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REVERSED-PHASE LIQUID CHROMATOGRAPHY OF ADRIAMYCIN AND DAUNORUBICIN AND THEIR HYDROXYL METABOLITES ADRIAMYCINOL AND DAUNORUBICINOL

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SUMMARY

Adriamycin and daunorubicin and their metabolites adriamycinol and daunorubicinol were separated by reversed-phase liquid chromatography using LiChrosorb RP-2, RP-8 and RP-18 as supports and acetone, acetonitrile and alcohols as organic modifiers in the mobile phase. The highest separation selectivity was obtained using a mobile phase containing low concentrations (< 20%) of acetonitrile. The length of the hydrocarbon chains of the surface-modified silica supports had no significant influence on the selectivity. The lowest capacity factor was obtained with 40–60% of organic solvent in the mobile phase. Increasing the length of the hydrocarbon chains of the supports increased the retention of the solutes.

INTRODUCTION

Adriamycin (A) and daunorubicin (D) are antracycline derivatives with antibiotic effects used for the treatment of neoplastic diseases[1–3]. They are metabolized to a great extent by reduction to adriamycinol (AOH) and daunorubicinol (DOH), respectively, which also have been reported to have cytotoxic activity[4,5]. The structures of the drugs and the metabolites are shown in Fig. 1.

Studies of the pharmacokinetics and the metabolism of adriamycin and daunorubicin require separation systems with high selectivity and high efficiency

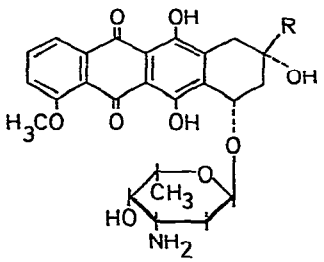

Fig. 1. Structural formulae. R = $-COCH_3$, daunorubicin; R = $-CH(OH)CH_3$, daunorubicinol; R = $-COCH_2OH$, adriamycin; R = $-CH(OH)CH_2OH$, adriamycinol.

because of the small differences in the chemical characters of the drugs and their corresponding reduced metabolites.

Methods for simultaneous determination of adriamycin and daunorubicin and their corresponding hydroxyl metabolites in biological fluids have been based on thin-layer chromatography (TLC)[6] or high-performance liquid chromatography (HPLC) with straight[7] or reversed[8,9] phases. However, no systematic studies for the optimization of the chromatographic conditions have been published.

This paper presents methods for this separation by reversed-phase liquid chromatography based on a systematic study of the influence of the support and the composition of the mobile phase on the selectivity and the retention. LiChrosorb RP-2, RP-8 and RP-18 were used as supports with aqueous mobile phases containing acetone, acetonitrile or alcohols as organic modifiers.

EXPERIMENTAL

Apparatus

The chromatographic detector was an LDC Spectromonitor I (cell volume 8 μl, path length 10 mm) operating at 500 nm. An LDC 711 Solvent Delivery System pump was used. The columns (length 150 and 50 mm, I.D. 4 mm, O.D. $\frac{1}{4}$ in.) were made of stainless steel. The column end fittings were modified Swagelok connectors. A Rheodyne (Model 70-10) injection valve was used with a sample loop of 100 μl.

Chemicals

Daunorubicin and daunorubicinol were obtained from Pharma Rhodia (Stockholm, Sweden) and adriamycin and adriamycinol were kindly supplied by Farmitalia (Milan, Italy). The drugs were used without further purification.

The mobile phases were prepared from acetone, acetonitrile, methanol, 2-propanol (Merck, Darmstadt, G.F.R.; Uvasol grade) or ethanol (spectroscopic grade) and distilled water. Phosphoric acid (Merck; p.a. grade) was added to a final concentration of 10^{-2} *M*.

The surface-modified chromatographic supports LiChrosorb RP-2, RP-8 and RP-18 (Merck) (mean particle diameter 5 μm) were used. According to the manufacturer, these supports are prepared from irregularly shaped, totally porous silica gels (LiChrosorb SI 60, specific pore volume 0.75 ml/g, specific surface area 500 m^2/g, for RP-2; LiChrosorb SI 100, specific pore volume 1.0 ml/g, specific surface area 300 m^2/g, for RP-8 and RP-18) by reaction with dimethyldichlorosilane (incomplete derivatization of RP-2), octylchlorosilane (RP-8) and octadecyldichlorosilane (RP-18).

Chromatographic technique

The chromatographic columns were packed by the balanced density slurry technique[10], using tetrachloroethylene as suspending medium. The slurry was forced into the column at a flow-rate of 9 ml/min or a pressure of 5000 psi, whichever was the limiting factor. The columns were washed with *n*-hexane and acetone (100 ml of each) before use.

Constant retention of the solutes was usually obtained after passage of less than 50 ml of mobile phase through the chromatographic system. The chromatographic system was thermostated at 25.0 $\pm$ 0.1°.

RESULTS AND DISCUSSION

The following abbreviations and symbols not defined in the text are used: k' = capacity factor; R_S = chromatographic resolution; S = solute; [R], [H_2O] = molar concentration of organic solvent and water, respectively, in the mobile phase.

Composition of mobile phase

The influence of the composition of the mobile phase on the retention and selectivity of the chromatographic system was studied with LiChrosorb RP-8 as the support and acidic mobile phases containing acetone, acetonitrile or alcohols as organic modifiers. Using this support, the capacity factors of the solutes could be determined with high accuracy for wide concentration ranges of the organic modifiers.

Retention of solutes

The capacity factors of the solutes were strongly dependent on the concentration of the organic modifier in the mobile phase, as can be seen in Fig. 2, which shows the relationship between the capacity factor and the molar concentration of acetone, acetonitrile and ethanol.

Many workers have reported a linear decrease in log k' with increasing concentration of organic modifier in the mobile phase[11–14], while others reported linear relationships between log k' and the logarithm of the molar concentration of the solvating component in the mobile phase[15–17]. In the latter instance, the retention of the solutes was regarded as being due to the formation of adducts with solvent molecules in the mobile phase in combination with adsorption to the support and/or distribution to a stationary phase[13,17].

The minima within the range $0.8 < \log [R] < 1.1$ in Fig. 2 might be due to solvation of the solutes in the mobile phases as adducts including both water and organic solvents, *i.e.*, in the form $S \cdot [H_2O]_P \cdot [R]_m$ (P and m are the numbers of molecules of water and organic solvent, respectively, in the adducts).

The retention of the solutes decreased with increasing hydrophobic character of the organic modifier in the mobile phase, studied using a homologous series of alcohols as modifiers (Fig. 3)[18,19].

Selectivity

The selectivity of the chromatographic systems varied with both the concentration and the nature of the organic solvent used as a modifier in the mobile phase. A decrease of the concentration of the organic modifier in the mobile phase within the range 20–90% (v/v) gave an increase of the selectivity of the chromatographic system. This is illustrated in Fig. 4, where the selectivity, expressed as log α (α is the separation factor relative to adriamycinol) is plotted against the concentration of acetone, acetonitrile and ethanol.

The highest selectivity was obtained using a low concentration of acetonitrile in the mobile phase. The excellent possibility of separating the four compounds is demonstrated in Fig. 5a, whereas only a small difference in retention was achieved using a high concentration of acetonitrile (Fig. 5b).

Acetone as modifier gave about the same selectivity as acetonitrile, but suffers from the drawback that it cannot be used with the commonly used UV detector

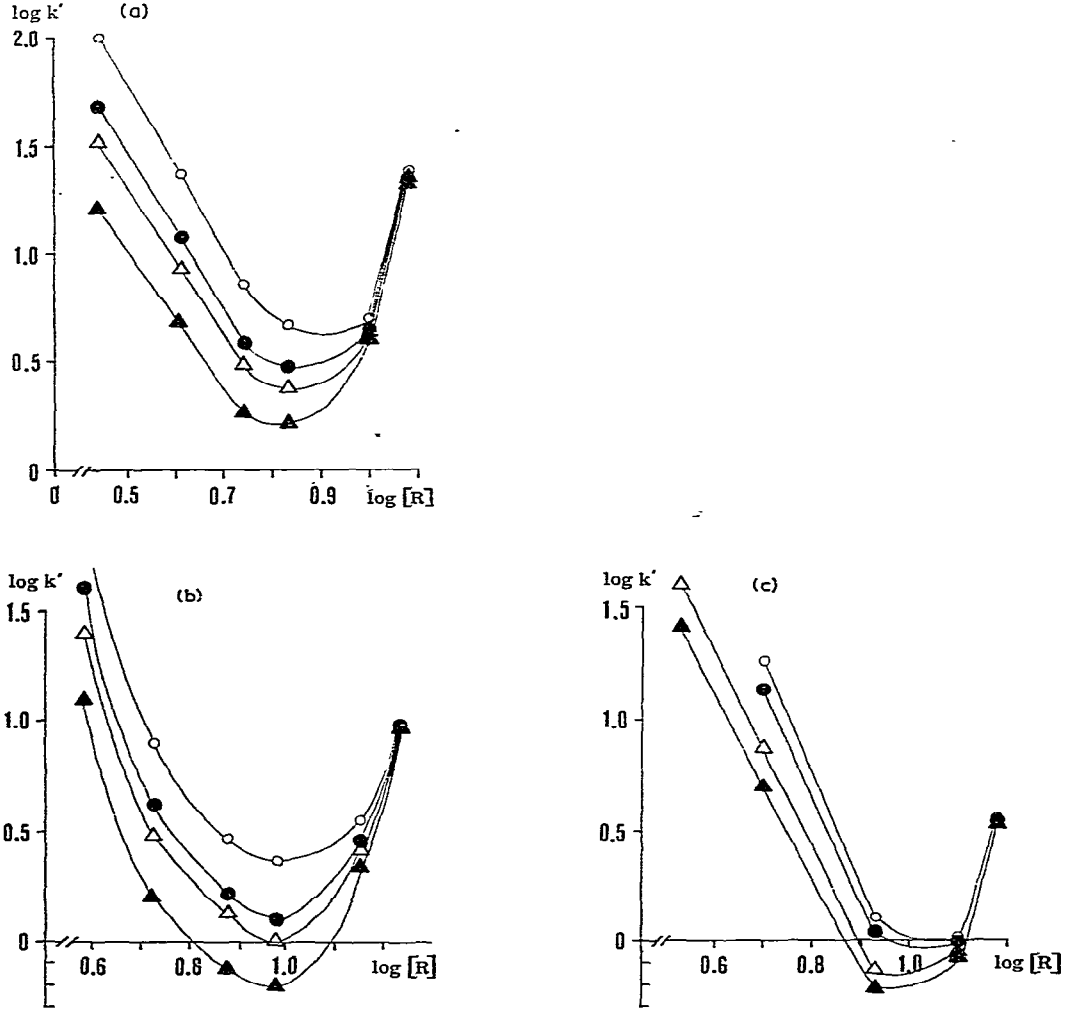

Fig. 2. Retention and mobile phase composition. Support: LiChrosorb RP-8 (5 μm). Mobile phase flow-rate: 1.7 mm/sec. Mobile phase: phosphoric acid (10^{-2} *M*) in water + organic modifier. Modifier (R): (a) acetone; (b) acetonitrile; (c) ethanol. Sample: 2.5 nmole of each solute in 100 μl of mobile phase. Solute: △, adriamycin; ▲, adriamycinol; ○, daunorubicin; ●, daunorubicinol.

measuring at 253.7 nm. Alcohols seem to be less suitable as organic modifiers in the separation of adriamycin or daunorubicin from corresponding reduced metabolites, as a considerable lower selectivity was found compared with acetone or acetonitrile (Figs.6 and 5a).

It was not possible to increase the separation of a drug and its metabolite by changing the hydrophobic character of the alcohol used as the organic modifier in the mobile phase (Fig. 7). For compounds differing by a hydroxyl group in the side-chain, *e.g.*, adiamycin and daunorbubicin, the separation factors increased with decreasing length of the alkyl chains of the alcohols.

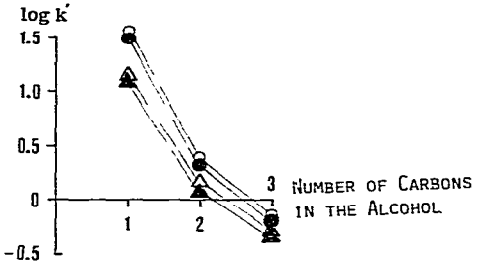

Fig. 3. Retention with alcohols as modifiers. Mobile phase compositions: methanol, [R] = 12.4, $[H_2O]$ = 27.8; ethanol, [R] = 8.6, $[H_2O]$ = 27.8; 2-propanol, [R] = 6.5, $[H_2O]$ = 27.8. Other conditions as in Fig. 2.

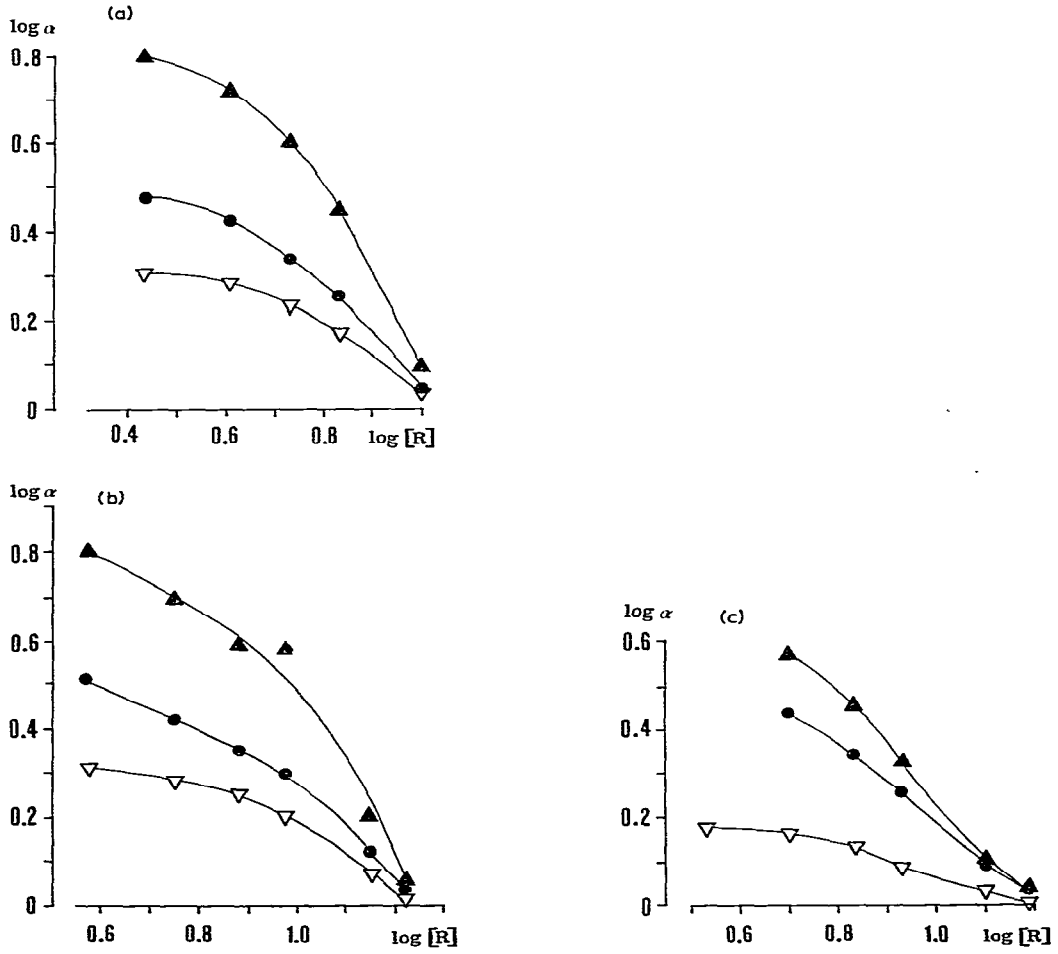

Fig. 4. Selectivity and mobile phase composition. ▲, $\alpha = k'_D/k'_{AOH}$; ●, $\alpha = k'_{DOH}/k'_{AOH}$; ▽, $\alpha = k'_A/k'_{AOH}$. Organic modifier: (a) acetone; (b) acetonitrile; (c) ethanol. Other conditions as in Fig. 2.

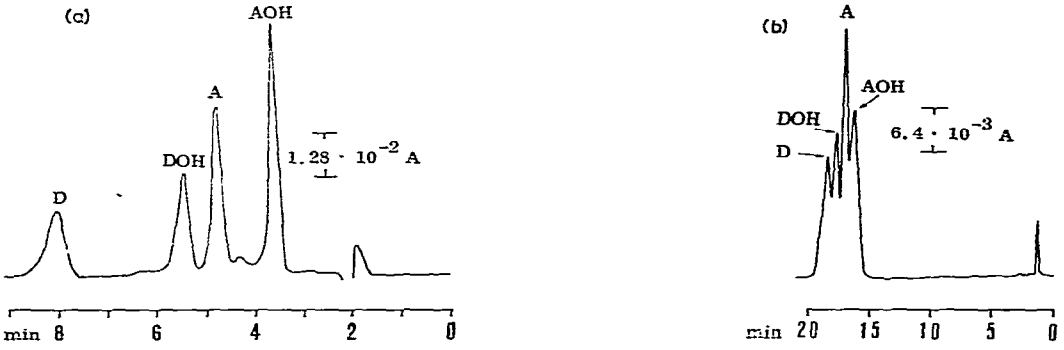

Fig. 5. Separation with acetonitrile in mobile phase. (a) [R] = 7.6, [H_2O] = 33.3; (b) [R] = 17.1, [H_2O] = 5.6. Other conditions as in Fig. 2.

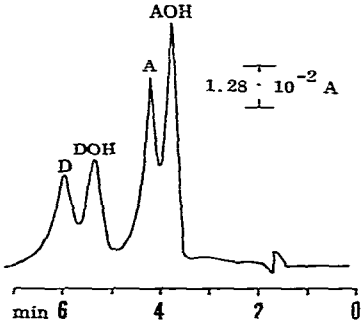

Fig. 6. Separation with ethanol as organic modifier. Mobile phase: [R] = 8.6, [H_2O] = 27.8. Other conditions as in Fig. 2.

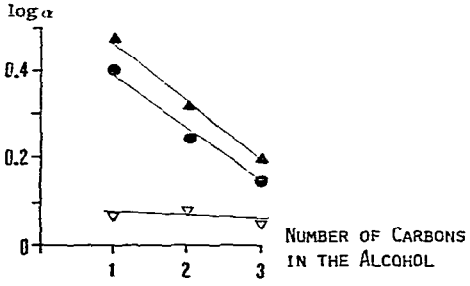

Fig. 7. Selectivity with alcohols in mobile phase. Mobile phases as in Fig. 3. Symbols as in Fig. 4. Other conditions as in Fig. 2.

Influence of supports on retention and selectivity

The influence of the support on the retention and selectivity of the chromatographic system was studied using LiChrosorb RP-2, RP-8 and RP-18 as supports and mobile phases containing various concentrations of acetonitrile as organic modifier. The retention of the solutes increased with increasing length of the alkyl carbon chains of the support, as has been found by other workers[20,21]. The most pronounced effect was found by changing the support from RP-2 to RP-8, giving an increase in log k'

of about 0.8 units. On changing from RP-8 to RP-18, the increase in log k' was 0.2–0.3 units.

The selectivity of the chromatographic system was not influenced to any significant extent by the number of alkyl carbon atoms bonded to the support, in contrast to reports in the literature[21,22] on an increase in selectivity with increasing length of the alkyl chains.

Optimization of the chromatographic system

The separating efficiency of the chromatographic systems was found to be almost independent of the length of the alkyl chains of the supports, the capacity factors of the solutes and the composition of the mobile phases (log [R] < 1.1) with a height equivalent to a theoretical plate of about 90 μm. Hence the maximum speed of the separation of two compounds, *e.g.*, a drug and its metabolite, is obtained when $k'_2 = 2$ (ref. 23).

The shortest separation time at a given flow-rate is obtained[23] at the minimum value of the function

$$f(\alpha, k'_2) = (4 R_S \alpha)^2 \cdot (\alpha - 1)^{-2} \cdot (1 + k'_2)^3 \cdot k'^{-2}_2$$

In this study, the capacity factors for the maximum separation speed and shortest separation time, however, seemed almost to coincide.

The number of theoretical plates required for a complete separation ($R_S = 1.5$) of adriamycin from adriamycinol and daunorubicin from daunorubicinol at optimal separation speed ($k'_2 = 2$) using acetonitrile as organic modifier in mobile phase are given in Table I for the different supports. The calculations are based on the relationships obtained between concentration of acetonitrile in the mobile phase, selectivity and retention of the chromatographic system (Figs. 2 and 4). Obviously, LiChrosorb RP-2 is the most suitable support for the separation of these drugs and their metabolites.

The importance of a high selectivity of the chromatographic system in combination with a high separating efficiency of the separation column is most pronounced when separating drugs and metabolites in biological samples. Interference from other drugs and endogenous compounds can be avoided with a minimum of clean-up of the extract from the biological sample prior to injection into the liquid chromatograph.

TABLE I

NUMBERS OF THEORETICAL PLATES REQUIRED FOR COMPLETE SEPARATION AT OPTIMAL SEPARATION SPEED

For chromatographic conditions, see Fig. 2b.

Support	*Number of theoretical plates*	
	Separation of daunorubicin from DOH	*Separation of adriamycin from AOH*
LiChrosorb RP-2	380	325
LiChrosorb RP-8	510	425
LiChrosorb RP-18	—*	700

* Separation at optimal speed not possible.

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