

Chiral Separation of Indapamide Enantiomers by Capillary Electrophoresis

Amelia Tero-Vescan¹, Gabriel Hancu^{2*}, Mihaela Oroian², Anca Cârje³

¹ Department of Biochemistry, Faculty of Pharmacy, University of Medicine and Pharmacy, Târgu Mureș, Romania.

² Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Medicine and Pharmacy, Târgu Mureș, Romania.

³ Department of Drug Analysis and Analytical Chemistry, Faculty of Pharmacy, University of Medicine and Pharmacy, Târgu Mureș, Romania.

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ABSTRACT

Purpose: Indapamide is probably the most frequently prescribed diuretic drug, generally being used for the treatment of hypertension. It contains a chiral center in its molecule; is marketed as a racemic mixture; but there are rather few studies regarding the pharmacokinetic and the pharmacological effect differences of the two enantiomers. Our aim was the development of a simple, rapid and precise analytical procedure for the chiral separation of indapamide enantiomers.

Methods: In this study capillary zone electrophoresis was used for the enantiomeric separation of indapamide using a systematic screening approach involving different native and derivatized; neutral and charged cyclodextrines as chiral selectors. The effects of pH value and composition of the background electrolyte, capillary temperature, running voltage and injection parameters have been investigated.

Results: After preliminary analysis a charged derivatized CD, sulfobutyl ether- β -CD, proved to be the optimum chiral selector for the enantioseparation. Using a buffer solution containing 25 mM disodium hydrogenophosphate – 25 mM sodium dihydrogenophosphate and 5 mM sulfobutyl ether- β -CD as chiral selector at a pH - 7, a voltage of + 25 kV, temperature 15°C and UV detection at 242 nm, we succeeded in the separation of the two enantiomers in approximately 6 minutes, with a resolution of 4.30 and a separation factor of 1.08.

Conclusion: Capillary zone electrophoresis using cyclodextrines as chiral selectors proved to be a suitable method for the enantioseparation of indapamide. Our method is rapid, specific, reliable, and cost-effective and can be proposed for laboratories performing indapamide routine analysis.

Introduction

Indapamide is a “thiazide-like” diuretic drug, generally used for the treatment of hypertension, alone or in combination with other antihypertensive drugs, as well as for the treatment of salt and fluid retention associated with congestive heart failure or edema.¹

The benzamide-sulfonamide-indole chemical structure of indapamide (4-chloro-N-(2-methylindolin-1-yl)-3-sulphamoylbenzamide) is presented in Figure 1.²

Its molecule contains both a polar sulfamoyl chlorobenzamide moiety and a lipid-soluble methylindoline moiety. It differs chemically from the thiazides in that it does not possess the thiazide ring system and contains only one sulfonamide group.

Indapamide appears to cause vasodilation, probably by inhibiting the passage of calcium and other ions (sodium, potassium) across membranes. Overall,

indapamide has an extra-renal antihypertensive action resulting in a decrease in vascular hyperreactivity and a reduction in total peripheral and arteriolar resistance.¹

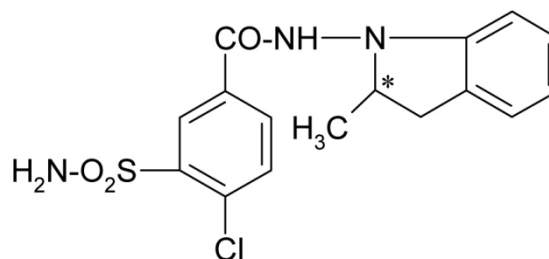


Figure 1. Indapamide chemical structure. The asterix denote the chiral center

*Corresponding author: Gabriel Hancu, Faculty of Pharmacy, University of Medicine and Pharmacy Târgu Mureș, GhMarinescu 38, 54000 Târgu Mureș, Romania. Email: g_hancu@yahoo.com

Indapamide possesses an asymmetric carbon atom adjacent to an amino group in its molecule, resulting in the existence of a S- and R-enantiomer, but is marketed as a racemic mixture. Despite the great prevalence of indapamide in modern therapy, studies' regarding the pharmacokinetics and the pharmacological effect differences of the two enantiomers are few and the results are inconclusive.³

Several chiral separation methods for the indapamide have been reported in recent years using especially high performance liquid chromatography (HPLC) methods.³⁻⁵ Capillary electrochromatography (CEC) was also used as an alternative to HPLC methods for the separation of indapamide enantiomers.⁶⁻⁸ But these methods require derivatization or the use of expensive chiral columns or chiral capillary packings.

Capillary electrophoresis (CE) has been found to be a powerful alternative to HPLC techniques as several chiral separation principles successfully applied in HPLC have been transferred also to CE. The main advantage of using CE in chiral separations is the small amounts of sample, chiral selector and solvents required. This permits the use of a large variety of chiral selectors and also makes it easy to change rapidly the chiral selector and the buffer electrolyte when screening for the suitable selector and electrophoretic conditions.^{9,10}

The most frequently used technique in chiral separations by CE is the capillary zone electrophoresis (CZE), with the direct addition of the chiral selector in the background electrolyte (BGE). Interaction between analytes and the chiral selector will depend on the stability of the formed diastereomeric complex. When a chiral selector is added to the BGE, the mobility of the complex will differ in most cases from the mobility of the free analyte. As a consequence, a difference in complex stability between two enantiomers, will result in a difference in the average velocity of these compounds.^{9,10}

Cyclodextrins (CD's) are by far the most popular chiral selectors used in CE. CD's are cyclic D-glucopolysaccharides, having a relatively hydrophobic interior cavity, while the outside of the rim is more hydrophilic. The inclusion mechanism is sterically selective because analytes must fit the size of the cavity, the diameter of which depends on the number of glucose units in the CD structure. Because of the chirality of the hydroxyls in the glucose molecules, which form the rim of the CD cavity, the inclusion complex formation will be chirally selective.^{11,12}

Our aim was the development of an alternative simple, rapid and cost-effective method for the chiral separation of indapamide enantiomers using a systematic screening of different native and derivatized CDs as chiral screening approach and the optimization of electrophoretic conditions in order to obtain a good chiral resolution in a short analysis time.

Materials and Methods

R,S-indapamide of pharmaceutical grade was purchased from Moehs Productos Quimicos (Barcelona, Spain). For the determination of carvedilol from commercial products we used Indapamid (Labormed, Romania) tablets containing 2.5 mg indapamide. The following reagents of analytical grade were used: phosphoric acid, sodium tetraborate, disodium hydrogenophosphate, sodium didydrogenophosphate (Merck, Germany), methanol, sodium hydroxide (Lach Ner, Czech Republic). Purified water was provided by a Milli-Q Plus water purification system (Millipore, USA).

As chiral selectors we used the following cyclodextrine (CD) derivatives of research grade: native neutral CD (α -CD, β -CD, γ -CD), derivatized neutral CD (hydroxypropyl- β -CD - HP- β -CD, randomly methylated β -CD - RAMEB), anionic substituted charged CD (sulfobutyl ether- β -CD - SBE- β -CD). All CDs were obtained from Cyclolab (Budapest, Hungary) with the exception of SBE- β -CD - Capsitol (Cydex, USA).

The experiments were made on an 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 μ m I.D uncoated fused silica-capillaries (Agilent, Germany). The electropherograms were recorded and processed by Chemstation 7.01 (Agilent, Germany). The pH of the buffer solutions was determined with the Terminal 740 pH-meter (Inolab). The UV spectrum of indapamide was recorded with Specord 210 spectrophotometer (Analytik Jena, Germany).

Indapamide sample stock solutions were prepared by dissolving the substance in methanol in a concentration of 100 μ g/ml and later diluted with the same solvent to the appropriate concentration. The samples were introduced in the system at the anodic end of the capillary by hydrodynamic injection. All samples and buffers were filtered through a 0.45 μ m syringe filter and degassed by ultrasound for 5 minutes before use.

Ten Indapamid tablets (each containing 2.5 mg indapamide) were weighed, and the net weight of each tablet was calculated. The tablets were powdered in a mortar, and an amount of powder equivalent to the average weight was accurately weighed, methanol was added to dissolve the active substance, and the solution was sonicated for 10 minutes. A sample of the tablet solution was then centrifuged at 3500 rpm for 10 minutes. The supernatant was diluted following the same procedure as for the preparation of the standard solution, before the CE analysis.

The capillaries were conditioned before use with 0.1 M sodium hydroxide for 15 minutes and with the background electrolyte used in the analysis for 15 minutes. The capillary was rinsed for 1 minute with 0.1M sodium hydroxide and buffer solutions before each electrophoretic separation.

The separation factors (α) 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 at half height (w_1 and w_2) were marked for the slow and fast migrating enantiomers, respectively.

Results and Discussion

Preliminary analysis

In the initial experiments the indapamide sample solution was injected in the absence of CDs and its effective electrophoretic 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 analyte.

We recorded previously the UV spectra of indapamide and found its absorption maximum at 242 nm, which was elected as detection wavelength in the CE separations. We applied some "standard" electrophoretic conditions for a CZE analysis: temperature 20 °C, applied voltage + 20 kV, injection pressure/time 50 mbar/3 sec, sample concentration 10 µg/ml.

In order to find the suitable conditions for the chiral separation of indapamide, a series of preliminary experiments were conducted at different pH and buffer compositions. In the preliminary analysis we used 25 mM phosphoric acid (pH – 2.1), 25 mM disodium hydrogenophosphate – 25 mM sodium didydrogenophosphate (pH – 7) and 25 mM sodium tetraborate (pH – 9.3) BGEs respectively and we modified the pH of the buffer by adding a 0.1M sodium hydroxide solution. Indapamide was detectable in an achiral environment over a pH range 5 to 10.

The type and concentration of CD added to BGE is of primary importance in achieving chiral resolution. 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.

The most important rule for chiral recognition is that the chiral selector must be compatible in size and structure to the analyte; a minimum of three molecular interactions has to occur. The size of the hydrophobic cavity is such that, in general, the α -CD can accommodate a single phenyl ring, while β -CD and the γ -CD can accommodate substituted single- and multiple ring systems. This inclusion alone is not enough for chiral recognition: interaction between substituents on the asymmetric center of the analyte and the hydroxyl groups on the CD-rim are also responsible for chiral recognition.

When using a phosphate buffer (pH – 5-8) no chiral separation was observed using native neutral CDs (α -CD, β -CD, γ -CD) or derivatized neutral CDs (HP- β -CD, RAMEB), as we observed only an increase in

migration times. The only CD, which exhibited obvious chiral interactions, was the anionic ionized SBE- β -CD. When using a borate buffer (pH – 8-11) no chiral separation was observed using α -CD, γ -CD and RAMEB, a slight peak splitting was observed for β -CD and HP- β -CD, and the best results were obtained again by using SBE- β -CD.

Consequently we can conclude that SBE- β -CD proved to be the optimum chiral selector for the separation of indapamide enantiomers.

Optimization of the analytical method

Stereoselectivity of the separation is influenced by several experimental parameters, such as CD type and concentration, ionic strength, pH of the BGE, capillary temperature, applied voltage, capillary length, addition of organic solvents and electro-osmotic flow (EOF).

The use of a charged CD derivative (SBE- β -CD) can play a more profound role in the chiral resolution mechanism; the electrostatic interactions with the analyte, the movement of the chiral selector in the opposite direction of the enantiomers and the possibility of separating uncharged compounds representing the main advantages.^{13,14}

SBE- β -CD contains four modified primary hydroxyl groups with a butyl chain and sulfonic groups; and due to its chemical properties is negatively charged and can be commonly used in CE over a wide pH range (2-11). Compared with neutral selectors, the effect of the concentration of the charged chiral selectors on the selectivity of enantioseparation can be more pronounced; a slight increase/decrease of the concentration of SBE- β -CD led to major overhauls of the migration times and chiral resolutions. In this work optimization of the concentration of the chiral selector was investigated experimentally for SBE- β -CD concentrations from 1 to 10 mM, we selected a concentration of 5 mM as higher concentration generated high currents and instability of the electrophoretic system.

An increase of the buffer concentration led to an increase of the migration times, but had no marked effect on the separation resolution.

Buffer pH is an important condition in CE separations, as the degree of dissociation of the charged selector, analyte charge, and the EOF are all affected by buffer pH. Indapamide is a basic drug with a pKa of 8.8; its net charge at pH between 3 to 5 is not significantly different, showing that analyte charge is insensitive to pH. It is, nevertheless, well known that the EOF is sensitive to pH in the range between 3.0 and 7.0; as it decreases considerably with decreasing pH. Indapamide was detectable at pH above 5.0 but did not elute toward the cathode when buffer pH was reduced to 3.0; this is indicative of a significant decrease in the EOF, and even reversal of the apparent mobility vector. It can also be seen that migration times decreased as the pH was increased from 5 to 11, while chiral resolution increased in the pH range 5 to 7 and deteriorated in the

pH range 9 to 11 (Figure 2). A neutral 25 mM disodium hydrogenophosphate – 25 mM sodium

didihydrogenophosphate at a pH – 7 was elected as the optimum BGE.

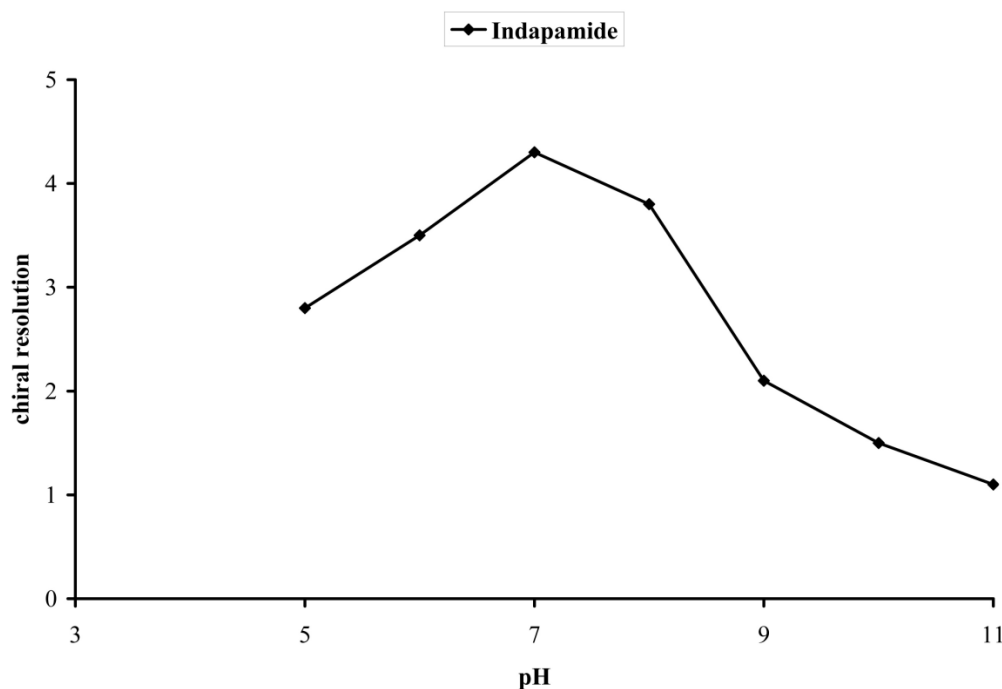


Figure 2. Effect of buffer pH on the indapamide chiral resolution

While the applied voltage had only a slight influence on the chiral resolution, temperature influenced strongly the separation efficiency and the enantiomeric resolution, as a decrease in temperature led to longer migration times but also to an increased chiral resolution. In order to obtain a satisfactory migration times and a high chiral resolution we combined the effects of these two secondary electrophoretic parameters, choosing an applied voltage of + 25 kV at a temperature of 15°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 selected an injection pressure of 50 mbar for 1 second.

Taking in consideration all these aspects we can conclude that the optimum electrophoretic conditions for the indapamide enantioseparation are: 25 mM disodium hydrogenophosphate – 25 mM sodium didihydrogenophosphate BGE, 5 mM SBE- β -CD chiral selector, buffer pH - 7, applied voltage + 25 kV, temperature 15°C, injection pressure/time 50 mbar/1 sec, UV detection at 242 nm. Applying the optimized electrophoretic conditions we succeeded in the separation of the two enantiomers in approximately 6 minutes, with a resolution of 4.30 and a separation factor of 1.08 (Figure 3).

Because we didn't have at our disposal pure indapamide enantiomers, we couldn't establish the migration order, consequently the two enantiomers

were called taking in consideration their migration order: enantiomer 1 and enantiomer 2.

Analytical performance

The analytical performances of the method were evaluated using the optimized electrophoretic conditions.

The RSD (relative standard deviation) for the migration times and peak areas was calculated by injecting consecutively (n = 6) a sample of 10 μ g/ml (Table 1).

Calibration plots were constructed by preparing standard solutions (n = 3) at six concentrations in a specific concentration range (concentration range: 2.5 - 50 μ g/ml). The regression equation and correlation coefficient are presented in Table 2.

The limits of detection (LOD) and quantification (LOQ) were estimated as: standard deviation of regression equation/slope of the regression equation multiplied by 3.3 and 10, respectively (Table 2).

The peaks obtained from tablets were similar to those from indapamide standard, no interference from the matrix had been observed. The content of a tablet was found to be 2.5 ± 0.06 mg (mean \pm SD, n = 6). Recovery was between 97.2 and 100.7%.

Conclusion

The development of new analytical methods for the separation and determination of enantiomers has attracted great interest in last twenty-five years, since it became evident that that the biological and

pharmacological activity of enantiomers can differ and is mostly restricted to one of the enantiomers. Therefore, there is considerable pressure to develop new analytical methods for enantiomer separation, for

enantiomeric purity control, pharmacological studies, pharmacodynamic investigations, clinical studies etc.

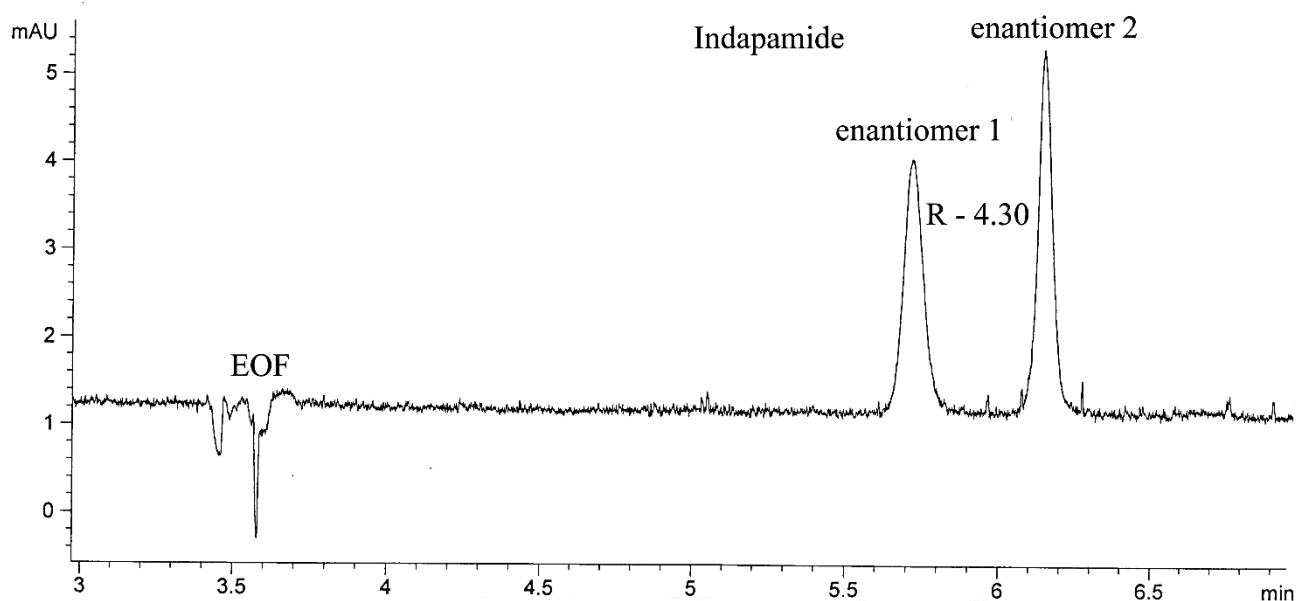


Figure 3. Capillary electrophoretic separation of indapamide enantiomers using SBE- β -CD as chiral selector (experimental conditions: BGE: 25 mM disodium hydrogenophosphate – 25 mM sodium dihydrogenophosphate, chiral selector: 5 mM SBE- β -CD, pH - 7, voltage + 25 kV, temperature 15°C, hydrodynamic injection 50 mbar/1 sec., sample concentration 10 μ g/ml, UV detection 242 nm)

Table 1. Analytical parameters of the indapamide chiral separation

Indapamide enantiomers	Migration time (min)	RSD migration time (%)	RSD peak area (%)	Electroforetic mobility ($\text{cm}^2/\text{kV min}$)
enantiomer 1	5.70	0.25	0.82	- 8.27
enantiomer 2	6.25	0.23	0.79	- 8.44

Table 2. Calibration data and LOD/LOQ values for indapamide chiral separation (calibration range: 2.5 - 50 μ g/ml)

Indapamide enantiomers	Regression equation	Correlation coefficient	LOD (μ g/ml)	LOQ (μ g/ml)
enantiomer 1	$y = 0.462x + 0.849$	0.992	1.85	5.25
enantiomer 2	$y = 0.446x + 0.818$	0.997	1.62	4.85

A simple, rapid, reproducible and accurate CZE method has been successfully developed for the enantioseparation of indapamide and applied for the determination of indapamide from tablets. The method uses a simple phosphate buffer and an anionic charged CD, SBE- β -CD, as chiral selector, producing the baseline separation of the two enantiomers with excellent chiral resolution with sharp peaks and relatively short analysis time. Highly satisfactory results were obtained from analysis of tablets, indicating the method is specific, accurate, and suitable for routine analysis of indapamide in pharmaceutical preparations.

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Conflict of Interest

The authors report no conflicts of interest.

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