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ORIGINAL ARTICLE

Synthesis and characterization of inclusion complex of the vasodilator drug minoxidil with β-cyclodextrin

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Abstract The inclusion complex of β-cyclodextrin and minoxidil (2,4-diamino-6-piperidinopyrimidine 3-oxide) was synthesized using two methods—kneading and freeze-drying—and characterized by UV-Vis spectroscopy, infrared spectroscopy, powder X-ray diffractometry, differential scanning calorimetry, thermal gravimetric analysis, and nuclear magnetic resonance spectroscopy. These techniques have demonstrated the existence of inclusion compound formation between the host and guest with a molar ratio of 1:1. The studies of solubility and the data obtained by nuclear magnetic resonance spectroscopy showed a weak interaction between the guest and the cyclodextrin molecules in solution.

Keywords Minoxidil · β-Cyclodextrin · Inclusion complex · NMR · ROESY · DOSY

Introduction

Cyclodextrins (CD) are cyclic oligomers of α-D-glucose, crystalline, non-hygroscopic, and torus-like macro cycles, formed by the action of certain enzymes on starch and have been studied extensively as a host molecule in supramolecular chemistry [1]. The glucose units are connected through glycosidic α-1,4 bonds [2]. The three most commonly used CD is: α-CD (having six glucose units), β-CD (seven glucose units), and γ-CD (eight glucose units). CD has a large intramolecular cavity; they have ability to include guest molecules altering their physical, chemical, biological and pharmacological properties through the formation of inclusion complexes. The size of the cavity of β-CD is more adequate to interact with a great number of molecules [1–4] and this CD was used in this study. The CD is often used to increase the aqueous solubility, stability and bioavailability of drugs [5, 6]. Besides, CD has numerous advantages as a host material: (1) they are biocompatible and are produced naturally in the enzymatic degradation of starch, (2) they are relatively cheap, and (3) are non-toxic, permitting applications in drugs, foods and cosmetics [1]. Minoxidil (MNX) is a drug that was initially developed as an anti-hypertensive agent by the Upjohn Company. Its first literature appearance was in 1968 and preliminary trials were first described in man in 1969, observing that it also could cause growth of hair, being able to revert baldness, acting as a hypertrichotic agent. However, its more important therapeutical indication is in the oral treatment of severe symptomatic or organ-damaging hypertension that cannot be controlled with other medicines. It acts as a potent peripheral vasodilator agent, decreasing the diastolic and systolic pressures through reduction of the vascular resistance to blood flow. It must concomitantly always be managed with diuretics to prevent fluid retention and congestive cardiac failure. Patients must be carefully monitored, as the intense and sudden decrease of arterial pressure can limit the sanguineous flow to the myocardium causing infart. MNX is also effective topically as a hair growth stimulant and is indicated for the treatment of *alopecia androgenetica* [7].

It is believed that encapsulation of this medicine with β-CD will increase the wettability and the solubility of the encapsulated drug for a supported and gradual release, maximizing its biodisponibility over time and reducing its

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direct contact with the gastric and duodenal mucous membranes. The aim of this paper is to confirm the formation of an inclusion complex between β-CD and MNX.

Two methods of encapsulation were tried using two different molar ratios: kneading (KN) and freeze-drying (FD), as well as their physical mixture (PM). It was possible to obtain the stability constant of the complex, and several techniques were applied to characterize and to prove its formation, including infrared spectroscopy, X-ray diffractometry, thermal gravimetric analysis, differential scanning calorimetry, UV-Vis spectroscopy and ^{1}H, DOSY and ROESY nuclear magnetic resonance spectrometry.

Experimental

Materials

The drug minoxidil was supplied by Galderma Brazil S/A, Code 110407-1, approved Lot number 03011818, validity until 11 JAN 2006 and used as received during the year 2005. The melting point obtained was (249 ± 2) °C using a Mettler FP5, MQAPF-301 apparatus from MicroQuímica Indústria e Comércio Ltda. The β-CD was purchased from AMAIZO (American Maize-Products Co.) and purified as previously published [8]. 28% (w/w) aqueous ammonium hydroxide solution and 99.5% ethyl alcohol, P.A., were purchased from LabSynth Ltda. Products for Laboratories. The 99.9% deuterium oxide was from Cambridge Isotope Laboratories, Inc.

Preparation of the physical mixtures (PM)

The physical mixtures were prepared using two molar ratios of β-CD and MNX, macerating in an agate mortar for 15 min.

Preparation of inclusion compounds

According to the freeze-drying (FD) method, a proportional amount of MNX was added to a solution of β-CD at 70 °C under stirring, forming a suspension. This was dissolved by addition of 14 drops of aqueous ammonium hydroxide and the solution was left at rest for 3 h to reach equilibrium. After this time, N_2 was bubbled (flow: 10 mL/min) through the solution to eliminate NH_4OH, until the pH reached 7. Then the sample was freeze-dried for approximately 72 h [9]. For the kneading (KN) method, both components were placed in an agate mortar with approximately 1 mL of water, macerated for 45 min in order to form a paste. After that, the sample was dried under vacuum for 48 h [3].

Characterization of PM and inclusion compounds

Infrared absorption spectroscopy was done using a model MB 102 Bomem FTIR, according to the KBr method. The X-ray diffraction studies were carried out using a Shimadzu—XRD diffractometer (model 6000), that provides Cu-Kα radiation ($\lambda = 1.5406$ Å), with 2θ ranging from 5 to 50°, operated with 40 kV and 30 mA, using the powder method.

The thermal gravimetric analyses were performed using the following devices: TA Instruments, model TA 5000 Hi 2950 Res TGA and TA Instruments, model TA 2050. The experimental conditions were the following ones: (a) under

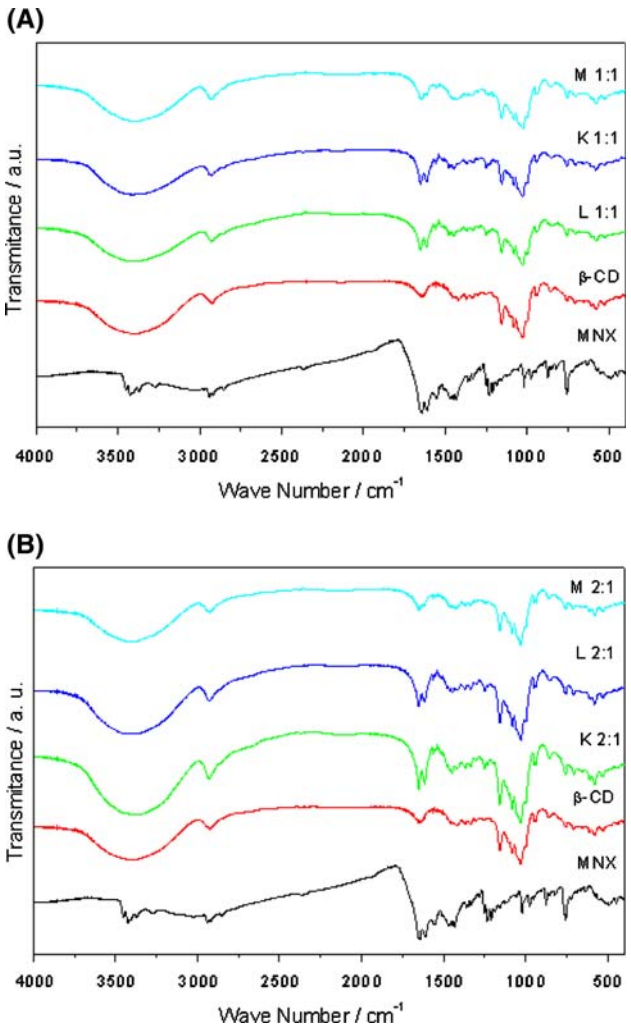

Fig. 1 IR spectra of MNX, β-CD, PM and the inclusion compounds in the molar ratios (**a**) 1:1; (**b**) 2:1

flowing argon, (b) heating rate of 10 °C/min and (c) range of heating 30–700 °C. The differential scanning calorimetry data were registered in a DuPont Instruments 910 DSC device under the following experimental conditions: (a) flowing argon, (b) heating rate of 10 °C/min and (c) range of heating 30–300 °C.

Determination of the molar ratio between MNX and β-CD by UV-Vis spectroscopy

The method used is known as the Job Method [10] and it was performed by preparing two stock solutions of concentration 5×10^{-5} mol/L of MNX and β-CD and mixing different ratios (1:0; 0.2:0.8; 0.4:0.6; 0.5:0.5; 0.6:0.4; 0.8:0.2; 0:1) of these solutions in order to obtain seven solutions with different ratios between the concentrations of MNX and β-CD, maintaining a constant final volume of 2 mL The measurements were obtained using a Hewlett Packard 8452A diode array spectrophotometer at a maximum absorbance wavelength of 230 nm.

Solubility studies

Solubility measurements were carried out as described by Higuchi and Connors [11] and consisted in measuring the increase of the solubility of a substance in water when the CD concentration is raised. In six 125 mL erlenmeyers, 100 mg of MNX were added to 10 mL of distilled water, making a supersaturated solution at 60 °C. The first solution was left only with the drug while a measured amount

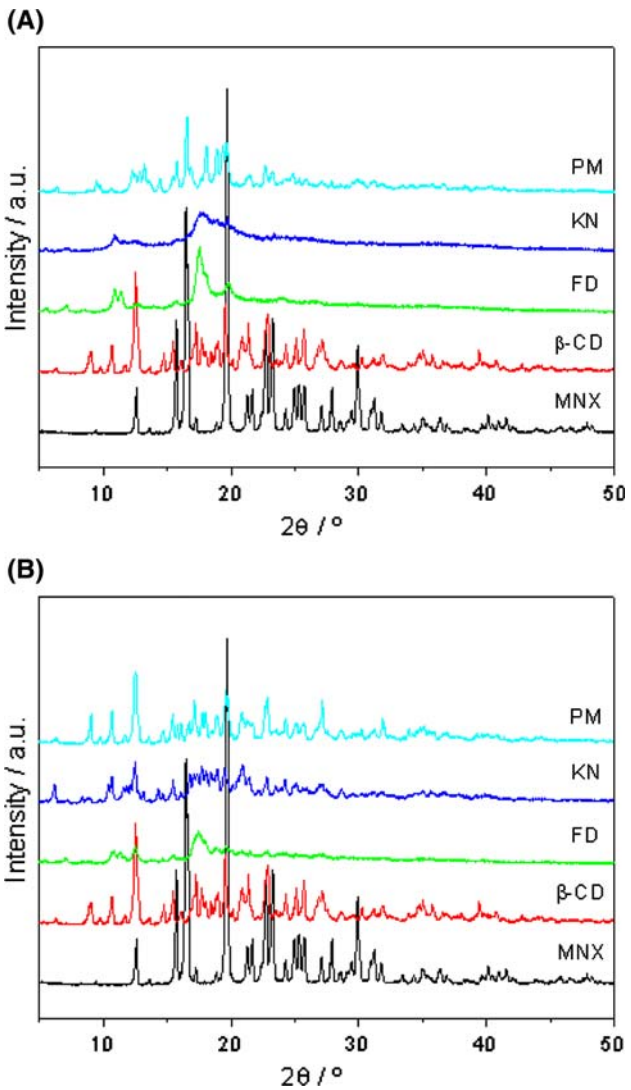

Fig. 2 Powder X-ray diffraction patterns of MNX, β-CD, PM and the inclusion compounds in the molar ratios (**a**) 1:1; (**b**) 2:1

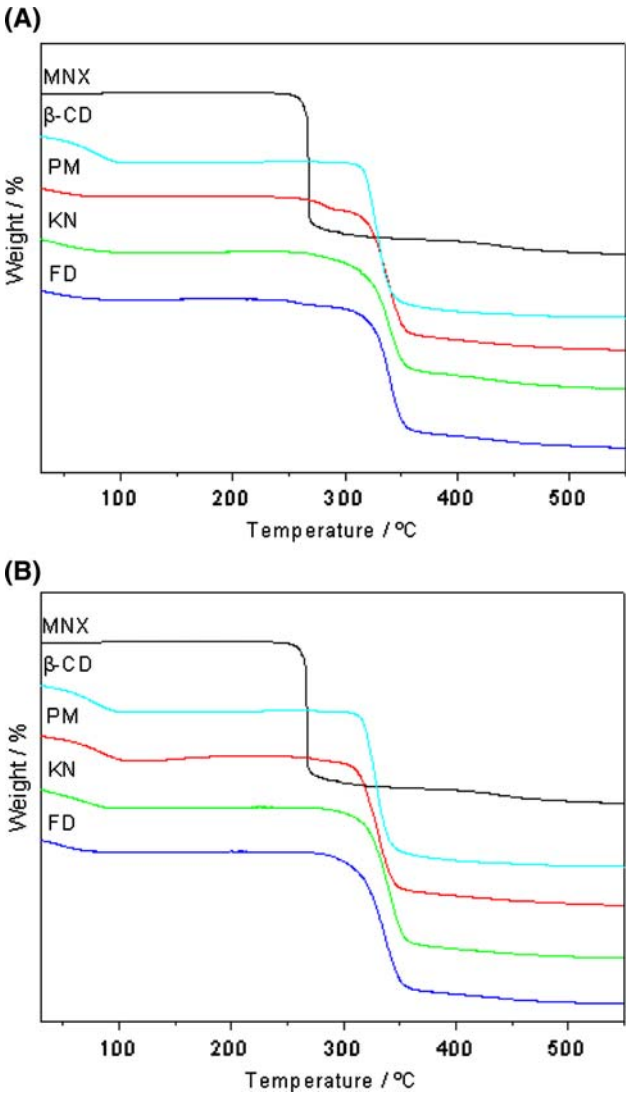

Fig. 3 Thermal analytical profiles of MNX, β-CD, PM and the inclusion compounds in the molar ratios (**a**) 1:1; (**b**) 2:1

of CD was added to each of the others in a way that their concentrations were 2, 4, 6, 8 and 10 mmol/L. The solutions were kept under agitation of 110 rpm, at 25 °C, during 24 h in a shaker (Incubator Shaker—Series 25—New Brunswick Scientific Co., Inc.). Each solution was then filtered with Milli-Q membrane (0.45 μm pore size). 50 μL of each filtered solution were diluted to 25 mL to assay the concentration by UV-Vis spectrophotometry.

NMR spectroscopy

All the experiments were run on a Varian INOVA-500 spectrometer (BO = 11 T), operating at 499,886 MHz for ^{1}H. The temperature was kept 25.0 ± 0.1 °C in all the experiments. The chemical shifts were referenced against the HOD resonance (δ = 4.67 ppm). The samples were prepared by dissolving of 2–4 mg of the FD complex in approximately 1 mL of D_2O. The signal of the solvent was used to stop the magnetic field and the radio frequency. The experiments were acquired using standards Varian software in the following described conditions and processed using the program VNMR of the equipment. To obtain the ^{1}H NMR spectra the conditions used were: pulse width (pw) = 6.1 s, acquisition time (at) = 3.3 s and acquisition delay (d1) = 3.0 s. The number of transients (nt) accumulated was 32 scans with line broading (lb) of 0.2 Hz. The ROESY experiment was carried out using the following parameters: at = 1.0 s; d1 = 3.0 s; nt = 1024 scans; lb = 1.0 Hz. The experiment was obtained applying a sequence of pulses 180° sel.–90° sel.—spin lock-FID, with mixing time of 500 ms, FIDs acquired through the sequence of pulses 90° sel. - spin lock-FID. A modulator generated the selective pulses and automatically attenuated the power and duration of the pulse. The pulse sequence used for DOSY was GCSTESL (DOSY Gradient Compensated Stimulated Echo with Spin Lock). In all the analyses 25 different pulsed gradient amplitude were used d1 = 6.1 s, at = 3.3 s, d1 = 3.0 s, nt = 32 and lb = 0.2 Hz.

Results and discussion

IR analysis

The inclusion compounds between β-CD and MNX were obtained by two methods, in addition to that prepared by physical mixing. A variety of different techniques were used to characterize them. The IR technique is not adequate to confirm inclusion compound formation, because the interactions between both molecules, the guest and the host, in these inclusion complexes are due to Van der Waals and hydrophobic forces and there is not a new true chemical bond formed, causing no significant alterations in these spectra. In the region 3600–3100 cm^{-1}, absorptions that characterize an intense and broad band corresponding to O–H stretching in the glucose units of the CD and the presence of water were observed. Because this band is wide and intense, it can mask bands that might be attributed to the drug. The broadening of the band at 3400 cm^{-1}, observed in all PM and complexes, suggests the occurrence of intermolecular interactions between drug and CD. The characteristic band present in the IV analysis of MNX due

Table 1 Mass loss (%); temperature interval (ΔT) and mass loss temperature (T) of MNX, β-CD, PM and the inclusion compounds β-CD:MNX

Sample	ΔT_1 (°C)	T_1 (°C) % w	ΔT_2 (°C)	T_2 (°C) % w	ΔT_3 (°C)	T_3 (°C) % w
MNX			260–269	267		
				−75.0		
β-CD	60–94	80			318–342	328
		−9.6				−76.5
KN 1:1	40–84	50			319–356	340
		−5.8				−66.0
KN 2:1	66–87	70			322–355	341
		−3.9				−72.5
FD 1:1	44–74	50	243–265	255	330–353	340
		−2.7		–2.8		−55.9
FD 2:1	43–72	49			315–353	337
		−4.4				−74.1
PM 1:1	39–68	46	262–287	278	331–356	345
		−3.0		–5.8		−44.6
PM 2:1	63–102	85			312–346	331
		−10.1				−74.8

to N–O around 1250 cm^{-1} is also covered in the others spectra. The IR spectra are shown in Fig. 1.

This figure shows alterations in the intensities and frequencies of some bands, caused by interactions between the encapsulated drug and the inner core of β-CD. New peaks and the disappearance of others, mainly in the region of 1660–1000 cm^{-1}, and in the fingerprint region were verified. Also, two well defined peaks between 1660–1640 cm^{-1} are observed in the spectra of the inclusion compounds, but were not observed for the PM. However, we can observe reduction of the band intensities, indicating that there are interactions between the drug and the CD molecules even for the PM.

X-ray diffraction analysis

The formation of the inclusion complex can be confirmed by the powder X-ray diffraction spectra. The powder X-ray diffraction patterns are in Fig. 2. We can observe that all diffractograms suggest that the complexes are less crystalline than pure β-CD and MNX, indicating some external or internal interaction between guest and host. The FD complex presents a greater reduction in crystallinity, indicating that encapsulation by the FD method is more efficient. The presence of some peaks in the sample KN 2:1 may be attributed to the insignificant amount of free CD remaining in the system. Also, the diffraction profiles of PM show reduction in crystallinity as well, even though it is possible to observe some regions that correspond to addition of the individual components. However, the disappearance of some peaks is observed in all scans, indicating that there is some interaction not only in the inclusion compounds but also in the PM.

Thermal analysis

TGA and DSC analyses are commonly used to characterize inclusion compounds with CD. The approach is the comparison of the thermal behavior of the individual components, their PM and the inclusion compounds [12]. The thermal analytical profile of MNX, β-CD, the inclusion compounds and PM are in Fig. 3. The data from TGA are shown in Table 1.

It is observed that there are two temperatures where mass loss occurs for the samples, except for PM 1:1 and FD 1:1, the first mass loss corresponding to the release of water and the second one to the decomposition of the sample. This suggests that the interaction between the two components in the solid state causes the protection of the drug and its decomposition takes place at higher temperatures. The samples PM 1:1 and FD 1:1 show a third region of mass loss near the drug decomposition temperature indicating that there is some free drug still present. Here, the TGA data indicate that there is also an interaction between drug and β-CD in the PM.

Figure 4 shows the DSC curves of MNX, β-CD, PM and the inclusion compounds in both molar ratios. The dehydration of β-CD occurs at 149 °C and the melting point of MNX was about 250 °C. The small endothermic peak around 250 °C observed only in PM show that the interactions were not as strong for these samples as for the inclusion compounds. It can be associated with the presence of a small portion of free drug in PM. As these peaks do not have the same characteristics as those for the free drug, an inclusion compound may be present. The other endothermic peaks correspond to dehydration in the system. The data from DSC are shown in Table 2.

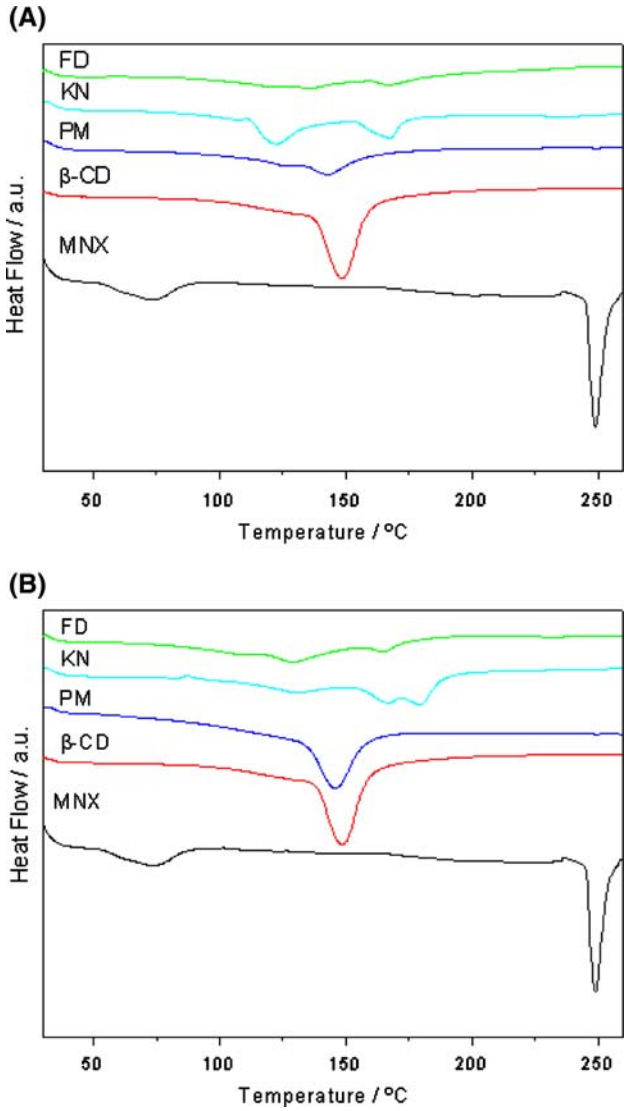

Fig. 4 DSC curves of MNX, β-CD, PM and the inclusion compounds in the molar ratios (**a**) 1:1; (**b**) 2:1

Table 2 Thermal data from DSC analysis of MNX, β-CD, PM and the inclusion compounds β-CD:MNX

Sample	$1T_i$ (°C) ΔH_1 (J/g)	T_1 (°C)	$2T_i$ (°C) ΔH_2 (J/g)	T_2 (°C)	$3T_i$ (°C) ΔH_3 (J/g)	T_3 (°C)	$4T_i$ (°C) ΔH_4 (J/g)	T_4 (°C)
MNX							247	250
							13.9	
β-CD			136	149				
			356.3					
KN 1:1			111	122	154	167		
			94.5		44			
KN 2:1	85	87	111.3	129	156	179		
	3.3		28.8		104.6			
FD 1:1			115	135	161	169		
			60.9		21.5			
FD 2:1			106	127	159	166		
			109.9		15.2			
PM 1:1			123	143			248	249
			159.3				2.4	
PM 2:1			131	145			248	249
			306.3				1.6	

Enthalpy (ΔH), initial temperature transition (Ti) and endothermic peak temperature (T)

Determination of the molar ratio

This experiment indicates that the molar ratio between the drug and CD in solution is 1:1, indicating that this is the ratio when the complex is at equilibrium in aqueous media. Figure 5 shows the plot of the data obtained, with the maximum of the curve at a molar ratio of 0.5:0.5.

Solubility studies

A linear increase in the solubility of the MNX with the increase of the concentration of β-CD was observed (Fig. 6), suggesting formation of the complex between the components. The stability constant $K_{1:1}$ calculated was 151 mol/L which indicates that the interaction between the two components in aqueous media is weak, since normally the order of magnitude for this kind of association is 10^3 mol/L [3]. This result is in agreement with Kim et al., where they found a stability constant of approximately 350 mol/L for the complex between the drug and hydroxipropyl-β-CD (HP-β-CD) and since the $K_{1:1}$ in the presence of HP-β-CD is usually bigger than in the presence of β-CD. [13]

^{1}H NMR spectroscopy analysis of the complex between MNX and β-CD

In this experiment, the behavior of the chemical shifts of the protons located inside the cavity was observed. Thakkar

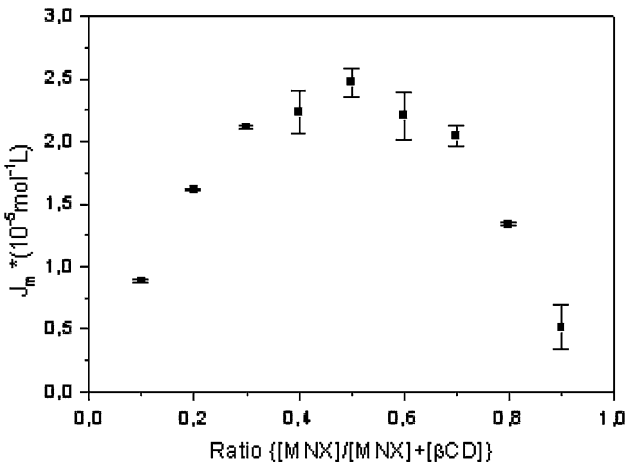

Fig. 5 Job method plot of β-CD:MNX. The maximum occurs at a molar ratio 1:1

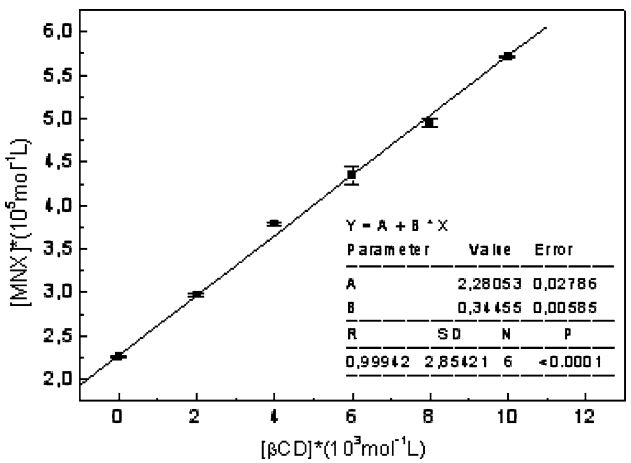

Fig. 6 Diagram of solubility. [MNX] versus [β-CD]

and Demarco [14] initiated NMR studies of CD complexes observing changes in the chemical displacements of the protons H3 and H5 located inside the cavity of α-CD in the presence of an aromatic substrate, due to the anisotropic effect of the aromatic ring. As the NMR signals of the external protons H1, H2 and H4 did not show any variation, they inferred that the guest molecule was inside the cavity. Thus, the chemical displacement of the CD signals induced by the presence of the guest is a first evidence of its inclusion in the CD [15]. The protons of MNX and β-CD were denominated in accordance with Fig. 7 and the results are reported in Tables 3 and 4.

According to Greatbanks and Pickford [16] when $\Delta\delta$ H3 > $\Delta\delta$ H5 the inclusion of the guest inside the cavity is partial and when $\Delta\delta$ H3 ≤ $\Delta\delta$ H5 the guest is included more deeply inside the cavity. The results show that partial inclusion of MNX occurs for 2:1 FD and total inclusion for 1:1 FD complexes.

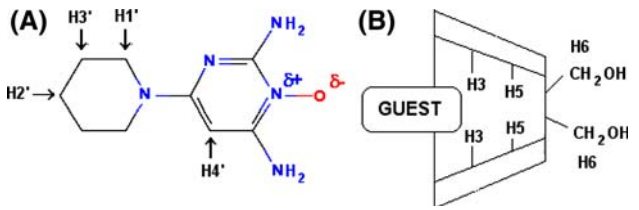

Fig. 7 (**a**) Denomination of the MNX protons; (**b**) Inclusion of a guest inside the CD cavity and denomination of β-CD protons

Table 3 ^{1}H NMR chemical shifts of β-CD protons in D_2O and their variation in the presence of MNX

H	β-CD	FD 1:1		FD 2:1	
	$\delta_{\beta\text{-CD}}$	$\delta_{\text{FD 1:1}}$	$\Delta\delta_{\text{FD 1:1}}$	$\delta_{\text{FD 2:1}}$	$\Delta\delta_{\text{FD 2:1}}$
1	4.960	4.965	0.005	4.965	0.005
2	3.545	3.550	0.005	3.555	0.010
3	3.830	3.820	−0.010	3.805	−0.025
4	3.490	3.480	−0.010	3.480	−0.010
5	3.720	3.710	−0.010	3.700	−0.020

$\Delta\delta = \delta_{\text{complexed}} - \delta_{\text{free}}$

Table 4 ^{1}H NMR chemical shifts of MNX protons in D_2O and their variation in the presence of β-CD

H	MNX	FD 1:1		FD 2:1	
	δ_{MNX}	$\delta_{\text{FD 1:1}}$	$\Delta\delta_{\text{FD 1:1}}$	$\delta_{\text{FD 2:1}}$	$\Delta\delta_{\text{FD 2:1}}$
1′	3.340	3.420	0.080	3.400	0.060
2′	1.535	1.570	0.035	1.595	0.060
3′	1.455	1.520	0.065	1.505	0.050

$\Delta\delta = \delta_{\text{complexed}} - \delta_{\text{free}}$

ROESY 1D

The experiment of ROESY in the one-dimensional version with selective pulses leads to a direct, fast and easily quantified analysis [17]. The ROESY spectrum provided information about which β-CD proton has an intermolecular correlation when the MNX protons H2′ and H3′ were selectively irradiated. It was not possible to irradiate the H1 proton because its signal is too close to the signals of the CD protons. The spectrum and the values of intermolecular rOes (%) are in Fig. 8.

DOSY

The diffusion coefficients (D) supplies important information on molecular organization and phase structure. This technique is non-invasive and it can provide individual multi-component translational diffusion coefficients with good precision. The sequence used was GCSTESL ("Gradient Compensated Stimulated Echo Spin Lock"), which is adequate when there is no exchange effect and modest gradients are enough for the acquisition. After correction of the base line and selection of the points, the diffusion coefficients were processed automatically and the values of each diffusion coefficient were calculated by the average between all the listed coefficients. The guest population (p) involved in the complexation was calculated from the observed diffusion coefficients of each component in the complexed and in the free form through the following equations [18, 19].

$$D_{\text{observed}} = p_{\text{free}}\, D_{\text{free}} + p_{\text{complexed}}\, D_{\text{complexed}} \quad (1)$$

where

$$p_{\text{free}} + p_{\text{complexed}} = 1 \quad (2)$$

then

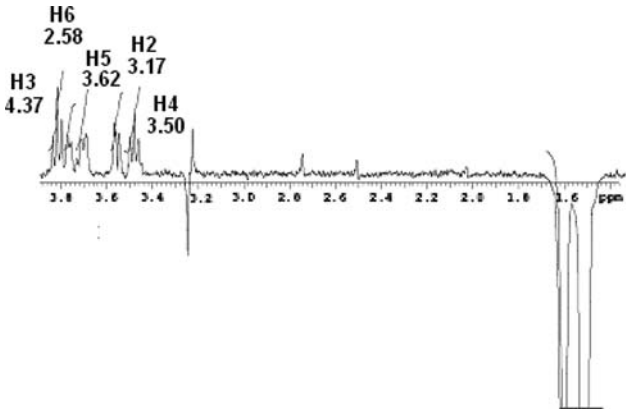

Fig. 8 ROESY 1D partial spectrum of β-CD and MNX at 25 °C (500 MHz; D_2O)

$$p_{free} = 1 - p_{complexed} \quad (3)$$

$D_{observed}$ corresponds to the MNX diffusion coefficient when β-CD is in the medium; D_{free} is the MNX diffusion coefficient in the absence β-CD; $p_{complexed}$ is the completely complexed MNX population and $D_{complexed}$ is the completely complexed MNX diffusion coefficient.

Substituting Eq. 3 in 1, considering that the completely complexed MNX diffusion coefficient is approximately the same as the completely complexed of β-CD diffusion coefficient and assuming that the observed β-CD diffusion coefficient is almost the same when it is in the free form, then:

$$D_{\beta-CD\,complexed} \approx D_{\beta-CD\,observed\,free} \approx D_{\beta-CD\,livre} \quad (4)$$

Substituting equation (4) in equation (1):

$$p_{complexed} = \frac{(D_{free} - D_{complexed})}{(D_{free} - D_{\beta-CD\,observed})} \quad (5)$$

The diffusion coefficients and percentages of the MNX population complexed with β-CD are in Table 5 and the NMR spectra are shown in Fig. 9. Analyzing the results

Table 5 Free and complexed MNX and β-CD diffusion coefficients and D_2O diffusion coefficient

Solution	D_{MNX} /10^{-10} m^2/s	D_{CD} /10^{-10} m^2/s^1	D_{OH}/10^{-10} m^2/s	$P_{complexed}$ (%)
MNX free	6.363 ± 0.019	–	22.538 ± 0.087	–
β-CD free	–	3.254 ± 0.024	21.943 ± 0.079	–
1:1 complex	3.565 ± 0.058	3.302 ± 0.021	23.935 ± 0.118	91.4 ± 0.8
2:1 complex	4.348 ± 0.023	3.361 ± 0.020	22.311 ± 0.076	67.1 ± 1.1

Percent values of the MNX population complexed with β-CD

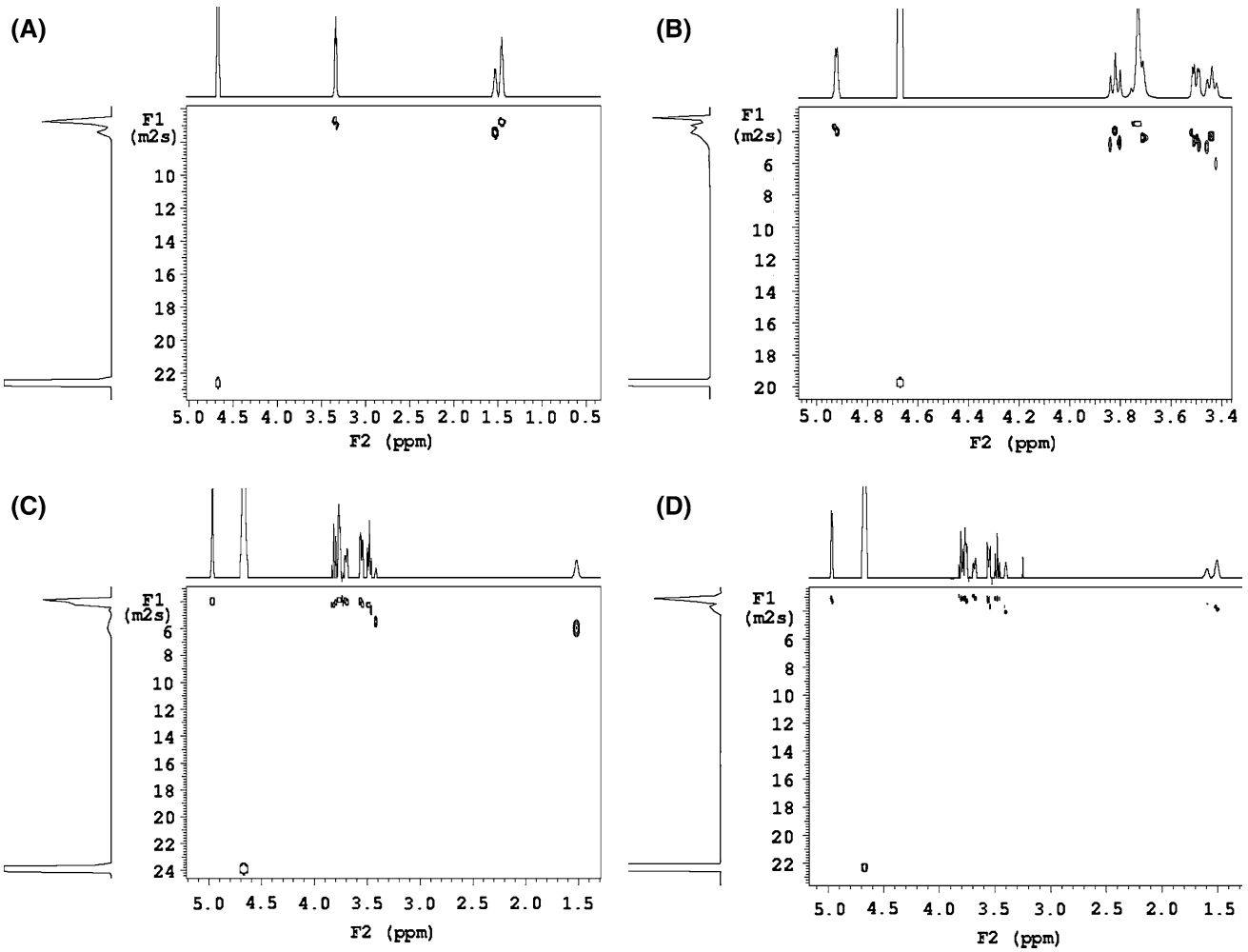

Fig. 9 NMR spectra of HR-DOSY (500 MHz; D_2O). (**a**) MNX; (**b**) β-CD; (**c**) FD 1:1 and (**d**) FD 2:1

it is verified that diffusion coefficients for HOD, β-CD and MNX population are quite distinct and they are very coherent, considering the size of the species in solution.

Conclusion

In summary, two different molar ratios inclusion compounds were prepared between β-CD and MNX by two methods. The formation of the inclusion compounds was investigated by many techniques. These results show interactions between the two components in the solid state even for the PM, even though the protection by β-CD and their interaction were much more intense in the inclusion compounds. The solubility studies showed that the interactions between the two components are weak. This was also confirmed by the NMR techniques, because the range of the chemical shifts was not very representative and the values of rOe were low. The ROESY results confirm how the drug molecule is spatially located inside the β-CD cavity and DOSY provided informations of the percentage of MNX encapsulated by the CD.

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