Cyclodextrine Screening for the Chiral Separation of Beta-blocker Derivatives

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The enantiomeric separation of nine frequently used β-blocker derivatives (atenolol, bisoprolol, carvedilol, labetalol, metoprolol, oxprenolol, pindolol, propranolol, sotalol) has been studied by capillary zone electrophoresis (CZE) using cyclodextrines (CDs) as chiral selectors directly added to the background electrolyte with the aim to establish the optimum experimental conditions for their chiral discrimination. Various native (α, β and γ-CD), alkylated (hydroxypropyl-β-CD and randomly methylated-β-CD) and anionic (carboximethyl-β-CD and sulfobuthylether-β-CD) CD derivatives were tested and electrophorectic parameters such as buffer composition, concentration and pH as well as CD type and concentration were investigated. All the studied β-blockers showed significant stereoselective interactions with one particular or with several CD derivatives. The results of the study underline the most important role of the differences in the physico-chemical and structural characteristics of the β-blockers derivatives while interacting with inclusion compounds.

Keywords: β-blocker derivatives, capillary electrophoresis, chiral separation, cyclodextrines, stereoselective interactions

The existence of asymmetric organic molecules have long been known, however, the pharmaceutical implications of racemic drugs administration have only been extensively recognized and studied in the last 25 years. Almost half of the drugs currently used in therapy have a chiral center in their molecule, but only approximately 25% of these are administered as stereochemically pure enantiomers. It is well established that in most cases the pharmacological effect of these substances is restricted to only one of the enantiomers, called eutomer, since there are many qualitative and quantitative differences in terms of absorption, distribution, metabolism or binding and affinity towards a receptor or protein between the two enantiomers, as the pharmacologically "inactive" enantiomer, called distomer, may cause undesirable or in some cases even toxic effects [1].

Development of pure enantiomers is currently more economically feasible and the understanding of the chiral structure of drug action sites is continually evolving, consequently the tendency of marketing pure enantiomers of chiral drugs increased. With this tendency the development of new improved methods for the separation and determination of enantiomers became a permanent necessity and also a challenge [2,3].

Capillary electrophoresis (CE) is an officinal method in the 8th edition of the European Pharmacopoeia [4], which is beginning to play a major role in the separation of chiral compound, being considered often superior to high-performance liquid chromatography (HPLC), accepted as the most universally applied method in pharmaceutical analysis [5].The advantages of using CE in chiral separations are related to its high resolving power, relatively short analysis time, rapid method development, low consumption of solvent, sample and chiral selector and especially with the high selectivity of choosing and changing the chiral selector. Another big advantage is that in CE, direct chiral separation is typically carried out by simply dissolving an optically pure additive in the buffer solution. [6-9].

In order to separate two enantiomers, these must come in contact with a chiral environment in order to form two diasteromeric complexes. According to the rule of three point's interactions of Dlagliesh, chiral recognition depends on at least three simultaneous interactions between the chiral molecule and the selector and at least one of these interactions must be stereoselective in order to allow enantiomer discrimination [9,10].

Electrophoretic separation of enantiomers to assure or enhance chiral purity can be achieved by the use of several selectors of great structural variety. Cyclodextrins (CDs) are without doubt the important and frequently used class of chiral selectors in CE, as they are multimodal selectors since multiple chiral interactions are possible by very different stereoselective mechanisms [11].

β-adrenergic antagonists, commonly termed β-blockers, are one of the most widely used and therefore extensively studied groups of drugs. Their clinical use ranges from cardiovascular disorders, such as angina pectoris and hypertension, to the therapy of migraine, glaucoma and hyperthyreosis, to mention only a few. All β-blockers that are currently in use in therapy posses an amino-alkanol side chain with an asymmetric carbon atom resulting in the existence of two enantiomers. Although, clinical studies showed there are differences between the pharmacological potency and effects of the two enantiomers, most β-blockers are still used in therapy as racemates (exception timolol – used as S-timolol) [12,13].

In order to offer a homogenous nomenclature for all β-blocking drugs throughout the article, we predominantly use the CIP rule nomenclature, since it defines the absolute configuration of a stereogenic center, in the present case a

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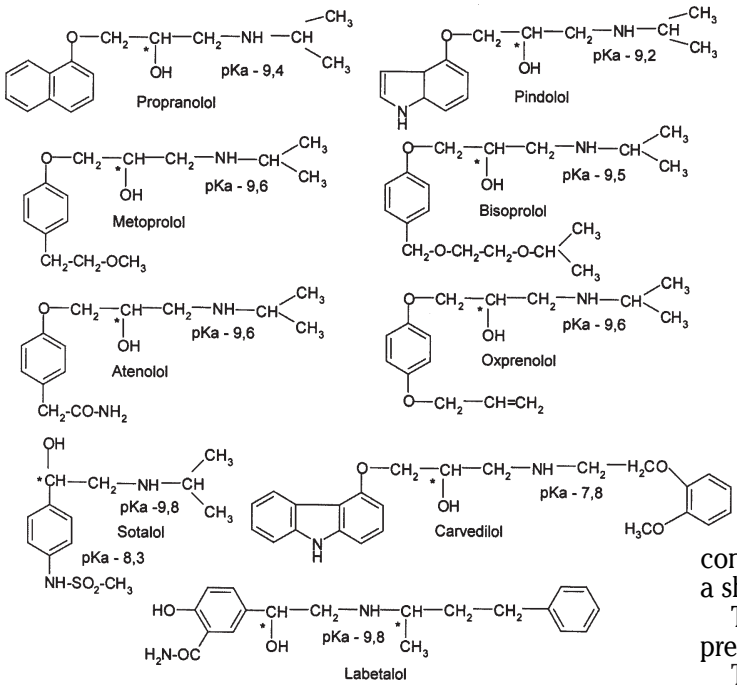

Fig. 1 The chemical structures of the studied β-blockers. The asterix denotes the chiral center

tetracoordinated carbon atom substituted by four different ligands. In the aryloxypropanolamine type compounds the d-enantiomers show the (R)-configuration, and the l-enantiomers show the (S)-configuration. (S)-enantiomers usually are orders of magnitude more potent in blocking adrenergic β-adrenergic receptors than the respective (R)-forms [14,15]. An exception to this rule is represented by sotalol where the asymmetric carbon atom is located in an ethanolamine type side chain. In this case, the priority of the four substituents changes according to the CIP rules so that (R)-sotalol (equivalent to l-sotalol) is much more effective as a β-blocker than (S)-sotalol (equivalent to d-sotalol) [16].

A particular case is represented by the β-blockers (labetalol, nadolol), which present two asymmetric carbons resulting in the existence of four stereoisomers (R,R), (S,S), (R,S) and (S,R); in the case of labetalol the (R,S) isomer being the more potent exhibiting mainly the β-adrenergic activity while the (S,S) isomer possesses rather an ±-adrenergic activity [17].

The chiral separation of β-blockers by CE using CDs as chiral selectors has been studied intensively in the las two decades. However, the optimal separation conditions are significantly different in several cases, and the explanation of these differences has not been clearly identified [18,19-21]. There are three main aspects that should be taken into consideration when explaining a chiral separation: the role of the chemical structure of the analyte, the effect of experimental parameters and the structure of the chiral selector [22,23].

Different CE techniques were reported for the chiral separation of different β-blockers, usually CZE methods using neutral CDs, charged CDs or dual CD systems as chiral selectors, but also CD-mediated micellar electrokinetic chromatography (CD-MEKCI) techniques have been also employed. Other chiral selectors such as: proteins, polysaccharides, crown ethers, macrocyclic antibiotics or chiral surfactans were also used more or less successfully for the enantioseparation of β-blockers [24,25].

Our aim was to develop rapid, simple and efficient methods for the chiral separation of several frequently β-blocker enantiomers using a systematic screening of different native and derivatized, neutral and ionized CDs as chiral selectors and the optimization of electrophoretic conditions in order to obtain enhanced chiral resolution in a short analysis time.

The chemical structures of the studied β-blockers are presented in figure 1.

The CD derivatives presented in this study were used before for the chiral separation of one or other β-blocker, but no comparative study has been reported in this field.

Experimental part

Chemicals and reagents

Nine frequently used β-blockers with different structural characteristics were analyzed in this study: R,S-atenolol (India), R,S-bisoprolol fumarate (Spain), R,S-carvedilol (India), R,R,S,S-labetalol hydrochloride (France), R,S-metoprolol tartrate (Spain), R,S-oxprenolol hydrochloride (Spain), R,S-pindolol (India), R,S-propranolol hydrochloride (Romania), R,S-sotalol hydrochloride (Spain).

For five of the studied chiral substances we had at our disposal also pure enantiomers: S-atenolol (India), S-carvedilol (India), R-propranolol (Spain), S-sotalol hydrochloride (Spain), S-metoprolol tartrate (Spain).

The following reagents of analytical grade were used: methanol, sodium hydroxide (Czech Republic), phosphoric acid, sodium tetraborate, disodium hydrogenophosphate, sodium didydrogenophosphate (Germany). Purified water was provided by a Milli-Q Plus water purification system (USA).

As chiral selectors we used the following CD derivatives of research grade: native neutral CDs (α-CD, β-CD, γ-CD), derivatized neutral CDs (hydroxypropyl-β-CD - HP-β-CD, randomly methylated β-CD – RAMEB), anionic substituted charged CD (carboxymethyl β-CD sodium salt – CM-β-CD, sulfobuthyl ether- β-CD sodium salt – SBE-β-CD). All CDs were obtained from (Hungary) with the exception of SBE-β-CD – (USA). The CDs in appropriate concentrations were added directly to the buffer solutions.

Instrumentation

The separations were carried out on a Agilent 6100 CE system (Agilent, Germany) equipped with a diode array UV detector. Separations were performed on a 48 cm length (40 cm effective length) x 50 μm I.D uncoated fused silica-capillaries (Germany). The electro-pherograms were recorded and processed by Chemstation 7.01 (Germany) software. The pH of the buffer solutions was determined with the Terminal 740 pH–meter (Inolab).

Sample preparation

Sample stock solutions were prepared by dissolving the substances in methanol in a concentration of 100 μg mL^{-1}

and later diluted with the same solvent to the appropriate concentration. All solutions were filtered with a 0.45μm pore size membrane filter, stored in a refrigerator at + 4 ^{0}C and degassed in an ultrasonic bath for 5 min before use. In all measurements hydrodinamic sample introductions have been used for injecting samples; the samples were injected at the anodic end of the capillary.

Electrophoretic procedure

The capillaries were preconditioned before use with 0.1 M sodium hydroxide for 10 min and with the background electrolyte (BGE) used in the analysis for 10 min. The capillary was rinsed for 1 min with 0.1M sodium hydroxide and buffer solutions before each electrophoretic separation to remove all substances, which may stick on capillary walls.

In the preliminary analysis we applied some "standard" electrophoretic conditions for a CE analysis: temperature 20°C, applied voltage + 25 kV, injection pressure/time 50 mbar/3 s, sample concentration 10 μg mL^{-1}.

UV-spectra of the analytes were recorded in a range of 200 and 400 nm, and the individual absorption maximum for each compound was elected as specific detection wavelength in the CE separations, using 210 nm as control wavelength.

Enantioselectivity evaluation

The separation factors (a) were calculated as the ratio of the migration times of the optical isomers, and the resolution (R) was obtained by the $R=2(t_2 - t_1)/(w_1 + w_2)$ equation, where the migration times (t_1 and t_2) and the peak-widths (w_1 and w_2) were marked for the slow and fast migrating enantiomers, respectively.

The separation factors and resolution parameters are characterizing the separation. A value above 1.04 for α and above 1.40 for R generally means baseline separation of the two enantiomers.

Results and discussions

Preliminary analysis

In order to find the suitable conditions for the chiral separation of the studied β-blockers, a series of preliminary experiments were conducted at different *p*H and buffer compositions. In the preliminary analysis we used 25 mM phosphoric acid (*p*H –2.1), 25 mM sodium didydrogenophosphate (*p*H – 5.0) 25 mM disodium hydrogenophosphate – sodium didydrogenophosphate (1:1) (*p*H – 7.0) and 25 mM sodium tetraborate (pH – 9.3) BGEs respectively and modified the *p*H of the buffer by adding a 0.1M sodium hydroxide solution.

The electrophoretic behavior and mobilities of the analytes were in close relationship to their pK_a values and structural characteristics.

β-blockers are basic compounds; consequently acidic pH values should be selected and used for their detection in an achiral environment. At low *p*H values, electroosmotic flow (EOF) is reduced, and this can increase resolution of the separation. *p*H values influence not only the EOF but also the charge of the analytes, and thus their effective electrophoretic mobility. The best results were obtained using a phosphate BGE at a pH between 2.5 and 3.5. But the use of a buffer with a *p*H > 4-5 can be also helpful in some cases because it enables the enantiomeric separation and allows sufficient EOF for the separation.

In the initial experiments the studied compounds were injected in the absence of CDs and their effective mobility was calculated. Then we performed the measurements using the same BGE, containing a relatively low amount of chiral selector in order to verify the decrease in the effective mobility of the analytes.

Initial concentration of 10 mM neutral CDs were added to the buffer solution, while for charged CDs we added a concentration of 5 mM in order to limit the increase of ionic strength which generated high currents and instability of the electrophoretic system. The solubility of β-CD is relatively low, when compared to that of α-CD and γ-CD; the maximum β-CD concentration used in this study was 20 mM.

Uncharged CDs are neutral from electrophoretic point of view; consequently the migrations of β-blockers enantiomers will be towards the cathode while the neutral CD will almost not move, because the EOF is close to zero.

The first requirement for inclusion-complexation is fitting the analyte into the CD cavity, thus selecting the appropriate CD is related to the shape and dimension of the analyte. If the analyte is too large it will not fit into the cavity and does not form the complex while if the analyte is too small the interaction with the CD cavity is not strong enough to allow chiral discrimination [26].

Clearly α-CD was not able to separate the racemic mixtures of the β-blockers because its cavity is too small, β-CD allowed chiral resolution for carvedilol and propranolol and γ-CD was not able to guest the studied analytes probaby because their molecules were too small. The use of derivatized CDs increased markedly stereoselective interactions, as chiral resolution was obtained for six β-blockers (atenolol, carvedilol, labetalol, oxprenolol, propranolol, sotalol) when using HP-β-CD and for all the nine derivatives (atenolol, bisoprolol, carvedilol, labetalol, metoprolol, oxprenolol, pindolol, propranolol, sotalol) when using RAMEB.

If complexation was observed but insuficient resolution was achieved, we increased the concentration of the chiral selector until satisfactory separation was obtained.

When using anionic CDs at strong acidic *p*H, no peaks were detected; because too weak EOF did not compensate for the negative electrophoretic mobility of the charged CD which interacted strongly with the β-blockers. Anionic CDs proved to be efficient for the separation of β-blockers only at a *p*H value above 5.0, the best results being obtained at *p*H 7.0. Stereoselective interactions were observed at *p*H – 7.0, with eight derivatives (atenolol, bisoprolol, carvedilol, metoprolol, oxprenolol, pindolol, propranolol, sotalol) when using CM-β-CD, respectively with six (bisoprolol, carvedilol, labetalol, metoprolol, oxprenolol, sotalol) when using SBE- β-CD.

The contrast between results obtained when using neutral respectively ionized CDs can be explained due to the contribution of ionic interactions between the negatively charged groups of the CD and the protonated amine group of the β-blocker.

Optimization of the analytical conditions

Stereoselectivity of the separation is influenced by several experimental parameters, such as CD type and concentration, ionic strength, *p*H of the BGE, capillary temperature, applied voltage, capillary length or the addition of buffer additives.

Chiral resolution is strongly influenced by the composition of the BGE, and thus the selection of the appropriate buffer system, its concentration, strength and *p*H should be carefully considered. Decreasing the ionic strength of the BGE generally caused a reduction of migration time and chiral resolution, probably due to electromigration dispersion, while increasing BGE concentration increased the migration time of the analytes; an upper limit for buffer concentration being dictated by

Nr.	β - blocker	pH	CD concentration [mM]	t1(min)	t2(min)	R	α
1.	Atenolol	2.5	20 mM HP-β-CD	9.9	10.2	0.88	1.03
		2.5	**20 mM RAMEB**	**10.8**	**11.3**	**1.04**	**1.04**
		7.0	10 mM CM-β-CD	11.2	11.4	0.90	1.01
2.	Bisoprolol	2.5	40 mM RAMEB	16	16.3	0.84	1.01
		7.0	10 mM CM-β-CD	16.5	16.8	1.10	1.01
		7.0	**5 mM SBE-β-CD**	**17.5**	**17.9**	**1.25**	**1.02**
3	Carvedilol	2.5	**10 mM β-CD**	**11**	**11.6**	**2.74**	**1.05**
		2.5	20 mM HP-β-CD	12.9	13.5	2.54	1.04
		2.5	30 mM RAMEB	15.3	15.9	1.98	1.04
		7.0	10 mM CM-β-CD	13.8	14.2	1.35	1.02
		7.0	5 mM SBE-β-CD	13.2	13.6	1.48	1.03
4	Labetalol	2.5	**20 mM HP-β-CD**	**13.2**	**13.7**	**1.27**	**1.03**
		2.5	30 mM RAMEB	15.9	16.4	1.48	1.03
		7.0	5 mM SBE-β-CD	16.4	16.8	1.20	1.02
5	Metoprolol	2.5	40 mM RAMEB	15.4	15.9	1.08	1.03
		7.0	10 mM CM-β-CD	17.8	18.1	1.08	1.01
		7.0	**5 mM SBE-β-CD**	18.2	18.6	1.28	1.02
6	Oxprenolol	2.5	**20 mM HP-β-CD**	**11**	**11.5**	**1.77**	**1.04**
		2.5	20 mM RAMEB	10	10.4	1.14	1.04
		7.0	10 mM CM-β-CD	11.4	11.8	1.25	1.03
		7.0	5 mM SBE-β-CD	12.8	13.2	1.35	1.03
7	Pindolol	2.5	**20 mM RAMEB**	**10.6**	**11**	**1.02**	**1.03**
		7.0	10 mM CM-β-CD	10.6	10.9	0.88	1.02
8	Propranolol	2.5	10 mM β-CD	9.4	9.8	1.13	1.04
		2.5	**10 mM HP-β-CD**	**11**	**11.5**	**1.46**	**1.04**
		2.5	20 mM RAMEB	13	13.4	1.21	1.03
		7.0	10 mM CM-β-CD	16.1	16.7	1.53	1.03
9.	Sotalol	2.5	20 mM HP-β-CD	9.3	9.7	0.93	1.04
		2.5	**30 mM RAMEB**	9.1	9.6	1.52	1.05
		7.0	10 mM CM-β-CD	12.4	12.9	1.48	1.04
		7.0	5 mM SBE-β-CD	12.1	12.5	1.45	1.03

Table 1
CAPILLARY ELECTROPHORETIC SEPARATION OF ENANTIOMERS OF β-BLOCKERS USING CD DERIVATIVES AS CHIRAL SELECTORS

the increase in current and resulting Joule heating which reduces the efficiency of the separation. The effect of the buffer concentration was studied in the range 25-100 mM; an optimum 50 mM buffer concentration was selected for the separations.

The addition of an organic solvent (methanol) to the BGE produced a negative effect on the binding constant of the inclusion-complex with CDs, causing decrease in chiral resolution.

The CD concentration plays a very important role in the chiral resolution, and should be carefully controlled in order to find the optimal experimental conditions. The chiral resolution usually increased with the increase of the CD concentration until optimum CD concentration was achieved and then decreased gradually.

The influence of the applied voltage over the resolution and migration times was studied in the range of 15–30 kV; optimum voltage was set at 25 kV. The effect of temperature was investigated in the range of 15–25 ^{0}C. An increase of the temperature caused a decrease in buffer viscosity, and thus a decrease in migration time. When the temperature is increased, resolution decreased, presumably due to limited solute-CD interaction; optimum temperature was set at 15^{0}C. A high injection pressure and a short injection time will increase chiral resolution; in order to obtain a quantifiable electrophoretic response and improve enantiomeric resolution we injection pressure of 50 mbar for 1 second.

The length of the capillary can also influence resolution between the two enantiomers, the use of a longer capillary increases resolution but also migration times.

The CD screening results using the optimized electrophoretic conditions are summarized in table 1.

The stereoisomers of labetalol were not fully resolved in any of the studied conditions, as the four optical isomers migrated always in two zones, with two aproximately equal peak areas.

It is intersting to notice that β-blockers with common structural characteristics (metoprolol – bisoprolol) exhibited very similar stereoselective interactions.

The migration order of the two enantiomers was determined by spiking and also by injecting the pure enantiomer using the selected chiral buffer BGE and the optimized analytical conditions. The results are summarized in table 2.

The migration order for a specific derivative was the same for all the studied CDs with whom it showed chiral interaction under the optimized analytical conditions. However for carvedilol and sotalol the migration order was opposite to the one of propranolol, metoprolol and atenolol, which can be explained by the differences in the chemical structures of the analytes.

Enantioseparation in CE requires that either the analyte or the CD is electrically charged.

The CDs interacts with the two enantiomers during the electrophoretic process, forming labile diasteroisomeric complexes; the separation of the two enantiomers can take place only if the two diasteroisomers formed possess different stability constants, causing the the diastero-isomers to move with different velocities. The chiral separation is obtained due to the formation of secondary bonds between the substituent groups of the chiral center of the analyte and those of the chiral selectors positioned outside the cavity (hydroxyl or modified hydroxyl groups). To explain and clarify recognition mechanism of chiral

Nr.	β - blocker	Enantiomer 1	Enantiomer 2
1	Atenolol	S(-)-atenolol	R(+)-atenolol
2	Carvedilol	R(+)-carvedilol	S(-)-carvedilol
3	Metoprolol	S(-)-metoprolol	R(+)-metoprolol
4	Propranolol	S(-)-propranolol	R(+)-propranolol
5	Sotalol	R(-)-sotalol	S(+)-sotalol

Table 2
MIGRATION ORDER OF THE STUDIED β-BLOCKER DERIVATIVES UNDER OPTIMIZED ELECTROPHORETIC CONDITIONS

separations, it is necessary to establish the structure and the main properties of the analytes and chiral selectors involved in the electrophoretic process [11, 20, 27].

Native CDs are neutral and hydrophilic and consequently migrate at the velocity of the EOF. When a charged compound forms a complex with a neutral CD, the mass/charge ratio and the mobility of the complexed form decreases compared with those of the free analyte. The differences between the equilibrium constants determines the ratio between the free and the complexed analyte; the separation of enantiomers occurring when the difference between equilibrium constants is large enough. The migration velocity of the complexed form will differ from that of the free molecule, because of the bigger size of the complex with the same charge as the free form [28].

When using derivatized CDs, chiral recognition is dependent on the derivatization of the hydroxyl groups on the CDs ring. By derivatization, hydrophobicity and charge can be altered and can influence the electrophoretic mobility and the complexing ability of the analyte.

Charged CDs can display a migration opposite to that of the free analytes, thus determining a more pronounced effect on the compounds they interact with, because they can act both as a chiral selector and a carrier of the inclusion complex. When CM β-CD was used as chiral selector, the weakly acidic carboxylic groups can be charged or uncharged depending on the *p*H of the BGE; at low *p*H (2-3) the carboxylic groups are protonated, and form hydrogen bonds with the analytes; while at *p*H > 4, the CD is charged due to the dissociation of the carboxylic substituents and has its own electrophoretic mobility, while forming inclusions complexes and allowing ion-pair interactions with analytes [24]. In contrast SBE-β-CD, due to its chemical properties (contains four modified primary hydroxyl groups with a butyl chain and sulfonic groups) is negatively charged over a wide *p*H range (2-11), and has a countercurrent mobility, showing increased interaction with the oppositely charged analytes, leading to an increase in analysis time. Severe band broadening was also observed, mainly due to electrodispersion, due to the high degree of substitution (average DS ~ 6.5) of the CD [29].

The chiral discrimination depends on the difference of interactions between CD derivative with R- and S-enantiomers. Therefore, the higher the affinity for the CD, the lower the overall electrophoretic mobility of the enantiomer. The faster migration times of one enantiomer indicates that this enantiomer has a weaker interaction with the CDs, while the interaction of the other is the stronger one.

Conclusions

The large number of publications dealing with different, stereoselective characteristics of chiral drugs including here β-blockers seems more and more to prove that stereoselectivity in pharmacological activity of these compounds is rather the rule than the exception. Development of new enantioselective analytical methods as well as preparative enantioseparation techniques and enantioselective synthesis, respectively, are the basis of 'stereoselective drug development.

The advantages of using CE in chiral separations are the small amounts of chiral selector and solvents required, which makes it easy to change the selector and the electrolyte when screening for a suitable selector and electrophoretic conditions. CE offers tremendous flexibility for enantiomeric separations, requiring only the addition of one or more chiral selector to the buffer solution; CDs an their derivatives remaining the most extensively used chiral additives.

CD type and concentration, *p*H value of the BGE and system temperature had a strong influence on the efficiency of the chiral separation. The changes in the concentration of the CDs and in the *p*H of the background electrolyte showed uneven effect on the resolution of the optical isomers of β-blockers.

CZE proved to be a rapid, specific, reliable and cost effective method which can be used succesfuly in laboratories performing routine chiral analysis of different β-blocker derivatives.

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