Evaluation of Variation of Acteoside and Three Major Flavonoids in Wild and Cultivated *Scutellaria baicalensis* Roots by Micellar Electrokinetic Chromatography

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Micellar electrokinetic chromatography (MEKC) conditions were developed to analyze the constituents of Scutellariae Radix (SR) and *Scutellaria baicalensis* roots. Using the MEKC method, the major flavonoid constituents of baicalin, baicalein and wogonin of wild and cultivated *S. baicalensis* roots were compared. In a preliminary comparison of electropherogram, one special peak was found in a wild sample but not in a 2-year-cultivated one. The compound corresponding to the peak was isolated and identified as a phenylethanoid glycoside, acteoside, by comparing the ^{1}H- and ^{13}C-NMR spectral data with that of the authentic compound. This is the first time acteoside has been isolated from the *Scutellaria* genus. It could only be found in SR derived from wild *S. baicalensis* roots and 4-year-cultivated plants, but not in plant materials cultivated for 3 years. Applying the MEKC method established in this study, rapid and simultaneous determinations of acteoside together with 3 flavonoids in samples were achieved. The method can thus be used for the quality control of SR in a shorter analysis period than HPLC.

Key words *Scutellaria baicalensis*; micellar electrokinetic chromatography; acteoside; HPLC; baicalin; capillary electrophoresis

Scutellariae Radix (SR: Huangqin in Chinese, Ogon in Japanese), dried root of *Scutellaria baicalensis* GEORGI (Labiatae), is a well-known traditional Chinese crude drug used as a remedy for inflammation, allergy, diarrhea, bronchitis and hepatitis.[2)] Although the source of SR in the past has been wild *S. baicalensis* roots, with the increasing demand and decline of the wild resource, cultivated plant roots have now became an additional resource. To assure safe and effective clinical use, the comparative study of wild and cultivated crude drugs is necessary. Measurement of crude drug constituents by HPLC for routine analysis is quite common, however, it requires huge volume of organic solvents and longer analysis time. As a part of our study program to develop an environmental friendly analysis for natural medicines,[3)] we have developed attempted a new method of analyzing constituents in the SR using micellar electrokinetic chromatography (MEKC). MEKC is environmentally friendly since the use of harmful solvents is greatly minimized compared to HPLC and the period for analysis is shorter.

In the process of our preliminary comparison of wild and cultivated *S. baicalensis* roots by MEKC, one peak was seen in a wild sample but not in a 2-year-cultivated one. This paper describes our detailed study on isolating the special peak to identify it as acteoside.[4)] Furthermore, the quantitative analysis of acteoside together with the 3 flavonoids of SR namely, baicalin, baicalein, and wogonin was performed in 9 wild and 10 cultivated samples using the MEKC method. To compare the analytical efficiency, each sample was also examined by the HPLC method.

Experimental

SR and Plant Materials The following 19 samples were collected and analyzed: Three SR prepared from cultivated *S. baicalensis* roots collected in China: No. 2389 (Laiyang, Shandong Province. 1-year-cultivated sample, Oct. 1999), No. 1200 (Anguo, Hebei Prov. 3-year-cultivated sample, Oct. 1998), and No. 1201 (Anguo, Hebei Prov. 3-year-cultivated sample, Oct. 1998). Five SR prepared from wild *S. baicalensis* roots collected in China: No. 1202 (Longhua, Hebei Province collected in Sep. 1998), No. 1203 (Lanqi, Hebei Prov. Sep. 1998), No. 2383 (Mengzi, Yunnan Prov. Apr. 1999), No. 2388 (Laiyang, Shandong Prov. Oct. 1999), and No. 2391 (Chengde, Hebei Prov. Oct. 1999). Seven cultivated *S. baicalensis* roots collected in China: No. 98002 (Laiyang, Shandong Prov. 1-year-cultivated sample, Jul. 1998), No. 98004 (Yushe, Shanxi Prov. 1-year-cultivated sample, Aug. 1998), No. 98008 (Yusha, ShanxiProv. 1-year-cultivated sample, Aug. 1998), No. 98001 (Laiyang, Shandong Prov. 2-year-cultivated sample, Jul. 1998), No. 98007 (Yushe, Shanxi Prov. 2-year-cultivated sample, Aug. 1998), No. 98011 (Longhua, Hebei Prov. 2-year-cultivated sample, Aug. 1998), and No. 98010 (Longhua, Hebei Prov. 3-year-cultivated sample, Aug. 1998). Two wild *S. baicalensis* roots collected in China: No. 98006 (Yushe, Shanxi Prov. Aug. 1998) and No. 98012 (Longhua, Hebei Prov. Aug. 1998). Voucher specimens of these 17 samples collected in China have been kept in the School of Pharmaceutical Sciences of Peking University. These samples were botanically identified and it was determined morphologically by one of the authors (Shao-qing Cai) whether the commercial SR had originated from wild plants or from cultivated plants.

The 2 commercial SR were purchased from Tochimoto tenkaido Co. (Osaka, Japan) and voucher samples are preserved in the Museum of Materia Medica, Toyama Medical and Pharmaceutical University, Toyama, Japan; they are registered as TMPW Nos. 20653 (Dongliao, Nei-Menggu: Hen-gon in Japanese, collected on Aug. 2000) and 20654 (Dongliao, Nei-Menggu: Sen-gon in Japanese, Aug. 2000).

Chemicals Baicalin, baicalein and wogonin were purchased from Wako Pure Chemicals Co., Ltd., Osaka. Sodium dodecyl sulphate (SDS), *p*-nitrophenol (internal standard, IS, in MEKC analysis), $Na_2B_4O_7$, and CH_3CN were purchased from Nacalai Tesque, Kyoto, Japan. All other chemicals were of analytical and/or HPLC grade.

Isolation of Acteoside SR (sample No. 2391, 1 kg) was extracted with MeOH (4 l, 3 times, for 2 h each) under reflux condition. The MeOH extract

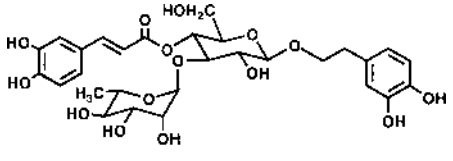

Fig. 1. Acteoside

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was concentrated under reduced pressure to obtain a viscous mass. About 1/5 of it was dissolved in MeOH–H_2O (4 : 1) and centrifuged at 3000 rpm for 15 min. The supernatant was chromatographed over Sephadex LH-20 and eluted with MeOH : H_2O (4 : 1) to obtain 5 fractions (SR-1 to SR-5). The SR-2 (1.23 g), which contained acteoside, was then chromatographed on a polyamide column eluting with water, and by gradually increasing the proportion of the EtOH 11 fractions (SB-2-1 to SB-2-11) were collected. The SR2-3 (12 mg) was applied on HPLC on a YMC-Pack ODS-AQ column and acteoside (5 mg: Fig. 1) was isolated and used as a reference.

The structure of acteoside was identified by direct comparison with the standard sample[4] and also together with its ^{1}H- and ^{13}C-NMR spectra taken on a JEOL JMN-LA400WB-FT.

Quantitative MEKC Analysis The powder (0.2 g) prepared from 5 roots of SR or plant materials in each sample was extracted with 5 ml of 50% aqueous EtOH by refluxing for 30 min, then centrifuged at 3000 rpm for 10 min. After removing the supernatant, the residue was extracted two times. All extract solution was combined, filtered through 0.45 μm Millipore followed by the addition of 0.8 ml of the internal standard solution (1 mg of *p*-nitrophenol in 1 ml of 50% aqueous EtOH. The solution was diluted to 20 ml with 50% aqueous EtOH and used for MEKC or HPLC analysis. The analytical procedure was repeated three times for each sample.

MEKC analysis was carried out on a Photal CAPI-3200 capillary electrophoresis system equipped with a UV detector. The column was a fused-silica capillary tube (82 cm×50 μm i.d., Photal CE0122) with the detection window placed at 70 cm. Other conditions were as follows: injection mode, siphon 25 mm, 30 s; applied voltage, 30 KV (constant voltage, positive to negative polarity); cartridge temperature, 25 °C; detector wavelength, 350 or 275 nm; and run time 11 min. The reported run buffer conditions[5] were used with minor modifications (pH: 8.73): 30 mM SDS, 10 mM NaH_2PO_4 and 13 mM $Na_2B_4O_7$. Before each run, the capillary was washed with 0.1 M NaOH for 60 s, with H_2O for 60 s and with buffer for 120 s.

The regression equations of calibration graphs for 4 compounds and their correlation coefficients are described in the legend of Table 1. Reproducibility was indicated by the coefficient of variation (CV), which was calculated from the peak height and migration time of acteoside in the standard solution by 3 measurements within one day. Reproducibility of peak height of acteoside each day was in also examined.

HPLC Analysis HPLC analysis of the 4 constituents in the extracts prepared by the above procedure was carried out on a JASCO HPLC system: pump unit (PU-1580), automatic sampler (AS-1550), dynamic mixer (HG-1580), column oven (CO-1565) and multiwavelength detector (MD-2010) with a column YMC-Pack ODS-AQ (5 μm, 150 mm×6 mm I.D.) were used. The mobile phase was composed of 1.5% AcOH (A) and CH_3CN (B), which was determined by the reported conditions.[6] The gradient elution was as follows: time 0 min A–B (88 : 12); time 30 min (84 : 16); time 45 min A–B (67 : 33); time 55 min A–B (52 : 48); time 60 min A–B (100 : 0). The flow-rate was 1 ml/min and the column temperature was maintained at 40 °C. The sample solution (20 μl) was injected and the detected wavelength was 331 and 275 nm. The HPLC was run on a JASCO auto injector with Borwin-PDA (Ver. 1.5) software.

Identification of 4 peaks in MEKC and HPLC was made by comparing their migration and retention times with those of authentic compounds and by UV spectra analyzed using a photodiode-array detector. Co-chromatography was also performed to compare with the authentic samples.

Results and Discussion

Electropherogram (Fig. 2) Constituents of the *S. baicalensis* roots were resolved by the MEKC method with a buffer containing 30 mM SDS, 10 mM NaH_2PO_4, and 13 mM $Na_2B_4O_7$. MEKC is one of the capillary electrophoresis methods using micelle formation reagents as SDS in order to separate compounds with various physicochemical characteristics. The SDS concentration under this buffer condition was modified from a previous report.[5]

As shown in Fig. 2, a peak was detected in the electropherogram of a wild sample (No. 2391) but not in a 2-year-cultivated one (No. 98011). It is reported that SR available in the market differs in chromatograms depending on the producing area or collection time, as well as whether it is cultivated or wild type.[7,8] The two samples examined were collected and cultivated in Hebei province in China, which is one of the major sources of SR.

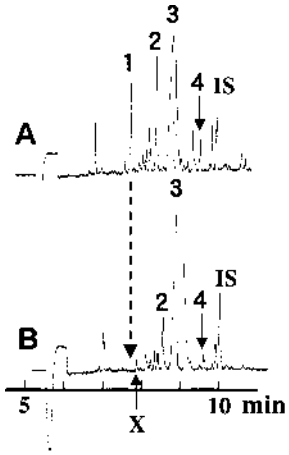

Fig. 2. Electropherograms of Wild (A) and 2-Year-Cultivated (B) Samples

1: acteoside, 2: baicalein, 3: baicalin, 4: wogonin, IS: *p*-nitrophenol (internal standard). (A): Commercial SR originating from wild *S. baicalensis* (sample No. 2391), (B): 2-year-cultivated *S. baicalensis* roots (sample No. 98011) in which acteoside (1) was not detected, as shown by broken line arrow. UV spectrum analysis confirmed that the peak X (λ_{max} 212, 233, 259, 290, 350 nm) was not acteoside (λ_{max} 224, 258, 295, 352 nm).

Identification of Acteoside We focused on the peak seen in the SR originating from wild *S. baicalensis* roots to elucidate its chemical structure. The MeOH–H_2O (4 : 1) soluble portion obtained from MeOH extract of commercial SR was subjected to Sephadex LH-20, polyamide column and preparative HPLC on a YMC-Pack ODS-AQ column to give the compound corresponding to the special peak in the electropherogram. ^{1}H- and ^{13}C-NMR data indicated the presence of phenylethanoid and caffeic acid and two sugar (glucose and rhamnose) moieties. Therefore, it was suggested to be a phenylethanoid glycoside derivative and identified as acteoside by comparing the ^{1}H- and ^{13}C-NMR data with that of the authentic compound.[4] Although phenylethanoid glycosides have been isolated from SR,[9,10] there are no reports of the presence or identifications of acteoside in SR.

Acteoside was reported to inhibit apoptosis in D-galactosamine and lipopolysaccharide-induced liver injury in mice,[11] and to have a potent antinephritic activity[12] and antiproliferative activity against tumor cell (B16F10).[13] Furthermore, anti-oxidant activity[14,15] of SR is regarded as a potential cure for hepatitis, inflammation and other chronic diseases. Therefore, acteoside with potent antioxidant properties could offer important biological functions of SR.

Quantitative MEKC Analysis (Table 1, Fig. 3) By applying the MEKC method using *p*-nitrophenol as the internal standard, 19 samples of cultivated and wild *S. baicalensis* roots and commercial SR were determined (Table 1). As shown in Fig. 3, acteoside was detected only in wild and 4-year-cultivated samples, although there are a few exceptions. The contents of acteoside in the 7 samples ranged from 17.5±0.1 mg/g (No. 1203, wild SR from Hebei Province) to 0.9±0.03 mg/g (No. 20654, wild SR from Nei-Menggu), while the contents of baicalin, an active major flavone constituent of SR, ranged from 209.8±0.1 mg/g (No. 2388, wild SR) to 97.5±0.8 mg/g (No. 98006, wild *S. baicalensis* roots). These quantitative data of baicalin are compatible with those determined by HPLC in a previous report.[8]

Table 1. Contents of 4 Constituents in Aceoside Containing Samples

Sample No	Acteoside	Baicalin	Baicalein	Wogonin
SR (W)				
1202	10.4±0.3	143.9±2.9	4.8±0.4	2.8±0.1
1203	17.5±0.1	133.3±1.5	3.8±0.2	3.2±0.1
2391	7.1±0.4	142.2±6.4	6.3±0.2	3.0±0.1
20653	9.0±0.2	149.1±8.5	4.2±0.2	1.7±0.1
20654	0.9±0.03	171.9±3.4	5.2±0.1	2.0±0.2
Sb				
98010 (C4)	4.3±0.2	153.8±1.8	3.5±0.3	2.0±0.2
98012 (W)	13.5±0.2	103.6±9.3	4.0±0.03	2.0±0.02

Each piece data (mg/g of sample) represents mean±S.D. (n=3) determinations by MEKC. Sample records are listed in Table 1. SR: commercial drugs (W: wild sample). Sb:plant materials of *S. baicalensis* roots (C4: 4-year-cultivated sample). The regression equations (y: peak-area ratio and x: concentration in mg/ml) of calibration curves and their correlation coefficients: $y=13.27x+0.05$ (r=0.988: for acteoside, linearity range: 0.005—0.100 mg/ml, limit of detection: 3 μg/ml), $y=3.29x-0.14$ (r=0.960: for baicalin, linearity range: 0.100—0.750 mg/ml), $y=25.80x-0.16$ (r=0.996: for baicalein, linearity range: 0.025—0.100 mg/ml), $y=11.387x+0.01$ (r=0.999: for wogonin, linearity range: 0.018—0.054 mg/ml). Some of the samples were applied in a two fold-diluted solution with eluted solvent.

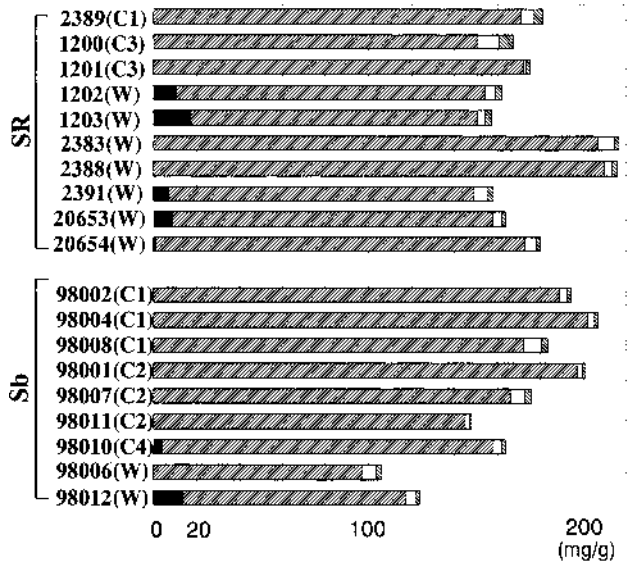

Fig. 3. Contents of Acteoside and Three Flavonoids as Determined by MEKC

Each value represents constituent (mg)/samples (g) (n=3). Figures represent sample number listed in Experimental. SR: commercial drugs. Sb: plant materials of *S. baicalensis* roots. C: cultivated samples. Number represents years of cultivation. W: wild samples. ■ acteoside, ▨ baicalin, ▩ baicalein, ▧ wogonin.

Recently SR prepared from cultivated and/or lateral roots of *S. baicalensis* has been circulated in the drug market, because they contain a higher level of baicalin than the wild type. In this study, it was proved that baicalin contents in cultivated samples (171.9±19.3 mg/g, n=10) are slightly higher than those in wild ones (150.9±39.6 mg/g, n=9) (though not significant). However, samples containing no acteoside have a significantly higher level of baicalin (173.3±31.9 mg/g, n=12) than samples containing acteoside (142.5±20.9 mg/g, n=7) ($p<0.10$). The former are cultivated samples and the latter are prepared from wild and 4-year-cultivated plants collected from a particular region such as Hebei and Shandong in China.

Hebei and Shandong have been the major source of traditional SR, and SR is called as Rehe-Huangqin in Chinese (Nekka-Ogon in Japanese) and Shandong-Huangqin (Santo-Ogon in Japanese) by wholesale dealers. SR is unique to those 2 regions which are referred to as Daodi-Yaocai in Chinese (Dochi-Yakuzai in Japanese), meaning a region famous for drugs. Present results show that these traditional SR prepared from wild *S. baicalensis* roots collected at Hebei and Shandong have a different composition of the 4 compounds than the SR prepared from cultivated *S. baicalensis* roots. Although baicalin contents are one of the valuable indices in quality control of SR, the composition of the constituents in SR might be even more important.

It is conjectured that age and region play an important in the number and content of SR constituents, and consequently determine its biological activity. Further research on the optimal age of cultivated SR and on comparative pharmacological studies using various types of SR and whether or not they contain acteoside are worth undertaking.

Table 2. Comparison of the Analytical Characteristics between MEKC and HPLC

	MEKC	HPLC
Solvent used (1 sample, 3 trials)	45 ml[a)]	270 ml[b)]
Time required/trial	12 min	60 min
Sample preparation and column washing time (for acteoside analysis)	15 min	30 min
S/N	36.4 ±7.5	36.2 ±1.7
CV (peak height within-day)[c)]	3.10%	1.58%
Symmetric factor[d)]	0.86±0.05	1.37±0.05
Recovery[e)]	0.99	1.02

a) (15 ml of sample solution in a bottle)×3 trials. *b*) [(1 ml/min)×60 min×3 trials]+column washing solvent (*ca.* 90 ml). *c*) Reproducibility of peak height for 3 d and migration time within one day in MEKC: 10.55 and 2.03%. *d*) Symmetry factor, tailing factor, is calculated by the equation: (the peak width a 1/20 of the peak height)/2×(the latter half width at 1/20 of the peak height). *e*) Recovery test was examined by addition of 0, 40, and 80 mg/g of aceteoside to the sample powder of No. 2391.

Comparison of MEKC with HPLC (Table 2) Measurement of constituents of SR by HPLC for routine analysis is quite common, however, the time required is longer than by MEKC. The MEKC analysis for the 4 compounds developed in this study can be completed within 12 min as shown in Fig. 2. HPLC analysis in this report to simultaneously examine acteoside and the 3 flavonoids required about 60 min, although HPLC analysis time for flavonoids alone in SR is reportedly about 30 min.[7)] Retention times of acteoside and baicalin in the present HPLC analysis were about 23 and 36 min and some unidentified peaks were detected at about 55—60 min.

To compare the analytical efficacy of MEKC with HPLC, the same sample (No. 2391) was used: acteoside content determined by MEKC was 7.1±0.4 mg/g, which did not differ greatly from that obtained by HPLC analysis (7.5±0.4 mg/g). The recovery in MEKC and HPLC were almost equal (Table 2). The analytical performances such as organic solvent volume used, S/N, CV value, symmetric factor and recovery in the two types of analyses were also compared. Since the symmetric factor of MEKC (0.86) was nearer 1.00 (symmetric) than that of HPLC (1.37), the peaks of the former are more symmetric. Solvent used in the MEKC analysis per sample in three trials was about one sixth that with the HPLC method, therefore, the consumption of harmful organic solvents can be largely decreased in MEKC analysis.

From this point of view, MEKC can be seen to be one of the environmentally-friendly analytical systems.

However, MEKC might not be beneficial for the micro-analysis of drug metabolites in blood, since reproducibility (CV values in Table 2) was slightly inferior to that in HPLC. Although MEKC is not suitable for preparative purposes to obtain constituents of crude drugs, the method described in the present paper could provide the same analytical needs in quality control of SR as by HPLC.

In summary, a MEKC analysis method using a minimal amount of organic solvent and shorter analysis time than HPLC was established to compare constituent variations of SR prepared from wild and cultivated *S. baicalensis* roots. In a preliminary comparison of electropherograms of samples, one special peak was seen in a wild sample but not in a 2-year-cultivated one. The compound corresponding to the peak was identified as acteoside by comparing the ^{1}H- and ^{13}C-NMR spectral data with that of the authentic compound. Acteoside together with baicalin, bailalein and wogonin of the 19 samples was examined quantitatively by MEKC, and acteoside was found in SR prepared from wild plants and 4-year-cultivated plants, but not in plant materials cultivated for 3 years (Fig. 3). These results revealed that the samples available in the market differ depending on producing area or collection time, as well as whether they are cultivated or wild type. Although further pharmaceutical comparisons are necessary, the present results suggest that SR prepared from *S. baicalensis* roots cultivated for 3 years are not equal to traditionally used SR in the acteoside contained.

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References and Notes

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