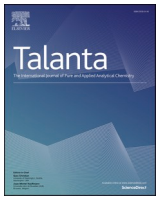
Talanta 253 (2023) 124020

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta



A chiral electrokinetic chromatography method for the separation and quantitation of licarbazepine and licarbazepine acetate in pharmaceutical formulations and urine samples

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ARTICLE INFO

Keywords:
Cyclodextrin
Electrokinetic chromatography
Enantiomeric separation
Licarbazepine
Pharmaceutical formulation
Urine

ABSTRACT

S-Licarbazepine acetate is a new antiepileptic that is quickly metabolized to S-licarbazepine which is the active principle. In this study, an enantioselective methodology enabling the simultaneous separation of licarbazepine acetate and licarbazepine by Electrokinetic Chromatography has been developed for the first time. After evaluating the potential of different chiral selectors, including bile salts and cyclodextrins, and selecting carboxymethyl-β-cyclodextrin as the most appropriate, a Box-Behnken experimental design was effectively applied for the optimization of the experimental separation conditions. Employing the best conditions, the four enantiomers were simultaneously separated (resolution values > 2.4) in less than 7 min. The evaluation of the figures of merit of the developed methodology showed to be suitable to determine both compounds. Finally, the EKC method was successfully applied in three different studies: (i) the quality control of the enantiopure pharmaceutical formulation, (ii) the monitoring of the stability and gastrointestinal digestion of the pharmaceutical formulation through a hydrolysis study, and (iii) the determination of licarbazepine enantiomers in urine samples.

1. Introduction

The existence of chiral molecules is relevant in different scientific disciplines including the bioanalytical and pharmaceutical fields since enantiomers with different configurations could play completely different biological functions. Due to the targets of drug action can discriminate between enantiomers, often the biological activity of a chiral compound may reside in one of its enantiomers. For this reason, the pharmaceutical industry promotes the commercialization of enantiopure drugs and, as a consequence, the chiral distinction and separation of enantiomers have received considerable attention.

S-licarbazepine acetate (S-licOAc), or eslicarbazepine acetate, is a new enantiopure drug employed in the treatment of epilepsy, a brain disorder characterized by an enduring predisposition to generate epileptic seizures [1]. This drug, which exerts its effect primarily as a classical blocker of voltage-gated sodium channels [1], is commercialized as S-enantiomer since so that S and R forms have patently different pharmacodynamic and pharmacokinetic properties being the enantiomer S the one with a better profile as an antiepileptic drug [1,2]. After oral administration, S-licOAc is metabolized, via rapid pre-systemic metabolic hydrolysis, solely to S-licarbazepine (S-licOH), its major active metabolite. Therefore, S-licOAc can be considered a pro-drug of S-licOH [3,4]. Subsequently, S-licOH undergoes a minor chiral inversion to R-licarbazepine (R-licOH) through a oxidation-reduction reaction of oxcarbazepine [2,5,6]. 90% of the S-licOAc administered (the usual once-daily dose ranges from 400 to 1200 mg) is mainly excreted in urine as S-licOH, R-licOH, and their glucuronide forms [3,5].

Several works reported the use of methods based on High Performance Liquid Chromatography (HPLC) with UV [7–15] or mass spectrometry (MS) detectors [7,16–18] to perform the chiral and/or achiral separation of different carbamazepine derivatives such as oxcarbazepine, licOH, or licOAc. These works were focused on the study of the biological effect of these compounds in humans or mice [8,14–18], the enantioselective synthesis of S-licOAc [12], or the determination of S-licOAc along with its impurities in bulk drugs or pharmaceutical

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https://doi.org/10.1016/j.talanta.2022.124020
Received 25 August 2022; Accepted 13 October 2022
Available online 17 October 2022
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dosage forms from an achiral [9,10] or chiral perspective [11]. Among all these works, just two of them have reported a stereoselective methodology to simultaneously separate licOH and licOAc enantiomers. El-Behairy and Sundby developed an HPLC method employing a mobile phase of cyclohexane/ethanol (1:1, v/v) and a Lux® cellulose-2 column. This methodology, which allowed the enantioseparation of RS-licOH and RS-licOAc (resolution values of 9.5 and 3.0, respectively) in 15 min, was employed for the monitoring of lipase-catalyzed kinetic resolution of RS-licOH [12]. On the other hand, two chiral HPLC methods were reported by Mone and Chandrasekhar for the estimation of R-licOAc in bulk drug samples of S-licOAc [11]. The enantioresolution of licOAc and licOH was achieved using a cellulose tris-(3,5-dichlorophenylcarbamate) column under polar organic mode or using a normal phase mobile phase. Even though the two methods showed to be suitable for the accurate estimation of R-licOAc in bulk drug samples up to a level of 0.1%, they were not applied to the analysis of real samples.

Yan et al. also demonstrated the potential of Supercritical Fluid Chromatography (SFC) to achieve the chiral separation of carbamazepine derivatives [19]. These authors developed a rapid method based on SFC-MS for the determination of oxcarbazepine and RS-licOH. Subsequently, the method was applied to a pharmacokinetic study in dog plasma samples.

Regarding the use of Capillary Electrophoresis (CE), one of the most potent analytical techniques in the field of chiral separations, just two works have described its use for the enantiomeric separation of RS-licOH [20,21]. In the former, Marziali et al. performed the separation of carbamazepine, oxcarbazepine, and five metabolites (including RS-licOH) by electrokinetic chromatography (EKC) using negatively charged cyclodextrins (CDs) as chiral selectors [20]. Using octakis-(2,3-dihydroxy)-6-sulfate-γ-CD, the RS-licOH enantiomers were baseline separated in 12 min whereas employing heptakis-6-sulfate-β-CD just a partial separation was achieved in 7 min. These methodologies were not applied to real samples. In the second work, Bocato et al. reported the simultaneous separation of oxcarbazepine and R- and S-licOH enantiomers by using β-cyclodextrin phosphate as chiral selector in EKC [21]. Under optimal conditions, the three compounds were separated in less than 8 min. The RS-licOH enantiomers were separated at baseline being S-licOH the first-migrating enantiomer. The methodology was applied to monitor the enantioselective biotransformation of oxcarbazepine, mediated by fungi, into its active metabolite S-licOH. To the best of our knowledge, the simultaneous chiral analysis of RS-licOAc and RS-licOH by CE has never been described before. Therefore, the aim of this work was to develop an innovative EKC methodology to carry out the simultaneous and fast separation of licOAc and licOH enantiomers. The most appropriate experimental conditions (in terms of resolution and analysis time) were chosen by employing a Box-Behnken experimental design. Subsequently, the developed methodology was applied to (i) the quality control of the enantiopure pharmaceutical formulation, (ii) monitor the stability and gastrointestinal digestion of the pharmaceutical formulation through a hydrolysis study, and (iii) determine licOH enantiomers in urine samples.

2. Materials and methods

2.1. Reagents and samples

Analytical grade chemicals and reagents were used, and ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

Boric acid, ammonium bicarbonate, sodium hydroxide (NaOH), dimethyl sulfoxide (DMSO), pancreatin (from porcine pancreas), and pepsin (from porcine gastric mucosa) were from Sigma (Saint-Louis, MO, USA). Hydrochloric acid (37%, v/v), formic acid, and methanol (MeOH) were from Scharlau (Barcelona, Spain). Di-sodium hydrogen phosphate anhydrous and dodecyl sulfate sodium salt (SDS) were from Panreac (Barcelona, Spain) and Merck (Darmstadt, Germany), respectively.

Regarding the chiral selectors, Methyl-γ-cyclodextrin (M-γ-CD, DS∼12), (2-hydroxypropyl)-γ-cyclodextrin (HP-γ-CD, DS∼4.5), heptakis (2,3,6-tri-O-methyl)-β-cyclodextrin (TM-β-CD), acetyl-β-cyclodextrin (Ac-β-CD, DS∼7), acetyl-γ-cyclodextrin (Ac-γ-CD, DS∼7), α-cyclodextrin sulfate sodium salt (S-α-CD, DS∼12), γ-cyclodextrin sulfate sodium salt (S-γ-CD, DS∼10), β-cyclodextrin phosphate sodium salt (P-β-CD, DS∼4), γ-cyclodextrin phosphate sodium salt (P-γ-CD, DS∼3.5), sulfobutylated-β-cyclodextrin sodium salt (SB-β-CD, DS∼6.3), succinyl-β-cyclodextrin (Succ-β-CD, DS∼3.4), succinyl-γ-cyclodextrin (Succ-γ-CD, DS∼3.5), carboxyethyl-β-cyclodextrin (CE-β-CD, DS∼3.5), carboxyethyl-γ-cyclodextrin (CE-γ-CD, DS∼3.5), carboxymethyl-α-cyclodextrin sodium salt (CM-α-CD, DS∼4.5), carboxymethyl-β-cyclodextrin sodium salt (CM-β-CD, DS∼3.5), and carboxymethyl-γ-cyclodextrin sodium salt (CM-γ-CD, DS∼3.5) were purchase from Cyclolab (Budapest, Hungary). (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD, DS∼0.6), γ-cyclodextrin (γ-CD), and β-cyclodextrin (β-CD) were from Fluka (Buchs, Switzerland). β-cyclodextrin sulfobutyl ethers sodium salt (CAPTISOL) was from CyDex Pharmaceuticals (Lawrence, KS, USA). α-cyclodextrin (α-CD), methyl-β-cyclodextrin (M-β-CD), heptakis (2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD), β-cyclodextrin sulfate sodium salt (S-β-CD, DS∼12–15), sodium cholate hydrate (SC), sodium deoxycholate (SDC), sodium taurocholate hydrate (STC), and sodium taurodeoxycholate hydrate (STDC) were provided by Sigma (Saint-Louis, MO, USA).

S-licOH and RS-licOH were purchased from MedChemExpress (Monmouth Junction, NJ, USA) and APExBIO (Houston, TX, USA), respectively. S-licOAc and R-licOAc were provided by Toronto Research Chemicals (Toronto, ON, Canada). The chemical structures of both compounds are shown in Fig. S1. The S-licOAc pharmaceutical formulation (containing 800 mg/tablet) was kindly provided by a Health Center of Guadalajara (Spain).

2.2. CE instrumentation and electrophoretic conditions

A 7100 CE system (Agilent Technologies from Palo Alto, CA, USA) equipped with a diode array detector (DAD) at 200 ± 4 nm with reference at 300 ± 80 nm and controlled by the ChemStation software was used to perform all the analyses. Uncoated fused-silica capillaries (effective length of 50 cm (total length of 58.5 cm) x 50 μm ID) from Polymicro Technologies (Phoenix, AZ, USA) were employed. Standards and samples were injected using 50 mbar for 5s.

Capillaries were initially conditioned by applying a pressure of 1 bar with 1 M NaOH, Milli-Q water, and buffer for 30, 15, and 60 min, respectively. Each day, the capillary was rinsed with 0.1 M NaOH (5 min), Milli-Q water (5 min), and buffer (30 min). Between analysis, it was rinsed for 3 min with the background electrolyte (BGE). When urine samples were analyzed it was necessary to wash the capillary each three injection with 0.1 M HCl (3 min), 1 min Milli-Q water (1 min), 0.1 M NaOH (3 min), Milli-Q water (1 min), and buffer 10 min.

2.3. Box-Behnken experimental design

The influence of the chiral selector concentration, voltage, and temperature on the separation of RS-licOH enantiomers was investigated using a Box-Behnken design. A total of 15 experiments (including three replicates of the center point) were performed in a randomized order. Each factor was tested at three levels: 1, 1.5, and 2% w/v for the chiral selector concentration; 20, 25, and 30 kV for the voltage; and 15, 20, and 25 °C for the temperature. The response variables were: the resolution value between the R and S-licOH enantiomers, and the migration time of S-licOH.

The model parameters were estimated using the software Statgraphics Centurion XVII- X64. The coefficient of determination (R^2), the lack-of-fit test, and the relative standard deviation (RSD), were used to evaluate the model adequacy. The theoretical optimal conditions, which

maximize the enantiomeric resolution and minimize the migration time, were provided by the software based on the criteria of the desirability function. Repetitions (n = 12) under the theoretical optimal conditions were performed experimentally to verify the study.

2.4. Preparation of BGEs and solutions

The different buffer solutions employed (100 mM borate buffer pH 9.0, 50 mM phosphate buffer pH 7.0, 50 mM ammonium bicarbonate pH 7.0, and 50 mM formate buffer pH 3.0) were prepared by dissolving the adequate amount of boric acid, disodium hydrogen phosphate, ammonium bicarbonate or formic acid in Milli-Q water and adjusting the pH to 9.0, 7.0 or 3.0, respectively with 1 M NaOH. BGEs containing CDs, SDS and/or bile sales were prepared dissolving the appropriate amounts of each one in the corresponding buffer.

Standard solutions of RS-licOH, S-licOH, R-licOAc, and S-licOAc were prepared dissolving appropriate amounts of each one in MeOH (final concentration of 1000 mg L^{-1}). These standard solutions were stored at −20 °C until their dilution to the final concentrations with MilliQ-water. Solutions were filtered (0.45 μm nylon syringe filters from GVS (Sanford, ME, USA)) and sonicated to avoid air bubbles.

2.5. Pharmaceutical solution preparation

The preparation of the pharmaceutical solutions of S-licOAc was performed by grinding and mixing homogeneously the content of 20 tablets. Considering the labeled amount of S-licOAc (800 mg/tablet) an appropriate amount of the powder obtained (11.7 mg) was dissolved (by ultrasonication for 10 min at room temperature) in 8.0 mL MeOH. Subsequently, the solution was centrifuged in a Centrifuge Megafuge 2.0R (Heraeus instruments) for 5 min at 4000 rpm and 20 °C. Then, the supernatant was separated and diluted to 10 mL with MeOH obtaining a final concentration of 1000 mg L^{-1}. This solution was diluted 1:10 (v/v) in MilliQ-water.

2.6. Hydrolysis studies of the pharmaceutical formulation

The hydrolysis study was performed under different conditions using in all cases three different replicate samples which were injected in triplicate.

1 mL of MeOH, 2 M HCl, or 0.1 M NaOH was added to 11.7 mg of the pharmaceutical powder (obtained from 20 tablets as it was described in the previous section). These solutions were stirred in a thermomixer (700 rpm, 1 h, room temperature) and centrifuged at 6000 rpm for 10 min. Supernatants were separated and diluted in MilliQ-water to a final volume of 2 mL (eq. 5000 mg L^{-1} S-licOAc). This procedure was also performed without using the centrifugation step. Afterward, 1 mL of these solutions were diluted to 5 mL with MeOH (eq. 1000 mg L^{-1} S-licOAc).

To carry out gastric digestion, 11.7 mg of the pharmaceutical powder were dissolved in 1 mL MilliQ-water at pH 2.0 (adjusted with 2 M HCl) plus 66.8 μL pepsin (5 mg mL^{-1} in MilliQ-water, enzyme/substrate ratio: 1/35). The solutions were stirred in a thermomixer at 700 rpm for 1 h at 37 °C. Then, the temperature was increased to 100 °C to stop the digestion and kept for 10 min. In the case of gastrointestinal digestion, this protocol continued adjusting the pH of the mixture to 8.0 with 1.0 M NaOH, adding 187.2 μL pancreatin (2.5 mg mL^{-1} in 0.1 M phosphate buffer pH 8.0, enzyme/substrate ratio: 1/25) and stirred in a thermomixer at 700 rpm for 2 h at 37 °C. Then, the temperature was increased to 100 °C and kept for 10 min. The solutions obtained both for gastric and gastrointestinal digestions were centrifuged at 6000 rpm for 10 min, and supernatants were diluted in MilliQ-water to a final volume of 2 mL (eq. 5000 mg L^{-1} S-licOAc). As before, the protocol was also performed without the centrifugation step. Finally, 1 mL of each solution was diluted to 5 mL with MeOH (eq. 1000 mg/L S-licOAc).

All the samples were diluted 1:10 (v/v) in MilliQ-water and filtered before their CE analysis.

2.7. Urine sample preparation

The amount of RS-licOH spiked to the urine sample was estimated considering: (i) the consumption of a dose of 800 mg S-licOAc per day [3,5], (ii) that the 90% of the amount ingested of S-licOAc is excreted in urine as approximately 62% S-licOH, 3% R-licOH and 35% derivates of glucuronic acid [2,3,5,22,23], and (iii) a volume of 2 L of urine excreted per day and a period of 72 h to eliminate completely the amount of drug ingested [2].

Thus, aliquots of 3 mL of urine samples (blank urine and urine spiked RS-licOH standards to obtain a final concentration of 3.0 mg L^{-1} R-licOH and 60.0 mg L^{-1} S-licOH) were submitted to a solid phase extraction (SPE) using Oasis™ HLB extraction cartridges (Waters, Milford, MA, USA). The cartridge was conditioned with MeOH (2 mL) and MilliQ-water (2 mL) before adding 3 mL of urine samples. Afterward, the cartridge was washed with MilliQ-water (0.5 mL) and the analyte was eluted with four portions of 0.5 mL of MeOH. These portions were combined and evaporated to dryness in a Concentrator from Eppendorf at 30 °C. The solid residue was reconstituted in 600 μL of MilliQ-water with a 10% of MeOH. Before CE analysis, an adequate volume of this solution was diluting in MilliQ-water with a 10% of MeOH to obtain the desired concentration for the injected solutions.

2.8. Data treatment

ChemStation software (Agilent Technologies) was employed to obtain the peak areas, resolution values, and migration times of each pair of enantiomers. Both the Box-Behnken design and the figures of merit of the developed methodology and statistical test were calculated and treated with STATGRAPHICS Centurion XVII- X64 and Microsoft Excel 365 programs. Figures displaying electropherograms and those that depict the analytes structures were performed using Origin 8.0 and ChemDraw Professional 15.0 software, respectively.

3. Results and discussion

3.1. Development of a CE methodology for the simultaneous enantiomeric separation of licOAc and licOH

LicOH, the active metabolite of licOAc, is a neutral compound throughout the pH range which is a key parameter to take into consideration to design an approach based on the use of CE to achieve its enantioselective separation. Bearing this in mind, the modes micellar electrokinetic chromatography (MEKC) and EKC can be considered as two possible alternatives.

First, different experiments were carry out using the MEKC mode. On the one hand, the discrimination potential of four different bile salt (SC, SDC, STC, STDC at concentrations of 50, 100, and 150 mM) was evaluated using a 100 mM borate buffer (pH 9.0), 20 kV, and 20 °C. In subsequent analyses, SC and SDC (at concentrations of 50, 100 and 150 mM) were mixed with a set of neutral CDs at 10 mM (α-CD, β-CD, γ-CD, HP-β-CD, HP-γ-CD, M-β-CD, M-γ-CD, DM-β-CD, TM-β-CD, Ac-β-CD, and Ac-γ-CD). On the other hand, MEKC analyses were also carried out by employing a mixture of a micellar system (SDS at concentrations ranging from 50 to 100 mM) with five different neutral CDs at 10 mM (HP-β-CD, M-β-CD, M-γ-CD, DM-β-CD, TM-β-CD). None of the MEKC approaches enabled the chiral separation of RS-licOH (data not shown).

To develop an EKC method, the potential of different negatively charged CDs as chiral selector was tested at two different pH values. In a first screening test, seven CDs (S-α-CD, S-β-CD, S-γ-CD, P-β-CD, P-γ-CD, SB-β-CD, and CAPTISOL) at 1.0% (w/v) in 50 mM formate buffer at pH 3.0 and working in negative polarity (−20 kV) were evaluated. In a second screening, the same CDs plus other seven CDs (Succ-β-CD, Succ-γ-CD, CE-β-CD, CE-γ-CD, CM-α-CD, CM-β-CD, CM-γ-CD) at 1.0% (w/v) in

Table 1
Migration times and resolution values (average value ± standard deviation, n = 3) obtained for the chiral separation of RS-licOH using as chiral selector different negatively charged CDs.

BGE	50 mM formate buffer pH 3.0			50 mM phosphate buffer pH 7.0		
Cyclodextrin (1% w/v)	$t_{S\text{-licOH}}$ (min)	$t_{R\text{-licOH}}$ (min)	Rs	$t_{R\text{-licOH}}$ (min)	$t_{S\text{-licOH}}$ (min)	Rs
S-α-CD	>60 min	>60 min	–	9.18 ± 0.01	–	–
S-β-CD	>60 min	>60 min	–	8.49 ± 0.03	–	–
S-γ-CD	55.3 ± 0.5	–	–	8.7 ± 0.3	–	–
CAPTISOL	21.4 ± 0.2	22.7 ± 0.2	2.8 ± 0.1	11.3 ± 0.1	11.7 ± 0.1	1.62 ± 0.03
SB-β-CD	20.8 ± 0.3	22.1 ± 0.3	2.4 ± 0.4	10.8 ± 0.1	11.2 ± 0.1	1.6 ± 0.2
P-β-CD	24.9 ± 0.4	28.4 ± 0.5	3.08 ± 0.02	11.80 ± 0.09	12.36 ± 0.09	2.8 ± 0.1
P-γ-CD	37.4 ± 0.2	35.8 ± 0.1	2.05 ± 0.06	9.55 ± 0.09	–	–
Succ-β-CD	NT	NT	NT	11.01 ± 0.03	11.23 ± 0.04	0.99 ± 0.06
Succ-γ-CD	NT	NT	NT	10.8 ± 0.2	–	–
CE-β-CD	NT	NT	NT	11.6 ± 0.1	11.9 ± 0.1	1.4 ± 0.1
CE-γ-CD	NT	NT	NT	11.94 ± 0.08	–	–
CM-α-CD	NT	NT	NT	8.61 ± 0.05	–	–
CM-β-CD	NT	NT	NT	9.69 ± 0.08	10.02 ± 0.08	1.84 ± 0.01
CM-γ-CD	NT	NT	NT	7.4 ± 0.1	–	–

Other experimental conditions: uncoated fused-silica capillary of 50 μm ID x 58.5 cm (50 cm of effective length); applied voltage, −20 kV at pH 3.0 and 20 kV at pH 7.0; temperature, 20 °C; injection by pressure, 50 mbar for 5 s.
> 60 min: no peaks were obtained in 60 min.
-: enantiomeric separation was not achieved.
NT: not tested under this condition.

50 mM phosphate buffer at pH 7.0 and working in positive polarity (+20 kV) were studied. As Table 1 shows, the use of CAPTISOL, SB-β-CD, P-β-CD, and P-γ-CD in acidic conditions allowed the enantiomeric separation of RS-licOH with resolution values (Rs) higher than 2.0 in analysis times from 22 to 36 min. In all these analyses, except in that carried out using P-γ-CD as chiral selector, the enantiomeric impurity (R-licOH) was the second-migrating enantiomer. When the experiments were performed at pH 7.0, the enantiomers were separated at times below 13 min with Rs from 0.99 to 2.8 (see Table 1). In this case, R-licOH migrates in the first place which is always the preferred situation. This fact together with the idea of developing a fast methodology with enough resolution was the reason for selecting phosphate buffer at pH 7.0 to perform further experiments.

Since the CD concentration can affect the enantioseparation, different percentages of those CDs that originated a Rs > 1.5 were tested. Fig. 1 represents the electropherograms obtained for the separation of RS-licOH enantiomers using CM-β-CD, P-β-CD, SB-β-CD, or CAPTISOL at percentages from 0.75 to 1.75% (w/v). In general, both the analysis time as the Rs increased when increasing the CD concentration. In all cases except for SB-β-CD, it was possible to achieve a Rs higher than 2.0 when percentages of 1.5% (w/v) were employed. Then, keeping this concentration for the three CDs (CM-β-CD, P-β-CD, and CAPTISOL), the effect of the buffer nature on the enantiomeric separation was also investigated. The results obtained using 50 mM ammonium bicarbonate, which has a lower conductivity than 50 mM phosphate buffer, demonstrated that a slight decrease in the analysis time took place in all cases (data shown in Table S1). Among the three CDs, CM-β-CD provided a shorter migration time and a good Rs so it was selected.

Before employing an experimental design to carry out a multivariate optimization of different experimental parameters in the enantiomeric separation of RS-licOH, it was necessary to establish an appropriate protocol for the capillary conditioning between analyses to achieve an adequate instrumental repeatability. The protocol used so far (based on the preconditioning with 0.1 NaOH (2 min), Milli-Q water (1 min), and BGE (3 min)) was compared with the use of a simple protocol based on the preconditioning with BGE for 3 min (data shown in Table S2). The use of the simple protocol improved the repeatability of peak areas although no variation was observed concerning migrations times. Then, taking into account the CM-β-CD migration, the interchanging of the inlet and outlet vials after each run was also investigated to improve the repeatability. In this way, after each run, the vials were interchanged to prevent the decrease of the CD concentration in the cathode vial whereas the concentration in the other reservoir would increase [24,25]. Following this strategy, a considerable improvement in the repeatability was observed with RSD values lower than 0.6% for migration times and 1.9% for peak areas when six consecutive injections were carried out. Even increasing the number of injections (n = 12) the RSD values were better than those obtained without interchanging vials (see Table S2). Another factor that should also be mentioned is the fact that without interchanging the vial, the current generated into the capillary decreased with the number of injections which affected the migration time so no more than six injections can be performed. However, the currents were stable in more than twelve consecutive injections when interchanging the vials. Taking into account that the baseline noise also increased after each run, no more than twelve injections can be done to keep the noise as low as possible. Based on these results, a preconditioning of 3 min with BGE and the interchange strategy were selected since this protocol favors repeatability and is advantageous from an economical point of view due to the cost of the chiral selector.

A Box-Behnken experimental design was performed for multivariate optimization of CM-β-CD concentration, applied voltage, and temperature. Unlike a trial-and-error approach, the use of an experimental design considers also the dependence among the optimized parameters. Table S3 displays the experimental matrix along with the results obtained for the two response variables (migration time of S-licOH and Rs). The parameters obtained in the models along with the statistical values for the goodness of fit are listed in Table 2 whereas the response surface plots of the two response variables are shown in Fig. S2. As can be seen in Table 2, the three factors studied can affect the migration time of S-licOH and Rs with a significant effect (p-value < 0.05). Among them, the term that mostly influenced the migration time is the voltage whereas the CM-β-CD concentration was the most important in the Rs. From the results obtained concerning the model, the following conclusions can be drawn: (i) the variability of both response variables explained by the model, which is indicated by the determination coefficient, is satisfactory (R^2 values of 0.997 for the migration time and 0.974 for the Rs), (ii) RSD values of the fit for both response variables were below 0.16, (iii) the RRSD values (which established the relative error of the fit and are calculated as a percentage of the mean value of the response) were 2.5% and 7.7% for the migration time and Rs, respectively. Moreover, the models showed to be appropriate to describe the data since the p-value of lack-of-fit test was higher than 0.05. Then, a multiple response optimization was carried out to determine the values of the factors which allowed to obtain the highest Rs in a lower time. In this way, the optimal levels of the factors were 2.0% CM-β-CD, 29.4 kV, and 15 °C. Under these conditions, a migration time of 5.6 min and a Rs of 2.3 were predicted by the model, with an overall desirability value of 0.947. These data were close to the values experimentally obtained (5.5 min and Rs of 2.4). which means that the predictive model allowed to attain a good prediction to obtain the separation of RS-licOH enantiomers in a short time with a high resolution.

Taking into account that S-licOH is the active metabolite of S-licOAc, it is interesting to investigate if the developed EKC methodology allowed

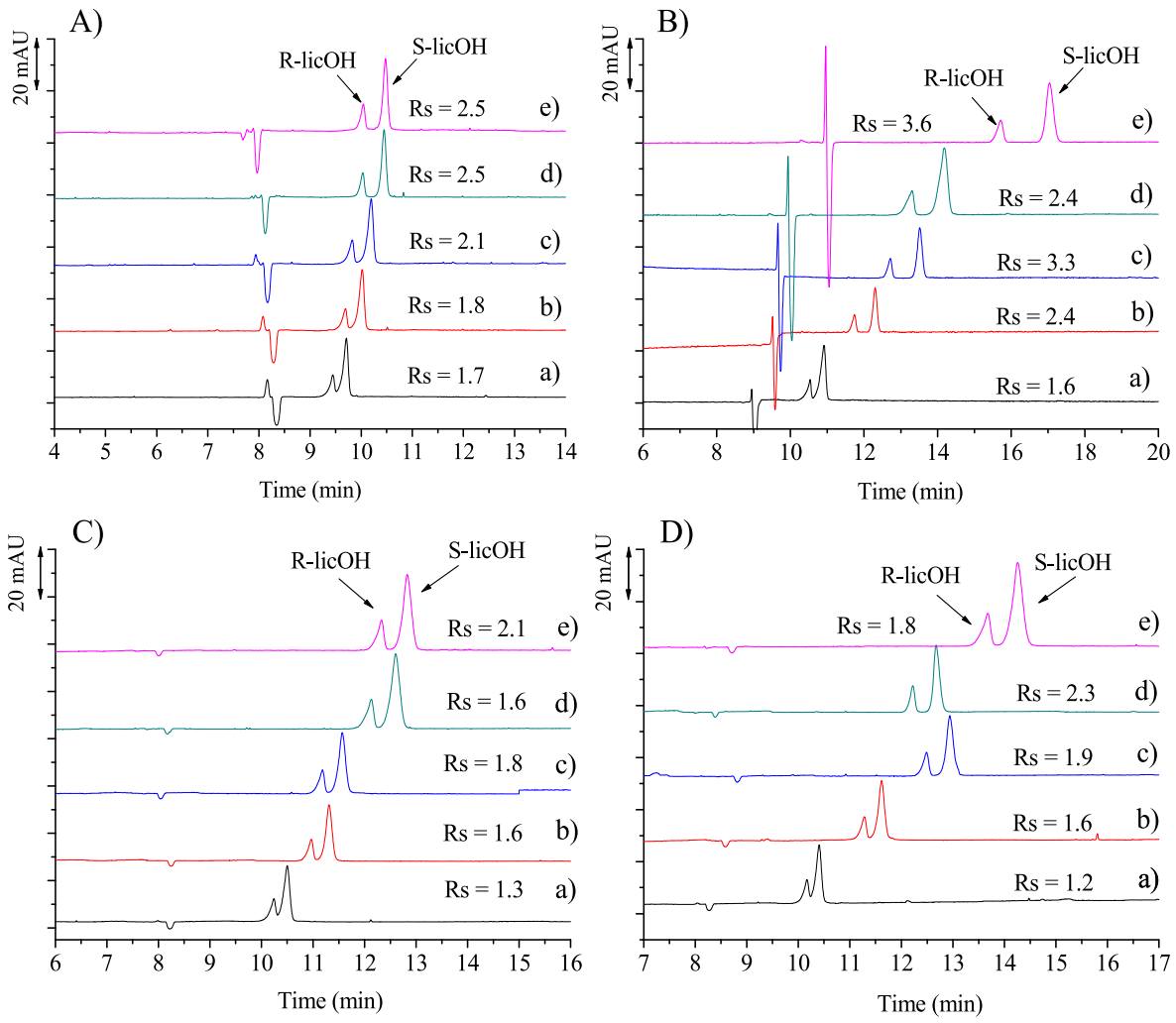

Fig. 1. Chiral separation of 50 mg L^{-1} R-licOH and 150 mg L^{-1} S-licOH using four negatively charged CDs at different percentages. A) CM-β-CD, B) P-β-CD, C) SB-β-CD, and D) CAPTISOL. Experimental conditions: BGE: CDs at a) 0.75% (w/v), b) 1.00% (w/v), c) 1.25% (w/v), d) 1.50% (w/v), and e) 1.75% (w/v) in 50 mM phosphate pH 7.0; uncoated fused-silica capillary of 50 μm ID x 50 cm (58.5 cm total length); applied voltage, 20 kV; temperature, 20 °C; UV detection at 200 ± 4 nm with reference at 300 ± 80 nm; injection by pressure, 50 mbar for 5 s.

Table 2
Coefficients (p-values from the analysis of variance) and statistics for the fit obtained by multiple linear regression models from the Box-Behnken experimental design.

	t S-licOH	Rs
Terms of the model		
Constant	25.16	1.1125
V (kV)	−0.825 (0.0009)	0.126 (0.0474)
T (°C)	−0.456 (0.0055)	−0.110 (0.0223)
[CM-β-CD] (%, w/v)	1.952 (0.0214)	0.988 (0.0143)
V x V	0.009 (0.1002)	−0.002 (0.5695)
V x T	0.006 (0.1969)	−0.002 (0.5049)
V x [CM-β-CD]	−0.099 (0.0865)	−0.019 (0.5585)
T x T	0.004 (0.3586)	0.002 (0.4899)
T x [CM-β-CD]	0.003 (0.9325)	0.004 (0.8969)
[CM-β-CD] x [CM-β-CD]	0.408 (0.3379)	0.068 (0.8324)
Statistics for goodness of fit of the model		
R^2	0.997	0.974
RSD	0.157	0.136
RRSD (%)	2.5	7.7
P	0.542	0.708

R^2: determination coefficient, RSD: relative standard deviation, *P*: p-value of the lack-of-fit test for the model, RRSD: relative standard deviation expressed as a percentage of the mean value of the response.

the simultaneous enantioseparation of RS-licOH and RS-licOAc to broaden the application range of the methodology. As it can be seen in Fig. 2, the simultaneous enantioseparation of RS-licOH and RS-licOAc with high resolution (Rs > 2.4) in an analysis time lower than 7 min was possible using the optimized conditions.

3.2. Method performance

Different figures of merit such as linearity, precision, limits of detection (LOD), and limits of quantification (LOQ) were evaluated according to the International Council on Harmonization (ICH) guidelines Q2 (R1) [26] to demonstrate the quality of the developed EKC methodology.

Linearity was determined using the external standard method by injecting in triplicate seven calibration levels of R-enantiomer and nine calibration levels of S-enantiomer on two different days. As Table 3 shows, correlation coefficients higher than 0.998 were obtained and all the confidence intervals (at 95% confidence level) for intercept included the 0 value and did not include the zero value for the slopes. Data for the four enantiomers fit to a linear model since in all cases the p-values from an ANOVA test were higher than 0.05. In addition, the response relative factors (RRF), (slope of the impurity (R-enantiomers)/slope of the active enantiomer (S-enantiomers)), were 1.02 for licOH and 0.90 for licOAc,

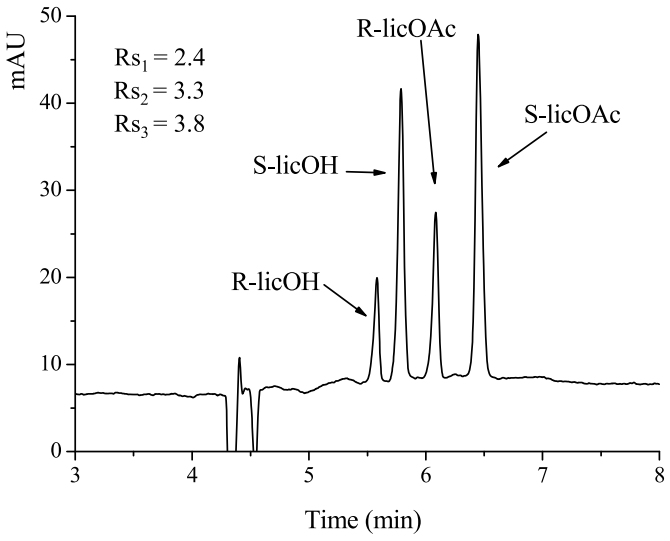

Fig. 2. Simultaneous chiral separation of RS-licOH and RS-licOAc by EKC. Experimental conditions: BGE, 2.0% (w/v) CM-β-CD in 50 mM ammonium bicarbonate buffer (pH 7.0); applied voltage, 29.4 kV; temperature, 15 °C. Other experimental conditions as in Fig. 1. Rs_1, Rs_2, Rs_3 correspond to the resolution value between consecutive enantiomers.

which indicates that the response of both enantiomers of licOH and licOAc can be considered equivalent according to the European Pharmacopoeia [27]. Therefore, the percentage of the R enantiomers can be established from the ratios between the areas of the R and S enantiomers.

Instrumental repeatability, method repeatability, and intermediate precision were calculated to evaluate method precision. To do that, standard solutions containing 10 mg L^{-1} R-enantiomers and 100 mg L^{-1} S-enantiomers were analyzed. Instrumental repeatability was assessed by six consecutive injections (n = 6) of a standard solution, the method repeatability was evaluated with three replicates of standard solutions injected in triplicate on the same day, and the intermediate precision was evaluated by injecting in triplicate three replicates of a standard solution during three different days. In all cases, RSD values were lower than 2.7% for migration times and 3.7% for peak areas as it can be observed in Table 3.

LODs and LOQs were experimentally calculated as the minimum concentration yielding a signal-to-noise ratio of 3 and 10 times, respectively. LODs of 1.2 mg L^{-1} for R-licOH and 1.3 mg L^{-1} for S-licOH, R-licOAc and S-licOAc, were calculated whereas LOQs were 4.1 mg L^{-1} for R-licOH, 4.2 mg L^{-1} for R-licOAc and, 4.3 mg L^{-1} for S-licOH and S-licOAc.

3.3. Applications

Once demonstrated the suitability of the developed method, it was applied in three different studies: (i) to carry out the quality control of a pharmaceutical formulation, (ii) to monitor the stability and gastrointestinal digestion of a pharmaceutical formulation through a hydrolysis study, and (iii) to determine RS-licOH in urine.

Table 3
Analytical characteristics of the method developed for the simultaneous chiral analysis of RS-licOH and RS-licOAc by EKC.

	R-licOH	S-licOH	R-licOAc	S-licOAc
External standard calibration method [a]				
Range	5–25 mg L^{-1}	5–175 mg L^{-1}	5–25 mg L^{-1}	5–175 mg L^{-1}
Slope ± t · S_a	0.92 ± 0.02	0.90 ± 0.02	0.90 ± 0.07	1.00 ± 0.03
Intercept ± t · S_b	−0.25 ± 0.26	−1.6 ± 2.3	0.005 ± 1.11	−0.10 ± 3.24
R	0.9999	0.9996	0.998	0.9993
p-value (ANOVA) [b]	0.9660	0.1243	0.0966	0.5308
Standard additions calibration method [c]	**Urine sample**		**Pharmaceutical formulation**	
Slope ± t · S_a	0.9 ± 0.1	0.8 ± 0.2	–	1.03 ± 0.01
Precision				
Instrumental repeatability [d]				
t, RSD (%)	0.1/0.8#	0.1/0.8#	1.0/1.5*	1.1/1.6*
A, RSD (%)	3.3/5.0#	3.3/1.0#	1.9/3.0*	1.8/3.5*
Method repeatability [e]				
t, RSD (%)	0.4/1.0#	0.4/1.1#	1.1/1.3*	1.2/1.4*
A, RSD (%)	3.1/5.2#	2.8/2.3#	2.6/3.6*	2.3/3.9*
Intermediate precision [f]				
t, RSD (%)	1.8/1.1#	1.9/1.2#	2.6/1.8*	2.7/1.9*
A, RSD (%)	3.7/5.7#	3.6/3.2#	2.8/2.7*	2.2/2.8*
Accuracy [g]				
Recovery	99 ± 9%	99 ± 7%	–	100 ± 5%
LOD [h]	1.2 mg L^{-1}	1.3 mg L^{-1}	1.3 mg L^{-1}	1.3 mg L^{-1}
LOQ [h]	4.1 mg L^{-1}	4.3 mg L^{-1}	4.2 mg L^{-1}	4.3 mg L^{-1}

#Urine sample.
*Pharmaceutical formulation.
[a] Seven or nine standard solutions at different concentration levels of R-enantiomer or S-enantiomer, respectively, injected in triplicate for two days.
[b] ANOVA p-value to confirm that the experimental data fit properly to linear models.
[c] Comparison of the confidence intervals for the slopes corresponding to the standard addition and the external standard calibration methods.
[d] Six consecutive injections (n = 6) of: a standard solution containing 10 mg L^{-1}, of R-enantiomers + 100 mg L^{-1}, of S-enantiomers, a urine sample containing 5 mg L^{-1}, R-licOH + 100 mg L^{-1}, S-licOH, or a pharmaceutical formulation containing 10 mg L^{-1}, R-licOAc + 100 mg L^{-1}, S-licOAc.
[e] Three solutions of a standard (containing 10 mg L^{-1}, of R-enantiomers + 100 mg L^{-1}, of S-enantiomers), a urine sample (containing 5 mg L^{-1}, R-licOH + 100 mg L^{-1}, S-licOH) or a pharmaceutical formulation (containing 10 mg L^{-1}, R-licOAc + 100 mg L^{-1}, S-licOAc) injected in triplicate on the same day (n = 9).
[f] Three different solutions of a standard (containing 10 mg L^{-1}, of R-enantiomers + 100 mg L^{-1}, of S-enantiomers), a urine sample (containing 5 mg L^{-1}, R-licOH + 100 mg L^{-1}, S-licOH) or a pharmaceutical formulation (containing 10 mg L^{-1}, R-licOAc + 100 mg L^{-1}, S-licOAc) injected in triplicate three different days (n = 9).
[g] Mean recovery obtained when the urine sample containing 5 mg L^{-1}, R-licOH + 100 mg L^{-1}, of S-licOH, or the pharmaceutical formulation containing 75 mg L^{-1}, of S-licOAc were spiked with known concentrations of RS-licOH and S-licOAc, respectively.
[h] LOD and LOQ experimentally obtained for a S/N = 3 or S/N = 10, respectively.

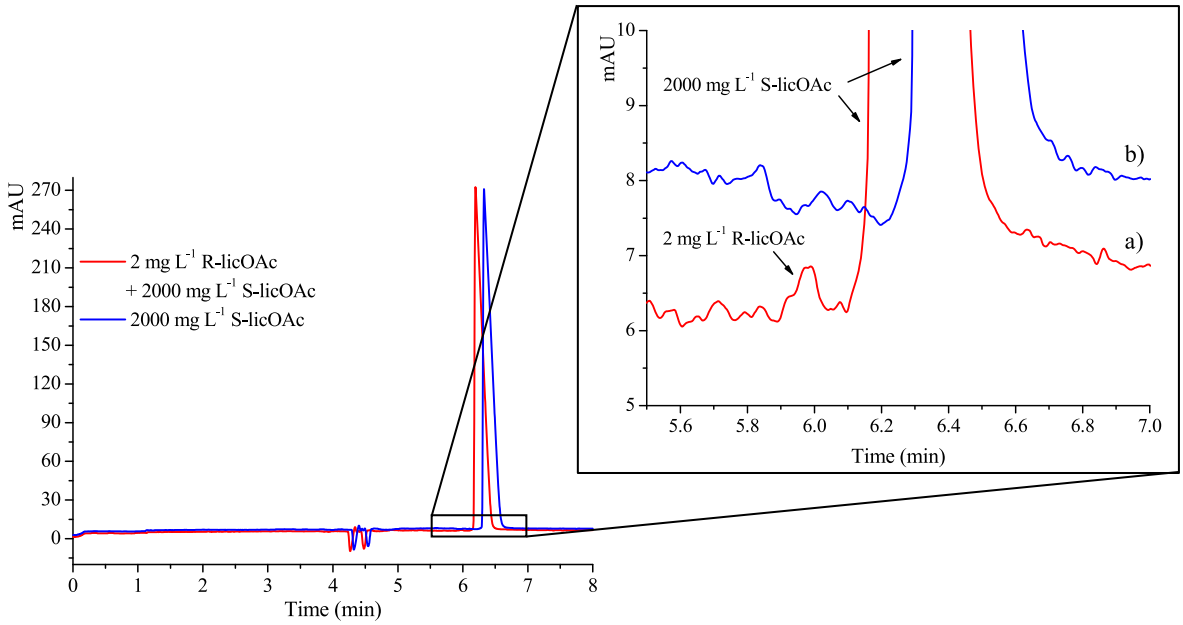

Fig. 3. Electropherograms obtained for a pharmaceutical formulation containing 2000 mg L^{-1} S-licOAc non-spiked (a) and spiked with 0.1% R-licOAc (b). Experimental conditions as in Fig. 2.

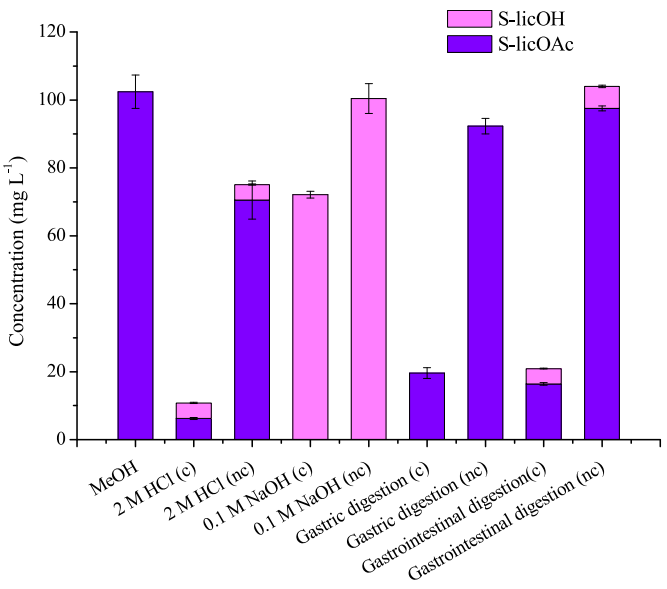

Fig. 4. Contents of S-licOAc and S-licOH determined after submitting the pharmaceutical formulation to different hydrolysis conditions. Experimental conditions as in Fig. 2. (c): a centrifugation step included in the sample preparation, (nc): centrifugation step not included in the sample preparation.

3.3.1. Determination of S-licarbazepine acetate in a pharmaceutical formulation

As it has been mentioned in the introduction, S-licOAc is an anticonvulsant drug. The quality control of this kind of enantiopure drug includes the control of the presence of enantiomeric impurities. In fact, the regulatory authorities establish that these impurities cannot exceed 0.1% in the enantiomerically pure formulation [28–30]. In this framework, the chiral methodology developed in this work acquires importance.

Before applying the EKC method, the existence of matrix interferences in the pharmaceutical formulation was investigated. Thus, a standard additions calibration method was achieved by adding five known amounts of S-licOAc to the pharmaceutical solution (containing 75 mg L^{-1} S-licOAc). Since there were no statistically significant differences between the confidence intervals obtained for the slopes and those obtained by the external calibration method (95% confidence level), it can be concluded that no matrix interferences existed (see Table 3). Thus, the external calibration method can be employed to carry out the quality control of the pharmaceutical formulation. The method's precision and accuracy were also determined for the pharmaceutical sample. The former was evaluated following the same procedure described in section 3.2 for standard samples. RSD values for R- and S-liOAc were lower than 1.9% for the migration time and lower than 3.9% for the peak areas. Accuracy was evaluated as the recovery obtained when the pharmaceutical formulation solution at a concentration of 75 mg L^{-1} S-licOAc was spiked with different amounts of S-licOAc. The recovery value was 100 ± 5% (Table 3). Moreover, the relative limit of detection (RLOD), calculated according to the LOD for R-licOAc and the nominal concentration injected for S-licOAc (2000 mg L^{-1}) was 0.065%.

Then, the EKC method was used to analyze the pharmaceutical formulation. Fig. 3, represents the electropherograms of the pharmaceutical formulation (S-licOAc) and the same sample spiked with a 0.1% of R-licOAc. The result obtained for the content of S-licOAc was 810 ± 32 mg S-licOAc per tablet. This value corresponds to a percentage of 101 ± 4% with respect to the labeled content. In addition, as it can be observed in Fig. 3, R-licOAc could not be detected so its concentration was below 0.1% according to the ICH guidelines.

3.3.2. Hydrolysis studies of the pharmaceutical formulation

To obtain a deeper understanding of S-licOAc stability and gastrointestinal digestion, a hydrolysis study of the pharmaceutical formulation was carried out. Thus, the sample was submitted to acid/basic media (employing HCl and NaOH solutions), gastric digestion (pepsin in acidic media), and gastrointestinal digestion (pancreatin in basic media after gastric digestion). subsequently, it was analyzed employing the developed EKC methodology.

All these experiments were conducted in two different ways. On the one hand, they were performed using a centrifugation step to remove the solid phase, and on the other hand, they were achieved without centrifugation before the dilution with MeOH. Fig. 4 depicts all the results obtained. As this figure shows, the use or not of the centrifugation

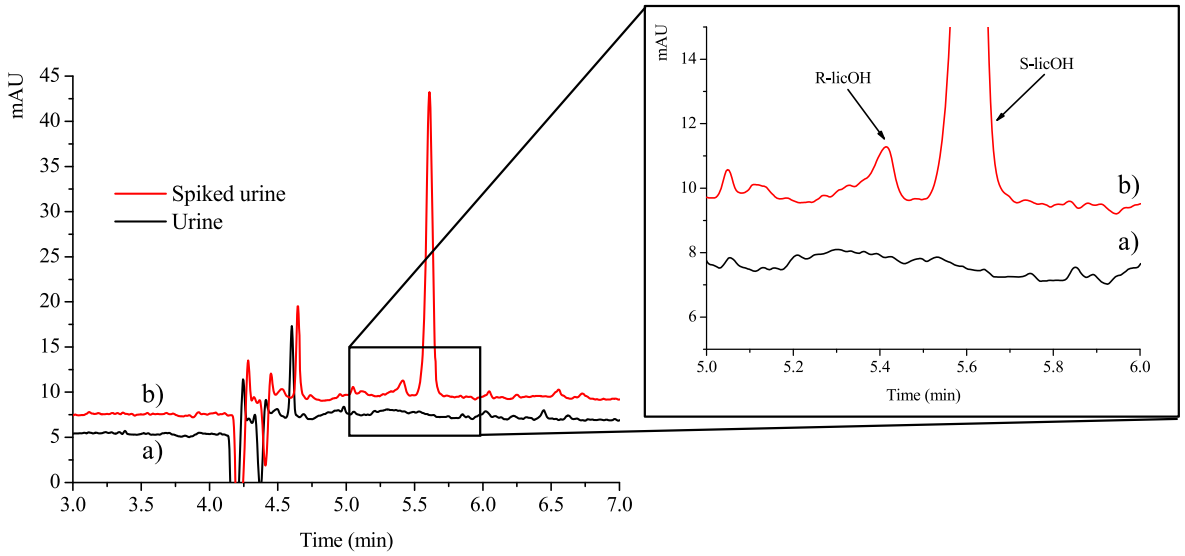

Fig. 5. Electropherograms corresponding to the urine sample. (a) Urine blank, (b) urine containing 5 mg L^{-1} R-licOH and 100 mg L^{-1} S-licOH. Experimental conditions as in Fig. 2.

step proved to be a relevant factor in the quantitative analysis of S-licOAc and S-licOH probably due to the different solubility of these species (log P (licOAc) = 1.99, log P (licOH) = 1.26). The pH of the medium was also a key parameter since under strongly acidic conditions a small amount of S-licOAc was hydrolyzed to S-licOH while under basic conditions it was totally hydrolyzed to S-licOH. The influence of pH was also observed then gastric digestion was employed. According to section 2.6, this experiment was carried out using the enzyme pepsin in an acidic media, but under these conditions, S-licOAc was not hydrolyzed to S-licOH. On the other hand, simulating complete gastrointestinal digestion, just a portion of S-licOAc was hydrolyzed to S-licOH.

The results obtained in this study indicated that the hydrolysis of S-licOAc to S-licOH was favored in strongly alkaline conditions. However, under the pH at which the simulated gastrointestinal digestion was performed (pH 8.0) the S-licOAc hydrolysis was not complete. This fact revealed that other biological factors must be involved in the hydrolysis, metabolism, and absorption of the pharmaceutical formulation.

3.3.3. Determination of RS-licarbazepine in urine

Analysis of urine samples may provide information about the degradation pathways and the elimination of drugs administered to patients. In this sense and bearing in mind that S-licOAc is a pro-drug of S-licOH [3], the EKC method developed in this work was also applied to the determination of RS-licOH in spiked urine (see section 2.7).

Two different SPE cartridges (Discovery C18 and Oasis HLB) were tested to carry out the extraction, clean-up, and pre-concentration of RS-licOH in urine. First, the cartridges were washed with 1 mL MilliQ-water and the analytes were eluted with three portions of 0.5 mL MeOH. Under these conditions, the recoveries obtained were 60 ± 5% for R-licOH and 75 ± 3% for S-licOH using Discovery C18, and 67 ± 3% and 92 ± 8% for R-licOH and S-licOH, respectively, using Oasis HLB. Since the HLB cartridge enabled to achieve not only better recoveries but also fewer interferences, it was chosen for further analysis. With the aim to improve the recoveries, the volume of MilliQ-water used to wash the cartridge was reduced to 0.5 mL, and the elution was achieved using four portions of 0.5 mL MeOH. In this way, recoveries of 84 ± 2% for R-licOH and 96 ± 10% for S-licOH were reached.

As in section 3.3.1, before applying the developed EKC methodology, the existence of matrix interferences was evaluated. In this case, the standard additions calibration method was performed by adding four different amounts of RS-licOH to the urine sample containing 5 mg L^{-1} R-licOH and 100 mg L^{-1} S-licOH. The comparison of the confidence intervals for the slopes obtained using the external standard calibration method and the standard additions calibration method (see Table 3) demonstrated that there were no statistically significant differences at a 95% confidence level. Method precision was also evaluated using the urine sample containing 5 mg L^{-1} R-licOH and 100 mg L^{-1} S-licOH. RSD values were lower than 1.2% and 5.7% for migration times and peak areas, respectively. The recovery values obtained when the urine samples containing 5 mg L^{-1} R-licOH and 100 mg L^{-1} S-licOH were spiked with different amounts of RS-licOH standard solutions were 99 ± 9% for R-licOH and 99 ± 7% for S-licOH.

Finally, the EKC method was applied to the analysis of RS-licOH in urine. The electropherograms obtained are shown in Fig. 5. As it can be observed in this figure, the developed methodology allowed to attain satisfactory results for the determination of both enantiomers at the minimum concentration excreted by a patient ingesting a normal dose of 800 mg of S-licOAc per day.

4. Conclusions

A chiral analytical methodology was developed to achieve, for the first time, the simultaneous separation of licOH and licOAc by EKC. After using a Box-Behnken experimental design to establish the optimal separation conditions, short analysis times and good resolution values were obtained using carboxymethyl-β-cyclodextrin (CM-β-CD) as chiral selector at a percentage of 2.0% in 50 mM ammonium bicarbonate buffer (pH 7.0). After evaluating the figures of merit of the developed methodology, it was applied to three different studies. On the one hand, the EKC method demonstrated its potential to carry out the quality control of an enantiopure pharmaceutical formulation. This study revealed that the content of S-licOAc agreed with the labeled one and that R-licOAc was not present at concentrations above 0.1% accomplishing the requirements established by the ICH guidelines. The EKC methodology was also employed to perform a hydrolysis study of the pharmaceutical formulation. From the results obtained, it can be concluded that strong alkaline conditions favor the hydrolysis of S-licOAc to S-licOH but the pH of gastric and intestinal juices seem not to be enough to give rise to the complete hydrolysis of S-licOAc so that other biological factors must be also involved in the hydrolysis, metabolism, and absorption of this pharmaceutical formulation. Finally, the chiral analysis of licOH enantiomers in a spiked urine sample was also carried out demonstrating the suitability of the developed methodology to determine both enantiomers at the minimum concentration excreted

by a patient ingesting a normal dose of S-licOAc. In this way, this methodology can be considered a relevant tool to carry out pharmacokinetics studies of S-licOAc.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Authors thank the Spanish Ministry of Science and Innovation for research project PID2019-104913 GB-I00, Agencia Estatal de Investigación, Referencia del Proyecto/AEI/10.13039/501100011033. S.S.F also thanks the University of Alcalá for her pre-doctoral contract.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2022.124020.

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