Journal of Chromatography, 461 (1988) 407–418
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1433

MOBILE PHASE OPTIMIZATION OF A UREA-LINKED CHIRAL STATIONARY PHASE FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF OPTICAL ISOMERS

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SUMMARY

A silica-based urea-linked chiral stationary phase, (*R*)-N-(1-naphthylethyl-N'-propylsilyl urea), was studied with binary, ternary, and single-component mobile phases, to determine the factors which most affect retention and selectivity. Binary mobile phases were prepared with hexane as the non-selective solvent, and 2-propanol, dichloromethane, or chloroform as the selective solvent. (±)-1-Phenylethyl-3,5-dinitrobenzamide was used as the enantiomer test mixture. Increasing the mobile phase strength reduced the retention of test solutes, while selectivity remained largely unchanged. Also, modifiers improved resolution and did not affect selectivity. Single-component mobile phases reduced the retention in the order of increasing solvent strength, selectivity being solvent-dependent.

INTRODUCTION

The resolution of optical isomers by high-performance liquid chromatography (HPLC) has become a much utilized technique. The importance of this technique is reflected in the fact that about 40% of all drugs used in therapy are chiral, and that their enantiomers differ to various degrees in that their pharmacological actions, as well as in their side-effects[1].

In a chiral solute mixture, the two enantiomers have identical internal energy before injection into the column and after elution from the column. The enantiomers can be separated if the chiral stationary phase (CSP) is able to produce a difference in internal energy between them. If there is chiral recognition between the solute molecules and the CSP, transient diastereomeric complexes of differing stability are formed. These transient complexes can be separated by chromatography.

Dalgliesh[2] was first to develop a mechanistic model for chiral recognition in a chromatographic system. The model postulates a three-point interaction between the CSP and the solute. Such interactions may consist of a combination of hydrogen bonding, dipole–dipole, π–π, and steric interactions, depending upon the system. Much understanding concerning the mechanism and the rationale for designing new CSPs has evolved from the work of Pirkle and co-workers[3,4], utilizing the three-point

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0021-9673/89/$03.50

interaction model. A two-point interaction model was proposed by Lochmüller and Ryall[5], and by Dobashi and Hara[6], while a single-point mechanism was proposed by Lochmüller *et al.*[7], and by Wainer *et al.*[8]. Lochmüller and Souter[9] further proposed an environmental chirality with no specific points of interaction, for certain systems. Such studies have greatly expanded the range of enantiomers that have been separated on a wide variety of CSPs and have paved the way for the development of newer and more useful CSPs.

In this paper, we discuss how mobile phase variations affect retention and selectivity for chiral resolution upon a commercial naphthyl urea-linked π-donor brush-type CSP. Studies were conducted with binary and ternary mobile phases, the latter prepared by adding the modifiers methanol and acetonitrile to the binary phases. In the binary mobile phases, hexane and heptane were used as non-selective solvents, while alcohols, chloroform, and dichloromethane were used as selective solvents. 1,2-Dichloroethane, acetonitrile and methyl *tert.*-butyl ether were used as single-component mobile phases.

EXPERIMENTAL

Materials

A Supelcosil™ LC-(*R*)-Naphthyl Urea, 250 × 4.6 mm I.D. column (NU column) was used for the chiral separations (Supelco, Bellefonte, PA, U.S.A.). The chiral solute test mixture (±)-1-phenylethyl-3,5-dinitrobenzamide (PEDNBA) was obtained from Supelco, all other reagents, standards and mobile phase solvents were obtained from Anachemia (Champlain, NY, U.S.A.). All solvents were of HPLC grade.

Mobile phase mixtures

All binary and ternary mobile phases were hand-mixed. All mobile phase changes were made by equilibrating the column with a minimum of 150 ml of mobile phase. Chromatography was carried out at ambient temperatures. Mobile phases used for these studies are listed in Table I.

TABLE I

MOBILE PHASE MIXTURES

Mobile phase components	*Composition (v/v)*	*Polarity, P*
Binary mobile phases[10]		
Hexane–2-propanol	90:10	0.48
	80:20	0.86
	75:25	1.05
	70:30	1.24
	50:50	2.00
Heptane–2-propanol	134:12	0.50
	100:25	0.94
	75:21	1.01
	75:25	1.13
	75:35	1.38
	52:48	1.98

TABLE I (*continued*)

Mobile phase components	*Composition (v/v)*	*Polarity, P*
Hexane–dichloromethane	50:50	1.60
	45:55	1.75
	40:60	1.90
	35:65	2.05
	33:67	2.11
	30:70	2.20
	25:75	2.44
Hexane–chloroform	200:300	2.50
	200:325	2.58
	150:300	2.77
	100:300	3.10
	50:300	3.53
	20:300	3.85
Ternary mobile phases		
Hexane–2-propanol–acetonitrile	225:75:0	1.05
	225:75:0.4	1.06
	225:75:1.0	1.07
	225:75:2.0	1.08
	225:75:3.0	1.10
	225:75:4.0	1.11
	225:75:6.0	1.14
Hexane–2-propanol–methanol	75:25:0	1.05
	75:25:0.2	1.06
	75:25:0.5	1.07
	75:25:0.7	1.08
	75:25:1.0	1.09
	75:25:2.0	1.13
Hexane–chloroform–methanol	360:640:0	2.55
	360:640:0.025	2.56
	360:640:0.06	2.57
	360:640:0.12	2.58
	360:640:0.2	2.61
	360:640:0.5	2.67
Hexane–chloroform–acetonitrile	360:640:0	255
	360:640:0.03	2.56
	360:640:0.06	2.57
	360:640:0.12	2.61
	360:640:0.25	2.63
	360:640:0.5	2.71
	360:640:0.9	2.85
Alcohols containing mobile phases		
Hexane-ethanol	80:20	0.94
Hexane–*n*-propanol	80:20	0.88
Hexane–*n*-butanol	80:20	0.86
Hexane–*n*-octanol	80:20	0.93
Hexane–isobutanol	80:20	0.90
Hexane–2-propanol	80:20	0.86

Apparatus

Chromatography was performed with a Waters (Waters Assoc., Milford, MA, U.S.A.) 590 pump, a WISP 710B injector with a Waters 720 system controller, a Kratos (Ramsey, NY, U.S.A.) Spectroflow 757 variable-wavelength detector set at 254 nm, and a SP™-4270 recorder/integrator from Spectra-Physics (San Jose, CA, U.S.A.).

RESULTS AND DISCUSSION

The chiral phase is a π-donor brush-type stationary phase. The chiral bonded phase (Fig. 1A) interacts with solute molecules through a number of different interactions, depending on the functionality of the solute and solvent molecules. When PEDNBA (Fig. 1B) is used as the solute mixture, the CSP–solute interactions consist of a combination of a π–π interaction between the π-accepting 3,5-dinitrobenzoyl group of the solute and the electron-rich naphthyl group of the CSP, a dipole interaction between the carbonyl group of the solute and the amido group of the CSP, and a dipole interaction between the phenyl group of the solute and the carbonyl group of the CSP. The strength of these interactions determines the degree of chiral recognition, as seen by the order of elution of the enantiomers and the difference in their retention. These reflect the differences in strength of the transient diastereomeric complexes formed between the CSP and the solute enantiomers.

Furthermore, the solvent–CSP interactions depend on the chemical properties of the solvent used. Hexane or heptane are non-polar, non-selective solvents, serving only to adjust the strength of the mobile phase. In the binary mobile phases, 2-propanol interacts with the CSP through reciprocal hydrogen bonding at the amide group. Dichloromethane is a dipolar molecule, and interacts with the carbonyl group through a dipole–dipole interaction. Chloroform is a hydrogen donor that interacts with the amide group through non-reciprocal hydrogen bonding at the carbonyl oxygen. Each of these interactions is capable of reducing the strength of the solute–CSP interaction.

This model only reflects the solvation interaction of the polar solvent, with the functional groups of the bonded stationary phase (Fig. 1A) or the chiral solute (Fig. 1B). Such interactions may alter chiral recognition by the disruption of chemical interactions and/or by solvation of the phase or solute to a degree that steric bulk prevents interaction. This model does not address solute and solvent interactions with

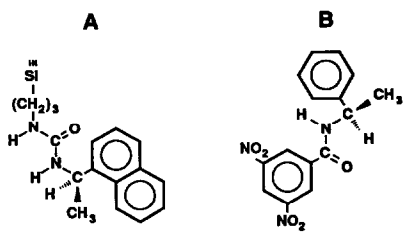


Fig. 1. Structures of the Supelcosil LC-(*R*)-naphthyl urea bonded-phase surface (A) and the PEDNBA test solute (B).

residual surface silanols upon the bonded phase support, as such interactions affect retention of each enantiomer equally and play no role in chiral recognition.

In this study, the effects of different binary and ternary mobile phases on retention and selectivity are examined. Retention of the enantiomers is reported as k'_{av}, the average capacity value for the enantiomers:

$$k'_{av} = \frac{k'_1 + k'_2}{2} \tag{1}$$

where k'_1 and k'_2 are the capacity factors of enantiomers 1 and 2, respectively. The difference in retention of the test solutes due to the binary and ternary mobile phases, dk', is reported as:

$$dk' = k'_{BMP} - k'_{TMP} \tag{2}$$

where k'_{BMP} and k'_{TMP} refer to the average capacity factors, k'_{av}, for the enantiomer pair in the binary and ternary mobile phases, respectively. Selectivity, α, is reported as:

$$\alpha = k'_2/k'_1 \tag{3}$$

For a further, in-depth discussion of general mobile phase optimization the work of Snyder *et al.*[11] is recommended. An in-depth discussion of the influence of mobile phase on k'_{av}, dk', and α for the NU column follows.

Binary mobile phases

Hexane–2-propanol and heptane–2-propanol. The non-polar solvents hexane and heptane by themselves play no active role in determining the strength of the solute–CSP interaction. Replacing hexane with heptane (Fig. 2A and B) produces no apparent difference in solute–CSP interaction, since, in each case, an increase in the 2-propanol concentration (increase in the mobile phase strength) produces a similar change in retention [k'_{av}]. Also, the selectivity (α) is similar with both mobile phases

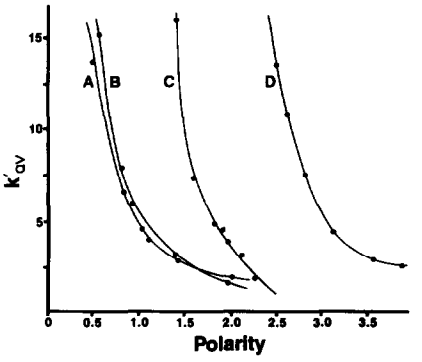


Fig. 2. Effect of mobile phase composition on retention of PEDNBA, showing k'_{av} *vs.* polarity for four binary mobile phases: hexane–2-propanol (A), heptane–2-propanol (B), hexane–chloroform (C), and hexane–dichloromethane (D). See Experimental for mobile phase compositions.

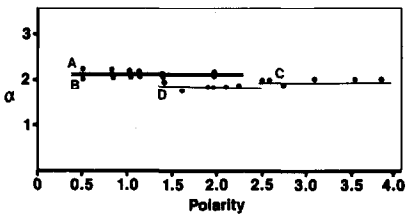


Fig. 3. Effect of mobile phase polarity on selectivity, showing α *vs.* polarity for four binary mobile phases: heptane–2-propanol (A), hexane–2-propanol (B), hexane–dichloromethane (C), and hexane–chloroform (D). See Experimental for mobile phase compositions.

(Fig. 3). This indicates that the stronger solvent, 2-propanol, competes more effectively at higher concentrations than the test solute for the polar sites on the CSP, thus causing a more rapid displacement of the latter. However, the difference in strength of the transient complexes remains equal, resulting in a constant α. Such effects are greatest for lower concentrations of 2-propanol (less than 0.5%), and fall off at higher concentrations. This could be due to nearly 1:1 interaction between 2-propanol and the CSP, indicating that solvation of the solute is not a major contributor to the reduction of k' with increasing 2-propanol concentration.

Hexane–dichloromethane. A trend similar to that discussed above is seen for hexane–dichloromethane mobile phases. In this case, a mobile phase polarity range of 1.6–2.4 gave k'_{av} reductions similar to those described before (Fig. 2C). This indicates that dichloromethane in a binary mobile phase produces a weaker mobile phase than 2-propanol. A higher mobile-phase strength is needed to elute the solute enantiomers. It is evident that, in competing with the solute–CSP interactions, the single dipole–dipole interaction produced by dichloromethane is not as effective as the reciprocal hydrogen bonding of 2-propanol.

Furthermore, when this mobile phase is used ($\alpha = 1.8$), the selectivity of the CSP is lower than with 2-propanol ($\alpha = 2.2$), but, as with hexane–2-propanol, α is independent of mobile phase strength. Selectivity is thus dependent on solvent type and not on mobile phase strength.

Hexane–chloroform. With chloroform in the mobile phase and polarity (P) ranging from 2.5 to 3.8, a higher mobile phase strength is required to produce a reduction in retention equivalent to that produced by the other binary mobile phases. This indicates that the proton-donating ability of chloroform is not as effective in reducing the overall solute–CSP interaction as the dipolar interaction of dichloromethane. The latter, in turn, is weaker than the reciprocal hydrogen bonding interaction of 2-propanol. In this case, also, the selectivity of the CSP remains constant ($\alpha = 2.0$) with increasing mobile phase strength, and is intermediate between that for 2-propanol and dichloromethane mobile phases.

From these observations, the active mobile phase components can be placed in the order 2-propanol > dichloromethane > chloroform for increasing solvent strength, and in the order 2-propanol > chloroform > dichloromethane for their ability to enhance the selectivity of the CSP. The minor variations in α, as observed in Fig. 3, also support the earlier statement that residuals play no significant role in chiral recognition influenced by polar mobile phase additives.

Ternary mobile phases

Hexane–2-propanol–methanol. Addition of methanol to hexane–2-propanol causes an initial increase in solute retention, k'_{av}, as seen in Fig. 4. In this figure k'_{av} and dk' are plotted against the change in polarity (ΔP) of the binary phase, caused by adding the modifier. As the methanol concentration increases from 0 to 2% (P increases from 0.01 to 0.05), dk' shows a negative deflection. The change in k'_{av} follows a similar but opposite trend, decreasing rapidly from 5.4 to 3.1. This implies that the change in k'_{av} is due entirely to the modifier. Addition of more than 2% methanol caused little additional change in retention.

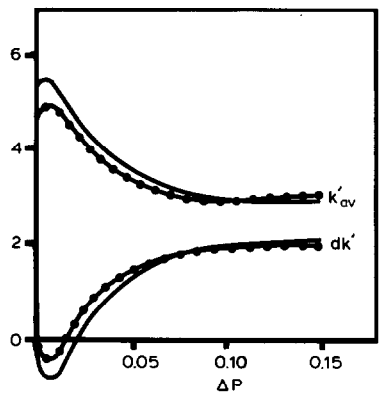


Fig. 4. Effect of mobile phase modifier concentration on PEDNBA retention, showing k'_{av} *vs.* ΔP and dk' *vs.* ΔP when hexane–2-propanol is modified with methanol (———) or acetonitrile (●—●). See Experimental for mobile phase compositions.

In Fig. 4, the increase in k'_{av} for $\Delta P < 0.02$ may be due to an initial interaction between 2-propanol and methanol, presumably weakening the solvent–CSP interaction and causing a stronger solute–CSP interaction. At higher concentrations, methanol displaces 2-propanol from the active sites on the CSP. When an equilibrium is established between methanol and the CSP, the methanol–solute competition lessens, and retention of the solute is no longer appreciably reduced by further addition of methanol.

Hexane–2-propanol–acetonitrile. When acetonitrile is added to a hexane–2-propanol mobile phase, in the same proportions as methanol, a similar trend is observed (Fig. 4): dk' initially decreases, then increases rapidly. A decrease in k'_{av} mirrors the increase in dk'. The initial interaction between acetonitrile and 2-propanol is somewhat smaller than that between methanol and 2-propanol, and the solvent–CSP interaction is larger, resulting in a reduction in the k'_{av} observed for acetonitrile. As for methanol, the fact that an increase in dk' corresponds to the decrease in k'_{av} shows that the decrease in k'_{av} is due solely to the increase in acetonitrile concentration.

It is interesting that the curves for methanol and acetonitrile in Fig. 4 flatten at approximately the same ΔP value (0.07). This could be related to the solvation strength of the solute–CSP interaction in the hexane–2-propanol binary phase.

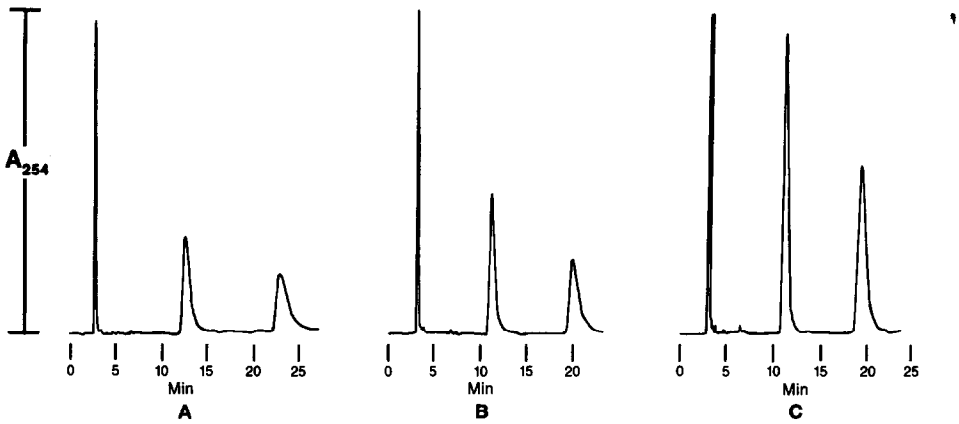


Fig. 5. Chromatograms showing the effects on retention and peak-shape, produced by adding 0.6% methanol (B) or acetonitrile (C) to hexane–2-propanol (75:25, v/v) (A).

Comparing the solvation interactions of methanol and acetonitrile, the effects caused by methanol are only minimally greater than with acetonitrile. The separation shown in Fig. 5A was obtained with hexane–2-propanol (75:25) as the mobile phase, while those in Fig. 5B and C were obtained by adding 0.6% methanol or acetonitrile, respectively. Both modifiers caused a reduction in k' and reduced peak tailing. Peak shape is most improved by using methanol, due to its stronger solvation effects.

Hexane–chloroform–methanol. When methanol is added to a hexane–chloroform mobile phase, k'_{av} decreases rapidly (Fig. 6) from 10.1 to 2.0 with a change in mobile-phase polarity of less than 0.02. As discussed previously, this decrease in k'_{av} is reflected in a corresponding increase in dk', indicating that the decrease in retention is due primarily to methanol in the mobile phase. Note that there is no initial methanol–chloroform interaction, as was seen with 2-propanol. Methanol competes directly with the solute for active sites on the CSP. Here, the drop in retention is more

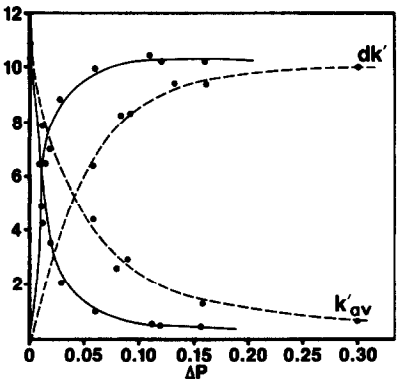


Fig. 6. Effect of mobile phase modifier concentration on PEDNBA retention, showing dk' *vs.* ΔP and k'_{av} *vs.* ΔP when hexane–chloroform is modified with methanol (———) or acetonitrile (– – –). See Experimental for mobile phase compositions.

rapid because methanol is a more interactive modifier in chloroform than in 2-propanol, and competes very strongly with the solute for active sites on the CSP.

Hexane–chloroform–acetonitrile. When acetonitrile is added to a hexane–chloroform mobile phase, k'_{av} drops less sharply than with methanol (Fig. 6), indicating that in this mobile phase, acetonitrile, although a polar modifier, is less interactive than methanol. Again, the corresponding rise in dk' indicates that the change in k'_{av} is due primarily to acetonitrile.

The difference in strength between methanol and acetonitrile is also seen in the polarity change required to cause k'_{av} to remain constant (flattened curve). For a similar decrease in retention, $\Delta P = 0.05$ and 0.17 for methanol and acetonitrile, respectively. Obviously, methanol is the stronger modifier.

The difference in the effects of the modifiers is seen in Fig. 7, in which the mobile phase is hexane–chloroform (38:62), and the modifier is 0.6% methanol or acetonitrile. Methanol has a much greater influence on retention and peak shape.

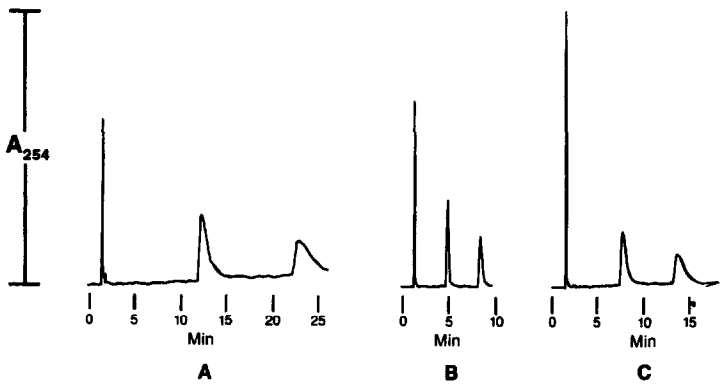
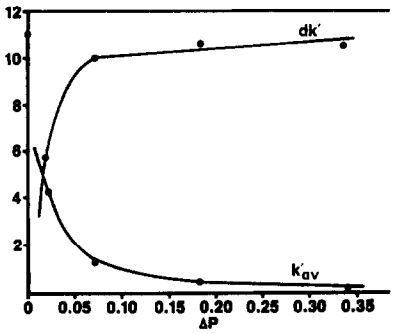


Fig. 7. Chromatograms showing the effects on retention and peak shape, produced by adding the methanol (B) or acetonitrile (C) to hexane–chloroform (A).

Hexane–dichloromethane–methanol. A ternary mobile phase, prepared by adding methanol to hexane–dichloromethane, shows a trend similar to that of the hexane–chloroform–methanol mobile phase (Fig. 8). This indicates that methanol is equally effective as a modifier in both binary mixtures, because it adjusts both to similar strengths. Retention becomes constant at $\Delta P = 0.05$.

Hexane–alcohol (80:20, v/v) binary phases

The effect of the steric bulk of mobile phase components on retention of the test solutes was investigated in mobile phases containing 20% (v/v) alcohol. Molarity differences for the various alcohols were not taken into account as the alcoholic portion of the varied mobile phases were in considerable molar excess when compared to the bonded stationary phase and chiral test solute in the chromatographic system. Fig. 9 shows that retention of the enantiomers increases, in a linear fashion, with the number of carbon atoms (steric bulk) of the alcohol in the mobile phase. Apparently, as steric interactions increase, the strength of the solute–CSP interactions decreases.



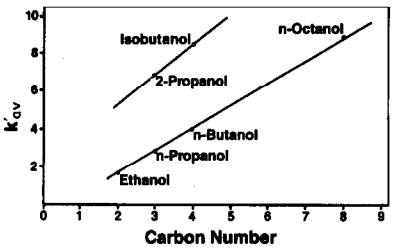


Fig. 8. Effect of modifier (methanol) concentration on PEDNBA retention, when added to a hexane–dichloromethane mobile phase. See Experimental for mobile phase compositions.

Fig. 9. Steric effect of alcohol modifiers on retention, showing k'_{av} *vs.* carbon number. Mobile phase, hexane–alcohol (80:20, v/v).

The two branched (hence more sterically hindered) alcohols produce greater retention of the test solute than their linear counterparts. In fact, 2-propanol and *n*-hexanol provide similar retention, and isobutanol provides retention similar to that from *n*-octanol. In other words, the branched alcohols provide retention similar to that of straight-chain alcohols having twice the carbon number. Compared to the linear alcohols, the steric bulk of the branched alcohols appears to reduce their ability to compete effectively with the solute for active sites on the CSP.

However, the branched alcohols impart greater selectivity to the CSP than their linear analogues. This is due to the fact that the branched alcohols allow the CSP to interact more strongly with the enantiomer that has the appropriate orientation, than with the less favorably oriented enantiomer. Chromatographically, the smaller alcohols allow better mass transfer than the larger alcohols (Fig. 10). Peak shapes in particular, are best with ethanol (Fig. 10A) or *n*-propanol (Fig. 10B).

Single-component mobile phases

Enantiomer separations obtained by using acetonitrile, 1,2-dichloroethane, and methyl *tert.*-butyl ether, as single-component mobile phases, are shown in Fig. 11. As expected, these solvents reduced retention of the test solutes in the order of increasing polarity: acetonitrile ($P = 5.87$) > 1,2-dichloroethane ($P = 3.5$) > methyl *tert.*-butyl ether ($P = 2.1$). The peak shape was best with acetonitrile and worst with methyl *tert.*-butyl ether. The strength of these solvents can be appropriately adjusted with weaker solvents, such as hexane.

General guidelines for method development

Although specific studies in this investigation were limited to one stationary phase and one test solute, these studies can serve as guidelines for developing methods, based on using the NU column or other π-donor, brush-type chiral stationary phases. The following summary of mobile phase trends, for PEDNBA, should apply generally for other chromatographic separations.

In the binary mobile phases, retention decreased when selective solvents were

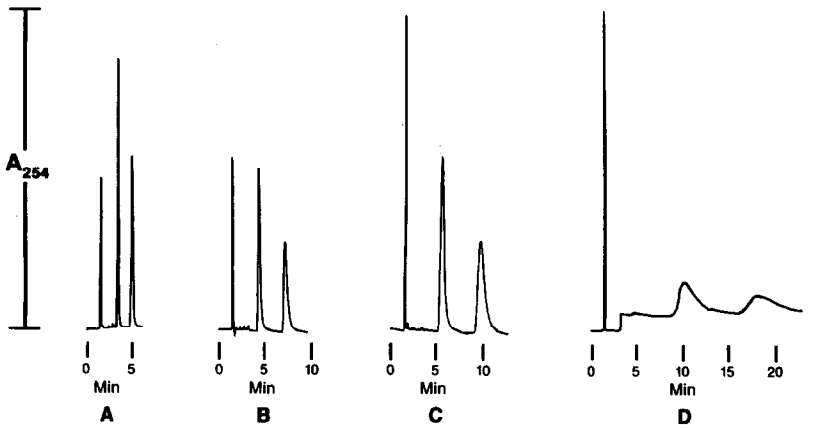


Fig. 10. Chromatograms showing the resolution of PEDNBA in hexane–ethanol (A), hexane–*n*-propanol (B), hexane–*n*-butanol (C), and hexane–*n*-octanol (D) (all 80:20, v/v).

added in the order 2-propanol > dichloromethane > chloroform (that is, proton acceptor > dipolar > proton donor). Also, k' increased with increasing chain length of *n*-alcohols when these were added (at the same concentration) to hexane. Branched alcohols caused longer retention than their linear analogues.

Addition of modifiers to the binary mobile phases caused a reduction in k' that corresponded with increasing modifier concentration. The largest decrease was seen with hexane–dichloromethane, and hexane–chloroform mobile phases to which methanol was added. In hexane–2-propanol, methanol and acetonitrile caused similar, but smaller, decreases in retention.

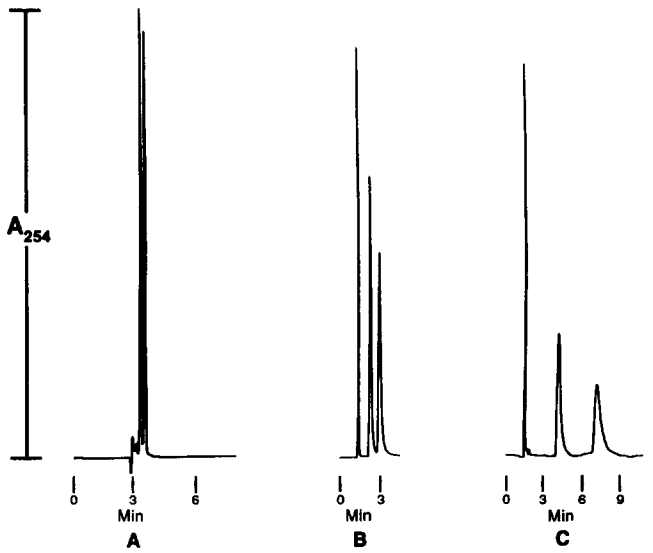


Fig. 11. Chromatograms showing the resolution of PEDNBA in the acetonitrile (A), 1,2-dichloroethane (B) and methyl *tert.*-butyl ether (C).

The separation factor for the isomers was largely independent of the composition of the mobile phase. Selectivity varied from 1.9 to 2.2 for 1,2-dichloroethane, acetonitrile, and methyl *tert.*-butyl ether single-component mobile phases, respectively. It was independent of component concentrations in all binary mobile phases and, in these cases, varied from 1.8 to 2.2. Selectivity was also unaffected by addition of small amounts of methanol or acetonitrile to a binary mobile phase. In all cases, sterically hindered (branched) alcohols gave higher α values than *n*-alcohols of the same carbon number.

Resolution of the isomers was greater in hexane–2-propanol than in any other binary mobile phase. Addition of modifiers increased resolution, methanol giving the largest increase, particularly in hexane–dichloromethane and hexane–chloroform.

CONCLUSIONS

The NU column shows good flexibility in use with numerous single-component, binary, and ternary mobile phases. Retention can be varied by changing mobile phase strength and composition. Selectivity was demonstrated to be mostly unaffected by changes in mobile phase strength, but was significantly influenced by mobile phase composition.

Organic modifiers, added to binary mobile phases at concentrations of less than 2%, influence both peak shape and retention. Methanol is a stronger mobile phase modifier than acetonitrile and exhibits a greater influence in a weak hexane–chloroform mobile phase than in hexane–2-propanol, suggesting that hydrogen bonding is the strongest modifier effect. At concentrations above 2.5%, such modifiers are only minimally effective at further reducing k'. Thus, a small quantity of a polar modifier can be used to modify a mobile phase to optimize a chiral separation.

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