J Incl Phenom Macrocycl Chem (2010) 68:453–459
DOI 10.1007/s10847-010-9809-1

ORIGINAL ARTICLE

A comparative study of inclusion complexes of flunarizine with alpha (α-CD) and beta-cyclodextrin (β-CD)

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Received: 22 February 2010 / Accepted: 21 May 2010 / Published online: 30 June 2010

Abstract A detailed NMR (^{1}H, COSY, and ROESY) spectroscopic study of complexation of Flunarazine (FL) with α- and β-CD was carried out. ^{1}H NMR titration studies confirmed the formation of FL/α-CD and FL/β-CD complexes as evidenced by chemical shift variations of the proton resonances of both the CDs and FL. The stoichiometry of the complexes was determined to be 1:2 (FL/α-CD) and 1:1 (FL/β-CD) and overall binding constants were also calculated. It was confirmed with the help of ROESY spectral data that only one of the F-substituted aromatic ring and phenyl ring penetrate the α-CD cavity while both F-substituted aromatic rings as well as phenyl ring penetrates the β-CD cavity during complexation. The binding modes of FL/CD cavity interactions derived from ROESY experimental data show that the resulting complex of FL with β-CD possesses better induced fit interaction as compared to α-CD, which is responsible for the enhanced molecular stability with β-CD in comparison to α-CD. The mode of penetration of guest into the CD cavity and structures of the complexes has been established.

Keywords Flunarazine · α-Cyclodextrin · β-Cyclodextrin · NMR spectroscopy · ROESY · COSY

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Introduction

Flunarizine dihydrochloride (FL), 1-[bis(4-Fluorophenyl)-methyl]-4-(3-phenyl-2-propenyl)piperazine. 2 HCl, is classified as a calcium channel blocker with calmodulin binding properties and histamine H1 blocking activity. It is very effective in the acute treatment of isosorbide dinitrate which induces migraine attacks and also helps to reduce the severity and duration of attacks of paralysis associated with the more serious form of alternating hemiplegia. Flunarizine is sparingly soluble in methanol, ethanol and chloroform, slightly soluble in water and stable for only 36 months at 25 °C [1, 2].

Cyclodextrins (CDs) are oligosaccharides composed of six to eight glucopyranose units bound by α-(1-4) linkages that are commonly named α-, β-, and γ-CD, respectively. β-CD, in particular, has an internal cavity shaped like a truncated cone. The interior of the cavity is relatively hydrophobic while the outer surface is quite hydrophilic because of the presence of numerous hydroxyl groups. By virtue of their shape and nature of cavity, CDs accommodate a variety of hydrophobic as well as hydrophilic molecules, or part of it, inside their cavity through non-covalent interactions to form inclusion complexes [3–5].

Today, the study of inclusion complex of pharmaceuticals with CDs is a subject of great interest because of their utility to improve the solubility, dissolution rate and bioavailability of poorly water soluble drugs [6, 7]. Furthermore, In order to use CDs as drug carrier systems, it is necessary to understand from the thermodynamics and kinetics points of view how the drug molecule interacts with the CD cavity. However, it is also important to determine the dynamics of entry and exit of guest molecules with CD cavity, because the knowledge of these rate constants has direct applications when using CD to perform

specific functions [8–10] For example, the dissociation rate constants define the lifetime of the guest in the CD cavities and may be directly related to the efficiency of drug delivery and protection. Thus, these types of studies are mainly addressed to clarify the following aspects: to prove that the inclusion takes place, to determine the stoichiometry of the complex and to determine the three dimensional structure of the complex.

^{1}H NMR spectroscopy is one of the most common useful techniques for investigating the stability and stoichiometry of the CDs complexes, particularly in the solution [11]. NMR spectra obtained from most CD complexes represent concentration weighted averages since exchanged between the free and complexed guest molecule was usually fast on the NMR time scale. Its main advantage is the possibility to use several independent signals for the determination of the association constants so being less prone to misinterpretations caused by minor impurities. The observed shift changes provide at the same time insight into the structure of the complexes, which is difficult to extract from UV or fluorescence titrations and impossible from calorimetric data [12]. 2D NMR spectroscopy has become an important tool for the investigation of the interactions between CDs and guest molecules. According to the relative intensities of these cross peaks, it is possible to estimate the orientation of the guest molecule within the CD cavity [13].

The aim of this work was to explore the use of CDs to form inclusion complexes with flunarizine to overcome the solubility and bioavailability problems of this drug. We investigated the complexation of flunarizine with α- and β-CD in aqueous solution by detailed NMR spectroscopic study and report here our results.

Experimental

All the ^{1}H NMR spectra were recorded on an Inova-500 instrument in D_2O at 25 °C and the chemical shift values (δ) are reported in ppm. No external indicator was used and HDO signal at 4.800 ppm was used as internal reference throughout this work. To determine the stoichiometry of the FL/β-CD complex, ^{1}H NMR shift data for Flunarizine and β-CD was obtained by keeping the overall concentration of the two components constant ([FL] + [β-CD] = 10 mM) while the molar ratio ([FL] or [β-CD]/([FL] + [β-CD]) was varied from 0 to 1. To determine the binding constant, ^{1}H NMR shift data for another set of six mixtures of FL and β-CD was obtained in which the concentration of β-CD was kept constant at 5 mM, due to lower solubility of β-CD, while that of FL was varied from 1.25 to 9.5 mM. The stoichiometry as well as binding constant of FL/α-CD complex was determined by Scott's method. For that, the concentration of FL was kept constant at 5 mM while the concentration of α-CD was varied from 1.15 to 9.5 mM. COSY and ROESY spectra were recorded for mixtures of FL with α- and β-CD with mixing time 200 ms and under spin lock conditions. Distinct peaks for bound and free form of the FL were not observed indicating the rapid exchange of FL between free and bound state on the NMR time scale.

Results and discussion

^{1}H NMR spectra of CDs

^{1}H NMR assignments of α- and β-CD proton resonances have been carried out with the help of COSY spectra. An examination of α- and β-CD protons in the ^{1}H NMR spectra of all the mixtures of FL and respective CD revealed significant upfield shift changes in the H-3′ and H-5′ proton resonances, which are positioned inside CD cavity, while the peaks of other protons show negligible chemical shift changes. These shift changes increase with increasing concentration of FL, in both the cases of α- and β-CD, which can be explained in terms of the ring current effect of aromatic ring/s penetrating the CD cavity and thus confirms the formation of FL/α-CD and FL/β-CD inclusion complexes in analogy to previous studies [14, 15]. The chemical shift change data for all the protons of β-CD in comparison to pure β-CD is given in Table 1 while Figs. 1 and 2 shows the expansions of ^{1}H NMR spectral regions of 1:1 mixtures of α- and β-CD with FL in comparison to pure α- and β-CD respectively. Figures 1 and 2 clearly indicate that the values of chemical shift changes (Δδ) increase from α-CD to β-CD which is probably due to the tight complexation of FL with β-CD in comparison to α-CD.

^{1}H NMR assignment of flunarizine

An unambiguous ^{1}H NMR spectral assignment, especially for aromatic protons, is required to establish the structure

Table 1 ^{1}H NMR chemical shift change (Δδ) data for β-CD protons, in the presence of FL

[FL]/[β-CD]	H-1′	H-2′	H-3′	H-4′	H-5′	H-6′
1.90	0.023	0.013	−0.275	−0.046	−0.304	−0.053
1.50	0.024	0.004	−0.273	−0.043	−0.299	−0.054
1.00	0.023	0.004	−0.257	−0.042	−0.285	−0.054
0.75	0.023	0.004	−0.225	−0.035	−0.255	−0.048
0.50	0.020	0.004	−0.164	−0.026	−0.189	−0.048
0.25	0.019	0.004	−0.099	−0.015	−0.116	−0.046

Negative values indicate upfield shift

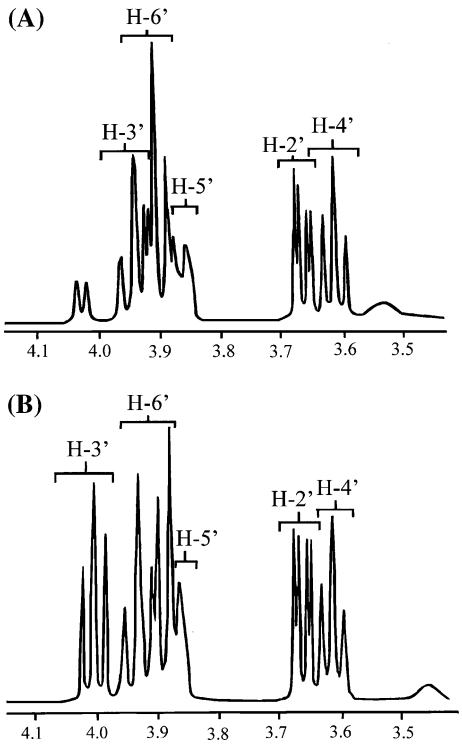

Fig. 1 Parts of ^{1}H NMR spectra (500 MHz) showing α-CD proton signals of **A** α-CD/FL mixture (1:1) in comparison to **B** pure α-CD

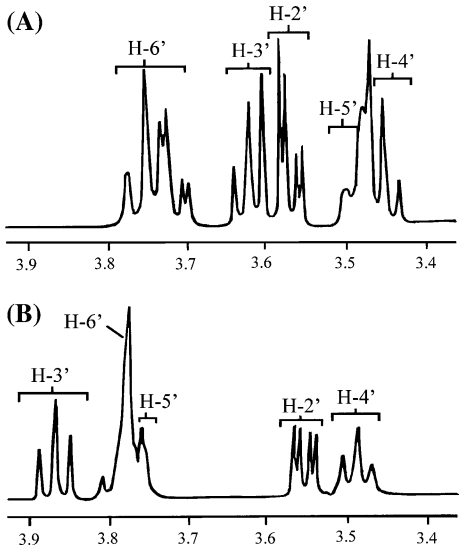

Fig. 2 Parts of ^{1}H NMR spectra (500 MHz) showing β-CD proton signals of **A** FL/β-CD mixture (1:1) in comparison to **B** pure β-CD

of inclusion complex. The ^{1}H NMR assignment of FL protons was made with the help of COSY (Fig. 3) and ROESY spectral data. The signal appearing at the lowest field as the multiplet, centered at 7.594, was assigned to H-2 as it shows cross correlation peaks with H-3 in ROESY spectrum. The triplet at 7.223 ($J = 8$ Hz) was assignable to H-1, due to the presence of cross peaks with H-2 in COSY

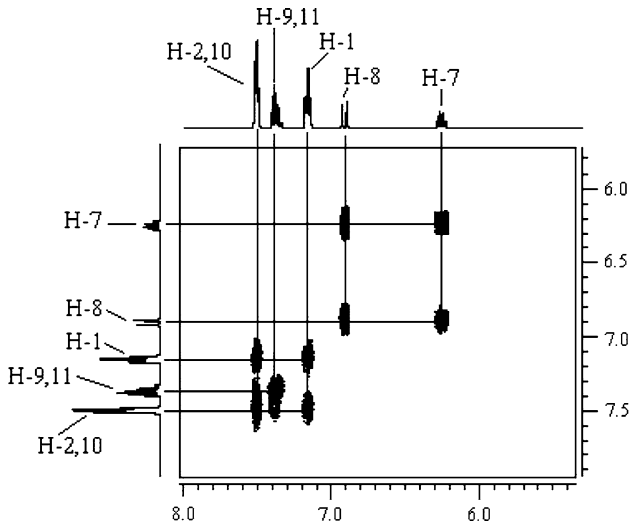

Fig. 3 Part of the COSY Spectrum (500 MHz) of a mixture of FL and β-CD

spectrum. The signal for H-9,11 of phenyl ring appeared as a multiplet centered at 7.449, integrating for three protons, this peak shows cross peaks with multiplet of H-2 signal in COSY spectrum, suggesting that H-10 are merged with H-2 signal. The doublet at 6.977 ($J = 16$ Hz) and multiplet at 6.355 were assigned to H-8 and H-7 respectively. The ^{1}H NMR spectral data of FL in the absence as well as in the presence of α-CD is given in Table 2.

^{1}H NMR chemical shift change data of flunarazine

Prominent changes in the position of signals of most of the protons of FL were observed in the presence of both α- and β-CD, which further increased with the increase in concentration of CDs. In the presence of β-CD, all aromatic protons moved upfield while in the case of α-CD shifted downfield, confirming the penetration of aromatic ring/s into the CD cavity driven by hydrophobic interactions. The upfield shift in FL aromatic protons is probably due to the interaction of fluoro group of F-substituted ring with 6′-OH of β-CD which may be explained in terms of close proximity of these protons with fluorine in free state while in complexed state this F-atom may interact with 6′-OH of β-CD and thus weakening the interaction between F-atom and aromatic protons of F-substituted ring. Moreover, the H-1 signal showed splitting in the presence of β-CD suggesting some chirality generated by β-CD cavity on inclusion. Parts of spectra showing all the studied protons of FL in the presence of α- and β-CD in comparison to pure FL are shown in Fig. 4 while chemical shift change data for all the studied protons of FL in presence of various amounts of α-CD is given in Table 2.

Table 2 ^{1}H NMR (500 MHz) chemical shift change ($\Delta\delta$, ppm) data for FL and [α-CD]/[FL] mixtures for all the protons of FL in the presence of α-CD in D_2O

Proton	[FL]	[αCD]/[FL] = 0.23	[α-CD]/[FL] = 0.35	[α-CD]/[FL] = 0.63	[α-CD]/[FL] = 0.86	[α-CD]/[FL] = 1.15	[α-CD]/[FL] = 1.90
H-1	7.223	0.009	0.019	0.036	0.055	0.056	0.094
H-2,10	7.594	0.003	0.007	0.015	0.023	0.027	0.045
H-9,11	7.449	0.002	0.003	0.005	0.019	0.023	0.039
H-8	6.977	0.002	0.004	0.012	0.024	0.039	0.044
H-7	6.355	0.002	0.005	0.013	0.027	0.044	0.045

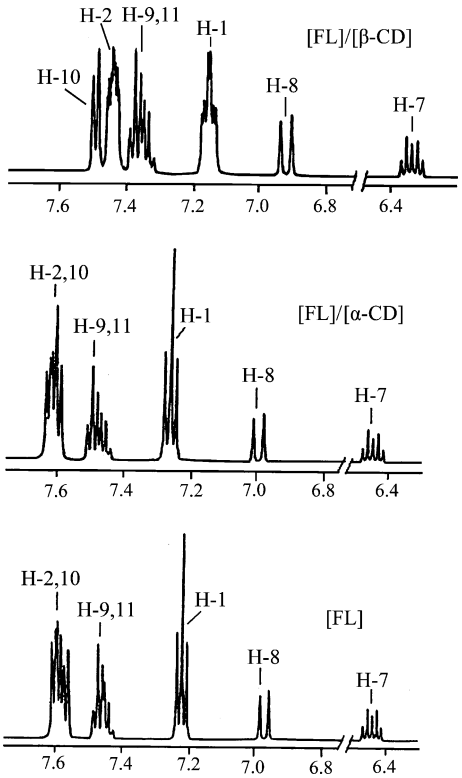

Fig. 4 Parts of ^{1}H NMR spectra (500 MHz) of mixtures of FL with α- and β-CD in comparison to pure FL

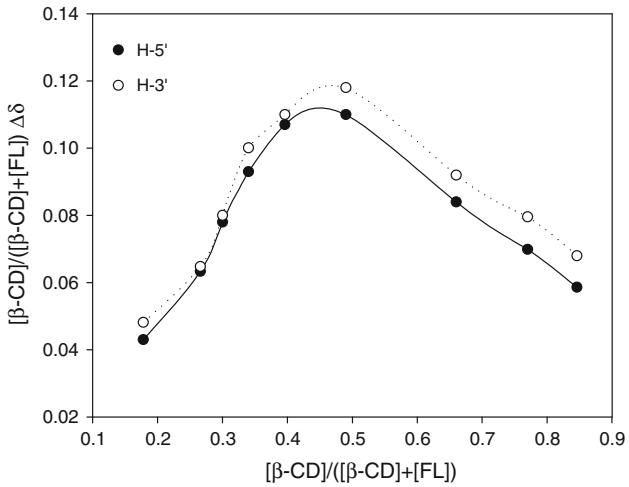

Fig. 5 Job's plot for the FL/β-CD complex corresponding to H-3′ and H-5′ protons of β-CD

Stoichiometry of the complex

To establish the stoichiometry of the FL/β-CD complex, the continuation method [16] (Job's) was used to follow the changes in the chemical shift of H-3′ and H-5′ protons of β-CD. Job's plot (Fig. 5) shows a maxima at [β-CD]/([β-CD] + [FL]) ratio (r) of 0.5 indicating the predominant existence of 1:1 stoichiometry within the range of concentration investigated.

The binding constant of the complex was determined by Scott's modification [17] of Benesi-Hilebrand method [18]. In Scott's equation,

$$[\mathrm{FL}]/\Delta\delta_{\mathrm{obs}} = [\mathrm{FL}]/\Delta\delta_{\mathrm{c}} + 1/K_{\mathrm{a}}\Delta\delta_{\mathrm{c}}$$

[FL] is the molar concentration of the guest, $\Delta\delta_{\mathrm{obs}}$ is the observed chemical shift change for a given [FL] concentration, $\Delta\delta_{\mathrm{c}}$ is the chemical shift change between a pure sample of complex and the free component at the saturation. A plot of chemical shift changes ($\Delta\delta$) for the β-CD protons against [FL] in the form of [FL]/$\Delta\delta$ versus [FL] gave a excellent linear fit (Fig. 6), also confirms the 1:1 FL/β-CD complex. The slope of the plot is thus equal to $1/\Delta\delta_{\mathrm{c}}$ and the intercept with the vertical axis to $1/K_{\mathrm{a}}\Delta\delta_{\mathrm{c}}$, allowing the estimation of K_{a}. The binding constant (K_{a}) was determined to be 179.2 M^{-1}.

Both the method's (Job's and Scott's) displayed the same result, which indicated that both the methods are equally effective for the determination of stoichiometry. Due to this, we used only the Scott's method to deduce the stoichiometry of the FL/α-CD. To determine the stoichiometry of the FL/α-CD, a plot for the chemical shift changes ($\Delta\delta$) of FL protons against the concentration of [α-CD] in the form of [α-CD] versus [α-CD]/[FL] was plotted, which should be linear for the 1:1 stoichiometry of the FL/α-CD complex. However, only a curve was obtained in our experiment (Fig. 6), which means that the assumption of 1:1 stoichiometry for FL/α-CD was not true.

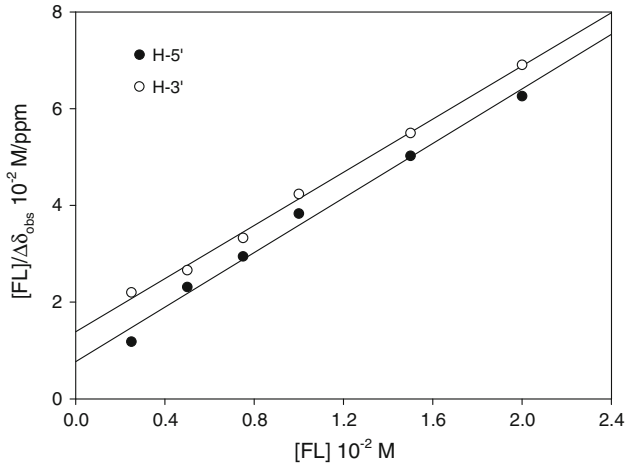

Fig. 6 Scott's plot of $[FL]/\Delta\delta_{obs}$ versus [FL] showing 1:1 stoichiometry for FL/β-CD complex

Furthermore, according to the Scott's equation for the 1:2 complexes [19]

$$[\mathrm{FL}]^2/\Delta\delta_{\mathrm{obs}} = [FL]^2/\Delta\delta_{\mathrm{c}} + 1/K_{\mathrm{a}}\Delta\delta_{\mathrm{c}}$$

a plot of $[\alpha\text{-CD}]^2/[\mathrm{FL}]$ versus $[\alpha\text{-CD}]^2$ was obtained which gave an excellent linear lines (Fig. 7), indicating the presence of 1:2 (FL/α-CD) complex within the concentration range of our experiment. According to the slope and intercept of the line, the binding constant was determined to be 95.7 M^{-1}.

ROESY spectral data

To clearly establish the geometry of the complex, among all the possible techniques, we select the 2D rotating frame ^{1}H-^{1}H nuclear Overhauser effect [19] (2D ROESY) because of its well known reliable results in these kinds of

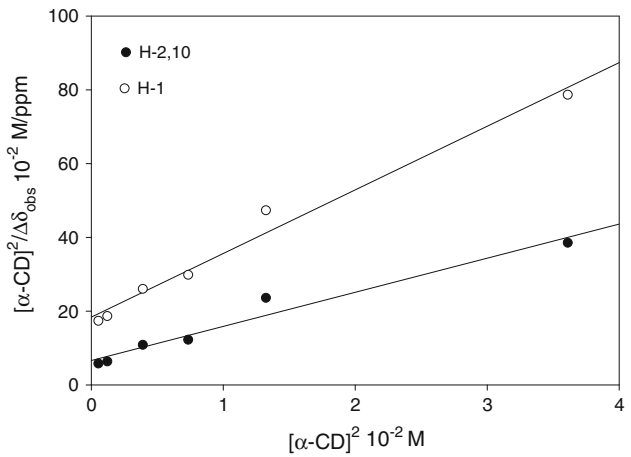

Fig. 7 Scott's plot of $[\alpha\text{-CD}]^2/\Delta\delta_{obs}$ versus $[\alpha\text{-CD}]^2$ showing 1:2 stoichiometry for FL/α-CD complex

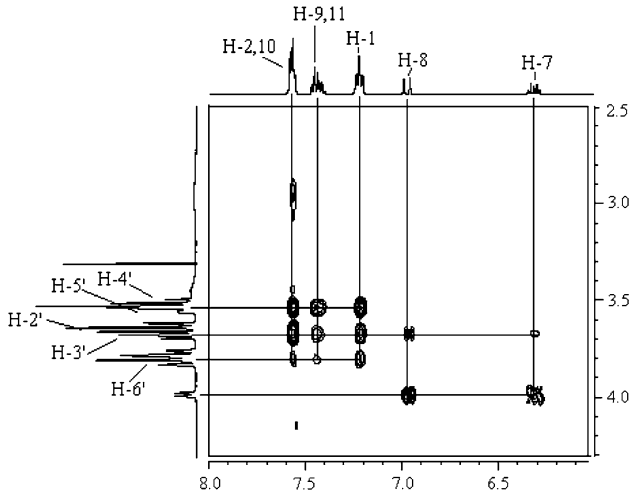

Fig. 8 A Partial ROESY (500 MHz) spectrum of a mixture of FL and β-CD showing dipolar interactions between FL and β-CD cavity protons

studies. The observation of the 2D ROESY spectrum has been given in Fig. 8.

ROESY spectrum displayed cross correlation peaks between all the aromatic protons of FL with H-3′ and H-5′ protons of β-CD, situated inside the β-CD cavity, confirming the involvement of all the aromatic rings in the complexation. The cross peaks between H-3 and H-7, 8 with H-3′ of β-CD, situated near the wider cavity, clearly indicate that both the F-substituted aromatic rings and phenyl ring penetrate from the wider side of β-CD cavity. Taking into account the 1:1 stoichiometry and the involvement of all the aromatic rings in the complexation confirms the presence of multiple equilibria between all the three aromatic rings of FL and β-CD. Figure 9 shows all the possible 1:1 FL/β-CD inclusion complexes present in solution.

Furthermore, in the ROESY spectrum (Fig. 10) of FL/α-CD mixture, F-substituted aromatic rings displayed cross connection peaks with α-CD cavity protons. This indicates the penetration of F-substituted aromatic ring with α-CD cavity. Moreover, H-8 and H-7 of FL also showed cross peaks with H-3′ and H-2′ protons of α-CD respectively, which implies that phenyl ring also penetrates deep from wider side of α-CD cavity (Fig. 10). Furthermore, the stoichiometry of the complex was found to be 1:2 (FL/α-CD). This confirms beyond doubt the penetration of phenyl ring and one of the F-substituted aromatic rings at a time. The plausible structure for the 1:2 (FL/α-CD) complex is shown in Fig. 11.

Conclusion

In the present study, we have demonstrated that flunarizine formed three 1:1 inclusion complexes with β-CD by

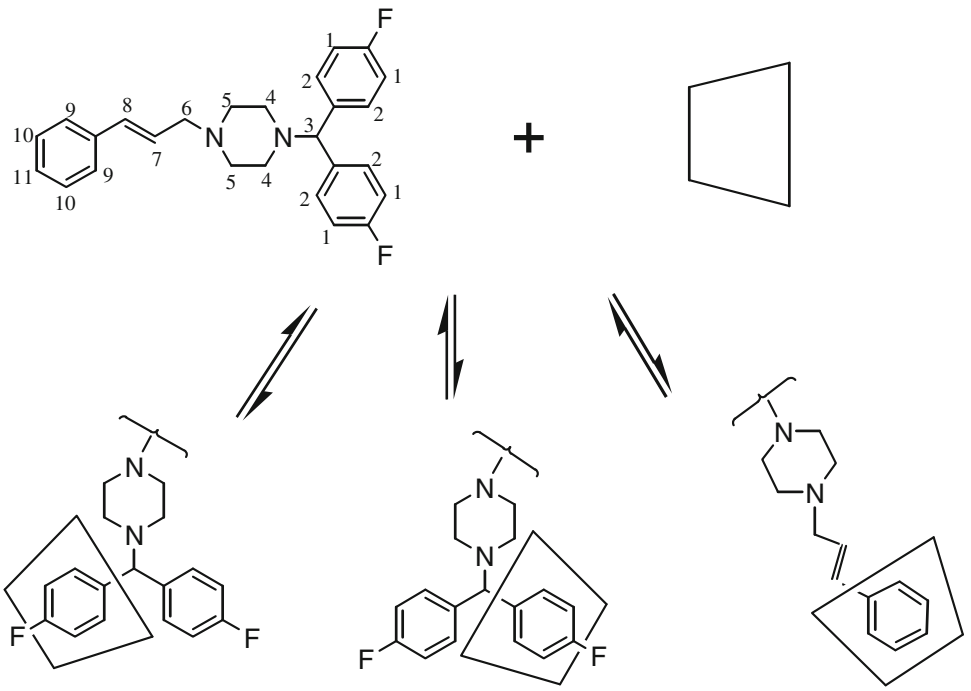

Fig. 9 Schematic representation of all the possible 1:1 inclusion complexes formed between FL and β-CD in D_2O

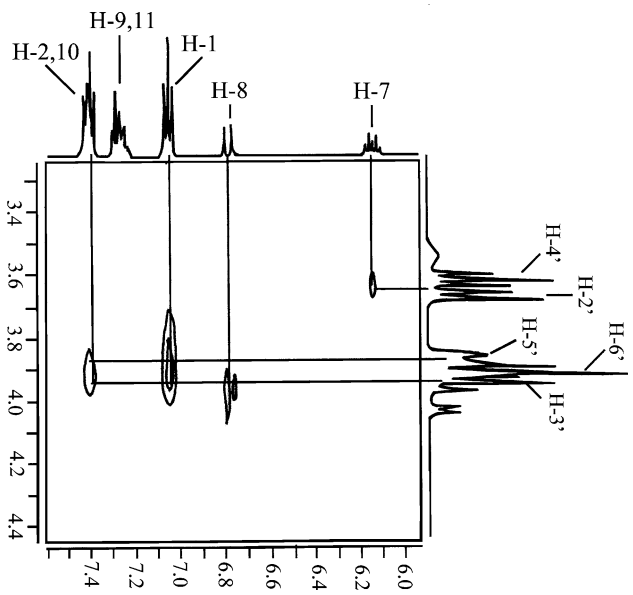

Fig. 10 A Partial ROESY (500 MHz) spectrum of a mixture of FL and α-CD showing dipolar interactions between FL and α-CD cavity protons

penetrating all the three aromatic rings into the β-CD cavity through wider rim side whereas with α-CD, it formed 1:2 (FL/α-CD) complex involving one of the F-substituted aromatic ring and phenyl ring through wider side of the α-CD cavity. The structures of all the complexes have been proposed taking into account the stoichiometry and ROESY spectral data. It is important to mention here

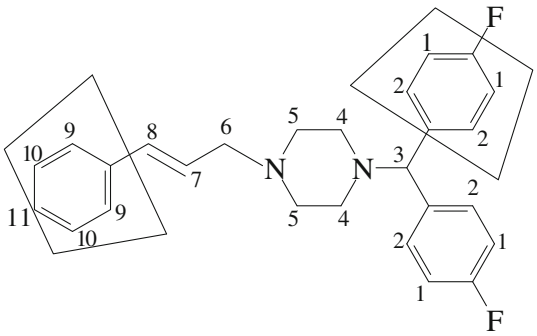

Fig. 11 Proposed structure of FL and α-CD complex

that the complex of flunarizine with α-CD (K_a = 95.7 M^{-1}) was found to be less stable in comparison to β-CD (K_a = 179 M^{-1}).

Acknowledgements Flunarizine and β-cyclodextrin were very kindly provided by Dr. Reddy's laboratory India, and Geertrui Haest, Cerestar Cargill, Belgium, respectively. We are highly grateful to Prof. S. W. Homans, Department of Biochemistry and Molecular Biology, University of Leeds, UK and Dr. P. De Waard, Department of Biophysics, Dreijenlaan, The Netherlands, for their help in obtaining some of the NMR data.

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