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Review

Reversal of elution order during the chiral separation in high performance liquid chromatography

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Dedicated to Professor Dr Gottfried Blaschke on the occasion of his 65th birthday.

Abstract

The elution order of the enantiomers is one of the most important topics in the field of chiral separations. The reversal of enantiomeric elution order, although rare, could be observed during the investigations of chiral discrimination in high performance liquid chromatography (HPLC). Scrutinizing these phenomena must be helpful in determining the optical purities because it is favorable to elute the minor enantiomeric impurity before the major component. In this mini-review, several examples of such unusual behavior will be described from the point of mechanistic rationale. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Chiral separation; Elution order; Enantiomer; Thermodynamic study; van't Hoff plots

1. Introduction

A variety of chiral stationary phases (CSPs) is now available for the separation of enantiomers by high performance liquid chromatography (HPLC), and they have proved to be very useful in the chromatographic resolution of racemic mixtures.

The elution order of the enantiomers is one of the most important topics in the field of chiral separations. In most cases, it is desirable to detect the minor component in the front of the major one in analyzing the synthetic chiral compounds. This is mostly due to the peaks not always being Gaussian and so-called tailing appearing in chromatography. For example, even an enantiomeric impurity as high as 1% was often impossible to detect when it is eluted as the second peak. It will be easily detected if it elutes as the first peak. In general, several studies showed that the limits of detection and quantification were lower and that reproducibility of the determination was higher for the first-eluting enantiomer [1]. This can be

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achieved easily by switching the antipode of the chiral selector [2,3], or by acquiring various kinds of CSPs [4,5].

The reversal of elution order of a particular pair of enantiomers from a given CSP, although rare, could be observed during the investigations of chiral discrimination in HPLC. It is clear that if the order of elution is reversed, it is because the relative stability of both adsorbates enantiomer/CSP have changed. Scrutinizing these phenomena must be helpful in determining the optical purities because it is favorable to elute the minor enantiomeric impurity before the major component.

In this mini-review, the present status of such unusual behavior occurred by the variation of the properties of the mobile phase from the point of mechanistic rationale is described.

2. Theory

Chromatographic retention depends upon the free energy of the partitioning process of the analyte between the mobile phase and the stationary phase. The van't Hoff plots are generally used in order to estimate the entropic and enthalpic contributions to these analyte interactions [6].

In the separation of enantiomers by chromatography, the separation factor, α, is determined by the difference between the free energy of adsorption of each enantiomer, this can be expressed by the following equation:

$$-\Delta\Delta G = RT \ln \alpha. \quad (1)$$

By an application of the Gibbs–Helmholtz equation; $\Delta G = \Delta H - T\Delta S$, an expansion of Eq. (1) yields as follows, where R is the gas constant, T is the absolute temperature of the column.

$$\ln \alpha = -\Delta\Delta H/RT + \Delta\Delta S/R. \quad (2)$$

Therefore, $\Delta\Delta G$ is itself composed of the terms of standard enthalpies and entropies of transfer from the mobile phase to the stationary phase.

In HPLC, the enantioseparations are dominated by enthalpic contributions in most cases, because the experiment is commonly performed at the comparatively low temperatures. The adsorption of enantiomers on the CSP usually features negative $\Delta\Delta H$ and $\Delta\Delta S$. These thermodynamic parameters are experimentally accessible and trends within these data can often provide valuable mechanistic information.

By collecting chromatographic data at different temperatures, one can plot the natural logarithm of separation factors (α) of the enantiomer against the reciprocal of absolute temperature at constant pressure. If this plot represents a linear inverse relationship between $\ln k'$ and temperature: (i) ΔH and ΔS are temperature independent; (ii) the enantiomers are retained in a single associative mechanism; (iii) a solvation–desolvation equilibrium does not obscure the association process of the enantiomers with CSP. Model van't Hoff plots for the separation of enantiomers on CSP are depicted in Fig. 1.

The slope of the line will then be proportional to the enthalpy difference, and $\ln \alpha = 0$ (the individual enantiomers will coelute) will give the temperature at which the enthalpy and entropy contributions cancel each other (isoelution temperature (T_{iso}))[6]. Above T_{iso}, an inversion in the elution order of the enantiomers is expected. Such phenomena have sometimes been observed in gas chromatography (GC) at relatively high temperature, because solvation effects are essentially absent [7]. HPLC is normally performed at relatively low temperature such as room temperature; little retention of analyte will be observed at high temperature. Therefore, the van't Hoff plot analysis in HPLC is generally less effective for predicting the enantiomeric elution order reversion than that in GC.

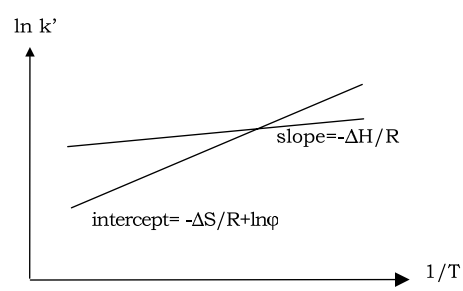

Fig. 1. Model van't Hoff plots for the separation of enantiomers on CSP. ϕ denotes the column phase ratio.

On the other hand, if stationary phase in HPLC undergoes a change in conformation at a certain temperature (transition temperature), the enthalpy and entropy of retention process will change, and the nonlinear van't Hoff plots will be obatined. Since both enthalpy and entropy of adsorption are influenced by any changes in solvation which accompany analyte adsorption, it is conceivable that mobile phase composition might influence not only the magnitude, but also the sign of $\Delta\Delta G$. This implies that the elution order of a particular pair of enantiomers from a given CSP in HPLC could be mobile phase dependent.

3. The reversal of elution order in Pirkle-type and other brush-type CSPs

The 'three-point-interaction model' applies mainly for the Pirkle-type CSP in the chiral discrimanation. According to the 'the three-point interaction model' proposed by Pirkle and Pochapsky [8], two interactions between the CSP and the analyte are much the same for each enantiomer, and one is generally different. This interaction difference is expressed in the term of $\Delta\Delta H$ in Eq. (2), whereas the former two interactions contribute to retention and not to chiral discrimination. This suggests that all the binding sites are nearly the same in the CSPs. Very small spacing variations may cause small perturbations of the dominant chiral recognition process. Inversions in the elution of the enantiomers are thus relatively rare and might occur only when the separation factor is generally small.

The first example on the inversion of the enantiomeric elution order in this type CSP was reported by Macaudière et al. [9]. They used the 3,5-dinitrobenzoyl (DNB) derivative of tyrosine as the analyte in order to investigate the separation on brush-type CSP including the thio-DNBTyr-A. When ethanol was utilized as the mobile phase, the elution order of the enantiomer was R/S. On the contrary, the enantiomeric elution order was S/R with chloroform as the mobile phase. They did not, however, investigate the thermodynamic approach on the chiral recognition. From these observations, they suggested that either solvation or conformation of both the solute and the CSP are affected by the change in the nature of the polar modifier. This may in turn alter the type of interaction involved during the chiral recognition process.

Pirkle and Murray first reported the temperature-dependent elution order reversal in π-basic proline-derived CSPs in 1993 [10]. They used (*RS*)-*N*-(3,5-dinitrobenzoyl)-α-phenylethylamine as the solute to investigate the response of the chromatographic behavior by changing the temperature on the CSP. When 20% 2-propanol in hexane was used as the mobile phase, the separation factor between enantiomers varied from 1.07 to 1.15 in 10–50 °C range, and at 0 °C the separation collapses, but −25 °C the separation is again clear ($\alpha = 1.11$), the enantiomers now eluting in the opposite order. The van't Hoff plots study resulting from this temperature are decidedly nonlinear and intersect at 0 °C. They suggested that with various kinds of modifiers including alcoholic and nonalcoholic mobile phase modifiers that this abnormal behavior resulted from the concentration and polarity of the alcoholic mobile phase modifier used.

This observation was explained as follows; prior to adsorption, polar sites in the solute and the CSP were solvated by the polar modifier in the mobile phase. Some desolvation of these sites used for chiral recognition was required to permit more effective solute-CSP interaction. This desolvation was energetically costly and decreased the binding energy of the solute relative to what would be observed in the nonsolvated gas phase situation. Because the more retained enantiomer normally used more polar site interactions with the CSP than did the less retained antipode, it typically had its retention reduced to a greater extent by these desolvation effects than did its antipode. As a consequence of this, enantioselectivity was usually reduced by such effects.

This desolvation might affect the conformations, and the selective solvation of specific sites which are essential to the chiral recognition process might change the strengths of the interactions at those sites, or new interactions might become important. They speculated that solvation affected weak interactions more profoundly than it did

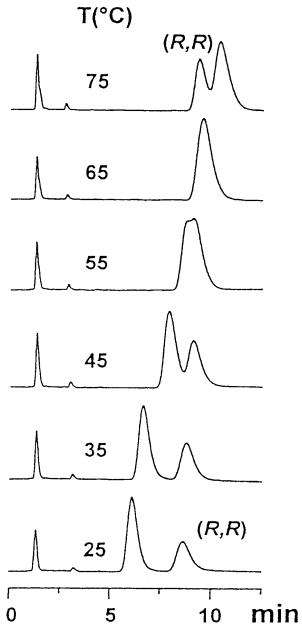

Fig. 2. Variable temperature chromatography of di-(3,5-dinitrophenyl)-carbamate derivative of *trans*-1,2-cycloheptanediols on a silica bound two-armed receptor CSP using 1% methanol in chloroform as eluent. (Reproduced by permission from Ref. [11].)

strong interactions. On this basis, a rationalization can be advanced which accounts for the ability of strong solvation to invert the elution order of the enantiomer.

The temperature dependent elution order of enantiomers of cyclic *trans*-1,2-diols on a silica bound two-armed receptor CSP have also been reported by Gasparrini et al. [11]. Retention of the enantiomers of six and seven membered diols showed an unusual response, i.e. the van't Hoff plot was nonlinear to temperature changes in the 25–85 °C range. The anomalous variation in retention with temperature led, for cyclohexan- and cycloheptanediols, to a reversal of the elution order above 65 and 75 °C, respectively (Fig. 2). These effects were observed with chloroform/methanol mobile phases and were absent with ethylacetate or acetonitrile as polar additives. Since the separation factor was not so small as the example in reference [10] in this case, it was suggested that this CSP would have relatively large conformational flexibility. This flexibility might be a potential source of nonlinearity for an $\ln k'$ versus $1/T$ plot.

4. The reversal of elution order of enantiomers in the protein bonded CSPs

A sort of 'chiral steric exclusion mechanism' and/or 'steric fit concept' involving only one binding interaction generally operates in the process of chiral discrimination on the protein-bonded CSP. The relative contributions made by each type of binding site to the overall retention of each enantiomer could depend upon mobile phase composition, temperature or both.

The reversal of the elution order in the protein-bonded CSP was first reported by Hermansson and Schill in 1988 [12]. They observed the inversion of the enantiomeric elution order for pseudoephedrine occurring on an α_1-acid glycoprotein-based (AGP) column with the addition of octanoic acid to the eluent. Variation of properties of the mobile phase, such as the concentration of a charged modifier, can be inferred from the changes in the chiral selectivity that even a reversal of the retention order of the enantiomers is obtained. These effects might be due to a blocking of one or more of several chiral bindig sites but a conformational change of the protein that gives rise to new stereoselective binding properties is a highly interesting alternative.

In 1990, Haginaka et al. observed the inversion of the elution order of propranolol (PP) and its ester derivatives (*O*-acetyl, -propyl, -butyl and -valerylPP) on an ovomucoid-bonded column [13]. In this study, the enantiomeric elution order was (*S*)/(*R*) for PP and (*R*)/(*S*) for the other four ester derivatives, when ethanol or 2-propanol was used as the organic modifier. When methanol or acetonitrile was used as the organic modifier, the inversion of the enantiomeric elution order was observed for *O*-valerylPP with the use of methanol, and for PP and *O*-propylPP with acetonitrile. Reversal of the enantiomeric elution order of these pharmaceutical substances occurred around eluent pH 5–7 in addition to the variation in the organic modifier used [14]. They have not scrutinized the contribution of the enthalpy

parameter to the enantioselectivity with a change in the mobile phase composition, and/or eluent pH. They suggested that there may be more than one binding site on this protein-bonded column, and/or that at least two chiral recognition mechanisms may operate on this CSP with regard to PP and its ester derivatives. Also, a conformational change in the ovomucoid-bonded structure might be caused by a change in eluent pH and/or addition of an organic modifier.

Along with a change in the pH or composition of the mobile phase, another way to control a protein-bonded chiral separation is to vary the column temperature. Loun and Hage have reported a typical chromatographic behavior of (*RS*)-warfarin on a human serum albumin (HSA)-immobilized column [15]. In their case, the enantiomeric elution order was inverted from (*R*/*S*) to (*S*/*R*) at about 15 °C. Based on only the thermodynamic data, this result was unexpected, because (*S*)-warfarin had a larger association constant than (*R*)-warfarin throughout the entire range of temperatures used. This discrepancy was caused by applying Eq. (2) to this phenomenon. The degree of separation predicted from the value of ΔH and ΔS was much larger than that actually measured. Better agreement was obtained when Eq. (2) was used, which combines the use of these thermodynamic constants along with the column binding capacities. According to Eq. (3), if the ratio of the solute binding sites is not constant with temperature, van't Hoff plots will be nonlinear.

$$\ln \Delta = -\Delta\Delta H/RT + \Delta\Delta S/R + \ln(m_{L2,T}/m_{L1,T}). \quad (3)$$

In this equation, $m_{L2,T}$ and $m_{L1,T}$ are the binding capacities for solutes 1 and 2 at the temperature being examined. Note that the above expression is reduced to Eq. (2) if the binding capacities for solutes 1 and 2 are identical. If the ratio $m_{L2,T}/m_{L1,T}$ is constant with temperature, then a plot of $\ln a$ versus $1/T$ will still be linear, as in Eq. (2). Eq. (3) can also be used if the value of $m_{L2,T}/m_{L1,T}$ changes with temperature; however, in this case, some nonlinear behavior in the resulting plot of $\ln \alpha$ versus $1/T$ might be observed.

Similar observations in the chiral separation of DL-tryptophan by a silica-immobilized bovine albumin column have been reported by Gilpin et al. [16].

5. The reversal of the elution order in the polysaccharide CSPs

Like the protein bonded CSP, the 'chiral steric exclusion mechanism' and/or 'steric fit concept' function in the chiral recognition in these CSPs'.

The reversal of enantiomeric elution order for the polysaccharide CSP was first reported by Okamoto et al. in 1991 [17]. They found that the reversal of the elution order of the enantiomers on a modified cellulose column (Chiralcel OJ) was associated with changes in the mobile phase modifiers during the investigation of the direct chromatographic enantioseparation of pyriproxyfen, an insect growth regulator. Like protein-bonded CSP, only one binding interaction such as the 'chiral steric exclusion mechanism' and/or 'steric fit concept' operates in the polysaccharide CSP. The contribution of the enthalpy parameter to the enantioselectivity by changing the mobile phase composition could not be estimated, because they have not scrutinized the temperature effect was not investigated in this study. There are two possibilities for this abnormal behavior: (i) the solvation or the conformation of either pyriproxyfen or the CSP is affected by the change in the nature of the modifier; (ii) an alteration in the steric environment of the chiral cavity in CSP could be induced by changing the modifier. Extensive nuclear Overhauser effect (NOE) experiments by ^{1}H NMR and circular dichroism (CD) measurements showed that the conformational changes of both the analyte and CSP did not occur with a fair degree of certainity. This phenomenon can be explained by the difference in the steric bulkiness around the hydroxyl moiety of the mobile phase modifier. The enantiomeric elution order was *S*/*R* with methanol, ethanol, 2-propanol, or *tert*-butanol as the mobile phase modifier, and the elution order was *R*/*S* with 1-propanol, 1-butanol, isobutanol, pentanols, or hexanols. Although the solvent polarity parameter

values for 1- and 2-propanol are virtually the same, the enantiomeric elution order was S/R with 2-propanol as the mobile phase modifier, and the elution order was R/S with 1-propanol. This shows that diastereomeric complex(es) between the CSP and the (R)-isomer may be more stabilized with less bulky alcohols such as methanol, ethanol, 2-propanol, or *tert*-butanol, as the mobile phase modifier, and that with bulkier alcohols such as 1-propanol, 1-butanol, isobutanol, pentanols, or hexanols, diastereomeric complex(es) between the CSP and the (S)-isomer may be more stabilized. The less bulky alcohols could be inserted into the cavity of the CSP more easily than the bulkier alcohols. The insertion of the mobile phase modifier into the chiral cavities of the CSP could induce changes in the dominant chiral recognition mechanism, leading eventually to inversion of the enantiomeric elution order. This suggested that at least two chiral binding- or recognition-sites are present in this CSP.

Although we have tried to find such phenomena in the separation of a variety of the racemic substances on the polysaccharide CSP, we could have observed only one example described above so far [18,19].

Posterior to their report, similar reversed enantiomeric elution order was reported by the Balmér et al. [20]. The inversion of the elution order was observed when a derivative of metoprolol, H170/40, a amino alcohol derivative with β-adrenergic blocking activity, was separated by Chiralcel OD containing cellulose *tris*(3,5-dimethylphenylcarbamate) as the CSP. They clearly showed that in this case the van't Hoff plot was linear and that T_{iso} decreased on adding water to the mobile phase consisting of 1-propanol/diethylamine/2,2,4-trimethylpentane. A preliminary study on thermodynamic approaches to the chiral discrimination employing an amylose-derivative CSP was also conducted by Kazusaki et al. [21]. In this study, enthalpies and entropies of the solute transfer (mobile to CSP) are calculated from retention data by evaluation of van't Hoff plots. Conformational changes of CSP, indicated by nonlinear van't Hoff plots, can be accompanied by the difference in analyte adsorption/desorption rates.

6. Conclusion

First of all, one should take into account the contribution of the mobile phase modifier to the enantioseparation, because one might also find the unusual effects described above. The importance of modifier selection has been pointed out by Stringham and Blackwell [6]. If one can find such a phenomenon, although very rare in HPLC, it will be important to understand the reasons for this behavior and to anticipate when such inversions of elution order are likely to occur. In addition, knowing the T_{iso} of certain analytes under given experimental conditions might be helpful in the determination of optical purities (if possible in HPLC experiments), as the elution order will be reversed, and other selectivity should be observed when increasing the temperature above T_{iso}. Finally, the author emphasizes that computational approaches should be introduced to elucidate the unusual behavior, because recent modelling systems can estimate the contribution of the solvent to some extent.

References

[1] J.A. Perryt, J.D. Rateike, T.J. Szczerba, J. Chromatogr. 389 (1987) 57.
[2] N. Oi, H. Kitahara, R. Kira, J. Chromatogr. 535 (1990) 213–227.
[3] M. Okamoto, K. Takahashi, T. Doi, J. Chromatogr. A 675 (1994) 244–247.
[4] T. Doyle, I.W. Wainer, J. High Res. Chromatogr. Chrom. Commun. 7 (1984) 38–40.
[5] A.M. Dyas, A.F. Fell, M.L. Robinson, Chirality 2 (1990) 120–123.
[6] R.W. Stringham, J.A. Blackwell, Anal. Chem. 69 (1997) 1414–1420.
[7] V. Schurig, J. Ossig, R. Link, Angew. Chem. 101 (1989) 197–198.
[8] W.H. Pirkle, T.C. Pochapsky, Chem. Rev. 89 (1989) 347–362.
[9] P. Macaudière, M. Lienne, M. Caude, R. Rosset, A. Tambuté, J. Chromatogr. 467 (1989) 357–372.
[10] W.H. Pirkle, P.G. Murray, J. High Resolut. Chromatogr. 16 (1993) 285–288.
[11] F. Gasparrini, F. Marini, D. Misiti, M. Pierini, C. Vallani, Enantiomer 4 (2000) 325–332.

[12] J. Hermansson, G. Schill, in: M. Zief, L.J. Crane (Eds.), Chromatographic Chiral Separation, Marcel Dekker, New York, 1988, p. 245.
[13] J. Haginaka, J. Wakai, K. Takahashi, H. Yasuda, T. Katagi, Chromatographia 29 (1990) 587–592.
[14] J. Haginaka, C. Seyama, H. Yasuda, K. Takahashi, J. Chromatogr. 598 (1992) 67–72.
[15] B. Loun, D.S. Hage, Anal. Chem. 66 (1994) 3814–3822.
[16] R.K. Gilpin, S.E. Ehtesham, R.B. Gregory, Anal. Chem. 63 (1991) 2825–2828.
[17] M. Okamoto, H. Nakazawa, J. Chromatogr. 588 (1991) 177–180.
[18] M. Okamoto, R. Sato, E. Nagano, H. Nakazawa, Agric. Biol. Chem. 55 (1991) 3151–3153.
[19] M. Okamoto, K. Takahashi, Y. Takimoto, H. Kishimoto, Pharmazie 55 (2000) 821–824.
[20] K. Balmér, P.-O. Lagerström, B.-A. Persson amd, G. Schill, J. Chromatogr. 592 (1992) 331–337.
[21] M. Kazusaki, H. Kawabata, H. Yatsukura, J. Liq. Chrom. Rel. Technol. 23 (2000) 2937–2946.