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Highlights

- An application of dynamic HPLC to the study of stereolabile chiral benzodiazepines.
- Dynamic HPLC on CSPs using water rich media is described.
- Energy barriers for enantiomer interconversion are obtained by computer simulation of plateau deformed HPLC plots.

Chromatographic separation of the interconverting enantiomers of imidazo- and triazole-fused benzodiazepines

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Abstract

The toolbox of medicinal chemists includes the 1,4-benzodiazepine scaffold as a "privileged scaffold" in drug discovery. Several biologically active small molecules containing a 1,4-benzodiazepine scaffold have been approved by the FDA for the treatment of various diseases, with most of them being used for their psychotropic effects. The therapeutic potential of 1,4-benzodiazepines has stimulated the interest of synthetic chemists in developing new synthetic strategies to a range of substituted analogues for biological evaluation. A structural variation of the classical benzodiazepine skeleton is observed e.g. in alprazolam, midazolam, and related benzodiazepines, which contain a 1,2,4-triazole or an imidazole ring fused to the benzodiazepine core. Irrespective of the presence of the fused heterocyclic ring, the seven-membered diazepine ring is far from planar, and its shape resembles a twist chair. Then, the unsymmetrical substitution pattern around the seven membered cycle renders these molecules chiral, as they lack any reflection-type symmetry element. However, chirality of this molecules is labile at room temperature, because a simple ring flipping process converts one enantiomer into the other, and 1,4-benzodiazepines exist as a mixture of rapidly interconverting conformational enantiomers in solution at or near room temperature. Physical separation of the interconverting enantiomers of diazepam and of other related 1,4-benzodiazepin-2-ones can be accomplished by low temperature HPLC on chiral stationary phases (CSPs). If the HPLC column is cooled down to temperatures where the interconversion rate is sufficiently low, compared to the chromatographic separation rate, distinct separated peaks can be observed, provided the CSP is sufficiently enantioselctive. The apparent rate constants for the on-column enantiomerization and the corresponding free energy activation barriers were obtained by simulation of exchange-deformed HPLC profiles using a computer program based on the stochastic model. Here we report on the dynamic HPLC investigations carried out on a set of fused imidazo and triazolo-benzodiazepines (alprazolam, midazolam, triazolam and estazolam) The experimental dynamic chromatograms and the corresponding interconversion barriers reported in this paper show that the third fused heterocyclic ring increase the energy barrier by 2 kcal/mol.

Keywords

Conformational enantiomers
Fused tricyclic benzodiazepines
HPLC on chiral stationary phases
Low temperature HPLC
Dynamic chromatography
Enantiomerization energy barriers

1. Introduction

Benzodiazepines, are a well-known class of pharmacologically active heterocyclic compounds with sedative, hypnotics, anxiolytic, and anticonvulsant properties [1]. the conformational chirality of the 1,4-benzodiazepine core of these molecules has been the focus of recent medicinal chemistry investigations that have pointed out its crucial role in determining their bioactivity: the interaction with both human serum albumin HSA and $GABA_A$ receptors is strongly stereodependent with a preference for the (M)-chiral conformation (see Fig. 2) as revealed by HSA induced circular dichroism for fast interconverting species like diazepam [2] and by direct $GABA_A$ receptors affinity measurements for single enantiomers of slowly interconverting diazepam derivatives [3] [4]. Recently, the 1,4-benzodiazepine structural motif has received specific attention in the field of epigenetics, with the discovery of a class of molecules acting as inhibitors of the interaction between Bromo and Extra-Terminal (BET) bromodomain proteins and their acetylated histone substrates [5]. Recent review reports have highlighted the central role that conformational chirality plays in contemporary medicinal chemistry [6], [7], [8], [9], [10]. Two principal classes of conformational enantiomers can be distinguished according to the energy barrier separating the two interconverting species. One class comprises those species featuring enantiomerization [11] energy barriers larger than 27 kcal/mol (atropisomers), featuring half-life time of the individual enantiomers covering the range from days to months or years at room temperature. The second class comprises stereochemically unstable species, with energy barriers smaller than 20 kcal/mol and featuring half-life times of the individual enantiomers in the range from minutes to fraction of seconds at room temperature. Chirality of drugs or drug-like molecules of the first class is relevant from the pharmaceutical point of view, stereochemical studies of bioactive molecules of the second class have only a pharmacological relevance that is related to their dynamic interaction with biological targets and can potentially show the intriguing phenomenon of enantiomeric selection or enrichment at the interaction site [12], [13].

The conformational preference of diazepam has been studied in detail, the three dimensional non-planar structure of the seven membered 1,4-diazepine ring being the principal focus, accompanied by measurements of the energy barrier for the interconversion of the two conformational enantiomers. Dynamic ^{1}H NMR has been the method of choice to measure the energy for interconversion, the method exploiting the non-equivalence of the two C_3 methylene hydrogens of diazepam that are diastereotopic under slow exchange conditions (at room temperature) and appear as a couple of doublets: at higher temperatures, the two doublets collapse and eventually coalesce at the coalescence temperature (T_c), yielding the rate constant at Tc and the associated energy barrier $\Delta G^{\neq}$ = 17.3 and 17.6 kcal/mol [14] (at 60 MHz in deuteropyridine and hexachlorobutadiene at T_c = 364 and 363 K) or $\Delta G^{\neq}$ = 18.0 ± 0.2 kcal/mol [15], [16], [17] (400 MHz in d_6-DMSO at T_c = 391 K). These barriers translate into a half life time of the order of 1-2.45 s at room temperature for the individual enantiomers of diazepam at room temperature. structurally related 1,4-benzodiazepin-2-ones (fig. 1), have comparable enantiomerization energy barriers. These are raised up to $\Delta G^{\neq}$ = 19.5 ± 0.2 kcal/mol when bulky substituents are located on N1.

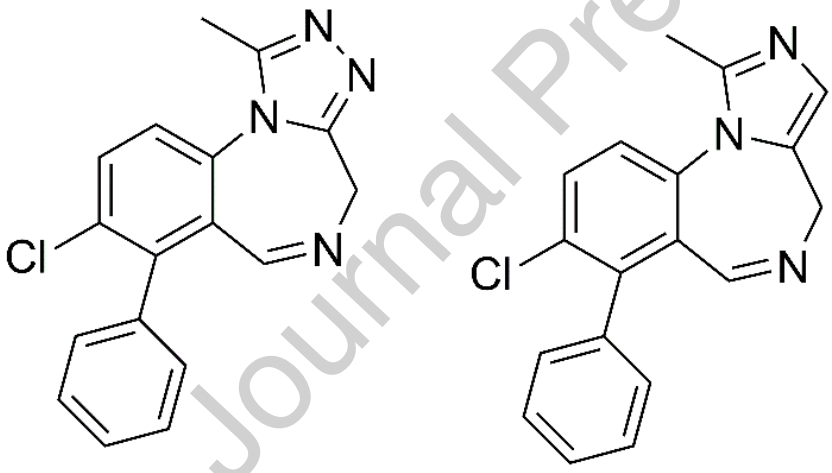

Fig. 1. Structure of [1,2,4]triazolo[4,3-a][1,4]benzodiazepine (left) and 4H-imidazo-[1,5-a[1,4]benzodiazepine (right) whose barriers separating the two conformational enantiomers are studied in this work.

Enantioselective HPLC separation of conformational enantiomers is an established technique to study this kind of conformers by off-column and on-column methodologies. Dynamic HPLC (DHPLC) can be employed to determine the enantiomerization barriers of those conformational enantiomers that interconverts on the same-time scale of the separation process. Typical chromatographic profiles with deformations of the peaks and

eventually the presence of a plateau between them, can be observed as the column temperature is increased or decreased, indicating an on-column interconversion process. Thus the chromatographic column is exploited as a reactor and the kinetic parameters of the stereochemical inversion process can be extrapolated by the shape of the peaks using different methods as for example the theoretical plate model, the stochastic model or the unified equation. In particular, in the stochastic model the chromatographic profile of two interconverting conformers results from the combination of the distribution functions of the non-interconverted species and the probability of density functions of the interconverted species[18]. The typical range of energetic barriers of interconversion that can be investigated by DHPLC goes from 25 kcal/mol to 14 kcal/mol, higher/lower barriers would requires extremely high/low temperatures, difficult to obtain for instrumental limits.

HPLC physical separation of the conformational enantiomers is thus possible at or near room temperature only for those benzodiazepine derivatives carrying bulky groups on N1 (^{i}Pr or ^{t}Bu). For diazepam ($\Delta G^{\neq}$ = 18.0 ± 0.2 kcal/mol) the physical separation of the conformational enantiomers was possible only at temperatures equal or lower than T = −15 °C .

This study presents the results obtained by variable sub-ambient temperature enantioselective HPLC of alprazolam, midazolam, triazolam and estazolam (fig. 2), carried out on a several of CSP (Chiralpak IA, Chiralpak AD, Chiralpak HAS) using a range of mobile phases ranging from organic solvent, to hydro-organic, to buffered aqueous. The dynamic exchanged experimental HPLC profiles were computer simulated and yielded the apparent rate constants and the associated free energy barriers for the on-column enantiomerization process. For the enantiomerization of estazolam the free energy barriers were also measured in solution by dynamic NMR in CD_3OD.

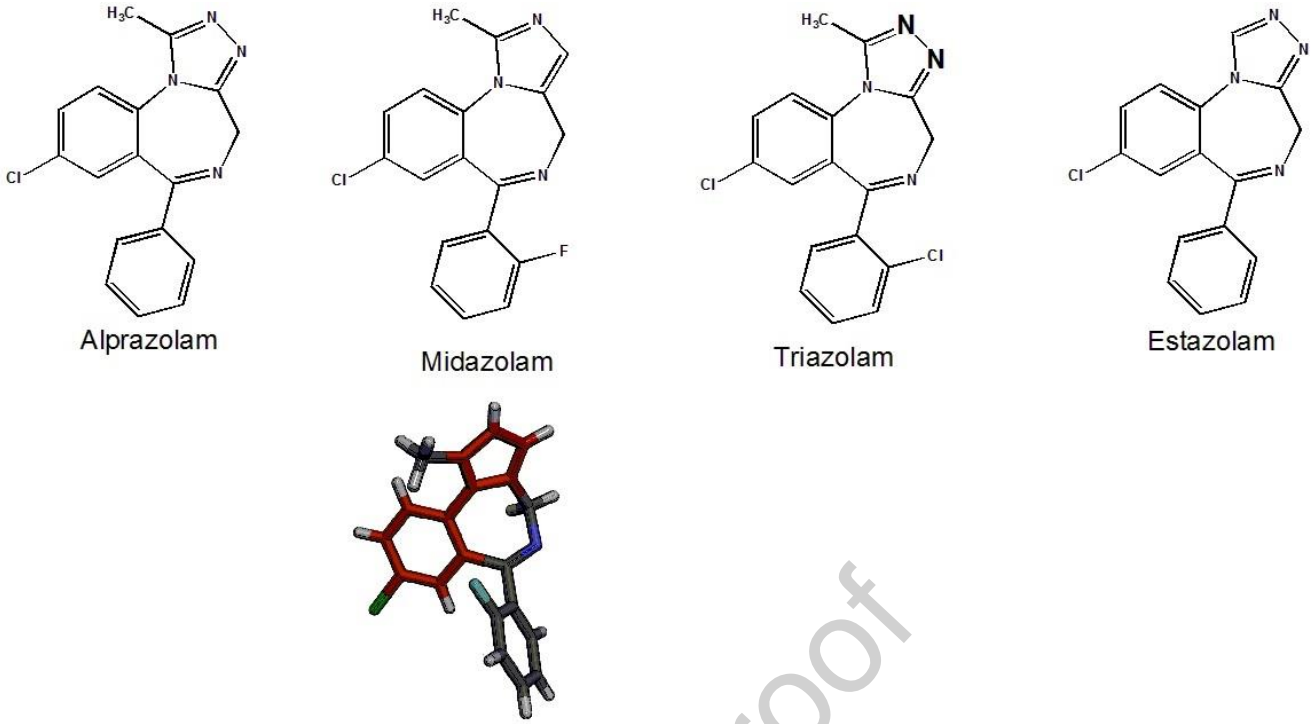

Fig. 2. Structure of 1,4-benzodiazepin-2-ones investigated in this work (top row) and of the (*R*,*R*) Whelk-O1 chiral stationary phase (bottom row, left). Polytube models of the one conformational enantiomer of midazolam is shown at the bottom. Crystallographic data for midazolam are reported in literature [19], [20].

2. Experimental

2.1. Materials

Samples of alprazolam, midazolam, tetrazepam and estazolam were kindly provided by F.I.S. – Fabbrica Italiana Sintetici S.p.A., Vicenza (Italy). HPLC-grade *n*-hexane, methanol, dichloromethane, were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Instrumentation and chromatographic methods

A Jasco PU-980 Intelligent HPLC pump equipped with a Rheodyne model 7725i 20 µl injector and coupled with a Jasco UV-975 UV/VIS detector was used for the low temperature HPLC runs. Data were collected using the Borwin software (Jasco, Europe). The Chiralpak IA, Chiralpak AD and Chiralpak HAS, 5 µm particle size, chromatographic column (250 mm × 4.6 mm I.D.) were purchased from Chiral technology Europe. HPLC runs were performed at flow rates of 1.0 ml min^{-1} and monitored by UV detection at 265 or

280 nm. Sub-ambient temperature chromatography was performed placing the chiral column in a homemade temperature control module (TCM); cooling of the TCM was provided by the expansion of liquid C02, controlled by a solenoid valve. Column temperature was maintained within ± 0.2 °C by means of an electronic controller. Samples were dissolved in the eluent and filtered through 0.4 μm membrane before injection. Simulations of variable-temperature experimental chromatograms were performed by Auto DHPLC y2k (Auto Dynamic HPLC) [21] based on the stochastic model. ^{1}H NMR spectra were recorded on a Bruker AC 300 P spectrometer, operating at 300.13 MHz for ^{1}H, equipped with a sample tube thermostat apparatus. Signals were referenced with respect to TMS (δ = 0.00 ppm)

3. Results and discussion

3.1. Dynamic chromatography at variable temperature

A preliminary screening of different chiral stationary phases for their ability to separate the conformational enantiomers of midazolam, was carried out under normal phase elution conditions and setting the column temperature at −25 °C. The best results in terms of enantioselectivity and overall resolution were obtained using a 250 mm × 4.6 mm column packed with the Chiralpak IA CSP, and using a mixture of n-hexane/CH_2Cl_2/MeOH 55/44/1 (v/v/v) as eluent delivered at a flow rate of 1.0 ml min^{-1}. When the HPLC analysis of midazolam on the Chiralpak IA CSP was carried out in a wider column temperature range, the usual phenomena of temperature dependent peak coalescence–decoalescence were observed in the temperature range spanning from 5 to 35 °C C (Fig. 3).

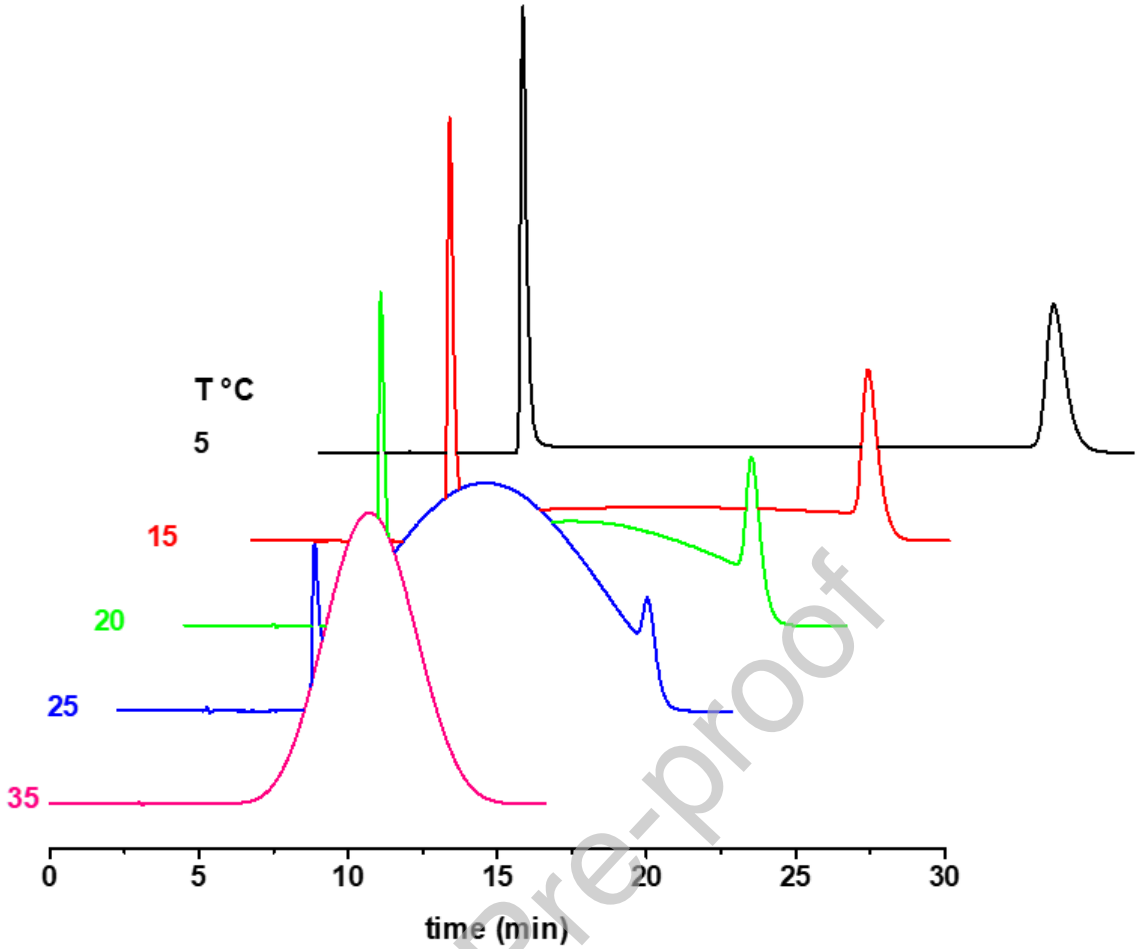

Fig. 3. Dynamic HPLC of midazolam. Column, Chiralpak IA 250 mm × 4.6 mm (5 μm); mobile phase, hexane/dichloromethane/methanol (55/44/1, v/v/v); flow rate, 1.0 ml min^{-1}; detection UV at 280 nm.

At 35 °C a single, unsplit broad peak was observed that eventually turn into two distinct peaks with an extensive plateau between them, indicative of on-column interconversion of the two enantiomers. Upon further cooling of the column down to 5 °C the plateau between the two peaks gradually diminished in intensity as a result of a lowered interconversion rate of the enantiomers compared to the separation rate. At 5 °C the on-column enantiomerization was completely frozen, and two equally intense well resolved peaks were observed for the two conformational enantiomers.

The dynamic HPLC traces recorded for alprazolam (Fig. 4) in the temperature range between -7°C and 20°C resembled those observed for midazolam, the only difference being the onset (at lower temperature) of complete decoalescence, due to the lower enantiomerization barrier.

Triazolam showed a similar behavior, when the column temperature was varied within the range between 10 °C and −25 °C: a single, averaged broad peak was observed at 20°C, then peak deformations and the presence of plateau due to intermediate exchange were observed between −10 and −20 °C, followed at lower temperatures by complete peak decoalescence (see fig. S1 in supporting information).

Estazolam behaved differently under otherwise identical experimental conditions (Fig. 5 left). A single broad peak was observed at -70°C column temperature using CH_2Cl_2/hexane/MeOH 67/32/1 (v/v/v) as eluent. Lowering the temperature of the column to -75°C generated an abnormally broad peak with a shoulder preceding the fronting of the peak, suggestive of the presence of enantioselectivity but a too fast on-column interconversion. At the same temperature of – 75°C and using the more polar eluent CH_2Cl_2/hexane/MeOH 75/24/1 the peak was anticipated but remained broad and unsplit, again with a shoulder on the first half. We then decided to use a shorter column packed with the same CSP (50*4.6 mm i.d.), with the intent to shorten the residence time of the enantiomers inside the column, and thus favoring their separation (see fig 5. right panel).

At column temperature of – 54°C a single broad peak was present. However, on the shorter column, peaks decoalescence was observed already at a column temperature of -66 °C and the plateau between the two peaks was further reduced on lowering the temperature down to – 70°C.

Dynamic ^{1}H NMR of estazolam dissolved in CD_3OD was used to confirm these findings. These experiments allowed us to study the enantiomer interconversion in a different temperature range in the absence of potentially perturbing effects of the CSP, and to study the effects, if any, of the solvent nature (protic/aprotic) on the energy barrier. The room temperature ^{1}H NMR spectrum (300 MHz) in CD_3OD of estazolam showed two distinct doublets close to 4.40 and 5.40 ppm, for the two C3 methylene protons (see supporting material fig. S6) that are diastereotopic at room temperature. Note that the ring inversion process is slow on the NMR time scale. Raising the temperature between 10 and 57 °C, enantiomer interconversion became increasingly faster, generating a broad peak that eventually coalescences and resharpens at the highest temperature explored. At the coalescence temperatures T_c = 44.3 °C in CD_3OD and the rate constant for the enantiomerization was calculated using Eq. (1):

(1) kc=2.22*[Δ*v*2+6(J_{AB})2]1/2

where Δ*v* (Hz) is the frequency difference between the two signals in the absence of exchange and J_{AB} is the coupling constant between A and B also in absence of exchange. These calculations yielded an energy barrier of interconversion 14.54 ± 0.1 kcal/mol. As expected, solvent and temperature have negligible effects on the enantiomerization

barrier, as is usually observed for thermal enantiomerization processes occurring by conformational changes.

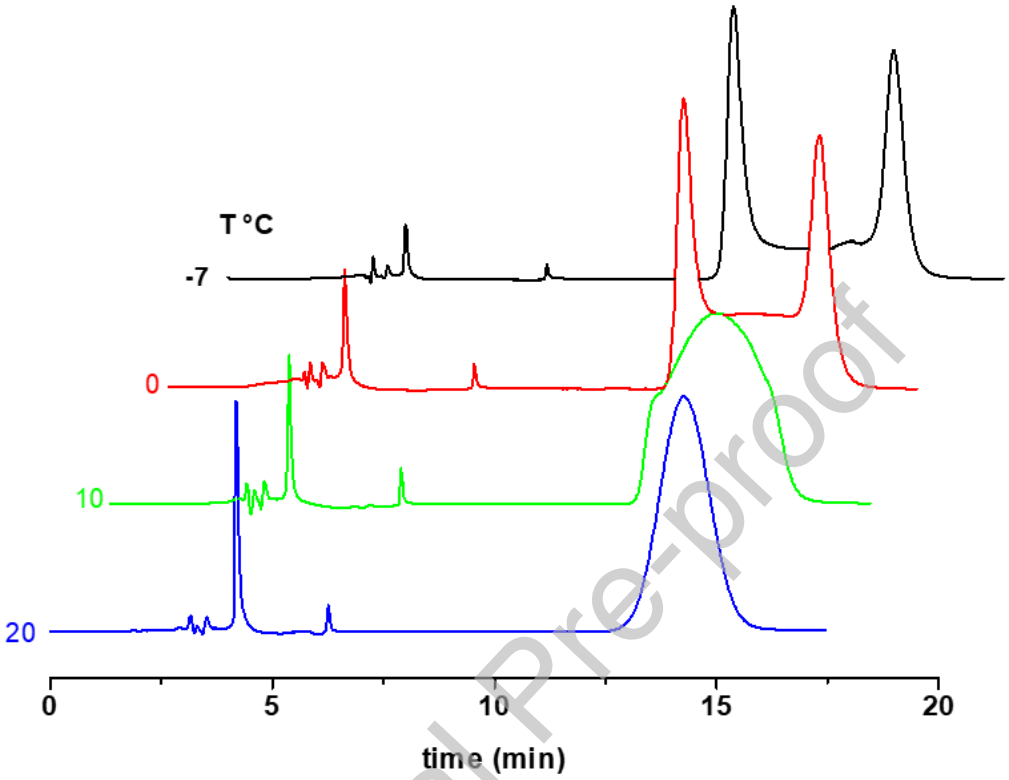

Fig.4. Dynamic HPLC of alprazolam. Column, Chiralpak IA 250 mm × 4.6 mm (5 μm); mobile phase, hexane/dichloromethane/methanol (55/44/1, v/v/v); flow rate, 1.0 ml min^{-1}; detection UV at 280 nm.

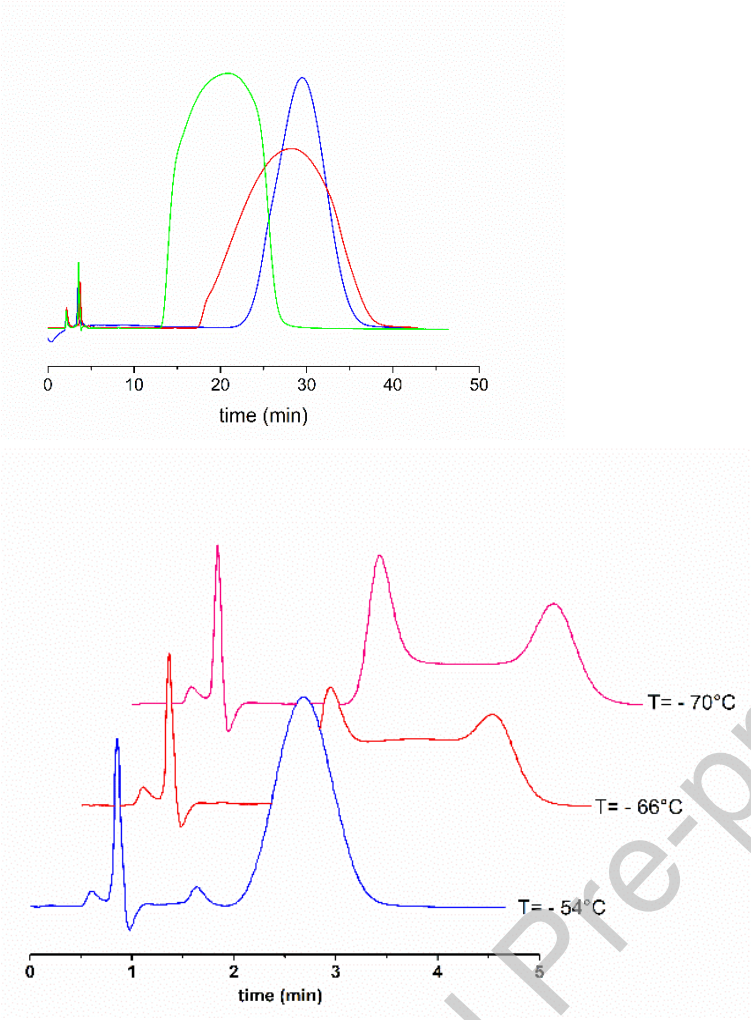

Fig.5. Left: Dynamic HPLC of estazolam. Columns Chiralpak IA 250 mm × 4.6 mm (5 µm) flow rate 1.0 ml min^{-1};. mobile phases, CH_2Cl_2/hexane//methanol (67/32/1, v/v/v), blue plot; T_{col} = -70°C; CH_2Cl_2/hexane//methanol (67/32/1, v/v/v) red plot; T_{col} = -75°C; CH_2Cl_2/hexane//methanol (75/24/1, v/v/v) T_{col} = -75°C, green plot. detection UV at 280 nm. Right: 50 mm × 4.6 mm (5 µm), mobile phase CH_2Cl_2/hexane//methanol (75/24/1, v/v/v) T_{col} = -54°C, -66°C, -70°C.

3.2 Dynamic chromatography with water rich eluent

The great versatility of Chiralpak IA in terms of eluent composition that the CSP tolerates, allowed us to explore the dynamic HPLC experiments in water rich media. Thus, we used a mobile phase made of acetonitrile/H_2O 70/30 and found that enantioselectivity was unaffected by the large amount of water in the eluent. Dynamic HPLC in the form of variable temperature was performed in the temperature range of 5-15 °C, yielding dynamically deformed plots with on-column interconversion nearly frozen at Tcol=5°C (figure S7). Next we explored the enantioresolution of midazolam on a column containing

immobilized human serum albumin on silica a CSP. In this case, the eluent was phosphate buffered water containing a small amount (10%) of 1-propanol as organic modifier. The eluent pH was adjusted to the values of pH = 5.7, 6.0, 7.0. In all cases we obtained good enantioselectivity, although peaks were strongly tailed, especially the second eluted one. In this case, dynamic HPLC traces with a plateau between the resolved peaks, were observed at T_{col} ranging from 10 to 20°C (see fig. S3, S4, S5 supporting information).

3.3 Dynamic chromatography and energy barriers

We determined the enantiomerization barriers of alprazolam, midazolam, triazolam and estazolam by dynamic HPLC on different CSPs. Interconversion profiles featuring plateau formation and peak broadening were generated by changing the column temperature and then simulated by the lab-made computer program Auto DHPLC y2k based on the stochastic model. Dynamic deformations of the experimental chromatograms were exploited to extract kinetic data for the enantiomerization process occurring during chromatography [22], [23], [24], [25], [26], [27], [28], [29], [30].

A selection of experimental (black traces) and simulated (red traces) chromatographic profiles used to extract kinetic data for the on-column interconversions can be found in supporting information fig. S2. A range of column temperatures between 15°C and -25°C was chosen for alprazolam, midazolam and triazolam: at these temperatures the three benzodiazepines gave dynamic plots with a visible interconversion plateau. On the other hand, Estazolam gave a dynamic plot with split peaks and a visible plateau between them only at lower temperatures (-70°C). The observed HPLC behavior of estazolam under otherwise identical conditions is clearly indicative of a lower enantiomerization barrier compared to the other benzodiazepines examined here.

Quantitative data for the on-column enantiomerizations were obtained by computer simulation of the exchange-modified HPLC plots, using a mathematical model based on the stochastic approach [26], [27]. The approach starts with the generation of an experimental plot with a sizeable plateau between the two resolved peaks. The simulation, in turn, uses tentative kinetic constants for the process in the mobile ($k_{m)}$ and stationary phases (k_s) as input parameters. The rate constant for the enantiomerization in the mobile and in the stationary phases and are in principle different because the stationary phase can exert a perturbing (retarding or activating) effect on the reversible process. Once the difference between the experimental and computed HPLC plots has reached the lowest possible value, the computational procedure returns the apparent rate constants for the

interconversion of the two enantiomers, k12a and k21a (the subscript numbers refer to the forward process converting the first eluted enantiomer into the second, k12a, and the backward process k21a). Each of these two rate constants is a weighted average value for the interconversion occurring in the mobile ad in the stationary phases, the average being computed according to the residence time of the enantiomers in the two phases. The two rate constants are different, in principle, because the more retained enantiomer interacts more strongly with the stationary phase, and in the adsorbed state, the ring flip process is more hindered compared to the less retained enantiomer, that forms a less structured complex with the CSP. The commonly observed retarding effect of the CSP on interconversion, causes the process leading from the second eluted to first eluted enantiomer to be faster, and this is what we found in this work, i.e. k21a<k12a.

Energy barriers were calculated from the obtained rate constants assuming a unitary transmission coefficient and the classical Eyring equation [25], [28]. Kinetic data and energy barriers are gathered in Table 1.

Table 1. Enantiomerization barriers obtained by DHPLC and computer simulation.

Compound	T (°C)	K^{app}_{1} (min^{-1})	K^{app}_{-1} (min^{-1})	$\Delta G^{\#\,app}_{1}$ (kcal/mol)	$\Delta G^{\#\,app}_{-1}$ (kcal/mol)
Midazolam Chiralpak AD Hex/IPA 70/30	15	0.25	0.047	20.07	21.02
	20	0.36	0.07_5	20.19	21.11
Midazolam Chiralpak IA Hex/CH_2Cl_2/MeOH 55/44/1	15	0.18	0.06	20.17	20.83
Midazolam Chiralpak IA ACN/H_2O 70/30	15	0.15	0.10	20.26	20.49
Midazolam Chiralpak HAS Phosphate buffer 0.1M pH=6.2/10%IPA	15	0.34	0.15	19.90	20.30
Triazolam Chiralpak IA Hex/CH_2Cl_2/MeOH 55/44/1	-25	0.11	0.06	17.49	17.77
Alprazolam Chiralpak IA Hex/CH_2Cl_2/MeOH 55/44/1	0	0.06	0.05	19.66	19.79
Estazolam Chiralpak IA Hex/CH_2Cl_2/MeOH 25/74/1	-70	0.27	0.16	13.92	14.14

T_c: column temperature, within ±0.2 °C. Energy barriers $\Delta G^{\neq}$ within ±0.02 kcal/mol.

When observing the energy barriers listed in Table 1, the lower values ΔG12≠ are the ones related to the conversion of the first eluted enantiomer into the second one, and are those closer to values recorded by other techniques in free solution (e.g. by D-NMR) in the absence of the usually retarding effect of the stationary phase. A large difference is observed between the energy barriers of alprazolam, midazolam, triazolam and that of estazolam: clearly, the presence of a methyl group no N1 of the diazepine ring raises the barriers by generating steric compression with the peri hydrogen of the benzene ring in the enantiomerization transition state.

Comparison of the enantiomerization energy barriers of benzodiazepines devoid of the third fused heterocyclic ring, our data show that the fused ring increases the barriers by about 2 kcal/mol, thus shifting to higher temperatures the onset dynamic HPLC profiles due to fast on-column interconversion. However, the observed barriers are still too low to permit a facile isolation of the individual enantiomers of fused imidazo and triazolo-benzodiazepines at room temperature.

4. Conclusions

Dynamic HPLC on chiral stationary phases of the conformational enantiomers of imidazo and triazolo-fused 1,4-benzodiazepin-2-ones has been used to study in detail the stereochemistry of this class of interesting molecules. The technique allowed us to perform the physical separation of the individual enantiomers and the measurement of the interconversion barriers by computer simulation of exchange-deformed HPLC plot. The enantiomerization barriers found for alprazolam, midazolam and triazolam are 2 kcal larger than those of similar 1,4-benzodiazepines lacking the third fused ring. Estazolam, devoid of the methyl group on N1, shows a considerably lower barrier due to minor steric compression in the transition state for ring flipping.

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Appendix A. Supplementary data

The following are the supplementary data to this article:

Authors credit Statement

Conceptualization, R.S., R.F., C.V.; methodology, A.I. and A.C.; software, M.P.; validation, R.S., C.V.; formal analysis, R.S., C.V.; investigation, R.S., C.V., A.C; resources, C.V.; data curation, M.P., A. I.; writing-original draft preparation, R.S, R.F, and C.V.; writing-review and editing, R.F. and C.V.; visualization, M.P.; supervision, R.F. and C.V.; project administration, C.V.; funding acquisition, C.V. All authors have read and agreed to the published version of the manuscript.

Declaration

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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