Journal of Inclusion Phenomena and Macrocyclic Chemistry **38:** 133–151, 2000.
© 2000 *Kluwer Academic Publishers. Printed in the Netherlands.*

Complexation of Voriconazole Stereoisomers with Neutral and Anionic Derivatised Cyclodextrins

PAUL K. OWENS[1,2,⋆], ANTHONY F. FELL[2], MICHAEL W. COLEMAN[3] and JOHN C. BERRIDGE[3]
[1] *Analytical Chemistry, Pharmaceutical Research and Development, Astra Zeneca R&D Mölndal, S-431 83, Mölndal, Sweden*
[2] *Pharmaceutical Analysis Research Unit, Pharmaceutical Chemistry, School of Pharmacy, University of Bradford, Bradford, BD7 1DP, UK*
[3] *Analytical Research & Development Department, Pfizer Central Research, Sandwich, Kent, CT13 9NJ, UK*

(Received: 4 June 1999; in final form 12 August 1999)

Abstract. A number of native, neutral derivatised and anionic derivatised cyclodextrins (CDs) were examined as chiral electrolyte additives in capillary electrophoresis (CE) to separate the four stereoisomers of the new antifungal agent, voriconazole. A very large difference in interaction between each diastereoisomer and the CDs was observed in the CE study, where enantioselectivity was easily obtained for one and extremely difficult to obtain for the other. Nuclear magnetic resonance spectroscopy (^{1}H-NMR) indicated a strong interaction between the easily separated diastereoisomer and each of the CDs with enantiomeric shift nonequivalence values of over 100 Hz obtained when using the anionic sulphobutylether-β-CD chiral solvating agent. In accordance with observations from the CE study, the opposite diastereoisomer indicated no shift nonequivalence at all. The nature of the complexation between the easily separated diastereoisomer and the anionic sulphobutylether-β-CD was also probed using a two-dimensional nuclear Overhauser enhancement experiment and a series of spin lattice relaxation time measurements. It was found that the enantioselective interaction occurred through the partial inclusion of a difluorophenyl group into the CD toroid which was also aided through a number of additional interactions between the drug molecule and the sulphobutylether derivatives outside the CD toroid.

Key words: capillary electrophoresis, nuclear magnetic resonance, charged cyclodextrin, voriconazole, diastereoisomer, shift nonequivalence

1. Introduction

Voriconazole is a new single enantiomer antifungal agent currently in clinical development which has potency against a broad range of fungal pathogens including *Aspergillus fumigatus* and *Candida krusei* [1–2]. The active agent, voriconazole (UK-109,496) has two chiral centres, as indicated in Figure 1 and has an absolute configuration of 2R, 3S. The opposite enantiomer (2S, 3R) is named UK-109,501. The diastereoisomer containing the mixture of voriconazole (UK-109,496) and its

⋆ Author for corresondence.

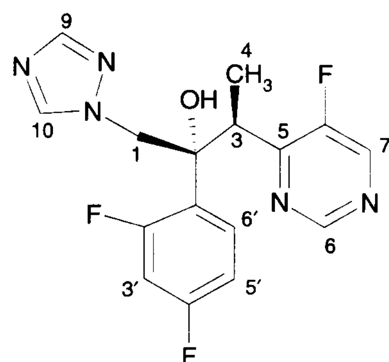

Figure 1. Chemical structure of the active antifungal agent, voriconazole (UK-109,496).

Table I. Chiral nomenclature for the four related voriconazole stereoisomers

Pfizer central research *in-house* name	Description	Absolute configuration
UK-109,496	The active drug, voriconazole	2R, 3S
UK-109,501	Opposite enantiomer of voriconazole	2S, 3R
UK-103,449	Mixture of voriconazole and enantiomer	2R, 3S and 2S, 3R
UK-103,451	The diastereoisomers of voriconazole	2R, 3R and 2S, 3S

enantiomer (UK-109,501) is called UK-103,449. The opposite diastereoisomer, a mixture of the 2R, 3R and 2S, 3S enantiomers is named UK-103,451. The nomenclature for the four voriconazole stereoisomers is shown in Table I.

Since this is a new drug entity, very little has been reported in the literature on its chromatographic or electrophoretic properties. In the first chromatographic report, a fully automated achiral method was developed for its direct analysis in human plasma using size exclusion chromatography [3]. The authors later developed a more suitable and robust achiral method employing acetonitrile precipitation prior to the chromatographic analysis [4]. In the first chiral chromatographic report concerning the stereoisomers of voriconazole, amylose-based chiral stationary phases were examined in the normal phase mode but it was necessary to employ an achiral-chiral method to completely separate the stereoisomers and related substances of voriconazole [5]. Recently, we reported the first chiral electrophoretic and chromatographic examination of the voriconazole stereoisomers employing cyclodextrin (CD) electrolyte and mobile phase additives, respectively [6]. In that report, although not described in the first chiral examination [5], we observed anomalous results when we compared the chiral chromatographic and electrophoretic properties of the two diastereoisomers of voriconazole (Table I). We found that, although the actual diastereoisomers were easily separated with similar capacity factors and electrophoretic mobilities in achiral liquid chromatography (LC) and

capillary electrophoresis (CE), respectively, the enantiomers of the UK-103,449 diastereoisomer could be easily separated without any problems, but the enantiomers of the opposite diastereoisomer, UK-103,451, were very difficult to separate.

One-dimensional (1D) NMR, can be used almost routinely now for investigating the stoichiometery, structure and stability of CD complexes, either prior or subsequent to chiral chromatographic or electrophoretic separation [7–12]. Additionally, the use of nuclear Overhauser enhancements (NOEs) and in particular two-dimensional (2D) NOE experiments in the rotating frame (ROESY) [13] which give positive and enhanced NOEs over the whole molecular range, have also been shown to be useful for examining the through-space interactions between drug and CD nuclei in a drug-CD complex [14–16]. Another approach which is often used to support 2D NMR experiments is the application of ^{1}H spin-lattice relaxation time (T_1) measurements which have been used by several groups to examine and corroborate 2D NMR experiments on drug-CD complexes [17–19]. These studies have shown that T_1 measurements can be used to obtain further information on the rotation and molecular assembly of a drug in a drug-CD complex [17–19].

In this paper, following our recent report on the surprising results indicating the differences in interaction between each diastereoisomer of voriconazole and the chiral chromatographic and electrophoretic systems [6], we report our subsequent nuclear magnetic resonance (NMR) examination on the nature of the interaction between the diastereoisomers and a number of native, neutral and anionic derivatised CDs. A series of 1D NMR experiments have been carried out in an attempt to explain the observed differences which are supported by a 2D NOE experiment and spin-lattice relaxation time measurements on one of the diastereoisomer-CD complexes.

2. Experimental

2.1. MATERIALS

The stereoisomers of voriconazole (Figure 1, Table I) and sulphobutyl-ether-CD (SBE-β-CD) sodium salt, DS = 6.5, were used as received from Pfizer Central Research (Kent, UK). α-CD, γ-CD and carboxymethyl-β-CD (CM-β-CD) sodium salt (DS = 5.3) were used as received from Wacker Chemicals Ltd. (Walton-on-Thames, Surrey, UK). β-CD, hydroxypropyl-β-cyclodextrin (HP-β-CD) (DS = 4.2) and hydroxyethyl-β-cyclodextrin (HE-β-CD) (MS = 11.2) were used as received from Aldrich Chemicals Co. Ltd. (Gillingham, Dorset, UK). SBE-β-CD(4) and SBE-β-CD(1) were gifts from CyDex Inc. (Kansas, USA) and were characterised by CE [20] as having DS values of 4.5 and 1.0, respectively. Deuterium oxide, 99.8% was obtained from Isotec Inc., Matheson, USA. Deuterium chloride (DCl), sodium deuteroxide (NaOD) and deuterated methyl alcohol (CD_3OD) were purchased from Fluorochem Ltd. (Old Glossop, Derbyshire, UK). Sodium dodecylsulphate (SDS), sodium dihydrogen orthophosphate dihydrate and sodium

dihydrogen orthophosphate anhydrous were purchased from BDH (Poole, Dorset, UK).

2.2. INSTRUMENTATION

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 degrees. 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 NMR experiments and T_1 measurements were obtained on the Varian Unity 500 NMR spectrometer, equipped with a 5 mm probe used for ^{1}H NMR observation. All spectra were recorded in D_2O solution. 2D ROESY spectra using a sweep window of 4898 Hz , acquisition time = 0.209 s, relaxation delay = 2 s, spin lock mixing time = 1 s and amplitude of spin lock = 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 = 20 s and acquisition time = 3.27 s. The interval between the 180 and 90 ° pulses was varied in the range 0.1 to 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 μm I.D. (50 cm effective length) obtained from Beckman Instruments (High Wycombe, Bukinghamshire, UK). The temperature of the cartridge was maintained constant throughout at 17 °C.

2.3. METHODS

A 400 MHz ^{1}H NMR spectrum was acquired initially for UK-103,449 and UK-103,451 in CD_3OD – 100 mM NaH_2PO_4 anhydrous at pD = 3.4 ± 0.1 (adjusted with deuterium chloride or deuterated sodium hydroxide as appropriate) (12.5 : 87.5 v/v) without any CD present. It was necessary to use an organic solvent to facilitate the dissolution of UK-103,449 and UK-103,451 since the solubility of these diastereoisomers is low in aqueous media. The assignment of the protons for both these diastereoisomers was previously performed (Unpublished data, Pfizer Central Research) and current results are in agreement with these. Additionally, the spin-lattice relaxation time measurements were used as an aid to confirm the spectral assignments. Subsequent to recording and assignment of proton signals

for the two diastereoisomers of voriconazole, spectra were recorded again for each diastereoisomer after the addition of CD in a 1 : 1 molar ratio under identical solvent conditions. CM-β-CD has a calculated acidic pK_a of 4.36 [12] and will have a different ionic state at pD 3.4 than at higher pD values. It was thus decided to acquire spectra for each diastereoisomer: CM-β-CD mixture both at pD 3.4 and 5.4 to determine if the different ionic state of this CD induced significantly different chemical shifts.

Two types of shift in the drug resonance signal were used to assess the interaction or complexation of each voriconazole diastereoisomer with each CD evaluated: (a) '*shift displacement* ($\Delta\delta$, Hz)' which is defined as 'the displacement value (up or downfield) of a singlet, or of a multiplet, after the addition of CD' and b) '*shift nonequivalence* ($\Delta\delta^*$, Hz)' which is defined as 'the magnitude of enantiomeric splitting of a singlet, or of a multiplet, after the addition of CD [12]. Shift displacement and nonequivalence values for split signals, doublets for example, were taken as the mean shift value between the two signals. The assignment of enantiomeric resonance signals in complex spectra was elucidated by examining the variation in splitting with CD concentration. In principle, each type of shift could be observed for a particular resonance either singly, or in combination. A number of spectra were acquired ($n = 10$) during a twelve hour period for similar mixtures, as recently reported by the present authors 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. It was concluded that relative standard deviation (RSD) values of 1.2% and 2.4% for shift displacement and shift nonequivalence, respectively, were acceptable when measuring drug resonance signals following complexation with a cyclodextrin [12].

The electrophoretic conditions used for assessing enantioselectivity are described in detail in an earlier report [6]. Enantiomeric separation 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 [21].

3. Results and Discussion

3.1. CAPILLARY ELECTROPHORESIS

Recently, we examined the use of CE and LC to develop a suitable routine method for the determination and separation of voriconazole and its related stereoisomers [6]. Since these stereoisomers are neutral, it was necessary to utilise a chiral micellar electrokinetic chromatography (MEKC) method when employing neutral CDs. Another approach was also adopted, the use of CDs that carried an overall anionic charge, SBE-β-CD and CM-β-CD. If complexation occurs between neutral enantiomers and an anionic CD, the drug will carry an effective charge and will thus migrate to a greater or lesser extent than the electroosmotic flow (EOF), thus creating the opportunity for a chiral separation. In conventional CE where the EOF

is in the cathodic direction, a CD carrying an overall cationic charge will result in the neutral drug enantiomers migrating before the EOF if complexation takes place; conversely, a CD carrying an anionic charge will result in the neutral drug enantiomers migrating after the EOF. It is thus not necessary to use a micellar system for the separation of neutral drug enantiomers when utilising a CD that carries an overall cationic or anionic charge [22].

The diastereoisomers, UK-103,449 and UK-103,451, were examined separately with either neutral CD modified MEKC using SDS surfactant or with the anionic SBE-β-CD and CM-β-CD. The migration times and separation index values obtained for each mixture on addition of these CDs to the electrolyte are shown in Table II. The enantiomers of UK-103,449 were completely separated using all the derivatised CD chiral selectors. The separation obtained for the UK-103,449 diastereoisomer and the corresponding electropherogram for UK-103,451 indicating no enantioselectivity is shown in Figure 2 when using (A) 20 mM HP-β-CD modified MEKC and (B) 1.0 mM SBE-β-CD. The active antifungal drug, voriconazole (UK-109,496) is a single enantiomer of the UK-103,449 diastereoisomer (Table I) and migrates before its opposite enantiomer (UK-109,501) when using either a neutral or an anionic CD. The effect of the CD derivative is clearly shown for UK-103,449 since the native β-CD only offered a 50% separation of this enantiomeric mixture. The most surprising data from these studies, however, were the contrasting results obtained for the two diastereoisomers. No enantioselectivity could be obtained at all for the UK-103,451 diastereoisomer when using either a neutral or an anionic CD in CE. This was in complete contrast to the results obtained for the UK-103,449 diastereoisomer where it was easily separated in CE using CD additives, but also in LC using both CD chiral stationary phases and mobile phase additives [6]. The differences between these two diastereoisomers were also highlighted in that report by the contrasting results obtained on an antibiotic chiral stationary phase in LC. It was possible to separate the enantiomers of the 'enigmatic' UK-103,451 diastereoisomer using the Chirobiotic V^{TM} vancomycin chiral stationary phase but only in the normal-phase mode. Surprisingly, it was not possible to develop a separation for the easier UK-103,449 diastereoisomer using this antibiotic phase in either reversed- or normal-phase LC.

It was clear from those CE and LC studies that the stereochemistry and thus the different overall three-dimensional spatial arrangement of each of the voriconazole stereoisomers was having an esoteric effect on chiral discrimination. It was thus decided to probe further the molecular interaction between these two diastereoisomers and each of the CDs examined in CE, or lack of it *per se*, using NMR spectroscopy.

3.2. NUCLEAR MAGNETIC RESONANCE

400 MHz 1D NMR spectra were obtained for each diastereoisomer separately, shown in Figure 3 for UK-103,449. The assignment of resonance signals and

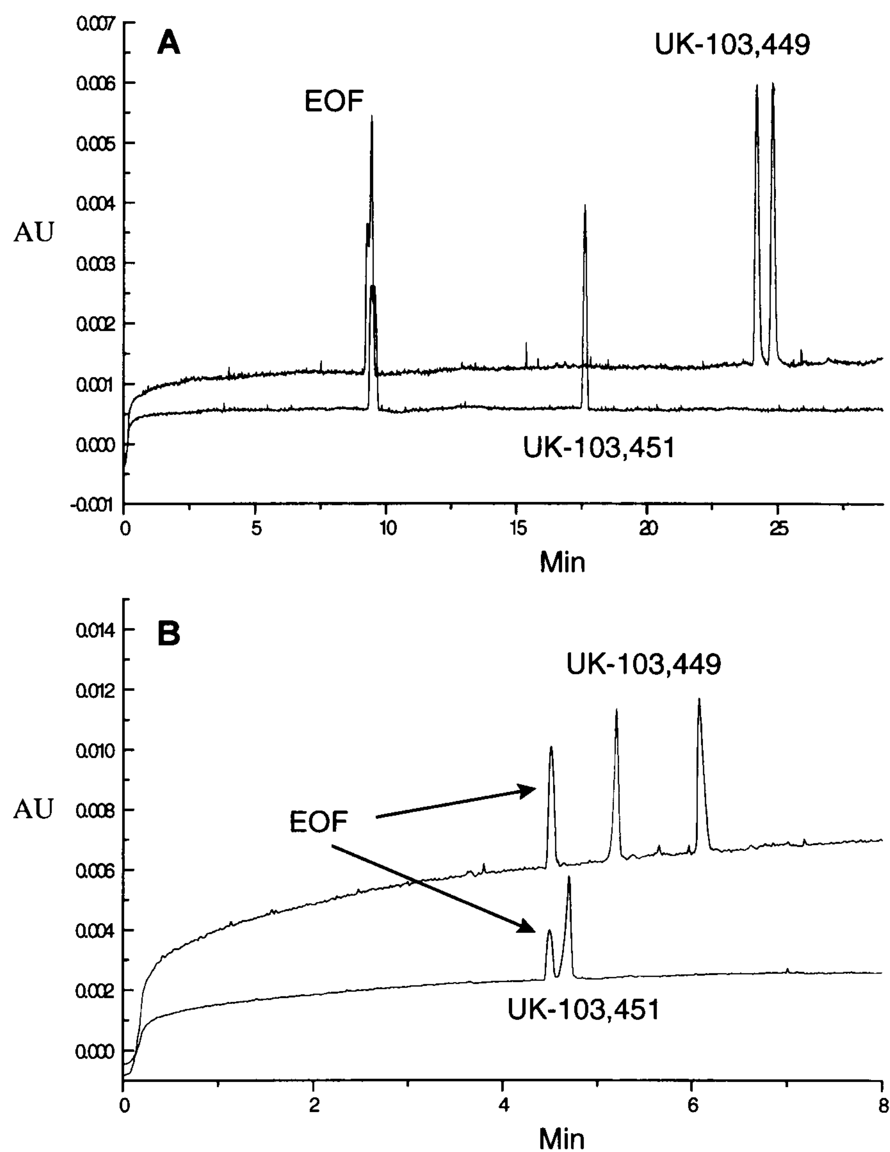

Figure 2. Chiral CE separation of the diastereoisomers, UK-103,449 and UK-103,451 using (A) 20 mM HP-β-CD modified MEKC and (B) 1.0 mM SBE-β-CD. Conditions: Fused silica capillary, 57 cm $\times$ 50 μm I.D. (50 cm effective length), 50 mM NaH_2PO_4 (pH 7.0) containing 50 mM SDS for (A) and CD as described, 214 nm, 17 °C and 15 kV.

Table II. Migration times and separation index values (P_i) for UK-103,449 and UK-103,451 when using CD modified MEKC and anionic CDs

CD additive	UK-103,449			UK-103,451		
	Migration		P_i	Migration		P_i
	t_{M1}	t_{M2}		t_{M1}	t_{M2}	
20 mM α-CD	35.4		0	26.4		0
20 mM β-CD	33.7	34.0	0.5	24.8		0
20 mM γ-CD	32.7		0.15	20.6		0
20 mM HP-β-CD	24.2	24.8	1.0	17.6		0
20 mM HE-β-CD	31.2	31.9	1.0	24.5		0
2.5 mM CM-β-CD †	8.1	8.5	1.0	7.6		0
1.0 mM SBE-β-CD	5.2	6.1	1.0	4.71		0

Fused silica capillary, 57 cm × 50 μm I.D. (50 cm effective length), 50 mM NaH_2PO_4 (pH 7.0) containing 50 mM SDS, 214 nm, 17°C, 15 kV.
† 50 mM NaH_2PO_4, pH 5.0.

500 MHz spin-lattice relaxation time measurements are shown in Table III. In addition to the use of spin-lattice relaxation time measurements for evaluating drug-cyclodextrin complexes [23–24] they may also be used, although a time consuming process, to help assign 1D resonance signals since the potential for dipole-dipole interactions contribute to the spin-lattice relaxation mechanisms leading to reduced T_1 times [25]. The T_1 value for the triazole hydrogen resonating at 7.61 ppm is twice that (6.8 s) for the hydrogen resonating at 8.15 ppm (3.4 s). Consequently the latter signal is assigned to H10 since it would be expected to have a shorter T_1 value due to its position relative to the methylene hydrogens (H1) and thus their contribution to the relaxation mechanism of H10. Similarly, the potential for dipole-dipole interactions between the pyrimidine fluorine and H7 atoms contribute to its shorter T_1 value (4.4 s) compared with that for H6 (7.4 s). 1D NMR spectra were subsequently acquired for each diastereoisomer of voriconazole separately, UK-103,449 and UK-103,451, each in a 1 : 1 molar mixture with each of the seven CDs chosen: α-CD, β-CD, γ-CD, HP-β-CD, HE-β-CD, CM-β-CD and SBE-β-CD under identical conditions to those when measured without a CD.

3.2.1. *Shift displacement*

It was decided to examine the two triazole hydrogen atoms (H9 and H10) and the two pyrimidine hydrogen atoms (H6 and H7, Figure 1, Table III) as possible diagnostic hydrogens for evaluating any observed shift displacement or shift nonequivalence since each appears as a singlet. These four singlets (7.61–8.85 ppm) were chosen on the basis that they were not overlapped by any CD resonance signals, or others arising from the drug itself. The three aromatic hydrogens (6.79–

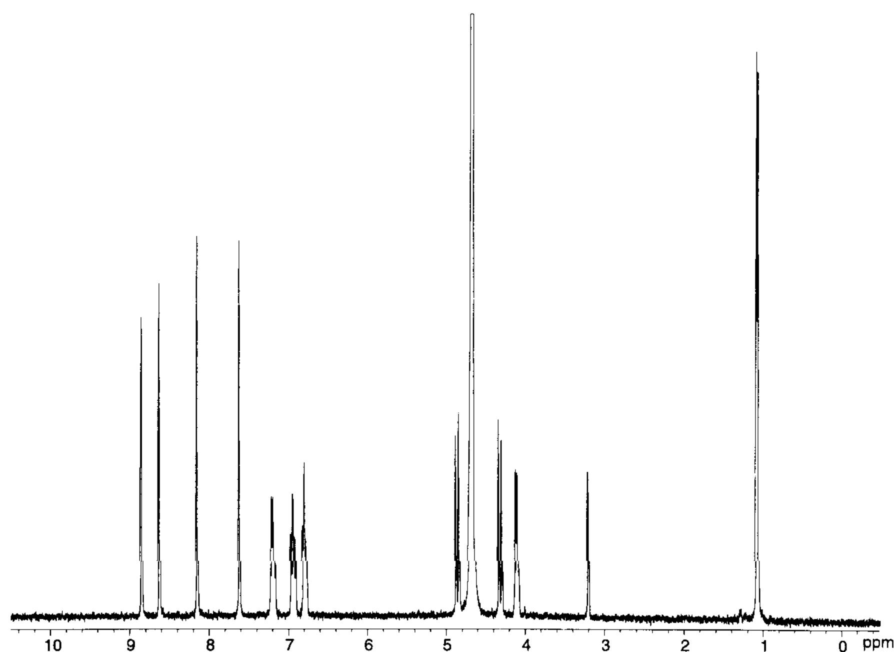

Figure 3. A 400 MHz NMR spectrum of the UK-103,449 diastereoisomer containing the active antifungal agent, voriconazole (UK-109,496).

Table III. 400 MHz proton resonance signal assignments and 500 MHz spin-lattice relaxation time (T_1) measurements for the UK-103,449 diastereoisomer of voriconazole. Relaxation time measurements were determined prior and subsequent to mixing with the SBE-β-CD in a 1 : 1 molar mixture

Chemical shift ppm	Multiplicity	Proton assignment	Free Molecule T_1 (s)	1 : 1 Mixture T_1 (s)
8.85	Singlet	6	7.4	2.4
8.62	Singlet	7	4.4	3.2
8.15	Singlet	10	3.4	not measured
7.61	Singlet	9	6.8	1.9
7.19	Quartet	6′	2.1	3.0
6.93	Triplet	3′	2.8	2.0
6.79	Triplet	5′	2.1	2.0
4.85	Doublet	1a	0.5	0.6
4.31	Doublet	1b	0.5	0.6
4.10	Quartet	3	1.1	0.8
1.07	Doublet	4	0.7	0.6

The estimated error in T_1 value is ± 0.2 s reported by the NMR spectrometer at 500 MHz.

Table IV. Shift displacement ($\Delta\delta$, Hz) values observed for UK-103,449 on complexation with cyclodextrins in a 1 : 1 ratio at pD 3.4

CD additive	H6	H7	H9	H10
α-CD	0.8 D	2.0 D	5.2 D	2.8 D
β-CD	26.8 U	4.0 U	6.8 U	36.0 D
γ-CD	15.6 D	15.2 D	6.0 D	0.8 D
HP-β-CD	50.0 U	5.6 U	20.0 U	65.2 D
HE-β-CD	40.0 U	5.2 U	18.4 U	49.2 D
CM-β-CD	29.2 U	8.0 U	16.0 U	31.2 D
CM-β-CD pD 5.4	22.8 U	5.2 U	9.6 U	30.0 D
SBE-β-CD	66.4 U	0.8 U	20.0 U	93.6 D
SBE-β-CD(4)	48.0 U	3.6 U	16.0 U	58.4 D
SBE-β-CD(1)	36.4 U	6.8 U	14.0 U	42.0 D

D = Downfield. U = Upfield.

7.19 ppm) were not chosen since they appear as a quartet and two triplets due to coupling with fluorine nuclei and thus may give complex multiplets on addition of CDs in comparison with the four singlets chosen. Shift displacement ($\Delta\delta$, Hz) values observed for each of the diagnostic singlets of UK-103,449 with respect to the same uncomplexed signals after the addition of each of the CDs in a 1 : 1 ratio at pD 3.4, are shown in Table IV. A representative 400 MHz NMR spectrum (6.0–9.5 ppm) for (A) the UK-103,449 diastereoisomer without any CD added and (B) after an equimolar addition of SBE-β-CD indicating shift displacement is shown in Figure 4. Voriconazole and CM-β-CD have measured basic and acidic pKa values of 1.76 and 4.36, respectively. It was thus decided to examine the chargeable CM-β-CD at two pD values, pD 3.4 and 5.4, to investigate if the degree of ionisation of this CD will influence any observed shifts for this neutral drug. The observed shifts in resonance signals at each pD value were calculated, relative to the uncomplexed resonance signal at that pD as outlined previously. It was also decided to investigate the batches of the anionic SBE-β-CD, characterised by different degrees of substitution (DS = 6.5, 4.5 and 1.0), as chiral reagents for the voriconazole studies.

The shift displacement values for the four singlets of UK-103,449 are generally much lower on addition of the native CDs than the derivatised CDs. Little or no interaction is observed between this diastereoisomer and α-CD, but shows an increase on changing to the larger γ-CD and yet another increase when the moderate sized β-CD is used. Here the size of the CD toroid is shown to have an effect on the nature of the complexation which is also reflected in the CE results shown in Table II, where β-CD is the more enantioselective additive followed by the larger γ-CD. The shifts observed on addition of the derivatised CDs indicate that the shift displacement values for the pyrimidine hydrogen H6 is consistently

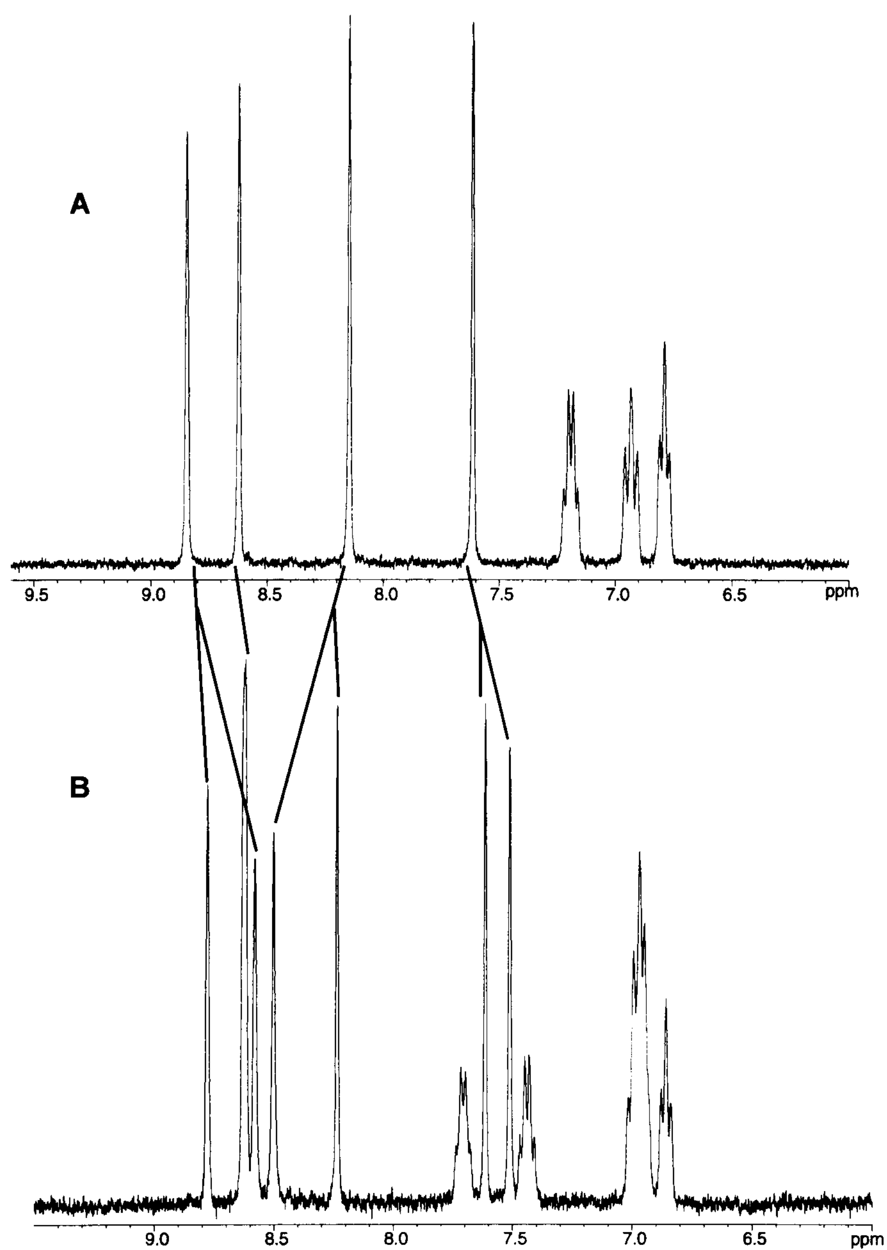

Figure 4. A representative 400 MHz NMR spectrum (6.5–9.5 ppm) for (A) the UK,103,449 diastereoisomer without any CD added and (B) after an equimolar addition of SBE-β-CD indicating shift displacement (coupled with shift non-equivalence).

higher than for the H7 hydrogen. This trend is also observed for the triazole hydrogen, H10, which is consistently higher than for the H9 hydrogen on addition of the derivatised CDs. These differences are particularly pronounced for the anionic SBE-β-CDs where 66.4 and 93.6 Hz are observed for H6 and H10, respectively, compared with 0.8 and 20.0 Hz for H7 and H9, respectively. These differences are not occurring for the native CDs which may indicate that the mechanism of interaction between UK-103,449 and each CD is occurring through the traditional insertion of the difluorophenyl group into the CD cavity and that its influence on H9 can only be observed when a derivatised CD is used. In that situation it is possible that side chains on the derivatised CDs are then interacting with parts of the triazole and the pyrimidine groups (not observed for the native CDs). It is also interesting to note, but difficult to interpret, that the triazole H10 is consistently shifted downfield compared to the upfield shifts observed by the other diagnostic singlets.

When considering the anionic CDs, there is no significant difference in shift displacement for any diagnostic singlet on changing the ionic state of the chargeable CM-β-CD. This may well indicate that little or no ionic interaction between UK-103,449 and the anionic CDs is taking place. For the three batches of SBE-β-CD characterised by different degrees of substitution, the results obtained for these voriconazole studies are in contrast to those obtained using the same CDs in an earlier study with the drug oxamniquine [20]. The observed shift displacement values decreased for three diagnostic singlets (H6, H9 and H10) on lowering the degree of substitution from the highly substituted SBE-β-CD to the medium substituted SBE-β-CD(4). This decrease in observed shift displacement value is continued further for the three singlets on using the even lower substituted SBE-β-CD(1). Interestingly, the shift displacement for the pyrimidine H7 singlet actually increases with decreasing SBE-β-CD degree of substitution.

Similar to that for UK-103,449, shift displacement ($\Delta\delta$, Hz) values were observed for each of the diagnostic singlets of the second diastereoisomer, UK-103,451, with respect to the same uncomplexed signals after the addition of each of the CDs. On examination of all these shift displacement values, however, it was clear that little differences appeared between each singlet for each CD and were all in the 4–8 Hz range. These data probably indicate that little or no interaction was taking place between any of the CDs studied and the UK-103,451 diastereoisomer. It is clear that the stereochemistry or more importantly the overall three-dimensional spatial arrangement of the voriconazole stereoisomers has a significant effect on their interaction with CD molecules. These were also the first data that supported and corroborated the CE and LC results where significant differences were observed between the diastereoisomers.

Similar elucidation studies for the complexation between fencamfamine diastereoisomers with native and derivatised CDs by NMR after initial examination by CE have recently been carried out by Thunhorst *et al.* [26]. Similar to the results shown above, lower shift displacement values for the resonance signals of one

fencamfamine diastereoisomer over the second when complexed with a derivatised β-CD and γ-CD were observed indicating that this phenomenon is not in itself unusual given that diastereoisomers have different physical and chemical properties. Although the differences were considered relatively large in that study, typically 10 Hz, they are relatively small compared to differences in shift values for the voriconazole diastereoisomers shown above.

3.2.2. *Shift nonequivalence*

Shift nonequivalence values ($\Delta\delta^*$, Hz), measured as the difference in the resonance position of each enantiomer, observed for each of the diagnostic hydrogen signals outlined above, after the addition of each CD with respect to the same uncomplexed signals, are shown in Table V. A representative 400 MHz NMR spectrum (6.0–9.5 ppm) for (A) the UK-103,449 diastereoisomer without any CD added and (B) after an equimolar addition of SBE-β-CD indicating shift displacement is shown in Figure 4. The data in Table V for shift nonequivalence of the four diagnostic singlets of UK-103,449 on addition of CDs in a 1 : 1 molar ratio, indicate enantioselective interaction between each of these CDs and this diastereoisomer. Similar to the shift displacement data (Table IV), the nature and size of the CD is shown to have a significant effect on the observed shift nonequivalence for diagnostic singlets of UK-103,449. The native β-CD was the only additive that offered any shift nonequivalence which compares favourably with the observed enantioselectivity in CE and LC when these CD additives are used as reported earlier [6]. The nature of the derivative side chain attached to the β-CD exerts a significant influence on the extent of the observed shift nonequivalence. When considering the neutral derivatised CDs, HP-β-CD and HE-β-CD, a significant increase in shift nonequivalence is observed for three diagnostic singlets over that observed for the corresponding native β-CD. The triazole hydrogen H10 and the pyrimidine hydrogen H6 are still showing larger shifts when compared to the corresponding H7 and H9 which is similar to that observed for the shift displacement. In fact, no shift nonequivalence is observed at all for the pyrimidine H7 hydrogen on addition of any neutral CD.

When considering the anionic CM-β-CD when mixed with UK-103,449 in a 1 : 1 molar ratio, the results are somewhat surprising. Shift nonequivalence values for diagnostic resonance signals for other drugs examined [12, 20] were consistently larger for this anionic CD when compared with other CDs examined. However, little or no difference in shift nonequivalence for the UK-103,449 singlets was observed when using this anionic CD (4.8–29.6 Hz) compared to those observed for the same singlets when using the corresponding native β-CD (9.6–22.8 Hz). In fact, shift nonequivalence values for this anionic CD were considerably less than those observed for the neutral derivatised CDs (32.0–62.4 Hz). Interestingly, this anionic CD did show shift nonequivalence for the pyrimidine H7 hydrogen (4.8 Hz) which may or may not be significant.

The shift nonequivalence data for the three batches of the anionic SBE-β-CD are complex and difficult to interpret. Once again, the triazole hydrogen H10 and

Table V. Shift nonequivalence ($\Delta\delta^*$, Hz) values observed for UK-103,449 on complexation with cyclodextrins in a 1:1 ratio at pD 3.4

CD additive	H6	H7	H9	H10
α-CD	–	–	–	–
β-CD	19.2	–	9.6	22.8
γ-CD	–	–	–	–
HP-β-CD	48.0	–	32.8	62.4
HE-β-CD	48.0	–	32.0	60.4
CM-β-CD	24.8	4.8	17.6	29.6
CM-β-CD pD 5.4	18.0	–	12.0	22.0
SBE-β-CD	63.5	–	40.8	106.4
SBE-β-CD(4)	46.0	–	25.2	56.4
SBE-β-CD(1)	28.8	4.4	16.4	33.6

(–) No shift nonequivalence was observed.

the pyrimidine hydrogen H6 show larger shifts when compared to the corresponding H7 and H9 hydrogens, similar to that observed for the shift displacement and shift nonequivalence when using the neutral CDs. The uniform decrease in shift nonequivalence values, from 40.8 to 16.4 Hz and from 106.4 to 33.6 Hz for the two triazole singlets, H9 and H10 respectively, and from 63.5 to 28.8 Hz for the pyrimidine hydrogen H6, when decreasing the degree of substitution of SBE-β-CDs probably indicates that (a) the enantioselective interaction between this drug mixture and these CDs probably occurs via inclusion of the difluorophenyl group into the CD toroid while the CD side chains interact with the triazole and pyrimidine groups as outlined above and (b) the degree of substitution is crucial in the enantiorecognition process.

It may also be worth noting that although high nonequivalence values can be obtained using chiral solvating agents other than CDs [27–28] the values shown here, over 106 Hz for the triazole H10 and the aromatic quartet $6'$ (shown in Figure 4) are reasonably large, particularily for a CD chiral solvating agent, considering values of 12.8 and 16.1 Hz were obtained for similar systems by our group [12, 20] and typically 20 Hz is normally considered significant by others [29–32].

Considering that the shift displacement data indicated little or no interaction between the opposite diastereoisomer UK-103,451 and any CD described previously, it was not surprising that no shift nonequivalence was observed at all for the UK-103,451 diastereoisomer on addition of each CD in a 1: 1 molar ratio. Once again this result supports the CE and LC data where significant differences between the diastereoisomers were observed. This small or zero interaction is particularly difficult to interpret since it was actually possible in the earlier CE study to separate the enantiomers of this diastereoisomer using the medium substituted SBE-β-

CD(4) (DS = 4.5) but with this CD alone and at a high concentration of 20 mM [6]. Higher shift displacement and nonequivalence values can be obtained when higher molar ratios (other than 1 : 1) of CD to drug are used but it was not possible due to SBE-β-CD(4) unavailability to examine the complexation between this CD and UK-103,451 further. It is likely that at significantly higher molar ratios small effects indicating a degree of interaction may have been observed but these would still have to be considered relative to any results obtained for UK-103,449 where even larger displacement and nonequivalence values would certainly be obtained at higher molar ratios.

3.3. TWO-DIMENSIONAL ROESY NUCLEAR MAGNETIC RESONANCE

It was decided to carry out a 2D ROESY NMR experiment on the mixture of UK-103,449 and the anionic SBE-β-CD to investigate the nature of the interaction between this diastereoisomer and the anionic CD. The SBE-β-CD was chosen since it offered the greater enantioselectivity in both the current spectroscopic study and also in the earlier CE and LC study [6]. 2D ROESY intramolecular cross peaks were observed between the three aromatic hydrogens and also between the chiral H3 and the methyl group (H4) of UK-103,449. A number of intramolecular cross peaks between the methylene hydrogens in the sulphobutyl side chain of the anionic SBE-β-CD were also observed. The strongest observed intermolecular cross peaks between UK-103,449 and the anionic SBE-β-CD arose from interaction between the aromatic H3′ and H5′ of UK-103,449 and the internal glucopyranose resonance signals of SBE-β-CD. Surprisingly, there were no intermolecular cross peaks between these SBE-β-CD glucopyranose signals and the aromatic H6′ of UK-103,449 which may indicate that the difluorophenyl group is only partially included into the CD hydrophobic cavity. These NOEs were confirmed by one-dimensional NOE experiments. This would support the 1D shift displacement and nonequivalence data where strong interactions were observed for each of the diagnostic singlets (non-aromatic hydrogen atoms) with each of the CDs. There were also a number of intermolecular cross peaks between the methylene hydrogens in the sulphobutyl side chains with the pyrimidine H6 and the triazole H9 hydrogens. Once again this may support the 1D NMR data which indicated that the pyrimidine H7 and the triazole H10 were strongly interacting at the top of the CD cavity (observed through high displacement and nonequivalence values) and that the remaining H6 and H9 were actually interacting with the CD derivative side chains. There were no intermolecular cross peaks between either the triazole or the pyrimidine hydrogens with the internal glucopyranose hydrogens of the SBE-β-CD indicating that competitive inclusion was not occurring.

It can be concluded from this 2D ROESY experiment that the interaction between UK-103,449 and the anionic SBE-β-CD occurs through a partial inclusion of the difluorophenyl group into the hydrophobic CD cavity. Additional interactions of the sulphobutyl side chains with the triazole and pyrimidine groups also

occur. Coupled with instrument and CD unavailability, it was thought that little information would be gained from a set of 2D experiments on the UK-103,451 diastereoisomer considering the poor results obtained using 1D dimensional NMR at 400 MHz.

3.4. SPIN-LATTICE RELAXATION TIME MEASUREMENTS

It was also decided to carry out a series of T_1 measurements to support the 2D ROESY experiment in determining the solution structure of the complex formed between UK-103,449 and SBE-β-CD. T_1 measurements were carried out for the hydrogen nuclei of UK-103,449 prior to and after the addition of one molar equivalent of the anionic SBE-β-CD and are shown in Table II. A reduction in spin-lattice relaxation time ($\approx$ 70%) is observed for the triazole hydrogen H9 and the pyrimidine hydrogen H6 of UK-103,449 on addition of equimolar SBE-β-CD. These large reductions are most likely resulting from increased dipole-dipole interactions between these two hydrogens and the methylene groups of the sulphobutyl side chains, which were also observed in the 2D ROESY experiment. These large reductions in T_1 time measurements observed for UK-103,449 when mixed with SBE-β-CD are considered significant since reported reductions of 40% by Irwin et al [24] and 38% by Azaroual-Bellanger and Perly [23] were all considered significant for analogous drug – neutral cyclodextrin systems. It is difficult to conclude, however, if the reductions for the remaining hydrogens of UK-103,449 ($\approx$ 5–27%) are significant. The lower T_1 values for this molecule and in particular the triazole and pyrimidine groups on addition of the SBE-β-CD, are most likely due to (a) inclusion of the difluorophenyl group into the CD cavity resulting in hydrogens tumbling more slowly in the larger drug-CD complex and (b) interactions between the two groups and the sulphobutyl side chains. The increase in T_1 for the aromatic H6′, observed on addition of SBE-β-CD is interesting but it is difficult to interpret its significance. The low T_1 value for this H6′ aromatic hydrogen in the free molecule of 2.1s would most likely be due to dipole-dipole interactions with the H5′ aromatic hydrogen. The conclusion from the 2D ROESY experiment, supported by 1D NOE spectroscopy, has shown that there was evidence that the aromatic hydrogens H3′ and H5′ are included into the CD cavity. There was no evidence, however, to suggest that the aromatic H6′ is included into the CD cavity. The increase in T_1 then for the aromatic H6′ could be due to the loss or a change in the dipole-dipole interactions between H6′ and H5′ as partial inclusion of the difluorophenyl into the CD occurs.

4. Conclusions

The complexation between the diastereoisomers of voriconazole, UK-103,449 and UK-103,451, and a number of native, neutral and anionic derivatised CDs has been examined in one and two dimensional NMR. These were carried out to help

rationalise the extraordinary differences in observed enantioseparation obtained in both CE and LC for these two diastereoisomers where complete separation was easily obtained for the former using almost all CDs in both techniques compared to the extreme difficulty in gaining an acceptable separation for the latter using any technique [6].

Observed differences in the shift of selected resonance signals coupled with large shift nonequivalence values on addition of CDs to these diastereoisomers in 1D NMR indicated that a strong degree of interaction occurred between a number of CDs and the enantiomers of UK-103,449. It was found that the dominating factor was the size of the cavity, since β-CD was shown to complex very strongly compared to decreasing interaction with the smaller and larger α-CD and γ-CD, respectively. Strong interactions with the derivatised CDs were also observed resulting in high shift displacement and nonequivalence values, over 100 Hz, which can be considered significant for a CD chiral solvating agent. To complement the 1D NMR shift displacement and nonequivalence data, the interaction between the UK-103,449 diastereoisomer and SBE-β-CD was explored further using a 2D ROESY experiment and a set of spin-lattice relaxation time measurements. It was concluded from these studies that the interaction occurred through the partial inclusion of the difluorophenyl group of UK-103,449 into the hydrophobic cavity of the SBE-β-CD. There was also strong evidence to indicate that this partial inclusion was also aided by additional interactions between the triazole and pyrimidine group of UK-103,449 and the sulphobutyl side chains of the SBE-β-CD outside the hydrophobic cavity.

Similar to the enantioseparation data obtained in CE and LC [6] little or no interaction was observed between any CD and the 'enigmatic' UK-103,451 diastereoisomer of voriconazole. Little or no shift displacement 4–8 Hz was observed, and no nonequivalence at all when each of the CDs was examined with this mixture. These results are particularly difficult to interpret given the extraordinarily high degree of interaction observed for its opposite diastereoisomer under identical conditions.

The inherent differences in the physical and chemical properties of diastereoisomers would dictate that some electrophoretic, chromatographic and spectroscopic differences should be detected. However, the extreme difference observed for the voriconazole diastereoisomers *in each* of these analytical techniques clearly indicate that they are indeed authentic and that the overall three-dimensional spatial arrangement of these stereoisomers is much more complex than what can be depicted in two dimensions. It may be possible that if the four stereoisomers, each represented by its correct absolute configuration, were viewed in three-dimensional space using crystallographic or molecular modelling techniques, that actual spatial differences between each moiety on each isomer may help to rationalise further the results obtained in our electrophoretic, chromatographic and spectroscopic studies.

Acknowledgements

Financial support from Pfizer Central Research (UK) for a Research Studentship (P.K.O.) is gratefully acknowledged. Colleagues at the *Pharmaceutical Analysis Research Unit* are thanked for technical support and David A. Brown, Physical Sciences, Pfizer Central Research (UK) is thanked for help with re-plotting the NMR spectra. Gifts of CM-β-CD from Wacker Chemicals Ltd., SBE-β-CD(4) and SBE-β-CD(1) from CyDex Inc. (Kansas, USA) are also acknowledged.

References

1. R. P. Dickinson, A. S. Bell, C. A. Hitchcock, S. Narayanaswami, S. J. Ray, K. Richardson, and P. F. Troke: *Bioorg. Medic. Chem. Lett.*, **6**, 2031 (1996).
2. M. V. Martin, J. Yates, and C. A. Hitchcock: *Antimicrob. Agents Chemother*. **41**, 13, (1997).
3. D. A. Stopher and R. Gage: *J. Chromatogr. B* **691**, 441 (1997).
4. R. Gage and D. A. Stopher: *J. Pharm. Biomed. Anal*. **17**, 1449 (1998).
5. R. Ferretti, B. Gallinella, F. La Torre and L. Zanitti, *Chromatographia* **47**, 649 (1998).
6. P. K. Owens, A. F. Fell, M. W. Coleman and J. C. Berridge, *Enantiomer*, **4**, 79 (1999).
7. A. F. Casy, A. D. Cooper, T. M. Jefferies, R. M. Gaskell, D.Greatbanks and R. Pickford: *J. Pharm. Biomed. Anal.* **9**, 787 (1991).
8. K. B. Lipkowitz, S. Raghothama and J. Yang: *J. Am. Chem. Soc.* **114**, 1554 (1992).
9. G. Uccello-Barretta, F. Balzano, A. M. Caporusso and P. Salvadori: *J. Org. Chem.* **59**, 836 (1994).
10. H. Grosenick, M. Juza, J. Klein and V. Schurig: *Enantiomer* **1**, 337 (1996).
11. B. Chankvetadze, G. Endresz and G. Blaschke: *J. Chromatogr. A* **717**, 245 (1995).
12. P. K. Owens, A. F. Fell, M. Kinns, M. W. Coleman and J. C. Berridge: *J. Pharm. Biomed. Anal.* **15**, 1603 (1997).
13. A. D. Bax and D. G. Davis, *J. Magn. Reson.* **63**, 207 (1985).
14. Y. Inoue, Y. Kanda, Y. Yamamoto, R. Chûjo and S. Kobayashi: *Carbohydr. Res.* **194**, c8 (1989).
15. J. Redondo, J. Frigola, A. Torrens and P. Lupon: *Magn. Reson. Chem.* **33**, 104 (1995).
16. T. Ndou, S. Mukundan and I. M. Warner: *J. Incl. Phenom.* **115**, 9 (1993).
17. M. Suzuki, H. Ohmori, M. Kajtar, J. Szejtli and M. Vikmon: *J. Incl. Phenom.* **18**, 255 (1994).
18. M. Suzuki, H. Takai, K. Tanaka, K. Narita, H. Fujiwara and H. Ohmori: *Carbohydr. Res.* **288**, 75 (1996).
19. S. Divakar, *J. Incl. Phenom.* **15**, 305 (1993).
20. P. K. Owens, A. F. Fell, M. W. Coleman, M. Kinns and J. C. Berridge: *J. Chromatogr. A* **797**, 149 (1998).
21. R. E. Kaiser, *Gas Chromatographie*, Geest and Portig, Leipzig (1960).
22. N. W. Smith, *J. Chromatogr. A* **652**, 259 (1993).
23. N. Azaroual-Bellanger and B. Perly, *Magn. Reson. Chem.* **32**, 8 (1994).
24. P. L. Irwin, J. N. Brouillette and K. B. Hicks, *Carbohydr. Res.* **269**, 20 (1996).
25. J. W. Akitt: *NMR and Chemistry. An Introduction to Modern NMR Spectroscopy*, London, pp. 78–98, Chapman and Hall (1992).
26. M. Thunhorst, Y. Otte, T. M. Jefferies, S. K. Branch and U. Holzgrabe: *J. Chromatogr. A*, **818**, 239 (1998).
27. E. Yashima, C. Yamamoto and Y. Okamoto: *J. Am. Chem. Soc.* **118**, 4036 (1996).
28. S. K. Latypov, J. M. Seco, E. Quiñoá and R. Riguera: *J. Org. Chem.* **61**, 8569 (1996).
29. K. H. Kim and Y. H. Park, *Int. J. Pharm.* **175**, 247 (1998).
30. Z. Aturki, C. Desiderio, L. Mannina and S. Fanali: *J. Chromatogr. A* **87**, 91 (1998).

31. G. Uccello-Barretta, A. Cuzzola, F. Balzano, R. Menicagli, A. Iuliano and P. Salvadori: *J. Org. Chem.* **62**, 827 (1998).
32. K. Csabai, M. Vikmon, J. Szejtli, E. Redenti, G. Poli and P. Ventura: *J. Incl. Phenom.* **31**, 169 (1998).