10

Thai J. Pharm. Sci. 32 (2008) 10-16

High-performance liquid chromatographic determination of asiatic acid in human plasma

Phensri Thongnopnua

Biomedical Analysis Research Unit, Faculty of Pharmaceutical Sciences,
Chulalongkorn University, Bangkok, Thailand
Corresponding author: +66(0) 2218 8308 E-mail address: tphensri@chula.ac.th

Abstract:

Asiatic acid, an active metabolite of asiaticoside, is one of the biological active triterpenoids in *Centella asiatica.* A single liquid-liquid extraction and high-performance liquid chromatographic (HPLC) method have been developed and validated for the quantification of asiatic acid in human plasma. A polymeric based reversed-phase C_{18} column was used for the separation of asiatic acid and acenaphthrene (internal standard) with a mobile phase composed of methanol and acetonitrile in ultra pure water (5:57:38, v/v), operated at the flow rate of 1.0 ml/min. The effluent was quantitated at 217 nm. The accuracy and precision of the method was confirmed for both intra-day and inter-day with the % bias and %RSD less than 8%. Recovery of asiatic acid from human plasma was 95.0%. The method would possibly be applicable for pharmacokinetic study of asiatic acid in human plasma following oral administration of *Centella asiatica* alcoholic extract or asiaticoside.

Keywords: Asiatic acid; Asiaticoside; *Centella asiatica;* Human plasma; HPLC; Triterpenoids

Introduction

Centella asiatica (L.) Urban. (Umbelliferae), commonly named Gotu Kola, is a small herbaceous perennial plant, preferably grown in damp swampy areas. The plant has been reported to possess antileprotic, antitumor, antistress, wound healing, antifilarial, antifeedant and antibacterial properties. This plant is also used as tonic in several Ayurvedic formulations [1-7]. These pharmacological efficacies have been proven to relate to four bioactive triterpenes from an alcoholic extract of *C. asiatica* leaves [8-10]. They are asiatic acid, madecassic acid, asiaticoside and madecassoside (Figure 1). Among these compounds, asiatic acid has been reported to be the most therapeutically active for collagen synthesis stimulation [11].

Although, a variety of clinical and pharmacodynamic studies [8-9, 12-15] on the extracts of *C. asiatica* have been presented, their pharmacokinetics were scarcely reported [16-17]. It has been clarified that after ingestion of *C. asiatica* alcoholic extract to human, the detectable concentration in plasma was asiatic acid, the metabolite product of asiaticoside. Asiaticoside is hydrolytic cleavage of the sugar moiety to become asiatic acid which is responsible for the therapeutic effects [16-17]. In pharmacokinetic study, the reliable bio-analytical method for determining asiatic acid in plasma is needed. The method of Gimoldi *et al.* [17] required 2 ml of plasma sample and double liquid-liquid extraction for asiatic

acid determination in human plasma. Although the method of Rush *et al.* [16] used only 1 ml of plasma sample, but the expendable solid-phase cartridge and combined gas chromatography/mass spectrometry were used. The method of Baek *et al.* [18] determined asiaticoside in rat plasma instead of asiatic acid.

This study presents the analytical method for asiatic acid in plasma utilizing only 350 µl of plasma sample with single liquid-liquid extraction. The method was fully validated to confirm its feasibility for further pharmacokinetic study.

Material and methods
Chemicals and reagents

Asiaticoside and asiatic acid (99.5% purity) were from Changzhou National Products Development Co., Ltd., China. Acenaphthrene (internal standard) was purchased from Sigma, USA. Ammonium chloride (99.5%) and tetrabutyl ammonium bisulfate were of analytical grade from Fluka, Argentina. A 85% phosphoric acid was supplied by Lab Scan, Thailand. For HPLC grade reagents, acetonitrile and methanol were purchased from Burdick & Jackson (Honey Well, Muskegon, MI, USA). Ethyl acetate (AR) was from Fischer Scientific, UK. The ultra pure water was used for HPLC analysis. Human plasma for analytical method development was supplied by the Plasma Division, Thai Red Cross Society, Thailand.
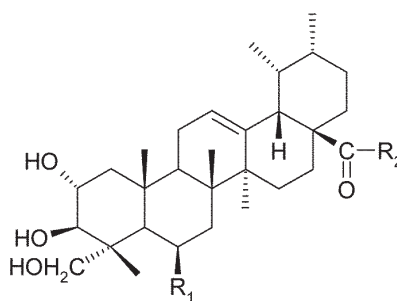
	R_1	R_2	Mol. formula	Mol. wt.
Asiaticoside	H	O-glu-glu-rham	$C_{48}H_{78}O_{19}$	958
Madecassoside	OH	O-glu-glu-rham	$C_{48}H_{78}O_{20}$	974
Madecassic acid	OH	OH	$C_{30}H_{48}O_6$	504
Asiatic acid	H	OH	$C_{30}H_{48}O_5$	488

Figure 1 Structure of asiaticoside, madecassoside, madecassic acid, and asiatic acid

12

Thai J. Pharm. Sci. 32 (2008) 10-16

Apparatus and chromatographic condition

The HPLC system (Water®; Water Corporation, MA, U.S.A.) consisted of Alliance 2695 Separations Module, 2487 dual absorbance detector and Empower workstation. The separation of compounds was made on an X-Terra® RP$_{18}$, 5 μm (150 mm x 4.6 mm, i.d.) at ambient temperature. The column was preceded by a Waters Sentry Guard Column X-Terra® RP$_{18}$, 3.5 μm (20 mm x 4.6 mm, i.d.).

The mobile phase comprised of methanol, acetonitrile and ultra pure water (5:57:38 v/v), delivered at the flow rate of 1.0 ml/min. The effluent was monitored by UV detector at the wavelength of 217 nm.

Standard solutions

Stock methanolic standard solution of asiatic acid and acenaphthrene as internal standard (IS) was separately prepared at the concentration of 1 mg/ml. These stock solutions were kept frozen and used within one month. The working standard solutions of IS, 0.10 μg/ml, was biweekly prepared from the stock solution. The serial dilution of methanolic asiatic acid as calibration solutions was also prepared biweekly.

The calibration for plasma samples was prepared by spiking plasma with standard asiatic acid solution at concentrations of 0.20, 0.60, 0.80, 1.0, 1.20, 1.40 and 1.60 μg/ml plasma.

Sample preparations

A 0.35 ml of blank human plasma was added into micro-tube containing 10 μl of standard asiatic acid solution. The tube was gentle vortex-mixed. Liquid extraction was preceded by subsequent adding 6.0 M aqueous ammonium chloride solution, 0.01 M tetrabutyl ammonium bisulfate solution, 85% phosphoric acid and ethyl acetate. After vortex-mixing, the mixture was rotated at 45 rpm for 30 min. The organic layer was separated after centrifuging at 13,500**x**g and dried under nitrogen gas. The residue was reconstituted with 80 ml methonolic acenaphthrene solution and injected 50 μl into the HPLC system.

Bio-analytical method validation

The developed analytical method was validated to ensure the linearity, accuracy and precision (intra-day and inter-day), sensitivity and specificity of the method. The stabilities of asiatic acid in plasma and solution as well as the processed analyte were also determined under specified conditions.

The calibration curve of asiatic acid in plasma was constructed from the peak area ratio (PAR) of asiatic acid to IS versus nominal asiatic acid concentrations in the range of 0.20-1.60 μg/ml. The reproducibility of linearity was confirmed by analyzing the other three series of spiked plasma standards of asiatic acid.

The lowest limit of quantitation (LLOQ) of asiatic acid in plasma was determined as the lowest non-zero concentration level, which could be accurately determined within the acceptable accuracy (% bias $\leq \pm$ 20%) and precision (%RSD $\leq$ 20%).

Spiked plasma standards of asiatic acid at the concentration of 0.4, 0.8 and 1.2 μg/ml were analyzed in a set of six replicates within one day for evaluating the intra-day accuracy and precision. For the inter-day assay, the same analysis was performed on six separate days. Imprecision and in accuracy were expressed as the percentage of %RSD $\leq$ 15% and %bias of $\leq \pm$15%, respectively.

Three replicates of spiked asiatic acid plasma standard at the concentrations of 0.40 and 0.80 μg/ml were used for short term (room temperature, 25 °C), long term (storage temperature, -48 °C) and freezed-thawed stability studies. The stability of asiatic acid and acenaphthrene (IS) stock solution as well as the processed analyte in autosampler (4 °C) were also quantified.

Results and discussion
Sample preparation

Asiatic acid is the ionized compound. Ethyl acetate extraction was promoted by pairing the ionized asiatic acid with tetrabutyl ammonium bisulfate. The efficiency of asiatic acid extraction was determined to be

approximately 95%. This sample preparation method is therefore appropriate for further analysis of asiatic acid in plasma.

In preliminary study, ethyl acetate, dichloromethane and chloroform were utilized as extracting solvents for asiatic acid in plasma. It was found that asiatic acid could be extracted by any of these three solvents with the efficiency of extraction of not less than 74 %. It was unfortunately that there were some interfering peaks when dichloromethane or chloroform was used as the extracting agent. However no interfering peak was observed from the chromatogram of ethyl acetate extraction. Therefore, ethyl acetate was selected as the appropriate extracting solvent for asiatic acid in plasma. The addition of tetrabutyl ammonium bisulfate as the ion-pairing agent into the plasma before extracting with ethyl acetate, could improve the efficiency of extraction to be 95%. This single step liquid extraction was efficient and less time consuming compared to the method of Gimoldi *et al.* [17]. In addition, the expense per sample of this single liquid extraction should be less than Rush's method that had to use disposable solid phase cartridge [16]. The plasma deproteinization as reported by Baek *et al.* [18] were also performed in this study. The percentage recoveries of asiatic acid post-deproteinization was determined to be less than 50%. Hence deproteinization would not be suitable for asiatic acid analysis in plasma. In all aforementioned reports, no any internal standard was used [16-18]. This study explored many chemical compounds that would be suited for internal standard. Finally, acenaphthrene was selected as an appropriate internal standard for controlling chromatographic condition. Therefore, this simple liquid-liquid extraction method was transferred to further method validation.

Chromatographic condition

XTerra® RP_{18} is the appropriate HPLC column for asiatic acid analysis in plasma comparing to many other C_{18} packing columns, tried in this study. With this polymeric based column, the retention of asiatic acid was not affected by the variation of mobile phase's pH. The initial mobile phase composition used, was

acetonitrile and ultra pure water. By adding buffer and varying the pH of mobile phase between 2.0 to 7.5, the retention and resolution of asiatic acid was not significantly affected. It was observed that the retention of asiatic acid was influenced by the proportional volume of acetonitrile. The addition of methanol into the composition of mobile phase could improve the resolution of asiatic acid. Therefore the final mobile phase was contained of methanol, acetonitrile and ultra pure water. Since mobile phase needed no buffer, this convenient mobile phase composition would gain more advantage than other previous reports [17-18], in term of less time consuming for column clean-up and equilibration.

Assay validation

The calibration curve of peak-area ratio of asiatic acid to IS versus spiked asiatic acid in plasma was linear in the concentration range of 0.2-1.60 µg/ml with a coefficient of determination (R^2) of 0.9937. The percentage relative standard deviation (%RSD) of slope and R^2 were 10.70 and 0.05%, respectively (Table 1), confirms the reproducibility of linear range of calibration curve. This concentration range would be used for the subsequent study of the validation parameters.

The intra-day and inter-day accuracy and precision of the method, evaluating by analyzing six spiked plasma samples containing three different concentrations of asiatic acid, are summarized in Table 2. The accuracy of the method was confirmed by the values of the percentage of bias within the same day and between six different days analysis that were within the ranges -7.45 to +0.47% and -2.16 to +3.03%, respectively. Meanwhile, the range of 2.56-3.08% RSD for intra-day analysis and 4.38-7.85% RSD for inter-day analysis supported the precision of the method.

The lowest limit of quantitation (LLOQ) for asiatic acid in plasma was determined to be 0.20 µg/ml. The accuracy and precision of this concentration was confirmed with the average bias of -4.50 and 16.73% RSD. The method is sensitive enough for analyzing asiatic acid in plasma.

The specificity of the analytical method was evidenced in the identical retention time of 19.6 min and 26.8 min for asiatic acid and IS in standard solution and in spiked plasma standard. No any detectable interference was observed in the chromatogram (Figure 2).

To determine the stability of asiatic acid in human plasma, a number of stability experiments were preformed. These are summarized in Table 3. Asiatic acid in plasma was stable at laboratory room temperature (23 ± 2 °C) for 8 hr and could be kept frozen (-48 ± 1 °C) for 25 days. Plasma samples could be frozen and thawed for up to three cycles without any deterioration of asiatic acid. Asiatic acid in the presence of in-processed solution in autosampler at 4 °C was stable for 18 hr. Meanwhile, the stock solution of asiatic acid and IS could be kept at -18 ± 2 °C up to 25 days.

Conclusion

The simple analytical method for asiatic acid in plasma was developed and validated. The method consisted of single ethyl acetate extraction and subsequent reversed-phase HPLC analysis with UV detection. The method was demonstrated to be specific, precise and accurate. It would possibly be applicable for pharmacokinetic study of asiatic acid in human plasma following asiaticoside administration.
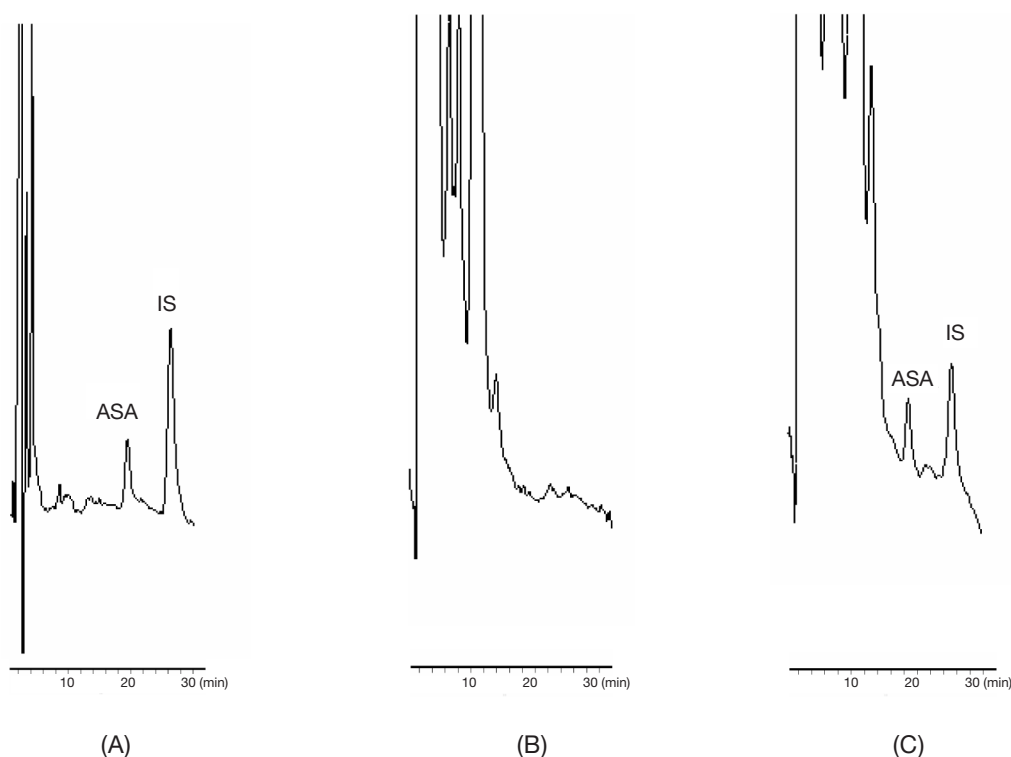
Acknowledgement

Figure 2 Chromatograms of asiatic acid (ASA) and internal standard (IS) in standard solution of ASA and IS (A), blank plasma (B) and standard plasma spiked with ASA and IS (C)

Table 1 Reproducibility of the calibration equation for asiatic acid analysis in plasma

Calibration equation PAR = a + b.Conc.	Slope (b)	Intercept (a)	R^2 value
Mean (n = 3)	0.44	0.12	0.9937
Standard deviation	0.05	0.04	0.0005
Relative standard deviation (%)	10.70		0.05

Table 2 Intra- and inter-day accuracy and precision of the determination of asiatic acid in plasma

Nominal plasma concentration (µg/ml)	Concentration analyzed (µg/ml) mean ± SD	Accuracy bias (%)	Precision RSD (%)
Intra-day (n=6)			
0.40	0.392 ± 0.014	+0.47	2.56
0.70	0.713 ± 0.022	-7.38	3.08
1.20	1.138 ± 0.030	-7.45	2.63
Inter-day (n=6)			
0.40	0.395 ± 0.031	+1.30	7.85
0.70	0.685 ± 0.030	-2.16	4.38
1.20	1.234 ± 0.088	+3.03	6.93

Table 3 Stability data of asiatic acid

Stability of asiatic acid	Condition	Mean recoveries ± SD (%) (n = 3)	RSD (%)
In human plasma	RT (23 ± 2°C) 8 hr	100.9 ± 6.82	6.76
	Frozen temp.(-48 ± 1°C) 25 d	97.40 ± 3.56	3.66
After three	Storage at -48°C, 12-24 hr;		
freeze-thaw cycles	Thawed at RT	103.5 ± 5.16	4.98
In processed analyte	4°C in auto sampler 18 hr	100.9 ± 6.82	6.76
In stock solution	25 d (-18 ± 2°C)		
	asiatic acid (1.0 mg/ml)	99.47 ± 2.28	2.30
	IS (0.10 µg/ml)	99.53 ± 0.30	0.30

References

[1]   E. Bailey. Treatment of leprosy, *Nature* 155: 601 (1945).

[2]   T.D. Babu, G. Kuttan, and J. Paddikala. Cytotoxic and antitumor properties of certain taxa of Umbelliferae with special reference to *Centella asiatica (L.)* Urban, *J. Ethnopharmacol.* 48: 53-57 (1995).

[3]   D.N.K. Sharma, R.L. Khosa, and J.P.N. Chansauria, M. Sahai. Antistress activity of *Tinospora cordifolia and Centella asiatica, Phytother. Res.* 10: 181-183 (1996).

[4]   Y. Asakawa, R. Matsuda, and T. Takemoto. Mono and sesquiterpenoids from *Hydrocotyl and Centella species, Phytochemistry* 21: 2590-2592 (1982).

[5]   T. Chakraborty, S.P. Sinha, and N.C. Sukul. Preliminary evidence of antifilarial effect of *Centella asiatica on canine dirolfilariasis, Fitoterapia* 67: 110-112 (1996).

[6]   R. Srivastava, and Y.N. Shukla. Disubstituted pyrone from *Centella asiatica, Indian J. Chem.* 36B: 963-964 (1997).

[7]   R. Srivastava, Y.N. Shukla, and M.P. Darokar. Antibacterial activity of *Centella asiatica, Fitoterapia* 68: 466-467 (1997).

[8]   A. Shukla, A.M. Rasik, G.K. Jain, R. Shankar, D.K. Kulshrestha, and B.N. Dhawan. *In vitro* and *in vivo* wound healing activity of asiaticoside isolated from *Centella asiatica, J. Ethnopharmacol.* 65: 1-11 (1999).

[9]   M.R. Cesarone, L. Incandela, M.T. De Sanctis, G. Belcaro, P. Bavera, M. Bucci, and E. Ippolito. Evaluation of treatment of diabetic microangiopathy with total triterpenic fraction of *Centella asiatica:* a clinical prospective randomized trial with a microcirculatory model, *Angiology* 52: S33-37 (2001).

[10] S. Sasaki, H. Shinkai, Y. Akashi, and Y. Kishihara. Studies of the mechanism of action of asiaticoside (Madecassol®) on experimental granulation tissue and cultured fibroblasts and its clinical application in systemic scleroderma, *Acta Dermato-Venereologica* 52: 141-150 (1972).

[11] F.X. Marquart, G. Bellon, P. Gillery, Y. Wegrowski, and J.P. Borel. Stimulation of collagen synthesis in fibroblast cultures by a tritepene extracted from *Centella asiatica, Connect. Tissue Res.* 24: 107-120 (1990).

[12] F. Bonte, M. Dumas, C. Chaudagne, and A. Meybeck. Influence of asiatic acid, madecassic acid and asiaticoside on human collagen I synthesis, *Planta. Med.* 60: 133-135 (1994).

[13] G. Belcaro. Microvascular evaluation by Laser-Doppler flowmetry of the effects of Centellase in the treatment of severe venous hypertension and leg ulcers, *Phlebology* 2: 189-195 (1987).

[14] L. Incandela, G. Belcaro, M.T.De Sanctis, M.R. Cesarone, M. Griffin, E. Ippolito, M. Bucci, and M. Cacchio. Total triterpenic fraction of *Centella asiatica* in the treatment of venous hypertension: a clinical, prospective, randomized trial using a combined microcirculatory model, *Angiology* 52: S61-67 (2001).

[15] L. Incandela, G. Belcaro, M.R. Cesarone, M.T. De Sanctis, E. Nargi, P. Patricelli, and M. Bucci. Treatment of diabetic microangiopathy and edema with total triterpenic fraction of *Centella asiatica* : a prospective, placebo-controlled randomized study, *Angiology* 52: S27-31 (2001).

[16] W.R. Rush, G.R. Murray, and D.J.M. Graham. The comparative steady-state bioavailability of the active ingredients of madecassol, *Eur. J. Drug. Metab. Pharmacok.* 18: 323-326 (1993).

[17] R. Grimaldi, F. De Ponti, L. D'Angelo, M. Caravaggi, G. Guidi, S. Lecchin, G.M. Frigo, and A. Crema. Pharmacokinetics of the total triterpenic fraction of *Centella asiatica* after single and multiple administrations to healthy volunteers: a new assay for asiatic acid, *J. Ethnopharmacol.* 28: 235-241 (1990).

[18] M. Baek, Y-S. Rho, and D-H. Kim. Column-switching high-performance liquid chromatographic assay for determination of asiaticoside in rat plasma and bile with ultraviolet absorbance detection, *J. Chromatogr. B.* 732: 357-363 (1999).