Rapid enantioseparation of amlodipine by highly sulfated cyclodextrins using short-end injection capillary electrophoresis

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ABSTRACT

Background and the purpose of the study: The use of highly sulfated cyclodextrins (HS-CDs) as chiral selectors in capillary electrophoresis (CE) has been examined for rapid and reproducible enantioseparation of the model drug amlodipine, a calcium channel blocker.

Materials and Methods: Fused silica capillaries with an inner diameter of 50 μm, and a total length of 45.5 cm (8.5 cm to the detector) were used. Capillaries were rinsed with polyethylene oxide (PEO) once daily. A systematic method development approach was conducted by modifying selected parameters such as the type and concentration of the chiral selector, the buffer pH and concentration of the background electrolyte.

Results: Baseline separation was achieved at low (i.e. 0.05%w/v) concentrations of HS-αCD, but migration time and peak area repeatability were more than 4% and 25% of the relative standard deviation (RSD), respectively. At higher concentrations (>0.3%) of HS-αCD, amlodipine was transported to the anode by the carrier ability of HS-αCD. In carrier mode, the migration order of enantiomers was reversed, the migration time was reduced and the peak area repeatability of analysis was improved. The optimum electrophoretic conditions for the stereoselective analysis of amlodipine were obtained in carrier mode with 25 mM sodium phosphate buffer containing 1.25% w/v of HS-αCD at pH 2.5 with an applied voltage of +15 kV. Under these conditions migration time was less than 3 min and within-day migration time and peak area repeatability, were less than 0.4% and 2.1% RSD, respectively.

Conclusions: Rapid enantioseparation was achieved with minimum variation in quantitative analysis. These optimized conditions are appropriate for the enantioselective analysis of amlodipine.

Keyword: Amlodipine, Chiral separation, Highly sulfated cyclodextrins, Carrier mode, Short-end injection

INTRODUCTION

The pharmacological, pharmacodynamic, and toxicological behavior of the enantiomers of chiral drugs can differ widely (1) . It is therefore important to develop enantioselective separation methods for studies on stereoselective pharmacokinetics and metabolism. Furthermore, enantioselective separation methods is required for quality control of pharmaceutical raw materials and finish products during their production, storage and application (2). Such methods should increasingly be robust and suitable for inclusion in exacting specification procedures. This is especially true for dihydropyridine (DHP) derivatives which have attracted interest as calcium channel blockers and are employed in a variety of cardiovascular diseases (3). The calcium channel blocking agent amlodipine (Figure 1) is classified as a basic DHP. While the S-enantiomer of amlodipine exhibits vasodilating properties, its R-amlodipine is inactive and thought to be responsible for pedal oedema observed with racemic amlodipine (4). In addition to longer duration of action of S-amlodipine it reduces the chances of reflex tachycardia and its clearance is subjected to much less inter-subject variation than R-amlodipine (5).

Capillary electrophoresis (CE) compared to other techniques has experienced enormous growth in the field of chiral separations and CE has several advantages: the high resolving power, low consumption of sample, solvent and chiral selector, as well as high flexibility in choosing and changing types of selectors (6). CE-based chiral analyses are greatly simplified by having the chiral selectors in the separation buffer which act as a pseudostationary phase. Chiral selectors are compounds which can

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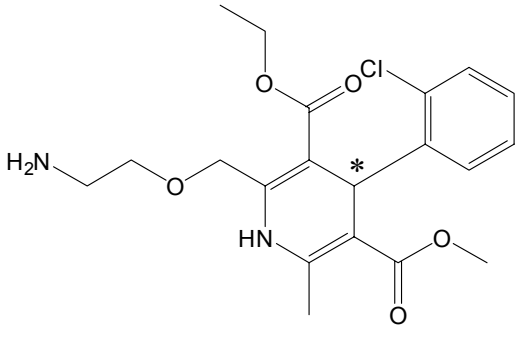

Figure 1. Amlodipine structure, pKa 8.6 (28).

stereoselectively recognize both enantiomers of the analyte via different binding constants. Different chiral selectors are employed to perform enantiomeric separations in CE. Cyclodextrins (CDs) whether native, neutral, or their ionic derivatives are by far the most common chiral selectors for the enantiomeric separation of chiral species by CE (7, 8). Cationic and amphoteric CDs are less common and only limited varieties are commercially available. Anionic CDs are widely used selectors for resolution of chiral compounds, especially for positively charged analytes. Among all the anionic CDs, sulfobutyl ether and in particular sulfated cyclodextrins have received the most attention and appear to be the most effective charged chiral selectors. However, these anionic CDs are relatively heterogeneous and may not provide reproducible results as chiral reagents (9). Highly sulfated cycldextrins (HS-CDs) are obtained as consistent products with reproducible performance as chiral selectors for CE, and contain a substantially higher degree of sulfation and less batch to batch variation. These selectors are used for enantioseparation of neutral, acidic, and basic compounds (10).

A successful separation of amlodipine enantiomers by using CE with a neutral CD, hydroxypropyl βCD (HP-βCD), and two anionic derivatives of βCD namely sulfobutylether βCD (SBE-βCD) and carboxy methyl βCD (CM-βCD) has been reported. (11). The enantioseparation of amlodipine by using HP-βCD and CM-βCD have been at pH 3.0, and SBE-βCD at pH 7.0 (11). However, no data pertaining to the experimental repeatability have been reported. Furthermore, the analysis times were more than 10 min. Other attempts for CE enantioseparation of amlodipine suffer from long analysis time (≥ 8.3min) and low resolution (≤ 1.73) (12, 13)

In the present work HS-CDs are explored for rapid, high resolution, and reproducible CE enantioseparation of amlodipine. The high selectivity of these chiral selectors enabled the short-end injection technique (14) to be used. In this technique, by injection of the sample at the end of the capillary which is the closest injection site to the detection window, separation length and as a result the migration times and analysis time are reduced.

MATERIALS AND METHODS

Reagents and Chemicals

All solutions were prepared in nanopure 18 M Ω water (Barnstead, Chicago, IL). HS-α, β, and γ CDs with an average degree of substitution of 11, 12, and 13, respectively were obtained from Beckman Coulter, Inc. (Fullerton, CA) as 20% w/v solutions in water. Running buffers containing HS-CDs (0.001-7.5%) were prepared by diluting the appropriate HS-CD stock solution (20% w/v) with phosphate buffer. Phosphate buffers were prepared by dissolving sodium phosphate monobasic monohydrate (EM sciences, Fort Washington, PA) to obtain a final concentration range of 6-75 mM, and adjusting the pH with 1 M orthophosphoric acid (BDH, Darmstadt, Germany) or 1 M sodium hydroxide (Sigma, Louis, MO). All capillaries were rinsed with conditioning solution containing 0.4% w/v polyethylene oxide (PEO, MW 300,000) as received from Beckman Coulter, Inc. A 5% v/v acetone (Fisher Scientific, Fair Lawn, NJ) solution in water was used as the neutral electroosmotic flow (EOF) marker. Racemic-amlodipine besylate(99.7%) was provided by Minoo Co. (Tehran, Iran). S-amlodipine besylate(99.5%) was donated from Cipla (Mumbai, India). Standards of 100 ppm of racemic-amlodipine and S-amlodipine were prepared in nanopure water.

Apparatus

Experiments were performed on a $HP^{3D}CE$ capillary electrophoresis system (Hewlett-Packard, Palo Alto, and CA. USA) equipped with a diode array detector. CE Chemstation software (Version A.06.01; Hewlett-Packard) was used for control and the data acquisition system. The data acquisition rate was 5 Hz, and the rise time was 0.1 s. Diode array detector was set at 254 nm. Untreated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) with a total length of 45.5 cm (8.5 cm to the detector), an inner diameter of 50 μm, were used. Samples were injected at the end of the capillary nearest to the detector by applying a pressure of -10 mbar for 5 seconds. Unless otherwise stated separations were performed at 15 kV which was experimentally determined to be within the linear portion of the Ohm's plot. Depending on the conditions, normal or reverse polarities were applied.

Under high pressure (950 mbar), each new capillary was pretreated by rinsing with 0.1 M NaOH for 10 min, water for 5 min, PEO solution for 1 min, and with running buffer for 5 min, sequentially. Between runs, the capillary was flushed with the running buffer for 2 min.

Throughout the investigation, the capillaries were

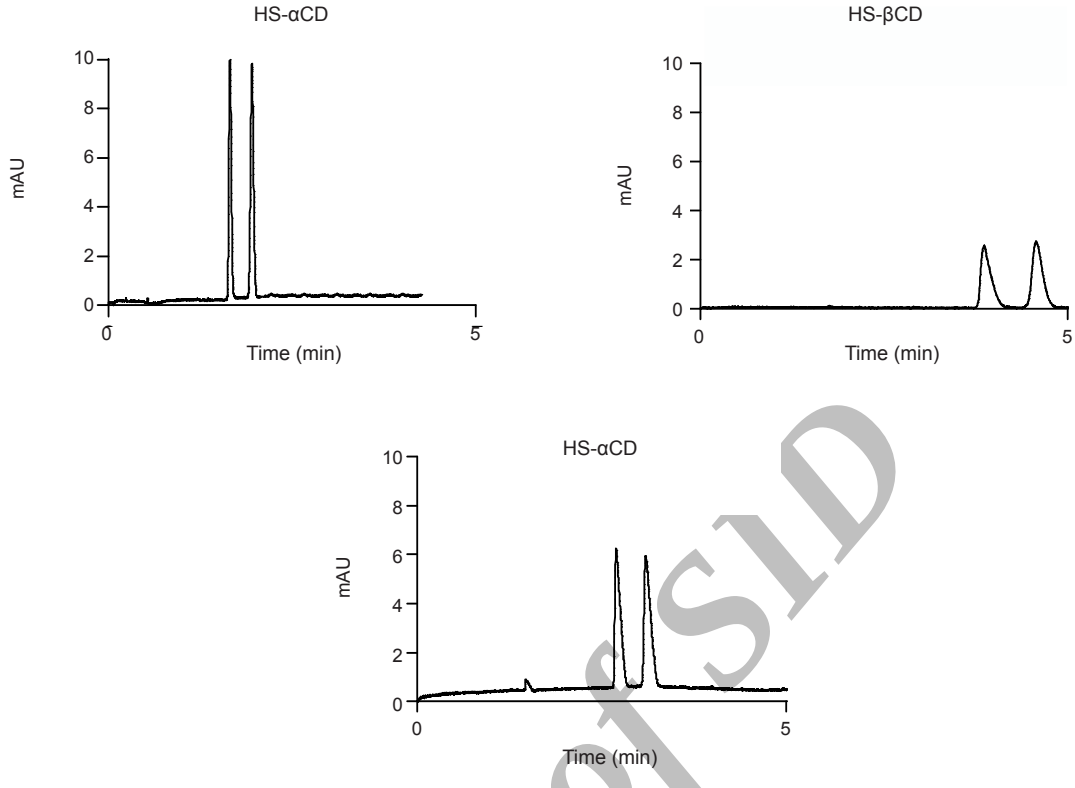

Figure 2. Effect of type of HS-CD on enantioseparation of amlodipine. Experimental conditions: buffer, 10 mM sodium phosphate (pH 2.5) containing 5%w/v of each α, β or γ HS-CDs; voltage, +15 kV; sample, 100 ppm amlodipine

rinsed with PEO once daily to obtain reproducible results. The pH was measured using a digital pH meter Model 445 (Corning, Acton, USA) calibrated with standards immediately prior to use.

Mobility Measurements

The electrophoretic mobility (μ) of a charged species was calculated using equation (1)

$$\mu = \frac{L_d \, L_t}{V} \left(\frac{1}{t_m} - \frac{1}{t_{EOF}} \right) \quad (1)$$

where t_m and t_{EOF} are the migration times of the analyte and the electroosmotic flow (EOF) marker, L_t the total length of the capillary (45.5 cm), L_d the detection length (8.5 cm), and V the voltage applied across the capillary. To determine migration time of the EOF, the neutral marker, acetone 5% v/v in water was injected into the capillary using hydrodynamic injection (-10 mbar) for 5 seconds.

RESULTS AND DISCUSSION

Type of HS-CD

HS-CDs are polynegatively charged and migrate towards the anode. On the other hand, the primary amine of amlodipine makes it positively charged and thus amlodipine inherently migrates toward the cathode. These opposite intrinsic mobilities enhance the effect of differences in enantiomer binding, making HS-CDs much more capable of resolving the enantiomers of amlodipine compared to the previously reported uncharged CDs (11, 15). The resolving power of HS-CDs could also be a result of the electrostatic interaction between the HS-CDs and the oppositely charged solute (16).

Typically, separations using HS-CDs are performed under conditions of suppressed EOF (10). Several methods have been developed to suppress EOF, including the use of low pH, high ionic strength, and zwitterionic additives. However, the most common approach has been to coat the capillary wall (17). Coatings can be broadly categorized as covalently linked polymeric coatings, physically adsorbed polymer coatings, or small-molecule additives. In this work, capillaries were coated dynamically with 0.4% w/v PEO (MW 300,000) solution to suppress the EOF and to decrease analyte adsorption (18).

To determine whether α, β or γ HS-CDs are mostly effective for enantiomeric separation of amlodipine, preliminary studies were performed by using solutions of 5% w/v of each HS-CD sodium phosphate buffer (10 mM) of pH 2.5. These conditions have been reported to be a good starting point for separation of analytes by HS-CDs (19). Under these conditions, the superior resolution was observed with HS-αCD

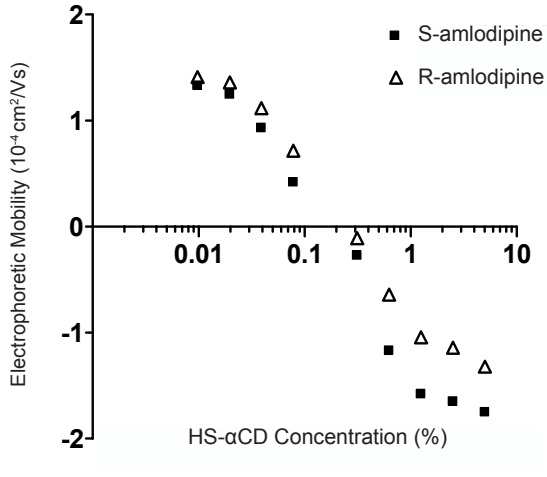

Figure 3. Effect of HS-αCD concentration on mobility of amlodipine enantiomers.
Experimental conditions: buffer, 25 mM sodium phosphate (pH 2.5) containing various concentrations of HS-αCD; voltage, -15kV for less than 0.1% HS-αCD and +15 kV for greater than 0.3% HS-αCD; sample, 100 ppm amlodipine; EOF marker, acetone 5%.

(Figure 2). Based on these results, HS-αCD was used as the chiral selector throughout the rest of this study.

Effect of HS-αCD concentration
As stated above, amlodipine (Figure 1) is positively charged under acidic and neutral conditions. Thus, in the absence of any anionic selector, amlodipine migrates electrophoretically towards the cathode. As shown in figure 3 the interaction between amlodipine, a monovalent cation, with HS-αCD, a polyanionic species, initially decreased the mobility of the drug and at about 0.1% HS-αCD the mobility of the amlodipine reached to zero. At this HS-αCD concentration, the net charge of the amlodipine/ HS-αCD complex is zero, i.e., the amlodipine is still only partially complexed by the HS-αCD, but to a degree by which the polyanionic charge of the HS-αCD balances the monocationic charge of amlodipine. As the selector concentration was further increased (above 0.3%), the net mobility of amlodipine became negative due to the "carrier effect" (20, 21) of HS-αCD. In the carrier mode a chiral selector is not only responsible for the enantioselectivity in separation system but also transports the resolved analytes towards the detector (21). Thus, it was necessary to change the polarity to detect amlodipine enantiomers. Resolution of analytes in complex matrices in carrier mode, due to higher selectivity of analysis, offer significant advantages. (21).
In non-carrier mode (0.05%w/v HS-αCD), the lower mobility of the R-enantiomer indicates a stronger interaction between this isomer and the selector. Owens et al. (11) have reported stronger complexation of the S-enantiomer with HP-βCD and CM-βCD. This complexation pattern is opposite to the result of this study. However, in another report it has been noted that even the location of the sulfate groups and the degree of sulfation of CD can reverse the migration order of enantiomers (22).
In carrier mode, and monitoring anodic migration, the more tightly complexed R-enantiomer has higher electrophoretic mobility and migrates to the detector before the S-enantiomer, causing a reversal of the order of the peaks (Figure 4b) relative to that observed at low CD concentration (Figure 4a). Reversal of migration order of enantiomers by increasing concentration of anionic selector for the cationic chiral analyte propranolol has been reported previously (23).
Greater separation efficiencies were observed when higher selector concentrations were empolyed (24). The impact of HS-αCD concentration on efficiency in terms of the number of theoretical plates is demonstrated in figure 5. In non-carrier mode (<0.1%w/v HS-αCD) increasing the selector concentration initially increased efficiency. However, after reaching a maximum at 0.04%w/v HS-αCD, due to the decrease in mobility of the amlodipine/ HS-αCD complex, efficiency decreased (Figure 3) and consequently greater longitudinal diffusion occured. In carrier mode (>0.1%w/v HS-αCD in Figure 5), increasing the HS-αCD concentration, consistent with the literature (24) gave better efficiency. However, the correlation between HS-αCD concentration and efficiency is not without limits. Increasing the selector concentration above 1.25% resulted in Joule heating due to the high ionic strength of the buffer. A similar biphasic relationship between selector concentration and resolution of the enantiomers was observed (data not shown).

Effect of sodium phosphate buffer concentration
Figure 6 shows the effect of the concentration of the pH 2.5 sodium phosphate buffer on the electrophoretic mobility of amlodipine. A significant increase in migration time and decrease in enantioselectivity was observed upon increasing the sodium phosphate concentration. The prominent role of ion exchange in the binding of cationic opiate alkaloids by HS-CD has been demonstrated (25). The basic equilibrium that proposed was:

$$B^+ + Na^+HS\text{-}CD \rightleftharpoons Na^+ + B^+HS\text{-}CD \qquad (2)$$

where B^+ represents the cationic (basic) drug and the sodium ion can either be in the complexed form (Na^+HS-CD) or free in solution (Na^+). It has been shown that approximately half of the sulfate groups on HS-CD are engaged in the ion exchange equilibrium(25). This is consistent with the observation that only about half of the sulfate

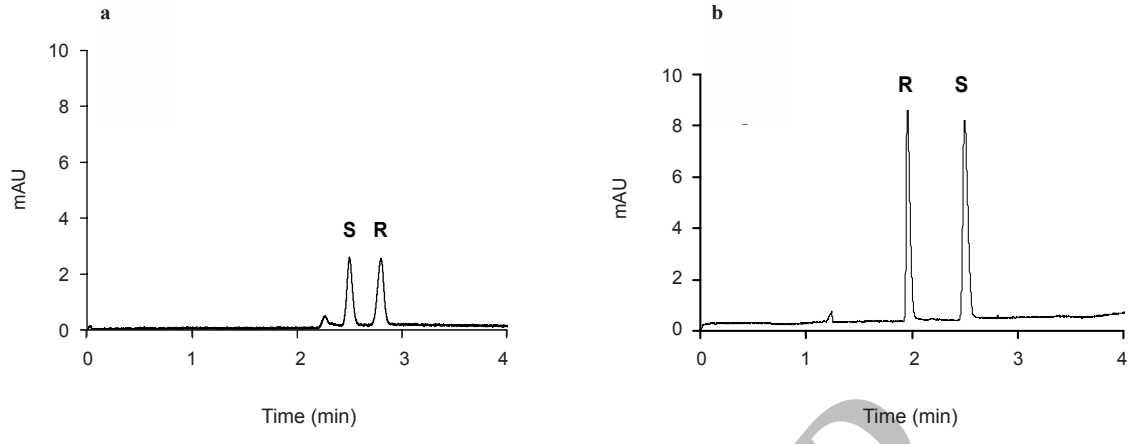

Figure 4. Enantioseparation of amlodipine in: a) non-carrier mode; and b) carrier mode.
Non-carrier mode (a) conditions: buffer, 25 mM sodium phosphate (pH 2.5) containing 0.05% HS-αCD; voltage, -15 kV; sample, 100 ppm amlodipine. Carrier mode (b) conditions: 25 mM sodium phosphate (pH 2.5) containing 1.25% HS-αCD; voltage, +15 kV; sample, 100 ppm amlodipine

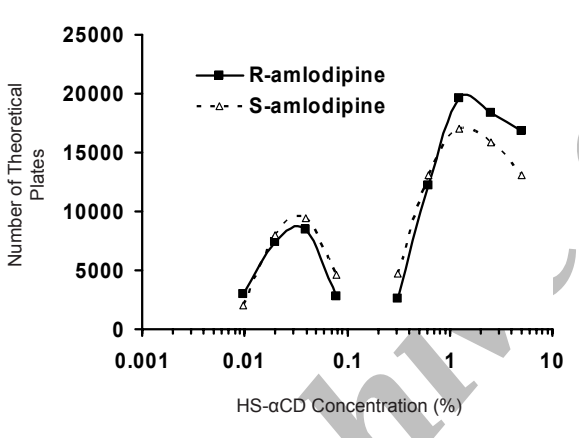

Figure 5. Effect of HS-αCD concentration on the efficiency of the separation of the enantiomers of amlodipine. Experimental conditions: as in Figure 3

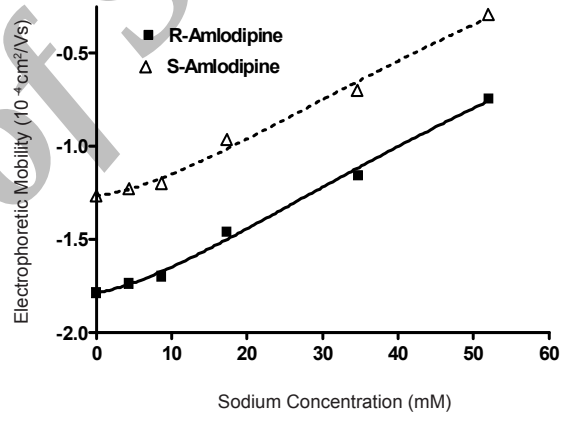

Figure 6. Effect of sodium concentration on mobility of the enantiomers. The curves are the fit of the data to the ion binding model (equation 3).
Experimental conditions: buffer, sodium phosphate (pH 2.5) containing 1.25% HS-αCD; voltage, +15 kV; sample 100 ppm amlodipine; EOF marker, acetone 5%.

groups on HS-CDs are free due to counter-ion condensation (19). The curves in Figure 6 are the fit of the data (n=6) for each enantiomer to the Zakaria et al. model (equation 3).
where $W_{\%}$ is the mass percent of the HS-CD, Q is the ion-exchange capacity of the selector, K_{ie} is the equilibrium binding constant for equilibrium 2, μ_{obs} is the observed mobility, μ_{bge} and μ_{cd} are the mobilities of the free analyte in the background

$$\mu_{obs} = \frac{1}{1+W_{\%} K_{ie} Q [Na^+]^{-X}} \times \mu_{bge} + \frac{W_{\%} K_{ie} Q[Na^+]^{-X}}{1+W_{\%} K_{ie} Q[Na^+]^{-X}} \times \mu_{cd} \quad (3)$$

electrolyte and analyte bound to the selector, respectively and X is the displacement ratio of Na ions by the analytes. $K_{ie} \times Q$ is treated as one parameter for fitting.
The determination coefficients (R^2) for fittings equation 3 to the behavior in figure 6 are 0.995 for R-amlodipine and 0.992 for S-amlodipine. The estimates for displacement (X) of Na ions by the amlodipine were statistically equivalent for the R and S-enantiomer data (1.43± 0.14) for the R-enantiomer and 1.52±0.19 for the S-enantiomer data set) which suggest that the displacement is not a 1:1 process as assumed in equilibrium 2. Similar results were reported by Zakaria in separation of opiate alkaloids by sulfated cyclodextrin (47).
From equilibrium 2 and the fitting of figure 6, it may be concluded that increasing the concentration of sodium ion decreases the binding between the drug and HS-CD. Under the experimental

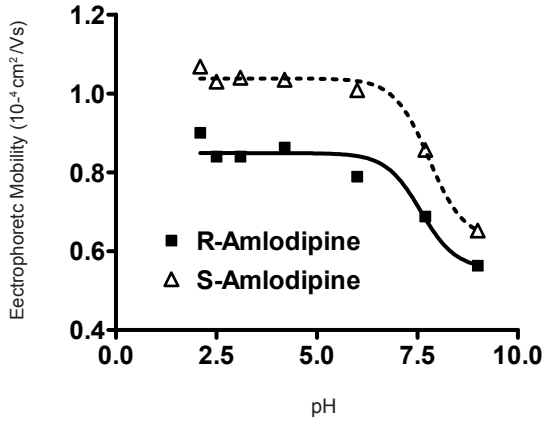

Figure 7. Effect of pH on mobility of amlodipine enantiomers. The curves are the fit of the data to the Sigmoidal dose-response equation. R^2 0.93 for R-amlodipine and 0.989 for R-amlodipine data-set; n=7 for each data-set. Experimental conditions: buffer, 25 mM sodium phosphate containing 0.05% HS-αCD; voltage, -15 kV; sample, 100 ppm amlodipine; EOF marker, acetone 5%.

conditions used for construction of figure 6, the separation is being performed in the carrier mode. Thus decrease in binding between the drug and HS-CD results in an increase in the observed electrophoretic mobility.

The efficiency increased with the concentration of the buffer up to 25 mM where 19500 and 17000 plates were observed for the R- and S-enantiomers of amlodipine, respectively. However, at higher buffer concentrations, the efficiencies decreased due to Joule heating. Typically, efficiencies for chiral separations by using HS-CDs are lower when short-end injection is used rather than a traditional longer capillary length to detector (26). The efficiencies which were obtained in this study are comparable to those achieved in chiral resolution of other drugs such as cetirizine with the sulfated cyclodextrins (24).

Effect of the running buffer pH

Buffer pH is one of the most important factors in CE as it may affect the charge of the analytes and the chiral selector, and thus the binding characteristics (27). The sulfated groups in HS-CDs are ionized over the entire pH range available in CE. Thus, the pH does not markedly affect the self-mobility of the chiral selector (20). However, amlodipine has a pKa of 8.6 (28) and so its charge state will vary dramatically with pH. Figure 7 shows the effect of pH on the electrophoretic mobility of amlodipine in the presence of 0.05% HS-αCD. Under acidic conditions (pH<6) amlodipine has a positive charge, and as a result its electrostatic interaction with the selector is predominant. As the pH becomes more alkaline than 6.0, the effective mobility decreases since amlodipine starts to deprotonate. More importantly with respect to enantioseparations, the difference between the mobilities of enantiomers, and consequently their resolution, decreases with pH over 6.0. Zhao et al. (29) observed similar loss in resolution with pH for synthetic phenylalanine products. The deprotonation of amlodipine decreases its electrostatic interaction with the selector (equilibrium 2) which causes a decrease in resolution (Figure 7). Also, in higher pH the EOF is stronger, allowing shorter time for enantioselective interactions between amlodipine and selector.

Carrier mode versus non-carrier mode

Separation of the enantiomers can be achieved in non-carrier mode, i.e., with low concentrations of the selector, but as illustrated in Figures 3 and 5 the resolution and efficiency are inferior to those achieved in carrier mode. For further comparison, the performance of the method was studied using the optimal HS-αCD concentrations for non-carrier (0.05%) and carrier (1.25%) modes determined previously.

The limit of detection (LOD) and quantification (LOQ) were estimated as three and ten times of the signal-to-noise ratio, respectively. The LOD of amlodipine enantiomers were 2 ppm in carrier mode and 7 ppm in non-carrier mode. The LOQ of amlodipine enantiomers were 5 ppm in carrier mode and 20 ppm in non-carrier mode. Table 1 shows the relative standard deviations (RSD) observed for migration times, efficiencies, and corrected peak area (peak area/migration time) for non-carrier mode analysis, both within day (n=10) and between days (n=6 days). Table 2 presents the same data for carrier mode separations.

Non-carrier mode displayed poor within-day and between-day repeatability, which results in poorer efficiencies (Figure 5) and poor LOD and LOQ. In the carrier mode, amlodipine is fully complexed and minor day-to-day variations in experimental conditions do not significantly affect the degree of complexation or consequent mobility. In non-carrier mode, however, amlodipine is only partially complexed and variation in experimental conditions can significantly affect the mobility. Therefore, determinations are better performed in carrier-mode rather than non-carrier mode.

CONCLUSION

This paper demonstrates the potential of HS-CDs for the chiral separation of a model chiral basic drug, amlodipine, using short-injection method in a capillary dynamically coated with PEO to suppress the EOF. Resolution was greatly affected by the type and concentration of the chiral selector, buffer ionic strength and pH. Among the three investigated HS-CDs, HS-αCD was the most effective for the enantioseparation of amlodipine. The optimum separation was achieved using a pH 2.5 running buffer containing 25 mM sodium phosphate containing 1.25% HS-αCD, and an applied voltage of +15 kV. Under these conditions,

Table 1. Performance of the method in non-carrier mode.

	Migration time (min)		Number of theoretical plates		Peak area /Migration time	
	R	S	R	S	R	S
Within-day repeatability [a]	2.7 (5.3) [c]	2.5 (4.5)	6000 (8.4)	6500 (8.8)	4.52 (25.2)	4.63 (28.6)
Between-day repeatability [b]	2.8 (10.7)	2.7 (8.9)	6500 (10.7)	6500 (12.3)	5.11 (33.3)	5.72 (36.5)

Experimental conditions: as in Figure 4a.
[a] Within-day repeatability: results from 10 experiments performed on the same day
[b] Between-day repeatability: results from 6 experiments performed on 6 days
[c] In parentheses %RSD

Table 2. Performance of the method in carrier mode.

	Migration time (min)		Number of theoretical plates		Peak area /Migration time	
	R	S	R	S	R	S
Within-day repeatability [a]	2.0 (0.30) [c]	2.6 (0.36)	20000 (2.3)	17500 (3.1)	10.85 (1.9)	10.62 (2.1)
Between-day repeatability [b]	1.9 (2.89)	2.6 (3.81)	19500 (6.9)	17500 (8.0)	10.46 (3.1)	10.15 (3.8)

Experimental conditions: as in Figure 4b.
[a], [b] and [c] as in Table 1.

enantioseparation was achieved in less than 3 min, with minimum variation in quantitative analysis. These optimized conditions are appropriate for the enantioselective analysis of amlodipine.

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