ANALYSIS OF BACLOFEN ENANTIOMERS AND IMPURITY A BY CHIRAL CAPILLARY ELECTROPHORESIS

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ABSTRACT

Baclofen, the selective $GABA_B$ receptor agonist, is used as a muscle relaxant for the treatment of spasticity associated with spinal cord injury. It is commercially available as a racemic mixture but only baclofen *R*-(-)-enantiomer is stereospecifically active. Additionally, (4*RS*)-4-(4-chlorophenyl)pyrrolidin-2-one is an important impurity in the synthesis and degradation pathway of baclofen. Analysis of baclofen enantiomers and its impurity is, therefore, important for the quality control of baclofen formulations. This research proposed a rapid and simple method for the simultaneous analysis of these compounds by chiral capillary electrophoresis (CCE). The CCE method was developed by varying buffer and chiral selector concentrations, pH of buffer, types of chiral selectors, amounts of organic solvents, temperature and voltage. α-Cyclodextrin was employed as a chiral selector, which enabled the separation of baclofen enantiomers. Dynamic coating of the capillary with 0.5% w/w polyethylene oxide (PEO) was necessary for the electroosmotic flow (EOF) suppression, which facilitated the separation of baclofen impurity. The optimized condition was in 100 mM sodium borate buffer (pH 9.9) containing 18 mM α-CD and 1% v/v acetonitrile using a fused-silica capillary dynamic coated with PEO, 64.5 cm total length with an effective length of 56 cm and inner diameter of 50 μ, hydrodynamic injection at 50 mbar for 6 s, temperature and applied voltage of 45 ^{o}C and 27 kV, respectively, and detection by UV absorbance at 220 nm. Baclofen enantiomers and its impurity were baseline separated (R_s = 2.7 and 44.8, respectively) in 10 min. The method was validated and the results showed good linearity (r^2 > 0.9994), precision (%RSD < 3.37%), and recovery (96-103.1%). The limits of detection and quatitation were 10 and 30 μg/ml for *S*-enantiomer, 7 and 30 μg/ml for *R*-enantiomer, and 2 and 5 μg/ml for baclofen impurity, respectively. The method was successfully applied for the analysis of baclofen enantiomers and its impurity in pharmaceutical raw materials and pharmaceutical formulations.

KEY WORDS: ENANTIOSEPARATION/ BACLOFEN/ CHIRAL CAPILLARY ELECTROPHORESIS

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การวิเคราะห์เอแนนทิโอเมอร์ของแบคโคเฟนและสารเจือปนโดยไคแรลแคปิลลารีอิเล็กโทรโฟริซิส
ANALYSIS OF BACLOFEN ENANTIOMERS AND IMPURITY A BY CHIRAL CAPILLARY ELECTROPHORESIS

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บทคัดย่อ

แบคโคเฟนเป็นยาคลายกล้ามเนื้อในกลุ่มอนุพันธ์ของกรดแกมมาอะมิโนบูทิริก (GABA) ออกฤทธิ์โดยจับกับ $GABA_B$ receptor ในสมองและไขสันหลัง มีประสิทธิภาพในการรักษาการหดเกร็งของกล้ามเนื้อที่มีสาเหตุมาจากการบาดเจ็บของไขสันหลัง วัตถุดิบที่ใช้ผลิตแบคโคเฟนอยู่ในรูปส่วนผสมระหว่างอาร์-(-)- และเอส-(+)-แบคโคเฟน แต่มีเพียงอาร์-(-)-แบคโคเฟนเท่านั้นที่ออกฤทธิ์ทางเภสัชวิทยา นอกจากนั้นในขบวนการสังเคราะห์และการเก็บรักษาแบคโคเฟนจะเกิดสารเจือปน คือ (4-อาร์เอส)-4-(4-คลอโรพีนิล)ไพโรริ-ดีน-2-โอน ดังนั้นงานวิจัยนี้จึงได้ทำการพัฒนาวิธีวิเคราะห์และประเมินวิธีวิเคราะห์ในการแยกเอแนนทิโอเมอร์ของแบคโคเฟนและสารเจือปนโดยไคแรลแคปิลลารีอิเล็กโทรโฟริซิส การพัฒนาวิธีวิเคราะห์ได้ศึกษาปัจจัยต่าง ๆ ที่มีผลต่อการแยกของสารทั้ง 3 ตัว ได้แก่ ชนิดของตัวคัดแยก (chiral selector) ความเข้มข้นของบัฟเฟอร์และตัวคัดแยก ค่าพีเอชของบัฟเฟอร์ ปริมาณตัวทำละลายอินทรีย์ อุณหภูมิและความต่างศักย์ไฟฟ้า จากการศึกษาพบว่าสภาวะที่เหมาะสมได้แก่ การใช้ 100 มิลลิโมลาร์ โซเดียมบอเรตบัฟเฟอร์ (พีเอช 9.9) ซึ่งประกอบด้วย 18 มิลลิโมลาร์ อัลฟา-ไซโคลเด็กซ์ทรินและอะซิโตไนไตรล์ (1% โดยปริมาตร) ความยาวของหลอดแคปิลลารีที่เคลือบด้วยโพลิเอทธิลลีนออกไซด์ (0.5% โดยน้ำหนัก) มีความยาวทั้งหมด 64.5 เซนติเมตร เส้นผ่านศูนย์กลางภายใน 50 ไมครอน ใช้การฉีดแบบไฮโดรไดนามิกที่ 50 มิลลิบาร์เป็นเวลา 6 วินาที อุณหภูมิและความต่างศักย์ไฟฟ้าเท่ากับ 45 องศาเซลเซียส และ 27 กิโลโวลต์ ตามลำดับ ตรวจวัดที่ความยาวคลื่นเท่ากับ 220 นาโนเมตร สภาวะดังกล่าวสามารถแยกสารทั้ง 3 ตัวได้อย่างสมบูรณ์ภายในเวลา 10 นาที โดยมีค่าการแยกชัด (resolution) เท่ากับ 2.7 และ 44.8 ตามลำดับ การประเมินวิธีวิเคราะห์ให้ค่าความสัมพันธ์เส้นตรงที่ดี ($r^2 > 0.9994$) และค่าความแม่นยำที่ดี (%RSD < 3.37%) ค่าความถูกต้องอยู่ในช่วงร้อยละ 96.0-103.1 ค่าลิมิตการตรวจหาและการวิเคราะห์ปริมาณเท่ากับ 10 และ 30 ไมโครกรัมต่อมิลลิลิตร สำหรับเอส-(+)-แบคโคเฟน 7 และ 30 ไมโครกรัมต่อมิลลิลิตร สำหรับอาร์-(-)-แบคโคเฟน 2 และ 5 ไมโครกรัมต่อมิลลิลิตร สำหรับ (4-อาร์เอส)-4-(4-คลอโรพีนิล)ไพโรริดีน-2-โอน ตามลำดับ วิธีวิเคราะห์ที่พัฒนาขึ้นและผ่านการประเมินมีประสิทธิภาพในการวิเคราะห์ปริมาณของแบคโคเฟนแอแนนทิโอเมอร์และสารเจือปนในวัตถุดิบและยาสำเร็จรูป

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LIST OF ABBREVIATIONS

ACN	acetonitrile
2-AIPHP-ß-CD	2-*O*-(2-aminoethyl-imino-propyl)-ß-*O*-hydroxypropyl-ß-cyclodextrin
APOC	1-(9-anthryl)-2-propyl chloroformate
A^o	angstrom
BGE	background electrolyte
α-CD	alpha-cyclodextrin
ß-CD	beta-cyclodextrin
γ-CD	gamma-cyclodextrin
CDR	chiral derivatizing reagent
CCE	chiral capillary electrophoresis
CNS	central nervous system
CRPS	complex regional pain syndrome
cm	centimeter
CE	capillary electrophoresis
CZE	capillary zone electrophoresis
CDs	cyclodextrins
[CS]	equilibrium concentration of the chiral selector
$[CS]_{opt}$	optimized concentration of the chiral selector
Cs	chiral selectors
CGTase	cyclodextrin glycosyltransferase
CM-ß-CD	carboxymethyl-ß-cyclodextrin
CSP	chiral stationary phase
CMC	critical micellar concentration
^oC	degree celcius
DAD	diode array detector
EOF	electroosmotic flow

LIST OF ABBREVIATIONS (Cont.)

EPMEs	potentiometric membrane electrodes
f	width at 5% peak height measured from the leading edge of the peak to a vertical line extrapolated from the peak apex
GABA	gamma-aminobutyric acid
GLC	gas-liquid chromatography
g	gram
GB	gigabyte
GC-EC	gas-liquid chromatography with electron capture detection
GC-MS	gas chromatography-mass spectrophotometry
HE-ß-CD	hydroxyethyl-ß-cyclodextrin
HP-ß-CD	hydroxypropyl-ß-cyclodextrin
HMMS	high-molecular-mass surfactant
HPMC	hydroxypropylmethylcellulose
HEC	hydroxyethylcellulose
HDMS	heptakis(2,3-di-methyl-6-sulfato)-ß-cyclodextrin
HPLC	high-performance liquid chromatography
I.D.	internal diameter
K	equilibrium constant of the complexation reaction of enantiomer
K_R	equilibrium constant of the complexation of reaction of R-enantimer
K_S	equilibrium constant of the complexation of reaction of R-enantimer
kV	kilovotage

LIST OF ABBREVIATIONS (Cont.)

ΔK	equilibrium constant difference of the complexation reaction of enantiomers
LIF	laser-induced fluorescence
LC/MS/MS	liquid chromatography-tandem mass spectrometry
LE-CE	ligand-exchange capillary electrophoresis
LA	labeled amount
LOD	limit of detection
LOQ	limit of quantification
MeOH	methanol
mM	millimolar
mbar	millibar
min	minute
ml	milliliter
Me-ß-CD	methyl-ß-cyclodextrin
MEKC	micellar electrokinetic chromatography
MHz	megahertz
MB	megabyte
mm	millimeter
NMR	nuclear magnetic resonance
N	theoretical plate
nm	nanometer
ODAS	octakis(2,3-di-acetyl-6-sulfato)-γ-cyclodextrin
O.D.	outside diameter
PEO	polyethylene oxide
PrAMCD	6^A-propylammonium-6^A-deoxy-ß-cyclodextrin chloride
pK_a	acid dissociation constant

LIST OF ABBREVIATIONS (Cont.)

poly(L-SUV)	poly(sodium N-undecylenyl-L-valinate)
PAA	polyacrylamide
PVA	polyvinyl alcohol
PVP	polyvinyl pyrrolidone
PEO	polyethylene oxide
PEG	polyethylene glycol
r^2	correlation coefficient
R_s	resolution
RSD	relative standard deviation
s/n	signal to noise ratio
s	second
SD	standard deviation
SDC	sodium deoxycholate
ST	sodium taurocholate
STDC	sodium taurodeoxycholate
SDVal	N-dodecanoyl-L-valinate
SDAla	sodium N-dodecanoly-L-alanine
SDGlu	sodium N-dodecanoyl-L-glutamate
SC	sodium cholate
SBE-ß-CD	sulfobutylether-ß-cyclodextrin
SPE	solid phase extraction
t_m	migration time
TDM	therapeutic drug monitoring
TLC	thin layer chromatography
TMA-ß-CD	2-hydroxy-3-trimethylammoniopropyl-ß-cyclodextrin
TF	tailing factor

LIST OF ABBREVIATIONS (Cont.)

USP	United State Pharmacopiea
UV	ultraviolet light
V	voltage
v	volume
w	peak width
$w_{1/2}$	temporal peak width at half height
$w_{0.05}$	peak width at 5% of peak height as measured from the front side of the peak to the tailing edge
W	watt
X_{found}	concentration of standard found in the spiked sample
X_{added}	concentration of standard added
μ	apparent electrophoretic mobility of enantiomer
μ_f	electrophoretic moblities of free solute
μ_c	electrophoretic moblities of complexed solute
$\Delta\mu$	electrophoretic mobility difference
μ_{avg}	average electrophoretic mobility
μ_{eof}	electroosmotic flow mobility
µm	micron
µg	microgram
µA	microampere
α	selectivity
$[\alpha]_D$	specific rotation

CHAPTER I
INTRODUCTION

Gramma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system (CNS),[1] can be divided into two major types, ionotropic ($GABA_A$ and $GABA_C$ receptors), and metabotropic receptors, which are G-protein-coupled receptor ($GABA_B$ receptors).[2] Baclofen [4-amino-3-(ß-chlorophenyl) butyric acid] is one of the stereoselective agonists for the $GABA_B$ receptor[3] and mostly used as a muscle relaxant in the treatment of spasticity occurring secondary to multiple sclerosis, tardive dystonia, cerebral and spinal cord injury, tetanus, cerebral palsy, stiff-person syndrome and complex regional pain syndrome (CRPS).[4] Commercially, it is available as a racemic mixture (Baclofene-Irex®, Lioresal®) but only *R*-(-)-enantiomer is stereospecifically active.[5,6] On the other hand, the *S*-(+)-enantiomer is almost inactive or even toxic.[7] Baclofen was firstly synthesized in 1962.[8] The synthesis pathways of both enantiomers of baclofen have been reported,[8-12] some of which involve an enzymatic reaction.[13,14] Recently, more studies have been described the developed methods for the enantioselective synthesis of chiral biologically active (*R*-enantiomer) of baclofen.[13-20]

(4*RS*)-4-(4-chlorophenyl)pyrrolidin-2-one is a major related product from the synthesis pathway of baclofen.[8-10] However, only *R*-(-)-enantiomer is an important lipophilic pro-drug related with GABA, showing its muscle relaxant activity, which has similar activity to baclofen.[8] Moreover, (4*RS*)-4-(4-chlorophenyl)pyrrolidin-2-one can also be occurred in a degradation pathway of baclofen.[21-23] These compounds are important impurities in the synthesis and the degradation pathway of baclofen. However, the enantioseparation of these impurities have not yet been described.

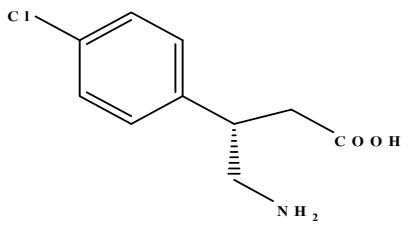
The determination of baclofen racemate in biological fluid have been achieved by several methods including gas-liquid chromatography (GLC) following derivatization or with electron capture detection (GC-EC),[24-26] gas chromatography-mass spectrophotometry (GC-MS),[27] high-performance liquid chromatography

(HPLC) with UV,[28,29] fluorescence[30,31] and electrochemical detection[32] after derivatization, SPE-HPLC with amperometric[33] and electrochemical detection[34,35] after derivatization, capillary electrophoresis (CE) with UV[36] and laser-induced fluorescence (LIF) detection[37-39] with derivatization. For chiral assay, different methods were developed such as GC-EC,[40] potentiometric membrane electrodes (EPMEs) based on maltodextrin[41] or α- and γ-CDs,[42] liquid chromatography-tandem mass spectrometry (LC/MS/MS),[43] HPLC[44,32,45-48] and CE.[48-57] Currently, CE has attracted attention as a favorable technique for the chiral separation because this technique offers several advantages such as high efficiency and selectivity, flexibility of method development, separation speed, minimized organic waste, low costs and small sample requirements.[58] Enantioseparation by CE bases on two approaches for the creation of distinctive migration of enantiomers, the indirect and the direct methods. For indirect method, enantiomeric resolution involves the coupling of the enantiomers with derivatizing agents to convert them into diastereomers.[59] In the direct method, chiral selectors (e.g., cyclodextrin, chiral surfactants, ligand exchange selectors, alkaloids, crown ether, natural macromolecules and chiral ion-pairing reagents)[60] are added as additives to the background electrolyte to covalently form diastereomeric selector-enantiomer complexes[58] that based on enantioselectivity of each enantiomer with chiral selector.[61] The aim of this work were to develop a CZE procedure for the enantioseparation of baclofen and its impurities by using native cyclodextrin. The influence of the types and concentrations of CDs, amounts of organic modifiers, concentrations and pH of buffer, voltage, and temperature on the baseline separation were discussed. The optimized method was validated and applied for the determination of baclofen and its impurities in raw material and pharmaceutical formulations.

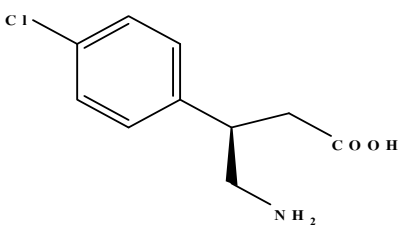
CHAPTER II
LITERATURE REVIEW

1. Baclofen

Baclofen, ß-[4-chlorophenyl] GABA, the *p*-chlorophenyl derivative of gamma-aminobutyric acid (GABA),[62] is an endogenous inhibitory neurotransmitter (Fig. 1).[1] It is commercially available as a racemic mixture but only the *R*-(-)-enantiomer is physiologically active.[4,62] Baclofen functions as a stereoselective agonist of the $GABA_B$ receptor and as such is used primarily for its anti-spasticity effects. Unlike the ligand-gated chloride ion channel $GABA_A$**,** $GABA_B$ is a G-protein-coupled receptor. Activation of the $GABA_B$ receptor results in reduced intraneuronal cAMP and decreased Ca^{2+} and increased K^+ conductance. The effect on Ca^{2+} conductance appears to be primarily presynaptic, whereas modulation of K^+ conductance is postsynaptic. The $GABA_B$ receptor is distributed in the central nervous system (CNS) in high density in the dorsal horn of spinal cord, thalamic nuclei, cerebellum, interpeduncular nucleus, and cerebral cortex. $GABA_B$ receptors are also found peripherally, notably in the gastrointestinal tract and the heart.[4]



R-(-)-enantiomer



S-(+)-enantiomer

Fig. 1 Chemical structures of baclofen.

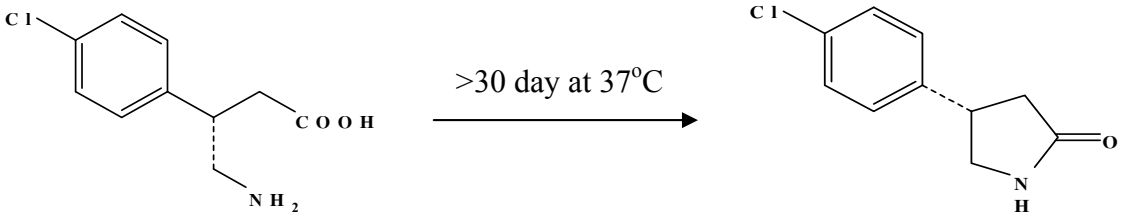
$GABA_B$ receptor agonism has been clinically exploited, mainly for its central anti-spasticity effects. Unlike GABA, baclofen is not rapidly cleared by the GABA reuptake mechanism and thus clinically valuable. Clinical efficacy has been demonstrated in a randomized, double-blind, placebo-controlled study in patients with severe spinal spasticity. Improvement in quality-of-life measures in patients with severe spasticity was demonstrated in another longitudinal, randomized, double-blind study. Baclofen has been used effectively in the treatment of spasticity associated with multiple sclerosis, cerebral palsy, tardive dystonia, cerebral and spinal cord injury, tetanus, stiff-person syndrome, and complex regional pain syndrome (CRPS).[4,63] Besides spasticity control, baclofen may have a role as an analgesic. Side effects of baclofen include sedation, weakness, and confusion.[62] Animal studies have established an antinociceptive role for $GABA_B$ agonism. No ramdomized clinical trials on the analgesic effects of intrathecal baclofen have been conducted. Anecdotal evidence suggests a beneficial effect of intrathecal baclofen in musculoskeletal pain secondary to spasticity, central pain, and CRPS.

Additional beneficial effects of baclofen include suppression of drug and alcohol craving in experimental and preliminary clinical findings, as well as antibronchospastic and antitussive activity. Baclofen is a slightly hydrophilic agent and is well absorbed orally, but crosses the blood-brain barrier ineffectively. In addition, it is uniformly distributed between spinal and supratentorail centers. Thus, treatment with oral baclofen has often been hampered by supraspinal side effects such as nausea, sedation, drowsiness, and fatigue.[4]

1.1 Synthesis and degradation of baclofen

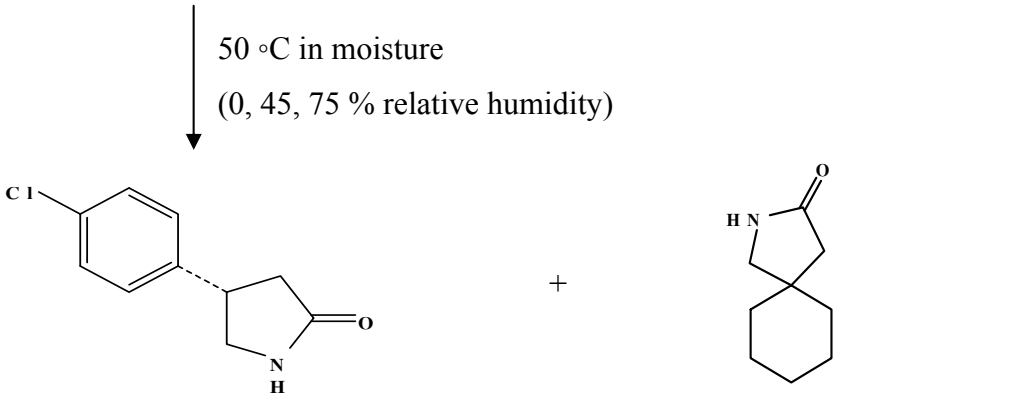
Baclofen was synthesized for the first time in 1962.[8] The synthesis pathways of both enantiomers of baclofen have been reported,[8-12] some of which involve an enzymatic reaction.[13,14] Recently, more studies have been described the developed methods for the enantioselective synthesis of chiral biologically active (*R*-enantiomer) of baclofen.[13-20] Several compounds can serve as key precursors for the synthesis of baclofen such as γ-nitroesters,[8] 4-aryl-1,2,5,6-tetrahydropyridines,[12] and pyroglutamic acid.[10] In the synthesis process of baclofen, the same precursors can be transformed into the corresponding (4*RS*)-4-(4-chlorophenyl)-2-pyrrolidone, which are important

related product of baclofen.[8-10] The studies of stability and compatibility profile of baclofen have been described in several literatures. It was found that baclofen in solid formulations stored at 50 °C under three different humidity (0, 45, 75 % relative humidity) can undergo degradation giving rise to the corresponding lactams-2-azaspiro[4,5]decan-3-one and (4*RS*)-4-(4-chlorophenyl)-2-pyrrolidone.[21] Moreover, baclofen in aqueous formulations stored at 37 °C give rise to little (4*RS*)-4-(4-chlorophenyl)-2-pyrrolidone[22-23] (Fig. 2).



(4*RS*)-amino-3-(4-chlorophenyl) Butanoic acid: Baclofen

(4*RS*)-4-(4-chlorophenyl)-2-pyrrolidone



(4*RS*)-4-(4-chlorophenyl)-2-pyrrolidone lactams-2-azaspiro[4,5]decan-3-one

Fig. 2 Degradation products of baclofen.

It seems that (4*RS*)-4-(4-chlorophenyl)-2-pyrrolidone are important impurities in the synthesis and the degradation pathway of baclofen and these impurities is an enantiomer compound (*R*- and *S*-form). However, only *R*-(-)-enantiomer is an important lipophilic pro-drug related with GABA, showing its muscle relaxant activity which has similar activity to baclofen.[8]

1.2 Analysis of baclofen and its impurity

The determination of baclofen is very important in order to achieve an optimal therapeutic drug monitoring (TDM). The determination of baclofen racemate in biological fluid have been achieved by several methods including gas-liquid chromatography (GLC) following derivatization or with electron capture detection (GC-EC),[24-26] gas chromatography-mass spectrophotometry (GC-MS),[27] high-performance liquid chromatography (HPLC) with UV,[28,29] fluorescence[30,31] and electrochemical detection[32] after derivatization, SPE-HPLC with amperometric[33] and electrochemical detection[34,35] after derivatization, capillary electrophoresis (CE) with UV[36] and laser-induced fluorescence (LIF) detection[37-39] with derivatization. For chiral assay, different methods were developed such as GC-EC,[40] potentiometric membrane electrodes (EPMEs) base on maltodextrin[41] or α- and γ-CDs,[42] liquid chromatography-tandem mass spectrometry (LC/MS/MS),[43] HPLC[44,32,45-48] and CE[48-57] as illustrated in Table 1 and 2, respectively.

The determination of (4*RS*)-4-(4-chlorophenyl)pyrrolidin-2-one, major related product of baclofen, by USP 31/NF26[64] method in raw material is based on the comparison of the intensity of the spots from the standard and assay solutions using TLC (limit 1 %). In tablets, the quantitation of (4*RS*)-4-(4-chlorophenyl)pyrrolidin-2-one is based on HPLC (limit 4 %). The quantition of these impurity have been reported by using MicroC18 column based on HPLC for the determination of impurity in baclofen power and tablets.[65]

Table 1 Selected HPLC methods for the analysis of baclofen.

Sample	Analyte	Mobile phase	Column	Condition	Detection	Ref.
Raw materials	Baclofen four analogues	Perchloric acid as mobile phase and methanol as organic modifier	Chiral Crownpack CR(+) column	Flow rate: 0.9 ml/min Injection volume: 10 µl	diode array detection at 200/220/225 nm	32
standard	Baclofen	Chiral mobile phase	Reverse-phase C18	Flow rate: 0.5 ml/min Injection volume: 10 µl	UV detection at 220 nm	45
Human plasma	Baclofen	Polar ionic mobile phase (PIM) containing MeOH:glacial acetic acid:triethylamine, (100:0.1:0.1,v/v)	Teicoplanin macrocyclic antibiotic (Chirobiotic T)	Flow rate: 0.5 ml/min Injection volume: 20 µl	UV detection at 220 nm	44
Human plasma	Baclofen	0.4 mM $CuSO_4$ in acetonitrile-20 mM sodium acetate (17:83)	Chirex 3216 chiral column	Flow rate: 1.1 ml/min Injection volume: 40 µl	UV detection at 220 nm	46
Biological materials	Baclofen and its fluoro analogue	n-hexane/dichloromethane/ Ethanol (100:9.4:1.6,v/v)	Silica gel column	Flow rate: 1.5 ml/min Injection volume: 50 µl	Fluorescence detection at 335/365 nm	47
-	Primary amines	0.1% $HC1O_4$ (v/v) at pH 1.9 with methanol (15:85)	Chiral Crownpak CR(+) column	Flow rate: 1.0 ml/min	UV detection at 210 nm	48

Table 2 Selected CE methods for the analysis of baclofen.

Method	Sample	Analyte	Chiral selector	Condition	Detection	Ref.
CE-UV	Raw material	Baclofen and racemic drugs	45 mmol/l Hydroxypropyl-α-CD	Running buffer: 100 mmol/l Sodium dihydrogenphosphast; pH 2.5 Voltage: 15 kV for 3 sec Polarity: Anode to cathode Temp: 25 ^{o}C Tube: Fused-silica capillaries (0.05 mm I.D.x 0.375 mm O.D.); total lengths 29 cm	200 or 210nm	52
MCE-LIF	Raw materials	Baclofen and chiral drugs	2 mM Neutral CDs	Running buffer: 20 mM Phosphate buffer; pH 9.3 Laser power: 20 mW (excitation source) Temp: 45 ^{o} to the chip	λ_{em} = 473 nm λ_{em} = 500 nm	53
CE-LIF	Raw materials and plasma	Baclofen	7 mM α-CD modified	Running buffer: 50 mM Sodium borate; pH 9.5 Voltage: 9 kV for 3 sec Laser power: 1 mW PT voltage: -800 V Tube: Electrophoretic capillaries (50 µm I.D.x 360 µm O.D.); total lengths 50 cm	λ_{ex} = 442 nm λ_{em} = 500 nm	54
CE-UV	Raw materials	Baclofen	10 mM ß-CD	Running Buffer: 50 mM Phosphate buffer; pH 7.0-acetonitrile (95:5 v/v) Voltage: 15 kV Tube: Fused-silica capillaries (75 µm I.D.); total lengths 60 cm	214 nm	51

Table 2 (cont.) Selected CE methods for the analysis of baclofen.

Method	Sample	Analyte	Chiral selector	Condition	Detection	Ref.
CE-UV	Raw materials	Baclofen and gabaergic ligand	3 % w/v of highly sulfated-ß-CD	Running Buffer: 25 mM Phosphate buffer; pH 2.5 (H_3PO_4 + TEA) Voltage: 0.40 kV/cm Temperature: 25 ^oC Polarity: Reverse polarity (cathodic injection) Tube: Capillaries coated with polyethylene oxide (50 µm I.D.); total lengths 52.2 cm	196/200 nm	49
CE-UV	Raw materials	Bacofen, phaclofen, saclofen and OH-saclofen	3 % w/v of highly sulfated-ß-CD	Running Buffer: 25 mM Phosphate buffer; pH 2.5 (H_3PO_4 + TEA) Voltage: 0.30 kV/cm Temperature: 25 ^oC Polarity: Reverse polarity (cathodic injection) Tube: Capillaries coated with polyethylene oxide (50 µm I.D.); total lengths 52.2 cm	200 nm	50
CE-UV and CE-LIF	Raw materials and human plasma	Baclofen	2 % w/v of highly sulfated-ß-CD	Running buffer: 50 mM Sodium borate; pH 9.5 Voltage: 30 kV for 6 sec at 50 mbar Temperature: 25 ^oC Polarity: Anode to cathode Laser power: 20 mW Tube: Uncoated fused-silica capillaries (50 µm I.D x 375 µm O.D.); total lengths 85 cm; effective length of 60 and 22 cm	λ_{ex} = 442 nm λ_{em} = 500 nm	55

Table 2 (cont.) Selected CE methods for the analysis of baclofen.

Method	Sample	Analyte	Chiral selector	Condition	Detection	Ref.
CE-UV	standard	Organic racemates that contain a primary amine functional group	Achiral crown ether plus CD	Running Buffer: 50 mM sodium dihydrogen-phosphate at pH 2.2 Voltage: 15 kV Polarity: Anode to cathode Tube: Fused-silica capillaries (75 μm I.D.); effective lengths 50 cm; total lengths 57 cm	254 nm	56
CE-UV	Raw materials	Chiral drug	45 mmol/l γ-CD	Running buffer: sodium dihydrogenphosphate at pH 2.5 Injection: 15 kV for 3 sec Polarity: Anode to cathode Temperature: 25°C Tube: Fused silica capillaries (0.05 mm ID, 0.375 mm OD) were coated with polyacrylamide on the inner surface	200/210 nm	57
CE-UV	standard	Primary amines	10 mM Chiral crown ether	Running Buffer: 20 mM Tris-H_3PO_4 at low pH values (pH 1.9-2.1) Voltage: 20 kV Temperature: 15 ^oC Polarity: Anode to cathode Tube: Uncoated capilly (75 μm I.D.); effective lengths 37 cm	210/235 nm	48

2. Chiral separation

Chirality (handedness) of enantiomeric molecules has become increasingly important in biotechnological, chemical, agricultural activities, and especially in pharmaceutical analysis. It is now known that those differences, which make molecules chiral, may result in different pharmacological effects in biological systems by determining the pathways of metabolism, disposition, and physiological effects.[66] Thus, analytical methods for the chiral resolution are most important. Enantioselective separations have been realized in all possible separation techniques with high resolution power and high efficiency, which include HPLC, GC, TLC. Recently, CE has become popular for separation of enantiomers.[61,67]

CE is a modern electrophoretic technique with a great potential for the separation of both charged and uncharged compounds. Its power comes from the high electric field that can be applied to the background electrolyte (BGE) used for the electrophoretic runs.[68] CE is especially suitable for enantiomeric separations because this technique offers several advantages such as high efficiency and selectivity, small sample requirements, flexibility of method development, separation speed, low costs, and short analysis time.[58]

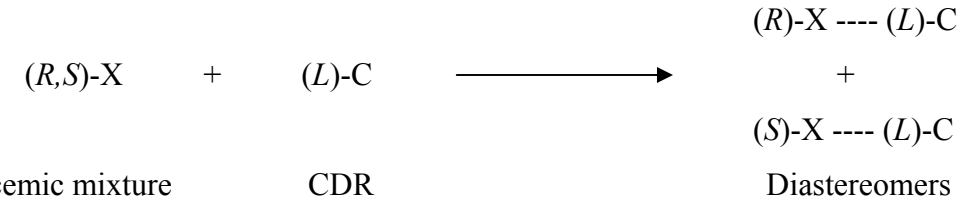
2.1 Chiral separation by CE

The separation mechanism of enantiomers by CE is similar to other enantiomer separation techniques, which is based on the formation of the correspondent diastereoisomers. These diastereoisomers can be easily separated because they possess different physicochemical properties. Two different procedures have been used for enantiomer resolution by CE, the indirect and the direct resolution approaches.

2.1.1 Indirect approach

The success of this approach involves the coupling of the enantiomers with an auxiliary chiral derivatizing reagent (CDR) to convert them into diastereomers. The diastereomers can then be separated by any achiral separation technique[61] because the

diastereomers possess different physicochemical properties. The reaction scheme may be illustrated as follows:



Recently, the developed chiral derivatization reagents for CE include (1*R*,2*R*)- and (1*S*, 2*S*)-N-[(2-isothiocyanato)cyclohexyl]-6-methoxy-4-quinolinylamide), *R*-(-)- or *S*-(+)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3 benzoxadiazole, and (+) and (-) 1-(9-anthryl)-2-propyl chloroformate (APOC).[69] Although this approach is widely used in HPLC and GC, it is less used in CE due to practical disadvantages and occurrence of the following problems: (a) the method is time consuming; (b) the method needs very pure chiral selectors as derivatizing agent; (c) the two enantiomers should react at the same rate; (d) the chemical structure of analytes should have the activating groups (nitrogen, hydroxyl, carboxylic); (e) reaction conditions should be appropriate in order to avoid stereo transformation of either chiral reagent, diastereoisomers or enantiomers and (f) the response of detector for the two diastereoisomers should be the same.[59] However, the indirect approach can be advantageously used in order to either increase the sensitivity by introducing fluorescent groups on the structure of analytes and/or to modify the chemical structure of analytes for advantageous interactions.[67]

2.1.2 Direct approach

The direct separation approach is successfully applied in CE for the enantiomeric separation. This approach is based on enantioselectivity of two enantiomers with chiral selector by adding a chiral selector to background electrolyte. Discrimination of enantiomers is considered to depend on a three-point interaction between one enantiomer and the chiral selector. At least one of these interactions must be stereochemmically dependent such that the other enantiomer can only from a less stable two-point complex[70] as Fig. 3.

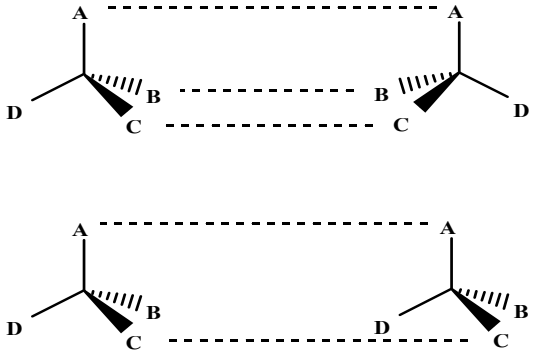


Fig. 3 Three-point interaction model.

During the electrophoretic run, the two enantiomers form labile diastereomeric complexes by intermolecular interactions forming non-covalent bonds (e.g., electrostatic ion-ion, ion-dipole, and dipole-dipole interactions, hydrogen-bonds, ¶-¶ interactions).[58] The labile complexes move toward the detector different velocities. Fig. 4 shows the scheme of enantioseparation of two anionic enantiomers (*D* and *L*) using cyclodextrin as chiral selector with suppressed electroosmotic flow where the *D*-isomer is more complexed than the *L*-isomer.

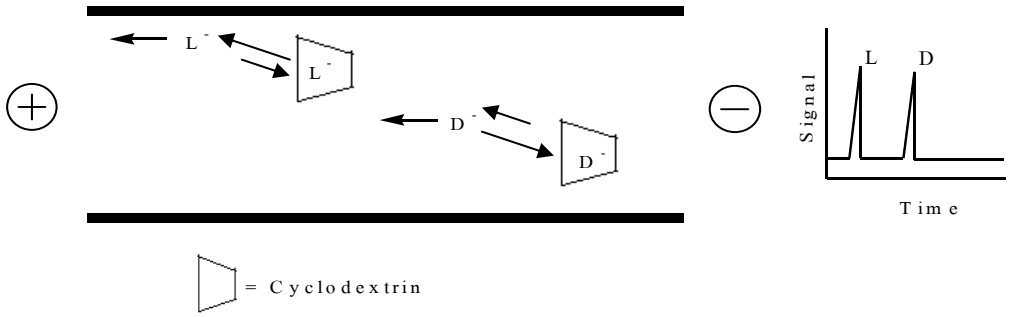


Fig. 4 Scheme of the enantioseparation of two anionic enantiomers (*D* and *L*) using cyclodextrin with suppressed electroosmotic flow. Adapted from [86].

2.1.2.1 Theory of enantioselective capillary electrophoresis

For the direct separation approach in CE, the enantiomers can be separated due to differences in their electrophoretic mobilities under the influence of an electric field. The relationship between mobility and chiral selector concentration can be expressed as[71]

$$\mu = \frac{\mu_f + \mu_c K [CS]}{1 + K [CS]} \quad (1)$$

Where: μ = Apparent electrophoretic mobility of enantiomer (cm^2/Vs)

μ_f and μ_c = The electrophoretic moblities of free and complexed solute (cm^2/Vs)

K = The equilibrium constant of the complexation reaction of enantiomer

[CS] = The equilibrium concentration of the chiral selector

When 1:1 stoichiometry of complexed between the chiral selector and the enantiomers is assumed, the mobility difference ($\Delta\mu$) which was applied to neutral cyclodextrins and charged enantiomers can be expressed by[58,71]

$$\Delta\mu = \frac{(\mu_f - \mu_c)\, \Delta K [CS]}{1 + (K_R + K_S) [CS] + K_R K_S [CS]^2} \quad (2)$$

Where: $\Delta\mu$ = The electrophoretic mobility difference (cm^2/Vs)

ΔK = The equilibrium constant difference of the complexation reaction of enantiomers

K_R and K_S = The equilibrium constant of the complexation reaction of (*R*)- and (*S*)-enantiomer

Both Eqs. (1) and (2) clearly emphasize that the type and concentration of chiral selector are primary parameter for optimization. Moreover, Eq. (2) also indicates that the enantioseparation can be achieved if there is complexation between the enantiomers and chiral selector. Hence, the two enantiomers should bind to the chiral selector to different extents in order to be separated.

The optimal concentration of chiral selector which is inversely related to the equilibrium constants by Eq. (3)[58]

$$[CS]_{opt} = \frac{1}{\sqrt{K_R K_S}} \quad (3)$$

Where: $[CS]_{opt}$ = The optimized concentration of the chiral selector

The resolution (R_s) is the key parameter that needs to be optimized and the resolution equation in CZE can be expressed as[58]

$$R_s = \left(\frac{\sqrt{N}}{4}\right)\left(\frac{\Delta K\,[CS]}{1 + (K_R + K_S)\,[CS] + K_R K_S\,[CS]^2}\right)\left(\frac{\mu_f - \mu_c}{\mu_{avg} + \mu_{eof}}\right) \quad (4)$$

Where:
- N = The number of theoretical plates
- R_s = Resolution value
- μ_{avg} = The average electrophoretic mobility (cm^2/Vs)
- μ_{eof} = The electroosmotic flow mobility (cm^2/Vs)

From Eq.(4), the resolution can be improved by optimizing type and concentration of chiral selector, type and concentration of running buffer, buffer pH, ionic strength and controlling EOF.

2.1.2.2 Chiral selectors (Cs)

Types of chiral selectors play the most important role in chiral separation by CE. Generally, not all of the novel chiral selectors become widely used in CE due to

different reasons such as availability, costs, compatibility and competitiveness with established chiral selectors. Many chiral selectors that are most frequently used in CE may be classified based on the type of chiral selectors (e.g., cyclodextrins, carbohydrates, and chiral crown ethers) and separation mechanism (e.g., inclusion-complexation, ligand exchange, chiral micelle, and affinity interaction).

A. Inclusion-complexation

A.1 Cyclodextrins

Cyclodextrins (CDs) are oligosaccharides constituted by several *D*-(+)-glucopyranose units. Despite the fact that cyclodextrins with 6-12 units have been separated, only those with 6, 7, and 8 glucopyranose units, named α-, ß-, and γ-CDs, are in frequent applied in e.g., drug composition, analytical chemistry, additives in food or tobaccos. Cyclodextrins are most popular of the many chiral selectors used in CE because they have many of the desirable features of the ideal chiral selector. Cyclodextrins were amongst the first chiral selectors employed in CE and their successful application has followed their use as chiral stationary phase in GC, TLC, and HPLC, and as mobile phase additives in TLC and HPLC.

Structure and properties of cyclodextrins

The solid state structures of α-, ß-, and γ-CDs have been determinate by numerous spectroscopic techniques including X-ray diffraction, solid state NMR, Infrared, and Raman spectroscopy.[72-74] Cyclodextrin structures in solution have been studied by both proton and ^{13}C NMR.[75] The chemical shifts and coupling constants of the cyclodextrin and guest analyte may change upon complexation, and these changes can be used to infer information about the orientation of the guest molecule within the cyclodextrin cavity. Cyclodextrin purity may be monitored by chromatographic techniques such as HPLC and TLC. Cyclodextrins are the general class of molecules composed of glucose units connected by α-1,4-glycosidic linkages to form a series of oligosaccharide rings. In nature, the enzymatic digestion of starch by cyclodextrin glycosyltransferase (CGTase) produces a mixture of cyclodextrins comprised of 6, 7, and 8 glucose units (α-, ß-, and γ-CDs). The source of the enzyme used for the

preparation of these compounds is a bacillus that Schardinger called *Bacillus macerans*.[76] Other bacteria containing the enzyme used for producing cyclodextrins have been studied. Cyclodextrin's shape is similar to a truncated cone, with a relatively hydrophobic cavity, and two opening of different sizes that are relatively hydrophilic owing to the presence of hydroxyl groups (primary and secondary). As an example, Fig. 5 shows a sketch of the structural features of cyclodextrins.

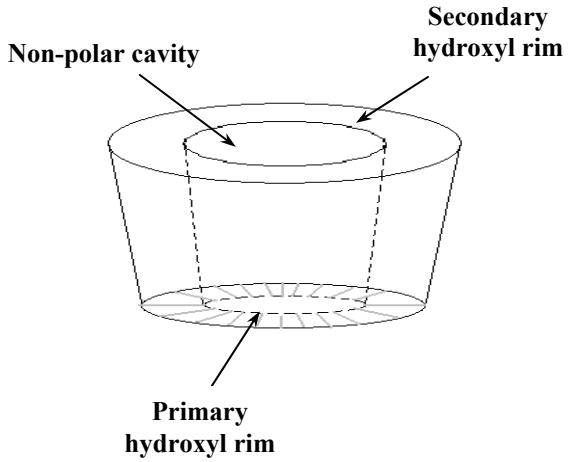
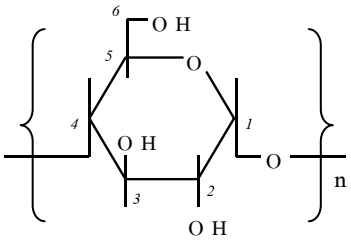


Fig. 5 Functional structural scheme of cyclodextrins. Adapted from [86].

Commercially, cyclodextrins are still produced from starch, but more specific enzymes are used to selectively produce consistently pure α-, ß-, and γ-CDs. Table 3 illustrates the main physical properties of the three native cyclodextrins. From the data shown in Table 3, it seems that ß-cyclodextrin exhibits the lowest solubility in water, the most used solvent in CE, which may give some limitations to the optimization of the separation methods.

Table 3 The main properties of native cyclodextrins.[68]



CD Type	Number of glucopyranose	$[\alpha]_D^{25}$	Cavity (A^o)		Molecular weight	Solubility (g/100 ml in water, 25 ^oC)
			Diameter	Depth		
α	6	+150.5	5.7	7.8	972	14.5
ß	7	+162.0	7.8	7.8	1135	1.85
γ	8	+177.4	9.5	7.8	1297	23.2

Cyclodextrin classes

Native cyclodextrins

Native cyclodextrins were the first cyclodextrins to be used in the separation of enantiomers by CE and are probably still the most widely used. Most early works centred around the parent α-, ß-, and γ-CDs, as Fig. 6 and 7 show the structures and dimensions of native cyclodextrins, respectively. Native cyclodextrins are neutral in charge and hence migrate with the same velocity as the electroosmotic flow (EOF). Complexation of neutral cyclodextrins molecule with an ionic species results in the formation of a charged complex, which can then migrate under the influence of electrophoresis and electroosmosis.[77]

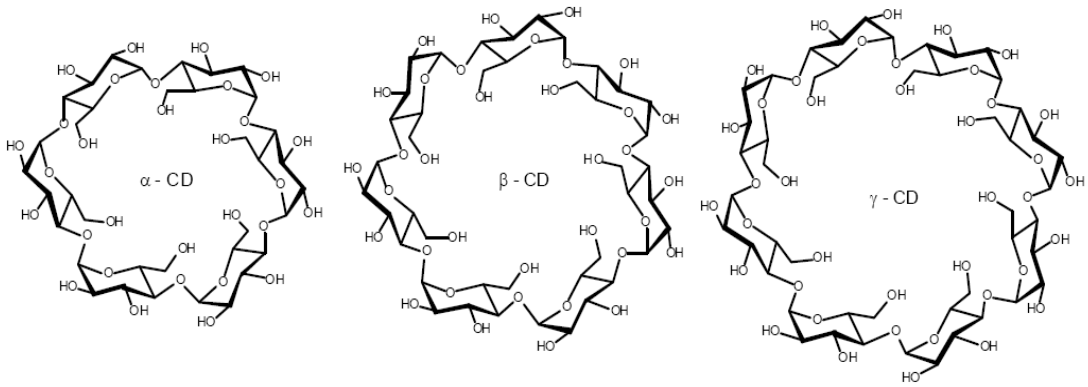


Fig. 6 Chemical structure of α-, ß-,γ-cyclodextrin. Reproduced from [76].

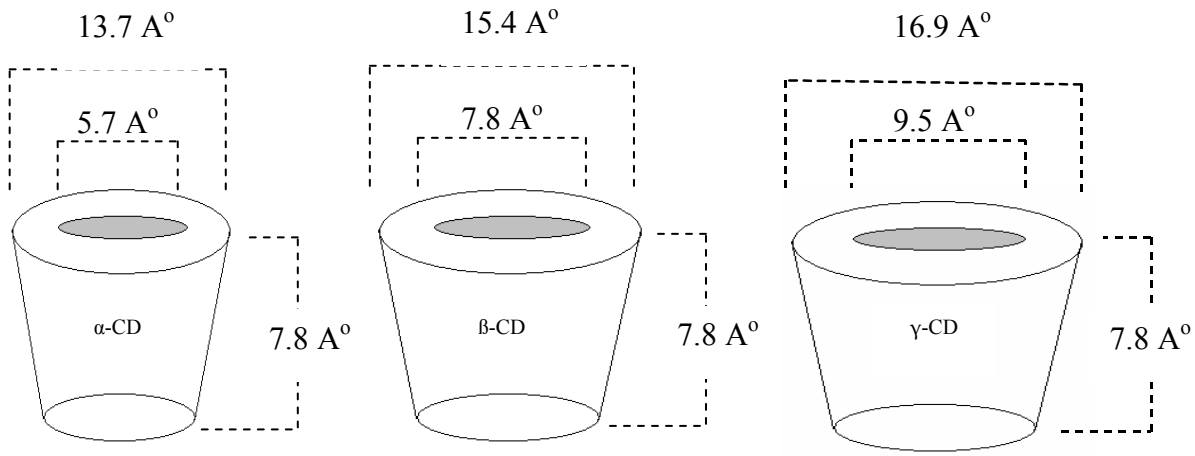


Fig. 7 Shape of α-, ß-,γ-cyclodextrin. Reproduced from [76].

Neutral derivatized cyclodextrins

Recently, derivatized neutral cyclodextrins became popular such as hydroxypropyl-ß-cyclodextrin (HP-ß-CD),[78,79] methyl-ß-cyclodextrin (Me-ß-CD), and hydroxyethyl-ß-cyclodextrin (HE-ß-CD).[58] The derivatized cyclodextrins exhibit different properties compared to the native cyclodextrins. The physicochemical properties of cyclodextrins can be enhanced by chemically modifying the outer rim hydroxyl groups at the 2, 3, and/or 6 position of each glucose unit can react with electrophilic compounds.[68] These modifications influence the overall hydrophobic character of the cyclodextrin, giving rise to changes in their cavity dimensions, their hydrogen-bonding ability, aqueous solubility, and stabilizing the inclusion complex

formed. Furthermore, charged cyclodextrins give rises to significant change in the enantioselectivity of the cyclodextrin, which are increasingly popular for the chiral separation in CE.[77]

Charged cyclodextrins

Charged cyclodextrins are required for the separation of the enantiomers of electrically neutral analytes, and are also beneficial for charged analytes.[72] Therefore, charged cyclodextrins carrying opposite charge to that of the analytes are very commonly used.

Cationic cyclodextrins

Cationic cyclodextrins are useful for the separation of the enantiomers of negatively charged drugs. Examples of cationic cyclodextrins include mono(6-amino-6-deoxy)-ß-cyclodextrin, 6-[(3-aminoethyl)amino]-6-deoxy-ß-cyclodextrin,[60] 6^A-propylammonium-6^A-deoxy-ß-cyclodextrin chloride (PrAMCD),[80] 2-hydroxy-3-trimethylammoniopropyl-ß-cyclodextrin (TMA-ß-CD),[81] and 2-*O*-(2-aminoethyl-imino-propyl)-ß-*O*-hydroxypropyl-ß-cyclodextrin (2-AIPHP-ß-CD).[82]

Anionic cyclodextrins

Anionic cyclodextrins are especially important for the enantiomers of positively charged drugs since the large number of pharmaceuticals which contain a basic functional group. Examples of anionic cyclodextrins include octakis(2,3-di-acetyl-6-sulfato)-γ-cyclodextrin (ODAS),[60,83] sulfobutylether-ß-cyclodextrin (SBE-ß-CD), carboxymethyl-ß-cyclodextrin (CM-ß-CD), and heptakis(2,3-di-methyl-6-sulfato)-ß-cyclodextrin (HDMS).[84] Anionic cyclodextrins are used much more often than cationic cyclodextrins and the number of anionic cyclodextrins synthesized is also much higher than the cationic cyclodextrins.

Polymerized cyclodextrins

Cyclodextrin can be polymerized by appropriate bi- or polyfunctional agents, which can couple with the hydroxyl groups of cyclodextrin to from oligomers, longer-

chain polymers, or crosslinked networks.[77] Agents for the preparation of cyclodextrin polymers include aldehydes, ketones, allyl halides, isocyanates, and epoxides.[76] The chemical and physical properties of these polymeric species differ in many ways from their monomeric analogs such as: (a) the polymerized cyclodextrins exhibit higher solubility; (b) the polymerized cyclodextrins is more rigid and has a different conformation to native cyclodextrin and (c) the high molecular mass of polymer causes a reduced effective mobility of the enantiomers due to lower mobility of the chiral selector, leading to a better enantioresolution.[85] For chiral separation in CE, these chiral polymeric derivatives show significant differences in enantioselectivity compared to monomeric cyclodextrins. Commercially available polymerized cyclodextrins can be either neutral or charged in nature.

Mechanism of enantioselectivity by cyclodextrins

The main mechanism of the analytes-cyclodextrin involves inclusion-complexation reaction. Since the cyclodextrin cavity possesses hydrophobicity, it exhibits higher affinity for the hydrophobic compound forming more stable complexes than compounds possessing lower hydrophobicity. Secondary, bonds between the analyte and the hydroxyl or modified hydroxyl groups on the rim of the cyclodextrin can stabilize the inclusion-complexes formed and considering that the primary and secondary hydroxyl groups are bound to asymmetric carbons, these interactions can be stereoselective and thus responsible for the separation of enantiomeric compounds.[86] Hence, the cyclodextrin type has a very important role in the separation.

Factors affecting the enantioselectivity using cyclodextrins

Varying the experimental conditions can cause important effects on chiral resolution in CE for improving peak shape and enhancing the stereoselectivity. Most simple approach can be done by modifying the cyclodextrin type and concentration, buffer concentration and its ionic strength, buffer pH, temperature, and the value of the applied voltage. Moreover, adding co-additives in background electrolyte and modifying the capillary wall can also control the EOF and improve the enantioselectivity.

Cyclodextrin type

The volume and the diameter of the cyclodextrin cavity are related to the number of glucose units in the ring. Stable and selective complexation of the enantiomers with the cyclodextrins is important. A wide variety of enantiomeric compounds can usually be separated by changing the type and the concentration of the cyclodextrin. Chemical derivatization of the cyclodextrins provides a choice of solubilities and selectivities. Usually, α-cyclodextrin is selected for analytes which lack an aromatic ring or contain a long alkyl chain.[72] Separations can be achieved for one aromatic ring structure (or similar size, and hydorphobicity) with ß-cyclodextrin. For substituted one ring or two ring structures, the use of derivatized forms of ß-cyclodextrin is suggested. Substituted two ring, three ring of larger structures usually require the use of derivatized ß-cyclodextrin or γ-cyclodextrin. Since predicting cyclodextrin type that will provide optimal resolution is not always obvious, it is advisable to attempt separations with several cyclodextrins during the methods development procedure.

Cyclodextrin concentration

The concentration of the chiral selector, cyclodextrin, is an important impact in peak resolution, the mobility and, in some instances, evens the migration order.[86] As the cyclodextrin concentration increases, the probability of complexation with the solute increases. Hence, usually leads to a higher enantiomertic separation factor, resolution, and migration times. These observations can be explained in terms of the formation of stronger inclusion complexes and a reduced electroendosmotic flow as a result of the increased viscosity of the buffer.[77] Once the optimal concentration has been reached, additional amounts of cyclodextrin might not improve resolution.

Buffer concentration and its ionic strength

The buffer concentration and ionic strength is a very important parameter to be controlled in chiral analysis by CE. The increase of ionic strength and/or concentration of the buffer influence the EOF and thus the migration time. However, higher ionic buffer strength is responsible for the increase in current that can cause a

reduction in efficiency.[86] It has been suggested that the decrease in EOF with increasing BGE concentration due to the increasing of the thickness of the diffusion of double layer at the inner capillary wall. Thus, the resolution is also increased with the increasing of the ionic strength.

Buffer pH

The buffer pH is an important factor for consideration in the separation of enantiomers by CE as it determines the charges on both the analyte and the cyclodextrin.[87] Altering the charged state of the analyte and chiral selector can affect both the degree and the enantioselectivity of inclusion. Hence, the buffer pH affects selectivity, efficiency, migration time, and resolution of enantiomeric separation. Moreover, changing in the buffer pH can also have effects on the magnitude of EOF and the enantiomeric migration order. The pH employed is primarily determined by the properties of the analyte such as the pK_a value.

Electric field strength

Vital parameter influencing migration times and peak efficiencies in cyclodextrin mediated chiral capillary electrophoresis are the applied field strength. Though, at high field strengths, excessive Joule heat is not efficiently dissipated, peak efficiency will decrease, as will resolution. Therefore, an effective temperature controlling system for the capillary is essential for allowing the maximum field strength to be applied for fast, well-resolved separations with high efficiency. It is important to note that, with field strengths less than 250 V/cm, separations may be inconveniently long with poor peak efficiencies.[87]

Temperature

The ability to maintain constant temperature during the separation (isotherm conditions) is important in order to achieve good migration time reproducibility.[88] Note, the increase of temperature causes a decrease of the buffer viscosity, thus a decrease of the migration time and chiral selectivity of the analytes, until very limited or no chiral recognition occurs.[86,88]

Organic solvent

The degree of inclusion of the analyte into the cyclodextrin cavity and the degree of enantioselectivity of the interaction can both be altered by the nature of the surrounding solvent system. It is hard to predict the effect of addition of an organic solvent to the buffer because it affects several variables such as viscosity, dielectric constant, and zeta potential. In addition, the effect of adding an organic solvent to the buffer depends on which and how much solvent is added. Organic solvents which mostly used in CZE are methanol, ethanol, and acetronitrile.

Modifying the capillary wall

Capillary can be permanently chemically coated or temporally coated by addition of appropriate surface active agents into the rinse and/or background electrolyte. This modification of the inner surface wall can reduce the analyte adsorption, eliminate the EOF, or reverse the EOF direction.[89] The surface active agents include polyacrylamide (PAA), polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), polyethylene oxide (PEO), polyethylene glycol (PEG), hydroxyethylcellulose (HEC), and hydroxypropylmethylcellulose (HPMC).[86,89]

PEO is a high molecular weight polymer (Fig. 8). PEO reduce the EOF and the interactions between analytes and the capillary wall surface, which could improve resolution and reproducibility.[90] The PEO molecules are held to the capillary walls only by weak interactions, such as hydrogen bonds and van der Waals forces.[91]

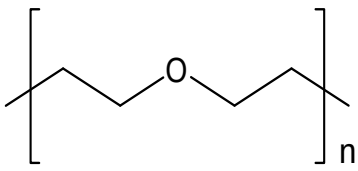


Fig. 8 The chemical structure of polyethylene oxide.

A.2 Chiral crown ethers

Crown ethers, (+)-18-crown-6 ether-tetracarboxylic acid are cyclic polyethers which from host-guest complexes with earth metal ions and primary ammonium cations (Fig. 9). The separation mechanism, when crown-ethers are employed, is based on inclusion-complexation stabilized by stereoselective interactions where the analyte fits the crown-ether cavity with the amino group (the hydrophilic part) on forming ion-dipole bonds with the oxygen atoms of the complexing agent or repulsion between the substituent groups of the asymmetric center of the analyte and carboxyl of the crown-ether derivative.[67,86] Hence the complexation mechanism is completely different to that with CDs. Mainly parameters influencing the stereoselectivity are chemical structure of enantiomers, pH and composition of the BGE.

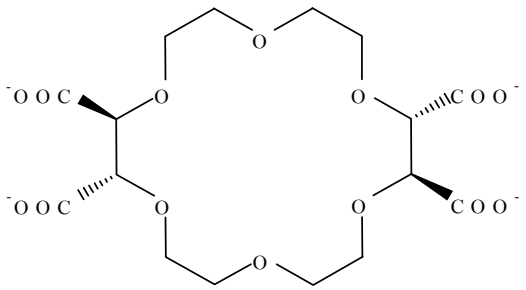
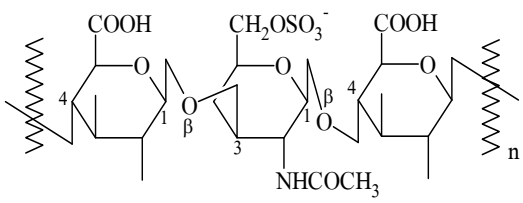


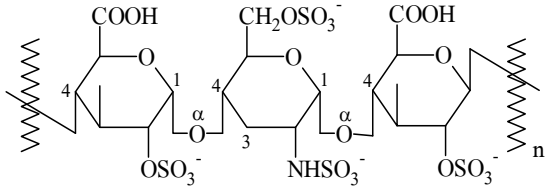
Fig. 9 Chemical structure of 18-crown-6 ether-tetracarboxylic acid.

A.3 Oligo- and Polysaccharides

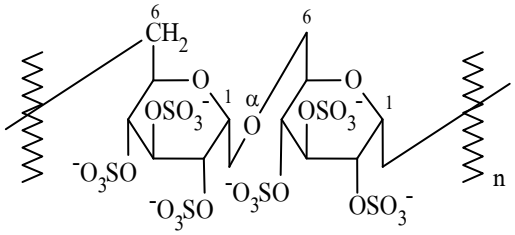
Apart from cyclodextrins, many other linear and cyclic oligo- and polysaccharides (dextrins, dextrans, and celluloses) are also presented as chiral selectors. Both of them received attention as chiral buffer additives in CE and some of polysaccharides such as celluloses have some success as chiral stationary phase in HPLC. Moreover, the derivatized polysaccharides have been used in CE for chiral separation such as dextran sulfate, heparin, and chondroitinsulfate C which illustrated in Fig. 10.[77]



Chondrotin sulphate C



Heparin



Dextran sulphate

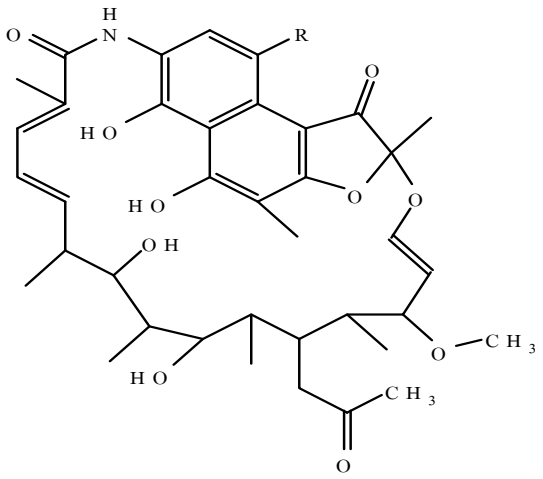
Fig. 10 Chemical structures of derivatized polysaccharides.

B. Affinity interactions

B.1 Macrocyclic antibiotics

Macrocyclic antibiotics have several asymmetric centers and many functional groups, allowing multiple interactions with the analytes. In addition to ionic interactions, hydrogen bonding, dipole-dipole, ¶-¶, hydrophobic interactions, and steric repulsion are the main effect involved in the enantioseparation mechanism. Macrocyclic antibiotics possess hydrophobic pockets which can include hydrophobic moieties, due to the presence of pendant polar arms, hydrogen bonds can be formed.[60] These compounds were first used as chiral selectors in TLC and HPLC.[77] Four groups of antibiotics have been introduced as chiral selectors in CE: (a) ansamycins (rifamycin B, rifamycin SV), (b) glycopeptides (vancomycin, ristocetin, and teicoplanin), (c) aminoglucoside antibiotics (streptomycin, fradiomycin and kanamycin), and (d) polypeptides. Chemical structure of rifamycin, vancomycin,

teicoplanin are shown in Fig. 11-13, respectively. Recently, another group of antibiotics, the macrolides, was investigated as chiral selector.[84]



rifamycin B (R, -OCH_2COOH) and rifamycin SV (R, -OH)

Fig. 11 Chemical structure of rifamycin.

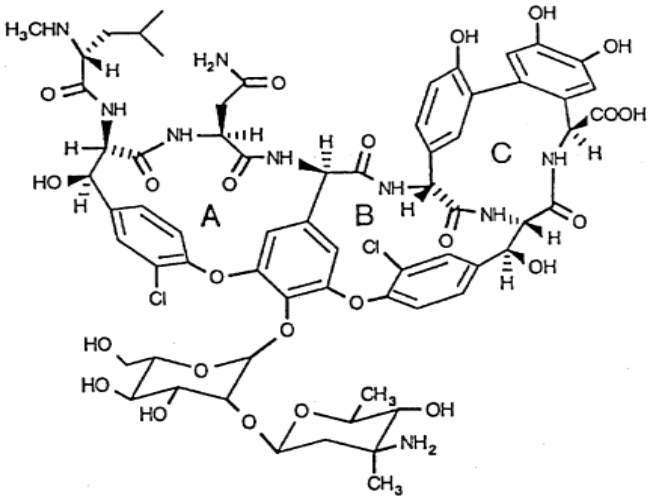


Fig. 12 Chemical structure of vancomycin.

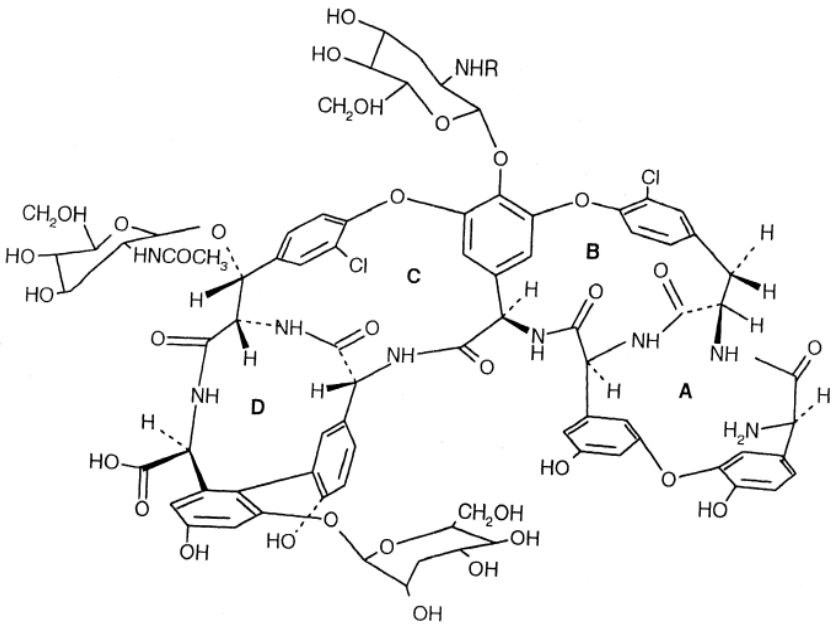


Fig. 13 Chemical structure of teicoplanin.

B.2 Proteins and glycoproteins

Proteins and glycoproteins are natural polymeric compounds that consists of amino acids or amino acids and sugars, both of which are chiral.[92] Despite all proteins have the possibility to discriminate a chiral molecule, only a limited number of proteins have been investigated in HPLC as chiral selectors for the enantiomer separation by immobilizing on a solid support forming a chiral stationary phase (CSP) such as bovine serum albumin, human serum albumin, α_1-acid glycoprotein, conalbumin, cellulose, ovomucoid, and casein.[60,77] In CE, proteins can be implemented in several ways in chiral separation. The simplest way is to dissolve them in the background electrolyte. There are various parameters influencing the chiral separations of various solutes in CE based on type of protein selectors, protein concentration, running buffer pH, modifier, ionic strength and displacer. Proteins can be positively or negatively charged depending on the pH applied. Their charges give them electrophoretic mobility and they can be used for the separation of basic and acidic analytes.

C. Ligand-exchange capillary electrophoresis (LE-CE)

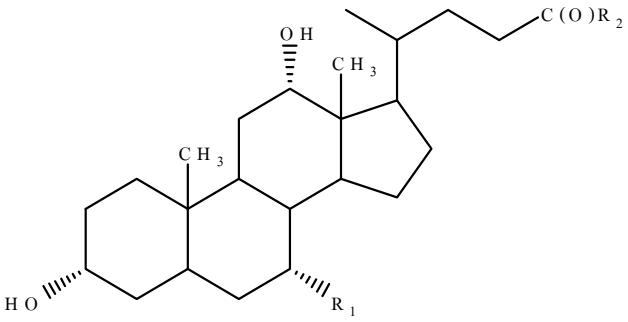
Ligand-exchange capillary electrophoresis has always been an important technique in chiral analysis of amino acids by using a chiral selector–metal complex as an additive to the background electrolyte. The separation mechanism of LE is based on the formation of diastereomeric ternary mixed metal complexes between ligand around the sphere of a central ion and the analytes. The central ion is a metal ion, which that the Cu (II) is used very often in different complexes such as Cu (II)-*L*-ornithine and Cu (II)-*L*-prolinamide.[84,93] Resolution is due to the difference in complex stability constants of the two mixed complexes with the analyte enantiomers.

D. Chiral micelles

Micellar electrokinetic chromatography (MEKC) is one of the most popular techniques in capillary electrophoresis for the chiral separation of uncharged compounds is based on the repart of the analytes between two phases, the aqueous and the micellar. A strong EOF is the driving force carrying the analytes to the detector while the surfactant, forming charged micells, is responsible for the selectivity of the separation.[67] In MEKC, the chiral separation can be obtained by two modes by using chiral surfactants or adding other chiral agents (cyclodextrin or derivatized cyclodextrin) to the achiral micellar buffer (CD-MEKC).[84,94]

D.1 Chiral surfactants

Surfactants are amphiphilic molecules composed of a polar head group and a hydrophobic tail. At concentrations above the critical micellar concentration (CMC), the detergent monomers aggregate to form micelles. The chiral separation of analytes is based on their partition coefficients between the chiral micelle phase and the electrolyte bulk phase. Chiral surfactants include (a) natural surfactants consists of bile salts, digitonins, and saponins, (b) monomeric synthetic surfactants such as N-dodecanoyl-*L*-valinate (SDVal), sodium N-dodecanoly-*L*-alanine (SDAla), and sodium N-dodecanoyl-*L*-glutamate (SDGlu), and (c) high-molecular-mass surfactant (HMMS) or polymeric surfactants such as poly(sodium N-undecylenyl-*L*-valinate); poly(*L*-SUV).[72,94]



Bile Salt	R_1	R_2
Sodium cholate (SC)	OH	ONa
Sodium deoxycholate (SDC)	H	ONa
Sodium taurocholate (ST)	OH	$NHCH_2CH_2SO_3Na$
Sodium taurodeoxycholate (STDC)	H	$NHCH_2CH_2SO_3Na$

Fig. 14 Chemical structure of bile salt.

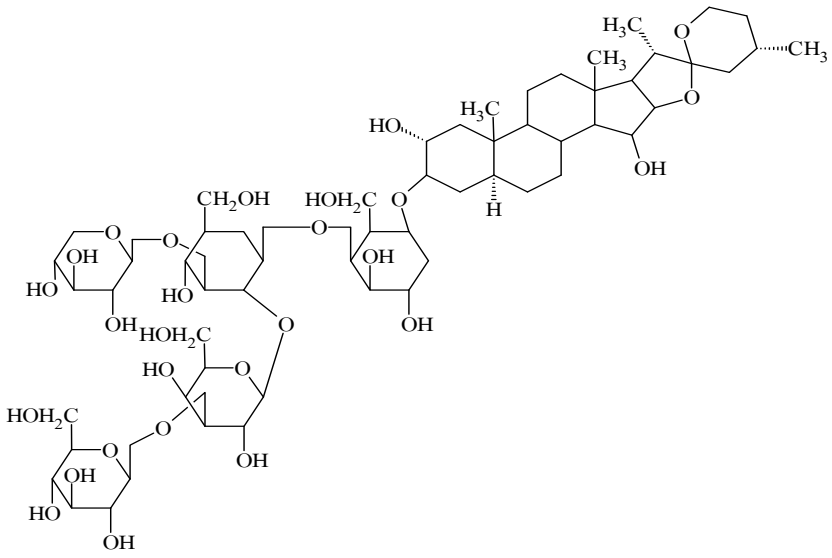


Fig. 15 Chemical structure of digitonin.

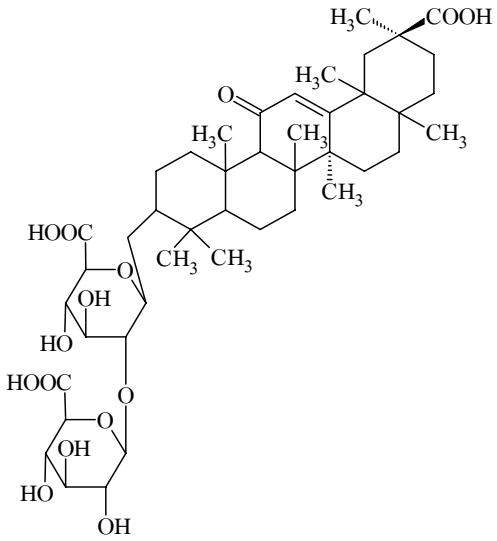


Fig. 16 Chemical structure of glycyrrihizic acid.

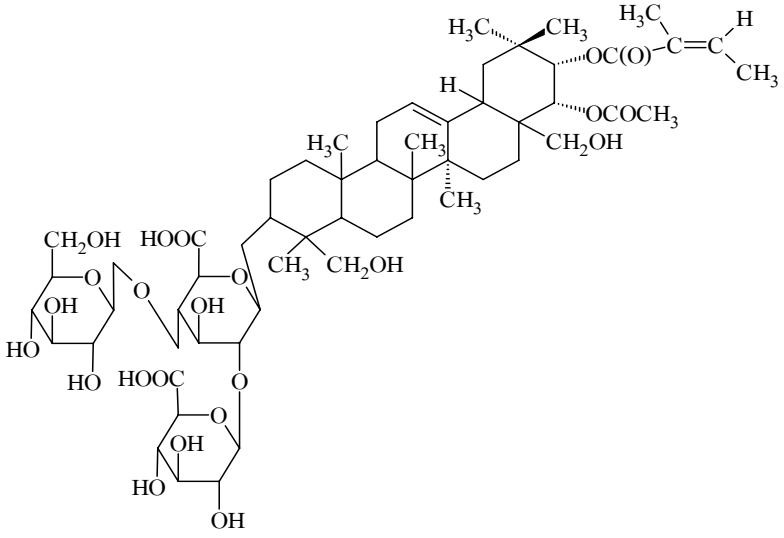


Fig. 17 Chemical structure of ß-escin.

CHAPTER III
MATERIALS AND METHODS

1. Chemicals and reagents

Name	Source/Supplier
(4*RS*)-4-(4-chlorophenyl)pyrrolidin-2-one	DQM (Strasbourg, France)
Acetic acid	Labscan Asia (Bangkok, Thailand)
Acetonitrile	Labscan Asia (Bangkok, Thailand)
Baclofen (raw material)	MacroPhar (Bangkok, Thailand)
Baclofen (raw material)	M & H Manufacturing (Samutprakarn, Thailand)
Baclofen pharmadica®	Pharmadica (Bangkok, Thailand)
Baclofen tablets:	
Baclosal®	M & H Manufacturing (Samutprakarn, Thailand)
Buffer pH 10.0	Ajaxchemical (Sydney, Australia)
Buffer pH 4.0	Ajaxchemical (Sydney, Australia)
Buffer pH 7.0	Ajaxchemical (Sydney, Australia)
Fenisal®	MacroPhar (Bangkok, Thailand)
Methanol (AR grade)	Labscan Asia (Bangkok, Thailand)
Methanol (HPLC grade)	Labscan Asia (Bangkok, Thailand)
Polyethylene oxide	Sigma (St. Louis Missouri, USA)
R-(-)-baclofen HCl	Sigma (St. Louis Missouri, USA)
S-(+)-baclofen HCl	Sigma (St. Louis Missouri, USA)
Sodium hydroxide AR	Mallinckrodt Baker (Xalostoc, Mexico)
Sodium tetraborate decahydrate	Antibioticos (Carlo erba, Mexico)
Sterile water for injection*	Thai Nakorn Patana (Nonthaburi, Thailand)
α-Cyclodextrin (cyclohexaamylose)	Sigma (St. Louis Missouri, USA)
β-Cyclodextrin	Sigma (St. Louis Missouri, USA)

* was indicated as water throughout the thesis

2. Instruments

Name	Source/Supplier
Analytical balance (AE 160)	Sartorius (Goettingen, Germany)
Capillary electrophoresis ^{3D} CE system	Agilent Technologies (Waldbronn, Germany)
Capillary, I.D. 50 μm, O.D. 375 μm	Polymicro Tecnologies (Arizona, USA)
Centrifuge Sorvall RC plus	N.Y.R. Limited Partnership (Bangkok, Thailand)
pH meter	Consort model C830 (Turnhout, Belgium)
Syringe filter nylon (13 mm, 0.2 μm)	Vertical Chromatography ((Bangkok, Thailand)
Ultrasonic bath (D-7700)	Elma (Singen, Germany)

3. Methods

3.1 Instrumentation and capillary

Capillary zone electrophoresis was performed on an Agilent Technologies instrument (^{3D} CE) system (model G1600A) and controlled by PC through Agilent ChemStation using a software version A.08 (G1601A) (Fig. 18). The software was designed to run on a compatible computer HP Pentium 4 (500 MHz, RAM 256 MB, hard disk 20 GB) under Microsoft® Window NT 4.0 operating environment and HP laserjet 4100 was used. An instrument was defined as running on a single time base, but could collect data from a number of different detectors simultaneously. The detector measured in the range of 190-600 nm (wavelength accuracy ± 2 nm) was a diode array detector, which consisted of a deuterium a lamp and detected by continuous emission. The regulation of high voltage was varied in the range of 0-30 kV (current 0-300 μA, power 0-6 W). The temperature control the capillary was varied from 5 to 60 ^{o}C (± 1 ^{o}C). The injection systems could be achieved by (a) applying pressure to sample vials (hydrostatic injection) and (b) applying voltage (electromigration injection).

Fig. 18 Instrument of capillary electrophoresis.

3.2 General procedure

The separation of baclofen enantiomers were carried out using fused-silica capillary with a total length of 64.5 cm, effective length of 56 cm and inner diameter of 50 μm and outer diameter of 375 μm. The preconditioning procedure for a new capillary tube, precondition, between runs and storage was described in Table 3. A new tube was required when the separation efficiency deteriorates and the migration time shifts more than 2 min.

The separation conditions were optimized by varying the chiral selector types and concentrations, buffer concentrations and pH, amount of organic solvents, temperature and applied voltage. Samples were injected into the anodic capillary inlet using 50 mbar pressures. Detection was performed using a UV-detector at a wavelength of 220 nm with a band width of 4 nm.

Table 4 The capillary conditioning procedure for the separation of baclofen enantiomers

Step	Duration	Solvent
New capillary:		
1. Rinse	20 min	1 N NaOH
2. Rinse	10 min	0.1 N NaOH
3. Rinse	10 min	Water
4. Rinse	10 min	BGE containing α-CD
Precondition:		
1. Rinse	10 min	1 N NaOH
2. Rinse	5 min	0.1 N NaOH
3. Rinse	10 min	Water
4. Rinse	10 min	BGE containing α-CD
Between run:		
1. Rinse	3 min	0.1 N NaOH
2. Rinse	2 min	Water
3. Rinse	5 min	BGE containing α-CD
Storage:		
1. Rinse	10 min	1 N NaOH
2. Rinse	10 min	Sterile water
3. Rinse	10 min	0.1 N NaOH
4. Rinse	10 min	Water
5. Rinse	10 min	MeOH
6. Rinse	10 min	Air

The separation of baclofen enantiomers and its impurity were carried out using fused-silica capillary dynamic coated with polyethylene oxide (PEO) with a total length of 50 and 64.5 cm (effective length of 41.5 and 56 cm, respectively), inner diameter of 50 μm and outer diameter of 375 μm. The preconditioning procedure for a new capillary, precondition, between runs and storage was described in Table 4. The separation conditions were optimized by varying buffer pH, amount of organic solvents, temperature, applied voltage, and injection time. Samples were injected into

the anodic capillary inlet using 50 mbar pressures. Detection was performed using a UV-detector at a wavelength of 220 with a band width of 4 nm.

Table 5 The capillary conditioning procedure for the separation of baclofen enantiomers and its impurity

Step	Duration	Solvent
New capillary:		
1. Rinse	20 min	1 N NaOH
2. Rinse	10 min	0.1 N NaOH
3. Rinse	10 min	Water
Precondition:		
1. Rinse	5 min	0.1 N NaOH
2. Rinse	1 min	Sterile water
3. Rinse	3 min	0.5 % w/v PEO
4. Rinse	1 min	Water
5. Rinse	5 min	BGE containing α-CD
Between run:		
1. Rinse	2 min	Water
2. Rinse	3 min	BGE without α-CD
3. Rinse	3 min	BGE containing α-CD
Storage:		
1. Rinse	5 min	Water
2. Rinse	10 min	1 N NaOH
3. Rinse	10 min	Water
4. Rinse	10 min	0.1 N NaOH
5. Rinse	10 min	Water

0.5% w/v PEO was prepared by dissolving the 0.125 g of PEO in 20 ml sterile water for injection, stirred for 10 h and adjusted to 25 ml. PEO solutions were diluted to 25 ml of volumetric flask.

3.3 Background electrolyte and standard preparation

For the optimization of baclofen enantiomers, stock buffer solutions (130 mM) were prepared by dissolving 12.395 g of sodium tetraborate decahydrate in sterile water for injection and sonicated for 20 min. Stock buffer solutions were adjusted to pH 9.25 by adding acetic acid and diluted to 250 ml. For the effect of buffer concentrations, stock of buffer solutions was diluted to 25-100 mM with sterile water for injection. For the effect of buffer pH, buffer solution (100 mM) were prepared by dissolving 3.8137 g of sodium tetraborate decahydrate in sterile water for injection and sonicated for 20 min. Stock buffer solutions were adjusted to pH 8.0-9.5 and diluted to 100 ml. Stock of BGE containing 25 mM of chiral selectors was freshly prepared. Briefly, 0.608 g of α-CD and 0.709 g of β-CD was weighed into a 25 ml volumetric flask and adjusted to volume with the borate buffer. The BGE containing 25 mM CD was diluted to 10-23 mM CD, added with 10% v/v ACN and diluted to 5 ml with borate buffer. For the effect of amount of ACN, different amounts of ACN (0-10% v/v) were added into the BGE containing 18 mM α-CD. All of solutions were kept at room temperature and filtered through a 0.2 μm membrane prior injection.

For the separation of baclofen enantiomers and its impurity, stock buffer solutions (100 mM) were prepared by dissolving 9.5343 g of sodium tetraborate decahydrate in sterile water for injection and sonicated for 20 min. Stock buffer solutions were adjusted to pH 9.7-9.9 by adding 1 N NaOH and diluted to 250 ml. 0.0876 g of α-CD was weighed into a 5 ml volumetric flask and adjusted to volume with the borate buffer. The BGE containing 18 mM CD and different amounts of ACN (0-10% v/v) was added into the BGE. All of solutions were kept at room temperature and filtered through a 0.2 μm membrane prior injection.

For the optimization of baclofen enantiomers, stock solutions of racemic mixture of (*R/S*)-baclofen was prepared by dissolving 5 mg of (*R/S*)-baclofen to a 10 ml volumetric flask and adjusted to volume with the borate buffer and sonicated for 10 min. Stock solutions were kept in the refrigerator at 8-10 ^{o}C. The mixture of (*R/S*)-baclofen solutions was diluted with 50% v/v ACN to obtain the concentration of 250 μg/ml and filtered through a 0.2 μm membrane prior injection.

For the optimization of (*R/S*)-baclofen and its impurity separation, stock solution of (*R/S*)-baclofen (100 μg/ml) was prepared by weighing 1 mg of (*R/S*)-

baclofen and dissolving in 100 mM borate buffer. Stock solution of impurity (50 µg/ml) was prepared by weighing 0.5 mg of (4*RS*)-4-(4 chlorophenyl) pyrrolidin-2-one and dissolving in 100 mM borate buffer. All of the stock solutions were sonicated for 10 min and kept in the refrigerator at 8-10 ^{o}C. Mixture of (*R/S*)-baclofen (50 µg/ml) and its impurity (5 µg/ml) solutions was added with different amounts of ACN (0-5% v/v) and filtered through a 0.2 µm membrane prior injection. Except for the effect of buffer pH, mixture of (*R/S*)-baclofen (50 µg/ml) and its impurity (5 µg/ml) solutions was prepared in the absence of ACN and filtered through a 0.2 µm membrane prior injection.

4. Optimization

4.1 Optimization for the separation of baclofen enantiomers

The effects of buffer concentrations, buffer pH, chiral selector types and concentrations, amounts of organic solvents, temperature and voltage on the separation of baclofen enantiomers were investigated.

4.1.1 Effects of chiral selector types

The influence of chiral selector types (e.g. α- and β-cyclodextrin) at different concentrations (10 and 18 mM) on the enantioseparation of baclofen was evaluated using a fused-silica capillary with a total length of 64.5 cm (effective length, 56 cm) x 50 µm ID in the BGE consisting of 100 mM sodium tetraborate (pH 9.25) and 10% v/v acetronitrile. The migration times, resolution, and selectivity were evaluated.

4.1.2 Effects of chiral selector concentrations

Cyclodextrin concentration is an important parameter for optimization of chiral separation because it influences to peak resolution, migration time and migration order. The influence of the cyclodextrin concentrations at 10-23 mM were studied using the BGE consisting 100 mM sodium tetraborate (pH 9.25) and 10% v/v acetronitrile.

4.1.3 Effects of buffer concentrations

Buffer concentration affects the EOF, which influences to migration time, and current.[86] The influence of BGE concentrations on the enantioseparation of the baclofen were studied using the BGE consisting of various sodium tetraborate concentrations (25-130 mM) containing the 18 mM cyclodextrin and 10% v/v acetronitrile.

4.1.4 Effects of buffer pH

Buffer pH has a significant effect on the electroosmotic flow because it influences the zeta potential. In chiral separation, the buffer pH significantly impacts the chiral selectivity.[66] The influence of buffer pH on the enantioseparation of the baclofen were varied in the range of 8.0-9.5 using 100 mM sodium tetraborate buffer containing the 18 mM cyclodextrin and 10% v/v acetronitrile.

4.1.5 Effects of amount of organic solvent

Addition of organic solvent can play an important role in the separation and the enantiomer resolution. The influence of organic solvent was studied using BGE consisting of 100 mM sodium tetraborate containing the 18 mM cyclodextrin and varied amount of acetronitrile (0-15% v/v).

4.1.6 Effects of temperature

The effect of temperature on the enantioseparation of the baclofen was studied in the range of 20-30 ^{o}C.

4.1.7 Effects of applied voltage

The effect of applied voltage on the enantioseparation of the baclofen was studied in a range of 15-30 kV.

4.2 Optimization for the separation of baclofen enantiomers and its impurity

These experiments were performed by using 100 mM sodium borate buffer containing 18 mM α-CD, fused-silica capillary dynamically coated with 0.5% w/v

PEO. Separation was carried out in a capillary with a total length of 50 cm (8.5 cm to the detector), an inner diameter of 50 μm and outer diameter of 375 μm using hydrodynamic injection of 50 mbar for 10 s. The initial temperature was 20 ^{o}C, and voltage was 25 kV, and a detection wavelength at 220 nm.

4.2.1 Effects of buffer pH

Effects of buffer pH on the separation of baclofen enantiomers and its impurity were observed in a range of 9.7-9.9 using 100 mM sodium borate buffer containing 18 mM α-CD.

4.2.2 Effects of amounts of acetronitrile

Effects of amounts of acetronitrile in the BGE and sample on the separation of baclofen enantiomers and its impurity were studied using 100 mM sodium borate buffer (pH 9.9) containing 18 mM α-CD. The influence of amounts of acetronitrile in BGE on the resolution of baclofen enantiomers and its impurity were studied in a range of 0-10% v/v acetronitrile. Moreover, the amounts of acetronitrile in sample solution affected the resolution and peak shape of baclofen enantiomers and its impurity were studied in a range of 0-5% v/v acetronitrile.

4.2.3 Effects of temperature and applied voltage

Effects of temperature and applied voltage on the separation of baclofen enantiomers and its impurity were simultaneously investigated in a range of 20-47 ^{o}C and 25-30 kV, respectively.

4.2.4 Effects of injection time

Effects of injections time on the separation of baclofen enantiomers and its impurity were studied in a range of 3-10 s using pressure at 50 mbar.

The optimum condition for the separation of baclofen enantiomers and its impurity was determined for the resolution, selectivity, tailing factor, and theoretical plate number. These parameters were calculated for the following equations.

$$R_s = \frac{2\,(t_2 - t_1)}{w_1 + w_2} \quad (5)$$

$$\alpha = \frac{\mu_2}{\mu_1} \quad (6)$$

$$TF = \frac{w_{0.05}}{2f} \quad (7)$$

$$N = 5.54 \left\{ \frac{t_m}{w_{1/2}} \right\}^2 \quad (8)$$

Where R_s = Resolution

t_m = Migration time (s or min)

w = Baseline peak width (min)

α = Selectivity

μ = Apparent electrophoretic mobility of enantiomer (cm^2/Vs)

TF = Tailing factor

N = Theoretical plate number

$w_{1/2}$ = Temporal peak width at half height (s or min)

$w_{0.05}$ = Peak width at 5% of peak height as measured from the front side of the peak to the tailing edge (s or min)

f = Width at 5% peak height measured from the leading edge of the peak to a vertical line extrapolated from the peak apex (s or min)

Suffixes 1, 2 = First and second migrating enantiomers

5. Method validation

The developed method was validated for the enantioseparation of baclofen and its impurity. Linearity and range, recovery, precision, limit of detection, and limit of quantitation were evaluated.

5.1 Linearity and range

The linearity of the method was determined by preparing standard curves for three different days on five different concentrations in a range of 50-500 µg/ml for baclofen enantiomers and 5-50 µg/ml for impurity. Three injections were made for each concentration. The least-square linear regressions of the analytes were obtained by plotting peak area, peak height, and normalized peak area versus concentrations. Linear regression equation, correlation coefficient (r^2) and %RSD were calculated from Excel®. The correlation coefficient should be ≥ 0.999.[95]

5.2 Recovery

Recovery of the method was investigated by standard addition. Standard of individual enantiomer of baclofen and its impurity at concentrations within 80-120% were spiked into a sample containing 400 µg/ml of baclofen. Each individual enantiomer of baclofen and its impurity concentration were injected in three replicates. Percent recoveries were calculated by the following equation.

$$\% \text{ recovery} = \frac{X_{found} \times 100}{X_{added}} \quad (6)$$

Where: X_{found} = the concentration of standard found in the spiked sample

X_{added} = the concentration of standard added

5.3 Precision

5.3.1 Injection precision

Injection precision was determined by repetitive injection (n = 9) at the middle point concentration of the calibration curve of baclofen and its impurity. The %RSDs

of migration time, peak area, normalized peak area, and peak height of individual enantiomer of baclofen and its impurity were calculated.

5.3.2 Intra-day precision

Intra-day precision was performed on three different concentrations of each individual enantiomer of baclofen and its impurity analyzed on the same day. The %RSDs of migration time, peak area, normalized peak area, and peak height of individual enantiomer of baclofen and its impurity were calculated.

5.3.3 Inter-day precision

Inter-day precision was performed by determining the three different concentrations of each individual enantiomers of baclofen and its impurity on six different days. The %RSDs of migration time, peak area, normalized peak area, and peak height of individual enantiomer of baclofen and its impurity were calculated.

Precision was assessed from the percent relative standard deviations (%RSDs), which was determined form the following formula:

$$\%RSD = \frac{SD \times 100}{\overline{X}} \quad (7)$$

Where: SD = the standard deviation from the mean value

$\overline{X}$ = the mean value

The value (%RSD) should be less than 2%.

5.4 Limit of detection (LOD) and Limit of quantification (LOQ)

The limit of detection (LOD) is the lowest amount of an analyte in a sample that can be detected, typically acceptable signal to noise ratio of ≥ 3. The limit of quantification (LOQ) is a characteristic of quantitative assays. It is the lowest amount of analyte in the sample that can be determined with acceptable precision and recovery with a signal to noise ratio of ≥ 10.

LODs and LOQs of each enantiomer of baclofen and its impurity were determined at signal to noise ratios of 3 and 10, respectively. Serial dilutions of

enantimers of baclofen over the range of 5-50 μg/ml and of its impurity over the range of 1-10 μg/ml were investigated (n = 3).

6. Application

The developed method was applied for the determination of *R*-(-) and *S*-(+)-baclofen and its impurity in two different brands of baclofen raw materials and three different brands of baclofen tablets.

For the determination of impurity, stock solutions of raw material were prepared by dissolving the powder in 100 mM borate buffer (pH 9.9) to obtain a concentration of 1 mg/ml. The solution was sonicated for 15 min. For tablets, twenty tablets of each brand were accurately weighed and ground to fine powder. A quantity of powder equivalent to 25 mg of baclofen was diluted with 100 mM borate buffer (pH 9.9) to obtain the concentration of 1 mg/ml. The solution (from tablets) was sonicated for 30 min and centrifuge for 10 min at 14,000 rpm and filtered through a 0.2 μ membrane prior analysis. For the determination of baclofen enantiomers, the working solutions of both raw material and tablets were diluted to 0.4 mg/ml and filtered through a filter paper prior analysis. Results were reported as % w/w for the determination of impurity (USP limit not more than 1% and 4% for raw material and pharmaceutical formulation, respectively) and percent label amount (USP limit within 99-101 and 90-110% of the labeled amount of $C_{10}H_{12}ClNO_2$ in raw material and pharmaceutical formulation, respectively) and ratio of *S:R* in the samples were calculated.

CHAPTER IV
RESULTS AND DISCUSSION

Capillary zone electrophoresis was developed for the separation of baclofen enantiomers and its impurity. Optimization of CCE conditions was performed by investigating factors affecting their separation such as the chiral selector types and concentrations, buffer concentrations and pH, organic solvents, temperature, applied voltage, and injection time. The optimum conditions were validated and applied for the determination of baclofen and its impurity in raw material and pharmaceutical formulations.

1 Optimization

The optimization of CCE condition for the separation of baclofen enantiomers and impurity was divided in two stages. Firstly, CCE conditions for the enantiomeric separation of baclofen were optimized. Secondly, the condition was further modified for the separation of all three analytes. The spectra of these analytes, with a maximum absorption at a wavelength of 220 nm, are shown in Fig. 19. The following factors were consecutively optimized: the chiral selector types and concentrations, buffer concentrations and pH, organic solvents, temperature, applied voltage, and injection time. Migration times (t_m), resolution (R_s), selectivity (α), peak width (w), and theoretical plate number (N) were evaluated.

1.1 Method development for the separation of baclofen enantiomers

The initial condition for enantioseparation of baclofen was performed using of 100 mM sodium borate buffer (pH 9.25) containing cyclodextrin and 10% v/v ACN, 20 ^{o}C, 25 kV, fused-silica capillary with a total length of 64.5 cm (effective length of 56 cm and inner diameter of 50 μm), hydrodynamic injection at 50 mbar for 3 s, and UV detection at 220 nm. A racemic mixture of (*R/S*)-baclofen was added with 50% v/v acetronitrile.[38,51]

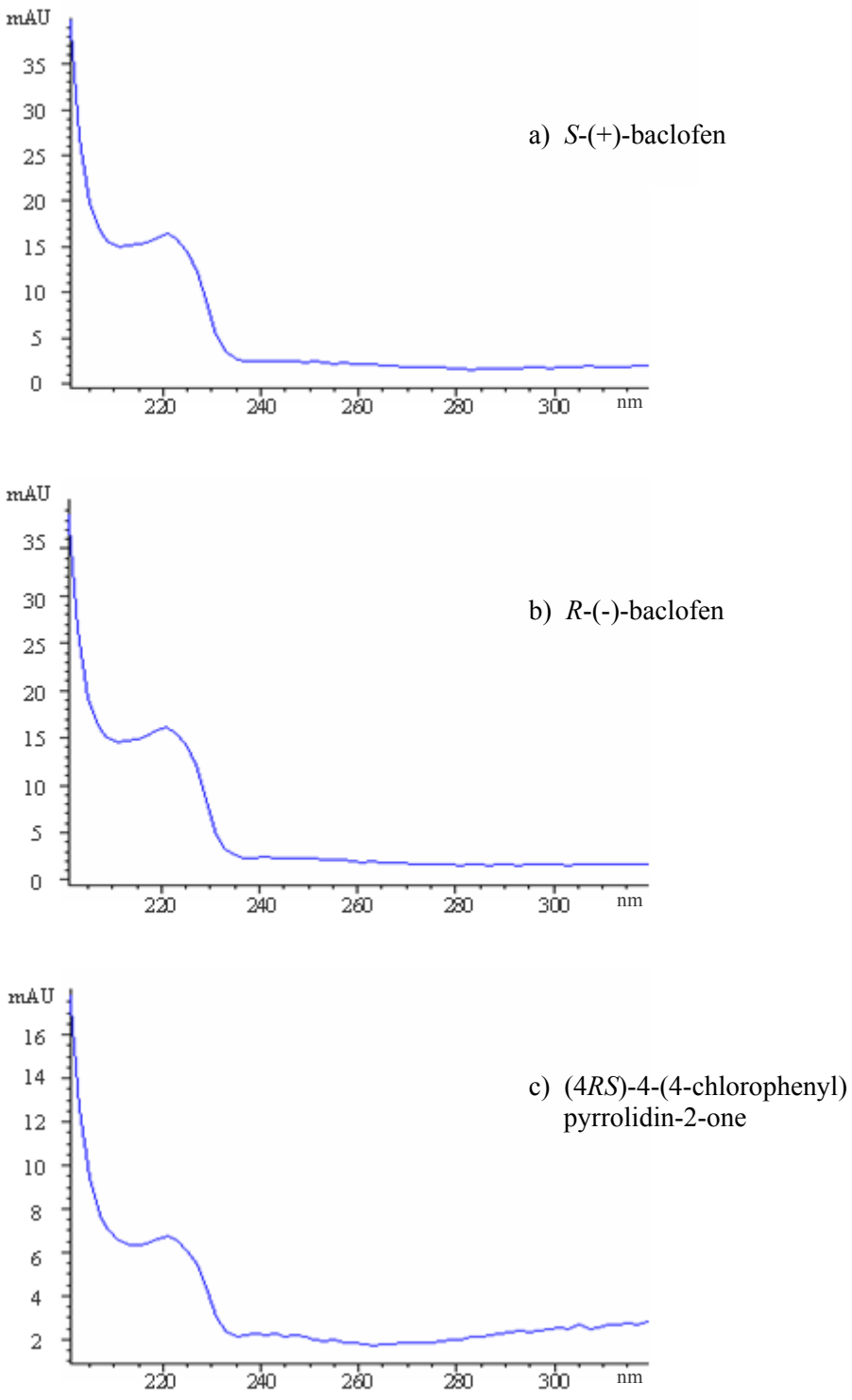


Fig. 19 UV spectra of the investigated analytes from the diode array detector of the capillary electrophoresis instrument. a) *S*-(+)-baclofen, b) *R*-(-)-baclofen, and c) (4*RS*)-4-(4-chlorophenyl) pyrrolidin-2-one.

1.1.1 Effects of the cyclodextrin types on the separation

Baclofen is an amphoterric molecule, with pK_a values of 3.87 and 9.62 for the carboxylic and amino groups, respectively.[3] At high pH values, the carboxylic group is deprotonated giving a negatively charged form. Therefore, the analytes move to anode site. While the neutral cyclodextrin (CD) move through the cathode site at the same rate as the electroosmotic flow. Thus, the use of neutral cyclodextrin appears to be a way to achieve the separation of their enantiomers.

The influence of the cyclodextrin types on the enantioseparation of baclofen was evaluated using α-CD and β-CD. Table 6 shows the t_m, R_s, α, w, and N for the enantioseparation of baclofen. Decreasing migration times of baclofen enantiomers was obtained when changing from α-CD to β-CD. Increasing α-CD concentration, increased the resolution of baclofen enantiomers, while β-CD could not provided the baseline separation because its cavity was too big to form the baclofen inclusion complexes. Therefore, α-CD was chosen as the chiral selector for enantioseparation of baclofen (Fig. 20).

Table 6 Effects of α-CD types on the separation of *R/S*-baclofen

CD type	Concentration (mM)	t_m (min)	R_s	α	w (*R*-baclofen) (min)	N (*R*-baclofen)
α-CD	10	14.30	0.5	1.01	0.19	31369
	18	14.35	2.1	1.02	0.08	201061
β-CD	10	12.58	0	-	0.10	99420
	18	9.56	0	-	0.08	77014

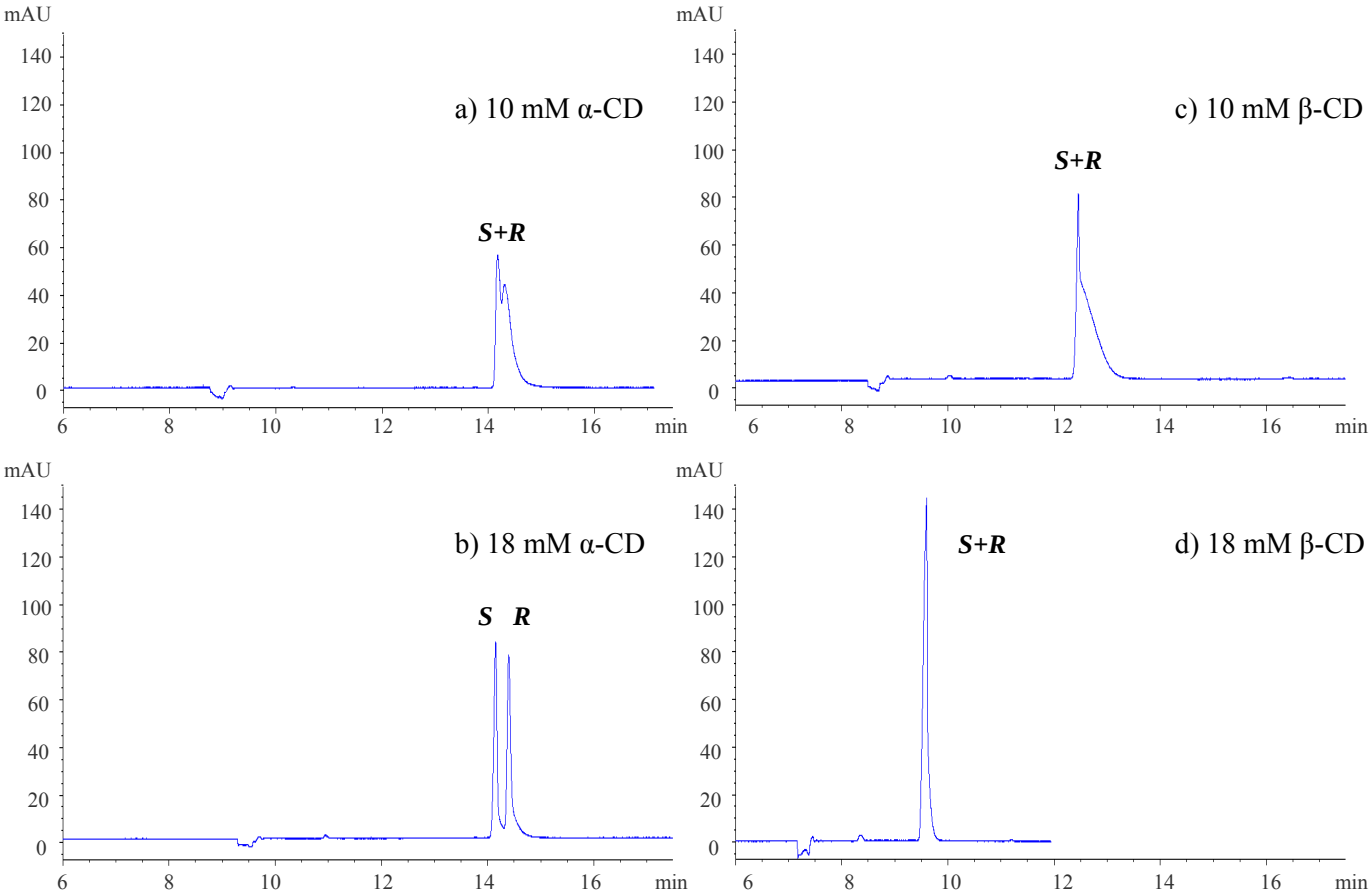


Fig. 20 Electropherogram of racemic mixture of (*R/S*)-baclofen (250 µg/ml). Conditions: 100 mM sodium borate buffer (pH 9.25) containing a) 10 mM α-CD, b) 18 mM α-CD, c) 10 mM β-CD, and d) 18 mM β-CD and 10% v/v ACN; fused-silica capillary, 64.5 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 3 s; temperature, 20 ^{o}C; voltage, 25 kV; detection by UV absorbance at 220 nm.

1.1.2 Effects of the α-CD concentration on the separation

Cyclodextrin concentration is an essential parameter for the optimization of chiral separation since it influences the peak resolution, the migration time, and in some instances, even the migration order.[62]

The influence of the α-CD concentration on the enantioseparation of baclofen was studied in the range from 10-23 mM (Fig. 21). Table 7 shows t_m, R_s, α, w, and N affecting by the α-CD concentration. Increasing α-CD concentration, the enantiomeric resolution increased. The resolution value of more than 2.0 was obtained at 18 and 20 mM of α-CD, but the completed baseline separation and better peak symmetry were achieved at 18 mM α-CD. However, higher α-CD concentration (23 mM) did not improve the resolution because of the increased buffer viscosity, which reduced the enantiomeric resolution. The effect of the CD concentration on the enantioseparation may be explained using the theoretical studies report by Wren and Rowe [96] and Penn et. al [97,98]. Electrophoretic mobility different ($\Delta\mu$) and resolution increase to the maximum with an increase in the CD concentration, and then decrease at the excess CD concentration. The optimum CD concentration is related to the equilibrium constants (K) as previously mentioned in Eq. (3).

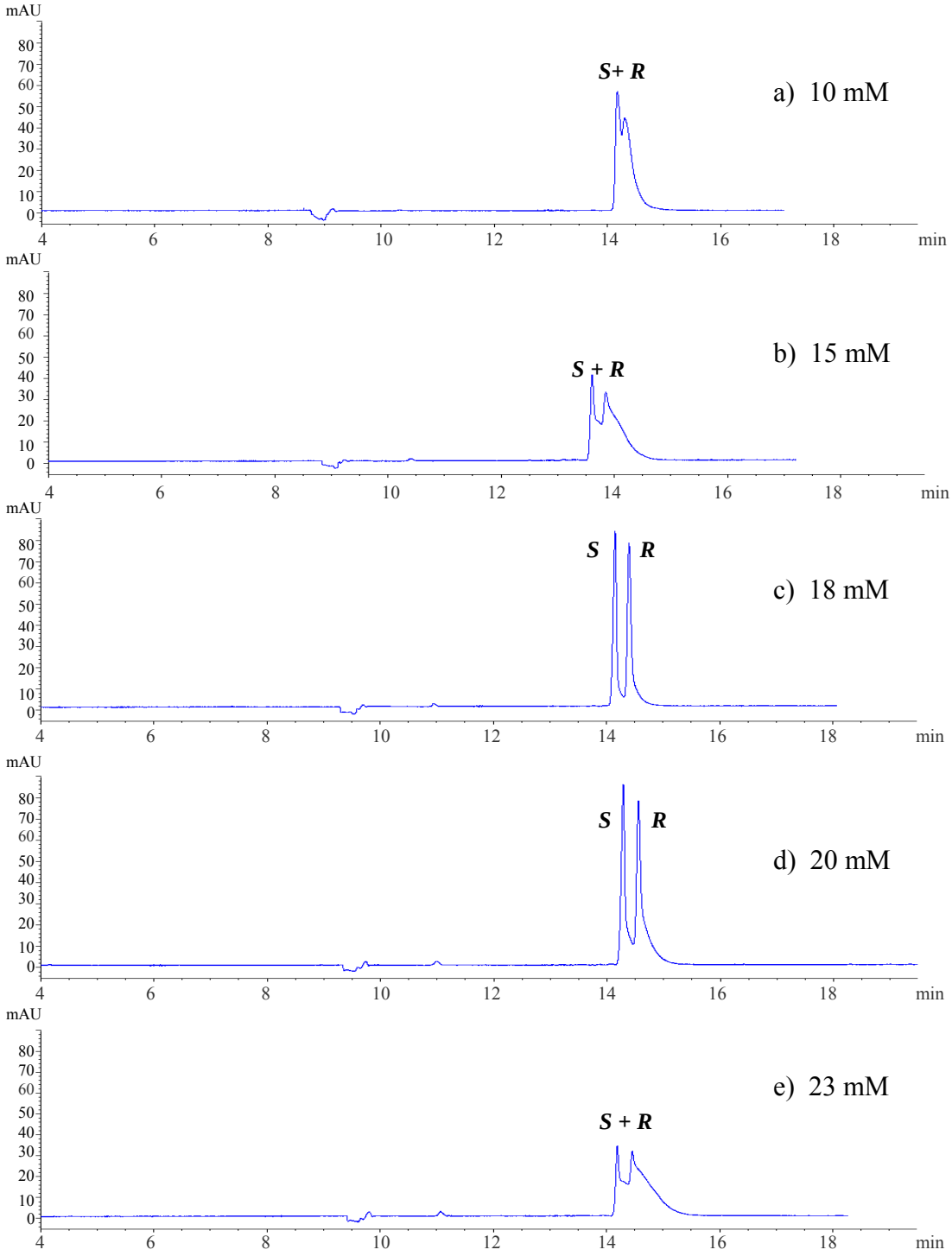


Fig. 21 Electropherogram of racemic mixture of (*R/S*)-baclofen (250 µg/ml). Conditions: 100 mM sodium borate buffer (pH 9.25) containing a) 10 mM, b) 15 mM, c) 18 mM, d) 20 mM, and e) 23 mM α-CD and 10% v/v ACN; and other CE condition as shown in Fig. 20.

Table 7 Effects of α-CD concentrations on the separation of *R/S*-baclofen

Alpha-CD (mM)	t_m (min)	R_s	α	w (*R*-baclofen) (min)	N (*R*-baclofen)
10	14.30	0.5	1.01	0.19	31369
15	13.99	0.9	1.02	0.26	21366
18	14.35	2.1	1.02	0.08	201061
20	14.20	2.1	1.02	0.08	187431
23	14.59	0.7	1.02	0.34	10241

1.1.3 Effects of the buffer concentration on the separation

Buffer concentration affects the EOF, which influences to migration time, and current.[82] Increasing buffer concentration, decreased the EOF because it lowers the zeta potential.

A borate buffer (pH 9.25) in the range of 25 to 130 mM $Na_2B_4O_7.10H_2O$ was investigated for the separation of the bacloen enantiomers (Fig. 22), using 18 mM α-CD and 10% v/v ACN in the buffer. Increasing buffer concentration slowed down the migration of baclofen. Table 8 shows analytical parameters of baclofen enantiomers at five different concentrations. The highest resolution of baclofen enantiomers was achieved at 100 mM sodium borate buffer containing 18 mM of α-CD and 10% v/v ACN.

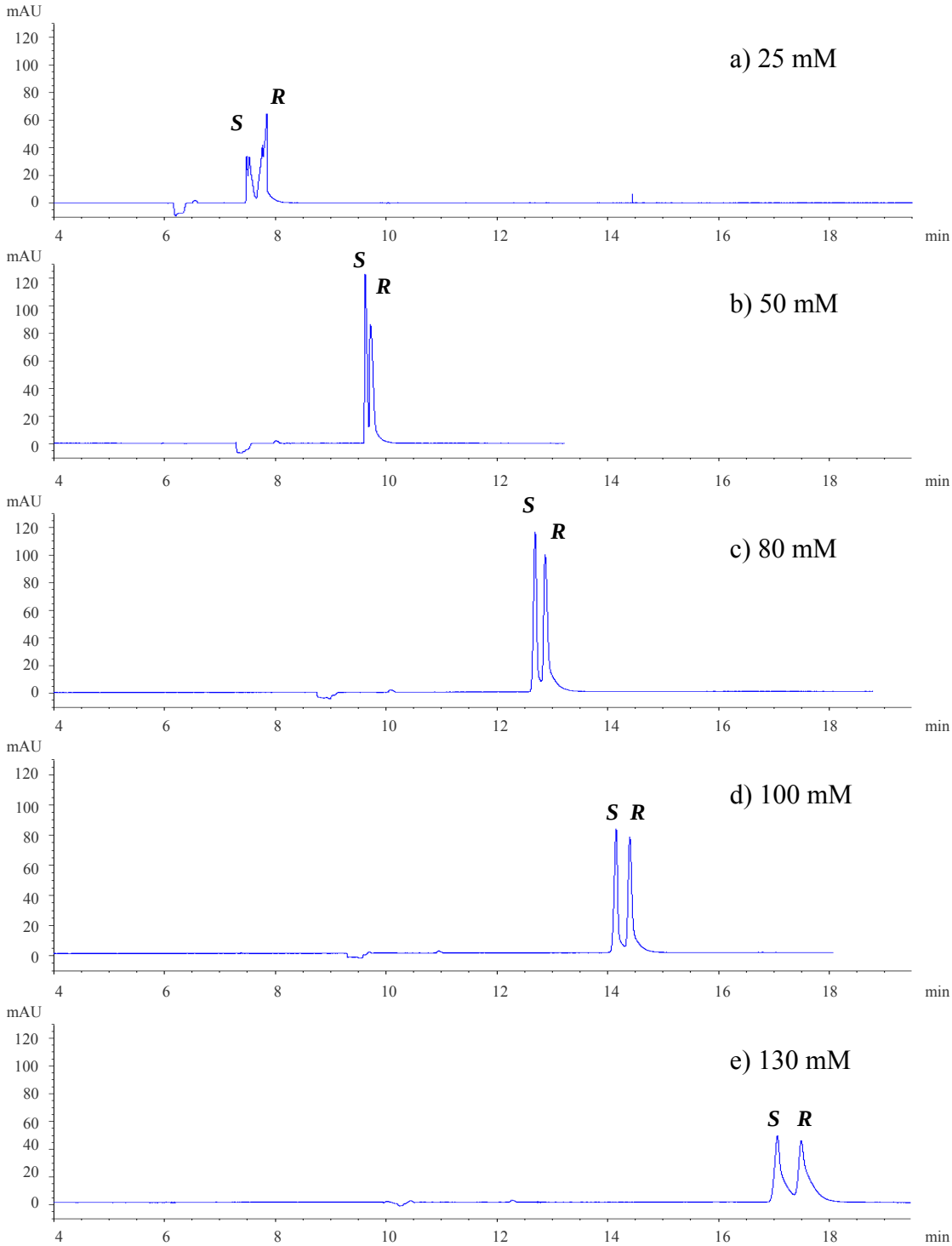


Fig. 22 Electropherogram of racemic mixture of (*R/S*)-baclofen (250 µg/ml). Conditions: a) 25 mM, b) 50 mM, c) 80 mM, and d) 100 mM, and (e) 130 mM sodium borate buffer (pH 9.25) containing 18 mM α-CD and 10% v/v ACN; fused-silica capillary, 64.5 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 3 s; temperature, 20 ^{o}C; voltage, 25 kV; detection by UV absorbance at 220 nm.

Table 8 Effects of the buffer concentrations on the separation of *R/S*-baclofen

BGE (mM)	t_m (min)	R_s	α	w (*R*-baclofen) (min)	N (*R*-baclofen)
25	7.92	2.0	1.05	0.11	30263
50	9.70	1	1.01	0.07	103129
80	12.74	1.6	1.01	0.07	168410
100	14.35	2.1	1.02	0.08	201061
130	17.55	1.7	1.03	0.19	54398

1.1.4 Effects of buffer pH on the separation

In enantiomeric separation by CE, the pH is an important factor for the consideration, especially for closely related compounds. Buffer pH affects the degree of dissociation of the analytes and the cyclodextrin, thus the buffer pH significantly impacts the complex selectivity of enantiomer with the cyclodextrin.[68]

The effects of buffer pH were observed in the range from 8.0-9.5 of sodium borate buffer containing 18 mM α-CD and 10% v/v ACN (Table 9). Increasing the buffer pH, increased the resolution of enantiomers. Fig. 23 shows the influences of buffer pH on the separation of baclofen enantiomers. A poorer separation was observed at pH 8.0 and the highest separation of baclofen enantiomers were obtained at pH 9.4. According to the theoretical studies report by Rawjee et. al [99-103], the effect of the buffer pH on the separation of weak acidic or basic enantiomers depend on ionic, or non-ionic forms that show enantiomeric discrimination with a particular neutral CD. If the former case, a change in the buffer pH can affect the enantioseparation, This implies that the negatively charged form of baclofen enantiomers has different in equilibrium constants.

Table 9 Effects of buffer pH on the separation of *R/S*-baclofen

Buffer pH	t_m (min)	R_s	α	w (*R*-baclofen) (min)	N (*R*-baclofen)
8.0	8.25	0	-	0.05	174891
9.3	14.17	0.8	1.02	0.22	22774
9.4	14.66	2.0	1.02	0.12	89549
9.5	16.96	1.1	1.03	0.29	19489

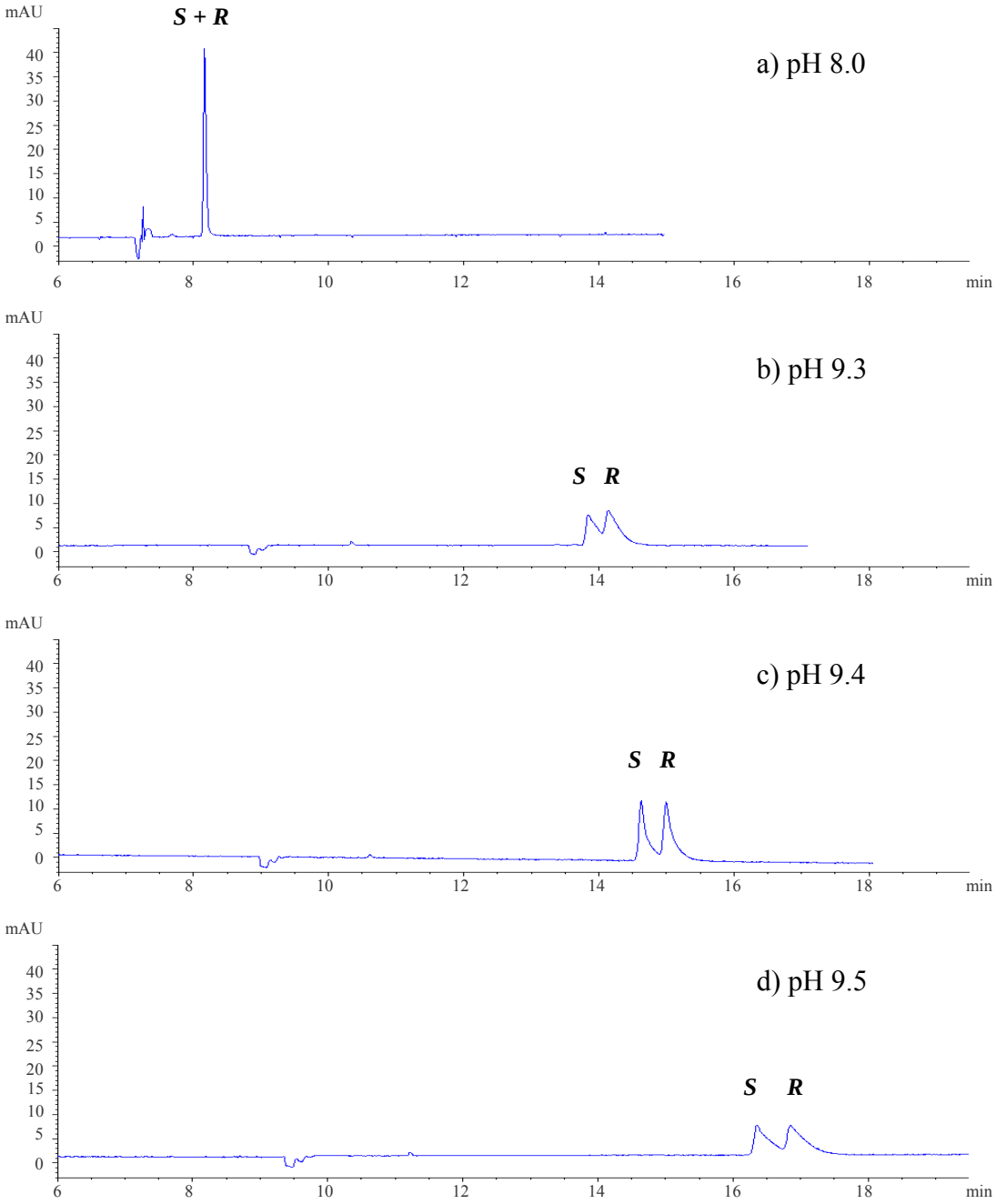


Fig. 23 Electropherogram of racemic mixture of (*R/S*)-baclofen (125 µg/ml). Conditions: 100 mM sodium borate buffer pH a) 8.0, b) 9.3, c) 9.4, and d) 9.5 containing 18 mM α-CD and 10% v/v ACN; fused-silica capillary, 64.5 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 10 s; temperature, 20 ^{o}C; voltage, 25 kV; detection by UV absorbance at 220 nm.

1.1.5 Effects of amounts of acetronitrile on the separation

Addition of an organic solvent can play an important role in the enantiomeric separation. The conductivity of the BGE and EOF was decreased by organic solvent such as MeOH and ACN through disruption of the order structure of the water molecules. Moreover, a combination of the decreased EOF (i.e., increased on-capillary time), decreased thermal diffusion, and improved analyte solubility results in enhancement of resolution. [83]

In this work, acetronitrile was used as organic solvent for separation of baclofen enantiomers. Influence of the amount of ACN on the enantioseparation was observed from 0-15% v/v of ACN in 100 mM sodium borate buffer containing 18 mM α-CD (Fig. 24). The optimum separation was obtained in the BGE contained 10% v/v ACN (Table 10). The addition of organic solvent in BGE can decrease or increase enantioseparation, depending on the CD concentration, as explained by Wren and Rowe [96] and Penn et. al [98]. 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. This implies that 18 mM α-CD may be above the optimum α-CD concentration, and therefore, improved resolution of baclofen enantiomers was observed with an increase in ACN concentrations. However, ACN in the BGE is needed to prevent precipitation of baclofen at high concentrations in the solution of real samples.

1.1.6 Effects of temperature on the separation

When temperature increases, shorter migration times are observed in CE. This is due to the simultaneous decrease of the viscosity of the electrolyte in capillary and increase of the EOF velocity.

The effect of the temperature of the enantioseparation was studied in the range from 20-30 ^{o}C (Fig. 25). The resolution of more than 1.5 was achieved at the temperature of 20-30 ^{o}C but the complete baseline separation of both enantioners were observed at 30 ^{o}C. Therefore, the capillary temperature was maintained at 30 ^{o}C to optimize the separation of baclofen enantiomers (Table 11).

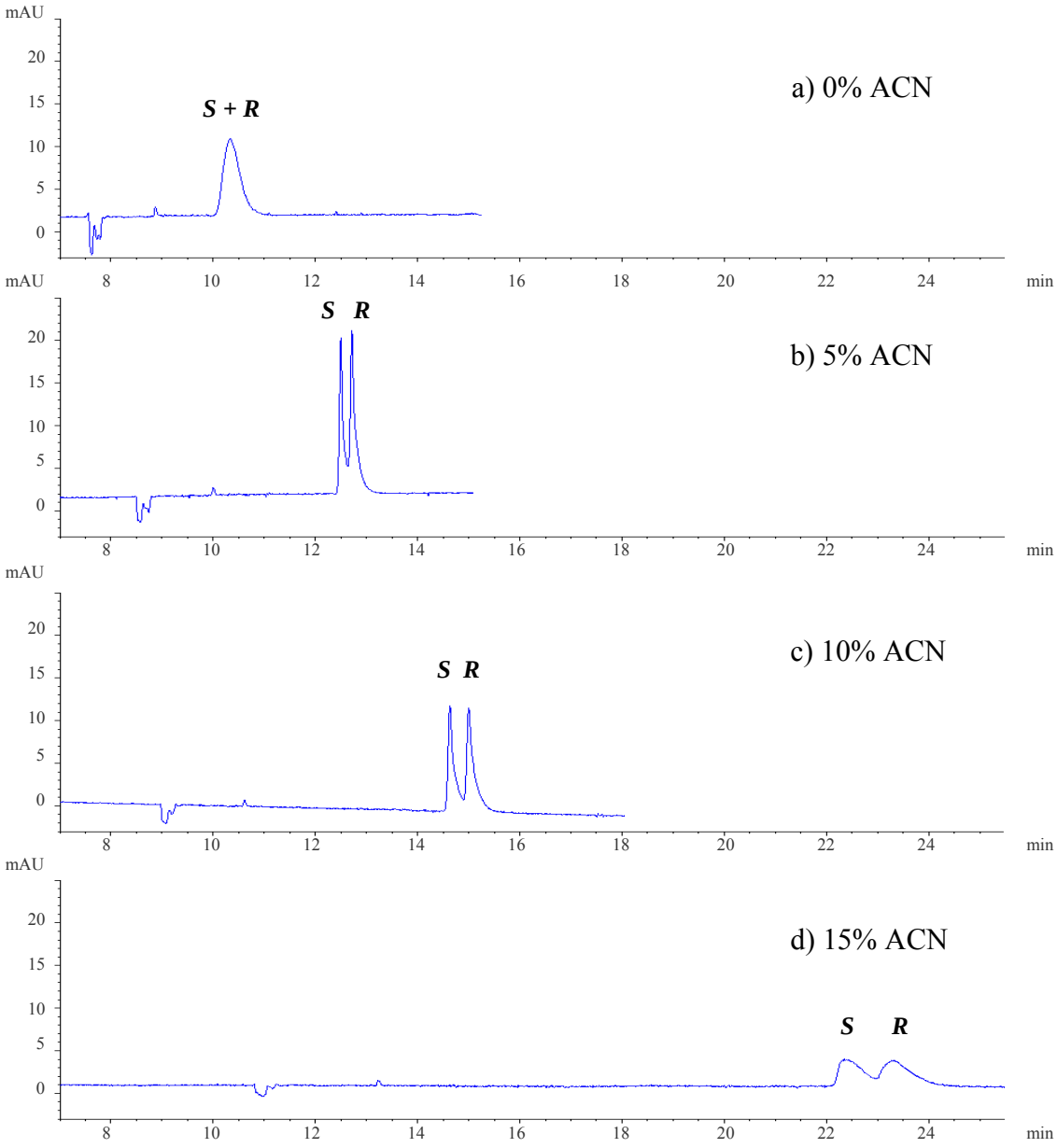


Fig. 24 Electropherogram of racemic mixture of (*R/S*)-baclofen (125 µg/ml). Conditions: 100 mM sodium borate buffer (pH 9.4) containing 18 mM α-CD and a) 0%, b) 5%, c) 10%, and d) 15% v/v ACN; fused-silica capillary, 64.5 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 10 s; temperature, 20 ^{o}C; voltage, 25 kV; detection by UV absorbance at 220 nm.

Table 10 Effects of amount of acetronitrile on the separation of *R/S*-baclofen

Amount of ACN (% v/v)	t_m (min)	R_s	α	w (*R*-baclofen) (min)	N (*R*-baclofen)
0	10.33	0	-	0.36	4562
5	12.75	1.4	1.02	0.11	92150
10	14.65	2.0	1.02	0.12	89549
15	23.33	1.0	1.04	0.66	6851

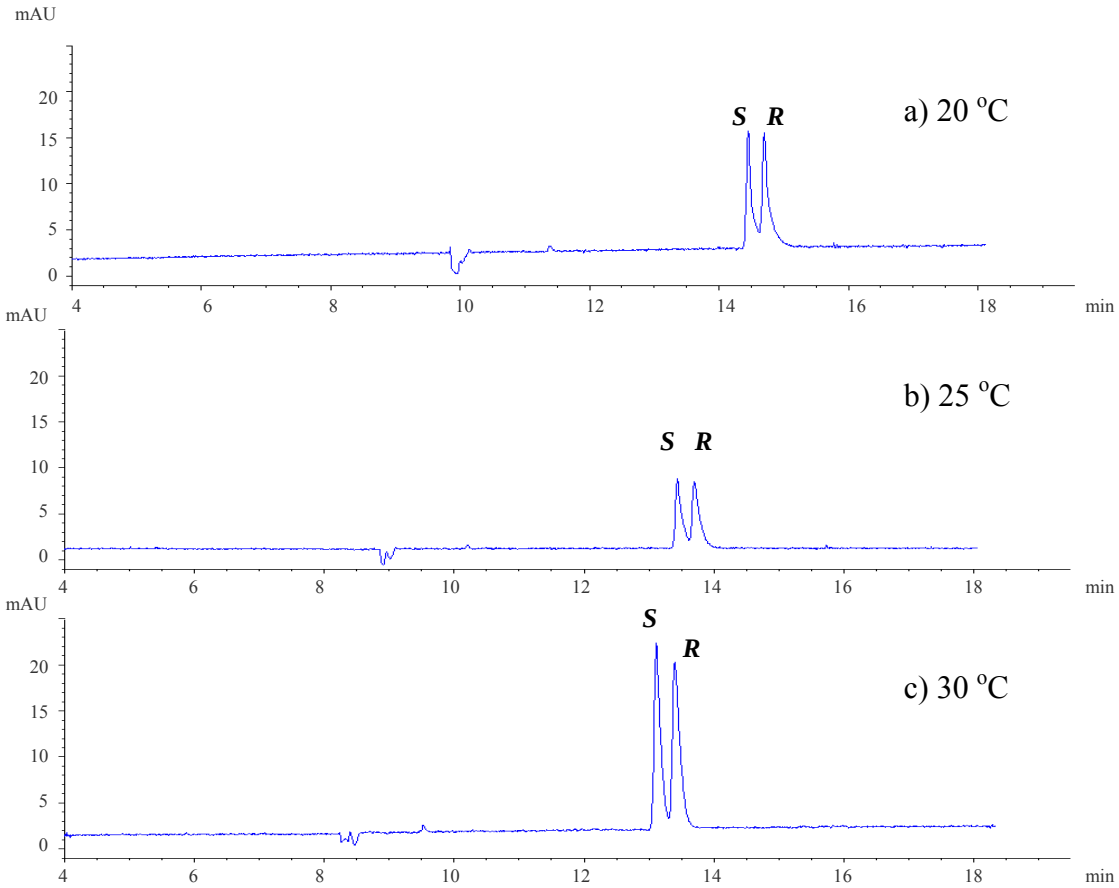


Fig. 25 Electropherogram of racemic mixture of (*R/S*)-baclofen (125 µg/ml). Conditions: 100 mM sodium borate buffer (pH 9.4) containing 18 mM α-CD and 10% v/v ACN; fused-silica capillary, 64.5 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 10 s; temperature, a) 20 °C, b) 25 °C, and c) 30 °C; voltage, 25 kV; detection by UV absorbance at 220 nm.

Table 11 Effects of temperature on the separation of *R/S*-baclofen

Temperature (^{o}C)	t_m (min)	R_s	α	w (*R*-baclofen) (min)	N (*R*-baclofen)
20	14.53	2.0	1.02	0.08	192138
25	13.69	1.5	1.02	0.11	86860
30	13.39	1.5	1.02	0.12	68957

1.1.7 Effects of applied voltage on the separation

Voltage has a great influence on CE separation. Both the EOF and electrophoretic velocities are directly proportional to the field strength, which affects to the migration time and resolution.

Fig. 26 shows effects of applied voltage on enantioseparation of baclofen**.** Interestingly, increasing applied voltage, the resolution improved. Table 12 reveals effects of the applied voltage on the migration time, resolution, and selectivity of baclofen enantiomers. The complete baseline separation of both enantioners were observed at 30 kV (R_s = 2.1). Therefore, the applied voltage was maintained at 30 kV to optimize the separation of baclofen enantiomers

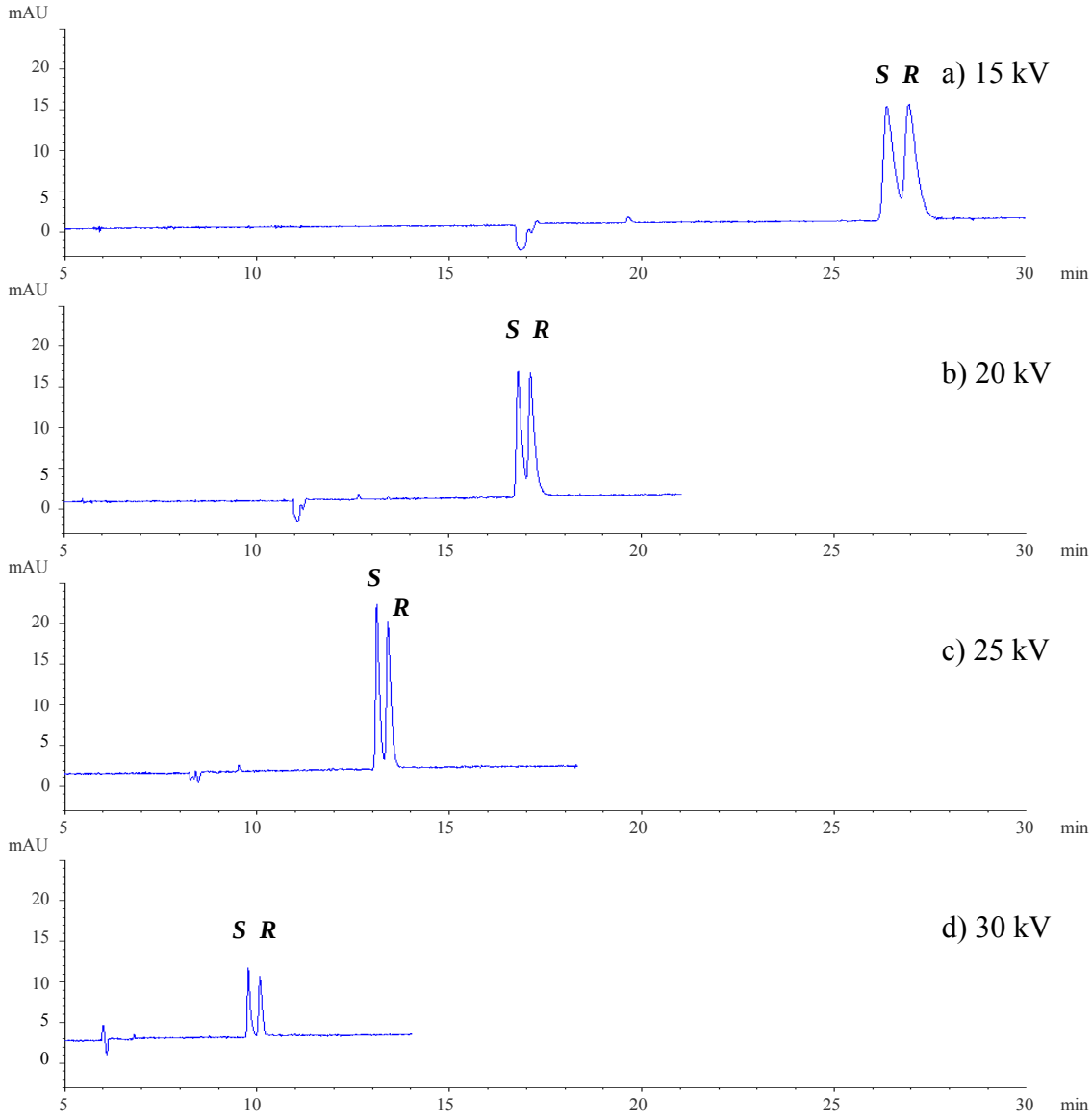


Fig. 26 Electropherogram of racemic mixture of (*R/S*)-baclofen (125 µg/ml). Conditions: 100 mM sodium borate buffer (pH 9.4) containing 18 mM α-CD and 10% v/v ACN; fused-silica capillary, 64.5 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 10 s; temperature, 30 °C; voltage, a) 15 kV, b) 20 kV, c) 25 kV, and d) 30 kV; detection by UV absorbance at 220 nm.

Table 12 Effects of applied voltage on the separation of *R/S*-baclofen

Applied voltage (kV)	t_m (min)	R_s	α	w (*R*-baclofen) (min)	N (*R*-baclofen)
15	26.92	1.1	1.02	0.32	40307
20	17.10	1.3	1.02	0.15	68929
25	13.39	1.5	1.02	0.12	68957
30	10.35	2.1	1.03	0.10	62099

In summary, the optimized conditions for enantioseparation of baclofen were performed by using 100 mM sodium borate buffer (pH 9.4) containing 18 mM α-CD and 10% v/v ACN, 30 °C, 30 kV, fused-silica capillary with a total length of 64.5 cm (effective length of 56 cm and inner diameter of 50 µm), hydrodynamic injection at 50 mbar for 10 s, and detected at 220 nm (Fig. 27).

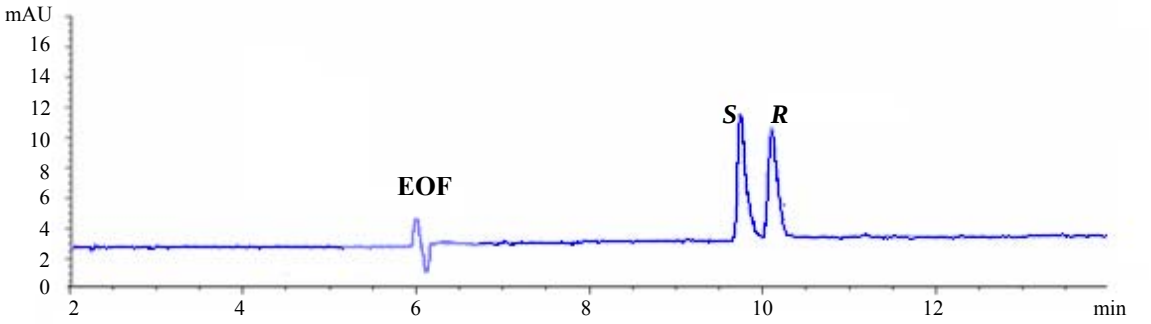


Fig. 27 Electropherogram of racemic mixture of (*R/S*)-baclofen (125 µg/ml). Conditions: 100 mM sodium borate buffer (pH 9.4) containing 18 mM α-CD and 10% v/v ACN; fused-silica capillary, 64.5 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 10 s; temperature, 30 °C; voltage, 30 kV; detection by UV absorbance at 220 nm.

1.2 Method development for the separation of baclofen enantiomers and its impurity

The optimum condition for the separation of baclofen enantiomer was applied for the separation of baclofen impurity, (4*RS*)-4-(4-chlorophenyl) pyrrolidin-2-one. However, the impurity could not be separated under this condition. Moreover,

reproducibility of the migration time for the impurity was poor and the system noise was unacceptable (Fig. 28). Consequently, next experiments focused on the separation of these three compounds.

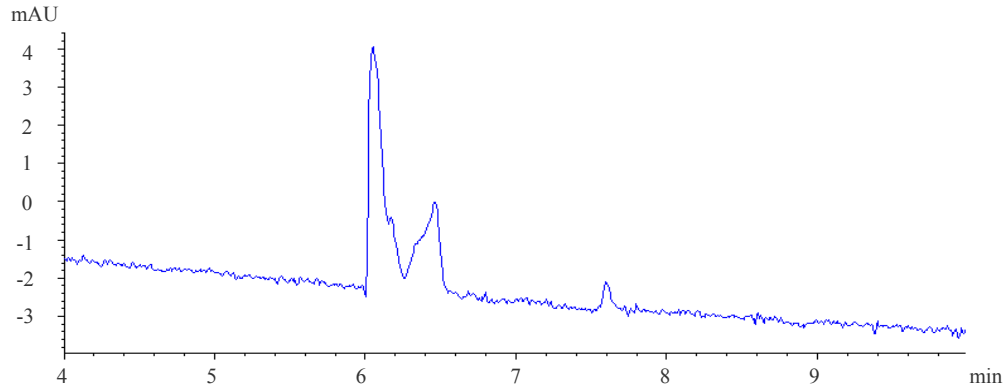


Fig. 28 Electropherogram of (4*RS*)-4-(4-chlorophenyl) pyrrolidin-2-one (25 µg/ml). Conditions: 100 mM sodium borate buffer pH 9.4 containing 18 mM α-CD and 10% v/v ACN; fused-silica capillary, 64.5 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 10 s; temperature, 30 ^{o}C; voltage, 30 kV; detection by UV absorbance at 220 nm.

The impurity of baclofen, (4*RS*)-4-(4-chlorophenyl) pyrrolidin-2-one, is rather neutral at pH 9.4 and it migrated with the EOF. Therefore the EOF needed to be controlled by coating the inner surface of the capillary, decreasing the temperature and the applied voltage, and varying the amount of ACN. Polyethylene oxide (PEO) is a high molecular weight polymer that was selected as a dynamic coating agent. This dynamic coating of the inner surface of the capillary was obtained by rinsing with a solution containing PEO in precondition process. Coating of PEO, reduced the EOF and the interactions between analytes and the capillary wall surface, which could improve resolution and reproducibility. The enantioresolution of baclofen have been achieved by using 50 cm x 50 µm ID capillary dynamically coated with PEO at low pH of phosphate buffer containing highly Sulfated-CD.[3,6] Therefore, buffer pH was investigated in a range of 9.7-9.9.

Thus, the experiments were initially performed by using 100 mM sodium borate buffer containing 18 mM α-CD; fused-silica capillary dynamic coated with PEO, 50 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at

50 mbar for 10 s; temperature, 20 °C; voltage, 25 kV; detection by UV absorbance at 220 nm with a band width of 4 nm.

1.2.1 Effects of buffer pH on the separation

Effects of buffer pH were observed in a range of 9.7-9.9 of sodium borate buffer containing 18 mM α-CD. Increasing buffer pH, increased the resolution of baclofen enantiomers (Fig. 30). Resolution value was calculated from peaks of *R*- and *S*-baclofen. The best separation of baclofen enantiomers and its impurity were obtained at 9.9 (Fig. 29).

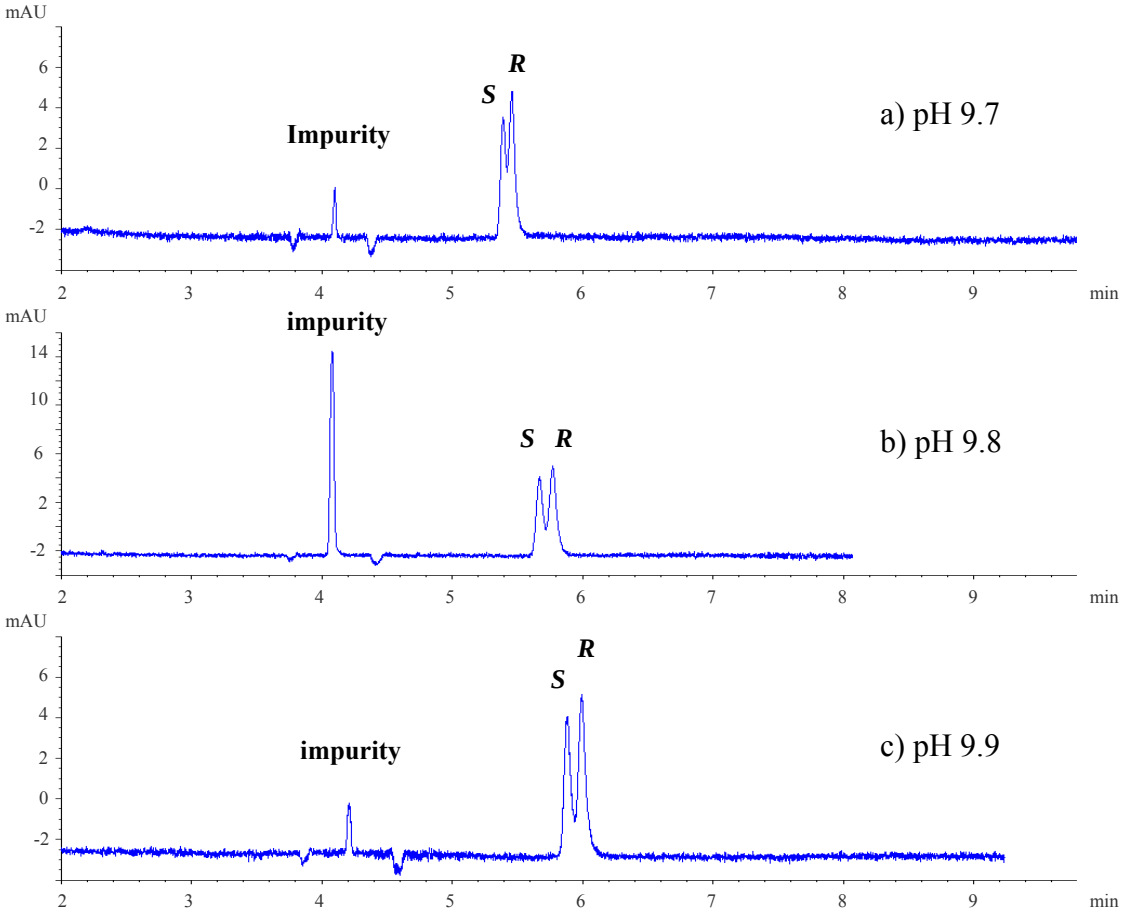


Fig. 29 Electropherogram of racemic mixture of (*R/S*)-baclofen (50 µg/ml) and its impurity (5 µg/ml, except in b) 50 µg/ml). Conditions: 100 mM sodium borate buffer pH a) 9.7, b) 9.8, c) 9.9 containing 18 mM α-CD; fused-silica capillary dynamic coated with PEO, 50 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 10 s; temperature, 20 °C; voltage, 25 kV; detection by UV absorbance at 220 nm.

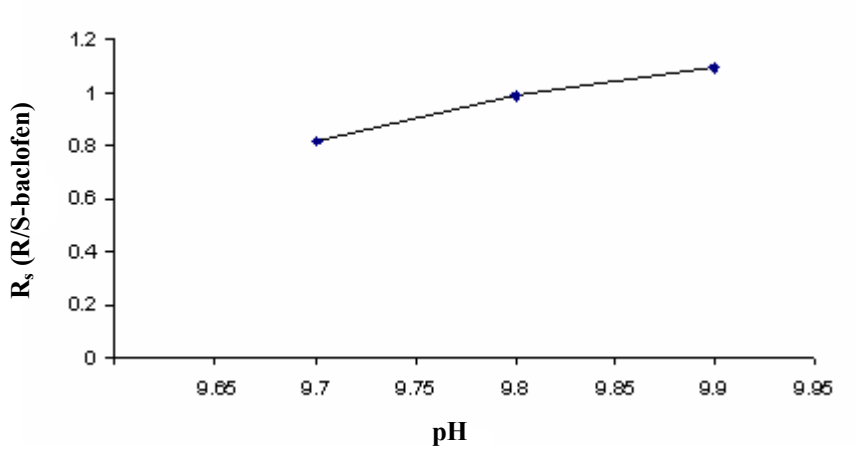


Fig. 30 Effects of buffer pH on the separation of racemic mixture of (*R/S*)-baclofen.

1.2.2 Effects of amounts of acetronitrile on the separation

The amount of ACN is an important parameter on the resolution of baclofen and peak shape of (4*RS*)-4-(4-chlorophenyl) pyrrolidin-2-one. The influence of ACN in buffer was studied in a range of 0-10% v/v ACN. Sample was prepared by mixing 49 µg/ml baclofen and 4.9 µg/ml impurity containing 2 % v/v ACN owing to adding ACN decrease the viscosity of mixture, therefore the complexation between the analytes and cyclodextrin is increased. The amounts of ACN in buffer impacted the resolution and the dispersion of baclofen peak but not significantly affected the peak shape of (4*RS*)-4-(4-chlorophenyl) pyrrolidin-2-one. Fig. 32 shows the influence of amounts of ACN in buffer on the resolution of baclofen enantiomers and peak width of *R*-enantiomer of baclofen. Although the dispersion of peak decreased when reduced the amount of ACN in buffer, the high resolution was observed at 5% v/v ACN. Therefore, the amount of ACN in buffer was performed at 5% v/v (Fig. 31).

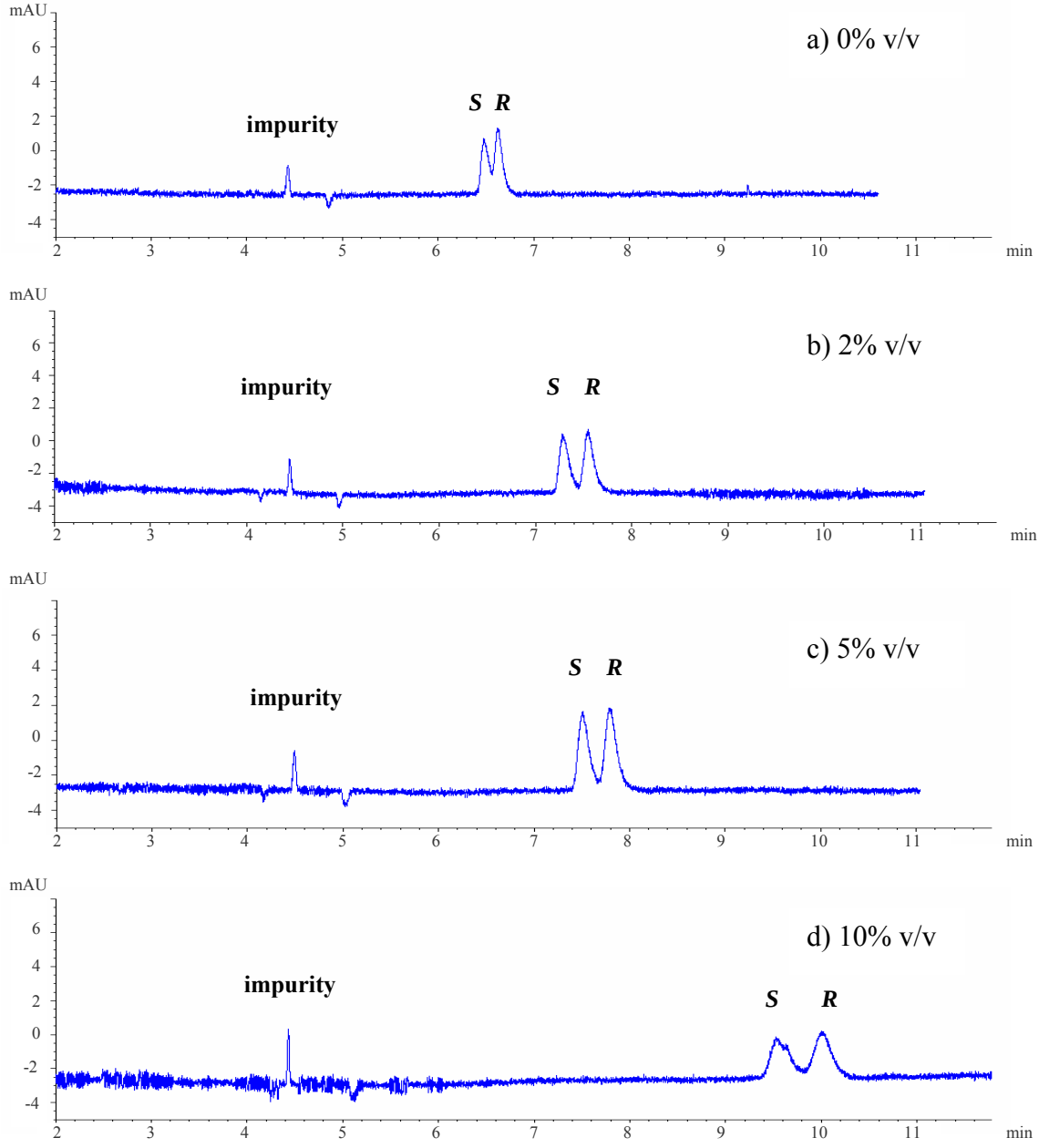


Fig. 31 Electropherogram of racemic mixture of (*R/S*)-baclofen (49 µg/ml) and its impurity (4.9 µg/ml). Conditions: 100 mM sodium borate buffer pH 9.9 containing 18 mM α-CD and a) 0%, b) 2%, c) 5%, d) 10% v/v ACN; fused-silica capillary dynamic coated with PEO, 50 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 10 s; temperature, 20 ^{o}C; voltage, 25 kV; detection by UV absorbance at 220 nm.

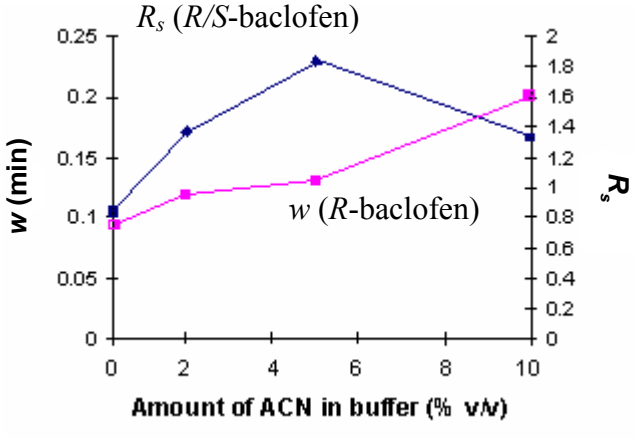


Fig. 32 Effects of amounts of ACN in BGE on the separation of racemic mixture of (*R/S*)-baclofen and its impurity.

Moreover, the amount of ACN in sample affected the resolution of baclofen enantiomers and peak shape of (4*RS*)-4-(4-chlorophenyl) pyrrolidin-2-one. The influence of ACN in sample was studied in a range of 0.5-2% v/v ACN. Fig. 33 shows that the amounts of ACN in sample significantly impacted the peak width of (4*RS*)-4-(4-chlorophenyl) pyrrolidin-2-one and the resolution of baclofen enantiomers. Increasing the amount of ACN in sample, decreased the width of (4*RS*)-4-(4-chlorophenyl) pyrrolidin-2-one peak. The high resolution of baclofen was obtained when 2% v/v ACN was added into the sample mixture (Fig. 34).

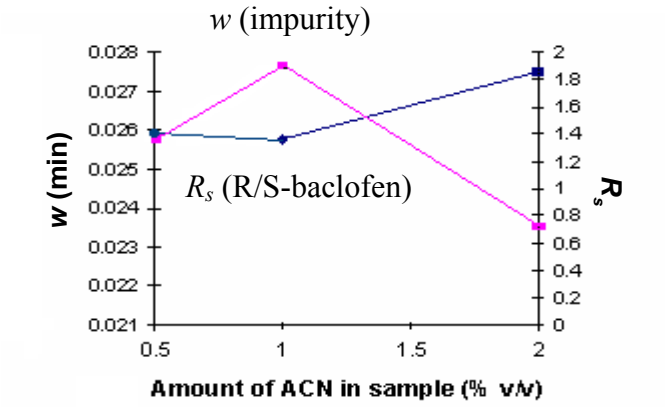


Fig. 33 Effects of amounts of ACN in sample on the separation of racemic mixture of (*R/S*)-baclofen and its impurity.

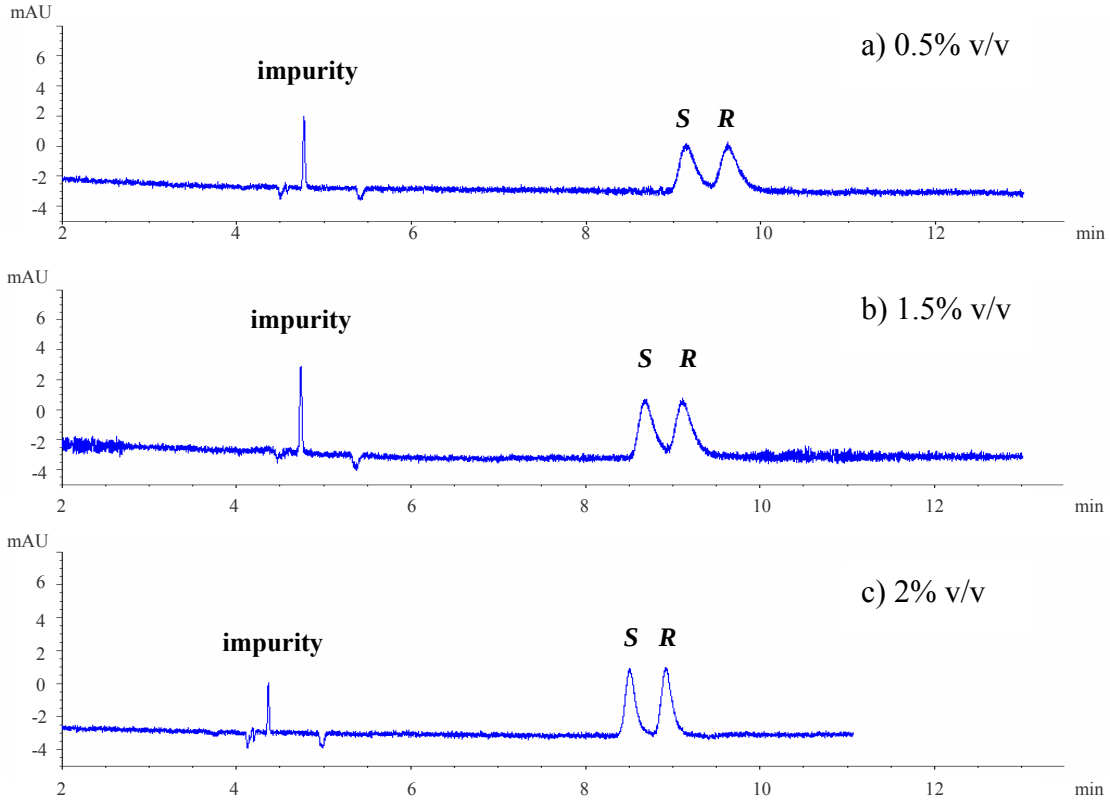


Fig. 34 Electropherogram of racemic mixture of (*R/S*)-baclofen (50 µg/ml) and its impurity (5 µg/ml). Conditions: racemic mixture of (*R/S*)-baclofen and impurity containing a) 0.5%, b) 1.5%, c) 2% v/v ACN; 100 mM sodium borate buffer pH 9.9 containing 18 mM α-CD and 5% v/v ACN; fused-silica capillary dynamic coated with PEO, 50 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 10 s; temperature, 20 ^{o}C; voltage, 25 kV; detection by UV absorbance at 220 nm.

1.3 Final optimization of CE condition for the separation of baclofen enantiomers and its impurity

In the previous sections, the CE condition still gave unsatisfactory separation of baclofen enantiomers and its impurity. Additionally, instrumental problem was encountered (e.g. expired of UV lamp for the DAD detector). Therefore, further optimization was needed. Moreover, the expired CE lamp may be resulting in sensitivity and system noises. Thus, a new lamp for the detector was replaced. So, further optimization was investigated by increasing length of capillary, temperature, and applied voltage, decreasing injection time, and varying amounts of ACN (narrow range). These experiments focused on the reducing of baclofen peak dispersion.

1.3.1 Effects of temperature and applied voltage on the separation and dispersion

Effects of temperature and applied voltage on the dispersion of baclofen peaks were simultaneously investigated. Increasing the temperature and applied voltage, slightly decreased the resolution of baclofen enantiomers, while also decreased the width of baclofen peaks (Table 13). The high resolution was obtained at 30 ^{o}C and 30 kV with baseline drift. However, increasing the temperature up to 45 ^{o}C and decreasing the voltage to 25 kV helped stabilizing the baseline. Therefore, the optimum temperature and applied voltage were maintained at 45 ^{o}C and 25 kV (Fig. 35).

Table 13 Effects of temperature and applied voltage on the separation and dispersion of baclofen and its impurity

Conditions	R_s (baclofen)	α (baclofen)	TF (*R*-baclofen)	w (*R*-baclofen) (min)	N (*R*-baclofen)
20 ^{o}C;25 kV	2.6	1.04	1.87	0.17	64176
30 ^{o}C;30 kV	2.3	1.04	1.19	0.13	42792
45 ^{o}C;25 kV	2.2	1.04	1.01	0.13	44876.5

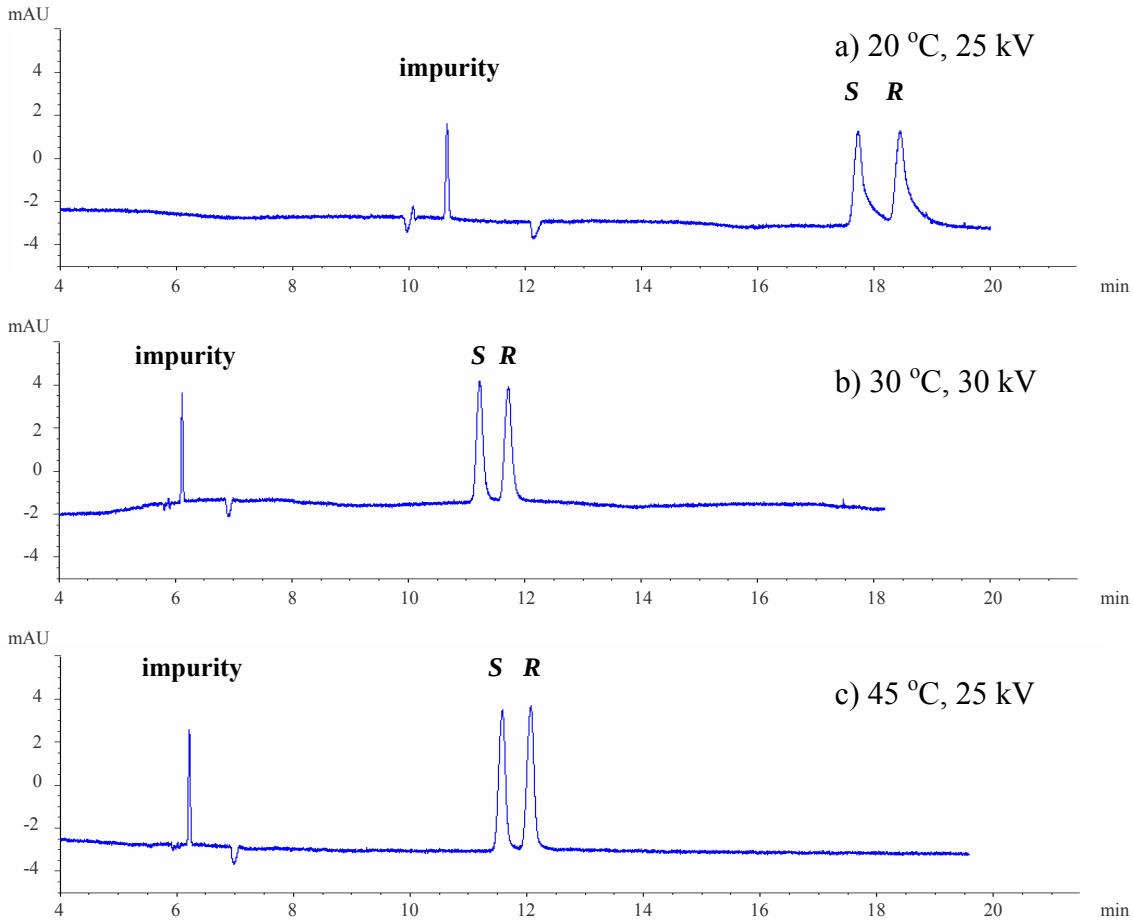


Fig. 35 Electropherogram of racemic mixture of (*R/S*)-baclofen (49 µg/ml) and its impurity (4.9 µg/ml). Conditions: 100 mM sodium borate buffer (pH 9.9) containing 18 mM α-CD and 5% v/v ACN; fused-silica capillary dynamic coated with PEO, 64.5 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 10 s; temperature and applied voltage, a) 20 °C, 25 kV, b) 30 °C, 30 kV, and c) 45 °C, 25 kV; detection by UV absorbance at 220 nm.

1.3.2 Effects of injection time on the separation and dispersion

In CE, sample plug length has a great influence on peak dispersion and separation efficiency. In the current work, the injections were studied in a range of 3 to 10 s using pressure at 50 mbar. Increasing the injection time, decreased the resolution of baclofen enantiomers, while the width of baclofen peaks increased (Fig. 37). Although the injection time of 3 s provided the highest resolution and smallest width, the signal intensity was low. Thus, the injection time of 6 s was selected as a compromise (Fig. 36).

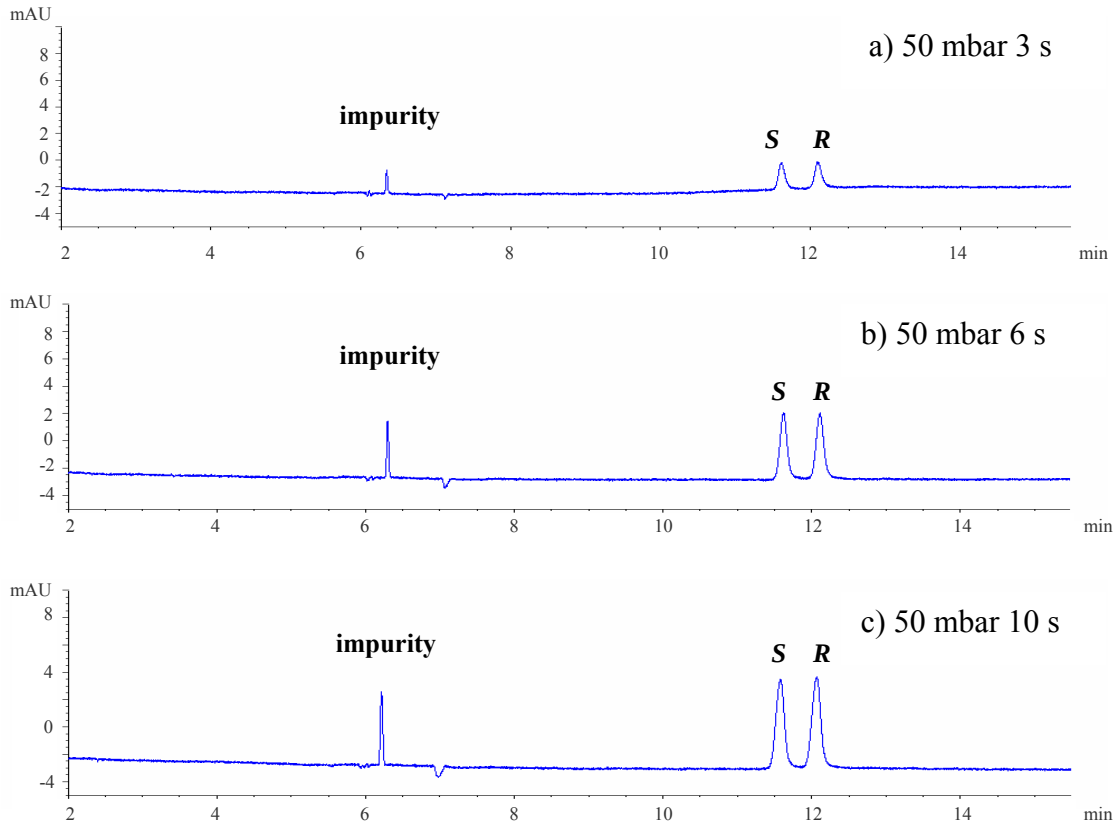


Fig. 36 Electropherogram of racemic mixture of (*R/S*)-baclofen (49 µg/ml) and its impurity (4.9 µg/ml). Conditions: 100 mM sodium borate buffer (pH 9.9) containing 18 mM α-CD and 5% v/v ACN; fused-silica capillary dynamic coated with PEO, 64.5 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for a) 3 s, b) 6 s, and c) 10 s; temperature and applied voltage, 45 ^{o}C, 25 kV; detection by UV absorbance at 220 nm.

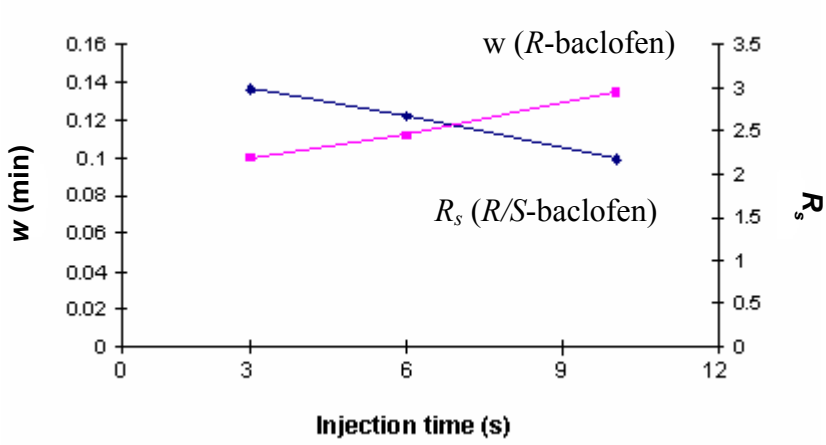


Fig. 37 Effects of injection time on the separation and dispersion of racemic mixture of (*R/S*)-baclofen and its impurity.

1.3.3 Effects of amounts of acetronitrile on the separation and dispersion

The influence of ACN in buffer was studied in a range of 0-5% v/v ACN. Addition of ACN in to the buffer generally increases the buffer viscosity and migration times of analytes. Consequently, the separating voltage was increased to 27 kV. Fig. 38 shows the influences of amounts of ACN in the buffer on the resolution and peak width of *R*-enantiomer of baclofen. The resolution of baclofen enantiomers were greater than 2.0 when the amounts of ACN was increased from 1-5% v/v, while the dispersion of peak decreased when decreasing the amounts of ACN in buffer. Therefore, the amount of ACN at 1% v/v was chosen as the optimized condition (Fig. 39).

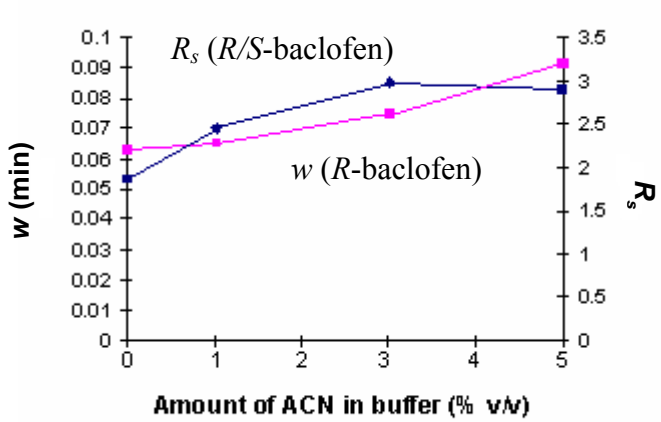


Fig. 38 Effects of amounts of ACN in BGE on the separation and dispersion of racemic mixture of (*R/S*)-baclofen and its impurity.

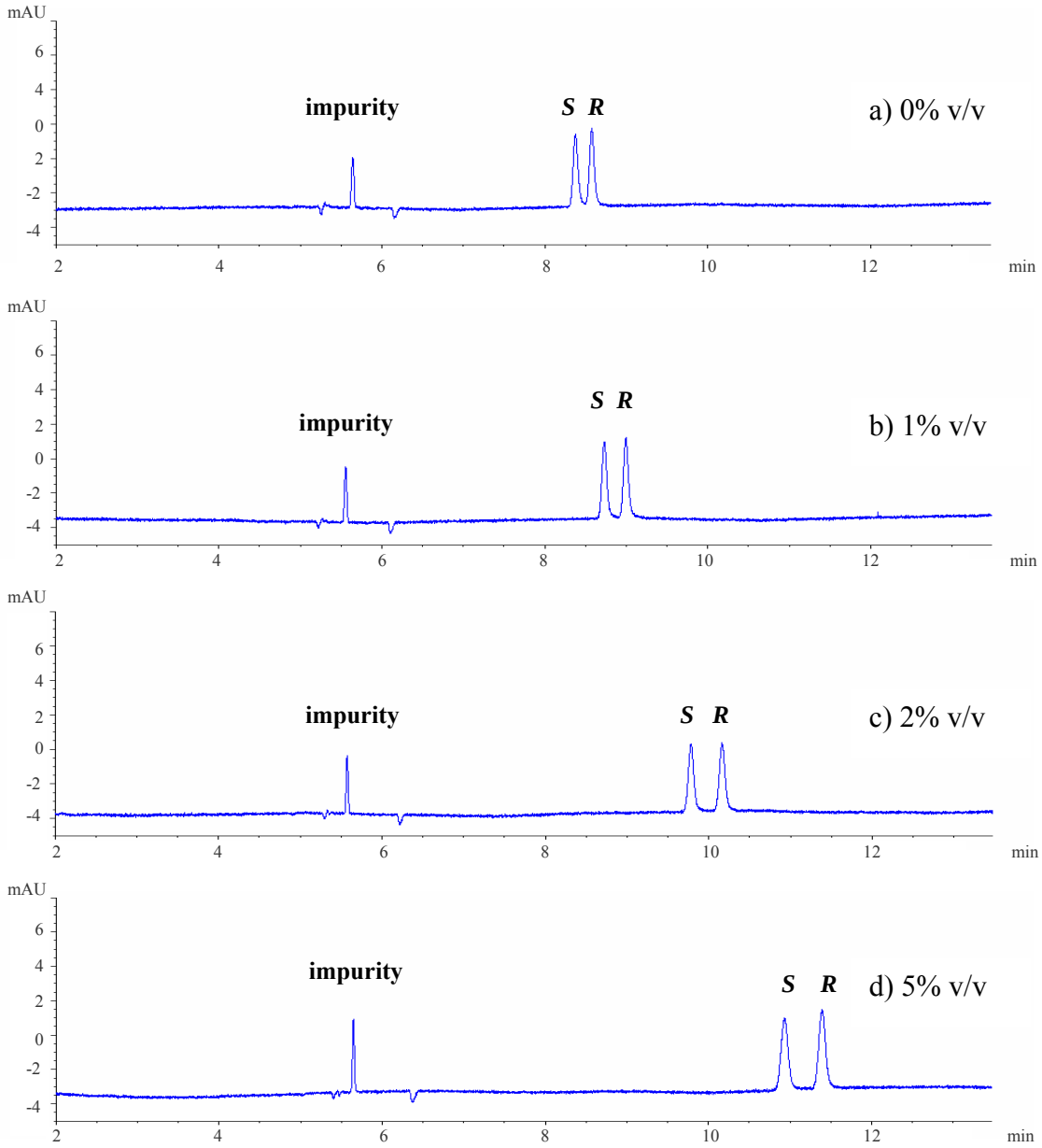


Fig. 39 Electropherogram of racemic mixture of (*R/S*)-baclofen (49 µg/ml) and its impurity (4.9 µg/ml). Conditions: 100 mM sodium borate buffer (pH 9.9) containing 18 mM α-CD and a) 0%, b) 1%, c) 3%, d) 5% v/v ACN; fused-silica capillary dynamic coated with PEO, 64.5 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 6 s; temperature, 45 ^{o}C; voltage, 27 kV; detection by UV absorbance at 220 nm.

The influence of ACN in sample was studied in a range of 0-4% v/v ACN. Fig. 40 shows that the amounts of ACN in sample significantly impacted the peak width of (4*RS*)-4-(4-chlorophenyl) pyrrolidin-2-one and the resolution of baclofen enantiomers. Decreasing the amounts of ACN in sample, decreased the width of (4*RS*)-4-(4-chlorophenyl) pyrrolidin-2-one peak, while the resolution of baclofen enantiomers increased. The best condition was obtained when ACN was absence (0 % v/v) for the sample solution (Fig. 41).

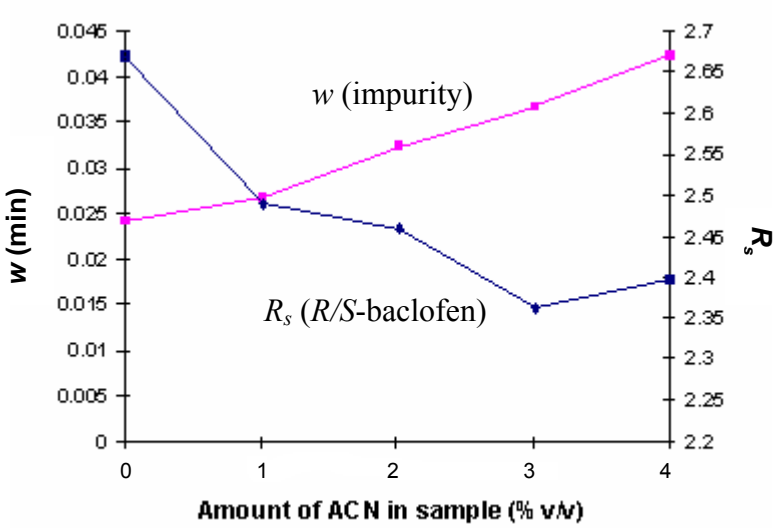


Fig. 40 Effects of amounts of ACN in sample on the separation and dispersion of racemic mixture of (*R/S*)-baclofen and its impurity.

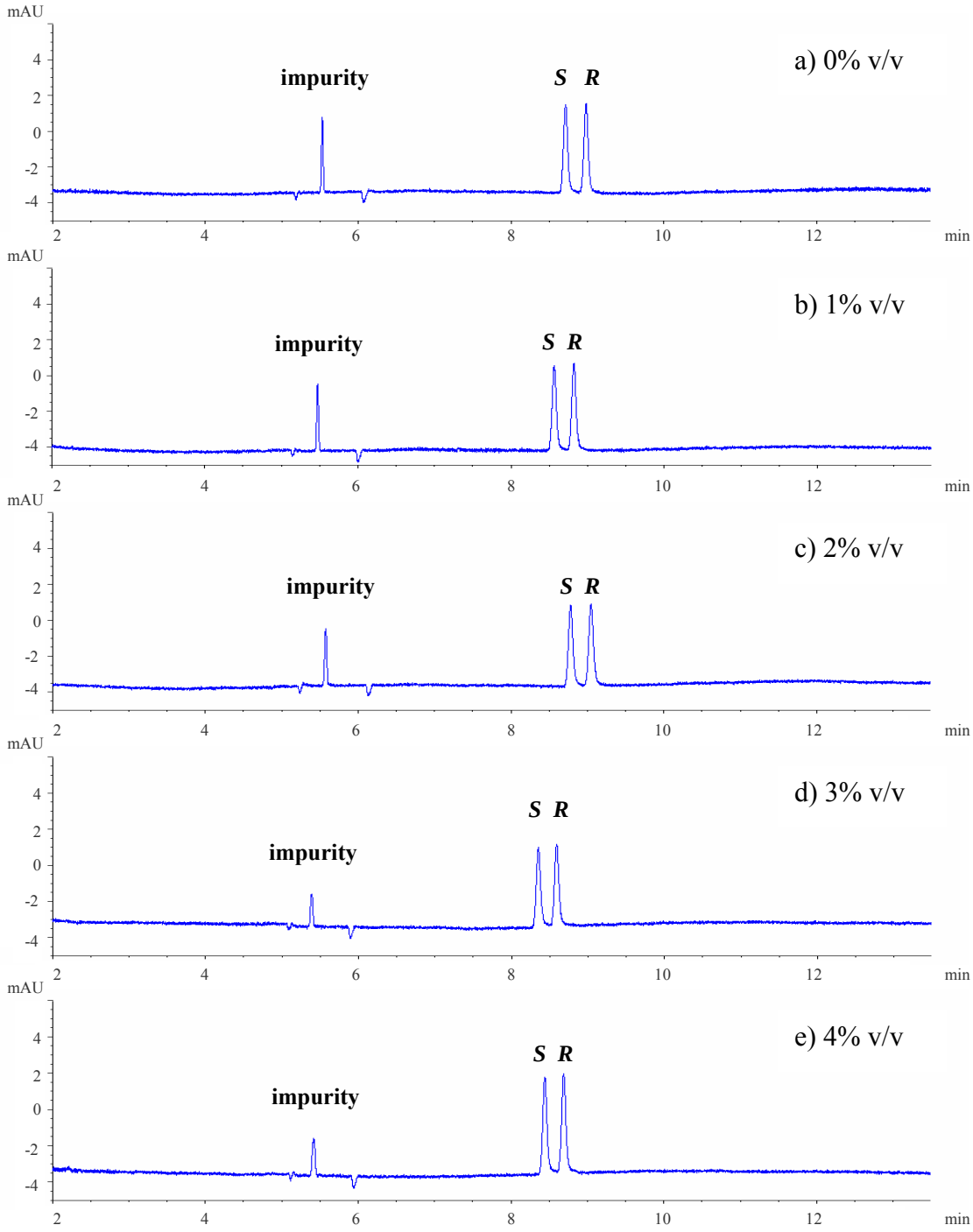


Fig. 41 Electropherogram of racemic mixture of (*R/S*)-baclofen (50 µg/ml) and its impurity (5 µg/ml). Conditions: racemic mixture of (*R/S*)-baclofen and impurity containing a) 0%, b) 1%, c) 2%, d) 3%, e) 4%v/v ACN; 100 mM sodium borate buffer (pH 9.9) containing 18 mM α-CD and 1% v/v ACN; fused-silica capillary dynamic coated with PEO, 64.5 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 6 s; temperature, 45 ^{o}C; voltage, 27 kV; detection by UV absorbance at 220 nm.

Finally, the optimum CE condition for the separation of baclofen enantiomer and its impurity was found 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, fused-silica capillary dynamic coated with PEO; 64.5 cm (effective length; 56 cm) x 50 µm I.D., injection of 50 mbar x 6 s and a detection wavelength at 220 nm (Fig. 42). The analytical parameters for each analyte were presented in Table 14.

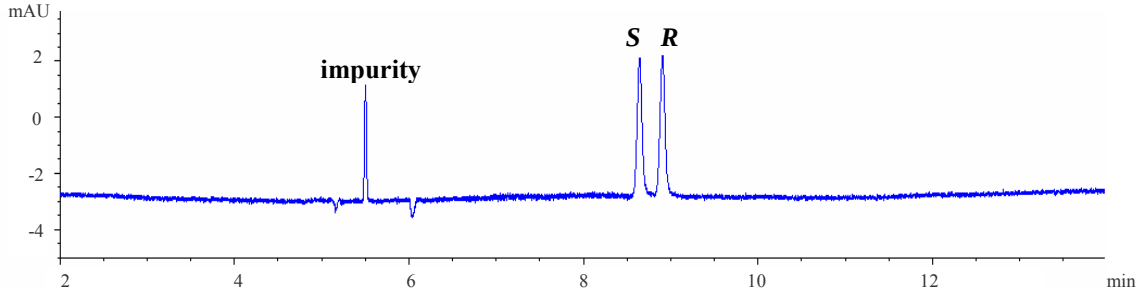


Fig. 42 Electropherogram of racemic mixture of baclofen (50 µg/ml) and its impurity (5 µg/ml). Conditions: 100 mM sodium borate buffer (pH 9.9) containing 18 mM α-CD and 1% v/v ACN; fused-silica capillary dynamic coated with PEO, 64.5 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 6 s; temperature and applied voltage, 45 ^{o}C, 27 kV; detection by UV absorbance at 220 nm.

Table 14 Migration times, resolution, selectivity, tailing factor, peak width, and theoretical plate number of the *R/S*-baclofen and its impurity

Analyte	t_m (min)	R_s	α	*TF*	*w* (min)	*N*
impurity	5.53	-	-	1.02	0.02	292744
S-(+)-baclofen	8.71	44.8	1.6	1.13	0.06	119533
R-(-)-baclofen	8.98	2.7	1.03	1.07	0.06	124695

Fig. 43 shows separate electropherogram of *S*-(+)- and *R*-(-)-baclofen, impurity ((4*RS*)-4-(4-chlorophenyl)pyrrolidin-2-one). It is evident that impurity migrated as the first peak since could not accommodate into the α-CD cavity. Additionally, the impurity was rather neutral and migrated solely by the EOF. Baclofen enantiomers

could accommodate into α-CD cavity to from inclusion complexes. However, *R*-(-)-baclofen migrated slightly later than the *S*-(+)-baclofen. The *R*-(-)-enantiomer might form a stronger inclusion complex with α-CD than *S*-(+)-inclusion complex.

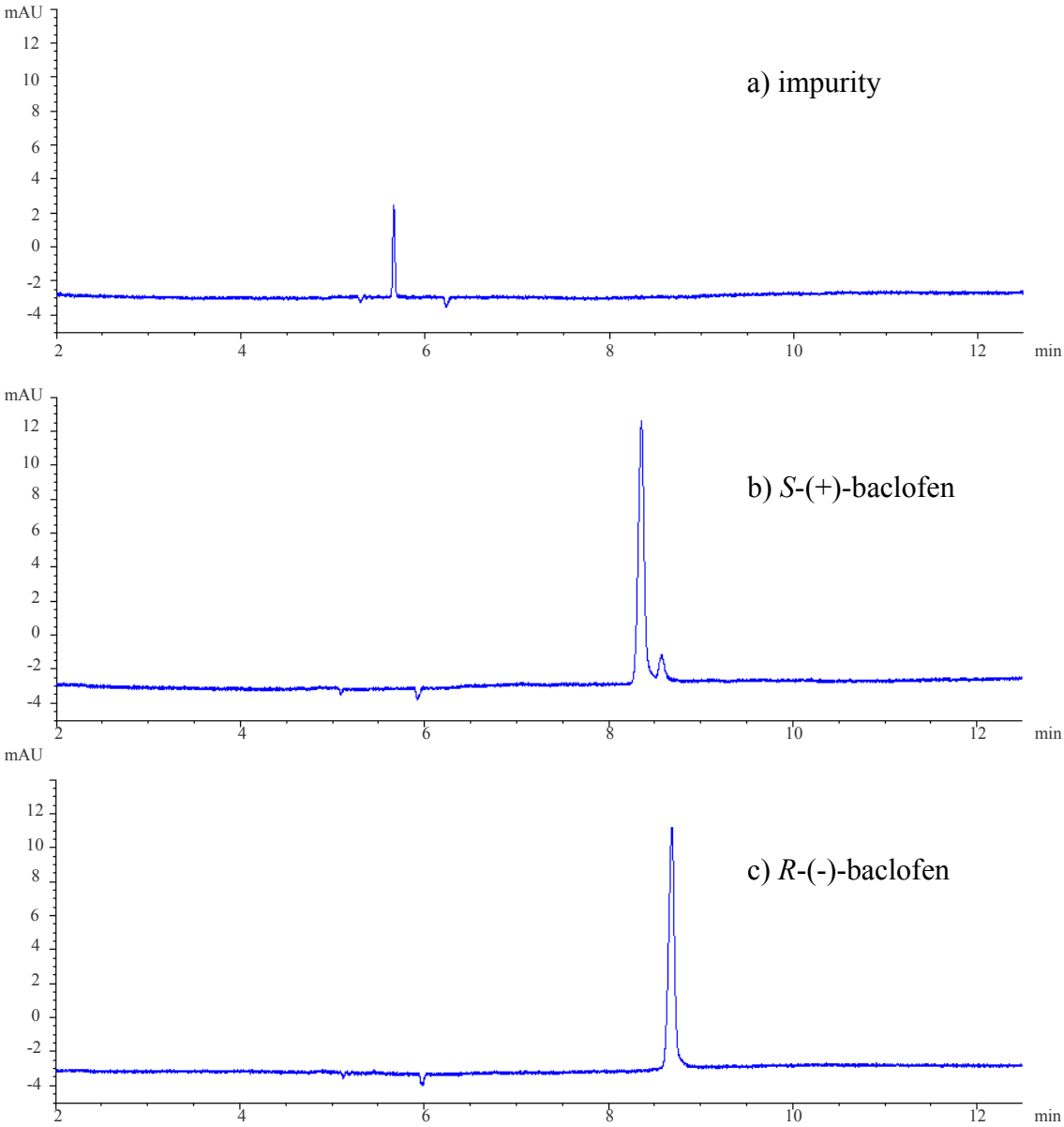


Fig. 43 Electropherogram of a) impurity (10 µg/ml), b) *S*-(+)-baclofen (100 µg/ml), and c) *R*-(-)-baclofen (100 µg/ml), obtained under optimized conditions.

2 Method validation

The optimized condition for separation of baclofen enantiomers and its impurity were validated by assessing the linearity, precision, recovery, limit of detection (LOD), and limit of quantitation (LOQ).

2.1 Linearity

Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. Range is the interval between the upper and lower levels of analyte that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. The linearity of method was performed by triplicate injection of standard mixture of baclofen enantiomers and impurity on three different days at five different concentrations in a range of 50-500 µg/ml for baclofen enantiomers and 5-50 µg/ml for impurity. The least-square linear regressions of the analytes were obtained by plotting peak area, peak height, and normalized peak area versus concentrations. Calibration curve parameters and statistics for the baclofen enantiomer and its impurity are in Tables 15-17 and all the raw data are shown in appendix B.

For the linearity of baclofen enantiomers and its impurity, the results show that good correlation existed between the peak area and concentration, and was selected for establishing the calibration curve. In summary, the calibration curves are found to be linear with the equation $y = 0.6061X + 5.3594$ ($r^2 = 0.9997$) and $y = 0.6981x + 5.688$ ($r^2 = 0.9997$) for *S*-(+)-baclofen and *R*-(-)-baclofen, respectively. For the impurity, the calibration curve of impurity was found to be linear with the equation $y = 0.6327X – 0.4461$ ($r^2 = 0.9994$).

Obviously, the correlation coefficient (r^2) of all analyte were within the acceptance criteria (r^2 should be 0.999 or greater).[90] Thus, these calibration curves could be used to analysis for enantioseparation of baclofen and its impurity.

Table 15 Calibration curve parameters and statistics of *S*-(+)-baclofen (*qn* = 3)

Curve	Slope	y-intercept	Correlation coefficient (r^2)
Peak area			
Day 1	0.6056	5.2594	0.9995
Day 2	0.6090	5.5924	0.9998
Day 3	0.6038	5.2262	0.9998
Average	0.6061	5.3593	0.9997
%RSD	0.44	3.78	0.02
Peak height			
Day 1	0.0991	5.1664	0.9645
Day 2	0.1024	4.5582	0.9939
Day 3	0.0917	4.9308	0.9943
Average	0.0977	4.8851	0.9842
%RSD	5.61	6.28	1.74
Normalized peak area			
Day 1	0.0739	0.3108	0.9981
Day 2	0.0735	0.638	0.9995
Day 3	0.0705	0.6817	0.9993
Average	0.0726	0.5435	0.9990
%RSD	2.56	37.30	0.08

Table 16 Calibration curve parameters and statistics of *R*-(-)-baclofen (n = 3)

Curve	Slope	y-intercept	Correlation coefficient (r^2)
Peak area			
Day 1	0.6917	5.819	0.9996
Day 2	0.7051	5.5361	0.9999
Day 3	0.6976	5.7088	0.9997
Average	0.6981	5.6880	0.9997
%RSD	0.96	2.51	0.02
Peak height			
Day 1	0.1216	5.2909	0.968
Day 2	0.1216	5.0456	0.9929
Day 3	0.1126	5.1621	0.9964
Average	0.1186	5.1662	0.9858
%RSD	4.38	2.38	1.57
Normalized peak area			
Day 1	0.0819	0.3246	0.9986
Day 2	0.0825	0.6261	0.9995
Day3	0.0789	0.7363	0.9992
Average	0.0811	0.5623	0.9991
%RSD	2.38	37.90	0.05

Table 17 Calibration curve parameters and statistics of impurity (n = 3)

Curve	Slope	y-intercept	Correlation coefficient (r^2)
Peak area			
Day1	0.6277	0.4444	0.9992
Day 2	0.6388	0.4271	0.9996
Day3	0.6315	0.4668	0.9994
Average	0.6327	0.4461	0.9994
%RSD	0.89	4.46	0.02
Peak height			
Day 1	0.4193	0.5695	0.9948
Day 2	0.4130	0.4682	0.9969
Day3	0.3908	0.0725	0.9981
Average	0.4077	0.3701	0.9966
%RSD	3.67	70.97	0.17
Normalized peak area			
Day 1	0.1192	0.1168	0.9988
Day 2	0.1206	0.0916	0.9994
Day 3	0.1148	0.0501	0.9998
Average	0.1182	0.0862	0.9993
%RSD	2.56	39.09	0.05

2.2 Recovery

The accuracy of the CCE method was evaluated using the percents of recoveries of baclofen and its impurity in pharmaceutical formulations. In the method of standard addition, known amounts of an analyte are spiked at the different levels into a sample matrix that already contain some (unknown) quantity of analyte. In this study, standard of *S*-(+) and *R*-(-)-baclofen in the range of 80-120% of assay concentrations were spiked into pharmaceutical formulation containing 400 µg/ml of baclofen. Each concentration was injected in three replicates under the optimized CE

condition. The recovery of baclofen and its impurity in tablet were shown in Tables 18-20. In tablet, the mean recoveries were 99.8-103.1, 99.6-101.5, and 96-96.4% for *S*-(+)-baclofen, *R*-(-)-baclofen and impurity, respectively.

Table 18 Recoveries of *S*-(+)-baclofen in pharmaceutical formulation ($n = 3$)

% added	Amount Added (µg/ml)	Total amount (µg/ml)	Amount found (µg/ml)	%Recovery
0	0	219.03	-	-
80	166.4	390.64	171.61	103.1
100	208	430.76	211.74	101.8
120	249.6	468.15	249.12	99.8
			Average	101.6
			SD	1.67
			%RSD	1.65

Table 19 Recoveries of *R*-(-)-baclofen in pharmaceutical formulation ($n = 3$)

% added	Amount Added (µg/ml)	Total amount (µg/ml)	Amount found (µg/ml)	%Recovery
0	0	210.05	-	-
80	176	385.39	175.34	99.6
100	208	417.35	207.30	99.7
120	249.6	463.37	253.32	101.5
			Average	100.3
			SD	1.07
			%RSD	1.06

Table 20 Recoveries of impurity in pharmaceutical formulation (n = 3)

% added	Amount Added (μg/ml)	Total amount (μg/ml)	Amount found (μg/ml)	%Recovery
80	26.24	26.24	25.19	96.0
100	32.80	32.80	31.50	96.0
120	39.36	39.36	37.95	96.4
			Average	96.1
			SD	0.24
			%RSD	0.25

2.3 Precision

Method reproducibility was determined by measuring repeatability of injection and intermediate precision (intra-day and inter-day precision) of the migration time, peak height, and normalized peak.

2.3.1 Injection precision

Injection precision was firstly investigated at the middle point of the calibration curve by nine replicated injection (n = 9). The %RSDs of peak area, peak height, and normalized peak area of baclofen enantiomers and its impurity are shown in Tables 21-23. %RSD calculated from peak area of the injection precision for *S*-(+)-, *R*-(-)-baclofen and the impurity were 1.43, 1.64 and 1.98, respectively. Obviously, %RSDs calculated from peak area were within the acceptance criteria (%RSD should be less than 2.0).

Table 21 Injection precision of *S*-(+)-baclofen at 200 µg/ml (n = 9)

Injection no.	*S*-(+)-baclofen			
	t_m (min)	Peak area (mAU*s)	Peak height (mAU)	Nor.peak area
1	8.68	131.07	19.49	15.11
2	8.53	131.23	19.50	15.38
3	8.68	130.30	19.09	15.00
4	8.35	131.33	21.81	15.73
5	8.65	127.92	19.77	14.78
6	8.20	128.10	21.00	15.61
7	8.26	127.98	20.47	15.49
8	8.11	128.83	21.00	15.89
9	8.11	126.09	20.68	15.55
Average	8.40	129.20	20.31	15.40
SD	0.24	1.85	0.90	0.36
%RSD	2.89	1.43	4.42	2.36

Table 22 Injection precision of *R*-(-)-baclofen at 200 µg/ml (n = 9)

Injection no.	*R*-(-)-baclofen			
	t_m (min)	Peak area (mAU*s)	Peak height (mAU)	Nor.peak area
1	8.92	146.99	23.26	16.47
2	8.78	149.71	23.42	17.05
3	8.93	147.00	23.20	16.46
4	8.58	147.75	26.42	17.22
5	8.90	147.16	23.95	16.53
6	8.43	143.40	25.39	17.01
7	8.49	147.25	24.95	17.34
8	8.32	145.10	25.55	17.43
9	8.32	141.86	25.09	17.04
Average	8.63	146.25	24.58	16.95
SD	0.26	2.40	1.16	0.38
%RSD	2.97	1.64	4.72	2.21

Table 23 Injection precision of impurity at 30 µg/ml (n = 9)

Injection no.	Impurity			
	t_m (min)	Peak area (mAU*s)	Peak height (mAU)	Nor.peak area
1	5.39	19.72	12.68	3.66
2	5.38	19.26	12.40	3.58
3	5.38	19.15	12.30	3.56
4	5.37	19.49	12.28	3.63
5	5.40	19.54	12.52	3.62
6	5.36	19.48	12.55	3.63
7	5.35	19.19	12.41	3.59
8	5.34	19.04	12.26	3.56
9	5.33	20.31	13.31	3.81
Average	5.37	19.46	12.52	3.63
SD	0.02	0.38	0.33	0.08
%RSD	0.41	1.98	2.60	2.12

2.3.2 Intra-day precision

Intra-day precision was examined at three different concentrations of 50, 200, and 500 µg/ml for baclofen enantiomers and 10, 30, and 50 µg/ml for the impurity on the same day. The %RSDs of migration time, peak area, peak height, and normalized peak area of baclofen enantiomers and its impurity were determined and the results are shown in Tables 24-26. %RSD calculated from peak area of the intra-day precision for *S*-(+)-, *R*-(-)-baclofen and the impurity were in range of 0.17-1.26, 0.27-0.67 and 0.24-1.22, respectively.

Table 24 Intra-day precision of *S*-(+)-baclofen (n = 3)

Injection no.	Conc. (µg/ml)	t_m (min)	Peak area (mAU*s)	Peak height (mAU)	Nor.peak area
1	50	8.31	35.58	7.72	4.28
2		8.31	36.49	7.66	4.39
3		8.34	36.11	7.68	4.33
Average		8.32	36.06	7.69	4.33
SD		0.01	0.45	0.03	0.05
%RSD		0.16	1.26	0.39	1.24
1	200	8.59	121.68	27.84	14.16
2		8.57	122.76	27.54	14.32
3		8.54	122.58	27.70	14.36
Average		8.57	122.34	27.69	14.28
SD		0.03	0.58	0.15	0.10
%RSD		0.31	0.47	0.55	0.72
1	500	8.18	308.73	51.92	37.74
2		8.19	308.10	51.47	37.62
3		8.19	309.17	51.07	37.76
Average		8.19	308.67	51.49	37.71
SD		0.004	0.54	0.43	0.08
%RSD		0.56	0.17	0.83	0.2

Table 25 Intra-day precision of *R*-(-)-baclofen (n = 3)

Injection no.	Conc. (μg/ml)	t_m (min)	Peak area (mAU*s)	Peak height (mAU)	Nor.peak area
1	50	8.55	40.22	8.73	4.71
2		8.55	40.66	8.54	4.75
3		8.58	40.16	8.59	4.68
Average		8.56	40.35	8.62	4.71
SD		0.01	0.27	0.10	0.036
%RSD		0.17	0.67	1.14	0.77
1	200	8.86	139.32	32.83	15.72
2		8.84	141.01	32.93	15.95
3		8.81	140.18	32.80	15.92
Average		8.84	140.17	32.85	15.86
SD		0.03	0.85	0.07	0.12
%RSD		0.33	0.60	0.21	0.78
1	500	8.43	350.60	63.02	41.59
2		8.44	352.43	62.37	41.78
3		8.43	351.21	61.66	41.65
Average		8.43	351.41	62.35	41.67
SD		0.003	0.93	0.68	0.09
%RSD		0.04	0.27	1.09	0.22

Table 26 Intra-day precision of impurity (n = 3)

Injection no.	Conc. (μg/ml)	t_m (min)	Peak area (mAU*s)	Peak height (mAU)	Nor.peak area
1	10	5.21	5.90	3.74	1.13
2		5.20	5.91	3.74	1.14
3		5.21	6.03	3.84	1.16
Average		5.21	5.94	3.77	1.14
SD		0.004	0.07	0.06	0.01
%RSD		0.09	1.22	1.55	0.01
1	30	5.43	18.86	11.19	3.47
2		5.43	19.00	11.33	3.50
3		5.43	18.92	11.13	3.49
Average		5.43	18.92	11.22	3.49
SD		0.003	0.07	0.10	0.01
%RSD		0.05	0.37	0.92	0.34
1	50	5.29	31.64	20.26	5.98
2		5.28	31.75	20.39	6.01
3		5.26	31.78	20.34	6.04
Average		5.28	31.72	20.33	6.01
SD		0.02	0.08	0.06	0.03
%RSD		0.30	0.24	0.30	0.52

2.3.3 Inter-day precision

Inter-day precision was examined at three different concentrations of 50, 200, and 500 μg/ml for baclofen enantiomers and 10, 30, and 50 μg/ml for the impurity on six different days. The %RSDs of migration time, peak area, peak height, and normalized peak area of baclofen enantiomers and its impurity were determined and the results are shown in Tables 27-29. %RSD calculated from peak area of the inter-day precision for *S*-(+)-, *R*-(-)-baclofen and the impurity were in range of 1.24-2.75, 0.88-3.82 and 1.30-4.56, respectively.

Table 27 Inter-day precision of *S*-(+)-baclofen ($n = 3$)

Day	Conc. (µg/ml)	t_m (min)	Peak area (mAU*s)	Peak height (mAU)	Nor.peak area
1	50	8.32	36.06	7.69	4.33
2		8.44	35.10	9.00	4.16
3		8.34	35.60	8.35	4.27
4		8.58	36.95	9.06	4.31
5		8.51	34.46	6.64	4.05
6		8.26	34.33	6.58	4.16
Average		8.41	35.43	7.88	4.21
SD		0.12	0.97	1.11	0.11
%RSD		1.47	2.75	14.09	2.56
1	200	8.57	122.34	27.69	14.28
2		8.30	125.27	23.24	15.09
3		8.49	124.59	22.07	14.67
4		8.37	128.00	20.42	15.30
5		8.32	125.74	22.79	15.11
6		8.64	127.45	22.64	14.76
Average		8.45	125.56	23.14	14.87
SD		0.14	2.05	2.44	0.37
%RSD		1.62	1.63	10.53	2.50
1	500	8.19	308.67	51.49	37.71
2		8.33	310.15	55.33	37.22
3		8.54	308.52	51.18	36.12
4		8.33	314.41	44.03	37.76
5		8.14	302.94	50.19	37.21
6		8.44	311.69	49.98	36.95
Average		8.33	309.40	50.37	37.16
SD		0.15	3.85	3.66	0.60
%RSD		1.79	1.24	7.27	1.61

Table 28 Inter-day precision of *R*-(-)-baclofen (n = 3)

Day	Conc. (µg/ml)	t_m (min)	Peak area (mAU*s)	Peak height (mAU)	Nor.peak area
1	50	8.56	40.35	8.62	4.71
2		8.70	39.94	10.10	4.59
3		8.59	40.58	9.43	4.72
4		8.84	41.80	10.17	4.73
5		8.77	38.57	7.53	4.40
6		8.50	37.14	7.17	4.37
Average		8.66	39.79	8.84	4.60
SD		0.13	1.52	1.29	0.15
%RSD		1.53	3.82	14.56	3.35
1	200	8.84	140.17	32.85	15.86
2		8.55	144.82	27.63	16.94
3		8.75	143.61	26.71	16.42
4		8.61	145.94	24.76	16.96
5		8.57	143.70	28.09	16.77
6		8.89	141.75	26.73	15.94
Average		8.70	143.33	27.80	16.48
SD		0.15	2.08	2.73	0.49
%RSD		1.68	1.45	9.82	2.98
1	500	8.43	351.41	62.35	41.67
2		8.60	357.77	64.79	41.63
3		8.82	356.19	61.65	40.39
4		8.57	353.59	56.23	41.28
5		8.39	351.23	62.20	41.89
6		8.70	349.86	62.25	40.22
Average		8.58	353.34	61.58	41.18
SD		0.16	3.10	2.84	0.71
%RSD		1.88	0.88	4.61	1.71

Table 29 Inter-day precision of impurity (n = 3)

Day	Conc. (µg/ml)	t_m (min)	Peak area (mAU*s)	Peak height (mAU)	Nor.peak area
1	10	5.21	5.94	3.77	1.14
2		5.36	6.59	4.19	1.23
3		5.34	5.87	3.79	1.10
4		5.37	5.92	3.79	1.10
5		5.45	6.30	4.09	1.16
6		5.28	6.07	3.99	1.15
Average		5.33	6.11	3.94	1.15
SD		0.08	0.28	0.18	0.05
%RSD		1.55	4.56	4.55	4.14
1	30	5.43	18.92	11.22	3.49
2		5.32	19.89	12.90	3.74
3		5.34	18.09	11.89	3.38
4		5.32	18.18	11.78	3.42
5		5.36	19.13	12.33	3.57
6		5.38	19.97	12.86	3.71
Average		5.36	19.03	12.16	3.55
SD		0.04	0.81	0.66	0.15
%RSD		0.77	4.24	5.42	4.23
1	50	5.28	31.72	20.33	6.01
2		5.40	32.27	21.84	6.0
3		5.422	31.04	19.71	5.72
4		5.28	31.39	21.14	5.94
5		5.3	31.48	21.09	5.94
6		5.31	31.69	21.02	5.96
Average		5.33	31.60	20.85	5.93
SD		0.06	0.41	0.74	0.10
%RSD		1.15	1.30	3.53	1.71

2.4 Limit of detection (LOD) and Limit of quantitation (LOQ)

LODs and LOQs of each enantiomer of baclofen and its impurity were determined at signal to noise ratios of 3 and 10, respectively, based on peak height. LOD and LOQ of *S*-(+)-baclofen were 10.0 µg/ml and 30.0 µg/ml, respectively (Fig. 44-45) and *R*-(-)-baclofen were 7.0 µg/ml and 30.0 µg/ml, respectively (Figs. 46-47). For the impurity, LOD and LOQ were 2.0 µg/ml and 5.0 µg/ml, respectively (Figs. 48-49). The precision of LOQ of baclofen enantiomers and impurity are indicated in Table 30-32. %RSD of LOQ for *S*-(+)-, *R*-(-)-baclofen and impurity were 6.70, 2.84 and 1.85, respectively. The LOD and LOQ values of this method show that the method is sensitive for the determination of baclofen enantiomers and its impurity in raw materials and formulations.

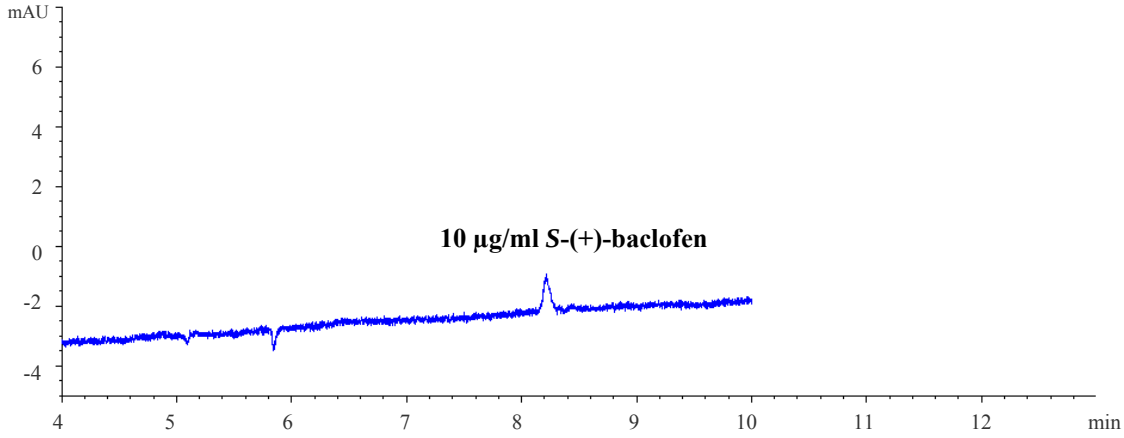


Fig. 44 Limit of detection of *S*-(+)-baclofen, obtained under optimized conditions.

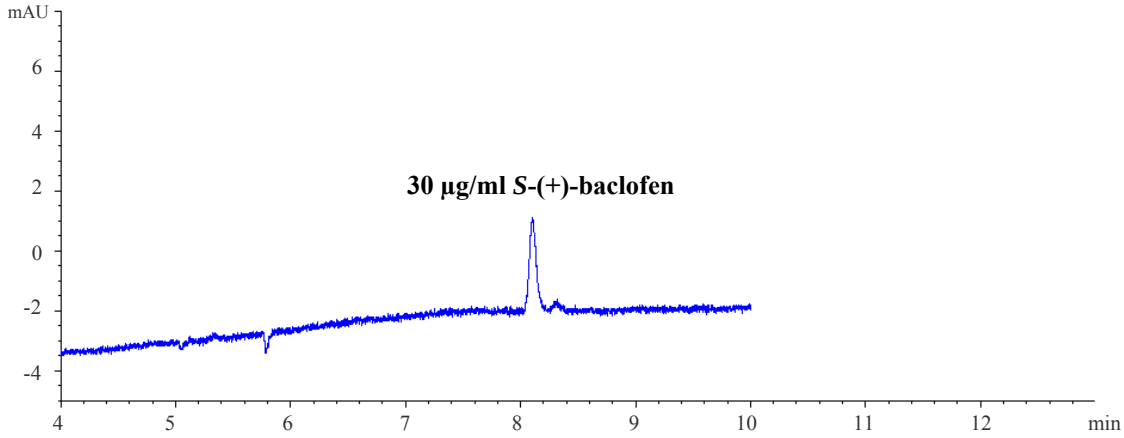


Fig. 45 Limit of quantitation of *S*-(+)-baclofen, obtained under optimized conditions.

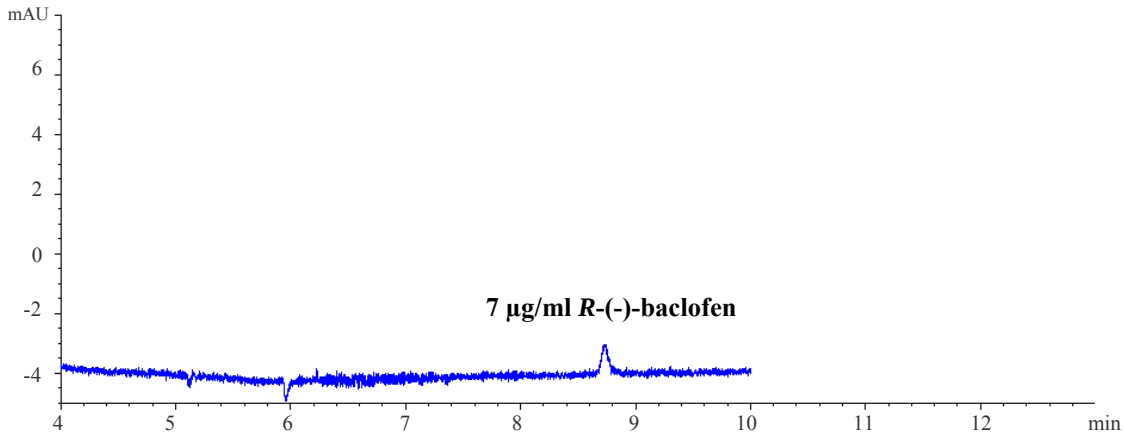


Fig. 46 Limit of detection of *R*-(-)-baclofen, obtained under optimized conditions.

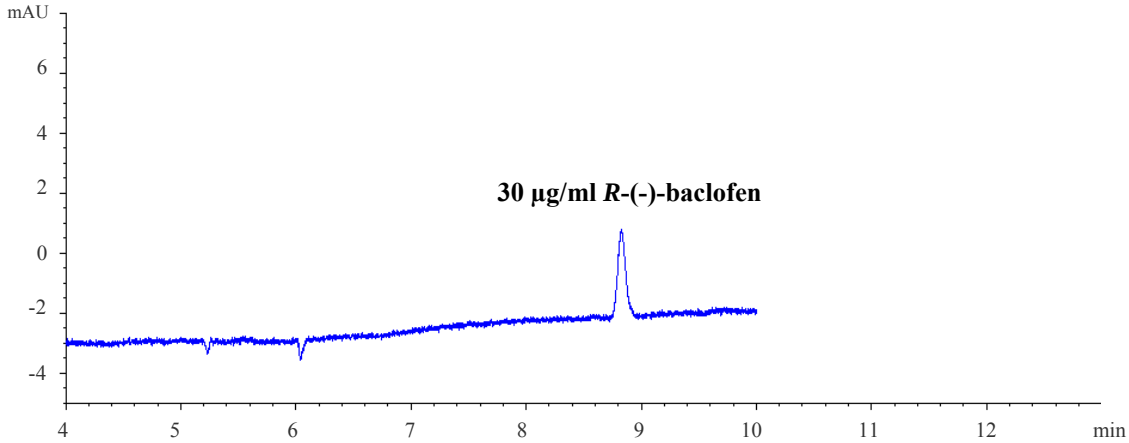


Fig. 47 Limit of quantitation of *R*-(-)-baclofen, obtained under optimized conditions.

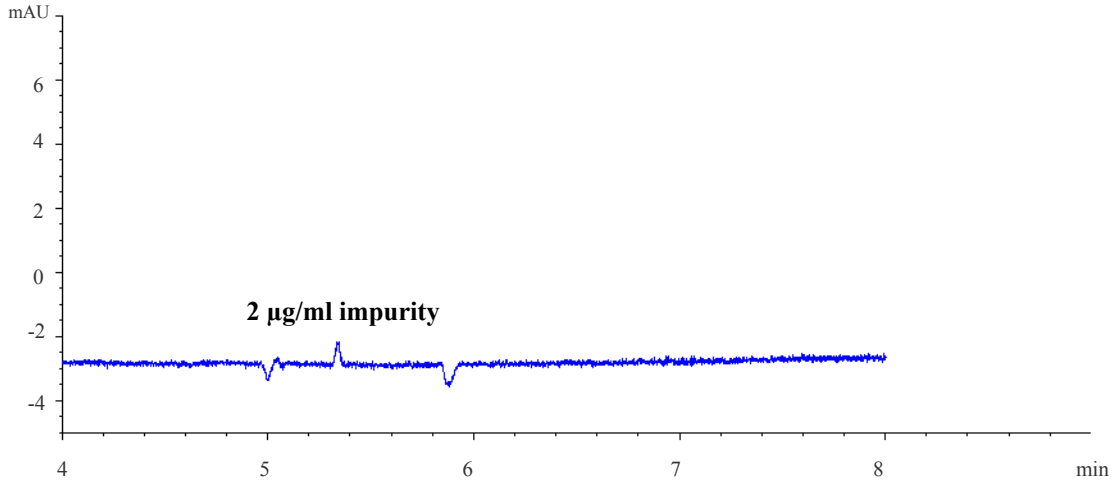


Fig. 48 Limit of detection of impurity, obtained under optimized conditions.

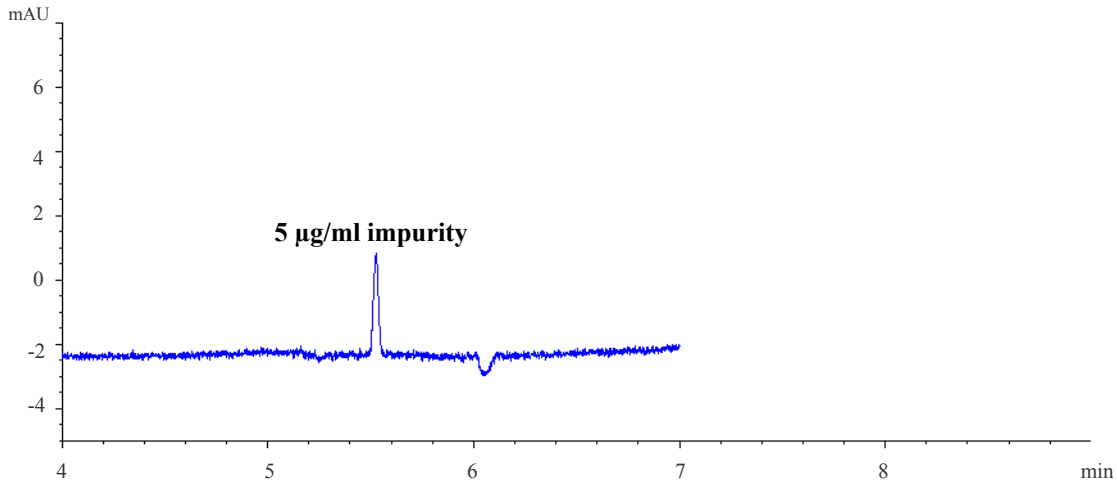


Fig. 49 Limit of quantitation of impurty, obtained under optimized conditions.

Table 30 Precision of limit of quantitation of *S*-(+)-baclofen at 30 µg/ml (n = 3)

Injection no.	t_m (min)	Peak height (mAU)
1	8.10	3.18
2	8.28	2.83
3	8.17	3.20
Average	8.18	3.07
SD	0.09	0.21
%RSD	1.11	6.70

Table 31 Precision of limit of quantitation of *R*-(-)-baclofen at 30 µg/ml (n = 3)

Injection no.	t_m (min)	Peak height (mAU)
1	8.82	2.98
2	8.86	3.07
3	8.61	3.15
Average	8.76	3.07
SD	0.14	0.09
%RSD	1.56	2.84

Table 32 Precision of limit of quantitation of impurity at 5 μg/ml (n = 3)

Injection no.	t_m (min)	Peak height (mAU)
1	5.50	3.08
2	5.51	3.03
3	5.52	3.15
Average	5.51	3.09
SD	0.01	0.06
%RSD	0.19	1.85

3 Determination of baclofen enantiomers and its impurity in raw materials and pharmaceutical formulations

The developed method was used to quantify baclofen enantiomers and its impurity in two different brands of baclofen raw materials and three different brands of baclofen tablets.

Fig. 50 shows electropherograms of baclofen enantiomers and its impurity in raw material. Fig. 50 b) revealed that the impurity could not be detected in the analyzed raw material. The %label amounts of total baclofen were 101.1 (%RSD = 0.89) and 99.3 (%RSD = 1.03) for raw material brand A and B, respectively. For the determination of impurity, quantify of impurity could not be detected (lower than LOD) of both brands (Table 33). Fig.51 shows electropherograms of the determination of baclofen enantiomers and its impurity in pharmaceutical formulation. Fig. 51 b) showed that the impurity was found in some baclofen tablets. The %label amounts of total baclofen 109.2 (%RSD = 0.89), 108.0 (%RSD = 1.52) and 107.9 (%RSD = 0.84) for brand A, B and C, respectively. The ratio of *S:R* in raw materials of both brand A and B were 1.07:1. The ratio of *S:R* in pharmaceutical formulations of brand A, B, and C were 1.06:1, 1.07:1, and 1.03:1. For the determination of impurity, it could not be detected in brand A. For brand B and C, it were 0.12 (%RSD = 1.95), and 0.39 (%RSD = 1.76), respectively, which were within limit of USP 31NF26.

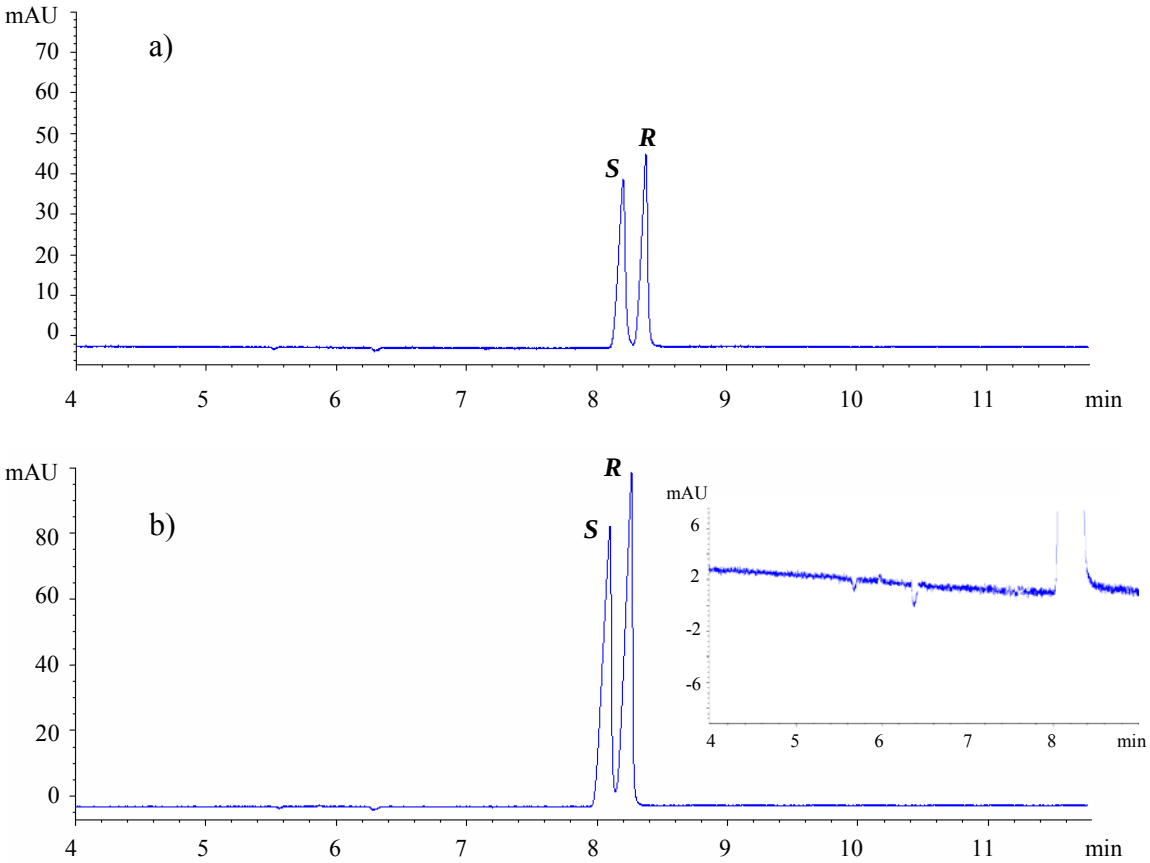


Fig. 50 Electropherograms of a) racemic mixture of (*R/S*)-baclofen (400 µg/ml), b) racemic mixture of (*R/S*)-baclofen (1,000 µg/ml) in raw material. Condition: 100 mM sodium borate buffer (pH 9.9) containing 18 mM α-CD and 1% v/v ACN; fused-silica capillary dynamic coated with PEO, 64.5 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 6 s; temperature and applied voltage, 45 ^{o}C, 27 kV; detection by UV absorbance at 220 nm.

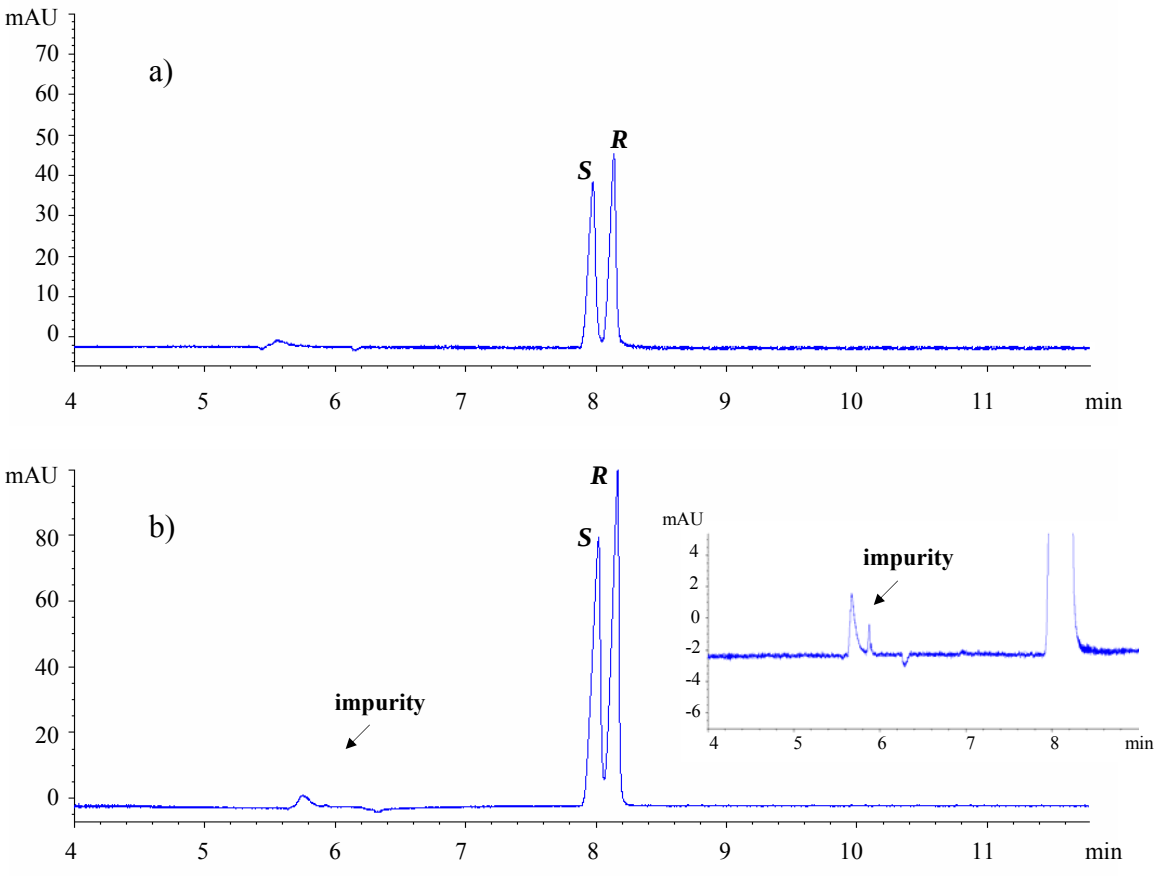


Fig.51 Electropherograms of a) racemic mixture of (*R/S*)-baclofen (400 µg/ml), b) racemic mixture of (*R/S*)-baclofen (1,000 µg/ml) in pharmaceutical formulation. Condition: 100 mM sodium borate buffer (pH 9.9) containing 18 mM α-CD and 1% v/v ACN; fused-silica capillary dynamic coated with PEO, 64.5 cm total length (8.5 cm to the detector), 50 µm ID; hydrodynamic injection at 50 mbar for 6 s; temperature and applied voltage, 45 ^{o}C, 27 kV; detection by UV absorbance at 220 nm.

Table 33 Assay the baclofen enantiomers and its impurity in raw materials and pharmaceutical formulations (n = 3)

Type	Analytes		Total of baclofen (%LA)	USP Limits	
	Impurity (%w/w)	Ratio *S:R*-baclofen		Baclofen (%LA)	Impurity (%w/w)
Raw material					
Brand A	N.D.	1.07:1	101.1 (0.89)	99.0-101.0%	≤1%
Brand B	N.D.	1.07:1	99.3 (1.03)	99.0-101.0%	≤1%
Tablet					
Brand A	N.D.	1.06:1	109.1 (0.89)	90.0-110.0%	≤4%
Brand B	0.1 (1.95)	1.07:1	108.0 (1.52)	90.0-110.0%	≤4%
Brand C	0.4 (1.76)	1.03:1	107.9 (0.84)	90.0-110.0%	≤4%

N.D. = Not detected

Number in ( ) = %RSD

CHAPTER V
CONCLUSION

The purpose of this work was to optimize, validate and apply a chiral capillary electrophoresis method for the determination of baclofen enantiomers and its impurity in baclofen raw materials and pharmaceutical formulations.

1. Optimization

Initially, the experiments were optimized for the separation of bacofen enantiomers. The experiments was investigated by varying different factors included chiral selector types and concentrations, buffer concentrations and pH, amount of organic solvents, temperature, applied voltage, and injection time. The optimized condition for the separation of baclofen enantiomers was found 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 x 10 s and a detection wavelength at 220 nm. The optimized condition was applied for the separation of baclofen impurity, (4*RS*)-4-(4 chlorophenyl) pyrrolidin-2-one, however, broad and overlapped peaks were obtained. The impurity peak migrated at the same time with the EOF since it was neutral under this condition and was swept out from a capillary by the EOF. Therefore, further optimization was needed. Next experiments were carried out by coating the capillary with PEO, varying the buffer pH, injection time, amounts of ACN in the buffer and sample solutions, temperature and voltage.

Finally, the optimum condition for the separation of baclofen enantiomers and its impurity was found 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 x 6 s and a detection wavelength at 220 nm. This optimized condition provided the completed baseline separation for baclofen enantiomers and its impurity within 10 min with R_s = 2.7 for *S*-(+)-/*R*-(-)-baclofen and 44.8 for *R*-(-)-baclofen and impurity , respectively, $\alpha \leq 1.6$, $TF \leq 1.13$, $w \leq 0.06$, and $N > 100{,}000$.

2. Method validation

Calibration curves of baclofen enantiomers and its impurity 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 > 0.9994) and lowest %RSDs for both slope and intercept for all analytes. The calibration curves equations were y = 0.6061X + 5.3594, y = 0.6981x + 5.688, and y = 0.6327X – 0.4461 for *S*-(+)-baclofen, *R*-(-)-baclofen, and impurity, respectively. Precision of the method was verified by injection, intra-day and inter-day precision. Precision of the migration times for all analytes was within 2.97%. Precision calculated from peak area gave the lowest %RSDs (0.51%) in most cases comparing to peak height and normalized peak area. Recoveries were performed by standard addition method in a range of 80-120% of the assay concentrations. Recoveries were 99.8-103.1, 99.6-101.5, and 96-96.4% for *S*-(+)-baclofen, *R*-(-)-baclofen, and its impurity, respectively. Sensitivity of baclofen enantiomers and its impurity were determined from LOD and LOQ at signal to noise ratios of 3 and 10, respectively, based on peak height. LOD and LOQ of *S*-(+)-baclofen were 10.0 and 30.0 µg/ml, respectively and *R*-(-)-bacolfen were 7.0 and 30.0 µg/ml, respectively. For the impurity, LOD and LOQ were determined to be 2.0 and 5.0 µg/ml, respectively. %RSDs of LOQ were less than 6.70%.

3. Determination of baclofen enantiomers and its impurity in raw materials and pharmaceutical formulations

The developed and validated CCE method was applied for the determination of baclofen enantiomers and its impurity in two different brands of raw materials and three different brands of pharmaceutical formulations.

In raw materials, the %label amounts of baclofen were 101.07 (%RSD = 0.89) and 99.31 (%RSD = 1.03) for brand A and B, respectively. In tablets, the %label amounts of baclofen were 109.18 (%RSD = 0.89), 108.01 (%RSD = 1.52) and 107.93 (%RSD = 0.84) for brand A, B and C, respectively. The ratio of *S:R* in raw materials of both brands were 1.07:1. The ratios of *S:R* in pharmaceutical formulations of brand A, B, and C were 1.06:1, 1.07:1, and 1.03:1, respectively.

For raw material brands A and B, the quantity of impurity was lower than LOD. For tablets, the impurity could not be detected in brand A, but was found in brands B (0.1 %w/w) and C (0.4 %w/w), which were within limit of USP31 (NF26).

In conclusion, the developed and validated CCE method was simple, rapid and efficient to use for routine analysis with excellent linearity, accuracy, precision and sensitivity in terms of LOD and LOQ. The method was successfully applied for the determination of baclofen and its impurity in raw materials and pharmaceutical formulations.

REFERENCES

1. Bettler B, Tiao JY. Molecular diversity, trafficking and subcellular localization of $GABA_B$ receptors. Pharmacol Ther. 2006; 110 (3): 533-543.
2. Johnston GA. $GABA_B$ receptor channel pharmacology. Curr Pharm Des. 2005; 11: 1867-1885.
3. Bowery NG. GABAB receptors and their significance in mammalian. Trends Pharmacol Sci. 1989; 10: 401-407.
4. Hayek SM, Joseph PN, Mekhail NA. Pharmacology of intrathecally administered agents for treatment of spasticity and pain. Seminars in Pain Medicine. 2003; 1(4): 238-241.
5. Kroin JS, Intrathecal drug administration: Present use and future trends. Clin Pharmacokinet. 1992; 22: 319–326.
6. Sawynok J, Dickson C. *D*-Baclofen: is it an antagonist at baclofen receptors?. Progr Neuropsychopharmacol Biol Psychiatry. 1984; 8: 729–731.
7. Hong YG, Henry JL. Effects of phaclofen and the enantiomers of baclofen on cardiovascular responses to intrathecal administration of *L*- and *D*-baclofen in the rat. Eur J Pharmacol. 1991; 196: 55-62, 267-275.
8. Felluga F, Gombac V, Pitacco G, Valentin E. A short and convenient chemoenzymatic synthesis of both enantiomers of 3-phenylGABA and 3-(4-chlorophenyl) GABA (baclofen). Tetrahedron: Asymmertry. 2005; 16: 1341-1345.
9. Chang MY, Sun PP, Chen ST, Chang NC. A facile synthesis of 3-aryl pyroglutamic acid. Facile synthesis of baclofen and chlorpheg. Tetrahedron Lett. 2003; 44: 5271-5273.
10. Camps P, Torrero DM, Sanchez L. Synthesis of both enantiomers of baclofen using (*R*)- and (*S*)-*N*-phenylpantolactam as chiral auxiliaries. Tetrahedron: Asymmertry. 2004; 15: 2039-2044.

11. Carpes MJS, Correia CRD. Heck arylations of *N*-acyl-3-pyrroline and *N*-acyl-1,2,5,6-tetrahydropyridine with aryldiazonium salts. Short syntheses of aryl γ- and δ-lactams, baclofen, homobaclofen and analogues. Tetrahedron Lett. 2002; 43: 741-744.
12. Chang MY, Pai CL, Kung YH. New synthesis of 3-arylpyrrolines. Tetrahedron Lett. 2006; 47: 855-859.
13. Mazzini C, Lebreton J, Alphand VR, Furstoss R. A Chemoenzymatie Strategy for the Synthesis of Enantiopure (*R*)-(-)-Baclofen. Tetrahedron Lett. 1997; 38: 1195-1196.
14. Wang MX, Zhao SM. Highly enantioselective biotransformations of 2-aryl-4-pentenenitriles, a novel chemoenzymatic approach to (*R*)-(−)-baclofen. Tetrahedron Lett. 2002; 43: 6617–6620.
15. Paraskar AS, Sudalai A. Co-catalyzed reductive cyclization of azido and cyano substituted a,b-unsaturated esters with NaBH4: enantioselective synthesis of (*R*)-baclofen and (*R*)-rolipram. Tetrahedron. 2006; 62: 4907–4916.
16. Nemoto T, Jin L, Nakamura H, Hamada Y. Pd-catalyzed asymmetric allylic alkylation with nitromethane using a chiral diaminophosphine oxide: (S,RP)-Ph-DIAPHOX. Enantioselective synthesis of (*R*)-preclamol and (*R*)-baclofen. Tetrahedron Lett. 2006; 47: 6577–6581.
17. Thakur VV, Nikalje MD, Sudalai A. Enantioselective synthesis of (*R*)-(−)-baclofen via Ru(II)–BINAP catalyzed asymmetric hydrogenation. Tetrahedron: Asymmetry. 2003; 14: 581–586.
18. Baldoli C, Maiorana S, Licandro E, Perdicchia D, Vandoni B. Michael addition of nitromethane to non-racemic chiral $Cr(CO)_3$ complexes of ethyl cinnamate derivatives: stereoselective synthesis of (*R*)-(-)-baclofen. Tetrahedron: Asymmetry. 2000; 11: 2007-2014.
19. Licandro E, Maiorana S, Baldoli C, Capella L, Perdicchia D. Enantioselective synthesis of (*R*)-(-)-baclofen using Fischer-type carbene anions. Tetrahedron: Asymmetry. 2000; 11: 975–980.
20. Resende P, Almeida WP, Coelho F. An efficient synthesis of (*R*)-(−)-baclofen. Tetrahedron: Asymmetry. 1999; 10: 2113–2118.

21. Cutrignelli A, Denora N, Lopedota A, Trapani A, Laquintana V, Latrofa A, Trapani G, Liso G. Comparative effects of some hydrophilic excipients on the rate of gabapentin and baclofen lactamization in lyophilized formulations. Int J Pharm. 2007; 332: 98–106.
22. Sitaram BR, Tsui M, Rawicki HB, Lam S, Courage P, Sitaram M, Chapman CB. Stability and compatibility of baclofen and morphine admixtures for use in an implantable infusion pump. Int J Pharm. 1995; 118: 181-189.
23. Sitaram BR, Tsui M, Rawicki HB, Lam S, Sitaram M. Stability and compatibility of intrathecal admixtures containing baclofen and high concentrations of morphine. Int J Pharm. 1997; 153: 13-24.
24. Knuttson E, Lindblom U, Martensson A. Plasma and cerebrospinal fluid levels of baclofen (Lioresal) at optimal therapeutic responses in spastic paresis. J Neurol Sci. 1974; 23: 473-484.
25. Degen PH, Riess W. The determination of gamma-amino-beta-(p-chlorophenyl)butyric acid (baclofen) in biological material by gas-liquid chromatography. J Chromatogr. 1976; 117(2): 399-405.
26. Kochak G, Honc F. Improved gas-liquid chromatographic method for the determination of baclofen in plasma and urine. J Chromatogr. 1984; 310(2): 319-326.
27. Swahn CG, Beving H, Sedvall G. Mass fragmentographic determination of 4-amino-3-p-chlorophenylbutyric acid (baclofen) in cerebrospinal fluid and serum. J Chromatogr. 1979; 162(3):433-438.
28. Harrison PM, Tonkin AM, McLean AJ. Determination of 4-amino-3-(p-chlorophenyl)butyric acid (baclofen) in plasma by high-performance liquid chromatography. J Chromatogr. 1985; 339: 424-428.
29. Rustum AM. Simple and rapid reversed-phase high-performance liquid chromatographic of baclofen in human plasma with ultraviolet detection. Application to a pharmacokinetic study. J Chromatogr. 1989; 487(1): 101-115.
30. Wuis EW, Dirks RJ, Vree TB, Van der Kleyn E. High performance liquid chromatographic analysis of baclofen in plasma and urine of man after

precolumn extraction and derivatization with o-phthaldialdehyde. J Chromatogr. 1985; 337(2): 341-350.

31. Tosunoglu S, Ersoy L. Determination of baclofen in human plasma and urine by high-performance liquid chromatography with fluorescence detection. Analyst. 1995; 120(2): 373-375.
32. Vaccher C, Berthelot P, Debaert M. Direct separation of 4-amino-3-(4-chlorophenyl)butyric acid and analogues, $GABA_B$ ligands, using a chiral crown ether stationary phase. J Chromatogr. 1993; 645: 95-99.
33. Wall GM, Baker JK. Determination of baclofen and alpha-baclofen in rat liver homogenate and human urine using solid-phase extraction, o-phthalaldehyde-tert.-butyl thiol derivatization and high-performance liquid chromatography with amperometric detection. J Chromatogr. 1989; 491(1): 151-162.
34. Millerioux L, Brault M, Gualano V, Mignot A. High-performance liquid chromatographic determination of baclofen in human plasma. J Chromatogr A. 1996; 729(1-2):309-314.
35. Flardh M, Jacobson BM. Sensitive method for the determination of baclofen in plasma by means of solid-phase extraction and liquid chromatography-tandem mass spectrometry. J Chromatogr A. 1999; 846(1-2):169-173.
36. Kowalski P, Chmielewska A, Konieczna L, Oledzka I, Zarzycki PK, Lamparczyk H. The bioequivalence study of baclofen and lioresal tablets using capillary electrophoresis. Biomed Chromatogr. 2004; 18(5): 311-317.
37. Chiang MT, Chang SY. Whang CW. Analysis of baclofen by electrophoresis with laser-induced fluorescence detection. J Chromatogr A. 2000; 877: 233-237.
38. Gu YS, Whang CW. Capillary electrophoresis of baclofen with argon-ion laser-induced fluorescence detection. J Chromatogr A. 2002; 972: 289-293.
39. Chang S., Yang H. Determination of baclofen by derivatization with anthracene-2,3-dicarboxaldehyde followed by capillary electrophoresis with laser-induced fluorescence detection. Chromatographia. 2003; 57: 825-829.

40. Sioufi A, Kaiser G, Leroux F, Dubois JP. Determination of the *S*-(+) and *R*-(-)-enantiomers of baclofen in plasma and urine by gas chromatography using a chiral fused-silica capillary column and an electron-capture detector. J Chromatogr A. 1988; 450(2):221-232.

41. Rat'ko AA, Determination of baclofen enantiomers in pharmaceutical formulations using maltodextrin-based enantioselective, potentiometric membrane electrodes. Il Farmaco. 2004; 59(12): 993-997.

42. Stefan van Staden RI, Rat'ko AA. Enantioselective, potentiometric membrane electrodes based on cyclodextrins: Application for the determination of *R*-baclofen in its pharmaceutical formulation. Talanta. 2006; 69(5): 1049-1053.

43. Goda R, Murayama N, Fujimaki Y, Sudo K. Simple and sensitive liquid chromatography-tandem mass spectrometry method for determination of the *S*-(+)- and *R*-(-)-enantiomers of baclofen in human plasma and cerebrospinal fluid.. J Chromatogr B. 2004; 801(2):257-264.

44. Hefnawy MM, Aboul-Enein HY. Enantioselective high-performance liquid chromatographic method for the determination of baclofen in human plasma. Talanta. 2003; 61: 667-673.

45. Weatherby RP, Allan RD, Johnston GAR. Resolution of the stereoisomers of baclofen by high performance liquid chromatography. J Neurosci Methods. 1984; 10(1): 23-28.

46. Zhu Z, Neirinck L. Chiral separation and determination of *R*-(-) and *S*-(+)-baclofen in human plasma by high-performance liquid chromatography. J Chromatogr B. 2003; 785(2):277-283.

47. Spahn H, Krauss D, Mutschler E, Enantiospecific high-performance liquid chromatographic (HPLC) determination of baclofen and its fluoro analogue in biological material. Pharm Res. 1988; 5(2): 107-112.

48. Hiroyuki N, Kouji N, Hideo N, Tadashi S. Separation of enantiomers and isomers of amino compounds by capillary electrophoresis and high-performance liquid chromatography utilizing crown ethers. J Chromatogr A. 1997; 757(1-2): 225-235.

49. Vaccher MP, Lipka E, Bonte JP, Vaccher C. Chiral capillary electrophoretic resolution of baclofen, gabaergic ligand, using highly sulfated cyclodextrins. Electrophoresis. 2004; 25(7-8): 1111-1120.

50. Vaccher MP, Lipka E, Bonte JP, Foulon C, Goossens JF, Vaccher C. Enantiomeric analysis of baclofen analogs by capillary zone electrophoresis, using highly sulfated cyclodextrins: Inclusion ionization constant pKa determination. Electrophoresis. 2005; 26(15): 2974-2983.

51. Ali I, Aboul-Enein HY. Optimization of the chiral resolution of baclofen by capillary electrophoresis using beta-cyclodextrin as the chiral selector. Electrophoresis. 2003; 24(12-13):2064-2069.

52. Koppenhoefer B, Epperlein U, Schlunk R, Xiaofeng Z, Bingcheng L. Separation of enantiomers of drugs by capillary electrophoresis V. Hydroxypropyl-α-cyclodextrin as chiral solvating agent. J Chromatogr A. 1998; 793: 153-164.

53. Gao Y, Shen Z, Dai Z, Lin B. Chiral separations on multichannel microfluidic chips. Electrophoresis. 2005; 26: 4774-4779.

54. Chiang MT, Chang SY, Whang C. Chiral analysis of baclofen by alpha-cyclodextrin -modified capillary electrophoresis and laser-induced fluorescence detection. Electrophoresis. 2001; 22(1):123-127.

55. Kavran-Belin G, Rudaz S, Veuthey JL. Enantioseparation of baclofen with highly sulfated beta-cyclodextrin by capillary electrophoresis with laser-induced fluorescence detection. J Sep Sci. 2005; 28(16):2187-2192.

56. Armstrong DW, Chang LW, Chang SSC. Mechanism of capillary electrophoresis enantioseparations using a combination of an achiral crown ether plus cyclodextrins. J Chromatogr A. 1998; 793(1): 115-134.

57. Koppenhoefer B, Epperlein U, Jacob A, Wuerthner S, Xiaofeng Z, Bingcheng L. Separation of enantiomers of drugs by capillary electrophoresis, part 7: Gamma-cyclodextrin as chiral solvating agent. Chirality. 1998; 10: 548-554.

58. Lammerhofer M. Chiral separations by capillary electromigration techniques in nonaqueous media. I Enantioselective nonaqueous capillary electrophoresis. J Chromatogr A. 2005; 1068(1):3-30.

59. Fanali S. Identification of chiral isomers by capillary electrophoresis. J chromatogr. 1996; 735: 77-121.
60. Gubitz G, Schmid MG. Recent progress in chiral separation principle in capillary electrophoresis. Electrophoresis. 2000; 21: 4112-4135.
61. Davankov VA. Analytical chiral separation methods. Pure Appl Chem. 1997; 69: 1469-1474.
62. Smith HS. Drugs for pain. Plliladelphia: Hunley & belfus; 2003.
63. Bowery NG. Pharmacology of $GABA_B$ receptors. In: Egebjerg J, Schousboe A, Larsen PK, editor. Glutamate and GABA receptors and transporters. London: Taylor & Francis; 2002. pp. 287-304.
64. The united states pharmacopeia (31/NF26). 26 ed. Rockville. webcom; 2008.
65. Gupta VD, Parasrampuria J. Quantitation of 4-(4-chlorophenyl)-2-pyrrolidinone in baclofen power and tablets. Drug Dev Ind Pharm. 1988; 14(11): 1623-1628.
66. Guttman A. Capillary electrophoresis separation of enantiomers by cyclodextrin array chiral analysis. In: Landers JP, editor. Hand book of capillary electrophoresis. 2 nd ed. New York: CRC Press; 1997. pp. 75-100.
67. Fanali S, Aturki Z, Desiderio C. New strategies for chiral analysis of drugs by capillary electrophoresis. Forensic Sci Int. 1998; 92: 137-155.
68. Fanali S. Use of cyclodextrins in capillary electrophoresis. In: Guzman NA, editor. Capillary electrophoresis technology. New York: Marcel dekker; 1993. pp. 731-741.
69. Gubitz G, Schmid MG. Chiral Separation by Chromatographic and Electromigration Techniques: Review. Biopharm Drug Dispos. 2001; 22: 291–336.
70. Porter WH. Resolution of chiral drugs. Pure & Appl Chem. 1991; 63 (8): 1119-122.
71. Stephen ACW. Theory of chiral separation in capillary electrophoresis. J Chromatogr. 1993; 636: 57-62.
72. Chromatographia: An international journal for rapid communication in chromatography, electrophoresis, and associated techniques. Chromatographic Supplement. 2001; 54:59-66.

73. Szejtli J. Cyclodextrins and their inclusion complexes. Budapest: Akademiai kiado; 1982.

74. Li S, Purdy WC. Cyciodextrins and Their Applications in Analytical Chemistry. Chem Rev. 992; 92: 1457-1470.

75. Schneider HJ, Hacket F, Rudiger V, Ikeda H. NMR Studies of Cyclodextrins and Cyclodextrin Complexes. Chem Rev. 998; 98: 1755-1785.

76. Szejtli J. Cyclodextrins and their inclusion complexes. Budapest: Akademiai Kiado; 1982.

77. Okafo GN. Separation of enantiomers in capillary electrophoresis. In: Camilleri P, editor. Capillary Electrophoresis: Theory and Practice. 2nd ed. New York: CRC Press; 1998. pp. 184-247.

78. Chankvetadze B, Schulte G, Blaschke G. Reversal of enantiomer elution order in capillary electrophoresis using charged and neutral cyclodextrins J Chromatogr A. 1996; 732: 183-187.

79. Sabbah S, Scriba Gerhard KE. Influence of the structure of cyclodextrins and amino acid sequence of dipeptides and tripeptides on the pH-dependent reversal of the migration order in capillary electrophoresis. J Chromatogr A. 2000; 894: 267-272.

80. Tang W, Muderawan IW, Ng SC, Chan HSO. Enantioselective separation in capillary electrophoresis using a novel mono-6^{A}-propylammonium-ß-cyclodextrin as chiral selector. Anal Chim Acta. 2006; 555: 63-67.

81. Bunke A, Jira. Use of cationic cyclodextrin for enantioseparation by capillary electrophoresis. J Chromatogr A. 1998; 798: 275-280.

82. Lin X, Zhu C, Hao A. Enantiomeric separations of some acidic compounds with cationic cyclodextrin by capillary electrophoresis. Anal Chim Acta. 2004; 517: 95-101.

83. Zhu W, Vigh G. Enantiomer separations by nonaqueous capillary electrophoresis using octakis(2,3-diacetyl-6-sulfato)-γ-cyclodextrin. J Chromatogr A. 2000; 892: 499-507.

84. Ha PTT, Hoogmartens J, Schepdael AV. Recent advances in pharmaceutical applications of chiral capillary electrophoresis. J Pharm Biomed Anal. 2006; 41: 1-11.

85. Amini A. Recent developments in chiral capillary electrophoresis and applications of this technique to pharmaceutical and biomedical analysis. Electrophoresis. 2001; 22: 3107-3130.

86. Fanali S. Controlling enantioselectivity in chiral capillary electrophoresis with inclusion-complexation. J Chromatogr A. 1997; 792: 227-267.

87. Landers JP, Oda RP. Introduction to capillary electrophoresis. In: Landers JP, editor. Hand book of capillary electrophoresis. 2 nd ed. New York: CRC Press; 1997. pp. 1-47.

88. Wang F, Khaledi MG. Enantiomeric separations by nonaqueous capillary electrophoresis. J Chromatogr A. 2000; 875: 277-293.

89. Elder D, Altria KD. Overview of the status and applications of capillary electrophoresis to the analysis of small molecules. J Chromatogr A. 2004; 1023: 1-14.

90. Niccolo DP, Giorgio P, Italbo F, Clementina M, Caterina O, Flaminia P, Giuseppe S, Vittorio S, Marco M. Use of capillary electrophoresis and poly(ethylene oxide) as the coating agent for the determination of substances related to heroin addiction and treatment. J Anal Toxicol. 2006; 30: 679-682.

91. Yeung ES, Preisler J. Characterization of nonbonded poly (ethylene oxide) coating for capillary electrophoresis via continuous monitoring of electroosmotic flow. Anal Chem. 1996; 68: 2885-2889.

92. Haginaka J. Enantiomer separation of drugs by capillary electrophoresis using proteins as chiral selectors. J Chromatogr A. 2000; 875: 235-254.

93. Schmid MG, Grobuschek N, Lecnik O, Gubitz G. Chiral ligand-exchange capillary electrophoresis. J Biochem Biophys. Methods. 2001; 48: 143-154.

94. Otsuka K, Terabe S. Enantiomer separation of drugs by micellar electrokinetic chromatography using chiral surfactants. J Chromatogr A. 2000; 875: 163-178.

95. Jenke DR. Chromatographic method validation: A review of current practices and procedures. II. Guidelines for primary validation parameters. J Liq Chrom & Rel Technol. 1996; 19(5): 737-757.

96. Wren SAC, Rowe RC. Theoretical aspects of chiral separation in capillary electrophoresis. J Chromatogr. 1992; 603: 235-241.

97. Penn SG, Goodall DM. Differential binding of tioconazole enantiomers to hydroxypropyl-β-cyclodextrin studied by capillary electrophoresis. J Chromatogr. 1993; 636: 149-152.

98. Penn SG, Bergstrom ET, Goodall DM. Capillary electrophoresis with chiral selectors: optimization of separation and determination of thermodynamic parameters for binding of tioconazole enantiomers to cyclodextrins. Anal Chem. 1994; 66: 2866-2873.

99. Rawjee YY, Staerk DU, Vigh G. Capillary electrophoretic chiral separations with cyclodextrin additives. I. Acid: chiral selectivity as a function of pH and the concentration of β-cyclodextrin for fenoprofen and ibuprofen. J Chromatogr. 1993; 635: 291-306.

100. Rawjee YY, Williams RL, Vigh G. Capillary electrophoretic chiral separations using β-cyclodextrin as resolving agent II. Bases: chiral selectivity as a function of pH and the concentration of β-cyclodextrin. J Chromatogr A. 1993; 652: 233-245.

101. Rawjee YY, Williams RL, Vigh G. Effects of pH hydroxypropyl β-cyclodextrin concentration on peak resolution in the capillary electrophoretic separation of the enantiomers of weak bases. J Chromatogr A. 1994; 688: 273-282.

102. Rawjee YY, Williams RL, Vigh G. Capillary electrophoretic chital separations using cyclodextrin additives III. Peak resolution surfaces for ibuprofen and homatropine as a function of the pH and the concentration of β-cyclodextrin. J Chromatogr A. 1994; 680:599-607.

103. Rawjee YY, Vigh G. A peak resolution model for the capillary electrophoretic separation of the enantiomers of weak acids with hydroxypropyl β-cyclodextrin-containing background electrolytes. Anal Chem. 1994; 66: 619-627.

APPENDIX A

Table 34 Analytical data from the effects of buffer pH on the separation of *R/S*-baclofen

Buffer pH	t_m (*R*-baclofen) (min)	R_s*	α*	w (*R*-baclofen) (min)	N (*R*-baclofen)
9.7	5.25	0.8	1.01	0.10	58081
9.8	5.78	1.0	1.02	0.11	51048
9.9	5.87	1.1	1.02	0.14	54387

Table 35 Analytical data from the effects of amounts of ACN in BGE on the separation of *R/S*-baclofen and its impurity

ACN in BGE (%v/v)	t_m (*R*-baclofen) (min)	R_s *	α*	w (*R*-baclofen) (min)	N (*R*-baclofen)
0	6.61	0.9	1.02	0.09	29923
2	7.66	1.4	1.04	0.12	23692
5	8.81	1.8	1.05	0.13	25490
10	9.99	1.3	1.05	0.20	13713

Table 36 Analytical data from the effects of amounts of ACN in sample on the separation of *R/S*-baclofen and its impurity

ACN in sample (%v/v)	t_m (impurity) (min)	R_s *	α*	w (impurity) (min)	N (impurity)
0.5	4.85	1.4	1.05	0.03	195476
1.5	4.83	1.4	1.05	0.03	169821
2.0	4.37	1.8	1.05	0.02	203030

Table 37 Analytical data from the effects of injection time on the separation and dispersion of *R/S*-baclofen and its impurity

Injection time (sec)	t_m (*R*-baclofen) (min)	R_s*	α*	w (*R*-baclofen) (min)	N (*R*-baclofen)
3	12.09	3.0	1.04	0.10	80998
6	12.09	2.7	1.04	0.11	65477
10	12.09	2.2	1.04	0.14	44877

Table 38 Analytical data from the effects of amounts of ACN in BGE on the separation and dispersion of *R/S*-baclofen and its impurity

ACN in BGE (%v/v)	t_m (*R*-baclofen) (min)	R_s*	α*	w (*R*-baclofen) (min)	N (*R*-baclofen)
0	8.57	1.9	1.02	0.06	106087
1	9.05	2.5	1.03	0.07	108359
3	10.16	3.0	1.04	0.08	97336
5	11.32	2.9	1.04	0.09	86131

Table 39 Analytical data from the effects of amounts of ACN on the separation and dispersion of *R/S*-baclofen and its impurity

ACN in sample (%v/v)	t_m (impurity) (min)	R_s*	α*	w (impurity) (min)	N (impurity)
0	5.53	2.7	1.03	0.02	292745
1	5.46	2.5	1.03	0.03	234278
2	5.58	2.5	1.03	0.03	165926
3	5.40	2.4	1.03	0.04	122123
4	5.40	2.4	1.03	0.04	91538

* R_s of *R/S*-baclofen

* α of *R*-baclofen

APPENDIX B

Table 40 Calibration data of *S*-(+)-baclofen for three different days

Day	Conc. (µg/ml)	t_m (min)	Peak area (mAU*S)	Peak height (mAU)	Nor.peak area
1	50	8.32	36.06	7.69	4.33
		(0.16)	(1.26)	(0.39)	(1.24)
	100	8.33	67.80	13.54	8.14
		(0.08)	(1.50)	(4.53)	(1.56)
	200	8.57	122.34	27.69	14.28
		(0.31)	(0.47)	(0.55)	(0.72)
	300	8.50	187.86	39.40	22.10
		(0.36)	(0.28)	(0.51)	(0.09)
	500	8.19	308.67	51.49	37.71
		(0.06)	(0.17)	(0.83)	(0.20)
Slope			0.6056	0.0991	0.0939
y-intercept			5.2594	5.1664	0.3108
SE (slope)			0.0075	0.0110	0.0018
SE (intercept)			2.1060	3.0780	0.5161
r^2			0.9995	0.9645	0.9981

SE = Standard error

Number in ( ) = %RSD

Day	Conc. (µg/ml)	t_m (min)	Peak area (mAU*S)	Peak height (mAU)	Nor.peak area
2	50	8.44	35.09	9.00	4.16
		(0.19)	(1.01)	(0.73)	(0.96)
	100	8.46	68.80	16.11	8.13
		(0.39)	(1.35)	(4.97)	(1.72)
	200	8.30	125.27	23.24	15.09
		(0.74)	(0.23)	(0.69)	(0.89)
	300	8.16	189.05	36.89	23.17
		(0.37)	(0.32)	(1.07)	(0.27)
	500	8.33	310.15	55.33	37.22
		(0.54)	(0.42)	(0.42)	(0.72)
Slope			0.609	0.1024	0.0735
y-intercept			5.5924	4.5582	0.6380
SE (slope)			0.0054	0.0046	0.0010
SE (intercept)			1.5190	1.2940	0.2728
r^2			0.9998	0.9939	0.9995

Day	Conc. (µg/ml)	t_m (min)	Peak area (mAU*S)	Peak height (mAU)	Nor.peak area
3	50	8.34	35.60	8.35	4.27
		(0.11)	(0.92)	(3.54)	(1.03)
	100	8.34	67.46	15.83	8.09
		(0.07)	(0.34)	(0.63)	(0.32)
	200	8.494	124.59	22.07	14.67
		(0.88)	(0.57)	(8.91)	(0.89)
	300	8.49	184.34	31.17	21.71
		(0.28)	(0.44)	(1.04)	(0.40)
	500	8.54	308.52	51.18	36.12
		(0.10)	(0.15)	(1.03)	(0.06)
Slope			0.6038	0.0917	0.0705
y-intercept			5.2262	4.9308	0.6817
SE (slope)			0.0055	0.0040	0.0011
SE (intercept)			1.5300	1.1260	0.2967
r^2			0.9998	0.9943	0.9993

Table 41 Calibration data of *R*-(-)-baclofen for three different days

Day	Conc. (µg/ml)	t_m (min)	Peak area (mAU*S)	Peak height (mAU)	Nor.peak area
1	50	8.56	40.35	8.62	4.71
		(0.17)	(0.67)	(1.14)	(0.77)
	100	8.57	76.81	15.53	8.96
		(0.09)	(1.71)	(4.33)	(1.80)
	200	8.84	140.17	32.85	15.86
		(0.33)	(0.60)	(0.21)	(0.78)
	300	8.77	215.79	46.95	24.61
		(0.38)	(0.21)	(0.29)	(0.28)
	500	8.43	351.41	62.35	41.67
		(0.04)	(0.27)	(1.09)	(0.22)
Slope			0.6917	0.1216	0.0819
y-intercept			5.8190	5.2909	0.3246
SE (slope)			0.0081	0.0128	0.0018
SE (intercept)			2.2760	3.5760	0.5029
r^2			0.9996	0.9680	0.9986

SE = Standard error

Number in ( ) = %RSD

Day	Conc. (µg/ml)	t_m (min)	Peak area (mAU*S)	Peak height (mAU)	Nor.peak area
2	50	8.70	39.94	10.10	4.59
		(0.20)	(1.22)	(1.07)	(1.03)
	100	8.72	77.67	18.31	8.91
		(0.38)	(0.77)	(5.42)	(1.14)
	200	8.55	144.82	27.63	16.94
		(0.78)	(0.40)	(0.85)	(1.17)
	300	8.41	218.33	44.20	25.97
		(0.38)	(0.26)	(0.66)	(0.15)
	500	8.60	357.77	64.79	41.63
		(0.57)	(0.27)	(0.44)	(0.82)
Slope			0.7051	0.1216	0.0825
y-intercept			5.5361	5.0456	0.6261
SE (slope)			0.0046	0.1216	0.0011
SE (intercept)			1.2840	5.0460	0.3130
r^2			0.9999	0.9929	0.9995

Day	Conc. (µg/ml)	t_m (min)	Peak area (mAU*S)	Peak height (mAU)	Nor.peak area
3	50	8.59	40.58	9.43	4.72
		(0.12)	(0.95)	(3.60)	(1.05)
	100	8.59	77.96	18.12	9.08
		(0.09)	(1.05)	(0.51)	(1.14)
	200	8.75	143.61	26.71	16.42
		(0.98)	(0.60)	(8.47)	(1.02)
	300	8.76	212.47	38.01	24.27
		(0.30)	(0.40)	(1.34)	(0.49)
	500	8.82	356.19	61.65	40.39
		(0.09)	(0.24)	(0.69)	(0.28)
Slope			0.6976	0.1126	0.0789
y-intercept			5.7088	5.1621	0.7363
SE (slope)			0.0068	0.0039	0.0013
SE (intercept)			1.9170	1.0880	0.3533
r^2			0.9997	0.9964	0.9992

Table 42 Calibration data of impurity for three different days

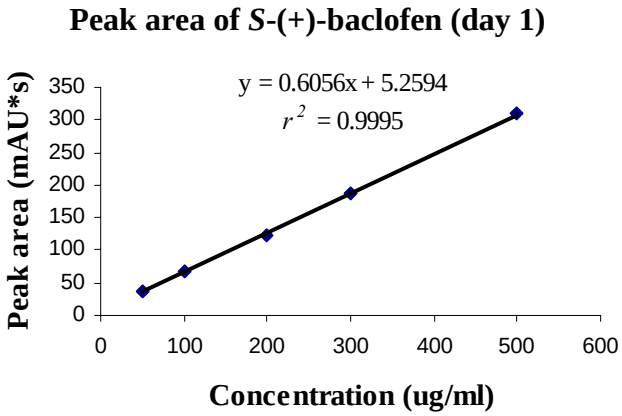
Day	Conc. (µg/ml)	t_m (min)	Peak area (mAU*S)	Peak height (mAU)	Nor.peak area
1	5	5.41	2.95	1.93	0.54
		(1.07)	(1.44)	(6.09)	(2.44)
	10	5.37	5.92	3.79	1.10
		(0.04)	(0.26)	(0.91)	(0.29)
	20	5.43	11.87	7.35	2.19
		(0.40)	(0.59)	(1.60)	(0.66)
	30	5.32	18.18	11.78	3.42
		(0.13)	(0.11)	(0.15)	(0.18)
	40	5.30	24.33	15.58	4.59
		(0.18)	(0.12)	(0.18)	(0.29)
	50	5.28	31.39	21.15	5.94
		(0.08)	(0.21)	(0.14)	(0.25)
Slope			0.6277	0.4193	0.1192
y-intercept			0.4444	0.5695	0.1168
SE (slope)			0.0090	0.0152	0.0021
SE (intercept)			0.2722	0.4621	0.0636
r^2			0.9992	0.9948	0.9988

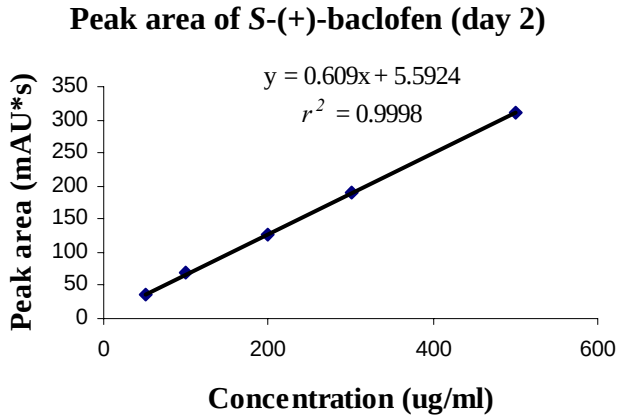
SE = Standard error

Number in ( ) = %RSD

Day	Conc. (µg/ml)	t_m (min)	Peak area (mAU*S)	Peak height (mAU)	Nor.peak area
2	5	5.32	2.92	1.88	0.55
		(0.02)	(0.15)	(0.80)	(0.16)
	10	5.21	5.94	3.77	1.14
		(0.09)	(1.22)	(1.55)	(1.23)
	20	5.34	12.12	7.56	2.27
		(0.72)	(0.36)	(3.42)	(0.98)
	30	5.43	18.92	11.22	3.49
		(0.05)	(0.37)	(0.92)	(0.34)
	40	5.29	24.82	16.44	4.69
		(0.24)	(0.15)	(1.82)	(0.39)
	50	5.28	31.72	20.33	6.01
		(0.30)	(0.24)	(0.30)	(0.52)
Slope			0.6388	0.4130	0.1206
y-intercept			0.4271	04682	0.0916
SE (slope)			0.0062	0.0116	0.0015
SE (intercept)			0.1911	0.3511	0.0445
r^2			0.9996	0.9969	0.9994

Day	Conc. (µg/ml)	t_m (min)	Peak area (mAU*S)	Peak height (mAU)	Nor.peak area
3	5	5.42	2.83	1.84	0.52
		(0.12)	(0.76)	(0.63)	(0.88)
	10	5.34	5.87	3.79	1.10
		(0.12)	(0.37)	(0.39)	(0.36)
	20	5.31	12.04	7.89	2.27
		(0.15)	(0.31)	(0.18)	(0.17)
	30	5.34	18.09	11.89	3.38
		(0.11)	(0.04)	(1.03)	(0.09)
	40	5.39	25.22	16.30	4.68
		(0.24)	(0.52)	(0.57)	(0.71)
	50	5.42	31.04	19.71	5.72
		(1.15)	(0.35)	(2.52)	(1.52)
Slope			0.6315	0.3908	0.1148
y-intercept			0.4668	0.0725	0.0501
SE (slope)			0.0078	0.0084	0.0008
SE (intercept)			0.2373	0.2563	0.0248
r^2			0.9994	0.9981	0.9998





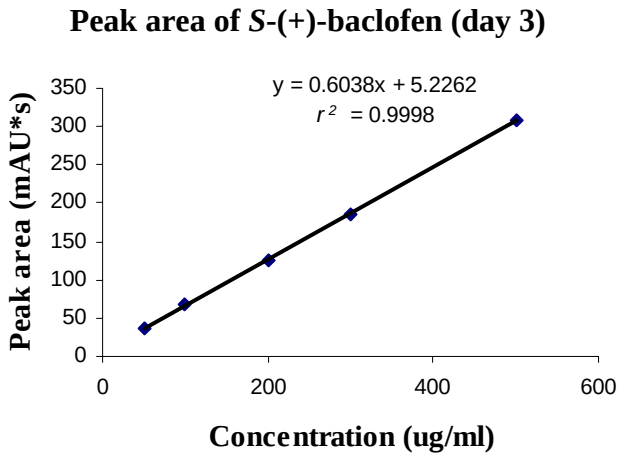
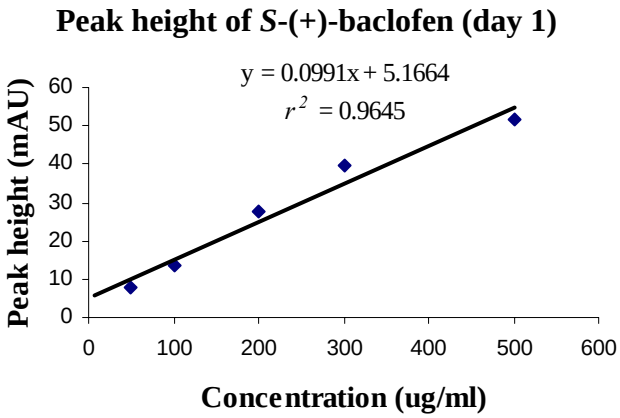
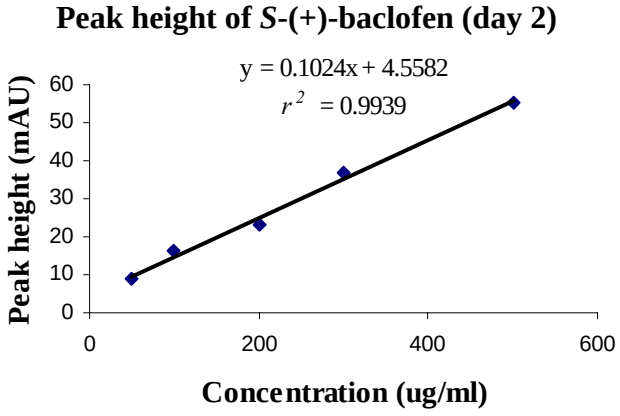


Fig. 52 Calibration data (peak area VS concentration) of *S*-(+)-baclofen for three different days.





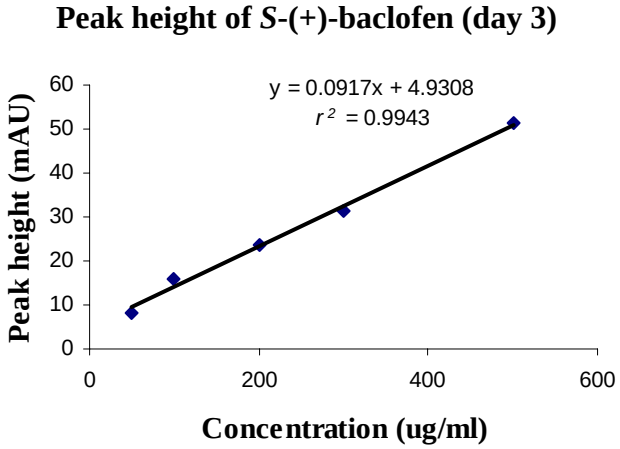
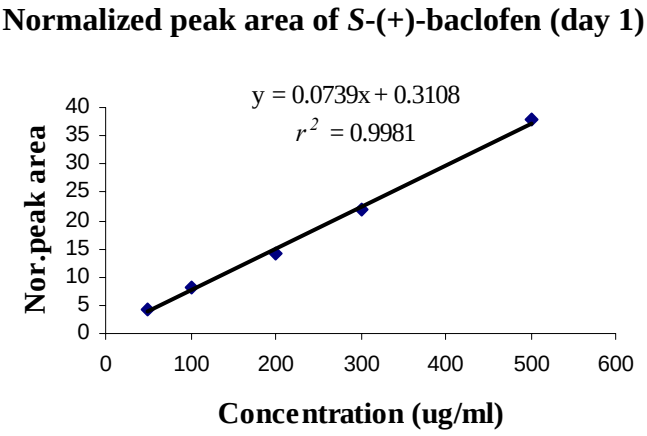
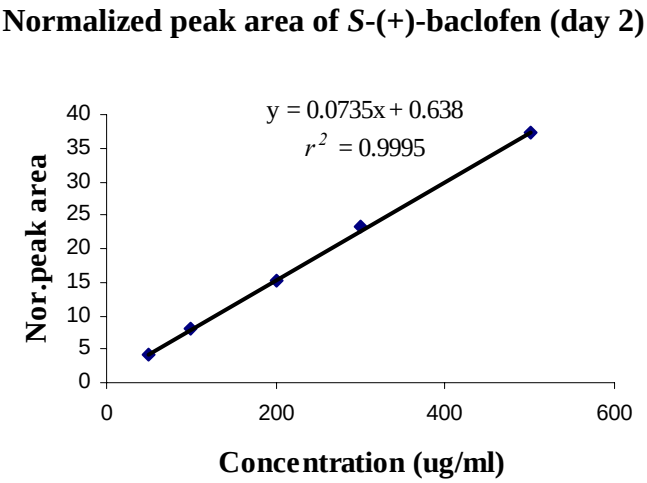


Fig. 53 Calibration data (peak height VS concentration) of *S*-(+)-baclofen for three different days.





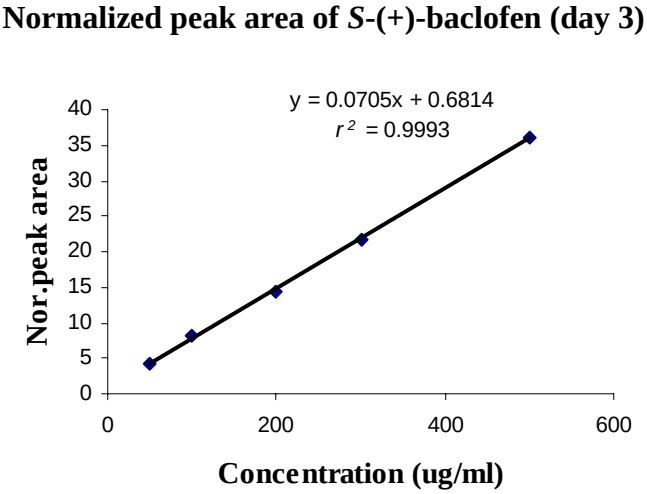
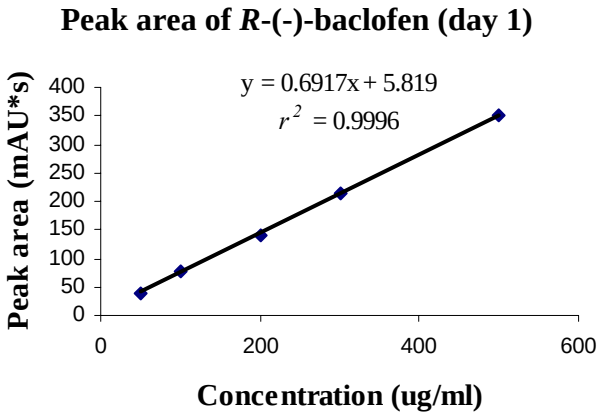
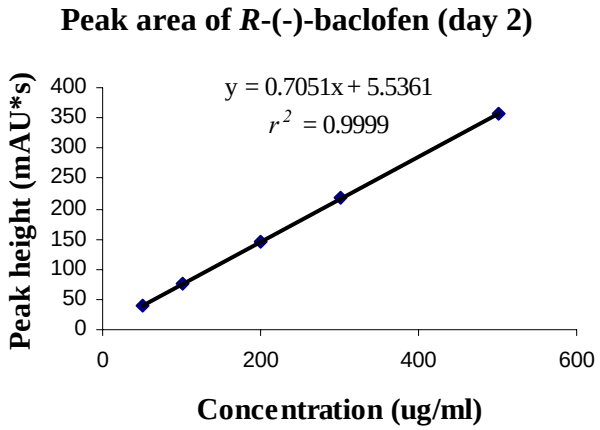


Fig. 54 Calibration data (normalized peak area VS concentration) of *S*-(+)-baclofen for three different days.





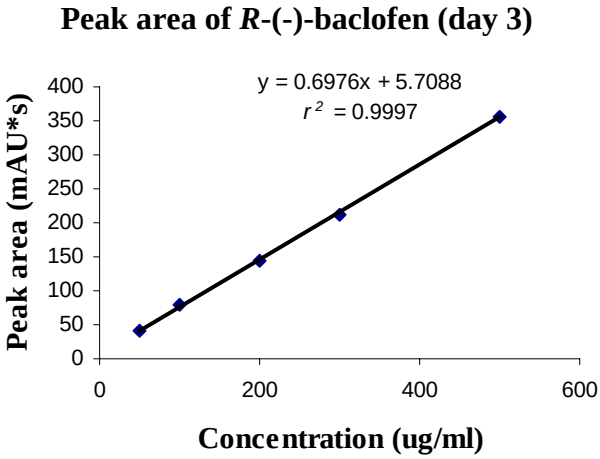
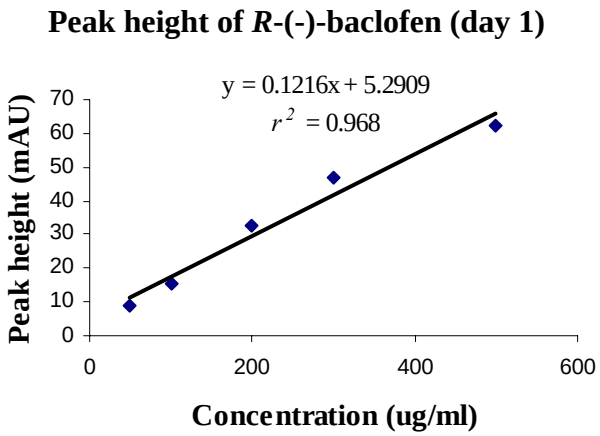
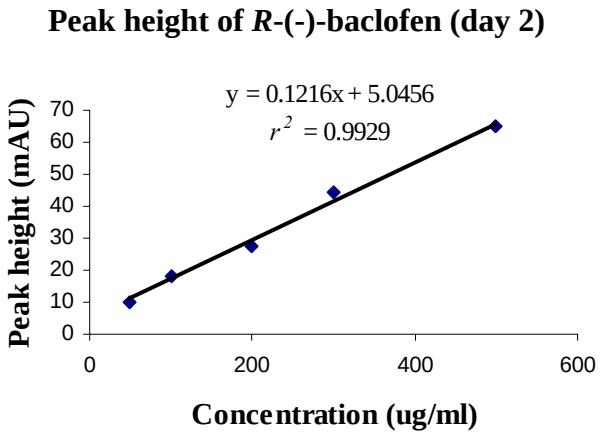


Fig. 55 Calibration data (peak area VS concentration) of *R*-(-)-baclofen for three different days.





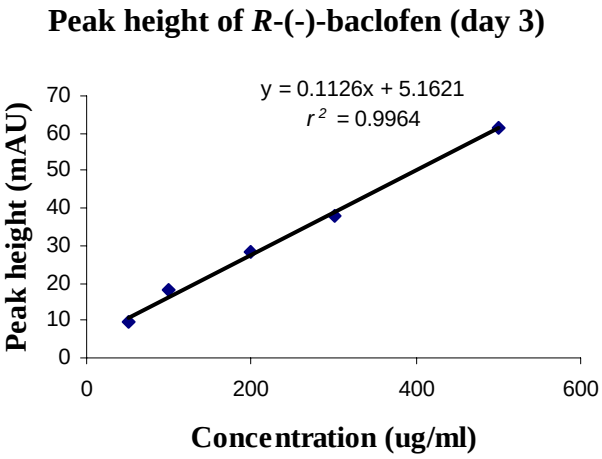
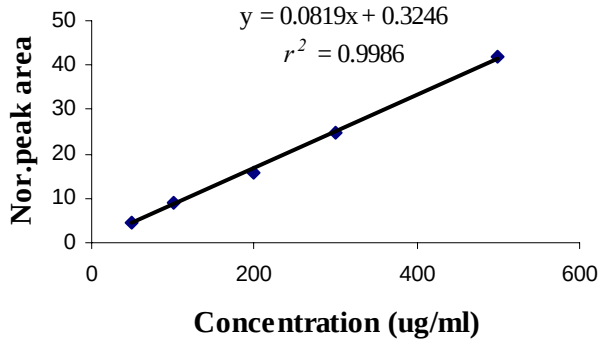
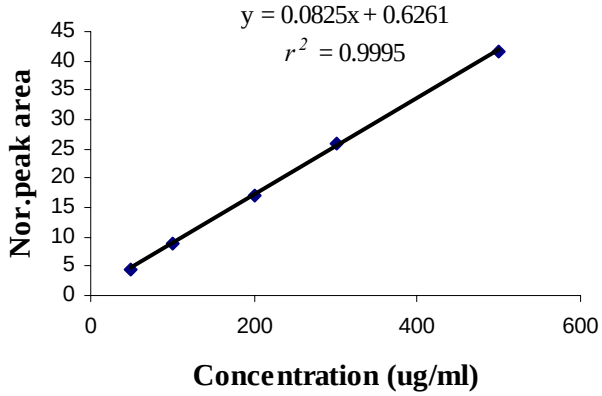


Fig. 56 Calibration data (peak height VS concentration) of *R*-(-)-baclofen for three different days.

Normalized peak area of *R*-(-)-baclofen (day 1)



Normalized peak area of *R*-(-)-baclofen (day 2)



Normalized peak area of *R*-(-)-baclofen (day 3)

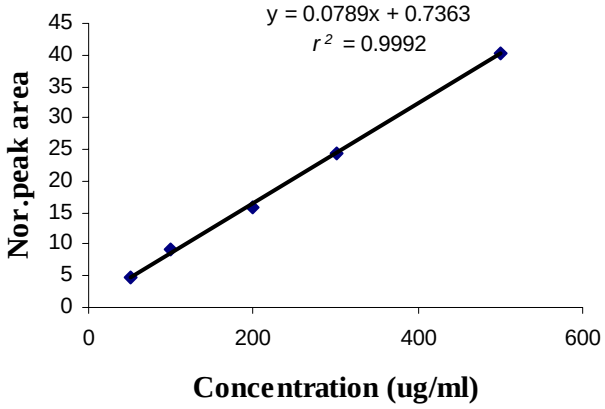


Fig. 57 Calibration data (normalized peak area VS concentration) of *R*-(-)-baclofen for three different days.

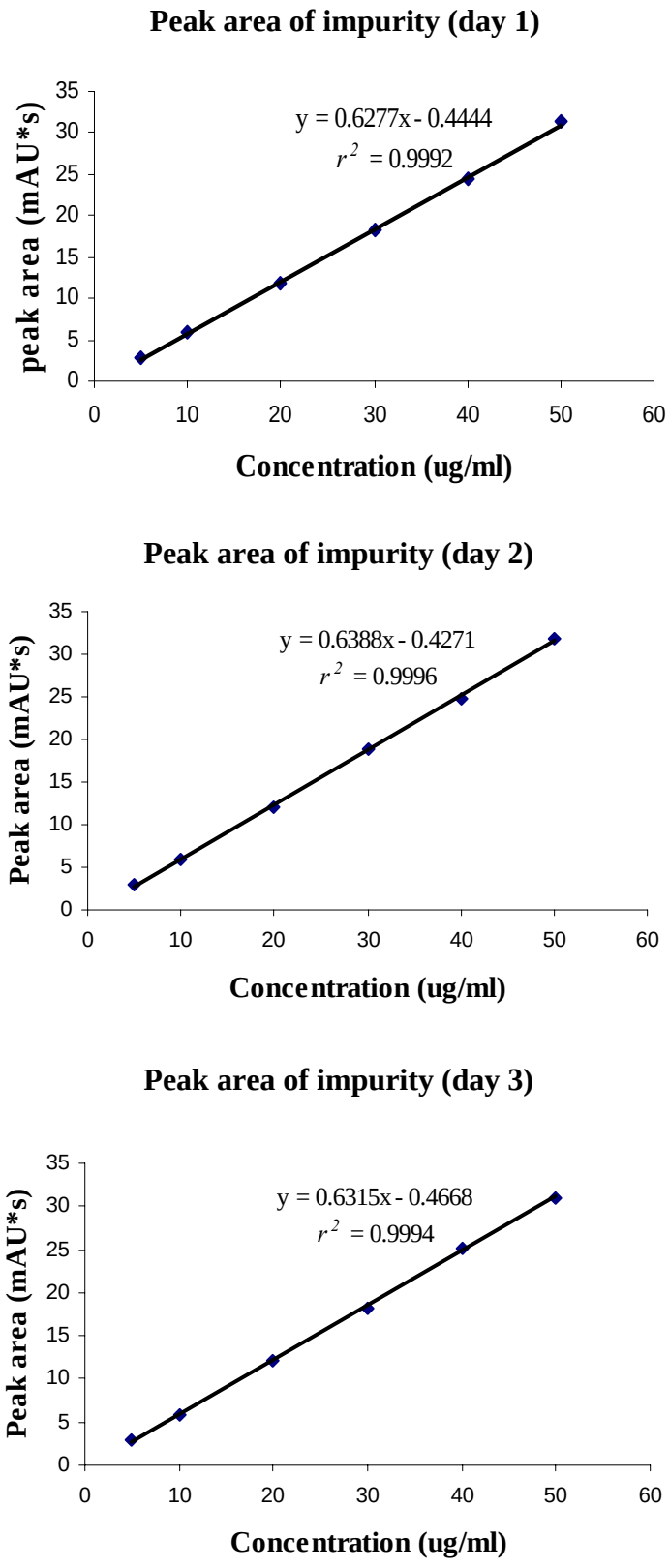


Fig. 58 Calibration data (peak area VS concentration) of impurity for three different days.

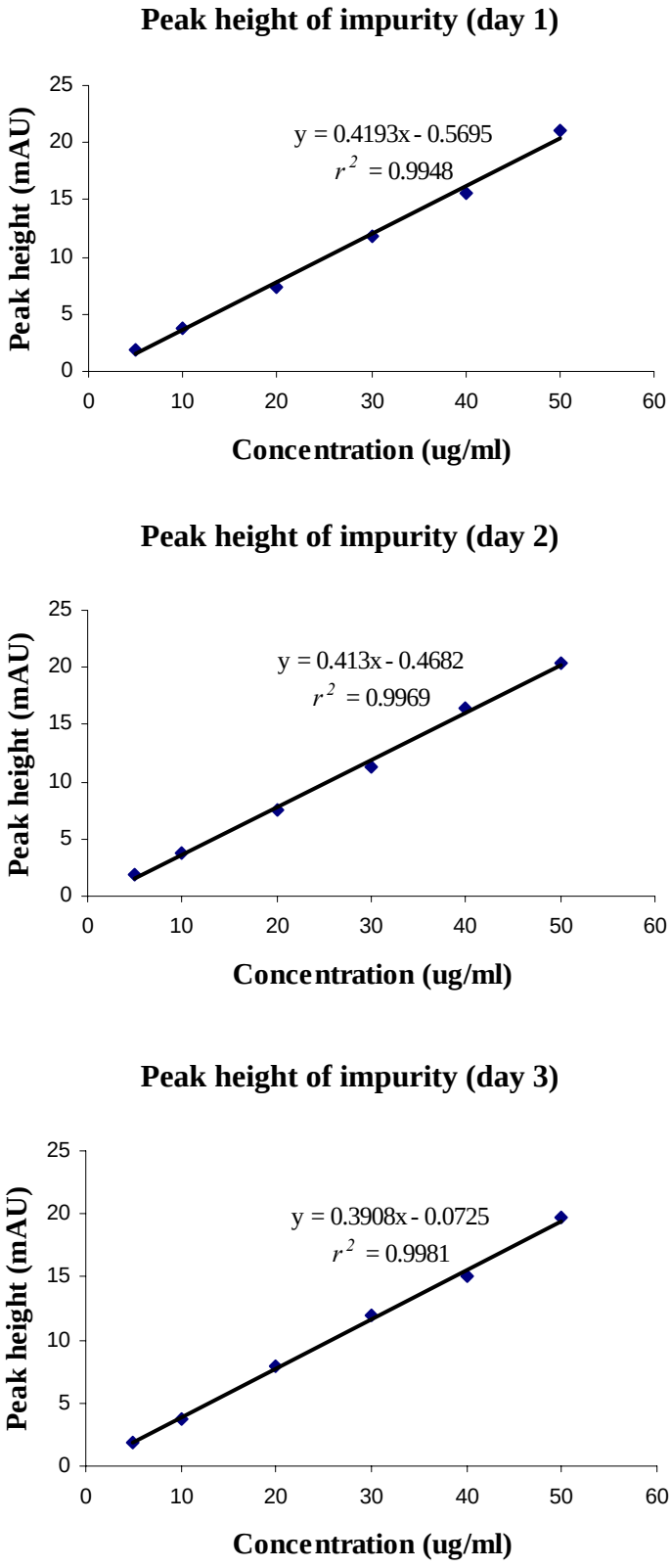


Fig. 59 Calibration data (peak height VS concentration) of impurity for three different days.

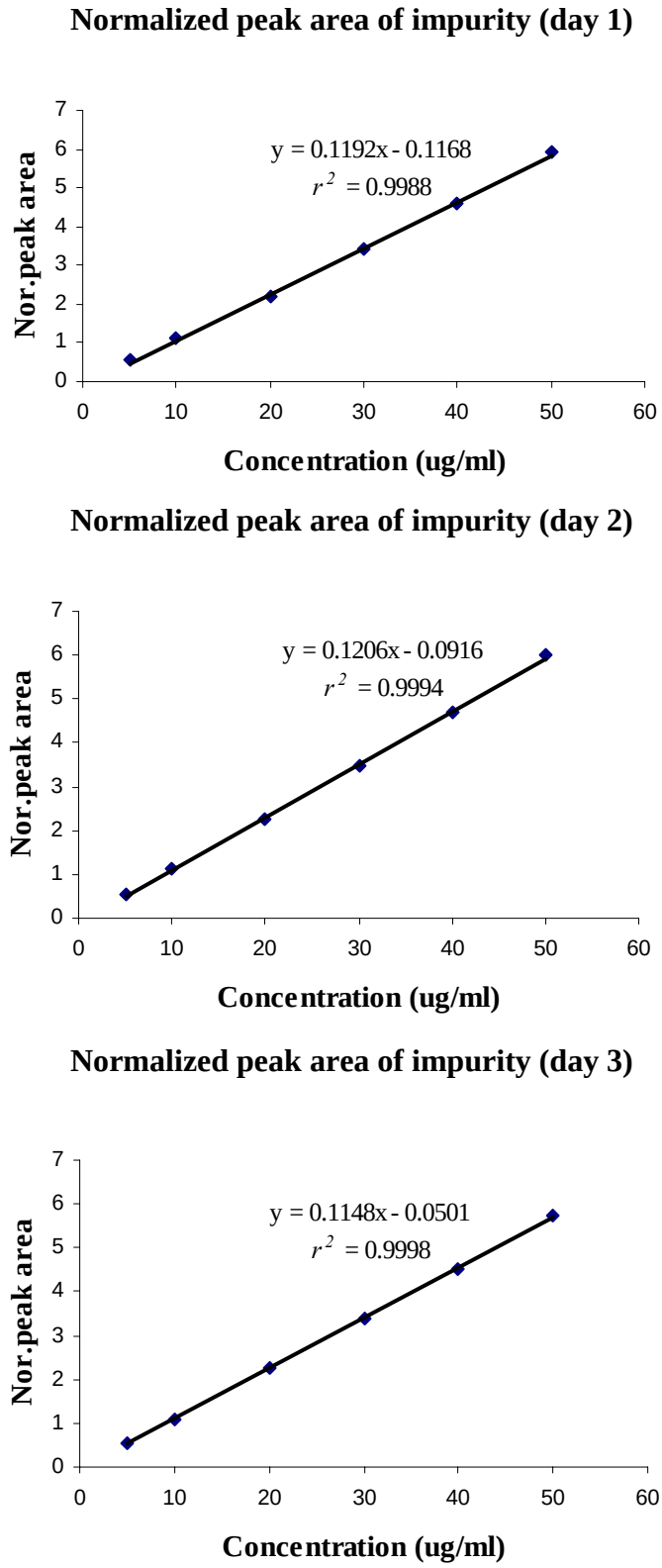


Fig. 60 Calibration data (normalized peak area VS concentration) of impurity for three different days.

APPENDIX C

Table 43 Regression data of *R/S*-baclofen and its impurity for assay the baclofen enantiomers and its impurity in raw materials and pharmaceutical formulation*

	Peak area	peak height	Normalized peak area
S-(+)-baclofen	y = 0.6634x + 8.564 (0.9995)	y = 0.1340x + 5.160 (0.9896)	y = 0.0824x + 0.973 (0.9996)
R-(-)-baclofen	y = 0.7419x + 10.367 (0.9997)	y = 0.1787x + 3.946 (0.9939)	y = 0.0902x + 1.183 (0.9995)
Impurity	y = 0.6965x + 0.099 (0.9991)	y = 0.4666x + 0.085 (0.9985)	y = 0.1328x + 0.015 (0.9996)

*numbers in parenthesis represent correlation coefficient (r^2)

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