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Dynamic high performance liquid chromatography on chiral stationary phases. Low temperature separation of the interconverting enantiomers of diazepam, flunitrazepam, prazepam and tetrazepam

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ABSTRACT

Diazepam and the structurally related 1,4-benzodiazepin-2-ones tetrazepam, prazepam and flunitrazepam are chiral molecules because they adopt a ground state conformation featuring a non-planar seven membered ring devoid of any reflection-symmetry element. The two conformational enantiomers of this class of benzodiazepines interconvert rapidly at room temperature by a simple ring flipping process. Low temperature HPLC on the Whelk-O1 chiral stationary phase allowed us to separate the conformational enantiomers of diazepam and of the related 1,4-benzodiazepin-2-ones, under conditions where the interconversion rate is sufficiently low, compared to the chromatographic separation rate. Diazepam, tetrazepam and prazepam showed temperature dependent dynamic HPLC profiles with interconversion plateaus indicative of on-column enantiomer interconversion (enantiomerization) in the temperature range between −10 °C and −35 °C, whereas for flunitrazepam on-column interconversion was observed at temperatures between −40 °C and −66 °C. Simulation of exchange-deformed HPLC profiles using a computer program based on the stochastic model yielded the apparent rate constants for the on-column enantiomerization and the corresponding free energy activation barriers. At −20 °C the enantiomerization barriers, $\Delta G^{\neq}$, for diazepam, prazepam and tetrazepam were determined to be in the range 17.6–18.7 kcal/mol. At −55 °C $\Delta G^{\neq}$ for flunitrazepam was determined to be in the 15.6–15.7 kcal/mol range. The experimental dynamic chromatograms and the corresponding interconversion barriers reported in this paper call for a reinterpretation of previously published results on the HPLC behavior of diazepam on chiral stationary phases.

1. Introduction

Benzodiazepines, and 1,4-benzodiazepin-2-ones in particular, form a well-known class of pharmacologically active heterocyclic compounds with sedative, hypnotics, anxiolytic, and anticonvulsant properties [1]. Recent medicinal chemistry investigations have sown that the conformational chirality of the 1,4-benzodiazepine core of these molecules plays a very important role in determining their bioactivity: binding to both human serum albumin HSA and $GABA_A$ receptors is strongly stereodependent and favors 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].

The central role that conformational chirality plays in modern medicinal chemistry has been the subject of recent review studies [4–6]. Depending on the energy barrier separating the two interconverting species, two major classes of conformational enantiomers can be distinguished. To the first one belong those species featuring enantiomerization [7] energy barriers larger than 27 kcal/mol (atropisomers), with half-life time of the individual enantiomers spanning from days to months or years at room temperature. The second class comprises stereochemically unstable species, with energy barriers separating the interconverting enantiomers smaller than 20 kcal/mol and featuring half-life times of the individual enantiomers ranging from minutes to fraction of seconds at room temperature. While chirality of drugs or drug-like molecules of the first class has a clear relevance from the pharmaceutical point of view, stereochemical studies of bioactive molecules of the second class have only a pharmacological relevance that is intrinsically linked to their dynamic interaction with biological targets and can lead to enantiomeric selection or enrichment at the interaction site [8,9].

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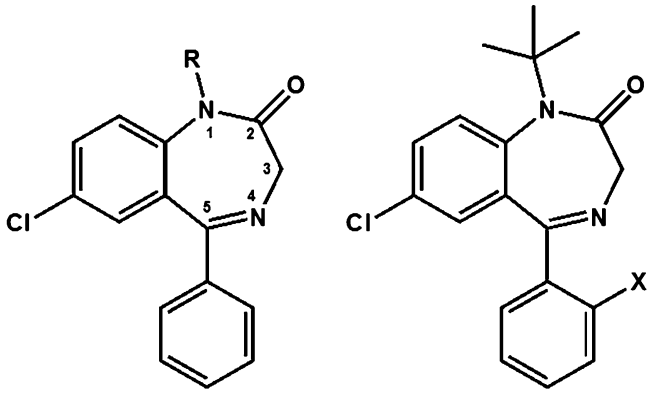

Fig. 1. Structure of 1,4-benzodiazepin-2-ones whose barriers separating the two conformational enantiomers are known from NMR or thermal racemization studies. R = CH_3, diazepam; R = CH_2Ph BDZ2; R = i-propyl BDZ3; R = $CHPh_2$ BDZ4; X = H BDZ5; X = F BDZ6.

The conformational behavior of diazepam has been the subject of several investigations, dealing with the determination of the three dimensional, non-planar structure of the seven membered 1,4-diazepine ring and with the measurements of the energy barrier for the interconversion of the two conformational enantiomers. Given the relatively fast interconversion at room temperature, dynamic ^{1}H NMR has been the method of choice to measure the energy for interconversion. In the ^{1}H NMR spectrum, the two C_3 methylene hydrogens of diazepam are diastereotopic under slow exchange conditions (at room temperature) and appear as a couple of doublets: upon temperature raising, the two doublets collapse and eventually coalesce at the coalescence temperature (T_c). From T_c the energy barrier was calculated as $\Delta G^{\neq}$ = 17.3 and 17.6 kcal/mol [10] (60 MHz in deuteropyridine and hexachlorobutadiene at T_c = 364 and 363 K) or $\Delta G^{\neq} = 18.0 \pm 0.2$ kcal/mol [11] (400 MHz in d_6-DMSO at T_c = 391 K). Using the more recently determined value of $\Delta G^{\neq} = 18.0 \pm 0.2$ kcal/mol for the ring inversion process [11–13], a half-life time $\tau_{(298)}$ between 1.24 and 2.45 s can be estimated for the conformational enantiomers of diazepam at room temperature. For structurally related 1,4-benzodiazepin-2-ones (Fig. 1), the energy barriers for the enantiomerization by ring flip reversal have been measured either by dynamic ^{1}H-NMR or by thermal racemization of isolated, long lived, enantiomers. The experimental results show that bulky N1 substituents increase the energy barrier: for BDZ2 $\Delta G^{\neq} = 19.5 \pm 0.2$ kcal/mol, and for BDZ3 and BDZ4 it is equal (BDZ4) or greater than 21.3 ± 0.2 kcal/mol. With a bulkier ^{t}butyl group on N1, the barrier in BDZ5 is estimated to be $\Delta G^{\neq}$ = 24 kcal/mol or larger, whereas in BDZ6 the fluorine atom on the non-fused phenyl ring lowers the barrier to $\Delta G^{\neq} \sim 21$ kcal/mol [4].

On the basis of the known energy barriers for the enantiomerization processes, HPLC separation of the conformational enantiomers is feasible at or near room temperature only for those benzodiazepine derivatives carrying bulky groups on N1 (^{i}Pr or ^{t}Bu). As a rule of thumb, separation of stereolabile enantiomeric species by chromatography can only be achieved if the separation and interconversion times are of the same order of magnitude [14–16]: assuming, as a lower limit, a separation time of 5 min on a standard HPLC column, the barrier of interconversion should be larger than 21 kcal/mol if the separation is carried out at room temperature. Simple calculations show that at 25 °C, the half-life time τ of a single enantiomer in such a case is around 4.6 min. In the presence of lower barriers, the two characteristic times (separation and interconversion) can only match at lower temperatures. For diazepam ($\Delta G^{\neq} = 18.0 \pm 0.2$ kcal/mol) the physical separation of the conformational enantiomers becomes possible only at temperatures equal or lower than $T = -15$ °C (assuming as upper value $\Delta G^{\neq}$ = 18.2 kcal/mol, $\tau_{(258)}$ = 5.5 min) or $T = -20$ °C (assuming as lower value $\Delta G^{\neq}$ = 17.8 kcal/mol, $\tau_{(253)}$ = 5.1 min).

This study presents the results obtained by low temperature enantioselective HPLC of diazepam, tetrazepam, prazepam and flunitrazepam carried out on a Whelk-O1 chiral stationary phase (see Fig. 2). Computer simulation of the dynamic exchanged experimental HPLC profiles yielded the apparent rate constants and the associated free energy barriers for the on-column enantiomerization process. In addition, the free energy barriers for the enantiomerization of flunitrazepam were also measured in free solution by dynamic NMR in d_6-DMSO and CD_3OD.

In the present work, examples of 1,4-benzodiazepin-2-ones were selected (i) to show the applicability of HPLC at low temperatures as a general method for the separation of fast-interconverting conformational enantiomers, (ii) to show the applicability of DHPLC combined with computer simulation to determine the unknown enantiomerization energy barriers for 1,4-benzodiazepin-2-ones related to diazepam (iii) to compare DHPLC results with independent NMR measurements.

2. Experimental

2.1. Materials

Samples of tetrazepam, prazepam and flunitrazepam were kindly provided by F.I.S. – Fabbrica Italiana Sintetici S.p.A., Vicenza (Italy). Diazepam was kindly provided by Istituto Superiore di Sanità, Rome, 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 (*R,R*)-Whelk-O1, 5 μm particle size, chromatographic column (250 mm × 4.6 mm I.D.) was purchased from Regis Technologies (Morton Grove, IL, USA). HPLC runs were performed at flow rates of 1.0 ml min^{-1} and monitored by UV detection at 280 nm. Column temperature was maintained within ±0.5 °C using a home-made cooling device.

Samples were dissolved in the eluent and filtered through 0.4 mm membrane before injection.

Simulations of variable-temperature experimental chromatograms were performed by Auto DHPLC y2k (Auto Dynamic HPLC) [17] that uses the stochastic model.

All the simulations in this article were performed with 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 thermostating apparatus. Signals were referenced with respect to TMS (δ = 0.00 ppm)

3. Results and discussion

3.1. Chromatographic retention and enantioselectivity

A preliminary screening of different chiral stationary phases for their ability to separate the conformational enantiomers of diazepam was carried out under normal phase elution conditions and fixing the column temperature at −35 °C. This low column temperature was chosen considering the known enantiomerization barrier of diazepam, $\Delta G^{\neq} = 18.0 \pm 0.2$ kcal/mol, and the associated