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Preparation and characterization of the inclusion complex of Baicalin (BG) with β-CD and HP-β-CD in solution: An antioxidant ability study

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ABSTRACT

The formation of the complexes of BG with β-CD and HP-β-CD was studied by UV–vis absorption spectroscopy, fluorescence spectra, Phase-solubility measurements and nuclear magnetic resonance spectroscopy (NMR) in solution. The formation constants (*K*) of complexes were determined by fluorescence method and Phase-solubility measurements. The results showed that the inclusion ability of β-CD and its derivatives was the order: HP-β-CD > β-CD. In addition, the experimental resulted confirmed the existence of 1:1 inclusion complex of BG with CDs.

The antioxidant ability studies of BG and CDs complexes were done. The results obtained indicated that the BG/HP-β-CD complex was the most reactive form, and then was the BG/β-CD complex; the last was BG. Special configuration of complex has been proposed on NMR technique.

1. Introduction

Investigations of molecular recognition have attracted much attention in supramolecular chemistry involving natural and artificial host–guest systems [1]. The inclusion process of pharmaceutical molecules with CDs usually results in a modulation of the physicochemical and pharmaceutical properties of guest molecules, such as increased solubility, improved chemical stability and bioavailability, reduced toxicity controlled-rate release and so on [2–4]. Therefore, it would be of great importance to comprehensively understand the inclusion behavior of molecules of pharmaceutical interests with CDs. Recently, various hydrophilic, hydrophobic and ionic cyclodextrin derivatives have been utilized to extend the physicochemical properties and inclusion capacity of natural cyclodextrin [5,6]. HP-β-CD is a water-soluble derivative of β-CD, which has been widely studied as a complexion agent for many pharmaceuticals. The ability of CDs to form inclusion complexes is highly affected by size, shape, hydrophobicity and the form of the guest's molecular.

Baicalin (BG), a flavonoid present in the root of *Scutellaria baicalensis* Georgi (Fig. 1), has attracted considerable attention because of the activities, such as antibacterial [7], anti-HIV activity [8], attenuating oxidative stress [9–11], inhibiting the growth of several types of cells [12–14] inducing cell death in human hepatocellular carcinonia cell [15] and in human promyelocytic leukemia HL-60 cells [16] and so on. However, in spite of the wide spectrum of pharmacological properties, its use in pharmaceutical filed is limited because of its poor solubility.

When the fluorescent guests are included in the CD cavity, the non-radiative decay processes of luminophores are significantly attenuated and hence fluorescence emission increased [17–19]. Due to its high sensitivity, selectivity and instrumental simplicity, fluorescence method has been used to investigate the phenomena of inclusion complexes and determine the association constants of complexes [19–21]. High resolution nuclear magnetic resonance (NMR) is also a powerful tool for studying CD complexes [22] that can provide not only quantitative information, but also detailed information on geometry of the complex.

The present work was designated to study the complexation of BG utilizing two different cyclodextrins (HP-β-CD and β-CD) to improve the solubility and to determine the effect of the complexation process on their antioxidant capacity.

2. Experimental

2.1. Apparatus and materials

UV-757CRT spectrophotometer (Shanghai Precision and Scientific Instrument Co. Ltd.); Fluorescence measurements were performed by F-2500 FL spectrofluoremeter (Hitachi) using 1 cm quartz cell and both the slits were set at 20 nm with the excitation

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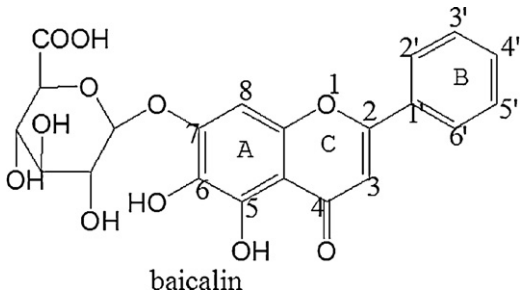

Fig. 1. The chemical structure of Baicalin.

wavelength at 270 nm and the emission at about 350 nm. All the NMR date was obtained on Bruker Avance DRX 300MHZ NMR spectrometer.

A stock solution of 1.0×10^{-4} mol/l BG (provided by Dr. Zhang and was purified by recrystallization) was prepared by dissolving and diluting its crystals in water. CDs and DPPH• were purchased from Sigma–Aldrich, Inc., St. Louis, MO. All other reagents were of analytical-reagent grade and were used without purification. Doubly distilled water was used throughout. All experiments were carried out at room temperature.

2.2. Procedure

A 0.1 or 1 ml aliquot of the stock solution of BG was transferred into a 10 ml volumetric burette, and then an appropriate amount of 1.0×10^{-2} mol/l CDs (β-CD and HP-β-CD) was added. The solution was diluted to a final volume of 10 ml with distilled water. The final mixture solution was dissolved thoroughly under ultrasonic for 30 min, and then equilibrated for 30 min at 20 ± 1 °C. The working solution was transferred into a 1 cm × 1 cm quartz cell to record absorption and fluorescence spectra. All measurements of absorption and fluorescence were made against a blank solution treated in the same way but without BG in a 1.0 cm quartz cell.

2.3. NMR measurements

1×10^{-4} mol/l BG and 1×10^{-4} mol/l CDs (HP-β-CD and β-CD) solutions with a volume ratio of 1:1 were mixed thoroughly. With D_2O as solvent, ^1H NMR spectra was obtained at 300.13 MHz with 10 μs as 90° pulse width. All experiments were performed at 20 ± 1 °C.

2.4. Phase-solubility study

Solubility measurements were based on the Phase-solubility technique [23]. Namely, excess amount of solid BG (8 mg) was added to a series of 10 ml stopper burette that contained increasing amount of CDs (1.0×10^{-2} mol/l, 0–9 ml, including β-CD and HP-β-CD). These obtained suspensions were shaken by ultrasonic method for 3 h at room temperature, and then were filtered after being placed for 7 days. The filtrate was diluted and analyzed through UV method. Phase-solubility profile was obtained by plotting the solubility of BG versus the concentration of CDs.

The apparent stability constant (K_s) of the complexes were calculated according to the following equation

$$K_s = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad (1)$$

where S_0 is the solubility of BG at room temperature in absence of CDs and slope means the corresponding slope of the Phase-solubility diagrams.

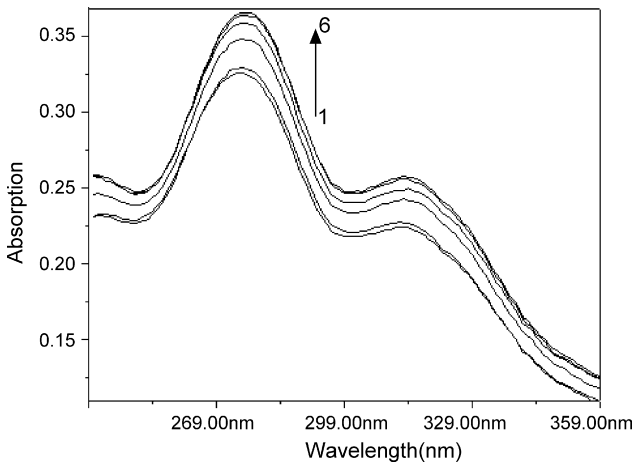

Fig. 2. The absorption spectra of 1.0×10^{-6} mol/LBG in the present of β-CD and the concentration of β-CD is $0–5 \times 10^{-3}$ M.

2.5. Determination of antioxidant activity by the scavenging of the stable radical DPPH•

The antioxidant activity was measured, wherein the bleaching rate of a stable free radical, DPPH• is monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH• absorbs at 517–520 nm, but upon reduction by an antioxidant or a radical species its absorption decreases.

A volume of 2 ml of 1.0×10^{-5} M DPPH• was used. Furthermore, DPPH• is insoluble in aqueous solution the scavenging study was performed in mixture of ethanol–water (20:80).

The reaction was started by addition of 1 ml of BG (1.0×10^{-5} M), BG/β-CD, and BG/HP-β-CD complex samples, which correspond to the 3 mM cyclodextrin concentration from the Phase-solubility studies. All the solution were balanced for 5 min in room temperature, then the bleaching of DPPH• was followed at 520 nm.

The decrease in absorbance at 520 nm was measured against a blank of ethanol–water (20:80) 1 and 2 ml 1.0×10^{-5} M DPPH• to estimate the radical scavenging capacity of each antioxidant sample. The results were expressed as percentage DPPH• elimination calculated according to the following equation [24]:

$$\text{AU} = \left[\frac{1 - A_s}{A_0}\right] 100, \quad (2)$$

where AU is radical-scavenging activity, A_s is absorbance of sample and A_0 absorbance of blank sample.

3. Results and discussion

3.1. UV spectroscopy

Fig. 2 shows the absorption spectra of BG in the absence and presence of CDs at room temperature. The absorption of BG varied significantly with the addition of β-CD. BG alone in water exhibited two absorption peaks at 275 and 314 nm, respectively. The increase in β-CD concentration from 1 to 5 m mol/l resulted in an increase in the absorption of BG. Simultaneously, as the β-CD increase a weak red shift of absorption peak at 275 nm and a weak blue shift of peak at 314 nm was observed. These might be partly attributed to the change of chromophore groups in BG molecular due to the complex formation between BG and β-CD through hydrophobic interaction, and suggested that the likely formation of an inclusion complex between BG and β-CD. Similar phenomena were observed for the HP-β-CD.

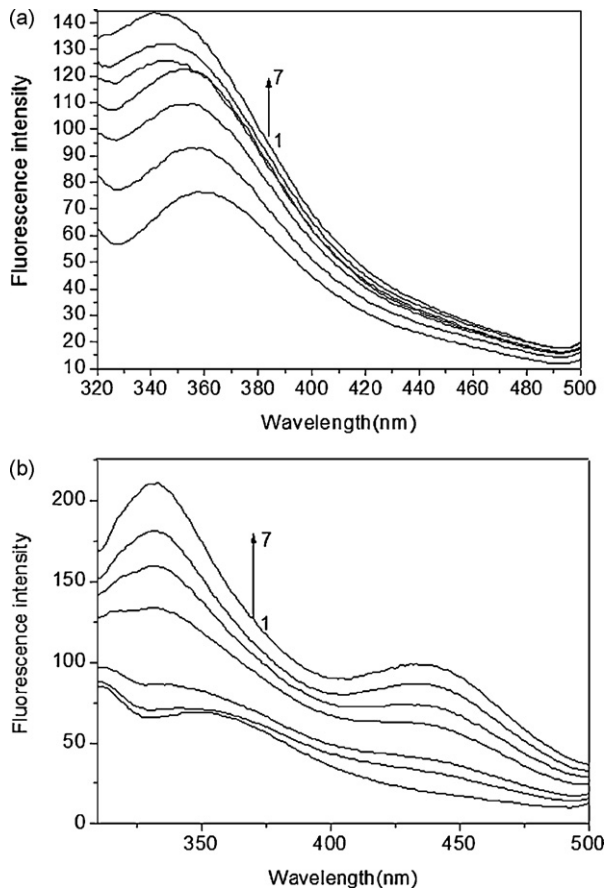

Fig. 3. Fluorescence emission spectra of 1.0×10^{-6} mol/l BG in CDs. a: β-CD (0–6.0 mM); b: HP-β-CD (0–6.0 mM).

3.2. Fluorescence study

Fig. 3 showed that adding CDs (including β-CD and HP-β-CD) to BG solution resulted in a significant enhancement of the fluorescence signal. The excitation wavelength was at 270 nm, the maximum emission wavelength at 358 and 349 nm, respectively. With the increasing of CDs, the emission wavelength appeared blue shift, and a new increasing emission wavelength was observed with the concentration increasing of HP-β-CD at about 430 nm. These suggested that the inclusion complexes were likely formed between BG and CDs. The CD cavity provided an apolar environment for the BG molecule and the motion of the BG molecule in the cavity was largely confined. Thus, the enhanced rigidity of the BG molecule resulted in an increase of its fluorescence quantum yield.

The inclusion formation constant (K) is a measure of the complexing power of CD. The formation constant and ratio of the complex were obtained from fluorescence data using the modified Benesi–Hildebrand equation [25]

$$\frac{1}{(F - F_0)} = \frac{1}{([\mathrm{CDs}]K\alpha)} + \frac{1}{\alpha} \tag{3}$$

where, F and F_0 represent the fluorescence intensity of BG in the presence and absence of CDs, respectively; K is a forming constant; α is a constant. Fig. 4 shows the double reciprocal plots of $1/(F - F_0)$ versus 1/[CD]. The good linear relationship obtained when $1/(F - F_0)$ were plotted against 1/[CDs] supports the existence of a 1:1 complex. These data suggested the inclusion ability of HP-β-CD was bigger than β-CD.

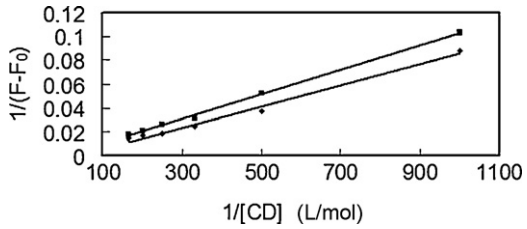

Fig. 4. Double reciprocal plots for BG complexes to β-CD or HP-β-CD. (♦): β-CD; (■): HP-β-CD.

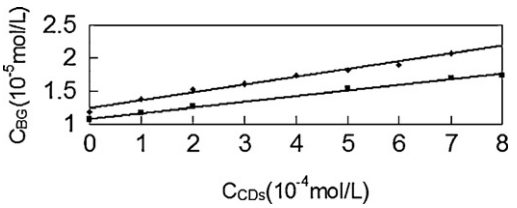

Fig. 5. Phase-solubility diagram of BG and CDs. (■) β-CD, (♦) HP-β-CD.

3.3. Phase-solubility measurements

Fig. 5 showed that CDs enhanced the poor aqueous solubility of BG, thus proving a certain degree of its inclusion complexation in aqueous solutions, the results observed showed a linear behavior for β-CD ($r^2 = 0.9927$) and HP-β-CD ($r^2 = 0.9842$), and consistent with 1:1 molecular complex formation for CDs and BG. The binding constant (K_s) of the complexes were shown in Table 1. As shown in Table 1, the binding constant and solubility of BG determined with CDs followed the rank order HP-β-CD > β-CD. The results were as the same as fluorescence results.

3.4. NMR measurements

To ascertain the structure of the inclusion complexes between BG and CDs, ^{1}H NMR spectroscopy studies of free drug and inclusion complexes were therefore undertaken. Figs. 6 and 7 illustrated the change of hydrogen atom of BG and CDs before and after forming the inclusion complexes. The difference in hydrogen chemical shift values between BG in the free and complexed state were presented

Table 1
Apparent stability constant (K_s) of BG inclusion.

CDs complex	Linear equation	K_s (M^{-1})	r^2
β-CD	$y = 0.0843x + 1.0864$	864	0.9927
HP-β-CD	$y = 0.1186x + 1.2338$	1143	0.9842

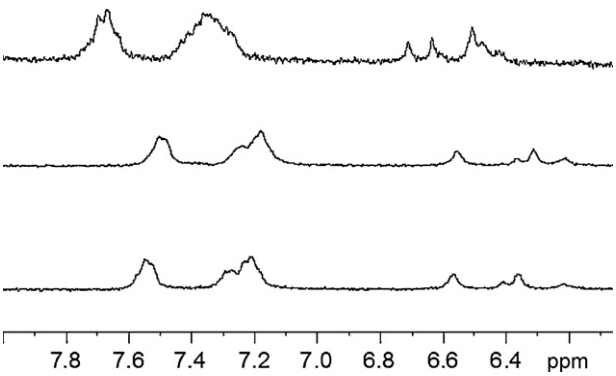

Fig. 6. ^{1}H NMR spectra of BG and inclusion complexes: the order were BG, BG/β-CD and BG/HP-β-CD from the below to the up.

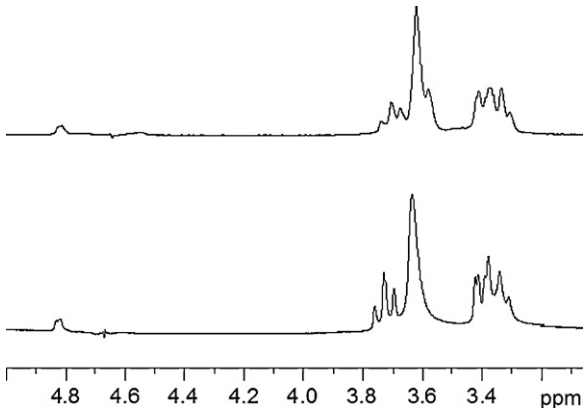

Fig. 7. ^{1}H NMR spectra of β-CD and BG/β-CD inclusion complex from the below to the up.

Table 2
The ^{1}H NMR chemical shifts corresponding to BG in the absence and presence of CDs in D_2O.

BG (H)	BG (δ_0)	BG/β-CD (δ_1)	$\Delta\delta_1$	BG/HP-β-CD (δ_2)	$\Delta\delta_2$
H-8	6.364	6.313	−0.051	6.636	0.272
H-3	6.573	6.554	−0.019	6.719	0.146
H-3′4′5′	7.218	7.179	−0.039	7.341	0.123
H-2′6′	7.552	7.502	−0.040	7.676	0.124

in Tables 2 and 3 showed the hydrogen chemical shift change values of CDs after forming the complexes.

It can be seen from the figures that the H-8, H-3, H-3′, H-4′, H-5′ and H-2′, H-6′ of BG exhibited larger chemical shifts, namely, the A, B and C ring of BG were all entered into the cavity of β-CD and HP-β-CD, because of the diminished freedom of rotation caused by the penetration of BG molecule into the CDs cavity. And the seam time, the H-5 of β-CD experienced larger chemical shift than H-3, which illustrated that the molecular of BG entered into the cavity of β-CD from the small ring-edge side of β-CD; while The H-3 of HP-β-CD experienced larger chemical shift than H-5, which illustrated that the molecular of BG entered into the cavity of HP-β-CD from the large port.

From all the above, the mechanism of complex between BG and CDs were shown as follow (Fig. 8).

3.5. *Scavenging study of DPPH• by free or complexed-BG*

DPPH• is a stable free radical generating a deep violet solution in organic solvents. Its progressive discoloration when in the presence of BG indicated that it is acting as an antioxidant.

Furthermore, since the mechanism of DPPH• reduction is known, the amount remaining of both reagents may be determined.

The rate of the DPPH•-scavenging reaction was measured by monitoring the decrease in absorbance at 520 nm due to DPPH•. Fig. 9 showed the consumption of DPPH• which indicates that the complexed BG with CDs were more effective than free BG, with the HP-β-CD complex (77.78) > β-CD complex (72.22) > free BG (38.89).

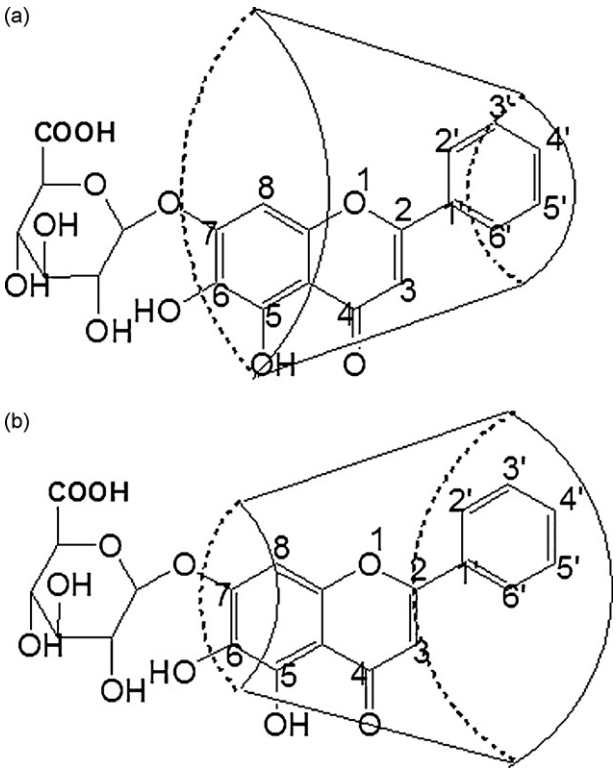

Fig. 8. The structure of inclusion complexes between BG and CDs. a: BG/HP-β-CD; b: BG/β-CD.

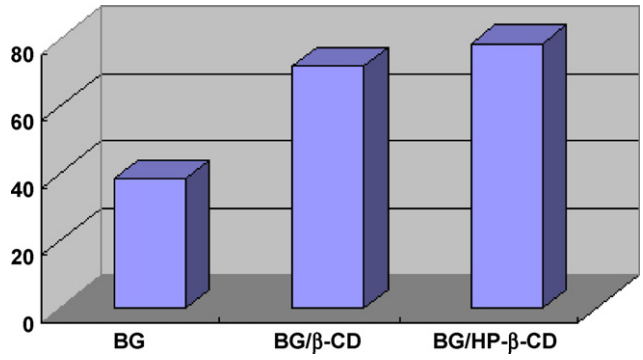

Fig. 9. The consume percentage of DPPH• in presence of BG free and complexes forms.

The scavenging ability was measured as a relative scavenging in presence of free or complex BG. Fig. 9 was in according with scavenging ability is related with enhanced solubility of BG. Also theses results indicated that the complexes formed maintained the BG antioxidant activity.

The antioxidant activity of phenolic compounds depends on the position and degree of hydroxylation, as well as the nature of radicals of the ring structure. Anti-oxidative activity is intensified by

Table 3
^{1}H NMR chemical shifts CDs and the inclusion complexes in D_2O.

CD	β-CD	BG/β-CD		HP-β-CD	BG/HP-β-CD	
(H)	(δ_0)	(δ_1)	$\Delta\delta_1$	(δ_0')	(δ_2)	$\Delta\delta_2$
H-4	3.376	3.379	0.003	3.260	3.259	−0.001
H-2	3.413	3.412	−0.001	3.346	3.344	−0.002
H-5	3.633	3.584	−0.069	3.461	3.466	0.005
H-6	3.633	3.619	−0.014	3.615	3.616	0.001
H-3	3.728	3.715	−0.013	3.749	3.764	0.015

the presence of a second hydroxy group, through the formation of an intramolecular hydrogen bond [26]. It might be that that the –OH positions of BG molecules is close enough to secondary –OH groups of CDs to form hydrogen bonds and contribute to antioxidant activity [27]. Therefore the formation of an "intramolecular" hydrogen bond of the inclusion complex is possible and consequently an increase of antioxidant capacity is expected.

4. Conclusion

The present study has demonstrated the inclusion complex interaction between BG with β-CD and HP-β-CD in the solution. Among CDs, the inclusion ability of HP-β-CD was stronger than that of β-CD. And the activity of eliminating free radical DPPH• were HP-β-CD inclusion complex > β-CD inclusion complex > free BG. In addition, the fluorescence spectroscopy and Phase-solubility measurements data showed the formation of 1:1 stoichiometric complex of BG with β-CD and HP-β-CD over the concentration range evaluated. Moreover, the study demonstrated that CDs severed as drugs carrier system in a dosage-controlled manner and can increase the solubility, stability and antioxidant activity of guest molecular. A mechanism was set up to expound the structure of the inclusion complexes.

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