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Temperature and enantioseparation by macrocyclic glycopeptide chiral stationary phases

Alain Berthod [a,*], Brian Lingfeng He [b], Thomas E. Beesley [b]

[a] *Laboratoire des Sciences Analytiques, Université de Lyon 1, CNRS 5180, 69622 Villeurbanne, France*
[b] *Advanced Separation Technologies Inc, Astec, P.O. Box 297, Whippany, NJ 07981, USA*

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Abstract

Seventy-one chiral compounds were separated on four macrocyclic glycopeptide chiral selectors: teicoplanin, its aglycone, ristocetin A and vancomycin, using three possible separation modes: reversed phase with methanol/buffer mobile phases, normal phase with hexane/ethanol mobile phases and polar ionic mode (PIM) with 100% methanol mobile phase with trace amounts of acid and/or base. These 148 separations were studied in a 5–45 °C temperature range. Peak efficiencies always increased with temperature, but in only 17% of the separations studied a small increase of the enantioresolution factor was observed. In the majority (83%) of the cases, the enantioresolution decreased or even vanished when temperature increased. All 148 Van't Hoff plots were linear showing that the selector did not change in the temperature range studied. The calculated enthalpy and entropy variations showed that the interaction of the solute with the stationary phase was always enthalpy driven with normal and reversed mobile phases. It could be enthalpy as well as entropy driven with PIM mobile phases strongly dependent on the solute. The plots of $\Delta(\Delta H)$ versus $\Delta(\Delta S)$ were linear in most cases (enthalpy entropy compensation). This observation cannot be used to give clear information on chiral recognition mechanisms, but it allowed identifying specific stationary phase–solute interactions because the points corresponding to the respective thermodynamic parameters were clearly delineated from the general compensation lines.
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1. Introduction

Macrocyclic glycopeptides were introduced at the 1994 Pittsburgh Conference by Armstrong as a new class of chiral selectors for all separation techniques [1–3]. In 10 years, this class of chiral stationary phase (CSP) established its usefulness especially in the separation of enantiomers containing an ionizable group on or close to the asymmetric center [3–9]. Macrocyclic glycopeptides are complex molecules produced by microorganisms in fermentation broths. Their structures contain functional groups that permit enantiomers to interact through π–π, hydrogen bonding, electrostatic interaction, as well as hydrophobic interactions and steric (repulsive) hindrance [5]. The macrocyclic parts of the glycopeptides can even interact with solutes forming inclusion complexes. Furthermore, in liquid chromatography (HPLC), these CSPs are multi-modal, i.e. they can work with polar hydro-organic mobile phases (reversed phase mode or RPLC), as well as low polar alkane–alcohol mobile phases (normal phase mode) and polar organic mobile phases (non-aqueous organic solvents) with traces of acetic acid and triethylamine to adjust the chiral selector ionization state. Indeed, it was observed that ionic interactions dominated in the chiral recognition mechanism of acid and/or base enantiomers [3,5,6,9]. The later mode is called the polar ionic mode (PIM) [9].

It was apparent from early observations, that temperature was an important parameter to control in HPLC [10]. Most often, an increase of the column working temperature produces at the same time a decrease of the solute retention times associated with an increase in peak efficiency [11]. The mobile phase viscosity also decreases so it may be possible to work with higher flow rates. Decreases of the solute retention times may be viewed as higher elution strength. It is then possible to work with less organic modifier in the mobile phase [12]. Technically, temperature regulation in HPLC was made possible by manufacturers of liquid

* Corresponding author. Tel.: +33 472 431 434; fax: +33 472 431 078.
E-mail address: berthod@univ-lyon1.fr (A. Berthod).

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chromatography material. With the advent of the Peltier devices the ability to regulate column temperature above as well as below room temperature became available to the market. It is even possible to perform reproducible programmed temperature gradients in HPLC [13].

The efficiency obtained with macrocyclic glycopetide CSPs is not as high as it can be observed with standard RPLC stationary phases. This is especially true for the second eluting enantiomer. Raising the temperature will increase the solute exchange rate, hence the chromatographic efficiency [14]. Since temperature changes can adversely affect the enantioselectivity factor, then the resolution factor, which combines both efficiency and retention differences, should be studied. The aim of this work is to evaluate from an experimental point of view the effect of temperature changes with four commercially available macrocyclic glycopeptide CSPs. A large set of chiral compounds known to be enantioseparated by the glycopeptide CSPs will be tested at different temperatures and with three possible mobile phases: polar aqueous mobile phases (RPLC), hexane–alcohol mobile phases (normal phase) and polar non-aqueous mobile phases (polar ionic mode).

2. Experimental

2.1. Chromatographic system

Two LC systems were used. Each one consisted of a Rheodyne 7125 valve with 20 μL loop, a LC10AS Shimadzu pump, a SPD10 UV detector and a CR5A integrator. The detection wavelength was most often 230 nm. A Jetstream Plus column thermostat (Astec, Whippany, NJ, USA) was used. This device is able to regulate column temperature from 5 to 85 °C using a microprocessor controlled, multi Peltier system.

2.2. Chiral columns

Four different 250 × 4.6 mm i.d. columns were used. They were a Chirobiotic T, Chirobiotic TAG, Chirobiotic R and Chirobiotic V columns (Astec) whose chiral selectors were, respectively, teicoplanin, teicoplanin aglycon, ristocetin A, and vancomycin. Their main physicochemical properties are listed in Table 1. They were comprehensively described in several articles [3–5,15]. The CSPs were prepared by bonding through multiple linking chains the chiral selectors to a 5 μm spherical porous silica gel [9,16]. Astec gives a maximum operating temperature of 70 °C but recommend to use very slow gradient temperature (lower than 2 °C/min). To be safe with the Chirobiotic columns, the optimum temperature change of + or −1 °C/min was always used.

2.3. Mobile phases

The RPLC mobile phases were methanol/triethylammonium acetate buffer (pH 4.1) in ratio varying from methanol 5% to 40% (v/v) with a flow rate of 0.9 or 1 mL/min. The normal mobile phases were mixture of hexane and ethanol in ratios varying from ethanol 10% to 50% (v/v) and a flow rates of 0.9 or 1 mL/min. PIM chromatography was performed with pure methanol or acetonitrile mobile phases containing small amounts (0.1% to 0.2% (v/v)) of acetic acid and triethylamine to adjust the stationary phase and/or analytes ionization state.

2.4. Solutes and chemicals

All solutes were selected as enantiomers known to be well separated by the studied CSP. Their structures can be found in the original publications [4,6,9].

HPLC-grade acetonitrile (ACN), methanol (MeOH), ethanol (EtOH), *n*-hexane (hex) and tetrahydrofuran (THF) were obtained from Fisher (St. Louis, MO, USA) and Solvent Documentation System (SDS, Peypin, France). Water was deionized and filtered through active charcoal and a 5 μm filter. Triethylamine (TEA) and acetic acid (AA) were from Sigma (St. Quentin Fallavier, France).

2.5. Theoretical background

Considering the solute exchange between the mobile and the stationary phase, ΔG°, the Gibbs free energy change of the solute phase transfer is expressed by:

$$\Delta G^\circ = -RT \ln\left(\frac{k}{\phi}\right) \qquad (1)$$

where k is the solute retention factor and ϕ is the column phase ratio (stationary phase volume over the mobile phase volume). Expressing the Gibbs energy using the enthalpy,

Table 1
Physicochemical characteristics of the four macrocyclic glycopeptides of the Chirobiotic columns

Chiral selector	Formula	fw (Da)	Asymmetric centers	Number of inclusion cavities	Number of sugar moieties	Ionizable groups[a]
Teicoplanin	$C_{88}H_{97}Cl_2N_9O_{33}$	1878	20	4	3	2
TAG[b]	$C_{58}H_{45}Cl_2N_7O_{18}$	1198	8	4	0	2
Ristocetin A	$C_{95}H_{110}N_8O_{44}$	2066	38	4	6	2
Vancomycin	$C_{66}H_{75}Cl_2N_9O_{24}$	1448	18	3	2	3

[a] Ionizable groups include carboxylic acids and amines excluding phenol groups.
[b] TAG stands for teicoplanin aglycon.

ΔH°, and entropy, ΔS°, changes, the retention factor depends on temperature according to:

$$\ln k = -\frac{\Delta H^{\circ}}{RT} + \frac{\Delta S^{\circ}}{R} + \ln \phi \qquad (2)$$

Slopes and intercepts of Van't Hoff plots ($\ln k$ versus $1/T$) will give the thermodynamic parameters of the solute overall phase transfer from the mobile to the stationary phase.

In the case of chiral separations, the enantioselectivity ratio, α, is the important parameter measuring the relative retention difference between the two enantiomers. The overall phase transfer of enantiomers is composed of an achiral and a chiral part. The later, $\Delta(\Delta G^{\circ})$, is responsible for the higher retention of the second enantiomer produced by the CSP. From Eqs. (1) and (2), it follows:

$$\Delta(\Delta G^{\circ}) = -RT \ln \alpha = \Delta(\Delta H^{\circ}) - T\Delta(\Delta S^{\circ}) \qquad (3)$$

that shows that the enantioselectivity factor, α, can be affected by temperature changes especially in entropy-dominated situation often encountered in chiral separations [14].

The equations show that the mobile phase void volume (improperly called dead volume), V_o, and the column phase ratio, ϕ, should be measured. It was found that uracil was not an acceptable unretained solute. The injection of blank mobile phase volumes produced visible detector fluctuations that were used as the void volume. All four 25 cm (4.6 mm i.d.) columns had void volumes between 2.5 and 2.8 mL. The ϕ parameter is always difficult to know with a high accuracy because the exact volume V_S, of the actually active stationary phase is not exactly known. The ϕ values that were taken to compute entropy variations were 0.086, 0.091, 0.089, and 0.090 for the teicoplanin (T), its aglycone (TAG), ristocetin (R), and vancomycin (V) Chirobiotic® columns, respectively. These approximate values are based on the carbon content of the chiral stationary phase and may not correspond to the actual active stationary phase volume. It should be noted that all listed entropy variation values will be biased by an equal amount of less than $R \ln \phi$ (Eq. (2)). It means that all ΔS listed values may be systematically up to $20\,\mathrm{J\,M^{-1}\,K^{-1}}$ too high. However, this possible bias cancels when working with the enantioselective $\Delta(\Delta S)$ entropy changes (Eq. (3)).

3. Results and discussion

3.1. General observations

A total of 148 enantiomeric separations involving four CSPs, three chromatographic modes and 71 different compounds was performed at temperatures from 5 to 45 °C. It is not recommended to work in the RPLC mode with Chirobiotic columns at temperature higher than 50 °C [9]. At high temperatures and in presence of water, the silica base of the stationary phase can be solubilized and/or the Si–O–Si–C bonds can be hydrolyzed.

For every combination of stationary phase and mobile phase, three to six different temperatures were tested. The retention, enantioselectivity, and enantioresolution factors along with the peak efficiency of the last eluted enantiomer were recorded and used to construct the corresponding Van't Hoff plots (Eq. (2)). With no exception, in this reduced temperature range (40 °C), the Van't Hoff plots for the two enantiomers of all compounds were linear with regression coefficient higher than 0.977. The enthalpy and entropy changes were calculated from the slopes and intercepts of the plots. Table 2 lists the results obtained for the teicoplanin CSP. Similar tables were prepared for three other CSPs and can be requested. (http://www.astecusa.com). It should be noted that Table 2 contains the data for only 40 different compounds. This is due to the fact that 31 compounds of the full set did not separate on the teicoplanin CSP. They were successfully separated on one of three other CSPs tested. Many compounds could be separated on more than one CSP and several chromatographic modes (e.g. mephenytoin was separated on the teicoplanin CSP in reversed and normal phase mode (Table 2), on the vancomycin CSP in reversed phase mode, and on the ristocetin CSP in the normal phase mode only (data not listed)).

The fact that all Van't Hoff plots were acceptably linear is an indication that there is no significant structural evolution of the macrocyclic glycopeptide chiral selectors in the 40 °C temperature range studied. It was shown that structural changes induced by temperature were producing non-linearity in the Van't Hoff plots [17]. Such structural changes were noted with some protein and carbohydrate CSPs [17,18]. This result confirms what was found by the Péter group [19,20].

3.2. Column temperature and retention

A surprising result was that, in few cases, the solute retention times did not decrease when the mobile phase temperatures rose; but increased somewhat. This observation was made for only 19 separations (13% of the whole set). All the compounds with this behavior were separated in the polar ionic mode. In all cases, the increases of retention times associated with the higher temperatures were very limited. Fig. 1, chromatograms D–F, shows the enantioseparation of terbutaline on the vancomycin CSP. The 40 °C temperature increase produced a mere 1.1 min higher retention time (15% or 1.6 s/°C). All increases of retention time observed in this study were close to or lower than 2 s/°C. It was shown that, working with the PIM mobile phases, minute changes in the acetic acid and/or triethylamine amount added to pure methanol could drastically change the CSP and solute ionization state and so, the chiral separation [2,6,9].

In most cases, the usual decrease of retention times associated with a rise in column temperature was observed. Fig. 1 shows some typical examples. From the retention point of view, the elution of the second enantiomer of

Table 2
Thermodynamic parameters obtained with the teicoplanin chiral stationary phase and the three elution modes

Compound	ΔH_1 (kJ M^{-1})	ΔH_2 (kJ M^{-1})	ΔS_1 (J M^{-1} K^{-1})	ΔS_2 (J M^{-1} K^{-1})	$\Delta(\Delta H)$ (kJ M^{-1})	$\Delta(\Delta S)$ (J M^{-1} K^{-1})	T_{iso} [a] (°C)
Reversed phase mode							
2-Phenoxypropionic acid	−27.2	−25.3	−77.5	−68.6	1.92	8.91	−58
3-Hydroxy-4-methoxy mandelic acid	−43.5	−39.9	−136	−115	3.48	21.0	−107
3-Phenylphthalide	−23.9	−29.3	−48.1	−61.3	−5.37	−13.2	133
4-Benzyl-3-propionyl-2-oxazolidinone	−23.1	−27.0	−49.1	−58.1	−3.95	−8.98	167
5-Methyl-5-phenyl hydantoin	−21.8	−27.9	−51.1	−64.6	−6.04	−13.5	174
Althiazide	−26.7	−27.4	−60.5	−62.2	−0.69	−1.75	120
Atrolactic acid	−30.9	−30.1	−93.9	−85.0	0.91	8.95	−172
Bendroflumethiazide	−26.8	−29.3	−65.5	−72.1	−2.44	−6.61	96
Chlorthalidone	−21.4	−22.0	−48.1	−48.6	−0.61	−0.46	1043
Devrinol	−20.8	−21.5	−36.6	−38.0	−0.66	−1.36	214
Flurbiprofen	−14.2	−15.2	−20.4	−22.9	−0.99	−2.43	135
Folinic acid	−64.1	−58.0	−200	−174	6.11	26.25	−40
Ibuprofen	−19.5	−20.8	−40.8	−44.1	−1.29	−3.26	124
Ketoprofen	−26.8	−27.5	−62.3	−63.8	−0.68	−1.56	162
Mandelic acid	−37.9	−29.2	−122	−78.7	8.71	43.4	−72
Mephenytoin	−19.4	−20.5	−43.0	−45.1	−1.17	−2.01	309
Methsuximide	−15.8	−16.8	−31.0	−33.5	−0.99	−2.43	135
α-Methyl-α-phenyl succimide	−20.5	−20.8	−46.6	−46.8	−0.33	−0.12	2414
Naproxen	−29.3	−30.0	−69.5	−71.3	−0.69	−1.75	120
Phensuximide	−17.9	−18.9	−38.8	−40.9	−0.97	−2.12	183
Thalidomide	−24.0	−24.7	−50.6	−52.4	−0.70	−1.85	102
Tropic acid	−24.4	−24.5	−68.1	−67.4	−0.03	0.68	(−322)
Warfarin	−23.7	−23.7	−45.5	−44.3	0.03	1.19	−246
Polar ionic mode							
Albuterol	6.0	4.8	43.0	40.5	−1.22	−2.51	212
Alprenolol	0.5	−0.45	25.2	23.1	−0.96	−2.02	201
Atenolol	−1.5	−2.5	26.0	23.7	−0.98	−2.33	150
4-Benzyl-2-oxazolidinone	−9.4	−9.4	−16.3	−13.6	0.00	2.74	(−273)
4-Benzyl-3-propionyl-2-oxazolidinone	−10.0	−10.3	−18.3	−16.6	−0.27	1.68	(−434)
Clenbuterol	3.9	2.1	36.3	32.5	−1.74	−3.87	177
Metaproterenol	5.1	2.9	41.5	36.0	−2.27	−5.45	144
Metoprolol	0.0	−1.3	25.2	22.1	−1.28	−3.15	134
Mianserin	−16.6	−17.3	−29.8	−31.3	−0.68	−1.56	162
Oxazepam	−6.9	−12.0	−11.0	−15.6	−5.07	−4.64	818
Oxprenolol	2.6	1.9	32.0	30.6	−0.67	−1.46	186
Pindolol	3.8	2.8	37.8	35.5	−0.97	−2.22	166
Propranolol	1.3	0.05	30.4	27.2	−1.28	−3.15	134
Sotalol	0.13	−0.52	29.4	28.3	−0.65	−1.08	324
Terbutaline	5.9	3.1	42.6	36.1	−2.83	−6.50	162
Normal phase mode							
Mephenytoin	−9.1	−9.1	−1.9	−1.4	0.00	0.56	(−273)
Methsuximide	−13.2	−12.9	−18.9	−17.4	0.35	1.55	−46
5-Methyl-5-phenyl hydantoin	−14.1	−20.4	−18.8	−31.2	−6.30	−12.4	234
α-Methyl-α-phenyl succimide	−10.8	−12.3	−15.5	−19.0	−1.52	−3.48	163
Phensuximide	−12.5	−13.1	−20.3	−21.7	−0.66	−1.36	214
3-Phenylphthalide	−11.0	−14.7	−18.8	−28.2	−3.64	−9.32	117

Teicoplanin Chirobiotic T column phase ratio (Eqs. (1) and (2)) $\phi = 0.086$. The subscripts 1 and 2 refer to the first and second eluting enantiomer, respectively.

[a] T_{iso}, theoretical temperature for elution order reversal. Values in parenthesis do not make sense (lower than the absolute 0 K).

benzoin methyl ether (chromatograms A–C) on the vancomycin CSP is 6.7 min faster at 35 °C compared to that at 15 °C. It is a 1/3 decrease of the retention time or an average decrease of 20 s/°C. Similarly, the retention of the second enantiomer of asparagine (chromatograms G–I) decreased from 8.13 to 5.88 min, a 28% drop or 7 s/°C, when the temperature rose from 15 to 35 °C. The maximum decrease in retention time was observed for 3-indolelactic acid on the ristocetin A CSP with a methanol/buffer mobile phase (Fig. 2). The retention time of the last eluted 3-indolelactic acid enantiomer decreased from 29.5 min down to 12.9 min, a 56% drop, for a 20 °C increase in temperature or −50 s/°C. The average decrease of retention time, considering all CSPs and mobile phases, is −14 s/ °C

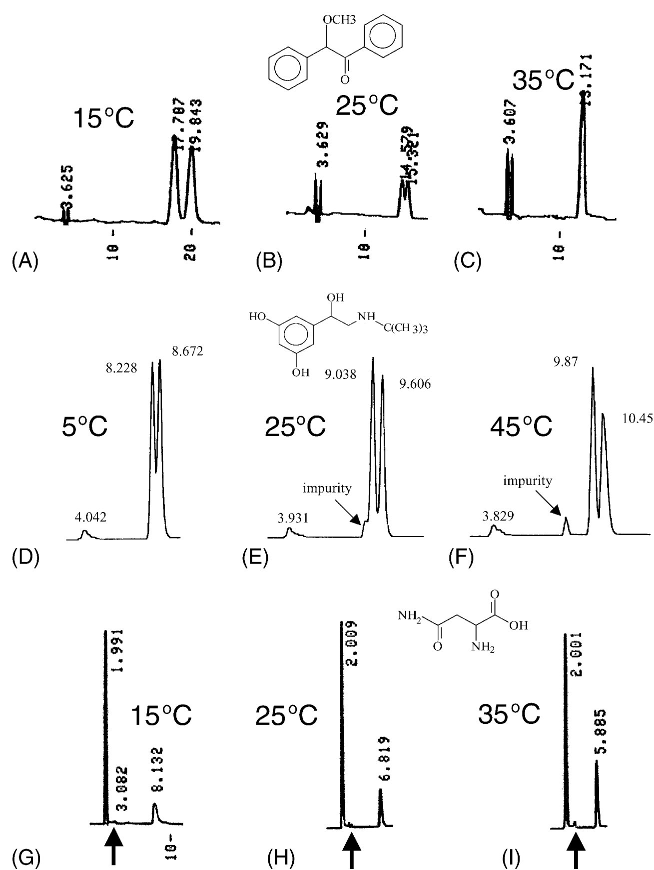

Fig. 1. Effect of temperature on enantioresolution. Chromatograms A–C: Decreased enantioseparation of the benzoin methyl ether racemate on vancomycin CSP. Column Chirobiotic V; mobile phase: methanol/0.01 M ammonium triethylacetate buffer at pH 6.0 20/80% (v/v) 0.9 mL/min. Chromatograms D–F: Increased retention and enantioresolution with the separation of the terbutaline racemate on vancomycin CSP. Column Chirobiotic V; mobile phase: PIM with 100% methanol with 0.1% acetic acid and 0.1% triethylamine, 0.9 mL/min. Chromatograms G–I: Decrease of resolution and increase of efficiency. Separation of the asparagine racemate on teicoplanin aglycon CSP. Column Chirobiotic TAG; mobile phase: methanol/0.01 M ammonium triethylacetate buffer at pH 4.1 30/70% (v/v) 0.9 mL/min. Detection: Light scattering detector. The arrows identify the dead volume position.

(or a minute of retention time variation for 4 °C of change in temperature).

3.3. Column temperature and resolution

With no exception, higher temperature increased peak efficiencies. This is well known and is one of the main reasons why separations are done at elevated temperature today [10]. For example, the efficiency of the more retained asparagine enantiomer was 2300 plates at 15 °C and 5000 plates at 35 °C (Fig. 1, chromatograms G–I). However, efficiency intervenes with its square root in the resolution equation. The doubling of the plate number will only increase the resolution factor, R_s, by 1.4 if other parameters, selectivity, and retention do not change.

Unfortunately, these chromatographic parameters do change. In only 17% of the experiments, a small increase of the resolution factor was observed with higher temperatures. For example, Fig. 1 (chromatograms D–F) shows the case of terbutaline separated on the vancomycin CSP. The resolution factor was 0.7 at 5 °C with an estimated peak efficiency of 4000 plates (fused peaks). R_s increased up to 1.3 at 35 °C, a close to baseline separation, with a peak efficiency of 6000 plates. It should be noted that the efficiency increase contributed only 25% to the R_s increase. The higher contribution came from the increase in selectivity and retention factors.

For the major part of the studied separations, as far as resolution is concerned, the increases in efficiency were obliterated by the selectivity and retention factor changes. For example, Fig. 1 (chromatograms A–C), shows the vanishing of the enantioseparation of benzoin methyl ether (R_s = 1.4, 0.6, and 0 at 15, 25, and 35 °C, respectively). R_s decreased from 10 to 7.5 in the separation of the asparagine enantiomers showed in Fig. 1, chromatograms G–H. Fig. 2 shows that the enantioselectivity, α, and retention factors, k_2, of 3-indolhydroxyacetic acid decrease, respectively, from 3.0 to 2.1 and 8.8 to 3.3. These drastic changes make the resolution factors decrease from 8.4 at 15 °C to 7.0 at 35 °C. Clearly, the huge increase in efficiency (from 1600 plates to 4000 plates) on the second 3-indole lactic acid peak just compensate part of the deleterious effect of the enantioselectivity reduction.

3.4. Thermodynamic studies

The mobile phase composition has a critical effect on the mechanism of solute interaction with the stationary phase. This is evidenced by the thermodynamic parameters obtained through temperature studies.

3.4.1. Polar aqueous mobile phases

Eighty-one enantiomeric separations involving 59 different compounds were resolved with methanol/buffer mobile phases on the four CSPs. The results obtained with the teicoplanin CSP are listed in Table 2 (23 compounds only). The full listing of results obtained with the three other CSP can be requested from the authors. With reversed mobile phases, all enthalpy and entropy variations are negative with all solutes and all four CSPs (e.g. Table 2 for the teicoplanin CSP). It is conclusive that with an aqueous mobile phase and Chirobiotic CSP's, the solute retention times will decrease when the temperature increases (Eq. (2)). There was one exception, bupivacaine, a piperidinecarboxamide anesthetic, that produced a positive change in both enthalpy and entropy on the vancomycin CSP with a 90% (v/v) citrate buffer mobile phase at pH 6.1. The retention time decrease with temperature was small (1.4 s/°C). At 0.9 mL/min, the retention time of the bupivacaine second enantiomer was 16.6 min at 45 °C reducing to 15.6 min at 5 °C.

For all solutes, at 25 °C (298 K) and with a polar reversed mobile phase, the weight of the enthalpic term, ΔH, in the change of Gibbs energy, ΔG, was similar or slightly higher

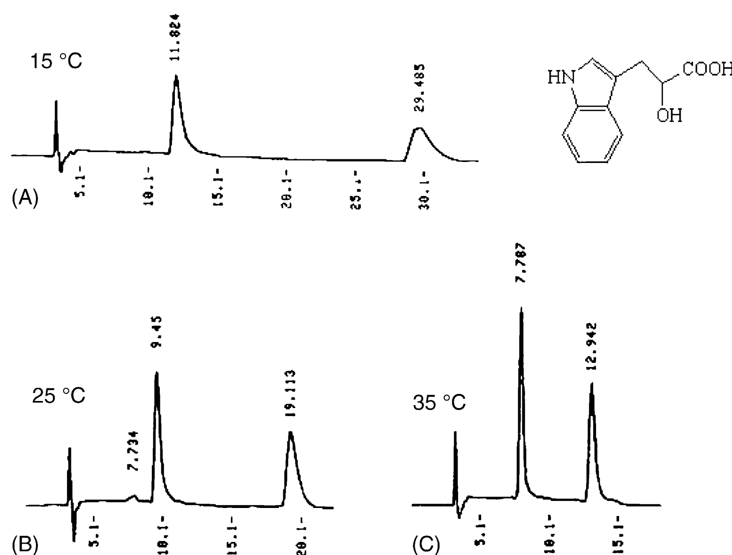

Fig. 2. Enantioseparation of the 3-indole lactic acid racemate on ristocetin A CSP. Column Chirobiotic R; mobile phase: methanol/0.01 M triethylammonium acetate buffer (pH 4.1) 40/60% (v/v), 1 mL/min.

than that of the entropic term, $T\Delta S$ (Table 2). In the case of bupivacaine on the vancomycin CSP, the $T\Delta S$ entropic terms of the first and second enantiomer were, respectively, 8.6 and 7.2 kJ mol^{-1} at 25 °C, three to six times higher than the corresponding enthalpic terms (3.1 and 1.2 kJ mol^{-1}). In this case, the stronger entropic term may be the sign of the formation of an inclusion complex between vancomycin and the most retained bupivacaine enantiomer.

The enantioselectivity factor, α, depends on the enantiomeric Gibbs energy, $\Delta(\Delta G)$ (Eq. (3)). It will increase with temperature only if the enthalpic contribution, $\Delta(\Delta H)$, is positive (Eq. (3)). Unfortunately this was rarely observed with reversed mobile phases. It was not observed on the ristocetin and vancomycin CSPs. It was observed with 25% (Table 2) and 7% of the compounds tested on the teicoplanin and TAG CSP, respectively. Surprisingly, the increase in enantioselectivity factor, α, was rarely associated with an increase of the resolution factor, R_s (Table 2).

A correlation was noted between the entropy and enthalpy contributions (Table 2). The higher and lower ΔH (and $\Delta(\Delta H)$) values were associated with higher and lower ΔS (and $\Delta\Delta S$) values. The enthalpy–entropy compensation study will fully detail this point later.

3.4.2. Non-aqueous PIM mobile phases

The enantiomers of 32 different compounds bearing ionizable groups were separated with 100% methanol mobile phases containing trace amounts of ionizing agents (PIM mobile phases) on the four CSPs. An approximately equal number of compounds produced negative or positive enthalpy and entropy variations. Actually all the compounds that showed a retention time increasing with temperature (i.e. a positive ΔH) were separated with a PIM mobile phase. Two points should be noted. (1) For all solutes and all CSPs, the signs of the entropy and enthalpy variation are the same, with only five exceptions: atenolol, metoprolol, and sotalol on the teicoplanin CSP (Table 2) and fluoxetine and labetalol on the vancomycin CSP (data not showed), all in PIM mode with 100% methanol mobile phases adjusted with trace amounts of acetic acid and triethylamine. (2) The relative weights of the entropic and enthalpic terms are strongly correlated to the sign of ΔH, the variation of enthalpy. All compounds with a positive enthalpy variation showed, at 25 °C, $T\Delta S$ term was 3–20 times higher than the ΔH term. All compounds with both a negative enthalpy and entropy variations showed, at 25 °C, the entropic term was comparable or up to about two times smaller than the enthalpic term.

In PIM mode, the $\Delta(\Delta H)$ variation was most often negative, meaning that the enantioselectivity factor was decreasing with temperature. Of the five compounds with a positive $\Delta(\Delta H)$ variation (one case, 4-benzyl-2-oxazolidinone, observed with teicoplanin, is listed in Table 2), only two showed a slight resolution increase with temperature. Fig. 1, chromatogram D–F, shows the separation of terbutaline on the vancomycin CSP with $\Delta(\Delta H)$ = 0.33 kJ M^{-1} and $\Delta(\Delta S)$ = 2.0 J M^{-1} K^{-1}. At 5 and 45 °C, the resolution factors were, respectively, 0.7 and 1.2. The unusual increase in retention times may be due to a slight shift of the ionization constant of the amine group of terbutaline and/or shifts in the ionization constants of groups on the vancomycin stationary phase.

3.4.3. Low polarity mobile phases (normal mode)

Few compounds were enantioseparated in the normal phase mode with hexane–ethanol mobile phases on the four CSPs. With no exception, all enthalpy and entropy variations were negative (Table 2). The weight of the enthalpy term was 1.3–3 times higher than that of the entropy term at 25 °C. In only one case: methsuximide separated on

teicoplanin (Table 2), the $\Delta(\Delta H)$ variation was positive. However, the small increase in enantioselectivity did not produce any resolution improvement.

3.4.4. Enthalpy–entropy compensation

Enthalpy–entropy compensation (EEC) studies are commonly used in chromatography to investigate retention mechanisms from a global thermodynamic point of view [19–24]. An EEC is demonstrated by a linear correlation between ΔH and ΔS. The slope of the linear plot is called the compensation temperature, T_c. At this temperature, the Gibbs energy of transfer from the mobile phase to the stationary phase is the same for all solutes. The enthalpy change is exactly compensated by the entropy change. The compensation temperature is regarded as a process characteristic and similar compensation temperatures for different equilibria are considered as an indication of mechanistic similarity [20,24].

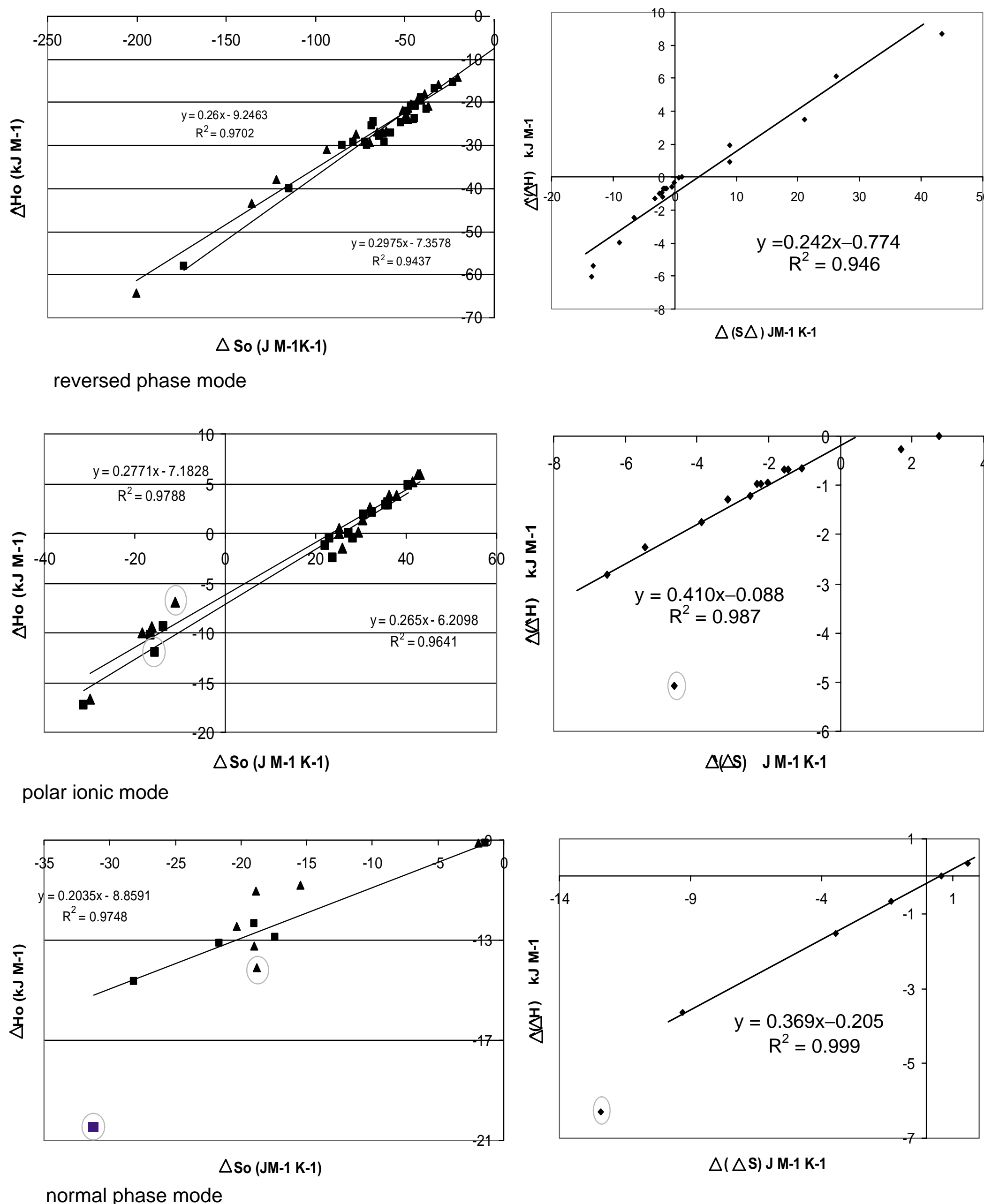

Fig. 3. Enthalpy–entropy compensation study on the teicoplanin chiral stationary phase. Left: compensation study for each enantiomers of Table 2; triangle: first enantiomer, square: last enantiomer. Right: compensation study on the enantioselective part of the retention mechanism. Top: methanol/water mobile phases (RPLC). Middle: PIM (100% methanol with adjusted ionization modifiers) mobile phases; compound oxazepam is excluded from the regression lines (circled). Bottom: hexane-ethanol mobile phases (normal mode); compound 5-phenyl-5-methyl hydantoin is excluded from the regression lines (circled).

In most cases of stationary phase–mobile phase associations, a correlation between the solute enthalpy and entropy terms, obtained as slope and intercept of the Van't Hoff plots, respectively, was observed. Fig. 3 shows the lines obtained with the results listed in Table 2 for the teicoplanin CSP plotted in the compensation form: ΔH versus ΔS. EEC is observed for the retention of both enantiomers in reversed phase and polar ionic mode. In normal phase mode, only the retention of the second enantiomer shows the EEC phenomenon (squares in Fig. 3 bottom left).

EEC is also observed on the enantioselectivity factor. However, in this case, there are compounds clearly standing out of the $\Delta(\Delta H)$ versus $\Delta(\Delta S)$ line. With the teicoplanin CSP and PIM mobile phases, oxazepam stands out of the line (circled in Fig. 3, middle). This point was excluded from the regression calculation. Similarly, 5-methyl-5-phenyl hydantoin was excluded from the regression calculation done with the data for the normal mobile phases (circled point in Fig. 3, bottom).

3.4.5. *Chiral recognition mechanism*

Table 3 lists the slopes, intercepts and corresponding compensation temperatures obtained with the EEC studies for all CSPs. Clearly, there is little if any EEC with the results ob-

Table 3
Entropy–enthalpy compensation studies with the four chiral selectors and the three elution modes

Chiral selector	Teicoplanin	TAG	Ristocetin	Vancomycin
Reversed phase mode				
Slope of first enantiomer	0.261	0.277	0.266	0.304
Intercept of first enantiomer	−9.24	−3.323	−5.14	−5.07
r^2	0.97	0.93	0.801	0.977
T_c (°C)	−12	4	−7	31
Slope of second enantiomer	0.298	0.292	0.274	0.309
Intercept of second enantiomer	−7.36	−5.34	−6.31	−5.24
r^2	0.944	0.944	0.752	0.979
T_c (°C)	25	19	1	36.3
Slope enantioselectivity	0.242	0.308	0.347	0.318
Intercept enantioselectivity	−0.774	−2.61	−0.656	−0.218
r^2	0.946	0.908	0.991	0.986
T_c (°C)	−31	35	74	45
Polar ionic mode				
Slope of first enantiomer	0.265	0.311	No EEC	0.286
Intercept of first enantiomer	−6.21	−4.69	No EEC	−3.95
r^2	0.964	0.988	No EEC	0.985
T_c (°C)	−8	38	No EEC	13.5
Slope of second enantiomer	0.277	0.309	0.419	0.2948
Intercept of second enantiomer	−7.18	−6.22	0.277	−4.51
r^2	0.979	0.992	0.978	0.994
T_c (°C)	4	36	146	22
Slope enantioselectivity	0.41	0.327	0.332	0.3316
Intercept enantioselectivity	−0.088	−0.458	−1.43	−0.219
r^2	0.987	0.963	0.983	0.998
T_c (°C)	137	54	59	59
Normal phase mode				
slope of first enantiomer	no EEC	no EEC	no EEC	no EEC
Intercept of first enantiomer	no EEC	no EEC	no EEC	no EEC
r^2	no EEC	no EEC	no EEC	no EEC
T_c (°C)	no EEC	no EEC	no EEC	no EEC
Slope of second enantiomer	0.204	no EEC	no EEC	no EEC
Intercept of second enantiomer	−8.86	no EEC	no EEC	no EEC
r^2	0.975	no EEC	no EEC	no EEC
T_c (°C)	−69	no EEC	no EEC	no EEC
Slope enantioselectivity	0.369	0.374	0.284	0.332
Intercept enantioselectivity	−0.205	−0.381	−0.31	−0.206
r^2	0.999	0.987	0.989	0.970
T_c (°C)	96	101	11	59

PIM: polar ionic mode; T_c is the theoretical compensation temperature in degree Celsius. No EEC means that the ΔH versus ΔS points were not forming a line ($r^2 < 0.7$).

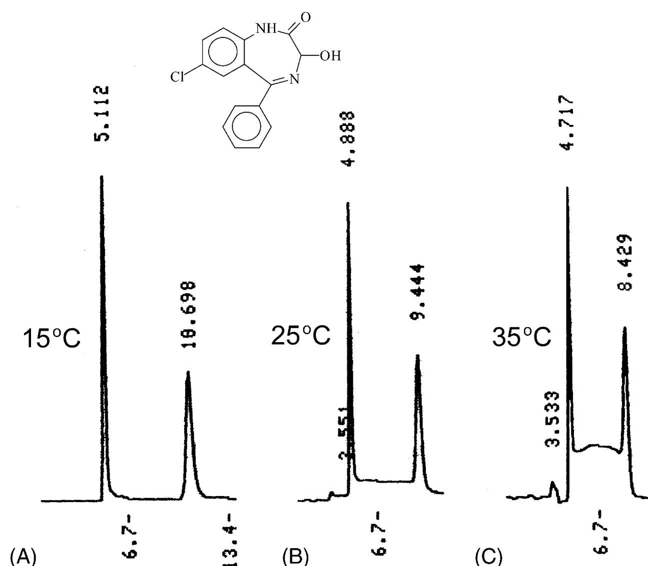

Fig. 4. Intracolumn enantiomer interconversion induced by temperature. Enantioseparation of the oxazepam racemate on teicoplanin CSP. Column Chirobiotic T; PIM mobile phase: 100% methanol with 0.2% acetic acid and 0.2% triethylamine, 0.9 mL/min.

tained with the normal mobile phases. This is an indication of the complexity of the solute–stationary phase interaction in this mode. It should also be noted that a small number of compounds can be separated by the somewhat polar macrocyclic glycopeptide CSPs with apolar normal mobile phases.

The surprising result of the EEC studies is that acceptable linearity was always obtained with the enantioselectivity factor, that is the plots of $\Delta(\Delta H)$ versus $\Delta(\Delta S)$. After excluding outsiders, these plots showed regression coefficients higher than 0.95 even with the normal phase mode. Most of the enantioselectivity compensation temperatures for the $\Delta(\Delta H)$ versus $\Delta(\Delta S)$ plots fell in the 10–70 °C range (Table 3). It is noted that two compensation temperatures are as cold as 30 °C and three others are close to or pass 100 °C. Great care should be taken before drawing any mechanistic conclusion using these results. Thirty years ago Krug et al. [25] pointed out that “detectable extrathermodynamic EEC effects are rare”. They recommended separating carefully the chemical from the statistical effect [26]. A study done with four to six points on a 40 °C temperature range can produce lines that are only tangents of non-linear phenomena on a hundredth degree or more width. So no further discussion on general enantiorecognition mechanism will be done with our results.

The positive point is that the compounds excluded because they did not stand on the EEC lines certainly separated with a mechanism differing significantly from the other compounds [27]. This was the case of oxazepam that was excluded from the EEC line done with the teicoplanin CSP and the PIM mobile phases. Observation from the oxazepam chromatograms showed that the increase of temperature produced intra-column racemization (Fig. 4). The “Batman”-shape of the two peaks tells that there is equilibrium between the two oxazepam enantiomers [28]. The time scale of the racemization reaction increases with temperature increasing the plateau region between the two peaks. Thalidomide was excluded from the EEC line because its representative point clearly stood out of the line. Its enantioselectivity factor was unusually high ($\alpha = 4.1$ at 5 °C down to 3.7 at 45 °C) producing impressive resolution factors (8.5 and 7.5, respectively). A strong and specific multi-point interaction site exists between one enantiomer of thalidomide and vancomycin. Other excluded compounds were 5-methyl-5-phenyl hydantoin with the vancomycin and teicoplanin CSPs both with a normal mobile phase and oxazepam on the TAG CSP and a PIM mobile phase. In these three cases, impressive enantioselectivity and resolution factors showed a key and lock effect between the CSP and one enantiomer. The separation of oxazepam on TAG also showed the racemization reaction above 25 °C.

3.4.6. Other effects

A surprising effect was observed with asparagines on the teicoplanin CSP. The elution of the asparagine L enantiomer occurred before the void volume (Fig. 1, G–I). This is an indication of repulsion of L-asparagine from the stationary phase and pores. At pH 4.1, asparagine is positively charged. The teicoplanin selector is also positively charged excluding the L-enantiomer with which it has no affinity. It retains the D-enantiomer that is also positively charged. This shows that the strength of the enantioselective interaction between the chiral selector and D-asparagine should be similar or higher than the electrostatic repulsion since it can overcome it. This interaction is necessarily an ionic interaction. No other statement can be made on the thermodynamic parameters obtained with asparagine because the retention times were used instead of retention factors for thermodynamic calculations.

4. Conclusion

Enantioresolution is much likely to be better at low temperature even though the retention times will be high and the peak efficiency lower. Once the separation is optimized, it may be possible to raise the temperature, slowly, to improve peak shapes and reduce retention times. Enantioresolution factor will be reduced as well. If the resolution factors are very high, like in the case of amino acid enantioseparations, the temperature can also be raised to improve efficiencies at the cost of a resolution decrease as long as baseline separation is maintained.

The second point is the critical role of the mobile phase in the chiral separation mechanism. The thermodynamic studies indicate that they take a broader approach in treating the solute interaction between the mobile and the stationary phase. Too often esthetical molecular modeling describes nice chiral recognition mechanisms completely ignoring the solvent role in liquid chromatography. This study shows that

the PIM mobile phases can produce enthalpy as well as entropy driven separations depending on the solute, when both polar hydro-organic (RPLC) and apolar hexane–ethanol (normal LC) mobile phases only produce enthalpy driven enantiomer separations. In all cases and within the temperature range studied, the macrocyclic glycopeptide stationary phases were not under any conformational transition.

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