Development of a high resolution mass spectrometry method for the determination of *danshensu* and salvianolic acid B

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Abstract

In this work, a highly sensitive ultrahigh-performance liquid chromatography and quadrupole Orbitrap high-resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS) method has been developed and validated by the quantifications of *danshensu* (DSS) and salvianolic acid B (SAB) in *Danhong* injection (DHI) in human plasma, urine and feces for the pharmacokinetic and excretion studies. The DSS and SAB were extracted from biological matrix by direct protein precipitation with fivefold volume of methanol and separated by a Waters CORTECS C18+ chromatographic column (100 × 2.1 mm, 2.7 μm) with gradient elution (mobile phase: methanol–H₂O, both containing 0.1% formic acid). The detection of analytes was achieved using negative ion ESI in Full MS/dd-MS² (data-dependent MS²) scan mode. The human subjects received either a single intravenous injection (i.v.) dose (containing 0.331 mg/kg DSS and 0.117 mg/kg SAB) or a single intramuscular injection (i.m.) dose (containing 0.663 mg/kg DSS and 0.234 mg/kg SAB) of DHI. For i.v., the $C_{\max}$, half-life ($t_{1/2}$) and $AUC_{0-\infty}$ were 906.2 ± 31.82 μg/L, 0.604 ± 0.103 h and 242.19 ± 18.24 μg/L × h for DSS and 1118.5 ± 35.64 μg/L, 0.893 ± 0.159 h and 277.63 ± 11.24 μg/L × h for SAB, respectively. For i.m., the $C_{\max}$, $T_{\max}$, $t_{1/2}$ and $AUC_{0-\infty}$ were 243.0 ± 29.1 μg/L, 0.67 h, 0.71 ± 0.03 h and 318.98 ± 7.62 μg/L × h for DSS and 119.4 ± 6.51 μg/L, 0.50 h, 1.08 ± 0.18 h and 185.63 ± 4.23 μg/L × h for SAB, respectively. Based on the $AUC_{0-\infty}$ obtained from i.m. and i.v. administrations, the absolute bioavailabilities (F) were estimated to be 65.9% for DSS and 33.4% for SAB.

Keywords *Danhong* injection · *Danshensu* · Salvianolic acid B · Pharmacokinetic · Excretion · Orbitrap

1 Introduction

Danhong injection (DHI) is a Chinese compound formula manufactured from two famous traditional Chinese medicines (TCMs) *Danshen* (*Salvia miltiorrhiza* Bge.) and *Honghua* (*Carthamus tinctorius* L.), both of which have been widely prescribed for the treatments of cardiovascular diseases over thousands of years in China. *Danshen* is the dried root of *Salvia miltiorrhiza* Bunge, which is a well-known TCM and widely used for treatments of cardiovascular diseases such as angina pectoris, myocardial infarction, and atherosclerosis. *Honghua*, derived from the dried flowers of *Carthamus tinctorius* Linn, is another TCM notable for its effects in therapies of cardiovascular

diseases [1–5]. Because *Danshen* is cold in nature whereas *Honghua* is warm according to the traditional Chinese medical theory, these two herbs have been frequently applied together to achieve a synergistic effect as well as to avoid side effects in clinical decoctions or Chinese patent medicines such as *Danhong* injection [6]. Nowadays in China, DHI has been commonly used in clinical treatments for the coronary heart disease, cardiac angina, acute myocardial infarction and organ protection. Restricted by their dosage forms, the hydrophilic components such as *danshensu* (DSS) and salvianolic acid B (SAB) in DHI are the main bioactive substances (chemical structures and ESI[−] full scan precursor ion mass spectra shown in Fig. 1) [7–9]. The DHI used in this study is produced by Heze

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Fig. 1 Full scan ESI[−] precursor ion mass spectra and the chemical structures of analytes

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The ever-increasing worldwide attentions to the pharmaceutical researches on TCMs require more investigations on the mechanism and pharmacokinetics of TCMs. However, the pharmacokinetic profiles of DHI in human are still unknown so far. Therefore, it is stringently necessary to develop reliable methods for the simultaneous determinations of the multiple bioactive components in DHI in biological samples of human.

Several methods have been developed for the determination of components in DHI using capillary electrophoresis (CE) [10], high-performance liquid chromatography (HPLC) [11–15] and liquid chromatography–mass spectrometry (LC–MS) [16–18]. All of the current LC–MS methods were fulfilled by coupling the liquid chromatography to a low resolution mass (LRMS) analyzer such as the ion trap (IT) or the triple-quadrupole (QqQ), while

the high resolution mass spectrometry (HRMS) has been applied to quantifications of various components of *Danhong* injection in guinea pig [19].

Orbitrap is the newest revolutionary HRMS analyzer [20]. As a new generation of the Orbitrap instruments, the Q-Orbitrap (hybrid quadrupole-orbitrap mass spectrometer) combines high-performance quadrupole precursor selection with high resolution and accurate mass (HR/AM) Orbitrap detection. Benefiting from its high resolution (> 100,000 FWHM), Orbitrap is able to well distinguish the matrix noises from the signals of the analytes, which significantly improves the sensitivity. In the present study, an ultrahigh-performance liquid chromatography (UHPLC) coupled with Q-Orbitrap HRMS was employed for the simultaneous assay of DSS and SAB in human biological samples and for the pharmacokinetic and excretion studies of the two components.

2 Materials and methods

2.1 Chemicals and reagents

DHIs (10 mL per division, lot number: 14061902) were purchased from Heze Buchang Pharmaceutical Co., Ltd. (Shandong, China). DSS (100%), SAB (96.0%) and diclofenac sodium (internal standard, IS, 100%) were obtained from National Institute for Food and Drug Control (Beijing, China). HPLC grade methanol, acetonitrile, formic acid, acetic acid, ammonium acetate and ammonium formate were all purchased from TEDIA Inc. (Fairfield, OH, USA). Ultrapure water (18.2 MΩ) was obtained from an ELGA-purelab Ultra system (High Wycombe, UK).

2.2 Instrumentation

The UHPLC-Q-Orbitrap HRMS system is comprised of an Accela 1250 LC pump, an Accela PDA detector and an Accela open auto-sampler coupled with a Q Exactive™ HR mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). XCalibur 2.2 software (Thermo Fisher Scientific, San Jose, CA, USA) was used for instrumental control and data processing, while Q Exactive 2.1 software (Thermo Fisher Scientific, San Jose, CA, USA) was used to control the mass spectrometer and for the tune application. Chromatographic separation was achieved using a CORTECS C18+ chromatographic column (100 × 2.1 mm, 2.7 μm) (Waters, Milford, USA). All centrifugations were performed on an Eppendorf 5415R Refrigerated Microcentrifuge (Eppendorf, Hamburg, Germany).

2.3 Healthy volunteers

The established bioanalytical method was applied to the pharmacokinetic and excretion studies of DSS and SAB in healthy volunteers. The clinical protocol was approved by the Medical Ethics Committee of the *Shandong Provincial Qianfoshan* Hospital prior to the study. Volunteers were given written informed consent to participate in the study according to the principles of the Declaration of Helsinki. Volunteers who submitted the agreements to take part in this project were all non-smokers and medically examined before the study. The human subjects were required to abstain from any other drug for at least 7 days prior to the starting of the test and to abstain from alcohol during the study.

2.4 Standard and QC sample preparation

2.4.1 Preparation of stock and working standard solutions

The stock standard solutions of DSS (1 mg/mL), SAB (1 mg/mL) and the IS (1 mg/mL) were prepared with methanol, respectively. The stock standard solutions of DSS and SAB were diluted with methanol to prepare a series of mixed working solutions. The stock solution of IS was diluted with methanol to make the 1 μg/mL working solution. Both of the stock and working solutions were kept at 4 °C away from light and brought to room temperature before use.

2.4.2 Preparation of quality control (QC) samples

Series of calibration standards (1, 5, 20, 50, 100, 500 and 1000 ng/mL for both DSS and SAB) were prepared by spiking 85 μL blank plasma, urine or feces with 5 μL mixed working solutions of different concentrations as mentioned above. QC samples at three levels of concentrations (low: 1.2 ng/mL; medium: 50 ng/mL; high: 800 ng/mL, for both DSS and SAB) were also independently prepared in the same way as was described above. The calibration standards and QC samples were freshly prepared before use.

2.5 Sample preparation

The human biological samples were taken out of –80 °C storage and thawed at room temperature. Then 5 μL of 20% formic acid solution and 5 μL of IS working solution (10 μg/mL) were added to 90 μL of biological matrices, which was followed by an addition of 500 μL methanol. The mixture was vortexed for 1 min and centrifuged at 13,000 rpm for 10 min. The supernatant (~400 μL) was collected and evaporated to dryness under vacuum at room temperature. The residue was reconstituted in 100 μL of initial mobile phase solution (methanol/H₂O = 10:90, v/v, 0.1% formic acid), vortexed for 1 min and centrifuged at 13,000 rpm for 10 min. Finally, the supernatant was injected for UHPLC-HRMS analysis.

2.6 Chromatographic conditions

The mobile phase was delivered at a flow rate of 300 μL/min using a gradient elution profile. The auto-sampler tray temperature, column oven temperature and injection volume were set to 20 °C, 30 °C and 5 μL, respectively. The mobile phase consisted of water (A) and methanol (B) that both contain 0.1% FA. The mobile phase was delivered with an elution gradient as follows: 0–1.0 min,

10% B; 1.0–5.0 min, 10% B → 80% B; 5.0–7.5 min, 80% B; 7.6–10 min, 10% B. The total runtime for each injection was 10 min.

2.7 Mass spectrometer conditions

High resolution MS using the Orbitrap mass spectrometer was carried out to detect the analytes. Nitrogen was employed as the sheath gas (40 units) and the auxiliary gas (15 units). Ion spray voltage, S lens RF level, capillary temperature and heater temperature were set to 2.5 kV, 50 V, 350 °C and 250 °C, respectively. The instrument was calibrated in the negative mode every 2 days using the manufacturer's calibration solutions. The analysis was performed in Full MS/dd-MS² (data-dependent MS²) mode. This type of scanning comprises a full MS scan followed by a dd-MS² scan with specific fragmentation energy. For the full MS scan, the selected scan range was from 100 to 750 *m/z* and the resolution was 70,000 (FWHM at 200 *m/z*), while the automatic gain control (AGC) and maximum injection time (IT) were set to 1.0e⁶ and 100 ms, respectively. For dd-MS² scan, the mass resolving power was set to 17,500 FWHM with a quadrupole isolation window of 0.4 Da. Other MS parameters for dd-MS² scan were applied as follows: AGC target, 2.0e⁵; maximum IT, 50 ms; under fill ratio, 1.0%; intensity threshold, 4.0e⁴; exclude isotopes, "on"; dynamic exclusion, 10.0 s. Precursor ions and corresponding normalized collision energies (NCEs) were stored in the method files. The deprotonated molecular ions [M–H][–] of DSS and SAB were detected at *m/z* of 197.0445 and 717.1462, while the optimal NCEs were 30 and 12, respectively.

2.8 Method validation

A thorough and complete method validation for assaying DSS and SAB in human plasma, urine and feces was done following the US Food and Drug Administration (FDA) guidelines [21]. The validation parameters included the selectivity, sensitivity, linearity, accuracy and precision, recovery, matrix effect and stability.

2.8.1 Selectivity

The selectivity of the method was determined by measuring the level of interfering components in six individual sources of a blank biological matrix.

2.8.2 Linearity and sensitivity

The amounts of DSS and SAB in the biological samples were quantified by using the ratio of the peak area of the analyte to that of IS as the assay parameter. The standard

curve was constructed with peak area ratios as a function of analyte concentrations, which were 1, 5, 20, 50, 100, 500 and 1000 ng/mL for both DSS and SAB. The standard curve was described in the form of $y = a + bx$. The LLOQ was determined as the lowest concentration of the calibration curve ($S/N > 10$).

2.8.3 Accuracy and precision

The accuracy and precision were calculated by determining QC samples at high, middle and low levels of concentration on three different validation days. The accuracy was measured by the relative error (RE) and the precision was measured by relative standard deviation (RSD). The acceptance values that were used for validations of RSD and RE were within 15%, except for LLOQ (within 20%).

2.8.4 Recovery

The extraction recoveries of DSS, SAB and IS from the biological matrices were determined by comparing the responses of DSS, SAB and IS in the biological matrices after extraction to those of the same concentration of analyte, which was spiked into the solution extracted from blank biological matrices. The recoveries of DSS and SAB were determined at three levels of concentration (high, medium, low), while IS at a single concentration of 50 ng/mL.

2.8.5 Matrix effect

The absolute matrix effect was determined by comparing the peak areas of analytes that were obtained from mobile phase spiked at low, middle and high concentrations (1.2, 50, 800 ng/mL, $n = 6$) and IS (50 ng/mL, $n = 6$) with spiked samples from the post-extraction blank human biological matrices.

2.8.6 Stability

The stability of the analytes in human plasma, urine and feces was investigated by quintuplicate determinations of QC samples at different concentrations (high, medium and low).

To measure the freeze and thaw stability, the QC samples were stored at –80 °C for 24 h and thawed at room temperature. The samples were refrozen for 24 h under the same conditions after being completely thawed, and this cycle was repeated three times. The concentrations of DSS and SAB were determined and the remaining percentage was calculated by comparing the concentrations with those obtained before freezing.

For short-term temperature stability, the QC samples were directly kept at room temperature for 8 h prior to analysis. For long-term stability, the QC samples were kept at -80°C for 14 days and thawed at room temperature prior to analysis. The concentrations were compared to the mean back-calculated values for the standards at the corresponding concentrations from the long-term stability testing on the first day.

The post-preparative stability was determined by repeatedly measuring the processed QC samples, which were kept in the auto-sampler (4°C) for 24 h. The concentrations of the samples were calculated on the basis of original calibration standards.

2.9 Content determination of DSS and SAB in DHI

To calculate the administration dosage, the contents of DSS and SAB in commercialized DHI were determined by an UHPLC-PDA method with the result of 1.16 and 0.409 mg/mL, respectively.

2.10 Pharmacokinetic and excretion studies of *Danhong* injection

Twelve male healthy volunteers were randomly divided into two groups (six human each group) for intravenous injection (i.v.) or intramuscular injection (i.m.), respectively. Volunteers received a single i.v. dose (containing 0.331 mg/kg DSS and 0.117 mg/kg SAB) or a single i.m. dose (containing 0.663 mg/kg DSS and 0.234 mg/kg SAB) of DHI after an overnight fasting. Volunteers were bled at each of the following time points: 0 (pre-dose), 0.033, 0.083, 0.167, 0.333, 0.5, 0.667, 1, 1.5, 2, 4, and 6 h. The blood samples were collected into 1.5 mL heparinized centrifuge tubes and kept on ice. Individual samples were centrifuged at 10,000 rpm for 10 min to retrieve the plasma. Each plasma sample was stored at -80°C until they were analyzed by UHPLC-HRMS.

Urine and feces samples were collected pre-dose and during the intervals of 0–1, 1–2, 2–4, 4–6, 6–8 and 8–12 h post-dose. The short intervals were adopted since the half-life ($t_{1/2}$) of DSS or SAB is relatively short. During these intervals, we recorded the collection as *zero* or *none* if there are no feces samples that can be collected. The volume of urine and the weight of feces were recorded; the sample amounts in each interval were recorded and compared and the cumulative sample amounts for each subject were calculated. The feces samples were homogenized with normal saline solution (250 mg feces/mL normal saline solution). The collected urine and feces samples were stored at -80°C .

2.11 Calculation

The chromatograms were processed using XCalibur 2.2 software developed by Thermo Fisher Scientific. All calculations were completed in Microsoft Excel 2010 (Microsoft Co., Redmond, WA, USA) and OriginPro 8.0 (OriginLab Co., Northampton, MA, USA). In order to acquire the pharmacokinetic parameters of DSS and SAB, the concentration–time curves were analyzed by DAS 2.0 Software (SFDA, Beijing, China). Data were expressed as mean $\pm$ SD.

3 Results and Discussion

3.1 Method development

3.1.1 Optimization of LC conditions

Chromatographic conditions, especially the composition of mobile phase, played a critical role in achieving good chromatographic behavior and appropriate ionization. Several kinds of mobile phase systems including methanol–ammonium acetate (5 mM), methanol–water (0.1% formic acid) and methanol–ammonium acetate (5 mM, containing 0.1% formic acid) were investigated. We found that the peak shapes obtained using above mobile phases were almost the same, except that the methanol–water (0.1% formic acid) provides higher sensitivity, which was thereby chosen as the mobile phase in this study. Several LC chromatographic columns such as the Waters ACQUITY UPLC BEH C_{18} column (100×2.1 mm, $1.7\ \mu\text{m}$), Waters CORTECS C_{18} column (100×2.1 mm, $2.7\ \mu\text{m}$) and Agilent Zorbax Eclipse plus C_{18} column ($100\ \text{mm} \times 2.1$ mm, $1.8\ \mu\text{m}$) were tested and compared during the initial investigation of the method. The Waters CORTECS C_{18} column (100×2.1 mm, $2.7\ \mu\text{m}$) generated a better chromatographic separation of the analytes compared to other columns and the best stability was achieved with the chosen content of the mobile phase as the RSD % of the retention time of the analyte was minimal. Therefore, the Waters CORTECS C_{18} column was used throughout this study. In addition, gradient elution was applied and the flow rate was optimized to be $300\ \mu\text{L}/\text{min}$. Chromatographic separation was accomplished on a CORTECS C_{18} chromatographic column (100×2.1 mm, $2.7\ \mu\text{m}$).

3.1.2 Optimization of HRMS conditions

As both the DSS and SAB possess carboxylic acid group, the negative ESI mode was used for the detection of the two compounds. All compounds were first analyzed individually in syringe infusion condition to obtain the deprotonated molecular ions and the optimal NCEs,

which were stored in the method files. For each analyte, the experimentally determined mass of the corresponding ion was evaluated towards the theoretical mass that was calculated by Xcalibur 2.1 software. Mass deviations were defined as $[(\text{measured mass} - \text{theoretical mass}) / \text{theoretical mass}] \times 10^6$ and expressed in ppm. In this work, under the optimized MS conditions, the deprotonated molecular ions $[M - H]^-$ of DSS and SAB were detected at m/z of 197.0452 and 717.1462, respectively. As shown in Table 1, mass deviations between the theoretical and experimental masses were less than 2 ppm for all analytes and IS, indicating a high level of mass accuracy provided by Orbitrap analyzer. In addition, MS^2 product ions were utilized for the additional confirmations of the analytes.

The parameters of the HESI source were optimized by manual tuning and the optimal parameters were summarized in Sect. 2.7. The detections of DSS and SAB were performed in the Full MS/dd- MS^2 mode, which includes a full scan event followed by MS/MS scan events of precursors in the inclusion list (Table 1). The use of an inclusion list with the mono-isotopic mass and specific NCEs of the precursors provided sensitive products as precursors are selected in the quadrupole. For the balance between selectivity and sensitivity, a mass resolution of 70,000 FWHM was selected for the full MS scan. For the dd- MS^2 scan, 35,000 FWHM was adopted to reduce the scan duration and ensure sufficient scan points of the full MS. The total ion chromatograms (TICs) were filtered through a mass tolerance of 5 ppm to reconstruct extracted ion chromatograms (XICs) (Fig. 2), which provides adequate selectivity. A mass window of 5 ppm was sufficient to decrease the backgrounds that arise from the complex matrix and to provide a sharp peak, which is representative of the specific compound without any interference (Fig. 3).

3.2 Method validation

3.2.1 Selectivity

A typical XIC of matrices spiked with 50 ng/mL DSS and SAB standards is shown in Fig. 2b. The results indicated that an excellent chromatographic separation was achieved, as the retention time (RT) of DSS, SAB and IS was 1.51 min, 4.96 min and 3.95 min, respectively. No interfering peaks

were detected at RTs of all analytes. The combination of RT and accurate mass provides highly selective detection of the compounds and powerful resolving performance to distinguish analytes from isobaric co-eluting compounds from the sample matrix.

3.2.2 Linearity and sensitivity

The calibration curves were obtained over the concentration range of 1–1000 ng/mL for both DSS and SAB in human biological matrices. The linearity of the calibration curves was evaluated by the determination coefficient (R^2). Determination coefficients (R^2) were higher than 0.9988 for all target compounds, indicating good linearity between the chromatographic peak area and the concentration of the analytes. The LLOQ for both DSS and SAB was 1 ng/mL. Such sensitivity has been proven to be satisfying for the pharmacokinetic and excretion studies of DHI in human. When it is compared with other methods, it can be concluded that the LLOQs provided by the present Q-Orbitrap method were much lower than those reported with LC-QqQ MS detection [16, 17].

3.2.3 Accuracy and precision

The results of intra- and inter-day accuracy and precision analyses performed at three levels (low, medium and high) are presented in Table 2. The averages of intra- and inter-day accuracy ranged from –2.8 to 4.1% and from –3.7 to 5.4%, respectively. The intra- and inter-day precision was in the range of 2.1–4.8% and 3.5–6.7%, respectively. These accuracy and precision results were within the acceptable criteria, showing that the developed method was reliable, reproducible and accurate for the quantitative analysis of DSS and SAB in human biological matrices.

3.2.4 Recovery and matrix effect

Recoveries were evaluated at three different concentration levels (low, medium and high). Five blank samples were spiked at each level and the results are indicated in Table 3. The recoveries of all compounds were $\geq 79.6\%$, indicating the reliability of the developed method.

Table 1 Elemental composition, accurate mass, mass deviation and NCEs of analytes and IS

Analyte	Elemental composition	Retention time (min)	Accurate mass		Mass deviation (ppm)	Normalized collision energy (NCE)
			Theoretical	Experimental		
DSS	C ₉ H ₉ O ₅	1.51	197.0455	197.0452	–1.52	30
SAB	C ₃₆ H ₃₀ O ₁₆	4.96	717.1461	717.1462	1.39	12
IS	C ₁₄ H ₁₀ Cl ₂ NO ₂	3.95	294.0099	294.0095	–1.36	30

Fig. 2 Representative XICs of blank plasma (**a**), plasma spiked with analytes and IS (**b**) and real plasma sample (**c**) obtained 5 min after an i.v. administration of DHI, respectively

Fig. 2 (continued)

It should be noted that the matrix components can reduce or enhance the responses of the ion source, and therefore matrix effect must be evaluated. The results of matrix effects evaluation are shown in Table 3. Signal suppression or enhancement effect was considered to be tolerable if the value of matrix effect was between 80 and 120%. As shown in Table 3, the matrix effects of analytes range from 83.7 to 102.7% with SDs < 7.3%, indicating that the human biological matrices have tolerable low suppression on the MS responses of the analytes.

3.2.5 Stability

The results of stability assessments were summarized in Table 4 and indicate that both DSS and SAB were stable in the auto-sampler (24 h) at 4 °C, on bench-top (8 h) at room temperature, through repeated three freeze/thaw cycles as well as under the frozen condition at -80 °C for 14 days. These results suggest that the established method for sample extraction, storage, and intermittent analysis were validated and suited for large-scale sample analysis.

3.3 Plasma pharmacokinetics of DHI

The plasma concentration–time course of the DSS and SAB in volunteers who were given a single i.m. or i.v. dose was

illustrated in Fig. 4. The non-compartmental pharmacokinetic parameters, as showed in Tables 5 and 6, were calculated from the plasma concentration versus time using DAS 2.0 software.

Figure 4 shows the plasma concentrations of DSS after i.m. or i.v. administration. For i.v., during the initial 0.5 h, the plasma concentration of DSS decreased rapidly and then reached an elimination phase at 1 h. During 1–4 h, the plasma concentration of DSS decreased slowly until below LLOQ. The C_{max} , half-life ($t_{1/2}$), $AUC_{0-\infty}$ and MRT_{0-t} are $906.2 \pm 31.82 \mu\text{g/L}$, $0.604 \pm 0.103 \text{ h}$, $242.19 \pm 18.24 \mu\text{g/L} \times \text{h}$ and $0.463 \pm 0.022 \text{ h}$, respectively. For i.m., plasma concentrations of DSS increased quickly to the C_{max} ($243.0 \pm 29.1 \mu\text{g/L}$) during the initial 0.67 h after dosing. Plasma concentrations decreased rapidly to $23.0 \pm 3.1 \mu\text{g/L}$ in the following 1.33 h, followed by a gradual decrease to LLOQ. The $t_{1/2}$ and MRT_{0-t} are both slightly longer than that of i.v. and are $0.71 \pm 0.03 \text{ h}$ and $1.24 \pm 0.11 \text{ h}$, respectively. This reveals that the residence time of DSS in vivo was very short for both i.m. and i.v. The above data also indicate that DSS in DHI show quick and short pharmacological effects in vivo. The absolute bioavailability (F) of DSS was evaluated as 65.9% ($F\% = [AUC_{0-\infty(i.m.)} \times Dose_{(i.v.)}] / [AUC_{0-\infty(i.v.)} \times Dose_{(i.m.)}] \times 100$) [22–24], based on the value of $AUC_{0-\infty}$ calculated from i.m. and i.v. administrations. As shown in Fig. 4 and Table 6, the plasma concentrations of SAB reached its C_{max} (119.4 ± 6.51) at 0.50 h (T_{max}), followed

Fig. 3 Product ion mass spectra of DSS (a) and SAB (b)

by a decrease to LLOQ for a long time (6 h) with the $t_{1/2}$ being 1.08 ± 0.18 h for i.m. However, the plasma concentrations of SAB underwent a faster decrease for i.v, as it is unable to be detected after 6 h. The absolute bioavailability of SAB is 33.4% based on the calculated $AUC_{0-\infty}$ values for i.m. and i.v.

Prior to our work, Fu et al. [25] have reported a pharmacokinetic study of DSS in human plasma after oral administration of *Danshen* granules and Shen and Cao [26] reported the simultaneous determination of seven components including DSS and SAB in human plasma following oral administration of *Danqi* tablets. The former evaluated the pharmacokinetic parameters $t_{1/2}$, MRT and $AUC_{0-\infty}$ of DSS as 1.65 ± 0.35 h, 2.19 ± 0.21 h and 491 ± 123 $\mu\text{g/L} \times \text{h}$, respectively. The latter found that the C_{max} , $t_{1/2}$, MRT and $AUC_{0-\infty}$ values are 400.21 ± 56.47 ng/mL, 2.47 ± 0.87 h, 5.35 ± 1.02 h and 2251.46 ± 183.32 $\mu\text{g/L} \times \text{h}$ for DSS and 260.54 ± 35.24 ng/mL, 3.64 ± 0.65 h, 5.87 ± 0.58 h and

1175.2 ± 185.32 $\mu\text{g/L} \times \text{h}$ for SAB; the DSS shows high plasma exposure after oral administration of *Danqi* tablets with AUC_{0-t} of 2247.51 ± 198.32 $\mu\text{g/L} \times \text{h}$. Taking the difference in dose into consideration (4.42 mg/kg vs. 0.663 mg/kg (i.m.) for DSS), the converted $AUC_{0-\infty}$ value of DSS in the literature of *Danqi* tablets (337 $\mu\text{g/L} \times \text{h}$) was pretty close to the $AUC_{0-\infty}$ value (318.98 ± 7.62 $\mu\text{g/L} \times \text{h}$) in the present study. There are significant differences in the $AUC_{0-\infty}$ values for SAB between the literature and our work.

There are several references that report the pharmacokinetics of DSS in *Danhong* lyophilized powder (DLPI) [16, 17]. After an intravenous dose of DLPI containing 0.29 mg/kg DSS, the pharmacokinetic parameters $t_{1/2}$, MRT and $AUC_{0-\infty}$ were determined as 0.58 ± 0.20 h, 1.36 ± 0.63 h and 398.65 ± 219.61 $\mu\text{g/L} \times \text{h}$, respectively. It should be noted that in this literature the $t_{1/2}$ (0.58 ± 0.20 h) is almost the same as that of the present study (0.604 ± 0.103 h). However, significant difference exists in the MRT values

Table 2 Intra- and inter-day accuracy and precision for the determination of DSS and SAB in human plasma, urine and feces ($n = 5$)

Sample matrix	Compound	QC concentration (ng/mL)	Intra-day		Inter-day	
			Accuracy (RE, %)	Precision (RSD, %)	Accuracy (RE, %)	Precision (RSD, %)
Plasma	DSS	1.2	3.6	4.8	5.4	6.7
		50	−2.8	3.4	−3.7	4.6
		800	−1.6	3.8	−2.5	5.2
	SAB	1.2	2.7	3.2	3.3	4.7
		50	4.1	4.3	4.2	6.0
		800	−2.2	2.1	−2.8	3.5
Urine	DSS	1.2	10.4	−6.3	12.0	−8.7
		50	6.5	3.5	8.7	−6.8
		800	4.3	2.8	6.4	6.0
	SAB	1.2	8.5	7.4	9.9	7.2
		50	5.7	−4.5	7.6	3.8
		800	6.2	3.7	9.4	6.5
Feces	DSS	1.2	5.8	−7.1	7.9	−4.4
		50	5.4	−4.5	8.4	−7.8
		800	3.2	2.0	8.2	−3.5
	SAB	1.2	9.2	8.4	10.3	8.5
		50	6.7	3.3	9.2	6.7
		800	8.9	2.1	10.7	7.1

Table 3 Matrix effect and extraction recovery for the assay of DSS, SAB and IS in human plasma, urine and feces ($n = 5$)

Sample matrix	Compound	QC concentration (ng/mL)	Matrix effect (Mean $\pm$ SD, %)	Extraction recovery (Mean $\pm$ SD, %)
Plasma	DSS	1.2	102.7 $\pm$ 7.2	85.2 $\pm$ 5.5
		50	88.6 $\pm$ 5.4	86.3 $\pm$ 3.2
		800	93.9 $\pm$ 7.3	91.8 $\pm$ 6.1
	SAB	1.2	94.3 $\pm$ 4.8	79.6 $\pm$ 6.5
		50	83.7 $\pm$ 6.0	84.1 $\pm$ 4.7
		800	88.5 $\pm$ 5.6	92.6 $\pm$ 3.5
Urine	IS	50	91.3 $\pm$ 3.1	87.5 $\pm$ 4.2
		1.2	86.2 $\pm$ 7.8	95.3 $\pm$ 6.4
		800	94.7 $\pm$ 16.1	98.1 $\pm$ 7.7
	DSS	50	91.4 $\pm$ 10.8	96.2 $\pm$ 4.8
		1.2	88.8 $\pm$ 14.1	93.0 $\pm$ 5.3
		800	90.2 $\pm$ 10.3	98.6 $\pm$ 4.4
Feces	SAB	50	94.4 $\pm$ 8.0	95.9 $\pm$ 6.8
		1.2	87.8 $\pm$ 8.5	86.4 $\pm$ 5.1
		800	84.3 $\pm$ 12.7	92.5 $\pm$ 5.8
	DSS	50	94.6 $\pm$ 8.6	93.6 $\pm$ 3.0
		800	86.0 $\pm$ 11.2	97.1 $\pm$ 9.4
		1.2	82.4 $\pm$ 6.8	85.2 $\pm$ 7.1
	IS	50	85.7 $\pm$ 9.2	89.6 $\pm$ 5.5
		800	90.6 $\pm$ 14.0	91.9 $\pm$ 6.4
			84.5 $\pm$ 7.9	84.0 $\pm$ 5.1

Table 4 Stability of DSS and SAB in human plasma, urine and feces ($n=5$)

Sample matrix	Compound	QC concentration (ng/mL)	Short-term stability (mean $\pm$ SD, %)	Post-preparative stability (mean $\pm$ SD, %)	Freeze and thaw stability (mean $\pm$ SD, %)	Long-term stability (mean $\pm$ SD, %)
Plasma	DSS	1.2	103.5 $\pm$ 4.6	96.7 $\pm$ 3.8	93.8 $\pm$ 5.6	95.0 $\pm$ 6.7
		50	98.1 $\pm$ 2.1	97.5 $\pm$ 3.4	95.6 $\pm$ 5.1	96.8 $\pm$ 4.6
		800	101.7 $\pm$ 1.2	102.2 $\pm$ 1.7	96.8 $\pm$ 3.7	98.4 $\pm$ 3.1
	SAB	1.2	97.3 $\pm$ 3.4	97.8 $\pm$ 4.5	95.4 $\pm$ 6.1	93.7 $\pm$ 7.4
		50	102.4 $\pm$ 3.2	101.3 $\pm$ 2.9	95.8 $\pm$ 4.6	94.9 $\pm$ 4.0
		800	99.1 $\pm$ 2.8	106.5 $\pm$ 2.5	97.5 $\pm$ 3.1	96.4 $\pm$ 5.6
	DSS	1.2	92.3 $\pm$ 5.8	94.3 $\pm$ 4.2	90.6 $\pm$ 7.2	98.7 $\pm$ 4.5
		50	93.5 $\pm$ 3.3	100.4 $\pm$ 3.6	87.2 $\pm$ 5.4	105.6 $\pm$ 2.7
		800	91.2 $\pm$ 5.4	102.7 $\pm$ 4.7	86.5 $\pm$ 7.0	101.2 $\pm$ 6.2
Urine	SAB	1.2	106.7 $\pm$ 3.5	98.2 $\pm$ 2.8	87.4 $\pm$ 6.7	97.1 $\pm$ 3.2
		50	92.6 $\pm$ 2.7	95.9 $\pm$ 5.3	93.4 $\pm$ 4.7	95.5 $\pm$ 4.4
		800	105.4 $\pm$ 4.8	97.3 $\pm$ 3.0	91.5 $\pm$ 8.6	105.2 $\pm$ 6.7
	DSS	1.2	92.4 $\pm$ 5.5	104.2 $\pm$ 3.6	91.5 $\pm$ 5.4	103.8 $\pm$ 3.5
		50	95.3 $\pm$ 3.8	101.7 $\pm$ 2.2	95.6 $\pm$ 5.7	95.5 $\pm$ 1.7
		800	92.0 $\pm$ 2.4	96.8 $\pm$ 3.1	94.2 $\pm$ 6.6	97.2 $\pm$ 4.5
	SAB	1.2	93.1 $\pm$ 3.5	95.2 $\pm$ 2.8	105.1 $\pm$ 4.7	107.7 $\pm$ 4.4
		50	94.3 $\pm$ 1.6	99.8 $\pm$ 1.6	103.6 $\pm$ 5.0	103.2 $\pm$ 3.1
		800	94.9 $\pm$ 5.2	102.6 $\pm$ 4.2	93.4 $\pm$ 4.7	104.3 $\pm$ 3.8

($P < 0.05$) between the literature (1.36 ± 0.63 h) and the present study (0.463 ± 0.022 h), indicating that the retention time of DSS in beagle dogs was much longer than that in human. Because the SD value of the $AUC_{0-\infty}$ in the literature was too high, it would be statistically meaningless to compare the $AUC_{0-\infty}$ parameter between the two studies. Since the literature did not investigate the pharmacokinetic profiles for i.m., the bioavailability was not given.

Wang et al. [18] have investigated the pharmacokinetics of SAB in DHI in rats and provided the plasma concentrations at each blood sampling time point. However, the pharmacokinetic parameters were not given. Zhan et al. [17] have reported the pharmacokinetics of SAB in DLPI in beagle dogs. After an intravenous dose of DLPI containing 0.91 mg/kg SAB, the pharmacokinetic parameters $t_{1/2}$, MRT and $AUC_{0-\infty}$ were evaluated as 0.848 ± 0.926 h, 1.27 ± 0.42 h and 2331.95 ± 595.31 $\mu\text{g/L} \times \text{h}$, respectively. Similar with the case of DSS, the $t_{1/2}$ values of SAB in this literature (0.848 ± 0.926 h) and in the present study (0.893 ± 0.159 h) were very close, while there exist statistically significant differences ($P < 0.05$) in the MRT values. Considering the difference of the dose (0.91 mg/kg vs. 0.117 mg/kg), the converted $AUC_{0-\infty}$ value of 299.82 $\mu\text{g/L} \times \text{h}$ from the literature was very close to that of the present study (277.63 ± 11.24 $\mu\text{g/L} \times \text{h}$). Via the above discussion, it can be concluded that there are no species differences in the total amount of drug in blood (AUC) and elimination rate ($t_{1/2}$) between the DSS and SAB in beagle dogs and those in human.

3.4 Excretion studies of DHI

The cumulative excretions of DSS and SAB in urine and feces after i.v. and i.m. administrations were investigated. The urinary and fecal cumulative excretions of DSS or SAB reached the plateau at 8 h after dosing. The results show that 37.4% or 0.635% of the administered dose of DSS was recovered in urine or feces, respectively. These indicate that the renal excretion was the dominant excretion pathway of DSS. On the contrary, 1.72% and 43.8% of the administered dose of SAB were recovered in urine and feces, respectively, which indicate that SAB was mainly excreted by feces. In this study, the relative molecular masses of DSS and SAB were 198 and 718, respectively, which was probably the reason for the difference in excretion pathways between DSS and SAB.

4 Conclusions

This paper presents an UHPLC-HRMS determination of DSS and SAB in human plasma, urine and feces with good sensitivity and selectivity. There was no endogenous interference with the analysis of the analytes. The developed and validated method was successfully applied for evaluation of the pharmacokinetic and excretion of DHI in human. The pharmacokinetic parameters $T_{\max}$, $t_{1/2}$ and MRT values show that DSS and SAB can be rapidly absorbed and quickly eliminated in plasma. The absolute bioavailabilities

Fig. 4 Mean plasma concentration–time curves of DSS in human given a single i.v. (a) or i.m. (b) administration of DHI ($n=6$). Mean plasma concentration–time curves of SAB in human given a single i.v. (c) or i.m. (d) administration of DHI ($n=6$)

Table 5 Pharmacokinetic parameters of DSS in human plasma following i.v. or i.m. administration of *Danhong Injection* ($n=6$)

Parameters	Mean $\pm$ SD (for i.v.)	Mean $\pm$ SD (for i.m.)
AUC_{0-t} ($\mu\text{g/L} \times \text{h}$)	241.26 ± 17.64	317.56 ± 8.01
$AUC_{0-\infty}$ ($\mu\text{g/L} \times \text{h}$)	242.19 ± 18.24	318.98 ± 7.62
$AUMC_{0-t}$ ($\mu\text{g/L} \times \text{h}$)	111.38 ± 2.85	394.34 ± 25.04
$AUMC_{0-\infty}$ ($\mu\text{g/L} \times \text{h}$)	137.74 ± 4.01	441.28 ± 34.68
MRT_{0-t} (h)	0.463 ± 0.022	1.24 ± 0.11
$MRT_{0-\infty}$ (h)	0.57 ± 0.026	1.39 ± 0.14
VRT_{0-t} (h^2)	0.593 ± 0.071	1.23 ± 0.14
$VRT_{0-\infty}$ (h^2)	1.027 ± 0.059	1.91 ± 0.27
$t_{1/2\alpha}$ (h)	0.604 ± 0.103	0.71 ± 0.03
$T_{\max}$ (h)	0.033 ± 0	0.67 ± 0
CL_z/F (L/h/kg)	0.017 ± 0.001	12.54 ± 0.3
V_z/F (L/Kg)	0.014 ± 0.001	12.94 ± 0.78
Zeta	1.166 ± 0.199	0.97 ± 0.04
$C_{\max}$ ($\mu\text{g/L}$)	906.2 ± 31.8	243.0 ± 29.1
Bioavailability (%)	65.9	

Table 6 Pharmacokinetic parameters of SAB in human plasma following i.v. or i.m. administration of *Danhong Injection* ($n=6$)

Parameters	Mean $\pm$ SD (for i.v.)	Mean $\pm$ SD (for i.m.)
AUC_{0-t} ($\mu\text{g/L} \times \text{h}$)	272.74 ± 14.05	181.04 ± 6.47
$AUC_{0-\infty}$ ($\mu\text{g/L} \times \text{h}$)	277.63 ± 11.24	185.63 ± 4.23
$AUMC_{0-t}$ ($\mu\text{g/L} \times \text{h}$)	135.54 ± 0.23	263.97 ± 5.82
$AUMC_{0-\infty}$ ($\mu\text{g/L} \times \text{h}$)	172.56 ± 0.07	345.31 ± 5.8
MRT_{0-t} (h)	0.498 ± 0.025	1.46 ± 0.02
$MRT_{0-\infty}$ (h)	0.622 ± 0.025	1.86 ± 0.07
VRT_{0-t} (h^2)	0.594 ± 0.011	2.05 ± 0.12
$VRT_{0-\infty}$ (h^2)	1.188 ± 0.051	4.08 ± 0.58
$t_{1/2\alpha}$ (h)	0.893 ± 0.159	1.08 ± 0.18
$T_{\max}$ (h)	0.033 ± 0	0.50 ± 0
CL_z/F (L/h/kg)	0.015 ± 0.001	21.55 ± 0.49
V_z/F (L/Kg)	0.019 ± 0.004	33.77 ± 6.47
Zeta	0.79 ± 0.141	0.65 ± 0.11
$C_{\max}$ ($\mu\text{g/L}$)	1118.5 ± 35.64	119.4 ± 6.51
Bioavailability (%)	33.4	

(F) were calculated to be 65.9% for DSS and 33.4% for SAB, indicating considerable oral absorptions for both DSS and SAB. Renal excretion was the dominant excretion pathway of DSS, while SAB was mainly excreted by feces. The data and results would provide useful information for the clinical applications of DHI and further studies of DHI preparation.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in this study.

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