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Short communication

Simultaneous determination of ginsenoside Rb_1 and Rg_1 in human plasma by liquid chromatography–mass spectrometry

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Abstract

A liquid chromatographic–mass spectrometric (LC/MS) method for the simultaneous determination of ginsenoside Rb_1 and Rg_1 in human plasma was developed. The method involved the protein precipitation followed by analysis of ginsenoside Rb_1 and Rg_1 in an Atlantis C_{18} column with the gradient elution of acetonitrile and ammonium formate (10 mM, pH 3.0) at a flow rate of 0.2 ml/min. The analytes were determined using electrospray negative ionization mass spectrometry in the selected ion monitoring mode. The standard curves for ginsenoside Rb_1 and Rg_1 were linear over the concentration range of 10.0–1000 ng/ml. The lower limit of quantification was 10.0 ng/ml using 100 μl plasma sample. The coefficient of variation of intra- and inter-day assays for ginsenoside Rb_1 and Rg_1 at three quality control levels ranged from 1.0 to 6.8% and 5.4 to 9.8%, respectively. Ginsenoside Rb_1 and Rg_1 were stable in blank human plasma at room temperature for 24 h and following three freeze–thaw cycles.

Keywords: Ginsenoside Rb_1; Ginsenoside Rg_1; LC/MS; Plasma

1. Introduction

Ginseng has been used as health products or natural remedies for a long time [1]. The pharmacological properties of ginseng are generally attributed to dammarane triterpene glycosides, called ginsenosides. Ginsenosides are classified into two groups, with 20(*S*)-protopanaxadiol (ginsenoside Ra_1, Rb_1, Rb_2, Rb_3, Rc, Rd) and 20(*S*)-protopanaxatriol (ginsenoside Re, Rg_1, Rg_2, Rh_1) based on their aglycone moieties [1,2]. The concentrations of ginsenoside Rb_1 and Rg_1 (Fig. 1) in ginseng radix are relatively higher than those of other ginsenosides [3]. The absorption, distribution, excretion and metabolism of ginsenosides in animals have been studied by using large oral doses of isolated individual ginsenoside Rb_1 or Rg_1 to rats [4–11]. However, there are a few reports on the pharmacokinetics of ginsenosides in humans after consumption of ginseng or ginseng preparations [5,12,13]. Methods for determination of ginsenosides in biological fluids include thin layer chromatography (TLC) [4–9], high performance liquid chromatography

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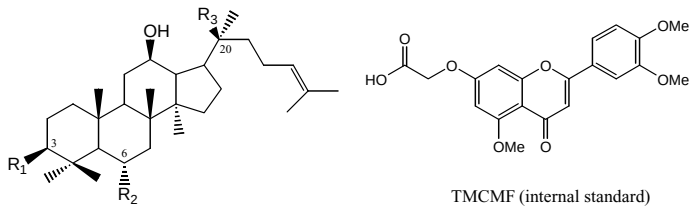

TMCMF (internal standard)

Compound	R1	R2	R3
Rb_1	-O-Glc-Glc	-H	-O-Glc-Glc
Rg_1	-OH	-O-Glc	-O-Glc

Glc : β-D-glucose

Fig. 1. Structures of ginsenoside Rb_1 and Rg_1, and TMCMF (I.S.).

(HPLC) with UV detection [5,9,10] or fluorescence detection [11], gas chromatography–mass spectrometry (GC/MS) [12] and liquid chromatography–mass spectrometry (LC/MS) with electrospray positive ionization [13]. Using the GC/MS method, 20(*S*)-protopanaxadiol and 20(*S*)-protopanaxatriol were measured as the genuine aglycones of ginsenosides in human urine [11]. TLC and HPLC methods with UV detection suffered from limited selectivity due to the presence of more than 20 structurally similar ginseng saponins and endogenous interferences from biological fluids. Liquid–liquid extraction with butanol [4,5] and the combination of protein precipitation and solid phase extraction [5–13] were used for sample preparation of ginsenoside Rb_1 and Rg_1 in biological fluids. The use of solid phase extraction in addition to protein precipitation in those reports [5–13] provided the cleanest extract in terms of selectivity but was the most complex and time-consuming.

The purpose of this study was to develop a simple, rapid and reliable LC/MS method for the simultaneous determination of two main saponins, ginsenoside Rb_1 and Rg_1 in human plasma using a simple protein precipitation method as sample preparation tool.

2. Experimental

2.1. Materials and reagents

Ginsenoside Rb_1 and Rg_1 were the gifts from Wonpharm Co. Ltd. (Iksan, Korea). 3′,4′,5-Trimethoxy-7-carboxymethyloxy flavone (TMCMF, I.S.) was given by Dong-A Pharm. Co. (Yongin, Korea). Hyosam® capsules (Wonpharm Co. Ltd.), a commercial ginseng preparation, were used for the application study. Acetonitrile and methanol (HPLC grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA) and the other chemicals were of the highest quality available.

2.2. Preparation of calibration standards and quality control samples

Primary stock solutions of ginsenoside Rb_1 and Rg_1, and TMCMF (1 mg/ml) were prepared in methanol. Working standard solutions of ginsenoside Rb_1 and Rg_1 were prepared by combining the aliquots of each primary solution and diluting with methanol. The working solution for I.S. (0.5 μg/ml) was prepared by diluting an aliquot of stock solution with methanol. All ginsenosides and TMCMF solutions were stored at ca 4 °C in polypropylene tubes in the dark when not in use.

Human plasma calibration standards of ginsenoside Rb_1 and Rg_1 (10.0, 20.0, 50.0, 100, 200, 500 and 1000 ng/ml) were prepared by spiking the working standard solutions into a pool of ten lots of drug-free human plasma. Quality control (QC) samples at 25.0, 150 and 750 ng/ml were prepared in bulk by adding 50 μl of the appropriate working standard solutions (2.5, 15 and 75 μg/ml) to drug-free human plasma (4950 μl). The bulk samples were aliquoted (100 μl) into polypropylene tubes and stored −20 °C until analysis.

2.3. Sample preparation

To a 100 μl aliquot of blank plasma, calibration standards and QC samples, 10 μl of I.S. working solution, 50 μl of 0.2 M zinc sulfate and 100 μl of acetonitrile were added. The samples were vortex-mixed for 5 min at high speed and centrifuged at 12,000 × *g* for 5 min. The supernatant was pipette transferred and was evaporated to the dryness in a Speed-Vac concentrator. The residues were dissolved in 30 μl of 30% acetonitrile in water by vortex-mixing for 2 min, transferred to injection vials, and 10 μl were injected onto a LC/MS system.

2.4. LC/MS analysis

The chromatographic system consisted of a Nanospace SI-2 pump, a SI-2 autosampler, a SI-2 column oven, a SI-2 switching valve and a S-MC system controller (Shiseido, Tokyo, Japan). The separation was performed on an Atlantis C_{18} column (5 μm, 2.1 mm i.d. × 100 mm, Waters Co., USA) using a gradient elution of (A) ammonium formate (10 mM, pH 3.0)-acetonitrile (95:5, *v*/*v*) and (B) acetonitrile at a flow rate of 0.2 ml/min: 25–32% mobile phase B for 4 min, 32–75% mobile phase B for 0.5 min, 75% mobile phase B for 4.5 min. The column and autosampler tray temperature was 40 and 4 °C, respectively. The eluent was introduced directly into the negative ionization electrospray source of a tandem quadrupole mass spectrometer (Quattro LC, Micromass UK Ltd. UK) from 2.5 min after the injection to decrease the contamination of ion source. The ion source and desolvation temperature were held at 120 and 350 °C, respectively. The optimum cone voltages were 70, 70 and 25 V for ginsenoside Rb_1, Rg_1 and TMCMF, respectively. Selected ion monitoring (SIR) mode was employed for the quantification: *m*/*z* 1107.6 for ginsenoside Rb_1, *m*/*z* 799.4 for ginsenoside Rg_1 and *m*/*z* 385.2 for TMCMF (I.S.). Peak areas for all components were automatically integrated using a MassLynx version 3.5 software (Micromass UK Ltd.).

2.5. Method validation

Batches, consisting of triplicate calibration standards at each concentration, were analyzed on three different days to complete the method validation. In each batch, QC samples at 25.0, 150 and 750 ng/ml were assayed in sets of six replicates to evaluate the intra- and inter-day precision and accuracy. The percentage deviation of the mean from true values, expressed as relative error (RE), and the coefficient of variation (CV) serve as the measure of accuracy and precision. The lower limit of quantification (LLOQ) was set at a level where the following criteria were met: signal-to-noise ratio $\geq$ 5.0 with RE $\leq \pm 20\%$ and CV $\leq$ 20% [14].

The absolute recoveries of ginsenoside Rb_1 and Rg_1 were determined by comparing the peak area of six extracted samples at the concentrations of 10.0, 25.0, 150 and 750 ng/ml with the mean peak area of recovery standards. Three replicates of each of the recovery standards were prepared by adding ginsenosides Rb_1 and Rg_1 or I.S. to blank human plasma extracts.

To evaluate the three freeze–thaw cycle stability and room temperature matrix stability, six replicates of QC samples at each of the low and high concentrations (25.0 and 750 ng/ml, respectively) were subjected to three freeze–thaw cycles or were stored at room temperature for 24 h before processing, respectively. Six replicates of QC samples at each of the low and high concentrations were processed and stored under autosampler conditions for 24 h were assayed to assess post-preparative stability.

2.6. Application

A healthy male volunteer, fasted for 12 h, received a single oral dose of six capsules of Hyosam®, each containing 250 mg ginseng powder fermented by intestinal bacteria, with 20 ml of water. Blood samples (2 ml) were withdrawn from the forearm vein at 0.5, 1, 2, 3, 4, 6 and 12 h post dosing, transferred to Vacutainer® tubes and centrifuged. Following centrifugation (3000 × *g*, 15 min, 4 °C), plasma samples were transferred to eppendorf tubes and stored at −70 °C prior to analysis. Drug concentrations were determined as the mean of duplicate samples.

3. Results and discussion

3.1. LC/MS

Ginsenoside Rb_1, Rg_1 and TMCMF (I.S.) were separated on an Atlantis C_{18} column using the linear

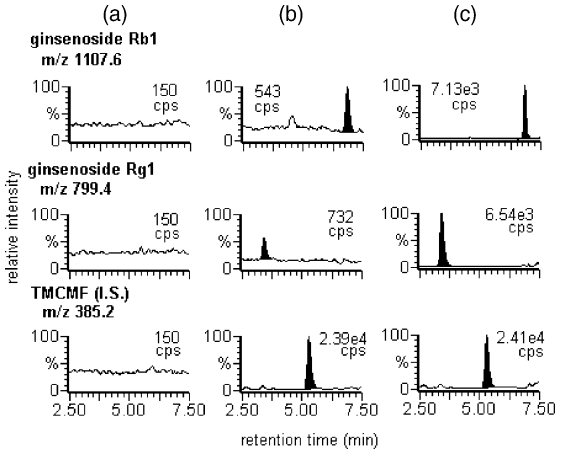

Fig. 2. Representative LC/MS chromatograms of: (a) a blank human plasma and a human plasma spiked with (b) 10.0 ng/ml and (c) 200 ng/ml of ginsenoside Rb_1 and Rg_1.

gradient of acetonitrile and ammonium formate (10 mM, pH 3.0). The retention times for ginsenoside Rb_1, Rg_1 and TMCMF were 6.8, 3.4 and 5.4 min, respectively (Fig. 2). The effects of pH of mobile phase on the ionization efficiency of ginsenoside Rb_1 and Rg_1 were evaluated in order to obtain the maximum sensitivity. The use of 10 mM ammonium formate adjusted to pH 3.0 as mobile phase gave the maximum sensitivity in LC/MS analysis of ginsenoside Rb_1 and Rg_1 in plasma samples.

Negative ion mode was more sensitive than positive ion mode for the ionization of ginsenoside Rb_1 and Rg_1. The electrospray ionization of ginsenoside Rb_1 and TMCMF (I.S.) with negative ion mode produced the molecular ion ($[M-H]^-$) at *m/z* 1107.6 and 385.2, respectively, without the presence of adduct ions and fragment ions. Ginsenoside Rg_1 exhibited $[M-H]^-$ at *m/z* 799.4 and formate adduct ion at *m/z* 845.4 in mass spectrum. The molecular ion of each compound was selected as SIR monitoring ion to obtain the selectivity and sensitivity for the determination of ginsenoside Rb_1 and Rg_1.

Fig. 2 shows the typical LC/MS chromatograms obtained from the analysis of blank human plasma and a human plasma sample spiked with ginsenoside Rb_1 and Rg_1 (10 or 200 ng/ml). In the analysis of blank plasma samples from seven different sources, there was no interference at the retention times of ginsenoside Rb_1, Rg_1 and TMCMF. Sample carryover effect was not observed.

3.2. Linearity

The calibration curves for ginsenoside Rb_1 and Rg_1 in plasma were linear over the concentration range 10.0–1000 ng/ml with correlation coefficient (γ^2) of 0.998 and 0.999 for ginsenoside Rb_1 and Rg_1, respectively. Linear regression analysis with a weighting factor of 1/concentration gave the acceptable accuracy and precision of the corresponding calculated concentrations at each level (Table 1). The CV values for the slopes of ginsenoside Rb_1 and Rg_1 indicated the repeatability of the method (Table 1).

3.3. Sensitivity

The lower limit of quantification (LLOQ) was set at 10.0 ng/ml for ginsenoside Rb_1 and Rg_1 using 100 μl of human plasma. As shown in Fig. 2b, the peak heights for ginsenoside Rb_1 and Rg_1 were at least five times than the background noise. The mean

Table 1
Calculated concentrations of ginsenoside Rb_1 and Rg_1 in calibration standards prepared in human plasma ($n = 9$)

Ginsenoside	Statistical variable	Theoretical concentration (ng/ml)							Slope	γ^2
		10.0	20.0	50.0	100	200	500	1000		
Rb_1	Mean (ng/ml)	10.3	19.9	46.2	90.4	198	494	1003	0.0029	0.998
	CV (%)	11.2	6.1	2.4	4.3	8.1	11.5	7.2	9.8	
	RE (%)	3.1	−0.7	−7.6	−9.6	−1.0	−1.2	0.3		
Rg_1	Mean (ng/ml)	11.3	20.3	47.1	89.1	192	495	1010	0.0024	0.999
	CV (%)	6.8	1.8	4.8	3.4	5.2	5.9	5.4	8.9	
	RE (%)	13.0	1.6	−5.8	−10.9	−4.0	−1.0	1.0		

Table 2
Precision and accuracy of ginsenoside Rb_1 and Rg_1 in human plasma quality control samples

Ginsenoside	Statistical variable	Intra-batch ($n = 6$)				Inter-batch ($n = 18$)		
Rb_1	Theoretical (ng/ml)	10.0	25.0	150	750	25.0	150	750
	Mean (ng/ml)	10.7	25.2	145	737	27.1	145	731
	CV (%)	2.1	6.8	5.0	6.0	8.3	5.4	7.9
	RE (%)	7.0	0.8	−3.3	−1.7	8.4	−3.3	−2.5
Rg_1	Theoretical (ng/ml)	10.0	25.0	150	750	25.0	150	750
	Mean (ng/ml)	10.9	24.8	149	747	25.7	147	737
	CV (%)	4.0	3.8	1.0	2.7	6.9	8.2	9.8
	RE (%)	9.0	−0.8	−0.7	−0.4	2.8	−2.0	−1.7

RE values were 7.0% for ginsenoside Rb_1 and 9.0% for ginsenoside Rg_1 at 10.0 ng/ml with CV of 2.1 and 4.0%, respectively (Table 2).

3.4. Intra- and inter-batch accuracy and precision

QC samples containing ginsenoside Rb_1 and Rg_1 were prepared and analyzed in assay batches 1–3. Table 2 shows a summary of intra- and inter-batch precision and accuracy. The intra-batch accuracy (RE) for ginsenoside Rb_1 and Rg_1 ranged from −3.3 to 0.8% at three different concentrations with the precision (CV) between 1.0 and 6.8%. The inter-batch accuracy (RE) for ginsenoside Rb_1 and Rg_1 ranged from −3.3 to 8.4% with the precision (CV) between 5.4 and 9.8% at three different concentrations. These results indicate that the present method has a satisfactory accuracy, precision and reproducibility.

3.5. Recovery

The absolute recoveries of ginsenoside Rb_1 and Rg_1 from spiked human plasma were determined at the concentrations of 10.0, 25.0, 150 and 750 ng/ml in six replicates. The recovery of ginsenoside Rb_1 at 10.0 ng/ml was 89.2 ± 7.7%; at 25.0 ng/ml, 94.0 ± 5.8%; at 150 ng/ml, 89.5 ± 7.6%; and at 750 ng/ml, 91.2 ± 3.8%. The recovery of ginsenoside Rg_1 was 94.1 ± 5.4%; at 25.0 ng/ml, 92.3 ± 8.6%; at 150 ng/ml, 96.1±7.7%; and at 750 ng/ml, 94.7±4.8%. The recovery of TMCMF (I.S.) at 50 ng/ml was 89.3 ± 3.2%. The protein precipitation with acetonitrile has been successfully applied to the sample preparation of ginsenoside Rb_1 and Rg_1 from plasma sample.

3.6. Matrix effect

Six samples of blank plasma from six independent sources spiked with ginsenoside Rb_1 and Rg_1 at 150 ng/ml were prepared and analyzed. CV and RE for ginsenoside Rb_1 were 4.5 and 0.9%, respectively. CV and RE for ginsenoside Rg_1 were 5.2 and −0.6%, respectively. These tight CV and RE values indicate no significant lot-to-lot variation in matrix effects.

3.7. Stability

Stability of ginsenoside Rb_1 and Rg_1 during sample handling (freeze–thaw and room temperature storage) and the stability of processed samples were evaluated (Table 3). Three freeze–thaw cycles and

Table 3
Stability of samples ($n = 6$)

Statistical variable	Theoretical concentration (ng/ml)			
	Ginsenoside Rb_1		Ginsenoside Rg_1	
	25.0	750	25.0	750
Three freeze and thaw stability				
Mean	25.7	741	24.4	751
CV (%)	5.2	7.1	4.4	3.9
RE (%)	2.8	−1.2	−2.4	0.1
Short-term temperature stability (24 h at room temperature)				
Mean	24.8	748	24.9	742
CV (%)	6.3	5.8	4.2	5.6
RE (%)	−0.8	−0.3	−0.4	−1.1
Post-preparative stability (24 h at room temperature)				
Mean	25.3	752	25.2	743
CV (%)	5.4	6.3	5.2	4.9
RE (%)	1.2	0.3	0.8	−0.9

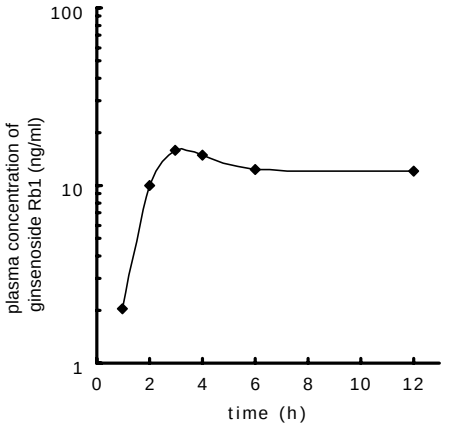

Fig. 3. Plasma concentration–time plot of ginsenoside Rb_1 following a single oral administration of Hyosam, a commercial ginseng preparation (1.5 g) in a male volunteer.

room temperature storage for 24 h before analysis had little effect on the quantification. Extracted QCs and calibration standards were allowed to stand at ambient temperature for 24 h prior to injection without affecting the quantification.

3.8. Application of method

The suitability of this method was proved in the pharmacokinetic study of ginsenoside Rb_1 and Rg_1 in human. Fig. 3 shows plasma concentration–time plot of ginsenoside Rb_1 after a single oral dose of a commercial ginseng preparation, Hyosam capsules (1.5 g) to a healthy male volunteer. The maximum concentration and the time to a maximum concentration of ginsenoside Rb_1 were 15.9 ng/ml and 3 h, respectively. However, ginsenoside Rg_1 was not detected in the plasma.

4. Conclusions

A sensitive and reliable LC/MS method has been described for the determination of ginsenoside Rb_1 and Rg_1 in human plasma using the protein precipitation as sample clean-up procedure. This method showed acceptable selectivity, sensitivity, precision, accuracy, linearity, recovery and stability of ginsenoside Rb_1 and Rg_1 in plasma samples. The present method was successfully applied to pharmacokinetic study for ginsenoside Rb_1 and Rg_1 after oral administration of a commercial ginseng preparation in human.

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