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MECHANISMS OF SEPARATION USING THE TERNARY MIXTURE DICHLOROMETHANE–ETHANOL–WATER IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The mechanisms of separation using the ternary system dichloromethane–ethanol–water in high-performance liquid chromatography were investigated. The use of an eluent saturated with water results in a separation based on partition of the component between a polar stationary phase, held in the pores of the porous support, and an apolar mobile phase percolating through the packed bed.

Selective separations according to an adsorption mechanism are obtained by using an eluent partially saturated with water, corresponding to a point outside the miscibility gap of the three solvents.

INTRODUCTION

In liquid–liquid partition chromatography, the mobile phase must be immiscible with the stationary phase. The use of solvent pairs with a large polarity difference follows as a matter of course. Huber and co-workers[1–4] introduced an elegant solution by using coexisting phases, formed by ternary mixtures of 2,2,4-trimethylpentane, ethanol and water. By an appropriate choice of the overall composition of the ternary mixture, the polarity differences between the phases and therefore the partition coefficients and selectivity can be varied over a wide range. Huber and co-workers[1,2] described an *in situ* coating technique, in which the apolar phase is pumped through a column filled with the bare support and the conjugated polar phase is presumably

deposited on this support. Part of the internal pore volume of the packing material is filled with the polar stationary phase. Essentially, the phase ratio is determined by the ratio of the interparticle (ε_i) and intraparticle (ε_u) void fractions.

Meijers *et al.*[5] used such a system for the assay of cortisol in plasma. A practical disadvantage of this method is the low solubility of corticosteroids in the mobile phase. Further, the high values of the partition coefficients require the use of packing materials of low internal volume.

Hesse and Hövermann[6] used an apolar layer of a ternary mixture of dichloromethane, ethanol and water as the eluent in a number of separation problems. In particular, they described a method for the determination of some corticosteroids in plasma, this eluent being a good solvent for corticosteroids[7]. It is assumed that a conjugated phase is deposited on the packing and that separations are based on partition between the apolar eluent and the polar conjugated phase in the pore system of the support (silica gel in this instance).

As we were interested in a routine procedure for the chromatographic determination of dexamethasone and cortisol in serum, we decided to evaluate the method of Hesse and Hövermann[6] for this purpose. However, columns were found to be unstable and a gradual decrease in the capacity ratios was observed. Similar results were obtained by Parris[8].

Therefore, it seemed useful to make a more detailed study of the behaviour and particularly of the separation mechanism in order to explain these findings. Several phase systems, both inside and outside the miscibility gap, were investigated and their chromatographic properties determined.

EXPERIMENTAL

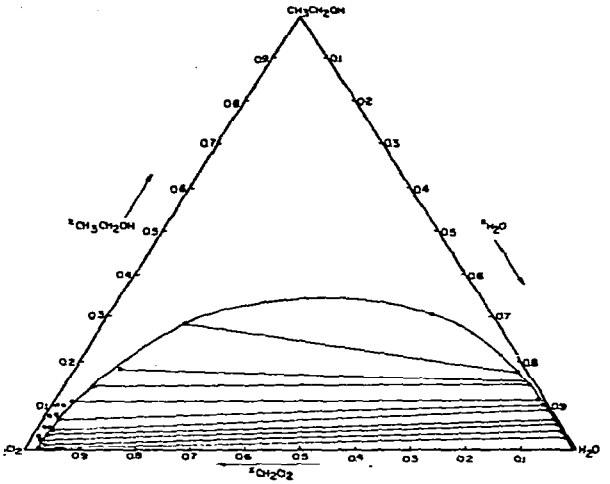
The liquid chromatograph was constructed in our laboratory and has been described in detail elsewhere[9,10]. LiChrosorb SI 60 (Merck, Darmstadt, G.F.R.) was used as the packing material and the columns were filled by the balanced-density slurry method. In a number of experiments glass columns of length 30 cm and I.D. 0.4 mm were used in order to observe optical changes in the packing material. These columns can be operated safely only at low pressures, and therefore 10-μm packings were used. Other experiments were carried out in 30 cm $\times$ 0.45 cm I.D. stainless-steel 316 columns filled with 5-μm packing materials. The columns were activated by the procedure described by Scott and Kucera[11].

The column and UV detector were thermostatted by using a circulating liquid. The container holding the eluent was also thermostatted within 0.1°.

All solvents used were obtained from Merck (pro analysi grade). With binary mixtures, the eluent was maintained at constant temperature for 24 h to ensure complete demixing. The conjugated polar phase was not removed from the eluent container when pumping the apolar phase through the column.

Partition coefficients of steroids were measured as described by Huber *et al.*[2].

Compositions of coexisting phase pairs were measured by gas chromatography. Fig. 1 shows the phase diagram for the ternary system dichloromethane–ethanol–water.



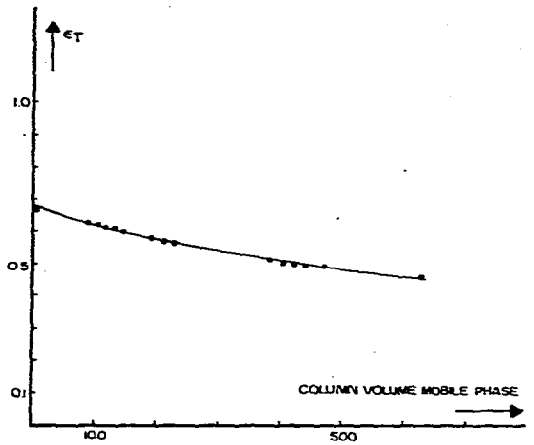


Fig. 1. Phase diagram for the ternary system dichloromethane–ethanol–water at ambient temperature. The dots outside the miscibility gap indicate the composition of systems 5–11 (see Table II).

Fig. 2. Total porosity *versus* number of column volumes of mobile phase pumped through the column.

RESULTS AND DISCUSSION

In a series of experiments, four phase systems (1–4 in Table I) having an overall composition within the miscibility gap were tested. The procedure for the activation of the silica gel according to Scott and Kucera[11] was completed by pumping dry dichloromethane through the column, followed by a mixture of dry dichloromethane and dry ethanol (50 ml) and finally a composition of dichloromethane, ethanol and water corresponding to the binodal curve in the phase diagram.

When the apolar phase was pumped through the column, the total porosity ε_T (internal and external void fraction) was determined as a function of the total pumped volume. The value of ε_T was calculated from the retention time of an inert component, t_{R0}, the observed volume flow-rate, F, and the volume of the empty column, V_0:

$$\varepsilon_T = \frac{F}{V_0} \cdot t_{R0}$$

TABLE I

COMPOSITION OF CONJUGATED PAIRS

Values are given in molar fractions (x).

Phase system	*Polar phase*			*Apolar phase*		
	x_{H_2O}	$x_{CH_3CH_2OH}$	$x_{CH\ Cl_2}$	$x_{H\ O}$	$x_{CH\ CH\ OH}$	$x_{CH\ Cl}$
1	0.969	0.031	0.0001	0.013	0.049	0.938
2	0.957	0.043	0.0004	0.017	0.055	0.928
3	0.958	0.042	0.0002	0.018	0.055	0.927
4	0.928	0.007	0.0012	0.035	0.152	0.813

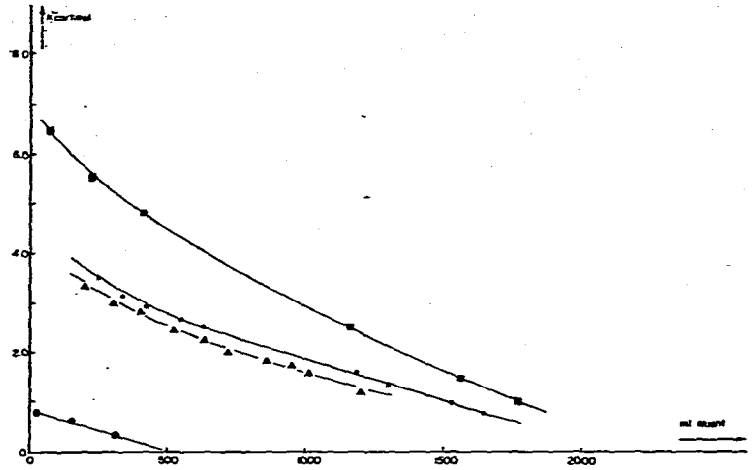


Fig. 3. Capacity factors of cortisol *versus* volume of mobile phase pumped through the column for phase systems 1 (■), 2 (•), 3 (▲) and 4 (●) (see Table I).

A regular decrease in ε_T was observed (Fig. 2). By measuring ε_T, the *in situ* coating can be followed.

The capacity ratios for some corticosteroids were determined at regular intervals; the results are shown in Fig. 3. Fig. 4 gives the chromatograms obtained after 70 and 520 column volumes, respectively.

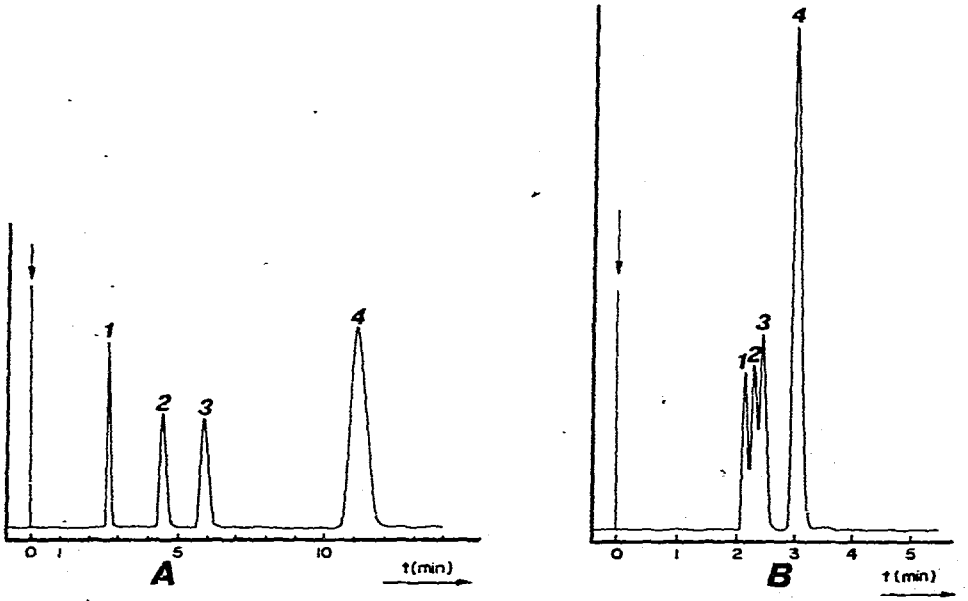


Fig. 4. Chromatogram of a text mixture of four steroids: (A) after 70 column volumes of mobile phase; (B) after 520 column volumes of mobile phase. Conditions: column, 300 × 4 mm I.D. glass; LiChrosorb Si 60, $\bar{d}_p = 10\,\mu$m; phase system, 2 (see Table I); volume flow-rate, $F = 0.95$ cm³/min; UV detection at 240 nm, 0.1 a.u.f.s. Peaks: 1 = progesterone; 2 = corticosterone; 3 = cortisone; 4 = cortisol.

Glass-walled columns were used for these experiments and we observed a distinct change in the appearance of the packing material during this pumping procedure. At first, the whole column packing showed a certain transparency. After pumping about 100 column volumes, the colour of the packing on the top of the column changed to white and this zone moved slowly down the bed as more eluent was pumped through the column. As soon as the white front reached the end of the column, separation was completely lost and the total porosity, ε_T, had decreased to a value of 0.4. The column could be regenerated by washing with acetone (30 ml) and dichloromethane (150 ml); the original value of ε_T was restored and the packing regained its original transparency.

The observed decrease in the column porosity, ε_T, corresponds to the deposition of a liquid phase in the pore system (pore condensation). The maximum column loading is obtained when ε_T reaches a value of 0.4.

Having obtained these results, it was decided to determine the partition coefficients of some steroids in separate batch experiments. For partition coefficients lower than unity and phase ratios nearly equal to unity, capacity ratios are expected to be small. It is chemically inexplicable that Hesse and Hövermann[6] and others[7,12,13] obtained separations with a liquid–liquid partition mechanism because the distribution is not in favour of the polar stationary phase. From these low values, which will result in capacity factors of less than unity, even for favourable phase ratios, it can be concluded that a separation of steroids by liquid–liquid partition chromatography is not possible.

Good and stable separation conditions, however, could be obtained by working under adsorption chromatographic conditions. These were realized by using an eluent that was only partially saturated with water. Therefore, a water-saturated mixture of dichloromethane and ethanol, corresponding to a point on the binodal curve of the phase diagram, was added to a water-free mixture of the same solvents in the same molar ratio (Table II).

The influence of the degree of saturation with water on retention was small,

TABLE II

COMPOSITION OF MOBILE PHASES OUTSIDE THE MISCIBILITY GAP

Values are given in molar fractions (x).

Degree of saturation (%)	*Composition of mobile phase*			*Phase system*
	$x_{CH_2Cl_2}$	$x_{CH_3CH_2OH}$	x_{H_2O}	
100	0.940	0.035	0.025	
40	0.960	0.030	0.010	5
100	0.930	0.047	0.023	
87	0.930	0.050	0.020	6
43	0.940	0.050	0.010	7
100	0.902	0.075	0.023	
87	0.906	0.074	0.020	8
43	0.915	0.075	0.010	9
100	0.859	0.110	0.030	
67	0.882	0.098	0.020	10
33	0.890	0.100	0.010	11

TABLE III

RETENTION CHARACTERISTICS FOR PHASE SYSTEMS OUTSIDE THE MISCIBILITY GAP

Phase system	*k'*		
	Corticosterone	*Cortisone*	*Cortisol*
5	0.54	0.79	1.86
6	0.80	1.32	3.34
7	1.14	1.46	3.87
8	0.61	0.79	1.87
9	0.70	0.70	1.76
10	0.02	0.02	0.33
11	0.27	0.27	0.60

especially at higher ethanol contents. When enough water was present to deactivate the strong hydroxyl sites on the silica gel, the selectivity is mainly determined by the ethanol content. The influence of the molar fraction of ethanol on the retention behaviour was measured (Table III). Fig. 5 shows an efficient separation (HETP = 20–25 μm for all components) of a test mixture of steroids, and indicates the excellent performance of adsorption chromatography using a eluent system consisting of dichloromethane, ethanol and water.

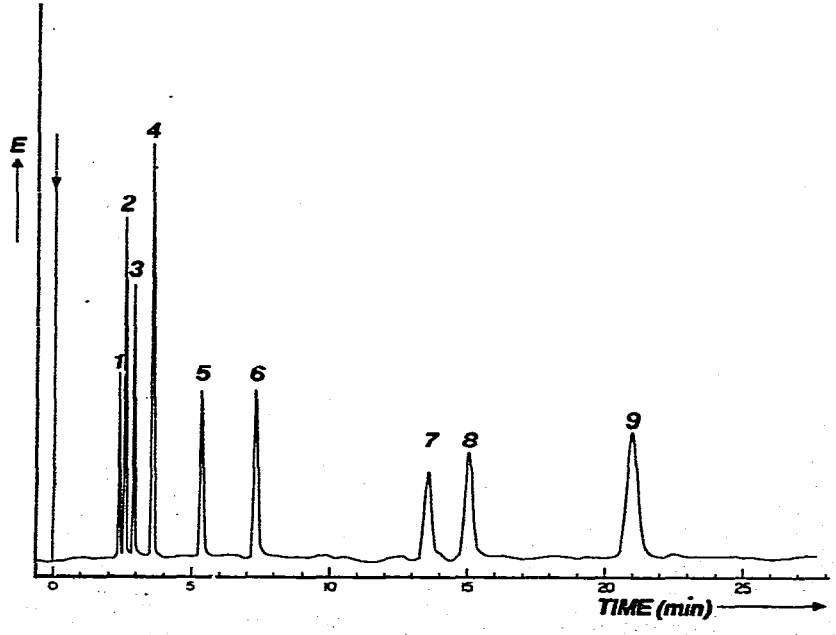


Fig. 5. Chromatogram of a test mixture of steroids. Conditions: column, stainless-steel 316, 300 × 4.6 mm I.D.; adsorbent, LiChrosorb SI 60, $\bar{d}_p = 5\,\mu$m; phase system, 7 (see Table II); volume flow-rate, 1.5 cm³/min; $\bar{u} = 0.2$ cm/sec; UV detection at 240 nm, 0.1 a.u.f.s. Peaks: 1 = monochlorobenzene; 2 = progesterone; 3 = 11-desoxycorticosterone; 4 = testosterone; 5 = corticosterone; 6 = cortisone; 7 = dexamethasone; 8 = cortisol; 9 = prednisolone.

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