Physicochemical Properties and Gastric Mucosa Irritation of Cantharidin-hydroxypropyl-β-cyclodextrin Inclusion Complex

AN Lin-na, DANG Yun-jie, HU Chun-hui, ZHU Chun-yan*

Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing 100193, China

Abstract: **Objective** To increase the solubility and relieve the mucous irritation of cantharidin (CA) by preparing cantharidin-hydroxypropyl-β-cyclodextrin (CA/HP-β-CD) inclusion complex. **Methods** The inclusion complex was prepared by co-evaporation method and characterized by differential scanning calorimetry (DSC), X-ray diffractometry (XRD), and nuclear magnetic resonance (NMR). **Results** The disappearance of CA as well as the shift of exothermic peaks shown in DSC results indicated the complexation phenomenon. XRD results showed that the crystalline CA pattern had disappeared, and in NMR results, H-5 shifted from δ 3.731 to 3.695 after complexation and H-2 shifted from δ 3.626 to 3.598, which suggested that part of the drug had entered the HP-β-CD cavity to form an inclusion complex. The solubility increased 10.3 times after complexation and the mucous irritation of CA was relieved remarkably. **Conclusion** Through complexation with HP-β-CD, the solubility and dissolution rate of CA are improved significantly, and the irritation of musous is relieved.

Key words: cantharidin; dissolution; hydroxypropyl-β-cyclodextrin; mucous irritation; solubility
DOI: 10.3969/j.issn.1674-6384.2012.03.009

Introduction

Mylabris is the dry body of *Mylabris phalerata* Pallas or *M. cichorii* Linn. recorded in *Chinese Pharmacopoeia 2010*. It is a Chinese materia medica with anticancer effect, which was first discovered and applied in medical treatment in China about 2000 years ago (Wang, 1989). Modern research indicated that cantharidin (CA, Fig. 1) is the main active component of mylabris (Wang, 1989). Pharmacologic studies proved that CA could significantly inhibit the growth of various implanted tumors on animal models because of its ability of interfering with the metabolism of nucleic acids and proteins in cancer cells. It has inhibitory effect on primary hepatoma and other carcinomas, such as uterine cervix cancer, nasopharyngeal carcinoma, cutaneous cancer, leukemia, and so on (Zhang, Ying, and Xiao, 2004; Efferth and Rauh, 2005; Liu and Zhang, 2006). In clinic, CA has demonstrated particular therapeutic efficacy in the treatment of cancer and some refractory diseases. Although CA presents good anticancer activity, the intense mucous irritation brought serious side effects and limited the use of CA in clinic. Oaks *et al* (1960) reported that someone had got severe oral mucous ulcerate after taking CA by mistake. In clinical therapeutics, many patients get gastrointestinal indisposition because of the mucous irritation. Moreover, our previous work indicated that the low oral bioavailability (Dang and Zhu, 2009) of CA might be correlated with its poor aqueous solubility. It is indeed possible to improve the bioavailability of CA by increasing its solubility.

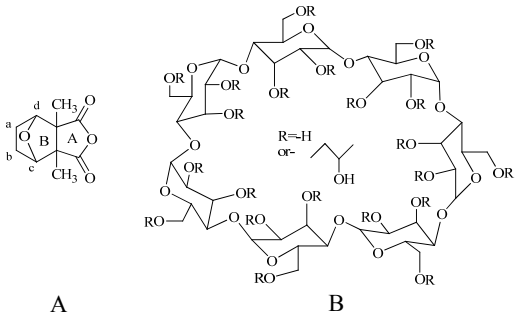

Fig. 1 Structures of CA (A) and HP-β-CD (B)

* Corresponding author: Zhu CY Address: No. 151 Malianwa North Road, Haidian District, Beijing 100093, China
Tel/Fax: +86-10-5783 3276 E-mail: cyzhu@implad.ac.cn
Received: September 15, 2011; Revised: November 23, 2011; Accepted: January 17, 2012
Fund: National S&T Major Special Project on Major New Drug Innovation (2009ZX09301-003&2008ZX09103-390)
Online time: May 18, 2012 Online website: http://www.cnki.net/kcms/detail/12.11410.R.20120518.1621.001.html

Cyclodextrins (CDs) as cyclic oligosaccharides are able to accommodate drug molecules in their cone-shaped cavity forming inclusion complexes which are stabilized by intermolecular forces such as hydrophobic interactions, van der Waals' forces, and hydrogen bonding. Inclusion could occur when the CD partially or fully entraps a guest compound in its cavity (Junco *et al*, 2002). Industrially-produced CDs include α, β, and γ types (comprised of six, seven, and eight glucose subunits, respectively), which have also been substituted at the hydroxyl proton or hydroxyl group to alter their physicochemical properties (Brewster and Loftsson, 2007). Complex formation with CD provides a way to increase the solubility, stability, and bio-availability of drugs. Hydroxypropyl-β-cyclodextrin (HP-β-CD), the alkylated derivative of β-CD, has attracted growing interest due to its improved complexing ability, greater water solubility, and less toxicity than β-CD.

The present study aimed to prepare and characterize the inclusion complex of CA with HP-β-CD and evaluate the mucous irritation of CA/HP-β-CD inclusion complex. Phase-solubility diagrams were used to evaluate the solubility of CA and its association constants with HP-β-CD. Differential scanning calorimetry (DSC), nuclear magnetic resonance (NMR), and X-ray diffraction (XRD) assays were used to characterize the CA/HP-β-CD inclusion. Solubility and dissolution studies *in vitro* provided the evidences for whether the oral bioavailability might be increased. Finally, experiments in rats were used to demonstrate whether the complexation with HP-β-CD could relieve the gastrointestinal irritation of CA.

This is an elementary study for the development of potential formulations of CA to be used in clinical therapeutics with better therapeutic effect and less untoward reaction. Moreover, with low aqueous solubility and low permeability, CA could be regarded as a model drug that the interaction between CA and HP-β-CD could offer the reference for other drugs with similar properties.

Materials and methods

Cantharidin reference substance (98% purity) was purchased from National Institute for Food and Drug Control (Beijing, China). Cantharidin (> 98% purity) used for preparation was purchased from Nanjing Zelang Medical Technological Development Co., Ltd. HP-β-CD (MS = 4.4) was purchased from Xi'an Deli Biology & Chemical Industry Co., Ltd. HPLC grade methanol was purchased from Fisher Scientific Co., Ltd. (USA). Absolute alcohol was of analytical grade, from Beijing Shiji (China), and all other reagents were of analytical grade. SD 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.

HPLC conditions

CA in samples was analyzed by HPLC (Dang and Zhu, 2009), using an LC-10A (Shimadzu, Japan) equipped with a C_{18} reversed-phase chromatographic column (250 mm × 4.6 mm, 5 μm). The column was kept at 30 ℃ throughout the analysis process, which used methanol-water (4:6) as mobile phase at a flow rate of 1.0 mL/min and the detection wavelength was 228 nm. As Fig. 2 shows, under the HPLC conditions, the presence of HP-β-CD did not interfere with the method used to analyze CA. Calibration curves of CA were linear over the concentration range of 16—160 μg/mL. Good linearity with a correlation coefficient r = 0.9999 was observed, and this method showed good precision and accuracy, the intra-day precision was less than 5.1%, and the inter-day precision was less than 4.8%.

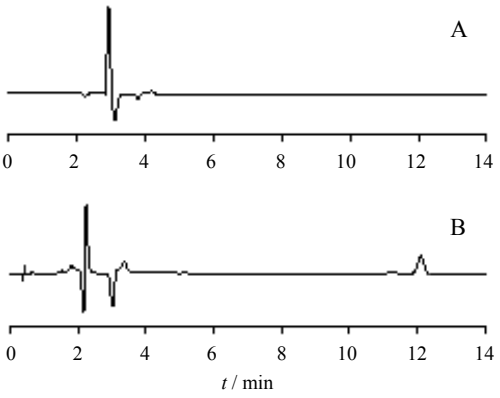

Fig. 2 HPLC chromatograms of blank (A) and CA (B)

Phase solubility studies

Phase solubility studies were carried out in water in triplicate. Excess amount (15 mg) of CA was added to aqueous solutions containing various concentration of HP-β-CD (1% to 20%). The suspensions were equilibrated at 37 ℃ under mechanical stirring for 72 h.

After equilibrium was achieved, the samples were then filtered (0.45 μm) and analyzed by HPLC. The presence of HP-β-CD did not interfere with the method used to analyze CA. The apparent stability constants (K_c) of CA/HP-β-CD inclusion complex were calculated from the slope of the phase solubility diagrams and the solubility of the pure drug in water (S_o), according to the following equation:

$$K_c = \text{slope}/S_o\,(1-\text{slope}) \quad (1)$$

Preparation of CA/HP-β-CD inclusion complex

Inclusion complex was prepared by co-evaporation method. Briefly, CA and HP-β-CD with 1:2 molar ratio were accurately weighed and dispersed in 80% ethanol and then stirred at 100 r/min for 2 h at 40 ℃. The complex solution was filtered through a 0.22 μm membrane filter and then dried at 60 ℃ in an oven. The dried masses were pulverized and sieved by a sieve, fractions ≤ 100 μmol/L were selected.

DSC analysis

The samples (3 mg) used for DSC (Exstar 6200, SII NanoTechnology Inc., Japan) were placed in aluminum crucibles. The experiments were carried out in the nitrogen (50 mL/min) and at a heating rate of 10 ℃/min over a range of 30－300 ℃. Thermograms were determined for the samples: CA, HP-β-CD, CA/HP-β-CD physical mixture, and CA/HP-β-CD inclusion complex.

X-Ray diffraction (XRD)

The X-Ray spectrums of CA, HP-β-CD, CA/HP-β-CD physical mixture, and inclusion complex were recorded by using powder XRD (PW3710, Philips Analytic, Holland) at room temperature. The diffractograms were recorded in the angle range between 2.5° and 70°, and the scanning speed was 4°/min.

NMR analysis

The formation of complex between CA and HP-β-CD was investigated by means of ^{1}H-NMR spectroscopy analysis. All ^{1}H-NMR spectra were obtained in a Bruker Avance III Spectrometer at 600 MHz. ^{1}H-NMR spectra for CA, HP-β-CD, and inclusion complex were obtained in $CDCl_3$ and D_2O, respectively.

Solubility analysis

Solubility studies were conducted as below: An excess amount of CA and CA/HP-β-CD inclusion complex (approximately 15 mg as CA) were placed in 10 mL distilled water, respectively. The mixtures were heated at 60 ℃ in a water bath. A vortex mixer was used to facilitate the solubilization and the mixtures were equilibrated at 25 ℃ for 72 h in water bath. The equilibrated samples were centrifuged at 3000 r/min for 15 min to remove the undissolved drug and the supernatant was determined by HPLC.

***In vitro* dissolution analysis**

The dissolution studies of CA and CA/HP-β-CD inclusion complex were performed in a dissolution apparatus using the paddle method according to *Chinese Pharmacopoeia 2010* apparatus No. 1 (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 ℃, with a paddle rotation speed of 50 r/min. Samples (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 fresh dissolution medium. The solutions were immediately filtered through 0.45 μm membrane filter and then the filtrate was determined by HPLC.

Mucous irritation experiment

Four groups of rats, which consisted of three rats, respectively, had free access to water but were fasted for 24 h before drug administration and 4 h after drug administration. All samples including physiological saline (negative control group), salicylic acid, free CA, and CA/HP-β-CD were dispersed separately in 2 mL physiological saline and then ig administrated. The rats were killed via CO_2 inhalation. The intact stomach was removed and then made into pathological section.

Results

Phase solubility analysis

CA was practically insoluble (89.14 μg/mL) in pure water at 25 ℃. Higher temperature and the addition of HP-β-CD both resulted in a significantly increased solubility. The stability constants of the inclusion complex were calculated using Eq (1), which were found to be 85.391 M^{-1} at 25 ℃, 28.982 M^{-1} at 40 ℃, and 24.459 M^{-1} at 55 ℃. With the temperature increasing, the K_c of CA/HP-β-CD inclusion complex decreased. The results indicated that higher temperature was not suitable for the formulation of inclusion complex. Meanwhile, the data demonstrated the

interaction between CA and HP-β-CD was weak. It was unfavourable for the stability but favorable for the drug dissolution.

DSC analysis

The ability of HP-β-CD to form inclusion complex with CA was confirmed by DSC. Fig. 3 showed thermograms for CA (Fig. 3A), HP-β-CD (Fig. 3B), CA/HP-β-CD physical mixture (Fig. 3C), and inclusion complex (Fig. 3D).

HP-β-CD and CA presented a specific characteristic endothermic peak each corresponding to their melting points (347.9 and 218.0 ℃, respectively) and for HP-β-CD, a peak corresponding to water loss was also observed (97.3 ℃). The CA/HP-β-CD physical mixture showed two endothermic peaks of 356.9 and 199.9 ℃, indicating an absence of interaction between CA and HP-β-CD upon simple mixing of the two solids. The CA/HP-β-CD inclusion complex presented only a single broaden peak at 344.3 ℃, in a different manner compared with pure CA, pure HP-β-CD or for their physical mixture. The disappearance as well as the shift of exothermic peaks shown in Fig. 3D is a clear indication of the complexation phenomenon. This analysis gave supporting evidences for the formulation of complex between CA and HP-β-CD.

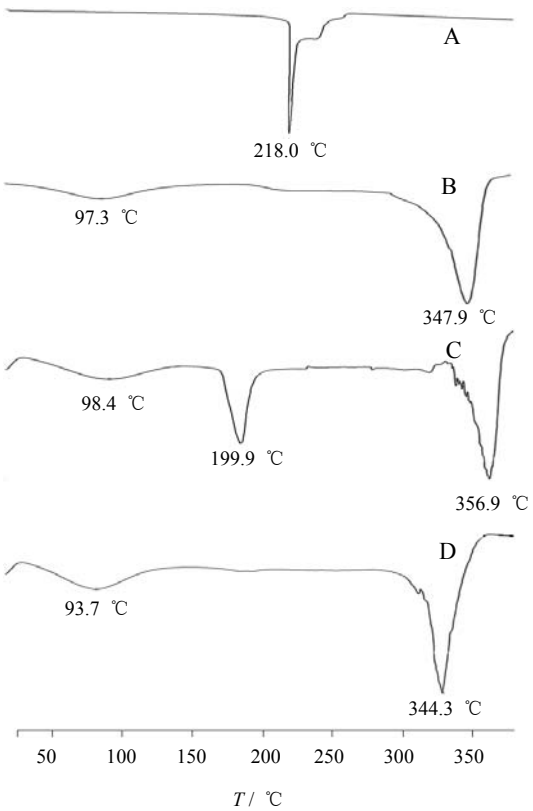

Fig. 3 DSC diagrams of CA (A), HP-β-CD (B), physical mixture (C), and CA-HP-β-CD (D)

NMR analysis

^{1}H-NMR is one of the most selected tools for the characterization of inclusion complex and for the demonstration of total or partial inclusion in the HP-β-CD cavity that occurred in a liquid medium. In the case of HP-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, these changes in chemical shifts of the protons in ^{1}H-NMR spectra that are due to shielding or deshielding effects. For the complexes, internal protons (H-3 and H-5) of the cavity were evaluated in ^{1}H-NMR spectra to detect the changes. As commercial HP-β-CD contains a mixture of HP-β-CD derivatives with different substitution degrees, a special characteristic of this spectrum is the presence of signals spread over a range of values.

In the present experiment (Fig. 4), H-5 shifted from δ 3.731 to 3.695 after complexation and H-2

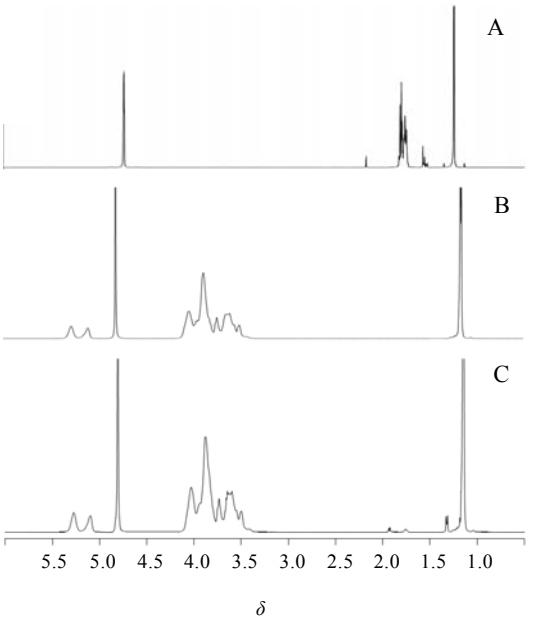

Fig. 4 ^{1}H-NMR spectrum of CA (A), physical mixture (B), and CA/HP-β-CD (C)

shifted from δ 3.626 to 3.598. The other protons shifted below δ 0.003. These results suggested that part of the drug had entered the HP-β-CD cavity to form an inclusion complex with the combination of H-5 and

part of the drug entrapped within the chains of the molecule with the combination of H-2.

XRD analysis

Powder XRD clearly confirmed the crystalline nature of CA while HP-β-CD was presented as an amorphous structure (Fig. 5). The CA/HP-β-CD physical mixture confirmed the superposition of the crystalline pattern of CA and the amorphous HP-β-CD diffraction. By contrast, the CA/HP-β-CD inclusion complex showed that the crystalline CA pattern had disappeared. The results demonstrated the interaction between CA and HP-β-CD had occurred.

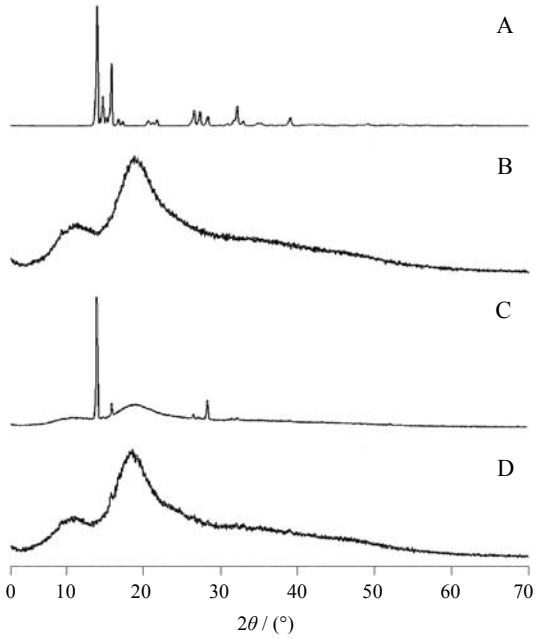

Fig. 5 Powder XRD of CA (A), HP-β-CD (B), physical mixture (C), and CA/HP-β-CD (D)

Solubility analysis

The solubility of free CA was (89.14 ± 1.2) μg/mL and the solubility of CA from inclusion complex was up to (918.14 ± 0.9) μg/mL. The solubility has increased by 10.3 times after complexation.

The interior of the cavity of HP-β-CD is hydrophobic, so it's necessary to provide a favorable environment for the inclusion of hydrophobic moiety of CA in aqueous solution. The exterior of the cavity of HP-β-CD, laced with hydroxyl groups, is hydrophilic (Wu, Lee, and Li, 2001). The hydrophilic groups outside the HP-β-CD may be the main reason why it could increase the solubility of CA. When inclusion complex formed, CA had higher solubility due to the interaction with HP-β-CD.

Our previous unpublished work demonstrated that the oral bioavailability of CA is correlated to its solubility. It is indeed possible to improve the bioavailability of CA with its higher solubility after complexation with HP-β-CD.

***In vitro* dissolution studies**

The dissolution results of CA and CA/HP-β-CD inclusion complex were illustrated in Fig. 6. 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 less than 20% after 4 h, while the CA amount dissolved from inclusion complex was more than 40% in 1 min and closed to totally after 4 h. The fast dissolution of the binary system of CA/HP-β-CD may be ascribed to the fact that CDs have the capability of improving the wet ability of powder materials and forming a rapidly soluble complex in solution.

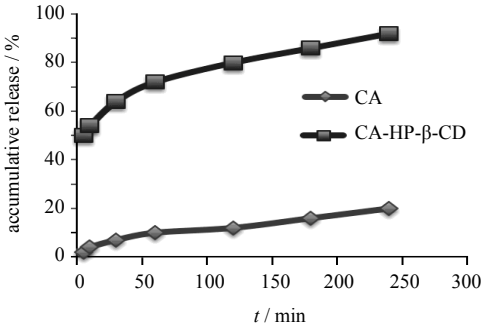

Fig. 6 Dissolution curves of CA and CA/HP-β-CD inclusion complex in PBS (pH 6.8) at 37.0 ℃ (*n* = 3)

Mucous irritation experiment

The results of pathological section showed in Fig. 7 indicated that free CA had gastrointestinal mucous irritation (Fig. 7C) and the irritation was relieved after complexation (Fig. 7D). It may be ascribed to the fact that drug had entered the HP-β-CD cavity. So it didn't touch the gastrointestinal mucous immediately. The present study provided the reference to the potential formulation of CA to be used in clinical with less untoward reaction.

Discussion and conclusion

As a Chinese materia medica with the anticancer effect, mylabris was first discovered and applied in practice in China. CA has demonstrated particular

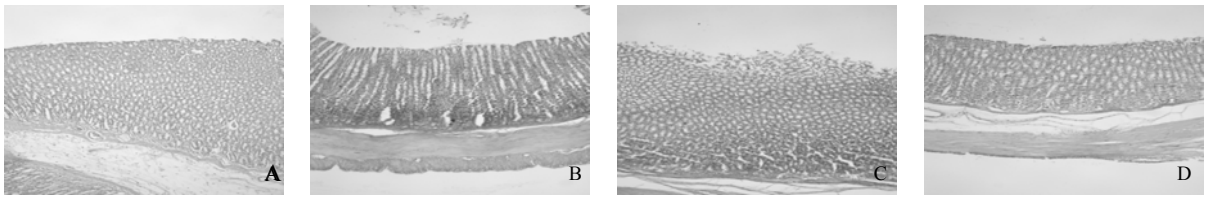

Fig. 7 Profiles of mucous irritation experiment

A: physiological saline B: salicylic acid C: CA D: CA/HP-β-CD

therapeutic efficacy in the treatment of cancer and some refractory diseases. Modern pharmacologic studies prove that CA could interfere with the metabolism of nucleic acids and the metabolism of proteins in cancer cells, significantly inhibit the growth of various implanted tumors in animal models, and has an inhibitory effect on primary hepatoma and certain other carcinomas, such as uterine cervix cancer, nasopharyngeal carcinoma, cutaneous cancer, and leukemia, and others as well (Zhang, Ying, and Xiao, 2004; Efferth and Rauh, 2005; Liu and Zhang, 2006).

There are now many kinds of mylabris-based pharmaceutical preparations in the Chinese market, such as compound Mylabris Injection (Aidi Injection, State Medical Permit No. Z52020236) and compound Mylabris Capsules (State Medical Permit No. Z19993294; State Medical Permit No. Z20003270; State Medical Permit No. ZF20000427), and all proved to have good anticancer effects.

CA is a partially water-soluble drug and displays poor intestinal absorption and low bioavailability (26.7%) in our former study. Several reports (Challa *et al*, 2005; Medlicott *et al*, 1998; Loftsson *et al*, 2005) have shown the advantages of using CD in pharmaceutical formulations to improve the bioavailability of drugs. Inclusion will occur when the CD 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 extensively utilized in pharmaceutical formulations to enhance drugs' bioavailability.

The results obtained from DSC, ^{1}H-NMR, and XRD studies demonstrated that the complex had been formed. Through formulation with HP-β-CD, the aqueous solubility of hydrophobic compound CA was remarkably improved and the mucous irritation of CA was significantly relieved.

This study is an elementary study for the development of potential formulations of CA to be used in clinical with better therapeutic effect and less untoward reaction. Moreover, with low aqueous solubility and low liposolubility, CA could be regarded as a model drug that the study of interaction between CA and HP-β-CD could offer the reference to other drugs with similar properties.

References

Brewster ME, Loftsson T, 2007. Cyclodextrins as pharmaceutical solubilizers. *Adv Drug Deliv Rev* 59: 645-666.

Challa R, Alka A, Javed A, Khar RK, 2005. Cyclodextrins in drug delivery: An updated review. *AAPS PharmSci Tech* 6: 329-357.

Dang YJ, Zhu CY, 2009. Determination of trace cantharidin in plasma and pharmacokinetic study in Beagle dogs using gas chromatography-mass spectrometry. *J Anal Toxicol* 33: 384-388.

Efferth T, Rauh R, 2005. Molecular modes of action of cantharidin in tumor cells. *Biochem Pharmacol* 69: 811-818.

Junco S, Casimiro T, Ribeiro N, Nunesda PM, Cabral MHM, 2002. A comparative study of naproxen-beta-cyclodextrin complexes prepared by conventional methods and using supercritical carbon dioxide. *J Incl Phenom Macrochem* 44: 117-121.

Liu GQ, Zhang KC, 2006. Method for determination of cantharidin from 5 ferment liquid of medicinal fungi using capillary gas chromatography. *J Food Sci Biotech* 25: 88-90.

Loftsson T, Jarho P, Másson M, Jvinen T, 2005. Cyclodextrins in drug delivery. *Expert Opin Drug Deliv* 2: 335-351.

Medlicott NJ, Foster KA, Audus KL, et al. 1998. Comparison of the effects of potential parenteral vehicles for poorly water soluble anticancer drugs (organic cosolvents and cyclodextrin solutions) on cultured endothelial cells (HUV-EC). *J Pharm Sci* 87: 1138-1143.

Oaks WW, Ditunno JF, Magnani T, Levy HA, Mills LC, 1960. Cantharidin poisoning. *Arch Intern Med* 105: 106-114.

Wang GS, 1989. Medical use of Mylabris in ancient and recent studies. *J Ethnopharmacol* 26: 147-162.

Wu YS, Lee HK, Li SFY, 2001. High-performance chiral separation of fourteen triazole fungicides by sulfated β-cyclodextrin-mediated capillary electrophoresis. *J Chromatogr A* 912: 171-179.

Zhang JP, Ying K, Xiao ZY, 2004. Analysis of gene expression profiles in human HL-60 cell exposed to cantharidin using cDNA microarray. *Int J Cancer* 108: 212-218.