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Biopharmaceutics classification of puerarin and comparison of perfusion approaches in rats

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ABSTRACT

The present study was conducted to characterize the biopharmaceutics classification system (BCS) category of puerarin in terms of intrinsic dissolution rate (IDR) and rat intestinal permeability and to investigate the poor intestinal absorption probably related to the drug metabolism in the gut wall of rats. Equilibrium solubility of puerarin was determined in various phosphate buffers and water, and IDR was estimated by measuring the dissolution of a non-disintegrating compact. Intestinal permeability (P_{eff} and P_{blood}) of puerarin was determined using the technology of *in situ* single-pass intestinal perfusion (SPIP) and intestinal perfusion with venous sampling (IPVS) in fasted rats. Metabolism of puerarin in intestinal tissue was tested by S9 incubation *in vitro*. The aqueous solubility of puerarin in phosphate buffers and water was good with a maximum solubility of 7.56 mg/mL at pH 7.4. Obtained IDR values of puerarin were in the range of 0.360–1.088 mg/min/cm^2, with maximum and minimum IDR value of pH 7.4 and pH 4.0, respectively. The P_{eff} was 1.252×10^{-5} cm/s determined by SPIP and the P_{blood} was 0.068×10^{-5} cm/s by IPVS in jejunum at puerarin 80 μg/mL. The metabolism rate of puerarin determined by the intestinal S9 fraction indicated that the gut wall metabolism of puerarin is one cause of poor absorption. According to the proposed classification of drugs and the results obtained from equilibrium solubility, IDR, P_{eff} and P_{blood}, it is concluded that puerarin could be categorized IV drug of the BCS based on its low solubility and low intestinal permeability values.

1. Introduction

The biopharmaceutics classification system (BCS) has been accepted and increasing implementation since it was carried out by Gordon L. Amidon (Amidon et al., 1995). With the adoption of the BCS by FDA (FDA, 2000) and EMA (EMA, 2007), the scientific framework of the BCS and its concept are widely accepted by more scientists. The provisional biopharmaceutical classification of herbs used in Western medicine (Waldmann et al., 2012) and Chinese herbal medicine (Fong et al., 2013) have been predicted by software programs. However, only few experimental studies of the BCS have concentrated on the active ingredients in herbs (Smetanova et al., 2009; Zhang et al., 2012). Puerarin (7,4′-dihydroxyisoflavone-8-glucopyranoside) is a major active ingredient in the Chinese herbal medicine, *Puerariae radix*, which comes from the kudzu root. Total cumulative amounts of the barren and its metabolites were only 3.6% excreted in the urine after oral administration (Yasuda et al., 1995). And lower blood concentration after administering puerarin or *Pueraria lobata* extract orally is found in many reports (Jiang et al., 2013; Li et al., 2013, 2006; Luo et al., 2011; Quan et al., 2007; Wang et al., 2013). Although all the investigations above carried out in the last few years show the poor oral absorption of puerarin, the basic physicochemical data coming from solubility and intrinsic dissolution rate (IDR) were still limited from the published literatures. And no data about the permeability of puerarin has been compared with different methods of *in situ* intestinal perfusion of rat. The BCS with aqueous solubility and intestinal permeability can be a useful tool to demonstrate the problem of absorption of puerarin. Furthermore, the categorization of puerarin by the BCS can be used to facilitate the future formulation development. In the present study, aqueous solubility, IDR property and intestinal permeability of puerarin were investigated, and the intestinal metabolism was also studied by incubation of intestinal S9 fractions. The overall objective of this

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research is to establish the basic framework in understanding of the BCS of the herbal active component, puerarin. And the approaches of permeability test by *in situ* intestinal perfusion of rat are compared.

2. Materials and methods

2.1. Materials

Puerarin was purchased from the Xi'an Zhongxin Biotechnology Co., Ltd. Shanxi, China. HEPES were provided by Amresco, U.S.A. HPLC (high-performance liquid chromatography) grade acetonitrile was obtained from Fisher Scientific. All other reagents were of analytical grade and were purchased from Sinopharm Chemical Reagent Co., Ltd. Shanghai, China, unless otherwise mentioned. Krebs–Ringer solution(2 L): 267 mM NaCl, 9.4 mM KCl, 32.6 mM $NaHCO_3$, 5.3 mM NaH_2PO_4, 0.4 mM $MgCl_2$, 6.7 mM $CaCl_2$, 15.6 mM Glucose. HEPES buffer (1.15% KCl, 100 mL): 20 mM HEPES, 15.4 mM KCl. The HPLC analyses were done using a Waters 515 series HPLC system (Milford, MA, USA).

2.2. Aqueous solubility

The equilibrium solubilities of puerarin were determined in buffers over a pH value ranging from 1.0 to 7.4 (0.1 mol/L HCl to pH 1.0, phosphate buffers was adjusted by H_3PO_4 or NaOH to pH 4.5, pH 6.8, pH 7.0 and pH 7.4). Excess puerarin was added to various buffer solutions in a vial placed in a water bath, then shaken at 37 °C. Samples were filtered through 0.45 μm membrane filter prior to analysis. The filtrate was diluted with corresponding buffer and the concentration of puerarin was determined by the validated HPLC method.

2.3. IDR measurement

A quantity of drug was prepared by compression of 200 mg of puerarin powder with a force of 8 Mpa for 1 min to make non-disintegrating compacts by using die and punch with a diameter of 8.0 mm. The surface area of the compacts was 0.5024 cm^2. After compression, compacts were placed in a molten beeswax-mold to ensure that the only one surface was exposed to the dissolution medium. Dissolution study was conducted using USP II dissolution apparatus with 900 mL dissolution media over the pH range 1.0–7.4 at a temperature of 37 °C with paddle rotate at 100 rpm. Samples were withdrawn with time intervals of 30 min through 0.45 μm syringe filters. The samples were analyzed by the validated HPLC method.

2.4. Animals and treatment

All animal experiments were performed at Beijing University of Chinese Medicine (BUCM) and conducted using protocols approved by the University Committee on Ethics in the Care and Use of Laboratory Animals, and the animals were housed and handled according to the Laboratory Animal Medicine guidelines of BUCM. Normal male Sprague-Dawley rats weighing 300–350 g were purchased from Vital River Laboratory Animal Technology Co., Ltd. China. Animals were kept under artificial light on a 12 h light/dark cycle and housed in rooms controlled between 23 ± 1 °C and 55 ± 5% relative humidity at the Laboratory Animal Center of BUCM. Rats were acclimated for at least 7 days with free access to animal chow and water before the study. Thenceforth were placed in individual cages with wide mesh floors and fasted overnight (water *ad libitum*) prior to the date of the experiment.

Rats were anesthetized with pentobarbital sodium (50 mg/kg) by intraperitoneal injection, then they were placed on a warming blanket and under a heating lamp to maintain body temperature during surgery and throughout the experiment. To sustain the anesthetic condition during the course, one third of the initial dose of pentobarbital sodium was administered throughout the remainder of the experiment.

2.5 In situ *single-pass intestinal perfusion (SPIP)*

SPIP studies were performed using established methods adapted from the literature (Ochsenfahrt and Winne, 1969). The abdomen of an anesthetized animal was shaved and a longitudinal midline incision of 3–4 cm was carefully made to expose the intestinal segments. The duodenum, jejunum and ileum were located respectively. The identification of the main parts of intestinal is: the duodenum from the pylorus to the ligament of Treitz, jejunum and ileum from the ligament of Treitz to ileocecal junction. As the difficulty to make a distinction between jejunum and ileum, the ileum segment was used about 10 cm long upwards ileocecal junction, the jejunum segment was selected about 10 cm in the middle of the ligament of Treitz to the site of ileum used. Careful handling to avoid disturbance of the intact blood supplying, the segment for surgery was located and the both ends were incised with a surgical scissors for cannula. Two silicone tubes were inserted through the small slits and sured. The segment was then rinsed with warm isotonic saline until the effluent was clear. As a means of expelling the remaining isotonic saline from the intestine, air was pumped slowly through the segment from a 50 mL airtight syringe. The exposed intestinal segment was kept moist by covering with a piece of sterilized gauze wetted with saline solution, and during the experiment, warm isotonic saline was also sprinkled on the gauze for many times by a syringe. After that, the inlet tubing was connected to a syringe pump (LSP02-1B Longer Pump, China). At the start of the study, the perfusion solution containing the drug was incubated in a 37 °C water bath to maintain the temperature. In order to assure a steady state, the perfusate was pumped at a flow rate of 0.2 mL/min for 30 min firstly. After reaching steady state, the perfused samples from the intestinal segment were collected at 15 min intervals up to 120 min. Following the termination of the experiment, the perfused intestinal segment was cut out, the length and radius of it were accurately measured. At last, animals were sacrificed by performing a bilateral thoracotomy.

2.6 In situ *intestinal perfusion with venous sampling (IPVS)*

The surgical procedures used to prepare the perfused rat jejunum with venous sampling are similar to those described methods adapted from the literature, with some modifications (Holmstock et al., 2012; Ochsenfahrt and Winne, 1969). Before perfusion surgical operation, the other five to seven rats were selected for donor blood per experiment, a total of 50–70 mL blood was obtained from the abdominal aorta with a heparinized syringe. The anticoagulation of blood collected from these donor rats was 1000 U/mL heparin solution. The blood incubated in a 37 °C water bath to maintain temperature was used to supply the blood loss via the mesenteric vein. The procedure of surgery preparation was the same as SPIP. Briefly, the left jugular vein of the anesthetized rat was exposed, isolated by blunt dissection, a cannula filled with heparinized saline (100 U/mL) was inserted approximately 1–2 cm into the vein and secured for the blood supply during the IPVS. Then the canal was connected to a peristaltic pump (BT 100-1F Longer Pump, China) which placed between the donor blood reservoir and the canal by a silicone tube filled with blood, and the other end of the silicone tube was immersed in the donor blood. The abdomen was shaved and a longitudinal midline incision of 3–4 cm was carefully made to expose the jejunum. The intestines were then placed to the rat's right, so that

the mesenteric vein was exposed. Then the distal end of mesenteric vein was isolated by blunt dissection, and the cannula with heparinized saline (100 U/mL) was intubated into mesenteric vein and secured with silk suture. Then, the cannula was also connected to the same peristaltic pump utilized for blood supply by the silicone tube. The blood reservoir was placed under the other end of the same silicone tube in order to collect the blood out of the vein. The procedure of intestinal perfusion was the same as SPIP, and the selected intestinal segment about 30 cm was corresponding to the operating mesenteric vein. After the perfusion reaching steady state, the proximal mesenteric vein was ligated with silk suture, and the distal cannula intubated into the mesenteric vein and the pump for blood supply was switched on immediately. The perfusion solution was perfused at a flow rate of 0.2 mL/min and the blood was supplied at a flow rate of 0.5 mL/min. During the perfusion, the perfusate from the outlet of the intestine was collected at 10 min intervals in pre-weighed 5 mL centrifuge tube and the blood from mesenteric vein was simultaneously collected in heparinized eppendorf tubes at 10 min intervals. Following the termination of the experiment, the perfused intestinal segment was removed, the length of the segment was measured without stretching and finally animals were sacrificed by performing a bilateral thoracotomy.

2.7. Metabolism experiments in rat intestinal 9000g supernatant (S9) fraction

The procedure for the intestinal S9 preparation followed previously published reports (Masaki et al., 2006; Saitoh et al., 1998). Briefly, the small intestines of the anesthetized rats were removed and cut into segments and rinsed with ice-cold 1.15% KCl. Intestines segments were then cut open, washed with ice-cold 1.15% KCl and the intestinal mucosa was stripped and homogenized with 3 volumes of 20 mM/L HEPES buffer (pH 7.4) containing 1.15% KCl in the ice-water bath. The tissue homogenate was centrifuged at 9000 g for 20 min at 4 °C and the supernatant was S9 fraction. The protein level was determined by means of Bradford with BSA as the standard substance. The preparations were dispensed into 0.5 mL per tube and stored at −80 °C immediately. The total 2 mL S9 was dispensed into a centrifuge tube per 0.2 mL and pre-incubated at 37 °C for 5 min. Then 0.2 mL puerarin (80 μg/mL, 40 μg/mL, 20 μg/mL) was added into the S9 solution respectively for 30 min at 37 °C. After incubation, 1 mL methanol was added into each tube to terminate the reaction. The mixtures were centrifuged at 1500 g for 5 min at 4 °C to get the supernatant. The supernatant was applied to the HPLC assay.

2.8. HPLC analysis

The used column was a Phenomenex C_{18} column (250 mm × 4.6 mm, i.d., 5 μm particle size) guarded with a precolumn .The mobile phase used in the analysis was a mixture of acetonitrile and water containing 1.0% glacial acetic acid (14:86, v/v). The flow rate was 1.0 mL/min and the detection was achieved at 250 nm. The injection volume was 20 μL with column oven at 30 °C. The linearity was evaluated by linear regression analysis with the linear concentration range (20.0–120.0 μg/mL), good correlation coefficient ($r^2 > 0.9995$).

2.9. Statistical analysis and calculations

2.9.1. Calculation of IDR

IDR is calculated by the following equation (Zakeri-Milani et al., 2009)

$$\mathrm{IDR} = \left(\frac{\mathrm{dw}}{\mathrm{dt}}\right)\left(\frac{1}{S}\right) = \frac{D\mathrm{Cs}}{h}$$

where IDR is the intrinsic dissolution rate (mg/min/cm^2); dw is the change in drug dissolved (mg); dt is the change in time (min); S is the surface area of the compact (cm^2); D is the diffusion coefficient (cm^2/s); Cs is the solubility (mg/cm^3) and h is the stagnant layer thickness (cm).

2.9.2. Calculation of effective permeability coefficient (P_{eff} and P_{blood})

The permeability coefficient is called P_{eff}, which means the disappearance of the compounds in the perfusate and P_{blood}, which means the appearance of the compounds in the mesenteric blood, respectively. They were calculated according to the following equations (Singhal et al., 1998; Zhang et al., 2008):

$$P_{\mathrm{blood}} = \frac{\Delta M_{\mathrm{B}}/\Delta t}{2\pi r L < C >}$$

$$P_{\mathrm{eff}} = \frac{-Q_{\mathrm{in}} \cdot \ln(C_{\mathrm{out}}/C_0)}{2\pi r L}$$

where Q_{in} is the perfusion rate entering the intestinal segment (cm^3/s); C_0 is the concentrations of the drug entering the intestinal segment (nmol/mL); C_{out} is the concentrations of the drug leaving the intestinal segment (nmol/mL); L is the length of the intestinal segment (cm); r is the radius of the intestinal segment (cm); $\triangle M_B/\triangle t$ is the appearance of drug in the blood was measured amount of drug appearing in the blood with time (nmol/s) divided by the surface area of the intestine ($2\pi rL$) and the logarithmic mean concentration of drug in the lumen $<C>$.

Because of the water absorption and efflux in the intestinal segments, concentrations determined by HPLC could not correct the changes in the water flux during each time interval. To provide an accurate measure of water flux correction, gravimetric method (Sutton et al., 2001) was utilized to calculate the net water flux and the P_{eff} was corrected for water transport as the following equation:

$$P_{\mathrm{eff}} = \frac{-Q_{\mathrm{in}} \cdot \ln(C_{\mathrm{out(cor)}}/C_0}{2\pi r L}$$

$$< C >= \frac{(C_{\mathrm{out(cor)}} - C_0)}{\ln(C_{\mathrm{out(cor)}}/C_0)}$$

$$C_{\mathrm{out(cor)}} = C_{\mathrm{out}}\frac{Q_{\mathrm{out}}}{Q_{\mathrm{in}}}$$

where $C_{out(cor)}$ is the corrected concentrations of the drug leaving the intestinal segment (nmol/mL); Q_{out} is the flow rate leaving the intestinal segment (cm^3/s); Q_{in} is the perfusate flow rate (cm^3/s).

Because of the difficulty of measuring the Q_{out}, the mass of the flows leaving the intestine at interval time was weighted by a balance, and the density was made by accurate determination of the mass of flows transferred in the 1000 μL volume delivered by the autopipette (Johnson et al., 2003), then the Q_{out} was calculated by the following equations:

$$Q_{\mathrm{out}=\frac{M_{\mathrm{out}}/D_{\mathrm{out}}}{t}}$$

where the M_{out} is the mass of the flow inlet and leaving the intestinal segment (g); the D_{out} is the density of flow inlet and leaving the intestinal segment (g/cm^3); t is the interval time of sampling (s).

2.9.3. Calculation of metabolism rate of intestinal S9

Metabolism rate of intestinal S9 was calculated by the equation as follow:

$$M = \frac{(C - C')}{C} \times 100\%$$

where C is the concentration of puerarin before incubation (nmol/mL); C' is the concentration of supernatant after incubation (nmol/mL).

Table 1
Solubility of puerarin in various buffers.

pH of buffers	Solubility (mg/mL)	Final pH
1.0	3.38	1.0
4.0	3.32	3.95
6.8	4.68	6.73
7.0	5.30	6.92
7.4	7.56	7.24
H_20	3.81	5.50

Data were analyzed by ANOVA. Significant differences between the two groups were detected by using HSD test. A probability level of $p < 0.05$ was set as the criterion of significance.

3. Results

3.1. Solubility of puerarin

Table 1 shows the results of the equilibrated solubilities of puerarin at 37 °C in various buffers. The aqueous solubility of the compound over the pH range of 1.0–7.4 was low and belonging to slightly soluble substance in reference to USP35 (USP35, 2012) with the maximum solubility of 7.56 mg/mL observed at pH 7.4.

3.2. IDR of puerarin

The IDRs of a total of the compound were measured over different pH range. These IDR data, consisting of five different IDR values, are tabulated in Table 2. During the dissolution test, Fig. 2 shows a typical linear plot of a cumulative dissolution versus time for puerarin at pH 1.0, 4.0, 6.8, 7.4 and H_2O. Dissolution data in three dissolution vessels indicate excellent reproducibility (CV less than 10%). Linearity was also good with a correlation coefficient of 0.99. IDR of puerarin was calculated to be 0.385, 0.360, 0.625, 1.088 and 0.448 mg/min/cm^2 at different aqueous dissolution media, respectively.

3.3. Permeability of puerarin

The intestinal permeability values obtained for puerarin are presented in Tables 3 and 4.

3.4. Metabolism in intestinal S9

According to the S9 section, the intestinal metabolism rate of puerarin was shown in Table 5.

Table 2
Intrinsic dissolution rate (IDR) of puerarin as a function of pH.

	Intrinsic dissolution rate (mg/min/cm^2)				
Puerarin	pH 1.0 0.385	pH 4.0 0.360	pH 6.8 0.625	pH 7.4 1.088	H_2O 0.448

Table 3
Effective intestinal permeability (P_{eff}) determined by SPIP.

Perfusate	P_{eff} (10^{-5} cm/s)		
	Duodenum	Jejunum	Ileum
Puerarin 80 μg/mL	2.014 ± 0.607^a	$1.252 \pm 0.172^{a,b}$	0.639 ± 0.150^c

The data were presented as mean ± SD ($n = 3$ for each group).
a,b,c, $p < 0.05$, the same letter not significantly different to the other intestinal segment.

Table 4
Intestinal permeability (P_{eff} and P_{blood}) determined by IPVS.

Experiment design	Permeability (10^{-5} cm/s)	
	P_{eff}	P_{blood}
SPIP	1.252 ± 0.172	
IPVS	1.11 ± 0.264	0.068 ± 0.003

The data were presented as mean ± SD ($n = 3$ for each group).
N.S.: not significantly different to IPVS.

Table 5
Metabolism rate of puerarin incubation by intestinal S9.

Concentration of puerarin before incubation (μg/mL)	Concentration of puerarin after incubation (μg/mL)	Metabolism rate (%)
81.28	77.80	4.28%
41.45	36.93	10.90%
23.03	20.18	12.38%

4. Discussion

Puerarin is weak acid compound and could be ionized at high pH condition. Puerarin mainly presents as a hydrophobic molecular form in weak acid condition and as a hydrophilic molecular form in weak alkali condition. Solubility of puerarin raised by the increase of the concentration of puerarin as shown in Fig. 1.

IDR is a rate phenomenon instead of an equilibrium phenomenon. Therefore, IDR might be expected to correlate more closely with drug dissolution dynamics *in vivo* than solubility. It should be noted that dose is considered in the classification of solubility by equilibrium solubility while the effect of the dose is not considered by IDR. Research data shows IDR to be a convenient, simple method to classify drugs. According to the results obtained and proposed classification of drugs, it is concluded that drugs could be categorized correctly based on their IDR. The test results obtained from this study show a good relationship between the IDR and BCS solubility classification with 1 mg/min/cm^2 and 0.1 mg/min/cm^2 as a class boundary (Yu et al., 2004; Zakeri-Milani et al., 2009) unless the dose is either extremely low or high where discrepancies may exist between the solubility and IDR methods. As we followed the experimental procedures of Zakeri-Milani et al. (Zakeri-Milani et al., 2009). Therefore, the IDR value of puerarin was lower than the borderline (1 mg/min/cm^2) except for IDR in pH 7.4 buffer. Puerarin could be classified as the compound with low solubility.

As shown in Table 3, intestinal site dependent absorption of puerarin could be found between duodenum, jejunum and ileum. The minimum and maximum P_{eff} obtain from SPIP were

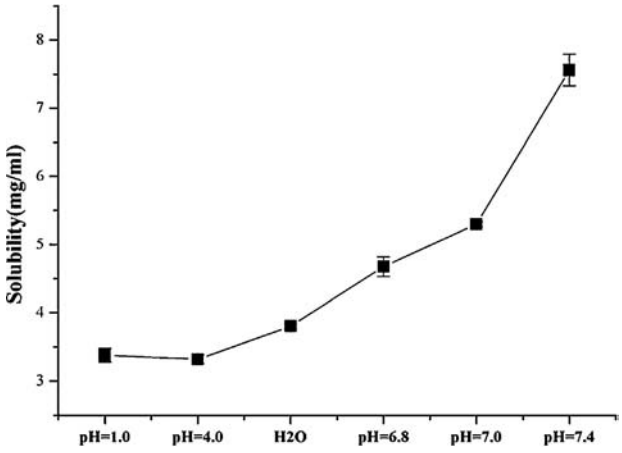

Fig. 1. Solubility of puerarin in various buffers.

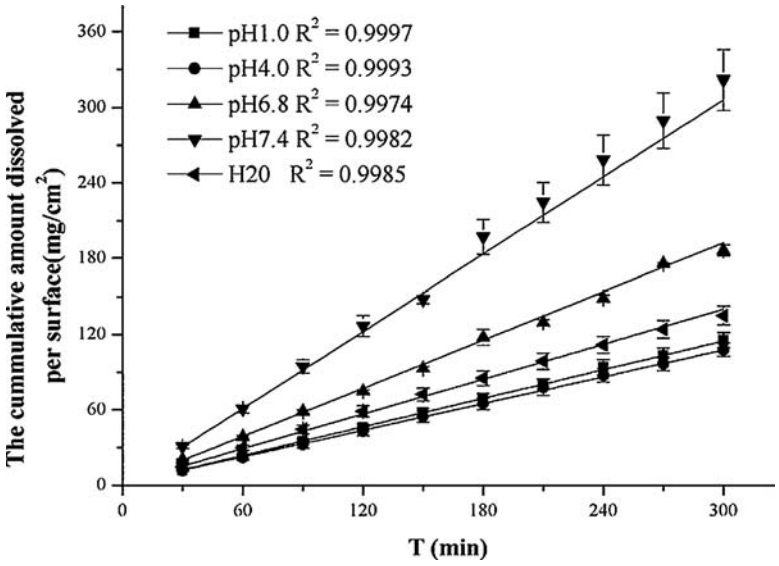

Fig. 2. Intrinsic dissolution rate (IDR) of puerarin at pH 1.0, 4.0, 6.8, 7.4 and H_2O.

determined to be 2.014×10^{-5} cm/s and 0.635×10^{-5} cm/s in the duodenum and ileum, respectively. In addition, the P_{eff} of puerarin in the duodenum was significantly greater than in the ileum ($p < 0.05$, ANOVA), and the permeability of jejunum was 1.252×10^{-5} cm/s in the middle of the permeability value of the duodenum and ileum. The results showed that puerarin could be absorbed in the whole small intestine and the permeability gradually decreased as the intestinal regions became progressively distal from duodenum to ileum. The effect of intestinal segments on the permeability of puerarin was described in Fig. 3.

Since the jejunum was the longest region in the small intestine and the main absorption site in general, the IPVS in the jejunum was carried out to profile the more detailed absorption procedure. The P_{eff} of puerarin in the jejunum determined by IPVS was not significantly different from by SPIP ($p = 0.491$, t-test). Interestingly, the P_{blood} in the jejunum was 0.068 (10^{-5} cm/s and 16-fold lower than the P_{eff} (1.114×10^{-5} cm/s) obtained from IPVS. The permeability of jejunum (P_{eff} and P_{blood}) determined by IPVS was shown in Fig. 4.

Respecting the large difference between the P_{eff} and P_{blood} in IPVS, the metabolism rate of puerarin was tested by S9 fraction to evaluate the extent of the absorption process of drugs affected by gut wall metabolism. Although the puerarin metabolic phenomenon existed in the jejunum, it was not completely to interpret the difference between the P_{eff} and P_{blood} that the metabolism rate of puerarin was 4.28% at puerarin 80.28 μg/mL. These results demonstrated that P_{eff} is the suitable parameter to evaluate the permeability of drug substance and P_{blood} is appropriate for learning the result of dispose in the course of absorption of intestine.

5. Conclusion

In this paper, puerarin was categorized as BCS class IV drug due to its low solubility and low permeability. SPIP was found to be applicable in investigating the permeability of drugs and was a more effective method to quantify parameters of the drug BCS property compared with IPVS. Because the data come from SPIP can reveal the permeability of chemical itself. The approach of IPVS not only profiled the drug absorption property of, but also revealed the information of drug deposition in the intestine. Maybe the IPVS method can be more suitable for some other BCS such as BDDCS (Wu and Benet, 2005).

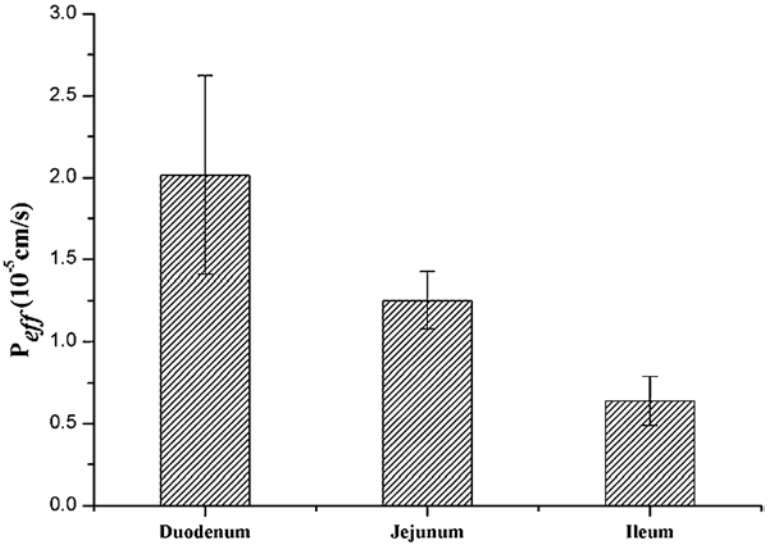

Fig. 3. Permeability of puerarin in three intestinal segments.

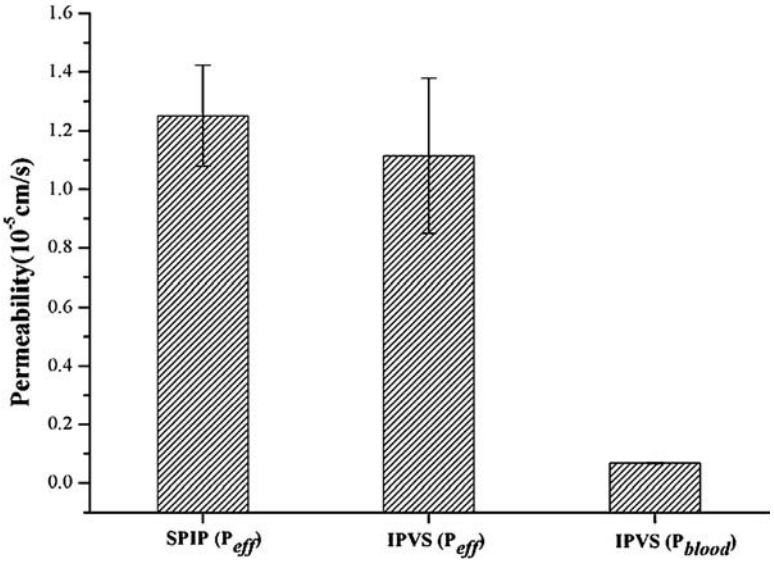

Fig. 4. Jejunum permeability (P_{eff} and P_{blood}) determined by SPIP and IPVS.

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