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Journal of Pharmaceutical and Biomedical Analysis
24 (2001) 1049–1054

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Simultaneous determination of quercetin, kaempferol and (*E*)-cinnamic acid in vegetative organs of *Schisandra chinensis* Baill. by HPLC

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Received 16 May 2000; received in revised form 6 November 2000; accepted 8 November 2000

Abstract

A reversed-phase high-performance liquid chromatographic (RP-HPLC) separation method with UV spectrophotometric detection has been developed for the determination of major components in leaves and caulomas of *Schisandra chinensis* Baill. The flavonols (quercetin and kaempferol) and (*E*)-cinnamic acid were analysed after extraction with alcohol from the dry plant material. Identification was based on retention times and UV spectra by comparison with commercial standards. Quercetin, kaempferol and (*E*)-cinnamic acid were separated within 12 min using acetonitrile–aqueous 0.05% *ortho*-phosphoric acid (40:60 v/v) mobile phase. The method has been successfully applied for the quantitative analysis of all three major components in several samples from different harvests using propylparaben as the internal standard.

Keywords: *Schisandra chinensis* Baill.; Reverse-phase high-performance liquid chromatography; Separation, Quercetin; Kaempferol; (*E*)-Cinnamic acid

1. Introduction

Schisandra chinensis Baill. (Magnoliaceae) is a perennial climber growing in China, Japan and the Far East of the Union of Independent States. The fruits and seeds of *S. chinensis* Baill. are a famous traditional Chinese medicine with mainly tonic, adaptogenic and hepatoprotective effect [1,2]. There are more than 25 species of the schisandra genus world-wide, most of which can be found in China. However, in the Chinese pharmacopoeia, only two species are recorded for medical use, *S. chinensis* Baill. and *S. spenanthera* Rehd. et Wils. Both have shown significant biological activity [3,4]. Fruits and seeds contain mainly dibenzo-[*a*,*c*]-cyclooctadiene lignans (schisandrins, schisandrols, gomisins), volatile oil containing monoterpens (borneol) and sesquiter-

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PII: S0731-7085(00)00539-2

pens (sesquicarene, (+)α-ylangene, α- and β-chamigrene, chamigrenal).

Recently *S. chinensis* has become a raw material for the preparation of nutraceuticals that are used by sportsmen for nutrition and as a preventive means against civilisation diseases of the cardiovascular system. The plant also grows well in Central Europe, but it rarely produces fruit. Therefore, the vegetative parts of the plant (leaves and caulomas) were subjected to phytochemical studies. More than 30 compounds were isolated and confirmed by MS, IR and NMR. It was found that the major components in leaves and caulomas are quercetin, kaempferol and (*E*)-cinnamic acid (Fig. 1). Their analysis, which for all compounds together was not described earlier, is discussed in this report.

Reversed-phase high-performance liquid chromatography (RP-HPLC) is the commonly used analytical separation technique for either phenolic compounds like flavonols or for various derivatives of benzoic acid. Due to the variability of column filling materials and solvent systems, RP-HPLC exhibits a great potential in separating complex mixtures of phenolic compounds [5–11].

The purpose of this study was to develop a novel high-performance liquid chromatography procedure for simultaneous determination of all three major components in vegetative parts of *S. chinensis* Baill.

2. Experimental

2.1. Standards and solvents

Quercetin (**I**), kaempferol (**II**) and (*E*)-cinnamic acid (**III**) were obtained from Aldrich (Steinheim, Germany), propyl-4-hydroxybenzoate (propylparaben) was used as the internal standard from Merck (Darmstadt, Germany). Methanol, acetonitrile (isocratic grade), ethanol, *ortho*-phosphoric acid, potassium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany) and acetic acid from Lachema (Neratovice, Czech Republic). Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). All the solutions were filtered through 0.45 μm membranes (Schleicher & Schuell, Germany) and degassed by an ultrasonic bath before use.

2.2. Chromatographic system

The HPLC system consisted of an isocratic pump HPP 5001 (Laboratory Instruments, Prague, Czech Republic), a Waters™ 486 detector and Waters 746 data module (Waters, Milford, MA), a LCI 30 injection valve (ECOM, Prague, Czech Republic) with a 10 μl loop. Analyses were

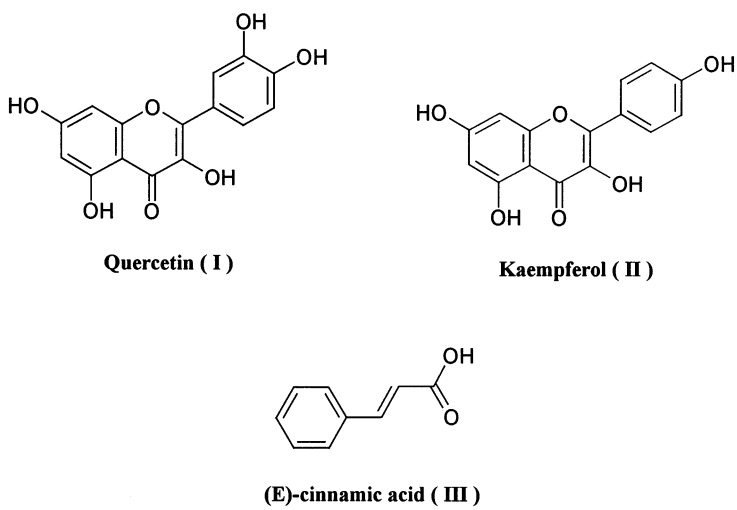

Fig. 1. Major components of *S. chinensis*.

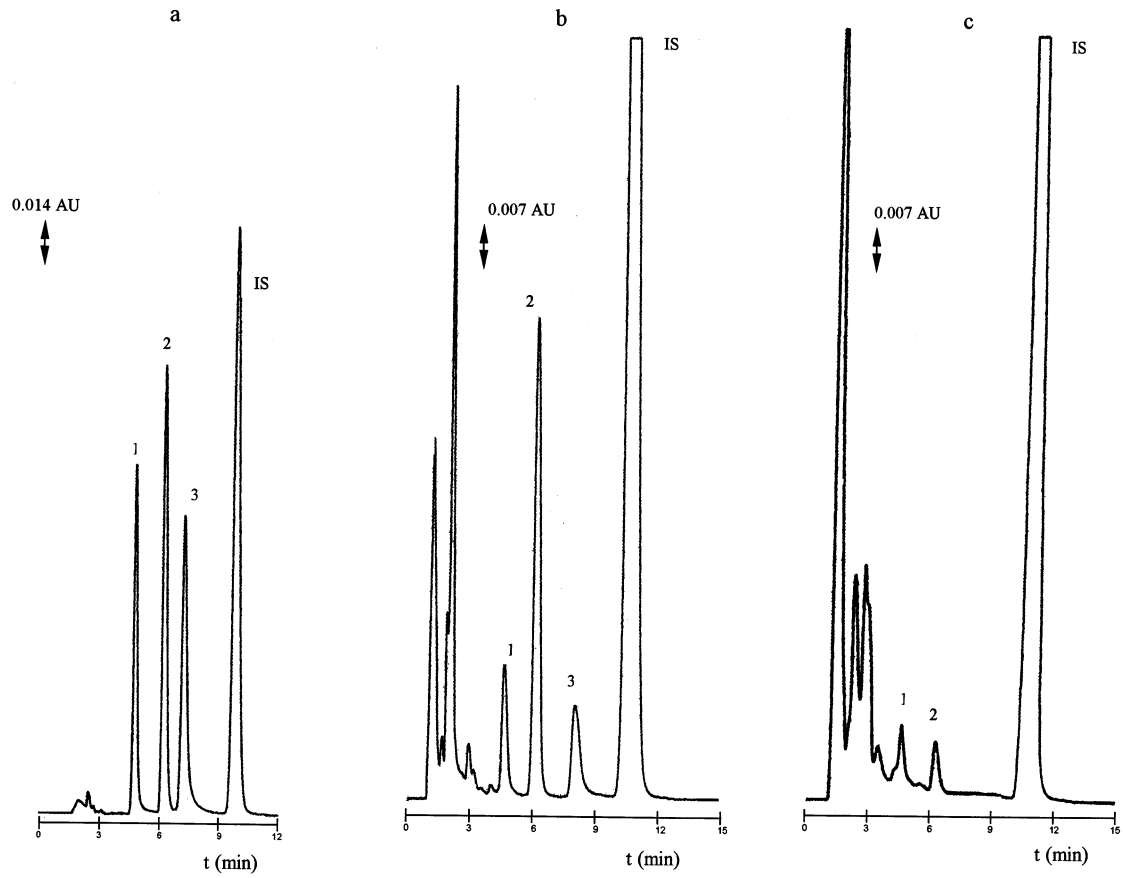

Fig. 2. HPLC chromatogram of (a) standard of flavonols (quercetin, kaempferol) and cinnamic acid; (b) leaves extract of *S. chinensis*; (c) caulomas extract of *S. chinensis*. Peaks: 1, quercetin; 2, cinnamic acid; 3, kaempferol; and IS, propyl-4-hydroxybenzoate (propylparaben); $\lambda = 260$ nm.

Table 1
Analytical characteristics of the calibration graphs

Compound	Range[a] (μg/ml)	Slope (b)	Intercept (a)	r[b]	LOD[c] (ng/ml)	LOQ[d] (ng/ml)
Quercetin	1–100	6.5736	−0.0123	0.9994	45	138
Kaempferol	0.5–75	6.2117	−0.0105	0.9993	50	153
Cinnamic acid	1.5–150	11.6693	−0.0094	0.9996	15	46

[a] No. of calibration points $n = 6$ (each injected in four times).
[b] Correlation coefficients of the regression equation $y = a + b \cdot x$ where x is the compound concentration (μg/ml) and y is the peak area.
[c] LOD, limit of detection.
[d] LOQ, limit of quantification.

performed on a 5 μm Separon SGX C18 150 × 3 mm ID glass column (Tessek, Prague, Czech Republic). The optimal mobile phase was a mixture of acetonitrile–aqueous 0.05% *ortho*-phosphoric acid (40:60 v/v). The flow rate was 0.5 ml/min. The analyses were monitored at $\lambda = 260$ nm.

2.3. Plant material

The plant were introduced from seeds (Botanic Gardens in Novosibirsk, Russia) and were grown in the north part of the Czech Republic. The samples of *Schisandra* leaves and caulomas were collected in July 1997–1999. Verification of the plant was done with herbarium vouchers. The collected plant material was immediately dried at ambient temperature and stored in a dry and dark place.

2.4. Sample preparation

About 0.25 g of dried and pulverised leaves (or 1 g of dried and pulverised caulomas) were refluxed with 25 ml methanol 98% (ethanol 95% for caulomas), the mixture was extracted under a regressive cooler for 15 min. After cooling, the extract was filtered through glass wool for sample clean up and diluted to 50 ml with methanol (ethanol for caulomas). Propylparaben (1 ml) (internal standard, 0.7 mg/ml) was added to the 5 ml volume of the extract and diluted to 10 ml with methanol. All samples were finally filtered through a 0.45 μm membrane (Schleicher & Schuell, Germany) and a 10 μl portion of these solutions were injected into the HPLC system.

3. Results and discussion

3.1. Optimization of separation conditions

Several solvent systems based on the various mixtures of acetonitrile or methanol with different acids (acetic, *ortho*-phosphoric) and salts (potassium dihydrogen phosphate) in different concentrations and ratios were described in the literature for the HPLC analysis of flavonols (**I**, **II**) [6-8,10,11] or (*E*)-cinnamic acid (**III**) [12] in different samples. No method was found in the literature for the simultaneous determination of all three compounds mentioned. Therefore, using an univariate method of optimization, a different composition of mobile phase was tested in order to achieve optimal separation in a relatively short time.

With respect to the separation efficiency and sensitivity, better results were achieved with acetonitrile instead of methanol. The use of *ortho*-phosphoric acid in the solvent system, based on

Table 2
Analysis of major compounds in leaves of *S. chinensis* Baill.

Harvest (year)	Content[a] (mg/g d.w.) ± S.D. ($n = 9$)					
	Quercetin	R.S.D. (%)	Kaempferol	R.S.D. (%)	Cinnamic acid	R.S.D. (%)
1997	1.535 ± 0.027	1.75	0.359 ± 0.009	2.55	3.824 ± 0.069	1.81
1998	1.140 ± 0.038	3.37	0.268 ± 0.004	1.38	3.131 ± 0.020	0.65
1999	1.294 ± 0.049	3.77	0.298 ± 0.005	1.81	2.515 ± 0.041	1.61

[a] Content is average from three samples, each injected in triplicate; d.w., dry weight.

Table 3
Analysis of major compounds in caulomas of *S. chinensis* Baill.

Harvest (year)	Content[a] (mg/g d.w.) ± S.D. ($n = 9$)					
	Quercetin	R.S.D. (%)	Kaempferol	R.S.D. (%)	Cinnamic acid	R.S.D. (%)
1997	0.341 ± 0.010	3.02	Not detected	–	0.116 ± 0.002	2.17
1998	0.303 ± 0.006	1.98	Not detected	–	0.099 ± 0.002	1.94
1999	0.389 ± 0.004	1.16	Not detected	–	0.260 ± 0.003	1.17

[a] Content is average from three individuals, each injected in triplicate; d.w., dry weight.

water–acetonitrile decreases the pH and gives a better separation for phenolic compounds. Therefore, different additions of *ortho*-phosphoric acid into the solvent system were tested. Because the stability of the column used was found to be lower at apparent pH* less than 2.0 for the mixed solvent system, finally the optimal addition of *ortho*-phosphoric acid was a compromise between stability of the column and separation efficiency and sensitivity of the determination. The apparent pH* of the mobile phase used (acetonitrile–aqueous 0.05% *ortho*-phosphoric acid 40:60 v/v) was finally 2.50. Retention parameters of the analytical method based on an optimal mobile phase for **I**, **II** and **III** were 4.76, 7.92 and 6.35 min; with tailing factors 1.34, 1.60 and 1.13, respectively.

3.2. Identification and quantification of compounds

The retention times and UV spectra of the peaks were compared with an authentic reference compound (Fig. 2). In order to check the linearity of the relationship between the peak area and the concentration, six standard solutions were prepared and diluted with methanol. Each of six different concentrations (1–100 μg/ml for **I**, 0.5–75 μg/ml for **II** and 1.5–150 μg/ml for **III**) of standard solutions were injected (10 μl) four times into the HPLC system. All the compounds showed good linearity ($r \geq 0.9993$) and obeyed Beer's law in the concentration ranges investigated (Table 1). The limits of detection (LOD) of selected compounds (**I**, **II** and **III**), determined with the UV detector at the operative wavelength of 260 nm, ranged from 15 ng/ml (**III**) to 50 ng/ml (**II**). Calculation of the detection limits for the compounds studied were based on a signal-to-noise ration of 3 throughout this work.

The limits of quantification (LOQ) ranged from 46 ng/ml for (**III**) to 153 ng/ml for (**II**) (Table 1). The results obtained for the limits of quantification confirm that the proposed HPLC method is sufficiently sensitive for the simultaneous determination of the flavonols (**I**, **II**) and (**III**) in this type of sample.

3.3. Analysis of the plant material

The results of the analysis of two flavonols (**I**, **II**) and (*E*)-cinnamic acid in leaves and caulomas of *S. chinensis* Baill. by the method described above are summarised in Tables 2 and 3. The R.S.D.s ($n = 9$) were between 1 and 3% except for that of quercetin in leaves (3.7%). The data presented here are average values of three analyses of three samples from three different harvests. In leaves, quercetin content is approximately four times higher than the level of **I** in caulomas and (*E*)-cinnamic acid content is about 20 times higher. No kaempferol (below detection limit of the method) was found in the extracts of caulomas. The highest amounts of free quercetin, kaempferol and (*E*)-cinnamic acid were in leaves of the 1997 harvest. The ratio of **I** and **II** in leaves was about 4.

4. Conclusion

Isocratic elution on a Separon SGX C18 column with a mobile phase composed of acetonitrile in water adjusted to an apparent pH* of 2.50 with *ortho*-phosphoric acid facilitated the separation of flavonols (quercetin and kaempferol) and (*E*)-cinnamic acid. This methodology was successfully applied for the novel procedure for the identification and the quantitative analysis of three major compounds in extracts of *S. chinensis* Baill. from leaves and caulomas. The results obtained were precise and the limits of detection were sufficiently low.

Acknowledgements

This work has been supported by the Internal Grant Agency of Charles University No. CEZ:J13/99:11600001 and 29/1998/B Ch/FaF.

References

[1] M. Pokert, Klinishe Chineschineshe Pharmakologie, Verlag fûr Medizin Dr. E. Fischer, Heidelberg, 1987, pp. 310–323.

[2] H.M. Chang, P.P.H. But, Pharmacology and Applications of Chinese Materia Medica, World Scientific, Singapore, 1986, pp. 278–295.
[3] W. Tang, G.F. Eisenbrand, Chinese Drugs of Plant Origin, Springer, Berlin, 1992, pp. 903–917.
[4] J.-S. Liu, Org. Chem. 4 (1987) 316–321.
[5] X.G. He, L.Z. Lian, L.Z. Lin, J. Chromatogr. A 757 (1997) 81–87.
[6] U. Justesen, P. Knuthsen, T. Leth, J. Chromatogr. A 799 (1998) 101–110.
[7] A. Crozier, E. Jensen, M.E.J. Lean, M.S. McDonald, J. Chromatogr. 761 (1997) 315–321.
[8] M. Keinänen, R. Julkunen-Tiitto, J. Chromatogr. A 793 (1998) 370–377.
[9] A. Escarpa, M.C. Gonzáles, J. Chromatogr. A 823 (1998) 331–337.
[10] M. Brolis, B. Gabetta, N. Fuzzati, R. Pace, F. Panzeri, F. Peterlongo, J. Chromatogr. A 825 (1998) 9–16.
[11] A. Rehwald, B. Meier, O. Sticher, J. Chromatogr. A 677 (1994) 25–33.
[12] C. Bocchi, M. Careri, F. Groppi, A. Mangia, P. Manini, G. Mori, J. Chromatogr. A 753 (1996) 157–170.