*Journal of Chromatography*, 467 (1989) 357–372
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 263

# RESOLUTION OF π-ACID RACEMATES ON π-ACID CHIRAL STATIONARY PHASES IN NORMAL-PHASE LIQUID AND SUBCRITICAL FLUID CHROMATOGRAPHIC MODES

## A UNIQUE REVERSAL OF ELUTION ORDER ON CHANGING THE NATURE OF THE ACHIRAL MODIFIER

P. MACAUDIÈRE, M. LIENNE, M. CAUDE* and R. ROSSET
*Laboratoire de Chimie Analytique de l'Ecole Supérieure de Physique et Chimie Industrielles de Paris, 10 Rue Vauquelin, 75231 Paris Cedex 05 (France)*
and
A. TAMBUTÉ
*Direction des Recherches et Etudes Techniques, Centre d'Etudes du Bouchet, BP No. 3, Le Bouchet, 91710 Vert-le-Petit (France)*
(First received October 6th, 1988; revised manuscript received January 10th, 1989)

## SUMMARY

The enantiomeric separation of a series of π-acid N-(3,5-dinitrobenzoyl) (DNB) derivatives of α-amino esters, α-aminoamides and α-amino alcohols was investigated in the normal-phase mode on several π-acid chiral stationary phases (CSPs) derived from (*S*)-DNB-tyrosine, (*R*)-DNB-phenylglycine and (*R*)-DNB-*p*-hydroxyphenylglycine (chiral selectors, CSs). CSs were covalently grafted on to either γ-mercaptopropylsilica gel (type-1 CSPs) or γ-aminopropylsilica gel (type-2 CSPs). A comparison of the selectivities obtained under liquid (LC) and subcritical fluid chromatographic (SubFC) modes indicated important differences in the stereoselectivity of the separation of α-aminoamide test solutes. Studies of the relationship between the nature of the achiral mobile phase (in LC) and enantioselectivity showed a unique reversal of elution order on changing from hexane–ethanol to hexane–chloroform (or hexane–methylene chloride) mobile phases. Finally, chiral recognition models are discussed. Difficulties in correlating chromatographic data (selectivity, elution order) with the proposed mechanisms are outlined. This study emphasizes the importance of the part played by the mobile phase during the separation process; the mobile phase can induce major changes in the conformation of the molecules, thus leading to different chiral recognition processes.

## INTRODUCTION

In previous papers[1,2], the syntheses and evaluations of new chiral stationary phases (CSPs) derived from tyrosine were presented. Various families of enantiomers could be resolved both in liquid chromatographic (LC) and subcritical fluid chroma-

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tographic (SubFC) modes. These CSPs were compared with closely related CSPs derived from phenylglycine, *p*-hydroxyphenylglycine and phenylalanine[2]. The choice of two methods of grafting on to silica gel (type 1 and 2 CSPs, Fig. 1) demonstrated considerable discrepancies in the chiral recognition ability of the corresponding CSPs. The relationship between enantioselectivity and solute and CSP structures was extensively studied in previous work[2]. It can be expressed by three simple rules: (a) solutes containing three or more attractive sites of interaction (SI) (amide dipole or $\pi$-donor groups) can be resolved on CSPs containing only two SI; if the CSP also contains numerous SI, it must then be sterically hindered; (b) solutes possessing few SI can be resolved on CSPs with multiple SI or on CSPs with a limited number of SI but of low steric hindrance; and (c) conformationally rigid and/or sterically hindered solutes can be resolved on CSPs containing various SI but with a conformation flexible enough to fit the solute structure.

The 3,5-dinitrobenzoyl (DNB) group confers a $\pi$-acid character to these CSPs. However, their ability to separate $\pi$-acid enantiomers was demonstrated with the resolution of a series of DNB $\alpha$-amino esters[2]. To our knowledge, very few examples of such separations have been described in literature; Wainer and Doyle[3] resolved a DNB derivative of 1-phenyl 2-aminopropane on (*R*)-DNBPG CSP with a very low selectivity ($\alpha = 1.03$) and Kip *et al.*[4] separated chiral selectors of $\pi$-acid CSPs on these ones. In this study we focused our investigations on this particular point. The scope of application was extended to DNB derivatives of $\alpha$-aminoamides, $\alpha$-amino alcohols and amines. The elution orders were not always consistent with the absolute configuration of the CSPs. Moreover, in contrast to common observations on the resolution of $\pi$-basic solutes[5], important differences in selectivity values were found between LC and SubFC depending on the solute or the CSP structures. These intriguing results led us to study in detail the influence of the nature and composition of the mobile phase on both selectivity and elution order in normal-phase liquid chromatography. A unique enantiomeric reversal of elution order occurred on changing the polar modifier from ethanol to chloroform (or methylene chloride). This is the first example of a reversal of elution order owing to achiral mobile phase modifiers (such an inversion has already been observed by Pirkle *et al.*[6] but between normal- and reversed-phase modes). We attempted to connect these results with the dominant character of the polar modifier (proton donor, proton acceptor or strong dipole) as defined by Snyder[7] according to the Rohrschneider solubility data[8]. Finally, several chiral recognition models are discussed.

## EXPERIMENTAL

### *Apparatus*

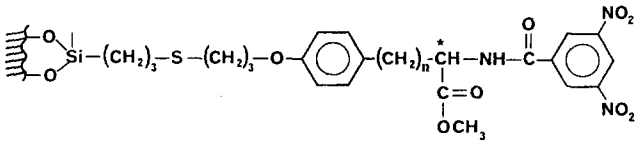
For LC, a modular liquid chromatograph (Gilson, Villiers-le-Bel, France) was used. The standard operating conditions were flow-rate 2 ml/min and temperature 25°C.

For SubFC, carbon dioxide, kept in a container with an eductor tube, was passed into a Model 303 pump (Gilson) through an ethanol cooling bath. The pump head (10 SC) was cooled in order to improve the pump efficiency. The inlet adaptor and cooling jacket were laboratory made. Polar modifiers were added by use of a second Gilson pump and mixed with carbon dioxide through a Gilson mixer (Model

802). A constant-temperature water-bath provided temperature control for the column. A Polychrom 9060 diode-array detector (Varian, Palo Alto, CA, U.S.A.) was used without modification. The pressure was monitored by a back-pressure regulator (TESCOM, Model 26-1700, GEC Composants, Asnières, France) connected in-line after the detector and maintained at 45°C by a water-bath. All results were recorded with a Shimadzu CR 3A integrator (Touzart et Matignon, Vitry-sur-Seine, France). The standard operating conditions were average column pressure 200 bar, temperature 25°C and average carbon dioxide flow-rate, 4.5 ml/min at 0°C.

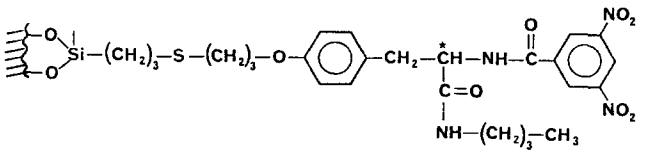
*Chiral stationary phases*

All the CSP structures are shown in Fig. 1. General procedures for the synthesis of the stationary phases derived from (*S*)-tyrosine [CSPs 1a (thio-DNBTyr-E), 1c (thio-DNBTyr-A) and 2a (DNBTyr)] were given in a previous paper[1]. The other CSPs were synthesized according to the experimental procedure for the corresponding tyrosine CSPs: CSP 1b (thio-DNBPG-E) was obtained like CSP 1a, starting from

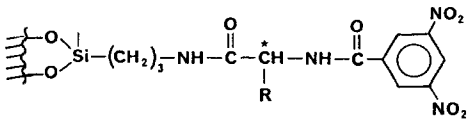


n=1 (S)-CSP 1a (*thio*-DNBTyr-E)

n=0 (R)-CSP 1b (*thio*-DNBPG-E)



(S)-CSP 1c (*thio*-DNBTyr-A)



(S)-CSP 2a : R = —CH$_2$— —O—(CH$_2$)$_2$—CH$_3$ (DNBTyr)

(R)-CSP 2b : R = — (DNBPG)

Fig. 1. Structure of the CSPs derived from (*S*)-tyrosine (CSPs 1a, 1c and 2a), (*R*)-*p*-hydroxyphenylglycine (CSP 1b) and (*R*)-phenylglycine (CSP 2b). Type 1 CSPs: grafting on to a γ-mercaptopropylsilica gel. Type 2 CSPs: grafting on to a γ-aminopropylsilica gel.

*p*-hydroxyphenylglycine, and CSPs 2b (DNBPG) were obtained like CSP 2a, starting from phenylglycine[9]. Grafting rates were calculated according to the elemental analyses for each CSP (Service Central de Microanalyse du CNRS, France): CSP 1a (0.18 mmol of chiral selector per gram of CSP), CSP 1b (0.22 mmol/g), CSP 1c (0.20 mmol/g), CSP 2a (0.32 mmol/g) and CSP 2b (0.28 mmol/g).

The CSPs were obtained starting from either γ-aminopropylsilica gel (LiChrosorb-$NH_2$ Si 60, 5 μm, type 2 CSPs) or LiChrosorb Si 60 (5 μm) modified with γ-mercaptopropyltrimethoxysilane (type 1 CSPs). Silica gels were purchased from Merck (Darmstadt, F.R.G.). If not indicated in the captions of figures, the analytical column size was 15 cm × 4.6 mm I.D.

Elution orders were determined by successive injections of racemic and enriched mixtures (in the *S* form) of test solutes.

*Mobile phase*

Carbon dioxide was N 45-grade (99.995% pure, Air Liquide, Alphagaz, Paris, France). Ethanol and *n*-hexane were of LiChrosolv grade, purchased from Merck. Chloroform [stabilized with 0.6% (w/w) of ethanol] and methylene chloride [stabilized with 0.1% (w/w) of ethanol] of analytical-reagent grade were purchased from Prolabo (Paris, France).

## RESULTS AND DISCUSSION

The structures of DNB racemates are shown in Fig. 2. A comparison of LC and SubFC stereoselectivities obtained on the different DNB CSPs is presented in Table I for some typical test solutes. A π–π interaction as a driving force can hardly be advocated during the formation of the transient diastereomeric complexes; the main attractive interactions involved are then dipole–dipole or hydrogen bonding. The lack of a directional π–π interaction gives the solutes various possibilities to approach the CSP; a noticeable influence of the steric hindrance in the vicinity of chiral centres on stereoselectivity can be expected.

Moreover, let us mention again[2] that the resolution of such π-acid compounds could not be achieved on the π-basic CSP designed by replacing the DNB group of CSP 1a by a 1-naphthoyl moiety [except for tyrosine derivative **2a** ($\alpha$ = 1.08) and phenylglycinol **4b** ($\alpha$ = 1.10)].

Several discrepancies between LC and SubFC are observed for α-aminoamides **2a** and **3** and phenylglycinol **4b** on CSPs 1a, 2a and 2b. A regular increase in selectivity is observed on CSP 2b in the SubFC mode whereas a significant decrease occurs on CSPs 1c and 2a for tyrosine **2a** and leucine **3** derivatives. For α-amino alcohols (**4**), an improvement in selectivity occurs on CSPs 1c and 2b.

*Elution order of enantiomers*

To complete the data in Table I, a study of the elution order of enantiomers was carried out (Table II). In Table II, the orders always refer to an (*S*)-CSP and to a hexane–ethanol mobile phase. Several comments can be made as follows: (a) for α-amino esters (**1**), inverted elution orders occur between CSPs derived from tyrosine and phenylglycine, whatever the grafting mode; (b) a surprising inversion is observed for α-aminoamides (**2**) and **3** between CSPs 1b and 2b [both derived from (*R*)-phenyl-

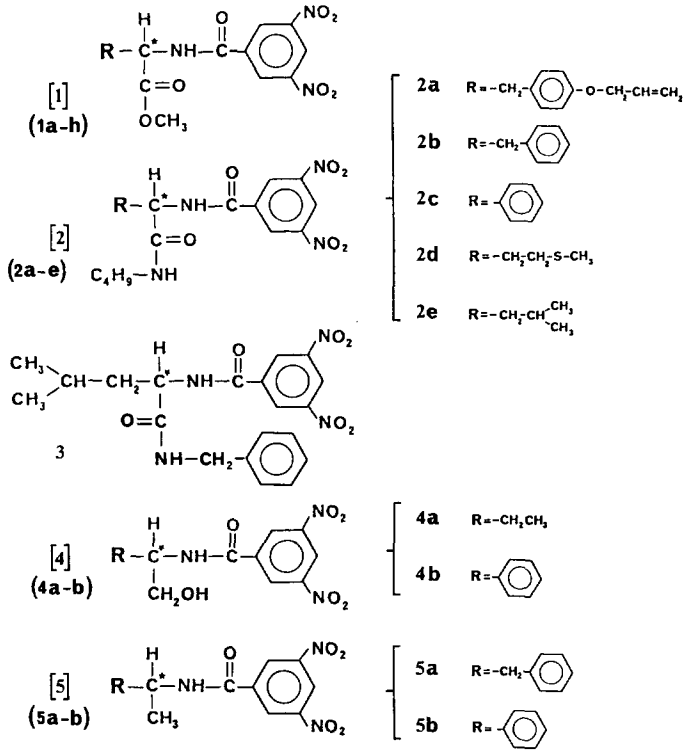



Fig. 2. Structures of 3,5-dinitrobenzoyl (DNB) derivatives of α-amino esters (**1**) (solutes **1a–h**; for R, see Fig. 3), α-aminoamides (**2**) and **3**, α-amino alcohols (**4**) and amines (**5**) investigated.

TABLE I

COMPARISON OF THE SELECTIVITY VALUES, α, OBTAINED UNDER LC OR SUBFC CONDITIONS FOR SOME TYPICAL DNB DERIVATIVES

LC mode: mobile phase, hexane–ethanol (85:15, v/v) with CSPs 1c and 2b, (90:10, v/v) with CSPs 1a and 1b and (92.5:7.5, v/v) with CSP 2a; flow-rate, 2 ml/min; temperature, 25°C; UV detection at 254 nm . SubFC mode: mobile phase, carbon dioxide–ethanol (93:7, w/w); flow-rate, 4.5 ml/min at 0°C; average column pressure, 200 bar; temperature, 25°C; UV detection at 254 nm. Values in italics indicate puzzling discrepancies between LC and SubFC results.

| *Solute* | *CSP* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *(S)-CSP 1a* | | *(R)-CSP 1b* | | *(S)-CSP 1c* | | *(S)-CSP 2a* | | *(R)-CSP 2b* | |
| | $\alpha_{LC}$ | $\alpha_{SubFC}$ | $\alpha_{LC}$ | $\alpha_{SubFC}$ | $\alpha_{LC}$ | $\alpha_{SubFC}$ | $\alpha_{LC}$ | $\alpha_{SubFC}$ | $\alpha_{LC}$ | $\alpha_{SubFC}$ |
| Leucine **1c** | 1.05 | 1.07 | 1.07 | 1.10 | 1.31 | 1.25 | 1.12 | 1.11 | 1.06 | 1.16 |
| Leucine **3** | 1.49 | 1.46 | 1.09 | nr[a] | *1.48* | *1.05* | *1.36* | *1.09* | *1.26* | *1.91* |
| Tyrosine **1h** | nr | 1.04 | nr | nr | 1.26 | 1.23 | 1.09 | 1.08 | 1.05 | 1.15 |
| Tyrosine **2a** | 1.52 | 1.45 | 1.17 | 1.12 | *1.23* | *nr* | *1.28* | *1.05* | *1.26* | *1.84* |
| Phenylglycine **1f** | 1.05 | 1.04 | 1.04 | 1.05 | 1.18 | 1.12 | 1.09 | 1.07 | nr | nr |
| 2-Aminobutanol **4a** | 1.06 | 1.04 | nr | nr | 1.04 | 1.18 | nr | nr | *1.18* | *1.54* |
| Phenylglycinol **4b** | 1.11 | 1.14 | 1.18 | 1.18 | *1.28* | *1.51* | 1.25 | 1.28 | *1.43* | *2.40* |

[a] No resolution.

TABLE II

ELUTION ORDERS OF DNB α-AMINO ESTER, α-AMINOAMIDE AND α-AMINO ALCOHOL DERIVATIVES

The elution orders are given with regard to an *S* configuration of each CSP and for hexane–ethanol mobile phase.

| *Solute as DNB derivative* | *(S)-CSP chiral selector* | | | | |
|---|---|---|---|---|---|
| | *CSP 1a DNB-tyrosine* | *CSP 1c DNB-tyrosine* | *CSP 2a DNB-tyrosine* | *CSP 1b DNB-p-hydroxy-phenylglycine* | *CSP 2b DNB-phenyl-glycine* |
| α-Amino esters (**1**) | (*R*,*S*) | (*R*,*S*) | (*R*,*S*) | (*S*,*R*) | (*S*,*R*) |
| α-Aminoamides (**2**, **3**) | (*R*,*S*) | (*R*,*S*) | (*R*,*S*) | (*R*,*S*) | (*S*,*R*) |
| α-Amino alcohols (**4**) | (*S*,*R*) | (*S*,*R*) | (*S*,*R*) | (*S*,*R*) | (*S*,*R*) |

glycine]; (c) only α-amino alcohols (**4**) display a regular elution order, whatever the CSP; and (d) the elution order of α-amino alcohols (**4**) is inverted compared with that of α-amino esters (**1**) or α-aminoamides (**2**) and **3** on CSPs derived from tyrosine.

*Comparison of resolution of a series of α-N-(3,5-dinitrobenzoyl)amino esters*

Selectivity values were plotted against the characterizing side-group, R, of various α-amino esters (**1**) on the five CSPs (Fig. 3). For these solutes, the sites of interaction are limited to the dinitrobenzamide dipole and the ester carbonyl group. As previously argued[2], the selectivity will be enhanced on a CSP with two easily accessible amide dipoles (CSP 1c). Steric hindrance due to the silica matrix on the aliphatic amide dipole (CSP 2a) slightly decreases the selectivity. Comparing now phenylglycine- and tyrosine-derived CSPs, the higher steric hindrance resulting from the presence of a phenyl instead of a benzyl group may account for the loss in selectivity and for the inversion of elution order observed from CSP 1a (or 2a) to CSP 1b (or 2b) (Table II); in fact, the accessibility to the chiral centre for phenylglycine CSPs is limited to one side only. Moreover, on tyrosine-derived CSPs, the occurrence of a weak $\pi$–$\pi$ overlapping between benzyl and dinitrobenzoyl moieties cannot be entirely disregarded; with a phenyl group directly attached to the asymmetric centre (CSPs 1b and 2b), good overlapping is not possible. Such a $\pi$–$\pi$ interaction can also be considered for solutes derived from tyrosine and phenylalanine, whatever the CSP.

Finally, on CSPs 1a, 1b, 2a and 2b, the structure of the R group has a minor effect on stereoselectivity, unlike previous observations on $\pi$-basic CSPs derived from tertiary phosphine oxides[10,11]. However, as CSP 1c combines the presence of multiple sites of interaction and a reduced steric hindrance, it is expected to be sensitive to the solute steric bulkiness. We indeed observed more significant variations in selectivity on this CSP in the amino ester series (**1**).

*Influence of the nature of the mobile phase*

*Chromatographic data.* Hitherto, selectivities were always similar in LC and SubFC on Pirkle-type CSPs[5,12], but the studies concerned the resolution of $\pi$-basic solutes on $\pi$-acid CSPs. In this work, dealing with the resolution of $\pi$-acid solutes on $\pi$-acid CSPs, the nature of the mobile phase appears to play a decisive part in the

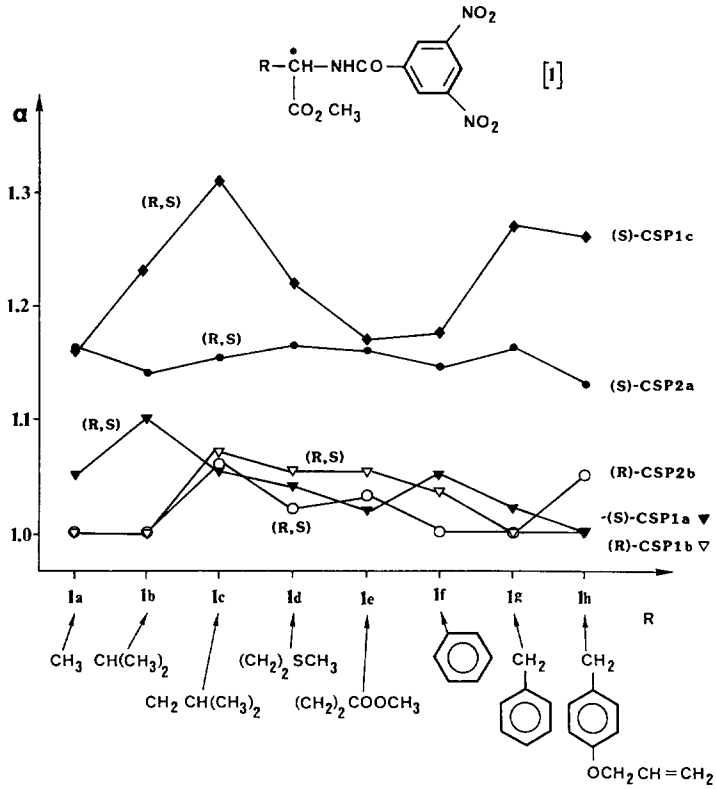



Fig. 3. LC resolution of DNB α-amino esters (**1**). Influence of the nature of the R substituent group on the selectivity α. Mobile phase, hexane–ethanol (85:15, v/v) with CSPs 1a, 1c and 2b (90:10, v/v) with CSP 1b and (92.5:7.5, v/v) with CSP 2a (the ethanol content was set to give a similar capacity factor, $k'_2$, for the last eluted enantiomer of a racemate on the five CSPs); flow-rate, 2 ml/min; temperature, 25°C; UV detection at 254 nm.

chiral recognition, as shown by the aforementioned discrepancies observed for aminoamides **2a** and **3** and amino alcohol **4b**. We therefore studied the influence in LC of the nature of the polar modifier on the stereoselectivity. The separation of four typical racemates derived from leucine ester **1c**, tyrosine ester **1h**, tyrosine amide **2a** and phenylglycinol **4b** was investigated.

TABLE III

SELECTIVITY PARAMETERS, AS DEFINED AND CALCULATED BY SNYDER[4] FROM SOLUBILITY DATA REPORTED BY ROHRSCHNEIDER

Values in italics indicate the dominant character of the solvent: $\chi_e$ (proton acceptor), $\chi_d$ (proton donor) and $\chi_n$ (strong dipole).

| *Polar solvent* | $\chi_e$ | $\chi_d$ | $\chi_n$ |
|---|---|---|---|
| Ethanol | *0.52* | 0.19 | 0.29 |
| Chloroform | 0.25 | *0.41* | 0.33 |
| Methylene chloride | 0.29 | 0.18 | *0.53* |

TABLE IV

INFLUENCE OF THE NATURE OF THE MOBILE PHASE ON THE RESOLUTION OF COMPOUNDS **1c, 1h, 2a** AND **4b** ON TYPE 1 CSPs (CSPs 1a, 1b AND 1c)

Values in italics indicate an inversion of elution order with regard to the hexane–ethanol mobile phase. Values between parentheses indicate the polar solvent content in hexane.

| *Solute* | *(S)-CSP 1a* | | | | *(S)-CSP 1c* | | | | *(R)-CSP 1b* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *$C_2H_5OH$ (15%)* | *$CHCl_3$ (%)* | *$CH_2Cl_2$ (%)* | *SubFC*[a] | *$C_2H_5OH$ (15%)* | *$CHCl_3$ (%)* | *$CH_2Cl_2$ (%)* | *SubFC*[a] | *$C_2H_5OH$ (10%)* | *$CHCl_3$ (%)* | *SubFC*[a] |
| Leucine **1c** | 1.04 | 1.45 (45) | 1.46 (40) | 1.07 | 1.31 | 1.86 (50) | 2.01 (40) | 1.25 | 1.07 | 1.12 (40) | 1.10 |
| Tyrosine **1h** | nr | 1.25 (40) | 1.26 (35) | 1.04 | 1.26 | 1.61 (35) | 1.71 (40) | 1.23 | nr | nr (55) | nr |
| Tyrosine **2a** | 1.45 | 1.87 (55) | 1.53 (60) | 1.45 | 1.23 | *1.51* (65) | *1.52* (90) | nr | 1.17 | nr (55) | 1.12 |
| Phenylglycinol **4b** | 1.10 | 1.08 (70) | nr (75) | 1.14 | 1.28 | 1.57 (85) | 1.36 (90) | 1.51 | 1.18 | 1.12 (70) | 1.18 |

[a] SubFC conditions as in Table I.

Ethanol, chloroform and methylene chloride were chosen according to their dominant character with regard to the selectivity parameters $\chi_e$, $\chi_d$ and $\chi_n$ (Table III)[7]. These parameters reflect the relative ability of a solvent to act as a proton acceptor ($\chi_e$), a proton donor ($\chi_d$) or a strong dipole ($\chi_n$). Ethanol can be mainly considered as a proton acceptor, chloroform as a proton donor and methylene chloride as a dipole.

Stereoselectivities, $\alpha$, observed with binary hexane–polar modifier mixtures are gathered in Table IV (type 1 CSPs) and Table V (type 2 CSPs). SubFC results are given again to allow easy comparison with LC. For each solute, the mobile phase composition was adjusted in order to maintain a similar capacity factor, $k'_2$, of the last eluted enantiomer on a given CSP (isoeluotropic mobile phases).

Two major observations can be made from these data on changing the polar modifier from ethanol to chloroform (or methylene chloride): (a) a unique reversal of elution order of enantiomers for tyrosine amide derivative **2a** on CSPs 1c and 2a; and (b) a systematic and noticeable increase in selectivity for ester derivatives **1c** and **1h**.

The chromatograms in Fig. 4a and c show the reversal of the elution order for tyrosine amide **2a** on CSP 1c. It is noteworthy that a complete loss of resolution is observed with the subcritical carbon dioxide–ethanol mobile phase (Fig. 4b). Regarding the stereoselectivity of this separation, SubFC can be considered as intermediate between the two LC binary mixtures, hexane–ethanol and hexane–chloroform (or methylene chloride). This intermediate behaviour is also observed for the resolution of phenylglycinol derivative **4b** (Fig. 5): $\alpha_{SubFC} = 2.40$ while $\alpha_{LC,C_2H_5OH} = 1.45$ and $\alpha_{LC,CHCl_3} = 3.37$. In LC, the increase in $\alpha$ has to be attributed to a strengthening of the stability of the (*R*)-CSP 2b–(*S*)-solute **4b** transient diastereomeric complex; a 190% increase in $k'_2$ is observed for only a 10% increase in $k'_1$ on changing from ethanol (15% in hexane) to chloroform (90% in hexane).

TABLE V

INFLUENCE OF THE NATURE OF THE MOBILE PHASE ON THE RESOLUTION OF COMPOUNDS **1c**, **1h**, **2a** AND **4b** ON TYPE 2 CSPs (CSPs 2a AND 2b)

Values in italics indicate an inversion of elution order with regard to the hexane–ethanol mobile phase. Values between parentheses indicate the polar solvent content in hexane.

| *Solute* | *(S)-CSP 2a* | | | | *(R)-CSP 2b* | | | |
|---|---|---|---|---|---|---|---|---|
| | *$C_2H_5OH$ (7.5%)* | *$CHCl_3$ (%)* | *$CH_2Cl_2$ (%)* | *SubFC*[a] | *$C_2H_5OH$ (15%)* | *$CHCl_3$ (%)* | *$CH_2Cl_2$ (%)* | *SubFC*[a] |
| Leucine **1c** | 1.12 | 1.50 (50) | 1.46 (50) | 1.11 | 1.06 | 1.18 (40) | 1.21 (50) | 1.16 |
| Tyrosine **1h** | 1.09 | 1.34 (50) | 1.32 (50) | 1.08 | 1.05 | 1.16 (40) | 1.17 (50) | 1.15 |
| Tyrosine **2a** | 1.28 | *1.14* (55) | *1.05* (65) | 1.05 | 1.26 | 2.00 (60) | 2.07 (65) | 1.84 |
| Phenylglycinol **4b** | 1.25 | 1.23 (95) | 1.23 (100) | 1.28 | 1.43 | 3.37 (90) | 2.88 (95) | 2.40 |

[a] SubFC conditions as in Table I.

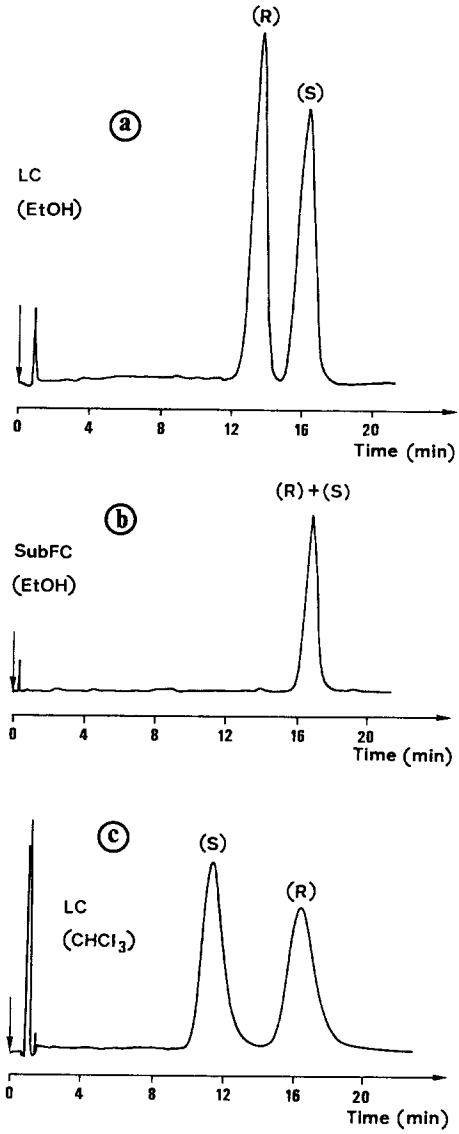



Fig. 4. Influence of the nature of the achiral mobile phase on resolution and elution order of tyrosine derivative **2a** enantiomers on (*S*)-CSP 1c. LC conditions: mobile phase, (a) hexane–ethanol (85:15, v/v) or (c) hexane–chloroform (35:65, v/v); flow-rate, 2 ml/min; temperature, 25°C; UV detection at 254 nm. SubFC conditions: mobile phase, (b) carbon dioxide–ethanol (93:7, w/w); flow-rate, 4.5 ml/min at 0°C; average column pressure, 200 bar; temperature, 25°C; UV detection at 254 nm. EtOH = Ethanol.

The inversion of the elution order with hexane–chloroform mobile phase was demonstrated for other aminoamides on CSP 1c, except for the phenylglycine derivative **2c** (Table VI). This last result is in good agreement with the regular elution order of tyrosine derivative **2a** observed on the phenylglycine-derived CSP 2b (Table V), whatever the mobile phase (reciprocality concept[13]).

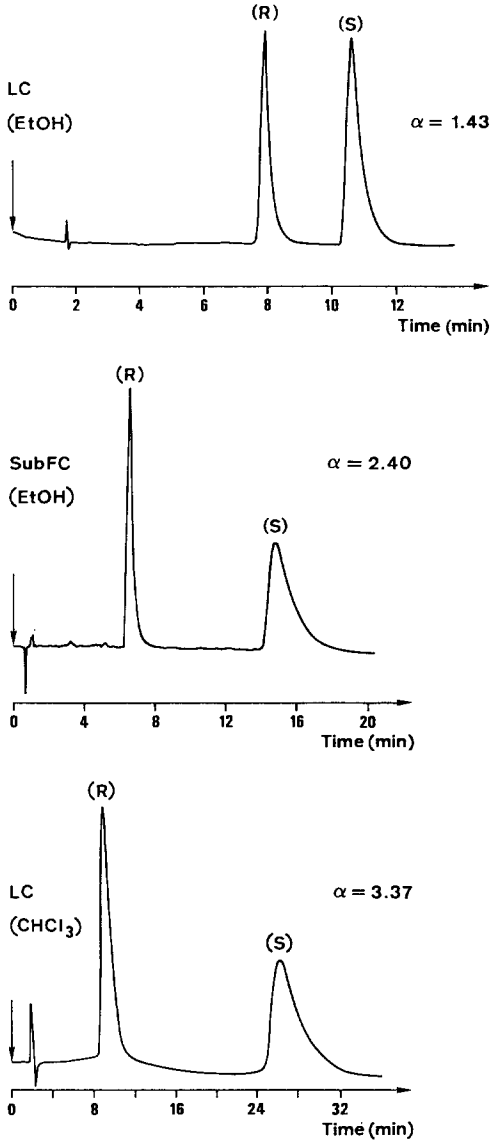



Fig. 5. Influence of the nature of the mobile phase on the resolution of phenylglycinol derivative **4b** on (*R*)-CSP 2b. LC conditions: mobile phase, hexane–ethanol (85:15, v/v) [$k'_{(R)}$ = 3.9, $k'_{(S)}$ = 5.6] or hexane–chloroform (10:90, v/v) [$k'_{(R)}$ = 4.3, $k'_{(S)}$ = 16.3]; flow-rate, 2 ml/min; temperature, 25°C; UV detection at 254 nm. SubFC conditions: mobile phase, carbon dioxide–ethanol (93:7, w/w); flow-rate, 4.5 ml/min at 0°C; average column pressure, 200 bar; temperature, 25 °C; UV detection at 254 nm.

*Relationship between elution order of α-aminoamides (**2**) and selectivity parameters of solvents.* The reversal of elution order was also checked using ternary mixtures, as shown in Fig. 6 (hexane–ethanol–chloroform) and Fig. 7 (hexane–ethanol–methylene chloride). The considerable decrease in the capacity factors, $k'_1$ and $k'_2$, has already been reported on CSP 2b with hexane–alcohol–chloroform mixtures for the resolu-

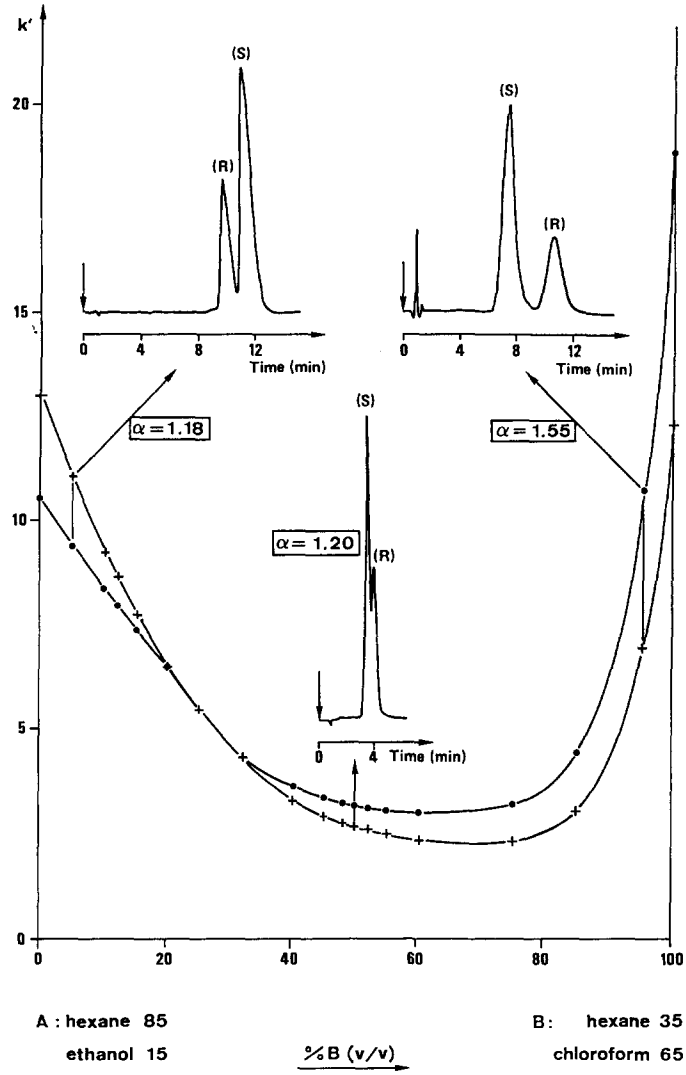



Fig. 6. Reversal elution order of **2a** enantiomers on (*S*)-CSP 1c on changing from hexane–ethanol (85:15, v/v) (solvent A) to hexane–chloroform (35:65, v/v) (solvent B) mobile phase. The capacity factors, *k'*, of (*S*)-**2a** (+) and (*R*)-**2a** (●) are plotted *versus* the content of binary mixture B in the ternary mixture A–B. The elution order was established using an enriched mixture of **2a** in the *S* form. Flow-rate, 2 ml/min; temperature, 25°C; UV detection at 254 nm.

tion of phosphine oxide enantiomers[14]. However, for the latter solutes, no inversion of elution order was observed.

Chloroform and methylene chloride display two different dominant characters ($\chi_d$ and $\chi_n$, respectively), although reversal of elution order was observed with both solvents. This leads us to attribute this phenomenon to the loss of the prevailing $\chi_e$ character in the mobile phase on changing from solvent A to B, to the benefit of either the $\chi_d$ or $\chi_n$ character. This is corroborated by the fact that, in the inversion region

TABLE VI

COMPARISON OF SELECTIVITY AND ELUTION ORDERS FOR AMINOAMIDES (2) AND 3 ON CSP 1c (*S*)-THIO-DNBTyr-A WITH HEXANE–ETHANOL (85:15, v/v) AND HEXANE–CHLOROFORM (35:65, v/v)

| *Solute* | *Hexane–ethanol (85:15, v/v)* | | | | *Hexane–chloroform (35:65, v/v)* | | | |
|---|---|---|---|---|---|---|---|---|
| | $k'_1$ | $k'_2$ | α | *Elution order* | $k'_1$ | $k'_2$ | α | *Elution order* |
| (4-Propyloxy)tyrosine **2a** | 12.94 | 15.86 | 1.23 | (*R*,*S*) | 10.67 | 16.11 | 1.51 | (*S*,*R*) |
| Phenylalanine **2b** | 7.42 | 9.02 | 1.40 | (*R*,*S*) | 11.03 | 13.42 | 1.22 | (*S*,*R*) |
| Phenylglycine **2c** | 7.00 | 9.74 | 1.39 | (*S*,*R*) | 5.38 | 18.44 | 3.43 | (*S*,*R*) |
| Methionine **2d** | 6.61 | 8.96 | 1.35 | (*R*,*S*) | 10.85 | 12.86 | 1.19 | (*S*,*R*) |
| Leucine **2e** | 3.18 | 4.79 | 1.51 | (*R*,*S*) | 11.63 | 12.75 | 1.10 | (*S*,*R*) |
| Leucine **3** | 6.75 | 9.42 | 1.39 | (*R*,*S*) | 15.66 | 20.30 | 1.30 | (*S*,*R*) |

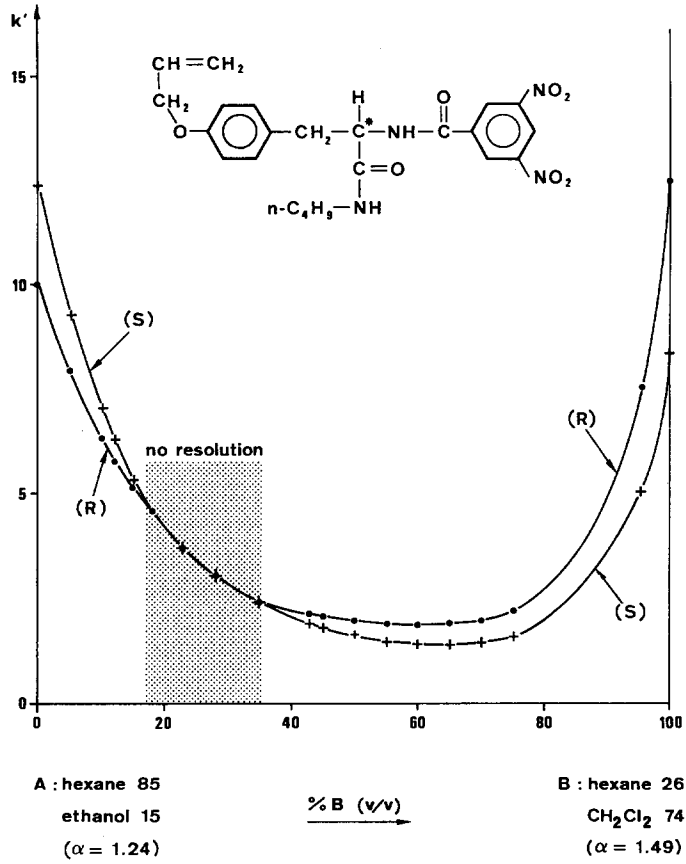



Fig. 7. Reversal of elution order of **2a** enantiomers on (*S*)-CSP 1c on changing from hexane–ethanol (85:15, v/v) (solvent A) to hexane–methylene chloride (26:74, v/v) (solvent B) mobile phase. The capacity factors, *k'*, of (*S*)-**2a** (+) and (*R*)-**2a** (●) are plotted *versus* the content of binary mixture B in the ternary mixture A–B. Analytical conditions as in Fig. 6.

TABLE VII

CALCULATION OF THE RELATIVE CONTRIBUTIONS $\chi_e$, $\chi_d$ AND $\chi_n$ FOR THE MOBILE PHASE COMPOSITION CORRESPONDING TO THE LIMITS OF THE INVERSION REGION WITH HEXANE–ETHANOL–CHLOROFORM OR HEXANE–ETHANOL–METHYLENE CHLORIDE TERNARY MIXTURES

Values in italics indicate the dominant character.

| *Mobile phase composition* | $\chi_e$ | $\chi_d$ | $\chi_n$ |
|---|---|---|---|
| Hexane–ethanol–chloroform: | | | |
| 18% B (76:12.3:11.7, v/v/v) | *0.39* | 0.30 | 0.31 |
| 35% B (67.5:9.8:22.7, v/v/v) | 0.33 | *0.35* | 0.32 |
| Hexane–ethanol–methylene chloride: | | | |
| 18% B (74.4:12.3:13.3, v/v/v) | *0.37* | 0.24 | *0.39* |
| 35% B (64.3:9.8:25.9, v/v/v) | 0.36 | 0.18 | *0.46* |

where no resolution is observed (18–35% of solvent B in the mobile phase, Figs. 6 and 7), at least two of the three contributions are of same order of magnitude (Table VII). Moreover, we can expect (from calculations in Table VII) that the balance point ($\alpha$ = 1.00) will arise for a content of solvent B close to 35% for chloroform ($\chi_e$ = 0.33 and $\chi_d$ = 0.35) and close to 18% for methylene chloride ($\chi_e$ = 0.37 and $\chi_n$ = 0.39). This assumption cannot be precisely confirmed owing to peak broadening on adding chloroform or methylene chloride to the mobile phase; the resulting decrease in efficiency contributes to the lack of resolution in the inversion region (*e.g.*, when $\alpha \leqslant 1.10$).

*Chiral recognition models.* The inversion region corresponds to the occurrence of two chiral recognition mechanisms working in opposite stereochemical senses and with similar contributions to the overall process[6]. Either solvation or conformation of both the solute and 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.

At this stage of investigation, it is impossible to ascribe a given mechanism to the resolution of a given solute on a given chromatographic system (CSP, mobile phase). Too many different factors are involved during the separation process and we failed to correlate the experimental data with observations inferred from the examination of CPK models[a]. Moreover, many of the results appeared to be contradictory from one CSP (or solute) to another. This has to be attributed to the lack of a clear driving force such as a strong $\pi$–$\pi$ overlapping which orientates the molecules inside the diastereomeric complex and is conformationally restrictive. However, general comments can be made as follows:

(a) The reversal of elution order was observed only for amide derivatives (**2**) (which contain two amide dipoles) on tyrosine-derived CSPs 1c and 2a (bearing also two amide dipoles); the multiplicity of possible sites of interaction may then favour the occurrence of competitive opposite-sense chiral recognition mechanisms, depend-

[a] CPK precision molecular models are improved versions of the Corey–Pauling models designed at the California Institute of Technology in the late 1940s, with new connectors by Dr. W. Koltun.

ing on the nature of the mobile phase. Alteration of the conformation of solutes and CSPs may play an important part in the inversion (intramolecular hydrogen bonding, solvation, etc.).

(b) The abnormal behaviour of phenylglycine amide **2c** and CSP 2b (derived from phenylglycine) remains unclear; we can assume that the phenyl group limits the access of the chiral centre to one side only, thus reducing the number of possible chiral recognition mechanisms.

(c) For phenylglycinol derivative **4b**, the existence of a prevailing hydrogen bonding interaction involving the hydroxyl group can explain the regular elution order on all the CSPs (Table II). Differences in $\alpha$ values according to the nature of the CSP or mobile phase are not yet explained.

(d) The influence of the nature of the polar modifier is not elucidated. It was not possible to connect the dominant character of a modifier with its ability to favour dipole–dipole or hydrogen bonding interactions. We can note, however, that alcohols will interact preferentially with NH moieties whereas chloroform, as a proton donor, will act with carbonyl groups. The resulting alteration of the conformations of the solute and CSP may induce changes in the dominant chiral recognition mechanism, leading eventually to inversion of the elution order of enantiomers.

## CONCLUSION

We have demonstrated the ability of π-acid DNB CSPs to resolve π-acid DNB racemates. However, the lack of π–π interactions as the driving force during the diastereomeric complex formation renders more difficult the understanding of chiral recognition mechanisms. Moreover, we found a reversal of elution order for a series of DNB aminoamide derivatives on just changing the polar modifier from ethanol to chloroform (or methylene chloride). This indicates the importance of a knowledge of the conformational state of both the solute and CSP during the chromatographic separation. We think that[15], together with chromatographic data, NMR studies of bimolecular solute–CSP complexes[16] would be useful means of elucidating chiral recognition processes, as they would also take into account the solvent contribution (even if the evaluation of the intrinsic role of the silica matrix still remains a problem).

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