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High-performance liquid chromatographic resolution of racemic 1,4-benzodiazepin-2-ones by means of a β-cyclodextrin silica bonded chiral stationary phase

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SUMMARY

A bonded β-cyclodextrin chiral stationary phase was used for the chromatographic resolution of a series of 1,4-benzodiazepin-2-ones (BDZs). This column can also be employed for the direct resolution of ionic BDZs as the hemisuccinate esters of 3-hydroxy-BDZs. Nuclear magnetic resonance spectroscopy was used to obtain preliminary information on the mechanism of chiral discrimination.

INTRODUCTION

In terms of prescribed volume, anxiolytic agents are one of the most important classes of drugs. By far the most widely used subgroup of anxiolytics are the 1,4-benzodiazepin-2-ones (BDZs)[1], several of which are chiral. Accordingly, stereochemical aspects of this group have received wide attention, in relation to both the chromatographic resolution of enantiomers[2–5] and also pharmacological studies on the individual isomers[6–9]. For 3-hydroxy-BDZs (which account for the majority of the therapeutically used chiral BDZs), the pure enantiomers cannot be easily recovered because they undergo racemization in aqueous medium[10,11]. Therefore, the relationship between their stereochemistry and pharmacological activity has been not investigated. Several studies have been reported on the enantioselective interaction of the esters of 3-hydroxy-BDZs with the receptor binding site[12,13].

BDZs are known to interact with cyclodextrins[14]. Evidence for their stereoselective complexation arises from the induced circular dichroism observed in the 1:1 complex between β-cyclodextrin and an achiral or a racemic BDZ[15]. This observation prompted us to use a β-cyclodextrin-based chiral stationary phase (CSP)[16] for the

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chromatographic resolution of several chiral members of this important class of compounds. The commercially available CSP Cyclobond I was efficient in the chromatographic resolution of several chiral BDZs, the enantiomeric separation of which, on other CSPs, has been previously reported[2–5]. For the first time, resolution was obtained for ionic BDZs, *i.e.*, the hemisuccinate esters of 3-hydroxy-BDZs. Simultaneous monitoring of the related hydrolysis product was also achieved. In addition, separation of the enantiomers (α = 1.1) was observed for two BDZs (uxepam and dihydrodiazepam), which are chiral by virtue of an asymmetrically substituted C-5 carbon atom. Finally, a preliminary NMR study was carried out using an aqueous solution of β-cyclodextrin and the hemisuccinate esters of 3-hydroxy-BDZs as a model for the chromatographic system.

EXPERIMENTAL

Chromatographic separation

The chromatographic separations were carried out with a Jasco Twincle apparatus connected to a Jasco Uvidec 100 variable-wavelength UV detector. Circular dichroism (CD) detection was provided by a Jasco J-500C spectropolarimeter equipped with a high-performance liquid chromatographic (HPLC) micro-cell and a doublet of lenses to focus the light beam in the sample compartment[3,17]. The two detectors were connected in series and both used at a fixed wavelength (254 nm). The measurements were carried out using mixtures of acetonitrile and acetate buffer (pH 4.2, 200 m*M*), or acetonitrile and phosphate buffer (pH 7.0, 100 m*M*) mixtures, at an isocratic flow-rate of 0.5 or 1 ml/min. A Cyclobond I column (25 × 0.46 cm I.D.) from Astec (Whippany, NJ, U.S.A.) was used for all the measurements, which were performed at room temperature. The solvents were HPLC-grade chemicals and were filtered and degassed before use.

Instrumentation

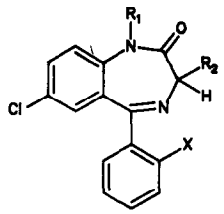
^{1}H NMR spectra were recorded on a Varian VXR-300 spectrometer operating at 300 MHz. All measurements were carried out in ^2H_2O solution at p^{2}H 7 and at 22°C.

Mass spectra were obtained using a VG 70-70E instrument. Melting points were determined with a Reichert Thermovar apparatus, and were uncorrected.

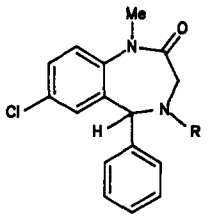
Preparation of the compounds

Compound **1**, **2** and **3** (see Fig. 1) were obtained by Soxhlet extraction (acetone) of commercial pharmaceutical formulations. The products obtained were characterized by NMR and mass spectrometry. The data were in accordance with the expected structure[18].

Compounds **4**, **5** and **10–13** were kindly provided by Prof. W. H. Pirkle (School of Chemical Sciences, University of Illinois at Urbana-Champaign, U.S.A.). Compounds **6**, **7** and **8** were prepared by acylation of **1**, **2** and **3**, respectively, with succinic anhydride in the presence of pyridine, according to the reported procedure for **6**[19]. The experimental conditions were changed for the recovery of the crude **6**, **7** and **8**. These compounds were extracted with chloroform from the hydrolysis mixtures and then recrystallized from ethyl acetate–hexane (1:1, v/v). Compounds **6**, **7** and **8** were



	R_1	R_2	X
1	H	OH	H
2	Me	OH	H
3	H	OH	Cl
4	H	OCOMe	H
5	H	$OCOBu^t$	H
6	H	$OCO(CH_2)_2COOH$	H
7	Me	$OCO(CH_2)_2COOH$	H
8	H	$OCO(CH_2)_2COOH$	Cl
9	H	OCOMe	Cl
10	H	Me	H
11	Me	Me	H
12	H	Pr^i	H
13	H	NEt_2	H



	R
14	H
15	$CONH_2$

Fig. 1. Compounds studied. Me = Methyl; Bu^t = *tert.*-butyl; Pr^i = isopropyl; Et = ethyl.

obtained (70–80% yield) as the crystalline powder having m.p. 152–154°C (lit.[19], 151–153°C), 170–172°C and 184–186°C, respectively. NMR and mass spectrometric data were in accordance with the structure.

The pure enantiomers of **6** were kindly provided by Ravizza (Milan, Italy). Compound **9** was a gift from Dr. E. Simon-Trompler (Central Institute for Chemistry, Budapest, Hungary). Compounds **14** and **15** were kindly provided by Chemical Works Gedeon Richter (Budapest, Hungary).

RESULTS AND DISCUSSION

Chromatographic data

The results for the resolution of **1–15** on Cyclobond I are reported in Tables I and II. Fig. 2 shows the separations of **5** and **15** as representative examples. Separation factors (α) up to 1.20 (Tables I and II) were observed for most of the compounds examined. The resolution was poor for **2**, **3** and **7** (Tables I and II): a broad peak was observed using the UV detection, whereas CD detection provided evidence of partial resolution and allowed the assignment of the elution order on the basis of the CD sign at 254 nm[3]. No resolution at all was obtained for **8**, **9**, **11** and **12**.

Several observations can be made regarding these results. Oxazepam (**1**) and its 3-ester derivatives (**4–6**) are well resolved (Table I). A relatively high on-column load (5–10 μg) was employed in order to monitor the CD signal and then to obtain direct stereochemical information. The efficiency of the CSP is affected by the on-column load and, under the experimental conditions adopted, resolution factors of 0.69 and

TABLE I

CHROMATOGRAPHIC RESOLUTION DATA ON CYCLOBOND I WITH ACETONITRILE–ACETATE BUFFER (pH 4.2) AS ELUENT AT 20°C

Compound	*Eluent (v/v)*	k'_1	α	*CD*[a]
1	15:85	2.5	1.17	–
2	10:90	5.0	1.0	–
3	10:90	4.9	1.0	–
4	10:90	4.0	1.15	–
5	15:85	10.2	1.15	–
6	15:85	4.9	1.08	–
7	15:85	6.6	1.0	–
8	15:85	7.6	1.0	
9	15:85	2.7	1.0	
10	10:90	6.6	1.04	–
11	10:90	8.5	1.0	
12	10:90	13.2	1.0	
13	10:90	2.7	1.05	–
14	10:90	3.5	1.08	+
15	10:90	6.0	1.10	+

[a] Sign of the CD at 254 nm for the first-eluted enantiomer.

0.75 were obtained for **5** (Fig. 2a) and **6** (Fig. 3), respectively. However, a baseline resolution can be achieved by using a one-tenth on-column load.

Temazepam (**2**) and its hemisuccinate ester (**7**) (which differ from the oxazepam analogues in that they have a methyl group in place of the hydrogen atom at N-1), exibit only partial chiral resolution on this CSP; separation is observed only by application of CD detection (Table I).

Lorazepam (**3**) and its 3-ester derivatives (**8** and **9**) show no chiral resolution at all on this CSP. The structure of these analytes differs from that of the corresponding oxazepam analogues in the presence of a chlorine atom at C-2′ of the benzene ring linked at C-5 (Fig. 1).

TABLE II

CHROMATOGRAPHIC RESOLUTION DATA ON CYCLOBOND I WITH ACETONITRILE–PHOSPHATE BUFFER (pH 7.0) AS ELUENT AT 20°C

Compound	*Eluent (v/v)*	k'_1	α	*CD*[a]
1	90:10	4.0	1.20	–
5	90:10	22.5	1.14	–
6	90:10	3.6	1.10	–
	95:5	8.8	1.12	–
7	90:10	4.4	1.0	
	95:5	12.3	1.0	
8	90:10	4.3	1.0	
	95:5	11.6	1.0	
14	90:10	5.5	1.10	+
15	90:10	5.3	1.11	+

[a] Sign of the CD at 254 nm for the first-eluted enantiomer.

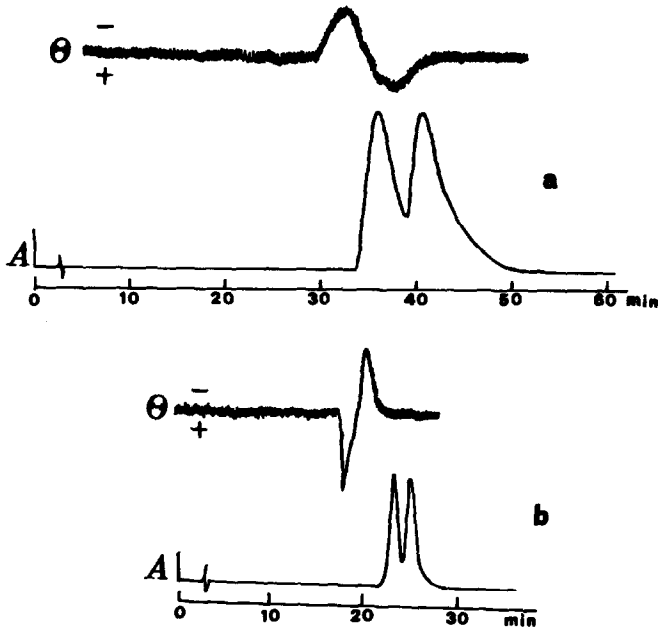


Fig. 2. Enantiomeric separation of **5** (a) (resolution, R_s = 0.69) and **15** (b) (R_s = 0.75) on Cyclobond I. UV (A) and CD (θ) detection at 254 nm. Eluent, acetonitrile–acetate buffer, pH 4.2 (15:85, v/v) (a) or (10:90, v/v) (b); 1 ml/min flow-rate.

1,4-BDZs which have the asymmetric carbon at the 5-position (dihydrodiazepam, **14** and uxepam, **15**) are resolved on the β-cyclodextrin-based CSP (Table I). The first-eluted enantiomers of **14** and **15** have a positive CD at 254 nm and correspond to (+)-(*S*)-dihydrodiazepam and (+)-(*R*)-uxepam, respectively. Interestingly, they adopt the same conformation of the seven-membered BDZ ring[20].

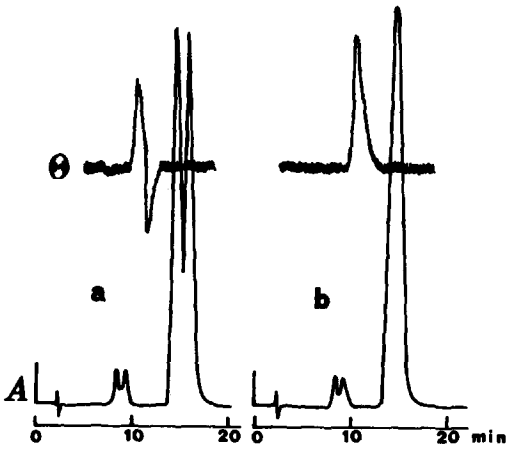
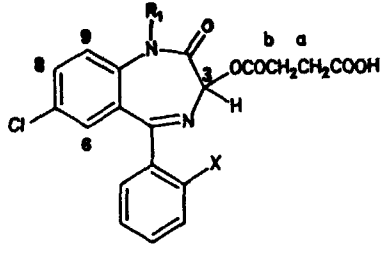


Fig. 3. Enantiomeric separation of **6** (a) (R_s = 0.75) and analysis of (*R*)-(−)-**6** (b) on Cyclobond I. IV (A) and CD (θ) detection at 254 nm. Eluent, acetonitrile–acetate buffer, pH 4.2 (15:85, v/v), 1 ml/min flow-rate.

TABLE III

VARIATION OF THE ^1H NMR CHEMICAL SHIFTS OF THE TWO ENANTIOMERS OF **6**, **7** AND **8** (5 m*M*) INDUCED BY INTERACTION WITH *β*-CYCLODEXTRIN (10 m*M*), ^2H_2O SOLUTION, PHOSPHATE BUFFER, p^{2}H 7.0



6: R_1 = H; X = H
7: R_1 = CH_3; X = H
8: R_1 = H; X = Cl

	6[a]			**7**		**8**	
Proton	$\Delta\delta(+)$	$\Delta\delta(-)$	$\lvert\delta(+)-\delta(-)\rvert$	$\Delta\delta(+)$, $\Delta\delta(-)$[b]	$\lvert\delta(+)-\delta(-)\rvert$	$\Delta\delta(+)$, $\Delta\delta(-)$[b]	$\lvert\delta(+)-\delta(-)\rvert$
a	−0.014	−0.003	0.011	−0.003, −0.003	0	+0.003, +0.003	0
b	+0.053	+0.048	0.005	+0.024, +0.033	0.009	+0.021, +0.021	0
3	−0.013	−0.013	0	−0.031, −0.023	0.008	N.d. N.d.	0
6	−0.109	−0.118	0.009	−0.083, −0.073	0.010	−0.048, −0.062	0.014
8	+0.065	+0.035	0.030	+0.033, +0.043	0.010	+0.042, −0.035	0.007
9	+0.088	+0.052	0.036	N.d. N.d.		+0.048, +0.056	0.008
CH_3				+0.030, +0.042	0.012		

[a] $\Delta\delta(+)$ = δ(complexed)−δ(free) for the (+)-enantiomer; $\Delta\delta(-)$ = δ(complexed)−δ(free) for the (−)-enantiomer; $\lvert\delta(+)-\delta(-)\rvert$ = absolute value of the chemical shift difference of the two enantiomers in the complexed form.

[b] For **7** and **8** only the racemates were available; therefore, it was not possible to distinguish the shifts due to (+)- or (−)-enantiomers.

This CSP allows the analysis of ionic BDZs, *i.e.*, the hemisuccinate esters of 3-hydroxy-1,4-benzodiazepin-2-ones (**6–8**) without derivatization of the carboxyl group.

This column appears to be particularly useful for the analysis of the 3-ester derivatives, because it allows the possibility of simultaneously monitoring the presence of their hydrolysis products. This method may be used to monitor the possible occurrence of hydrolysis during receptor binding studies[21]. Significant differences have been observed in the receptor affinity values of the esters of **1**, depending on the preparation of the synaptosomal membrane, *i.e.*, depending on the presence of brain esterases[22]. As shown in Fig. 3, simultaneous measurement of the chemical and optical purity of **6** is possible[21]. The resolution can be improved by injecting smaller amounts of sample[21], although under these conditions CD detection became impracticable. In Fig. 3a the chromatographic analysis of racemic **6** is reported and the resolution peaks of the hydrolysis product, *i.e.*, **1**, is present at a shorter retention time. Interestingly, we can observe the racemization of **1**; in Fig. 3b, the chromatographic analysis of (*R*)-(−)-**6** is reported, and again two peaks are observable at shorter retention times, owing to the presence of racemic **1**.

NMR analysis

To elucidate the nature of the interaction between the substrate and the chromatographic support, we carried out an investigation by ^{1}H NMR spectroscopy of the complexes formed between the BDZ hemisuccinate esters, **6–8** and β-cyclodextrin, a system which can be assumed to be a free solution model of the CSP.

^{1}H NMR spectra (Table III) of the three BDZs were recorded in ^2H_2O solution at p^{2}H 7 (phosphate buffer, 22°C) in the free state and in the presence of a 2-fold molar excess of β-cyclodextrin. Spectral analysis led to the following observations.

Variations of the chemical shifts of proton nuclei of **6–8** are caused by interaction with the cyclodextrin. This observation provides evidence for the formation of inclusion complexes between cyclodextrin and benzodiazepine derivatives. The changes in the chemical shifts are different for the two complexed enantiomers. With **6**, for which the two pure enantiomers were available, the (+)-enantiomer showed a larger complexation shift than the (−)-isomer. This indicates that the former is more tightly complexed within the cyclodextrin cavity than is the latter. This result agrees with the data for elution order, (+)-**6** being the more retained antipode on the β-cyclodextrin column. Both the actual molecular location of the protons which differentiate in the diastereoisomeric pair and the magnitude of the diastereoisomeric shift observed are different for **6**, **7** and **8** (Table III).

It is the aromatic protons of **6–8** that are the most affected by interaction with β-cyclodextrin, the diastereoisomeric shift being larger for these protons than for those bonded to the alkyl chain. On this basis, it is reasonable to postulate that the three hemisuccinate esters enter the cyclodextrin torus by means of the hydrophobic aromatic nuclei. The magnitude of the above effects follows the trend **6** > **7** > **8**. Therefore, **6** (both enantiomers) forms more stable inclusion complexes with β-cyclodextrin than does **7** or **8**, at least under these experimental conditions.

The mode of interaction between the polar groups of the cyclodextrin and those of the BDZs are probably different in all three instances. On interaction of racemic **6** with β-cyclodextrin, duplication of the alkyl chain protons is observed, whereas the

proton H-3 bonded to the chiral centre does not differentiate. It therefore appears that the carboxyl group of **6** is probably involved in a electrostatic interaction with external OH groups of the β-cyclodextrin molecule. With **7** no relevant differences are found in the chemical shifts of alkyl chain protons, whereas the H-3 and N-methyl resonances duplicate in the two complexed enantiomers. With **8**, duplication of resonances is not observed for alkyl chain protons or the H-3 proton.

CONCLUSIONS

The β-cyclodextrin-based CSP was efficient in the chromatographic resolution of a series of 1,4-benzodiazepin-2-ones. This method is particularly useful for ionic BDZs (*i.e.*, the hemisuccinate esters of 3-hydroxy-BDZs), which can be analysed without derivatization of the carboxyl group. This method also provided a means of assessing the degree of hydrolysis that may occur when esters are used in *in vitro* pharmacological studies.

On the basis of the chromatographic data for the hemisuccinate esters of the 3-hydroxy-BDZs and of the NMR data for the soluble model, the chiral discrimination of β-cyclodextrin appears to be related to the interaction of the aromatic moiety of the substrate with the hydrophobic cavity of the oligosaccharide and of the hemisuccinic chain with the hydrophilic residues outside the cavity.

ADDITIONAL NOTE

A successful optical resolution of 3-hydroxy-BDZs was reported using β-cyclodextrin as a mobile phase additive[23]. In the same paper, partially successful attempts were made to resolve the enantiomers of 3-hydroxy-BDZs on a β-cyclodextrin-based CSP.

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