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Analysis of Inclusion Complexes of Clotrimazole and Differently Substituted α, β and γ Cyclodextrins by NMR Spectroscopy

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Abstract: *Clotrimazole, a widely used imidazole-type antifungal agent and its cyclodextrin complexes were studied. Stoichiometries, structures and stability constants of the inclusion complexes with fourteen different cyclodextrin derivatives were characterized using NMR spectroscopy as primary technique. The cyclodextrin complexes were found to be of high stability (logK > 2). 2D ROESY NMR spectra revealed the formation of isomeric complexes with every cyclodextrin studied. The highest stability constant was observed with sulfobutylated β-CD, and interestingly enough the heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin showed the lowest stability.*

Keywords*: Clotriomazole, cyclodextrin, inclusion complex, stability, NMR*

1. Introduction

Clotrimazole, 1-(*o*-Chloro-*α,α*,-diphenylbenzyl)-imidazole (CTZ), is a first generation imidazole derivative antifungal drug, used topically in the treatment and prophylaxis of disseminated and deep organ candidiasis and for the treatment of tinea infections. Besides being an antifungal, there are promising results on its use against sickle cell anemia, malaria, beriberi, tineapedis, Chagas disease and cancer [1-3].

CTZ is suitable for both topical and oral administration as a free base.

Clotrimazole can cause allergies, which are also treated with antihistamines [4,5].

Its four aromatic rings provide the molecule with extreme lipophilicity [1].

The constitutional formula and numbering of CTZ are in Figure 1.

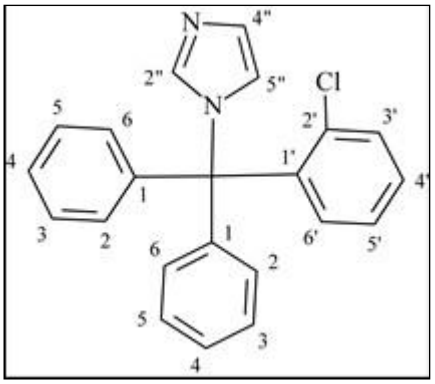

Figure 1. Constitutional formula and numbering of CTZ

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Clotrimazole has an extremely low water solubility (0.49 μg/mL) [6] and very high octanol/water partition coefficient (logP = 5.9) [7], indicating its viability in lipophilic media only. Since CTZ is reported to be a weak base ($pK_a \approx 6$) [8,9], its uncharged form is predominant at physiological pH in biological fluids.

Earlier attempts to enhance the solubility and the concomitant bioavailability of CTZ were made by cyclodextrin complex formations [10-12, 6, 13], applying native β-CD, (2-hydroxypropyl)-β-CD, methyl-β-CD, heptakis(2,6-di-O-methyl)-β-CD and native α- and γ-CD.

All the reported studies investigated the complex formation with the unprotonated form of CTZ. By these means, even a several hundred-fold solubility increase would only result in a solubility around 0.1 mg/ml, which is still not high enough. On the other hand, the protonation of CTZ increases the solubility by several orders of magnitude, which can be further improved by cyclodextrin complex formation. Moreover, one could expect more stable complexes between negatively charged CDs and the positively charged CTZ.

Here we report the inclusion complex formation of the protonated form of clotrimazole, with fourteen different cyclodextrins: native α, β and γ-cylclodextrin, (2-hydroxypropyl)- α, β and γ-cylclodextrin, methyl- α, β and γ-cylclodextrin, sulfobutyl ether-α, β and γ-cylclodextrin sodium salt, heptakis(2,6-di-O-methyl)- β- cylclodextrin and heptakis(2,3,6-tri-O-methyl)- β- cylclodextrin.

To determine the complex stoichiometry and stability, NMR-CD titrations were run, the composition of the complexes was evaluated by Job's method of continuous variation [14-17].

To explore the host-guest geometry of the CD-complexes, 2D ROESY spectra were recorded, since the relative volume integrals of intermolecular correlation peaks are measures of through-space distances between adjacent protons of the host and guest molecules.

2. Materials and methods

Materials

Clotrimazole was from Sigma-Aldrich. The deuterated solvents (D_2O, DMSO-d6) were purchased from Tokyo Chemicals Inc. Cyclodextrins (α-cyclodextrin (ACD), (2-hydroxypropyl)-α-cyclodextrin (HPACD, degree of substitution (DS) = 3.8), methyl-α-cyclodextrin (RAMEA, DS = 10.9), sulfobutyl ether α-cyclodextrin sodium salt (SBACD, DS = 4.3), β-cyclodextrin (BCD), (2-hydroxypropyl)-β-cyclodextrin (HPBCD, DS = 4.6), methyl-β-cyclodextrin (RAMEB, DS = 12.6), heptakis(2,6-di-O-methyl)- β-cyclodextrin (DIMEB), heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin (TRIMEB), sulfobutyl ether β-cyclodextrin sodium salt (SBBCD, DS = 6.3) γ-cyclodextrin (GCD), (2-hydroxypropyl)-γ-cyclodextrin (HPGCD, DS = 4.1) and methyl-γ-cyclodextrin (RAMEG, DS = 13.2), sulfobutyl ether γ-cyclodextrin sodium salt (SBGCD, DS = 4.0)) were from Cyclolab Ltd (Budapest, Hungary). All other solvents, reagents of analytical grade were obtained from commercial suppliers and used without further purification. Bidistilled water was used in all experiments.

Methods

^{1}H-NMR-CD-titrations

All NMR measurements were carried out on a Varian VNMRS spectrometer (600 MHz for ^{1}H). Spectra were recorded at 25 ^oC and referenced to the DMSO signal (2.700 ppm). Titrations were carried out in a medium of 5 v/v% DMSO, 5 v/v% D_2O and 90 v/v% H_2O. DMSO in 5% was needed because of the poor solubility of CTZ even in acidic solutions.

A stock solution containing 20 mM CTZ was prepared in DMSO. The background medium contained 5 % (v/v) D_2O and 0.01 M HCl, the ionic strength was set to 0.15 M using KCl. The CD solutions were also prepared using this background media. 30 μl of the CTZ stock solution was mixed with different volumes of CD stock solution, and filled with the background media to a total volume of 600 μl. Spectra were recorded after 24 hours, in order to reach the equilibrium state. The solvent signals were suppressed by the WET pulse sequence. 64 transients were accumulated and 32768 data points were collected for each spectrum.

Determination of complex stoichiometry

Solutions were prepared from CTZ and the appropriate host α, β and γ CD in complementary amounts, to make up a 1 mM total concentration. The solutions were mixed in different ratios, and the ^{1}H NMR spectra were recorded after 24 hours.

Determination of the structure of inclusion complexes formed

Solutions containing 2 mM CTZ and 5 mM α-CD, β-CD or γ-CD were prepared in D_2O containing 0.02 M DCl. The structures of the complexes were determined from 2D ROESY experiments, where 64 ^{1}H spectra were accumulated for 512 increments. The mixing time was 300 ms and the number of data points was 2048.

Molecular modelling of the complexes

Structures of CTZ and the five single isomer cyclodextrins were drawn in Hyperchem 8.0 software. The 3D structures of the molecules were optimized using the semi-empirical PM3 method using the Polak-Ribiere conjugate gradient algorithm, and energies of the molecules were also calculated. In the docking procedure CTZ was placed near the wider rim of CD, and the structure of the inclusion complex was optimized using the MM+ molecular mechanics method. This structure was optimized further by the semi-empirical PM3 method, which is widely used in cyclodextrin chemistry [18]. The energy of complexation was calculated as follows: $\Delta E_{\text{complexation}} = E_{\text{complex}} - \left(E_{\text{CTZ}} + E_{\text{CD}}\right)$. A large negative value means the formation of a complex of high stability.

3. Results and discussions

NMR assignments of clotrimazole

The assignment of the signals was based on chemical shifts, multiplicity patterns, 2D 1H-13C HSQC, -HMBC measurements. The values are listed in Table 1. The chemical shift of the central, quaternary, aliphatic carbon is 78.9 ppm.

Table 1. ^{1}H and ^{13}C chemical shifts, multiplicity and some coupling constants of clotrimazole in 0.01 M DCl in D_2O/DMSO-d6 (s: singlet, m: multiplet, d: doublet, t: triplet)

	^{1}H		^{13}C
Position	δ (ppm)	multiplicity	δ (ppm)
1			139.3
2,6	7.12	d (J = 7.6 Hz)	130.7
3,5	7.33	m	129.4
4	7.35	m	129.9
1′			135.4
2′			138.1
3′	7.44	d (J = 8.2 Hz)	133.3
4′	7.27	t (J = 8.2 Hz)	128.4
5′	7.40	t (J = 8.1 Hz)	132.0
6′	7.07	d (J = 8.1 Hz)	132.1
2″	8.66	s	137.5
4″	7.42	s	120.5
5″	7.26	s	124.6

Stoichiometry of the complexes

Stoichiometry of the CD complexes was determined by the continuous variation method of Job [15], where the chemical shift changes (Δδ) weighted by the mole fraction of CTZ were plotted against the mole fraction of CTZ.

The chemical shift change extrema were found at $\chi = 0.5$ (Figure 2), indicating the classical 1:1 stoichiometry of the complexes.

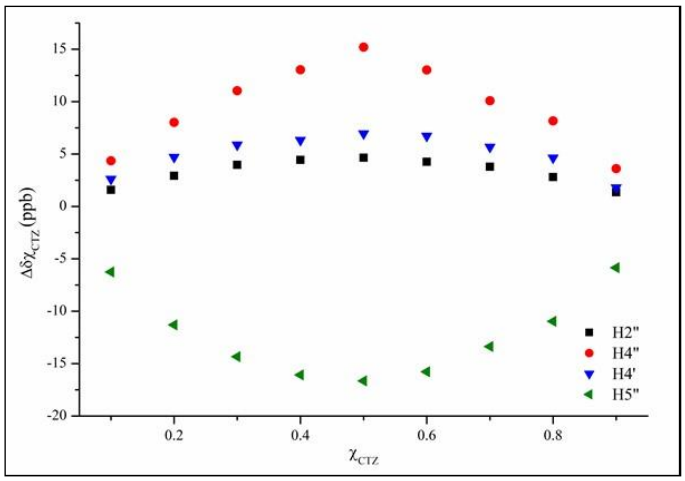

Figure 2. Job's plot of selected protons of clotrimazole with β-cyclodextri.

Stability constants of the complexes

The stability constants were determined by NMR titrations, where the CTZ concentration was kept constant and the CD concentrations were changed. Since complex formation is a fast process on the NMR chemical shift timescale, only one common peak of a particular NMR nucleus can be observed. The observed chemical shift is the weighted average of the free and complexed CTZ, which can be expressed in terms of the analytical concentrations of the reactants and the limiting chemical shifts [19]:

$$\delta^{obs} = \delta_{CTZ} + \Delta\delta \frac{[CTZ]_T + [CD]_T + \frac{1}{K} - \sqrt{\left([CTZ]_T + [CD]_T + \frac{1}{K}\right)^2 - 4[CTZ]_T[CD]_T}}{2[CTZ]_T} \quad (1)$$

where δ^{obs} is the observed chemical shift of the NMR nucleus in question, δ_{CTZ} is the chemical shift of the uncomplexed CTZ, $\Delta\delta$ is the chemical shift change upon complexation, K is the stability constant of the complex, $[CTZ]_T$ and $[CD]_T$ are the analytical concentrations of clotrimazole and cyclodextrin, respectively.

Typical ^{1}H NMR-CD titration curves are in Figure 3.

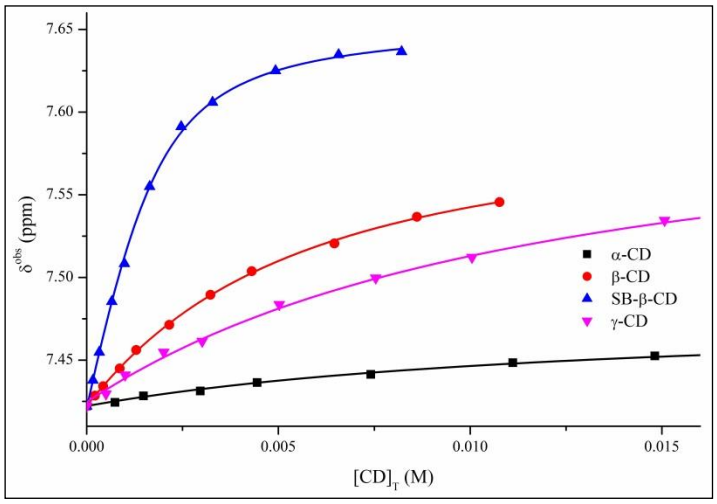

Figure 3. ^{1}H NMR-CD profiles of H4" nuclei with various cyclodextrins

The stability constants were evaluated by fitting Eq. (1) to the δ^{obs} *vs.*$[CD]_T$ datasets. The results are listed in Table 2.

Table 2. Stability constants of CTZ with various cyclodextrins, in terms of logK units.

	logK		logK		logK
α-CD	1.94 ± 0.01	β-CD	2.36 ± 0.01	γ-CD	1.97 ± 0.03
HPACD	2.04 ± 0.01	HPBCD	2.50 ± 0.02	HPGCD	1.95 ± 0.02
RAMEA	2.67 ± 0.02	RAMEB	2.63 ± 0.02	RAMEG	2.07 ± 0.01
SBACD	2.97 ± 0.02	SBBCD	3.29 ± 0.01	SBGCD	2.49 ± 0.03
		DIMEB	3.16 ± 0.03		
		TRIMEB	1.28 ± 0.03		

Substitution of the CD in α-CD derivatives increases the stability of the complexes: the (2-hyroxypropyl)-derivate is 25 % more stable than the native α-CD-CTZ complex, while the RAMEA-CTZ complex has a 5 times higher stability constant. Introducing negative charges on the cyclodextrin will increase the stability by one order of magnitude, because of the electrostatic attractions between the sulfonate groups and the positively charged imidazole ring of CTZ.

For β-CD derivatives, the same order of stability can be observed: the (2-hydroxypropyl)–derivative is slightly more stable than the native β-CD-CTZ complex, while methylation causes a significantly enhanced stability. The negatively charged SBBCD forms the complex of highest stability, due mainly to the electrostatic interactions. Surprisingly enough, stability of the DIMEB–complex is almost as high as the stability of the SBBCD complex, although no electrostatic interactions can occur. On the other hand, the permethylated derivative (TRIMEB) forms a complex of very low stability, the difference between the two single–isomer methyl-derivate is striking: the CTZ-DIMEB complex is approximately 75 times more stable than the CTZ-TRIMEB complex.

In case of γ-CD derivates, the neutral substituents had only a small effect on the stability of the complexes, while SBGCD complexes were 0.5 logK units more stable than the neutral ones.

Structure of the complexes

The approximate complex structures were derived from 2D ROESY NMR data and molecular modelling. The intermolecular ROESY cross-peaks indicate the vicinity of certain protons, within 5 Å, providing sound basis to elucidate the structural orientation of the clotrimazole moieties in the cavity of the host cyclodextrins.

ROESY spectra of CTZ were recorded with the five single-isomer cyclodextrins (ACD, BCD, GCD, DIMEB and TRIMEB). Cross peaks with the α-CD were found between the protons in the phenyl and 2-chlorphenyl rings and the H3 and H5 signals of CD. The cross-peaks were stronger between H3 and the CTZ protons. This clearly indicates that two isomeric complexes coexist: either one of the phenyl rings or the 2-chlorphenyl ring immerses from the wider rim into the CD cavity.

The chemical shifts of the cyclodextrin H3 and H5 protons in CTZ-BCD complex are overlapping, but clear cross peaks can be found between the phenyl and the chlorophenyl protons and the cyclodextrin protons. An additional cross-peak is observed between the cyclodextrin H6 signals and the overlapping phenyl 3, 5 and 4 protons. This means that two isomeric complexes are formed, similarly to α-CD, but the phenyl ring immerses deeper into the cavity than the 2-chlorophenyl ring.

The CTZ-DIMEB has the highest stability among the complexes studied with neutral CDs, indicating that, there must be further interactions that stabilize the complex. This is also corroborated by the 1H NMR spectrum, since the doublet signal of protons 2 and 6 in CTZ split into two doublets, due to the prochiral nature of CTZ. If one of the two phenyl rings gets into the CD cavity, the two rings become nonequivalent, and in a chiral environment, such as the cyclodextrin cavity, the isomers become diastereomers. Further cross peaks are visible in the ROESY spectrum of CTZ-DIMEB complex between the 2-methyl protons of DIMEB and the CTZ protons 3, 4, 5, 3', 5', 4", 5", meaning that CTZ enters into the cavity from the wider rim. Since a clear cross peak can be seen between the 6-

methyl protons of DIMEB and hydrogens 2, 3, 5 of CTZ, the phenyl ring immerses deeper into the cavity than in case of CTZ-BCD complex (Figure 4). The 3D structures of the ACD, BCD and DIMEB complexes are similar, the largest difference can be found in their stability. Latter was confirmed by molecular modelling, the complexation energies are listed in Table 3.

Table 3. Complexation energies of CTZ-CD complex isomers.

	ΔE (kcal/mol)	
	Isomer A (Figure 4)	Isomer B (Figure 4)
ACD	-10.17	-11.54
BCD	-13.41	-14.19
DIMEB	-26.69	-28.09

The results are in excellent agreement with the stability constants: not only the order of stability ($K_{ACD}<K_{BCD}<K_{DIMEB}$), but also the differences in the energy are proportional to the differences in the stability constants ($\Delta \log K_{ACD\text{-}BCD} \approx 0.4$ and $\Delta \log K_{BCD\text{-}DIMEB} \approx 0.8$).

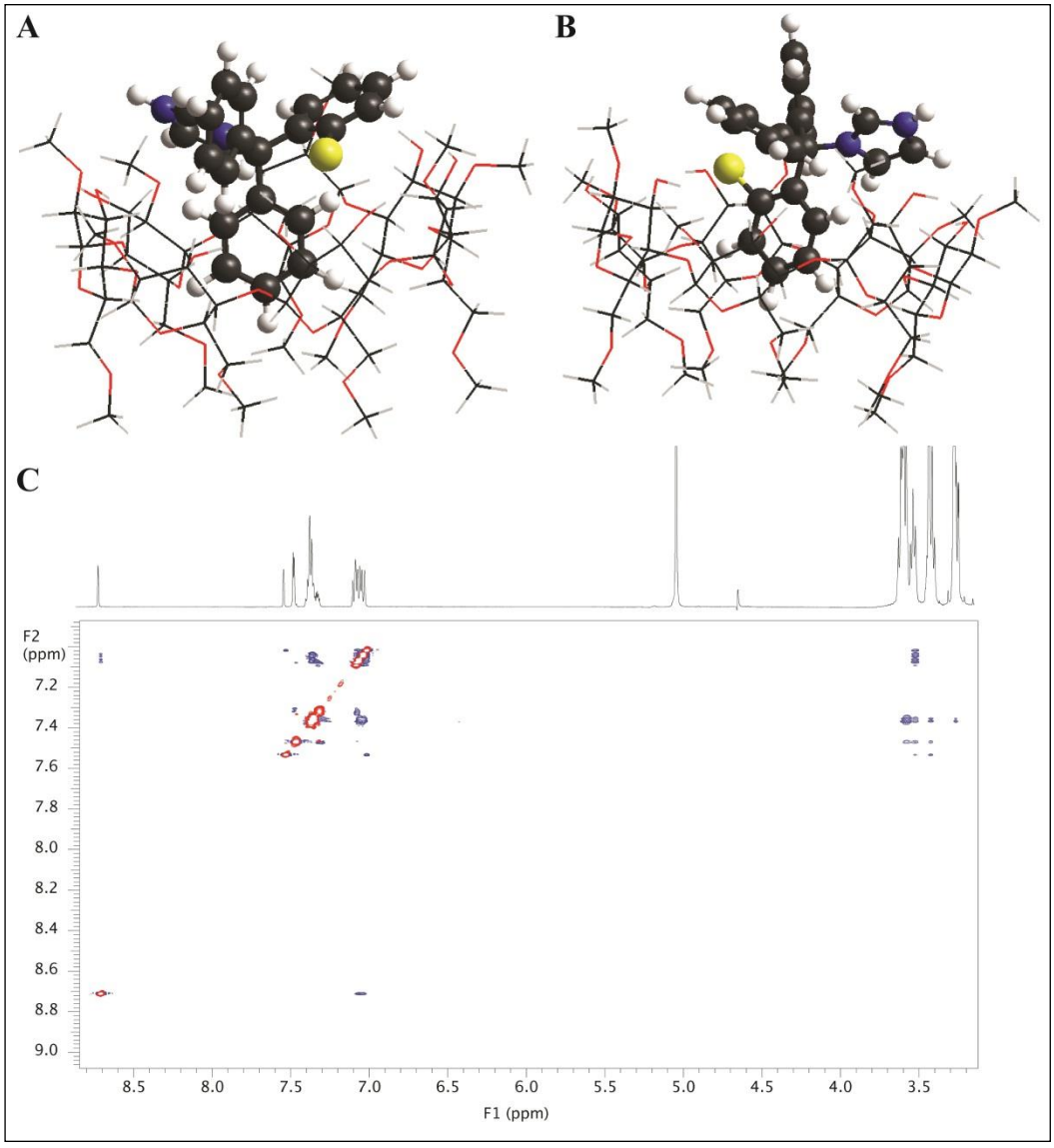

Figure 4. A, B: Structures of the two isomeric CTZ-DIMEB complexes
C: Part of ^{1}H-^{1}H ROESY spectrum of the complex

The TRIMEB complex has the lowest stability constant. On the ROESY spectrum of the complex only the 3, 4, 5 protons have cross peaks with the cyclodextrin H3 and H5 protons, indicating the existence of one inclusion complex only. These CTZ protons have also cross peaks with all the three methyl (2,3 and 6) and H4 protons of CD (Figure 5**C**). The most probable explanation for this phenomenon is the outer binding. The protons of the 2-chlorophenyl ring have cross peaks only with the 3-methyl protons of TRIMEB, thus they are not located in the cavity, only associated from the outside to the CD (Figure 5**B**).

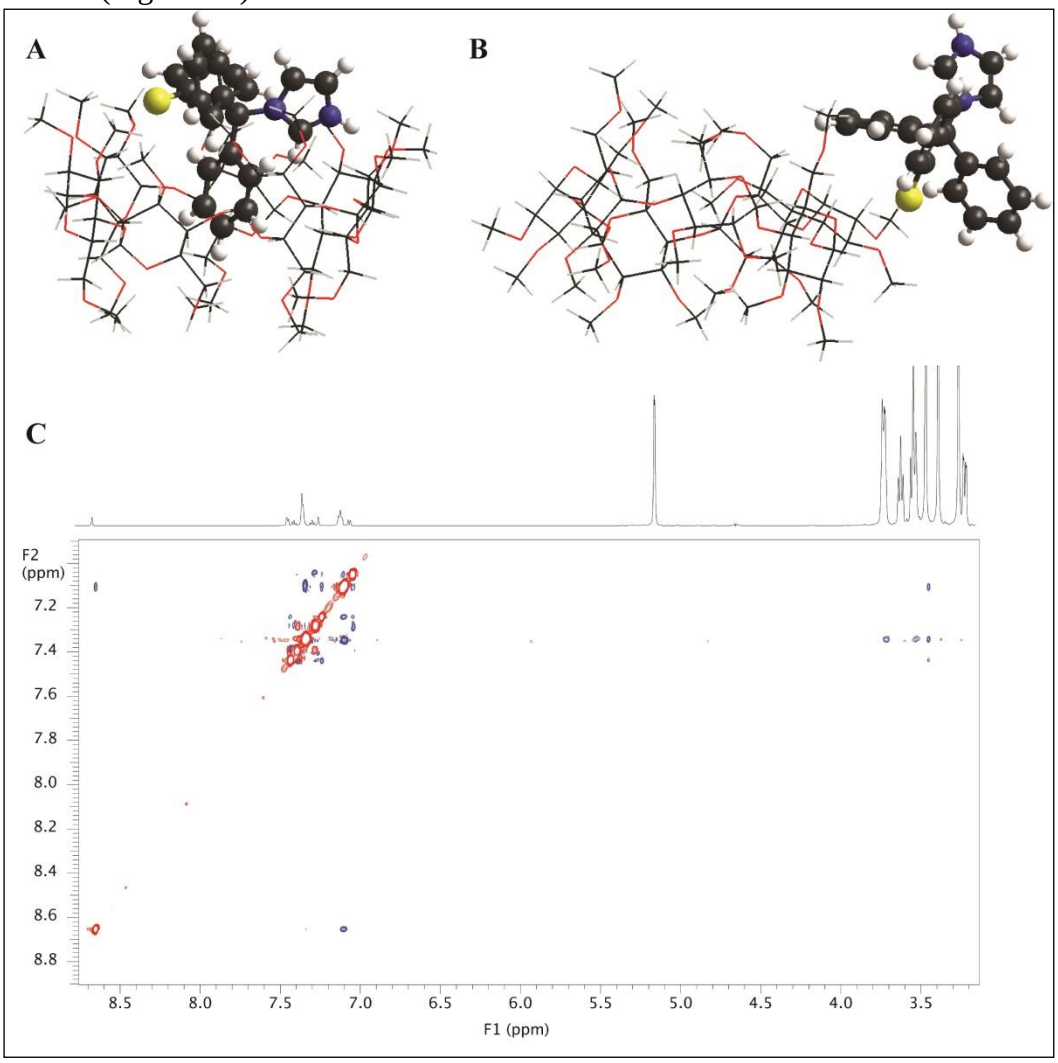

Figure 5. A, B: Structures of the two isomeric CTZ-TRIMEB complexes.
C: Part of ^{1}H-^{1}H ROESY spectrum of the complex

In case of γ-CD all CTZ protons have cross-peaks with the cyclodextrin H-3 proton and weaker cross peaks were observed with cyclodextrin H-5 proton (Figure 6**B**). These observations, and the appearance of new intramolecular cross peaks between protons 2 and 2'; 2' and 2", 2 and 2", 2 and 5' indicate that two rings simultaneously immerse into the cavity, due the larger diameter of the CD cavity (Figure 6**A**).

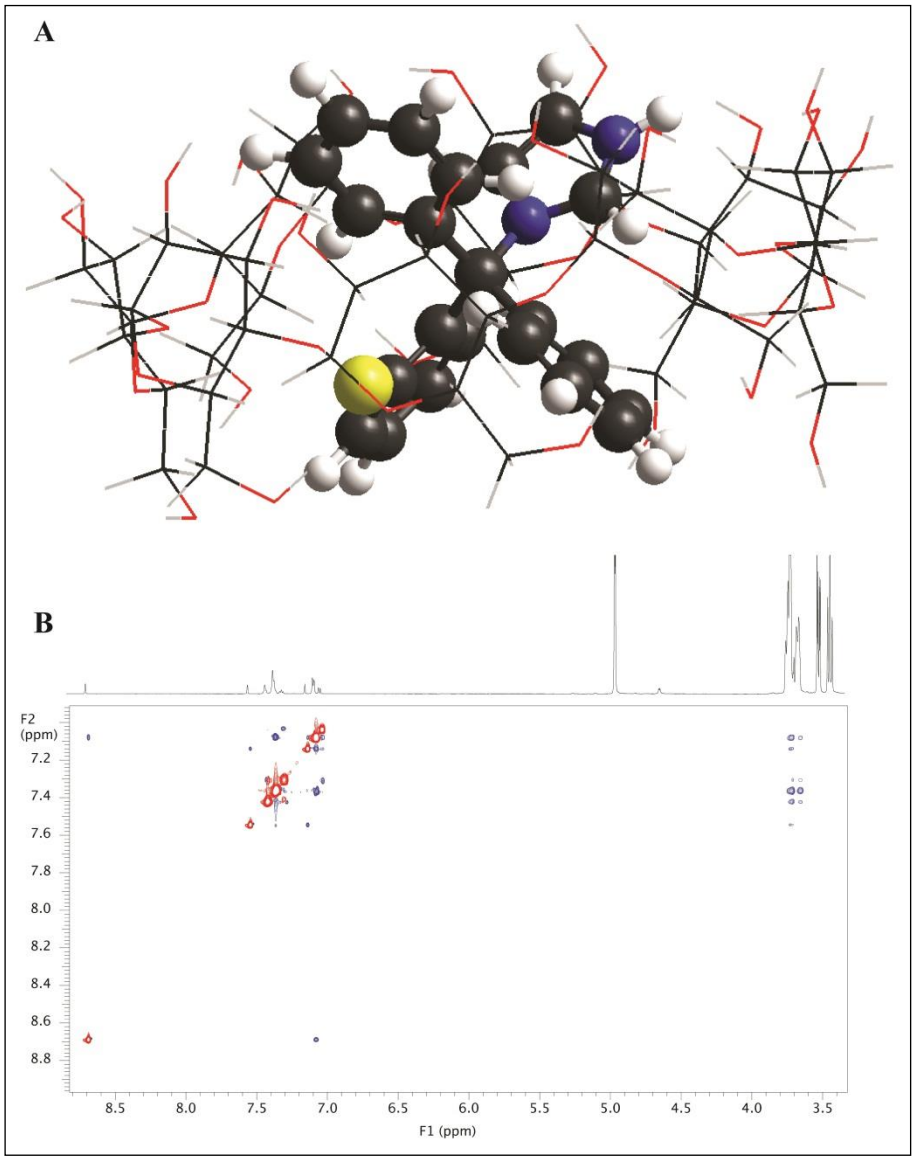

Figure 6. A: Structures of the CTZ-GCD complexes.
B: Part of ^{1}H-^{1}H ROESY spectrum of the complex

4.Conclusions

Protonated clotrimazole forms stable complexes with each of the α, β and γ cyclodextrins. Substitution of the cyclodextrins usually increases the stability of the complexes by 0.2 – 1 logK units. The largest increase was found by introducing negatively charged side chain, like sulfobutyl groups. Interestingly the CTZ-DIMEB complex is of unexpectedly high stability, while the CTZ-TRIMEB complex has a stability one order of magnitude lower, compared to native β-CD. NMR experiments and molecular modelling also corroborated the existence of isomeric complexes The calculated complexation energies are in good agreement with NMR data: the intermolecular cross peaks of the more stable isomer have larger volume integrals. Such stable cyclodextrin complexes can largely increase the water solubility of clotrimazole, enabling the development of new pharmaceutical formulations for improved bioavailability.

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