Determination of Four Active Saponins of *Panax Notoginseng* in Rat Feces by High-Performance Liquid Chromatography

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Abstract

A method is developed for the determination of ginsenoside Rg_1, Rb_1, Rd, and notoginsenoside R_1 of *Panax notoginseng* (PNS) in rat feces after oral and intravenous administration of total saponins of PNS. The fecal samples are treated with organic extraction and solid-phase extraction prior to high-performance liquid chromatography. The calibration curves for the four saponins are linear in the given concentration ranges. The precision of the method is in the range of 1.0–10.0% (relative standard deviation), and the accuracy is between 80.0% and 110%. The recoveries of this method are all over 75%. This method is successfully applied to the analyses of fecal samples of rats treated with PNS.

Introduction

Panax notoginseng (PNS) Burk F.H. Chen (Araliaceae) is one of the most popular traditional Chinese medicines. It has been used to treat coronary heart disease, cardiac angina, and apoplexy in clinics (1–3). The total saponins of PNS have been regarded as the principal components manifesting the pharmacological activities. Like most traditional Chinese medicines, the effectiveness and safety of PNS is based on historical experiences. Thus, it is important to carry out the preclinical study, including absorption, distribution, metabolism, and excretion of PNS to provide evidences for its efficacy in clinics.

In a previous study (4), it was reported that the cumulative urinary excretions of notoginsenoside R_1, ginsenoside Rg_1, Rb_1, and Rd (Figure 1) are all lower than 10% of the dose after oral administration of PNS. It is therefore necessary to study the fecal excretion of PNS, which is useful for illuminating the excretion routes of PNS. Furthermore, there is no literature regarding the fecal excretion after administration of PNS. Odani et al. (5,6) determined the contents of ginsenoside Rg_1 and Rb_1 in rat feces with a thin-layer chromatography method after the administration of isolated individual ginsenosides.

In the present paper, a simple and sensitive pretreatment and high-performance liquid chromatography (HPLC) method were first developed to determine the contents of the four saponins in fecal samples after dosing of PNS. The results revealed that considerable amounts of saponins, in the unchanged form, are excreted in the feces from dosed rats.

Experimental

Chemicals and reagents

HPLC-grade acetonitrile was purchased from Merck Company (Merck, Darmstadt, Germany). Deionized water was purified by Milli-Q system (Millipore, Bedford, MA). Methanol was analytical grade from Beijing Reagent Co. Ltd (Beijing, China). An Extract-

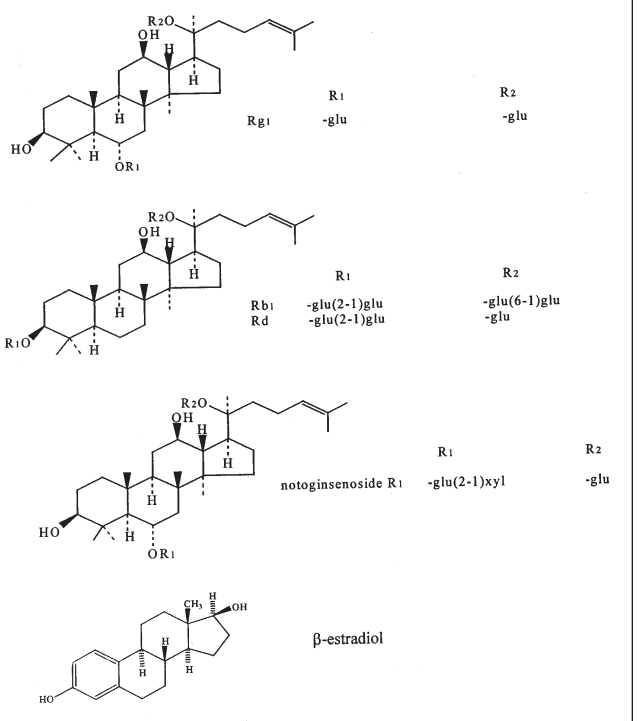

Figure 1. The structures of the four saponins in PNS and the internal standard.

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clean C_{18} solid-phase extraction (SPE) cartridge column was purchased from Alltech Company (Deerfield, IL).

Ginsenosides Rg_1 and Rb_1 were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Notoginsenoside R_1 and ginsenoside Rd were kindly provided by Professor Yinjie Chen (Shenyang Pharmaceutical University, Shenyang, China). β-Estradiol (purity ≥ 97%) was purchased from Fluka Chemical Company (Buchs, Swizerland). The total saponins of PNS, phytochemically obtained from the roots of PNS, were purchased from Wanfang Co. Ltd. (Wenshan, Yunnan, China). The injection prepared from PNS was manufactured by Kangyuan Pharmaceutical Co., Ltd. (Inner Mongolia, China).

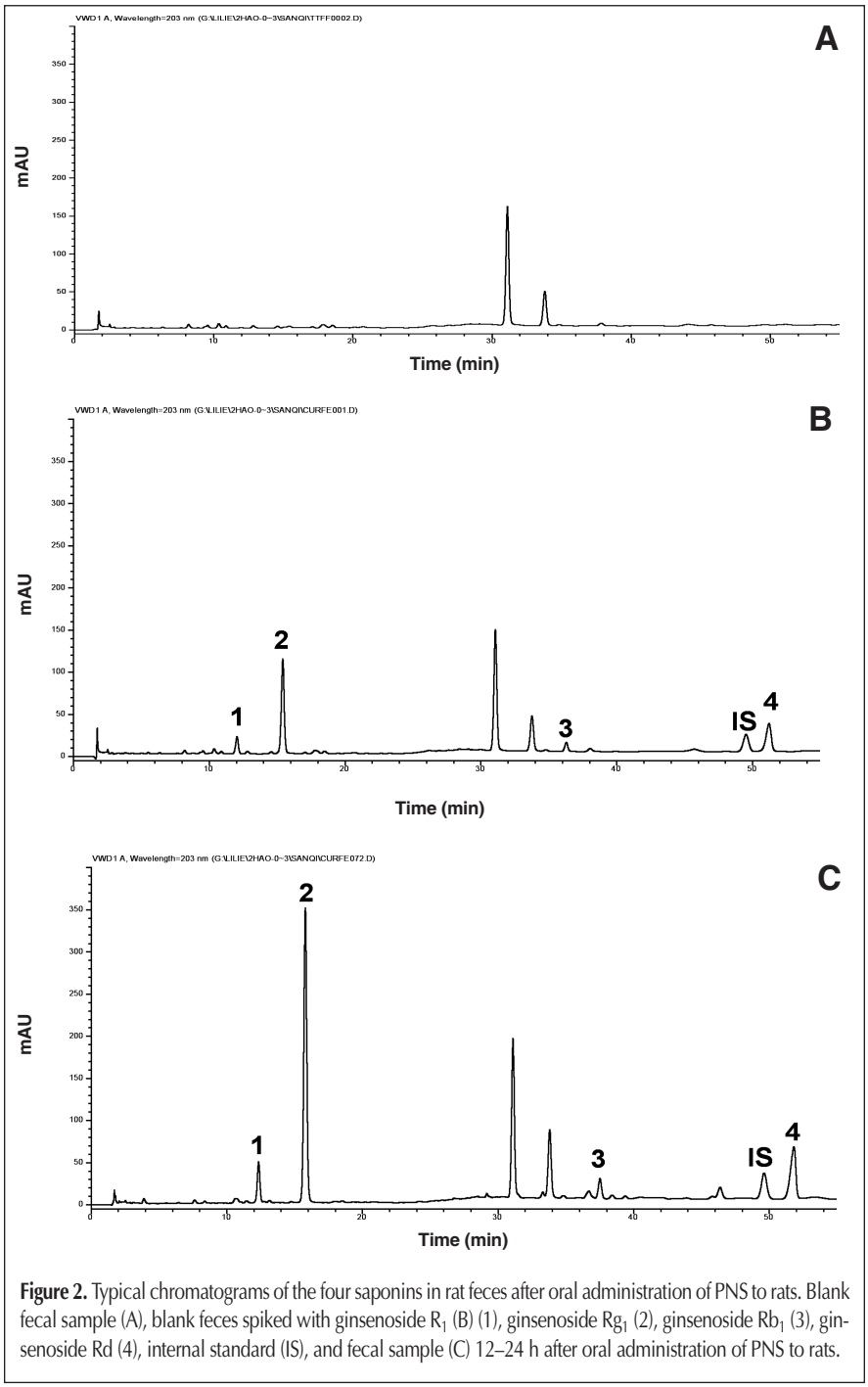

Figure 2. Typical chromatograms of the four saponins in rat feces after oral administration of PNS to rats. Blank fecal sample (A), blank feces spiked with ginsenoside R_1 (B) (1), ginsenoside Rg_1 (2), ginsenoside Rb_1 (3), ginsenoside Rd (4), internal standard (IS), and fecal sample (C) 12–24 h after oral administration of PNS to rats.

Instrumentation and chromatographic conditions

An Agilent 1100 liquid chromatography system (Agilent Technologies, Waldbronn, Germany), equipped with a quaternary solvent delivery system, autosampler, and UV detector, was used. The column configuration consisted of an Agilent Zorbax SB-C_{18} reversed-phase column (250- × 4.6-mm, 5 µm) and an Aglient Zorbax SB-C_{18} guard column (20- × 4-mm, 5 µm). UV absorption was measured at 203 nm.

Gradient elution of the analytes was performed using water (A) and acetonitrile (B). The initial condition was 20% B (v/v), linearly changed to 25% B at 20 min. The mobile phase composition was 31% B at 25 min. Over the next 30 min, the percentage of mobile phase B increased linearly to 40%. The total run time was 55 min. The column temperature was maintained at 35°C and the flow rate was 0.8 mL/min.

Animals and sample collection

Sprague-Dawley rats (220–230 g) from the Laboratory Animal Center of Peking University Health Science Center (Beijing, China) were randomly placed in individual metabolic cages. An 80 mg/mL PNS aqueous solution was administered orally with gavage at a dose of 80 mg/100 g to rats deprived of food but with free access to water for 12 h (800 mg PNS was easily dissolved in 10 mL distilled water after ultrasonication for 10 min). For intravenous experiments, an 8 mg/mL solution of PNS in 0.9% saline was given via the femoral vein at a dose of 50 mg/kg to rats. Feces were collected –12 to 0 h predose and 0–5, 5–11, and 11–24 h postdose. Feces were stored immediately at –18°C until processing.

Preparation of solutions and quality control samples

A stock solution of ginsenosides Rg_1, Rb_1, Rd, and notoginsenoside R_1 was prepared by dissolving the reference substances in a mixture of acetonitrile–water (1:1, v/v) to the final concentration of 1.0 mg/mL for ginsenoside Rb_1 and notoginsenoside R_1, 3.0 mg/mL for ginsenoside Rg_1, and 2.0 mg/mL for ginsenoside Rd. Working solutions were prepared by appropriate dilution of the stock solution with the mixture of acetonitrile–water (1:1, v/v) to appropriate concentrations. The internal standard (β-estradiol) was dissolved in acetonitrile to give a concentration of 100 µg/mL. All solutions were stored at –10°C and found to be stable for at least 1 month.

Quality control (QC) fecal samples were prepared at three concentrations (high, medium, and low) for the four saponins by adding appropriate working solutions to drug-free fecal homogenates and processed as fecal samples.

Fecal sample preparation

Fecal samples were weighted and homogenized in twice distilled water. An aliquot (~ 1.5 g of feces) of fecal homogenates, 10 mL of acetonitrile, and 100 µL of internal standard solution were combined in a glass tube and vortex mixed for 5 min, then centrifuged at 3000 rpm for 15 min. The supernatant was removed and evaporated to dryness under a nitrogen gas flow. The residue was dissolved in 2 mL of water. The solution was applied to an Extract-clean C_{18} cartridge column, which was preconditioned by passing through 5 mL methanol followed by 5 mL water before loading. The solid-phase cartridge was washed with 10 mL water and 4 mL 20% (v/v) aqueous methanol solution in that order, followed by elution with 5 mL 70% aqueous methanol solution. The eluate was evaporated at ambient temperature to dryness under a stream of nitrogen and reconstituted in 400 µL of 50% aqueous acetonitrile solution. A 5-µL aliquot was injected into the HPLC system for analysis. The calibration standards and quality control samples were applied to the same processing procedure described previously.

Assay validation

Linearity

The calibration curves consisted of at least seven points for the four saponins. These curves were prepared by adding 100 µL of internal standard solution and 600 µL working solutions in drug-free fecal samples and submitted to the same analytical procedure described for fecal sample preparation. Four calibration curves with standards in duplicate were analyzed in two separate runs. The peak-area ratios of the four saponins to internal standard were calculated and plotted against the concentrations of the four saponins in feces. The calibration curves were calculated by weighted (1/*x*: reciprocal of the concentration) least squares linear regression (7), and the correlation coefficient (*r*-value) was used to assess the linearity of the calibration curves. For every calibration curve, the calibration concentrations were back-calculated from the peak area of the analytes. The deviation from the nominal concentration was less than 6%.

Precision and accuracy

The intra- and interday variability was assessed by analyzing QC samples. QC samples were prepared from the stock solutions at three different concentrations: 6.30, 78.75, and 31.50 µg/g for notoginsenoside R_1; 3690, 495, and 198 µg/g for ginsenoside Rg_1; 840, 105, and 42 µg/g for ginsenoside Rb_1; and 2640, 330, and 132 µg/g for ginsenoside Rd. The intraday precision and accuracy were calculated by analyzing QC samples at three levels of concentration in five replicates. The analyses for calculation of interday accuracy and precision were performed for four consecutive days.

The precision was evaluated by intra- and interday percent relative standard deviation (%RSD) (within ± 15% for all concentrations). The accuracy is a measure of the systematic error and bias. It is defined as the agreement between the measured concentration and nominal value. Accuracy of the method is calculated from the expression:

$$\% \text{ bias} = \text{measured value/nominal value} \times 100 \qquad \text{Eq. 1}$$

which should be within 75% and 115% for all the concentrations.

Limit of quantitation

The limit of quantitation (LOQ) was determined during the

Table I. Intra- and Interday Variability for the Assay of the Four Saponins of PNS in Feces

	Intraday precision (*n* = 5)			Interday precision (*n* = 4)		
Spiked concentration (µg/g)	Measured concentration (µg/g)	RSD* (%)	Accuracy† (%)	Measured concentration (µg/g)	RSD (%)	Accuracy (%)
Notoginsenoside R_1						
630	549.43 ± 14.67	2.7	87.2	554.02 ± 10.54	1.9	87.9
78.75	81.17 ± 2.07	2.6	103.1	79.41 ± 2.24	2.8	100.8
31.5	33.55 ± 0.99	2.9	106.5	33.63 ± 0.74	2.2	106.8
Ginsenoside Rg_1						
3960	4155.35 ± 83.99	2.0	104.9	4028.13 ± 163.07	4.0	101.7
495	513.37 ± 6.77	1.3	103.7	527.26 ± 17.00	3.2	106.5
198	204.83 ± 13.21	6.4	103.4	190.83 ± 16.75	8.8	96.4
Ginsenoside Rb_1						
840	807.91 ± 36.73	4.5	96.2	787.16 ± 33.34	4.2	93.7
105	108.96 ± 3.25	3.0	103.8	110.82 ± 2.57	2.3	105.5
42	44.94 ± 3.17	7.1	107.0	44.43 ± 1.83	4.1	105.8
Ginsenoside Rd						
2640	2175.69 ± 56.71	2.6	82.4	2199.00 ± 38.41	1.7	83.3
330	355.22 ± 7.37	2.1	107.6	339.37 ± 19.22	5.7	102.8
132	111.87 ± 5.73	5.1	84.8	111.20 ± 4.19	3.8	84.2

* Relative standard deviation.
† Accuracy = [1 – (nominal concentration – mean of measured concentration)/nominal concentration] × 100.

evaluation of the linear range of the calibration curve (8). For the concentration to be accepted as the LOQ, the percent deviation of the nominal concentration and the RSD have to be within the range of ± 20% and less than 20%, respectively.

Recovery and stability

Known concentrations of the four saponins (high, medium, and low) and 150 µL internal standard solution were added to the drug-free fecal suspension. They were processed and analyzed according to the described method. Equivalent amounts of the analytes were dissolved in a 50% aqueous solution of acetonitrile. The absolute recoveries of the four saponins were assessed by comparison of the peak area obtained by direct injection of pure standard solutions to that obtained by injection of extracts of fecal samples. Each concentration of fecal samples was prepared in four replicates. The QC samples were used to examine the stability of the fecal samples. They were stored at room temperature for 48 h and –10°C for 10 days, then the samples were analyzed.

Results and Discussion

Chromatography

Simple and reproducible separation of the analytes was achieved using a silica, reversed-phase column. The pretreatment procedure, involving the removal of the interferences derived from the biological matrix, is important for the specificity, accuracy, and precision of the analytical procedure. SPE was suitable and applied for the extraction of the drug from the biological matrix with high selectivity and efficiency. It was proven to be suitable for the extraction of the drug from the biological matrix with high selectivity and efficiency. Figure 2 shows representative chromatograms obtained from the analysis of blank fecal samples and unknown fecal samples of rats administered orally or intravenously with PNS. Chromatographic profiles indicated that the four saponins and internal standard are well separated from each other without endogenous interferences. Determination of the presence of the four active ginsenosides in fecal samples is based on matching retention time and maximum UV absorption with known standards.

In this experiment, β-sitosterol, digtoxin, and β-estradiol were compared. It was found that β-estradiol was the most suitable internal standard, which was structurally close to the four saponins and possessed analogous behavior with the analytes during both the sample preparation procedure and detection in the HPLC system.

Linearity and limit of quantitation

The calibration graphs were linear over the concentration ranges of 30.0–1300 µg/g for notoginsenoside R_1, 200–12000 µg/g for ginsenoside Rg_1, 40–800 µg/g for ginsenoside Rb_1, and 100–2800 µg/g for ginsenoside Rd, respectively. It provided typical linear regression for the four saponins: notoginsenoside R_1, $Y = 0.0011X - 0.012$ ($r^2 = 0.9999$); ginsenoside Rg_1, $Y = 0.00065X + 0.116$ ($r^2 = 0.9995$); ginsenoside Rb_1, $Y = 0.0008X - 0.0089$ ($r^2 = 0.9993$); and ginsenoside Rd, $Y = 0.00078X - 0.0003$ ($r^2 = 0.9994$). Y is the area ratio of analyte versus internal standard, and X is the concentration of the analyte. The limit of quantitation was 1.0 µg/g for notoginsenoside R_1, 1.5 µg/g for ginsenoside Rg_1, 1.1 µg/g for ginsenoside Rb_1, and 1.3 µg/g for ginsenoside Rd.

Precision and accuracy

The results of inter- and intraday precision and accuracy are shown in Table I. The precision (coefficient of variation) of the method in rat feces was within the acceptable limits (less than

Table II. Recovery of the Four Saponins in PNS from Urine

Saponins	Spiked concentration (µg/g)	Recovery*,† (%)	RSD (%)
Notoginsenoside R_1	525.0	91.5 ± 0.6	0.7
	210.0	86.8 ± 2.2	2.5
	52.5	76.5 ± 1.2	1.6
Ginsenoside Rg_1	3300.0	89.0 ± 1.3	1.5
		1320.090.3 ± 2.7	3.0
	330.0	82.0 ± 1.3	1.6
Ginsenoside Rb_1	700.0	84.2 ± 0.5	0.6
	280.0	82.4 ± 0.9	1.1
	70.0	81.4 ± 2.3	2.8
Ginsenoside Rd	2200.0	85.2 ± 0.4	0.5
		880.078.7 ± 0.3	0.4
	220.0	81.0 ± 2.2	2.8

* Recovery = [1 – (spiked concentration – measured concentration)/spiked concentration] × 100.
† All values quoted are the mean ± SD (n = 4).

Table III. Stability of the Four Saponins in Rat Urine Stored at –10°C and at Ambient Temperature (n = 4)

QC Concentration (µg/g)	Stored at ambient temperature			Stored at –10°C		
	Before storage	After storage	Recovered (%)	Before storage	After storage	Recovered (%)
Notoginsenoside R_1						
525.0	523.9	476.7	91.0	524.1	474.8	90.6
210.0	208.6	177.5	85.1	208.7	181.4	86.9
2.5	53.4	44.2	82.8	52.9	50.4	95.8
Ginsenoside Rg_1						
3300	3301.0	2984.1	90.4	3304.0	2993.4	90.6
1320	1324.0	1132.0	85.5	1321.0	1163.8	88.1
330.2	325.6	298.2	91.6	330.4	289.4	87.6
Ginsenoside Rb_1						
700.0	702.5	562.0	80.0	701.6	566.9	80.8
280.0	281.5	240.1	85.3	281.1	278.0	98.9
70.5	71.2	81.0	113.8	71.6	59.1.	82.5
Ginsenoside Rd						
2200	2204.0	1961.6	89.0	2201.0	1961.1	89.1
880.2	881.9	978.0	110.9	880.5	807.4	91.7
220.1	222.3	188.1	84.6	223.4	244.0	109.2

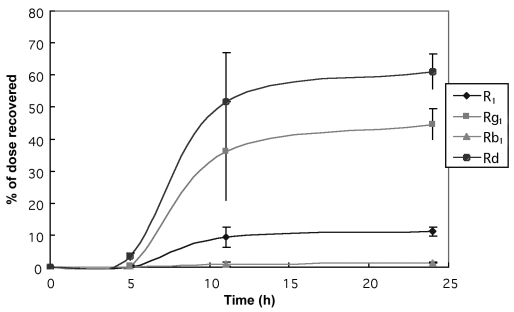

Figure 3. Cumulative excretions of notoginsenoside R_1 (◆), ginsenoside Rg_1 (■), ginsenoside Rb_1 (▲), ginsenoside Rd (●) in feces after oral administration of PNS saponins to rats. Each point with bar represents mean ± standard deviation; (n = 3) is the percentage of the administered dose of PNS.

Table IV. Concentrations of the Four Saponins Found in Feces after Oral Administration of PNS to Rats (n = 3)

Tested saponins	Concentration found (μg/g) 0–5 h	5–11 h	11–24 h
Notoginsenoside R_1	ND*	533.43 ± 110.19	734.79 ± 171.42
Ginsenoside Rg_1	42.75 ± 19.28	5886.97 ± 714.62	9179.63 ± 1979.4
Ginsenoside Rb_1	ND	187.05 ± 60.86	485.33 ± 119.63
Ginsenoside Rd	101.75 ± 36.27	1995.42 ± 303.28	2571.9 ± 770.03

* ND = not detected.

15%). The percent accuracy was within ± 15% deviation of the nominal values both for intra- and interday assays.

Recovery and stability

The recoveries of notoginsenoside R_1, ginsenoside Rg_1, Rb_1 and Rd from spiked fecal samples were calculated by comparing the peak area of extracted samples with those obtained from the analysis of corresponding, directly-injected standards. The results are shown in Table II.

The stability study showed that the four saponins were stable in feces at room temperature for 48 h and –10°C for at least 10 days. The results are shown in Table III.

Application of the method to fecal samples

The main routes of drug excretion in quantitative terms are the renal and biliary (hepatic) ways. The renal excretion is particularly important in the elimination of more polar compounds and drug metabolites. Compounds strongly bound with serum albumin are more easily excreted into bile than into urine (6). Substances excreted into the feces are mainly unabsorbed, orally-ingested drugs or those which are excreted in the bile and not reabsorbed by the gut (9).

In a previous study (4), the cumulative urinary excretion of notoginsenoside R_1, ginsenoside Rg_1, Rb_1, and Rd within 48 h was 1.79%, 5.14%, 0.75%, and 6.62%, respectively. It seems that urinary excretion is not the main excretion route for the four saponins after dosing of PNS. The time variations of fecal excretions of the four saponins after oral administration of PNS to rats are shown in Figure 3. The cumulative fecal excretions of notoginsenoside R_1, ginsenoside Rg_1, Rb_1, and Rd within 24 h are 11.1%, 44.66%, 1.39%, and 61.11%, respectively. Table IV shows the concentrations of the four saponins in fecal samples collected 0–24 h after dosing of PNS to rats. The highest concentration of fecal samples was observed at 11 to 24 h.

The concentrations of the four saponins are too low to be detected after intravenous dosing of PNS, which indicates that the four saponins are mainly excreted in urine or in bile, and it may be hypothesized that the four saponins excreted in bile may be reabsorbed in the gut or decomposed or metabolized in the intestine. Previous work supports this assumption (6). However, this hypothesis needs to be proven by further investigation.

Conclusion

The chromatographic system described in this work has been shown to be applicable to the determination of the four saponins in feces after administration of PNS to rats. The HPLC analysis and the method, based on SPE, showed adequate sensitivity, selectivity, and reproducibility. It could be used in further investigations on the absorption, distribution, metabolism, and excretion of PNS saponins.

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