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ORIGINAL ARTICLE

Inclusion of α-lipoic acid in β-cyclodextrin. Physical–chemical and structural characterization

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Abstract The inclusion of α-lipoic acid (LA) in β-cyclodextrin (β-CD) by increasing the aqueous solubility and photostability can enhance its medicinal use in the oral administration. Different preparation methods were employed to obtain an α-lipoic acid-β-cyclodextrin (LA-β-CD) inclusion complex and to determine the physical–chemical characteristics and the interactions present in this compound. The formation of the solid inclusion compound was confirmed by X-ray powder diffraction, differential scanning calorimetry (DSC) and infrared spectroscopy (FTIR). FTIR and DSC data confirm the new obtained compound. The crystalline structure of this compound belongs to the monoclinic system with four molecules in the unit cell. ^{1}H NMR spectroscopic method was employed to study the inclusion process in aqueous solution. Job plots derived from the ^{1}H NMR spectral data demonstrated an 1:1 stoichiometry of the inclusion complex in liquid state. 2D NMR data suggest the orientation of LA with the carboxyl group near to narrower rim of the β-CD.

Keywords Cyclodextrin · DSC · Inclusion compound · Lipoic acid · Molecular spectroscopy (FTIR, ^{1}H NMR) · X-ray powder diffraction

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Introduction

Conventional drugs are usually formulated for the immediate release of the medicinal substances and for obtaining the desired therapeutic effect. Oxidative stress has been associated with both the ageing process and the development of age-dependent tissue degenerative pathologies. Beneficial effects of antioxidant therapies to abrogate the deleterious consequences of elevated free radicals are implicated in disease prevention and cost-effective strategy. Previous data have shown protective effects of the classic natural antioxidant LA against oxidative stress and aging.

Cyclodextrins (CD) are a naturally occurring cyclic polysaccharides, consisting of six to eight [1] up to several hundreds [2, 3] α-D-(1→4)-linked glycosyl units, obtained from starch by enzymatic reaction. They have conical cavities capable of selectively incorporating a great variety of organic guests to form inclusion complexes in aqueous solutions. The cavity within CD is hydrophobic, therefore, upon inclusion into the CD cavity, the chemical and spectral properties of the guest molecule can be affected significantly. The molecular formula of β-cyclodextrin (β-CD) is presented in Fig. 1a.

The guest molecules are usually maintained in positions in the β-CD cavity via non-covalent hydrophobic interactions, hydrogen or van der Waals bonds. The enclosed molecule is located in the cavity of the host molecule without affecting the framework structure of the host. Some chemical and physical properties of guest molecules are affected by the complex formation. These include alteration of the solubility of the guest compound, stabilization against the effects of light, heat, and oxidation. In some applications, more benefits are obtained by complexation with CD [1, 4–8].

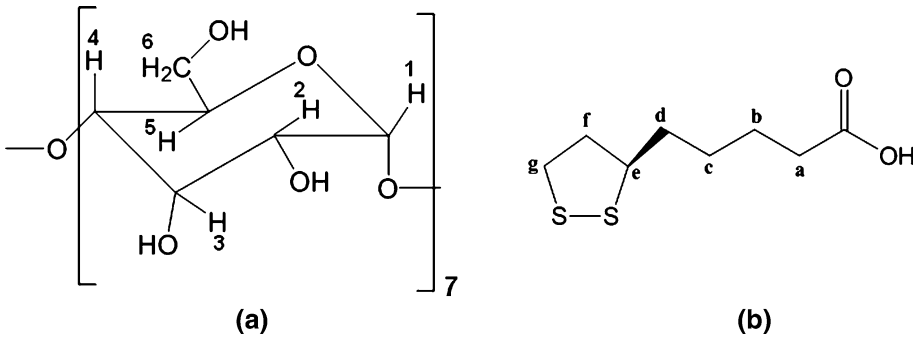

Fig. 1 Chemical structure and numbering scheme for β-CD (**a**) and α-LA (**b**)

α-Lipoic acid (LA) is a disulfide derivative of octanoic acid that forms an intramolecular disulfide bond in its oxidized form (Fig. 1b). High electron density resulting from special position of the two sulfur atoms in the 1,2-dithiolane ring confers upon LA a high tendency for reduction of other redox-sensitive molecules according to environmental conditions [9]. LA shows structural similarity to octanoic acid, a medium chain fatty acid. Since it is an important cofactor for oxidative decarboxylation, LA presents antioxidant properties and is successfully used in prevention and treatment of several diseases, such as diabetic polyneuropathy, cataract formation, radiation injury or heavy metal intoxications [10]. LA is an essential cofactor in mitochondrial dehydrogenase reactions, functions as an antioxidant and reduces oxidative stress [11]. It occurs naturally as a coenzyme in both prokaryotic and eukaryotic cells, as well as in plants, and animals including humans [12]. In the intracellular environment, two or more enzymes reduce the exogenous LA to dihydrolipoic acid. The dihydrolipoic acid can regenerate or recycle the antioxidants CoQ (ubiquinol), vitamins C and E, and glutathione [13]. LA and dihydrolipoic acid are efficiently transported in and out of both mitochondria and cells. The α-LA/dihydrolipoic acid couple, called "universal antioxidant", it fulfills several criteria used to evaluate the antioxidant potential as well as preventive or therapeutic applications of a compound such as specificity of free radical quenching, metal chelating ability, interaction with other antioxidants, effects on gene expression, absorption and bioavailability, concentration in tissues, cells, and extracellular fluid, and location [13].

Although there are several papers [14–18] dedicated to the investigation of LA with different CD or their derivatives, the authors employed different methods (infrared spectroscopy (FTIR), X-ray powder diffraction (XRPD) and thermal analysis) to evidence the inclusion compound formation. NMR ROESY was used to determine the architecture of the inclusion complex and UV–Vis spectroscopy or capillary electrophoresis for stoichiometry and stability constant determination.

A patent [19] was found, being dedicated to the preparation and investigation of LA-α-CD. There is already a commercial product [20] of LA with HP-β-CD.

Despite of this cited literature, the inclusion compound of β-CD with LA prepared by the most common methods (co precipitation (*co*) and freeze-drying (*fd*) ones) will be studied. Also, the determination of the stability constant by using a non linear method applied to ^{1}H NMR chemical shifts of the protons involved in the complexation process is aimed. The aim of this paper was to obtain using different preparation methods an α-lipoic acid-β-cyclodextrin (LA-β-CD) inclusion complex, to determine the physical–chemical characteristics and the interactions present in this compound.

Materials and methods

Materials

DL-α-LA (purity 99 %) was purchased from Sigma-Aldrich Chemie GmbH, (Germany). The β-CD (having $\leq$15 % in water weight) was purchased from Cyclolab (Hungary) and both compounds were used without further purification. The ethanol (purity 99.8 %) was obtained from Chemical Company, Romania. The heavy water used for NMR experiments was obtained from Romag-Prod, Romania.

Methods

The inclusion compounds were prepared by different methods as follows:

Preparation of solid compound by co: an accurately weighted quantity of 206 mg of LA was dissolved in 5 ml ethanol and 1,294 mg of β-CD in 10 ml distilled water, respectively. Very slowly under continuous stirring, the two solutions were then mixed. The stirring was continued another 24 h at room temperature, succeeded by evaporation and drying at 38 °C.

Preparation the solid compound by fd: 1,294 mg of β-CD was dissolved in distilled water under continuous stirring and after complete dissolution the 206 mg of LA was added. The mixture was stirred for 1 day and the complex was obtained by freeze drying method in an Alpha 1–2 LD type freeze dryer.

LA-β-CD complexation was characterized by powder XRPD, FTIR, ^{1}H and 2D NMR spectroscopy and DSC.

Infrared spectroscopy

Infrared spectra obtained for solid samples were recorded in the 4,000–400 cm^{-1} frequency range with a resolution of 4 cm^{-1} using a FTIR JASCO 6100 spectrometer. KBr discs were prepared by compressing blends corresponding to 0.8 mg of the samples and 150 mg of potassium bromide.

Differential scanning calorimetry

The experiments were carried out with a Shimadzu DSC-60 differential scanning calorimeter using Shimadzu TA-WS60 and TA60 2.1 version system software for data acquisition and analysis. Non hermetic aluminium pans, in which 1–2 mg of sample were accurately weighed, and then just covered with the lid, were used to perform the experiments. The samples were heated from room temperature up to 350 °C under flowing nitrogen flux, the heating rate being 10 °C min^{-1}.

X-ray powder diffractometry

XRPD patterns were collected with Bruker D8 Advance diffractometer in the $2\theta = 2$–40^o angular domain using Cu Kα1 radiation. In order to increase the resolution a monochromator was used to eliminate Kα2 radiation.

^{1}H NMR spectroscopy

^{1}H NMR spectra were obtained with Bruker Avance 500 spectrometer after at least 15 min of thermal equilibration at 25 °C. The spectrometer was operated at 500.1325 MHz, with the following parameters: 32 K data points, 10.1 μs pulse of 90°, 2 s delays between scans (16 scans) and a digital resolution of 0.588 Hz point^{-1}. The chemical shifts were expressed in parts per milion (ppm) relative to the chemical shift of HOD signal located at 4.700 ppm.

2D NMR spectroscopy

The 2D NMR spectrum at 500 MHz was obtained on a solution with $rG = 0.7$ through standard Bruker software. The conditions for ROESY phase-sensitive spectra via time proportional phase incrementation were: presaturation of residual HDO signal, spectral widths of 6.8 ppm in both dimensions with a resolution of 0.83 and 1.66 Hz point^{-1} in f2 and f1, respectively and a mixing time of 300 ms. The experiment was performed using 4,096 data points in f2 and 2,048t1 increments with 16 scans per t1 value and a relaxation period of 2 s. A sine function (SSB = 2) was applied in f1 and f2 before Fourier transformation.

Results and discussion

FTIR spectroscopy

The infrared spectra of inclusion compounds, see Fig. 2, were analyzed and compared with the spectra of the pure compounds.

The LA does not present any bands in this spectral domain, so the broad band between 4,000 and 3,000 cm^{-1} is due only to O–H stretching vibrations of the β-CD molecule. The stretching frequency of the O–H group, located at ~3,392 cm^{-1} in pure β-CD is shifted to 3,386 (*co* product) and 3,383 cm^{-1} (*fd* product), respectively due to the increasing of hydrogen bonds number during the complexation process. One cannot neglect also the expulsion of the water molecules from the β-CD cavity [21, 22].

The differences, which appear in the 1,800–1,500 cm^{-1} spectral region (see Fig. 3), were analyzed in terms of the implication of different molecular groups of the guest molecules in the inclusion process. The following vibrational modes of LA molecule were affected by complexation process: υ_{as}(C = O) located at 1,691 cm^{-1} in pure

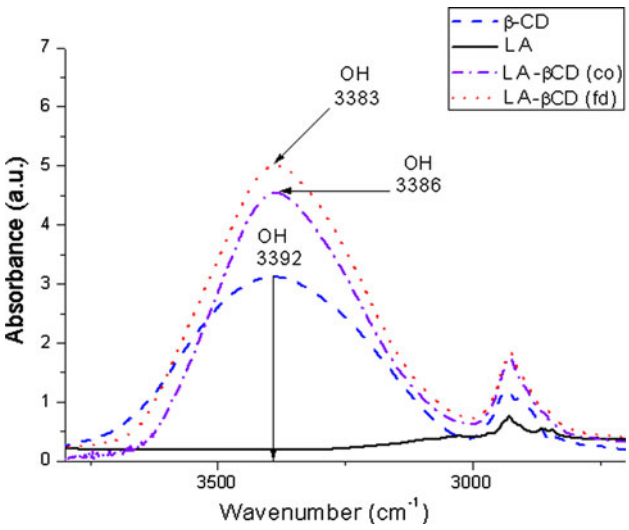

Fig. 2 FTIR spectra of pure α-LA, β-CD and of the inclusion compounds of LA with β-CD obtained by *co* and *fd*, in the 3,800–2,700 cm^{-1} spectral domain

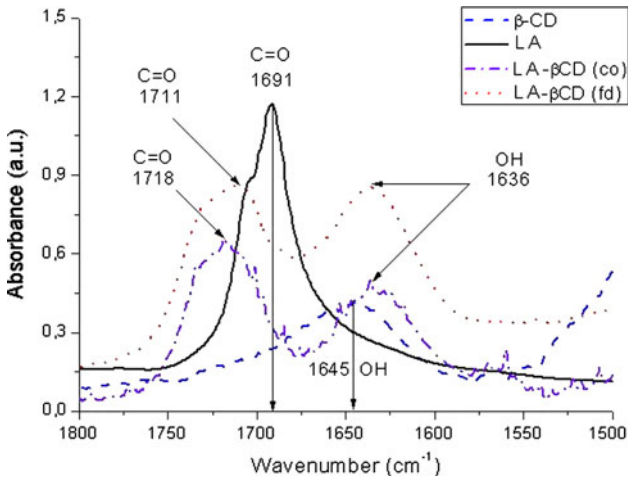

Fig. 3 FTIR spectra of pure α-LA, β-CD and of the inclusion compounds of LA with β-CD obtained by *co* and *fd*, in the 1,800–1,500 cm^{-1} spectral domain

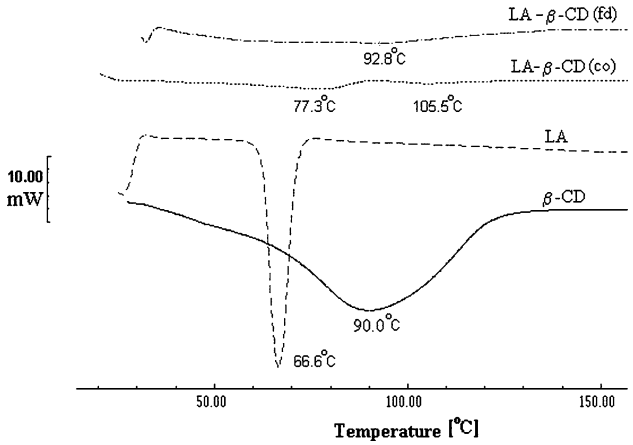

Fig. 4 DSC thermograms of α-LA, β-CD and of the corresponding inclusion compounds

drug spectrum is shifted to 1,711 cm^{-1} in the spectra of inclusion compound obtained by freeze drying respectively to 1,718 cm^{-1} in the spectrum of inclusion compound obtained by *co* method [14]. The splitting of the $\upsilon_{as}(C = O)$ band can bee assigned to the coexistence of different association type species present in the solid state. The higher frequency shift can be ascribed to the destruction of strong hydrogen bonding structure in uncomplexed drug after inclusion compound formation with β-CD. The υ(OH) (bending mode) located at 1,645 cm^{-1} in pure β-CD spectrum is shifted to 1,636 cm^{-1} in spectra of inclusion compounds obtained by different methods. The formation of new hydrogen bonds between β-CD and LA can explain these spectral differences.

These vibrational band changes certify the inclusion compound formation and offer an idea about the mechanism of the inclusion process.

Differential scanning calorimetry

DSC reveals some information on solid-state interactions between drug and CD. The DSC thermograms of pure components and of LA-β-CD inclusion compounds are presented in Fig. 4.

The endothermic peaks are identified during the dehydration and melting processes and for each peak the entire peak area was used to calculate the enthalpy of various thermal effects.

The curves of β-CD revealed a broad endothermic signal from 71 to 120 °C, with a $\Delta H = 293$ kJ mol^{-1}, that corresponds to the loss by evaporation of the water molecules existing as residual humidity ($t < 100$ °C) as well as those included in the cavity ($t > 100$ °C) [23, 24].

The DSC curve of LA presents a sharp endothermic peak at 66 °C, with a $\Delta H = 43$ kJ mol^{-1}, corresponding to the melting, following to the degradation of the compound.

The DSC curve of the *co* LA-β-CD inclusion compound presents two broad endotherms between 59 and 89 °C due to loss of non-bounded water molecules and another one for the temperature range from 99 to 122 °C corresponding to the bounded water molecules with ΔH values of about 38.79 ± 0.3 kJ mol^{-1} and of about 9.02 ± 0.1 kJ mol^{-1}, respectively. The LA melting peak disappears from the thermal profile.

The thermogram of the LA-β-CD inclusion compound obtained by *fd* method shows a single weak endothermic peak at 92 °C with ΔH of 34.82 ± 0.2 kJ mol^{-1} corresponding to the loss of water molecules still present in this complex. This small peak might indicate that by integrating of LA molecule inside the nanocavity of β-CD the major removal of water molecules occurs. The LA melting peak disappears from the thermal profile [14].

Both inclusion compounds of LA with β-CD, obtained either by freeze drying or by *co* techniques, showed a decreasing in dehydration endotherm peaks compared to the thermal profile of β-CD and of LA and clearly they correspond to different solid state structures. Generally, both inclusion compounds do not present the characteristic melting endotherm of LA. These findings might be considered as an evidence of molecular interactions between LA and β-CD components within the inclusion complex supporting also the complex formation.

X-ray powder diffraction

The crystal structure of LA was already reported [25]. In Fig. 5, the XRPD patterns are shown for β-CD, LA, and for their inclusion complexes, LA-β-CD, for the 1:1 molar

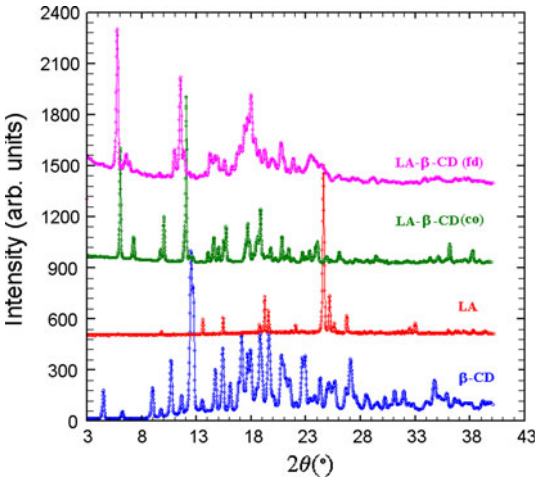

Fig. 5 X-ray patterns of: α-LA, β-CD, and the products obtained by *co* and *fd* procedures

ratio, prepared by the two different methods, by *co* and by *fd*.

XRPD patterns of LA, β-CD and of the products obtained by *co* and *fd* procedures were compared (Fig. 5). From the powder diffraction patterns one can see that inclusion compounds are formed in different degree. Both crystalline and amorphous phases are present in the obtained compounds. The difference between these patterns is due to different ratios between crystalline and amorphous phases. The highest degree of formation is obtained for *co* procedure.

From powder pattern indexing by using Dicvol method [26] it was established that LA-β-CD inclusion compound obtained with *co* method crystallized in monoclinic system, having following lattice parameters: $a = 18.8929$ Å; $b = 24.4634$ Å; $c = 15.7376$ Å and $\beta = 110.5195^{\circ}$. The unit cell volume is $V = 6812.183$ Å^3, the space group obtained from reflections systematic absences is $C2$. The calculated density is $d_C = 1.371$ g/cm^3 if we consider four molecules in the unit cell which is characteristic for this space group.

^{1}H NMR

The inclusion complexes of β-CD with the active substance LA were prepared in D_2O starting from equimolar stock solutions (of 10×10^{-3} mol/dm^3) of the host (H, β-CD) and the guest (G, LA). Complex stoichiometry was determined by the method of continuous variation using the chemically induced shifts of both the host and guest protons. For this purpose, several mixtures having different molar ratios at constant volume of LA and β-CD were prepared. The sum of the total concentration was maintained constant, $M = ([\beta\text{-CD}]_t + [\text{LA}]_t)$ (where the total index refers to the total concentration), being equal with 10×10^{-3} mol/dm^3. The molar ratio of the guest G, $r_G = [\text{G}]_t/([\beta\text{-CD}]_t + [\text{G}]_t)$ varies between 0 and 1 with an interval of 0.1. The chemical shifts of the pure LA, β-CD and of their 1:1 inclusion complex are presents in Table 1.

Table 1 The chemical shifts δ (ppm) for the pure species and the inclusion complex

Proton	LA δ (ppm)	β-CD δ (ppm)	IC (1:1) δ (ppm)	Δδ (ppm)
Ha	2.118		2.308	0.190
Hc	1.362		1.469	0.107
Hd	1.537		1.633	0.096
Hf	2.434		2.472	0.038
Hg	3.157		3.227	0.070
H2		3.583	3.642	0.059
H3		3.900	3.992	0.092
H4		3.517	3.592	0.075
H5		3.776	3.834	0.058
H6		3.811	3.872	0.061

By using the well-known Job plots [27] for different protons of LA and of β-CD, an 1:1 stoichiometry of the inclusion complex was obtained, see Fig. 6.

The association constant for the 1:1 complex was evaluated by a nonlinear least-squares curve-fitting regression analysis of the observed chemical shift changes of the LA and of β-CD NMR lines using the program CONSTEQ [28]. The value of the association constant K obtained on this way is 149 M^{-1}. There are several papers [14–17] that reported inclusion complexes of various CD, prepared in different pH conditions. The stability constant for inclusion complex with β-CD varied between 600 (obtained by phase solubility method [14]), 1,645 M^{-1} (capillary electrophoresis [16]) and 8,912 M^{-1} (obtained by UV–Vis measurements [15]) depending on experimental conditions. All these methods are linear and take into account a global size whereas the ^{1}H NMR method looks at the protons implied in the complexation process.

2D NMR spectroscopy

In order to establish the orientation of the LA molecule inside β-CD cavity, 2D NMR experiments were performed. The 2D ROESY spectrum for the LA-β-CD (1:1 molar ratio) complex in D_2O is presented in the Fig. 7.

The Hg hydrogen of LA (located at 3.227 ppm) interact with all hydrogens of the β-CD (H2, H3, H4, H5 and H6 located at 3.64, 3.91, 3.59, 3.79 respectively 3.87 ppm) giving the corresponding cross-peaks (see Fig. 7). The intensities of these cross peaks indicate a stronger interaction with H2, H3 and H6 of β-CD. The Hf proton of

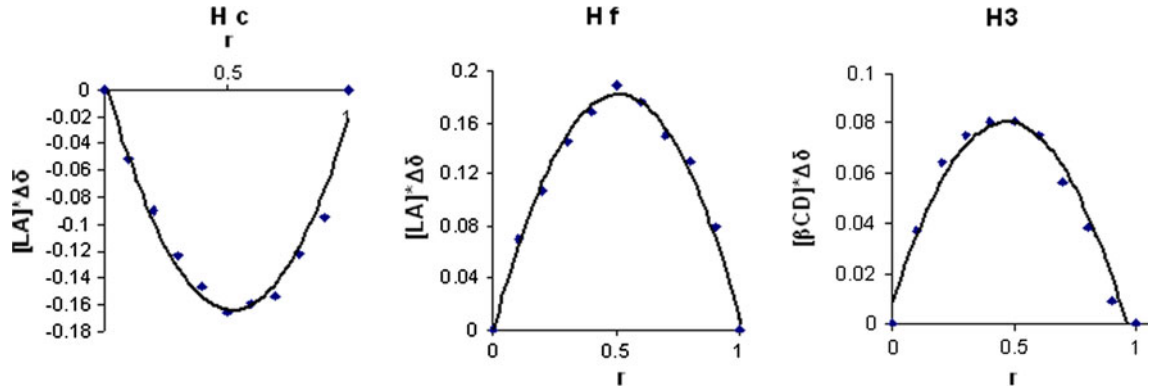

Fig. 6 *Job plots* of the Hc and Hf protons of the LA and of H3 proton of the β-CD

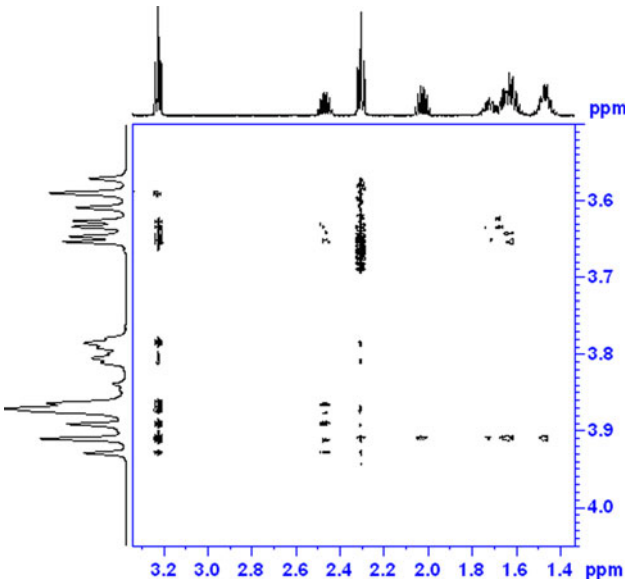

Fig. 7 500 MHz 2D ROESY symmetrized spectrum of LA-β-CD complex showing the interaction between Ha, Hb, Hc, Hd, Hf, Hg of LA and H2, H3, H4, H5, H6 of β-CD

LA (2.472 ppm) shows weak interactions with H2, H3 and H6 of β-CD. Ha of LA shows strong interactions with the H2 and H4 protons of β-CD. Hd of LA interact with H3 and H4 protons of the β-CD [18]. These interactions confirmed both the internal and external inclusion complex formation between LA and β-CD [16]. The cross-peak intensities suggest the orientation of LA with the carboxyl group near to narrower rim of the β-CD.

Conclusions

Based on XRPD data, a new quite crystalline inclusion compound was obtained by *co*; in the case of freeze dried product, a mixture of amorphous and crystalline phases was reported. The $\nu(C=O)$ FTIR vibrational mode shift certify the inclusion compound formation. The decreasing of dehydration endothermic peak of CD and the disappearance of the melting peak of the LA in DSC thermogram of the LA-β-CD compound demonstrate also the inclusion compound formation.

Based on ^{1}H NMR spectra an 1:1 stoichiometry was established for the inclusion complex. The stability constant was determined from ^{1}H NMR data by a non linear method taking into account only the protons H3 and H2 of the β-CD and Hf and Hc of the LA that present chemical shifts due to their implying in inclusion process. The already reported value was obtained as a global value obtained from phase solubility method. 2D NMR experiments confirmed both the internal and external inclusion complex formation between LA and β-CD, and suggest the orientation of LA molecule with its carboxylic group near to the β-CD narrower rim torus.

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