An LC–MS/MS method for determination of bioactive components of licorice and Semen Strychni in rat plasma: application to a pharmacokinetics study

Running title: Determination of licorice and semen strychni components

Min Zhang[1,2]; Email: csuzhangmin@163.com

Yang Deng[1,2]; Email: deng14789918527@126.com

Chao Wang[1,2]; Email: 15573299941@163.com

Hua-Lin Cai[1,2]; Email: ghostspecialist@163.com

Jing-Wen[1,2]; Email: nuliwen5832@163.com

Ping-Fei Fang[1,2*]

*Corresponding author

Department of Pharmacy, the Second Xiangya Hospital, Central South University, Renmin Road 139# Changsha 410011, China; fangpingfei@163.com, fax: 0731-85292098

Bi-Kui Zhang[1,2]; Email: zhbk68@163.com

Huan-De Li[1,2]; Email: lihuande1953@126.com

Miao Yan[1,2]; Email: yanmiaocsu@126.com

1. Department of Pharmacy, the Second Xiangya Hospital, Central South University, Changsha 410011, PR China；

2. Institute of Clinical Pharmacy, Central South University, Changsha 410011, PR China

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Key words: Semen Strychni, Licorice, Bioactive ingredient, LC-MS/MS, Pharmacokinetics.

Abstract

Semen Strychni is known for its treatment of rheumatic arthritis with a low therapeutic index. Licorice contributes a lot in herb detoxification according to the traditional Chinese medicine theory. A simple, rapid and sensitive liquid chromatography–mass spectrometric method was developed and validated for simultaneous determination of main bioactive ingredients in licorice and Semen Strychni in rat plasma. Using moclobemide and cyproterone acetate as the internal standards, the analytes were pretreated via protein precipitation with methanol. An Ultimate AQ-C18 column (3.0 μm, 3.0×100 mm) was employed for chromatographic separation, combining with gradient elution. The mobile phase consisted of 0.07% formic acid and 0.12% ammonium acetate in aqueous phase (A) and acetonitrile in organic phase (B). The elution program was as follows: 0–0.5 min, 20% B; 0.5–1 min, 20–60% B; 1–7 min, 60–85% B; and 7–7.5 min, returned to 20% B, then continued to 12 min. Selected reaction monitoring was performed in both positive and negative ESI. Positive mode was adopted for detection of strychnine, brucine and moclobemide, while negative mode was used for glycyrrhizic acid, glycyrrhetinic acid, liquiritigenin, isoliquiritigenin, liquiritin and cyproterone acetate. The method was validated for specificity, linearity, matrix effect, recovery, precision, accuracy and stability. The results show that this method is sensitive, accurate and robust for biological matrix analysis. Moreover, the proposed method was applied to a pharmacokinetic study in Sprague-Dawley rats for investigating the mechanism of which liquorice detoxifies Semen Strychni.

Key words: Semen Strychni, Licorice, Bioactive ingredient, LC-MS/MS, Pharmacokinetics.

1 Introduction

Semen Strychni, the dried seeds of Strychnos nux-vomica L. (Loganiaceae), has been officially listed in Chinese Pharmacopoeia[1]. The pharmacological activities of Semen Strychni, including its improving blood circulation[2], alleviating rheumatic arthritics and relieving cancer pain[3], as well as the anti-inflammatory, anti-angiogenesis effects[4], can mainly be attributed to strychnine and brucine, two main alkaloids in *Semen Strychni*[5]. However, the use of Semen Strychni is limited by the fatal neurotoxicity of these alkaloids[1]. In fact, the toxicity of Semen Strychni could be relieved if processed properly. Traditionally, thermal treatments with oil or sand bath is an effective way to reduce toxicity[1]. Furthermore, licorice was reported to have detoxic effect through compatibility or herb–herb interactions[6]. Because of the unsatisfactory therapeutic index and obvious individual difference of strychnine, brucine was chosen as a probe drug to investigated the pharmacokinetics interaction with licorice, and the underlying detoxification mechanism was explored.

Glycyrrhiza, one of the most popular traditional Chinese medicines(TCM), has been widely used in China for its medical potential[7], in the treatment of asthma with coughing[8], liver function improvement[9] and especially, drug toxicity relieving[10-12] according to TCM theory. Phytochemical investigations reveal that flavones and triterpenes are principal components responsible for the main pharmacological activities of glycyrrhiza[13]. Liquiritigenin, isoliquiritigenin, liquiritin are typical flavone compounds, and glycyrrhizic acid, glycyrrhetinic acid regard as the main triterpenes[7]. Recent studies demonstrated that licorice showed a remarkable interaction with strychnine and brucine[6], which may be an underlying detoxification mechanism of licorice. However, the disposition *in vivo* and herb-herb interaction of principal components in licorice and Semen Strychni remains to be clarified by pharmacokinetic study. Therefore, it is of great significance to establish an effective and sensitive method for simultaneously determination of multiple bioactive compounds in Semen Strychni and liquorice in plasma samples.

As far as we know, no method has been documented for the simultaneous determination of flavonoids, triterpenes from liquorice and alkaloids from Semen Strychni *in vivo*. Several analytical methods for quantitative determination of the flavonoids, triterpenes or alkaloids have been described, including high performance liquid chromatography with ultraviolet detection (HPLC-UV)[3], capillary zone electrophoresis coupled to time-of-flight mass spectrometry[14], liquid chromatography-tandem mass spectrometric (LC/MS)[15, 16] and high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC/MS/MS)[17]. Recently, a rapid, sensitive HPLC with ultrasound-assisted mixed cloud

point extraction method was developed and validated to simultaneously quantify strychnine, brucine, strychnine *N*-oxide and brucine *N*-oxide in rat plasma simultaneously, in which the LLOQ is 1 ng/mL[18]. Besides, an LC–MS/MS method with high sensitivity (0.5 ng/mL for strychnine, brucine, 0.1 ng/mL for strychnine *N*-oxide and brucine *N*-oxide) was also established[19]. On the other hand, systematical quantitative analysis of the active constituents of licorice has been reported both in vitro[20] and in vivo samples[15, 21]. Nevertheless, the sensitivity of liquiritigenin (LLOQ 12.5 ng/mL), isoliquiritigenin (LLOQ 12.5 ng/mL) and liquiritin (LLOQ 7.5 ng/mL) in previously reported methods were not enough to determine the plasma concentration of rats in our research for pharmacokinetic study[21]. Furthermore there were no published method that could comprehensively and concurrently measuring the principal components of licorice and Semen Strychni, not to mention studying their pharmacokinetic behaviors and herb-herb interaction effects.

In the present study, a sensitive, rapid and robust HPLC-MS/MS method was developed to simultaneously determine seven components in plasma samples, including two alkaloids (brucine and strychnine), two triterpenes (glycyrrhizic acid and glycyrrhetinic acid), and three flavonoids (liquiritigenin, isoliquiritigenin and liquiritin). The quantitative method was then successfully applied to a pharmacokinetic study after oral administration of brucine and subsequent (30 minutes later) licorice extracts for detoxification in Sprague-Dawley rats. The findings would be beneficial for evaluating the ADME and exploring the underlying detoxification mechanism of licorice, as well as the safety clinical application of Semen Strychni.

2. Materials and methods :

2.1 Chemicals and reagents

Standards including strychnine, brucine, glycyrrhetinic acid, moclobemide, cyproterone acetate were purchased from the National Institute for Food and Drug Control (Beijing, China). Glycyrrhizic acid, liquiritin, liquiritigenin, isoliquiritigenin were obtained from Chengdu Preferred Biological Technology Co. Ltd (Chengdu, China). Methanol, acetonitrile (HPLC grade) were from ACS Company (Poole,UK). Formic acid (HPLC grade) was from ROE scientific INC (Network USA). Ultra-pure water was deionized and purified by a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents were of analytical grade.

2.2 Preparation of standard solutions and internal standards

Stock solutions of seven reference compounds and the internal standards (ISs) were weighed accurately and dissolved in methanol at 0.50 mg/mL. Moclobemide, cyproterone acetate were chosen as IS1 and IS2 for alkaloid and flavonoids, triterpenes, respectively. The working standard solutions were obtained by mixing seven stock solutions and then being diluted with acetonitrile to a series of appropriate concentrations. The ISs were mixed first, then prepared by diluting stock solutions with acetonitrile to final concentrations of 10 ng/mL (moclobemide) and 5 μg/mL (cyproterone acetate) as work solutions. All solutions were stored at 4℃ during analysis.

2.3 Sample preparation

Plasma sample (100 μL) was pipetted into 1.5 mL EP tube, in addition to 100 μL of the IS work solution and 900 μL of methanol. After mixing for 3 min and centrifugation at 15000 rpm (20627 *g*) for 10 min, an aliquot of 1.05 mL from the supernatant was transferred and subsequently evaporated to dryness by vacuum concentration. The residue was dissolved by 50% acetonitrile and followed with blending for 3 min and centrifugation for 5 min at 15000 rpm (20627 *g*). Finally, the supernatant was transferred for LC-MS/MS analysis.

2.4 Instrumentation and chromatographic conditions

The analytes were determined by an LC-20A HPLC system (SHIMADZU, Kyoto, Japan), coupled with an 4000 triple-quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA). An Ultimate AQ-C18 column (3.0 μm, 3.0×100 mm, Welch Materials Inc, MD, USA) was used for chromatographic separation. The mobile phase was a gradient elution of A (0.07% ammonium acetate and 0.12% formic acid aqueous) and acetonitrile (B). The elution program was as follows: 0–0.5 min, 20% B; 0.5–1 min, 20–60% B; 1–7 min, 60–85% B; and 7–7.5 min, returned to 20% B, then continued to 12 min. The initial flow rate was 0.35 mL/min, the column temperature was 40 ℃ and the injection volume was 5 μL. The MS was operated in SRM mode. The strychnine, brucine and IS1 were detected in a positive ESI mode, while the others were in negative ion mode. The main MS parameters in ESI^+ was as

follows: curtain gas at 30; collision gas at high level; ionspray voltage at 4500 V; turbo gas temperature at 550 °C; dry gas at 50; nebulizer gas at 50; cell exit potential at 10; entrance potential at 10 and the dwell time was 100 ms. The values of MS parameters in ESI^- were similar to ESI^+ mode except for the ionspray voltage, which was 4000 V. The declustering potential (DP), collision energy (CE) and the SRM transitions were individually optimized for each compound (Table 1).

2.5 Method validation

The analytical method was validated for selectivity, linearity, matrix effects, extraction recovery, precision, accuracy and stability.

2.5.1 Specificity

Specificity was tested by analyzing blank plasma, blank plasma spiked with strychnine, brucine, glycyrrhetinic acid, glycyrrhizic acid, liquiritin, liquiritigenin, isoliquiritigenin and internal standards, and plasma samples from rats after oral administration of the brucine and licorice extracts.

2.5.2 Linearity and lower limit of quantification (LLOQ)

Blank plasma was added with reference compounds stock solutions to prepare a series of calibration standard samples which ranged widely (Table 2). Then the plasma samples were prepared as described in sample preparation part (2.3). The peak areas ratios (analyte/IS) and plasma concentrations were fitted to the linear regression (1/X as weighting factor). The LLOQ was defined as the lowest plasma concentration in the calibration curve.

2.5.3 Matrix effect and extraction recovery

The matrix effect was assessed by comparing the peak area percentage of analytes in standard working solution with extracted and dried blank plasma redissolving with mixed standard working solution. The extraction recovery was evaluated by using three levels of plasma QC samples (blank plasma spiking with combined standard working solution) and extracted blank plasma spiked with the combined standard working solution. The peak area percentage of anlytes in QC samples and extracted blank plasma spiking with standard

working solution was determined as extraction recovery.

2.5.4 Precision and accuracy

Replicates of QC samples at three levels were prepared for intra-day assay accuracy and precision. And the samples were prepared and analyzed on three consecutive days for the inter-day variations. The precision was described as relative standard deviation (RSD) and the accuracy was exhibited as relative error (RE). Meanwhile, the accuracy and recovery rate of anyltes at LLOQ was also reported.

2.5.5 Stability

Stability of anyltes was evaluated by QC samples in different conditions, including three freeze-thaw cycles at -70℃ (freeze) and room temperature (thaw), and placing them on the bench top for 5 h at room temperature. The long-term storage stability was also determined after storage for 21 days at -70℃.

2.6 Application in pharmacokinetic study

This study was approved by the Animal Care & Use Committee of Central South University. All experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals (Chinese Council). Male Sprague-Dawley rats (300-320 g) were purchased from Hunan SJA Laboratory Animal Co. Ltd (Hunan, China). The animals were housed in a temperature-controlled environment（22±2 °C） under a 12/12 h light/dark cycle with optionally access to food and water for a week. The rats were fasted overnight (12-14 h) before dosing and had free access to water throughout the experimental period. The brucine was dissolved in 0.5 M HCl, then adjusted pH to 6.5 by 0.5 M NaOH and diluted to 4 mg/mL with water. The liquorice was extracted in a traditional way. In short, crude licorice (54 g) was soaked for 12 h and decocted in 250 mL of water for 2 hours twice. The decoctions were filtered, combined and concentrated to a final concentration of equivalent to 1.8 g of crude drug per milliliter (glycyrrhizic acid, 17.75 mg/mL; glycyrrhetinic acid, 3.55 μg/mL; liquiritigenin, 0.867 mg/mL; isoliquiritigenin, 0.261 mg/mL, liquiritin, 5.26 mg/mL). The animals were randomly divided into 3 groups (n = 8 for each group) before treatment. One group rats received a single oral administration of 40 mg/kg of brucine and 30 min later, a gavage of liquorice extract brucine (18 g/kg) was given. The other two groups were respectively administrated with liquorice extract or brucine alone.

Blood samples were collected from ophthalmic venous plexus at 0, 0.833, 0.25, 0.58, 0.75, 1, 2, 3, 5, 7, 9, 11, 24, 35, 48 h after oral administration of brucine. The plasma samples were centrifuged at 3700 rpm for 10 min. All these samples were stored at −70℃ until analysis.

2.7 Data analysis

Pharmacokinetic parameters were assessed by the DAS 3.2.8. t_{max}, and C_{max} were obtained directly from the plasma concentration-time curve. Other parameters were counted by non-compartment modeling using the DAS software.

3. Results and discussion

3.1 Chromatography development

Chromatography development included the composition of the mobile phase, flow rate and injection volume. The acetonitrile was chosen as the organic phase to obtain higher eluting ability for the analytes and less pressure on the LC system. The formic acid in mobile phase could get a better peak shape and enhance the ion response of analytes. Strychnine and brucine were alkaloids, so formic acid could not only regulate the pH of mobile phase but also promote ionization of the analytes in the ESI^+ mode. On the other hand, ammonium acetate was used to improve the ionization efficiency and sensitivity in the ESI^- mode. Therefore, the ratio of formic acid to ammonium acetate in the aqueous phase was very important to achieve good chromatographic behavior and appropriate ionization in both the ESI^+ mode and ESI^- mode. Finally, the optimized mobile phase of this method constituted of 0.07% (10 mmol) ammonium acetate and 0.1% formic acid in the aqueous phase which adjusted the pH to 6.5. To achieve a short run time for the analysis, the flow rate of mobile phase was set at 0.35 mL/min. The injection volume of 1, 2 and 5 μL were investigated, and 5 μL was chosen as the optimal injection volume for its better instrument response of analytes.

The selection of ISs is very important for the analysis of target compounds. To find a compound that could ideally mimic the analytes and serve as an appropriate IS, we screened several compounds. Finally, moclobemide and cyproterone acetate were chosen as the ISs in positive and negative scan modes, respectively. Moclobemide shares similar physicochemical property and chromatographic behavior with strychnine and brucine. Cyproterone acetate is a typical steroid with proper chromatographic behavior for the determination of triterpenes and flavonoids since itsretentio n locates in between (Fig 1).

3.2 Sample preparation

Initially, we used pure acetonitrile as the precipitant, but it produced unsatisfied recovery rate of glyccirizhic acid and glycyrrhetinic acid. Therefore a combination of acetonitrile and methanol mixture was utilized as the agent for deproteinization of plasma. We explored a series of ACN/MeOH proportion to find a satisfactory mixed solvent (Supplement table 1). Obviously, ACN/MeOH (1:9, v/v) showed the optimum recovery rate for the analytes and ISs. Plasma concentrations of analytes ranged widely, so we also screened the suitable precipitant volume. Ultimately, decuple volume of protein precipitant was elected instead of triple or quintuple volume, in case of incomplete extraction.

3.3 Mass spectrometry

To optimize MS parameters, pure compounds (1.0 μg/mL in methanol) were individually injected into MS instrument using the ESI source and operating in the SRM scan mode. The signal intensity of brucine and strychnine obtained in positive mode was much higher than that of negative mode. On the contrary, the response of glycyrrhizic acid, glycyrrhetinic acid, liquiritigenin, isoliquiritigenin and liquiritin were stronger in the positive ion mode. Parameters such as DP, CE, and the most abundant ions were optimized to obtain highest intensity of deprotonated molecules of the analytes and ISs (Table 1).

Additionally, because of the different properties from the flavonoids and triterpenes, the alkaloids need to be detected separately. Recently, UHPLC–ESI/MS/MS and HPLC–Q-TOF-MS method were reported to simultaneous qualitative analysis of flavonoids and alkaloids in both positive and negative ESI[22,23]. In this paper, we firstly measured the alkaloids, flavonoids and triterpenes in licorice and Semen Strychni simultaneously via ESI mode switch in different retention periods, which not only achieved approving chromatograph separation (Fig 1), but also had sufficient sensitivity for analytes.

3.4 Method validation

3.4.1 Specificity

Selectivity was investigated using blank rat plasma, blank plasma spiking with analytes and IS and plasma samples after oral administration of brucine and liquorice extract, which were evaluated for interference. As shown in Fig. 1, no significant interference peak around the retention times of the analytes and IS was observed, which fulfilled the guideline of bioanalytical method validation.

3.4.2 Linearity and lower limit of quantification (LLOQ)

The calibration curves, correlation coefficients, LLOQ and linear ranges of the seven analytes in plasma are shown in Table 2. The calibration curves exhibited good linearity and covered wide ranges. The LLOQ of glycyrrhetinic acid (2.55 ng/mL), liquiritin (0.65 ng/mL), liquiritigenin (0.28 ng/mL), isoliquiritigenin (0.13 ng/mL) in our method was excellent. The recovery rate, accuracy and precision in LLOQ were also measured. It turned out that the recovery rate was within 87.2% to 114.3%. The RE value of accuracy and the RSD value of precision was less than 15.0%.

3.4.3. Matrix effect and extraction recovery

Both matrix effect and extraction recovery were tested using QC samples at three levels of concentrations. The results are listed in Table 3. The matrix effect ranged from 87.48% to 114.18%, suggesting that there was no significant ion suppression or enhancement in this LC–MS/MS method. As for extraction recovery, all the percentages varied between 83.09% and 107.04%, which indicated a stable and relatively high extraction efficiency in the plasma sample preparation.

3.4.4 Precision and accuracy

Accuracy and precision was assessed by QC samples at low, medium and high concentrations in five replicates on the same day and other three consecutive days. The precision was expressed as relative standard deviation (RSD), and the accuracy was calculated by relative error (RE). The results of intra-day and inter-day precision and accuracy for the determined QC samples are exhibited in Table 4. The intra-day precision of all these analytes were between 1.00% and 10.83%, whereas the inter-day precision ranged from 3.42% to 11.62%, which matched the requirements of biological sample analysis. The intra-day accuracy ranged from -10.00% to 10.83% and the inter-day accuracy were between -11.57% and 12.79%, indicating excellent accuracy of the present method.

3.4.5 Stability

Briefly, the stability of analytes was estimated as recovery rate and the results are presented in Table 5. All of the samples displayed applicable recoveries ranging from 85% to 115% and suitable variation under different stability conditions including three freeze-thaw cycles, short-term (25℃, 5 h) place and long-term (-70℃, 21 days) store. The data indicates a satisfactory stability of analytes in the process of the whole experiment.

3.5 Application in a pharmacokinetic study

The validated LC-MS/MS method was employed to evaluate pharmacokinetic behaviors of brucine, glycyrrhetinic acid, glycyrrhizic acid, liquiritin, liquiritigenin, isoliquiritigenin after oral administration of brucine and/or licorice extract. The plasma concentration-time profiles of all analytes are presented in Fig 2 and Fig 3. The pharmacokinetic parameters of the analytes calculated by non-compartmental model are summarized in Table 6.

In the present study, the pharmacokinetic parameters of brucine after a single dose of 40 mg/kg were consistent with those reported in the literature. Brucine was absorbed quickly and the peak concentration of brucine occurred at around 0.4 hours after the oral administration. It was eliminated with an average $t_{1/2}$ of 2.6 hours in rats, which were in close accordance with the data reported previously[4,19,24]. When brucine was coadministered with single dose of liquorice water extract, the C_{max}, AUC_{0-t}, $AUC_{0-\infty}$ of brucine were significantly decreased by 30.0%, 22.9% and 21.8%, respectively. The PK alterations by liquorice water extract might

be related to two aspects: activation of metabolic enzyme system, ingredient interaction.

CYP3A4, one of the most important metabolic enzymes in the CYP450. There are a wide range of xenobiotics or endogenous chemicals can be metabolized by CYP3A4. Previous research had reported that alkaloids like brucine were mainly metabolized by CYP3A4 in liver and intestines[25]. In the present study, the reduction of C_{max} and AUC of brucine were observed in liquorice treatment group, which might partly account to the inducement of CYP3A4. Documents demonstrated that glycyrrhetic acid and glycyrrhetinic acid, two of the main components in Radix Glycyrrhizae, can activating the function of CYP3A4[26]. Thus, we can infer that the inducement of CYP3A4 by Radix Glycyrrhizae might accelerate the metabolism of brucine. Unexpectedly, in this study, treatment with licorice had hardly any influence on the $t_{1/2}$ of brucine. It might be due to the insufficient exposure time of liquorice. As it suggested, glycyrrhetinic acid, glycyrrhizic acid had effect on mRNA expression and activities of the CYP3A4 enzymes after a 7-days administration, rather than a sigle oral administration[27].

Pharmacokinetic studies of glycyrrhizic acid confirmed that glycyrrhizic acid was hydrolysed by intestinal bacteria and produced glycyrrhetinic acid and glucuronic acid[28]. Glucuronidation played a role in phase II metabolism, which could conjugate with toxic substance such as brucine, and facilitate its elimination. Besides, liquorice may generate chemical precipitations with alkaloids[12]. Meanwhile, brucine had a great role in the $AUC_{0-\infty}$ of glycyrrhizic acid, the AUC_{0-t}, $AUC_{0-\infty}$, $t_{1/2}$ of liquiritigenin, and the AUC_{0-t}, $AUC_{0-\infty}$ of liquiritin, suggesting significant drug interaction between brucine and liquorice. And glycyrrhizic acid, liquiritigenin, liquiritin were the most possible effective constituents in liquorice.

There were some pharmacokinetic studies in those five main components of liquorice in rats[15, 17,21,29-31]. Although liquiritin, liquiritigenin and isoliquiritigenin were all flavones ingredients and had similar chemical constructions, they had different pharmacokinetic characteristics. Liquiritigenin and isoliquiritigenin presented the phenomenon of double peaks and the liquiritin appeared in a single peak; it suggested that they might have multiple absorption. It might attribute to the hydrolysis of liquiritin and isoliquiritin, respectively. Analogously, glycyrrhetinic acid had a weak peak at about 30 min and a much stronger peak around 7.0 h (Fig 2). As we known, there were only bits of glycyrrhetinic acid in licorice extract and the plasma glycyrrhetinic acid mainly came from glycyrrhizic acid.

4 Conclusion

In this work, a simple, rapid, reliable LC–MS/MS method has been established and successfully applied to a multi-component pharmacokinetic study. Seven components including brucine, strychnine, glycyrrhizic acid, glycyrrhetinic acid, liquiritigenin, isoliquiritigenin and liquiritin were rigorously validated and accurately determined in rat plasma. This method is suitable for pharmacokinetic and toxicological studies of the analytes and, shortly afterwards, could also be further applied to investigate the metabolic mechanism of Semen Strychni and liquorice *in vivo*. Furthermore, the C_{max} and AUC of brucine decreased significantly after a sigle licorice water extract treatment, which might keep the plasma concentration away from the lethal dose.

Abbreviations

ADME: absorbed, distributed, metabolized and excreted, CE: collision energy, DP: declustering potential, ESI: electrospray ionization, HPLC-UV: high performance liquid chromatography with ultraviolet detection, IS: internal standard, LC-MS/MS: liquid chromatography—tandem mass spectrometric, LLOQ: lower limit of quantification, SRM: selected reaction monitoring, QC: quality control, RE: relative error, RSD: relative standard deviation, TCM: traditional Chinese medicine.

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Author Contributions

Min Zhang and Ping-Fei Fang designed the study, wrote the protocol and prepared the manuscript. Yang Deng and Min Zhang contributed to establish the LC-MS/MS method and accomplish pharmacokinetic analysis. Chao Wang and Jing Wen helped in the animal experiment. Hua-Lin Cai, Bi-Kui Zhang, Huan-De Li and Miao Yan corrected the draft of the manuscript. All authors contributed to have approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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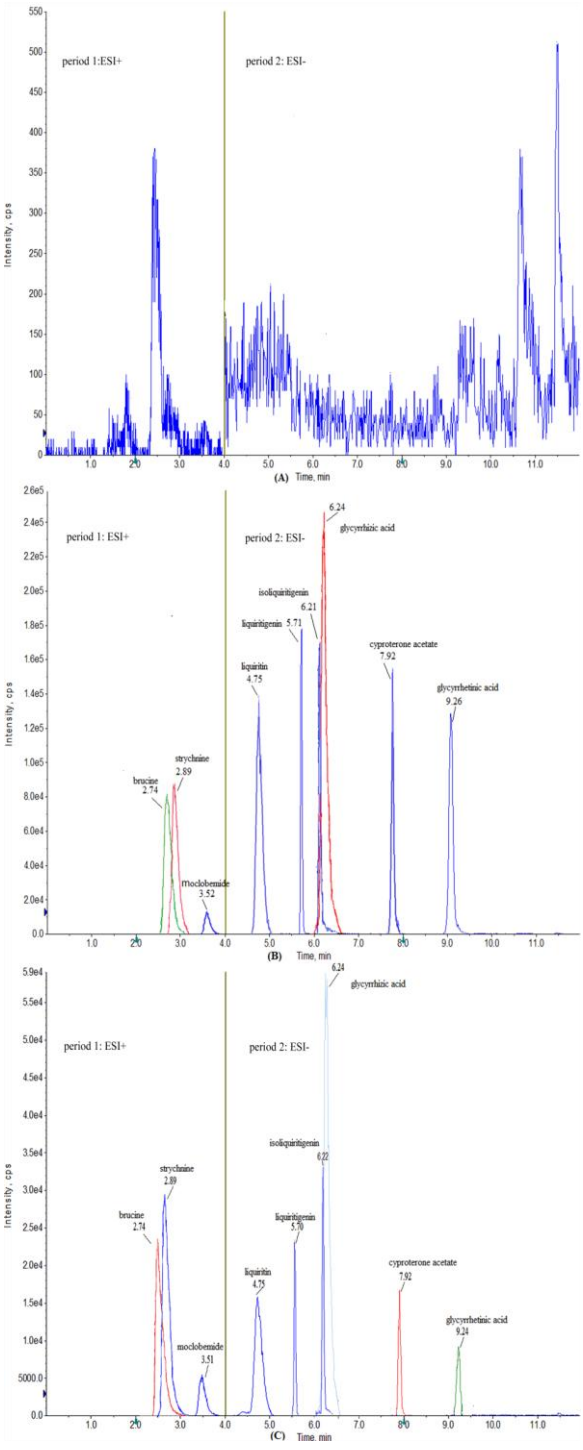

Figure 1. Typical chromatograms of seven active components and internal standards in plasma samples. A: Blank plasma B: Blank plasma added with standard references. C: Representative chromatograms of rat plasma after 0.75 h of administrated brucine and 0.25 h for licorice extract.

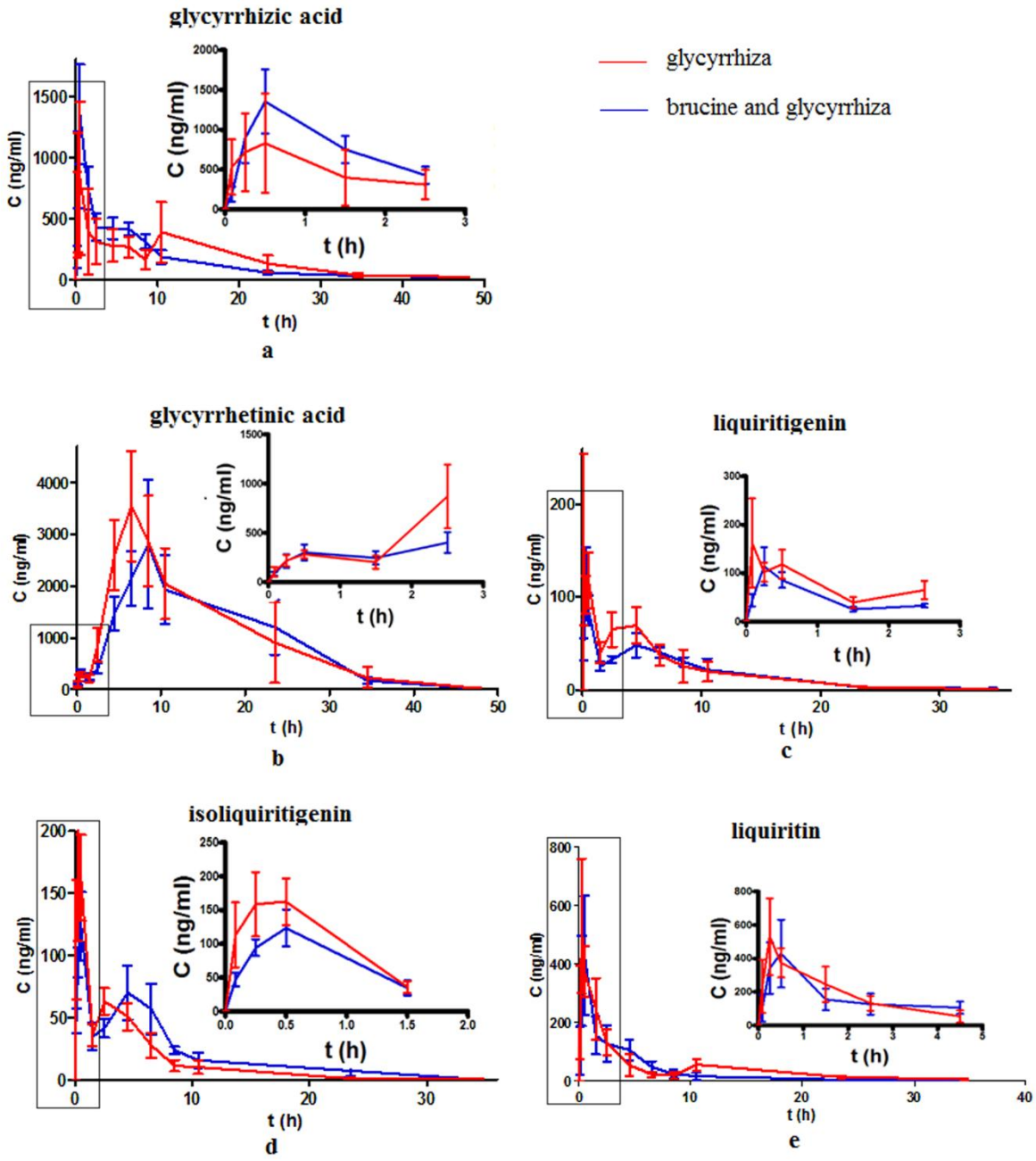

Figure 2. Mean plasma concentration–time curves of glycyrrhizic acid (a), glycyrrhetinic acid (b), liquiritigenin (c), isoliquiritigenin (d), liquiritin (e) in rats following single intragastric administration of licorice water extractive or brucine and licorice water extractive (40 mg/kg brucine and 18 g/kg licorice extract, n=8, mean±SD).

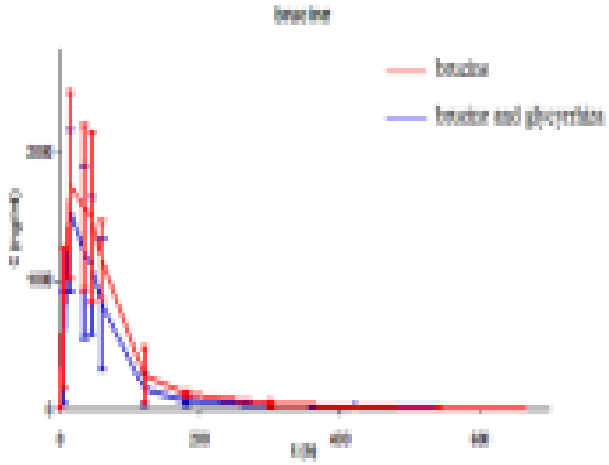

Figure 3. Mean plasma concentration–time curves of brucine in rats following single intragastric administration of brucine or brucine and licorice water extractive (40 mg/kg brucine and 18 g/kg licorice extract, n=8, mean±SD).

Table 1. Parameters for the detection of analytes in SRM mode for LC–MS/MS analysis.

Analytes	t_R (min)	SRM transitions	Declustering Potential (eV)	Collision energy (eV)
Strychnine	2.89	335.3→184.3	103.8	52.08
Brucine	2.74	395.2→324.3	114.0	44.0
Moclobemide (IS1)	3.52	269.2→181.9	109.8	25.4
Glycyrrhetinic acid	9.26	469.4→425.5	−90	−50
Glycyrrhizic acid	6.24	821.7→351.2	−70	−54
Liquiritin	4.75	417.3→254.9	−78	−26.8
Liquiritigenin	5.71	255.2→118.9	−79	−38.2
Isoliquiritigenin	6.21	255.1→118.8	−79	−38.2
Cyproterone acetate (IS2)	7.915	416.9→355.2	−84	−23

Table 2. Standard curves, linear ranges, correlation coefficients, LLOQ and LLOQ accuracy and precision of the 7 investigated compounds in blood samples (n=5).

Analytes	Calibration curve	R	Linear range (ng/mL)	LLOQ (ng/mL)	Recovery rate (%)	Accuracy (%, RE)	Precision (%, RSD)
Strychnine	Y=0.0584X+0.0142	0.9994	0.27—267.50	0.27	88.89	-11.11	10.21
Brucine	Y=0.0721X+0.0978	0.9982	0.97—2037.00	0.97	87.21	-12.78	14.61
Glycyrrhetinic acid	Y=0.048X+0.558	0.9999	2.55—5100.00	2.55	98.75	-1.25	0.76
Glycyrrhizic acid	Y=0.00499X-0.0855	0.9978	31.25—1500.00	31.25	109.27	9.27	8.41
Liquiritin	Y=0.125X+0.142	0.9999	0.65—810.00	0.65	87.38	-12.62	8.75
Liquiritigenin	Y=0.653X+0.876	0.9998	0.28—270.00	0.28	114.29	14.29	10.13
Isoliquiritigenin	Y=0.999X+1.11	0.9975	0.13—108.00	0.13	93.84	-6.15	3.67

Table 3. Matrix effect and extraction recovery for the determination of strychnine, brucine, glycyrrhetinic acid, glycyrrhizic acid, liquiritin, liquiritigenin, isoliquiritigenin in QC samples (n=5).

Analytes	Added concentration (ng/mL)	Matrix effect (%)	Extraction recovery (%)
Strychnine	2.1	114.81	92.33
	35.0	95.42	91.75
	214.0	104.28	83.09
Brucine	1.9	111.05	106.35
	203.7	91.21	87.31
	1500.0	114.50	87.05
Glycyrrhetinic acid	20.4	113.16	98.16
	255.0	89.46	88.78
	4080.0	102.75	91.68
Glycyrrhizic acid	65.1	88.83	97.80
	375.0	93.28	90.39
	1250.0	87.48	86.97
Liquiritin	1.080	109.80	107.04
	64.80	91.25	90.11
	756.0	94.94	97.99
Liquiritigenin	1.1	105.45	100.89
	27.0	104.56	97.89
	216.0	107.40	89.59
Isoliquiritigenin	1.08	96.00	101.35
	10.80	97.31	88.18
	97.20	96.26	96.12