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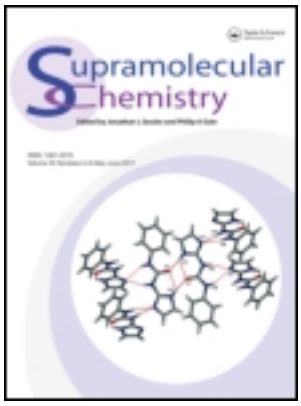

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Investigation on the inclusion behaviour of baicalein with β-cyclodextrin and derivatives and their antioxidant ability study

Jianbin Chao [a], Jian Su [a], Jinxia Li [a], Wei Zhao [a], Shuping Huang [a] & Rui Du [b]
[a] The Institute of Applied Chemistry of Shanxi University, Taiyuan, 030006, China
[b] Department of Engineering, Shanxi Coal Mining Administrators College, Taiyuan, 030006, China

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Investigation on the inclusion behaviour of baicalein with β-cyclodextrin and derivatives and their antioxidant ability study

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[a]*The Institute of Applied Chemistry of Shanxi University, Taiyuan 030006, China;* [b]*Department of Engineering, Shanxi Coal Mining Administrators College, Taiyuan 030006, China*

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The formation of the complexes of baicalein (Ba) with β-cyclodextrin (β-CD) and β-CD derivatives (HP-β-CD and Me-β-CD) was studied by UV–vis absorption spectroscopy, fluorescence method, nuclear magnetic resonance spectroscopy and phase-solubility measurement. The solid–inclusion complexes of Ba with CDs were synthesised by the co-precipitation method. The characterisations of the solid–inclusion complexes have been proved by infrared spectra and differential scanning calorimetry. Experimental conditions including the concentration of various CDs and media acidity were investigated in detail. The results suggested that the inclusion ratio of HP-β-CD with Ba was the highest among the three kinds of CDs. The binding constants (*K*s) of the inclusion complexes were determined by fluorescence method and phase-solubility measurement. Kinetic studies of DPPH· with Ba and CDs complexes were also done. The results indicated that the Ba/HP-β-CD complex was the most reactive form.

Keywords: cyclodextrins; baicalein; inclusion complex

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1. Introduction

Herbal medicines have a long history in medical practice and health care, especially in a number of Asian and African countries. Now, herbal medicines have expanded globally and gained considerable attention because of low toxicity and good therapeutical performance. *Scutellaria baicalensis* Georgi is one commonly used herbal medicine in China and other East Asian countries. Baicalein (Ba, Figure 1) is one of the main active components, which has a variety of interesting activities such as antibacterial (*1*), anti-HIV activity (*2*), attenuating oxidative stress (*3–5*), inhibiting the growth of several types of cells (*6–8*) and inducing cell death in human hepatocellular carcinoma cell (*9*) and in human promyelocytic leukaemia HL-60 cells (*10*). However, one disadvantage of this compound is its low-water solubility, leading to limited use in pharmaceutical field.

Cyclodextrins (CDs) are polysaccharides made of six to eight D-glucose monomers connected at one and four carbon atoms. They have the property of forming inclusion complex with various guests' molecules with suitable polarity and dimension because of their special molecular structure – hydrophobic internal cavity and hydrophilic external surface. They have been widely used in pharmaceutical industries, in analytical purposes and as models for protein and enzymes (*11, 12*). Formation of the inclusion complex can increase the guest's stability against hydrolyses, oxidation, photodecomposition and dehydration, and the water solubility (*13–15*). However, parent CDs have relatively low solubility and molecular binding abilities, which limit their further use. On this basis, modification of CDs is one of the 'hot' topics in recent host–guest supramolecular chemistry, and CD derivatives have been successfully utilised to extend the natural CD (*16, 17*). Hydroxypropyl-beta-cyclodextrin (HP-β-CD), which is a water-soluble derivative of β-CD, and methyl-beta-cyclodextrin (Me-β-CD), which is no harm to human, were selected as other two host molecules.

The inclusion of Ba with biomacromolecule, for example, DNA (*18*) has been researched by many groups, but the inclusion of Ba with CDs has not been investigated. As such, this paper has definite guidance purport in clinic pharmaceutics. In this study, not only the inclusion process was characterised by fluorescence and NMR, but also the solubility was improved and the effect of the complexation process on their antioxidant capacity was determined. A new theory based on new drug carrier system is demonstrated.

2. Experimental section

2.1 Apparatus and materials

The absorption and fluorescence measurements were performed with a UV-757CRT spectrophotometer (Shanghai Precision & Scientific Instrument Co., Ltd, Shanghai, China) and a F-2500 FL spectrofluorometer (Hitachi, Tokyo, Japan), respectively. Both excitation and emission bandwidths were at 20 nm obtained with Bruker

*Corresponding author. Email: chao@sxu.edu.cn

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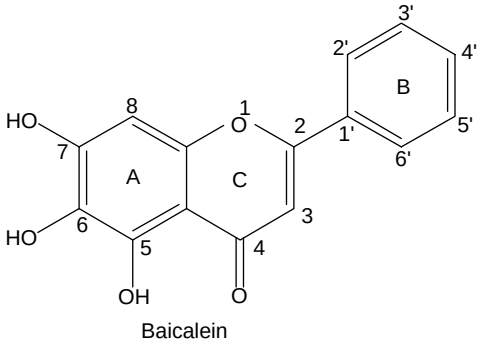

Figure 1. The chemical structure of Ba.

Avance DRX 300 MHz superconducting NMR spectrometer, and D_2O was used as the solvent. Infrared (IR) spectra were obtained with PerkinElmer FT-1730 IR spectroscopy using KBr pelleting. The range of spectra was from 400 to 4000 cm^{-1}. Differential scanning calorimetry (DSC) analyses were carried out in the temperature range from 30 to 500°C in a stream of nitrogen atmosphere on DSC-60 thermal analyzer (Shimadzu, Japan). During experiments, aluminium crucibles were used. The weight of the sample was 5 mg. The heating rate was 10°C/min, and the flow rate of nitrogen atmosphere was 20 ml/min.

The stock solution of 1.0×10^{-4} mol/l Ba (purchases from Nanjing Tcm Institute of Chinese Materia Medica, Nanjing, China; >99%) was prepared by dissolving and diluting its crystals in water. The stock concentrations of CDs were 1.0×10^{-2} mol/l. Phosphate-buffered saline (PBS; 0.2 mol/l) was used to control the pH value of the media. CDs and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich, Inc. (St Louis, MO, USA). All other reagents were of analytical-reagent grade and were used without purification. Double distilled water was used throughout. All experiments were carried out at room temperature.

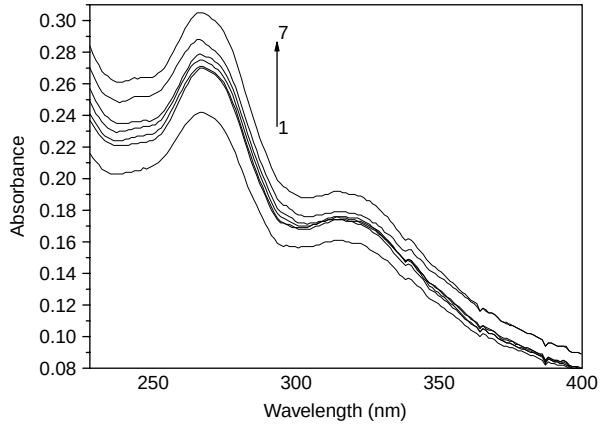

Figure 2. The absorption spectra of 1.0×10^{-5} mol/l Ba in the presence of β-CD. The concentration of β-CD (M): $0–6.0 \times 10^{-3}$.

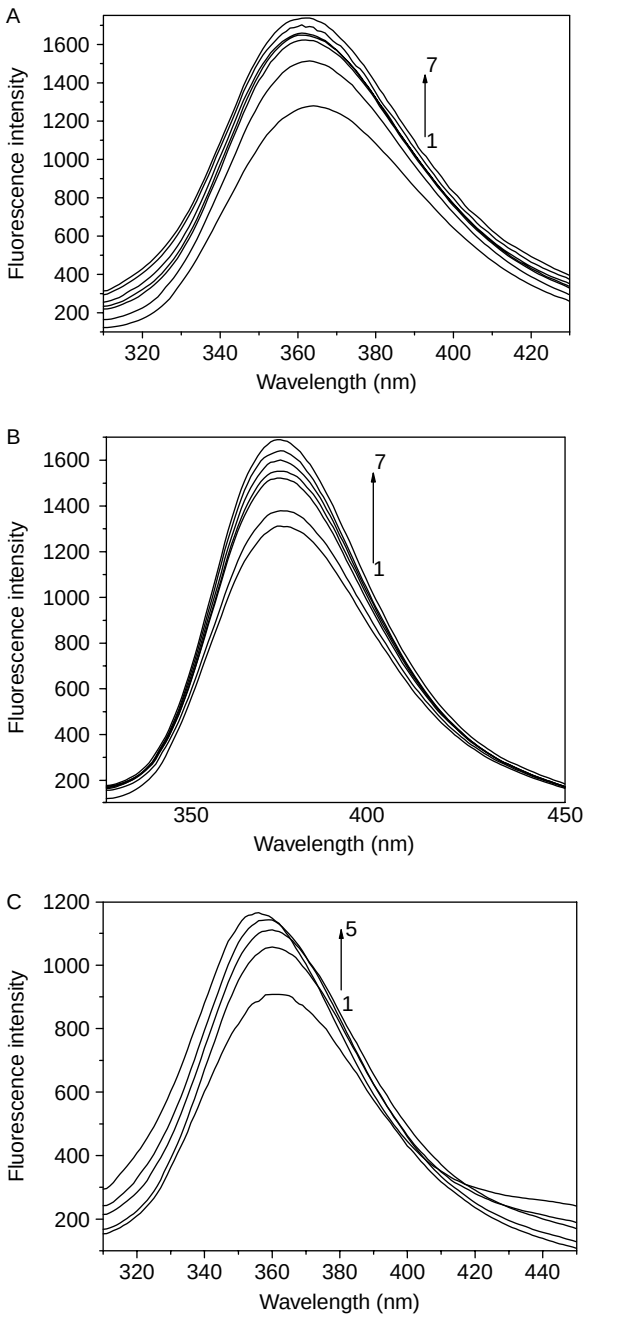

Figure 3. Fluorescence emission spectra of 1.0×10^{-5} mol/l Ba in three kinds of CDs solution. (A) β-CD; (B) HP-β-CD and (C) Me-β-CD. CD concentration (M): $0–6.0 \times 10^{-3}$.

2.2 Procedure

A 1 ml aliquot of the stock solution (1.0×10^{-4} mol/l) of Ba was transferred into a 10 ml volumetric cuvette, and an appropriate amount of 1.0×10^{-2} mol/l CDs (β-CD, HP-β-CD and Me-β-CD) was added. The pH was controlled by

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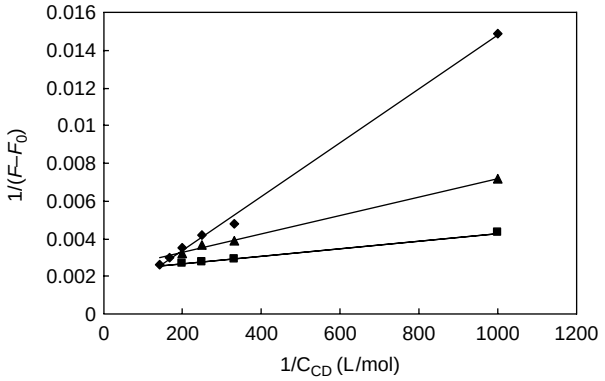

Figure 4. Double reciprocal plots of Ba complexes to β-CD or HP-β-CD or Me-β-CD. (◆) β-CD; (■) HP-β-CD and (▲) Me-β-CD.

Table 1. Apparent stability constants (*K*s) of Ba inclusion.

CDs complex	Linear equation	*K*s (M^{-1})	r^2
β-CD	$y = 1E\text{-}05x + 0.0005$	50	0.9974
HP-β-CD	$y = 2E\text{-}06x + 0.0023$	1150	0.9981
Me-β-CD	$y = 5E\text{-}06x + 0.0023$	460	0.9949

0.2 M PBS. The mixed solution was diluted to the final volume with distilled water and ultrasonically handled for 30 min, and then equilibrated for 30 min at room temperature. Excitation bandwidth was obtained at 270 nm, and fluorescence emission was obtained at about 360 nm. All measurements of absorption and fluorescence were made against a blank solution treated in the same way but without a CD in a 1.0 cm quartz cell.

2.3 NMR measurements

All the concentrations of Ba and CDs solution were 1.0×10^{-4} mol/l, and Ba solution is diluted with CDs solutions, respectively, at the volume ratio of 1:1. ^{1}H NMR of Ba solution as well as its inclusion complexes solutions was also performed to get further evidence.

2.4 Phase-solubility study

Solubility measurements were based on the phase-solubility technique (*19*). For example, an excess amount of solid Ba (6 mg) was added to a series of 10 ml stopper volumetric cuvette that contained an 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 filtered after being placed for 7 days. This filtrate was diluted and analysed through UV method. Phase-solubility profile was obtained by plotting the solubility of Ba versus the concentration of CDs.

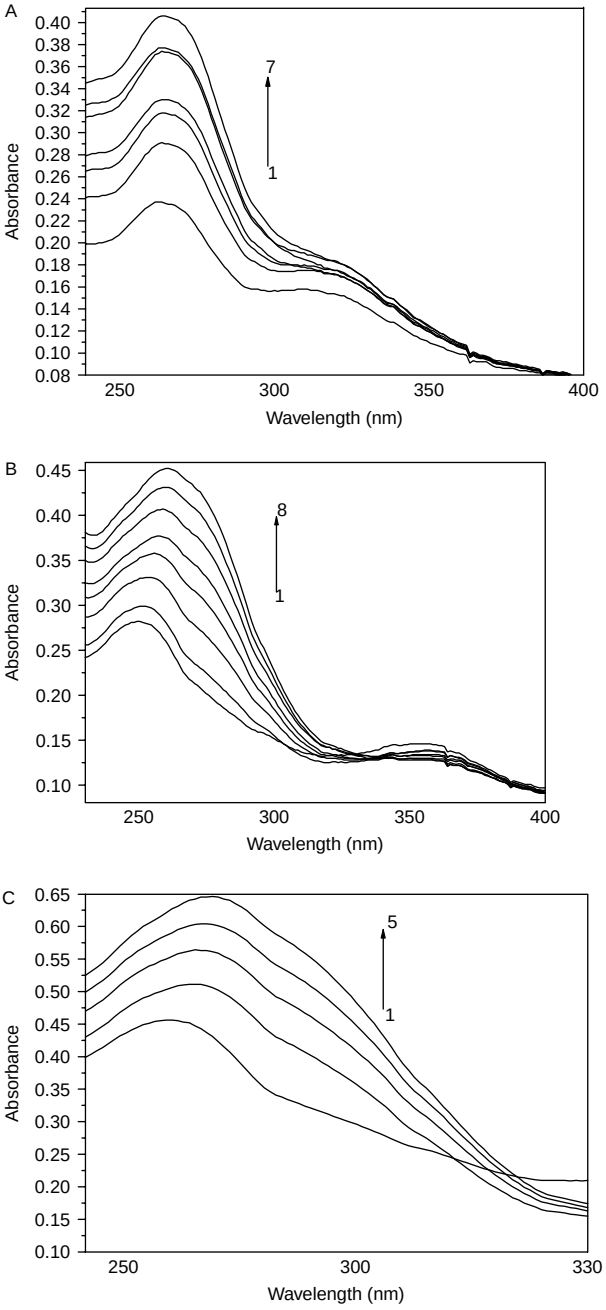

Figure 5. Absorption of 1.0×10^{-5} mol/l Ba at different pH values in HP-β-CD. The pH is (A) 3.1; (B) 6.5 and (C) 8.9.

The apparent stability constant (*K*) of the complexes was calculated according to the following equation:

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

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

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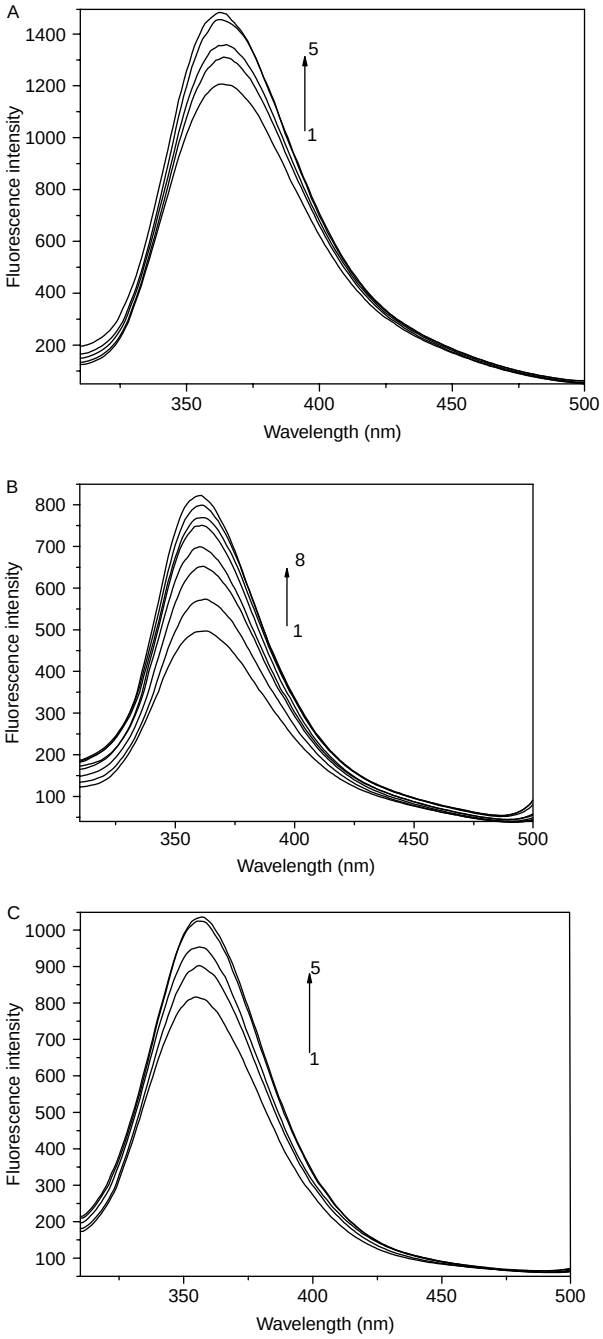

Figure 6. Fluorescence emission spectra of 1.0×10^{-5} mol/l Ba at different pH values in β-CD. The pH is (A) 3.1, (B) 6.5 and (C) 8.9.

Table 2. The binding constants (*K*s) at different pH value solutions.

		PH		
CD	*K*s	3.1	6.5	8.9
β-CD	50	312	132	344
HP-β-CD	1150	1150	900	–
Me-β-CD	460	300	100	200

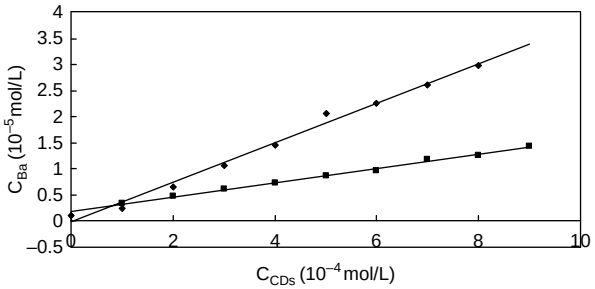

Figure 7. Phase-solubility diagram of Ba and CDs. (■) β-CD and (◆) HP-β-CD.

Table 3. Apparent stability constants (*K*s) of Ba inclusion.

CDs complex	Linear equation	*K*s (M^{-1})	r^2
β-CD	$y = 0.1362x + 0.1941$	500.29	0.9962
HP-β-CD	$y = 0.3784x - 0.0183$	5247.86	0.9914
Me-β-CD	$y = 0.2331x + 0.2291$	613.79	0.9914

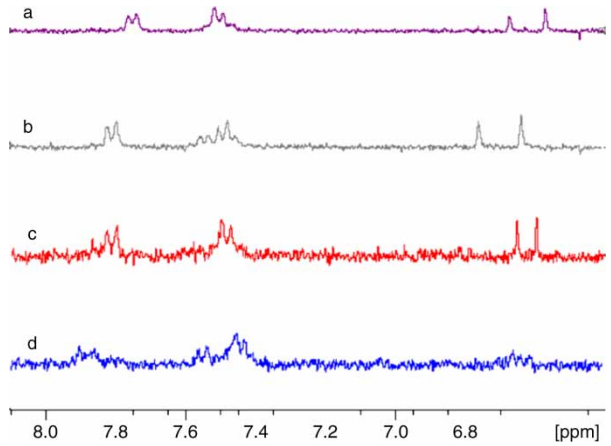

Figure 8. ^{1}H NMR spectra of Ba (d), and Ba complexed with Me-β-CD (a), HP-β-CD (b), and β-CD (c).

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, and the scavenging study was performed in a mixture of ethanol–water (20:80).

The reaction was started by the addition of 1 ml of Ba (1.0×10^{-5} M), Ba/β-CD and Ba/HP-β-CD complex samples, which correspond to the 3 mM CD concentration from the phase-solubility studies. The bleaching of DPPH· was followed at 520 nm.

Table 4. ^{1}H NMR chemical shifts corresponding to Ba in the absence and presence of CDs in D_2O.

	δ_{free} (ppm)	$\delta_{complexed}$ (ppm)			$\triangle\delta$ ($\delta_{complexed} - \delta_{free}$) (ppm)		
	Ba	β-CD	H-β-CD	Me-β-CD	β-CD	H-β-CD	Me-β-CD
8-H	6.613	6.597	6.641	6.572	0.034	−0.028	0.041
3-H	6.671	6.654	6.763	6.675	0.017	−0.092	−0.004
3′4′5′-H	7.454	7.501	7.481	7.517	−0.047	−0.027	−0.063
2′6′-H	7.905	7.828	7.823	7.766	0.077	0.082	0.139

Table 5. ^{1}H NMR chemical shifts CDs and the inclusion complexes in D_2O.

	δ_{free} (ppm)			$\delta_{complexed}$ (ppm)/Ba			$\triangle\delta$ ($\delta_{complexed} - \delta_{free}$) (ppm)		
	β-CD	H-β-CD	Me-β-CD	β-CD	H-β-CD	Me-β-CD	β-CD	H-β-CD	Me-β-CD
3-H	3.817	3.858	3.702	3.814	3.857	3.695	0.003	0.001	0.007
5-H	3.727	3.720	3.502	3.727	3.707	3.490	0.000	0.013	0.012
6-H	3.727	3.607	3.502	3.696	3.586	3.472	0.031	0.021	0.030

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The decrease in absorbance at 520 nm was measured against a blank of ethanol–water (20:80) with 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 (*20*):

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

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

2.6 Preparation of solid complexes of Ba with CDs

Accurately, weighed 0.0567 g β-CD or 0.069 g HP-β-CD was placed into a 50 ml conical flask and 10 ml of distilled water was added, stirred, then 0.0135 g Ba was added into a 50 ml beaker and 10 ml of distilled water was added and electromagnetically stirred until it was dissolved. Then CDs solution was poured slowly into stirred Ba solution, which was continuously stirred for 12 h at room temperature. The reaction mixture was put into refrigerator for 24 h. Precipitate was filtrated by G4 sand filtering funnel and washed with distilled water. After drying in an oven at 60°C, white powdered products were obtained. This is inclusion complex of Ba with CDs.

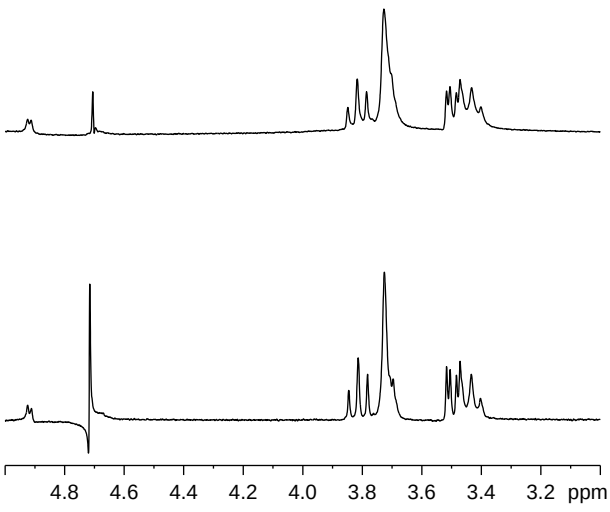

Figure 9. ^{1}H NMR spectra of β-CD and Ba/β-CD inclusion complex from the bottom to the top.

3. Results and discussion

3.1 UV spectroscopy

The putative formation of complex of Ba with β-CD in aqueous solution was characterised by UV spectroscopy. Figure 2 shows the absorption spectra of Ba in the absence and presence of β-CD. The absorption peaks of Ba itself were 272 and 319 nm. With an increasing concentration of β-CD from 1 to 6 mmol/l, an increasing absorption that depended on the concentration of β-CD was observed. Simultaneously, with an increasing concentration of β-CD, weak blue shift appeared in the absorption wavelength of 272 nm and a line was formed in the absorption wavelength of 319 nm. These changes might be partly attributed to the shielding of chromophore groups in Ba molecule due to the complex formation between Ba and β-CD through hydrophobic interaction. The nuclear energy is counteracted partially because of the repellency between the electrons. So, these changes might be partly attributed to the shielding of chromophore groups in Ba molecule due to the complex formation between Ba and β-CD through hydrophobic interaction. Similar phenomena were observed for the other two CDs. The absorption peaks of the inclusion of Me-β-CD were 260 and 359 nm, whereas the absorption peaks of the inclusion of HP-β-CD were the same as that of β-CD. All of these suggested that the inclusion complexes were formed between Ba and CDs.

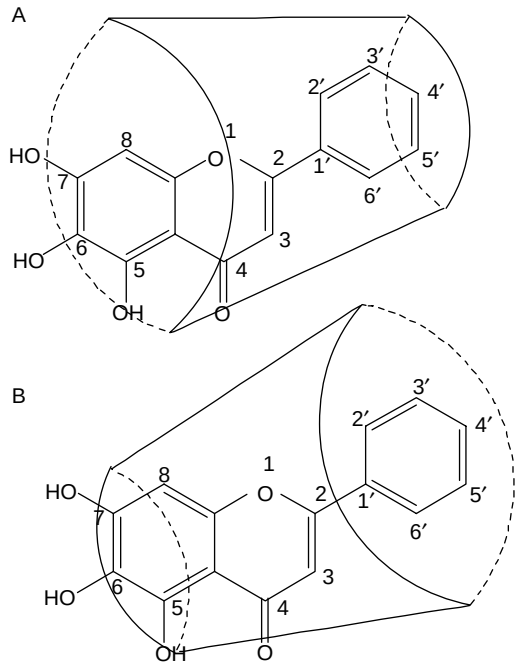

Figure 10. The structure of inclusion complexes between Ba and CDs. (A) Ba/β-CD and (B) Ba/HP-β-CD.

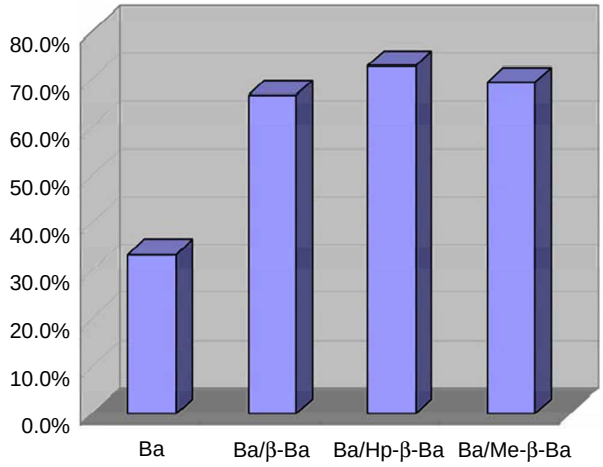

Figure 11. Consumption percentage of DPPH· in the presence of free and complex Ba forms.

3.2 Fluorescence study

Figure 3 shows the fluorescence spectra of Ba in the absence and presence of CDs (including β-CD, HP-β-CD and Me-β-CD). The maximum excitation and emission wavelengths were 270 and 363 nm, respectively. Addition of different CDs (β-CD, HP-β-CD and Me-β-CD) caused significant enhancement of Ba fluorescence intensity. From Figure 3, it can be seen that fluorescence intensity was enhanced with an increasing concentration of CDs. These data suggested that stable complexes were formed between CDs and Ba. The CD cavity provided an apolar environment for Ba molecule, which increases the quantum yield of the fluorescence of Ba.

The formation constant (K) and the ratio of the complex were calculated from these data by using the modified Benesi–Hildebrand equation (*21*)

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

where F and F_0 represent the fluorescence intensity of Ba in the presence and absence of CDs, respectively, K is a forming constant and α is a constant. Figure 4 shows the double reciprocal plots of $1/(F - F_0)$ versus 1/[CD]. They exhibit good linearity. These implied that the inclusion complexes have a stoichiometry of 1:1. The values of K are shown in Table 1. As such, the inclusion ability was HP-β-CD > Me-β-CD > β-CD.

3.3 Influence of pH

Figure 5 shows the effect of pH on the absorption spectra of Ba in the presence of HP-β-CD. At pH 3.1, the absorption peaks were kept at constant and compared with that without any PBS, while at pH 6.5 and pH 8.9, the absorption peaks of 272 and 319 nm formed different shifts, respectively. At the same time, adding CDs to Ba solution resulted in noticeable increase in absorption signals.

Figure 6 shows the effect of pH on the fluorescence spectra on Ba in the presence of β-CD. The excitation wavelengths were 270 nm. At pH 3.1 and pH 6.5, the emission wavelengths were the same as that without any PBS, whereas at pH 8.9, the emission wavelength shifted to lower wavelength at 355 nm. Simultaneously, the increasing concentration of CDs resulted in the enhancement of fluorescence signals. The ability of inclusion in different pH values is shown in Table 2.

3.4 Phase-solubility measurements

In Figure 7, it can be seen that CDs enhanced the poor aqueous solubility of Ba, thus proving a certain degree of its inclusion complexation in aqueous solutions. The results observed showed a linear behaviour for β-CD ($r^2 = 0.9962$) and HP-β-CD ($r^2 = 0.9914$), and consistent with 1:1 molecular complex formation for CDs and Ba. The binding constant (K) of the complexes is shown in Table 3. As shown in Table 3, the binding constant and solubility of Ba determined with CDs followed the rank order HP-β-CD > β-CD. The results were the same as that of fluorescence results.

3.5 NMR measurements

To ascertain the structure of the inclusion complexes between Ba and CDs, ^{1}H NMR spectroscopy studies of free drug and inclusion complexes were therefore undertaken.

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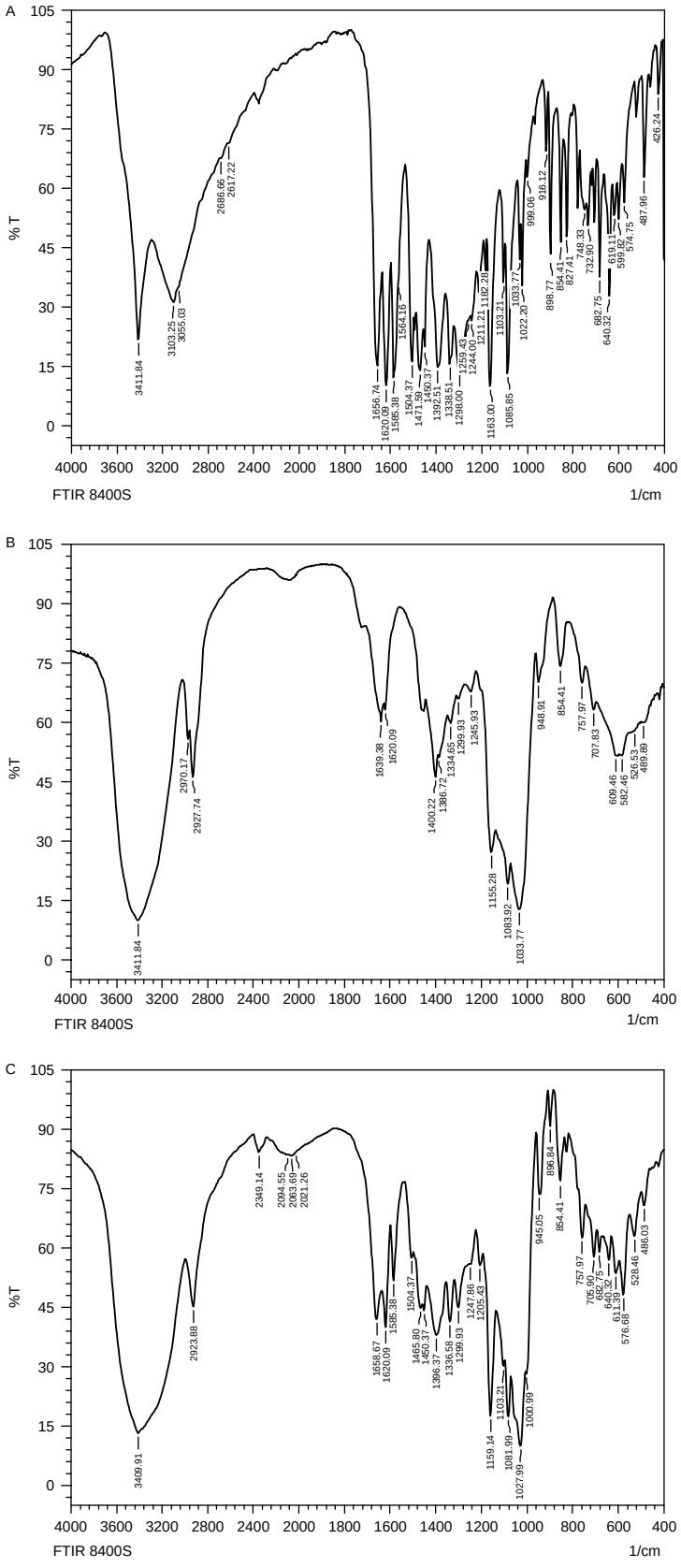

Figure 12. IR spectra of (a) Ba, (b) HP-β-CD and (c) Ba/HP-β-CD inclusion complex.

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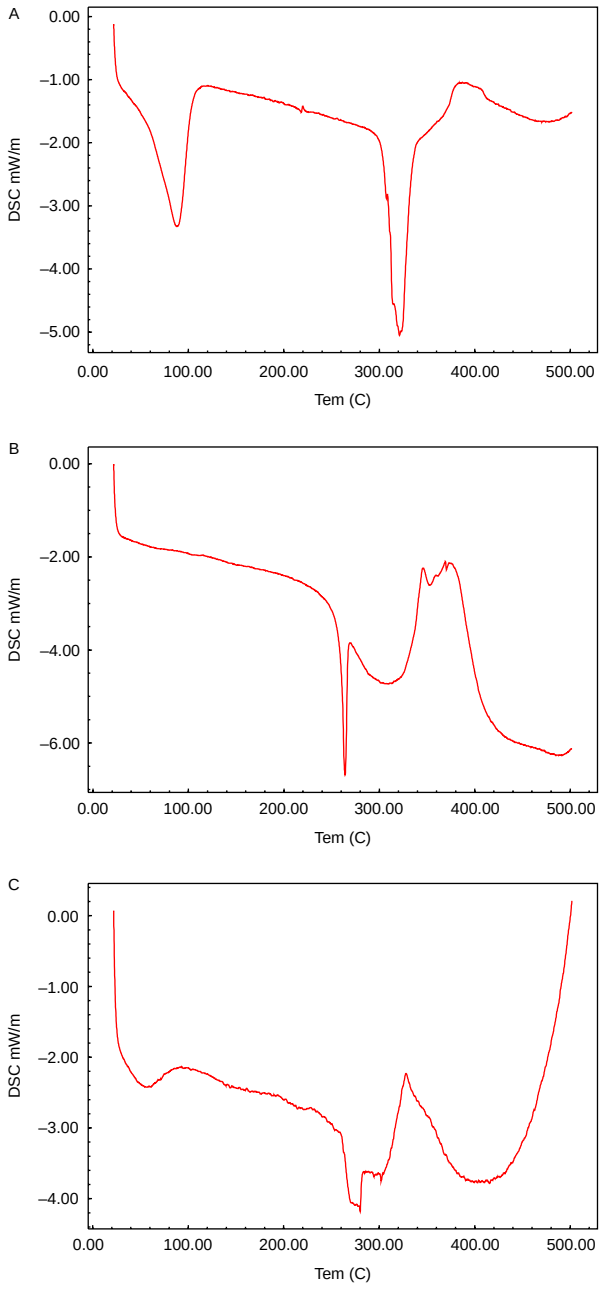

Figure 13. Thermal spectra of (a) β-CD, (b) Ba and (c) Ba/β-CD inclusion complex.

Figures 8 and 9 illustrate the change of hydrogen atom of Ba and CDs before and after forming the inclusion complexes. The difference in hydrogen chemical shift values between Ba in the free and in the complexed state is presented in Table 4. Table 5 shows 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 Ba exhibited larger chemical shifts, namely the A, B and C rings of Ba were all entered into the cavity of β-CD and HP-β-CD. The H-3 of β-CD had larger chemical shift than H-5, which illustrated that the molecule of Ba entered into the cavity of β-CD from the large port; whereas the H-5 of HP-β-CD had larger chemical shift than H-3, which illustrated that the molecule of Ba entered into the cavity of HP-β-CD from the vintage port.

From the above discussion, the mechanism of inclusion complexes between Ba and CDs is shown in Figure 10.

3.6 Scavenging study of DPPH· by free or complexed Ba

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

Furthermore, since the mechanism of DPPH· reduction is known, the amount remaining from both the 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·. Figure 11 shows the consumption of DPPH·, which indicates that the complexed Ba/CDs were more effective than the free Ba, with the HP-β-CD complex (72.22) > Me-β-CD complex(69.24) > β-CD complex (66.67) > free Ba (33.33). The scavenging ability was measured as a relative scavenging in the presence of free or complex Ba. Figure 11 shows the scavenging ability related to the enhanced solubility of Ba. Also, these results indicated that the complexes formed maintained the Ba antioxidant activity.

The antioxidant activity of phenolic compounds depends on the position and degree of hydroxylation, as well as on the nature of radicals of the ring structure. Antioxidative activity is intensified by the presence of a second hydroxy group through the formation of an intramolecular hydrogen bond (*22*). It might be that the —OH positions of Ba molecules are close enough to secondary —OH groups of CDs to form hydrogen bonds and contribute to antioxidant activity (*23*). Therefore, the formation of an 'intramolecular' hydrogen bond of the inclusion complex is possible, and consequently an increase in antioxidant capacity is expected.

3.7 IR spectra studies

Compared IR spectra of Ba, HP-β-CD and complex of HP-β-CD with Ba, as in Figure 12, showed that the absorption intensity of C═O group and phenyl ring gave rise to changes. The absorption intensity of C═O and phenyl ring in inclusion complex were weaker than in Ba and have a right shift; therefore, it can be deduced that C═O and phenyl ring in Ba were included into the cavity of HP-β-CD. And the same phenomenon was observed for β-CD (the figure has not been given).

3.8 DSC studies

The DSC curves of Ba, HP-β-CD and inclusion complex are shown in Figure 13. From Figure 13, it can be seen that

DSC curves of inclusion complex with the DSC curves of Ba and HP-β-CD were different. This proved that the new inclusion complex was formed. And the same phenomenon was observed for β-CD (the figure has not been given).

4. Conclusion

This study has demonstrated the inclusion complex interaction between Ba with β-CD and its derivatives. The major factors of affecting guest/host binding are size matching between CD and guest, and the hydrophobicity of the guest molecule. Among the CDs examined, HP-β-CD was the most suitable for the inclusion of Ba, which suggested that HP-β-CD cavity supplies a more hydrophobic environment. And the activities of eliminating free radical DPPH· were HP-β-CD inclusion complex > β-CD inclusion complex > free Ba. In addition, the fluorescence spectroscopy and phase-solubility measurement data have shown the formation of a stable 1:1 stoichiometric complex of Ba with CDs (β-CD, HP-β-CD and Me-β-CD). CDs can be used as a guest complex agent, which act as a substrate reservoir in a dosage-controlled manner and can increase the solubility, stability and antioxidant activity of guest molecule. Moreover, this study demonstrated that CDs served as drug carrier systems.

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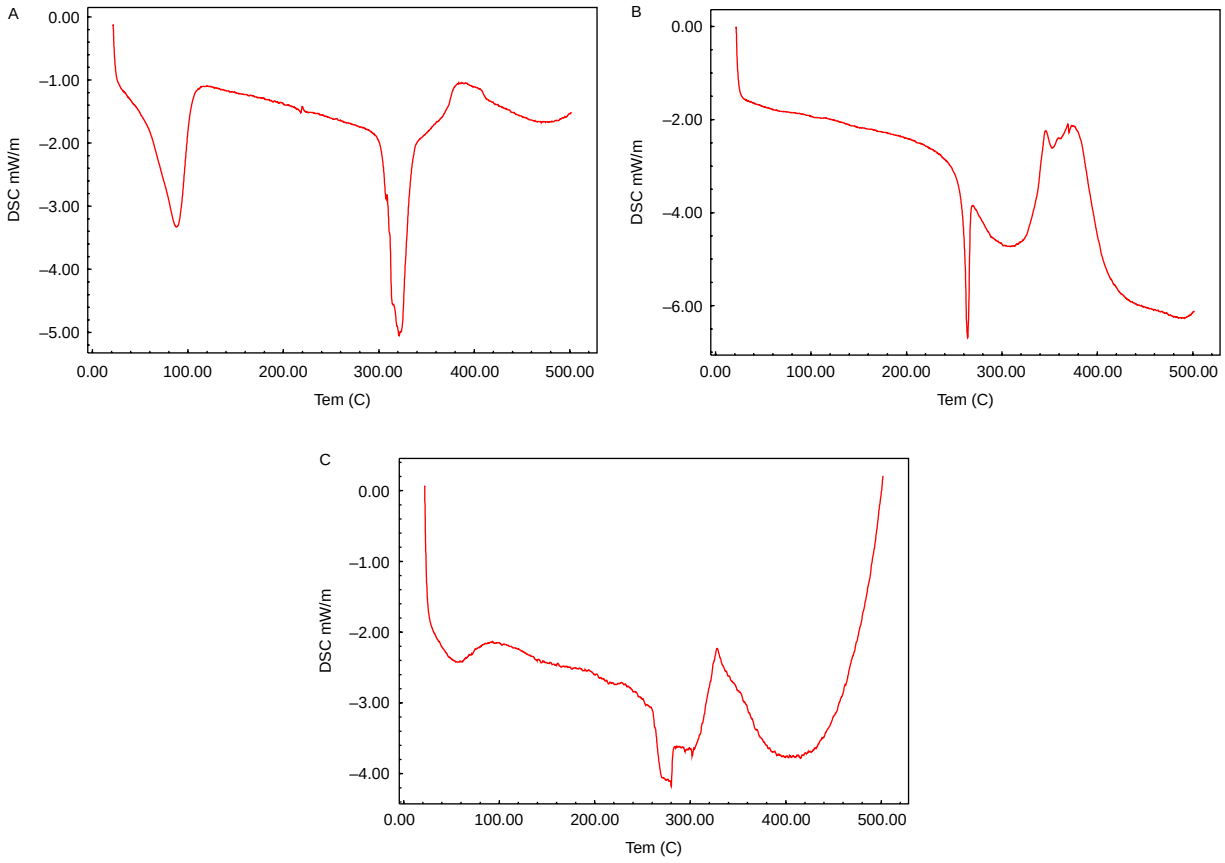

The DSC curves of Ba, HP-β-CD and inclusion complex are shown in Figure 13. From Figure 13, it can be seen that DSC curves of inclusion complex with the DSC curves of Ba and HP-β-CD were different. This proved that the new inclusion complex was formed. And the same phenomenon was observed for β-CD (the figure has not been given).

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