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Ultra-Performance Liquid Chromatography and Time-of-Flight Mass Spectrometry Analysis of Ginsenoside Metabolites in Human Plasma

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Abstract

American ginseng is a commonly used herbal medicine in the United States. When ginseng is taken orally, its active components, ginsenosides, are reportedly biotransformed by intestinal microbiota. Previous pharmacokinetic evaluations of ginseng in humans have focused on its parent constituents. However, the metabolites, especially those transformed by intestinal microbiota, have not been carefully studied. We used an ultra-performance liquid chromatography/time-of-flight mass spectrometry (UPLC/TOF-MS) method to determine 15 ginsenosides and/or metabolites and their bioavailability in humans. Six healthy human subjects received a single oral dose of 10 g of American ginseng root powder, after which samples of their blood were collected at 0, 2, 4, 7, 9 and 12 h for measurement of ginsenoside/metabolite levels in plasma. Ginsenosides Rb1, Rd, Rg2 and compound K (C-K) were detected in human plasma samples at different time points. The Rb1 concentration peak was 19.90 ± 5.43 ng/ml at 4 h. C-K was detected from 7 h to 12 h with 7.32 ± 1.35 ng/ml at 12 h. Since the last time point was at 12 h, C-K peak level was not observed. The areas under the concentration curves (AUC) from 0 to 12 h were 155.0 ± 19.5 ng•h/ml for Rb1 and 26.4 ± 6.4 ng•h/ml for C-K, respectively. The gradual decrease of Rb1 levels and the delayed increase in levels of C-K observed in human subjects supported previous reports that enteric microbiota played a key role in transforming Rb1 to C-K.

Keywords

Panax quinquefolius; American Ginseng; Microbiota Metabolism; Ginsenoside Rb1; Compound K; UPLC/TOF-MS Analysis; Human Plasma Concentration

Introduction

Ginseng has been used for thousands of years in Asian countries. There are three major species of ginseng, *Panax ginseng* C.A. Meyer or Asian ginseng, *Panax notoginseng* (Burk.) F.H. Chen or notoginseng, and *Panax quinquefolius* L. or American ginseng (Christensen, 2009; Kim *et al.*, 2009; Taira *et al.*, 2010; Qi *et al.*, 2011b). Compared to the long history of use and the copious amount of research on Asian ginseng and notoginseng (Ang-Lee *et al.*, 2001; Ng, 2006; Lin *et al.*, 2010), studies of American ginseng, especially in humans, are limited (Yuan *et al.*, 2004).

It is believed that the active compounds in American ginseng are ginsenosides, a group of triterpene glycosides, and more than 90 of them have been isolated (Qi *et al.*, 2011a). The pharmacological effects of American ginseng have been observed in the central nervous, cardiovascular, endocrine, and immune systems (Attele *et al.*, 1999; Yuan *et al.*, 2004; Wang *et al.*, 2007).

American ginseng is commonly taken orally. After oral consumption, ginsenosides may be biotransformed by intestinal microbiota (Laparra and Sanz, 2010). When this happens, ginseng metabolites, not the natural ginsenosides, are bioactive (Kong *et al.*, 2009).

In American ginseng root, the major constituent is ginsenoside Rb1. In some cases, the proportion of ginsenoside Rb1 is over 50% of the total (Wang *et al.*, 2006; Qi *et al.*, 2011a). Ginsenoside Rb1 can be metabolized by intestinal microbiota before absorption. Ginsenosides Rd, F2 and compound K are the identified gut bacterial metabolites, of which compound K (C-K) is a major metabolite (Paek *et al.*, 2006; Qi *et al.*, 2011a). Thus, the C-K in plasma samples must be determined for a bioavailability study of ginseng. Because plasma levels of American ginseng compounds are relatively low, the usual HPLC/UV or HPLC/MS methods are not sufficiently sensitive to detect their metabolites in biological samples. With its high speed of chromatographic separation, ultra-performance liquid chromatography (UPLC) has emerged as a powerful method of analysis in pharmacokinetic studies. Existing HPLC conditions can be directly transferred to UPLC without reducing resolution (Guengerich and Rendic, 2010). Combining UPLC with a time-of-flight mass spectrometry (TOF-MS) detector makes trace compound analysis rapid, sensitive, and selective with peak-assignment certainty for trace compounds (Guillarme *et al.*, 2010). With this method, ginsenoside metabolites at very low concentrations are detected in complex biological samples.

In this study, after a single oral administration of American ginseng powder, we collected plasma samples from 6 healthy human subjects. We established a UPLC/TOF-MS method to determine 15 ginsenosides and metabolites (Fig. 1) simultaneously and to evaluate the bioavailability of ginsenosides and/or metabolites of American ginseng.

Materials and Methods

Chemicals

HPLC grade acetonitrile, methanol, ethanol and *n*-butanol were purchased from Fisher Scientific (Norcross, GA). Standards of ginsenosides Rb1, Rb2, Rc, Rd, Re, and Rg1 were purchased from Indofine Chemical Company (Somerville, NJ); ginsenosides Rb3, Rg2, 20(*R*)-Rg2, Rg3, 20(*R*)-Rg3, Rh1, Rh2 and pseudoginsenoside F11 were purchased from Delta Information Center for Natural Organic Compounds (Xuancheng, AH, China). Ginsenoside compound K (C-K) was obtained from ChromaDex. The internal standard (I.S.), digoxin, was obtained from Sigma-Aldrich. All standards were of biochemical-reagent grade and at least 95% pure as confirmed by HPLC.

Plant Material

Dried American ginseng (*Panax quinquefolius* L.) root was obtained from Roland Ginseng, LLC (Wausau, WI). The voucher samples were deposited at the Tang Center for Herbal Medicine Research at University of Chicago (Chicago, IL). The root was pulverized and passed through a 40 mesh screen to prepare the powder sample. Based on our assay, the American ginseng root contained the following ginsenosides (in milligrams per gram): Rb1 31.7, Rb2 0.7, Rb3 0.9, Rc 4.4, Rd 9.9, Re 17.2, Rg1 2.4, Rg2 0.1 and pseudoginsenoside F11 1.7. Other ginsenosides were not detected.

Human Subjects and Study Design

With approval from the Institutional Review Board at the University of Chicago, six adult volunteers (five males and one non-pregnant female) were enrolled in this clinical trial. Subjects were screened for drug abuse disorders or medical contraindications for participation in the study. The volunteers underwent a physical examination, and each participant completed a health history questionnaire.

At time 0, all subjects took 10 g of American ginseng powder with a cup of water. Venous blood samples were drawn at 0, 2, 4, 7, 9 and 12 h for analysis of compound levels in plasma. The samples were centrifuged and plasma was transferred to polypropylene tubes. All samples were immediately chilled on ice and stored at -80°C until analysis.

Analytical System and Chromatographic Conditions

Concentrations of ginsenosides and metabolites in plasma were determined using the UPLC/TOF-MS method. Chromatographic analysis was performed on an Agilent 1200 series (Agilent, Germany) liquid chromatographic system equipped with a binary pump, micro degasser, auto plate sampler, and a thermostatically controlled column compartment. Chromatographic separation was carried out at 25°C on an Agilent ZORBAX Extend- C_{18} UPLC column (2.1×50 mm, $1.8 \mu\text{m}$). For the mobile phase we used 0.1% formic acid in water (A) and acetonitrile (B). Gradient elution started with 82% solvent A and 18% solvent B, changed to 21% B for 6 min, then changed to 26% B for 1 min and held for 2 min; changed to 27% B for 4 min and held for 1 min; changed to 30% B for 3 min; changed to 36% B for 5 min; changed to 50% B for 3 min and held for 2 min; changed to 33% B for 5 min. The flow rate was kept at 0.4 ml/min, and the sample volume injected was set at $2 \mu\text{l}$.

The TOF-MS analysis was performed in the full-scan modes with an electrospray ionization (ESI) source and the mass range was set at mass to charge ratio (m/z) 100–1500 in negative mode. The conditions used for the ESI source were as follows: drying gas (N_2) flow rate, 10.0 l/min; drying gas temperature, 330°C ; nebulizer, 35 psig; capillary voltage, 3500 V; fragmentor, 120 V; skimmer voltage, 60 V; octapole DC1, 37 V; octopole RF, 250 V. The acquisition and analysis of data were controlled by Agilent LC-MS TOF software, version A.01.00 and Applied Biosystems/MDS-SCIEX Analyst QS software, respectively.

Sample Preparation and Analysis

Before analysis, 700 μl of human plasma was mixed with 17.5 μl of acetic acid and 20 μl of digoxin (10 $\mu\text{g}/\text{ml}$) and vortexed for 1 min at room temperature. The plasma sample was diluted with 3 ml of saline and homogenized. The homogenate was purified by solid-phase extraction (SPE) with a Sep-Pak C8 Vac 3cc 500 mg cartridge (Waters, Milford, MA). The cartridge was placed on an SPE vacuum manifold, cleaned with 2 ml of methanol and then preconditioned with 4 ml of water. The homogenate was applied to the SPE cartridge, washed with 5 ml of water, and air dried. The ginsenosides and metabolites were then slowly eluted from the cartridge with 2 ml of methanol into a glass vial; the elution was evaporated to dryness at 40°C under nitrogen and dissolved in 200 μl of methanol for

analysis. A calibration curve was used to calculate the compound concentration in samples. The curve plots the concentration of the standard (10 ng/ml, 100 ng/ml and 1000 ng/ml) against the area ratio of tested compound/internal standard. The known concentration is placed on the x-axis; and the measured area ratio, on the y-axis. The area ratio of the samples is applied to the curve and the concentration is found from the x-axis to determine the calculated concentration.

Pharmacokinetic and Data Analysis

Pharmacokinetic data were analyzed using pharmacokinetic software (Kinetic 4.4; Thermo Electron Co., Waltham, MA). All data are expressed as the mean \pm standard error (S.E.). A one-way ANOVA determined whether the results had statistical significance when applicable. The level of statistical significance was set at $P < 0.05$.

Results

To determine the 15 ginsenosides/metabolites simultaneously, a UPLC/TOF-MS method using a 2.1×50 mm reverse phase column was developed. With 0.1% formic acid in water and acetonitrile in the mobile phase with gradient elution, we observed an acceptable resolution for the 15 ginsenosides/metabolites (Fig. 2A and 2B).

Using the ESI negative ion mode, we found molecular ion peaks $[M-H]^-$ (m/z 1107.5947) and $[M+2^*HCOO]^{2-}$ (m/z 599.3008) of ginsenoside Rb1, and $[M+HCOO]^-$ (m/z 667.4432) of C-K. The mass spectra of Rb1, C-K and the internal standard are shown in Fig. 2C. For the MS spectrum of Rb1, the first and second abundant fragment ions were molecular ion peaks (m/z 599.3008 and 1107.5947). The most abundant fragment ion for C-K was the molecular ion (m/z 667.4432). For the internal standard, the most abundant fragment ion was the molecular ion $[M+HCOO]^-$ (m/z 825.4255).

The target compounds in samples were identified by comparison with the retention time and accurate mass data of reference compounds. Since the composition of human plasma samples is complex, at the same retention time of tested compounds, peaks of other unidentified compounds were observed. For example, at 28.3 min (the retention time of C-K), a strong overlapping peak was observed in the total ion chromatograms (TICs) of human plasma samples (Fig. 3A). With traditional UV and MS detectors, C-K is difficult to determine in the plasma samples.

Using the TOF-MS detector, analytical selectivity was improved because high-resolution provided exact mass to reconstruct extracted ion chromatograms (XICs) over nominal mass chromatograms (1 Da mass range). Mass interferences with analytes having the same nominal mass and chromatographic retention time can be identified by UPLC/TOF-MS analyses. Ginsenosides Rb1, Rd, Rg2 and compound K were detected in human plasma samples. However, Rd and Rg2 were not detected in all the plasma of every subject; Rb1 and C-K were. Fig. 3 shows the representative chromatograms of human plasma samples with internal standards, as well as the extracted ion chromatograms of ginsenoside Rb1, C-K and the internal standard extracted from the complex biological sample for quantification. Under chromatographic conditions, the target compounds were separated with overlapped peaks in plasma and were accurately determined.

Plasma concentrations of ginsenoside Rb1 and C-K after oral administration of American ginseng powder are shown in Table 1. As shown in Fig. 4, Rb1 concentration peak was at 4 h after administration of ginseng powder (19.90 ± 5.43 ng/ml). C-K was not detected at 2 or 4 h. The plasma concentration of C-K increased from 7 h to 12 h. The highest detected C-K

plasma concentration was 7.32 ± 1.35 ng/ml at 12 h. Because the concentration of C-K was not determined after 12 h, C-K concentration peak was not detected.

Pharmacokinetic parameters reflecting the bioavailability of American ginseng powder in subjects were calculated. There was one Rb1 concentration peak after the oral administration of ginseng powder. The peak plasma concentrations (C_{\max}) are shown in Table 1. The area under the concentration curve (AUC) for Rb1 from 0 to 12 h was 155.0 ± 19.5 ng•h/ml. For C-K, C_{\max} was not detected, and the AUC from 0 to 12 h was 26.4 ± 6.4 ng•h/ml. At each plasma sample point, the concentration of Rb1 was significantly higher than that of C-K. From 7 h to 12 h, plasma concentrations of C-K increased continuously, suggesting that the plasma concentration of C-K may have increased notably had we used additional time points.

Discussion

Ginseng is one of the world's most widely used medicinal plants and one of the best selling natural products (Li *et al.*, 2009; Jang and Shin, 2010; Qi *et al.*, 2011b). A big challenge in ginseng research is the complexity of the chemical constituents in different plants. A wide range of analytical methods are needed to fully characterize the ginsenosides and their metabolites in ginseng (Christensen, 2009; Qi *et al.*, 2011b). The ginsenoside profile of American ginseng is distinct from that of Asian ginseng. Yet compared to the many studies of Asian ginseng, relatively few biomedical and pharmacokinetic studies focus on American ginseng.

Like many other herbal medicines, American ginseng is almost always taken orally. Taken orally, its bioavailability is relatively low because absorption of the parent compound is incomplete and it converts to its metabolites. Previous evaluations of American ginseng have focused only on its parent constituents, i.e., ginsenosides Rb1 and Re (Andrade *et al.*, 2008; Lee *et al.*, 2008). In the gut, the main metabolic pathways include deglycosylation reactions in ginseng saponins by intestinal microbiota via stepwise cleavage of the sugar moieties (Tawab *et al.*, 2003; Hasegawa, 2004; Liu *et al.*, 2009). The identified major metabolite of the ginsenosides of Asian ginseng is C-K. However, whether C-K is a primary metabolite of American ginseng saponins has not been proven.

In this study, we analyzed the ginsenosides and their metabolites in human plasma using ultra-performance liquid chromatography (UPLC) with time-of-flight mass spectrometry (TOF-MS) (Dan *et al.*, 2008; Li *et al.*, 2010a). Compared to conventional HPLC, UPLC, which uses short columns packed with sub-2 μm particles, has significant advantages in high-speed separation and rapid resolution (Dan *et al.*, 2008; Muth *et al.*, 2008; Li *et al.*, 2010a). TOF-MS provides accurate mass measurement, high resolution, and selectivity, which makes this technique attractive for the qualitative and quantitative analysis of chemical constituents in complex biological samples (Li *et al.*, 2010a; Li *et al.*, 2010b).

Our 6 subjects took 10 g of American ginseng powder orally, and plasma samples were collected from 0 h to 12 h. Using the UPLC/TOF-MS method, ginsenosides Rb1, Rd, Rg2 and C-K were detected in the plasma samples. Ginsenoside Rb1 and C-K were detected from all plasma samples at different time points. The t_{\max} of Rb1 was 4 h. From 7 h, C-K level gradually increased. Since we collected the plasma samples for only 12 h, the t_{\max} and C_{\max} of C-K was not determined. C-K with delayed t_{\max} compared to the parent compounds indicated that intestinal microbiota are critical for transforming Rb1 to C-K. From this study, American ginseng was well tolerated at doses up to 10 g in 6 healthy volunteers. Data from our study suggest that enteric microbiota are responsible for metabolism of parent ginsenosides to C-K.

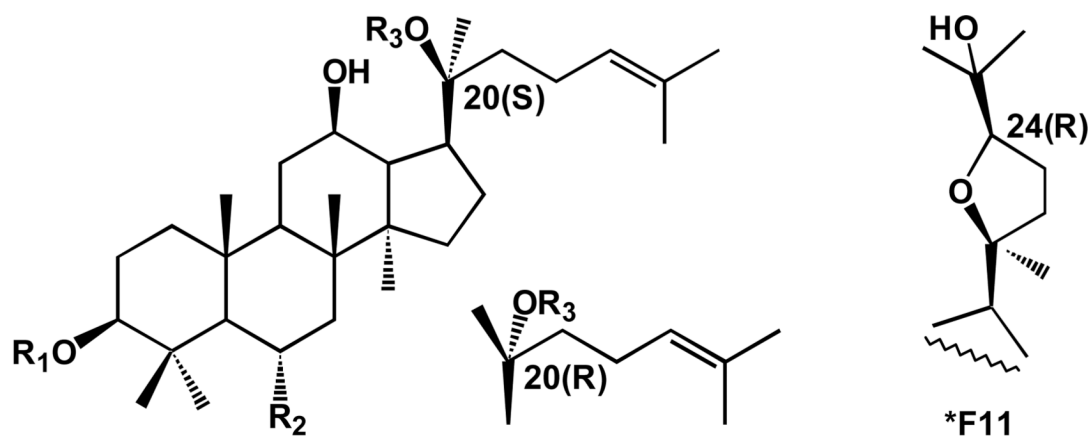
Acknowledgments

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Compound	R ₁	R ₂	R ₃
Ginsenoside Rb1	-glc ² - ¹ glc	-H	20(<i>S</i>), -glc ⁶ - ¹ glc
Ginsenoside Rb2	-glc ² - ¹ glc	-H	20(<i>S</i>), -glc ⁶ - ¹ ara(pyr)
Ginsenoside Rb3	-glc ² - ¹ glc	-H	20(<i>S</i>), -glc ⁶ - ¹ xyl
Ginsenoside Rc	-glc ² - ¹ glc	-H	20(<i>S</i>), -glc ⁶ - ¹ ara(fur)
Ginsenoside Rd	-glc ² - ¹ glc	-H	20(<i>S</i>), -glc
Ginsenoside Re	-H	-O-glc ² - ¹ rha	20(<i>S</i>), -glc
Pseudoginsenoside F11	-H	-O-glc ² - ¹ rha	24(<i>R</i>), *F11
Ginsenoside Rg1	-H	-O-glc	20(<i>S</i>), -glc
Ginsenoside Rg2	-H	-O-glc ² - ¹ rha	20(<i>S</i>), -H
20(<i>R</i>)-ginsenoside Rg2	-H	-O-glc ² - ¹ rha	20(<i>R</i>), -H
Ginsenoside Rg3	-glc ² - ¹ glc	-H	20(<i>S</i>), -H
20(<i>R</i>)-ginsenoside Rg3	-glc ² - ¹ glc	-H	20(<i>R</i>), -H
Ginsenoside Rh1	-H	-O-glc	20(<i>S</i>), -H
Ginsenoside Rh2	-glc	-H	20(<i>S</i>), -H
Compound K	-H	-H	20(<i>S</i>), -glc

Figure 1.
Chemical structures of 15 tested American ginseng saponins and metabolites.

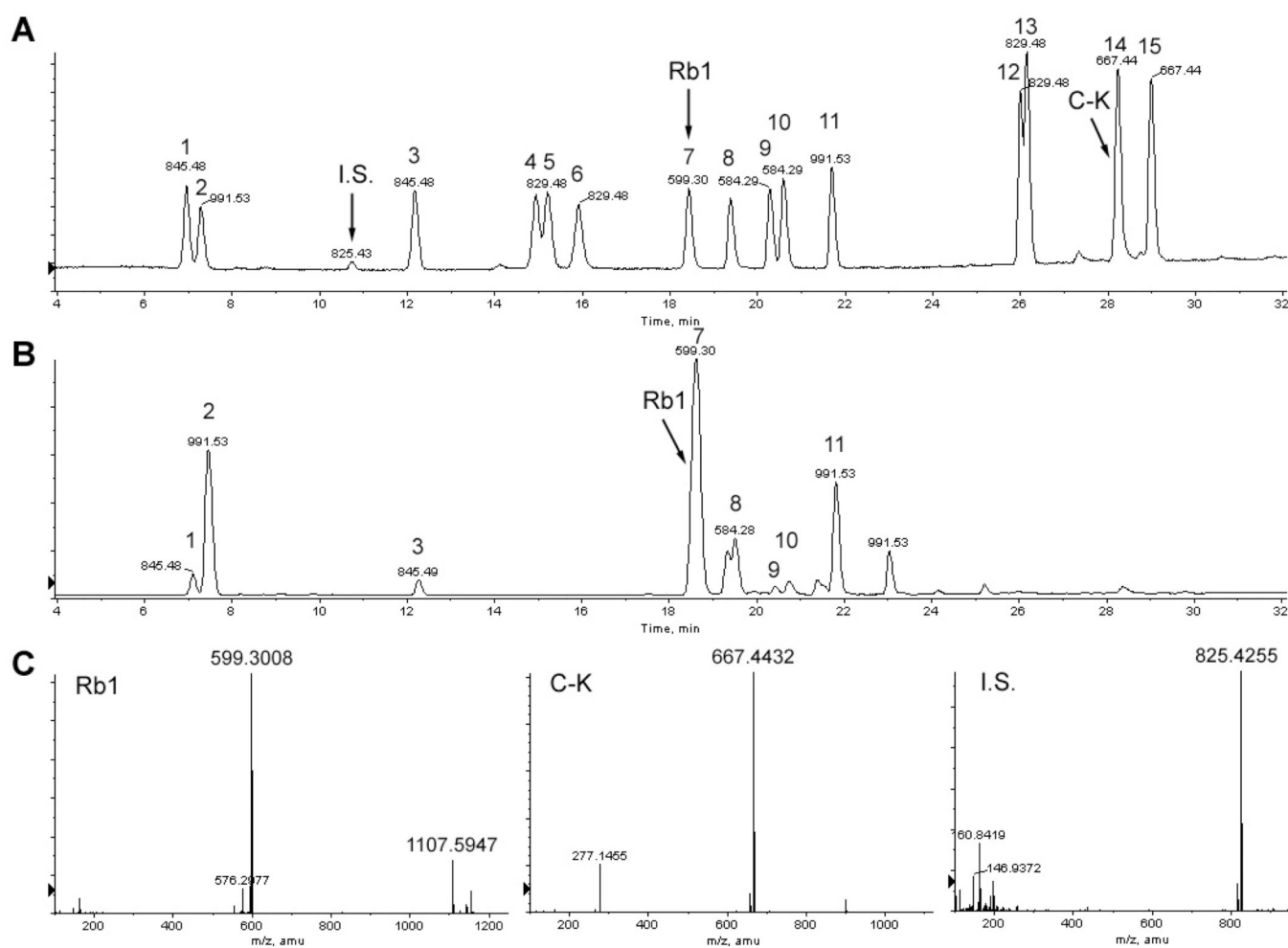


Figure 2.

UPLC/TOF-MS analysis of American ginseng saponins and metabolites. (A) Total ion chromatogram (TIC) of 15 standards of parent saponins and metabolites; (B) American ginseng root extract. Saponin peaks: 1, Rg1; 2, Re; 3, pseudoginsenoside F11; 4, Rh1; 5, Rg2; 6, 20(*R*)-Rg2; 7, Rb1; 8, Rc; 9, Rb2; 10, Rb3; 11, Rd; 12, Rg3; 13, 20(*R*)-Rg3; 14, C-K; and 15, Rh2; (C) TOF mass chromatograms of ginsenoside Rb1, compound K (C-K) and internal standard (I.S.).

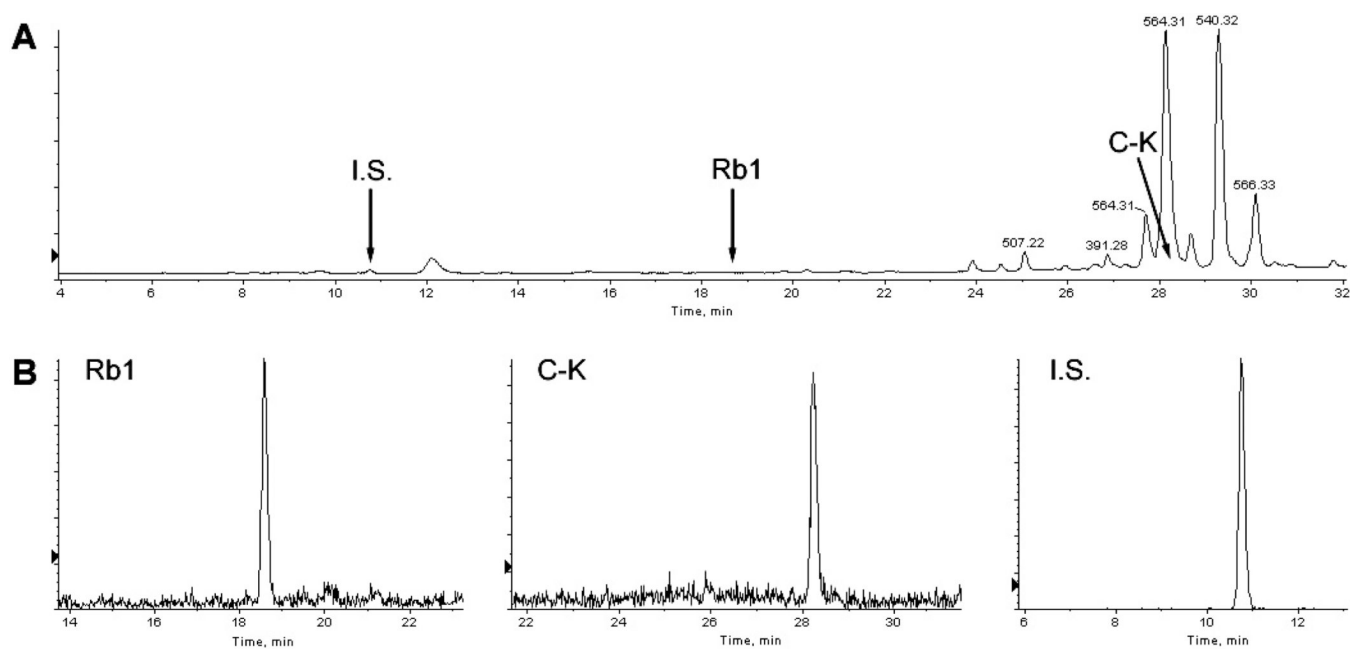


Figure 3. Representative UPLC chromatograms of American ginseng saponins and metabolites in human plasma sample. (A) Total ion chromatogram (TIC) of a plasma sample. C-K was overlapped by other compounds. (B) Extracted ion chromatograms (XIC) of ginsenoside Rb1, C-K, and I.S. from the plasma matrix for quantification with a 0.01 Da mass window in negative modes. XIC at m/z 599.30–599.31 for Rb1, m/z 667.44–667.45 for C-K, and m/z 825.42–825.43 for I.S.

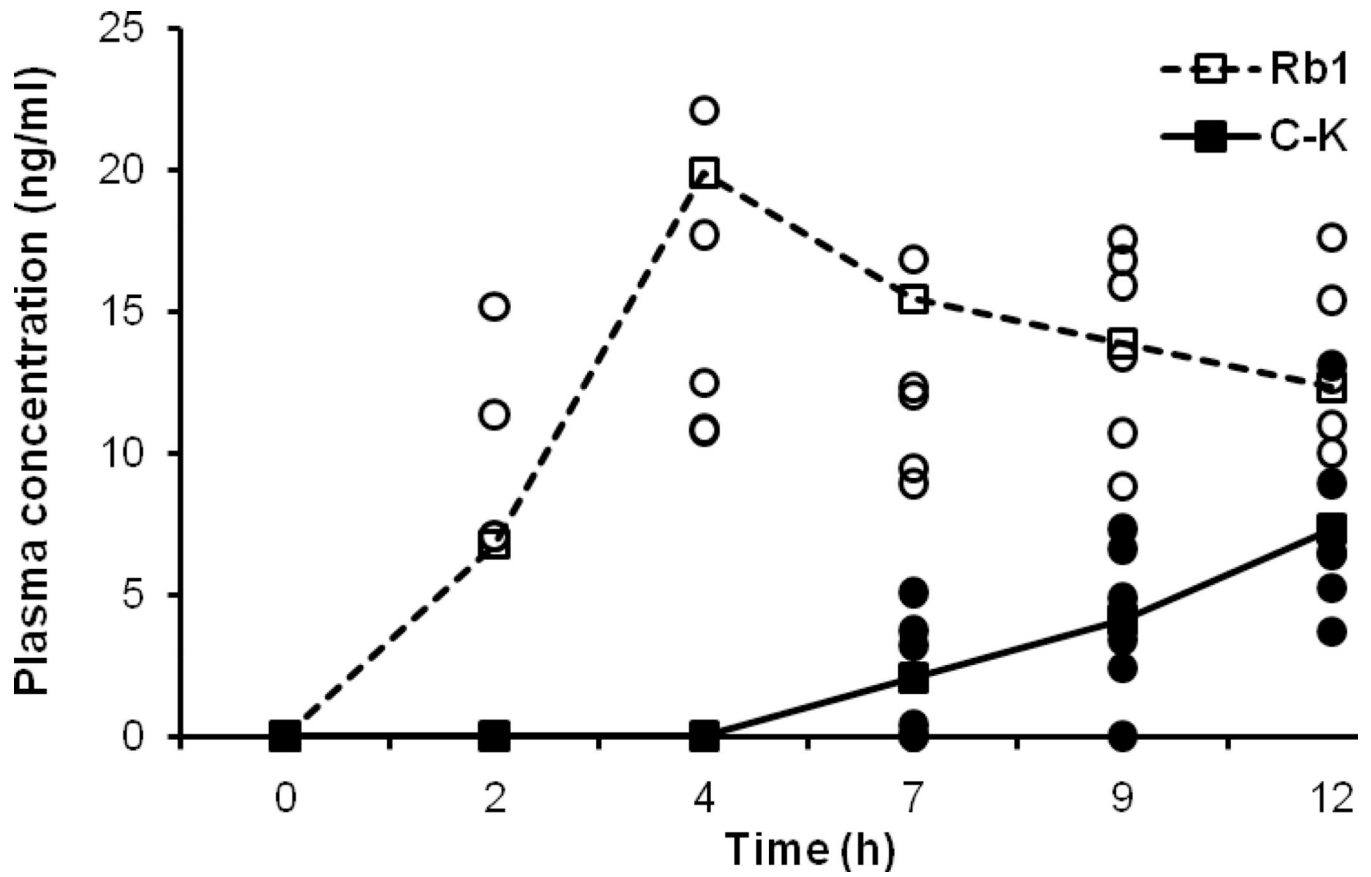


Figure 4. Concentrations of Rb1 and C-K in human plasma. Open circles are individual points of Rb1 plasma concentrations; open squares represent mean values of Rb1; solid circles are individual points of C-K plasma concentrations; solid squares represent mean values of C-K.

Table 1

Concentrations of Ginsenoside Rb1 and Compound K in Human Plasma Samples (n = 6)

	Ginsenoside Rb1 (ng/ml)						Compound K (ng/ml)					
	0	2	4	7	9	12	0	2	4	7	9	12
Time (h)	0	2	4	7	9	12	0	2	4	7	9	12
Subject 1	0	11.36	45.48	12.31	17.55	12.61	0	0	0	0	0	3.71
Subject 2	0	0	17.71	9.46	16.79	17.60	0	0	0	0	4.88	6.50
Subject 3	0	7.06	10.83	12.03	10.72	10.01	0	0	0	0.39	3.42	6.44
Subject 4	0	0	10.77	33.03	13.42	7.26	0	0	0	5.09	7.31	13.09
Subject 5	0	15.16	22.10	16.85	15.91	15.40	0	0	0	3.23	6.62	8.93
Subject 6	0	7.05	12.49	8.93	8.83	10.99	0	0	0	3.72	2.42	5.25
Mean	0	6.77	19.90	15.44	13.87	12.31	0	0	0	2.07	4.11	7.32
S.E.	0	2.47	5.43	3.70	1.43	1.53	0	0	0	0.90	1.12	1.35