Journal of Chromatography, 204 (1981) 43–48
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,226

REVERSED-PHASE LIQUID–LIQUID COLUMN CHROMATOGRAPHY OF ORGANIC ACIDS WITH A COMPLEXING STATIONARY PHASE CONTAINING TRI-*n*-OCTYLPHOSPHINE OXIDE

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SUMMARY

A reversed-phase system for the liquid chromatographic separation of hydrophilic organic acids was developed. A solution of a strong hydrogen acceptor, tri-*n*-octylphosphine oxide, in *n*-decane was coated on LiChrosorb RP-8 as support, and aqueous buffer solutions were used as mobile phases. Three methods for loading the stationary phase on to the support were tested. The retention of the solutes can be described by a liquid–liquid distribution model, based on complex formation between the solutes and the hydrogen acceptor in the stationary phase. The retention and the separation selectivity can be regulated by the concentration of the hydrogen acceptor in the stationary phase and by the pH of the mobile phase.

INTRODUCTION

Hydrophilic acids are difficult to retain in reversed-phase liquid–liquid chromatography, both as acids and as ion pairs. A considerable increase in retention can be obtained by addition of a complexing agent, *e.g.* a strong hydrogen acceptor, to the stationary organic phase. The enhancement of the degree of extraction of carboxylic acids in liquid–liquid extractions after the addition of tri-*n*-octylphosphine oxide (TOPO) to the organic solvent has been studied[1]. In the present study, we used TOPO in the stationary phase of liquid–liquid chromatographic systems for the separation of organic acids of physiological importance.

Complexing stationary phases have been extensively used in the reversed-phase liquid–liquid chromatography of metal ions, often termed extraction chromatography[2]. The complexing agents have been neutral compounds, such as TOPO, or charged compounds such as long-chain amine salts and quaternary ammonium salts ("liquid ion exchangers"). The stationary phase has been either the pure complexing agent or a solution of it in an organic diluent, and the mobile phases have been aqueous solutions.

These reversed-phase systems have been applied in column chromatography as well as in paper and thin-layer chromatography, and have been used mainly for

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the separation of ionic compounds. Applications to the separation of organic substances seem to be few. Some compounds of biochemical origin[3], and sulphonic acids[4], were separated in columns with a "liquid ion exchanger" in the stationary phase. Similarly, steroidal glucosiduronic acids were separated by paper chromatography[5]. Aminophenols were separated with a stationary phase of di-(2-ethylhexyl)-phosphoric acid (HDEHP) in chloroform[6]. Rapid separations of sulphonic acids, carboxylic acids and phenols were obtained by high-pressure column chromatography with tri-*n*-octylamine as the stationary phase[7].

Up to now, most of the column chromatographic work has been done with supports of large particle diameter, which give columns of relatively low efficiency. However, Kraak and Huber[7] used a 5–10-μm support, which was dry packed after coating with the stationary phase. Horwitz *et al.*[8,9] made very efficient columns for the separation of radioactive metal ions by coating HDEHP dissolved in dodecane on a 5-μm support, the coated support being slurry packed (suspended in the eluent) by a rather specialized technique. Modern microparticulate liquid chromatographic supports are usually slurry packed[10] suspended in organic liquids that are miscible with an organic stationary phase. This means that the stationary phase has to be coated on the already packed column by *in situ* methods. This has been done with the neutral extractant tributyl phosphate, which was coated as the undiluted liquid on a 5-μm support for the separation of carboxylic acids[11] and catecholamines[12].

In the present work, we have been able to apply, by *in situ* coating, a stationary phase consisting of a solution of the neutral organophosphorus extractant TOPO in a hydrocarbon; the support was a hydrophobic microparticulate silica. We report the retention of aromatic carboxylic acids and phenol. One of the advantages of this separation system is that it can be extended to other types of complexing agents dissolved in the stationary phase, whereby the separation selectivity can be changed and different types of solutes can be retained. Further, the retention can be varied within wide limits by altering the concentration of the complexing agent.

CHROMATOGRAPHIC SYSTEM

Our system has LiChrosorb RP-8 (7 μm) as support, a solution of TOPO in *n*-decane as stationary phase and aqueous buffer solutions with different pH (ionic strength 0.1) as mobile phase. The columns are of length 150 mm and I.D. 4.6 mm.

COATING OF THE SUPPORT WITH STATIONARY PHASE

Three techniques were tested for the coating of the support with the stationary phase: pumping, injection and precipitation.

In the first method (*cf.* ref. 13), the column is equilibrated with the stationary phase by pumping (*ca.* 50 ml); the mobile phase is then pumped through the column until the excess of stationary phase is removed. With decane as stationary phase, it took about 1 week before the system was stable. The amount of stationary phase deposited in the pores of the support was very reproducible, but the method was limited to stationary phases with relatively low viscosity.

Injection of small volumes of stationary phase while the mobile phase was

being pumped (*cf.* ref. 11) was a rapid coating technique. It gave, however, unstable systems with decreasing capacity ratios and a much lower retention than systems prepared by the pumping method.

In the precipitation method (*cf.* ref. 14), the column was first equilibrated with a solution of the stationary phase in acetone (3:1). The mobile phase was then pumped through the column; the acetone dissolved and the stationary phase was precipitated on the support. With this method, only a low degree of coating could be obtained.

A fourth method, based on adsorption of the stationary phase from the mobile phase[15], was not usable, as the solubility of the stationary phase in the eluent was too low.

The results presented below were obtained on columns prepared by the pumping method. So far, the columns have been used only over short periods (not more than 10 days). The systems were stable with respect to the volume of the mobile phase (V_m). Thus, the phase ratio (V_s/V_m) can be considered constant (V_s = volume of stationary phase). The capacity ratios changed by less than 1% per day.

RETENTION

Model

When an acidic solute (HX) is retained by distribution between an aqueous mobile phase and a stationary organic liquid phase containing a complexing agent (TOPO), the capacity ratio can be expressed by:

$$k'_{HX} = \frac{V_s}{V_m} \cdot \frac{K_{D(HX)} \cdot K_{HXTOPO_n} \cdot [TOPO]_o^n}{(1 + K'_a/a_{H^+})} \quad (1)$$

(The subscript o refers to the organic phase; symbols without subscript relate to the aqueous phase.)

where $K_{D(HX)} = \frac{[HX]_o}{[HX]}$ is the distribution constant, and $K_{HXTOPO_n} =$

$$= \frac{[HXTOPO]_o}{[HX]_o \cdot [TOPO]_o^n}$$

is the formation constant of the complex between the solute and the complexing agent. The number of TOPO molecules in the complex is n. The model is based on the assumption that one single complex is formed, which contains as many TOPO molecules as the number of hydrogen-donating groups in the solute.

According to this model, the retention can be regulated by (1) the nature and concentration of the complexing agent and (2) the pH of the aqueous phase.

Results

The effect of TOPO in the stationary phase is demonstrated in Fig. 1A. A linear relationship is found between $\log k'_{HX}$ and $\log [TOPO]_o$. This is in accordance with eqn. 1, which can be transformed into:

$$\log k'_{HX} = \log K + n \cdot \log [TOPO]_o \quad (2)$$

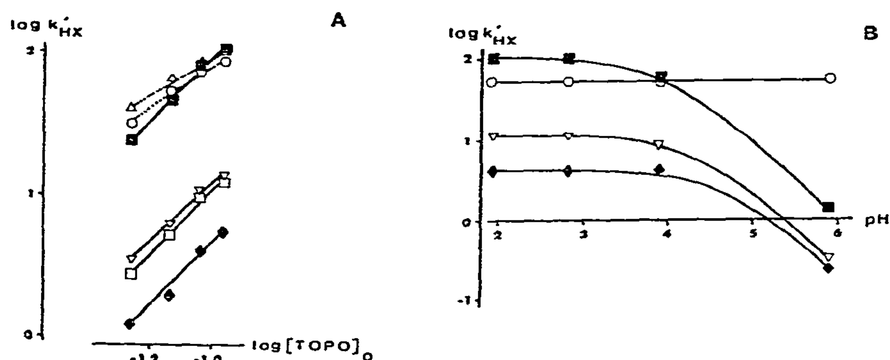

Fig. 1. Influence of TOPO (A) and pH (B) on the retention. Solutes: △, benzoic acid; ○, phenol; ■, 3-hydroxybenzoic acid; ▽, 3-hydroxy-4-methoxybenzoic acid; □, 4-hydroxyphenylacetic acid; ⧫, 5-hydroxyindole-3-acetic acid. A: Stationary phase, TOPO in decane; eluent, formate buffer solution of pH 3.9. B: Stationary phase, 0.1 *M* TOPO in decane; eluents, phosphate and formate buffer solutions.

in which

$$K = \frac{V_s}{V_m} \cdot \frac{K_{D(HX)} \cdot K_{HXTOPO_n}}{(1 + K'_a/a_{H^+})}$$

The slope of the line represents n, and $K_{D(HX)} \cdot K_{HXTOPO_n}$ can be evaluated from the intercept using the known values of a_{H^+} and K'_a.

The results are presented in Table I, which also gives results obtained by batch-extraction experiments with the same liquid phases. There is a fairly good agreement between the values of n and of log $K_{D(HX)} \cdot K_{HXTOPO_n}$ obtained by the chromatographic and by the batch-extraction experiments, indicating that the retention is mainly governed by liquid–liquid distribution. The found and the expected values of n also agree well. For benzoic acid and phenol, each containing one hydrogen-donating group, n is close to unity, whereas it rises to *ca.* 2 when a phenolic and a carboxylic group are present. The indole function in 5-hydroxyindole-3-acetic acid, which might be weakly hydrogen donating, does not affect the value of n.

TABLE I

COMPLEX FORMATION WITH TOPO

Chromatographic conditions: $V_s/V_m = 0.7$; pH 3.9.

Solute	*log $K_{D(HX)} \cdot K_{HXTOPO_n}$*		*Number of TOPO molecules in complex (n)*		
	Chrom.	*Batch*	*Chrom.*	*Batch*	*Expected*
5-Hydroxyindole-3-acetic acid	3.14		2.3		2
4-Hydroxyphenylacetic acid	3.59	3.56	2.2	2.0	2
3-Hydroxy-4-methoxy-benzoic acid	3.41		2.0		2
3-Hydroxybenzoic acid	4.54	4.81	2.2	2.0	2
Benzoic acid	3.64		1.3		1
Phenol	3.46		1.4		1

The retention and the separation selectivity depend on the complex formation constants and on n.

Fig. 1B shows the relationship between the retention of the acids and the pH of the mobile phase. The carboxylic acids are protolyzed on increase of pH, and, in accordance with the model, the capacity ratio then decreases. The retention of the weak acid phenol ($pK'_a = 9.9$) is not affected in the pH region studied. The pK'_a values of the solutes can be estimated from the retention data in Fig. 1B by use of equation 1. The values found (Table II) are in fairly good agreement with the literature values.

Further details on the chromatographic system and on the liquid–liquid distribution of organic acids with TOPO in the organic phase will be given in forthcoming papers.

TABLE II

PROTOLYSIS OF SOLUTES

Temperature: 25°C. Ionic strength: 0.1. The literature values are corrected for the ionic strength.

Solute	*pK'_a*	
	Chrom.	*Literature*[16]
5-Hydroxyindole-3-acetic acid	4.65	—
4-Hydroxyphenylacetic acid	4.38	—
3-Hydroxy-4-methoxybenzoic acid	4.35	4.38
3-Hydroxybenzoic acid	4.07	3.98
Phenol	—	9.90

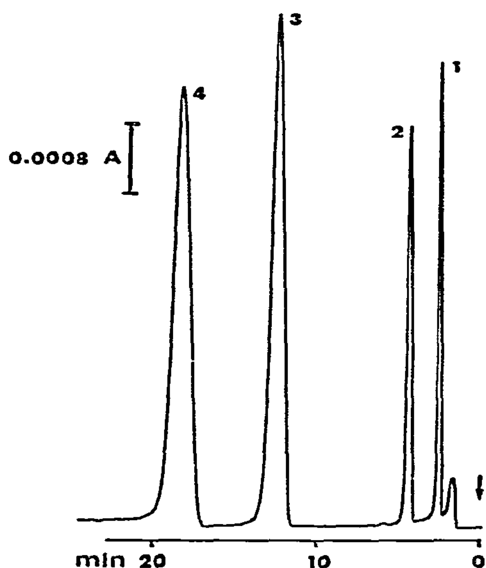

Fig. 2. Separation of hydrophilic aromatic acids. Stationary phase: 0.15 *M* TOPO in *n*-decane. Mobile phase: phosphate buffer solution of pH 1.8; 0.8 ml/min. Solutes: 1 = 4-hydroxy-3-methoxymandelic acid; 2 = 3,4-dihydroxymandelic acid; 3 = 3-hydroxymandelic acid; 4 = 5-hydroxyindole-3-acetic acid.

SEPARATIONS

The separation of some hydrophilic aromatic acids is demonstrated in Fig. 2. The mandelic acid derivatives, differing only in the number of aromatic hydroxy groups, are well separated.

CONCLUSIONS

(1) The pumping method is suitable for the coating of LiChrosorb RP-8 with a hydrophobic liquid stationary phase.

(2) The retention of acidic solutes can be described by a liquid–liquid distribution model, based on complex formation between the solutes and TOPO in the stationary phase.

(3) The retention depends on the number of TOPO molecules in the complex and on the magnitude of the complex formation constant. This gives possibilities for controlling retention and selectivity.

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