An integrated strategy using UPLC-QTOF-MS^E and UPLC-QTOF-MRM (Enhanced target) for pharmacokinetics study of wine processed Schisandra Chinensis fructus in rats

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Abstract

Currently the pharmacokinetic (PK) research of herbal medicines is still limited and facing critical technical challenges on quantitative analysis of multi-components from biological matrices which often accompanied by lacking of authentic standards and low concentration. This present work contributes to the development of an integrated strategy for extensive pharmacokinetics assessments, and a selective and sensitive method independent of authentic standards for multi-components analysis based on the use of ultra-performance liquid chromatography/ quadrupole-time-of-flight/MS^E (UPLC-TOF-MS^E) and UPLC-TOF- MRM (Enhanced target). Initially, phytochemicals were identified by UPLC-TOF-MS^E analysis, subsequently the identified components were matched with authentic standards and pre-classified, and UPLC-QTOF-MRM method optimized and developed. To guarantee reliable results, three rules are necessary: (1). detection with a mass error of less than 5 ppm; (2). same class chemical compositions with structural high similarity between analytes with and without authentic reference substance; (3). a matching retention time between TOF- MRM mode and TOF-MS^E within 0.2 min. The developed and validated method was applied for the simultaneous determination of 12 lignans in rat plasma after administered with wine processed Schisandra Chinensis fructus (WPSCF) extract. Such an approach was found capable of providing extensive pharmacokinetic profiles of multi-components absorbed into blood after oral administrated with WPSCF extract. The results also indicated that significant difference in pharmacokinetics parameters of dibenzocyclooctadiene lignans was observed between schizandrin and gomisin compounds. For lignans, the absorption via gastrointestinal tract were all rapid and maintained relatively long retention time, especially for schisantherin A and schisantherin B with higher plasma exposure.

Keywords: herbal medicine; pharmacokinetics; QTOF- MRM; QTOF-MS^E; wine processed Schisandra Chinensis fructus

1. Introduction

Herbal medicine has a long therapeutic history over thousands of years and is currently still serving many of the health needs of a large population of the world [1]. Numerous studies have demonstrated that herbal extracts as a whole or multiple herbs in complex formulations offer better efficacies than equivalent doses of individual active ingredients when used alone [2]. Over the last decade, this mono-substance therapy model has gradually shifted toward the adoption of combination therapies, in which multiple active components are employed [3]. It is reasonable to expect that the inspired investigation for multi-components, multi-targets and multi-mode of action will be of great attraction in the future drug discovery.

Moreover, pharmacokinetics (PK) of multi-components is initial and important for our understanding of potential multi-target treatment approaches. As it is well known that PK properties plays a significant role throughout the development of new drug. Unfortunately, the current research on herbal PK is still limited to a few components which pure substances often could be isolated and obtained.

The herbal pharmacokinetics requires that the *in vivo* dynamics process of the complex system of multi-components should be comprehensively characterized. Conventional methods for quantification and PK studies rely mainly on HPLC-MS, HPLC-MS/MS, or UPLC-MS/MS technologies. Under this condition, the bottleneck formed largely due to lack of pure reference substances.

Currently, LC-coupled to triple-quadrupole mass spectrometry (QqQ-MS), operating in multiple-reaction monitoring (MRM) mode and this technique has been the first choice for quantitative analysis because of its high sensitivity and selectivity

against a complex matrix background [4-7]. The limitations of QqQ-based method are obvious, such as low-resolution (LR), inability for untargeted analysis, and dependencies on reference substances. High-resolution (HR) MS, e.g., time-of-flight (TOF) or tandem quadrupole-TOF (QTOF) can provide specificity based on accurate mass analysis [8, 9] and has the capability to resolve these limitations of LRMS. The use of HRMS enables rapid, selective, and robust analysis in both qualitative and quantitative applications. Several studies have been performed in recent years using TOF-MS [10], Orbitrap-MS [11], and TOF-MS^E [12, 13] mode. Nandkishor S. Chindarkar *et al.* evaluated a QTOF-MS in five different modes and compared their quantitative performance for small molecule. Their results showed quantitative data obtained by TOF-enhanced target and MS^E performed better than the other modes [14].

In this study, we proposed a strategy appropriate for comprehensive pharmacokinetics study using UPLC-QTOF-MS^E and UPLC-QTOF- MRM (Enhanced target) (Fig 1.). This novel strategy would opens up the possibility of expanding PK profiles of TCM when often facing quantitative difficulties of lacking corresponding reference substances. To guarantee reliable results, three rules are necessary for approaches based on UPLC-QTOF-MRM: (1). detection with a mass error of less than 5 ppm [15]; (2). same class chemical compositions with structural similarity between analytes with and without authentic reference substance; (3). a matching retention time between QTOF-MRM mode and QTOF-MS^E within 0.2 min. Initially, MS^E data collection mode adopted is helpful to identify components of herbal medicines with high mass resolution for precursor and fragmentation ions. Subsequently pre-classification of identified components carried out ensures the subsequent determining error of these compounds without reference substances to

minimum. UPLC-QTOF-MRM (Enhanced target) is adopted in quantitative analysis for its sensitivity advantage. In this mode, a precursor ion is selected and the TOF pusher is synchronized with the precursor or a product ion to maximize duty cycle for a target m/z range. This 100% duty cycle should result in an increase in response, sensitivity and selectivity. It is appropriate for low concentration analysis of multi-components in complex biological specimens and applied in pharmacokinetics of herbal medicine.

As a famous traditional Chinese medicine (TCM), Schisandra Chinensis fruceetus (SCF) is commonly used as herbal medicine in many oriental countries and officially listed in different pharmacopoeias [16-18], used for chronic cough and asthma, enuresis, frequency of urinator, palpitation and insomnia [16]. In order to meet the need of clinical application, TCM is usually used after processing as decoction pieces called Yinpian in Chinese for increasing diversity, or reducing the toxicity of crude drugs [1, 2]. SCF was used commonly after special processing methods with wine, honey, and vinegar for clinic treatment [19]. Thousands of years of clinical practice showed that SCF through different processing methods, the focal points of therapy have changed. After steamed with rice wine, the effect of nourishing Yin and tonifying kidney strengthened, the efficacy of insomnia exhibited more superiority [20]. Although ancient practitioners recognized these conversions for therapeutic effects in processed TCM, the possible mechanisms are rather complex and not fully understood. For the wine processed SCF, little relevant knowledge was acquired. Pharmacokinetic (PK) profiles are the basic and first step for better understanding the advantage and particularity of TCM processing on pharmacological and clinical effects.

The current methods for determining lignans in *Schisandra chinensis* or in biosamples include HPLC [21,22], LC-MS[23], and UFLC-MS/MS [24,25]. These methods measured several various lignans based on UV or triple-quadrupole mass spectrometry detector limited by unavailable reference substances. Considering the similar structure characteristics of lignans ingredients in *Schisandra chinensis*, especially for many isomer constituents, we developed this rapid, sensitive and reliable method based on UPLC-QTOF-MS^E and UPLC-QTOF-MRM, which the high resolution mass information was obtained and the potential quantitative capacity of UPLC-QTOF was validated. We applied this strategy to pharmacokinetics study of wine processed *Schisandra Chinensis* fructus in rats and obtained more comprehensive PK profiles. Compared to previous reports, the pharmacokinetics information of 4 or 6 lignans of SCF [25, 26], in this research, the PK profiles of major 12 lignans components are acquired simultaneously firstly and can be used for further studies. More importantly, the integrated strategy addressing the critical problems underlined in the field of herbal medicine pharmacokinetics could provide more holistic information for the other TCM and herbs studies.

2. Materials and methods

Chemicals and materials

SCF originated from Benxi City, Liaoning Province were purchased from Yifeng Pharmacy. Reference standard schisandrin, Schisandrol B, Schizandrin A, Schisandrin C, SchizandrinB, Schisantherin A and internal standard bifendate (IS) (>99% purity) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Schisantherin B, Gomisin D, Angeloylgomisin H and Gomisin J (>99% purity) were purchased from Chengdu Must Bio-technology co., Ltd. Gomisin G (>99% purity) were purchased from Chengdu Push Bio-

technology co., Ltd. Acetonitrile, (HPLC grade), was purchased from Fisher Scientific (Fairlawn, NJ, USA). Formic acid (for MS, $\geq 99\%$) was purchased from Sigma-Aldrich Co.(St Louis, USA). Other reagents all were of analytical purity. Deionized water was prepared by Milli-Q ultrapure water system (Millipore, Milford, MA).

Animals and treatments

Male pathogen-free Wistar rats ($n=6$, 180-200 g) were provided by the Hunan Silaikejingda Laboratory Animal Co., Ltd. (Changsha, PR China). These animals were housed under controlled conditions ($22 \pm 2^\circ\text{C}$, RH $50 \pm 20\%$) with a natural light-dark cycle for 3 days before the experiment was carried out. All the rats were fasted with only water provided for 12 hours prior to experimentation. All procedures were performed in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China. Wine processed SCF extract was solved in 0.5% sodium carboxymethyl cellulose (CMC-Na) to get an evenly dispersed suspension and was orally administrated to rats at 8.8g/kg.

Liquid chromatography conditions

Chromatographic separation was achieved on a Welch Ultimate[®] UHPLC XB-C₁₈ Column (1.7 μm , 100×2.1 mm) using 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The column temperature was maintained at 40°C , the autosampler temperature was set at 4°C , and the injection volume was 2 μL . The mobile phase (delivered at 0.4 mL/min) consisted of solvent A and solvent B. Elution conditions were as follows: 0-5min, B from 20% to 30%; 5-15min, from 30% to 60%; 15-20 min, from 60% to 90%; 20-23 min, maintained 90%; 23-25 min, from 90% to 20%. The total run time was 25 min.

Mass spectrometry conditions

Qualitative and quantitative determination was performed using Waters Acquity I-CLASSTM (Waters Corporation, Milford, MA, USA) UPLC coupled with Synapt G2-SiTM (Waters Corporation, Manchester, UK) tandem mass spectrometry.

The mass spectrometer operated in MS^E mode from 100 to 1000 Da with a 0.2 s scan time, using a desolvation temperature of 600°C, source temperature of 120°C, and cone voltage of 40 V. The mass spectrometer was calibrated across the mass range 100-1500 Da using a solution of sodium formate. Data were centroided in positive mode and mass corrected during acquisition using an external reference (Lock-SprayTM) comprising a 1 µg/mL solution of leucine enkephalin infused at 5 µL/min which was sampled continuously at 30s intervals generating a reference ion at 556.2771. The MS^E mode comprises two interleaved full scan functions, one acquired under low-energy conditions (function 1) set 4V to obtain accurate mass data for intact precursor ions and one acquired under high-energy conditions (function 2) with a ramp of 20-50 V to obtain product ions and corresponding accurate mass data.

The ESI source parameters in MRM (Enhance target) mode were as MS^E mode. Mass spectrometric parameters, viz. the cone voltage (V) and collision energy (CE), for each analyte are listed in **Table 1**. The MRM scan padding and isotope cluster were used.

Preparation of wine-processed SFC and its extract

The dried SFC (100 g) were mixed with rice wine (20 g) then moistening for 1 h. When the wine was completely absorbed, the mixture was steamed 3h with the color of the crude surface was purplish black or dark brown. The final product obtained after desiccating thoroughly at 60°C.

The dosing solutions used for all animal studies were prepared by the wine-processed SFC (100g), which was extracted with 95% ethanol at reflux for 2 h 3 times. After concentration in vacuum, the extract was subsequently dried under reduced pressure conditions, which was the intragastric solution. The wine-processed SFC extract (WPSFC-extract) was stored in the refrigerator at 4 °C.

Preparation of WPSFC-extract sample solution

The WPSFC-extract sample was accurately weighed (1 g), and transferred into 25 mL volumetric flasks. They were extracted with 25 mL of methanol in an ultrasonic bath for 30 min at room temperature. The solid particles were filtered from the solution and methanol replenished; then the solution 1mL was transferred into 5mL volumetric flasks following 1mL bifendate (IS) solution (5 µg/mL), and additional methanol were added to make up to the volume. Then it was centrifuged at 16,000 rpm for 10 minutes. An aliquot of 2 µL of the final testing samples was injected onto the UPLC–QTOF-MS^E system for analysis.

Preparation of standard solution and quality control samples

Accurately weighed standards were dissolved in methanol to prepare stock solutions consisting of 200 µg/mL for schisandrin, SchizandrinB, Schisantherin A and Angeloylgomisin H, 100 µg/mL for schisandrol B, Schizandrin A, Schisandrin C, Gomisin D and Gomisin G, 400 µg/mL for Schisantherin B

A series of working solutions of these analytes was freshly prepared by diluting the mixed standard solutions with methanol in appropriate ratios to yield seven concentrations of 50 – 6250 ng/mL for schisandrin, SchizandrinB, Schisantherin A and Angeloylgomisin H with quality control (QC) setting at 100, 1000, 5000ng/mL; 25-3125 ng/mL for schisandrol B, Schizandrin A, Schisandrin C, Gomisin D and

Gomisin G with QC setting at 50, 500, 2500 ng/mL; 100-12500 ng/mL for Schisantherin B with QC setting at 200, 2000, 10000ng/mL. All solutions were stored at 4 °C.

Preparation of plasma samples

One hundred μ L aliquots of plasma from rats were transferred to a 2.5mL EP together with 20 μ L IS solution, 20 μ L methanol. After vortex shaking for 30s, 1mL ether was added. The analytes and IS were extracted from plasma by vortexing and shaking for 3 min. Then the samples were centrifuged at 15,000 rpm for 10 minutes. The supernatant was quantitatively transferred to a 5 mL glass tube. The eluent was evaporated to dryness under N₂ for about 20 minutes at 45 °C. The residues were reconstituted in 100 μ L acetonitrile and were centrifuged at 16,000 rpm for 10 minutes. An aliquot of 2 μ L of the final testing samples was injected onto the UPLC–QTOF-MRM system for analysis.

Method validation

The assay for 10 lignans compounds in rat plasma which have authentic reference substances was validated in accordance with the guidance for the bioanalytical methods validation [27]. The calibration was performed by least-squares linear regression of the ratio of peak area of each analyte to the IS versus the nominal standard concentration with a weighted factor. The LLOQ was defined as a reproducible lowest possible concentration, linear with the calibration curves having a relative error (R.E. %) below 20 % and accuracy between 80 and 120 %.

To assess the intra-day accuracy and precision of the method, three concentrations levels of QC solution were spiked into plasma, with 6 replicates at each concentration.

The extraction recoveries were determined by comparing the peak area obtained from plasma samples spiked before extraction with those from plasma samples spiked after extraction. The matrix effect was investigated by comparing the absolute peak area of control plasma extracted and then spiked with a known amount of drug to that of neat standard samples at equivalent concentrations. The same procedure was performed for IS.

Stability of QC samples was assessed by analyzing samples stored at -20°C for 1 month, subjected to three freeze (-20°C) thaw (room temperature) cycles, and stored at room temperature for 24 hours. Samples were considered to be stable if the deviation from the nominal concentration was within $\pm 15.0\%$.

Application to pharmacokinetic study

After fasting for 12 hours, six rats for getting blood samples were deprived of food for 12 h with water before the experiment and were administrated the WPSFC-extract at an oral dose of 8.8g/kg. Blood samples (about 150 μ L) were collected in heparinized tubes via the postorbital venous plexus veins from each rat before administration (0 h) and 0.17 h, 0.33 h, 0.5 h, 1.0 h, 2.0 h, 4.0 h, 6.0 h, 8 h, 12 h, 24 h, 36 h, 48 h, 60 h, 72 h after administration, and were immediately centrifuged and stored at -20 °C until analysis.

Data processing

Data acquisition was achieved with MassLynx version 4.1 (Waters Corp., Milford, MA, USA). MS^E data viewer (Waters, Manchester, UK) was used for visualization and alignment of low- and high-energy information. QuanLynx (Waters, Manchester, UK) was used to integrate chromatograms of identified compounds. Statistical analyses were performed using SPSS 11.0 (SPSS Inc., Chicago, IL). Significant differences were considered significant when test p values were less than 0.05. All pharmacokinetic parameters were processed by noncompartmental analysis using DAS 3.0 software (Chinese Pharmacological Society). The elimination half-life ($t_{1/2}$) was determined directly by the graphic method (log c vs. t). Maximum plasma concentration (C_{max}) and the time needed to reach C_{max} (t_{max}) were directly calculated from the plasma concentrations obtained. The area under the plasma-concentration time curve [$AUC_{0-\infty}$] was calculated by the trapezoid method with extrapolation to infinity. This parameter was used to calculate the apparent clearance [$CL/F = \text{dose} / AUC_{0-\infty}$], apparent distribution volume [$V_z/F = CL / \beta \cdot F$], and mean retention time [$MRT_{(0-\infty)} = AUMC / AUC$].

3. Results and discussion

Identification of lignans in WPSFC-extract by UPLC-TOF-MS^E analysis

A positive ESI source was chosen based on preliminary experiments for its stronger response and more ion fragments information. UPLC-QTOF-MS^E detection collects data using two scan functions, which provides a unique way to incorporate fragment ion information without the need for precursor ion selection. Identification was based on the presence of one characteristic m/z ion obtained with the low collision energy function and fragment ions obtained with the high collision energy

function, both with mass errors of less than 5 ppm permitted. The typical total ion chromatograms of SCF and wine-processed SCF were shown in **Fig.2**.

From **Table 2**, in the low energy function, the base peak were $[M+Na]^+$ as parent ions for most dibenzocyclooctadiene lignans, which prone to emerge in-source dissociation. In some mass spectrum, such as schisandrin, schisandrol B, regular $[M+H]^+$ ion was not found, however, special $[M-e]^+$ ion formed by losing a electron. Such free radical ion m/z was even numbers because of neutral molecule missing electron. Here we took representative lignans from five classification groups respectively as examples to describe the fragmentation process in details.

Schisandrin: In the low energy spectrum, it shows two peaks, the sodium adduct ion $[M+Na]^+$ at m/z 455.2045, the radical ion $[M-e]^+$ at m/z 432.2140 . Further confirmation of schisandrin was provided by the high-energy function, which gives a spectrum resulting from fragmentations. At m/z 415.2115 was detected the fragment identified as $[M-H_2O+H]^+$, at m/z 384.1932 we assigned a fragment due to the further loss of methoxy group corresponding to $[M-H_2O-OCH_3+H]^+$. Thus, based on the parent and characteristic fragment ions information, it was identified to schisandrin.

Gomisin D: In the low energy spectrum, it shows two peaks, the protonated adduct ion $[M+H]^+$ at m/z 531.2213, the sodium adduct ion $[M+Na]^+$ at m/z 553.2052 , which elemental composition of $C_{28}H_{34}O_{10}$. Further MS^2 fragmentations showed characteristic product ions at m/z 401.1605 and 383.1497 by losing lactonic ring residue and one molecular water sequentially.

Angeloylgomisin H: eluted at 13.06 min gave an $[M+Na]^+$ ion at m/z 523.2301. In the high energy scan, 483.2318 $[M-H_2O+H]^+$, 401.1869 $[M+H-C_4H_6COOH]^+$ and

383.1853 $[M - C_4H_6COOH - H_2O + H]^+$ were observed as the main characteristic mass fragments.

Schisantherin A: The low-energy spectrum shows a base peak at m/z 537.2097 $[M+H]^+$ and 559.2020 $[M+Na]^+$. At the high energy scan, fragment ions at m/z 415.1673 $[M - C_6H_5COOH + H]^+$ and m/z 371.1404 $[M - C_6H_5COOH - C_2H_4O + H]^+$ were produced by losing side chain of benzoic acid and acetaldehyde sequentially.

Schizandrin A: The molecular ions at m/z 401.1962 $[M+H]^+$, and 423.1683 $[M+Na]^+$ at the retention time of 7.98 min, which elemental composition was $C_{24}H_{32}O_6$. The product ion at m/z 370.1732 was 31 Da less than parent ion indicated $[M - OCH_3 + H]^+$ fragmentation. Besides, there was a characteristic fragment ion $[M+H-CH_3]^+$.

A total of major 16 constituents were identified, which retention time, molecular formula, parent and fragmentation ions information were summarized in [Table 2](#). The maximum mass errors between measured and calculated values were all less than 5 ppm, which guaranteed high resolution and good accuracy.

Match and pre-classify identified components by authentic reference standard

In this study, 10 lignans constituents were confirmed by authentic reference standard (ARS) which defined as I components. The others identified components lacking ARS were II components, including Gomisin E, γ -Schizandrin B. In addition, tigloylgomisin H, angeloylgomisin Q, 6-O-benzoylgomisin-O, and gomisin J were not detected in rat plasma after administration, might mainly due to its low contents in WPSFC-extract or poor oral bioavailability.

Based on the structural similarity rule, the above 12 compounds were classified into five types as group 1 to group 6 (**Fig.3**). Then it showed that Gomisin E, γ -Schizandrin B lacking ARS falls into the category of group 2 and 4 respectively. In subsequent determination on UPLC-QTOF-MRM, these II components can be quantified relying on corresponding I components. Similarity in chemical structure could ensure the extraction efficiency, chromatographic behaviors, and mass spectra responses of these II components are closely to corresponding I components with authentic reference standard. This was precondition and guarantee for accurate determination of compositions lacking ARS.

Determination of 12 lignans in WPSFC-extract by UPLC-QTOF-MRM when compared with RRLC-MS-MS (MRM)

To calculate the dose to be administered, the amounts of 12 lignans in WPSFC-extract solution were quantitatively determined using UPLC-QTOF-MRM. Compared to traditional triple quadrupole mass spectrometer methods, the advantages of using novel UPLC-QTOF-MRM for quantification are obvious, such as high mass resolution capability to achieve selective/sensitivity. Herein, we sought to investigate whether the UPLC-QTOF-MRM technique would be a good choice for the extensive quantity analysis of multiple components. For this purpose, a separate MRM-based RRLC-MS-MS (Agilent 1290 RRLC coupled to 6460 QQQ MS) was developed specifically for comparing the results with UPLC-QTOF-MRM mode of analysis.

Considering the reproducibility and independence of the two methods, different determination conditions applied in LC-MS/MS method. Chromatographic separation was achieved on an AcquityTM UPLC BEH C₁₈ column (1.7 μ m, 100 $\times$ 2.1 mm), (Waters, USA). The mobile phase, elution conditions and mass spectrometric

parameters, viz. the fragmentor voltage (FV) and collision energy (CE), for each analyte are listed in **Table S1**. The 10 lignans contents results of WPSFC-extract quantified by the different two methods were presented in **Table S2**. The typical chromatograms of mixed standard substances and WPSFC-extract solutions are shown in **Fig. S1**. The comparison of results from UPLC-QTOF-MRM and RRLC-MS-MS was possessed by SPSS 16.0 (Statistical Package for the Social Science) using Independent Samples T-test, $p < 0.05$ was considered statistically significant for all the tests. As clearly shown in **Table S2**, the determination results of all the 10 lignans in both assays were no significant differences, suggesting identical contents in WPSFC-extract from both assays.

From the quantitative analysis of 10 lignans (**Fig.3**) with authentic standards, the UPLC-QTOF-MRM provided accurate measurement as RRLC-MS-MS. As have previously described Gomisin E and γ -Schizandrin B lacking ARS as II components (**Fig. 3**), their concentrations could be calculated in accordance with Gomisin D and Schizandrin B calibration curves adopting internal standard (IS) respectively. The following equation applied: II components level (ng/mL) = ((II components / IS peak area ratio) / (corresponding I components / IS peak area ratio)) $\times$ corresponding I components absolute level. The relative quantification was done with peak area ratios of II components and IS. The II components / IS peak area ratios were calculated to reduce variability furthermore. The application of I and II components matching, and furthermore IS correcting, ensured the determination accuracy of those components without pure standards.

Development of the UPLC-QTOF-MRM method

Based on the identification results of lignans by UPLC-QTOF-MS^E analysis, the criteria followed to confirm the presence of 12 components in UPLC-QTOF-MRM were: (1) difference in retention time relative to the adjacent peak must be within 0.2 min for the both analysis modes and (2) mass accuracy must be <5 ppm.

For I compounds, the qualitative ion pairs (precursor ion product ion) and quantitative ion pair (precursor ion product ions) of the analytes were selected based on their full scan mass spectra obtained by directly infusing standard solutions into the mass spectrometer ESI source under resolution mode. Two transitions between the precursor ion and the most abundant product ions were monitored for the identification of each compound, and the ion pair with relatively higher intensity was selected for quantification. To obtain maximum sensitivity of these 10 I compounds, two important parameters viz., the cone voltage (CV) and collision energy (CE) were optimized. The other II components, Gomisins E and γ -Schizandrin B, due to lacking standard solutions, their MRM transitions selection firstly according to fragment ions occurred in QTOF-MS^E, and then under QTOF-MRM mode the response intensity of these various ion pairs compared. Subsequently, the most appropriate MRM transitions for II components were confirmed. In virtue of the high similarity of chemical structure, the CV and CE parameters for II components were adopted in light of corresponding I compounds respectively. The product ion spectra, monitored transitions, optimized CV and CE parameters of the twelve lignans and IS were shown in **Fig.4** and **Table 1**.

Method Validation

The detection of 12 analytes and IS by MRM mode was highly selective with no interference. Typical chromatograms of blank plasma, blank plasma spiked with 10

analytes and IS, samples from rats after oral administration of WPSFC-extract are shown in **Fig.5**. The linearity of the calibration curve was determined by plotting the peak area ratio (y) of analyte to IS versus the nominal concentration (x) of analyte using weighted ($1/x^2$) least square linear regression. The calibration curve test range was found to be adequate for the concentrations observed from the analysis of collected plasma samples, and 10 analytes showed linearity over the low and high concentration range with $r^2 > 0.99$. The linear ranges, regression equations and correlation coefficients are shown in **Table 3**. The LLOQs of analytes in plasma were 5-20 ng/mL for 10 lignans analytes. The intra-day precision, inter-day precision and accuracy results of the tested samples were all within the acceptable criteria ($RSD\% < 15\%$; $RE\%$ less than $\pm 15\%$), shown in **Table 4**, which indicated that the present method had remarkable reproducibility with acceptable accuracy and precision. In the study, $\text{Recovery (\%)} = (\text{Peak area of analyte after extraction} / \text{peak area of analytical standard solution}) \times 100$. The mean extraction recoveries of the analytes from rats plasma were all more than 75.0% at different concentration levels (**Table 4**), and the average extraction recovery of IS was 83.0%. The matrix effects of ten analytes were in the range of 85.9–96.8%, and the matrix effects of IS was 92.4%, indicating a negligible matrix effect on the ionization of the analytes. TOF-MRM mode could provide sensitivity and large linear dynamic range often needed for quantitative studies.

Pharmacokinetic study

For pharmacokinetic of herbal medicines with multiple-components, one bottleneck is that the authentic standards of many herbal components proved very difficult to obtain. The ingredients as secondary metabolites have structural homology in biosynthesis process of medical plant providing a premise to quantitative

determination of compounds similar to those with authentic standards. High-resolution mass, QTOF-MS provides feasibility to deep insights into herbal pharmacokinetics with complex components. However, either QTOF-MS or QTOF-MS^E method is full scan mode, which sensitivity is far less than QQQ-MS, and often cannot satisfy bioanalysis demands of herbal components for low-level exposure *in vivo*. Therefore, we sought to develop a UPLC-QTOF-MRM (enhanced target) assay based methodology achieving high mass resolution and analytical sensitivity for following the pharmacokinetics of main components after herbal ingestions.

Based on the powerful UPLC-QTOF-MRM assay and this approach, the plasma pharmacokinetic profiles of 12 lignans after oral administration of wine processed *Schisandra Chinensis fructus* were successfully characterized (**Fig. 6**). From these plasma profiles, the estimated pharmacokinetic parameters calculated by DAS 3.0 software were listed in **Table 5**. In this study, plasma concentration and PK characteristics of these twelve lignans were firstly simultaneously determined and described. As shown in **Table 5**, **Fig. 7**, there were similarities between their PK parameters, but most were distinctive, suggesting the structurally related different pharmacokinetic properties of lignans components. (1): The 12 dibenzocyclooctadiene lignans were all rapidly absorbed and into blood after oral administration, with the T_{max} values less than 3.5 h; (2): The $T_{1/2}$ and $MRT_{(0-\infty)}$ value of gomisins G, and angeloylgomisin H were much lower than other lignans, indicating they were prone to be metabolized and elimination *in vivo*. Unlike these two gomisin compounds, schizandrin A, schizandrin B, γ -schizandrin B, and schizandrin C were eliminated slowly and had longer dwell time *in vivo*. (3): the differences of V_z/F values between these 12 lignans were more than 60 times, as gomisins G, angeloylgomisin H, schisantherin A, and schisantherin B less than 6 L/Kg, while

gomisin E was 143.2 ± 24.0 L/Kg. The phenomenon indicates the biodistribution of lignans ingredients varies greatly due to their different tissue affinity and selectivity. Compared to schizandrin A, schizandrin B, γ -schizandrin B, this diversity performed significantly in gomisin compounds. (4): Owing to the AUC values were closely related with the administration dose, the AUC/dose values were adopted for better comparison. Three levels were observed for the $AUC_{0-t}/dose$ (gh/L) values of 12 lignans after oral administration, in which gomisin E was poor, gomisin D, gomisin G, Angeloylgomisin H, schizandrin A, schizandrin B, γ -schizandrin B, and schizandrin C were medium, schisantherin A and schisantherin B were relatively best. Besides, compared to raw *Schisandra Chinensis fructus* [25], the absorption phase in rats after oral administration of wine processed extract also exhibited quickly, however, the elimination phase was relatively slowly and residence time was prolonged which brought better oral exposures.

4. Conclusion

Pharmacokinetics plays an important role, and can maximally improve efficiency of developing new drugs. For herbal medicines with complex ingredients, the *in vivo* action investigation needs more precise and comprehensive pharmacokinetic information which is still blocked by the lacking of powerful analytical technology. The lacking of authentic standards and low concentration in biosamples are great and longstanding obstacle on charactering herbal pharmacokinetics. To address this problem, we developed an integrated approach for extensive pharmacokinetics study based on UPLC-TOF-MS^E and UPLC-TOF- MRM (Enhanced target). This novel, rapid and sensitive UPLC-QTOF-MRM method was firstly developed and validated for the simultaneous determination of 12 lignans in rat plasma. Then this approach was successful to obtain the important pharmacokinetic characteristics of multi-

components absorbed into blood after oral administrated with WPSCF extract. Thus, this integrated strategy on addressing the critical problems underlined in the field of herbal pharmacokinetics, will contribute an approach to the highly selective and sensitive determination of target and nontarget components lacking of authentic compounds in the further study of herbal medicine.

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Fig. 1.

Fig.2

Fig.3

Dibenzocyclooctadiene Lignans Structure classification	
Group1	(Schisandrin) (Schisandrol B)
Group 2	(Gomisin D) (Gomisin E *)
Group 3	(Angeloylgomisin H)
Group 4	(Schizandrin B) (γ-Schizandrin B*) (Schizandrin A) (Schisandrin C)
Group 5	(Schisantherin B) (Gomisin G)
Group 6	(Schisantherin A)

Note:* a component lacking authentic reference standard

Fig.4

pos

pos

pos

pos

pos₀

Fig.5

Fig.6

Fig. 7

An integrated strategy for determination of multi-components from biological matrices with or without authentic standards

Table 1 Optimized TOF-MRM parameters analytes

Analytes	Parent ion	cone voltage (v)	CE(eV)	Fragment ions
Schisandrin C	385.1647	40	15	385.1555
Schisandrin	455.2045	40	20	415.2048
Schisandrol B	439.1726	40	20	399.1716
Angeloylgomisin H	523.2301	40	20	401.1849
Gomisin G	559.1939	40	20	415.1685
Schizandrin A	439.2095	40	15	417.2202
Schizandrin B	423.1784	40	15	401.1878
Schisantherin A	559.1836	40	20	415.1637
Gomisin D	553.2046	40	20	401.1515
Schisantherin B	537.2091	40	20	415.1665
Bifenndate(IS)	441.0800	40	25	329.0552

Table 2. Identification of chemical constituents from *Schisandra chinensis* fruit in positive mode

NO.	t _R /min	Identification	Molecular	Parent Ion	ppm	Fragment Ion
1	10.28	Schisandrin	C ₂₄ H ₃₂ O ₇	432.2140[M-e] ⁺ , 455.1999[M+Na] ⁺	-1.9	415.2044, 400.1794, 384.1856, 369.1677
2	11.10	Gomisin D	C ₂₈ H ₃₄ O ₁₀	531.2234[M+H] ⁺ , 553.2052[M+Na] ⁺	-3.2	485.2178, 401.1513, 383.1497, 341.0915,
3	11.36	Gomisin J	C ₂₂ H ₂₈ O ₆	389.1959[M+H] ⁺	1.5	374.1705, 357.1702, 319.1186, 287.0922
4	11.59	Schisandrol B	C ₂₃ H ₂₈ O ₇	439.1647[M+Na] ⁺	-3.0	399.1716, 369.1595, 353.1382
5	13.06	Angeloylgomisin H	C ₂₈ H ₃₆ O ₈	523.2301[M+Na] ⁺	-1.3	483.2318, 401.1869, 383.1853, 359.1125
6	13.36	Tigloylgomisin H	C ₂₈ H ₃₆ O ₈	523.2302[M+Na] ⁺	-1.1	483.2377, 401.1959, 369.1693
7	13.57	Angeloylgomisin Q	C ₂₉ H ₃₈ O ₉	553.2408[M+Na] ⁺	-1.1	431.2059, 400.1864, 387.1794
8	13.87	Gomisin G	C ₃₀ H ₃₂ O ₉	537.2098[M+H] ⁺ , 559.1920[M+Na] ⁺	-3.1	415.1678, 371.1667, 310.1091
9	14.69	Schisantherin A	C ₃₀ H ₃₂ O ₉	537.2097[M+H] ⁺ , 559.2020[M+Na] ⁺	-5.0	415.1673, 371.1389, 340.1193, 325.0963

10	14.79	Schisantherin B	C ₂₈ H ₃₄ O ₉	515.2280[M+H] ⁺ , 537.2091[M+Na] ⁺	-0.2	496.3388, 415.1666, 385.1644
11	15.85	Gomisin E	C ₂₈ H ₃₄ O ₉	515.2241[M+H] ⁺	-3.7	385.1560, 355.1439, 316.0835, 285.0606
12	16.97	Schizandrin A	C ₂₄ H ₃₂ O ₆	417.2206[M+H] ⁺ , 439.2095[M+Na] ⁺	-4.3	417.2202, 316.1200, 301.0962
13	17.67	γ-Schizandrin B	C ₂₃ H ₂₈ O ₆	401.1957[M+H] ⁺ , 423.1683[M+Na] ⁺	-1.7	401.1874, 386.1722, 370.1732, 300.0999
14	17.87	Schizandrin B	C ₂₃ H ₂₈ O ₆	401.1962[M+H] ⁺ , 423.1683[M+Na] ⁺	-0.5	401.1874, 386.1722, 370.1732, 300.0999
15	18.32	Schisandrin C	C ₂₂ H ₂₄ O ₆	385.1647[M+H] ⁺	-2.6	358.3572, 285.0642, 227.0571
16	18.50	Schisantherin D	C ₂₉ H ₂₈ O ₉	521.1807[M+H] ⁺	-1.0	399.1432, 385.1295, 368.1257, 341.1389

Table 3

Linear ranges, regression equations and correlation coefficients of multi-components in plasma.

Components	Linear range (ng/mL)	Linear regression equation (*10 ⁻³)	Correlation coefficient (<i>r</i> ²)
Schisandrin C	5-625	y=30.172x-17.562	0.9969
Schisandrin	10-1250	y=3.659x+3.349	0.9989
Schisandrol B	5-625	y=1.297x+3.851	0.9955
Gomisin D	5-625	y=1.432x-0.491	0.9989
Gomisin G	5-625	y=4.429x+5.389	0.9973
Angeloylgomisi H	10-1250	y=4.081x+4.643	0.9991
Schizandrin A	5-625	y=13.336x+12.767	0.9979
Schizandrin B	10-1250	y=11.519x+14.727	0.9985
Schisantherin A	10-1250	y=4.319x+8.368	0.9995
Schisantherin B	20-2500	y=3.607x+3.362	0.9979

Table 4

Summary of accuracy, precision, recovery and matrix effect of ten lignans in plasma
($n = 6$).

Components	Spiked (ng/mL)	Concentration Measured (ng/mL)	Inter-day (%, RSD)	Intra-day (%, RSD)	Accuracy (%, RE)	Extraction Recovery (%,mean±SD)	Matrix Effect (%,mean±SD)
Schisandrin C	10	9.5	4.4	8.6	-5.2	91.9±3.4	103.3±2.2
	100	95.4	4.1	4.0	-4.6	90.8±2.9	100.9±1.3
	500	526.9	3.1	4.8	5.4	94.4±0.8	99.7±0.8
Schisandrin	20	22.5	5.8	5.0	12.1	89.3±9.3	85.1±3.9
	200	223.5	5.6	4.5	11.7	86.2±3.8	95.7±4.3
	1000	1041.1	4.8	12.1	4.1	95.4±1.3	94.2±5.2
Schisandrol B	10	9.6	8.4	6.3	-3.9	91.3±5.3	90.9±7.2
	100	102.4	7.2	10.3	2.4	92.1±4.6	97.8±2.9
	500	527.3	6.1	9.3	5.5	92.1±2.8	93.9±3.8
Gomisin D	10	9.9	6.4	1.6	-0.6	94.8±3.3	103.2±6.6
	100	101.9	6.8	4.8	1.9	89.6±6.4	97.5±6.0
	500	503.9	5.4	6.2	0.8	94.6±6.3	95.3±5.3
Gomisin G	10	9.7	7.5	4.9	-3.0	94.7±4.5	95.7±4.0
	100	98.0	6.6	6.9	-2.0	96.4±2.2	98.9±1.8
	500	476.4	3.6	6.0	-4.7	96.6±1.9	93.9±0.9
Angeloylgomisi H	20	18.4	3.0	6.7	-7.9	92.8±2.1	93.9±3.0
	200	203.2	5.9	3.8	1.6	93.5±2.9	96.5±4.7
	1000	1113.9	2.1	1.2	11.4	95.6±0.9	97.0±0.6
Schizandrin A	10	9.8	6.9	1.7	-2.0	90.2±6.4	97.0±4.5
	100	102.6	4.8	9.1	2.6	89.0±4.7	93.7±3.5
	500	526.0	5.3	2.2	5.2	93.5±4.7	93.7±4.1
Schizandrin B	20	20.7	6.7	10.1	3.4	94.5±2.9	95.4±1.8

	200	199.0	6.1	10.7	-0.5	91.6±5.8	93.0±7.4
	1000	1032.0	4.9	3.0	3.2	92.2±1.9	95.4±3.4
Schisantherin A	20	20.5	5.9	10.6	2.7	93.4±3.3	94.4±2.7
	200	216.1	3.6	7.2	8.0	91.7±2.4	96.9±2.4
	1000	1084.8	3.1	4.9	8.5	96.2±0.9	96.5±0.6
Schisantherin B	40	38.4	4.0	7.0	-4.1	95.7±1.3	95.1±2.0
	400	410.0	4.5	6.4	2.5	95.9±1.5	96.6±2.8
	2000	2002.3	3.5	4.6	0.1	98.3±3.4	95.6±4.1

Table 5

The main pharmacokinetics parameters of twelve lignans after oral administration of extract of WPSCF (mean ± SD).

	C _{max}	T _{max}	T _{1/2}	AUC _{0-∞}	MRT _(0-∞)	Vz/F	AUC _{0-∞} /Dose
	(µg/L)	(h)	(h)	(µg/L*h)	(h)	L/Kg	(g/L*h)
Schisandrin	647.4±199.8	1.7±0.6	7.5±0.2	5418.3±752.2	10.2±0.6	6.2±1.9	1865.8
Schisandrol B	239.8±20.3	2±0	13.5±0.7	4932.0±386.3	23.0±0.8	13.3±1.7	1474.9
Gomisin D	496.9±70.5	3.3±1.2	13.4±2.5	13378.8±1700.8	26.6±1.1	23.1±5.4	844.6
Gomisin E	388.0±110.9	1.7±0.6	11.8±1.5	7707.6±366.2	21.8±1.8	143.2±24.0	119.98
Gomisin G	233.0±18.2	2±0	3.7±0.1	1781.8±163.7	7.2±0.3	2.1±0.1	2531.0
Schisantherin B	573.6±20.4	1.3±0.6	13.0±1.6	6676.9±381.9	16.4±0.9	5.5±0.3	3448.8
Angeloylgomisin H	718.8±160.4	1±0	3.5±0.3	4153.1±471.4	6.4±0.2	4.4±0.6	1151.1
Schizandrin A	416.4±13.2	2±0	12.7±2.4	5957.6±478.8	19.2±2.3	14.7±3.6	1277.4
Schisandrin C	181.2±64.9	6±0	12.6±0.9	5091.2±342.1	30.5±0.8	13.2±0.7	1377.5
γ-SchizandrinB	182.2±55.6	2.7±1.2	15.1±1.6	4324.5±394.1	30.8±2.3	25.0±2.3	877.5
SchizandrinB	219.8±23.7	1.7±0.6	14.7±3.3	4879.1±412.7	26.2±2.9	27.6±7.8	780.9
Schisantherin A	491±15.8	1.3±0.6	12.7±1.9	7298.2±689.0	18.8±0.9	2.5±0.5	7539.4