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Artigo

IMPROVEMENT OF GENISTEIN CONTENT IN SOLID GENISTEIN/β-CYCLODEXTRIN COMPLEXES

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Genistein:β-cyclodextrin complexes with high drug loading (19.22%) were prepared by freeze-drying and characterized by differential scanning calorimetry and hydrogen nuclear magnetic resonance spectroscopy. The spatial configuration of the complex was proposed by means of 2D-NOESY experiment combined with molecular modeling. According to the results obtained, the interaction of genistein with β-cyclodextrin in a 1:1 complex is supposed to occur mainly through the insertion of the guest A-ring in cyclodextrin cavity, without rule out the possibility of inclusion through the B-ring, as previously reported in the literature.

Keywords: genistein; β-cyclodextrin; computer molecular mechanics.

INTRODUCTION

Genistein (GEN) is one of the major and most extensively studied soybean isoflavones.[1] This drug has revealed special interest for local and systemic delivery due to its antioxidant[2-4] and estrogenic[5,6] properties. However, its activities are conditioned to the aglicone form, which presents reduced aqueous solubility.[7,8] This fact limits its application in the pharmaceutical field and the complexation of this isoflavone with cyclodextrins (CDs) has emerged as an interesting alternative for the improvement of GEN aqueous solubility. CDs are cyclic oligosaccharides consisting of variable glucopyranose units linked by α-(1,4) bonds, with relative water solubility. They can form inclusion complexes with many size-suitable guest molecules due to their unique molecular structure with hydrophobic cavity and hydrophilic outer face. This system provides technological advantages to formulations, especially solubility increment and stability improvement. β-CD, with seven glucose units, is the most used cyclodextrin by the pharmaceutical industry because of its low cost, expired patent and suitable cavity size.[9-13]

The association of GEN with CDs was firstly investigated by Crupi *et al*.,[7] who performed phase-solubility studies with β-CD, hydroxypropyl-β-CD and methyl-β-CD and employed FTIR-ATR in the analysis of the 1:1 solid complexes obtained by co-precipitation method. In the same way, Stancanelli *et al*.[8] prepared GEN/hydroxypropyl-β-CD complexes in liquid media followed by UV-VIS spectrophotometry and circular dichroism analysis. More recently, Daruházi *et al*.[14] reported the obtention of GEN/β-CD complexes employing kneading method. The complexes were characterized using ^{1}H-NMR and computer modeling studies, which allowed to propose a good size-wise correlation between GEN and the geometry of the βCD cavity. The corresponding complexes presented a GEN content of 9.9% (w/w) with 1:2 guest:host stoichiometry. This low GEN content in the solid complexes may, however, represent a limitation for its incorporation in hydrophilic vehicles, semisolid or solid dosage forms, considering the high molecular weight of β-CD.

In this context, in view to obtain solid complexes containing higher GEN content, the present work was designed to perform the complexation of GEN with β-CD in aqueous media, followed by separation of the soluble host:guest association, which was freeze-dried. Additionally, the NMR and molecular mechanics calculation were employed in order to demonstrate the spatial configuration of the GEN:β-CD complexes and their corresponding state of energy.

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EXPERIMENTAL

Genistein (certificate standard 98%, w/w) was purchased from Sigma-Aldrich (St. Louis, MO, USA). β-cyclodextrin was gently provided by Roquette Frères (Lestrem, France). The HPLC-grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Dimethylsulfoxide-*d6* was supplied by Cambridge Isotope Laboratories (Andover, MA, USA). Ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). All the other chemicals were of analytical grade.

Chromatographic conditions and method validation[15]

GEN assay was performed using a Shimadzu LC-10A (Shimadzu, Kyoto) equipped with a LC-10AD pump, an automatic SPD-10A flow controller, an SIL-10A autosampler equipped with a 20 μL loop, a LC10 integrator, and a SPD-10A UV-Vis variable-wavelength detector. For evaluation of the peak purity, a Waters 2690 Separation Module with autosampler, software Empower, coupled to a Waters 996 Photodiode array-UV Scanning Detector was employed. Samples were analyzed on a Phenomenex Synergi Fusion RP 18 chromatographic column (250 x 4.60 mm, 4 μm) guarded by a Fusion RP (4 x 3 mm) precolumn. The HPLC conditions were as follows: isocratic mobile phase constituted of methanol-water-acetonitrile (75:25:05, v/v/v), flow rate of 1.0 mL/min, detection wavelength at 270 nm. The method was validated with respect to specificity, linearity, precision, accuracy and detection and quantification limits.

Phase-solubility studies[16]

Excess amounts of GEN (1.5 mg) and β-CD at different molar ratios (1:0, 1:1, 1:2 and 1:3) were dispersed in water (2.5 mL). These dispersions were stirred in a water bath (Ika®-Werke EH4 Basic) at 37

°C, during 48 h. After this period, each dispersion was cooled down to room temperature, filtered through a 0.45 µm pore diameter membrane to volumetric flask of 5 mL, and the volume was made up with water. Aliquots from each solution were withdrawn and diluted with methanol for further analysis by the HPLC method. This procedure was repeated three times for each GEN:β-CD molar ratio. The mean and standard deviation of three different sets of experiments are presented.

Preparation and characterization of solid complex and corresponding physical mixture

The 1:1 molar ratio was chosen for preparing the GEN:β-CD complex in aqueous media by stirring in a water bath at 37 °C, during 48 h. After this period, the dispersion was cooled down to room temperature, filtered through a 0.45 µm pore diameter membrane and the supernatant was freeze-dried at –60 °C and $2x10^{-1}$ Torr. The corresponding physical mixture was obtained by vortex mixing GEN and β-CD in eppendorf tube.

The solid GEN:β-CD complex and the GEN/β-CD physical mixture were characterized with respect to drug assay (using a validated HPLC method); thermal behavior (in a Shimadzu DSC-60 equipment, around 1 mg, under N_2 atmosphere from 30 to 300 ° C at 10 °/min); and hydrogen nuclear magnetic resonance (^{1}H NMR spectra were acquired in a Bruker DRX400- Avance spectrometer operating at 400 MHz, equipped with a ϕ5 mm inverse probe with z-gradient coil, using deuterated dimethylsulfoxide as solvent and TMS as internal standard).

To delineate the spatial configuration of the complex, 2D-NOESY experiment (mixing time = 600 ms) was combined with molecular modeling. Calculations were performed by Molecular Mechanics (MM2) at 300 K, using Chem3D Ultra 9.0 (CambridgeSoft), and the simulation was obtained by the insertion of GEN in vertical position into the β-CD cavity, through the secondary or primary hydroxyl group rim and perpendicularly to its diameter.

RESULTS AND DISCUSSION

LC method validation

GEN was eluted at approximately 4.4 min. The method was specific for genistein assay from CD complexes; linear in the range 1.0-50.0 µg/mL ($r^2 > 0.999$; with 4.6% R.S.D. of the slope), precise (within day and between day R.S.D. < 2.89 and 1.44%, respectively), and accurate (R.M.E. < 3.91% for quality control samples prepared using independent stock solutions at 7, 15 and 30 µg/mL). Detection and quantification limits were 0.086 and 0.26 µg/mL, respectively.

Phase-solubility studies

Figure 1 presents the improvement of GEN aqueous solubility in the presence of increasing amounts of β-CD. This linear increase ($r^2 > 0.99$) in GEN solubility classifies the phase-diagram as A_L type, as already observed by Crupi *et al.*.[7] The apparent stability constant determined for the GEN/β-CD complex was 3492 ± 769 mol L^{-1} and suggests a relatively strong interaction between both molecules.

Characterization of solid complex and corresponding physical mixture

The 1:1 molar ratio was chosen for preparation of the solid complex in aqueous media, followed by filtration of soluble fraction, and freeze-drying the supernatant. The solid complex presented a GEN content of 19.22% (w/w). The corresponding physical mixture was prepared in the same molar ratio.

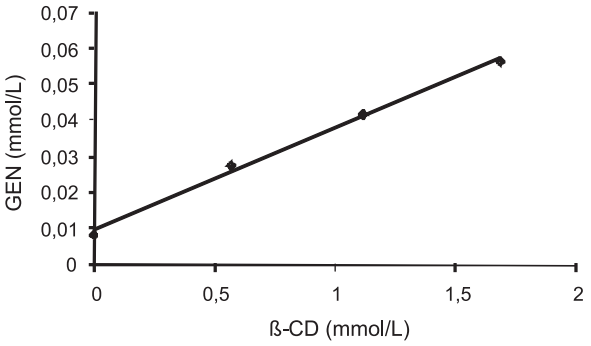


Figure 1. *Phase-solubility diagram of genistein associated to* β*-cyclodextrin*

Figure 2 presents the thermograms of GEN, β-CD, GEN:β-CD complex and GEN/β-CD corresponding physical mixture. As can be observed, GEN presents a unique endothermic peak, characteristic of melting point, at 305 °C, in agreement with previous literature.[17] β-CD presents a broad band at around 100 °C, characteristic of loss of water, an endothermic event at 220 ºC, corresponding to an irreversible transformation process within the β-CD molecule, and the melting point at 312 ºC.[18] In the GEN/β-CD physical mixture, the intensity of the peak corresponding to the melting point of GEN is reduced and its position displaced, while in the GEN:β-CD solid complex, the melting point of GEN can be no longer observed. The peak reduction was expected since the amount of GEN in the physical mixture is very small (19.22%). On the other hand, these thermal events (peak displacement or disappearance) observed in GEN melting peak are indicative of physical interaction with β-CD, which seems to be stronger in the complex form.

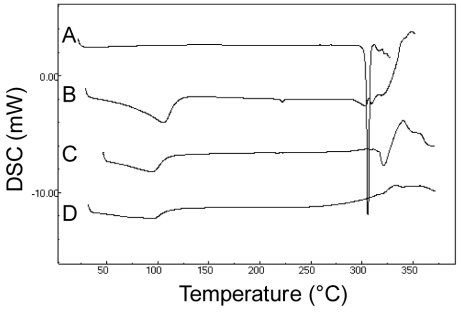


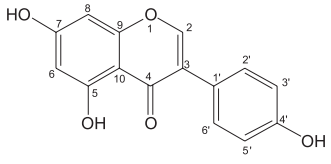
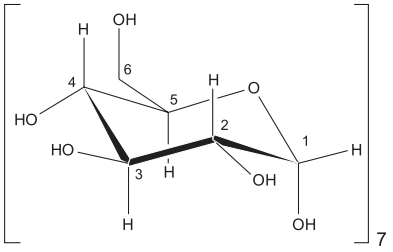
Figure 2. *Thermograms of: (A) GEN; (B)* β*-CD; (C) GEN/*β*-CD physical mixture and (D) GEN:*β*-CD complex*

The host:guest interactions were also studied by the comparison between ^{1}H NMR spectrum of GEN and ^{1}H NMR spectrum of GEN:β-CD complex (Figure 3 and Table 1) in DMSO-d_6 solution. Some loss of resolution in the spectral lines of the NMR spectra can be observed, due to the complexation effects with the β-CD molecule. Nuclear Overhauser Effects observed between the hydrogens H6 (δ 6.23) of the A-ring of GEN,[19] and the hydrogens located inside the β-CD molecule (H3, δ 3.61-3.67 and H5, δ 3.55-3.61),[20] as detected in 2D-NOESY experiments (Figure 4), indicate the formation of the GEN:β-CD inclusion complex.

These data suggest that GEN A-ring may be placed into the β-CD cavity, suggesting the complexation with a 1:1 stoichiometry. In a recent report, Daruházi *et al.*[14] found that solely genistein B-ring would be included into the β-CD cavity and the mode of insertion would occur from the wider rim side (H-5 proton).

Computer molecular mechanics (MM2) method was used to get information about the supramolecular geometry of the GEN:β-CD complex. Four possible inclusion compound models

Table 1. ^{1}H NMR spectral data for genistein and β-CD (400 MHz, DMSO-d_6)

GEN		β-CD	
			
Hydrogen	δ (ppm)	Hydrogen	δ (ppm)
H-2	8.32	H-1	4.83
H-6	6.23	H-2	3.28-3.30
H-8	6.38	H-3	3.61-3.67
H-2' and H-6'	7.36-7.38	H-4	3.32-3.38
H-3' and H-5'	6.81-6.83	H-5	3.55-3.61
H-5 OH	13.00	H-6	3.61-3.67
		OH (2)	5.70
		OH (3)	5.66
		OH (6)	4.44

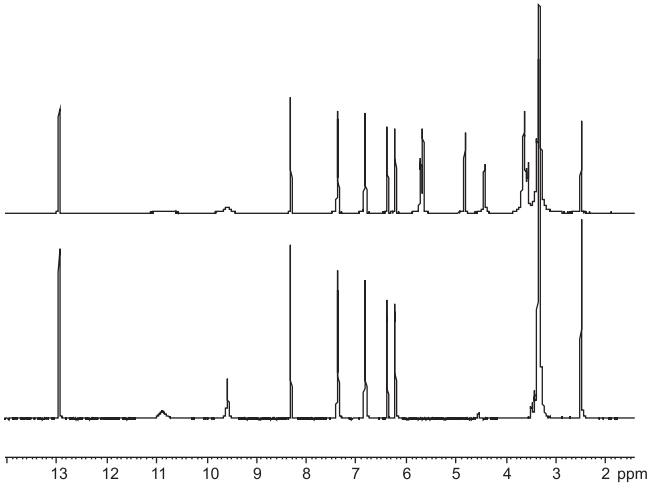


Figure 3. *^{1}H NMR of (A) GEN/β-CD physical mixture, (B) GEN:β-CD complex (400 MHz, DMSO-d_6)*

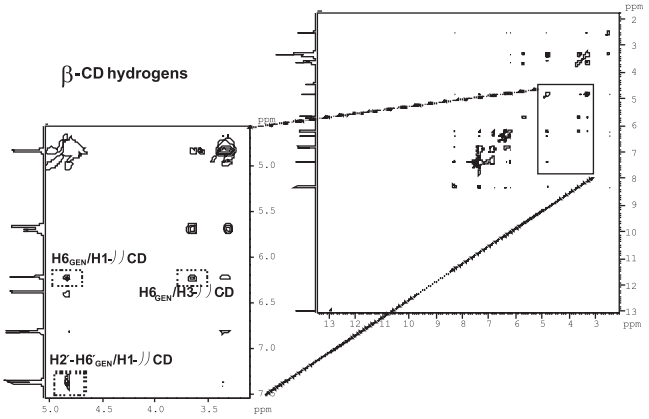


Figure 4. *Two-dimensional NOESY spectrum of the GEN:β-CD complex (400 MHz, DMSO-d_6)*

may be formed from the interaction of one β-CD and one GEN molecule. The A or B-ring of GEN can be included into the CD cavity either through the secondary hydroxyl groups edge (model I) or through the primary hydroxyl groups edge (model II) (Figure 1S in supplementary material), although the insertion through the wider rim was slightly more favorable. These orientation and conformation were obtained according to energy characteristics, being the local minimum energy for the first model the lowest (Table 2) being in according to the our RMN data as well as to that reported by Daruházi *et al*..[14]

Table 2. Energy (kJ mol^{-1}) results of conformation optimization for four models of β-CD/GEN assemblies

	A-ring and Secondary OH rim	A-ring and Primary OH rim	B-ring and Secondary OH rim	B-ring and Primary OH rim
Stretch	9.4098	10.4528	9.1768	9.6501
Bend	59.0777	63.2763	58.7786	59.1749
Stretch-bend	5.1769	5.7028	5.0838	5.2291
Torsion	25.2005	24.1641	24.1616	24.9817
Non-1,4VDW	-80.9680	-82.2887	-81.8595	-78.4156
1,4VDW	97.8079	96.6259	97.4524	96.6593
Dipole/Dipole	-28.1228	-29.1519	-27.3311	-25.3463
Total energy	87.5820	88.7812	85.4626	91.9930

CONCLUSIONS

The method for obtaining a GEN/β-CD complex yielded a two fold higher GEN content than that firstly reported.[14] This excellent result represents an important advantage for its incorporation in cosmetics or pharmaceutical solid or semi-solid dosage forms, considering the high molecular weight of the complex. Additionally, the applied techniques revealed strong evidence of interaction between GEN and β-CD, either for the GEN:β-CD complex or for the GEN/β-CD physical mixture. The spatial configuration of the complex proposed by means of NMR and molecular modeling revealed that the complexation occurs mainly through the insertion of the A-ring into the cyclodextrin cavity, however the possibility of inclusion through the B-ring, as previously reported in the literature, can not be excluded,

considering the slight differences in the total energy presented by the inclusion of A-ring or B-ring.

SUPPLEMENTARY MATERIAL

Available in http://quimicanova.sbq.org.br, with free access. Figure 1S. Complexation simulation of genistein through the secondary (b: A-ring; d: B-ring) and primary (a: A-ring; c: B-ring) rims of β-cyclodextrin cavity.

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