Simultaneous Determination of 17 Ginsenosides in Rat Urine by Ultra Performance Liquid Chromatography–Mass Spectrometry with Solid-Phase Extraction

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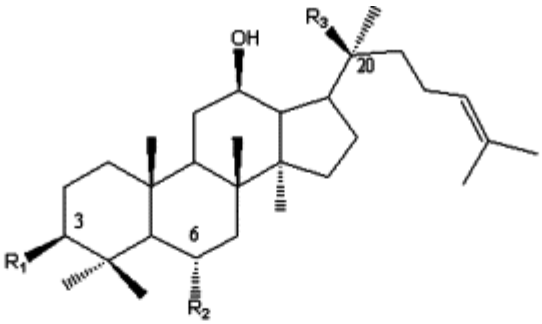
Abstract:

A rapid analytical method for quantifying 17 ginsenosides in rat urine by ultra performance liquid chromatography (UPLC) coupled to electrospray ionization mass spectrometry (ESI-MS) is described. All analytes were extracted by solid-phase extraction optimized to obtain good recovery and quantified using digoxin as an internal standard. ESI-MS was optimized for different cone voltages at positive ionization mode to allow simultaneous analysis of all analytes in a relatively short time. Qualitative methodological considerations, including the linear range, precision, limit of quantification, limit of detection, recovery and sensitivity are also provided.

Article:

INTRODUCTION

Species of *Panax*, including *P. ginseng*, *P. quinquefolium* L. and *P. notoginseng*, have been used for thousands of years and are now accepted as common medicinal herbs all over the world. More than 30 ginseng saponins (ginsenosides) have been isolated as the main biologically active constituents of ginseng [1,2]. There are two main maternal dammarane skeletons, protopanaxatriol (PPT) and protopanaxadiol (PPD) [3,4]. After oral administration, PPT ginsenosides are hydrolyzed to ginsenoside Rh_1 and PPD ginsenosides are converted primarily to compound-K via stepwise cleavage of the sugar moieties by intestinal bacteria [4,5]. Consequently, most ginsenosides could be found in both plasma and urine of human or animal after administration. Some of these glucosides and their biologically active metabolites (see Fig. 1) have been separated and analyzed by HPLC-UV, LC–MS, or GC–MS [6-9]. However, a method for simultaneous rapid separation and determination of ginsenosides (including Rg_1, Rb_1, Re, Rb_2, Rc, Rg_2) as well as secondary saponins and aglycones has yet to be described. Ginsengs and ginseng products consist of multiple components that may act synergistically once ingested *in vivo*. Thus, in order to clarify the pharmacological and toxicological properties of ginsengs, it is important to monitor active constituents as many as possible when profiling the ginsenoside composition of plant, product and biofluid.



Compound	R_1	R_2	R_3	m/z	Cone Voltage (V)
Rb_1	-O-Glc$^{2-1}$Glc	-H	-O-Glc$^{6-1}$Glc	1131.80	120
Rb_2	-O-Glc$^{2-1}$Glc	-H	-O-Glc$^{6-1}$Arap	1102.00	120
Rc	-O-Glc$^{2-1}$Glc	-H	-O-Glc$^{6-1}$Araf	1102.00	120
Rd	-O-Glc$^{2-1}$Glc	-H	-O-Glc	969.80	100
Re	-OH	-O-Glc$^{2-1}$Rha	-O-Glc	969.80	100
Rg_1	-OH	-O-Glc	-O-Glc	823.80	90
Rf	-OH	-O-Glc$^{2-1}$Glc	-OH	823.80	90
Rg_2	-OH	-O-Glc$^{2-1}$Rha	-OH	807.90	90
F_2	-O-Glc	-H	-O-Glc	807.90	90
Rg_3	-O-Glc$^{2-1}$Glc	-H	-OH	807.90	90
Digoxin	/	/	/	803.80	90
Rh_1	-OH	-O-Glc	-OH	661.80	80
F_1	-OH	-OH	-O-Glc	661.80	80
C-K	-OH	-H	-O-Glc	645.80	80
PPT	-OH	-OH	-OH	499.60	75
PPD	-OH	-H	-OH	483.60	75

Abbreviation: Glc, -D-glucopyranosyl; Arap, -L-arabinopyranosyl; Arafp, -L-arabinofuranosyl; Rha, -L-rhamnopyranosyl.

Figure 1: Chemical structures and *m*/*z* values of ginsenosides and their main degradation products in addition to the cone voltage setting according to *m*/*z* [*M* + Na].

Since many ginsenosides are of similar polarity and not easy to be chemically derivatized, it is difficult to detect multi-ginsenosides simultaneously by HPLC-UV or GC–MS. Consequently, LC–MS is becoming the most suitable and convenient instrument for ginsenoside analysis [8]. However, multi-components analysis requires better separation which often results in longer retention times and consumption of more mobile phase. The recently developed ultra performance liquid chromatography (UPLC) system offers a solution to this problem. The ACQUITY UPLC instrument (Waters Corporation, USA) is a commercially available UPLC system with full instrument modifications, including advancements in the solvent delivery module and new column technology, which utilizes pressure-tolerant 1.7 μm hybrid particles containing a bridged ethylsiloxane/silica structure allowing the Acquity to run routinely at pressures up to 15,000 psi [10]. The Acquity sample manager has also been modified to permit injection of as little as 1 μL using a needle-in-needle probe [10]. The combination of these technological developments produces increased sensitivity, improved peak resolution, and a significant reduction in analysis time compatible with rapid throughput [11,12].

We have established a rapid UPLC–MS quantification method for simultaneous multi-component analysis of ginsenosides and their metabolites in rat urine. The precision, linearity, limit of quantification (LOQ), limit of detection (LOD), recovery and sensitivity of the analytical method have been systematically evaluated. Effects of the solid-phase extraction process, UPLC elution, and mass conditions on ginsenoside separation and detection in spiked standards and urine samples are also investigated.

EXPERIMENTAL PROCEDURES

Materials and reagents

The internal standard, digoxin, and Rg_1, Rb_1, and Re ginsenosides were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Rf, Rb_2, Rg_2(S), Rg_2(R), Rc, Rd, Rh_1, Rg_3(S), Rg_3(R), C-K, F_1, F_2, PPT and PPD were obtained from Wuhu DELTA Co. (Anhui, China) and Sanqi Research Institute of Wenshan Prefecture (Yunnan, China). Total ginsenosides were purchased from Hangzhou Greensky Biological Tech. Co. Ltd. HPLC-grade methanol and acetonitrile were purchased from Merck (New Jersey, USA). Water used in the experiments was generated by a Milli-Q Ultra-pure water system (Millipore, Billerica, USA). Other chemicals (analytical grade) were purchased from Sigma (St. Louis, MO, USA). Oasis^{TM} HLB, 1cc, solid-phase extraction (SPE) cartridges and Manifold for Extraction Cartridge were purchased from Waters Co. (Milford, MA, USA).

Apparatus and operating conditions

Chromatographic separation was carried out on an ACQUITY UPLC^{TM} system from Waters Co. using an ACQUITY UPLC^{TM} BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm particle size). The column temperature was 40 °C, the flow rate was 0.35 mL min^{-1}, and the injection volume was 5 μL. The mobile phase consisted of water (A) and acetonitrile (B). The initial composition was 95% A and 5% B. A gradient elution was performed where phase B increased linearly from 0 to 50% over the first 6 min, then increased to 90% over 3 min, then increased to 95% in 1 min, was maintained for 2 min, and finally returned to the initial composition in 1 min. The column was re-equilibrated for another 1 min before the next injection.

Mass spectrometry was carried out on a Micromass–ZQ mass spectrometer (Waters Co.) using the positive electrospray ionization (ESI) mode (data analysis software MassLynx V4.1). Nitrogen was used as the nebulizer, desolvation and cone gas. The mass spectrometer was operated at a capillary voltage of 3.5 kV, desolvation gas flow of 700 L h^{-1}, cone gas flow of 50 L h^{-1}, desolvation temperature of 350 °C, source temperature of 120 °C, LM resolution of 15.2, HM resolution of 14.5, RF of 0 V, ion energy of 0.5 V, and multiplier voltage of 650 V. In the selected ion recording (SIR) mode, the cone voltage was set to different voltages according to different *m*/*z* (see Fig. 1).

Sample collection and preparation

Eight male Sprague-Dawley rats weighing 200 ± 20 g (8 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China) and individually housed in metabolic cages. Rats received total ginsenosides orally at a daily dose of 100 mg kg^{-1} from days 1 to 13. Blank 24-h urine samples were collected on day 0. Test urine samples were collected after ginsenoside administration on days 7, 10, and 13. The fresh urine samples were

immediately centrifuged at 3500 rpm for 10 min at room temperature in order to remove particle contaminants, and then supernatants were stored at −20 °C pending analysis. The urine volume and body weight of each rat were recorded over the entire course of the experiment.

HLB solid-phase extraction cartridges were equilibrated with 1 mL of methanol and 2 mL of water, then 700 μL of urine supernatant with 50 μL of internal standard solution (1 μg mL^{-1}) was passed through the cartridge under gentle vacuum. The cartridges were then washed five times with water and dried. Finally, the cartridge was eluted twice with 0.2 mL of 95% methanol (a mixture of 95% methanol and 5% of water) then mixed. After centrifugation at 20,000 × *g* for 10 min at 4 °C, 5 μL of the supernatant was injected into the UPLC/ESI-MS.

Method validation

Stock solutions for the 17 analytes were prepared separately in DMSO (dimethyl sulfoxide). Digoxin stock solution was prepared in pyridine. Working solutions were prepared in methanol by mixing known amount of all the compounds together. To simulate real sample analysis, a series of standard solutions including quality control (QC) samples were prepared using blank rat urine specimen spiked with known serial amounts of the 17 analytes and then extracted by SPE (prepared as described in Section 2.3) prior to UPLC–MS analysis (Table 1). The limits of detection and quantitation for each compound were estimated by the low concentration standard solutions mentioned above and the dilutions, when the target signal peak's signal-to-noise (S/N) ≥ 3 and ≥ 9. Based on the LOQ level, concentration scale and linear regression equation was established, where the regression coefficient (r^2) of each analyte's linear regression equation should be >0.99.

Table 1: Linearity for each component LOD and LOQ of spiked urine

Compound	Linear range (μg mL^{-1})[a]	*n*	r^{2}[b]	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
Rb_1	0.01–1	6	0.997541	2	8
Rb_2	0.01–1	6	0.997516	3	10
Rc	0.01–1	6	0.996406	4	12
Rd	0.01–1	6	0.998446	3	10
Re	0.02–2	6	0.99812	8	20
Rg_1	0.02–2	6	0.998321	6	18
Rf	0.02–2	6	0.996542	8	20
Rg_2(S)	0.05–5	6	0.997618	16	50
Rg_2(R)	0.05–5	6	0.997818	15	45
F_2	0.02–2	6	0.999299	8	20
Rg_3(S)	0.02–2	6	0.998446	6	18
Rg_3(R)	0.02–2	6	0.998848	8	20
Rh_1	0.05–5	6	0.998095	15	45
F_1	0.02–2	6	0.993589	8	20
C-K	0.05–5	6	0.995013	16	50
PPT	0.05–5	6	0.99955	18	55
PPD	0.05–5	6	0.998898	16	50

[a]Each standard stock solution of test compounds was carefully prepared in DMSO (1 mg mL^{-1}) and stored at −4 °C.
[b]Regression coefficients were calculated for linearity ranging at the concentration listed here.

In order to discover possible interference among analytes, specificity was determined by comparing QC samples with blank rat urine that had undergone the same pretreatment and analysis. Precision was calculated as the relative standard deviation (R.S.D.). Intraday precision and accuracy were evaluated by analyzing triplicates of three QC samples (low, middle, and high concentrations) over the course of a single day (Table 2). Interday variation was evaluated on the same QC samples on three different days. The freeze–thaw stability study was also carried out

every 7 days for a month on the same three QC samples stored at −20 °C in the dark. As biological specimen analysis, all these R.S.D. should be <15% [8].

Table 2: Recovery and precision (R.S.D.) (n = 3)

Compound	Spiked level (ng mL^{-1})	Recovery (%)		Intraday (ng mL^{-1})		Interday (ng mL^{-1})	
		Mean	R.S.D.	Mean	R.S.D. (%)	Mean	R.S.D. (%)
Rb_1	10.3	90.6	8.8	9.4	9.6	9.5	10.8
	103.2	92.1	7.2	103.7	8.2	106.8	11.3
	1032.0	94.5	5.2	1028.5	6.1	983.1	9.6
Rb_2	10.6	89.4	7.9	9.6	10.8	9.2	9.8
	106.4	93.1	6.6	96.4	9.7	93.7	10.4
	1064.0	93.9	5.0	995.3	9.1	978.0	9.5
Rc	10.2	92.5	7.3	10.2	11.2	10.5	10.6
	102.4	94.0	5.6	104.6	9.9	102.8	10.3
	1024.0	93.2	6.7	986.1	8.6	987.4	9.4
Rd	10.4	85.1	10.2	10.5	9.0	9.7	9.2
	104.3	82.9	9.4	92.8	9.6	95.0	8.7
	1043.0	84.7	7.9	973.0	9.4	1032.8	9.4
Re	20.5	78.3	9.2	18.5	9.5	19.4	10.8
	205.3	77.0	8.3	209.3	9.7	207.6	9.1
	2052.0	80.4	6.9	2016.8	9.0	1988.7	9.3
Rg_1	20.4	75.6	6.4	21.6	8.6	19.8	10.8
	204.3	75.1	7.1	195.3	9.4	208.9	9.2
	2043.0	76.5	5.8	2076.9	7.8	2058.4	8.7
Rf	20.2	77.6	7.0	19.6	8.6	19.8	11.8
	202.3	76.0	8.9	208.6	8.2	195.7	8.9
	2023.0	76.3	8.6	2043.9	7.9	2067.2	10.3
Rg_2(S)	50.4	81.5	9.6	47.8	10.3	50.4	9.9
	503.6	82.8	8.1	505.4	9.4	506.1	8.6
	5036.0	84.3	8.4	5060.1	9.0	5078.2	9.5
Rg_2(R)	50.6	82.6	9.9	48.1	9.9	48.7	10.5
	505.7	85.4	8.6	486.4	8.2	492.3	9.7
	5057.0	84.2	7.9	4957.2	7.8	5078.6	8.4
F_2	20.4	83.1	6.2	20.7	11.0	20.4	10.3
	203.8	85.8	5.4	195.4	9.7	208.9	10.1
	2038.0	82.6	3.0	2061.0	8.9	1992.6	9.0

Rg_3(S)	20.6	76.4	9.3	20.7	10.8	19.2	11.4
	206.2	75.3	7.6	185.6	9.3	190.8	10.7
	2062.0	75.6	7.2	1953.2	9.5	2037.9	9.6
Rg_3(R)	20.4	81.8	6.4	21.8	9.8	20.5	10.6
	203.7	82.3	5.8	186.3	9.0	194.6	9.4
	2037.0	83.4	4.7	2052.4	7.6	2089.5	8.9
Rh_1	50.8	82.5	8.0	49.6	9.1	51.7	9.7
	508.3	83.3	6.2	514.3	6.8	506.2	8.1
	5083.0	86.9	6.5	4962.7	5.7	5067.3	8.5
F_1	20.3	82.1	7.3	20.5	10.2	19.7	9.8
	202.6	85.4	5.0	191.4	9.4	208.9	9.7
	2026.0	83.6	6.8	1994.9	8.0	2013.6	9.2
C–K	50.4	77.4	11.6	53.1	8.6	50.1	10.6
	503.7	75.0	8.7	510.8	8.9	490.5	9.7
	5037.0	79.7	8.3	4990.5	8.1	5027.8	6.8
PPT	50.4	78.4	8.7	48.6	9.7	51.7	11.8
	504.0	76.3	7.4	488.3	8.3	506.2	8.8
	5040.0	75.1	6.1	4903.7	7.2	5068.1	7.9
PPD	50.3	75.4	6.7	52.8	9.9	49.3	10.6
	503.1	75.5	5.3	492.4	8.6	506.9	8.6
	5031.0	74.9	5.8	4937.0	7.5	5061.7	8.4

Extraction recovery was evaluated for the same QC samples by comparing peak area of interest from SPE pretreated sample with the same concentration of matrix-matched solutions. The QC samples were also used to further optimize the extraction method.

RESULTS AND DISCUSSION

UPLC/MS conditions

The ACQUITY UPLC provided a rapid, high resolution, economical method for multi-ginsenoside analysis (Fig. 2). Relative to HPLC–MS methods described in previous studies [13,14], UPLC reduced analytical time by at least 75%, thus significantly conserving eluent. All constituents presented good peak capacity with a 14-min gradient. In theory, the UPLC column requires a higher flow rate (above 0.6 mL min^{-1}) to gain super separation and the electrospray source can accommodate flow rates up to 1.0 mL min^{-1}. However, flow into the ion source of mass should be below 0.2 mL min^{-1} to increase ionization efficiency and ameliorate ion suppression which significantly influences analyte sensitivity. In order to obtain greater sensitivity and higher resolution simultaneously, we tested several different flow rate conditions before deciding on a flow rate of 0.35 mL min^{-1} as the best compromise. Most ginsenosides possessed similar polarity and showed almost dull peaks by isocratic elution, while gradient

elution resulted in better separation and lower LOQ. During gradient elution optimization, we found that hydrophilic substances eluted early and if the gradient changed too rapidly, Rg_1 and Re were easily buried by other hydrophilic impurities in urine. Therefore, we selected a gentle gradient and short analysis time in order to achieve effective elution and minimize matrix effects. Formic acid was added to the mobile phase at 0.1%, but it greatly reduced peak signal response with little enhancement of separation. Therefore pure water generated by Milli-Q and acetonitrile were chosen for the mobile phase.

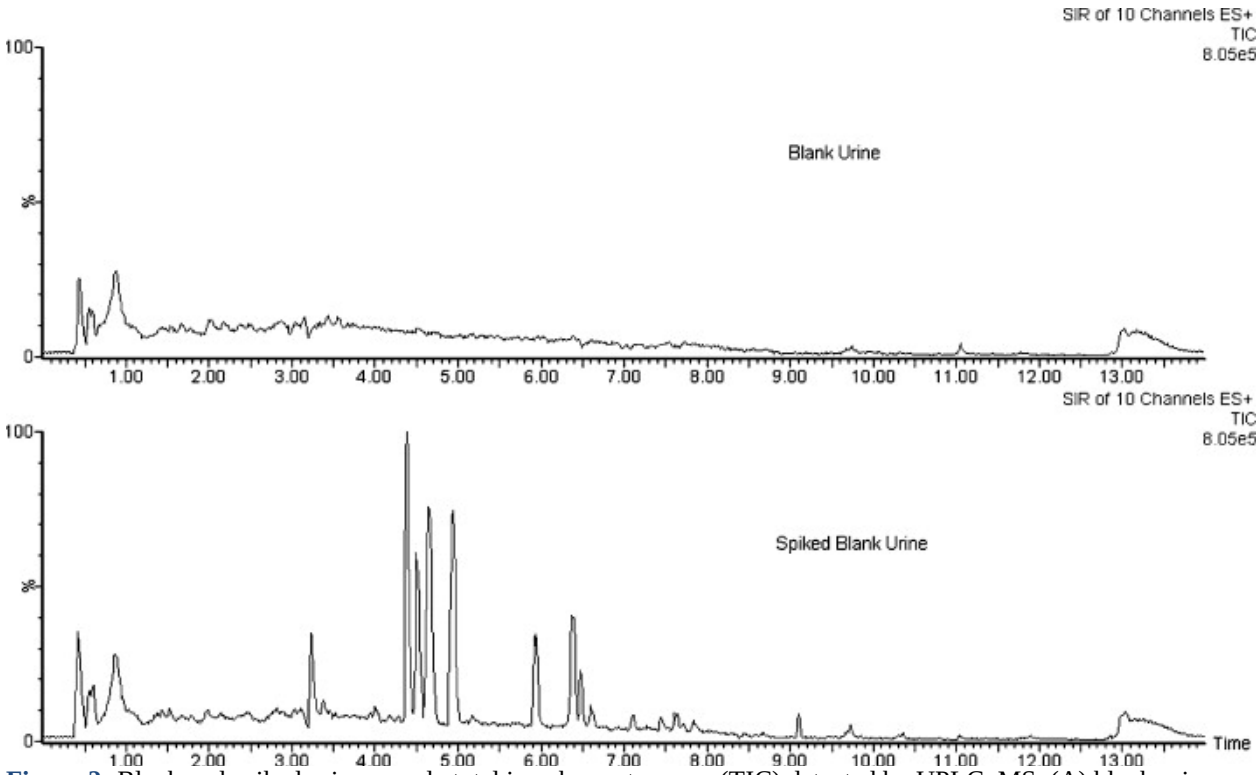


Figure 2: Blank and spiked urine sample total ion chromatograms (TIC) detected by UPLC–MS: (A) blank urine specimen; (B) spiked urine specimen (PPT and PPD 2.0 μg mL^{-1}, others 0.2 μg mL^{-1}).

In preliminary tests, standard substances were tuned at positive and negative modes directly in ZQ Mass with mobile phase. For all compounds, $[M + Na]^+$, $[M + K]^+$, and $[M - H]^-$ were found in the positive and negative ion spectra, respectively, but $[M + Na]^+$ was the most abundant, so the adduct ion of $[M + Na]^+$ was selected as the target *m*/*z*. For electrospray mass spectrometry, a flow rate of 0.35 mL min^{-1} showed better resolution but more interference by solvents, so the desolvation gas flow was set at 700 L h^{-1}, cone gas flow at 50 L h^{-1}, and desolvation temperature at 350 °C to remove redundant solvents. We had tried the other ionization mode atmospheric pressure chemical ionization (APCI), of the instrument and found that, although APCI could accommodate a higher flow (<2.0 mL min^{-1}) the intensive ionization style did not fit for the current simultaneous analysis of ginsenosides. Most of the analytes are saponins which have the instable glucosidic bonds connected with different glycons, as seen in Fig. 1. APCI source provides a higher temperature and more intense ionization leading to the glucosidic bonds cleavage and therefore failure of simultaneous quantification of ginsenosides with different glycons. Such an ionization mode is more suitable for low molecular weight compounds with non-polarity, which, in this case, do not represent the characteristics of ginsenosides. Therefore,

the ESI ionization mode was chosen for this study. In order to gain strong quasi-molecular ion response in spectrum (Fig. 1), cone voltage was optimized to values ranging from 75 to 120 V according to the different *m*/*z* of the analytes, which ranged from 483.6 to 1131.8.

Sample preparation

Interference from endogenous biochemical compounds presents a problem for analysis of biological specimens. Solid-phase extraction is a rapid, convenient sample treatment method for detecting metabolites in plasma and urine [15]. We compared the absolute extraction recovery of C_8 (Alltech Co., USA), C_{18}, and HLB SPE cartridges with the same matrix-matched samples. As seen in Fig. 3, extraction recovery of Rg_1 and Re was better with C_8 than others. However, recovery of the other compounds, including the internal standard, was significantly greater with HLB and C_{18} than C_8 cartridges. We assumed that the discrepancy reflected the polarity affinity of different chemical bonded phases and functional groups. For most target compounds, HLB cartridges provided both high recovery with less discrimination and better reproducibility under batch operations due to hydrophile–lipophile balanced packing material. Therefore, HLB SPE cartridges were chosen for this study. If the focus of study is analysis of origin saponins, such as Re and Rg_1, the C_8 cartridge is a good choice for sample treatment.

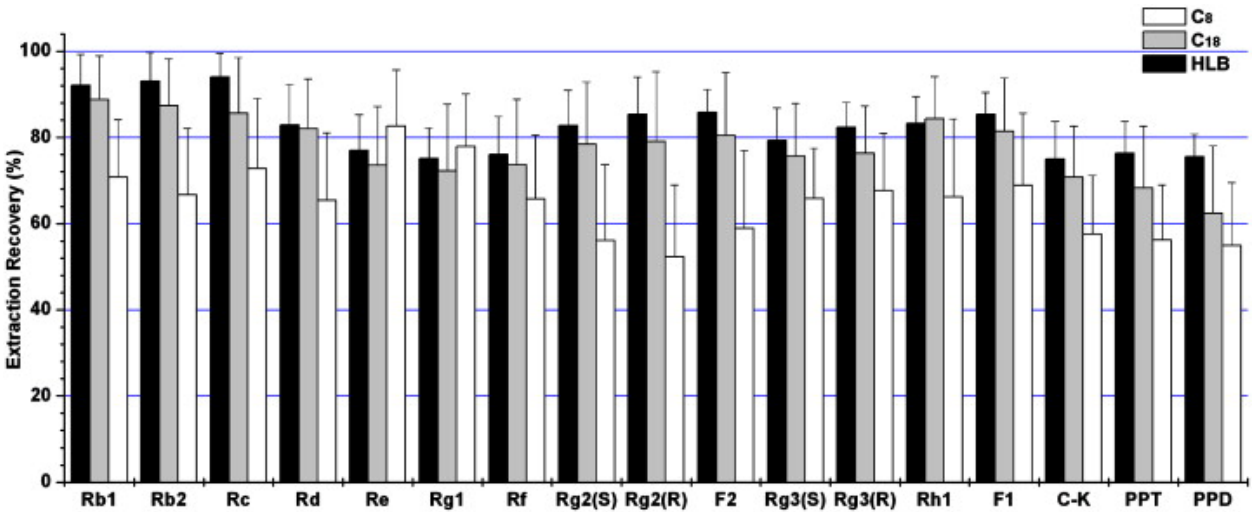


Figure 3: Comparison of the extraction recovery of the 17 analytes spiked in blank urine after extraction with HLB, C_{18} and C_8 SPE cartridge.

Loading speed was important for extraction, hence we investigated sample loading times ranging from 5 to 60 s. For all substances, extraction efficiency was similar when loading times were ≥40 s. Thus, we selected 40 s. Methanol concentrations ranging from 80% (a mixture of 80% methanol and 20% water) to 100% were tested for the final elution. After weighing the efficiency for each substance, 95% methanol was chosen as the eluent for the validation study.

Method validation

As shown in Fig. 2, blank urine did not produce interference under our working conditions. Both the analytes and internal standard could be detected on their own selected ion chromatograms without mutual interference (Fig. 4). Linearity of response was evaluated using matrix-matched standards ranging from 0.01 to 1 μg mL^{-1} for Rb_1, Rb_2, Rc and Rd; 0.02 to 2 μg mL^{-1} for Re, Rg_1, F_1, F_2, Rg_3(S), Rf and Rg_3(R); 0.05 to 5 μg mL^{-1} for Rg_2(S), Rg_2(R), Rh_1, C-K, PPT and

PPD as listed in Table 1. Calibration curves for each test compound were adjusted using the internal standard. The regression coefficients of determination (r^2) generally approached 1.000 (≥0.99) for all compounds tested. We determined the LOD and LOQ for each compound by analyzing signal-to-noise ratios ≥3 and ≥9. The S/N ratio calculation was carried out displaying the peak-to-peak values by Masslynx software. Occasionally, the actual quantification limit was less than the lowest spiked concentration, which had a higher S/N value. The lowest spiked concentration was used as the quantification limit in these instances.

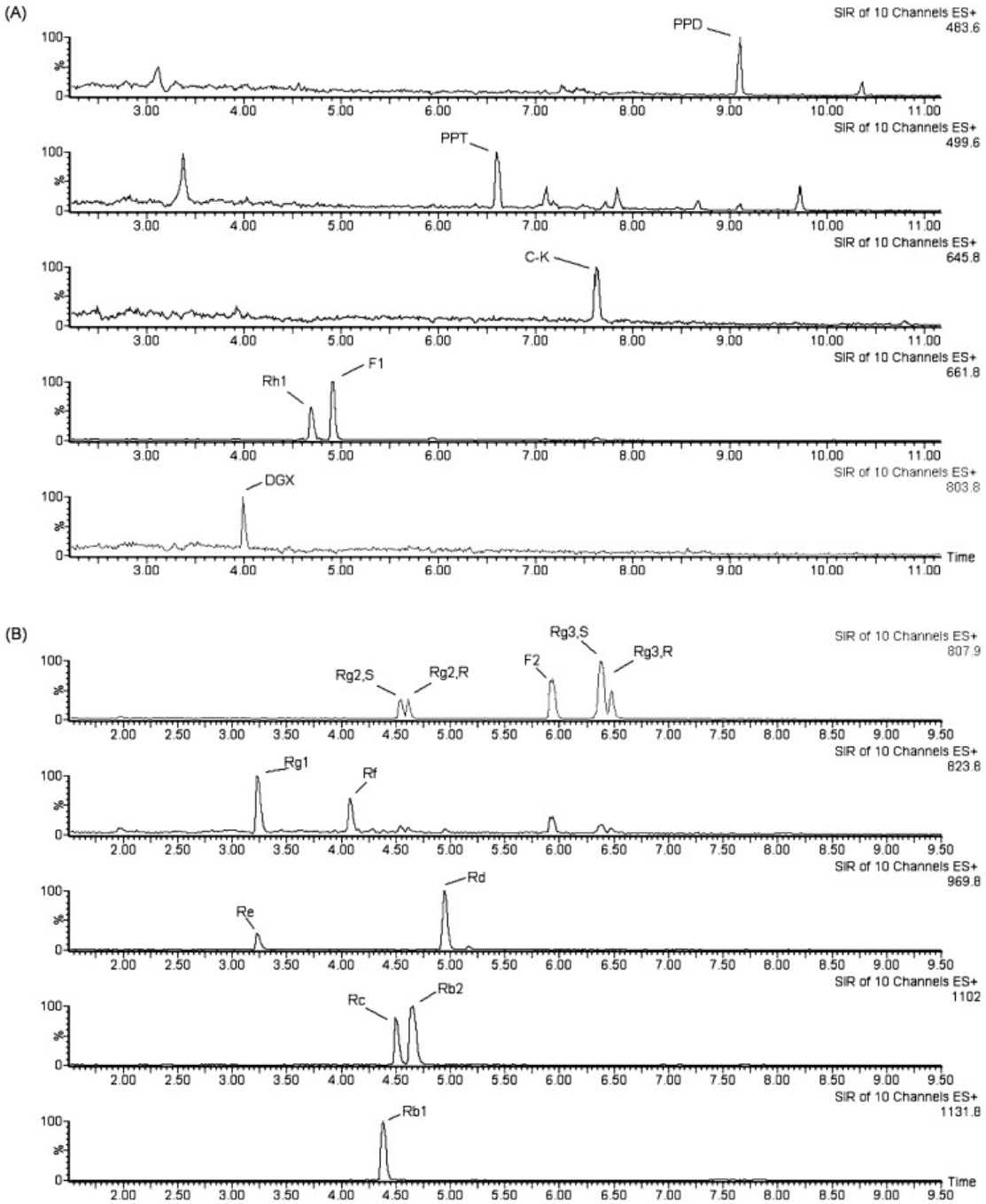


Figure 4: SIR chromatograms of blank urine specimen spiked with the 17 analytes (PPT and PPD 2.0 μg mL^{-1}, others 0.2 μg mL^{-1}). (A) *m/z* from 483.8 to 803.8; (B) *m/z* from 807.9 to 1131.8.

Recovery, intraday and interday precision were evaluated by analyzing triplicate samples of three concentrations of rat urine, as shown in Table 2. Recovery, intraday and interday performance, expressed as R.S.D., was less than 12% (the acceptance criteria is <15%) for all concentrations within the standard curve [8]. Similar results were obtained by two different operators.

The freeze/thaw stability of rat urine samples was studied by storing urine spiked with known amounts of each of the 17 standard substances at −20 °C. Samples were thawed at 37 °C and analyzed. The R.S.D. of the 17 analytes were less than 12% (the acceptance criteria is <15%) during freeze/thaw tests, indicating the substances were stable when stored at −20 °C then thawed at 37 °C.

Application of the method

Urine samples collected from rats orally administered total ginsenosides were analyzed using the method described above. As expected, all 17 ginsenosides were detected in all samples. A typical chromatogram is displayed in Fig. 5. Excretion of the 17 ginsenosides over a 24 h period was quantified on days 7, 10, and 13 (Fig. 6). The results demonstrate that our method can be applied for the detection and quantification of ginsenosides and their metabolites.

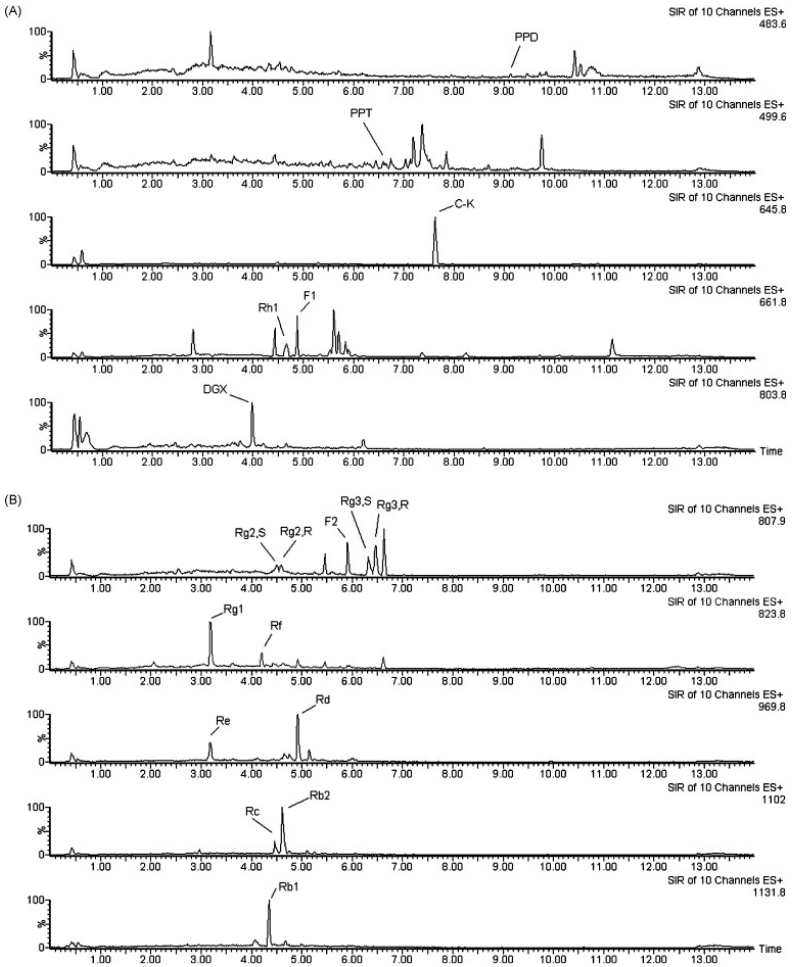


Figure 5: SIR chromatograms of 24-h urine sample after administration of the total ginsenosides. (A) *m/z* from 483.8 to 803.8; (B) *m/z* from 807.9 to 1131.8.

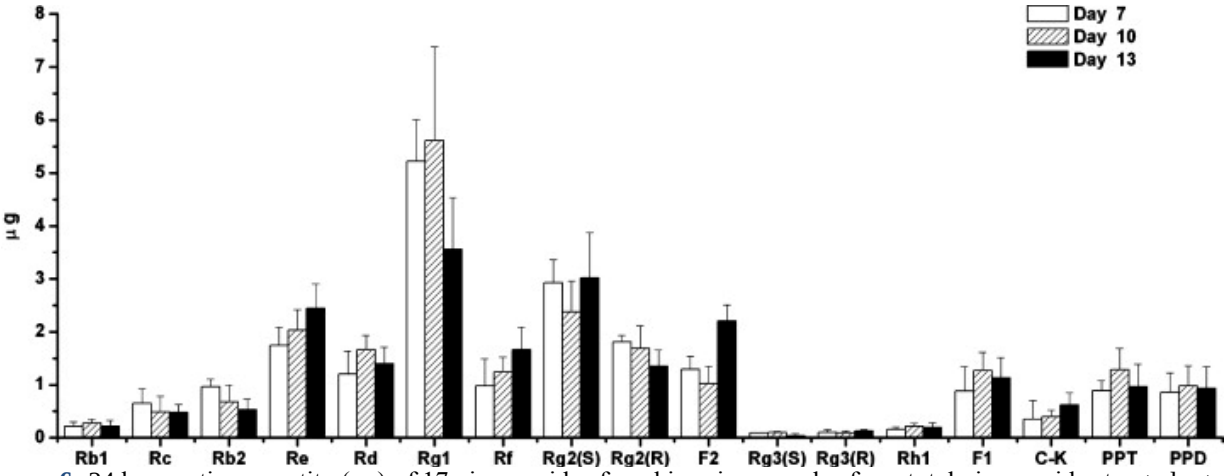


Figure 6: 24 h excretion quantity (μg) of 17 ginsenosides found in urine samples from total ginsenosides treated rats (n = 8).

CONCLUSIONS

A UPLC–MS method for rapid separation and quantification of 17 ginsenosides has been established and used to analyze rat urine. Target analytes were isolated by HLB solid-phase extraction, analyzed simultaneously, and precisely quantified using an internal standard. Compared to other methods, this procedure requires shorter analysis time and less solvent, while providing at least comparable sensitivity. We have shown that UPLC–MS technology is superior for characterizing and quantifying complex ginsenosides, secondary glucosides and aglycones. With SPE pretreatment, this method could be used to profile ginsenosides and their metabolites in ginseng pharmacokinetic and metabolism studies.

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