The Effect of Compound Danshen Dripping Pills on the Dose and Concentration of Warfarin in Patients with Various Genetic Polymorphisms

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ABSTRACT

Purpose: The combination of warfarin and compound Danshen dripping pill (CDDP) is helpful for patients with both coronary heart disease (CHD) and atrial fibrillation (AF). The main adverse drug reaction of warfarin is bleeding because of its narrow therapeutic index. The safety of a combination therapy with warfarin and CDDP is always a concern. Our previous research showed that the combination of warfarin and CDDP improved the quality of life for patients with both CHD and AF. This study describes the changes in dose and concentration of warfarin necessary and evaluates bleeding risk when warfarin is given concomitantly with CDDP.

Methods: An ultra-performance liquid chromatography–MS/MS method with a chiral column was developed to assay the concentration of S-warfarin and R-warfarin in human plasma simultaneously. The method was applied to compare the concentration of warfarin in patients taking warfarin combined with CDDP and without CDDP. International normalized ratio (INR) values were monitored to evaluate bleeding risk. Paired *t* tests were then used to compare the dose and the concentration in 2 periods. Moreover, patients with *VKORC1*, *CYP2C9*3*, *CYP4F2*, *EPHX1*, and *PROC* gene polymorphisms were evaluated to determine interactions.

Findings: The results indicate that the dose of warfarin had no significant change with or without CDDP. Also, the peak concentrations of S-warfarin and total warfarin were significantly different in *CYP4F2 C/C* patients, but there was no significant difference identified in other genetic groups. No bleeding occurred in the study.

Implications: The dose of warfarin would be sustainable when combined with CDDP, because CDDP did not affect concentration of warfarin significantly in most patients and the change of INR was not significant.

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Key Words: compound Danshen dripping pills, concentration, dose, gene, warfarin.

INTRODUCTION

Coronary heart disease (CHD) is a critical risk factor for atrial fibrillation (AF) and a strong predictor for cardiovascular morbidity and mortality.[1,2] AF is always accompanied by CHD.[3] The incidence of CHD in patients with AF ranges from 24.0% to 46.5%.[4] AF is the most common arrhythmia found in clinical practice. Approximately 4.5 million people in the European Union and 2.3 million people in

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North America reportedly have paroxysmal or persistent AF.[5] The main hazard of AF is thromboembolic complications. The death rate in patients with AF may be reduced to 38% with the use anticoagulants,[6] and ~70%−80% of patients with AF are suitable for long-term use of warfarin, which has been the first-line anticoagulant, although optional agents are now available.[7,8]

Compound Danshen dripping pill (CDDP) functions by activating blood circulation and removing blood stasis, and it is a representative Chinese patent drug. CDDP is composed of *Salvia miltiorrhiza* (Danshen), *Radix notoginseng* (Sanqi), and *Borneolum syntheticum* (Bingpian) and has been widely administered to treat coronary arteriosclerosis, hyperlipidemia, angina pectoris, and other cardiovascular diseases.[9–11] The active ingredient of CDDP is the salvianolic acids, including danshensu (DSS), protocatechuic aldehyde, rosmarinic acid, and salvianolic acid A, among others. The Chinese Pharmacopoeia has stipulated that the content of DSS could not be < 0.10 mg/grain. The fingerprints of CDDP and plasma concentration of DSS and protocatechuic aldehyde in humans are shown in Supplemental Figures 1 and 2, respectively (see the online version at https://doi.org/10.1016/j.clinthera.2019.04.006).

The combination of warfarin and CDDP is a solution for patients with both CHD and AF because warfarin decreases the incidence of thromboembolic complications and CDDP relieves the symptoms of CHD. The main adverse drug reaction of warfarin is bleeding due to its narrow therapeutic index.[12] To guide the prescription of warfarin accurately, many studies have been performed. A number of factors, including physiological (ie, body weight, age, body surface area), pharmacologic (ie, vitamin K intake, drug−drug interactions), and genetic (ie, *VKORC1*, *CYP2C9*, *CYP4F2* polymorphisms), have been proven to be associated with the warfarin anticoagulant response, especially the genetic polymorphisms.[13] Whether CDDP affects the metabolism of warfarin and increases the bleeding risk in patients were considered. Warfarin is a racemic mixture of R- and S-enantiomers; the anticoagulant effect is mainly attributed to the S-isomer, which has 3- to 5-fold the potency of the R-form.[14] Although S-warfarin is the dominant species for activity of anticoagulation, the contribution of R-warfarin should not be ignored.[15] One study was conducted that analyzed the interactions between CDDP and warfarin in rats,[16] but the result of interactions in humans has not been shown.

We developed pharmacokinetic and pharmacodynamic models to evaluate the effect of CDDP on the distribution and excretion of warfarin.[17] In the present article, we discuss 4 concentrations of warfarin over each period (1 trough concentration [before the administration of warfarin], 1 near-peak concentration [1.5 h], and 2 on elimination phase) to illustrate the concentration influence of CDDP on warfarin. Moreover, these 4 concentrations are compared in different gene subtypes to show the interaction with genetic polymorphism. The international normalized ratio (INR) was monitored throughout the entire study to evaluate bleeding risk.

MATERIALS AND METHODS

Chemicals and Materials

The reference standard of warfarin was obtained from Tokyo Chemical Industry Co, Ltd (Tokyo, Japan), and tolbutamide (internal standard [IS]) was obtained from MilliporeSigma (Burlington, Massachusetts). The purity of these reference standards was 98.0% and 99.6% for warfarin and IS, respectively. Distilled water prepared with demineralized water was used throughout the experiment. Methanol and acetonitrile of HPLC grade was from Thermo Fisher Scientific (Fair Lawn, New Jersey), and ammonium acetate was provided by MilliporeSigma. The drug warfarin was manufactured by Orion Corporation (Espoo, Finland), and CDDP was manufactured by Tianjin Tasly Group Co, Ltd (Tianjin, China).

Patients and Genotyping

The study protocol was approved by the institutional review board of the Second Affiliated Hospital of Tianjin University of Traditional Chinese Medicine, and the study followed the guidelines of the Declaration of Helsinki and Tokyo for humans. Informed consent forms were given before enrollment, and the participants were free to withdraw from the study at any time.

All participants were administered warfarin to treat long-time CHD and AF. According to the previous reports, we determined those genes that affected the

metabolism of warfarin.[18,19] The genotyping of *VKORC1*, *CYP2C9*3*, *CYP4F2*, *EPHX1*, and *PROC* were then detected in this study. All genotyping was performed by using gene sequencing methods. *VKORC1* was classified as rs9923231 by detecting the 1173 C>T variant, *CYP2C9*3* was classified as rs1057910 by detecting the 1075 A>C variant, *CYP4F2* was classified as rs2108622 by detecting the C>T variant, *EPHX1* was classified as rs2292566 by detecting the G>A variant, and *PROC* was classified as rs5936 by detecting the G>T variant.

Instrumentation and Chromatographic Conditions

An ACQUITY ultra-performance liquid chromatography (UPLC) system equipped with a degasser, an autosampler, and a binary pump (Waters Corporation, Milford, Massachusetts) was used for the UPLC system. The Triple Quad 5500 MS/MS system equipped with a turbo ion spray source was from AB Sciex (Foster City, California). All the operations, acquisition, and analysis of data were performed by using Analyst version 1.6.2 (AB Sciex).

An Astec CHIROBIOTIC V column (250 × 4.6 mm internal diameter, particle size 5 μm; MilliporeSigma) with acetonitrile 5-mm ammonium acetate in water (30:70, v/v; isocratic elution) as the mobile phase were used for the chromatographic separation of S-warfarin, R-warfarin, and IS. The flow rate was 0.5 mL/min, and the sample injection volume was 2.00 μL.

The detection of warfarin was performed by using multiple reaction monitoring (MRM) with negative mode. The ion spray voltage was set at −4500 V. The source temperature was set at 600 °C; curtain gas, gas 1, and gas 2 (all gases, nitrogen) were set at 30, 55, and 50 psi, respectively. Quantitative parameters are listed in Table I.

Preparation of Standard Solutions

The stock solutions of warfarin and IS were prepared in methanol at the concentration of 2.00 mg/mL. They were further diluted with 30% acetonitrile/water to make working solutions at the concentration of 1.00−40.0 μg/mL for warfarin. IS was diluted to a concentration of 5.00 μg/mL with 30% acetonitrile/water as the working solution. All the solutions were stored at 4 °C.

The plasma concentration of the standards were prepared by adding the blank human plasma with the corresponding mixed standard solutions. The final plasma concentrations were 50.00, 100.0, 250.0, 500.0, 1000, 1500, and 2000 ng/mL for R-warfarin and S-warfarin. Quality control (QC) samples were prepared in the same fashion (90.00, 900.0, and 1800 ng/mL).

Sample Preparation

Before analysis, all calibration standards, QC samples, and frozen samples were thawed and allowed to equilibrate at room temperature. Before spiking, the samples were vortexed for 10 s; 5.00 μL of IS and 5.00 μL of methanol were added to 45.0 μL of plasma, the mixture were extracted with 150.0 μL of acetonitrile, with subsequent vortex mixing for 3 min and centrifugation for 10 min at 14,000 rpm. The supernatant was further diluted by taking 20.00 μL to 380.0 μL mobile phase solution, then 2.00 μL of the mix solution was injected into the LC-MS/MS system for analysis.

Method Validation

The method was validated by using selectivity, calibration curve, lower limit of quantitation (LLOQ), carryover, accuracy and precision, recovery, matrix effect, and stability, according to the Bioanalytical Method Validation Guidance in the

Table I. List of selected multiple reaction monitoring parameters, declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP) for warfarin and the internal standard (IS).

Compound	Q1	Q3	DP (V)	EP (V)	CE (V)	CXP (V)
Warfarin	307.1	161.1	−100	−10	−26	−17
Tolbutamide (IS)	269.1	170.0	−90	−10	−24	−17

Chinese Pharmacopoeia and the US Food and Drug Administration.

Specificity was calculated by comparing areas of analytes in blank plasma from 6 different human plasma samples versus the areas of analytes in LLOQ. The goal was to determine if there was significant interference at the retention time of R-warfarin, S-warfarin, and IS.

The linearity for R-warfarin and S-warfarin with a total of 7 calibration standards (50.00, 100.0, 250.0, 500.0, 1000, 1500, and 2000 ng/mL) were evaluated by plotting a calibration model with analytes-to-IS peak area ratios versus the calibration standard concentration. Least squares linear regression with a weighted ($1/x^2$) factor was applied for the models. Each run included 2 independent calibration curve runs for 3 different analytical batches: $y = ax + b$ was applied for the calibration models, where x = the concentration of R-warfarin or S-warfarin in plasma, y = the peak area ratio of warfarin/IS, a = slope, and b = intercept. If the calibration correlation coefficient r was >0.99, and the residuals were within ±20% at the LLOQ and 15% at all other calibration levels, the calibration model would be accepted.

LLOQ was determined as the lower limit of quantitation, which must have a signal-to-noise ratio >10. The accuracy (which was within ±20%) and the precision (which was between 80% and 120%) seem to be acceptable.

The carryover was analyzed by measuring the area of blank plasma, which were followed by the upper limit of quantitation for 3 runs. It was considered negligible if the peak area of blank plasma was less than ±20% of the area of LLOQ.

The intraday/interday accuracy and precision were conducted at 3 QC levels (low/medium/high, in 6 replicates) within the same run and on 3 consecutive validation runs. Accuracy was defined as percent relative error (RE) and precision was defined as the percent relative SD (RSD).

The absolute extraction recoveries of warfarin were also determined at 3 QC levels (low/medium/high, in 6 replicates). They were calculated by comparing the mean area of 3 QC level samples with the area of freshly prepared unextracted standards in a mixture of 30% acetonitrile/water. They were also evaluated at 1 concentration level for IS and at 3 QC levels for warfarin.

The matrix effect was evaluated by using matrix factor (MF) with 6 lots of matrix. MFs were calculated by comparing the peak area of warfarin or IS spiked into the treated blank plasma versus the peak area in pure standard solutions of warfarin or IS at 3 QC levels. The IS-normalized MF was defined as the ratio of warfarin MF to IS MF, with acceptable IS-normalized MF (RSD) < 15%.

The stability tests of warfarin were evaluated by analyzing spiked samples at each QC level with 3 repetitions under different conditions: room temperature for 24 h, autosampler (4 °C) for 24 h, after 3 freeze—thaw cycles, and at –70 °C for 90 days.

Dose and Concentration Influence

The clinical trial consisted of 2 periods. In the first period, eligible patients took warfarin, and this dose was mediated by regular determinations of INR to guarantee that the INR value was 2—3. When the INR value was stable, patients would take the same dose of warfarin for 2 weeks. Participants then entered into the second period. They would take warfarin and the CDDP (10 grains, 3 times per day) for not less than 4 weeks. According to the affected INR value, the dose of warfarin was re-adjusted until the INR value was stable. The dose of warfarin was then retained for 2 weeks. At the end of each period, 4 blood samples (each 3 ml) were collected for determination of warfarin concentration. The 4 sampling times were 1 trough concentration (before the administration of warfarin), 1 near peak concentration (1.5 h), and 2 on elimination phase. The concentrations of R- and S- warfarin were then determined by using the UPLC-MS/MS method with a chiral column. The concentration of total warfarin was the sum of the R- and S-enantiomers.

The paired t test was used to compare the dose and the concentration difference of the 2 periods. Moreover, patients with gene polymorphisms were assessed to study the interaction between different genes.

RESULTS

Patients and Genotyping

A total of 64 patients with both CHD and AF were recruited from 4 hospitals from 2013 to 2016 in Tianjin; 59 patients completed the trial. All the participants enrolled in this study were Chinese. The percentage of male and female subjects was 64.41%

(n = 38) and 35.59% (n = 21), respectively. The mean age of these patients was 63 years (range, 49–79 years). The genetic polymorphisms of *VKORC1*, *CYP2C9*3*, *CYP4F2*, *EPHX1*, and *PROC* are presented in Table II.

Method Validation

Specificity

The retention time for R-warfarin, S-warfarin, and IS were 7.95, 8.78, and 6.99 min, respectively. The areas of analytes in blank plasma from 6 different human plasma samples with the areas of analytes in LLOQ were all <20%. There was no significant interference observed. Fig. 1 displays typical multiple reaction monitoring chromatograms of blank plasma, plasma spiked with LLOQ, and NO. 1 human plasma samples before oral administration of warfarin.

Linearity, LLOQ, and Carryover

A linear relationship was found when plotting peak area ratios (analyte/IS) against warfarin concentrations. The relationship was found to be linear for R- and S-warfarin under the concentration range. The mean values for the regression parameters and the correlation coefficient of R-warfarin were $y = 2.5500\ x + 0.0067$ ($r = 0.9957$); for S-warfarin, they were $y = 2.4976\ x + 0.0038$ ($r = 0.9976$).

The maximum intra-RE of LLOQ in plasma in 3 runs of R-warfarin and S-warfarin was –2.58% and 10.63%, respectively. The inter-RE of LLOQ of R-warfarin and S-warfarin was 0.14% and 8.34%; inter-RSD was 12.71% and 7.57%.

After the highest calibrator (upper limit of quantitation), a blank plasma sample was injected. The warfarin peak areas in the blank samples were <20% of LLOQ areas, resulting in an acceptable carryover effect.

Table II. Genetic information summary for subjects.

Characteristic	Value
No. of patients	59
No. of plasma concentration points	404
VKORC1	
T/T	54 (91.53%)
C/T	5 (8.47%)
*CYP2C9*3*	
A/A	51 (86.44%)
A/C	8 (13.56%)
CYP4F2	
C/C	35 (59.32%)
C/T	19 (32.20%)
T/T	5 (8.47%)
EPHX1	
A/A	2 (3.39%)
A/G	33 (55.93%)
G/G	24 (40.68%)
PROC	
G/G	11 (18.64%)
T/T	13 (22.03%)
T/G	35 (59.32%)

Precision and Accuracy

The intraday and interday precision and accuracy were investigated by analyzing QC samples (low/medium/high) (Table III). Intraday and interday precision and accuracy of R-warfarin and S-warfarin were well within acceptance criteria (±15%), indicating satisfactory results for accuracy and precision of warfarin.

Recovery and Matrix Effect

Table III presents the recovery results of R-warfarin and S-warfarin that were obtained at the 3 concentration levels. The recoveries of R-warfarin were 91.10%, 93.48%, and 99.05%; for S-warfarin, they were 93.14%, 93.42%, and 97.94%. These results indicate that recoveries of warfarin were consistent and not concentration dependent.

The matrix effect was calculated at the 3 QC levels by IS-normalized MF. The RSDs in Table III were all <6.03%. The results indicate that the endogenous matrix did not affect the determination of warfarin and IS.

Stability

The results of the stability experiments under different storage conditions were all within the acceptable limit (Table III). The results indicate that warfarin was stable in plasma samples kept for 24 h at room temperature, 24 h at autosampler (4 °C), after 3 freeze–thaw cycles, and at –70 °C for 90 days.

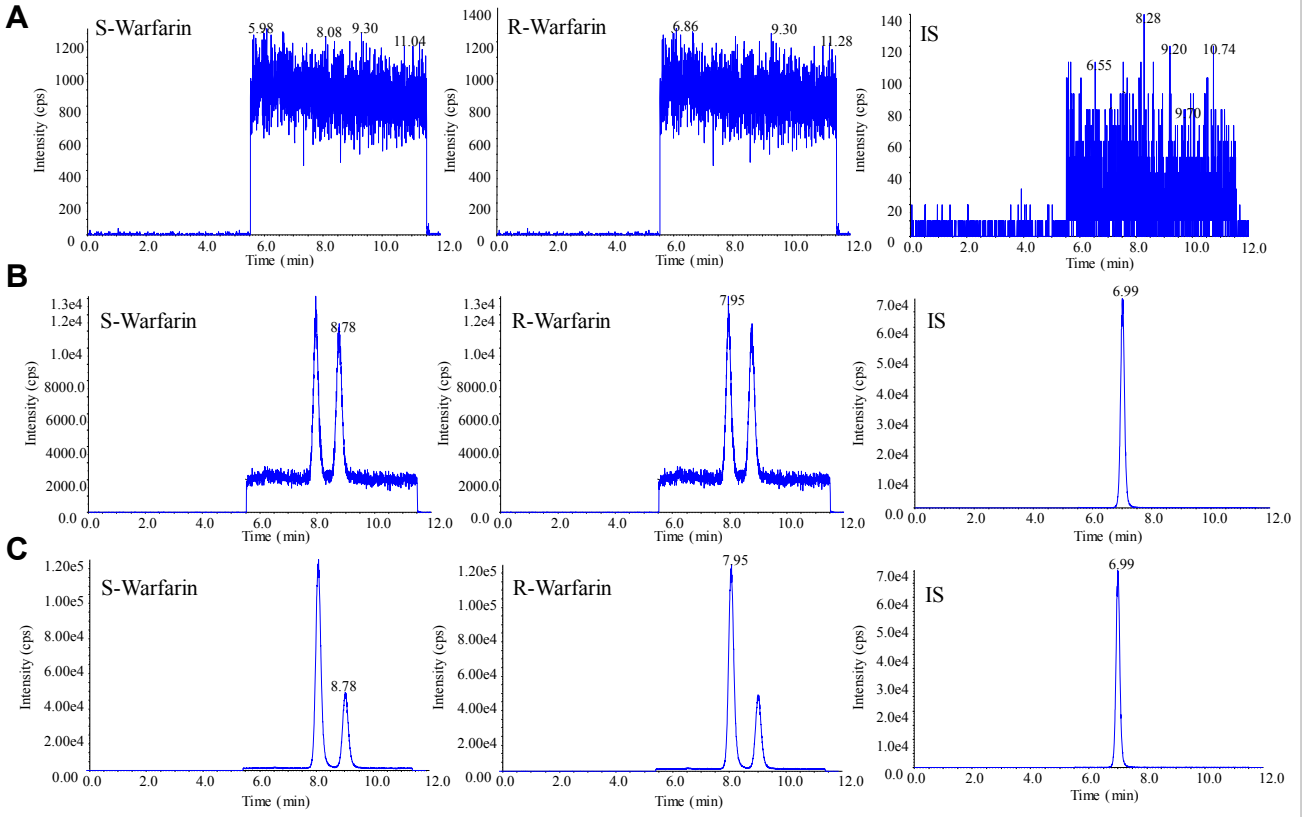

Fig. 1. Typical multiple reaction monitoring chromatograms of S-warfarin, R-warfarin, and internal standard (IS). (A) Blank plasma. (B) Plasma spiked with warfarin and IS (lower limit of quantitation). (C) NO. 1 human plasma samples before oral administration of warfarin.

Dose and Concentration Influence

The dose comparisons of warfarin between the 2 periods with or without CCDP are shown in Table IV. and Fig. 2. For all patients, the mean (SD) dose of warfarin was 3.41 (1.11) mg in the first period and 3.39 (0.98) mg in the second period, which was not significantly different. For the different genetic groups, the *P* values from the paired *t* test were all >0.05, indicating that there was no significant dose change between 2 periods in the various genetic groups.

The concentration of R-warfarin, S-warfarin, and total warfarin are shown in Table V and Fig. 2. Because of the huge variation in blood collection time of the third and fourth concentrations designed for the calculation of the population pharmacokinetic parameters,[16] both of them were not included in the concentration comparison. The first (trough) and the second (peak) concentration of R-warfarin, S-warfarin, and total warfarin were compared between the 2 periods by using a paired *t* test. For the trough concentration, there was no significant difference between all these patients. The peak concentration of S-warfarin in *CYP4F2 C/C* was significantly different between the 2 periods ($P = 0.033$). The result for total warfarin was similar ($P = 0.040$).

DISCUSSION

Patients and Genotyping

It has been reported that *VKORC1*, *CYP2C9*3*, *CYP4F2*, *EPHX1*, and *PROC* could influence the anticoagulation of warfarin.[8,13,20–23] Moreover, both *EPHX1* and *PROC* may alter the pharmacokinetic and pharmacodynamic variables of warfarin and thus further affect the warfarin maintenance dose.[8,24] Because the patients in this study had serious heart

Table III. The linearity, precision and accuracy, absolute extraction recovery, matrix effect, and stability of warfarin.

Variable	R-Warfarin LQC	R-Warfarin MQC	R-Warfarin HQC	S-Warfarin LQC	S-Warfarin MQC	S-Warfarin HQC
Calibration ($y = ax + b$)	$y = 2.5500x + 0.0067$ ($r = 0.9957$)			$y = 2.4976x + 0.0038$ ($r = 0.9976$)		
Intraday accuracy RE%	−9.22	−5.37	−0.82	−8.30	−6.02	3.60
	−0.61	1.77	2.83	3.20	0.54	5.41
	3.04	4.47	8.83	3.75	8.44	7.72
Intraday precision RSD%	5.21	5.96	8.69	5.39	6.16	5.32
	6.09	7.07	8.07	3.18	8.16	6.81
	8.34	6.94	4.93	5.32	4.40	2.82
Interday accuracy RE%	−2.30	0.30	3.60	−0.50	1.00	5.60
Interday precision RE%	8.37	7.61	7.92	7.24	8.48	5.17
Absolute recovery (mean,%)	91.10	93.48	99.05	93.14	93.42	97.94
Matrix effect RSD%	6.03	2.75	0.49	2.80	5.03	0.64
24-h room temperature RE%	6.21	7.43	5.32	1.27	3.84	5.03
24-h autosampler RE%	2.23	3.23	1.54	−0.57	1.24	2.31
Three freeze−thaw cycles RE%	−0.28	3.23	4.67	−1.37	3.65	4.31
−70 °C for 90 d RE%	8.23	6.42	7.48	3.67	7.89	4.69

HQC = high quality control; LQC = low quality control; MQC = medium quality control; RE = relative error; RSD = relative SD.

disease, it is hard to recruit more. Nevertheless, the population gene distribution was consistent with previous reports.[20–23]

Method Validation

Optimization of Electrospray Ionization−MS/MS

In this study, MS/MS operation parameters were carefully optimized for measurement of R- and S-warfarin in plasma. The full-scan precursor/product ions of warfarin were finished by injecting the suitable standard solutions into the mass spectrometer. Some parameters such as curtain gas, gas 1, gas 2, IS voltage, declustering potential, and collision energy were optimized. The other parameters were adopted for the recommended value of the instrument.

Optimization of Chromatographic Conditions

Because warfarin is a racemic mixture of R-enantiomer and S-enantiomer, they must be completely separated. As a result, a chiral column was used to improve their separation. In addition, the column avoided a signal suppression effect, which is suitable for the separation and retention of racemic warfarin.

Acetonitrile was chosen as the organic phase compared with methanol because it gave a lower background noise and a higher mass spectrometric response. Furthermore, the addition of 5-mm ammonium acetate in the mobile phase improved the peak shape of warfarin. However, the retention time of the analytes was easilye changed by gradient; the different ratio of the mobile phase had a strong impact on the separation of the enantiomer. If 20% acetonitrile was used as the mobile phase, the retention time exceeded 10 min. If 40% acetonitrile was used as the mobile phase, R-warfarin and S-warfarin could not be well separated. Thus, 30% acetonitrile/5-mm ammonium acetate in water was chosen as the mobile phase. In addition, R- and S-enantiomers were perfectly detected with sharp peak shape and high separation.

Dose and Concentration Influence

The increasing use of herbal medicinal products by individuals who are also taking prescription medicines suggests that adverse herb−drug

Table IV. The warfarin dose comparison between 2 periods.

Variable	Dose (Mean [SD]), mg		*P*
	First Period	Second Period	
All Patients	3.41 (1.11)	3.39 (0.98)	0.808
VKORC1 T/T	3.31 (0.98)	3.32 (0.94)	0.973
VKORC1 C/T	4.28 (1.87)	4.05 (1.14)	0.573
*CYP2C9*3 A/A*	3.49 (1.11)	3.45 (0.95)	0.653
*CYP2C9*3 A/C*	2.89 (1.01)	3.00 (1.15)	0.604
CYP4F2 C/C	3.30 (0.84)	3.37 (0.73)	0.531
CYP4F2 C/T	3.35 (1.37)	3.15 (1.13)	0.217
CYP4F2 T/T	4.20 (1.46)	4.30 (1.37)	0.587
EPHX1 A/A	3.38 (1.59)	2.25 (1.06)	0.205
EPHX1 A/G	3.28 (1.15)	3.29 (0.88)	0.915
EPHX1 G/G	3.61 (1.03)	3.65 (1.04)	0.538
PROC G/G	3.70 (1.58)	3.98 (1.31)	0.351
PROC T/T	3.15 (1.01)	2.91 (0.85)	0.190
PROC T/G	3.42 (1.02)	3.40 (0.86)	0.824

In first period, patients took warfarin only; in the second period, patients took warfarin and compound Danshen dripping pill. $P < 0.05$ is regarded as significant.

interactions may be of significant public health consequence.[25] A herb–drug interaction study is the key tool for determination of adverse drug reactions of herbal products and conventional drugs.[26] However, it is difficult to avoid side effects arising from the simultaneous intake of herbal products and drugs. Some unacceptable risks must be specifically considered in the most vulnerable populations, such as the elderly affected by heart diseases. A serious approach to solving this problem would be to provide accurate information on potential risks arising from a prolonged use of herbal products; medical supervision is always recommended.[27] It is thus challenging and important to conduct research on the herb–drug interaction.

Many reports have shown that subjects treated with warfarin should avoid taking herbal products that remove blood stasis due to the high risk of bleeding; these products include *Panax ginseng*, *Salvia miltiorrhiza*, and *Radix notoginseng*.[28,29] CDDP is composed of *Salvia miltiorrhiza*, *Radix notoginseng*, and *Borneolum syntheticum*, which functions to promote blood circulation and remove blood stasis.[9,10] In this study, we assessed the herb–drug interaction between warfarin and CDDP in patients of different gene polymorphisms. Five genes, *VKORC1*, *CYP2C9*3*, *CYP4F2*, *EPHX1*, and *PROC*, reportedly affect the dose and concentration of warfarin. The trough and peak concentration of warfarin were tested to evaluate interactions between CDDP and warfarin in patients with various genotyping. There was no significant difference in dose of warfarin and the trough concentration between the 2 periods, not only in all patients but also in each of the 5 gene polymorphisms. In *CYP4F2 C/C* patients, the peak concentrations of S-warfarin and total warfarin were significantly increased after administration of CDDP. The peak concentration of S-warfarin changed from 403.5 (161.3) ng/mL to 467.1 (216.8) ng/mL and that of total warfarin changed from 1213.2 (363.7) ng/mL to 1379.4 (459.8) ng/mL. Moreover, the monitored INR values were all <3.0; thus, there was no bleeding reported in our study.

The dose of warfarin was regulated according to the INR value, and the increase from 3.30 (0.84) mg to 3.37 (0.73) mg probably accounted for the little proportion change of peak concentration in the first and second periods. Furthermore, *Salvia miltiorrhiza* comprises the major content in CDDP that is rich in phenolic acids and different levels of tanshinones.[30] It has been reported that the steady-state plasma concentration of warfarin in rats was increased by 23% when Danshen was co-administered due to the inhibition of warfarin hydroxylation by major tanshinones in Danshen (*Salvia miltiorrhiza*).[28,29] Although the peak concentration of S-warfarin and total warfarin increased significantly, no cases of bleeding were reported. It is challenging to explain the phenomena due to the multicomponent property of Chinese patent medicines. Nonetheless, it has been confirmed that CDDP does not increase the bleeding risk of warfarin in patients.

In our previous study,[17] we developed some population pharmacokinetic models to assess the effect of CDDP on warfarin. The results showed that CDDP did not influence the pharmacokinetic characteristic of warfarin and the INR stability of warfarin in most patients with CHD. However, some genetic subtypes require further investigation. In present study, the subtypes of *VKORC1*, *CYP2C9*3*, *CYP4F2*, *EPHX1*, and *PROC* were

Table V. The concentration comparison of S-warfarin, R-warfarin, and total warfarin between the 2 periods (values are given in nanograms per milliliter).

Variable	S-Warfarin			R-Warfarin			Total Warfarin		
	First Period	Second Period	*P*	First Period	Second Period	*P*	First Period	Second Period	*P*
Concentration 1									
All patients	374.3 (200.5)	379.7 (238.4)	0.834	807.6 (286.9)	823.1 (279.1)	0.737	1181.9 (417.9)	1202.8 (461.7)	0.755
VKORC1 T/T	355.4 (186.9)	367.5 (236.1)	0.658	784.6 (281.6)	811.7 (282.1)	0.571	1140.0 (393.9)	1179.2 (456.0)	0.575
VKORC1 C/T	632.4 (242.0)	546.3 (249.3)	0.120	1122.3 (157.3)	978.6 (208.7)	0.501	1754.7 (350.8)	1524.8 (429.4)	0.387
CYP2C9*3 A/A	352.5 (171.0)	352.9 (206.3)	0.988	816.5 (297.4)	831.5 (280.7)	0.778	1169.0 (406.8)	1184.4 (441.2)	0.839
CYP2C9*3 A/C	512.6 (320.9)	549.1 (365.8)	0.514	751.2 (221.8)	769.8 (288.1)	0.726	1263.8 (517.6)	1318.9 (611.4)	0.596
CYP4F2 C/C	391.2 (193.5)	417.7 (272.0)	0.472	824.0 (276.2)	871.3 (284.7)	0.487	1215.2 (403.0)	1289.0 (498.9)	0.448
CYP4F2 C/T	306.6 (178.3)	278.2 (97.0)	0.468	679.1 (227.0)	668.0 (211.4)	0.854	985.6 (337.5)	946.2 (247.3)	0.682
CYP4F2 T/T	537.9 (308.5)	510.8 (284.8)	0.692	1260.7 (128.6)	1113.3 (65.3)	0.261	1798.6 (127.3)	1624.2 (349.0)	0.143
EPHX1 A/G	338.4 (217.4)	362.6 (228.3)	0.985	746.1 (276.8)	809.4 (250.0)	0.721	1084.5 (442.1)	1172.0 (412.9)	0.841
EPHX1 G/G	402.4 (135.7)	403.5 (264.8)	0.709	890.0 (292.7)	864.1 (319.8)	0.366	1292.4 (3330.0)	1267.7 (542.8)	0.393
PROC G/G	332.1 (193.3)	347.5 (150.4)	0.389	631.6 (306.4)	799.9 (349.2)	0.159	963.7 (410.7)	1147.3 (392.0)	0.227
PROC T/T	498.3 (262.4)	450.9 (290.2)	0.535	932.8 (187.0)	838.5 (267.6)	0.765	1431.1 (365.9)	1289.4 (470.9)	0.653
PROC T/G	339.3 (162.4)	361.6 (238.8)	0.658	806.9 (296.7)	823.4 (275.0)	0.571	1146.3 (406.9)	1185.1 (406.9)	0.575
Concentration 2									
All patients	428.3 (209.2)	452.7 (220.6)	0.237	861.4 (332.4)	911.6 (353.8)	0.177	1289.7 (496.1)	1364.3 (532.3)	0.169
VKORC1 T/T	403.3 (177.8	433.5 (203.5)	0.174	823.4 (275.9)	882.0 (309.6)	0.143	1226.7 (396.3)	1315.6 (465.1)	0.128
VKORC1 C/T	702.6 (351.4	663.3 (322.3)	0.372	1279.8 (620.6)	1237.0 (658.5)	0.596	1982.4 (951.9)	1900.2 (959.5)	0.446
CYP2C9*3 A/A	421.9 (200.6	432.3 (210.3)	0.622	889.5 (337.1)	923.3 (363.3)	0.405	1311.4 (500.0)	1355.6 (542.3)	0.442
CYP2C9*3 A/C	472.8 (280.5	595.1 (258.3)	0.105	665.0 (232.2)	830.0 (292.3)	0.071	1137.8 (481.1)	1425.0 (497.0)	0.082
CYP4F2 C/C	403.5 (161.3	467.1 (216.8)	0.033*	809.8 (261.1)	912.3 (292.4)	0.072	1213.2 (363.7)	1379.4 (459.8)	0.040*
CYP4F2 C/T	429.9 (275.9	395.1 (224.7)	0.296	867.4 (437.7)	823.2 (442.0)	0.374	1297.3 (681.0)	1218.3 (640.5)	0.329
CYP4F2 T/T	562.1 (213.6	544.8 (230.3)	0.557	1132.8 (238.1)	1173.1 (308.8)	0.527	1694.9 (359.4)	1717.9 (477.8)	0.737
EPHX1 A/G	387.3 (223.8	429.2 (213.5)	0.069	817.7 (383.1)	902.4 (382.3)	0.124	1205.0 (573.7)	1331.6 (554.1)	0.082
EPHX1 G/G	444.8 (146.8	457.8 (225.9)	0.740	906.8 (262.0)	924.8 (330.1)	0.715	1351.7 (343.4)	1382.6 (522.4)	0.717
PROC G/G	426.0 (197.5	442.8 (195.7)	0.790	737.4 (361.3)	858.0 (403.9)	0.456	1163.4 (505.4)	1300.8 (552.9)	0.515
PROC T/T	507.6 (239.6	472.1 (255.0)	0.402	891.4 (244.2)	822.4 (235.4)	0.232	1399.0 (401.7)	1294.5 (417.2)	0.280
PROC T/G	402.4 (202.1	448.8 (221.9)	0.071	884.5 (352.1)	955.6 (375.2)	0.072	1286.9 (527.8)	1404.5 (571.7)	0.056
Concentration 3									
All patients	461.1 (199.8	437.4 (196.7)	0.263	916.5 (328.3)	870.3 (323.6)	0.302	1377.6 (484.5)	1307.7 (463.9)	0.258

(*continued on next page*)

Table V. (*Continued*)

Variable	S-Warfarin			R-Warfarin			Total Warfarin		
	First Period	Second Period	*P*	First Period	Second Period	*P*	First Period	Second Period	*P*
VKORC1 T/T	442.7 (190.3	426.0 (192.6)	0.460	886.4 (306.3)	849.6 (288.9)	0.436	1329.1 (449.6)	1275.6 (419.4)	0.414
VKORC1 C/T	663.8 (215.9	562.8 (226.3)	0.071	1246.8 (429.5)	1097.6 (607.1)	0.337	1910.6 (606.4)	1660.4 (818.0)	0.216
CYP2C9*3 A/A	446.4 (188.1	424.1 (188.9)	0.345	930.9 (335.5)	889.9 (335.9)	0.416	1377.3 (486.1)	1314.0 (480.4)	0.364
CYP2C9*3 A/C	563.9 (265.9	530.7 (242.9)	0.431	815.4 (275.1)	732.9 (181.6)	0.253	1379.4 (518.6)	1263.5 (357.4)	0.227
CYP4F2 C/C	423.7 (174.2	442.1 (211.9)	0.458	840.6 (283.1)	859.0 (297.3)	0.776	1264.3 (412.3)	1301.1 (441.0)	0.659
CYP4F2 C/T	481.9 (237.7	401.7 (178.6	0.077	943.7 (378.8)	824.2 (382.2)	0.098	1425.7 (574.4)	1225.9 (527.7)	0.082
CYP4F2 T/T	607.6 (167.7)	518.5 (163.5)	0.143	1259.6 (180.2)	1071.6 (250.8)	0.026	1867.2 (241.1)	1590.1 (340.5)	0.013
EPHX1 A/G	431.9 (211.8)	421.5 (185.5)	0.664	860.9 (338.5)	866.0 (341.2)	0.937	1292.7 (518.8)	1287.5 (469.2)	0.950
EPHX1 G/G	473.6 (129.3)	453.7 (218.4)	0.548	984.3 (309.5)	887.9 (309.6)	0.077	1457.9 (389.0)	1341.6 (478.9)	0.167
PROC G/G	499.9 (169.6)	478.6 (174.5)	0.758	868.5 (404.1)	925.6 (420.3)	0.802	1368.4 (533.5)	1404.2 (504.7)	0.901
PROC T/T	558.5 (274.6)	467.4 (198.4)	0.115	981.8 (272.5)	809.4 (183.4)	0.025	1540.2 (492.6)	1276.8 (274.1)	0.038
PROC T/G	418.3 (169.6)	416.4 (204.7)	0.935	907.5 (331.6)	875.8 (338.3)	0.375	1325.8 (473.9)	1292.3 (510.7)	0.539
Concentration 4									
All patients	440.3 (212.3)	434.2 (225.3)	0.824	874.9 (341.6)	904.9 (343.4)	0.587	1315.1 (508.1)	1339.1 (512.8)	0.751
VKORC1 T/T	416.7 (204.5)	406.4 (192.8)	0.713	835.7 (321.0)	866.0 (303.6)	0.603	1252.4 (475.0)	1272.4 (430.9)	0.797
VKORC1 C/T	652.9 (176.8)	684.4 (365.7)	0.814	1227.0 (361.6)	1255.7 (524.3)	0.899	1879.9 (500.8)	1940.0 (847.1)	0.865
CYP2C9*3 A/A	435.1 (201.1)	407.2 (180.3)	0.310	900.9 (339.4)	904.9 (340.2)	0.946	1336.0 (501.8)	1312.1 (478.4)	0.762
CYP2C9*3 A/C	486.8 (333.5)	677.0 (439.8)	0.069	640.7 (302.3)	905.6 (426.7)	0.055	1127.5 (605.1)	1582.6 (813.4)	0.053
CYP4F2 C/C	407.2 (162.5)	417.8 (227.2)	0.703	852.9 (307.6)	901.4 (317.0)	0.586	1260.1 (421.7)	1319.1 (478.8)	0.591
CYP4F2 C/T	421.1 (216.1)	397.1 (162.9)	0.473	808.7 (390.4)	784.9 (349.8)	0.665	1229.8 (573.5)	1182.1 (474.4)	0.576
CYP4F2 T/T	693.0 (332.5)	649.1 (324.7)	0.856	1215.8 (188.7)	1315.5 (137.5)	0.579	1908.8 (460.7)	1964.6 (454.1)	0.891
EPHX1 A/G	438.8 (245.7)	426.7 (240.8)	0.756	858.3 (360.9)	909.8 (359.3)	0.501	1297.0 (568.7)	1336.5 (539.9)	0.702
EPHX1 G/G	416.1 (127.7)	438.7 (214.8)	0.526	890.7 (332.5)	916.1 (333.8)	0.763	1306.8 (419.8)	1354.8 (503.5)	0.674
PROC G/G	370.8 (122.3)	420.9 (130.2)	0.205	762.4 (420.3)	1020.7 (491.5)	0.324	1133.2 (511.3)	1441.6 (536.4)	0.296
PROC T/T	541.2 (241.0)	553.9 (321.9)	0.873	887.5 (300.4)	893.4 (316.8)	0.957	1428.7 (452.2)	1447.2 (565.4)	0.920
PROC T/G	425.2 (215.5)	400.4 (202.4)	0.475	896.9 (343.2)	881.8 (322.0)	0.789	1322.2 (530.2)	1282.2 (503.6)	0.628

In the first period, patients took warfarin only; in the second period, patients took warfarin and compound Danshen dripping pill; *P* values are the results of paired *t* tests for the 2 periods.

Concentration 1 means trough concentration (before the administration of warfarin); Concentration 2 means near peak concentration (1.5 h); and concentrations 3 and 4 were 2 concentrations on elimination phase of warfarin.

* The one different between the 2 periods; EPHX1 A/A were not listed because the number is < 3.

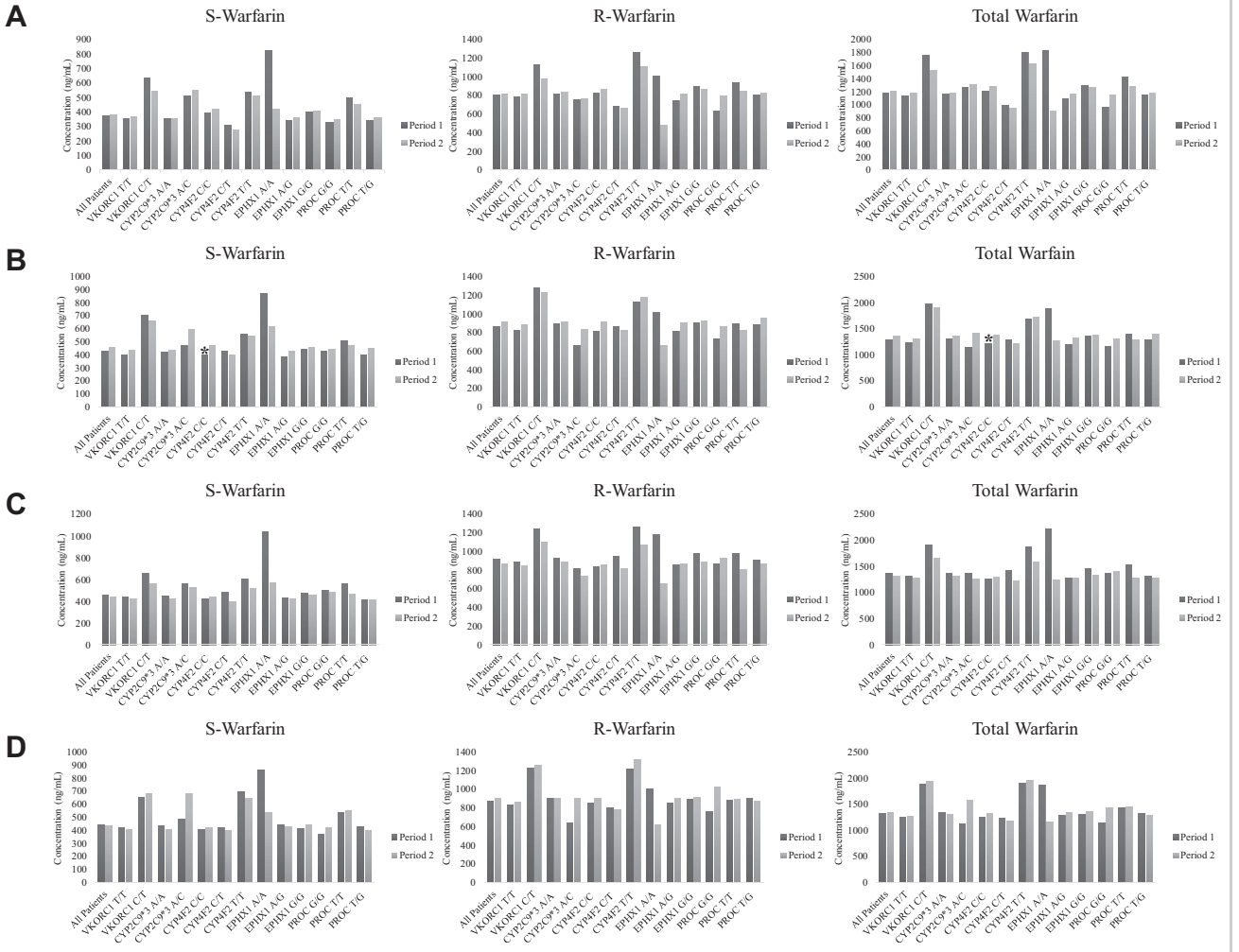

Fig. 2. The concentration comparison of S-warfarin, R-warfarin, and total warfarin between the 2 periods. (A) Concentration 1 means trough concentration (before the administration of warfarin). (B) Concentration 2 means near peak concentration (1.5 h). *$P < 0.05$ (P value was the result of paired t test for the 2 periods. (C, D) Concentration 3 and 4 were two concentrations on elimination phase of warfarin.

investigated, and the role of *CYP4F2 C/C* was confirmed. Further research should be performed to explore the mechanism of interaction between CDDP and warfarin in the subgroup of *CYP4F2 C/C*.

CONCLUSIONS

A robust and sensitive UPLC-MS/MS method with a chiral column for simultaneous quantitation of S-warfarin and R-warfarin in human plasma has been developed and completely validated. The method was applied to compare the concentration of warfarin in patients taking warfarin with and without CDDP. The results of clinical research indicate that there was no significant difference in warfarin dose between 2 periods, but there was a significant difference in the peak concentrations of S-warfarin and total warfarin in *CYP4F2 C/C* patients. One of the limitations of this study was that the interaction of warfarin and CDDP was investigated in Chinese participants only. In addition, the plasma concentrations of active ingredients in CDDP were not measured in this

study. Further studies are warranted to fully understand the interaction between the active ingredients of CDDP and warfarin.

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CONFLICTS OF INTEREST

The authors have indicated that they have no conflicts of interest regarding the content of this article.

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