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International Journal of Pharmaceutics 312 (2006) 137–143

INTERNATIONAL JOURNAL OF PHARMACEUTICS

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Preparation, characterization and in vivo evaluation of formulation of baicalein with hydroxypropyl-β-cyclodextrin

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Received 31 August 2005; received in revised form 5 January 2006; accepted 9 January 2006
Available online 3 February 2006

Abstract

The interaction of 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) and a poorly water-soluble flavonoid, baicalein (Ba), chemically 5,6,7-trihydroxy flavone in solution and solid-state was studied. Ba/HP-β-CD solid systems were prepared by freeze-drying method. The formation of Ba/HP-β-CD complex in aqueous solution was demonstrated by UV spectroscopy, while Ba/HP-β-CD co-lyophilized product was characterized by differential scanning calorimetry (DSC) and X-ray diffractometry (XRD). Through complexation with HP-β-CD, the solubility of Ba in neutral aqueous solution was improved significantly. The phase-solubility profile was A_P-type, indicating the formation of higher-order complexes or complex aggregates. Ba/HP-β-CD solid powders were amorphous and show a significantly improved dissolution rate in comparison with free Ba. Comparison of the pharmacokinetics between Ba/HP-β-CD co-lyophilized product and free Ba was also performed in rats. The concentration of Ba and its mainly conjugated metabolite, 7-*O*-glucuronide of baicalein (BG) in rat plasma was determined by HPLC method. The in vivo results show that Ba/HP-β-CD co-lyophilized product exhibits the similar pharmacokinetics as that of free Ba after intravenous administration. Ba/HP-β-CD co-lyophilized product displays earlier $t_{\max}$ and higher $C_{\max}$ of BG than free Ba after oral dosing. By comparing the $AUC_{0-\infty}$ of BG between oral dosing, the relative bioavailability of Ba/HP-β-CD co-lyophilized product to free Ba was 165.0%, which highlighted the evidence of significantly improved bioavailability of formulation of Ba with HP-β-CD.

Keywords: Baicalein; 2-Hydroxypropyl-β-cyclodextrin; Solubilization; Co-lyophilized product; Pharmacokinetics

1. Introduction

Baicalein (Ba), a flavonoid present in the root of Scutellaria baicalensis Georgi, has attracted considerable attention because it has a variety of interesting activities such as antibacterial (Kubo et al., 1981), anti-HIV activity (Wu et al., 2001), attenuating oxidative stress (Shao et al., 1999; Shao et al., 2002; Gao et al., 2001), inhibiting the growth of several types of cells (Qain et al., 1990; Huang et al., 1994; Inoue and Jackson, 1999) and inducing cell death in human hepatocellular carcinoma cell (Matsuzaki et al., 1996) and in human promyelocytic leukemia HL-60 cells (Li et al., 2004). However, one disadvantage of this compound is its low water solubility. In spite of the wide spectrum of pharmacological properties, its use in pharmaceutical field is limited.

In recent years, cyclodextrin complexation has been successfully used to improve the solubility, chemical stability and bioavailability of a number of poorly soluble compounds. Cyclodextrins are cyclic non-reducing, non-hygroscopic, water-soluble oligopolysaccharides, which present a hydrophobic cavity with the appropriate size to accommodate another molecule forming an inclusion compound through host–guest interactions. Formation of the inclusion complex can increase the guest's stability against hydrolyses, oxidation, photodecomposition and dehydration. Upon inclusion, the water solubility of the guest can increase as well as its bioavailability (Szejtli, 1998; Uekama et al., 1998; Cortes et al., 2001). However, the application of cyclodextrin in the pharmaceutical field is limited by its rather low aqueous solubility, which led to a search for more soluble derivatives of cyclodextrins. Recently, various hydrophilic, hydrophobic and ionic cyclodextrin derivatives have been successfully utilized to extend the physicochemical properties and inclusion capacity of natural cyclodextrin (Hirayama and Uekama, 1999; Ono et al., 2001).

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doi:10.1016/j.ijpharm.2006.01.011

2-Hydroxypropyl-β-cyclodextrin (HP-β-CD) is a water-soluble derivative of β-cyclodextrin, which has been widely studied as a complexing agent for many pharmaceuticals. Complexation of drugs with HP-β-CD has been shown to increase their solubility and stability in aqueous solutions (Brewster et al., 1991; Szejtli, 1991; Bekers et al., 1991).

In the present study, with an aim to increase the aqueous solubility, dissolution properties and bioavailability of Ba, the complex of Ba with HP-β-CD was prepared by freeze-drying method. Solubility studies were performed by phase solubility analysis. UV spectroscopy was measured to evaluate the formation of Ba/HP-β-CD complex in aqueous solution. On the other hand, information about Ba/HP-β-CD co-lyophilized product was obtained by differential scanning calorimetry (DSC) and powder X-ray diffractometry (XRD). A dissolution study was performed to evaluate the improved solubility and dissolution properties of Ba/HP-β-CD co-lyophilized product in comparison with pure drug. In order to evaluate the improved bioavailability of the lyophilized product, the pharmacokinetics of free Ba and Ba/HP-β-CD co-lyophilized product in rats post-intravenous or oral administration had been characterized and compared. The plasma concentration of Ba and Baicalin (BG), one of the mainly conjugated metabolites of Ba that has the homologous pharmacological effects with Ba, was simultaneously assayed by a modified HPLC method.

2. Materials and methods

2.1. Materials

Ba and BG were kindly provided by Professor Che (Peking University). 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD) was purchased from Taixin Yiming Fine Chemicals Co., Ltd. (Jiangsu, China). Propyl paraben used as an internal standard and PEG 400 were purchased from Wulian Chemicals (Shanghai, China). Methylcellulose was purchased from Shangyu Haishen Chemical Co., Ltd. (Zhejiang, China). Methanol for HPLC analysis was obtained from Merck (Darmstadt, Germany). All other reagents were commercially available and used as received.

2.2. HPLC method

The Angilent 1100 HPLC system was used in the drug analysis. The reversed-phase HPLC column (Diamonsil ODS, 4.6 mm × 250 mm, 5 μm particle size) equipped with a guard column (C_{18}, 4.6 mm × 10 mm) was employed in the study.

2.2.1. In vitro Ba quantification

The mobile phase consisted of methanol and 0.05% (v/v) phosphoric acid solution in a ratio of 55:45 (v/v), and the column temperature was 40 °C. The detection wavelength for Ba was set at 280 nm and the flow rate was 1.0 ml/min.

2.2.2. Metabolic pharmacokinetics analysis

A HPLC-method originally described by Zhang et al. (2004) was used with some modifications. The mobile phase, consisting of methanol and 0.05% (v/v) phosphoric acid solution, was run using a linear gradient elution program as shown in Table 1. The detection wavelength was set at 270 nm.

Table 1
HPLC mobile phase of detection of Ba, BG and internal standard (propyl paraben)

Time (min)	Flow rate (ml/min)	Percentage of each eluent (%) (v/v)	
		Methanol	0.05% (v/v) phosphoric acid solution
0	1.0	40	60
20	1.0	60	40
25	1.0	60	40
30	1.0	40	60
35	1.0	40	60

2.3. Preparation of Ba/HP-β-CD co-lyophilized product

The solid system composed of Ba and HP-β-CD was prepared by lyophilization technique. Briefly, 400 mg Ba was dispersed into 30 ml HP-β-CD aqueous solution (200 mM), and the obtained suspension was stirred magnetically at 80 °C for 2 h. After the suspension was filtered through a 0.22 μm PTFE filter, the filtrate was lyophilized.

2.4. Characterization of Ba/HP-β-CD complex

2.4.1. UV spectroscopy measurement

Complex formation composed of Ba and HP-β-CD in water was studied using the spectral shift method. The concentration of Ba was 4.5 μg/ml while the HP-β-CD concentration varied from 1 to 10 mmol/L. The mixtures were stirred for 6 h and recording the UV absorption spectra with a TU-1800PC spectrophotometer (Beijing Purkinje General Instrument Co., Ltd.) was conducted. As a control, the absorbance of free Ba in ethanol was also determined.

2.4.2. Differential scanning calorimetry (DSC)

DSC curves of the Ba raw material, HP-β-CD, Ba/HP-β-CD co-lyophilized product as well as the physical mixture, which was obtained by pulverizing in a glass mortar and carefully mixing in the same weight ratio as the lyophilized complex, were measured with a DSC instrument (Model DSC Q100, New Castle, USA). Samples were accurately weighed and heated in closed aluminium crimped cells at a rate of 10 °C min^{-1} between 50 and 340 °C temperature range under a nitrogen flow of 40 ml min^{-1}. Duplicate determination was carried out for each sample.

2.4.3. X-ray powder diffractometry (XRD)

The powder samples were packed in the X-ray holder from the top prior to analysis. X-ray powder diffraction patterns were recorded on a Rigaku-D/MAX-2550PC diffractometer using Ni-filtered, Cu Kα radiation, a voltage of 40 kV and a 300 mA current. The scanning rate employed was 0.02° s^{-1} over a 2θ range of 5–70°.

2.5. Phase solubility study

Solubility measurements were based on the phase solubility technique established by Higuchi and Connors (Higuchi and Connors, 1965). With a standard procedure, excess amount of solid Ba was added to a series of 50 ml stoppered conical flasks that contained increasing amounts of HP-β-CD (0–337.2 mM). These obtained suspensions were shaken at 25 ± 0.5 °C. After 48 h, aliquots were withdrawn, filtered (0.22 μm pore size) and assayed by HPLC for drug content. Each experiment was carried out in triplicate (R.S.D. < 3%). Phase-solubility profile was obtained by plotting the solubility of Ba versus the concentration of HP-β-CD.

2.6. Dissolution studies

Dissolution profiles of free Ba and Ba/HP-β-CD co-lyophilized product were evaluated according to the CP2000 method (Appendix C, No.1 method). Briefly, 10 mg of free Ba or equivalent amount of Ba/HP-β-CD co-lyophilized product in capsules was added to 0.9 L hydrochloric acid (0.1 M) at 37.0 °C in a ZRS8G Dissolution Apparatus (Precision Instruments, Tianjing Universituy). At predetermined times, suitable aliquots were withdrawn, filtered (0.8 μm pore size) and HPLC analyzed for Ba content.

2.7. Pharmacokinetics in rat plasma

Female Sprague–Dawley rats (200 ± 10 g) were fasted for 12 h before drug administration. Water was freely available. For intravenous administration, Ba/HP-β-CD co-lyophilized product was redissolved in water and the free Ba was dissolved in PEG400 aqueous solution (50%). The intravenous bolus was given to rats ($n = 5$) via the tail vein at a dose of 10 mg/kg. Blood samples were collected and centrifuged at 3, 5, 10, 15, 20, 30, 60, 120, 240, 360, 480, 720 and 1440 min post-dosing. For the assay of Ba and BG, in 100 μl plasma sample, 5 μl ascorbic acid solution (200 mg/ml) and 300 μl internal standard (propyl paraben, 1 μg/ml) in methanol were added. After centrifugation, the supernatant was withdrawn and solvent was evaporated under N_2 gas. The dryness was dissolved with 100 μl mobile phase then 20 μl was subjected to HPLC analysis. For oral administration, Ba/HP-β-CD co-lyophilized product was redissolved in water and free Ba was suspended in 1% methyl cellulose solution. Ba or Ba/HP-β-CD administration was carried out via gastric gavage at a dose of 75 mg/kg. Blood samples were collected at 5, 10, 30, 60, 90, 120, 180, 240, 300, 360, 480, 720, 1440 and 2880 min post-dosing. The procedure of blood samples' treatment was the same as that described above. The pharmacokinetic parameters were calculated by using DAS (Drug and Statistics) VER 2.0 software (http://www.drugchina.net).

3. Results and discussion

3.1. UV spectroscopy

The putative formation of complex of Ba with HP-β-CD in aqueous solution was characterized by UV spectroscopy. Fig. 1 shows the effect of HP-β-CD concentration on the spectra of Ba in aqueous solution. The absorption spectrum of Ba varied significantly with the addition of HP-β-CD. Ba alone in ethanol exhibits two absorption peaks at 276 and 323 nm. The increase in HP-β-CD concentration from 1 to 10 mmol/L resulted in a decrease in the absorbance of Ba. These changes might be partly attributed to the shielding of chromophore groups in Ba molecule due to the complex formation between Ba and HP-β-CD through hydrophobic interaction. Simultaneously, as the HP-β-CD increased a red shift of absorption peak at 323 nm and a blue shift of peak at 276 nm was observed with the broadening and deformation of the two peaks, which might also suggest the possibility of an interaction between B and HP-β-CD as a result of a partial shielding of the chromophore electrons in the HP-β-CD cavity (Chow and Karara, 1986).

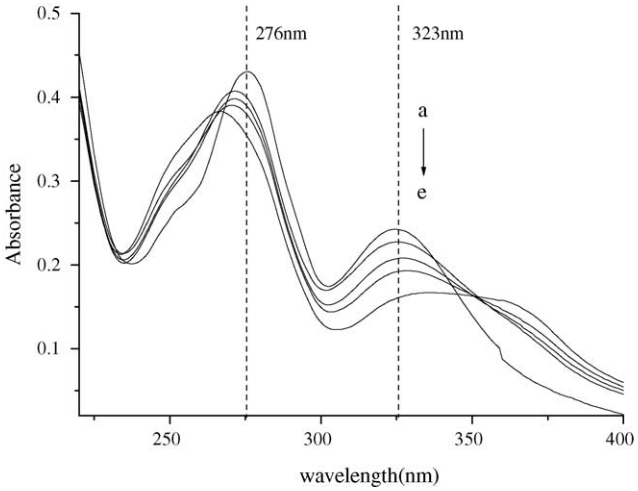

Fig. 1. The effect of HP-β-CD concentration on the UV absorbance of Ba in aqueous solution; the HP-β-CD concentration was (a) 0 mmol/L, (b) 1 mmol/L, (c) 2 mmol/L, (d) 6 mmol/L and (e) 10 mmol/L, respectively.

3.2. Differential scanning calorimetry (DSC)

The thermal curves of pure components and different drug–cyclodextrin systems are shown in Fig. 2. The DSC results presented in Fig. 2(a) demonstrate an endothermic peak for Ba at 270.08 °C, which corresponds to the melting point of Ba. In Fig. 2(b), (c) and (d), DSC measurements show one broad endothermic peak near 80 °C corresponding to the release of water of HP-β-CD and a small endothermic peaks at 210 °C corresponding to the transconformation of the molecule and the irregular peaks above 300 °C corresponding to the decomposition of HP-β-CD (Kohata et al., 1993). In the DSC curve of the Ba/HP-β-CD co-lyophilized powder, the fusion peak of Ba is not observable, indicating the interaction of Ba and the CD cavity, which in turn leads to an almost complete loss of crystallinity of this binary system. Moreover, the thermal curve of the physical mixtures shows approximately the same thermal behavior. Similar result was reported previously (Beraldo et al., 2002). The disappearance of the drug-melting peak of physical mixture might be due to the drug amorphization during the DSC run in the presence of amorphous carrier. Moreover, the vicinity

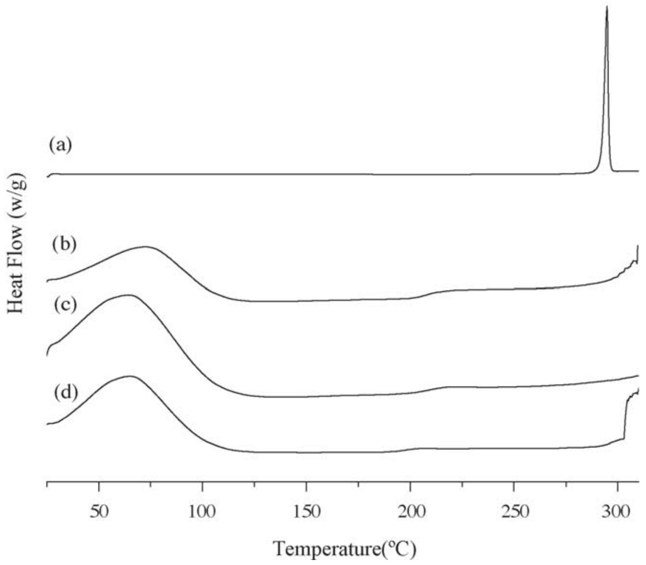

Fig. 2. DSC curves of (a) Ba, (b) physical mixture of Ba and HP-β-CD, (c) HP-β-CD and (d) Ba/HP-β-CD co-lyophilized product.

of melting peak of Ba and the decomposition temperature of HP-β-CD might be also responsible for this result.

3.3. X-ray powder diffractometry (XRD)

Fig. 3 shows the X-ray diffraction patterns of Ba, HP-β-CD, physical mixture and Ba/HP-β-CD co-lyophilized product. The Ba XRD powder diffraction pattern shown in Fig. 3(a) displayed sharp peaks, which is the characteristic of a crystalline compound. In contrast, HP-β-CD shown in Fig. 3(c) was amorphous lacking crystalline peaks. Some drug crystallinity peaks were still detectable in the physical mixture shown in Fig. 3(b), the diffractogram consisted mostly of the HP-β-CD character, but some of the Ba characteristics remained. Compared to the diffraction patterns of pure Ba and HP-β-CD, the diffractogram of the co-lyophilized product shown in Fig. 3(d) was superimposable with that of the amorphous HP-β-CD shown in Fig. 3(c). These results, confirm that Ba is no longer present as a crystalline material and its HP-β-CD solid complexes exhibit complete amorphization.

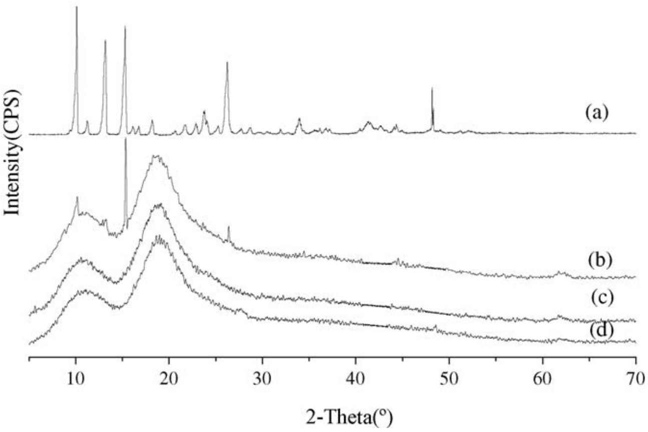

Fig. 3. XRD patterns of (a) Ba, (b) physical mixture of Ba and HP-β-CD, (c) HP-β-CD and (d) Ba/HP-β-CD co-lyophilized product.

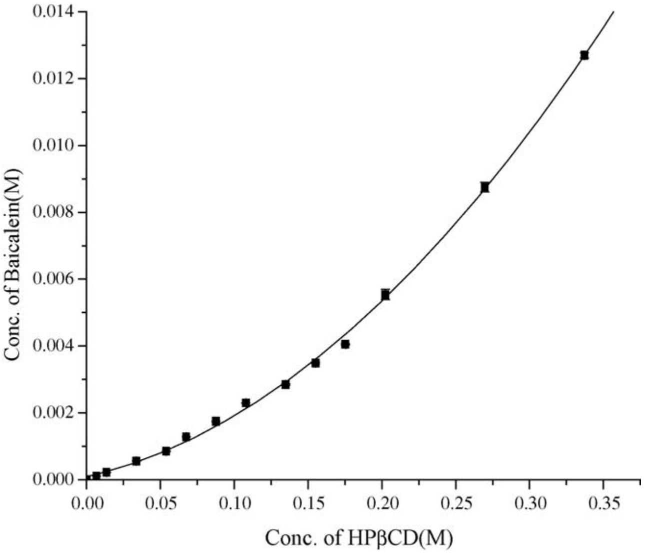

Fig. 4. Higuchi phase solubility diagram of Ba and HP-β-CD, the HP-β-CD concentration was in the range of 0–337.16 mM.

3.4. Phase solubility studies

Inclusion stoichiometry, i.e. the molar ratio of Ba to HP-β-CD, on the other hand, was determined by phase solubility study. The phase-solubility diagram of Ba in aqueous HP-β-CD solution is shown in Fig. 4. It could be observed that the solubility of Ba increased as the concentration of HP-β-CD increased. The phase solubility diagram of Ba with HP-β-CD resulted in an A_P-type Higuchi phase solubility diagram ($r = 0.999$) similar to that reported by Higuchi and Connors (1965). The curve showed a positive deviation from the straight line of the A_L-type where complexes formed were of the first order with regards to the host molecule. Therefore, the complexes formed, in the A_P-type Higuchi phase solubility diagram were presented to a higher order with respect to cyclodextrin. Additionally, this may indicate the formation of 1:1 and 1:2 stoichiometric ratios of Ba/HP-β-CD complexes (Higuchi and Connors, 1965). At the higher concentrations of HP-β-CD, complexation between more than one HP-β-CD molecule and one guest molecule was likely to have occurred. Additionally, A_P-type phase-solubility diagram also indicates the formation of complex aggregates that could solubilize additional amount of the guest molecules through non-inclusion complexation or formation of micelle-like structures (Loftsson et al., 2002, 2004).

3.5. Dissolution studies

The dissolution profiles of Ba and Ba/HP-β-CD co-lyophilized product are illustrated in Fig. 5. At each time point, Ba amount dissolved from co-lyophilized powder was significantly higher than that of free Ba. The amount dissolved from free Ba was less than 2 wt.% after 2 h, while the Ba amount dissolved from co-lypholized powder was more than 90 wt.% in

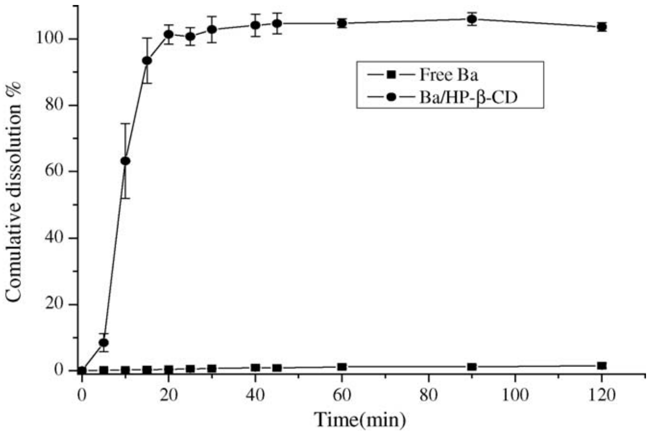

Fig. 5. Dissolution profiles of free Ba and Ba/HP-β-CD co-lyophilized product in 0.1 M hydrochloric acid at 37.0 °C.

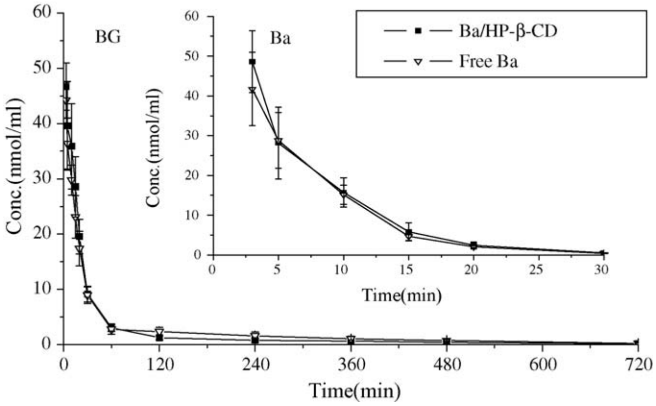

Fig. 7. Mean (± S.D.) plasma concentration–time profiles of Ba and its conjugate BG after intravenous administration of Ba/HP-β-CD co-lyophilized product or free Ba to rats at a dose of 10 mg/kg, respectively, $n = 5$.

20 min. This fast dissolution of the binary system of Ba/HP-β-CD was ascribed to the fact that cyclodextrins have the capability of improving the wettability of powder materials and forming a rapidly soluble complex in solution.

3.6. Pharmacokinetics in rat plasma

Fig. 6 shows the typical chromatograms of Ba in plasma. A good linear relationship was obtained for both Ba and BG with concentration ranging from 0.05 to 25 μg/ml (Ba: $y = 0.40952x - 0.00657$, $r = 0.9998$; BG: $y = 0.19597x - 0.00753$, $r = 0.9997$). The R.S.D. of both Ba and BG for inter-day and intra-day precision and accuracy at low, medium and high concentration (0.1, 1 and 10 μg/ml) was blow 11.6%. The recoveries of Ba and BG at the up three concentrations were 88.3%, 86.4%, 88.7% and 84.2%, 82.7%, 80.3%, respectively. The LOQ and LOD for Ba were 0.03 and 0.05 μg/ml; for BG were 0.05 and 0.03 μg/ml.

Mean plasma concentration of Ba and its conjugate BG after intravenous administration of Ba was shown in Fig. 7. The metabolic pharmacokinetics study of Ba was conducted according to the method previously reported (Lai et al., 2003). The results demonstrated that Both Ba in co-lyophilized powders and its free form were quickly metabolized to BG in vivo. After 30 min of the dosing, the concentration of Ba reduced to a very low level (near to the LOD), which was kept for about to 8 h. Compared to Ba, the elimination of conjugate BG was relative slow, which could still be detected 12 h post-dosing. Moreover, by using t-test for statistical comparison, the AUC (mg min L^{-1}) of Ba and BG of Ba/HP-β-CD complex and free Ba in Table 2 suggest no significant deviation.

After oral dosing of free Ba and Ba/HP-β-CD co-lyophilized product, the parent form of Ba in plasma was detected in a very low level (near the LOD), while the conjugates of Ba were almost exclusively circulating in the bloodstream. The mean plasma concentration–time curves of BG, the mainly conjugated metabolites of Ba, are shown in Fig. 8 and the pharmacokinetic parameters of BG are listed in Table 3. Examining the results from the group analysis, the mean $C_{\max}$ of BG was significantly higher for Ba/HP-β-CD c-olyophilized product than that of free Ba, and the $t_{\max}$ of BG of Ba/HP-β-CD co-lyophilized product was significantly earlier than that of free Ba. By comparing of the $\mathrm{AUC}_{0-\infty}$ of BG between free Ba and Ba/HP-β-CD co-lyophilized product, the relative bioavailability of free Ba to Ba/HP-β-CD co-lyophilized product was 165.0%. The oral dose of Ba given in this study was different from that of the intravenous bolus, therefore, based upon dose correction, the comparison of $\mathrm{AUC}_{0-\infty}$ of BG after oral single dose was compare with the sum $\mathrm{AUC}_{0-\infty}$ of BG and Ba after intravenous

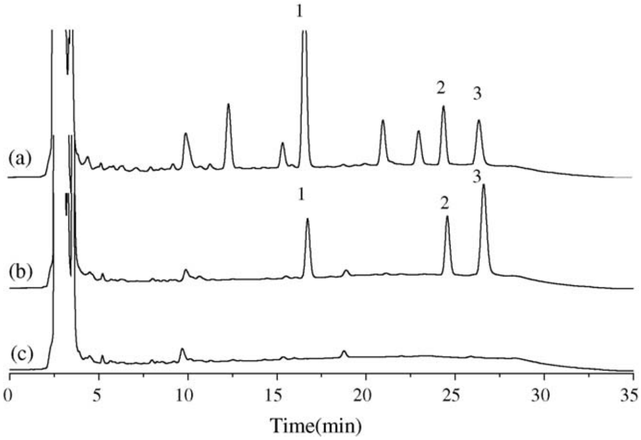

Fig. 6. Chromatograms of (a) plasma sample collected at 15 min post-dosing, (b) plasma mixed with Ba and BG and internal standard and (c) blank plasma: (1) BG; (2) internal standard (propyl paraben); (3) Ba.

Table 2
The AUC (mg min L^{-1}) of Ba and its conjugate BG after intravenous administration of Ba/HP-β-CD co-lyophilized product or free Ba to five rats in a dose of 10 mg/kg, respectively

	Ba/HP-β-CD (mean ± S.D.)	Free Ba (mean ± S.D.)	P
$\mathrm{AUC}_{0-\infty}$ of Ba (nmol min mL^{-1})	761.4 ± 424.1	611.4 ± 112.6	0.482
$\mathrm{AUC}_{0-\infty}$ of BG (nmol min mL^{-1})	1561.7 ± 228.2	1814.4 ± 320.9	0.193

Table 3
Pharmacokinetic parameters of conjugate BG after oral administration of Ba/HP-β-CD co-lyophilized product or free Ba to nine rats in a dose of 75 mg/kg, respectively

	$AUC_{0-\infty}$ (nmol min mL^{-1})	C_{max} (nmol mL^{-1})	t_{max} (min)	Absolute bioavailability (%)
Free baicalein	9516.9 ± 2191.7	16.6 ± 3.7	90.0 ± 34.6	52.3
Ba/HP-β-CD	15707.4 ± 8053.0	33.8 ± 24.1	7.5 ± 2.7	86.4

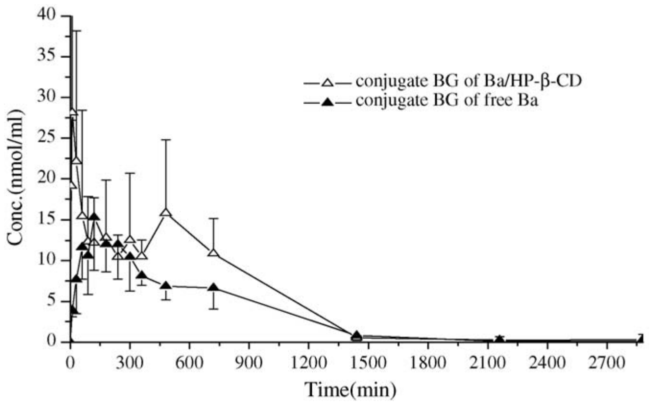

Fig. 8. Mean (± S.D.) plasma concentration–time profiles of conjugate BG after oral administration of Ba/HP-β-CD co-lyophilized product or free Ba to rats at a dose of 75 mg/kg, respectively, $n = 9$.

bolus. The absolute bioavailability of free Ba and Ba/HP-β-CD co-lyophilized product was 52.3% and 86.4%, respectively.

Moreover, after oral dosing, for both free Ba and Ba/HP-β-CD co-lyophilized product, the individual kinetic curve of BG exhibited multiple peaks. After oral dosing of Ba, extensive conjugation metabolism (i.e. BG) occurred during the first pass at gut and liver, and enterohepatic circulation of BG was reported in rats previously (Xing et al., 2005). These might be the reasons to the appearance of multiple peaks.

4. Conclusions

Through formulation with HP-β-CD, the aqueous solubility of extremely hydrophobic compound Ba was improved markedly (from 0.43 μg/ml in water to 2.37 mg/ml in 40% HP-β-CD solution) in neutral aqueous solution. Taken the results together obtained from UV spectroscopy, DSC, X-ray diffraction studies suggest the formation of complex both in aqueous solution and in the solid-state. Additionally, the freeze-dried powder of Ba/HP-β-CD complex had excellent re-dissolution ability. Our previous studies on Ba indicated that the solid Ba was more stable than it in solution. Therefore, the preparation of co-lyophilized product was a good way to improve both the aqueous solubility and the stability of Ba. Moreover, the solid system of Ba and HP-β-CD prepared by freeze-drying was completely amorphous and displayed the best dissolution performance. Comparison of metabolic pharmacokinetics of Ba and its co-lyophilized product with HP-β-CD in rats indicated that Ba/HP-β-CD co-lyophilized product had earlier t_{max}, higher C_{max} and bioavailability. In conclusion, our results demonstrated that formulation Ba with HP-β-CD significantly improved its solubility, and the resultant product could improve the dissolution properties and oral bioavailability of Ba.

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