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Research Article

Cyclodextrine Screening for the Chiral Separation of Amlodipine Enantiomers by Capillary Electrophoresis

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Abstract

Purpose: Amlodipine is a long acting, dihydropyridine type calcium channel blocker frequently used in the treatment of hypertension and coronary insufficiency. The calcium channel blocking activity resides primarily in the S-amlodipine enantiomer, while R-amlodipine is a potent inhibitor of smooth muscle cell migration.

Methods: In this study capillary electrophoresis was applied for the enantiomeric separation of amlodipine using different native and derivatized; neutral and charged cyclodextrines as chiral selectors. The effects of pH and composition of the background electrolyte, concentration and type of chiral selector, capillary temperature, running voltage and injection parameters have been investigated.

Results: Stereoselective interactions were observed when using α-CD, β-CD, HP-β-CD, RAMEB, CM-β-CD and SBE-β-CD. Optimized separation conditions consisted on a 50 mM phosphate buffer, pH – 3.0, 20 mM RAMEB as chiral selector, + 25 kV applied voltage, 15°C temperature and UV detection at 238 nm. Using the optimized electrophoretic conditions we succeeded the chiral separation of amlodipine enantiomers in approximately 6 minute, the order of migration being R-amlodipine followed by S-amlodipine. The method was successfully applied for the determination of amlodipine enantiomers from commercially available pharmaceuticals. The linearity range, limits of detection and quantification, precision and accuracy were determined and the results obtained confirmed that the method was suitable for this purpose.

Conclusion: It can be concluded that the proposed capillary electrophoresis methods can be useful for routine pharmaceutical applications with benefits of its effectivity, simplicity, short analysis time and low consumption of analytes, solvents and chiral selectors.

Introduction

Amlodipine, 2-[(2-aminoethoxy) methyl]4(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid 3-ethyl 5-methyl ester (AML), is a second generation 1,4-dihyropyridine type calcium-channel blocker.[1]

Structurally AML compared with the prototypical molecule nifedipine, is an asymmetric ester; in which a methyl group is replaced by an amino-ether substituent, the nitro group is substituted with chlorine and also one of the ester groups is different. AML posses in its structure an asymmetric carbon atom, resulting in the existence of a S- and R-enantiomer, but is marketed as a racemic mixture (1:1) mixture of R-(+)- and S-(–)-AML). It is usually marketed and is officinal in pharmacopoeias as a benzene sulfonic acid salt (besylate).[2,3]

The chemical structure of amlodipine is presented in Figure 1.

AML is used in the treatment of chronic stable angina and in the management of mild-to-moderate essential hypertension. AML inhibits the movement of calcium ions into vascular smooth muscle cells and cardiac muscle cells; reducing blood pressure by relaxing arterial smooth muscles, which decreases total peripheral resistance. In angina AML increases blood flow to the heart muscles, but the precise mechanisms by which AML relieves angina is not fully understood, because dihyropyridine type calcium-channel blockers are more selective for arteries than the muscular tissue of the heart, but the calcium ion channels of the heart are not of the dihydropyridine-type.[4]

Structural features of AML give the molecule physicochemical and pharmacokinetic properties that are unique among calcium-channel blockers. AML is absorbed gradually after oral administration (peak plasma levels 6-12 h after administration) and has an absolute bioavailability of approximately 60%. Low clearance and a high volume of distribution give AML a long elimination half-life, and mean effective plasma levels are maintained with once-daily doses.[5]

S-(-)-AML and R-(+)-AML do not have the same level of antagonistic effect on the calcium channel receptor; as the S-enantiomer of AML is active while the R-

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enantiomer is considered to be inactive in terms of calcium channel blocking activity. S-(-)-AML has 1000 fold stronger calcium channel blocking activity than R-(+)-AML, being therefore responsible for all of the calcium-channel blocker mediated pharmacodynamic action of AML.[6] Furthermore R-(+)-AML has shown to release nitric oxide in the peripheral blood vessels which may lead to peripheral edema.[7] In addition the longer duration of action of S-(-)-AML reduces the chances of reflex tachycardia and its clearance is subjected to much less inter-subject variation than R-(+)-AML.[8]

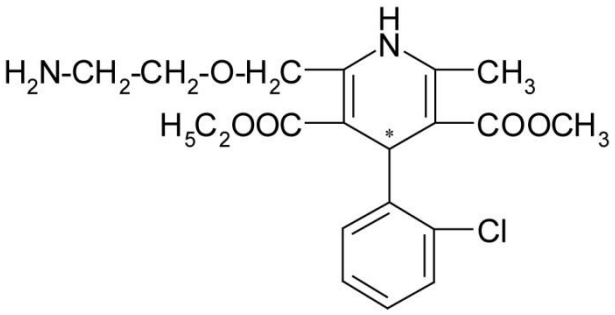

Figure 1. Chemical structure of amlodipine. The asterix denote the chiral center

It is obvious, from above mentioned facts regarding the stereochemistry of AML, that the control of enantiomeric composition of pharmaceuticals containing AML is important.

Analytical methods used so far for the enantiomeric separation of pharmaceutical substances include high performance liquid chromatography (HPLC), thin-layer chromatography (TLC), gas chromatography (GC), supercritical fluid chromatography (SFC) and capillary electrophoresis (CE). CE offers several advantages over other techniques such as HPLC, including: rapid method development, high separation efficiency, low sample, buffer and chiral selector consumption and especially the high selectivity in choosing and changing the chiral selector.[9]

In most cases in CE, a direct method of enantioseparation is used, by simply adding the chiral selector to the background electrolyte (BGE). Chiral separation is based upon the formation of diastereomeric complexes between the optical isomer and the chiral selector and can be obtained only if these complexes have different equilibrium constants of complex formation. Therefore, complex formation will result in an average velocity of the analyte, which is different from the velocity of the free analyte. As a consequence, a difference in complex stability between the two optical antipodes will result in a difference in the average velocity of these compounds. In order to maximize enantioselectivity, one should obviously maximize this difference in average velocity between the two optical antipodes.[9]

Chiral selectors are compounds, which can stereoselectively, recognize both enantiomers of the analyte via different binding constants. CDs whether native or derivatized, neutral or ionic are by far the most common chiral selectors employed in CE for the enantiomeric separation of chiral substances. CD modified CE has been proved to be extremely effective in the resolution of racemic drugs including here dihydropiridine derivatives.[10]

Also chiral separation of AML has been studied using CE methods and different CD derivatives as chiral selectors, but the optimal separation conditions significantly differ in several cases, and the basis of the differences has not been clearly identified.[11-16]

CE has been applied as method of separation and determination of AML enantiomers from pharmaceutical preparations,[14] serum,[12] urine[13] and also in pharmacokinetic studies.[13]

Taking in consideration the aspects regarding the strong connections between stereochemistry and pharmacodynamic effects of AML, elaboration of new methods for its enantiomer separation becomes a necessity and also a permanent challenge.

This study describes a screening of various native and derivatized cyclodextrines for the development of a simple, rapid an efficient method for the chiral separation of AML enantiomers and the optimization of electrophoretic conditions in order to obtain an enhanced chiral resolution in a short analysis time.

Materials and Methods

Chemicals

The racemic mixture R,S–amlodipine besylate and the pure enantiomer S-amlodipine besylate of pharmaceutical grade were obtained from Fako Ilaclari A.Ş (Istanbul, Turkey). The pure enantiomer of a β-blocker, namely S-propranolol used as an internal standard (IS) was obtained from Moehs Productos Quimicos (Barcelona, Spain). The following reagents of analytical grade were used: phosphoric acid (Pernix Pharma, Hungary), methanol, sodium hydroxide (Lach Ner, Czech Republic), sodium tetraborate, disodium hydrogenophosphate, sodium didydrogenophosphate (Merck, Germany). 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 (carboxymethyl-β-CD - CM-β-CD, sulfobuthyl ether- β-CD – SBE-β-CD). All CDs were obtained from Cyclolab (Budapest, Hungary) with the exception of SBE-β-CD - Capsitol (Cydex, USA).

For the determination from commercial pharmaceutical preparations we used Norvasc tablets (Pfizer, USA) each tablet containing 10 mg AML, obtained from a local pharmacy.

Instrumentation

CE determinations were performed on a Agilent 6100 CE system (Agilent, Germany) equipped with a diode

array UV detector. The data was processed using a Chemstation 7.01 (Agilent, Germany) software. Separation was performed in a 48 cm (40 cm effective length) x 50 μm I.D. uncoated fused silica-capillaries (Agilent, Germany). The pH of the buffer solutions was measured with the Terminal 740 pH–meter (Inolab). The UV spectrum of AML was recorded with Specord 210 spectrophotometer (Analytik Jena, Germany).

Sample preparation

AML sample stock solutions were prepared by dissolving the substance in methanol in a concentration of 100 μg/ml and later diluted 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 μm syringe filter and degassed by ultrasound for 5 minutes before use.

Ten Norvasc tablets (each containing 10 mg AML) 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 of a single tablet was accurately weighed, methanol was added to dissolve the active material, and the solution was diluted to 25 mL with the same solvent in a volumetric flask and sonicated for 10 minutes. A sample of tablet solution was then centrifuged at 3500 rpm for 10 minutes. The supernatant was diluted using the same procedure as for preparation of standard solution, before CE analysis.

CE procedures

The capillaries were conditioned before use with 0.1 M sodium hydroxide for 30 minutes and with the BGE used in the analysis for 30 minutes. Before each analysis the capillary was washed and conditioned by rinsing for 1 minute with 0.1 M sodium hydroxide and then for 1 minute with the BGE.

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 sec, sample concentration 10 μg/ml.

Previously we recorded the UV spectra of AML and found three absorption maximum at 214, 232 and 360 nm.

Enantioselectivity evaluation

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 (w_1 and w_2) were marked for the slow and fast migrating enantiomers, respectively.

Results and Discussion

Preliminary study

Electrophoretic mobilities and ionization behavior of analytes are the key factors driving separations in CE. Knowledge of these basic physicochemical properties of analytes gives valuable information about their nature and makes it easier to choose appropriate experimental conditions for their separation.[17]

In order to find the suitable conditions for the chiral separation of AML, a series of preliminary experiments were conducted in an achiral system with different buffer compositions at different pH values. In the preliminary analysis we used: 25 mM phosphoric acid (pH –2.1), 25 mM sodium didydrogenophosphate (pH – 5.0), 25 mM disodium hydrogenophosphate – sodium didydrogenophosphate (1:1) (pH – 7.0) and 25 mM sodium tetraborate (pH – 9.3) BGE respectively and we modified the pH of the buffer by adding a 0.1M sodium hydroxide solution.

AML is a basic compound, due to its amino-ether substituent, and has a pKa value of 9.1, consequently an acidic BGE should be suitable for its determination.[11,16]

In the pH range between 2.5-5.0 we identified a well-shaped peak after the injection of an AML sample; as it is well established the fact that at low acidic pH values the influence of electroosmotic flow (EOF) is minimum, and the analyte will migrate mostly through its own electrophoretic mobility. In the pH range between 6.0-8.0 AML migrates very close or even with the EOF, its own electrophoretic mobility being very low. Under acidic conditions (pH < 6.0) AML is positively charged, but as the pH becomes more alkaline, the effective mobility decreases because AML starts to deprotonate.

Initial concentration of 10 mM neutral CDs were dissolved in the BGE, 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 size of the hydrophobic cavity is such that, in general, the α-CD can accommodate a single phenyl ring, while β-CD and γ-CD can include substituted single- and multiple ring systems. But 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.[10]

Stereoselective interactions were observed for α-CD, β-CD, HP-β-CD, RAMEB and CM-β-CD over a pH range between 2.5-5.0, and for SBE-β-CD over a pH range between 7.0-9.0.

We noticed that the difference between the electrophoreic mobilities of enantiomers, and consequently chiral resolution, decreases at a pH over 5.0 when using uncharged CDs as chiral selectors. Also, in a BGE with higher pH the effect of EOF on the separation is stronger, allowing shorter time for enantioselective interactions between AML and selector.

At low acidic pH values, the anionic charged SBE-β-CD is negatively charged while AML is positively charged, so the SBE-β-CD will move towards the anode while AML enantiomers will move towards the cathode, resulting in very long migration times and no apparent chiral interactions. At pH values above 7.0, SBE- β-CD is positively charged and will move in the direction of EOF, while AML electrophoretic mobility is very low,

consequently certain stereoselective interactions can be observed.[18]

Method optimization

One of the most important factors to be considered in a chiral CE analysis is the concentration of the chiral selector in the BGE. The optimum concentration depends on the binding affinity of stereoisomers with the chiral selector. At a low CD concentration, no separation of the enantiomers is possible because there are not enough chiral selectors available to form the complexes. Conversely, at a high CD concentration, the enantiomers may be completely complex and do not result in separation. Lower concentrations of anionic CDs in comparison with neutral CDs were used in order to obtain the same chiral resolution. The optimum concentration for the studied CDs and pH of the BGE are presented in Table 1.

The chemical composition and the concentration of the buffer can affect the baseline stability, peak shape and separation selectivity. While the buffer concentration increased from 25 to 50 mM, the enantiomer separation improved slightly whereas the concentration changed in the range of 50 to 100 mM, almost no influence on the separation could be observed. A slight increase in analyte migration time due to a decrease in the EOF with increasing buffer concentration was also observed. Hence a 50 mM buffer concentration was selected for the optimal enantioseparation method.

Running voltage did not have a strong effect on the resolution; an optimum potential of + 25 kV was chosen for the analysis. The effect of capillary temperature on chiral separation is related to the thermodynamic behavior of the analyte-chiral selector complex during the chiral separation. A decrease in temperature led to longer migration times but also to an increased chiral resolution. The capillary temperature for analysis was chosen at 15°C in order to obtain adequate resolution of the enantiomers and reasonable analysis time.

A high injection pressure and a short injection time will increase chiral resolution; consequently we selected an injection pressure of 50 mbar for 1 second.

The migration order of the two enantiomers was determined by spiking and also by injecting the pure enantiomer using the selected chiral buffer BGE. The slower migration time of S-(-)-AML indicates a stronger interaction between this enantiomer and the selected chiral selector, which leads to a slower movement of this enantiomer towards the detector.

The results obtained with different CDs as chiral selectors using the optimized analytical conditions are presented in Table 1.

Table 1. Capillary electrophoretic separation of AML enantiomers using different CD derivatives as chiral selectors

CD	CD concentration [mM]	pH	t (min) R-AML	t (min) S-AML	R	α
α-CD	20	3.0	5.70	6.10	1.40	1.05
β-CD	15	3.0	6.30	6.45	0.86	1.02
HP-β-CD	20	3.0	4.50	4.90	2.17	1.08
RAMEB	20	3.0	5.40	5.90	2.48	1.09
CM-β-CD	10	5.0	9.50	9.90	1.55	1.04
SBE-β-CD	5	7.0	12.70	13.80	2.59	1.08

Taking in consideration both chiral resolution and also migration times we considered that the best results were obtained when using 20 mM RAMEB as chiral selector.

We can conclude that the optimum electrophoretic conditions for the AML enantioseparation are: 50 mM phosphate BGE, buffer pH – 3.0, applied voltage + 25 kV, temperature 15°C, injection pressure/time - 50 mbar/1 sec, UV detection 238 nm. Using the optimized electrophoretic conditions presented above we succeeded in the separation of AML enantiomers in less than 6 minutes, the order of migration being S-(-)-AML followed by R-(+)-AML with a resolution of 2.48 and a separation factor of 1.09 (Figure 2).

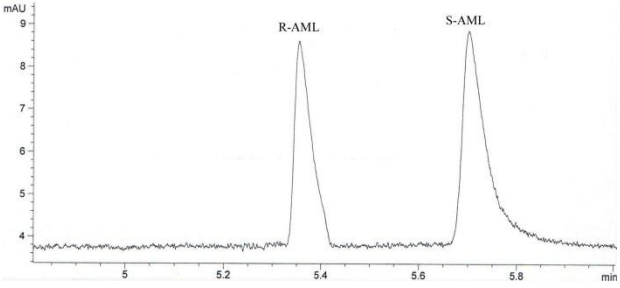

Figure 2. Capillary electrophoretic separation of AML enantiomers using RAMEB as chiral selector (experimental conditions: BGE: 25mM phosphate buffer, chiral selector: 20 mM RAMEB, pH – 3.0, voltage + 25 kV, temperature 15°C, hydrodynamic injection 50 mbar/1 sec., sample concentration 10 μg/ml, UV detection 238 nm)

Analytical performance

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

The relative standard deviations (RSD) for the migration times and peak areas was calculated by injecting consecutively (n = 6) a sample of 10 µg/ml (Table 2).

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

Table 2. Analytical parameters of the AML chiral separation

AML enantiomers	Migration time (min)	RSD migration time (%)	RSD peak area (%)	RSD peak height (%)
R-AML	5.40	0.25	0.97	1.02
S-AML	5.90	0.27	0.99	1.10

Table 3. Calibration data and LOD/LOQ values for AML chiral separation (calibration range: 2 - 50 µg/ml)

AML enantiomers	Regression equation	Correlation coefficient	LOD (µg/ml)	LOQ (µg/ml)
R-AML	y = 0.051x + 0.744	0.997	2.31	7.88
S- AML	y = 0.052x + 0.868	0.997	2.43	8.24

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 3).

Quantification was accomplished on the basis of AML to IS peak-area ratios [i.e. (peak area of STL)/(peak area of IS)].

In order to demonstrate that the developed method can be used in true samples, we applied the optimized conditions for the enantiomeric separation of AML from Norvasc tablets, each tablet containing 10 mg AML. The peaks obtained from the tablets were similar to those from AML standard and there was no interference from the matrix. The content of a tablet was found to be 9.85 ± 0.20 mg (mean ± SD, n = 6). The content of AML, obtained by the proposed method, was in a good agreement with that declared by the manufacturers.

The accuracy of the method was determined by analysis of two quality-control samples, at concentrations within the calibration range, prepared from AML standard solution, at different, known, concentrations. Recovery was between 99.8 and 101.8% for drug substance and between 97.9 and 100.2% for drug product.

Conclusion

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, BGE pH and composition have strong effects on the chiral resolution of AML.

When using an acidic BGE, since AML is cationic, the mobility of the enantiomers will be in the direction of EOF; consequently neutral CDs can be used successfully for the enantioseparation. In an alkaline BGE, AML effective electrophoretic mobility will be low, and the substance will migrate very close to the EOF; consequently anionic CD (SBE- β-CD) can be used as chiral selector for the enantioseparation. Lower pH values proved to be favorable for increasing separation efficiency, enantioresolution and decreasing analysis time. The affinity of S-(-)-AML towards the studied CDs is stronger of that of the R-(+)-AML, for both neutral and anionic CDs.

CE proved to be a rapid, specific, reliable and cost-effective method for the chiral separation of AML enantiomers and can be useful for laboratories performing routine analysis.

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Ethical Issues

Not applicable.

Conflict of Interest

The authors report no conflicts of interest.

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