Heliyon 9 (2023) e12689

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Consecutive baicalin treatment relieves its accumulation in rats with intrahepatic cholestasis by increasing MRP2 expression

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ARTICLE INFO

Keywords:
Intrahepatic cholestasis
Baicalin
Pharmacokinetics
Accumulation
MRP2

ABSTRACT

Baicalin, an important flavonoid isolated from *Scutellaria baicalensis* Georgi, is a Chinese herb widely used in clinical practice. We previously reported the *in vivo* accumulation of baicalin in rats with intrahepatic cholestasis (IHC) after a single dose. However, the effects of the long-term administration of baicalin on its pharmacokinetics are unknown. Thus, we investigated the disposition of baicalin in normal rats and those with IHC after single and multiple consecutive administrations. In addition, we further investigated the effect of baicalin on multidrug resistance protein 2 (MRP2) *in vivo* to explore the underlying mechanism. In our study, the liquid chromatography-mass spectrometry (LC-MS) method established to determine baicalin concentrations in rat blood was simple, specific, and with linearity (R^2 = 0.9980) in the range of 1.01–506.00 μg/mL. The relative standard deviations (RSD) for intra-day and inter-day precision were not more than 10.55%, and the intra-day and inter-day accuracies were 94.94%–109.13%. The recovery rate and stability were in line with the requirements of the quantitative analysis of biological samples as stated in the Chinese Pharmacopoeia (2020 Edition). Compared with that in normal rats, the C_{max} and $t_{1/2}$ increased significantly in EE-induced rats with IHC, whereas the clearance (CL) decreased after a single administration of baicalin. However, the area under the curve decreased, CL increased, and the $t_{1/2}$ was shortened after the continuous administration of baicalin in the IHC rat model compared with the single administration of baicalin, and the pharmacokinetic characteristics were similar to those in normal rats. Moreover, MRP2 expression increased in rats with IHC with the continuous administration of baicalin. Continuous baicalin intervention could effectively reduce its accumulation in rats with IHC, and the mechanism may be attributed to its enhancement of MRP2 expression.

1. Introduction

Intrahepatic cholestasis (IHC) is a common liver disorder caused by various reasons, such as virus infections, drugs, and alcohol. It is characterized by elevated serum transaminase and bile acid levels [1,2]. If not treated on time, it can further develop into liver cirrhosis, liver fibrosis, and even liver cancer. The current pharmacotherapy of IHC includes ursodeoxycholic acid (UDCA) and S-adenosylmethionine (SAMe) [3,4]. However, some patients respond poorly to these drugs.

Traditional Chinese medicine has been used for thousands of years in China. It has attracted considerable attention of scholars and

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https://doi.org/10.1016/j.heliyon.2022.e12689
Received 11 August 2022; Received in revised form 16 December 2022; Accepted 22 December 2022
Available online 3 January 2023

researchers, as it involves treatment using naturally occurring compounds and is associated with low costs and extremely low toxicity [5]. Baicalin is a flavonoid and an active component derived from the roots of *Scutellaria baicalensis* Georgi (Lamiaceae). It has several pharmacological effects, including anti-inflammatory, anticancer, antioxidant, cardioprotective, cerebrovascular-protective, hepatoprotective, and renoprotective effects [5,6]. We previously reported that baicalin could protect against IHC by improving bile acid homeostasis and oxidative stress damage [7].

Drug pharmacokinetics plays an important role in guiding the selection of clinical drug dosages and optimizing dosing regimens [6]. Baicalin is known to exhibit different degrees of accumulation in animal models of diabetes [8], ulcerative colitis [9], and cerebral ischemia-reperfusion [10]. In the animal model of fever, the concentration and bioavailability of baicalin are significantly reduced [11]. We previously reported that a significant increase *in vivo* exposure led to delayed *in vivo* clearance (CL) after a single dose of baicalin in rats with IHC [12]. However, the effects of the long-term administration of baicalin on its pharmacokinetics are unknown.

Baicalin is used as a specific substrate of multidrug resistance protein 2 (MRP2) [13]. Changes in MRP2 activity may affect the distribution and metabolism of baicalin *in vivo*. We have found that continuous treatment with baicalin can reverse the decreased expression of MRP2 in rats with IHC [7]. Therefore, we hypothesized that the pharmacokinetics of baicalin in IHC may be different when used as a single dose versus in the long term.

In this study, we used liquid chromatography-tandem mass spectrometry (LC-MS) to determine the concentration of baicalin in the plasma of rats with IHC after single or multiple administrations to preliminarily explore the differences in the pharmacokinetic characteristics of baicalin in IHC. Furthermore, we studied the effect of baicalin on MRP2 activity *in vivo*. Our findings suggest that the continuous administration of baicalin could restore its pharmacokinetic characteristics in rats with IHC and that the restoration may be associated with enhanced MRP2 expression.

2. Materials and methods

2.1. Chemicals and reagents

Baicalin (purity ≥99.0%) and naringenin (purity ≥98%) were obtained from the National Institutes for Food and Drug Control. 17α-Ethynylestradiol (EE) (purity ≥99.8%) and pentobarbital sodium (purity ≥99.0%) were purchased from Sigma-Aldrich. Antibodies against MRP2 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Absin Biochemical Company (Shanghai, China). Methanol, acetonitrile, and other chemicals and reagents were of analytical grade.

2.2. Animal experiments and protocols

Adult male Sprague-Dawley rats (weighing 220 ± 20 g) were purchased from the Center of Experimental Animal of Tongji Medical School, Huazhong Science and Technology University. All animal studies were approved by the Ethics Committee on Animal Experimentation of Tongji Medical School, Huazhong Science and Technology University (TJH-202106008). Animal experiments were conducted in accordance with the National Institutes of Health guide for the care and use of laboratory animals. Rats were maintained on a standard laboratory pellet diet, provided access to food and water *ad libitum*, and maintained on a 12-h/12-h automatically timed light/dark cycle. All rats were acclimatized for one week before commencing experiments.

All rats were randomly divided into the following 4 groups ((n = 10): A (Single baicalin), B (Multiple baicalin), C (EE + Single baicalin), and D (EE + Multiple baicalin). The rat model of IHC was established by subcutaneously injecting EE propylene glycol solution for 5 d as reported in a previous study [7]. Baicalin was intragastrically administered single dose (200 mg/kg) after the EE injection, and successive intragastrically administrated for 5 d parallelly with the EE injection.

After the last treatment of EE or baicalin, blood was drawn from the orbital canthal venous plexus at 0.13, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, and 36 h and collected into 1.5 mL tubes containing heparin and immediately centrifuged at 4000 rpm for 10 min. The plasma and hepatic tissue were collected and stored at −80 °C.

2.3. Determination of baicalin content in plasma samples using LC-MS

2.3.1. Mass spectrometry conditions

The mass spectrometry conditions were electrospray ion source (ESI), positive ion mode (+), and multiple ion reaction monitoring (MRM). Ion pair monitoring and other optimal parameters of baicalin and the internal standard (naringenin) are shown in Table 1.

2.3.2. Chromatographic conditions

The stationary phase was a C_{18} chromatographic column (5 μm, 4.6 × 150 mm, Waters XTERRA® RP18), mobile phase A was an aqueous solution containing 0.1% formic acid, mobile phase B was methanol (A:B = 20:80, v/v), and the elution time was 5 min. The

Table 1
Ion pair and MS parameters of baicalin and naringenin (internal standard [IS]).

	Q1	Q3	DP	CE
baicalin	447.0	271.3	80	22
naringenin	273.0	153.2	103	28

flow rate was 0.8 mL/min, the injection volume was 10 μL, and the column temperature was 30 °C.

2.3.3. Processing of plasma samples

Plasma samples of rats stored at −80 °C were thawed at 25 °C. To 50 μL of the plasma sample, 200 μL of a methanol solution of 1.009 μg/mL of naringenin was added. The samples were vortexed for 3 min and centrifuged at 12,000 rpm for 10 min. Next, 200 μL of the plasma was blow-dried in a stream of nitrogen, followed by LC-MS (AB Sciex, the USA).

2.4. Western blotting

Western blotting of protein samples was performed as described in our previous study [14]. Briefly, the total protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membrane. After blocking for 90 min with 5% nonfat milk in Tris-buffered saline with Tween 20, the membranes were incubated overnight at 4 °C with primary antibodies directed against MRP2 (1:1000) and GAPDH (1:1000). Next, membranes were incubated with HRP-conjugated secondary antibodies (1:2500) for 1 h at room temperature. Protein expression was detected using enhanced chemiluminescence (ECL) ECL kit (MedChemExpress, China) and the membranes were imaged using a BOX Chemi XRQ imaging system (SynGene, Cambridge, United Kingdom).

2.5. Statistical analysis

LC-MS data were collected and analyzed using Analyst 1.6.1. All data are expressed as mean ± standard deviation and were analyzed using SPSS version 19.0. One-way ANOVA and Dunnett's *t*-test were used to determine statistical differences between groups. $p < 0.05$ indicates statistical difference.

3. Results

3.1. Method validation

Briefly, the LC-MS method to determine baicalin in plasma samples was specific and efficient. Baicalin and naringenin could be readily identified in the ESI positive-ion mode (baicalin: *m/z* 447.0 → 271.3; naringenin *m/z* 273.0 → 153.2). Ion maps are shown in Fig. 1A and B.

The peak shape of baicalin could be affected by the mobile phase ratio and flow rate. To improve the peak shape, a water (0.1% formic acid)-methanol (20:80, v/v) system was selected for elution, and the flow rate was set to 0.8 mL/min. Baicalin and naringenin exhibited good peak shape and stable retention time (Rt) under these conditions. The Rt of baicalin was 2.47 min and that of naringenin was 2.04 min (Fig. 2C). These results showed that endogenous substances did not interfere with the peaks of baicalin and naringenin while analyzing rat plasma (Fig. 2A–C).

Next, we determined the precision, accuracy, and recovery validation of baicalin in the quality control (QC) samples at concentrations of 1.01, 2.53, 101.20, and 404.80 μg/mL. The intra-day accuracy and precision were 97.99%–105.40% and 0.95%–6.49%, respectively, and the inter-day accuracy and precision were 94.94%–109.13% and 3.47%–10.55%, respectively (Table 2). In addition, the recoveries of baicalin were 86.02%–103.78% in QC samples with concentrations of 1.01, 2.53, 101.20, and 404.80 μg/mL. QC samples prepared at concentrations of 2.53, 101.20, and 404.80 μg/mL were stored at −80 °C for 7 d, frozen, thawed at 25 °C 3 times, and stored in the autosampler for 24 h. Results from the analysis revealed that baicalin was stable in the QC samples (RSD: 1.29%–10.87%) (Table 3). All samples were in line with the requirements for quantitative analysis of biological samples as stated in the 2020 Edition of the Chinese Pharmacopoeia.

3.2. Pharmacokinetics of baicalin in rats with IHC

We investigated the pharmacokinetics of baicalin in normal rats and those with IHC after both single and continuous administration. The drug-time curves of baicalin show a double-peak phenomenon in normal rats and those with IHC (Fig. 3). As shown in Table 4, the peak value (T_{max}) was attained in 9–10 h in rats in the normal group after a single dose of 200 mg/kg of baicalin (group A), following which, baicalin levels in blood decreased slowly. The area under the curve $(AUC)_{(0\text{-}t)}$ and $AUC_{(0\text{-}\infty)}$ of the multiple-dose group (group B) in normal rats were higher than those of rats in group A and reached 3766.63 ± 692.70 and 3870.72 ± 755.24 μg/mL·h, respectively, whereas the T_{max} was approximately 10 h.

Compared with group A, the C_{max}, $AUC_{(0\text{-}t)}$, and $AUC_{(0\text{-}\infty)}$ of baicalin were significantly increased in rats in the EE + single baicalin group (group C) ($p < 0.01$). The $t_{1/2}$ was significantly prolonged to 10.23 ± 4.48 h in group C and the CL decreased to approximately 0.35 times compared with group A ($p < 0.001$). The above results showed that the pharmacokinetics of baicalin changed significantly in rats with IHC after a single dose of baicalin and manifested as the peak concentration of baicalin *in vivo* was increased. There was a decrease in CL as the exposure was increased significantly.

Compared with group C, continuous baicalin pretreatment could significantly reduce the AUC of baicalin in group D, which manifested as a drop to 4123.18 ± 1261.29 $(AUC_{(0\text{-}t)})$ and 4144.14 ± 1255.25 μg/mL·h $(AUC_{(0\text{-}\infty)})$. The T_{max} was significantly shorter than that in group C ($p < 0.05$). The CL increased and the $t_{1/2}$ was significantly reduced to 3.75 ± 1.06 h, which was close to that in group A. However, the $AUC_{(0\text{-}t)}$, $AUC_{(0\text{-}\infty)}$, $t_{1/2}$, and CL between groups B and D were not significantly different ($p > 0.05$). These results

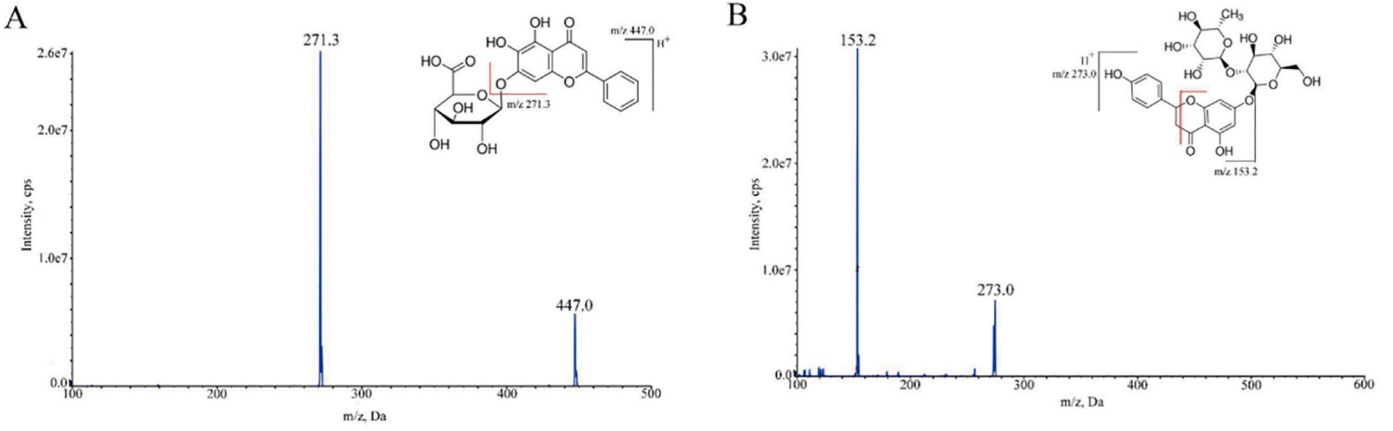

Fig. 1. Full scan mass spectra of (A) baicalin and (B) naringenin.

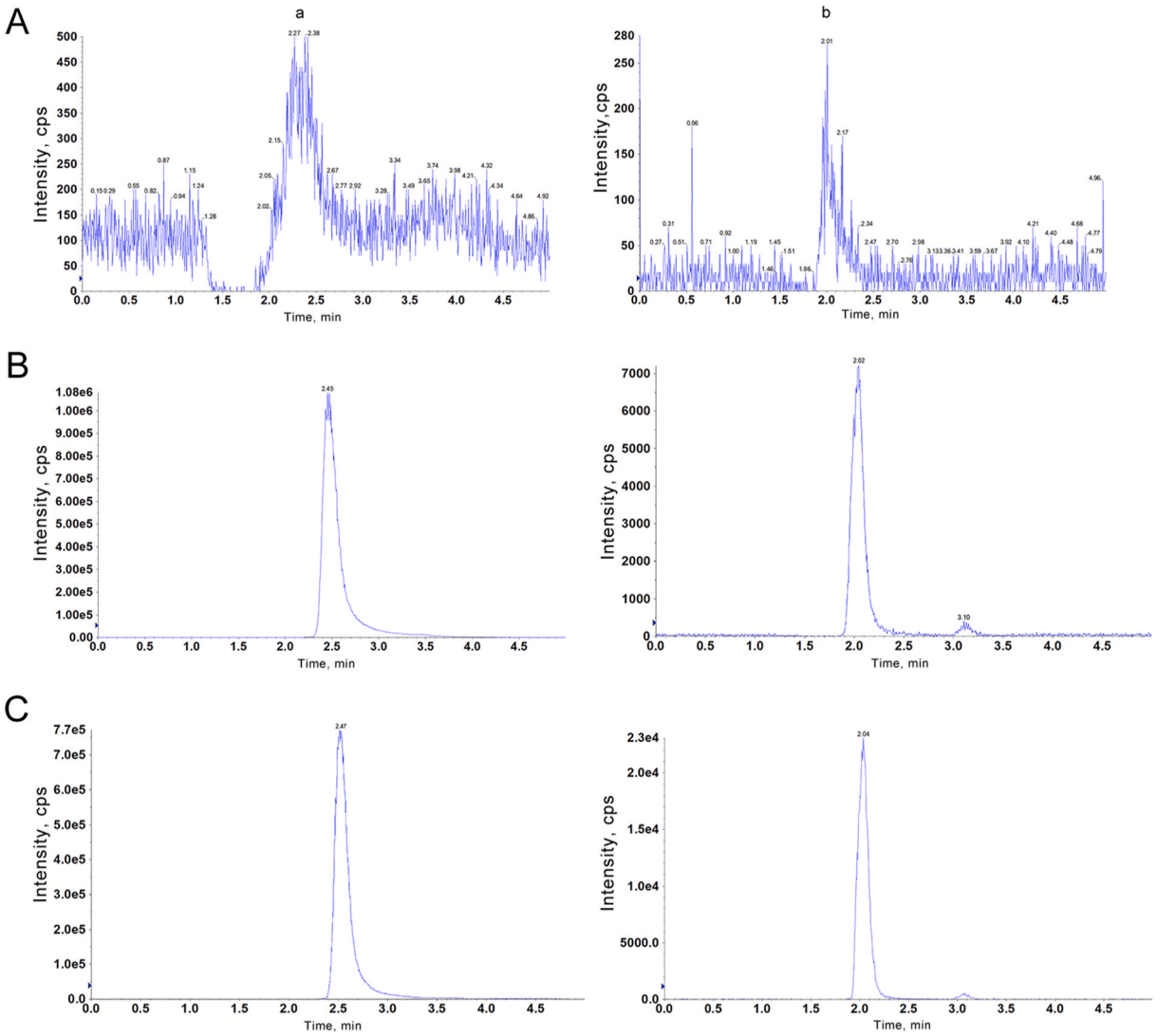

Fig. 2. Representative multiple reaction monitoring chromatograms for baicalin (a) and naringenin (b) in rat plasma samples. (A) Blank plasma sample; (B) plasma spiked with baicalin using 202.40 μg/mL baicalin and naringenin; (C) plasma sample 30 min after the oral administration of baicalin and naringenin.

indicated that the continuous administration of baicalin may significantly improve the accumulation of baicalin by increasing its CL and reducing the AUC in rats with IHC.

3.3. Effects of different administration modes of baicalin on MRP2 in rats with IHC

MRP2 plays an important role in bile acid regulation and drug transport in the liver, and a dysfunction or gene mutation is may result in cholestatic liver diseases [15,16]. Besides, baicalin is a specific substrate for MRP2, and decreased MRP2 activity in IHC may inhibit baicalin efflux, thereby increasing its AUC *in vivo* [17]. In this study, we investigated the effect of single and multiple

Table 2
Accuracy, RSD, and recovery of baicalin in quality control samples.

Concentration (μg/mL)	Intra-day(n = 5)		Inter-day(n = 5)		Recovery(%)(n = 3)
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	
1.01	104.06	6.49	100.71	10.55	103.78
2.53	98.26	5.48	99.28	3.47	91.12
101.20	105.40	6.03	109.13	4.23	86.02
404.80	97.99	0.95	94.94	6.30	86.47

Table 3
Stability of baicalin in quality control samples.

Concentration (μg/mL)	A		B		C		D	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
2.53	108.38	3.91	110.84	5.38	93.49	9.35	106.28	5.55
101.20	98.22	10.12	95.39	2.90	107.58	1.29	97.45	10.87
404.80	101.29	2.73	98.32	6.45	102.39	5.34	98.41	3.09

Note: A: −80 °C cryopreservation for 7 days; B: −80 °C repetitive freeze-thawing; C: Maintained at room temperature for 6 h; D: Stored in the autosampler for 24 h.

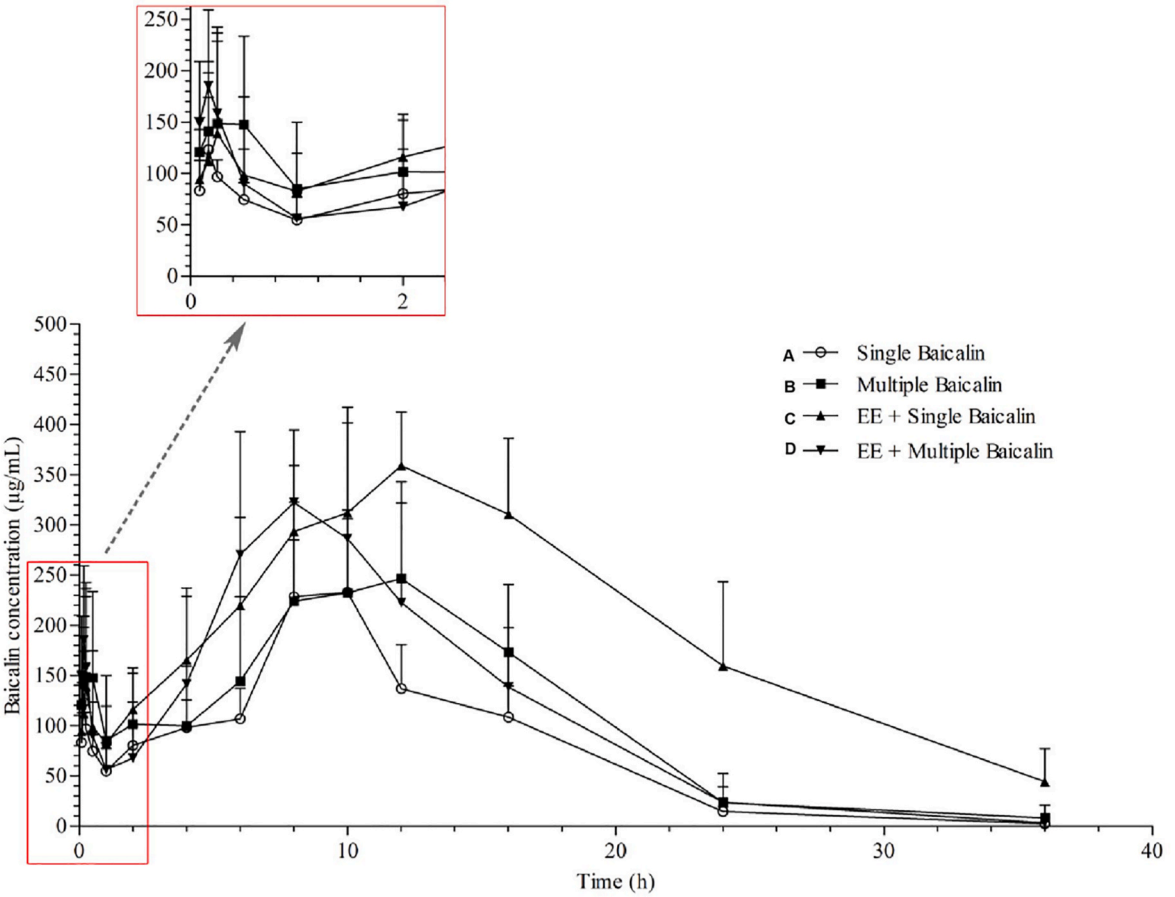

Fig. 3. Plasma concentration-time curves of baicalin in rats (mean ± SD, (n = 10). (A) Single baicalin group; (B) multiple baicalin group; (C) EE + single baicalin group; (D) EE + multiple baicalin group.

Table 4
Pharmacokinetic parameters of baicalin.

Parameters	Control + single baicalin (A)	Control + multiple baicalin (B)	EE + single baicalin (C)	EE + multiple baicalin (D)
$AUC_{(0-t)}$ (μg/mL*h)	2697.03 ± 593.49	3766.63 ± 692.70*	6981.63 ± 1540.03***	4123.18 ± 1261.29$^{\#\#\#}$
$AUC_{(0-\infty)}$ (μg/mL*h)	2714.93 ± 591.46	3870.72 ± 755.24*	8026.16 ± 2022.48***	4144.14 ± 1255.25$^{\#\#\#}$
C_{max} (μg/mL)	248.76 ± 78.05	282.24 ± 60.58	383.29 ± 63.18**	363.63 ± 93.58
T_{max} (h)	9.14 ± 1.07	10.29 ± 2.43	9.18 ± 4.21	7.71 ± 1.80$^{\#\#}$
CL (mL/h/kg)	76.86 ± 17.54	53.29 ± 10.13*	26.86 ± 8.51***	51.57 ± 12.95$^{\#\#}$
$t_{1/2}$ (h)	3.83 ± 1.47	4.15 ± 3.27	10.23 ± 4.48**	3.75 ± 1.06$^{\#\#\#}$

Data values are depicted as mean ± SD, n = 10. *p < 0.05, **p < 0.01, ***p < 0.001 vs A group, $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001 vs C.

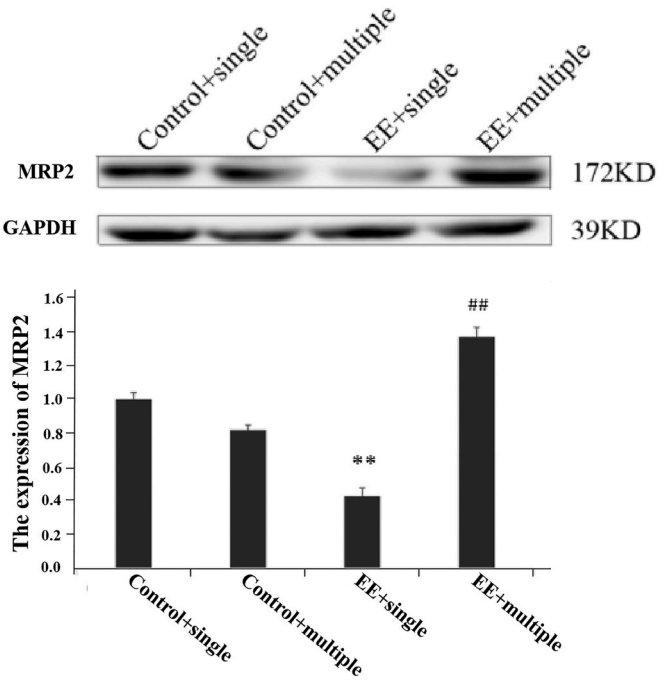

Fig. 4. Effect of different administration modes of baicalin on MRP2. There were no significant differences in MRP2 expression in normal rats after single and multiple baicalin doses. MRP2 expression was reduced in rats with IHC after a single dose of baicalin. Compared with that achieved using a single dose, the downregulation of MRP2 was improved in IHC after multiple doses of baicalin ((n = 10). ** was $p < 0.01$ versus (vs) single baicalin group, ## was $p < 0.001$ vs EE + single baicalin group.

consecutive administration of baicalin on MRP2 in a rat model of IHC. As shown in Fig. 4, MRP2 expression in normal rats was not significantly different after single or multiple consecutive administration of baicalin. MRP2 down-regulation was not alleviated in rats with IHC after a single administration of baicalin ($p < 0.01$). However, MRP2 expression was significantly enhanced in rats with IHC after the continuous administration of baicalin. These results indicated that the continuous administration of baicalin could improve MRP2 expression, which may effectively improve its CL and reduce its accumulation and residue *in vivo*.

4. Discussion

In this study, an LC-MS method was established to determine plasma baicalin levels in rats. This method had good specificity, sensitivity, precision, and accuracy. We determined changes in the pharmacokinetic characteristics in normal rats and those with IHC after single as well as multiple administration of baicalin. We found that exposure to baicalin was significantly increased and the CL was reduced in rats with IHC after a single administration of baicalin, which was in accordance with findings from our previous study [12]. However, continuous administration of baicalin effectively decreased the exposure and increased the CL and MRP2 expression in rats with IHC. These phenomena may be related to the MRP2-enhancing effect of baicalin.

EE exposure can disrupt liver homeostasis and impact the pharmacokinetic profile of drugs. Studies show that the pharmacokinetics of certain drugs, including methotrexate, morphine, and metformin, are altered in EE-induced IHC [18,19]. Studies have demonstrated that the disposal processes of baicalin *in vivo*, such as its hydrolysis in the gastrointestinal tract [20], enterohepatic circulation [21], carrier-mediated transport through cell membranes (13) and excretion in the bile and urine [22], are complex. Thus, bile flow can affect the fate of baicalin *in vivo*. In IHC, deceased bile flow results in a reduction in the total amount of baicalin in the bile, thereby decreasing the CL of baicalin. Moreover, alterations in transporter expression and activity in the cholestatic liver are considered the main factors affecting drug pharmacokinetics. MRP2 is an efflux transporter responsible for the transport of numerous endogenous compounds and xenobiotics including taurocholic acid, methotrexate, and baicalin [23]. About half of the administered baicalin is excreted into the bile by MRP2 and flows into the gut to complete enterohepatic circulation [24]. MRP2 knockdown significantly increases plasma baicalin concentration [25], whereas increasing MRP2 expression or activity significantly enhances baicalin CL and reduces its oral availability [17]. Thus, MRP2 plays an important role in mediating the bile efflux of baicalin. In this study, we found that MRP2 expression was decreased in rats with IHC, resulting in an increase in the AUC, a decrease in CL, and a significant prolongation of its $t_{1/2}$ *in vivo*. Continuous administration of baicalin could significantly promote MRP2 expression (Fig. 4), thereby increasing bile flow, promoting its own enterohepatic circulation, increasing CL *in vivo*, and reducing accumulation.

Since baicalin is frequently prescribed with other medications, understanding the pharmacokinetics of baicalin is of importance for clinical applications. Furthermore, pharmacokinetic changes of baicalin under different pathological conditions indicate clinical considerations of drug safety and the possible requirement of individualized therapy. Thus, we explored the pharmacokinetics of

baicalin in IHC, aiming to provide valuable reference for its clinical application and individualized medication. According to our results, the clinical dose of baicalin should be carefully determined when used to treat patients with ICH. However, our study still has some limitations. Baicalin undergoes extensive first-pass metabolism, has low bioavailability and a short half-life due to the glycosyl group on the ring. Although many studies have been conducted on the pharmacokinetics of baicalin, there are still some uncertainties regarding its *in vivo* process and effects, which need more studies to elucidate.

In conclusion, the pharmacokinetics of baicalin was significantly different in rats with IHC with single and multiple administration of baicalin. Continuous administration of baicalin significantly improved baicalin accumulation *in vivo*. Its pharmacokinetic parameters were gradually restored to normal levels likely due to enhanced MRP2 activity. However, the regulatory mechanism of baicalin in modulating MRP2 expression needs to be further explored.

Ethics approval number for the animal study

TJH-202106008.

Author contributions

Yue Zu: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Yanan Liu and Lulu Lan: Performed the experiments; Analyzed and interpreted the data. Chen Zhu: Contributed reagents, materials, analysis tools or data; Chengliang Zhang and Dong Liu: Conceived and designed the experiments.

Funding statement

Dr. Chengliang Zhang and Dr. Dong Liu were supported by National Natural Science Foundation of China [82073939].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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