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The Effects of Danshen (*Salvia miltiorrhiza*) on Warfarin Pharmacodynamics and Pharmacokinetics of Warfarin Enantiomers in Rats

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Abstract

The effects of Danshen (*Salvia miltiorrhiza*), a popular traditional Chinese medicinal herb on the pharmacokinetics and pharmacodynamics of *R*- and *S*-warfarin stereoisomers were studied in rats.

After a single oral dose of racemic warfarin (2 mg kg^{-1}), treatment with oral Danshen extract (5 g kg^{-1}, twice daily) for 3 days significantly altered the overall pharmacokinetics of both *R*- and *S*-warfarin and increased the plasma concentrations of both enantiomers over a period of 24 h and the prothrombin time over 2 days. At steady-state levels of racemic warfarin (0·2 mg kg^{-1} day^{-1} for 5 days) the 3-day treatment of Danshen extract (5 g kg^{-1}, twice daily) not only prolonged the prothrombin time but also increased the steady-state plasma concentrations of *R*- and *S*-warfarin.

The results indicate that Danshen extracts can increase the absorption rate constant, area under plasma concentration-time curves, maximum concentrations and elimination half-lives, but decreases the clearances and apparent volume of distribution of both *R*- and *S*-warfarin. The pharmacokinetic and pharmacodynamic interactions of warfarin during co-treatment with Danshen extract observed in this study indicate an explanation for the clinically observed incidents of exaggerated warfarin adverse effects when traditional Chinese medicinal herbs or herbal products such as Danshen and Danggui (observed in a previous study) were co-administered.

In general the traditional Chinese medicinal (TCM) herbs available to the public can be classified into two categories: crude herbs or herbal drug preparations and health food products (Working Party 1991). Some of these crude herbs are included as part ingredients in composite prescriptions for treating illnesses by TCM practitioners. Nevertheless these products, whether as foods or drugs, are freely available to the public without restriction (Hong et al 1992). Anecdotal observations pointed out that patients on regular warfarin treatment who took TCM products as food supplements experienced exaggerated warfarin adverse effects (Lo et al 1992). Danshen (*Salvia miltiorrhiza*) is one of the most popular TCM herbs used as food supplements in the form of boiled broth by patients recovering from surgical operations. We have previously observed that co-administration of Danshen extract prolonged the prothrombin time and increased the bioavailability of warfarin at steady state in rats (Lo et al 1992).

In the present study we investigated further whether Danshen treatment would affect the pharmacokinetics of the *R*- and *S*-enantiomers of warfarin, using the same rat model after oral doses of racemic warfarin.

Materials and Methods

Preparation of Danshen aqueous extracts

The Danshen root, purchased locally in Hong Kong, was authenticated pharmacognostically by the Chinese Medicinal Material Research Centre at the Chinese University of Hong Kong according to the Chinese Pharmacopoeia (1992). The authentication tests included morphological examination and identification of content of tanshinone II using thin-layer chromatography according to the Chinese Pharmacopoeia. The preparation of Danshen aqueous extract was previously reported (Lo et al 1992). Briefly the root (1 kg) was initially cut into small pieces and soaked in double glass-distilled water (3 L) overnight at room temperature (25 ± 1°C). A further 500 mL water was added and the contents were boiled for 3 h in a glass flask. After cooling, the mixture was filtered through a double-layer bandage and the filtrate was collected and left overnight at room temperature. The supernatant was then pipetted out and the precipitate was filtered once again using filter paper. The filtrate was combined with the supernatant. The combined contents were concentrated by freeze-drying after dispensing into individual glass vials, which were stored at −20°C before use. After thawing the residue was reconstituted in water to make up to approximately 2 g dried weight of Danshen root per mL before use.

Chemicals and reagents

Warfarin was obtained from Sigma Chemical Co. (St Louis, MO, USA). Acenocoumarol, the internal standard, was supplied as a gift from Ciba-Geigy, Basle, Switzerland. HPLC grade methanol was purchased from Mallinckrodt Speciality Chemical Co. (Paris, KT, USA). Diethylether (freshly distilled before use), acetone, chloroform and quinidine (AnalaR grade) were obtained from E. Merck (Darmstadt, Germany). AnalaR grade hydrochloric

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acid (2 mM) was washed with ether and water was double glass-distilled before use. The thromboplastin reagent, simplastin, was supplied by Organon Teknika Corp. (Durham, NG, USA).

Separation of R- *and* S-*enantiomers of acenocoumarol and warfarin*

R- and *S*-Acenocoumarol and *R*- and *S*-warfarin were obtained by a modification of a chemical separation method (West et al 1961). Racemic warfarin (15·4 g) and quinidine (16·2 g) were dissolved in a mixture of chloroform (100 mL) and acetone (150 mL) at 60°C with constant stirring.

The solution was left overnight at −20°C. The resulting precipitate after filtration was separated as the quinidine-*S*-warfarin salt while the filtrate (filtrate A) was further processed to obtain the quinidine-*R*-warfarin. The purified *S*-warfarin salt (by recrystallization in acetone at −20°C) was dissolved in 0·5 M sodium hydroxide (50 mL). The resulting alkaline solution was first partitioned with chloroform (25 mL) to remove quinidine and then acidified with excess 1 M hydrochloric acid to precipitate *S*-warfarin, which was purified by recrystallization in pre-warmed 80% aqueous acetone solution. The yield of *S*-warfarin was 2·6 g.

The filtrate A from the initial resolution procedure was concentrated to 50 mL by rotating evaporation at < 60°C. The concentrate diluted with acetone (50 mL) was left at −20°C overnight to ensure complete separation of quinidine-*S*-warfarin as precipitate, while the filtrate was concentrated to dryness at 60°C under nitrogen. The dried residue, containing the partially resolved *R*-warfarin, was dissolved in chloroform (50 mL) and extracted into 0·5 M sodium hydroxide (100 mL) which was subsequently acidified with excess 1 M hydrochloric acid. The precipitate was dissolved in a solution of boiling ethanol (50 mL) containing quinidine (6·7 g). Diethylether (150 mL) was added to the cooled solution and left at −20°C overnight. The quinidine-*R*-warfarin salt was collected by filtration and purified by recrystallization twice in ethanol (50 mL) and diethylether (50 mL). The purified *R*-warfarin salt was subjected to the same treatment as that for the *S*-warfarin salt to obtain *R*-warfarin. The yield was 1·6 g.

Apparatus

The high-performance liquid-chromatographic (HPLC) system for the assay of warfarin consisted of a Hewlett-Packard 1050 series pumping system and multiple wavelength detector (Waldbronn, Germany), a Model 7125 syringe loading sample injector (Rheodyne Inc., Cotati, CA, USA) and a chart recorder (Linseis, Germany). The analysis of eluates was carried out by passing through a Hiber pre-column (50 mm × 4·6 mm i.d., packed with Perisorb RP-8 30-μm C8 reverse phase packing) obtained from E. Merck, and a Chiralcel OC analytical column (250 mm × 4·6 mm i.d.) which was purchased from Daicel Chemical Industries Ltd (Tokyo, Japan). The operation conditions for the HPLC system were: mobile phase, consisting of hexane/ethanol (4 : 1, v/v) filtered through a 0·22-μm filter and simultaneously degassed under vacuum; flow rate 1 mL min^{-1}; ambient temperature 25 ± 1°C; detection wavelength 217 nm. During the assay the mobile phase was recycled and under these conditions, there was complete separation of *R*- and *S*-warfarin and *R*- and *S*-acenocoumarol.

The apparatus for measurement of prothrombin time was a Coagulometer KC 4A with CR-A computer and stainless-steel balls (no. Z03200), plastic tubes (no. 834011) and glass tubes (15 mm i.d.) as accessories, which were obtained from Heinrich Amelung GmbH, Lehbrinksweg, Lemgo-1-Lieme, Germany. Other apparatus used included: 10-mL capacity centrifuge tubes with well-fitted, Teflon-lined screw caps (Sovirel, Levallois-Peter, France), and 15-mL capacity evaporation tubes with finely tapered bases. All glassware was cleaned by soaking overnight in a 2·5% solution of Extran (E-Merck) in water, then thoroughly rinsed with methanol, hot tap-water and distilled water. The tubes were subsequently silanized by rinsing with 3% hexamethyldisilazane in chloroform to minimize possible loss of drug due to adsorption to the glass walls (Chan & Dehghan 1978).

Animal studies

Male Sprague-Dawley rats, 250–300 g were allowed free access to standard diet and water, except when fasted overnight before administration of drugs and for four hours afterwards. Blood samples (0·6 mL) were collected at intervals from the tail vein. The freshly-drawn blood was mixed thoroughly with 3·8% sodium citrate (60 μL) in a ratio of 9 : 1. After centrifugation for 10 min at 3000 *g*, the plasma was collected for HPLC assay and prothrombin-time measurements.

Single dose study. The rats (n = 10) were treated with a fixed dose of Danshen extract (5 g kg^{-1}) intraperitoneally twice daily. Physiological saline was used in the control group (n = 10). After three days of treatment with Danshen extract, a single oral dose of warfarin (2 mg kg^{-1}, dose volume = 0·3 mL) was administered. Blood samples (0·6 mL) were collected at 0, 2, 4, 8, 12, 24, 36, 48 and 72 h post-dose. The plasma was separated by centrifugation (10 min at 3000 *g*) and stored at −20°C before HPLC analysis.

Steady state study. The rats (n = 10) were treated orally with warfarin (0·2 mg kg^{-1} day^{-1}, dose volume = 0·3 mL) daily to maintain a steady state concentration. After five days of treatment, the steady state was reached and maintained by monitoring the prothrombin-time values. The rats were then treated with Danshen extract (2 g kg^{-1}, dose volume = 0·3 mL) intraperitoneally twice daily for three days with daily warfarin treatment being continued. Blood samples (0·6 mL) were collected daily and the plasma was separated as soon as possible by centrifugation for prothrombin-time measurement and storage at −20°C before HPLC analysis.

Prothrombin-time (PT) measurement

The one-stage prothrombin-time test was performed. A sufficient volume of reconstituted simplastin was pre-warmed to 37°C in a water bath. Plasma (0·1 mL) was added to the plastic tube and incubated at 37°C for 1 min. The pre-warmed simplastin (0·2 mL) was then added and clotting time noted. The clotting time was recorded in seconds. The test was duplicated for each sample to ensure accuracy.

HPLC assay for warfarin

The warfarin concentrations in plasma samples were determined by a modification of an HPLC method to monitor simultaneously *R*- and *S*-warfarin (Chan & Woo 1988). The internal standard, racemic acenocoumarol (500 ng in 25 μL ethanol), was added to 0·2 mL plasma in a 10-mL glass centrifuge tube. Methanol (300 μL) was added to precipitate protein, followed by glass-distilled water (1·5 mL) and 2 M hydrochloric acid (0·5 mL). Diethyl ether (7 mL) was added for extraction. The mixture was then mixed with the aid of an automatic shaker for 15 min and subsequently centrifuged for 15 min at 3000 *g* to break the emulsion. The ether extract was collected and transferred to a 15-mL evaporation tube with tapered base. The extract was evaporated to dryness at 45°C in a water bath with a gentle stream of nitrogen. The residue was then dissolved in methanol (80 μL). A 35-μL aliquot was injected into the HPLC. The detection limits of the assay for *R*- and *S*-warfarin were 8 and 4·8 ng mL^{-1}, respectively, and the inter-batch assay at 750 ng mL^{-1} of drugs was reproducible and precise with a coefficient of variation of 8·3%.

Pharmacokinetic analysis

The plasma concentration–time data were analysed by a BASIC computer program, PKCALC, which performs standard statistical and pharmacokinetic analysis of multi-subject data sets (Shumaker 1986). The concentration–time data associated with the terminal phase of the plasma concentration–time curve were evaluated by the program for calculating the regression techniques. The absorption rate constant (K_a) was derived using residual analysis. The elimination rate constant (k_e) was obtained directly from regression analysis. The elimination half-life ($t_{\frac{1}{2}}$) and the total body clearance (CL) were calculated from the following equations:

$$t_{\frac{1}{2}} = 0{\cdot}693/k_e \quad (1)$$

$$CL = \text{Dose}/AUC \quad (2)$$

where AUC represents the total area under the plasma warfarin concentration–time curve between time zero and infinity. The AUC was calculated using the linear trapezoidal approximation from time 0 to t, the last sampling time; the additional area from t to ∞ was calculated as C_t/k_e where C_t is the measured warfarin concentration at the last sampling time t and k_e is the elimination rate constant previously defined.

The apparent volume of distribution Vd was calculated as:

$$Vd = CL/k_e \quad (3)$$

The maximum plasma warfarin concentration (C_{max}) and the time taken to achieve that concentration (T_{max}) were directly obtained from the experimental observations.

Statistical analysis

Experimental data and derived parameters were analysed by unpaired Student's *t*-test. A value was considered significantly different from the control value if it fell outside the 95% confidence limits of the latter ($P < 0{\cdot}05$).

Results

The effect of Danshen on the pharmacodynamics of warfarin

The baseline prothrombin times in control rats (n = 10) without any drug treatment were within the range 6·0–6·6 s (s.d. = 0·4 s). After plasma concentrations of warfarin reached steady state the prothrombin times were prolonged to 10 s and above. Fig. 1 illustrates the mean time-course of prothrombin times in rat treated with and without Danshen extract at steady-state plasma concentrations of warfarin. The values were significantly increased from control (12·9 ± 1·2 s) at day 6 (17·5 ± 2·6 s), day 7 (20·8 ± 2·9 s) and day 8 (23·9 ± 3·4 s).

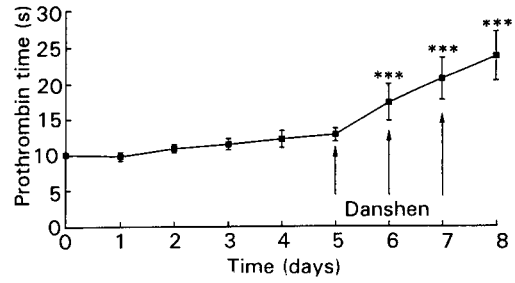

FIG. 1. Time course of mean (± s.d.) prothrombin time in rats treated with and without Danshen extract at steady state of racemic warfarin. ***$P < 0{\cdot}001$.

The effects of Danshen on the warfarin pharmacokinetics after a single oral dose

Fig. 2 shows the mean time course of plasma concentrations of *R*-warfarin and *S*-warfarin after oral administration of racemic warfarin (2 mg kg^{-1}) in 10 rats. No significant differences were found in the K_a, C_{max}, T_{max}, or Vd values between *R*- and *S*-warfarin, whilst the total AUC and $t_{\frac{1}{2}}$ of *S*-warfarin (115·2 ± 24·5 μg h mL^{-1}; 23·6 ± 4·6 h, respectively) were significantly greater than those of *R*-warfarin (81·5 ± 16·8 μg h mL^{-1}; 17·5 ± 2·2 h, respectively) and the clearance (CL) of *S*-warfarin (9·1 ± 2·4 mL h^{-1}) was significantly lower than that of *R*-warfarin (12·8 ± 3·1 mL h^{-1}) (Table 1).

Danshen treatment significantly increased the plasma concentrations of *R*-warfarin (Fig. 3a) and *S*-warfarin (Fig. 3b) in the rats treated with intraperitoneal Danshen extract twice daily for 3 days. Significant differences in most of the pharmacokinetic parameters of *R*- and *S*-warfarin with the exception of T_{max}, between the saline control group and the Danshen-treated group of rats were observed after oral administration of racemic warfarin (Table 1). Thus Danshen treatment increased absorption rates, total AUC values, elimination half-lives, and decreased apparent

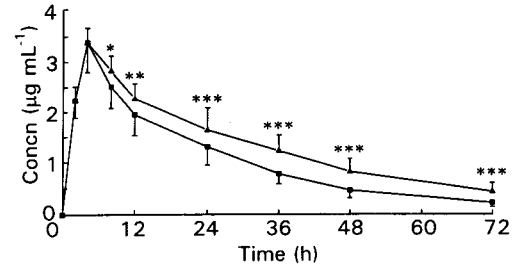

FIG. 2. Time course of mean (± s.d.) plasma concentrations of *R*-warfarin (■) and *S*-warfarin (▲) after oral administration of racemic warfarin (2 mg kg^{-1}) to 10 rats. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$.

Table 1. Mean (±s.d.) pharmacokinetic parameters of *R*-warfarin and *S*-warfarin after oral administration of racemic warfarin (2 mg kg^{-1}) to rats with or without 3-day treatment of intraperitoneal Danshen aqueous extract (5 g kg^{-1} dry weight of herb, twice daily).

Parameter	*R*-warfarin		*S*-warfarin	
	Saline	Danshen	Saline	Danshen
AUC (μg h mL^{-1})	81·5 ± 16·8*	223·1 ± 72·6***	115·2 ± 24·5*	236·4 ± 63·5***
C_{max} (μg mL^{-1})	3·4 ± 0·6	6·0 ± 1·2***	3·4 ± 0·3	5·5 ± 0·9***
T_{max} (h)	4·0 ± 0·0	4·0 ± 0·0	4·0 ± 0·0	4·0 ± 0·0
$t_{\frac{1}{2}}$ (h)	17·5 ± 2·2*	25·5 ± 4·8***	23·6 ± 4·6*	30·6 ± 3·7***
Vd (mL)	322·0 ± 71·1	179·8 ± 45·3***	301·9 ± 23·7	199·5 ± 44·3***
CL (mL h^{-1})	12·8 ± 3·1*	5·0 ± 1·8***	9·1 ± 2·4*	4·5 ± 1·2***
k_a (h^{-1})	2·07 ± 0·05	2·20 ± 0·05***	2·06 ± 0·02	2·18 ± 0·04***

***$P < 0·005$ comparing control group (n = 10) with Danshen-treated group (n = 10). *$P < 0·005$ comparing data within the control group.

volume of distribution and clearance of both *R*- and *S*-warfarin (Table 1).

The effects of Danshen on the steady state concentrations of R- *and* S-*warfarin*

Fig. 4 illustrates the time course of mean (± s.d.) plasma concentrations of *R*-warfarin and *S*-warfarin at steady state. Significant increases in the mean *R*-warfarin concentrations from day 6 to day 8 (day 5 control: 0·96 ± 0·07 μg mL^{-1}; day 6: 1·14 ± 0·12 μg mL^{-1}; day 7: 1·30 ± 0·21 μg mL^{-1} and day 8: 1·43 ± 0·20 μg mL^{-1}) were observed after Danshen treatment. Similarly the mean *S*-warfarin concentrations were increased from day 6 (1·14 ± 0·09 μg mL^{-1}) through day 7 (1·25 ± 0·09 μg mL^{-1}) to day 8 (1·39 ± 0·15 μg mL^{-1}) compared with the steady-state concentration at day 5 (1·00 ± 0·07 μg mL^{-1}).

Discussion

It was observed in our previous study (Lo et al 1992) that Danshen itself did not alter prothrombin times but increased the maximum prothrombin time after a warfarin dose (41·1 ± 3·4 to 49·9 ± 4·2 s). The present steady-state data demonstrate the pharmacodynamic potentiation of warfarin by Danshen. Danshen is a popular TCM herb used for many years in China for treatment of cardiovascular abnormalities such as angina pectoris and myocardial infarction (Liu 1983). The use of Danshen aqueous extract, as an intravenous injection, by orthodox physicians can be traced back to the 1960s (Lei & Chiou 1986) when capsules of Danshen were used for oral prophylaxis of angina in cardiac patients in the provinces of Eastern China. Some of these preparations are freely available to the public in China, Hong Kong and the United Kingdom. If warfarin is prescribed for anticoagulant therapy, the use of Danshen either as a prescribed medicine or self-medication is not recommended because of this pharmacodynamic interaction.

The mechanisms responsible for the interaction between Danshen and warfarin may be related to the effects of

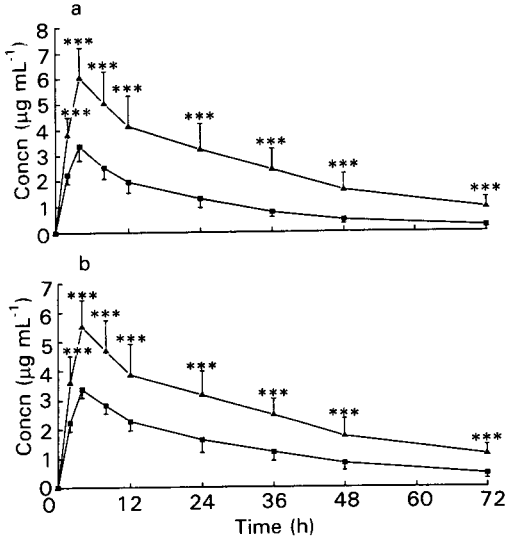

FIG. 3. Time course of mean (± s.d.) plasma concentrations of *R*-warfarin (a) and *S*-warfarin (b) after oral administration of racemic warfarin (2 mg kg^{-1}) in 10 rats (▲ Danshen treated rats; ■ saline control rats). ***$P < 0·001$.

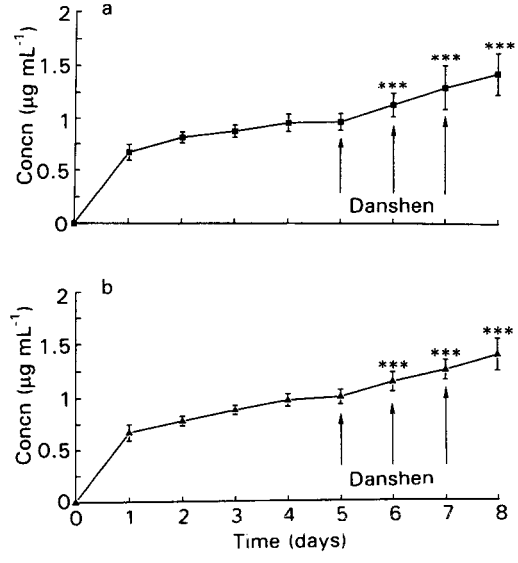

FIG. 4. Time course of mean (± s.d.) plasma concentrations of *R*-warfarin (a) and *S*-warfarin (b) at steady state of racemic warfarin in rats (n = 10). ***$P < 0·001$.

Danshen on warfarin kinetics. Our previous study observed that Danshen increased the bioavailability of racemic warfarin and affected its elimination (Lo et al 1992). The present study indicates that after the administration of a single oral dose of racemic warfarin to rats no significant differences were found in K_a, C_{max}, T_{max}, and Vd between *R*- and *S*-warfarin. Significantly higher AUC and longer elimination $t_{\frac{1}{2}}$ with lower clearance of *S*-warfarin were also observed (see control rats data, Table 1). These results confirm the earlier findings by Baars et al (1990) that *S*-warfarin had a longer elimination $t_{\frac{1}{2}}$ (38·5 ± 4·3 h) than that of *R*-warfarin (24·5 ± 2·9 h) in rats and the *S*-enantiomer was a more effective anticoagulant. In man, *S*-warfarin has a shorter half-life (18 to 35 h) and is more potent than *R*-warfarin (Breckenridge & Orme 1973). Danshen treatment was shown to affect the pharmacokinetics of a single oral dose of warfarin (Table 1). With the exception of T_{max}, all other pharmacokinetic parameters of warfarin were affected in the presence of Danshen. Danshen increased absorption rates and the relative bioavailabilities of both *R*- and *S*-warfarin (Fig. 3, Table 1). The increase in AUC was related to a decrease in clearance and apparent volume of distribution and the prolonged elimination $t_{\frac{1}{2}}$ for both enantiomers (Table 1). The consequence of these changes were reflected in the exaggeration of pharmacological effects which became apparent at steady-state warfarin concentrations (Fig. 4). Increases in plasma concentrations of *R*- and *S*-warfarin were similar.

Several chemical entities have been isolated from Danshen extracts. These include tanshinones I, IIA and IIB, isotanshinones I and II, cryptotanshinone, isocryptotanshinone, methyltanshinonate, hydroxytanshinone IIA, miltirone, 1-dihydrotanshinone I, salviol, protocatechnic aldehyde, protocatechnic acid, β-(3,4-dihydroxyphenyl) lactic acid and vitamin E (Tang & Eisenbrand 1992). These chemical compounds, either singly or in combination, are responsible for the pharmacological actions. Intravenous Danshen extract increased coronary blood flow and lowered coronary resistance but increased myocardial oxygen consumption in anaesthetized cats (Chen 1981). Danshen extract counteracted pituitrin-induced ECG abnormalities due to myocardial ischaemia in rabbits similar to the action of injected β-(3,4-dihydroxyphenyl) lactic acid (Wang et al 1980). Tanshinone IIA sodium sulphonate or Danshen root decoction inhibited adenosine diphosphate-induced platelet aggregation in myocardial infarct patients. Human platelet aggregation induced by adrenaline was inhibited by Danshen root decoction possibly due to fibrinolytic activity resulting in inhibition of platelet aggregation (Tang & Eisenbrand 1992). Danshen root extract reduced the spontaneous motor activity of mice and potentiated the hypnotic action of chlorpromazine, chloral hydrate and cyclobarbitone (Chen et al 1979). The anti-anxiety effects of Danshen are useful for treatment of angina. It is likely that the drug interactions between warfarin and Danshen extract observed in the present study are consequences of Danshen's effects on the circulation and cardiovascular system and its effects on the elimination of both *R*- and *S*-warfarin.

R- and *S*-Warfarin are metabolized by different metabolic pathways (Lewis et al 1974; Banfield et al 1983). *R*-Warfarin is predominantly reduced by soluble enzymes to warfarin alcohols, while *S*-warfarin is mainly metabolized by a cytochrome P450-dependent, mixed function oxidase. Further investigations are required to elucidate the mechanisms of interactions between Danshen, or isolated pure compounds from its extracts, and warfarin enantiomers. The results of this study suggest that an important interaction between Danshen and warfarin is likely to occur in patients who self-medicate with Danshen.

Acknowledgements

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