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Enantiomeric separation of baclofen by capillary electrophoresis tandem mass spectrometry with sulfobutylether-β-cyclodextrin as chiral selector in partial filling mode☆

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ABSTRACT

Capillary electrophoresis (CE) coupled to tandem mass spectrometry was applied to the chiral separation of baclofen using sulfobutylether-β-cyclodextrin chiral selector in partial filling counter current mode. On-line UV detection was simultaneously used. Method optimization was performed by studying the effect of cyclodextrin and BGE concentration as well as sheath liquid composition on analyte migration time and enantiomeric resolution. The cyclodextrin showed stereoselective complexation towards baclofen enantiomers, allowing chiral resolution at low concentration. The CE capillary protrusion from the ESI needle relevantly affected the chiral resolution and the analyte migration time. Complete enantiomeric separation was obtained by using 0.25 M formic acid BGE containing 1.75 mM of chiral selector and water/methanol (30:70, v/v) 3% formic acid as sheath liquid. The method exhibited a LOD of 0.1 μg/mL (racemic concentration) in MS^3 product ion scan mode of detection and was applied to the analysis of racemic baclofen in pharmaceutical formulations.

1. Introduction

Baclofen (4-amino-3-*p*-chlorophenylbutyric acid) is a chemical analogue of γ-amino-butyric acid (GABA) used as skeletal muscle relaxant for the treatment of spasticity or multiple sclerosis. Its molecule contains a chiral center and thus exists in two enantiomeric forms (Fig. 1). Baclofen is administered as racemic mixture drug, although only the *R*-(−)-enantiomer is stereospecifically active on the $GAGA_B$-receptors [1,2]. The enantiomeric forms of baclofen showed different pharmacodynamic and toxicological properties. It was reported that *R*-(−)-enantiomer is 100-times more active than the *S*-(+)-enantiomer, but also more toxic [2–4].

The analytical methods so far used for the separation of baclofen enantiomers include gas chromatography [5], liquid chromatography [1,2], liquid chromatography/tandem mass spectrometry [6] and capillary electrophoresis [7–18]. One paper reports the determination of baclofen enantiomers using potentiometric membranes electrodes based on maltodextrins [19].

Capillary electrophoresis (CE) is a powerful analytical technique in the field of enantiomeric separation, however only few papers report the use of chiral CE coupled to mass spectrometry detection [20,21]. The direct mode of enantiomeric separation is the mostly used, allowing the formation of labile and reversible diastereoisomeric complexes during the run. The chiral selector is simply dissolved in the running buffer in few quantities making possible the use of expensive chiral selectors at low costs.

Chiral separation of baclofen was first obtained by CE in β-cyclodextrin modified MEKC at neutral pH and UV detection after 1-cyano-2-substituted-benz[*L*]isoindol derivatization [7] and later with α-cyclodextrin [8] and highly sulfated β-cyclodextrin [9] at alkaline pH after naphtalene-2,3-dicarboxyaldehyde (NDA) derivatization. In the last two papers LIF detection was used allowing a limit of detection of baclofen in plasma of 10 ng/mL and 50 nM concentrations, respectively. The stereoselective

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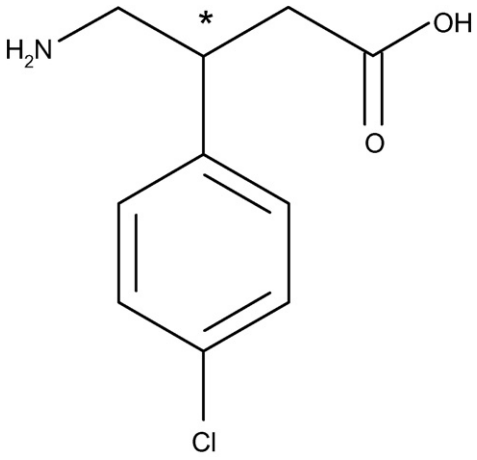

Fig. 1. Molecular structure of baclofen. *Chiral center.

separation of baclofen in multichannel microfluidic chips with γ- and hydroxypropyl-α-cyclodextrins as chiral selectors after derivatization with fluorescent tag (FITC) was recently published [10].

Direct chiral resolution of underivatised baclofen enantiomers was obtained in CE by using different native [11,12] and uncharged modified cyclodextrins [13–15], charged crown ether [16] and cyclodextrins/crown ether combination [17]. All these methods used UV detection but only one reported the limit of detection (LOD) corresponding to 0.1 mg/mL for baclofen [12]. Recently, highly sulfated cyclodextrin were used for the separation of baclofen enantiomers at acidic pH in counter current mode. A method LOD and LOQ in UV of 0.13 and 0.5 μg/mL, was respectively reported [18]. The sulfobutylether-β-cyclodextrin was tested as chiral selector for baclofen enantiomers only in one paper and provided only a partial resolution [12].

To our knowledge no papers report the chiral and/or achiral analysis of baclofen by CE in coupling with mass spectrometry detection (CE–MS). The CE–MS coupling expands the applicability of CE technique in drug analysis allowing obtain sensitive detection together with molecular mass and structural information of the analytes [22–25]. With respect to the numerous CE–MS applications recently reported in the field of analysis of pharmaceuticals and metabolites, only a few are enantioselective methods, probably due to the incompatibility of the chiral selectors with the MS analytes ionization and detection [24,26].

The use of chiral selectors of opposite charge of the analyte allows to perform enantiomeric separation in partial filling counter current mode (PFCC). The partial filling technique (PFT) was first introduced by Valtcheva et al. [27] and successfully applied in CZE to increase the detection sensitivity when using chiral selector with strong UV absorption [28–31]. In PFT only part of the capillary is filled with the chiral selector that is never reaching the detection path. In the counter current mode the chiral selector and the analytes due to the opposite charge migrate in opposite direction, offering a powerful enantioselective capability at low concentration [27,32,33]. As reviewed [34–36], PFCC is particularly advantageous in CE–MS to perform chiral separations avoiding MS detector contamination.

Aim of this paper was to develop a new and high specificity analytical method by CE in coupling with tandem mass spectrometry detection for the separation of baclofen enantiomers using sulfobutylether-β-cyclodextrin (SBEβCD) as chiral selector in partial filling counter current mode (PFCC).

2. Experimental

2.1. Chemicals

Methanol LC–MS was purchased from Riedel-de Haën, Sigma–Aldrich Laborchemikalien GmbH, Seelze, Germany. Ultrapure water was obtained from P.Nix Power System apparatus, Human, Seoul, Korea. Formic acid, Baker analysed, 98%, was from Mallinckrodt Baker B.V. (Deventer, Holland). Sodium hydroxide pellets, pro-analysis, and hydrochloric acid 37% GR were from Merck, Darmstadt, Germany. (±)-Baclofen and *S*-(−)-baclofen hydrochloride reference standards were from Sigma–Aldrich (St. Louis, MO, USA). Advasep® β-cyclodextrin sulfobutyl ether 4 (SBEβCD), sodium salt, substitution degree 5.5, was from CyDex Pharmaceuticals, Inc., Lenexa, USA. The stock (1 mg/mL) solutions of baclofen racemic mixture and pure *S*-enantiomer were prepared in 10 mM hydrochloric acid water solution and stored at 4 °C (1-month period) or −20 °C for period longer than 1 month. Further dilutions were made by water. L-Tyrosine (L-Tyr) from Pierce Chemical Co (Rockford, IL, USA) was used as internal standard (IS). Concentrated (1 mg/mL) L-Tyr solutions were prepared in 20 mM NaOH and diluted with water.

2.2. Apparatus

Capillary electrophoresis automated apparatus was from Agilent Technologies (Waldbronn, Germany) equipped with diode array UV detector and external nitrogen pressure. The CE apparatus was coupled to the Esquire 3000 plus mass spectrometer (Bruker Daltonics, Bremen, Germany) via a coaxial sheath liquid electrospray ionization (ESI) interface (Agilent Technologies, Waldbronn, Germany). The sheath liquid was delivered by an external syringe pump (Cole Palmer, Vernon Hills, IL, USA) at a constant flow rate of 180 μL/h. Nebulizing and drying gas (nitrogen) were set at 6.0 psi and 4.0 L/min, respectively. Dry gas temperature was 300 °C. Mass spectrometry capillary voltage was 4000 V. Separations were performed in 50 μm I.D., 375 μm O.D. fused silica uncoated capillaries (Composite Metal Services, Hallow, Worcs., UK) of total length of 79.5 cm. Effective length was 21.5 cm for UV detection and 79.5 cm for MS detection.

Tandem mass spectrometry (MS^2) detection of the analytes was performed in product ion scan mode by activating the Multiple Reaction Monitoring (MRM) windows using an isolation width of ±4.0 *m*/*z* and fragmentation amplitude of 1.0 V in positive ionization and normal resolution scan. When MS^3 detection was used the selected fragments isolation width was 3.0 *m*/*z* with fragmentation amplitude of 1.0 V. The acquisition of the MS^2 or MS^3 extracted ion current (EIC) signals was made in 50–300 *m*/*z* mass range using a maximum accumulation time of 100 ms and a set target value of 50,000 and by activating the ion charge control (ICC) function. The IS was detected in MS^2 product ion scan mode.

The temperature of the CE–MS assembly cartridge was set at 25 °C. The CE running voltage was 25 kV (positive polarity). Samples were injected at the anodic end at 50 mbar × 10 or 20 s followed by BGE injection at 50 mbar × 15 s. Formic acid solutions at different concentrations were used as BGE and were daily prepared. Different water/methanol (v/v) mixtures containing formic acid were used as sheath liquid solutions.

Before starting with CE experiments, new capillaries were conditioned using the following procedure: (1) water (5 bar × 2 min); (2) 0.1 M sodium hydroxide (5 bar × 20 min); (3) water (5 bar × 5 min). Between runs the capillary in use was rinsed with water (5 bar × 1 min) and BGE (5 bar × 1.5 min) and partially filled with the BGE containing the chiral selector at lower pressure (flush function, about 940 mbar) × 1.1 min, corresponding to a

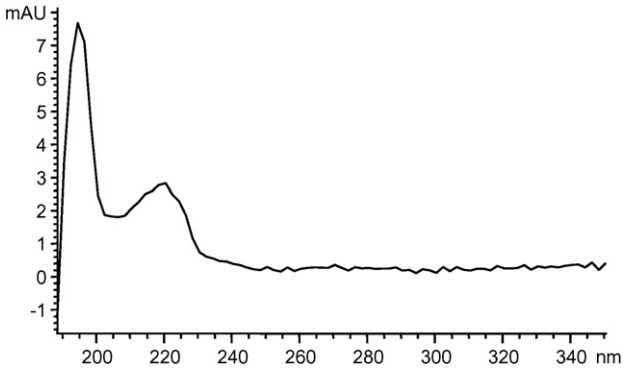

Fig. 2. UV spectrum of baclofen.

capillary filled length of 70 cm. Every four runs the capillary was rinsed with water (5 bar × 0.5 min), 0.1 M sodium hydroxide (5 bar × 0.7 min) and water (5 bar × 0.7 min).

2.3. Preparation of the BGE and the chiral selector

In order to increase method repeatability and to avoid errors due to the daily weight of few cyclodextrin milligrams, a study was performed to investigate the stability of SBEβCD aqueous working solutions during 4 days of storage at −20 °C and 5 days at 4 °C. Each day six samples of SBEβCD solution containing the double concentration in use, e.g. three stored at 4 °C and three at −20 °C were diluted with the BGE and checked for the enantiomeric resolution of baclofen enantiomers. The repeatability of Rs for $n = 15$ total analysis (4 °C) and $n = 12$ (−20 °C) showed RSD values of 6.62% and 6.81%, respectively. It was concluded that the aqueous solution of SBEβCD was stable for 5 days at 4 °C. In our study the cyclodextrin solution was then weekly prepared and stored accordingly.

2.4. Analysis of pharmaceutical formulation: sample preparation

Samples from two different pharmaceutical preparations commercially available containing 10 and 25 mg tablet baclofen content were prepared by weighting, crushing and mixing three tablets for each preparation. An aliquot of 15 mg of the resultant powder was dissolved in 10 mL of 10 mM HCl solution and extracted by magnetic stirring for 10 min at room temperature followed by ultrasonic bath for 5 min. The solution was therefore vortex mixed, filtered (0.45 μm, nylon filter) and the filtrate stored at −20 °C. Before the analysis the extract was thawed at room temperature, vortex mixed and added of the IS (L-Tyr, final concentration 5 μg/mL) before 1:50 dilution with water.

3. Results and discussion

With respect to the chromatographic method were UV wavelengths not lower than 220 nm are commonly used, CE can improve the limit of detection of aromatic molecules due to its compatibility with as low UV wavelengths as 195 or 200 nm. Particularly for baclofen, the use of 195 nm wavelength can increase its detectability in accordance with its UV absorption. In fact, as it can be observed in Fig. 2, the baclofen UV spectrum shows two maximum absorption wavelengths at 195 and 220 nm, 195 nm being more than two times higher. Alternatively to UV, the use of high sensitivity detectors (e.g. LIF), derivatization procedures or MS detection can be applied. The use of MS can strongly increase the analytes detection sensitivity due to its high specificity, especially in MS/MS detection scan mode.

In this paper CE in coupling with ion trap mass spectrometry was for the first time applied for optimizing the chiral separation of baclofen using the negatively charged SBEβCD as chiral selector in PFCC mode. On-line UV detection was also simultaneously used, however the UV and MS detection path lengths were remarkably different, e.g., 21.5 and 79.5 cm, respectively.

Preliminary experiments of CE–MS for the analysis of baclofen were performed in 0.5 M formic acid in positive polarity and full scan positive ESI ionization in absence of chiral selector. According to its pK_a value of 3.9 [37], under these conditions the baclofen was migrating as positively charged compound towards the cathodic end of the capillary coupled to the ion-trap spectrometer via a coaxial sheath liquid interface. A mixture of water/methanol (30:70, v/v) containing 0.1% formic acid was used as sheath liquid solution at a flow rate of 180 μL/h. MS capillary voltage was set at 4000 V in positive ESI ionization mode.

The use of ion trap MS allowed to increase the method selectivity by using MS^2 or MS^3 product ion scan modes. Fig. 3 shows the MS^2 and MS^3 spectra of baclofen. In the MS^2 spectra of baclofen the main fragment ion at m/z 196.9 was corresponding to the neutral loss of ammonia from the parent ion at m/z 214.2. The isolation and fragmentation of the product ion at m/z 196.9 generated the MS^3 fragments at m/z 178.9 and 151.1 by the further neutral loss of water and formic acid, respectively. During method optimization MS^2 detection was used.

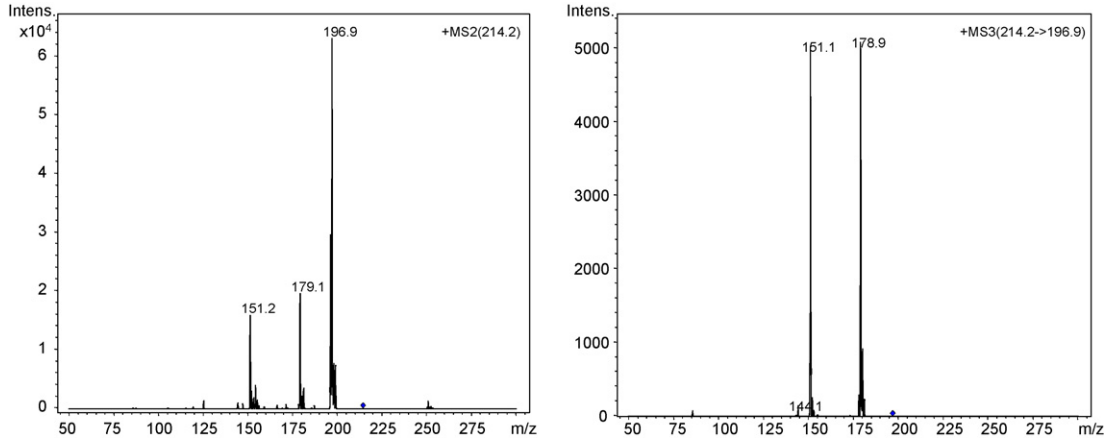

Fig. 3. MS^2 and MS^3 production ion spectra of baclofen.

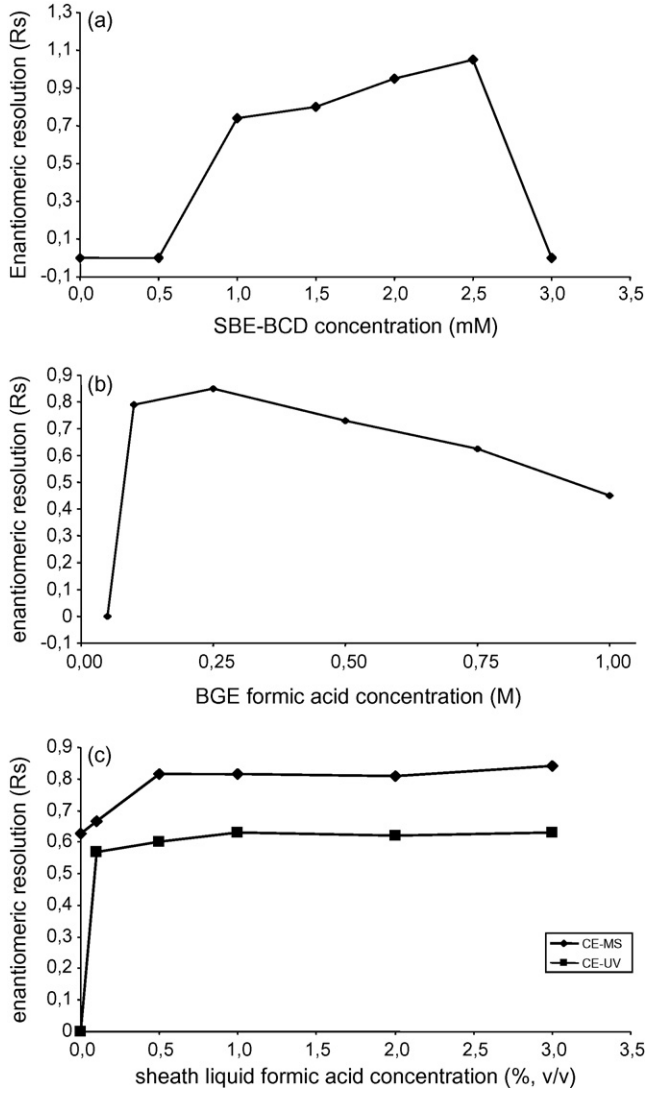

Fig. 4. Effect on baclofen enantiomeric resolution (Rs) of SBEβCD concentration (panel a) and BGE formic acid concentration (panel b) in MS detection, and of sheath liquid formic acid concentration in both UV and MS detection (panel c). Baclofen racemic concentration 4.0 μg/mL. For the experimental conditions see Section 2.

In order to separate baclofen enantiomers the direct mode of chiral separation was used by dissolving into the BGE the SBEβCD and using the partial filling mode (PF) of separation. The SBEβCD possesses a negative charge in the entire pH range opposite to that of baclofen at acidic pH. In PF only part of the capillary was filled with the chiral selector during the pre-run conditioning. Once the voltage was applied in positive polarity mode, the SBEβCD was migrating in the opposite direction of baclofen (counter current mode) far from the MS capillary outlet end, avoiding source contamination and analyte signal suppression during ionization. The use of a chiral selector of opposite charge of the analytes is suitable for CE–MS applications and is particularly effective in resolving enantiomers by using very few quantities of chiral selector.

Chiral resolution (Rs) was calculated by using equation (1):

$$\mathrm{Rs} = 2\frac{t_{m2} - t_{m1}}{w_2 + w_1} \quad (1)$$

where t_m and w are the migration time and the peak width of the enantiomers at the base, respectively.

The SBEβCD was added to the 0.5 M formic acid BGE at 1.5 mM concentration. Although the low chiral selector content, the baclofen enantiomers showed a different complexation that resulted in a significant but not baseline chiral resolution (Rs = 0.86). To increase the Rs, the formic acid content of the sheath liquid was increased to 2% (v/v). With respect to the 0.1%, the 2% of formic acid produced a slight increase of the chiral separation and was therefore selected for further investigations.

The optimization of the method to obtain the complete enantiomeric separation of baclofen required the careful investigation of the effect of several parameters influencing either the CE separation or the MS detection, e.g., the effect of cyclodextrin and BGE concentration, the sheath liquid composition, partial filling length, detection scan mode and capillary exit length. The use of both the UV and MS detection on the same analytical run was important in evidencing the critical parameters involved in the optimization of chiral separation by CE–MS methods.

3.1. The effect of chiral selector concentration

The effect of SBEβCD concentration on Rs was studied in the range 0.5–3.0 mM (e.g., 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM) in PF mode by filling 70 cm capillary length. In UV electropherograms the electroosmotic flow (EOF) baseline disturbance could also be monitored. The increase of the SBEβCD concentration produced the increase of the EOF and baclofen migration times, probably due to both the increase of the BGE viscosity and chiral selector complexation (data not shown).

Fig. 4(panel a) shows the effect of SBEβCD on Rs in MS detection. Enantiomeric resolution was not observed at 0.5 mM of SBEβCD. At 1.0 mM SBEβCD the Rs was 0.74 and increased up to 1.05 at 2.5 mM (maximum value of Rs observed). At 3.0 mM resolution was completely lost. The same effect was observed in UV detection, considering that a lower Rs was always recognized due to the shorter detection path length. By increasing the cyclodextrin concentration the analytical windows between the baclofen peak and the EOF UV disturbance become shorter due to the analyte chiral selector complexation. As a result the baclofen cationic mobility decreased up to be almost completely lost at 3.0 mM where the peak was co-migrating with the EOF baseline disturbance (data not shown). The higher cyclodextrin concentration increased the complexation degree producing a prolonged residence time of the analyte into the chiral selector resulting in longer baclofen migration time. Being the chiral selector of opposite charge of the analyte, the inclusion mechanism produced a decrease of the cationic mobility of baclofen. At higher SBEβCD concentrations a reversed mobility could be expected. However, concentrations higher than 3.0 mM were not investigated due to the strong increase of the BGE current incompatible with the maximum current limit allowed (e.g. 50 μA) for CE–MS methods in the instrument in use.

The observance of a maximum of Rs in a selected range of chiral selector concentration is common using cyclodextrins [38]. At 3.0 mM SBEβCD the observed loss of Rs was probably due to both the decrease of the baclofen mobility and its co-migration with the EOF that negatively influenced the counter current chiral recognition process based on both inclusion complexation and intermolecular Coulomb interactions [33].

Although 2.0 and 2.5 mM chiral selector concentrations showed the highest values of Rs, they were not selected for investigating other effects because at these concentrations the baclofen peaks were too close to the EOF disturbance and, in addition, the CE current was too high to study other effects, e.g. the effect of BGE concentration. For this reason 1.5 mM (Rs = 0.80) was the cyclodextrin concentration selected to investigate the effect of the BGE concentration and sheath liquid composition.

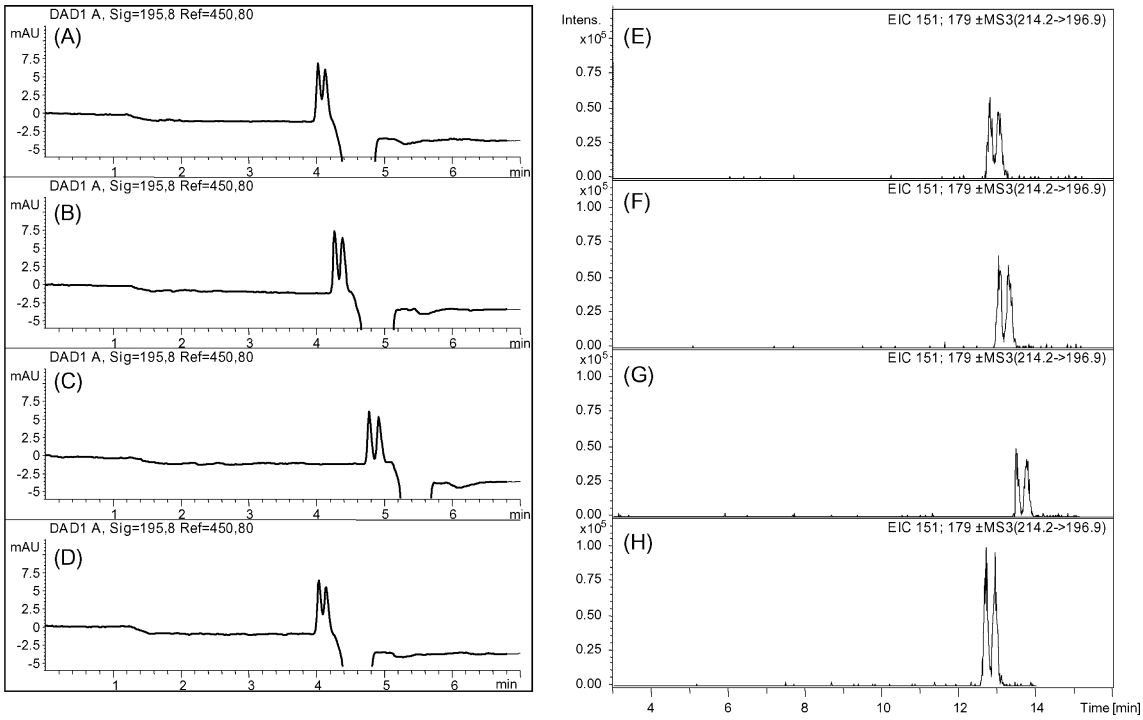

Fig. 5. Effect of CE capillary exit protrusion from the ESI needle on baclofen enantiomeric separation in UV and MS detection at +1 (A and E), +2 (B and F), +3 (C and G) and +4 (D and H) micro-regulation screw tally marks. UV detection wavelength: 195 nm. MS^3 EIC electropherograms: 214.2 → 196.9 → 151.2 + 179.0. Baclofen racemic concentration 4.0 μg/mL. Sample injection: 50 mbar × 10 s. For the experimental conditions see Section 2.

3.2. The effect of BGE concentration

Different formic acid aqueous solutions at 0.05, 0.10, 0.25, 0.50, 0.75 and 1.0 M concentrations were tested as BGE in presence of 1.5 mM of SBEβCD. Fig. 4 (panel b) shows the effect of BGE concentration on Rs in MS detection. At 0.05 M formic acid the baclofen did not exhibit enantiomeric resolution. From 0.10 to 1.0 M of formic acid the enantiomers showed a separation with a maximum of Rs at 0.25 M. The same effect on Rs was also observed in UV detection. In UV it was additionally observed that the decrease of the formic acid concentration produced a decrease of baclofen mobility (data not shown). Differently, the EOF migration time was not influenced by the change in formic acid concentration. The faster migration of baclofen at higher formic acid concentrations, probably due to its increased dissociation, reduced the time of interaction between the cyclodextrin and the analyte resulting in lower enantiomeric resolution. At 0.05 M the baclofen and the EOF were co-migrating and, as result, the enantiomers were unresolved.

The 0.25 M formic acid BGE provided the highest Rs in presence of 1.5 mM SBEβCD, however the chiral resolution was not baseline still. Under these conditions the increase of SBEβCD concentration to 2.0 mM did not further improve the Rs.

3.3. The effect of formic acid sheath liquid content

The role of the sheath liquid is to provide both the electrical contact with the capillary during CE and the analyte electrospray ionization in the source and is strongly influencing the peak efficiency and the sensitivity, by a dilution process of the CE eluate. Its choice is one of the most important parameter influencing the MS detection of the analytes but also their CE separation and migration times due to the possible formation of ionic boundaries [39]. In this work a water/methanol (30:70, v/v) mixture was selected as sheath liquid solution in absence or in presence of different formic acid concentrations, namely, 0.1, 0.5, 1.0, 2.0 and 3.0% (v/v). Fig. 4 (panel c) shows the effect of sheath liquid formic acid content on Rs in UV and MS detection. In absence of formic acid the UV Rs was just visible but not measurable. It slightly increased (Rs = 0.57) by the addition of 0.1% formic acid and did not exhibit a strong improvement at higher content of formic acid (Rs = 0.63 at 3.0% formic acid). The analytes migration time increased from 0 to 0.1% formic acid content and then remained unchanged at all the other formic acid concentrations studied (data not shown). The same effect was also recognized for the EOF peak. In MS detection a different effect was observed. The analyte migration time showed a slight increase from 0 to 0.5% of formic acid and than a slight decrease from 0.5 to 3%

Table 1
Intra- and inter-day precision and accuracy data

Analyte	Calibration level	Nominal concentration (μg/mL)	Intra-assay precision (n = 6 runs)		Intermediate precision (n = 5 days)		Intra-assay accuracy	
			S.D.	RSD (%)	S.D.	RSD (%)	Value (%) ±S.D.	RSD (%)
R-Baclofen	2	0.2	0.02	5.09	0.02	7.34	99.3 ± 5.1	5.09
	4	0.4	0.03	4.30	0.03	3.99	96.8 ± 4.1	4.30
S-Baclofen	2	0.2	0.02	6.25	0.01	1.54	93.1 ± 5.8	6.25
	4	0.4	0.01	2.42	0.04	6.26	85.2 ± 2.1	2.42

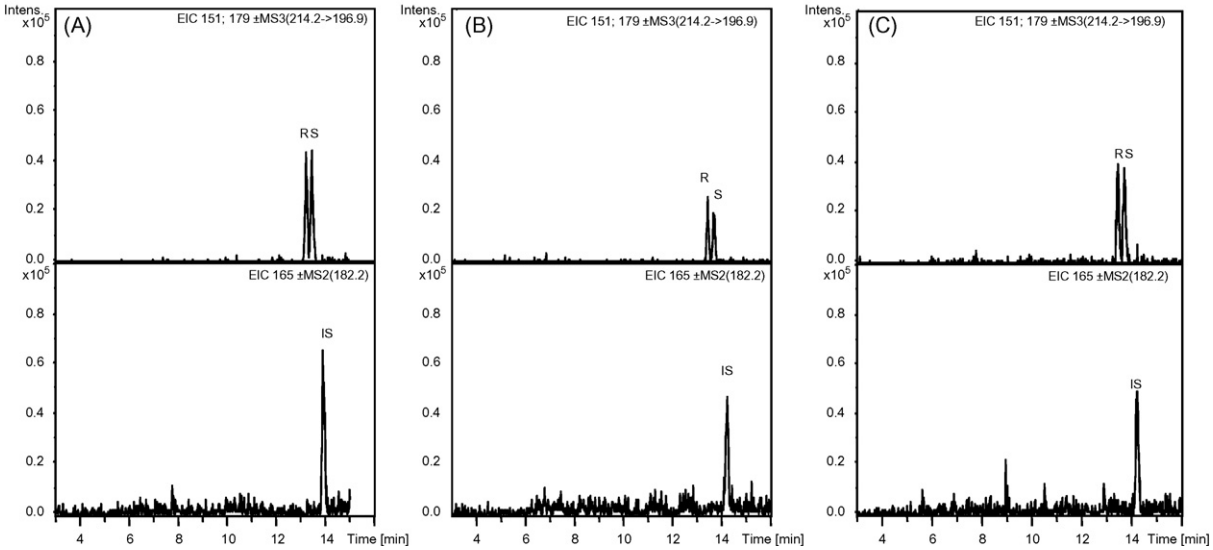

Fig. 6. MS^3 EIC electropherograms of the analysis of baclofen enantiomers in standard mixture (A) and in pharmaceutical formulations with different racemic drug dosages of (B) 10 mg and (C) 25 mg (tablet content) under the optimum experimental conditions. BGE: 0.25 M containing 1.75 mM SBEβCD in PF mode (70 cm filled capillary path). Sheath liquid: 70% methanol, 3% formic acid. Capillary exit at +3. Sample injection: 50 mbar × 20 s. IS: L-Tyr, 5.0 μg/mL final concentration. *R*- and *S*-baclofen enantiomers (3.75 μg/mL racemic concentration in the standard mixture). For other experimental conditions see Section 2.

(data not shown). In MS detection baclofen Rs was observed both in absence and in presence of formic acid. It increased from 0.63 to 0.82 at 0 and 0.5% formic acid, respectively, and then exhibited an almost stable value showing a slight maximum (Rs = 0.84) at 3.0%.

The main effect of formic acid addition was observed on the analyte MS peak intensity. It strongly decreased from 0 to 0.1% of about the 50% (data not shown). From 0.1 to 0.5% the peak signal intensity was not further affected. Although the lower analyte signal intensity produced, the water/methanol (30:70, v/v) mixture containing the 3.0% of formic acid was selected as the optimum sheath liquid solution owing to the shortest analysis time and the highest Rs exhibited. The use of a sheath liquid without formic acid was excluded due to unstable ESI current, poor reproducible results and low enantiomeric resolution.

Finally, the effect of the use of lower methanol content in the sheath liquid, e.g., water/methanol 50:50 (v/v), 3% formic acid, was also compared. The lower methanol content did not have remarkably effects on analyte migration time and Rs, but poorer signal intensity was observed.

3.4. The effect of PF capillary length

In order to increase the enantiomeric resolution of baclofen, the effect of the length of the PF was studied by filling 70, 72, 76 cm capillary paths with 1.5 mM SBEβCD solution in 0.25 M BGE. Considering that the total capillary length was 79.5 cm, filled lengths longer than 76 cm were not investigated to prevent accidental ESI source contamination by the chiral selector. It was surprising to observe that the more the capillary path length filled with cyclodextrin the same the Rs and analyte migration time. It was therefore decided to fill 70 cm of the capillary as previously performed.

3.5. Optimum experimental conditions

Under the optimum experimental conditions found, namely, 0.25 M formic acid BGE and sheath liquid of water/methanol (30:70, v/v) containing 3.0% (v/v) of formic acid, the effect of cyclodextrin concentration was deeper investigated to find the maximum of Rs. Different SBEβCD concentrations, namely, 1.0, 1.25, 1.50, 1.75 and 2.0 mM, were added and tested for Rs. The effects produced were the same as previously discussed in the paragraph of CD concentration effect. The increase of CD concentration produced an increase of the migration times and of Rs. The maximum Rs was found at 1.75 mM with Rs values of 0.86 and 1.08, in UV and MS detection, respectively.

Under the optimum conditions the enantiomeric migration order was established by adding the pure *S*-enantiomer to the racemic mixture. The *S*-enantiomer corresponded to the slower migrating peak.

3.6. Method sensitivity and effect of capillary exit length

Compared to UV, the MS detection in MS^2 product ion scan mode provided a comparable method sensitivity. Differently, the MS^3 product ion scan mode of detection, using the $214.2 \rightarrow 196.9 \rightarrow 151.1 + 179.0$ transition, produced higher signal to noise ratio (s/n) with respect to UV detection. A baclofen solution of 0.4 μg/ml showed s/n ratios of 48.0 and 3.8 (instrumental values) in MS^3 and UV detection (LOD value), respectively. The MS^3 product ion scan mode was therefore used for further experiments.

Before evaluating the sensitivity of the method, the effect of the entity of the protrusion of the CE capillary from the ESI needle (capillary exit) on analyte migration time and Rs was studied. Its regulation is important for ensuring a stable ESI current, good analyte ionization and reproducible results. It was regulated by using the tally marks on the micro-regulation screw on the ESI needle interface at +1, +2, +3 and +4 (capillary exit values). During method optimization the +2 value was used. The +1, +2 and +3 selected regulations provided acceptable and stable values of the ESI current. The +4 showed the most unstable ESI current.

It was interesting to observe that the capillary exit exhibited a relevant effect on Rs both in UV and MS detection. Fig. 5 shows the UV and MS^3 EIC electropherograms of the chiral separation of baclofen obtained at +1, +2, +3 and +4 capillary exit values. The increase of the capillary exit produced the increase of the Rs and baclofen migration time from +1 to +3 capillary exit values. The Rs increased from 0.74 to 0.80 (UV and MS Rs values, respectively, at +1 value) to its maximum value of 1.12 and 1.25 (UV and MS Rs values,

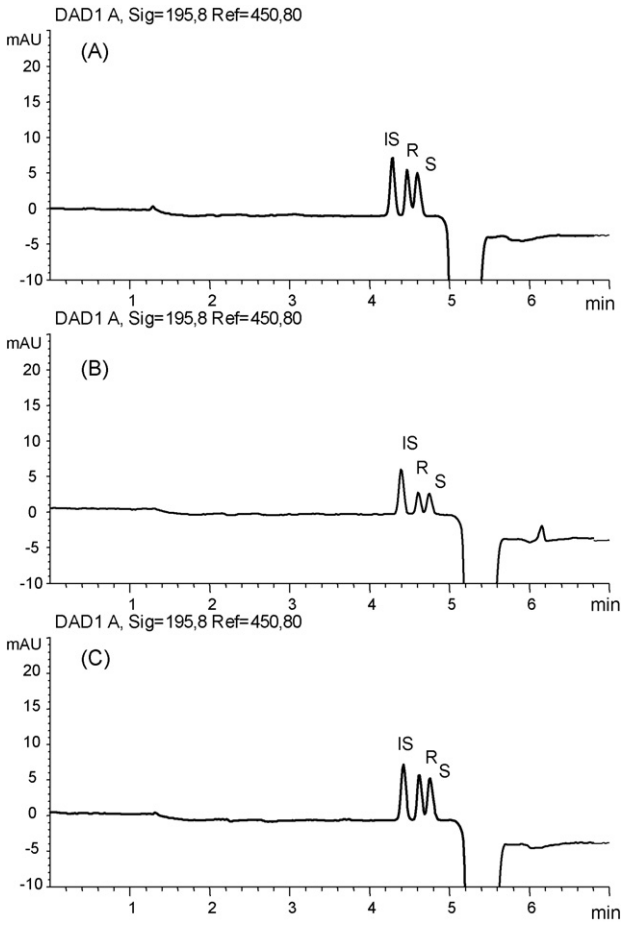

Fig. 7. UV electropherograms of the analysis of baclofen enantiomers in standard mixture (A) and in pharmaceutical formulations with different racemic drug dosages of (B) 10 mg and (C) 25 mg (tablet content) under the optimum experimental conditions. Experimental conditions as in Fig. 6.

respectively) at +3 using formula (1) for calculation. However using the formula (2):

$$\mathrm{Rs} = \frac{1}{4}\sqrt{N}\frac{\Delta\mu}{\bar{\mu}} \quad (2)$$

that takes into account the peak efficiency (N) and analytes apparent mobility (μ_{app}), the resulting Rs was 1.50. The use of μ_{app} includes the detrimental effect of cathodic EOF on Rs of positively charged compounds. The peak efficiency was calculated using the formula (3):

$$N = 5.54\left(\frac{t_m}{w_{1/2}}\right)^2 \quad (3)$$

and was corresponding for baclofen enantiomers to 174,000 number of theoretical plates per meter (average values of the two enantiomers).

At +4 the migration time and Rs both decreased. The recognized effects were similar in UV and MS detection, however in MS the capillary exit additionally influenced the peak height producing different responses. The capillary exit seemed to be a critical parameter to be optimized, strongly influencing the enantiomeric separation and the ionization efficiency of the analyte. Probably, under the experimental conditions used, e.g. sheath liquid type and flow and nebulizer gas pressure, the +3 seemed the optimal value ensuring good baclofen ionization efficiency and minimum influence of possible sheath liquid moving boundaries and nebulizer gas suction effect on Rs.

Particularly interesting was to observe that, despite the longer separation capillary path length, the chiral resolution observed in MS detection was often lower than expected concerning that observed in the corresponding UV analysis. Probably, the diffusion of the sample zone during the run and the counter current migration of the chiral selector had to be taken into account. A possible loss of the CE separation efficiency at the electrospray ionization in source and/or during the ion trap detection could not be excluded. These observations evidenced the difficulty in transferring chiral methods from CE–UV to CE–MS techniques and the need of different optimization criteria.

Under these conditions the MS method sensitivity were evaluated. The method provided an LOD value ((s/n) = 3) of 0.1 μg/mL (0.47 μM) racemic concentration in MS^3 detection by injecting the sample at 50 mbar × 20 s.

3.7. Method validation

The developed CE–MS method was tested for quantitation by evaluating the intra-day and inter-day precision and method linearity using the internal standard method. L-Tyrosine was used as reference compound (I.S.) and detected in MS^2 product ion scan mode. Baclofen enantiomers were detected and quantified calculating the ratio of analyte/IS peak area from the MS^3 (baclofen, 214.2 → 196.9 → 151.1 + 179.0 transition) and MS^2 (I.S., 182.2 → 165 transition) EIC plots generated by the intensities of the main product ions.

Method linearity was evaluated with three different calibration curves for each enantiomer over the range of 1.0–5.0 μg/mL (racemic concentration) baclofen standard compound using 5 calibration levels in duplicate runs. The three curves were performed on three different days using two different capillaries. The I.S. final concentration in calibrating solutions and samples was 5.0 μg/mL. The MS detector showed a linear response in the studied concentration range with regression equations of $y = 338.93 \pm 3.44x + 0.005 \pm 0.008$, $r = 0.993 \pm 0.0045$ and $y = 371.80 \pm 17.34x - 0.017 \pm 0.027$, $r = 0.998 \pm 0.00006$ ($n = 3$) for the first and the second migrating enantiomer, respectively.

Calibration levels 2 and 4, corresponding to 2.0 and 4.0 μg/mL racemic concentration levels were tested for intra-assay ($n = 6$ consecutive runs) precision and accuracy and intermediate precision ($n = 5$ days, using a different calibration sample per day in duplicate runs) of the analyte/IS peak area ratio value. The data are reported in Table 1. Levels 2 and 4 were selected because corresponding to

Table 2
quantitative analysis of baclofen enantiomers in pharmaceutical preparations of different dosage[a]

Commercial formulation (tablet content (total labelled weight))	*R*-Baclofen		*S*-Baclofen		Total weight	
	mg	RSD (%)	mg	RSD (%)	mg	RSD (%)
Baclofen 10 mg	5.75 ± 0.27	4.79	5.50 ± 0.37	6.69	11.25 ± 0.29	2.61
Baclofen 25 mg	12.46 ± 0.98	7.90	11.33 ± 0.77	6.78	23.78 ± 1.57	6.62

[a] Average values of two determinations (two/three runs per determination).

the concentrations values closest to the final level of baclofen in the two pharmaceutical preparations analysed (see below in the text).

Method recovery and accuracy was evaluated by spiking the pharmaceutical preparation of baclofen at 10 mg declared tablet dosage with 2.0 μg/mL final racemic concentration of the standard compound and following the extraction procedure reported in Section 2. The method recovery was calculated on duplicate determinations in duplicate runs and was in the range 92–106% and 92–112% for *R*- and *S*-baclofen, respectively. The accuracy was 88–108% and 86–104% for *R*- and *S*-baclofen, respectively.

3.8. Analysis of baclofen enantiomers in pharmaceutical formulations

The optimized method was applied to the analysis of baclofen enantiomers in pharmaceutical preparations commercially available with different therapeutic dosages. As described in details in Section 2, the sample preparation followed a very rapid procedure involving a simple extraction step before the CE–MS analysis. Fig. 6 shows the MS^3 EIC electropherograms of the chiral analysis of baclofen in standard mixture (panel A) and in two different pharmaceuticals formulations declared to contain 10 (panel B) and 25 (panel C) mg of racemic drug, respectively. Fig. 7 shows the relative electropherograms in UV detection. By comparing the analysis of baclofen in presence of IS in MS and UV detection, respectively in Figs. 6 and 7, it was interesting to observe the different migration order of L-Tyr and baclofen enantiomers. In UV the L-Tyr was migrating faster, the reversed was observed in MS detection. This was probably due to a diverse interaction of the analytes with the chiral selector that differently influenced the analytes velocities. During migration on the longer capillary path the counter current migration of the cyclodextrin have to be considered.

The analysis of baclofen in pharmaceutical preparation did not show matrix interferences and confirmed the racemic presence of *R*- and *S*-baclofen enantiomers. Table 2 reports the quantitative analysis of baclofen enantiomers in two different pharmaceutical preparations at 10 and 25 mg tablet dosage.

4. Conclusions

CE in coupling with MS detection was for the first time applied to the enantiomeric separation of baclofen. On-line UV detection was simultaneously used. The method optimization required the careful investigation of several physico-chemical parameters both influencing the CE separation and the MS detection and evidencing the difficulties in transferring the optimized separation from UV to MS mode of detection. The effect of cyclodextrin concentration showed a maximum of the enantiomeric resolution and a complexation that relevantly influenced the baclofen mobility. The optimum experimental conditions were found at relatively low formic acid BGE content (0.25 M) using a sheath liquid containing 70% of methanol and 3% of formic acid and 1.75 mM SBEβCD concentration in 70 cm filled capillary path. The entity of the CE capillary exit from the ESI needle strongly influenced the analyte migration time and the enantiomeric resolution and it showed an optimum at +3 micro-regulation set value.

Under the optimized conditions the baclofen enantiomers showed baseline separation. The method exhibited LOD values of 0.4 (UV) and 0.1 (MS^3) μg/mL racemic concentrations, in UV and MS detection, respectively.

The method was successfully validated evaluating linearity, intra-assay and intermediate precisions, method accuracy and recovery and applied to the quantitative analysis of baclofen in pharmaceutical formulations of different dosage. The analysis of baclofen in pharmaceutical formulation did not require a particularly high sensitivity and could be successfully allowed by both the UV and the MS detection. The MS^3 detection scan mode provided higher sensitivity and selectivity to the analytical method particularly interesting for its potential application in pharmacokinetic studies. In fact, the method showed a limit of detection in MS that largely matches the sensitivity requirements for baclofen analysis in urine [5].

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