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Review

Unusual effects of separation conditions on chiral separations

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Abstract

Unusual effects in liquid chromatographic separations of enantiomers on chiral stationary phases are reviewed with emphasis on polysaccharide phases. On protein phases and Pirkle phases reversal of the elution order between enantiomers due to variation of temperature and mobile phase composition has been reported. Most of the nonanticipated observations have dealt with the widely used polysaccharide phases. Reversed retention order and other stereoselective effects have been observed by variation of temperature, organic modifier and water content in nonpolar organic mobile phases.

Keywords: Reviews; Enantiomer separation; Chiral stationary phases, LC; Mobile phase composition

Contents

1. Introduction

Usual or expected effects of separation conditions on chiral resolution are by no means as obvious as would be desirable but this makes it a fascinating field. A couple of chromatographic dependences, for example, the relationship between separation factor, column efficiency and resolution is applicable for chiral chromatography as well. However, the possibility to resolve a pair of enantiomers is not easy to predict from their chemical structure even with the knowledge about the macro-structure of the chiral stationary phase (CSP). The rate of success to achieve resolution is fairly high due to the large number of commercially available CSPs. Among

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these, the polysaccharide phases developed by Okamoto and coworkers [1,2] have been found to be very versatile tools. Other widely used chiral phases are the derivatised tartaric acid CSP (Kromasil-TBB) [3], the α_1-acid glycoprotein by Hermansson [4], the Pirkle phases [5] cyclodextrins [6], polyacrylamides [7] and glycopeptides such as vancomycin, teicoplanin and ristocetin [8] are also quite useful in the separation of optical isomers. In retrospective, it may be possible from retention data to elucidate the correlation between analyte structure and stereoselectivity of the stationary phase as promoted by Pirkle [9] for his brush type CSPs and by Lindner [10] for CSPs of quinine based anion-exchangers. The interaction between the CSP and the analyte and the discrimination of one enantiomer relative to the other one is a matter of great interest. NMR and computational studies have been performed in order to help understand the chiral discrimination in chromatography. The different molecular modelling methods were reviewed a few years ago by Lipkowitz [11], who discussed the limitations of those approaches taken, due to experimental conditions being omitted but also foresaw new possibilities owing to improved computational tools. Attempts to elucidate the types of interactions present between the enantiomers and the structurally more complex polymeric CSPs have resulted in some insight [12,13]. However, the lack of knowledge about the geometry of the solute accessible binding structures in the CSP and the multiple types of cavities involved has to date hindered a detailed understanding of the chiral discrimination process at a molecular level.

2. Chiral phases

Unusual effects in chiral separations due to changes in temperature or composition of the mobile phase are often rationalized as conformational changes of the stationary phase, influencing the interaction with the solute molecule. The separation factor, α, is determined by the difference in the enthalpy and entropy of adsorption of the optical isomers to the CSP and the thermodynamic parameters are means to describe observed effects on the retention and resolution of the enantiomers without detailed explanation of effects.

2.1. Pirkle phases

For brush-type CPSs it is less likely to find unusual effects such as inversion of the enantiomers due to changes in mobile phase composition or temperature as pointed out by Pirkle [14]. Compared to protein or cellulose based phases, the brush-type phases immobilized on the silica support are more homogeneous and despite spatial variations all binding sites are of similar nature. Caude et al. [15] studied 3,5-dinitrobenzoyl derivatives of α-amino esters and found that the elution order of the enantiomers of one of the compounds was reversed when ethanol as organic modifier was exchanged for chloroform or dichloromethane in a hexane mobile phase. Pirkle reported on an unusual temperature effect for the enantiomers of the conformationally rigid spirolactam [16]. With low content of 2-propanol in hexane mobile phase, the retention first decreased, then increased and then decreased again when the temperature was raised. In a later study Pirkle and Murray [14] observed the inversion of the elution order of the enantiomers of 3,5-dinitrobenzoyl α-phenethylamine when temperature was successively changed. The observed effect of temperature was dependent on the content and polarity of the organic modifier in the mobile phase.

2.2. Protein phases

Protein based CSPs with aqueous mobile phases usually show decreased retention with increased temperature or increased amount of organic modifier in the mobile phase. Gilpin and coworkers [17] found that the retention of D-tryptophan on a CSP of bovine serum albumin immobilized on silica increased with decreasing temperature as expected, while for L-tryptophan there was first an increase in retention reaching a maximum, which was followed by a slight decrease. Similarly, Isaksson and coworkers [18] observed in their studies on cellulase CBH I CSP that the two enantiomers of propranolol behaved differently on variation of the temperature. Increase in the temperature decreased the retention of the (*R*)-enantiomer, which eluted first, while the (*S*)-enantiomer became more retained resulting in a larger α-value at 45°C compared to 10°C. When investigating the separation of the enantiomers of

sotalol, Fulde and Frahm [19] found on the same kind of CSP, a reversal of the retention order in the range of 17–28°C. The retention time of the (*S*)-enantiomer rapidly decreases with increasing temperature while the retention of the (*R*)-enantiomer is only slightly affected by temperature changes. A reversal in the retention order was also seen by using acetonitrile instead of 2-propanol as organic modifier at 23°C. A change in the enantiomeric retention order was also seen on an ovomucoid-bonded silica column [20] when ethanol or 2-propanol as organic modifier was exchanged for methanol or acetonitrile. The compounds studied were propranolol and its ester derivatives. Karlsson and coworkers [21] found that the elution order of the enantiomers of mosapride and its metabolite was inverted at 30 and 20°C respectively, but also when they changed pH of mobile phase buffer solution at 20°C. This study was performed on a α_1-acid glycoprotein (Chiral AGP) phase which was also used to separate the enantiomers of felodipine [22], whereby the retention was increased for the (*R*)-isomer and decreased for the (*S*)-isomer with increasing pH.

2.3. Polysaccharide phases

The major part of this paper will deal with polysaccharide CSPs developed by Okamoto and coworkers [1,2] since they show the broadest applicability so far and also most reported unusual effects. These have mainly come from studies in the normal-phase mode which is quite dominating although for some of the cellulose and amylose based CSPs aqueous mobile phases were introduced a few years ago [23]. Recently polar organic mobile phases have attracted a lot of interest for chiral separations [24] not least as start-up mobile phase to evaluate enantioselectivity. In general, the retention of the analytes is very much influenced by the amount of polar modifier in the apolar mobile phase, while the separation factor often remains relatively constant [25]. The retention of the two enantiomers is thus influenced in a similar way and the solvent molecules compete with the solute molecules for the specific adsorption sites on the CSP but solvation in the mobile phase will also increase with increased modifier content. This is in accordance with achiral liquid chromatography and that is also valid for temperature effects where increased temperature commonly gives decreased retention.

2.3.1. Effects of sample amount

An inversion of the capacity factor of a pair of atropisomers was observed by Rousell and coworkers [26] when they increased the sample amount by liquid chromatography on microcrystalline triacetylcellulose. For an analytical scale sample the first eluted enantiomer was dextrorotatory, whereas for semipreparative scale injection it was levorotatory as followed by a polarimetric detector.

2.3.2. Temperature effects

Optimization of enantioselectivity for separation on polysaccharide stationary phases should always include investigation of temperature dependence, since this can enhance the separation factor needed for partial or complete resolution of the two enantiomers. Küsters et al. succeeded to separate sulphoxide enantiomers on Chiralcel-OB by increasing the column temperature from 20 to 50°C [27] and a partial resolution was achieved for the compound, rolipram, on Chiralcel OD by going from 20 to 60°C [28]. The opposite effect was seen by Smith et al. [29], where a decrease in temperature from 42 to 0°C partially resolved the enantiomers of a structural analogue of a potassium channel activator on Chiralcel OD. A compound having a closely related structure showed peak broadening but the separation factor of the resolved enantiomers was almost unchanged. Under supercritical fluid chromatographic conditions, Yaku et al. found on the Chiralcel OF column [30] that the separation of the *trans*-enantiomers of diltiazem improved with increased temperature, while chiral separation was improved for the *cis*-enantiomers by decrease in temperature. The latter behaviour was observed for both *cis*- and *trans*-enantiomers in normal-phase liquid chromatography. The effects observed were attributed to differences in the steric environment around the phenyl groups of the diltiazem isomers between the supercritical fluid and liquid chromatographic mode. Cabrera and Lubda [31] found on β-cyclodextrin, chemically bonded to silica, that the chiral separation of oxazepam was improved by decreased temperature but for mephobarbital by increased temperature.

2.3.3. Solvent effects

Increased concentration of the polar organic modifier, most often an alkanol or acetonitrile, leads to decreased retention as expected. Among the alcohols, usually C_1–C_4, the primary ones with straight chains are stronger eluents on a molar basis than the secondary and tertiary alkanols having a branched alkyl chain. This is largely in agreement with achiral liquid chromatography. However, as far as enantioselectivity is concerned, it is difficult to predict which solvent will be the most favourable modifier in order to achieve sufficient resolution or give maximum separation. In retrospective, it may be possible to draw some conclusions on the interactions involved in the chiral discriminating process. However, retention order is difficult to predict given the complexity of the derivatized polysaccharide CSPs.

Retention order may be inversed changing from one mobile phase modifier to another one as shown by Gaffney et al. [32] for 2-phenoxy propanoic acid methyl ester. On Chiralcel OD with hexane as nonpolar solvent, ethanol and 2-propanol gave separation of the two enantiomers and the same elution order, use of 1-propanol gave no resolution and 1-butanol reversed the elution order. Inversed enantiomeric retention was also found for an insecticide on Chiralcel OJ due to the different properties of the alcohols used as modifiers in the mobile phase [33]. Balmér and coworkers separated enantiomers of omeprazole and other substituted benzimidazoles on a Chiralpak AD column [34]. Using ethanol as a modifier in hexane, the (−)-enantiomer eluted first with an α-value of 1.8 but with 1- or 2-propanol as the mobile phase modifier, the retention order was changed and the (+)-isomer eluted first with a separation factor of 1.1–1.3 (Fig. 1). Consequently, mixtures of ethanol and propanols (4 and 10% respectively) resulted in co-elution of the enantiomers. Methanol gave the same retention order as ethanol but was only used together with propanols due to low solubility in hexane. An interesting effect was observed for timoprazole, a structural analogue of omeprazole when the content of methanol was gradually increased in a mobile phase consisting of 20% 2-propanol in hexane. A methanol concen-

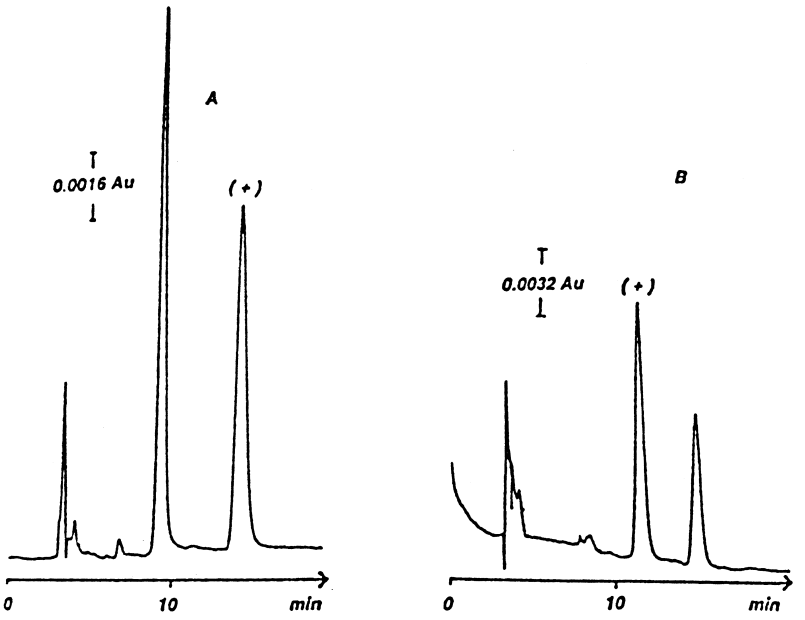

Fig. 1. Enantioselective separation of omeprazole on Chiralpak AD column, flow-rate 1.0 ml/min. (A) Mobile phase ethanol–hexane (35:65, v/v), $k'_1 = k'_{(-)} = 2.66$, $\alpha = 1.72$; (B) mobile phase 2-propanol–hexane, (14:86, v/v) $k'_1 = k'_{(+)} = 4.16$, $\alpha = 1.20$. (Reproduced with permission from Ref. [34]).

tration below 6% had very little effect on the separation factor (α~1.2). At concentrations above 6%, the (−)-enantiomer behaved as expected, i.e. an increase in the methanol content of the mobile phase decreased retention. However, the retention of the more retained (+)-enantiomer increased by 80% as the methanol content was increased from 6 to 8%. A larger α-value of about 2.2 was attained and valid at least up to addition of 20% of methanol (Fig. 2). This was interpreted as a competition between methanol and 2-propanol in the mobile phase, whereby 2-propanol adsorbed on the CSP is displaced by methanol resulting in the drastic effect on the retention of one of the enanantiomers. If this induced conformational changes in the CSP, these were found to be reversible on lowering the methanol content again. A reversal in the elution order of the two enantiomers of a substituted benzylmorpholinone compound was observed by Wang and Chen on changing the polar modifier from 2-propanol to ethanol [35]. This effect was seen both on Chiralpak AS and Chirlapak AD but not on Chiralcel OD. Furthermore, none of the three CSPs showed a reversal in the elution order of the optical isomers of two structurally related analytes which underlines the complexity of this kind of stereoselective effect.

2.3.4. Water content

Most reports on the effect of organic modifiers in the nonpolar mobile phase have not considered the influence of the water content. This is not necessarily of any great importance for the enantiomeric pairs resolved with sufficient selectivity. However, in certain instances and for difficult separations it may be crucial to control the water content and decisive for successful separation of the enantiomers. These effects have so far mainly been reported or studied for a number of amino alcohol compounds on the Chiralcel OD. For almokalant, an amino alcohol with one asymmetric carbon atom and one sulphoxide group, Balmér and coworkers [36] found that the presence of a moderate amount of water in the mobile phase enabled the resolution of all 4 isomers. Thus, the presence of about 400 mg water per litre mobile phase consisting of 15% 1-propanol and 0.1% diethylamine in hexane, decreased the retention of the (*R*)-(+)-isomer resulting in the complete separation of the isomers. Without control of water content a lot of confusion may occur due to lack of consistency for this kind of separation. For metoprolol, another amino alcohol, separated also on Chiralcel OD, the influence of the water content was studied in more detail [37]. The (*R*)-enantiomer

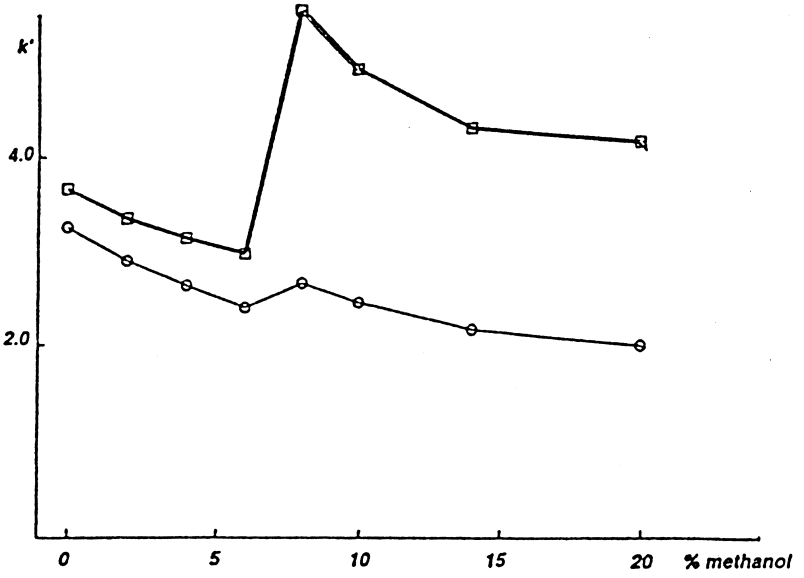

Fig. 2. Influence of the addition of methanol to 2-propanol–hexane (20:80, v/v) mobile phase on the capacity factors, k', of the isomers of timoprazole. Chiralpak AD column. □=(+)-Isomer; O=(−)-isomer. (Reproduced with permission from Ref. [34]).

eluted first and was not affected significantly by increasing the water content from 200 to 1400 mg/l. However, the retention of the (*S*)-enantiomer decreased resulting in a decrease in a from 2.3 to 1.4 (Fig. 3). A major metabolite, α-hydroxymetoprolol, has an additional asymmetric carbon and thus 4 isomers, where the water content had to be in the range 600–800 mg/l to enable complete separation (Fig. 4). Also, for this compound the two (*R*)-isomers are only slightly affected by the water content.

In a more extensive study, the separation of the optical isomers of metoprolol and three of its structural analogues was investigated [38].The compound, H 170/40, which had an α-value of around 1 was of particular interest, since inversion of the retention order of its two enantiomers could be induced by

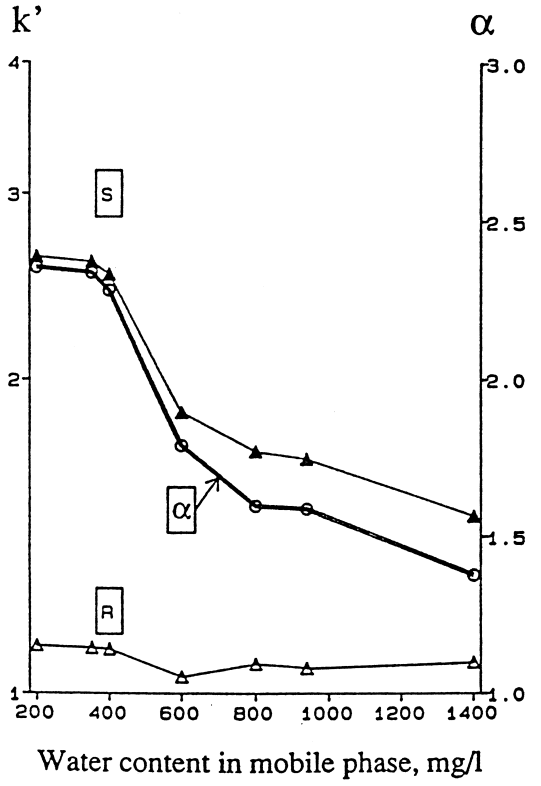

Fig. 3. Effect of water content in the mobile phase on retention (k') and separation factor (α) of (*R*- and (*S*)-metoprolol. Mobile phase: 10% 1-propanol and 0.1% diethylamine in hexane; temperature, 30°C; flow-rate, 0.75 ml/min. (Reproduced with permission from Ref. [37]).

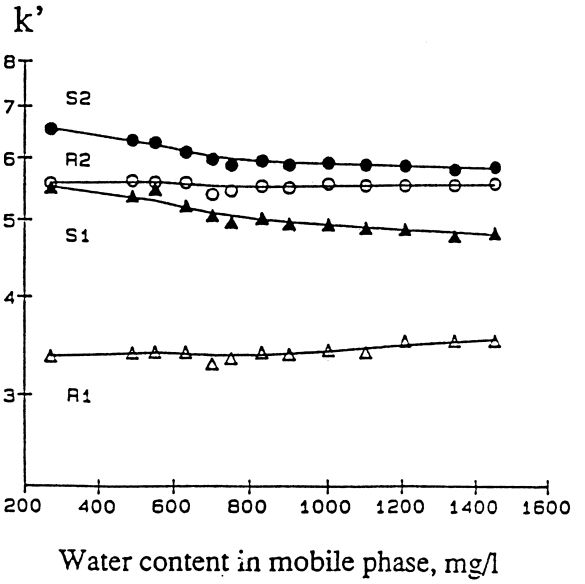

Fig. 4. Effect of water content on retention (k') of the isomers of (*R*)- and (*S*)-α-hydroxymetoprolol. Mobile phase: 10% 1-propanol and 0.1% diethylamine in hexane; temperature, 30°C; flow-rate, 0.75 ml/min. (Reproduced with permission from Ref. [37]).

different means. When the concentration of 1-propanol was increased from 10 to 30% in a mobile phase consisting of hexane containing 10 m*M* diethylamine and 1 g/l water, a reversal of the elution order was obtained (Fig. 5). Using constant alcohol concentration and varying the water content from 0 to 1600 mg/l, the retention of the (*R*)-enantiomer was almost unaffected, while the more retained (*S*)-enantiomer showed decreasing capacity factor up to 1000 mg water per litre resulting first in coelution and finally inversed elution order (Fig. 6). Increasing temperature from 15 to 55°C also reversed the retention order as seen in Fig. 7. However, this was dependent on the water content in the mobile phase as illustrated by the Van't Hoffs plot in Fig. 8. Balmér et al. suggested that the observed interdependent effects could be linked and were due to the extent of accessible free water in the mobile phase. The alcohol is responsible for solvation of the water in the mobile phase and the degree of solvation is affected by the type and content of the alkanol as well as by the temperature. In a recent study, Karlsson and coworkers [39] used a statistical experimental design to study the influence of 2-pro-

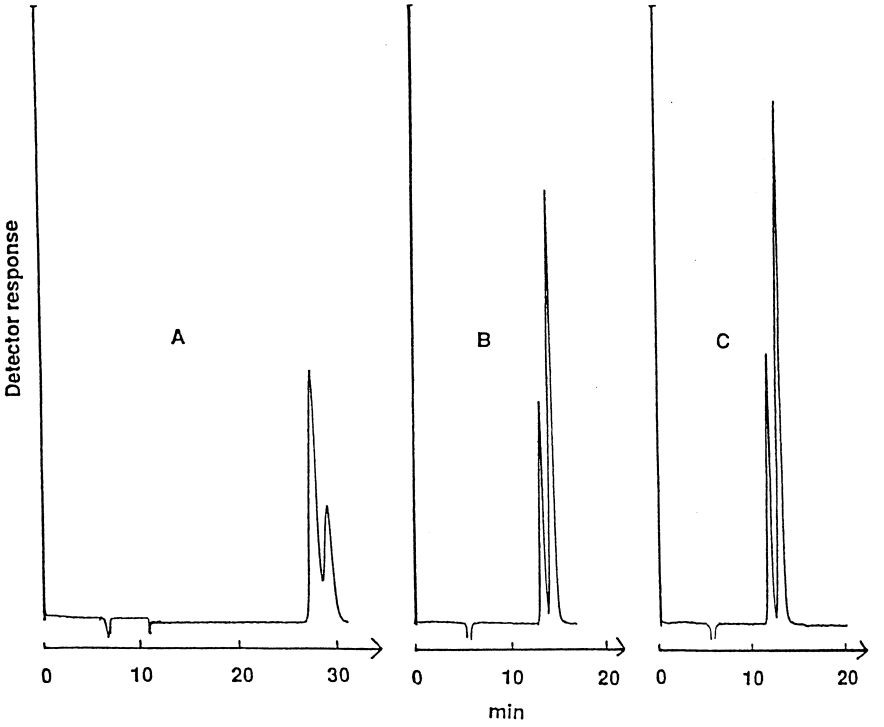

Fig. 5. Effect of propan-1-ol on the resolution of enantiomers of H 170/40. Mobile phase: 1 g/l water, 10 m*M* diethylamine in hexane and propan-1-ol. Solutes: *R*- and *S*-enantiomers in the ratio 1:2. (A) 0.67 mol/l propan-1-ol ($\alpha = 0.94$); (B) 1.33 mol/, ($\alpha = 1{,}13$); (C) 2.0 mol/l ($\alpha = 1.17$). (Reproduced with permission from Ref. [38]).

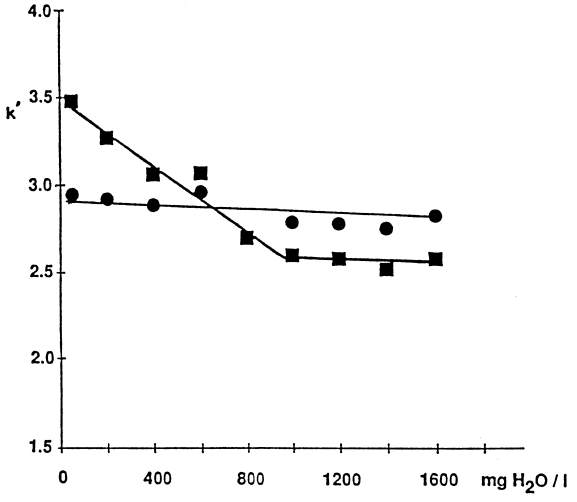

Fig. 6. Influence of water on the retention of the enantiomers of H 170/40. Mobile phase: 1.0 mol/l propan-1-ol, 10 m*M* diethylamine and water in hexane. Temperature, 30°C; flow-rate, 0.5 ml/min. ■ = *S*-Enantiomer; ● = *R*-enantiomer. (Reproduced with permission from Ref. [38]).

panol content and temperature on the enantioseparation of several metoprolol analogues on Chiralcel OD. They also included acetic acid as a parameter in the multivariate design experiments and found that the retention order was reversed for one of the compounds studied when going from 0 to 25 m*M* acetic acid in the mobile phase.

3. Conclusion

Unusual effects on the separation of optical isomers and elution order have been observed due to interaction of the polar mobile phase additives with the different binding sites available on the CSP or with the enantiomer molecules. Furthermore, temperatures may in some cases affect the geometry of the stereodiscriminating sites in the CSP due to conformational changes in the polymer structure and also the solvation of the CSP and/or the analyte, resulting in non-anticipated changes in the selectivity

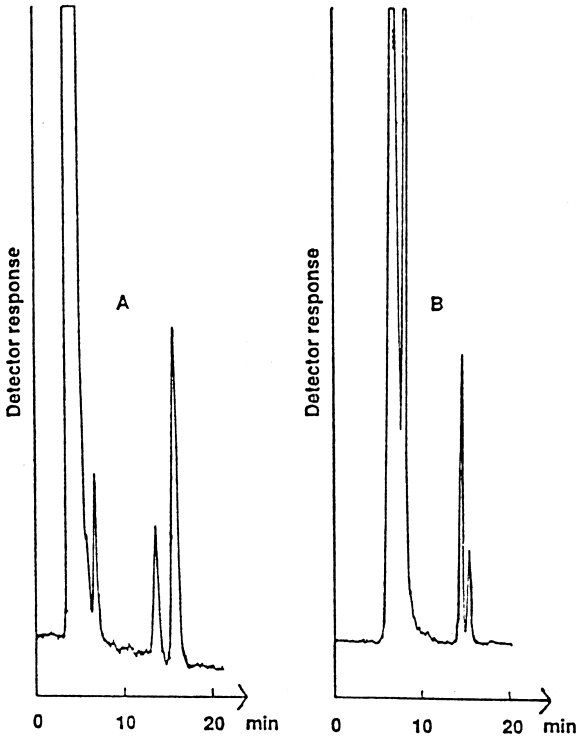

Fig. 7. Effect of temperature on the resolution of the enantiomers of H 170/40 on Chiralcel OD. Mobile phase: 1.0 mol/l propan-1-ol, 10 m*M* diethylamine and 1 g/l water in 2,2,4-trimethylpentane. Solutes: *R*- and *S*-enantiomers in the ratio 2:1. (A) 15°C, flow-rate 1.0 ml/min; (B) 55°C, flow-rate 0.5 ml/min. (Reproduced with permission from Ref. [38]).

and the reversal of the elution order of the enantiomers.

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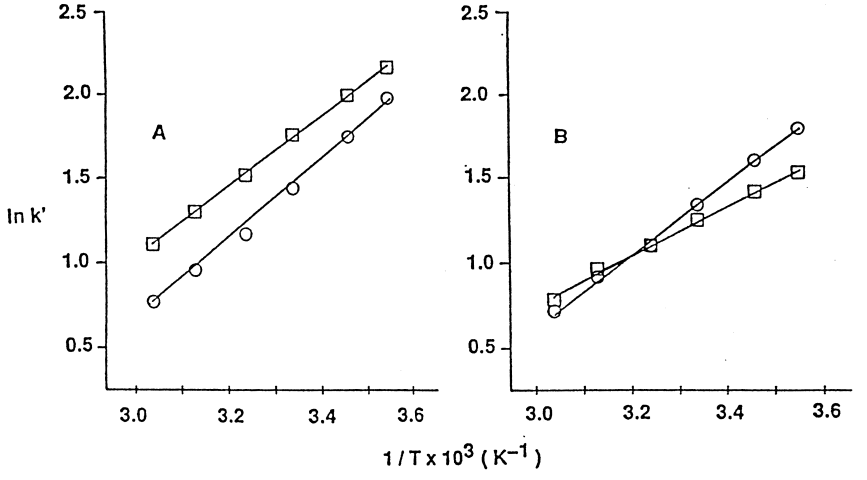

Fig. 8. Van't Hoff plots for the enantiomers of H 170/40. Temperature range, 8–55°C. Mobile phase, 0.93 mol/l propan-1-ol, 10 m*M* diethylamine in 2,2,4-trimethylpentane. Flow-rate, 0.5 ml/min. (A) No water added; (B) 1 g/l water added to the system (□) *S*-Enantiomer; (O) *R*-enantiomer. (Reproduced with permission from Ref. [38]).

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