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Quantification of *Panax notoginseng* saponins metabolites in rat plasma with *in vivo* gut microbiota-mediated biotransformation by HPLC-MS/MS

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[ABSTRACT] *Panax notoginseng* saponins (PNS) are the major components of *Panax notoginseng*, with multiple pharmacological activities but poor oral bioavailability. PNS could be metabolized by gut microbiota *in vitro*, while the exact role of gut microbiota of PNS metabolism *in vivo* remains poorly understood. In this study, pseudo germ-free rat models were constructed by using broad-spectrum antibiotics to validate the gut microbiota-mediated transformation of PNS *in vivo*. Moreover, a high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) was developed for quantitative analysis of four metabolites of PNS, including ginsenoside F1 (GF1), ginsenoside Rh2 (GRh2), ginsenoside compound K (GCK) and protopanaxatriol (PPT). The results showed that the four metabolites could be detected in the control rat plasma, while they could not be determined in pseudo germ-free rat plasma. The results implied that PNS could not be biotransformed effectively when gut microbiota was disrupted. In conclusion, gut microbiota plays an important role in biotransformation of PNS into metabolites *in vivo*.

[KEY WORDS] *Panax notoginseng*; Gut microbiota; HPLC-MS/MS; Saponins; Ginsenoside compound K; Protopanaxatriol

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Introduction

Panax notoginseng saponins (PNS), such as notoginsenosides and ginsenosides, are the major components of *P. notoginseng*, which is a famous traditional Chinese medicine and has been clinically used for over 400 years in East Asian countries [1-2]. They possess a variety of biological activities, including anti-tumor activity [3], antidepressant [4], anti-aging [5], anti-inflammatory [6-7], immunological modulated activity [8] and neuroprotective effects [9]. However, most PNS are poorly absorbed into circulatory system with extremely low oral bioavailability, and hardly transported into target tissue [10-13]. Therefore, most PNS seem like the prodrugs that could be metabolized or transformed *in vivo* to yield some metabolites with pertinent pharmacological effects.

Trillions of gut microbes are a complex community, which inhabit in the human gastrointestinal tract and play an important role in the metabolism of xenobiotics, including altering pharmacokinetic process, bioavailability, pharmacological activities and toxicities [14-16]. Moreover, some studies have found that diet or drugs, such as antibiotics, can affect the profile and function of gut microbiota [17-19]. Recently, more and more studies have verified that saponins, such as *American ginseng* saponins, *Panax ginseng* extract and *P. notoginseng* saponins could be biotransformed by gut microbiota *in vitro* [18-21]. Metabolites, such as ginsenoside compound K (GCK) and ginsenoside Rh2 (GRh2), have shown better bioavailabilities and pharmacological activities [22-27]. These metabolites seem to be the active compounds. Therefore,

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These authors have no conflict of interest to declare.

it is essential to understand the exact role of gut microbiota in biotransformation for further study of PNS. However, despite the fact that biotransformation of saponins by gut microbiota was verified *in vitro*, there were few related researches *in vivo*. It was reported that only ginsenoside Rb1 had been validated for its bioconversion with gut microbiota *in vivo* using pseudo germ free (GF) mice [28-29]. Due to the complexity of components and chemical structure of PNS, there is limited study about validation of PNS transformed by gut microbiota *in vivo*.

In this study, Ampicillin sodium, metronidazole, neomycin sulphate and vancomycin hydrochloride were used to constructed chronic pseudo GF rat model (Chronic group), while ceftriaxone sodium and gentamicin sulfate were used for acute pseudo GF rat model (Acute group). A HPLC-MS/MS was developed for quantitative analysis of the metabolites of PNS. GF1, PPT, GCK and GRh2 were determined as the main metabolites, because they have been found as highly abundant products of PNS incubating with gut microbiota in vitro in our previous work [18]. Our results showed that the four metabolites could be detected in the control rat plasma, while their concentrations were below the limit of detection in pseudo GF rat plasma. It implied that PNS could not be metabolized effectively when gut microbiota was disrupted. Taking together, gut microbiota might play an important role in biotransformation of PNS into metabolites *in vivo*. In addition, GCK and PPT were the most abundant metabolites of PNS *in vivo*.

Materials and methods

Materials and reagents

Ampicillin sodium, metronidazole, neomycin sulphate and vancomycin hydrochloride were obtained from BBI Life Sciences Co., Ltd. (Shanghai, China). Gentamicin sulfate and ceftriaxone sodium were supplied by Beijing Huamai Biotechnology Co., Ltd. (Beijing, China). GF1, GRh2, and PTT were provided by Baoji Herbest Bio-Tech Co., Ltd. (Shaanxi, China), GCK and digoxin (the internal standard, IS) were bought from Chengdu Push Bio-technology Co., Ltd. (Sichuan, China). The purity of all compounds was determined by HPLC (≥ 98%), and their chemical structures were shown in Fig. 1. Dichloromethane, ethyl acetate and n-butanol were analytical reagent and purchased from Sino-pharm Chemical Reagent Co., Ltd (Shanghai, China). Ammonium acetate and formic acid (HPLC-grade) were obtained from CNW technologies GmbH (Dusseldorf, Germany). HPLC-grade acetonitrile (ACN) and methanol were supplied by Merck Company (Darmstadt, Germany). Deionized water (18 MΩ·cm^{-1}) was prepared by a Milli-Q Ultrapure water system (Milford, MA, USA).

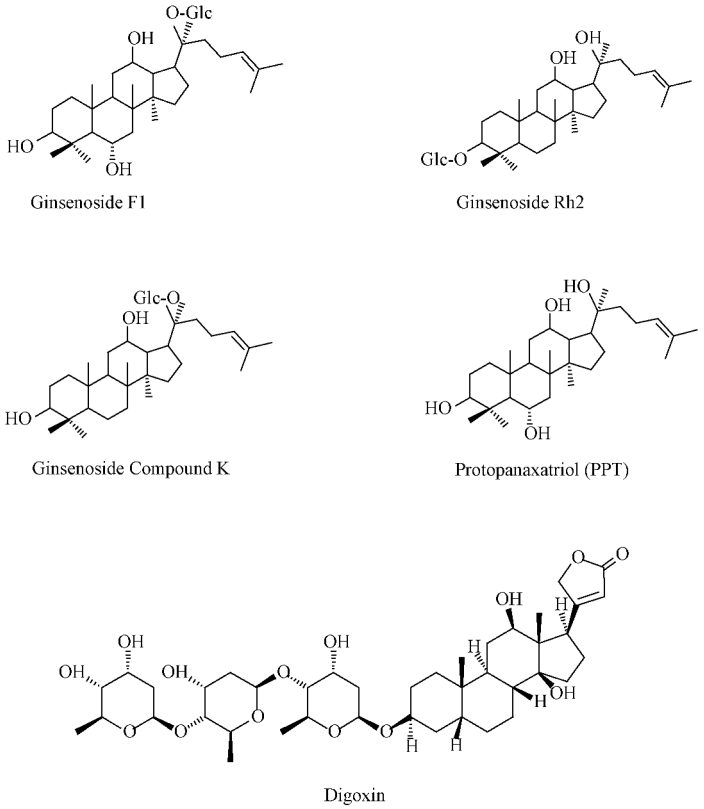

Fig. 1 Chemical structures of the analytes and IS

Panax notoginseng saponins

The air-dried root of *P. notoginseng* was bought from Wenshan City (Yunnan, China), and authenticated by Dr. SHAO Li from Hunan University of Chinese Medicine (Hu-

nan, China). A voucher specimen (Lot: PNR-20170302-1) has been deposited in the Department of Clinical Pharmacology, Xiangya Hospital, Central South University (Hunan, China). The extraction method of PNS was same as our previous work, and the extraction ratio was 19.2% [18].

Animals

Fifteen male Sprague-Dawley rats (6–8 week, 200 ± 20 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China) with a license No. 43004700044390. All rats were housed in a room with a 12/12 h light/dark cycle, where the temperature (22 ± 2 °C) and relative humidity (60% ± 5%) were controlled. All animals were free access to standard diet and water, and acclimated to the laboratory environment for seven days before beginning experiments. The animals study protocol was approved by the Ethical Committee of Animal Experiments of Central South University.

Study design

The rats were divided into three groups randomly, normal control group, acute group and chronic group with five rats in each group. The chronic models were constructed using broad-spectrum antibiotics solution (containing 1 g·L^{-1} ampicillin sodium, 1 g·L^{-1} metronidazole, 1 g·L^{-1} neomycin sulphate and 0.5 g·L^{-1} vancomycin hydrochloride in sterile water) set as the drinking water and provided enough to each cage for four consecutive weeks [30]. The acute rat models were established by orally administration of ceftriaxone sodium (350 mg·kg^{-1}) and gentamicin sulfate (126 mg·kg^{-1}) twice a day for six consecutive days [31]. The control group was orally administered with physiological saline. And the feces were collected at the end of antibiotics administration. Two days later after the end of modeling, the rats were fasted overnight with free access to water. All rats were orally administrated with PNS extract in dose of 1.535 g·kg^{-1} (equivalent to 8 g·kg^{-1} for *P. notoginseng*). Blood samples were collected via the jugular vein at 0, 2, 4, 6, 8, 12, 24, 48 h after drug administration. The plasma samples were then separated by centrifugation at 4000 r·min^{-1} for 10 min and stored at –80 °C until use.

Microbial DNA was extracted from rat fecal samples using the E.Z.N.A.Stool DNA kit (Omega Bio-TEK, USA). DNA concentration was quantified using Nanodrop 2000 spectrophotometer (Shimadzu, Japan).

Instruments and condition

HPLC system consisted of a SHIMADZU Nexera X2 HPLC system with LC-30AD pumps, SIL-30AC autosampler, CBM-20A communications bus module and CTO-20AC column oven (Tokyo, Japan). The chromatographic separation was achieved at gradient elution of 0.2% formic acid in water (A) and ACN (B) at a flow rate of 0.3 mL·min^{-1} on an Phenomenex LUNA C_{18} (2) Reversed Phase (150 mm × 2.0 mm, 5 μm). The gradient profile was optimized below, 0–2 min: 35%–65% B, 2–4 min: 65%–70% B, 4–7 min: 70%–72% B. The injection volume was 10 μL and the temperature of column was set at 40 °C.

The mass spectroscopic analysis was performed on AB Sciex Triple Quad ™ 6500 mass spectrometers equipped with electrospray ionization (ESI). The following mass spectrometer parameters were selected in positive ion mode: spray voltage, 5500 V; temperature, 350 °C; collision gas, 10 psi; curtain gas, 20 psi; ion source gas 1, 50 psi; ion source gas 2, 50 psi. The detection for each compound was performed in the multiple reaction monitoring (MRM) mode.

Table 1 Calibration curves and LLOQ of four analytes in the rat plasma

Compounds	Linear range (ng·mL^{-1})	R^2	Regression equation	LLOQ (ng·mL^{-1})
GF1	0.210–100.6	0.9959	$Y = 0.0185\,x + 0.000\,575$	0.210
GRh2	0.217–104.2	0.9931	$Y = 0.0178\,x + 0.002\,41$	0.217
GCK	0.241–115.6	0.9960	$Y = 0.0016\,x + 0.000\,948$	0.241
PPT	1.168–93.4	0.9952	$Y = 0.00346\,x + 0.001\,1$	1.17

Preparation of calibration standards and quality control (QC) samples

The primary stock solutions of GRh2 (1.042 mg·mL^{-1}), GCK (1.156 mg·mL^{-1}), GF1 (1.006 mg·mL^{-1}) and PPT (0.934 mg·mL^{-1}) were prepared by dissolving each compound in methanol, respectively. Working solutions of the four compounds were prepared by diluting the stock solutions appropriately with methanol–water (V/V = 1 : 1) and stored at 4 °C. The plasma samples for standard calibration curves and the QC samples were spiked 5 μL of working solution with 45 μL of blank rat plasma. The solution of digoxin (IS) was diluted to 1.134 μg·mL^{-1} using methanol–water and stored at 4 °C.

Sample preparation

The metabolites of PNS in the rat plasma were extracted by liquid-liquid extraction (LLE). Ethyl acetate and water saturated *n*-butanol were used for extracting GF1 and GRh2. 50 μL of plasma added 5 μL of IS solution and 100 μL of buffer solutions (containing 0.1% formic acid and 25 mmol·L^{-1} ammonium acetate) was continuously extracted with ethyl acetate and water saturated *n*-butanol (300 μL, respectively) for 10 min using a vortex. The two organic phases were mixed together and dried at 55 °C under nitrogen. Then, the residues were re-dissolved in 50 μL of methanol-water.

Five hundreds μL of dichloromethane was applied for extraction of GCK and PPT in 50 μL of plasma added with 5 μL of IS solution and 100 μL of buffer solutions. The organic phase was dried at 55 °C under nitrogen and the residues were

re-dissolved in 50 μL of methanol-water before analysis.

Method validation

Sensitivity, linearity, selectivity, precision, accuracy, extraction recovery, matrix effect and stability were validated in this method, according to the FDA guidelines for bioanalytical method validation.

Table 2 The intra- and inter-day precision and accuracy of four analytes in rat plasma

Compounds	c (ng·mL^{-1})	Intra-day (n = 6)		Inter-day (n = 18)	
		Accuracy (%)	Precision (RSD %)	Accuracy (%)	Precision (RSD %)
GF1	0.503	108.83	8.59	104.25	6.40
	8.05	97.88	11.31	97.61	9.65
	80.5	97.02	13.40	100.34	8.44
GRh2	0.521	105.34	5.08	102.76	7.39
	8.34	100.26	11.11	98.20	10.72
	83.4	102.15	14.55	104.12	7.29
GCK	0.578	102.93	2.52	106.11	4.81
	9.25	95.68	9.01	99.65	8.40
	92.5	113.83	5.01	105.07	8.71
PPT	1.87	108.20	5.97	106.06	7.08
	7.47	108.13	9.86	103.87	5.40
	74.7	98.97	13.88	103.4	9.76

c means the final concentration of analytes in the rat plasma

Selectivity

Six blank rat plasma from six different rats, and the same blank rat plasma spiked with IS or with analytes and IS were analyzed to evaluate the selectivity. The peak areas around retention time of four analytes in the blank plasma must be less than 20% of peak areas of blank plasma spiked with analytes, and were less than 5% of the mean peak areas of blank plasma spiked with IS. In addition, there must be a good chromatographic separation between IS and analytes, and the IS did not disturb the determination of the four analytes.

Linearity and low limits of quantification (LLOQ)

The calibration curves were conducted in a weighted ($1/X^2$) linear least squares regression model, and were fitted using analyte/IS peak area ratios versus concentrations of the standards. And the correlation coefficient (R^2) of calibration curves must be greater than 0.99. The lowest concentration in the calibration curve was usually considered as the LLOQ, whose signal-to-noise ratios (S/N) were about 10 at least. Six replicates of LLOQ samples were analyzed, and the concentration was acceptable if accuracy was within 80%–120% of the nominal concentration.

Precision and accuracy

Relative standard deviation (RSD) was used to assess the precision. Accuracy was evaluated with the percentage of the observed concentration to the known true concentration. Six replicates of the LQC, MQC, HQC samples and two sets of calibration standards were assayed to evaluate the intra- and inter-day precision and accuracy. The inter-day precision and accuracy were conducted by analyzing QC samples in three consecutive days. RSD of intra- and inter-day must be less than 15%, and compared with nominal concentration, the mean concentration of accuracy at each level must be within 85%–115%.

Extraction recovery and matrix effect

QC samples at low, medium and high concentrations (six replicates per level) were analyzed to evaluate extraction recovery. The extraction recovery was calculated by comparing peak areas of QC plasma samples with post-extracted blank plasma sample spiked with the same concentration of analytes and IS in the QC samples. The matrix effect was calculated by comparing peak areas of post-extracted blank rat plasma samples spiked with analytes and IS, with the working solutions which contained equivalent amounts of analytes and IS. The precision of matrix effect must be less than 20%.

Stability

Six replicates of QC samples at three concentrations were prepared and stored at –80 °C for sixty days. These QC samples were analyzed to evaluate long-term stability. Six replicates of QC plasma samples at three concentrations were frozen at –80 °C and thawed at room temperature (25 °C). The freeze/thaw cycles were operated for three times and then, the QC samples were analyzed to evaluate the freeze/thaw cycle stability. The precision must be less than 15%, and the accuracy must be within 85%–115% of the nominal standard.

Application to quantification

Fifteen male rats were orally administered with PNS dissolved in purified water in dose of 1.535 g·kg^{-1}. Blood samples were collected at 0, 2, 4, 6, 8, 12, 24, 48 h. Plasma samples were obtained and stored at –80 °C after centrifugation. Four metabolites of PNS, GF1, GRh2, GCK and PPT in the rat plasma were detected by the validated HPLC-MS/MS. Plasma samples at every sampling time were analyzed to study pharmacokinetic process of the four metabolites.

Results

Model evaluation

The pseudo GF rat models were evaluated through the microbial DNA concentration shown in Fig. 3. There was significant decrease in DNA levels both in model groups

compared with control group. Therefore, these results implied that the total amounts of microbes were extremely small in the intestinal cavity of pseudo GF rat models due to gut microbiota was disrupted by antibiotic treatments.

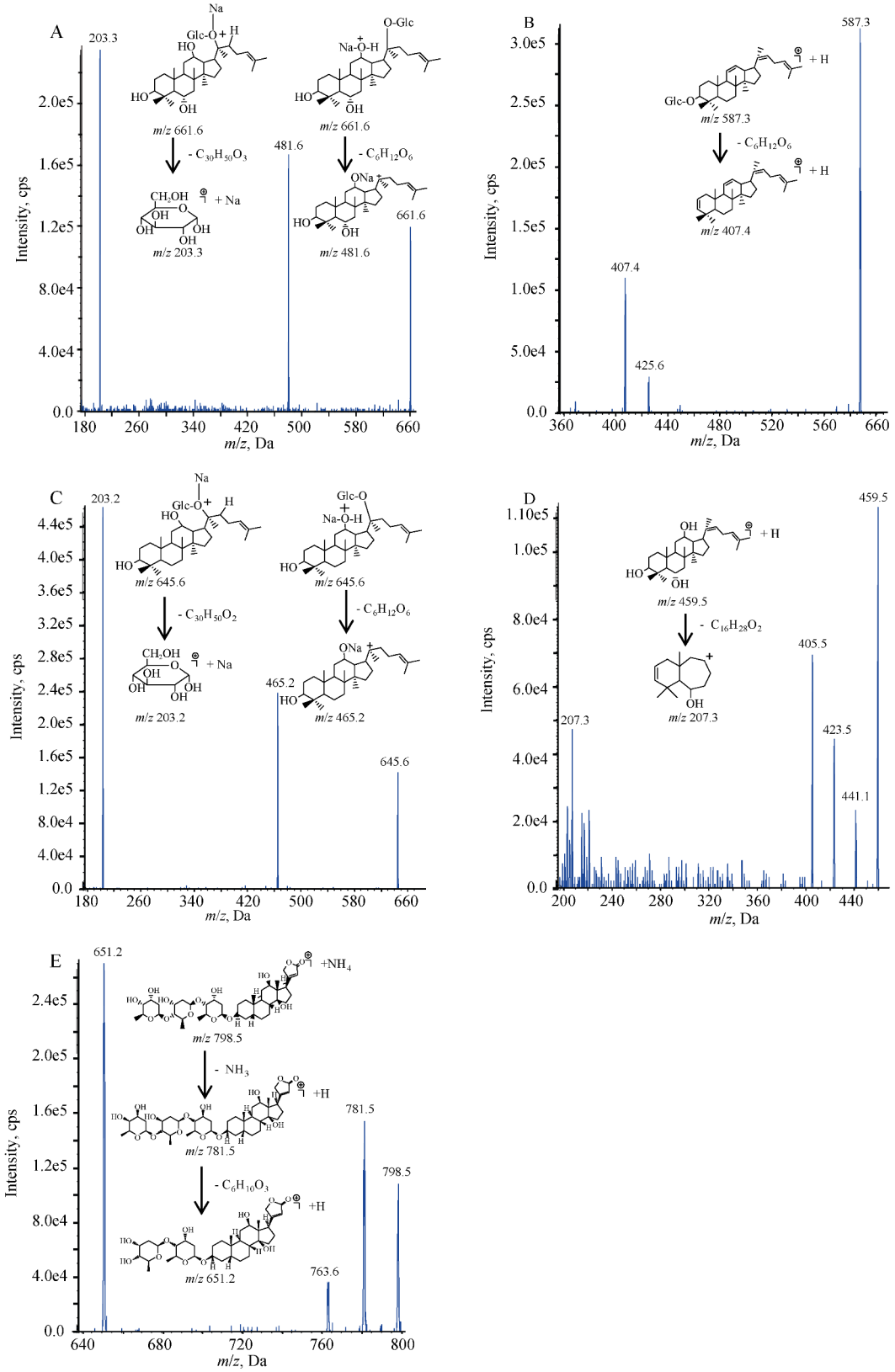

Fig. 2 Mass scan spectra and fragmentation pathways of GF1 (A), GRh2 (B), GCK (C), PPT (D) and digoxin (E) in positive ion mode

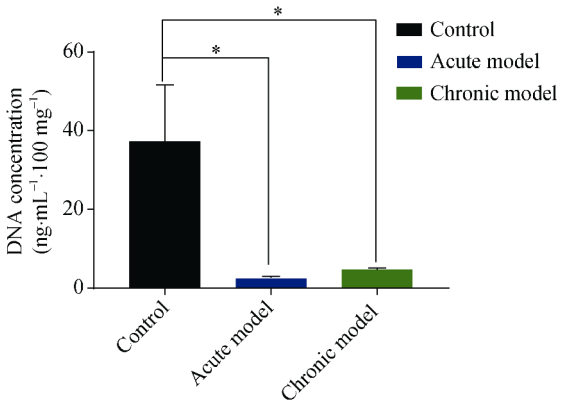

Fig. 3 Microbial DNA concentration in the feces from control, acute model and chronic model group (mean ± SEM, $^{*}P < 0.05$ *vs* control)

Methods validation

Selectivity

This method displayed a good selectivity for the detection of all analytes. Fig. 4 showed the typical MRM chromatograms of blank rat plasma and blank rat plasma spiked with analytes or with IS. There was no significant endogenous interference around the chromatographic regions of analytes and IS in all blank rat plasma samples. Also, baseline separation has been achieved between IS and analytes.

Linearity and LLOQ

Linearity range, correlation coefficients, regression equations and LLOQ of the four analytes in rat plasma were shown in table 1. All calibration curves showed good linearity ($R^2 \geqslant 0.993$ for all four analytes), and the LLOQ of GF1, GRh2, GCK and PPT were 0.210, 0.217, 0.241 and 1.17 ng·mL^{-1}, respectively. The method was highly sensitive for determining the metabolites in plasma sample.

Precision and accuracy

The results of intra- and inter-day precision and accuracy of four analytes in rat plasma were listed in Table 2. Intra- and inter-day precision of GF1, GRh2, GCK and PPT in three levels of QC samples were ranged from 6.4%–13.4%, 5.1%–14.6%, 2.6%–9.1%, 5.4%–13.9%, respectively. The intra- and inter-day accuracy of GF1, GRh2, GCK and PPT in three levels of QC samples were ranged from 97.0%–108.8%, 98.2%–105.3%, 95.7%–113.8%, 99.0%–108.2%, respectively. The results demonstrated that the method was performed with good precision and accuracy.

Extraction recovery and matrix effect

The results of extraction recovery and matrix effect were listed in table 3. The extraction recovery of GF1, GRh2, GCK and PPT in three levels of QC samples were ranged from 94.8%–104.1%, 98.0%–106.8%, 75.3%–77.5%, 92.5%–101.0%, respectively. Deviation of all four analytes at three levels was within 10%. It indicated that this method showed high and reproducible extraction recovery, which be able to extract the desired analytes well. The matrix effect of GF1, GRh2, GCK and PPT in three levels of QC samples were ranged from 86.1%–102.4%, 99.3%–112.0%, 85.1%–86.6%, 86.6%–114.3%, respectively. The matrix effect of all analytes was acceptable, and it showed that there were no endogenous substances significantly suppressed or enhanced the ionization of all analytes.

Stability

The results of stability were shown in table 4. Comprehensive analysis of long-term stability and freeze/thaw stability, the precision of GF1, GRh2, GCK and PPT in three levels of QC samples were ranged from 1.7%–9.7%, 6.7%–11.4%, 5.7%–10.6%, 4.6%–11.5%, respectively. And the accuracy of GF1, GRh2, GCK and PPT in three levels of QC samples were ranged from 87.3%–109.6%, 95.2%–104.5%, 91.7%–103.9%, 90.6%–105.8%, respectively. The results indicated that all analytes were stable after storing at –80 °C for 60 days or 3 freeze-thaw cycles.

Quantitative analysis

Four metabolites (GF1, GRh2, GCK and PPT) of PNS in plasma of each group after oral administration of PNS extract were analyzed using the validated method successfully. Fig. 5 presented the representative MRM chromatograms of four metabolites from control group, acute model group and chronic model group, respectively, after oral administration of PNS extract. In both model groups, the concentration of four metabolites were all below the limit of detection at any sampling time, while there were relative high responses of four metabolites in control group and their concentrations were listed in Table 5. As the biotransformed metabolites by gut microbiota, the plasma concentrations of all analytes were so low that could only be detected in 12 h, except that GCK could be quantified in 8 h, 12 h and 24 h. The maximum plasma concentrations of four metabolites were 0.64 ± 0.12 ng·mL^{-1}, 0.30 ± 0.11 ng·mL^{-1}, 3.60 ± 1.62 ng·mL^{-1}, and 2.19 ± 0.41 ng·mL^{-1}, respectively. It indicated that GCK and PPT were the most abundant metabolites of PNS, which could be bio-conversed by gut microbiota and absorbed into blood in normal rat among 48 h.

Discussion

In our study, a HPLC-MS/MS in MRM mode showed a high specificity. Positive ion mode was used for the mass detection to achieve higher sensitivity, compared with negative ion mode. 0.2% formic acid was added into mobile phase to keep the analytes positively charged easily. In this condition, precursor ions were set as $[M + Na]^+$ for GF1 (*m/z* 661.4), $[M + Na]^+$ for GCK (*m/z* 645.7), $[M - 2H_2O + H]^+$ for GRh2 (*m/z* 587.2), $[M - H_2O + H]^+$ for PPT (*m/z* 459.6) and $[M + NH_4]^+$ for IS (*m/z* 798.5). Mass scan spectra and fragmentation pathways of four analytes and IS were shown in Fig. 2.

For construction of pseudo GF models, antibiotics or antimicrobials were applied in some studies. It has been verified that antibiotics can change the profile and function of gut

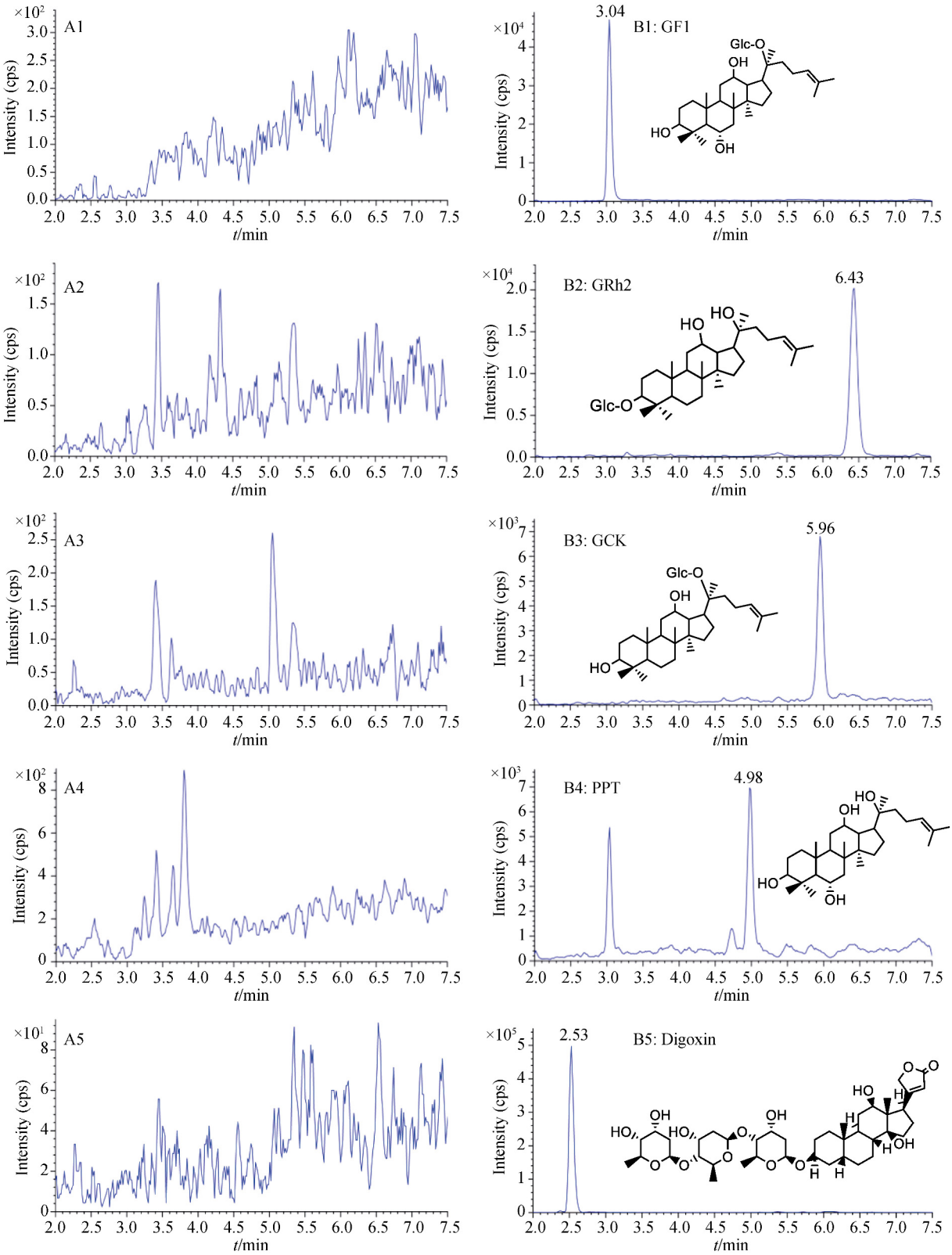

Fig. 4 Typical MRM chromatograms of blank rat plasma (A) and blank rat plasma spiked with IS or with analytes (B) in positive ion mode. B1, B2, B3, B4 and B5 were typical MRM chromatograms of blank rat plasma spiked with GF1 (5.03 ng·mL^{-1}), GRh2 (5.21 ng·mL^{-1}), GCK (5.78 ng·mL^{-1}) and PPT (4.67 ng·mL^{-1}) or IS (1.134 μg·mL^{-1}), and A1, A2, A3, A4 and A5 were the corresponding chromatograms of blank rat plasma. Time marked on the peak was the retention time of corresponding analytes

Table 3 Extraction recovery and matrix effect of four analytes in rat plasma (mean ± SD)

Compounds	c (ng·mL^{-1})	Recovery (%) (n = 6)	Matrix effect (%) (n = 6)
GF1	0.503	101.63 ± 0.04	89.43 ± 0.07
	8.05	104.09 ± 0.07	102.37 ± 0.09
	80.5	94.85 ± 0.08	86.09 ± 0.13
GRh2	0.521	97.98 ± 0.11	112.01 ± 0.09
	8.34	106.77 ± 0.03	104.97 ± 0.02
	83.4	104.20 ± 0.04	99.26 ± 0.02
GCK	0.578	77.51 ± 0.07	86.22 ± 0.10
	9.25	77.54 ± 0.08	85.10 ± 0.07
	92.5	75.30 ± 0.02	86.64 ± 0.04
PPT	1.87	92.46 ± 0.07	114.34 ± 0.14
	7.47	100.04 ± 0.04	89.44 ± 0.04
	74.7	100.95 ± 0.06	86.64 ± 0.10

Table 4 Long-term stability and freeze/thaw stability of four analytes in rat plasma

Compounds	c (ng·mL^{-1})	Long-term stability (n = 6)		Freeze/thaw stability (n = 6)	
		Accuracy (%)	Precision (RSD %)	Accuracy (%)	Precision (RSD %)
GF1	0.503	109.58	3.97	91.90	1.65
	8.05	96.77	9.64	103.18	9.55
	80.5	92.15	4.57	87.30	2.43
GRh2	0.521	97.74	7.88	95.20	6.82
	8.34	100.56	6.66	97.65	7.53
	83.4	104.46	7.05	99.64	11.34
GCK	0.578	102.70	7.84	103.91	5.63
	9.25	96.82	10.53	102.84	6.68
	92.5	95.61	7.62	91.73	5.90
PPT	1.87	94.47	6.45	104.28	8.53
	7.47	103.48	11.50	100.45	4.61
	74.7	105.78	4.55	90.63	9.15

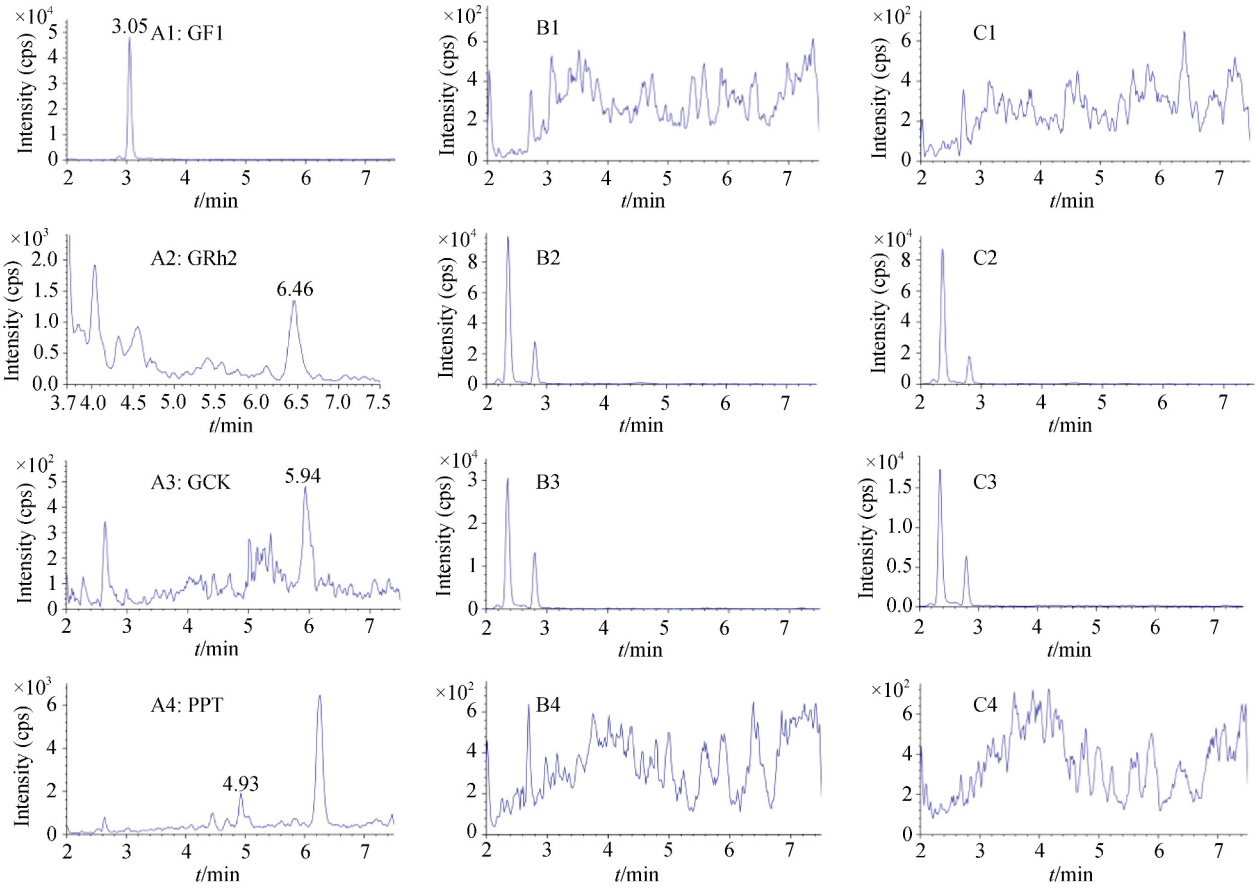

Fig. 5 Typical MRM Chromatograms of GF1, GRh2, GCK and PPT in the plasma of normal control group (A), acute model group (B) and chronic model group (C) after oral administration of PNS. A1, A2, A3, A4 were typical MRM chromatograms of GF1, GRh2, GCK and PPT in plasma from control group, respectively. B1, B2, B3, B4 were typical MRM chromatograms of four analytes in acute group, respectively. C1, C2, C3, C4 were typical MRM chromatograms of four analytes in chronic group, respectively

Table 5 Plasma concentrations of four metabolites in control group after oral administration of PNS extract (mean ± SD)

t/h	Metabolites (ng·mL^{-1})			
	GF1	GRh2	GCK	PPT
2	–	–	–	–
4	–	–	–	–
6	–	–	–	–
8	–	–	0.48 ± 0.29	–
12	0.64 ± 0.12	0.30 ± 0.11	3.60 ± 1.62	2.19 ± 0.41
24	–	–	0.74 ± 0.10	–
48	–	–	–	–

Short line means that the metabolites were below the detection limit.

microbiota [19, 30]. To construct pseudo GF rat models, rats were treated with a four-week or six-day course of different broad-spectrum antibiotics in our study. Metronidazole was widely used in the treatment of anaerobic infections, and vancomycin hydrochloride was only against Gram-negative bacterium. Gentamicin sulfate was an aminoglycoside antibiotic against Gram-negative bacterium by preventing bacterial protein synthesis, and neomycin sulphate and ceftriaxone sodium was against both Gram-positive and Gram-negative bacterium. Through the results of determination of microbial DNA concentration, Combination use of broad-spectrum antibiotics disrupted rat gut microbiota effectively both in the acute models and chronic models.

To analyze the results of biotransformation of PNS *in vivo*, combined with our previous study [18], PNS could be metabolized by normal gut microbiota. Moreover, the metabolites could be absorbed into rat plasma and detected. However, in model groups, no or few metabolites could be effectively generated by antibiotic-altered gut microbiota. Therefore, these results indicated that gut microbiota in health was essential for the biotransformation of PNS *in vivo*. PNS cannot be metabolized effectively in model groups. It may be associated with the decreased enzyme levels and activities, due to enough enzymes mediated biotransformation can no longer be secreted from disrupted gut microbiota.

In control group, the plasma concentrations of four metabolites were so low that could only be detected at some sampling time. Compared with other study [21, 32-34], the LLOQ of four metabolites in this method were more sensitive to detect metabolites in plasma. Therefore, the reason why metabolites at some earlier sampling time not be detected is that no or very little metabolites were generated during the first few hours after oral administration. Besides, Metabolites of PNS *in vivo* were excreted through feces at all time. Individual differences of rats affected the results, too.

The pharmacokinetics parameters of four metabolites were unable be calculated for evaluating their pharmacokinetics behavior in this study, due to the concentrations of four metabolites at some sampling time were below the detection limit. Analyzing plasma concentrations of them detected at 12 h after oral administration, however, GCK and PPT were still the most abundant metabolites.

Conclusion

PNS can be metabolized effectively to yield GF1, GRh2, GCK and PPT in control group with normal gut microbiota profile, while PNS cannot be metabolized in pseudo GF rat models. The gut microbiota plays an essential role in the transformation of PNS *in vivo*. Moreover, GCK and PPT are the most abundant metabolites of PNS *in vivo* among 48 h.

Abbreviation

ACN, acetonitrile; ESI, electrospray ionization; FDA, food drug administration; GCK, ginsenoside compound K; GF, germ free; GF1, ginsenoside F1; GRh2, ginsenoside Rh2; HPLC-ESI-MS/MS, high performance liquid chromatography-electrospray ionization tandem mass spectrometry; HQC, high quality control; IS, internal standard; LLE, liquid-liquid extraction; LLOQ, low limits of quantification; LQC, low quality control; MQC, medium quality control; MRM, multiple reaction monitoring; PNS, *panax notoginseng* saponins; PPT, propanaxatriol; QC, quality control; RSD, relative standard deviation; SD, Standard deviation.

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