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Review

Dynamic HPLC on chiral stationary phases: A powerful tool for the investigation of stereomutation processes

Dynamic HPLC on enantioselective stationary phases has become a well-established technique to investigate chiral molecules with internal motions that result in stereoinversion and occur on the time scale of the separation process. Kinetic parameters for the on-column interconversion phenomena can be extracted from experimental peak profiles by computer simulation or by direct calculation methods. The technique has been used in a wide range of temperatures and is complementary in scope to dynamic NMR spectroscopy.

Keywords: Dynamic HPLC / Chiral stationary phases / Stereomutation, enantiomerization / Computer simulation

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1 Introduction

The direct HPLC separation of enantiomers on chiral stationary phases is nowadays one of the preferred modes to quantitatively characterize chiral compounds in terms of enantiomeric composition, and to obtain discrete amounts of pure, single enantiomers on large scales. Enantiomer separation is realized through non-covalent, reversible formation of diastereomers as the two enantiomers of the analyte interact with the chiral, enantiomer-enriched selector present in the system. Reversible interactions of the individual enantiomers with the stationary phase are usually referred to as primary equilibria [1 – 5].

The enantioselectivity has a thermodynamic origin and is simply related to a difference in the stability of the diastereomeric species formed by the two enantiomers and the chiral selector. A simple equation (Eq. 1) relates chromatographically derived enantioselectivity α with this energy difference. This value may approach the true value of the thermodynamic enantioselectivity of the selector-analyte-solvent system when the association with the chiral selector governs the retention of the enantiomers in the chromatographic system and other, non-selective types of interactions are negligible.

$$\Delta\Delta G^{\circ}_{2,1} = \Delta G^{\circ}_{2} - \Delta G^{\circ}_{1} = -RT\ln(k'_2/k'_1) = -RT\ln\alpha_{2,1} \quad (1)$$

where $\delta\Delta G^{\circ}_{2,1}$ is the difference in the interaction Gibbs energies between the two enantiomers and the system selector, R is the gas constant, T is the absolute temperature and $\alpha_{2,1}$ is the ratio of the retention factors k'_2 and k'_1 of the two enantiomers.

Both enantioselective and unspecific association of the enantiomers with the stationary phase may have a retarding or activating effect on the internal molecular motions of stereolabile chiral species during their passage through the chromatographic column.

2 Theory of dynamic HPLC of chiral stereolabile compounds

In recent years enantioselective HPLC has been extended to the investigation of stereolabile compounds. Dynamic HPLC (DHPLC) in the form of variable temperature or variable flow chromatography can be employed to determine the enantiomerization (the reversible isomerization of one enantiomer into the other) barrier of chiral stereolabile species when the interconversion takes place at the time scale of the separation process [6 – 12].

This technique has been recently used for the study of internal molecular dynamics of a range of chiral, stereolabile compounds, and for the determination of the pertinent kinetic parameters. Attractive features of this

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Abbreviations: **CSP**, chiral stationary phase ; **DHPLC**, dynamic HPLC

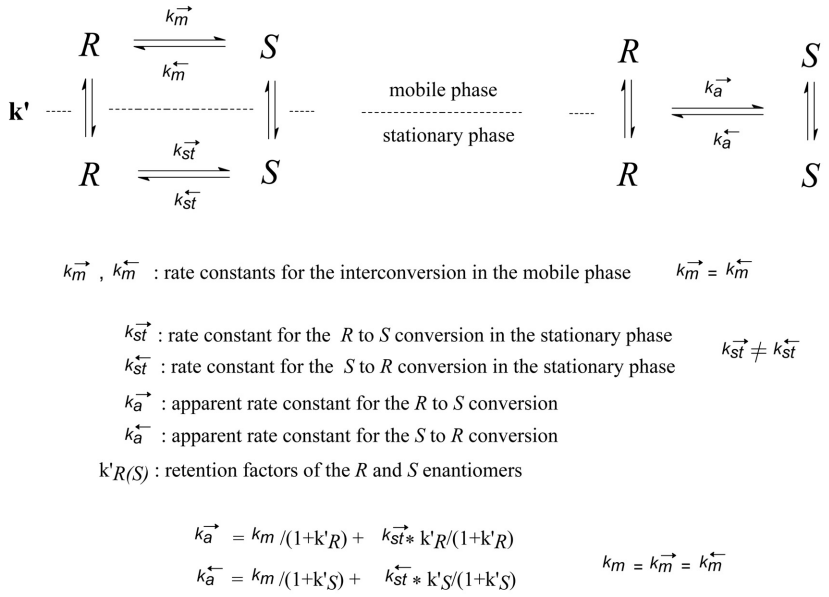

Figure 1. Primary (vertical arrows) and secondary (horizontal arrows) equilibria taking place during chromatography of two interconvertible enantiomers *R* and *S* on a CSP. Left: actual interconversions occurring in the mobile and stationary phases, with *R* enantiomer eluting first; right: the apparent equilibria.

technique are: (i) only small amounts of racemic sample are required, (ii) a wide range of working temperatures are available, and (iii) the interconverting species are physically separated and, under certain circumstances, they can be further characterized, *e.g.*, by chiroptical methods.

A dynamic chromatographic experiment entails two resolvable enantiomers that interconvert on the separation time scale. The HPLC time scale extends from seconds in the cases of fast HPLC on short columns to hours when standard columns and low linear velocities of the eluent are used. For a given column format (column dimensions and particle size of the packing material), typical peak shape deformations are observed in response to changes in the column temperature and/or in the flow rate of the eluent. Peak shape characteristic of an on-column interconversion are usually observed as a profile between the two resolved peaks that does not reach the baseline (plateau).

The resulting peak shapes contain the necessary information to extract pertinent kinetic parameters related to the exchange process between the two species.

Several methods (the theoretical plate model [8], the stochastic model [7 – 10], the continuous flow model [13], peak deconvolution methods [10], approximation functions [10], and unified equation [12]) have been used to extract kinetic data from experimental elution profiles, and each method exploits a different theoretical framework to describe the dynamic system in which primary and secondary equilibria take place inside the column (Fig. 1).

The theoretical plate model describes the chromatographic separation as a discontinuous process, assuming that all steps proceed repeatedly in separate uniform sections of a column consisting of *N* plates. Every theoretical plate is considered as a distinct chemical reactor in which three events take place: distribution of the species between the mobile and stationary phases, interconversion in both phases, and shifting of the mobile phase to the next plate [8]. The stochastic model describes the chromatographic separation using time-dependent distribution functions [7, 10]. The elution profile of two interconverting species is given by the sum of the distribution functions of the non-interconverted species and the probability of density functions of the interconverted species. In the continuous flow model, general analytical expressions based on the Damköhler number describe the chromatographic mass distribution at the column outlet for a solute subject to secondary equilibria [13].

The effects of changes in some critical parameters (k_m, k_{st}, retention) on the appearance of the chromatographic profiles are illustrated in Figs. 2 – 4, where the theoretical plate model is used to generate typical plots for a chiral stereolabile analyte resolved on a chiral stationary phase (CSP).

Figure 5 shows the individual traces of two interconverting enantiomer that are concomitantly separated on a CSP: Tailing of the first and fronting of the second eluted enantiomers are due to analyte molecules that have undergone at least one stereochemistry inversion during elution.

Recent applications of DHPLC to the study of chiral stereolabile compounds (Fig. 6) will be illustrated here, with a special emphasis on two aspects of the technique: (i) the effect of the stationary phase on the interconver-

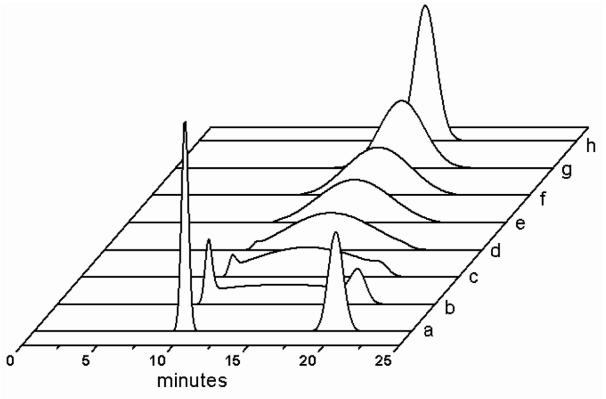

Figure 2. Transition from slow to fast exchange at constant retention and efficiency. Simulation parameters from **a** to **h**: $k_m = k_{st}$ = 0.1; 0.2; 0.3; 0.4; 0.5; 1.0; 5.0 min^{-1}; $N_1 = N_2 =$ 1500; t_0, t_1, $t_2 =$ 2.2; 10.0; 20.0 min.

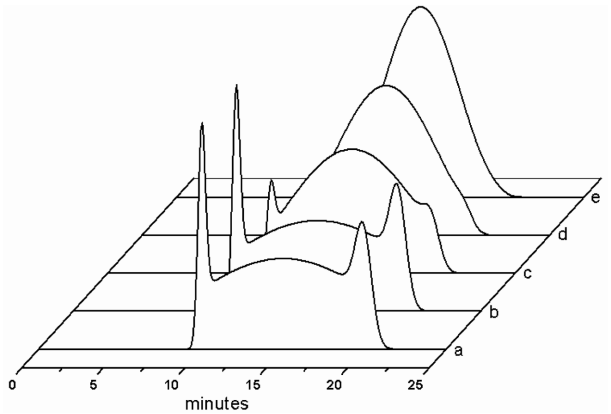

Figure 3. Stationary phase effects. Transition from intermediate to fast exchange at constant retention and efficiency. Simulation parameters: k_m fixed at 0.2 min^{-1}; from **a** to **e**: $k_{st} = 0.0$; 0.1; 0.2; 0.3; 0.5 min^{-1}; $N_1 = N_2 = 1500$; t_0, t_1, $t_2 = 2.2$; 10.0; 20.0 min.

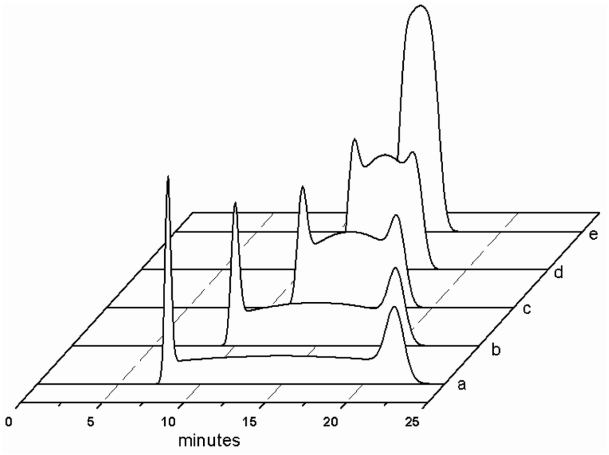

Figure 4. Retention and selectivity effects. Transition from intermediate to fast exchange at constant efficiency and kinetic parameters. Simulation parameters: $k_m = k_{st} =$ 0.1 min^{-1}; $N_1 = N_2 = 1500$; $t_0 = 2.2$ min; from **a** to **e**: t_1, $t_2 =$ 8.0, 22.0; 10.0, 20.0; 12.0, 18.0; 13.0, 17.0; 14.0, 16.0.

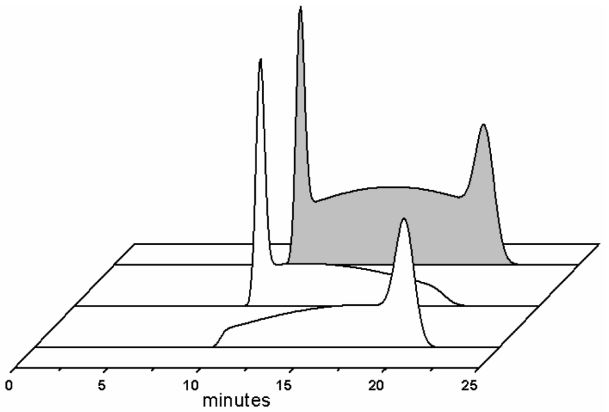

Figure 5. Simulated plots showing separate peak profiles for the first and second eluted peaks and their convolution to give the total profile featuring the interconversion plateau between the resolved peaks.

sion process, and (ii) cryo-HPLC study of stereolabile compounds featuring low barrier to interconversion.

3 Stationary phase effects

The apparent rate constants, $k_a^{\rightarrow}$ and $k_a^{\leftarrow}$, determined by simulation methods are related to those involved in the actual equilibria occurring in the mobile phase, $k_m^{\rightarrow} = k_m^{\leftarrow}$, and in the stationary phase, $k_{st}^{\rightarrow}$ and $k_{st}^{\leftarrow}$. The apparent rate constants are not in principle equal to the rate constants of the interconverting processes occurring in the mobile and stationary phases. The potential perturbing effect of the stationary phase on the interconversion can be studied by combining the DHPLC experiment with independent measurements that yields the rate constants in free solution. Off-column thermal racemization experiments are conveniently performed on single enantiomers that are first collected by enantioselective HPLC and then converted back to the racemate, while monitoring enantiomer composition as a function of time by chromatography or chiroptical methods (polarimetry or circular dichroism spectroscopy). Alternatively, free solution rate constants are extracted from dynamic NMR (DNMR) experiments. However, the NMR time scale is different and dynamic spectra are observed across a higher temperature range compared to DHPLC.

For a series of axially chiral tertiary amides of 1-naphthoic acid, the whole set of rate constants was obtained by a combination of DHPLC and off-column thermal racemization experiments. Tertiary amides of 1-naphthoic amides have a ground state conformation in

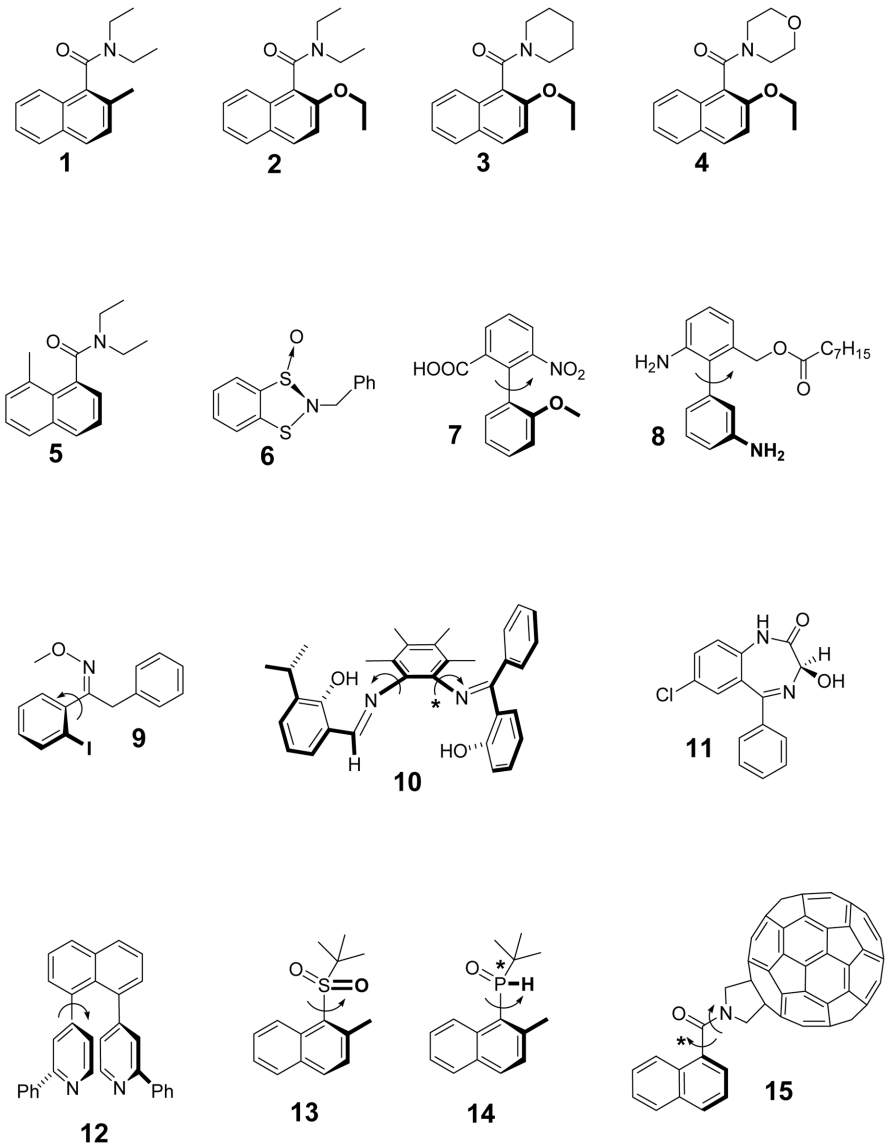

Figure 6. Structures of stereolabile compounds investigated by DHPLC.

which the aromatic and carboxamide planes are twisted at about right angles, thus rendering the molecule chiral. Rotation around the Ar-CO bond interconverts the two conformational enantiomers, and the substitution pattern at the 2 and 8 positions of the naphthyl ring regulates the enantiomerization rate. Tertiary amides of 1-naphthoic acid are not isolable at room temperature (enantiomerization barriers $\Delta G^{\ddagger}$ lower than 20 kcal/mol), whereas tertiary amides of 2-methyl, 2-ethoxy and 8-methyl substituted 1-naphthoic acid are atropisomeric, *i.e.*, physically separable at room temperature ($\Delta G^{\ddagger}$ between 21 and 24.8 kcal/mol).

The individual enantiomers of atropisomeric amides **1**–**5** were isolated at room temperature by HPLC on the Whelk-O1 CSP and subjected to off-column thermal racemization at temperatures between 318 and 348 K [14, 15]. The enantiomerization rate constants obtained from these experiments were used as fixed input parameters for the computer simulations of exchange broadened chromatograms of **1**–**5**, collected on the Whelk-O1 CSP, using different combinations of eluent flow rate and temperature that yielded a visible plateau between the resolved peaks. Comparison between off-column and chromatographically derived rate constants showed that the enantiomerization process is always faster in free solution (k_m) compared to the adsorbed state (k_{st}), with differences between the energy barriers in the two phases in the 0.3–1.3 kcal/mol range. The inhibitory effects of the stationary phase were found to be small for the first eluted enantiomer and more pronounced for the second eluted one. This finding is consistent with the assump-

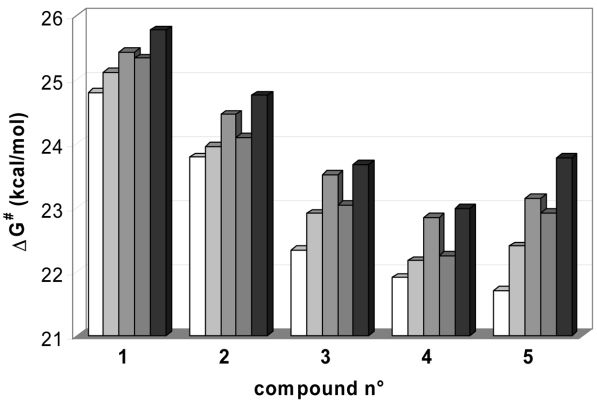

Figure 7. Stationary phase effects observed in the DHPLC experiments on amides **1**–**5**. For each compound vertical bars are (white to black): free energy of activation in the mobile phase, forward (conversion from first to second eluted) and backward (conversion from second to first eluted) apparent free energies of activation, forward and backward free energies of activation in the stationary phase.

tion that, in the solute-selector complex, the transition state for the enantiomerization (180° rotation of the carboxamide fragment around the C_{Ar}-CO bond) cannot be reached unless the hydrogen bond between the carbonyl oxygen of the analyte and the selector amidic NH is broken. This interaction, detected both in solution and in the solid state in similar systems, is more pronounced for the second eluted enantiomer and leads to a higher energy barrier for the conversion of the second into the first eluted enantiomer (Fig. 7). A similar study was performed on the chiral *N*-benzyl-1,3,2-benzodithiazole 1-oxide **6**, whose stereolabile enantiomers were resolved by HPLC on the Whelk-O1 CSPs [16, 17]. Comparison of the activation barriers for the R/S inversion measured by thermal racemization of the isolated enantiomers in free solution and by DHPLC (*i.e.*, in the presence of the stationary phase) revealed only a small, deactivating effect of the solid sorbent on the enantiomerization process ($\Delta\Delta G^{\ddagger}$ = 0.5 kcal/mol at 308.9 K, $\Delta\Delta G^{\ddagger}$ = 0.8 kcal/mol at 320.7 K). In these studies, on-column and off-column experiments were performed using the same solvents and exploring the same temperature ranges. Related studies on axially chiral compounds, whose R/S interconversions were observed by concomitant DHPLC and off-column thermal racemization or DNMR experiments, revealed that free solution processes are only marginally affected by the HPLC stationary phases. In addition, solvent and temperature effects were unimportant, as expected for intermolecular motions occurring through single bond rotation processes. The biphenyl derivatives **7** and **8** were resolved by HPLC on Chiralpak AD columns and studied by variable temperature HPLC. The enantiomers of **7** gave two well-resolved peaks, with an intermediate plateau-like region clearly visible when the column temperature was in the 15–25°C range [18]. The dynamic chromatographic profiles changed in a flow and temperature dependent manner and such dynamic deformations of the experimental chromatograms were exploited to extract the pertinent kinetic data. Computer simulations of exchange-deformed elution profiles obtained at 25°C and using eluent flow rates of 1.0 and 0.5 mL/min gave the apparent rate constants for the on-column R/S interconversion an the associated barriers $\Delta G^{\ddagger}_{12}$ = 21.8 kcal/mol and $\Delta G^{\ddagger}_{21}$ = 21.9 kcal/mol at 24°C, using hexane/2-propanol (95:5) + 0.1% CF_3COOH as eluent. Off-column racemization of the individual enantiomers of **7** in methanol/water (5:1) + 0.1% CF_3COOH was studied by circular dichroism spectroscopy. Following the decay of the circular dichroism signal as a function of time, the energy barrier to enantiomerization at T = 9°C was found $\Delta G^{\ddagger}$ = 21.8 kcal/mol, indicating that temperature, solvent and the HPLC stationary phase have little effect on this enantiomerization. Similar results were obtained for the biphenyl derivative **8**, for which the DHPLC experiment [eluent hexane/isopropanol (80:20) at T = −5°C] gave an averaged energy barrier $\Delta G^{\ddagger}$ = 19.7 kcal/mol, close to the NMR (micellar aqueous phase, T = 90°C) value of $\Delta G^{\ddagger}$ = 18.3 kcal/mol [19].

The enantiomers of the O-alkyl aryl oxime **9** were resolved by HPLC on Chiralcel OD using hexane/isopropanol (98:2) as eluent [20]. The DHPLC experiment carried out in the temperature range between −40 and −70°C gave the averaged energy barrier of 15.0 kcal/mol, that correlates well with the value of 15.9 kcal/mol determined by DNMR in toluene-d_8. The non-symmetrical salophen ligand **10** enantiomerizes through a two-step process in which the C_{Ar}-N bonds rotate sequentially, with the rate determining step represented by the slower rotation around the more hindered bond (indicated by the asterisk in Fig. 6): in this case DHPLC carried out on Chiralcel OD using hexane/isopropanol/methanol as eluent and DNMR in DMSO-d_6 gave the energy barriers $\Delta G^{\ddagger}$ = 21.8 kcal/mol and 21.4 kcal/mol, respectively [21].

Oxazepam **11** is a well-studied chiral compound, whose enantiomers easily interconvert near room temperature in a range of solvents. Free solution enantiomerization barriers are in the 21.6–22.6 kcal/mol range, while barriers extracted by DHPLC are 21.6–21.7 kcal/mol (on Nucleodex-β-PM, permethylated β-CD chemically bonded to silica, using a 6:4 mixture of phosphate buffer and methanol as eluent, T range 20–60°C) [22] and between 20.7 and 22.1 kcal/mol (on Chirobiotic R, Chirobiotic T, Whelk-O1 CSPs under HPLC or SFC conditions at 45°C) [10]. It has to be pointed out that enantiomerization of oxazepam **11** (and of related 3-hydroxy-1,4-benzodiazepin-2-ones) does not occur through simple bond rotations, but presumably proceeds *via* protonation of the 3-hydroxy-group and elimination of water with generation

of an intermediate carbocation that undergoes keto-enol tutomerization. Accordingly, DHPLC is able to correctly model also on-column interconversions that require reversible bond breaking and formation, as well as complex processes involving charged intermediate species.

The interconversion between *syn* and *anti* rotamers of **12** (diastereoisomers, the *anti* rotamer is chiral) is fast at ambient temperature and the energy barrier to rotation about the naphthylpyridyl axis was determined by DNMR ($\Delta G^{\ddagger}$ = 17.5 kcal/mol at 40.3°C in $CDCl_3$). The *syn*/*anti* isomers were separated by HPLC on an achiral cyano column [hexanes/EtOH (3:2) as eluent] in the temperature range between −65.0 and −43°C and the corresponding dynamic experiments gave the averaged energy barrier in the 16.2 – 16.4 kcal/mol range. A strictly linear Eyring plot was obtained by combining the DHPLC and DNMR experiments that spanned a temperature range of about 100°C, demonstrating the two methods are complementary and can be used in concert to extract structural and dynamic information on enantiomer and diastereomer interconversions [23].

4 DHPLC on CSPs at low temperature

DHPLC has found wide applications in the study of slow molecular motions with energy barriers up to 25 kcal/mol, barriers that are beyond the usual range of DNMR. Fast interconversion processes can be studied by DHPLC as well, but the typical characteristic time of the technique (minutes) poses a lower limit for the energy barrier of interconversion at around 14 kcal/mol. Such energy barrier translates into half-life times for the individual enantiomers in the millisecond range at temperatures close to 25°C. To bring these processes on the same time scale of the fastest HPLC separation (seconds), the column has to be cooled down to temperatures around −60°C, whereas slower HPLC separations that are complete within minutes require temperatures as low as −80°C. Cryo-chromatography at temperatures ranging from −50° down to −80°C presents some practical and instrumental difficulties, mainly related to the increased viscosity of the mobile phases. Higher viscosities in turn result in a large pressure drop across the column, with limitations in the range of available linear velocities of the eluent. The back pressure of a packed column can be estimated using Eq. 2, that combines the Darcy law and the Kozeny-Carman equation for the specific column permeability for the velocity of the eluent in a laminar flow system (Eq. 2) [24], where the pressure drop (ΔP) is proportional to the length of the column (L), the eluent viscosity (η), the column linear velocity (u) and the flow resistance factor (Φ), and inversely proportional to the square of the particle diameter (d_p)

$$\Delta P = L\,\eta\,u\,(\Phi/d_p^2) \quad (2)$$

For typical normal-phase HPLC solvents, a temperature jump from 25°C to −80°C results in a three- to fourfold increase in both viscosity and pressure drop for a given combination of column format and linear velocity of the eluent.

However, decreasing the column temperature has additional ramifications that include changes in retention, selectivity, and efficiency. As a general rule, retention and enantioselectivity increase as the column temperature is lowered. Enthalpic and entropic contribution to retention and selectivity are described using expressions (Eq. 3) and (Eq. 4), respectively, where R is the gas constant, T the absolute temperature of the column, ϕ is the column phase ratio, α the enantioselectivity, ΔH and ΔS are the enthalpy and entropy changes for the solute-selector interaction and δΔH and δΔS are the differences in enthalpy and entropy changes between the two enantiomers.

$$\ln k' = -\Delta H/RT + \Delta S/R + \ln\phi \quad (3)$$

$$\ln\alpha = -\delta\Delta H/RT + \delta\Delta S/R \quad (4)$$

The interaction of enantiomeric solutes with the stationary phase usually features negative ΔH and ΔS, *i.e.*, the process is exothermic and accompanied by entropy loss, as expected for a simple bimolecular association process; under these conditions, retention decreases with increasing temperature. The linear inverse relationship between lnα and temperature (Eq. 4) is explained by entropy effects: The diastereomeric solute-selector combination having the greatest degree of simultaneous bonding gives the more stable (negative δΔH term) complex but is also expected to lose the most degrees of freedom (positive δΔS). Hence, temperature reduction decreases the importance of the adverse entropy term and increases enantioselectivity [25].

At sub-ambient temperatures column efficiency degrades rapidly, mainly because adsorption/desorption events, mass transport between phases and diffusion inside the stationary phase are slowed down. Although mass transfer problems erode column efficiency at low temperatures, the increase in the magnitude of α more than offsets the loss in column performance.

When sufficient retention is obtained at a given low temperature (*i.e.*, temperature and eluent composition are fixed parameters), analysis time can be reduced by controlling the flow rate and/or the column format. Available column formats range from standard 25-cm length packed with 5-μm particles (25,5 format) to short 5-cm columns packed with 3-μm particles (5,3 format).

Several literature examples show that in practice cryo-HPLC with analysis times in the range of few minutes is

conveniently carried out using either classical 25,5, 10,5 or 5,3 column formats.

One example of enantioselective HPLC at extremely low temperature is represented by the separation of the rapidly interconverting enantiomers of naphthyl sulfone **13**. The experimental setup consisted of a 250 × 4.6 column packed with 5-μm Whelk-O1 CSP, a mobile phase based on methylene chloride/methanol (98:2) delivered at 2.0 mL/min, and a 1 m long connecting tube used as mobile phase precooler [26]. Room temperature HPLC of **13** gave a single sharp peak that remained sharp down to −60°C, indicating fast interconversion on the time scale of the separation (about 2 min). Further cooling of the column at 208 K resulted in peak broadening and splitting. At −70°C, the two peaks were separated but on-column enantiomerization was still occurring at this temperature. On the other hand, the dynamic process was slow on the HPLC time scale at −80°C, where the two peaks are baseline resolved. A similar dynamic behavior was observed for the corresponding 1,5-disulfone, where the two stereogenic Ar-SO_2 axes generate a *meso* and two enantiomerically related chiral conformers. Interconversion between the three species was frozen on the HPLC time scale at −80°C, giving two equally intense peaks corresponding to the chiral species, along with a major peak corresponding to the *meso* form [27].

An additional example of cryogenic DHPLC of fast interconverting species is given by the secondary phosphine oxide **14** [28]. The two stereogenic elements present in **14** (the configurationally stable P atom and the stereolabile P-Ar axis) generate two configurational enantiomers, each of which can generate two distinct diastereomers that rapidly interconvert at room temperature by rotation around the P-Ar axis.

The residual enantiomers of **14** at room temperature were resolved by HPLC on 10 × 0.4-cm column packed with 5-μm (*R,R*)-DACH DNB CSP. While room temperature HPLC separation of the stereostable enantiomers of **14** was straightforward, the separation of the rotamers of each enantiomer was expected to be quite difficult, given the low interconversion energy barrier $\Delta G^{\ddagger}$ = 14.8 kcal/mol measured by DNMR. The use of a short column had in this case a positive effect as it shortened the analysis time and reduced the operating backpressure due to increased eluent viscosity at the required low temperature. Indeed, as the column temperature was lowered to about −50°C the two peaks showed considerable broadening due to on-column exchange. An interconverting region preceding the peaks appeared between −60°C and −70°C, consistent with a reduced rotamer interconversion rate on the separation time scale. Further cooling of the column to −83°C prevented on-column exchange, and allowed a complete separation of the four species,

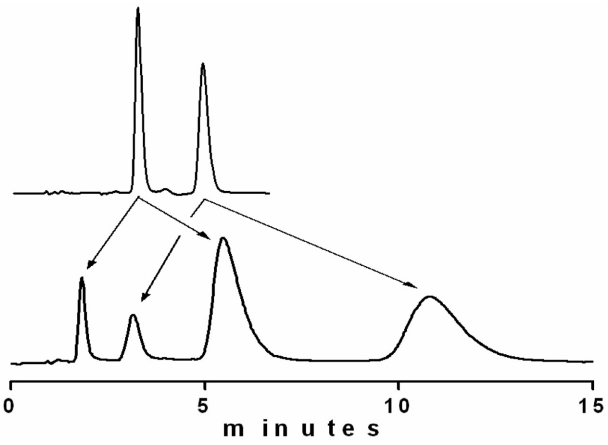

Figure 8. HPLC of secondary phosphine oxide **14** on a 100 × 4-mm column packed with 5-μm DACH-DNB CSP. Eluent: CH_2Cl_2/MeOH (99:1), flow rate 1.5 mL/min. Temperatures 25°C (top) and −83°C (bottom).

which no longer interconverted during their passage through the column, as evident from the absence of any plateau between the resharpened peaks (Fig. 8). The same low-temperature experiment performed on the individual enantiomers (isolated by HPLC at ambient temperature) yielded two peaks for each enantiomer, corresponding to the diastereomeric rotamers.

Computer simulations of the chromatographic profiles carried out on the individual enantiomers of **14** yielded the isomerization barriers $\Delta G^{\ddagger}$ = 14.8 kcal/mol for the enantiomer eluted first at room temperature and $\Delta G^{\ddagger}$ = 15.1 kcal/mol for the remaining species. In the first case the value was identical to that obtained by DNMR, whereas in the second case the value was higher due to a small deactivating effect of the stationary phase. The relative amounts of the two diastereomeric conformers and the energy barriers separating them, as measured by DNMR and DHPLC methods, are in good agreement, in spite of the different temperature ranges at which the experiments have been performed.

A short column packed with 3.5 μm silica-bonded chiral phase was used for the resolution of the stereolabile enantiomers of tertiary amide **15**, separation that required a column temperature as low as −70°C [29]. As the column temperature was raised in 5°C increments, the two chromatographic peaks gradually broadened and a plateau appeared between the resolved peaks, indicating accelerated on-column interconversion of the two enantiomers. At −40°C a complete peak coalescence due to fast exchange was observed. Computer simulation of the exchange-broadened elution profiles at −70°C (Fig. 9, bottom traces), gave the averaged apparent rate constant for the enantiomer interconversion (C-CO bond rotation) and the corresponding energy barrier $\Delta G^{\ddagger}$ = 14.3 kcal/

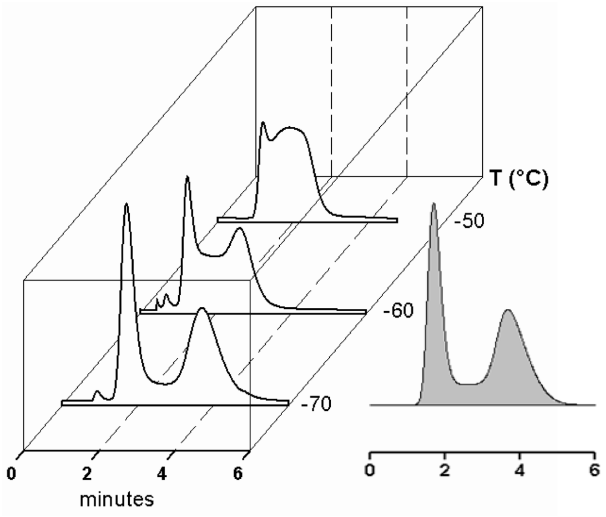

Figure 9. DHPLC traces of tertiary amide **15** on a 50 × 4 mm column packed with 3.5-μm DACH-DNB CSP. Eluent: CH_2Cl_2/MeOH (98:2), flow rate 2.0 mL/min. Simulated plot on the right at −70°C.

mol, in close agreement with the DNMR value of 13.5 kcal/mol extrapolated at the same temperature.

5 Conclusions

Enantioselective DHPLC is now a well-established technique for the investigation of the stereochemical stability of chiral compounds. The DHPLC experiment coupled with computer simulation of the experimental profiles can yield valuable information on interconverting species (rate constants for the dynamic process, conformer distribution for non-enantiomeric interconverting species, chiroptical data with circular dichroism or polarimetric detectors) and requires only small amounts of racemic material. Prerequisite for a successful experiment is the separability of the species on proper stationary phases, with the separation rate matching the rate of the on-column interconversion. The technique is suited to study on-column interconversions that exchange analyte stereochemistry and that occurs either through single bond rotations or by bond breaking-formation processes [30 – 36].

The experimental parameters that can be varied to reach this situation are the column temperature, the eluent flow rate and the column format. Column temperature has the greatest effect on the relative rates of the two processes (separation and interconversion) as it can be set from 80°C down to −80°C: the molecular internal motions that can be studied in this range of temperatures have energy barriers spanning from 14 to 25 kcal/mol. For low temperature runs, a solvent of low viscosity should be chosen to avoid excessive deterioration of the column kinetic performances and to reduce operational pressure. The eluent flow rate can also be adjusted to fine-tune the separation and interconversion rates. The linear velocity of the eluent can be varied by approximately an order of magnitude, so the impact on the separation rate is much smaller than the effect of temperature on the interconversion rate. Enantioseparations of stereolabile compounds can be carried out on the time scale of few minutes at cryogenic temperatures using either standard columns (25,5 format) or short columns packed with small particle CSPs (5,3 format) used in conjunction with high eluent flow rates.

The ease with which key experimental parameters are changed and controlled (temperature, eluents, eluent flow rate) makes DHPLC the ideal approach to extract kinetic data for solute isomerizations that occur with energy barriers between 14 and 25 kcal/mol. Considering the time scale of the experiments, DHPLC is complementary to DNMR in that it can reveal the existence of exchange phenomena, and yield the corresponding exchange rates for molecular systems with slow internal motions. In addition, the physical separation of the interconverting species can be fruitfully exploited to extend their characterization by chiroptical methods. Yet, when the DHPLC and DNMR techniques can be used in combination, the quality and quantity of information achievable on the dynamic system are greatly extended compared to each of the two techniques alone.

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