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Chiral 1,4-benzodiazepin-2-ones: relationship between stereochemistry and pharmacological activity*

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Abstract: Pure enantiomers of 3-substituted-1,4-benzodiazepin-2-ones, obtained by HPLC resolution on chiral stationary phases, show significant differences in their pharmacological activity. The occurrence of biotransformation during the pharmacological test is monitored using a new chromatographic method. The reliability of the pharmacological activity data is discussed.

Keywords: *Chiral stationary phases; benzodiazepinones; stereochemistry; pharmacological activity; biotransformation; circular dichroism.*

Introduction

Nowadays it is well documented that the pharmacological activity of two enantiomers may be quite different [1, 2]. Thus, a complete stereochemical characterization of chiral drugs is essential, in order to avoid tremendous consequences as in the case of thalidomide [1], and to have a better insight into their *in vivo* distribution and metabolism [2]. The availability of efficient chiral chromatographic separation techniques [3], together with the use of chiroptical spectroscopies [4], provides a very powerful tool to approach the problem. Chiral separation on a semipreparative scale often allows suitable amounts of the pure enantiomers to be obtained to carry out pharmacological tests. Sensitive and selective analytical methods are used in order to check the enantiomeric purity of the drugs examined in the experimental conditions of the pharmacological test, as well as to monitor the possible occurrence of their biotransformation [2].

In this paper we present the chromatographic separation of a series of 3-substituted 1,4-benzodiazepin-2-ones (BDZs), belonging to a class of widely used anxiolytics [5]. Different chiral stationary phases (CSPs) were used either on analytical or on a semipreparative scale. The pharmacological activity of the pure enantiomers has been determined *in vitro* by displacement of radioactively labelled Flunitrazepam from the

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specific BDZs binding site [6]. Moreover, in the case of two esters of oxazepam (a 3-hydroxy-BDZ), a chromatographic method has been developed to monitor, in the same run, the enantiomeric composition of the esters and the presence of the related hydrolysis product. Indeed, a hydrolysis reaction can occur during the pharmacological test, due to the presence of esterases in the synaptosomal fractions of brain membrane preparations [7, 8]. This reaction can strongly affect the results of the pharmacological test because of the high affinity of the produced oxazepam [8]. The aforementioned analytical method establishes the presence of esterase activity in the synaptosomal fractions and allows an evaluation of the hydrolysis product.

Experimental

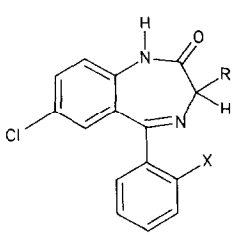
Chemicals

Oxazepam (**1**) and lorazepam (**2**) (see Chart 1) were obtained by Soxhlet extraction (acetone) of commercial pharmaceuticals. The products obtained were characterized by NMR and mass spectrometry. The data were in accordance with the structure [9]. Compounds **3**, **5**, **7** and **8** were kindly provided by Prof. W. H. Pirkle (School of Chemical Sciences, University of Illinois at Urbana-Champaign, USA). Compound **4** was kindly provided by Dr E. Simon-Trompler (Central Research Institute for Chemistry, Budapest, Hungary). The pure enantiomers of **6** were kindly provided by Ravizza SpA, Milan, Italy. Racemic **6** was prepared by acylation of **1** with succinic anhydride in the presence of pyridine, according to the reported procedure [10]. Compound **6** was obtained in 80% yield as a crystalline powder having m.p. 152–154°C. NMR and mass spectrometry data were in accordance with the structure.

Chiral stationary phases

An analytical ionic Pirkle column (CSP I), i.e. (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine ionically bonded to a γ-aminopropylsilanized silica column, was prepared *in situ* using a 5 μm silica NH_2 column (250 × 4 mm i.d.) from Merck, Darmstad, FRG, following the procedure reported in the literature [11]. A semipreparative ionic Pirkle column (CSP II) was prepared *in situ* following the same procedure [11], using a 5 μm γ-aminopropylsilanized silica column (250 × 20 mm i.d.) from Spherisorb, Queensferry, UK.

Two commercially available 250 × 4 mm i.d. columns were used: Chiralpack OT(+) (CSP III), a column with (+)-poly-(triphenylmethyl methacrylate) coated on phenylsilanized silica, from Daicel Chem. Ind.; Cyclobond I (CSP IV), a β-cyclodextrin bonded silica column from Astec, USA. The CSP V, SiSQuinmei, was obtained by reacting *N*-



	R	X
1	OH	H
2	OH	Cl
3	OCOMe	H
4	OCOMe	Cl
5	$OCOBu^t$	H
6	$OCO(CH_2)_2COO^-$	H
7	Me	H
8	NEt_2	H

Chart 1

methylquininium iodide with γ-mercaptopropylsilanized silica, prepared starting from LiChrosorb Si60 (5 μm, Merck, Darmstad, FRG) and (3-mercaptopropyl)-trimethoxy-silane, as described elsewhere [4]. A 125 × 4 mm i.d. column was slurry packed (MeOH) with this material by conventional techniques [4].

Chromatographic resolution

The analytical separations were carried out using a Jasco Twincle apparatus equipped with a Jasco Uvidec 100-V variable wavelength detector. CD detection was obtained by means of a Jasco J-500C spectropolarimeter, equipped with a micro-HPLC cell and a doublet of lenses to focus the light beam in the sample compartment [12]. The two detectors were connected in series and set at 254 nm. The semipreparative separations (1–5 mg/150 μl each injection) were carried out using a Perkin–Elmer series 2 liquid chromatograph coupled to a LC 75 spectrophotometer detector. The HPLC systems were operated at room temperature, at a flow rate of 0.5–1 ml min^{-1} or at 9 ml min^{-1} for analytical or semipreparative columns, respectively. All solvents used for the preparation of the mobile phase mixtures were HPLC grade (from Merck, Darmstad, FRG) and were filtered and degassed before use.

Instrumentation

The UV and CD spectra of the samples were obtained by using a Perkin–Elmer Lambda 9 Spectrophotometer and a Jasco-J600 Spectropolarimeter, respectively. NMR spectra were acquired on a Varian Gemini-200 spectrometer. Mass spectra were recorded on a VG 70–70E instrument. Melting points were determined with a Reichert-Thermovar apparatus.

Pharmacological test: binding to the BDZ receptor

The ability of the pure enantiomers to displace specific ^{3}H-Flunitrazepam binding from bovine brain membranes was tested. Tritiated Flunitrazepam (FNZ) was obtained from New England Nuclear, Du Pont (FRG) and had a specific activity of 76.9 Ci $mmol^{-1}$ and a radiochemical purity >99%. Bovine brain synaptosomal membranes were prepared from bovine cortex in accordance with ref. [13]. The membrane preparations were subjected three times to a freeze–thaw cycle and washed by suspension and centrifugation in a 50 mM Tris–HCl buffer pH 7.4, in order to reduce the esterase activity [8]. The BDZ receptor binding activity was tested following a literature procedure [13]. Different concentrations of the compounds were incubated in triplicate together with 100 μl of the membrane suspension (0.4–0.5 mg of protein) and 0.4 nM ^{3}H-Flunitrazepam for 45 min at 4°C in the Tris–HCl buffer (final volume of 500 μl). The samples were then diluted with 5 ml ice-cold buffer and immediately filtered under reduced pressure through glass fiber filter disks (Whatman GF/B). The filters were washed with the same buffer, dried and shaken in plastic vials with the scintillation liquid (HP Beckman: 0.01 M KOH; 8:0.4 v/v). The radioactivity on the filters was determined using a Beckman LS 1800 scintillometer. Specific ^{3}H-FNZ binding was determined after subtraction of non-specific binding (5–10%), estimated by incubating membranes and ^{3}H-FNZ in the presence of 10 μM Diazepam. The concentration of the investigated compound that inhibits specific ^{3}H-FNZ binding by 50% (IC_{50}) is the mean (±SEM) determined by log-probit analysis of the results obtained for five concentrations of the compound, each analysis being performed in triplicate (EBDA Program, G. A. McPherson 1983).

Hydrolysis of the esters

The hydrolysis occurring during incubation with the brain membranes was monitored for compounds **3** and **6** using CSPs I and IV, respectively, by the following procedure: **3** and **6** (as a racemic mixture or as pure enantiomers) were incubated with 300 μl of the membrane suspension in Tris–HCl buffer (final volume 1.5 ml, BDZ concentrations 12 μM for **3**, 10 μM and 0.5 mM for **6**) at 4 or 22°C. After 45 min (as in the binding test) and at later times, the compounds were extracted (after acidification up to pH 2.5 for ionic compound **6**) with three volumes of diethylether [7] three times and evaporated. The material was redissolved in the mobile phase, filtered, and aliquots (≈30%) were chromatographed on the CSPs. Blank experiments showed that the extraction did not affect the ratio of the ester (**3** or **6**) to the related hydrolysis product (**1**). The results are given for each compound as the per cent composition of the recovered BDZs (assumed 100%), calculated from the ratio of the peak area of the individual peaks to that of the sum of the peak areas of the ester and of the hydrolysis product. Hydrolysis in samples extracted immediately after incubation did not exceed 2%.

Results and Discussion

Chromatographic resolution

Several CSPs have been used in the present investigation in order to obtain suitable amounts of the pure enantiomers (0.1–2 mg) for the *in vitro* pharmacological tests. Furthermore, the optical purity of the collected fractions was determined before their utilization. For this purpose, chromatographic analytical methods were optimized in order to monitor even a few per cent of the less abundant enantiomer. This can be achieved by using CSPs which show a reversal of the normal elution order for the class of compounds being examined (Table 1). By choosing the CSP the less abundant enantiomer of the BDZs can be eluted first, making its determination easier. CSPs (I–V) have been used for the analytical resolution of compounds **1–8** and good separation factors (up to 1.6) were obtained in all the cases (Table 1). The resolution of **5** on CSP III is shown in Fig. 1a, as a representative example.

Table 1
Chromatographic resolution data of compounds **1–8** on CSPs I–V

Compound	CSPs I* and II†			CSP III‡			CSP IV§			CSP V‖		
	K_1	α	CD¶	K_1	α	CD¶	K_1	α	CD¶	K_1	α	CD¶
1				1.5	1.0		2.5	1.17	–	2.7	1.18	–
2				1.5	1.0	+	4.9	1.0	–	12.5	1.48	–
3	6.34†	1.12†	–	2.08	1.3	+	4.0	1.15	–	10.0	1.12	–
4	4.81*	1.16*	–	1.61	1.32	+	2.7	1.0		8.0	1.0	–
5				1.2	1.6	+	10.2	1.15	–	10.8	1.05	–
6				3.31	1.3	+	4.9	1.08	–			
7	2.30†	1.20†	–				6.6	1.04	–	10.8	1.12	–
8				4.75	1.2	+	2.7	1.05	–	1.5	1.29	–

*250 × 4 mm column, eluent ex-$CHCl_3$–IPA, 85:5:10 v/v/v, 1 ml min^{-1}.
†250 × 20 mm column, eluent ex-IPA, 80:20 v/v, 9 ml min^{-1}.
‡250 × 4 mm column, eluent ex-IPA 90:10 or 95:5 v/v in the case of **8**, 0.5 ml min^{-1}.
§250 × 4 mm column, CH_3CN–acetate buffer pH 4.2 10:90 v/v for **2**, **3**, **7** and **8**, and 15:85 v/v for **1**, **4**, **5** and **6**, 1 ml min^{-1}.
‖125 × 4 mm column, eluent ex-IPA 80:20 v/v for **1**, **2**, **3** and **4**, eluent ex-$CHCl_3$–IPA 175:20:5 v/v/v for **5** and **7**, eluent ex-CH_2Cl_2–IPA 100:10:20 v/v/v for **8**; 1 ml min^{-1}.
¶CD sign at 254 nm of the first eluted enantiomer.

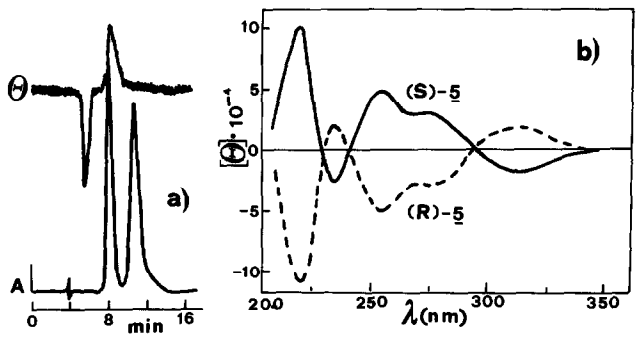


Figure 1
(a) Chromatographic resolution of **5** on CSP III. Eluent hexane–2-propanol (90:10 v/v). Absorption and CD detection at 254 nm. (b) CD spectra of the recovered pure enantiomers of **5** (0.2 mM solutions in ethanol, 0.5 mm quartz cell, room temperature).

CSP I is reported [14] to be efficient for the resolution of **1–3**, **5**, **7** and **8**. In the present investigation CSP I was used for the resolution of **3**, **4** and **7**, with hexane–2-propanol mixtures as mobile phase (1 ml min^{-1}). CSP III was successfully used for the resolution of **3–6** and **8**, with hexane–2-propanol mixtures, 0.5 ml min^{-1}, as mobile phase.

CSP IV was efficient in the resolution of **1**, **3** and **5–8** using a CH_3CN–acetate buffer pH 4.2 (0.2 M) mixtures, 1 ml min^{-1}. This CSP has been employed for the first time in the resolution of BDZs and appeared particularly useful for the chiral chromatographic resolution of ionic BDZs (P. Salvadori *et al.*, submitted for publication).

CSP V was used for separating racemates of **1–5**, **7** and **8**. Good resolution factors were obtained for all the samples examined. In the case of **4** a single peak was observable with the UV detector, while the CD detector allowed a partial resolution to be monitored.

The semipreparative ionic Pirkle column (CSP II) was utilized for the resolution of **3** and **7**, allowing the recovery of *ca* 2 mg of the two enantiomers per run. It is well known that compounds **1** and **2** undergo racemization in a very short time in aqueous solutions [15]. Thus, they were not considered for the investigation of the relationship between stereochemistry and pharmacological activity.

The fractions collected of compounds **3–5**, **7** and **8**, obtained either using semipreparative CSP II (one run) or analytical columns (five to ten runs), were analysed for their optical purity (o.p.) using analytical CSPs. Only samples with o.p. >97% have been used for the pharmacological tests. The absolute configuration of the resolved antipodes were deduced from the sign of the CD at 254 nm [4]. The complete CD spectra were recorded on line (P. Salvadori *et al.*, in *Chirality and Biological Activity*, Alan R. Liss, New York, in press) by entrapping the eluting enantiomers ($\geq$5 μM) in the cell of the spectropolarimeter used as detector. Stock solutions in ethanol were prepared (actual concentrations were determined by the absorbance at 230 nm) for the binding assays. The CD spectra of these solutions were recorded immediately before their use, in order to check the optical purity of the samples (see Fig. 1b).

Binding of the BDZs to the receptor

The binding affinities of the pure enantiomers of the investigated compounds are shown in Table 2. The data are reported as the IC_{50} ±SEM values for the displacement of

Table 2
Inhibition of specific ^{3}H-Flunitrazepam binding to synaptosomal preparation from bovine brain cortex

Compound	IC_{50}* (*S*)-enantioner	IC_{50}* (*R*)-enantioner
3	95 ± 14	520 ± 82
4	13 ± 2	46 ± 13
5	740 ± 130	>10,000
6	254 ± 51	551 ± 74
7	78 ± 5	>10,000

*IC_{50} = nM concentration (±SEM) for 50% displacement of ^{3}H-Flunitrazepam.

specific ^{3}H-FNZ binding from the synaptosomal membranes. The IC_{50} values >10 μM are not reported because at these concentrations (indicating a very low activity) the data may be affected by precipitation of the compound under investigation. Our results confirm the already reported [16–20] higher affinity to the receptor (i.e. lower IC_{50}) of the (*S*)-enantiomers of 3-substituted 1,4-benzodiazepin-2-ones, with respect to the (*R*)-enantiomers. The two enantiomers of **7** show the highest difference in IC_{50} among the BDZs tested here; the pure antipodes of the same compound have already been tested giving similar results [19]. Compound **8** showed a very low affinity (IC_{50} >10 μM) as the racemic mixture, and the pure enantiomers were not tested.

The other data concern the results for esters of oxazepam (**3**, **5**, **6**) and lorazepam (**4**). Among these as far as can be ascertained the receptor affinity of the pure enantiomers of only **6** has been investigated [8, 17]. The others have been investigated as racemic forms [8]. In the present study a large difference was found between the activity of the two pure enantiomers of the esters of **1** (in particular **3** and **5**). The same behaviour was observed for **4**, where the phenyl ring is 2′-chloro substituted. In this case, the (*R*)-enantiomer showed an unusually high receptor affinity. However, the possibility of the occurrence of hydrolysis during the binding test [8] must be taken into account, as this is responsible for the different IC_{50} values reported for **6** [8]. In order to inhibit the esterase activity [8], the synaptosomal membranes were subjected to a freeze–thaw cycle three times and washed, and a low temperature (4°C) was used for the incubation. In addition, with a new analytical method the amount of oxazepam produced from the two enantiomers was monitored. In this way, the preparation of the membranes can be optimized and, further, the receptor affinity data can be rationalized considering the actual amount of 3-hydroxy-BDZ present (under the same experimental binding conditions, IC_{50} of **1** was 26 ± 2.6 nM). This procedure could provide reliable methodology for investigating the relationship between stereochemistry and pharmacological activity of these prodrugs.

Hydrolysis of the esters

The hydrolysis caused by brain esterases has already been examined [7, 8] for compounds **3** and **6** as the racemic form. A BDZ concentration close to that suitable for the binding test was used and the experiment employed ^{14}C-labelled esters and TLC. In addition, using concentrated solutions and CD spectroscopy, the enantiomer differentiation of the hydrolysis reaction has been demonstrated [7, 8].

The chromatographic analysis of **3** and **6** in the presence of **1**, using the CSPs I and IV, respectively is shown in Fig. 2a, b. This procedure allowed the enantiomeric and the

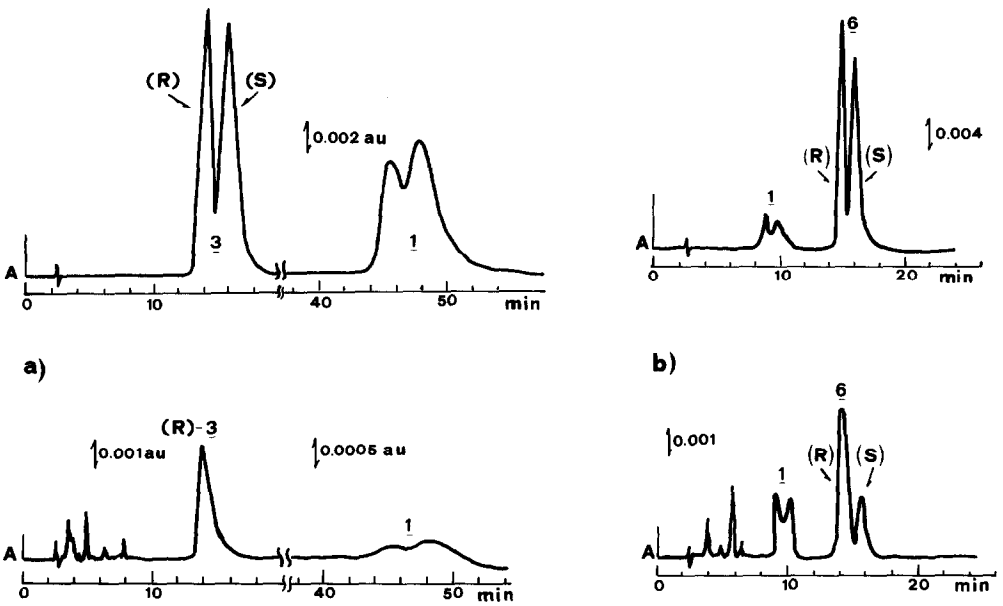


Figure 2
(a) Chromatographic analysis of **1** + **3** mixture, as standard solution (top) and as the mixture extracted from the synaptosomal membranes (10 μM of **3**, 3 h at 22°C, total BDZs injected estimated *ca* 3 μg) (bottom). (b) Chromatographic analysis of **1** + **6** mixture, as standard solution (top) and as the mixture extracted from the synaptosomal membranes (0.5 mM of **6**, 24 h at 22°C, total BDZs injected estimated *ca* 3 μg) (bottom).

chemical composition of the drug under investigation to be examined simultaneously; therefore, the chromatographic data can be used to monitor the extent of hydrolysis which occurs during the incubation with the membrane preparation.

The data obtained for **3** and **6** from the chromatographic determination of their enantiomeric composition and of their hydrolysis products are summarized in Table 3. The analysis was performed on the diethylether extraction of the incubation mixture, obtained under different conditions (i.e. temperature and time of incubation). These results show that at 4°C and after 45 min (binding conditions) the amount of **1** is low (from 5 to 10%). Moreover, differences can be observed in the extent of hydrolysis of the two enantiomers, in particular after longer incubation and at 22°C. For racemic **3**, after 3 h at 22°C only the peak of the (*R*)-enantiomer and the peaks of the hydrolysis product were observed (Fig. 2a), even if a low per cent of (*S*)-**3** cannot be excluded. The same experiment carried out on the pure (*R*)-**3** showed only 8% of **1**. Under the same conditions (3 h, 22°C) **6** showed quite a low content of **1** (Table 3). On the other hand, the analysis of the biotransformation utilizing a higher concentration of **6** (0.5 mM) and longer incubation times (up to 36 h) showed a high content of **1** and a significant enantiomer differentiation of the hydrolysis reaction (Table 3 and Fig. 2b).

The application of the above chromatographic method to monitor the extent of the hydrolysis reaction under the binding test conditions avoids the use of radioactive material. Furthermore, the method appears very promising for studying the enantiomer-differentiating biotransformation of the BDZ esters *in vitro* and, potentially, it could also be a useful tool for the study of BDZ metabolism *in vivo*.

Table 3
Hydrolysis reaction of the esters during the incubation with synaptosomal fractions

Compound*	Recovered BDZs†	45′ (4°C)	45′ (22°C)	3 h (22°C)
(*R*)-(*S*)-**3**	**1**	11%	{	36%
[10 μM]	(*R*)-**3**	50%		62%
	(*S*)-**3**	39%		
(*R*)-**3**	**1**	5%		8%
[10 μM]	(*R*)-**3**	95%		92%
(*R*)-(*S*)-**6**	**1**	8%	8%	12%
[12 μM]	(*R*)-(*S*)-**6**	92%	92%	88%
(*R*)-**6**	**1**	6%	7%	n.d.
[12 μM]	(*R*)-**6**	94%	93%	n.d.
(*S*)-**6**	**1**	9%	11%	10%
[12 μM]	(*S*)-**6**	91%	89%	90%
	(22°C)	6 h	24 h	36 h
(*R*)-(*S*)-**6**	**1**	7%	27%	36%
[0.5 mM]	(*R*)-**6**	50%	51%	50%
	(*S*)-**6**	43%	22%	14%

*The **1** content before incubation was, for all the compounds, ≤2%.
†Composition (%) of the recovered BDZs, evaluated by chromatographic analysis, for different experimental condition, i.e. temperature and time of incubation.

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