Systemic Exposure to and Disposition of Catechols Derived from *Salvia miltiorrhiza* Roots (Danshen) after Intravenous Dosing DanHong Injection in Human Subjects, Rats, and Dogs

Meijuan Li, Fengqing Wang, Yühong Huang, Feifei Du, Chenchun Zhong, Olajide E. Olaleye, Weiwei Jia, Yanfen Li, Fang Xu, Jiajia Dong, Jian Li, Justin B. R. Lim, Buchang Zhao, Lifu Jia, Li Li, and Chuan Li

Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China (M.L., F.W., F.D., C.Z., O.E.O., W.J., F.X., J.D., J.J., J.B.R.L., L.L., C.L.); University of Chinese Academy of Sciences, Shanghai, China (M.L., L.L., C.L.); Second Affiliated Hospital, Tianjin University of Traditional Chinese Medicines, Tianjin, China (Y.H., Y.L.); Buchanan Pharmacy Group, Xi’an, Shaanxi Province, China (B.Z., L.J.); and Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China (C.L.)

Received October 1, 2014; accepted February 10, 2015

ABSTRACT

DanHong injection is a Danshen (*Salvia miltiorrhiza* roots)-based injectable solution for treatment of coronary artery disease and ischemic stroke. Danshen catechols are believed to be responsible for the injection’s therapeutic effects. This study aimed to characterize systemic exposure to and elimination of Danshen catechols in human subjects, rats, and dogs receiving intravenous DanHong injection. A total of 28 catechols were detected, with content levels of 0.002–7.066 mM in the injection, and the major compounds included tanshinol, protocatechualdehyde, salvianolic acid B, rosmarinic acid, salvianolic acids A and D, and lithospermic acid with their daily doses ≥10 μmol/subject. After dosing, tanshinol, salvianolic acid D, and lithospermic acid exhibited considerable exposure in human subjects and rats. However, only tanshinol had considerable exposure in dogs. The considerable exposure to tanshinol was due to its having the highest dose, whereas that to salvianolic acid D and lithospermic acid was due to their relatively long elimination half-lives in the human subjects and rats. Protocatechualdehyde and rosmarinic acid circulated in the bloodstream predominantly as metabolites; salvianolic acids A and B exhibited low plasma levels with their human plasma metabolites little or not detected. Tanshinol and salvianolic acid D were eliminated mainly via renal excretion. Elimination of other catechols involved hepatobiliary and/or renal excretion of their metabolites. Methylation was found to be the primary metabolism for most Danshen catechols and showed intercompound and interspecies differences in rate and degree in vitro. The information gained here is relevant to pharmacological and toxicological research on DanHong injection.

Introduction

Traditionally, herbal medicines are orally administered. However, there are 134 types of herbal injections that are approved by the China Food and Drug Administration (China FDA) for clinical use. The herbal injections are prepared from a single herb or from a mixture of several herbs and contain multiple bioactive compounds. The Chinese pharmaceutical industry is required to manufacture herbal injection products in compliance with good manufacturing practice. Unlike orally administered herbal medicines, herbal injections are injected directly into the bloodstream and potentially have immediate effects. Many approved herbal injections are extensively used in Chinese hospitals for treating cardiovascular or infectious diseases and for alleviating related symptoms; these herbal products appear to exhibit comparable incidence of side effects with synthetic drug injections. However, active compounds responsible for the therapeutic effects have remained poorly defined for most herbal injections. This hampers investigating the mechanisms of action, optimizing the herbal therapies, and assessing the safety and associated potential herb-drug interactions. Bioactive herbal compounds that exhibit considerable levels of body exposure after administration are most likely to account for the pharmacological effects of an herbal injection and form the basis of therapeutic efficacy. Accordingly, pharmacokinetic (PK) studies should be designed for herbal injections and implemented.
in human subjects and laboratory animals to reveal body exposure to the herbal compounds after dosing. The body exposure to herbal compounds depends on the doses of the compounds from the administered injection and their pharmacokinetics and disposition after dosing. In the first step of PK studies of an herbal injection, three questions should be answered: 1) what chemical compounds from the herbal injection are injected into the bloodstream and how much are they dosed? 2) Which herbal compounds and herb-related metabolites exhibit considerable systemic exposure after dosing and how long are their elimination half-lives? 3) How are the herbal compounds that are considerably dosed from the injection principally eliminated? The first step of PK studies provides information on which compounds derived from the herbal injection are worth further investigation with respect to pharmacokinetics, pharmacology, and toxicology. The follow-up PK studies should focus on these important herbal substances and should be carried out to define their detailed pharmacokinetics and associated interindividual variations, to characterize their principal routes of elimination, to quantify the contribution of enzymes and transporters to their pharmacokinetics and disposition, and to assess associated potential herb-drug and herb-herb interactions. Here, the aforementioned “first-step” PK studies were designed for DanHong injection and implemented in human subjects, rats, and dogs.

DanHong injection is prepared from a 3:1 mixture of *Salvia miltiorrhiza* roots (Danshen in Chinese) and *Carthamus tinctorius* flowers (Honghua). It is an injectable solution available as a sterile, nonpyrogenic parenteral dosage form for intravenous injection. This herbal injection is approved by the China FDA for the treatment of atherosclerotic coronary artery disease and acute ischemic stroke. Several reports on meta-analyses of randomized trials show that DanHong injection is effective in treating angina pectoris, congestive heart failure, and acute ischemic stroke (Peng et al., 2010, 2011; Yang and Zeng, 2012). DanHong injection contains multiple water-soluble polyphenols of Danshen-origin (Liu et al., 2013; Li et al., 2013; Xie et al., 2014). The Danshen polyphenols contain one or more ortho-dihydroxy benzene rings, known as catechols, and have one or more carboxylic acid functional groups, except for protocatechuic aldehyde. They are caffeic acid derivatives, occurring as monomers (such as tanshinol and caffeic acid), dimers (such as rosmarinic acid and salvianolic acid D), trimers (such as salvianolic acid A and lithospermic acid), and tetramers (such as salvianolic acid B) (Jiang et al., 2005; Li et al., 2009). Cell- and isolated-tissue-based studies have shown that Danshen catechols exhibit vasodilating, endothelial protective, cardioprotective, antiinflammatory, antioxidant, and anti-inflamatory properties; tanshinol and other Danshen catechols also decreased blood pressure in rats and attenuated venular thrombosis, cardiac hypertrophy, and methionine-induced hyperhomocysteinemia in rats (Jiang et al., 2005; Han et al., 2008; Cao et al., 2009; Ho and Hong, 2011; Wang et al., 2013a). Despite increasing knowledge of the chemistry and pharmacology of DanHong injection, the systemic exposure to and disposition of its bioactive compounds after dosing remain to be elucidated. This report presents the Danshen-derived chemical profile of DanHong injection and the systemic exposure to and elimination of Danshen catechols. It also presents comparative in vitro metabolic stability of Danshen catechols toward various metabolic reactions and their in vitro methylation profiles. The information gained here will be relevant to the optimization of DanHong injection-based therapy.

**Materials and Methods**

**DanHong Injection.** DanHong injection was manufactured by Heze Buchang Pharmaceutical Co., Ltd. (Heze, Shandong Province, China) with a China FDA ratification no. of GuoYaoZhenZi-Z20026866. Each milliliter of DanHong injection was prepared from 0.75 g of *Salvia miltiorrhiza* roots Danshen and 0.25 g of *Carthamus tinctorius* flowers (Honghua). To prepare the herbal injection, Danshen and Honghua were mixed and extracted with water. After filtration and concentration under reduced pressure, the concentrated extract was precipitated with ethanol and centrifuged. The resulting supernatant was treated with active carbon, evaporated under reduced pressure to remove the ethanol, adjusted to pH 6.5–7.5, ultrafiltered, and sterilized. The finished product of DanHong injection was standardized to contain not less than 0.35 mg/ml concentration of tanshinol and protocatechuic aldehyde combined. Samples of six DanHong injection lots (lot numbers: 13010137, 13072016, 13082026, 13092035, 13102030, and 13122024) were obtained for analysis, and all the products were manufactured in 2013. DanHong injection from lot number 13011037 was used in the current human and animal studies.

**Chemicals and Reagents.** Analytical reference standards of caffeic acid; lithospermic acid; protocatechuic acid; protocatechuic aldehyde; rosmarinic acid; salvianolic acids A, B, C, and D; tanshinol; chrysin, (−)-epicatechin; 7-hydroxyflavone; isovanillin; isovanillin acid; and vanillic acid were purchased from Tauto Biotech (Shanghai, China). The purity of the preceding compounds was ≥98%. Pentobarbital was obtained from Shanghai Westang Biotechnology (Shanghai, China).

**-adenosynmethylamine (SAM), uridine 5′-diphospho-glucuronic acid, 3′, 5′-phosphoadenosine-5′-phosphosulfate, tris-hydroxymethyl-aminomethane, nicotinamide adenine dinucleotide (NAD†), taurocholic acid, formic acid, and methanol were obtained from Sigma-Aldrich (St. Louis, MO). Rat liver cytosol and microsomes and dog liver cytosol and microsomes were prepared in-house by differential centrifugation. Human liver cytosol and microsomes were obtained from Corning Genest (Woburn, MA). (−)-Epicatechin was used as the positive control to assess the catechol-O-methyltransferase (COMT) activities of the human, rat, and dog liver cytosols (Zhu et al., 2000; Li et al., 2012), and the in vitro $1/2$ values were 0.46, 0.08, and 0.21 hour, respectively. 7-Hydroxyflavone was used as the positive control to assess the sulfotransferase (SULT) activities of the human, rat, and dog liver cytosols (Li et al., 2012; Hu et al., 2013), and the in vitro $1/2$ values were 0.07, 0.29, and 0.10 hour, respectively. Chrysin was used as the positive control to assess the UDP-glucosyltransferase (UGT) activities of the human, rat, and dog liver microsomes (Li et al., 2012; Hu et al., 2013), and the in vitro $1/2$ values were 0.08, 0.05, 0.05 hour, respectively. Isovanillin was used as the positive control to assess the aldehyde dehydrogenase (ALDH) activities of the human, rat, and dog liver cytosols (Panoutsopoulos et al., 2004), and the in vitro $1/2$ values were 0.04, 0.05, and 0.05 hour, respectively.

**Human Study.** The protocol for human study was approved by the ethics committee of clinical investigation at Second Affiliated Hospital of Tianjin University of Traditional Chinese Medicine (Tianjin, China). The study was registered in Chinese Clinical Trials Registry (www.chictr.org) with a registration number of ChiCTR-ONRC-13003571. Healthy volunteers gave written informed consent to participate in the study. The human subjects were considered to be in good health on the basis of medical history, physical examination, vital signs measurement, electrocardiogram, and clinical laboratory tests. They were required to be aged between 18 and 35 years, have a body mass index between 19 and 24 kg/m², be a nonsmoker, and not be allergic to products prepared from Danshen and/or Honghua. Synthetic drugs and herbal products were prohibited starting from 2 weeks before the study began until the end of the study period. In addition, alcoholic beverages were prohibited starting from 2 days before the study began until the end of the study period.

A single-period, open-label human study was performed at a national clinical research center of the Second Affiliated Hospital of Tianjin University of Traditional Chinese Medicine (Tianjin, China). Six male subjects were asked to fast overnight before receiving a 120-minute intravenous infusion dose of DanHong injection (40 ml/person, a label daily dose) the next morning. Before dosing, 40 ml of DanHong injection was diluted in 250 ml of 5% glucose injection (GuoYaoZhenZi-H12002001; China Oswalu Pharmaceutical Co., Ltd., Tianjin, China). Serial blood samples (∼1 ml, collected in heparinized tubes) were taken from an antecubital vein catheter at 0, 0.25, 0.5, 1, 2, 2.5, 3, 3.5, 5, 11, and 24 hours after the intravenous infusion was started. The blood samples were heparinized and centrifuged to obtain plasma fractions. 0.1 ml aliquots of the resulting plasma samples of the same time point were pooled and stored at −70°C until analysis. Urine samples were collected predose and at 0–2, 2–6, 6–10, and 10–24 hours postdose and weighed. One milliliter aliquots of the urine samples within the same time interval were pooled before storage at −70°C without use of
were centrifuged to obtain the plasma fractions and the resulting plasma samples (0.5 ml) were collected from three dogs into heparinized tubes. The blood samples were promptly centrifuged to obtain the plasma fractions and the plasma samples (0.5 ml) were collected from the same collection period from different rats that did not undergo any surgical treatment were housed individually in the unidirectional airflow room at 20–24°C and relative humidity between 30% and 70% with a 12-hour light/dark cycle. Rats were given filtered tap water ad libitum and allowed to acclimate to the facilities and environment for 3 days before use. Rats received in-house femoral–artery-cannulation for blood sampling or bile duct cannulation for bile sampling (Chen et al., 2013; Cheng et al., 2013). After surgery, rats were housed individually and allowed to regain their preoperative body weights before further use. During the bile collection period, a sodium taurocholate solution (pH 7.4) was infused into the duodenum of bile-duct-cannulated rats at 1.5 ml/h. After use, rats were euthanized with CO₂.

Male beagle dogs (7.8–9.0 kg) were obtained from the Teaching and Research Farm of Agricultural College, Shanghai Jiao Tong University (Shanghai, China) and were individually housed in stainless steel cages (1.7 × 1.0 × 0.9 m), which could be used to collect urine samples. The dogs were maintained at a controlled temperature of 20–24°C, relative humidity between 40% and 70%, and a 12-hour cycle of light and dark. They were given commercial diets, except for an overnight fasting period before dosing, and filtered tap water ad libitum. The dogs were maintained at the facilities for 2 weeks before use.

Rat Studies. Conscious and freely moving rats received a single intravenous bolus dose of DanHong injection at 4 ml/kg. The dose of the herbal injection for the rats was derived from the clinical daily dose (40 ml/person) according to dose normalization by body surface area (Reagan-Shaw et al., 2008). Serial blood samples (~150 μl; immediately before and 5, 15, and 30 minutes and 1, 2, 4, 6, 8, 11, and 24 hours after dosing) were collected from three femoral cannulated rats into heparinized tubes. The blood samples were centrifuged to obtain the plasma fractions and the resulting plasma samples (0.05 ml) of the same time point were pooled. Bile samples were collected from three bile duct cannulated rats before and 0–1, 1–2, 2–4, 4–6, and 6–8 hours after dosing. Three rats that did not undergo any surgical treatment were housed individually in metabolic cages. Urine samples were collected before and 0–4, 4–8, and 8–24 hours after dosing; samples (0.5 ml) of the same collection period from different rats were pooled and stored at −70°C until analysis.

Dog Study. Dogs received a single intravenous bolus dose of DanHong injection at 1.2 ml/kg. The dog dose was derived from the clinical daily dose of the herbal injection (40 ml/persoon) according to dose normalization by body surface area (Reagan-Shaw et al., 2008). Serial blood samples (~500 μl; immediately before and 5, 15, 30 minutes and 1, 2, 4, 6, 8, 11, and 24 hours after dosing) were collected from three dogs into heparinized tubes. The blood samples were centrifuged to obtain the plasma fractions and the resulting plasma samples (0.1 ml) of the same time point were pooled. Urine samples were also collected from the dogs before and 0–4, 4–8, and 8–24 hours after dosing. The urine samples (0.5 ml) from the same collection period were pooled. The samples from dogs were stored at −70°C until analysis.

In Vitro Metabolism Studies. Comparative metabolic stability of several Danshen catechol compounds toward COMT, SULT, UGT, and ALDH (ALDH for protocatechualdehyde only) was assessed with human, rat, and dog liver cytosols and microsomes. The test Danshen catechol compounds were tanshinol, protocatechualdehyde, protocatechuic acid, salvinorin A, licochalconec acid, licochalconolic acid, and licochalcone D. Before use, the COMT, SULT, and ALDH activities of the liver cytosols were assessed with the reference compounds (−)-epicatechin, 7-hydroxyflavone, and isovanillin, respectively; the UGT activities of liver microsomes was assessed with chrysirin. The liver cytosol was fortified with an appropriate cofactor (SAM for methylation, 3′-phosphoadenosine-5′-phosphosulfate for sulfation, and NAD⁺ for oxidation), and the liver microsomes were fortified with uridine 5′-diphospho-glucuronic acid for glucoronidation. The reaction was initiated by adding the test compound (10 μM) at 37°C and terminated at 0, 15 (for rat enzyme-mediated reactions only), 30 (for dog enzyme-mediated reactions only), and 60 minutes (for human enzyme-mediated reactions only) by adding a double volume of ice-cold methanol. The time of incubation for tanshinol was double that for the other Danshen catechol compounds. Negative control reactions were carried out by incubating mixtures that excluded the cytosol or the microsomes. The quenched incubation was centrifuged at 1309 g for 10 minutes, and 5 μl of the supernatant was analyzed by liquid chromatography/mass spectrometry. Metabolic stability was calculated as percentage of unchanged compound remaining after incubation. When determining the metabolic stability of protocatechualdehyde toward COMT and SULT present in human liver cytosol, isovanillin (100 μM) was used to inhibit the catalytic activity of the coexisting ALDH.

In vitro methylation was further examined individually for the preceding Danshen catechol compounds by incubating with human, rat, and dog liver cytosols fortified with SAM. The incubation broth contained 0.5 mg protein/ml liver cytosol, 50 mM Tris-HCl buffer (pH 7.4), 1 mM diithiothreitol, 2 mM MgCl₂, and 1 mM SAM and was preincubated for 3 minutes at 37°C before initiation of reaction by adding the test compound (10 μM). After incubation for 0, 5, 15, 30, 60, 90, and 120 minutes at 37°C, the reactions were terminated by adding a double volume of ice-cold methanol. After centrifugation, the supernatant (5 μl) was analyzed to profile unchanged substrate and methylated metabolites. In the determination of in vitro t½, the concentrations of the test Danshen catechol in the incubation broth over time were calculated as percentage of compound remaining, using the initial concentration (the incubation time, 0 minutes) as 100%. The slope of the linear regression from log percentage remaining versus incubation time relationship, i.e., −k, was used in the calculation of in vitro t½, according to the following equation:

\[ t_{1/2} = \frac{0.693}{k} \]

Analysis of Danshen Catechol Compounds and the Metabolites. A Waters Synapt G2 high definition time-of-flight mass spectrometer (TOF-MS; Manchester, UK) was interfaced via a Zspray/LockSpray ESI source with a Waters Acuity UPLC separation module (Milford, MA). The mass spectrometer was operated in resolution mode giving a resolving power of around 20,000. The ESI source worked in the negative ion mode under the following conditions: capillary voltage at −2.5 kV, source temperature of 120°C, desolvation gas at 800 l/hour and 400°C, sampling cone at 25 V, and extraction cone at 4.0 V. The mass spectrometer was externally calibrated over a range of m/z 50–1000 using a 5 mM sodium formate solution at 20 μl/min and mass shifts during acquisition were corrected using leucine enkephalin (m/z 554.2615) as a lockmass. MS² data acquisition (in centroid mode, m/z 50–1000) was achieved using a trap collision energy of 4 V, a trap collision energy ramp of 15–30 V simultaneously, and a scan time of 0.3 seconds. Chromatographic separation of Danshen catechol compounds in DanHong injection was achieved on a Waters Acuity UPLC HSS T3 1.8-μm column (100 × 2.1 mm i.d.; Dublin, Ireland; kept at 45°C) using a mobile phase that consisted of solvent A (methanol/water, 1:99, v/v, containing 25 mM formic acid) and solvent B (methanol/water, 99:1, v/v, containing 25 mM formic acid). The mobile phase was delivered at 0.3 ml/min. A gradient program was used as follows: 0–2 minutes, at 2% solvent B; 2–32 minutes, from 2 to 98% solvent B; 32–37 minutes, at 98% solvent B; and 37–42 minutes, at 2% solvent B. For quantitative analysis of Danshen compounds, a modified gradient elution program with a mobile phase flow rate of 0.3 ml/min was used as follows: 0–10 minutes, from 2 to 70% solvent B; 10–12 minutes, from 70 to 98% solvent B; and 12–15 minutes, at 2% solvent B. Sample preparation for human, rat, and dog samples was performed using methanol as precipitant with a volumetric precipitant-to-sample ratio of 3:1. After precipitation and centrifugation, the supernatants were reduced to dryness by centrifugal evaporation under reduced pressure. The residues were reconstituted in 50% methanol for LC/TOF-MS²-based analysis.

Profiling of Danshen compounds in the samples of DanHong injection was conducted. The detected compounds were identified by comparing with the reference standards, with respect to accurate molecular mass, fragmentation, and chromatographic retention time. When the reference standard was not available, the compound identification was achieved by comparing the compound’s accurate molecular mass, fragmentation, and elution order with the reported data of Danshen compounds. To support the metabolite profiling, an Accelrys metabolite database (version 2012.1; Accelrys, Inc., San Diego, CA) was used to get prior knowledge of likely metabolic pathways of Danshen compounds. Metabolite
profiling was conducted with the human, rat, and dog samples according to the molecular mass gains or losses predicted for the possible metabolites compared with those of the parent compounds and according to the generation of fragment ions from the precursor deprotonated molecules by collision-induced dissociation. The MS fragmentation data were also used to assign the metabolite structures. Methylated metabolites were characterized by the comparative fragmentation profiles of metabolite and parent compound. Glucuronides and sulfates were characterized by neutral losses of 176 and 80 Da, respectively. Quantification of Danshen catechols and the metabolites in the biologic samples was performed using the available reference standards for calibration or using the calibration curve of an analog (also called “brother compound”) that bore close structural similarity to the analyte (Li et al., 2012). By using the reference standards, matrix-matched calibration curves (13.7–10,000 nM) were constructed using weighted linear regression of the peak area against the corresponding nominal analyte concentration (nM), which showed good linearity ($r^2 > 0.99$). Assay validation was carried out according to the United States Food and Drug Administration guidance on bioanalytical validation (2013) to prove that the bioanalytical assays were reliable for the intended applications.

**PK Data Analysis.** The area under plasma concentration-time curve up to the last measured time point (AUC0-t) was calculated by the trapezoidal rule. The AUC0-t was generated by extrapolating the AUC0-t to infinity using the elimination rate constant ($k$) and the last measured concentration. The elimination half-life ($t_{1/2}$) was calculated using the relationship $0.693/k$. The cumulative amounts excreted into urine (Cum.A) and bile (Cum.Ab) were calculated by numeric integration of the amount excreted per collection interval. The fractions of dose excreted into urine ($f_{e-U}$) and bile ($f_{e-B}$) were established by Cum.A/dose and Cum.Ab/dose, respectively.

**Results**

### Danshen Catechols Present in DanHong Injection

Three literature references by Liu et al. (2007), Wang et al. (2007), and Li et al. (2009) give the most comprehensive information about chemical constituents present in Danshen. Among the Danshen constituents, hydrophilic catechols and lipophilic diterpene quinones are pharmacologically relevant. However, only the Danshen catechols, but not the diterpene quinones, were detected in DanHong injection. This is probably because of water extraction of the herb involved in manufacturing the injection. As shown in Table 1 and Fig. 1, a total of 28 Danshen catechol compounds were detected in six lots of DanHong injection with tanshinol (6) present in the highest content level (6.139–7.066 mM). Other major Danshen compounds present were protocatechuic aldehyde (1; 1.474–1.840 mM), salvianolic acid B (26; 1.014–1.245 mM), rosmarinic acid (10; 0.684–0.795 mM), salvianolic acids A (16; 0.563–0.886 mM) and D (13; 0.467–0.704 mM), litoriopsideic acid B (22; 0.235–0.322 mM), salvianolic acid B isomer (25; 0.159–0.227 mM), and salvianolic acid E (27; 0.136–0.154 mM). These were major Danshen catechols, which have LogP values of −0.28–2.69 (calculated using Pallas software, Pallas 3.7.1.1; CompuDrug International, Sedona, AZ). The remaining Danshen compounds, which were also detected in the herbal injection samples, were salvianolic acid B isomer (24); litoriopsideic acid isomers (18, 19, and 20); caffeic acid (3); protocatechuic acid (2); salviiflaside (17); salvianolic acids H/L (21), F (7), J (23), G (8), and C (15); salvianolic acid C (11) and salvianolic acid C isomer (12); isosalvianolic acid C (14); prolpitomperisic acid (9); isoferic acid (5); ferulic acid (4); and 4-methoxyl salvianolic acid B (28). These minor Danshen compounds exhibited content levels of 0.002–0.076 mM with LogP values of 0.17–3.02. Lot-to-lot variability of DanHong injection was also investigated with the major constituents ($>0.136$ mM). The herbal injection showed consistency in the content levels of 6, 1, 26, 10, and 27 from lot to lot. This was indicated by relative standard deviations of 4.3–9.4%. Such relative standard deviations values for 16, 13, 22, and 25 were greater, i.e., 12.0–16.3%.

DanHong injection (lot number, 13011037) was used in the current study; the content levels of tanshinol (6), protocatechuic aldehyde (1), salvianolic acid B (26), rosmarinic acid (10), salvianolic acid A (16), salvianolic acid D (13), and litoriopsideic acid (22) were 7.066, 1.833, 1.013, 0.769, 0.886, 0.704, and 0.247 mM, respectively. Clinical daily dose levels of the Danshen catechols from the herbal injection could be divided into three classes: >100, 10–100, and <10 μmol/sample (Fig. 1C). The dose level of 6 was 282.8 μmol/sample, which was 52.7% of the total dose of Danshen catechols detected in the injection (Fig. 1D); 6 was a Danshen catechol of class I. The dose levels of 1, 26, 10, 16, 13, and 22 were 10.0–73.2 μmol/sample, the sum of which accounted for 40.7% of the total dose of Danshen catechols detected in the injection; these compounds were of class II. The remaining Danshen catechols were of class III with dose levels of 0.1–7.2 μmol/sample, the sum of which accounted for only 6.6% of the total dose of Danshen catechols detected in the injection.

### Unchanged Danshen Catechols Detected in Plasma and Urine Samples of Human Subjects Receiving an Intravenous Infusion Dose of DanHong Injection

In the current study, pooled human plasma samples and pooled human urine samples were used for the detection of unchanged Danshen catechols and related metabolites. This was because the detection of Danshen compounds depended on factors, such as assay sensitivity and interindividual variations in the elimination mediated by drug metabolizing enzymes and/or drug transporters. The samples were pooled to minimize the possibility of failed detection of important herbal compounds and their metabolites due to the potential influence of interindividual variation in their elimination. A total of 11 Danshen catechols were detected in human plasma samples during and after intravenous infusion of DanHong injection; they were not detected in the control plasma samples before dosing. Tanshinol (6) was detected in considerable amount in the plasma samples after dosing started. Although salvianolic acid D (13) and litoriopsideic acid (22) were Danshen catechols of class II with content levels being only 10.0 and 3.5% of that of 6, respectively, their levels of systemic exposure were comparable with that of 6 (Fig. 2A). The other Danshen catechols detected in plasma, including salvianolic acids B (26) and A (16), rosmarinic acid (10), and protocatechuic acid (2), were at low AUC0-t levels (Fig. 2A). The observed high levels of systemic exposure to 13 and 22 were probably associated with their longer $t_{1/2}$ values (2.5 and 5.7 hours, respectively) compared with those of 6 (0.6 hour) and the other Danshen catechols (around 0.2 hour). Only trace amounts of protocatechuic aldehyde (1), litoriopsideic acid isomers (19 and 20), and salvianolic acid C (15) were detected in plasma. Plasma concentrations of 6, 13, and 22 over time during and after an intravenous infusion dose of DanHong injection in human subjects are shown in Supplemental Fig. 1.

A considerable amount of tanshinol (6) from intravenously dosed DanHong injection was recovered in human urine, with a $f_{e-U}$ of 63.3%. Although the $f_{e-U}$ value of salvianolic acid D (13; 62.7%) was almost the same as that of 6, its $Cum.A_{e-U,0–24h}$ was only 8.6% of that of 6 (Fig. 2B). The difference in urinary $Cum.A_{e-U,0–24h}$ between the two catechols was associated mainly with the difference in their content levels in DanHong injection; the content level of 13 was 10.0% of that of 6 (Fig. 1B). Only 3.8% of litoriopsideic acid (22) from intravenously dosed DanHong injection was excreted unchanged into the urine, and its $Cum.A_{e-U,0–24h}$ was quite low (Fig. 2B). Other plasma Danshen catechols (including salvianolic acid B (26) and rosmarinic acid (10)) were also detected in urine, with $f_{e-U}$ values of 0.2 and 18.2%, respectively; salvianolic acid A (16) was not detected in urine.

**Metabolites of Danshen Catechols Detected in Plasma and Urine Samples of Human Subjects Receiving the Intravenous Infusion Dose of DanHong Injection.** Metabolites detected in the human samples after dosing were those of the major Danshen catechols. As
shown in Fig. 3A, the major Danshen catechols could be divided into three categories according to their circulating patterns: 1) tanshinol (6), salvianolic acid D (13), and lithospermic acid (22): circulating mainly as parent compound with minor or major metabolites; 2) protocatechuic aldehyde (1) and rosmarinic acid (10): circulating in the bloodstream predominantly as metabolites with a minor parent compound; and 3) salvianolic acids B (26) and A (16): only minor parent compound circulating in the bloodstream with metabolites very little or not detected. The major metabolites of Danshen catechols were the methylated metabolites and their further sulfated and glucuronized products. Sulfates and glucuronides of Danshen catechols were also detected in plasma after dosing. Table 2 shows LC/TOF-MS²-based detection and measurement of Danshen catechols present in DanHong injection. (A) Chromatogram of DanHong injection; (B) average content levels of Danshen catechols in different lots of DanHong injection; (C) clinical daily doses of Danshen catechols from intravenous DanHong injection (lot number, 13011037) in mmol/subject; (D) percentages of individual catechols in total content level of Danshen catechols in DanHong injection. Danshen catechols (shown as compound ID) in (B) and (C) are ranked according to their average content levels in different lots of the herbal injection; their names and detection information are shown in Table 1.
metabolites of 10 included M10M-G-2, M10M-G, M10M-S-1, M10M-S-2, M10M-G, and M10M-G-2; these metabolites were eliminated considerably via renal excretion with a total Cum.A1-1/2 values ranging from 0.2 to 0.8 hour.

In rats, both tanshinol (6) and salvianolic acid D (13) were eliminated mainly via renal excretion (fB,0.5 4.7%, respectively), rather than hepatobiliary excretion (fB, 1.2%), after dosing DanHong injection (Fig. 2, D and E). Their major metabolites M6M, M6G, and M13M exhibited lower levels of systemic exposure than their respective parent compounds (Fig. 3C). These metabolites were also eliminated mainly via renal excretion (Fig. 3, D and E); the total Cum.A1-1/2 values of M6M and M6G and Cum.A1-1/2 values of M13M were 29.1 and 20.0% of the amounts of 6 and 13, respectively, dosed from DanHong injection. Lithospermic acid (22) was not eliminated mainly via renal excretion (fB, 2.7%) or hepatobiliary excretion (fB, 1.2%) (Fig. 2, D and E). Its metabolites M22M and M22M were substantially detected in rat plasma and bile after dosing (Fig. 3, C and E). The hepatobiliary excretion of these metabolites were significantly greater than their renal excretion (Fig. 3, D and E); their total Cum.A1-1/2 values and total Cum.A1-1/2 values were 76.9 and 4.7%, respectively, of the amount of 22 dosed from DanHong injection. Protocatechuic aldehyde (1) was eliminated mainly via oxidation into protocatechuic acid (2) followed by further methylation and sulfation; sulfation also considerably occurred in rats for 1 (Fig. 3, D and E). The urinary recovery of 2 and the follow-up metabolites (M2M-S, M2-S, and M2-S) in rats exceeded the amount of 2 dosed from DanHong injection by 109-fold, which was markedly greater than that observed in human subjects (28-fold). This was, at least in part, because the rat diet contained a considerable amount of p-hydroxybenzoic acid esters can be hydrolyzed and hydroxylated into protocatechuic acid (Wang et al., 2013b). Rosmarinic acid (10) was eliminated more extensively via renal excretion (fB, 25.2%) than hepatobiliary excretion (fB, 0.12%) (Fig. 2, D and E). Unlike in the human subjects, 10 exhibited a higher level of systemic exposure than its metabolites (M10M-G-2, M10M-G-1, and M10M-G-2) in rats (Fig. 3C). These metabolites were eliminated via both renal and hepatobiliary excretion (Fig. 3, D and E); their total Cum.A1-1/2 values and total Cum.A1-1/2 values were 29.0 and 49.4%, respectively, of the amount of 10 dosed from DanHong injection. Salvianolic acid B (26) was eliminated more extensively via renal excretion.
hepatobiliary excretion ($f_{EB}$, 8.1%) than renal excretion ($f_{EU}$, 1.0%) (Fig. 2, D and E). Its major metabolites included M26M, M26M·1, M26M·2, M26M·3·1, and M26M·3·2; neither sulfate nor glucuronide of 26 was detected. These metabolites were eliminated mainly via hepatobiliary excretion rather than renal excretion (Fig. 3, D and E); their total $\text{Cum.AUC}_{0-8h}$ and total $\text{Cum.AUC}_{0-24h}$ was 90.1 and 0.6%, respectively.

![Fig. 3. Systemic exposure to and excretion of metabolites of major Danshen catechols (tanshinol (6), protocatechuic aldehyde (1), rosmarinic acid (10), salvianolic acid A (16), salvianolic acid D (13), lithospermic acid (22), and salvianolic acid B (26)) in human subjects (A and B), rats (C–E), and dogs (F and G) receiving intravenous DanHong injection (lot number, 13011037). The parent Danshen catechols are ranked according to their average content levels (Fig. 1B), and their respective metabolites are shown on the right (in a left-to-right ranking order of methylated metabolites, sulfates and glucuronides of methylated metabolites, sulfates and glucuronides of parent compounds, and oxidized metabolites). Only metabolites (shown as metabolite ID) exhibiting plasma AUC0-8h values and/or excretory amounts, which were 10% of those of their respective parent compounds at least in one species, are shown in this figure. The metabolite ID is used to indicate the compound as a metabolite, its parent compound, the type of metabolism, and metabolite isomer. For instance, “M10” in M10M·S·1 denotes “metabolite of Danshen catechol 10 (rosmarinic acid)”; the subscript number and letter “M” denotes “dimethylation”; the subscript letter “S” denotes “sulfation”; and the last subscript number “1” denotes “the first eluted metabolite isomer.” The subscript letter “G” for other metabolite IDs denotes “glucuronidation.” Red/pink, black/gray, and green/light green bars represent the plasma, urinary, and biliary levels of the parent Danshen catechols/their metabolites, respectively. The symbol “/C2” (in red) denotes the Danshen compounds that was not detected. The names of metabolites and associated detection information are shown in Table 2 (for human metabolites) and Supplemental Tables 1 (for rat metabolites) and 2 (for dog metabolites). The human subjects received a 120-minute intravenous infusion dose of the herbal injection at 40 ml/subject. The rats and the dogs received an intravenous bolus dose of the herbal injection at 4 and 1.2 ml/kg, respectively. Because of the sample pooling, no standard deviation is shown.
**TABLE 2**

Detection of major metabolites of tanshinol (6), protocatechaldehyde (1), rosmarinic acid (10), salvianolic acid A (16), salvianolic acid D (13), and lithospermic acid (22) in human plasma and/or urine samples collected during and after an intravenous infusion dose of DanHong injection.

<table>
<thead>
<tr>
<th>ID*</th>
<th>Parent compound/metabolite</th>
<th>LC/TOF-MSE Data</th>
<th>Molecular Mass</th>
<th>Molecular Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>min</strong></td>
<td><strong>[M–H]</strong>^-1 (m/z)**</td>
<td><strong>Fragmentation profile</strong>^-1 (m/z)**</td>
</tr>
<tr>
<td><strong>Metabolites of tanshinol (6)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Tanshinol</td>
<td>6.61</td>
<td>197.0455</td>
<td>179.0346, 135.0454, 123.0453</td>
</tr>
<tr>
<td>M6M</td>
<td>Methyltanshinol</td>
<td>9.90</td>
<td>211.0611</td>
<td>193.0507, 150.0323, 134.0371</td>
</tr>
<tr>
<td><strong>Metabolites of protocatechaldehyde (1)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Protocatechaldehyde</td>
<td>8.36</td>
<td>137.0240</td>
<td>109.0311, 108.0233</td>
</tr>
<tr>
<td>2</td>
<td>Protocatechaldehyde</td>
<td>7.07</td>
<td>153.0194</td>
<td>109.0295</td>
</tr>
<tr>
<td>M2M</td>
<td>Methylprotocatechaldehyde</td>
<td>10.8</td>
<td>167.0348</td>
<td>123.0451</td>
</tr>
<tr>
<td>M2M-G1</td>
<td>Methylprotocatechaldehyde glucuronide</td>
<td>8.04</td>
<td>343.0660</td>
<td>167.0345, 123.0443</td>
</tr>
<tr>
<td>M2M-G2</td>
<td>Methylprotocatechaldehyde glucuronide</td>
<td>8.96</td>
<td>343.0667</td>
<td>167.0345, 123.0441</td>
</tr>
<tr>
<td><strong>Metabolites of protocatechaldehyde sulfate (10)</strong></td>
<td>10</td>
<td>5.2</td>
<td>232.9767</td>
<td>153.0193, 109.0295</td>
</tr>
<tr>
<td><strong>Metabolites of rosmarinic acid (16)</strong></td>
<td>16</td>
<td>18.41</td>
<td>493.1127</td>
<td>313.0716, 295.0611</td>
</tr>
<tr>
<td>M10M1</td>
<td>Methylrosmarinic acid</td>
<td>16.26</td>
<td>373.0918</td>
<td>197.0446, 175.0392, 179.0346</td>
</tr>
<tr>
<td>M10M2</td>
<td>Dimethylrosmarinic acid</td>
<td>18.16</td>
<td>373.0842</td>
<td>197.0446, 175.0392, 179.0346</td>
</tr>
<tr>
<td>M10M2S</td>
<td>Dimethylrosmarinic acid sulfate</td>
<td>19.47</td>
<td>387.1076</td>
<td>211.0600, 193.0492, 175.0388</td>
</tr>
<tr>
<td>M10M2S-G1</td>
<td>Dimethylrosmarinic acid glucuronide</td>
<td>21.33</td>
<td>467.0649</td>
<td>387.1076, 211.0605, 175.0392</td>
</tr>
<tr>
<td>M10M2S-G2</td>
<td>Dimethylrosmarinic acid glucuronide</td>
<td>21.44</td>
<td>467.0650</td>
<td>387.1085, 211.0601, 175.0388</td>
</tr>
<tr>
<td>M10M2S-G3</td>
<td>Dimethylrosmarinic acid glucuronide</td>
<td>17.17</td>
<td>563.1411</td>
<td>387.1085, 211.0601, 193.0484</td>
</tr>
<tr>
<td>M10M2S-G4</td>
<td>Dimethylrosmarinic acid glucuronide</td>
<td>17.39</td>
<td>563.1405</td>
<td>387.1093, 211.0607, 193.0501</td>
</tr>
<tr>
<td><strong>Metabolites of salvianolic acid A (16)</strong></td>
<td>13</td>
<td>16.81</td>
<td>473.1127</td>
<td>313.0716, 295.0611</td>
</tr>
<tr>
<td>M13M1</td>
<td>Methylsalvianolic acid A glucuronide</td>
<td>18.62</td>
<td>683.1606</td>
<td>507.1295, 355.0576, 295.0599</td>
</tr>
<tr>
<td>M13M2</td>
<td>Dimethylsalvianolic acid A glucuronide</td>
<td>19.03</td>
<td>683.1611</td>
<td>507.1285, 355.0573, 295.0598</td>
</tr>
<tr>
<td>M13M2-G1</td>
<td>Dimethylsalvianolic acid A glucuronide</td>
<td>19.46</td>
<td>683.1616</td>
<td>507.1295, 355.0573, 295.0598</td>
</tr>
<tr>
<td>M13M2-G2</td>
<td>Dimethylsalvianolic acid A glucuronide</td>
<td>19.76</td>
<td>697.1771</td>
<td>521.1448, 327.0865, 309.0760</td>
</tr>
<tr>
<td><strong>Metabolites of salvianolic acid D (13)</strong></td>
<td>13</td>
<td>18.41</td>
<td>493.1127</td>
<td>313.0716, 295.0611</td>
</tr>
<tr>
<td>M13M3</td>
<td>Methylsalvianolic acid D</td>
<td>17.79</td>
<td>431.0891</td>
<td>387.1073, 211.0608, 175.0392</td>
</tr>
<tr>
<td><strong>Metabolites of lithospermic acid (22)</strong></td>
<td>22</td>
<td>16.90</td>
<td>537.1037</td>
<td>493.1141, 313.0725, 295.0625</td>
</tr>
<tr>
<td>M22M</td>
<td>Methylthiolithospermic acid</td>
<td>18.00</td>
<td>551.1198</td>
<td>507.1316, 327.0869, 309.0774</td>
</tr>
<tr>
<td>M22M</td>
<td>Dimethylthiolithospermic acid</td>
<td>19.22</td>
<td>565.1342</td>
<td>521.1436, 327.0879, 309.0753</td>
</tr>
</tbody>
</table>

respectively, of the amount of 26 dosed from DanHong injection. Salvianolic acid A (16) was eliminated more extensively via hepatobiliary excretion (f_eB, 93.6%) than renal excretion (f_eU, 3.9%) (Fig. 2, D and E). Unlike in the human subjects, the metabolites M16M-G1, M16M-G-G1, M16M-G-G2, and M16M-G2 appeared to have higher levels of systemic exposure than unchanged 16 in rats (Fig. 3C). In addition to these plasma metabolites, M16M-G-G2, M16M-G-G3, and M16M-G2 were considerably detected in bile. Like the parent compound, the detected metabolites were eliminated mainly via hepatobiliary excretion (Fig. 3, D and E); their total Cum.A_x,B,G,3_6h was 71.8% of the amount of 16 dosed from DanHong injection. Supplemental Table 1 shows LC/TOF-MS^2-based detection of the individual metabolites of Danshen catechols in rat plasma, urine, and/or bile after dosing DanHong injection.

In dogs, only plasma tanshinol (6) was considerably detected after dosing DanHong injection (Fig. 2F). Unlike in the human subjects and in the rats, plasma salvianolic acid D (13) and lithospermic acid (22) were detected at low levels in dogs after dosing. Their t_{1/2} values were 0.2 and 0.5 hour, respectively, which were shorter than that of 6 (1.0 hour). Salvianolic acids B (26) and A (16) and rosmarinic acid (10) were also detected in plasma but at low levels of systemic exposure, with t_{1/2} of 0.2–0.3 hour (Fig. 2F). Only trace amounts of protocatechaldehyde (1) was detected in canine plasma. The f_eU values of 6, 13, and 22 were 34.5, 43.6, and 5.8%, respectively (Fig. 2G).
length of time. Among the human hepatic enzyme-mediated metabolic reactions, the rates of methylation of the test compounds were greater than those of associated sulfation and glucuronidation, except for protocatechuic aldehyde and salvianolic acid A (Fig. 5A). Sulfation and glucuronidation of salvianolic acid D, methylation, and salvianolic acid B appeared to be negligible, because the sulfates and glucuronides were not detected after incubation. Glucuronidation and oxidation of protocatechuic aldehyde were significantly faster than its methylation. Glucuronidation of salvianolic acid A was significantly faster than its methylation. Similar scenarios were observed for the rat and dog hepatic enzyme-mediated metabolic reactions, except for rat COMT-mediated methylation of protocatechuic aldehyde comparably as fast as the ALDH-mediated oxidation (Fig. 5, B and C). The in vitro catalytic activities of the rat and dog enzymes appeared to be higher than those of the human enzymes. Accordingly, the incubation times used for the rat and dog enzymes to mediate metabolism reactions were shorter than those of the human enzymes, i.e., 15 minutes (30 minutes for tanshinol only), 30 minutes (60 minutes for tanshinol only), 60 minutes (120 minutes for tanshinol only), respectively.

The rates of hepatic COMT-mediated methylation of the different test Danshen catechol compounds were considerably different (Fig. 6A). This is indicated by in vitro IC50 values, which were ranked as follows: salvianolic acid D (0.31 hour) ≈ lithospermic acid (0.41 hour) ≈ protocatechuic acid (0.44 hour) < salvianolic acid B (0.73 hour) < rosmarinic acid (1.23 hours) < salvianolic acid A (3.25 hours) ≈ protocatechuic aldehyde (3.71 hours) ≈ tanshinol (3.73 hours). The results of in vitro metabolite profiling of the incubations indicated that the methylation degree was catechol moiety number dependent and incubation time dependent. Tanshinol, protocatechuic aldehyde, and protocatechuic acid contain only one catechol moiety; only monomethylated metabolites (M6M, M1M, and M2M) were detected for these Danshen catechol compounds. Rosmarinic acid and lithospermic acid contain two unsubstituted catechol moieties. Both monomethylated (M10M1, M10M2, and M22M) and dimethylated metabolites (M19M10 and M22M2) of rosmarinic acid and lithospermic acid were found to be predominant, and the formation of dimethylated metabolites was significantly delayed compared with that of monomethylated metabolites. Salvianolic acid D contains one unsubstituted catechol moiety and one substituted catechol moiety; it was converted predominantly into a monomethylated metabolite (M13M). Its dimethylated metabolite was detected at very low levels. Salvianolic acid B contains three unsubstituted catechol moieties and salvianolic acid A contains two unsubstituted catechol moieties and one substituted catechol moiety. Human COMT-mediated methylation of these catechol compounds resulted in the formation of monomethylated (M16M1, M16M2, and M26M) and dimethylated metabolites (M16M1M1, M16M2M2, and M26M2M2), but only trace amounts of the trimethylated metabolites were detected. The rates of methylation mediated by the rat and the dog COMTs were significantly faster than those by the human enzyme (Fig. 6, B and C). Because of this faster methylation by the rat COMT, trimethylated metabolites (M16M1M1, M16M2M2, M26M1M1, and M26M2M2) were significantly detected for salvianolic acids A and B (Fig. 6B). For the dog COMT, these trimethylated metabolites were also considerably detected only after 120-minute incubation (Fig. 6C). For the rat hepatic COMT-mediated methylation, the in vitro IC50 values were ranked as follows: lithospermic acid (0.04 hour) = salvianolic acid B (0.04 hour) = rosmarinic acid (0.04 hour) ≈ salvianolic acid D (0.05 hour) < salvianolic acid A (0.09 hour) ≈ protocatechuic acid (0.12 hour) < protocatechuic aldehyde (0.24 hour) < tanshinol (0.94 hour).

For the dog hepatic COMT-mediated methylation, the in vitro IC50 values were ranked as follows: lithospermic acid (0.10 hour) ≈ salvianolic acid D (0.13 hour) < protocatechuic acid (0.25 hour) ≈ rosmarinic acid (0.28 hour).
hour) \approx \text{salvianolic acid B (0.30 hour)} \approx \text{salvianolic acid A (0.39 hour)} < \text{protocatechuic aldehyde (1.36 hours)} < \text{tanshinol (1.94 hours)}.

Discussion

Danshen catechols are pharmacologically important for DanHong injection, because they have been reported to have multiple antianginal properties. Because systemic exposure to compounds after dosing an herbal injection depended on their dose levels and elimination kinetics, defining the correlations between exposure and influencing factors are important in PK research on the injection. The analysis of DanHong injection revealed a total of 28 Danshen catechols present. Because of their different content levels, these Danshen catechols could be divided into three classes according to their daily dose levels from the

Fig. 5. Comparative in vitro metabolic stability of Danshen catechols (including tanshinol, protocatechuic aldehyde, protocatechuic acid, salvianolic acid B, rosmarinic acid, salvianolic acid A, salvianolic acid D, and lithospermic acid) toward metabolism reactions mediated by COMT, SULT, UGT, and ALDH (ALDH, for protocatechuic aldehyde only). Human, rat, and dog liver cytosols containing COMT, SULT, and ALDH were used to induce methylation, sulfation, and oxidation of Danshen catechols, whereas human, rat, and dog liver microsomes containing UGT were used to induce glucuronidation of the compounds. Green, brown, black, and purplish-red semi-open bars denote the data of Danshen catechols undergoing methylation, sulfation, glucuronidation, and aldehyde dehydrogenation, respectively. Green, brown, black, and purplish-red open bars denote the data of positive controls, i.e., \((-\text{-epicatechin for COMT-mediated methylation, 7-hydroxyflavone for SULT-mediated sulfation, chrysin for UGT-mediated glucuronidation, and isovanillin for ALDH-mediated aldehyde dehydrogenation, respectively. Values represent the means \pm standard deviations (n = 3).}

Fig. 6. Methylation of Danshen catechols (including tanshinol, protocatechuic aldehyde, protocatechuic acid, salvianolic acid B, rosmarinic acid, salvianolic acid A, salvianolic acid D, and lithospermic acid) mediated by COMT in SAM-fortified human (A), rat (B), and dog liver cytosols (C) over time. Blue lines/blue open circles denote the substrate compounds. Green lines/green open circles, green lines/green semi-open circles, and green lines/green solid circles denote monomethylated, dimethylated, and trimethylated metabolites, respectively. For the methylation of protocatechuic aldehyde, isovanillin was used to inhibit the catalytic activity of ALDH in human liver cytosol. Values represent the means \pm standard deviations (n = 3).
intra venous injection. Tanshinol (6; 282.8 μmol/subject) was the only Danshen catechol of class I (>100 μmol/subject). Protocatechuic aldehyde (1), salvianolic acids B (26) and A (16), rosmarinic acid (10), salvianolic acid D (13), and lithospermic acid (22) had dose levels of 10.0–73.2 μmol/subject; they were Danshen catechols of class II (10–100 μmol/subject). The other 21 minor Danshen catechols (0.1–7.2 μmol/subject) were of class III (<10 μmol/subject). In the human subjects receiving DanHong injection, Danshen catechols of classes I and II were all detected in plasma. However, only few Danshen catechols of class III, including lithospermic acid isomers (19 and 20; 5.2 and 3.9 μmol/sub ject, respectively) and protocatechuic acid (2; 1.9 μmol/subject), were detected in human plasma after dosing. Similar compound dose-dependent detection of plasma Danshen catechols took place in rats and dogs.

Among the detected Danshen catechols, tanshinol (6), salvianolic acid D (13), and lithospermic acid (22) exhibited the most significant systemic exposure in the human subjects receiving DanHong injection, suggesting that these herbal compounds are worth special consideration in pharmacological research on DanHong injection. Unlike 6 (the only Danshen catechol of class I), 13 and 22 were Danshen catechols of class II and exhibited content levels in the dosed injection lower than the other catechols of the same class. The considerable systemic exposure to 13 and 22 appeared to be correlated with their elimination half-lives (t 1/2, 2.5 and 5.7 hours, respectively) significantly longer than those of 6 and the other Danshen catechols of class II (0.2–0.6 hour). Rats and dogs are laboratory animals extensively used in cardiovascular pharmacological research. Rats receiving DanHong injection exhibited an exposure profile of unchanged Danshen catechols similar to the human subjects; 6, 13, and 22 exhibited levels of systemic exposure higher than the other Danshen catechols. In the rats, the t 1/2 values of 13 and 22 (around 2 hours) were longer than those of 6 and the other Danshen catechols of class II (<1 hour). Unlike the human subjects and the rats, dogs exhibited considerable systemic exposure to 6 only after dosing DanHong injection. In the dogs, 13 and 22 exhibited short t 1/2 (<0.5 hour). The observed PK interspecies difference should be considered in pharmacological research of DanHong injection. In PK studies of other herbal medicines, significant differences in elimination half-life have also been found to cause considerably different systemic exposure to herbal compounds; long elimination half-life may considerably counterbalance influence of poor oral bioavailability on systemic exposure to an herbal compound (Liu et al., 2009; Chen et al., 2013; Jiang et al., 2015). With rapidly increasing application of analytical techniques, such as liquid chromatography/mass spectrometry, in research on herbal medicines, content levels of compounds in herbal formulations and their elimination half-lives have become more and more available in literature; such data of herbal compounds could be used together as potential PK marker to predict their systemic exposure after dosing herbal injections.

In addition to the systemic exposure to unchanged Danshen catechols, their elimination was also investigated in the current study. After dosing DanHong injection, tanshinol (6), salvianolic acid D (13), and lithospermic acid (22) circulated mainly as parent compounds with minor or major metabolites. Elimination of 6 and 13 mainly involved renal excretion of the parent compounds, whereas 22 was eliminated mainly via hepatic uptake and methylation followed by hepatobiliary excretion of the metabolites. Unlike these three catechols, protocatechuic aldehyde (1) circulated in the blood stream predominantly as metabolites, rather than the parent compound, and was first transformed into protocatechuic acid (2), which was then eliminated by conversion into sulfates and methylated glucuronides followed by renal excretion. Rosmarinic acid (10) also circulated in the blood stream predominantly as metabolites, rather than the parent compound. This catechol was eliminated mainly via metabolism, and only about 20% of dosed 10 was eliminated via renal excretion of the parent compound. The metabolism of 10 involved formation of dimethylelated products and their sulfates and glucuronides; these metabolites were eliminated comparably via renal excretion and hepatobiliary excretion. Salvianolic acids A (16) and B (26) were detected at low plasma levels, and their plasma metabolites exhibited little or no detection in the human subjects. These two Danshen catechols appeared to be eliminated mainly via hepatobiliary excretion of glucuronized and methylated metabolites of 16 and methylated metabolites of 26. Zhang et al. (2004) reported several hepatobiliary metabolites of salvianolic acid B in rats, including 3-monomethyl-, 3″'-dimethyl-, 3″''-dimethyl-, and 3″',3″''-trimethyl-salvianolic acid B.

The Danshen catechols have poor membrane permeability, except for protocatechuic aldehyde (Lu et al., 2008). During elimination, these compounds tend to necessitate transporter-mediated basolateral uptake from blood as the first step in hepatobiliary and/or renal tubular elimination. Hepatic OATP1B3 and renal OAT1 were found to mediate cellular uptake of Danshen catechols (the details to be published elsewhere). Because of their catechol moieties, most Danshen catechols underwent COMT-mediated methylation as the primary metabolic pathway in humans, rats, and dogs. Significant intercompound and interspecies differences in rate and degree of methylation were observed for Danshen catechols. Hepatic methylation of tanshinol by human COMT was significantly slower than those of salvianolic acid D and lithospermic acid, demonstrating their in vitro t 1/2, 3.73, 0.31, and 0.41 hour, respectively. However, the elimination t 1/2 of tanshinol (6; 0.6 hour) after dosing DanHong injection in human subjects was significantly shorter than those of salvianolic acid D (13; 2.5 hours) and lithospermic acid (22; 5.7 hours). This is probably because the transporter-mediated hepatic and/or renal tubular uptake from blood, rather than the methylation, was the rate-limiting step in the elimination of these Danshen catechols. Although methylation of drugs often hampers their pharmacological activities (Goldstein et al., 2003), the antiangiogenic properties of the major methylated metabolites of Danshen catechols are unknown.

Methylation of xenobiotics with catechol moieties involves COMT-catalyzed transfer of methyl groups from SAM to the catechol hydroxyl groups in the presence of Mg 2+ (Zhu et al., 2000). This produces S-adenosylhomocysteine, which can be further converted to homocysteine by S-adenosylhomocysteine hydrolase in the liver and kidneys (to less extent). An abnormally high blood concentration of homocysteine is recognized as a risk factor for vascular diseases and neurodegenerative conditions (McCully, 2007; Vogel et al., 2009; Smith et al., 2010). This has raised concerns regarding untoward effects of hepatic methylation of xenobiotics on the blood concentration of homocysteine. A typical example is hyperhomocysteinemia, which is caused by chronic levodopa treatment of patients with Parkinson’s disease (Kuhn, et al., 1998; Müller et al., 1999; Miller et al., 2003). The Danshen catechols from intravenous DanHong injection undergo COMT-mediated methylation in the body. Further investigation of the relationship between methylation of Danshen catechols and hyperhomocysteinemia risk may facilitate hazard identification for this herbal injection. This may also improve understanding of the endothelial protection properties of Danshen catechols (Chan et al., 2004; Cao et al., 2009; Yang et al., 2010; Zhang et al., 2013).

In summary, Danshen catechols present in DanHong injection could be divided, according to their daily dose levels, into three classes (>100, 10–100, and <10 μmol/subject). Tanshinol (6) was the only Danshen catechol of class I; protocatechuic aldehyde (1), salvianolic acids B (26) and A (16), rosmarinic acid (10), salvianolic acid D (13), and lithospermic acid (22) were of class II; the other 21 minor Danshen catechols were of class III. Unchanged Danshen catechols of classes I and
II were all detected in plasma after intravenous dosing DanHong injection; only three catechols of class III were detected in plasma but at very low levels. Only 6, 13, and 22 exhibited considerable systemic exposure in human subjects receiving the injection. These three catechols exhibited considerable systemic exposure also in rats after dosing, but only 6 had considerable exposure in dogs. The considerable exposure to 6 was due to its highest daily dose, whereas that to 13 and 22 was due to their relatively long elimination half-lives. The catechols 1 and 10 circulated in the blood stream predominantly as metabolites rather than as the parent compounds; 16 and 26 were detected at low plasma levels with their plasma metabolites little or not detected in the human subjects. Both 6 and 13 were eliminated mainly via renal excretion of the parent compounds; 22 was eliminated mainly via hepatobiliary excretion of its methylated metabolites. The other Danshen catechols were eliminated mainly via hepatobiliary and/or renal excretion of their metabolites. COMT-mediated methylation was found to be the primary metabolism for most Danshen catechols and showed intercompound and interspecies differences in rate and degree. Because the only known mechanism of homocysteine production involves SAM-dependent methylation, further studies are warranted to fully characterize the methylation of Danshen catechols and to address safety concern associated with possible alteration of blood concentrations of homocysteine related to using catechols and to address safety concern associated with possible alteration of blood concentrations of homocysteine related to using DanHong injection.

Authorship Contributions

Participated in research design: C. Li, L. Li, Huang.


Contributed new reagents: Zhao, L. Jia.

Performed data analysis: C. Li, L. Li, M. Li.

Wrote or contributed to the writing of the manuscript: C. Li, L. Li, M. Li, Ohaley, Lim.

References


At ASPET Journals on August 23, 2017 dmd.aspetjournals.org Downloaded from