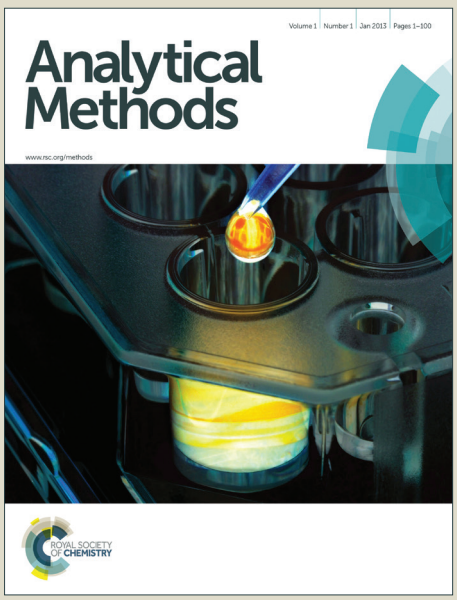
Analytical Methods

Accepted Manuscript



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Simultaneous determination of benzoylmesaconine and piperine in rat plasma after oral administration of Naru-3 pill by an Ultra Fast Liquid Chromatography - tandem mass spectrometry method and its application to a comparative pharmacokinetic study

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Abstract

A rapid, sensitive and reliable Ultra Fast Liquid Chromatography - tandem mass spectrometry (UFLC-MS/MS) method has been developed for simultaneous quantitation of benzoylmesaconine and piperine in rat plasma after oral administration of Naru-3 pill. Naru-3 is a well-known traditional Mongolian medical analgesic formula, which contains three Chinese herb powder - Aconiti Kusnezoffii Radix Cocta, Piperis Longi Fructus and Chebulae Fructus. After addition of brucine (internal standard, IS), 100 μL of plasma were extracted by liquid-liquid extraction with methyl *tert*-butyl ether, and then the two analytes and IS were separated on a Venusil MP C18 column (100mm × 2.1mm, 3.0 μm) at 30 ºC with a gradient program of 0.1% formic acid - methanol in water as the mobile phase. UFLC-MS/MS system coupled with an electrospray ionization source was performed in multiple reaction monitoring mode. The linear range was 0.075 - 15 ng/mL for benzoylmesaconine and 5 - 1000 ng/mL for piperine, respectively. Linearity, accuracy, precision, recovery and matrix effect of the two analytes were all within satisfaction. The validated method was successfully applied to compare pharmacokinetic profiles of the analytes in rat plasma after oral administration of three compatibility-composition suspensions designed according to Naru-3 pill. The pharmacokinetic data obtained by the method indicated that some ingredients in Naru-3 pill would have influence on the pharmacokinetic behavior of the two analytes.

Keywords: Benzoylmesaconine; Piperine; Naru-3 pill; Pharmacokinetics; Ultra Fast Liquid Chromatography - tandem mass spectrometry

1 Introduction

Rheumatoid arthritis (RA) is a systemic, chronic inflammatory disease associated with not only a slowly progressive disease limited to the joins, but rather an increased prevalence of systemic disease, which may cause disability, decrease of life quality and even mortality of patients.[1-3] Thus RA would carry "social costs" as well as economic burden.[4] Naru-3 pill, a well-known and widely-used traditional Mongolian medical analgesic formula, was originally described in a traditional Mongolian medical book "Zhi Gao Yao Fang". It has the efficacy of subsiding swelling, expelling wind, dispelling cold and analgesia.[5] During an extremely long period of clinical practice, its excellent efficacy in RA treatment has been demonstrated.[6]

Attributed to the complexity, unknown effective ingredients and herbs combination of the formula, Traditional Chinese Medicine (TCM) preparation is commonly used to treat various complex diseases. Naru-3 pill contains three Chinese herb powder with a ratio of 1:0.6:2 (*w/w/w*), Aconiti Kusnezoffii Radix Cocta (AKRC, the processed products of the dried root of *Aconitum kusnezoffii* Reichb.), Piperis Longi Fructus (PLF, the dried clusters of *Piper longum* L.) and Chebulae Fructus (CF, the dried fruit of *Terminalia chebula* Retz. or *Terminalia chebula* Retz. var. *tomentella* Kurt.). AKRC is the key component of anti-inflammatory and analgesic in the Naru-3 prescription. Meanwhile, extreme toxicity exists in all Aconitum herbs which have a narrow therapeutic window between therapeutic and toxic doses.[7] Diester diterpenoid alkaloids (DDAs), such as aconitine, mesaconitine, and hypaconitine, are considered as the chief toxic ingredients in the plant *Aconitum* spec.. To ensure therapeutic safety, only carefully processed root of aconites could be used clinically, since after processed, most of the DDAs changed to monoester diterpenoid alkaloids (MDAS).[8] Some studies reported that MDAs have much less toxicity than DDAs.[9-10] Additionally, as one of the vital MDAs in AKRC, benzoylmesaconine exhibited activities in anti-inflammatory and analgesia. In TCM, PLF could dispel cold by warming stomach and could be helpful for RA patients. Piperine, a major active ingredient in PLF, has been widely reported as an effective constituent for the treatment of ache and inflammation.[11-12] When drugs co-administered with piperine, enhancement of

bioavailability and improvement of therapeutic effects have been demonstrated by a plenty of studies[13-15]. CF, rich in tannins and phenolic acid,[16] is commonly used combining with AKRC to reduced AKRC's toxicity. Benzoylmesaconine and piperine were chosen as target markers to investigate the possible influence of the drug-drug interactions on the pharmacokinetic behavior between single herb and different compatibility-composition groups after oral administration of different herb powder. The combination of Chinese Material Medica might produce a synergistic effect or antagonistic action. Moreover, the pharmacokinetics of the ingredients in prescription might be influenced by the combination.[17-18] Therefore, the study of the composite prescription theory would make a considerable contribution to the therapeutic application of traditional Chinese medicine formulae, especially the poisonous ones. In this study, three groups (AKRC, AKRC: PLF 1:0.6 and AKRC: PLF: CF 1:0.6:2) were designed following the original ratio of Naru-3 pill to investigate the possible pharmacokinetic differences of the two target markers among the three groups.

Several quantitation methods have been demonstrated for determination of *Aconitum* alkaloids and piperine in vitro, including CE-electrochemiluminescence (CE-ECL) technology[19] and high-performance liquid chromatography coupled with photodiode array detector system (HPLC-DAD).[20] In the vivo study, the content of components was at fairly low level; as a consequence, it required a more sensitivity method to meet the analysis requirements. In particular, LC-MS and LC coupled with tandem mass spectrometry (LC-MS/MS) have been proposed for the determination of aconitum alkaloids[21-22] or piperine,[23] separately. Benzoylmesaconine was the maximum alkaloid of AKRC. Simultaneous determination of benzoylmesaconine and piperine has not been reported. Therefore, a simple, fast and highly sensitive UFLC-MS/MS method has been developed and completely validated for the first time for simultaneous determination of benzoylmesaconine and piperine in rat plasma after oral administration of AKRC, AKRC:PLF and AKRC:PLF:CF herb powder. The result of the pharmacokinetics study might improve the clinical rational application of Naru-3 pill.

2 Experimental

2.1 Animals

Eighteen pathogen-free male Wistar rats (200 ± 20g) supplied by Liaoning Changsheng Biotechnology Co., Ltd. (Benxi, China) were kept in an environmentally controlled room for at least 7d before the experiments. The experimental animals were randomly and averagely divided into 3 groups by giving different compatibility-composition drugs orally. The animal study was carried out in accordance with the Guideline for Animal Experimentation of Shenyang Pharmaceutical University, and the protocol was approved by the Animal Ethics Committee of the institution.

2.2 Chemicals and Materials

Aconiti Kusnezoffii Radix Cocta was purchased from Yaodu pharmaceutical Co., Ltd. (Anguo, China), Piperis Longi Fructus was purchased from Tong-Ren-Tang TCM store (Shenyang, China) and Chebulae Fructus was purchased from Kangmei pharmaceutical Co., Ltd. (Puning, China). All the crude drugs were authenticated by associate professor Ying Jia (Department of Pharmacognosy, Shenyang Pharmaceutical University, Shenyang, China). All the three herbs were finely powdered. The reference standards of benzoylmesaconine (Figure 1a) and brucine (Figure 1c, IS) were acquired from the National Institutes for Food and Drug Control (purity both >98%, Beijing, China). Piperine (Figure 1b, purity >97%) was obtained from Sigma-Aldrich (St. Louis, MO). Methanol and acetonitrile of HPLC grade was from Fisher Scientific (Fair Lawn, NJ, USA). HPLC grade reagents such as formic acid, acetic acid, isopropanol, ethyl acetate, methyl *tert*-butyl ether and ammonia water were provided by Shandong Yuwang Industrial Co., Ltd. (Yucheng, China). Distilled water prepared with demineralized water was used throughout the study.

<Figure 1>

2.3 Instruments and LC-MS/MS Conditions

The assay was carried out on a Prominence™ LC-20A UFLC XR system equipped with the following components: a binary pump, a degasser, a thermostatted auto sampler and a thermostatted column compartment (Shimadzu, Japan), and a 4000 QTRAP™ linear ion trap triple stage quadrupole tandem mass spectrometer equipped with a turbo ion spray (TIS) source (AB Sciex, Foster City, CA, USA). Instrument control, acquisition and quantification of data were all operated using the Analyst software (version 1.5.2, AB Sciex, USA). Benzoylmesaconine, piperine, and IS were separated on a Venusil MP C18 column (100mm×2.1mm, 3.0 μm) at 30 ºC. The mobile phase consisted of 0.1% formic acid in water (A) and methanol (B) at a flow rate of 0.4 mL/min in a total run time of 7.0 min. The UFLC gradient program was as follows: 80% A 0-0.5 min; 80%-25% A 0.5-1.7 min; 25%-10% A 1.7-1.8 min; 10%-2% A 1.8-3.0 min; 2% A 3-4 min; 80% A 4-7 min. The autosampler was set at 4 ºC with an injection volume of 2 μL. The analytes and IS were measured in the multiple reaction monitoring mode (MRM) using electrospray ionization (ESI) operated in the positive-ion mode under the following source conditions: curtain gas, 20 psi; gas 1, 50 psi; gas 2, 50 psi (all gases: nitrogen) with a source temperature of 500 ºC; ion spray voltage, 5500 V. Quantitative parameters are listed in Table 1.

<Table 1>

2.4 Sample Preparation

2.4.1 Preparation of administration solution

In the study, the dosage of experimental groups were as follows: AKRC group was administrated with AKRC 1.5 g/kg; following original ratio, the AKRC-PLF group was administrated with AKRC 1.5 g/kg-PLF 0.9 g/kg; the Naru-3 group was administrated with 1.5 g/kg, 0.9 g/kg and 3 g/kg for AKRC, PLF and CF respectively. AKRC contains 0.11 % benzoylmesaconine and PLF contains 2.85% piperine. So the dose of benzoylmesaconine and piperine was 1.65 mg/kg and 25.65 mg/kg. The dosage has also been used in the previous pharmacodynamic experiment and has been proved effective. The herb powder were evenly mixed, dissolved in 0.5% CMC-Na solution and then supersonicated for 15 min to make the final intragastric administration suspension at the dose of 10 mL/kg.

2.4.2 Preparation of Standard Solutions and Quality-Control Samples

The stock solutions of benzoylmesaconine and piperine were prepared in methanol at the concentration of 1.0 μg/mL and 10 μg/mL for each. They were further diluted with methanol to make a series of mixed working solutions at the concentration of 0.3-60 ng/mL for benzoylmesaconine and 20-4000 ng/mL for piperine and quality control (QC) solutions at three levels containing 0.75, 6, 48 ng/mL for benzoylmesaconine and 50, 400, 3200 ng/mL for piperine. The working solution of IS was 1.0 μg/mL. All the stock solutions were stored at 4 ºC.

The calibration standard samples of benzoylmesaconine (0.075, 0.15, 0.75, 1.875, 3.75, 7.5, 15 ng/mL) and piperine (5, 10, 50, 125, 250, 500, 1000 ng/mL) were prepared by adding appropriate amounts of the mixed working standard solution and IS working solution (10 μL) to blank rat plasma (100 μL).

Daily QC samples at three levels were prepared in the same process (0.187, 1.5, 12 ng/mL for benzoylmesaconine and 12.5, 100, 800 ng/mL for piperine).

2.4.3 Preparation of rat plasma samples

After thawing the plasma samples at room temperature for 30min and vortexing for 30 s, 10 μL of IS and 50 μL 10% ammonia solution *v*/*v* were spiked into plasma samples (100 μL) by vortexing for 30 s, and then the mixture was extracted with 1 mL of methyl *tert*-butyl ether by vortexing for 3 min. After centrifuging at 13000 *g*, 4 ºC for 5 min, the upper organic phase was transferred to a clean centrifuge tube and evaporated to dryness under a gentle stream of nitrogen at 35 ºC. The residue was reconstituted in 100 μL methanol by vortex-mixing for 1 min, supersonicated for 3 min then centrifuged at 13000 *g* for 5min. Finally, 2 μL supernatant was injected into the UFLC-MS/MS system for analysis.

2.5 Method validation

The method was validated following the European Medicines Agency (EMA) guideline on bioanalytical Method Validation[24] and the currently accepted US-FDA Bioanalytical Method Validation Guidance.[25] Method validation was with respect to selectivity, lower limit of quantification, calibration curve, accuracy and precision, recovery, matrix effect and stability.

2.5.1 Selectivity

The specificity was verified by comparing chromatograms of blank plasma from six different rats, with blank plasma spiked with benzoylmesaconine, piperine and IS, and plasma samples obtained 20 min after oral administration of Naru-3 pill.

2.5.2 Linearity and sensitivity

The calibration curves of the UFLC-MS/MS method for the test compounds were evaluated by analyzing a series of standard plasma samples at concentrations 0.075-15 ng/mL for benzoylmesaconine and piperine 5-1000 ng/mL using least-square linear regression of two analytes-to-IS peak area ratios versus the normalized concentration of the calibration standard with a weighted factor ($1/x^2$). The lower limit of quantification (LLOQ) were defined as the lowest plasma concentration which gave a signal to noise ratio (S/N) >10 as well as an acceptable accuracy within ±20% and the precision below 20%.

2.5.3 Accuracy and precision

The intra-batch accuracy and precision were evaluated by analyzing five replicates at three QC levels of the same batch. The inter-batch precisions were carried out by analyzing the respective QC samples between three different batches.

2.5.4 Recovery and matrix effect

The recoveries of the two analytes were determined by comparing the peak area ratios obtained from rat plasma samples spiked with the known amount of standards with those obtained from the true concentrations of pure standard solutions at three QC levels. The recovery of IS from rat plasma was evaluated at a concentration of 1 μg/mL for brucine by the same method.

The matrix effect of the two analytes was measured at three QC levels by comparing the peak response in the presence of matrix (calculating by analyzing blank plasma extraction with analytes) to the peak response of pure standard solutions. The IS was measured in the same way.

2.5.5 Stability

Several different storage conditions such as at room temperature for 12 h, at -20 ºC for at least 7 days, after three freeze-thaw cycles and 8 h after prepared at 4 ºC were chosen to evaluate the stability in plasma at three QC levels.

2.6 Pharmacokinetic study

The animals fasted for 12 h with free access to water prior to the oral administration of experimental drugs. The eighteen rats were divided randomly into 3 groups and orally administrated with different suspension, respectively. Animal blood (approx. 0.3 mL) were collected from the suborbital vein and transferred into heparinized tubes before administration and at 0.167, 0.33, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 36, 48 h after administration, and then immediately centrifuged at 12000 *g* for 5min. The supernatant was transferred into clean tubes and stored at -20 ºC for later analysis.

The pharmacokinetic parameters (AUC, C_{max}, $T_{1/2}$ and T_{max}) of the analytes were calculated by DAS 2.1 software package (Chinese Pharmacological Society). Comparison of pharmacokinetic among 3 groups was performed by SPSS 16.0 (SPSS Inc. Chicago, IL, USA), using independent samples t-tests after their natural logarithmic transformation test for AUC, C_{max}, nonparametric statistical test (Mann-Whitney U test) for $T_{1/2}$ and T_{max}. $p<0.05$ was considered statistically significant difference for all the tests. All data were presented as mean ± SD.

3 Results and discussion

3.1 Preparation of rat plasma samples

A simple and adequately stabilized sample preparation method is the premise for the development of a new method. Liquid-liquid extraction (LLE) technique was applied in the study. Several extractants such as isopropanol, ethyl acetate, methyl *tert*-butyl ether and isopropanol-ethyl acetate in different ratios (1:1, 1:2, 2:1, *v/v*) had been tested in the study. Methyl *tert*-butyl ether could increase the recovery of benzoylmesaconine [from 30.23% (isopropanol-ethyl acetate 2:1, *v/v*) to 59.11%], which was helpful for the study. Meanwhile methyl tert-butyl ether required the shortest time for dryness. The two analysts and IS all belonged to alkaloids, so appropriate pH and organic solvent played a vital role in sample preparation. The alkaline condition could increase the recoveries of the analysts. In our preliminary experiment, 10%, 20%, 50% ammonia solution [*v/v*, prepared by $NH_3 \cdot H_2O$ (25% g/g) and H_2O], and 0.5 M/L NaOH were spiked to the plasma respectively, and the result showed that ammonia solution exhibited high recoveries for both of the two analysts and the concentrations of $NH_3 \cdot H_2O$ has just a little influence on the improvement of the recoveries for the two analysts. For example, the recovery ranged from 68.22% to 59.85% for benzoylmesaconine after addition 10%, 20% and 50% ammonia solution. On the contrary, NaOH solution showed a very low recovery for benzoylmesaconine. It might attribute to the instable structure of benzoylmesaconine, which could lead to a structure broken in strong alkaline condition. Finally, addition of 50 μL of 10% ammonia solution and 1 mL of methyl tert-butyl ether into 100 μL plasma was selected for the sample preparation for the high recoveries for both of the two analysts.

3.2 LC-MS/MS optimization

The mobile phase played a critical role in achieving good chromatographic behavior and appropriate ionization. In consideration of the properties of the analysts, addition of 0.1% formic acid enhanced the sensitivity and improved the peak shape compared with no additives. Methanol-water system was chosen as the organic modifier due to higher response and lower background noise than acetonitrile. A pair of precursor and product ion with steady and high response were chosen to quantitate the

analytes and IS in ESI positive ion mode and the full-scan mass spectrums of the analysts and IS after direct injection into the mass spectrometer were obtained (Figure 2). Some parameters such as gas 1, gas 2, curtain gas, DP, EP, CE and CXP were optimized in the meantime. The other parameters were adopted for the recommended value of the instrument.

In pharmacokinetic study, a proper IS is necessary to control extraction, injection, and ionization variability. In the study, matrine, diazepam, berberine, and brucine were tested as IS, and brucine was eventually chosen due to its stable recovery, appropriate chromatographic retention time, and ionization response, which were similar to those of the analytes in positive mode.

<Figure 2>

3.3 Method validation

3.3.1 Selectivity

There was no endogenous interference observed from the blank plasma at the relevant retentions. The retention times were about 2.77, 3.52 and 2.42 min for benzoylmesaconine, piperine and IS, respectively (Figure.3).

<Figure 3>

3.3.2 Linearity and LLOQ

Calibration curves of two analytes (0.075-15 ng/mL for benzoylmesaconine and 5-1000 ng/mL for piperine) were established by weighted ($w = 1/x^2$) linear regression analysis. The parameters of regression equations of the analytes were as follows: y=4.99 × $10^{-3}x$ + 0.10 × 10^{-4}(r=0.9961) for benzoylmesaconine; y=6.768 × $10^{-2}x$ + 2.822 × 10^{-1}(r=0.9962) for piperine. x stood for the concentration of analytes in plasma (ng/mL); y represented the peak area ration of analytes and IS. The LLOQ of analytes in plasma were 0.075 ng/mL with RSD 4.34% and RE 1.3% for benzoylmesaconine and 5 ng/mL with RSD 3.08% and RE -7.8% for piperine.

3.3.3 Precision and accuracy

The intra- and inter-day precision (presented as RSD) and accuracy (presented as RE) were evaluated by replicate analysis at least five QC samples at three different levels. All the data are shown in Table 2. The results for both benzoylmesaconine and piperine were all within acceptance criteria, indicating that the accuracy and precision of the method satisfied the stipulation of bioanalytical method validation.

<Table 2>

3.3.4 Recovery and matrix effect

The mean absolute recoveries of benzoylmesaconine and piperine were all more than 60% at different concentration levels (Table 2), and the mean recovery of IS was 70.3%.

The matrix effect of the analytes was evaluated by the RSD of the IS-normalized MF which were all no more than 15% (Table 2).

The results indicated that the process of extraction was stable and efficient, and the endogenous has no effect on the quantification of both the two analytes.

3.3.5 Stability

The results of stability experiments of benzoylmesaconine and piperine in rat plasma under various conditions are listed in Table 3. Room temperature for 12 h, -20 ºC for at least 7 days, after three freeze-thaw cycles and 8 h after prepared at 4 ºC were chosen to evaluate the stability in plasma at three QC levels.

<Table 3>

3.3.6 Pharmacokinetics study

The validated method was successfully applied to the comparative pharmacokinetic study of benzoylmesaconine and piperine in rat plasma after administration of different compatibility-composition groups mentioned above. The mean concentration-time curves are illustrated in Figure 4. The pharmacokinetic parameters are given in Table 4.

According to Table 4, for benzoylmesaconine, when administration of AKRC combined with PLF, the AUC increased significantly, but the C_{max} decreased remarkably. The $T_{1/2}$ became slightly longer than administration of AKRC powder alone and the T_{max} changed barely. After mixing the CF powder to prepare the Naru-3 suspension, the AUC of benzoylmesaconine was reduced obviously, and the T_{max} prolonged significantly comparing with Group A.

Numerous studies have reported that piperine, which is the main medicinal ingredient of PLF, could enhance bioavailability of many drugs by inhibiting P-gp[13] or cytochrome P.[26-27] Therefore it might contribute to the increase of $AUC_{(0-t)}$, $AUC_{(0-\infty)}$ of benzoylmesaconine in group B.

In traditional Mongolian medicine, application of AKRC combined with CF was commonly used to reduce the toxicity of AKRC. An existing popular view for the explanation of the compatibility mechanism is that the acid compounds in CF could peculiarly conjunct with the alkaline compounds in AKRC. Thus, the decrease of benzoylmesaconine in $AUC_{(0-t)}$, $AUC_{(0-\infty)}$ and C_{max} after administration of Naru-3 suspension might be caused by CF. The similar compatibility could also be found in the TCM such as the Aconiti Lateralis Radix Praeparata - Glycyrrhizae Radix et Rhizoma herb-pair.[28]

<Table 4>

<Figure 4>

The variation of pharmacokinetics parameters of piperine were not the same as benzoylmesaconine. Comparing group C with group B, the $AUC_{(0-t)}$, $AUC_{(0-\infty)}$ and C_{max} of piperine increased distinctly, and the $T_{1/2}$ and T_{max} shortened. Piperine is a weak alkaloid with pKa 2.4, hence

it might lead to that the conjunction of the constituents in PLF and CF might not be as tight as that in the AKRC and CF combination, and some studies have proved that CF could increase gastric emptying time.[29] This might also have an influence on the altering of piperine pharmacokinetic parameters.

More than one plasma concentration peak could be found obviously from Figure 4. Previously study confirmed that some drugs could store in tissues when the concentration of the drug in plasma was much lower than that of tissues. It could transfer from tissues to plasma, and this might cause another absorption peak in plasma.[30] Meanwhile intrahepatic circulation could also be responsible for the appearance of the second peak. Moreover, herb powders were the experimental material in the study. As a consequence, the constituents in deep layer dissolving from herb powders would take more time than that in surface layer.[31] It might be another conceivable reason for the multimodal phenomenon.

4 Conclusion

In summary, a simple, specific and sensitive LC-MS/MS method has been developed and completely validated with a satisfactory selectivity, precision, stability and a simple sample preparation for simultaneous determination of benzoylmesaconine and piperine in rat plasma after oral administration of Naru-3 pill. The method was successfully employed to a comparative pharmacokinetic behavior of benzoylmesaconine and piperine in rat plasma after administration of different components combination of Naru-3 for the first time. The results indicated that the absorption of benzoylmesaconine increased when AKRC was applied combined with PLF, comparing with administration of AKRC herb powder alone, whereas the absorption decreased in Naru-3 group. On the contrary, the absorption of piperine in Naru-3 group was greater than that of the group with administration of AKRC combined with PLF. The chemical and pharmacology properties of compositions of AKRC, PLF and CF would have a key influence on the change of pharmacokinetic behavior of benzoylmesaconine and piperine in different components combination

of Naru-3. The study could clarify the compatibility mechanism of Naru-3 decoction, and would be helpful to the application of Naru-3 pill for treatment of rheumatoid arthritis.

Acknowledgement

This study was financially supported by Liaoning Innovative Research Team in University (LNIRT, Grant No. LT2013022).

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Figure Legends:

Figure 1 Chemical structures of benzoylmesaconine (a), piperine (b), and IS (c).

Figure 2 Full scan product ion spectrums of benzoylmesaconine (a), piperine (b) and IS (c).

Figure 3 MRM chromatograms of a blank plasma sample (a), a blank plasma sample spiked with benzoylmesaconine, piperine and IS (b) at 0.75 ng/mL, 50 ng/mL, 1.0 μg/mL, respectively, and a plasma sample 0.33 h after oral administration of Naru-3 suspension (c). MRM transitions named m/z 590.4→105.1, 286.2→201.1 and 395.2→324.2, for benzoylmesaconine, piperine, and IS, respectively.

Figure 4 Mean plasma concentration-time profiles for benzoylmesaconine (a),(b) and (c), piperine (d) and (e) in rat plasma after oral administration of different powder suspensions (n=6). Group A for AKRC group; Group B for AKRC-PLF (1:0.6); Group C for AKRC-PLF-CF (1:0.6:2). Insets show initial 1 h profiles for the two analytes.

Table 1

List of selected MRM parameters, declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP) for each analyte and IS.

Analyte	Transitions	DP	EP	CE	CXP
Benzoylmesaconine	590.4→105.1	120	10	60	10
Piperine	286.2→201.1	110.8	10	27.5	12.6
Brucine (IS)	395.2→324.2	125.2	10	43.3	20.6

Table 2

Summary of accuracy, precision, recovery and matrix effect of benzoylmesaconine and piperine in rat plasma (n=6).

Analytes	Concentration (ng/mL)	Intra-day RSD%	Inter-day RSD%	Accuracy RE%	Recovery (%,mean±SD)	Matrix effect (RSD, %)
benzoylmesaconine	0.187	3.7	4.3	-7.18	68.14±5.3	4.9
	1.5	1.9	3.5	-8.3	73.27±2.4	4.9
	12	3.6	6.9	-5.69	75.70±3.4	6.3
piperine	12.5	1.5	5.5	13.4	85.0±2.6	3.9
	100	1.9	2.4	-9.1	88.6±2.4	12.3
	800	1.1	0.4	-13.0	90.5±1.6	10.7

Table 3

Summary of stability of benzoylmesaconine and piperine under various storage conditions (n=6).

Condition	Benzoylmesaconine			Piperine		
	concentration (ng/mL)	RE(%)	RSD(%)	concentration (ng/mL)	RE(%)	RSD(%)
Room temperature for 12 h						
	0.187	-3.2	3.6	12.5	12.5	1.2
	1.5	-9.5	1.4	100	-14.3	1.0
	12	-6.2	1.7	800	-9.6	2.4
Frozen for 7 days						
	0.187	2.7	2.4	12.5	13.3	3.8
	1.5	-3.4	1.9	100	-5.3	1.8
	12	-7.4	3.8	800	-6.5	1.5
Three freeze-thaw cycles						
	0.187	-2.5	10.8	12.5	9.8	4.7
	1.5	4.3	1.5	100	-3.7	3.4
	12	-2.9	1.6	800	-6.4	13.8
4 ºC in autosampler for 8h in processed samples						
	0.187	-3.3	8.8	12.5	5.0	9.2
	1.5	4.5	2.3	100	2.4	2.5
	12	-6.3	5.5	800	-12.5	7.7

Table 4

Pharmacokinetic parameters of benzoylmesaconine and piperine in rat plasma (n=6) after oral administration three compatibility-composition suspensions designed according to Naru-3 pill.

Data	Benzoylmesaconine			Piperine	
	Group A[1]	Group B[2]	Group C[3]	Group B[2]	Group C[3]
$AUC_{(o-t)}$ (ng h/mL)	36.28±4.86	42.73±12.55*	30.33±4.94#	3764±724	5738±1159#
$AUC_{(o-\infty)}$ (ng h/mL)	39.72±7.84	49.58±12.97	33.75±4.70#	3842±724	5883±1227#
C_{max} (ng/mL)	5.75±1.58	3.25±0.52*	4.58±1.61	382.1±72.8	446.6±91.7
$T_{1/2}$ (h)	13.90±4.27	16.91±3.17	14.35±2.92	8.56±1.03	8.40±1.30
T_{max} (h)	0.29±0.08	0.25±0.10	0.5±0.00*, #	0.38±0.16	0.25±0.10

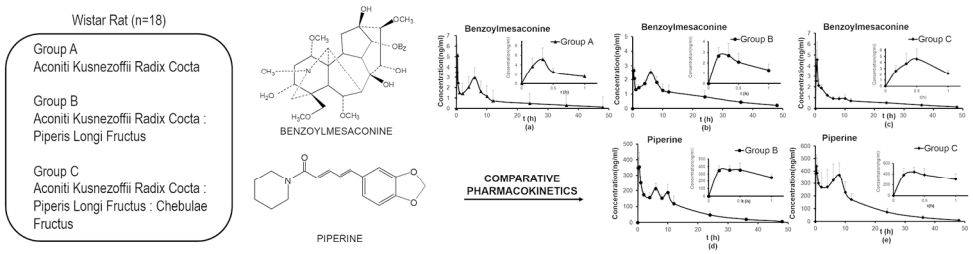
* means compare with Group A, $p<0.05$

means compare with Group B, $p<0.05$

[1] AKRC

[2] AKRC-PLF (1:0.6)

[3] AKRC-PLF-CF (1:0.6:2)



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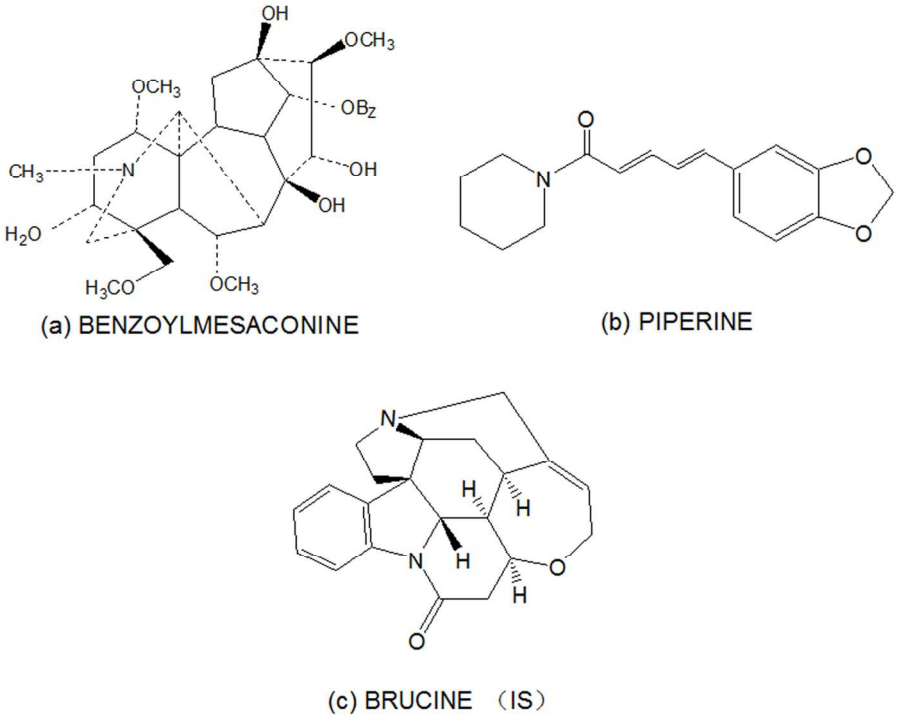

Figure 1 Chemical structures of benzoylmesaconine (a), piperine (b), and IS (c).
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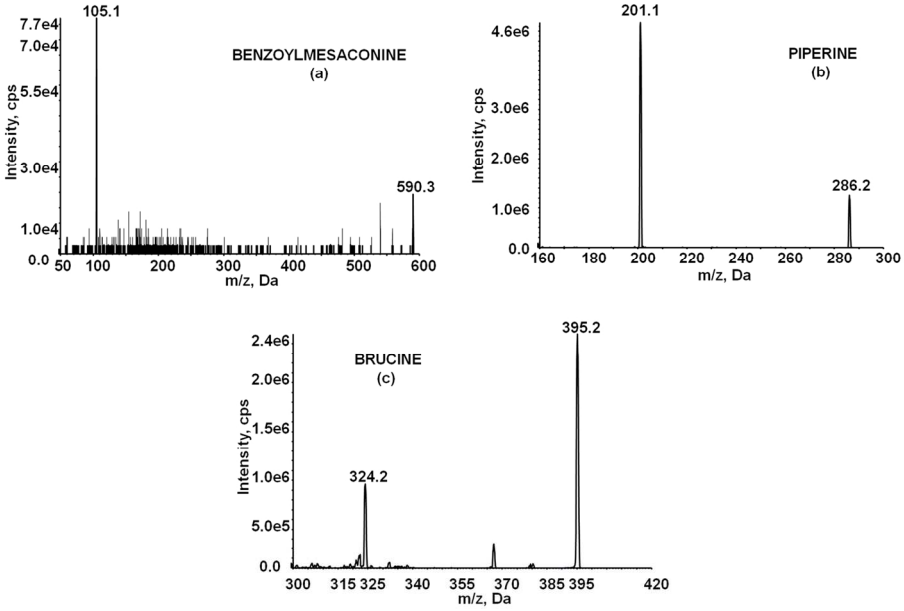

Figure 2 Full scan product ion spectrums of benzoylmesaconine (a), piperine (b) and IS (c).
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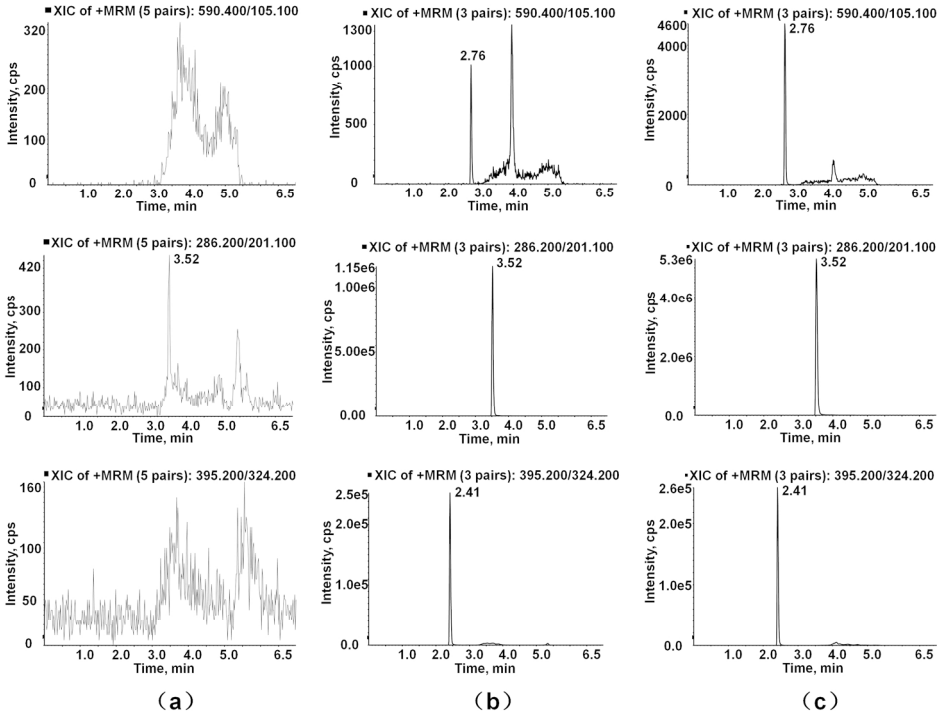

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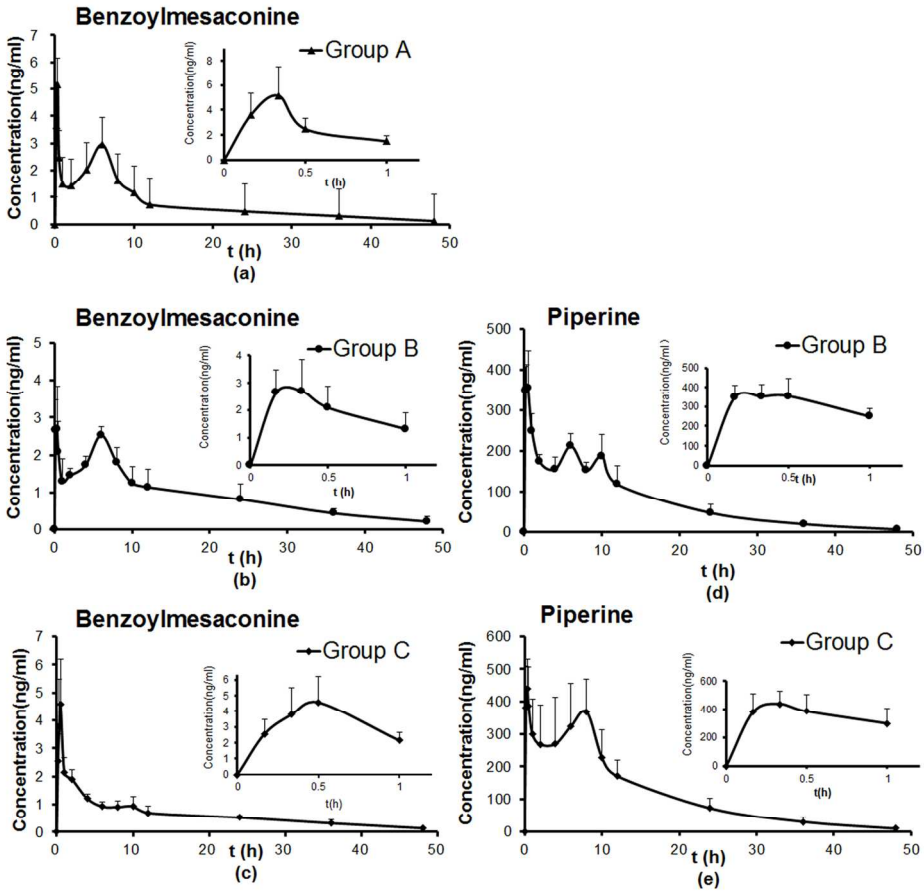

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162x154mm (300 x 300 DPI)