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^{1}H-NMR and molecular modelling techniques for the investigation of the inclusion complex of econazole with α-cyclodextrin in the presence of malic acid

M.T. Faucci, F. Melani, P. Mura *

Dipartimento di Scienze Farmaceutiche, Universita di Ferenze, Via G. Capponi 9, 50121, Firenze, Italy

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Abstract

Carrying on a study where the combination of α-cyclodextrin and malic acid was found to be the most effective in improving the solubility of econazole, an antifungal drug very poorly water soluble, in the present work ^{1}H-NMR and nuclear overhauser effect (NOE) experiments and molecular modelling studies were performed to gain insight into the interactions in solution between such three components and the structure of the supposed multicomponent complex. Findings demonstrated that two different complexes can be simultaneously present in solution involving, respectively, the inclusion of econazole monochloro-phenyl group within the host cavity from the primary hydroxyl side of the cyclodextrin cavity, or that of the other phenyl group through the opposite side of the cavity. It was shown that also malic acid is strictly involved in the molecular assembly of the complex, particularly through interactions with primary hydroxyl groups of the cyclodextrin molecule. Molecular modelling studies allowed to elaborate possible geometric models of the multicomponent complex and to select the more energetically favourable conformations which complied better with experimental data. Results suggested the possible formation in solution of stable oligomeric aggregates constituted by the repeated concatenation of the three components.

Keywords: Econazole; α-Cyclodextrin; Malic acid; Multicomponent complexes; 1H-NMR; NOE; Molecular modelling

1. Introduction

Econazole (1-[2- (4-chlorophenyl) methoxy] -2-(2,4- dichlorophenyl) ethyl)-1H-imidazole, is a potent broad-spectrum antifungal agent applied topically, in the treatment of infections of the skin, hair and mucous membranes, and administered by mouth or by intravenous infusion in the treatment of systemic fungal infections. Unfortunately, its very low water solubility (about 5 μg/ml at 25°C) gives rise to problems in preparation of pharmaceutical formulations and limits its therapeutic applications and bioavailabilty. We previously reported that complexation with alfa- and

* Corresponding author. Tel.: +39-55-240776; fax: +39-55-240776.
E-mail address: mura@farmfi.scifarm.unifi.it (P. Mura)

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beta- cyclodextrins improved both water solubility and antifungal activity of econazole [1]. However, the greatest enhancement of solubility attainable for econazole by this conventional complexation method was not enough to reach the drug concentration commonly used in marketed pharmaceutical liquid dosage forms (1%w/v), not even at the saturation concentration of cyclodextrin. Subsequently, we showed that the combined use of hydroxyacids and cyclodextrins have a synergistic effect on the hydrosolubility of the drug [2], thus making it possible to obtain and even exceed the concentration level of econazole usually utilized in liquid formulations, even using lower concentrations of cyclodextrin. Similar positive effects on cyclodextrin solubilization due to the addition of certain low molecular weight acids or hydroxyacids have also been reported for other base-type drugs [3–5]. However we showed that the best cyclodextrin-acid combination for an optimal drug solubilization was related neither to the corresponding salt nor binary complex solubility and that the optimal drug:Cd:acid molar ratio was different for the various systems examined [6]. Therefore, steric factors probably play a significant role in the performance of the system. The most effective combination was that with α-Cd and malic acid, which combined the greatest solubilizing efficiency with the highest apparent stability constant of the hypothesized ternary complex, measured according to the solubility method of Higuchi and Connors [7].

The aim of this work, therefore, was to gain insight into the interactions in solution between the components (econazole, α-Cd and malic acid) and the structure of the supposed multicomponent complex, through ^{1}H-NMR and nuclear overhauser effect (NOE) spectroscopy. Molecular modelling was used as a complement to the experimental results, since the combination of NMR and NOE experimental data with molecular mechanic calculations can often be a very useful means for learning the geometry of cyclodextrin complexes [8–10]. Agreement between the two techniques in fact leads better understanding of the evolution of the supramolecular structure.

2. Experimental

2.1. Materials

Econazole (1-[2-(4-chlorophenyl)methoxy]-2-(2,4-dichlorophenyl)ethyl) 1H-imidazole, ECO) was kindly donated by Italfarmaco (I-Genova); α-cyclodextrin (α-Cd) and malic acid were purchased from Sigma (St Louis, MO).

2.2. ^{1}H-NMR studies

^{1}H NMR spectra (VARIAN GEMINI 200 operating at 200 MHz) of pure components and their equimolar combinations were taken in D_2O by adding the minimum amount of HCl to dissolve ECO (pH ≈ 2.5). The concentration of each component was 0.035 M. All spectra were recorded with a 5 mm tube, without degassing, at 25 ± 1°C. Chemical shifts were measured relative to the peak at 4.74 ppm, due to the solvent (D_2O).

The NOE experiments were performed in spin-lock conditions. The NOE DIFF program (VARIAN, version 6.3A) was used to directly obtain spectra showing only the increasing of the signals. The percent of presaturation (%p) was calculated from the ratio between the intensity of the presaturated signal of NOE DIFF spectrum and that of the presaturated signal of the reference spectrum. The percent of NOE was calculated from the following equation:

$$\%\mathrm{NOE} = \frac{2 \times In}{Ir \times \%p} \times 100$$

where In is the intensity of NOE signal, Ir that of the corresponding signal in the reference spectrum and%p the percent of presaturation. Only the NOE DIFF spectra with presaturation values ranging between 80 and 110% were considered significant.

2.3. Molecular modelling

Analysis and modelling of the structures of ECO, α-Cd, malic acid and of binary (ECO-Cd) and ternary (ECO-Cd-acid) complexes were carried out using the INSIGHT 97.0 program from Biosym Technologies (Biosym/MSI, San Diego,

CA) [11]. ECO and malic acid molecules were built-up directly using the builder routine of the INSIGHT program. The molecular structure of α-Cd [12] was obtained from crystallographic parameters provided by the Cambridge Crystallographic Data Centre (Cambridge, UK). Conformational energy was evaluated applying the Discover program, version 97.0 (Biosym/MSI, San Diego, CA) run on the IBM RISC 6000. The force field calculations were performed using the AMBER method [13,14]. In addition, the original AMBER method was modified adding specific parameters for carbohydrates according to Homans [15]. Each molecule was subjected to a simulated annealing process from 900 to 0 K, performing iterations until the minimum of energy conformation was found. Among the various possible conformations for ECO and malic acid, those which complied better with experimental NMR and NOE data were selected. Complexes were obtained by assembling the components in their minimum energy conformations and then subjecting the overall structure to a stochastic conformational research by a simulated annealing process.

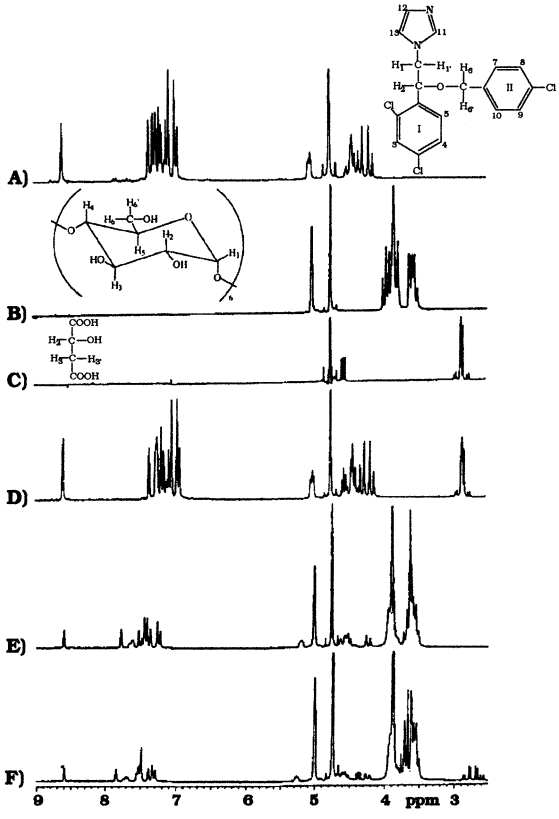

Fig. 1. ^{1}H-NMR spectra of (a) ECO; (b) α-Cd; (c) malic acid; (d) ECO-malic acid 1:1 mol/mol; (e) ECO-α-Cd 1:1 mol/mol; (f) ECO-α-Cd-malic acid 1:1 mol/mol.

Table 1
Changes in the chemical shifts (δ ppm) for econazole (ECO) protons in the presence of equimolar concentrations of mailc acid and/or α-Cd[a]

Proton	δ	$\Delta\delta_{(+\text{malic acid})}$	$\Delta\delta_{(+\_\alpha\text{-Cd})}$	$\Delta\delta_{(+\text{mailc acid-}\alpha\text{-Cd})}$
H-1	4.37	0.00	+0.09	+0.13
H-1′	4.47	0.00	+0.12	+0.15
H-2	5.03	0.00	+0.16	+0.22
H-6	4.15	−0.01	+0.08	+0.09
H-6′	4.30	−0.02	−0.07	−0.05
H-7, H-10	6.97	−0.02	+0.45	+0.35
H-8, H-9	7.10	−0.04	+0.14	+0.50
H-3	7.37	0.00	+0.39	+0.47
H-5	7.19	−0.01	+0.31	+0.35
H-4	7.16	−0.03	+0.29	+0.36
H-11	8.60	0.00	0.00	0.00
H-12	7.30	−0.03	+0.32	+0.39
H-13	7.26	−0.01	+0.10	+0.13

[a] Protons with the same number are on the same C atom but, being magnetically unequivalent, have different chemical shifts; the attribution of the chemical shift to one or the other proton has been made arbitrarily.

3. Results and discussion

3.1. ^{1}H-NMR spectroscopy

^{1}H-NMR spectra of pure components and of equimolar binary and ternary systems of ECO with α-Cd and malic acid are shown in Fig. 1. The changes in the chemical shifts induced on ECO proton signals in binary and ternary systems are shown in Table 1, whereas those induced on α-Cd and malic acid protons are shown in Table 2.

As for the binary systems, the spectrum of ECO-acid mixture was the simple superimposition of those of the corresponding individual components. On the contrary, ECO signals displayed significant changes in the presence of α-Cd. In

particular, the downfield shifts of the protons of both phenyl groups were particularly evident, suggesting their involvement in hydrophobic interactions with the Cd. The downfield displacement is indicative of weaker interactions (deshielding effect due to Van der Waals forces) with the hydrogen atoms [16]. Conversely, H-11 proton of ECO did not show any chemical shift, suggesting no involvement in the complexation of the imidazolic moiety. Finally, the protons H-3 and H-5 of α-Cd (located within the cavity) experienced diagnostic chemical shifts, typical of occurred inclusion, whereas the protons located on the exterior of the torus (H1, H2 and H4) were unaffected. This confirms that ECO only interacts with the inside of the Cd cavity and thus that occlusion really occurs.

The changes observed on ECO signals made it possible to establish that either of the ECO phenyl moieties can be inserted into the cavity of α-Cd. On the other hand, the changes in both H-3 and H-5 protons of α-Cd could indicate that the insertion can occur both from the secondary or the primary hydroxyl side of the cavity. However, since α-Cd, due to its reduced dimensions, is generally considered a one-binding site ligand with respect to most guest molecules (the secondary hydroxyl end of the cavity being the binding site) [17], the data could be simply indicative of a deep penetration of the host in the Cd cavity. It was in fact shown that the α-Cd dimension allows the phenyl ring to be fully enclosed with rather tight (Van der Waals) contacts [17].

Table 2
Changes in chemical shifts (δ ppm) for α-Cd and malic acid protons in equimolar binary and ternary systems with econazole (ECO)

Sample	Proton	δ	$\Delta\delta_{(+ECO)}$	$\Delta\delta_{ternary\ system}$
α-Cd	H-1	5.01	0.00	0.00
α-Cd	H-2	3.59	0.00	0.00
α-Cd	H-3	3.94	−0.27	−0.23
α-Cd	H-4	3.54	0.00	0.00
α-Cd	H-5	3.78	+0.15	+0.15
α-Cd	H-6	3.85	+0.03	+0.03
α-Cd	H-6′	3.85	+0.03	+0.03
Malic acid	H-2	4.56	−0.01	−0.16
Malic acid	H-3	2.80	0.00	−0.16
Malic acid	H-3′	2.90	0.00	−0.09

In the ternary system a further downfield shift of aromatic signals of ECO was observed, whereas the imidazolic proton was unaffected also in this case. Inerestingly, the signals of malic acid, which were unchanged in binary systems with ECO, showed an appreciable downshift, indicating its involvement in interactions with the Cd molecule and therefore a possible role in a multicomponent complex formation.

NOE experiments were then performed to further investigate the interactions between the components in solution and to elucidate the geometry of the inclusion complex. In fact, since NOE regards the dipolar interactions between spatially close protons (< 4 Å), it can provide useful information about the structure of the supramolecular system present in solution. NOE DIFF analysis was performed also on the free drug to obtain the definite attribution of its ^{1}H-NMR signals and to determine its conformation.

As for drug-acid systems, presaturation of ECO signals did not cause any NOE effect on malic acid protons and viceversa. On the contrary, in the case of drug-Cd systems, presaturation of aromatic ECO signals gave evident NOE enhancements on α-Cd inner protons H-3 and H-5, thus definitively confirming drug inclusion in the Cd cavity. Similar effects were observed also in ternary systems. The most significant spectra of the ternary system are collected in Fig. 2, whereas the percent of signal increase related to the various NOE increments observed is reported in Table 3. Interestingly, it was observed that presaturation of H-7 and H-10 protons of ECO (located on the mono-chloro phenyl moiety (II)) evidenced an intense NOE on H-5, H-6 and H-6′ of α-Cd, whereas presaturation of H-3 proton (located on the di-chloro-phenyl moiety (I)) induced a NOE effect only on H-3 of α-Cd (and not on H-5). These results confirmed the interaction of both inner protons of α-Cd with the host; however, in light of NOE experiment results, this effect was not due to a deep penetration into the host molecule, as previously hypothesized, but instead to a different possibility of inclusion mode of the two phenyl moieties. NOE findings in fact seem to

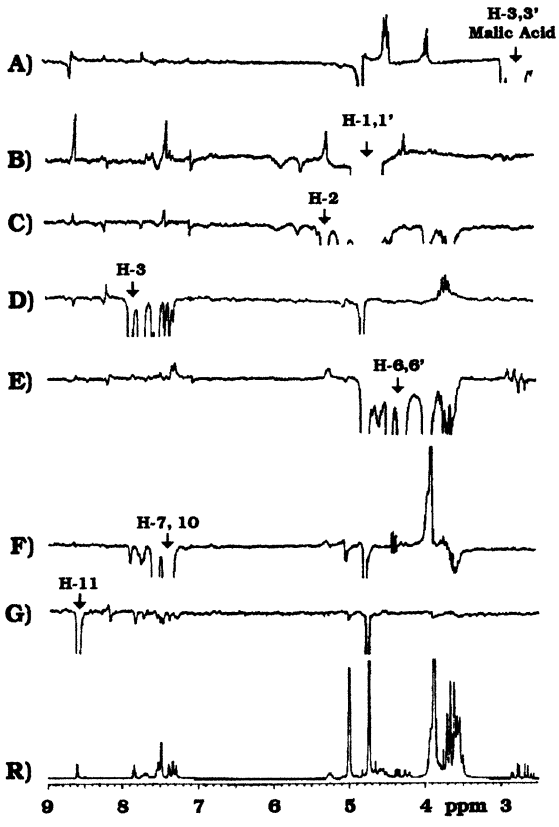

Fig. 2. NOE difference spectra of 1:1:1 ECO-α-Cd-malic acid ternary system: (A) saturating H-3 and H-3′ of malic acid; (B) saturating H-1 and H-1′ of ECO; (C) saturating H-2 of ECO; (D) saturating H-3 of ECO; (E) saturating H-6 and H-6′ of ECO; (F) saturating H-7 and H-10 of ECO; (G) saturating H-11 of ECO. R = reference ^{1}H-NMR spectrum of the ternary system.

indicate that the phenyl group II enters the narrower (primary hydroxyl) rim, whereas the phenyl group I enters the wider (secondary hydroxyl) rim of the cavity. Therefore two different 1:1 complexes can be simultaneously present in solution, each one involving the inclusion of a different part of the ECO molecule. Presaturation of aliphatic and imidazolic ECO signals did not evidence any NOE effect on Cd protons, further confirming that only the phenyl moieties are involved in the inclusion complex.

Moreover it should be noted that a significant NOE effect on H-6 and H-6′ protons of α-Cd was observed as a consequence of presaturation of H-3 and H-3′ protons of malic acid, confirming its involvement in the ternary complex formation. Furthermore, since presaturation of imidazolic H-11 of ECO did not cause any NOE effect on α-Cd, it is likely that the imidazolium ring could be situated at some distance from Cd protons and its basic nitrogen is therefore free for interacting with the carboxylic groups of malic acid.

3.2. Molecular modelling

Experimental data were then utilized to elaborate possible geometric models of the multicomponent complex through molecular modelling technique.

The Discover program was used to find the minimum energy conformation of ECO. A systematic conformational research was performed which led to the choice of the conformations that, relaxed at the minimum of energy, complied better with NOE findings relative to the drug in the free state or in the ternary complex (Fig. 3). The energies of these conformations were 43.9 and 41.8 kcal/mol, respectively.

Table 3
Percent of NOE increments for econazole (ECO), α-Cd and malic acid protons in the equimolar ternary system

Presaturated proton(s)	NOE effect	
	Proton	Percent (%)
H-3, H-3′ malic acid	H-2 malic acid	11.7
H-3, H-3′ malic acid	H-6, H-6′ α-Cd	0.8
H-1, H-1′ ECO	H-2 ECO	15.2
H-1, H-1′ ECO	H-6, H-6′ ECO	10.1
H-1, H-1′ ECO	H-11 ECO	8.8
H-1, H-1′ ECO	H-13 ECO	8.0
H-1, H-1′ ECO	H-7, H-10 ECO	1.4
H-2 ECO	H-11 ECO	1.8
H-2 ECO	H-13 ECO	1.4
H-3 ECO	H-3 α-Cd	1.1
H-6, H-6′ ECO	H-2 ECO	20.5
H-6, H-6′ ECO	H-7, H-10 ECO	12.5
H-6, H-6′ ECO	H-11 ECO	3.2
H-6, H-6′ ECO	H-3 ECO	3.0
H-7, H-10 ECO	H-5 α-Cd	2.7
H-7, H-10 ECO	H-2 ECO	5.8
H-7, H-10 ECO	H-6, H-6′ α-Cd	4.2
H-7, H-10 ECO	H-11	1.3
H-11 ECO	–	–

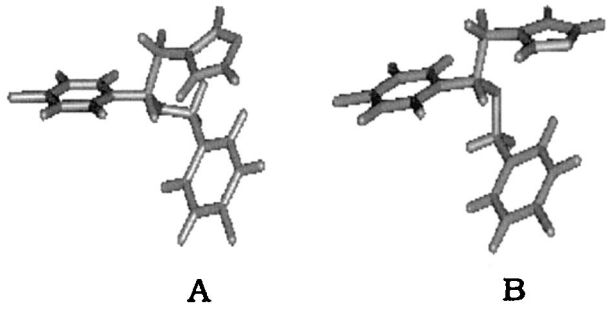

Fig. 3. Lowest-energy conformations for ECO molecule in the free state (A) or in the ternary system (B).

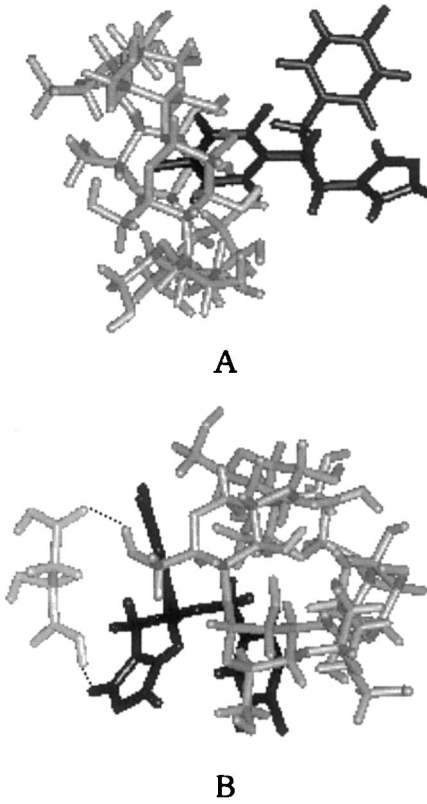

Fig. 4. Proposed models for the 1:1 ECO-α-Cd inclusion complex: (A) the di-chloro-phenyl group of ECO enters the wider (secondary hydroxyl) rim of the Cd cavity; (B) the mono-chloro-phenyl group of ECO enters the narrower (primary hydroxyl) rim; malic acid interacts with the primary hydroxyl groups of Cd and the imidazole moiety of ECO.

As for the malic acid molecule, the most stable conformation found for the pure substance through the systematic conformational research was that with the carboxylic groups in cis position, differently from the structure observed by X-ray diffraction analysis, where these groups were in trans position [18]. Molecular dynamic experiments showed that only the malic acid in trans position can interact with ECO and α-Cd molecules, positioning itself in spatial proximity of both H-6 and H-6′ protons of α-Cd and the basic nitrogen of imidazolic moiety of ECO, in agreement with the NOE findings.

Moreover, molecular modelling showed that, of the two possible modes of ECO inclusion in the α-Cd cavity, only that with the mono-chloro phenyl group included (primary hydroxyl side of the Cd rim cavity) allows the best fit of the third component, i.e. the malic acid. In fact only in this situation can the latter simultaneously interact with both the primary hydroxyl groups of α-Cd and the imidazolic moiety of ECO (Fig. 4).

Finally, molecular modelling calculations also indicated, on the basis of the two possible modes of inclusion of ECO in the α-Cd cavity, the possible formation in solution of oligomeric stable aggregates where the ECO molecule bridges thanks to its phenyl groups two cyclodextrin molecules, each in its turn interacting on the opposite side of the cavity with another drug molecule. The overall structure is further stabilized by the malic acid molecules which place themselves as go-betweens for the α-Cd and ECO molecules, so that they can interact with both primary hydroxyl groups of the Cd cavity, by hydrogen bonds, and the basic nitrogen of the ECO imidazolium ring, by a salt bridge (Fig. 5).

4. Conclusion

NMR and NOE experiments demonstred the effective inclusion complex formation between ECO and α-Cd, showing that two different complexes can be simultaneously present in solution involving, respectively, the inclusion of ECO phenyl group with one chlorine atom within the host cavity from the O-6 side, or that of the other phenyl group through the opposite side of the α-Cd cavity. Moreover, NOE experiments give

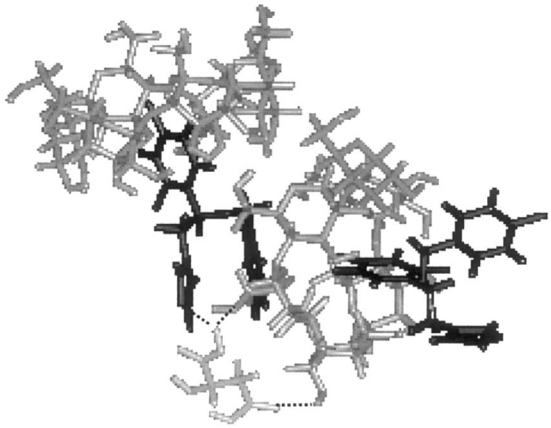

Fig. 5. Proposed model for the formation of oligomeric aggregates constituted by the repeated concatenation of the three components (ECO, α-Cd and malic acid).

important information about the structural characteristics of the ECO-α-Cd-malic acid multicomponent complex, demonstrating that malic acid is strictly involved in the molecular assembly, particularly through interactions with primary hydroxyl groups of the α-Cd molecule. Among the more-energetically favourable complex geometries proposed by molecular modelling studies, those which complied better with NMR and NOE experimental findings were selected. Results suggested the possible formation of stable oligomeric aggregates constituted by the repeated concatenation of the three components.

Acknowledgements

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