Enantioseparation of baclofen with highly sulfated β-cyclodextrin by capillary electrophoresis with laser-induced fluorescence detection

BELIN-KAVRAN, Gamze, RUDAZ, Serge, VEUTHEY, Jean-Luc

Abstract
The enantioseparation of baclofen (4-amino-3-p-chlorophenylbutyric acid) was achieved by CE-LIF with highly sulfated β-CD (HS-β-CD) as chiral selector. Naphthalene-2,3-dicarboxaldehyde was used for the derivatization of nonfluorescent baclofen. HS-β-CD (2%) containing 50 mM borate buffer at pH 9.5 was chosen as the optimal running electrolyte and applied to the analysis of baclofen enantiomers in human plasma. The linearity of calibration curves (R² 0.998) for R-(−)- and S-(+)-baclofen was in the 0.1-2.0 M concentration range. After a simple ACN-protein precipitation, the LOD of baclofen in plasma sample was found as low as 50 nM.

Reference

DOI : 10.1002/jssc.200500100

Available at:
http://archive-ouverte.unige.ch/unige:3629

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Key Words: Baclofen; Capillary electrophoresis; Chiral separation; Highly sulfated β-cyclodextrin; Laser-induced fluorescence detection

Received: March 2, 2005; revised: May 3, 2005; accepted: May 6, 2005

DOI 10.1002/jssc.200500100

1 Introduction

CE has become a powerful separation technique especially for the chiral analysis of drug substances. For these, it is necessary to add a chiral selector into the running electrolyte. Native CDs [1–4], derivatized CDs [5–9], crown ethers [10, 11], and bile salts [12] are widely used as chiral selectors but CDs are preferred since they resolve a variety of drug substances. Furthermore, CDs are commercially available, UV transparent at short wavelength length usually used to monitor the enantiomers, cheap, and not toxic. In particular, successful applications of sulfated CDs to a wide range of analytes, including weak and strong acids and bases as well as zwitterions, have been reported by Evans and Stalcup [13].

Baclofen is a muscle relaxant and an antispastic agent working directly in the brain and spinal cord [14] and is generally administered as a racemate. However, since the $R$(-) enantiomer is more active and toxic than the $S$(-) enantiomer, the chiral separation of baclofen in biological fluids is very important in order to achieve an optimal therapeutic drug monitoring (TDM). The analytical methods reported for the determination of baclofen include GC [15, 16], HPLC [17–23], and CE [24, 25]. These techniques were coupled to MS [15, 23], electron capture [16], UV [17, 18], fluorescence [19, 20], electrochemical [21, 22], and LIF detection modes [24, 25]. All these methods were only used for the determination of baclofen racemate. For chiral analysis, different methods were developed such as GC with electron capture detection [26, 27] as well as HPLC coupled with fluorescence [28, 29], UV [30, 31], and MS detection [32]. As reported in the literature, there are only few papers dedicated to the chiral separation of baclofen by CE with derivatized CDs [33] and crown ethers [34, 35] in acidic media with UV detection. However, to our knowledge, there is only one study presenting LIF detection [36] and α-CD as chiral selector. In comparison to UV detection, LIF provides lower detection limits and better selectivity, thus increasing the applicability of CE to biological and clinical analyses. In this work, different chiral selectors such as HS-α-, β-, and γ-CD were investigated in the enantioseparation of racemic baclofen mixture by CE-LIF. The effects of organic modifiers, buffer concentration, nature, and concentration of HS-CDs were investigated. Developed electrophoretic conditions were finally applied to the analysis of baclofen enantiomers in human plasma.

2 Experimental

2.1 Instrumentation

CE experiments were performed on an Agilent HP 3000 CE system (Waldbronn, Germany) equipped with an on-column UV-visible diode-array detector and a LIF detector ZETALIF (Picometrics, Ramonville, France) connected to an Omnicrome (Chino, CA, USA) He-Cd laser source (20 mW, $\lambda_{\text{ex}} = 442$ nm, $\lambda_{\text{em}} = 500$ nm) (Fig. 1). A CE Chemstation (Agilent) was used for system control and analyses. Borwin data acquisition system (JMBs Developments, Grenoble, France) was used for data collection. The separation was performed in an uncoated fused-silica capillary (Composite Metal Service, Worcestershire, UK).
of 50 μm ID × 375 μm OD with a total length of 85 cm and an effective length of 60 and 22 cm for LIF and UV detections, respectively. Temperature was set at 25°C and capillaries were initially treated with 1 M NaOH, 0.1 M NaOH, deionized water, and running electrolyte for 30, 10, 5, 10 min, respectively. Injections were performed hydrodynamically for 6 s at 50 mbar (1 bar = 10^5 Pa). The applied voltage was set at 30 kV at the anodic side. Acetone was used as EOF marker.

2.2 Chemicals

*R-(–) Enantiomer of baclofen and its racemic form were obtained from Sigma (St. Louis, MO, USA). D-(+) Norephedrine · HCl, potassium cyanide (KCN), and acetone were purchased from Fluka (Buchs, Switzerland). Naphthalene-2,3-dicarboxaldehyde (NDA) was from Aldrich (Milwaukee, WI, USA). HS-α-, β-, and γ-CD (20% w/v) aqueous solutions were purchased from Beckman (Fullerton, CA, USA). Boric acid and NaOH were obtained from Acros Organics (NJ, USA) and Merck (Darmstadt, Germany), respectively. Methanol (MeOH), ethanol (EtOH), and ACN were of analytical grade and purchased from Panreac (Barcelona, Spain). Stock solutions of baclofen (2 mM), D-(+) norephedrine · HCl (2 mM), and KCN (20 mM) were prepared in 50 mM borate buffer at pH 9.5. NDA derivatization reagent (2 mM) was prepared in MeOH. Ultrapure water was supplied by a Milli-Q RG purification unit from Millipore (Bedford, MA, USA). The running electrolyte was borate buffer (50 mM) adjusted to pH 9.5 with 1 M NaOH and degassed in an ultrasonic bath for 10 min. Between runs, the capillary was flushed with the running electrolyte for 2 min.

2.3 Derivatization procedure

NDA was used as a fluorescent probe for the derivatization of nonfluorescent baclofen. It provides high complex stability and good sensitivity at 442 nm, especially for primary amine. Derivatization was performed in the dark in the presence of CN⁺, which acts as a nucleophilic agent [37]. Two micromolar (200 μL) racemic baclofen in a dye solution containing 0.4 mM (100 μL) KCN and 0.4 mM (100 μL) NDA in 50 mM borate buffer at pH 9.5 was kept at room temperature for 30 min to obtain stable fluorescence intensity for 24 h. Before injection, NDA-baclofen solution was diluted to the desired concentration with running electrolyte and filtered through a 0.45 μm pore-size microfilter (Supelco, Bellefonte, PA, USA). Figure 2 shows the derivatization reaction of baclofen with NDA.

2.4 Plasma sample

Plasma obtained from blood samples by centrifugation and stored at −20°C was deproteinized by centrifugation at 8500 × g for 15 min after the addition of ACN (1:1; v/v). Supernatant liquid (200 μL) was derivatized with 200 μL of the dye solution as described in Section 2.3 and filtered before injection. Plasma samples containing different amounts of baclofen and internal standard (IS) were prepared by spiking the plasma with a known amount of racemic baclofen and IS before the deproteinization step.

2.5 Calculation of separation parameters

Resolution ($R_s$) was calculated as

$$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2} \quad (1)$$

where $t_1$, $t_2$ and $w_1$, $w_2$ are migration times and peak widths of the enantiomers, respectively. Selectivity ($α$) was calculated as

![Figure 1. Instrumental design of CE-LIF.](image1)

![Figure 2. Derivatization reaction of baclofen with NDA.](image2)
\[ \alpha = \frac{\mu_{\text{eff}2}}{\mu_{\text{eff}1}} \]

where \( \mu_{\text{eff}2} \) and \( \mu_{\text{eff}1} \) are effective mobilities of the enantiomers obtained from the following equation.

\[ \mu_{\text{eff}} = \mu_0 - \mu_{\text{app}} = \frac{Ld}{V} \left( \frac{1}{t_0} - \frac{1}{t} \right) \]

Here, \( \mu_0 \) shows the electroosmotic mobility (EOM) measured with the injection of a neutral marker (acetone) and \( \mu_{\text{app}} \) shows the apparent mobility measured with the total length of capillary (\( L, \) cm), capillary length to the detector (\( L_d, \) cm), applied voltage (\( V, \) V), migration time of the neutral marker (\( t_0, \) s), and migration time of the analyte (\( t, \) s).

### 3 Results and discussion

#### 3.1 Effect of chiral selector

In this work HS-CDs were used as chiral selectors. A 0.0–4.0% concentration range of HS-CDs was prepared in borate buffer at pH 9.5. Indeed, Gu and Whang [25] showed that derivatization of primary amines was pH dependent and the best results were obtained at pH 9.5. For this reason, a borate buffer set at pH 9.5 was used as derivatization solvent and running electrolyte for a stable baclofen-NDA derivative and repeatable results. As reported in Fig. 3, preliminary investigations showed that no separation occurred with HS-\( \alpha \)-CD even at higher concentrations. HS-\( \gamma \)-CD was able to resolve baclofen enantiomers but HS-\( \beta \)-CD was found to be the most effective chiral selector for this separation. In Table 1, selectivity (\( \alpha \)) and resolution (\( R_s \)) values obtained by increasing HS-\( \beta \)-CD content in the buffer electrolyte are reported. The generated current was measured between 9 and 115 \( \mu \)A as a function of HS-\( \beta \)-CD concentration. By increasing the chiral selector concentration, baclofen enantiomers were better resolved according to the charged resolving agent migration (CHARM) model [38]. Anionic chiral additives presented a negative effective electrophoretic mobility because of their multiply negative charges, while the EOF at pH 9.5 was directed toward the cathodic end. Baclofen enantiomers interacted differently with the chiral selector and moved in the opposite direction of the EOF. As seen in Fig. 3, the addition of 2% HS-\( \beta \)-CD into the running electrolyte was found sufficient for the enantioseparation of a racemic baclofen mixture. Since HS-\( \alpha \)-CD has the smallest cavity, it might be too small to form baclofen complexes. Due to the larger cavity size of HS-\( \gamma \)-CD, baclofen

**Table 1.** Selectivity (\( \alpha \)) and resolution (\( R_s \)) values depending on HS-\( \beta \)-CD content

<table>
<thead>
<tr>
<th>HS-( \beta )-CD [%] v/v</th>
<th>( \alpha )</th>
<th>( R_s )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1.000</td>
<td>0.00</td>
</tr>
<tr>
<td>0.3</td>
<td>1.000</td>
<td>0.00</td>
</tr>
<tr>
<td>0.6</td>
<td>1.005</td>
<td>1.32</td>
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<tr>
<td>1.0</td>
<td>1.011</td>
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</tr>
<tr>
<td>1.5</td>
<td>1.016</td>
<td>2.32</td>
</tr>
<tr>
<td>2.0</td>
<td>1.024</td>
<td>4.22</td>
</tr>
<tr>
<td>3.0</td>
<td>1.030</td>
<td>7.05</td>
</tr>
<tr>
<td>4.0</td>
<td>1.035</td>
<td>8.00</td>
</tr>
</tbody>
</table>

**Figure 3.** Effect of HS-CDs on the enantioseparation of racemic baclofen mixture. Running electrolyte: (a) 2% HS-\( \gamma \)-CD, (b) 2% HS-\( \alpha \)-CD, and (c) 2% HS-\( \beta \)-CD containing 50 mM borate (pH 9.5). \( V = 30 \text{kV} \); injections: 6 s at 50 mbar; detection: LIF at 442 nm excitation and 500 nm emission wavelengths.
enantiomers gain a higher flexibility and cannot reach optimal interaction with the selector. For this reason, HS-β-CD allows the best enantioseparation.

3.2 Buffer concentration and applied voltage

Borate buffers (10, 20, 50, and 100 mM) containing 2% HS-β-CD at pH 9.5 were used to separate a racemic baclofen mixture. The change in the borate buffer concentration from 10 to 50 mM increased $R_s$ and $\alpha$ values. At the higher buffer concentration (100 mM), enantiomer migration times increased slightly while efficiencies decreased. Higher buffer concentration probably induced a more stable inclusion complex inside the hydrophobic cavity of the selector, as already described in the literature [39]. Therefore, 50 mM was finally chosen as the best concentration. The applied voltage was set at 30 kV. By decreasing the voltage, peak resolutions increased but enantiomer migration times and peak widths also increased while efficiencies decreased. Temperature was set at 25°C and was only partially controlled since a long part of the capillary was outside the CE instrument for the LIF connection, as seen in Fig. 1.

3.3 Effect of organic solvent

Organic solvent in BGE influences parameters such as buffer viscosity, charge and mobility of analytes, solvation, EOF, and electrostatic interactions between the chiral selector and the solute as well as the BGE components, etc. Thus, MeOH, EtOH, and ACN were added at a low concentration (0–10% v/v) to BGE. Table 2 shows the effect of these solvents on enantiomeric resolution. The organic modifiers in BGE caused a general increase of efficiency. Besides, organic solvents generally cause a decrease of analyte complexation with the CD due to the analyte–organic solvent competition in fitting the hydrophobic chiral selector cavity. However, in several cases, improvement of enantiomer resolution was observed [39–42]. By adding an organic solvent, the interaction between baclofen and HS-β-CD was reduced and migration times increased.

3.4 Migration order

Pure $R$-(−)-baclofen was used to confirm the enantiomeric separation order. With HS-β-CD, $R$-(−) enantiomer of baclofen migrated faster than $S$-(+)-enantiomer, indicating a strong interaction between $S$-(+)-baclofen and HS-β-CD while the migration order was inverted with HS-γ-CD (Fig. 4). As mentioned by Chankvetadze et al. [42], the reversal of the enantiomeric migration order (EMO), using CD with opposite chiral recognition ability, is a powerful technique. Furthermore, in the analysis of nonracemic mixtures of enantiomers, it is desirable to detect the minor component before the major one since the peak tailing does not allow the detection of enantiomeric impurity as the second peak even at high percentages [43]. In this case, reversal of EMO was simply obtained by adjusting the chiral selector cavity size. This possibility has already been demonstrated for the EMO reversal of 1-(9-fluorenyl) ethyl chloroformate (FLEC) isomers with β- and γ-CD [44]. Hydrogen-bonding opportunity between the oxygen atoms

Table 2. Selectivity ($\alpha$) and resolution ($R_s$) values depending on the organic modifier content

<table>
<thead>
<tr>
<th>ACN [%]</th>
<th>$R_s$</th>
<th>$\alpha$</th>
<th>MeOH [%]</th>
<th>$R_s$</th>
<th>$\alpha$</th>
<th>EtOH [%]</th>
<th>$R_s$</th>
<th>$\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.37</td>
<td>1.007</td>
<td>5</td>
<td>2.61</td>
<td>1.015</td>
<td>5</td>
<td>1.89</td>
<td>1.011</td>
</tr>
<tr>
<td>10</td>
<td>1.32</td>
<td>1.003</td>
<td>10</td>
<td>3.57</td>
<td>1.011</td>
<td>10</td>
<td>1.01</td>
<td>1.007</td>
</tr>
</tbody>
</table>

Figure 4. Migration order of baclofen enantiomers. Running electrolyte: (a) 2% HS-β-CD, (b) 2% HS-γ-CD containing 50 mM borate (pH 9.5). Other conditions are the same as in Fig. 3.
Enantioseparation of baclofen with highly sulfated \( \beta \)-cyclodextrin in acetal linkage of HS-CDs and the carboxyl group of \( S-(+) \)-baclofen enantiomer is probably more effective with HS-\( \beta \)-CD than HS-\( \gamma \)-CD [45].

### 3.5 Application in biological samples

Enantioseparation of a standard racemic baclofen mixture under the optimum conditions is shown in Fig. 3c. Figure 5 shows the applicability of this method to human plasma. \( D-(+) \)-Norephedrine \( \cdot \) HCl was used as IS to improve analysis precision and accuracy. Blank plasma sample (Fig. 5a) did not show any interference on the migration time of baclofen-NDA derivative and IS. Other plasma components were observed after the separation of baclofen enantiomers. In order to assess interindividual selectivity, three different types of plasma samples from healthy volunteers were investigated. All the detected plasma endogenous components migrated after the baclofen enantiomers without any interfering effect. Electrophoretic patterns of these three plasma samples were almost identical as in Fig. 5, except for the number and relative peak size of some extraneous plasma peaks. The observed baseline noise with plasma can be attributed to the different ionic strengths of the tested samples and/or temperature variability due to the experimental setup [46]. Most plasma components containing amino acids were removed by ACN-protein precipitation. After the simple sample preparation, LOD of baclofen was found as low as 50 nM (S/N = 3) and 30 nM (S/N = 3) in plasma and standard baclofen solution, respectively. RSD values on peak areas of a plasma sample spiked with 1.0 \( \mu M \) racemic baclofen were 4.8 and 5.3\% (\( n = 10 \)) for \( R-(--) \)- and \( S(++) \)-baclofen, respectively. RSD values on migration time were inferior to 0.4\% (\( n = 10 \)) for both enantiomers. Calibration curves for \( R-(--) \)- and \( S(++) \)-baclofen were achieved in the 0.1–2.0 \( \mu M \) concentration range after the replicate injection of the spiked plasma sample at eight different concentrations. Parameters for calibration curves in plasma were \( y = 15.637x - 90.936 \), \( R^2 = 0.9996 \) for \( R-(--) \)-baclofen and \( y = 15.49x - 91.153 \), \( R^2 = 0.9989 \) for \( S(++) \)-baclofen. In these equations, \( y \) shows normalized peak area, the ratio of migration time corrected peak area of analytes to IS, \( x \) the theoretical concentration of the spiked standards in \( \mu M \), and \( R^2 \) the determination coefficient. LOQ of baclofen racemate in plasma sample was found as 0.175 \( \mu M \) (S/N = 10). The mean recoveries of \( R-(--) \) and \( S(++) \)-baclofen in plasma sample spiked with 1.0 \( \mu M \) baclofen were 98 and 97\% (\( n = 4 \)), respectively. In comparison with published data [36] using native \( \alpha \)-CD, this work investigated the effect of HS-CD chiral selectors on the enantioseparation of baclofen and the method was applied to plasma samples. Results showed good enantiomer resolution and short analysis time without any disturbance from endogenous plasma components.

### 4 Concluding remarks

The enantioseparation of baclofen with HS-\( \beta \)-CD by CE-LIF was achieved within a short analysis time. NDA was used for the derivatization of nonfluorescent baclofen. Because of the high sensitivity afforded by the LIF detection, LOD and LOQ values in the analysis of baclofen in the plasma sample were very low. Furthermore, the simple sample preparation procedure with ACN-protein precipitation afforded a suitable method for routine analyses.

### Acknowledgment

We would like to thank Werner Kloeti (Laboratory of Mass Spectrometry, University of Geneva, Switzerland) for his technical support during the connection of LIF to the CE instrument.
5 References