Inclusion Complex of Cantharidin with β-Cyclodextrin: Preparation, Characterization, *In Vitro* and *In Vivo* Evaluation

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ABSTRACT: Because of low aqueous solubility and slow dissolution rate, cantharidin has a low oral bioavailability. Our research aims to prepare the inclusion complex of cantharidin and β-cyclodextrin (β-CD) and accomplish characterization, *in vitro* and *in vivo* evaluation. CA-β-CD inclusion complex was prepared by saturated solution method. The CA was demonstrated by HPLC *in vitro* experiment and by GC-MS *in vivo* experiment. CA-β-CD inclusion complex was characterized by differential scanning calorimetry (DSC), X-ray diffractometry (XRD), and nuclear magnetic resonance (NMR). Through complexation with β-CD, the solubility of CA in neutral aqueous solution was improved significantly. CA-β-CD inclusion complex also shows a significantly improved dissolution rate in comparison with free CA. Comparison of the pharmacokinetics between CA-β-CD inclusion complex and free CA was performed in rats. The *in vivo* results show that CA-β-CD inclusion complex has earlier t_{max}, higher $C_{max,}$ and higher bioavailability than free CA after oral dosing. By comparing the AUC_{0-t} of CA and CA-β-CD inclusion complex, the relative bioavailability of CA-β-CD inclusion complex to free CA was 506.3%, which highlighted the evidence of significantly improved bioavailability of formulation of CA with β-CD. Thus, this β-CD-based drug delivery system should be an effective oral dosage form to improve oral bioavailability of CA. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 123: 1557–1562, 2012

Key words: cantharidin; β-cyclodextrin; inclusion complex; drug delivery

INTRODUCTION

As the description in pharmacopoeia of people's republic of china, Mylabris is the dry body of *Mylabris phalerata Pallas* or *Mylabris cichorii Linnaeus*.[1] According to the record in Shen Nong's Herbal, a famous book about the traditional Chinese medicine written in ancient China, Mylabris has the biological action of eliminating the toxic material, eroding the mycosis, removing the blood stasis, and dispersing the obstruction and lumps, thus it could be used to treat such diseases as carbuncle-abscess, tinea, ulcer, mycosis, and so on. As a traditional Chinese anticancer medicine, Mylabris was first discovered and applied in practice in China, its usage can be traced back to 2000 years ago.[2] Now, there are many kinds of Mylabris-based pharmaceutical preparations in China market, such as compound Mylabris injection and compound Mylabris capsules, all of them have been proved to have good anticancer effect.

Modern research indicates that cantharidin (CA, Fig. 1) is the main active component of Mylabris. The CA crystalline was first isolated from *Lytta vesicatoria* by a French pharmacist, Robiquet, in 1810.[3] Modern pharmacologic studies prove that cantharidin can interfere with the metabolism of nucleic acids and of proteins in cancer cells, significantly inhibit the growth of various implanted tumor on animal models. It has inhibitory effect on primary hepatoma and other carcinomas, such as uterine cervix cancer, nasopharyngeal carcinoma, cutaneous cancer, leucemia, etc.[4–6] In clinic, cantharidin has been demonstrated particular therapeutic efficacy in the treatment of cancer and some refractory diseases. Because of its good treatment effectiveness for cancer, especially for terminal cancer, it is necessary to lucubrate CA. On the basis of our previous experiments, CA is a partially water-soluble drug and displays poorly intestinal absorption and low bioavailability (26.7%) in our former study.

Several reports[7–12] have shown the advantages of using Cyclodextrin in pharmaceutical formulations to improve the bioavailability of drugs. β-CD are cyclic oligosaccharides consisting of six or more

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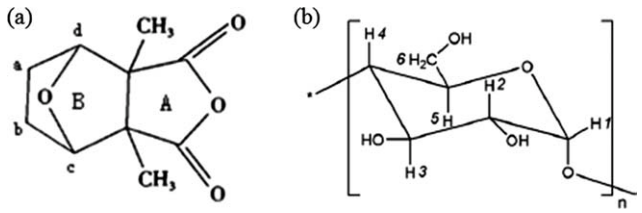

Figure 1 Structure of (a) cantharidin and (b) β-CD.

α-1,4-linked d-glucopyrannose units. There is no evidence that CDs are harmful for human health. Non-toxic effect level for oral administration of β-cyclodextrin was established to be 0.7–0.8 g/kg/day in rats and about 2 g/kg/day in dogs.[13] Inclusion will occur when the cyclodextrin partially or fully entraps a guest compound in its cavity and the inclusion complexes overcome undesirable physicochemical properties including low aqueous solubility, poor dissolution rate, and limited drug stability. CDs have been utilized extensively in pharmaceutical formulations to enhance drugs' bioavailability

Our previous experiments have provided a simple and sensitive method for the analysis of CA in blood.[14] In this article, we describe the GC–MS method based on electron ionization mode for determination of CA. This study aims to prepare, characterize, and collect information about the gastrointestinal absorption of the inclusion complex of CA with β-cyclodextrin.

MATERIALS AND METHODS

Materials

Cantharidin standard preparation (98% purity, 110783-200503) was purchased from National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Cantharidin used for preparation was purchased from Nanjing Zelang Medical Technological Development Co. Ltd. (>98% purity) (Nanjing, China). The internal standard, clofibrate (IS) (purity above 99%) was purchased from Alexis Biochemicals (Lausanne, Switzerland). *β*-cyclodextrin were purchased from Shandong Jingong Biology & Chemical Industry Co. Ltd (China). HPLC grade methanol was purchased from Fisher Scientfic Co. Ltd. (USA). Absolute alcohol was analytical reagent grade, from the BEIJING SHIJI (Beijing, China), all other reagents were of analytical grade. Sprague-Dawley rats (SCXK (jing) 2007-0001), male, healthy, weighing 190–210 g, were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) for the animal experiment

Preparation of the physical mixtures of CA with β-CD

The physical mixtures (PM) of CA and HP-β-CD were prepared by mixing individual components in 1 : 2 molar ratio that had previously been sieved through sieve no. 60.

Preparation of the CA-β-CD inclusion complex

The CA-β-CD inclusion complex was prepared by saturated solution method. The required β-CD was dissolved in an amount of water approximately equivalent to its solubility at 50°C and the proper amount of CA was dissolved in absolute ethanol. The CA solution was added to β-CD aqueous solution slowly in a mole ratio of 1 : 2. These dispersions were stirred in a water bath at 50°C for 2 h. After this period, the dispersions were cooled in 4°C for 24 h and then filtered through a 0.45 μm pore diameter membrane. The precipitate dried at 60°C in an oven.

Differential scanning calorimetry

Samples in DSC (Exstar 6200, SII NanoTechnology, Japan) using aluminum crucibles with ~ 3 mg of samples in a nitrogen (50 mL/min) and a heating rate of 10°C/min in the temperature range of 30–300°C. Thermograms were determined for the samples: CA, β-CD, CA/β-CD physical mixture, and CA-β-CD inclusion complex.

Nuclear magnetic resonance

The formation of a complex between CA and β-CD was investigated by means of ^{1}H-NMR spectroscopy analysis. All ^{1}H-NMR spectra were obtained in a Bruker Avance spectrometer at 600 MHz. ^{1}H spectra for CA, β-CD, and inclusion complex were obtained in $CDCl_3$ and D_2O, respectively. Data were collected without an external reference to avoid possible interactions with β-CD.

X-ray analysis

The X-Ray spectrum of CA, β-CD, CA/β-CD physical mixture, and inclusion complex were recorded by using power X-Ray diffractometer (PW3710, Philips Analytic, Holland) at room temperature. The diffractograms were recorded in the angle range between 2.5°and 60°, and the scanning speed was 4°/min.

Solubility study

Solubility study was conducted by placing an excess amount of CA (~ 15 mg) in 10 mL distilled water, and the mixture was heated at 60°C in a water bath to facilitate the solubilization using a vortex mixer. Mixtures were equilibrated at 25°C for 72 h in a water bath. The equilibrated samples

were centrifuged at 3000 rpm for 15 min to remove the undissolved CA. The supernatant was determined by HPLC, using an LC-10A (Shimadzu, Japan) equipped with a C-18 reversed-phase chromatographic column (250 × 4.6 mm; 5 μm particle size) conditioned. The column was kept at 30°C throughout the elution process, which used a mobile phase consisting of methanol and water 4 : 6 (v/v) at a flow rate of 1.0 mL/min and the detection wavelength set to 228 nm. The presence of β-CD did not interfere with the method used to analyze CA.

In vitro dissolution study

The dissolution rate studies of CA and CA-β-CD inclusion complexes were performed in triplicate in a dissolution apparatus using the paddle method (ChP, Type II). Powders (amount equivalent to 30 mg of CA) were placed in 900 mL of PBS (pH 6.8) at 37.0°C ± 0.1°C, with a paddle rotation speed of 50 rpm. Samples of 5 mL were withdrawn at time intervals of 1, 5, 10, 30, 60, 120, 240, and 360 min. The volume of dissolution medium was adjusted to 900 mL by replacing each 5 mL aliquot withdrawn with 5 mL of fresh dissolution medium. The solutions were immediately filtered through 0.45 μm membrane filter, determined by HPLC.

In vivo experiment

Two groups consist of six rats were allowed free access to water but were fasted for 24 h before drug administration and 6 h after drug administration. CA and CA-β-CD inclusion complex were dispersed in 2 mL physiological saline and then administered orally. The doses were 4 mg/kg[15] as CA. Blood samples were withdrawn from the jugular vein of rats at 0.5, 1, 2, 3, 4, and 6 h after dosing. In aliquots of 0.2 mL of plasma samples from rats in a disposable Eppendorf tube, were added with 200 μL hydrochloric acid (6*M*), 28 ng of IS (10 μL of the 2.8 μg/mL) and 2 mL ethyl acetate. After vortexing for 90 s, the tube was centrifuged at 15,000 rpm for 15 min. The supernatant (1.4 mL) was transferred to a clean tube and evaporated to dryness under stream of nitrogen gas at 30°C. The residue was dissolved by 100 μL ethyl acetate and the ethyl acetate was injected into GC-MS system for analysis.

The area under the curve (AUC) was calculated by linear trapezoidal rule from zero to the last plasma concentration. The maximum plasma concentration, C_{max}, and the time of its occurrence, T_{max}, were compiled from the concentration–time data.

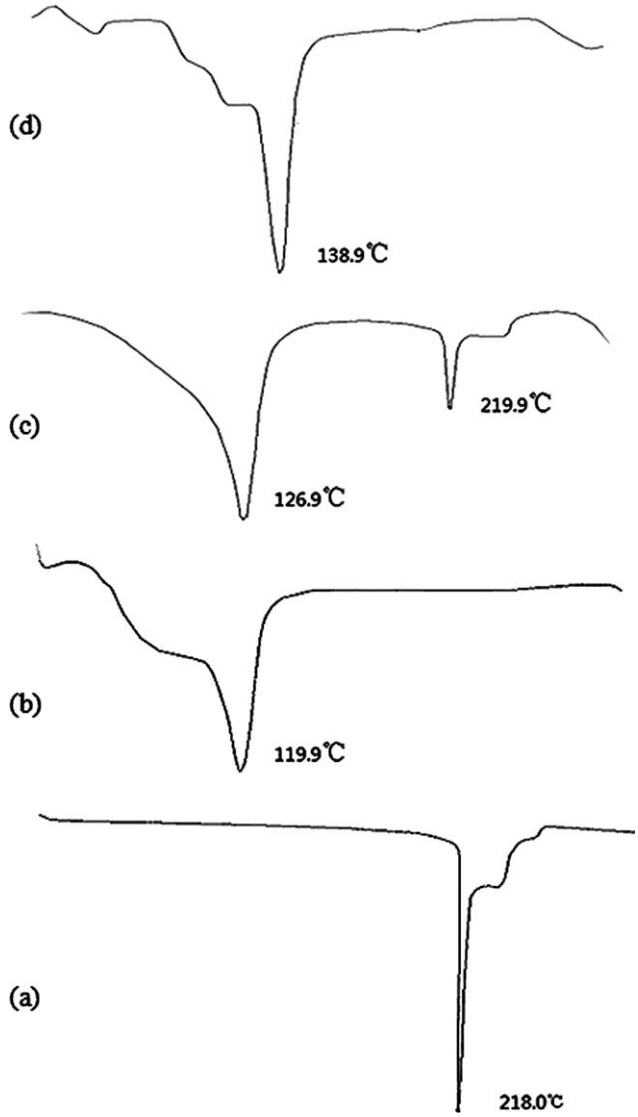

Figure 2 The DSC diagrams. (a) Cantharidin; (b) β-CD; (c) physical mixture; and (d) inclusion complex.

RESULT AND DISCUSSION

Differential scanning calorimetry

The ability of β-CD to form inclusion complexes with CA was confirmed by differential scanning calorimetry (DSC).[16,17] Figure 2 shows thermograms for CA [Fig. 2(A)], β-CD [Fig. 2(B)], CA/β-CD physical mixture [Fig. 2(C)], and solid complex CA-β-CD [Fig. 2(D)]. β-CD and CA presented a characteristic endothermic peak each corresponding to their melting point (218.0°C and 119.9°C, respectively). The CA/β-CD physical mixture shows two endothermic peaks of intermediate temperatures (219.9°C and 126.9°C), indicating an absence of interaction between CA and β-CD upon simple mixing of the two solids, but there is a little shift of the melting point of β-cyclodextrin from 119.9 to 126.9, which may results from the mixing force. The mixing force

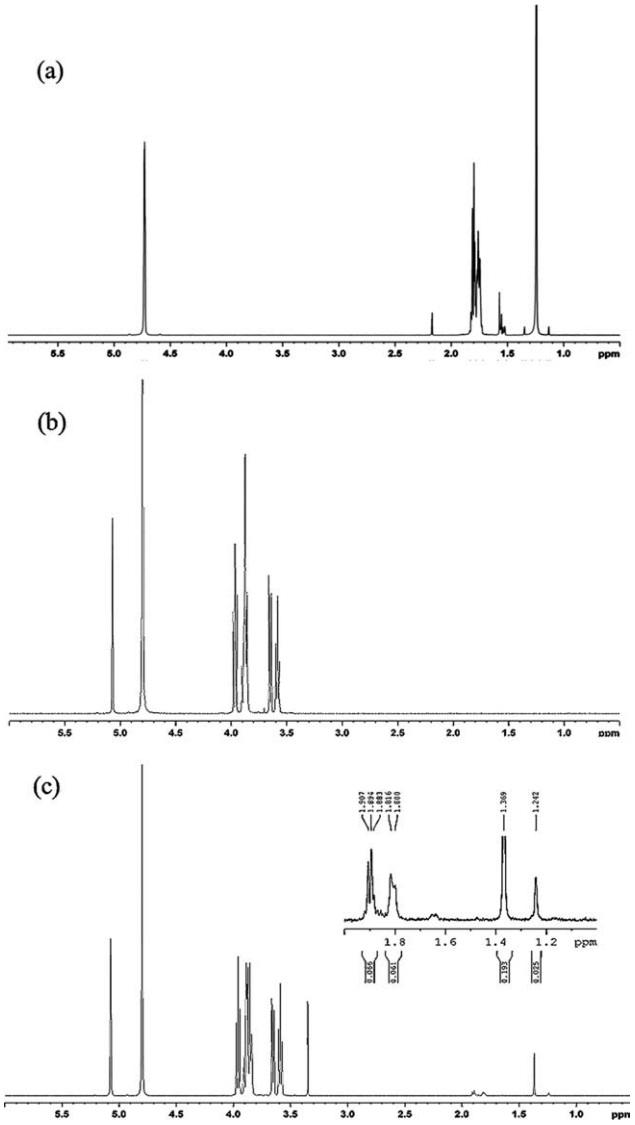

Figure 3 ^{1}H-NMR spectra of (a) CA, (b) β-CD, and (c) CA-β-CD.

may cause some degree of interaction as the reviewer said. The CA-β-CD inclusion complex presents only a single peak at 138.9°C, in a different manner than those observed for the pure CA, pure β-CD or for their physical mixture. The disappearance as well as the shift of exothermic peaks of drugs is a clear indication of the complexation phenomenon explaining the absence of the fusion peak of pure CA (218.0°C) in the thermogram showed in Figure 2(D). These analyses gave supporting evidences for the complexation of CA with β-CD.

Nuclear magnetic resonance spectroscopy

^{1}H NMR is one of the most selective tools for the characterization of inclusion complexes and for the demonstration of total or partial inclusion in the β-CD cavity that occurs in a liquid medium.[18,19] In the case of β-CD, it is important to demonstrate whether the drug is included in the cavity or entrapped within the chains of the molecule. During complexation, the chemical environment of some protons changes, and this results in changes in chemical shifts of ^{1}H-NMR lines of the protons that are due to shielding or deshielding effects. For the complexes, internal protons of the cavity, which were H-3 and

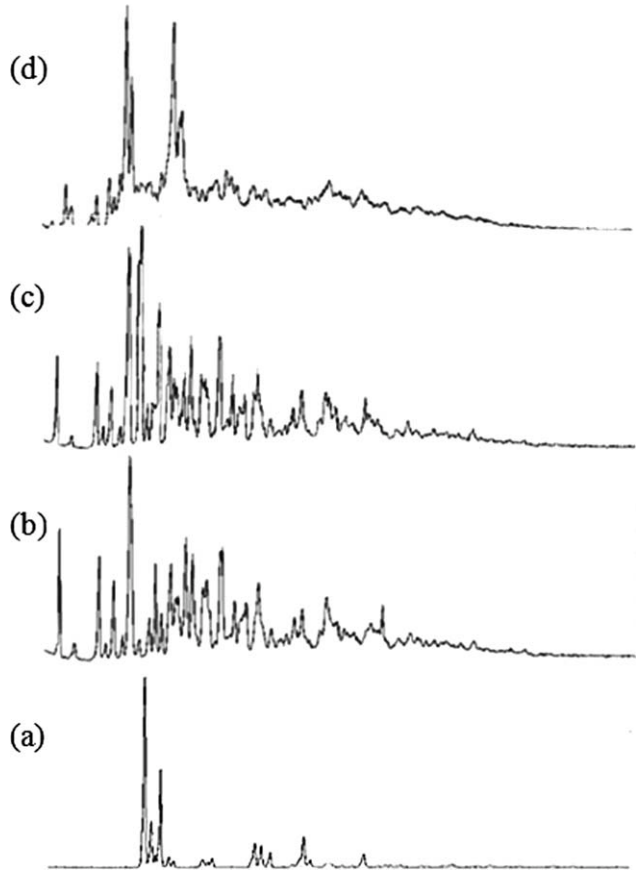

Figure 4 Powder X-ray diffractograms. (a) Cantharidin; (b) β-CD; (c) physical mixture; and (d) inclusion complex.

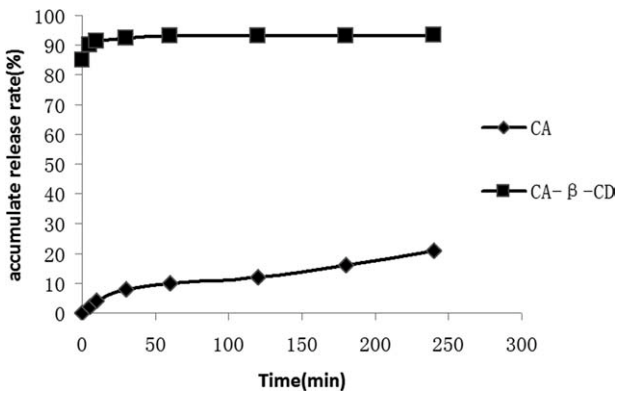

Figure 5 Dissolution profiles of free CA and CA-β-CD inclusion complex in PBS (pH 6.8) at 37.0°C ($n = 3$).

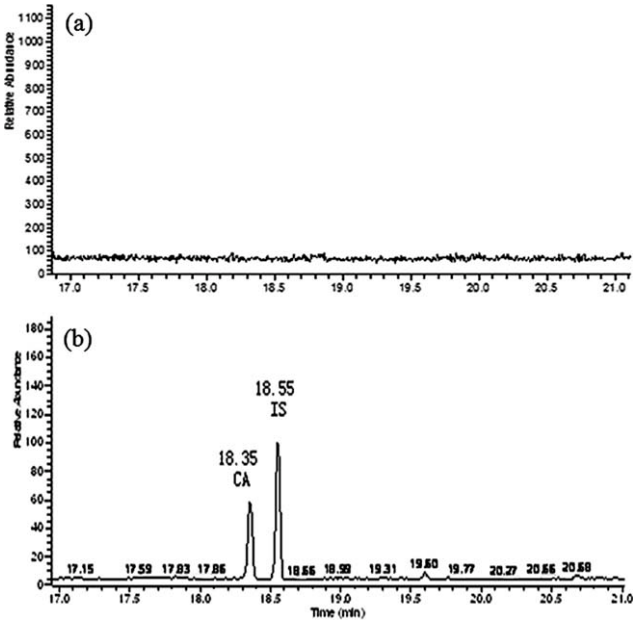

Figure 6 Representative SIM (m/z = 128) chromatograms of CA and IS (n = 5). (a) A blank plasma and (b) plasma spiked with CA and IS.

H-5, were evaluated for changes in ^{1}H NMR spectra (Fig. 3). In the present experiment, H-5 shifted from 3.871 to 3.906 but H-3 did not display any shifts before and after complexation. These results suggest that the drug has entered the β-CD cavity to form an inclusion complex. The hydrogen Hc and Hd (δ = 4.73) of ring B lost their equivalence in the presence of the β-CD. The Ha and Hb from δ = 1.79 to δ = 1.88, which is a strong evidence of the inclusion of ring B in the β-CD cavity. The interaction between ring A and β-CD can't be observed for there is no proton on ring A.

X-ray analysis

Powder X-ray diffractograms clearly confirmed the crystalline nature of CA, whereas β-CD was presented another crystalline structure [Fig. 4(A,B), respectively].[20] The CA/β-CD physical mixture confirmed the superposition of the crystalline pattern of CA and β-CD diffraction [Fig. 4(C)]. By contrast, the CA-β-CD inclusion complex showed that the crystalline CA and β-CD pattern has disappeared, and a new XRD spectrum formed. The exchange of spectra showed the interaction has formed.

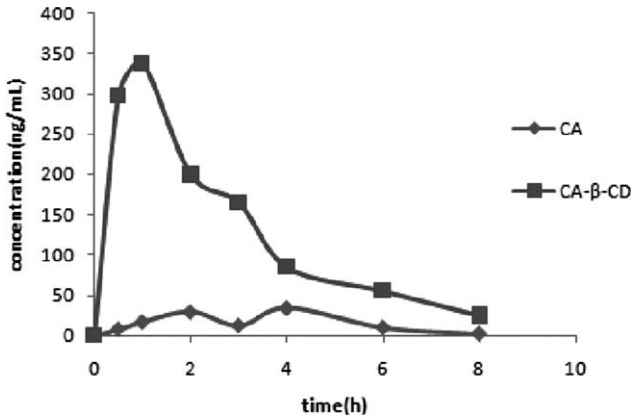

Figure 7 Mean (±SD) plasma concentration–time profiles after oral administration of CA-β-CD inclusion complex and free CA to rats at a dose of 4 mg/kg, respectively (n = 3).

Solubility study

The solubility of free CA is 89.14 ± 1.2 mg/mL and the solubility of CA from inclusion complex is up to 1055.29 ± 1.6 mg/mL. The solubility has increased 11.8 times after complexation.

In vitro dissolution study

The dissolution data of CA and CA-β-CD inclusion complex are illustrated in Figure 5. At each time point, CA amount dissolved from inclusion complex was significantly higher than that of free CA. The amount dissolved from free CA was <20% after 4 h, whereas the CA amount dissolved from inclusion complex was >90% in 10 min. This fast dissolution of the binary system of CA-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.

In vivo experiment

Figure 6 shows the typical chromatograms of CA in plasma. A good linear relationship with linearity ranged from 2.14 ng/mL to 314.2 ng/mL. Mean plasma concentration of CA after oral administration of CA and CA-β-CD inclusion complex was shown in Figure 7 and the pharmacokinetic parameters of CA was show in Table I.After 30 min of the dosing, the parent form of CA in plasma was detected in a

TABLE I
Pharmacokinetic Parameters of CA and CA-β-CD in Rats (n = 5)

	C_{max} (ng/mL)	T_{max} (h)	$AUC_{0\sim t}$(ng/mL)·h	AUC (%)
CA	66.22 ± 23.63	2.33 ± 0.47	187.37 ± 76.09	100.00
CA-β-CD	355.63 ± 81.66	1.33 ± 0.578	948.72 ± 289.12	506.34

very low level, whereas the concentration of CA from CA-β-CD inclusion complex were almost 40 times to free CA. The mean plasma concentration–time curves of CA are shown in Figure 7. Examining the results from the group analysis, the mean C_{max} of CA from inclusion complex was significantly much higher than that of free CA, and the t_{max} of CA of CA-β-CD inclusion complex was significantly earlier than that of free CA. By comparing of the AUC_{0-t} between free CA and CA-β-CD inclusion complex the relative bioavailability of CA-β-CD inclusion complex to free CA was 506.3%.

CONCLUSION

Through formulation with β-CD, the aqueous solubility of hydrophobic compound CA was improved markedly in neutral aqueous solution. Taken the results obtained from DSC together, ^{1}H-NMR, X-ray diffraction studies suggest the formation of complex. Comparison of metabolic pharmacokinetics of CA and its inclusion complex with β-CD in rats indicated that CA-β-CD inclusion complex had earlier t_{max}, higher $C_{max,}$ and bioavailability. In conclusion, our results demonstrated that formulation of CA with β-CD significantly improved its solubility, and the resultant product could improve the dissolution properties and oral bioavailability of CA. This study provides perspectives for future experiments using this inclusion complex of CA with β-CD to improve its therapeutic efficacy.

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