Plasma and Urinary Tanshinol from Salvia miltiorrhiza (Danshen) Can Be Used as Pharmacokinetic Markers for Cardiotonic Pills, a Cardiovascular Herbal Medicine

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ABSTRACT:
Cardiotonic pills are a type of cardiovascular herbal medicine. To identify suitable pharmacokinetic (PK) marker(s) for indicating systemic exposure to cardiotonic pills, we examined the in vivo PK properties of putatively active phenolic acids from the component herb Danshen (Radix Salviae miltiorrhizae). We also performed in vitro and in silico assessments of permeability and solubility. Several phenolic acids were investigated, including tanshinol (TSL); protocatechuic aldehyde (PCA); salvianolic acids A, B, and D; rosmarinic acid; and lithospermic acid. Plasma TSL exhibited the appropriate PK properties in dogs, including dose-dependent systemic exposure in area under concentration-time curve (AUC) and a 0.5-h elimination half-life. In rats, more than 60% of i.v. TSL was excreted intact into the urine. In humans, we found a significant correlation between the urinary recovery of TSL and its plasma AUC. The absorption rate and bioavailability of TSL were not significantly different whether cardiotonic pills were given p.o. or sublingually. The gender specificity in plasma AUC disappeared after body-weight normalization, but the renal excretion of TSL was significantly greater in women than in men. PCA was predicted to be highly permeable according to in vitro and in silico studies; however, extensive presystemic hepatic elimination and degradation in the erythrocytes led to extremely low plasma levels and poor dose proportionality. Integrated in vivo, in vitro, and in silico studies on the other phenolic acids showed poor gut permeability and nearly undetectable levels in plasma and urine. In conclusion, plasma and urinary TSL are promising PK markers for cardiotonic pills at the tested dose levels.

The use of herbal therapies is escalating worldwide. However, the basis for the therapeutic effects is often poorly interpreted, and the safety, dose, and potential herb-drug interactions require better estimation (Fugh-Berman, 2000; De Smet, 2002; Lazar, 2004). Unlike most synthetic drugs, herbal medicinal products usually contain numerous chemical constituents, especially traditional Chinese medicines that often use a combination of multiple herbs.

Determining which constituents of an herbal product have favorable drug-like properties will extend our knowledge of the basis of pharmacological efficacy and safety. An herbal constituent can be defined as drug-like when it possesses the desired potency, a wide safety margin, and appropriate pharmacokinetic (PK) properties and exists in adequate abundance in the herbal product. A deficit in these properties limits the usefulness of the herbal constituent for the herbal product. For a drug, the pharmacologic effect is attained when the drug or its active metabolite reaches and sustains an adequate concentration at an appropriate site of action; this hypothesis should also be applied to the herbal product. Both the dose levels and fates of active constituents in the body govern their target-site concentrations after administration of an herbal product. The relevant PK properties include ability of an herbal chemical to be absorbed from the site of administration and to pass through multiple biological barriers to reach the action target, sufficient metabolic stability to achieve therapeutically meaningful systemic and target-site concentrations, and appropriate metabolic liability to be eliminated effectively by the excretory processes.

ABBREVIATIONS: PK, pharmacokinetic; TSL, tanshinol; PCA, protocatechuic aldehyde; SAA, salvianolic acid A; SAB, salvianolic acid B; SAD, salvianolic acid D; RMA, rosmarinic acid; LSA, lithospermic acid; LC/MS/MS, liquid chromatography/tandem mass spectrometry; AUC, area under concentration-time curve; Cltotal, total plasma clearance; MRT, mean residence time; E1h, hepatic extraction; CLR, renal clearance; P-gp, P-glycoprotein; MRP2, multidrug resistance-associated protein 2; fu, unbound fraction in plasma; LogD, distribution coefficient D; TPSA, topological polar surface area; NROTB, number of rotatable bonds.
Measuring systemic exposure to an herbal medicinal product is important for understanding the link between the product consumption and the medicinal effects. To implement this, the PK properties of the active constituents and major chemical constituents should be evaluated. Active constituents that possess favorable PK properties, including a significant dose-dependent systemic exposure and an appropriate elimination half-life, qualify as PK markers for the herbal product. When the active constituent is unknown or a suitable assay is not feasible, the major chemical constituents or the main metabolites detected in plasma or urine may be evaluated as surrogate PK markers. PK markers may be used to show systemic exposure to the herbal product in animals and/or humans. For a multiherb product, identification of PK markers derived from each component herb is important for evaluating the combination rationality and for investigating possible synergistic interactions between the component herbs. Such studies are also relevant to designing rational dosage regimens, evaluating potential herb-drug or herb-herb interactions, and developing new formulations.

Cardiotonic pills (Fufang Danshen Diwan) are an herbal medicine recognized in the official Chinese Pharmacopoeia (The State Pharmacopoeia Commission of China, 2005); they contain three component herbs, i.e., *Radix Salviae miltiorrhizae* (Danshen or red root sage), *Radix Notoginseng* (Sanqi or sanchi ginseng), and *Borneolum* (Bingpian or borneol), and they are indicated for angina pectoris in coronary heart disease. The pills are prepared by combining concentrated aqueous extracts of Danshen and Sanqi and pulverized Bingpian, mixing well with heated polyethylene glycols, and dripping the mixture into cool liquid paraffin to yield pills. In cardiotonic pills, the main constituents of Danshen are phenolic acids, including tanshinol (TSL); protocatechuic aldehyde (PCA); salvianolic acids A, B, and D (SAA, SAB, and SAD); rosmarinic acid (RMA); and lithospermic acid (LSA); the constituents of Sanqi are triterpene saponins, including ginsenosides Rb1, Rd, Rg1, and notoginsenoside R1; and the constituent of Bingpian is borneol (Fan et al., 2006; Liu et al., 2006). As specified in the Chinese Pharmacopoeia, each pill (~25 mg) should contain ≥0.1 mg of TSL. Cardiotonic pills are generally well tolerated and have rare side effects, usually mild stomach discomfort and temporary dizziness. The annual sales volume of cardiotonic pills has exceeded ¥1 billion Chinese (~$140 million U.S.) since 2002. This herbal medicine is also available as a prescription or an over-the-counter drug in countries including Singapore, Republic of Korea, India, the United Arab Emirates, Russia, Cuba, and South Africa, and as a dietary supplement in the United States.

To help better understand the role of cardiotonic pills in coronary heart disease, we have implemented relevant PK studies. The current study focused on estimating the PK properties of the main phenolic acids from Danshen. The phenols received our attention because they have properties known to benefit heart function, including antioxidation, antiblood coagulation, coronary artery dilatation, and cell protection (Wang et al., 2007). Notably, we identified plasma and urinary TSL as PK markers for this herbal medicine.

### Materials and Methods

**Chemicals and Materials.** Purified TSL, PCA, RMA, and LSA were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Purified SAA, SAB, and SAD were obtained from the Phytochemistry Department of the Shanghai Institute of Materia Medica (Shanghai, China). The purity of these compounds was ≥99.0%, and their chemical structures are illustrated in Fig. 1.

Cardiotonic pills comprised *Radix S. miltiorrhizae*, *Radix Notoginseng*, and *Borneolum* (lot 20040710, expiration 07/2008; Tianjin Tasly Pharmaceutical Co. Ltd., Tianjin, China). Each pill (24.7 ± 1.1 mg) contained the following amounts of Danshen phenolic acids: 160 μg of TSL, 61.3 μg of PCA, 75.1 μg of SAA, 76.4 μg of SAB, 87.5 μg of SAD, 35.7 μg of RMA, and 14.8 μg of LSA, as measured by liquid chromatography/tandem mass spectrometry (LC/MS/MS). An injectable cardiotonic solution was prepared by diluting an aqueous extract of cardiotonic pills (Tianjin Tasly Pharmaceutical Co. Ltd., Tianjin, China). Each pill (24.7 ± 1.1 mg) contained the following amounts of Danshen phenolic acids: 160 μg of TSL, 61.3 μg of PCA, 75.1 μg of SAA, 76.4 μg of SAB, 87.5 μg of SAD, 35.7 μg of RMA, and 14.8 μg of LSA, as measured by liquid chromatography/tandem mass spectrometry (LC/MS/MS). An injectable cardiotonic solution was prepared by diluting an intermediate fluid extract of the cardiotonic pill (Tianjin Tasly Pharmaceutical Co. Ltd., Tianjin, China) in a 20-fold volume of physiological saline and then passing it through a 0.22-μm nylon filter. The prepared cardiotonic solution contained the following amounts of Danshen phenolic acids: 1.50 mg/ml TSL, 0.419 mg/ml PCA, 0.393 mg/ml SAA, 1.17 mg/ml SAB, 1.07 mg/ml SAD, 0.333 mg/ml RMA, and 0.237 mg/ml LSA. In addition, a separate portion of purified TSL or PCA...
was dissolved in physiological saline and then filtered (0.22-μm) to prepare an injectable solution of 2 mg/ml.

Experimental Animals. Animal studies were conducted according to protocols approved by the Review Committee of Animal Care and Use at the Shanghai Institute of Materia Medica. Male beagle dogs (7.8–9.0 kg) were purchased from the Teaching and Research Farm of Shanghai Jiao Tong University (Shanghai, China) and were individually housed in stainless steel cages (1.7 × 1.0 × 0.9 m). Male Sprague-Dawley rats (300–350 g) were obtained from Shanghai Laboratory Animals Co. (Shanghai, China) and were housed in rat cages (48 × 29 × 18 cm, 3 rats/cage). The animals were maintained on controlled temperature (20–24°C), relative humidity (40–70%), and a 12-h cycle of light and dark. The animals were given commercial diets, except for an overnight fasting period before dosing, and filtered tap water ad libitum. The rats were acclimated to the facilities for 1 week before use and the dogs for 2 weeks.

Study in Beagle Dogs. The dogs were randomly assigned to three treatment groups (3 dogs/group) to receive a single p.o. administration of cardiotonic pills at a dose of 2, 4, or 8 pills/kg. The doses were delivered in gelatin capsules, and the animal doses were derived according to the daily dose commonly given clinically in humans. After a 7-day washout period, the dogs of the intermediate-dose group received a single i.v. bolus of the cardiotonic solution at 0.50 ml/kg (in the left forelimb vein). Then, after another 7-day washout period, the TSL solution was given to the same group in a single i.v. bolus at 0.32 ml/kg. Serial blood samples (~250 μl collected in heparinized tubes) were taken from the right forelimb vein at 0, 5, 10, 20, 30 min and 1, 2, 4, and 6 h. The collected blood samples were placed on ice. After centrifugation at 6000g for 2 min, plasma fractions were decanted and then frozen at −70°C until analyzed. For dosing and blood sampling, the dogs were temporarily restrained in slings.

Studies in Rats. Three rat studies were performed as a supplement to the dog and human studies. In the first study, four rats were housed in Nalgene (Respirometics, Murraysville, PA) metabolic cages (1 rat/cage) with urine collection tubes frozen to −15°C for sample stability. After an i.v. bolus dose of the TSL solution (2 mg/kg), urine samples were collected over 24 h. All the urine samples were weighed before storage at −70°C.

In another study, six rats were randomly assigned to two groups. The animals were anesthetized with an i.p. injection of pentobarbital at 50 mg/kg and maintained under anesthesia throughout the blood sampling period. In each group, either the portal vein or the right femoral vein was cannulated for a 15-min intraportal or i.v. infusion of the TSL solution (0.6 mg/rat, 20 μl/min) using a PHD 2000 microdialysis infusion pump (Harvard Apparatus, Holliston, MA). Serial blood samples (~120 μl collected in heparinized tubes) were taken at 0, 5, 15, 20, 30, and 45 min and 1, 2, and 4 h from a carotid artery catheter after initiation of infusion. Plasma samples were prepared as described above. Similar study was also performed for PCA.

In the third study, rats under ether anesthesia were given a single i.v. dose of TSL solution (2 mg/kg) and then sacrificed by bleeding at the abdominal aorta at 0, 15, 30 min and 1 and 2 h after dosing. Three animals were used for each sampling time. Selected tissues (the heart, brain, liver, and kidney) were excised, rinsed in ice-cold saline, blotted, and weighed. The tissues were homogenized in 4 volumes of ice-cold saline, and homogenate samples were stored at −70°C until analysis.

Study in Human Subjects. Six male and six female healthy volunteers were enrolled in this study after providing written informed consent. The study was approved by the Ethics Committee of Clinical Investigation at Tianjin University of Traditional Chinese Medicine (Tianjin, China). Subjects were between 21 and 26 years old and within 15% of ideal body weight. The volunteers were determined healthy with regard to medical history, physical examination, electrocardiogram, and routine clinical laboratory tests. The female volunteers were negative for menstruation and pregnancy. Subjects who had a significant history of drug or food allergy or intolerance to cardiotonic pills were excluded. Any over-the-counter or prescription drugs were prohibited 2 weeks before and through the end of the study period. In addition, any alcohol-, caffeine-, tea-, or citrus-containing beverages or foods were prohibited 2 days before and through the end of each study period.

A single-dose, randomized, two-way crossover study was designed to compare the pharmacokinetics of TSL, particularly the absorption profiles, after p.o. or sublingual administration of cardiotonic pills. Subjects were randomized to use 30 cardiotonic pills by either swallowing whole pills with 100 ml of water (p.o. route) or placing the pills underneath the tongue without swallowing for 15 min (sublingual route). After a 10-day washout period, the subjects received cardiotonic pills by the alternate route. All the subjects were in a fasted state from midnight of the previous evening. Food consumption was not allowed until 4 h after dosing, and strenuous physical activity was not allowed during the study. Serial blood samples (~1 ml, collected in heparinized tubes) were taken from the antecubital vein catheter at 0, 5, 10, 15, 20, and 30 min and 1, 2, 4, and 6 h after initiating drug administration. The collected blood samples were placed on ice and then centrifuged to obtain plasma samples, as described above. Additionally, serial urine samples were collected in ascobic acid–treated bottles predose and at 0 to 1, 1 to 3, 3 to 6, 6 to 8, 9 to 12, and 12 to 24 h postdose. All the urine samples were weighed before storing.

PK Data Analysis. Plasma PK parameters were estimated by a noncompartmental method using the Kinetics 2000 software package (version 3.0; InnaPhase Corp., Philadelphia, PA). The AUCmax and the Tpeak were observed values with no interpolation. The area under concentration-time curve up to the last measured time point (AUC0–t) was calculated by the trapezoidal rule. The AUC0–inf was generated by extrapolating the AUC0–t to infinity using the k and the last measured concentration. The t1/2 was calculated using the relationship 0.693/k. The total plasma clearance (CLtot,p) for i.v. dosing or the CLi.v./F for p.o. dosing was estimated by dividing the administered dose by the AUC0–t. The Vss for i.v. dosing or Vss/F for p.o. dosing was estimated by multiplying the CLi.v. or CLi.v./F, respectively, by the mean residence time (MRT). The F of TSL was calculated according to the following equation:

\[F = \frac{[\text{AUC}_{i.v.} \times \text{Dose}_{p.o.}] - [\text{AUC}_{p.o.} \times \text{Dose}_{i.v.}]}{[\text{AUC}_{p.o.} \times \text{Dose}_{i.v.}] \times 100\%}\]

where the AUCp.o. was the plasma AUC0–inf value of TSL measured after a p.o. dose of cardiotonic pills was given to a dog, and the AUCi.v. was such a value measured after a bolus i.v. dose of the TSL solution was delivered to the same animal. The hepatic extraction (Ei.v.) was calculated by comparing the AUC values obtained after i.v. and intraportal infusions according to the following equation:

\[E_{i.v.} = \frac{1 - ([\text{AUC}_{i.v.}] - ([\text{AUC}_{p.o.}] \times \text{Dose}_{i.v.}])}{100\%}\]

where the AUCi.v. or the AUCp.o. was the mean of three rat measurements after intraportal or i.v. infusion, respectively.

Dose proportionality studies on the AUC0–inf and the Cmax were conducted by regression of log-transformed data (power model) (Smith et al., 2000). The correlation coefficient (r2), slope, and 90% confidence intervals for slope were calculated, and inferences were made based on the theoretical slope of 1 and the confidence limits of 0.84 to 1.16.

The urinary PK parameters included the cumulative amount excreted during urine collection period (CumA,) calculated by numeric integration of the amount excreted per collection interval. The fraction of administered compound excreted unchanged in urine (f)e was established by CumA,/Dose, and the renal clearance (CLr) was calculated as CumA,/AUC.

The exposure profiles of TSL in human subjects after administration of cardiotonic pills were compared for normal p.o. and sublingual routes in terms of the mean Cmax, Tpeak, AUC0–inf and CumA, values. All the results are expressed as the arithmetic mean ± S.D. A two-tailed Student’s t test or a Wilcoxon rank sum test was performed, where appropriate, to compare the PK parameter estimates of the two administration routes. The Pearson correlation test was used to study the association between plasma TSL AUC0–inf and relative urinary excretion (CumA, values). A p value <0.05 was considered statistically significant.

Stability in Canine Erythrocytes and Plasma. To assess the stability of PCA in erythrocytes and plasma, heparinized whole blood (5 ml, freshly collected from dogs and maintained at 37°C) was spiked with PCA to yield an initial blood concentration of 150 ng/ml. The spiked blood sample was incubated at 37°C and was sampled in 500-μl aliquots at 5, 10, 30, and 60 min. After sampling, the blood samples were immediately centrifuged at 6°C to separate the plasma and the blood cells for analysis. After removing leukocytes and residual plasma, the erythrocytes were lysed by sonication on ice. The concentrations of PCA were measured both in the lysed erythrocytes and in the plasma to determine the in vitro degradation t1/2 of the compound. As a control,
the metabolic stability of PCA was also measured after PCA was spiked at 150 ng/ml into plasma alone. In addition, the blood/plasma ratio was determined for all the tested Danshen phenolic acids from the ratio of the spiked blood concentration to the measured plasma concentration.

**Permeability Study in Caco-2 Monolayers.** Caco-2 cells (passage number 34–36, American Type Culture Collection, Manassas, VA) were cultured at 37°C in an atmosphere of 5% CO2 and 90% relative humidity in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% minimal essential medium nonessential amino acids. After harvesting at 90% confluence, the cells were seeded onto 0.4-
micron Millicell-PCF filter inserts (12-mm diameter, Millipore, Cork, Ireland) at a seeding density of 10^5 cells/cm^2. The culture media were changed after seeding and every other day thereafter. The integrity of the Caco-2 monolayers in 21 days postseeding was monitored by measuring the transepithelial electrical resistance, and only monolayers with values ≥400 Ω·cm^2 were used. The monolayers were washed three times with prewarmed Hanks’ balanced salt solution (37°C) before use. Bidirectional transport experiments were conducted in triplicate for each Danshen phenolic acid. The compounds were individually added to the donor compartment at a concentration of 50 μM in Hanks’ balanced salt solution. After 1 h of incubation, samples were collected from both the receiver compartment and the donor compartments, and the recovery of the tested compound was determined. The monolayer permeability and the expression of ATP-dependent drug transporters P-glycoprotein (P-gp) and multidrug resistance-associated protein 2 (MRP2) were checked as described previously with commonly used probe compounds and specific inhibitors (Hubatsch et al., 2007). The Papp values expressed in centimeters per second was calculated according to the following equation:

\[
P_{\text{app}} = \frac{\Delta Q/\Delta t}{A \times C_{\text{in}}}
\]

where the ΔQ/Δt is the linear appearance rate of the tested compounds on the receiver side, the A is the surface area of the cell monolayer, and the C_{\text{in}} is the initial concentration of the tested compound on the donor side.

**Plasma Protein Binding.** A rapid ultrafiltration method (13,362 g) was used to assess the unbound fraction in plasma (f_u) of TSL in rat and human plasma. TSL was tested at concentrations of 12.3, 36.9, 111, and 333 ng/ml. Non-specific binding of TSL to the ultrafiltration membrane was negligible.

**In Silico Assessment of Permeability and Solubility.** Chemoinformatic approaches were also used to assess the absorption properties of the tested Danshen phenolic acids. The aqueous solubility and the distribution coefficient D (LogD) at a given pH were calculated with ACD/ aqueous solubility version 8.02 and ACD/LogD version 8.02 software, respectively, via the ACD/I-Lab service (Advanced Chemistry Development Inc., Toronto, ON, Canada). The topological polar surface area (TPSA), number of hydrogen bond donors, number of hydrogen bond acceptors, and number of rotatable bonds (NROTIB) were determined using the Molinspiration Property Calculator (Molinspiration Chemoinformatics, Bratislava, Slovak Republic). Lipinski’s Rule of Five (Lipinski et al., 1997) was used as a guide to predict the compound permeability. Compound permeability was also assessed with regard to molecular-surface properties (TPSA and NROTIB). The aqueous solubility S was expressed in molar.

**LC/MS/MS Analysis.** A validated bioanalytical method was used based on LC/MS/MS, which was capable to measure simultaneously TSL, PCA, SAA, SAB, SAD, RMA, and LSA. The sample cleanup involved ethyl acetate–based extraction of hydrochloride acid–treated biological samples (100 μl, including those of plasma, plasma water, erythrocyte homogenate, urine, or cell culture media).

The LC/MS/MS system consisted of an ACQUITY UPLC separation module (Waters, Milford, MA) and a TSQ Quantum triple stage quadrupole mass spectrometer (Thermo Fisher, San Jose, CA) with an electrospray ionization interface. The chromatographic separation was achieved on an Agilent Eclipse Plus 5-μm C18 column (50 × 2.1 mm i.d.; Agilent, Chadds Ford, PA). The mobile phases (delivered at 0.4 ml/min) consisted of water (containing 25 mM formic acid) for A and acetonitrile/isopropanol (80:20, v/v, containing 25 mM formic acid) for B. A binary escalation gradient elusion was performed, which consisted of five isocratic segments, i.e., 0 to 2.5 min at 1%, 2.5 to 10 min at 10% B, 10 to 10.5 min at 50% B, 10.5 to 12 min at 80% B, and 12 to 14 min at 1% B. Following each isocratic segment, B immediately changed to the next condition. The precursor-to-product ion pairs used for selected reaction monitoring in the negative-ion electrospray ionization mode of TSL, PCA, SAA, SAB, SAD, RMA, and LSA were m/z 197→135, 137→108, 493→185, 717→519, 417→197, 359→161, and 537→295, respectively. The linear dynamic ranges for determination of the tested compounds were 8 to 2000 ng/ml, except for SAA and SAB (16–2000 ng/ml). Assay validation was conducted to show that the performance characteristics of the newly developed method were suitable and reliable for the intended applications.

**Results**

**Absorption and Pharmacokinetics of Danshen Phenolic Acids in Dogs.** PK data were calculated for the Danshen phenolic acids after an i.v. bolus of the cardiotonic solution was given to dogs (Table 1). Plasma levels of the Danshen phenolic acids decreased rapidly after dosing, indicated by the short t_{1/2} observed between 0.31 and 1.08 h. The CL_{tot,p} values of TSL, SAA, SAB, SAD, RMA, and LSA ranged from 0.48 to 1.22 l/h·kg, which fluctuated around the average canine hepatic and renal plasma flow rates (0.93 and 0.65 l/h·kg, respectively) (Davies and Morris, 1993). The distribution of these Danshen phenolic acids within the blood was basically limited to the plasma, indicated by the blood/plasma concentration ratios between 0.50 and 0.68. The mean V_{SS} values for TSL, SAA, SAB, SAD, RMA, and LSA ranged from 0.22 to 0.33 l/kg; values less than canine total body water (0.60 l/kg) suggested these compounds were predominantly restricted to the extracellular fluid. However, SAA had a V_{SS} of 0.93 l/kg, indicative of a relatively greater tissue distribution. PCA was exceptional because it was detected at much lower plasma levels (70.7–88.4 ng/ml at 5 min), had a much faster CL (3.83 l/h·kg), and had a larger V_{SS} (4.64 l/kg) than the other Danshen phenolic acids.

In contrast to the results from the i.v. administration, after p.o. administration of cardiotonic pills to dogs, only TSL was detected in plasma at dose levels of 2, 4, and 8 pills/kg. Although the PCA content (0.49 mg) in eight pills was comparable with that of TSL (1.28 mg), the plasma concentrations of PCA were very low, with a mean C_{max} of 18.7 ng/ml for PCA compared with 1034 ng/ml for TSL. The other compounds, including SAA, SAB, SAD, RMA, and LSA, in canine plasma were below the quantification limits (16 ng/ml for SAA or SAB or 8 ng/ml for the other compounds) of our bioanalytical methods.
Plasma TSL concentration versus time curves after p.o. administration of cardiotonic pills at different dose levels (2–8 pills/kg, each pill contained 0.16 mg of TSL) are shown in Fig. 2, and the PK parameters are summarized in Table 2. TSL reached its C_{max} within 0.5 h after p.o. administration, indicating that this phenolic acid was rapidly absorbed. The TSL t_{1/2} was 0.40 to 0.55 h, which was comparable with those observed after i.v. administrations of the injectable TSL solution (0.44 h) and the injectable cardiotonic solution (0.51 h). Log-transformed plots of AUC_{0→∞} and C_{max} versus the tested dose of cardiotonic pills were analyzed with power regression (Fig. 2). The results indicated that AUC_{0→∞} increased linearly over the cardiotonic pill dose range with a correlation coefficient (R^2) of 0.975, a slope of 1.008, and a 90% confidence interval of 0.892 to 1.123 (falling completely in the critical range of 0.839 to 1.169), whereas the C_{max} of TSL was directly related to dose but nonlinearly. The mean systemic bioavailability of TSL after p.o. administration of cardiotonic pills was around 40%.

When TSL was given i.v. to dogs in the cardiotonic solution (Table 1), it displayed PK behavior similar to that observed when it was given i.v. as pure TSL (Table 2); this suggested that the other herbal constituents and metabolites did not significantly affect TSL concentrations. Collectively, plasma TSL of Danshen origin was a valid PK marker for cardiotonic pills after p.o. administration to dogs at doses of 2 to 8 pills/kg.

Eliminations and Tissue Distribution of TSL in Rats. More than 60% of i.v. administered TSL was excreted in an intact form into rat urine, suggesting that renal excretion provided a major elimination route for the compound. The CL_{R} value exceeded the product of the rat glomerular filtration rate (0.31 l/h/kg) and f_{u} (75%) by ~6.0-fold, indicating that active tubular secretion of TSL occurred in rats. In addition, after intraportal or i.v. infusion of the TSL solution to rats, a comparison of AUCs was used to determine the extent of presystemic hepatic elimination. The results showed that the mean E_{Re} in rats was only about 5%, indicating that TSL underwent minimal presystemic hepatic elimination.

A rapid tissue distribution was observed, indicated by the highest concentrations of TSL measured at 5 min after i.v. administration. Tissue and plasma concentrations at 5 min postdose were ranked as follows: kidney (29,755 ng/g) > plasma (2630 ng/ml) > heart (325 ng/g) > liver (165 ng/g) > brain (38 ng/g). The concentrations of TSL in the studied tissues decreased rapidly at rates similar to those observed in plasma. At every sampling time, the maximum concentrations were measured in the kidney, a major eliminating tissue for TSL. Moreover, the t_{1/2} in the kidney was almost the same as that in plasma (both around 0.27 h), whereas the data for the other tissues were about 0.20 h. Thus, plasma TSL decrease reflected the compound decrease in the body; this is an advantageous property for human PK studies.

Comparative Pharmacokinetics of TSL after p.o. or Sublingual Administration of Cardiotonic Pills to Human Subjects. All 12 human subjects completed the entire protocol. After administration of cardiotonic pills via either p.o. (swallowing) or sublingual routes, TSL was the only Danshen phenolic acid detected in plasma and urine samples. Plasma and urinary PK parameters of TSL are summarized in Table 3, and the plasma concentration-time curves are illustrated in Fig. 3. Comparisons were made between the two administration routes and between women and men.

TSL was not measured in the first 0.2 h following the p.o. administration of cardiotonic pills; then TSL was absorbed rapidly, reaching peak levels at approximately 1.3 h after dosing. In women, the plasma C_{max} values of TSL ranged from 19.3 to 49.8 ng/ml and were significantly higher than those observed in men (range 14.6–27.1 ng/ml, p < 0.05). In addition, the mean AUC_{0→6h} in women was 72.8 h·mg/ml, also significantly greater than that observed in men (43.3 h·mg/ml, p < 0.05). However, when the dose was corrected for body weight, the gender difference was insignificant in these data. In
addition, no significant gender differences were observed in the dose-independent plasma PK parameters $t_{1/2}$, MRT, $C_{\text{SS}}$, $V_{\text{SS}}$, and $F$. As to urine PK data, the renal excretion of intact TSL during the 12-h period following dosing was significantly greater in women than in men with respect to $Cum.A$ and $f_e$ ($p < 0.05$). However, mean $CL_R$ did not exhibit gender difference, indicative of 0.50 ± 0.13 l/h · kg in women and 0.45 ± 0.03 l/h · kg in men, which accounted for 23 to 46% of the total systemic $CL_{\text{r,SS}}$. As was found in rats, TSL might also be subject to renal secretion in humans; $CL_R$ values for TSL were greater than the product of the glomerular filtration rate (0.11 l/h) and $f_e$ (85%) by about 5-fold.

As shown in Table 3, the two different dosing regimens of cardiotonic pills given at the same dosage resulted in comparably high and robust plasma concentrations of TSL. In both women and men no significant differences were observed between sublingual and p.o. administration routes in the PK data, including $AUC_{0\rightarrow t}$, $T_{\text{peak}}$, $C_{\text{max}}$, $t_{1/2}$, MRT, $C_{\text{SS}}/F$, $V_{\text{SS}}$, $Cum.A$, $f_e$, and $CL_R$. Because of the small number of human subjects, there was insufficient power for determining bioequivalence of the two dosing regimens.

Irrespective of the administration routes, the urinary $Cum.A_{0\rightarrow t}$ plotted as function of the plasma $AUC_{0\rightarrow t}$ (Fig. 3) revealed a significant correlation (Pearson correlation coefficient $= 0.86$, $p < 0.001$, $n = 24$). A similar correlation was found between an abbreviated $Cum.A_{0\rightarrow t}$ and $AUC_{0\rightarrow t}$, with a Pearson correlation coefficient $= 0.74$ and $p < 0.01$ ($n = 24$).

Intake and Elimination of PCA by Canine Erythrocytes and Its Preischemic Hepatic Elimination in Rats. Because of our findings that the $CL_{\text{r,p}}$ of PCA exceeded the canine hepatic plasma flow rate (1.09 l/h/kg) by 3.5-fold and that only about 1% of i.v. administered PCA was excreted intactly into rat urine, we examined the distribution and stability of the compound in canine erythrocytes and plasma. As shown in Fig. 4, 5 min after PCA was added to heparinized whole blood (150 ng/ml, 37°C), the compound was extensively distributed into and decayed in erythrocytes, resulting in reduced plasma concentrations (101 ng/ml in plasma and 68 ng/ml in erythrocytes) compared with the theoretical initial plasma concentration of 259 and 0 ng/ml erythrocytes. Thereafter, the concentrations of PCA in plasma and in erythrocytes decreased, indicated by the short $t_{1/2}$ values of 0.29 h in plasma and 0.17 h in erythrocytes. However, when PCA was spiked into plasma, it was stable without any obvious decrease in the measured concentration. These results, together with the high membrane permeability of PCA (shown below in the Caco-2 data), suggested that...
the low plasma levels and the rapid $\text{CL}_{\text{tot},p}$ were caused, at least in part, by extensive uptake and degradation by erythrocytes. In addition, we also found that PCA underwent substantial presystemic hepatic elimination in rats, showing the mean $E_H$ of about 60%.

Permeability and Solubility of the Danshen Phenolic Acids Assessed In Vitro and In Silico. Table 4 summarizes the $P_{\text{app}}$ values measured in Caco-2 cell monolayers for the Danshen phenolic acids. These data were compared with those of reference compounds, including antipyrine, atenolol, caffeine, doxorubicin, norfloxacin, rani-
tidine, reserpine, and testosterone. A sigmoid correlation was obtained between the percentage of dose absorbed after p.o. administration in humans and the corresponding log of the $P_{\text{app}}$ value measured in Caco-2 cell monolayers (data not shown). Based on this finding, a compound might be absorbed poorly, moderately, or well with a $P_{\text{app}}$ value of $\lesssim 10^{-6}$, $1 \times 10^{-6}$ to $10 \times 10^{-6}$, or $>10 \times 10^{-6}$ cm/s, respectively. Accordingly, the measured $P_{\text{app}}$ values indicated that the membrane permeability of TSL and that of PCA were moderate and good, respectively, but the other Danshen phenolic acids were poorly transported. The Caco-2 cells had high expression levels of the mul-
tidrug transporters P-gp and MRP2. Comparable bidirectional $P_{\text{app}}$ values, which were not affected by the initial concentration on donor side ($C_0$; data not shown), suggested a passive diffusion mechanism for TSL and PCA across the Caco-2 monolayer. The ratios of the $P_{\text{app}}$ values for basolateral$\rightarrow$apical and apical$\rightarrow$basolateral were $\sim 2.5$ for SAD; this was probably caused by the extremely low transport, and the low concentrations measured may have affected the accuracy. Metabolism of the compounds was not detected in the study.

The preceding Caco-2 data suggested that the gastrointestinal ab-
sorption of the Danshen phenolic acids was characterized by passive diffusion. Accordingly, we performed a chemoinformatic assessment of physicochemical properties to further understand the mechanisms

**Table 4**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>$P_{\text{app}}$ ($\times 10^{-6}$ cm/s)</th>
<th>Apical$\rightarrow$Basolateral</th>
<th>Basolateral$\rightarrow$Apical</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSL</td>
<td>$1.22 \pm 0.14$</td>
<td>$0.903 \pm 0.044$</td>
<td></td>
</tr>
<tr>
<td>PCA</td>
<td>$12.1 \pm 0.3$</td>
<td>$21.8 \pm 0.6$</td>
<td></td>
</tr>
<tr>
<td>SAA</td>
<td>$0.0595 \pm 0.0114$</td>
<td>$0.0554 \pm 0.0052$</td>
<td></td>
</tr>
<tr>
<td>SAB</td>
<td>$0.0200 \pm 0.0050$</td>
<td>$0.0377 \pm 0.0197$</td>
<td></td>
</tr>
<tr>
<td>SAD</td>
<td>$0.0662 \pm 0.0082$</td>
<td>$0.164 \pm 0.069$</td>
<td></td>
</tr>
<tr>
<td>RMA</td>
<td>$0.523 \pm 0.301$</td>
<td>$0.550 \pm 0.003$</td>
<td></td>
</tr>
<tr>
<td>LSA</td>
<td>$0.0816 \pm 0.0275$</td>
<td>$0.0413 \pm 0.0274$</td>
<td></td>
</tr>
</tbody>
</table>
governing intestinal absorption. Table 5 summarizes the calculated molecular and structural descriptors of the Danshen phenolic acids. The tested compounds could be defined as highly soluble, indicated by the calculated aqueous solubility values at pH 5.0 (6–3789 mM) or pH 7.0 (102–5051 mM). The solubility values were greater than the initial concentrations (C0) in the Caco-2 study (0.05 mM) and the compound-specific concentrations calculated for human p.o. dosing (0.008–0.242 mM) (Varma et al., 2004) or for sublingual dosing (0.412–12.1 mM). TSL had favorable properties for supporting acceptable permeability, including molecular mass (<500 Da), hydrogen-bonding capacity (number of hydrogen bond acceptors + number of hydrogen bond donors <12, TPSA <140 Å2), and molecular flexibility (NROTB <10) (Veber et al., 2002). The lipophilicity of TSL at pH 7.0 was poor compared with the value at pH 5.0, suggesting that the duodenum might be a favorable absorption site for the compound. PCA showed the best permeability potential among the tested Danshen phenolic acids with regard to the molecular mass, hydrogen-bonding capacity, lipophilicity, and molecular flexibility. SAA, SAB, SAD, RMA, and LSA possessed unfavorable traits, including total hydrogen bond counts ranging from 13 to 25, TPSA values ranging from 145 to 278 Å2, and LogD ranging from 3.8 to 9 (except for SAA); these traits underlie their poor permeability. In addition, SAD had the unfavorable traits of high molecular mass (718 Da) and molecular flexibility (NROTB = 14).

Discussion

In the current study we evaluated the PK properties of the phenolic acids from Danshen after p.o. administration of cardiotonic pills. For comparison, the injectable cardiotonic solution, as well as pure TSL, was administered i.v. We found that TSL was detected in canine plasma, the total exposure increased linearly over the dose range 2 to 8 pills/kg, and the peak exposure was directly related to the dose. TSL was absorbed rapidly from the gastrointestinal tract (Tmax = 0.5 h) and had an acceptable bioavailability (40%). A short plasma half-life (t1/2 = 0.5 h) indicated that TSL was rapidly eliminated from the body. The elimination rate of plasma TSL was comparable with that in different rat tissues, and its concentration in the heart was about 1/10 of the plasma concentration. Based on these PK properties, we conclude that plasma TSL from Danshen is a suitable PK marker for cardiotonic pills administered p.o. at clinical doses in both animals and humans.

There was good correlation in humans between the urinary recovery (Cum. A0–12h) of TSL and its plasma AUC0–6h, independent of the administration route chosen for cardiotonic pills. Our rat studies indicated that a substantial fraction (>60%) of i.v. administered TSL was eliminated by renal excretion. We also found that TSL concentrations were quite high in the rat kidney compared with plasma or other tissues. Accordingly, we conclude that urinary TSL can also be a reliable surrogate PK marker in humans for cardiotonic pills. Of clinical importance, we found that the correlation between the plasma AUC0–6h and Cum. A0–3h was comparable with that using Cum. A0–12h; the mean Cum. A0–3h accounted for approximately 73% of mean Cum. A0–12h. Compared with plasma TSL, urinary TSL possesses some merits as a PK marker in humans because it is noninvasive, less expensive, and straightforward to measure.

Several presystemic processes can affect the bioavailability of herbal chemicals. These include the solubility in the gastrointestinal fluid, membrane permeability, degradation in the gastrointestinal tract, transporter-mediated intestinal efflux, presystemic gut wall metabolism, and presystemic hepatic metabolism. In addition to TSL, we tested several other phenolic acids (PCA, SAA, SAB, SAD, RMA, and LSA) in the cardiotonic pills. However, these Danshen phenolic acids were only slightly or not detectable in canine or human plasma after p.o. administration of cardiotonic pills at the tested dose levels. The Caco-2 and chemoinformatic data suggested that PCA possesses better membrane permeability than TSL and good aqueous solubility. However, plasma and urine levels of PCA were much lower than those of TSL after p.o. administration of cardiotonic pills, despite the comparable amounts of PCA and TSL present in the administered herbal medicine. The systemic exposure to PCA showed poor dose proportionality. We found that PCA was subject to extensive presystemic hepatic elimination and degradation in the erythrocytes. Purified PCA can be biotransformed in vivo into protocatechuic acid (Xu et al., 2007a); aldehyde oxidase, xanthine oxidase, and aldehyde dehydrogenase may mediate the metabolism (Panoutsopoulos and Beedham 2005). In the current study, we found the concentration of protocatechuic acid in plasma and urine was in the low nanogram per milliliter range after administration of cardiotonic pills. Notably, we found that PCA was subject to extensive presystemic hepatic elimination and degradation in the erythrocytes. Collectively, plasma PCA was not considered an adequate PK marker for cardiotonic pills at the tested dose level because of its poor PK properties.

Unfavorable hydrogen bond capacities of SAA, SAB, SAD, and LSA were associated with poor membrane permeability, which could result in the low bioavailability from the gastrointestinal tract. This might explain our limited detection of these Danshen phenolic acids in plasma and urine of animals or humans after p.o. administration. Our results are consistent with the PK studies by Zhang et al. (2004), who revealed that the oral bioavailability of SAB was extremely low (0.02%), and Wang et al. (2008), who reported that the oral bioavailability of SAA was only 1.15%. Although we found that RMA showed potentially better membrane permeability than SAA, SAB, SAD, and LSA, this compound was also poorly detected in plasma and urine at the tested dose levels of cardiotonic pills. This result is also consistent with several studies that suggested intact RMA was poorly absorbed in the small intestine (Baba et al., 2004), and the majority of the compound was degraded in the colon by the gut microflora (Nakazawa and Ohsawa, 1998; Baba et al., 2005). In addition, we did not detect TSL in plasma or urine in rats p.o. receiving SAA, SAB, SAD, RMA, and LSA on separate occasions, which is in agreement with the observation with p.o. SAB in rats by Xu et al. (2007b).

As illustrated in the product label, cardiotonic pills can be taken either p.o. or sublingually. Sublingual administration of a drug may bypass some exposure to the presystemic drug-metabolizing enzymes and transporters that is encountered when the drug is p.o. administered by swallowing. In addition, the bioavailability of a drug taken p.o. may be more variable because of functional polymorphisms involved in presystemic metabolism and transportation. Accordingly, we performed a crossover, random-order, open-label human study to exam-
ine whether sublingual administration of cardiotoxic pills might change the bioavailability or absorption rate of the Danshen phenolic acids. Our results indicated that the sublingual administration did not improve the poor bioavailability of SAA, SAB, SAD, RMA, and LSA.

This finding was not surprising, considering the poor PK properties exhibited by these compounds in the Caco-2 cells. Although PCA was subject to extensive preemostic hepatic elimination, its bioavailability was not improved by the sublingual administration; this may attributed to, at least in part, the significant intake and elimination of the compound by the erythrocytes. Focusing on TSL, we found that the sublingual administration showed a bioavailability comparable with p.o. administration. The plasma C_{max}, T_{peak} and AUC of TSL, as well as the urinary C_{um,A,C}, were not significantly different between the sublingual and p.o. routes. This result was supported by our findings that in rats a high percentage of TSL escaped first-pass hepatic elimination, and in Caco-2 cells there was no indication of TSL transport by P-gp or MRP2. The finding that TSL was absorbed similarly after sublingual and p.o. administrations of cardiotoxic pills may be clinically relevant for patients with swallowing difficulties. However, further studies with the other component herbs in cardiotoxic pills, Sanqi and Bingpian, are required to confirm this potential benefit; in addition, the practical benefit of the sublingual route of administration should be studied in patients.

The therapeutic strategy for relief of angina is based on improvement of the balance between myocardial oxygen supply and demand. TSL has been shown to dilate coronary arteries (Dong and Jiang, 1982), inhibit platelet aggregation (Li et al., 1983), improve microcirculation (Cheng et al., 1987), and protect the myocardium from reperfusion injury of ischemic heart (Han et al., 2008). These cardiovascular actions may be produced primarily by TSL inhibition of extracellular Ca^{2+} entry into both cardiac cells (Cao et al., 2003) and vascular smooth muscle cells (Lam et al., 2007). Other effects of TSL may also include scavenging oxygen-free radicals (Zhao et al., 2008) and protecting the endothelial cells against homocysteinemia (Chan et al., 2004). In the current study, we identified plasma TSL and urinary TSL as PK markers of p.o. administered cardiotoxic pills based on PK properties evaluated in vivo (in experimental animals and in humans), in vitro, and in silico. However, our study did not address whether TSL is a principal factor in the putative antianginal actions of cardiotoxic pills.

In summary, although an herbal product usually contains numerous chemical constituents, its principal medicinal factors most likely possess favorable drug-like properties. In the current study, we examined the PK properties of putatively active phenolic acids from Danshen to identify suitable compound(s) that could indicate systemic exposure to p.o. cardiotoxic pills. We found that plasma TSL was a suitable PK marker and urinary TSL was a surrogate PK marker for p.o. cardiotoxic pills. The other Danshen phenolic acids were unsuitable because of unfavorable PK properties. In addition, sublingual administration of the pills neither changed the absorption rate and bioavailability of TSL nor improved the poor bioavailability of the other tested Danshen compounds compared with the p.o. administration. Three points are worth mentioning: 1) in addition to PK properties, the dose level, dosing form, and route of administration affect the suitability of an herbal constituent measured in plasma or urine as a PK marker for the herbal product; 2) PK markers from the other component herbs also need to be identified, and a combination of PK markers will provide a more complete characterization of the systemic exposure to cardiotoxic pills; and 3) although a substantial fraction (>60%) of i.v. TSL was eliminated in intact form by renal excretion, the biotransformation of this Danshen phenolic compound remains to be revealed.

References

Baba S, Osakaba N, Natsume M, and Terao J (2004) Orally administered rosmarinic acid is present as the conjugated and/or methylated forms in plasma, and is degraded and metabolized to conjugated from of caffeic acid, ferulic acid and m-coumaric acid. Life Sci 75:165–178.


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