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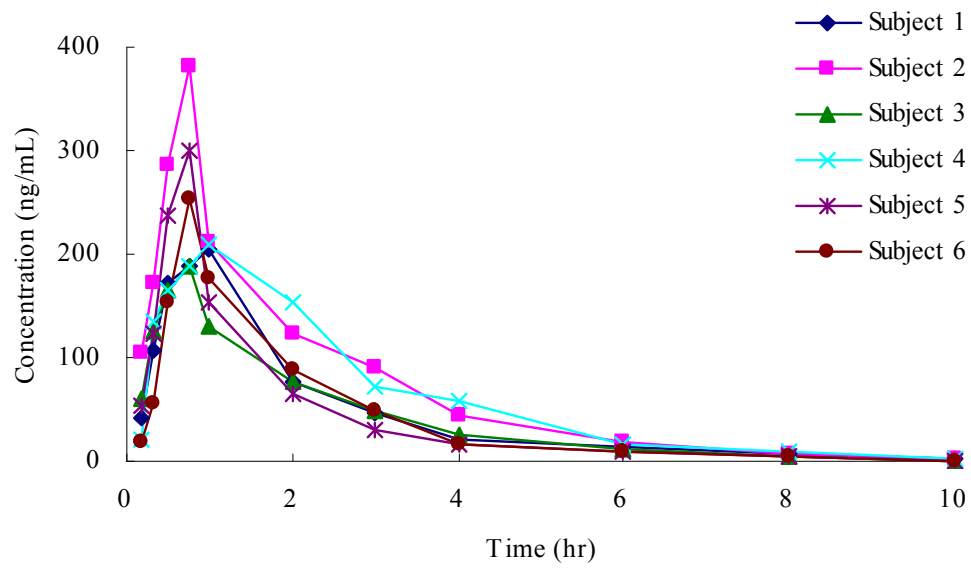
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Individual plasma concentration–time curves of danshensu in Chinese healthy subjects after oral administration of Danshen granules (n=6)



**Application of a Simple and Rapid LC-MS/MS Method for Determination of Danshensu
in Human Plasma for an Oral Pharmacokinetic Study of Danshen Granules in Chinese**

Healthy Subjects

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ABSTRACT

A simple and rapid liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) method was developed and validated for the determination of danshensu in plasma of Chinese healthy subjects after oral administration of Danshen granules. After liquid-liquid extraction (LLE) with ethyl acetate, danshensu and chloroamphenicol (internal standard, IS) were separated on an Agilent Zorbax XDB-C₁₈ column using a gradient mobile phase consisting of water (0.1% formic acid)-acetonitrile (0.1% formic acid) at a flow rate of 0.45 mL/min. The detection was performed in multiple reaction monitoring (MRM) mode, using the transitions of m/z 196.9→134.8 and m/z 320.9→151.9 for danshensu and chloroamphenicol, respectively. The method was linear over the range of 0.50–500 ng/mL using only 100 μ L of plasma and the lower limit of quantification (LLOQ) was 0.50 ng/mL. The intra-day and inter-day precisions (% RSD) were all less than 15% and the accuracies (% RE) were within the range of \pm 15%, and recoveries were between 85.0% and 115%. The validated method was successfully applied to an explorative pharmacokinetic study of danshensu in Chinese healthy subjects after oral administration of Danshen granules. After oral administration, T_{\max} and C_{\max} values of danshensu were found to be 0.83 ± 0.13 hr and 257 ± 73.8 ng/ml, respectively. Plasma concentrations declined with $t_{1/2Z}$ of 1.65 ± 0.35 hr.

Key Words: Danshensu; LC-MS/MS; Pharmacokinetics; Danshen Granules

1. Introduction

Danshen granules is a kind of Chinese medicine approved by China State Food and Drug Administration (SFDA) for treatment of cardiac dysfunction, which is composed of only one traditional Chinese medicines *Radix Salviae Miltiorrhizae* (Chinese name Danshen). *Radix Salviae Miltiorrhizae*, the dried root of *Salviae Miltiorrhizae* Bunge, is a commonly used natural medicine for treating coronary heart disease, cerebrovascular disease, hepatitis, hepatocirrhosis, chronic renal failure, dysmenorrheal and neurosasthenic insomnia in China and Southeast Asia [1–3].

There are all kinds of components existing in Danshen granules, including danshensu, tanshinone, cryptotanshinone, isotanshinones, miltirone etc. Among them, danshensu is usually considered to be one of the main therapeutic components for cardiac dysfunction. Also, danshensu is now used as one marker compound for the quality control of Danshen granules. Therefore, danshensu will be used as a target biomarker compound for the human pharmacokinetic evaluation of Danshen granules [4-5].

For the quantification of danshensu in complicated biological samples, liquid chromatography coupled to fluorescent [6], UV [7-9] or MS [9-16] methods have been reported depending on the required sensitivity, the biological matrix, and the applied pretreatment/workup procedures. HPLC-UV and fluorescent methods were general analytical methods for determination of danshensu in biological fluids, however they both showed poor sensitivity (lower limit of quantification (LLOQ) usually above 0.1 µg/mL). LC–tandem mass spectrometry (LC–MS/MS) has been used to identify and quantify danshensu in biological fluids with LLOQ of about 5-50 ng/mL [11, 12, 16]. Nevertheless, methodological improvements for the quantitative determination of genistein in biological fluids are still needed. And, to our best knowledge, no bioanalytical methods have been reported for the quantification of danshensu in human plasma after oral administration of Danshen granules till now. Furthermore, no PK results of

Danshen granules in human subjects have been reported.

In present study, a simple and rapid liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated for the quantification of danshensu in the plasma of Chinese healthy subjects, and the validated method has been successfully applied to an explorative pharmacokinetic study of danshensu in Chinese healthy subjects following oral administration.

2. Experimental

2.1. Materials and reagents

Reference standard of sodium danshensu (Purity 100%, Lot No. 110855-200809) was purchased from National Institutes for Food and Drug Control (Beijing, China). Chloroamphenicol (Purity 99.5%, Lot No K0350706) was purchased from China Institute of Veterinary Drugs Control (Beijing, China) used as the internal standard (IS). The chemical structures of danshensu and chloroamphenicol are shown in Fig. 1. Danshen Granules (1.5 g/pouch, equivalent to 10 grams of *Radix Salviae Miltiorrhizae*, Batch No 1304002H) were purchased from China Resources Sanjiu Medical & Pharmaceutical Co., Ltd (Shenzhen, Guangdong, China). Methanol and acetonitrile of HPLC-grade were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (Purity 96%) was purchased from Tedia Company Inc. (Fairfield, OH, USA) and Dikma Technologies (Beijing, China), respectively. Deionized water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA) and other common chemicals were provided by standard commercial sources and were of the highest quality available. Pooled plasma from healthy subjects (danshensu-free and anti-coagulated with ETDA-2Na) was prepared in our laboratory.

2.2. LC–MS/MS instrumentation and analytical conditions

The HPLC system consisted of an LC-20AD pump, a DGU-20 A₃ degasser, an SIL-20AC autosampler and a CTO-20A column oven (Shimadzu, Japan). The HPLC separation was performed on an Agilent Zorbax

XDB-C₁₈ column (2.1 mm × 50 mm, 3.5 μm) with a gradient elution by a mobile phase consisting of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) with following gradient: 0.00 min 2% B, 0.50 min 2% B, 2.00 min 98% B, 3.00 min 98% B, 3.01 min 2% B, 4.50 min 2% B, with the flow rate of 0.45 mL/min. The injection volume was set to be 10 μL.

The HPLC system was coupled with an API 4000 Qtrap mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) via a Turbo IonSpray ionization interface. Following optimization of the setting parameters, the ESI source was operated in negative mode with the curtain, nebulizer and turbo-gas (all nitrogen) set at 15, 65 and 55 psi, respectively. The source temperature was 600°C and the ion spray needle voltage was -4200 V. The mass spectrometer was operated at unit resolution for Q1 and low resolution for Q3 in the multiple reaction monitoring mode, with a dwell time of 150 ms per multiple reaction monitoring channel. The collision activated dissociation (CAD) gas level was set at medium. Two MRM transitions (m/z 196.9→134.8, danshensu; m/z 320.9→151.9, IS) were recorded and used for quantification. The optimized collision energies for the transitions of danshensu and IS were set at -24 and -20 eV, respectively. The declustering potential (DP) was set at -70 and -50 eV for danshensu and IS, respectively. Data were collected and analyzed by the Analyst Data Acquisition and Processing software (Version 1.5.2, Applied Biosystems/MDS Sciex, Concord, ON, Canada).

2.3. Preparation of calibration standards and quality control (QC) samples

Stock standard solution of danshensu at 1.00 mg/mL was prepared in duplicate by dissolving the accurately weighed reference standards in DMSO for preparation of calibration standards and QC, respectively. Series of standard combined dilutions were prepared in methanol at 5.00, 10.0, 20.0, 50.0, 200, 500, 2000 and 5000 ng/mL for danshensu. Calibration standards were prepared at 0.50, 1.00, 2.00, 5.00, 20.0, 50.0, 200 and 500 ng/mL by adding 10 μL of standard combined dilutions to 100 μL of analyte-free plasma of

healthy subjects. The QC samples were similarly prepared at concentrations of 1.00, 20.0 and 400 ng/mL for low, medium and high QC, respectively. IS working solution at 2000 ng/mL was prepared by diluting the chloroamphenicol stock solution (1.00 mg/mL) with methanol/water (1:1, v/v). All the solutions were kept refrigerated (4°C) and were brought to room temperature before use.

2.4. Sample preparation

After thaw at room temperature for about 30 min and vortex for 30 s, aliquots of 100 µL plasma were mixed with 10 µL of methanol (or standard or QC solution), 10 µL of IS solutions (2000 ng/mL chloroamphenicol in methanol/water (1:1, v/v)), 20 µL of 1 mol/L HCl solution and 600 µL of ethyl acetate. After vortex for 1 min and then centrifugation at 10000 g for 10 min, aliquots of 450 µL supernatants were removed and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residues were dissolved in 100 µL of the mixture of methanol and water (1:1, v/v), and then transferred to HPLC vials. A volume of 10 µL of this solution was then injected onto the column for LC–MS/MS analysis.

2.5. Method validation

The method was validated for selectivity, linearity, precision and accuracy, matrix effect, recovery and stability according to the FDA guidelines [17].

To investigate the selectivity, one pooled batch of blank plasma from six different healthy subjects and several spiked plasma samples at LLOQ level were pretreated and analyzed in parallel. The obtained responses were compared with those of LLOQ. The peak area of coeluting interferences should be less than 20% of the peak area of the LLOQ.

Calibration standards were prepared and analyzed in duplicate in three consecutive days. The peak area ratios (danshensu versus IS) versus the nominal concentrations of danshensu were calculated to construct the calibration curves. The calibration curves were fitted via a 1/x² weighted linear least-squares

regression model.

The accuracy and precision of the method were determined by analyzing the QC samples at three concentrations in six replicates on three consecutive days. The accuracy and precision are expressed in terms of relative error (% RE) and relative standard deviation (% RSD), respectively. The intra and inter-day precision should not exceed 15% for all three QC levels. Accuracy (% RE) should be within \pm 15% for QC samples.

The recovery of danshensu was determined at three QC levels by comparing the mean peak areas of QC samples ($n = 6$) with those of the blank plasma samples ($n = 3$) spiked with working solutions after pretreatment. The recovery of IS was determined using a similar method.

To evaluate the matrix effect in the experiment, six different lots of blank plasma were pretreated and then spiked with QC solutions. Chromatographic peak areas of each analyte from the spike-after-pretreatment samples were compared to those of the solution standards at equivalent concentrations [18]. In the present study, the matrix effect was evaluated at three concentration levels for each analyte. The matrix effect for IS was determined in a similar way at 200 ng/mL. Inter-subject variability at the matrix effect should be less than 15% [19].

In present study, the stability of danshensu was investigated by analyzing replicates ($n = 3$) of plasma samples at three concentration levels, which were exposed to different conditions (processed, time and temperature). The analyte was considered stable under the prescribed conditions only if 85–115% of the nominal concentrations were found.

2.6. Method application

This validated LC-MS/MS method would be applied to determination of plasma concentrations of danshensu in Chinese healthy subjects. The clinical studies were approved by the Ethics Committees of

Beijing Friendship Hospital. Six healthy Chinese subjects were enrolled in this explorative study to investigate the safety, tolerability, and pharmacokinetics of Danshen granules. All the subjects have signed the informed consent forms prior to the clinical trials. Blood samples (about 500 μL /time point) were collected as the following time points: pre-dose and at 10, 20, 30, 45 min and 1, 2, 3, 4, 6, 8, and 10 hr after oral dose of Danshen granules (1 pouch, 0.5 g/pouch). Plasma samples were separated by centrifugation (5000 g for 10 min) and then stored at -20°C until analysis.

Pharmacokinetic parameters including half-life ($t_{1/2Z}$), maximum plasma time (t_{max}) and concentration (C_{max}), area under concentration–time curve (AUC_{0-t} and $\text{AUC}_{0-\infty}$), mean residence time (MRT) of danshensu were analyzed by non-compartmental method using Drug and Statistics (DAS) 2.0.1 pharmacokinetic program (Chinese Pharmacology Society, Beijing, China). All mean results were expressed as arithmetic mean \pm standard deviation (SD).

3. Results and Discussion

3.1. Optimization of LC-MS/MS conditions

There are carboxyl and hydroxyl groups in the structure of danshensu. Hence, negative ion detection mode was finally chosen after experiment confirmation. The full-scan product ion mass spectra and fragmentation patterns of danshensu and chloroamphenicol are shown in Fig. 1. In the Q1 full scan experiment, distinct deprotonated molecules were observed for danshensu (m/z 196.9), and no significant adductive ions were detected. Usually the most abundant fragment ion was selected in the MRM transitions. For danshensu, the most abundant ion was observed at m/z 178.8 (a dehydrated ion). However, the background noise of the transition (m/z 196.9 \rightarrow 178.8) was relative higher than that of the transition (m/z 196.9 \rightarrow 134.8). Therefore, the MRM transition of m/z 196.9 \rightarrow 134.8 was finally chosen as it provided a better signal-to-noise ratio (S/N), reproducibility and response than other transitions.

During the optimization of chromatographic conditions, danshensu might be extensively retained on several kinds of columns due to its physical-chemical properties. To achieve symmetric peak shapes and shorten chromatographic running time compromising the potential matrix effect mostly arising from all kinds of coexisting components, the mobile phase consisting of acetonitrile with 0.1% formic acid and water with 0.1% formic acid was used on a Zorbax XDB C18 column. By applying current gradient, danshensu and IS were eluted between early eluting hydrophilic components and later eluting hydrophobic components, while maintaining a relative short analysis time of 4.5 min. All these hydrophilic and hydrophobic components were considered to be potentially response-suppressing matrix components. As shown in Fig. 2, the retention times for danshensu and IS were 2.27 and 2.60 min, respectively. The chromatograms show baseline separation of danshensu and the internal standard without any interference from endogenous plasma components and other coexisting components in Danshen Granules.

3.2. Sample preparation

Considering the complexities of Danshen Granules and human plasma matrix, protein precipitation (PPT) was firstly excluded to be an applicable sample preparation method, because PPT is well known as a non-selective purification method, which may introduce high amounts of endogenous components and can cause signal suppression or enhancement (i.e. matrix effect), especially with an ESI ionization source. Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) techniques were further investigated to be utilized in the sample preparation of human plasma. However, due to thousands of plasma samples would be analyzed during the clinical trials, relative economic LLE technique had become our first and preferred consideration for its economic and efficiency. In the present experiment, different liquid-liquid extraction conditions were evaluated including different pH modifiers and extraction solvents. Three organic extraction solvents (diethyl ether, dichloromethane and ethyl acetate) were evaluated. Among them, the last

one (ethyl acetate) yielded the best clean-up of the plasma samples and highest and most stable recovery values. It was found that addition of 1 mol/L HCl solution could significantly increase the extraction of danshensu.

3.3. Selection of internal standard

An appropriate internal standard is usually required in LC-MS/MS analysis in order to eliminate the effects from matrix and the pretreatment efficiency. Usually stable isotope-labeled internal standard is the optimal choice, however, stable isotopically internal standard of danshensu is difficult to be obtained. Other naturally compounds, which may be present in Danshen granules, should not be used as internal standard. In this study, chloroamphenicol, a chemical synthetic compound was selected as the internal standard (IS); its chromatographic behavior and extraction efficiency were similar to that of danshensu, and in addition, there were no interferences from the analytes and endogenous substances.

3.4. Method validation

The validated method was high selective for the target analyte because no significant interference was observed in the blank plasma samples from six different sources. Fig. 2 shows the typical chromatograms of danshensu and chloroamphenicol (IS) in a blank human plasma sample, and blank plasma sample spiked with danshensu at the LLOQ level (0.50 ng/mL) and IS (200 ng/mL), a plasma sample collected at 1 hr after oral administration of 1.5 g of Danshen Granules (1 pouch) to healthy subjects.

The linear regression of the peak ratios versus concentrations were fitted over the plasma concentration ranges of 0.50–500 ng/mL for danshensu. The typical linear regression equation of the calibration curves generated during the validation was as follows: $y = 0.00216x + 0.000729$ ($r = 0.9975$), where y represents the peak area ratio of danshensu to the IS, and x is the nominal concentration of danshensu. The LLOQ of danshensu was determined to be 0.50 ng/mL with acceptable accuracy (RE 0.63%) and precision (RSD

8.57%).

Table 1 summarizes the intra and inter-day precision and accuracy values for the QC samples. The intra and inter-day precisions for danshensu were less than 12.4%, while accuracy was within $\pm 12.0\%$. The accuracy and precision data indicate that method is reliable and reproducible.

The recovery results of danshensu at 1.00, 20.0, and 400 ng/mL were 92.5%, 90.8% and 95.1%, respectively. The recovery of IS at 200 ng/mL was estimated to be 88.4%.

The matrix effects for danshensu determined at 1.00, 20.0, and 400 ng/mL were 85.4%, 84.7% and 89.6%, respectively. The matrix effect for IS determined at 200 ng/mL was 87.1%. The inter-subject variability of matrix effects for each analyte was below 12.5%. As a result, the matrix effects for danshensu and IS were negligible in present conditions.

The stability results were presented in Table 3. Danshensu at the three concentrations tested had acceptable stabilities after three cycles of freeze-thaw, at room temperature for 24 hr and at -20°C for 1 month with the % RE values being within $\pm 15\%$, indicating danshensu remained stable during pretreatment, chromatography and sample storage processes in human plasma samples.

3.5. Pharmacokinetics

The validated method was successfully applied to an explorative pharmacokinetic study of danshensu in Chinese healthy subjects after oral administration of Danshen Granules. Individual and mean plasma concentration–time curves of danshensu in Chinese healthy subjects after oral administration of 1.5 g of Danshen Granules (1 pouch) (n=6) are presented in Fig. 3. After oral administration of Danshen granules, T_{\max} and C_{\max} values of danshensu were found to be 0.83 ± 0.13 hr and 257 ± 73.8 ng/ml, respectively. Plasma concentrations declined with $t_{1/2Z}$ of 1.65 ± 0.35 hr. $\text{MRT}_{0-\infty}$ was calculated as 2.19 ± 0.21 hr. The AUC_{0-t} and $\text{AUC}_{0-\infty}$ values obtained were 485 ± 122 and 491 ± 123 ng/mL*hr, respectively.

4. Conclusion

The optimized method was validated to guarantee the reliable determination of danshensu in the plasma of Chinese healthy subjects. The LLOQ of the method is 0.50 ng/mL for danshensu using 100 μ L of human plasma sample. Relative simple and economic liquid-liquid extraction procedure and the running time of no more than 6 min allowed sample throughput of 15–18 samples per day and thus made the method easily applied to clinical trials.

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Table Captions:

Table 1 Precision and accuracy of the assay method for danshensu in human plasma

Table 2 Matrix effects and recoveries of danshensu in human plasma (n=5)

Table 3 Stability of danshensu in human plasma (n=5)

Table 4 Pharmacokinetic parameters of danshensu in healthy subjects determined after oral administration of Danshen granules (n=6)

Table 1 Precision and accuracy of the assay method for danshensu in human plasma

Day	No.	Low	Medium	High
		1.0 ng/mL	20 ng/mL	400 ng/mL
Day 1	Mean ± SD	0.88 ± 0.07	18.7 ± 2.31	418 ± 37.4
	RSD (%)	7.95	12.4	8.95
	RE (%)	-12.0	-6.50	4.50
Day 2	Mean ± SD	1.09 ± 0.11	22.3 ± 1.34	426 ± 10.5
	RSD (%)	10.1	6.01	2.46
	RE (%)	9.00	11.5	6.50
Day 3	Mean ± SD	1.12 ± 0.10	22.1 ± 1.45	386 ± 16.5
	RSD (%)	8.93	6.6	4.27
	RE (%)	12.0	10.5	-3.50
Inter-Day	Mean ± SD	1.03 ± 0.10	21.0 ± 1.57	410 ± 16.4
	RSD (%)	9.71	7.46	4.00
	RE (%)	3.00	5.17	2.50

Table 2 Matrix effects and recoveries of danshensu in human plasma (n=5)

Spiked concentration (ng/mL)	Matrix effect (%)	Mean \pm SD (%)	Recovery (%)	RSD (%)
1.00	85.4		92.5	10.5
20.0	84.7	86.6 \pm 2.65	90.8	5.16
400	89.6		95.1	8.47

Table 3 Stability of danshensu in human plasma (n=5)

Stability conditions	Added Conc.	1.00 ng/mL	20.0 ng/mL	400 ng/mL
Three freeze–thaw cycles	Mean ± SD	1.13 ± 0.07	20.8 ± 2.31	423 ± 19.4
	RSD (%)	6.19	11.1	4.59
	RE (%)	13.0	4.00	5.75
Room temperature for 24 hr	Mean ± SD	1.09 ± 0.13	22.2 ± 2.58	406 ± 36.3
	RSD (%)	11.9	11.6	8.94
	RE (%)	9.00	11.0	1.50
Storage at –20°C for 1 month	Mean ± SD	0.87 ± 0.10	21.4 ± 3.11	416 ± 35.6
	RSD (%)	11.5	14.5	8.56
	RE (%)	-13.0	7.00	4.00

Table 4 Pharmacokinetic parameters of danshensu in healthy subjects determined after oral administration of Danshen granules (n=6)

PK Parameters	Unit	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Mean	SD
AUC _(0-t)	ng/mL*hr	434	670	389	609	400	406	485	122
AUC _(0-∞)	ng/mL*hr	444	676	391	616	405	413	491	123
MRT _(0-t)	hr	2.11	2.10	2.09	2.39	1.75	1.92	2.06	0.21
MRT _(0-∞)	hr	2.33	2.18	2.14	2.50	1.88	2.13	2.19	0.21
t _{1/2z}	hr	1.85	1.42	1.25	1.45	1.73	2.22	1.65	0.35
T _{max}	hr	1.00	0.75	0.75	1.00	0.75	0.75	0.83	0.13
C _{max}	ng/mL	204	382	189	210	301	253	257	73.8

Figure Captions:

Fig. 1. Product ion spectra and fragmentation patterns of $[M-H]^-$ ions: (A) Danshensu using a CE of -24 eV; (B) Chloroamphenicol using a CE of -20 eV.

Fig. 2. Typical MRM chromatograms of danshensu and chloroamphenicol (IS) in the plasma of healthy subjects: (A) blank plasma sample; (B) blank plasma spiked with danshensu at the LLOQ level (0.50 ng/mL) and IS (200 ng/mL); (C) plasma sample collected at 1 hr after oral administration of 1.5 g of Danshen granules (1 pouch) to healthy subjects.

Fig. 3. Individual (A) and mean (B) plasma concentration–time curves of danshensu in Chinese healthy subjects after oral administration of 1.5 g of Danshen granules (1 pouch) (n=6).

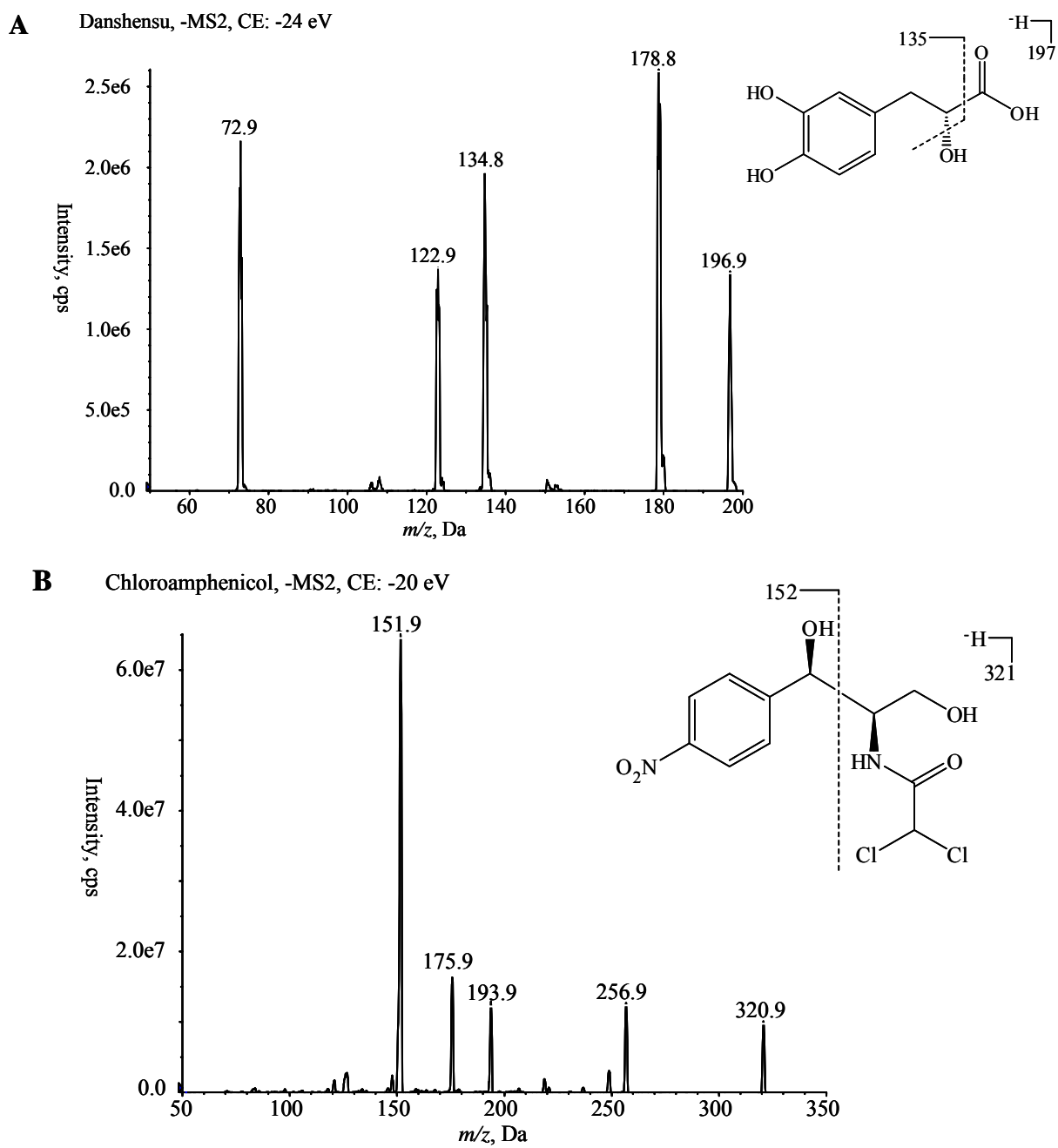


Fig. 1. Product ion spectra and fragmentation patterns of [M-H]⁻ ions: (A) danshensu using a CE of -24 eV;

(B) chloroamphenicol using a CE of -20 eV.

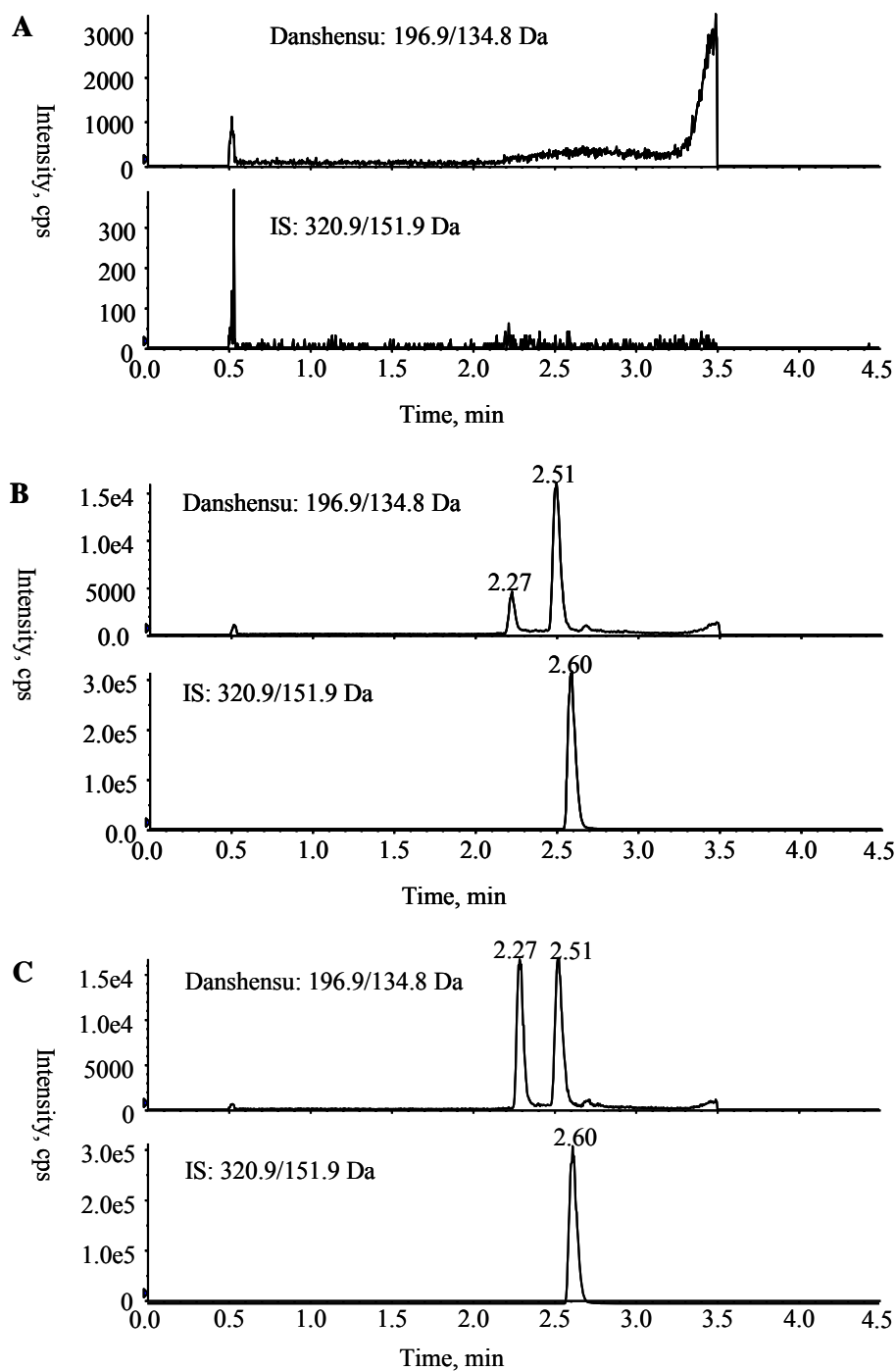


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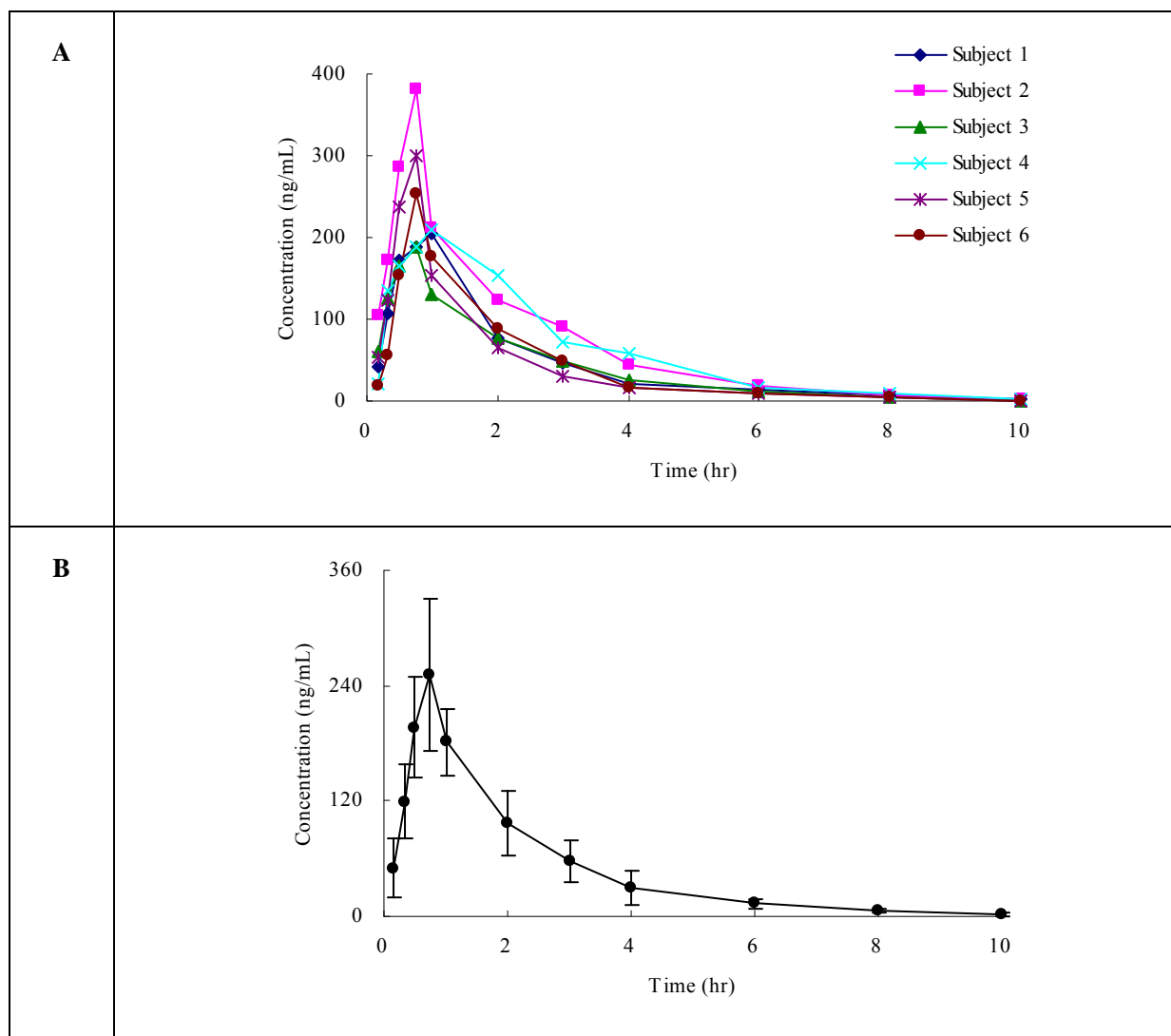


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