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Preparative resolution of saclofen and hydroxysaclofen with analytical-scale high-performance liquid chromatography

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Abstract

The preparative enantiomeric resolution of two analogues of baclofen: saclofen and hydroxysaclofen, potent γ-aminobutyric acid$_B$ ($GABA_B$) antagonists, was obtained by HPLC with an analytical crown ether column (CR+) using isocratic conditions and multiple repetitive injections. The preparative separation was optimized by adjusting the sample size, by detecting the sample where its UV absorbance was low and from a scale-up of the analytical method. The analytical separation was modified to provide a convenient method for the isolation of the two enantiomers. The best analytical results obtained were $\alpha=3.68$, $R_s=20.96$ with H_2O–CH_3OH (90:10) at 0.5 ml/min and 20°C for hydroxysaclofen. Suitable fractionation allowed the isolation of pure (>99%) enantiomers for pharmacological testing.

Keywords: Enantiomer separation; Preparative chromatography; Saclofen; Hydroxysaclofen

1. Introduction

The inhibitory neurotransmitter γ-aminobutyric acid (GABA) acts through at least two distinctly different receptor sites named $GABA_A$ and $GABA_B$ receptors [1,2]. Baclofen [4-amino-3-(4-chlorophenyl)butyric acid], one of the only selective agonists for the $GABA_B$ receptor [3], presents stereospecific biological properties: its *R*-enantiomer being much more potent than its *S*-enantiomer [4].

Phaclofen [3-amino-2-(4-chlorophenyl)propylphosphonic acid] (**2**), saclofen [3-amino-2-(4-chlorophenyl)propylsulfonic acid] (**3**) and hydroxysaclofen [3-amino-2-(4-chlorophenyl)-2-hydroxy-propylsulfonic acid] (**4**) (Fig. 1) have been recently developed by changing the carboxylic acid group to a phosphonic or a sulfonic one to furnish potent $GABA_B$ antagonists. They are currently used as biological probes to understand the functions of the receptor. These compounds all present a chiral center at C-3. To investigate the pharmacodynamic properties of each enantiomer, in a first step quantities in excess of 5–10 mg of the relevant compounds are generally required and chiral HPLC [11] has been recognized as a useful methodology for this purpose offering the advantage of furnishing both enantiomers. In previous papers we recently described the analytical enantiospecific chromatographic separation of the agonist baclofen (**1**) [5] and of the antagonists **2**, **3**, and **4** [6] with a chiral crown ether moiety (CR+) as chiral selector and perchloric acid as mobile phase.

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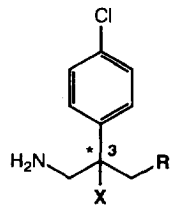

(1)	R = CO_2H	X = H	baclofen
(2)	R = PO_3H_2	X = H	phaclofen
(3)	R = SO_3H	X = H	saclofen
(4)	R = SO_3H	X = OH	hydroxy-saclofen

Fig. 1. Chemical structures.

In this article we report the extension of this work to the preparative direct enantiomeric separation of the potent molecules **3** and **4**. First the analytical method was modified with the use of water or water–methanol as mobile phase; then a direct scale-up from the analytical to the preparative mode was achieved by only slight changes in the chromatographic conditions [13,14].

2. Experimental

2.1. Reagents and materials

Compounds **3** and **4** have been purchased from Tocris–Cookson (Langford, Bristol, UK). Water used was purified through a Milli-Q unit. The methanol was gradient grade from Merck. The perchloric acid was of analytical grade from Prolabo. All the solutions, were filtered (0.45 μm), degassed and purged with helium. The pH of $HClO_4$ was 2. The mobile phases used were: eluent A, H_2O (100); eluent B, H_2O–CH_3OH (90:10); eluent C, $HClO_4$ (100); eluent D, $HClO_4$– CH_3OH (90:10) and they were prepared as previously described [6].

2.2. Analytical chromatography

Chromatographic resolution was accomplished with a LKB Model 2249 metering pump and the detection with a HP 1040 photodiode array spectrophotometer connected to a HP 9000 S300 computer. The column eluate was monitored at 200, 220, and 225 nm (4-nm bandwidth) with a reference at 550 nm (100-nm bandwidth). The column was a 150 × 4 mm I.D. Crownpak CR(+) (5 μm) column (Daicel Chemical Industries, Baker, France). The sample loop was 10 μl and was made using a Rheodyne 7125 injector. Mobile phase elution was made isocratically and the flow-rate was 0.5 or 0.9 ml/min. The temperature of the column was controlled by the circulation of water through a jacket surrounding the column. The temperature was measured in the water bath.

2.3. Preparative chromatography

Preparative chromatographic resolutions were run with the same column and instrumentation that was described for the analytical work. The column eluate was monitored at 220, 225, 230, and 254 nm (4-nm bandwidth) with a reference at 550 nm (100-nm bandwidth) in order to prevent detector saturation. The apparatus was equipped with a 1-ml loop. The racemic compounds **3** and **4** were dissolved in water (ca. 4 mg/ml) by heating and ultrasonication and then filtered through a 0.45-μm membrane prior to loading.

3. Results and discussion

The analytical HPLC data are summarised in Table 1. For **3** and **4** adequate resolution can be easily achieved using a H_2O–CH_3OH (90:10) solution as mobile phase. An example of an analytical chromatogram (λ = 225 nm) for compound **4** is given at a 0.9 ml/min flow-rate and 40°C temperature (Fig. 2). The comparison is made with pure enantiomers obtained after preparative separation. The designation of k'_S and k'_R for baclofen as the first and the second peaks respectively was proposed by analogy with the chromatography, under similar conditions, of authentic samples of its enantiomers [5,6] and has been recently confirmed with phaclofen whose preparative chromatographic resolution has been recently published [7]. Thus, following Cahn–Ingold–Prelog rules, first and second peaks are k'_S and k'_R for **3** but k'_R and k'_S for **4**. Regulation of

Table 1
Analytical HPLC: retention times (t, min) capacity factors (k') selectivity of resolution (α) and resolution (R_s) of **3** and **4**

Compound	Eluent	Flow-rate (ml/min)	Temperature 20°C						Temperature 40°C					
			t_1	k'_1	t_2	k'_2	α	R_s	t_1	k'_1	t_2	k'_2	α	R_s
3	A	0.5	20.74	7.7	44.19	17.6	2.28	10.62						
	A	0.9	11.67	7.8	24.82	17.7	2.27	9.38						
	B	0.5	14.16	4.9	31.79	12.4	2.50	14.67						
	B	0.9	7.76	4.8	17.58	12.2	2.53	10.10	4.40	2.3	7.25	4.4	1.93	4.60
	C	0.9							7.60	4.7	12.12	8.1	1.72	4.79
	D	0.9							5.08	2.6	8.20	4.9	1.85	6.04[a]
4	A	0.5	17.26	6.5	50.37	20.9	3.22	13.70						
	A	0.9	9.72	6.4	28.18	20.3	3.20	11.04						
	B	0.5	12.28	4.2	38.84	15.3	3.68	20.96						
	B	0.9	6.82	4.1	21.65	15.3	3.70	12.04	3.75	1.8	7.66	4.8	2.61	6.81
	C	0.9							6.71	3.9	12.96	8.5	2.17	6.12
	D	0.9							4.69	2.4	9.30	5.7	2.40	7.92[a]

Eluent A: H_2O (100); eluent B: H_2O–CH_3OH (90:10); eluent C: $HClO_4$ pH 2 (100); eluent D: $HClO_4$ pH 2–CH_3OH (90:10).
Concentration ca. 1.6 m*M* (ca. 4–5 mg/10 ml) equivalent to 16 nmoles (ca. 4 μg) injected in a 10-μl loop.
[a] Ref. [6].

retention and resolution can be made either by addition of an organic modifier to the mobile phase, or by varying the pH or the temperature.

The enantioselectivity factor (α) and resolution (R_s) slowly increase while significant retention factors (k') reduction are observed with increased concentration of methanol [8,10]. The lower the temperature is, the better the resolution and enantioselectivity become (α, R_s increase); as might be expected the resolution increased at the expense of lengthened retention times as well as broadened peak shape [8–10]. The resolution (R_s) and the retention factors (k' show a decrease with increasing pH (comparison between eluent D and eluent B) while the enantioselectivity factor (α) increases [8,10]. It is claimed that an acidic mobile phase is necessary to assure protonation both (i) of the carboxylic acid group to avoid repulsion by the electronegative oxygen-containing crown ether ring and (ii) of the primary amino group to form the ammonium ion which fits into the cavity of the crown ether to form two diasteroisomeric host–guest complexes which induce discrimination between enantiomers. In the present study the acidic mobile phase does not seem

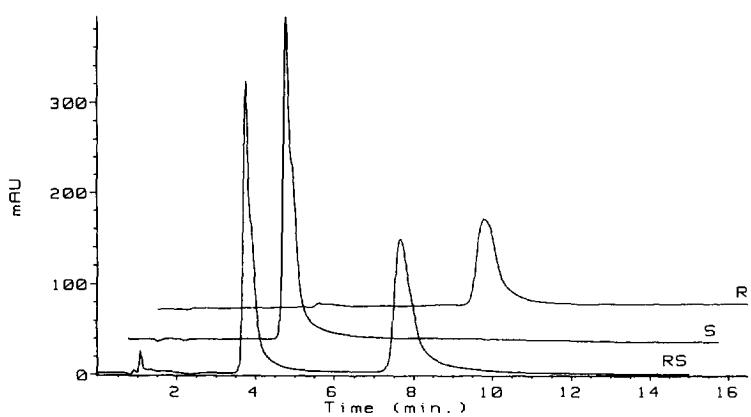

Fig. 2. Analytical chromatographs of racemic (*R,S*)-**4**; (*S*)-**4**; (*R*)-**4**. (λ=225 nm). Eluent, H_2O–CH_3OH (90:10); temperature, 40°C; flow-rate, 0.9 ml/min; concentration, ca. 1.6 m*M* (ca. 4–5 mg/ 10 ml) equivalent to 16 nmoles (ca. 4 μg) injected; pressure, 1200 p.s.i. (the chromatograms are displaced 0.8 min from each other). The *R*-enantiomer eluted first.

to be essential since, in all cases, the chromatographic separations remain very satisfactory.

When developing an analytical method for scale-up to the preparative mode [11,13,14] it is desirable to have a resolution factor, R_s, greater than 2, an enantioselectivity factor, α, greater than 1.2 and a retention factor, k', of less than 10, the latter because of the shorter run times obtained and the consequent increase in the throughput (mg/h of chemical) for the separation [11]. The preparative HPLC resolution of **3** and **4** can be easily achieved using H_2O as mobile phase: it provides the best compromise between resolution, separation time and solvent comsumption (and its consequent elimination). A chromatogram of a preparative separation of **4** (λ = multiwavelengths) is given at a 0.5 ml/min flow-rate and a temperature of 20°C (Fig. 3) with baseline resolution: the preparative retention times are 16 and 30 min (tops of the peaks), to be compared to the retention times (17 and 50 min) of the analytical method (For **3** the retention times are, respectively, 17 and 25 min in the preparative and 21 and 44 min in the analytical modes). This decrease, in the times corresponding to the tops of the peaks, is due to mass-overloading of the column. The theory demonstrates that the end times of elution are the same both in preparative and analytical loadings but that the retention times generally decrease with increasing loadings [13]. A good reproducibility during twelve injections over several days was observed. A loading-study showed that the maximum amount of racemate the column could tolerate without compromise of the resolution was around 4–5 mg for **4** (and 3–4 mg for **3**) which corresponds to the "touching-band" method. So a total of 50 mg of **4** was resolved by ca. 4-mg portions by elution with water. The locations of the cut points were determined by use of the detector response levels at multiwavelengths rather than time. The collected fractions from each of the two peaks were pooled and lyophilized to give white residues. The first peak furnished 25 mg (100% yield) and the second one, 23 mg (92% yield) (For **3** the yields were 100 and 80%, respectively). Following this simple work-up procedure, the values for the specific rotation [α] were +15.0 (*S*) and −13.3 (*R*), respectively [For **3** the [α] values were +13.7 (*S*) and −11.9 (*R*)]. They were determined by dissolving the samples in water at a concentration of c = 0.48% in a cell of 100-mm pathlength, at 18°C, using a Perkin-Elmer 241 polarimeter set at the sodium D-line (589 nm). Those results are comparable to literature data: for (*S*)-phaclofen (**2**), [α]=+15 [7]; for saclofen (**3**), [α]= +16.9 and −12.1, but in this last case the absolute configuration of the enantiomers, prepared through stereospecific synthesis, was not described [12]. Analytical determination of the enantiomeric purity of the isolated enantiomers of **3** and **4** were performed on the same column and materials. Fig. 2 illustrates the enantioseparation of racemate **4** and its

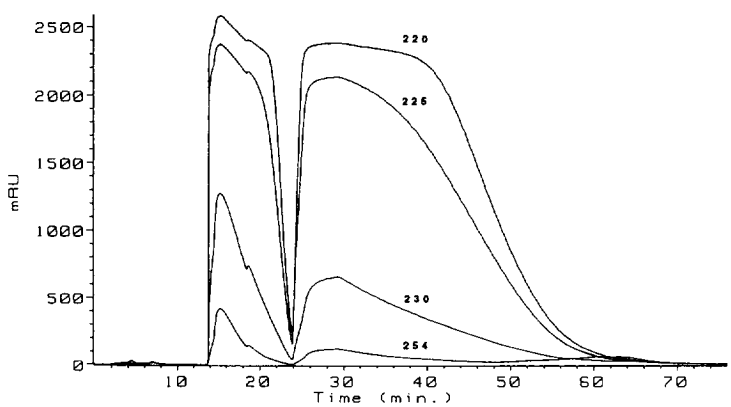

Fig. 3. Preparative chromatograph of **4** at multiwavelengths. Eluent, H_2O; temperature, 20°C; flow-rate, 0.5 ml/min; concentration, ca. 16 m*M* (ca. 4–5 mg/ml) equivalent to 16 μmoles (ca. 4 mg) injected; pressure 880 p.s.i.

comparison with its two enantiomers. Their structures were confirmed by ^{1}H and ^{13}C NMR spectroscopy at 300 MHz and were identical to their racemates. Bioassays of the resolved enantiomers are now under investigation. The *R*-(−)-saclofen **3** and the *S*-(−)-hydroxy-saclofen **4** are the active enantiomers [15].

The good separation of optical isomers of **3** and **4** offers a simple and direct approach to the provision of sufficient material for pharmacological testing. The high capacity of the CR column enabled the isolation of roughly 25 mg of each enantiomer from twelve successive injections within one hour. Moreover this method is suitable since it avoids the consequences due to the use of perchloric acid: (i) the equipment corrosion and (ii) the problems caused by the additives in the mobile phase which have to be removed from the desired solute after the separation.

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