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Solution structure of loperamide and β-cyclodextrin inclusion complexes using NMR spectroscopy

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Abstract. Loperamide (LPR) is a synthetic, poorly water soluble, peripherally acting opiate agonist drug used for the treatment of diarrhea. Major challenges in formulating this drug for clinical applications include solubility enhancement and improved stability in biological systems. Cyclodextrins (CDs) are chiral, truncated cone shaped; cyclic oligosaccharides that can encapsulate a variety of poorly water soluble drug molecules into inclusion complexes, thereby increasing their stability and solubility. ^{1}H NMR spectroscopic studies showed the inclusion complexation between β-CD and LPR, based on the upfield shift changes in the β-CD cavity protons (H-3′ and H-5′) and downfield shift changes in the guest (LPR) protons. 2D COSY spectral data was used for assignment of β-CD as well as LPR protons and 2D ROESY spectral data to know the inclusion of LPR inside the β-CD cavity. The 1 : 1 stoichiometry and overall association constant (K_a) were determined by using Scott's plot method to be 68·805 M^{-1}. 2D ROESY spectral data suggest that the inclusion of aromatic rings of LPR in β-CD cavity can be from narrower as well as the wider rim side and the six possible 1 : 1 LPR : β-CD inclusion complexes have been proposed. Thus, we anticipate that complexation of LPR with β-CD would increase its solubility and stability in biological system.

Keywords. Cyclodextrin; inclusion complex; loperamide; NMR; COSY; ROESY.

1. Introduction

Loperamide (LPR), imodium, chemically known as 4-(*p*-chlorophenyl)-4-hydroxy-N, N-dimethyl-α,α-diphenyl-1-piperidinebutyramide (figure 1), is a synthetic peripherally acting opiate agonist and is used primarily to treat diarrhea.[1] LPR and its salts are light sensitive and its solubility in water is 0·000837 mg/ml. β-Cyclodextrin (β-CD) can be used as a suitable solubilizing agent, which increases the solubility of LPR and/or its salts in the aqueous solutions.

CDs are cyclic, truncated cone shaped, chiral oligosaccharides composed of α-(1 $\rightarrow$ 4) glucopyranose unit (figure 1). The three industrially important forms, alpha ($-\alpha$), beta ($-\beta$) and gamma ($-\gamma$) CDs, are composed of 6, 7 and 8 glucopyranose units respectively. The outer periphery of the macrocyclic ring is hydrophilic due to the presence of numerous hydroxyl groups. The internal cavity of CDs is relatively hydrophobic and can encapsulate a variety of compounds especially pharmaceuticals.[2] The structural feature of CDs suggests that the primary hydroxyls are present at the wider rim side while secondary at the narrower rim side of the truncated cone. The hydrophobic cavity of β-CD is lined with H-3′ and H-5′ protons and their NMR signal is more influenced than the other β-CD protons (H-1′, 2′, 4′, 6′).

CDs and their inclusion complexes are widely applicable industrially as well as naturally. They are extensively employed in chiral separation/recognition studies. The use of most of the pharmaceuticals are limited due to their lower solubility, poor bioavailability and interestingly enantiomeric properties, as one of its enantiomer is usually inactive. Majority of the pharmaceutical companies develop pharmaceuticals by mixing drug with CDs, which improve their bioavailibity. Apart from the consumer applications of CDs, it is also useful in protein binding.

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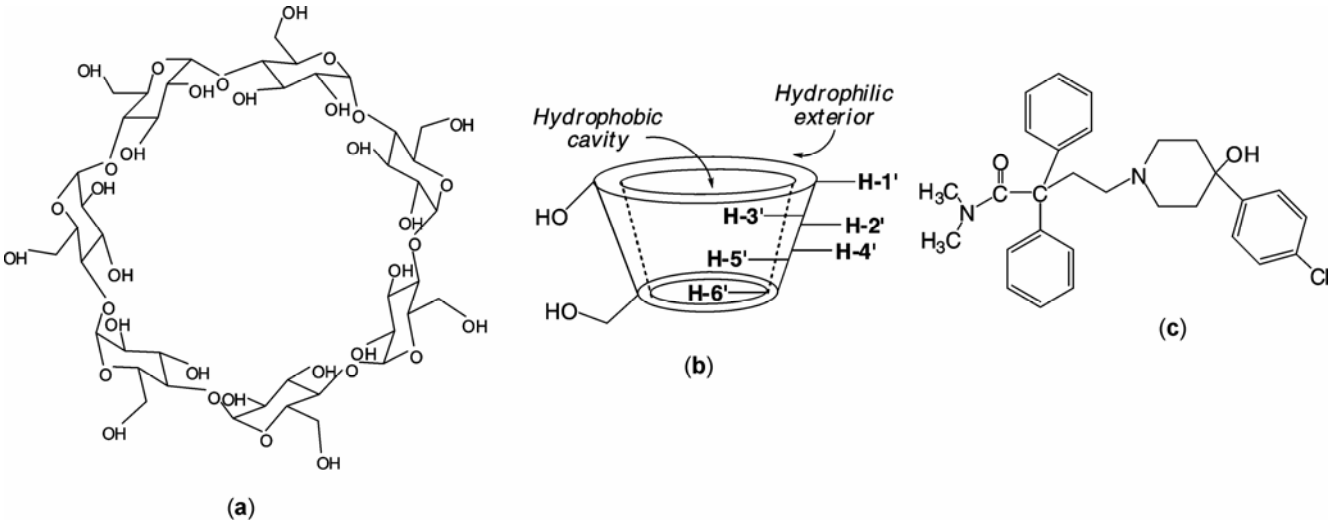

Figure 1. (**a**) Chemical structure diagram of the host β-cyclodextrin (β-CD); (**b**) truncated cone shape of β-CD and position of its different protons; and (**c**) guest loperamide (LPR).

NMR spectroscopy is one of the most efficient tools for CD complexation studies.[2,3] On the basis of ^{1}H NMR chemical shift pattern of CD and guest molecule, the possibility of inclusion complex formation can be concluded. The stoichiometry as well as association/binding constant of guest: CD complexes can be easily calculated from NMR titration results.[2–5] Recently, the use of 2D NMR techniques has been carried out for such type of studies.[2,3] The assignment of the CD and guest molecule can be done from the 2D COSY spectral studies. 2D ROESY spectral data give information about the part of the guest included inside the CD cavity, the mode of penetration, i.e. either from narrower or wider rim side, the depth of penetration and orientation of the guest molecule.

Our group is interested in the complexation studies of pharmaceutical compounds with β-cyclodextrin.[6] In the present communication, we explore the mechanism of loperamide and β-cyclodextrin complexation in aqueous solution. The primary experimental tool for the study being NMR experiments like ^{1}H NMR, 2D COSY and 2D ROESY.

2. Experimental

2.1 *Materials*

Loperamide was obtained form Cadila pharmaceuticals, India while β-cyclodextrin was kindly gifted form DKSH India Pvt. Ltd. and these were used as received.

2.2 *Methods*

All the ^{1}H NMR and 2D NMR (COSY, ROESY) spectra of pure β-CD, pure LPR and β-CD: LPR mixtures were obtained at a temperature of 300 K on a Varian Inova-500 MHz spectrometer using 5 mm HCX ^{1}H-detection NMR probe. For resonance assignment, ^{1}H–^{1}H 2D COSY, ^{1}H–^{1}H 2D ROESY experiments were performed for a 1 : 1 mixture of LPR : β-CD using standard pulse sequences. The 2D ROESY spectrum was collected with mixing time of 500 ms under spin lock condition. ^{1}H NMR spectra of five samples of mixture of β-CD and LPR with LPR/β-CD molar ratios ranging from 0·2 to 1·2 were recorded. The concentration of β-CD was kept constant at 10 mM while that of LPR was varied. The LPR/β-CD molar ratios were calculated by direct integration of appropriate signals. As, there was no separate peak for free as well as complexed form of LPR, it is presumed to undergo rapid exchange between free and bound state on the NMR time scale. The chemical shift values reported in δ (ppm) were calculated with reference to residual solvent (D_2O) resonance at 4·800 ppm. Chemical shift changes ($\Delta\delta$) were calculated according to the formula: $\Delta\delta = \delta_{(\text{complex})} - \delta_{(\text{free})}$.

2.3 *Phase solubility measurements*

Solubility studies were carried out according to the method reported by Higuchi and Connors.[7] Excessive amount of LPR was introduced into flasks con-

taining 10 ml of β-CD solutions of increasing concentrations (0·2 to 1·2 M). The flasks were sealed and shaken on a rotary shaker at 27°C, until the equilibria were reached. The solutions were filtered through 0·22 μm pore size nitrocellulose filters (mdi Membrane Technologies, India). Aliquots of the filtrates were suitably diluted, and the concentration of LPR was determined by UV spectroscopy (Shimadzu UV-2450, UV-Vis spectrophotometer).

2.4 *Stoichiometry and the association constant (K_a) determination*

The stoichiometry and the association constant (K_a) of the LPR: β-CD complexes were determined by using Scott's method.[8] This method is a modification of Benesi–Hildebrand equation.[9] Equation (1) refers the Scott's equation:

$$[\text{LPR}]/\Delta\delta_{obs} = [\text{LPR}]/\Delta\delta_{max} + \Delta\delta_{max}/K_a \quad (1)$$

where [LPR] is the molar concentration of the guest, $\Delta\delta_{obs}$ the observed chemical shift change for a given [LPR] concentration, $\Delta\delta_{max}$ the chemical shift change between a pure sample of complex and the free component at saturation.

In this procedure, the plot of chemical shift changes ($\Delta\delta$) for the β-CD protons against [LPR] in the form of $[\text{LPR}]/\Delta\delta_{obs}$ versus [LPR] (referred to as *y*-reciprocal plot) should be linear for 1 : 1 inclusion complexes. The slope of the plot thus equals to $1/\Delta\delta_{max}$ and the intercept with the vertical axis to $\Delta\delta_{max}/K_a$, allowing the estimation of association constant (K_a).

3. Results and discussion

The resonance assignment of β-CD protons was made on the basis of their specific shapes, ^{1}H NMR, 2D COSY and 2D ROESY spectral data. The most influenced protons of β-CD is H-3′ and H-5′ and shows the significant upfield shift changes while other β-CD protons (H-1′, 2′, 4′, 6′) display the insignificant shift changes in the presence of varying amount of LPR. Figure 2 shows the expansion of a part of ^{1}H NMR spectra showing free β-CD protons and LPR: β-CD mixture. The upfield shift of β-CD cavity protons was mainly due to magnetic anisotropy affects in the β-CD cavity, arising due to the inclusion of a π electron-rich group. Such a group in LPR molecule being a phenyl ring indicates its inclusion in the β-CD cavity. Furthermore, the magnitude of shift changes of these β-CD protons increased with the increase in concentration of LPR. The chemical shift (δ) data of β-CD protons with and without LPR is given in table 1.

An unambiguous resonance assignment of LPR protons was required to ascertain which one of the ring/s or all aromatic rings are involved in complexation. The assignment of LPR protons was made with the help of ^{1}H NMR, 2D COSY and 2D ROESY spectral data because some of the signals which appeared, completely merged in the spectrum of the pure LPR and separated in the presence of β-CD helping in assignment. NMR signals of all the aromatic protons (H-9 to H-22) of LPR in LPR: β-CD mixtures merged with one another because the chemical shifts of two phenyl and one chlorophenyl ring were nearly same. Two triplets at $\delta = 1{\cdot}97$ and $\delta = 2{\cdot}21$ were assigned for H-7_a/H-6_a and H-7_e/H-6_e protons, totally integrating four protons of piperidine ring, two axially$_{(a)}$ and two equatorially$_{(e)}$ arranged, and it showed the cross correlation peaks with each other in 2D COSY spectrum. Two singlets at $\delta = 2{\cdot}33$ and $\delta = 2{\cdot}95$ were assigned for two methyl protons (H-1 and H-2) and it did not show any cross correlation peak to any signal. Similarly, two singlets at $\delta = 2{\cdot}63$ and $\delta = 2{\cdot}76$ were assigned for two methylenic protons (H-3 and H-4), totally integrating four protons and it showed the cross correlation

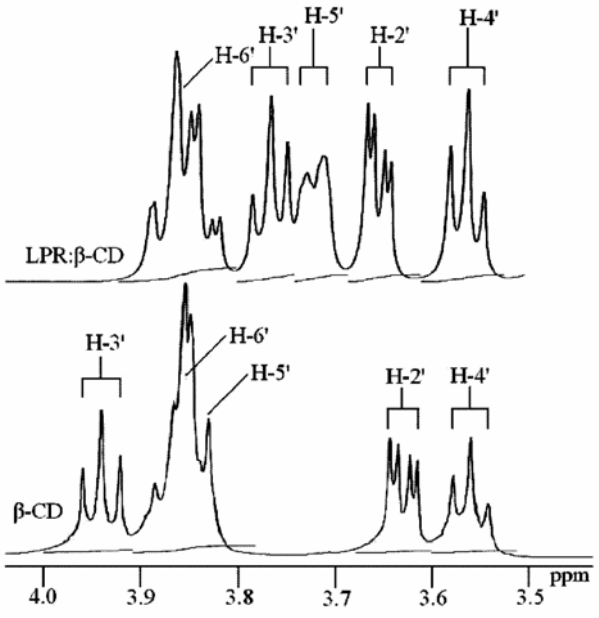

Figure 2. Expansion of a part of ^{1}H NMR data (500 MHz), showing free β-CD protons and LPR: β-CD mixture.

Table 1. ^{1}H NMR (500 MHz) chemical shift (δ) data of pure β-CD and β-CD: LPR mixtures.

Sample	H-1′	H-2′	H-3′	H-4′	H-5′	H-6′
Pure β-CD	5·051	3·633	3·940	3·562	3·830	3·851
β-CD/LPR = 0·2	5·043	3·627	3·849	3·558	3·752	3·848
β-CD/LPR = 0·4	5·039	3·631	3·776	3·561	3·691	3·846
β-CD/LPR = 0·6	5·042	3·630	3·718	3·553	3·641	3·850
β-CD/LPR = 0·8	5·050	3·597	3·667	3·456	3·606	3·846
β-CD/LPR = 1·2	5·048	3·641	3·587	3·558	3·548	3·850

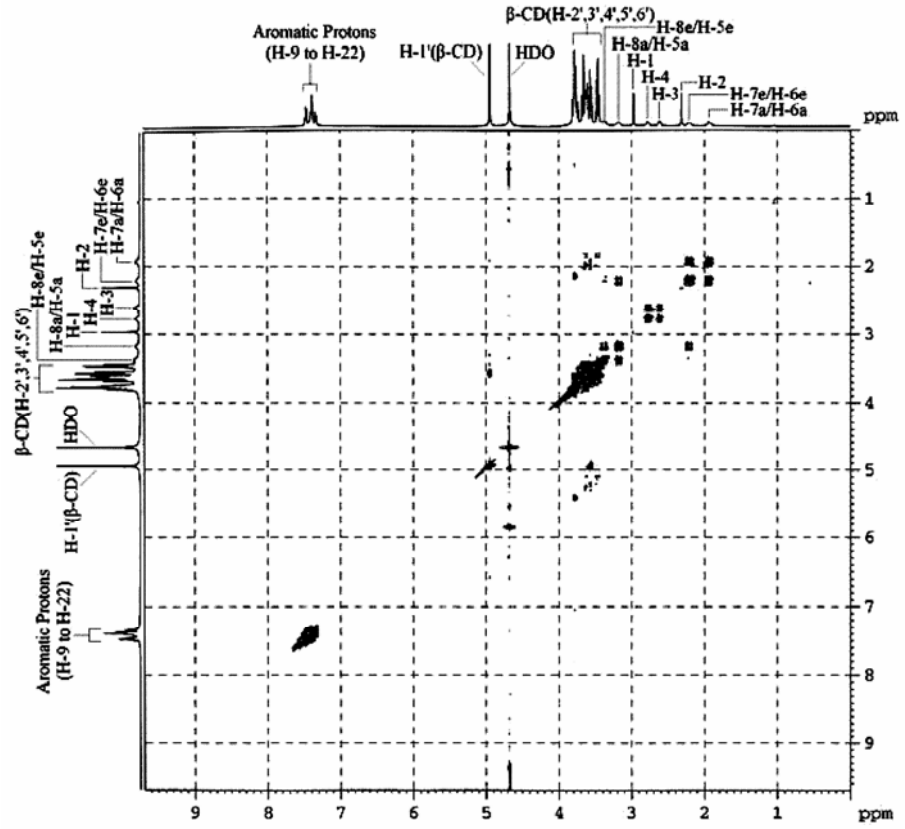

Figure 3. 2D COSY (500 MHz) spectra of LPR: β-CD mixture (1 : 1) showing the ^{1}H–^{1}H cross-correlation peaks.

peak with each other. Another two triplet signals at $\delta = 3{\cdot}17$ and $\delta = 3{\cdot}37$ were assigned for H-8_a/H-5_a and H-8_e/H-5_e protons, totally integrating four protons of piperidine ring, two axially$_{(a)}$ and two equatorially$_{(e)}$ arranged and similarly it showed the 2D COSY cross correlation peaks with each other. Also, H-7_e/H-6_e showed the cross correlation peak with the H-8_e/H-5_e. Moreover, all the aromatic rings' protons showed cross peaks with each other. Figure 3 displays the 2D COSY spectra of LPR: β-CD mixture, showing ^{1}H–^{1}H COSY cross correlation peaks.

In presence of β-CD, all the aromatic protons of LPR show significant downfield shift changes while other LPR protons show insignificant shift changes. The chemical shift (δ) data of LPR protons with and without β-CD are given in table 2. The upfield shift of β-CD cavity protons and downfield shift changes in aromatic protons of guest LPR, is a clear indication of LPR: β-CD inclusion complexes in analogy to our previous studies,[2,3,6] but it is difficult to know which part of aromatic guest penetrates into β-CD cavity. Since the ^{1}H NMR data was not conclusive

Table 2. ^{1}H NMR (500 MHz) chemical shift (δ) data of free LPR and LPR: β-CD mixtures.

Proton	Pure guest	G/H = 0·2	G/H = 0·4	G/H = 0·6	G/H = 0·8	G/H = 1·2
H-1	2·953	2·958	2·957	2·960	2·963	2·961
H-2	2·338	2·347	2·342	2·331	2·331	2·353
H-3	2·635	2·642	2·632	2·641	2·624	2·774
H-4	2·762	2·772	2·762	2·775	2·770	2·774
H-5_a	3·174	3·192	3·178	3·182	3·175	3·183
H-5_e	3·372	3·373	3·376	3·374	3·372	3·373
H-6_a	1·977	1·985	1·972	2·008	1·933	2·004
H-6_e	2·212	2·222	2·213	2·215	2·211	2·212
H-7_a	1·970	1·981	2·001	2·002	1·973	2·001
H-7_e	2·210	2·231	2·251	2·210	2·190	2·212
H-8_a	3·171	3·180	3·182	3·173	3·171	3·433
H-8_e	3·371	3·387	3·362	3·368	3·372	3·433
Aromatic region	7·261–7·506	7·292–7·537	7·314–7·561	7·332–7·580	7·350–7·618	7·393–7·643

a, Axial protons; e, equatorial protons; [G] = guest LPR; [H] = host β-CD

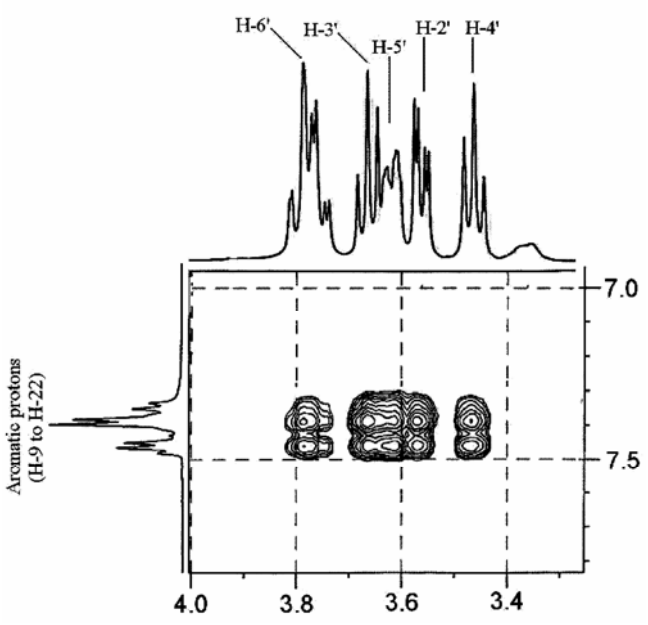

Figure 4. Expanded region of 2D ROESY (500 MHz) spectra of LPR: β-CD mixture (1 : 1) showing the ^{1}H–^{1}H NOE's between β-CD protons and aromatic protons of LPR.

of inclusion of guest molecule inside the β-CD cavity, we performed the 2D ROESY spectral studies for confirming our results. It helped us to establish the structure of LPR: β-CD inclusion complexes in solution. Expansion of a part of 2D ROESY spectral data showing ^{1}H–^{1}H cross connection peaks between host β-CD and guest LPR is shown in figure 4.

The ROESY spectral data shows that all the aromatic protons of the guest LPR are close in space to cavity protons (H-3′ and H-5′) as well as to other protons of β-CD. This suggests that the aromatic rings of LPR are included in the hydrophobic cavity of β-CD. Moreover, strong cross connection peaks between H-6′ (located at the mouth of narrower rim), H-2′ and H-4′ (located at outer periphery) were also observed which is a clear indication of the inclusion of guest LPR from narrower as well as wider rim side. Two phenyl rings and one chlorophenyl ring show equal intensity of NOEs with β-CD cavity protons (H-3′ and H-5′), thus suggesting close proximity of former with latter. From the aforementioned observations, we concluded that there is no preference for inclusion of a particular aromatic ring inside the β-CD cavity. Since –Cl is hydrogen bonded to rim –OH, it must be positioned near the narrower rim suggesting the penetration of chlorophenyl ring from the wider rim but nothing can be said with certainty about the mode of penetration of phenyl ring, but it seems more likely to be from the wider rim side.

On the basis of 2D ROESY spectral data, 1 : 1 stoichiometry and chemical shift (δ) observed in ^{1}H NMR spectra of pure β-CD, pure LPR and β-CD : LPR mixture, it can be supposed that LPR forms six 1 : 1 β-CD : LPR inclusion complexes in aqueous solution. The structures for the six 1 : 1 LPR: β-CD inclusion complexes are proposed as shown in figure 5.

The solubility of LPR increased linearly with an increase in the concentration of β-CD, giving A_L type of solubility diagram as reported by Higuchi and Connors,[7] indicating the formation of soluble complexes of presumable 1 : 1 stoichiometry. The

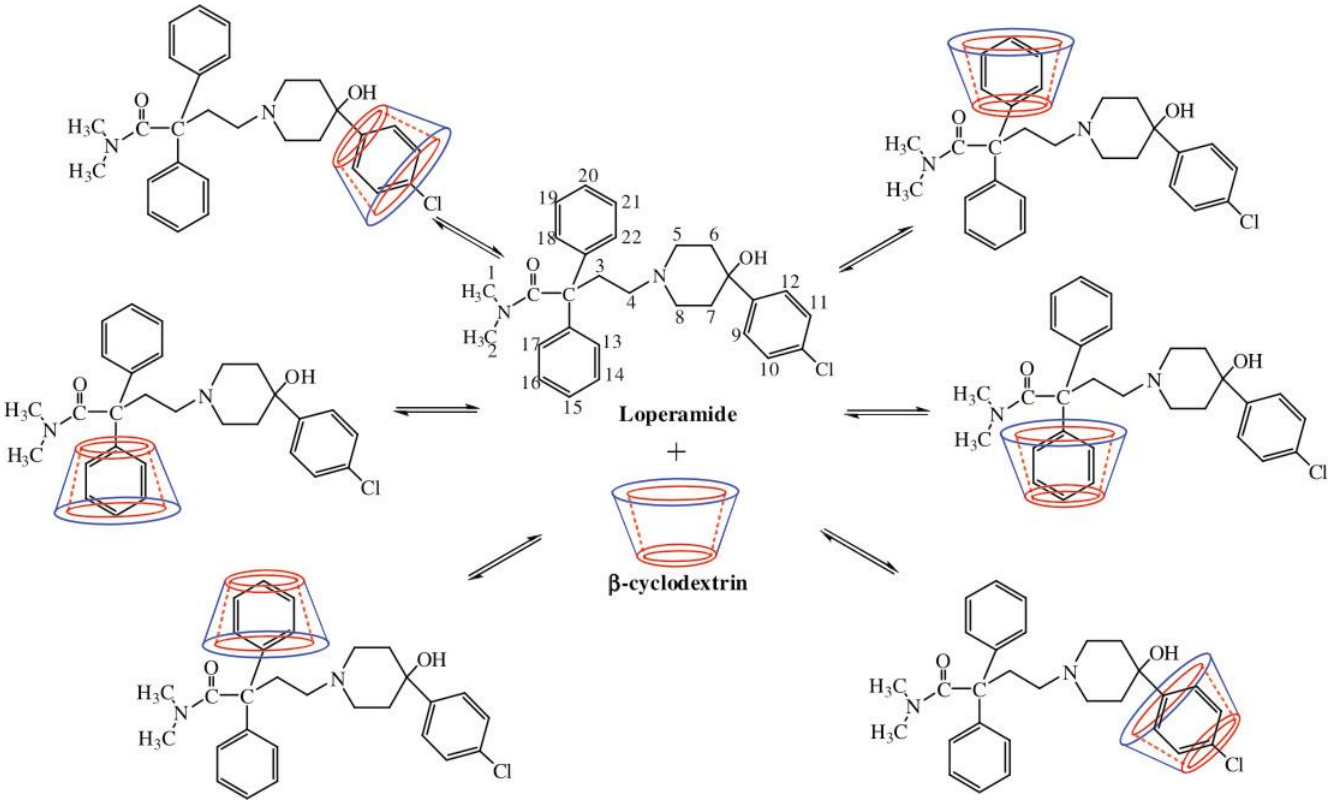

Figure 5. Structures of six possible 1 : 1 LPR: β-CD inclusion complexes.

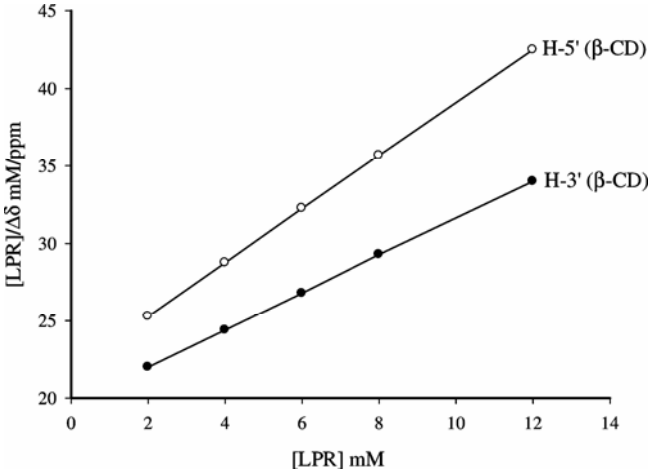

Figure 6. A typical Scott's plot for LPR: β-CD inclusion complexes showing 1 : 1 stoichiometry.[8] (Overall association constant (K_a) = 68·805 M^{-1}.)

increase in solubility of LPR is due to the one or more molecular interaction between guest LPR and host β-CD.

The association constant was estimated by Scott's plot technique[8] (figure 6) and the overall association constant (K_a) was calculated to be 68·805 M^{-1} (average of two independent K_a values).

4. Conclusion

The inclusion complex formation between LPR and β-CD, the stoichiometry and the association constant, were assessed by NMR observation in aqueous solution. The insertion of LPR molecule into β-CD cavity was demonstrated by changes in ^{1}H NMR chemical shift values. NMR chemical shift change data was treated to determine 1 : 1 stoichiometry for the LPR : β-CD inclusion complexes and the measured association constant was consistent with efficient complexation. On the basis of observed 2D ROESY spectral data a structure for the LPR: β-CD inclusion complexes was proposed. Spectral data suggested that the aromatic moiety of LPR is most probably included inside the hydrophobic cavity of β-CD in aqueous solution. The inclusion can be from wider as well as narrower rim side. This study also proves the pertinence of NMR spectroscopy as an important tool for studying drug : CD interactions with a better discussion of supramolecular assem-

blies in solution and also to characterize structurally the inclusion compounds formed in aqueous solution. The aqueous solubility of LPR has been improved in solution through complexation with β-CD.

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The abbreviations used

LPR, loperamide; β-CD, β-cyclodextrin; ^{1}H NMR, proton Nuclear Magnetic Resonance spectroscopy; 2D, two-dimensional; COSY, COrrelation SpectroscopY; ROESY, ROtating frame Overhauser Effect SpectroscopY; NOE, Nuclear Overhauser Effect.

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