Research Article

Simultaneous Determination of Five Marker Components In Preparations of Si-Wu-Tang By Micellar Electrokinetic Capillary Chromatography

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ABSTRACT

Si-Wu-Tang (SWT), a Traditional Chinese Medicine (TCM) formula, has long been used in eastern Asia for the treatments of gynecological diseases. A simple, sensitive and accurate micellar electrokinetic chromatography (MEKC) method is developed and validated for simultaneous determination of five marker constituents (acteoside, paeoniflorin, ferulic acid, tetramethylpyrazine and benzoic acid) in the Chinese patent medicine SWT. Factors affecting the separation of these marker constituents were estimated including pH, concentration of boric acid and sodium dodecyl sulfate (SDS). A carrier composed of 30 mM boric acid and 75 mM SDS (pH was adjusted to 10 with 0.1M NaOH) is found to be the most suitable electrolyte for this separation. The five constituents in SWT can be easily determined within 10 min. The developed method was validated for intra- and inter-day variability, recovery and detection limit. The linear correlation coefficients, intra-day and inter-day variability, and recovery were R≥0.9991, R.S.D≦1.9 %, and 98.7-100.6 %, respectively. The detection limit of acteoside, paeoniflorin, ferulic acid, tetramethylpyrazine and benzoic acid was 1.55 μg/mL, 1.18 μg/mL, 0.19 μg/mL, 0.68 μg/mL and 0.31 μg/mL , respectively. The method was applied to quantitatively determine five constituents in 6 brands of concentrated SWT preparations on the Taiwanese market, and these were successfully estimated to be 0.63 ~2.51 mg/g. The results indicate that the established assay method is suitable for quality control of SWT preparations.

Keywords: MEKC, acteoside, paeoniflorin, ferulic acid, tetramethylpyrazine.

INTRODUCTION

Si-Wu-Tang (SWT), one of the most important Chinese patent medicines, is a Traditional Chinese Medicine (TCM) formula which has long been used in eastern Asia for the treatments of gynecological diseases[1]. SWT is a representative tonic formula in TCM, possessing the ability to improve a deficiency

of blood, promote blood circulation, regulate menstruation and relieve pains. Recent studies showed that SWT was useful for the inhibition of cutaneous inflammatory diseases[2] and it could improve the working memory performance impaired by scopolamine in the eight-arm radial maze task and in the T-maze delayed alternation task in rats[3]. SWT consists of four herbs: Radix Paeoniae Alba, Radix Angelicae Sinensis, Rhizoma Chuanxiong and Radix Rehmanniae. Paeoniflorin and benzoic acid are constituents in the roots of *Paeonia alba*, while ferulic acid is the marker compound in the roots of *Angelica sinensis* and *Ligusticum chuanxiong*. It was reported that paeoniflorin and ferulic acid are the two active compounds of SWT[4]. Acteoside and tetramethylpyrazine are marker components of Radix Rehmanniae and Rhizoma Chuanxiong, respectively. Acteoside (1), paeoniflorin (2), ferulic acid (3), tetramethylpyrazine (4), and benzoic acid (5) are five select marker constituents of SWT, their structural formulae are shown in Fig. 1.

In Taiwan and China, there are totally hundreds of medicinal manufacturers who produce SWT and its derivative varieties, such as Shi Quan Da Du Tang, Xiang Fu SWT, San Huang SWT, Tao Hong SWT, Chih Yu Tang, Ba Zhen Tang, etc. Suitable assay methods are therefore in demand for quality control purpose. Several methods have been developed to determine one component in SWT. High performance liquid chromatography (HPLC) is a common method for determination of paeoniflorin and ferulic acid[5,6], paeoniflorin[7] and ferulic acid[8]. However, this HPLC method using gradient elution needs at least 35 min for a single run. It is quite necessary that efforts to develop a simpler and suitable analytical method that can assay as many bioactive ingredients as possible.

Capillary electrophoresis (CE) was first described by Joegenson and Luckacs in 1981[9], its application to the determination of a variety of samples has become increasingly widespread because of its short analysis time, high separation efficiency, and minimal sample volume requirement[10]. The simultaneous determination of tetramethylpyrazine and ferulic acid in SWT by Capillary zone electrophoresis (CZE) was reported by Shaoping et al[11]. However, none of these methods have been carried out to simultaneously separate these five compounds of SWT. In this study, we developed a micellar electrokinetic chromatography (MEKC) method for simultaneous determination of five marker components (acteoside, paeoniflorin, ferulic acid, tetramethylpyrazine and benzoic acid) in SWT.

MATERIALS AND METHODS

MATERIALS

Sodium chloride, boric acid, benzoic acid, and p-Hyroxy benznic acid were obtained from E. Merck (Schuchardt, Germany). Acteoside were obtained from ChromaDex ™. (USA). Ferulic acid was obtained from Sigma-Aldrich. (USA). Tetramethylpyrazine were obtained from Alfa Aesar. (USA). Paeoniflorin were obtained from Fusol Material Co., LTD. (Tainan, Taiwan). Sodium dodecyl sulfate (SDS) were obtained from Nacalai (Koyto, Japan). Water used in all experiments was purified by a Millipore Milli-QSP60 system (Bedford, MA). All other

chemicals were of reagent grade.

Apparatus and conditions

The experiments were performed on a Beckman Coulter's capillary electrophoresis instrument P/ACE ™ MDQ (Beckman Coulter Taiwan Inc., Taiwan Branch). A fused silica capillary (50 μm i.d. × 40 cm) was used with UV detection at 230 nm for acteoside, paeoniflorin, ferulic acid and benzoic acid, and 214 nm for ligustrazine. The separation voltage and temperature were set at 20 kV and 25℃, respectively. Factors affecting the separation of these marker constituents were studied including pH, boric acid concentration and sodium dodecyl sulfate (SDS) for optimizing the assay conditions.

Determination of five marker components in SWT preparations

Five grams of 5 brand's concentrated SWT preparations on the Taiwanese market were accurately weighed and extracted with 50 mL of 50% ethanol for 0.5 h in an ultrasonic bath. The extraction was repeated three times. The extracted solutions were combined and concentrated to dryness. Before sample injection, the extract was diluted to 100 mL with 50% ethanol and filtered through a 0.45μm filter after the addition of a 5 mL of internal standard solution (10 mg of p-Hyroxy benznic acid in 25 mL of 50% ethanol).

RESULTS AND DISCUSSION

Pre-test of analytical study

Before conducting the MEKC experiments, preliminary studies using the capillary zone electrophoresis (CZE) were attempted. Different compositions of the electrolyte solution were tested for optimization. The peaks of tetramethylpyrazine and electroosmosis flow (EOF) were always crowded together with serious overlapping (Data not shown). MEKC was first reported by Terabe et al. in 1984[12], and since then, it has been used successfully to both charged and neutral compounds[13-16]. A negatively charged surfactant such as sodium dodecyl sulfate (SDS) or sodium decyl sulfate was usually added to the background electrolyte to improve the selectivity of separation[17]. MEKE was thus applied to study the optimizing conditions for simultaneous determination of five marker constituents (acteoside, paeoniflorin, ferulic acid, tetramethylpyrazine and benzoic acid) in SWT. Optimization conditions were carried out by studying the effects of three factors including pH, concentration of boric acid and sodium dodecyl sulfate (SDS) relevant to migration time and peak resolution.

Effect of boric acid concentration

In order to study the effect of boric acid concentration on the separability, a range of 10 ~ 50 mM boric acid were used in the presence of 75 mM SDS and pH 10. The results obtained are given in Fig. 2. The migration times and the overall resolution of all the components increase with increasing buffer concentrations. Higher buffer concentration also led to a higher viscosity coefficient of the solution. A quantity of 30 mM boric acid was found to produce a good resolution. Too high a buffer concentration decreased the resolution, probably because of the increase of the Joule heat.

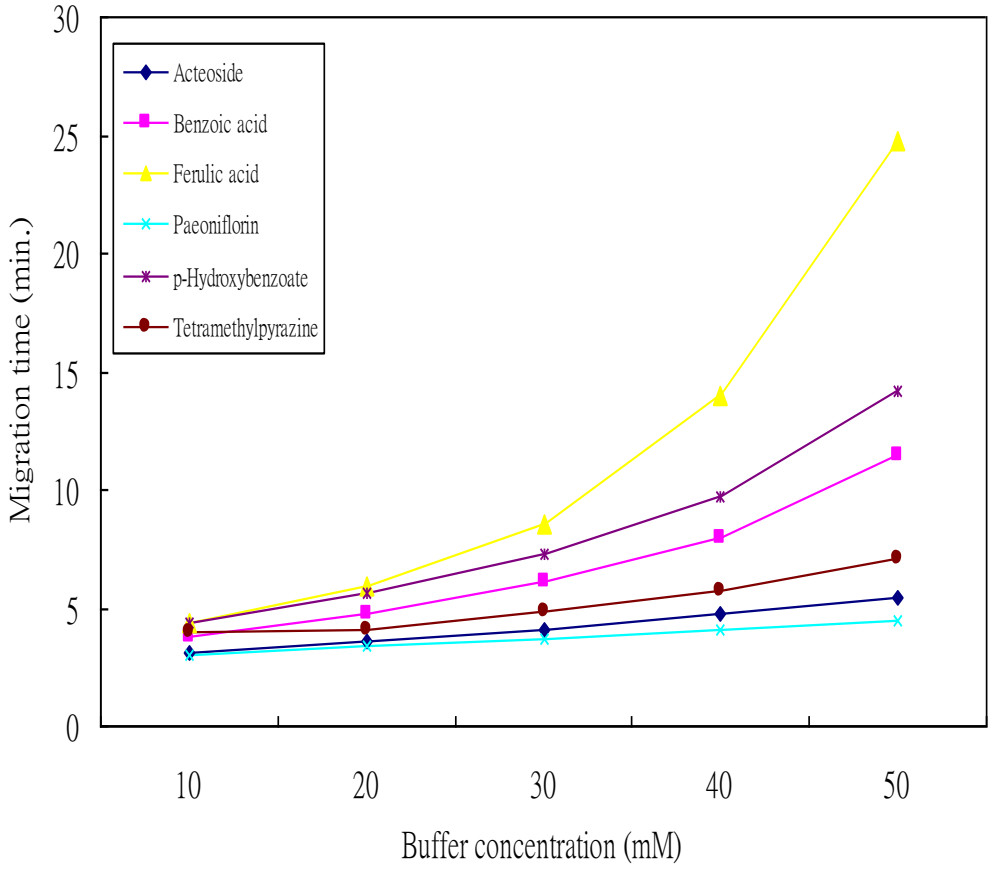

Fig. 2: The effects of boric acid concentration on migration times.

◆ Acteoside; × Paeoniflorin; ▲ Ferulic acid; ● Tetramethylpyrazine; ■ Benzoic acid; * p-Hydroxybenzoate (internal standard)

Effect of pH

The pH is the most important factor for peak separation in CE as well as in HPLC. The pH of the buffer solution affects electro-osmotic flow as well as the overall charge of the analytes. The effects of pH on migration times were investigated from pH 8.5 to 10.5 in the presence of 30 mM boric acid and the results were shown in Fig. 3. In addition to ferulic acid, the migration times of other four marker components slight increased with the increasing of the buffer pH. At pH 10, the five constituents can be well separated within a relatively short time.

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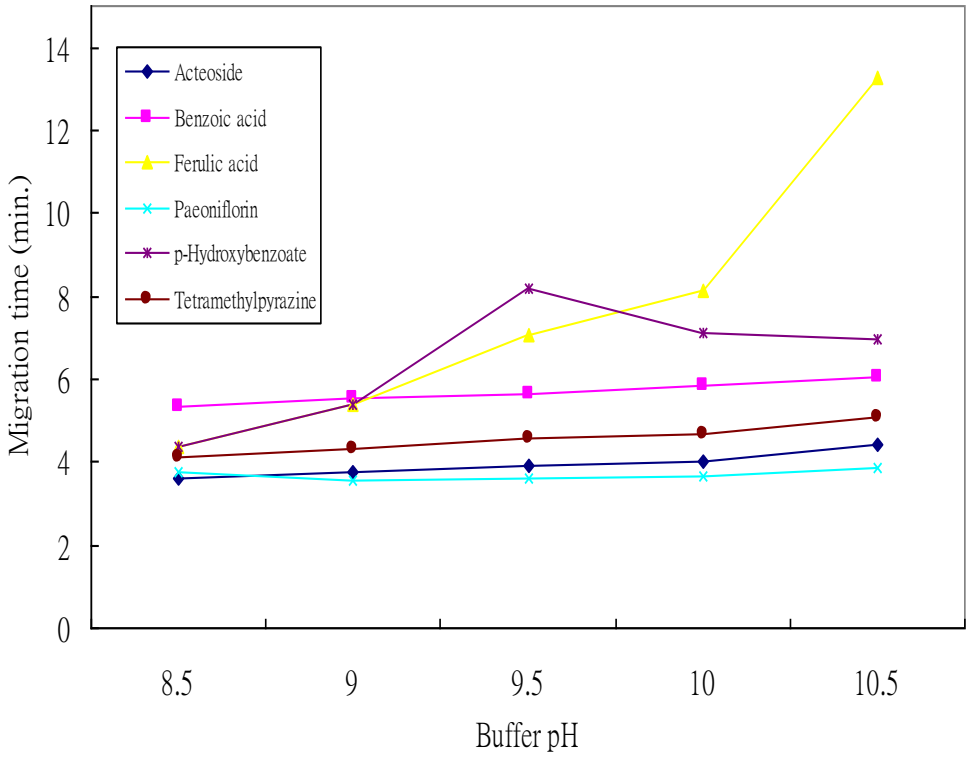

Fig. 3: The effects of pH on migration times of marker components. ◆ Acteoside; × Paeoniflorin; ▲ Ferulic acid; ● Tetramethylpyrazine; ■ Benzoic acid; * p-Hydroxybenzoate (internal standard).

Effect of SDS

A range of 25 ~ 100 mM SDS were applied in the presence of 30 mM boric acid at pH 10 to study the effect of SDS concentration on the separability of these marker components. The results were shown in Fig. 4. The migration times of all the constituents increase with SDS concentrations increased. The five marker constituents were completely separated from 75 to 100 mM SDS, however, higher SDS concentration gave a poor baseline and prolonged the analysis time. Therefore, 75 mM SDS was chosen in this work. From the above results, a carrier composed of 30 mM boric acid and 75 mM SDS (pH was adjusted to 10 with 0.1M NaOH) was found to be the most suitable electrolyte for this separation. The electropherogram of these marker components in SWT was shown in Fig. 5. The five constituents and IS could be successfully determined within 10 min.

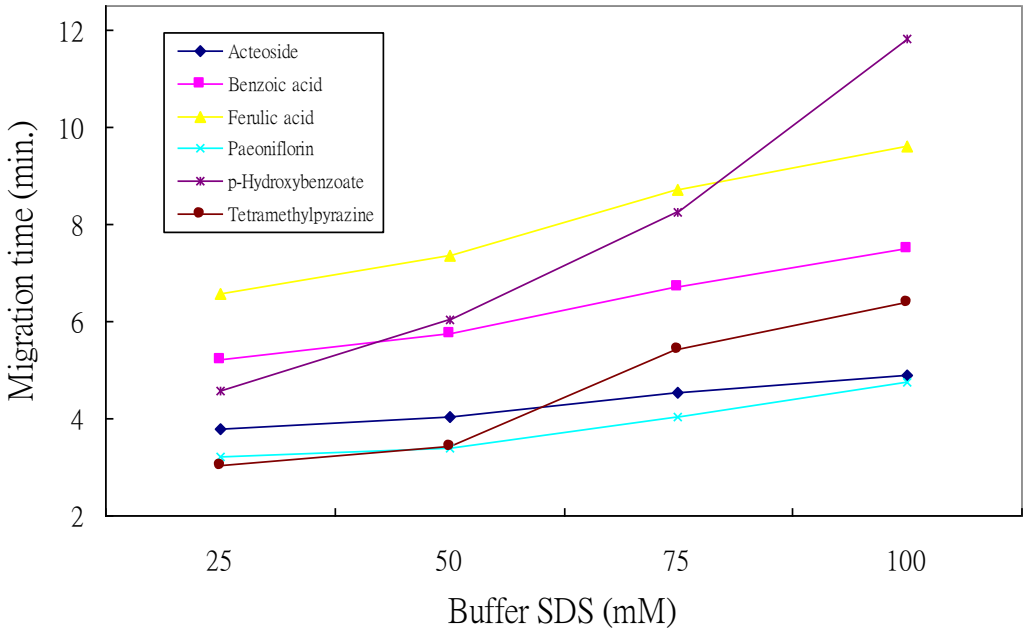

Fig. 4: The effects of SDS concentration on migration times of marker components. ◆ Acteoside; × Paeoniflorin; ▲ Ferulic acid; ● Tetramethylpyrazine; ■ Benzoic acid; * p-Hydroxybenzoate (internal standard)

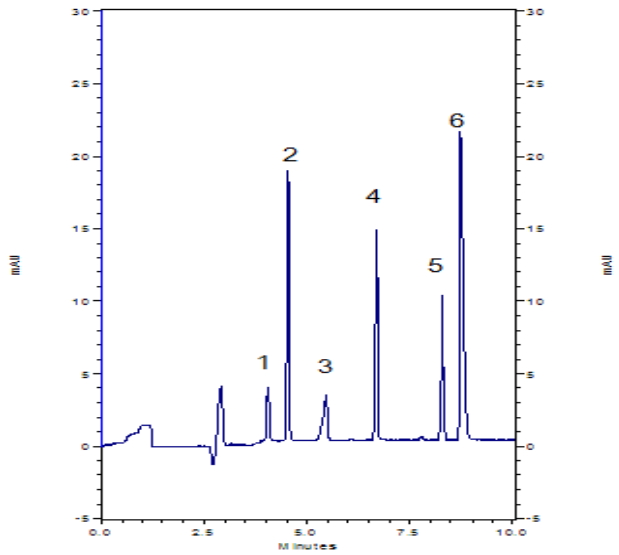

Fig. 5 Electropherogram of five marker components in SWT. (1) Paeoniflorin, (2) Acteoside, (3) Tetramethylpyrazine, (4) Benzoic acid, (5) p-Hydroxybenzoate (internal standard) and (6) Ferulic acid. Electrophoresis conditions: 30 mM boric acid; 75 mM SDS; pH 10; fused silica capillary (50 µm i.d. × 40 cm); applied voltage, 20 kV; run time, 10min; UV detection at 230 nm; temperature, 25 ℃.

Method validation and simultaneous determination of five marker constituents in SWT preparations

The measurements of intra- and inter-day variability were utilized to assess the precision of the developed assay. The relative standard deviation (R.S.D) values of the peak area ratios for five replicate injections intra-day at the concentrations of 10 ~ 200 μM were 1.0~1.5% for paeoniflorin, 0.8~1.0% for acteoside, 0.7~1.6% for tetramethylpyrazine, 0.6~1.0% for benzoic acid, and 1.0~1.8% for ferulic acid, respectively. The inter-day variability was examined over three days by performing five replicates each day. The R.S.D values inter-day at the concentrations of 10 ~ 200 μM were 1.2~1.4% for paeoniflorin, 1.1~1.3% for acteoside, 0.9~1.9% for tetramethylpyrazine, 1.1~1.7% for benzoic acid, and 1.6~1.8% for ferulic acid. The recovery was tested by adding known amounts of the five marker constituents at the concentrations of 200 μM. The mean recoveries (n = 3) of paeoniflorin, acteoside, tetramethylpyrazine, benzoic acid and ferulic acid were 100.6, 99.8, 98.7, 99.7 and 99.9%, respectively. All the tailing factors of the peaks were very close to 1. The limits of detection (LOD) and quantification (LOQ) were separately determined in five replicate determinations at a signal-to-noise ratio (S/N) of 3 and 10, respectively. The LOD were 1.18 μg/mL for paeoniflorin, 1.55 μg/mL for acteoside, 0.68 μg/mL for tetramethylpyrazine, 0.31 μg/mL for benzoic acid and 0.19 μg/mL for ferulic acid. The LOQ were 3.89 μg/mL for paeoniflorin, 5.12 μg/mL for acteoside, 2.24 μg/mL for tetramethylpyrazine, 1.02 μg/mL for benzoic acid and 0.63 μg/mL for ferulic acid. Calibration graph (peak-area ratio, y, versus concentration, x, μg/mL) was constructed in the range 4.72–94.38 μg/mL for paeoniflorin, 6.21–124.28 μg/mL for acteoside, 1.36–27.18 μg/mL for tetramethylpyrazine, 1.21–24.28 μg/mL for benzoic acid and 1.94–38.72 μg/mL for ferulic acid. The regression equations of these curves and their correlation coefficients were calculated as follows: paeoniflorin, $y = 0.0173x - 0.1600$ ($r = 0.9994$); acteoside, $y = 0.0261x + 0.1050$ ($r = 0.9991$); tetramethylpyrazine, $y = 0.0111x - 01113$ ($r = 0.9996$); benzoic acid, $y = 0.0201x - 0.0507$ ($r = 0.9995$); ferulic acid, $y = 0.0289x - 0.0252$ ($r = 0.9997$). As the above results, the linear correlation coefficients for these five marker components were $R \geq 0.9991$. The established and validated method was further applied to quantitatively determine five constituents in 6 brands of concentrated SWT preparations on the Taiwanese market. As shown in Table 1, the contents of acteoside, paeoniflorin, ferulic acid, tetramethylpyrazine and benzoic acid were successfully estimated to be 0.63 ~2.51 mg/g. The results indicated that the established assay method is suitable for quality control of SWT preparations.

Table 1: Contents of five marker components in SWT preparations (n=6).

A~B : Six brands of concentrated SWT preparations on the Taiwanese market

Marker components	Contents (mg/g)						
	A	B	C	D	E	F	Mean ± SD
Paeoniflorin	3.14	1.27	2.96	2.75	2.68	2.23	2.51 ± 0.68
Acteoside	1.01	0.39	0.97	0.84	0.95	0.70	0.81 ± 0.23
Tetramethylpyrazine	0.76	0.32	0.76	0.63	0.77	0.52	0.63 ± 0.18
Benzoic acid	1.38	0.58	1.34	1.82	1.86	1.02	1.33 ± 0.49
Ferulic Acid	1.23	0.50	1.16	1.03	1.05	0.88	0.98 ± 0.26

In conclusion, we have developed a MEKC method after optimization of factors affecting the separation of these marker constituents were estimated including pH, concentration of boric acid and sodium dodecyl sulfate (SDS). The method was demonstrated to satisfy validation items including linearity, precision, sensitivity and accuracy for simultaneous determination of five marker components in SWT preparations. This simple and rapid method is reliable and applicable for quality control of SWT preparations on the market.

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