Enhancement effect of geniposide on solubility and intestinal absorption of baicalin

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Abstract: Geniposide and baicalin, the main components of Huangqin-Zhizi herb pair, have been combined to increase the efficacy. To reveal the underlying compatibility mechanism of these two components, the synergistic effects of geniposide on the enhancement of solubility, apparent oil-water partition coefficient, and intestinal absorption of baicalin were investigated. The equilibrium solubility and apparent oil–water partition coefficient of baicalin in different solvents were determined through the shake-flask and high-performance liquid chromatography with diode array detection methods. The intestinal absorption of baicalin was investigated through the in situ single-pass intestinal perfusion method. When combined with different amounts of geniposide, the solubility and apparent oil-water partition coefficient of baicalin improved to 98.74-159.03μg/mL and 0.24-0.29, respectively, which were respectively 1.25-2.02-fold and 1.6-1.9-fold higher than those parameters in the baicalin-only control. The intestinal absorption study indicated that geniposide was an absorption-enhancer for baicalin and significantly increased the absorption rate constant value and the apparent absorption constant value of baicalin, especially in duodenum and jejunum when the compatibility concentrations were 1:1 and 1:2. Geniposide had synergistic effects in enhancing the solubility, apparent oil-water partition coefficient, intestinal absorption of baicalin. The study results provide scientific information elucidating the compatibility mechanism of the Huangqin-Zhizi herb pair and its primary components.

Keywords: Baicalin; geniposide; compatibility; solubilization; intestinal absorption.

INTRODUCTION

Herb-pairing in traditional Chinese medicine (TCM), as the relatively fixed composition of two herbs for clinical medical application, is a basic form of compatibility in Chinese herbal formulae (Deng *et al*., 2012). Many previous studies have confirmed the applicability of TCM herb pairs in increasing the efficacy of drugs and reducing the toxicity, primarily through the antagonistic or synergistic effects of different components (Xu *et al*., 2006; Zhang *et al*., 2010; Peng *et al*., 2013; Jiang *et al*., 2011). In TCM, *Scutellaria baicalensis* Georgi and *Gardenia jasminoides* Ellis have been widely used as a herb pair to treat jaundice and chronic liver disease induced by high fever and damp-heat. Baicalin combined with geniposide has been reported to attenuate the development of atherosclerosis and (Liao *et al*., 2014; Liu *et al*., 2014) and protect against ischemia-reperfusion injury (Zhang *et al*., 2006; Guo *et al*., 2011), but the compatibility mechanism of the two components is not clear.

Baicalin, as one of the main components in the Huangqin-Zhizi herb pair (*S. baicalensis* and *G. jasminoides*), is isolated from *Scutellariae radix* and exhibits numerous pharmacological effects, including anti-inflammatory (Burnett *et al*., 2007; Altavilla *et al*., 2009) and antioxidant (Guo *et al*., 2011) properties. However, the low bioavailability and absorption of baicalin were caused by its poor transmembrane permeability and solubility (Gong *et al*., 2009; Gong *et al*., 2008). Geniposide, an active compound in *G. jasminoides* Ellis, is widely known for its favourable water-solubility and is typically combined into sustained-release or controlled-release preparations (Zhang *et al*., 2009; Li *et al*., 2013).

In recent years, research on the compatibility mechanism of main components has become a new model to elucidate the compatibility mechanism of herb pairs (Sheng *et al*., 2013; Li *et al*., 2015; Bi *et al*., 2016). Many previous compatibility studies about the Huangqin–Zhizi herb pair mostly focused on its chemical composition changes (Liu *et al*., 2012; Bi *et al*., 2011) and pharmacological effects (Li *et al*., 2012), but few studies have evaluated the synergistic effect of the main components. Therefore, the present study investigated the enhancement effect of geniposide and baicalin on the basis of both physicochemical properties and intestinal absorption.

MATERIALS AND METHODS

Materials

The equipments used for this study comprised Agilent-1100 series HPLC (Agilent Technologies, Shanghai, China), thermostatic shaking gas bath (THZ-92C;

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Shanghai Boxun Industry and Commerce Co., Ltd, Shanghai, China) and peristaltic pump (BT100-2J; Baoding Longer Precision Pump Co., Ltd, Hebei, China) (Jin *et al*., 2014).

Chemicals

Geniposide (Lot No.:12070541, purity ≥98%) and Baicalin (Lot No.:13042635, purity ≥98%) were purchased from Shanghai Tauto Biotech Co. (Shanghai, China). Geniposide extract (purity ≥98%) was prepared in the laboratory of an experiment center for teaching and learning at Shanghai University of Traditional Chinese Medicine. HPLC grade solvents were purchased from Anhui Fulltime Specialised Solvents and Reagents Co. (Anhui, China). All other chemicals were purchased from local chemical suppliers.

Animals

Male Sprague-Dawley rats used in the experiment were bred in the central animal facility of Shanghai University of TCM (Shanghai Sipper BK Laboratory Animals Ltd., License: SCXK (Shanghai 2013-0016). All the animal studies were conducted in accordance with the guidelines of the Institutional Animal Ethical Care Committee. All effort was made to minimise the suffering of animals.

Chromatographic conditions

A Dikma C_{18} column (250×4.6 mm, 5μm) was used for sample analysis with a gradient eluent composed of 0.2% phosphoric acid (A) and acetonitrile (B) (From 0~13min, A was 87.5%, from 13~23min, A was 87.5%→45%, from 23~30min, A was 45%). The flow rate of mobile phase was 1mL/min. The column temperature was 30°C. The detection wavelengths for geniposide and baicalin were 238nm and 280 nm, respectively. The injection volume was 20μL.

Preparation of standard solutions

Baicalin and geniposide were dissolved in methanol to prepare standard solution 1, which contained 0.382 mg/mL baicalin and 0.732mg/mL geniposide. Baicalin and geniposide were dissolved in methanol to prepare standard solution 2, which contained 0.36mg/mL baicalin and 0.245 mg/mL geniposide (Jin *et al*., 2014).

Composition of buffer systems

The composition of Krebs-Ringer (K-R) solution was as follows: $CaCl_2$ (0.37 g/L), NaCl (7.8 g/L), glucose (1.4 g/L), KCl (0.35 g/L), $NaHCO_3$ (1.37 g/L), NaH_2PO_4 (0.32 g/L) and $MgCl_2$ (0.02 g/L).

Phosphate buffered saline (PBS) solution, which consisted of 13.6g of KH_2PO_4, was dissolved in 1L of distilled water, which was prepared into a series of PBS solutions (pH 2.0, 3.0, 4.0, 5.0, 6.0, 6.8, 7.0, 7.4, 8.0 and 9.0) by using 0.1 mol/L NaOH or 10% phosphoric acid.

Preparation of sample solutions

Determination of solubility of baicalin

An excess of baicalin (0.0515 g) was added to 5mL of distilled water or a PBS solution at various pH levels or to geniposide solutions with different concentrations (0.0103, 0.0206, or 0.0412g/mL) in a 10-mL Eppendorf tube, and the resulting suspension was shaken for 72h (37°C, 120 rpm) in a thermostatic shaking gas bath. The obtained values were the means of triplicate measurements. The sample solutions were centrifuged for 10 min (12 000 r/min) and filtered through a 0.45-μm hydrophilic membrane. The subsequent filtrates were collected and determined through HPLC for a solubility analysis.

Determination of oil-water partition coefficient of baicalin

An excess of baicalin (5.15g) was added to 50mL of water-saturated n-octanol and shaken constantly for 24h. Subsequently, 1mL of the resulting supernatant was added to 9mL of n-octanol-saturated water, n-octanol-saturated PBS solution, or n-octanol-saturated geniposide solution (0.0103, 0.0206, 0.0412g/mL respectively) in 15mL tubes. The two phases were mutually equilibrated by shaking them in a thermostatic shaking gas bath for 72h (37°C, 120 rpm). The tubes were centrifuged for 10 min (12 000 r/min). The aqueous and organic phases were collected and filtered through 0.45-μm hydrophilic and hydrophobic membranes, respectively and determined through HPLC for an analysis of the apparent oil–water partition coefficient.

Intestinal absorption test

Each baicalin enema was prepared by diluting a solution composed of 0.4g of vitamin C and 1mL of baicalin ethanol solution (14.66mg/mL) to 200mL of K-R solution. The mixed enemas of baicalin and geniposide were prepared by diluting a solution, which consisted of 0.4g of vitamin C, 1mL of baicalin ethanol solution (14.66mg/mL) and 1mL of geniposide ethanol solutions with various concentrations (14.66, 29.32 and 58.64 mg/mL) to 200mL of K-R solution.

Intestinal absorption study

The intestinal absorption study was conducted using an experimental design similar one that Zhenggen Liao *et al*. described (Liao *et al*., 2014). Male Sprague-Dawley rats weighing 200-220g were gave free access to water and food until 12 h prior to the experiment. Rats were placed on a heated pad to maintain normal body temperature and anaesthetised with 20% ethyl carbamate (1.6 mg/g). The abdomen was opened with a midline longitudinal incision, the segments of duodenum, jejunum, ileum, and colon (10 cm) were perfused with saline to wash the contents. After the contents in the segments were washed, the duodenum, jejunum, ileum, and colon (10cm) segments were perfused (0.2mL/min) with an enema for 30 min. Intestinal perfusate was collected at 45, 60, 75, 90, 105, 120 min. After the experiment, the lengths and inside

diameters of the cut segments were measured. Perfusate samples were filtered with a 0.45-μm microporous membrane and analysed through HPLC. The effect of geniposide on the intestinal absorption of baicalin was measured in the same manner.

The intestinal absorption rate constant (K_a) and the apparent absorption coefficient of the drugs (P_{app}) were determined using the following mathematical expression (Li *et al*., 2015). The terms *Ka,c* and *Papp,c* are the means of six samples from 45 to 120 min.

$K_{a,c} = （1\text{-}C_{out}Q_{out}/C_{in}Q_{in}）\cdot Q/V$

$P_{app,c} = \text{-}QIn（C_{out}Q_{out}/C_{in}Q_{in}）/2\pi r \cdot l$

STATISTICAL ANALYSIS

Three samples were used for every experiment, and the data are expressed as the mean ± standard deviation. Statistical analysis was performed through a one-way ANOVA by using SPSS version 18.0. A $p<0.05$ was considered statistically significant.

RESULTS

Solubility analysis

Calibration curves (Jin et al., 2014)

Two analytes were diluted to seven different concentrations by using methanol solutions to plot the calibration curves. The calibration curves were established by plotting the peak area (*Y*) versus the concentration (*X*) of each component. The regression equations of baicalin and geniposide were $Y = 52\,198X + 53.674$ ($r = 0.9998$) and $Y=26\,588X+102.12$ ($r = 0.9998$), respectively. The linear ranges of baicalin and geniposide were 3.82–382μg/mL and 7.32–732 μg/mL, respectively.

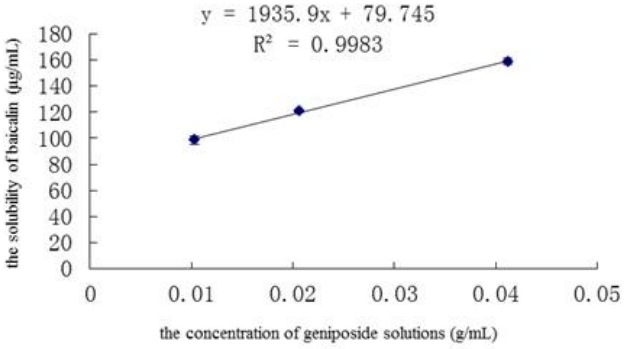
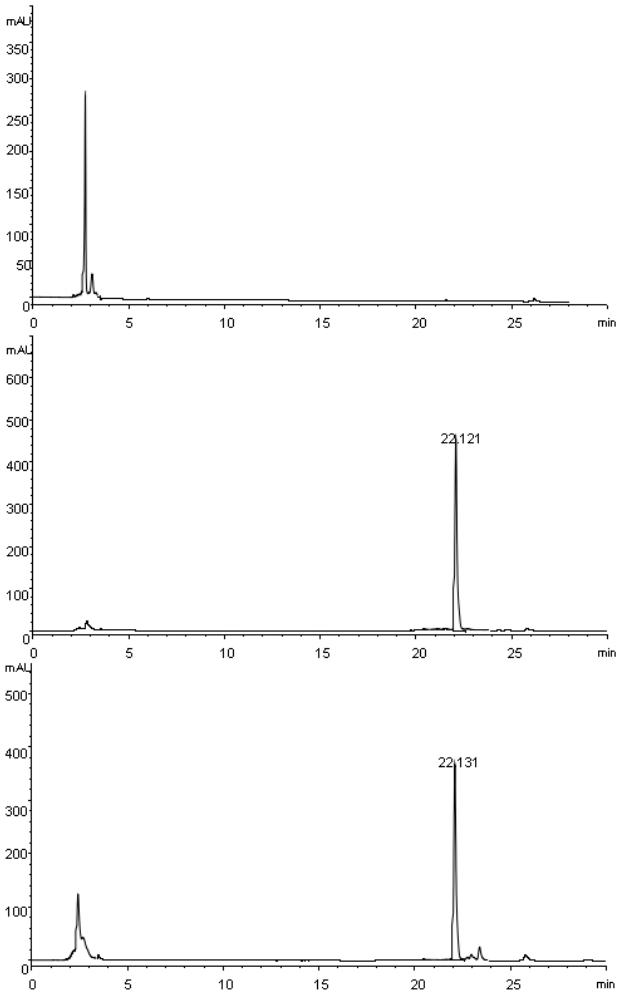


Fig. 1: Solubilisation effect of geniposide on baicalin

Precision, reproducibility and stability

As shown in table 1, the precision of the baicalin and geniposide was acceptable. The reproducibility values ($n = 6$) of the baicalin and geniposide were 0.0245mg/mL ±2.9% and 2.1460mg/mL ±2.8%, respectively. The stability ($n = 3$) of the baicalin and geniposide at 48 h was 1060.9 (peak area) ±1.6% and 106 333.2 (peak area) ± 2.3%, respectively.



a: blank enema; b: standard solution (baicalin: 0.00764mg/ml); c: baicalin enema (0.0366mg/ml)

Fig. 2: HPLC chromatogram of baicalin in 280nm

Determination of the equilibrium solubility of baicalin

As shown in table 2, the equilibrium solubility of the baicalin was 78.88μg/mL in pure water. With the increase of pH of the PBS solutions, the solubility of the baicalin increased to 36 721.09μg/mL, indicating that the baicalin was more soluble in a basic solution than in an acidic solution.

Solubilisation effect of geniposide on baicalin

The solubilisation effect (SE) of geniposide on baicalin is expressed as the ratio of solubility of baicalin in the geniposide solutions to that in pure water, which was determined using the following formula (Ou *et al*., 2009)

$SE = S/S0$

As shown in table 3 and fig. 1, the solubility of the baicalin was concentration-dependent in the geniposide solutions. When combined with different amounts of geniposide, the solubility of the baicalin improved to 98.74-159.03μg/mL, which was 1.25-2.02-fold higher

than it was in water. This indicated that geniposide could effectively enhance the solubility of baicalin.

Determination of the oil–water partition coefficient of baicalin

The oil-water partition coefficient was calculated with the following formula:

$P_{app}=C_{oil}/C_{water}$

P_{app}: The oil–water partition coefficient; C_{oil}: The concentration of baicalin in organic phase at equilibrium (μg/mL); C_{water}: The concentration of baicalin in aqueous phase at equilibrium (μg/mL).

As shown in table 4, the oil–water partition coefficient of baicalin was 0.15 (lg*Papp* = -0.83) in the n-octanol-saturated water. Under acidic conditions, baicalin is a type of molecule with weak polarity, which is easy to dissolve in the organic phase. With the increase in the pH of the PBS solutions, the oil–water partition coefficient of baicalin decreased because of the dissociation of baicalin molecules. The geniposide solution was slightly acidic, which improved the liposolubility of baicalin; therefore, the oil–water partition coefficient of the baicalin in the geniposide solution was 0.24-0.29, which was 1.6-1.9-fold higher than it was in the n-octanol-saturated water.

In situ single-pass perfusion studies

Calibration curves

Ethanol solutions with two analytes were diluted by the K–R solution to five different concentrations to plot the calibration curves. The calibration curves were established by plotting the peak area (*Y*) versus the concentration (*X*) of each component. The regression equations of baicalin and geniposide were $Y = 43\,522X + 145.42$ (r=0.9997) and $=26\,109X + 72.76$ (r=0.9998), respectively. The linear ranges of baicalin and geniposide were 1125-18 000μg/mL and 765-12 270μg/mL, respectively.

Blank interference experiment

The blank enema (K–R solution), standard solution (baicalin: 0.00764mg/mL), and baicalin enema (0.0366 mg/ml) were analysed to investigate the interference of K-R solution on analytes. The results in fig. 2 show that the K-R solution had no interference effect on the analytes.

Stability study of baicalin and geniposide in the blank enema (K-R solution)

The mixed solution containing baicalin and geniposide with K–R solution as solvent was heated in a thermostatic shaking gas bath at 37°C for 10 h and analysed at 0, 1, 2, 4, 6, and 10 h. The mean concentration of baicalin (n = 6) in 10 hours was 0.2381mg/mL, when the relative standard deviation (RSD) was 3.3%. The average concentration of geniposide (n=6) in 10 hours was 0.2270mg/mL, when the RSD was 3.1%. The results showed that the mixed solution was stable in the blank enema.

Recovery experiment

The recovery is expressed as the ratio of the measured concentration to the actual concentration. The mean recovery (Mean (rate) ± RSD (%), n=3) of baicalin with different concentrations (0.0225, 0.09 and 0.36 mg/mL) were 96%±2.6%, 95% ±0.9% and 100.5% ±2.5%, respectively. The mean recovery (Mean(rate) ± RSD(%), n = 3) of geniposide with different concentrations (0.015, 0.061, and 0.245mg/mL) were 90.8%±2.4%, 102.9% ± 0.1%, and 98.7% ±1.2%, respectively (Wang *et al.*, 2011).

Intestinal absorption study

As shown in fig. 3 and table 5, Baicalin was absorbed in all four intestinal segments, with the cumulative amounts ordered as follows: colon > ileum > jejunum > duodenum. The k_a and P_{app} of baicalin respectively increased to 2.6- and 3.2-fold when the compatibility concentration was 1:1 in the duodenum. The k_a of baicalin increased to 3.3-fold when the compatibility concentration was 1:2 in the jejunum, and the P_{app} of baicalin increased to 2.8-fold when the compatibility concentration was 1:1 in the jejunum. The results indicated that geniposide was an absorption-enhancer for baicalin that significantly increased the k_a and P_{app} of baicalin, especially in the jejunum and duodenum when the compatibility concentration was 1:1 and 1:2, respectively.

DISCUSSION

Drug absorption is a highly complex process that depends on both the physicochemical properties of the drug and physiological conditions of the body (Song *et al*., 2004). Solubility, permeability and intestinal absorption characteristics are considered the most critical factors affecting drug absorption. Therefore, considerable effort has been expended to improve these properties and achieve desirable drug absorption (Zhang *et al*., 2014; Zhao *et al*., 2013).

The molecular structure of baicalin, a primary component in the Huangqin-Zhizi herb pair, shows that both flavones and glucuronide can form intramolecular hydrogen bonds, resulting in poor water-solubility and low oral bioavailability (Zhang *et al*., 2009). Geniposide has a high solubility in water and a low absorption rate in the gastrointestinal tract. The diffusion mechanism of geniposide might involve active diffusion or facilitated diffusion but not passive diffusion (Ou *et al*., 2009). Current methods to improve the bioavailability and solubility of insoluble drugs include adding a solubiliser, cosolvent, or surfactant; preparing solid dispersions, β cyclodextrin inclusion complexes, or micro emulsion and employing superfine grinding technology. However, employing the compatibility of herb pairs or its main components, such as the combination of geniposide and baicalin, has become a unique method for enhancing the

Table 1: Intra-day and inter-day precision of the two constituents

Compound	Concentration (mg/mL)	Intra-day precision Mean (peak area) ±RSD (%) (n=6)	Inter-day precision Mean (peak area) ±RSD (%) (n=6)
Baicalin	0.00382	225.7±1.4%	230.8±2.2%
	0.0382	2843.4±2.1%	2238.5±2.9%
	0.382	18515.0±2.9%	17864.3±2.4%
Geniposide	0.00732	258.6±3.0%	230.8±2.9%
	0.0732	2786.0±1.9%	2340.2±1.8%
	0.732	19171.7±1.4%	19740.4±2.6%

Table 2: The equilibrium solubility of baicalin in water and different phosphate buffers (n=3)

Solvent	Solubility ($\mu g \cdot mL^{-1}$)
water	78.88
pH2.0PBS	15.96
pH3.0PBS	53.41
pH4.0PBS	256.94
pH5.0PBS	612.92
pH6.0PBS	5861.26
pH6.8PBS	6010.28
pH7.0PBS	5655.39
pH7.4PBS	33255.86
pH8.0PBS	36721.09
pH9.0PBS	36069.93

Table 3: Solubilisation effect of geniposide on baicalin (n=3)

Geniposide	0.0103g/mL	0.0206g/mL	0.0412g/mL
S($\mu g \cdot mL^{-1}$)	98.74	121.04	159.03
SE(S/S0)	1.25	1.53	2.02

Table 4: Apparent oil– water partition coefficient of baicalin in water and different buffer solutions and geniposide solutions at 37°C (n=3)

Solvent	P_{app}	lg P_{app}	RSD/%
water	0.15	-0.83	4.41
pH2.0PBS	32.30	1.51	0.45
pH3.0PBS	14.94	1.17	1.85
pH4.0PBS	7.17	0.85	0.08
pH5.0PBS	1.64	0.21	3.85
pH6.0PBS	0.19	-0.70	0.69
pH6.8PBS	0.08	-1.07	0.75
pH7.0PBS	0.09	-1.04	0.89
pH7.4PBS	0.11	-0.96	1.98
pH8.0PBS	0.08	-1.07	1.77
pH9.0PBS	0.10	-0.99	0.68
Geniposide solution (0.0103g/mL)	0.28	-0.55	3.46
Geniposide solution (0.0206g/mL)	0.29	-0.54	2.78
Geniposide solution (0.0412g/mL)	0.24	-0.61	1.07

solubility (Lu *et al*., 2014; Yang *et al*., 2015) and absorption (Jiang *et al*., 2014; Zhang *et al*., 2013) of insoluble drugs.

Rat in situ single-pass intestinal perfusion systems are widely used in studying the intestinal absorption of drugs. Such systems can simulate the physiological environment of the human body without destroying the circulatory system of the experiment subject and fully guarantee the reliability of the experimental results (Zakeri-Milani *et al*., 2007). Because the permeability values are influenced by water absorption and secretion during perfusion, previous

Table 5: Effects of geniposide on the absorption of baicalin in different segments ($\bar{x}$±s, n=3)

group	segment	K_a/×10^{-2}min^{-1}	P_{app}/×10^{-2}cm·min^{-1}
Baicalin (0.0733mg/ml)	duodenum	0.6626±0.0062	0.1018±0.0225
	jejunum	0.6688±0.0030	0.1243±0.0087
	ileum	0.8167±0.0618	0.1270±0.0070
	colon	0.7261±0.0528	0.1585±0.0136
Baicalin:Geniposide=1:1 (0.0733mg/ml:0.0733mg/ml)	duodenum	1.7487±0.0539**	0.3269±0.0074**
	jejunum	1.8525±0.0719**	0.3475±0.0247**
	ileum	0.8674±0.0479	0.2103±0.0125**
	colon	0.9526±0.0194**	0.1808±0.00619*
Baicalin:Geniposide =1:2 (0.0733mg/ml:0.1466mg/ml)	duodenum	1.4338±0.0203**	0.2379±0.0105**
	jejunum	2.2189±0.1014**	0.3142±0.0140**
	ileum	1.0733±0.0498**	0.1577±0.0188*
	colon	0.9511±0.0306**	0.1936±0.0073**
Baicalin:Geniposide =1:4 (0.0733mg/ml:0.2932mg/ml)	duodenum	0.7553±0.0390*	0.0995±0.0025
	jejunum	1.3308±0.0156**	0.2421±0.0041**
	ileum	1.0874±0.0615**	0.1731±0.0080**
	colon	0.7307±0.0157	0.1680±0.0058

*P<0.05, **P<0.01 VS. Baicalin group

studies have typically used phenol red to indicate the net water flux. However, phenol red is not a nonabsorbable marker; consequently, it interferes with the intestinal transport of some compounds and disturbs their analytical determination. Therefore, the gravimetric method (Nie *et al*., 2005) was employed in the present study to correct the perfusion volume to avoid the interference of a marker.

Moreover, both baicalin and geniposide are respectively metabolised into baicalein and genipin by intestinal flora. Studying the compatibility of metabolites might be an alternative method for investigating the intestinal absorption mechanism of geniposide and baicalin.

The present study emphasised the synergistic effect of geniposide on physicochemical properties and the intestinal absorption of baicalin to reveal the compatibility mechanism of the Huangqin-Zhizi herb pair and its primary components. Furthermore, the relationship between solubilisation and molecular structure changes and the intestinal transport mechanism at the molecular level should be studied in the future.

In conclusion, adding geniposide showed a markedly higher improvement in the solubility, apparent oil–water partition coefficient, and intestinal absorption of baicalin than when biacalin was used alone. This study was a crucial investigation for the compatibility mechanism of main components in Huangqin-Zhizi herb pair, and might provide a feasible and generalised design for researching the compatibility mechanism of other herb pairs.

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