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SELECTION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS IN PHARMACEUTICAL ANALYSIS

II. OPTIMIZATION FOR SELECTIVITY IN NORMAL-PHASE SYSTEMS

M. GAZDAG, G. SZEPESI* and K. FÁBIÁN-VARGA
Chemical Works of Gedeon Richter, Ltd., P.O. Box 27, 1475 Budapest 10 (Hungary)
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SUMMARY

Optimization of selectivity in normal-phase chromatography was studied. Using the same optimization criteria ($R_{s,min}$, D_{min}, R_{sb} and R_{sa}) as in Part I the possible combination of "solvent strength" optimization by variation of the percentage of polar modifier in the less polar mobile phase and "solvent type" optimization by measuring the solvent selectivity of chloroform, acetonitrile, tetrahydrofuran and dioxane was examined. From the results it can be concluded that by replacement of medium-polarity solvents (chloroform, acetonitrile, tetrahydrofuran and dioxane) in the mobile phase, the selectivity of the separation can be significantly improved as the elution order is a function of solvent type belonging to Class P. In the separation of a steroid mixture dioxane provides the best properties for improving band spacing.

INTRODUCTION

In Part I[1] we considered optimization for selectivity in reversed-phase systems. Such optimization with normal-phase systems is more complex for two main reasons, as studied extensively by Snyder and co-workers[2–5]: solvent–solute localization and its effect on solvent selectivity.

As a continuation of our work in Part I[1], the combination of "solvent strength" and "solvent type" optimization in normal-phase systems has been studied.

EXPERIMENTAL

The same instrumentation (HP 1090A) as described in Part I[1] was used. Separations were performed on a LiChrosorb Si 60 (5 μm) column (250 × 4.6 mm I.D.) (Chrompack, Middelburg, The Netherlands). The eluent flow-rate was 1 ml/min and the steroids (as listed in Table I in Part I[1]) were detected at 254 nm. Other experimental details can be found in Part I[1].

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TABLE I

ESTABLISHMENT OF ELUENT COMPOSITION FOR INITIAL ISOCRATIC SEPARATIONS

Abbreviations: B1 = phosphate buffer (pH 2.2); B2 = 10 m*M* ammonium carbonate buffer; THF = tetrahydrofuran.

Sample characterization			*Eluent selection*	
Solubility	*Polarity*	*Basicity*	C_{18}	*Si 60*
Lipophilic	Non-polar	Neutral	Acetonitrile–water (8:2)	Hexane–chloroform (98:2)
	Medium	Neutral	Methanol–water (7:3)	Hexane–isopropanol (99:1)
	Medium	Proton donor	THF–water (1:1)	Hexane–isopropanol (99:1)
	Medium	Proton acceptor	Methanol–water (6:4)	Hexane–isopropanol (99:1)
	Medium	H-bond formation	Acetonitrile–water (6:4)	Hexane–isopropanol (99:1)
	Polar	Proton donor	Methanol–water (4:6)	Hexane–isopropanol (98:2)
	Polar	Proton acceptor	THF–water (3:7)	Hexane–isopropanol (98:2)
	Polar	H-bond formation	Acetonitrile–water (4:6)	Hexane–isopropanol (98:2)
Hydrophilic	Medium	Proton donor	Acetonitrile–B1 (6:4)	Chloroform–methanol–glacial acetic acid (95:5:1)
	Medium	Proton acceptor	Acetonitrile–B2 (6:4)	Chloroform–methanol (95:5)
	Polar	Proton donor	Acetonitrile–B1 (3:7)	Chloroform–isopropanol–glacial acetic acid (9:1:1)
	Polar	Proton acceptor	Acetonitrile–B2 (3:7)	Chloroform–isopropanol–diethylamine (9:1:1)
	Polar	Neutral	Methanol–water (3:7)	Hexane–isopropanol (9:1)
	Ionic	Cationic	Special techniques	
	Ionic	Anionic	Special techniques	

RESULTS AND DISCUSSION

Optimization criteria

The same optimization criteria as discussed in Part I[1], $R_{s,\min}$, R_{sb}, R_{sa} and $D_{\min}$, were applied.

Optimization for selectivity in normal-phase systems

The importance of the contribution of solvent–solute localization to solvent selectivity effects in separations performed on silica and alumina columns using less polar eluents (liquid–solid chromatography) has been investigated in detail by Snyder and co-workers[2–7]. A quantitative model for solvent–solute localization and its effects on solvent selectivity was also established. With respect to solvent localization capability, the solvents were classified into three groups as follows[3]:

(a) Class N: less polar solvents that show no tendency for localization at low volume fractions in the mobile phase (molecules possessing no functional group).

(b) Class P: more polar solvents that cannot self-hydrogen bond and that can localize at low volume fractions in a mobile phase containing only one polar functional group or only one heteroatom, *e.g.*, alkyl ethers, ketones, nitriles (sub-class Pa); or the molecule is aromatic (*e.g.*, pyridine), multifunctional (*e.g.*, dioxane) or the polar functional group contains more than one heteroatom (sub-class Pb).

(c) Class AB: amphotheric molecules that can self-hydrogen bond and that are fairly polar (alcohols, carboxylic acids, etc.).

As a result of the comprehensive studies described in the literature[2–7], predictions of solvent strength for binary, ternary and quaternary eluent systems can be made.

Three important features of sample molecules can provide useful information for their chromatographic characterization: the lipophilic and hydrophilic character of the compounds to be tested (solubility in hexane, chloroform, chloroform–ethanol and water); polarity of the compounds (depending on the functional group attached to the basic skeleton); and basicity relating to the proton donor or acceptor properties and to hydrogen bond formation ability of the functional groups with the solvents in the eluent.

The first eluent compositions in both systems can be chosen on the basis of sample characterization, as discussed above and shown in Table I.

The data in Table I are based on our practical experience and some examples of its use have been published[8–13]. However, it should be emphasized that the applicability of the eluent compositions indicated in Table I has some limitations, as follows:

(i) The retention data obtained from the first experiments can be considered only as a starting point for the optimization of eluent composition, as discussed in detail in Part I and here. The prediction of correct percentage of the organic component in an initial study seems impossible in most instances.

(ii) The elutropic strength of the mobile phase compositions in Table I is higher than can be expected on the basis of the chromatographic characterization of the sample to exclude the possibility that late-eluting bands may not show up in initial isocratic separations.

(iii) Reversed-phase retention data vary considerably with the molecular weight of the sample. In our practice the data in Table I may be applicable to the separation of compounds in the molecular weight range 100–700.

(iv) For the separation of highly polar samples necessitating the use of a reversed-phase mobile phase with a very low organic solvent content (less than 10%), the data in Table I cannot be applied.

When using the recommended eluent compositions for normal-phase chromatography in Table I to establish the experimental conditions for initial isocratic runs, the application of two-component eluent systems containing an apolar (Class N) and a polar (Class AB) constituent is proposed. The "solvent strength" optimization[1,14] was started with two binary eluent compositions (hexane–isopropanol and chloroform–isopropanol).

Plots of log k' (capacity factor) *vs.* volume fraction of isopropanol in hexane (Fig. 1) and in chloroform (Fig. 2) indicate a non-linear correlation between the data points, as shown together with the corresponding window diagrams. Using the linearizing equation of Soczewinski[15] a significant deviation was also found. However, using the iterative lattice method[16] for the optimization of ternary mobile phases the window diagrams are corrected with the retention data from the experimental runs

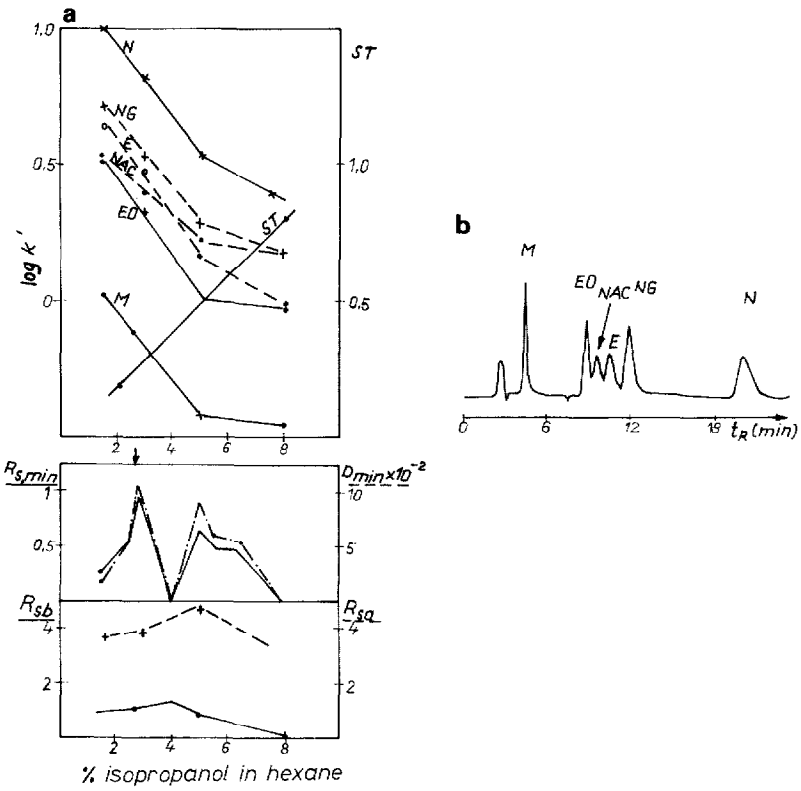

Fig. 1. Plots of log k' *vs.* volume fraction of isopropanol in hexane to illustrate the resolution of the steroid samples. (a) Window diagram; (b) chromatogram of model mixture [hexane–isopropanol (97.25:2.75); eluent strength calculated from the data in Table II according to eqn. 1, ST = 0.275]. Column: LiChrosorb Si 60 (5 μm) (250 × 4.6 mm I.D.); flow-rate, 1 ml/min; detection at 254 nm. Compounds and abbreviations: norethindrone (N), ethinylestradiol (E), norgestrel (NG), estrone (EO), norethindrone acetate (NAC) and mestranol (M). Solid lines, $R_{s,min}/R_{sb}$; broken lines, D_{min}/R_{sa}. t_R = Retention time.

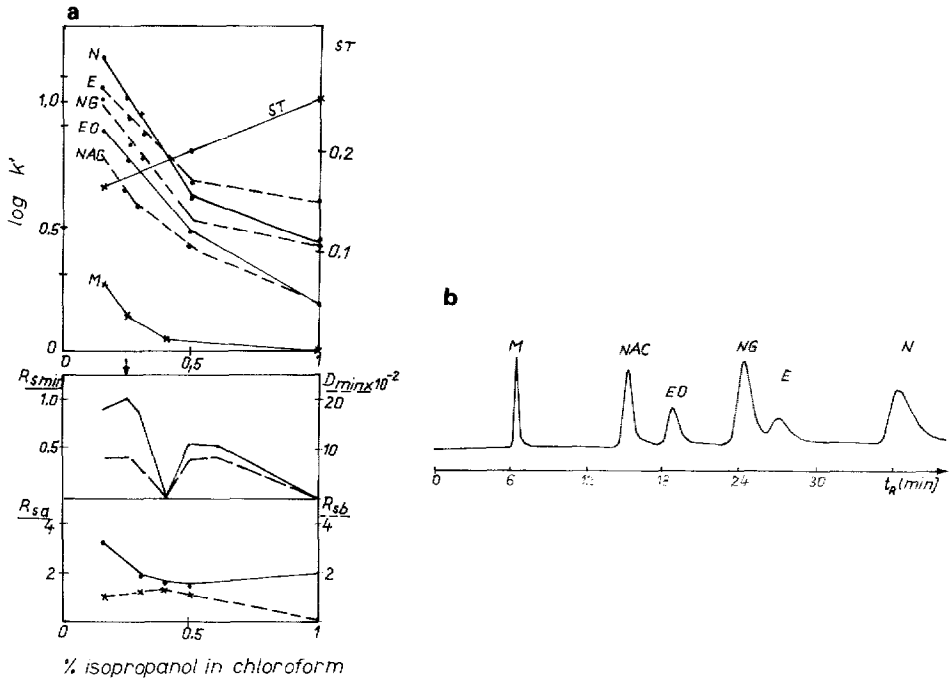

Fig. 2. Plots of log k' *vs.* volume fraction of isopropanol in chloroform. (a) Window diagram; (b) chromatogram of model mixture [chloroform–isopropanol (99.75:0.25); ST = 0.18]. Details as in Fig. 1.

step by step, assuming a linear correlation between the measured data points of the log k' *vs.* volume fraction of isopropanol plots.

Regarding the hexane–isopropanol eluent, three optima can be found (isopropanol concentrations 2.75, 4.9 and 6.4%). Similarly to the procedure described in Part I[1], norgestrel (NG) was chosen as main component when the values of R_{sb} and R_{sa} have been calculated. The optimum found at 2.75% isopropanol was selected for further experiments, giving the best values for $R_{s,min}$ and D_{min} and acceptable values for R_{sb} and R_{sa} (system A). A chromatogram obtained with this eluent is shown in Fig. 1.

Similar results were obtained with chloroform–isopropanol eluents. From the three optima (isopropanol concentration 0.25, 0.5 and 0.6%) the first was selected (system B) as it gave the highest $R_{s,min}$ value (the values for D_{min}, R_{sb} and R_{sa} were similar). A chromatogram obtained with this eluent is shown in Fig. 2.

Comparing the two selected systems, it can be concluded that both systems provide acceptable $R_{s,min}$ values (about 1.0), and system A gave better R_{sa} and worse R_{sb} values. NG is well separated from N and poorly separated from E; with system B the reverse applies, NG being separated well from EO (better R_{sb}) and poorly from E (R_{sb}).

The study was continued using the iterative lattice method[16]. The initial eluent composition for the first experimental run with a ternary eluent was selected from the window diagram shown in Fig. 3 [A–B (40:60)]. The resulting chromatogram is also shown in Fig. 3.

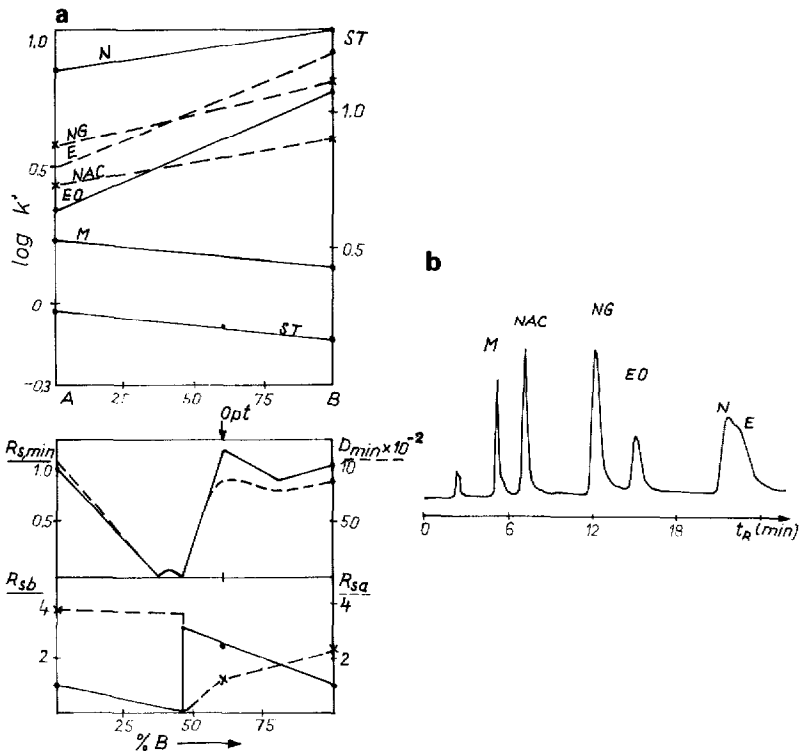

Fig. 3. Window diagram for the selection of initial three-component eluent mixture. System A, hexane–isopropanol (97.25:2.75); system B, chloroform–isopropanol (99.75:0.25). (a) Window diagram; (b) chromatogram obtained with the initial ternary eluent mixture [hexane–chloroform–isopropanol (38.90:59.85:1.25); ST = 0.215]. Details as in Fig. 1.

Contrary to expectation, a poor separation for N and E was obtained ($R_{s,min}$ = 0.54). The window diagram was corrected using the retention data from this run, resulting in a new optimum [A–B (80:20)], as shown in Fig. 4 together with the chromatogram obtained with this eluent system.

As shown in Fig. 4, the separation with this newly selected eluent does not also meet expectation, and no improvement in the separation efficiency was achieved. Re-correcting the window diagram again with the retention data, the next optimum was A–B (58:42), as shown in Fig. 5 together with the chromatogram obtained.

Fig. 5 shows that the separation was improved (N and E can be separated) and the system seems to be applicable for purity testing ($R_{s,min}$ = 0.82, R_{sb} = 4.2 and R_{sa} = 2.3).

The experiment was continued using an eluent composition indicated by the corrected window diagram shown in Fig. 6 [A–B (65:35)]. A better separation was achieved ($R_{s,min}$ = 0.88, R_{sb} = 3.66 and R_{sa} = 2.33) and this composition of mobile phase, corresponding to hexane–chloroform–isopropanol (63.2:34.9:1.9), is considered to be optimal because on re-correction of the window diagram the same optimum was obtained (Fig. 7).

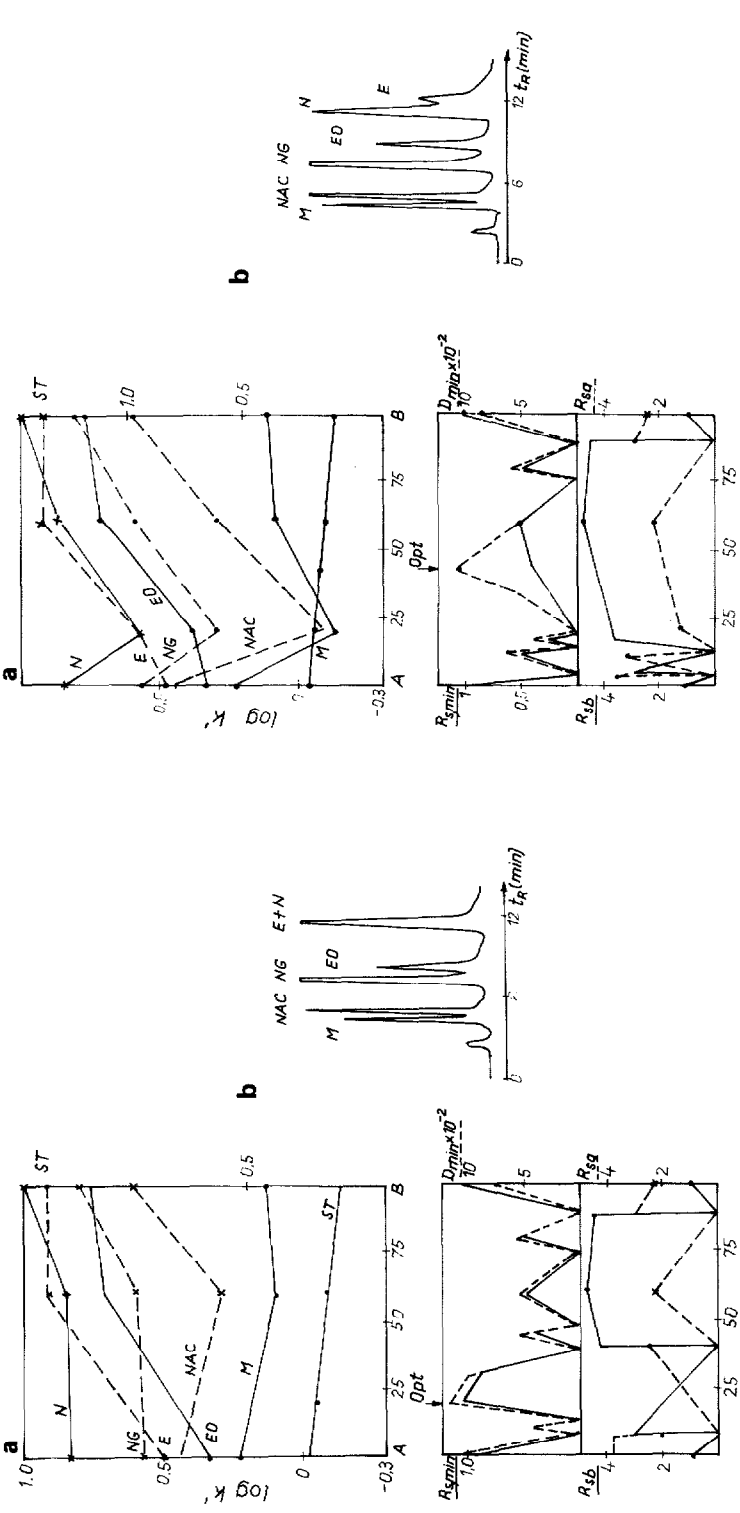

Fig. 4 First correction of window diagram (a) based on the retention data of the resulting chromatogram (Fig. 3b). (a) Window diagram; (b) chromatogram obtained at the selected optimum. Mobile phase composition in (b): hexane–chloroform–isopropanol (77.8:19.95:2.25); ST = 0.255. Details as in Fig. 1.

Fig. 5. Second correction of window diagram (a) based on the retention data of the resulting chromatogram (Fig. 4b). (a) Window diagram; (b) chromatogram obtained at the selected optimum. Mobile phase composition in (b): hexane–chloroform–isopropanol (56.4:41.9:1.7); ST = 0.233. Details as in Fig. 1.

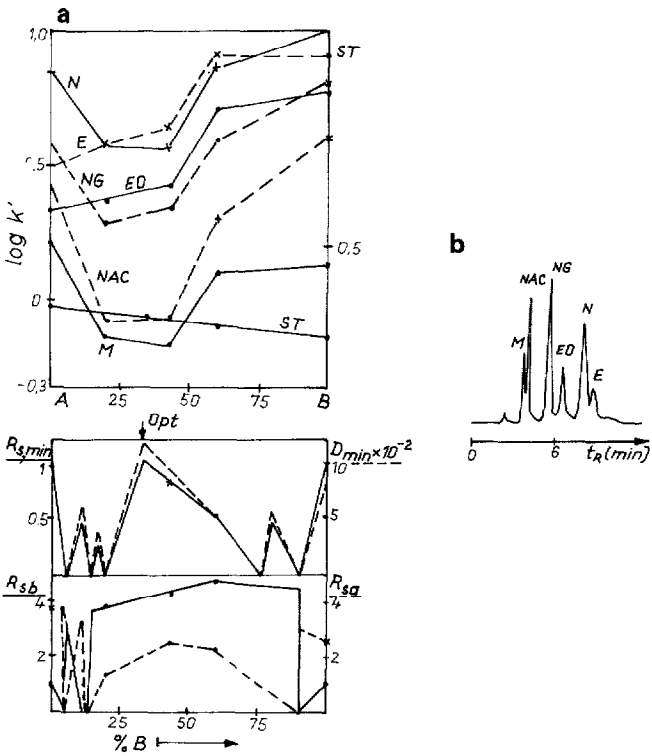

Fig. 6. Third correction window diagram (a) based on the retention data of the resulting chromatogram (Fig. 5b). (a) Window diagram; (b) chromatogram obtained at the selected optimum. Mobile phase composition in (b): hexane–chloroform–isopropanol (63.2:34.9:1.9); ST = 0.242. Details as in Fig. 1.

To improve further the selectivity of the separation, replacement of chloroform with other solvents belonging to the same group of solvents (Class P) in the ternary mobile phase was studied. As a practical approach, the elutropic strength of ternary mobile phases was calculated using an equation similar to that used in reversed-phase chromatography[17]:

$$ST = \sum s_i v_i \tag{1}$$

where ST is the elutropic strength of the eluent, s_i is the strength of individual solvents and v_i is the percentage volume fraction of the solvent in the eluent.

When the elutropic strengths of the eluents were calculated according to literature data[18] some deviations were observed. Based on our recent experimental data[9,11,12] obtained for different groups of compounds separated using different mobile phase compositions, an empirical order for the solvent strengths was established. The solvent strengths of two solvents were fixed (hexane = 0, isopropanol = 10) and the other solvents were placed in order by calculating the eluent strength according to eqn. 1, considering the concentrations of other solvents providing similar retentions for the same components.

Some large deviations in the solvent strengths were found (Table II) when our

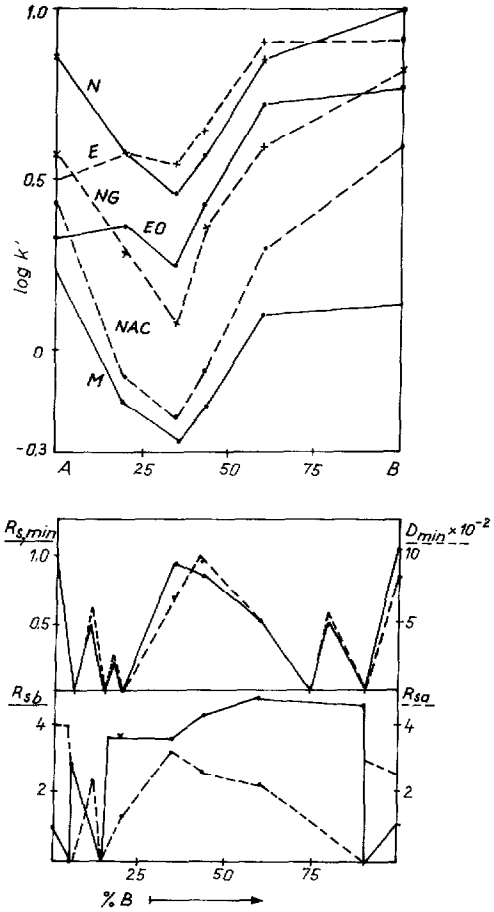

Fig. 7. Re-corrected window diagram.

TABLE II

SOLVENT STRENGTHS OF DIFFERENT SOLVENTS

Solvent	*Solvent strength*	
	Experimental	*Literature*[18]
Hexane	0	0.01
Dichloromethane	0.11	0.42
Chloroform	0.15	0.40
Dioxane	2.50	0.56
Acetonitrile	4.00	0.65
Tetrahydrofuran	4.50	0.45
Methanol	6.70	0.95
Isopropanol	10.0	0.82

data were compared with the data published in the literature[18]. According to our findings the solvent strength of chlorinated hydrocarbons (chloroform and dichloromethane) seems to be weaker than expected. Similarly, the order of solvent strengths for solvents belonging to Class P is different. According to our previous assumption, the significant deviations may be due to the change in separation mechanism with binary and ternary mobile phase compositions from liquid–solid adsorption to liquid–liquid partition chromatography as a result of the dynamically coated thin liquid layer formed on the silica surface.

A more likely explanation was given by Snyder[19] based on two effects. The first is derived from the fact that very low concentrations of strong solvents (Class AB) are used and under these conditions the strong (localizing) solvent has a much higher strength than normal, as was shown by Snyder and co-workers[3,4,6]. Second, all polar solvent systems have large secondary solvent effects (interactions between solute and solvent molecules in the adsorbed phase), resulting in deviations from the predicted solvent strength.

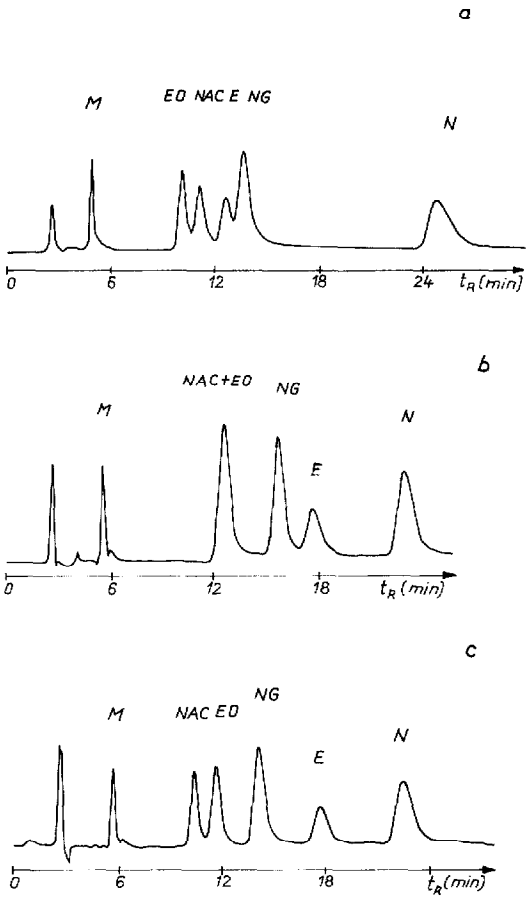

Fig. 8. Replacement of chloroform with other solvents belonging to Class P. (a) Hexane–tetrahydrofuran–isopropanol (97:1:2); ST = 0.245; (b) hexane–acetonitrile–isopropanol (97:1:2); ST = 0.240; (c) hexane–dioxane–isopropanol (96:2:2); ST = 0.250. Details as in Fig. 1.

Based on this explanation, the solvent strength values in Table II can be considered to represent a good practical approach, particularly for low concentrations of the polar modifier.

As a continuation of our experiments, the possible replacement of chloroform with tetrahydrofuran, acetonitrile and dioxane in the eluent (the calculated eluent strength according to eqn. 1 is 0.24) was studied. The chromatograms are shown in Fig. 8.

Comparing the chromatograms shown in Fig. 8, two important conclusions can be drawn. First, similar retentions were obtained for NG, M and N (first, last and main components). This seems to support our expectation regarding the calculation possibilities of elutropic strength. Second, replacement of chloroform with dioxane

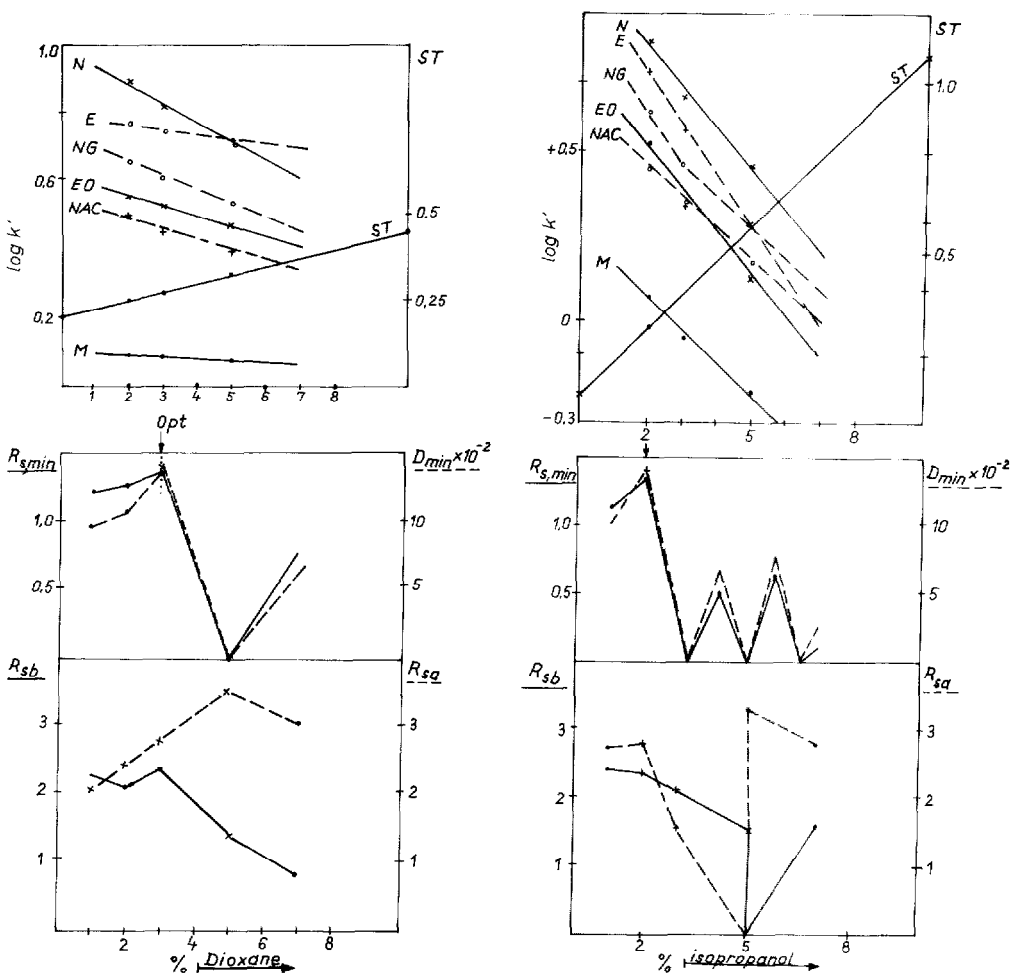

Fig. 9. Plots of log k' *vs.* volume fraction of dioxane in the mobile phase. Mobile phase: hexane–dioxane containing 2% of isopropanol. Details as in Fig. 1.

Fig. 10. Plots of log k' *vs.* volume fraction of isopropanol in the mobile phase. Mobile phase: hexane–isopropanol containing 3% of dioxane. Details as in Fig. 1.

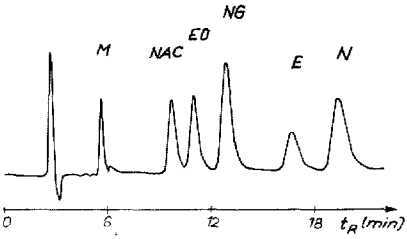

Fig. 11. Chromatogram obtained with the optimal eluent composition. Mobile phase: hexane–dioxane–isopropanol (95:3:2); ST = 0.275. Details as in Fig. 1.

can significantly improve the selectivity of the separation, and therefore this system was subjected to further investigations.

Plots of log k' *vs.* concentration of dioxane using a constant concentration of isopropanol (2%) and of log k' *vs.* concentration of isopropanol using a constant concentration of dioxane (3%) are shown in Figs. 9 and 10, respectively. The data show that the mobile phase hexane–dioxane–isopropanol (95:3:2) can be considered optimal. The chromatogram obtained with this eluent is shown in Fig. 11.

CONCLUSIONS

Six different mobile phase compositions (two binary and four ternary systems) were compared with respect to their selectivity. The elution orders obtained with these systems are shown in Fig. 12.

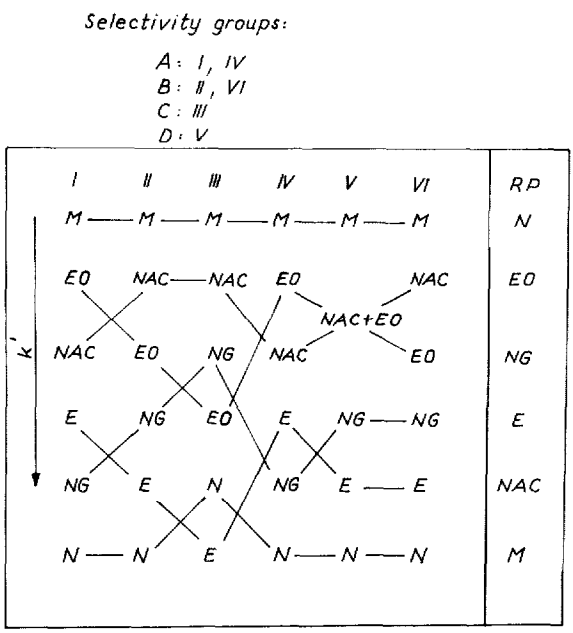

Fig. 12. Dependence of elution order on eluent composition. RP = Reversed phase. Solvent systems: I = Hexane–isopropanol; II = chloroform–isopropanol; III = hexane–chloroform–isopropanol; IV = hexane–tetrahydrofuran–isopropanol; V = hexane–acetonitrile–isopropanol; VI = hexane–dioxane–isopropanol.

All the ternary systems differ from each other, which supports our recent experience that the difference in selectivity of solvents available for normal-phase chromatography is greater than that of solvents applied in reversed-phase chromatography, and this can advantageously be used to improve the selectivity of separations.

This was the main reason why normal-phase chromatography was selected as a second alternative approach for optimizing HPLC separations in pharmaceutical analysis. The elution order with the optimal reversed-phase system is also indicated in Fig. 12.

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