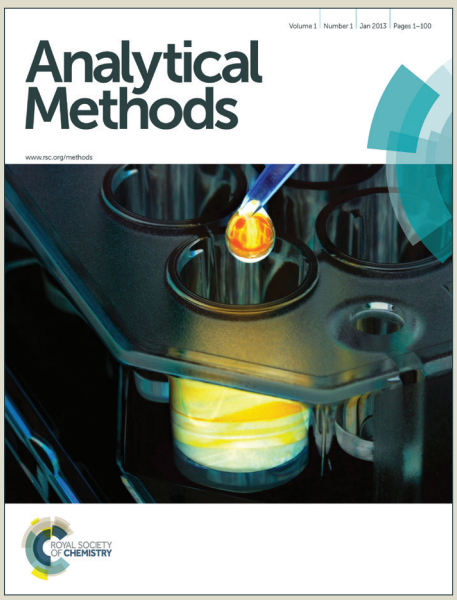
Analytical Methods

Accepted Manuscript



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Development and validation of an UPLC-MS/MS method for determination of jujuboside B in rat plasma and its application to the pharmacokinetic and bioavailability studies

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Abstract： Jujuboside B (JuB) is a main bioactive saponin constituent of Ziziphi Spinosae Semen. The compound is used clinically as an anti-insomnia and anti-anxiety medicine. In this study, a sensitive, simple, and rapid ultra performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) was developed and validated to quantify JuB in rat plasma. A simple protein-precipitation method was used to extract JuB from rat plasma samples. Jujuboside A was used as an internal standard (IS). Chromatographic separation was performed using Acquity HSS T3 column. The mobile phase consisted of acetonitrile and 0.1% formic acid in water with a flow rate of 0.3 mL/min. Identification and quantification were performed through electrospray ionization in a negative mode with multiple reaction monitoring of JuB and IS transitions of *m/z* 1043.3→911.5 and *m/z*1205.6→1073.4, respectively. The calibration curve was linear in the range of 0.1 ng/mL to 1000 ng/mL (R^2=0.990) with a limit of detection of 0.03 ng/mL. The extraction recoveries of JuB was 90.3 %to 95.7 % and the precisions of intra-and inter-day were less than 11.5 %. The matrix effect of JuB at three different concentrations ranged from 93.5 % to 95.9 % with a standard deviation of < 5%. The inter- and intra-day assay accuracies were 86.7 % to 94.3 % and 93.3 % to 95.7 %, respectively. The pharmacokinetic processes of JuB fit in the one-compartment model of *p.o* administration and two-compartment model of *i.v* administration. The absolute oral bioavailability of JuB in rats was only 3.6 %. Indeed, the proposed method was successfully applied to analyze the pharmacokinetic and bioavailability of JuB in rats after JuB was administered *p.o* and *i.v*.

Key Words: Jujuboside B; pharmacokinetics; bioavailability; UPLC-MS/MS

1. Introduction

Jujuboside B (JuB, Fig.1), a dammarane triterpenoid saponin, is the main active ingredient in Ziziphi Spinosae Semen derived from *Zizphus jujuba* Mill *vars pinosus*(Bunge) Hu ex H F Chou [1]. JuB has exhibits beneficial activities, such as anti-insomnia and anxiolytic properties[2], inhibitory effect on platelet aggregation [3], and anti-tumor activity [4]. JuB also is also one of the main active ingredients of Chinese medicine preparations, such as granules, capsules, decoction, pills and oral liquid [5]. As such, the of JuB application in food and clinical medicine has been extensively explored. Despite extensive research on JuB bioactivity, limited information is available regarding the pharmacokinetic profile of JuB. Therefore, the pharmacokinetic properties and bioavailability of JuB should be evaluated and described.

HPLC has been applied to detect the JuB contentin raw materials or pharmaceutical preparations. Sun *et al* [6]and Gu *et al* [7] developed HPLC-UV and HPCE to determine JuB in raw materials. These techniques have been applied to evaluate the quality of raw materials and verify true or false identification. Du *et al* [8], Zhang *et al* [9], and Zhang *et al* [10] established HPLC-ELSD to measure JuB concentration in Semen soups, Suanzaoren dropping pills, and raw materials. The sensitivity of this assay is at a microgram level. To improve analytical sensitivity and

low selectivity, Zhang *et al* [11], Zhao *et al* [12], Liu *et al* [13], and Zhao *et al* [14] developed LC-MS/MS quantified JuB in Semen Ziziphi Spinosae. The linear range was found at 10 to 2000 ng/mL, suggesting that mass spectrometry significantly improves assay sensitivity. To the best of knowledge, no analytical method that determines the JuB content of in biological samples has been developed. In this study, a simple, rapid, and sensitive ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) was established and validated to determine JuB in rat plasma. The proposed method was successfully applied to analyze the pharmacokinetics and bioavailability of JuB in rats after this substance was administered *p.o* and *i.v* . This study provided reference for further clinical applications of JuB.

2.Experimental

2.1 Reagents and materials

Jujuboside A (JuA, internal standard (IS)) and JuB were purchased from Chengdu Munster Biotechnology Co., Ltd (China), and the purity of each compound was > 98 %. Methanol and acetonitrile (Merck, Germany) were of HPLC grade and ultra-pure water was obtained from the Milli-Q system (Millipore, Bedford, MA, USA). Formic acid was purchased from Sigma (Shanghai,China). All other chemicals and reagents were of analytical grade.

2.2 Analytical system

Acquity UPLC system (Waters, Milford, MA, USA) coupled with a triple-quadrupole

tandem Waters Quattro Micro mass spectrometer was used for sample analysis. LC separation was performed on an Acquit HSS T3(2.1 mm × 100 mm, 1.8 μm) with a security guard column, maintained at 35 ℃. The mobile phase consisted of acetonitrile (A) and 0.1 % (v/v) formic acid in water (B) with flow rate of 0.3 mL/min. A linear gradient of mobile phase was set as follows: 70 % (B) over 0 - 0.6 min；70 % - 60 % (B) over 0.6 - 0.8 min；60 % - 10 % (B) over 0.8 - 3.2 min；10 % (B) over 3.2 - 4.8 min；10 %- 70 % (B) over 4.8 - 5.0 min；70 % (B) over 5.0 - 6.0 min. The samples were maintained at 4 ℃ in the auto-sampler, and a 5 μL of the sample was injected into the UPLC system.

A mass spectrometer with an electrospray ionization (ESI) interface in a negative ion mode (ESI⁻) was used for quantitative analysis, with acquisition in multiple reaction monitoring (MRM) mode. The MRM analysis was conducted by monitoring the precursor ion to produce ion transitions of *m/z* 1043.3→911.5 for JuB and *m/z* 1205.6→1073.4 for IS. The optimized electrospray conditions were as follows: capillary voltage 3.2 kV, cone voltages of 80 V and 100 V for JuB and IS, respectively; source temperature, 110 ℃; desolvation temperature, 500 ℃, and desolvation gas flow (nitrogen), 800 L/h.

2.3 Preparation of standard and quality control (QC) samples

Stock solutions were separately prepared by dissolving accurately weighed standard reference compounds of IS and JuB in methanol. The standard solutions of JuB and IS were prepared to obtain concentrations of 100 and 4 μg/mL in methanol,

respectively. The analytical standard and QC samples were prepared as follows. The standard working solution (10 μL) was evaporated to dryness by a gentle stream of nitrogen, and 100 μL of blank rat plasma was added. The final calibration concentration ranged from 0.1 ng/mL to 1000 ng/mL. The QC samples were prepared at concentrations of 0.3, 50 and 800 ng/mL.

2.4 Sample preparation

IS (10 μL, 4 μg/mL) was placed in a 1.5 mL eppendorf tube and dried with a flow of nitrogen gas at 30 °C. The residue was added to the rat plasma (100 μL) and vortexed for 30 s. Afterward, 300 μL of acetonitrile was added. The mixture was vortexed for 3 min and ultrasonicated for 1 min; the mixtures were centrifuged at 15,000 *g* and 4 °C for 15 min. The supernatant that was filtered across a membrane (5 μL) was injected into the UPLC–MS/MS system for analysis.

2.5 Method validation

The specificity of the method was tested by comparing the chromatograms of blank rat plasma samples, plasma samples spiked with the analytes and IS, and plasma samples after *p.o* and *i.v* administration of JuB. Blank rat plasma samples were investigated for endogenous interference, followed by spiking with IS for the interference of IS. Calibration curves were obtained by plotting the measured peak area ratios of analyte to IS. The standard curve was fitted to linear regression ($y = ax + b$) by using $1/x$ as the weighting factor. The LLOQ of the analyte is the lowest

concentration with signal/noise ≥10, which could be quantitatively determined with the precision and accuracy (≤ 20%). The limit of detection (LOD) was defined as the amount that could be detected with a signal/noise ratio ≥3. The intra- and inter-day precision and accuracy were distinguished by examining three different concentrations of QC samples on the same day and on five consecutive days, respectively. Precision was evaluated by relative standard deviation (RSD %) and accuracy as (mean measured concentration/spiked concentration) ×100 %.

The extraction recoveries of JuB at the three QC levels were determined by comparing the responses obtained from extracted QC samples with those obtained from reference standards spiked in post-extracted blank rat plasma at the same concentrations. The matrix effects were evaluated by comparing the peak areas obtained from the samples in which the extracted matrix was spiked with standard solutions to those obtained from the reference standard solutions at the same concentration. Stability experiments were performed to evaluate the stability of the analyte in the rat plasma under different conditions. Short-term stability was determined by keeping the QC samples at room temperature for 12 h. Long-term stability was evaluated by analyzing samples stored at −20 °C for 15 days, 4 °C in a refrigerator for 24 h and three freeze–thaw stability cycles.

2.7 Pharmacokinetic application

Twelve male Sprague-Dawley (SD) rats (weight 200 ± 20 g) were purchased from the Experimental Animal Center of Wenzhou Medical University and were cared for

under a controlled environment at 25 ± 1 °C, relative humidity of 50 ± 10 % and 12 h day/light cycle with free access to the standard laboratory food and water. All animal studies were in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals in Wenzhou Medical University (No. 2013-152). After a 12 h fast prior to the experiment, the rats were administered with JuB at 15 mg/kg for *p.o* and 1.5 mg/kg for *i.v* administration. Blood samples were collected from the tail vein at 0.083, 0.167, 0.333, 0.5, 0.75, 1, 2, 4, 8, 12 and 24 h after administration. The blood samples were immediately transferred to heparinized tubes and centrifuged at 5000 rpm for 10 min. The supernatant was then transferred into 1.5 mL Eppendorf tubes and stored at −20 °C prior to analysis.

3. Results and discussion

3.1 Method development

The choice of IS is very important to obtain good accuracy and precision of the analyte. Given its similarity in chemical structure, chromatographic behavior, extraction efficiency, and ionization with JuB, JuA was set as the IS. Initially, isocratic elution was used to acquire the chromatogram of JuB, but the resolution and sensitivity was not suitable for determination of JuB and IS in rat plasma. The gradient elution of the mobile phase was then offered and the acquired chromatograph had a more symmetrical peak shape, thus enhancing the sensitivity and resolution in the chromatography. Meanwhile, we also have investigated various solvent systems composed of methanol and acetonitrile, as well as different buffers, such as formic

acid, ammonium formate, and acetic acid to obtain the appropriate retention time, best resolution, and optimal sensitivity. Acetonitrile and 0.1 % (v/v) formic acid in water was finally chosen as the organic modifier because it led to lower background noise and the best resolution. Experiments were also performed with different LC columns such as BEH C18 (2.1×100 mm, 1.7 μm), BEH Shield C18 (2.1×100 mm, 1.7 μm) and HSS T3 (2.1×100 mm, 1.8 μm). HSS T3 column was finally selected for the chromatographic separation because of the better peak shape,. Therefore, the best combinations of peak shape and retention time were achieved using HSS T3 under the gradient elution with a mobile phase of acetonitrile and 0.1 % (v/v) formic acid. Typical chromatograms are shown in Fig. 2.

3.2 Specificity

The acquired chromatograms of blank plasma, blank plasma spiked the standard solution and IS, and rat plasma sample are shown in Fig. 2. Given the described chromatographic conditions, JuB and IS were simultaneously detected and their retention times was approximately 3.24 and 3.00 min, respectively. No significant interference from the rat plasma was found during the retention times of either JuB or IS. The results indicated that the method exhibited good specificity and selectivity, and the method was applied to plasma samples for the pharmacokinetic study.

3.3 Extraction recovery and matrix effects

The extraction recoveries and matrix effects of JuB and IS are shown in Table 1. At

low, medium and high QC samples, the extraction recoveries of JuB were greater than 90 % with a standard deviation of less than 5 %. The extraction recovery of IS at 50 ng/mL was 95.9 ± 4.9 %. In addition, the matrix effect of JuB at three concentrations ranged from 93.5 % to 95.9 % with standard deviation less than 5 %. The matrix effect of IS at 50 ng/mL was 94.0 ± 7. 5 %. The recovery and matrix effect of the determination of JuB and IS in rat plasma was acceptable according to the FDA' guideline.

Table 1 Extraction recovery and matrix effect of JuB in rat plasma (mean ± SD, n=6)

Spiked plasma Concentration (ng/mL)	Extraction recovery (mean ± SD)	Matrix effect (mean ± SD)
0.3	90.3 ± 3.2	94.0 ± 2.5
50	94.1 ± 3.4	93.5 ± 4.0
800	95.7 ± 4.5	95.9 ± 4.5

Table 2 Intra- and inter-day accuracy and precision for JuB in rat QC samples.

Spiked concentration (ng/mL)	Intra-day concentration (mean ± SD)	Precision (% RSD)	Accuracy (%)	Inter-day concentration (mean ± SD)	Precision (%RSD)	Accuracy (%)
0.3	0.28 ± 0.04	11.4	93.3	0.26 ± 0.03	11.5	86.7
50	47.40 ± 3.40	6.8	94.8	45.75 ± 2.36	4.7	91.5
800	765.46 ± 28.86	3.6	95.7	754.75 ± 30.6	3.8	94.3

3.4 LOD, LLOQ, linearity, accuracy, and precision

The calibration curves ranged from 0.1 ng/mL to 1000 ng/mL using nine calibration

standards. The regression equation for calibration curves in plasma was y = 1.0622 *x* + 0.1639 (R^2=0.990), where y is the peak-area ratio versus concentration, and *x* is the JuB concentration . The calibration curves provided a reliable response for JuB. The LOD and LLOQ of JuB were 0.03 and 0.1 ng/mL, respectively. The intra- and inter-day precisions and accuracies of the assay are presented in Table 2. The RSD of the intra- and inter-day assays were less than 11.4 % and 11.5 %, respectively. The intra- and inter-day assay accuracies was 93.3 %to 95.7 % and 86.7 % to 94.3 %, respectively.

3.5 Stability

QC samples at three different concentrations were analyzed in five replicates to investigate the stability of JuB. JuB was stable in the rat plasma after this substance was stored at room temperature for 4 h, 4 °C in the autosampler for 24 h and at −20 °C for 15 days, or after this substance was subjected to three repeated freeze–thaw cycles. Stability test results are summarized in Table 3. Thus, the samples remained stable analysis.

Table 3 Stability of JuB in rat plasma (mean ± SD, n=5)

Storage conditions	Concentration (ng/mL)	Measured (mean ± SD)	RSD (%)
Room temperature for12	0.3	0.35 ± 0.04	13.3
h	50	45.03 ± 2.08	4.2
	800	760.98 ± 26.70	3.3
Three freeze/thawcycles	0.3	0.34 ± 0.03	13.3

	50	45.82 ± 2.77	5.5
	800	761.00 ± 14.90	1.9
Keeping at 4 °C for 24 h	0.3	0.27 ± 0.03	13.3
	50	46.30 ± 2.47	5.4
	800	769.01 ± 25.08	3.1
Long-term stability(at	0.3	0.26 ± 0.03	13.0
-20 °C for 15 days)	50	46.04 ± 2.32	4.6
	800	763.30 ± 25.04	3.1

Table 4 Pharmacokinetic parameters of JuB in two groups ($\bar{X}$ ± S, n=6)

Parameter	*i.v.*(1.5 mg/kg)	*p.o.* (15 mg/kg)
$t_{1/2\alpha}$ (h)	0.35 ± 0.09	
$t_{1/2\beta}$ (h)	4.92 ± 5.92	
$t_{1/2}$ (h)		2.61 ± 0.88
V (L/kg)	3.12 ± 3.11	1139.16 ± 1114.90
CL (L/h/kg)	0.68 ± 0.14	351.93 ± 358.73
$AUC_{(0-t)}$ (μg/L×h)	2254.70 ± 409.36	64.20 ± 41.20
C_{max} (μg/L)	475.51 ± 106.21	1.81 ± 0.48
T_{max} (h)	0.08 ± 0.00	0.46 ± 0.17

3.6 Pharmacokinetic analysis

The developed UPLC-MS/MS method was applied to the pharmacokinetic analyze of JuB after *p.o* and *i.v* administration of 15 and 1.5 mg/kg, respectively. The mean plasma concentration–time profiles of JuB are illustrated in Fig.3. The major pharmacokinetic parameters of JuB are calculated by a two - compartment model for the *i.v* administration and one - compartment model for *p.o* administration (Table 4).

For *i.v* group, T_{max} is 0.08 ± 0.00 h and $t_{1/2\alpha}$ (0.35 ± 0.09) h is much smaller than $t_{1/2\beta}$ (4.92 ± 5.92) h, suggesting that the plasma concentration of JuB quickly declined in a few minutes. The *CL* (0.68 ± 0.14) L/h/kg and *V* (3.12 ± 3.11) L for JuB were small numbers, whereas $AUC_{(0-t)}$ (225.50 ± 40.94) μg/L*h and $AUC_{(0-\infty)}$ (228.82 ± 44.75) μg/L*h were large numbers, indicating that JuB was mainly distributed in the plasma and was eliminated slowly after *i.v* administration, thus possibly achieving good treatment effect. For the *p.o* group, T_{max} was 0.46 ± 0.37 h and C_{max} was 21.31 ± 14.03 μg/L. $AUC_{(0-t)}$ (64.20 ± 41.20) μg/L*h. The $AUC_{(0-\infty)}$ (81.64 ± 61.81) μg/L*h were small numbers, whereas *CL*(351.93 ± 358.73) μg/L*h and *V* (1139.16 ± 1114.90) L were large numbers, indicating that JuB was mainly distributed in the tissues and body metabolism was faster. The absolute bioavailability (F) is the dose-corrected area under *AUC* non-intravenous divided by *AUC* intravenous. For a drug administered by the oral route (*p.o*), F is calculated as follows: $F = 100 \times [(AUC_{p.o} \times Dose_{i.v})/ (AUC_{i.v} \times Dose_{p.o})]$. The bioavailability of JuB in rats was only 3.6 %.

4. Conclusion

This paper establishes a simple, sensitive and rapid UPLC-MS/MSmethod for the determination of JuB in rats, and this assay was applied to the pharmacokinetic and bioavailability studies, which provided a basis for further guidance on the clinical application of JuB.

Acknowledgements

The authors acknowledge financial support from the Nature Foundation Committee of Zhejiang Province, China (LQ13H280001 & Y2110407) and Traditional Chinese Medicine Research Project of Zhejiang Province (2011ZA071).

References

1. M. Jinjie, L. Ping and M. Baiping, *J Int Pharm Res*, 2011, 38, 206-211.
2. X. Wang, B. Yang, H. Sun and A. Zhang, *Anal Chem*, 2012, 84, 428-439.
3. E. J. Seo, S. Y. Lee, S. S. Kang and Y. S. Jung, *Phytother Res*, 2013, 27, 829-834.
4. M. Y. Xu, S. Y. Lee, S. S. Kang and Y. S. Kim, *J Nat Prod*, 2014, 77, 370-376.
5. W. Heping, W. Jianming and W. Dongni, *Chin Pharm J*, 2006, 41, 173-175.
6. S. Sun, H. Liu, S. Xu, Y. Yan and P. Xie, *J Pharm Anal*, 2014, 4, 217-222.
7. G. Jin and L. Ping, *Chin J Drug Appl Monit*, 2007, 5, 38-40.
8. D. Chenhui, Y. Yan, X. Haiyuan, P. Xiangpei, Z. Bin and P. Miaorong, *Chin J Pharm Anal*, 2012, 32, 1545-1549.
9. Z. Yanqing, J. Junbo, Z. Mingchun and L. Chagjiangsheng, *Chin Tradit Herb Drugs*, 2009, 40, 234-235.
10. M. Zhang, Y. Zhang and J. Xie, *J Pharm Biomed Anal*, 2008, 48, 1467-1470.
11. Q. Y. Zhang, H. T. Yang, H. Shi, Z. Y. Zhang and L. T. Zhang, *Chinese Traditional and Herbal Drugs*, 2015, 46, 123-127.
12. X. Zhao, L. Xie, H. Wu, W. Kong and M. Yang, *Anal Methods-Uk*, 2014, 6, 5856-5864.
13. J. Liu, B. Chen and S. Yao, *Talanta*, 2007, 71, 668-675.
14. J. Zhao, S. P. Li, F. Q. Yang, P. Li and Y. T. Wang, *J Chromatogr A*, 2006, 1108, 188-194.

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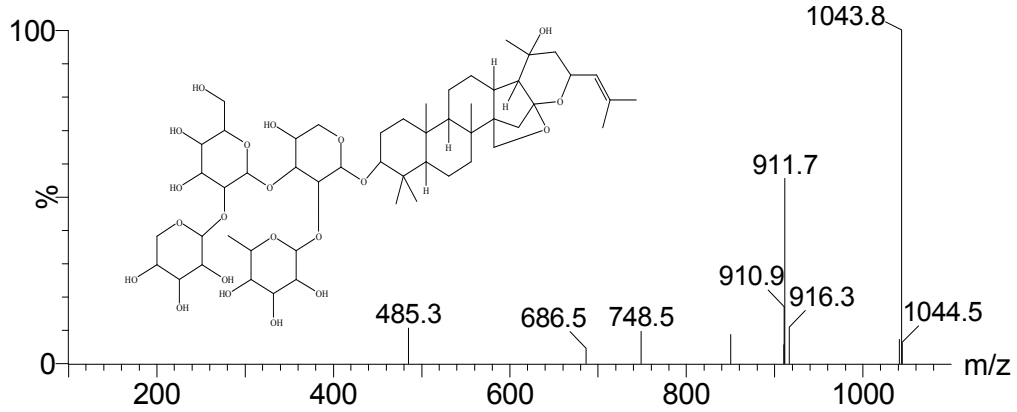

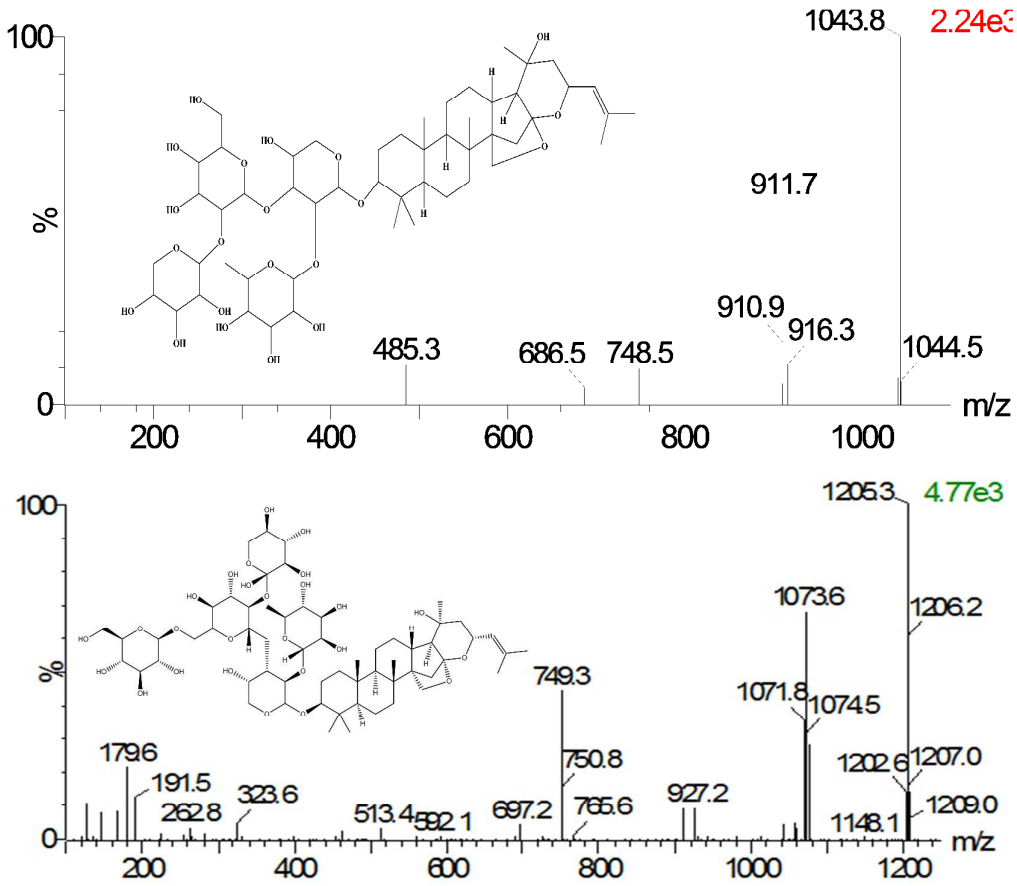

Fig.1. Chemical structure and positive ionization electrospray mass scan of JuB

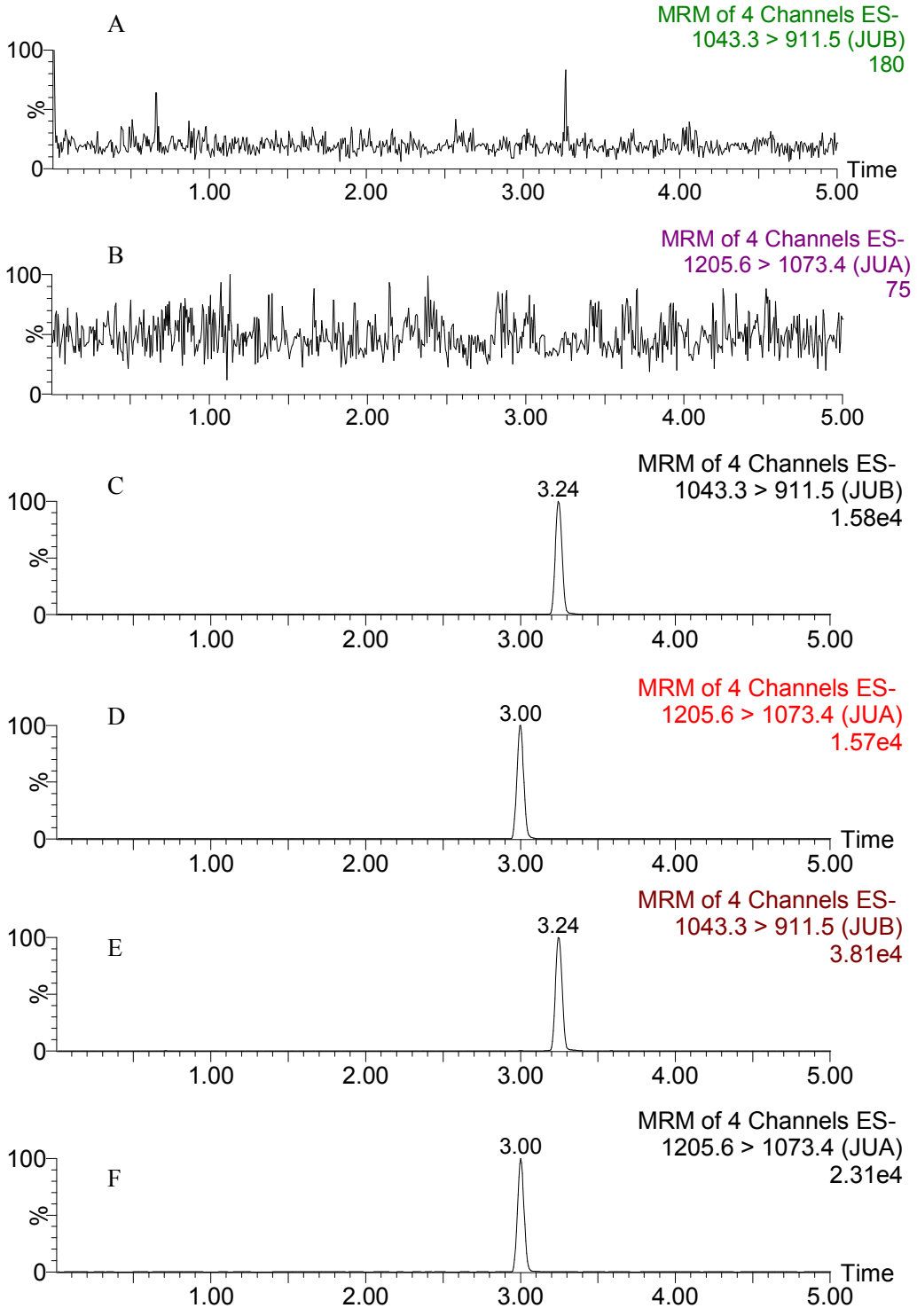

Fig.2. Typical MRM chromatograms of JuB (A) and JuA (B) in rat blank plasma; rat plasma spiked with 50 ng/mL of JuA and IS (C&D); a 0.5 h plasma sample following intravenous injection of 1.5 mg/kg to rats (E&F).

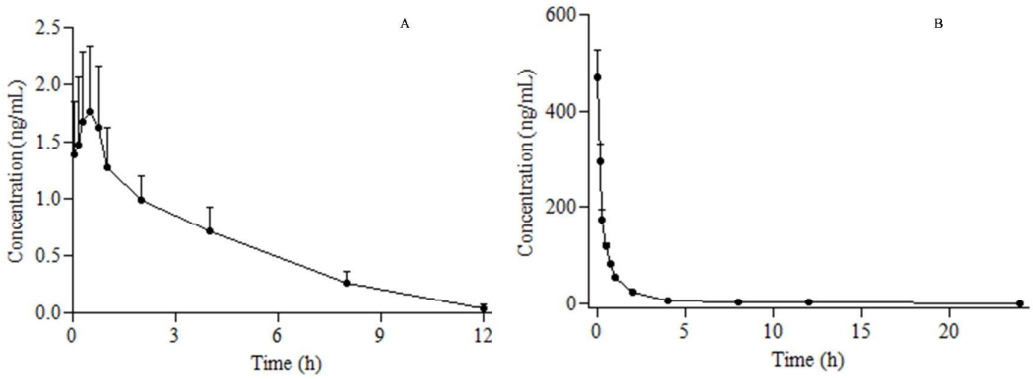

Fig.3. Plasma concentration-time plots of JuB after *p.o* (A) and *i.v* (B) administration