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Direct separation of 4-amino-3-(4-chlorophenyl)butyric acid and analogues, $GABA_B$ ligands, using a chiral crown ether stationary phase

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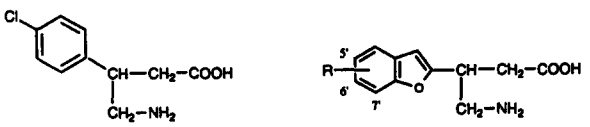
ABSTRACT

The direct resolution of baclofen (β-*p*-chlorophenyl-γ-aminobutyric acid) and a series of four analogues was achieved by HPLC on an enantioselective crown ether column, CR(+). Chromatography was carried out with perchloric acid as mobile phase and methanol as organic modifier. The effects of temperature, pH, eluent composition and substituents are discussed. The absolute configuration is attributed. The best results were obtained with baclofen ($\alpha = 1.48$; $R_s = 8.07$).

INTRODUCTION

The neutral amino acid γ-aminobutyric acid (GABA) is an inhibitory neurotransmitter concerned with the control of neuronal activity in the mammalian central nervous system and with the regulation of many physiological mechanisms [1]. Within the central and peripheral nervous systems, GABA has been shown to act through at least two distinctly different receptor sites [2]. These are termed $GABA_A$ and $GABA_B$ receptors, with different binding properties [3,4]. Until now, β-*p*-chlorophenyl-GABA (baclofen) was the only selective agonist for the $GABA_B$ receptor. Analogues of baclofen have been synthesized and tested for $GABA_B$ receptor affinity [5,8]. The enantiomers of baclofen were found to have different properties, the (*R*)-(−)-enantiomer being about 100 times more active than the (*S*)-(+)-enantiomer [3,6]. We recently described [7] potent and specific $GABA_B$ receptors antagonists [5]: β-(substituted benzo[*b*]furanyl)GABA (**1–4**), analogues of baclofen, now commercially available as racemates (**1** and **2** from Tocris Neuramin, Langford, Bristol, UK) (Fig. 1).

Stereoselective analyses of mixtures of enantiomers of amino acids, such as GABA, have largely employed high-performance liquid chromatography (HPLC) [9]. Separation is possible directly through the use of chiral eluents [chiral solvating agents (CSAs)] or chiral stationary



baclofen

(1) R = H
(2) R = $(CH_3O)5'$
(3) R = $(CH_3O)6'$
(4) R = $(CH_3O)7'$

Fig. 1. Structures of baclofen and compounds **1–4**.

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phases (CSPs) or indirectly through derivatization, leading to diasteromers, with chiral reagents [chiral derivatizating agents (CDAs)].

Baclofen has been extensively studied in analytical chromatography using HPLC with UV [10,11], fluorimetric [12] or radioactivity [13] detection, TLC [14], GC–MS [15] and GLC [16]. For optical resolution of the baclofen enantiomers, GC with electron-capture detection on a CSP [17], HPLC with UV detection on a Pirkle-type CSP after achiral derivatization [18], UV detection on a normal phase after chiral derivatization [19], fluorimetric detection after chiral derivatization [20,21], radioactivity detection [22] using a CSA and polarimetry on a CSP [18] have been described. A recently introduced phase uses a chiral crown ether moiety as chiral selector to resolve amino acids; discrimination between enantiomers relies on the formation of two diasteroisomeric inclusion complexes between the ammonium ion moiety of the amino acid and the chiral crown ether entity of the stationary phase. An acidic mobile phase ensures the protonation to form the ammonium ion which fits in the cavity. Perchloric acid is recommended because of the better resolution and low UV absorption. In this paper we describe the direct separation of baclofen and an optimization study on compounds **1–4**.

EXPERIMENTAL

Chromatography

Analytical HPLC was carried out with an LKB Model 2249 metering pump. Detection was performed with a Hewlett-Packard HP 1040 photodiode-array spectrophotometer connected to an HP 9000 S300 computer. The detection wavelengths were 200, 220 and 225 nm. The column was a 150 × 4 mm I.D. Crownpack CR(+) (5 μm) column (Daicel Chemical Industries, Baker, Paris, France). The sample loop was 10 μl and was made using a Rheodyne Model 7125 injector. Elution was carried out isocratically using perchloric acid as the mobile phase diluted to obtain the required pH. An organic modifier (methanol) was included in the mobile phase. The flow-rate was 0.9 ml/min. The temperature of the column was controlled by circulating water through a jacket surrounding the column. Temperature was measured in the water-bath and was in the range 10–40°C.

Reagents and materials

Baclofen was kindly supplied by Ciba-Geigy. Compounds **1–4** were prepared as described previously [7]. Water was purified through a Milli-Q unit (Millipore). Methanol was of gradient grade from Merck and perchloric acid was of analytical-reagent grade from Prolabo. The required pH was obtained after dilution as described in the technical notice: concentrated acid (70%, 13.6 g) was diluted with 1 l of water to give a solution of pH 1, and further dilutions furnished solutions of pH 2 (100 ml to 1 l), pH 1.3 (500 ml to 1 l) and pH 1.1 (794 ml to 1 l). All the solutions were filtered (0.45 μm), degassed and purged with helium. All amino acids were dissolved in the mobile phase to a concentration of about 1.6 m*M* (which corresponds to $1.6 \cdot 10^{-8}$ mol injected) and passed through a 0.45-μm membrane filter prior to injection.

RESULTS AND DISCUSSION

The enantiomeric separation of baclofen and compounds **1–4** on the crown ether CSP is summarized in Table I. For baclofen adequate resolution can easily be achieved (Figs. 2 and 3). The lower the temperature, the better the resolution becomes (α and R_s increase). Moreover, a high temperature (40°C) and addition of methanol (10%) give excellent results. A faster run could be obtained with sufficient resolution and enantioselectivity, but the limiting parameter was the column pressure [<2200 p.s.i. (1 p.s.i. = 6894.76 Pa)]. The designation of k'_S and k'_R as the first and second peaks under similar conditions was verified by chromatographing authentic samples of the enantiomers of baclofen (Fig. 3). The elution of the *R* isomer prior to the *S* isomer was the expected order of elution for almost all amino acids on the CR(+) column (Daicel information). Using the crown ether phase we were able to determine the minor enantiomer of baclofen at levels of less than 1%.

For the (benzo[*b*]furanyl)-GABA compounds **1–4**, addition of methanol as an organic modifier

TABLE I

ANALYTICAL HPLC: CAPACITY FACTORS (k') SELECTIVITY OF RESOLUTION (α) AND RESOLUTION (R_s) OF BACLOFEN AND COMPOUNDS 1–4

Capacity factor $k' = (t_x - t_0)/t_0$, separation factor $\alpha = (t_2 - t_0)/(t_1 - t_0)$ and peak resolution $R_s = 2(t_2 - t_1)/(w_1 + w_2)$, where w = peak width at baseline, t_0 = retention time of an unretained compound and t_x retention times of compounds (S or R).

Compound	Mobile phase[a]	Temperature 30°C				Temperature 40°C			
		k'_S	k'_R	α	R_s	k'_S	k'_R	α	R_s
Baclofen	A	25.4	42.0	1.65	8.07	17.4	25.8	1.48	6.11
	B	8.4	16.1	1.92	8.01	7.2	11.3	1.57	5.78
1	B	37.57		1	–	20.75		1	–
	C	53.91		1	–				
	D	64.54		1	–				
2	B	53.74	60.43	1.12	1.99	29.16	32.62	1.12	1.75
3	B					28.68	30.87	1.07	1.06
4	B					31.32	33.58	1.07	1.21

[a] A = $HClO_4$ (pH 2); B = $HClO_4$ (pH 2)–CH_3OH (90:10); C = $HClO_4$ (pH 1.3)–CH_3OH (90:10); D = $HClO_4$ (pH 1.1)–CH_3OH (90:10).

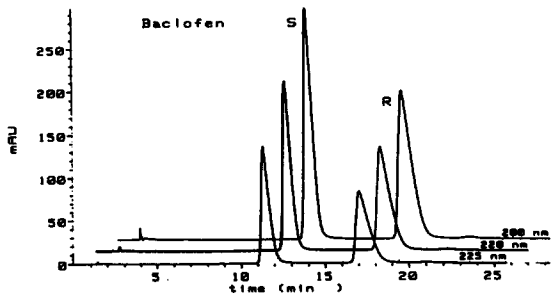

Fig. 2. Chromatograms of baclofen at three different wavelengths (225, 220 and 200 nm). Pseudo-three-dimensional representation. Eluent, $HClO_4$ (pH 2)–CH_3OH (90:10); temperature, 30°C; for other conditions, see Experimental.

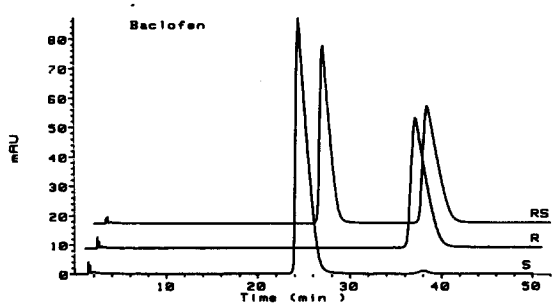

Fig. 3. Chromatograms of racemic (R,S)-baclofen and (R)- and (S)-enantiomers (λ = 225 nm). Pseudo-three-dimensional representation. Eluent, $HClO_4$ (pH 2); temperature, 40°C; for other conditions, see Experimental.

and an increase in temperature were necessary to decrease the capacity factors k'. Under the same eluting conditions (40°C, eluent B), **1–4** are much more retained (k'_S = 20.75, 29.16, 28.68 and 31.32, respectively) than baclofen (k'_S = 7.2). These compounds should be more hydrophobic than baclofen but the literature data for the log P values of chlorophenyl and benzofuranyl moieties are very similar (2.81 and 2.67 respectively, where P is the partition coefficient measured in n-octanol–water) [23]. With **1–4**, hydrogen bonding is possible between the ammonium group and the oxygen of the benzofuran ring [24], which could perhaps explain the increase in retention times. Compound **1** (without a methoxy group) is much less retained than **2–4** but poor resolution (shoulder) was observed even at low temperature (10 or 20°C) and low pH (1.1), where the product seems to be definitely retained on the stationary phase. These differences in retention behaviour are almost certainly due to differences in the hydrophobic nature between **1** (k' = 20.75) and **2–4** (k'_S = 29.16, 28.68 and 31.32, respectively) (40°C, eluent B) (Fig. 4) and **1** (k' = 37.57) and **2** (k'_S = 53.74) (30°C, eluent B). This may be due to the hydrophobicity of the methoxy moiety [23].

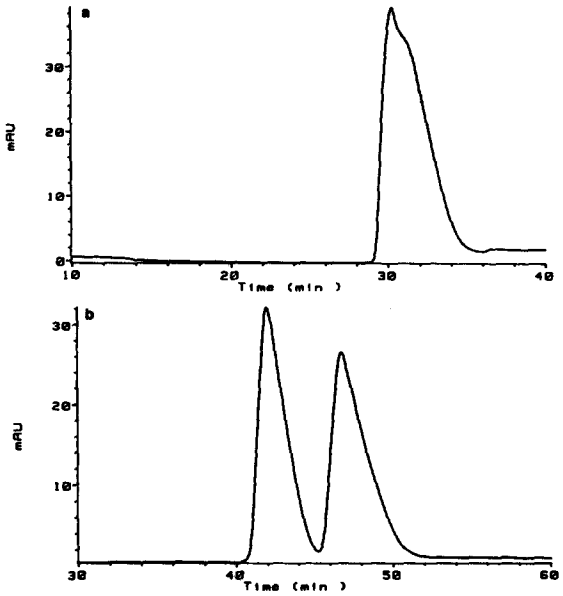

Fig. 4. Chromatograms of (a) **1** and (b) **2** ($\lambda = 225$ nm). Eluent, $HClO_4$ (pH 2)–CH_3OH (90:10); temperature, 40°C; for other conditions, see Experimental.

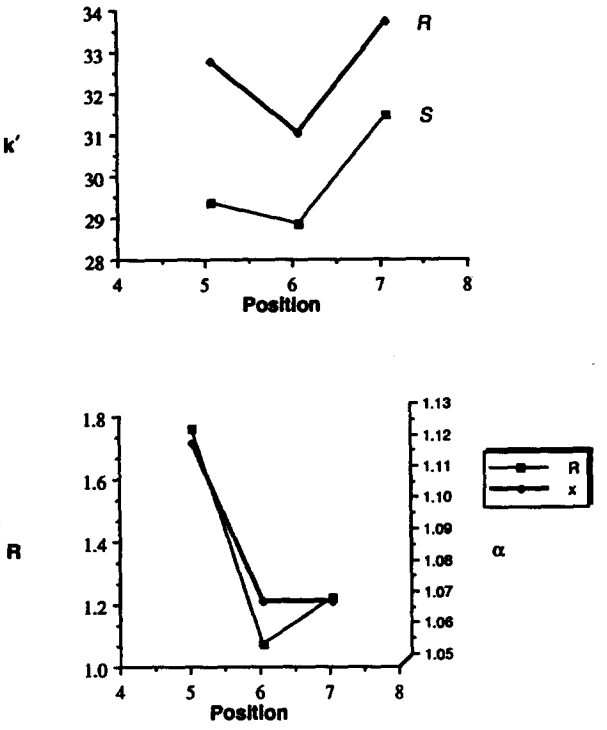

Fig. 5. Variations of the capacity factor (k'), enantioselectivity (α) and resolution (R_s) with the methoxy position in compounds **2–4**.

Steric bulk may be another factor that affects the separation; it can either enhance or decrease the enantioselectivity. This depends on whether or not a bulky group prevents the ammonium group from forming a strong inclusion complex. The presence of the methoxy moiety on the heteroaromatic ring seems necessary to observe separation (**1**, $\alpha = 1$) (Fig. 4) and the position of this group changes the enantioselectivity (**2–4**, $\alpha = 1.12$, 1.07 and 1.07, respectively) (40°C, eluent B). The 5-substitution seems more favourable (Fig. 5). The data in Table I also show that **1** is progressively more retained with decreasing pH.

Preparative separation of the enantiomers of baclofen was easily achieved by HPLC after two-step derivatization [19]. Hydrolysis of the diastereoisomers obtained led to the corresponding enantiomers, whose optical purity was measured by chiral HPLC. The same procedure was applied to the benzo[*b*]furanyl-GABA compounds **1–4**. Attribution of the absolute configuration of their diastereoisomers was made by comparison with baclofen diastereoisomers (relative ^1H and ^{13}C NMR chemical shifts, analytical HPLC) [25]. After hydrolysis the recovered enantiomers have a configuration fully in accordance with the order of elution; the least and most retained enantiomers are *S* and *R*, respectively. The good separation of the optical isomers of **2** makes the chromatographic method suitable for determining optical purity and for studies on pharmacological distribution.

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