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Physicochemical properties of bergenin

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Physicochemical properties of bergenin, namely the solid state characteristics, solution stability, dissociation constant (pK_a), solubility and octanol/water partition coefficient (Log P) were investigated. The results showed that the drug was not hygroscopic and was stable against heat and humidity in the solid state, but sensitive to hydrolysis in the neutral and alkaline solution with a pseudo first-order kinetics. The compound exhibited pK_{a1} of 5.46 ± 0.13 and pK_{a2} of 5.74 ± 0.18. The solubilities in buffers were slight (e.g. 1.29 ± 0.044 mg/ml (pH 1.0), 1.08 ± 0.057 mg/ml (pH 3.0) and 1.22 ± 0.058 mg/ml (pH 5.0), at 25 °C), but increased considerably at high temperatures (e.g. 8.76 ± 0.039 mg/ml (pH 1.0), 6.75 ± 0.095 mg/ml (pH 3.0) and 7.80 ± 0.075 mg/ml (pH 5.0), at 60 °C) with chemically stability. The Log P ranged from -1.06 ± 0.033 to -1.19 ± 0.044 at pH 1.0–6.0 at 37 °C, which demonstrated the poor lipophilicity of bergenin at acidic pHs.

1. Introduction

Bergenin (a C-glucoside of 4-*O*-methyl gallic acid) is the major component of the traditional Chinese medicine *Bergenia crassiflora* (Saxifragaceae). It has been said to be effective in relieving cough and treating some gastrointestinal diseases such as gastric ulcer, diarrhea and constipation (Abe et al. 1980). In addition, it has been reported to have anti-inflammatory (Nazir et al. 2007), anti-arrhythmic (Pu et al. 2002), anti-HIV (Paciente et al. 1996), hepatoprotective (Kim et al. 2000), neuroprotective (Takahashi et al. 2003), wound healing (Kimura et al. 2007), and trypanocidal activities (Nyasse et al. 2004). Bergenin has been used widely over a century with very few adverse reactions reported (Wang and Ma 2002).

Bergenin

Despite those advantages, the low bioavailability of bergenin has puzzled people for many years. It was reported that after oral administration in humans, bergenin was absorbed quickly but incompletely with a short half life and low bioavailability. The C_{max} appeared at 1–4 h in plasma and at 2–7 h in urine after IM administration in dogs (Jiangsu New College Med. 1999). Shi et al. (2006) concluded that the low bioavailability might be attributed to the easy degradation of bergenin in the digestive system. But no further studies of bergenin stability after oral administration have been reported.

Nowadays, bergenin is commercially available in various pharmaceutical forms, such as tablets, dripping pills, soft capsules, etc. Several new pharmaceutical methods, such as the solid dispersion, have been developed for promoting the drug's solubility and dissolution so as to enhance the bioavailability (Sun et al. 1987). But unfortunately, few publications address consequences of these approaches. Yet the effects of other fundamental physicochemical properties, such as stability, dissociation constant (pK_a) and partition coefficient, on the formulation and *in vivo* absorption are not clear. Therefore, it is important to investigate physicochemical properties of bergenin to explore the possible causes of the low oral bioavailability and put forward some corresponding suggestions. To this end, this paper shows the results of a study carried out to establish the main properties of bergenin, which will be of great value for the enhancement of the drug's bioavailability and the development of new oral dosage forms.

2. Investigations, results and discussion

Powder X-ray diffraction patterns of sample A, B and C are shown in Fig. 1. Crystalline forms of bergenin showed sharp diffraction peaks with high intensity and the majority of peaks were consistent for all the three samples. The results indicated that the architecture of the crystal forms for all of them were almost the same. The solubility test and hygroscopicity test did not modify the arrangement of molecules in samples.

The weight increase of bergenin stored at 57.5% ~ 98% R.H (25 °C) are shown in Fig. 2. It was demonstrated that the bulk drug gained weight very slightly (0.39% ~ 0.43% w/w), even at the highest humidity (0.40% w/w, 98% R.H). Since bergenin used in this study was in crys-

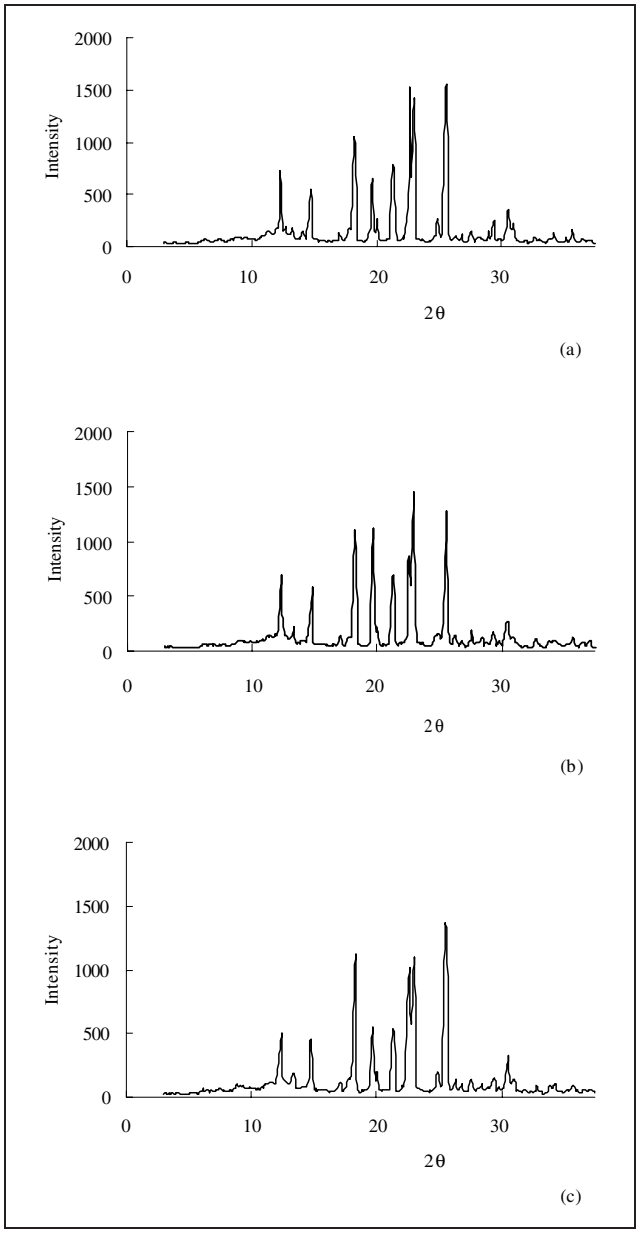

Fig. 1: Powder X-ray diffraction patterns of bergenin obtained from different environments (a) sample A: the commercial bulk; (b) sample B: excess solids remaining at the conclusion of solubility test; (c) sample C: solids of hygroscopicity test after 12 days

talline state, according to Ohta's report (2000), the water is taken up predominantly by surface adsorption, which leads to the low hygroscopicity. Furthermore, Ye et al. (2004) have demonstrated that there are one intra- and six intermolecular hydrogen bonds which form an extensive hydrogen-bonding network within the crystal, thus it is not expected to have a great number of energetic (active) sites that favor water sorption (Carvajal and Staniforth 2006). Generally, the low water uptake of bergenin suggested good stability to moisture during formulation and storage.

The effect of humidity and heat on the stability of bergenin was tested at 75% R.H (40 °C) for 4 weeks. Neither color change nor deliquescence of the drug was inspected visually. The contents were $101.31 \pm 0.018\%$ relative to control, which implied that bergenin was stable under the conditions applied.

The stability of the aqueous solutions were tested at both 25 °C and 37 °C in various buffers (pH 1.0, 3.0, 5.0, 7.0

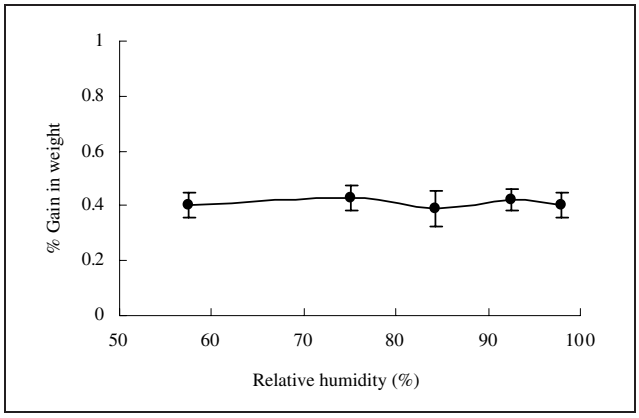

Fig. 2: Adsorption isotherms of bergenin

and 8.0). Degradation was observed under the neutral and alkaline conditions, while the drug was shown to be stable at acidic pHs (1.0, 3.0 and 5.0). The susceptibility of bergenin to decomposition was determined as a decrease concentration during the time course of the experiment (Fig. 3). The results indicated that bergenin was susceptible to degradation at neutral-basic pH conditions and the rates of degradation increased with increase in pH. Also, increase in temperature markedly accelerated the degradation process. At 37 °C, the remaining amounts of bergenin after 24 h incubation at pH 7.0, 8.0 were 49.3% and 3.59%, respectively, where as it was 82.7% and 51.4% at 25 °C.

In order to characterize the kinetics of the compound degraded at various pH values at the two temperatures, the logarithmic concentrations of the residual bergenin in each sample were plotted as a function of time (Fig. 3). The linear behavior of these plots indicated that degradation followed pseudo first-order kinetics at two tested temperatures. The pH dependencies of the overall first-order degradation rate constants of bergenin are shown in Table 1. At 25 °C, the observed reaction rate constant, k_{obs}, was 7.9×10^{-3} h^{-1} at pH 7.0 ($r = 0.977$), while it was approximately 4-fold higher (28.2×10^{-3} h^{-1}) at pH 8.0 ($r = 0.998$). As the degradation rate increased at higher temperature (37 °C), k_{obs} rose up to 27.6×10^{-3} h^{-1} (pH 7.0, $r = 0.989$) and 135.5×10^{-3} h^{-1} (pH 8.0, $r = 0.997$).

The HPLC chromatogram showed that a new peak was detected in the aqueous samples at pH 7.0 and 8.0. This compound was apparent after 2 h incubation at 25 °C and 30 min at 37 °C. The corresponding peak area on the chromatogram increased with time significantly, 19-fold at 12 h and 31-fold at 24 h, compared with that at 0 h. It can be deduced that the degradation product was more hydrophilic than bergenin since it was less retained by the C_{18} column and eluted at 2.3 min without interfering with bergenin peak.

However, as an isocoumarin (Kobayashi and Mejìa 2005), some properties of bergenin are similar to that kind of coumarin, which contains an α-pyrone ring with an α,β-unsaturated lactone structure. The lactone of coumarin tends to hydrolyze in alkaline medium to form a salt of coumarin acid, and the acidification of the salt solution causes the recovery of the cyclic structure, which contributes to the acidic stability indirectly (Wu and Wu 2003a). Considering the hydrolytic splitting of the lactone, bergenin could undergo the same alkaline hydrolysis as coumarin, thus the presence of a hydrophilic product on the chromatograms would be expected.

To determine the degradation mechanism of bergenin, both the degradation product and the parent drug were

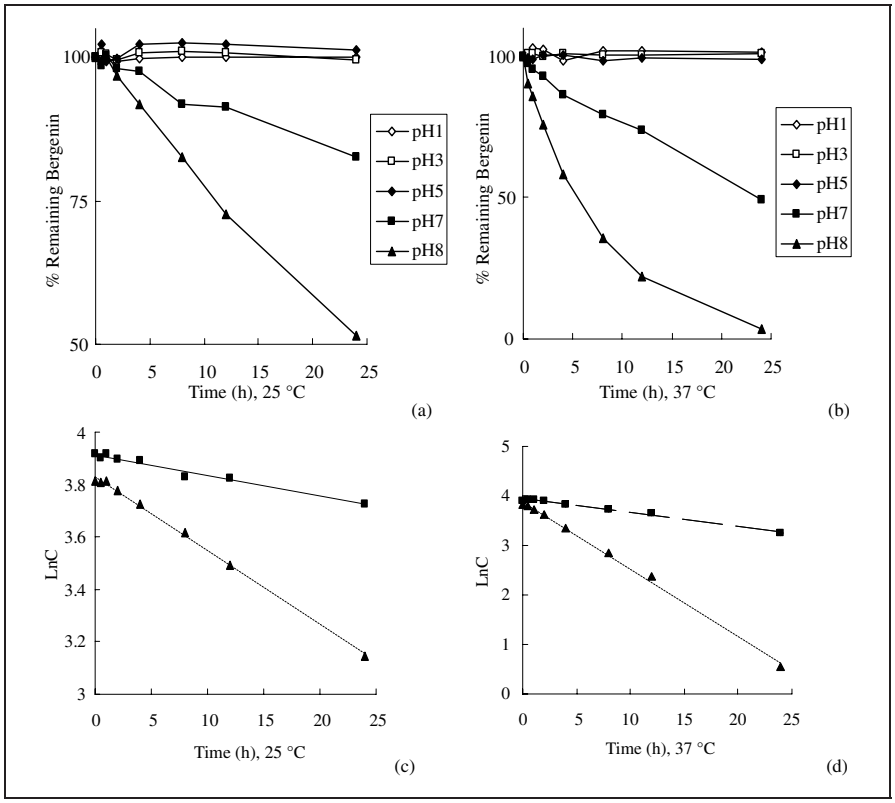

Fig. 3:
The pH-profile stability of bergenin at 25 °C (a), (c); 37 °C (b), (d); pH 1 (—◇—), pH 3 (—□—), pH 5 (—◆—), pH 7 (—■—), pH 8 (—▲—)

identified by MS analysis corresponding to peaks observed in the LC chromatograms. The MS spectrum associated with the new peak confirmed that it coincided with the hydrolytic product with m/z 347.2 of $[M + H^+]$ ion, while the mass spectrum associated with the parent drug with m/z 329.3 of $[M + H^+]$ ion. The results indicated that the degradation pathway of bergenin in alkaline media was the hydrolysis of the lactone bond.

Therefore, the low oral bioavailability of bergenin could be partially attributed to its instability in neutral and alkaline physiological environments, such as the intestinal tract and the blood, which was in agreement with the conclusions of Shi et al. (2006) and Qin et al. (2007). Also, certain acidic substances, such as tartrate, malate and citric acid, could be considered to be added into the formulation as a stabilizer to avoid drug's decomposition during the preparation of dosage forms and after oral administration.

The titration curve showed a biprotic acid profile with two potential jumps (Fig. 4). Two dissociation constants were derived as $pK_{a1} = 5.46 \pm 0.13$ and $pK_{a2} = 5.74 \pm 0.18$, indicating a very weak acidic property. Then it could be predicted that the molecules of bergenin could mostly ionize in neutral and alkaline physiological environments. Since the non-ionized molecules are more lipid soluble (Luger et al. 1996), the weak acidic property will favor its absorption in the upper gastrointestinal tract.

The results of solubility studies at different pHs and temperatures are provided in Table 2. The data obtained at pH 7.0 and 8.0 were excluded from this study because bergenin underwent a pH sensitive decomposition at neutral and alkaline pHs. At each temperature, the solubilities changed but were not proportional to the pHs. At 37 °C, the solubility at pH 3.0 (2.13 ± 0.061 mg/ml) was lower than that at pH 1.0 (2.40 ± 0.089 mg/ml) and pH 5.0 (2.53 ± 0.094 mg/ml). Besides, the low solubility of bergenin can be explained by the low hygroscopicity of the crystalline form of the

Table 1: Observed rate constants and $t_{1/2}$ for the degradation of bergenin

Temperature (°C)	pH of PBS	$k_{obs} \times 10^{-3}(h^{-1})$	$t_{1/2}$(h)
25	7	7.9	50.13
	8	28.2	14.04
37	7	27.6	14.35
	8	135.5	2.92

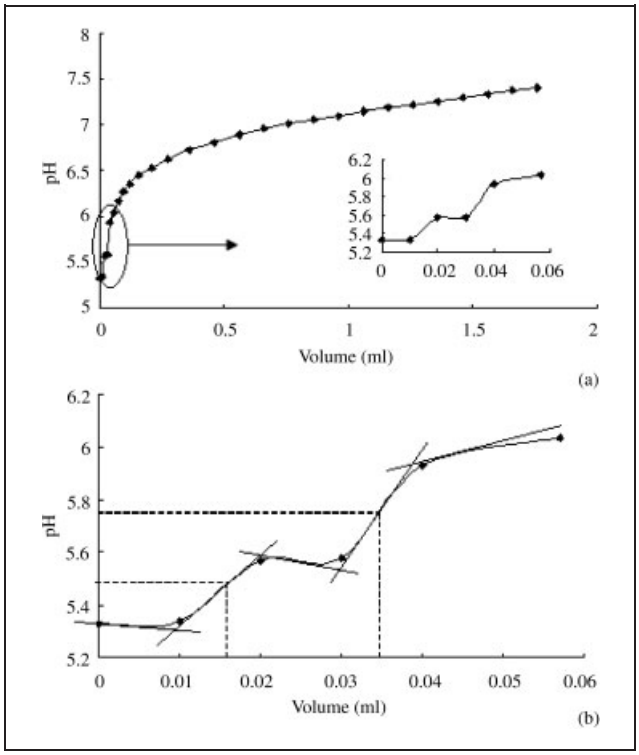

Fig. 4: The potentiometric titration curve of bergenin (a) over the pH range (pH 5.3 ∼ pH 7.4); (b) over the pH range (pH 5.3 ∼ pH 6)

Table 2: Solubilities of bergenin in aqueous solutions at different pHs and temperatures (n = 3)

Temperature (°C)	Solubility in buffers of different pH (mg/ml)		
	1	3	5
25	1.29 ± 0.044	1.08 ± 0.057	1.22 ± 0.058
37	2.40 ± 0.089	2.13 ± 0.061	2.53 ± 0.094
45	3.82 ± 0.068	2.96 ± 0.077	3.52 ± 0.065
50	4.52 ± 0.041	3.99 ± 0.026	5.80 ± 0.057
60	8.76 ± 0.039	6.75 ± 0.095	7.80 ± 0.075

drug substance and the presence of the C-glucoside structure, which results in poor solubility in many solvents such as water, ethanol et al. (Wu and Wu 2003b). According to Posti et al. (2000), drugs having limited solubility (below 1%) in the gastrointestinal fluids often exhibit poor or erratic absorption. Thus, new dosage forms with promoting solubility and dissolution are feasible to enhance the oral bioavailability. This is also the reason why people chose the solid dispersion for promoting bergenin's solubility and dissolution (Sun et al. 1987).

On the other hand, the effect of increasing temperature on solubility was much more significant than that of pH. The solubility of drug at 60 °C was approximately 7 times better than that at 25 °C and 2 times than 37 °C, indicating that this process was endothermic. Thermodynamic parameters of solution were obtained by determining solubilities at five different temperatures. The natural logs of the solubilities were plotted versus the reciprocal of the absolute temperatures (van't Hoff plot; Fig. 5). The change in enthalpy and entropy of solution, $\Delta_{sol}H$ and $\Delta_{sol}S$, respectively, were calculated from the slope and intercept of the van't Hoff plot by linear regression Eq. (1).

$$\text{Ln } S = \Delta_{sol}S/R - \Delta_{sol}H/(RT) \quad (1)$$

The van't Hoff plots for pH 1.0 and pH 3.0 were quite the same, thus could not be distinguished very well in Fig. 5.

The $\Delta_{sol}H$ of solution was 44 kJ mol^{-1}, a positive value, which means that the crystal lattice energy outweighed the solvation energy (Perlovich 2007) and confirmed the endothermic solvation process of bergenin.

The $\Delta_{sol}S$ of solution was 0.15 kJ $mol^{-1}K^{-1}$, suggesting an overall increase in the systemic disorder which was

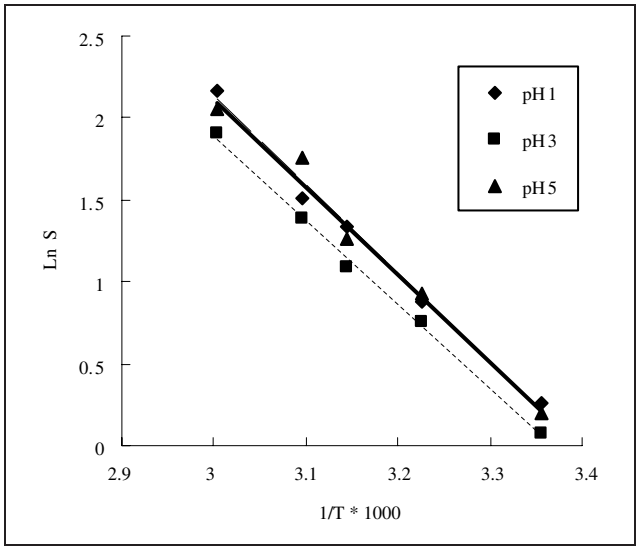

Fig. 5: The van't Hoff plot of Ln S vs. 1/T for bergenin at pH 1 (—◆—); pH 3 (—■—); pH 5 (—▲—)

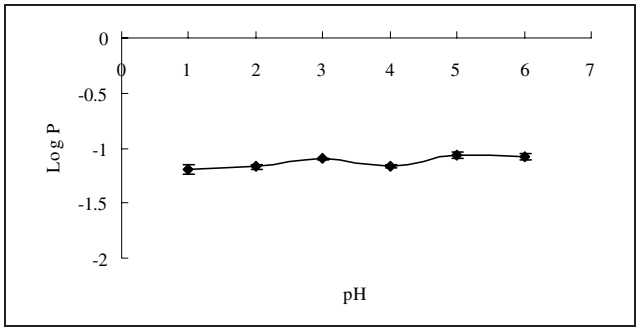

Fig. 6: The pH-partition coefficients profile of bergenin at different pHs

most likely due to the increasing of the thermal motion of drug's molecules (Hou and Zhan 2003).

Besides, according to Noyes-Whitney's equation, increase in temperature will accelerate the diffusion of drug molecules from the diffusion layer into the inner of the solution. Also temperature is an important factor influencing solubility. Taking these facts together, increase in temperature might be helpful for the bergenin formulation especially liquid preparations.

The lipophilicity of compounds exerts a key role in determining their penetration into cells, intestinal absorption and membrane permeability (Langloisa et al. 2005). After oral administration, a drug will encounter pH values from about 1.0 to 8.0 (Balbach and Korn 2004). In this study, the data obtained from pH 7.0 and 8.0 were of no significance because of drug's decomposition, therefore partition coefficients of bergenin were calculated at acidic pHs only. As can be seen from the pH-partition profile (Fig. 6), the Log P values were not high, ranging from -1.06 ± 0.033 to -1.19 ± 0.044, indicating poor membrane permeability (Hartmann et al. 2004), which lead to the poor absorption. The changes of the Log P values with the increasing pHs were very slightly, which was consistent with its characteristics as a weak acid. Thus, certain absorption enhancers may be considered during the formulation process.

In conclusion, various physicochemical properties of bergenin were investigated in this study for the first time. The solid state of bergenin was characterized by the PXRD and few transformation of crystal structure was observed. The drug substance was stable against heat/humidity, but sensitive to hydrolysis in the neutral and alkaline solution with pseudo first-order kinetics. The solubility values and apparent partition coefficients demonstrated that bergenin was neither highly hydrophilic nor lipophilic with chemical properties of a weak acid. Among these parameters, unfavorable ones including solution instability, inadequate aqueous and lipid solubility may be responsible for the low bioavailability of bergenin. In addition, some suggestions have been provided, which will be useful for bergenin's biopharmaceutical study and formulation development.

3. Experimental

3.1. Chemicals and reagents

Bergenin was purchased from Sichuan Dianhon Medical Development Co. Ltd (Chengdu, China) with chemical purity of 101.53%. Methanol and *n*-octanol (both guaranteed reagents) were supplied by Shanghai Luzhong Labor & Trade Co. Ltd (Shanghai, China) and Tianjin Meiling Labor & Trade Co. Ltd (Tianjin, China), separately. All other chemicals were analytical grade.

3.2. Chromatographic system

The analysis of bergenin was based on a reversed phase HPLC method. The HPLC instrument employed was Alltech™ (Alltech Technologies,

USA) LC system with a model 426 pump, a model UV-VIS-201 absorbance detector. The output signal was monitored and processed using a AllChrom™ Plus Chromatograph Data System (version 3.1.4, Multilink Services Co. Ltd). The columns were Dikma Diamonsil® C_{18} (150 × 4.6 mm, 5 μm) and Dikma EasyGuard 6101 C_{18} kit guard column. The mobile phase was a mixture of methanol-water (30 : 70, v/v, pH 2.5) at a flow rate of 1 ml/min. The wavelength was set at 275 nm. All analyses were performed at 30 °C.

3.3. Calibration curve

Stock solutions of bergenin (1 mg/ml) with different pH values were prepared by dissolving certain amounts of the drug in different buffer solutions. The calibration curve was constructed by appropriate dilution of stock solutions with corresponding buffers. The concentration range was 1.25–100 μg/ml.

3.4. Liquid chromatograph-mass spectrometry (LC-MS)

Bergenin and its degradation product were identified using an API3000 LC–MS/MS mass spectrometer (Applied Biosystems Inc., USA) equipped with ESI sources. The mobile phase and the columns used were identical to that described in the above section of HPLC analysis.

3.5. Powder X-ray diffraction (PXRD)

The PXRD pattern was obtained using a Rigaku D/max-γA 33466 diffractometer with a vertical goniometer in θ/2θ geometry. The X-ray generator was operated at 40 kV and 100 mA. Samples were scanned from 5 to 50°, 2θ, at a rate of 1° min^{-1} using Cu kα radiation. Samples used for PXRD were the commercial bulk of bergenin (A), excess solids remaining at the conclusion of solubility test (B), and solids of hygroscopicity test after 12 days (C).

3.6. Moisture sorption studies

Solid samples were stored under controlled temperature and humidity conditions to investigate the ability of the drug to uptake water from the environment. The relative humidities (R.H) at 25 °C were prepared using saturated solutions with known R.H values (57.5%, 75%, 84.26%, 92.5%, and 98%) in desiccators (Stokes 1949).
Certain amounts of bergenin were weighed and put into open clear glass bottles, which were exposed to the desired R.H. The gains in weight of samples were determined up to the saturation humidity. At each investigated R.H, samples were prepared in triplicate.

3.7. Solid state stability

The stability test was carried out under the conditions of 40 °C/75% R.H. The drugs were kept in the humidity chambers for up to 4 weeks. The samples were prepared in triplicate and analyzed by HPLC. The peak area of non-degraded bergenin relative to the control as a percent was reported.

3.8. Stability in aqueous solutions at different pH values

The degradation of bergenin was investigated in different buffer solutions in the pH range 1.0–8.0 at 25 °C and 37 °C.
Firstly, bergenin was dissolved in buffer solutions to prepare stock solutions with a concentration of 1 mg/ml in duplicate. Then the stock solutions were incubated at 25 °C or 37 °C using a thermostatted water bath (Model HH · Sy21-Ni6, Changyuan laboratory apparatus Co., Beijing, China). Samples were collected at intervals of 0, 0.5, 1, 2, 4, 8, 12 and 24 h by taking 50 μl from the stock solution and immediately diluted to a final volume of 1 ml with appropriate buffer solutions. The concentrations of bergenin in the samples were determined by HPLC.

3.9. Dissociation constants

Due to the low solubility and poor stability in aqueous media, the dissociation constants, pK_a, were determined by potentiometric titration in non-aqueous solution of absolute methanol. The titration was performed with an automatic potentiometric titrator (Model DL53, METTLER TOLEDO, Zurich, Switzerland) fitted with a non-aqueous composite electrode (Model DG 113-SC). Bergenin was dissolved in 50 ml absolute methanol (0.4×10^{-3} M) and then titrated with 0.1 M sodium methylate. The titration curve was drawn by plotting pH versus the volume of the titrant. The pK_a values were said to correspond to the inflexion point of the curve.

3.10. Solubility determinations

Solubility studies were performed with the help of a rotating sample holder at 25, 37, 45, 50 and 60 °C (Model QZX-C, Dongming medical apparatus Co., Harbin, China). Suspensions were prepared in triplicate by adding an excess quantity of bergenin to screw capped glass conical flasks (50 ml) containing the buffers (pH 1.0, 3.0 and 5.0). The flasks were shaken mechanically for 24 h. At various intervals, samples were drawn and filtered through a 0.45 μm filter paper. The filtrates were diluted with buffers (1 : 50) and analyzed by HPLC. The solubility values were calculated according to the calibration curves.

3.11. Partition coefficients

Partition coefficients of bergenin at different pHs were measured between *n*-octanol and buffers (pH 1.0–6.0) using the shake-flask technique. The organic and aqueous phases were mutually pre-equilibrated. Bergenin were dissolved in the aqueous buffers to give a final concentration of 40 μg/ml. And then, equal volumes (5 ml) of the aqueous solution and *n*-octanol were mixed and placed in a shaking thermostat (Model QZX-C, Dongming medical apparatus Co., Harbin, China) at 37 °C for 24 h. After equilibration, the samples were centrifuged for 5 min at 5000 rpm for phase separation. The concentrations of drug in aqueous phase were assayed by HPLC before and after the partition equilibrium and the partition coefficients were calculated according to Eq. (2):

$$P = C_o/C_w = (C_{wi} - C_{we})/C_{we} \quad (2)$$

(P: partition coefficient, C_o: concentration in octanol, C_w: concentration in buffer, C_{wi}: initial concentration in buffer, C_{we}: concentration in buffer at equilibrium).
All values presented were the mean of three independent determinations.

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