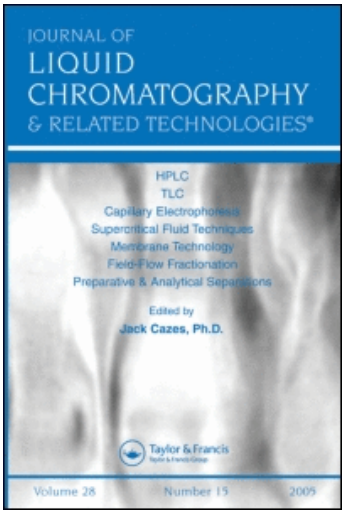

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Separation and Determination of Asiaticoside, Asiaticoside-B and Madecassoside in *Centella asiatica* Total Triterpenoid Saponins by HPLC

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Abstract: A high performance liquid chromatographic method was developed for the separation and determination of asiaticoside and madecassoside isomers (asiaticoside-B and madecassoside) in *Centella asiatica* products. The method involved separation by reversed phase chromatography on an Atlantis T3 C_{18} column using water-acetonitrile-methyl tert-butyl ether (80:18:2:0.1, 0.1% acetic acid, by volume) as mobile phase. The isomers of asiaticoside-B and madecassoside were separated with high resolution by the developed method. The method's linearity, precision, and recovery were validated. The effects of stationary phase and mobile phase composition on the separation of madecassoside isomers were also investigated. Experimental results indicated that the Atlantis T3 C_{18} column offered enhancing retention and resolution to the polar isomers, and the method could be referenced for the separation of other triterpenoid saponins isomers.

Keywords: Asiaticoside, *Centella asiatica*, HPLC, Isomer, Madecassoside, Separation

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INTRODUCTION

Centella asiatica (L.) urban, also known as gotu kola and Indian pennywort, is a traditional herbal medicine used in Asiatic countries for hundreds of years. It contains many bioactive triterpenoid saponins, mainly asiaticoside, asiaticoside-B and madecassoside.[1–3] Their chemical structures are shown in Figure 1, in which madecassoside and asiaticoside-B is a pair of isomeric compounds. Triterpenoid saponins from *Centella asiatica* are widely used to treat leprosy, wound healing, psoriasis, ulceration, eczema, cancer, and arthritis, etc.[4–8] Recent study revealed that these triterpenoid saponins exhibited different pharmacological activity and toxicity.[9] Thus, an effective method for the separation and determination of triterpenoid saponins from *Centella asiatica* is needed.

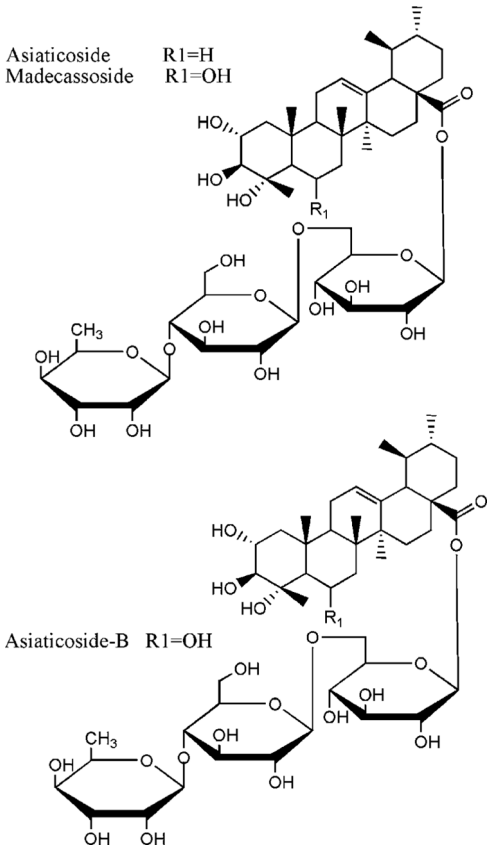

Figure 1. Chemical structures of madecassoside, asiaticoside-B and asiaticoside.

Triterpenoid saponins are complex molecules consisting of non-sugar aglycone coupled to sugar chain units. Due to the fact that saponins usually occur in plants as a mixture of structurally related forms with very similar polarities, their separation and determination still remains a challenge.[10–11] High performance liquid chromatography (HPLC) using C_{18} as stationary phase and UV or evaporative light scattering as the detector is the most effective method for the separation and determination of saponins in plant extracts.[10–15] In our previous works, we also presented a reversed phase HPLC method with mass spectrometry for the separation and determination of soyasaponins.[16–17] However, many of previous reported HPLC systems failed to separate the isomers of triterpenoid saponins, e.g., asiaticoside-B and madecassoside.[18–21] That is because triterpenoid saponins are highly polar compounds, and the structural and polar difference between aglycone isomers is weakened due to existence of highly polar sugar chain units. Up to now, there have been few studies on the separation of triterpenoid saponins isomers (asiaticoside-B and madecassoside, etc.).

In this study, the separation of asiaticoside, asiaticoside-B, and madecassoside by HPLC were conducted. The effects of the stationary phase and the mobile phase composition on the separation of madecassoside isomers were discussed. A specific HPLC method for the determination of madecassoside and asiaticoside in *Centella asiatica* products was presented.

EXPERIMENTAL

Materials

Standard asiaticoside (purity: 98%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Standard madecassoside (purity: 88%) was purchased from Sigma. *Centella asiatica* total triterpenoid saponins were purchased from Guangxi Changzhou Natural Products Development Co., Ltd, Nanning, China. HPLC grade acetonitrile, methyl tert-butyl ether (MTBE) and methanol were purchased from Tedia Company, USA. Pure water was purchased from Hangzhou Wahaha Group Co., Ltd, Hangzhou, China. All other chemicals (analytical grade) were commercially available and used without further purification unless otherwise stated.

HPLC System

Experiments were conducted on a Waters HPLC system consisting of a 1525 binary HPLC pump, 717plus autosampler, 2487 UV-vis detector

and thermostat. Chromatographic columns tested were Nova-Pak C_{18} (150×4.6 mm I.D., 5 μm, Waters), Symmetry C_{18} (250×4.6 mm I.D., 5 μm, Waters), Sunfire C_{18} (250×4.6 mm I.D., 5 μm, Waters) and Atlantis T3 C_{18} (250×4.6 mm I.D., 5 μm, Waters). All the separations were carried out at a flow rate of 1 mL min^{-1}, the column temperature of 30°C and a wavelength of 203 nm. The sample injection volume was 10 μL. The composition of mobile phase was indicated in the article.

Sample Preparation

The *Centella asiatica* total triterpenoid saponins were accurately weighed (100 mg) and dissolved in 10 mL methanol-water mixture (50:50, v/v). The solution was filtered through a 0.45 μm filter before use.

Standard solutions of madecassoside and asiaticoside were prepared as follows. Standards asiaticoside or madecassoside were accurately weighed (10 mg) and dissolved in 10 mL methanol-water mixture. Further standard solutions with other concentrations were prepared by diluting the stock solution with methanol-water mixture.

RESULTS AND DISCUSSION

Method Development

Several reversed phase C_{18} columns, Nova-Pak C_{18}, Symmetry C_{18}, Sunfire C_{18}, and Atlantis T3 C_{18}, were tested for the separation of asiaticoside, asiaticoside-B, and madecassoside, with acetonitrile-water (25:75, v/v) as mobile phase. One of the C_{18} column, Atlantis T3 C_{18}, uses an optimized silica particle (optimization of pore size, ligand type and density, and endcapping) that prevents the hydrophobic silica pores of expelling highly polar aqueous eluents thus, enhancing retentive performance in aqueous conditions.[22] All of the columns were successful in the separation of asiaticoside and madecassoside. However, as shown in Figure 2, chromatograms just show the peak splitting of asiaticoside-B and madecassoside using Nova-Pak C_{18}, Symmetry C_{18}, and Sunfire C_{18} column as stationary phases, and neither achieved baseline separation. The best separation performance of asiaticoside-B and madecassoside was obtained using Atlantis T3 C_{18} as stationary phase (250×4.6 mm I.D., 5 μm). This is because asiaticoside-B and madecassoside are highly polar compounds, and the Atlantis T3 C_{18} column offers increased retention to these polar compounds as compared to other C_{18} columns (as indicated in Figure 2).

In order to achieve a satisfactory separation of asiaticoside-B and madecassoside, the composition of the mobile phase was optimized and

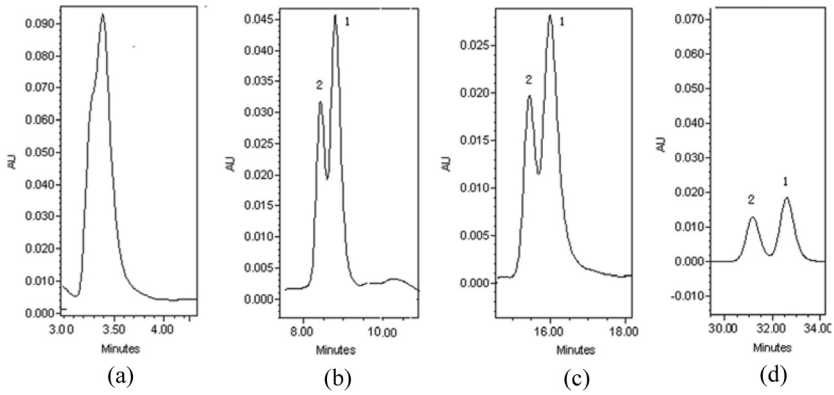

Figure 2. Separation of asiaticoside-B (2) and madecassoside (1) on different C_{18} column with acetonitrile-water (25:75, v/v) as mobile phase. Chromatographic conditions: Nova-Pak C_{18} (a), Symmetry C_{18} (b), Sunfire C_{18} (c) and Atlantis T3 C_{18} (d) respectively; a flow rate of 1.0 mL/min; 30°C; 203 nm.

the effect of the mobile phase on the separation was studied. The separation was initially carried out using different volumetric ratio of methanol and water as mobile phase on the Atlantis T3 C_{18} column. As shown in Figure 3, the retention and resolution of asiaticoside-B and madecassoside increases with the decrease of methanol content in the mobile phase. However, in spite of the retention time of two isomeric

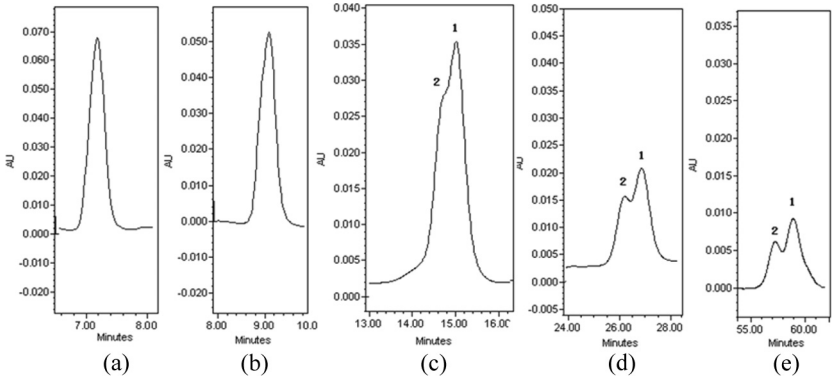

Figure 3. Effect of water and methanol ratio on the separation of asiaticoside-B (2) and madecassoside (1) on Atlantis T3 C_{18} column. Chromatographic conditions: the volumetric ratio of water to methanol is 45:55 (a), 50:50 (b), 55:45 (c), 60:40 (d) and 65:35 (e), respectively; a flow rate of 1.0 mL/min; 30°C; 203 nm.

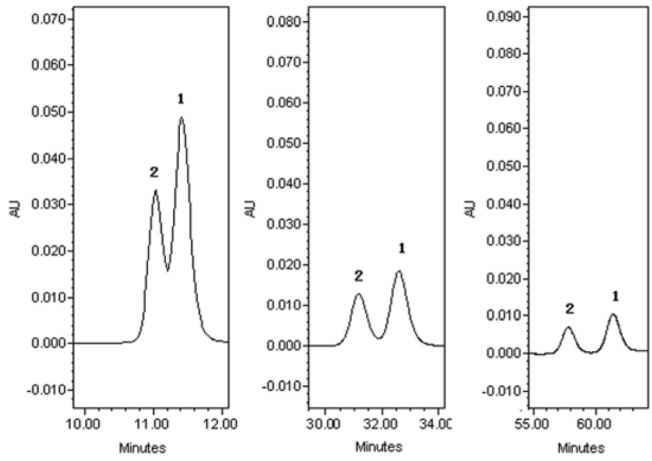

Figure 4. Effect of water and acetonitrile ratio on the separation of asiaticoside-B (2) and madecassoside (1) on Atlantis T3 C_{18} column. Chromatographic conditions: the volumetric ratio of water to acetonitrile is 65:35 (a), 75:25 (b) and 80:20 (c) respectively; a flow rate of 1.0 mL/min; 30°C; 203 nm.

compounds being near 58 min with water-methanol (65:35, v/v) as mobile phase, the chromatogram just shows peak splitting, and baseline separation hasn't been achieved. Replacing methanol with acetonitrile, the separation performance enhanced remarkably, as indicated in Figure 4. The retention and resolution of asiaticoside-B and madecassoside both increase with the decrease of acetonitrile content. Baseline separation was achieved when the mobile phase was water-acetonitrile (80:20, v/v). However, the time needed for analysis was too long. To shorten the analysis time, MTBE was added to the acetonitrile-water mixture as a modifier. Figure 5 shows the results obtained; the retention time of the two isomeric compounds decrease dramatically with increasing of the concentration of MTBE, whereas resolution only has a light drop from 1.85 to 1.73.

The optimized chromatographic conditions for the separation of asiaticoside, asiaticoside-B, and madecassoside are: Atlantis T3 C_{18} column (250×4.6 mm I.D., 5 μm, Waters) as the stationary phase and a mobile phase of water-acetonitrile-MTBE (80:18:2, by volume) with 0.1% acetic acid for improving the peak shape. By the developed HPLC method, baseline separation of asiaticoside-B and madecassoside was obtained and the retention time of asiaticoside-B, madecassoside, and asiaticoside was 24.7, 26.6, and 52.4 min, respectively.

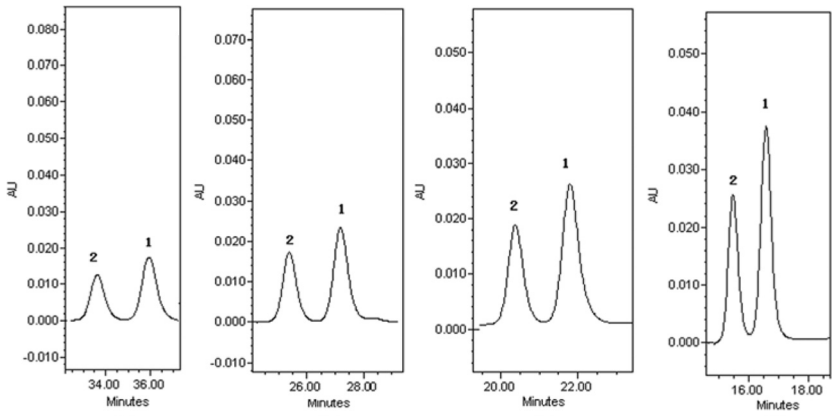

Figure 5. Effect of MTBE concentration on the separation of asiaticoside-B (2) and madecassoside (1) on Atlantis T3 C_{18} column. Chromatographic conditions: the volumetric ratio of water, acetonitrile and MTBE is 80:18:1 (a), 80:18:2 (b), 80:18:3 (c) and 80:18:4 (d) respectively; a flow rate of 1.0 mL/min; 30°C; 203 nm.

Method Validation

Linearity

The calibration curves for asiaticoside and madecassoside were constructed by respectively analyzing a series of asiaticoside and madecassoside standard samples. Table 1 gives the linear equation, linear range, and correlation coefficients for the two compounds, where y is the values of peak area (mv sec) and x is the concentration of the standard compounds (mg mL^{-1}). Good linearity is found in the investigated concentration range for each of the compounds examined.

Precision

Assay precision was evaluated by repeatedly analyzing the standard solutions of asiaticoside or madecassoside five times using the developed

Table 1. Linear regression data of asiaticoside and madecassoside

Compounds	Linear range (mg mL^{-1})	Regression equation	Correlation coefficient
Asiaticoside	0.0545–0.545	$Y = 2836840 \times -26962$	0.9992
Madecassoside	0.072–0.5412	$Y = 2937530 \times -6275$	0.9999

method. The relative standard deviation for asiaticoside and madecassoside is 1.13% and 1.16%, respectively.

Recovery

For the recovery experiments, known amounts of standard asiaticoside or madecassoside were added to the sample, and then it was analyzed three times by the developed HPLC method. The average recovery obtained was 98.5% and 98.9% for asiaticoside and madecassoside, respectively. Considering the results of linearity, precision, and recovery, the developed HPLC method is accurate and reliable.

Analysis of the Sample of Total Triterpenoid Saponins

The developed HPLC method was applied to the determination of asiaticoside and madecassoside in *Centella asiatica* total triterpenoid saponins. The contents of asiaticoside and madecassoside are 26.7% and 25.53%, respectively. The peak location of madecassoside was not interfered by asiaticoside-B in the sample.

CONCLUSIONS

Asiaticoside-B and madecassoside were separated with high resolution on Atlantis T3 C_{18} column using water-acetonitrile-MTBE (80:18:2, containing 0.1% acetic acid, by volume) as mobile phase. The kinds of C_{18} stationary phase and mobile phase composition have remarkable effects on the separation performance of asiaticoside-B and madecassoside. The Atlantis T3 C_{18} column offers an enhancing retention to these polar triterpenoid saponins, and it is suitable for the separation of asiaticoside-B and madecassoside. Mobile phase composed by acetonitrile-water is superior to methanol-water mixture, and the resolution of asiaticoside-B and madecassoside increases with decreasing the content of acetonirile.

The developed HPLC method is specific for the simultaneous analysis of asiaticoside and madecassoside in *Centella asiatica* products with good sensitivity, precision, and repeatability, and the analytical result of madecassoside is not interfered by asiaticoside-B in the sample. The developed method can be referenced for the analysis of other triterpenoid saponins isomers.

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REFERENCES

1. Singh, B.; Rastogi, R.P. A reinvestigation of the triterpenes of *Centella asiatica* III. Phytochemistry **1969**, *8*, 917–921.
2. Sahu, N.P.; Roy, S.K.; Mahato, S.B. Spectroscopic determination of structures of triterpenoid trisaccharides from *Centella asiatica*. Phytochemistry **1989**, *28*, 2852–2854.
3. Matsuda, H.; Morikawa, T.; Ueda, H.; Yoshikawa, M. Medicinal foodstuffs. XXVII. Saponin constituents of gotu kola (2): Structures of new ursane- and oleanane-type triterpene oligoglycosides, centellasaponins B, C, and D, from *Centella asiatica* cultivated in Sri Lanka. Chem. Pharm. Bull. **2001**, *49* (10), 1368–1371.
4. Gnanapragasam, A.; Yogeeta, S.; Subhashini, R.; Ebenezar, K.K.; Sathish, V.; Devaki, T. Adriamycin induced myocardial failure in rats: Protective role of Centella asiatica. **2007**, *294* (1–2), 55–63.
5. Gnanapragasam, A.; Ebenezar, K.K.; Sathish, V.; Govindaraju, P.; Devaki, T. Protective effect of *Centella asiatica* on antioxidant tissue defense system against adriamycin induced cardiomyopathy in rats. Life Sci. **2004**, *76* (5), 585–597.
6. Sampson, J.H.; Raman, A.; Karlsen, G.; Navsaria, H.; Leigh, I.M. *In vitro* keratinocyte antiproliferant effect of *Centella asiatica* extract and triterpenoid saponins. Phytomedicine **2001**, *8* (3), 230–235.
7. Shukla, A.; Rasik, A.M.; Jain, G.K.; Shankar, R.; Kulshrestha, D.K.; Dhawan, B.N. In vitro and in vivo wound healing activity of asiaticoside isolated from *Centella asiatica*. J. Ethnopharmacol. **1999**, *65* (1), 1–11.
8. Cheng, C.L.; Koo, M.W.L. Effects of *Centella asiatica* on ethanol induced gastric mucosal lesions in rats. Life Sci. **2000**, *67* (21), 2647–2653.
9. Bouillon, A.L.; Paris, G.S. Method for preparing a centelia asiatica extract rich in madecassoside and in terminoloside. US patent 2006/0106206A1 2006.
10. Oleszek, W.; Bialy, Z. Chromatographic determination of plant saponins—An update (2002–2005). J. Chromatogr. A. **2006**, *1112*, 78–91.
11. Ha, Y.W.; Nab, Y.C.; Seo, J.J.; Kima, S.N.; Linhardt, R.J.; Kim, Y.S. Qualitative and quantitative determination of ten major saponins in Platycodi Radix by high performance liquid chromatography with evaporative light scattering detection and mass spectrometry. J. Chromatogr. A **2006**, *1135*, 27–35.
12. Rupasinghe, H.P.V.; Jackson, C.J.; Poysa, V.; Berardo, C.D.; Bewley, J.D.; Jenkinson, J. Soyasapogenol A and B distribution in soybean (*Glycine max* L. Merr.) in relation to seed physiology, genetic variability, and growing location. J. Agric. Food Chem. **2003**, *51* (20), 5888–5894.
13. Müller, A.; Ganzera, M.; Stuppner, H. Analysis of phenolic glycosides and saponins in *Primula elatior* and *Primula veris* (primula root) by liquid

chromatography, evaporative light scattering detection and mass spectrometry. J. Chromatogr. A **2006**, *1112*, 218–223.
14. Schaneberg, B.T.; Mikell, J.R.; Bedir, E.; Khan, I.A. An improve HPLC method for quantitative determination of six triterpenes in *Centella asiatica* extracts and commercial products. Pharmazie **2003**, *58*, 381–384.
15. Pan, J.; Kai, G.Q.; Yuan, C.X.; Jin. R.S. Separation and determination of the structural isomers of madecassoside by HPLC using β-cyclodextrin as mobile phase additive. Chromatographia **2007**, *66* (1–2), 121–123.
16. Jin, M.C.; Yang, Y.W.; Su, B.G.; Ren, Q.L. Determination of soyasaponins Ba and Bb in human serum by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. J. Chromatogr. B **2007**, *846*, 169–175.
17. Jin, M.C.; Yang, Y.W.; Su, B.G.; Ren, Q.L. Rapid quantification and characterization of soyasaponins by high-performance liquid chromatography coupled with electrospray mass spectrometry. J. Chromatogr. A **2006**, *1108*, 31–37.
18. Inamdar, P.K.; Yeole, R.D.; Ghogare, A.B. Determination of biologically active constituents in *Centella asiatica*. J. Chromatogr. A **1996**, *742*, 127–130.
19. Verma, R.K.; Bhartariya, K.G.; Gupta, M.M.; Kumar, S. Reverse-phase high performance liquid chromatography of asiaticoside in *Centella asiatica*. Phytochem. Anal. **1999**, *10*, 191–193.
20. Jia, G.T.; Lu, X.Y. Enrichment and purification of madecassoside and asiaticoside from *Centella asiatica* extracts with macroporous resins. J. Chromatogr. A **2008**, *1993*, 136–141.
21. Aziz, Z.A.; Davey, M.R.; Power, J.B.; Anthony, P.; Smith, R.M.; Lowe, K.C. Production of asiaticoside and madecassoside in *Centella asiatica in vitro* and *in vivo*. Biologia Plantarum **2007**, *51* (1), 34–42.
22. t'Kindtl, R.; Alaerts, G.; Heyden, Y.V.; Deforce, D.; Bocxlaer, J.V. Broad-spectrum separations in metabolomics using enhanced polar LC stationary phases: a dedicated evaluation using plant metabolites. J. Sepn. Sci. **2007**, *30*, 2002–2011.

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