Low Temperature Dynamic Chromatography for the Separation of the Interconverting Conformational Enantiomers of the Benzodiazepines Clonazolam, Flubromazolam, Diclazepam and Flurazepam

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Abstract: Benzodiazepines (BZDs) are an important class of psychoactive drugs with hypnotic-sedative, myorelaxant, anxiolytic and anticonvulsant properties due to interaction with the GABA\(_\text{A}\) receptor in the central nervous system of mammals. BZDs are interesting both in clinical and forensic toxicology for their pharmacological characteristics and potential of abuse. The presence of a non-planar diazepine ring generates chiral conformational stereoisomers, even in the absence of stereogenic centers. A conformational enrichment of BZD at the binding sites has been reported in the literature, thus making interesting a stereodynamic screening of a wide range of BZDs. Herein, we report the investigation of three stereolabile 1,4-benzodiazepine included in the class of “designer benzodiazepines” (e.g., diclazepam, a chloro-derivative of diazepam, and two triazolo-benzodiazepines, flubromazolam and clonazolam) and a commercially available BZD known as flurazepam, in order to study the kinetic of the “ring-flip” process that allows two conformational enantiomers to interconvert at high rate at room temperature. A combination of low temperature enantioselective dynamic chromatography on chiral stationary phase and computer simulations of the experimental chromatograms allowed us to measure activation energies of enantiomerization (\(\Delta G^\ddagger\)) lower than 18.5 kcal/mol. The differences between compounds have been correlated to the pattern of substitutions on the 1,4-benzodiazepinic core.

Keywords: conformational enantiomers; chirality; chiral stationary phases; benzodiazepines; low temperature dynamic chromatography; enantiomerization

1. Introduction

Chirality is a common property in pharmaceutically active compounds [1], and the investigation of the stereoselectivity in the interaction of drugs with their biological target is a fundamental step in drug design. The constant increase in the request for enantiomerically pure active compounds in the pharmaceutical market [2] urges for the improvement of stereoselective analytical and preparative methodologies and stereochemo stability studies [3,4]. Benzodiazepines are one of the most prescribed drugs in the world for their sedative, anxiolytic and anticonvulsant properties [5,6] but are also known for their many adverse effects and potential of abuse [7–9].

The seven membered ring of the 1,4-benzodiazepine core lacks planarity and adopts a boat conformation; the chirality that derives from it has been the focus of many recent investigations in the last few decades [10–13]. The two possible boat-shaped conformations are non-superimposable mirror images, so the compound is chiral despite the fact that it lacks a chiral center.

Conformational enantiomers of 1,4 benzodiazepines interconvert via a ring inversion mechanism with an energetic barrier calculated for diazepam of about 17.6 kcal/mol [12].
The interest in investigating the chirality of BZD results from the evidence of a stereoselective association with both human serum albumin HSA and GABAa receptors with the (M)-chiral conformation as the preferential one in the interaction, as revealed by has-induced circular dichroism measured for fast interconverting species such as diazepam [14,15] and by the direct measurements of GABAa receptors’ affinity of single enantiomers of slowly interconverting derivatives [16,17]. Recently, many reviews pointed out the role of conformational chirality in modern medicinal chemistry [18–25]. Basically, a classification of conformationally chiral compounds, also known as atropisomers, can be made based on the energetic barriers of interconversion ($\Delta G^\text{ǂ}$) between the stereoisomers: the first group comprises those species whose single enantiomers feature half-lives of racemization covering the range from days to months or years at room temperature with $\Delta G^\text{ǂ}$ values larger than 27 kcal/mol. Drugs or drug-like molecules of the first group are stereochemically stable enough to be developed as single enantiomers. A second group includes stereochemically less stable compounds, characterized by energetic barriers smaller than 20 kcal/mol, featuring half-lives of racemization for the individual enantiomers spanning from minutes to seconds (or fractions of seconds) at room temperature. Thus, atropisomers that equilibrate with short half-lives, if selected as candidates in drug design, will be developed to be administered as an equilibrating mixture. In this context, to achieve information on the rate of racemization or the equilibrium ratio for diastereoisomers is essential for understanding the results of spectroscopic and chromatographic analysis of the investigated drug. Specifically for benzodiazepines, HPLC separation of the enantiomers is achievable at or near room temperature, only for those derivatives that present a pattern of substitution that increases the energy barrier, for example, bulky groups on N1 (iPr or iBu).

Dynamic NMR and dynamic HPLC (coupled with computational techniques) are two of the most common methods to measure the free energy of interconversion between stereoisomers [26–33]. Dynamic HPLC with chiral stationary phase (CSP) is successfully employed in the determination of the enantiomerization barriers of fast interconverting enantiomers that exchange on a time-scale comparable to that of the separation process. In a typical dynamic HPLC experiment, the racemic mixture of the studied compound is analyzed while the temperature of the column is increased or decreased by defined step observing the coalescence of the peaks into a single one at higher temperatures. Typical dynamic elution profiles feature a plateau between the peaks corresponding to the interconverting enantiomers. In these conditions, the chromatographic column behaves as a reactor and by peak shape analysis supported by mathematical models as the theoretical plate model, the stochastic model or the unified equation [34–36], it is then possible to extrapolate the kinetic parameters of the stereochemical inversion process. Specifically, the chromatographic profile of two interconverting conformers is the result, in the stochastic model, of the probability of density functions of the interconverted species combined with distribution functions relative to the non-interconverted species.

This methodology allows the investigation of a typical range of energy barriers of interconversion spanning from 25 kcal/mol to 14 kcal/mol; higher or lower barriers would require extreme temperatures that are usually not well tolerated by the most of stationary phases.

This study presents the results obtained by low temperature dynamic HPLC experiments with chiral stationary phase for flubromazolam, clonazolam, diclazepam, three designer benzodiazepines that recently appeared on the market of substances of abuse, and flurazepam, a hypnotic BZD (Figure 1). Experiments carried out both on polysaccharide-based and Pirkle type CSP (Chiralpak IA and (R,R)-WhelkO1, respectively) aimed at developing an analytical method to separate the enantiomers of the studied BZD in order to measure the $\Delta G^\text{ǂ}$ values of enantiomerization, following an experimental procedure previously reported in the literature for analogous compounds [37–40]. The dynamic exchanged experimental HPLC profiles were computer simulated using the mathematical stochastic model to give the kinetic parameters for the on-column enantiomerization process.
2. Materials and Methods

2.1. Chemicals and Materials

All solvents were HPLC grade purity and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Specimens of flubromazolam, clonazolam, diclazepam and flurazepam were already present in our laboratories from previous studies and were obtained and purified by extraction from tablets containing 25 mg, 0.5 mg, 1 mg and 30 mg of active ingredient, respectively. Tablets were smashed and the powder treated with CH$_2$Cl$_2$. The solution was separated from the precipitate by filtration. The isolated compounds were analyzed by mass spectrometry with an ESI-LTQ Orbitrap (see SI for spectra) purchased from Thermo Scientific (Waltham, MA, USA).

2.2. HPLC Measurements

Analytical chromatography was performed using a Jasco (Tokyo, Japan) HPLC system with a universal Rheodyne 20 µL injector and Jasco PU 980 pump. Detection was conducted using a Jasco UV975 detector. Low temperature dynamic HPLC experiments were performed using a homemade cooling device and a cooling mixture composed of acetone and dry ice. The CSP (R,R)-Whelk-O1 (250 × 4.6 mm, L. × I.D., 5 µm particle size) was purchased from Regis Technologies Inc. The CSP Chiralpak IA (250 × 4.6 mm, L. × I.D., 5 µm particle size) was purchased from Chiral Technologies Europe (Illkirch-Graffenstaden, France). Samples for the analysis were prepared by dissolving 1 mg in 1 mL of mobile phase. All chromatographic measurements were replicated three times to ensure reproducibility.

2.3. Simulation of Dynamic Chromatograms

Simulations of variable temperature experimental chromatograms presenting a dynamic profile were performed with Auto DHPLC y2k (Auto Dynamic HPLC) (Rome, Italy), using the stochastic model. Both chromatographic and kinetic parameters can be automatically optimized by a simplex algorithm until the best agreement between experimental and simulated dynamic chromatograms is obtained.

3. Results

3.1. Dynamic Chromatography on CSP

A preliminary screening of different CSPs to investigate the ability to separate the conformational enantiomers of compounds 1–4, was carried out under normal phase elution conditions with the column temperature set at −30 °C.

For the two triazole-fused 1,4 benzodiazepines 1 and 2, the best results in terms of enantioselectivity and overall resolution were obtained with a 250 × 4.6 mm (L. × I.D.) col-
umn packed with the Chiralpak IA CSP, and using a mixture of n-hexane/CH$_2$Cl$_2$/MeOH 55/44/1 (v/v/v) as eluent, a flow rate of 1.0 mL min$^{-1}$ and a UV–VIS detector set at 265 nm. The elution profile of flubromazolam (Figure 2a) features two well resolved peaks, corresponding to the conformational enantiomers, while a plateau is present between the two peaks corresponding to the enantiomers of clonazolam (see SI). Measurements performed at different temperatures for both flubromazolam and clonazolam, show the typical dynamic behavior, with the height of the plateau that increases by temperature and the peaks that eventually turn into a single broad and asymmetric peak at higher temperatures, where the exchange between the two conformational enantiomers is faster.

Figure 2. Elution profiles registered for flubromazolam with the CSP Chiralpak IA: (a) elution profile registered with a column temperature of $-30^\circ$C, two peaks almost resolved at the baseline; (b) variable temperature chromatograms show progressive decoalescence of the peaks from 25°C to $-50^\circ$C.

For both flubromazolam and clonazolam, at 25°C a single unsplit peak was observed, that progressively broadens and turns into two distinct peaks separated by a plateau that becomes evident while decreasing the temperature of the column, indicating the presence of on-column interconversion of the two enantiomers. Upon further cooling of the column, down to $-50^\circ$C, the plateau that separates the peaks gradually decreased in intensity as a result of a progressively slower interconversion of the enantiomers if compared to the separation process. At $-50^\circ$C, the on-column enantiomerization resulted basically locked, as two equally intense and well resolved peaks were observed for the two enantiomers. A plot with the experimental chromatograms of flubromazolam registered at different temperatures is shown in Figure 2b (see SI for clonazolam chromatograms).

The best analytical conditions for the separation of diclazepam were achieved using a different chiral stationary phase, the (R,R)-Whelk-O1. A 250 × 4.6 mm (L. × I.D.) column was used with an eluent composed by n-hexane/CH$_2$Cl$_2$/MeOH in a proportion 60/60/1 (v/v/v) at a flow rate of 1 mL/min. In these conditions, with a column temperature of $-30^\circ$C the elution profile features a single broad peak, which indicates a possible selectivity for the enantiomers coupled to a fast exchange process. It was necessary to decrease the temperature down to $-50^\circ$C to observe a sign of decoalescence into two peaks connected by a curved plateau (Figure 3a). The same CSP allowed one to successfully separate the single enantiomers of flurazepam with an elution profile that, unlike clonazolam, featured two peaks connected by a low plateau already at $-30^\circ$C. Given the presence of a tertiary amine in the chemical structure of flurazepam, it was necessary to add a base as organic modifier in the mobile phase; specifically, in the eluent composed by n-hexane/CH$_2$Cl$_2$/MeOH 60/60/1, 1% triethylamine was added. Decreasing the temperature of the chromatographic column down to $-50^\circ$C the two peaks resulted as baseline-resolved (Figure 3b).
able to treated species com-

experiments on flurazepam from 25 to 50 C (a) Dynamic chromatography experiments on diclazepam from 15 C to –50 C; (b) dynamic chromatography experiments on flurazepam from 25 to –50 C.

3.2. Calculation of Energy Barriers of Enantiomerization

The enantiomerization barriers of flubromazolam, clonazolam, diclazepam and flurazepam were determined by coupling dynamic HPLC with computational simulation of the experimental chromatograms. Interconversion profiles featuring plateau formation and peak broadening were simulated by Auto DHPLC y2k, a stochastic model-based computer software developed in our laboratories. Dynamic profiles of the deformed experimental chromatograms were studied to extract kinetic parameters for the enantiomerization process that occurs during the chromatographic analysis [41–46]. A collection of the experimental (black traces) and simulated (red traces) chromatograms that have been from which it was possible to measure the kinetic data for the on-column interconversions are gathered in Figure 4. Gibbs free energies of enantiomerization for the on-column interconversions of the four compounds were calculated by computer simulating the exchange-modified HPLC plots. The method involves a mathematical model based on the stochastic approach and the simulation starts generating a simulated chromatographic profile with a plateau between the two resolved peaks while using as input parameters speculative kinetic constants for both the interconversion processes that take place in the mobile (km) and in the stationary phase (ks). The two rate constants for the enantiomerization in the mobile phase and in the stationary phase are different from each other due to the perturbing effect of the stationary phase that may slow down or, the other way around, accelerate the reversible process. Finally, when the difference between the experimental and the computed HPLC profiles has reached the minimum value, the computational procedure gives the computed apparent rate constants for the interconversion of the two corresponding enantiomers, kapp1,2 and kapp2,1 (1,2 for the process that considers the interconversion of the first eluted enantiomer into the other one and 2,1 for the opposite process). Each one of these rate constants is a weighted average value calculated for the interconversion that occurs in the mobile and in the stationary phase, according to the residence time of the two enantiomers in the phases. The two kinetic rate constants slightly differ from each other; in particular, the most retained enantiomer features the lowest value, presumably because it engages a stronger interaction with the chiral stationary phase. As a result, in the adsorbed state, the ring flip-mediated interconversion process is more hindered if compared to the less retained enantiomer, which engages a less stable complex with the CSP. As expected, the retarding effect of the CSP on interconversion, causes the enantiomerization of the second eluted towards the first eluted enantiomer to be faster. Energy barriers (ΔG°) were calculated from the obtained rate constants assuming a unitary transmission coefficient and the classical
Eyring equation, all the free energies have been calculated with an error of ±0.02 kcal/mol. The kinetic rate constant and free energies of enantiomerization are gathered in Table 1.

![Figure 4. Simulated profiles (red traces) of the experimental chromatograms (black traces): (a) flubromazolam – 20 °C; (b) clonazolam – 40 °C; (c) diclazepam – 50 °C; (d) flurazepam – 30 °C. Tc: column temperature, within ±0.2 °C. Energy barriers ΔG\(_f^i\) within ±0.02 kcal/mol.](image)

| Compound      | T\(_\text{column}\) °C | k\(_\text{app,1,2}\) (min\(^{-1}\)) | k\(_\text{app,2,1}\) (min\(^{-1}\)) | ΔG\(_{1,2}^i\) (kcal/mol) | ΔG\(_{2,1}^i\) (kcal/mol) |
|---------------|--------------------------|-----------------------------------|-----------------------------------|---------------------------|---------------------------|
| flubromazolam | –10                      | 0.160                             | 0.109                             | 18.43                     | 18.63                     |
|               | –20                      | 0.045                             | 0.030                             | 18.34                     | 18.74                     |
|               | –30                      | 0.013                             | 0.009                             | 18.17                     | 18.36                     |
| clonazolam    | –30                      | 0.076                             | 0.052                             | 17.35                     | 17.54                     |
|               | –40                      | 0.036                             | 0.024                             | 17.34                     | 17.52                     |
|               | –50                      | 0.026                             | 0.018                             | 17.12                     | 17.29                     |
| diclazepam    | –20                      | 0.170                             | 0.107                             | 15.53                     | 15.73                     |
|               | –30                      | 0.070                             | 0.047                             | 17.39                     | 17.58                     |
|               | –40                      | 0.022                             | 0.014                             | 17.20                     | 17.38                     |

ΔG\(_f^i\) values are all intended ±0.02 kcal/mol and temperatures ±0.5 °C.

Considering the free energy barriers reported in Table 1, the lower values of ΔG\(_f^i\) are those related to the conversion of the first eluted enantiomer into the second eluted; these values are usually closer to the ones calculated by other techniques (not affected by the influence of the CSP). Significantly, the lowest value was found for diclazepam, that has only a ΔG\(_f^i\) of about 15.5 kcal/mol. For this compound, it was possible to observe a dynamic profile with two peaks and a plateau in between at –50 °C, and we were not able to reach a baseline-separated profile. Flubromazolam has the highest ΔG\(_f^i\) found in this work, slightly above 18 kcal/mol at –10 °C, compatible with the presence of a triazole-fused ring in N1 that increases the steric hindrance, raising the energy necessary for the ring flip interconversion process. Clonazolam has a structure that differs from flubromazolam for the substituents at C7 and C2’, and a ΔG\(_f^i\) that results in a decrease by
almost 1 kcal/mol, probably because of electronic effects. Flurazepam has a similar value, indicating a comparable effect between the branched aminic group and the triazole portion.

4. Conclusions

All ∆G‡ values calculated were <18.5 kcal/mol and differences between compounds were found to be structure-correlated, involving steric and electronic effects. In particular, the triazole fused with the diazepine ring, and substituents in the C7 and C2′ position, such as nitro-group and halogens, have a role in stabilization or destabilization of enantiomers. The method applied in this work proved effective in studying this class of stereolabile compounds and the HPLC with chiral stationary phases allowed one to observe at low temperature the decoalescence of peaks corresponding to conformational enantiomers. The short half-life for these isomers makes it impossible to isolate and characterize them singularly at room temperature. More details please check Supplementary Materials.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/sym13061012/s1, Figure S1: variable temperature chromatograms, Figures S2–S5: Experimental (black traces) and simulate chromatograms (red traces) at different temperatures, Figure S6: ESI-MS spectra of flubromazolam, clonazolam and diclazepam.

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