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Simultaneous separation of the enantiomers of cizolirtine and its degradation products by capillary electrophoresis ☆

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Abstract

The simultaneous enantioselective separation of (±)-cizolirtine and its impurities: (±)-*N*-desmethylcizolirtine, (±)-cizolirtine-*N*-oxide and (±)-5-(α-hydroxybenzyl)-1-methylpyrazole was investigated by capillary electrophoresis. Electrokinetic chromatography with carboxymethyl-β-CD (CM-β-CD) and sulfobutyl-ether-β-CD was tried, showing good enantioseparation but poor chemical selectivity. The four racemic pairs were baseline separated, in a single run, by cyclodextrin-modified micellar electrokinetic chromatography. The migration buffer composition was: (60 m*M* hydroxypropyl-β-cyclodextrin–150 m*M* sodium dodecyl sulfate–50 m*M* disodium tetraborate, pH 9.2, in water)–butanol (95:5, v/v). Work was done to determine the effect of buffer components and their optimal concentration on selectivity. The method was validated with respect to enantioselectivity of cizolirtine as well as its degradation products and separation selectivity between the different components. Linearity, limit of detection, limit of quantitation and precision were also determined. This method is suitable for the enantiomeric purity determination and stability control of cizolirtine (racemic mixture or enantiomers) and its degradation products. Examples of electropherograms of (*R*)-cizolirtine degraded under stressed conditions are shown.

Keywords: Pharmaceutical analysis; Enantiomer separation; Stability studies; Cizolirtine; Cyclodextrins; Neutral cyclodextrins; Charged cyclodextrins

1. Introduction

Cizolirtine, (±)-5-[α-(2-dimethylaminoethoxy)benzyl]-1-methyl-1H-pyrazole (**1**) is a new potent non opioid analgesic agent, that inhibits substance P release [1]. This compound is currently in phase II clinical trials for acute and neuropathic pain.

Cizolirtine has a stereogenic center and, therefore, the racemic mixture and the enantiomers should be considered in activity, pharmacokinetic and toxicological studies [2,3]. Thus, provided that enantiomers have been synthesized [4–6], methods to determine their chemical and enantiomeric purity are needed [2,3,7].

Usually the chemical purity profile of a drug is determined by high-performance liquid chromatography (HPLC). However, when a simultaneous enantioselective separation is required, capillary electro-

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phoresis (CE) is preferred, because of its higher separation efficiency and wide variety of available chiral additives.

Applications have been described to determine impurities in drugs [8–13] and for stereoselective analysis of single enantiomers besides other impurities [14,15], by CE. Nevertheless, since in CE separation of analytes depends on their charge density differences, the selective migration of neutral compounds can only be accomplished if a charged complexing agent is added to the migration buffer, in other words, through electrokinetic chromatography (EKC) [16].

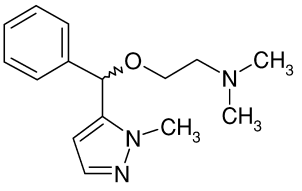
The goal of this work is the optimization of a CE method, for the simultaneous enantioselective analysis of (±)-cizolirtine and its degradation products: (±)-*N*-desmethyl cizolirtine (**2**), (±)-cizolirtine-*N*-oxide (**3**), and the product of ether bond breakdown in **1**: (±)-5-(α-hydroxybenzyl)-1-methylpyrazole (**4**) (see Fig. 1 for structures). Using this method we wish to achieve, in a single run, the discrimination between the enantiomers of the four racemic mixtures and their chemical separation.

In this target mixture, compounds **1** and **2** behave as cations in a wide range of pH values (calculated pK_a 9.6 and 10.5, respectively). Compound **3** behaves as neutral, except at acid pH, where it acts as a cation (approximate pK_a 4.5) [17]. And **4** is practically neutral (calculated pK_a 2.4). Therefore, this mixture must be determined by EKC.

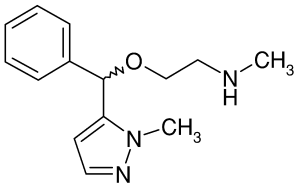
A widely used subtype of EKC is micellar electrokinetic chromatography (MEKC) with ionic surfactants [16,18–21]. If MEKC is used for the separation of enantiomers, chiral ionic surfactants may be used. However, usually, an achiral ionic micellar agent, combined with a chiral neutral selector, are preferred. In the majority of cases, separations are achieved by cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC) [22,23].

Among all types of available CE chiral selectors, CDs are a good choice for the separation of these racemic pairs [14,24,25]. Chiral recognition capability of these agents is related to the selective formation of inclusion complexes with molecules having a hydrophobic part. Compounds **1**–**4** have two aromatic rings close to the chiral center that fit well in the CD cavity, especially with β-CD [26]. It should be mentioned that previously, a CE method for the enantioselective analysis of cizolirtine was developed with hydroxypropyl-β-CD as chiral selector [27]. Further work showed that this CD was also a good chiral agent for the degradation products **1**, **2** and **3**.

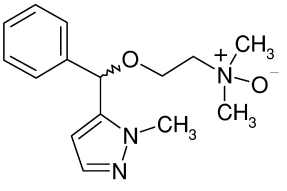
Direct electrokinetic chromatography with charged



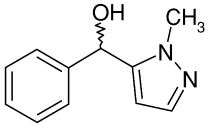
(±)-cizolirtine **(1)**



(±)-N-desmethylcizolirtine **(2)**



(±)-cizolirtine-N-oxide **(3)**



(±)-5-(α-hydroxybenzyl)-1-methylpyrazole **(4)**

Fig. 1. Structures of the compounds studied.

CDs, as simultaneous chiral and chemical selectors (CD-EKC), is another strategy that could be applied [28–35]. Neutral analytes acquire a charge, when fitted into the ionic CD cavity. Moreover, negatively charged CDs strongly interact with cationic species [29,31,33].

In an attempt to achieve the simultaneous enantioselective separation of cizolirtine and its degradation products, the two aforementioned approaches were tried. With CD-MEKC, a method was optimized and validated.

2. Experimental

2.1. CE system

The experiments were performed on a HP ^{3D}CE capillary electrophoresis system (Hewlett-Packard, Waldbronn, Germany), with an on-column UV diode array detector and Chemstation data handling software. An uncoated bare fused-silica capillary of 64.5 cm (56 cm inlet to detector)×50 μm I.D. was used (Hewlett-Packard). The temperature was 20 °C. UV detection was done at 205 nm. The applied voltage was 20 kV with an initial ramp of 1 kV/s. Injection was performed on the anodic side, except for experiments with anionic CDs at acidic pH, in which injection was done at the cathode and the applied migration voltage was −20 kV. The sample was introduced into the capillary hydrodynamically, at 50 mbar over 5 s. Daily, the capillary was activated with a 5 bar flush of water (5 min), 0.1 *M* NaOH (10 min), water (5 min), and buffer (15 min). Before each EKC analysis, the capillary was flushed with the migration buffer (5 min). In MEKC, the preconditioning was water (1 min), 0.1 *M* NaOH (3 min), water (1 min), and buffer (5 min). The buffer was changed every 8–10 injections. After changes of the buffer composition, the capillary was rinsed with new buffer (15 min for minor variation and 30 min for major change). The first injection of each series was a blank of sample solvent.

2.2. Samples, chemicals, reagents and general procedures

(±)-Cizolirtine citrate, (+)-(*R*)-cizolirtine citrate, (−)-(*S*)-cizolirtine citrate, (±)-*N*-desmethylcizolirtine oxalate, (+)-(*R*)-*N*-desmethylcizolirtine oxalate, (±)-cizolirtine-*N*-oxide, (+)-(*R*)-cizolirtine-*N*-oxide, (±)-5-(α-hydroxybenzyl)-1-methylpyrazole and (+)-(*R*)-5-(α-hydroxybenzyl)-1-methylpyrazole were synthesized in Laboratorios Dr. Esteve (Barcelona, Spain). Absolute configuration of the enantiomers was determined by X-ray diffraction analysis. Working solutions were prepared in methanol–water (15:85, v/v).

$Na_2HPO_4 \cdot 2H_2O$, $NaH_2PO_4 \cdot H_2O$, $Na_2B_4O_7 \cdot 10 H_2O$, H_3PO_4 (85%), NaOH, methanol, ethanol, acetonitrile, *n*-butanol, triethanolamine, were all analytical grade, from Scharlau (Barcelona, Spain), or Merck (Darmstadt, Germany). Deionized water was obtained with a Milli-Q Plus system (Millipore, Bedford, MA, USA). Sodium dodecyl sulfate (SDS) and β-cyclodextrin (β-CD) were purchased from Fluka (Buchs, Switzerland). Heptakis(2,3,6-tri-*O*-methyl)-β-CD (TM-β-CD, M_r 1430) was from Sigma (St. Louis, MO, USA), hydroxypropyl-β-CD (HP-β-CD, M_r 1540, mixture of approximate degree of substitution, D.S., 7 groups/ring,) was from Aldrich (Milwaukee, WI, USA), carboxymethyl-β-CD (CM-β-CD, M_r 1309, mixture of D.S. 3 groups/ring,) was purchased from Cyclolab (Budapest, Hungary) and sulfobutyl ether-β-CD (SBE-β-CD, M_r 1976, mixture of D.S. 4 groups/ring) was from Bioscience Innovation (Lawrence, KS, USA).

Borate buffer of pH 9.2 was directly prepared by dissolving disodium tetraborate in water. When used at other pH values, it was adjusted with sodium hydroxide or boric acid diluted in the salt solution. Phosphate buffers of pH 6 and 7 were prepared by dissolving in water monosodium and disodium phosphate salts at appropriate concentrations. Phosphate buffers at acidic pH were prepared diluting phosphoric acid in water and adjusting the pH with triethanolamine. Buffers were filtered by a 0.45 μm nylon membrane. In MEKC experiments, the organic solvent was added to an aliquot of the filtered buffer. Prior to use, the working buffer was degassed.

Migration buffer of the definitive method was prepared by dissolving 1.907 g of disodium tetraborate decahydrate in 90 ml of water, then 4.326 g of SDS and 9.24 g of HP-β-CD were added. The volume was adjusted to 100 ml and the pH of the solution was measured, being 9.25±0.05. The aque-

ous solution was filtered through a 0.45 μm membrane filter and mixed with butanol in the proportion of 95:5 (v/v).

Peak identification was done by spiking with standards. For quantitative purposes, area was normalized by migration time [9].

Resolution (R_s) was calculated according to the equation:

$$R_s = 2 \cdot (t_2 - t_1)/(W_1 + W_2)$$

where t_1 and t_2 are the migration times of the two enantiomers and W_1, W_2 are their respective peak widths at peak base.

Theoretical pK_a values were calculated with pK_{calc} 3.2 (Pallas for Windows 3.0) software of Compudrug International (San Francisco, CA, USA)

3. Results and discussion

3.1. CD-EKC approach

EKC with negatively charged CDs was originally described by Terabe et al. in 1985 [28]. Since then, as these CD derivatives have become available, numerous applications have been published [24,33].

A spacer between the CD cavity and the ionic group presumably increases interaction possibilities between the CD and the analyte. Thus, among all commercialized anionic CDs, CM-β-CD and SBE-β-CD were screened. SBE-β-CD has a strongly acidic group that is deprotonated over the entire pH range applied in electrophoresis. While the carboxylic group of CM-β-CD has a pK_a around 4.5, it is only totally deprotonated at pH values equal or higher than 6.5 [30,33].

Experiments with these two CDs were done at pH 9.2 and 6, and also at pH 3 with SBE-β-CD (Table 1). Analytes migrated towards the cathode at pH 9.2 and 6, while with SBE-β-CD at pH 3, the compounds moved in the direction of the anode. With anionic CDs, two migration directions are possible. Usually, when the buffer pH is high, the EOF vector surpasses that of the anionic CD; but when the buffer pH is low, the electrophoretic mobility of the negatively charged CDs wins, and reversed migration of the complexed analytes occurs [28,29,35].

We were not able to achieve the separation of the four compounds, nor that of cizolirtine with respect to its degradation products, under any of the conditions tested (Table 1).

SBE-β-CD appeared to have better separation efficiency than CM-β-CD. For instance, at pH 9.2 and 10 m*M* CD, R_s values better than 1.5 were obtained for racemates of **1**, **2** and **4** with SBE-β-CD; whereas R_s values of 0.9, 0.3 and 0.6 were,

Table 1
Results of CD-EKC experiments with CM-β-CD and SBE-β-CD

Migration buffer		t_m (min)				Separation	
CD	Buffer	(±)-**1**	(±)-**2**	(±)-**3**	(±)-**4**	Chemical	Chiral
CM-β-CD, 10 m*M*	Tetraborate, 50 m*M*, pH 9.2	12.33 12.52	12.70 12.77	11.95 –	11.88 12.04	**1**, **2**	Partial **1**, **2**, **4**
	Phosphate, 50 m*M*, pH 6	26.17 27.03	30.41 –	28.42 28.87	25.58 26.15	**2**, **3**	Baseline **1** Partial **3**, **4**
SBE-β-CD, 10 m*M*	Tetraborate, 50 m*M*, pH 9.2	17.47 18.28	16.98 17.47	15.19 15.43	17.82 18.98	**3**	Baseline **1**, **2**, **4** Partial **3**
	Phosphate, 50 m*M*, pH 6	17.37 17.72	18.08 18.31	17.51 17.72	20.81 22.43	**4**	Baseline **4** Partial **1**, **2**, **3**
	Triethanolamine phosphate, 25 m*M*, pH 3*	17.63 18.35	17.15 17.76	17.15 17.42	15.29 15.92	**4**	Baseline **1**, **2**, **4** Partial **3**

*Reversal of electroosmotic flow and polarity. Other conditions as described in the Experimental section.

respectively, obtained with CM-β-CD. However, none of these two CDs produced baseline separation of enantiomers of **3** at any of the tested pH values.

3.2. CD-MEKC approach

Since the CD-EKC approach did not work properly, CD-MEKC was tried.

The principal factors that should be taken into account in a CD-MEKC method optimization are [21,36,37]: pH, concentration/type of surfactant and cyclodextrin derivative. SDS was chosen as micellar agent, because it had good separation capability for cizolirtine and its degradation products (see Fig. 2). The effect of pH, cyclodextrin derivative, cyclodextrin concentration and SDS concentration on selectivity was investigated.

The main difficulty we had was the separation of the enantiomers of **3**. When using a migration media composed of buffer, SDS micelles and CD, this racemate appeared as a unique wide distorted peak, in all compositions tried. The addition of butanol to the migration buffer greatly improved peak shape and resolution. For this reason, in all the optimization tests discussed the migration buffer contained butanol.

3.2.1. Method optimization

3.2.1.1. pH

The buffer pH plays an important role in the migration direction and the retention of analytes. When SDS is used, it is a common practice to apply neutral to basic pH values, to ensure that all the species migrate in the direction of the cathode [37].

A phosphate buffer of pH 7 and borate buffers with pH values 8.3, 8.7, 9.2, 9.6 were screened (Fig. 3). Attempts were done at pH values 10, 10.5 and 11 with mixed phosphate–borate buffers, but reproducible migration could not be obtained for racemate **2**. In all experiments, the buffer had 60 m*M* HP-β-CD–100 m*M* SDS and 5% (v/v) butanol.

The behaviour of non charged racemic pairs of **3** and **4** was identical at all pH values tested: enantiomers of **4** had an R_s better than 1.5, whereas

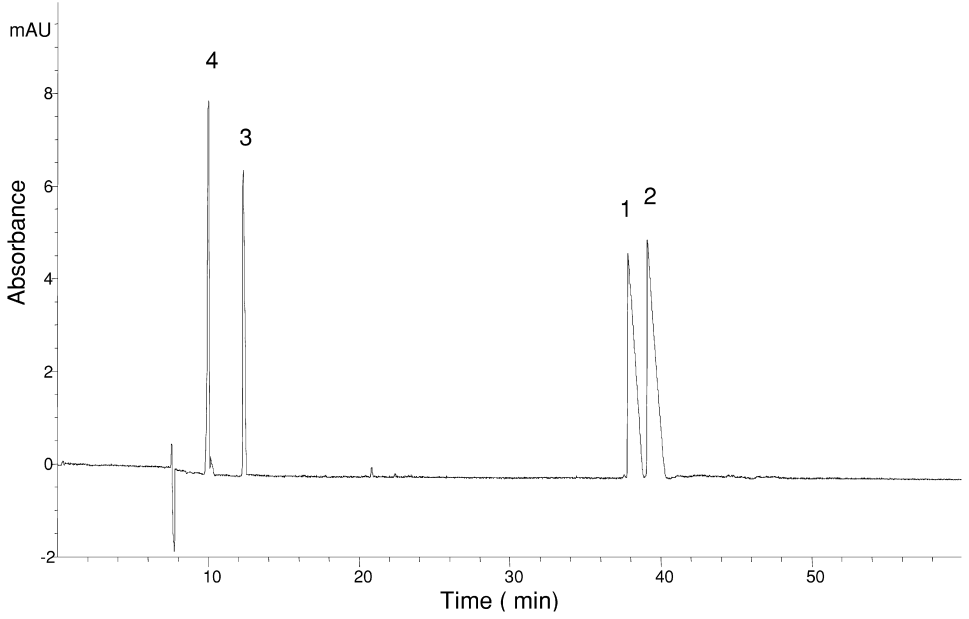


Fig. 2. SDS selectivity for cizolirtine and its degradation products. Run buffer: 10 m*M* SDS–50 m*M* disodium tetraborate, pH 9.2. Sample 25 μg/ml in methanol–disodium tetraborate 25 m*M* at pH 9.2 (15:85, v/v). Other conditions as in the Experimental section.

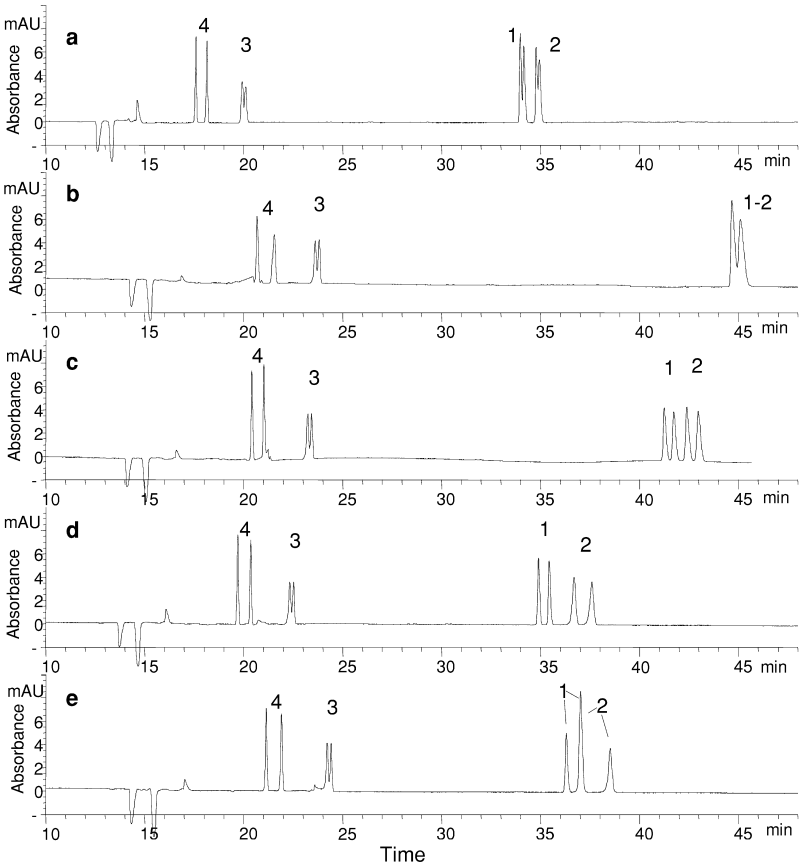


Fig. 3. pH effect on the separation of the four racemic mixtures. Run buffer: (60 m*M* HP-β-CD–100 m*M* SDS, buffer)–butanol (95:5, v/v). Buffer: (a) 50 m*M* sodium phosphate at pH 7.0, (b) 50 m*M* disodium tetraborate at pH 8.3, (c) 50 m*M* disodium tetraborate at pH 8.7, (d) 50 m*M* disodium tetraborate pH 9.2 (not adjusted), (e) 50 m*M* disodium tetraborate pH 9.6. Sample: 25 μg/ml. Other conditions as in the Experimental section.

enantiomers of **3** were only partially separated (R_s 0.6).

In the range of the studied pH values, **1** and **2** showed high sensitivity to pH changes. 9.2 gave the best results, affording R_s 2.5 for both pairs. It is known that, in critical separations of very closely related structures, but different pK_a values, a buffer pH near or between their pK_a provides the best results [15]. **1** and **2** have calculated pK_a values of 9.6 and 10.5, respectively. Thus, complete separation of these two enantiomeric pairs at pH 9.2 is presumably due to their different non ionized/ionized ratio.

3.2.1.2. Cyclodextrin derivative

Native β-CD and its neutral derivatives TM-β-CD and HP-β-CD were tested (Fig. 4).

With β-CD and TM-β-CD neither enantiomeric discrimination, nor chemical separation of the mixture was obtained. This behaviour suggests that inclusion constants in these CDs are practically equal for all racemic pairs.

With HP-β-CD, baseline separation of racemates of **1**, **2** and **4**, was obtained. The introduction of a propyl spacer between the CD cavity and the external hydroxyls enlarges the cavity size, resulting in more inclusion flexibility [38,39]. This effect may

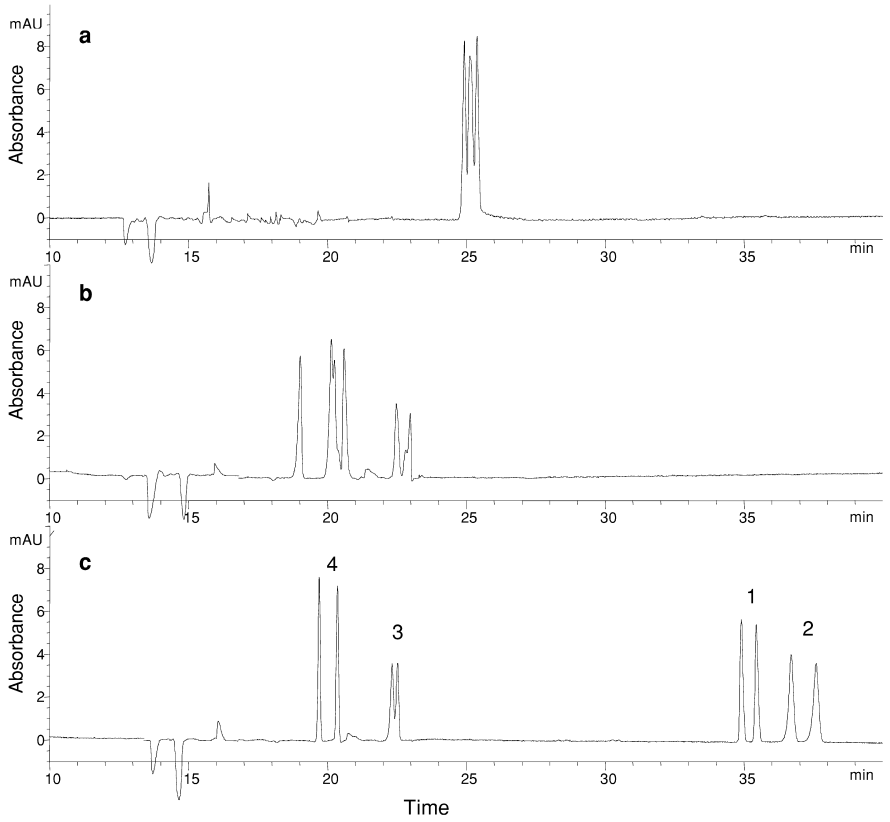


Fig. 4. Effect of the CD derivative on the chiral recognition of the four racemates. Run buffer: (60 m*M* CD–100 m*M* SDS–50 m*M* disodium tetraborate, pH 9.2)–butanol (95:5, v/v). (a) β-CD, (b) TM-β-CD, (c) HP-β-CD. Sample: 25 μg/ml. Other conditions as in the Experimental section.

improve selectivity, as happened with our target compounds.

Resolution of enantiomers of **3** was partial (R_s 0.7). However, this racemate was perfectly separated (R_s 2.3) with HP-β-CD in free zone capillary electrophoresis at pH 2.5 (Fig. 5). These dramatic enantioseparation differences are related to the ionic state of **3,** which behaves as a cation at pH 2.5, while it is neutral at pH 9.2, under the conditions of CD-MEKC (Fig. 4c).

3.2.1.3. HP-β-CD concentration

The chiral selector concentration is an important optimization parameter. If concentration is too low, not all analytes can interact with the selector; but if it is too high both enantiomers of a pair will complexate, although they have different binding constants [15,24]. The adequate separation occurs in the mid range concentration.

Optimal HP-β-CD concentration was adjusted in relation to 100 m*M* SDS and 5% (v/v) butanol (Fig. 6). 100 m*M* HP-β-CD was clearly an excessive level. 60 m*M* of the CD allowed enough retention of **1** and **2** to obtain four perfectly separated peaks (R_s of enantiomeric pairs 2.3 and 2.4, respectively). Lower levels of HP-β-CD (40 m*M*) greatly increased migration time and led to lower enantioseparation for racemates of **1** and **2** (R_s 0.8 and 1.5, respectively).

With respect to non charged species, almost no effects of CD concentration were observed for **3**, while the optimal CD concentration for racemic **4**, appeared to be lower than 40 m*M*. However, this was

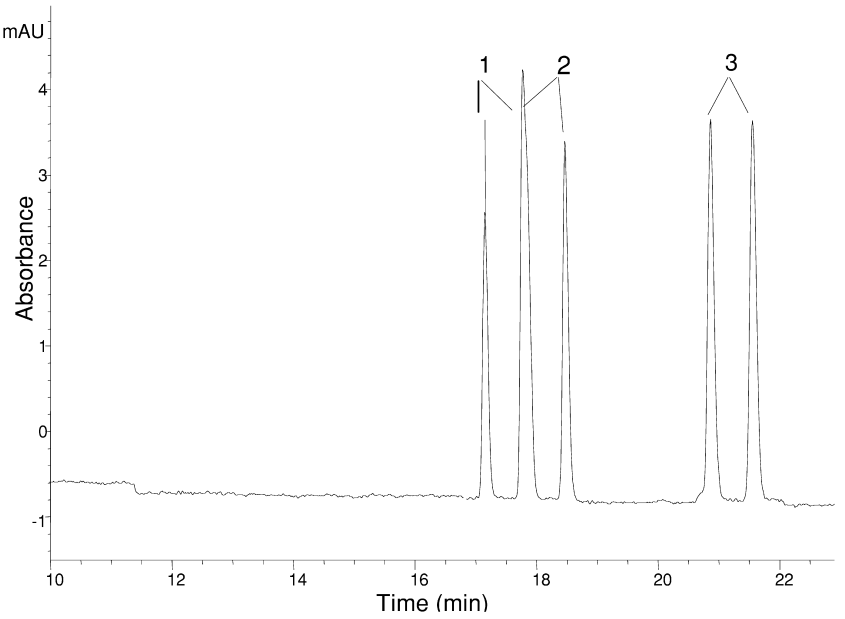


Fig. 5. Separation of racemates of **1**, **2** and **3** with HP-β-CD in free solution capillary electrophoresis. Run buffer: 25 m*M* HP-β-CD–100 m*M* sodium phosphate, pH 2.5. Sample: 25 μg/ml. Other conditions as in the Experimental section.

not relevant for the separation, because with both 40 and 60 m*M* CD, enantiomers were resolved with an R_s of 4. Hence, the optimal concentration for the enantiomeric resolution of the more critical racemates of **1** and **2** was chosen (60 m*M*).

3.2.1.4. Organic solvent

As explained before, an organic solvent was added to the migration buffer, because separation of racemate **3** at pH 9.2 with a buffer composed of SDS–HP-β-CD and borate was zero.

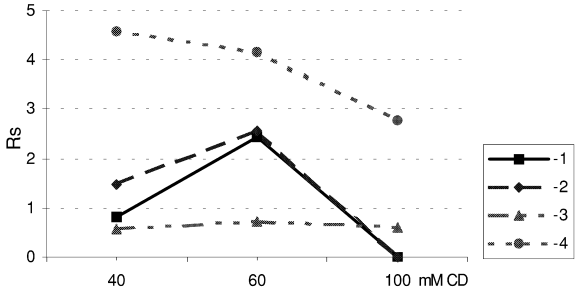


Fig. 6. CD concentration effect on the resolution of the four racemic pairs. Run buffer: (HP-β-CD–100 m*M* SDS–50 m*M* disodium tetraborate, pH 9.2)–butanol (95:5, v/v). Other conditions and R_s calculation as in the Experimental section.

Examples of significant improvement of critical MEKC and CD-MEKC separations, by the addition of small amounts of an organic solvent to the run buffer, have been described [8,12,23]. Organic solvents decrease the dielectric constant (ϵ) and, generally, increase viscosity (η). Provided that electroosmotic mobility is proportional to ϵ/η [40], the addition of organic solvent diminishes the electroosmotic flow (EOF) and enlarges the migration window which in turn increases selectivity. Moreover, the organic solvent interacts and modifies analytes distribution between the micelle, the cyclodextrin and the aqueous phase, with unpredictable effects on selectivity.

Electroosmotic velocities (ν_{EOF}) were calculated from the first distortion in the baseline (sample solvent had 15% of methanol) and they were (Fig. 7): 0.085 cm/s (aqueous buffer); 0.076 cm/s (buffer with 5% of acetonitrile); 0.070 cm/s (5% of methanol); 0.066 cm/s (5% of ethanol) and 0.068 cm/s (5% of butanol). Then, the four solvents produced an enlarged migration window, as expected (Fig. 7).

Nevertheless, no significant resolution changes

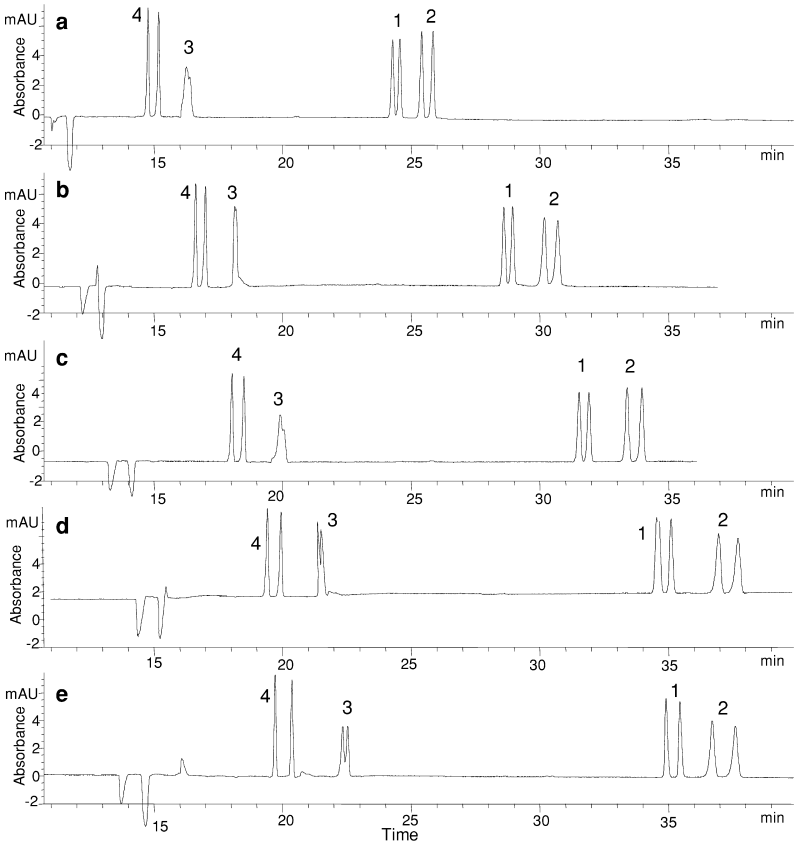


Fig. 7. Effect of organic solvent on the separation of the four racemates. Run buffer: (60 m*M* HP-β-CD–100 m*M* SDS–50 m*M* disodium tetraborate, pH 9.2)–organic solvent (95:5, v/v). (a) None, (b) acetonitrile, (c) methanol, (d) ethanol, (e) butanol. Sample: 25 μg/ml. Other conditions as in the Experimental section.

were obtained with the addition of 5% acetonitrile or methanol. When ethanol or butanol were added, chiral discrimination of racemate **3** was greatly enhanced, with higher R_s for the latter (0.6 and 0.7, respectively). The effect was the same for the other species, although less relevant. Experiments with lower and higher butanol percentage were carried out with negative results (not shown).

There are two possible mechanisms to explain the enantioselectivity improvement obtained with ethanol and butanol: competition of the solvent with analytes for the CD inclusion and/or increased analytes solubilization in the aqueous buffer [22,24].

3.2.1.5. SDS concentration

If only chemical separation of cizolirtine and its degradation products was required, 10 m*M* SDS would have been adequate (Fig. 2).

With the addition of the chiral agent (HP-β-CD) to the run buffer, partition into the micelle was dramatically lowered and the migration velocity greatly increased. Thus, to maintain chemical selectivity, higher SDS concentration was required.

Moreover, since a buffer with 100 m*M* SDS and 5% butanol did not allow the complete separation of **3** enantiomers, the SDS concentration was increased further, producing longer migration time for all the analytes and improved separation of neutral species (Fig. 8). Finally, with 150 m*M* SDS, R_s 1.5 was obtained for the critical enantiomeric pair **3**.

Summarizing, a CD-MEKC buffer composed of (60 m*M* HP-β-CD–150 m*M* SDS–50 m*M* disodium

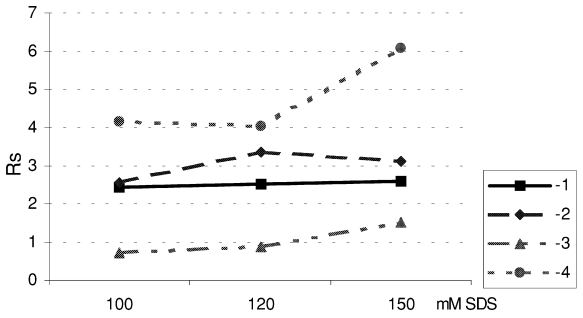


Fig. 8. Effect of SDS concentration on enantiomer separation. Run buffer: (60 m*M* HP-β-CD–SDS–50 m*M* disodium tetraborate, pH 9.2)–butanol (95:5, v/v). Other conditions and R_s calculation as in the Experimental section.

tetraborate, pH 9.2)–butanol (95:5, v/v), allowed the complete and simultaneous chemical and chiral separation of (±)-cizolirtine, and its degradation products racemates.

3.2.2. Method validation

Parameters of validation were determined to establish the suitability of definitive conditions for the analysis of real samples [41].

The four racemic pairs were baseline resolved in one injection (Fig. 9). Moreover, the efficiency of cizolirtine racemate separation permitted the determination of the enantiomeric purity of the two enantiomers (Fig. 10). The resolving power of this method is an example of the potential of CE to afford challenging separations.

Calibration curves (normalized area versus concentration), for the assay of cizolirtine enantiomers (0.8–400 μg/ml), as well as for the impurity enantiomers (0.8–40 μg/ml), were determined. The straight lines obtained had correlation coefficients better or equal to 0.997 (Table 2).

Repeatability was determined for a mixture of 400 μg/ml (100%) cizolirtine enantiomers and 0.8 μg/ml (equivalent to 0.2% with respect to nominal concentration) of enantiomers of impurities. It was also checked in almost stereochemicallly pure (*R*)-cizolirtine and (*S*)-cizolirtine samples (400 μg/ml, 99.7:0.3 major/minor enantiomer) (Tables 3 and 4). The relative standard deviation (RSD) of cizolirtine assay was less than 3%. At 0.3% level of minor cizolirtine enantiomer and 0.2% of the enantiomers of degradation products **2**, **3**, and **4**, RSD was less than 10% [41]. Within-day variation of migration time was between 2 and 3%. It could probably be improved by working with an internal standard.

In CE, good precision is not easily attainable [12,42,43]. Variability in the migration time of this method can be produced by dynamic changes in the buffer phases and/or the status of the capillary wall. Slight fluctuations of electroosmotic velocity were

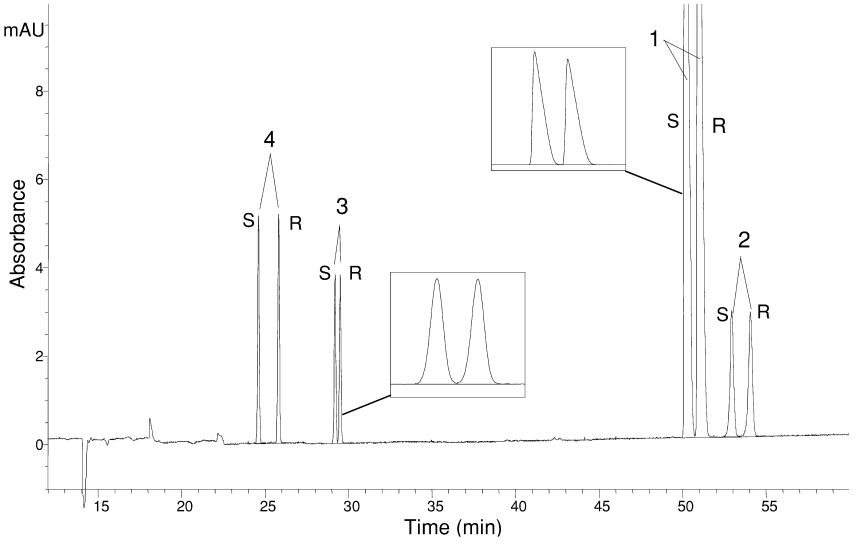


Fig. 9. Selectivity of the definitive method. Run buffer: (60 m*M* HP-β-CD–150 m*M* SDS–50 *M* disodium tetraborate, pH 9.2)–butanol (95:5, v/v). Sample: 800 μg (±)-cizolirtine and 40 μg racemic impurities/ml. Other conditions as in the Experimental section.

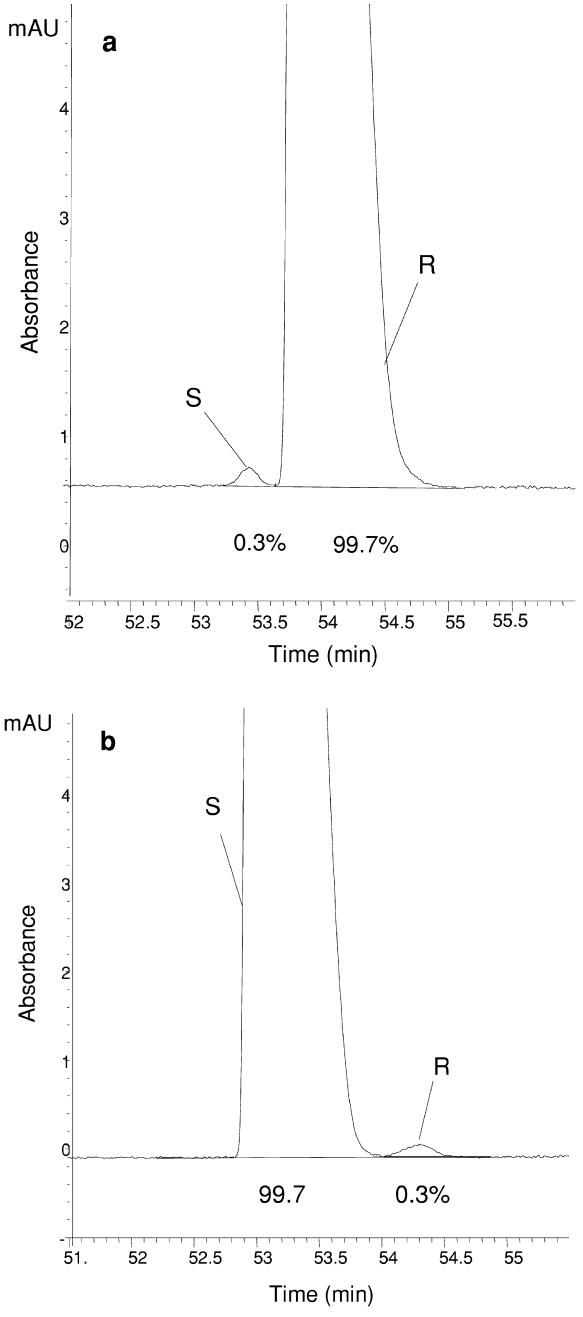


Fig. 10. Electropherograms of spiked samples of: (a) (*R*)-cizolirtine and (b) (−)-(*S*)-cizolirtine, both 99.7% enantiomeric purity. Other conditions as in Fig. 9.

also observed, but no relevant changes in the current intensity occurred (approx. 55 μA). Complexity of the buffer composition, and viscosity produced by the high concentration of SDS and butanol may be the cause of migration time instability.

The limit of detection (LOD) determined at a signal/noise ratio of 2, was 0.05% for **3** enantiomers and 0.1% for all other peaks.

3.2.3. Examples of real samples

As part of preformulation studies, an aqueous solution of (*R*)-cizolirtine was exposed to stressed conditions of pH and oxidation (Fig. 11).

At basic pH, (*R*)-cizolirtine (*R*-**1**) slowly oxidized to (*R*)-cizolirtine-*N*-oxide (*R*-**3**), and lost an *N*-methyl group giving (*R*)-desmethylcizolirtine (*R*-**2**) (Fig. 11a).

At acid pH, the ether bond of (*R*)-cizolirtine hydrolyses with racemization to (±)-5-(α-hydroxybenzyl)-1-methylpyrazole [(±)-**2**]. This result indicates that the ether breakdown takes place through a carbocation intermediate (Fig. 11b).

In oxidizing media and neutral pH, (*R*)-cizolirtine degrades to *R*-cizolirtine-*N*-oxide (*R*-**3**) (Fig. 11c).

4. Conclusions

In the EKC optimization approach, SBE-β-CD and CM-β-CD were good chiral resolving agents, but poor chemical selectors, for our target compounds.

In this application, CD-MEKC provided better selectivity-tuning than CD-EKC. Selective conditions for the simultaneous separation of the four racemic pairs, (±)-cizolirtine and racemates of its three degradation products, were developed.

The main optimization variables were pH, micellar agent and CD derivative. Absolute and relative buffer component concentrations also played a significant role. The final migration buffer was composed of SDS, HP-β-CD, disodium tetraborate and butanol. SDS gave adequate chemical selectivity. HP-β-CD provided the enantioselective separation of (±)-cizolirtine, (±)-*N*-desmethylcizolirtine and 5-(α-hydroxybenzyl)-1-methylpyrazole. The organic solvent contributed to baseline resolution of cizolirtine-*N*-oxide enantiomers.

Table 2
Linearity of cizolirtine and derivatives

Compound	Range (μg/ml)	Slope (mean±SD)	Intercept (mean±SD)	SE_y	Correlation coefficient (r^2)
(+)-*R*-**1**	0.8–400	$6.3586\cdot10^{-4}\pm3.7171\cdot10^{-6}$	$2.97\cdot10^{-4}\pm6.86\cdot10^{-4}$	$2.217\cdot10^{-3}$	0.99945
(−)-*S*-**1**	0.8–400	$6.4343\cdot10^{-4}\pm3.6925\cdot10^{-6}$	$2.88\cdot10^{-4}\pm6.81\cdot10^{-4}$	$2.203\cdot10^{-3}$	0.99947
(+)-*R*-**2**	0.8–40	$7.2464\cdot10^{-4}\pm2.7869\cdot10^{-6}$	$-1.7\cdot10^{-4}\pm5.6\cdot10^{-5}$	$1.32\cdot10^{-4}$	0.99988
(−)-*S*-**2**	0.8–40	$7.4145\cdot10^{-4}\pm3.2795\cdot10^{-6}$	$-2.2\cdot10^{-4}\pm6.59\cdot10^{-5}$	$1.56\cdot10^{-4}$	0.99984
(+)-*R*-**3**	0.8–40	$7.5072\cdot10^{-4}\pm5.0126\cdot10^{-6}$	$3.057\cdot10^{-5}\pm1.01\cdot10^{-4}$	$2.4\cdot10^{-4}$	0.99964
(−)-*S*-**3**	0.8–40	$7.5313\cdot10^{-4}\pm3.9527\cdot10^{-6}$	$1.04\cdot10^{-5}\pm8.0\cdot10^{-5}$	$1.89\cdot10^{-4}$	0.99977
(+)-*R*-**4**	0.8–40	$11.9851\cdot10^{-4}\pm6.1842\cdot10^{-6}$	$3.1\cdot10^{-5}\pm1.23\cdot10^{-4}$	$2.92\cdot10^{-4}$	0.99978
(−)-*S*-**4**	0.8–40	$12.3381\cdot10^{-4}\pm5.464\cdot10^{-6}$	$7.69\cdot10^{-5}\pm1.09\cdot10^{-4}$	$2.58\cdot10^{-4}$	0.99984

$n=10$, $y=$area/migration time, $x=$μg analyte/ml.

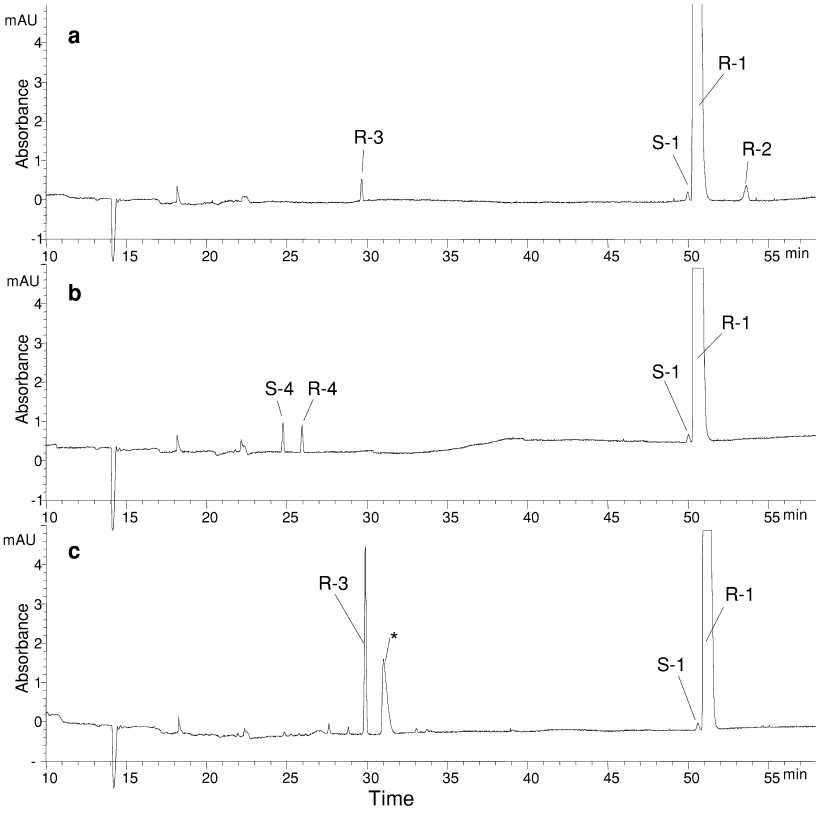


Fig. 11. Electropherograms of degraded real (+)-(*R*)-cizolirtine samples obtained under stressed conditions. (a) Target compound dissolved in 0.1 *M* NaOH (pH 12), and stored for 31 days at 60 °C. (b) Target compound dissolved in 0.1 *M* HCl (pH 1) and stored 1 day at room temperature. (c) Target compound dissolved in 0.3% (v/v) H_2O_2–100 m*M* sodium phosphate, pH 7, and stored for 6 days at room temperature. Final sample concentration: 800 μg/ml. Other conditions as in Fig. 9. * Blank signal.

Table 3
Within-day precision of cizolirtine analysis

Compound	Enantiomer ratio		RSD (%)	
	Mean±SD (%)	RSD (%)	Area/t_m	t_m (min)
(±)-**1**	50.28±0.10	0.2	2.3	1.6
	49.72±0.2	0.2	2.4	1.7
(−)-(*S*)-**1**	99.66±0.02	0.02	2.8	2.6
	0.34±0.02	6.7	3.0	7.8
(+)-(*R*)-**1**	99.73±0.02	0.02	2.5	1.8
	0.27±0.02	8.8	2.4	8.9

800 mg/ml racemic, 400 μg/ml enantiomer (100%), $n=6$.

Table 4
Within-day precision of degradation product analysis at limit of quantitation level

Compound	RSD (%)	
	Area/t_m	t_m (min)
(−)-(*S*)-**2**	5.1	2.5
(+)-(*R*)-**2**	3.5	2.7
(−)-(*S*)-**3**	5.1	2.4
(+)-(*R*)-**3**	6.9	2.4
(−)-(*S*)-**4**	4.0	2.0
(+)-(*R*)-**4**	3.7	2.1

0.8 mg/ml (0.2%), $n=6$.

The method has good linearity for cizolirtine assay (racemic or enantiomers), and for the quantitation of the impurities. Limits of detection and quantitation are between 0.1–0.05% and 0.2–0.3%, respectively, and precision is acceptable. Therefore, this method is suitable for the stereochemical and chemical purity determination, as well as for stability control of cizolirtine (racemic mixture or pure enantiomer), and its potential degradation products.

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References

[1] I. Alvarez, F. Andreu, J. Buxens, M. Colombo, A. Dordal, M. Fort, B. Gutiérrez, A.J. Farré, Methods Find. Exp. Clin. Pharmacol. 22 (2000) 211.
[2] FDA Policy Statement on Stereoisomeric Drug Products, FDA 92D-0211, Fed. Reg. 57 (1992) 2249.
[3] CPMP Note For Guidance: Investigation of Chiral Active Substances, III/3501/91 Final, Commission of the European Communities, Brussels, 1993.
[4] J. Frigola, J. Berrocal, R. Cuberes, V. Gotor, Int. Pat. Publ. WO 97/20817 (1997).
[5] A. Torrens, J. Frigola, Int. Pat. Publ. WO 99/02500 (1999).
[6] A. Torrens, J. Frigola, Int. Pat. Publ. WO 99/07684 (1999).
[7] Guideline of Directive 75/318/ECC, Pharmaceutical Legislation of European Communities, 3A, 1998, p. 57.
[8] M.J. Hilhorst, G.W. Somsen, G.J. de Jong, J. Pharm. Biomed. Anal. 16 (1998) 1251.
[9] K.D. Altria, J. Chromatogr. 646 (1993) 245.
[10] A. Yesilada, B. Tozkoparan, N. Gökhan, L. Öner, M. Ertan, J. Liq. Chromatogr. Rel. Technol. 21 (1998) 2575.
[11] O.M. Denk, D.G. Watson, G.G. Skellern, J. Chromatogr. A 895 (2000) 9.
[12] E. Castellanos Gil, A. Van Shepdael, E. Roets, J. Hoogmartens, J. Chromatogr. A 895 (2000) 43.
[13] D. Altria, Y.L. Chanter, J. Chromatogr. A 652 (1993) 459.
[14] D.J. Skanchy, G. Xie, R.J. Tait, E. Luna, C. Demarest, J.F. Stobaugh, Electrophoresis 20 (1999) 2638.
[15] E.C. Rickard, R. Bopp, J. Chromatogr. A 680 (1994) 609.
[16] H. Nishi, J. Chromatogr. A 735 (1996) 57.
[17] A. Albert, E.P. Serjeant, in: The Determination of Ionization Constants, 3rd ed., Chapman and Hall, London, 1984, p. 151, Chapter 9.
[18] C.J. Sciacchitano, B. Mopper, J.J. Specchio, J. Chromatogr. B 657 (1994) 395.
[19] H. Nishi, S. Terabe, J. Chromatogr. A 735 (1996) 3.
[20] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Anal. Chem. 56 (1984) 111.
[21] S. Terabe, J. Pharm. Biomed. Anal. 10 (1992) 705.
[22] H. Nishi, T. Kufuyama, J. Chromatogr. 553 (1991) 503.
[23] S. Terabe, Y. Miyashita, Y. Ishihama, O. Shibata, J. Chromatogr. 636 (1993) 47.
[24] S. Fanali, J. Chromatogr. A 792 (1997) 227.
[25] G. Gübitz, M.G. Schmid, J. Chromatogr. A 792 (1997) 179.
[26] J. Redondo, M.A. Blázquez, A. Torrens, Chirality 11 (1999) 694.
[27] A. Torrens, J.A. Castrillo, J. Frigola, L. Salgado, J. Redondo, Chirality 11 (1999) 63.
[28] S. Terabe, H. Ozaki, K. Otsuka, T. Ando, J. Chromatogr. 332 (1985) 211.
[29] B. Chankvetadze, G. Endresz, G. Blaschke, Chem. Soc. Rev. 25 (1996) 141.
[30] X. Ren, Y. Dong, Y. Liu, A. Huang, H. Liu, Y. Sun, Z. Sun, Chromatographia 50 (1999) 363.
[31] G. Xie, D.J. Skanchy, J.F. Stobaugh, Biomed. Chromatogr. 11 (1997) 193.
[32] N.W. Smith, J. Chromatogr. A 652 (1993) 259.

[33] T. de Boer, R.A. de Zeeuw, G.J. de Jong, K. Ensing, Electrophoresis 21 (2000) 3220.
[34] S.L. Tamisier-Karolak, M.A. Stenger, A. Bommart, Electrophoresis 20 (1999) 2656.
[35] A.M. Stalcup, K.H. Gahm, Anal. Chem. 68 (1996) 1360.
[36] A.T. Balchunas, M.J. Sepaniak, Anal. Chem. 59 (1987) 1466.
[37] R. Weinberger, in: Practical Capillary Electrophoresis, Academic Press, San Diego, CA, 1993, p. 147, Chapter 7.
[38] J. Sepaniak, R.O. Cole, B.K. Clark, J. Liq. Chromatogr. 15 (1992) 1023.
[39] A. Aumatell, R.J. Wells, D.K.Y. Wong, J. Chromatogr. A 686 (1994) 293.
[40] I.E. Valkó, H. Sirén, M.L. Riekkola, LC–GC Int. March (1997) 190.
[41] J.M. Green, Anal. Chem. News Features May 1 (1996) 305A.
[42] B. Nickerson, S. Scypinski, H. Sokoloff, S. Sahota, J. Liq. Chromatogr. 18 (1995) 3847.
[43] H. Wu, Ch. Huang, S. Chen, S. Wu, J. Chromatogr. Sci. 37 (1999) 24.