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Short communication

The enhancement of isoflavones water solubility by complexation with modified cyclodextrins: A spectroscopic investigation with implications in the pharmaceutical analysis

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Abstract

The improvement of isoflavones bioavailability by complexation with chemically modified cyclodextrins (CyDs) has been exploited to analyse the drug/macrocycle binding affinity by a conventional method with new useful measures. Genistein (Gen) and daidzein (Daidz) were investigated in aqueous medium and in presence an amount of (2-hydroxypropyl)-β-cyclodextrin (HP-β-CyD) at different host/guest molar ratios. The solubility in pure water, $\sim 3 \times 10^{-6}$ M for Gen and $\sim 10 \times 10^{-6}$ M for Daidz, was obtained by distributing the of guest molecule between water and the organic solvent. The stoichiometric ratios and stability constants describing the extent of formation of the complexes have been determined by phase-solubility UV–vis measurements and confirmed by circular dichroism data. These results have implications in the determination of the carrier's capacity for the complexation of the drug in water solution.

Keywords: (2-Hydroxypropyl)-β-cyclodextrin; Isoflavones; UV–vis spectroscopy; Circular dichroism; Binding constant; Water solubility measurements

1. Introduction

Phytoestrogens are diverse groups of plant-derived compounds that structurally or functionally mimic mammalian estrogens and show potential benefits for human health. There are several classes of phytoestrogens: steroidal estrogens, found in a few plants and the more ubiquitous phenolic estrogens, isoflavone, coumestanes and lignans. Aglicones, genistein (Gen) and daidzein (Daidz) are naturally occurring isoflavones that have shown estrogenic activity.

Interest for these substances originated from epidemiological findings indicating that they may provide protection against chronic diseases, such as hormone-dependent cancers [1] and disorders of the cardiovascular system. Subsequent studies revealed that Gen and Daidz exhibit multiple pharmacological effects, with the result that at present the isoflavones are emerging as active ingredients for nutraceuticals or as prospective drug candidates [2–4]. These activities, along with low toxicity, make isoflavones important candidates for experimental anticancer therapy, and thus new lead compounds for drug design.

Nevertheless, Gen and Daidz seem to have some disadvantages, which considerably limit their potential clinical utility. These include rapid in vivo metabolism and excretion, low serum level after oral administration, insufficient targeting of cancer cells, and poor solubility in water that remarkably reduces their bioavailability.

Complexation with cyclodextrins (CyDs) represents a valid method to improve the physico-chemical characteristics and reduce the limitations of these substances.

Inclusion in CyD cavity modifies the physico-chemical properties of guest molecules [5]; for instance, the solubility of

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the highly insoluble guest is enhanced, labile guests are stabilized against the degradative effects of oxidation and visible or UV light and heat, volatility and sublimation are controlled, incompatible compounds can be physically isolated and chromatographically separated. Moreover, the inclusion phenomenon can allow for taste modification by masking off flavours, unpleasant odours and for the controlled release of drugs and flavours. Therefore, CyDs are used in food [6], pharmaceuticals [7], cosmetics [8], environment protection [9], bioconversion [10], packing and the textile industry [11].

Many drugs are poorly soluble in the commonly used pharmaceutical solvents and the use of solvent mixtures is often exploited as a method to increase their solubility. Reliable solubility values are of primary importance to test the effectiveness of such systems.

To date, the thermodynamic stability of the CyD/drug systems have been studied by evaluation of the constant binding from UV–vis, circular dichroism, fluorescence, calorimetry, NMR, electron spin resonance (ESR), potentiometry, chromatography, capillary electrophoresis, etc. [12].

Generally, the water solubility of a free drug (S_0) is determined by means of electronic and emission spectroscopy employing linear regression analyses from the phase-solubility diagram. In literature, the macrocycle concentration for solubility phase study have been used in a range in which, probably, there are molecular aggregates [13].

Lee and co-workers [14] have evaluated the inclusion efficiency of Gen and other guests in β-CyD and HP-β-CyD in 1 wt% solutions (∼9 mM). In particular, above 5 mM in the case of β-CyD, non-consideration of the formation of aggregates could invalidate the quantitative analysis.

The aim of this work was to outline an accurate method to determine the complexation constants for isoflavones with HP-β-CyD starting from an experimental technique which measures the S_0 parameter. The stoichiometric ratios and stability constants describing the extent of formation of the complexes have been determined by UV–vis phase-solubility measurements and confirmed by circular dichroism (CD) data. The concentration values of macrocycle have been chosen in the range (0–9 mM) in which, as verified by light scattering measurements, the presence of CyD aggregates were not registered.

2. Experimental

2.1. Materials

The following reagents and solvents were used: genistein (4′,5,7-trihydroxyisoflavone, $C_{15}H_{10}O_5$, FW 270.24) from Sigma Aldrich Chemie® (Genay, France); daidzein (4′,7-trihydroxyisoflavone, $C_{15}H_{10}O_4$, FW 254.2) from Sigma Aldrich Chemie® (Genay, France); (2-hydroxypropyl)-β-cyclodextrin (HP-β-CyD, FW ≈1170.0, m.p. ≈ 278 °C dec., degree of substitution ≈0.6), from Fluka Chemie (Switzerland); 1-butanol (HPLC grade) from Merck (Germany). They were employed without any further purification. Water used throughout the study was double-distilled and deionised, then filtered through 0.22 μm Millipore® GSWP filters (Bedford, USA). Solutions to be analysed were previously filtered through 0.45 μm Sartorius Minisart®-SRP 15 PTFE filters (Germany).

2.2. Apparatus

2.2.1. UV–vis and circular dichroism spectroscopy

UV–vis absorption spectra were obtained with a Perkin-Elmer UV-VIS double beam spectrophotometer mod. Lambda 45 (resolution, 0.001 absorbance units; signal-to-noise ratio, 1×10^{-4}). The pathlength of the quartz cell was 10.00 mm except when explicity indicated. For each measurement the baseline was established by placing an aqueous solution of each cyclodextrin in the reference compartment at the same concentration of the sample. All measurements were carried out at 25.0 ± 0.01 °C. The CD spectra were collected using a JASCO J-500A spectropolarimeter equipped with a 150 W Xenon lamp. The instrument was interfaced with a PC for CD signals reading. The measurements were performed at 25 ± 0.1 °C and the samples were contained in rectangular quartz cuvettes of 2 mm pathlength.

All data shown represent the average of at least, three determinations.

2.2.2. Determination of isoflavones water solubility

All the solutions of isoflavones were prepared in butanol and the extinction molar coefficient (ε) was determined. Experiments were carried out in triplicate and all are in good agreement within the experimental error; the data reported are average values.

Equivalent volumes of each organic solution, previously prepared for calibration curves, and pure water were put on a separatory funnel and shaken for 15 min; they were then kept standing until the total separation of the two solvents was achieved. Later on, the organic phase of all solutions was analysed spectrophotometrically and each experiment was repeated until a constant value of absorbance was reached.

2.2.3. Phase-solubility measurements

Phase-solubility studies were performed with a Telesystem stirring bath thermostat 15.40 with a Telemodul 40 °C control unit which allowed an accuracy of 0.01 °C. A fixed initial amount of Gen (185 μM) and Daidz (18 μM) exceeding their solubility, were added to unbuffered aqueous solutions of HP-β-CyD (0.0 to 9.0 mM), then sonicated (15 min) in a Bandelin RK 514 water bath (Berlin, Germany). The flasks were sealed to avoid changes due to evaporation and magnetically stirred for 3 days in a thermostated bath at 25.0 ± 0.01 °C, shielded from light to prevent any degradation of the molecules. After the equilibrium was reached (about 72 h), the suspensions were filtered. An aliquot from each vial was withdrawn by 1 mL glass syringe (Poulten & Graf GmbH, Germany) and assayed spectrophotometrically to evaluate the amount of isoflavone dissolved. Experiments were carried out in triplicate and solubility data were all consistent. The data were averaged and used to determine the binding constant for Gen/HP-β-CyD and Daidz/HP-β-CyD complexes formation, by UV–vis as well as by CD spectroscopy.

Isofl	R_1	R_2	R_3
Gen	OH	H	H
Daidz	H	H	H

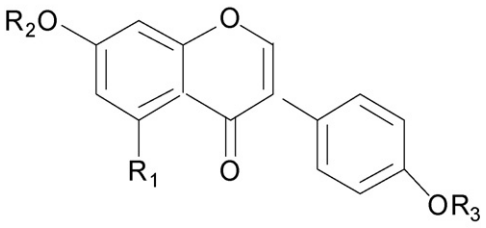

Fig. 1. Sketched structures of investigated isoflavones.

3. Results and discussion

3.1. Determination of isoflavones water solubility

In literature, Gen and Daidz (Fig. 1) are reported as substances poorly soluble in water. To determine the solubility of these isoflavones in pure water a method of distribution in water/organic solvent was used, after having already calculated the ε of isoflavones in organic solvent (butanol as organic solvent was chosen because of the good compromise between its poor miscibility with water, good solubility of isoflavones and its low volatility). In Fig. 2 (see inset) carries the straight lines of Gen ($y = 0.99x - 4.53$) and Daidz ($y = 0.86x - 3.70$) after which the molar extinction coefficients in butanol were obtained in accordance with the equation $\varepsilon = 10^{-\text{intercept}}$ ($\varepsilon_{\text{Gen}} = 33{,}700 \pm 200\,\text{cm}^{-1}\,\text{M}^{-1}$ and $\varepsilon_{\text{Daidz}} = 4900 \pm 100\,\text{cm}^{-1}\,\text{M}^{-1}$). Once ε in organic solvent was obtained, the concentration in the same organic phase was achieved before and after shaking, and the difference between these concentrations gave the S_0 value of isoflavones in water ($S_{0\text{Gen}} = 3 \pm 0.5\,\mu\text{M}$; $S_{0\text{Daidz}} = 10 \pm 3\,\mu\text{M}$). Then the molar extinction coefficient in water was determined by measuring the absorbance value at S_0 ($A_0 = \varepsilon_0 S_0 d$), namely in the absence of HP-β-CyD.

3.2. Phase-solubility studies

In the literature, the solubility of the free guest in water is often determined by strong dilution of a less-soluble substance (in the range 1–5 μM) or by linear regression analyses from a phase-solubility diagram. In the present paper the conventional method [15,16] has been adopted to determine the binding constant of isoflavones to HP-β-CyD. Two steps are used: the measurement of S_0 value by means of the distribution equilibrium of the guest between immiscible solvents (i.e. water–butanol), and the measurement of ε of the complex, which in this case is strongly different from that of free isoflavones. The absorbance of the isoflavone in water solution increases with the amount of HP-β-CyD (Fig. 2), suggesting that the apparent solubility of the isoflavone is enhanced by a binding process with HP-β-CyD.

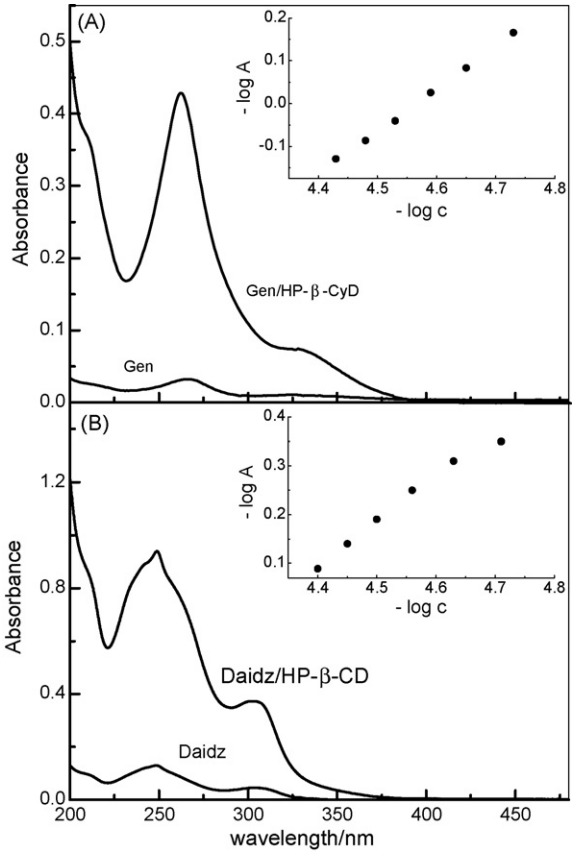

Fig. 2. UV–vis absorbance of Gen (A) ($d = 1$ mm) and Daidz (B) alone in water and in presence of HP-β-CyD (4.0 mM). *Inset*: The calibration curves of Gen and Daidz in butanol are reported ($\varepsilon_{\text{Gen}} \cong 33{,}500\,\text{cm}^{-1}\,\text{M}^{-1}$ and $\varepsilon_{\text{Daidz}} \cong 5000\,\text{cm}^{-1}\,\text{M}^{-1}$).

From the plateau in the absorbance and optical density values (see insets of Fig. 3) measured at different amounts of HP-β-CyD it is possible to obtain the concentration of the complexed isoflavone, by considering the S_0 values obtained with the debate method that correspond to the concentration of Gen and Daidz in pure water.

In the presence of a given amount of HP-β-CyD, the absorbance of the isoflavone is $A = \varepsilon_0 d S_0 + \varepsilon_c d[\text{R·CyD}] = A_0 + \varepsilon_c d[\text{R·CyD}]$, where ε_0 and ε_c are the molar extinction coefficient of the isoflavone in water and of the complex isoflavone/HP-β-CyD, respectively, S_0 is the solubility of the isoflavone in water, d the length of the optical cell and [R·CyD] the concentration of the complex. By considering that ε_0 is measured as previously explained, the concentration of the complexed isoflavone is obtained through the determination of ε_c as $(A_{\text{plateau}} - A_0)/[d(C - S_0)]$, A_{plateau} being the saturated absorbance value for which all the available isoflavone concentration, $(C - S_0)$, is in the complexed form (that is, for the saturated absorbance value, the concentration of the complex [R·CyD] is $C - S_0$). For the complexes Gen/HP-β-CyD and Daidz/HP-β-CyD were $\varepsilon_c = 19{,}000 \pm 1000\,\text{cm}^{-1}\,\text{M}^{-1}$ and $\varepsilon_c = 95{,}000 \pm 5000\,\text{cm}^{-1}\,\text{M}^{-1}$, respectively.

Fig. 3 shows the linear dependence of the concentration of the complexed isoflavone on the amount of HP-β-CyD, suggesting the formation of 1:1 complexes; the change of slope occurs

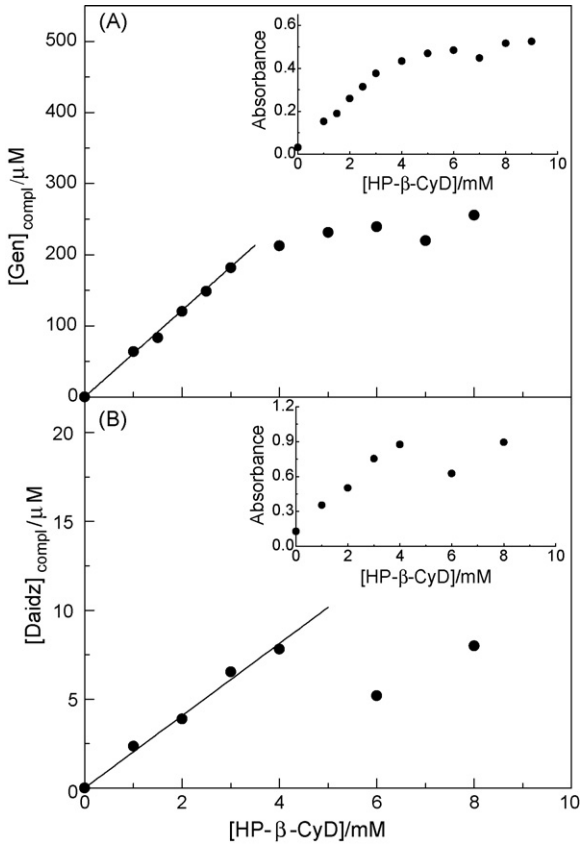

Fig. 3. Concentration of complexed Gen (A) and Daidz (B) at different amounts of HP-β-CyD (the slope gives the binding constant values of $K_{Gen} \cong 20{,}000\ M^{-1}$ and $K_{Daidz} \cong 210\ M^{-1}$). *Inset*: Reports their phase-solubility diagram obtained by UV–vis spectra (path length $d = 1$ mm for Gen/HP-β-CyD).

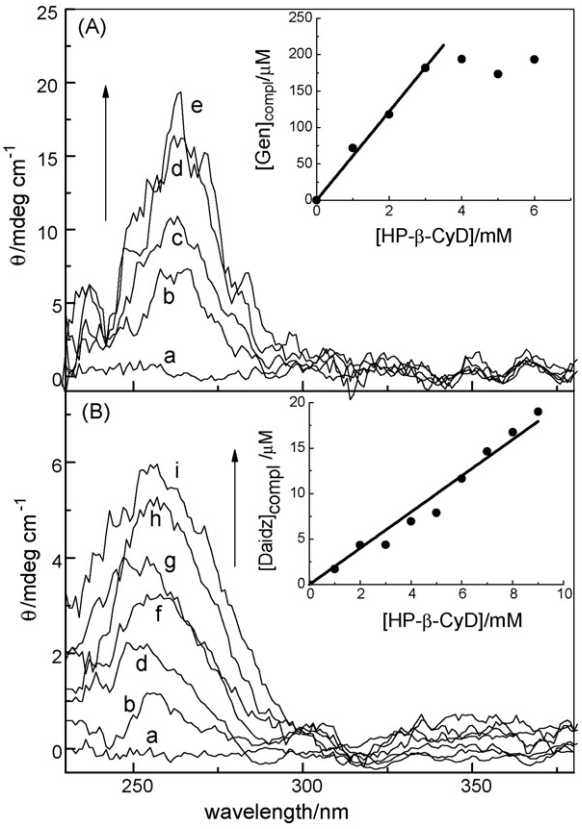

Fig. 4. ICD spectra of Gen (10^{-4} M) (A) and Daidz (10^{-4} M) (B) at different amounts of HP-β-CyD; the letters a, b, c, d, e, f, g, h and i indicate the amount of HP-β-CyD 0, 1, 2, 3, 4, 5, 6, 7 and 9 mM, respectively. *Inset*: Shows the concentration of the complexed isoflavones as a function of HP-β-CyD; the slope of the plots gives the binding constant value of $K_{Gen} \cong 19{,}000\ M^{-1}$ and $K_{Daidz} \cong 190\ M^{-1}$ for Gen and Daidz, respectively (the plateau value of ICD of Daidz is different from that for the absorbance because of the different initial amount of Daidzein).

when all the available isoflavone concentration ($C - S_0$) is in the complexed form. From the slope, α, of the linear fit the binding constant can be evaluated through $K = \alpha/(S_0(1-\alpha))$ [16]: $K_{Gen} = 20{,}000 \pm 4000\ M^{-1}$ and $K_{Daidz} = 210 \pm 40\ M^{-1}$.

3.3. Circular dichroism investigation

The formation of the isoflavones/HP-β-CyD complexes is more evident from circular dichroism spectra, shown in Fig. 4, which are more explicative than UV–vis in the case of chiral species. The spectra show the presence of an induced circular dichroism (ICD) band in correspondence with the absorption band of both isoflavones centred at 256 nm for Gen/HP-β-CyD and at 262 nm for Daidz/HP-β-CyD complexes solutions. The ICD band amplitude (A_{cd}) increases with the amount of HP-β-CyD used indicating that the concentration of the complex increases progressively. By adopting the same approach as in the UV–vis measurements, the concentration of the complexed isoflavones (see the insets of Fig. 4) can be obtained: $[R{\cdot}CyD] = A_{cd}/[\theta_c d]$, where $\theta_c = A_{cd(plateau)}/[d(C - S_0)]$ is the molar ellipticity of the complex ($\theta_c \cong 90^\circ\ cm^{-1}\ M^{-1}$ for Gen/HP-β-CyD and $\theta_c \cong 350^\circ\ cm^{-1}\ M^{-1}$ for Daidz/HP-β-CyD). In this case, the integrated area of the spectra minimized errors due to the noise of the experimental data. The values of the binding constants are in good agreement with those obtained by the UV–vis method ($K_{Gen} = 19{,}000 \pm 4000\ M^{-1}$ and $K_{Daidz} = 190 \pm 40\ M^{-1}$).

According to the Kirkwood–Tinoco theory of polarizabilities, the ICD Cotton positive effect suggests that both isoflavones can reside inside the cavity which will have the axis of symmetry parallel to the transition moment axis of chromophores [17,18]. Alternatively, a positive ICD can be ascribed to the isoflavones located outside the cavity possessing axis aligned perpendicularly to the drug moment axis [19]. In the case of β-CyD/rutin complex [20,21], on the basis of NMR and molecular modelling results, the observed positive ICD band suggests that the phenyl portion (single ring of molecule) could be included in the cavity. Furthermore, as previously reported [22], the changes of band-shapes in FTIR-ATR spectroscopy were ascribed to partial inclusion of phenyl moieties of Gen in the CyD cavity. FTIR-ATR (Fourier transform infrared used in attenuated total reflectance geometry) spectroscopy revealed also differences in the O–H stretching profile, and in particular in the C=O, C=C, C–O–C and C–O stretching vibrations. Finally, the out-of-plane bending C–H band of the phenyl group disappears by suggesting that this portion is hindered in the CyD cavity [22].

Comparison between the bands of the free Gen and CyD, and the physical mixture showed different profiles with respect

to the complex indicating the hindering of the aromatic moiety, probably, due to a close fit to the cavity.

4. Conclusions

A conventional method with new useful measures was employed in order to determine accurately the binding constant of isoflavones/HP-β-CyD inclusion complexes.

The focal points of this investigation were: (i) the absence of aggregation of host macrocycle in the range of concentration used; (ii) the estimation of water solubility of the free substance by distribution between two immiscible solvents. With this procedure the phase-solubility study by UV–vis and ICD can give more reliable results for the binding affinity constant. In addition the induced band in CD spectra would indicate the inclusion of isoflavones inside the CyD cavity, confirming the hypothesis previously reported on flavonoid/β-CyD system [20,21]. These findings can be helpful to compare the physical chemical peculiarities of systems with their pharmaceutical properties.

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