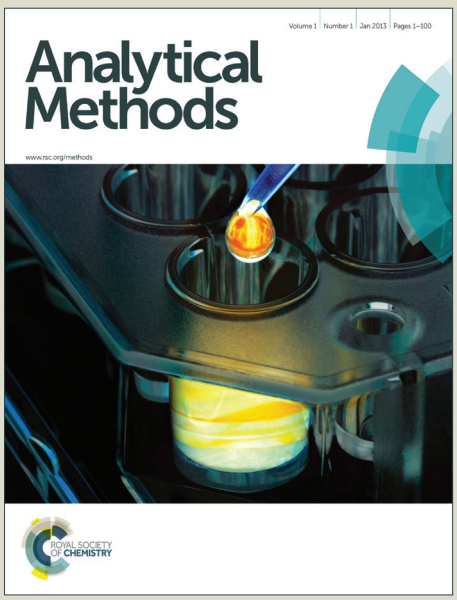
Analytical Methods

Accepted Manuscript



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Pharmacokinetic study of liquiritin in rat serum using molecularly imprinted solid-phase extraction combined with RP-HPLC

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[Abstract]: A method using molecularly imprinted solid-phase extraction combined with RP-HPLC-UV was developed for determination of Liquiritin(LQR) in rat plasma after oral administration of Compound Liquorice Tablets(CLT). To this end, molecularly imprinted polymers were prepared by *in situ* polymerization, using LQR as the template, acrylamide as the functional monomer, and ethylene glycol dimethacrylate as crosslinker, whose solid-phase extraction properties for Liquiritin were evaluated by HPLC-UV. The enrichment and extraction recovery were investigated in the study after the extraction procedure was optimized. The recovery was larger than 85.0% and the RSD was less than 10.0%. Calibration curve was expressed as y=16004x-1.864 (r=0.996) with a linear range of 0.007μg·mL^{-1}~1.683 μg·mL^{-1}. Besides, the T_{max} was 0.5 h for Liquiritin and the C_{max} was 0.8 μg·mL^{-1} after oral administration of Compound Liquorice Tablets. In addition, the pharmacokinetic parameters were calculated by two-compartment model. The results indicated that the monolithic polymer pipette presented a good extraction efficiency for Liquiritin. At the same time, the results indicated the index components could basically reveal the pharmacokinetic behavior. This study was aimed to explore an approach that could be applied to promulgate the pharmacokinetic and provide more information for the clinical application.

[Keywords]: Liquiritin; Compound Liquorice Tablets; MI-SPE-HPLC-UV; Pharmacokinetic

1. Introduction

Compound Liquorice Tablets(CLT) are applied extensively in clinical whose main functions are antitussive and expectorant. It is widely received by majority of patients for its smaller side effects and inexpensive feature[1]. Licorice is the sole medicinal herbs of CLT. Nevertheless, Liquiritin(LQR) is one of the bioactive ingredients existing in the root of licorice, which has been reported to possess a variety of pharmacological activity, such as anti-depressed[2], anti-Alzheimer's Disease [3], in addition, antioxidant and liver detoxification[4]. There are so many pharmacological effects that it is often studied by scholars. According to reports in the literatures, the frequently detected methods were HPLC-UV[5-7], SPE-HPLC[8], UPLC[9-10], Electrochemistry[11], Fluorescence spectroscopy[12]. However, Few documents reported any methods determining the content of LQR from the traditional Chinese medicine with solid phase extraction(SPE) especially in biological samples. To enrich, purify, or remove LQR from a mixture, selective separation is necessary because of the coexistence of compounds with similar functional groups[13]. However, isolation and purification of LQR from natural products by molecularly imprinted solid phase extraction(MISPE) have not previously been reported at present. Meanwhile, early research had shown that orally administered LQR could be metabolized into liquiritigenin[14]. The low level of LQR in the blood has been a bottleneck in its pharmacokinetic study. Therefore, it is necessary to develop a sensitive and convenient method to determine LQR in plasma in the studies of pharmacokinetics and clinical application.

SPE, which is a mature technique[15-17], has been widely used for biological fluids[18-20]. It is used to purify and pre-concentrate the analytes. At the same time, molecularly imprinted polymers(MIP) are synthetic material with the capability of selective recognition of targeted compounds, which have specific binding sites with complementary sizes,

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shapes, and functional groups to the template, and involve a mechanism of interaction based on molecular recognition[21-22]. Moreover, *in situ* polymerization of polymer based monoliths has several advantages over silica based ones, such as easy preparation and convenient introduction of function groups in monolith[23]. Researches on 96-well pipette-tips with monolithic methacrylate sorbent plug[24-25] and monolithic micro-extraction tips by photopoly merization[26] validated the more flexibility of preparing polymer monoliths. MISPE is an efficient approach for separation and clean-up of target compound present at low concentrations or in complex matrixes[27], which may have the advan -tages of both MIP, with high selectivity, and SPE, with the most convenience. The technique is attracting for separation and purification of natural products[22]. MISPE has not been used for separation of LQR in CLT.

In this paper, MIP were prepared by *in situ* polymerization, using LQR as the template, acrylamide(AM) as the functional monomer, and ethylene glycol dimethacrylate (EDMA) as cross -linker. Separation of LQR in CLT was carried out by MISPE. A new method using MI-SPE-HPLC-UV for the determination of LQR in rat plasma demonstrate its applicability in analyzing the pharmacokinetic parameters of LQR after a single oral administration of CLT, so as to provide reliable data to support the safety in clinical.

2. Experimental

2.1 Chemicals and Reagents

CLT were purchased from GuangZhou Bai Yun Shan Pharmaceutical General Factory (Guangzhou, China); Ethylene glycol dimethacrylate (EDMA, 98%, Acros Organics,US), N,N-DimethylFormamide(DMF) acrylamide(AM), and 2-(dimethylamino) ethylmetha- crylate(DMAM) were purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). Toluene, Azobisisobutyronitrile(AIBN), polyethylene glycol 400 (PEG, AR grade, MW 400) were obtained from Tianjin Kermel Chemical Reagent Co. Ltd. (Tianjin, China). LQR was supplied by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and acetonitrile were of chromatographic grade (Darm stadt, Germany). Water was prepared in ultrapure water system (MillIpore, American). All other reagents were of analytical grade.

2.2 Instrument and Equipment

HPLC system was from Agilent Technologies (1200 series LC system). The HPLC consisted of a G1311A quaternarypump, a G1316A thermostatted column compartment, a G1322A degasser, a G1314B VWD and a G1328B manual injector (with 20μL loop). The Microcentrifuge was purchased from Ther -mo Electron corporation(Beijing, China). Super silent adjustable air pump was obtained from Guang dong hailea group Co. Ltd(Guang zhou, China). Moreover, JSM-6330F Field Emission Scanning Eletron Microscope and AUW220D electronic balance were gained from Japan Electronics Co.Ltd(Qingdao, China) and Shimadzu (Guangzhou, China); In addition, 80-2Centrifuge and HH-S thermostat water bath were acquired from the Gongyi City to Instrument Co.Ltd(Gongyi, China).

2.3 Dosage Regimen

Male Sprague-Dawley rats weighed 250-300g were supplied by Laboratory Animal Center of Guangzhou University of Traditional Chinese Medicine (scxk: 2008-0020). All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Guang Zhou University of Chinese Medicine, Guang Zhou, China, and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals ("Laboratory Animal Management Regulations" and "Experimental Animal Permit Management Regulations (Trial)"). The rats were kept in an air conditioned animal quarter at 22-24°C and a relative humidity of 50±10%, which had accessed to standard laboratory food and water. The rats were fasted and given free access to water for 12 h prior to experiments. Gavaged 20 $mL \cdot kg^{-1}$ at equivalent dosage(Weigh the CLT 40 tablets to 20 mL, per tablet containing 112.5 mg of licorice extract powder), blood samples were collected into a heparinized tube via the oculi chorioideae vein prior to intragastric gavage and at 0.17 h, 0.25 h, 0.5 h, 0.75 h, 1 h, 1.5 h, 2 h, 4 h, 8 h, 12 h and 24 h after oral administration. After centrifu gating at 4000 rpm for 15 min, the layers of plasma were transferred to clean tubes and stored at-20 °C.

2.4 The preparation of Molecularly Imprinted Polymers

The pre-polymerization solution consisted of LQR(5.01 mg), AM (6.8 mg), DMF (400 μL), Dodecanol (400 μL), EDMA (90 μL), PEG 400 (100 μL), and were dissolved in 1.1 mL acetonitrile. The initiator AIBN (2.0 mg) was added to the pre-polymerization solution. The resulting solution was purged with nitrogen gas for 10 min to remove oxygen. A polypropylene pipette of 5 mL was filled with toluene to pretreat its inner surface after the taper end was flame-sealed. After that, 2 mL pre-polymerization solution was introduced into the pipette and the other end of the pipette was sealed immediately. The polymerization was carried out for 24 h by setting pipette in a water bath at 60 °C. Finally, a plug of monolithic polymer column was prepared by *in situ* polymerization in the pipette. After both sealed ends of the pipette were cut off, the monolithic column in pipette was set on a SPE digital control instrument for being rinsed with methanol thoroughly in order to remove the porogenic solvents and any other unreacted monomers under vacuum condition. The finally obtained pipette containing monolithic polymer plug was used as SPE cartridge for the following SPE experiments. The reaction results were illustrated as follows (Fig.1 and Fig.2).

Fig.1. Photograph of the monolithic polymer SPE column.

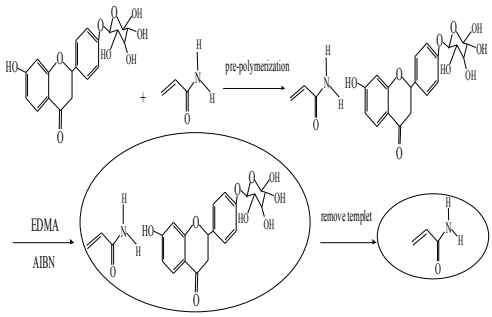

Fig.2. The reaction principle

2.5 The Scanning Electron Microscopy (SEM) of Monolithic Column

Monolithic column was activated after eluting of the template and cutting its cross-section, then characterizing the microstructure of the monolithic column by cold field SEM before the samples properly been treated (Fig.3). The column contained a pretty uniform voids and larger pore size, compared with several photographs. It proved that the column had a good permeability. Due to the dense and intricate network structure formed in the monolithic column, it expressed strong mechanical strength and resisted greater pressure.

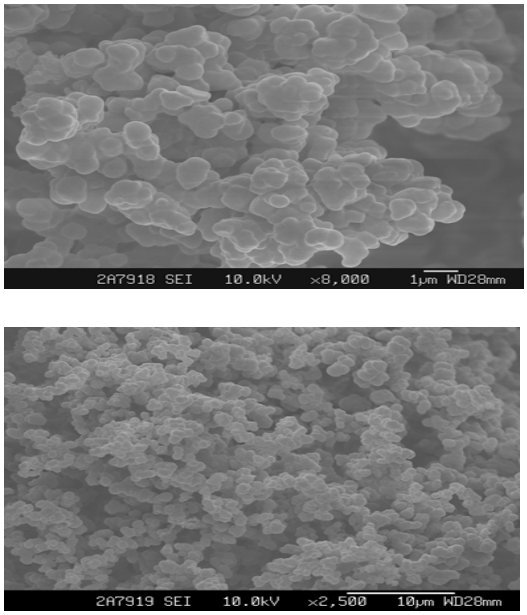

Fig.3. SEM image for the section of monolithic polymer (8000 and 2500 times magnification).

2.6 Imprinted Activated and Template Column Eluted

The cartridge was eluted with 10% acetic acid in acetonitrile (V/V), which was detected by HPLC-UV and traced detection until it is undetectable template molecule. The preprocessing of monolithic column was as follows: 0.5% ammonia adjusted the pH until neutral, water leaching, and eventually the whole column was stored in the water, to be on the kind, with a flow rate of 0.10 mL·min^{-1}.

2.7 Plasma Sample Preparation

The volume of 200 μL plasma was spiked with 1.0 mL methanol. The mixture was vortexed for 1.0 min. After centrifugation at 12,000 rpm for 15 min, the supernate was transferred to a new tube and dried under a stream of nitrogen. The residue was vortex-mixed with 100 μL methanol for 30 s and then passed through the molecularly imprinted column. The cartridge was eluted with 8.0 mL in acetonitrile containing 10% acetic acid after rinsing with 2.0 mL pure water and washing with 1.0 mL of 5% methanol in water (V/V). The eluate were dried under a stream of nitrogen at 40 °C. The mixture was vortex-mixed with 100 μL methanol for 60 s and centrifuged at 12,000 rpm for 15 min to precipitate protein. 20 μL supernateant were injected into the HPLC system for quantitative analysis.

2.8 HPLC Chromatographic Conditions

A Diamonsil C_{18} column (250 mm×4.6 mm, 5 μm) and Guard Column: XB-C_{18} (5 μm, Welch Materials) were applied to chromatography separations, which were performed at 35°C with the detecting wavelength set at 276 nm and flow rate at 1.0 $mL{\cdot}min^{-1}$. Mobile phase were composed of 0.03% acetic acid in water (V/V) (A) and acetonitrile (B). The composition of mobile phase (V/V) within an analysis term for rat plasma sample changed as follows Table 1.

Table 1 The liquid phase elution conditions

T(min)	0.03% acetic acid in water (V/V) (A)%	acetonitrile (B)%
0	90	10
10	87	13
15	82	18
20	70	30
30	60	40
40	60	40
55	90	10

2.9 Pharmacokinetics

The rats were almost given orally with 6.0 $g{\cdot}kg^{-1}$ CLT which was equivalent to 74 $mg{\cdot}kg^{-1}$ of LQR (Contents of LQR in CLT is about 3.7 mg per tablet[28]). Plasma collected in different time points. Average plasma concentration-time curve (Fig.4), and the pharmacokinetic parameters (Table 2) were calculated (DAS 3.1, China). The area under plasma concentration-time curve (AUC_{0-t}) to the last measurable plasma concentration (C-t) was estimated by using the linear trapezoidal rule. The area under the plasma concentration-time curve to time infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-\infty} = AUC_{0-t} + C_t/K_e$. The mean residence time (MRT) was calculated as $AUMC_{0-\infty}/AUC_{0-\infty}$. Data were expressed in mean and standard deviation (SD) for each group. The validated method was successfully applied to pharmacokinetic study for LQR in rat plasma after a single oral administration of CLT.

3. Results and discussions

3.1 Specificity

The representative chromatograms of LQR and blank plasma, blank plasma spiked with LQR and plasma sample obtained after oral administration of CLT decoction are shown in Fig.4. The retention time of LOQ is about 22 min. Under the established chromatographic condition, no endogenous interference from the plasma was observed and the analyte were not interfered with each other.

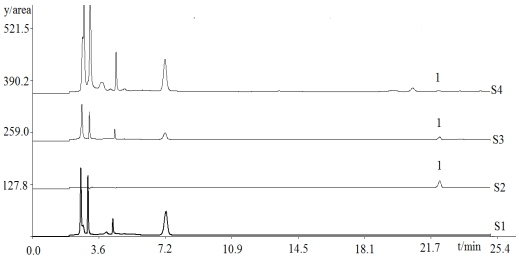

Fig.4. S_1: Blank plasma; S_2: LQR(0.168 $\mu g{\cdot}mL^{-1}$) ; S_3: Blank serum containing LQR; S_4: Rat plasma after 0.5 hour oral administration of CLT

3.2 Calibration Curve and Linear Range

According to the handling of plasma samples(2.7). The reference standard of LQR was accurately weighed to 10 mL volumetric flask, the 10mL volumetric flask was filled to mark with methanol (obtaining 520$\mu g{\cdot}mL^{-1}$ stock solution). The calibration curves ranged from 0.007 $\mu g{\cdot}mL^{-1}$ to 1.683 $\mu g{\cdot}mL^{-1}$. The series of standard solution concentr ations were 0.007 $\mu g{\cdot}mL^{-1}$, 0.017 $\mu g{\cdot}mL^{-1}$, 0.067 $\mu g{\cdot}mL^{-1}$, 0.168 $\mu g{\cdot}mL^{-1}$, 0.337 $\mu g{\cdot}mL^{-1}$, 0.673 $\mu g{\cdot}mL^{-1}$, and 1.683 $\mu g{\cdot}mL^{-1}$. Pressing the operation of the processing of the plasma sample, the regression equation in plasma

was y=16004x-1.864 (n=5), in which y is the peak-area, and x is the concentration of LQR. The correlation coefficient (r) was 0.996. The method was found to be sufficiently sensitive for the determination of LQR in rats. The lower limit of quantification(LOQ) of the method was 0.052 $\mu g \cdot mL^{-1}$(signal-to-noise >10).

3.3 The Recoveries and Precision Test

On the basis of plasma sample processing operations(2.7). The accuracy and precision of the method were evaluated with QC sample at concentrations of 1.683 $\mu g \cdot mL^{-1}$, 0.168 $\mu g \cdot mL^{-1}$, 0.017 $\mu g \cdot mL^{-1}$. According to the processing and determination of the plasma samples, the precision of intra-day and inter-day were obtained. The results were 4.4% (n=5), 0.7% (n=5), 5.7% (n=5). 3.2% (n= 5), 5.6% (n=5), 2.9% (n=5) for the low, medium and high QC samples, respectively. Mean recoveries of LQR rat plasma were 88.7% (RSD=2.4%), 94.1% (RSD=0.8%), 90.5% (RSD=3.7%), respectively. These results indicated that the method possessed the acceptable accuracy and high reproducibility.

3.4 Stability

The stability of LQR in plasma was evaluated under different storage conditions at concentrations of 1.683 $\mu g \cdot mL^{-1}$, 0.168 $\mu g \cdot mL^{-1}$, 0.017 $\mu g \cdot mL^{-1}$ levels. It was found that LQR was stable in rat plasma after being stored at room temperature for 24 h, the RSD were 3.2%, 1.2%, 2.3%(n=5), respectively. After repeating three freeze-thaw cycles and being stored at -20°C for one week, the RSD were 4.0%, 1.5%, 3.3% (n=5). In addition, the samples were found to be stable in repeated freezing, in accordance with the processing and determination of plasma samples, the concentrations of LQR content RSD were 1.5%, 2.8%, 3.02%; In brief, The results showed that the plasma samples of LQR had a good stability under the above conditions.

3.5 Optimization of The Column and Analysis of The Real Sample

Different washing and eluting steps were investigated to optimize the process of selective extraction, It was important to apply a washing step immediately after loading biological sample on the sorbent because it could reduce most of the interferences in the separation of the analyte. Different washing solvents with different polarity (pure water, 5% methanol in water(V/V), 10% methanol in water(V/V)) were investigated. Besides, three kinds of elution processes(pure methanol, 5% acetic acid in acetonitrile (V/V) and 10% acetic acid in acetonitrile (V/V)) were checked. Finally, we chose 5% methanol in water (V/V) as the leaching solvent and 10% acetate in acetonitrile (V/V) as eluent judging by the imprinted column retention and adsorption effects.

The blood samples of 200 μL were selected and compared with the sample before passing through the column. Apparently, the results were enriched a lot of times. The peak area of LQR in real sample was just 0.9 calculating in one rat(n=3). Meanwhile, the corres ponding concentration of LQR was determined as 3.45 ng (n =3, RSD =4.8%), which could not reach the LOQ. So, the action greatly limit the detecting methods. However, our analysis objects couldn't effectively convey and reflect by HPLC-UV in chromatograms (Fig.5-S2). By analysing the content of target compound after being directly injected, the area of LQR was attained 38.9(n=3), which was expressed at the concentration of 50.9ng collecting the blood samples of 400 μL. By the enrichment, we could fairly conduct quantitative analysis with the low content of LQR in rat serums and we can distinctly identify the target peak(Fig.5-S3). In addition, the LQR can effectively selective adsorption by MI-SPE. Therefore, the way we made could solved the analyte of low content in serum. Furthermore, it had made great practical significance. The way of synthesis was not only so simple but also contained obvious effect for the enrichment. It seems suitable to apply a bigger volume of polymer monolith in pipette as SPE device to extracting more drug molecules from serum for HPLC analysis. Besides, the establishment of the method provided many parmacokinetic parameters for CLT, which could effectively guide the reasonable application in clinical.

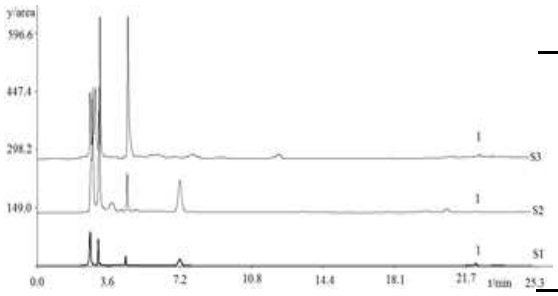

Fig.5. S1: Blank serum containing LQR; S2: The blood sample of 200μL after 0.5 hour oral administration of CLT; S3: The Preconcentration of 400μL blood sample after dealing with SPE cartridge

3.6 Results of Pharmacokinetic Study

The validated MI-SPE-HPLC-UV method was applied to a pharmacokinetic study of LQR in rats after oral administration of CLT. The pharmaco -kinetic parameters were calculated by two -compartment model presented in Table 2. As seen from Table 2, the T_{max} was 0.5 h for LQR, which demonstrated that LQR was quickly absorbed into the body and also quickly eliminated after oral administration of CLT. In addition, the concentration of LQR at 24 h plasma samples was below limit of detection(LOD). However, the C_{max} of was 0.8 $\mu g \cdot mL^{-1}$ after oral adminis tration of CLT. Meanwhile, the little component into blood of LQR quickly converted to other biologically active substances. Therefore, the pharma cokinetic studies need to be further investigated.

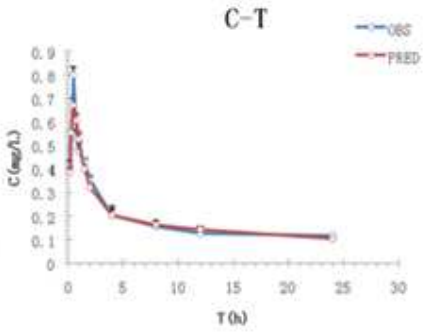

Fig.6. The plasma concentration-time curve administrated LQR

Table2.Pharmacokinetic parameters of CLT in rat plasma after gavaging 74 $mg \cdot kg^{-1}$ of LQR (n=5)

Parameters	Unit	Mean±SD
T_{max}	H	0.50±0.00
C_{max}	$\mu g \cdot mL^{-1}$	0.80±0.03
$t_{1/2}\beta$	H	27.49±10.46
V_1/F	$L \cdot kg^{-1}$	80.36±7.08
CL/F	$L \cdot (h \cdot kg)^{-1}$	7.97±3.41
AUC(0-t)	$mg \cdot (L*h)^{-1}$	8.51±4.41

4. Conclusions

In the present work, a MI-SPE-HPLC-UV method for the quantification of LQR in plasma was developed and validated. Meanwhile, The MIP was prepared in acetonitrile using LQR as template molecule and AM as functional monomer. In the end, the composite of the porogen were DMF and Dodecanol, which had a better ability to identify LQR. Mechanistic studies indicated that the recognition sites came from hydrogen bonding, possibly causing the system of benzoyl groups between LQR and acrylamide[29]. Owing to the presence of hydrogen, the competitive reagents had a significant effect on the recognizable performance of the polymer. MIP were prepared by in *situ* polymerization, reacting in the 5 mL pipette, which was feasible and contained potential applications. MISPE is an efficient approach for separation and clean-up of target compound presenting at low concen trations or in complex matrixes. It can be envisaged that the developed monolithic polymer was suitable to enrich other target molecules with the similar structure as LQR. Further investigations on its application of other flavonoid compounds in plasma are in progress in our group.

In addition, the pharmacokinetic parameters of LQR were preliminary studied. The developed method was employed for the first time to a pharmacokinetic study of CLT. Results of the present study indicated that the pharmacokinetic information were needed for the design and interpretation of toxicity and clinical studies. A two-compartment model (weight=1) was selected for pharmacokinetic modeling, as it best fits the datas obtained from rats using DAS software. The pharmacokinetic parameters of LQR were presented in Table 2. The T_{max} was 0.5 h for LQR and AUC(0-t) was 8.51±4.41 $mg \cdot (L*h)^{-1}$. However, according to the previous study[30], the results showed that the T_{max} of LQR was 0.25 h, which were a little difference with previous studies. The high bioavailability of LQR in our study may

cause by the animal species differences, different pharmaceuticals or individual variations. Besides, the analytical method in our study shows high sensitivity and selectivity for the quantitation of LQR in rat plasma. Thus, it may be presumed that the detetion limit of LQR may contribute to the precision in pharmacokinetic study.

In summary, a simple, specific and sensitive MI-SPE-HPLC-UV method was established and validated for determination of LQR in rat plasma. The pharmacokinetic results provided reliable data to support the safety in clinical. In adition, the method was a very valuable reference as clinical instruction of CLT.

5. Acknowledgments

The authors express their gratitude for the financial supports from the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry (NO.44140022) and the financial supports of the National Natural Science Foundation of China (No.[2011]1139).

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A table of contents entry

[Title]:Pharmacokinetic study of liquiritin in rat serum using molecularly imprinted solid-phase extraction combined with RP-HPLC

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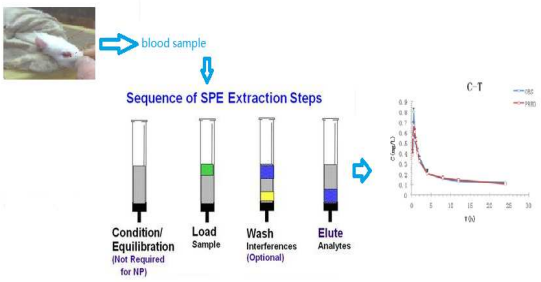
[Abstract]: This study was aimed to explore an approach that could be applied to promulgate the pharmacokinetic and provide more information for the clinical application. A new method using MI-SPE-HPLC-UV for the determination of LQR in rat plasma demonstrate its applicability in analy zing pharmacokinetic parameters of LQR after a single oral administration of CLT, so as to provide reliable data to support the safety in clinical. In the form of a chart can be expressed as follows.



[Keywords]: