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Development and validation of an LC-MS/MS method for determination of compound K in human plasma and clinical application

Jung Soo Kim[1-3#], Yunjeong Kim[1,4#], Song-Hee Han[1,4], Ji-Young Jeon[1,4], Minho Hwang[1,4], Yong-Jin Im[1,4], Jung Hyun Kim[1,5], Sun Young Lee[1,6], Soo-Wan Chae[1,4,7], and Min-Gul Kim[1,3,4*]

[1]Biomedical Research Institute of Chonbuk National University Hospital, Jeonju 561-712, Korea
[2]Department of Pediatrics, Chonbuk National University Medical School, Jeonju 561-180, Korea
[3]Research Institute of Clinical Medicine, Chonbuk National University, Jeonju 561-712, Korea
[4]Clinical Trial Center, Chonbuk National University Hospital, Jeonju 561-712, Korea
[5]Department of Medical Nutrition Therapy, Chonbuk National University Medical School, Jeonju 561-180, Korea
[6]Department of Radiation Oncology, Chonbuk National University Hospital, Jeonju 561-712, Korea
[7]Department of Pharmacology, Chonbuk National University Medical School, Jeonju 561-180, Korea

A rapid, sensitive and selective analytical method was developed and validated for the determination of compound K, a major intestinal bacterial metabolite of ginsenosides in human plasma. Liquid-liquid extraction was used for sample preparation and analysis, followed by liquid chromatography tandem spectrometric analysis and an electrospray-ionization interface. Compound K was analyzed on a Phenomenex Luna C18 column (100×2.00 mm, 3 μm) with the mobile phase run isocratically with 10 mM ammonium acetate-methanol-acetonitrile (5:47.5:47.5, v/v/v) at a flow rate of 0.5 mL/min. The method was validated for accuracy (relative error <12.63%), precision (coefficient of variation <9.14%), linearity, and recovery. The assay was linear over the entire range of calibration standards i.e., a concentration range of 1 ng/mL to 1,000 ng/mL (r^2 >0.9968). The recoveries of compound K after liquid-liquid extraction at 1, 2, 400, and 800 ng/mL were 106.00±0.08%, 103.50±0.19%, 111.45±5.21%, and 89.62±34.46% for intra-day and 85.40±0.08%, 94.50±0.09%, 112.50±5.21%, and 95.87±34.46% for inter-day, respectively. The lower limit of quantification of the analytical method of compound K was 1 ng/mL in human plasma. The developed method was successfully applied to a pharmacokinetic study of compound K after oral administration in ten of healthy human subjects.

Keywords: *Panax ginseng*, Compound K, Fermented Korean red ginseng, LC-MS/MS, Pharmacokinetics

INTRODUCTION

Panax ginseng Meyer (Araliaceae) is frequently used as traditional herbal medicines in Asia. The main molecular components responsible for the medicinal actions of ginseng are ginsenosides. Ginsenoside are classified into 20(S)-protopanaxadiol (ginsenoside Rb1, Rb2, Rg3, Rc, and Rd) and 20(S)-protopanaxatriol (ginsenoside Re, Rg1, Rg2, and Rh1) groups based on their aglycone moieties [1]. Protopanaxadiol ginsenoside Rb1, Rb2, and Rc are mostly metabolized to compound K ([20-*O*-(β-D-glucopyranosyl)-20(S)-protopanaxadiol]) (Fig. 1A) by intestinal bacteria after oral administration in human via the following hydrolytic transformation pathway [2-

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[#]These authors contributed equally to this work.

[*]**Corresponding author**

E-mail: mgkim@jbctc.org
Tel: +82-63-250-2532, Fax: +82-2-63-250-2349

5]: ginsenoside Rb1→ginsenoside Rd→ginsenoside F2→compound K [6]. Compound K is easily absorbed by the human body and may be produced from fermented Korean red ginseng that is used for enhancing the preservation of steamed and dried raw fresh ginseng. Recently, interest in compound K has increased because of its biological activity. Compound K has been reported to exhibit various pharmacological activities *in vitro* and *in vivo* and to have anti-cancer [7-9], anti-inflammation [10,11], anti-diabetes [12,13], and anti-allergic effects [14,15]. However, despite intensive research on the bioactivity of ginsenosides pharmacokinetic study has not been investigated.

Many methods have been published for ginsenoside assay from ginseng extract, including thin layer chromatography [16], HPLC with UV and evaporative light scattering [17,18], and gas chromatography [19]. The bioanalytical methods for ginsenoside in blood and urine are HPLC with UV detector [20] and LC-MS [21]. These analytical methods had some limitations, such as long analysis times to improve the separation for analysis of various ginsenosides and excessive use of mobile phase reagents. The HPLC method has become the most widely used for the quantification of compound K but it takes much time and many reagents. Lately, faster analytical methods for ginsenosides have been developed and applied in biological samples. LC tandem spectrometry (LC-MS/MS) is capable of detecting desired components using the multiple reaction monitoring method, can analyze small volumes of ginsenosides in biological matrix such as urine and plasma, saves time, and is more convenient.

The determination for compound K was reported in blood after the oral administration of ginsenosides Rb1, Rb2 or Rc [14,22,23]. The conventional published method for the pharmacokinetics of compound K was conducted mostly in rats, mice or dogs and involved the direct oral administration or intra vascular injection of analyst. In humans after oral administration of ginseng preparation, compound K, which is not originally present in the preparation, was detected in plasma and urine [2,24,25]. However, there are no data on the pharmacokinetic properties, including oral absorption, distribution, and elimination of compound K in humans using LC-MS/MS.

The purpose of this study was to develop and validate and LC-MS/MS analysis method for the determination of compound K in human plasma, and to evaluate the pharmacokinetics of compound K after oral administration to healthy Korean subjects (n=10).

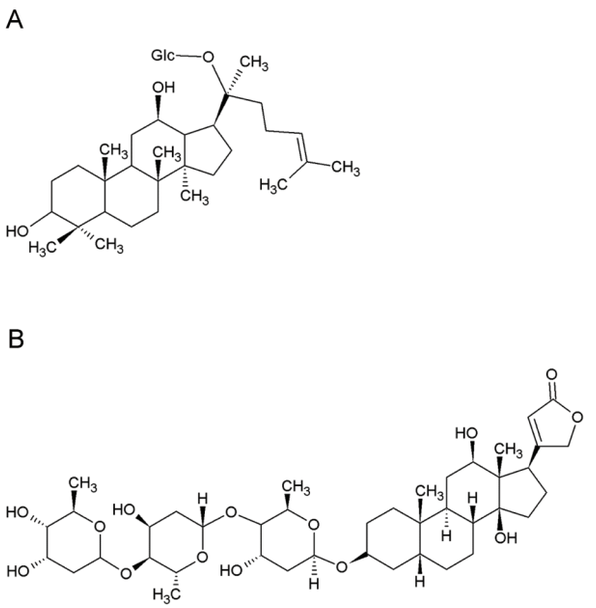

Fig. 1. Chemical structure of (A) compound K and (B) digoxin (internal standard).

MATERIALS AND METHODS

Materials

Compound K and fermented Korean red ginseng extracts were purchased from Metabolab (Seoul, Korea) and digoxin (internal standard [IS], [Fig. 1B]) from Fluka (Ronkonkoma, NY, USA). HPLC grade water, methanol and acetonitrile for LC-MS/MS were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The ethyl acetate used as extraction organic solvent was purchased from Duksan (Ansan, Korea). The ammonium acetate (purity 98.0%) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of standard solutions and quality control samples

Stock standard solutions of compound K and digoxin (IS) were prepared in methanol at a concentration of 1 mg/mL and stored at -20°C. Working standard solutions of compound K and IS were prepared by dilution of stock solutions in methanol and stored at -20°C. The working solutions of compound K were diluted by methanol before preparations of 10, 20, 50, 100, 1,000, 4,000, 8,000, and 10,000 ng/mL. The plasma calibration curves were prepared at concentrations of 1, 2, 5, 10, 100, 400, 800, and 1,000 ng/mL by spiking 90 μL of blank plasma with 10 μL of working standard of each analyte at appropriate concentration (to make up to 100 μL total sample

volume). To each sample was added 100 μL of 10 mM potassium phosphate buffer (pH 7.4) and 900 μL of ethyl acetate. Then, the samples were vortexed for 5 min, centrifuged for 10 min and concentrated by speed vacuum evaporator (Eppendorf AG 22331, Hamburg, Germany). The residue was reconstituted in 100 μL of mobile phase and 70 μL of sample was added to 10 μL of IS (1 μg/mL). The samples were transferred to injection vials, and 10 μL were injected onto an LC-MS/MS system for analysis.

The quality control (QC) samples were independently prepared in blank plasma at four levels of concentrations for compound K: 1 ng/mL (limit of quantification), 2 ng/mL (low concentration QC, LQC), 400 ng/mL (medium concentration QC, MQC), and 800 ng/mL (high concentration QC, HQC).

Sample preparation

The plasma samples (100 μL) were added to 100 μL of 10 mM potassium phosphate buffer (pH 7.4) and 900 μL of ethyl acetate. The mixture was vortex mixed and centrifuged according to the above condition for the preparation of the standard solution. The samples were concentrated, reconstituted and added to IS. Then, 10 μL aliquots of samples were injected into the LC-MS/MS system.

Instrumentation and analytical conditions

Analysis and validation were performed using an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA), interfaced with a mass spectrometer equipped with an electrosparay-ionization source operated in the positive mode (API4000; AB SCIEX, Foster City, CA, USA). Chromatographic separations were performed using a Luna C18 (2.0×100 mm inner diameter, 3 μm particle size; Phenomenex, Torrance, CA, USA) at a column temperature of 30°C. In order to optimize the mobile phase, triplicate LC-MS/MS analyses of a mixture containing 500 ng/mL of compound K and 500 ng/mL of IS in methanol:water (50:50) were performed consecutively using each of the investigated mobile phase combinations. All chromatographic separations were performed using each mobile phase combination at a flow rate 0.5 mL/min. The 10 mM ammonium acetate/methanol/acetonitrile (5/47.5/47.5, v/v/v) mixture was the optimum condition in terms of sensitivity and peak shape of compound K and digoxin [26].

The following parameters were optimized for compound K MS analysis: curtain gas 20 psi, collision gas 5 psi, ion spray voltage 5500 V, and temperature 350°C. The confirmation ion transitions for quantification were m/z 621.494→161.0 for compound K and 779.395→649.500 for digoxin.

Validation of the analytical method

The method was validated for selectivity, linearity, precision, accuracy and recovery. The selectivity of the method was investigated by comparing chromatograms of extracted blank plasma obtained from six different human plasma samples spiked with compound K and IS to ensure that it was free of interference at the retention time of compound K. The intra-day precision and accuracy were determined within one day by analyzing five sample replicates at concentrations of 1 (lower LQC, LLQC), 2 (LQC), 400 (MQC), and 800 ng/mL (HQC) for compound K. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the QC samples to their respective nominal values, expressed as percentages. The inter-day precision and accuracy were determined on five separate days at identical concentrations. A signal-to-noise ratio of three is generally accepted for estimating limit of detection (LOD) and signal-to-noise ratio of ten is used for estimating LOQ. Based on residual standard deviation of the response and the slope, the LOD for compound K was 0.3 ng/mL and LOQ was 1 ng/mL. To quantify for the loss of the compounds during the sample treatment process, the recovery of the method was determined as the ratio of the peak area of extracted QC samples after a full assay process to that from direct injection of equivalent concentrations of compounds in methanol. An eight-point standard curve ranging from 1 ng/mL to 1,000 ng/mL of compound K having calibration standards and used in a manner similar to the peak area ratios of the target ions of compound K to those of the IS was compared with weighted ($1/x^2$) least-squares calibration curves in which the peak area ratios of the calibration standards were plotted versus their concentrations.

The intra- and inter-day accuracies were determined by replicate analysis of the QC samples and at LOQ were extracted from the sample batch. Accuracy was defined as the percent relative error (RE, %) and the assay precision was calculated by the percent coefficient of variation (CV, %). The intra- and inter-day recoveries of compound K in blank plasma were determined by comparing the response of the analytes extracted from the replicate QC samples (n=5) at LLQC, LQC, MQC, and HQC with the response of analytes from post-extracted plasma standard samples at the equivalent concentrations.

Clinical application

This method was applied for clinical trial and was designed as an open label, single dose study, which was approved by the institutional review board of the Chonbuk National University Hospital (permission no. CUH 2011-09-022). The clinical trial was performed in the Clinical Trial Center, Chonbuk National University Hospital in Korea. All participants filled out the informed consent form before participating in this study. Ten young, healthy males (age, 24.9±2.7 years; weight, 68.1±6.3 kg) participated as study subjects and took a 5 g of fermented Korean red ginseng extract (containing 2.1 mg/g of Rg1 and Rb1, and 0.5 mg/g of compound K) dissolved in 100 mL of water as a single oral dose. Blood sample collected pre-dose and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8, 10, 12, 24, and 48 h post dose. The blood samples were centrifuged at 3,000 rpm for 10 min, and the plasma samples were stored at -70°C until LC-MS/MS analysis. Pheonix WinNonlin 6.2 (Pharsight Corporation, Cary, NC, USA) was used for pharmacokinetic analysis of the data by using a noncompartmental approach.

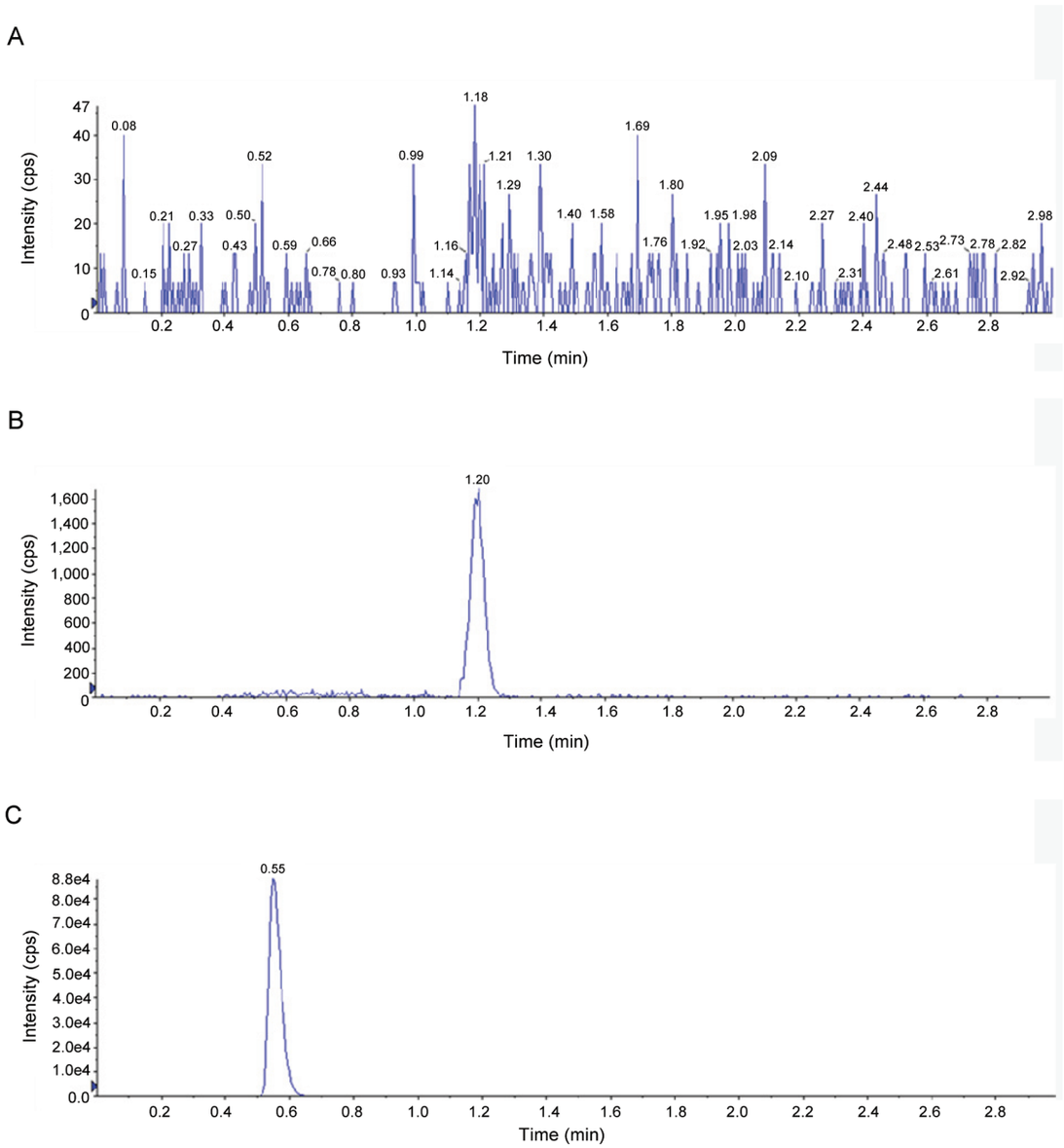

Fig. 2. Representative chromatograms of compound K and digoxin in blank and spiked human plasma (compound K, m/z 621.494→161.0; digoxin, m/z 779.395→649.500): (A) blank, (B) compound K spiked at lower limit of quantification (1 ng/mL), and (C) digoxin spiked (100 ng/mL).

RESULTS AND DISCUSSION

Method validation

An LC-MS/MS method for the estimation of compound K in human plasma was developed and validated. Representative chromatograms of compound K in blank plasma, LLOQ, and IS are shown in Fig. 2 for selectivity. The results of these analyses were used to evaluate the linearity range of quantification and LOQ, set to the lowest point of the calibration curve. The intra-day precision and accuracy for compound K at LLOQ (1 ng/mL) were 8.30% (CV) and 6.00% (RE), respectively. The plasma was validated over a concentration range of 1 to 1,000 ng/mL. A good linearity was obtained with r^2 always larger than 0.995 (y=0.013x+0.0076 [y, peak area; x, concentration {ng/mL}], r^2=0.9968). The precision of the determination was evaluated in both MS/MS acquisition methods according to the calculated percentage (CV) of the replicated compound K sample solutions. The precision and accuracy results are reported in Table 1. Intra-day precision (CV) ranged from 1.17% to 8.30%, and intra-day accuracy (RE) ranged from -10.38% to 11.45%. Inter-day precision (CV) ranged from 1.16% to 9.14%, and intra-day accuracy (RE) ranged from -8.66% to 12.63%. To evaluate these determination, four points of the calibration curve (at LLQC, low, medium, high concentration) were processed by the aforementioned liquid-liquid extraction method and then analyzed in triplicate. The recovery was evaluated by processing 1, 2, 400, and 800 ng/mL of compound K concentration. The recovery was expressed by the percent recovery reported in Table 2. Recovery rates were >85.4%, which was consistent over the concentration range 2 to 800 ng/mL at both intra- and inter-day. The intra- and inter-day precisions and accuracies below 15% indicated that in stabilized human plasma samples, compound K concentrations can be determined with reasonable precision and accuracy and that these methods can be used for pharmacokinetic studies. Wang et al. [27] reported ginsenoside metabolite by ultra performance liquid chromatography-time of flight/mass spactrometry after pretreatment using solid phase extraction. In this study, it was simple and fast pretreatment by protein precipitation method. The retention time of compound K was long at 28.3 min than that of current method (1.2 min). To analysis of many samples, it is required rapid sample preparation and short analysis time. In conclusion, this method demonstrated good linearity, precision, and accuracy, and consumed less time, which is helpful in pharmacokinetic study for fermented Korean red ginseng in humans.

Table 1. Intra- and inter-day precision and accuracy (n=5) of compound K in human plasma quality control samples

Statistical variable	Concentration (ng/mL)			
	1	2	400	800
Intra-day				
Mean	1.06	2.07	445.80	717.00
CV (%)	8.30	4.74	1.17	4.81
RE (%)	6.00	3.50	11.45	-10.38
Inter-day				
Mean	0.96	1.98	450.50	730.70
CV (%)	9.14	4.96	1.16	4.72
RE (%)	-3.75	-1.15	12.63	-8.66

RE (%)=100xstandard deviation/mean.
RE, relative error; CV, coefficient of variation.

Table 2. Recovery (n=5) of compound K in human blank plasma quality control samples at intra- and inter-day

Concentration (ng/mL)	Recovery (%)	
	Intra-day	Inter-day
1	106.00±0.08	85.40±0.08
2	103.50±0.19	94.50±0.09
400	111.45±5.21	112.50±5.21
800	89.62±34.46	95.87±34.46

Each value is presented as mean±SD.
Recovery (%)=100×(amount found–amount contained)/amount added.

Clinical application

The study showed that LC-MS/MS could be a useful method with fast analysis time for the rapid and sensitive analysis of even small sample amounts of compound K.

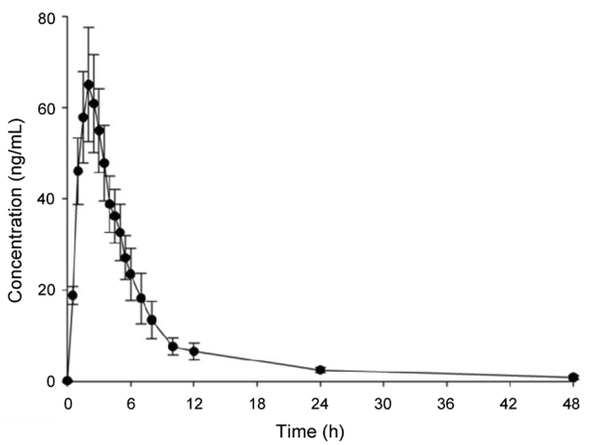

Fig. 3. Mean plasma concentration-time profiles of compound K after oral administration of fermented Korean red ginseng extracts in ten healthy Korean subjects. Each point represents the mean±standard error.

Table 3. Pharmacokinetic parameters of compound K after a single oral administration of fermented Korean red ginseng

Subject ID	C_{max} (ng/mL)	T_{max} (h)	AUC_{last} (ng·h/mL)	AUC_{inf} (ng·h/mL)	$t_{1/2}$(h)
1	68.9	2.5	341.4	348.5	9.9
2	85.2	1.5	440.9	447.6	9.3
3	117.0	2.0	911.4	942.8	10.9
4	16.5	1.5	89.3	89.8	3.6
5	142.0	2.0	774.5	911.5	23.2
6	78.7	2.5	459.6	483.7	13.9
7	55.8	2.0	297.1	309.1	6.9
8	49.3	1.0	249.3	259.8	7.3
9	30.4	2.0	180.3	191.1	8.3
10	31.8	1.5	245.9	273.1	5.9
Mean	67.6	1.85	399.0	425.7	9.9
SD	39.7	0.47	260.8	287.8	5.5

The developed method was applied to a pharmacokinetic study of compound K in ten healthy Korean subjects. The plasma concentration-time profile of compound K following administration of fermented Korean red ginseng is shown in Fig. 3 and exhibits multiexponential decay kinetics. The pharmacokinetic parameters derived from plasma concentration-time profiles of compound K are summarized in Table 3. C_{max}, T_{max}, AUC_{last}, and AUC_{inf} were 67.6±39.7 ng/mL, 1.85±0.47 h, 399.0±260.8 ng·h/mL, and 425.7±287.8 ng·h/mL, respectively. The half-life ($t_{1/2}$) of compound K was 9.9±5.5 h. In this study, the half-life in human plasma differed from that in rat plasma [28]. Because there are no data for the pharmacokinetic property, including oral bioavailability and dose-linearity of compound K in human, this method can apply to them. After oral administration, analysis of compound K in human urine and feces as well as plasma will help to find the exact metabolic pathway of compound K. In the future study, to evaluate overall ginseng metabolism in human body, simultaneous determination of many metabolites including compound K will be necessary. Hence, more research is required regarding compound K with the current analytic method.

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