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HPLC resolution of C5 chiral 4,5-dihydro-1,4-benzodiazepines: stereochemical characterization and enantioselective $GABA_A$ receptor binding

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Abstract

HPLC resolution on chiral stationary phases has been successfully employed to obtain single enantiomers of C5 chiral 4,5-dihydro-1,4-benzodiazepines and to determine the enantiomeric composition of the collected stereoisomeric fractions. The absolute configuration of the prevailing enantiomer has been assigned on the basis of the circular dichroism spectra, as compared with that of the structural analogue (5R)- and (5S)-dihydrodiazepam. The single enantiomers, assayed for their binding to the central nervous system receptor, showed relatively low affinity but significant differences in displacing radioactively labelled flunitrazepam from specific benzodiazepine site. GABA shift experiments allowed the classification of these benzodiazepines as partial agonist or antagonist. © 1997 Elsevier Science B.V.

Keywords: Stereochemistry; Chiral chromatography; Chiral benzodiazepines; Enantioselective receptor binding; GABA-benzodiazepine-chloride ionophore receptor complex

1. Introduction

δ-Aminobutyric acid (GABA), an inhibitory neurotransmitter, interacts with specific receptors on post-synaptic neuronal membranes which control anion selective channels in the mammalian central nervous system (CNS) [1,2]. The efficacy of this interaction appears to be controlled by allosteric modulatory sites closely related to the $GABA_A$ subtype receptor, which bind the 1,4-benzodiazepines (BDZs) [3–6], a class of drugs extensively used in clinical practice for their anxiolytic, muscle relaxant, hypnotic and anti-convulsant properties [7]. Many papers report the structure-activity relationship of BDZs as well as the influence of the stereochemistry on the receptor function [8–17], most of the reports concerning 3-substituted BDZs. Thus, when the BDZ is chiral, the single enantiomers have to be prepared

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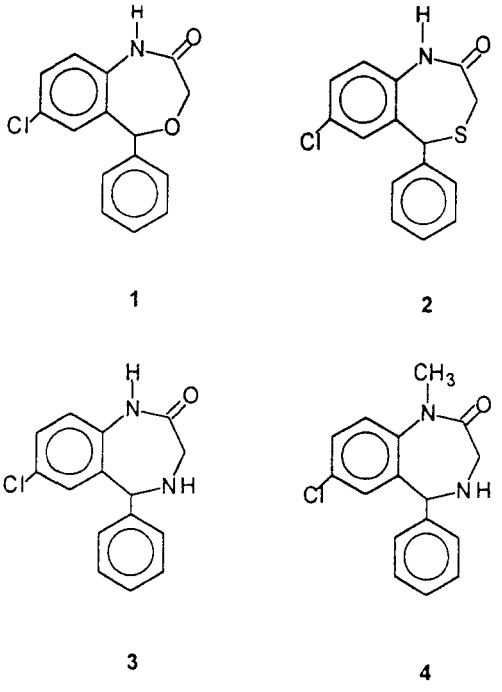
and the enantiomeric fractions characterized for their enantiomeric composition and absolute configuration of the prevailing enantiomer. Their stereochemical characterization is essential in order to have a better insight on the structural features which are important in determining the chiral discrimination and to develop new drugs with higher specificity in the pharmacological action.

In the present paper the interaction to the (CNS) receptors of a series of C5-chiral-4,5-dihydro-1,4-benzodiazepines (C5-BDZs) (Scheme 1) resolved as pure enantiomers by preparative chiral HPLC, is studied. The study of the interaction of the BDZ with the $GABA_A$ receptor and chloride channel is utilized to profile the differences between enantiomeric BDZs as agonist, antagonists and inverse agonist. Quantifiable differences in coupling the BDZ to the $GABA_A$ can be determined by measuring the GABA shift in the affinities of BDZs to displace [^{3}H]flunitrazepam [4,13,18,19]. Thus, the influence of the stereochemistry on the pharmacological profile on the BDZ receptors ligands has been investigated as GABA ratio determination.



Scheme 1.

2. Materials and methods

[^{3}H]flunitrazepam (specific activity = 83 Ci mmol^{-1} was obtained from DuPont (New England Nuclear division, Germany). Other chemicals were reagent grade and from commercial suppliers. BDZs were kindly provided as racemates by Prof M. Simonyi, Hungarian academy of sciences, Budapest, Hungary.

The HPLC resolution of 1–3 was obtained with the Chiralcel OD (Daicel 25 × 0.4 cm. i.d.) at room temperature. The mobile phase was hexane-2-propanol mixture (80/20, v/v), 0.5 ml min^{-1}. A chiralcel AD (Daicel 25 × 0.4 cm i.d.) and a HSA column (Shandon, 15 × 0.4 cm i.d.) were also used. The mobile phases were hexane/2-propanol, 90/10 (v/v), 0.5 ml min^{-1} and phosphate buffer 0.1 M pH 7.4/1 propanol, 90/10 (v/v), 0.8 ml min^{-1}, respectively.

The chromatographic system consisted of a Jasco 887-PU HPLC pump and a Jasco multi-340 multi channel detector. The same system was used for the preparative resolution of 1–3 by low loading repetitive injections, using a Reodyne model 7125 injector with a 20 μl loop. Simultaneous absorption (UV) and circular dichroism (CD) detection was carried out using a Jasco J700 spectropolarimeter (set at 254 nm) equipped with a micro HPLC cell (volume 8 μl) and a doublet of lenses to focus the light beam in the sample compartment.

The chromatographic retention of the solutes was followed at 255 nm and reported as the capacity factor (k'), where k' is defined as $(t_{BDZ} - t_0)/t_0$ (t_{BDZ} = retention of the BDZ enantiomer; t_0 = retention of a non retained solute). The enantioselectivity (α), where $\alpha = k'_2/k'_1$, was also calculated (k'_2 and k'_1 are the capacity factors of the second and the first eluted enantiomer, respectively).

CD spectra were carried out with a Jasco J-600 spectropolarimeter. UV spectra were obtained using a Perkin-Elmer Lambda 9 spectrophotometer. CD and UV were carried out at room temperature, with ethanol as solvent and 1–0.01 cm cells.

Membrane preparation was obtained essentially as previously described [20]. Bovine cerebral cortex was homogenized in 10 vol. (w/vol.) of 0.32 M ice-cold sucrose containing protease inhibitors. The homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C. The resultant pellet was discarded and the supernatant was recentrifuged at $48\,000 \times g$ for 30 min at 4°C. The resultant pellet was osmotically shocked by suspension in 10 vol of 50 mM Tris–HCl buffer at pH 7.4 containing protease inhibitors and recentrifuged at $48\,000 \times g$ for 30 min at 4°C. The pellet was suspended in 50 mM Tris–HCl buffer at pH 7.4 and used for BDZ binding assays as reported previously [21].

The membrane suspension (0.5 mg of proteins) was incubated in triplicate with a 0.4 nM [^{3}H]flunitrazepam at 0°C (45 min in the buffer, with a final vol of 0.5 ml). After incubation the samples were diluted at 0°C with 5 ml of the assay buffer and were immediately filtered under reduced pressure through glass fibber filter disk (Whatman GF/B). The filters were washed with 5 ml of the buffer. The filter disks were than placed in polypropylene scintillation vials together with 8 ml of Ready Protein Beckman scintillation cocktail: the radioactivity of the filters was determined by a Beckman LS 1800 scintillometer. The BDZ derivatives were dissolved in ethanol/buffer and the same mixture was present in the blank experiments. Nonspecific binding was determined by incubation in the presence of 10 μM diazepam. The estimation of the proteins was based on the method of Lowry et al., after solubilization with 0.75 N NaOH [22]. Bovine serum albumin was utilized as standard. The concentrations of the investigated compounds that inhibit specific [^{3}H]flunitrazepam binding by 50% (IC_{50}) were determined by log-probit analysis with six concentrations of the displacers, each performed in triplicate. The membranes used for the determination of the GABA ratio were exhaustively washed [23]. IC_{50} determination for the GABA ratio values were carried out in the absence and in the presence of 10 μM GABA. The inhibition constants of the unlabelled ligands (K_i) can be derived according to the equation of Cheng and Prussoff ($K_i = IC_{50}/1 + [L]/K_d$) [24]. The ligand dissociation constant (K_d) of ^{3}H-flunitrazepam was 1.8 nM.

3. Results and discussion

3.1. Chromatographic resolution and stereochemical characterization

The enantioselective separation of 1–3 was obtained by HPLC upon three different chiral stationary phases(CSPs): a Chiralcel OD, a Chiralcel AD and a HSA based column. Base line resolution (α values up to 3.30) was obtained for the three samples on chiralcel OD, using hexane/2-propanol as mobile phase (Table 1). In Fig. 1 the resolution of 3 is reported as an example. Good enantioselectivity was observed also with the chiralcel AD (Table 1). The highest enantioselectivity was obtained in the resolution of 2 with hexane/2-propanol as mobile phase, while only partial resolution was obtained in the case of 3, in the same experimental conditions. As far as HSA column is concerned a base line resolution was obtained in the case of 2, while only partial resolution was obtained for 1 and 3 (Table 1).

The use of the detection system measuring UV and CD signals, simultaneously [25], allowed to determine the elution order of 1, 3 on the various chiral stationary phases (CSPs).The elution order on the different columns is reported in Table 1. In the case of the Chiralcel OD the enantiomer with positive CD at 254 nm was eluted first for 1 and 3, while it was the more retained one for 2. Compound 1 shows the same elution order on Chiralcel OD and Chiralcel AD, while an inversion was observed in the analysis of 2 and 3. Different elution order was observed for 2, with respect to 1 and 3, also on the HSA column, being the enantiomer with the positive CD eluted first for 2 and the more retained for 1 and 3. The elution order observed for 3 on the HSA column is in agreement with the higher affinity reported for the (S) enantiomer of its structural analogue dihydrodiazepam, 4 [26]. As far as compound 3 is concerned, the relationship between sign of the CD and absolute configuration can be empirically done by comparison with 4. In the case of 4, where a methyl group replaces the hydrogen on the N1 atom, the relation between CD and absolute configuration has been already established [27], being the (+)(5S)-4 the enantiomer with

Table 1
HPLC resolution of 1–3 on various chiral stationary phases

Compound	Column	K'_1	α	CD[a]	Mobile phase[b]
	Chiral. OD	2.2	1.13	(+)	a
1	Chiral. AD	7.1	1.85	(+)	b
	HSA	14.0	1.0	(−)	c
	Chiral. OD	1.5	3.30	(−)	a
2	Chiral. AD	2.5	1.58	(+)	d
	HSA	22.4	1.24	(+)	e
	Chiral OD	1.9	1.51	(+)	a
3	Chiral AD	3.8	1.0	(−)	b
	HSA	1.3	1.0	(−)	f

[a]CD sign of the first eluted enantiomer at 254 nm.
[b](a) Hexane/2-propanol 80/20 (v/v), 0.5 ml min^{-1}; (b) hexane/2-propanol 90/10 (v/v), 0.5 ml min^{-1}; (c) phosphate buffer (50 mM, pH 7)/1-propanol, 94/6 (v/v) 1 ml min^{-1}; (d) hexane/2-propanol 90/10 (v/v) 0.8 ml min^{-1}; (e) phosphate buffer (50 mM, pH 7)/1-propanol 92/8 (v/v), 0.5 ml min^{-1}; (f) phosphate buffer (50 mM, pH 7)/1-propanol 94/6 (v/v) 0.8 ml min^{-1}.

positive CD at 254 nm. Thus the (5S) configuration can be assigned to the less retained enantiomer of 3 on chiralcel OD, in the experimental condition adopted, taking into account the high similarity of the two structures. The structural analogues 1 and 2 show a similar behaviour of the CD spectra with respect to 3 as observed in Fig. 2 where the CD spectra of the enantiomeric fraction of 1–3, more retained on Chiracel OD, are reported. By studying a series of 4,5-saturated-5-aryl 1,4-benzodiazepin-2-one derivatives, Snatzcke et al. suggested that the sign of the first strong CD band appearing between 270 and 250 nm is determined by the helicity, M or P [28], of the seven member 1,4-benzodiazepin-2-one ring [27]. Positive and negative sign correspond to M and P helicity, respectively [27]. Taking into account the similarity of the CD spectra of the single enantiomers of 3 with those of its S4 and N4 analogues, we can tentatively consider the same source for the optical activity in the examined spectral region and thus assigning the absolute configuration at C5 on the basis of the sign of the 260 nm CD band. Thus the (5S) configuration can be tentatively assigned to the less retained enantiomer of 1 and the more retained enantiomer of 2.

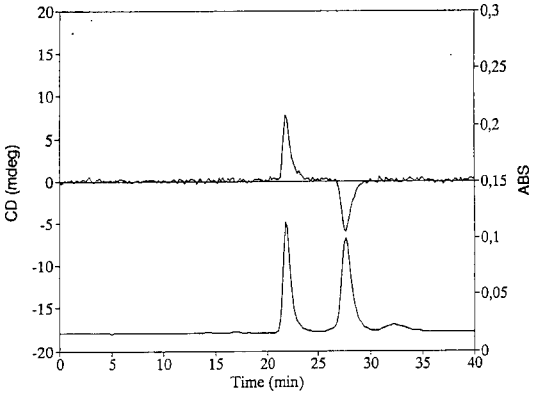


Fig. 1. HPLC resolution of *rac*-3 on chiralcel OD. Mobile phase: hexane/2-propanol 80/20 (v/v), 0.5 ml min^{-1}. CD (upper HPLC profile) and absorption (lower HPLC profile) detection at 254 nm.

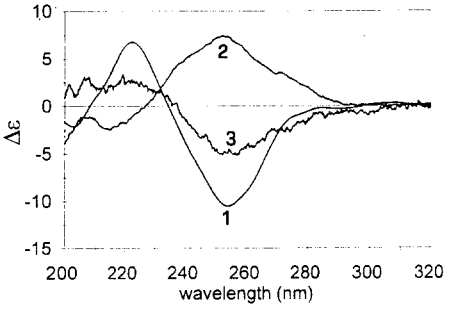


Fig. 2. CD spectra of the more retained enantiomers of 1–3 on chiralcel OD. The collected fractions were dried under vacuum and then analysed in ethanol solution, 0.1 cm cell.

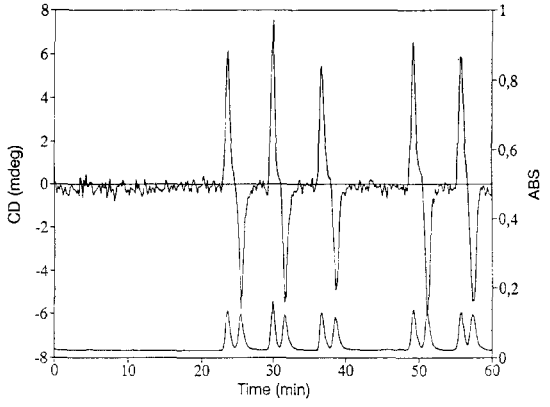


Fig. 3. HPLC preparative resolution of *rac*-1 on chiralcel OD: repetitive injections of 10 μg every 8–10 min. Mobile phase: hexane/2-propanol 80/20 (v/v), 0.6 ml min^{-1}. CD (upper HPLC profile) and absorption (lower HPLC profile) detection at 254 nm.

The chiralcel OD column was used also for the preparative resolution of 1–3. In practice, low loading repetitive injections (10 μg each injection, one injection every 8–10 min) allowed to collect about 200–250 μg of each enantiomeric fraction in 10 h. In Fig. 3 the preparative resolution of 1 is reported, as an example. The enantiomeric composition of the obtained fractions was determined by their re-injection on column. Only samples with enantiomeric excess higher than 98% were directly used for the pharmacological test. As an example the analysis of a sample of (5S)-1 on

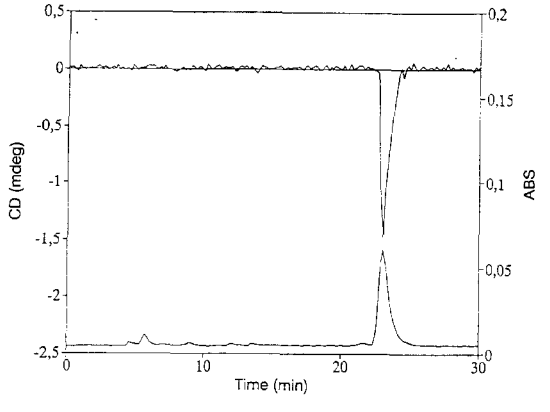


Fig. 4. HPLC analysis of (5R)1, the more retained enantiomer on chiralcel OD. Mobile phase hexane/2-propanol 80/20 (v/v), 0.6 ml min^{-1}. CD (upper HPLC profile) and absorption (lower HPLC profile) detection at 254 nm.

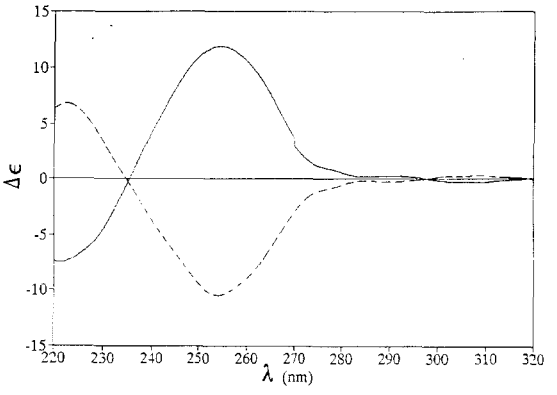


Fig. 5. CD spectra of (5S)-1 (continous line) and (5R)-1 (broken line), immediately before and after their use for binding assay: c 0.5 mM, EtOH, 0.01 cm cell. The spectra of each enantiomer were superimposable.

chiralcel OD is reported in Fig. 4. An enantiomeric excess (e.e) of 98.4% was evaluated for that a fraction. Higher e.e. (99.1%) was obtained for (5R)-1. The enantiomeric fractions of 2 and 3 resulted, in practice, enantiomerically pure because of the high enantioselectivity obtained in the experimental condition adopted (Table 1).

CD was also used to check the stereochemical stability of the samples. Actually the CD spectra of the single enantiomers of 1–3 were carried out after their HPLC preparation and immediately before their use in the receptor binding experiments. The enantiomeric fractions resulted stable for months. This procedure, i.e. to check the stereochemical stability of the compounds, is essential to ensure the reliability of the receptor binding experiments.

3.2. Binding assays

Stock solutions in ethanol were prepared for the binding assays. Absorption and CD spectra of these solutions were recorded immediately before their use, in order to check the concentration and the enantiomeric excess of the sample. Further CD analysis can be used to check the stereochemical stability of the sample in the experimental conditions adopted for the binding experiments. Thus CD spectra of the enantiomeric fractions of 1, carried out immediately before and after the

binding assay, resulted identical (Fig. 5). The ability of the enantiomeric fractions, and of the racemate, to displace specific [^{3}H]flunitrazepam binding was studied in membrane prepared from bovine cerebral cortex with a radioligand concentration of 0.4 nM. The concentration of the compounds able to give 50% inhibition of [^{3}H]flunitrazepam binding (IC_{50}) are reported in Table 2. In the case of 1 the single enantiomers showed comparable activity in displacing the radioligand from bovine brain membranes, being a little higher that of the enantiomeric fraction showing negative CD signal at 260 nm. It has to be observed that only slight differences in the receptor affinity have been previously reported for dihydrodiazepam, 4 [10], and for other C5 chiral benzodiazepines [14]. By contrast significant differences in the displacing activity were observed for the single enantiomers of 2 and 3 being the enantiomeric fractions with positive CD at 260 nm those showing the higher displacing activity (Table 2).

A convenient in vitro method to roughly differentiate compounds interacting with the $GABA_A$ receptor into agonist, inverse agonist or antagonist is the GABA shift or GABA ratio, i.e. the ratio between the receptor affinity of the ligand, measured as the concentration able to inhibit 50% of [^{3}H]flunitrazepam binding (IC_{50}), in the absence and in the presence of GABA. The presence of GABA enhances the affinity of an agonist, decreases the affinity of an inverse-agonist, while the affinity of an antagonist is unaffected [18,19,29,30]. Although these data concern receptor binding, the GABA ratio could be useful for predicting the pharmacological profile of $GABA^A$ ligands, according to the literature [29,30]. On these bases the results (Table 2) indicate that the examined compounds 1–3 behave as antagonist or partial agonist. Binding data of diazepam, standard as agonist, RO 15-1788, standard as antagonist, and β-carboline-3-carboxylic acid methylester (βCCM), standard as inverse-agonist are also reported in Table 2.

Table 2
Inhibition of [^{3}H]flunitrazepam binding and GABA Ratio

Compound	IC_{50}^a	GABA ratio[b]
(+)(−) 1	4.57 ± 0.35 μM	1.05
(+) 1	6.03 ± 0.51 μM	1.13
(−) 1	4.48 ± 0.32 μM	1.05
(+) (−) 2	8.93 ± 0.70 μM	1.10
(+) 2	6.17 ± 0.43 μM	1.02
(−) 2	>10 μM	n.d.
(+)(−) 3	2.59 ± 0.15 μM	1.18
(+) 3	1.49 ± 0.05 μM	1.13
(−) 3	3.22 ± 0.23 μM	1.35
Diazepam	13 ± 2 nM	1.50
βCCM	1.3 ± 0.1	0.65
RO 15-1788	0.5 ± 0.02	1.0

[a]Concentrations for 50% inhibition (IC_{50}) are means ± S.E.M. of six experiments.
[b]IC_{50} (compound)/IC_{50} (compound + 10 μM GABA).

The enantioselectivity in the binding of 1,4 BDZs to CNS GABA-receptor has been explained on the basis of the selectivity of the receptor binding sites for one of the two M and P conformations of the seven member ring of the drug. Similar conformational behaviour is present for 4-5-saturated derivatives of 1,4-benzodiazepine-2-ones where the M helicity results in (5S) absolute configuration. On this basis the enantiomeric fractions assumed in M conformation appears to have the higher affinity in the case of 2 and 3, in agreement with the behaviour of 3-substituted BDZs. The reverse appears the case of 1, where the enantiomer expected in the P-conformation presents a higher affinity to the receptor, even if the difference between the enantiomers is low.

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