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ORIGINAL ARTICLE

Preparation, characterization, and pharmacokinetics of the inclusion complex of genipin-β-cyclodextrin

Yi Lu[1], Tong Zhang[2], Jiansheng Tao[1], Guang Ji[1] and Shaomin Wang[3]

[1]*College of Chinese Material Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai, PR China,* [2]*Experiment Center for Teaching, Shanghai University of Traditional Chinese Medicine, Shanghai, PR China and* [3]*Institute for Food and Drug Control, Shanghai, PR China*

Abstract

Objective: The aim of this study was to prepare the inclusion complex of genipin (GP) and β-cyclodextrin (β-CD) with improved stability, solubility, and bioavailability and to study the pharmacokinetics of β-CD inclusion complex in mice. *Methods*: Lyophilization was employed in the preparation of the inclusion complex of GP–β-CD, whose formation was confirmed by infrared, ultraviolet, differential scanning calorimetry, X-ray diffraction, and phase solubility method. Comparative studies on the in vitro solubility and stability and in vivo evaluation of GP in mice were investigated. Liquid–liquid extraction was used for the isolation of GP in the assay of its concentration. After injection in the caudal vein at equal doses of the inclusion complex of free GP, the drug concentration in mice plasma at fixed time after administration was determined by high-performance liquid chromatography method. *Results*: The results demonstrated that GP–β-CD solid powders showed improved stability and solubility in aqueous solution, when comparing with free GP. The results of the in vivo study showed that the inclusion complex of GP–β-CD exhibited the dissimilar pharmacokinetics from that of free GP after intravenous administration. The inclusion complex of GP–β-CD displayed longer $MRT_{0-\infty}$ and higher $AUC_{0-\infty}$ than free GP did. *Conclusions*: The relative bioavailability of the inclusion complex of GP–β-CD to free GP was 305.3%, which demonstrated that GP formulations containing β-CD significantly increased the bioavailability.

Key words: β-*Cyclodextrin; genipin; inclusion complex; pharmacokinetics; solubility; stability*

Introduction

Genipin (Figure 1), a metabolite derived from the herbal medicine *Gardenia*'s active ingredient, genipinside, is an iridoid, which can be prepared in vitro by enzymic hydrolysis method.[1,2] GP has attracted considerable attention because it has a variety of interesting activities such as choleretic effects,[3,4] preventing fulminant hepatic failure,[5] anti-inflammatory effects,[6,7] repairing myocardial defects,[8] inducing hepatoma cell apoptosis optosis,[9] antifibrosis,[10] preventing neurotoxicity,[11] and antithrombotic effects.[12] However, one undesired property of this compound lies in its instability in aqueous solution, especially in alkaline solution. Despite the wide range of the pharmacological properties, its application in pharmaceutical field is limited. Therefore, it is essential to find an appropriate way to improve its stability, so as to facilitate its clinical application.

In recent years, cyclodextrin complexation has been successfully used to improve the chemical stability and bioavailability of a number of drugs. Cyclodextrins (CDs) are cyclic nonreducing, nonhygroscopic, water-soluble oligosaccharides composed of α-(1→4)-linked α-D-glucosyl units,[13] which present hydrophobic cavities with the appropriate size to accommodate another molecule by forming inclusion compounds through host–guest interactions. Formation of the inclusion complex can increase the guest molecule's stability against hydrolysis, oxidation, photodecomposition, and dehydration. Upon inclusion, the use of CDs in pharmaceutical formulations has been shown to enhance drug stability, solubility, and bioavailability.[14–22]

Address for correspondence: Tong Zhang, Experiment Center for Teaching, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, PR China. Tel: 86-021-51322318. E-mail: zhangtdmj@sohu.com

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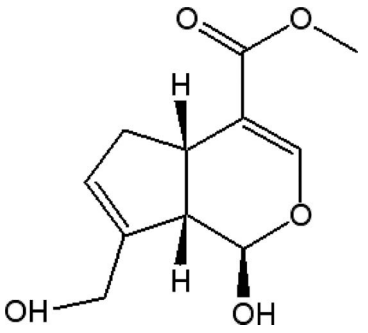

Figure 1. The chemical structure of genipin.

In this study, with an aim to improve the stability, solubility, and bioavailability of GP, the inclusion complex of GP–β-CD was prepared by lyophilization method. The formation of GP–β-CD complex was evaluated by infrared (IR) spectroscopy, ultraviolet (UV) spectra, differential scanning calorimetry (DSC)[27–30], X-ray diffractometry (XRD), and phase solubility method. A stability-comparing test of the inclusion complex of GP–β-CD and free GP in buffer with the pH of 6.8 was performed. In addition, the pharmacokinetics study of free GP and the inclusion complex of GP–β-CD in mice via intravenous administration had been carried out to compare their bioavailability, whereas a sensitive high-performance liquid chromatography (HPLC) method was developed to assay the concentration of GP in mice plasma.

Materials and methods

Materials

GP was purchased from Challenge Bioproducts Co., Ltd. (Taiwan, China). β-CDs were supplied by China Pharmaceutical (Group) Shanghai Chemical Reagent Co. Ltd. (Shanghai, China). Puerarin (internal standard, IS) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile and methanol for HPLC analysis were obtained from Merck (Darmstadt, Germany). All other reagents and solvents were of analytical grade. Distilled water was used throughout the experiment.

In vitro GP quantification

The Agilent 1100 HPLC system was used in the concentration assay. The reversed-phase HPLC column (Kromasil 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 method. The mobile phase, consisting of acetonitrile and water in a ratio of 15:85 (v/v), was filtered through 0.45-μm nylon filter (Alltech Associates, Inc., Deerfield, IL, USA) and degassed in an ultrasonic bath (Branson, Shanghai, china) before use. All samples were analyzed at a flow rate of 1 mL/min after injected into a 20-μL loop, with UV detection wavelength for GP at 238 nm and the column temperature 30°C.

Preparation of the inclusion complex of GP–β-CD

The inclusion complex of GP–β-CD was prepared by lyophilization method. Briefly, 50 mg GP was dispersed into 30 mL β-CD aqueous solution (7.5 mM), and the obtained suspension was magnetically stirred for 2 hours at room temperature. After the suspension was filtered through a 0.22-μm polytetrafluoroethylene (PTFE) filter, the filtrate was lyophilized.

Characterization of the inclusion complex of GP–β-CD

IR spectra measurement

Fourier transform infrared (FTIR) spectra of GP, β-CD, GP–β-CD physical mixture, and the inclusion complex of GP–β-CD were taken with a AVATAR 330 FTIR spectrophotometer (Thermo Nicolet Corporation, Waltham, MA, USA). The prepared KBr disks contained 0.01 g of sample per 0.1 g, scanning from 400 to 4000 cm^{-1} spectral region.

UV spectroscopy

Inclusion complex formation between GP and β-CD in water was studied by the spectral shift method. In the sample inclusion complex, the concentration of GP was 2 μg/mL whereas the β-CD concentration varied from 2 to 15 mmol/L, respectively. After the mixtures were stirred for 4 hours, the UV absorption spectra were recorded by a UV-visible spectrophotometer (Hp8453E, China Hewlett-Packard Co., Ltd., Beijing, China). As a control, the absorbance peak of free GP in aqueous solution was also obtained.

Differential scanning calorimetry

The thermal behaviors of the samples of GP–β-CD, the physical mixtures at the molar ratio of 1:1, free GP, and β-CD were studied by DSC (NETZSCH STA 409 PC/PG, German). The weighed samples were heated in sealed aluminum pans with empty aluminum pans sealed as reference, over the temperature range of 25–300°C, at a rate of 1.80°C/min. Nitrogen was used as the carrier gas. The sample size was 1.938, 3.080, 2.610, and 3.370 mg.

X-ray powder diffractometry

Powder X-ray diffraction (XRD) patterns were recorded on an X-ray diffractometer (Rigaku-D/MAX-2550PC, Rigaku Corporation, Tokyo, Japan) using a Ni-filtered Cu K(α) radiation under a voltage of 40 kV and a

current of 100 mA. The scanning rate employed was $0.02°s^{-1}$ over the angle 2θ range of 0–50°. The XRD patterns of free GP, inclusion complex, and their physical mixtures were recorded.

Phase solubility study

The aqueous solubility of GP at various concentrations of β-CD was studied according to the method reported by Higuchi and Connors.[23] The excess amount of GP was added to β-CD aqueous solutions (50 mL) and shaken at room temperature with increasing concentrations (0–15 mmol/L), for a period of 48 hours, until equilibrium was attained. The samples were prepared by filtering through a 0.22-μm PTFE filter and assayed by HPLC for GP content.

The phase-solubility diagram was obtained by plotting the solubility of GP versus the concentration of β-CD. The apparent 1:1 stability constant of the inclusion complex of GP–β-CD was calculated from the phase-solubility diagram:

$$K_c = \frac{\text{slope}}{S_o(1-\text{slope})}$$

where K_c is the stability constant (M^{-1}), slope is obtained from the linear relationship between the concentration of GP and β-CD and S_0 is GP solubility (25°C, M). Each experiment was carried out in triplicate ($RSD < 3\%$).

In vitro stability assessment

The inclusion complex of GP–β-CD (5 μg/mL of GP) and free GP (5 μg/mL) were dissolved in aqueous solution at a temperature of 50°C. The concentration of GP in both solutions was assayed by HPLC at proper time intervals for 48 hours to compare the stability.

Pharmacokinetics in mouse plasma

Pharmacokinetic studies were performed on male Kunming mice (22 ± 2 g) obtained from the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine (Shanghai, China). The animal experimentation was approved by the Animal Ethics Committee of Shanghai University of TCM (Shanghai, China). Before the experiment, all the mice were adaptively fed for 1 week, 12-hour fasting for and free drinking before the experiment. Seventy mice were randomly divided into two groups, each group with 35 mice. For intravenous administration, the inclusion complex of GP–β-CD and the free GP were redissolved in liquor natrii chloridi isotonicus. The intravenous bolus was given to mice (n = 5) in the caudal vein at a dose of 10 mg/kg mice. Blood samples from mice were collected via retro-orbital sinus into heparinized Eppendorf tubes at 5, 10, 15, 30, 45, 60, and 90 minutes after administration. Then the samples were immediatedly prepared by centrifugation.

To assay GP, 200 μL plasma sample, after adding 20 μL hydrochloric acid (2 N) and 50 μL internal standard (Puerarin, 1.1 μg/mL) and vortexing for 1 minute, was extracted with 3 mL of acetic ether by vortex mixing for another 2 minutes and centrifuged at 4000 rpm for 5 minutes in 5-mL centrifuge tubes. After centrifugation, the organic supernatant was poured into a clean tube and evaporated to dryness under a gentle stream of nitrogen. The resulting dry products were redissolved with 200 μL mobile phase, and then 20 μL of it was subjected to HPLC analysis. The pharmacokinetic parameters were calculated by DAS VER 2.0 (Drug and Statistics software, which was developed by Mathematical Pharmacology Professional Committee of China and authorized and supported by China's State Food and Drug Administration).

Results and Discussion

IR spectroscopy

The FTIR spectra of wave number from 4000 to 400 cm^{-1} are shown in Figure 2. The pure GP has intense absorptions peaks at 3397.6, 3242.2, 1681.4, 1621.7, 1443.21, and 1301.30 cm^{-1}, which are the characteristic peaks for identification. The spectrum of pure β-CD showed characteristic peaks at 3404.6 cm^{-1}. The spectrum of the physical mixture is well matched with that of β-CD spectrum in about 2944.1 cm^{-1} and the crystalline drug spectrum in about 3396.6 cm^{-1}, which indicated that no interaction occurred during simply physical mixing of the free drug and β-CD. The spectrum of the inclusion complex of GP–β-CD shows a broad peak at around 3400 cm^{-1} and the less-intense absorption at around 1681, 1621, 1443, and 1301 cm^{-1}, with the disappearance of the sharp absorptions at 3400 and 3242 cm^{-1}, which were observed in the spectrum of GP. It led us to the result that some groups of GP might be included in the cavity of β-CD molecules to form the inclusion complex.

UV spectroscopy

The inclusion complex composed of GP and β-CD in aqueous solution was characterized by using UV spectroscopy. Figure 3 illustrates the effects of β-CD's concentration on the spectra of GP in aqueous solution. Absorption peak of GP in aqueous solution is exhibited at 238 nm. The absorbance of GP varied significantly with the addition of β-CD. The increase in β-CD's

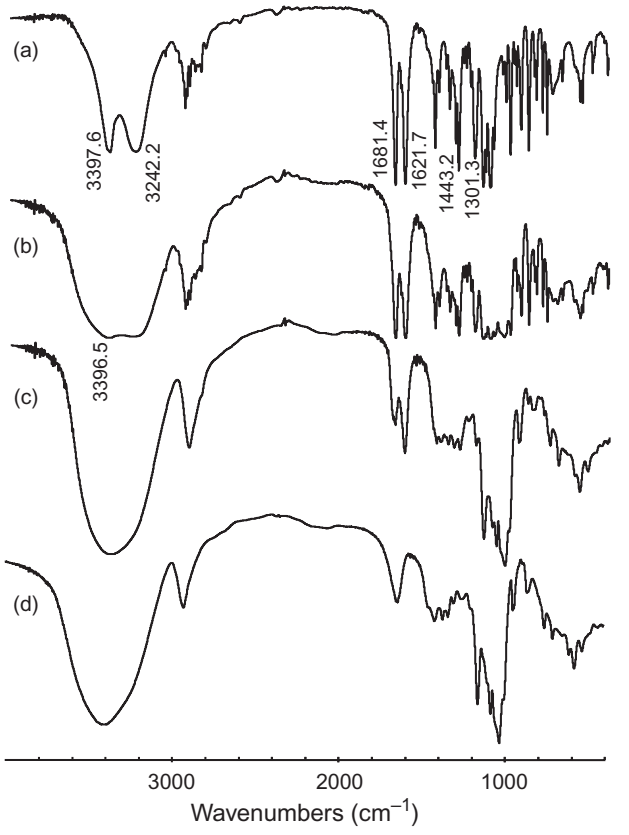

Figure 2. IR graphs of (a) GP, (b) physical mixture of GP–β-CD, (c) the inclusion complex of GP–β-CD, and (d) β-CD.

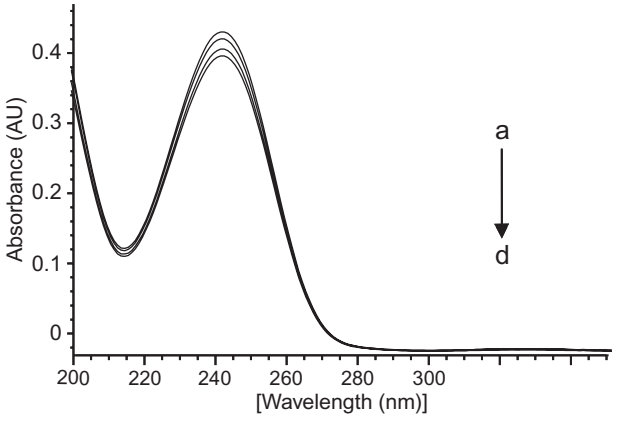

Figure 3. The effect of β-CD concentration on the UV absorbance of GP in aqueous solution; the β-CD concentration was (a) 2 mmol/L, (b) 4 mmol/L, (c) 6 mmol/L, and (d) 15 mmol/L, respectively.

concentration from 2 to 15 mmol/L resulted in a different decrease in the absorbance of GP, compared with that of the free GP aqueous solution at equal concentration. These changes might be partly attributed to the shielding of chromophore groups in GP molecule, which is possibly the results of the complex formation between GP and β-CD through hydrophobic interaction.[24]

Differential scanning calorimetry

The thermograms of β-CD, GP, the inclusion complex, and the physical mixture are shown in Figure 4. The DSC diagram of GP exhibited a sharp endothermic peak at 123.7°C, indicating the melting point of GP. Another sharp peak at 220.5°C was attained in the diagram, which might be a degradation temperature of GP. The thermogram of the inclusion complex showed a sharp endothermic peak at the dissociation temperature of 214.8°C, without any trace of GP's melting peak of 123.7°C, which indicated that guest–guest interactions were completely replaced with guest–host interactions and presented the formation of the complexation. However, after the investigation of the diagram of the physical mixture, it could be deduced that the melting peak of GP still existed, which decreased to 110.7°C. The DSC resulted gave the evidence that free GP was included within the central cavity of the β-CD molecule in the conclusion complex, whose thermal property was partly similar to β-CD.

X-ray powder diffractometry

X-ray powder diffraction patterns of plain GP, physical mixture, corresponding to the inclusion complex and β-CD, are shown in Figure 5. The XRD of physical mixture was observed to be almost the overlap between that of β-CD and that of free GP, which indicated that in the physical mixture there was no formation of a new crystal form. However, compared with the diffractogram of

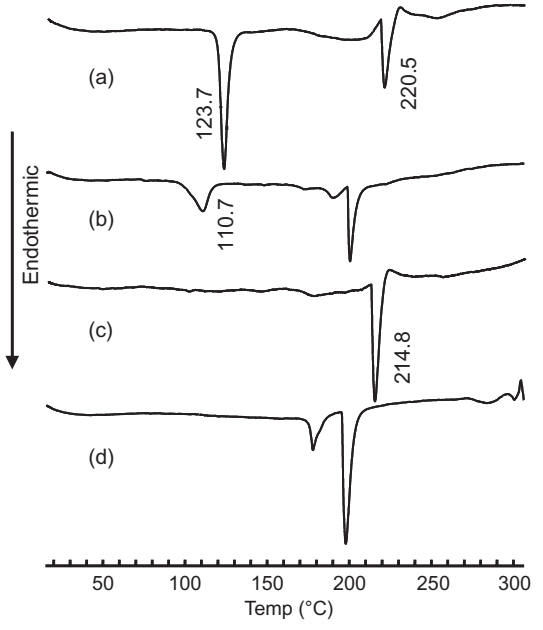

Figure 4. DSC thermogram of (a) GP, (b) physical mixture of GP–β-CD, (c) the inclusion complex of GP–β-CD, and (d) β-CD.

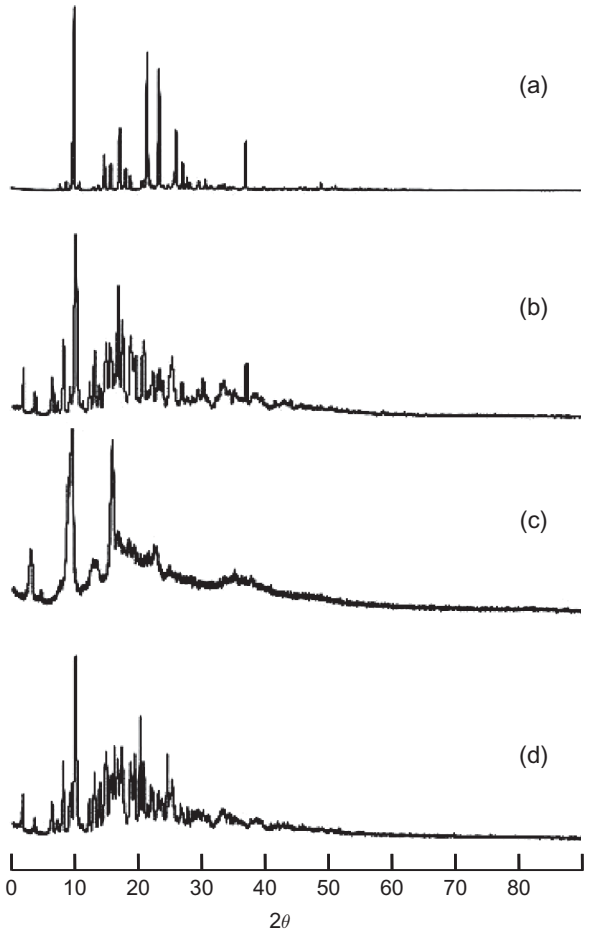

Figure 5. XRD patterns of (a) GP, (b) physical mixture of GP–β-CD, (c) the inclusion complex of GP–β-CD, and (d) β-CD.

GP and the physical mixture, some sharp peaks were missing, whereas other new dispersed peaks were detected in the XRD pattern of GP–β-CD inclusion system, which strongly suggested the formation of the inclusion complex.

Phase solubility studies

The solubility of GP in distilled water was affected by the presence of β-CD. The obtained GP's apparent solubility showed a linear relationship with β-CD concentration (Figure 6) and conformed to Higuchi and Connors's results and could be classified as A_L type, where complexes formed were of the first order with regard to the host molecule. The slope of solubility diagram was less than 1; it was therefore presumed that the solubility increase could be attributed to the formation of the 1:1 complex. Stability constant value (K_c) obtained for the GP–β-CD complex was 497 M^{-1}, which indicated that the GP–β-CD complex at a 1:1 ratio is relatively stable.[21]

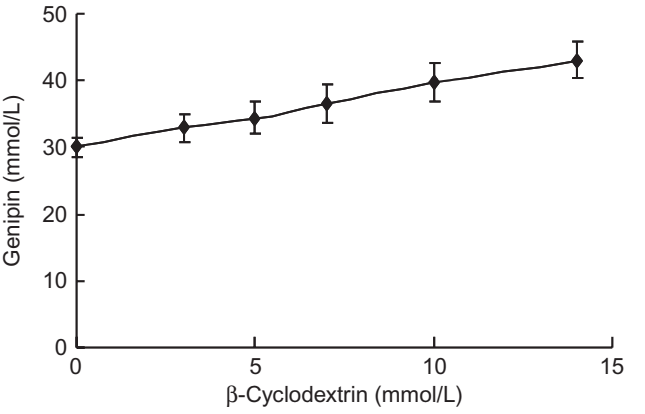

Figure 6. Phase solubility diagram of GP in 0–15 mmol/L β-CD aqueous solution (mean ± SD, $n = 3$).

In vitro dissolution studies

The in vitro dissolution profiles of the drug and inclusion complex are shown in Figure 7. The solubility of complexes was much better compared with the drug alone. The solubility profile of the complex showed that 73.0% drug was released in 3 hours while that of the pure drug showed 58.7%. This enhancement can ascribe to the more hydrophilic property of the systems, which may reduce the interfacial tension between the drug and the dissolution media.[25] Moreover, the GP–β-CD inclusion complex dissolves more rapidly than the drug at the early stage of the dissolution process. Hence, it can act on the hydrodynamic layer surrounding the drug particles, resulting in an in situ inclusion process that improves the solubility of the drug.[25] In fact, the systems containing a greater amount of β-CD dissolve relatively faster. Therefore, it must be pointed out that the freeze-dried powder of GP–β-CD complex at the molar ratio of 1:1 showed improved drug solubility compared with its free drug.

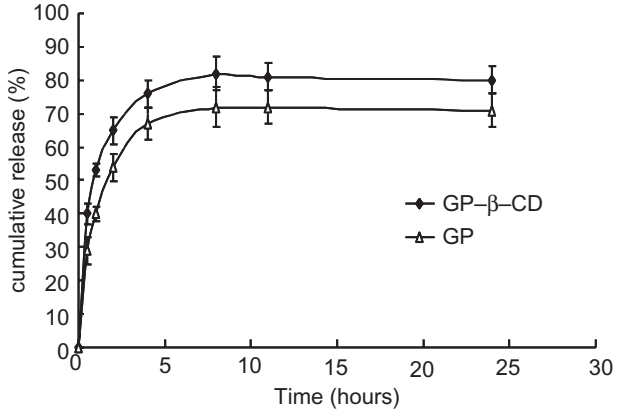

Figure 7. Dissolution profile of GP and the inclusion complex of GP–β-CD (mean ± SD, $n = 3$).

In vitro stability

The in vitro stability of the drug and inclusion complex is shown in Figure 8. The result demonstrated that the inclusion complex formation of GP and β-CD could significantly improve the stability of GP in aqueous solution, with the data of 85.2% of GP detected in the inclusion complex solution, while only 18% in the free drug solution at the temperature of 50°C after 48 hours.

Pharmacokinetics in mouse plasma

The representative HPLC chromatograms of a plasma sample after intravenous administration at a dose of 10 mg/kg of GP are shown in Figure 9. The calibration curve of GP was linear over the concentration range of 0.052–20.96 μg/mL, fitting the equation: y = 2.3995x + 55.3743 (r = 0.9992). The relative standard deviation (RSD) obtained for interday and intraday precision, at low, medium, and high concentration solutions (0.055, 1.1, and 20.0 μg/mL, respectively), was less than 8.5%. The recoveries of GP at the three concentrations (n = 5) were 105 ± 8.9%, 85.6 ± 7.3%, and 81.3 ± 6.4%, respectively. The LOQ and LOD for GP were 0.05 and 0.04 μg/mL, respectively.

Results of GP in the plasma (Figure 10) showed significant difference between the groups treated with the inclusion complex of GP–β-CD and with free GP. In the

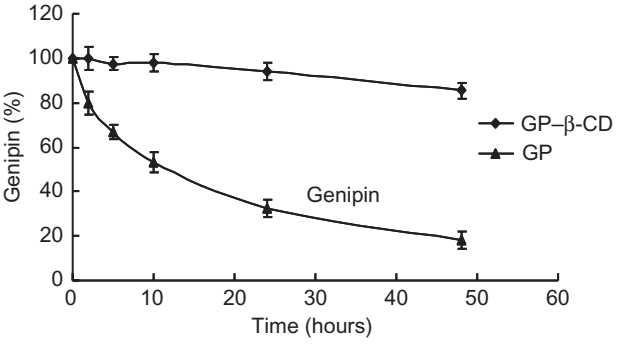

Figure 8. Stability of GP and the inclusion complex of GP–β-CD (mean ± SD, $n = 3$).

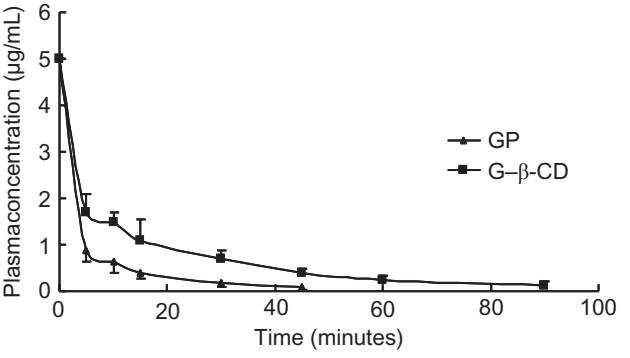

Figure 10. Mean GP concentration-time curve in mouse plasma after injection of GP (10 mg/kg) to mice. Each point indicates means ± SD of five mice.

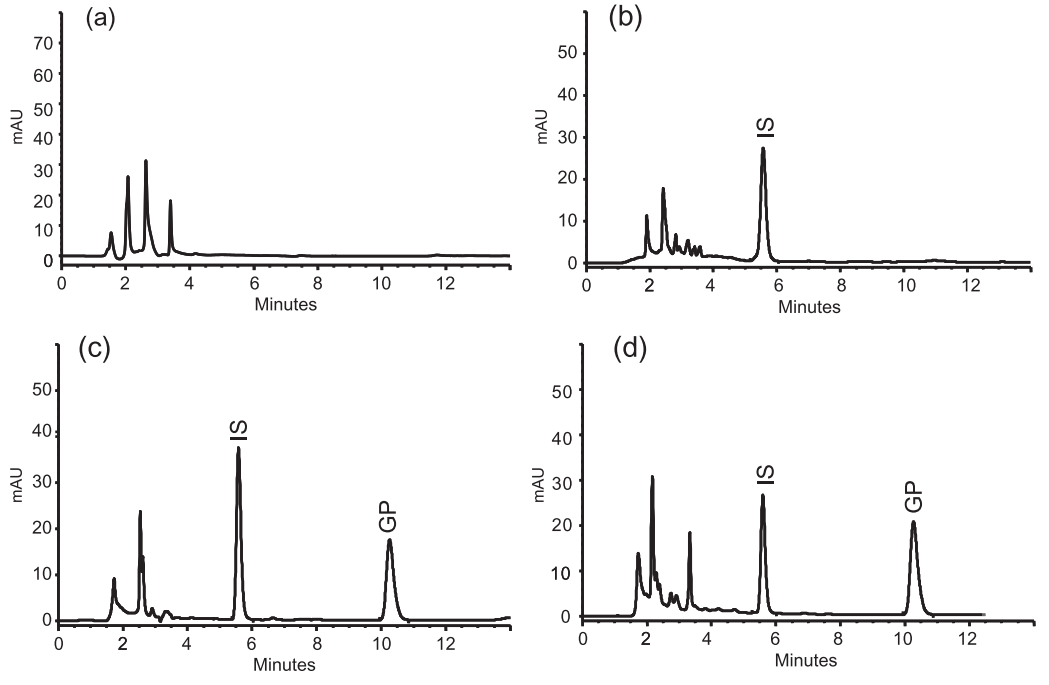

Figure 9. (a) Blank plasma; (b) blank plasma with IS (8 μg/mL); (c) a representative chromatogram of GP (5 μg/mL) and IS (8 μg/mL) added to plasma; (d) representative chromatogram of GP and IS in pharmacokinetic sample. IS, internal standard, Puerarin.

Table 1. Pharmacokinetic parameters following intravenous administration of the inclusion complex of GP-β-CD or free GP to mice in a dose of 10 mg/kg.

Pharmacokinetic parameters	GP	GP–β-CD
AUC_{0-t} (mg/L)	0.882	1.71*
AUC_{0-t} (mg/L h)	0.304	0.934*
$AUC_{0-\infty}$ (mg/L h)	0.321	0.98*
MRT_{0-t} (hours)	0.202	0.402*
$MRT_{0-\infty}$ (hours)	0.259	0.522*
$t_{1/2z}$ (hours)	0.078	0.354*
CL_z (L/h/kg)	31.122	10.208*
V_z (L/kg)	8.115	5.212

* $P < 0.01$ versus GP.

free GP group, GP was quickly metabolized with the reduced concentration of GP to a very low level (close to the LOD) after 45 minutes of the administration. However, in the inclusion complex group, GP seemed to keep relatively higher concentration level and eliminate slowly in vivo, which could still be detected 1.5 hours post-i.v. The pharmacokinetic parameters and statistical results are listed in Table 1. By comparison, the CL_z of the inclusion complex of GP–β-CD and free GP suggests significant deviation ($P < 0.01$) between the two groups and the reduced CL_z increased $t_{1/2z}$, MRT, and AUC in the inclusion complex of GP–β-CD group. Examining the results from the group analysis, the $t_{1/2z}$ and MRT of GP of the inclusion complex of GP–β-CD group was significantly prolonged than that of free GP group ($P < 0.01$). By comparing the $AUC_{0-\infty}$ of GP between the groups, the relative bioavailability of the inclusion complex of GP–β-CD to free GP was as high as 305.3%.

Conclusion

The influence of solvent's pH environment on the stability of GP aqueous solution was also investigated, through detecting the GP concentration after preparing some 11.8 μg/mL GP solutions by different pH buffers (pH 3.8 PBS, pH 5.3 PBS, pH 7.2 PBS, pH 9.1 PBS) and putting them at room temperature for about 24 hours. The resulting detected concentrations of GP decreased to 82.8%, 79.1%, 68.3%, and 12.3% of the initial concentration, respectively, which showed significant influence of pH on GP aqueous solution. The results showed that GP was more stable in acid solution (pH ≤ 5.3) whereas it degraded quickly in alkalized buffer solutions. So, it could be deduced that GP was degraded in plasma, which was a weak alkalized environment with the pH of 7.4. Moreover, GP is unstable and prone to be hydrolyzed in aqueous solution,[26] which was verified in our experiment. Our studied on GP indicated that the inclusion complex of GP–β-CD was more stable than free GP in aqueous solution, which had physicochemical characteristics differentiating it from GP. In addition, through inclusion with β-CD, the dissolution of GP was also improved to some extent in aqueous solution.

Complexes formation has been studied. The differences in UV spectra of GP–β-CD complex and of the free GP indicated the interaction of the drug with β-CD, that is, the formation of complex occurred. The difference of IR spectroscopy between the inclusion complex of GP–β-CD and the physical mixture of GP–β-CD notified the formation of the inclusion complex. The SEM photomicrographs of the inclusion complex formed by GP and β-CD, of the physical mixture of the crystalline substance, implied the variations of the crystalline structure, which hinted the formation of the inclusion complex. The results obtained from XRD studies suggested the formation of complex between GP and β-CD. The solid system of GP and β-CD prepared by lyophilization method presented a good stability and solubility performance. The results obtained by different characterization techniques clearly indicated that the lyophilization led to the formation of complexes between GP and β-CD. Moreover, the comparison of pharmacokinetics of GP and its complex with β-CD in mice indicated that the inclusion complex of GP–β-CD had longer $t_{1/2z}$ and $MRT_{0-\infty}$, and better bioavailability. In conclusion, the results indicated that the inclusion complex could significantly improve GP's stability and dissolution properties in vitro, and bioavailability in vivo, which allowed us to conclude that the complex of GP with β-CD is quite preferable and of an important reference to the clinical application of GP, leading to a better therapeutic prospect.

Acknowledgments

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Declaration of interest: The authors report no conflicts of interest.

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