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Pharmacokinetics of a Ginseng Saponin Metabolite Compound K in Rats

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ABSTRACT: The absorption, dose-linearity and pharmacokinetics of compound K, a major intestinal bacterial metabolite of ginsenosides, were evaluated *in vitro* and *in vivo*. Using the Caco-2 cell monolayers, compound K showed moderate permeability with no directional effects, thus suggesting passive diffusion. After intravenous dose (i.v.; 1, 2, and 10 mg/kg), no significant dose-dependency was found in *Cl* (17.3–31.3 ml/min/kg), V_{ss} (1677–2744 ml/kg), dose-normalized *AUC* (41.8–57.8 μg · min/ml based on 1 mg/kg) and $t_{1/2}$. The extent of urinary excretion was minimal for both i.v. and oral doses. The extent of compound K recovered from the entire gastrointestinal tract at 24 h were 24.4%–26.2% for i.v. doses and 54.3%–81.7% for oral doses. Following oral administration (doses 5–20 mg/kg), dose-normalized *AUC* (based on 5 mg/kg) was increased at the 20 mg/kg dose (85.3 μg · min/ml) compared with those at lower doses (4.50–10.5 μg · min/ml). Subsequently, the absolute oral bioavailability (*F*) was increased from 1.8%–4.3% at the lower doses to 35.0% at the 20 mg/kg dose. The increased *F* could be related to the saturation of carrier-mediated hepatic uptake and esterification of compound K with fatty acids in the liver.

Key words: compound K; pharmacokinetics; rats; bioavailability; Caco-2 cells

Introduction

Ginseng, the root of *Panax ginseng* C.A. Meyer, has been used as a health product or natural remedy for a long time. More than 25 dammarane-type saponins have been identified as the characteristic ingredients of white and red Ginseng and are classified into 20(*S*)-protopanaxadiol (ginsenoside Rb_1, Rb_2, Rb_3, Rc, Rd) and 20(*S*)-protopanaxatriol (ginsenoside Re, Rg_1, Rg_2, Rh_1) groups based on their aglycone moieties [1–3]. Protopanaxadiol ginsenoside Rb_1, Rb_2 and Rc are metabolized to compound K (Figure 1) by intestinal bacteria in humans as well as rats [4–13]. Compound K shows various pharmacological activities *in vitro* as well as *in vivo*: antimetastatic or antitumoric activity [7,10,14–17], reduction of doxorubicin toxicity in mouse testis [18], antiallergic effect [12,19] and hypothalamo–pituitary–adrenal axis-modulating activity [20].

A better understanding of the pharmacokinetics and bioavailability of herbal medicinal products (HMP) can link data from pharmacological assays to clinical effects and also help in designing rational dosage regimens [21]. Further studies are needed to characterize the bioavailability and pharmacokinetics of HMP in order to fully take advantage of the therapeutic potential. The pharmacokinetics and metabolism of compound K after oral administration of a large dose

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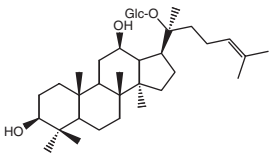

Figure 1. Chemical structure of compound K

of ginsenoside Rb_1 [4-6,8] or compound K [7,9,23] to mice or rats have been performed. Compound K, an intestinal bacterial metabolite of ginsenosides, is further biotransformed to biologically active fatty acid-conjugates [7]. After oral administration of ginseng preparation to humans, compound K, which is not originally present in the preparation, was detected in plasma and urine [4,5,13]. However, there are no data for the pharmacokinetic property, including oral bioavailability and dose-linearity of compound K.

The purpose of this study was to evaluate the pharmacokinetics of compound K after an i.v. administration at doses of 1, 2 and 10 mg/kg and an oral administration at doses of 5, 10 and 20 mg/kg of the drug to rats.

Materials and Methods

Chemicals

Compound K, which was isolated as reported previously [5] from ginseng powder fermented with *Lactobacillus casei* strain Hasegawa (FERM BP-10123), and lithospermic acid B dimethylester (internal standard) were supplied from Wonpharm Co. Ltd (Iksan, Korea) with >99.0% purity. Acetonitrile and ethyl acetate (HPLC grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA), and other chemicals were of HPLC grade or the highest quality available.

Animals

Male Sprague–Dawley rats (weighing 210–260 g, 8 weeks of age) were purchased from Charles River Company Korea (Biogenomics, Seoul, Korea). The animals were housed in an air conditioned room at a temperature of $23^\circ \pm 2^\circ$C, with a relative humidity of 55% ± 10%, an illumination intensity of 150–300 lux, a frequency of air ventilation of 15–20 times/h, and a 12 h illumination (07:00–19:00). Food and water were supplied *ad libitum*. The rats were fasted for 18 h before experiments with the exception of free access to water. All animal procedures involving animal care were approved by the Wonkwang University Animal Care and Use Committee.

Compound K transport in Caco-2 cell monolayers

Caco-2 cells were cultured at 37°C in minimum essential medium, supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/ml penicillin and 0.1 mg/ml streptomycin in a humidified 37°C incubator with 5% carbon dioxide. Caco-2 cells (passage number 35) were seeded at a density of 60 000 cells/cm^2 onto polycarbonate membranes of Transwells™ (12 mm i.d., 0.4 μm pore size, Corning Costar, Cambridge, MA, USA). The medium was changed the day after seeding, and every other day thereafter. The medium was added to both apical and basolateral compartments. The cell monolayers were used approximately 21 days post-seeding. To ensure the cell monolayer integrity, the transepithelial electrical resistance (TEER) was measured using an EVOM Epithelial Tissue Voltamometer and an Endohm-12 electrode (World Precision Instruments, Sarasota, FL, USA). Caco-2 cells with TEER values $\geqslant 300\,\Omega \cdot cm^2$ were used for transport experiments. The cell monolayers were incubated in transport buffer (HBSS with 10 mM glucose and 25 mM HEPES adjusted to pH 7.4) for 30 min at 37°C. Compound K solutions in transport buffer (5–50 μM) were added to the apical side (for apical to basolateral permeability measurements) or to the basolateral side (for basolateral to apical permeability measurements) of the inserts. For apical to basolateral transport, the inserts were moved to a well with fresh transport buffer at various times over a 1.5 h period and the aliquots were removed from each well. For basolateral to apical transport, the transport media in the insert were removed and replaced with 0.5 ml of fresh transport buffer at various times over a 1.5 h period, and the aliquots were stored at −20°C until analysis. The concentrations of compound K were determined by our previous LC/MS method [22]. At the completion of all experiments, TEER was measured to ensure

cell monolayer integrity and viability had not been adversely affected by the experimental conditions. The apparent permeability coefficients (P_{app}, cm/s) were calculated using $P_{app} = (dQ/dt)/(A \cdot C_D)$ where Q is the amount of compound transported over time t of the experiment, A is the surface area of the porous membrane in cm^2, and C_D is the initial concentration of compounds added to the donor compartment. Metoprolol (50 μM) and atenolol (50 μM) were added to the apical side as markers of high and low permeability, respectively.

Protein binding test

The extent of binding of compound K to rat plasma proteins was investigated by spiking rat plasma with compound K at 1 and 10 μg/ml and then allowing equilibration to take place for 30 min at 37°C prior to ultrafiltration. A 500 μl aliquot of equilibrated plasma solution was placed in a filter unit of Centrifree® ultrafiltration device with YMT membrane (Millipore, Bedford, MA, USA) and centrifuged at 3000 *g* for 30 min to separate the protein-bound from the free compound K. A 50 μl aliquot of each ultrafiltrate was vortex-mixed with 5 μl of the internal standard (lithospermic acid B dimethylester) working solution and 900 μl of ethyl acetate. After centrifugation at 5000 *g* for 5 min, the organic layer was evaporated to the dryness under nitrogen at 35°C. The residues were dissolved in 25 μl of 75% acetonitrile in water and 10 μl were injected onto a LC/MS system for the determination of the concentration of free compound K.

Intravenous and oral administration of compound K to rats

The rats were cannulated with polyethylene tubing (PE-50, Clay Adams, Parsippany, NJ, USA) in the right jugular and left femoral veins under light ether anesthesia. Compound K was dissolved in a mixture of DMSO:PEG400:DDW (1:4:5, v/v) and administered to rats by bolus injection over 1 min *via* the femoral vein at doses of 1, 2 and 10 mg/kg ($n = 7$ each). Blood samples (0.25 ml) were collected *via* the jugular vein at 0 (to serve as a control), 1 (at the end of i.v. administration), 5, 15, 30, 45 min, and 1, 1.5, 2, 3, 4, 6, 8 and 10 h after drug administration. After centrifugation of blood samples, 100 μl aliquots of plasma samples were collected and stored at −70°C. Urine samples were collected over a period of 0–24 h. After measuring the exact volume of the urine samples, two 100 μl aliquots of each sample were stored at −70°C. Approximately 0.3 ml of heparinized 0.9% NaCl-injectable solution (20 units/ml) was used to flush the cannula immediately after each blood sampling to prevent blood clotting. At the end of experiment (24 h), each rat was killed by cervical dislocation and the entire gastrointestinal (GI) tract (including its contents and feces) was removed, transferred into a beaker containing 50 ml of methanol (for the extraction of compound K), and cut into small pieces using scissors. After stirring with a glass rod, two 100 μl aliquots of the supernatant were collected from each beaker and stored at −70°C until drug analysis.

For oral dosing experiments, compound K dissolved in a mixture of DMSO:PEG400:DDW (1:4:5, v/v) was administered to rats at doses of 5 ($n = 7$), 10 ($n = 8$) and 20 mg/kg ($n = 10$) by oral gavage. Blood samples (0.25 ml) were collected at 0 (to serve as a control), 15, 30 and 45 min, and 1, 1.5, 2, 3, 4, 6, 8, 10 and 24 h after drug administration. The plasma, urine and GI samples were handled in a similar manner as described above.

LC/MS analysis of compound K

The concentrations (or amounts) of compound K were determined by our previous LC/MS method [22]. To 100 μl aliquots of the biological samples, 10 μl of the internal standard (lithospermic acid B dimethylester) working solution and 100 μl of 10 mM phosphate buffer (pH 7.4) were added. The samples were extracted with 900 μl of ethyl acetate in 1.5 ml polypropylene tubes by vortex-mixing for 5 min at high speed and centrifuged at 5000 *g* for 5 min at room temperature. The organic layer was transferred and evaporated to the dryness under nitrogen at 35°C. The residues were dissolved in 40 μl of 70% acetonitrile in water by vortex-mixing for 2 min, transferred to injection vials, and 10 μl were injected onto a LC/MS system.

For LC/MS analysis, the chromatographic system consisted of a Nanospace SI-2 pump, a SI-2 autosampler and a S-MC system controller (Shiseido, Tokyo, Japan). The separation was performed on an Atlantis C_{18} column (5 μm, 2.1 × 100 mm, Waters, Ireland) using a mixture of acetonitrile-ammonium formate (10 mM, pH 3.0) (75:25, v/v) at a flow rate of 0.2 ml/min. The column and autosampler tray temperature were 30°C and 4°C, respectively. The analytical run time was 4 min. The eluent was introduced directly into the tandem quadrupole mass spectrometer (Quattro LC, Micromass UK Ltd, Manchester, UK) through the negative ionization electrospray interface. The ion source and desolvation temperature were held at 120°C and 350°C, respectively. The optimum cone voltages for ionization of compound K and lithospermic acid B dimethylester (internal standard) were 60 V and 50 V, respectively. Selected ion monitoring (SIR) mode was employed for the quantification: *m/z* 621.5 for compound K and *m/z* 745.2 for lithospermic acid B dimethylester. Peak areas for all components were automatically integrated using MassLynx Version 3.4 (Micromass UK Ltd, Manchester, UK). The standard curves were linear over the concentration range of 2.0–1000 ng/ml. The coefficient of variation and relative error values for quality control samples containing compound K were 3.5–7.8% and −4.6–5.4%, respectively, at 6.0, 40.0 and 400 ng/ml ($n = 15$).

Pharmacokinetic and statistical analysis

The plasma concentration *vs* time data were analysed by a non-compartmental method using the nonlinear least squares regression program WinNonlin (Scientific Consulting Inc., Cary, NC, USA). The area under the plasma concentration–time curve (*AUC*) and the area under the first moment curve (*AUMC*) were calculated using the trapezoidal rule extrapolated to infinity. The terminal elimination half-life ($t_{1/2}$), the systemic clearance (*Cl*), mean residence time (*MRT*) and volume of distribution at steady state (V_{ss}) were obtained. The extent of absolute oral bioavailability (*F*) was estimated by dividing *AUC* after oral dose by *AUC* obtained after i.v. administration of 10 mg/kg dose. The peak plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) after oral administration were also obtained.

All data were expressed as the mean ± standard deviation (SD). Three means for unpaired data were analysed by the Duncan's multiple range test of Social Package of Statistical Sciences (SPSS) posteriori analysis of variance (ANOVA). A p value of less than 0.05 was considered to be statistically significant.

Results and Discussion

The Caco-2 cell monolayers, the well-studied model for assessing drug absorption [24], were used to evaluate the rates of compound K in both apical to basolateral and basolateral to apical directions. The P_{app} values for compound K in both directions and at four concentrations were calculated and are summarized in Table 1. The *in vitro* Caco-2 permeability of compound K ($3–6 \times 10^{-6}$ cm/s) was intermediate between the high permeability standard metoprolol (28×10^{-6} cm/s) and low permeability standard atenolol (0.25×10^{-6} cm/s). There was no significant difference between P_{app} values for compound K in the two directions and at the initial concentrations of compound K (5–50 μM). These results indicate that compound K crossed the Caco-2 cell monolayers at a moderate rate through a direction-independent, passive diffusion mechanism. A Caco-2 transport rate of $>2 \times 10^{-6}$ cm/s is considered equivalent to complete absorption of pharmaceutical agent from the human intestine [24]. Therefore, moderate P_{app} values ($3–6 \times 10^{-6}$ cm/s) of compound K

Table 1. Apparent permeability coefficients of compound K through Caco-2 cell monolayers in both apical to basolateral and basolateral to apical directions from cells grown on filter inserts suspended in culture ($n = 6$, mean ± SD)

	P_{app} ($\times 10^{-6}$ cm/s)	
Concentration (μM)	Apical to basolateral	Basolateral to apical
5	6.6 ± 1.5	5.3 ± 1.3
10	4.1 ± 1.2	4.8 ± 1.1
20	3.0 ± 0.7	4.2 ± 0.9
50	4.1 ± 0.9	3.3 ± 0.7

across the Caco-2 monolayers indicate that compound K should be well absorbed by the intestine. These results also support that compound K was shown to be absorbed rapidly from gastrointestinal tract after oral administration of compound K to rats [7,9].

The extent of compound K to rat plasma protein was investigated using the ultrafiltration method. The plasma free fraction of compound K was 0.002 ± 0.001 at 1 and 10 μg/ml, suggesting that compound K was highly protein-bound in plasma.

After i.v. administration of compound K, mean plasma concentration–time curves declined in a polyexponential manner for all three doses studied (Figure 2), with mean terminal half-lives of 222, 298 and 224 min for 1, 2 and 10 mg/kg doses, respectively (Table 2); the half-lives were not significantly different among the three doses. The dose-normalized (based on 1 mg/kg) *AUC*s of compound K were independent of i.v. doses studied; the values were 41.8 ± 29.0, 57.8 ± 3.4 and 48.7 ± 18.8 μg·min/ml for 1, 2 and 10 mg/kg doses, respectively. As could be expected from the *AUC* results, the *Cl*s were also independent of i.v. doses studied; the values were 31.3, 17.3 and 23.1 ml/min/kg for 1, 2 and 10 mg/kg, respectively (Table 2). The calculated V_{ss} values were 1677–2744 ml/kg, thus suggesting considerable tissue distribution of compound K. In addition, other pharmacokinetic parameters of compound K listed in Table 2 were not significantly different among three i.v. doses studied. These data indicate that the pharmacokinetic parameters of compound K are linear within the i.v. dose ranges studied (1–10 mg/kg). The contribution of renal clearance to *Cl* of compound K was negligible; the percentage of an i.v. dose of compound K excreted in a 24 h urine sample as unchanged drug (Ae_{0-24h}) was less than 0.009% for all three doses studied (Table 2), indicating that most of intravenously administered compound K was eliminated *via* a nonrenal route. In contrast, the percentages of the i.v. dose recovered from the entire gastrointestinal tract at 24 h as unchanged drug (GI_{24h}) were 26.2%, 25.2% and 24.4% of i.v. dose for 1, 2 and 10 mg/kg, respectively (Table 2), indicating that the contribution of biliary excretion of unchanged

Table 2. Pharmacokinetic parameters of compound K after i.v. injection of various doses to rats ($n = 7$)[a]

Parameter	1 mg/kg	2 mg/kg	10 mg/kg
Body weight (g)	270 ± 9.9	284 ± 16.0	262 ± 11.1
$t_{1/2}$ (min)	222 ± 82.5	298 ± 77.9	224 ± 115
AUC (μg·min/ml)[b]	41.8 ± 28.9	115.6 ± 6.8	486.8 ± 188.2
MRT (min)	81.1 ± 31.8	96.1 ± 54.8	79.9 ± 40.5
Cl (ml/min/kg)	31.3 ± 14.8	17.3 ± 1.0	23.1 ± 8.4
V_{ss} (ml/kg)	2744 ± 2050	1677 ± 1001	1757 ± 750
Ae_{0-24h} (% of dose)	0.008 ± 0.008	0.009 ± 0.002	0.006 ± 0.002
GI_{24h} (% of dose)	26.2 ± 3.2	25.2 ± 10.3	24.4 ± 10.4

[a] Values expressed as mean ± SD.
[b] Dose-normalized (1 mg/kg) *AUC*s were compared by statistical analysis.

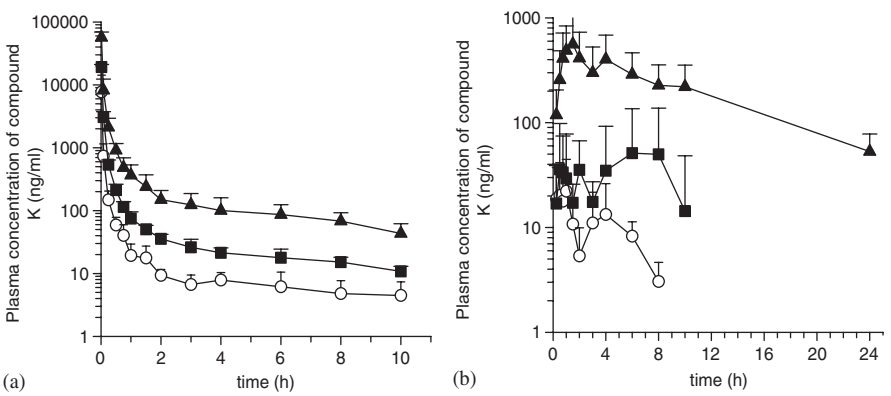

Figure 2. Mean plasma concentration–time profiles of compound K in rats after (A) intravenous injection of the drug at 1 (○), 2 (■) and 10 (▲) mg/kg ($n = 7$) and (B) oral administration of the drug at 5 (○, $n = 7$), 10 (■, $n = 8$) and 20 (▲, $n = 10$) mg/kg. Vertical bars represent standard deviation

compound K to Cl_{NR} of compound K was considerable. These results support the findings of Hasegawa *et al*. [7], that is, compound K was selectively accumulated into the liver and excreted in bile after i.v. injection of compound K to mice and rats but free from the biliary excretion, *ca*. 24 mol% of dosed compound K was metabolized to the pharmacologically active metabolites, fatty acid compound K esters *via* esterification with fatty acids such as stearate, oleate and palmitate in the liver.

After oral administration of compound K at doses of 5, 10 and 20 mg/kg to rats, the mean plasma concentration–time profiles of compound K are shown in Figure 2; the relevant pharmacokinetic parameters are listed in Table 3. The percentages of oral dose of compound K recovered from the entire gastrointestinal tract at 24 h as an intact drug (GI_{24h}) were 54.3%, 81.7% and 77.5% for 5, 10 and 20 mg/kg, respectively (Table 3). Based on the moderate intestinal permeability of compound K ($3–6 \times 10^{-6}$ cm/s) across Caco-2 cell, these high GI_{24h} values might result not from poor absorption but from biliary (including gastrointestinal) excretion of compound K. These results support that the reports of Hasegawa *et al*. [7]: orally administered compound K was absorbed mainly from the small intestine followed by its accumulation in the liver where biliary excretion and esterification of compound K occurred. The plasma concentration–time curves of compound K exhibited distinct multiple peaks after oral administration and compound K was excreted in the bile, suggestive of the involvement of enterohepatic recirculation. The identification of enterohepatic recirculation may be required by a comparison of *AUC*s obtained after oral administration of compound K in normal and bile-duct cannulated rats. The dose-normalized (based on 5 mg/kg) C_{max} value (181 ng/ml) at 20 mg/kg was significantly higher than those at 5 (28 ng/ml) and 10 (39 ng/ml) mg/kg doses. The dose-normalized (based on 5 mg/kg) *AUC* (44.7 μg · min/ml) of compound K at 20 mg/kg was also significantly larger than those at 5 (4.5 μg · min/ml) and 10 (10.5 μg · min/ml) mg/kg doses (Table 3). Hence, the *F* values were also dependent of the oral doses; the values 1.8%, 4.3% and 35.0% for oral doses of 5, 10 and 20 mg/kg, respectively (Table 3). The low absolute oral bioavailability (*F*) values could be due to the considerable biliary excretion and the hepatic metabolism of compound K *via* esterification. The significant increase of *F* value at 20 mg/kg may be due to the saturation of carrier-mediated hepatic uptake and the esterification of compound K. Previous report suggested that compound K is uptaken to the liver cells *via* non-specific diffusion and Na^+/taurocholate cotransporting polypeptide- and organic cation transporter-mediated transport using isolated hepatocytes [23]. Compound K was below detection limit in urine for 5, 10 and 20 mg/kg oral doses (Table 3). All other pharmacokinetic parameters of compound K listed in Table 3 were also comparable among different oral doses.

Table 3. Pharmacokinetic parameters of compound K after oral administration of various doses to rats[a]

Parameter	5 mg/kg ($n = 7$)	10 mg/kg ($n = 8$)	20 mg/kg ($n = 10$)
Body weight (g)	245 ± 14.6	254 ± 18.4	224 ± 11.5
AUC (μg · min/ml)[b]	4.50 ± 3.86	21.0 ± 28.8	341.0 ± 201.8[c]
T_{max} (min)	124 ± 162	191 ± 196	151 ± 122
C_{max} (μg/ml)[b]	0.028 ± 0.023	0.078 ± 0.098	0.726 ± 0.386[c]
Ae_{0-24h} (% of dose)	BD[d]	BD[d]	BD[d]
GI_{24h} (% of dose)	54.3 ± 21.5	81.7 ± 26.8	77.5 ± 15.3
F (%)	1.8	4.3	35.0

[a] Values expressed as mean ± SD.
[b] Dose-normalized (5 mg/kg) *AUC*s were compared by statistical analysis.
[c] 20 mg/kg was significantly different ($p < 0.05$) from 5 and 10 mg/kg.
[d] Below detection limit.

In summary, the dose-independent pharmacokinetic parameters of compound K were observed after i.v. administration at doses of 1–10 mg/kg to rats. However, dose-dependent pharmacokinetic parameters of compound K were evaluated after oral administration of 5–20 mg/kg to rats. Using the Caco-2 cell monolayers, compound K would be absorbed by the intestine at a moderate rate by means of direction-independent, passive diffusion. The mean *F* values of compound K ranged from 1.8% to 35.0% over an oral dose range of 5–20 mg/kg. These results could result from the saturation of hepatic uptake and hepatic metabolism of compound K *via* esterification at 20 mg/kg doses.

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