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Gut microbiota-mediated deglycosylation of ginsenoside Rb_1 in rats: In vitro and in vivo insights from quantitative ultra-performance liquid chromatography-mass spectrometry analysis

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Abstract:

Ginsenoside Rb_1, an ingredient of the herbal medicine *Panax ginseng*, possesses a variety of biological activities. Gut microbiota mediated deglycosylation of ginsenoside Rb_1 has long been proposed to play an important role in mediating its pharmacological effects. However, the major active ginsenoside components after the gut microbial transformation have not been clearly identified. To provide pharmacokinetic clues, we firstly established a rapid and sensitive ultra performance liquid chromatography (UPLC) tandem mass spectrometry method for targeted analysis of ginsenoside Rb_1-related components (ginsenoside Rd, F_2, Rg_3, Rh_2 and, compound K) in biological samples. We then performed a comprehensive quantitation of ginsenoside Rb_1 and its metabolites in rat plasma after oral administration of ginsenoside Rb_1. *In vitro* microbial hydrolysis of ginsenoside Rb_1 was further investigated to help elucidate the *in vivo* findings. The results indicated that ginsenoside Rb_1 and ginsenoside Rd could be detected within 72 hours after oral administration of ginsenoside Rb_1. Other metabolites, like compound K only appeared at a very low concentration in the circulation. *In vitro* hydrolysis study revealed that the formation of ginsenoside Rg_3 and Rh_2 were relatively low compared with the ginsenoside Rd，F_2 and compound K. Ginsenoside Rb_1 was rapidly hydrolyzed to ginsenoside Rd and the formation of F_2 from Rd paralleled compound K from F_2. Together, our work suggested that ginsenoside Rd could be the major circulating active metabolite of ginsenoside Rb_1 mediated by gut microbial deglycosylation in rats.

Key Words: ginsenoside Rb_1; ultra-performance liquid chromatography-tandem

mass spectrometry; pharmacokinetics; gut microbiota

1. Introduction

Ginseng, the root of *Panax ginseng C.A. Meyer*, is one of the best known herbal medicines [1-2]. Now, ginseng is considered as the most used and top-selling herbal medicine in the world as modern pharmacological studies indicate that its extract exerts a wide range of therapeutic effects on immune function[3], cancer[4], diabetes[5], cardiovascular diseases[6], brain ischemia[7], and improving cognitive performance[8]. Ginseng saponins, namely ginsenosides, have been regarded as the primary constituents responsible for ginseng's pharmacological actions[9]. Approximately 60 different ginsenoside forms have been isolated and identified from the root of *panax ginseng*. These molecules can be classified according to their sapogenins as 20(*S*)-protopanaxadiol type (ppd-type) and 20(*S*)-protopanaxatriol type (ppt-type)[10].

Previous reports[11-12] showed that ginsenosides were usually characterized with poor absorption and extensive metabolism which could be classified into three pathways such as gastric acid-mediated hydrolysis or hydration[13], gut microbiota-mediated decomposition[14] and CYP450 enzymes-mediated oxygenation[15]. The deglycosylation of ginsenosides is the most important metabolic pathways occurring *in vivo*[16]. After metabolism into aglycones, ginsenosides could be absorbed more easily and produce better pharmacological effects[17]. The glycosidases have a considerable effect on deglycosylation reactions, such as β-D-glucosidase[18], α-L-

rhamnosidase[19] and β-D-xylosidase[20]. A large number of bacterial strains including Lactobacillus[21], Bifidobacterium[22], Streptococcus thermophilus[23] and Bacteroides thetaiotaomicron[24] have been revealed to have the glycosidases.

Ginsenoside Rb_1 is a major component of ginseng, which was found to possess many pharmacological activities. Ginsenoside Rb_1 and its metabolites (ginsenoside Rd, F_2, Rg_3, Compound K (C-K), Rh_2) are PPD-type ginsenosides and have the same glycoside site (C-3 site, C-20 site) [25]. After oral administration, ginsenoside Rb_1 was converted primarily to aglycone via stepwise cleavage of the sugar moieties by the gut microbiota[26]. And the main metabolic pathways of ginsenoside Rb_1 by gut microbiota has been proposed (Fig.1). Also，the pharmacokinetic study of ginsenoside of Rb_1 and its active metabolites C-K have been studied in mice, rats or humans[27-29]. However, these studies failed to gain more comprehensive pharmacokinetic profiles of ginsenoside Rb_1 and its deglycosylated metabolites. Also the actual biotransformation and metabolic studies of ginsenoside Rb_1 in gut microbiota needed to be well clarified.

Thus, in this study, a UPLC–MS/MS method was developed and further applied in the pharmacokinetic studies of ginsenoside Rb_1 and its metabolites in rat plasma after oral administration of ginsenoside Rb_1. The metabolic profiles of ginsenoside Rb_1 in gut microbiota suspension were also studied to classify the active components that are responsible for the pharmacological effect of ginsenoside Rb_1.

2. Materials and methods

2.1. Chemicals and reagents

Ginsenoside Rb_1, Rd, F_2, Rg_3, C-K and Rh_2 (purity 98.0%) were obtained from the College of Chemistry, Jilin University (Changchun, China). Digoxin (internal standard, IS) (purity 99.0%) was purchased from the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). Their structures are given in Fig.1. HPLC-grade methanol and acetonitrile were purchased from Merck (New Jersey, USA). Water used in the experiments was generated by a Milli-Q System (Millipore Corporation, Germany). Other chemicals were all of analytical grade.

2.2 UPLC-MS/MS conditions

UPLC-MS/MS analyses were performed using a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, Milford, MA, USA) including a binary solvent pump and a cooled autosampler. The chromatographic separation was performed using an Acquity^{TM} UPLC BEH C_{18} analytical column (50mm×2.1mm, 1.7μm; Waters, Milford, MA, USA) and column temperature was maintained at 40°C. The mobile phases consisted of 1mM ammonium formate (A) and acetonitrile (B). The initial composition was 90% A and 10% B, with a gradient elution program as follows: 0-4 min, 10-35%B; 4-8 min, 35-70%B; 8-9 min, 70-10% B; 9-10, 10% B. And the flow rate was set at 0.25mL/min and the injection volume was 3μL.

The detector was an Acquity Xevo TQD mass spectrometer (Waters Corporation) using an electrospray ionization (ESI) source operated in negative ion mode. Ultrapure nitrogen was used as both the nebulizing gas and the desolvation gas. The

parameters in the source were set as follows: capillary voltage, 3.0 kV; desolvation temperature, 450°C; cone gas flow, 50 L/h; desolvation gas flow, 1000 L/h. Table 1 shows the optimized multiple reaction monitoring (MRM) parameters, including the precursor-to-product ion transitions, cone voltage, collision energy for each analyte. The dwell time was automatically set by the software. Masslynx 4.1. Software (Waters Corporation, Milford, MA) was used to process data. Quantification was based on peak area.

2.3. Preparation of standards, internal standard and quality control (QC) samples

Standard stock solution at concentration of 10.15, 5.46, 5.03, 5.20, 5.16, and 5.02μg/mL were prepared in methanol for ginsenoside Rb_1, Rd, F_2, Rg_3, C-K, and Rh_2, respectively. Working solutions were prepared by appropriate dilution of the stock solutions with a mixture of methanol to provide nine standards of desired concentrations. The IS solution was prepared at a final concentration of 0.8μg/mL in methanol. All the solutions were stored at 4°C.

Calibration standard samples were prepared by spiking blank rat plasma with the working solutions to obtain concentration of 2.03-1015.0 ng/mL for ginsenoside Rb_1, 2.18-1092.0 ng/mL for ginsenoside Rd, 1.61- 804.8 ng/mL for ginsenoside F_2, 1.66-832.0 ng/mL for ginsenoside Rg_3, 1.03-516.0 ng/mL for compound K, 1.00- 502.0 ng/mL for ginsenoside Rh_2, respectively. For the validation of the method, three concentration levels of quality control (QC) plasma samples were prepared containing ginsenoside Rb_1 (3.0, 40.0, 800.0 ng/mL), ginsenoside Rd (3.0, 40.0 800.0 ng/mL), ginsenoside F_2 (2.4, 32.0, 640.0 ng/mL), ginsenoside Rg_3(2.4, 32.0, 640.0 ng/mL),

ginsenoside C-K (1.5, 20.0, 400.08 ng/mL), ginsenoside Rh_2 (1.5, 20.0, 400.08 ng/mL). All QC samples were extracted on each analysis day with the same procedures for plasma samples as described below.

2.4. Sample preparation

In our study, a liquid-liquid extraction method was used to prepare plasma samples. A 50μL aliquot of plasma sample was transferred into a 1.5mL plastic test tube together with 10μL of I.S. working solution (0.8μg/mL). The sample was briefly mixed for about 30s and 1mL water-saturated *n*-butanol: ethyl acetate (3:1, v/v) was added. The mixture was vortexed for 3 min. After centrifugation at 9,000 × *g* for 5 min, 800 μL of the supernatant was transferred to another clean tube and evaporated to dryness at 40°C in a Speed Vacplus Model vacuum drier (ThermoFisher). The residue was dissolved in 50μL methanol and centrifuged at 21,000 × *g* for 10 min. A 3 μL aliquot of the supernatant was injected into the UPLC-MS/MS system for analysis.

2.5. Method validation

The method was validated by specificity, linearity, lower limits of quantification (LLOQ), precision, accuracy, matrix effect, recovery and stability，according to the Food and Drug Administration guidelines[30].

To investigate assay specificity, six lots of blank plasma from individual rats were analyzed for excluding any endogenous co-eluting interference by comparing the blank plasma with corresponding plasma samples spiked with each analyte standard and I.S. using the liquid-liquid extraction and UPLC-MS/MS procedures.

The matrix effect on the ionization of ionization of analytes and I.S. was tested

by comparing the peak area of post-extraction blank plasma samples spiked with analytes at three QC levels and I.S.(80.4 ng/mL) with the same concentration resolved in the methanol. If the ratio <85% or >115%, it indicated that ionization suppression or enhancement is implied.

The extraction recovery experiments were estimated by comparing the peak area of extracted spiked samples at three QC levels and I.S.(80.0 ng/mL) with those corresponding pure standards without extraction.

The linearity of the method needed at least five sets of calibration curves to assay the concentration of analytes in rat plasma. The peak area ratio(Y) of analyte/I.S. and the concentration of each analyte(X) were used to construct the calibration curve formula using weighted ($1/x^2$) linear least squares regression model. The LLOQ was determined as the lowest concentration point of the standard curve, which could be detected with a signal-to-noise ratio more than 10. The acceptable accuracy and precision of LLOQ is defined within 20% according to the results of six replicates.

The intra-batch and inter-batch precision and accuracy were evaluated by analysis of three QC samples (n=6) in three validation batches. Concentrations were calculated from the calibration curve. The precision was defined as the relative standard deviation (RSD, %) of replicate measurements within 15%. The accuracy was obtained as the ratio of calculated concentration versus actual value, and considered to be in the range of 85-115%.

Stability experiments were performed under different conditions. Six replicates of analytes at low, medium and high concentrations were assessed. The various

conditions storing QC samples included at room temperature for 12 h, at −20°C for 4 weeks, three freeze-thaw cycles and at 4°C for 24 h in autoinjection, respectively.

2.6. Pharmacokinetics study in rats

The study was used developed UPLC-MS/MS method to investigate the pharmacokinetic behavior study of ginsenoside Rb_1 and its metabolites in rats. Specific-pathogen-free (SPF) Sprague-Dawley rats (male, 200-250 g) were obtained from the Animal Ethics Committee of the Nantong University (Certificate No. SCXK-2008-0010). The rats were kept in wire cages at 50 ± 10% humidity and 20–22 °C with free access to food and water and fasted for 12h before the experiment. Animal welfare and experimental procedures were strictly in accordance with the guide for the care and use of laboratory animals.

Rats (n=6) were orally administrated at a dose of 80mg/kg of ginsenoside Rb_1. This dose was selected based on our preliminary experiment to give more comprehensive plasma concentration–time profiles of ginsenoside Rb_1 and related metabolites. Blood samples (150 μL) were collected with heparinized tubes at the time points of 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 24, 36, 48 and 72 h from the ophthalmic veins. All blood samples were immediately centrifuged at 2,000 × *g* for 10min and 50 μL of plasma samples were transferred and stored at -20°C until analysis. The maximum plasma concentration (C_{max}) and time to maximum concentration (T_{max}) were obtained directly from the plasma concentration-time plots. The area under the concentration-time curve (AUC), terminal elimination half-life ($T_{1/2}$) mean retention time (MRT), apparent volume of distribution (VZ/F) and

clearance (CL) were calculated by non-compartmental analysis using DAS 3.2.6 software (Chinese Pharmacological Association, China).

2.7. Hydrolysis of ginsenoside Rb_1 in gut microbiota suspension

Fresh feces (approximately 0.8g) of six Sprague-Dawley rats were collected and stored at -80°C until use. Feces were carefully mixed with a spatula and suspended with ice-cold phosphate buffer solution (1:9, v/v). After centrifuged in 500 g for 5min at 4°C, the supernatant was collected and stored at -80°C.

The thawed gut microbiota suspension (100 μL) was transferred to 1.5 mL clean plastic test tubes, ginsenoside Rb_1 was added to a final concentration of 20 μM with ice-cold 0.1 mM PBS in a volume of 200 μL. The mixture was incubated in water bath at 37°C. Samples at 0, 10, 20, 30, 45 min, 1, 1.5, 2, 4, 8, 12 and 24h, were stopped by 1mL ice-cold water-saturated *n*-butanol: ethyl acetate (3:1, v/v) containing 80ng digoxin (I.S.) The samples were vortexed for 3min and centrifuged at 18,000 × *g* for 10min. A 100μL aliquot supernatant was evaporated to dryness at 40°C, reconstituted with 100 μL methanol, vortexed for 3min and centrifuged at 21,000 × *g* for 10min. A 3 μL supernatant was injected into the UPLC-MS/MS system for analysis.

3. Results and discussion

3.1. Method development

The response of analytes to ESI was greater sensitivity and selectivity in MRM mode. The full-scan ion spectra of the analytes after direct injection in methanol suggested that much higher sensitivity was observed in the negative mode than in the

positive mode. The most abundant predominant ions for ginsenoside Rb_1, Rd, F_2, Rg_3, C-K, Rh_2 and digoxin were $[M-H]^-$ ions with *m/z* of 1107.44, 945.33, 783.34, 783.34, 621.22, 621.22 and 779.18, respectively. The fragment ions, collision energy and cone voltage which significantly influence the sensitivity were automatically optimized. The ion pairs *m/z* 1107.44→179.01, 945.33→783.22, 783.34→621.24, 783.34→621.24, 621.22→160.87, 621.22→160.87 and 779.18→649.11 for ginsenoside Rb_1, Rd, F_2, Rg_3, C-K, Rh_2 and digoxin were finally selected to give higher responses in the MRM mode.

For the simultaneously determination of all analytes and I.S., a gradient elution program was employed in a Acquity™ UPLC BEH C_{18} analytical column. A linear gradient 10-35% acetonitrile from 0min to 4min were crucial for the separation of all analytes and I.S., especially for ginsenoside F_2 and Rg_3. As ginsenoside F_2 and Rg_3. are isomers sharing the same aglycone with the minor difference in the terminal sugar moiety at C-3 and C-20 position.

According to various kinds of extraction procedures [11,14,15], liquid-liquid extraction with water-saturated *n*-butanol and ethyl acetate was considered to be an appropriate method, which took into account the recoveries of low-polarity and high-polarity analytes. The preliminary experiments tried to use different proportions of the two reagents to explore an optimal extraction method. And the results demonstrated that water-saturated *n*-butanol: ethyl acetate (3:1, v/v) can be selected as the most suitable extraction solvent, which achieved stable and acceptable recoveries.

3.2. Method validation

3.2.1. Specificity

The specificity of the method was evaluated by analyzing individual blank plasma samples from six different rats. All analytes and I.S. were found no apparent interferences from endogenous substances at the retention times of ginsenoside Rb_1 (t_R=4.70min), Rd (t_R=5.30min), F_2 (t_R=6.27min), Rg_3 (t_R=6.78min), C-K (t_R=7.91min), Rh_2 (t_R=8.26min) and I.S. (t_R=4.01min). Representative chromatograms of blank plasma, plasma spiked with analytes and I.S., the plasma sample at 12h after oral administration of 80mg/kg ginsenoside Rb_1 were shown in Fig. 2.

3.2.2. Linearity and lower limits of quantification

The calibration curves of six analytes exhibited good linearity with correlation coefficients (*r*) within the range from 0.9920 to 0.9998. The LLOQs were sufficient for quantitative detection of analytes in the pharmacokinetic studies. The LLOQs for ginsenoside Rb_1, ginsenoside Rd, ginsenoside F_2, ginsenoside Rg_3, Compound K and ginsenoside Rh_2 were 2.03, 2.18, 1.61, 1.66, 1.03, and 1.00 ng/mL, respectively. The results of the mean regression equation of calibration curves, linear ranges, R^2, LLOQ and LLOD were shown in Table 2.

3.2.3. Accuracy and precision

Table 3 summarized the intra- and inter-batch accuracies and precisions of ginsenoside Rb_1, Rd, F_2, Rg_3, C-K and Rh_2 at three different concentration levels. The accuracy and precision of the method were evaluated by analyzing six replicates of the QC samples at three concentrations on three batches. The intra-batch and inter-batch precisions ranged 1.76–11.18% and 4.86–11.46%, respectively. The accuracy

derived from QC samples was between 92.37% and 108.72% for each QC level of the six analytes. The assay values on both intra- and inter-batch were all within the acceptable range.

3.2.4. Recovery and matrix effect

The extraction recoveries of ginsenoside Rb_1, Rd, F_2, Rg_3, C-K, Rh_2 at three concentration levels and the IS were all in the range from 48.2% to 79.7%. These results demonstrated that the values were all in the acceptable ranges. The mean matrix effects derived from QC samples were between 92.8-113.2% and I.S. was 101.9%, which demonstrated no significantly endogenous matrix effect. The matrix effects of ginsenoside Rb_1, Rd, F_2, Rg_3, C-K, Rh_2 and I.S. in rat plasma were shown in Table 3.

3.2.5. Stability

All stability results were conducted under three levels of QC samples. The analytes in rat plasma were all stable for 12h at room temperature, 4 weeks at −20℃, three cycles of freeze-thaw and 24h in autoinjection at 4℃. These results (Table 4) indicated that there were no stability-related problems during routine analysis in the pharmacokinetic study.

3.3. Application to pharmacokinetics study

The validated method was successfully applied to the pharmacokinetic study of ginsenoside Rb_1 and its metabolites in rat plasma following a single oral administration (80mg/kg) to rats. The plasma concentration-time profiles were presented in Fig. 3. The main pharmacokinetic parameters were shown in Table 5.

The results of T_{max} in oral administration study indicated that the absorption of ginsenoside Rb_1 was very slow. On the basis of the experimental results shown in Table 6, some metabolites of ginsenoside Rb_1 could also be detected in rat plasma. The primary metabolite ginsenoside Rd was observed all the time after oral administration of ginsenoside Rb_1. Additionally, ginsenoside C-K appeared between 8-24 hours after oral administration of ginsenoside Rb_1.

4.4. Metabolic profiles of ginsenoside Rb_1 in gut microbiota suspension

Gut microbiota mediated deglycosylation of ginsenoside Rb_1 could provide more insights to help understand the plasma pharmacokinetic profiles of ginsenoside Rb_1 and its deglycosylated metabolites. As shown in Fig 4, ginsenoside Rb_1 could be rapidly hydrolyzed to ginsenoside Rd, as indicated by the disappearance of ginsenoside Rb_1 and appearance Rd after 2 hours incubations. The formation of ginsenoside Rg_3 from Rd was relatively low as compared with that of F_2, which indicated that, β-glucosidases in rat gut microbiota may prefer to hydrolysing the sugar moiety at the C-3 hydroxyl group than that at the side chain. A similar phenomenon could be seen in the formation of C-K and Rh_2 from F_2. These data indicated that, the gut microbiota mediated deglycosylation of the ginsenoside Rb_1 in rats occurred at C-20 until only one sugar remained and then the deglycosylation preferentially took place at C-3. Taken together, our *in vitro* hydrolysis study of ginsenoside Rb_1 was well correlated with the pharmacokinetics profiles of ginsenoside Rb_1 and its metabolites in plasma (Fig 3. and Table 6).

5. Conclusions

In summary, a sensitive and rapid UPLC-MS/MS method for simultaneous determination of ginsenoside Rb_1 and its deglycosylated metabolites, ginsenoside Rd, F_2, Rg_3, C-K and Rh_2 in biological samples was developed and validated. Pharmacokinetics of ginsenoside Rb_1 after oral administration revealed that the ginsenoside Rb_1 had poor bioavailability. The gut microbiota mediated deglycosylation of ginsenosoide Rb_1 may play an important role for its low bioavailability and its pharmacological effects.

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Table

Table 1 The main parameters of ginsenoside Rb_1, its main deglycosylation metabolites ginsenoside Rd, F_2, Rg_3, C-K, Rh_2, and digoxin (I.S.) obtained in UPLC-MS/MS.

Components	M.F.	M.W.	Channel	Cone voltage	Collision energy
Ginsenoside Rb_1	$C_{54}H_{92}O_{23}$	1108.6	1107.44→179.01	94	64
Ginsenoside Rd	$C_{48}H_{82}O_{18}$	946.6	945.33→783.22	90	40
Ginsenoside F_2	$C_{42}H_{72}O_{13}$	784.5	783.34→621.24	52	28
Ginsenoside Rg_3	$C_{42}H_{72}O_{13}$	784.5	783.34→621.24	52	28
Ginsenoside C-K	$C_{36}H_{62}O_8$	622.4	621.22→160.87	62	26
Ginsenoside Rh_2	$C_{36}H_{62}O_8$	622.4	621.22→160.87	62	26
Digoxin	$C_{41}H_{64}O_{14}$	779.2	779.18→649.11	72	34

Table 2 The linearity, LLOD and LLOQ of the assay for ginsenoside Rb_1, Rd, F_2, Rg_3, C-K, Rh_2.

Components	Calibration curves (y=ax+b)	Linear range (ng/ml)	R^2	LLOQ (ng/ml)	LLOD (ng/ml)
Ginsenoside Rb_1	y = 0.0026x − 0.0037	2.03-1015.00	0.999	2.03	0.77
Ginsenoside Rd	y = 0.0038x + 0.0022	2.18-1092.00	0.9999	2.18	0.60
Ginsenoside F_2	y = 0.0047x + 0.0054	1.61-804.80	0.996	1.61	0.45
Ginsenoside Rg_3	y = 0.0024x + 0.0043	1.66-832.00	0.995	1.66	0.75
Ginsenoside C-K	y = 0.0049x − 0.0010	1.03-516.00	0.998	1.03	0.42
Ginsenoside Rh_2	y = 0.0030x + 0.0021	1.00-502.00	0.993	1.00	0.63

Table 3 Intra-batch and inter-batch accuracy and precision and matrix effects of LC/MS/MS determination of ginsenoside Rb_1, Rd, F_2, Rg_3, C-K and Rh_2 in rat plasma

Components	Spiked conc. (ng/mL)	Intra-Batch(n=6)			Inter-Batch(n=18)			Matric effect (%)
		Measured Conc. (ng/mL)	Accuracy (%)	Precision (%)	Measured Conc. (ng/mL)	Accuracy (%)	Precision (%)	
Ginsenoside Rb_1	2.03	2.14±0.17	105.42	7.94	1.92±0.22	95.05	11.46	103.4
	40.0	41.86±2.70	104.66	6.44	42.37±3.37	105.92	7.96	108.4
	800.0	771.83±56.50	96.48	7.32	785.50±58.78	98.19	7.48	105.8
Ginsenoside Rd	2.18	2.02±0.17	92.66	8.42	2.31±0.12	105.96	5.19	107.2
	40.0	36.95±2.39	92.37	6.48	38.21±2.89	95.52	7.55	99.4
	800.0	853.96±25.57	106.74	2.99	854.17±48.66	106.77	5.70	92.8
Ginsenoside F_2	1.61	1.52±0.11	94.41	7.28	1.58±0.08	98.13	5.06	105.8
	32.0	31.80±1.85	99.37	5.80	32.56±1.99	101.74	6.10	109.7
	640.0	648.61±51.97	101.34	8.01	616.09±50.05	96.26	8.12	100.2
Ginsenoside Rg_3	1.66	1.61±0.18	96.98	11.18	1.72±0.14	104.82	8.14	104.9
	32.0	33.05±2.37	103.30	7.18	31.70±3.38	99.06	10.67	98.7
	640.0	680.19±28.92	106.28	4.25	695.82±53.55	108.72	7.70	97.2
Ginsenoside C-K	1.03	1.08±0.09	104.85	8.33	1.00±0.09	97.09	9.00	105.8
	20.0	20.01±1.77	100.04	8.85	21.13±2.10	105.65	9.93	105.7
	400.0	422.67±7.42	105.67	1.76	433.42±25.88	108.35	5.97	103.5
Ginsenoside Rh_2	1.0	1.04±0.11	104.02	10.58	1.06±0.10	106.12	9.43	106.8
	20.0	18.77±0.73	93.85	3.88	19.44±0.95	97.21	4.86	108.0
	400.0	371.08±15.39	92.77	4.15	394.62±26.74	98.65	6.78	113.2

Table 4 The stability test of ginsenoside Rb_1, Rd, F_2, Rg_3, C-K and Rh_2 in rat plasma

Components	Spiked	At room temperature for	At −20°C for 4 weeks (n=6)	After three freeze-thaw	At 4°C for 24h in

		12h (n=6)				cycles (n=6)		autoinjection (n=6)	
	Conc. (ng/mL)	Measured Conc. (ng/mL)	Accuracy (%)	Measured Conc. (ng/mL)	Accuracy (%)	Measured Conc. (ng/mL)	Accuracy (%)	Measured Conc. (ng/mL)	Accuracy (%)
Ginsenoside Rb_1	3.0	3.21±0.34	106.94	3.10±0.29	103.49	2.96±0.19	98.66	3.14±0.26	104.57
	40.0	39.48±2.24	98.69	42.10±3.33	105.25	37.87±1.75	94.67	41.67±2.86	104.18
	800.0	777.18±44.53	97.15	796.46±38.49	99.56	842.94±60.65	105.37	794.12±70.33	99.26
Ginsenoside Rd	3.0	2.86±0.32	95.32	3.00±0.23	99.99	3.19±0.34	106.36	2.82±0.27	93.84
	40.0	40.69±2.87	101.74	39.50±2.67	98.76	42.53±2.42	106.32	42.38±1.07	105.95
	800.0	849.85±17.88	106.23	833.63±20.02	104.20	819.81±45.17	102.48	847.11±40.52	105.89
Ginsenoside F_2	2.4	2.59±0.18	107.95	2.32±0.23	96.96	2.51±0.22	104.76	2.56±0.10	106.67
	32.0	33.29±1.40	104.02	35.28±1.77	110.25	33.21±0.60	103.78	30.24±1.68	94.51
	640.0	610.20±24.60	95.34	710.46±44.10	111.01	678.02±24.61	105.94	697.58±62.21	109.00
Ginsenoside Rg_3	2.4	2.34±0.23	97.50	2.61±0.27	108.58	2.39±0.28	99.49	2.28±0.19	95.19
	32.0	31.80±1.84	99.37	30.31±1.11	94.73	29.43±1.78	91.97	31.38±1.52	98.06
	640.0	694.78±70.58	108.56	703.49±67.19	109.92	645.47±24.03	100.85	670.38±55.35	104.75
Ginsenoside C-K	1.5	1.42±0.07	94.96	1.42±0.14	94.45	1.51±0.15	100.99	1.57±0.17	104.90
	20.0	19.80±1.70	98.99	21.10±0.82	105.50	18.79±1.41	93.96	20.09±1.53	100.46
	400.0	404.59±9.84	101.15	427.21±40.30	106.80	421.41±32.66	105.35	434.49±22.65	108.62
Ginsenoside Rh_2	1.5	1.65±0.19	110.44	1.63±0.15	108.90	1.39±0.06	92.91	1.43±0.07	95.02
	20.0	21.77±1.31	108.84	21.74±1.44	108.69	21.11±1.68	105.54	19.01±1.18	95.03
	400.0	401.08±36.13	100.27	402.18±37.97	100.54	390.93±24.17	97.73	389.72±18.36	97.43

Table 5 The pharmacokinetic parameters of ginsenoside Rb_1 after oral administration of 80mg/kg

to rats (n=6, mean ± SD)

Parameters	Oral
T_{max}(h)	5.60±1.67
C_{max}(μg/L)	1480.24±600.32
$AUC_{(0-t)}$(μg/L*h)	36112.98±10847.49
$AUC_{(0-\infty)}$(μg/L*h)	37885.52±10801.89
$MRT_{(0-t)}$(h)	22.05±1.13
$MRT_{(0-\infty)}$(h)	25.85±1.98
$T_{1/2}$z(h)	16.48±2.75
Vz/F(L/kg)	56.44±27.57
CLz(L/h/kg)	2.28±0.73

Table 6 Ginsenoside Rb_1 and its metabolites detected in rats plasma after oral administration (black circle represents ginsenosides can be quantitatively detected, short line represents ginsenosides can't be detected).

Components / Time(h)	Ginsenoside Rb_1	Ginsenoside Rd	Ginsenoside F_2	Ginsenoside Rg_3	Ginsenoside C-K	Ginsenoside Rh_2
0.25	●	●	—	—	—	—
0.5	●	●	—	—	—	—
0.75	●	●	—	—	—	—
1	●	●	—	—	—	—
1.5	●	●	—	—	—	—
2	●	●	—	—	—	—
4	●	●	—	—	—	—
6	●	●	0-3.32ng/mL	—	0-1.51ng/mL	—
8	●	●	0-1.99ng/mL	—	0-1.16ng/mL	—
12	●	●	0-1.67ng/mL	—	0-1.30ng/mL	—
24	●	●	—	—	0-1.11ng/mL	—
36	●	●	—	—	—	—
48	●	●	—	—	—	—
72	●	●	—	—	—	—

Figures

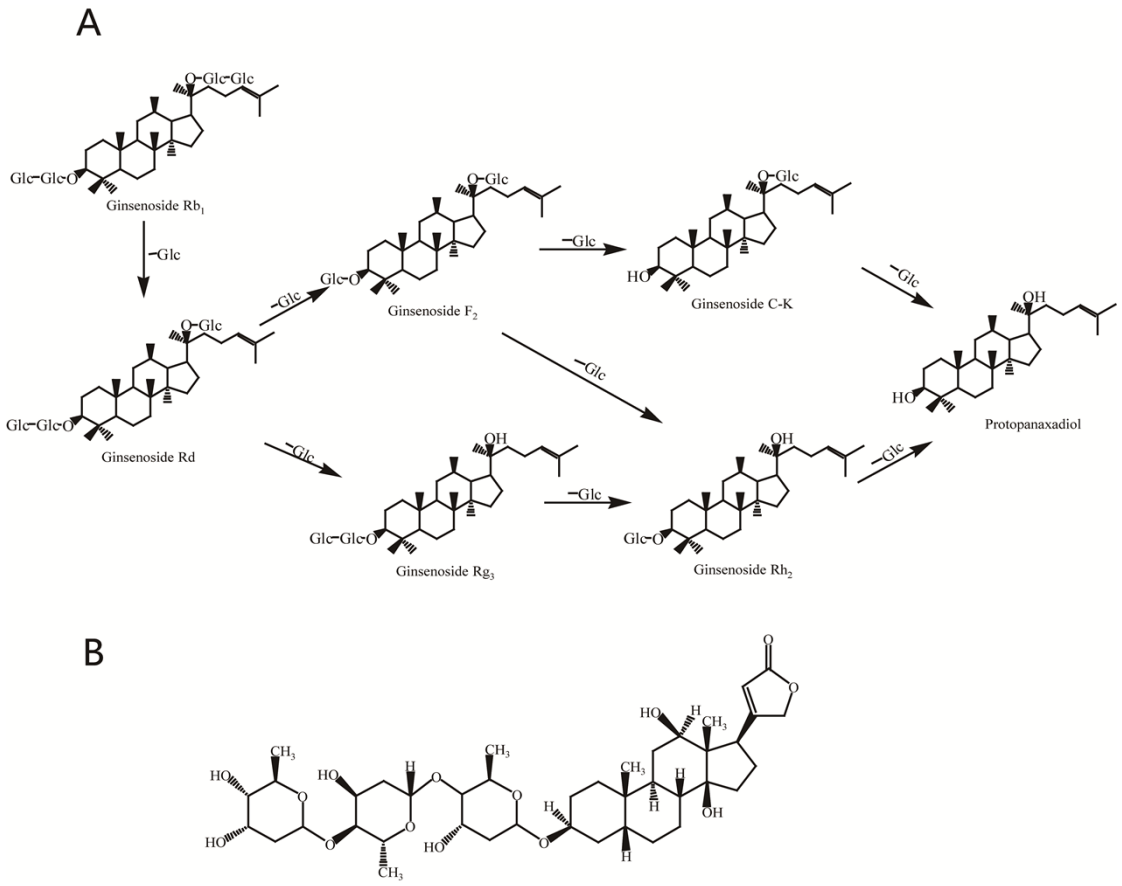

Fig1. (A) Metabolic pathways of ginsenoside Rb_1 by gut microbiota.(B) Chemical structures of digoxin (internal standard).

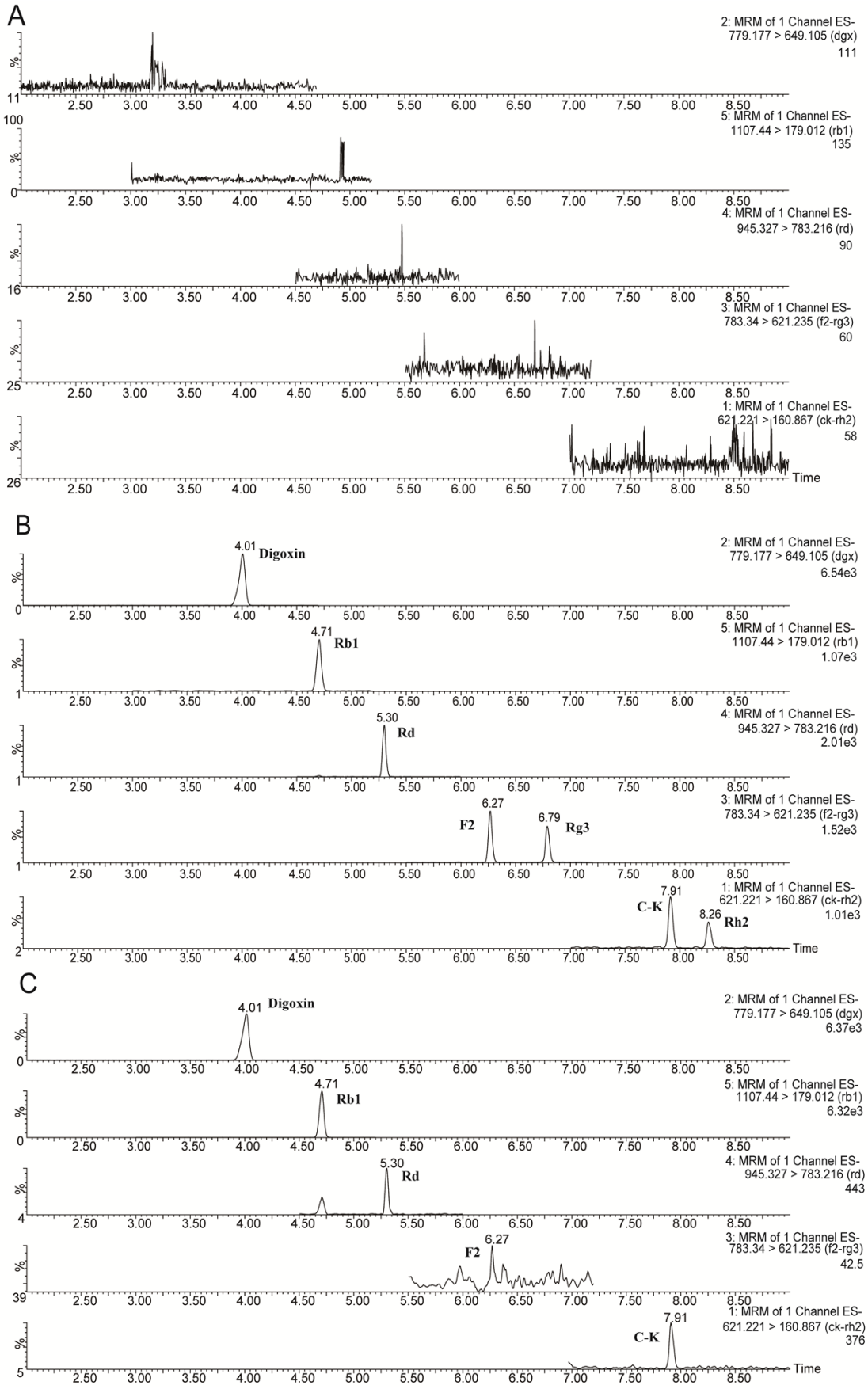

Fig 2. Typical MRM chromatograms of ginsenoside Rb_1, Rd, F_2, Rg_3, C-K, Rh_2 and

digoxin. (A) a blank rat plasma sample, (B) a blank plasma spiked with ginsenoside Rb_1(40.6 ng/mL), Rd (43.68 ng/mL), F_2 (32.19 ng/mL), Rg_3 (33.28 ng/mL), C-K (20.64 ng/mL), Rh_2 (20.08 ng/mL) and I.S.(80.4 ng/mL), respectively, (C) a rat plasma sample obtained at 12h after oral administration of ginsenoside Rb_1(80 mg/kg) .

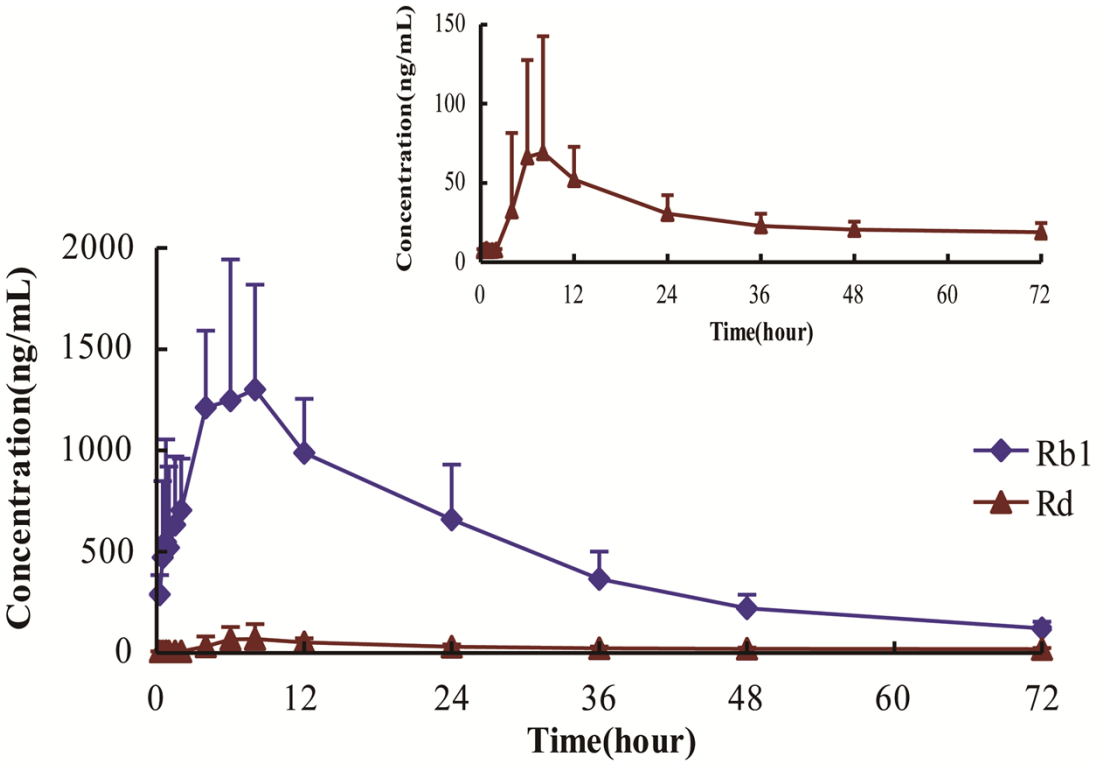

Fig 3. The mean plasma concentration-time curves of ginsenoside Rb_1 and Rd after oral administration of 80mg/kg ginsenoside Rb_1 (mean ± SD, n=6). For i.g. experiment, the inset represents the concentration-time profile of ginsenoside Rd in detail.

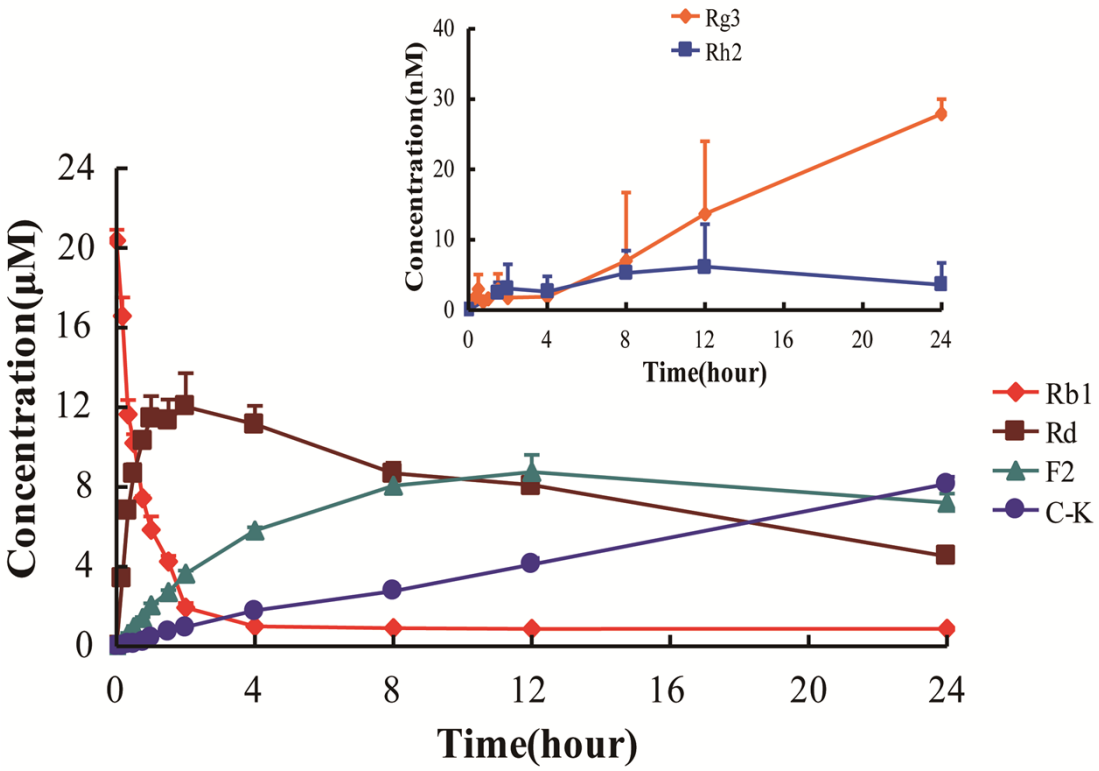

Fig 4. Hydrolysis of ginsenoside Rb_1(20 μM) in SD rats fecal suspension.