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Enantiomeric separation of ivabradine by cyclodextrin-electrokinetic chromatography. Effect of amino acid chiral ionic liquids

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19 **ABSTRACT**

20 A chiral methodology was developed by electrokinetic chromatography (EKC) to ensure the quality
21 control of ivabradine, a novel anti-ischemic and heart rate lowering drug commercialized as a pure
22 enantiomer. The enantiomeric separation of ivabradine was investigated using different anionic and
23 neutral cyclodextrins (CDs) and amino acid-based chiral ionic liquids (CILs) as sole chiral selectors.
24 Baseline separation was only achieved with sulfated CDs, and the best enantiomeric resolution was
25 obtained with sulfated-γ-CD. Under the optimized conditions, ivabradine enantiomers were separated in
26 6 min with a resolution of 2.7. Nuclear magnetic resonance experiments showed a 1:1 stoichiometry for
27 the enantiomer-CD complexes and the same apparent and averaged equilibrium constants for both of
28 them (1.31 ± 0.17 mM^{-1}), which suggested that the chiral separation was not determined by the stability
29 of the complexes but by their different mobilities. The combined use of sulfated-γ-CD and different CILs
30 as dual separation systems was investigated, resulting in a significant increase in the resolution. The best
31 enantioseparation for ivabradine was obtained adding 5 mM $[TBA]^{+}[L\text{-}Asp]^{-}$ to 50 mM formate buffer
32 (pH 2.0) containing 4 mM sulfated-γ-CD. Nevertheless, since good separation resolution was also
33 obtained by using sulfated-γ-CD as sole chiral selector, the analytical characteristics of this method were
34 evaluated, showing good recovery (98% and 103% for S- and R-ivabradine, respectively) and precision
35 values (RSD < 5% for instrumental repeatability, < 6% for method repeatability and < 7% for
36 intermediate precision). The limits of detection (LODs) were 0.22 and 0.28 μg mL^{-1} for S- and R-
37 ivabradine, respectively, and the method enabled to detect a 0.1% of the enantiomeric impurity, allowing
38 to accomplish the requirements of the International Conference on Harmonisation (ICH) guidelines.
39 Finally, the method was applied to the analysis of a pharmaceutical formulation of ivabradine. The
40 content of R-ivabradine was below the LOD and the amount of S-ivabradine was in agreement to the
41 labeled content.

42 **Keywords:** Capillary electrophoresis, electrokinetic chromatography, ivabradine, chiral ionic liquids,
43 enantioseparation, cyclodextrins

44 **1. Introduction**

45 Chirality is a relevant issue in the pharmaceutical field. Pharmaceutical laboratories must justify
46 the commercialization of a new drug, either as a pure enantiomer or as a racemic mixture, because of
47 the potentially different enantioselective bioactivity of a chiral drug. For this reason, chiral
48 methodologies need to be developed to ensure quality control and to monitor the presence of
49 enantiomeric impurities in pharmaceutical formulations. Indeed, according to the International
50 Conference on Harmonisation (ICH), to regulate drug enantiomeric impurities, chiral methodologies
51 must be able to detect the amount of an enantiomeric impurity in a level lower than 0.1% in relation
52 to the active enantiomer [1].

53 Capillary electrophoresis (CE) is a powerful technique for separating enantiomers and analyzing
54 enantiomeric impurities, which offers many advantages such as simplicity, high resolution power,
55 versatility and high separation efficiency. For these reasons, it has been extensively applied in the
56 pharmaceutical field to achieve the enantioseparation of a wide range of drugs [2]. Moreover, this
57 technique requires small sample volume, low reagent consumption and low operating costs, thus it is
58 considered an environmentally friendly technique. There are different CE modes for chiral analysis,
59 being the most widely used the electrokinetic chromatography (EKC) where a chiral selector is added
60 to the separation buffer. A wide variety of chiral selectors can be used [2] among which cyclodextrins
61 (CDs) are still by far the most commonly employed [3]. Dual systems composed by two chiral
62 selectors have also been investigated as a means to improve resolution and peak efficiency. In this
63 context, there is a current trend to evaluate chiral ionic liquids (CILs) as new potential chiral selectors
64 [4-7], when used as sole chiral selectors, as chiral ligands or in dual separation systems [7]. Several
65 works have studied the synergistic effect obtained by combining CILs and CDs in CE to perform the
66 chiral separation of drugs [8-16] and in all of them it was observed that the combination of both chiral
67 selectors increased the enantiomeric resolution and improved the selectivity.

68 Ivabradine is a novel anti-ischemic and heart rate lowering drug indicated for the treatment of
69 chronic heart failure with reduced ejection fraction [17]. It was approved by the European Medicines
70 Agency (EMA) in 2005 and by the Food and Drug Administration (FDA) in 2015 [18, 19]. This drug
71 was developed as an alternative to other antianginal drugs such as β-blockers and calcium channel
72 blockers, since these compounds exhibit adverse events because of their negative ionotropic effects
73 [20-22]. Ivabradine selectively inhibits the "funny" channel pacemaker current (I_f) in the sinoatrial
74 node, what slows heart rate and increases blood flow to the myocardium without affecting cardiac
75 contractility. In contrast, β-blockers and calcium channel blockers reduce both heart rate and
76 contractility [23]. Ivabradine is the (+)-enantiomer (S configuration) of the racemic benzocyclobutane
77 derivative S 15544 compound. During its development, ivabradine was named (+) S 16257 and it was
78 compared with its enantiomer, named (-) S 16260 (R configuration) [24]. It was observed that both
79 isomers equally reduced heart rate. However, contrary to (+) S 16257, the (-) S 16260 enantiomer
80 significantly prolonged the action potential duration of ventricular preparation, which is a potential
81 proarrhythmic effect. In addition, (-) S 16260 increases the QT interval corrected (QTc) for heart rate
82 in a dose-dependent manner, this indicating a direct effect on ventricular repolarization, in contrast
83 to the absence of effect of (+) S 16257 on the QTc [24, 25]. Therefore, because of its
84 electrophysiological selectivity, (+) S 16257 was chosen for clinical development, becoming
85 ivabradine. Thus, ivabradine, whose chemical name is [3-(3-{[((7S)-3,4-
86 dimethoxybicyclo[4,2,0]octa-1,3,5-trien-7-yl)methyl]methylamino}propyl)-1,3,4,5-tetrahydro-7,8-
87 dimethoxy-2*H*-3-benzazepin-2-one], is marketed as a pure enantiomer under different brand names.

88 The aim of this work was to develop a CE methodology to achieve for the first time the
89 enantioseparation of ivabradine, as to the best of our knowledge, it has not been reported in any
90 previous work. For this purpose, CDs and CILs were investigated as sole chiral selectors and also
91 combined in dual separation systems. It is worth mentioning, that the use of some of the CILs
92 employed in this work ($[TBA]_2^+[L\text{-}Glu]^-$, $[TMA]^+[L\text{-}Glu]^-$ and $[TMA]^+[L\text{-}Lys]^-$) have never been

93 reported before in CE. Moreover, nuclear magnetic resonance (NMR) experiments were carried out
94 in order to obtain information about the stoichiometry and the apparent and averaged equilibrium
95 constants of the enantiomer-CD complexes. Finally, the analytical characteristics of the optimized
96 method using sulfated-γ-CD as chiral selector were evaluated and it was applied to the enantiomeric
97 determination of ivabradine in a pharmaceutical formulation.

98 **2. Materials and methods**

99 *2.1. Chemicals, reagents and standard solutions*

100 Ortho-phosphoric acid 85% was purchased from Scharlau Chemie (Barcelona, Spain), dimethyl
101 sulfoxide (DMSO) was obtained from Merck (Darmstadt, Germany), formic acid, deuterated water
102 (D_2O, >98%D) and sodium 3-trimethylsilyl-(2,2,3,3-tetradeutero)propionate (TSP-Na) were from
103 Sigma-Aldrich (St. Louis, MO, USA). The water used was obtained from a Millipore Milli-Q-System
104 (Bedford, MA, USA).

105 Anionic CDs: sulfated-β-CD was from Fluka (Buchs, Switzerland), sulfated-α-CD, sulfated-γ-
106 CD, succinyl-β-CD, succinyl-γ-CD, phosphated-β-CD, sulfobutylated-β-CD, carboxymethyl-α-CD
107 and carboxymethyl-γ-CD were purchased from Cyclolab (Budapest, Hugary) and carboxymethyl-β-
108 CD was obtained from Sigma-Aldrich.

109 Neutral CDs: β-CD, heptakis (2,3,6-tri-*O*-methyl)-β-CD and (2-hydroxy)propyl-β-CD were
110 purchased from Fluka, α-CD, heptakis (2,6-di-*O*-dimethyl)-β-CD and methyl-β-CD were from
111 Sigma-Aldrich, γ-CD, randomly substituted-methyl-β-CD, acetylated-β-CD, acetylated-γ-CD and (2-
112 hydroxypropyl)-γ-CD were obtained from Cyclolab.

113 Amino acid-based CILs employed in this work $[TBA]^+[L\text{-}Asp]^-$, $[TMA]^+[L\text{-}Asp]$, $[TBA]^+[L\text{-}$
114 $Arg]^-$, $[TMA]^+[L\text{-}Arg]^-$, $[TBA]^+[L\text{-}Iso]^-$, $[TMA]^+[L\text{-}Iso]^-$, $[TBA]^+[L\text{-}Lys]^-$, $[TMA]^+[L\text{-}Lys]^-$,

115 $[TBA]_2^+[L\text{-}Glu]^-$, $[TBA]^+[L\text{-}Glu]^-$ and $[TMA]^+[L\text{-}Glu]^-$, were synthesized by the Center for Applied
116 Chemistry and Biotechnology (CQAB) from the University of Alcalá.

117 (*S*)-Ivabradine was purchased from Sigma-Aldrich, (*R*)-Ivabradine was obtained from Toronto
118 Research Chemicals Canada (North York, ON, Canada). Fig. 1 shows the structure of ivabradine.
119 The pharmaceutical formulation of ivabradine was from a laboratory authorized to commercialize
120 this drug. According to its label, it contained 5 mg of ivabradine per capsule.

121 Stock standard solutions of ivabradine enantiomers (1000 mg L^{-1}) were prepared in DMSO and
122 stored at -20 ˚C. Working standard solutions containing the analytes at different concentration levels
123 were prepared by appropriate dilution of the stock solutions with Milli-Q water until desired
124 concentration. For the pharmaceutical solution, the content of 2 drug capsules was dissolved in an
125 appropriate volume of DMSO and sonicated until homogenization. Afterwards, it was filtered through
126 a 0.45 µm nylon filter and diluted with Milli-Q water to the required concentration.

127 *2.2. CE conditions*

128 CE experiments were performed on an Agilent 7100 CE system (Agilent Technologies,
129 Waldbronn, Germany), equipped with a diode array detector (DAD) operating at 200 nm (bandwidth
130 20 nm) including a reference wavelength of 286 nm (bandwidth 20 nm). The system was controlled
131 by the HP^{3D} CE ChemStation software from Agilent Technologies. Separation was achieved using
132 50 mM formic buffer (pH 2.0) containing 4 mM of sulfated-γ-CD as BGE and an uncoated fused-
133 silica capillary of 50 µm I.D. with a total length of 58.5 cm (50 cm effective length) from Polymicro
134 Technologies (Phoenix, AZ, USA). Injections were performed by applying 50 mbar for 5 s, and the
135 optimum electrophoretic separation was achieved at 25 ˚C in negative-polarity mode (-30 kV). At the
136 beginning of each working day, the capillary was flushed with 0.1 M sodium hydroxide, Milli-Q
137 water, 0.1 M HCl and BGE during 10, 5, 2 and 10 min, respectively. To ensure repeatability between

138 injections, the capillary was conditioned 2 min with 0.1 M sodium hydroxide, 2 min with Milli-Q
139 water, 2 min with 0.1 M HCl and 2 min with BGE.

140 *2.3. NMR experiments*

141 NMR experiments were performed with a Varian INNOVA 500 NMR System (Palo Alto, CA,
142 USA), fitted with a CHX 1H/13C/15N-31P probehead, z-gradient module and variable temperature
143 unit. The spectrometer resonance frequency for 1H was 499.61 MHz. All NMR experiments were
144 done at 25ºC.

145 **3. Results and discussion**

146 *3.1. Development of a CE methodology for the enantiomeric separation of ivabradine*

147 Ivabradine is a basic drug (pKa 9.37); therefore, an acidic BGE was chosen to assure
148 quaternization (protonated form of ivabradine). With the aim of evaluating the enantiodiscrimination
149 power of different CDs towards ivabradine, a screening test was carried out using a 50 mM phosphate
150 buffer at pH 3.0. The CDs tested were 5 anionic (sulfated-α-CD, sulfated-β-CD, sulfated-γ-CD,
151 phosphated-β-CD, sulfobutylated-β-CD), 11 neutral (α-CD, β-CD, γ-CD, heptakis (2,6-di-*O*-
152 dimethyl)-β-CD, heptakis (2,3,6-tri-*O*-methyl)-β-CD, randomly substituted-methyl-β-CD, methyl-β-
153 CD, acetylated-β-CD, acetylated-γ-CD, (2-hydroxy)propyl-β-CD, (2-hydroxy)propyl-γ-CD), and a
154 group of 5 CDs that are charged at pH values above 4.5, and so they are also neutral at pH 3.0
155 (succinyl-β-CD, succinyl-γ-CD, carboxymethyl-α-CD, carboxymethyl-β-CD, and carboxymethyl-γ-
156 CD). All CDs were tested at a concentration of 10 mM, employing a voltage of -20 kV and a
157 temperature of 20 ˚C. Succinyl-β-CD, succinyl-γ-CD, carboxymethyl-α-CD, carboxymethyl-β-CD,
158 and carboxymethyl-γ-CD were also investigated at pH 5.0 to guarantee complete anion formation.
159 Among all the CDs studied, only sulfated-α-CD, sulfated-β-CD and sulfated-γ-CD enabled the
160 separation of ivabradine enantiomers, providing resolution values of 1.2 (25 min), 0.9 (27 min) and
161 4.5 (18 min), respectively. When using these three CDs, the first-migrating enantiomer was the active

162 principle (*S*-ivabradine) and the second-migrating enantiomer the enantiomeric impurity (*R*-
163 ivabradine). Sulfated-γ-CD was chosen as chiral selector since it provided the highest resolution value
164 in the shortest analysis time.

165 Once sulfated-γ-CD was selected as chiral selector, different experimental variables, such as CD
166 concentration, buffer composition, pH, working temperature and voltage were optimized. The effect
167 of the sulfated-γ-CD concentration was studied in the 2 to 10 mM range (2, 3, 4, 5 and 10 mM). It
168 was observed that the analysis time increased when the CD concentration decreased (Table S1). In
169 fact, when using 2 mM sulfated-γ-CD, the separation was not achieved in less than one hour.
170 Regarding resolution, it improved when increasing the CD concentration from 3 to 4 mM, then it
171 decreased at 5 mM and finally increased again up to 10 mM. Since resolution was the same for 4 and
172 10 mM (Table S1), 4 mM was selected in order to minimize the amount used of this selector. The
173 influence of the buffer nature was evaluated by comparing phosphate and formate buffers (pH 3.0) at
174 a 50 mM concentration under the same experimental conditions. Although the resolution decreased
175 to 2.5 when formate buffer was used (see Table S2), this buffer was chosen for further experiments
176 since it enabled to obtain the enantiomeric separation in less time (17 min) than phosphate buffer (29
177 min). When the concentration of formate buffer was increased to 100 mM, the resolution increased
178 but separation time took longer, so a 50 mM buffer concentration was employed (Table S2). The
179 effect of the buffer pH was also investigated (2.0, 2.5 and to 3.0). It was seen that at pH values lower
180 than 3.0 the analysis time slightly increased but resolution values were higher (Fig. 2a), for which
181 reason a pH 2.0 value was selected. The influence of the temperature on the enantiomeric separation
182 was also studied in the range from 15 to 25 ˚C (see Fig. 2b). Despite a temperature of 20 ˚C provided
183 the highest resolution values, 25 ˚C was chosen as optimum value since the separation was achieved
184 in shorter analysis times. Finally, the separation voltage was varied from -20 to -30 kV and it was
185 shown that increasing the voltage improved the enantiomeric resolution and decreased the analysis
186 time (Fig. 2c). Therefore, it was decided to use a -30 kV voltage to achieve the enantiomeric

187 separation. Under these optimized conditions, the separation of ivabradine enantiomers was achieved
188 in 6 min with a resolution value of 2.7 (Fig. 3a).

189 *3.2. Study of the ivabradine-sulfated-γ-CD interactions by NMR*

190 NMR experiments were carried out to study the interactions between ivabradine and sulfated-γ-
191 CD. The stoichiometry ratio of the complex between ivabradine enantiomers and the CD was
192 determined by constructing the Job's plot [26, 27]. This method involves keeping constant the total
193 molar concentration of both binding partners, while varying the analyte's mole fraction. A stock
194 solution with both ivabradine enantiomers enriched with the *S*-enantiomer (75/25, v/v) and containing
195 the CD (5 mM sulfated-γ-CD in 50 mM formate buffer at pH 2.0 in D_2O with 1 mM TSP-Na) was
196 prepared. Solutions with a different ivabradine mole ratio (0.2, 0.4, 0.5, 0.6, 0.6, 0.8 and 1.0) were
197 prepared by mixing the appropriate volumes of the stock solution under the same temperature
198 conditions. The signal of TSP-Na (δ 0.00 ppm) was used as internal reference for chemical shift
199 measuring. No signal splitting upon complexation was observed in the different solutions (Fig. 4),
200 suggesting that apparently there were no substantial differences in the complexation of each
201 ivabradine enantiomer with the CD. Since the Job's plot of ivabradine and sulfated-γ-CD gave a
202 maximum at a mole fraction value of 0.5 (Fig. 4), a 1:1 stoichiometry was assigned to the complexes
203 formed.

204 When the stoichiometric ratios between the enantiomer and the chiral selector are 1:1, the
205 apparent and averaged equilibrium constants (K) can be calculated using the Scott's equation [28]:

206
$$\frac{[selector]}{\Delta\delta obs} = \frac{[selector]}{\Delta\delta s} + \frac{1}{K\,\Delta\delta s}$$

207 where, [selector] is the molar concentration of the chiral selector, $\Delta\delta_{obs}$ is the chemical shift difference
208 between the ^{1}H signals with and without the presence of the chiral selector at a given concentration
209 and $\Delta\delta_{s}$ is the chemical shift difference at a saturation concentration of the chiral selector. According

210 to this, the Scott's plot for the ivabradine/sulfated-γ-CD system was obtained with the same stock
211 solutions used in the Job's plot. In this case, the concentration of ivabradine was kept constant at 0.4
212 mM, while the concentration of sulfated-γ-CD ranged from 0.5 to 4.0 mM. Again the signal of TSP-
213 Na was used as internal reference. As with the Job plot, no signal splitting was either observed.
214 Therefore, the Scott's plot resulted in a unique straight line with a good linear regression ($R^2 = 0.996$),
215 from which a K value of 1.31 ± 0.17 mM^{-1} for both ivabradine enantiomers was calculated. This
216 implies that both enantiomers form complexes with very similar stability, this suggesting that their
217 separation by CE is not determined by the affinity of either enantiomer towards the chiral selector but
218 by the electrophoretic mobility of each complex as reported before for other drugs [29].

219 *3.3. Evaluation of the effect of amino acid chiral ionic liquids (CILs)*

220 Eleven different amino acid-based CILs were tested combined in dual systems with sulfated-γ-
221 CD to evaluate the chiral discrimination potential of these mixtures towards ivabradine. It is worth to
222 mention that the use of some of the CILs synthesized in this work ($[TBA]_2^+[L\text{-}Glu]^-$, $[TMA]^+[L\text{-}Glu]^-$
223 and $[TMA]^+[L\text{-}Lys]^-$) in chiral CE has never been reported before. CILs were evaluated at different
224 concentration levels (5, 10 and 30 mM) in combination with 4 mM sulfated-γ-CD in order to
225 investigate a possible synergistic effect allowing to improve resolution and selectivity. The results
226 revealed that as the added concentration of CIL increased, the separation resolution significantly
227 increased, but so did the analysis time (Table 1). In general, the same trend was observed for the
228 different CILs, and a 5 mM CIL concentration was enough to achieve good resolution in short analysis
229 times. Indeed, for some CILs ($[TBA]^+[L\text{-}Arg]^-$, $[TMA]^+[L\text{-}Arg]^-$, $[TBA]^+[L\text{-}Lys]^-$, $[TBA]_2^+[L\text{-}Glu]^-$,
230 $[TBA]^+[L\text{-}Glu]^-$ and $[TBA]^+[L\text{-}Glu]^-$) it was not possible to obtain results at a 30 mM concentration.
231 Generally, in combination with the CD, TBA CILs enabled to achieve the enantiomeric separation in
232 shorter analysis times and with similar resolution than their analogs with TMA, except in the case of
233 the CILs with isoleucine, in which $[TMA]^+[L\text{-}Iso]^-$ was more effective than $[TBA]^+[L\text{-}Iso]^-$ (Table
234 1). From all the CILs assayed, best results were obtained with $[TBA]^+[L\text{-}Asp]^-$, $[TMA]^+[L\text{-}Iso]^-$ and

235 $[TMA]^+[L\text{-}Asp]^-$, being the dual system with $[TBA]^+[L\text{-}Asp]^-$ the most effective, since the
236 enantioseparation took place with almost twice the resolution (5.1) than that using only sulfated-γ-
237 CD as sole chiral selector (2.7) and in an analysis time increased just in 1 min (Fig. 3b). It has to be
238 indicated that when all the CILs were evaluated as sole chiral selectors under the optimized CE
239 conditions, both in positive and negative polarity, at different concentration levels (5, 10 and 30 mM)
240 in 50 mM formate buffer (pH 2.0), no direct enantioselectivity towards ivabradine was encountered.
241 No peaks were detected in negative polarity, whereas only one peak was observed in positive polarity
242 for every CIL (except for 30 mM $[TMA]^+[L\text{-}Arg]^-$ and 30 mM $[TMA]^+[L\text{-}Iso]^-$ in which no peaks
243 were detected).

244 *3.4. Analytical characteristics of the CE method developed*

245 Since none of the CILs investigated provided an inversion in the enantiomer migration order that
246 would have made possible that the first-migrating enantiomer was the enantiomeric impurity of
247 ivabradine, and taking into account that the resolution obtained using sulfated-γ-CD alone as chiral
248 selector in the separation buffer (2.7) was enough to achieve a relative limit of detection (RLOD)
249 enabling to assess the accomplishment of ICH regulations [1], the analytical characteristics of the CE
250 method developed using sulfated-γ-CD as sole chiral selector were evaluated. In fact, when the RLOD
251 was calculated under these conditions, it was shown that it was lower than 0.1% (see Fig. 5) which is
252 the maximum percentage of the enantiomeric impurity allowed by ICH guidelines. Selectivity,
253 linearity, the existence of matrix effects, accuracy, precision and limits of detection (LODs) and
254 quantification (LOQs) were also evaluated in order to demonstrate the suitability for the quality
255 control of pharmaceutical formulations.

256 Selectivity was assessed by the analysis of the ivabradine pharmaceutical formulation under the
257 optimized separation conditions. No interfering peaks caused by the excipients present in the capsule
258 were found, therefore selectivity was appropriate. Linearity was established from eight concentration

259 levels plotting corrected peak areas versus the analytes concentration in μg mL^{-1}. As shown in Table
260 2, good linearity was achieved with R^2 values ≥ 0.993 for both enantiomers. Moreover, confidence
261 intervals for the slopes did not include the zero value, while the confidence intervals for the intercept
262 included it (in both cases for a 95% confidence level). Additionally, the ANOVA test confirmed that
263 experimental data fit properly to a linear model (p-values > 0.05 for *S*- and *R*-ivabradine). The
264 existence of matrix effects was investigated by comparing the confidence intervals for the slopes
265 obtained by the external standard calibration method and the standard additions calibration method
266 (eight known amounts of *S*- and *R*- ivabradine were added to a pharmaceutical formulation sample
267 solution containing a constant concentration of *S*-ivabradine). No statistically significant differences
268 were found between the slopes obtained from each calibration method (for a 95% confidence level).
269 Therefore, as no matrix interferences could be noticed, the external standard calibration method could
270 be used to quantify the content of ivabradine in pharmaceutical formulations. In addition, the response
271 relative factor (RRF), which is calculated by dividing the slopes ($slope_{impurity}$ / $slope_{active\ principle}$), was
272 found to be between 0.8 and 1.2, which is in accordance with what the European Pharmacopoeia
273 establishes [30] to demonstrate that *S*- and *R*-ivabradine responses are equivalent. Therefore, the
274 percentage of *R*-ivabradine could be determined from the ratio between the areas of *S*- and *R*-
275 ivabradine.

276 The accuracy was evaluated as the recovery obtained from six solutions of the pharmaceutical
277 formulation containing 75 μg mL^{-1} of *S*-ivabradine (according to the labelled amount) spiked with 2
278 and 75 μg mL^{-1} of *R*- and *S*-ivabradine, respectively. As Table 2 shows, good recovery values were
279 obtained, since the 100% value was included in all cases. Precision was evaluated in terms of
280 instrumental repeatability, method repeatability and intermediate precision. Instrumental repeatability
281 was assessed by performing six consecutive injections of a pharmaceutical sample solution containing
282 75 μg mL^{-1} of *S*-ivabradine and spiked with 2 μg mL^{-1} of *R*-ivabradine. RSD values were lower than
283 5% and 0.2% for corrected peak areas and migration times, respectively (Table 2). Method

284 repeatability was evaluated through the analysis of three replicates of a pharmaceutical sample
285 solution containing 75 μg mL^{-1} of *S*-ivabradine and spiked with 2 μg mL^{-1} of *R*-ivabradine injected
286 in triplicate in the same day. In this case, RSD values were lower than 5.6% and 0.6% for corrected
287 peak areas and migration times, respectively (Table 2). Finally, intermediate precision was assessed
288 by the analysis of three replicates of a solution of the pharmaceutical formulation containing 75 μg
289 mL^{-1} of *S*-ivabradine and spiked with 2 μg mL^{-1} of *R*-ivabradine injected in triplicate in three
290 consecutive days. RSD values were lower than 6.9% and 1.7% for corrected peak areas and migration
291 times, respectively (Table 2).

292 LODs and LOQs were calculated as the minimum concentration yielding a signal to noise (S/N)
293 ratio of 3 and 10 times, respectively. LODs were 0.22 and 0.28 μg mL^{-1}, and LOQs 0.73 and 0.93 μg
294 mL^{-1} for *S*- and *R*-ivabradine, respectively (Table 2). As it has been previously indicated, the RLOD
295 enabled to detect 0.1% of ivabradine enantiomeric impurity (using a nominal value of 300 μg mL^{-1}
296 for *S*-ivabradine), so according to the ICH regulations [1] the method can be applied to the analysis
297 of the enantiomeric impurity.

298 *3.5.Quantitation of ivabradine in pharmaceutical formulations*

299 Once demonstrated the feasibility of the developed CE method for the enantiomeric determination
300 of ivabradine, it was applied to the analysis of an ivabradine pharmaceutical formulation. The results
301 obtained revealed a content of 5.2 ± 0.3 mg per capsule of *S*-ivabradine (corresponding to 103 ± 6 %
302 of the labeled content), which is in agreement with the labeled amount. On the other hand, *R*-
303 ivabradine was not detected in the pharmaceutical formulation, so its concentration was below the
304 LOD of the method or was not present in the sample (Fig. 5).

305 **4. Conclusions**

306 The first enantiomeric separation of ivabradine is presented in this work. The use of sulfated-γ-
307 CD as chiral selector in the separation buffer under the optimized CE conditions enabled the

separation of ivabradine enantiomers in 6 min with a chiral resolution of 2.7. NMR experiments were carried out in order to study the interactions between ivabradine enantiomers and sulfated-γ-CD. The stoichiometry of the complexes was found to be 1:1 for each ivabradine enantiomer, while the apparent and averaged equilibrium constants were the same for both ivabradine enantiomers (1.31 ± 0.17 mM^{-1}), suggesting that the chiral separation was due to the different electrophoretic mobility of the enantiomer-CD complexes, rather than to their stability. Moreover, the effect of different amino acid CILs on the enantiomeric separation of ivabradine was investigated showing the existence of a synergistic effect when combining the CILs and sulfated-γ-CD. Nevertheless, as no inversion of the migration order was observed when combining CILs and sulfated-γ-CD and a good enantiomeric resolution and efficiency were obtained using just sulfated-γ-CD as chiral selector, the analytical characteristics of this methodology were evaluated, obtaining good performance for the enantiomeric analysis of ivabradine in a pharmaceutical formulation and enabling to detect up to 0.1% of its enantiomeric impurity, allowing to assess the accomplishment of ICH guidelines.

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414 **Figure Captions**

415 **Fig. 1** Structure of Ivabradine.

416 **Fig. 2** Influence of the buffer pH (a), the temperature (b) and the separation voltage (c) on the migration
417 time of the first ivabradine enantiomer and on the chiral resolution obtained by CE using 4 mM sulfated-
418 γ-CD in 50 mM formate buffer.

419 **Fig. 3** Electropherograms corresponding to the chiral separation of 75 and 25 μg mL^{-1} of S- and R-
420 ivabradine, respectively, using 4 mM sulfated-γ-CD as sole chiral selector (a) and dual system based on
421 the combination of 4 mM sulfated-γ-CD and 5 mM $[TBA]^{+}[Asp]^{-}$ (b). Experimental conditions: 50 mM
422 formate buffer (pH 2.0); uncoated fused-silica capillary 58.5 cm (50 cm to the detector window) x 50
423 μm ID; UV detection at 200 nm; applied voltage -30 kV; temperature 25 ˚C; injection by pressure, 50
424 mbar for 5 s.

425 **Fig. 4** NMR spectra (a) and Job's plot (b) for ivabradine with sulfated-γ-CD.

426 **Fig. 5** Electropherograms corresponding to the LOD of R-ivabradine (0.3 μg mL^{-1}) in the presence of
427 300 μg mL^{-1} of S-ivabradine (a) and a pharmaceutical formulation with 500 μg mL^{-1} of S-ivabradine
428 according to the labeled content (b). Experimental conditions as in Fig 3.

Table 1. Migration times and chiral resolution of ivabradine enantiomers obtained by CE using as chiral selector 4 mM sulfated-γ-CD alone and in dual systems with CILs at different concentrations.

Chiral selector	CIL concentration (mM)	t_1 (min)	t_2 (min)	R_s
4 mM sulfated-γ-CD	-	5.6	5.9	2.7
4 mM sulfated-γ-CD + $[TBA]^+[L\text{-}Asp]^-$	5	6.7	7.1	5.1
	10	9.1	9.8	5.5
	30	16.0	17.7	6.4
4 mM sulfated-γ-CD + $[TMA]^+[L\text{-}Asp]^-$	5	6.8	7.2	4.3
	10	8.2	8.7	5.1
	30	25.8	32.0	19.6
4 mM sulfated-γ-CD + $[TBA]^+[L\text{-}Arg]^-$	5	8.8	9.5	5.3
	10	16.5	18.1	5.8
	30	-	-	-
4 mM sulfated-γ-CD + $[TMA]^+[L\text{-}Arg]^-$	5	9.6	10.3	5.5
	10	15.9	17.5	6.5
	30	-	-	-
4 mM sulfated-γ-CD + $[TBA]^+[L\text{-}Iso]^-$	5	7.4	7.8	4.9
	10	8.6	9.2	5.3
	30	15.3	16.7	6.4
4 mM sulfated-γ-CD + $[TMA]^+[L\text{-}Iso]^-$	5	6.7	7.1	4.9
	10	8.7	9.4	5.7
	30	18.7	21.0	7.9
4 mM sulfated-γ-CD + $[TBA]^+[L\text{-}Lys]^-$	5	10.4	11.3	6.3
	10	16.2	18.0	8.2
	30	-	-	-
4 mM sulfated-γ-CD + $[TMA]^+[L\text{-}Lys]^-$	5	12.2	13.3	6.6
	10	14.9	16.8	7.7
	30	24.9	28.8	8.9
4 mM sulfated-γ-CD + $[TBA]_2^+[L\text{-}Glu]^-$	5	9.3	9.9	5.9
	10	11.2	12.1	6.3
	30	-	-	-
4 mM sulfated-γ-CD + $[TBA]^+[L\text{-}Glu]^-$	5	8.7	9.3	7.0
	10	10.0	10.9	7.3
	30	-	-	-
4 mM sulfated-γ-CD + $[TMA]^+[L\text{-}Glu]^-$	5	9.4	10.1	6.6
	10	13.4	14.8	7.3
	30	-	-	-

Experimental conditions: BGE: chiral selectors in 50 mM formate buffer (pH 2.0), applied voltage -30 kV, 25 ˚C, injection by pressure 50 mbar for 5 s of sample.
t_1: time of the first-migrating enantiomer (S-ivabradine)
t_2: time of the second-migrating enantiomer (R-ivabradine)

Table 2. Analytical characteristics of the CE methodology developed for the determination of ivabradine enantiomers using sulfated-γ-CD as chiral selector.

	S-Ivabradine	**R-Ivabradine**
External standard calibration method [a]		
Range	10 - 200 µg mL^{-1}	0.5-5 µg mL^{-1}
Slope ± t × S_{slope}	1.10 ± 0.05	1.21 ± 0.05
Intercept ± t × $S_{intercept}$	4.68 ± 4.70	-0.02 ± 0.14
R^2	0.994	0.995
p-value of ANOVA [b]	0.2755	0.1838
Standard additions calibration method [c]		
Range	0 - 112.5 µg mL^{-1}	0 - 5 µg mL^{-1}
Slope ± t × S_{slope}	1.01 ± 0.07	1.24 ± 0.04
R^2	0.993	0.998
p-value of ANOVA	0.2399	0.3023
Accuracy [d]		
Recovery	98 ± 9%	103 ± 9%
Precision		
Instrumental repeatability [e]		
t, RSD (%)	0.20	0.21
A_c, RSD (%)	1.86	5.00
Method repeatability [f]		
t, RSD (%)	0.50	0.64
A_c, RSD (%)	2.10	5.55
Intermediate precision [g]		
t, RSD (%)	1.63	1.74
A_c, RSD (%)	5.40	6.88
LOD [h]	0.22 µg mL^{-1}	0.28 µg mL^{-1}
LOQ [i]	0.73 µg mL^{-1}	0.93 µg mL^{-1}

A_c: corrected area

[a] Eight standard solutions at different concentration levels injected in triplicate for 3 consecutive days.

[b] p-value of ANOVA to state that experimental data fit properly to linear models.

[c] Addition of eight known amounts of S- and R-ivabradine to a pharmaceutical sample solution containing a constant concentration of S-ivabradine.

[d] Evaluated as the mean recovery obtained from six pharmaceutical sample solutions (n=6) containing 75 µg mL^{-1} of S-ivabradine (as labelled amount) spiked with 2 and 75 µg mL^{-1} of R- and S-ivabradine, respectively.

[e] Six consecutive injections (n=6) of a pharmaceutical sample solution containing 75 µg mL^{-1} of S-ivabradine (as labelled amount) spiked with 2 µg mL^{-1} of R-ivabradine.

[f] Three pharmaceutical sample solutions containing 75 µg mL^{-1} of S-ivabradine (as labelled amount) spiked with 2 µg mL^{-1} of R-ivabradine injected in triplicate in the same day (n=9).

[g] Three pharmaceutical sample solutions containing 75 µg mL^{-1} of S-ivabradine (as labelled amount) spiked with 2 µg mL^{-1} of R-ivabradine injected in triplicate in three different days (n=9).

[h] Calculated as the concentration yielding a S/N ratio of 3.

[i] Calculated as the concentration yielding a S/N ratio of 10.

Fig. 1

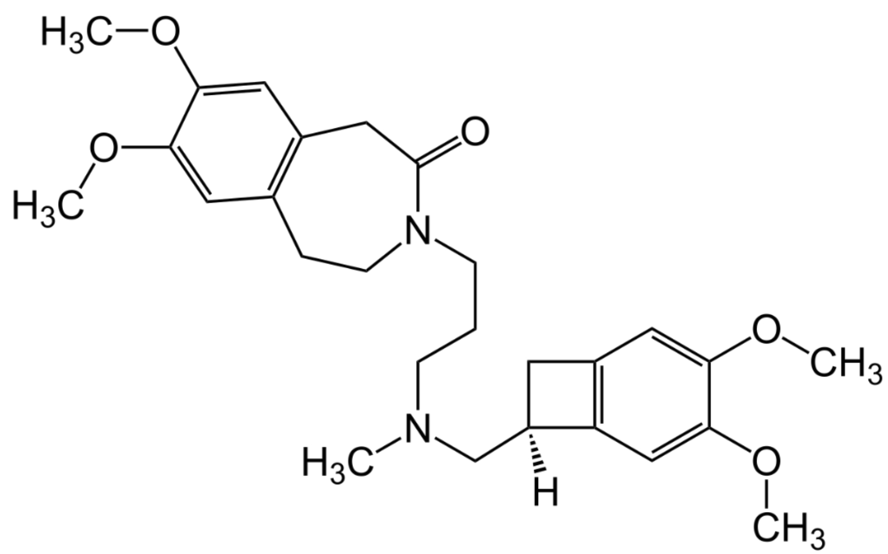


Fig. 2

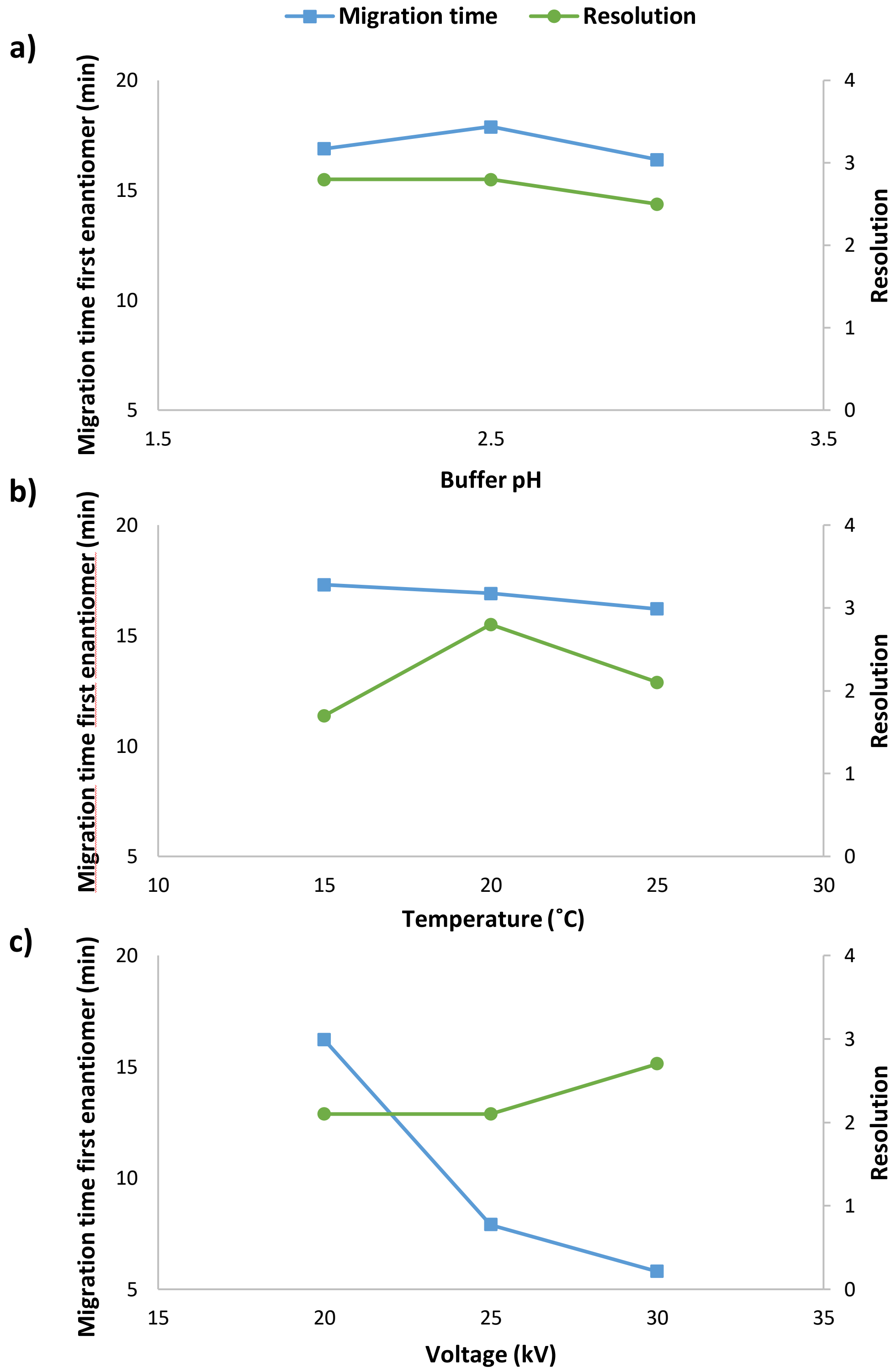


Fig. 3

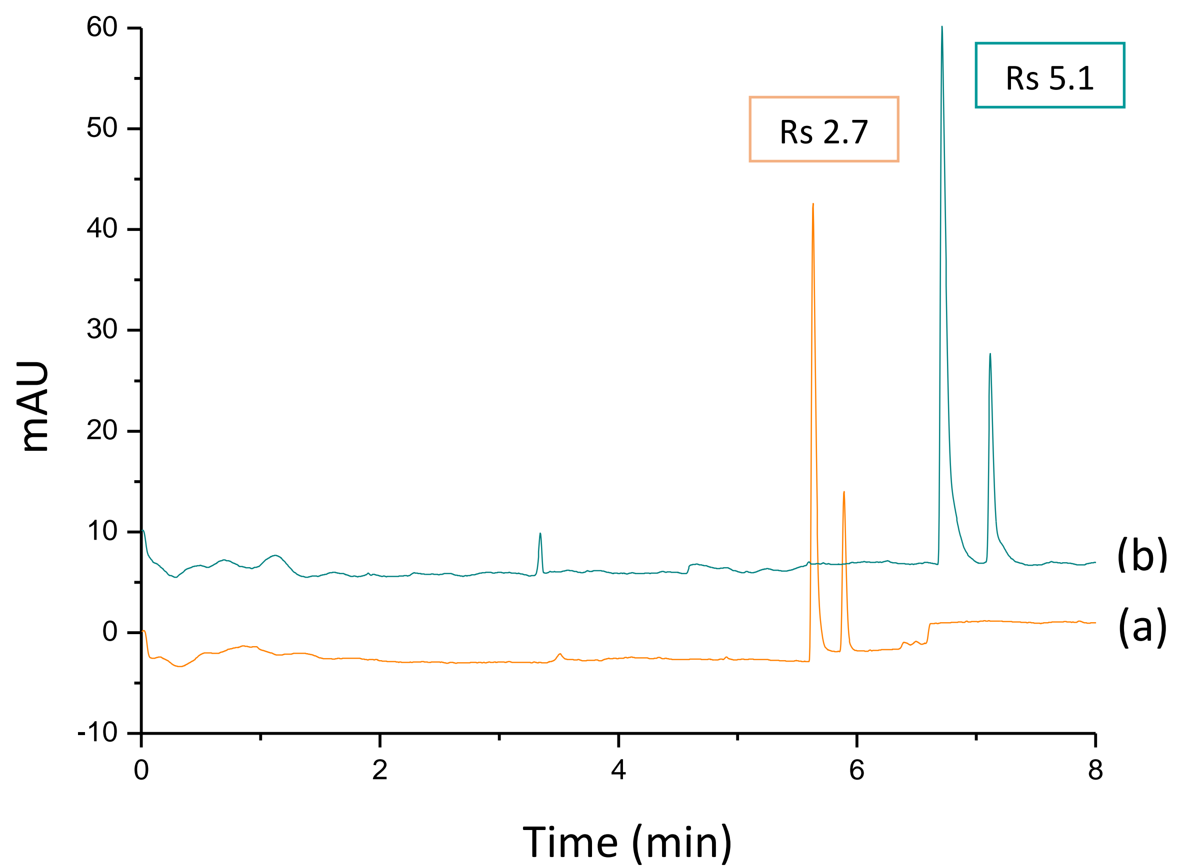
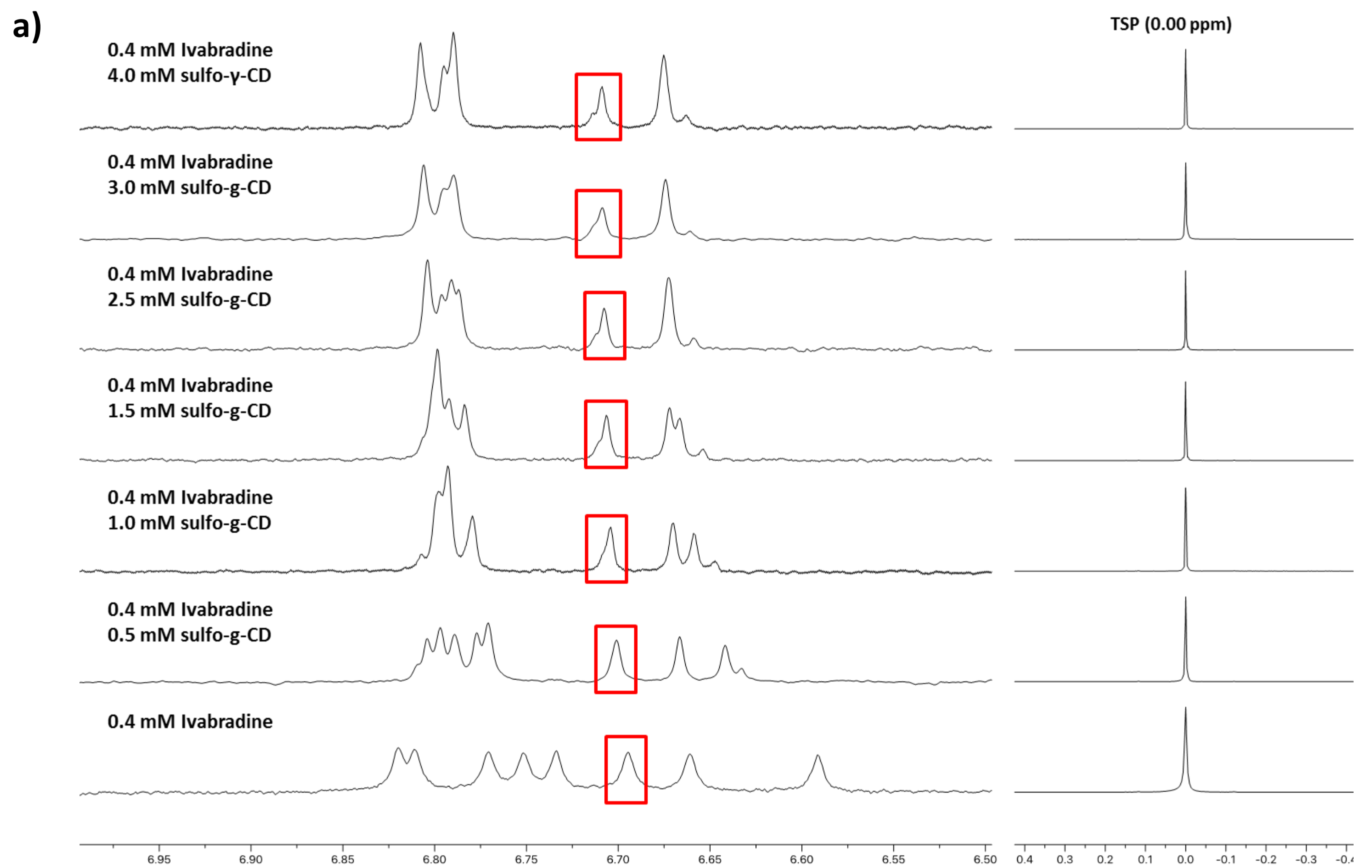


Fig. 4

a)



b)

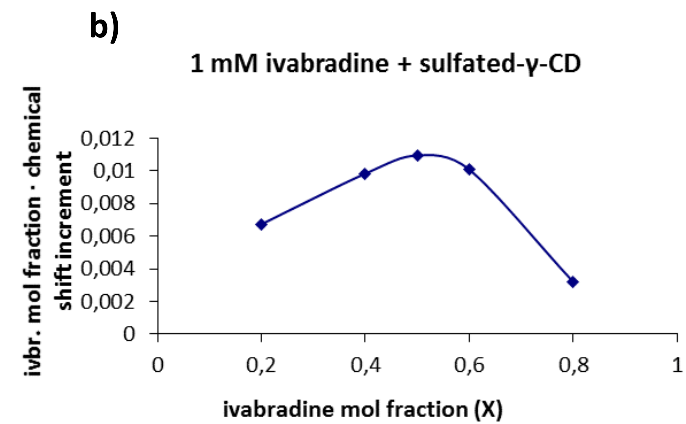
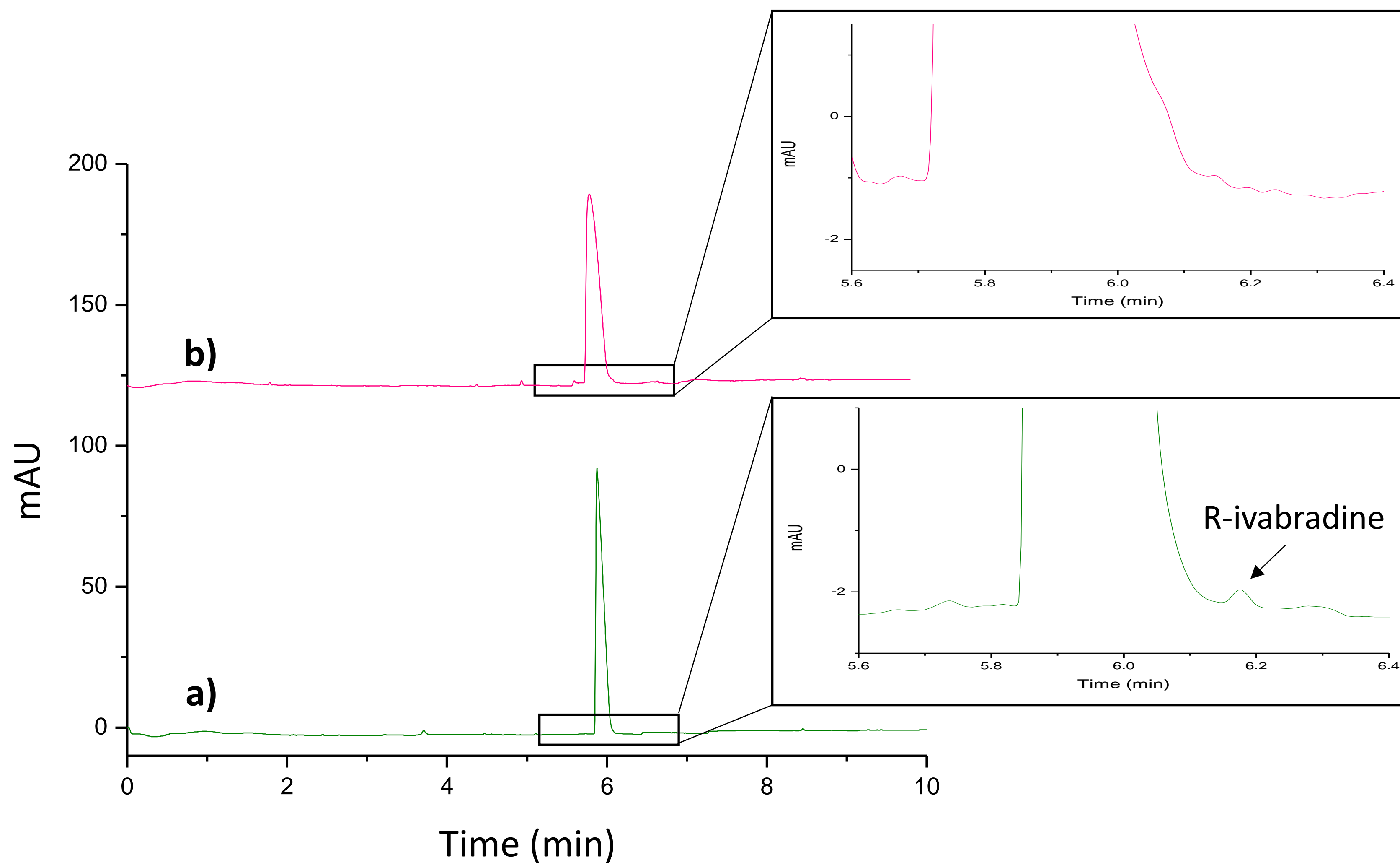


Fig. 5



Supplementary Material

Enantiomeric separation of ivabradine by cyclodextrin-electrokinetic chromatography. Effect of amino acid chiral ionic liquids

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Table S1. Migration times and chiral resolution of ivabradine enantiomers obtained by CE using sulfated-γ-CD at different concentration values.

sulfated-γ-CD concentration (mM)	**t_1 (min)**	**t_2 (min)**	**R_s**
2	> 60	> 60	-
3	44.3	47.5	4.3
4	26.9	28.8	4.5
5	22.6	23.9	4.0
10	16.6	17.4	4.5

Experimental conditions: BGE: sulfated-γ-CD in 50 mM phosphate buffer (pH 3.0), applied voltage -20 kV, 20 ˚C, injection by pressure 50 mbar for 5 s of sample.
t_1: time of the first-migrating enantiomer (S-ivabradine)
t_2: time of the second-migrating enantiomer (R-ivabradine)

Table S2. Migration times and chiral resolution of ivabradine enantiomers obtained by CE using 4 mM sulfated-γ-CD in different buffer conditions.

Buffer	**t_1 (min)**	**t_2 (min)**	**R_s**
50 mM formate buffer (pH 3.0)	16.4	16.8	2.5
100 mM formate buffer (pH 3.0)	20.7	21.5	2.9

Experimental conditions: BGE 4 mM sulfated-γ-CD in buffer solution, applied voltage -20 kV, 20 ˚C, injection by pressure 50 mbar for 5 s of sample.
t_1: time of the first-migrating enantiomer (S-ivabradine)
t_2: time of the second-migrating enantiomer (R-ivabradine)