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CHIRAL SEPARATION OF A BASIC DRUG WITH TWO CHIRAL CENTERS BY ELECTROKINETIC CHROMATOGRAPHY FOR ITS PHARMACEUTICAL DEVELOPMENT

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Highlights:

- CE method to separate four stereoisomers of a drug development as single enantiomer
- Two in-capillary preconcentration techniques allow sensitivity gains of 3000-fold
- CE method enables the control of stereoisomeric impurities of a new drug substance
- CE method enables to investigate the chiral stability of a new drug product
- CE method enables to investigate the in vivo metabolism of a single enantiomer drug

Abstract

A chiral method using capillary electrophoresis was developed for the separation of the four stereoisomers of a new chiral substance currently undergoing drug development as single enantiomer. After the selection of highly sulfated β-CD as chiral selector, an exhaustive study on the influence of several experimental variables on the resolution was performed, being the substitution degree of the CD a very decisive factor. Run time and resolutions were about 20 min and higher than 2.0, respectively. The method was validated in terms of selectivity, linearity, accuracy, precision, and limits of detection and quantitation according to the requirements of the International Conference on Harmonisation for the determination of the chiral purity of a drug substance. The usefulness of the method was demonstrated in the control of stereoisomeric impurities in raw material as well as in the determination of the chiral stability of the drug in the solid state and in dosage forms used in safety assessment. Finally, the chiral method was used to investigate the possible *in vivo* inversion in biological samples.

Keywords: chiral drug, enantiomeric purity, chiral stability, electrokinetic chromatography with cyclodextrins.

1. Introduction

The development of racemic mixtures of chiral compounds is not favoured within the pharmaceutical industry [1]. Two facts are responsible for one of the most important trends in the pharmaceutical field in the last decade which is the use of pure enantiomers as new drug substances: on one hand, it is known that the chiral nature of living systems has strong implications for biologically active compounds interacting with them; on the other hand, the majority of active pharmaceutical ingredients (API) contain chiral centers that are crucial for the pharmacological activity of the drug [2]. Thus, when a chiral drug is administered as a racemic mixture, one enantiomer can have the desired pharmacologic effect whereas the other stereoisomers, considered enantiomeric impurities, could not have effects or could have undesired effects [3]. In order to prevent the marketing of chiral drugs as racemic mixtures, their potential drawbacks must be perfectly studied [4].

Establishing appropriate specifications for the drug substance is key to ensure the quality of pharmaceuticals on the market. In the case of established drugs, specifications may be provided by the relevant pharmacopoeia. However, in the case of new drugs only various international guidelines of the International Conference on Harmonisation (ICH) [5] have been developed in an effort to instigate global requirements for registration of pharmaceuticals. Thus, according to the guideline on specifications for chemical drug substances (ICH Q6A) [6], the control of antipode enantiomers of drug substances, developed as single enantiomeric forms, should be considered in the same manner as for the other impurities. Chiral impurities in pharmaceutical samples may occur as side-products of the synthetic process, as a result of inversion of chiral centers due to chemical degradation of the drug substance or both.

Thus on the one hand, the Q3A ICH guideline on impurities in new drug substances define certain thresholds for the content of impurities above which they should be identified and/or qualified [7]. On the other hand, stereospecific degradation of chiral drug substances should be investigated according to the recommended conditions for stability testing of new drug substances described in the ICH guideline Topic Q1A [8], and the evaluation of stability data shown in the ICH guideline Topic Q1E [9]. Therefore, it will be necessary to establish the need for control of chiral impurities in the drug substance or finished product specifications, and the analytical methodologies required for the determination of chiral compounds in pharmaceutical samples are a key component for the successful development of chiral drugs.

Currently, the methods to control the purity of the enantiomers of chiral drugs have been performed by HPLC for reasons of robustness and familiarity of analysts with this technique, while CE has been established as a good complement or an alternative to HPLC. However, CE has experienced a great grown in the pharmaceutical industry, particularly seen in the field of chiral analysis, for reasons concerning to its high efficiency, versatility, wide application range, reduced sample pre-treatment, and cheapness (a low consumption of selector and reagents) with respect to HPLC techniques [10-12]. The most common approach for the chiral separation of basic compounds in CE is the CE mode named Electrokinetic Chromatography (EKC) [13]. The most widely chiral selectors used in EKC are cyclodextrins (CDs) due to their ready availability and broad applicability [12, 14-18]. There are currently a wide range of both neutral and charged derivatized CDs commercially available, which increased chiral discrimination of enantiomers can be expected due to an increased degree of asymmetry [17].

The compound studied in this work is a basic chiral drug undergoing drug development for its commercialization like single enantiomeric drug. This compound possesses two stereogenic centers leading to four stereoisomers *RR*, *SS*, *SR*, and *RS*. The *SR* isomer shows an interesting pharmacological activity as immunomodulator for cancer treatment being the other three isomers chiral impurities, two diastereoisomers (*SS* and *RR* configuration) and an enantiomer (*RS* configuration). For this reason, it is of particular importance to develop a chiral method to assess the ~~optical~~ enantiomeric purity of this drug, i.e., a quality control method to separate and measure the individual diastereoisomers and enantiomer, unwanted stereoisomers, which may be present as impurities or degradation products in the drug substance and in the drug product or generated via metabolism in biological systems.

In this work, the separation of the four stereoisomers of this new drug was carried out by EKC using a CD as chiral selector. After the selection of the most suitable CD, the influence of several experimental conditions on the enantiomeric resolution was investigated. The method developed was validated following ICH Q2 guidelines [19] for the quantitation of the API and the other stereoisomers considered impurities in raw material always bearing in mind the need to measure low levels (≤ 0.1%) of stereoisomers in the presence of an excess of the majority enantiomer. Finally, the usefulness of the method was demonstrated in the control of stereoisomeric impurities in raw material as well as in the determination of the chiral stability of the drug in the solid state and in dosage forms used in safety assessment. Likewise, the chiral method was used to investigate the possible *in vivo* inversion in metabolism studies.

2. Materials and methods

2.1 Reagents

All reagents employed were of analytical grade. α-, β- and γ-CD, methyl-β-CD (degree of substitution (DS): 1.7-1.9), (2-hydroxy)propyl-β-CD (DS~4.2), heptakis-(2,6,-di-*O*-methyl)-β-CD, heptakis-2,3,6-tri-O-methyl-β-CD, highly sulfated β-CD (HS-β-CD) (DS: 12-14) and carboxymethyl-β-CD (DS~3) from Fluka (Buchs, Switzerland); HS-β-CD from Aldrich (St. Louis, MO, USA) (DS: 12-14); HS-β-CD from Sigma (St. Louis, MO, USA) (DS: 18); and acetylated-β-CD (DS 7), succinyl-β-CD (DS~3.5), succinyl-γ-CD (DS~3.5), carboxymethyl-α -CD (DS~3), carboxymethyl-γ-CD (DS ~3), carboxyethyl-β-CD (DS ~3) and carboxyethyl-γ-CD (DS ~3) all from Cyclolab (Budapest, Hungary), were used as chiral selectors. These selectors together with orthophosforic acid from Merck (Darmstadt, Germany) were used for the CE running buffers at the pHs indicated. ~~Pure~~ Triethanolamine ~~solution~~ from Riedel-de Haën (Seelze, Germany) was used to adjust the pH of the buffers. Distilled water was deionized by using a MilliQ system (Millipore, Bedford, MA, USA). Sodium hydroxide from Merck and hydrochloride acid from Scharlau (Barcelona, Spain) were used to rinse of the capillary. Acetonitrile, methanol, and 2-propanol from Merck, and ethanol from Scharlau were used like additives in running background electrolyte (BGE).

2.2 Samples

Solutions of racemic mixture, single stereoisomers, and analytical standard of the API (SR-enantiomer, purity > 99 %) were directly prepared by dissolving them in Milli-Q water up to the desired concentration.

Dosing forms of the API (SR-enantiomer) were obtained from Pharmaceutical Development Unit (University of Basque Country, Vitoria, Spain). The drug products were injectable formulations (powder to be reconstituted with water at 50 µg/mL).

Rat plasma samples were obtained from RCC CIDA (Barcelona, Spain). A single dose of 5 mg kg^{-1} body weight of the chiral drug (SR-enantiomer) was administered in each rat for the in vivo metabolism study. Blood samples were withdrawn at 5, 30, 45, and 60 min after drug ingestion. The samples were centrifuged and ~~plasma~~ harvested plasma was stored at -20ºC until the assay was performed.

2.3 CE conditions

CE experiments were carried out with an HP^{3D} CE instrument (Agilent Technologies, Waldbron, Germany) equipped with an on-column DAD working at 260 nm with a bandwidth of 5 nm. The instrument was controlled by a PC running the 3D-CE ChemStation from Agilent Technologies. Separation was carried out in a common uncoated fused-silica capillaries of 50 μm ID (375 μm OD) with different lengths, 48.5 cm and 80.5 cm total length from Composite Metal Services (Worcester, England). The electrophoretic separation was achieved with a voltage ranging from -15 to -30 kV (reverse-polarity mode) and 25 kV (normal polarity mode). The temperature of the capillary was tested in the range from 15 to 30 °C.

Conventional injections were made at the cathodic end using a pressure of 5 kPa (50 mbar) for 5 s with capillaries of 48.5 cm, and for 15 s or 150 s with capillaries of 80.5 cm, in the latter case the sample dissolved in water were injected between plugs of 200 mM phosphate buffer (pH 2.5) to obtain a focus effect of the compound bands. Other types of injections to improve the sensitivity by in-capillary preconcentration are described in section 3.3.

Before the first use, the capillary was conditioned by flushing with 1 M NaOH for 30 min, then with water for 5 min, followed with 0.1 M HCl for 3 min, and finally with the running buffer for 60 min. When a BGE was changed, the capillary was

conditioned with the new BGE for 30 min. Between injections, the capillary was rinsed with water for 3 min, then with 0.1 M orthophosphoric acid for 3 min, and finally with BGE for 3 min to maintain an adequate repeatability of run-to-run injections. At the end of the working day, the capillary was rinsed with water for 3 min, then with 0.1 M orthophosphoric acid for 3 min, and finally with water for 3 min to keep it all night with water inside. A pressure of 0.1 MPa (1 bar) was applied in all steps.

Buffer solutions were prepared diluting the appropriate volume of orthophosphoric acid with Milli-Q water, adjusting the pH to the desired value with pure trietanolamine before completing the volume with water to get the desired buffer concentration. Finally, BGEs were prepared dissolving the appropriate amount of CD in the buffer solution.

All these solutions (buffers, standards, and samples) were filtered prior to use through 0.45 mm pore size disposable nylon filters from Titan (Eatontown, NJ, USA).

2.4 Bioanalytical procedure for plasma samples

The rat plasma samples were centrifuged at 20ºC and 2500 rpm for 5 min and then 200 μL of the supernatant was transferred into an eppendorf tube, added 1 mL of water grade Milli-Q and vortex. The extract was loaded to an Oasis® MCX solid-phase extraction (SPE) cartridge (1 cc (30 mg) Mixed-mode Cation-Exchange sorbent from Waters, Milford, MA, USA) which had been previously conditioned with 1mL of methanol and 1mL of Milli-Q grade water, respectively. After loading the sample on the cartridge, the column was washed with 2 mL of 0.1 M HCl and then 2 mL of methanol. Finally, the chiral drug was eluted with 1 mL of 10% NH_4OH in methanol freshly prepared solution. The eluate from the SPE column was evaporated to dryness under a

stream of nitrogen at 50 ºC. The residue was reconstituted with 1 mL of Milli-Q grade water and transfered into an injection microvial to be injected into the CE system.

3. Results and discussion

The chiral substance studied in this work is a very tough molecule. In fact, it is novel pyroglutamic acid derivative with a quaternary ammonium in an aromatic ring which makes prediction much more difficult (see **Figure 1**). In previous experiments, the resolution of the four isomers could not be accomplished by chiral HPLC using four chiral stationary phases (CSPs): *Chiral-AGP* ~~from Chrom Tech (Apple Valley, MN, USA)~~ used in reversed-phase, ~~one of the most likely CSPs with the broadest applicability of all chiral columns available today,~~ and three CSPs ~~from Daicel (Chiral Technologies Europe, Strasbourg, France)~~, the *Chiralcel OG* used in normal phase, and *Chiralpak QD-AX* and *QN-AX* classified as weak chiral anion exchangers.

3.1 Method development

An evaluation of the physical and chemical properties of the analyte such as solubility in water (over 2 mg/mL), UV absorbance (260 nm wavelength for maximum), and basicity was carried out. Because of the cationic character of the analyte at pH < 6.0, the use of neutral or anionic CDs was considered in this work.

Although it is known that sulfated CD lead to higher enantioresolution in most cases for cationic analytes, based on our own experience, a first screening test of CDs to explore the conditions giving the best chiral resolution (faster, economical and robust separation) was established. Sixteen neutral or anionic CDs (see section 2.1) were tested at a concentration of 2.5 % (w/v) except for β-CD that was used at 1.5 % (w/v) due to its low solubility, and for HS-β-CD whose strong electrostatic interaction with cationic

compounds made recommendable its use at lower concentrations, e.g., 1.0 % (w/v). The experiments were conducted at pH 2.0 and 4.5 in 50 mM phosphate buffer on a single CD system. Phosphate buffer was selected due to its protective effect against interactions of cationic analytes with the capillary wall at low pH values [20, 21]. A low pH (pH 2.0) was tested because for chiral discrimination of basic solutes, the use of this pH gives rise to a lower adsorption of analytes to the capillary wall, shorter equilibration times for the capillary and higher reproducibility for migration times. All CDs were tested at pH 2.0; however, only the CDs modified with carboxyl groups (e.g., carboxyalkyl- or succinyl- CDs) were tested at pH 4.5 because a good selectivity has been reported for these CDs when a partial dissociation of acidic groups took place at the operating pH [22, 23]. All experiments were carried out with a separation voltage of +25 kV except for HS-β-CD which required a reverse polarity (-25 kV). Due to the virtually ~~null~~ no EOF at pH 2.0 and high electrophoretic mobility of HS-β-CD towards the anode, electrophoretic runs were performed in the reversed-polarity mode, with basic analytes being carried out to the detector by their association with the negatively charged CD due to their high electrostatic interaction.

The results showed that in most of the studied conditions only the separation between the diastereomers of the chiral drug was possible (two peaks). Only using HS-β-CD at pH 2.0 the chiral discrimination of the four stereoisomers was obtained (partial separation) so it was chosen as chiral selector to develop the chiral method. These results demonstrate once again that in the case of cationic analytes, anionic CDs have greater enantioselectivity due to extra ionic interactions and ion-pair formation in addition to hydrophobic inclusion in the CD [24, 25]. The higher enantioselectivity in this case is also related to the increase in mobility difference between the free and complexed forms of the analyte enantiomers.

For optimisation of the separation media an exhaustive study on the influence of several experimental conditions on the enantiomeric resolution was performed in order to improve the separation. First, the influence of the buffer pH was investigated in the range from 2.0 to 3.5. Analysis time increased ~~by large amounts~~ significantly with the pH due to the increase in the cathodic EOF in opposite direction to the electrophoretic mobility of the analyte-CD complex towards the anode. However, increasing the pH at 2.5 enabled to obtain the best enantiomeric resolution in a reasonable migration time, showing that a small change in the degree of ionization of the analytes favour the interactions governing enantioselectivity. At pH = 2.5, a study on the influence of the buffer concentration in the range from 10 to 100 mM was also performed. Resolution was improved by increasing electrolyte concentration due to a decrease in peak tailing. However, the concentration should not be raised excessively as this will increase the analysis time and current intensity. Taking into account a compromise between the resolution and the analysis time, a buffer concentration of 50 mM at pH 2.5 was chosen.

An essential step in the development of a chiral method is the optimisation of the concentration of the chiral selector. The effect of the HS-β-CD concentration on the resolution among the four stereoisomers and on their migration times was studied in the range from 0.5 to 4.0 % (w/v). The results showed that at concentrations of 0.5 % the stereoisomers were not detected in the reversed-polarity mode. However, when normal polarity was used, the basic analytes were detected in the cathode but without enantioseparation. On the other hand, an increase in the amount of HS-β-CD increased analyte-CD complexation by shifting the equilibrium and migration was more rapid, but the enantioresolution was compromised if the concentration was too high. Hence, a CD concentration of 2.5 % w/v was chosen as a compromise between resolution and run time also providing good peak shape.

Besides the concentration of the selected chiral selector, the effect on the enantioresolution of its degree of substitution (number of groups generally attached randomly per molecule of native CD to form the derivative), and the batch of HS-β-CD used was studied. The results showed that the same chiral discrimination was obtained using two different batches of HS-β-CD (one of Aldrich and another Fluka) both with DS between 12-14 sulphate molecules. Nevertheless, using HS-β-CD with 18 sulphate molecules supplied from Sigma, smaller migration times were obtained, but the chiral separation of the four stereoisomers was not possible since only the diastereomers of the chiral drug were resolved. As a consequence, HS-β-CD with SD between 12-14 sulphate molecules supplied from Fluka was chosen for further experiments.

To finish the optimization of the separation medium, the use of different additives was considered. Thus, the possibility of adding a neutral CD (5% (w/v) of α-CD or methyl-β-CD) or different organic modifiers (10 % (v/v) of acetonitrile, methanol, ethanol or 2-propanol) to the BGE was tested. However, a decrease in the enantiomeric resolution and an increase in the analysis time were observed.

Under the optimised conditions (50 mM phosphate buffer at pH 2.5 containing 2.5 % (w/v) of HS-β-CD with DS between 12-14), the migration order for the stereoisomers was investigated by injecting separately each pair of stereoisomers *trans* and *cis* at a 1:3 concentration ratio. The order of affinity of each stereoisomer towards HS-β-CD as follows: *RR* > *SS* > *RS* > *SR*. Note that this migration order favours the detection of the minor enantiomeric impurity (*RS* isomer) because it migrates before the ~~majority compound~~ major peak (*SR* isomer).

Finally, the effect of some instrumental variables that may have a significant impact on the chiral resolution, such as the operating temperature, the applied voltage, and the capillary length, was studied. On the one hand, the effect of the temperature on

the enantiomeric separation was tested in the range from 15 to 30ºC. An increase in the capillary temperature resulted in a decrease of migration times for all stereoisomers due to a smaller electrolyte viscosity, but it also produced a decrease in resolution values. On the other hand, the influence of the applied voltage and the possibility to improve the resolution increasing the length of the capillary keeping constant the electric field was also studied. **Figure 2** shows that an increase in the capillary length to 80.5 cm together with an increase in the applied voltage to -30 Kv resulted in an improvement in the enantioresolution which originated an excellent baseline separation.

After selecting the optimal conditions of the chiral method and before studying its performance, it was necessary to define a suitable protocol for the conditioning of the capillary to achieve a good repeatability of migration times and peak areas. On the one hand, washing the capillary with 0.1 M phosphoric acid was essential to favour better repeatability in peak area, forcing a subsequent conditioning with BGE to equilibrate the capillary wall with the separation media favouring better time repeatability. On the other hand, due to the migration of the charged CD, the possibility of interchanging the inlet and outlet vials after each run was investigated to improve method precision [24]. Hence, the vials with 400 µL of BGE were interchanged after each run automatically to prevent the decrease in the CD concentration in the cathode vial during the run and its increase in the other reservoir to evaluate the instrumental repeatability. Migration times decreased in the case where the vials were not interchanged (from 21.8 to 18.2 min). In contrast, when interchanging the vials, much better repeatability in terms of migration times was observed (see **Table 1**). However, the best repeatability was obtained when separately grouping the 6 values corresponding to odd injections and the 6 values corresponding to even injections in addition to interchanging the vials. The RSD values obtained in this case were similar to those obtained without reusing BGE.

Investigations were conducted to determine how often the same BGE could be used. After 40 injections along a working day interchanging vials, good repeatability in migration times (RSD = 1.7 %) and corrected peak areas (RSD = 5.7 %) was obtained. Even better repeatability was obtained when separately grouping the values corresponding to odd and even injections (RSD ≤ 1.1 % for migration times and RSD ≤ 4.4 % for corrected peak areas with n = 20). After a working day, a new BGE should be used to keep the baseline noise as low as possible. Hence, interchanging the vials should be routinely done, protocol that not only favours the repeatability of the analysis, but also is very interesting economically (because of the high cost of the chiral selector) and provides a greater number of free positions in the equipment for sample vials facilitating the automation of the analysis.

3.2. Method performance

The chiral method developed was validated in terms of selectivity, linearity, repeatability and intermediate precision, accuracy by means of recovery studies, and limits of detection (LOD) and quantitation (LOQ). All these parameters were evaluated according to the recommendations of the ICH [19] and the US Pharmacopeia. The component to be analyzed was the API (*SR*, majority stereoisomer and main compound), while the other stereoisomers (minority stereoisomers and related compounds) are its impurities.

A selective method to assess the ~~optical~~ enantiomeric purity of a drug substance must accurately measure the single enantiomer of interest even in the presence of potential sample components, i.e., interferences such as stereoisomeric impurities, other synthetic impurities or synthetic precursors, or degradation products that may be

expected to be present in the sample matrix. When all interferences are not available, stressed stability samples are often specified in validation protocols to evaluate the selectivity [26]. Thus, the impurity profiles should be compared in samples sufficiently stressed to provoke at least 10-15 % degradation. Among all the tested conditions [26], the use of 0.1 M NaOH and heating at 80 ºC during 24 hours resulted in a significant degradation (≈ 30 %) of the *SR*, being the inversion to *SS* the most important degradation as shown in **Figure 3**. This Figure shows that an adequate selectivity for the determination of the enantiomeric purity of the *SR* was obtained (resolution for all peaks ≥ 2). In addition, peak purity tests corresponding to *SR* in this stressed sample using spectral evaluation by DAD showed that the API peak was not attributable to more than one component (mean purity value of 999.464). As a consequence, the chiral method developed was selective for the determination of the enantiomeric purity of the drug substance studied, over other compounds such as synthetic impurities, synthetic precursors, or degradation products that may be present in the sample matrix.

The linearity was assessed as described in **Table 2**. Calibration curves were established by plotting corrected peak areas (peak area to migration time ratio) versus concentrations in mg/mL or µg/mL. First, the linearity for API was proven over the whole range analyzing fourteen concentrations, from 0.05 % to 150 % of the nominal assay concentration (0.5 mg/ml). Satisfactory results were obtained with a determination coefficient (R^2) of 99.97 %, an y-intercept not differing statistically from zero, a slope differing statistically from zero, a relative standard deviation (RSD in %) for all the generated response ratios (area/concentration) of only 1.2 %, and the analysis of variance for the calibration line revealing that the lack of fit did not differ statistically from the pure error (p-value, 0.265), confirming that a straight line was a suitable model. Second, the linearity for the enantiomeric impurity (*RS*) and for one

diastereomeric impurity (*SS*) was established for low concentration levels with seven standard solutions in the range from 0.05 to 5 % of the nominal API concentration. Acceptable linearity was obtained, with determination coefficients higher than 99.9 %, both confidence intervals at 95 % included the zero value for the intercept and statistically differed from zero for the slope, being RSD for response ratios < 5 % at low concentration levels for minority compounds.

The Relative Response Factors (RRFs) were obtained by dividing the slopes of the calibration lines for each stereoisomeric impurity and the slope obtained for the API, obtaining RFFs between 0.8 to 1.2 in agreement with the requirements of the European Pharmacopoeia. Therefore, the response for minority stereoisomers was equivalent to that for the main stereoisomer, being not necessary to correct their values by the factor, i.e., the percentages of stereoisomeric impurities can be determined from the ratio of the areas for the active enantiomer and its stereoisomeric impurities.

The precision of the whole method was evaluated in terms of repeatability and intermediate precision, both expressed as RSD (%) as described in **Table 2**. The results for corrected peak areas and migration times showed an acceptable precision in all cases.

In order to test the accuracy of the method, recovery studies were achieved for the API (*SR* isomer), the enantiomeric impurity (*RS* isomer) and one of the two diastereomeric impurities (*SS* isomer). Recoveries within the range 98.0-104.5 %, with a good mean recovery of 99.8 % and acceptable RSD (< 6 %) were obtained (see **Table 2**). To determine the accuracy for the impurities, the concentration level was set at 0.5 % of the nominal API concentration. Mean recoveries of 100.9 % and 104.5 % with acceptable RSD (< 10 %) were obtained.

According to the requirements of Q2 ICH guidelines [19], we have applied the following equations to calculate the limits of detection (LOD) and quantitation (LOQ):

$$LOD = \frac{3.3 \cdot s_{y-\mathrm{intercept}}}{Slope} \qquad LOQ = \frac{10 \cdot s_{y-\mathrm{intercept}}}{Slope}$$

In addition, the values estimated by this approach were verified experimentally. It can be concluded that the LODs and LOQs for API and their impurities were about 0.15 µg/mL and 0.5 µg/mL, respectively.

On the other hand, in an analytical method optimized to determine a high enantiomeric purity, it is also practical to use a limit of detection for the minor enantiomer in the presence of the major enantiomer, designated as the relative limit of detection (RLOD), which was determined according to the following equation [27]:

$$RLOD = \frac{LOD_{Min}}{C_{Maj}^{Max}} \times 100$$

where LOD_{Min} is the LOD for the minor enantiomer and C_{Maj}^{Max} the maximum concentration of the major enantiomer that can be injected without loss of the resolution or solubility problems (i.e., at nominal concentration). The RLOD value defined is a measure of the minimum enantiomeric impurity that can be detected in samples containing high enantiomeric excess. In this work this value was 0.03 % for all the impurities.

3.3 Applications

Prior to phase I clinical investigations, the analytical method developed was applied to the determination of the "chiral purity" of the drug substance and to the determination of its "chiral stability" in the solid state, in its dosage forms and in biological samples.

3.3.1 Drug substance and product analysis

The enantiomeric purity testing of drug substance was achieved for three batches of raw material (see **Figure 4**). The values obtained for the enantiomeric purity and for the percentage of impurities are given in the same figure. On the other hand, since interconversion of enantiomers can occur with time (inversion of chiral centers due to chemical degradation), it is necessary to evaluate the chiral stability of the drug substance. Thus, an accelerated testing to evaluate the solid state chiral stability during storage of the drug substance was performed according to the ICH guideline Q1A [8]. For example, the impurity profiles of API samples at time 0 and after 6 months at 40 ºC and 75 % of humidity (most drastic storage conditions) showed that a significant degradation did not occur, only a slight inversion (≈ 0.5 %) of the *SR* enantiomer to *SS* diastereoisomer described above in **Figure 3** took place, confirming that the most likely inversion should affect only ~~to~~ one chiral center [28].

On the other hand, given the low content of the formulations under development (50 µg/mL, 10 times smaller than the nominal concentration in the analysis of raw material), it was necessary to apply an in-capillary preconcentration technique for the analysis of these formulations. This technique is named sweeping-EKC, which is defined as the picking and accumulating of analytes by the charged pseudostationary phase, such as sulphate-CDs, that penetrates the sample zone during application of a voltage [29]. In this approach, the concentration of the sample solution must be much

lower than that of the BGE and this requirement is achieved given the composition of the intravenous formulations (injectable) analyzed. As a result, in comparison with a normal injection (hydrodynamic injection during 15 s) about 10-fold improvement in detection sensitivity was obtained when a hydrodynamic injection during 150 s was carried out. The impurity profiles of the formulation samples at time 0 and after 1 month at 40 ºC and 75 % of humidity (most drastic storage conditions) showed that the chiral inversion at two chiral centers did not occur, only a slight inversion (< 2 %) of the *SR* enantiomer to *SS* diastereoisomer described above took place (see **Figure 5**), i.e., inversion at only one chiral center.

These results were very interesting because if inversion of the chiral center has been observed in these early studies, then drug metabolism and pharmacokinetics should determine the extent of exposure to the unwanted stereoisomers in the safety studies.

3.3.2 In vivo samples analysis

Finally, the chiral method was applied to investigate for possible *in vivo* inversion in metabolism samples, i.e., if unwanted stereoisomers may be present as degradation products generated via metabolism in biological systems. Thus, a special clinical study with representative plasma extracts, selected at appropriate time points from the regular phase I studies (5, 30, 45 and 60 minutes), was used for this purpose. First, it was necessary to carry out a treatment of the biological sample by SPE to remove matrix components. To do this, a standard protocol for cationic compounds in plasma samples, as described in Section 2.4, was applied. The chiral drug was eluted with 1 mL of 10% NH_4OH in methanol, and in order to remove residues of this matrix that could interfere with the injection protocol used for analysis, the eluate was

evaporated to dryness and reconstituted in water. Second, in response to the sensitivity problem due to the extremely low concentration levels of these biological samples, a novel method named cation-selective exhaustive injection (CSEI)-sweep-EKC that combines two in-capillary preconcentration techniques in CE, sample stacking with electrokinetic injection (field-amplified sample injection, FASI) and sweeping, afforded the detection of all stereoisomers (positively chargeable analytes) at ppb levels [30]. The main idea was to selectively introduce by FASI as many organic cations as possible from a very diluted sample solution, with electrokinetic injection performed for a longer period of time than typical, and to focus the resulting zone by sweeping using a charged pseudostationary phase (i.e., sulfated CDs). This in-capillary preconcentration technique was applied according to the protocol developed by Quirino and Terabe [30] (described in **Figure 6**), but optimizing the filling time of the capillary with high-conductivity buffer (step (ii) in Figure 6B) at along with the time of the electrokinetic injection (step (iv) in Figure 6B) to achieve maximum sensitivity without damaging enantioresolution, 100 and 300 s, respectively. Note that about 250-fold improvement in detection sensitivity was obtained using CSEI-sweep-EKC technique when it was compared with sweeping-EKC technique (see **Figure 6**).

The electropherograms obtained for the biological samples showed that the levels of undesired stereosiomers of the API (*SR* enantiomer, and *SS* or *RR* diastereomers) were negligible as a result of metabolism (see **Figure 7**). It can be emphasized that these results are very important in the development of the new chiral drug, because if the single-dose pharmacokinetic study had shown a rapid inversion of the drug *in vivo*, then the decision to develop the drug as a single isomer should be re-evaluated.

4. Concluding remarks

A chiral EKC methodology was successfully developed and validated in this work for enantiomeric purity testing of a new chiral drug with two chiral centers which is currently undergoing drug development as single enantiomer. Since only one of the four stereoisomers of this drug shows an interesting pharmacological activity, the other three isomers are considered undesired impurities. After a screening of sixteen CDs (neutral and anionic), only sulfated CD gave the adequate chiral discrimination. In addition, not only the type of CD employed as chiral selector was critical but also its substitution degree. The difficulty of resolving the four stereoisomers required an optimization process, being necessary to optimize the pH and buffer concentration, concentration and degree of substitution of the CD as well as the temperature, applied voltage and capillary length separation.

The developed chiral EKC method was validated and successfully applied to the analysis of the new drug in raw material, allowing to establish diastereomeric and enantiomeric impurities up to 0.03 %, as well as to establish the chiral stability of the drug in the solid state. Note that a protocol of interchanging between inlet and outlet vials was necessary to maintain the repeatability of migration times, thereby allowing to reuse the expensive separation buffer throughout a complete day obtaining excellent repeatability for migration times (RSD $\leq$ 2 %) as well as for corrected peak areas (RSD $\leq$ 6 %).

The analysis of dosage forms with low dose of API required the use of an in-capillary preconcentration technique (sweeping-EKC), which allowed using a hydrodynamic injection for 150 s to achieve about 10-fold improvement in sensitivity. Another in-capillary preconcentration technique (CSEI-sweep-EKC) was necessary to

analyse biological samples. This last technique enabled a significant improvement in sensitivity, about ~3000-fold compared with the usual injection technique.

Therefore, the performance of this method enables to accomplish with the requirements of the ICH guideline entitled 'Specifications for New Drug Substances and Products: Chemical Substances', which indicates that suitable controls should be imposed during the synthesis to assure chiral purity, controlling that there is no racemization on storage of the drug substance, during its manufacture or *in vivo* metabolism.

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Conflicts of interest

Authors declare that they have no conflict of interest

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Figure captions

Figure 1. Structure of the studied chiral drug. Chiral center is denoted by *.

Figure 2. Effect of the length of the capillary on the chiral separation of API. Conditions: **a)** capillary, 50 μm x 48.5 cm; applied voltage, -20 kV (-82 μA) **(b)** capillary, 50 μm x 80.5 cm; applied voltage, -30 kV (-75 μA). Other conditions: 1.0 % (w/v) HS-β-CD in phosphate buffer (pH 2.5); injection by pressure at 5 kPa (50 mbar) for 5 s; temperature, 15º C; UV detection at 260 nm; applied voltage.

Figure 3. Electropherograms corresponding to an API solution (0.5 mg/mL) compared with the same sample stressed by hydrolysis with 0.1 M NaOH and 80 ºC during 24 hours. Experimental conditions: BGE, 2.5% (w/v) HS β-CD in 50 mM phosphate buffer (pH 2.5); uncoated fused-silica capillary, 80.5 cm (72 cm to the detector) x 50 μm ID;

UV detection at 260 nm; applied voltage, -30 kV (-75 µA); temperature, 15º C; injection by pressure at 5 kPa (50 mbar) for 15 s.

Figure 4. Electropherograms corresponding to four different batches of API raw material. Enantiomeric purity for API and percentages of stereoisomeric impurities are included. Experimental conditions as in Figure 3.

Figure 5. Electropherogram showing the impurity profiles of a drug product (intravenous formulation with 0.05 mg/mL of API) with SR-isomer at time 0 and after 1 month at 40 ºC and 75 % of humidity. Enantiomeric purity for API and percentages of stereoisomeric impurities are included. Injection by pressure at 5 kPa (50 mbar) for 150 s. Other experimental conditions as in Figure 3.

Figure 6. Comparison of two in-capillary preconcentration techniques: (A) Sweep-EKC analysis of a mixture of the four stereoisomers dissolved in water, hydrodynamic injection at 5 kPa (50 mbar) for 150 s between plugs (5 kPa for 5 s) of 200 mM phosphate buffer (pH 2.5); (B) CSEI-sweep-EKC analysis of a mixture of the four stereoisomers dissolved in water, injection according to the following steps: (i) the capillary was filled with the BGE without CD (50 mM phosphate buffer at pH 2.5), (ii) a 200 mM phosphate buffer at pH 2.5 was introduced for 100 s at 5 kPa (50 mbar); (iii) a plug of water was introduced for 5 s at 5 kPa (50 mbar), (iv) +30 kV was applied for 300 s to inject the sample contained in a microvial at the inlet end and BGE without CD at the outlet end of the capillary, (v) the voltage was shifted to -30 kV for normal CE separation with the BGE at both ends of the capillary. Other experimental conditions as in Figure 3.

Figure 7. Electropherograms by CSEI-sweep-EKC showing an *in vivo* pharmacokinetic study of the SR-isomer. Rat plasma samples were withdrawn at 5, 30, 45, and 60 min after drug ingestion, and they were injected following the steps described in Figure 3B after their clean up by SPE (see section 2.4). Other experimental conditions as in Figure 3.

Table 1. Study of the precision of the method. Experimental conditions: BGE, 2.5% (w/v) HS β-CD in 50 mM phosphate buffer (pH 2.5); uncoated fused-silica capillary, 80.5 cm (72 cm to the detector) x 50 μm ID; UV detection at 260 nm; applied voltage, -30 kV; temperature, 15º C; injection of API (0.5 mg/mL) by pressure at 5 kPa (50 mbar) for 15s. Volume of vial, 400 μL.

Instrumental repeatability		
Protocol	**Time, RSD %**	**Corrected area, RSD%**
Without interchanging vials Consecutive injections, n=6	6.9	4.4
With interchanging vials Consecutive injections, n=6	1.9	1.5
With interchanging vials Consecutive injections, n=12	2.0	2.1
With interchanging vials Odd injections, n=6	0.33	2.1
With interchanging vials Even injections, n=6	0.43	1.8
Without reusing the BGE	0.20	1.4

Table 2. Performance characteristics of the proposed method. Experimental conditions as in Table 1.

PARAMETERS	**API (*SR*)**		**Enantiomeric impurity (*RS*)**		**Diastereomeric impurity (*SS*)**	
Linearity [a]	*0.25 µg/mL-0.75 mg/mL*		*0.25 µg/mL -15 µg/mL*			
R^2	99.97%		99.97%		99.94%	
y-intercept ± *t* $s_{y\text{-intercept}}$	-0.053 ± 0.094		-0.0004 ± 0.0052		-0.0020 ± 0.0051	
slope ± *t* s_{slope}	49.0 ± 0.3		44.7 ± 0.8		41.8 ± 1.0	
RSD (%) for response ratios	1.2		4.0		4.8	
RRF	---		0.91		0.85	
Precision						
Repeatability [b]						
Protocol	Time	A_C	Time	A_C	Time	A_C
Consecutive injections, n=18	1.5 %	4.5 %	1.8 %	5.7 %	1.5 %	5.0 %
Odd or even injections, n=9	0.4 – 0.6 %	3.7 – 5.1%	0.4 – 0.7 %	4.0 – 5.9 %	0.4 – 0.6 %	3.8 – 4.6 %
Intermediate precision [c]						
Protocol	Time	A_C	Time	A_C	Time	A_C
Consecutive injections, n=18	3.8 %	7.3 %	4.0 %	5.7 %	4.0 %	4.4 %
Accuracy [d]						
Recovery (average ± RSD)	99.8 ± 5.9 %		100.9 ± 9.5		104.5 ± 9.1	
LOD [e]	0.15 µg/ml		0.16 µg/ml		0.17 µg/ml	
LOQ [e]	0.45 µg/ml		0.48 µg/ml		0.52 µg/ml	
RLOD [e]	0.03 %		0.03 %		0.03 %	

[a] The linearity was assessed by analyzing at least seven standard concentrations, three replicates at each concentration, and each one was injected in triplicate.
[b] Six samples at two concentration levels (0.5 mg/mL for API , and 0.5 % of this nominal level for stereoisomeric impurities) were prepared, and each one was injected in triplicate.

[c] The same samples analyzed to evaluate the repeatability were injected in triplicate on three different days with the same equipment, but using two different capillaries and two lots of HS-β-CD.
[d] Six replicates were prepared at the nominal concentration level (0.5 mg/mL) for API and each one was injected in triplicate. In the case of the *impurities*, 0.5 % of the nominal concentration level of API was added to the samples.
[e] According to equations described in section 3.2.

Figure 1

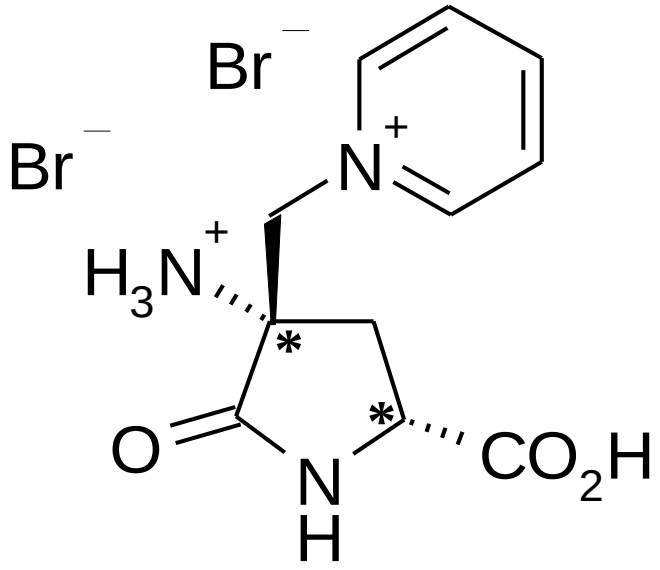

Figure 2

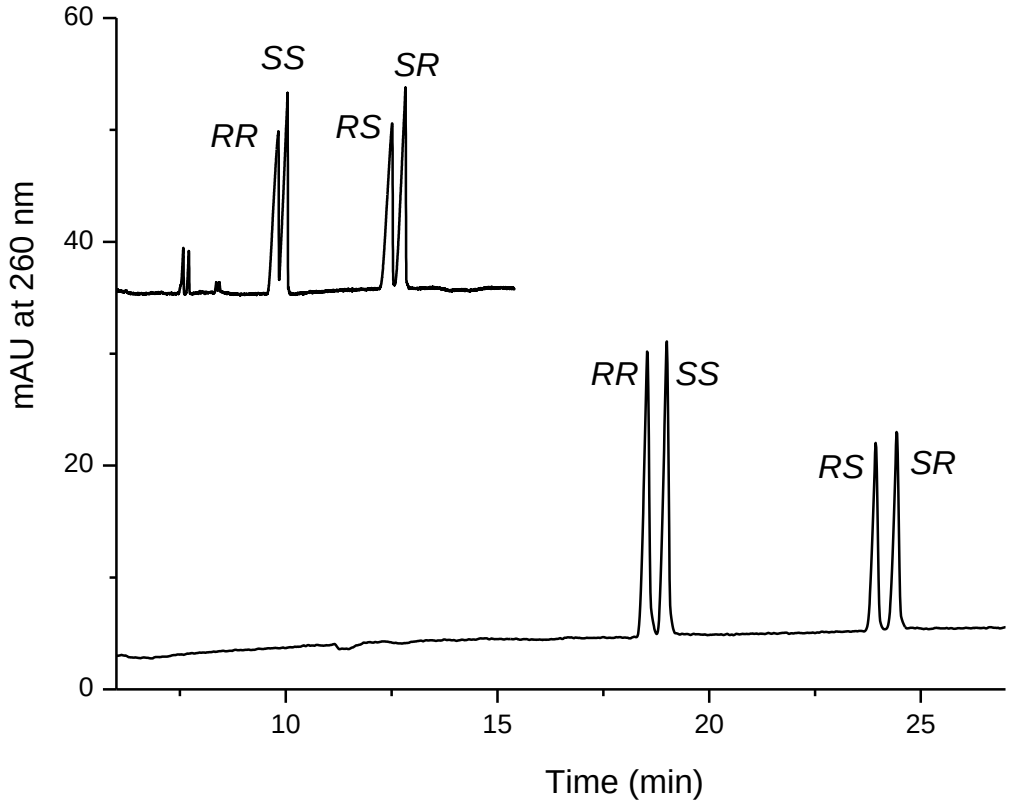

Figure 3

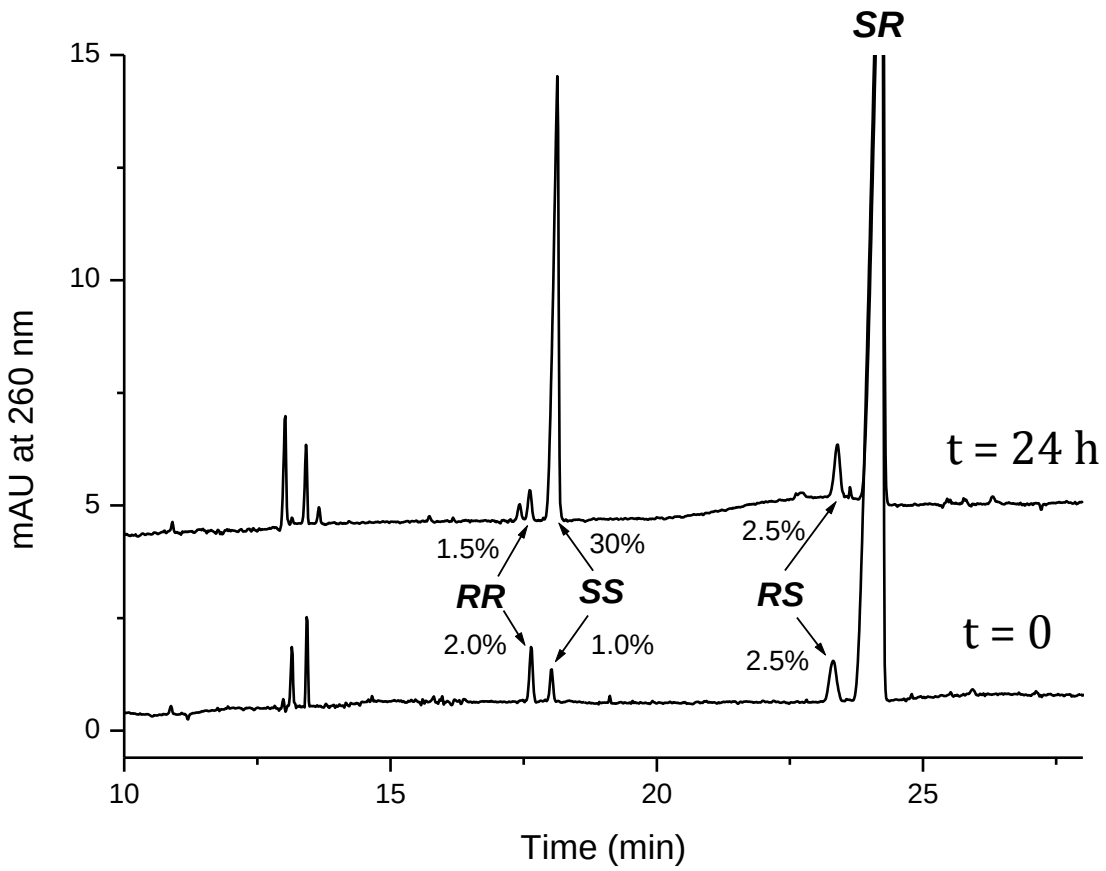

Figure 4

	Batch 1	Batch 2	Batch 3
RR	6.9 %	1.7 %	0.3 %
SS	1.9 %	2.1 %	1.6%
RS	15.4 %	3.6 %	0.4 %
SR	75.8 %	92.6 %	97.7 %

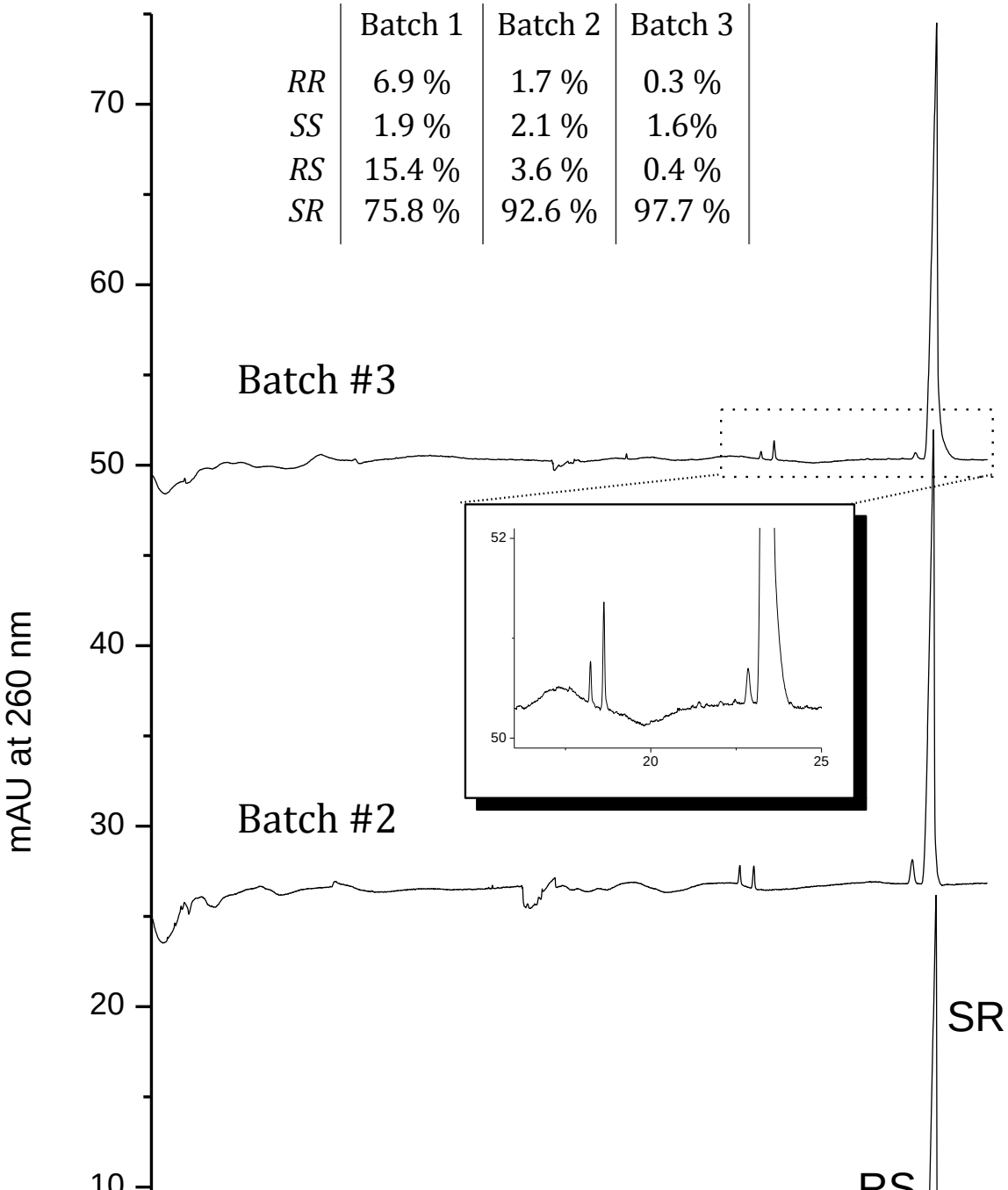

Figure 5

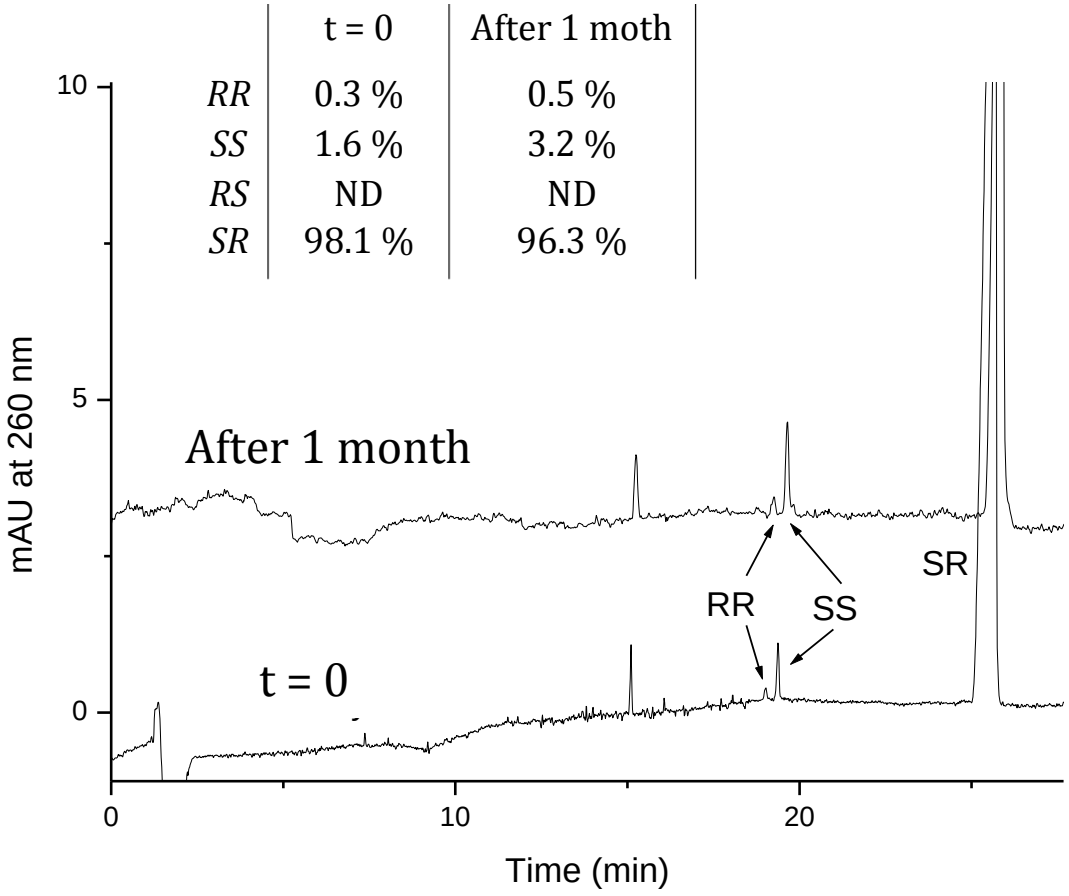

Figure 6

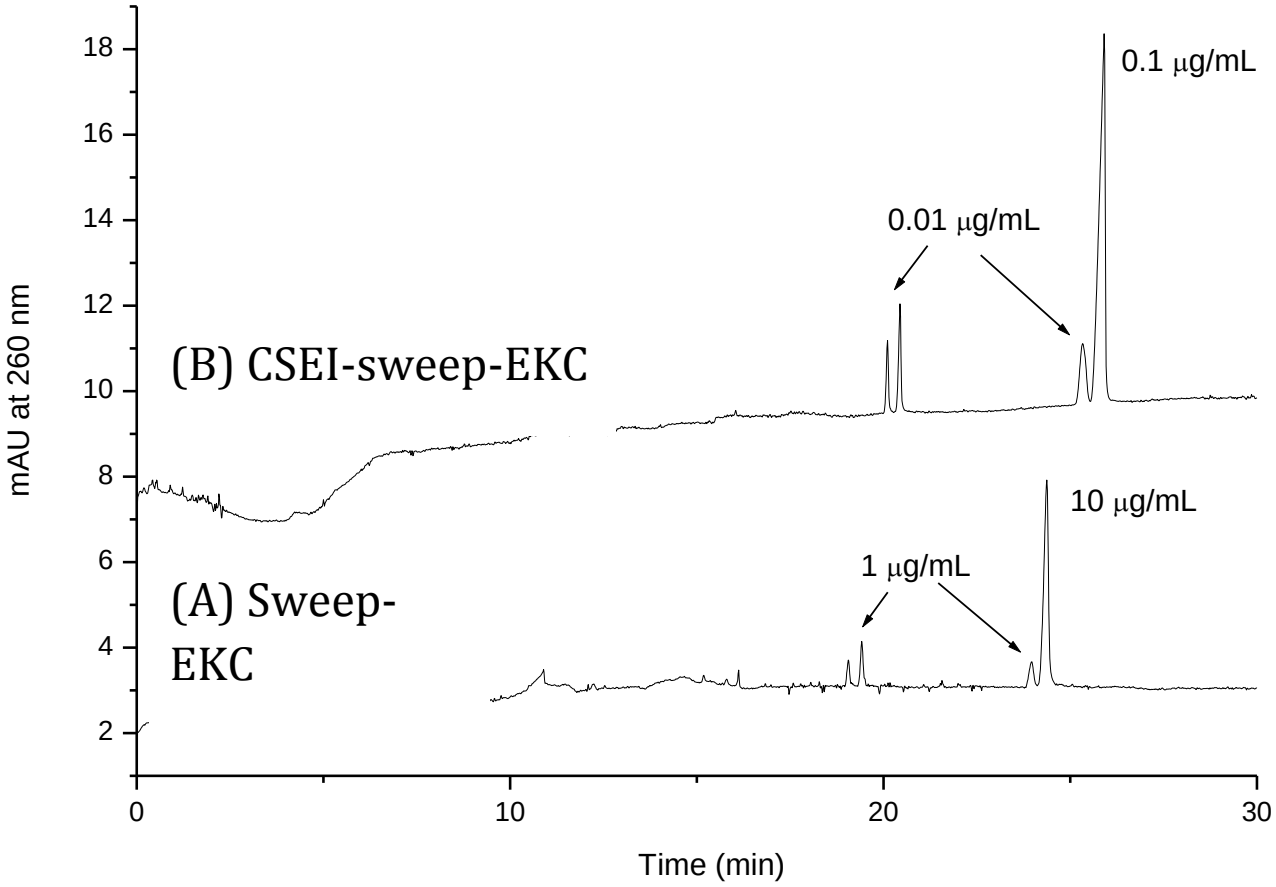

Figure 7