Combinatorial Metabolism Notably Affects Human Systemic Exposure to Ginsenosides from Orally Administered Extract of Panax notoginseng Roots (Sanqi)\textsuperscript{S}

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ABSTRACT

Ginsenosides are medicinal ingredients of the cardiovascular herb Panax notoginseng roots (Sanqi). Here, we implemented a human study (ChiCTR-ONC-09000603; www.chictr.org) to characterize pharmacokinetics and metabolism of ginsenosides from an orally ingested Sanqi-extract (a 1:10 water extract of Sanqi) and the human plasma and urine samples were analyzed by liquid chromatography-mass spectrometry. Plasma and urinary compounds derived from ginsenosides included: 1) intestinally absorbed ginsenosides Ra3, Rb1, Rd, F2, Rg2, and notoginsenoside R1; and 2) the deglycosylated triol, and their oxidized metabolites. The systemic exposure levels of the first group compounds increased as the Sanqi-extract dose ingested Sanqi-extract (a 1:10 water extract of Sanqi) and the human plasma and urine samples were analyzed by liquid chromatography-mass spectrometry. Plasma and urinary compounds derived from ginsenosides included: 1) intestinally absorbed ginsenosides Ra3, Rb1, Rd, F2, Rg2, and notoginsenoside R1; and 2) the deglycosylated triol, and their oxidized metabolites. The systemic exposure levels of the first group compounds increased as the Sanqi-extract dose increased, but those of the second group compounds were dose-independent. The oxidized metabolites of 20(S)-protopanaxadiol and 20(S)-protopanaxatriol represented the major circulating forms of ginsenosides in the bloodstream, despite their large interindividual differences in exposure level. The metabolites were formed via combinatorial metabolism that consisted of a rate-limiting step of ginsenoside deglycosylation by the colonic microflora and a subsequent step of sapogenin oxidation by the enterohepatic cytochrome P450 enzymes. Significant accumulation of plasma ginsenosides and metabolites occurred in the human subjects receiving 3-week sub-chronic treatment with the Sanqi-extract. Plasma 20(S)-protopanaxadiol and 20(S)-protopanaxatriol could be used as pharmacokinetic markers to reflect the subjects’ microbial activities, as well as the timely-changes and interindividual differences in plasma levels of their respective oxidized metabolites. The information gained from the current study is relevant to pharmacology and therapeutics of Sanqi.

Introduction

In China, the tradition of herbal medicine has prospered over millennia and is still accepted by physicians, patients, and the regulatory authority. However, the scientific standards for accepting the value of an herbal product as a medicine fall below the existing requirements for establishing the value of synthetic drugs. China has announced an ambitious attempt to bring the practice of traditional medicine into line with modern standards (Qiu, 2007).

Phytochemists traditionally work in concert with ethnopharmacologists in herbal medicine research, where whole-herb extracts, as well as the individual compounds isolated and purified from the herbs, are applied to in vivo and/or in vitro pharmacological assessments. However, the studies often fail to address which chemical ingredients present are the medicinal principles responsible for the pharmacological effects of the investigative herbal medicine. The most abundant constituents present in an herbal medicine do not necessarily produce the highest in vivo concentrations after dosing (such as plasma concentrations, tissue concentrations, or the like), while in vivo measured herbal metabolites are usually absent in the dosed medicine (Lu et al., 2008; Liu et al., 2009; Li, 2012; Li et al., 2012; Yan et al., 2012). In other words, there can be significant differences in nature and relative levels between the chemical ingredients present in an herbal medicine and the botanical compounds circulating after dosing. Accurate and complete information on pharmacokinetics and metabolism is vital to pharmacological research for identification of the medicinal principles. Accordingly, pharmacokinetic (PK) scientists are needed in herbal medicine research to work with ethnopharmacologists and phytochemists.

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ABBREVIATIONS: AUC\textsubscript{0-\textit{t}}, area under concentration-time curve to the last measured time point; CLR, renal clearance; C\textsubscript{max}, maximum plasma concentration; Cum\textsubscript{A\textsubscript{r}}, cumulative amount excreted; HLM, human liver microsomes; MRT, mean residence time; P450, cytochrome P450; PK, pharmacokinetic; PO, per oral; ppt-type, 20(S)-protopanaxadiol type; ppt-type, 20(S)-protopanaxatriol type; T\textsubscript{max}, time taken to achieve the maximum concentration.

1457
in a manner similar to that for drug discovery. Different from early practice based only on pharmacological assessment of a range of chemicals, current drug discovery is normally achieved by combining medicinal chemistry and pharmacology together with pharmacokinetics, which has resulted in improved candidate selection for preclinical and clinical development (Pritchard et al., 2003). PK and metabolism studies of an herbal medicine tell the ethnopharmacologists which herb-derived compounds are worth further evaluation.

Sanqi (the dried root of *Panax notoginseng*, family Araliaceae) has been commonly used in Chinese traditional medicine to eliminate blood stasis, to arrest bleeding, to cause subsidence of swelling, and to alleviate pain (Chinese Pharmacopoeia Commission, 2010). The medicinal herb is usually administered orally in such forms as powders, aqueous extracts, and teas, in daily dose ranging from 2 g to 30 g of root but averaging 3–9 g. In addition, injections prepared from Sanqi-extract have also been approved for clinical use in China. The triterpene saponins ginsenosides are believed to be the pharmacologically active ingredients of Sanqi and can be classified according to their structures as 20(S)-protopanaxadiol type (ppd-type) and 20(S)-propanaxatriol type (ppt-type) (Christensen, 2009; Lü et al., 2009). The major saponins present in Sanqi include the ppd-type ginsenosides Rα3, Rb1, and Rd and the ppt-type ginsenosides Re, Rg1, and notoginsenoside R1. Recently, we investigated intestinal absorption and disposition of ginsenosides in rats receiving an orally (PO) administered Sanqi-extract and found that poor membrane permeability, rapid biliary excretion, and colonic microflora-induced deglycosylation were the key factors affecting the rat systemic exposure to ginsenosides (Liu et al., 2009). The major ginsenosides measured in rat plasma were long-circulating ppd-type ginsenosides Rα3, Rb1, and Rd, which can be used as pharmacokinetic markers (PK markers) to substantiate rat systemic exposure to the Sanqi-extract. Meanwhile, ppt-type ginsenosides Re, Rg1, and notoginsenoside R1, as well as the deglycosylated products of both the types, were subject to rapid biliary excretion that limited their systemic exposure levels. Interspecies differences in biliary excretion (Mahmood and Sahajwalla, 2002; Lai, 2009) and colonic microflora-induced metabolism (Nicholson et al., 2005; Sousa et al., 2008) complicate the extrapolation to human clinical data. We implemented a human study to characterize pharmacokinetics and metabolism of ginsenosides from an orally ingested Sanqi-extract. One of our important findings is that several metabolites derived from ginsenosides were measured as major circulating compounds after dosing. These metabolites, absent in the administered herbal extract, were formed via combinatorial metabolism that consisted of a rate-limiting step of ginsenoside deglycosylation induced by the colonic microflora and a subsequent step of sapogenin oxidation mediated by the enterohepatic cytochrome P450 enzymes (P450 enzymes).

**Materials and Methods**

A detailed description of materials and experimental procedures is provided in the Supplemental Data, Materials and Methods.

**Materials.** Sanqi-extract, for PO administration to human subjects, was a 1:10 water extract, i.e., 2 kg of dried roots of *Panax notoginseng* (Sanqi) was used to make 20 l of the final extract. The prepared Sanqi-extract contained ginsenosides Rg1 (2680 μM), Rb1 (1377 μM), Rd (366 μM), Re (254 μM), Rb1 (103 μM), Rα3 (76 μM), Rg2 (60 μM), F1 (48 μM), F2 (18 μM), Fg3 (17 μM), Re (14 μM), Rf (13 μM), Rc (3 μM), and Rb2 (1 μM), as well as notoginsenosides R1 (360 μM), Rg5/Rg6 (69 μM), and 20-gluco-notoginside Rf (33 μM). Only one batch of Sanqi-extract was prepared and used for the whole study. The content levels of ginsenosides in Sanqi-extract were measured immediately after preparation and after storage at 4°C for 1 and 3 months. Chemical stability was defined as the retention of ≥85% of the initial levels. The herb extract was used after shaking well to ensure thorough mixing.

Purified ginsenosides 20(S)-protopanaxadiol and 20(S)-protopanaxatriol were obtained from the National Institutes for Food and Drug Control (Beijing, China) or other commercial sources, the purity of which exceeded 98%. Pooled human liver microsomes (HLM) of Chinese origin were obtained from the Research Institute for Liver Diseases (Shanghai, China). Single cDNA-expressed human cytochrome P450 enzymes were obtained from BD Gentest (Woburn, MA).

**Human Study.** The protocol for human study was approved by the Ethics Committee of Clinical Investigation at the 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-ONC-09000603. The informed consent was obtained from each of twelve male and twelve female healthy volunteers (22–26 years).

A two-period, open-label, single-center study was performed at the National Clinical Research Center of the hospital (Tianjin, China; Fig. 1). In period 1, all the subjects were randomly assigned to one of three dosage groups, with four male and four female subjects per group. Each subject was asked to fast overnight before receiving a single PO dose of Sanqi-extract (90, 180, or 270 ml/subject) the next morning. The low dose level was derived from the dose

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**Fig. 1.** Flowchart for the two-period, open-label, single-center human study. Twenty-four subjects were randomly assigned to one of three dosage groups (four males and four females per group). After the random assignment, the subjects were renumbered before receiving a PO dose of Sanqi-extract at 90 (the male subjects m1–m4 and the female subjects f1–f4), 180 (m5–m8 and f5–f8), or 270 ml/subject (m9–m12 and f9–f12). In addition, the subjects m1–m4, after 3-day wash-out period following the first acute PO treatment with Sanqi-extract, continued to receive multiple PO doses of the herb extract at 90 ml/subject per day for 3 weeks. Their blood and urine samples were collected on days 11, 18, and 24. HF/RF, hepatic function and renal function.
level recommended by the Pharmacopoeia of the People’s Republic of China. The intermediate and high dose levels were also used clinically. Serial blood samples (~1 ml collected in heparinized tubes) were taken from an antecubital vein catheter at 0, 0.5, 1.5, 3, 4.5, 8, 12, 15, 24, 30, 38, 48, and 56 hours after dosing. The blood samples were then centrifuged at 3000g for 5 minutes, and the resulting plasma fractions were frozen at −70°C until analysis. Meanwhile, serial urine samples were also collected pre-dose and at 0–3, 3–6, 6–10, 10–14, 14–24, 24–32, 32–40, 40–48, and 48–72 hours postdose, and weighed prior to storage at −70°C without use of any preservative. In period 2 after 3-day wash-out period following the first acute PO treatment with Sanqi-extract, the four male subjects of the low dose group continued to receive a subchronic PO treatment with Sanqi-extract at 90 ml/subject per day for 3 weeks. On days 11, 18, and 24 of period 2, the blood and urine samples were collected according to the preceding time schedules, except for sampling on days 11 and 18, which were only performed 0–24 hours after the dosing. The hepatic and renal function of subjects was monitored before the single dose of Sanqi-extract of period 1 and 72 hours after dosing, as well as on days 4, 12, 15, 19, 20, 21, 22, 23, and 25 (before the daily dose of Sanqi-extract) of period 2. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin/globulin (A/G) ratio, total bilirubin (TBil), and direct bilirubin (DBil) were monitored as liver function markers for the human subjects, while serum creatinine (CRE) and blood urea nitrogen (BUN) were also assessed to monitor renal function. The reference ranges indicating normal liver or kidney function were: ALT, 0–40 IU/l; AST, 0–40 IU/l; TP, 64–87 g/l; A/G ratio, 1.2–2.5; TBil, 0–17.1 μM; DBil, 0–8.0 μM; CRE, 22–106 μM; and BUN, 1.7–8.3 mM.

In Vitro Metabolism Studies. Pure 20(S)-protopanaxadiol, 20(S)-protopanaxatriol, and compound-K at 2 μM were incubated with HLM in presence of the cofactor(s) NADPH, uridine 5′-diphospho-glucuronic acid (UDPGA), or both to assess metabolic stability and to support metabolite characterization. A variety of cDNA-expressed human P450 enzymes (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5) were used for the enzyme identification. To determine the enzyme kinetics, 20(S)-protopanaxadiol (1.56–200 μM) or 20(S)-protopanaxatriol (0.08–200 μM) were incubated with NADPH-fortified HLM under linear metabolite formation conditions performed in duplicate in 96-well plates.

Liquid Chromatography-Mass Spectrometry Analyses. An AB Sciex API 4000 Q Trap mass spectrometer (Toronto, Canada) interfaced via a Turbo V ion source with a Waters Acuity UPLC separation module (Milford, MA) was used for metabolite profiling and quantification for study purposes. The human plasma and urine samples (50 μl) were processed with 150 μl of methanol before measurement. Meanwhile, in vitro metabolism study samples (100 μl) were treated with an equal volume of methanol to stop the reaction before analysis. Validation of the quantification assays with the reference standards available were implemented according to the US Food and Drug Administration guide on bioanalytical method validation (http://www.fda.gov/cder/guidance/index.htm) to demonstrate that their performance characteristics were reliable for the intended use.

Data Analysis. PK parameters were determined by noncompartmental method using Kinetic 5.0 (Thermo Fisher Scientific, Philadelphia, PA) and shown as means ± standard deviation. Dose proportionality assessment was conducted by the regression of log-transformed data (the Power Model) with the criterion that was calculated according to the method of Smith et al. (2000). Michaelis constant (Km) and maximum velocity (Vmax) values were determined by nonlinear regression analysis using the Michaelis-Menten equation (rate of metabolism formation as a function of substrate concentration) using GraFit software (version 5; Eirihacus Software Ltd., Surrey, UK). The ratio of Vmax and Km was used to calculate in vitro intrinsic clearance (CLint). The Shapiro-Wilk test of normality was used to check the distribution shape of systemic exposure levels of ginsenoside-derived compounds in area under concentration-time curve to the last measured time point (AUC0-t). The Kendall’s tau-b coefficients were calculated using PASW Statistics, Version 18, software (SPSS Inc., Chicago, IL) to estimate whether the systemic exposure levels of a certain ginsenoside-derived compound were positively or negatively correlated to those of another ginsenoside-derived compound. Correlation was statistically significant at P < 0.01 level (two-tailed).

Results

Safety of Sanqi-Extract. No serious adverse event was observed during the human study. After a PO dose of Sanqi-extract at 90, 180, or 270 ml/subject, none of the liver or kidney function indicators exceeded the upper normal levels. During the subsequent multiple dose treatment, the four male subjects (m1–m4) receiving Sanqi-extract at 90 ml/subject per day had hepatic or renal function indicators within the normal ranges during the study period, except for some transient elevation of ALT for subjects m2–m4. Subject m3 had elevated ALT (54–79 IU/l) on days 15–22. This subject withdrew from the study after day 18 to let his elevated ALT return to the normal level (<40 IU/l).

Appearance of Plasma and Urine Ginsenosides and Metabolites after Oral Administration of Sanqi-Extract. Ginsenosides and their deglycosylated products occurred in human plasma and urine after a PO dose of Sanqi-extract (270 ml/subject) and were compared with the profiles of the control plasma and urine samples before dosing. As shown in Fig. 2B, Ppd-type ginsenoside Rb1 was the most abundant plasma Sanqi saponin absorbed, while other saponins measured in plasma were ppt-type ginsenosides Rg3, Rh2, as well as ppt-type ginsenosides Re, Rg2, F1, Rg1h, and notoginsoside R1. These plasma ginsenosides were also excreted into urine (Fig. 2C), except ginsenosides Rg3, Rh2. Meanwhile, 20-gluco-ginsenoside Rf and notoginsenoside R23E were measured in urine but not in plasma. Although ppt-type ginsenoside Rg1 had a higher content level in the administered Sanqi-extract than ginsenoside Rb1, its plasma abundance was significantly lower than that of the ppd-type ginsenoside (Fig. 2B). However, urinary ginsenoside Rg1 was the most abundant of all the ginsenosides excreted into urine (Fig. 2C). Notably, the deglycosylated products compound-K, 20(S)-protopanaxadiol, and 20(S)-protopanaxatriol, absent in the ingested Sanqi-extract (Fig. 2A), also occurred substantially in plasma with 20(S)-protopanaxatriol, being ~3 more abundant than plasma ginsenoside Rb1 (Fig. 2B).

Although 20(S)-protopanaxadiol and 20(S)-protopanaxatriol circulate significantly in the bloodstream, they are not measured in the urine samples. To understand the elimination of the deglycosylated products, their metabolism was further assessed. A total of 29 metabolites of the deglycosylated products were detected in the plasma samples (Fig. 2D). Plasma M8, M11, and M12 had their abundances 1.2–11 times higher than 20(S)-protopanaxatriol, while the abundances of plasma M3–M7, M9, M10, and M13–M22 were 7–72% of that of the sapogenin. Meanwhile, a total of 29 urinary metabolites were measured (Fig. 2E). The plasma metabolites M17–M22 were absent in urine, while the urinary metabolites M30 and M31–M35 were not measured in plasma. The most abundant metabolites in urine were M8 and M6, i.e., 131% and 90% of urinary ginsenoside Rg1, respectively, while urinary M3, M5, M7, M11–M13, M23–M25, M27, M29, and M35 were only 10–43%. It is worth mentioning that the preceding plasma and urine metabolites (M1–M35) were absent in the administered Sanqi-extract. As to the ginsenosides present in Sanqi-extract, their oxidized or conjugated metabolites were not measured in the human samples.

In Vitro Metabolism of 20(S)-Protopanaxadiol, 20(S)-Protopanaxatriol, and Compound-K. To help characterize the preceding in vivo metabolites (M1–M35), in vitro metabolism studies were implemented with HLM. 20(S)-Protopanaxadiol or 20(S)-protopanaxatriol were labile when incubated with NADPH-fortified HLM (in vitro 1/2, 6 and 4 minutes, respectively; Supplemental Table 1). Furthermore, both the sapogenins were quite stable with UDPGA-fortified HLM (>120 minutes). These data suggested that the oxidation of the sapogenins was rapid in the liver, while the glucuronidation was relatively slow.
Compound-K was slowly metabolized with the human microsomal enzymes (>120 minutes). According to the in vitro data (Supplemental Table 1), M16–M22, M34, and M35 were characterized as the oxidized metabolites of 20(S)-protopanaxadiol, while M3–M15, M23–M29, and M31–M33 were derived from 20(S)-protopanaxatriol. M1, M2, and M30 were the metabolites of compound-K. Fig. 3 depicts the proposed metabolic pathways of the deglycosylated products in the humans.

Humans CYP3A4 and CYP3A5 were the major enzymes responsible for the oxidation of 20(S)-protopanaxadiol or 20(S)-protopanaxatriol (Fig. 4). In addition, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 could also mediate the oxidation of 20(S)-protopanaxadiol. The Michaelis constants of HLM toward the formation of M20/M21/M22 from 20(S)-protopanaxadiol and those for M8/M10/M11/M12 from 20(S)-protopanaxatriol were 4.0–6.6 and 0.2–1.1 \( \mu M \), respectively (Supplemental Table 2).

**Plasma Pharmacokinetics and Urinary Excretion of Ginsenosides and their Major Metabolites after Oral Administration of Sanqi-extract.** Fig. 5 shows plasma level-time profiles of the major ginsenosides after a PO dose of Sanqi-extract in human subjects, as well as those of the deglycosylated products (compound-K, 20(S)-protopanaxadiol, and 20(S)-protopanaxatriol) and the oxidized...
metabolites of the sapogenins, while the plasma PK parameters are summarized in Supplemental Tables 3–5. After dosing at 270 ml/subject, the plasma concentrations of ppd-type ginsenosides Ra3, Rb1, and Rd increased up to maximum plasma concentration ($C_{\text{max}}$) 3.1, 27.9, and 9.3 nM, respectively, with $T_{\text{max}}$ of 8–12 hours and then slowly decreased with $t_{1/2}$ of 33–57 hours. Their mean-residence-time (MRT) values were 50–82 hours. The $T_{\text{max}}$ and MRT of ppd-type ginsenoside F2 were 13 hours and 22–29 hours, respectively. The renal excretion of ginsenosides Ra3, Rb1, Rd, and F2 was slow with renal clearance ($CL_R$) of 0.01–0.04 l/h and their urinary cumulative amount excreted (Cum.Ae(0→72h)) accounted for only ~0.01% of the compound doses from the PO-ingested Sanqi-extract. The AUC$_{0–56h}$ of ginsenosides Ra3, Rb1, Rd, and F2 increased in Sanqi-extract dose-related manners (90–270 ml/subject), but the dose proportionality was inconclusive (Fig. 6). Compound-K exhibited a significantly higher mean AUC$_{0–56h}$ than ginsenoside Rb1, demonstrating 4749 versus 749 nM·h after dosing at 270 ml/subject, while the former’s interindividual difference in AUC$_{0–56h}$ was significantly larger than that of the latter. Meanwhile, compound-K had a $T_{\text{max}}$ of 10–30 hours and an MRT of 23–26 hours. The average $CL_R$ value of compound-K was very low (0.01 l/h). Significant interindividual difference in plasma occurrence was also observed with 20(S)-protopanaxadiol. The AUC$_{0–56h}$ value of compound-K or 20(S)-protopanaxadiol correlated poorly with the dose of Sanqi-extract.

**Fig. 3.** Proposed metabolism pathways of compound-K, 20(S)-protopanaxadiol, and 20(S)-protopanaxatriol in humans. The metabolites in brackets are proposed intermediates, which were not measured in the samples of human plasma (HP) or human urine (HU). The metabolites M1, M2, and M3 were the glucuronides of M20, M21, and M22, respectively, which were measured in the samples of rat bile (RB) after i.v. administration of 20(S)-protopanaxadiol. Black and red colors denote metabolites of Ppd-type ginsenosides and Ppt-type ginsenosides, respectively.
In addition, compound-K or 20(S)-protopanaxadiol were correlated poorly with ginsenosides Rα3, Rb1, Rd, and F2 in AUC0–7h for the individual subjects (Fig. 7). Compound-K was also correlated poorly with 20(S)-protopanaxadiol.

At the Sanqi-extract dose 270 ml/subject, ppt-type ginsenoside Rg1 and notoginsenoside R1 had \( T_{\text{max}} \) of 6–7 hours and \( C_{\text{max}} \) values of 10.2 and 1.4 nM, respectively. Their MRT values were 7–13 hours. The renal excretion of these ppt-type ginsenosides was rapid with CLR of 5.6 and 4.0 l/h, respectively, while their urinary Cum.Ae(0–24h) accounted for 0.06% of the compound doses from Sanqi-extract. Unlike synthetic drugs, herbal medicines are complex chemical mixtures. Benefits of herb therapy are normally based on a range of

Discussion

Unlike synthetic drugs, herbal medicines are complex chemical mixtures. Benefits of herb therapy are normally based on a range of
activities of compounds working together. Accordingly, absorption, distribution, and metabolism (ADME)/PK study of an herb medicine involves investigation of multiple botanical compounds to link the medicine administration and the medicinal benefits. An earlier systematic ADME/PK study of a Sanqi-extract was performed in rats (Liu et al., 2009), which provided the indispensable guidance for us to implement strategically this follow up multicompound human PK study.

Similar to the situation in rats, the ppd-type ginsenosides Ra3, Rb1, and Rd from PO dosed Sanqi-extract had long elimination half-lives (Liu et al., 2009), which provided the indispensable guidance for us to implement strategically this follow up multicompound human PK study.

![Mean plasma concentration-time profiles of ginsenosides and their major metabolites after a single PO dose of Sanqi-extract at 90 (green solid lines), 180 (blue solid lines), or 270 ml/subject (red solid lines). The subject f12 (receiving Sanqi-extract at 270 ml/subject; red dashed lines) exhibited marked extensive but delayed formation of 20(S)-protopanaxadiol and 20(S)-protopanaxatriol, and their oxidized metabolites.](Fig. 5)
(>30 hours) in the human subjects and significant accumulation in AUC0–24h during the multiple-dose treatment. Their systemic exposure levels increased in a Sanqi-extract dose-dependent manner (90–270 ml/subject). These plasma ginsenosides were considered to be Sanqi-extract dose-dependent PK markers to substantiate human systemic exposure to the oral herb extract. Unlike the situation in rats, the human systemic exposure levels of the ppt-type ginsenoside Rg1, and notoginsenoside R1 and the ppd-type ginsenoside F1 increased in Sanqi-extract dose-dependent manners. The term “r” denotes the correlation coefficient. The ratio of dose-normalized geometric mean AUC values is given before the parentheses, while the numbers in the parentheses are the statistically derived 90% confidence interval. The critical interval was 0.797–1.203.

Fig. 6. AUC0–56h values of compound-K, 20(S)-protopanaxadiol, 20(S)-protopanaxatriol, the ppt-derived metabolites (M16, M17, and M19–M22), and the ppt-derived metabolites (M4–M8 and M10–M15) being Sanqi-extract dose-independent. The exposure values were measured in human subjects (solid circles, the male subjects; open circles, the female subjects) receiving a PO dose of Sanqi-extract at 90 (green circles), 180 (blue circles), or 270 ml/subject (red circles). Unlike the metabolites’ situations, AUC0–56h values of ginsenoside Ra3, Rb1, Rd, F2, Rg1, and notoginsenoside R1 increased in Sanqi-extract dose-dependent manners. The term “r” denotes the correlation coefficient. The ratio of dose-normalized geometric mean AUC values is given before the parentheses, while the numbers in the parentheses are the statistically derived 90% confidence interval. The critical interval was 0.797–1.203.
F₂ also increased in Sanqi-extract dose-related manners. Poorer biliary excretion in humans than in rats (Mahmood and Sahajwalla, 2002; Lai, 2009) likely accounted for the interspecies differences. Accordingly, plasma ginsenosides Rg₁, F₂ and notoginsenoside R₁ could also be used as Sanqi-extract dose-dependent PK markers in humans.

The most important finding of this human study was the substantially measured plasma metabolites that resulted from the combinatorial metabolism of ginsenosides (consisting of the saponin deglycosylation by the colonic microflora and the subsequent sapogenin oxidation by the enterohepatic P450 enzymes). Deglycosylation of ginsenosides via action of the colonic microflora has been demonstrated in vitro and in

![Fig. 7. Strong positive correlations existing in AUC₀–56 h between 20(S)-protopanaxadiol and its oxidized metabolite (M16, M17, M19, M20, M21, or M22), between 20(S)-protopanaxatrol and its oxidized metabolite (M4, M5, M6, M7, M8, M10, M11, M12, M13, M14, or M15), and between 20(S)-protopanaxadion and 20(S)-protopanaxatrol in the human subjects (solid circles, the male subjects; open circles, the female subjects) receiving a single PO dose of Sanqi-extract at 90 (green circles), 180 (blue circles) or 270 ml/subject (red circles). Meanwhile, no significant correlation occurred in AUC₀–56 h between compound K and ginsenoside Ra₃, Rb₁, Rd, or F₂, between 20(S)-protopanaxadiol and ginsenoside Ra₃, Rb₁, Rd, or F₂, between 20(S)-protopanaxatrol and notoginsenoside R₁, ginsenoside Rg₁, or F₁. r, correlation coefficient.](downloaded_from_dmd.aspetjournals.org_at_ASPET_Journals_on_April_20,_2017)
vivo for years (Hasegawa et al., 1996; Tawab et al., 2003; Yang et al., 2006) and the human P450-mediated oxidation of the sapogenins has also been assessed in vitro recently (Kasai et al., 2000; Hao et al., 2010; Li et al., 2011). Unlike the situation in humans, these metabolites were poorly detected in rat plasma after PO dose of Sanqi-extract (Liu et al., 2009). The human colon is heavily bacterial-colonized as compared with the rat colon (Sousa et al., 2008), which likely accounts for the observed metabolism interspecies difference. The amount of variation in the colonic microflora is considerable between human individuals (Nicholson et al., 2005). In this study, we observed marked interindividual differences in formation of the deglycosylated compound-K, 20(S)-protopanaxadiol, and 20(S)-protopanaxatriol. Notably, the systemic exposure levels of oxidized metabolites of the sapogenins, regardless of the sex of subject or the Sanqi-extract dose level, were well correlated with those of the sapogenins, rather than the levels of the ginsenosides absorbed (Figs. 7 and 9). The colonic microflora-mediated deglycosylation appeared to be the rate-limiting step in the combinatorial metabolism of ginsenosides. As shown in Fig. 5, although the $T_{\text{max}}$ values of compound-K, 20(S)-protopanaxadiol, and 20(S)-protopanaxatriol were delayed ~20 hours as compared with those of the ginsenosides, these deglycosylated products and their oxidized metabolites were almost identical in $T_{\text{max}}$. Even if the gastrointestinal transit time (normally 4–5 hours) to colon is taken into consideration, these data still suggested that the deglycosylations were significantly slower than the enterohepatic oxidations. In addition, the deglycosylation activities of the colonic microflora appeared to be induced by the
unabsorbed ginsenosides during the subchronic treatment with Sanqi-extract. Despite the structural similarity, multiple P450 enzymes mediated the oxidation of 20(S)-protopanaxadiol, whereas only CYP3A4 and CYP3A5 were implicated in the metabolism of 20(S)-protopanaxatriol. This finding suggests that the 20(S)-protopanaxadiol oxidation is relatively refractory to the effects of P450-inhibitors. The oxidized metabolites from the sapogenins were likely cleared via renal excretion and/or glucuronidation-associated biliary excretion (Supplemental Fig. 1).

Besides the preceding dose-dependent PK markers, we further proposed two other concepts of dose-independent PK markers for Sanqi-extract. The colonic microflora mediating the ginsenoside deglycosylation played a key role in the combinatorial metabolism. Plasma compound-K, 20(S)-protopanaxadiol, and 20(S)-protopanaxatriol could be used as
PK markers to reflect the interindividual differences in deglycosylation activities of the colonic microflora. This proposal is also supported by in vitro formation of these deglycosylated products from ginsenosides via action of the human fecal microflora under anaerobic conditions (Hasegawa et al., 1996). In addition, the plasma concentration of 20(S)-protopanaxadiol and the plasma levels of oxidized metabolites (M16–M22) concomitantly increased up to maxima and then decreased with almost the same $T_{max}$ and MRT values. The same situation also occurred with 20(S)-protopanaxatriol and its oxidized metabolites (M4–M8 and M10–M15). Significant correlations in AUC (Figs. 7 and 9) between 20(S)-protopanaxadiol and the ppd-derived metabolites and those between 20(S)-protopanaxatriol and the ppt-derived metabolites suggested that the plasma sapogenins could be used as PK markers to reflect the timely-changes and interindividual differences in plasma levels of their respective oxidized metabolites. In pharmacological studies of herbal medicines, the intensity of pharmacological response was often found to be correlated poorly with the dose. Unlike the absorbed ginsenosides, the plasma levels of the ppd-derived or ppt-derived metabolites were dependent on the human individual’s deglycosylation activities of colonic microflora rather than the Sanqi-extract dose. The information gained and the associated PK markers identified may be implicated when the dose-response relationship is assessed for Sanqi in humans.

Poor oral bioavailability of many herbal constituents is often associated with their low levels of systemic exposure, which impedes translation of many in vitro pharmacological activities of the compounds to the therapeutic effects of herbal medicines (Zhang et al., 2012). However, we found that ppd-type ginsenosides Ra3, Rb1, and Rd from a PO-ingested Sanqi-extract had considerably high plasma levels in rats despite their oral bioavailability (Liu et al., 2009). These ppd-type ginsenosides were long-circulating because their elimination kinetics was associated with high metabolic stability and slow biliary and urinary excretion. In this human study, we found that the deglycosylated products compound-K, 20(S)-protopanaxadiol, 20(S)-protopanaxatriol, and their many oxidized metabolites had high plasma levels and accumulated substantially with repeated doses of Sanqi-extract. Assessing the pharmacological activities of these herbal compounds with significant exposure levels after dosing is important for evaluation of oral Sanqi’s therapeutic values. Accordingly, additional studies are planned to make pharmacological assessments of the herbal metabolites found in this study.

Considerable hepatotoxicity and nephrotoxicity were observed in rats receiving multiple PO doses of “notoginseng total saponin” (containing ginsenosides Rg1, Rb1, Rd, Re, and notoginsenoside R1) at 1600 mg/d per kilogram for 13 weeks (Ma and Dai, 2011). Our earlier rat Sanqi study indicated that the major ppt-type ginsenosides Rg1, Re, and notoginsenoside R1, as well as the ppd-type compound-K, were subject to rapid biliary excretion via an active transport mechanism and had high liver levels (Liu et al., 2009). In this study, no significant hepatotoxicity and nephrotoxicity were observed for the human subjects receiving oral Sanqi-extract, except for the subject m3. Although this subject had obviously increased ALT during the multiple-dose treatment, his plasma levels of ginsenosides and the metabolites were not high as compared with the other subjects. According to dose normalization by body surface area (Reagan-Shaw et al., 2008, the daily doses (800–1600 mg/kg) in the rat study by Ma and Dai (2011) were about 20–40 times greater than the daily dose for our subchronic treatment of the human subjects m1–m4 with Sanqi-extract (90 ml/subject). Although the potential effects of ginsenosides and their metabolites on the hepatic and renal function need to be confirmed by more studies, it is nonetheless wise to exercise caution when high dose levels of Sanqi-extracts or the associated pharmaceutical products are administered for a long period.

In pharmacological assessment of an herbal medicine, it is important to understand not only its chemical content but also which herb-derived compounds circulate considerably in the bloodstream after dosing. In this study, notable differences were found between the circulating compounds derived from ginsenosides after PO ingestion of Sanqi-extract and the ginsenosides present in the extract. Our study provides information for the ethnopharmacologists on which Sanqi-derived compounds are worth further evaluation, while it also tells the phytochemists which Sanqi-derived compounds should be prepared for the pharmacological and safety assessments. In summary, 20(S)-protopanaxadiol, 20(S)-protopanaxatriol, and their oxidized metabolites, as well as compound-K, represent major circulating forms of ginsenosides in the human bloodstream after PO ingestion of Sanqi-extract. However, the systemic exposure levels of these metabolites are of large interindividual variance, which depends on the deglycosylation activities of the colonic microflora of individual subjects, rather than the Sanqi-extract dosage size. Compound-K, 20(S)-protopanaxadiol, and 20(S)-protopanaxatriol measured in human plasma can be used as PK markers to reflect the microbial activities of human individuals. In addition, plasma 20(S)-protopanaxadiol and 20(S)-protopanaxatriol can also serve as PK markers to indicate the timely-changes and interindividual differences in plasma levels of their respective oxidized metabolites. The ppd-type ginsenosides Ra3, Rb1, Rd, and F2, as well as the ppt-type ginsenosides Rg2 and notoginsenoside R1, can be used as Sanqi-extract dose-dependent PK markers to substantiate human systemic exposure to the orally ingested extract. Most plasma ginsenosides, as well as their metabolites, accumulate substantially in the systemic circulation during multidose treatment with oral Sanqi-extract.

**Authorship Contributions**

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**Conducted experiments:** Hu, Yang, Cheng, Huang, Du, Wang, Niu, Xu.

**Performed data analysis:** Li, Hu, Yang, Cheng, Jiang.

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**References**


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