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Determination of Polar Constituents in Scrophulariae Radix by Micellar Electrokinetic Capillary Chromatography

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ABSTRACT

A rapid method for simultaneous determination of three marker constituents, 2-(3-hydroxy-4-methoxyphenyl) ethyl 1-O-[α-L-arabinopyranosyl (1→6)]-feruloyl (1→4)-α-L-rhamnopyranosyl (1→3)-β-D-glucopyranoside (SN-A), harpagoside (SN-B) and cinnamic acid (SN-C) in Scrophulariae Radix by micellar electrokinetic capillary chromatography was developed. In this study, the effects of analytical conditions, including buffer pH, buffer electrolyte, temperature, applied voltage and organic modifier concentration, on separations were studied. The optimal chromatographic conditions were obtained with a buffer composed of 20 mM sodium tetraborate and sodium dihydrogen phosphate containing 100 mM sodium cholate and 10% acetonitrile at pH 7.5. Propylparaben was used as an internal standard and analytes were detected at 280 nm. The linear calibration range of SN-A, SN-B and SN-C were 20.0-320.0 μg/ml (r=0.9994), 9.6-153.6 μg/ml (r=0.9994) and 1.6-25.6 μg/ml (r=0.9996), respectively. The recoveries of these markers were SN-A: 103.5 ± 0.4%, SN-B: 102.4 ± 2.9% and SN-C: 102.4 ± 1.9%. The relative standard deviations of the three marker substances for intraday and interday analyses were 0.55-1.71% and 1.36-3.83%, respectively.

Key words: **pharmaceutical analysis, Scrophulariae Radix, harpagoside; MEKC.**

INTRODUCTION

Traditional Chinese medicines have been used for centuries, and have been widely adopted for clinical use. The effectiveness and safety are some yet uncertain, hence proper methods for quality control are needed. For this reason, standardized methods to assess traditional Chinese medicines are as important as the discovery of new active principles.

Scrophulariae Radix (Chinese name: Xuanshen) is the dried root of *Scrophularia ningpoensis* and *S. buergeriana* (Scrophulariaceae) and is a commonly used Chinese herb. It is administered to allay thirst in febrile disease, in macula, pharyngolarygitis and constipation[1]. In our previous paper, the isolation and identification of three polar constituents,

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2-(3-hydroxy-4-methoxyphenyl) ethyl 1-O-[α-L-arabinopyranosyl(1→6)]-feruloyl(1→4)-α-L-rhamnopyranosyl(1→3)-β-D-glucopyranoside (SN-A), harpagoside (SN-B) and cinnamic acid (SN-C) from the root of *Scrophularia ningpoensis* was reported[2]. For the determination of the above constituents in Scrophulariae Radix, an HPLC method was also developed[2].

Capillary electrophoresis (CE) is a recently developed technique with the advantages of requiring only a short time for analysis and a small amount of sample. In addition, the capillary can be easily and thoroughly cleaned, which is important especially when large numbers of samples are involved and for quality control in pharmaceutical plants. According to Sheu *et al.*[3-5] CE produced very good results when it was used to analyze Chinese herbs.

Although the HPLC method developed for the detemination of the three Scrophulariae Radix constituents had good repeatability and reproducibility, CE method is particularly interesting for the advantages mentioned above. A rapid and simple CE method for routine quantitative analysis was established. In this study we report on an analysis method using micellar electrokinetic capillary chromatography (MEKC) for determination of the same three constituents: SN-A, SN-B and SN-C. The suitabilities of MEKC and HPLC methods are compared and discussed.

MATERIALS AND METHODS

I. *Reagents and Materials*

2-(3-hydroxy-4-methoxyphenyl) ethyl 1-O-[α-L-arabinopyranosyl (1→6)]-feruloyl (1→4)-α-L-rhamnopyranosyl (1→3)-β-D-glucopyranoside (SN-A, purity 96.3%) and harpagoside (SN-B, purity 97.1%) were isolated from the root of *Scrophularia ningpoensis*[2]. The structures of the marker constituents are shown in Fig. 1. Sodium cholate, propylparaben and sorbic acid were purchased from Sigma (St. Louis, MO, USA), cinnamic acid, sodium tetraborate and sodium dihydrogenphosphate from Nacalai (Kyoto, Japan). Acetonitrile (HPLC grade) was purchased from Labscan (Dublin, Ireland). Phosphoric acid was analytical reagent grade. Ultrapure distilled water with a resistivity greater than 18 MΩ was used. Seven crude drug samples of Scrophulariae Radix were obtained from local markets in Taipei and cut into small pieces.

II. *Preparation of Sample Solution for MEKC*

Two grams of Scrophulariae Radix were extracted with 30% alcohol (25 ml, three times) by reflux at 80℃, each for 30 min[2]. The extracts were combined and filtered into a volumetric flask and 30% alcohol was added to make 100 ml of stock solution. Ten ml of this stock solution was concentrated under reduced pressure to dryness and the residue was then dissolved to 10 ml with run buffer. An aliquot of 2.5 ml of stock solution and 0.2 ml of propylparaben solution (0.5 mg/ml) was placed into a volumetric flask and made up to 5 ml with run buffer. This solution was filtered through a 0.45 μm syringe filter (Gelman, Ann Arbor, MI, USA) before use.

III. *Preparation of Sample Solution of HPLC*

An aliquot of 2.5 ml of the stock solution and 0.03 ml of sorbic acid solution (0.2 mg/ml) was placed into a 5 ml volumetric flask and filled with 30% alcohol. This solution was filtered through a 0.45 μm syringe filter (Gelman) before use.

IV. *Apparatus and Conditions*

(I) *CE System*

The analysis was carried out on a Beckman P/ACE 5500 capillary electrophoresis system equipped with a photodiode array detector set at 280 nm and a 67 cm × 75 μm I.D. uncoated capillary (Beckman) with the detection window placed at 60 cm. The conditions were as follows: sampling time, 4 sec, hydrostatic; run

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time, 20 min; applied voltage, 25 kV (constant voltage, positive to negative polarity); and temperature, 25℃. The run buffer was a solution containing 100 mM sodium cholate, 10% acetonitrile and 20 mM sodium dihydrogenphosphate solution which was adjusted to pH 7.5 with 20 mM sodium tetraborate. The electrolyte was filtered through a 0.45 μm syringe filter (Gelman) before use. All experiment was cleaned sequentially with: 1% (v/v) sodium hydroxide solution, 1 min; water, 1 min; 10 mM sodium dodecyl sulfate, 1 min; and water, 2 min. Gold software (Beckman) was used for system control and data handling.

(II) *HPLC System*

HPLC was conducted with a Hitachi Model L-6200 Intelligent pump system equipped with a Hitachi Model L-3000 photodiode array detector and a Shimadzu SIL-9A autoinjector. The detector was set at 280 nm. Satisfactory separation of the marker substances was obtained with a reversed phase column (Cosmosil $5C_{18}$-AR, 5 μm, 15 cm × 4.6 mm I.D., Nacalai, Kyoto, Japan) eluted at a rate of 1 ml/min with a linear solvent gradient of A-B (A=acetonitrile; B=1% (v/v) acetic acid) varying as follows: 0 min, 15:85 and 44 min, 73:27.

V. *Preparation of Standard Solution*

To prepare standard solutions containing SN-A, B and C, an appropriate amount of internal standard solution was added to an accurately weighed amount of SN-A, SN-B and SN-C standard dissolved in run buffer for MEKC and 70% methanol for HPLC. The various concentrations of SN-A, SN-B and SN-C were within the ranges 20.0-320.0, 9.6-153.6 and 1.6-25.6 μg/ml, respectively, for MEKC

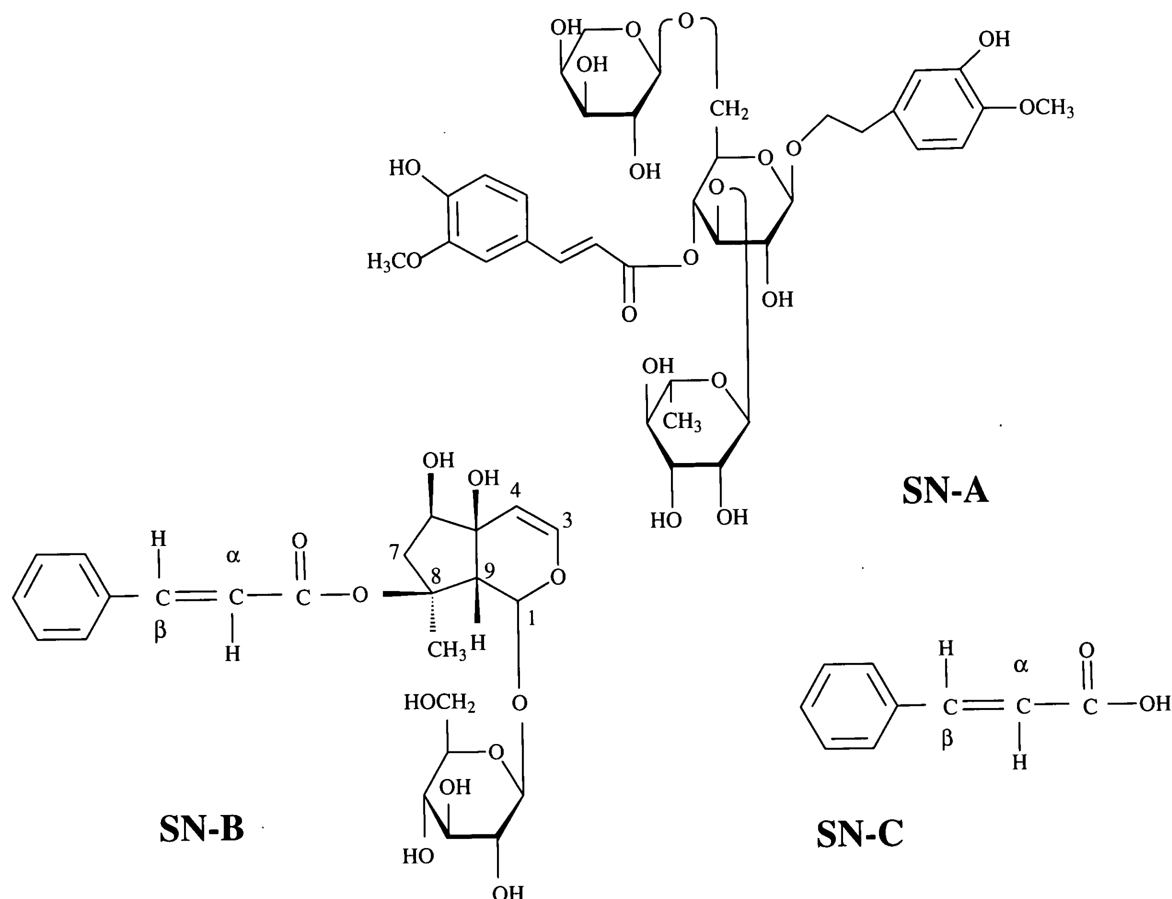

Figure 1. Structures of marker constituents.

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and HPLC. Calibration graphs of the peak-area ratios versus concentration were plotted subsequent to linear regression analysis.

VI. *Solution for Recovery Studies*

Three different concentrations of markers, 16.0, 32.0 and 64.0 μg/ml for SN-A, 6.4, 12.8 and 25.6 μg/ml for SN-B, 1.6, 3.2 and 6.4 μg/ml for SN-C, were added to each sample solution. To each solution a suitable amount of internal standard was added to yield a final concentration of 20 μg/ml of propylparaben.

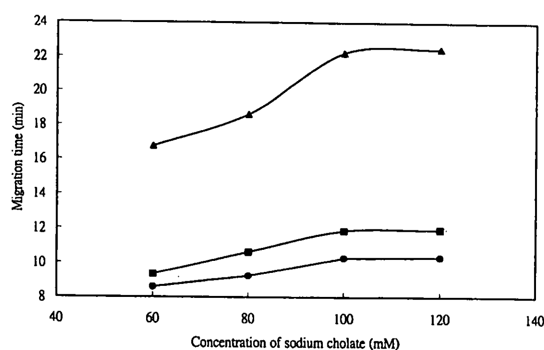

Figure 2. Effect of sodium cholate on migration time. All experiments were conducted at a voltage of 25 kV across the 67 cm × 75 μm I.D. uncoated capillary filled with borate-phosphate buffers of different sodium cholate concentration at pH 7.5; cartridge temperature, 25℃; detection wavelength, 280 nm. (●) SN-A; (■)SN-B; (▲)SN-C.

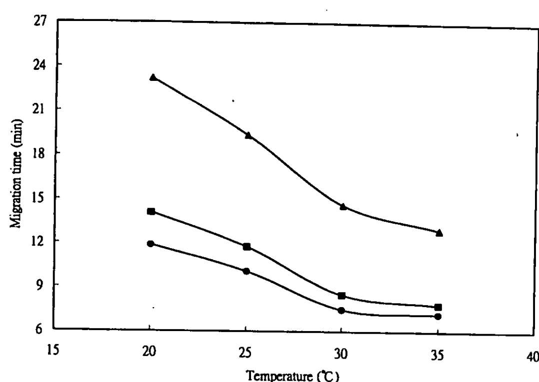

Figure 3. Effect of temperature on migration time. The carrier was 20 mM borate-phosphate containing 100 mM sodium cholate and 10% acetonitrile at pH 7.5. The experiments were conducted at cartridge temperatures of 20, 25, 30 and 35℃. Symbols as in Fig 2.

All samples were filtered through a 0.45 μm syringe filter (Gelman) and injected for MEKC analysis to calculate the concentration of SN-A, SN-B and SN-C from their calibration graphs.

RESULTS AND DISCUSSION

Traditional Chinese medicine is usually prepared by water decoction, with the high polarity constituents being a major part of the decoction. The chemical components in the decoction are very complex, so that analysis is not easy. MEKC has proved to be a highly efficient separation method for the determination of constituents with high polarity, whether the

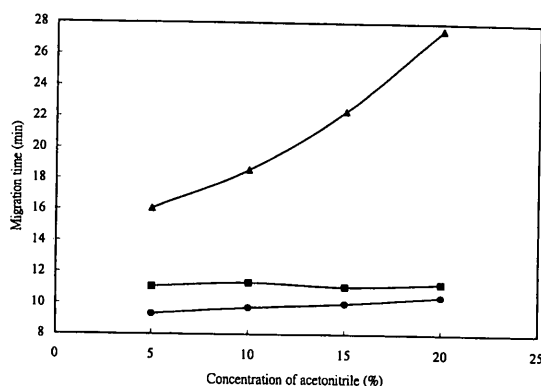

Figure 4. Effect of acetonitrile concentration on migration time. The carrier was 20 mM borate-phosphate containing 100 mM sodium cholate at pH 7.5 and different acetonitrile concentration. Symbols as in Fig 2.

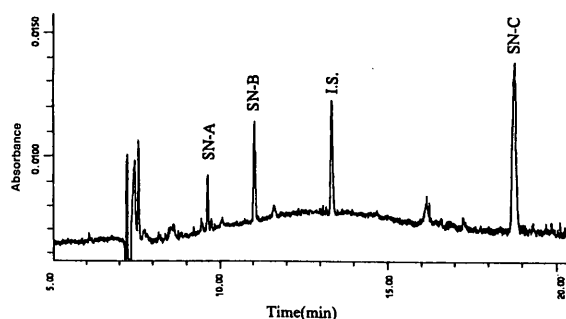

Figure 5. CE electropherogram of Scrophulariae Radix (commercial sample no. 2). Peak identity: same as Fig 1. IS= internal standard (propylparaben).

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compounds are positive, negative or neutral. Previously, we reported the results of HPLC method for determination of the marker constituents. The contents of SN-A, SN-B and SN-C in 24 crude drugs of Scrophulariae Radix, were 0.53-17.10, 0-1.75, and 0.58-5.35 mg/g, respectively(2). In this study, two neutral compounds (SN-A and SN-B) and one acidic compound (SN-C) were used as marker constituents of Scrophulariae Radix.

I. *Analytical Conditions*

The detection wavelength of 280 nm was chosen because the absorption maxima of SN-B, SN-A and SN-C are at 280, 287 and 267 nm, respectively.

In the study of Scrophulariae Radix crude drug, we found the marker constituents, SN-A, SN-B and SN-C, were successfully separated in a single run by MEKC under optimized conditions. The separation was achieved by optimizing the pH of the buffer, sodium cholate concentration, temperature, voltage and acetonitrile concentration.

Preliminary experiments were first conducted at pH 6, 7, 8 and 9 (20 mM NaH_2PO_4 and 20 mM $Na_2B_4O_7$) without sodium cholate in the electrophoretic medium. In all electropherograms, SN-A and SN-B overlapped. With the addition of sodium cholate, the constituents in the sample could be separated on the basis of their relative affinity for the micellar environment. For these reasons, a buffer system with suitable amounts of sodium cholate, NaH_2PO_4 and $Na_2B_4O_7$ was chosen.

In order to study the effect of sodium cholate concentration, temperature and organic solvent concentration, these parameters were varied. The results are shown in Fig. 2-4. Fig. 2 shows how separation was improved by the application of sodium cholate as the micelle-forming agent. Four electrolyte systems containing different sodium cholate concentrations (60, 80, 100 and 120 mM) at pH 7.5 were used to study the effect of sodium cholate concentration on the selectivity of the separation. Although SN-A, SN-B and SN-C were completely separated at all four concentrations, when the sample solution was determined, the SN-A peak was still not free of interference from co-existing constituents at 60 and 80 mM sodium cholate. Concentrations of both 100 and 120 mM offered good separation, but the running time was shorter at 100 mM sodium cholate. In Fig. 3, a buffer solution composed of 100 mM sodium cholate, 20 mM NaH_2PO_4 and $Na_2B_4O_7$ at pH 7.5 was used to study the effect of temperature (20, 25, 30 and 35℃) on the selectivity of the separation. According to expectation, the migration times of SN-A, SN-B and SN-C were reduced when the temperature was increased. Once again, however, although separation was complete at all temperature, the SN-A peak in the sample solution was still subject to interference; only at 25℃ was the sample solution SN-A peak interference-free. In Fig. 4, a buffer solution composed of 100 mM sodium cholate, 20 mM NaH_2PO_4 and $Na_2B_4O_7$ at pH 7.5 was used with different acetonitrile concentrations (5, 10, 15 and 20%) to study the effect of organic solvent on the selectivity of the separation. The 10% solution gave the best resolution; co-

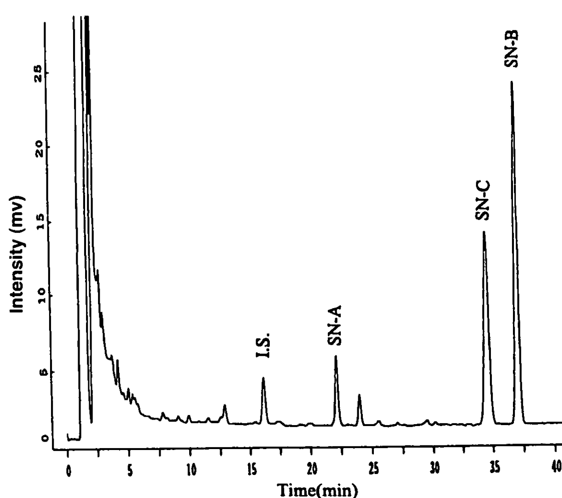

Figure 6. HPLC chromatogram of Scrophulariae Radix (commercial sample no. 2). Peak identity: same as Fig 1. IS= internal standard (sorbic acid)

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existing constituents interfered with SN-A peak at other acetonitrile concentrations.

With an electrolyte containing 100 mM sodium cholate, 20 mM NaH_2PO_4 and $Na_2B_4O_7$ and the cartridge temperature and voltage set at 25℃ and 25 kV, the migration

Table 1. Intraday and interday assay variations of SN-A, SN-B and SN-C

Marker constituents	Concentration (μg/ml)	Intraday[a] R.S.D. (%)	Interday[a] R.S.D. (%)
SN-A	20.0	1.55	3.83
	80.0	0.84	3.20
	320.0	1.20	2.65
SN-B	9.6	0.57	3.79
	38.4	0.95	3.32
	153.6	1.15	1.36
SN-C	1.6	1.50	3.16
	6.4	0.55	2.18
	25.6	1.71	3.29

[a]n=6.

Table 2. Recoveries of SN-A, SN-B and SN-C in Scrophulariae Radix

Marker constituents	Amount added (μg/ml)	Amount measured[a] (μg/ml)	Recovery (%)	Mean ± S.D. (%)	R.S.D. (%)
SN-A	16.0	16.6	103.6	103.5 ± 0.4	0.4
	32.0	33.2	103.8		
	64.0	65.9	102.9		
SN-B	6.4	6.7	104.7	102.4 ± 3.0	2.9
	12.8	12.6	98.1		
	25.6	26.7	104.3		
SN-C	1.6	1.6	101.6	102.4 ± 2.0	1.9
	3.2	3.2	100.5		
	6.4	6.7	105.1		

[a]n=3.

Table 3. Reproducibility of separation of marker constituents

Marker constituent	HPLC R.S.D. (%) (n=3)		MEKC R.S.D. (%) (n=3)	
	Retention time	Amount measured	Migration time	Amount measured
SN-A	0.06	1.00	0.22	1.11
SN-B	0.01	1.72	0.28	2.80
SN-C	0.04	2.95	0.21	1.22

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Table 4. Contents of SN-A, SN-B and SN-C in commercial Scrophulariae Radix by MEKC and HPLC

Marker constituent	Commercial sample	MEKC (mg/g)	HPLC (mg/g)
SN-A	1	1.70	1.79
	2	2.09	2.00
	3	1.97	1.91
	4	1.12	1.06
	5	1.67	1.63
	6	0.70	0.65
	7	1.45	1.45
SN-B	1	0.45	0.46
	2	0.60	0.60
	3	1.46	1.42
	4	1.22	1.22
	5	1.44	1.41
	6	0.33	0.34
	7	0.67	0.70
SN-C	1	0.16	0.16
	2	0.32	0.32
	3	0.11	0.11
	4	0.28	0.29
	5	0.27	0.27
	6	0.07	0.07
	7	0.27	0.28

times of the marker constituents, SN-A, SN-B and SN-C and the internal standard (propylparaben) were 9.6, 11.0, 18.9 and 13.3 min, respectively (Fig. 5). As the sample solutions were injected directly and analyzed, the results were as good as those obtained and the analysis could also be completed within 19 min. This is only half the separation time required by HPLC (for comparison, a chromatogram is shown in Fig. 6). Since the use of organic solvents to dissolve the sample can influence the separation enormously[6], the running buffer was used as solvent for the standards and samples in all MEKC experiments. Samples dissolved with running buffer rather than 70% methanol led to improvements in peak shape, migration time and baseline.

II. *Calibration Graphs for SN-A, SN-B and SN-C*

Calibration graphs consisted of SN-A, SN-B and SN-C in the ranges 20.0-320.0 μg/ml, 9.6-153.6 μg/ml and 1.6-25.6 μg/ml, respectively. The regression equations of these curves and their correlation coefficients were calculated as follows: SN-A, $Y=148.15X+0.79$ ($r=0.9994$); SN-B, $Y=27.80X+0.58$ ($r=0.9994$); SN-C, $Y=5.30X+0.24$ ($r=0.9996$).

III. *System Suitability Tests*

The intraday and interday coefficients of variation were less than 1.7 and 3.8%, respectively (Table 1), indicating that the precision as well as accuracy of this assay was satisfactory. Recovery studies of SN-A, SN-B and SN-C gave recoveries ranging from 98.1 to 105.0% (Table 2).

IV. *Precision of MEKC and HPLC*

The reproducibility (relative standard deviation) of the two methods, on the basis of peak-area ratios for three replications, were 1.00-2.95% for HPLC and 1.11-2.80% for MEKC. The relative standard deviation of the retention time or migration time of each peak for three replicate injections was 0.01-0.06% (HPLC) and 0.21-0.28% (MEKC). The data for individual constituents are given in Table 3.

V. *Detection Limits of Marker Constituents*

A signal three times higher than the peak noise height was regarded as the detection limit. The detection limits of SN-A, SN-B and SN-C detected by HPLC and MEKC were 0.25 and 5.0 μg/ml; 0.02 and 1.2μg/ml; and 0.01 and 0.4 μg/ml, respectively. Although HPLC is more sensitive than MEKC, this is but the many other advantages offered by MEKC.

VI. *Determination of Marker Constituents in Scrophulariae Radix*

Traditional Chinese medicines are usually prepared by boiling with water. However, the extractability of the constituents from

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Scrophulariae Radix with water alone were less than with water containing an organic solvent. As in our previous paper(2), the best extraction solvent is 30% ethanol solution.

Sample solutions were analyzed by MEKC and HPLC under the described conditions, and the peaks were identified by comparison with authentic samples of Scrophulariae Radix. By substituting the peak area ratio of the individual peaks for Y in the above equations, the contents of the markers in the 7 crude drug samples of Scrophulariae Radix were obtained (Table 4). The MEKC analyses indicated SN-A, SN-B and SN-C contents of 0.70-2.09, 0.33-1.46 and 0.07-0.32 mg/g, respectively. These values were near the contents range found in 24 crude drug samples by HPLC, and thus show that both of the proposed methods are relatively suitable for the determination of these marker constituents.

In conclusion, this study has shown that MEKC can be applied successfully to analyze marker constituents of Scrophulariae Radix. This technique offers high separation efficiency, rapid analysis, low running costs and is aqueous rather than organic solvent based. All these are advantages over traditional chromatographic procedures.

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玄參藥材中高極性成分之毛細管電泳定量法之探討

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摘　要

本研究利用毛細管電泳儀，分析玄參藥材中之高極性成分2-(3-hydroxy-4-methoxyphenyl) ethyl 1-O-[α-L-arabinopyranosyl (1→6)]-feruloyl (1→4)-α-L-rhamnopyranosyl (1→3)-β-D-glucopyranoside (SN-A)，harpagoside (SN-B)及cinnamic acid (SN-C)含量，以20 mM sodium tetraborate及sodium dihydrogen phosphate調配爲pH 7.5含100mM sodium cholate及 10% 乙腈之溶液爲緩衝液，內部標準品爲propylparaben，檢測波長爲280 nm，得到良好的分析結果。SN-A、SN-B及SN-C三成分之相關係數(r)分別爲：SN-A 濃度20.0～320.0 μg/ml, (r=0.9994)；SN-B濃度9.6～153.6 μg/ml, (r=0.9994) 及SN-C 濃度1.6～25.6 μg/ml), (r=0.9996)，均呈現良好線性關係。添加回收率試驗結果，SN-A爲103.5 ± 0.4%，SN-B爲102.4 ± 2.9%，SN-C爲102.4 ± 1.9%。三種指標成分之同日間及異日間試驗之相對標準差，分別爲0.55～1.71%及1.36～3.83%，顯示再現性佳。本研究並探討分析條件中緩衝液之pH值、界面活性劑之濃度、溫度、電壓及修飾劑，對上述成分移動時間之影響。

關鍵詞：玄參，哈巴俄苷，毛細管電泳，中藥分析。