NMR Studies of the Inclusion Complexes Between Ezetimibe and Cyclodextrins

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Ezetimibe, the antihyperlipidemic drug of poor bioavailability was complexed with native and derivatized cyclodextrins.The complexes were characterized in terms stability, stoichiometry and structure using various 1D and 2D solution NMR spectroscopic techniques. The complexes were found to be of moderate stability (logK<3). The least stable inclusion complex is formed with β-cyclodextrin, while the ezetimibe-methylated-β--cyclodextrin has a 7-fold higher stability. The results can be useful to improve the poor water-solubility and the concomitant bioavailability of ezetimibe.

Keywords: ezetimibe, cyclodextrin, inclusion complex, Job plot, stoichiometry

Ezetimibe, (3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxy-phenyl) azetidin-2-one (EZE, fig. 1) is a strong cholesterol absorption inhibitor that prevents its absorption by inhibiting the passage of dietary and biliary cholesterol across the intestinal wall without affecting the absorption of fat-soluble vitamins, triglycerides or bile acids[1, 2]. It acts on Niemann-Pick-C1-Like (NPC1L1) transporter proteins in the brush boarder of enterocytes and macrophages[3]. It also demonstrates anti-inflammatory and immunomodulatory effects and influences the expression of certain antigens[4, 5].Its therapeutic use has been shown to be safe and effective among patients after cardiac, renal and liver transplant as well as in HIV patients [5].

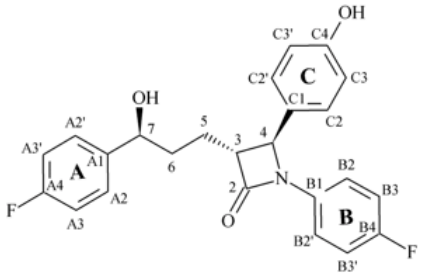

Fig. 1. Structure, numbering and ring indexing of EZE

An inherently limiting factor of its efficacy is the poor water-solubility and the concomitantly low bioavailability.In fact, its water solubility is 1.2 μg/mL at 25°C, and its bioavailability is 35-65 % [6].

Due to its therapeutic significance, several attempts have been made to increase the aqueous solubility and the dissolution characteristics of EZE, including solid dispersion[7], co-crystal formation [6, 8], liquisolid techniques[9], self-nano emulsifying drug delivery system(SNEDDS) [10] and cyclodextrin (CD) complexation [11].Only a few doubtfuldata were published on the stability of EZE-CD complexes [12, 13], bearing the contradiction that EZE forms a 1:2 complex with HP-β-CD, in spite of the dimension of the given stability constants (M^{-1}) necessitates a 1:1 stoichiometry.

Nevertheless, it has been shown that complex formation with (2-hydroxypropyl)-β-CDcan increase the solubility of EZE approximately 15 times [11].

In order to elaborate a systematic strategy to significantly improve the bioavailability of EZE, it was our objective to clarify the stoichiometry, stability and structure of EZE with CDs of different cavity size and chemical modifications. We have therefore studied the inclusion complex formation of EZE with seven cyclodextrins: β-CD, (2-hydroxypropyl)-β-CD (HPB), methyl-β-CD (RAMEB), (2,3,6-tri-O-methyl)-β-CD (TRIMEB), γ-CD, (2-hydroxy-propyl)-γ-CD (HPG), methyl-γ-CD (RAMEG).

The stoichiometry and the stability of the complexes were determined by NMR-CD titrations. The stoichiometry was evaluated by Job's method of continuous variation [14]. The approximate structure of the complexes were elucidated by 2D ROESY NMR spectra, and visualized by the Hyperchem software. CD complexes are often characterized in the solid by phase by X-ray diffraction [15] and thermoanalytical methods, such as DSC or thermogravimetry [16-18]. NMR spectroscopy, on the other hand, is capable of characterizing complexes in solution phase [19].

Experimental part

Materials

Ezetimibe was a generous gift of Sandoz. The deuterated solvents (D_2O, DMSO-d6) were purchased from Tokyo Chemicals Inc. Cyclodextrins (β-cyclodextrin, (2-hydroxypropyl)-β-cyclodextrin (degree of substitution, DS = 4.6), methyl-β-cyclodextrin (DS = 12.6), heptakis(2,6-di-O-methyl)-β-cyclodextrin, heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin, γ-cyclodextrin, (2-hydroxypropyl)-γ-cyclodextrin (DS = 4.1) and methyl-γ-cyclodextrin (DS=13.2)) were from Cyclolab Ltd.,Budapest, Hungary.All the other solvents, reagents of analytical grade were obtained from commercial suppliers and used without further purification. Bidistilled water was used in every experiment.

^{1}H-NMR-CD-titrations

All measurements were done on a Varian VNMR spectrometer (600 MHz for ^{1}H). Spectra were recorded at 25°C and referenced to the ^{13}C satellite signal of DMSO (2.700 ppm). Titrations were carried out in a medium of 25 v/v% DMSO, 5 v/v% D_2O and 70 v/v% H_2O.

A stock solution of10 mM EZE was prepared in DMSO. The background medium contained 20 % (v/v) DMSO, 5 % (v/v) D_2O, the ionic strength was set to 0.15 M using KCl.

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The CD solutions were also prepared using this background media. 30 µL of the EZE 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 h, 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 for the Job's experiments were prepared from EZE andthe complexing CD in complementary amounts, to make up a 0.5 mM total concentration. The solutions were mixed in different ratios, and the ^{1}H NMR spectra were recorded after 24 h.

Structural characterization of the inclusion complexes formed

Solutions containing 0.5 mM EZE and 10 mM β-CD, γ-CD and DIMEB were prepared with 25 v/v% DMSO-d_6 and 75 v/v% D_2O. Host-guest proton-proton vicinities were determined by 2D ROESY experiments, where128^{1}H spectra were accumulated for 512 increments. The mixing time was 300 ms and 2048data points were collected.

Results and discussions

NMR assignments of ezetimibe

The assignment of the signals was based on chemical shifts, multiplicity patterns, 2D ^{1}H-^{13}C HSQC, -HMBC measurements. The values are listed in table 1.

Stoichiometry of the inclusion complexes

The stoichiometry of the CD complexes was determined by the continuous variation method of Job [14], in which the chemical shift changes (Δδ) weighted by the molar fraction of EZE were plotted as a function the molar fraction of EZE.

The chemical shift change extremawere found at χ=0.5 in each case (fig. 2), indicating the classical 1:1 stoichiometry of the complexes.

Stability of the complexes

The observed chemical shift (δ^{obs}) of a particular NMR nucleus is the weighted average of the non- complexed and the complexed forms:

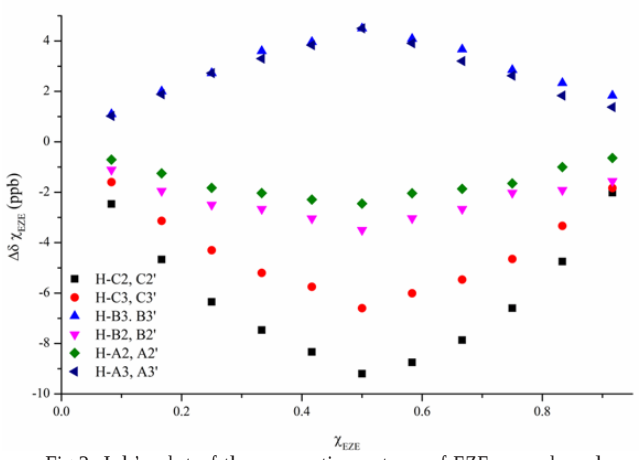


Fig.2. Job's plot of the aromatic protons of EZE complexed with β-CD

$$\delta^{obs} = \delta_{EZE}\chi_{EZE} + \delta_{EZE-CD}\chi_{EZE-CD} \quad (1)$$

where δ_{EZE} and δ_{EZE-CD} are the chemicals shifts, χ_{EZE} and χ_{EZE-CD} are the molar fractions of the noncomplexed and complexed forms, respectively.

The molar fractions can be expressed from the formula of the stability constant:

$$K = \frac{[EZE-CD]}{[EZE][CD]} \quad (2)$$

where [EZE-CD], [EZE], [CD] are the equilibrium concentrations of the complex, EZE and the CD, respectively. None of the equilibrium concentrations can be measured directly, only the analytical concentrations ($[EZE]_T$, $[CD]_T$) are known. The analyticalconcentrations are the sums of the non-complexed and complexed EZE and CD as shown in equations and below:

$$[EZE]_T = [EZE] + [EZE-CD] \quad (3)$$

$$[CD]_T = [CD] + [EZE-CD] \quad (4)$$

Introducing equations (3) and (4) into (2) and rearranging, the concentration of the complex can be expressed in terms of the analytical concentrations of EZE and CD and the stability constant:

	^{1}H		^{13}C
Position	δ(ppm)	multiplicity	δ(ppm)
2			167.3
3	3.05	m	59.4
4	4.77	d (J=2.4 Hz)	59.6
5	1.70; 1.80	m	24.5
6	1.70	m	36.4
7	4.46	dd (J=6.8 Hz, J=8.3 Hz)	71.0
A1			142.1 ($^4J_{C-F}$=2.8 Hz)
A2, A2'	7.27	dd ($^3J_{H-H}$=8.6 Hz; $^4J_{H-F}$=5.5 Hz)	127.5 ($^3J_{C-F}$=7.8 Hz)
A3, A3'	7.11	dd ($^3J_{H-H}$=$^3J_{H-F}$=8.6 Hz)	114.6 ($^2J_{C-F}$=20.9 Hz)
A4			161.0 ($^1J_{C-F}$=240.9 Hz)
B1			134.0 ($^4J_{C-F}$=2.6 Hz)
B2, B2'	7.21	dd ($^3J_{H-H}$=8.6 Hz; $^4J_{H-F}$=5.5 Hz)	118.2 ($^3J_{C-F}$=22.0 Hz)
B3, B3'	7.12	dd ($^3J_{H-H}$=$^3J_{H-F}$=8.6 Hz)	115.8 ($^2J_{C-F}$=20.9 Hz)
B4			158.0 ($^1J_{C-F}$=239.6 Hz)
C1			127.8
C2, C2'	7.22	d (J=8.5 Hz)	127.5
C3, C3'	6.75	d (J=8.5 Hz)	115.7
C4			157.5

Table 1
^{1}H AND ^{13}C CHEMICAL SHIFTS, MULTIPLICITIES AND SOME COUPLING CONSTANTS OF EZETIMIBE IN DMSO-d6

Abbreviations: m, d and dd stand for multiplet, doublet, and doublet of doublets, respectively.

$$[EZE-CD]=\frac{[EZE]_T+[CD]_T+\frac{1}{K}-\sqrt{\left([EZE]_T+[CD]_T+\frac{1}{K}\right)^2-4[EZE]_T[CD]_T}}{2} \quad (5)$$

Combining eqs. (1) and (5), the observed chemical shift becomes a function of the analytical concentrations and the stability constant:

$$\delta^{obs}=\delta_{EZE}+\Delta\delta\frac{[EZE]_T+[CD]_T+\frac{1}{K}-\sqrt{\left([EZE]_T+[CD]_T+\frac{1}{K}\right)^2-4[EZE]_T[CD]_T}}{2[EZE]_T} \quad (6)$$

where $\Delta\delta$ is the chemical shift change upon complexation (δ_{EZE}-δ_{EZE-CD}). The analytical concentration of EZE ($[EZE]_T$)was kept constant during the titrations. The stability constants of the different cyclodextrin complexes were calculated by fitting eq. (6) to the δ^{obs} vs. $[CD]_T$ datasets. The fitting was done by the OriginPro 8.0 software. Titration curves of H-C2,C2' are in figure 3.

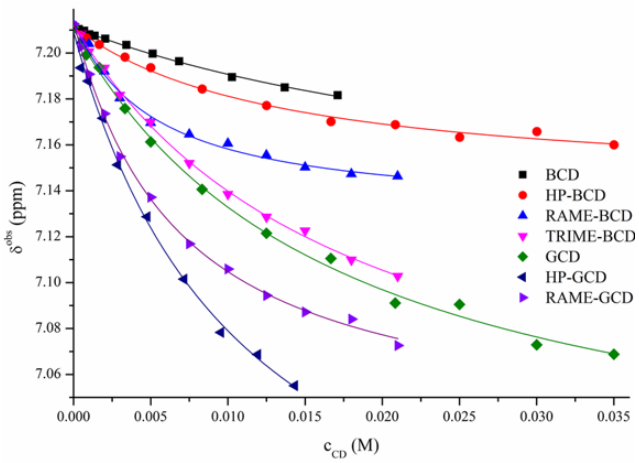


Fig. 3. δ^{obs}*vs.*$[CD]_T$ plots of H-C2 protons of EZE. The computer fits are the solid lines

The results are listed in table 2.

Cyclodextin	logK
β-CD	1.51 ± 0.06
HP- β-CD	1.84 ± 0.05
RAMEB	2.33 ± 0.04
TRIMEB	1.72 ± 0.04
γ-CD	1.78 ± 0.04
HP- β-CD	2.01 ± 0.03
RAMEG	2.16 ± 0.03

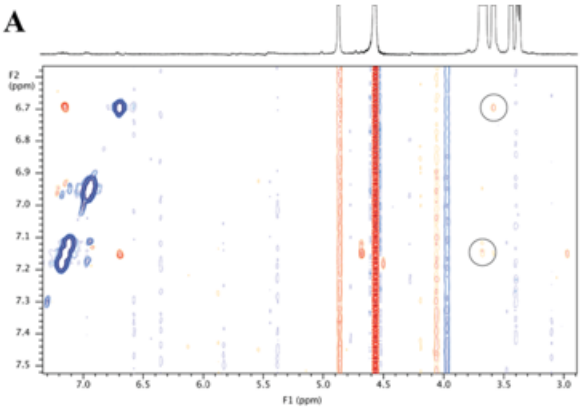
Table 2
STABILITY CONSTANTS OF EZE WITH THE CYCLODEXTRINS, EXPRESSED IN LogK UNITS

Structure of the inclusion complexes

The structures of the inclusion complexes were investigated with the three single isomer CDs: β-CD, γ-CD and DIMEB.

In the ROESY spectra only weak intermolecular cross peaks were observed between H-C2, C2' protons of EZE and the H-5 protons of CD and H-C3, C3' of EZE and H-3 protons of CD. This clearly indicates that EZE enters from the wider rim, the most stable complex is formed with the phenolic ring (ring **C**). Part of the ROESY spectrum and the approximate structure of the inclusion complex are shown in figure 4.

ROESY spectrum of EZE-γ-CD complex is similar to that of EZE-DIMEB, cross peaks can be found between the aromatic protons of rings **B** and **C** of EZE and H-3, H-5 protons of γ-CD (fig. 5A). Since Job's plots did not show any sign of complex formation with a stoichiometry other than



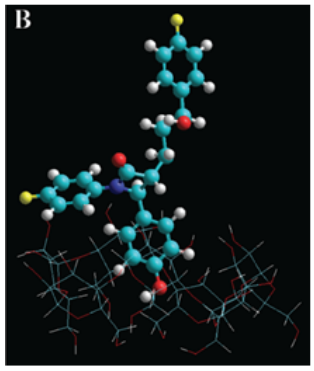
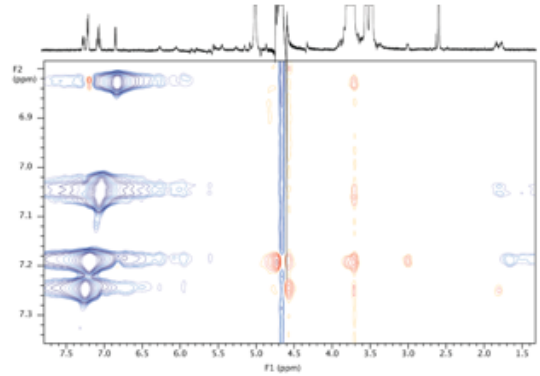


Fig. 4. A. Part of ROESY spectrum of EZE-β-CD complex. B. Structure of the EZE-β-CD inclusion complex



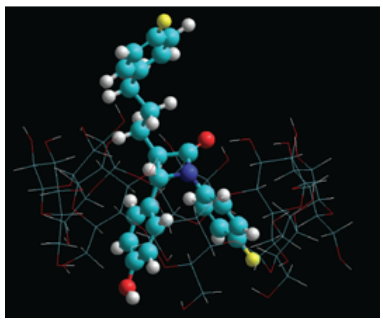


Fig. 5. A. Part of ROESY spectrum of EZE-GCD complex. B. Structure of the GCD inclusion complex

1:1 and the chemical shifts of all aromatic protons changed monotonously up to 70-fold excess of γ-CD in the process of the CD titration, we can the two aromatic rings are located in together in the CD cavity (fig. 5B). This was confirmedby intramolecular ROESY cross-peaks between H-B3,B3' and H-C3, C3' and also the H5 and H6 protons of ezetimibe are in the vicinity of the protons of the inner cavity of γ-CD.

Conclusions

Although the 3 aromatic rings of EZE have the theoretical potential to occupy the cavity of even 3 CDs, Job's plots verify the formation of 1:1 complexes only, indicating the repulsion or steric hindrance of nearby CDs.

Nevertheless, since every CD studied forms a sufficiently stable complex with EZE, the enhancement of

bioavailability by inclusion complex formation is a real, quantified possibility, especially with RAMEB and RAMEG.

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