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Reverse-phase High Performance Liquid Chromatography of Asiaticoside in *Centella asiatica*

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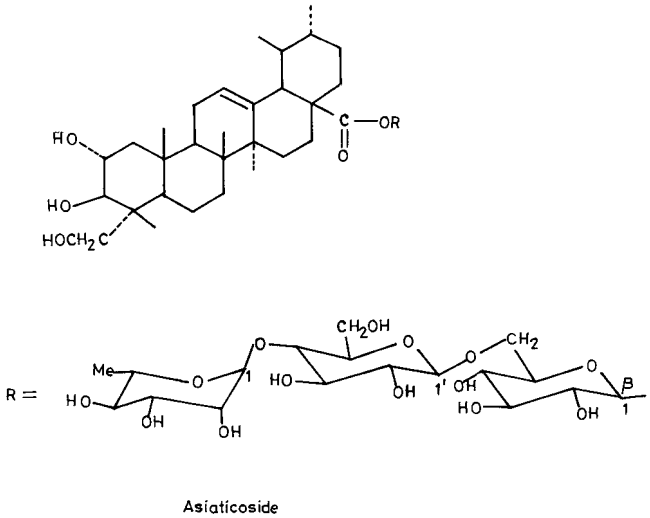
A rapid and simple reverse-phase high performance liquid chromatographic method has been developed for the quantitative determination of asiaticoside, a pharmacologically active constituent from *Centella asiatica*. Using an octadecyl silane-packed column eluted with water (containing 1% trifluoroacetic acid):methanol (30:70, v/v), asiaticoside was well resolved from other constituents. These optimized analytical conditions allowed the determination of asiaticoside with a recovery of 97%. Copyright © 1999 John Wiley & Sons, Ltd.

Keywords: Anti-leprosy; asiaticoside; reverse-phase high performance liquid chromatography; *Centella asiatica*; Umbelliferae.

INTRODUCTION

Centella asiatica (syn. *Hydrocotyle asiatica*), a plant of the family Umbelliferae, is a weakly scented species occurring in parts of India, Sri Lanka, China, Indonesia, Malaysia, Australia and Southern and Central Africa. It has been used in traditional medicine in India for the treatment of leprosy, varicose veins, ulcers, lupus and certain eczemas, and of mental retardation since prehistoric times (Sharma *et al.*, 1985; Kartnig, 1988).



Asiaticoside

Infusions or poultices of *C. asiatica* have been used in Europe since the eighteenth century for the treatment of lesions of leprosy (Wolfram, 1965; Kartnig, 1988). The drug is also reported to possess anti-epileptic activity (Moharana and Moharana, 1994). Clinical trials have shown that extracts of *C. asiatica* heal wounds, burns and ulcerous abnormalities of the skin, cure stomach and duodenal ulcers, and are effective in the treatment of leprosy, lupus, scleroderma and diseases of the veins (Kartnig, 1988). Many commercial drug preparations of *C. asiatica* are available in West Germany and France (Kartnig, 1988). Asiaticoside, a trisaccharide triterpene, has been identified as the most active compound in the plant associated with the healing of wounds and duodenal ulcers, whilst the triterpene saponins are also reported to possess immunomodulatory properties (Plohmann *et al.*, 1994).

Along with the use of *C. asiatica* in medicine, the plant is also finding acceptance as a vegetable. Several groups are in the process of domesticating and developing cultivars of this species. In order to screen the germplasm and segregating populations of *C. asiatica*, a fast and accurate analytical procedure is required for estimating the asiaticoside content of individual plants. There are only a few reports on the quantitative determination of triterpenes in *C. asiatica* (Meng and Zeng, 1988; Gunther and Wagner, 1996). The TLC-densitometric procedure of Meng and Zeng (1988) lacks precision in comparison with HPLC. In our series of studies concerned with the development of HPLC procedures for the rapid determination of plant drugs (Verma *et al.*, 1990, 1996; Gupta *et al.*, 1993), we have developed a rapid, sensitive and accurate reverse-phase HPLC method for the analysis of asiaticoside in *C. asiatica*. Using this method, a solvent suitable for the extraction of asiaticoside from *C. asiatica* plant material has been identified.

EXPERIMENTAL

Apparatus. The gradient liquid chromatographic system (model LC-10A series; Shimadzu, Tokyo, Japan) consisted of two LC-10AD pumps controlled by a CMB-10A interface module, a model 7725i manual injector valve (Rheodyne) equipped with a 20 μL sample loop, and a multi-dimensional UV–VIS detector (model SPD-10A). Data were collected and analysed using a class LC-10

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work station equipped with an HP-DeskJet printer. Solvents were filtered by using a Millipore system (Bangalore, India) and the analyses were performed on a CLC-ODS (M) column (250 × 4.6 mm i.d.; Shimadzu).

Reagents. The reagents used were HPLC grade (Spectrochem, Bombay, India) and were filtered through a Millipore filter (0.45 μm) prior to use. Asiaticoside was isolated and its structure was confirmed by comparison of spectroscopic data with those reported earlier (Mahato *et al.*, 1987), and by TLC co-elution with an authentic sample. For this, dried aerial material of *C. asiatica* (400 g) was obtained from the Institute's experimental field plots: a voucher specimen of the plant is deposited in the Institute's herbarium (no. 9219). The material was extracted with methanol at 20°C and the extract was fractionated into hexane soluble (13.4 g), chloroform soluble (0.4 g) and butanol soluble (9.5 g) fractions. The butanol soluble fraction was chromatographed on a silica gel column using mixtures of chloroform and methanol of increasing polarity as eluting solvent. Fractions from chloroform:methanol (85:15) afforded pure asiaticoside.

HPLC conditions. The composition of the mobile phase was optimized by varying the percentage of methanol in water: the following conditions were optimal: mobile phase, water [containing 1% trifluoroacetic acid (TFA)]: methanol (30:70, v/v); flow-rate, 1 mL/min; column temperature, 26°C; detector wavelength, 220 nm.

Extraction procedure. Aerial material of *C. asiatica* (1 g) was oven dried at 35–50°C and defatted with *n*-hexane (3 × 20 mL); the hexane insoluble material was extracted with methanol (3 × 20 mL; 4 h), filtered, concentrated under vacuum and the volume was made up to 3 mL with methanol. Samples were filtered through a Millipore filter and a known amount of each extract was subjected to HPLC under the above conditions. The contents of asiaticoside were calculated with the aid of a calibration graph obtained using a stock solution of pure asiaticoside (2 mg/mL, prepared in methanol), different volumes of which were analysed using the HPLC conditions as above. The areas under the peaks (*X*, arbitrary units) and the corresponding concentrations (*Y*) were used to plot the calibration graph.

Table 1. The chromatographic performance of a Shimadzu CLC-ODS column in the separation of asiaticoside from an extract of *Centella asiatica*

Composition of mobile phase[a]	Number of theoretical plates	Capacity factor	Recovery (%)	Resolution (factor)	Separation (factor)
25:75	4181	0.53	84	1.85	1.40
30:70	5472	0.86	97	3.68	1.60
35:65	4880	1.74	75	0.76	1.15

[a] Ratio of water (containing 1% TFA): methanol in the mobile phase.

RESULTS AND DISCUSSION

Selection of the mobile phase

Using an ODS HPLC column, the composition of the mobile phase was optimized by employing different proportions of methanol in water (containing 1% TFA). As an effective measure of column performance, the number of theoretical plates was at a maximum when

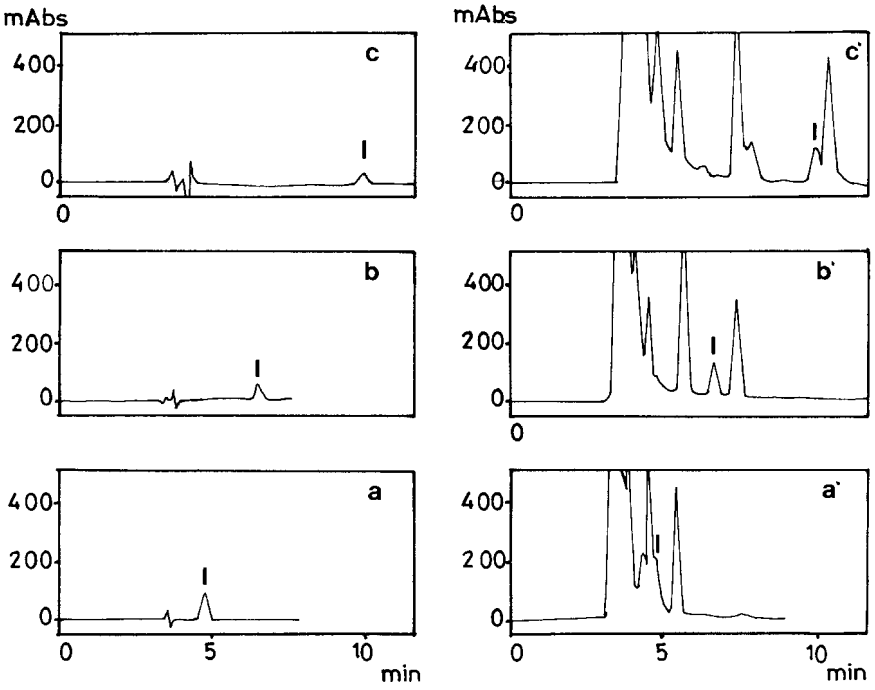

Figure 1. Reverse-phase HPLC separation of standard asiaticoside (I; traces A, B and C) and compound I in admixture with an extract of *Centella asiatica* (traces A′, B′ and C′) using a Shimadzu CLC-ODS column and UV detection at 220 nm. The mobile phases employed were: A, A′, water (containing 1% TFA): methanol (25:75); B, B′, water (containing 1% TFA): methanol (30:70); C, C′, water (containing 1% TFA): methanol (35:65), all at flow rates of 1 mL/min.

Table 2. The effect of the extracting solvent on the recovery of asiaticoside from dried material of *Centella asiatica*

Extracting solvent	Determined content of asiaticoside (% dry weight) ± standard error ($n = 4$)
Chloroform	0.310 ± 0.003
Ethyl acetate	0.300 ± 0.010
Methanol	0.360 ± 0.010
Ethanol	0.040 ± 0.003
Water	0.040 ± 0.004

30% water in methanol was used. The resolution and separation factors were also maximum using this solvent system (Table 1). Figure 1 illustrates the separation of asiaticoside from other components of a plant extract using different compositions of mobile phase. Peak overlapping and poor baseline separation were observed using 25% and 35% water in methanol, but with 30% water (containing 1% TFA) in methanol a clear baseline separation of asiaticoside (retention time 6.6 min) with a maximum recovery of 97% was achieved. In order to examine recovery rates, known amounts of a stock solution of pure asiaticoside were added to extracts of *C. asiatica* in each mobile phase and the quantitative determination was repeated three times.

Selection of the solvent for extraction

Five different solvents (chloroform, ethyl acetate, methanol, ethanol and water) were used separately for plant extraction. The processing of the extracts for HPLC was performed in a manner similar to that reported for the methanol extraction earlier: the means of four determinations in each case are presented in Table 2. The extraction of asiaticoside was very poor with ethanol and water: more efficient extractions were obtained with chloroform, ethyl acetate and methanol, the maximum being with the latter solvent (asiaticoside content determined as 0.36% dry weight; coefficient of variation 4.39%).

Linearity of calibration

The calibration graph for asiaticoside was linear in the range of 1–30 μg, and the regression equation was $Y = 1.53099 \times 10^{-5} X - 0.0107107$ ($r = 0.996874$).

The quantitative reverse-phase HPLC method described above has proved to be simple and rapid in its application to a large number of plant samples. Its use has led to the identification of a germplasm rich in asiaticoside (results to be published elsewhere).

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