Use of choline chloride-D-sorbitol deep eutectic solvent as additive in Cyclodextrin-Electrokinetic Chromatography for the enantiomeric separation of lacosamide

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Abbreviations: CD (Cyclodextrin); ChCl (Choline chloride); DES (Deep eutectic solvent); DS (Average degree of substitution); EKC (Electrokinetic chromatography); EMA (European Medicines Agency); EtGly (Ethylene glycol); EtOH (Ethanol); FDA (Food and Drug Administration); HBA (Hydrogen-bond acceptor); HBD (Hydrogen-bond donor); ICH (International Council for Harmonisation); IL (Ionic Liquid); MeOH (Methanol); MW (Molecular weight); RLOD (Relative limit of detection); RRF (Response relative factor); SFC (Supercritical fluid chromatography).

Highlights

- The use of the chiral deep eutectic solvent ChCl-D-sorbitol in CE is reported

- The enantiomeric separation of lacosamide was achieved for the first time by CD-EKC

- The effect of different variables on the enantiomeric separation was investigated

- An enantiomeric resolution of 2.8 was obtained for lacosamide

- Quantitation and purity testing of (*R*)-lacosamide in a pharmaceutical formulation

ABSTRACT

The potential of chiral deep eutectic solvents to enhance the chiral discrimination in Cyclodextrin-Electrokinetic Chromatography is demonstrated in this work. With this aim, a method enabling the enantiomeric separation of the antiepileptic drug lacosamide was developed. After a screening using 12 cyclodextrin derivatives, succinyl-β-CD was chosen due to its higher discrimination power to separate lacosamide enantiomers. The effect of different variables, such as cyclodextrin concentration, buffer concentration, temperature and separation voltage, on the enantiomeric separation of lacosamide, was studied. As the maximum enantiomeric resolution achieved under the optimized conditions was lower than 1.5, the effect of the addition of methanol or different deep eutectic solvents (choline chloride – ethylene glycol, choline chloride – urea, choline chloride – D-glucose, and choline chloride – D-sorbitol) as additives to the separation medium was investigated. The best results in terms of enantiomeric resolution and analysis time were obtained using choline chloride – D-sorbitol at a 0.5 % (w/v) in a 100 mM borate buffer (pH 9.0) with 18 mM succinyl-β-CD. Under these conditions, lacosamide enantiomers were separated with a resolution value of 2.8. Analytical characteristics of the developed method were evaluated and demonstrated to be adequate to apply the methodology to the enantiomeric analysis of lacosamide in a pharmaceutical formulation.

Keywords: Cyclodextrin, Deep eutectic solvent, Electrokinetic chromatography, Enantiomeric separation, Lacosamide.

1. Introduction

Since the dramatic discovery of the different pharmacological and toxicological effects associated to thalidomide enantiomers, chiral analysis of drugs has become an important subject in the pharmaceutical field due to the risk that racemic drugs may entail for human health [1]. This concern stems from the fact that many of the commercialized drugs have stereogenic centres, so they can exist as one or more pairs of enantiomers which sometimes may exhibit different biological, pharmacokinetic and pharmacodynamic activities [2]. Indeed, usually the desirable pharmacological activity of the drug resides only in one of the enantiomers. For this reason, nowadays, the trend is to commercialize enantiomerically pure drugs only containing the active enantiomer responsible for the pharmaceutical activity [3]. Therefore, within the quality control of these drugs, it is of utmost importance to control the possible presence of enantiomeric impurities. Accordingly, the International Council for Harmonization (ICH), the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) have established several guidelines to monitor the presence of enantiomeric impurities, which cannot exceed 0.1 % in the enantiomerically pure formulation [4-6]. Consequently, sensitive chiral analytical methods need to be developed to ensure the quality control of drugs commercialized as pure enantiomers.

In this context, different chromatographic and electrophoretic separation techniques can be employed for chiral analysis, such as high-performance liquid chromatography (HPLC), gas chromatography (GC), supercritical fluid chromatography (SFC) and capillary electrophoresis (CE). Although HPLC is the most popular among them, CE stands out as a powerful alternative due to its well-known inherent advantages, such as high separation efficiency, easy change of separation conditions and minimum consumption of reagents and samples (which confers to CE the properties of sustainable

and environmentally friendly analytical technique) [7]. Among the different CE modes, electrokinetic chromatography (EKC) is by far the most employed in chiral analysis of drugs due to its great versatility [3, 7, 8]. This CE mode involves adding a chiral selector into the background electrolyte (BGE). In this sense, a wide variety of chiral selectors can be used, such as cyclodextrins (CDs), antibiotics, crown ethers, proteins, polysaccharides, surfactants, ligand-exchange complexes, etc. [8-10]. However, although all of them can be effectively employed, CDs are still the most frequently used due to their availability, diversity, universality, and high discrimination power [8-12]. Nonetheless, sometimes it is not possible to achieve a satisfactory enantioseparation by only using one single chiral selector. In this case, dual systems involving the combination of two chiral selectors can help improving resolution and peak efficiency [12]. For this reason, the search of new additives, which can be added in combination with CDs into the BGE, has attracted a considerable interest in recent years as a way of improving enantioseparations. Thus, the synergistic effect of dual systems involving the combination of CDs with other additives, e.g. crown ethers, polysaccharides, or ionic liquids (ILs) has been evaluated to improve the enantioseparation of different chiral compounds [9, 13-21]. In drug analysis, increasing the enantiomeric resolution is of special interest in order to make possible the detection of the minor enantiomer (the enantiomeric impurity) in the presence of a high concentration of the active enantiomer to accomplish the ICH guidelines.

On the other hand, nowadays, the current trend is to develop environmentally friendly analytical procedures meeting the Green Analytical Chemistry principles. In this sense, the use of green solvents, mainly represented by ILs and deep eutectic solvents (DESs) has received considerable attention in the last years among researchers. The addition of ILs as additives to improve the enantioseparation of chiral drugs in

EKC has been extensively studied [21-23] as previously mentioned; however, DESs have barely been applied in CE [24, 25]. DESs are eutectic mixtures of a hydrogen-bond acceptor (HBA) and a hydrogen-bond donor (HBD) [23, 25-30]. Quaternary ammonium, tetralkylammonium or phosphonium salts are usually employed as the HBA, whereas the HBD are often carboxylic acids, amines, polyols, or carbohydrates. The synthesis of DESs, unlike ILs, is always simple and environmentally friendly, as it does not involve using any organic solvent, since the two solid components are just mixed and heated until a homogeneous liquid is formed. Consequently, the H-bond interactions established between the two components lead to decrease the melting point of the mixture, even at room temperature. Therefore, compared with ILs, DESs are cheaper, more environmentally friendly, and easier to obtain [26, 29]. Despite these advantages, to the best of our knowledge, up to date only two works have reported the use of DESs as additives to the separation buffer in CE [24, 25]. Mu *et al.* demonstrated that the combination of different DESs with β-CD enabled to improve the separation of different chiral drugs (zopiclone, salbutamol and amlodipine) by EKC [24]. More recently, Deng *et al.* carried out the characterization and combination of five DESs with different β-CD derivatives to study the improvement of the chiral separation of five different model drugs (tropicamine, homatropine, ofloxacin, atenolol and propranolol) [25]. In this study, the effect of adding separately each initial component of the DESs into the BGE was also evaluated since it has been recently suggested that water-soluble DESs are dissociated when they are added as additives in aqueous mobile phases, resulting in the decomposition of DESs into their initial components [31]. Authors observed that adding individually choline chloride or ethylene glycol (the DES initial components) as additives, also provided the enantioseparation of the target analytes.

Nevertheless, they concluded that the use of DESs lead to a better enantioresolution values than those achieved using their individual components [25].

Lacosamide is a chiral antiepileptic drug, which has recently been approved in Europe and USA for adjunctive treatment of partial-onset seizures and diabetic neuropathic pain [2]. It presents one stereogenic centre, so it only has one pair of enantiomers. The (*R*)-enantiomer is the one with the pharmacological activity, whereas the (*S*)-configuration is inactive. To date, just three works have reported the chiral separation of lacosamide by HPLC in normal phase [32, 33] and reversed phased [34] using polysaccharide coated chiral columns but its separation by EKC has never been described before.

Hence, the aim of this work was to develop for the first time an environmentally friendly chiral methodology by EKC to achieve the enantioseparation of lacosamide. For this purpose, different CDs were investigated as sole chiral selectors. Moreover, the effect of the addition of different DESs to the separation medium containing a CD was studied. For the DES enabling the highest enantiomeric resolution for lacosamide, results were compared with those obtained when adding to the BGE the initial components of the DES. The developed method was applied to the quality control and purity testing of lacosamide in a pharmaceutical formulation.

2. Materials and methods

2.1. Reagents and samples

Boric acid, sodium hydroxide, choline chloride (ChCl), ethylene glycol (EtGly), D-glucose and D-sorbitol were obtained from Sigma (St. Louis, MO, USA). Methanol (MeOH), hydrochloric acid and urea were purchased from Scharlab S.L. (Barcelona,

Spain) and ethanol (EtOH) was from Panreac (Barcelona, Spain). The water employed was purified and obtained from a Millipore Milli-Q system (Bedford, MA, USA).

Sulfated-β-CD (average degree of substitution (DS) ~ 12, molecular weight (MW) 2462 g/mol), was purchased from Fluka (Buchs, Switzerland). Sulfated-α-CD (DS ~ 12, MW 2198.10 g/mol), sulfated-γ-CD (DS ~ 14, MW 2726.20 g/mol), succinyl-β-CD (DS ~ 3.5, MW 1323 g/mol), succinyl-γ-CD (DS ~ 3.5, MW 1647.55 g/mol), phosphated-β-CD (DS ~ 4, MW 1542.90 g/mol), sulfobutylated-β-CD (DS ~ 6.3, MW 2131.66 g/mol), carboxymethyl-α-CD (DS ~ 3.5, MW 1253 g/mol), carboxymethyl-γ-CD (DS ~ 3.5, MW 1472 g/mol), 2-carboxyethyl-β-CD (DS ~ 3.5, MW 1464.35 g/mol) and 2-carboxyethyl-γ-CD (DS ~ 3.5, MW 1549.55 g/mol) were obtained from Cyclolab (Budapest, Hungary) and carboxymethyl-β-CD (DS ~ 3, MW 1375 g/mol) was from Sigma-Aldrich (St. Louis, MO, USA).

(*R*)-lacosamide was obtained from European Directorate for the Quality of Medicines (Strasbourg, France) and (*S*)-lacosamide purchased from Clearsynth Labs LTD (Mumbai, India). The pharmaceutical formulation of lacosamide was obtained in its injectable form from a laboratory authorized to commercialize this drug. According to its label, it contained 10 g/L of lacosamide.

2.2. CE conditions

CE experiments were carried out on an Agilent 7100 CE system (Agilent Technologies, Waldbronn, Germany) with a diode array detector (DAD) operating at 200 nm (bandwidth 4 nm). The system was controlled by the HP^{3D} CE ChemStation software from Agilent Technologies. The enantiomeric separation was performed in an uncoated fused-silica capillary of 50 µm I.D. with a total length of 58.5 cm (50 cm effective length) from Polymicro Technologies (Phoenix, AZ, USA) and using 100 mM

borate buffer (pH 9.0) containing 18 mM of succinyl-β-CD and 0.5 % (w/v) of ChCl-D-sorbitol as BGE. The injection was performed by applying 50 mbar for 5 s, and the optimum electrophoretic separation was achieved at 15ºC and +25 kV. At the beginning of each working day, the capillary was flushed (applied 1 bar of pressure) with 0.1 M sodium hydroxide, Milli-Q water and BGE during 5, 5 and 30 min, respectively. To ensure repeatability between injections, the capillary was conditioned by flushing the capillary 2 min with 0.1 M hydrochloric acid, 1 min with Milli-Q water, 2 min with 0.1 M sodium hydroxide, 1 min with Milli-Q water and 4 min with BGE.

2.3. Preparation of solutions, DESs, and samples

Buffer solutions were prepared by dissolving the appropriate amount of boric acid in Milli-Q water to obtain the desired concentration and adjusting the pH to 9.0 with 1 M sodium hydroxide. BGEs were prepared by adding the desired concentration of MeOH or DES to the borate buffer and dissolving the adequate amount of succinyl-β-CD in an aliquot of those solutions.

In this work, 4 different DESs were synthesized using ChCl as HBA with different compounds as HBD (EtGly, urea, D-glucose or D-sorbitol) and following the molar ratios and conditions previously described in the literature [23, 35]. In the case of ChCl-EtGly and ChCl-urea, 1 g ChCl was mixed with 0.89 g of EtGly or 0.86 g of urea (molar ratio of 1:2), respectively, and stirred for 30 min in a water bath at 70-80ºC. However, for the synthesis of ChCl-D-Glucose and ChCl-D-Sorbitol, the molar ratios were 2:1 and 1:1, respectively. Both mixtures were stirred for 4 h in a water bath at 70-80ºC. All DESs were stored at room temperature in a dark place until their dilution with the appropriate buffer solution.

Stock standard solutions of lacosamide enantiomers (1000 mg/L) were prepared in EtOH and stored at -20ºC. Working standard solutions at different concentration levels were prepared by appropriate dilution of the stock solutions with Milli-Q water until the desired concentration. For the pharmaceutical solution, an appropriate volume of the injectable drug was measured and diluted with Milli-Q water to achieve the desired concentration. Afterwards, it was filtered through a 0.45 μm nylon filter.

2.4. Data treatment

All data files were treated with ChemStation software from Agilent Technologies to obtain migration times, peaks areas and resolution values between adjacent peaks. Analytical characteristics data and statistical tests were treated using the STATGRAPHICS Centurion XVII-X64 and Microsoft Excel programs. Also, Origin 8.0 software was employed to compose the figures with different electropherograms.

3. Results and conclusions

3.1. Enantiomeric separation of lacosamide by CD-EKC

Lacosamide is a neutral drug except at very basic pH (pKa 12.47) [36], thus a screening test of 12 anionic CDs (sulfated-α-CD, sulfated-β-CD, sulfated-γ-CD, phosphated-β-CD, sulfobutylated-β-CD, carboxymethyl-α-CD, carboxymethyl-β-CD, carboxymethyl-γ-CD, 2-carboxyethyl-β-CD, 2-carboxyethyl-γ-CD, succinyl-β-CD and succinyl-γ-CD) was performed using a 25 mM borate buffer at pH 9.0 in positive polarity mode to select the most suitable chiral selector to achieve the enantiomeric separation of lacosamide. All CDs were evaluated at a concentration of 10 mM, using a voltage of +20 kV and a temperature of 20ºC. Among all the CDs assayed, only sulfated-α-CD, phosphated-β-CD and succinyl-β-CD allowed to obtain a partial separation of lacosamide enantiomers (resolution values <0.4). With the aim of

improving the separation, the temperature was decreased at 15ºC and different voltages were tested (+20, +25 and +30 kV) with these three CDs. Nevertheless, with these modifications the separation only improved using succinyl-β-CD at 15ºC and +25 kV (a resolution value of 0.5 was reached). Therefore, this CD was chosen as chiral selector for further experiments.

Afterwards, the effect of the succinyl-β-CD concentration was evaluated in the 2 to 20 mM range (2, 5, 10, 15 and 20 mM) at 15ºC and +25 kV. Chiral discrimination was only observed from a concentration of 10 mM onwards (**Fig. S1** in supplementary material). The resolution values obtained were 0.5 (19.0 min), 0.7 (26.3 min) and 0.7 (29.1 min) for 10, 15 and 20 mM, respectively, and the analysis time increased with the CD concentration. Therefore, since the highest resolution achieved was the same for 15 and 20 mM, 15 mM was selected as the most suitable CD concentration as it provided shorter analysis time and enabled to use a reduced amount of CD.

The influence of the borate buffer concentration was also studied in the range from 10 to 200 mM (10, 25, 50, 100, 150 and 200 mM) at pH 9.0. The best separation of lacosamide enantiomers was obtained using 100 mM borate buffer at pH 9.0, achieving a resolution value of 1.3. The buffer concentrations below 100 mM provided little resolution while concentrations higher than 100 mM did not provide separation. Subsequently, the influence of the temperature and the voltage on the enantiomeric separation was also evaluated in the range from 15 to 25ºC (15, 20 and 25ºC) and from +20 to +30 kV (+20, +25 and +30 kV), respectively, using 15 mM of succinyl-β-CD and 100 mM borate buffer at pH 9.0 (**Table 1**). It was observed that the analysis time was more affected by the voltage than by the temperature. Increasing the voltage decreased the analysis time, whereas only varying the temperature barely modified the migration times. The best resolution values were achieved at 15 and 20ºC with both

voltages of +20 and +25 kV. Nevertheless, higher resolution values and shorter analysis times were achieved with +25 kV, and although a temperature of 20ºC provided shorter analysis time, 15ºC was chosen as optimum value since the separation resolution achieved was slightly higher (**Table 1**). Under these optimized conditions, the separation of lacosamide enantiomers was achieved in less than 15.5 min with a resolution value of 1.3.

Table 1. Migration times and resolution values for lacosamide enantiomers obtained by EKC using 15 mM succinyl-β-CD in 100 mM borate buffer (pH 9.0) at different temperatures and voltages.

Temperature (ºC)	Voltage (kV)	t_1 (min)	t_2 (min)	R_s
	+20	16.8	17.1	1.1
15	+25	15.1	15.4	1.3
	+30	9.8	9.9	0.7
	+20	16.9	17.2	1.1
20	+25	14.5	14.7	1.2
	+30	10.3	10.5	0.8
	+20	17.7	18.0	0.8
25	+25	13.8	14.1	0.7
	+30	11.1	11.3	0.7

Experimental conditions: uncoated fused-silica capillary 58.5 cm (50 cm to the detector window) x 50 µm ID; injection by pressure 50 mbar for 5 s of sample.
t_1: time of the first-migrating enantiomer
t_2: time of the second-migrating enantiomer

3.2. Effect of the addition of an organic modifier or a DES

With the aim of improving the enantiomeric separation of lacosamide, the effect of the addition of an organic modifier in the BGE was investigated. However, previous to this study, the conditioning of the capillary between injections was modified to improve the repeatability obtained until this moment. Conditioning was carried out by flushing the capillary with 0.1 M hydrochloric acid (2 min), Milli-Q water (1 min), 0.1 M sodium hydroxide (2 min), Milli-Q water (1 min) and BGE (4 min) since repeatability

significantly improved although under these conditions the analysis time and the enantiomeric resolution were reduced ($R_s < 1.0$). Then, different percentages of MeOH (5, 10 and 20 %) were added into the BGE. It was observed that the addition of MeOH originated an increase in the analysis time and in the enantiomeric resolution that was maximum at a 20 % MeOH (R_s 1.4). However, as this value of enantiomeric resolution was still low, the effective length of the capillary was increased from 50 to 60 cm. The enantiomeric resolution improved with the capillary of 60 cm and best separation was obtained when a 20% of MeOH was added to the BGE (**Fig. 1**).
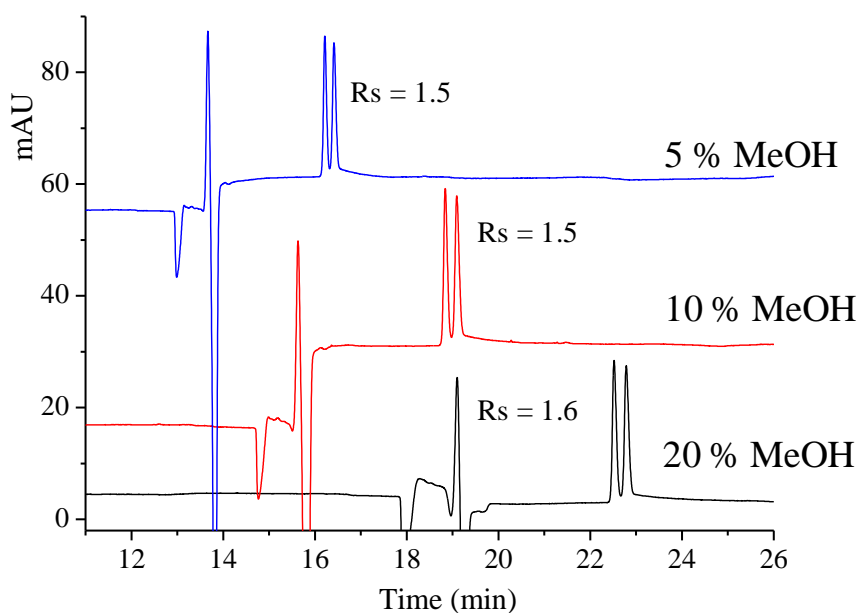

Figure 1. Electropherograms showing the influence of the addition of different concentrations of methanol into the BGE on the chiral separation of racemic lacosamide (200 mg/L) using an uncoated fused-silica capillary of 68.5 cm (60 cm to the detector window) x 50 µm ID. Other experimental conditions: BGE: 100 mM borate buffer (pH 9.0) containing 15 mM succinyl-β-CD; UV detection at 200 nm; applied voltage +25 kV; temperature 15ºC; injection by pressure, 50 mbar for 5 s.

Under these conditions, an analysis time of 22.9 min and an enantiomeric resolution of 1.6 were obtained. The enantiomer migration order was also studied by injecting under these conditions a lacosamide standard enriched with each of its enantiomers. As shown in **Fig. S2** (see supplementary material)**,** the active principle ((R)-lacosamide) was the first-migrating enantiomer while the enantiomeric impurity ((S)-lacosamide) was the second-migrating enantiomer.

The effect of the addition of different types of DESs (two achiral and two chiral DESs) at a percentage of 1 % (w/v) into the BGE (**Table 2**) was also investigated using a capillary with an effective length of 50 cm. Among the four DESs tested (ChCl-EtGly, ChCl-urea which are achiral, and ChCl-D-Glucose and ChCl-D-Sorbitol which are chiral), the best results in terms of enantiomeric resolution were obtained using ChCl-D-sorbitol as additive, although this DES originated the highest analysis time (resolution value of 3.2 in 38.3 min).

Table 2. Influence of the addition of different DES to the BGE.

DES 1 % (w/w)	t_1 (min)	t_2 (min)	R_s
ChCl-EtGly	28.1	28.8	1.8
ChCl-urea	27.3	27.9	1.7
ChCl-D-glucose	33.4	34.4	2.2
ChCl-D-sorbitol	38.3	40.0	3.2

Experimental conditions: uncoated fused-silica capillary 58.5 cm (50 cm to the detector window) x 50 µm ID. BGE; 15 mM succinyl-β-CD in 100 mM borate (pH 9.0), temperature, 15ºC; voltage, +25 kV; UV-detection: 200 nm; injection by pressure 50 mbar for 5 s of sample.
t_1: time of the first-migrating enantiomer
t_2: time of the second-migrating enantiomer

Nevertheless, the repeatability between injections worsened due to the addition of DESs to the BGE. For this reason, the concentration of ChCl-D-sorbitol was reduced to 0.5 % (w/v). With this change, the repeatability improved, and the analysis time was reduced to 26.6 min with a resolution value of 2.6. Moreover, to improve the sensitivity,

the influence of the injection time (5, 10, 15 and 20 s applying 50 mbar) was evaluated. It was observed that the resolution decreased as the injection time increased (data not shown). However, sensitivity did not improve significantly, so 5 s was chosen as the optimum injection time. Despite the addition of ChCl-D-sorbitol improved the separation of the enantiomers of lacosamide, the resolution was still not enough to detect the 0.1 % impurity ((*S*)-lacosamide) of (*R*)-lacosamide. Therefore, to achieve this objective, the concentration of succinyl-β-CD was increased from 15 mM to 18 mM, which enabled to obtain an enantiomeric resolution of 2.8 in less than 35 min (**Fig. 2**).

In summary, by extending the capillary length to 60 cm and adding 20 % MeOH to the BGE, the separation of lacosamide enantiomers using 15 mM succinyl-β-CD was achieved in 22.6 min (resolution value of 1.6), while adding 0.5 % ChCl-D-sorbitol to the BGE (with 18 mM succinyl-β-CD) the enantiomeric separation of lacosamide was achieved in less than 35 min with an enantiomeric resolution of 2.8. Despite obtaining a longer analysis time, this DES was chosen as additive in the BGE since an enantiomeric resolution of 2.8 was obtained and the detection of a 0.1 % of (*S*)-lacosamide with respect to the major enantiomer was possible (**Fig. 2)**, which is widely accepted as a minimum requirement for the enantiomeric purity control by the ICH guidelines [4].

The results obtained by the developed EKC methodology were compared with those previously achieved by HPLC in which the enantioseparation of lacosamide was performed in less than 20 min with resolution values higher than 4.2 [32-34]. Considering the high cost of chiral columns, the high consumption of organic solvents and the fact that two of these works used a mobile phase with a high content of hexane (a not environmentally friendly solvent), the EKC method developed in this work can be considered a potent and sustainable alternative providing a more cost effective and

environmentally friendly methodology for the chiral separation of lacosamide and for the evaluation of its enantiomeric purity in pharmaceutical formulations.
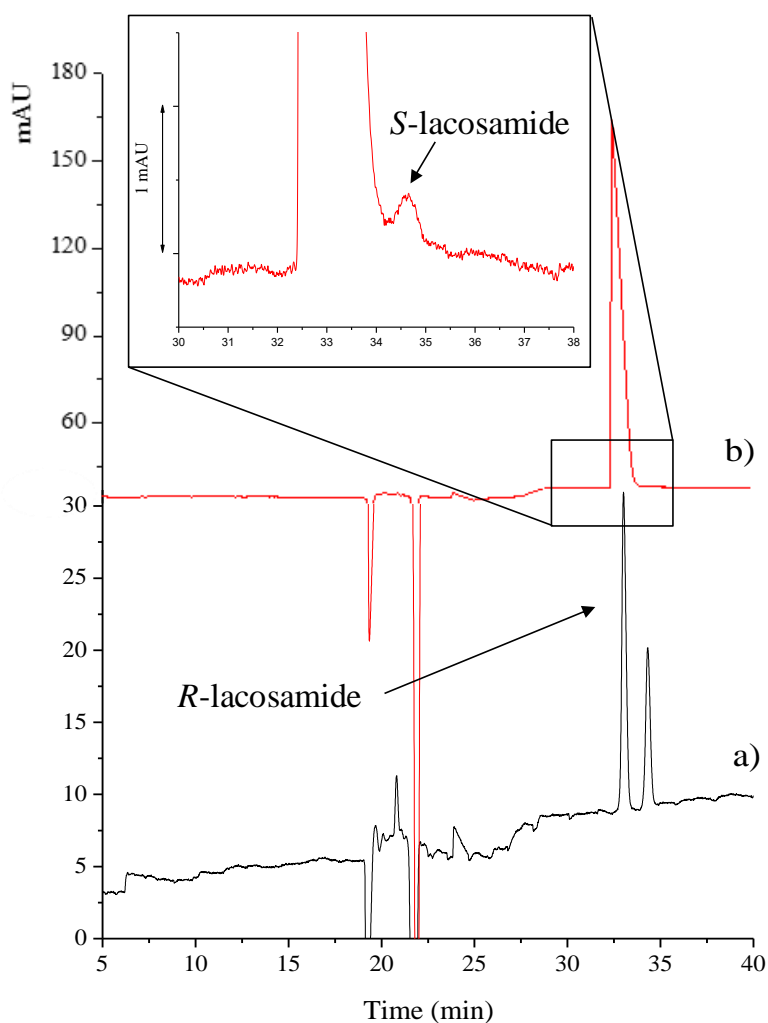

Figure 2. Electropherograms corresponding to the chiral separation of: (a) a standard solution 100 mg/L in (*R*)-lacosamide and 50 mg/L in (*S*)-lacosamide; (b) a standard solution 2000 mg/L in (*R*)-lacosamide and 2 mg/L in (*S*)-lacosamide. Experimental conditions: 100 mM borate buffer (pH 9.0) + 18 mM succinyl-β-CD + 0.5 % (w/v) ChCl-D-sorbitol; uncoated fused-silica capillary 58.5 cm (50 cm to the detector window) x 50 µm ID; UV detection at 200 nm; applied voltage +25 kV; temperature 15ºC; injection by pressure, 50 mbar for 5 s.

3.3. Effect of the addition of the individual components of the DES

In order to investigate the effect of the addition of the individual components of choline chloride-D-sorbitol DES on the enantiomeric separation of lacosamide, compared to the use of the ready-made DES, a study was achieved by using in the separation medium both DES individual components, each individual component separately and the ready-made DES. All these components were added to the BGE at the same percentages (w/v) as those employed in the synthesis of the DES. Results are shown in **Fig. 3** and **Table S1** (see supplementary material).
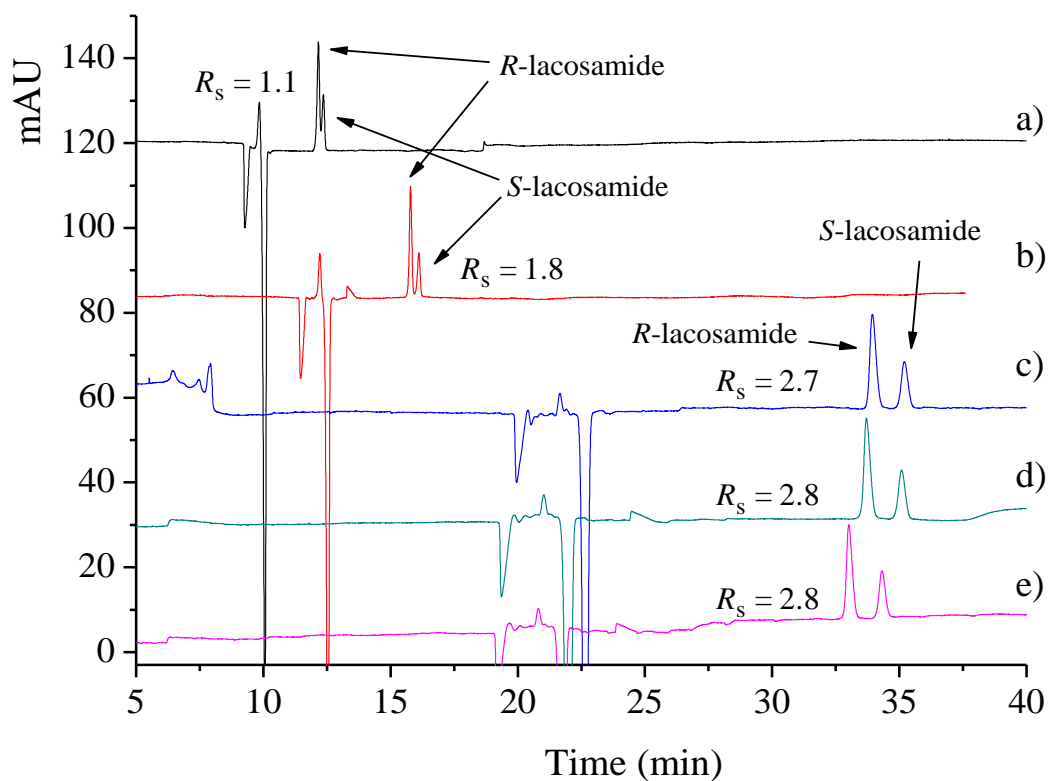

Figure 3. Electropherograms corresponding to the separation of (*R*)-lacosamide (100 mg/L) and (*S*)-lacosamide (50 mg/L) when using succinyl-β-CD as chiral selector alone in the BGE (a) or combined with: 0.284 % (w/v) D-sorbitol (b); 0.216 % (w/v) ChCl (c); 0.216 % (w/v) ChCl + 0.284 % (w/v) D-sorbitol (d); and 0.5 % (w/v) ChCl-D-sorbitol DES (e). Other experimental conditions as in Figure 2.

As it can be observed in **Fig. 3**, the addition of ChCl, ChCl plus D-sorbitol or DES to the separation medium containing succinyl-β-CD enabled to improve considerably the separation of lacosamide enantiomers compared with the absence of the DES or its components (resolutions increased from 1.1 to 2.7 with the addition of ChCl and to 2.8 with both individual components of DES added together or the DES). In these three cases, higher migration times were obtained (ranging from 34.1 to 35.5 min) in comparison with the use of succinyl-β-CD alone in the separation buffer (Rs 1.1 in an analysis time of 12.4 min). However, the addition of D-sorbitol to the separation medium containing the CD had a small effect on both migration times and enantiomeric resolution for lacosamide (resolution 1.8 in 16.2 min). The increase in the migration times observed when ChCl is present in the separation medium could be justified taking into account its possible adsorption to the capillary wall [25] originating a decrease in the EOF. This fact could improve the enantiomeric separation by favoring the interactions between the CD and the enantiomers that could take place for a longer time. In fact, as shown in **Fig. 3**, the resolution obtained using ChCl was close to the values obtained when the components of the DES were added together (ChCl plus D-sorbitol) or when the synthesized DES was added to the separation medium. In these last two cases, no significant differences were observed for the resolution values obtained, suggesting that the DES could be total or partially dissociated into its initial components due to the destruction of the hydrogen bonds in the DES chemical structure induced by the presence of water in the aqueous separation medium [31]. Therefore, in the case of choline chloride-D-sorbitol DES, results show that a very similar effect on the separation of lacosamide enantiomers is caused by the addition of the DES, its individual components together or ChCl alone to the separation medium.

The results obtained in this work agree with those recently published by *Deng et al.* [25]. In this study, the enantiomeric resolution of different chiral drugs was shown to be improved after the addition of ChCl-EtGly DES. Likewise, when they only added ChCl into the BGE instead of ChCl-EtGly DES, the enantioseparation also improved compared to the addition of the CD alone. However, in this case the resolution was not as good as that obtained when adding the DES.

Based on the fact that migration times were slightly shorter when using the DES than when using its individual components, and that the DES had been easily synthesized and was completely available in the laboratory, the DES was selected as additive to evaluate the analytical characteristics of the method developed according to the ICH guidelines. Nonetheless, further investigation in future works will be carried out to better understand and explore the effect of the addition of DESs or their initial components when they are used as additives to improve chiral separations in EKC.

3.4. Analytical characteristics of the developed CD-EKC method

With the aim of demonstrating the suitability of the CD-EKC methodology for the quality control of pharmaceutical formulations, analytical characteristics such as selectivity, linearity, accuracy, precision, detection limits (LOD) and quantification limits (LOQ) were evaluated.

As **Table 3** shows, good linearity (correlation coefficient values of 0.998) was achieved from plotting corrected peak areas versus six concentrations levels of each lacosamide enantiomer. Also, the ANOVA test confirmed that data fit properly to a linear model since both p-values obtained were higher than 0.05. Additionally, in both cases, confidence intervals (at a 95% confidence level) for the slopes did not include the zero value and the confidence intervals for the intercepts included it.

Table 3. Analytical characteristics of the chiral EKC method developed for the determination of lacosamide.

	(R)-Lacosamide	(S)-Lacosamide
External standard calibration method [a]		
Range	15-90 mg/L	15-90 mg/L
Slope $\pm$ t $\cdot$ S_a	0.098 $\pm$ 0.008	0.102 $\pm$ 0.008
Intercept $\pm$ t $\cdot$ S_b	-0.23 $\pm$ 0.38	-0.10 $\pm$ 0.41
Correlation coefficient	0.998	0.998
p-value (ANOVA) [b]	0.31	0.10
Matrix interferences [c]		
Slope $\pm$ t $\cdot$ S_a	0.087 $\pm$ 0.006	-
Accuracy [d]		
Recovery	98 $\pm$ 5 %	-
Precision		
Instrumental repeatability [e]		
t, RSD (%)	0.4	0.4
A, RSD (%)	4.1	3.9
Method repeatability [f]		
t, RSD (%)	1.8	1.8
A, RSD (%)	4.4	4.5
Intermediate precision [g]		
t, RSD (%)	1.8	1.8
A, RSD (%)	5.8	7.1
LOD [h]	1.5 mg/L	1.4 mg/L
LOQ [h]	5.1 mg/L	4.8 mg/L

[a] Six standard solutions at different concentration levels injected in triplicate.
[b] p-value for ANOVA to confirm that experimental data fit properly to linear model.
[c] Comparison of the confidence intervals for the slopes corresponding to the standard addition and the external standard calibration methods for (R)-lacosamide.
[d] Accuracy was evaluated as the recovery obtained from a pharmaceutical solution containing 40 mg/L of (R)-lacosamide (as labeled amount) with the addition of 25, 50, 75 and 100% of (R)-lacosamide standard.
[e] Six consecutive injections (n = 6) of a standard solution containing 40 mg/L of each drug enantiomer.
[f] Three standard solutions containing 40 mg/L of each drug enantiomer injected in triplicate (n = 9) on the same day.
[g] Three standard solutions containing 40 mg/L of each drug enantiomer injected in triplicate on three different days (n = 9).
[h] LOD and LOQ obtained for a S/N ratio = 3 or a S/N ratio = 10, respectively.

Moreover, to evaluate the existence of matrix interferences in the pharmaceutical formulation, a standard addition calibration method was performed to compare the confidence interval for the slope with the slope confidence interval obtained by the external calibration method. For this purpose, different known amounts of (R)-

lacosamide were added to the pharmaceutical sample solution (containing a constant concentration of (*R*)-lacosamide 40 mg/L). Data showed no statistically significant differences between the slopes for a 95 % confidence level. Therefore, under the optimal conditions, no matrix interferences were found, so the external standard calibration method is adequate for the quantification of lacosamide in pharmaceutical formulations. The response relative factor (RRF) was calculated by dividing the slope of the impurity ((*S*)-lacosamide) between the slope of the active enantiomer ((*R*)-lacosamide) resulting in a value of 1.04. Thus, since it was between the range established by the the European Pharmacopoeia [37], the response of both enantiomers of lacosamide can be considered equivalent, so that the percentage of the (*S*)-enantiomer can be established from the ratio between the areas of (*R*)- and (*S*)-enantiomers.

Accuracy of the developed EKC method for (*R*)-lacosamide was evaluated as the recovery obtained when the pharmaceutical solution (40 mg/L (*R*)-lacosamide) was spiked with different amounts (10, 20, 30 and 40 mg/L) of (*R*)-lacosamide standard. Adequate recovery value was obtained since the 100 % value was included (98 ± 5 %).

Precision of the EKC method was evaluated in terms of instrumental repeatability, method repeatability and intermediate precision (using a standard solution of 40 mg/L of each lacosamide enantiomer). Six consecutive injections of the standard solution determined the value of instrumental repeatability (*n*=6), method repeatability was assessed by performing three replicate standard solutions injected in triplicate on the same day (*n*=9), and intermediate precision was evaluated through the analysis of three standard solutions injected in triplicate in three consecutive days (*n*=9). Finally, the RSD values obtained (see **Table 3**) were lower than 1.8 % and 7.1 % for migration times and corrected peak areas, respectively.

LODs and LOQs were experimentally calculated as the minimum concentration yielding a signal to noise (S/N) ratio of 3 and 10 times, respectively. LODs of 1.5 mg/L and 1.4 mg/L were achieved for (R)-lacosamide (first-migrating enantiomer) and (S)-lacosamide (second-migrating enantiomer), respectively. Regarding LOQs, values of 5.1 mg/L for (R)-lacosamide and 4.8 mg/L for (S)-lacosamide were obtained. In addition, the relative limit of detection (RLOD) was 0.07 % which was calculated according to the LOD for (S)-lacosamide and the nominal concentration injected for (R)-lacosamide (2000 mg/L).

3.5. Analysis of a lacosamide pharmaceutical formulation

Once demonstrated the suitability of the developed EKC method for the enantiomeric separation and quantification of lacosamide, it was applied to the determination of lacosamide in a pharmaceutical formulation. **Fig. 4** shows the electropherograms corresponding to the lacosamide pharmaceutical formulation and the same sample spiked with a 0.1 % of its enantiomeric impurity ((S)-lacosamide).

The adequate selectivity of the developed EKC methodology was demonstrated by the absence of interfering peaks in the electropherograms. Results obtained revealed an average amount of 9.4 ± 0.6 g/L (R)-lacosamide in the pharmaceutical solution (labeled as 10 g/L content of (R)-lacosamide) corresponding to a percentage of 94 ± 6 % of the labeled amount. Moreover, as it is observed in **Fig. 4**, (S)-lacosamide was not detected, so its concentration was below a 0.1 % with respect to the majority enantiomer as established by the ICH guidelines.
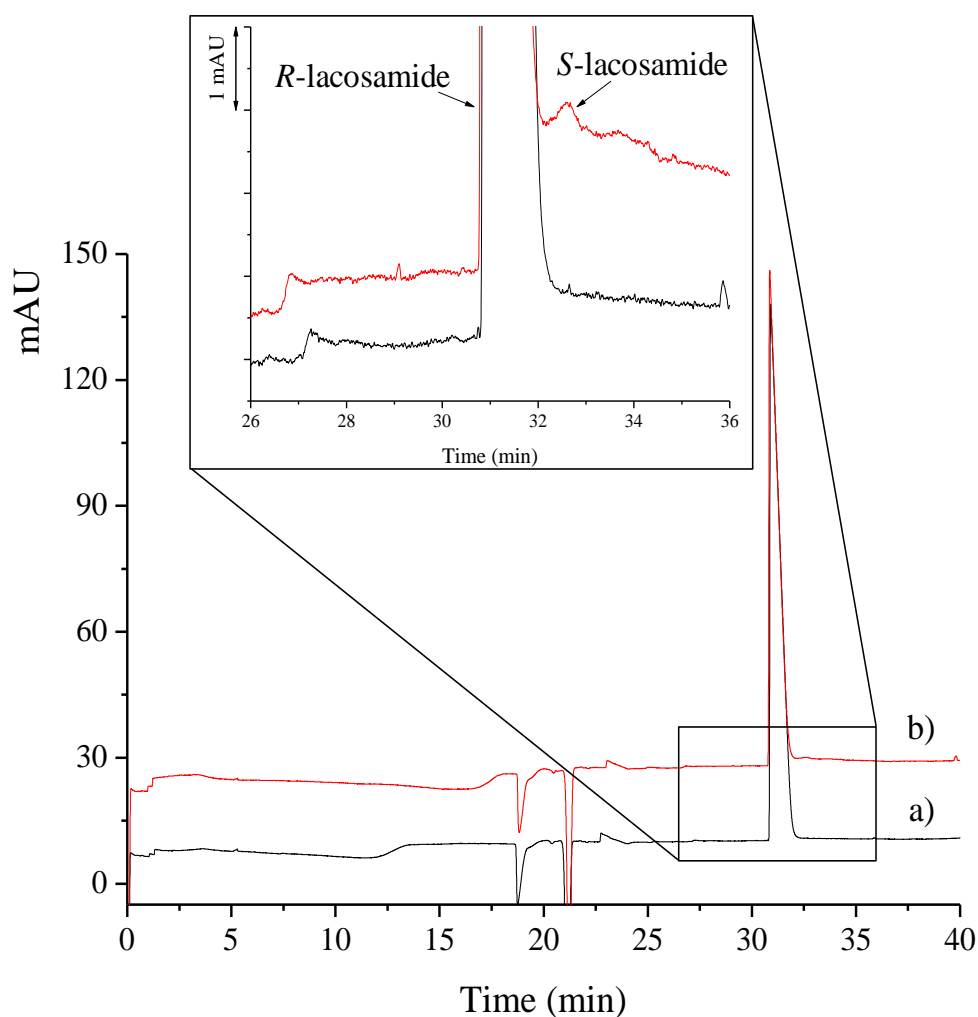
Figure 4. Electropherograms corresponding to the chiral separation of: (a) a pharmaceutical formulation containing 2000 mg/L of (*R*)-lacosamide; (b) the same as in (a) spiked with 2 mg/L of (*S*)-lacosamide. Experimental conditions as in Figure 2.

4. Conclusions

The use of ChCl-D-sorbitol in CE is described for the first time in this work. The combination of 18 mM succinyl-β-CD with 0.5 % (w/v) ChCl-D-sorbitol in 100 mM borate buffer at pH 9.0 enabled the separation of lacosamide enantiomers with a resolution value of 3.1. This is the first time that the enantiomeric separation of lacosamide is described by EKC. The combined system CD/ChCl-D-sorbitol was needed to reach a resolution value high enough to carry out the analysis of enantiomeric impurities in pharmaceutical formulations. The evaluation of the analytical

characteristics of the developed EKC method demonstrated adequate selectivity, linearity, precision, and accuracy with LODs of 1.5 mg/L for (*R*)-lacosamide, 1.4 mg/L for (*S*)-lacosamide, and a RLOD of 0.07 % for S-lacosamide. The developed methodology enabled to detect up to 0.1% of the enantiomeric impurity in a lacosamide pharmaceutical formulation thus accomplishing the requirements established by the ICH guidelines for the determination of enantiomeric purity. The application of the developed method to the quantitation and purity testing of (*R*)-lacosamide in a pharmaceutical formulation revealed that the content measured of the (*R*)-enantiomer was in agreement with the labeled content and that the (*S*)-enantiomer was not present at concentrations above 0.1 % of the majority enantiomer.

Acknowledgments

Declaration of interest

Declarations of interest: none.

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Supplementary Material

Use of choline chloride-D-sorbitol deep eutectic solvent as additive in Cyclodextrin-Electrokinetic Chromatography for the enantiomeric separation of lacosamide

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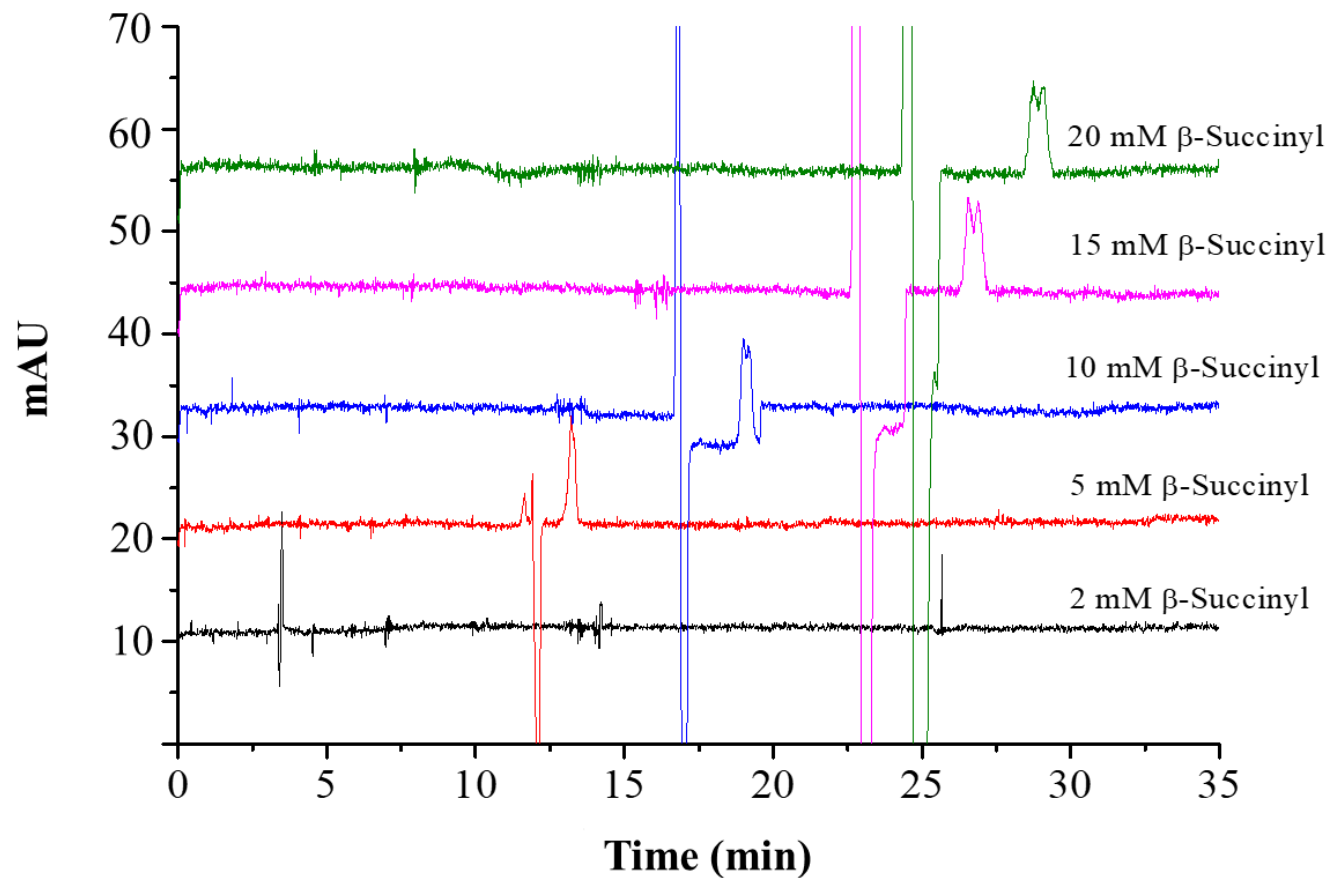*E-mail address:* mluisa.marina@uah.es

Figure S1. Electropherograms corresponding to the enantiomeric separation of racemic lacosamide using succinyl-β-CD at different concentrations. Experimental conditions: 25 mM borate buffer (pH 9.0); uncoated fused-silica capillary 58.5 cm (50 cm to the detector window) x 50 μm ID; UV detection at 200 nm; applied voltage 25 kV; temperature 15 ˚C; injection by pressure, 50 mbar for 5 s.

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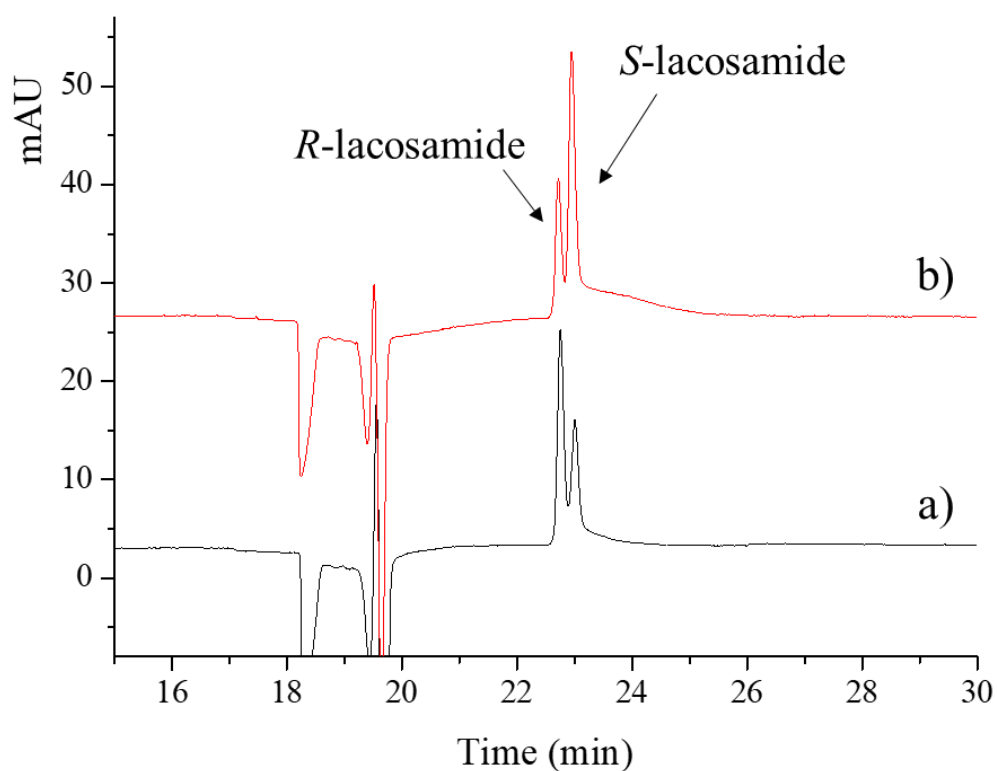
Figure S2. Electropherograms corresponding to the chiral separation of a racemic
lacosamide standard solution enriched in (R)-lacosamide (100 and 50 mg/L of (R)-
lacosamide and (S)-lacosamide, respectively) (a) and a standard solution enriched in
(S)-lacosamide (100 and 50 mg/L of (S)-lacosamide and (R)-lacosamide, respectively)
(b). Experimental conditions: BGE, 100 mM borate buffer (pH 9.0) containing 15 mM
succinyl-β-CD and 20 % (v/v) methanol; uncoated fused-silica capillary 68.5 cm (60 cm
to the detector window) x 50 µm ID; UV detection at 200 nm; applied voltage +25 kV;
temperature 15ºC; injection by pressure, 50 mbar for 5 s.

12

13

14

15 **Table S1.** Effect of the addition of ChCl-D-sorbitol deep eutectic solvent or its
16 components on the migration times and resolution values for lacosamide enantiomers
17 using succinyl-β-CD as chiral selector in the BGE.

BGE	t_1 (min)	t_2 (min)	R_s
succinyl-β-CD	12.2	12.4	1.1
succinyl-β-CD + D-sorbitol	15.9	16.2	1.8
succinyl-β-CD + ChCl	34.2	35.5	2.7
succinyl-β-CD + ChCl with D-sorbitol	33.6	35.0	2.8
succinyl-β-CD + DES ChCl-D-sorbitol	32.8	34.1	2.8

18 Experimental conditions: uncoated fused-silica capillary 58.5 cm (50 cm to the detector window) x 50
19 µm ID. BGE: 18 mM succinyl-β-CD in 100 mM borate (pH 9.0) without or with a 0.284 % (w/v) of D-
20 sorbitol; 0.216 % (w/v) ChCl; 0.284 % (w/v) D-sorbitol plus 0.216 % (w/v) ChCl; or 0.5 % (w/v)
21 ChCl-D-sorbitol deep eutectic solvent; temperature, 15ºC; voltage, +25 kV; UV-detection: 200 nm;
22 injection by pressure 50 mbar for 5 s of sample.
23 t_1: migration time for the first-migrating enantiomer
24 t_2: migration time for the second-migrating enantiomer
25