Comparison of the curdione in brain of blood stasis pregnant rats and its offsprings with the normal group rats by UPLC-Q-TOF-MS

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Abstract: Curdione is a main active component of curcuma rhizomes (Ezhu), which shows an excellent antithrombotic activity. In this study, the concentration of Curdione in pregnant rats and their offspring brain was determined using ultra-performance liquid chromatography-quadrupole time-of-flight/mass spectrometry (UPLC-Q-TOF-MS) method. The water extraction then alcohol precipitation extract from Ezhu was administered through tail intravenous injection. The pharmacokinetic parameters were analyzed to compare the differences between the blood stasis group rats and normal group rats. Using Schisandrol A as an internal standard, samples were extracted using dichloromethane and isopropanol (90:10, *v/v*). Calibration plot was linear over the range of 0.5-200μg·mL^{-1} for Curdione in brain with the lower quantification limit being 0.5μg·mL^{-1}. The recoveries of Curdione and IS from brain were more than 93.31% and 90.90% separately. The RSD for both intra- and inter-day precision were <6.49%, RE were -14.84%~-2.8%. The pharmacokinetic parameters C_{max} and AUC among the four kinds of rats had significant difference. The Curdione distributed in rat brain in model group is less than normal group. Ezhu medicine may show the therapeutic effect but not the reproductive toxicity on mother or unborn baby to cure the pregnant women under the adaptive symptoms.

Keywords: UPLC/QTOF/MS, Curdione, Schisandrol A, Pharmacokinetics, brain.

INTRODUCTION

Successful pregnancy depends on sufficient uteroplacental circulation. The pregnant women will easily lead to venous thrombosis and anti-clotting abnormalities because of the unique physiological and changed endocrine. Recent researches showed that anticoagulated function abnormality is closely related to pregnancy loss and other maternity complications. To the women who have anticoagulation defects, the pregnancy complications incidence rate is 49-65%, while only 18-22% of normal pregnant women, the risk increased by 3-8 times (Kupferminc *et al*., 1999; Brenner, 1999).

Although, the thrombosis state may increase the dangerous consequences of spontaneous abortion, limited fetal development, even result in fetal death, we must take into account the toxicity of the medicine on the mother and unborn fetus (Bitsadze *et al*., 2013; Torkzad *et al*., 2010; Shah *et al*., 2014). Traditional Chinese medical theory of "YOU GU WU YUN" stemed from "Huang Di Nei Jing" is a standard of medication safety for pregnancy. The meaning is that, medicine will show the therapeutic effect but not the reproductive toxicity on mother or unborn baby to cure the pregnant women under the adaptive symptoms.

Therefore, the medical scientists were focusing on developing the natural, efficient and non-toxic antithrombotic medicine for the blood stasis pregnant women. *Curcuma zedoaria* (Ezhu in Chinese) (Chinese Pharmacopoeia Commission, 2010), a genus of rhizomatous herbaceous species, is a traditional herbal medicine, has a variety of activities (Zorofchian *et al*., 2014), such as anti-flammatory (Manhas *et al*., 2014; Kuptniratsaikul *et al*., 2014; Agarwal *et al*., 2013), anti-microbial (Revathi and Malathy, 2013), some protective effects (Kim *et al*., 2014; Ahmad *et al*., 2014; Rahim *et al*., 2014; Hao *et al*., 2014), Immunostimulatory (Yue *et al*., 2010), anti-viral activity (Dong *et al*., 2013). Recently, many anti-cancer activities of Curcuma have been reported (Liu *et al*., 2014; Li *et al*., 2014; Chen *et al*., 2011; Gao *et al*., 2014; Lu *et al*., 2012). Among these activities, Curcuma is the most widely used to treat blood stagnation and accumulation for a long time in China. It was commonly used in clinical treatment of thrombosis and used since ancient times to remove blood stasis and to alleviate pain (Srivastava, 1989; Chen *et al*., 2011). Chemical constituent studies (Tiwari *et al*., 2014; Asem and Laitonjam, 2014; Kikuchi *et al*., 2014; Zhang *et al*., 2014) have indicated that essential oils, such as β-elemene, Curcumol and Curdione are major bioactive components in Ezhu. Among these compounds, Curdione is the most effective ingredient of anticoagulation and antithrombotic effects. Many modern pharmacological studies have shown that Curdione is the main active ingredient to remove blood stasis: Curdione has inhibition activity to ADP-induced platelet aggregation of rabbit in vitro (Xia *et al*., 2006); Curdione have anti-clotting and regulate blood rheology effect had been proved by determining the blood rheology and coagulation activities of stasis in model rats (Xia *et al*., 2012). So the increasing incidence and severity of blood stasis during pregnancy prompted further development of Curdione.

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There are many methods to determine the Curdione in vitro and vivo experiments, such as UPLC/QTOF/MS (Lv *et al*., 2014), capillary electrophoresis (Anubala *et al*., 2014), GC-MS (Deng *et al*., 2006), LC-MS (Ma *et al*., 2012), and GC method (Sun *et al*., 2012). UPLC/QTOF/MS is a very useful tool in identifying and quantifying the components for its high resolution and accuracy. We developed a UPLC/QTOF/MS method for quantifying Curdione accurately in rat brain, and showed that it is an ideal tool for the pharmacokinetic study of complex biological samples obtained after intravenous injection of Ezhu herbs extract to pregnant rats. Understanding of the pharmacokinetics of Curdione during pregnancy is critical to optimize the therapeutic dose. No pharmacokinetic study of Curdione after intravenous injection to pregnant rats of Curcuma extract has ever been reported. Due to ethical and safety concerns the study is conducted on the pregnant rats.

We developed and validated a sensitive, accurate, and UPLC/QTOF/MS method to measure Curdione in rat brain tissue. And through this method, whether the disease status can affect the pharmacokinetic behavior of Curdione in rat brain could be explored. This research will through pharmacokinetics provide scientific experimental basis and clarify the modern connotation of the theory "YOU GU WU YUN", which has important theoretical and practical significance. It also can help construct the traditional Chinese medical toxicity system, which was important for the development of traditional Chinese medical theory and clinical practice.

MATERIALS AND METHODS

Chemicals and materials

Acetonitrile and methanol were supplied by Dikma (Beijing, China), and formic acid was obtained from Yuwang limited company (Shandong, China). Stock standards of Curdione (>98.5%, batch No. 20120313) and internal standard Schisandrol A (>98.5%, batch No. 0764-200109) were purchased from National Institutes for Food and Drug Control, Beijing, China. Curcuma herb was identified by Lina Guo from Qiqihar Medical University, School of Pharmacy in Heilongjiang. TGL-16MC instrument from Hangzhou Aipu Instrument & equipment limited company (Zhejiang, China) was used to centrifuge.

Animals

About 30 Wistar female rats weighting 180-220g were obtained from the Beijing Vital River Lavoratory Animal Technology Limited Company, animal certificate No.: SCXK 2007-0001 (Beijing, China). Wistar female rats were randomized into normal group and blood-stasis model group. The rats were maintained in an air-conditioned animal facility.

Preparation of cold coagulation blood-stasis model rats: 1g·L^{-1} adrenaline hydrochloride was diluted with normal saline to 0.5g·L^{-1} as the injection solution. On the first day, 0.2mL solution was injected to thigh subcutaneously every 4 hour for three times. In every dosing interval, rats were immersed in ice-cold (0-2) water for cold stimulation for 2 times, 4 minutes every time. On the second day, the same dose was injected subcutaneously every 4 hour for two times. In the interval, rats were immersed in ice-cold (0-2) water for 3 minutes. It was continued in the consecutive two weeks. Then, modeling experiment was carried out one time every two days in the next two weeks. The criteria of successful blood-stasis model rats showed that the rats were chilled, huddled and decreased activity. At the meanwhile, the claw tail showed purpura symptom.

Preparation of blood-stasis pregnant rats: the normal and model group female rats were put in the cage with male rats (the ratio of female: male is 2:1) separately. In the next morning, we check the sperm through vaginal smearing using microscopy. We identified the day we detected the sperm as the gestational day (GD 0). If the female rats were not checked out the sperm, they should be returned to the male rats' cage again to make sure the pregnant rats model were successfully established.

Preparation of samples

Preparation of herb extract sample for vein injection

Curcuma herb of 20.0g from Leqing (Zhejiang, China) were weighed accurately and crushed. Curcuma was boiled with 100mL water for 60min, totally two times. The two decoctions were combined and cooled down. The combined decoction was added with 50% ethanol (20mL) to extract the active ingredients. The solution was sealed and refrigerated for 24 hour, then filtrated and removed the ethanol under low pressure condition to make sure the final concentration of injection solution was 1.0g·mL^{-1}. The solution was stored in 4 refrigerator.

Preparation of brain samples for analysis

Before the rats gave birth, about GD 20, rats were subcutaneously injected a single dose of Curcuma extraction and killed by breaking neck at different time to get out the brain tissue. Brain tissue samples of 0.3g were accurately weighed. The brain samples were homogenized in normal saline (1mL). The homogenate was then mixed with IS (100μL, 20μg·mL^{-1}), followed by vigorous vortexing for 1min. Extracting solution consisted of dichloromethane and isopropanol (*v/v*, 90:10, 3mL) was added into the homogenate, followed by vortexing for 2 min and centrifuging at 10000 r·min^{-1} for 10 min. The supernatant was transferred to a new tube and evaporated under a gentle stream of nitrogen in bath at 37 . The residue was dissolved in methanol (100μL), vigorously vortexed for 1min, disposed with ultrasonic for 5min and then centrifuged at 10000 r·min^{-1} for 10 min. The supernatant was used for further UPLC/QTOF/MS analysis.

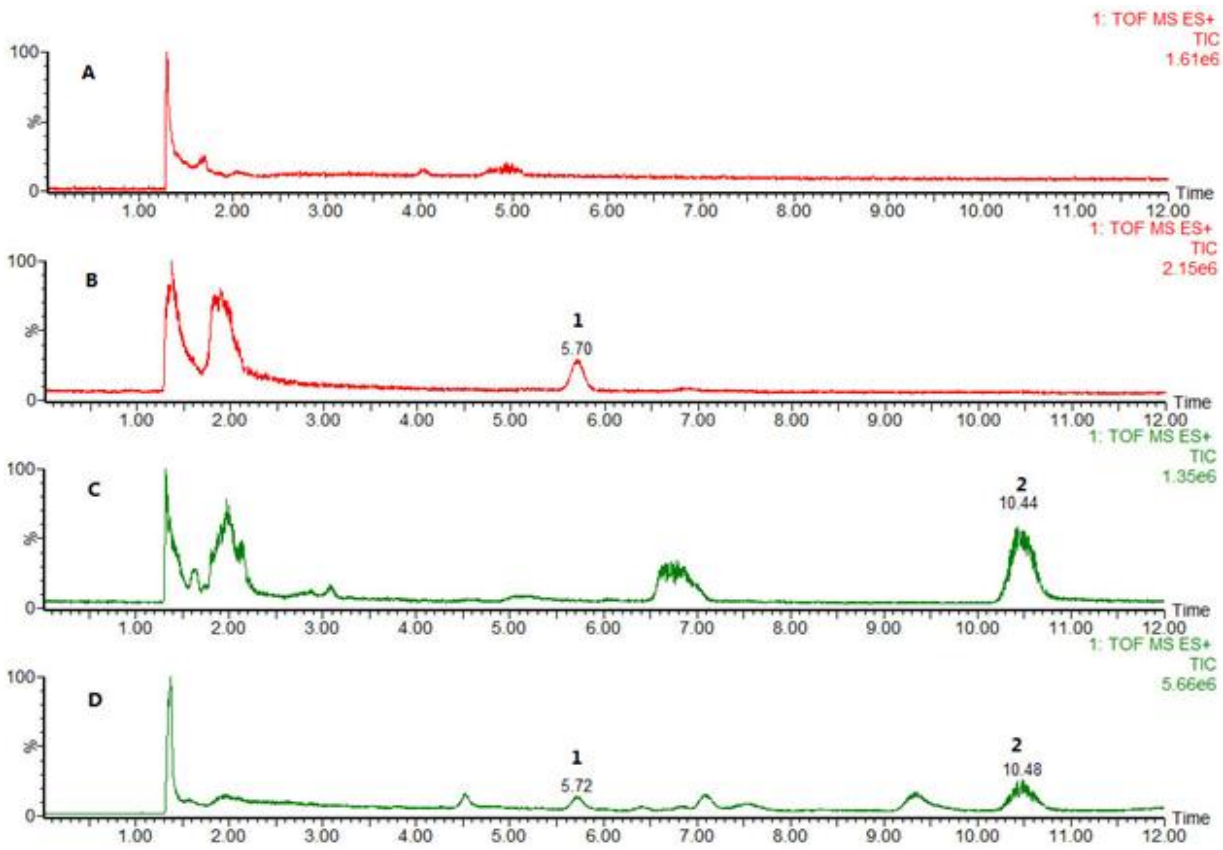

Fig. 1: Typical total ion chromatograms of (A) blank brain; (B) blank brain spiked with IS; (C) blank brain spiked with Curdione; and (D) brain sample after intravenous injection 10 min: peak 1 (5.7 min): Schisandrol A (IS); peak 2 (10.4 min): Curdione.

Preparation of calibration standards and quality control (QC) samples for analysis

Standard stock solution of Curdione and the IS were prepared by dissolving in methanol at 1.0 and 0.5mg·mL^{-1}, respectively. To make sure the concentrations of curdione in maternal and offspring rats's brain were all in the range of standard curve, working solutions of Curdione were obtained by mixing blank rat brain with stock solution at final concentrations of 0.5, 2.5, 5, 10, 25, 50 and 200μg·mL^{-1}. QC samples at low (2.5μg·mL^{-1}), medium (10μg·mL^{-1}), and high (50μg·mL^{-1}) concentration of Curdione were prepared by adding Curdione to blank rat brain and treating them as above. QC samples were used to evaluate recovery, precision, and stability. A final solution of internal standard (IS) consisting of Schisandrol A was prepared in deionized water at a concentration of 20μg·mL^{-1}.

UPLC/QTOF/MS conditions

For quantitative analysis, we used a Waters ACQUITY UPLC/Q-Xevo G_2-TOF-MS instrument. Chromatographic separation was carried out on an Agilent Extend-C_{18} reverse phase column (2.1×150 mm, 1.8μm) with a guard column (2.1×5mm, 1.8μm). Column temperature was maintained at 30℃. Mobile phase delivered at a flow rate of 0.5mL·min^{-1}, consisted of 0.1% aqueous formic acid (*v/v*, A) and acetonitrile (B) at an initial mixture of 40% B, increased to 55% B in a linear gradient over 5 min, then increased to 90% B from 5min to 15min, followed by column re-equilibration for 5min for next sample run. Total run time per sample was 20min and all injection volumes were 5μL.

Detection was used MS-ESI instrument in positive ion mode and full-scan mode with the range set at *m/z* 50-1000. Quantitation was performed by monitoring their respective *m/z* values. The nitrogen drying gas was at a flow rate of 10L·min^{-1} with capillary temperature of 300 . A nebulizer gas (nitrogen) pressure was 50 psig, a spray voltage of 4000V, a skimmer voltage of 150V. Waters Masslynx software (version 4.0) was used to process data in Total-Ion Chromatography (TIC) mode.

Method validation

Precision and accuracy

Precision and accuracy of the method was determined using QC samples. For determining the intra-day accuracy and precision, a replicate analysis of the QC samples of Curdione was carried out on the same day. The run consisted of a calibration curve and five replicates at (2.5

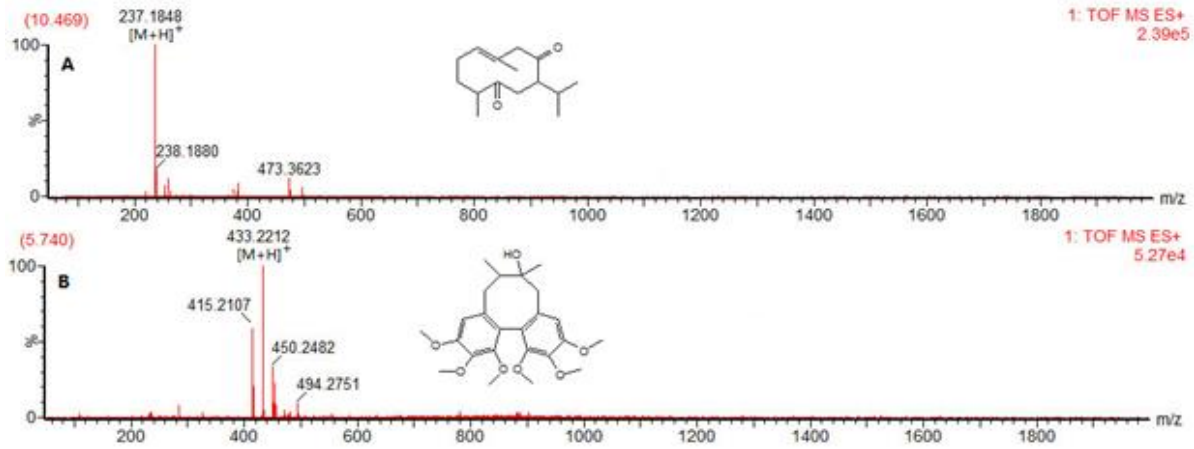

Fig. 2: Counts vs. Mass-to-charge (m/z) in MS spectra of Curdion (A) and Schisandrol A (IS, B)

Table 1: Intra-day and inter-day assay precision and accuracy for Curdione

Spiked amount /(μg·mL^{-1})	Intra-assay (Mean ± S.D.)			Inter-assay (Mean ± S.D.)		
	Determined/(μg·mL^{-1})	RE%	RSD%	Determined/(μg·mL^{-1})	RE %	RSD %
2.5	2.21±9.34	11.38	4.22	2.20±14.28	11.97	6.49
10	8.74±34.30	12.59	3.92	8.51±34.67	14.84	4.07
50	48.60±37.37	2.8	0.77	48.26±44.72	3.47	0.93

Table 2: Stability of Curdione under various storage conditions

Conditions	Concentration (μg·mL^{-1}, Mean±SD)		
	2.5	10	50
Autosampler ambient 24h	2.37±1.34	9.86±1.02	51.47±1.35
Ambient 24h	2.04±0.27	9.91±1.93	50.03±0.22
Three freeze-thaw cycles	2.34±0.74	9.68±1.48	50.36±0.99
-20ºC, 30 days	2.01±1.70	9.45±1.15	49.34±1.56

μg·mL^{-1}), medium (10μg·mL^{-1}), and high (50μg·mL^{-1}) QCs. The relative standard deviation (RSD) was used to express the precision. The lower limit of detection (LLOD) was determined by the minimum value with a signal-to-noise (S/N) ratio of ⩾ 3:1. The miminum value of the calibration was the lower limit of quantitation (LLOQ). The accuracy was evaluated with RE%=(*E*-*T*)/*T*×100, which *E* represented the calculated value, *T* represented its true value. This accuracy precision was evaluated and expressed using relative standard deviation (RSD%), which was calculated using the equation RSD%=SD/M×100.

Stability

The stability of Curdione in rat brain was evaluated by triplicate analyses of QC samples at 2.5, 10 and 50 μg·mL^{-1}. Short-term stability was determined by exposure of spiked samples to room temperature for 24h. The freeze-thaw stability was evaluated after three complete freeze-thaw cycles on consecutive days. The long-term stability was assessed after storage at -20º for 30 days.

Analyte recovery determination

The extraction recovery was determined by comparing the area of samples spiked before extraction to those spiked after extraction. The recovery samples were processed using extraction method and concentrations were determined by UPLC/QTOF/MS as described in section 2.5.

Pharmacokinetic application

Ezhu (2.5g/kg) was intravenously injected to rats. Brain samples (0.3g) were collected at 0, 10 and 30 min and 1, 2, 3 and 4 h after dosing. The obtained brain was stored at -20℃. The Curdione concentration in brain vs time data was analyzed using DAS 2.0 software.

STATISTICAL ANALYSIS

The concentration of Curdione in mother and unborn rats brain in model and normal groups were described using mean value ± Standard Deviation (x ± s). The significance of difference comparing the model and normal groups was analyzed using SPSS 13.0 software through T-test and a $p<0.05$ was considered significant.

RESULTS

Development of UPLC/QTOF/MS method and Selectivity

Acetonitrile and water with formic acid were chosen as mobile phases, which produced sensitivity for Curdione detection. Under this experimental condition, the representative chromatograms of blank brain, blank brain spiked with Curdione, the IS and the real brain sample were shown in fig. 1. There is no interfering endogenous substance observed at the retention time of the analyte or the IS.

Identification of Curdione and IS in rat brain by UPLC/QTOF/MS

The retention times and mass spectra were used to compare with the standards to identify the target compounds. The molecular formulae were established by positive mode electrospray ionization (+ESI), ions such as $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$. Data acquisition and processing were conducted with the Masslynx analysis software, extracting the m/z 237.1848 ion of Curdione (fig. 2A) and the m/z 433.2212 ion for Schisandrol A (fig. 2B).

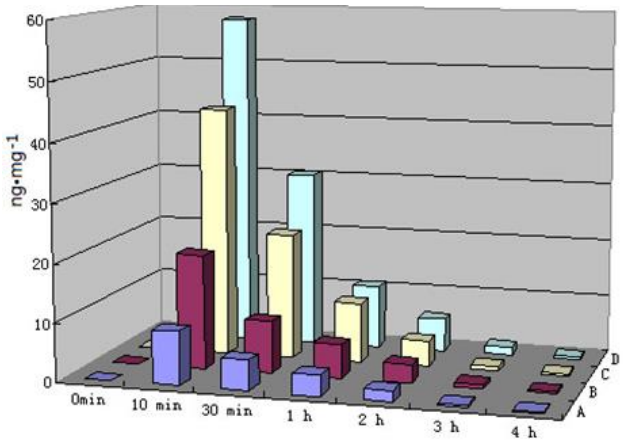

Fig. 3: Brain concentration-time bar chart of Curdione in rat brain (n=3) (A: model unborn rats; B: normal unborn rats; C: model mother rats; D: normal mother rats)

Linearity, accuracy and precision

The peak area ratios of Curdione to IS were plotted against analyte concentrations and standard curves fitted to equations by linear regression with a weighting factor of the reciprocal concentration squared ($1/x^2$) in the concentration range of 0.5-200μg·mL^{-1}. The equation was Y=0.0361X+0.0083, the correlation coefficient (R^2) was 0.9983.

The data for precision and accuracy for the method were listed in table 1. The intra-day RSD for the three concentrations of Curdione QC samples was <4.22% with the accuracy within the range of 2.8 to 12.59%. The inter-day RSD was <6.49% with the accuracy within the range of 3.47 to 14.84%. The LLOD and LLOQ were determined to be 0.15μg·mL^{-1} and 0.5μg·mL^{-1} separately.

$$RE\% = \frac{|\text{Measured value - true value}|}{\text{True value}} \times 100$$

Recovery of analytes

The extraction recovery of Curdione was calculated by analyzing three replicates at 2.5, 10 and 50μg·mL^{-1}. The mean recovery of Curdione was more than 93.31%, and the recovery from the IS at 20μg·mL^{-1} was more than 90.90%. The recovery efficiency for Curdione showed good reproducibility with a RSD% below 20. The matrix effect for standard Curdione was between 90 and 110%.

Stability

The result showed that Curdione spiked into rat brain samples were stable at room temperature for 24h, at -20 for 30 days, and through the whole three freeze-thaw cycles. The stability of Curdione in the sample solvent residing in the auto sampler was also observed over a 24 h period. The result of the stability experiments are listed in table 2.

Analysis of rat brain tissue samples

The optimized UPLC/QTOF/MS analytical method was applied to quantify the Curdione concentrations and satisfy the requirements for Curdione analysis in rat brain. The results can demonstrate the pharmacokinetic characteristics of Curdione in rats. After intravenous injection with a 1mL dose of extract, Curdione in brain was determined within 24h. The brain concentration vs time bar chart after injection is shown in fig. 3 and the main pharmacokinetic parameters between the normal and model groups were summarized in table 3.

The brain concentration at 0.167 h (T_{max}) was an observed value in the fig. The areas under the concentration curve-time curves was arranged in descending order, normal mother rats > model mother rats > normal unborn rats > model unborn rats. The half-life ($T_{1/2}$) of the four kinds of rats was calculated to be less than 0.593 h, indicating that Curdione was rapidly eliminated in these rats after intravenous injection.

Statistical analysis

The P value between model and normal in unborn rats and mother rats group were 0.009 (P<0.01) and 0.021 (P<0.05) separately. So the result showed that there is extremely significant difference in unborn rats group and the significant difference in mother rats group.

DISCUSSION

In this study, we have successfully applied the processing conditions and extraction procedure to determine the Curdione in rat brain. Electrospray ionization (Jarmusch *et al.*, 2016) in positive or negative ion mode for Curdione

Table 3: The main pharmacokinetic parameters after intravenous injection of single-dose

Parameters	Model unborn rats (A)	Normal unborn rats (B)	Model mother rats (C)	Normal mother rats (D)
$T_{1/2}$ (h)	0.61	0.666	0.593	0.642
T_{max} (h)	0.167	0.167	0.167	0.167
C_{max} (ng·mg^{-1})	9.2**	19.67	43.16*	57.97
$AUC_{(0-t)}$ (h·ng·mg^{-1})	9.534**	17.381	33.149*	42.821
$AUC_{(0-\infty)}$ (h·ng·mg^{-1})	9.648**	17.668	33.314*	43.255

**P<0.01 (A *vs* B); *P<0.5 (C *vs* D); $T_{1/2}$, Half-life; T_{max}, time to reach maximum brain concentration; C_{max}, maximum brain concentration; AUC, area under the concentration curve-time curve.

detection in rat brain was first evaluated. We found that the positive ion detection resulted in a better signal-to-noise ratio.

Secondly, peak shape and low interference were considered to optimize the chromatographic conditions. The methanol, acetonitrile, water and 0.1% formic acid in water and a variety of ratios and gradient strategies were chosen to optimize the mobile phase. Finally, acetonitrile and water with 0.1% formic acid were chosen as the mobile phase components for gradient elution on the basis of peak symmetry and sharpness. Different C_{18} columns (Ruiz-Angel *et al.*, 2014) were chosen to offer the best chromatographic resolution, speed, sensitivity and selectivity.

In conclusion, we established an UPLC/QTOF/MS method (Tang YN, *et al.*, 2015) to quantify the concentration of Ezhu extract in rat brain after intravenous injection. The Curdione was extracted from brain samples using dichloromethane and isopropanol. The result demonstrated that the optimized UPLC/QTOF/MS method was a simple and sensitive approach for the pharmacokinetic study of complex biological samples.

Curdione, one of the major sesquiterpene compounds from Rhizoma Curcumae, has been shown to exhibit multiple bioactive properties (Xia Q *et al.*, 2012). In this study, we investigated the antithrombotic activity, especially in the pregnant rats. It will cure the blood stasis of the maternal rat and will not damage its infant rat on the basis of the theory of traditional Chinese Medicine "YOU GU WU YUN". The concentration of Curdione in brain in model group is significantly lower than normal group. The result in this study shows that the disease status can affect the distribution of medicine in brain. We reveal the scientific connotation of "YOU GU WU YUN" through pharmacokinetic method: during the application of Chinese clinical medicine, some toxic potent Chinese herbal medicines will show mainly therapeutic effect, not the toxicity in disease situations.

ACKNOWLEDGMENTS

The authors appreciate the financial support from the National Natural Science Foundation of China (No. 81403173 and No. 81273640), China Postdoctoral Science Foundation (No. 2014M551291) and Research project of Health Department of Heilongjiang Province (No. 2012-327).

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