Journal of Chromatography, 208 (1981) 78–82
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

Note

Reversed-phase ion-pair chromatography of tetracyclines on a LiChrosorb NH_2 column

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(Received November 19th, 1980)

Tetracyclines are strongly adsorbed on reversed-phase columns having bonded alkyl chains[1]. The addition of alkylammonium ions to the mobile phase has been shown to result in competition with the solutes for adsorption sites on the supports, leading to an increase in peak symmetry and column efficiency[2,3]. Moreover, the addition of ammonium compounds to the mobile phase has been shown to be a powerful tool for the regulation of the retention and thus for optimization of the separation conditions[1].

The aim of the present work was to study the retention behaviour and selectivity in a chromatographic system using a support having a chemically bonded amino group, LiChrosorb NH_2, for the separation of tetracyclines, under conditions where the amino group is protonated. The tetracyclines were retained as ion pairs with 1-hydroxy-2,3-diisobutylbenzenesulphonic acid (HIBS) dissolved in mobile phases comprised of acetonitrile in 10^{-1} *M* phosphoric acid.

EXPERIMENTAL

Apparatus

The pump was an LDC 711 solvent delivery system. The columns were of stainless steel (150 mm × 4 mm I.D. × $\frac{1}{4}$ in.) and equipped with modified Swagelok® connectors and Altex stainless-steel frits (2 μm).

The chromatographic detector used was an LDC Spectromonitor III with 10.0-mm path length and a cell volume of 8 μl, operating at 357 nm. A Rheodyne (Model 70-10) injection valve with a sample loop of 100 μl was used.

Chemicals

The LiChrosorb RP-8 and LiChrosorb NH_2 supports (E. Merck, Darmstadt, G.F.R.) had mean diameters of 5 and 10 μm, respectively.

The mobile phase contained acetonitrile (E. Merck, Uvasol) and phosphoric acid (E. Merck, p.a.) in a final concentration of 10^{-1} *M*. In some experiments 1-hydroxy-3,5-diisobutylbenzenesulphonic acid (HIBS), kindly supplied by Dr. P. O. Lagerström, AB Hässle (Mölndal, Sweden), was used as counter ion in the mobile phase.

Tetracycline (ACO Läkemedel AB, Solna, Sweden), chlortetracycline (Glaxo

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Läkemedel AB, Mölndal, Sweden), demeclomycine (Cyanamid Nordiska AB, Stockholm, Sweden), doxycycline (AB Ferrosan, Malmö, Sweden), metacycline (Roerig AB, Täby, Sweden) and oxytetracycline (Pfizer AB, Täby, Sweden) were used as obtained from the suppliers.

A 1-μg amount of each tetracycline dissolved in 10 μl of 10^{-1} *M* phosphoric acid was injected into the liquid chromatograph.

Chromatography

The chromatographic columns were packed by the slurry packing technique using glycerol–methanol (1:3) as suspension medium[4]. The slurry was forced into the column at a flow-rate of 9 ml/min or a pressure of 5000 p.s.i., whichever was the limiting factor. The mobile phases were passed through the chromatographic system until constant retention was obtained. Usually less than 50 ml were required. The experimental temperature was 25 $\pm$ 0.5°C throughout this study. In all experiments a flow-rate of 1.0 ml/min was used.

The interstitial volume of the columns was determined by injection of 1 μl of 1.0 *M* phosphoric acid or 1 μl of acetonitrile. All values of the capacity factor presented are the means from at least three determinations.

The concentration of HIBS in the mobile phase was determined spectrophotometrically at 285 nm. The molar absorptivity of HIBS in a mobile phase containing 20% acetonitrile was $3.43 \cdot 10^3$ l·mol^{-1}·cm^{-1}.

RESULTS AND DISCUSSION

Retention of tetracyclines with HIBS in mobile phase

The tetracyclines were not retarded on the LiChrosorb NH_2 support when mobile phases containing 0–90% acetonitrile in 0.1 *M* phosphoric acid were used. The tetracycline peak even appeared in front of the solvent peak, *e.g.*, when the mobile phase contained 20% acetonitrile the tetracycline and solvent peaks had retention volumes of 0.82 and 1.11 ml, respectively. Under these conditions the ammonium groups on the support are protonated[5]. By the addition of a counter ion, HIBS, to the mobile phase the tetracyclines could be retarded on the LiChrosorb NH_2 column (Fig. 1).

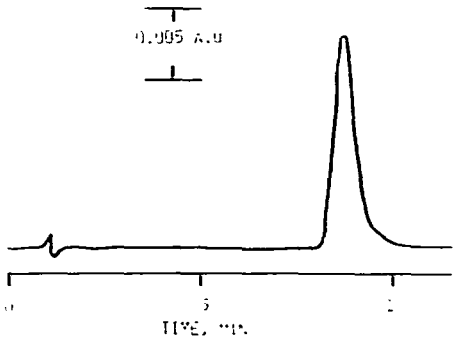

Fig. 1. Chromatogram of oxytetracycline on LiChrosorb NH_2. Mobile phase: 10% acetonitrile in 0.1 *M* phosphoric acid with $4 \cdot 10^{-3}$ *M* HIBS. Other conditions as in the Experimental.

The retention of the tetracyclines was strongly dependent upon concentration of HIBS in the mobile phase, as studied with a mobile phase containing 10% acetonitrile as organic modifier (Fig. 2). The retention order was similar to that obtained using reversed-phase supports with chemically bonded alkylcarbon chains[1].

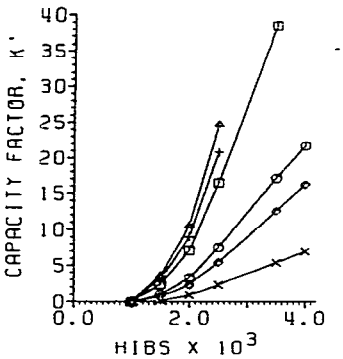

Fig. 2. Dependence of retention on the concentration of HIBS (*M*) in the mobile phase. Support: LiChrosorb NH_2. Mobile phase: 10% acetonitrile in 0.1 *M* phosphoric acid with addition of HIBS. Solutes: □, chlortetracycline; ○, demeclomycin; △, doxycycline; +, metacycline; ×, oxytetracycline; ◇, tetracycline. Other conditions as in the Experimental.

The selectivity of the chromatographic system showed a slight increase with decreasing concentration of HIBS in the mobile phase. The retardation of tetracyclines as ion pairs with HIBS decreased with increasing concentration of acetonitrile in the mobile phase. This effect could be compensated for by increasing the concentration of HIBS (Table I): approximatively the same capacity factors were obtained in systems with 0% acetonitrile–10^{-3} *M* HIBS and 10% acetonitrile–$2 \cdot 10^{-3}$ *M* HIBS, respectively. On further increase of the acetonitrile concentration to 20%, a 10^{-2} *M* solution of HIBS was required to yield capacity factors of the same order of magnitude.

The retention of the tetracyclines on the LiChrosorb NH_2 support when HIBS had been added to the mobile phase may be due to at least two effects: an increase of the hydrophobicity of the support surface as the result of ion-pair formation of the amino groups of the support and HIBS in the mobile phase; formation of HIBS–tetracycline ion pairs which are adsorbed on the solid phase.

TABLE I
RETENTION OF TETRACYCLINES AS ION PAIRS WITH HIBS

Tetracycline	*Capacity factor, k'*		
	0% Acetonitrile–10^{-3} M HIBS	*10% Acetonitrile–$2 \cdot 10^{-3}$ M HIBS*	*20% Acetonitrile–10^{-2} M HIBS*
Chlortetracycline	7.6	7.1	11.8
Demeclomycine	3.6	3.2	6.6
Doxycycline	11.5	10.5	15.1
Metacycline	9.9	9.0	13.4
Oxytetracycline	0.70	0.91	2.7
Tetracycline	3.1	2.3	4.7

Adsorption of acetonitrile

On alkyl-bonded reversed-phase supports the amount of adsorbed modifier (acetonitrile, methanol, etc.) increased with increasing concentration in the mobile phase, with a concomitant decrease of the interstitial volume of the columns[6,7]. In contrast to the results obtained with the LiChrosorb RP-8 column, no variation of the interstitial volume with the concentration of acetonitrile in the mobile phase was found for the LiChrosorb NH_2 column (Table II). It can therefore be concluded that acetonitrile is adsorbed only to a minor extent on the LiChrosorb NH_2 support.

TABLE II
EFFECT OF CONCENTRATION OF ACETONITRILE IN MOBILE PHASE ON INTERSTITIAL VOLUME

Acetonitrile in mobile phase (%)	*Interstitial volume, V_m (ml)**	
	LiChrosorb NH_2	*LiChrosorb RP-8*
0	1.06 ± 0.01	1.36 ± 0.01
20	1.11 ± 0.01	1.14 ± 0.01
80	1.09 ± 0.02	0.797 ± 0.006

* Mean ± standard deviation (n = 20).

Adsorption of HIBS

The coating of the LiChrosorb NH_2 with HIBS was followed by spectrophotometric measurement of the mobile phase after passage through the chromatographic column. The amount of adsorbed HIBS decreased with increasing concentration of acetonitrile in the mobile phase (Fig. 3). The coating process is fast, and seems to be complete after passage of less than ten column volumes of mobile phase, *cf.*, ref. 6.

The amount of titratable amino groups was $9.8 \cdot 10^{-4}$ mole per gram of support as determined by potentiometric titration of LiChrosorb NH_2 with 0.1 M HCl. It can therefore be concluded that under the conditions used in Fig. 3 the ammonium groups on the support are masked to only a minor extent by adsorbed HIBS.

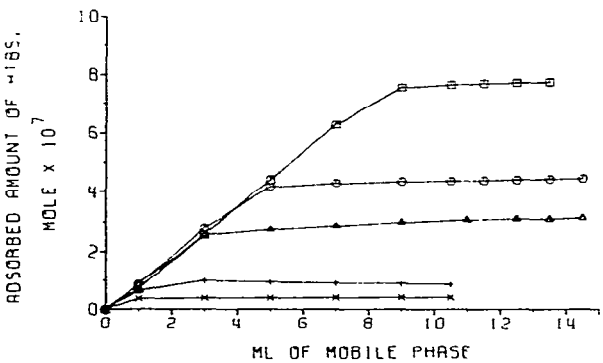

Fig. 3. Adsorption of HIBS to the support LiChrosorb NH_2 (0.567 g). Mobile phase: 10^{-4} M HIBS and acetonitrile in 0.1 M phosphoric acid. Concentration of acetonitrile in mobile phase: □, 0%; ○, 5%; △, 10%; +, 40%; ×, 60%. Other conditions as in Experimental.

CONCLUSIONS

Reversed-phase ion-pair chromatography on a support with an amino phase bonded to the silica gel (LiChrosorb NH_2) has proved to be a suitable technique for the isolation of tetracyclines. The capacity factor was easily regulated by the concentration of acetonitrile and/or the concentration of counter ion in the mobile phase.

ACKNOWLEDGEMENT

The author thanks Dr. Hans Ehrsson for valuable discussions of the manuscript. The excellent technical assistance of Miss Britta Ekqvist is gratefully acknowledged.

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