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Simultaneous Determination of Seven Bioactive Constituents from *Salvia miltiorrhiza* in Rat Plasma by HPLC-MS/MS: Application to a Comparative Pharmacokinetic Study

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Abstract: The roots of *Salvia miltiorrhiza* (Danshen) is a precious herbal medicine used to treat cardiovascular diseases. This study establishes a high-performance liquid chromatography-tandem mass spectrometric (HPLC-MS/MS) method to quantify seven bioactive constituents from Danshen in rat plasma simultaneously. Chromatographic separation is performed on an Agilent Eclipse Plus C18 column (150 mm × 2.1 mm, 5 µm), utilizing a gradient of acetonitrile and 0.2% formic acid aqueous solution as the mobile phase, at a flow rate of 0.6 mL/min. We conduct a tandem mass spectrometric detection with electrospray ionization (ESI) interface via multiple reaction monitoring (MRM) in both positive and negative ionization mode. Our results show that a linear relationship is established for each analyte of interest over the concentration range of 0.5–300 ng/mL with $r \geq 0.9976$. The validated method is successfully used to compare the pharmacokinetic properties of crude and wine-processed Danshen extract orally administered to rats. C_{max} of tanshinone IIA, C_{max}, and $AUC_{0\text{-}t}$ of dihydrotanshinone I decrease significantly ($p < 0.05$) in the wine-processed group. No significant changes for other compounds are observed. These results might provide meaningful information for the further application of wine-processed Danshen and understanding of wine-processing mechanisms.

Keywords: *Salvia miltiorrhiza*; HPLC-MS/MS; pharmacokinetics; wine-processed

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1. Introduction

Bioactive components of the roots of *Salvia miltiorrhiza* (Danshan) have been developed into various formulations clinically to treat microcirculatory disturbance-related diseases, such as heart disease, chronic hepatitis, diabetes, early cirrhosis, cerebral ischemia, and cancer [1–3]. Chemical investigation of Danshen in the past few years has revealed that two major types of secondary metabolites were responsible for its therapeutic effects: lipophilic tanshinones and hydrophilic phenolic acids [4,5]. These components exhibited multiple biological activities via different mechanisms. For example, tanshinone IIA and dihydrotanshinone I could enhance autophagy and induce proteasomal degradation of the Tau protein, resulting in increased Amyloid-β clearance and decreased Tau phosphorylation, making them potential candidates for AD treatment in the future [6,7]. Cryptotanshinone and tanshinone I exhibit vigorous antiviral activities against SARS-CoV-2 with IC_{50} values of 5.63 and 2.21 µmol/L, respectively [8]. Salvianolic acids, rosmarinic acid, caffeic

acid, ferulic acid, and lithospermic acid are four representative hydrophilic phenolic acids isolated from Danshen [9,10]. Among them, salvianolic acid A can reduce cardiotoxicity induced by arsenic trioxide through decreasing cardiac mitochondrial injury and shows great potential against cancer cells via targeting various signaling pathways [11,12]. Rosmarinic acid, ferulic acid, and lithospermic acid play several biological roles, including free radical scavenger, inhibitor of prooxidant enzymes that catalyze free radical production, and enhancer of scavenger enzyme [13–15].

Pharmacokinetic description of the bioactive components in Danshen will provide scientific evidence regarding their properties of adsorption, distribution, metabolism, and excretion in vivo. Understanding the pharmacokinetic properties of each component enables prescribers to choose appropriate dose and dose intervals to ensure the safety and efficiency of drug application. HPLC–MS/MS method has been extensively applied to determine the pharmacokinetic profiles of different bioactive components from Danshen because of its high sensitivity and specificity [16–18]. An efficient UPLC/MS/MS method that employs isocratic elution and positive/negative ionization switching analysis, has been established for the determination of four salvianolic acids and four tanshinones in Danshen simultaneously in 2 min [19]. However, several aspects still need to be improved for their application in biological samples. Higher sensitivity and efficiency, as well as a larger concentration range for the quantification, remain to be achieved by optimizing the HPLC-MS/MS and sample pretreatment conditions.

Wine-processing is a traditional method for treating herbal medicine before clinical use to achieve multiple purposes: modify the taste, reduce the toxicity, and enhance the biological activity [20,21]. Wine-processed Danshen has been reported to possess enhanced blood—quickening and stasis—transforming, as well as antimicrobial activities. Although the crude and wine-processed Danshen exhibited different chemical profiles, detailed exploration of the chemical basis behind medicinal property changes after the processing has not been performed yet [22–24]. Moreover, a comparison between pharmacokinetic properties of crude and wine-processed Danshen in vivo, which is meaningful for their clinical reasonable application and understanding the wine-processing mechanism, has not been characterized yet.

In the current work, a sensitive and efficient method for simultaneous quantification of seven bioactive constituents from Danshen in plasma samples was developed and applied to compare the pharmacokinetic properties of crude and wine-processed Danshen extract orally administered to rats. Hydrophobic tanshinones, including tanshinone IIA, cryptotanshinine, and dihydrotanshinone I, as well as hydrophilic phenolic acids, including salvianolic acid A, rosmarinic acid, ferulic acid, and lithospermic acid (Figure 1), were simultaneously determined for the first time at a concentration range of 0.5–300 ng/mL with $r \geq 0.9976$ in 8 min. Pharmacokinetic parameters, such as AUC, C_{max}, and $t_{1/2}$, were determined and compared. This will provide information about the influence of wine-processing on the pharmacokinetics, as well as the pharmacological activity of Danshen bioactive components.

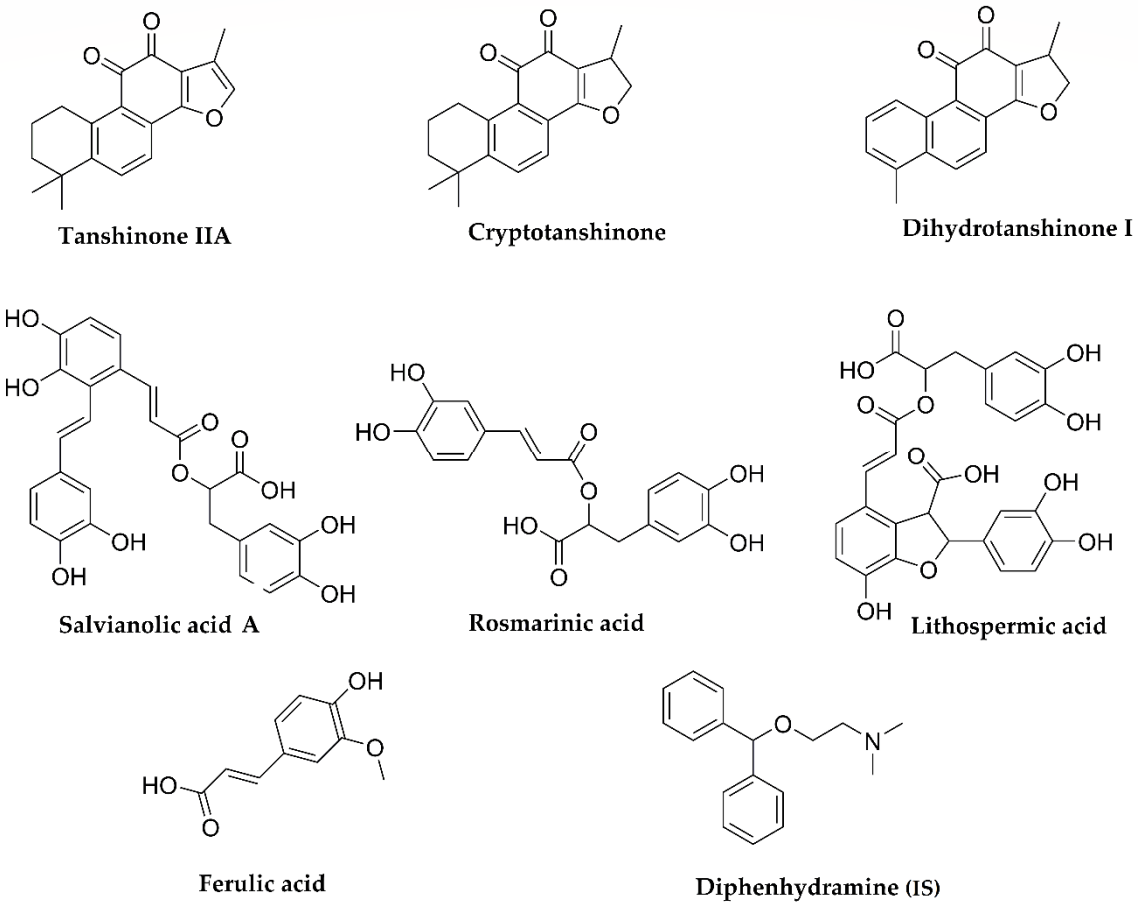

Figure 1. Chemical structure of seven components and internal standards (IS): Tanshinone IIA; cryptotanshinone; dihydrotanshinone I; salvianolic acid A; rosmarinic acid; lithospermic acid; ferulic acid and diphenhydramine (IS).

2. Materials and Methods

2.1. Chemical Reagents

Danshen were collected from Jinan Green Chinese Herbal Pieces Co., (Jinan, Shandong, China) and authenticated by Professor Xu Lingchuan in the field. The wine-processed Danshen were collected from Shandong Jianlian Shengjia Traditional Chinese Medicine Co. (Jinan, Shandong, China) and authenticated by Professor Xu Lingchuan. Tanshinone IIA (Lot: 110766-200416), cryptotanshinone (Lot: 110852-201307), rosmarinic acid (Lot: 11871-201303), ferulic acid (Lot: 110773-200608), and diphenhydramine (Lot: 100066-200807) were purchased from China National Institutes for Food and Drug Control. Salvianolic acid A (Lot: B20260), lithospermic acid (Lot: B21683), and dihydrotanshinone I (Lot: B20357) were purchased from Shanghai Yuanye Biotechnology Co. Ultra-pure water and chemicals, such as acetonitrile and formic acid of analytical grade purity, were used in the experiment.

2.2. Instruments, Liquid Chromatography, and Mass Spectrometry Conditions

A Nanospace SI-2 HPLC system (Shiseido, Japan) equipped with a NASCA 5100 autosampler, a vacuum degasser unit, and a binary pump, and an Agilent C18 column (150 mm × 2.1 mm, 5 μm) were selected for chromatographic analysis. An API 5500 Q-Trap triple quadrupole mass spectrometer (AB SCIEX, Concord, ON, Canada) equipped with a TurboIonSpray source was used for mass detection. Data were processed on Analyst 1.5.2 software package. The solvent flow was diverted from the MS after the first minute of the gradient. Samples were ionized using an electrospray ion (ESI) source in both positive and negative mode. The ionization voltage for positive and negative mode was +4.5 kV and −4.0 kV, respectively. The source temperature was set at 55 °C. Nitrogen was used as the curtain gas (35 psi), nebulizer gas (GS1, 55 psi), and turbo gas (GS2, 55 psi).

2.3. Preparation of Calibration Standards, Internal Standard (IS), and Quality Contro (QC) Samples

To prepare the stock solution of calibration standards and QC samples, all compounds were dissolved in acetonitrile to a final concentration of 1 mg/mL. The stock solutions were serially diluted with acetonitrile to prepare the working solutions of calibration standards and QC samples. The calibration standards were prepared by spiking a specific volume of the working solutions into the corresponding biological samples to yield a final concentration of 0.5, 1, 3, 5, 10, 30, 50, 100, and 300 ng/mL, respectively. Diphenhydramine at 400 ng/mL was used as the IS working solution. The low, medium, and high levels of QC samples containing analytes at the concentration of 0.5, 5, and 240 ng/mL were prepared in the same manner. All the solutions were stored at 4 °C for further use.

2.4. Preparation of Crude and Wine-Processed Danshen Extract

Extracts were prepared according to the following procedures. Powder of crude and wine-processed Danshen were firstly extracted twice by heating reflux at 80 °C for 2 h in 80% ethanol, and the extract was concentrated with rotary evaporation under vacuum at 60 °C. The residual was then extracted twice with water by heating reflux at 80 °C for 2 h and concentrated in the same manner. Extract obtained after the four extractions were combined and dried, and then crushed into powder for further experiments. The contents of tanshinone IIA, cryptotanshinone, dihydrotanshinone I, salvianolic acid A, rosmarinic acid, ferulic acid, and lithospermic acid were 28.3 ± 0.08, 19.6 ± 0.22, 64.0 ± 1.36, 217.0 ± 2.32, 505.0 ± 4.91, 14.9 ± 0.22, 1240.0 ± 13.5 ng/mg in crude Danshen extract, and 33.5 ± 1.13, 23.1 ± 1.07, 72.3 ± 1.39, 166.0 ± 3.56, 564.0 ± 4.97, 16.5 ± 0.43, 1010.0 ± 4.32 ng/mg in wine-processed Danshen extract, respectively.

2.5. Preparation and Handling of Biological Samples

An aliquot of 10 μL IS working solution (400 ng/mL) and 200 μL acetonitrile were added into a 20 μL plasma sample. The mixture was vortex mixed for 5 min to precipitate the proteins and then centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was transferred to a 1.5 mL Eppendorf tube, and 5 μL of the supernatant was injected into the HPLC-MS/MS system for analysis.

2.6. Method Validation

The HPLC-MS/MS method was developed and validated according to the Guidance for Industry Bioanalytical Method Validation [25]. The full validation, including selectivity, linearity, accuracy, precision, matrix effect, extraction recovery, and stability, was carried out in the plasma matrix.

2.6.1. Selectivity

To evaluate the selectivity of the method, the chromatogram of blank plasma samples from six different lots of rats was compared with those of corresponding plasma samples spiked with a standard solution of the seven analytes, and plasma samples after oral administration of Danshen extract.

2.6.2. Linearity and Lower Limit of Quantification (LLOQ)

The final concentration of calibration standards for plotting the calibration curve was 0.5, 1, 3, 5, 10, 30, 50, 100, and 300 ng/mL. The curve was plotted with the mass concentration of each drug in the plasma on the abscissa (X), and the peak area ratio of the drug to the IS on the ordinate (Y). The weighting factor $1/x^2$ was used for the best fit line of $y = kx + c$ using linear regression analysis. The correlation coefficient (r) of 0.995 or better was considered as the best response for quantification analysis. The concentration of analytes in the QCs or test samples was calculated based on the regression parameters obtained from the calibration curves. The analyte response at the LLOQ should be at least 10 times of blank response.

2.6.3. Accuracy, Precision, and Recovery

Intra-day precision and accuracy were estimated at three different QC levels, i.e., 0.5, 5, and 240 ng/mL, by analyzing six replicates in a single day. The inter-day precision was determined by analyzing the three different QC samples on nine different runs in three consecutive days. Relative error (RE) and related standard deviation (RSD) were used to evaluate the accuracy and the precision, respectively. The mean values for RE and RSD should be within 15% of the actual value except at LLOQ, where it should not deviate from the mean value by more than 20%.

The recovery of each analyte at three different concentrations was evaluated by comparing the relative peak area of the analyte spiked in the rat plasma to that of the standard directly dissolved in solvent at the same concentration. The ratio gives the recovery.

2.6.4. Stability and Matrix Effect

The stability of the analytes was evaluated in triplicates at three different QC levels: 0.5 ng/mL, 5 ng/mL, and 240 ng/mL. A freeze-thaw stability experiment was performed by subjecting the QC samples to three freeze and thaw cycles from −80 °C to room temperature. Samples were left overnight in an autosampler setting at 4 °C or at room temperature for 4 h to evaluate their autosampler stability and benchtop stability. Bias was calculated against the freshly prepared QC samples. Samples with a difference within ±15% were considered stable. The matrix effect on the quantification of analytes was evaluated by comparing the peak area ratio of the analyte spiked in plasma samples to that of the standard directly dissolved in solvent at the same concentration.

2.7. Application to a Pharmacokinetic Study

Male Wistar rats (220–230 g, n = 12) used in the experiments were supplied by the Lab Animal Center of Shandong University (Grade II, Certificate No. SYXK 2013-0001). Rats fasted for 12 h before drug administration and for a further 2 h after dosing, and have free access to water during experiments. The experimental protocol was approved by the University Ethics Committee and conformed to the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985). The rats were randomly divided into two groups (six in each group) and conducted with a single oral dose of crude and wine-processed Danshen extract suspended in water (10 g/kg), respectively.

After oral administration, 150 µL of blood samples were collected from the jugular vein at different time points (before dosing and at 0.25, 0.5, 1.0, 1.5, 2.0, 4.0, 4.5, 6.0, 12.0, 24.0, and 48.0 h post-dosing) and put into heparinized tubes, which were centrifuged at 3000× *g* for 10 min at 4 °C. The supernatant was stored at −80 °C and analyzed using the method described above within one month. The plasma concentration of different drugs at different time points was determined based on the standard curve. The plasma drug concentration was plotted on the ordinate, and time was on the abscissa, yielding the concentration–time curves.

Parameters, including the peak plasma concentration (C_{max}) and time to peak concentration (T_{max}), were obtained from experimental observations. The other pharmacokinetic parameters were analyzed using the program TOPFIT (version 2.0; Thomae GmbH, Germany) according to a non-compartmental model. The linear trapezoidal rule to approximately the last point was used to calculate the area under the plasma concentration–time curve ($AUC_{0\text{-}t}$). Dividing the area under the first moment–time curve ($AUMC_{0\text{-}t}$) by the area under the curve ($AUC_{0\text{-}t}$) yields the mean residence time (*MRT*). Total oral body clearance (*CL/F*) was calculated using the following equation: $CL/F = \text{dose}/AUC_{0\text{-}t}$. All results were expressed as mean ± standard deviation (SD). Statistical comparisons between different groups were analyzed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) by an analysis of variance (ANOVA).

3. Results and Discussion

3.1. Method Development

3.1.1. Optimization of the HPLC-MS/MS Conditions

Methanol-water or acetonitrile-water as the mobile phase in the gradient elution system was firstly compared in the aspects of retention time, signal intensity, and resolution. We found that the acetonitrile-water mobile phase system yielded chromatographic peaks with better resolution and intensity than methanol-water did. The addition of 0.2% formic acid into the mobile phase significantly increased the signal intensity of the eight compounds and improved their peak shapes. Thus, the acetonitrile (A)/water (B) with 0.2% formic acid was selected as the elution solvent system. The HPLC gradient system was set up as follows—13% of A for 0.5 min, then linearly increased to 90% of A in 2.5 min, and finally a decrease to 13% of A in 0.5 min prior to column re-equilibration. The total running lasted 8 min.

The standard solution of the seven analytes and the IS was separately introduced into the ESI source in either positive or negative ionization mode to determine the pattern of the most abundant ions in these compounds. The four phenolic acids showed an intense signal response and less noise in negative mode, while the three tanshinones and IS provided higher signal intensity in positive mode. Based on these results, we designed a segment-program to detect the eight compounds with the most vital signals in both positive and negative modes. Optimal MS parameters for the MRM scan mode were listed in Table 1.

Table 1. MRM transitions and parameters for detecting tanshinone IIA, cryptotanshinone, dihydrotanshinone I, salvianolic acid A, rosmarinic acid, ferulic acid, lithospermic acid, and diphenhydramine.

Analytes	ESI Mode	Precursor Ion (*m*/*z*)	Product Ion (*m*/*z*)	DP	CE
Tanshinone IIA	positive	295.1	277.2	130	26
Cryptotanshinone	positive	297.1	279.1	100	29
Dihydrotanshinone I	positive	279.1	261.2	200	22
Salvianolic acid A	negative	493.3	295.1	−100	−24
Rosmarinic acid	negative	359.1	293.1	−150	−33
Ferulic acid	negative	193.1	134.1	−100	−26
Lithospermic acid	negative	537.1	493.1	−68	−11
IS	positive	256.2	167.1	200	16

Abbreviations: ESI, electrospray ion; CE, collision energy; DP, declusting potential.

3.1.2. Optimization of Extraction Procedure

Protein precipitation, solid-phase extraction, and liquid phase extraction are three commonly used methods for sample extraction. Protein precipitation using organic solvents has been widely accepted because of its high efficiency, convenience, and low cost [26,27]. In the current study, different organic solvents for protein precipitation were attempted during sample preparation. Finally, acetonitrile was selected because of its excellent efficiency, preferable recovery (87.3~105.6%), and little matrix effect. In the case of methanol, the recovery of rosmarinic acid, and lithospermic acid is relatively poor.

3.2. Method Validation

3.2.1. Specificity

Figure 2 shows the typical MRM chromatograms of blank plasma, blank plasma spiked with the seven analytes and IS, and the sample collected from rats at 1h after oral administration of crude Danshen extract. Peaks of all the seven components and IS, at the retention times of 4.89 min (tanshinone IIA), 4.45 min (cryptotanshinone), 4.00 min (dihydrotanshinone I), 2.10 min (salvianolic acid A), 2.09 min (rosmarinic acid), 1.97 min (ferulic acid), 2.09 min (lithospermic acid) and 2.23 min (IS), did not interfere with those from endogenous substances.

3.2.2. Linearity and LLOQ

The linear ranges, regression equations, and correlation coefficients of the seven analytes are shown in Table 2. All calibration curves exhibited good linearity over the concentration range from 0.5 ng/mL to 300 ng/mL with correlation coefficient (r) ranging from 0.9956 to 0.9976. Based on a signal peak-to-noise ratio = 10, the LLOQs for all the eight compounds were 0.5 ng/mL.

Table 2. Linear ranges, regression equations, and correlation coefficients of tanshinone IIA, cryptotanshinone, dihydrotanshinone I, salvianolic acid A, rosmarinic acid, ferulic acid, and lithospermic acid in rat plasma.

Analytes	Concentration Range (ng/mL)	Regression Equation	Correlation Coefficient (r)
Tanshinone IIA	0.5−300	Y = 0.828X + 0.0137	0.997
Cryptotanshinone	0.5−300	Y = 0.125X + 0.0744	0.996
Dihydrotanshinone I	0.5−300	Y = 0.0695X − 0.0205	0.997
Salvianolic acid A	0.5−300	Y = 0.00478X + 0.0137	0.996
Rosmarinic acid	0.5−300	Y = 0.00434X + 0.00434	0.998
Ferulic acid	0.5−300	Y = 0.00332X + 0.00174	0.997
Lithospermic acid	0.5−300	Y = 0.000721X + 0.00376	0.996

3.2.3. Precision, Accuracy

The intra-day and inter-day precisions (RSD) of the seven analytes at three different concentration levels ranged from 2.2% to 9.3%, while the accuracy (RE) of the samples ranged from −2.1% to 6.4% (Table 3). All these values were within the acceptable range, implying that the method was reproducible and reliable.

Table 3. Summary of precision, accuracy, recovery, and matrix effect of tanshinone IIA, cryptotanshinone, dihydrotanshinone I, salvianolic acid A, rosmarinic acid, ferulic acid, and lithospermic acid in rat plasma (n = 9, three consecutive days).

Analytes	Spiked (ng/mL)	Intra-Day Precision RSD (%)	Inter-Day Precision RSD (%)	Intra-Day Accuracy RE (%)	Recovery Mean ± SD (%)	Matrix Effect Mean ± SD (%)
Tanshinone IIA	0.5	6.2	7.4	6.4	87.4 ± 5.0	109.1 ± 3.4
	5.0	5.2	5.8	4.3	89.4 ± 6.5	105.4 ± 4.2
	240	3.4	4.2	4.7	91.7 ± 4.2	110.3 ± 2.5
Cryptotanshinone	0.5	7.8	8.9	2.7	85.3 ± 7.6	102.5 ± 1.7
	5.0	5.9	4.2	−1.5	89.2 ± 3.4	92.3 ± 2.5
	240	4.7	5.2	4.6	91.3 ± 6.1	97.4 ± 6.4
Dihydrotanshinone I	0.5	8.1	6.7	−2.1	89.4 ± 6.9	91.4 ± 7.1
	5.0	3.5	5.5	6.2	91.2 ± 7.5	93.9 ± 2.0
	240	4.3	5.7	5.5	92.6 ± 8.4	98.1 ± 5.5
Salvianolic acid A	0.5	7.2	9.3	3.2	106.7 ± 8.7	96.4 ± 4.1
	5.0	6.8	5.7	0.8	87.9 ± 2.4	106.5 ± 3.4
	240	4.6	3.6	2.9	105.4 ± 4.7	103.7 ± 3.6
Rosmarinic acid	0.5	4.1	5.2	4.5	94.6 ± 4.2	89.4 ± 5.7
	5.0	2.4	2.2	1.4	92.7 ± 5.1	91.6 ± 6.8
	240	3.3	5.8	2.6	102.5 ± 7.8	95.5 ± 1.5
Ferulic acid	0.5	9.2	8.7	5.5	87.3 ± 5.4	112.4 ± 2.4
	5.0	6.5	6.7	6.2	91.6 ± 4.7	104.8 ± 5.1
	240	4.7	5.5	5.4	96.5 ± 6.4	105.4 ± 2.8
Lithospermic acid	0.5	7.1	6.5	2.7	105.6 ± 8.7	92.4 ± 3.4
	5.0	5.6	7.6	5.5	96.8 ± 7.9	89.7 ± 4.8
	240	2.8	4.0	6.2	90.9 ± 6.2	86.7 ± 6.5
IS	400	N.D.	N.D.	N.D.	94.7 ± 5.4	N.D.

3.2.4. Extraction Recoveries and Matrix Effects

The extraction recoveries and matrix effects of the investigated analytes in rat plasma are shown in Table 3. The recovery rates of the investigated analytes ranged from 87.3% to 105.6%, with SD values lower than 8.7%, 7.9%, and 8.4% at three concentration levels,

respectively. The matrix effects of the eight analytes of interest varied from 86.7% to 112.4%, with SD values lower than 7.1%, 6.8%, and 6.5% at low, medium, and high concentrations, respectively. These results suggested that the sample processing method for extraction of analytes from biological samples exhibited high efficiency, and the recoveries for all the eight analytes were in acceptable ranges. No significant matrix effect existed.

3.2.5. Stability

All the seven analytes were stable in rat plasma under different experimental conditions: 4 °C for 24 h (RE: −9.6~9.2%, RSD ≤ 9.8%), −80 °C for 30 days (RE: −8.8~9.2%, RSD ≤ 8.9%), and after three freeze thaw cycles at −20 °C (RE: −3.3~7.2%, RSD ≤ 8.6%). All the results were within the acceptance criteria of ±15% deviation from the nominal concentration (Table 4).

Table 4. Stability of tanshinone IIA, cryptotanshinone, dihydrotanshinone I, salvianolic acid A, rosmarinic acid, ferulic acid, and lithospermic acid in rat plasma samples (n = 5).

Analytes	Spiked (ng/mL)	Stability at 4 °C for 24 h		Stability at −80 °C for 30 Days		Freeze-Thaw Stability	
		(RSD, %)	(RE, %)	(RSD, %)	(RE, %)	(RSD, %)	(RE, %)
Tanshinone IIA	0.5	6.8	4.0	4.1	3.6	6.7	3.1
	5.0	5.9	−2.4	4.8	6.8	5.9	−2.8
	240	6.5	−2.6	1.6	2.0	3.0	4.8
Cryptotanshinone	0.5	8.0	−7.2	8.9	5.6	8.3	2.4
	5.0	3.6	9.2	6.5	6.4	7.0	6.4
	240	3.2	2.4	5.9	−3.7	2.8	1.9
Dihydrotanshinone I	0.5	9.8	−2.4	7.4	−5.2	6.7	−1.2
	5.0	4.5	5.6	4.3	0.8	3.7	4.8
	240	1.9	1.9	4.0	3.2	3.0	4.3
Salvianolic acid A	0.5	5.2	6.0	6.0	1.2	6.4	2.8
	5.0	6.1	2.4	3.6	7.2	6.8	−2.8
	240	1.7	−0.9	4.8	−3.6	4.6	−3.3
Rosmarinic acid	0.5	8.2	−1.2	6.7	−8.8	4.9	−1.2
	5.0	2.9	3.2	5.1	4.8	2.8	7.6
	240	1.3	2.5	3.6	4.1	3.0	4.3
Ferulic acid	0.5	3.7	−9.6	7.6	6.0	8.0	7.2
	5.0	5.3	5.6	5.2	5.6	7.3	4.0
	240	3.2	3.6	6.2	1.5	4.4	3.0
Lithospermic acid	0.5	7.2	4.8	3.5	1.6	4.0	8.4
	5.0	3.5	8.4	4.2	9.2	8.6	3.6
	240	3.0	0.9	5.4	2.3	2.9	3.9

3.3. Pharmacokinetic Study

The above-validated method was successfully applied to a comparative pharmacokinetic study in rats after oral administration of crude and wine-processed Danshen extract at a dose of 10 g/kg. The mean plasma concentration-time profiles of seven analytes are plotted and presented in Figure 3. The main non-compartmental pharmacokinetic parameters are listed in Table 5.

Based on the concentration–time curves and the pharmacokinetic parameters, the effect of wine-processing on the pharmacokinetics of the seven ingredients of Danshen in the rats were examined. As shown in Figure 3, the plasma concentration of all the components reached maximum quickly (T_{max} < 1 h), and wine-processing did not change the T_{max} values significantly. Compared to the crude Danshen group, the C_{max} of all the hydrophobic tanshinones decreased to some extent, and a significant decrease was observed for tanshinone IIA and dihydrotanshinone I ($p < 0.05$) in the wine-processed Danshen group. As to the hydrophilic acids, we did not see any obvious differences in C_{max} between the two groups. In addition, the $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$ of dihydrotanshinone I decreased significantly; *CL/F* of salvianolic acid A and dihydrotanshinone I increased significantly in the wine-processed Danshen group.

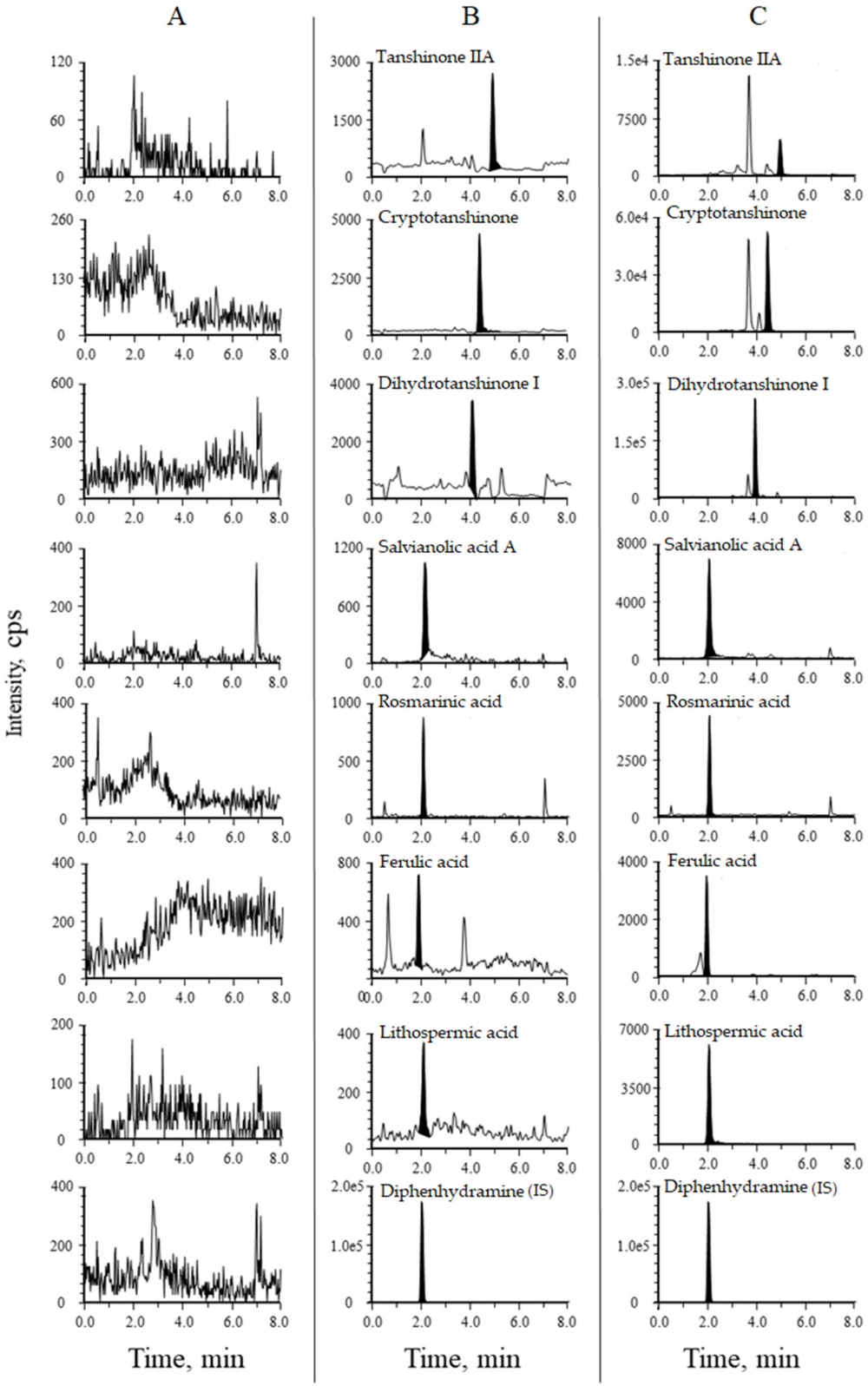

Figure 2. Representative MRM chromatograms of blank rat plasma (**A**); blank plasma spiked with seven analytes and IS (**B**); rat plasma sample collected at 1 h after oral administration of Danshen extract (**C**). Tanshinone IIA, cryptotanshinone, dihydrotanshinone I and IS were detected in positive mode. Salvianolic acid A, rosmarinic acid, ferulic acid, and lithospermic acid were detected in negative mode.

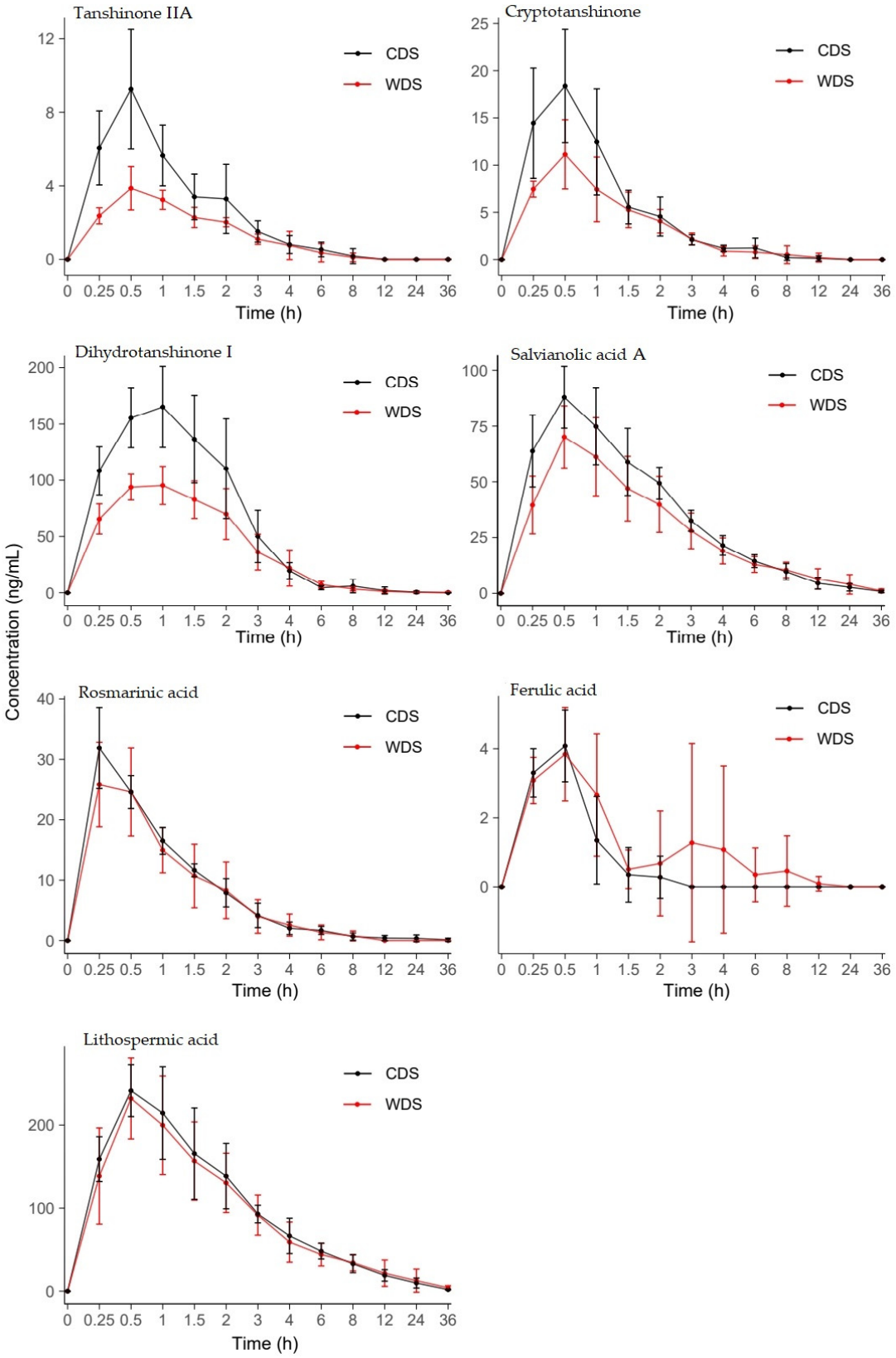

Figure 3. Mean (±SD, n = 6) plasma concentration-time profiles of tanshinone IIA, cryptotanshinone, dihydrotanshinone I, salvianolic acid A, rosmarinic acid, ferulic acid, and lithospermic acid in rat plasma after oral administration of crude danshen (black) and wine-processed Danshen extracts (red). CDS, crude Danshen; WDS, wine-processed Danshen.

Table 5. Pharmacokinetic parameters of tanshinone IIA, cryptotanshinone, dihydrotanshinone I, salvianolic acid A, rosmarinic acid, ferulic acid, and lithospermic acid in rats after oral administration of crude Danshen and wine-processed Danshen (n = 6, mean ± SD) [a].

	Analytes	Doses (mg/kg)	$AUC_{0\text{-}t}$ [b] (ng·h/mL)	$AUC_{0\text{-}\infty}$ (ng·h/mL)	C_{max} (ng/mL)	CL/F (L/h/kg)	T_{max} (h)	MRT (h)	$T_{1/2}$ (h)
CDS	Tanshinone IIA	0.283	15.80 ± 5.62	15.80 ± 5.62	**9.36 ± 3.17** *	21.23 ± 9.60	0.58 ± 0.19	1.70 ± 0.29	1.73 ± 0.44
	Cryptotanshinone	0.196	30.92 ± 11.95	30.97 ± 12.01	18.48 ± 5.91	7.36 ± 2.81	0.46 ± 0.09	1.97 ± 0.54	2.82 ± 1.56
	Dihydrotanshinone I	0.64	**446.17 ± 94.44** *	**449.09 ± 92.53** *	**173.67 ± 31.12** *	**1.49 ± 0.28** *	0.83 ± 0.24	2.73 ± 1.39	3.34 ± 3.58
	Salvianolic acid A	2.17	343.16 ± 57.06	354.79 ± 56.29	87.92 ± 13.82	**6.50 ± 1.10** *	0.50 ± 0.00	5.84 ± 0.93	9.12 ± 2.48
	Rosmarinic acid	5.05	57.63 ± 12.38	61.58 ± 16.73	31.88 ± 6.69	93.35 ± 27.27	0.25 ± 0.00	4.05 ± 3.05	6.65 ± 6.91
	Ferulic acid	0.149	3.41 ± 1.35	3.41 ± 1.35	4.14 ± 1.00	49.94 ± 17.00	0.58 ± 0.19	0.69 ± 0.23	N.A.
	Lithospermic acid	12.40	1093.00 ± 179.05	1116.94 ± 183.96	249.00 ± 35.22	11.65 ± 1.88	0.67 ± 0.24	6.89 ± 1.14	7.33 ± 1.74
WDS	Tanshinone IIA	0.335	9.62 ± 2.85	9.60 ± 2.86	**3.94 ± 1.12** *	37.56 ± 9.28	0.67 ± 0.24	2.08 ± 0.80	2.06 ± 0.98
	Cryptotanshinone	0.231	23.78 ± 8.35	24.54 ± 9.77	11.59 ± 3.63	10.95 ± 3.71	0.54 ± 0.22	2.51 ± 1.60	3.29 ± 3.47
	Dihydrotanshinone I	0.723	**300.84 ± 78.25** *	**304.75 ± 79.61** *	**101.95 ± 15.35** *	**2.57 ± 0.70** *	0.67 ± 0.24	2.84 ± 0.97	4.30 ± 4.81
	Salvianolic acid A	1.66	340.75 ± 65.30	364.27 ± 70.23	73.57 ± 14.13	**17.27 ± 3.82** *	0.58 ± 0.19	8.03 ± 3.17	10.26 ± 4.30
	Rosmarinic acid	5.64	47.58 ± 12.78	47.62 ± 12.79	27.32 ± 6.88	132.83 ± 55.11	0.33 ± 0.12	1.91 ± 0.71	1.98 ± 1.36
	Ferulic acid	0.165	3.73 ± 1.63	3.73 ± 1.63	4.28 ± 1.25	55.9 ± 28.50	0.40 ± 0.12	0.62 ± 0.16	N.A.
	Lithospermic acid	10.1	1058.43 ± 256.90	1131.25 ± 227.92	238.67 ± 49.66	10.16 ± 2.58	0.58 ± 0.19	7.19 ± 1.61	10.51 ± 5.10

*, $p < 0.05$ compared with Crude group; [a], was assessed by the Kruskal–Wallis test; [b], significantly changed pharmacokinetic parameters in both groups are in bold; N.A., not applicable. CDS, crude Danshen; WDS, wine-processed Danshen.

4. Discussion and Conclusions

Danshen extract has been widely utilized in the treatment of cardiovascular-related diseases clinically [28]. Processing crude Danshen with wine has long been considered to enhance its biological activities; however, the mechanisms supported by adequate evidence are not clear yet. In the current study, we developed and optimized an HPLC-MS/MS method to simultaneously quantify seven bioactive components of Danshen in rat plasma for the first time. A model in which both positive and negative ions were detected simultaneously was built without losing the specificity and sensitivity (LLOQ = 0.5 ng/mL). Besides, the complete analysis takes a period as short as 8 min. With the sensitive method, a small quantity of biological sample (5 μL) at each time point was needed for the analysis, and no additional pre-column derivatization of the compounds was required. By employing a simple one-step protein precipitation approach for sample pretreatment and a relatively quick procedure for chromatographic separation (running time = 8 min), we were able to rapidly analyze the samples in a highly efficient manner. The establishment of the method is valuable for the quick quantification of various Danshen active components in biological samples, providing information about their pharmacokinetical behavior in vivo, explaining their efficiency model. Moreover, the study will direct the development of an analytical method for therapeutic monitoring the components, ensuring the safety and effectiveness of drug application.

Pharmacokinetic comparison between crude and wine-processed Danshen revealed that all the active ingredients in Danshen extract were absorbed fastly in vivo. This is consistent with their clinical application in the treatment of acute cardiovascular diseases. However, we observed significant decreases for C_{max} of tanshinone IIA and dihydrotanshinone I, and AUC for dihydrotanshinone I in wine-processed Danshen group. These results were completely contrary to our initial expectations: increased contents of tanshinone IIA and dihydrotanshinone I in wine-processed Danshen would result in higher plasma maximum concentration and absorption. By referring to previous literature, we speculate that the results might be attributed to the faster tissue distribution of the components in wine-processed Danshen group after initial absorption. As reported, processing of *Chuanxiong Rhizoma* with wine significantly decreased the $AUC_{0\text{-}t}$ values of its four active compounds, while increased their apparent volume of distribution (Vd), indicating wider tissue distribution [29]. Wine-processing also modified the distribution model of some flavonoids in *Radix scutellariae*: increased distribution in the rat upper-energizer tissues (lung and heart) and decreased distribution in the rat middle-and lower-energizer tissues (spleen, liver, and kidney) [30]. Thus, wine-processing might enhance the biological activity of Danshen by accelerating its distribution into target tissues. Detailed mechanisms about drug distribution into different tissues still need to be further investigated in subsequent studies.

Regarding the four hydrophilic acids, no significant differences between the pharmacokinetic parameters were found between the crude and wine-processed Danshen. A second peak was observed in the plasma-concentration profile of ferulic acid in the WDS group. We then did literature searching and found that the bimodal phenomenon was widely observed in pharmacokinetic profiles of constituents from traditional herbal medicine, which was probably due to distribution, reabsorption, and enterohepatic circulation [31,32]. Therefore, we guess that the second peak for ferulic acid in WDS might also be attributed to the above reasons. Further study is required to demonstrate the underlying mechanism. Our findings indicate that wine-processing of Danshen may modify the pharmacokinetic properties of some active components. However, the modification is limited, which might restrict its application in clinics. Since processing traditional herbal medicines is time-consuming and sometimes expensive, comparative study of the in vivo efficiency and pharmacokinetics between crude and processed medicines is rather necessary and meaningful. Our finding will provide useful suggestions for the doctors about the reasonable application of crude or processed Danshen.

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