Simultaneous determination of R-(−)-, S-(+)-baclofen and impurity A by electrokinetic chromatography

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ABSTRACT

A rapid method for the simultaneous analysis of R-(−)-, S-(+)-baclofen and impurity A, (4RS)-4-(4-chlorophenyl) pyrrolidin-2-one, by electrokinetic chromatography was established. The optimized condition was in 100 mM sodium borate buffer (pH 9.9) containing 18 mM α-cyclodextrin (CD) and 1% (v/v) ACN using a fused-silica capillary dynamically coated with polyethylene oxide (PEO), with an effective length of 56 cm and an inner diameter of 50 μm, hydrodynamic injection at 50 mbar for 6 s, temperature of 45 °C, applied voltage of 27 kV and UV detection at 220 nm. Baseline separation of all analytes was achieved within 9 min ($R_s > 2.7$) with the migration order of impurity A, S-(+)- and R-(−)-baclofen. The method showed good linearity ($r^2 > 0.999$ in a range of 5–50 μg/mL for impurity A and 50–500 μg/mL for baclofen enantiomers), precision (%RSDs < 3.37%) and recoveries (100.3% for R-(−)-baclofen, 101.6% for S-(+)-baclofen and 96.1% for impurity A). Detection and quantitation limits were 10 and 30 μg/mL for both enantiomers, and 2 and 5 μg/mL for the impurity, respectively. The method was efficient for the determination of baclofen enantiomers and impurity A in pharmaceutical raw material and formulations due to its reliability, speed and simplicity.

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1. Introduction

Baclofen, [4-amino-3-(β-chlorophenyl) butyric acid] (Fig. 1), is an analog of γ-aminobutyric acid (GABA), which is an inhibitory neurotransmitter. Baclofen is one of the stereoselective agonists for the GABA_B receptor [1] and mostly used as a muscle relaxant in treatments of spasticity occurring secondary to multiple sclerosis, tardive dystonia, cerebral and spinal cord injury, tetanus, cerebral palsy, stiff-person and complex regional pain syndromes (CRPS) [2]. The drug is commercially available as a racemic mixture, although baclofen enantiomers have different properties. R-(−)-baclofen is stereospecifically active for GABA_B receptor, but S-(+)-baclofen is almost inactive, sometime toxic and even antagonizes effects of R-(−)-enantiomer [3,4]. The R-(−)-enantiomer decreases heart rates and arterial pressure, while the S-(+)-enantiomer increases arterial pressure with no effects on heart rates [5]. (4RS)-4-(4-chlorophenyl) pyrrolidin-2-one (impurity A, Fig. 1) is a related product from the synthesis of baclofen by a chemoenzymatic method using 3-(4-chlorophenyl)-4-nitrobutyric acid methyl ester as a precursor [6]. Moreover, impurity A increases during storage [7]. Thus, this compound is an important impurity in the synthesis and the degradation pathways of baclofen.

Consequently, determination of baclofen enantiomers and impurity A are necessary from pharmacological and pharmaceutical aspects.

United State Pharmacopoeia (USP) recommends non-aqueous titration and high performance liquid chromatography (HPLC) for the assay of baclofen in raw material and in pharmaceutical dosage forms, respectively [8]. In addition, USP suggests thin layer chromatography (TLC) and HPLC for testings of impurity A in baclofen raw material and tablets, respectively [8]. British Pharmacopoeia (BP) proposes non-aqueous titration for the assay of baclofen raw material and HPLC for impurity A testing [9].

Chiral separation of baclofen by HPLC can be achieved by either derivatization or using chiral stationary phase. For instance, Bhushan et al. derivatized baclofen with Marfey's reagent prior reversed-phase HPLC [10]. De Veredas et al. used continuous chromatographic separation of baclofen precursor, (N-Boc-4-[p-chloro-phenyl]-2-pyrrolidone) in a simulated moving bed using polysaccharide carbamate as chiral stationary phases [11]. Goda et al. employed a chiral CROWNPAK CR(+)® column for the LC–mass spectrometry (MS) of baclofen in human plasma and cerebrospinal fluid [12]. Zhu and Neirinck [13] and Hefnawy and Aboul-Enein [14] used chiral HPLC columns (Phenomenex chirex® and Chirobiotic T, respectively) for the determination of baclofen enantiomers in human plasma. Vaccher et al. developed chiral crown ether stationary phases for HPLC analyses of baclofen and several analogs [15–17].

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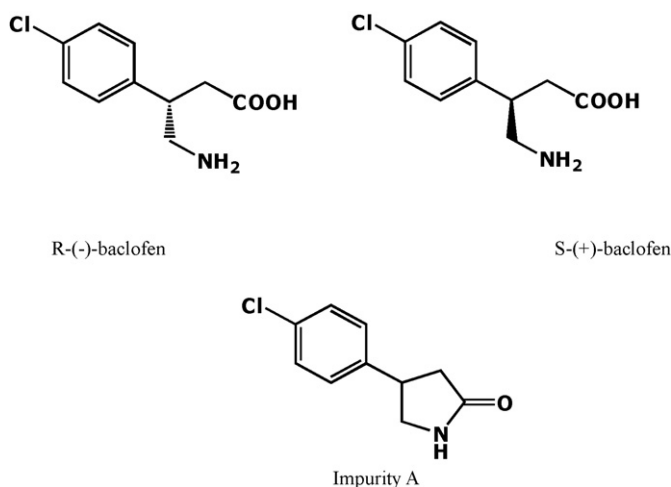 E-mail address: pylll@mahidol.ac.th (L. Suntornsuk).

Fig. 1. Structures of R-(−)-, S-(+)-baclofen and impurity A, (4RS)-4-(4-chlorophenyl) pyrrolidin-2-one.

Enantioseparation of chiral compounds by electrokinetic chromatography (EKC) is now well established and popular due to its high efficiency, flexibility, speed and low solvent and sample consumption as described in recent reviews [18–23]. EKC of baclofen is mostly performed by a direct method (*i.e.* addition of chiral selectors in to the background electrolyte (BGE) using cyclodextrins (CDs) and their derivatives as chiral selectors. For example, Desiderio et al. developed CE-MS (mass spectrometry) for enantioseparation of baclofen in pharmaceutical formulations in 0.25 mM formic acid containing 1.75 mM sulfobutylether-β-CD in water/methanol (30/70, v/v) providing a resolution (R_s) of 1.08 and a limit of detection (LOD) of 0.1 μg/mL in 14 min [24]. Kavran-Belin et al. [25] and Chiang et al. [26] proposed CE-laser induced fluorescence (LIF) for the separation of derivatized baclofen enantiomers (naphthalene-2,3-dicarboxaldehyde) in human plasma using 2% highly sulfated-β-CD (HS-β-CD, giving a R_s of 4.2, LOD of 50 mM and t_m of 11 min) and α-CD (giving a R_s of 1.1, LOD of 10 ng/mL and t_m of 14 min), respectively, as chiral selectors,. Ali et al. established EKC of baclofen in 50 mM phosphate buffer (pH 7.0) containing 10 mM β-CD and 5% (v/v) ACN leading to a R_s of 1.0 and LOD of 100 μg/mL in 60 min [27]. Vaccher et al. demonstrated the enantioseparation of baclofen (R_s > 1.6) in 25 mM phosphate buffer (pH 2.5) containing 3% (w/v) HS-β-CD using PEO coated capillaries [28,29]. The method revealed good resolution (R_s > 3.0), repeatability (%RSDs < 3.0%), sensitivity (LOD = 0.13–0.18 mg/L) and short analysis time (t_m ∼ 8.5 min) [28], which could be applied for the determinations of inclusion ionization constant pK_a of baclofen analogs [29]. Up to date, only one paper described a HPLC method for the analysis of impurity A in baclofen raw material and tablets using a micro-C_{18} column as stationary phase and 26% (v/v) ACN in 0.01 M potassium dihydrogen phosphate buffer as mobile phase [30].

This research aims to develop a simple, rapid and efficient EKC method for analysis of baclofen enantiomers and impurity A. Simultaneous analysis of these three analytes has not been reported. Optimization of EKC condition was divided into two steps, first for separation of baclofen enantiomers and then for the resolution of all three analytes. Effects of BGE ionic strength and pH, chiral selector types and concentrations, amounts of organic solvents, capillary coating, temperature and voltage were optimized. The method was validated and applied for the analysis of baclofen enantiomers and impurity A in baclofen raw material and tablets.

2. Experimental

2.1. Chemicals

R-(−)- and S-(+)-baclofen, α- and β-CD and PEO (polyethylene oxide) were from Sigma (St. Louis Missouri, USA). Impurity A, (4RS)-4-(4-chlorophenyl)pyrrolidin-2-one, was from DQM (Strasbourg, France). Sodium tetraborate was purchased from Antibioticos (Carlo erba, Mexico). Baclofen raw material and tablets were gifted from local manufacturers (Bangkok, Thailand). Acetonitrile were HPLC grade, all other reagents were of analytical reagent grades and water was sterile water for injection.

2.2. Instrumentation

EKC separations were performed on a ³DCE instrument model G1600A (Agilent Technologies, Waldbronn, Germany) and controlled by a PC through Agilent ChemStation Plus software version A.08 (G1601A). Separations were carried out using fused-silica capillaries with a total length of 64.5 cm, an effective length of 56 cm, and an internal diameter of 50 μm (Polymicro Technologies (Arizona, USA). Detection was performed with a diode-array detector using a wavelength at 220 nm with a bandwidth of 4 nm. All experiments were carried out in a positive mode (anode at the inlet and cathode at the outlet). New capillaries were conditioned with 0.1N NaOH, water and BGE for 20, 10 and 10 min, respectively. For enantioseparation of baclofen, capillaries were pre-conditioned by rinsing with 0.1N NaOH, water and BGE for 10, 5, and 10 min, respectively. For separations of baclofen enantiomers and impurity A, capillaries was pre-conditioned (every 20 injections) by rinsing with 0.1N NaOH, water, 0.5% (w/v) polyethylene oxide (PEO), water and BGE 5, 1, 3, 1 and 5 min, respectively. Between runs, capillaries were rinsed with 0.1N NaOH, water and BGE for 3, 2 and 5 min, respectively. pH meter was Consort model C830 (Turnhout, Belgium).

2.3. Background electrolyte, standard and sample preparations

The BGE for EKC of baclofen enantiomers was performed in sodium tetraborate (5–100 mM, pH 8.0–9.5) containing different chiral selectors (*i.e.* 10–23 mM α-CD or β-CD) and varied amount of ACN (0–10%, v/v). For the separation of baclofen enantiomers and impurity A, 100 mM sodium tetraborate (pH 9.7–9.9) containing varied amount of ACN (0–5%, v/v) was employed. The BGE was adjusted to desired pH with 1N acetic acid or 1N NaOH. The BGE containing CD was prepared daily by appropriate dilutions of CD stock solutions and the buffer, filtered and degassed prior CE analyses. 0.5% (w/v) PEO was prepared by dissolving PEO in water and stirred for 10 h.

For enantioseparation of baclofen, stock standard solutions of baclofen racemate (0.5 mg/mL) were prepared in 100 mM sodium tetraborate (pH 9.25) and diluted with 50% (v/v) ACN to 250 μg/mL. For baclofen enantiomer and impurity A separation, stock solutions of baclofen racemate (100 μg/mL) and impurity A (50 μg/mL) were prepared and diluted with the 100 mM sodium tetraborate (pH 9.9, buffer A) to 50 and 5 μg/mL, respectively.

Baclofen raw material was dissolved with buffer A to obtain a concentration of 1 mg/mL, sonicated for 15 min and used for impurity A analyses. Twenty tablets of baclofen tablets were accurately weighed and ground to a fine powder. A quantity of powder equivalent to 40 mg of baclofen was diluted with buffer A to obtain a concentration of 1 mg/mL, sonicated for 30 min, centrifuge for 10 min at 14,000 rpm and used for impurity A analyses. Solutions of baclofen raw material and tablets (1 mg/mL) were diluted to 0.2 mg/mL with buffer A and used for quantitation of baclofen enantiomers. Standard and sample solutions were kept
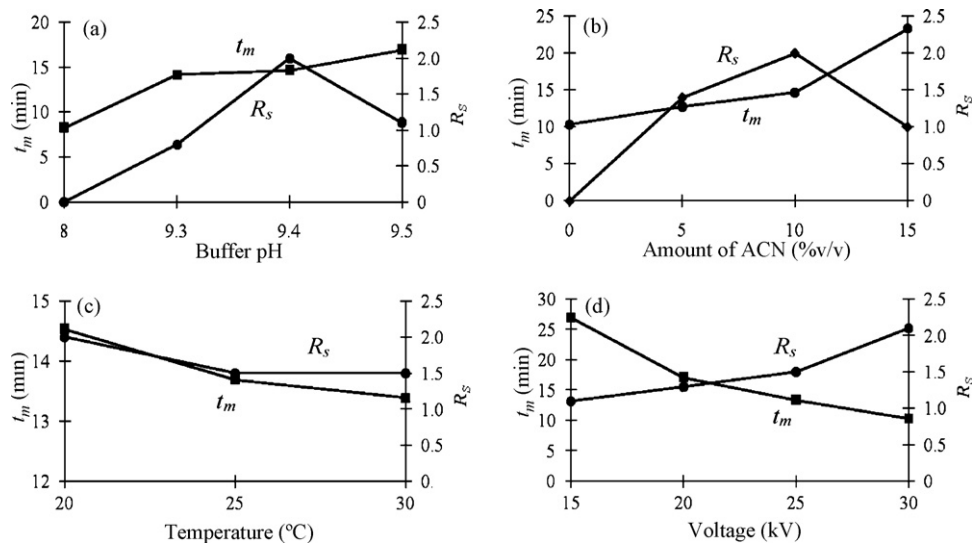
Fig. 2. Effects of (a) buffer pH, (b) amounts of ACN in BGE, (c) temperature, and (d) voltage on the resolution and migration time of the second enantiomers (125 μg/mL). *Condition*: 100 mM sodium borate buffer (pH (a) 8.0–9.5, (b)–(d) 9.4) containing 18 mM α-CD and (a), (c) and (d) 10% and (b) 0–15% (v/v) ACN; capillary, 64.5 cm total length (8.5 cm to the detector), 50 μm ID; hydrodynamic injection at 50 mbar for 10 s; temperature, (a) and (b) 20 °C, (c) 20–30 °C and (d) 30 °C; voltage, (a)–(c) 25 kV and (d) 15–30 kV; detection by UV absorbance at 220 nm.

in a refrigerator at 8–10 °C, filtered and degassed before CE injections.

2.4. Analytical parameter calculation and migration order

R_s was calculated from $2(t_2 - t_1)/(w_1 + w_2)$, α from t_2/t_1, TF from $w_{0.05}/2f$ and N from $5.54 (t_x/w_{0.5})^2$, where t_x is the enantiomer migration time, w_x is baseline peak width (in time unit) of analyte x, $w_{0.05}$ and $w_{0.5}$ are peak width at 5 and 50% of peak height, respectively, and f is peak width from leading edge of peak to the intercept of a perpendicular line dropped from the peak maximum to the base. Identification of migration order of the resolved R-(−)-, S-(+)-baclofen and impurity A was confirmed by running the electropherograms of the individual pure compound under identical separating conditions.

2.5. Method validation

Method linearity was performed by injecting five different concentrations of baclofen enantiomers and impurity A in ranges of 50–500 and 5–50 μg/mL, respectively, on three different days. Three injections were made for each concentration. Linear regression equation, correlation coefficients (r^2) and %RSDs of slope and intercept were calculated from Excel®.

Injection precision was determined by repetitive injections ($n = 9$) of the mid-points of calibration curves of baclofen (200 μg/mL) and impurity A (30 μg/mL). Intra-day precision was performed on different concentrations of baclofen enantiomers (50, 200 and 500 μg/mL, $n = 3$) and impurity A (10, 30 and 50 μg/mL) and analyzed on the same day. Inter-day precision was performed by determining three different concentrations of baclofen enan-
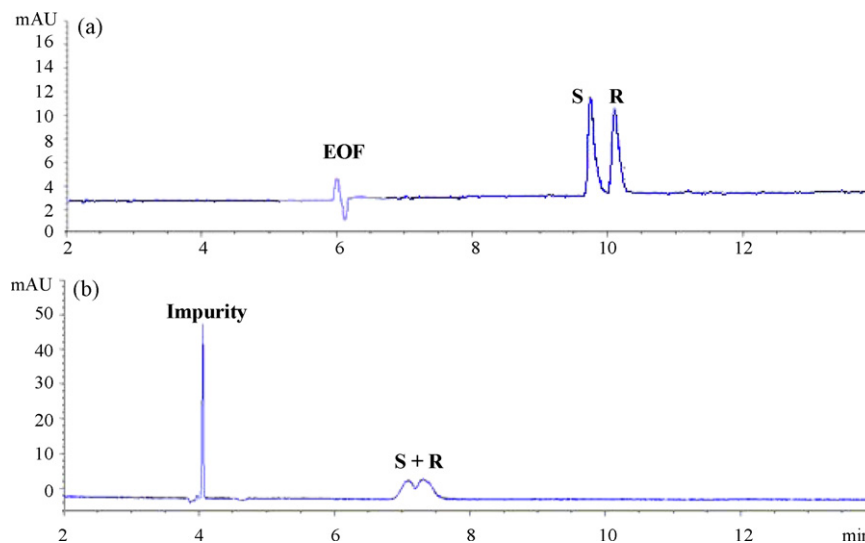

Fig. 3. Electropherograms of (a) baclofen enantiomers (125 μg/mL) and (b) baclofen enantiomers (100 μg/mL) and impurity A (50 μg/mL). *Condition*: 100 mM sodium borate buffer (pH 9.4) containing 18 mM α-CD and 10% (v/v) ACN; capillary 64.5 cm total length (8.5 cm to the detector), 50 μm ID, (a) uncoated and (b) dynamically coated with 0.5% (w/v) PEO; hydrodynamic injection at 50 mbar for 10 s; temperature: (a) 30 and (b) 20 °C; voltage: (a) 30 and (b) 25 kV; detection by UV absorbance at 220 nm. Peak identification: S = S-(+)-baclofen, R = R-(−)-baclofen and EOF = electroosmotic flow.
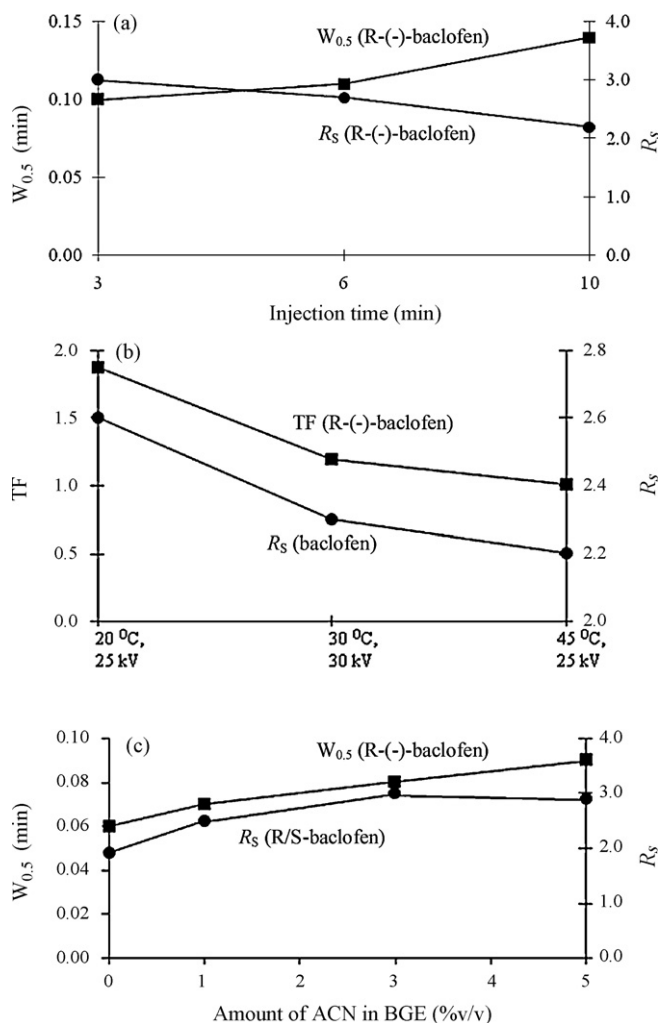
Table 1

Analytical parameters of baclofen enantiomers and impurity A under the optimized condition.

Analyte	t_m (min)	N	$w_{0.5}$ (min)	R_s	α	TF
Impurity A	5.53	292,744	0.02	–	–	1.02
S-(+)-baclofen	8.71	119,533	0.06	–	–	1.13
R-(−)-baclofen	8.98	124,695	0.06	2.7	1.03	1.07

t_m = migration time, N = number of theoretical plate, $w_{0.5}$ = peak width at half height, R_s = resolution, α = selectivity and TF = tailing factor.

Fig. 4. Effects of (a) injection time, (b) temperature and voltage and (c) amounts of ACN in the BGE on the separation of baclofen (49 μg/mL) and impurity A (4.9 μg/mL). *Condition*: 100 mM sodium borate buffer (pH 9.9) containing 18 mM α-CD and (a) and (b) 5% and (c) 0–5% (v/v) ACN; capillary, 64.5 cm total length (8.5 cm to the detector), 50 μm ID; hydrodynamic injection at 50 mbar for (a) 3–10 s, (b) and (c) 6 s; temperature, (a) and (c) 45 °C and (b) 20–45 °C; voltage, (a) 25 kV, (b) 25, 30 kV and (c) 27 kV; detection by UV absorbance at 220 nm.

tiomers (50, 200 and 500 μg/mL) and impurity A (10, 30 and 50 μg/mL) on different days (*n* = 6). Precision was assessed from percent relative standard deviations (%RSDs) of migration time, peak area, normalized peak area, and peak height of baclofen enantiomers and impurity A.

Recovery (R) of the method was performed by spiking different concentrations of baclofen enantiomers and impurity A (80, 100,
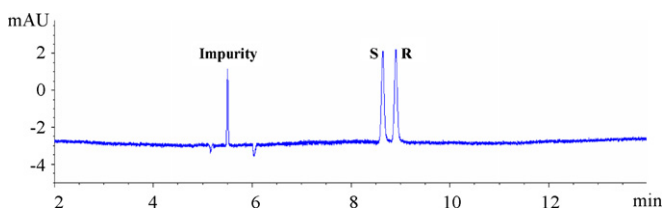

Fig. 5. Electropherogram of baclofen (50 μg/mL) and impurity A (5 μg/mL) under the optimized conditions. *Condition*: 100 mM sodium borate buffer (pH 9.9) containing 18 mM α-CD and 1% (v/v) ACN; capillary 64.5 cm total length (8.5 cm to the detector), 50 μm ID, dynamically coated with 0.5% (w/v) PEO; hydrodynamic injection at 50 mbar for 6 s; temperature: 45 °C; voltage: 27 kV; detection by UV absorbance at 220 nm. Peak identification: S = S-(+)-baclofen, R = R-(−)-baclofen.

and 120% of the nominal concentration) into the sample solutions (*n* = 3). The nominal concentrations were selected from the midpoint of the calibration curves, which were 200 μg/mL for baclofen enantiomers and 30 μg/mL for impurity A. Recoveries were calculated by ratios of amount found and amount added.

Serial dilutions of baclofen enantiomers (5–50 μg/mL) and impurity A (1–10 μg/mL) were investigated for LODs and LOQs determinations. LODs and LOQs are defined as signal to noise ratios (S/N) of 3 and 10, respectively.

3. Results and discussion

3.1. Chiral EKC optimization

Baclofen is an amphoteric molecule with pK_a in water of 3.9 (at the carboxylic acid group) and 9.6 (at the amine group) [31]. EKC conditions for enantioseparation of baclofen were firstly investigated and in the final optimization, impurity A was included. Basic BGE was employed to facilitate the migration of impurity A since it is rather neutral. Native CDs were used because of their low cost and availability in our laboratory and was found to be efficient.

3.1.1. Separation of baclofen enantiomers

Initial EKC conditions, modified from Ref. [25], for enantiomeric separations of baclofen were in borate buffer (pH 9.25). Effects of chiral selector types on the separation were studied using α- and β-CDs at 10 and 18 mM. Baclofen enantiomers migrated as a single peak at 12 and 9 min, when 10 and 18 mM β-CD were used as a chiral selector, respectively. 10 mM α-CD could partially separate the enantiomers in 14 min (R_s = 0.5), while 18 mM α-CD gave well separated peaks at 14.0 and 14.4 min for S-(−)- and R-(+)-baclofen, respectively (R_s = 2.2) (data not shown). The cavity diameter of α-CD was smaller than β-CD (0.49 vs. 0.62 nm) [32] and could efficiently accommodate baclofen enantiomers. The two enantiomers formed distinct diasteriomeric inclusion complexes with α-CD that possessed different physicochemical properties resulting in different migrations. The R-(−)-enantiomer might form the more stable inclusion complex than the S-(+)-enantiomer, which made it migrate later. Effects of α-CD concentrations (10–23 mM) were investigated since degree of enantiomeric resolutions can be varied with chiral selector concentrations [21–23]. Results show that the resolution increased from 0.5 to 2.1 when α-CD concentrations were increased from 10 to 18 mM and then dropped to 0.7 when 23 mM α-CD was added into the BGE (electropherograms not shown). Low α-CD concentrations (10 and 15 mM) were not sufficient for the resolution of baclofen enantiomers. Poor enantiomeric resolution at higher concentrations of α-CD (20 and 23 mM) might due to increased BGE viscosity and reduced conductivity [21]. These results are in good agreement with the theoretical studies reported by Wren and Wren [33] and Penn et al. [34,35]. A maximum R_s was observed at 18 mM α-CD and was chosen as the optimal concentration.

Ionic strength or ionic concentrations of buffer affects BGE viscosity and conductivity hence enantiomeric resolution, selectivity

Table 2
Regression data of baclofen enantiomers (50–500 μg/mL) and impurity A (5–50 μg/mL).

	Peak area	Peak height	Normalized peak area
Impurity A	$y = 0.633x + 0.446$	$y = 0.408x + 0.370$	$y = 0.118x + 0.086$
%RSDs of slope	0.89	3.67	2.56
%RSDs of intercept	4.46	71.0	39.1
r^2	0.999	0.997	0.999
S-(+)-baclofen	$y = 0.606x + 5.359$	$y = 0.098x + 4.885$	$y = 0.073x + 0.543$
%RSDs of slope	0.44	5.61	2.56
%RSDs of intercept	3.78	6.28	37.3
r^2	0.999	0.984	0.999
R-(−)-baclofen	$y = 0.698x + 5.688$	$y = 0.119x + 5.166$	$y = 0.081x + 0.562$
%RSDs of slope	0.96	4.38	2.38
%RSDs of intercept	2.51	2.38	37.9
r^2	0.999	0.986	0.999

Table 3
Injection, intra-day and inter-day precision data of baclofen enantiomers and impurity A.

	%RSDs			
	t_m	Peak area	Peak height	Normalized peak area
Injection precision ($n = 9$)				
Impurity A	0.41	1.98	2.60	2.12
S-(+)-baclofen	2.89	1.43	4.42	2.36
R-(−)-baclofen	2.97	1.64	4.72	2.21
Intra-day precision ($n = 3$)				
Impurity A	0.15	0.61	0.92	0.29
S-(+)-baclofen	0.18	0.63	0.59	0.72
R-(−)-baclofen	0.18	0.51	0.81	0.59
Inter-day precision ($n = 6$)				
Impurity A	1.16	3.37	4.50	3.36
S-(+)-baclofen	1.63	1.87	10.63	2.22
R-(−)-baclofen	1.70	2.05	9.67	2.68

and electromigration dispersion [21,32]. Varying the BGE ionic strength (50–130 mM sodium borate buffer, pH 9.25), slight effects on the separation selectivity ($\alpha = 1.01$–1.03) was observed. However, longer migration time from 9.70 to 17.55 min was obtained (electropherograms not shown), which might due to the more stable inclusion complex inside the hydrophobic cavity of the chiral selector [36]. Increasing the BGE ionic strength from 50 to 100 mM enhanced the R_s from 1.0 to 2.1, but broad peaks ($w_{0.5} = 0.19$ min) with lower resolution ($R_s = 1.7$) and sensitivity were obtained at 130 mM. The proper ionic strength was at 100 mM sodium borate.

Baclofen enantiomers migrated as anions under the investigated pHs, thus changing pH affected the enantiomeric discrimination with α-CD, which is a neutral CD [37–41]. This implies that the negatively charged form of baclofen enantiomers have different interaction with α-CD resulting in distinct binding constants and stabilities of the diasteriomeric complexes. R_s of baclofen enantiomers increased from 0 to 2.0 when pH was increased from 8.0

to 9.4 then decreased to 1.1 at pH 9.5 (Fig. 2(a)). Thus, pH 9.4 was selected.

Addition of organic solvents (*e.g.* ACN and MeOH) into the BGE containing CD affects enantiomeric resolution, inclusion complexes with analytes, solubility, BGE viscosity, migration times and peak dispersion [32,33,35]. If the CD concentration is above the optimum value, the addition of organic solvent will improve the peak resolution. If it is below the optimum value, the lost of peak resolution will be obtained. Without ACN in the BGE, baclofen enantiomers were eluted as one peak at 10 min. Increases of ACN significantly improved the R_s to 1.4 (5% ACN) and 2.0 (10% ACN) (Fig. 2(b)), while the selectivity of 1.02 was obtained at both concentrations. This implies that 18 mM α-CD may be above the optimum concentration and, therefore, improved resolution of baclofen enantiomers was observed with an increase in ACN concentration. However, further increasing the ACN to 15% (v/v) worsen the peak shapes and resolution ($R_s = 1.0$) due to peak broadening ($w_{0.5} > 0.66$ min). An appropriate amount of ACN (10%, v/v) enhanced the enantiomer resolution and reduced peak width ($w_{0.5} = 0.12$ min) due to the decreasing of inclusion complex and capillary wall interaction [27].

Effects of instrumental factors on the separation of baclofen enantiomers, including temperature (20–30 °C) and separating voltage (15–30 kV), were evaluated. Separation selectivity remained the same ($\alpha = 1.02$) under these conditions. Increasing of temperature decreased the resolution and migration time (Fig. 2(c)), but increasing of voltage enhanced the resolution with reduced migration time (Fig. 2(d)). Interestingly, greater mobility differences ($\Delta\mu_e$) of the enantiomers increased at elevated temperature and voltage (data not shown). Therefore, temperature of 30 °C and voltage of 30 kV were used to obtain the enantioseparation of baclofen providing the highest R_s (2.0), $\Delta\mu_e$ (0.35×10^{-9} cm²/V s) and shortest migration time (10.35 min).

Enantiomeric resolution of baclofen was in 100 mM sodium tetraborate buffer (pH 9.4) containing 18 mM α-CD and 10% (v/v) ACN using voltage of 30 kV, temperature of 30 °C, injection of 50 mbar for 10 s and detection at 220 nm (Fig. 3(a)).

Table 4
Recovery data of baclofen enantiomers and impurity A ($n = 3$).[a]

%Added	Impurity A			S-(+)-baclofen			R-(−)-baclofen		
	Amount added (μg/mL)	Amount found (μg/mL)	%R	Amount added (μg/mL)	Amount found (μg/mL)	%R	Amount added (μg/mL)	Amount found (μg/mL)	%R
80	26.2	25.2	96.2 (1.06)	166.4	171.6	103.1 (1.91)	176.0	175.3	99.6 (1.50)
100	32.8	31.5	96.0 (0.31)	208.0	211.7	101.8 (0.39)	208.0	207.3	99.7 (0.0)
120	39.4	37.9	96.2 (1.17)	249.6	249.1	99.8 (0.58)	249.6	253.3	101.5 (0.45)
LOD (μg/mL)	2			10			10		
LOQ (μg/mL)	5 (6.70)			30 (2.84)			30 (1.85)		

[a] Numbers in parentheses represent %RSDs.

Table 5
Assay data of baclofen enantiomers and impurity A in raw material and tablets (n = 3).[a].

	Impurity A (%w/w)	% Determined amount	Ratio of S-(+):R-(−)-baclofen
Raw material			
Brand A	ND	101.1 (0.89)	1.07:1
Brand B	ND	99.3 (1.03)	1.07:1
USP limits	1%	99.0–101.0	–
BP limits	1%	98.0–101.0	–
Tablet			
Brand C	ND	109.1 (0.89)	1.06:1
Brand D	0.12 (1.95)	108.0 (1.52)	1.07:1
Brand E	0.39 (1.76)	107.9 (0.84)	1.03:1
USP limits	4%	90.0–110.0	–

[a] ND = not detected (LOD = 2 µg/mL), numbers in parentheses represent %RSDs.

3.1.2. Separation of baclofen enantiomers and impurity A

The ultimate goal of this work was to obtain the baseline separation of baclofen enantiomers and impurity A by EKC. The condition in Fig. 3(a) was used for the separation of impurity A, however, a broad peak overlapped with the EOF was obtained (electropherogram not shown). The impurity could not fit into the α-CD cavity and migrated at the same velocity with the EOF since it was rather neutral under this condition and was swept out from the capillary by the EOF. Consequently, controlling of the EOF could play an important role on the separation of the impurity. Addition of polymer such as hydroxypropylmethylcellulose (HPMC), polyvinylalcohol (PVA) and PEO reduces the EOF, increases viscosity and eliminates or reduces analyte adsorption [28,29,32]. Although Vaccher et al. [28,29] proposed acidic BGE (pH 2.5) for the enantioseparation of baclofen, basic BGE (pH > 9.25) was currently employed to facilitate the migration of impurity A in the coated capillary. With the reduced EOF, baclofen enantiomers were attracted to the anodic site (*i.e.* inlet) due to negative charges on their molecules, thus lower temperature (20 °C) and voltage (25 kV) were employed to ensure their separation of baclofen. Results showed that, with the coated capillary (*i.e.* reduced EOF), the impurity migrated as a sharp peak at 4.2 min, but baclofen enantiomers
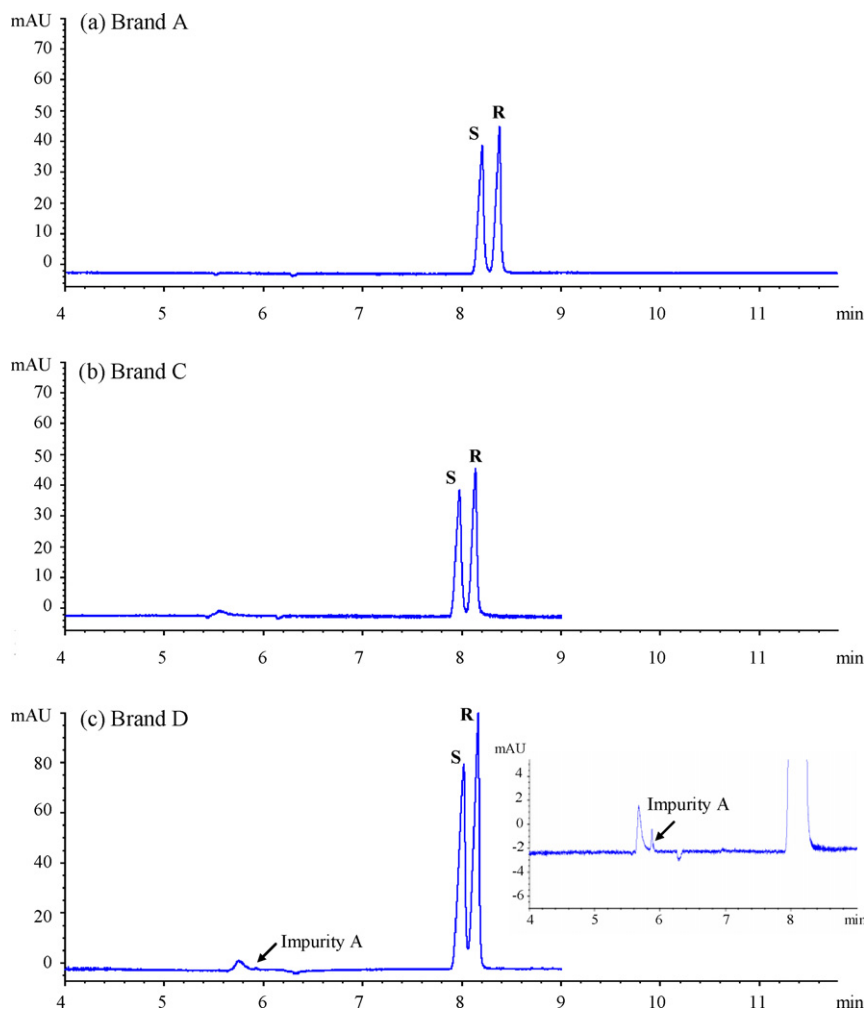

Fig. 6. Electropherograms of baclofen enantiomers (400 µg/mL) in (a) raw material brand A, (b) tablet brand C and (c) baclofen enantiomers (1000 µg/mL) and impurity A (0.12%, w/w) in tablet brand D. Condition and peak identification: see Fig. 5.

overlapped at 7.0 min (Fig. 3(b)). Interestingly, increasing pH of the BGE from 9.4 to 9.9 enhanced the enantiomer resolution from 0.8 to 1.0. Therefore, dynamically coated capillaries and 100 mM sodium borate buffer (pH 9.9) were employed for the remaining experiments.

The dynamically coated capillary enabled the migration of impurity A as the first peak, which was well separated from the overlapped peaks of baclofen enantiomers ($\Delta t_m \sim 3$ min). Therefore, other factors (*e.g.* injection time, temperature, voltage and amounts of ACN in the BGE) were re-investigated to obtain enantioseparation of baclofen. To optimize the chiral resolution and peak shapes, sample loading of 50 mbar for 3–10 s was investigated. Decreases of R_s from 3.0 to 2.2 were observed when injection times were increased from 3 to 10 s (Fig. 4(a)) due to peak broadening. Additionally, at 3 s, the signal was too low (~ 2 mAU), while 10 s gave broad peaks with $w_{0.5}$ of >0.14 min. Thus, an injection time of 6 s was chosen as a compromised leading to a R_s of 2.7 and $w_{0.5}$ of 0.11 min. Influences of temperature and voltage on the separation of baclofen enantiomers and impurity A were simultaneously studied to reduce electromigration dispersion and migration times. Separation of the analytes could be achieved at all investigated temperature and voltage (20 °C/25 kV, 30 °C/30 kV and 45 °C/25 kV) with $R_s > 2.2$, however, tailing peaks of baclofen enantiomers (TF = 1.9) was observed at 20 °C/25 kV. At 30 °C/30 kV and 45 °C/25 kV, comparable R_s (2.2–2.3), migration time (12 min), selectivity (1.04) and TF (1.0–1.2) were obtained (Fig. 4(b)). The latter was selected since 30 kV caused baseline drift and the current almost reached the instrument limit (300 μA). A slight increase of voltage from 25 to 27 kV could reduce the migration time to 9 min without baseline and current problems. Therefore, 45 °C/27 kV was used for further optimization. Organic solvents significantly affect enantioseparation as previously mentioned. Amounts of ACN in the BGE was re-adjusted since 5 and 10% (v/v) ACN still provided broad peaks of baclofen. Decreasing amounts of ACN from 5 to 0%, reduced the migration time from 11.3 to 8.5 min and $w_{0.5}$ from 0.09 to 0.06 min (Fig. 4(c)). Presences of 1, 3 and 5% (v/v) ACN in the BGE gave $R_s > 2.5$, however, without ACN the R_s greatly reduced to 1.9. Therefore, 1% (v/v) ACN was employed giving a R_s of 2.7 and $w_{0.5}$ of 0.06 min in short time (9 min).

Finally, the optimum CE condition for the separation of baclofen enantiomers and impurity A was in 100 mM sodium tetraborate buffer (pH 9.9) containing 18 mM α-CD and 1% (v/v) ACN using a voltage of 27 kV, temperature of 45 °C, injection of 50 mbar for 6 s and detection at 220 nm (Fig. 5). Table 1 represents analytical parameters of the analytes.

3.2. Method validation

Calibration curves of baclofen enantiomers and impurity A were established in ranges of 50–500 and 5–50 μg/mL, respectively. Regression data calculated from peak area, peak height and normalized peak area showed that peak area provided the best correlation coefficients ($r^2 > 0.999$) and lowest %RSDs for both slope and intercept for all analytes (Table 2). Precision of the method was determined by injection, intra- and inter-day precision and %RSDs of the migration times for all analytes was within 2.97% (Table 3). Additionally, precision calculated from peak area gave lowest %RSDs (<3.37%) in most cases comparing to peak height and normalized peak area. Mean recoveries were 96.1% for impurity A, 101.6% for S-(+)- and 100.3% for R-(−)-baclofen with %RSDs of <1.91% (Table 4). The LODs and LOQs were less than 10 and 30 μg/mL, respectively, for all analytes (Table 4). The %RSDs for the LOQ were within 2.84 for the baclofen enantiomers and 6.70% for impurity A. Analytical performance characteristics revealed that the method was efficient for the quantitation of baclofen enantiomers and impurity A in pharmaceutical products.

3.3. Applications

Applications of the developed method were performed in two different brands of raw materials and three different brands of tablets. Results from the analysis of baclofen enantiomers and impurity A in raw material and tablets are shown in Table 5 and typical electropherograms are presented in Fig. 6. Matrices from tablets did not interfere since no extra peaks were observed during the analysis. The ratios of S-(+)- and R-(−)-baclofen were 1.07:1 in raw material and varied from 1.03 to 1.07:1 in tablets. The investigated samples generally contained S-(+)- more than R-(−)-enantiomer in both raw material and tablets. Assay of baclofen in raw material and tablets showed % determined amount of 99.3–101.1 and 107.9–109.1%, respectively, which were within limits of USP and BP. Impurity A could not be detected in raw material brand A, B and tablet brand C, however, tablets brand D and E contained small amounts of the impurity, but still were within USP limit. The impurity A in brand D and E was confirmed by spiking the sample with standard impurity A and increased of signals was observed in the spiked samples.

4. Conclusion

This work demonstrates the simultaneous separation of baclofen enantiomers and impurity A by EKC using α-CD as the chiral selector. Enantioseparation of baclofen could be achieved in an uncoated capillary, however, when the impurity was included, manipulation of the EOF was required. Controlling of the EOF by coating the capillary with PEO significantly improved the separation efficiency of the impurity. The amount of impurity A can be accurately quantified with less time and labor consuming than methods described in the pharmacopoeias. Resolution of baclofen enantiomers ($R_s = 2.7$) and migration time of the second enantiomer ($t_m = 9.0$ min) were comparable or, in some cases, better than those reported in literatures [24–29]. The LODs of 2 μg/mL for impurity A and 10 μg/mL for baclofen enantiomers were acceptable for analyses of these compounds in pharmaceutical raw material and products. This is the first time that an EKC method is developed for the separation of baclofen enantiomers and its impurity in a single run. The method is efficient, fast, simple and low cost, which is important for the routine quality control of the drug in pharmaceutical manufacturers.

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