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Research Article
Comparative Pharmacokinetics and Metabolic Profile of Rhein Following Oral Administration of Niuhuang Shang Qing Tablets, Rhubarb and Rhein in Rats

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Abstract

Background and Objective: Niuhuang Shang Qing tablet (NHSQ) is commonly used in traditional Chinese medicine preparation and rhein is a bioactive component in NHSQ for the anti-inflammatory effect. Up to now, there was no study done on pharmacokinetic (PK) of rhein after oral NHSQ and also no study on metabolic profile of rhein *in vivo*. The aim of this study was to investigate PKs of rhein, explore the reasons of PK differences after oral administration of NHSQ, rhubarb and rhein in rats, identify the metabolites of rhein in multi-biosamples and establish the metabolic profile of rhein. **Materials and Methods:** Male and female Sprague-Dawley rats were given NHSQ, rhubarb and rhein orally administration. Samples from the plasma were collected at different times for PK analysis and samples from plasma, urine and internal organs (brain, heart, liver and kidney) were collected for metabolic profile analysis. **Results:** The C_{max} and AUC of rhein were significantly increased by rhein group, rhubarb group and NHSQ groups, the reason was that rhubarb, peppermint and chrysanthemum in NHSQ could produce additive, synergetic effects. Glucuronidation was the main metabolic pathway for rhein, followed by methylation and sulfation. Rhein and its metabolites distributed extensively in plasma, kidney, urine, liver and heart, anthrones could pass through blood-brain barrier more easily than anthraquinones. **Conclusion:** The NHSQ was superior to rhubarb or rhein in PKs by rhein-various ingredients synergistic interactions in NHSQ. Extensive metabolism of rhein occurred in rats.

Key words: Niuhuang Shang Qing tablets, rhein, rhubarb, comparative pharmacokinetic, metabolic profile

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Traditional Chinese Medicines (TCMs) have been used for preventing and treating various diseases for thousands of years. Nowadays, the TCM preparation is the main application mode of TCM because of its convenience of taking and carrying. Niuhuang Shangqing tablet (NHSQ) is a TCM preparation with the effect of clearing away heat and detoxifying, which is widely used in clinical and officially recorded in Pharmacopoeia of P.R. China (Ch.P)[1]. The NHSQ is composed of nineteen herbs, including *Bovis Calculus Artifactus*, *Rheum palmatum* L. (rhubarb), *Mentha haplocalyx Briq* (peppermint), *Chrysanthemum morifolium* (chrysanthemum), *Schizonepetae spica*, *Angelicae dahuricae radix*, *Ligusticum chuanxiong* Hort, *Gardenia jasminoides* Ellis, *Coptischinensis* Franch, *Phellodendri chinensis cortex*, *Scutellaria baicalensis* Georgi, *Forsythia suspense* Vahl, *Paeoniae rubra radix*, *Angelicae sinensis radix*, *Rehmannia glutinosa* Libosch, *Platycodon grandiflorum*, *Glycyrrhizae Radix et Rhizoma*, *Gypsum fibrosum* and *Borneolum syntheticum.* Among them, rhubarb is one of the major ingredients. The pharmacological studies showed that rhubarb has antibacterial[2], antiviral[3], anti-inflammation[4], anticancer[5], antioxidative[6], immunoregulatory effects[7] and it could activate endothelial nitric oxide synthase[8]. The anthraquinones isolated from rhubarb are known to be the main bioactive constituents. Among them, rhein is considered to be the main existence form of anthraquinones *in vivo* and chosen as one of the marker components to assess the quality of rhubarb in China. It is reported that rhein has many pharmacological effects, including hepatoprotective, nephroprotective, anti-inflammatory, antioxidant, anticancer and antimicrobial activities[9] and its pharmacological effects lay the foundation for the treatment of hepatic disease[10], diabetes[11], atherosclerosis[12] and various cancers, such as tongue cancer[13], lung cancer[14] and so on.

It has been reported that pharmacology and pharmacokinetic studies on traditional Chinese medicine (TCM) could illustrate the principle of pharmacokinetic compatibility from the essence leading to the changes of effective substances *in vivo*[15-18]. Moreover, the research on serum pharmacochemistry of TCM and bioavailability *in vivo* which is helpful to study drug interaction of main efficacious components mediated by metabolic enzymes, transport proteins or plasma protein binding in the course of absorption, distribution, metabolism and excretion (ADME)[19-20]. Therefore, it is necessary to study the pharmacokinetics and metabolic profile of TCM.

Up to now, there have been many reports for the pharmacokinetic (PK) or comparative PKs of rhein. These reports revealed that the different herbaceous compatibility and preparation method could affect the PK characteristics of rhein and lead to different PK parameters but these reports didn't investigate why these differences are produced[21-24]. Moreover, phase II metabolic routes of glucuronide and sulfation are reported to be the main metabolic pathway of rhein[25-28] and the conjugated metabolites (glucuronides and sulfates) possess a more potent free-radical scavenging activity than the prototypes of rhubarb anthraquinones[29,30], implying these conjugates would contribute to drug efficacy. Therapeutic and pharmacological effects of TCM are usually attributed to synergism or diminished the possible adverse reactions among multiple herbs and constituents. The synergistic effect resulting from the combinatorial intervention is often accompanied by pharmacokinetic (PK) changes of the individual components. There are many reports of rhein PK, but there have been no any reports of rhein PK after oral NHSQ, little is known about what and how the active ingredients of NHSQs which have complex ingredients, influenced the PK of rhein. In addition, it remains unclear about the metabolic profile of rhein. Therefore, this study was to develop a rapid, simple and accurate high-performance liquid chromatography with diode array detection (HPLC-DAD) method to determine the rhein in rat plasma, investigate the PK parameters after rats were given oral NHSQs, rhubarb and pure rhein, elucidate the reasons of PK changes of rhein. Moreover, in order to better understand the *in vivo* metabolic fate of rhein, the metabolites of rhein in multi-biosamples were identified by the ultra high-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF-MS/MS) method and the metabolic profile of rhein was established.

MATERIALS AND METHODS

Experimental location and duration: Experimental studies were carried out at School of Pharmacy, Nanchang University. The collections of samples and studies took over a period of one year (February, 2016-March, 2017).

Chemicals and materials: The reference standards of 1, 8-dihydroxyanthraquinone as Internal Standard (IS), rhein, aloe-emodin and chrysophanol were obtained from the National Institute for Food and Drug Control (Beijing, China). The purity of these reference standards were all more than 98%. HPLC-grade methanol, acetonitrile and formic acid, phosphoric acid and ethyl acetate of analytical grade were products of Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Purified water used throughout the study was commercially available (Wahaha®, Hangzhou Wahaha Co., Ltd,

China). Niuhuang Shang Qing tablets (No 20160103 ($NHSQ_A$), 20160304 ($NHSQ_B$), Wantong pharmaceutical Co., Ltd of Jilin, China) and Niuhuang Shang Qing tablet (No. 20160125 ($NHSQ_C$), Jinchen pharmaceutical Co., Ltd of Shanghai, China) were purchased from a local drugstore. Rhubarb (*Rheum palmatum* L.), *Mentha haplocalyx Briq* and *Chrysanthemum morifolium* were purchased from Beijing Tongrentang (Beijing, China), which were identified by Prof. Yun Ling (Nanchang University).

Animals: Male and female Sprague-Dawley (SD) rats (220-240 g) were purchased from the Laboratory Animal Center of Nanchang University (Nanchang, China) and were fed with the standard feed and drinking water, controlling the room temperature at 18-26°C and relative humidity at 50±10% on a 12 h light/dark cycle to acclimatize the environment for 7 days and then fasted for 12 h with free access to water before the experiment. All animal experiments were performed in compliance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of Jiangxi province, China and approved by Animal Research Ethics Committee of Nanchang University (NCDXSYDWLL-201780).

Measurement of rhein by HPLC: The concentration of rhein was determined using HPLC with diode array detection (Shimadzu, Japan). The chromatographic separations were performed on an Inertsil® ODS-3 column (150×4.6 mm, 5 μm, GL Science, Japan). The mobile phase consisted of methanol: 0.2% phosphoric acid (80:20; v/v). The flow rate of the mobile phase under isocratic elution was kept at 1.0 mL min^{-1}. The injection volume was 10 μL and the chromatographic run time was 20.0 min. Detection wavelength of analyte and internal standard was set at 254 nm.

Validation of PK method: The specificity of the method was demonstrated by comparing the chromatograms of blank plasma, plasma spiked with rhein, plasma spiked with 1, 8-dihydroxyanthraquinone as Internal Standard (IS), plasma spiked with rhein and IS as well as the plasma samples after oral administration of NHSQ, rhubarb and rhein. The Lower Limit of Quantification (LLOQ) was defined as the lowest concentration of the standard curve, giving a signal-to-noise ratio of 6:1. The intra-day precision and accuracy were carried out by determining five replicate QC samples at three different concentration (low, medium and high) levels on the same day. The inter-day precision and accuracy were assessed by analyzing five replicate QC samples at three concentrations on three consecutive days. The extraction recovery from rat plasma was determined at three concentrations by comparing the peak area ratio of rhein in spiked plasma sample with that sample to which rhein had been added after extraction. The stability of rhein in rat plasma was tested with QC samples stored at -20°C for 7 days. Freeze-thaw stability in rat plasma were determined with QC samples after three freeze-thaw cycles (from -20°C to room temperature). Short-term stability was determined by thawing the frozen samples and maintaining them at room temperature for 24 h. Post-preparation stability was tested by storing them at 4°C for 24 h.

Pharmacokinetic study of rhein: The dose of NHSQ for translation from human to animal is recommended by the Methodology of Pharmacological Experiment [31] and the study on rhein PK in human[32]. The SD rats were divided randomly into five groups (n = 18/group) and assigned to receive drug suspension by oral administration at doses of 4.85 g kg^{-1} ($NHSQ_A$), 5.00 g kg^{-1} ($NHSQ_B$), 5.87 g kg^{-1} ($NHSQ_C$), 0.49 g kg^{-1} (rhubarb) and 1.35 mg kg^{-1} (rhein), respectively. Serial blood samples were collected from the orbital sinus vein at 0, 0.083, 0.167, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 18 h in heparinized tube and then centrifuged at 4000 rpm for 10 min at room temperature, the supernatant was frozen at -20°C for HPLC analysis. The pharmacokinetic parameters were estimated by a compartment model method using Drug and Statistics 2.1.1 (DAS 2.1.1) software package (Chinese Pharmacological Society).

Measurements of components in plasma after oral administration of aloe-emodin, chrysophanol, peppermint and chrysanthemum: In order to analyse the sources of rhein *in vivo* after oral administration of NHSQ, the components absorbed in rat plasma after oral administration of aloe-emodin, chrysophanol, peppermint and chrysanthemum were determined by HPLC. 12 Male and female SD rats were randomly divided into four groups (three per group) and orally administered peppermint suspensions (43.75 g kg^{-1}), chrysanthemum suspensions (10.00 g kg^{-1}), aloe-emodin suspensions (5.23 mg kg^{-1}) and chrysophanol suspensions (5.00 mg kg^{-1}), respectively. Blood samples were collected in heparinized tubes before drug administration and at 2 h after drug administration. The plasma samples were immediately centrifuged at 4000 rpm for 10 min, the supernatant was frozen at -20°C for HPLC analysis.

Metabolic profile study of rhein: Fourteen rats were randomly assigned to four groups: three administration groups (A, B and C) and a blank control group, administration groups were assigned to receive rhein by oral administration at the dose of 1.35 mg kg^{-1}. The serial plasma samples in

group A (n = 3) were collected from the orbital sinus vein in heparinized tube at 0.25, 0.5, 1, 2, 4, 6, 8 and 12 h after oral dosed with rhein. The rats of group B (n = 3) were housed in metabolic cages and accepted urine samples were collected for 0-4, 4-8 and 8-12 h after oral dosed with rhein. The rats of group C (n = 5) were anaesthetized by diethyl ether and meanwhile, the brain, heart, liver and kidney were rapidly dissected and removed blood with ice-cold physiological saline at 0.5, 1, 2, 4 and 6 h after oral dosed with rhein.

Identification of metabolites of rhein: The metabolites of rhein were separated and identified by the ultra high-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF-MS/MS) which consisted of Shimadzu-20AD coupled with a AB Triple TOF 5600-1 mass spectrometer. The chromatography was carried out on an Eclipse Plus C18 column (4.6×100 mm, 3.5 μm, Agilent). The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B). Separation was performed by gradient elution as follows: 0-8 min, 5-10% B; 8-15 min, 10-30% B; 15-29 min, 30-33% B; 29-35 min, 33-34% B; 35-50 min, 34-56% B; 50-60 min, 56-95% B; 60-65 min, 95-5% B. The flow rate was set at 0.3 mL min^{-1}. The injection volume of the test sample was 5 μL and the chromatographic run time was 65.0 min. The UV spectra were recorded from 190-800 nm and the detection wavelength was set at 254 nm. The temperatures of the analytical column and auto-sampler were maintained at 40 and 4°C, respectively.

Statistical analyses: All results are expressed as the Msean±standard deviation. Differences in pharmacokinetic parameters among groups were tested by one-way analysis of variance (ANOVA) using SPSS 16.0 (SPSS IBM, Armonk, NY, USA). A probability level of $p<0.05$ was considered as the criterion of significance.

RESULTS

Method validation of PK: The representative chromatograms of blank and spiked plasma with analytes and IS were shown in the Supplementary Fig. S1. No interfering peak was detected at the retention times of the analyte and IS,

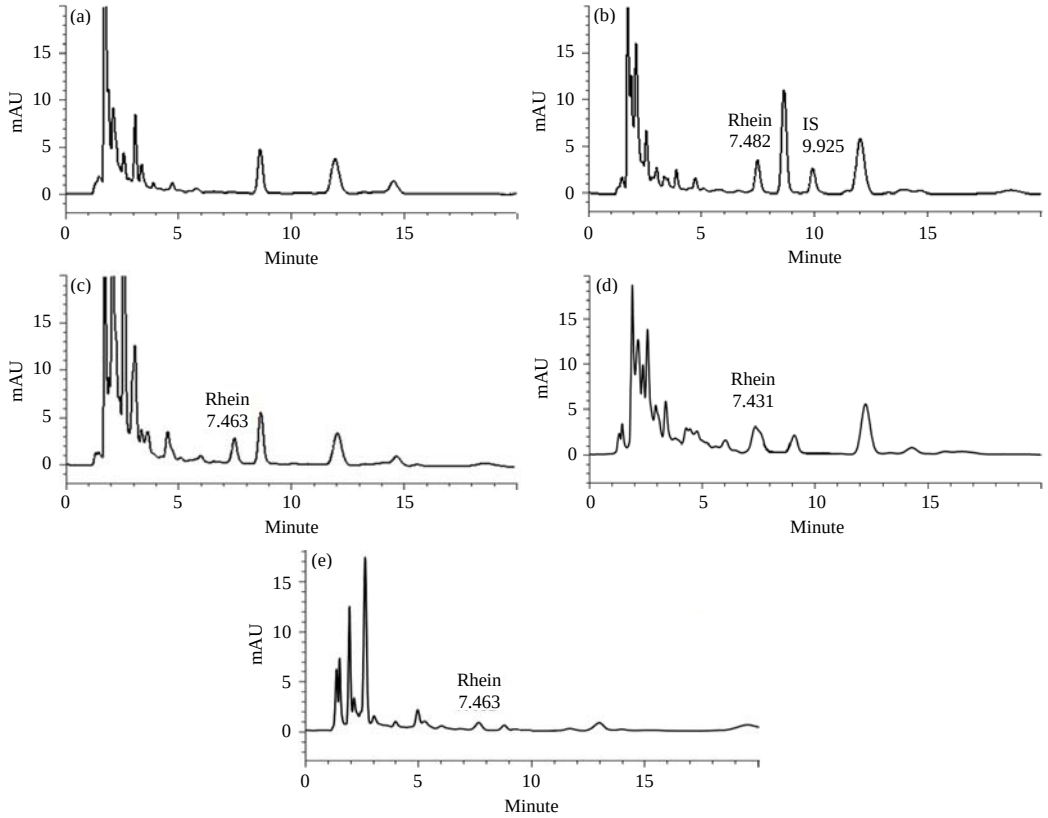

Fig. S1(a-e): HPLC chromatograms of rat plasma samples: (a) Blank plasma samples, (b) Plasma spiked with rhein and IS, plasma samples collected at 30 min in rats after oral administration of (c) NHSQ, (d) Rhubarb and (e) Rhein

indicating no interference from the endogenous substances in plasma. The high value of the correlation coefficient (r^2 = 0.9991) of the regression line and small values of the intercept exhibited good linearity of the calibration graphs. The typical line regression equation was Y = 1.1902X+0.0452. The LLOQ for rhein in plasma matrix was 50 ng mL^{-1}. The intra-day and inter-day precisions and accuracies of QC samples at three concentration levels were listed in Table S1. The Relative Standard Deviation (RSD) of intra-day precision ranged between 8.3 and 11.9% and Relative Error (RE) of accuracy ranged between 0.4 and 6.0%. Inter-day precision (RSD) ranged between 12.8 and 14.7% and accuracy (RE) ranged between 3.2 and 13.3%, respectively. The average extraction recoveries of the QC samples at three concentration levels were presented in Table S2. The recovery of rhein ranged from 86.5-106.3%. The results of stability experiments were displayed in Table S3. The data showed that rhein was stable in rat plasma for 7 days at -20°C, 1 day at room temperature, three freeze-thaw cycles. Moreover, the post-extracted samples were stable in auto-sampler vials at 4°C for 24 h.

Pharmacokinetic analysis: The mean plasma concentration-time profiles of rhein after oral dosage with $NHSQ_A$, $NHSQ_B$, $NHSQ_C$, rhubarb and rhein to six individual rats for each group were illustrated in Fig. 1. The main pharmacokinetic parameters of rhein in rat plasma were calculated and shown in Table 1 and the AUC, T_{max}, $t_{1/2}$, C_{max}, CLz/F and Vz/F were considered as the representative parameters to compare the differences of $NHSQ_A$ group, $NHSQ_B$ group, $NHSQ_C$ group, rhubarb group and rhein group and the histogram of the representative pharmacokinetic parameters for each group were illustrated in Fig. 2.

As shown in Fig. 2, the AUC, C_{max}, T_{max}, CLz/F and Vz/F of the herbal formulae groups ($NHSQ_A$, $NHSQ_B$, $NHSQ_C$) were significantly ($p<0.05$) different from that of the single herb group (rhubarb) and pure compound group (rhein), among them, the AUC and C_{max} significantly increased and T_{max}, CLz/F and Vz/F significantly decreased. The overall trends of C_{max} and AUC were NHSQ groups>rhubarb group>rhein group, the trends of T_{max}, CLz/F and Vz/F were NHSQ groups rhubarb group<rhein group. The C_{max} of NHSQ groups were 2.9-4.4 times compared with rhein group and 1.7-2.6 times compared with rhubarb group, the AUC of NHSQ groups were 2.4-3.3 times compared with rhein group and 1.4-1.9 times compared with rhubarb group. The rhein of $NHSQ_A$ group, $NHSQ_B$ group, $NHSQ_C$ group were reached C_{max} after 0.236, 0.333 and 0.264 h, respectively and were ahead 4.1, 2.9 and 3.6 fold to that of rhein group. Rhein was more rapidly eliminated from rat plasma with $t_{1/2}$ of 5.51-13.66 h in NHSQ groups than rhein group with 17.98 h.

Table S1: Intra-day and inter-day precision and accuracy for determination of rhein in rat plasma (n = 5)

Added concentration	Measured value (μg mL^{-1})	Precisions (RSD %)	Accuracy (RE %)
Intra-day precision and accuracy (n = 5 replicate samples)			
0.05	0.0518±0.0062	11.9	+3.6
0.5	0.4980±0.040	8.3	-0.4
5	5.3000±0.51	9.7	+6.0
Inter-day precision and accuracy (n = 3 days of replicate samples)			
0.05	0.0530±0.0073	13.8	+6.0
0.5	0.5160±0.076	14.7	+3.2
5	5.6600±0.73	12.8	+13.3

Values are expressed as Means±SD, RSD: Relative standard deviation

Table S2: Extraction recoveries of rhein and IS in rat plasma (n = 5)

Analyte	Spiked-concentration (μg mL^{-1})	Recovery (%) (Mean±SD)	RSD (%)
Rhein	0.05	106.3±12.6	11.9
	0.5	86.5±7.10	8.3
	5	96.6±12.6	13.1
IS	2	91.2±11.4	12.5

RSD: Relative standard deviation

Table S3: Stability of rhein in rat plasma (n = 5)

Spiked concentration (μg mL^{-1})	-20°C for 7 days		Three freeze-thaw cycle		25°C for 24 h		4°C for 24 h	
	Measured (μg mL^{-1})	RSD (%)	Measured (μg mL^{-1})	RSD (%)	Measured (μg mL^{-1})	RSD (%)	Measured (μg mL^{-1})	RSD (%)
0.05	0.0506±0.0030	6.0	0.0510±0.0064	12.6	0.0528±0.0045	8.5	0.0543±0.0048	8.9
0.5	0.538±0.075	14.0	0.512±0.065	12.7	0.511±0.058	11.3	0.525±0.071	13.5
5	5.40±0.50	9.3	5.29±0.74	14.0	5.48±0.60	10.9	5.38±0.31	5.8

Values are expressed as Means±SD in 5 rats, RSD: Relative standard deviation

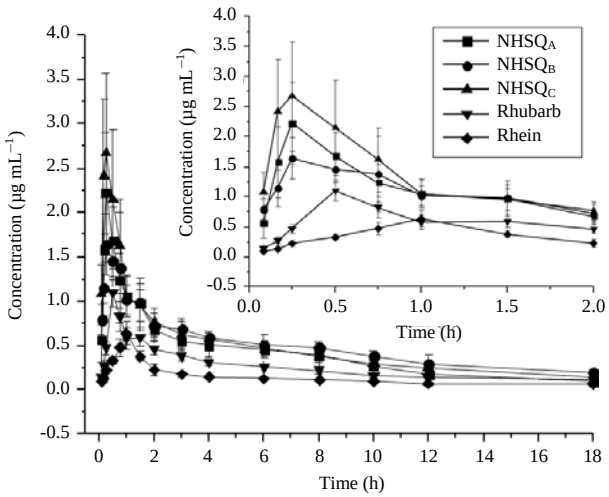

Fig. 1: Mean plasma concentration-time profile of rhein after oral dosed

Each point represents Mean±SD (n = 6)

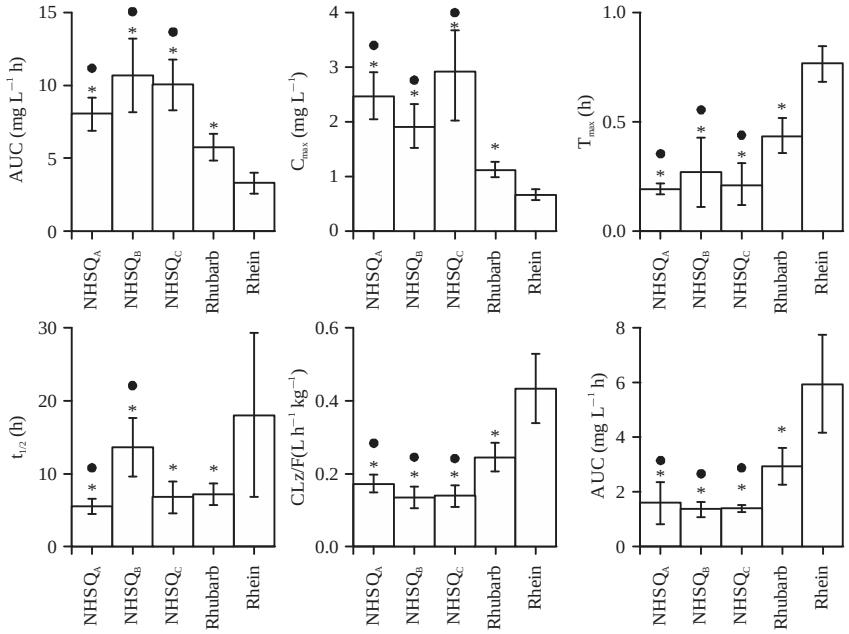

Fig. 2: The histogram of the pharmacokinetic parameters of rhein in rat plasma after oral administration

*p<0.05, $NHSQ_A$, $NHSQ_B$, $NHSQ_C$, rhubarb compared with rhein, •p<0.05, $NHSQ_A$, $NHSQ_B$, $NHSQ_C$ compared with rhubarb

Table 1: Pharmacokinetic parameters of rhein in rats after oral administration of NHSQ, Rhubarb and Rhein, respectively (n = 6)

Pharmacokinetic parameters	$NHSQ_A$ (4.85 g kg^{-1})	$NHSQ_B$ (5.00 g kg^{-1})	$NHSQ_C$ (5.87 g kg^{-1})	Rhubarb (0.49 g kg^{-1})	Rhein (1.35 mg kg^{-1})
AUC (mg L^{-1} h)	7.96±1.17*#	10.66±2.57*#	9.99±1.74*#	5.72±0.92*	3.25±0.69
AUMC	37.94±7.56*#	54.22±12.20*#	46.03±7.90*#	27.51±3.48*	14.34±1.34
MRT (h)	7.91±2.15*#	10.40±3.03	9.07±1.33*#	11.25±2.54	13.25±5.15
CLz/F (L h^{-1} kg^{-1})	0.17±0.03*#	0.13±0.03*#	0.14±0.03*#	0.24±0.04*	0.43±0.09
$t_{1/2}$ (h)	5.51±1.00*#	13.66±4.01*#	6.69±2.20*	7.14±1.57*	17.98±11.28
T_{max} (h)	0.236±0.034*#	0.333±0.204*#	0.264±0.123*#	0.542±0.102*	0.958±0.102
C_{max} (mg L^{-1})	2.462±0.427*#	1.903±0.405*#	2.922±0.736*#	1.116±0.141*	0.662±0.094
Vz/F (L kg^{-1})	1.594±0.774*#	1.349±0.267*#	1.388±0.125*#	2.941±0.67*	5.942±1.791

Data expressed as Mean±SD (n = 6), The doses of $NHSQ_{A, B, C}$ (4.85 g kg^{-1}, 5.00 g kg^{-1}, 5.87 g kg^{-1}) and rhubarb (0.49 g kg^{-1}) are equivalent to a rhein administration dose of 1.35 mg kg^{-1}. AUC: Area under the plasma concentration-time curve, AUMC: Area under the first moment curve, MRT: Mean residence time, CLz/F: Plasma clearance, $t_{1/2}$: Half-life, T_{max}: The time point of maximum plasma concentration, C_{max}: The peak plasma concentration of a drug after administration, Vz/F: Apparent volume of distribution. One-way ANOVA followed by Dunnett's test was used for statistical analysis (*p<0.05, $NHSQ_A$, $NHSQ_B$, $NHSQ_C$, Rhubarb compared with Rhein, #p<0.05, $NHSQ_A$, $NHSQ_B$, $NHSQ_C$ compared with Rhubarb)

Components in plasma after oral administration of aloe-emodin, chrysophanol, peppermint and chrysanthemum: By comparing the HPLC chromatograms of rat plasma samples with that of the reference standards of rhein, aloe-emodin, chrysophanol (Fig. 3a), rhein could be found in aloe-emodin group (Fig. 3b) and chrysophanol group (Fig. 3c), indicating aloe-emodin and chrysophanol could be metabolized to rhein. In addition, rhein could be found in peppermint group (Fig. 3d) and chrysanthemum group (Fig. 3e), indicating the rhein in rat plasma would be come from peppermint and chrysanthemum except for rhubarb.

Identification of metabolites of rhein: To identify the metabolites in multi-biosamples, the MS/MS fragment ions were detected in negative mode and positive mode. Total eleven absorbed components (one prototype and ten metabolites) of rhein were identified by comparison with blank biological samples. The identification results of metabolites of rhein were displayed in Table 2. Among them, M2 (rheinanthrone-1, 8-O-diglucuronide) and M5 (methoxy-rhein-O-glucuronide) were new metabolites of rhein which have not been reported previously. M2 (t_R = 18.66 min) showed an $[M-H]^-$ ion at m/z 621.1134, which was 352 Da

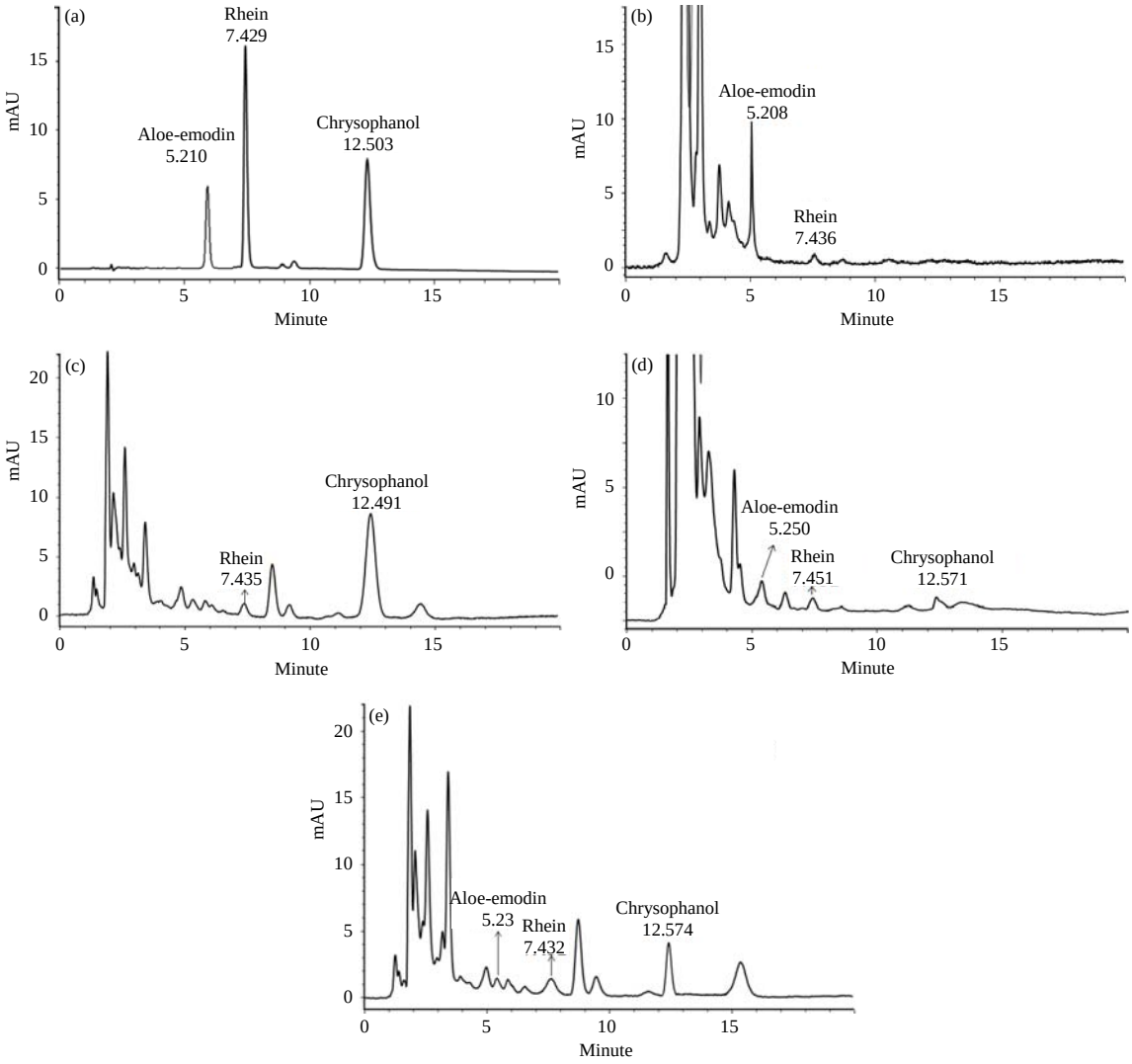

Fig. 3(a-e): HPLC chromatograms of aloe-emodin, rhein and chrysophanol (a) Reference standards of aloe-emodin, rhein and chrysophanol, (b) Plasma sample after oral administration of aloe-emodin, (c) Plasma sample after oral administration of chrysophanol, (d) Plasma sample after oral administration of *Mentha haplocalyx Briq* and (e) Plasma sample after oral administration of *Chrysanthemum morifolium*

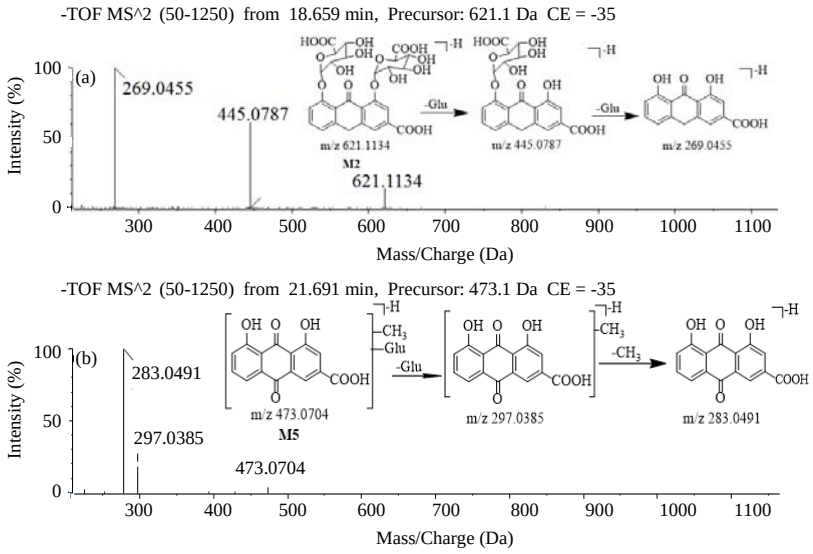

Fig. 4(a-b): Product ion spectra and proposed fragmentation pathways of (a) M2 and (b) M5

Table 2: Identification result of metabolites of rhein in multi-biosamples

No.	t_R/min	[M-H]-/[M+H]+ (m/z) Detected	ppm	Fragment ions (m/z)	Molecular formula	Mass (Da)	Identification	Distribution
M0	46.13	283.0238	-1.8	(-) 283.0238, 239.0342, 211.0389, 183.0442	$C_{15}H_8O_6$	284.0321	Rhein	p, h, l, k, u
M1	17.79	459.0563	-1.3	(-) 459.0563, 283.0233, 239.0332, 211.0374	$C_{21}H_{16}O_{12}$	460.0642	Rhein-8-O-glucuronide	p, b, h, k, u
M2	18.66	621.1134	-0.8	(-) 621.1134, 445.0787, 269.0455	$C_{27}H_{26}O_{17}$	622.1170	Rheinanthrone-1, 8-O-diglucuronide	p, b, h, l, k, u
M3	19.83	459.0559	-2.1	(-) 459.0560, 283.0228, 239.0325, 211.0379	$C_{21}H_{16}O_{12}$	460.0642	Rhein-1-O-glucuronide	p, l, k, u
M4	20.40	362.9810	3.0	(-) 362.9810, 283.0228, 239.0329, 211.0384, 183.0438	$C_{15}H_8O_9S$	363.9889	Rhein-8-O-sulfate	p, h, l, k, u
M5	21.69	473.0704	-4.3	(-) 473.0704, 297.0385, 283.0491	$C_{22}H_{18}O_{12}$	474.0798	Methoxy-rhein-O-glucuronide	p, h, l, k, u
M6	22.39	362.9816	-2.6	(-) 362.9816, 283.0227, 239.0329, 211.0379, 183.0435	$C_{15}H_8O_9S$	363.9889	Rhein-1-O-sulfate	p, k, u
M7	25.63	459.0917	-3.4	(-) 459.0915, 283.0592, 268.0359, 113.0249	$C_{22}H_{20}O_{11}$	460.1006	Methoxy-rheinanthrone-O-glucuronide	p, b, h, l, k, u
M8	31.41	445.0764	-2.2	(-) 445.0741, 269.0432, 197.0591	$C_{21}H_{18}O_{11}$	446.0849	8-Glucorhein	p, b, h, l, k, u
M9	39.40	297.0397	-2.6	(-) 297.0372, 283.0482, 225.0538	$C_{16}H_{10}O_6$	298.0477	Methoxy-rhein	p, b, h, l, k, u
M10	55.07	269.0427	-4.7	(-) 269.0427, 225.0536, 197.0591, 169.0563	$C_{15}H_{10}O_5$	270.0528	Rheinanthrone	p, b, l, k, u

p: Plasma; b: Brain; h: Heart; l: Liver; k: Kidney; u: Urine

higher than that of M10 and the other fragment ions at m/z 445.0787, 269.0455, the loss ions of 176.0347 Da and 176.0332 Da, which suggested that M2 was a diglucuronidation of rheinanthrone, hence M2 was identified as rheinanthrone-1, 8-O-diglucuronide. The proposed fragmentation pathways of M2 are shown in Fig. 4a. M5 (t_R = 21.69 min) showed an [M-H]$^-$ ion at m/z 473.0704 and the other fragment ions at m/z 297.0385, 283.0491, the loss ions of 176.0319 Da and 13.9894 Da indicated it was the glucuronide combined methyl conjugates, so M5 was tentatively identified as methoxy-rhein-O-glucuronide, the proposed fragmentation pathways of M5 are shown in Fig. 4b.

Metabolic profile of rhein: Five metabolic pathways, including reduction, glucuronidation, methylation, sulfation, glucosidation were involved in the metabolism of rhein, among them, the main metabolic pathway was glucuronidation, followed by methylation and sulfation (Fig. 5). The two novel metabolic routes of rhein were revealed, the first was rhein could be converted to phase I metabolite (anthrone, M10) via reduction, followed by diglucuronidation to form phase II metabolite M2, the second was rhein could be converted to combined metabolite M5 of glucuronidation and methylation at 1, 8 positions of anthraquinones. The graphic of tissue distribution for rhein and its metabolites is shown in Fig. 6. Rhein was distributed to all examined tissues, except for brain, where no rhein was detected, which indicated that rhein is liable to be absorbed into blood but could not pass through the blood-brain barrier (BBB). Although there was no rhein in the brain, one phase I metabolite (anthrone, M10) and five conjugated metabolites of glucuronidation, glucosidation and methylation (M1, M2, M7-M9) were distributed in brain. The tissues with the most

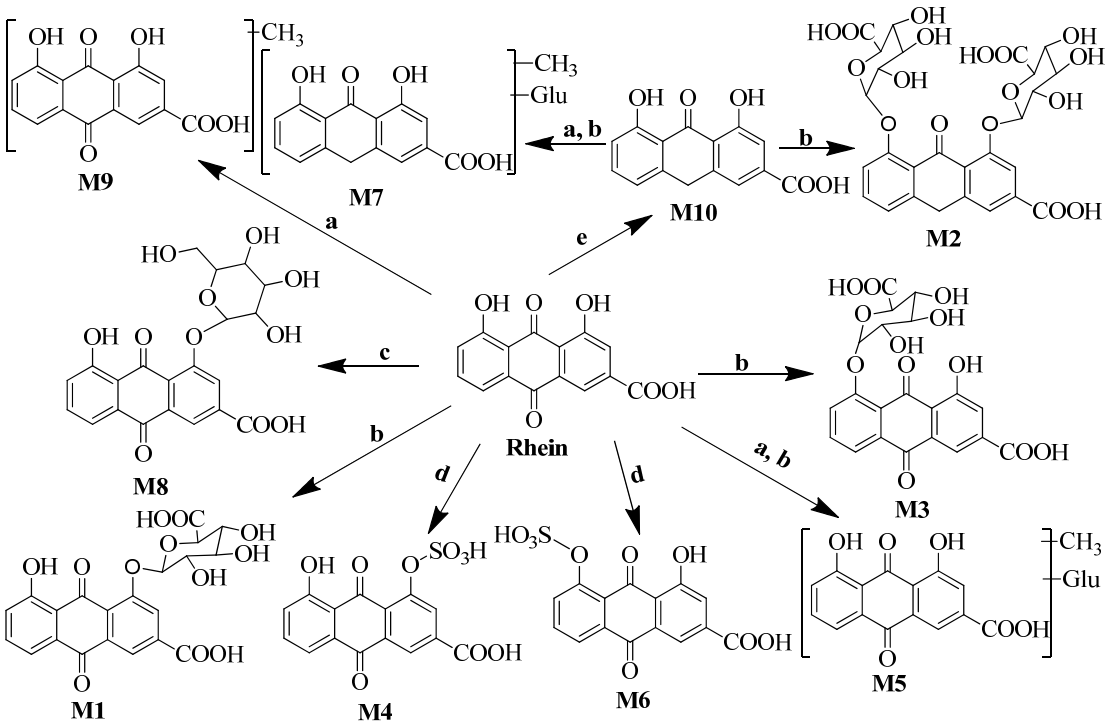

Fig. 5: Proposed metabolic pathways of rhein
a: Methylation, b: Glucuronidation, c: Glucosidation, D: Sulfation, E: Reduction

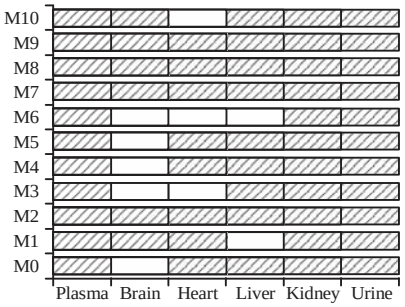

Fig. 6: Tissue distribution of rhein and its metabolites

metabolites distribution are plasma, kidney and urine in which all the metabolites were identified, which suggesting that all the metabolites of rhein exist in plasma, accumulate in kidney and then excrete with urine. In addition to the kidneys, there are abundant metabolites in the liver, in which, a total of 8 metabolites, including M2-M5, M7-M10 were distributed.

DISCUSSION

In the present study, the PK characteristic of rhein was distributed as opened two-compartment model in rats after oral administration of NHSQs, rhubarb and rhein respectively, which was consistent with other compound preparations[33,34]. The rhein showed a single, stable peak in concentration-time profile and achieved its maximum plasma concentration within 0.236-0.333 h after oral administration of NHSQs. The maximum plasma concentration was reached more rapidly than that of Dahuang Huanglian Xiexin Decoction, decoction of Fuzi Xiexin Tang (T_{max} around 1h)[35,36], Dahuang Fuzi Tang (T_{max} around 0.5 h)[37], Xiao-Cheng-Qi Decoction (T_{max} around 20.5 h)[38] and similarly with that of Quyu Qingre granules (T_{max} around 0.30h)[39], Chaiqin-Chengqi-Tang (T_{max} around 0.31 h)[40], Rhubarb-Radix scutellariae (T_{max} around 0.28 h)[41]. The T_{max} (around 0.54 h) of rhein after oral administration of rhubarb was longer than that of rhubarb extract (T_{max} around 0.20 h)[42], indicating that administration of rhubarb and rhubarb extract could result in difference on PK characteristics of rhein. The results of comparative PKs indicated the C_{max} and AUC of NHSQ groups were significantly increased compared with that of rhubarb group and rhein group which were consistent with the results of comparative PKs after administration of San-Huang-Xie-Xin-Tang (SHXXT), rhubarb and rhein[22], Chaiqin-Chengqi-Tang and Rhei Rhizoma[40], Nao Mai Tong formula and Rhubarb[43] but different from that of Da-Huang-Mu-Dan-Tang (RPD), rhubarb and rhein[23]. These studies demonstrated that different herbaceous compatibility has different effects on the PK characteristics of rhein, some

herbal formulae could promote the absorption of rhein, while some could suppress the absorption of rhein.

In the present study, two reasons were demonstrated for the increase in C_{max} and AUC. Firstly, the rhein after oral administration of NHSQs was not only come from rhubarb but also from peppermint and chrysanthemum, Secondly, aloe-emodin and chrysophanol which are common components in rhubarb, peppermint and chrysanthemum, could be metabolized to rhein, also lead to the increase of C_{max} and AUC of rhein. In addition, the effect of rhein-various ingredients interactions in NHSQs might promote the elimination of rhein and lead to less T_{max}, $t_{1/2}$. In conclusion, the NHSQ containing various ingredients have an obvious impact on the contribution of a specific chemical to absorption, among the herbs of NHSQ, rhubarb, peppermint and chrysanthemum produce additive, synergetic effects which showed an improved PK characteristics, that is higher C_{max} and AUC, shorter T_{max}, $t_{1/2}$ which was consistent with other TCM formula, such as Da-Cheng-Qi decoction[44] and Xiexin decoction[45]. This demonstrated that the administration of NHSQ would maintain higher plasma concentrations during therapeutic window than that of rhein or rhubarb, which is preferable for effective treatment.

The results of metabolic profile indicated extensive metabolism of rhein occurred in rats. Comparison of the number of conjugates showed an order of glucuronides >methides>sulfates>glucoside. The glucuronides were the dominant *in vivo* metabolite forms of rhein and distributed widely in plasma, brain, heart, liver, kidney and urine. The rhein was not found in brain but its six conjugates found in brain, which were glucuronide (M1), glucoside (M8), methides (M9), diglucuronide of anthrone (M2), methoxy glucuronide of anthrone (M8), anthrone (M10). This suggested that rhein conjugate by glucuronidation, glucosidation and methylation to form conjugated metabolites, which could improve the polarity and allow for easier diffusion and pass through BBB, a further study is warranted to investigate potential pharmacological effect or toxicity of the six metabolites on brain. More significantly, although rhein does not pass through BBB, when it is metabolized as anthrone or anthrone conjugates, they could both pass through BBB, suggesting that anthrones could pass through BBB more easily than anthraquinones. To ascertain the mechanism of this phenomenon, more detailed studies are needed.

CONCLUSION

The PK properties of rhein differed significantly after oral administration of NHSQ compared with that of rhubarb and rhein. The NHSQ was superior to rhubarb or rhein in PKs by synergetic effects of rhubarb, peppermint and chrysanthemum. Rhein could be metabolized by reduction, glucuronidation, methylation, sulfation, glucosidation and the glucuronides were the dominant *in vivo* metabolite forms of rhein and distributed widely in plasma, brain, heart, liver, kidney and urine. The present studies provide a significant basis for further development of the NHSQ and guidance for the clinical applications of NHSQ.

SIGNIFICANCE STATEMENTS

This study uncovered effects of complex ingredients in NHSQs on the PK of rhein and found the reasons for the increase of C_{max} and AUC of rhein after oral administration of NHSQ compared with that of rhubarb and rhein. Two metabolites of rhein were discovered *in vivo* for the first time and two novel metabolic routes of rhein were revealed. The metabolic profiles of rhein in rats indicated rhein and its metabolites distributed extensively *in vivo*, anthrones could pass through BBB more easily than anthraquinones. This study will help the researcher to uncover the *in vivo* metabolic fate of rhein that many researchers were not able to explore. Thus a new theory on comparative pharmacokinetics and metabolism of rhein may be arrived at.

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