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Note

Investigation of conditions of the preparative liquid chromatographic separation of cardiac glycosides present in *Digitalis lanata*

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Digitalis lanata contains glycosides constituting important pharmaceutical agents and for this reason their analysis by high-performance liquid chromatography (HPLC) has been described in numerous papers[1–5]. However, there are no examples of preparative-scale separations in larger columns.

From point of view of the requirements for large-scale preparative chromatography, *e.g.*, with columns of 100 mm I.D., particular attention should be paid to the systems described by Erni and Frei[5], which comprised silica gel as a stationary phase and methylene chloride–methanol–water as a mobile phase; glycosides are highly soluble in the mobile phase and both phases are readily available and economically priced. These authors altered the eluting strength of the mobile phase depending on the glycosides to be separated by varying the amount of methanol added to methylene chloride saturated with water (*ca.* 0.18%). An example of a separation of certain groups of cardiac glycosides using a mobile phase containing as much as 1.2% of water was also reported. However, the rôle of water in this system and the rules for selecting its content were not elucidated.

The purpose of the present work was to determine the effect of water on the selectivity, efficiency and especially the loadability of the discussed systems.

EXPERIMENTAL

Apparatus

A Model KB-51 liquid chromatograph KABiD-ZOPAN (Warsaw, Poland) equipped with 25 cm × 4.6 mm I.D. column and a UV-254 detector with maximum sensitivity of 0.01 a.u. was employed. The preparative chromatographic unit comprised a 50 cm × 100 mm I.D. stainless-steel column and two pumps with an output of 135 l/h at pressures up to 40 atm, one delivering the mobile phase and the other the sample solution. This unit was equipped with an automatic system of sampling valves and fraction collector valves and a UV-254 detector with a maximum sensitivity of 0.01 a.u. connected to the bypass line.

Analytical columns were packed with LiChrosorb 60 (E. Merck, F.R.G.), particle diameter, d_p = 10 μm, using a suspension method. Preparative columns were filled with silica gel Si 60 (E. Merck), d_p = 40–63 μm, by a dry method.

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Reagents

The mixtures of lanatosides were obtained by preconcentration and partial purification of a methanol extract from *Digitalis lanata*. Mixture I comprised 22% of lanatoside A, 12% of lanatoside B and 64% of lanatoside C. Mixture II contained 65, 12 and 17% of lanatosIides A, B and C, respectively. The remaining mixtures comprised less polar impurities and water. Methylene chloride (pure) was purified by distillation over potassium bicarbonate and calcium chloride. The methanol used was of analytical reagent grade.

Procedure

Lanatoside standards and their mixtures were accurately weighed and dissolved in mobile phases prior to use. The concentrations of the mixtures and individual components in the solutions are listed in Table I. The aim of such a selection of concentrations was to enable a high loading of the system with a sample while maintaining identical concentrations of one of the components (LC) in all solutions.

The solvents were dried over molecular sieve 5A and used for the preparation of solutions containing 9% methanol and selected amounts of water and methylene chloride.

All data presented in the diagrams are the averages from at least four independent experiments. The method based on the segment determined on the baseline of peaks by tangents to inflection points was selected as the most suitable one for calculating the number of theoretical plates in columns.

TABLE I

THE CONTENT OF INDIVIDUAL COMPONENTS IN SOLUTIONS

Solution	*Content of component in solution (g/μl)*		
	Lanatoside A(LA)	*Lanatoside B(LB)*	*Lanatoside C(LC)*
4% of mixture I	$8.8 \cdot 10^{-6}$	$4.8 \cdot 10^{-6}$	$2.5 \cdot 10^{-5}$
15% of mixture II	$9.7 \cdot 10^{-5}$	$1.8 \cdot 10^{-5}$	$2.5 \cdot 10^{-5}$
2.5% of LA, LB, LC standards		$2.5 \cdot 10^{-5}$	

RESULTS AND DISCUSSION

It follows from the chromatograms presented in Fig. 1 that the separation of lanatoside mixtures depends on the presence of water in the system. The separations in the systems described previously[5], in which the mobile phase was prepared using methylene chloride saturated with water, *i.e.*, containing *ca.* 0.2% water, were admittedly better than separations in anhydrous systems, yet increasing the water content to close to saturation of the mobile phase yields much better results. This is due to the effect of water on the selectivity and efficiency of the system (Figs. 3 and 4). In contrast, a decrease of the methanol content in an anhydrous system results merely in an increase in all capacity coefficients without a change in the selectivity and efficiency of the system.

The increase in water content brings about a substantial increase in capacity

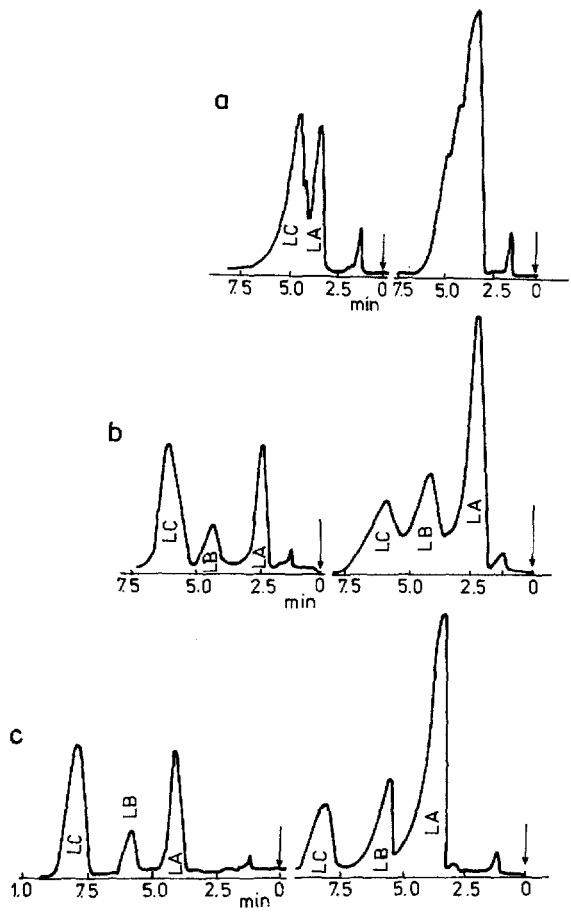

Fig. 1. Separation of lanatoside mixtures I and II. Column: 25 cm × 4.6 mm I.D. packed with LiChrosorb, 10 μm. Sample size: 100 μl of a 4% solution of mixture I and 100 μl of a 15% solution of mixture II. Mobile phases: a, methylene chloride–methanol (91:9); b, methylene chloride–methanol–water (90.8:9:0.2); c, methylene chloride–methanol–water (90:9:1). Flow-rate: 2 cm^3/min.

(Fig. 2) and selectivity coefficients (Fig. 3) with a simultaneous increase in the efficiency of the system (Fig. 4). The influence of water on the selectivity and efficiency parameters is most pronounced in the case of the most polar lanatoside C.

On the basis of the results reported it can be presumed that this is not a typical adsorption system. In order to confirm the partition mechanism of interfacial mass transfer, lanatosides were separated in a system in which the mobile phase was saturated with water (1.2%), whereas the stationary phase was previously supersaturated with water by passing through the column 30 dead volumes of a slightly turbid phase containing 1.5% of water. All substances were eluted as a single peak with capacity factor, $k' = 0$.

The present results indicate that the examined system is neither a typical adsorption system nor a typical partition system. It may be concluded that the basic retention mechanism is adsorption of the lanatoside molecules on the surface layer

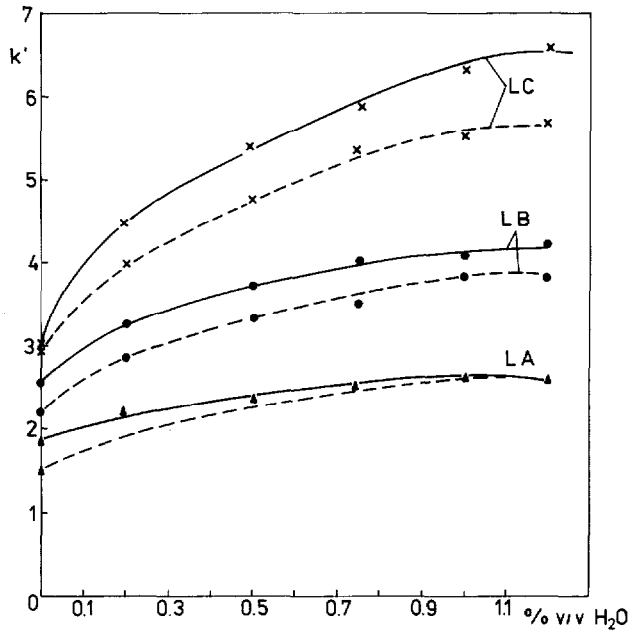

Fig. 2. The effect of the water content in a methylene chloride–methanol–water mobile phase containing 9% methanol on the capacities of lanatosides. Relative sample loadings: ———, $4.7 \cdot 10^{-4}$ g/g; ---------, $3.2 \cdot 10^{-3}$ g/g. Other conditions as in Fig. 1.

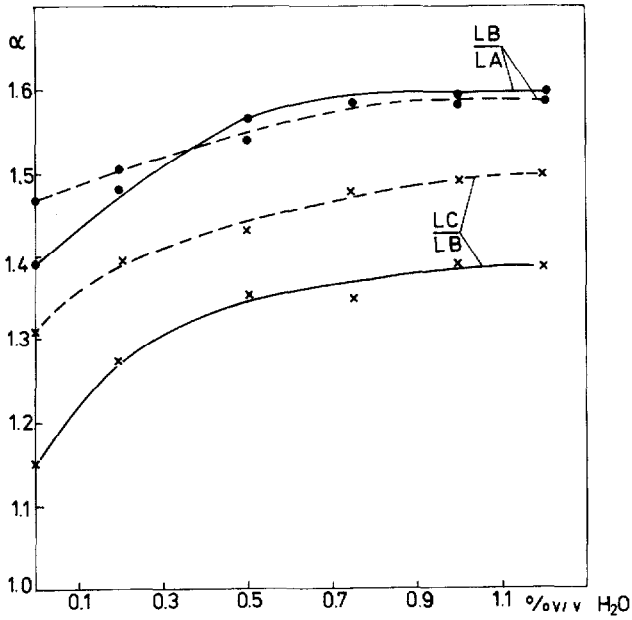

Fig. 3. The dependence of selectivity coefficients, d, of lanatosides on the water content in the mobile phase. Conditions as in Fig. 2.

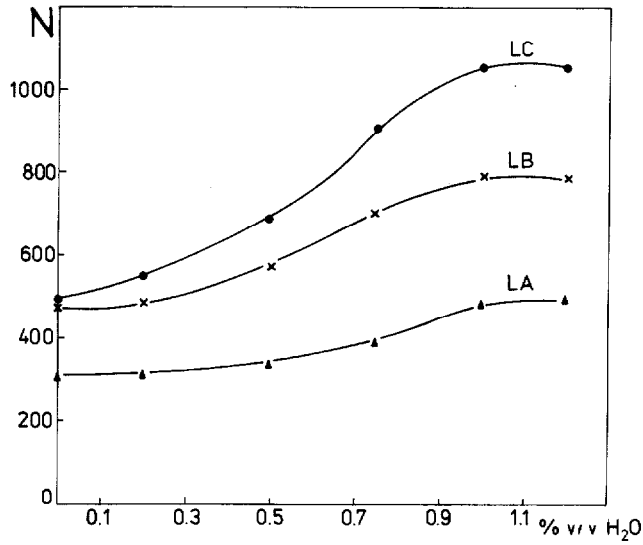

Fig. 4. The efect of the water content in the mobile phase on the efficiency of the systems. Conditions as in Fig. 2. Relative sample loading, $4.7 \cdot 10^{-4}$ g/g.

of water deposited on the silica gel. An excessive amount of water filling the pores of the silica gel particles decreases the surface area of the adsorbent and results in a decrease in retention. Further support for such a mechanism is provided by the effect of the relative sample load on the system efficiency (Fig. 5). Although the efficiency decreases rapidly both in an anhydrous system and in that containing 1% of water,

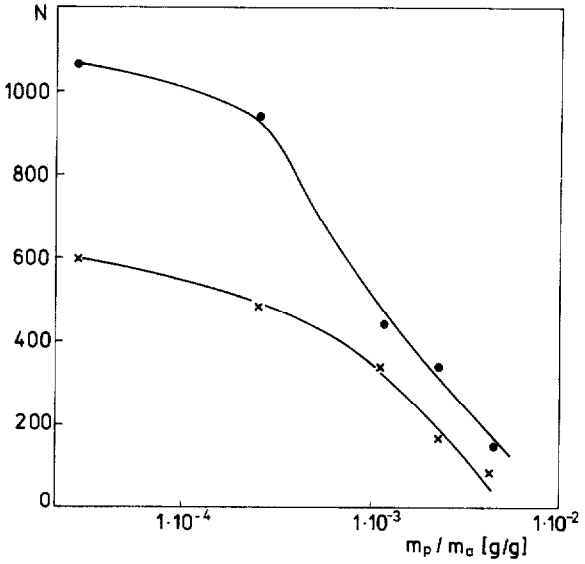

Fig. 5. The effect of the relative loading of the system with sample on its efficiency. Sample: lanatoside C (2.5% solution). Mobile phases: ●—●, containing 1% water; ×—×, anhydrous. Other conditions as in Fig. 1. m_p = Mass of sample; m_a = mass of adsorbent.

at a loading of $5 \cdot 10^{-3}$ g/g there is a two-fold difference in the efficiency, which is significant for the separation effectiveness.

A separation of lanatosides in a system loaded in this manner using 50 cm × 100 mm I.D. columns is shown in Fig. 6.

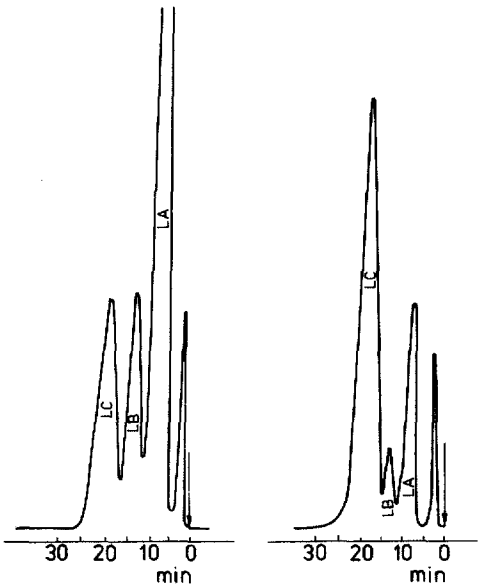

Fig. 6. Chromatographic separation of mixtures I and II of lanatosides A, B and C in a 50 cm × 100 mm I.D. column. Sample size: 500 cm^3 (concentrations as in Table I). Mobile phase: methylene chloride–methanol–water (90.5:8.5:1). Flow-rate: 1.5 dcm^3/min.

The observed advantageous influence of a high content of water in the mobile phase on the selectivity and efficiency of the system permits one to conclude that the ultimate choice of the eluent strength for the separation of a certain group of glycosides occurring in *Digitalis lanata* should be performed by selection of the methanol content in a mobile phase containing the maximum amount of water.

The following advantages of the discussed systems: the possibility of high sample loading up to 10^{-2} g/g, the ease of adjustment of the eluting power of the mobile phase, the appropriate volatility of the mobile phase, the ease of column regeneration by washing with methanol, as well as economical reasons determine their suitability for the separation of cardiac glycosides from natural extracts in preparative systems on an arbitrary scale.

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