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Determination of two C21 steroidal glycosides of Baishouwu and their metabolites in mice plasma and tumor homogenate by liquid chromatography–tandem mass spectrometry

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ABSTRACT

For the evaluating of the absorption, tumor affinity and metabolism of wilfoside C3N and wilfoside C1N (two main C21 steroidal glycosides from Baishouwu), an LC–MS/MS method was developed. Plasma or tumor homogenate samples were extracted by liquid–liquid extraction with ethyl acetate after internal standard (ginsenoside Rh2) spiked. The separation was performed by a Luna C18 column (3.0 μm, 2.0 mm × 50 mm) with gradient elution. The method was fully validated and successfully applied to determine the concentrations of the parent drugs and metabolites after intragastric administration of wilfoside C3N and wilfoside C1N to mice respectively.

1. Introduction

Baishouwu is a commonly used herbal drug and has been used as a tonic for enriching vital essence and enhancing immunity in China and other Asian countries for over one thousand years. Modern pharmacological studies showed that the herb possessed various activities: anti-tumor [1], gastroprotection [2], prevention against free radical damage [3]. Up to now, only its anti-tumor activity has been well studied and C21 steroidal glycosides are considered to be its main active agents [4–6].

Natural products have attracted the interests of many scientists due to their low toxicity and excellent pharmacological activities. Many scientific studies have been carried out and the in vitro experiments showed that large quantity of them possessed significant anti-tumor activities. But little of them came into market and the main obstacle was the unsatisfactory in vivo properties, such as poor oral absorption, low tumor targeting and fast elimination. In vivo properties, especially the oral absorption properties, decide the fate of candidates in their new drug development process. It is well recognized that more than 50% of drug candidates are rejected due to poor pharmacokinetic properties, so for the development and scientific usage of natural products, it is necessary to study the in vivo properties of their main constituents. To our knowledge only one paper about the pharmacokinetics of one component of the herb, caudatin 3-*O*-methyl-β-D-cymaropyranoside, was reported [7]. The constituents of C21 steroidal glycosides in this herb are complex and most of them show good pharmacological activities. Moreover, the content of caudatin 3-*O*-methyl-β-D-cymaropyranoside in Baishouwu is rather low, so it is obvious that the published paper about the in vivo properties of them could not give sufficient instruction for the further developments and scientific clinical usage of the herbal drug. Furthermore, the oligosaccharide portions of these glycosides are deoxy sugar and the effects of deoxy sugars on the absorption and tumor affinity properties of drugs remains unclear. To uncover these effects might be useful for the development and chemical modification of these C21 steroidal glycosides. Then, the in vivo properties of these C21 steroidal glycosides should be studied, and accordingly a sensitive and selective analytical method needs to be established.

Wilfoside C3N (Caudatin 3-*O*-β-D-cymaropyranosyl-(1 → 4)-α-L-diginopyranosyl-(1 → 4)-β-D-cymaropyranoside) and wilfoside

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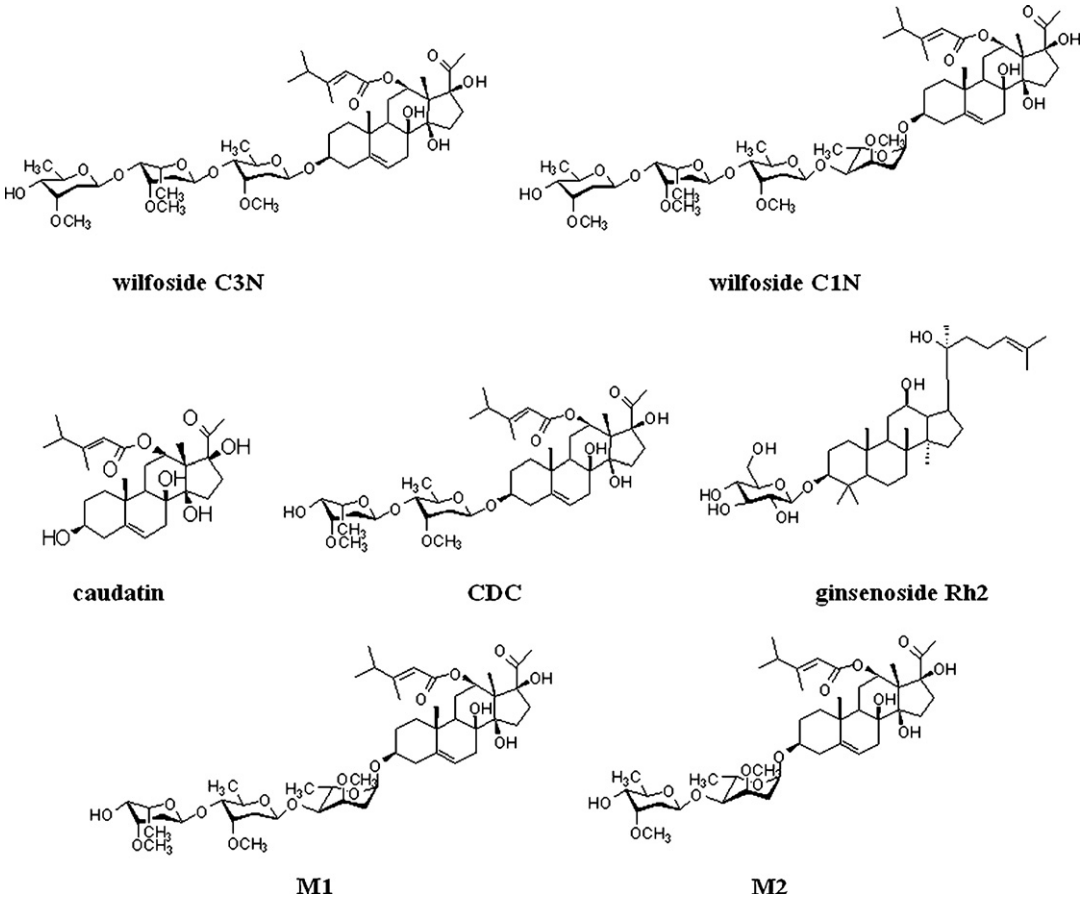

Fig. 1. Chemical structures of wilfoside C3N, wilfoside C1N, caudatin, CDC, ginsenoside Rh2 (IS) and presumed chemical structures of two metabolites of wilfoside C1N.

C1N (Caudatin 3-*O*-*α*-L-cymaropyranosyl-(1 → 4) -*β*-D-cymaropyranosyl-(1 → 4)-*α*-L-diginopyranosyl-(1 → 4)-*β*-D-cymaropyranoside) (Fig. 1) are the two most abundant and active C21 steroidal glycosides in the herb Baishouwu. In this paper we developed an LC–MS/MS method for the determination of them in mice plasma and tumor homogenate. Caudatin 3-*O*-*α*-L-diginopyranosyl-(1 → 4)-*β*-D-cymaropyranoside (CDC, a diglycoside of caudatin) and caudatin (the aglycone) presumed to be metabolites of wilfoside C3N were also simultaneously quantified. Two metabolites of wilfoside C1N were found and the concentrations of them were estimated by the methods of their isomerides, wilfoside C3N and CDC. The method has been successfully applied to assay the plasma and tumor homogenate concentrations of the parent drugs and their possible metabolites after wilfoside C3N and wilfoside C1N were intragastrically administered to tumor-bearing mice respectively.

2. Experimental

2.1. Chemicals and reagents

Wilfoside C3N, wilfoside C1N, Caudatin 3-*O*-*α*-L-diginopyranosyl-(1 → 4)-*β*-D-cymaropyranoside and caudatin were extracted from *Cynanchum bungei* Decne and refined in our laboratory (>98.5% purity, identified by NMR and MS). Ginsenoside Rh2 (>99% purity, I.S.) were purchased from National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). HPLC-grade ethyl acetate was purchased from Tianjin Kermel Chemical Reagents Development Centre (Tianjin, China). HPLC-grade acetonitrile was purchased from Merck Company (Darmstadt, Germany). HPLC-grade formic acid was purchased from Tedia Company Inc. (Fairfield, USA). All other reagents were of analytical grade.

2.2. Instrumentation

An Agilent 6410A triple quadrupole LC–MS system (Agilent Corporation, MA, USA) equipped with G1311A quaternary pump, G1322A vacuum degasser, G1329A autosampler and G1316A therm. Column compartment was used for all analyses. The system was controlled by MassHunter software (Agilent Corporation, MA, USA).

2.3. Chromatographic conditions

The separation was performed by a Luna C18(2) column (3.0 μm, 2.0 mm × 50 mm, Phenomenex Inc., MA, USA) with mobile phase consisted of solvent A (acetonitrile) and solvent B (0.1% aqueous formic acid, v/v). Gradient elution was as follows: initial 0–2 min, linear changed from A–B (55:45, v/v) to A–B (95:5, v/v), then hold at A–B (95:5, v/v) for 3 min. The column temperature was set at 35 °C. The flow rate was 0.3 mL/min and sample injection volume was 10 μL.

2.4. Mass spectrometric condition

Ionization was achieved using electrospray in the negative mode with the spray voltage set at 4000 V. Nitrogen was used as nebulizer gas and nebulizer pressure was set at 0.276 Mpa with a source

Table 1
Optimized MRM (multiple reaction monitoring) parameters for caudatin, CDC, wilfoside C3N, wilfoside C1N and ginsenoside Rh2.

	Precursor ion	Fragmentor energy (V)	Collision energy (eV)	Product ion
Caudatin	489.3	135	15	127.2
CDC	777.5	180	15	127.2
Wilfoside C3N	921.8	200	16	127.2
Wilfoside C1N	1065.7	200	16	127.2
Ginsenoside Rh2	667.5	180	23	621.2

temperature of 105 °C. Desolvation gas (nitrogen) was heated to 350 °C and delivered at a flow rate of 10 L/min. For collision-induced dissociation (CID), high purity nitrogen was used as collision gas at a pressure of about 0.1 MPa. Quantitation was performed using multiple reaction monitoring (MRM) mode, Table 1 shows the optimized MRM parameters for detected drugs and IS. The peak widths of precursor and product ions were maintained at 0.7 amu at half-height in the MRM mode.

2.5. Preparation of stock and working solutions

The standard stock solutions of wilfoside C3N, wilfoside C1N, CDC, caudatin and ginsenoside Rh2 were prepared in methanol to a final concentration of 100 μg/mL. Working solutions were prepared by further diluting the stock solutions with 55% aqueous acetonitrile to 2.50, 5.00, 10.0, 25.0, 50.0, 100.0 and 250.0 ng/mL for caudatin, 10.0, 25.0, 50.0, 250.0, 500, 2500, 5000 and 25,000 ng/mL for CDC and wilfoside C3N, and 25.0, 50.0, 250.0, 500.0, 2500, 5000 and 25,000 ng/mL for wilfoside C1N. Working solution of IS was prepared by diluting the stock solution of ginsenoside Rh2 with 55% aqueous acetonitrile to 1000 ng/mL. All the working solutions were kept at 4 °C.

2.6. Preparation of standard and quality control (QC) samples

Calibration curves were prepared by spiking 100 μL of blank plasma or tumor homogenate each with 20 μL of one of the above mentioned working solutions to produce the calibration point equivalent to 0.50, 1.00, 2.00, 5.00, 10.0, 20.0 and 50.0 ng/mL of caudatin, 2.00, 5.00, 10.0, 50.0, 100.0, 500.0, 1000 and 5000 ng/mL of CDC and wilfoside C3N, and 5.00, 10.0, 50.0, 100.0, 500.0, 1000 and 5000 ng/mL of wilfoside C1N.

The QC samples were prepared using separately prepared stock solution of each analyte to obtain the plasma or tumor homogenate concentrations of 1.00, 5.00 and 20.0 ng/mL for caudatin, of 5.00, 100.0 and 1000 ng/mL for CDC and wilfoside C3N, and of 10.0, 100.0 and 1000 ng/mL for wilfoside C1N, representing low, medium and high concentration of QC samples, respectively. The spiked plasma samples (standard and QC samples) were pretreated and detected on each analytical batch along with the unknown samples.

2.7. Sample preparation

To a 100 μL aliquot of plasma or tumor homogenate sample, 20 μL of 55% aqueous acetonitrile and 20 μL of the IS working solution were added. Samples were then vortex-mixed for 30 s and extracted with 3.5 mL ethyl acetate by vortex-mixing for 1.0 min. After centrifugation at 2000 × *g* for 10 min, the upper organic layer was transferred to another tube and evaporated to dryness at 45 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μL 55% aqueous acetonitrile followed by vortex-mixing for 1.0 min and centrifugation at 2000 × *g* for 10 min. Then, a 10 μL aliquot of supernatant was injected onto the LC–MS/MS system.

2.8. Assay validation

Comparison of the chromatograms of the blank and the spiked mice plasma or tumor homogenate was used to assay the selectivity of the method. In order to develop a reliable and reproducible method, the matrix effect was also investigated.

Blank mice plasma and water were pretreated separately as the same procedure of plasma sample, and the plasma matrix residue and water residue were got. Then these resides were reconstituted in 100 μL 55% aqueous acetonitrile containing each analyte at three QC levels, separately. After vortex-mixed for 1.0 min and centrifugated at 2000 × *g* for 10 min, a 10 μL aliquot of supernatant was injected onto the LC–MS/MS system. Peak areas of each analyte in the two kinds of matrix were A and B. Another QC samples pretreated with exactly the same procedure as described in Section 2.7 were also assayed and the peak areas were defined C. Matrix effects were evaluated by dividing A by B and the extraction recoveries were determined by dividing C by A. The matrix effects of tumor homogenate and extraction recoveries from the matrix were also evaluated with the above mentioned method.

Calibration curves of each analytes were run on three separate days. They were constructed from the peak area ratios of each analyte to IS versus plasma concentrations using a $1/x^2$ weighted linear least-square regression model.

Six replicates of QC samples at three levels of each analyte were included in each run to determine the intra-day and inter-day precision of the assay. The accuracy was determined as the percentage difference between the mean detected concentrations and the nominal concentrations. The lower limit of quantification (LLOQ) is defined as the lowest concentration of standard that can be measured with an acceptable accuracy and precision (≤20% for both parameters).

The stability of five analytes in plasma and tumor homogenate was assessed by analyzing triplicate QC samples stored for 6 h at ambient temperatures, three cycles of freezing at −20 °C and thawing and stored for 1 month at −20 °C, respectively. The stability of four analytes as well as IS in reconstituted extract at room temperature for 24 h was also assessed. Concentrations following storage were compared with freshly prepared samples of the same concentrations.

2.9. Application of the analytical method

ICR mice (male, 14–16 g), were purchased from the Shanghai SLAC Laboratory Animal Company Ltd. (Shanghai, China). The animals were maintained in controlled conditions of 22 ± 1 °C with food and water ad libitum. All animal treatments were strictly in accordance with the National Institutes of Health Guide to the Care and Use of Laboratory Animals. The experiments were carried out with the approval of the Committee of Experimental Animal Administration of the University. S180 tumor cells (2.0×10^6 per mouse) in 0.9% NaCl solution was inoculated intradermally into the right axilla of ICR mice. Drugs were administered at the 10th day after the tumor cell inoculation and the tumor tissues were about 2.0 g. These 50 mice were randomly assigned to 2 groups according to the body weight and each group contained 25 of them. Mice of one group received wilfoside C3N intragastrically at a single dose of 50 mg/kg (2.0 mg/mL, suspended in 0.5% sodium carboxymethyl cellulose (CMC–Na)). Mice of the other group received wilfoside C1N at the same dose. Five mice were sacrificed at each time point (1, 2, 4, 8 and 12 h) and blood and tumor tissues were collected. The blood samples were placed in heparinized tubes and immediately centrifuged and then the plasma samples were immediately stored at −20 °C until analysis. Tumor tissues were excised, rinsed 2 times in ice-cold physiological saline. Then they

were blotted dry with filter paper, accurately weighted, cut into slices and homogenated in ground glass tissue grinder after adding the appropriate amount of 0.9% NaCl solution (3 mL/g tissue). The uniform homogenates were immediately stored at −20 °C until analysis.

3. Results

3.1. LC–MS/MS optimization

The four analytes and ginsenoside Rh2 (IS) were at first characterized by MS scan and MS–MS product ions to ascertain their precursor ions and to select product ions for use in MRM mode, respectively. To get the richest relative abundance of precursor ions and product ions, the parameters for fragmentor energies and collision energies were optimized, and the MRM transition were chosen to be m/z 921.8 → 127.2 for wilfoside C3N, m/z 1065.7 → 127.2 for wilfoside C1N, m/z 489.3 → 127.2 for caudatin, m/z 777.5 → 127.2 for CDC and m/z 667.5 → 621.2 for ginsenoside Rh2. Fig. 2 shows the spectra of full scan product ion of precursor ions of the four analytes and IS.

Formic acid was added to the mobile phase for enhancing the abundance of precursor ions. After optimization, a proper concentration of formic acid was chosen. Further addition of formic acid

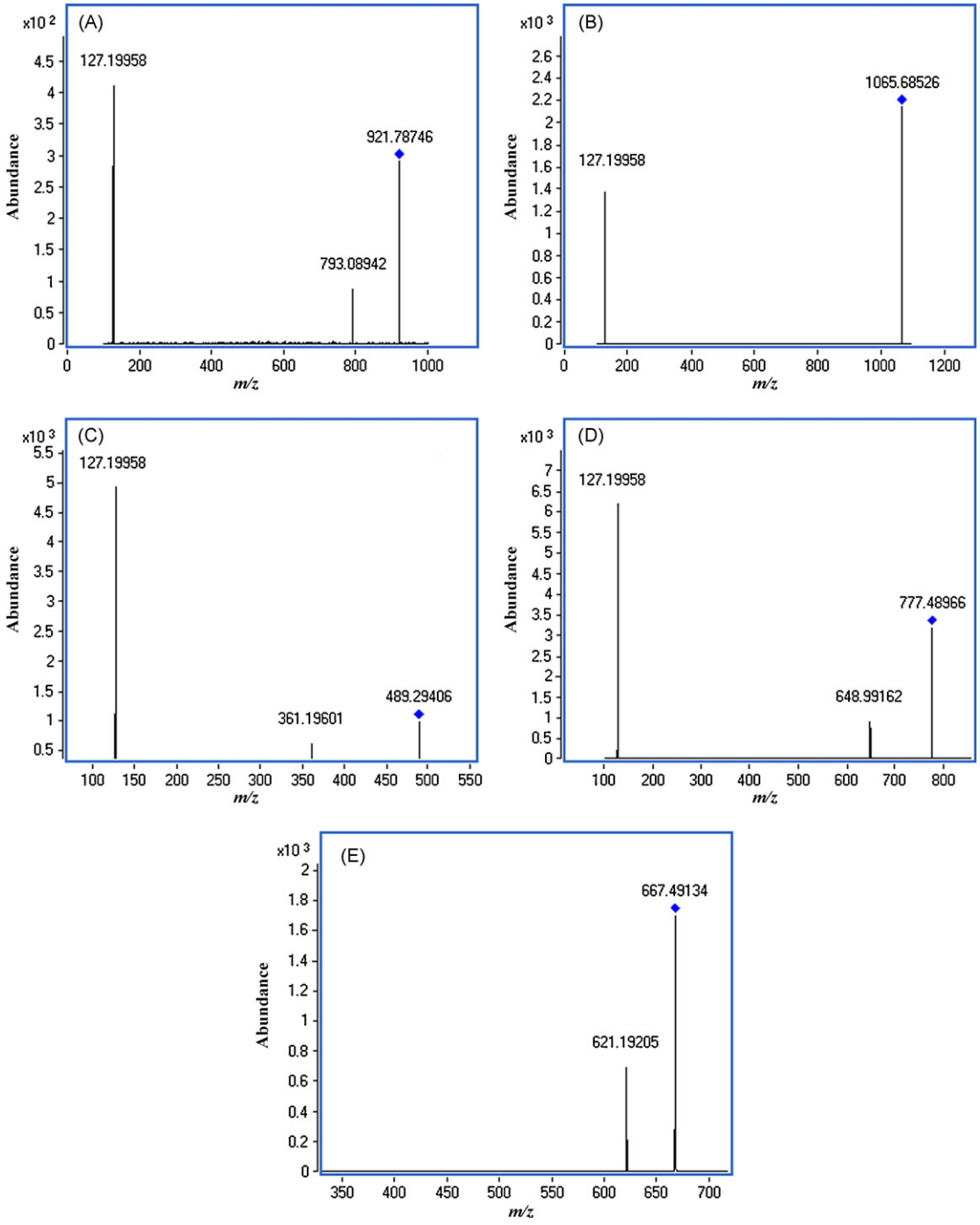

Fig. 2. Spectra of full scan product ion of precursor ions of wilfoside C3N (A), wilfoside C1N (B), caudatin (C), CDC (D) and ginsenoside Rh2 (E).

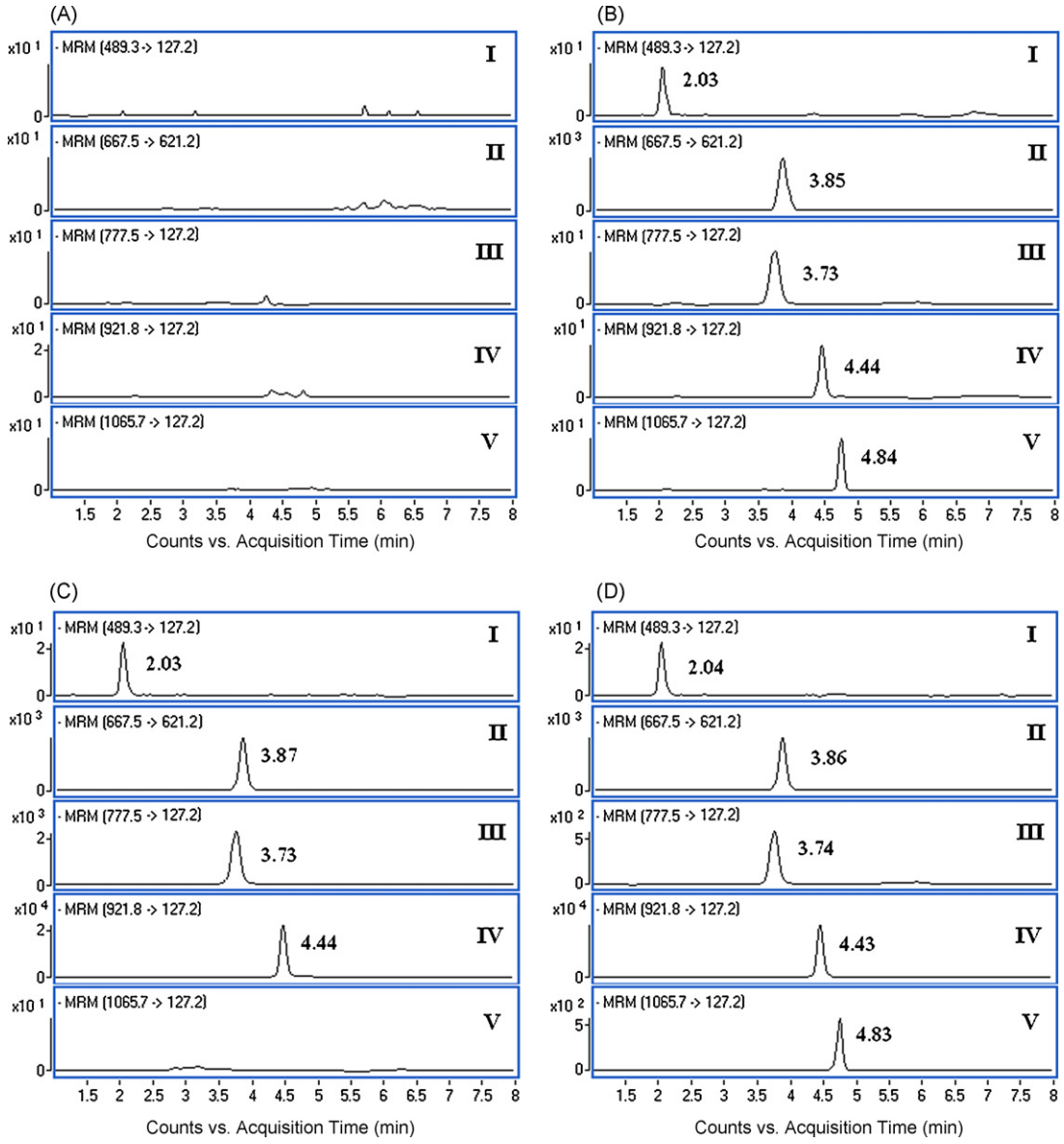

Fig. 3. Representative MRM chromatograms of caudatin (I), ginsenoside Rh2 (II, IS), CDC or M2 (III), wilfoside C3N or M1 (IV) and wilfoside C1N (V). (A) A blank mice plasma sample, (B) a blank mice plasma sample spiked with wilfoside C3N, wilfoside C1N, caudatin, CDC at the lower limit of quantification and IS, (C) plasma sample from a mouse at 2 h after intragastric administration of wilfoside C3N at a dose of 50 mg/kg and (D) plasma sample from a mouse at 2 h after intragastric administration of wilfoside C1N at a dose of 50 mg/kg.

would do little help for the production of precursor ions and would do more harm to the column.

3.2. Selection of internal standard

An internal standard should be used when performing MS quantitation. An appropriate internal standard will control for extraction, HPLC injection and ionization variability. In this method ginsenoside Rh2 was chosen as internal standard for its similar chemical structure, extraction recovery, ionization response in ESI mass spectrometry and chromatographic retention time with those of analytes. It was also stable during the period of pretreatment and assaying of the plasma samples. Ginsenoside Rh2 is not contained in Baishouwu which means this method does not tend to be inter-

Table 2
Extraction recoveries of the liquid–liquid extraction method ($n = 6$) for caudatin, CDC, wilfoside C3N and wilfoside C1N.

	Extraction recovery in plasma (%)			Extraction recovery in tumor homogenate (%)		
	Low	Medium	High	Low	Medium	High
Caudatin	89.8 ± 5.3	90.6 ± 4.5	90.5 ± 4.0	88.9 ± 6.1	88.7 ± 5.6	89.3 ± 4.2
CDC	91.6 ± 4.9	91.9 ± 3.4	92.1 ± 2.4	91.3 ± 5.7	91.5 ± 5.2	91.8 ± 4.6
Wilfoside C3N	94.8 ± 5.1	94.7 ± 4.2	95.2 ± 2.0	94.4 ± 6.7	94.0 ± 6.3	94.6 ± 5.8
Wilfoside C1N	95.8 ± 4.5	96.1 ± 3.8	96.3 ± 2.2	95.4 ± 6.3	95.7 ± 5.8	95.8 ± 5.3

fered when the method is adapted for wider application. Moreover, it is commercially available, which makes other researchers easy to repeat the method.

3.3. Method validation

3.3.1. Assay selectivity and matrix effect

LC–MS/MS method has high selectivity because only selected ions produced from selected precursor ions are monitored. Comparison of the chromatograms of the blank and the spiked rat plasma (see Fig. 3) indicated no significant interference at the retention times of the analytes and the IS.

The results of matrix effect experiments showed that there was no significant difference between the peak areas of samples prepared from mice plasma or tumor homogenate and from water which indicated that no co-eluting unseen compounds significantly influenced the ionization of the analytes and IS.

3.3.2. Linearity of calibration curves and lower limit of quantification

The calibration curves for spiked mice plasma or tumor homogenate containing each analyte were all linear over the ranges defined in Section 2.6 with correlation coefficients (r) > 0.995. Typical equations for the calibration curves were as follows:

Wilfoside C3N	$Y = 4.43 \times 10^{-3}X + 1.25 \times 10^{-3}$	$r = 0.9976$
Wilfoside C1N	$Y = 1.69 \times 10^{-3}X + 4.45 \times 10^{-4}$	$r = 0.9992$
CDC	$Y = 6.73 \times 10^{-3}X + 1.74 \times 10^{-3}$	$r = 0.9997$
Caudatin	$Y = 2.52 \times 10^{-2}X + 4.83 \times 10^{-3}$	$r = 0.9985$

Where X is the plasma concentration of each analyte (ng/mL) and Y is the peak area ratios of each analyte to IS.

The lower limits of quantification for determination of caudatin, CDC, wilfoside C3N and wilfoside C1N in mice plasma or tumor homogenate, is defined as the lowest concentration analyzed with accuracy within ±20% and a precision ≤20%, were 0.50, 2.00, 2.00 and 5.00 ng/mL respectively. These limits are sufficient for the determination of them in mice plasma and tumor homogenate following intragastric administration of wilfoside C3N or wilfoside C1N. The limits of detection (LOD) were calculated on the basis of a signal-to-noise ratio of 3:1, resulting in 0.15, 0.50, 0.75 and 1.25 ng/mL for caudatin, CDC, wilfoside C3N and wilfoside C1N, respectively.

3.3.3. Assay precision and accuracy

Intra- and inter-day precision was assessed from the results of QCs. The mean values and RSD for QC samples at three concentration levels were calculated over three validation runs. Six replicates of each QC level were determined in each run. The accuracy of the method was determined by calculating the percentage deviation observed in the analysis of QCs and expressed as the relative error (R.E.). The method showed good precision and accuracy. The intra- and inter-day precisions were measured to be below 9.7% and 11.4%, respectively, with relative errors from −4.2% to 3.4%. All intra- and inter-day precision and accuracy were acceptable.

3.3.4. Extraction recovery

The extraction recoveries of the analytes under the liquid–liquid extraction conditions were summarized in Table 2. The recovery of the internal standard was 91.2 ± 2.4% in mice plasma (n = 6) and 90.5 ± 3.7% in mice tumor homogenate (n = 6).

The extraction recoveries were high and stable for all analytes and IS.

3.3.5. Analyte stability

The stability of caudatin and its three glycosides in mice plasma and tumor homogenate were investigated. The analytes were found to be stable in mice plasma and tumor homogenate stored for 1 month at −20 °C and in reconstituted solution (55% aqueous acetonitrile, v/v) at room temperature for 24 h (<15% reduction). The analytes were found to be stable in plasma after three freeze–thaw cycles from −20 °C to room temperature with a reduction of less than 15%. The analytes were also shown to be stable in mice plasma or tumor homogenate at room temperature for at least 6 h with a reduction of less than 15%. The stability of analytes and IS in stock and working solutions was also investigated and no obvious reduction was found after storage at 1–4 °C for 2 months.

3.4. Application of the analytical method

When wilfoside C3N was administered to tumor-bearing mice, wilfoside C3N, CDC and caudatin were found in plasma and tumor homogenate and were quantified (Fig. 4). The concentrations of the parent drug both in plasma and in tumor homogenate were high from 1 to 4 h after drug administration. CDC, which was supposed to be a metabolite of wilfoside C3N, was also found in large amount in both the assayed tissues. The highest concentration of CDC was found in the plasma sampled 1 h after intragastric administration of its parent drug. So we supposed that it was metabolized from wilfoside C3N by enzymes other than bacterium of intesti-

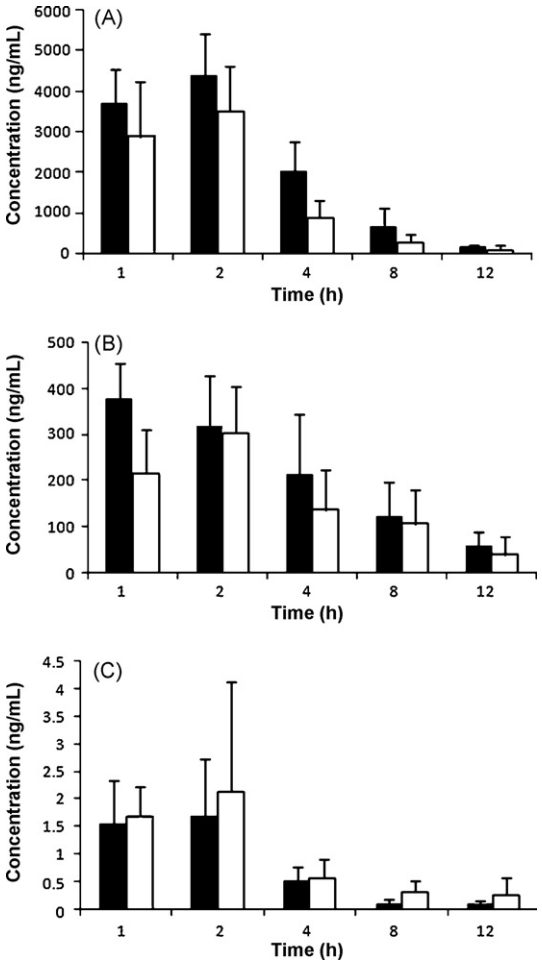

Fig. 4. Plasma (■) and tumor tissue homogenate (□) concentrations of wilfoside C3N (A), CDC (B) and caudatin (C) after intragastric administration of wilfoside C3N to tumor-bearing mice at a dose of 50 mg/kg.

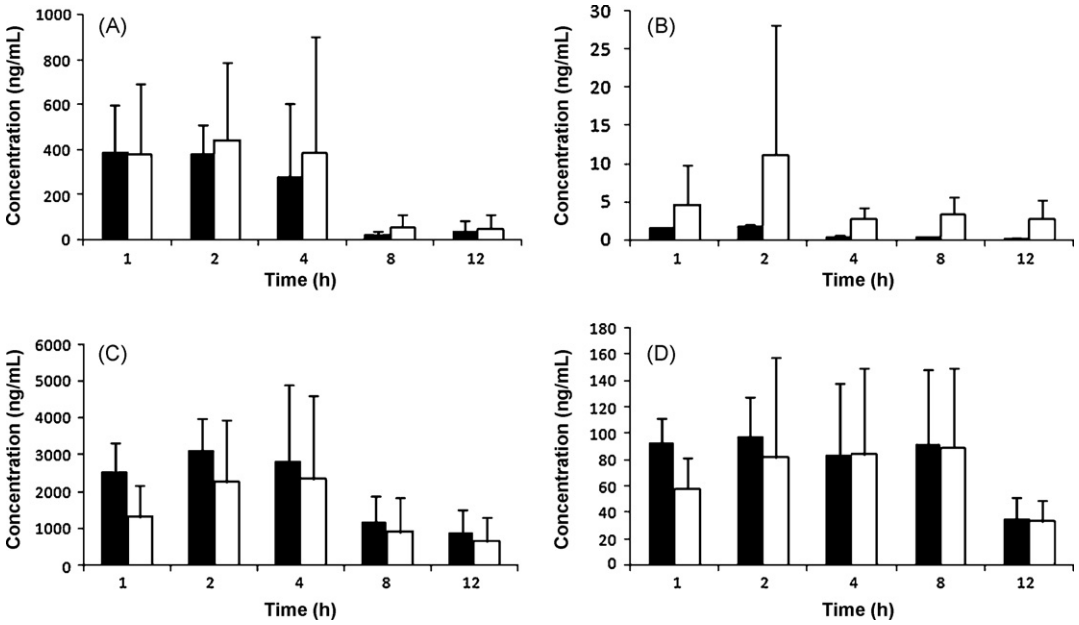

Fig. 5. Plasma (■) and tumor tissue homogenate (□) concentrations of wilfoside C1N (A), caudatin (B), M1 (C) and M2 (D) after intragastric administration of wilfoside C1N to tumor-bearing mice at a dose of 50 mg/kg.

nal tract. The concentrations of caudatin, the aglycone metabolite of wilfoside C3N, were low both in plasma samples and tumor homogenate samples. The concentrations of the parent drug and its two metabolites in mice plasma were close to those in tumor homogenate at the same sampling times, add to that the drug concentrations were diluted by about 3.5 folds when the tumor tissues were homogenated, we concluded that these components showed good affinity for tumor tissues.

When wilfoside C1N was administered to tumor-bearing mice, wilfoside C1N and caudatin were found and quantified (Fig. 5). The concentrations of wilfoside C1N in mice plasma and tumor homogenate were only about 10% of those of wilfoside C3N when it was administered, which means wilfoside C1N was not well absorbed or it might undergo more significant first-pass metabolism. The concentrations of caudatin, the aglycone metabolite of wilfoside C1N, were still low both in plasma samples and tumor homogenate samples. Good affinity of the parent drug and its metabolite for tumor tissues was also found.

3.5. Estimate the concentrations of metabolites of wilfoside C1N

After wilfoside C1N was intragastrically administered to tumor-bearing mice, two main metabolites were found in both plasma and tumor homogenate samples. They had the same retention times, molecular weights and fragmentation regularities as those of wilfoside C3N and CDC. So we assumed that the two main metabolites were caudatin triglycoside (M1) and caudatin diglycoside (M2) (Fig. 1). The constructions of oligosaccharide portions of wilfoside C3N and M1 are different, but they had limited influence on the response of the two analytes, so we estimated the concentrations of M1 using the quantify LC–MS/MS method of wilfoside C3N. The concentration of M2 was also estimated using the quantify LC–MS/MS method of CDC.

When wilfoside C1N was given to mice intragastrically, much more M1 than the original drug were found either in the plasma or in the tumor tissue (Fig. 5). The amounts of M1 in plasma and tumor homogenate were close to those of wilfoside C3N when wilfoside C3N was administered. The concentrations of M2 in both tissues assayed were not high comparing with those of M1. These results indicated that the length of oligosaccharide portions showed great effect on the absorption of the glycosides and the oligosaccharide portions with three deoxy sugars might be proper.

4. Discussion

The oligosaccharide portions of most glycosides from natural resource are glucosyl group, and they can affect the in vivo properties of drugs, such as oral absorption [8,9], bile efflux [10], and uptake or efflux in tumor cells [11]. But most oligosaccharide portions of these C21 steroidal glycosides are deoxy sugars and the effect of them on the in vivo properties of drugs remains unclear. The plasma and tumor concentrations of wilfoside C3N were high when the drug was intragastrically administered, though the absolute bioavailability was not evaluated by us, the results indicated that the absorption was not poor. “Rules of 5” [12,13] which instructs the development of new drugs indicated that 90% of the marketed oral drugs had molecular weight less than 500, calculated log *P* less than 5, sum of hydrogen bond acceptors (as a sum of N and O) less than 10. When two or more of these limits are exceeded, poor absorption and permeation are more likely. The rules indicate that wilfoside C3N should be poorly absorbed which was contrary to the result of our experiment. In the membrane of epithelial cells of intestine, glucose transporters play a crucial role in the selective sugar absorption [14,15]. Not only monosaccharides but also glucosides and galactosides are actively absorbed via these transporters [16]. So the possibility for the drug to be absorbed by active transportation is high.

Two main metabolites of wilfoside C1N were found in mice plasma and tumor tissue and the concentration of one metabolite was supposed to be higher than that of the parent drug, so it is necessary to quantify them. But these two compounds are not contained in the herb of Baishouwu. Due to the lack of specific hydrolytic enzyme, we tried to get them by hydrolyzing wilfoside C1N using acid, but failed. Considering the two metabolites were isomeric compounds of wilfoside C3N and CDC, we evaluated the possibility of estimating the concentration of them using the quantify method of two known compounds. Take wilfoside C3N and M1 as an example. Since the hydroxyl groups on their aglycone portions

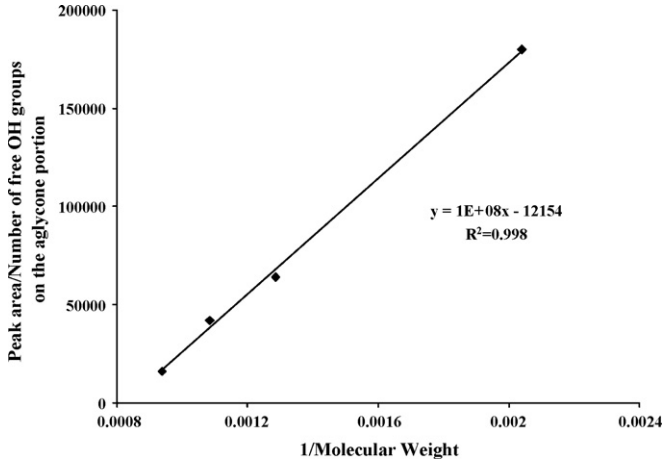

Fig. 6. Relationship between molecular weights of wilfoside C3N, wilfoside C1N, caudatin, CDC and their peak areas. The smaller figure is an expansion of the last three data points.

were prone to lose a proton, they were both monitored by negative mode. So the number of hydroxyl groups on the aglycone portions might influence the ionization of analytes and further peak areas of them. Intensities of four analytes with different oligosaccharide portions and numbers of hydroxyl groups on the aglycone portion were compared. A good correlation between peak area/number of hydroxyl group on the aglycone portion and reciprocal of molecular weight was found (Fig. 6). So we assumed that the length of oligosaccharide portions instead of their components affected the intensity of the analyte in LC–MS/MS. Thus, we believed that to estimate the two metabolites using the methods of their isomerides was feasible.

Wilfoside C3N showed a much better absorption than wilfoside C1N and the triglycoside metabolite of wilfoside C1N was found in mice plasma in large quantity. So the length of oligosaccharide portion plays an important role in the absorption of the drug. We assumed that the absorption of glycosides of caudatin with a longer sugar chain would be poor and the in vitro evaluation of their pharmacological activity would be meaningless. The results could also give some instructive advice for the modification of poorly absorbed drugs: glycolization by biotransformation in plant cell or organ cultures of Baishouwu.

5. Conclusion

An LC–MS/MS method was developed and validated for the determination of wilfoside C3N, wilfoside C1N and their metabolites. This method was sensitive, rapid, with high accuracy and met all requirements in bioanalytical method. The analytical method has been successfully applied to assay the plasma and tumor concentrations of the analytes mentioned above after intragastric administration of wilfoside C3N and wilfoside C1N to tumor-bearing mice, respectively. Moreover, two metabolites of wilfoside C1N were estimated by the methods of their isomerides. The high concentrations of wilfoside C3N and triglycoside metabolite in mice plasma and tumor homogenate indicate that the length of oligosaccharide portions shows great effect on the absorption of the glycosides. The results could also give some instructive advice for the pharmacological experiments on the C21 steroidal glycosides of Baishouwu and for the modification of other poorly absorbed drugs.

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References

[1] L. Shan, W.D. Zhang, C. Zhang, R.H. Liu, J. Su, Y. Zhou, Phytother. Res. 19 (2005) 259.
[2] L. Shan, R.H. Liu, Y.H. Shen, W.D. Zhang, C. Zhang, D.Z. Wu, L. Min, J. Su, X.K. Xu, J. Ethnopharmacol. 107 (2006) 389.
[3] Z.Z. Guo, Y.C. Lu, X.Q. Liu, Zhong Xi Yi Jie He Za Zhi 8 (1988) 282.
[4] Y.R. Peng, Y.B. Li, X.D. Liu, J.F. Zhang, J.A. Duan, Phytomedicine (2008).
[5] D.Y. Wang, H.Q. Zhang, X. Li, Yao Xue Xue Bao 42 (2007) 366.
[6] H. Wang, Q. Wang, R.K. Srivastava, S.S. Gong, L. Lao, J.D. Fondell, J.B. Wang, Altern. Ther. Health Med. 9 (2003) 62.
[7] Y. Peng, Y. Li, D. Wang, X. Liu, J. Zhang, S. Qian, J. Duan, Biomed. Chromatogr. 22 (2008) 575.
[8] T. Mizuma, Y. Nagamine, A. Dobashi, S. Awazu, Biochim. Biophys. Acta 1381 (1998) 340.
[9] A.L. Sesink, I.C. Arts, V.C. de Boer, P. Breedveld, J.H. Schellens, P.C. Hollman, F.G. Russel, Mol. Pharmacol. 67 (2005) 1999.
[10] I.C. Arts, A.L. Sesink, M. Faassen-Peters, P.C. Hollman, Br. J. Nutr. 91 (2004) 841.
[11] A. Waki, H. Kato, R. Yano, N. Sadato, A. Yokoyama, Y. Ishii, Y. Yonekura, Y. Fujibayashi, Nucl. Med. Biol. 25 (1998) 593.
[12] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Adv. Drug Deliv. Rev. 46 (2001) 3.
[13] M.C. Wenlock, R.P. Austin, P. Barton, A.M. Davis, P.D. Leeson, J. Med. Chem. 46 (2003) 1250.
[14] L.A. Drozdowski, A.B. Thomson, World J. Gastroenterol. 12 (2006) 1657.
[15] T. Puntheeranurak, B. Wimmer, F. Castaneda, H.J. Gruber, P. Hinterdorfer, R.K. Kinne, Biochemistry 46 (2007) 2797.
[16] T. Mizuma, K. Ohta, S. Awazu, Biochim. Biophys. Acta 1200 (1994) 117.