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Short communication

LC–MS/MS method for determination of hederacolchiside E, a neuroactive saponin from *Pulsatilla koreana* extract in rat plasma for pharmacokinetic study

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ABSTRACT

A simple, rapid, and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was applied to pharmacokinetic study of a neuroactive oleanolic-glycoside saponin, hederacolchiside E from SK-PC-B70M, a standardized extract of *Pulsatilla koreana* in rat. Rat plasma samples were pretreated by protein precipitation with acetonitrile, eluted from C_{18} column, and analyzed using electrospray ionization (ESI)-MS/MS in negative ion mode. Digoxin was used as an internal standard. The standard curves were linear ($r > 0.997$) over the concentration ranges of 2–500 ng/mL. The intra- and inter-day precisions were measured to be below 9% and accuracy between 90 and 111% for all quality control samples at 2, 20, 100, and 500 ng/mL ($n = 5$). The lower limits of quantification (LLOQ) for hederacolchiside E was 2 ng/mL and the limit of detection (LOD) 0.5 ng/mL using 20 μL of plasma sample. Subsequently, hederacolchiside E was determined in rat plasma samples after oral administration of SK-PC-B70M. The mean maximum plasma concentrations of hederacolchiside E were 0.07, 0.13, and 0.36 μg/mL and the mean areas under the plasma concentration versus time curve 0.56, 1.27, and 6.46 μg h/mL at doses of 100, 200, and 400 mg/kg, respectively, which indicated non-linear pharmacokinetic pattern. In conclusion, this method was successfully applied to the pharmacokinetic study of hederacolchiside E after an oral administration of SK-PC-B70M to rats.

1. Introduction

Pulsatilla koreana Nakai (Ranunculaceae) is a traditional Korean herbal medicine, which has been used to treat amoebic dysentery and malaria [1]. Recently, it has been reported that a standardized extract of *P. koreana*, SK-PC-B70M, improves scopolamine-induced impairments of memory consolidation and spatial working memory [2,3] and SK-PC-B70M is under development as an agent for treatment of Alzheimer's disease. The major constituents of SK-PC-B70M are known to be oleanolic-glycoside saponins, and especially, hederacolchiside E has been reported to be a major biologically active compound of SK-PC-B70M [3–5]. The content of hederacolchiside E in SK-PC-B70M was determined to be more than 30% in our preliminary study. Hederacolchiside E is considered to be a marker compound for SK-PC-B70M. Accordingly, determination of hederacolchiside E in biological samples such as plasma is necessary for pharmacokinetic study of SK-PC-B70M as a preclinical or clinical investigation.

There are many reports on analysis of triterpene saponins such as ginsenosides in biological samples using liquid chromatography–mass spectrometry (LC–MS) or liquid chromatography–tandem mass spectrometry (LC–MS/MS) [6–10]. Differently to the reported saponins, hederacolchiside E is polyglycosides with 6 glycoside moieties at oleanolic acid backbone. A few papers regarding the analytical methods for polyglycoside saponins using LC–MS/MS have been published.

In this investigation, a sensitive, simple, and rapid method using LC–MS/MS was developed to determine hederacolchiside E in rat plasma samples. Subsequently, application to the pharmacokinetic study of SK-PC-B70M in rats was presented.

2. Experimental

2.1. Materials

Hederacolchiside E and SK-PC-B70M were provided from SK chemicals (Suwon, Korea). The purity of hederacolchiside E was assayed to be higher than 99% by LC–MS analysis. Content of hederacolchiside E in SK-PC-B70M was determined to be 31.2% by the LC–MS/MS analysis method developed in this study. Digoxin was

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purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Acetonitrile (ACN), tetrahydrofuran (THF), and formic acid (FA) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). HPLC-grade water was prepared using a Direct-Q 3UV Ultrapure Water System (Millipore; Bedford, MA, USA).

2.2. LC–MS/MS conditions

The LC/MS system consisted of a Nanospace SI-2 HPLC system (LC pumps 3101, degasser 3010, column oven 3014, and autosampler 3133; Shiseido Co., Ltd., Tokyo, Japan) with an API4000 triple quadrupole mass spectrometer (Applied Biosystems-SCIEX, Concord, Canada), equipped with a turboionspray source. Chromatographic separation was achieved on a Hydrosphere C_{18} column (2.0 mm × 50 mm, 3 μm; YMC Co., Ltd., Kyoto, Japan). The HPLC mobile phases consisted of (A) 0.1% FA:THF (90:10) and (B) ACN:0.1% FA:THF (80:10:10). A linear gradient program was used with flow rate of 0.5 mL/min. Initial composition of solvent (B) was 25%. Solvent (B) was gradually increased 60% for 3 min, maintained for 0.5 min and followed by a 1.5 min re-equilibration. Injection volume was 2 μL. Column oven temperature was set at 50 °C. Electrospray ionization (ESI) was performed in the negative mode. Nitrogen (99.999%) was used as the nebulizing, turbo spray and curtain gas, with the optimum values set at 60, 60 and 20 (arbitrary units), respectively. Nebulizer temperature was 450 °C. For optimizing multiple reaction monitoring (MRM) condition, a precursor ion and daughter ions were scanned at the range of m/z 400–1300. The precursor–product ion pairs used in MRM mode were: m/z 1365 → 895 (hederacolchiside E) and m/z 779 → 649 (IS, digoxin).

2.3. Preparation of calibration standards and quality control (QC) samples

The stock solution of hederacolchiside E was prepared in dimethyl sulfoxide and serially diluted to give working solutions of 20, 50, 100, 200, 500, 1000, 2000, and 5000 ng/mL in methanol. The IS (digoxin) solutions (50 ng/mL) was prepared in methanol. All stock solutions and working solutions were stored at 4 °C.

Calibration standards and QC samples were prepared by spiking 50 μL of working solutions into 450 μL of rat blank plasma. Calibration standards were obtained with concentrations of 2, 5, 10, 20, 50, 100, 200, and 500 ng/mL. QC samples were obtained with concentrations of 2, 20, 100, and 500 ng/mL. Aliquots (50 μL) were prepared from the bulk samples and stored at −20 °C until analysis.

2.4. Sample preparation

Twenty microliters of rat plasma samples were mixed with 10 μL of internal standard solution and 40 μL of ACN using a boltex mixer and centrifuged at 13,000 × g for 5 min. The supernatant was removed and transferred to LC–MS vial.

2.5. Method validation

The analytical method was validated according to the "Guidance for Industry, Bioanalytical Method Validation" presented by US Food and Drug Administration (2001). The calibration curve of hederacolchiside E in rat plasma was generated by plotting the peak area ratio for analyte to internal standard versus the concentrations in the standard-spiked plasma by least-square linear regression. The calibration curve was obtained using eight calibration standards, and each standard was prepared in triplicate.

The intra-day coefficient of variation (CV) and accuracy of the method were evaluated by the analysis of five plasma samples spiked with four concentration levels of hederacolchiside E (2, 20, 100, and 500 ng/mL as final concentrations). The CV and accuracy for inter-day assay were assessed at the same concentrations, and repeated for five different days. The precision was evaluated as the relative standard deviation of the mean expressed as percent (coefficient of variation: %CV) for each sample. The accuracy was calculated at each concentration to the nominal concentration multiplied by 100%, both using the intra- and inter-day precision data.

For freeze and thaw stability test, three aliquots at each of the low (10 ng/mL) and high (500 ng/mL) concentrations were stored at −70 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same condition. The freeze–thaw cycle was repeated two more times, and then analyzed on the third cycle. For short-term stability, the QC samples were thawed at room temperature and kept at this temperature for 4 or 10 h and then analyzed. For long-term stability, the QC samples were stored at −70 °C for 1-week or 2-weeks, thawed and then analyzed.

2.6. Animal treatments

Male Sprague–Dawley rats (250–280 g) were obtained from the Samtako Inc. (Osan, Korea). The animals were acclimated for at least 1 week. The animal quarters were strictly maintained at 23 ± 3 °C and 50 ± 10% relative humidity. A 12 h light and dark cycle was used with an intensity of 150–300 lx. All animal procedures were followed based on a guideline recommended by the Society of Toxicology (USA) in 1989. SK-PC-B70M was dissolved in water and orally administered to rats (n = 5). The rats were fasted overnight before drug administration and until 6 h after dosing.

2.7. Application to pharmacokinetic study

The developed LC–MS/MS method was applied to investigate the plasma concentration–time profile of hederacolchiside E after an oral administration of 100, 200, and 400 mg/kg of SK-PC-B70M to rats. Heparinized samples of blood (0.2 mL) were collected at 5, 15, 30, and 45 min and 1, 2, 4, 6, 8, 10, 12, 24, 36, and 48 h post-dose. Plasma was harvested after centrifugation and stored frozen at −20 °C until analyzed. Plasma-concentration data for individual rats were analyzed by a non-compartmental method using WinNonlin (Scientific Consulting, Inc., Lexington, KY, USA). The area under the plasma concentration–time curve (AUC) was calculated using the trapezoidal rule extrapolated to infinite time. The terminal elimination half-life ($t_{1/2}$), mean residence time (MRT), the peak plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were obtained directly from the experimental data.

3. Results and discussion

3.1. Investigation of LC–MS/MS condition of analytes

The mass response of hederacolchiside E in ESI was evaluated in both positive and negative ionization modes. The negative mode yielded signals of the deprotonated molecular ion $[M\text{-}H]^-$ more than 10-fold higher compared with the response for the protonated molecular ion in the positive mode for hederacolchiside E. As shown in Fig. 1, hederacolchiside E and digoxin produced the $[M\text{-}H]^-$ ions at m/z 1365 and 779, respectively. In the product ion scan, the main product ion for the $[M\text{-}H]^-$ precursor ions of hederacolchiside E was observed at m/z 895 with neutral losses of two glucoses and one rhamnose moiety via cleavage of the ester

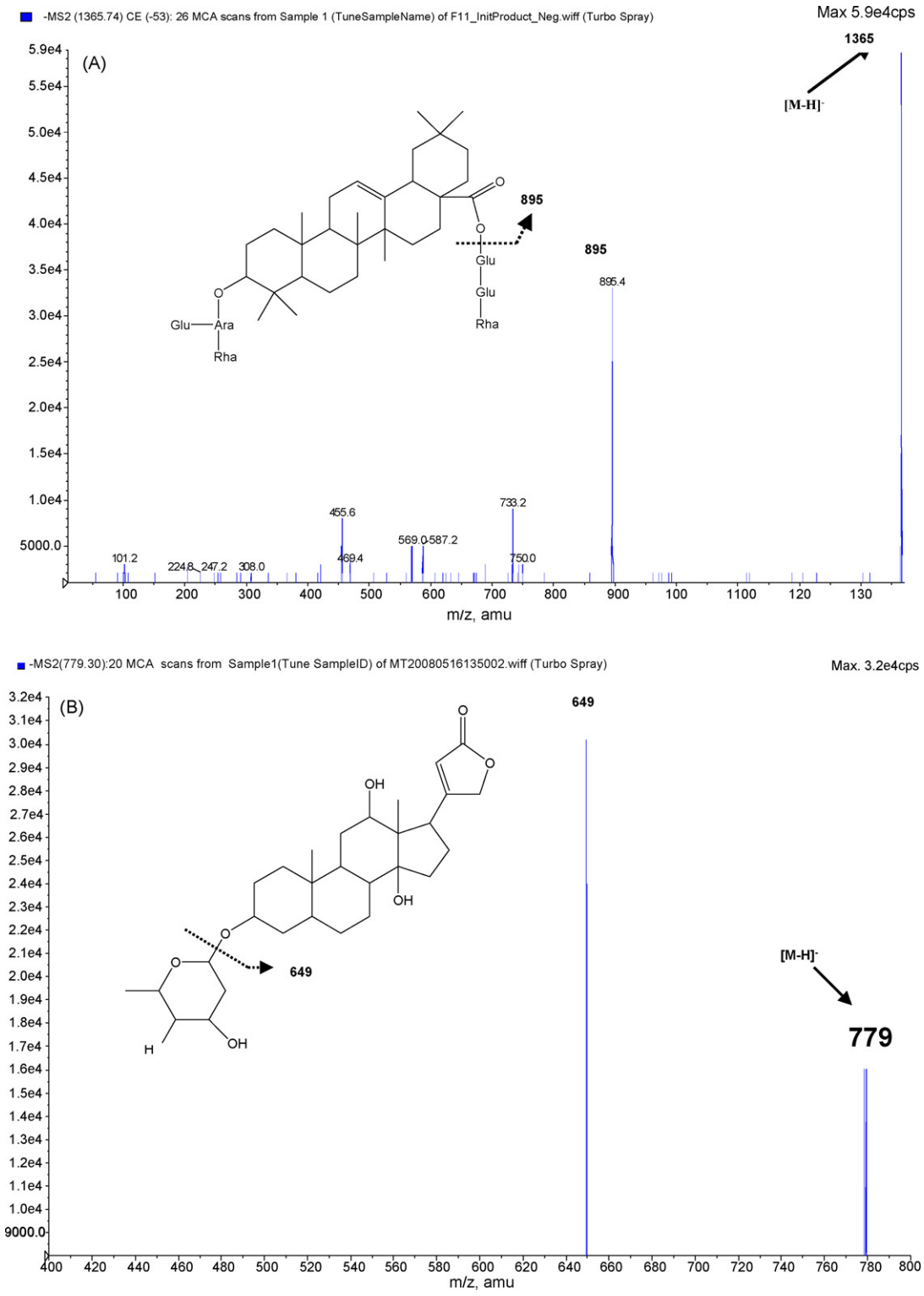

Fig. 1. Product ion mass spectra of hederacolchiside E and digoxin (IS).

bond. Digoxin showed the main product ion at *m*/*z* 649, suggesting loss of one digitoxoside moiety. Subsequently, the optimization of chromatographic separation was conducted on Hydrosphere C_{18} column with gradient mobile phase. Adding 10% THF to ACN–0.1% FA solvent system reduced a peak width and peak tailing and minimized carry-over effects. High flow rate of 0.5 mL/min allowed a rapid elution of analytes within 2 min.

3.2. Method validation

3.2.1. Specificity

The specificity of the method was presented by comparing MRM chromatograms of hederacolchiside E and digoxin for a blank rat plasma sample, a spiked plasma sample, and a plasma sample from a rat 0.5 h after oral administration of SK-PC-B70M. As shown in

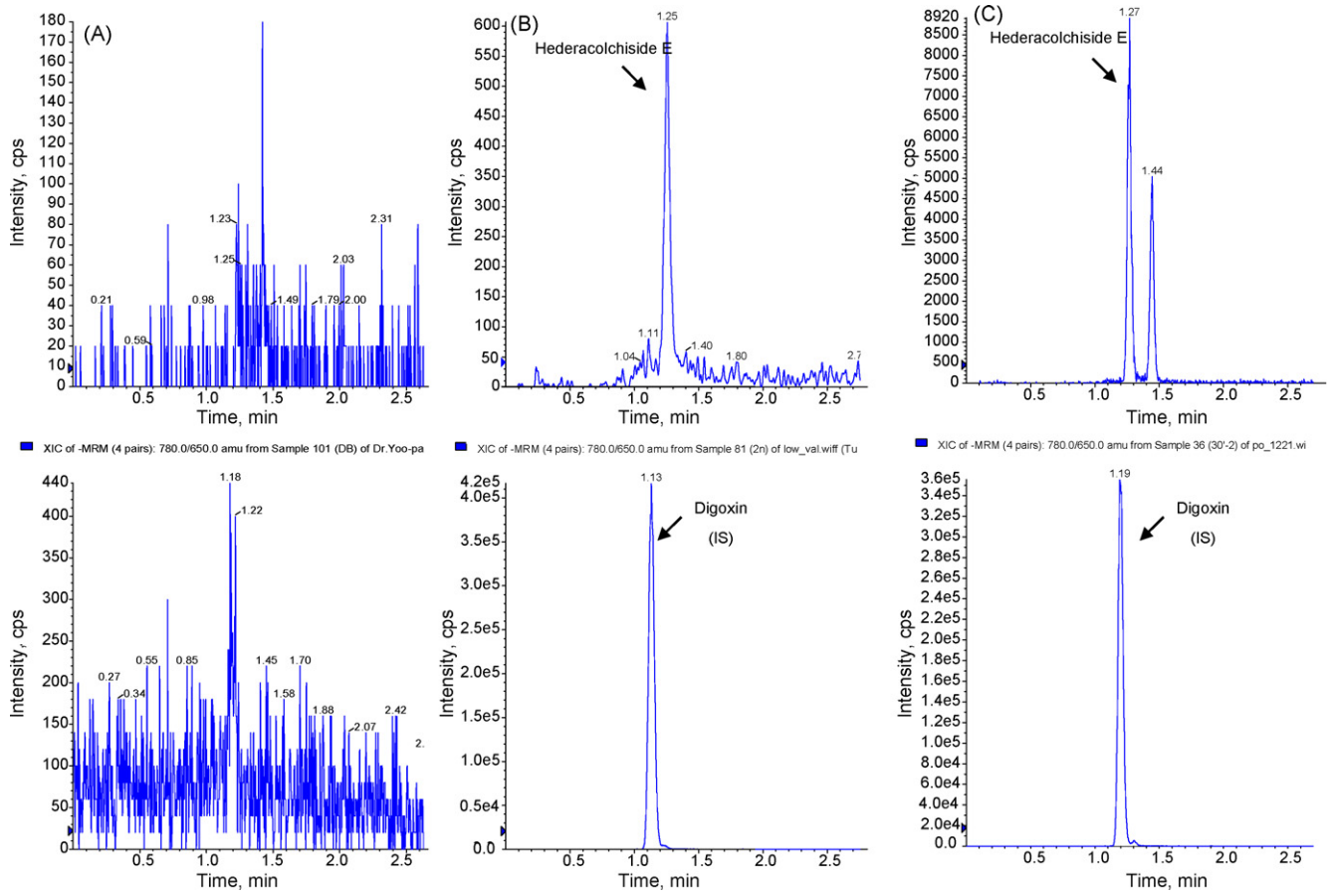

Fig. 2. MRM chromatograms of (A) a rat blank plasma sample, (B) blank plasma sample spiked with 2 ng/mL of hederacolchiside E and IS, and (C) plasma sample obtained 30 min after oral administration of SK-PC-B70M at a dose of 400 mg/kg.

Fig. 2, no significant peaks interfering with hederacolchiside E or digoxin were observed in the blank rat plasma. In the plasma sample collected from the dosed rat, another peak was detected at 1.4 min in the MRM condition for hederacolchiside E, which was thought to be an isomeric compound of hederacolchiside E.

3.2.2. Linearity and lower limit of quantitation (LLOQ)

Linear calibration curves with correlation coefficients greater than 0.997 were obtained in the concentration ranges 2–500 ng/mL in 20 μL of plasma (Table 1). The LLOQ defined as the lowest concentration analyzed with accuracy within 80–120% and a precision ≤20%, was 2 ng/mL in 20 μL of plasma in the present analytical system. The limit of detection (LOD) was determined to be 0.5 ng/mL with a signal to noise ratio of 3.

3.2.3. Precision and accuracy

Precision and accuracy of the assay were determined by replicate analyses ($n = 5$) of QC samples at four concentrations, by performing the complete analytical runs on the same day and also on five consecutive days. The intra-day precisions were less than 9% and the accuracy between 88.5 and 103% for all QC samples. The inter-day precisions were less than 5% and the accuracy between 93.5 and 111% for all QC samples. These results indicated that the present method has the acceptable accuracy and precision. The results are summarized in Table 2.

Table 2
Intra- and inter-day accuracy and precision for hederacolchiside E in rat plasma QC samples.

Statistical variables	Theoretical concentration (ng/mL)							
	Intra-day ($n = 5$)				Inter-day ($n = 5$)			
	2	20	100	500	2	20	100	500
Mean	2.05	17.7	90.4	481	2.12	18.7	108	553
CV (%)	8.97	3.15	3.88	3.34	4.72	4.81	3.48	4.26
Accuracy (%)	103	88.5	90.4	96.1	106	93.5	109	111

3.2.4. Stability

The stability of hederacolchiside E in rat plasma was investigated under a variety of storage and process conditions. Hederacolchiside

Table 1
Back-calculated concentrations of hederacolchiside E in calibration standards prepared in rat plasma.

Statistical variables	Theoretical concentration (ng/mL)								Calibration curve	Regression coefficiency (r)
	2	5	10	20	50	100	200	500		
Mean	1.94	5.11	10.6	19.0	48.1	94.6	210	497	$y = 0.00017x + 0.00202$	0.999
CV (%)	4.63	4.81	12.8	5.69	3.54	4.62	5.40	2.50		
Accuracy (%)	96.8	102	105	95.0	96.1	94.6	105	99.5		

Table 3
Short-term and long-term stabilities of hederacolchiside E in rat plasma samples.

Theoretical concentration (ng/mL)	Short-term stability						Long-term stability					
	4 h			10 h			1-week			2-week		
	Mean	Accuracy (%)	CV (%)	Mean	Accuracy (%)	CV (%)	Mean	Accuracy (%)	CV (%)	Mean	Accuracy (%)	CV (%)
10	9.79	97.9	2.1	9.73	97.3	2.1	9.72	97.2	10.9	9.74	97.4	4.6
500	487	97.5	4.4	510	102	2.5	514	103	0.9	501	100	0.6

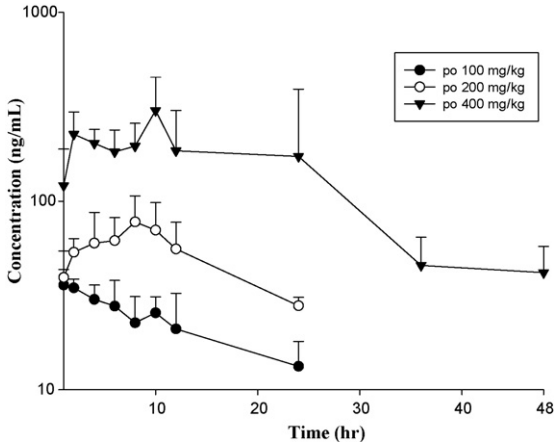

Fig. 3. Mean plasma concentration–time plots of hederacolchiside E after oral administration of SK-PC-B70M at doses of 100, 200, and 400 mg/kg to male SD rats. Each point represents mean ± SD ($n = 4$).

Table 4
Pharmacokinetic parameters of hederacolchiside E after oral administration of SK-PC-B70M at a dose of 400 mg/kg to male SD rats.

Parameter	SK-PC-B70M		
	100 mg/kg	200 mg/kg	400 mg/kg
AUC (μg h/mL)	0.56 ± 0.10	1.27 ± 0.27	6.46 ± 4.1
MRT (h)	9.46 ± 0.61	10.1 ± 0.41	16.1 ± 2.3
T_{max} (h)	0.38 ± 0.14	5.69 ± 4.13	11.5 ± 9.1
C_{max} (μg/mL)	0.07 ± 0.04	0.13 ± 0.08	0.36 ± 0.11
$T_{\lambda 1/2}$ (h)	31.1 ± 37.2	19.0 ± 18.5	28.9 ± 19.9

E was found to be stable in rat plasma with the acceptable accuracy (103 and 105%) and precision (6.3 and 4.2%) at 10 and 500 ng/mL, respectively, after three freeze/thaw cycles. The compound was also shown to be stable after 4 and 24 h in rat plasma sample at room temperature and 1-week and 2-week at −70 °C (Table 3).

3.2.5. *Pharmacokinetic studies of SK-PC-B70M*

The plasma samples collected after the oral administrations of SK-PC-B70M were analyzed by the present LC–MS/MS method and consequently the major bioactive compound of SK-PC-B70M, hederacolchiside E, was successfully determined. Fig. 3 shows mean plasma concentration–time curves of hederacolchiside E ($n = 4$) after oral administration of SK-PC-B70M at doses of 100, 200, and 400 mg/kg; the pharmacokinetic parameters are presented in Table 4. After the oral administration of SK-PC-B70M, the mean maximum plasma concentrations of hederacolchiside E were 0.07, 0.13, and 0.36 μg/mL and the mean areas under the plasma concentration versus time curve 0.56, 1.27, and 6.46 μg h/mL at doses of 100, 200, and 400 mg/kg, respectively, which indicated non-linear pharmacokinetic pattern. The mean apparent plasma half-life of terminal elimination phase were estimated to be 19.0–31.1 h.

4. Conclusions

In conclusion, the LC/MS/MS method for the analysis of neuroprotective oleanolic-glycoside saponin, hederacolchiside E in rat plasma was developed and validated. This method allowed a rapid and sensitive analysis with a small amount of plasma sample and a simple preparative step. Subsequently, the method was successfully applied to a pharmacokinetic study of a standardized *P. koreana* extract, SK-PC-B70M in rats.

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