.D.) was packed with Li-Chrosorb Si 60 (5 μm) particles.

A column (guard column) identical with the chromatographic column was installed between the pump and the loop injector in order to saturate the mobile phase with silica.

Using the method of Chalmers and Sinclair[14] for assaying silicate in the presence of phosphate, it was found that an eluent consisting of methanol–0.2 *M* potassium phosphate (pH 7.5)–water (50:5:45) containing 0.0025 *M* of CTMA bromide operated at 1.0 ml/min dissolves *ca.* 10 mg of silica per litre, which was found to be in good agreement with the observed loss from the guard column. All of the silica is therefore dissolved from the guard column, which is in accordance with the fact that the analytical columns show excellent stability. With a guard column installed, an analytical column has lasted for 6 months of daily use without deterioration.

The use of the columns at pH values above 8 for longer periods of time is not recommended because of the increasing solubility of silica gel with increasing pH.

RESULTS AND DISCUSSION

Because of the low solubility of long-chain amines at higher pH values, CTMA bromide was chosen for the preliminary experiments. The influence of the concentration of CTMA bromide in the mobile phase on the retention of some neutral, cationic and anionic compounds was investigated. Further, the effect of the concentration and pH of the buffer, the methanol concentration and the nature of the quaternary ammonium compound in the eluent was investigated.

Concentration of CTMA bromide

When adding increasing amounts of CTMA bromide the retention of the anionic and non-ionic compounds increases, whereas the behaviour of the cationic compounds differs depending on the nature of the cationic group and on other functional groups (if any) in the molecule.

The retention of a quaternary ammonium compound (N-methylimipramine) decreases linearly with increasing amounts of CTMA bromide, indicating a simple competition between the two cations for the ionic sites on the silica gel[15]. At the same

time the retentions of the corresponding primary, secondary and tertiary amines increase, leading to the conclusion that at least two different retention mechanisms are involved: (1) competition (ion exchange) between the ammonium compounds for the ionic sites on the ionized silica gel and (2) reversed-phase partition between a lipophilic "stationary phase" formed by CTMA ions adsorbed on to the silica gel and the more hydrophilic mobile phase.

Other amines with groups with strong hydrogen-accepting properties in the molecule show decreasing retention with increasing concentration of CTMA, indicating very little or no partition to the lipophilic "stationary phase".

Non-ionic compounds show increasing retention with increasing concentration of CTMA bromide, which can be explained by assuming a reversed-phase mechanism as outlined above.

The anionic compounds also show increasing retention with increasing amounts of CTMA bromide. This again can be explained as a reversed-phase chromatographic separation in which the substances probably migrate as ion pairs with the CTMA ion. The order of elution of the anionic and non-ionic compounds is in agreement with similar separations made in reversed-phase chromatography on chemically alkyl-bonded phases[10].

Buffer concentration

The retention of all compounds tested decreases with increasing concentration of the buffer. This may be explained as a competition between the potassium ion and the CTMA ion for the ionic sites on the silica gel. An increase in the concentration of potassium ion reduces the amount of lipophilic "stationary phase", and thereby reduces the retention of the chromatographed compounds. The retention of the quaternary ammonium compound again decreases linearly with increasing buffer concentration, which supports the assumption that the only mechanism involved in the retention of this substance is ion exchange.

Amount of organic phase

Decreasing the polarity of the mobile phase by adding increasing amounts of methanol (Fig. 1) results in a decrease in the retention of most of the compounds tested. However, for the quaternary ammonium compound and for normorphine the retention increases. The explanation for this may in part be that increasing amounts of methanol decrease the amount of CTMA ions adsorbed on to the silica gel, giving a more polar stationary phase. At the same time solvation of the cations decreases.

Changing the pH value

The main result of an increase in the pH of the mobile phase is an increase in the retention of most of the compounds tested (Fig. 2). This is probably due to the increasing ionization of the silica gel and, as a consequence, an increasing adsorption of CTMA ions on to the silica gel. The expected capacity ratios (k') should then form a S-shaped curve if plotted against pH between 5 and 9 as the pK_a value for silica gel is about 7[16]. This tendency is seen for some compounds, but for others the retention passes through a maximum at pH between 7 and 8. This last phenomenon seems to be very complex and will require further study before an explanation can be given.

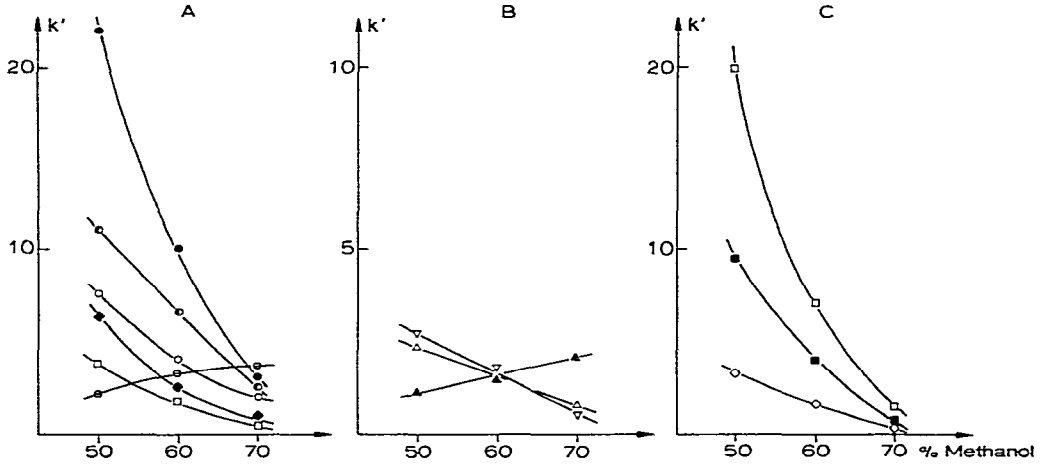


Fig. 1. k' values *versus* percentage of methanol in the mobile phase [25 volumes of 0.2 *M* potassium phosphate (pH 7.7) and water + methanol to 100 volumes] containing 1 g/l of CTMA bromide. A: ⊕, N-methylimipramine chloride; ●, imipramine; ◐, desmethylimipramine; ○, didesmethylimipramine; ◇, ethylbenzene; ⊡, toluene. B: ▽, thebaine; △, morphine; ▲, normorphine. C: □, estrone 3-sulphate; ■, estrone; ◇, estrone 3-β-D-glucuronide.

Nature of the quaternary ammonium ion

Having made all the above investigations with CTMA bromide in the mobile phase as the reversed-phase-forming agent, some other quaternary ammonium compounds were tested in the same way. Table I shows a decrease in the reversed-phase effect with decreasing chain length of the quaternary ammonium compound. At the same time an increase in the k' values of the quaternary ammonium compound and the three polyfunctional amines occurs, indicating a corresponding change in the ion-exchange effect and the polarity of the stationary phase.

When symmetrical quaternary ammonium compounds with a number of

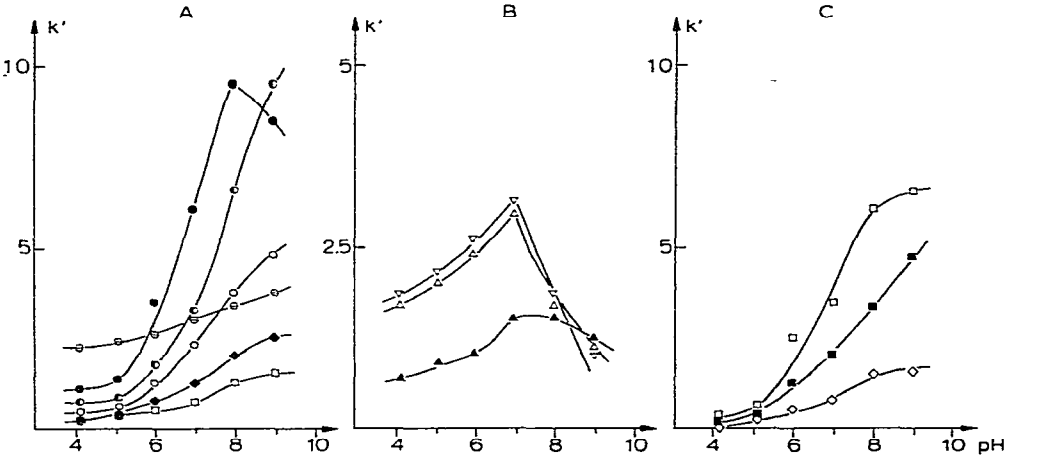


Fig. 2. k' values *versus* pH of the buffer in the mobile phase (methanol–water–0.2 *M* potassium phosphate, 60:15:25, containing 1 g/l of CTMA bromide). Sample identification as in Fig. 1.

TABLE I

k VALUES FOR A NUMBER OF TEST SUBSTANCES FOR MOBILE PHASES (METHANOL–WATER–0.2 *M* POTASSIUM PHOSPHATE BUFFER (pH 7.0), 60:15:25) CONTAINING DIFFERENT QUATERNARY AMMONIUM COMPOUNDS

The molar concentrations of each quaternary ammonium compound was 0.0027 *M*.

Sample	*Quaternary ammonium compound (bromides)*					
	CTMA	*TTMA*	*DTMA*	*THA*	*TBA*	*None*
Toluene	0.7	0.2	0.2	0.2	0.2	0.2
Ethylbenzene	1.3	0.3	0.2	0.2	0.2	0.2
Didesmethylimipramine	2.3	1.7	1.5	1.0	1.0	1.0
Desmethylimipramine	3.3	2.9	2.3	2.5	1.4	1.5
Imipramine	6.0	5.9	4.5	3.5	3.3	3.1
N-Methylimipramine chloride	3.3	5.9	5.9	5.9	5.9	5.7
Thebaine	3.1	4.5	4.7	5.8	5 8	4.6
Morphine	2.9	4.0	4.6	5.5	5.5	5.0
Normorphine	1.6	2.6	2.8	2.1	2.1	2.8
Estrone	1.7	0.7	0.3	0.2	0.2	0.1
Estrone sulphate	3.5	0.3	0.1	0.1	0.1	0.0
Estrone glucuronide	0.9	0.1	0.0	0.0	0.0	0.0

carbon atoms corresponding to that of CTMA are used, no reversed-phase effect is seen, and the retention of all test substances in this instance is very similar to the system in which no quaternary ammonium compound is added to the mobile phase. This may possibly be due to steric hindrance of these globular cations, preventing them from adsorption on to the ionized silica gel.

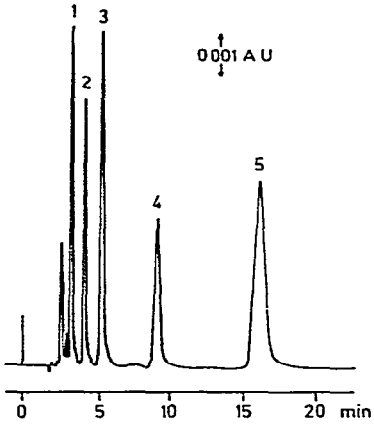


Fig. 3. Separation of aromatic hydrocarbons. Mobile phase: methanol–water–0.2 *M* potassium phosphate (pH 8.0) (60:35:5) containing 0.80 g/l of CTMA. Flow-rate, 1.0 ml/min. Peaks: 1 = benzene; 2 = toluene; 3 = ethylbenzene; 4 = 2-methylnaphthalene; 5 = phenanthrene.

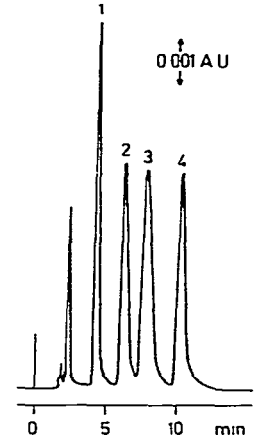
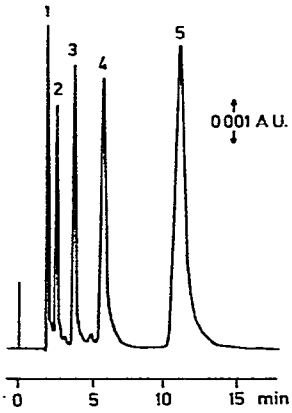


Fig. 4. Separation of aliphatic amines and a quaternary ammonium compound. Mobile phase: methanol–water–0.2 *M* potassium phosphate (pH 7.7) (60:15:25) containing 0.20 g/l of CTMA. Flow-rate, 1.0 ml/min. Peaks: 1 = didesmethylimipramine; 2 = desmethylimipramine; 3 = imipramine; 4 = N-methylimipramine ion.



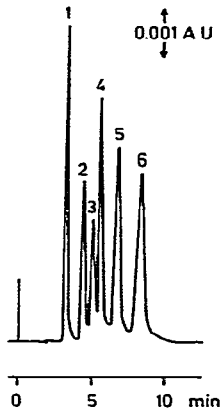
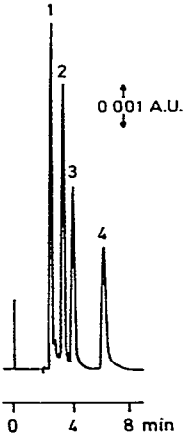


Fig. 5. Separation of sulphonic acids. Mobile phase: methanol–water–0.2 *M* potassium phosphate (pH 8.0) (60:15:25) containing 0.6 g/l of CTMA. Flow-rate, 1.0 ml/min. Peaks: 1 = sulphanilic acid; 2 = *p*-toluenesulphonic acid; 3 = 2-naphthalenesulphonic acid; 4 = anthraquinone-2-sulphonic acid; 5 = 9,10-dimethoxyanthracene-2-sulphonic acid.

Fig. 6. Separation of barbiturates. Mobile phase: methanol–water–0.2 *M* potassium phosphate (pH 8.0) (50:45:5) containing 0.80 g/l of CTMA. Flow-rate, 1.0 ml/min. Peaks: 1 = 5,5-diethylbarbituric acid; 2 = 5-allyl-5-ethylbarbituric acid; 3 = 5,5-dipropylbarbituric acid; 4 = 5-cyclohexenyl(1)-1,5-dimethylbarbituric acid; 5 = 5-ethyl-5-isopentylbarbituric acid; 6 = 5-ethyl-1-methyl-5-phenylbarbituric acid.

Applications

The separation of non-ionic compounds is demonstrated in Fig. 3, and Fig. 4 shows the separation of a primary, a secondary, a tertiary and a quaternary ammonium compound that differ only in having zero, one, two or three methyl groups on the nitrogen atoms. Increasing substitution results in increasing retention.



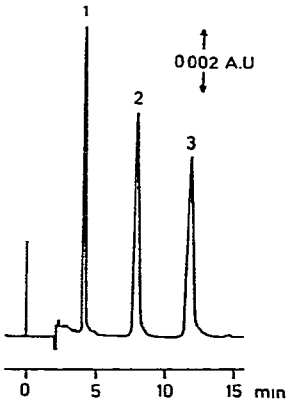


Fig. 7. Separation of carboxylic acids. Conditions as in Fig. 3. Peaks: 1 = *p*-aminobenzoic acid; 2 = acetylsalicylic acid; 3 = benzoic acid; 4 = salicylic acid.

Fig. 8. Separation of estrone and its conjugated metabolites. Mobile phase: methanol–water–0.2 *M* potassium phosphate (pH 7.7) (60:15:25) containing 0.80 g/l of CTMA. Flow-rate, 1.0 ml/min. Peaks: 1 = estrone 3-β-D-glucuronide; 2 = estrone; 3 = estrone 3-sulphate.

Fig. 5 shows the separation of some sulphonic acids, and the separation of barbituric acids has also been performed in the reversed-phase mode (Fig. 6).

When separating aromatic carboxylic acids (Fig. 7), benzoic acid is eluted later than acetylsalicylic acid, which can be explained by steric hindrance by the acetyl group, preventing good ion-pair formation. *para*-Substitution of the benzoic acid with an amino group leads to an even shorter retention time.

Fig. 8 shows the separation of estrone and two of its metabolites, the 3-sulphate and the 3-β-D-glucuronide.

This chromatographic technique has been used for analyses of drugs in biological samples, and direct injections of urine and solvent-precipitated serum samples (more than 1000 injections) on to the column have been performed with less than a 15% change in the performance of the column.

CONCLUSION

It is possible to separate non-ionic compounds using a kind of reversed-phase chromatography on unmodified silica gel at pH values in the aqueous phase above the pK_a value of the silica gel, when a long-chain asymmetric quaternary ammonium salt (*e.g.*, cetyltrimethylammonium bromide) is added to the mobile phase. The eluent should be saturated with silica.

A pure ion-exchange mechanism is probably involved when chromatographing a quaternary ammonium compound in such a system, but when the corresponding primary, secondary and tertiary amines are separated, the mechanism seems to be more complex and reversed-phase partition is assumed to play a part. Anionic compounds are also chromatographed well in the system.

If a monolayer of CTMA ions is dynamically adsorbed to the silica gel with the hydrocarbon chains pointing away from the silica surface, the results found may be explained by (1) reversed-phase partition between the hydrophobic layer on the silica gel and a more polar mobile phase, (2) cation exchange on the surface of the silica gel and (3) reversed-phase ion-pair chromatography of ion pairs between anions and CTMA ions.

If more than a monolayer of CTMA ions is adsorbed on the silica surface, one must assume that the molecules in the secondary layer will be oriented with the quaternary ammonium groups towards the mobile phase and the long hydrocarbon chains immersed in the hydrophobic part of the primary layer of CTMA ions. If this were so, one could expect a decrease in the retention time of cationic and non-ionic compounds proportional to the degree of secondary layer formed due to repulsive forces. The present experiments indicate that only a monolayer is formed.

One of the advantages of this approach, which is not more complicated to perform, over methods using chemically bonded reversed-phase materials is that much cheaper column materials are used. Further, the different methods for chemical derivatization of silica give reversed-phase materials that differ in selectivity. As the use of dynamically modified silica involves no derivatization step, this problem would be expected to be diminished.

Work is in progress to compare silica packings from different manufacturers and investigations on the amounts of long-chain quaternary ammonium compounds adsorbed on to the surface of silica gel are being carried out.

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