Absorption and disposition of ginsenosides after oral administration of *Panax notoginseng* extract to rats

Houfu Liu, Junling Yang, Feifei Du, Xiumei Gao, Xutao Ma, Yuhong Huang, Fang Xu, Wei Niu, Fengqing Wang, Yu Mao, Yan Sun, Tong Lu, Changxiao Liu, Boli Zhang, Chuan Li

*Shanghai Institute of Materia Medica* (H.L., J.Y., F.D., F.X., W.N., F.W., Y.M., Y.S., T.L., Chu.L) and *Graduate School* (H.L., Chu.L), *Chinese Academy of Sciences, Shanghai, China*; *Tianjin University of Traditional Chinese Medicine, Tianjin, China* (X.G., X.M., Y.H., B.Z.); *Tianjin State Key Laboratory of Pharmacodynamics and Pharmacokinetics, Tianjin Institute of Pharmaceutical Research, Tianjin, China* (Cha.L)

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Running Title: ADME OF SANQI GINSENOSIDES

Address correspondence to: Dr. Chuan Li, Shanghai Center for DMPK Research, Shanghai Institute of Materia Medica, SIBS, Chinese Academy of Sciences, 555 Zuchongzhi Road, Zhangjiang Hi-Tech Park, Shanghai 201203, China. E-mail: chli@mail.shcnc.ac.cn

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ABBREVIATIONS: AUC, area under concentration-time curve; CL_b, biliary clearance; CL_R, renal clearance; CL_tot,p, total plasma clearance; Cum.A_e, cumulative amount excreted during sampling period; ESI, electrospray ionization; i.v., intravenous; LC/MS/MS, liquid chromatography/tandem mass spectrometry; MRP2, multidrug resistance-associated protein 2; NROTB, number of rotatable bonds; P_app, apparent permeability coefficient; P-gp, P-glycoprotein; PK, pharmacokinetic; p.o., peroral; S, aqueous solubility at a given pH; t_1/2, elimination half-life; TPSA, topological polar surface area.
ABSTRACT:

*Panax notoginseng* (Sanqi) is a cardiovascular herb containing ginsenosides that are believed responsible for the therapeutic effects of Sanqi. This study aimed to evaluate rat exposure to ginsenosides following p.o. administration of Sanqi extract and to identify the key factors affecting their absorption and disposition. Ginsenosides were administered to rats, either in the form of Sanqi extract or as pure chemicals. The ginsenosides Ra₃, Rb₁, Rd, Re, Rg₁ and notoginsenoside R₁ were the major saponins present in the herbal extract. Systemic exposure to ginsenosides Ra₃, Rb₁, and Rd following p.o. administration of the extract was significantly greater than that of the other compounds. Considerable colonic deglycosylation of the ginsenosides occurred, but the plasma levels of deglycosylated metabolites were low in rats. Poor membrane permeability and active biliary excretion are the two primary factors limiting systemic exposure to most ginsenosides and their deglycosylated metabolites. In contrast to other ginsenosides, biliary excretion of ginsenosides Ra₃ and Rb₁ was passive. Meanwhile, the active biliary excretion of ginsenoside Rd was significantly slower than that of other saponins. Slow biliary excretion, inefficient metabolism, and slow renal excretion resulted in long-circulating and thus relatively high exposure levels for these three ginsenosides. Due to these reasons, plasma ginsenosides Ra₃, Rb₁ and Rd were identified as pharmacokinetic markers for indicating rat systemic exposure to Sanqi extract. This is a systematic investigation of the absorption and disposition of ginsenosides from an herb, the information gained from which is important for linking Sanqi administration to its medicinal effects.
Introduction

Herbs have been used for medicinal purposes in China for millennia, and traditional Chinese medicine still plays an important role in Chinese healthcare. The dried root of *Panax notoginseng* (family Araliaceae) is an important Chinese medicinal herb known as Sanqi. In traditional Chinese medicine, Sanqi is indicated for analgesia and hemostasis (*Chinese Pharmacopoeia Commission, 2005*). The herb is also used to treat patients with angina and coronary artery disease (*Mashour et al., 1998*); the pharmacological mechanism may be as a selective calcium ion antagonist that may interact with the receptor-operated calcium ion channel (*Kwan, 1995*). Sanqi also exerts protective effects on the cardiovascular system (*Ng, 2006*).

Most of Sanqi’s bioactivities are believed to be associated with triterpene saponins derived mainly from the tetracyclic dammarane. These compounds, known as ginsenosides, can be classified according to their structures as 20(S)-protopanaxadiol type (ppd-type) and 20(S)-protopanaxatriol type (ppt-type). The ppd-type ginsenosides possess sugar moieties at the C-3 and/or C-20 positions, while ppt-type ginsenosides have a hydroxyl group at C-3 and sugar moieties at C-6 and/or C-20. The major saponins present in Sanqi include the ppd-type ginsenosides Ra3, Rb1 and Rd and the ppt-type ginsenosides Re and Rg1, and notoginsenoside R1. These ginsenosides contain 2–5 sugars. Although ginsenosides are also prominent constituents of other *Panax* species, the relative amounts of the saponin constituents present in Sanqi differ from those in Asian ginseng (*P. ginseng*) and American ginseng (*P. quinquefolius*). Sanqi contains approximately equivalent amounts of ginsenosides Rb1 and Rg1, whereas ginsenoside Rb1 is often more abundant than ginsenoside Rg1 in Asian and American ginsengs. In addition, Sanqi contains a substantial amount of notoginsenoside GR1, which is also different from Asian and American ginsengs.
The pharmacological effects of ginsenosides vary and can even be oppositional. In contrast to the anti-angiogenic effects of the ppd-type ginsenoside Rb₁, the ppt-type ginsenoside Rg₁ has angiogenic properties (Sengupta et al., 2004). However, the lack of quantitative data regarding the absorption, distribution, metabolism, and excretion of ginsenosides has hindered investigation of the pharmacological activities. Earlier PK studies have focused on the development of bioanalytical assays for ginsenosides and their application to preliminary PK assessments (Xu, et al., 2003; Li et al., 2004a,b; Li et al., 2006; Li et al., 2007a,b). The PK profiles and disposition of ppd-type and ppt-type ginsenosides after administration of Sanqi extracts remain largely unknown. Some studies suggested low oral bioavailability for ginsenosides Rb₁ and Rg₁, only 0.1% and 2%, respectively (Odani et al., 1983a,b), whereas these values were reported to be 4% and 18%, respectively, by others (Xu et al., 2003). The intestinal microflora can degrade ginsenosides via cleavage of the sugar moieties (Hasegawa et al., 1996; Akao et al., 1998; Tawab et al., 2003). However, the effects of deglycosylation on the systemic exposure to ginsenosides are inconclusive.

The objective of this study was to gain understanding about comparative systemic exposure and PK properties of various ginsenosides from Sanqi and about the key factors that affect their absorption and disposition. We also investigated the influence of colonic deglycosylation on systemic exposure to the ginsenosides. The information gained from the study is indispensable for evaluating the contributions of the ginsenosides to the medicinal effects of Sanqi, for assessing potential herb-drug or herb-herb interactions, and for developing Sanqi-based pharmacotherapeutics. To this end, ginsenosides were administered to rats, either in the form of Sanqi extract or as pure compounds. Multiple in vivo, in vitro, and in silico approaches were integrated to determine ginsenoside exposure, absorption, and disposition.
Materials and Methods

Chemicals and reagents. Ginsenosides Rb₁ (GRb₁; molecular mass: 1108), Rd (GRd; 946), Rg₃ (GRg₃; 784), F₂ (GF₂; 784), Rb₂ (GRb₂; 622), Re (GRe; 946), Rg₁ (GRg₁; 800), Rf (GRf; 800), F₁ (GF₁; 638), notoginsenoside R₁ (NGR₁; 932), compound-K (C-K; 622), protopanaxadiol (Ppd; 460), and protopanaxatriol (Ppt; 476) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ginsenosides Ra₃ (GRA₃; 1240), Rc (GRC; 1078), and 20-glucosyl-Rf (20gRf; 962) were obtained from Fengshanjian Co., Ltd. (Kunming, China). Ginsenoside Rh₁ (GRh₁; 638) was obtained from Wuhu Delta Co., Ltd. (Wuhu, China). Ginsenoside Rg₂ (GRg₂; 784) was purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China). The compound purity exceeded 98%.

Hydroxypropylmethyl cellulose was obtained from Colorcon (Shanghai, China). N,N-dimethylacetamide, Cremophor, and taurocholic acid were purchased from Sigma-Aldrich (St Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM), penicillin-streptomycin, and MEM non-essential amino acids were obtained from Gibco Invitrogen Cell Culture (Grand Island, NY). Fetal bovine serum was purchased from Hyclone (Logan, UT). Hank’s balanced salts solution (HBSS), atenolol, rhodamine123, verapamil hydrochloride, and sulfasalazine were obtained from Sigma-Aldrich. MK571 was obtained from Calbiochem (San Diego, CA). HPLC-grade organic solvents, antipyrine, and sodium carboxymethylcellulose (CMC-Na) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Preparation of Sanqi extract and individual ginsenosides. The dried roots of *P. notoginseng* (Sanqi) were obtained from Shanghai Huayu Chinese Herbs Co., Ltd. (Shanghai, China). For p.o. administration, 400 g of Sanqi were pulverized, mixed,
and steeped in 2400 ml of drinking water for 0.5 h at room temperature before 1 h sonication-enhanced extraction. The extract was separated by filtration and the residue was re-extracted with 1600 ml of water. The pooled extract was concentrated under reduced pressure at 40°C and was modified with hydroxypropylmethyl cellulose at 0.3% (g/ml) before adding water to 800 ml to yield Sanqi extract. The extract was stored at −15°C pending use.

For i.v. administration, GRa3, GRb1, GRc, GRd, GF2, GRg3, C-K, GRh2, Ppd, 20gRf, GRe, NGR1, GRg1, GRf, GRg2, GRh1, GF1, and Ppt were prepared individually as injectable 1.0-mM solutions in 0.5% N,N-dimethylacetamide, 9.5% Cremophor, and 90% saline.

Experimental animals. Rat studies were conducted according to protocols approved by the Review Committee of Animal Care and Use at the Shanghai Institute of Materia Medica (Shanghai, China). Male Sprague-Dawley rats (220–250 g; Shanghai SLAC Laboratory Animal Co., Shanghai, China) were housed in rat cages (48 × 29 × 18 cm³) in a unidirectional airflow room under controlled temperature (20–24°C), relative humidity (40–70%), and a 12-h light-dark cycle. Filtered tap water was available ad libitum, and the rodents were given commercial rat chow ad libitum except for the overnight period prior to dosing. Rats were acclimated to the facilities and environment for 7 days prior to the experiments.

Plasma pharmacokinetic studies in rats. Rats were randomly assigned to different groups (3 rats/group) to receive a p.o. dose of Sanqi extract at 4, 8, or 16 ml/kg (via gavage). In addition, bolus i.v. doses of the injectable solutions of individual ginsenosides, including GRa3, GRb1, GRc, GRd, GF2, GRg3, C-K, GRh2, Ppd, 20gRf, GRe, NGR1, GRg1, GRf, GRg2, GRh1, GF1, and Ppt, at 5 μmol/kg were given to rats (2 or 3 rats/compound) through the tail vein. Serial blood samples (~60
µl; 0, 5, 15 (p.o. only), 30 min, and 1 (i.v. only), 2, 4 (i.v. only), 6, 8 (p.o. only), 10, 12 (p.o. only), 15, 24, 36, and 48 h] were collected in heparinized tubes from the orbital sinus under light ether anesthesia. The blood samples were centrifuged at 3000g for 5 min and the plasma fractions were decanted and frozen at −70°C until analysis.

In a multiple-dose study, rats received Sanqi extract at a daily p.o. dose of 4 ml/rat. Four rats per time points were chosen randomly for blood sampling on days 1, 8, 15, or 22. Serial blood samples (~60 µl; 0, 15, 30 min, and 2, 6, 8, 10, 12, 15, and 24 h post dose) were collected and centrifuged to yield plasma fractions.

**Excretion studies in rats.** Rats were housed in Nalgene metabolic cages (1 rat/cage) and received p.o. Sanqi extract at 8 ml/kg (via gavage). Urine and feces samples were collected from four rats at 0–4, 4–10, and 10–24 h on day 1 and every 24 h over the next 3 days. The urine and feces collection tubes were frozen at −15°C to keep collected samples stable. All excretory samples were weighed before storage at −70°C. The thawed feces samples were homogenized in 9 volumes of saline for analysis. Similar urinary/fecal excretion studies were performed for individual ginsenosides administered in i.v. boluses of 5 µmol/kg.

For bile sampling, rats were anesthetized with ether, the bile duct was cannulated using polyethylene tubing, and bile was collected at 0–1, 1–3, 3–6, 6–12, 12–18, 18–24, 24–30, 30–36, 36–42, and 42–48 h after a p.o. dose of Sanqi extract at 8 ml/kg (vial gavage; 3 rats/collection interval). Sodium taurocholate solution (pH 7.4) was infused into the duodenal cannula (~1 ml/h) during bile collection. The bile samples were weighed and stored at −70°C. Similar biliary excretion studies were performed for individual ginsenosides administered in i.v. boluses of 5 µmol/kg.

**Tissue distribution study in rats.** Rats under ether anesthesia were sacrificed by bleeding (~10 ml blood; the resulting plasma samples used for analysis) the
abdominal aorta at 0, 5, 15 min, 1, 2, 4, 24, or 48 h (3 rats/time point) post i.v. bolus of GRb1, GRg1, or C-K at 5 µmol/kg (via tail vein). Samples of abdominal adipose, testicle, bladder, spleen, stomach, small intestine, large intestine, kidney, liver, lung, heart, and brain were excised and then rinsed in ice-cold saline before gently blotting on absorbent paper and weighing. The large tissues were homogenized in 4 volumes of ice-cold saline, whereas the small ones were prepared in 9 volumes. The homogenates were stored at −70°C.

**Pharmacokinetic data processing.** Plasma PK parameters were determined by non-compartmental and compartmental methods using the Kinetica 2000 software package (version 3.0; Philadelphia, PA). The $C_{\text{max}}$ and $T_{\text{max}}$ were observed values without interpolation. The area under concentration-time curve up to the last measured time point ($\text{AUC}_{0 \rightarrow t}$) was calculated using the trapezoidal rule. The value of $\text{AUC}_{0 \rightarrow \infty}$ was generated by extrapolating $\text{AUC}_{0 \rightarrow t}$ to infinity using the elimination rate constant and the last measured concentration. The biliary clearance (CLb) and the renal clearance (CLR) were calculated from the cumulative amount excreted ($\text{Cum.A}_e$) in the bile and in the urine, respectively, divided by the plasma AUC. Dose proportionality studies on $\text{AUC}_{0 \rightarrow \infty}$ and $C_{\text{max}}$ were conducted by the regression of log-transformed data (power model) (Smith et al., 2000).

**Cell culture and transport study in Caco-2 monolayers.** Caco-2 cells (American Type Culture Collection, Manassas, VA) were cultured as described previously (Dai et al., 2008). Bidirectional transport experiments were conducted in triplicate at 2–50 µM for the individual ginsenosides in HBSS. Dimethyl sulfoxide was used as cosolvent to improve the solubility of one- or two-sugar containing ginsenosides, Ppd, and Ppt, but its final concentration did not exceed 1% (v/v). After incubation for 2 h at 37°C, 50-µl samples were collected from both the receiver and
donor compartments, and the compound recovery was determined. The apparent permeability coefficient \( (P_{\text{app}}) \) was expressed in cm/s and the efflux ratio \( (P_{\text{app(basolateral→apical)}}/P_{\text{app(apical→basolateral)}}) >3 \) was considered to be a positive result, suggesting that the tested compound was an in vitro substrate for the efflux transporter(s). The P-gp substrate rhodamine 123 exhibited substantial directional preference with the efflux ratio of 51.6, which was significantly reduced to 2.3 in the presence of the P-gp inhibitor verapamil. For sulfasalazine, a MRP2 substrate, the presence of the MRP2 inhibitor MK571 caused reduction of efflux ratios from 137 to 4.8. These data suggested the normal presence of the efflux transporters in the Caco-2 cell monolayers used.

**In silico assessment of permeability and solubility.** Chemoinformatic assessment of the physicochemical properties governing intestinal absorption was performed for ginsenosides and their aglycones. Aqueous solubility \((S)\) was calculated with ACD/aqueous solubility v8.02 via the ACD/I-Lab service (Advanced Chemistry Development Inc., Toronto, Canada). The octanol-water partition coefficient \((\log P)\) was calculated using Pallas software (Pallas 3.6, CompuDrug International, Sedona, AZ). The total hydrogen bond count (donors and acceptors), topological polar surface area \((\text{TPSA})\), and number of rotatable bonds \((\text{NROTB})\) were determined using Molinspiration Property Calculator (Molinspiration Cheminformatics, Bratislava, Slovak Republic). Lipinski’s Rule of 5 (Lipinski et al., 1997) and the molecular surface properties (total hydrogen bond count, TPSA, and NROTB) (Veber et al., 2002) were used to predict the permeability of the tested compounds.

**LC/MS/MS bioassays.** Validated bioanalytical methods were used to measure ginsenosides and their metabolites. Biological samples \((20 \, \mu\text{L})\), including plasma, tissue homogenate, excretory, and cell culture medium samples, were precipitated
with 80 μL of methanol followed by centrifugation at 21885g for 5 min before LC/MS/MS analysis. TSQ Quantum mass spectrometer (Thermo Fisher, San Jose, CA) was interfaced via an ESI probe with a liquid chromatograph (Agilent, Waldbronn, Germany). The LC separation was achieved on a Phenomenex® Synergi 5 μm C18 column (Torrance, CA). The LC mobile phases were CH₃OH/H₂O (2:98, v/v, containing 0.08 mM HCOONH₄) for A and CH₃OH/H₂O (98:2, v/v, containing 0.08 mM HCOONH₄) for B, which were used for pulse gradient chromatography (Wang et al., 2007). The gradient parameters including the start proportion, the elution proportion, the elution proportion segment, and the column equilibrium segment were 15% B, 100% B, 5.4 min, and 4.4 min, respectively, except for excretory samples requiring a 5-min start proportion segment. ESI and collision energy were optimized to maximize generation of the sodiated or protonated molecules and to produce the characteristic product ions, respectively. The precursor-to-product ion pairs used for selected reaction monitoring of GRa₃, GRb₁, GRc, GRd, GF₂, GRg₃, C-K, GRh₂, Ppd, 20gRf, GRe, NGR₁, GRG₁, GRf, GRG₂, GRh₁, GF₁, and Ppt were m/z 1263→497, 1131→365, 1101→335, 969→789, 807→627, 807→365, 645→203, 623→407, 461→443, 985→365, 969→789, 955→775, 823→643, 823→365, 807→349, 639→621, 661→203, and 477→109, respectively. An online motorized six-port divert valve was used to introduce the LC eluent flow to the ion source for data acquisition over the period of 3–9 min, except for excretory samples (8–14 min). Matrix-matched calibration curves were constructed for the analytes (1.37 or 12.3–3000 ng/ml) using weighted linear regressions of the analyte peak area against the corresponding nominal concentrations of the analyte (ng/ml), which showed good linearity (r² >0.99).
Results

**Rat systemic exposure to ginsenosides from p.o. Sanqi extract.** Major ginsenosides in Sanqi extract were the ppd-type GRα3 (4.6 mM), GRβ1 (23.2 mM), and GRd (6.9 mM) and the ppt-type GRe (5.2 mM), NGR1 (7.9 mM), and GRg1 (33.1 mM). 20gRf and GRh1 were present at lower levels (2.2 mM for both). In addition, several minor ginsenosides, including GRc, GF2, GRg3, GRf, GRg2, and GF1 were at the levels of 0.1–0.5 mM. No other ginsenosides or aglycones were detected in Sanqi extract.

As shown in Fig. 1, the ppd-type GRα3, GRβ1, and GRd were measurable in rat plasma up to 48 h following p.o. administration of Sanqi extract (4–16 ml/kg), whereas the ppt-type GRe, NGR1, and GRg1 were measurable only within 24 h. Of the other ginsenosides, the ppd-type GRc, GF2, and GRg3 were only measured in nanomolar plasma levels at 6–10 h after dosing at 8 and/or 16 ml/kg. The ppt-type GRf, GRg2, GRh1, and GF1, except for 20gRf, were not detected in plasma. Compound-K (C-K; ppd-type) was also measured in rat plasma with delayed occurrence, i.e., 6 h after dosing. C-K was not contained in the administered Sanqi extract; it was a colonic deglycosylated product of the bulky ppd-type ginsenosides (Hasegawa et al., 1996; Akao et al., 1998). We found that C-K could be measured in rat feces following p.o. administration of purified GRβ1 or GRd.

**(Insert Figure 1 Here)**

The maximal plasma concentrations of the ppd-type GRα3, GRβ1, or GRd occurred 6–10 h after dosing. The exposure levels of GRα3, GRβ1, and GRd in AUC\(_{0\rightarrow\infty}\) and \(C_{\text{max}}\) increased directly with Sanqi extract dose from 4 to 16 ml/kg but nonlinearly. However, no significant dose-exposure relationship was observed for C-K. In addition, we also observed a strong linear relationship from a scatter plot of
logAUC\(_{0\rightarrow\infty}\) of GRa\(_3\), GRb\(_1\), and GRd versus their corresponding logarithmic compound doses from the ingested extract (\(r^2 = 0.96; n = 27\); Fig. 1). The mean oral bioavailability of GRa\(_3\), GRb\(_1\), and GRd after administration of the extract was quite low: 0.1–0.2%. Due to their long \(t_{1/2}\), i.e., 13, 18, and 13 h for GRa\(_3\), GRb\(_1\), and GRd, respectively, significant increases in systemic exposure were observed in the multiple-dose study (Fig. 1). The plasma AUC\(_{0\rightarrow24h}\) and \(C_{\text{max}}\) of these ppd-type ginsenosides increased from day 1 to day 15 and the values (corrected for rat body weight) on day 15 were 2.1–2.6-times higher than those on day 1. However, the systemic exposure levels on day 22 increased only 1.3–1.9-fold compared with those on day 1. The reason for these decreases remains to be explored.

Double or triple peaks occurred in the plasma-concentration time courses of the ppt-type GRe, NGR\(_1\), and GRg\(_1\) following p.o. dose of Sanqi extract (Fig. 1). Despite the interanimal variability, the peak appearance patterns for GRe, NGR\(_1\), and GRg\(_1\) for a given rat were almost identical. The \(F\) values of these ppt-type ginsenosides ranged from 0.2 to 0.6%. Dislike the preceding ppd-type compounds, the systemic exposure levels of GRe, NGR\(_1\), and GRg\(_1\) were poorly correlated with the p.o. extract dosage. In addition, the AUC\(_{0\rightarrowt}\) values of GRe, NGR\(_1\), and GRg\(_1\), normalized according to the corresponding compound doses from the extract, were about 1/10 of those of the ppd-type compounds.

Collectively, although their total amounts present in Sanqi extract were comparable, the systemic exposure levels of the ppd-type ginsenosides GRa\(_3\), GRb\(_1\), and GRd were significantly greater than those of the ppt-type ginsenosides GRe, NGR\(_1\), and GRg\(_1\). The long \(t_{1/2}\) of these ppd-type compounds counteracted the unfavorable effects of their poor \(F\) on the systemic exposure levels.

Permeability and solubility of ginsenosides and their deglycosylated
products. Because of the poor $F$, the permeability and solubility of individual ginsenosides were assessed in vitro and in silico to gain understanding of the mechanisms governing intestinal absorption of ginsenosides. Because it had been reported that ginsenosides could be stripped of their sugar moieties by the colonic microflora (Hasegawa et al., 1996; Akao et al., 1998; Tawab et al., 2003), some monoglycosides and aglycones were also included in the assessment. As shown in Fig. 2, the $P_{\text{app}}$ values of ginsenosides measured in Caco-2 monolayers suggested that the membrane permeability of most ginsenosides was poor with the $P_{\text{app}}$ values $<3 \times 10^{-7}$ cm/s. GF$_2$, C-K, GRh$_2$, GRh$_1$, and GF$_1$ possessed slightly increased $P_{\text{app}}$ values (around $1 \times 10^{-6}$ cm/s); the $P_{\text{app}}$ values of Ppd and Ppt were about $2 \times 10^{-6}$ cm/s. The comparable bidirectional $P_{\text{app}}$ values of most the ginsenosides and their aglycones suggested passive diffusion as the transport mechanism, except for GF$_2$ and GF$_1$, which exhibited efflux ratios 10.0 and 48.7, respectively. The efflux ratio of GF$_2$ was significantly reduced to 2.1 and 2.2, in the presence of verapamil and MK571, respectively, suggesting that this ginsenoside might be the substrate of both P-gp and MRP2. Similar situation occurred for GF$_1$, i.e., the efflux ratio was reduced to 12.0 and 9.6 with verapamil and MK571, respectively.

(Insert Figure 2 Here)

In silico assessment suggested that many ginsenosides could be defined as soluble (Fig. 2). $S$ values ranged from $160 \times 10^{-6}$ to $17,000 \times 10^{-6}$ M, which were significantly greater than the highest initial concentrations in the Caco-2 study ($50 \times 10^{-6}$ M). However, the solubility significantly decreased for compounds with fewer or no sugar moieties attached. The $S$ values of GF$_1$ and GRh$_1$ were $86 \times 10^{-6}$ and $75 \times 10^{-6}$ M, respectively, whereas those of GRg$_3$, C-K, GRh$_2$, Ppd, GRg$_2$, and Ppt ranged from $0.08 \times 10^{-6}$ to $9 \times 10^{-6}$ M. The $S$ values did not vary significantly as pH
changed. On the other hand, most the ginsenosides, except C-K, GRh₂, Ppd, GF₁, GRh₁, and Ppt, had unfavorable traits underlying poor membrane permeability, including high total hydrogen bond counts ranging from 22 to 44 (donors and acceptors; favorable $\leq 12$), high TPSA from 219 Å to 436 Å (favorable value $\leq 140$ Å), and high flexibility ranging from 10 to 18 (favorable value $\leq 10$). Reducing the number of sugar moieties decreased hydrogen-bonding capacity, molecular flexibility, and molecular mass, but also reduced solubility significantly (Fig. 2). In addition, the lipophilicity of the Ppd and Ppt aglycones was significantly higher ($\text{Log} P$, 5.4 and 4.4, respectively) than that of the ginsenosides ($\text{Log} P$, $-0.1$–$3.1$).

Collectively, our in vitro observations and in silico calculations suggested that the poor intestinal absorption of the ginsenosides could be attributed to poor membrane permeability, which was influenced by the increased sugar number. Although these traits were improved for the deglycosylated products, significant increases in intestinal absorption appeared to be limited by their lowered solubility.

Structure-dependent disposition of i.v. administered ginsenosides in rats.

In order to understand the variations in the systemic exposure to ginsenosides following p.o. administration of Sanqi extract, pure ginsenosides and their aglycones were administered as i.v. boluses. As shown in Fig. 3, the systemic exposure levels of the ginsenosides were directly related to their $t_{1/2}$. GRₐ, GR₉, GRc, and GRd had significantly longer $t_{1/2}$ values (7.5–19.8 h) than the other ginsenosides (0.2–3.2 h). The wide variation in $\text{CL}_{\text{tot.p}}$ was the primary reason for the considerable differences in $t_{1/2}$. The tested ginsenosides could be divided into two groups according to $\text{CL}_{\text{tot.p}}$: (1) the 3–5 sugar-containing ppd-type ginsenosides GRₐ, GR₉, GRc, and GRd ($\text{CL}_{\text{tot.p}}$, 4–20 ml/h/kg) and (2) the ppt-type (352–2718 ml/h/kg) and the minor ppd-type ginsenosides (505–2296 ml/h/kg, except for GF₂ being 66 ml/h/kg).
contrast to $CL_{tot,p}$, the $V_{ss}$ values for most ginsenosides fell within the range of 93 ml/kg for GRg3 to 533 ml/kg for GRg1, which was between the rat plasma volume (31 ml/kg) and the rat total body water volume (670 ml/kg) (Davies and Morris, 1993). The $V_{ss}$ value of GF1 was 808 ml/kg. Ppd and Ppt had short $t_{1/2}$ values of 1.5 and 0.2 h, respectively, as well as high $CL_{tot,p}$ (1482 and 6960 ml/h/kg, respectively) and large $V_{ss}$ (2016 and 1716 ml/kg, respectively).

Collectively, the notable differences in systemic exposure among ginsenosides were primarily related to their variations in $CL_{tot,p}$, and the clearance mechanisms were most likely subject to structure-specific recognition. Analysis of the structural features of the compounds suggested that the attachment of 4 for more sugar moieties significantly reduced the rate of elimination. In addition, the sugar attachment site appeared to be also relevant as demonstrated by the significant difference in $CL_{tot,p}$ between the ppd-type GRd and the ppt-type GRe, NGR1, and 20gRf (Fig. 3).

(Insert Figure 3 Here)

Elimination pathways of ginsenosides in rats receiving Sanqi extract. To identify the primary elimination pathways that influenced systemic exposure to the ginsenosides, we evaluated the biliary and renal excretion profiles of the ginsenosides, as well as and the metabolism, in rats. In addition, fecal samples were also analyzed to monitor intestinal nonabsorption, biliary excretion, and/or colonic deglycosylation of ginsenosides. As shown in Fig. 4, the ppd-type ginsenosides (GRa3, GRb1, and GRd) and the ppt-type ginsenosides (NGR1, GRe, and GRg1) exhibited significant differences in excretion profiles. In contrast to the rat systemic exposure levels (as per AUC), the cumulative amounts of the ppt-type ginsenosides excreted intact into bile and urine, corrected according to the corresponding compound doses from Sanqi extract, were significantly greater than those of the ppd-type ginsenosides.
As shown in Fig. 4, the most abundant fecal compounds in rats were GRg₁ (Cum.Aₑ, 39.1 μmol/kg), C-K (20.9 μmol/kg), and GF₁ (7.1 μmol/kg). We speculated that the high levels of these compounds in feces resulted from the colonic microflora stripping the sugar moieties from the ginsenosides in the extract. Their absence (C-K) or presence at a very low level (GF₁) in Sanqi extract supports this hypothesis. The contribution of colonic deglycosylation of major ppt-type ginsenosides to the fecal GRg₁ was supported by two lines of evidence: (1) fecal recovery of GRg₁ following p.o. administration of pure GRg₁ was significantly lower than that following p.o. administration of Sanqi extract (at the same GRg₁ dose) in the same rats, and (2) p.o. administration of pure 20gRf, GRe, and NGR₁ generated fecal GRg₁. Meanwhile, poor intestinal absorption and rapid biliary excretion accounted for the relatively low plasma levels of GRg₁, C-K, and GF₁. Although both of the aglycones Ppd and Ppt were detected in fecal samples following p.o. administration of Sanqi extract, their fecal Cum.Aₑ values were quite low: 0.4 and 0.2 μmol/kg, respectively. Ppd and Ppt were not detected in plasma, bile, or urine, suggesting that they were two minor metabolites in negligible quantities.

To further elucidate the excretion profiles of ginsenosides, individual pure compounds were i.v. administered to rats at 5 μmol/kg. We confirmed that biliary excretion was the major elimination route for most ginsenosides, demonstrating the percentage of i.v. dose excreted intact into bile 43–100%. The ginsenosides were excreted rapidly via active biliary transport, indicative of high bile-to-plasma distribution ratios (AUCₘ₈/AUCₚₐ₉ₐₐₙ, 22–1907). The AUCₘ₈/AUCₚₐ₉ₐₐₙ ratio of GRd was 4.4. However, the biliary excretion of GRa₃, GRb₁, and GRc were slow and their AUCₘ₈/AUCₚₐ₉ₐₐₙ ratios ranged from 0.2 to 0.4. As to renal excretion, most
ginsenosides were slow and passively excreted into rat urine. GRg₃ and monoglycosides were detected in negligible amounts in urine. However, when corrected for plasma protein binding, the unbound CL_R of NRG₁, 20gRf, and GRg₁, 497–1007 ml/h/kg, exceeded the rat glomerular filtration rate of 314 ml/h/kg (Davies and Morris, 1993), suggesting that some active tubular secretion occurred in rats. Figure 5 shows the excretion profiles of GRb₁ and GRg₁, which could represent the two types of typical situations among the tested ginsenosides. About 10% and 40% of i.v. administered GRb₁ were excreted intact into rat bile and urine, respectively, whereas the corresponding fractions for GRg₁ were around 73% or 18%, respectively. The CL_B and CL_R were 1840 and 427 ml/h/kg, respectively, for GRg₁, but only 1.0 and 3.5 ml/h/kg, respectively, for GRb₁.

As to the metabolism of i.v. administered GRb₁, the monooxidized metabolite, the deglycosylated metabolite (GRd), and the monooxidized metabolite of GRd were measured in rat bile and urine (Fig. 5). These metabolic reactions were catalyzed by enzymes in rat tissues such as the liver. About 1.8% and 1.5% of i.v. administered GRb₁ were eliminated very slowly as the tissue-deglycosylated metabolite GRd via bile and urine, respectively, and the values would continue to increase beyond 48 h for bile and 96 h for urine. The tissue-oxidation of GRb₁ appeared to take place faster than the tissue-deglycosylation, but to lesser extent. We estimated that in rats about 10–15% of i.v. administered GRb₁ might be ultimately eliminated via these metabolic pathways. As for i.v. administered GRg₁, only one monooxidized metabolite was detected in rat bile and urine, which accounted for about 0.2% and 0.1% of the dose, respectively. The significantly minimal tissue-metabolism observed for GRg₁ could have resulted from, at least in part, the rapid biliary and renal excretions. Recently, Qian et al. also detected the oxidation and deglycosylation products of GRb₁ in rat
Collectively, rapid and extensive biliary excretion was a key factor limiting the systemic exposure to most ginsenosides from Sanqi extract, which appeared to involve active secretion mechanisms. However, the ppd-type GRα3, GRβ1, GRc, and GRd circumvented or significantly reduced the active secretion into bile. Renal excretion also contributed considerably to the elimination of some ginsenosides including GRα3, GRβ1, GRc, GRd, NRG1, 20gRf, and GRg1, but via different transportation mechanisms. There was considerable deglycosylation of unabsorbed ginsenosides by the colonic microflora, but the colonic metabolism did not significantly improve overall systemic exposure to ginsenosides from orally administered Sanqi extract. The relative contributions of tissue-deglycosylation and tissue-oxidation to overall elimination of ginsenosides appeared to be poor.

**Comparative tissue exposure to i.v. administered GRβ1, GRg1, and C-K.**

To address whether the observed differences in plasma concentration among ginsenosides reflect well the situation in tissues, a comparative study of tissue distribution was performed with GRβ1, C-K, and GRg1, which was based on measurements in whole rat tissue. These three compounds were selected as prototype compounds for the study, because GRβ1 and GRg1 were the ppd-type and the ppt-type ginsenosides, respectively, present in the greatest amounts in Sanqi extract and because their GRβ1, C-K, and GRg1 were the compounds measured in large amounts in rat plasma or excretory samples (Fig. 4). To ease comparisons, each investigational compound was administered as an i.v. bolus of 5 μmol/kg. Following dosing, all compounds transferred rapidly from blood to tissues with T_{max} values of 0.1–2.7 h for different tested tissues. Plasma and tissue concentrations of GRβ1 at 5 min post dose
(C<sub>5min</sub>) were in the rank order of plasma (50 μM) > liver (22 μM) > kidney (19 μM) > heart (16 μM) > lung (12 μM) > bladder (5.1 μM) > spleen (4.7 μM) > testicle (3.8 μM) > large intestine (3.2 μM) > small intestine (2.7 μM) > stomach (2.2 μM) ≈ adipose (1.9 μM) > brain (0.8 μM). The tissue AUC values of GRb<sub>1</sub> versus the corresponding plasma value are shown in Fig. 6.

Consistent with the differences observed for plasma, both the C<sub>5min</sub> and AUC of GRb<sub>1</sub> in the tissues were much greater than the corresponding levels for GRg<sub>1</sub> or C-K. The tissue t<sub>1/2</sub> values of GRb<sub>1</sub> (14–31 h) were comparable with the corresponding plasma data (20 h), which were significantly longer than those of GRg<sub>1</sub> (0.2–1 h; except for the kidney 22 h) or C-K (0.3–6 h). Despite its generally low levels in tissue, GRg<sub>1</sub> exhibited a relatively high C<sub>5min</sub> and AUC in the liver (70 μM and 28 μM·h, respectively) compared with the corresponding plasma data (11 μM and 2 μM·h, respectively). Similar phenomena were observed for C-K: 276 μM and 242 μM·h, respectively, in the liver versus 29 μM and 11 μM·h, respectively, in plasma. The hepatic uptake of GRg<sub>1</sub> and C-K could be associated with the active biliary secretion mediated by the hepatic transporters. In addition, renal uptake of GRg<sub>1</sub> was also extensive, demonstrating a relatively high C<sub>5min</sub> (86 μM) and AUC (99 μM·h) as compared with the corresponding plasma data. In agreement with their unfavorable properties regarding membrane permeability, GRb<sub>1</sub>, GRg<sub>1</sub>, and C-K were poorly delivered to the CNS.

(Insert Figure 6 Here)

Collectively, the significant differences in the plasma levels among ginsenosides could reflect the situations in the tissues. In addition, the tissue distribution data for GRg<sub>1</sub> and C-K support the possible involvement of active secretion mechanisms in the hepatic and/or renal elimination of the compounds.
Discussion

Bioactive constituents with favorable PK properties and existing in adequate abundance in a medicinal herb are most likely to account for the pharmacological effects of the herb and to form the basis of its therapeutic efficacy. Our current and recent studies (Lu et al., 2008) indicate that both animals and humans are exposed significantly to some but not all constituents of an herbal medicine after dosing and that the PK profiles, like the pharmacological activities, can be used as a “sieve” to assess the importance and usefulness of the individual herbal constituents. Such PK studies are essential for understanding the link between the herb consumption and the pharmacological effects and for identifying the medicinal principles from the chemical constituents in an herbal medicine.

In contrast to Western medicines that normally contain only one active ingredient, herbal medicines contain numerous chemical constituents. Multi-component PK studies of an herbal medicine can be challenging. Such studies are complicated by the great diversity of the constituents with regard to both chemical structure and content. Variations in chemical structures result in different physiochemical properties and PK profiles among the constituents, whereas the variations in content result in dissimilarities in constituent dosages. Some important goals for PK studies of an herbal medicine are to (1) measure systemic exposure to the administered medicine, (2) identify suitable “PK markers” indicative of exposure to the medicine, (3) compare the PK properties of the constituents, (4) understand the pathways that influence their systemic exposure, (5) analyze the quantitative structure-PK relationships for the constituents and metabolites, and (6) assess differences in PK properties between the animal species and humans. It is worth mentioning that the constituents in an herbal medicine are often structurally related, which may be divided
into more than one class. Analysis of quantitative structure-PK relationships (QSPKR) of herbal chemicals may gain insight into the role of molecular properties and/or functional group presentation in the PK of compounds, and may help understand the PK trends within the compound series and anticipate which homologues have favorable PK properties compatible with the pharmacological activities. The key role that structure-based differentiation can play in the absorption and disposition of herbal constituents highlights that the QSPKR analysis is a vital component of the multi-component PK study. In the current study, QSPKR analysis helped us identify key factors affecting the systemic exposure to different ginsenosides and their deglycosylated products following administration of Sanqi extract.

The PK properties of constituents contained in an herbal medicine can be quite different. When bioactive constituents from an herb are measurable in a biosample, such as plasma or urine, and have favorable PK properties that could be used to substantiate systemic exposure to the herb, they are referred to as “PK markers” of the herb (Lu et al., 2008). Identification of PK markers for herbs could be helpful in the design and interpretation of toxicity and clinical studies, as well as in the evaluation of potential herb-drug or herb-herb interactions. Here, we measured rat systemic exposure to ginsenosides and their deglycosylated products following p.o. administration of Sanqi extract and compared their PK properties. We found that the ppd-type GRa₃, GRb₁, and GRd measured in plasma were suitable PK markers of p.o. administered Sanqi extract in rats. The conclusion was made on the basis of the dose-dependent systemic exposures and PK properties of these ginsenosides. In contrast, the other plasma ginsenosides, including the ppt-type GRe, NGR₁, and GRg₁ and the metabolite ppd-type C-K, were not the PK markers for Sanqi extract because of their low exposure levels and poor dose proportionality. Although the ppt-type
Ginsenosides were excreted into the rat urine in substantial amounts, large inter-animal variation prevented the use of the urinary compounds as surrogate PK markers. Meanwhile, renal excretion of GRa3, GRb1, GRd, and C-K was very slow.

Poor membrane permeability was a major factor limiting intestinal absorption of ginsenosides, which was attributed mainly to the sugar moieties increasing the hydrogen bond count, polar surface area, and molecular flexibility of the molecules to unfavorable levels. Furthermore, reducing the sugar moiety content appeared not to improve significantly intestinal absorption, because of the associated decrease in solubility. This is supported by our rat data that the fecal Cum.Ae values of the deglycosylated products exceeded substantially the corresponding sum of biliary and urinary Cum.Ae. In addition, the poor permeability could also partially explain our observations in the tissue distribution study. In most cases, the tissue concentrations of ginsenosides were lower than the corresponding plasma levels, and the compounds persisted in the organ capillaries and the organ interstitial fluid, rather than the organ cells. However, the liver concentrations of most ginsenosides were significantly higher than the plasma levels. This can most likely be attributed to some uptake transporters mediating active transport of the ginsenosides into the hepatic cells.

Most of the ginsenosides and their deglycosylated products were subject to rapid extensive biliary excretion through active transport, resulting in their short t1/2 values and low systemic exposure levels. Meanwhile, renal excretion of most ginsenosides is slow and its role in the overall elimination could be minimized by rapid extensive biliary excretion. There were two kinds of exception, i.e., (1) slow but extensive renal excretion because of slow biliary excretion for GRa3, GRb1, and GRc and (2) rapid renal excretion involving active tubular secretion for NRG1, 20gRf, and GRg1. In addition, the contribution of ginsenoside metabolism to their overall elimination was
also limited. The metabolism occurring in rat tissues, such as the liver, included deglycosylation and oxidation. The tissue-deglycosylation was found to be quite slow, while the tissue-oxidation was rapid. Compared with the minor tissue-deglycosylation, the pre-absorption deglycosylation of ginsenosides mediated by the gut microflora appeared to be relatively extensive. Our data (Fig. 4) suggested that the colonic deglycosylation of the ppd-type ginsenosides in rats occurred at C-20 until only one glucose remained, i.e., GRd, and then deglycosylation preferentially took place at C-3, followed by stripping of the last glucose moiety at C-20 to yield the aglycone Ppd. Meanwhile, the initial colonic deglycosylation of the ppt-type ginsenosides appeared to occur preferentially at C-6 to yield GRg1. Further deglycosylation occurred at either C-6 or C-20 to form GF1 or GRh1, respectively, which was followed by stripping of the last glucose moiety at C-20 or C-6, respectively, to yield the aglycone Ppt.

The attachment of four and five sugar moieties in the ppd-type GRa3 and GRb1, respectively, appeared to play a key role in blocking access to the biliary transporters responsible, resulting in slow biliary excretion of the two compounds (CLB: 1.5 and 1.0 ml/h/kg, respectively) in rats. GRd had a significantly slower CLB value (19 ml/h/kg) than the other three-sugar containing ginsenosides, 20gRf, GRe, and NGR1 (258–775 ml/h/kg), suggesting that the sugar attachment sites also influenced the active biliary excretion. In addition, GRa3, GRb1, and GRd underwent limited metabolism and slow renal excretion. Slow elimination of these bulky ppd-type ginsenosides made them long-circulating, which counteracted the unfavorable effect of poor intestinal absorption on their systemic exposure.

GRb1 and GRd have been reported to possess activities associated with the putative cardiovascular effects of Sanqi. GRb1 has estrogenic activity (Lee et al., 2003) via a mechanism independent of estrogen receptor-binding (Cho et al., 2004). GRb1
also has antioxidant activity (Liu et al., 2003). GRd can dilate vascular muscle by blocking Ca$^{2+}$ influx through receptor- and store-operated Ca$^{2+}$ channels in the muscle cells (Guan et al., 2006). The pharmacological activities of GRa$_3$ remain to be understood.

Drug exposure is a crucial determinant of drug response, and therefore its efficacy and safety. In the current study, we compared the systemic exposure to putatively active ginsenosides from p.o. administered Sanqi extract and investigated the relevant mechanisms governing exposure to ginsenosides. In summary, our data indicate that poor membrane permeability and rapid and extensive active biliary excretion are two primary factors limiting systemic exposure to most Sanqi ginsenosides and their deglycosylated metabolites. The major ginsenosides measured in the plasma of rats were long-circulating GRa$_3$, GRb$_1$, and GRd, because of their slow biliary excretion. These plasma ginsenoside can be used as suitable PK markers for Sanqi extract. Considerable colonic deglycosylation occurred but the systemic exposure to the metabolites was low. The PK profiles of the Sanqi ginsenosides in humans were comparable to those found in rats. Slight interspecies differences could have resulted from humans being relatively poor biliary excretors compared with rats. The details of the human study will be reported elsewhere.
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Footnotes

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Fig. 1. Plasma concentration-time profiles of ginsenosides in rats following p.o. administration of Sanqi extract. In the single-dose study, rats received Sanqi extract at 4, 8, or 16 ml/kg. In the multiple-dose study, rats received Sanqi extract at 4 ml/rat/day for 22 days. The right upper panel shows good logarithmic compound dose-exposure relationship among the bulky ppd-type ginsenosides GRa3, GRb1, and GRd, but the doses of other ginsenosides did not correlate with their exposures.

Fig. 2. Relationship between the sugar-substitution in ginsenosides and their PK properties that limit intestinal absorption. GF2 and GF1 exhibited efflux ratios greater than 8 in Caco-2 monolayers.

Fig 3. Relationship between the sugar-substitution in ginsenosides and their disposition profiles in rats following i.v. administrations. The i.v. dose of each individual compound was 5 μmol/kg. The PK parameters were calculated from the plasma concentration-time curves.

Fig. 4. Rat excretion profiles of ginsenosides and their deglycosylated products following p.o. administration of Sanqi extract. The p.o. dose of Sanqi extract was 8 ml/kg. The red numbers 3, 6, and 20 in parentheses indicate the deglycosylation sites in the ginsenosides. Note that the measured urinary Cum.Ae values of C-K, GF1 and GRh1 could be greater than the actual values due to fecal contamination (which contained substantially high amounts of the compounds) during sample collection.

Fig. 5. Rat excretion profiles of i.v. administered GRb1, GRg1, and their metabolites. The i.v. dose of each individual compound was 5 μmol/kg. Both the oxidized (Ox-) and deglycosylated metabolites were measured. GRd was the only measurable deglycosylated metabolite of i.v. administered GRb1, whereas no deglycosylated metabolite of GRg1 was detected in urine or bile following i.v. administration of the
compound.

**Fig. 6.** Tissue distribution profiles of i.v. administered GRb1, GRg1, and C-K in rats. The i.v. dose of each individual compound was 5 μmol/kg. The plasma $V_{ss}$ values for i.v. administered GRb1, GRg1, and C-K in rats were 220, 533, and 441 ml/kg, respectively.
Figure 2
Figure 3
Individual dose from Sanqi extract:

- Plasma: AUC
- Urine: Cum. A
- Feces: Cum. A

- Intraperitoneal injection
- Deglycosylation
- Intestinal absorption

Compd. R1 R2
GRa3 Glc2-Glc Glc6-Glc3-Xyl
GRb1 Glc2-Glc Glc6-Glc
GRc Glc2-Glc Glc6-Ara
GRd Glc2-Glc Glc
GF2 Glc Glc
C-K H Glc
Ppd H H

Compd. R1 R2
GRg1 Glc2-Glc H
GRh2 Glc H

Compd. R1 R2
GRg2 Glc2-Rha H
GRf H Glc
GRh1 Glc H
Figure 5
Figure 6