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Use of ^{1}H-NMR spectroscopy to determine the enantioselective mechanism of neutral and anionic cyclodextrins in capillary electrophoresis[1]

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Abstract

One-dimensional (1D) and two-dimensional (2D) ^{1}H nuclear magnetic resonance (NMR) techniques have been used to investigate the chiral recognition process in capillary electrophoresis (CE) for seven different cyclodextrins (CDs) with the calcium channel blocker amlodipine as a model compound. These include five neutral CDs (α-CD, β-CD, γ-CD, hydroxypropyl-β-CD and hydroxyethyl-β-CD) and two anionic CDs (sulphobutyl-ether-β-CD and carboxymethyl-β-CD) where mixtures of amlodipine with each of the seven CDs were examined by 1D NMR in deuterated phosphate buffer at pD 3.4. The resonance shift of signals with added CD, relative to the CD-free position (shift displacement, $\Delta\delta$) and shift non-equivalence ($\Delta\delta$*) of enantiomeric signals shifted relative to each other after addition of CD were examined for non-overlapped protons of amlodipine. The possible correlations of NMR shift non-equivalence data with chiral separation in CE for amlodipine have been critically assessed. Qualitative differences in the 1D NMR shifts and enhanced enantioselectivity in CE were observed for amlodipine with sulphobutyl-ether-β-CD. Further experiments on the through-space interactions using 2D rotating frame nuclear Overhauser effect spectroscopy (ROESY) indicated that there was no association between internal glucopyranose hydrogen atoms and the aromatic hydrogens of amlodipine. This gives evidence for the aromatic ring not being included in this CD. Moreover, data from spin–lattice relaxation times (T_1) measured for amlodipine in the free state and after addition of the anionic sulphobutyl-ether-β-CD indicate that the aromatic moiety of amlodipine is not included into the sulphobutyl-ether-β-CD cavity. There is evidence that it interacts with the sulphobutyl side chains, and may adopt a preferred orientation outside the sulphobutyl-ether-β-CD toroid itself. © 1997 Elsevier Science B.V.

Keywords: Amlodipine; Capillary electrophoresis; Chiral; Cyclodextrin; Nuclear magnetic resonance; Rotating frame overhauser effect spectroscopy; Spin-lattice relaxation times

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1. Introduction

Liquid chromatography (LC) has been widely used with cyclodextrins (CDs) as chiral mobile phase additives (CMPAs) and chiral stationary phases (CSPs) for the resolution of enantiomers [1,2]. However, the high efficiency, versatility, easy handling, automation and low costs of capillary electrophoresis (CE) has lead to its increasing use as the technique of choice for the resolution of enantiomers and their impurities [3–5]. The majority of chiral separations in CE are achieved by addition of a chiral selector to the running buffer. These include chiral metal complexes [6], crown ethers [7], antibiotics [8], chiral surfactants [9] chiral mixed micelles [10], but in most cases a CD [3].

CDs are toroidal in shape containing six (α-), seven (β-), or eight (γ-) D(+)-glucopyranose units bonded through α-(1,4) linkages. The interior of the CD cavity is relatively hydrophobic, which allows chiral separations to be developed through formation of enantioselective interactions. These may involve the formation of inclusion complexes with guest analytes, aided by hydrogen bonding at the larger annulus of the CD cavity.

Native CDs (α-, β- and γ-) have been successfully used as bonded CSPs in LC [2], or as MPAs in LC [1] and CE [11]. However, a range of derivatised CDs with a neutral derivative randomly bonded to the primary or secondary hydroxyl positions with a defined degree of substitution (DS) has shown enhanced degrees of enantioselectivity, both as bonded CSPs in LC [12] or as additives in CE [13]. These neutral derivatised CDs are thought to provide not only extra sites for hydrogen bonding at the larger annulus of the CD cavity, but additional steric interactions as well.

Recently, the chargeable anionic carboxymethyl-β-cyclodextrin (CM-β-CD) was first used in CE not as a chiral selector but as a pseudo-stationary phase, comparable with a surfactant, for the separation of neutral aromatic isomers [14]. CM-β-CD was used later as a chiral selector in CE [15], allowing the reversal of migration order of enantiomers compared with that for the neutral CDs, permitting the accurate quantitation of both enantiomers, where otherwise poor separation is achieved [16]. CM-β-CD has also been successfully used as a MPA in LC for the resolution of doxazosin enantiomers [17]. Stability constants calculated for doxazosin and CM-β-CD based on those of Sybilska et al. [18] were shown to be much larger than those calculated by that group for a range of barbiturates with the native β-CD. Studies on the ionic nature of doxazosin and CM-β-CD based on systematic variation in pH indicated that the ionic nature of both selector and selectand played a crucial role in enantioseparation [17].

Another CD bearing a net charge, sulphobutylether-β-cyclodextrin (SBE-β-CD) has recently been characterised [19] and widely studied [20]. This CD offers greater resolution using lower concentrations with reduced retention times in LC [21] and lower migration times in CE [20]. Exploitation of the net charge and thus the electrophoretic mobility of SBE-β-CD has resulted in its use for reversing the migration order of enantiomers. It has also being used in combination with neutral CDs, for the separation of enantiomers and geometrical isomers in CE [22].

The use of one-dimensional (1D) ^{1}H nuclear magnetic resonance (NMR) is now well established as a powerful tool for investigating the through-space interactions both inside and outside a CD cavity between a chiral analyte and a CD [23–25]. Examination of the chemical shifts observed for both the analyte and the CD on mixing have proved useful for investigating the stoichiometry, stability and structure of cyclodextrin complexes [26,27]. One-dimensional nuclear Overhauser effect (NOE) experiments have been used to demonstrate spatial proximity of cyclodextrin and substrate hydrogens within a complex [28]. Two-dimensional (2D) nuclear Overhauser effect spectroscopy (NOESY) [29] and rotating frame nuclear Overhauser effect spectroscopy (ROESY) experiments, which give positive and enhanced NOEs over the whole molecular weight range, have been found particularly useful for species such as analyte–CD complexes [30,31]. ROESY has been applied previously to a range of neutral CDs as a probe

for selecting an appropriate CD for the chiral separation of amlodipine in CE [32].

The work of Behr and Lehn [33] pioneered the use of ^{13}C spin–lattice relaxation time (T_1) measurements for examining the dynamic properties between 2-methylcinnamate, 3-methylcinnamate and 3-*tert*-butylphenate with α-CD. Suzuki et al. have subsequently used these ^{13}C data to estimate the portion of an azo dye that interacted with a host CD molecule [34–36]. Recently, ^1H T_1 measurements calculated for naringin aromatic hydrogens have shown a 10–25% reduction after complexation with β-CD [37]. Similar studies on methylene hydrogens for three nitramine explosives have shown a 5–40% reduction in T_1 values on complexation with the native α-, β- and γ-CDs [38]. A 30% reduction in the average T_1 value of *N*-acetyl-L-tryptophan was observed after addition of deuterated β-CD [39]. Irwin et al. have measured the T_1 values for the glycosidic hydrogens of β-CD and have shown an approximately 40% reduction in value on complexation with chlorogenic acid [40].

In the present work, 1D ^1H-NMR is used as a probe to investigate any interactions that occur between racemic amlodipine and seven CDs. These included the native α-, β- and γ-CDs, the neutral derivatised hydroxypropyl-β-cyclodextrin (HP-β-CD) and hydroxyethyl-β-cyclodextrin (HE-β-CD), the anionic SBE-β-CD and the anionic CM-β-CD. These seven CDs were subsequently investigated as chiral selectors added to the background electrolyte in CE for the separation of amlodipine enantiomers. Further investigations were carried out with these data to determine if a correlation could be drawn between chemical shifts observed in the 1D ^1H-NMR spectrum of amlodipine after addition of a CD, and enantioseparation in CE. The 1D ^1H-NMR data indicated that different interactions were occurring between racemic amlodipine with the neutral CDs than with the anionic SBE-β-CD. A comprehensive series of 2D ROESY experiments recently carried out [32] with racemic amlodipine and five neutral CDs has shown that inclusion of the aromatic moiety probably occurs in the cavity of the neutral HP-β-CD. Similar 2D ROESY experiments were therefore carried out in the present work to determine if analogous interactions occurred for racemic amlodipine with the anionic SBE-β-CD. Spin–lattice relaxation times T_1 were also measured for amlodipine in the free state and when complexed with the anionic SBE-β-CD at different mole ratios to evaluate and support the ROESY experiments.

2. Experimental

2.1. Chemicals and reagents

Racemic amlodipine maleate (Fig. 1) and SBE-β-CD sodium salt, DS = 6.3 (Fig. 2), were used as received from Pfizer Central Research (Kent, UK). α-CD, γ-CD and CM-β-CD sodium salt (DS = 5.3) (Fig. 2) were used as received from Wacker Chemicals (Walton-on-Thames, Surrey, UK). β-CD, HP-β-CD (MS = 4.2) and HE-β-CD (DS = 11.2) were used as received from Aldrich (Gillingham, Dorset, UK). Deuterium oxide, 99.8%, was obtained from Isotec, Matheson, USA. Sodium dihydrogen orthophosphate dihydrate and sodium dihydrogen orthophosphate anhydrous were purchased from BDH (Poole, Dorset, UK).

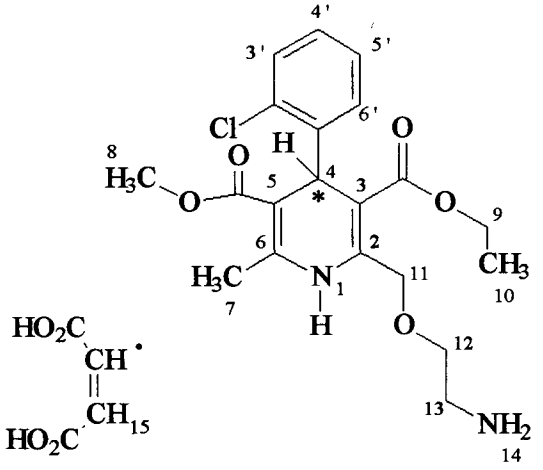
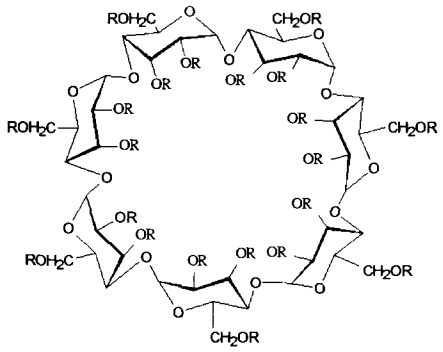


Fig. 1. Structure of amlodipine maleate.



(a) R = H or $-CH_2-CH_2-CH_2-CH_2SO_3^- Na^+$
a b c d

(b) R = H or $-CH_2COO^- Na^+$

Fig. 2. Structure of (a) SBE-β-CD and (b) CM-β-CD.

2.2. 1D NMR spectroscopy

2.2.1. Equipment

The 1D NMR spectra were obtained on a Unity INOVA-400 NMR spectrometer operating at 399.96 MHz for ^1H. Thirty-two scans were acquired for each sample with a sweep width of 8000 Hz and a pulse width of 30.2°. The temperature was controlled at 30 ± 0.1°C. All 1D resonance spectra were referenced to the internal HOD signal at 4.66 parts per million (ppm).

2.2.2. Methods

Amlodipine, 0.005 M, and amlodipine:CD mixtures (1:2 ratio; molar ratios are used throughout) were prepared in 100 mM NaH_2PO_4 deuterium oxide buffer, pD = 3.4 ± 0.1, adjusted with deuterium chloride or deutrated sodium hydroxide as appropriate. Listed pD values were obtained by adding 0.4 to the pH meter reading, in accordance with the work of Glasoe and Long [41]. CM-β-CD has a calculated pK_a of 4.36 and may have a different ionic state at pD 3.4 than at higher pH values. It was thus decided to acquire spectra of amlodipine and the amlodipine:CM-β-CD mixture (1:2 ratio) both at pD 3.4 and 5.4 to determine if a possible different ionic state of this CD induced significantly different chemical shifts. A number of spectra were acquired ($n = 10$) during a 12 h period for two mixtures, amlodipine:CM-β-CD and amlodipine:α-CD in a 1:2 ratio in order to determine the statistical reliability of the shift data. This was to assess the significance of any small shifts observed and also to evaluate the stability of the sample over that time.

2.3. Capillary electrophoresis

2.3.1. Equipment

The Beckman P/ACE 5510 (Fullerton, CA, USA) equipped with a UV detector operated at 214 nm was used. The electrophoretic experiments were performed in an uncoated fused-silica capillary 57 cm × 50 μm i.d. (50 cm effective length) obtained from Beckman Instruments (High Wycombe, Bukinghamshire, UK). The temperature was maintained constant throughout at 17°C.

2.3.2. Methods

The capillary was conditioned initially for 1 h with 1 M NaOH and 20 min with water. Sodium dihydrogen phosphate dihydrate adjusted to the appropriate pH with orthophosphoric acid and sodium hydroxide as appropriate was used as electrolyte. CDs were prepared in this electrolyte as required for the final run buffer. The capillary was washed for 1 min with 0.1 M NaOH and 3 min with run buffer prior to each run. Amlodipine (≈ 100 μg ml^{-1}) was prepared each day in deionised water from a 1 mg ml^{-1} stock prepared in electrolyte. The dissolution of samples in 10% buffer is known to favour sample stacking in CE. All samples and buffers were sonicated and filtered through a 0.45 μm filter (Anachem, Luton, Bedfordshire, UK). It was also decided to investigate the use of CM-β-CD at both pH 3.0 and 5.0 to determine if a potential different ionic state of this CD resulted in increased or decreased enantioselectivity when used as a chiral selector in CE.

2.4. 2D ROESY spectroscopy and spin–lattice relaxation times

2.4.1. Equipment

The Varian Unity 500 NMR spectrometer equipped with a 5 mm probe was used for ^1H-

NMR observation in D_2O solution. 2D ROESY spectra were obtained using a sweep window of 4898 Hz, acquisition time was 0.209 s, relaxation delay was 2 s, spin lock mixing time was 1 s and amplitude of spin lock was 4735 Hz. Offset compensation was used to eliminate the dependency of the amplitude of rotating frame nuclear Overhauser effect (ROE) cross peaks on the transmitter frequency offset. States–Haberkorn phase cycling with 2048 data points in F2 and 1024 data points in F1 was used to acquire the data, which were processed using linear prediction in F1 and Gaussian apodisation in both dimensions. The inversion recovery technique was used to measure the spin–lattice relaxation times with relaxation delay of 20 s and acquisition time of 3.27 s. The interval between the 180° and 90° pulses was varied in the range 0.1–20 s. The data were processed using the Varian VNMR software. No correction was made for viscosity effects.

2.4.2. Methods

Amlodipine, 0.005 M, and amlodipine:SBE-β-CD mixtures were prepared as described in Section 2.2.2. 2D ROESY spectra were acquired for a mixture of amlodipine:SBE-β-CD in a ratio of 1:1. Spin–lattice relaxation times were measured for amlodipine hydrogens and compared to the same hydrogens in 1:1, 1:2 and 1:3 mixtures of amlodipine:SBE-β-CD.

3. Results and discussion

3.1. 1D NMR spectroscopy

Two types of shift in the resonance signal can be observed after complexation of a chiral solute with a CD: (a) the displacement (up- or down-field) of a singlet, or of a multiplet, defined as a 'shift displacement' ($\Delta\delta$ ppm); and (b) the enantiomeric splitting of a singlet, or of a multiplet, defined as a 'shift non-equivalence' ($\Delta\delta^*$ ppm) [42]. In principle, each type of shift could be observed for a particular resonance either singly, or in combination.

A spectrum was acquired initially for racemic amlodipine maleate in the absence of CD at pD 3.4 ± 0.1 shown in Fig. 3. The assignments of multiplets are shown in Table 1 and are in agreement with those found earlier [32]. Spectra for amlodipine:CD mixtures in a 1:2 ratio were then acquired under identical conditions for each of the seven CDs.

The ^1H chemical shift range of CDs is narrow (3.5–5.1 ppm) and consequently the multiplets do not overlap the aromatic, aliphatic or higher-field alkyl proton regions of amlodipine. It was thus possible to investigate the following non-overlapped signals: the aromatic hydrogens (7.12–7.41 ppm), the chiral proton (5.27 ppm), and two methyl proton systems (2.22 and 1.09 ppm) of amlodipine in detail. It has been shown previously that the addition of a racemate to β-CD led to upfield shifts of CD cavity proton signals, indicating that these hydrogens were shielded by the inclusion of aromatic groups [43]. However, in the 1D NMR spectra, overlap of the complex multiplets from both amlodipine and CD in the 3.5–5.1 ppm region did not allow a detailed study of these particular glucopyranose cavity hydrogens on addition of racemic amlodipine.

3.1.1. Shift displacement ($\Delta\delta$ ppm)

Shift displacement ($\Delta\delta$ ppm) values observed for each of the non-overlapped amlodipine proton systems outlined above, after the addition of each of the seven CDs with respect to the same uncomplexed signals, are shown in Table 2. This is also illustrated in Figs. 4 and 5 for 10-CH_3 and 7-CH_3 respectively. These data show evidence of interaction of amlodipine with each of the seven CDs, with a substantial shift displacement in the multiplets either up- or down-field. The magnitude of these shift displacements on addition of both the neutral and anionic CDs are of the same order as each other, and are similar to those reported for other CD systems with the exception of γ-CD. Shift displacement of the aromatic hydrogens on addition of γ-CD are an order of magnitude larger than the others and are all up-field. This may indicate that the aromatic moiety of amlodipine is included deeper into the larger γ-CD hydrophobic cavity (9.5 Å), than for either α-CD

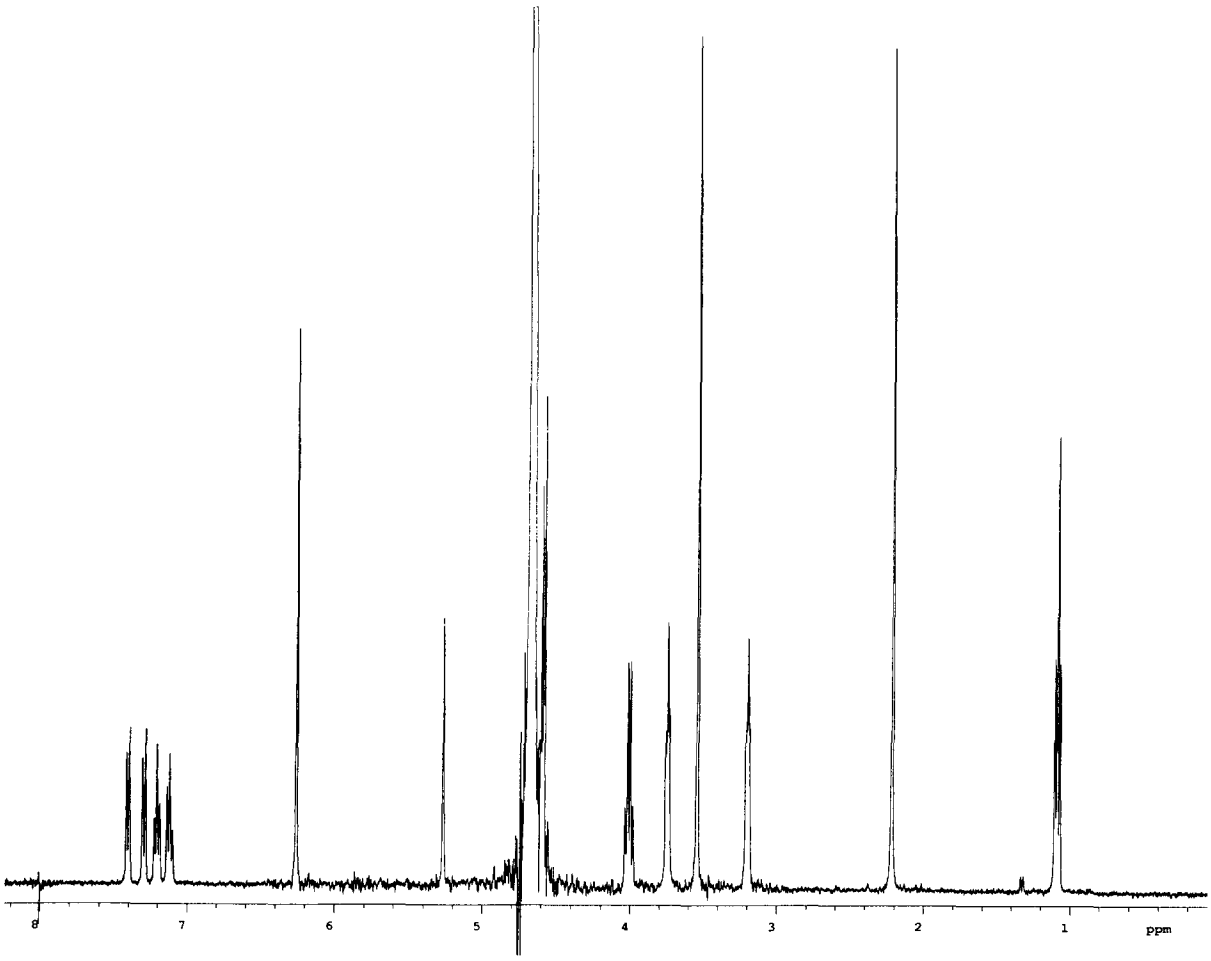


Fig. 3. The 400 MHz NMR spectrum of amlodipine maleate.

(5.6 Å) or β-CD (7.8 Å), considering that all other experimental conditions are fixed. This phenomenon is not consistent with theories previously reported for the γ-CD complexes with orange II, where an increase in the diameter of the CD cavity led to smaller high-field shifts than those observed for the corresponding β-CD complex [44]. The anionic SBE-β-CD was the only cyclodextrin to produce a down-field shift displacement for all four amlodipine aromatic hydrogens studied, indicating that there may be a difference in the mode of interaction between amlodipine and the neutral CDs, compared with that for this anionic CDs. It is important to note that, for amlodipine ($pK_a = 9.02$), a considerable reduction in shift displacement was observed for the aromatic signals alone on changing the ionic state of CM-β-CD ($pK_a = 4.36$) from pD 3.4 ($\approx$ 10% CD ionised) to pD 5.4 ($\approx$ 90% CD ionised). From these observations, it is reasonable to postulate that the aromatic moiety of amlodipine is included into the CM-β-CD cavity. This considerable change in shift displacement was not observed for the aliphatic hydrogens studied.

3.1.2. *Shift non-equivalence ($\Delta\delta$* ppm)*

Shift non-equivalence ($\Delta\delta$* ppm) values observed for each of the non-overlapped amlodipine

Table 1
^{1}H assignments of racemic amlodipine maleate

Chemical shift (ppm)	Multiplicity	Proton assignment
1.09	Triplet	10
2.22	Singlet	7
3.2	Triplet	13
3.54	Singlet	8
3.74	Triplet	12
4.01	Quartet	9
5.27	Singlet	4
6.28	Singlet	Maleate
7.12	Triplet	5′
7.20	Triplet	4′
7.30	Doublet	3′
7.40	Doublet	6′

proton systems outlined above, after the addition of the seven CDs with respect to the same uncomplexed signals, are shown in Table 3. This is illustrated again in Figs. 4 and 5 for 10-CH_3 and 7-CH_3 respectively. Any shift non-equivalence ($\Delta\delta$*) observed for a particular signal was measured as the difference in the resonance position of each enantiomer. The separated signals have not yet been attributed to their respective enantiomers. No shift non-equivalence was observed for any hydrogens studied, either aliphatic or aromatic, on addition of β-CD. The methyl hydrogens of the ethyl ester side chain (H10) were the only signals which displayed shift non-equivalence with α-CD. Shift non-equivalence was observed, however, on addition of γ-CD for two methyl hydrogens systems (H7 and H10) and for all of the aromatic hydrogens of amlodipine.

The nature of the derivative side chain attached to the β-CD exerts a significant influence on the type and extent of shift non-equivalence for the aromatic hydrogens in amlodipine. This is specifically observed for SBE-β-CD and HE-β-CD where shift non-equivalence of aromatic hydrogens in amlodipine is observed for two and one hydrogen, respectively. For these CDs, no detectable shift non-equivalence was observed for the other aromatic hydrogens studied (Table 3) with the exception of H4′, where multiplet overlap interfered with the measurement of a small shift non-equivalence. For CM-β-CD, a similar situation was observed due to complex overlapping multiplets both at pD 3.4 and 5.4, namely that it was not possible to measure quantitatively the significant shift non-equivalence that was detected for all aromatic hydrogens. For HP-β-CD it was clear that there was no shift non-equivalence for the H6′ or H3′ hydrogens and the shift non-equivalence observed for H4′ and H5′ hydrogens could not be quantified because overlapping multiplets were too complex.

The influence of the derivative side chain attached to the β-CD can also be clearly seen on examination of the shift non-equivalence data for the amlodipine aliphatic hydrogens. Multiplets due to the hydroxypropyl side chains of HP-β-CD and the 10-CH_3 group of amlodipine overlapped in the region 1.07–1.11 ppm.

Table 2
Shift displacements ($\Delta\delta$, ppm) observed for racemic amlodipine on complexation with cyclodextrins

CD additive	H3′	H4′	H5′	H6′	H4	H7	H10
α-CD	0.015 U	0.007 D	0.007 U	0.058 D	0.021 D	0.007 D	0.055 D[a]
β-CD	0.059 U	0.055 U	0.024 U	0.049 U	0.094 D	0.045 D	0.059 D
γ-CD	0.210 U	0.402 U	0.615 U	0.425 U	0.096 D	0.090 D	0.055 D
HP-β-CD	0.041 U	0.030 U	0.010 U	0.047 U	0.060 D	0.029 D	Obstructed
HE-β-CD	0.021 U	0.013 U	0.011 D	0.015 U	0.038 D	0.013 D	0.004 U
SBE-β-CD	0.020 D	0.058 D	0.069 D	0.039 D	0.056 D	0.061 D	0.011 U
CM-β-CD	0.034 U	0.090 U	0.0150 D	0.053 U	0.092 D	0.042 D	0.029 D
CM-β-CD (pD 5.4)	0.011 U	0.031 U	0.014 D	0.020 U	0.085 D	0.046 D	0.039 D[b]

D, Down-field; U, up-field. The actual chemical shift can be obtained by adding or subtracting these shift displacement values to or from the chemical shift of uncomplexed amlodipine in Table 1.
[a] 0.055 ± 0.0007, R.S.D. = 1.24% ($n = 10$).
[b] 0.039 ± 0.0011, R.S.D. = 2.9% ($n = 10$).

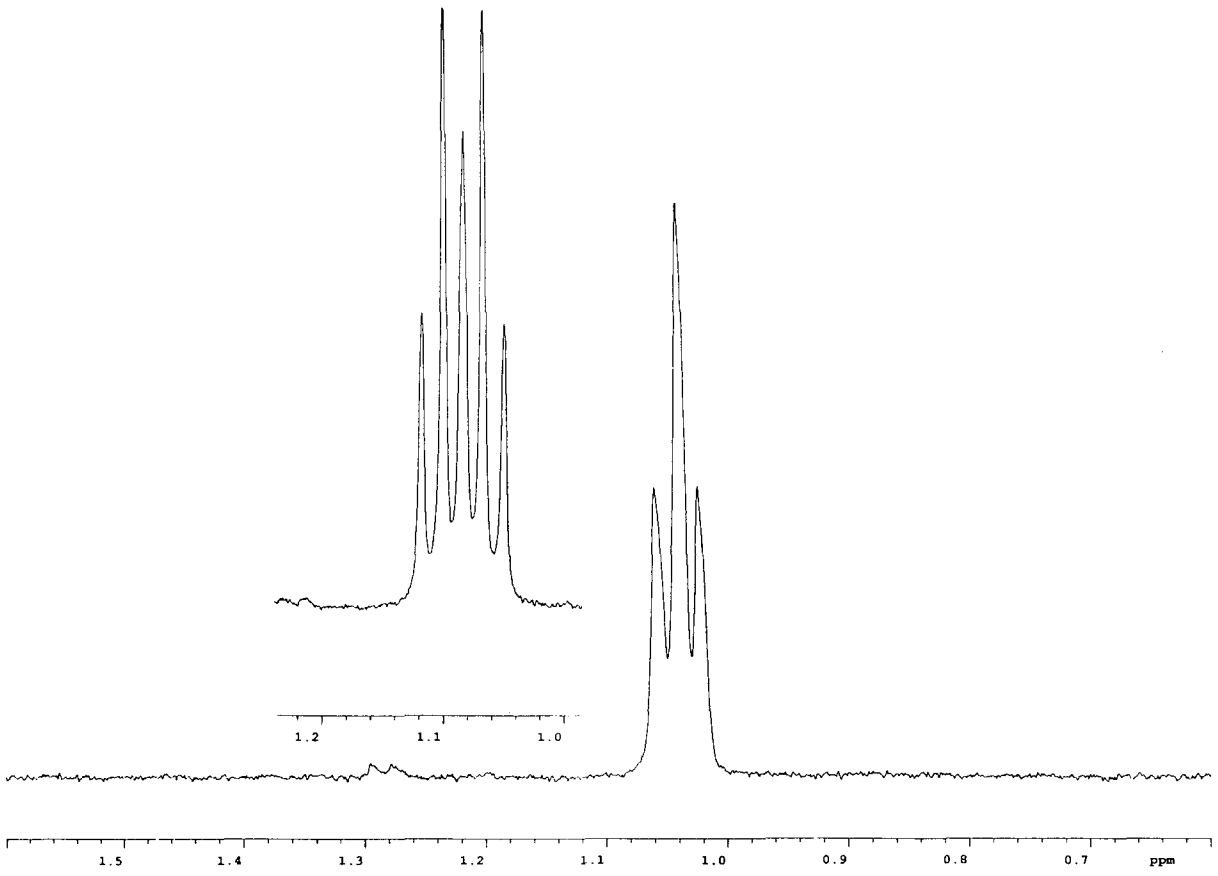


Fig. 4. The 400 MHz NMR spectrum (0.6–1.6 ppm) indicating the 10-CH_3 triplet of amlodipine before mixing with CM-β-CD in a 1:2 ratio, and (insert) the down-field shift displacement (coupled with shift non-equivalence) after mixing.

Consequently, no quantitative or qualitative data for these hydrogens could be obtained for this CD. Shift non-equivalence was observed for the methyl hydrogens of the ethyl ester side chain, (10-CH_3) on addition of HE-β-CD. Shift non-equivalence was also observed for a different methyl group on addition of SBE-β-CD, the methyl attached to the 6-position of the 1,4-dihydropyridine ring (7-CH_3). This significant spatial difference in interaction between a neutral and the anionic CD may be another indication of different mode (or modes) of interaction between these CDs with amlodipine. Shift non-equivalence was observed for all aliphatic hydrogen groups on addition of CM-β-CD both at pD 3.4 and 5.4. CM-β-CD was the only cyclodextrin to exhibit shift non-equivalence for all the hydrogens of amlodipine studied.

Since the above data represent amlodipine:CD mixtures in a 1:2 molar ratio, spectra for amlodipine:CD mixtures were also acquired in a 1:1 molar ratio under identical conditions for each of the seven CDs. The observed shift non-equivalence values were considerably lower than those for the 1:2 ratio mixtures. Moreover, increasing the amount of CD to give molar ratios greater than 1:2 would be expected to yield a further increase in the shift non-equivalence values.

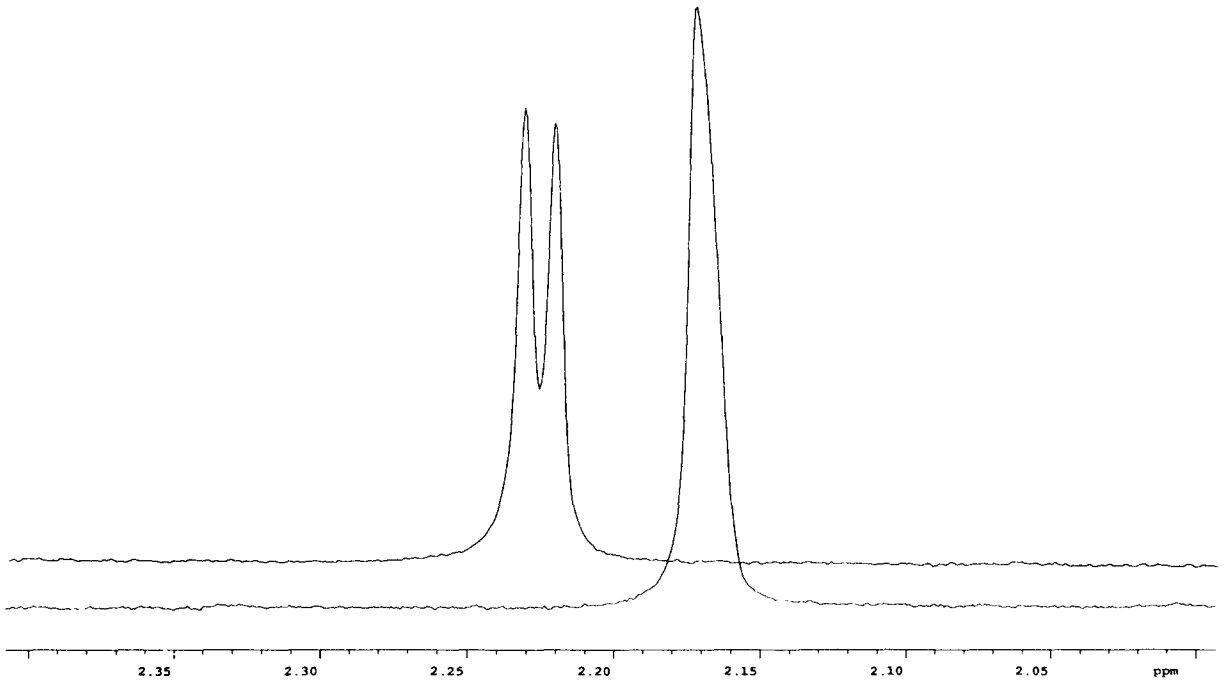


Fig. 5. The 400 MHz NMR spectrum (2.0–2.4 ppm) indicating the 7-CH_3 singlet of amlodipine before mixing with CM-β-CD in a 1:2 ratio and (overlaid) the down-field shift displacement (coupled with shift non-equivalence) after mixing.

3.2. Capillary electrophoresis

The separation index used is the Kaiser peak separation index, P_i, which is defined as the average valley depth expressed as a ratio to the average peak height of the two enantiomeric peaks [45].

3.2.1. Neutral cyclodextrins

Racemic amlodipine maleate was run initially using 20 mM NaH_2PO_4 dihydrate electrolyte, pH 3.0, without any CD and finally with 20 mM of each of the five neutral CDs added to the electrolyte. The migration times and separation index values obtained for amlodipine enantiomers on addition of these CDs to the electrolyte are shown in Table 4. This represents a direct comparison of the properties of each neutral CD for the separation of amlodipine enantiomers, where the electrophoretic conditions selected were identical, using typical values for this chiral base. These conditions could have been optimised further; nevertheless, HP-β-CD and HE-β-CD were shown to be significantly more enantioselective (both $P_i = 1.0$) than the corresponding native CDs. It is interesting to note that β-CD alone displays no enantioselectivity compared with HP-β-CD and HE-β-CD.

3.2.2. Anionic cyclodextrins

The addition of the anionic SBE-β-CD and CM-β-CD to the electrolyte altered the ionic strength of the run buffer, resulting in extremely high currents (> 100 μA) at 20 kV and consequently very poor peak shapes were observed. A reduction in the applied voltage to 15 kV was thus necessary in order to reduce the background current to an acceptable level (< 100 μA). However, at 15 kV running at pH 3.0, the electro-osmotic flow (EOF) was extremely low and was found to be insufficient to elute the anionic analyte–cyclodextrin complexes from the capillary in a reasonable analysis time. This is primarily due to the anionic species migrating against the EOF to the

Table 3
Shift non-equivalence ($\Delta\delta$*, ppm) observed for racemic amlodipine on complexation with cyclodextrins

CD additive	H3′	H4′	H5′	H6′	H4	H7	H10
α-CD	—	—	—	—	—	—	0.013[a]
β-CD	—	—	—	—	—	—	—
γ-CD	*	*	0.018	0.018	—	0.011	0.013
HP-β-CD	—	*	*	—	—	—	Not resolved
HE-β-CD	—	—	0.018	—	—	—	0.015
SBE-β-CD	0.015	*	0.017	—	—	0.007	—
CM-β-CD	*	*	*	*	0.013	0.010	0.032
CM-β-CD (pD 5.4)	*	*	*	*	0.012	0.010	0.032[b]

(—) No shift non-equivalence was observed at all; (*) Shift non-equivalence was observed but was not possible to measure due to resonance overlap.
[a] 0.013 ± 0.0003, R.S.D. = 2.4% ($n = 10$).
[b] 0.032 ± 0.0002, R.S.D. = 0.59% ($n = 10$).

anode. It would have been possible to reverse the EOF with the addition of amine additives and thus would allow the complex to be eluted in a reasonable time. This approach was not adopted however as this may have affected any possible correlations that could be drawn between the NMR and CE studies. It was thus decided to increase the EOF by increasing the pH of the running buffer to 7.0 for studies with SBE-β-CD. For similar reasons, the optimum pH for CM-β-CD was selected as pH 5.0. SBE-β-CD is strongly ionised at all pH values studied as is amlodipine with a pK_a of 9.02. It is thus reasonable to postulate that their ionic state would be expected to be comparable at pH 7.0 and 3.0. Hence, it should be possible to compare the enantioselectivity in CE at pH 5.0 and 7.0 with the NMR interaction data at pH 3.0.

Table 4
Migration times and Kaiser separation index values (P_i) for amlodipine enantiomers on addition of neutral CDs to 20 mM NaH_2PO_4 dihydrate, pH 3.0 ± 0.1, run at 20 kV

CD additive	Migration time (min)		P_i
	t_{M1}	t_{M2}	
Achiral	7.0	7.0	—
20 mM α-CD	8.78	8.89	0.91
20 mM β-CD	10.24	10.24	0
20 mM γ-CD	9.67	9.75	0.31
20 mM HP-β-CD	10.28	10.47	1.0
20 mM HE-β-CD	10.13	10.44	1.0

Table 5 describes the electrophoretic conditions used for studying the resolution of amlodipine enantiomers with the anionic CDs added to the electrolyte. Surprisingly, separation of amlodipine enantiomers was achieved with the addition of only 1.0 mM SBE-β-CD to the electrolyte (pH 7.0), a 20-fold decrease in concentration relative to that for the neutral CDs. At that pH, amlodipine alone has an overall positive charge and elutes before the EOF as shown Fig. 6(a), where a slug of methanol was added to the sample as a marker for the EOF. However, the SBE-β-CD-amlodipine complex is characterised by an overall negative charge as illustrated in Fig. 6(b), where the enantiomers elute after the EOF. Fig. 7 illustrates the effect of concentration and pH on the separation of amlodipine enantiomers when CM-β-CD is used as a chiral selector. It is clear that reducing

Table 5
Migration times and Kaiser separation index values (P_i) for amlodipine enantiomers on addition of anionic CDs to 20 mM NaH_2PO_4 dihydrate run at 15 kV

CD additive	pH	Migration time (min)		P_i
		t_{M1}	t_{M2}	
Achiral	7.0	6.25	6.25	—
1.0 mM SBE-β-CD	7.0	14.1	15.1	1.0
15 mM CM-β-CD	3.0	20.2	23.01	1.0
2.5 mM CM-β-CD	3.0	14.87	16.87	1.0
2.5 mM CM-β-CD	5.0	5.32	5.46	1.0

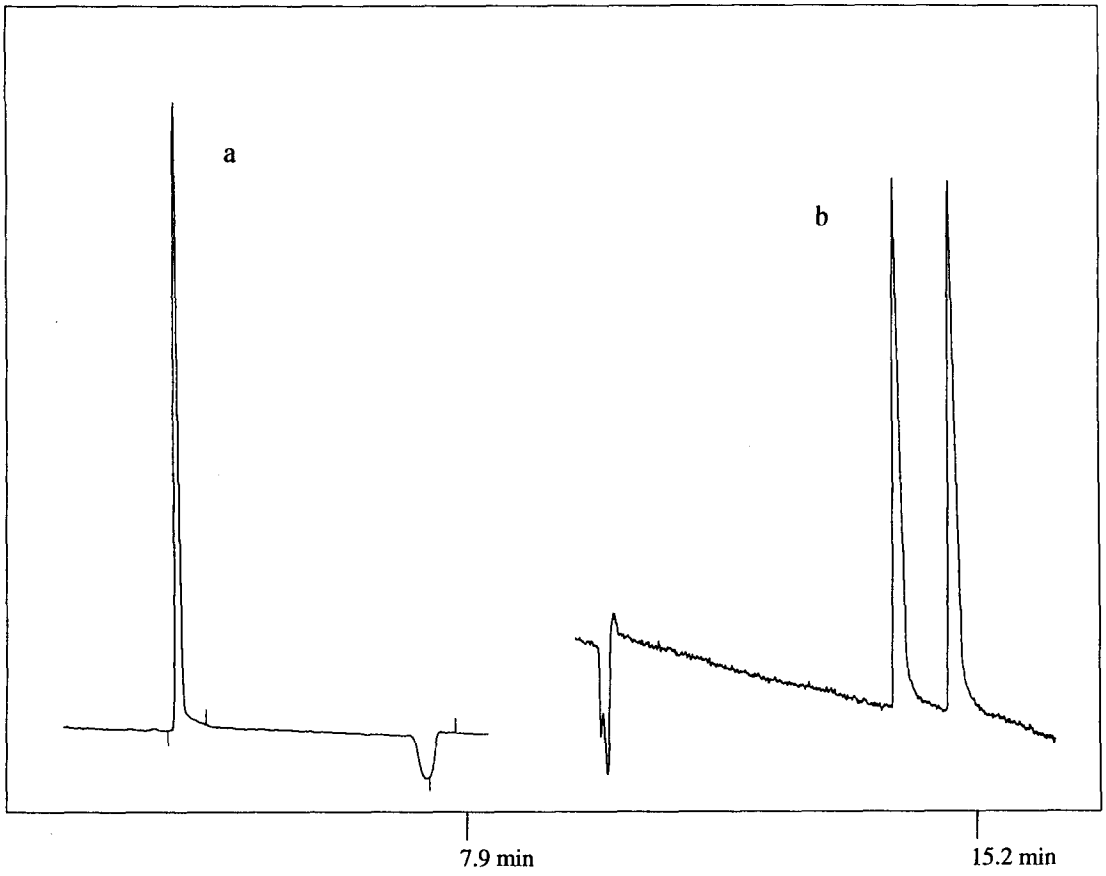


Fig. 6. Separation of amlodipine enantiomers with 20 mM NaH_2PO_4 dihydrate (pH 7.0) run at 15 kV, 17°C using: (a) an achiral run and (b) 1.0 mM SBE-β-CD added.

the selector concentration led to reduced migration times and ultimately reduced resolution (R_s: 6.2–4.8). Increasing the pH to 5.0 resulted in a further loss of resolution ($R_s = 1.3$), although enantioselectivity was still retained. This reduction in migration time and loss in resolution is considered to result from the larger EOF value observed at pH 5.0.

3.3. Possible correlations between NMR and CE

It was of interest to explore whether there were any possible correlations between these two powerful chiral discrimination techniques. Potential correlations between shift non-equivalence ($\Delta\delta$* ppm) data obtained for racemic amlodipine with the seven CDs studied, and enantiorecognition (P_i) in CE are summarised in Table 6. The 10-CH_3 group which is a triplet in the spectrum of amlodipine was chosen for examination. This multiplet was totally non-overlapped and can be regarded as a diagnostic signal for identifying potential enantioselectivity in CE.

3.3.1. Neutral cyclodextrins

Shift non-equivalence values observed for both α-CD and γ-CD show a good correlation with separation in CE. Although the magnitudes of these shifts are the same (0.013 ppm), the separation in CE varies for each. It is expected that the enantioselectivity for both these CDs would be higher after optimisation, as already reported for

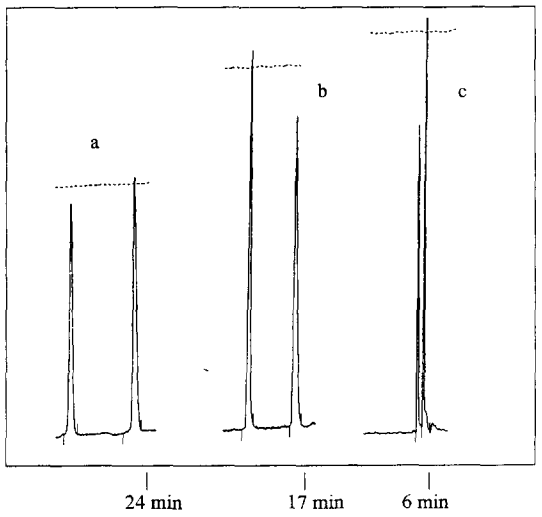


Fig. 7. Separation of amlodipine enantiomers with 20 mM NaH_2PO_4 dihydrate run at 15 kV, 17°C using: (a) 15 mM CM-β-CD (pH 3.0), (b) 2.5 mM CM-β-CD (pH 3.0) and (c) 2.5 mM CM-β-CD (pH 5.0).

α-CD [46]. No shift non-equivalence at all was observed for β-CD for any proton system; this is reflected in the CE studies, where no separation was observed using β-CD. Shift non-equivalence was observed on addition of HE-β-CD (0.015 ppm), again for this diagnostic proton system, which corresponds to separation of amlodipine enantiomers in CE. Due to resonance overlap between HP-β-CD signals and the diagnostic amlodipine signal, no possible correlation can be drawn between NMR shifts and the enantioselectivity observed in CE ($P_i = 1.0$).

Table 6
Possible correlations of shift non-equivalence ($\Delta\delta$*, ppm) data for amlodipine and CDs with enantiorecognition in capillary electrophoresis (P_i)

CD additive	Methyl (10): $\Delta\delta$*	CE: P_i
α-CD	0.013	0.91
β-CD	—	—
γ-CD	0.013	0.31
HP-β-CD	Obstructed	1.0
HE-β-CD	0.015	1.0
SBE-β-CD	—	1.0
CM-β-CD	0.032	1.0
CM-β-CD (pH 5)	0.032	1.0

3.3.2. Anionic cyclodextrins

In the case of the anionic CM-β-CD, an increase in shift non-equivalence is observed (0.032 ppm) relative to that for the neutral derivatised and native CDs (0.013–0.015 ppm). This greater shift non-equivalence correlates well with the very large separation observed ($R_s = 4.8$) despite there being nearly a 10-fold decrease in CM-β-CD concentration as shown in Fig. 7(b).

The behaviour of the anionic SBE-β-CD is, however, anomalous. When added to the electrolyte at a 20-fold decrease in concentration, this yielded a separation ($P_i = 1.0$) in less than 16 min. However, by contrast with CM-β-CD and the neutral CDs, no shift non-equivalence was observed at all for the diagnostic methyl proton of the ethyl ester side chain (10-CH_3 in Fig. 1). However, the 7-CH_3 attached to the 1,4-dihydropyridine ring did display shift non-equivalence.

γ-CD was the only other neutral CD to affect that particular methyl group. Shift non-equivalence was observed for three aromatic hydrogens, however, after the addition of SBE-β-CD, indicating some type of interaction with this moiety. It can be concluded from these data that there may be a correlation between shift non-equivalence data from specific hydrogens and enantiorecognition in CE. These methyl hydrogens (10-CH_3) may be considered diagnostic for amlodipine interacting with CDs. These data may also indicate that despite enantioseparation observed for racemic amlodipine in CE, there may be a different mode of interaction taking place between amlodipine and the anionic SBE-β-CD, relative to the mode (or modes) for the neutral CDs examined. To investigate this possibility further, 2D ROESY NMR studies on the through-space interactions and T_1 measurements were carried out.

3.4. 2D ROESY NMR

2D ROESY experiments have been reported in studies on the interactions between amlodipine maleate and five neutral CDs (α-CD, β-CD, γ-CD, HP-β-CD and HE-β-CD) [32]. It was concluded from these studies that amlodipine formed

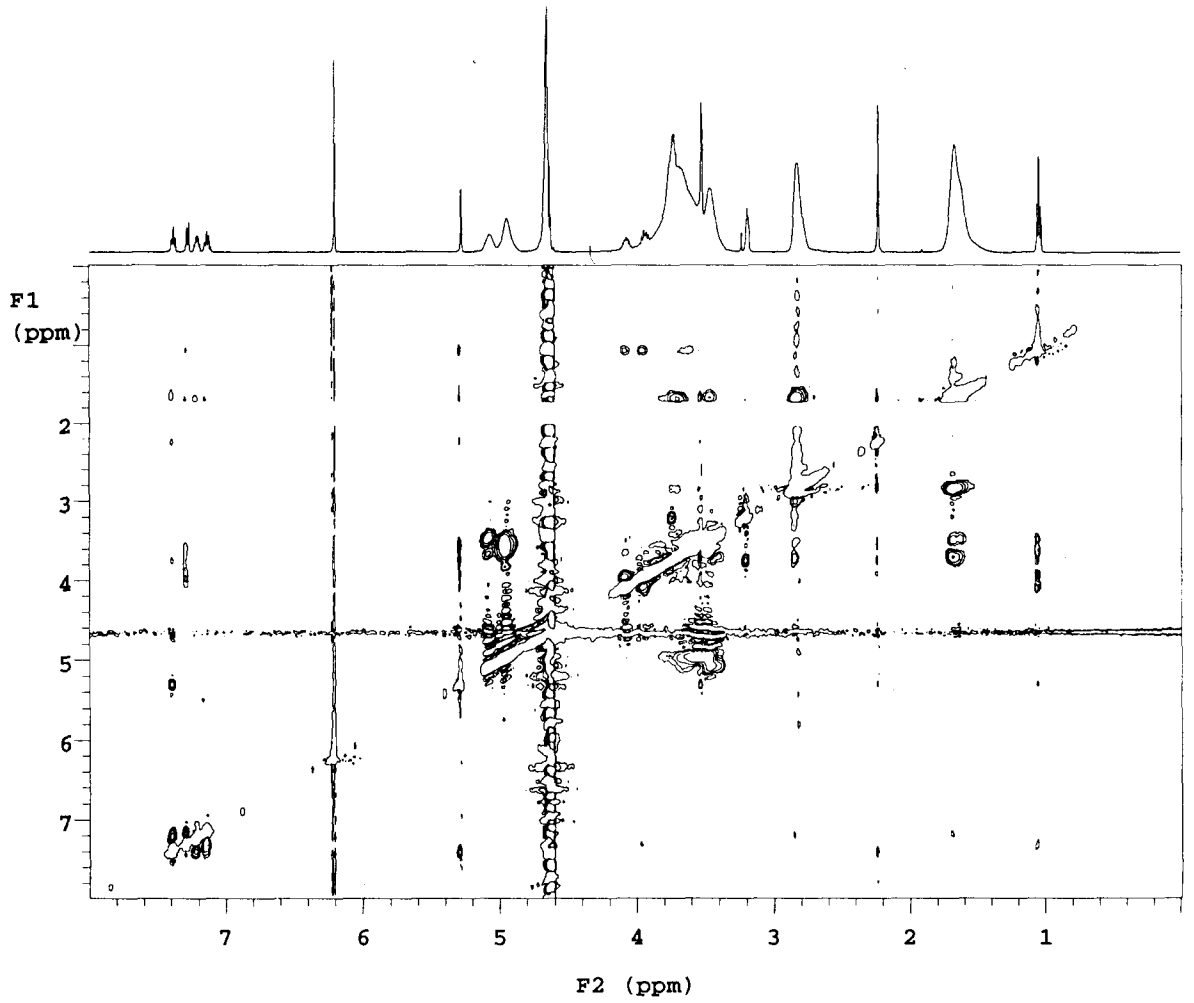


Fig. 8. The 500 MHz 2D ROESY NMR spectrum of amlodipine (0.005 M) after addition of an equimolar amount of SBE-β-CD.

an inclusion complex with each of the five CDs by insertion of the aromatic moiety into the CD cavity. It was also suggested that the carboxyl groups of amlodipine could interact via hydrogen bonding either with the secondary hydroxyl groups or with the neutral derivative at the larger annulus of the CD cavity which may have assisted the inclusion process.

Since no significant difference in the interaction data for SBE-β-CD was observed in the 1D NMR at 400 MHz as a function of CD concentration, other than the magnitude of the shifts observed, a molar ratio of SBE-β-CD:amlodipine corresponding to 1:1 was adopted for a series of 2D NMR experiments. The 500 MHz 2D ROESY spectrum of a 1:1 mixture of amlodipine and SBE-β-CD is shown in Fig. 8. In this spectrum, cross-peaks are observed between hydrogens which are close in space. There are intramolecular cross-peaks between hydrogens both in SBE-β-CD and in amlodipine. There are also weak intermolecular cross-peaks in the F1 dimension (which are present in the F2 dimension at a lower intensity) between the aromatic hydrogens in amlodipine and the b, c-CH_2 hydrogens of the sulphobutyl ether side chains of SBE-β-CD (in the 1.85–1.65 ppm region).

Table 7
Spin–lattice relaxation time (T_1) measurements for amlodipine protons and amlodipine: SBE-β-CD mixtures

Amlodipine assignment	Free molecule T_1 (s)	1:1 Mixture T_1 (s)	1:2 Mixture T_1 (s)	1:3 Mixture T_1 (s)
13-CH_2	1.0	0.8	0.7	Not measured
12-CH_2	1.0	1.3	1.2	Not measured
10-CH_3	1.6	1.3	1.2	1.2
8-CH_3	1.0	1.3	1.3	Not measured
7-CH_3	1.0	1.4	1.5	1.6
H4	3.1	2.1	2.0	1.9
H6′	2.6	1.6	1.5	1.5
H5′	1.6	1.5	1.5	1.5
H4′	1.7	1.5	1.5	1.6
H3′	1.7	1.8	1.8	1.9

The estimated errors in the T_1 values are ± 0.1 s.

The pair of overlapping cross-peaks between the H6′ aromatic hydrogens of amlodipine and the CD multiplets in the 3.5–4.1 ppm region are more difficult to explain, since the corresponding H3′ cross-peak to this region is very weak and there are no detectable cross-peaks between H4′, H5′ hydrogens and the same hydrogens. The most intense of the H6′ cross-peaks apparently derives from the 9-CH_2 group in amlodipine and is probably intramolecular in origin. This suggests that there is a preferred orientation for the aromatic ring in interactions with SBE-β-CD. The second weaker cross-peak could be between H6′ and the a-CH_2 groups of the sulphobutyl ether side chains in SBE-β-CD. There are no cross-peaks between any amlodipine hydrogens and the glucopyranose hydrogens in SBE-β-CD of the β-CD. These data suggest that interaction between amlodipine and SBE-β-CD probably occurs at the surface of the larger annulus of the CD cavity. This involves the sulphobutyl ether side chains interacting with the phenyl moiety of amlodipine and this is consistent with the 1D NMR data. There is no direct evidence for an ion-pair interaction occurring, but it should be noted that there was also no direct evidence for an inclusion complex per se.

3.5. Spin–lattice relaxation times

Recently, spin–lattice relaxation times (T_1) were measured for aromatic hydrogens of naringin before and after the addition of β-CD [37]. A reduction in these values of about 10%, all the same order of magnitude, was observed, from which the authors concluded that all aromatic hydrogens had been included into the β-CD cavity. In the work of Cahill and Bulusu [38], the reduction in T_1 values for nitramine hydrogens on addition of CD molecules was reported to be due to increased correlation time, τ_c, confirming the restricted rotation of hydrogen atoms as a result of complexation in an aqueous solution.

In the present work, T_1 measurements were obtained for each amlodipine hydrogen and compared to the T_1 values for the same hydrogens in 1:1, 1:2 and 1:3 amlodipine:SBE-β-CD mixtures as shown in Table 7. In the 1:1 mixtures, taking into account the normal degree of variability associated with T_1 data, it is probable that the changes for the chiral H4 ($\approx -32\%$) and for the aromatic H6′ ($\approx -38\%$) protons are significant.

The large decreases observed for H4 and H6′ may well indicate that the aromatic ring is adopting a preferred orientation relative to the chiral H4 proton, due to complexation with the anionic SBE-β-CD. This is consistent with the 2D ROESY data, where it was postulated that the aromatic moiety of amlodipine was not included into the CD cavity but was shown to interact with the sulphobutyl side chains at the wider annulus of the cavity.

These data fall within the extreme narrowing limit condition both for the free state and the complexed molecules as for the systems of Cahill

and Bulusu [38]. Hence, it would be anticipated that T_1 should be independent of measurement field strength. Taken together, these T_1 data confirm that there is a shift in the relative position of H4 and H6′ protons and a reduction in their inter-nuclear distances. These observations support the findings of Cahill and Bulusu with analogous complexation systems using native CDs [38]. Much smaller changes were subsequently observed in the 1:2 and 1:3 mixtures; these were not considered significant.

4. Conclusions

One-dimensional ^{1}H-NMR has been successfully used in examining shift displacement and shift non-equivalence data observed for amlodipine hydrogens after separate addition of seven CDs under identical conditions. The two types of shift observed were examined qualitatively and quantitatively to interpret the nature of interactions between amlodipine, five neutral CDs and two anionic CDs. Shift differences were observed between (a) each of the three native CDs, (b) neutral derivatised (HP-β-CD and HE-β-CD) and the corresponding native β-CD and (c) the neutral CDs and the anionic CDs.

These differences were explored further using 2D ROESY experiments which measured the proximity and degree of interaction between the amlodipine aromatic hydrogen group and the glucopyranose hydrogens within the SBE-β-CD cavity. There were no through-space interactions observed between these two groups, indicating that an inclusion complex in the classical sense was not being formed. However, there was also no evidence for a complementary ion-pair interaction. There was evidence for interaction of the aromatic moiety of amlodipine with the sulphobutyl ether side chains at the wider annulus of the β-CD cavity. Although there was no evidence for an ion-pair interaction, it is thought that an ionic interaction between cationic amlodipine and the anionic sulphobutyl side chains could be responsible for the absence of any extensive inclusion process. It is possible that entry to the larger annulus of the SBE-β-CD cavity is sterically hindered by the presence of multiple sulphobutyl moieties. Similar studies with SBE-β-CD having a lower DS are currently being undertaken to investigate this possibility further.

Spin–lattice relaxation measurements were made for amlodipine hydrogens before and after the addition of the anionic SBE-β-CD. A significant reduction in relaxation time was observed for the aromatic atom H6′, indicating that the aromatic moiety of amlodipine may adopt a preferred orientation when added to the SBE-β-CD. This would be consistent with the 2D ROESY data.

The enantioselectivity of seven CD additives, two of which were anionic, has been explored for the chiral separation of amlodipine in CE. These CE studies indicated that for the chiral separation of amlodipine, the native CD of choice was α-CD ($P_i = 0.9$). This selector was not as enantioselective as the neutral derivatised CDs, HP-β-CD or HE-β-CD (both $P_i = 1.0$). This study has highlighted the influence of the side chain attached to the β-CD, as the native β-CD showed no enantioselectivity at all. However, when an anionic side chain was attached to the same β-CD, enantioselectivity was observed at considerably lower concentrations (1.0 mM for SBE-β-CD and 2.5 mM for CM-β-CD). This may be attributable to an electrophoretic effect where the overall complex remains on the capillary for a longer period of time, but recent results on this anionic CM-β-CD as an MPA in LC [17] and the larger shifts observed in NMR in the present work may indicate that additional sites for hydrogen bonding, increased steric interactions and additional ionic interactions with the anionic derivative, or a combination of these three factors may be more influential.

This is the first time, to our knowledge, that 2D ROESY or ^{1}H T_1 measurements have been carried out with the anionic SBE-β-CD. The shifts in NMR and the enantioseparation observed in CE have shown good correlation for amlodipine indicating the potential usefulness of 1D NMR for predicting chiral recognition in CE. This also applies to the process of choosing a chiral selector in LC. The usefulness of these powerful techniques, together with 2D NMR, will become in-

creasingly important for evaluating complex enantioselective mechanisms as novel CDs are introduced where the enantioselective mechanism may no longer follow a simple inclusion process.

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References

[1] J. Debowski, D. Sybilska, J. Jurczak, Journal of Chromatography 237 (1982) 303–306.
[2] D.W. Armstrong, W. Demond, Journal of Chrom. Science 22 (9) (1984) 411–415.
[3] S. Fanali, Journal of Chromatography 545 (1989) 437–444.
[4] S.A.C. Wren, R.C. Rowe, Journal of Chromatography 603 (1989) 235–241.
[5] K.D. Altria, D.M. Goodall, M.M. Rogan, Chromatographia 34 (1992) 19–24.
[6] E. Grassman, J.E. Kuo, R.N. Zare, Science 230 (1985) 813–814.
[7] R. Kuhn, F. Stoecklin, F. Erni, Journal of Chromatography 33 (1992) 32–36.
[8] D.W. Armstrong, K.L. Rundlett, J.R. Chen, Chirality 6 (1994) 496–509.
[9] H. Nishi, T. Fukuyama, M. Matsuo, S. Terabe, Journal of Microcolumn Separations 1 (1989) 234–241.
[10] K. Otsuka, S. Terabe, Electrophoresis 11 (1990) 982–984.
[11] M.J. Sepaniak, R.O. Cole, B.K. Clark, Journal of Liquid Chromatography 15 (1992) 1023–1040.
[12] A.M. Stalcup, S.C. Chang, D.W. Armstrong, J. Pitha, Journal of Chromatography 513 (1990) 181–194.
[13] M. Heuermann, G. Blaschke, Journal of Chromatography 648 (1993) 267–274.
[14] S. Terabe, H. Ozaki, K. Otsuka, T. Ando, Journal of Chromatography 332 (1985) 211–217.
[15] J. Crommen, M. Fillet, I. Bechet, P. Hubert, Journal of Pharmaceutical and Biomedical Analysis 14 (8–10) (1996) 1107–1114.
[16] J. Crommen, M. Fillet, I. Bechet, G. Schomburg, P. Hubert, 7th International Symposium on Pharmaceutical and Biomedical Analysis, Osaka, Japan, 1996.
[17] P.K. Owens, A.F. Fell, M.W. Coleman, J.C. Berridge, Chirality, 8 (1996) 466–476.
[18] D. Sybilska, in A.M. Kristulovic (Ed.), Chiral Separations By HPLC, Applications to Pharmaceutical Compounds, Cyclodextrin Additives, Ellis Horwood/Wiley, Chichester, 1989, pp. 147–172.
[19] R.J. Tait, D.J. Skanchy, D.P. Thompson, N.C. Chetwyn, D.A. Dunshee, R.A. Rajewski, V.J. Stella, J.F. Stobaugh, Journal of Pharmaceutical and Biomedical Analysis 10 (1992) 615–622.
[20] C. Dette, S. Ebel, S. Terabe, Electrophoresis 15 (1994) 799–803.
[21] P.K. Owens, A.F. Fell, M.W. Coleman, J.C. Berridge, Chirality (1996) in press.
[22] I.S. Lurie, R.F.X. Klein, M.J. LeBelle, T.A. Dal Cason, R. Brenneisen, R.E. Weinberger, Analytical Chemistry 66 (1994) 4019–4026.
[23] D. MacNicol, D. Rycroft, Tetrahedron Letters 25 (1977) 2173–2176.
[24] D. Greatbanks, R. Pickford, Magnetic Resonance in Chemistry 25 (1987) 208–215.
[25] A.F. Casy, A.D. Mercer, Magnetic Resonance in Chemistry 26 (1988) 765–774.
[26] P. Job, Ann. Chim. (Paris) (Série 10) 9 (1928) 113–203.
[27] Y. Yamamoto, Y. Inoue, Carbohydrate Research 8 (1989) 29–46.
[28] R. Bergeron, R. Rowan III, Inorganic Chemistry 5 (1976) 425–436.
[29] Y. Yamamoto, M. Onda, M. Kitagawa, Y. Inoue, R. Chujo, Carbohydrate Research 167 (1987) 11–16.
[30] A.A. Bothner-By, R.L. Stephens, J. Lee, C.D. Warren, R.W. Jeanloz, Journal of the American Chemical Society 106 (1984) 811–813.
[31] Y. Inoue, Y. Kanda, Y. Yamamoto, R. Chujo, S. Kobayashi, Carbohydrate Research 194 (1989) c8–c12.
[32] T.S. Small, Ph.D. Thesis, University of Bradford, UK, 1995.
[33] J.P. Behr, J.M. Lehn, Journal of the American Chemical Society 98 (1976) 1943.
[34] M. Suzuki, J. Szejtli, L. Szente, Carbohydrate Research 192 (1989) 61.
[35] M. Suzuki, H. Ohmori, M. Kajtar, J. Szejtli, M. Vikmon, Journal of Inclusion Phenomena 18 (1994) 255–264.
[36] M. Suzuki, H. Takai, K. Tanaka, K. Narita, H. Fujiwara, H. Ohmori, Carbohydrate Research 288 (1996) 75–84.
[37] S. Divakar, Journal of Inclusion Phenomena 15 (1993) 305–316.
[38] S. Cahill, S. Bulusu, Magnetic Resonance in Chemistry 31 (1993) 731–735.
[39] N. Azaroual-Bellanger, B. Perly, Magnetic Resonance in Chemistry 32 (1994) 8–11.
[40] P.L. Irwin, J.N. Brouillette, K.B. Hicks, Carbohydrate Research 269 (1996) 201–216.
[41] P.K. Glasoe, F.A. Long, Journal of Physical Chemistry 64 (1960) 188–190.
[42] P.K. Owens, A.F. Fell, M.W. Coleman, M. Kinns, J.C. Berridge, 8th International Symposium on Chiral Discrimination, Edinburgh, Scotland, 1996.

[43] A.F. Casy, A.D. Cooper, T.M. Jefferies, R.M. Gaskell, D. Greatbanks, R. Pickford, Journal of Pharmaceutical and Biomedical Analysis 9 (10–12) (1991) 787–792.
[44] M. Suzuki, Y. Sasaki, Chemical and Pharmaceutical Bulletin 32 (3) (1984) 832–838.
[45] R.E. Kaiser, Gas Chromatographie, Geest and Portig, Leipzig, 1960.
[46] T.S. Small, A.F. Fell, M.W. Coleman, J.C. Berridge, Chirality 7 (1995) 226–234.