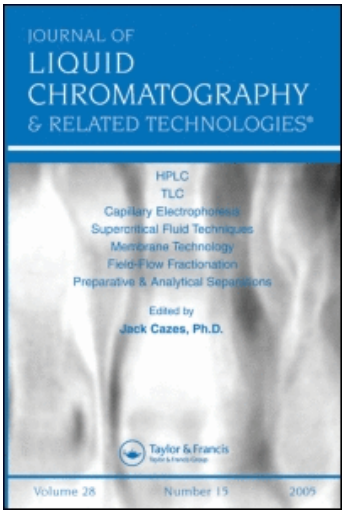

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Abstract: We developed a liquid chromatography-tandem mass spectrometric (LC-MS/MS) method for the determination of a ginseng saponin metabolite, IH901 or compound K (20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol) in rat plasma. The method involves protein precipitation by acetonitrile and HPLC separation of the sample extracts on a reversed-phase column (X-terra™ RP18) with isocratic elution of 20 mM ammonium acetate and acetonitrile (30:70, v/v), at a flow rate of 0.2 mL/min. The MS analysis was performed by electrospray positive ionization mass spectrometry using multiple reaction-monitoring mode. The mass transitions of IH901 and prednisolone (internal standard) were m/z 640 → 425 and 361 → 343, respectively. The standard curves for IH901 were linear over the concentration range of 2.0–500 ng/mL. The lower limit of quantification was 2 ng/mL and the limited of detection was 1 ng/mL for IH901. The coefficient of variation of intra- and inter-day assays at five quality control levels ranged from 2.0 to 13.9% and the accuracy varied between 93.2 and 110.5%. This method was used to determine IH901 in plasma samples after the oral administration of a single 30 mg/kg dose on SD rats.

Keywords: IH901, Compound K, 20-O-β-D-Glucopyranosyl-20(S)-protopanaxadiol, LC-MS/MS, Rat plasma

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INTRODUCTION

Ginseng (the root *of Panax ginseng* C.A. Meyer, Araliaceae) has been used for over 2000 years as neutraceuticals or herbal remedy in the Far East, and has gained popularity in the West during the last decade. Pharmacological properties of ginseng and its constituents have been demonstrated on the central nervous system (CNS), nuroendocrine function, carbohydrate, and lipid metabolism, immune function, and the cardiovascular system.[1,2] There are extensive literatures on physiologically active components of ginsengs, which include ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, and fatty acids. But the major active components are attributed to ginseng saponins called ginsenosides, which are mainly triterpenoid dammarane glycosides. Two groups of ginsenosides are 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]

Ginseng saponins are poorly absorbed in spite of their beneficial effects on humans.[3] Ginseng saponins are metabolized by intestinal bacteria when they are orally administered.[4,5] The main metabolite of protopanaxadiol ginseng saponins was 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol, referred to as IH901 or compound K (Fig. 1). In particular, the metabolite itself has pharmacological activities such as antitumor, antigenotoxic, antiallergic, and antiplatelet aggregation effects.[6–8] Because IH901 is currently being evaluated for a promising new herbal drug, a sensitive analytical method for the compound in blood samples was urgently needed for pharmacokinetic and toxic studies with animals.

Methods for the determination of ginsenosides or their metabolites in biological fluids include thin layer chromatography (TLC),[9] high performance liquid chromatography (HPLC) with UV detection,[10] evaporative light scattering,[11] or fluorescence detection,[12] gas chromatography–mass spectrometry (GC/MS),[13] enzyme immunoassay (EIA),[14] and liquid chromatography–mass spectrometry (LC/MS).[15–17]

EIA methods show higher sensitivity than chromatographic methods, but it is difficult to acquire suitable antibodies. TLC and HPLC methods with short-wavelength UV detection suffered from limited selectivity and

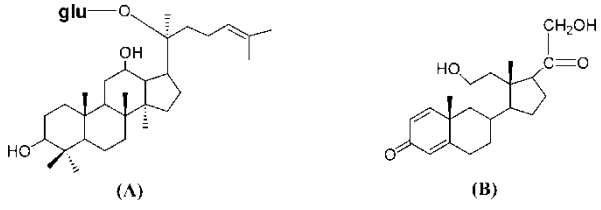

Figure 1. Structures of IH901 (20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol) and prednisolon (internal standard).

sensitivity, due to the presence of more than 20 structurally similar ginseng saponins and high endogenous background interferences from biological fluids. HPLC methods with fluorescence or UV detection after labeling need extensive work-up procedures for biological samples.

Recently, LC-tandem mass spectrometric methods (LC-MS/MS) have appeared for the assay of ginsenosides in ginseng extracts or products,[18,19] or in biological fluids,[20] but no reports are available yet for IH901. The purpose of this work is to develop a simple and sensitive LC-MS/MS method to determine IH901 in rat plasma for pharmacokinetic studies. The assay method was validated and used to obtain pharmacokinetic profiles of the IH901 in rat plasma.

EXPERIMENTAL

Materials and Reagents

The IH901 standard (purity, 99.0–101.0%) was supplied by the Il-Wha Pharm. Co. (Seoul, Korea). Prednisolone, which was used as an internal standard, was purchased from Sigma Co. (St. Louis, MO, USA). Acetonitrile was obtained from Fisher Scientific Co. (Fairlawn, NJ, USA). All other chemicals were of the highest quality available and used without further purification. Water was passed through Millipore Milli-RO4 and Milli-Q water purification systems (Bedford, USA). Mobile phase was filtered through 0.2 μm membrane filters (Phenomenex, CA, USA) and ultrasonically degassed prior to use. Micro tubes (2.0 mL) used for sample mixture were purchased from Axygen (Central Avenue Union, CA, USA).

Preparation of Standards and QC Samples

Stock solutions of IH901 (100 μg/mL) and prednisolone (10 μg/mL) were freshly prepared in methanol and stored at 4°C. Standard solutions were prepared by serial dilution of respective stock solutions with methanol to required concentrations prior to use. Calibration standards (CS) were prepared by spiking respective standard solutions in drug free rat plasma at concentrations of 0 (blank), 2, 5, 10, 50, 100, and 500 ng/mL. Quality control (QC) samples were prepared with blank plasma independently, at the concentrations of 2, 5, 10, 50, 100, and 500 ng/mL, respectively.

Sample Preparation

A 100 μL aliquot of plasma sample was accurately measured into a 2-mL microtube with teflon-lined cap, followed by spiking with 10 μL of internal standard (10 μg/mL). The mixture was vortex-mixed for 1 min, then

500 μL of acetonitrile was added. The mixture was then vortex-mixed for 5 min at high speed and centrifuged at 10000 × g for 3 min. The resulting supernatant liquid was transferred to another micro tube and then was evaporated to dryness under a stream of nitrogen at room temperature. The residues were reconstituted in 100 μL of freshly prepared mobile phase and then 10 μL aliquots were injected onto a LC/MS/MS system.

Chromatography

The chromatography system consisted of an Agilent 1100 series, equipped with binary pump, autosampler, degasser (Palo Alto, CA, USA). Separation was performed on a Waters X-terra^{TM} C_{18} column (2.1 mm i.d. × 150 mm, 5 μm) with isocratic elution of 20 mM ammonium acetate-acetonitrile (30:70, v/v), at the flow rate of 0.2 mL/min at room temperature. The HPLC effluents were introduced directly into the positive electrospray ionization source.

LC-MS/MS Analysis

The plasma concentration of IH901 was quantified using liquid chromatography-mass spectrometry with a Sciex API 3000 triple-quadrupole tandem mass spectrometer (Applied Biosystems, MDS Sciex, Concord, Canada), equipped with a Turbo-IonSpray interface to generate positive ions $[M + H]^+$. The ion spray interface was operated in the positive ion mode at 5.5 KV with the turbo gas temperature at 350°C. The operating conditions were optimized by flow injection of a mixture of all analytes and were determined as follows: nebulizing gas flow, 1.46 L/min (setting 12); auxiliary gas flow, 6.1 L/min; curtain gas flow, 0.95 L/min (setting 7); orifice voltage, 53 V; ring voltage, 400 V; collision gas (nitrogen) pressure, 3.58×10^{-5} Torr (setting 7). Quantitation was performed with multiple reaction monitoring (MRM) of the precursor and related product ions by an internal standard method, computing peak area ratios with a weighting factor of $1/x$. The characteristic ion transitions used for IH901 and internal standard were m/z 640 → m/z 425 and m/z 361 → m/z 343, respectively (collision energy 20 eV, dwell time 200 ms). Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by Analyst software (version 1.4).

Method Validation

Six QC samples were analyzed on four different days to complete the method validation. Batches of QC samples at 2, 5, 10, 50, 100, and 500 ng/mL were assayed in sets of four replicates to evaluate the intra- and inter-day

precision and accuracy. The extraction recoveries were calculated by comparing the peak areas of QC samples obtained through clean-up procedure to the peak areas measured using identical concentrations of standard IH 901 solution.

Pharmacokinetic Analysis

All animals were administered a single oral dose of IH901 at 30 mg/kg to SD rats (male, 250 ± 1.2 g, Semtaco, Seoul, Korea). Bloods (200 μL) were taken before and 0.16, 0.3, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, and 48 h after drug administration. The plasma was separated by centrifugation at 1000 × g for 10 min, and stored at −20°C until analysis.

A non compartmental pharmacokinetic analysis was performed on plasma concentrations using WinNonlin Version 2.1 (Pharsight, Mountainview, CA)]. Levels lower than the limit of quantitation was taken as zero. Maximum plasma concentration (C_{max}) and its corresponding time to C_{max} (T_{max}) were obtained through direct observation of plasma concentration-time curves. Area under the plasma concentration time curves from time zero to time of the last quantifiable concentration ($AUC_{0\text{-last}}$) was calculated by the trapezoidal rule, whereas $AUC_{0\text{-}\infty}$ was calculated according to $AUC_{0\text{-}\infty} = AUC_{0\text{-last}} + C_{last}/k$, where k is the slope of the terminal phase of the plasma concentration curve using log-transformed concentrations and C_{last} is the last concentration higher than the limit of quantitation. Plasma half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k$.

RESULTS AND DISCUSSION

Mass Spectra

The LC-MS/MS condition was optimized for detection of IH901 and prednisolone (I.S.) during the direct infusion of analyte and I.S., each 100 ng/mL in the mobile phase at the flow rate of 10 μL/min. Precursor ions of IH901 and prednisolone with the corresponding product ions were determined from spectral intensities obtained during the infusion of standard solutions into a mass spectrometer using an electrospray ionization source, which operated in positive ionization mode with collision nitrogen gas in Q2 of a MS–MS system. IH901 produced the most abundant ion at m/z 640 ($[M + NH_4]^+$) and I.S., at m/z 361 ($[M + H]^+$). The molecular adduct ions were made collision with nitrogen in Q2 to exhibit several product ions as shown in Fig. 2. From this fragment, the most intensive ions at m/z 425 ($[M + H]^+$-Glucose-H_2O) for IH901 and at m/z 342 ($[M + H]^+$-H_2O) for I.S were scanned in Q3 for the LC-MS analysis.

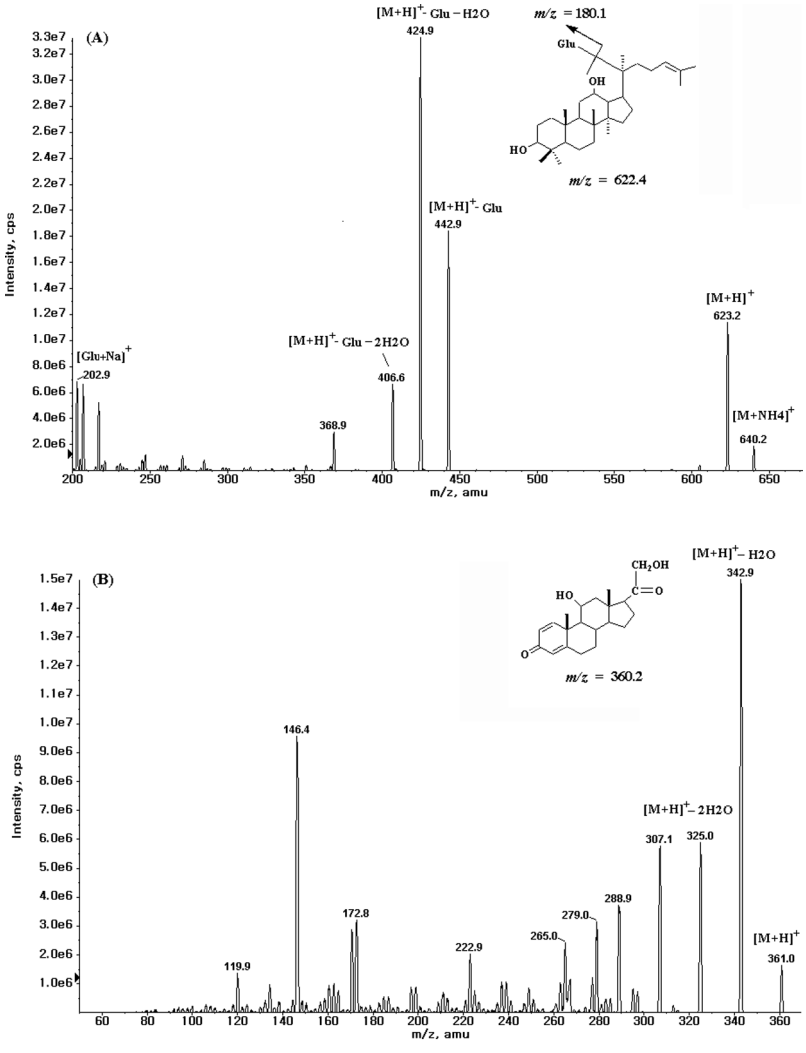

Figure 2. Mass-mass spectra of IH901 (A) and prednisolone (B) using electrospray ionization mode.

Determination of IH901

The retention time of IH901 and internal standard were 3.2 and 2.1 min, respectively, and the total run time was within 7 min. There was no interfering peaks at the elution times for either IH901 or internal standard. Figure 3 show representative chromatograms for drug-free rat plasma (A), a blank plasma spiked with 2 ng/mL IH901 and 200 ng/mL I.S. (B) and a plasma sample 2 h after oral

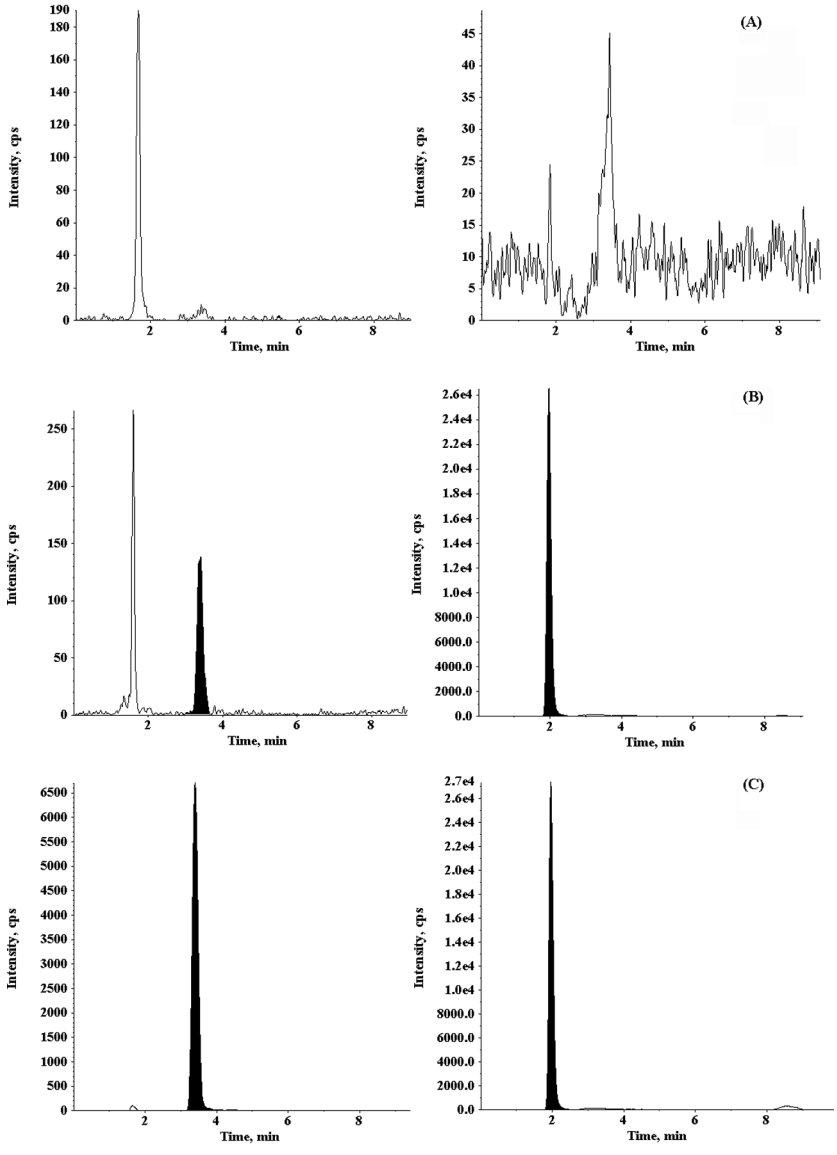

Figure 3. Representative chromatograms of IH901 (left) and prednisolone (right) from rat plasma. (A) Blank plasma (B) Plasma spiked with 5 ng/mL of IH901 and 200 ng/mL of internal standard. (C) Plasma sample 2 h after oral administration of IH901 at 30 mg/kg on SD rats.

administration of IH901 at 30 mg/kg to SD rats (C), where the drug concentration of the sample plasma was determined to be 98.5 ng/mL IH901. Since no late-eluting peaks were observed, regeneration of the LC column using a gradient elution step was not necessary. Efficient removal of protein with acetonitrile resulted in low background level. Reconstitution of the sample did not cause any type of clog to tubes or needle sprayer owing to non precipitated proteins.

Recovery

Recovery of IH901 was determined using QC samples at 2, 5, 10, 50, 100, and 500 ng/mL with replicate determinations (n = 7) made at each concentration. Table 1 shows that the extraction recoveries ranged over 95% with C.V. values of 5.8–8.8% for the entire range of the standard curve. The protein precipitation with acetonitrile has been successfully applied to the sample preparation of IH901 from plasma sample.

Linearity and Sensitivity

The calibration curve for IH901 was linear over a concentration range of 2–500 ng/mL (32.0 *f*mol to 8.0 *p*mol on-column) with correlation coefficients (r) of 0.9993. The mean equation of the regression line was $y = (0.012 \pm 0.001)x + (0.024 \pm 0.070)$. The limit of detection (LOD) was 1 ng/mL (16 *p*mol on-column) with a signal-to-noise ratio of 3:1. The lower limit of quantification (LLOQ) was set at 2 ng/mL (32.0 *p*mol on-column) with a precision of 9.5% for peak area measurements using 10 μL injections to the LC system.

Precision and Accuracy

The intra- and inter-day precision and accuracy were determined by analyzing the QC samples at five different concentrations each for 4 batches (Table 2).

Table 1. Recovery of IH901 in rat plasma (n = 7)

Nominal concentration (ng/mL)	Recovery (%) (mean ± S.D.)
2	101.2 ± 5.9
5	103.3 ± 7.4
10	95.3 ± 8.8
50	96.0 ± 7.0
100	97.3 ± 6.8
500	96.7 ± 5.8

Table 2. Intra- and inter-day precision and accuracy from quality control samples

Nominal concentration (ng/mL)	Measured concentration (ng/mL)	RSD (%)	Accuracy (%) (mean ± S.D.)
Intra-day assay (n = 4)			
2	2.03	8.8	101.2 ± 5.8
5	5.2	5	105.4 ± 9.9
10	9.5	9	95.4 ± 2.9
50	48.4	4.5	96.8 ± 0.8
100	100	4.5	100.0 ± 1.1
500	498.7	9.7	99.7 ± 2.9
Inter-day assay (n = 4)			
2	2.1	9.5	102.5 ± 6.2
5	5.1	9.7	103.3 ± 7.4
10	9.3	11.4	93.5 ± 5.2
50	48.9	12.7	97.8 ± 2.0
100	99.4	11.7	99.4 ± 0.5
500	507.9	13.9	101.5 ± 2.0

The intra- and inter-day precision (RSD) were 4.5–9.7% and 9.7–13.9%, respectively. In both cases, the accuracy was in the range of 93.5–105.4% over the entire concentration range with SD values less than 13.9%. The results are within the acceptable criteria for precision and accuracy.

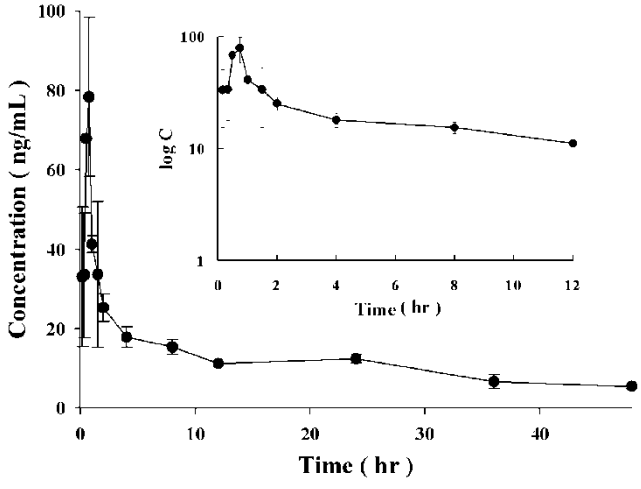

Figure 4. Rat plasma concentration-time profile after oral administration of IH901 at 30 mg/kg S.D. rats. Each point represents mean ± S.D. (n = 6). Insert shows the profile up to 12 h (log scale).

Table 3. Pharmacokinetic parameters of IH-901 after a single 30-mg/kg oral administration to six rats

Parameters	Mean ± S.E*
C_{max} (ng/mL)	84.7 ± 20.8
T_{max} (h)	0.625 ± 0.170
$AUC_{0-48\,h}$ (ng h/mL)	654 ± 18.9
$AUC_{0-\infty}$ (ng h/mL)	739 ± 24.4
$t_{1/2}$ (h)	28.1 ± 3.79

*Standard error.

Pharmacokinetics of IH901

Figure 4 shows the time course of the mean rat plasma concentration of IH901 after oral administration at 30 mg/kg dose to SD rats. The mean pharmacokinetic parameters are listed in Table 3. The mean terminal half-life ($t_{1/2}$) was 28.1 h; maximum plasma concentration (C_{max}) was 84.7 ng/mL; time to maximum concentration (T_{max}) was 0.625 h; the area under the plasma concentration-time curves, the $AUC_{0-48\,h}$ and $AUC_{0-\infty}$, were 654 ± 18.9 and 739 ± 24.4 ng/mL/h each, respectively.

In conclusion, we have developed a validated LC-MS/MS method for the determination of a ginsenoside metabolite, IH901 (or Compound K) in rat plasma. The analytical method described here allows the quantitative recovery and reliable measurement of the target analyte in a blood sample. This method, using a simple clean-up procedure, showed good specificity, precision, accuracy, and recovery characteristics. Finally, this method has been successfully applied to pharmacokinetic studies of IH901 in rat plasma.

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