Screening of cyclodextrins by nuclear magnetic resonance for the design of chiral capillary electrophoresis separations

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Abstract

High-field one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy and capillary electrophoresis (CE) are examined to investigate the process of chiral recognition occurring between different cyclodextrins (CDs) and the racemic anti-schistosomiasis drug, oxamniquine. Five neutral CDs (α-CD, β-CD, γ-CD, hydroxypropyl-β-CD and hydroxyethyl-β-CD) and two anionic CDs, carboxymethyl-β-CD (CM-β-CD) and sulphobutyl ether-β-CD (SBE-β-CD) were selected for these NMR and CE studies. Three batches of the anionic CD [SBE-β-CD(7), SBE-β-CD(4) and SBE-β-CD(1)] differing in their degree of substitution (6.5, 4.5 and 1.0, respectively), were also examined in these studies. In the 1D NMR studies, the shift displacement values ($\Delta\delta$ Hz), after addition of each of the nine CDs in a 1:1 molar ratio, the diagnostic aromatic singlets indicated interaction between oxamniquine and each of the CDs. With regard to shift non-equivalence ($\Delta\delta^*$) for the two aromatic singlets, the anionic CDs were the only agents to show any interaction. It was also found that by manipulating the sample pH value, the ionic state of both selector and selectand had an influence on the enantiorecognition process. The complex between oxamniquine and the SBE-β-CD(7) was subsequently examined using 2D rotating frame nuclear Overhauser effect spectroscopy (ROESY), and the complexation process was found to involve an inclusion mechanism. Spin-lattice relaxation time (T_1) measurements confirmed this observation. In the CE studies, each of the nine CDs was examined as a chiral electrolyte additive for the enantioseparation of racemic oxamniquine. Complete separation was not found when using any of the neutral CDs, but in contrast to the anionic CDs, CM-β-CD and the SBE-β-CD(4), displayed a 95 and 100% separation, respectively. Interestingly, the higher and lower derivatised SBE-β-CD selectors yielded 10% and zero separation, respectively. This is strong evidence that the degree of substitution has a critical effect on the enantiorecognition process. Good correlation between the NMR shift non-equivalence data for oxamniquine and the degree of enantioseparation was observed in CE. The neutral CDs displayed no shift non-equivalence for oxamniquine and no enantioseparation was found. However, for the anionic CDs a significant shift non-equivalence was observed and this corresponded to a significant enantioseparation in CE. © 1998 Elsevier Science B.V.

Keywords: Nuclear magnetic resonance spectrometry; Enantiomer separation; Cyclodextrins; Oxamniquine

1. Introduction

The separation, detection and quantification of chiral drug enantiomers is now recognised as an important field in the pharmaceutical industry. The use of capillary electrophoresis (CE) for chiral drug discrimination has attracted considerable attention as a result of its high efficiency, versatility, low running costs and speed of analysis [1]. Chiral separations in CE are normally achieved by the addition of a chiral

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selector to the running buffer. These include chiral metal complexes [2], crown ethers [3], glycopeptides [4], chiral surfactants [5], chiral mixed micelles [6], acyclic carbohydrates [7], but in most cases a cyclodextrin (CD) is used [8,9].

Native CDs are cyclic oligosaccharides which have a toroidal shape built up from six (α-), seven (β-) or eight (γ-) D(+)-glucopyranose units bonded through α-(1,4) linkages. Cyclodextrins contain 18 (α-CD), 21 (β-CD) or 24 (γ-CD) hydroxyl groups, respectively, which can be modified chemically. The C6-OH groups are the most reactive while the C3-OH are the least reactive. A native CD host molecule can be derivatised (e.g. at 21 sites on β-CD), thereby altering its physical and chemical properties and thus the nature of any interaction with a guest molecule. Native CDs, and CDs derivatised with a neutral sidechain, have been used extensively as chiral selectors in CE [1]

Recently, however, considerable attention has focused on the use of CDs derivatised with a chargeable sidechain for chiral recognition in both LC and in CE [10–12]. These chargeable CDs in CE have been shown to offer enhanced enantioselectivity in shorter analysis times at significantly lower concentrations than for neutral CDs [12]. Additionally, chargeable CDs have been utilised for controlling drug enantiomer migration order, a feature that may be advantageous when quantitatively determining trace levels of a drug distomer in the presence of other impurities and formulation components [13].

The degree of substitution (DS) (i.e. the average number of derivative molecules per CD molecule) of neutral derivatised CDs is well recognised as a factor that can influence enantioselectivity in LC, GC and CE [14–16]. In CE, the DS of the anionic sulphobutyl ether-β-cyclodextrin (SBE-β-CD) has been shown to be particularly influential when it was used as a chiral selector for separating the enantiomers of bidisomide and related impurities [17]. Interestingly, a recent report on the use of another similar anionic CD, the sulphoethyl ether-β-cyclodextrin, as a chiral selector for R/S-1,1'-binaphthyl-2,2'-diyl hydrogenphosphate in CE, demonstrated that in this case its DS was "a factor with rather low importance" [18]

High-field nuclear magnetic resonance spectroscopy (NMR) has also been used for chiral drug

discrimination based on the use of chiral derivatising agents, chiral lanthanide shift reagents or chiral solvating reagents [19]. CDs have been used since 1977 as chiral shift reagents [20] and have been explored by several groups since for chiral drug discrimination [21–27]. NMR has also been effective for investigating the stoichiometry, structure and stability of cyclodextrin complexes [28]. Job plots of the variation of chemical shift with drug cyclodextrin ratio have been used to determine complex stoichiometry. Nuclear Overhauser enhancements (NOE) and in particular two-dimensional (2D) NOE experiments in the rotating frame (ROESY) which give positive and enhanced NOEs over the whole molecular range, have been useful for examining the through-space interactions between drug and CD nuclei in a drug–CD complex [29–32].

Recently ^{1}H spin-lattice relaxation time (T_1) measurements have also been used by several groups to examine and corroborate 2D NMR experiments on drug–CD complexes [33–39]. These studies have shown that T_1 measurements can be used to obtain information on the rotation and molecular assembly of a drug in a drug–CD complex. It has also been suggested that T_1 measurements can actually be used as a probe for selecting the CD which binds most strongly to a particular enantiomer in a racemate [37].

In the present work, CE and one-dimensional (1D) NMR have been used to discriminate between the enantiomers of oxamniquine, an anti-schistosomiasis drug (Fig. 1), using five neutral and two charged CDs. The neutral α-CD, β-CD, γ-CD, hydroxypropyl-β-CD (HP-β-CD) and hydroxyethyl-β-CD (HE-β-CD) and the anionic carboxymethyl-β-CD (CM-β-CD) and SBE-β-CD have been examined as chiral selectors, when added to the electrolyte in CE and as chiral shift reagents in NMR. Three batches of the anionic SBE-β-CD, each having a
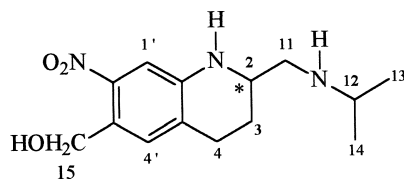

Fig. 1. Structure of oxamniquine.

different DS, have been characterised in CE using indirect UV detection methods as previously reported [40,41]. The performance of each batch was subsequently examined as a chiral selector for oxamniquine both in CE and in NMR, to examine the possible effect of DS on the chiral discrimination.

The nature of the interaction between oxamniquine and the anionic SBE-β-CD has been examined using 2D ROESY experiments and T_1 measurements. The overall data from these 1D NMR, 2D NMR and CE experiments are critically assessed to examine if there is a basis for a correlation to be drawn between the spectroscopic and separative methods for chiral drug discrimination.

2. Experimental

2.1. Reagents and materials

Racemic oxamniquine and SBE-β-CD sodium salt, DS=6.5, were used as received from Pfizer (Kent, UK). α-CD, γ-CD and CM-β-CD sodium salt (DS=5.3) were used as received from Wacker (Walton-on-Thames, Surrey, UK). SBE-β-CD(4) and SBE-β-CD(1) were gifts from CyDex (Kansas, USA). Benzoic acid, tris(hydroxymethyl)-aminomethane (Tris), β-CD, HP-β-CD (DS=4.2) and HE-β-CD (MS=11.2) were used as received from Aldrich (Gillingham, UK). Deuterium oxide (99.8%) was obtained from Isotec (Matheson, USA). Sodium dihydrogenorthophosphate dihydrate and sodium dihydrogenorthophosphate anhydrous were purchased from BDH (Poole, UK).

2.2. Apparatus

The 1D NMR spectra were obtained on a Unity INOVA-400 NMR spectrometer operating at 399.96 MHz for [1]H. 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).

The 2D experiments and T_1 measurements were obtained on a Varian Unity 500 NMR spectrometer. A 5-mm probe, was used for ^{1}H NMR observation in

D$_2$O solution. 2D ROESY spectra were obtained using the following conditions: a sweep width window of 4898 Hz; acquisition time, 0.209 s; relaxation delay, 2 s; spin-lock mixing time, 1 s; and spin-lock amplitude, 4735 Hz. Off-set 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, with Gaussian apodisation in both dimensions. The inversion recovery technique was used to measure the spin-lattice relaxation times: relaxation delay, 20 s; and acquisition time, 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.

Capillary electrophoresis was carried out using a Beckman P/ACE 5510 (Fullerton, CA, USA) equipped with a UV detector operated at 214 nm. The electrophoretic experiments were performed in an uncoated fused-silica capillary 57 cm (50 cm effective length)×50 μm I.D. obtained from Beckman Instruments (High Wycombe, UK). The temperature of the cartridge was maintained constant throughout at 17°C.

2.3. Methods

Oxamniquine, 0.005 M, and oxamniquine–CD mixtures (in a 1:1 ratio; molar ratios were used throughout) were prepared in 100 mM NaH$_2$PO$_4$ deuterium oxide buffer at pH 3.0±0.1, adjusted with deuterium chloride or deuterated sodium hydroxide, as appropriate. Although Glasoe and Long [42] suggest that pD values should be used with solutions in D$_2$O, for practical comparative purposes, it was decided to utilise the pH value for all drug–CD mixtures in the present NMR studies. Thus the degree of ionisation of drug and anionic CD at specific pH values could be readily calculated from the measured pK_a values. CM-β-CD which has a calculated acidic pK_a of 4.36 would be expected to be much less strongly ionised at pH 3.0 than at pH values higher than 5.0. It was thus decided to acquire spectra for oxamniquine and the oxamniquine–CM-

β-CD mixture (1:1 ratio), both at pH 3.0 and at pH 5.0, to determine whether changes in the ionic state of this CD induced significantly different chemical shifts. A number of spectra were acquired ($n=10$) during a 12-h period for similar mixtures, as recently reported by the present authors [27], to determine the statistical reliability of the shift data. This was done to assess the significance of any small shifts observed and also to evaluate the stability of the sample over that time.

The capillary was conditioned initially for 1 h with 1 M NaOH and 20 min with water. Sodium dihydrogenphosphate dihydrate adjusted to the appropriate pH with orthophosphoric acid and sodium hydroxide as appropriate was used as electrolyte. CDs were prepared in this electrolyte at the required concentration 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. Oxamniquine (ca. 100 μg/ml) was prepared each day in deionised water from a 1 mg/ml stock prepared in electrolyte. Samples were prepared in 10% buffer to favour sample stacking and were introduced to the capillary by pressure injection.

For quantitative measurements, corrected peak areas were used for calculating the DS of the three SBE-β-CD batches. All samples and buffers were sonicated and filtered through a 0.45-μm filter (Anachem, Luton, UK). It was also decided to investigate the use of CM-β-CD at both pH 3.0 and at 5.0 to determine if the ionic state of this CD affected enantioselectivity when used as a chiral selector in CE. Any enantiomeric separation observed was evaluated using 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 [43].

2.4. Cyclodextrin characterisation

There are a number of analytical techniques available for determining the DS (i.e. the average number of derivative substituents per CD molecule) of a derivatised CD: 1D and 2D proton and carbon NMR, Fourier transform-infrared (FT-IR), and elemental analysis have all been used to characterise CDs and calculate their DS values [44]. Fast atom bombardment mass spectrometry (FAB-MS) was

used to characterise the neutral hydroxypropyl derivatives of α-CD and β-CD [45]. FAB-MS, electrospray-ionisation time-of-flight mass spectrometry (ESI-MS) and matrix-assisted laser-desorption/ionisation mass spectrometry (MALDI-TOF-MS) have also been used to calculate the molecular weight, DS and purity of a range of anionic CDs [46]. The DS values calculated for the anionic CDs studied were found to be in good agreement with those calculated using the CE method.

The DS value of a CD, which characterises the median value of the substitution pattern, was first calculated in CE for two batches of the anionic sulphobutyl ether-β-cyclodextrin, taking advantage of the fact that sulphobutyl derivatives carry a negative charge [40]. These two CD batches were found to contain a mixture of positional and regio isomers, containing from one (mono) to as many as ten (deca) sulphobutyl derivatives per CD molecule. The CE method of analysis showed complete resolution of mono- to deca-substituted SBE-β-CD, for both batches, with average DS values of 4.0 and 7.0 calculated for each batch. These DS values were confirmed by NMR and elemental analysis. The method was fully evaluated later by the same group, using benzoic acid in the running buffer and detecting each CD by indirect UV [41].

The three batches of SBE-β-CD used in this study were characterised by different values of DS, and will be referred to as follows: SBE-β-CD, SBE-β-CD(4) and SBE-β-CD(1). The DS value for each SBE-β-CD was established by CE, using the above method [41]. SBE-β-CD and SBE-β-CD(4) were shown to be mixtures of isomers containing the mono-substituted through to the deca-substituted CD, as shown in Fig. 2a Fig. 2b. The average DS value for each of these two mixtures was 6.5 (subsequently confirmed at Pfizer Central Research by NMR and elemental analysis), and 4.5, respectively. These values were calculated by using corrected peak area values for each peak in the electropherogram and expressing each as a percentage of the total corrected substitution envelope area. On the other hand, SBE-β-CD(1) was shown to consist primarily of the mono-substituted CD with only ca. 0.74% of the di-substituted CD present, as shown in Fig. 2c. The peak for the mono-substituted derivative shows slight signs of splitting. This probably corresponds to the
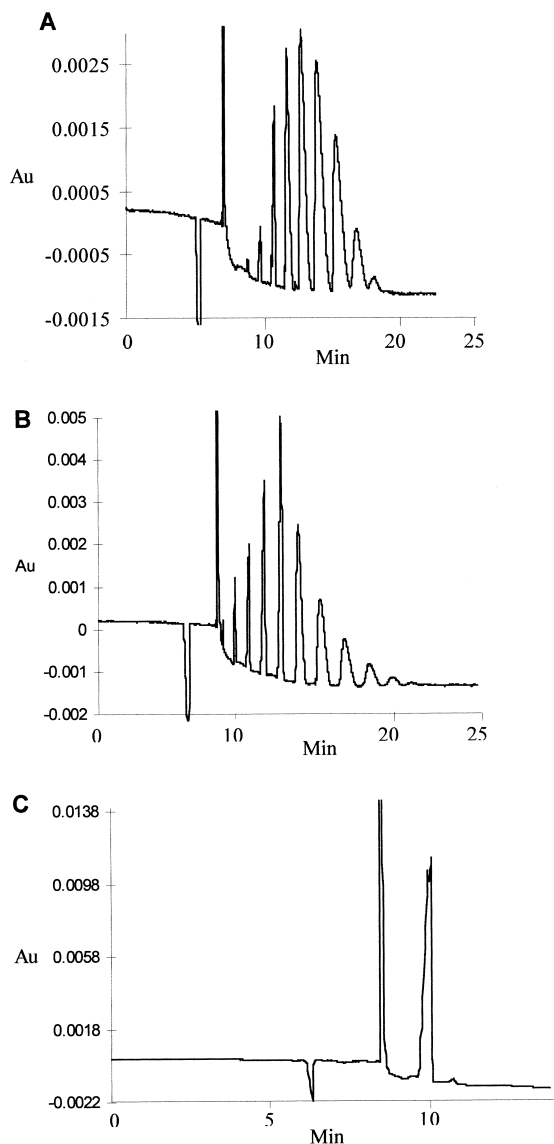
Fig. 2. Separation of the (a) SBE-β-CD (DS=6.5), (b) SBE-β-CD(4) (DS=4.5) and (c) SBE-β-CD(1) (DS=1.0) mixtures using 30 mM benzoic acid made with 100 mM Tris buffer and adjusted to pH 6.3. Indirect UV at 200 nm, run at 30 kV and 25°C.

three overlapping positional isomers, partially resolved to give two peaks, since potentially three positional isomers for the mono-substituted CD can exist. The three sites for substitution on a glucopyranose unit are at C2, C3 and C6. The overall substitution patterns for these three SBE-β-CD batches are in agreement with those found experimen-

tally for comparable batches [17]. Indeed, the DS values or the median values of the substitution pattern, for the three batches studied, were also found to be comparable.

3. Results and discussion

3.1. Nuclear magnetic resonance

3.1.1. One-dimensional NMR

A spectrum was acquired initially for racemic oxamniquine in the absence of CD at pH 3.0 shown in Fig. 3. The assignments for the resonance signals observed are shown in Table 1. Spectra for oxamniquine–CD mixtures in a 1:1 ratio were then acquired under identical conditions for each of the seven CDs. Two types of shifts for racemic drug resonance signals can be observed after addition of a CD chiral selector: (a) the displacement (up or downfield) of a singlet, or of a multiple, defined as a *shift displacement* ($\Delta\delta$); and (b) the enantiomeric splitting of a singlet, or of a multiplet, defined as a *shift non-equivalence* ($\Delta\delta^*$) [27]. In principle, each type of shift could be observed for a particular resonance either singly, or in combination.

3.1.2. Shift displacement ($\Delta\delta$)

It was decided to examine the aromatic H1′ and H4′ hydrogen atoms of oxamniquine (each as a singlet) as possible diagnostic protons for evaluating any shift displacement observed (i.e. shift of the singlet up or downfield). These two singlets were chosen on the basis that the interaction of a chiral guest molecule with a CD is generally considered to occur through inclusion of an aromatic moiety of the guest into the CD host molecule, and also because these singlets were not overlapped by any CD resonance signals, or others arising from oxamniquine itself.

Shift displacement ($\Delta\delta$ Hz) values observed for each of the aromatic oxamniquine hydrogen atoms with respect to the same uncomplexed signals, are shown in Table 2, after the addition of each of the nine CDs in a 1:1 ratio at pH 3.0, respectively. These data show evidence of interaction of oxamniquine with each of the CDs, with substantial shift displacement of the singlets either up or downfield. More-
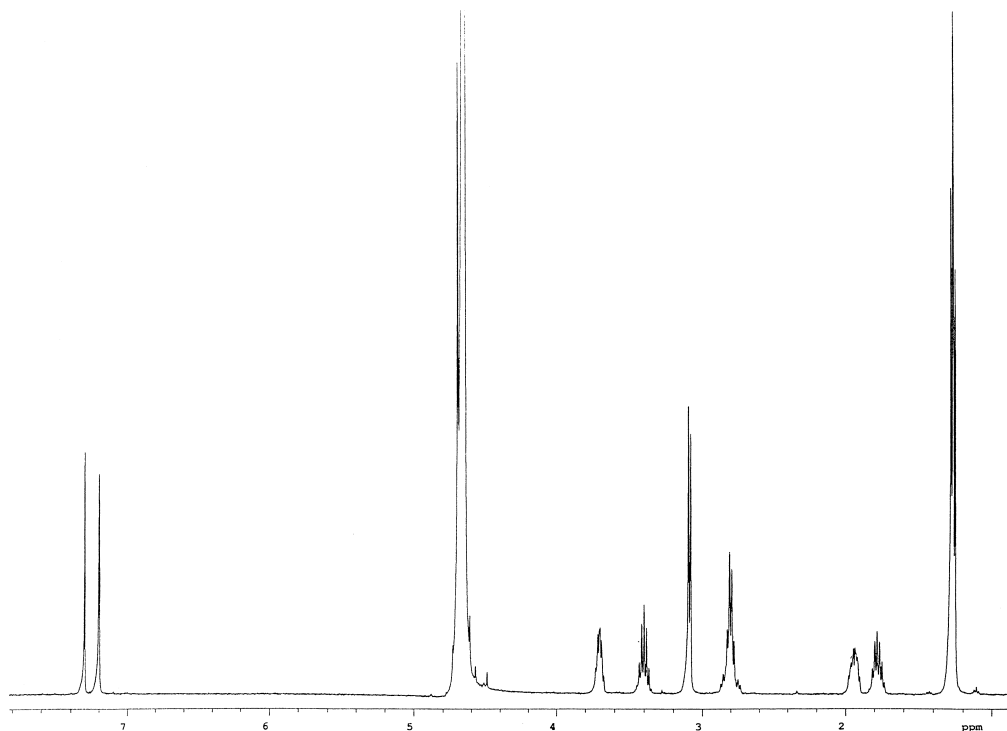
Fig. 3. NMR spectrum (400 MHz) of oxamniquine.

over, the observed shift displacement for the ox-amniquine aromatic hydrogens after addition of each CD in a 1:1 molar ratio, is of the same order of magnitude as that previously reported for amlodipine aromatic hydrogens after the addition of the same CDs in a 1:2 molar ratio [27]. Thus it may be possible that a stronger association or interaction is occurring between oxamniquine and these CDs than that observed for amlodipine with the same CDs.

The shift displacement for the two oxamniquine aromatic resonance signals in a 1:1 molar mixture with CM-β-CD is illustrated in Fig. 4.

The shift displacement values shown in Table 2, for oxamniquine aromatic hydrogens on addition of the CDs, clearly indicate that each of the nine CDs interacts with this molecule, but each to a different extent. α-CD is the only CD to show downfield shift displacement for both the diagnostic hydrogens. The

Table 1
^{1}H assignments of racemic oxamniquine resonance signals

Chemical shift (ppm)	Multiplicity	Proton assignment
1.26	triplet	13,14
1.78, 1.92	multiplet	3
2.81	multiplet	4
3.09	doublet	11
3.40	multiplet	12
3.70	multiplet	2
4.66	obscured by HOD signal	15
7.20	singlet	4′
7.30	singlet	1′

Table 2
Shift displacements ($\Delta\delta$, Hz) observed for racemic oxamniquine on complexation with cyclodextrins in a 1:1 ratio at pH 3.0

CD additive	H1′	H4′
α-CD	2.9 D	2.7 D
β-CD	14.4 U	0.5 U
γ-CD	21.0 U	1.7 D
HP-β-CD	5.4 U	6.6 D
HE-β-CD	2.4 U	6.8 D
CM-β-CD	24.2 U	7.1 D
SBE-β-CD	11.2 U	29.0 D
SBE-β-CD(4)	23.3 U	12.4 D
SBE-β-CD(1)	16.7 U	3.2 D

D, downfield; U, upfield.

magnitudes of these shifts are also quite small compared to those for other CDs, where a large shift is observed for at least one of the diagnostic aromatic hydrogens. With α-CD, it is possible that the nitro and hydroxymethyl substituents attached to the aromatic moiety of oxamniquine precluded any inclusion process into the smaller cavity (5.7 Å), as compared to the larger cavities of β-CD (7.8 Å) or γ-CD (9.5 Å).

Each of the other eight CDs showed an upfield shift for the H1′ hydrogen. The values for the shift displacement for this hydrogen on addition of the native and the six derivatised CDs are comparable, with the exception of HE-β-CD and HP-β-CD (2.4 and 5.4 Hz, respectively). This is also true for the neutral and anionic CDs for this hydrogen, where the order of magnitude of shift displacements are comparable. It is interesting to note that the SBE-β-CD(4) showed a significantly larger shift displacement for H1′ (23.3 Hz) than did the more highly derivatised SBE-β-CD (11.2 Hz), or the less derivatised SBE-β-CD(1) (16.7 Hz). Typical values of standard deviation (S.D.) ($n=10$) for data on this instrument were found to be S.D.=0.3–0.4 Hz for a shift displacement of ca. 22 Hz.

By complete contrast to H1′, the H4′ singlet showed a downfield shift displacement for each of the nine CDs, with the exception of β-CD. The extent of the shift displacements for H4′ on addition of the three native CDs are somewhat lower than those for the derivatised CDs, with the exception of the mono-derivatised SBE-β-CD(1), indicating a stronger interaction for oxamniquine with these CD derivatives, either through hydrogen bonding, ionic

interactions or simply steric effects. Comparing the shift displacements for the H4′ singlet by neutral CDs with those for the anionic CDs, it becomes apparent that there may be a different mode (or modes) of interaction taking place. The shift displacement observed for the H4′ resonance signal when using the anionic SBE-β-CD (29.0 Hz) and SBE-β-CD(4) (12.4 Hz) are significantly larger than the highest shift displacement observed when using any neutral CD (6.8 Hz). It is also interesting to note that for H4′, the SBE-β-CD showed a larger shift displacement than its lower derivatised counterpart SBE-β-CD(4), which is in contrast to the shifts observed for H1′ with these CDs.

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

It was also decided to examine the aromatic H1′ and H4′ hydrogen atoms of oxamniquine (each appearing as a singlet) as diagnostic protons for evaluating any shift non-equivalence (i.e. enantiomeric splitting of the singlet to two resonance signals). Shift non-equivalence ($\Delta\delta*$ Hz) values observed for each of the oxamniquine aromatic hydrogen atoms with respect to the same uncomplexed signals are shown in Table 3, after the addition of the nine CDs in a 1:1 ratio at pH 3.0. The shift non-equivalence for the two oxamniquine aromatic resonance signals in a 1:1 molar mixture with CM-β-CD is illustrated in Fig. 4. Shift non-equivalence values observed for each particular singlet were measured as the difference in the resonance position of each enantiomer with respect to each other.

The shift non-equivalence values observed for both H4′ and H1′, shown in Table 3, give clearer evidence (than the shift displacement data for the same signals) on the differences observed between the neutral CDs and the anionic CDs. No shift non-equivalence was observed at all for either H1′ or H4′ on addition of any neutral CD, either native or derivatised at pH 3.0. This is in complete contrast to the selectivity observed for the anionic CDs, where shift non-equivalence of the H1′ resonance signal was observed after addition of all anionic CDs.

These observed shifts clearly indicate that the nature of the derivative is a crucial factor in determining the interaction of oxamniquine with these CDs. A number of interesting observations can also be made regarding the magnitude of these shift
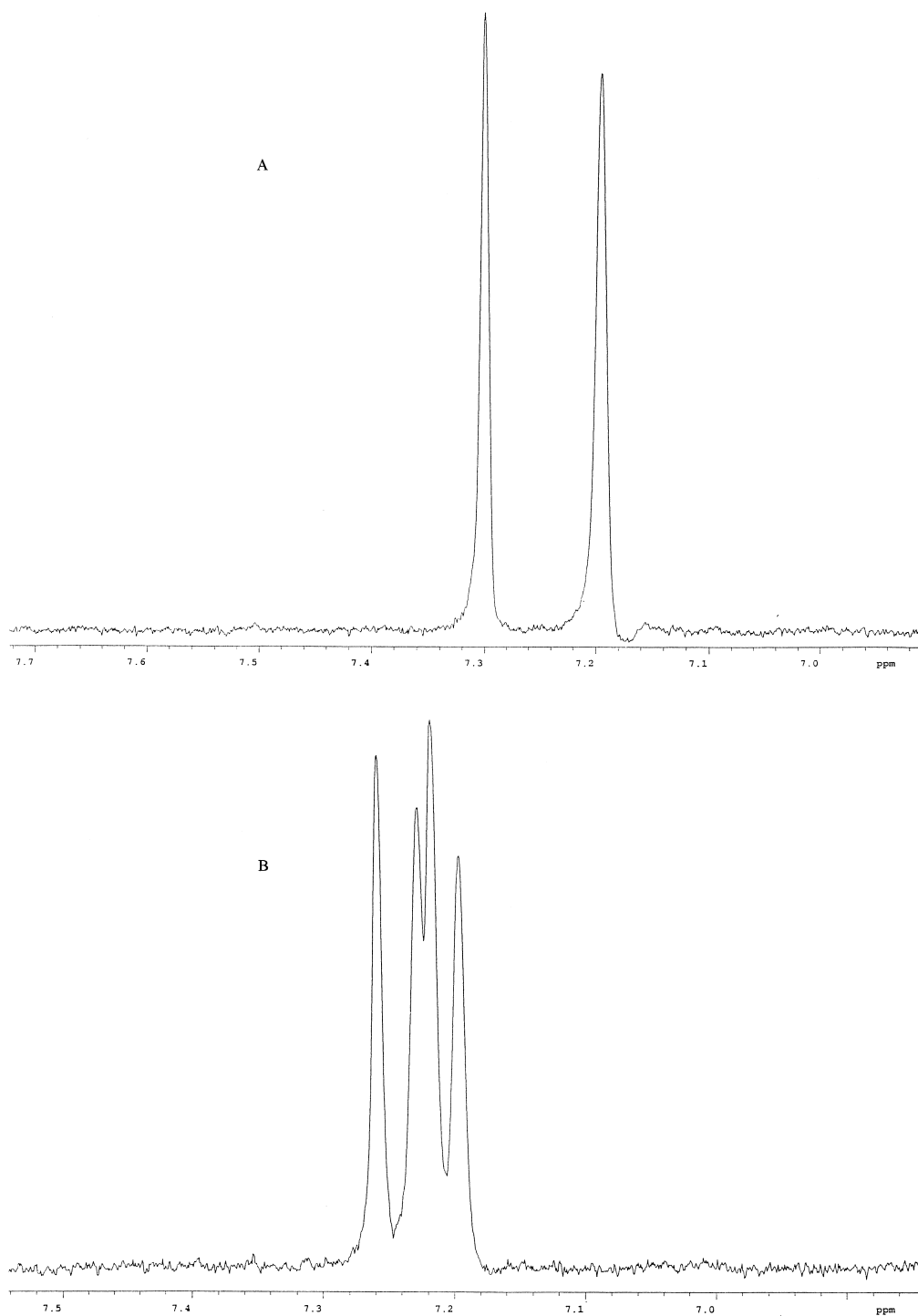
Fig. 4. NMR spectrum (400 MHz) of (A) oxamniquine (6.9–7.7 ppm) indicating the two aromatic singlets and (B) oxamniquine (6.9–7.5 ppm) after mixing with CM-β-CD in a 1:1 molar ratio indicating shift displacement (coupled with shift non-equivalence).

Table 3
Shift non-equivalence values ($\Delta\delta$*, Hz) observed for racemic oxamniquine on addition of cyclodextrins in a 1:1 ratio at pH 3.0

CD additive	H1′	H4′
α-CD	—	—
β-CD	—	—
γ-CD	—	—
HP-β-CD	—	—
HE-β-CD	—	—
CM-β-CD	16.1	12.7
SBE-β-CD	8.3	—
SBE-β-CD(4)	12.0	—
SBE-β-CD(1)	4.7	—

(—), no shift non-equivalence was observed.

non-equivalence values. The anionic CM-β-CD was the only CD to show shift non-equivalence for both H1′ and H4′ resonance signals. The magnitude of these shifts for H1′ and H4′ were 16.1 and 12.7 Hz, respectively, which were larger than those observed for any of the anionic SBE-β-CDs. It may be reasonable then to postulate that the anionic CM-β-CD would be the chiral selector of choice for enantiorecognition in CE or LC.

Comparing the selectivity of the anionic SBE-β-CDs as regards the H1′ signal, it may also be postulated that the degree of substitution of this CD is crucial. The SBE-β-CD(4) showed larger shift displacement and shift non-equivalence values for H1′ than did both the higher and lower substituted mixtures of this CD. It is thus reasonable to conclude that the degree of substitution is a crucial factor in any enantiorecognition observed in CE or LC for oxamniquine.

3.1.4. Ionic considerations

The differences in interaction between several drugs and a neutral CD compared with an anionic CD were recently evaluated by Okimoto et al. [47]. In that study, the role of charge was investigated during the interaction of charged and uncharged drugs with the neutral HP-β-CD and the anionically charged SBE7-β-CD. Using the phase solubility method, binding studies were carried out for a number of charged and uncharged drugs, both cationic and anionic, with these two CDs. The authors concluded from these studies that ''the binding constants for the neutral forms of the drugs studied were always greater with SBE-β-CD than with HP-

β-CD, while the binding constants for the cationic drugs studied with SBE-β-CD were significantly superior to those for HP-β-CD''.

It is thus important when considering any ionic CD and its interaction with a drug, to consider the ionic state of *both* drug *and* CD at the working pH or (pD) value. Oxamniquine has a measured basic pK_a value of 8.62 corresponding to its secondary amine. It will also have a much lower basic pK_a value (≈ 3.0) corresponding to the 1,2,3,4-tetrahydroquinoline. CM-β-CD has a measured acidic pK_a value of 4.36. For technical reasons, the three differently substituted SBE-β-CDs do not have reliable measured pK_a values. However, it is generally considered that these SBE-β-CDs are fully anionic at pH values >2, as a result of the strong sulphonic acid moiety.

Shift displacement and shift non-equivalence values for the oxamniquine singlets were observed at pH 3.0. At this pH, both oxamniquine and SBE-β-CD are essentially fully ionised, compared with CM-β-CD which is $\approx 10\%$ ionised. It therefore seems likely that the magnitude of these shifts may be enhanced, or decreased, on changing the ionic state of *either* oxamniquine *or* CM-β-CD. For the oxamniquine–SBE-β-CD mixtures, however, only the ionisation state of oxamniquine is altered by pH.

The shift displacement and shift non-equivalence values observed for oxamniquine on addition of anionic CDs at pH 3.0 were also made at two higher pH values: pH 5.0 for CM-β-CD and pH 7.0 for SBE-β-CD. These data are presented in Table 4. The

Table 4
Shift displacement ($\Delta\delta$, Hz) and shift non-equivalence values ($\Delta\delta$*, Hz) observed for racemic oxamniquine for evaluating the effect of ionic state when mixed with anionic CDs in a 1:1 molar ratio

CD additive	H1′		H4′	
	$\Delta\delta$	$\Delta\delta$*	$\Delta\delta$	$\Delta\delta$*
SBE-β-CD, pH 3.0	11.2 U	8.3	29.0 D	—
SBE-β-CD, pH 7.0	15.6 U	8.8	29.8 D	—
SBE-β-CD(4), pH 3.0	23.3 U	12.0	12.4 D	—
SBE-β-CD(4), pH 7.0	21.7 U	7.8	11.4 D	—
SBE-β-CD(1), pH 3.0	16.7 U	4.6	3.2 D	—
SBE-β-CD(1), pH 7.0	20.5 U	5.4	5.4 D	—
CM-β-CD pH 3.0	24.2 U	16.1	7.1 D	12.7
CM-β-CD pH 5.0	22.0 U	6.8	1.0 D	—

(—), no shift non-equivalence was observed.

observed shifts in resonance signals at each pH value were calculated, relative to the uncomplexed resonance signal at that pH; any shift non-equivalence observed was measured as the difference in resonance signal position between each enantiomer, as noted above.

The considerable reduction in shift displacement and shift non-equivalence values observed for the two diagnostic aromatic singlets of oxamniquine, H1′ and H4′, on changing the ionic state of CM-β-CD from pH 3.0 (≈4% CD ionised) to pH 5.0 (≈80% CD ionised) are considered significant. This reduction is particularly noticeable for H4′, where a 7-fold decrease in shift displacement and a complete loss of shift non-equivalence is observed. Considerable reductions in shift displacement and shift non-equivalence are also observed for H1′ when increasing the pH from 3.0 to 5.0 with CM-β-CD. However, the shift displacement and shift non-equivalence values observed for the diagnostic singlets after addition of SBE-β-CD are considered to be not significant. This may be attributed to little change in the ionisation state of the secondary amine moiety of oxamniquine on changing the pH from 3.0 to 7.0.

The significant reduction in shift values for oxamniquine with the anionic CM-β-CD at elevated pH may indicate the presence of some ionic or ion-pair association between this drug and CD. At pH 5.0, oxamniquine is essentially fully cationic as a result of protonation of the secondary amine, while the CM-β-CD is ≈80% anionic. This may result in an ion-pair association between the cationic oxamniquine and anionic carboxymethyl sidechains of CM-β-CD. It is possible then that this ion-pair association may be effectively precluding the total inclusion of the aromatic ring into the CM-β-CD toroid, leading to the lower shift values observed (Table 4). At pH 3.0, however, oxamniquine is still fully cationic, whereas the CM-β-CD is only ≈4% anionic. This would be expected to result in reduced or indeed the absence of, an ion-pair association between oxamniquine and the CM-β-CD, leading to a greater extent of inclusion and thus higher shift values.

From these observations, it is reasonable to postulate that at pH 3.0 the aromatic moiety of oxamniquine is probably being included into the CM-β-CD toroid. The increased enantioselectivity observed for this anionic CD over any other CD studied at pH 3.0 may *not* thus arise from the ionic moiety, but rather from the presence of the carboxyl group and thus its potential for stronger hydrogen bonding, or steric interactions, with oxamniquine compared with the unsubstituted secondary hydroxyl groups or derivatised CDs.

3.1.5. 2D ROESY NMR

A similar set of 2D ROESY experiments to those recently reported [27] was studied to investigate the mode of interaction or complexation between oxamniquine and the anionic SBE-β-CD. 2D ROESY experiments were carried out on a mixture of oxamniquine and the anionic SBE-β-CD(7) in a 1:1 molar ratio. A 2D ROESY spectrum of a 1:1 mixture of oxamniquine and SBE-β-CD at 500 MHz is shown in Fig. 5. In this spectrum, cross-peaks are observed between hydrogens which are close in space. Intermolecular cross-peaks between hydrogens both in SBE-β-CD and in oxamniquine are observed (ca. 3.8–4.0 ppm). This provides some evidence for inclusion complexation, through the presence of weak intermolecular cross-peaks observed in the F1 dimension (these peaks are also present in the F2 dimension), between the two aromatic hydrogens of oxamniquine and the internal glucopyranose hydrogens of the SBE-β-CD(7). This mode of complexation is also supported by the *absence* of any intermolecular cross peaks between the aromatic hydrogens of oxamniquine and the sulphobutyl methylene hydrogens.

These data can be interpreted as indicating that an inclusion complex is formed between oxamniquine and the anionic SBE-β-CD by insertion of the aromatic ring into the CD cavity. These data also indicate that steric hindrance arising from the sulphobutyl derivatives of the more highly derivatised SBE-β-CD(7), is not likely to occur for oxamniquine. There is also no clear indication from this spectrum that ion-pair association is or is not taking place between oxamniquine and SBE-β-CD. It would be reasonable to postulate that, in addition to the inclusion process, an ion-pair association could also be taking place. This would effectively result in higher binding constants for each oxamniquine enantiomer with this anionic CD, compared with the
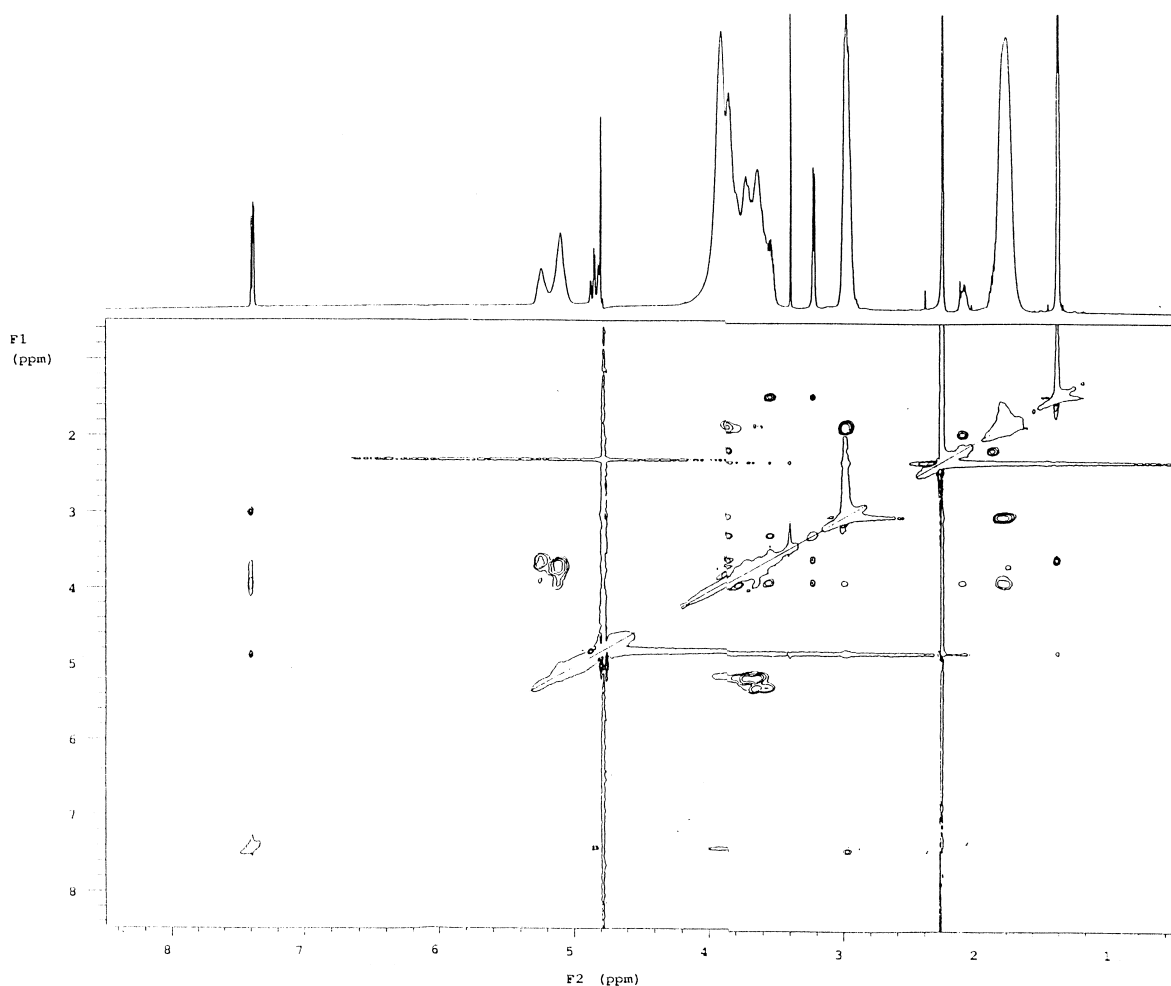
Fig. 5. NMR spectrum (500 MHz, 2D ROESY) of oxamniquine (0.005 *M*) after addition of SBE-β-CD in a 1:1 molar ratio.

neutral CDs, which would be expected to show greater 1D NMR shifts.

Interestingly from a similar set of 2D ROESY experiments for amlodipine with this SBE-β-CD(7), it was concluded that a complete inclusion complex involving insertion of the aromatic ring into the CD cavity, was probably *not* taking place [27]. This lower degree of inclusion complexation may be attributable to steric effects, where the sulphobutyl sidechains of the SBE-β-CD(7) (DS=6.5) could be 'crowding' the wider annulus of the toroid, thus hindering the inclusion process. There was some evidence for this theory with NOE interactions between the sulphobutyl sidechains and the aromatic

ring of amlodipine [27]. Another more plausible theory for the lack of inclusion of amlodipine with this CD was that an ion-pair association was probably formed between the anionic sulphobutyl derivatives and the cationic amlodipine molecule.

These data with SBE-β-CD for oxamniquine indicating that steric hindrance is not a major factor, suggest that it is less likely that steric hindrance is occurring for the interaction with amlodipine. It seems likely then, that the lack of any significant inclusion of the amlodipine molecule into the SBE-β-CD cavity, could be due to ionic or ion-pair association between the cationic amlodipine and the anionic SBE-β-CD.

3.1.6. Spin-lattice relaxation times

Spin-lattice relaxation time (T_1) measurements were acquired for each oxamniquine hydrogen and compared to the T_1 values for the same hydrogens in a mixture of oxamniquine–SBE-β-CD (1:1 molar ratio) as shown in Table 5. The large reductions in T_1 values observed for the aromatic H1′ (67.5%) and H4′ (31.6%) of oxamniquine on mixing with the SBE-β-CD support the 2D ROESY data that an inclusion complex is been formed. The T_1 value for H1′ in the free molecule (7.7 s) is significantly larger than that measured for the second aromatic hydrogen H4′ (1.9 s). Dipole–dipole interactions are known to contribute and assist the spin-lattice relaxation mechanism for hydrogen nuclei in a molecule. The isolation of the H1′ hydrogen from other hydrogens and thus the absence of any possible dipole–dipole interactions, accounts for the large spin-lattice relaxation time observed. On complexation with SBE-β-CD however, its T_1 value is reduced significantly to 2.5 s due to dipole–dipole interactions with the internal glucopyranose hydrogens of the CD. These observations support the findings of Cahill and Bulusu [37] with analogous complexation systems using native CDs.

3.2. Capillary electrophoresis

3.2.1. Neutral cyclodextrins

Racemic oxamniquine was run using 50 m*M* NaH$_2$PO$_4$ dihydrate electrolyte, pH 3.0, initially with no CD added, and finally with 20 m*M* of each of the five neutral CDs added to the electrolyte. The migration times and separation index values obtained for oxamniquine enantiomers on addition of these CDs to the electrolyte are shown in Table 6. These data represent a direct comparison of the properties of each neutral CD for the separation of oxamniquine enantiomers, where the electrophoretic conditions selected were identical.

The data in Table 6 indicate the difficulty in separating the enantiomers of oxamniquine in CE. The derivatised HP-β-CD was the only neutral CD to display any measurable separation at 20 m*M*. All these CDs, with the exception of β-CD due to its poor solubility, were also investigated at a concentration of 25 m*M* where enhanced enantioselectivity was observed for both HP-β-CD and HE-β-CD, with peak separation indices of 0.45 and 0.1, respectively. The native α-CD and γ-CD did not show any enantioselectivity when used at a concentration of 25 m*M*.

3.2.2. Anionic cyclodextrins

The anionic CM-β-CD and SBE-β-CD were investigated as chiral selectors in CE for racemic oxamniquine. It was necessary to investigate SBE-β-CD at pH 7.0, as the EOF (in the cathodic direction) was found to be insufficient at pH 3.0 to allow the analyte-SBE-β-CD complex to migrate towards the detector. This is because the SBE-β-CD migrates *against* the EOF in the anodic direction. Table 7 describes the electrophoretic conditions used for studying the resolution of oxamniquine enantiomers with these anionic CDs added to the electrolyte.

The difficulty of separating the enantiomers of oxamniquine is seen once again when using the anionic CDs in CE. Separation is achieved in a

Table 5
Spin-lattice relaxation time (T_1) measurements for the hydrogen atoms of oxamniquine alone and for the oxamniquine–SBE-β-CD mixture

Oxamniquine assignment	T_1 (s)	
	Free molecule	1:1 mixture
H1′	7.7	2.5
H4′	1.9	1.3
7-H	1.2	1.0
12-H	1.6	1.1
11-CH$_2$	0.5	0.5
5-CH$_2$	0.5	0.7
6-CH$_2$	0.5	0.5
13-CH$_3$, 14-CH$_3$	0.9	0.7

The estimated error in T_1 value is ±0.09 s (±0.2 s for H1′) reported by the NMR spectrometer at 500 MHz.

Table 6
Migration times and separation index values (P_i) for oxamniquine enantiomers–50 m*M* NaH$_2$PO$_4$ dihydrate, pH 3.0, run at 20 kV

CD additive	Migration time (min)		P_i
	t_{M1}	t_{M2}	
None		5.89	—
20 mM α-CD		6.82	0
20 mM β-CD		8.19	0
20 mM γ-CD		7.67	0
20 mM HP-β-CD	8.43	8.50	0.37
20 mM HE-β-CD		8.3	Shoulder

Table 7
Migration times and separation index values (P_i) for oxamniquine enantiomers–50 mM NaH$_2$PO$_4$ dihydrate run at 15 kV

CD additive	pH	Migration time (min)		P_i
		t_{M1}	t_{M2}	
3 mM CM-β-CD	3.0	16.5	16.75	0.95
2.5 mM CM-β-CD	3.0	16.6	16.9	0.85
None	7.0	3.61		—
1 mM SBE-β-CD	7.0	4.46		0
5 mM SBE-β-CD	7.0	6.56	6.64	0.1
10 mM SBE-β-CD	7.0	7.5		0
15 mM SBE-β-CD	7.0	8.0		0

Table 8
Migration times and separation index values (P_i) for oxamniquine enantiomers on changing the DS of SBE-β-CD–50 mM NaH$_2$PO$_4$ dihydrate, pH 7.0, run at 15 kV

CD additive	Migration time (min)		P_i
	t_{M1}	t_{M2}	
1 mM SBE-β-CD[a]	4.46		0
15 mM SBE-β-CD	8.0		0
1 mM SBE-β-CD(4)[b]	4.03	4.15	0.2
5 mM SBE-β-CD(4)	5.7	5.8	0.45
10 mM SBE-β-CD(4)	6.24	6.4	0.83
15 mM SBE-β-CD(4)	7.0	7.2	0.98
1 mM SBE-β-CD(1)[c]	3.7		0
15 mM SBE-β-CD(1)	3.9		0

Actual DS values for these SBE-β-CDs are: [a]6.5; [b]4.5; and [c]1.0.

reasonable analysis time with the addition of only 3 mM CM-β-CD to the electrolyte, illustrated in Fig. 6. This represents an increase in separation, although at a very significant decrease in concentration relative to that for the neutral CDs (25 mM). The electrophoretic parameters for this separation were investigated further, but any change from these initial conditions resulted in a loss of enantioselectivity. Surprisingly, no practicable separation of oxamniquine enantiomers was achieved with the addition of SBE-β-CD to the electrolyte over a range of concentrations (1–15 mM).

The separation of oxamniquine enantiomers was explored further with three batches of SBE-β-CD characterised by different DS values. Table 8 illustrates the effect of changing the substitution pattern of SBE-β-CD on the chiral separation of oxamniquine. Reducing the DS of SBE-β-CD was shown to have a pronounced effect on the chiral separation of oxamniquine. The SBE-β-CD with higher substitution offered little enantioselectivity at any con-
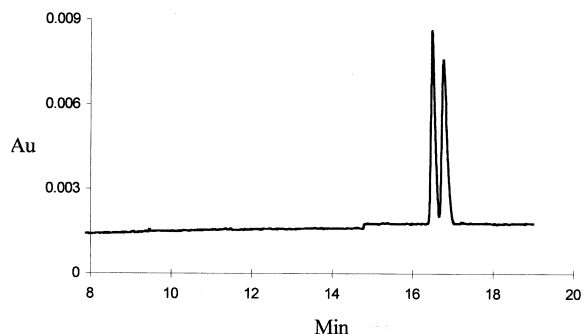

Fig. 6. Separation of oxamniquine enantiomers: 50 mM NaH$_2$PO$_4$ (pH 3.0) containing 3.0 mM CM-β-CD run at 15 kV.

centration, which is in complete contrast to that shown by the lower substituted SBE-β-CD(4). In fact, by reducing the applied voltage to 10 kV, the enantiomers of oxamniquine were fully separated in 13 min using 15 mM SBE-β-CD(4) at pH 7.0, as shown in Fig. 7. The enantioselectivity shown by SBE-β-CD(4) for oxamniquine may be attributable to reduced steric hindrance at the larger annulus of the CD cavity, allowing a stronger inclusion complex to be formed. A reduction in mobility of the SBE-β-CD(4) in the anodic direction (against the EOF) with a consequent increased mobility towards the detector, appears to be a crucial factor influencing this separation.

These results indicating the influence of CD degree of substitution on the enantiorecognition process for oxamniquine are in agreement with those found in previous LC and GC studies using neutral CD bonded stationary phases [14,15]. They also agree with the findings of others when using neutral CDs as chiral selectors in CE [16]. Surprisingly however, a recent report on the first use of another anionic derivative, sulphoethyl-β-CD, as a chiral selector for R/S-1,1′-binaphthyl-2,2′-diyl hydrogenphosphate in CE, claimed that ''the spacer arm length and substitution pattern of this CD are factors with little importance'' [18]. Szejtli's group has studied the effects of separating conditions with emphasis on the degree of substitution of a CD, when using the anionic CM-β-CD in both LC and CE [48]. Their studies showed that the substitution pattern has a significant effect on the resolution of
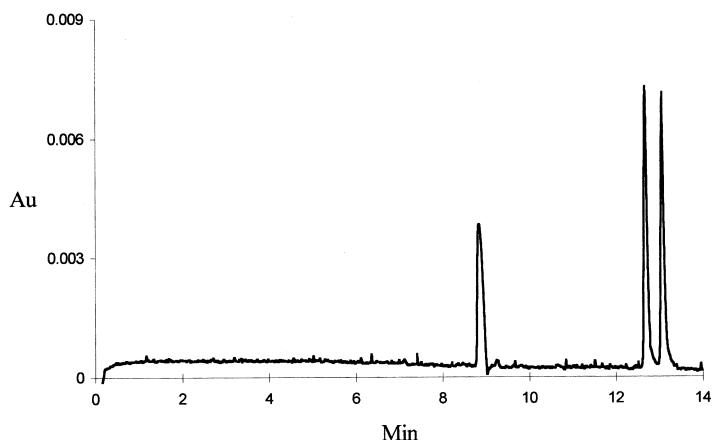
Fig. 7. Separation of oxamniquine enantiomers: 50 mM NAH$_2$PO$_4$ (pH 7.0) containing 15 mM SBE-β-CD(4) run at 10 kV.

racemic basic drugs, and they conclude that "the use of well characterised CD derivatives is crucial".

3.3. Possible correlations between NMR and CE

Possible correlations between the shift non-equivalence values observed for the two aromatic hydrogens of oxamniquine and the optimum chiral separation found for each CD studied in CE are shown in Table 9. A number of correlations may be made from these data. No shift non-equivalence values were observed for any of the native CDs, which is fully reflected in the CE data where no separation was observed. Surprisingly, this was not the case for the neutral derivatised CDs, where some separation was found for HP-β-CD (albeit less than

Table 9
Possible correlations of shift non-equivalence ($\Delta\delta^*$, Hz) data for oxamniquine and CDs with enantiorecognition in capillary electrophoresis (P_i)

CD additive	$\Delta\delta^*$		P_i
	H1′	H4′	
α-CD	—	—	0
β-CD	—	—	0
γ-CD	—	—	0
HP-β-CD	—	—	0.45
HE-β-CD	—	—	0.1
CM-β-CD	16.1	12.7	0.95
SBE-β-CD	8.3	—	0.1
SBE-β-CD(4)	12.0	—	1.0
SBE-β-CD(1)	4.7	—	0

50%), even though no shift non-equivalence was observed.

CM-β-CD has shown the largest shift non-equivalence value for oxamniquine and was the only CD to resolve the H4′ resonance signal. This corresponds to a 95% chiral separation in CE. It may be possible that this separation could be further optimised using chemometric methods, similar to those reported earlier [49]. It is also possible that the DS of CM-β-CD (DS=5.3) could be playing a crucial role in the enantiorecognition process, as already shown for oxamniquine with SBE-β-CD in the present work.

The data for SBE-β-CD as a chiral selector for racemic oxamniquine may represent an example of the potential of NMR for choosing an appropriate CD chiral selector in CE. The lower substituted SBE-β-CD(1) when used as a chiral selector in the 1D NMR studies, shows evidence of interaction with the H1′ hydrogen of oxamniquine, giving a shift non-equivalence value of 4.7 Hz; however, it shows no enantioselectivity in CE. This shift non-equivalence value is increased to 8.3 Hz when using the highly substituted SBE-β-CD(7) as a selector in the NMR studies, and this corresponds to an increase in the chiral separation in CE. This trend is continued for the SBE-β-CD(4) chiral selector, where the observed shift non-equivalence value is increased further to 12 Hz, corresponding to a complete chiral separation in CE.

The nature and extent of complexation between oxamniquine and SBE-β-CD(7) could not be quan-

tified using the 2D NMR experiments, but one strong possibility is that SBE-β-CD(4) also involves an inclusion complexation. It would thus be interesting to investigate the complexation between each ox-amniquine enantiomer and the three differently sub-stituted SBE-β-CD derivatives quantitatively, using similar calorimetric methods to those employed on native CDs [50]. These studies showed a correlation between equilibrium constants determined by titra-tion calorimetry and resonance signal shifts in NMR for 29 related drugs with α-CD and β-CD. It would be interesting to determine whether or not a good correlation, or correspondence, between a spectro-scopic, separative and a thermal method of analysis could be demonstrated for the enantiomeric binding of oxamniquine to SBE-β-CD characterised by dif-ferent DS values.

4. Conclusions

One-dimensional ^{1}H NMR has been successfully used in examining shift displacement and shift non-equivalence data observed for two diagnostic aro-matic hydrogens of oxamniquine after separate addi-tion of seven CDs under identical conditions. The two types of shift observed were examined to interpret the nature of interactions between oxam-niquine with five neutral CDs and two anionic CDs. Similar 1D NMR experiments were also carried out to evaluate the effect of the degree of substitution of the anionic SBE-β-CD.

Shift displacement data indicated interaction of oxamniquine with each of the CDs studied, but conclusions could not be drawn from the magnitude of these shifts in terms of indicating a suitable CD chiral selector for subsequent CE studies. Shift non-equivalence data for oxamniquine aromatic hydro-gens indicated quite clearly, however, that signifi-cantly lower interaction was taking place between oxamniquine and the neutral CDs compared with that for the anionic CDs. These differences observed in the 1D NMR were subsequently reflected in the CE studies, where no separation was obtained for any of the native CDs, and only poor separation was obtained for neutral derivatised HP-β-CD. These data were in contrast to the data for anionic CM-β-CD, however, where large shift non-equivalence

values were observed for both aromatic hydrogens and this corresponded to a 95% separation of ox-amniquine enantiomers when using this CD in CE.

The SBE-β-CD data were anomalous however, since the shift non-equivalence values observed did not correspond to a satisfactory separation of ox-amniquine in CE. On examination by NMR of the effect of the degree of substitution of these CDs, it was shown that on lowering the DS value from 6.5 to 4.5, an increase in shift non-equivalence from 8.3 to 12.0 Hz was observed. In the CE studies, this decrease in DS of SBE-β-CD also corresponded to an increase in enantioselectivity, which went from zero separation to 98% separation. The complexation mechanism between oxamniquine and the anionic SBE-β-CD was examined using 2D ROESY experi-ments and T_1 measurements. Both techniques gave concordant results indicating that complexation was taking place via inclusion of the aromatic ring of oxamniquine into the SBE-β-CD cavity. It is im-portant that these results be interpreted in relative terms, however, since the SBE-β-CDs are mixtures and the complex between one defined SBE-β-CD and oxamniquine is not examined, but of a mixture of complexes.

A further conclusion that can be drawn from these NMR and CE studies between oxamniquine and CDs is that the degree of substitution of derivatised CDs, both neutral and ionic, has a highly significant effect on the resolution of enantiomers indicating the necessity for their characterisation before use.

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