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Original Article

Monitoring unbound warfarin in drug combination therapy by pharmacokinetics and fluorospectrometry

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ABSTRACT

Objective: Monitoring the unbound drug concentration in blood in combination therapy is necessary, because its concentration determines the efficacy of drug therapy. This study was designed to explore the effect of Dan Hong Injection (DHI) on the unbound warfarin using two approaches including an *in vivo* pharmacokinetic and *in vitro* fluorescence studies.
Methods: The effect of DHI on the pharmacokinetic properties of the unbound warfarin was investigated by a microdialysis sampling method coupled with LC–MS/MS. The effect of DHI and salvianolic acid B (SaB) on warfarin binding with bovine serum albumin (BSA) was conducted by fluorescence spectrometry.
Results: The $AUC_{0\text{-}tn}$ of warfarin with DHI group was higher than that of warfarin alone group. The result showed that DHI could increase the concentration of unbound warfarin in rat blood, which may be due to the competition between warfarin and DHI as well as its components binding to serum albumin. The competition process was demonstrated by fluorescence study.
Conclusion: Combination therapy of DHI with warfarin could enhance the release profile of warfarin from serum protein.

1. Introduction

With the increasing development in traditional Chinese medicine and western medicine, combination therapies involving these medicines are used widely in clinical practice (Yue et al., 2013). Also, the interactions of traditional Chinese medicine and western medicine have become one of the interesting topics in the field of pharmacy and medicine (Zhen, Kong, & Ren, 2014).

It is a common clinical phenomenon that warfarin, which is commonly used to prevent and treat thrombotic and embolic disorders in clinics (Neidecker, Patel, Nelson, & Reardon, 2012), is often used in combination with Dan Hong Injection (DHI), a typical blood circulation promoting TCM (He et al., 2012; Sun, Fan, & Han, 2015), for the treatment of cardiovascular diseases in China (Li et al., 2016). The use of warfarin is very challenging due to its narrow therapeutic index (Kimmel, 2015; Alarifi et al., 2016; Kinoshita et al., 2016; Connolly et al., 2013). This makes it difficult to administer correct dose of warfarin consequently rendering combination of warfarin and DHI even more difficult.

Binding of a drug to serum albumin in plasma has a significant influence on the efficacy of drug therapy (Zeitlinger, Derendorf, & Mouton, 2011), and this process is competitive. Warfarin is so highly bound to plasma albumin to the extent that any increase in unbound warfarin concentrations will cause a significant increase in its effects and potential toxic effects. Thus, there is the need to monitor the free warfarin concentration. Microdialysis have been introduced into the rapidly growing field of ligand-protein binding assays as a promising alternative for measuring of the "true unbound" concentration *in vivo* (Oravcova, Bo, & Lindner, 1996).

In this study, a method based on microdialysis combined with LC–MS/MS to detect the pharmacokinetic profile of the unbound warfarin was developed. Then fluorescence spectroscopy was used to further validate the results of the pharmacokinetic study. To the best of our knowledge, it is the first essay to explore the change of unbound warfarin *in vivo* by microdialysis combined with LC–MS/MS method. And this study will provide some suggestions for rational clinical use of warfarin and DHI.

2. Materials and Methods

2.1. Reagents, chemicals, and solution

Acetonitrile of chromatographic grade was purchased from Merck (Darmstadt, German). $CaCl_2$, KCl, and NaCl were obtained

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from Kermel Chemical Reagent Co., Ltd. (Tianjin, China), and used in the preparation of perfusate for blood microdialysis-Ringer's solution (14.7 mmol/L NaCl, 0.2 mmol/L $CaCl_2$, and 0.4 mmol/L KCl). Salvianolic acid B (SaB) and warfarin were supplied from the National Institute for Food and Drug Control (Beijing, China). DHI and Warfarin Tablet were purchased respectively from Shandong Danhong Pharmaceutal Co., Ltd. (Shandong, China) and Qilu Pharmaceutical Co., Ltd. bovine serum albumin (BSA) was obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China) and was used without further purification. Water was purified with a Millli-Q system (Millipore, Bedford, MA, USA).

2.2. Apparatus and instruments

A validated bioanalytical method was established based on LC–MS/MS, which was able to measure the concentration of warfarin in the microdialysates. The LC–MS/MS system was made up of an Agilent 6430 Quad LC–MS/MS (QQQ) equipped with electro-spray ionization (ESI) source (Agilent, Santa Clara, CA, USA), installed with Agilent Mass Hunter acquisition software version B.03.01 and analysis workstation software version B.03.02/Build 3.2.170.25, and an Agilent 1200 model (Agilent, Santa Clara, CA, USA) connected with quaternary pump, auto-sampler, column oven and degasser. The microdialysis system (CMA, Stockholm, Sweden) mainly consisted of a CMA/402syringe pump-Dual syringe perfusions. All of the fluorometric experiments were recorded on a multifunctional microplate reader (Perkin-Elmer Corporation USA).

2.3. Microdialysis combined with LC–MS/MS

2.3.1. Chromatographic and mass spectrometry conditions

Chromatographic separation was performed on Agilent Zorbax Eclipse Plus C_{18} (2.1 mm × 150 mm, 5 μm). The mobile phase consisted of water (A) and acetonitrile (B) at a flow rate of 0.3 mL/min with the separation temperature at 25 °C. Gradient elution was set as follows: 0–5 min, 10.0%–65.0% B; 5–7 min, 65.0%–65.0% B; 7–10 min, 65.0%–73.8% B; 10–12 min, 73.8%–90.0% B; 12–14 min, 90.0%–10.0% B. The sample injection volume was 1 μL for analysis.

The mass spectrometry analysis was operated in the negative-ion electro-spray ionization mode for warfarin. The optimized MS parameters were designed as follows: capillary voltage, 4000 V; nebulizer, 35 psi; drying gas (N_2) flow rate, 10 L/min with a temperature at 350 °C. The following transitions were obtained: *m/z* 306.9–*m/z* 161.1 for warfarin.

2.3.2. Animals and microdialysis experiments

Males special pathogen free Sprague-Dawley rats [$n = 12$, (180–220) g] were obtained from the Beijing HFK Bioscience Co., Ltd. [Approval No:SCXK (jing) 2014-0004]. Animals were housed to acclimatize environmentally for one week in groups with four per standard cage, on 12 h light/dark cycle; room temperature was maintained at 20–25 °C and relative humidity kept at 40%–70%. Rats were fasted for 12 h but drunk water freely before the experiment.

Before surgery, rats were anesthetized with ethyl carbamate (14 g/kg, ip) and remained anesthetized during the experimental period. Surgical sites were shaved and disinfected with 75% alcohol. The probe for blood sampling (CMA 20, membrane length, 10 mm, molecular weight cut-off of 20 kDa) was implanted in the jugular vein/right atrium and then perfused with Ringer's solution at a flow rate of 2.0 μL/min.

2.3.3. Recovery of microdialysis probe

The *in vitro* recoveries of probes were determined prior to the *in vivo* calibration by the gain and by loss method (MacKichan, 1989), respectively. A retrograde calibration method was utilized to estimate the recovery rates *in vivo* (MacKichan, 1984). Details of validation procedure are given in supporting information.

2.3.4. Preparation of standard and quality control (QC) samples

In order to prepare the calibration samples and quality control (QC) samples, a stock solution of 1 mg/mL of warfarin was dissolved in blank microdialysates. Standard stock solutions were stored at 4 °C, and then diluted with blank microdialysates to appropriate concentrations. Calibration standard solutions of warfarin at concentrations ranging from 1ng/mL to 50 ng/mL were prepared in blank microdialysates prior to each analytical run. The quality control (QC) samples used in the validation and the pharmacokinetic study were prepared similar to the samples for calibration at three levels for 2, 20, and 40 ng/mL.

2.3.5. Method validation

The LC–MS/MS detection method was validated to establish the selectivity, linearity and sensitivity, lower limit of quantification (LLOQ), and stability.

For the pharmacokinetic study, the selectivity of the LC–MS/MS method was assessed by blank blood microdialysates and spiked blood microdialysates with warfarin for the peak interference.

The calibration curves were established by testing warfarin dissolved in blank microdialysates ranging from 1ng/mL to 50 ng/mL with an external standard method by plotting the normalized concentration of the calibration standard against the chromatographic peak area using least-square linear regression with weighted ($1/X^2$). The signal-to-noise ratio (S/N) of 10 was set as the threshold for calculating the LLOQ which is defined as the lowest concentration on the calibration curve.

The intraday and interday variabilities were assessed with the spiked QC sample at three concentration levels (2, 20, and 40 ng/mL), and six samples were prepared for each concentration level and parallel injections were made six times on the same day and on three consecutive days. The validation was performed in blank blood microdialysate solutions. The relative standard deviation (RSD) served as the measure of precision, while the accuracy was expressed in terms of the relative error (RE).

Stability validation was used to evaluate the stability of analytes in rat microdialysates, at three concentration levels (2, 20, and 40 ng/mL) under a variety of storage and handling conditions, which included the short-term stability, long-term stability, and freeze–thaw stability. The short-term stability was assessed by analyzing QC samples stored at 4 °C for 24 h. The long-term stability was assessed by evaluating the QC samples at −70 °C for 7 d. For freeze–thaw stability, QC samples were subjected to three complete freeze–thaw cycles and analyzed on consecutive days.

2.3.6. Administration programs and pharmacokinetic data analysis

Rats were divided into two groups: the warfarin only group (1 mg/kg, orally) and combined warfarin (1 mg/kg) and DHI (1 mL/100 g) group which was injected via caudal vein. After a post-surgical stabilization period for 2.0 h subsequent to the successful implantation of probes, the rats were administered orally with warfarin dissolved in water and caudal vein injection of DHI. The microdialysis samples of blood were collected every 20 min for 12 h. The samples were kept at 4 °C and analyzed within 24 h.

The drug concentration data (after being converted to free form concentrations) were calculated by the computer program "Drug and Statistics 1.0" (DAS 1.0; Medical College of Wannan, China).

2.4. Fluorospectrometry studies

BSA stock solution (0.1 mmol/L) was prepared in a physiological aqueous solution with 100 mmol/L phosphate buffer, pH 7.2–7.4.

The preparation of SaB (0.1 mmol/L) was similar with BSA stock solution. The stock solution of warfarin (10 mmol/L) was prepared in methanol. All of the stock solutions were kept at 4 °C in the dark.

A simple experiment was carried out prior to the formal fluorescence experiments. The specific fluorescence experimental operation and precautions obtained from literature (Hao et al., 2015).

3. Results and discussion

3.1. Recovery of warfarin from microdialysis probes

Recoveries of blood probes for warfarin at three concentrations (6, 45, and 75 ng/mL) *in vitro* and *in vivo* were shown in Table 1. The RSDs between recoveries by gain and by loss of warfarin were 2.31%, 2.37%, and 1.68% at the three different concentration levels, respectively, which indicated that *in vitro* recoveries by gain and by loss were equivalent to the warfarin. Therefore, the recoveries determined by loss can be used for the calibrations of the probes (Zhang et al., 2011). In addition, no significant difference was observed at different concentration levels, and the RSD of 4.75% suggested that the *in vivo* recoveries of blood probes in rat may be irrelevant to the concentration of warfarin for the MD experiments, resulting in the average *in vivo* recovery of (83.6 ± 3.0)%.

Table 2
Summary of precision and accuracy from QC sample in blank microdialysate (n = 6).

Concentrations / (ng·mL^{-1})	Inter-day		Intra-day	
	RE / %	RSD / %	RE / %	RSD / %
2	96.12	3.23	103.33	1.753
20	105.02	0.92	106.23	0.86
40	96.88	3.12	102.69	2.03

dialysates did not interfere with the analytes at retention time at 10.7 min.

The standard calibration curve for warfarin in rat blank microdialysates was linear over the range of 1 ng/mL–50 ng/mL and the regression equation was $y = 19.907x + 7.4188$ ($r^2 = 0.999$). LLOQ for warfarin was approximately 1 ng/mL.

The precision and accuracy data were summarized in Table 2, the data indicated that the present LC–MS/MS method had good accuracies and reproducibilities for warfarin at three levels of concentrations. The precision was assessed by calculating the relative standard deviation (RSD) which was not more than 3.23% and the accuracy expressed as the relative error (RE) were within 96.12%–106.23%. These accuracy and precision values were well within the acceptance criteria of bioanalysis.

Table 1
Recoveries of blood microdialysis probes for warfarin (n = 3).

Concentrations / (ng·mL^{-1})	*In vitro*			*In vivo*		
	Recovery by gain / %	Recovery by loss / %	RSD between by gain and by loss / %	Recovery / %	RSD / %	Average / %
6	85.60 ± 2.2	84.20 ± 1.7	2.31	86.70 ± 1.9		
45	84.70 ± 2.8	83.10 ± 0.5	2.37	83.30 ± 3.2	4.75	83.60 ± 3.0
75	78.03 ± 4.3	78.90 ± 4.2	1.68	79.70 ± 4.8		

3.2. Validation of LC–MS/MS coupled with microdialysis method

The proposed LC–MS/MS method was used to test the selectivity by comparing the representative chromatograms of blank blood microdialysates, spiked blood microdialysates with warfarin criterion, and blood microdialysates sample collected 2 h after oral administration of warfarin (1 mg/kg). Fig. 1 showed the typical chromatograms indicating that the biological matrix from rat blood

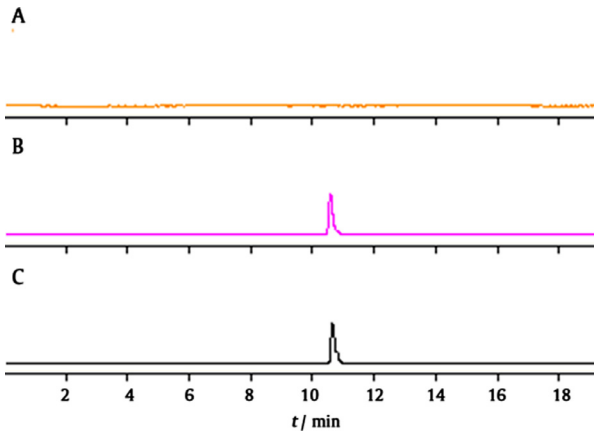

Fig. 1. LC–MS/MS chromatograms of blank blood microdialysates (A), blank blood microdialysates with warfarin criterion (50 ng/mL) (B), and blood microdialysates collected 2 h after administration of warfarin (C).

The stability results presented in Table 3 showed that warfarin was stable after 24 h at 4 °C in auto-sampler with RSD < 6.31%, −70 °C for 7 d with RSD all <12.7%, and after three freeze–thaw cycles with RSD all <4.23%. The stability results indicated that all the collected microdialysates were stable during microdialysates storage and occasional analytical process.

3.3. Pharmacokinetic study

Fig. 2 showed the real concentration-time profile of unbound warfarin in rat blood for the warfarin and warfarin with DHI groups. The concentration-time data were analyzed by non-compartmental method and the pharmacokinetic parameters were summarized in Table 4. It was showed intuitively from the pharmacokinetic curves of unbound warfarin in blood that the real concentration of unbound form maintained a stable state for 12 h after oral administration of warfarin alone, while a maximum concentration peak appeared in the combined administration group. In addition, warfarin at the administration dose of 1 mg/kg, the $AUC_{0\text{-tns}}$ of the groups of warfarin alone and co-administration with DHI (1 mL/kg) were (363.79 ± 183.09) and (432.90 ± 229.67) ng/mL/h. Compared with the administration of warfarin alone it was found that $AUC_{0\text{-tn}}$ in blood of warfarin were increased after co-administration with DHI, which demonstrated that DHI could increase the exposure amount of unbound warfarin in the body, so the concentration of unbound drug was increased. This increase was likely caused by the effect of DHI on the metabolism and plasma protein binding capacity of warfarin, nothing to do with absorption process of warfarin because of DHI administration via a tail vein. Actually, it has been reported that warfarin belongs to “restrictively cleared drug”, which the effect

Table 3
Stability study of warfarinin blank microdialysate ($n = 6$).

Conditions	2 / (ng·mL^{-1})		20 / (ng·mL^{-1})		40 / (ng·mL^{-1})	
	RE / %	RSD / %	RE / %	RSD / %	RE / %	RSD / %
Autosampler (24 h at 4°C)	105.29	1.71	106.98	6.31	105.43	2.37
Long-term (7 d at −70°C)	97.26	12.73	107.74	0.713	96.95	1.65
Three cycles (freeze–thaw)	90.92	4.233	110.89	1.56	111.1	1.09

Table 4
Pharmacokinetic parameters of unbound warfarin of rat blood about an oral warfarin alone group and combination with DHI administration group ($n = 6$).

Parameters	warfarin alone	warfarin with DHI
T_{max} / h	1.12 ± 0.57	1.23 ± 0.41
C_{max} / (ng·mL^{-1})	52.93 ± 31.70	66.51 ± 31.21
$AUC_{0\text{-}tn}$ / (ng·mL^{-1}·h^{-1})	363.79 ± 183.09	432.90 ± 229.67

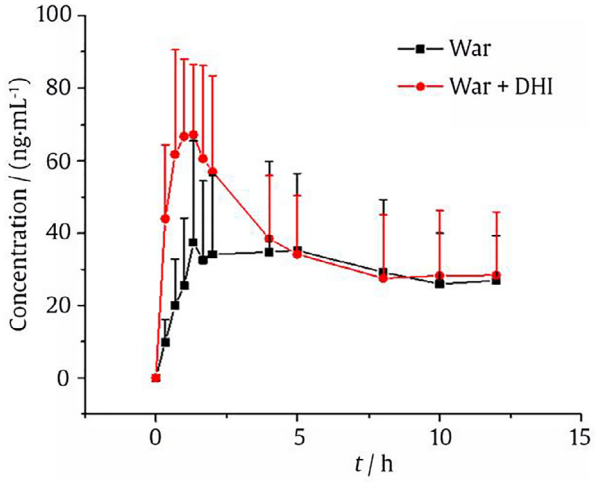

Fig. 2. Mean concentration based on *in vivo* recovery time about an oral warfarin alone group alone and combination with DHI administration group ($n = 6$).

of binding to plasma protein on the metabolic clearance of drug was greater than that of the enzyme (Mehranfar, Bordbar, & Parastar, 2013; Ranjan, Diffley, Stephen, Price, & Walton, 2002). In addition, it is well known that warfarin is completely absorbed after oral administration and highly bound to site I of albumin with a high affinity. Therefore, we concluded based on the results of our study that DHI, especially some of its components in DHI, possibly competed with warfarin for binding to plasma proteins, thereby increasing the concentration of unbound warfarin in the blood when combined with DHI. We further conducted an *in vitro* fluorescence studies to verify the results of our pharmacokinetic studies and assumptions.

3.4. Fluorospectrometry study

From our pre-experiments, when the excitation wavelength was fixed at 280 nm, BSA had a strong fluorescence emission band at 355 nm. As shown in Fig. 3, decreasing of the fluorescence intensity value of BSA followed with the successive addition of warfarin and unchanging peak shape at 355 nm showing that warfarin could quench the fluorescence of BSA possibly through the static process, but DHI and SaB did not change the quenching mechanism. Furthermore, from our previous study, SaB was the most highly bound component of DHI to serum and its binding site was the same as warfarin (Hao et al., 2015). Therefore, we deduced that SaB could displace warfarin from its binding site consequently, leading to the concentration of free warfarin rising greatly, which could result in changes in its PK properties and even possible toxic reactions effects.

Generally speaking, fluorescence quenching spectra and quenching type could be analyzed by the well known Stern–Volmer Eq. (1) (Wang, Zhang, Fu, Chen, & Zhang, 2003) and the modified Stern–Volmer Eq. (2) (de Lange, 2013) to confirm the mechanism, as shown in Fig. 4.

$$F_0/F = Kq\zeta_0(D) + 1 = K_{sv}(D) + 1 \quad (1)$$

$$F_0/\Delta F = F_0/(F_0 - F) = 1/fa + 1/(fa\mathrm{Ka}(D)) \quad (2)$$

As can be seen in Fig. 4A, the curves of F_0/F *versus* (D) had a good linearity when the concentration of warfarin ranged from 0 to 1.6 μmol/L, which suggested that quenching type of warfarin to BSA should be single static or dynamic quenching regardless of DHI and SaB. Furthermore, in Table 5, the binding parameter K_q of BSA, BSA-DHI and BSA-SAB by warfarin obtained from the slope of equation at 37 °C were (4.63×10^{12}) L/ mol/s, (3.13×10^{12}) L/mol/s, and (3.52×10^{12}) L/ mol/s, respectively, all of

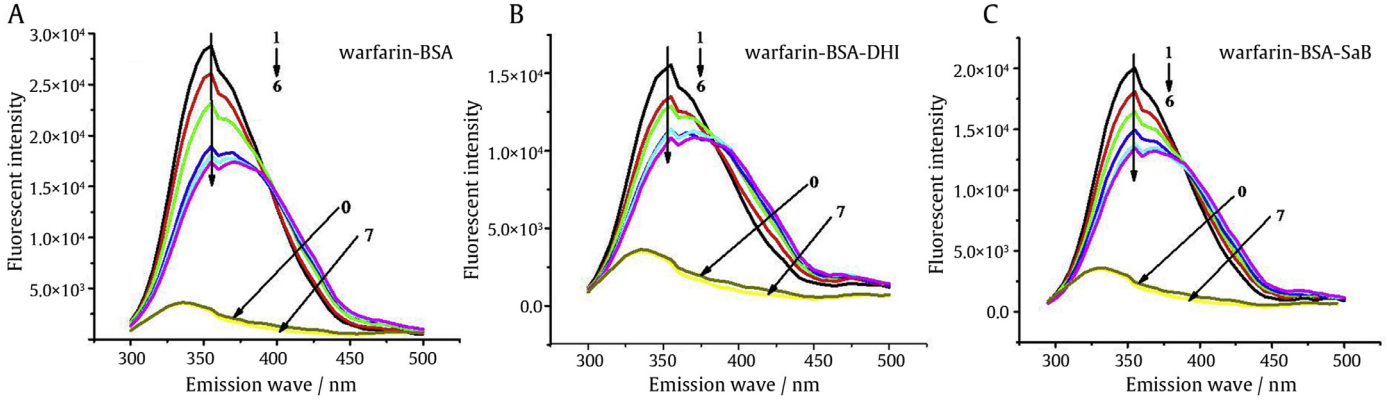

Fig. 3. Fluorescence quenching spectra of warfarin to BSA with and without DHI and SaB.
Note: $C_{BSA} = C_{SaB} = 5$ μmol/L; DHI 200 times dilution; 1→6 $C_{warfarin}$ (μmol/L) = 0, 0.4, 0.8, 1.2, 1.4, 1.6; 0→PBS, PH 7.2-7.4; 7→warfarin.

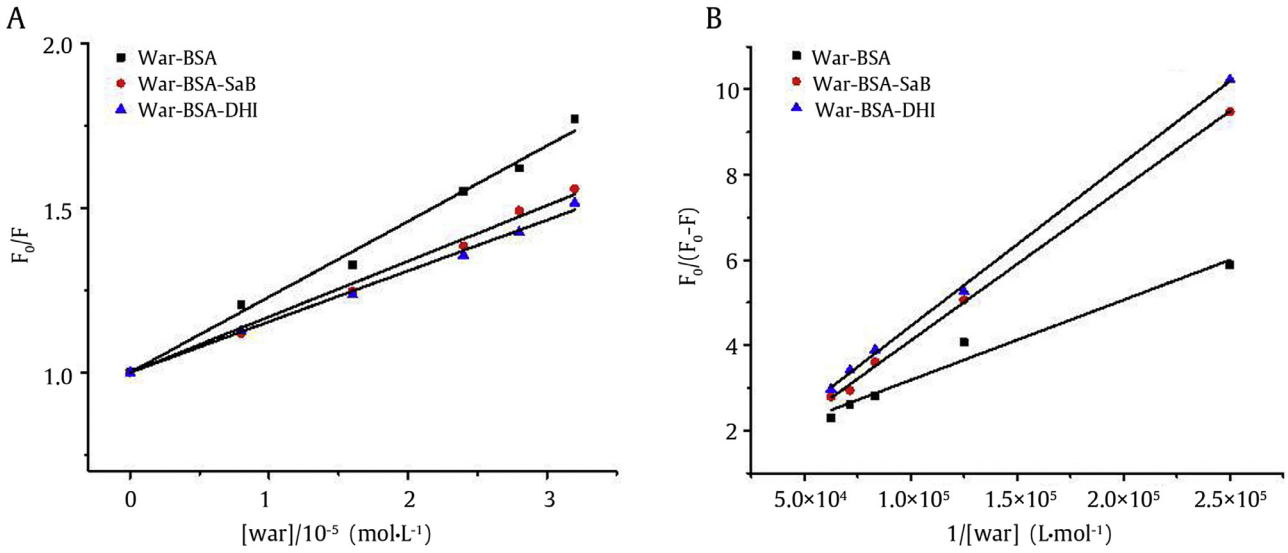

Fig. 4. Stern–Volmer (A) and modified Stern–Volmer (B) curves of fluorescence quenching of BSA, BSA-SaB, and BSA-200 diluted DHI caused by warfarin.

Table 5
Correlation parameters of warfarin-BSA with and without DHI and SaB.

Systems	K_q /($\times 10^{12}$ L·mol^{-1}·s^{-1})	K_{sv} /($\times 10^4$ L·mol^{-1})	K_a /($\times 10^4$ L·mol^{-1})
Warfarin-BSA	4.63	4.63	6.97
Warfarin-BSA-DHI	3.09	3.09	1.62
Warfarin-BSA-SAB	3.52	3.52	1.46

which were larger than that of a variety of the quenchers to biological macromolecules (2.0×10^{10} L/mol/s) (Zhang et al., 2015). Obviously, the quenching process of warfarin to BSA and BSA with the coexistence of DHI and SaB were static quenching in the linear range.

From Fig. 4B, it was shown that under certain warfarin concentrations, the curves of $F_0/(F_0–F)$ *versus* 1/(D) were linear, which showed that there were obviously characters of static quenching. As shown in Table 5, the binding parameter K_a obtained from the Eq. (2) for warfarin (6.97×10^4 L/mol) was larger than that in the presence of DHI (1.62×10^4 L/mol) and SaB (1.46×10^4 L/mol). The correlation parameters indicated that warfarin and BSA binding capacity decreased in the presence of DHI and SaB because of the much higher values of K_q, K_{SV}, and K_a. We concluded that the binding capacity of SaB to SAB was greater than that of warfarin, which resulted in the increase of the free concentration of warfarin, which subsequently may lead to efficacy enhancement or toxic and side effects increase due to its narrow therapeutic index.

4. Conclusions

In this study, the influence of DHI on the pharmacokinetic properties of unbound warfarin in rats was investigated by LC–MS/MS–MD. Furthermore, fluorescence spectra studies on the interaction between warfarin and BSA by the influences of DHI and SaB were conducted to provide a vitro explanation for the effect of DHI on the unbound warfarin concentration. This was the first essay to explore the change of unbound warfarin in the absence of DHI both *in vivo* and *in vitro*.

From the pharmacokinetic results, the unbound concentration of warfarin was higher with DHI than that warfarin alone. It was likely to increase the concentration of unbound warfarin when it was combined with DHI. In the fluorescence spectra studies, warfarin could quench the intrinsic fluorescence of BSA through a static quenching procedure regardless of the presence of DHI and SaB. The ability of warfarin to bind to BSA decreased due to the addition of DHI and SaB which led to an increase in the concentration of the unbound warfarin in plasma, which was consistent with results of the pharmacokinetic studies.

Based on this study, possible drug interactions could result in potentiating the anticoagulant effect of warfarin and/or even increasing the risk of bleeding when warfarin is combined with DHI or drugs containing SaB in clinical practice. Therefore, it is necessary to monitor the dosage of warfarin closely, maintaining optimum anticoagulation therapy for all patients treated with warfarin and DHI or any other drugs containing SaB. In addition, further studies are required to confirm our findings and to clarify the mechanism of interaction between these drugs, which may provide a significant guidance for clinical drug combination.

Conflict of interest

The authors declare no conflict of interest.

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