Frontiers in Pharmaceutical Analysis
Volume 2 Issue 2 2026

Frontiers in Pharmaceutical Analysis
Volume 2 Issue 2 2026

A spectroscopic study with implications for pharmacological analysis on the improvement of isoflavones' water solubility through complexation with modified cyclodextrins

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Article Info

Received: 7-1-2026 Revised:10-2-2026 Accepted:13-3-2026 Published:17-4-2026

Abstract

The enhanced bioavailability of isoflavones through complexation with chemically modified cyclodextrins (CyDs) has been utilized to analyze the drug/macrocycle binding affinity using a traditional approach and new practical metrics. In aqueous medium and with varying host/guest molar ratios of (2-hydroxypropyl)-β-cyclodextrin (HP-β-CyD), genistein (Gen) and daidzein (Daidz) were studied. By dividing the guest molecule between water and the organic solvent, the solubility in pure water was found to be approximately 3 × 10−6 M for Gen and approximately 10 × 10−6 M for Daidz. Phase-solubility UV–vis measurements and circular dichroism data have corroborated the stoichiometric ratios and stability constants that describe the degree of complex formation. The determination of the carrier's ability to compound the medication in aqueous solution is affected by these findings.

Keywords: β-cyclodextrin (2-hydroxypropyl); UV-vis spectroscopy; isoflavones; Water solubility easurements, binding constant, and circular dichroism

Introduction

Phytoestrogens are a broad class of compounds generated from plants that resemble mammalian estrogens in structure or activity and may be beneficial to human health. There are numerous kinds of phytoestrogens, including the more common phenolic estrogens, isoflavone, coumestanes, and lignans, and steroidal estrogens, which are present in a small number of plants. Naturally occurring isoflavones that have demonstrated estrogenic activity include aglicones, genistein (Gen), and daidzein (Daidz). Epidemiological results suggesting that these compounds may offer protection against chronic diseases, including hormone-dependent malignancies [1] and cardiovascular disorders, sparked interest in them. The isoflavones are currently emerging as active ingredients for nutraceuticals or as promising medication candidates as a consequence of subsequent research showing that Gen and Daidz exhibit numerous pharmacological actions [2–4]. Isoflavones are significant prospects for experimental anticancer therapy and, consequently, novel lead molecules for drug creation due to these actions and their low toxicity. However, Gen and Daidz appear to have several drawbacks that significantly restrict their potential therapeutic use. These include low serum levels following oral administration, fast in vivo metabolism and excretion, inadequate cancer cell targeting, and poor water solubility that significantly lowers their bioavailability. Complexation with cyclodextrins (CyDs) is a legitimate way to enhance these molecules' physico-chemical properties and lessen their limits.

The physico-chemical properties of guest molecules are altered by inclusion in the CyD cavity [5]. For example, the highly insoluble guest's solubility is increased, labile guests are stabilized against the degradative effects of oxidation, visible or UV light, and heat, volatility and sublimation are controlled, and incompatible compounds

can be physically isolated and chromatographically separated. Additionally, the inclusion phenomenon can enable the regulated release of medications and flavors as well as taste alteration by masking off flavors and offensive odors. Consequently, food [6], pharmaceuticals [7], cosmetics [8], environmental protection [9], bioconversion [10], packaging, and the textile industry [11] all employ CyDs.

Solvent mixes are frequently used to boost the solubility of medications that are poorly soluble in the pharmaceutical solvents that are widely utilized. To evaluate the efficacy of such systems, reliable solubility data are crucial. Thus far, the evaluation of the constant binding from UV–vis, circular dichroism, fluorescence, calorimetry, NMR, electron spin resonance (ESR), potentiometry, chromatography, capillary electrophoresis, etc. has been used to study the thermodynamic stability of the CyD/drug systems [12].

A free drug's (S0) water solubility is often determined using electronic and emission spectroscopy and linear regression studies from the phase-solubility diagram. The macrocycle concentration for solubility phase research has been employed in a range that most likely contains molecular aggregates in the literature [13].The inclusion efficiency of Gen and other guests in β-CyD and HP-β-CyD in 1 weight percent solutions (about 9 mM) has been assessed by Lee and colleagues [14]. Specifically, in the instance of β-CyD, failure to take aggregate formation into account could render the quantitative analysis invalid above 5 mM.

The objective of this work was to describe a precise procedure for determining the complexation constants for isoflavones with HP-β-CyD, beginning with an experimental method that measures the S0 parameter. Circular dichroism (CD) data has verified the stoichiometric ratios and stability constants characterizing the degree of complex formation, which have been established using UV–vis phase-solubility studies. The macrocycle concentration values were selected within the range of 0–9 mM, where the absence of CyD aggregates was confirmed by light scattering studies.

1. Experimental

Materials

The following solvents and reagents were used: 1-butanol (HPLC grade) from Merck (Germany); daidzein (4′,7-trihydroxyisoflavone, C15H10O4, 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). They were used without any additional purification. The water used in the study was filtered via 0.22 µm Millipore® GSWP filters (Bedford, USA) after being double-distilled and deionized. Prior to analysis, the solutions were filtered through

Apparatus

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 base-line was established by placing an aqueous solution of each cyclodextrin in the reference compartment at the same concen- tration 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 read- ing. 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 deter- minations.

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 pre- pared 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 anal- ysed spectrophotometrically and each experiment was repeated until a constant value of absorbance was reached.

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 ther-mostated 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 consis- tent. 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

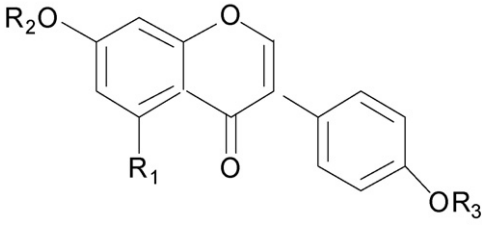

Fig. 1. Sketched structures of investigated isoflavones.

2. Results and discussion

2.1. Determination of isoflavones water solubility

In literature, Gen and Daidz (Fig. 1) are reported as substances poorly soluble in water. To determine the sol- ubility 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_{0Gen} = 3 ± 0.5 μM; S_{0Daidz} = 10 ± 3 μM). 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.

Phase-solubility studies

In the literature, the solubility of the free guest in water is often determined by strong dilution of a less-soluble sub- stance (in the range 1–5 μM) or by linear regression analyses from a phase-solubility diagram. In the present paper the con- ventional 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 distribu- tion 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 sol- ubility 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 ($\varepsilon_{Gen} \simeq$ 33,500 $cm^{-1}\,M^{-1}$ and $\varepsilon_{Daidz} \simeq$ 5000 $cm^{-1}\,M^{-1}$).

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 $A = \varepsilon_0 d S_0 + \varepsilon_c d[R \cdot CyD] = A_0 + \varepsilon_c d[R \cdot CyD]$, 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 $[R \cdot CyD]$ 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 $(A_{plateau} - A_0)/[d(C - S_0)]$, $A_{plateau}$ being the saturated absorbance value for which all the available isoflavone con- centration, $(C - S_0)$, is in the complexed form (that is, for the saturated absorbance value, the concentration of the com- plex $[R \cdot CyD]$ is $C - S_0$). For the complexes Gen/HP-β-CyD and Daidz/HP-β-CyD were ε_c = 19,000 ± 1000 $cm^{-1}\,M^{-1}$ and ε_c = 95,000 ± 5000 $cm^{-1}\,M^{-1}$, respectively.

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

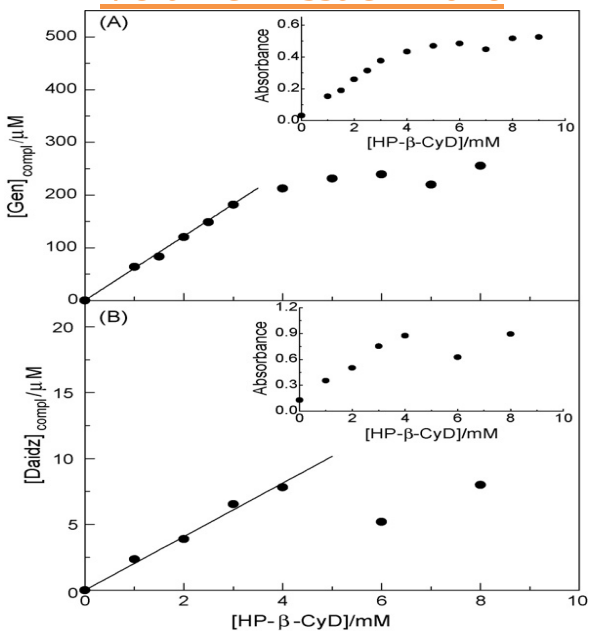

Fig. 4. ICD spectra of Gen (10^{-4} M) (A) and Daidz (10^{-4} M) (B) at different amounts of HP-β-CyD; the letters a, b, c, d, e, f, g, h and i indicate the amount of HP-β-CyD 0, 1, 2, 3, 4, 5, 6, 7 and 9 mM, respectively. *Inset*: Shows the concentration of the complexed isoflavones as a function of HP-β-CyD; the UV–vis spectra (path length d = 1 mm for Gen/HP-β-CyD).

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 ± 4000 M^{-1} and K_{Daidz} = 210 ± 40 M^{-1}.and K = 190 ± 40 M^{-1}).]: slope of the plots gives the binding constant value of $K_{Gen} \simeq$ 19,000 M^{-1} and $K_{Daidz} \simeq$ 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).obtained by the UV–vis method (K_{Gen} = 19,000 ± 4000 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 com- plex 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 ($\theta_c \simeq 90^\circ$ cm^{-1} M^{-1} for Gen/HP-β-CyD and $\theta_c \simeq 350^\circ$ 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 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 perpendicu- larly 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 cav- ity. Furthermore, as previously reported [22], the changes of band-shapes in FTIR-ATR spectroscopy were ascribed to par- tial 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.

3. Conclusions

The binding constant of isoflavones/HP-β-CyD inclusion complexes was accurately determined using a traditional approach with new useful measurements. The investigation's main focuses were (i) the lack of host macrocycle aggregation within the concentration range employed; and (ii) the evaluation of the free substance's water solubility by distribution between two immiscible solvents. This procedure can provide more accurate results for the binding affinity constant in the phase-solubility investigation by UV-vis and ICD. Furthermore, the inclusion of isoflavones within the CyD cavity would be indicated by the induced band in CD spectra, supporting the hypothesis previously reported on the flavonoid/β-CyD system [20,21]. These results can be useful in comparing the pharmacological qualities of systems with their physical chemical features.

Acknowledgement

We are appreciative of the scientific assistance provided by Norberto Micali (CNR-Istituto per i Pro-cessi Chimico-Fisici, Messina).

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