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Micellar electrokinetic chromatography for separation of a mixture of coptis alkaloids, scute flavonoids, and rhubarb anthraquinones and bianthrones

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Abstract

Coptidis Rhizoma, Scutellariae Radix, and Rhei Rhizoma are three common Chinese herbs. There are many herbal formulas which contain either two or all three of the herbs mentioned above. Their bioactive components have already been identified, respectively. However, there is no report about separation of the 13 bioactive constituents of the three herbs at the same time. In order to assess these constituents of related Chinese herbal preparations, a micellar electrokinetic chromatography method was developed. While buffer pH and surfactant concentration affected the resolution of separation, acetonitrile percentage was found to significantly influence the resolution, peak shape, and elution window. Optimum separation of 13 compounds was achieved at pH 7.3 using a buffer mixture of 70% (v/v) 3 mM di-sodium tetraborate, 10 mM sodium dihydrogen phosphate, and 50 mM sodium deoxycholate with 30% (v/v) acetonitrile. When applying the developed method to analyze a model preparation, San-huang-xie-xin-tang, which contains all three herbs, 8 of the 13 bioactive constituents, could be determined. The present study proposed a method to assess San-huang-xie-xin-tang within short analysis time and also provided a possible starting point to evaluate related herbal preparations containing Coptidis Rhizoma, Scutellariae Radix, and Rhei Rhizoma.

Keywords: Micellar electrokinetic chromatography; Coptidis Rhizoma; Scutellariae Radix; Rhei Rhizoma

1. Introduction

Berberine, coptisine, and palmatine are three main constituents of coptis alkaloids from Coptidis Rhizoma, whose antimicrobial activity has been supported by numerous reports [1]. Recently, Coptidis Rhizoma was demonstrated to possess protective effects against peroxynitrite-induced damage and the effect was mainly attributable to the constituent alkaloids, including berberine, coptisine, and palmatine, especially berberine [2]. Besides, the common and distinct genes responsible for the antiproliferative activities of purified berberine and Coptidis Rhizoma were already identified [3]. The antitumor effects of Scutellariae Radix and its components, the scute flavonoids, baicalein, baicalin, and wogonin, were also investigated using human bladder cancer cell lines (KU-1 and EJ-1) and a murine bladder cancer cell line (MBT-2). The results suggested that Scutellariae Radix may become an attractive and promising treatment for bladder cancer [4]. Rhei Rhizoma (rhubarb) was one of the ancient and best known Chinese herbal medicines with the effect of purgation, purging heat, loosening the bowels, curing gastric and renal disorders, removing bacterial dysentery, removing heat from the blood, clearing toxins away, promoting blood circulation, removing blood stasis, and so on [5]. Among the various constituents isolated from rhubarb, anthraquinone derivatives (including aloe-emodin, chrysophanol, physcion, emodin, and rhein) and bianthrone derivatives (sennoside A and sennoside B) were accepted as important active components [5,6].

There are several kinds of Chinese herbal preparations containing Coptidis Rhizoma, Scutellariae Radix, and Rhei Rhizoma [7]. For example, Da-huang-huang-lian-xie-xin-

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tang contains Coptidis Rhizoma and Rhei Rhizoma while San-huang-xie-xin-tang contains all three herbs mentioned above. Therefore, to assure the quality of these herbal preparations, it is essential to develop a simple method to assess their bioactive components.

Due to its high resolution, short analysis time, and low solvent and sample consumption, capillary electrophoresis (CE) possesses a great capability for separation of drugs. Many methods applying CE have been proposed for the analysis of coptis alkaloids [8,9], or of scute flavonoids [10–12], or of rhubarb anthraquinone derivatives [5,6,10], respectively. However, only some experiments have demonstrated the separation of their combinations. For example, to analyze the herb couple of scute and coptis, Li and Sheu have developed a micellar electrokinetic chromatography (MEKC) method to simultaneously determine six scute flavonoids and four coptis alkaloids [13]. In analyzing the herbal combination of the three herbs, Sheu and Lu have determined six bioactive constituents by a MEKC method [14] while Liu et al. assessed three components using two-marker technique by a capillary zone electrophoresis method [7].

Since micellar electrokinetic chromatography is especially powerful for the separation of complex mixtures such as natural products and Chinese herbal preparations [10], it was chosen to analyze the mixtures of coptis alkaloids, scute flavonoids, and rhubarb anthraquinones and bianthrones. The method may be applicable to different herbal combinations which contain two or all three of the herbs mentioned above. In previous reports [7,14], only either three or six constituents out of three herbs could be identified and determined. In order to test the applicability of our developed method, San-huang-xie-xin-tang, an herbal remedy which contains all three herbs, was used as the model preparation. The remedy described in ancient Chinese book "Jin-Gui-Yao-Lue" was originally used to treat those patients with epigastric fullness, flushing, restlessness, constipation, and a hard pulse [15–17]. It was found that the formula could significantly improve the hyperkinetic states of the cardiovascular system without apparent side effects so as to become a promising antihypertensive agent [15–17].

In the present study, we developed a MEKC method for separation of coptis alkaloids, scute flavonoids, and rhubarb anthraquinone and bianthrone mixtures, and the 13 bioactive constituents could be separated. While applying the method on a model preparation containing all three herbs, 10 components were found, and 8 of which could be determined.

2. Experimental

2.1. Chemicals and materials

Di-sodium tetraborate, sodium dihydrogen phosphate, and hydrochloric acid were purchased from Merck (Darmstadt, Germany). Methanol was from J. T. Baker (Phillipsburg, NJ, USA). Acetonitrile (ACN) was from Tedia (Fairfield, OH, USA). Sodium deoxycholate, aloe-emodin, emodin, rhein, and berberine chloride were from Sigma (St. Louis, MO, USA). Tetrandrine (used as internal standard, I.S.), baicalin, and baicalein were from Aldrich (Milwaukee, WI, USA). Sodium hydroxide, coptisine chloride, palmatine chloride, sennoside A, sennoside B, and wogonin were from Wako (Osaka, Japan). Chrysophanol was from Fluka (Buchs, Switzerland). Physcion was from Extrasynthese (Genay, France). San-huang-xie-xin-tang was from Sun Ten Pharmaceutical Co. (Taipei, Taiwan).

2.2. Preparation of standard solutions

Standard working solutions of berberine chloride, palmatine chloride, aloe-emodin, chrysophanol, sennoside B, sennoside A, emodin, and rhein were prepared in 70% (v/v) ACN at a concentration of 25 μg/ml, while coptisine chloride, physcion, baicalein, baicalin, and wogonin were prepared in 70% (v/v) ACN at concentrations of 40, 20, 20, 15, and 10 μg/ml, respectively. The I.S. was dissolved in 70% (v/v) ACN and used at a final concentration of 100 μg/ml.

2.3. Extraction of the model preparation

An amount of 0.2509 g of San-huang-xie-xin-tang sample powder was ultrasonically extracted with 3 ml of 70% (v/v) methanol for 30 min and centrifuged at 1500 × *g* for 10 min. The extraction procedure was repeated three times. The extracts were combined and diluted to 10 ml with 70% (v/v) methanol, then 6 ml of which was evaporated using a rotary vacuum evaporator. The concentrated residue was dissolved in 4 ml of 70% (v/v) ACN and filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA). Since the amount of the constituents differed to a great extent, the solution was then properly diluted or concentrated so as to make the concentration of each component as close as possible to the center of the linearity range. The I.S. solution was added in equal amount before use.

2.4. Apparatus and conditions

The experiments of capillary electrophoresis were carried out with Beckman P/ACE^{TM} MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA, USA) equipped with photodiode array detector. Separations were performed using a 59 cm (49 cm effective length) × 50 μm i.d. fused-silica capillary from Polymicro Technologies (Phoenix, AZ, USA). The new capillary was pre-conditioned prior to use by flushing successively with 1.0 M sodium hydroxide for 20 min, 0.2 M sodium hydroxide for 20 min, de-ionized water for 20 min, and running buffer for 20 min. At the beginning of each day, the capillary was flushed by 0.2 M sodium hydroxide for 10 min and running buffer for 10 min. Before each experiment, the capillary was washed by 0.2 M sodium hydroxide for 5 min and de-ionized water for 5 min, and then equilibrated with running buffer for 5 min.

Operation conditions were as follows: voltage, 30 kV; temperature, 30 °C; injection time, 5.0 s (hydrodynamic, 0.7 psi); detection wavelength, 270 nm. The optimum running buffer composition contained 70% (v/v) of the mixture of 3 mM di-sodium tetraborate, 10 mM sodium dihydrogen phosphate, and 50 mM sodium deoxycholate with the pH value of 7.3 adjusted by using 0.1 M hydrochloric acid and 30% (v/v) of ACN. ACN was added prior to use. All the solutions were filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA) before use, except for ACN. After adding ACN, running buffer was not filtered again to avoid evaporation loss.

3. Results and discussion

3.1. Method development

All of the 13 components to be analyzed are shown in Fig. 1. These components include three coptis alkaloids, i.e., coptisine, berberine, and palmatine, three scute flavonoids, i.e., wogonin, baicalin, and baicalein, five rhubarb anthraquinones, i.e., aloe-emodin, chrysophanol, physcion, emodin, and rhein, and two rhubarb bianthrone glycosides, i.e., sennoside A and sennoside B. In a previous study, six bioactive components were successfully separated using 60% of buffer solution containing 4.25 mM sodium borate, 15 mM sodium dihydrogen phosphate, and 50 mM sodium cholate with 40% of acetonitrile [14]. Although 13 components could not be separated simultaneously with this composition, the peak shapes were much better than those using tris or histidine buffer in our pre-experiments. Since the mixture of sodium borate and sodium dihydrogen phosphate seemed to be suitable for separation, a series of experiments containing different proportions of these two buffer components were tested. The mixture of 3 mM di-sodium tetraborate with 10 mM sodium dihydrogen phosphate was found to be the most suitable one. Besides, although being similar in structure, sodium deoxycholate was found to give better resolution and peak shapes of aloe-emodin, chrysophanol, and physcion than sodium cholate. In a previous study analyzing nine rhubarb components using experimental design in our laboratory, buffer pH, sodium deoxycholate concentration, and acetonitrile percentage were demonstrated to be the critical factors for optimization [18]. Since 7 out of the 13 components to be separated belonged to rhubarb anthraquinones and bianthrones in the present study, these three parameters were further investigated to search for optimized conditions.

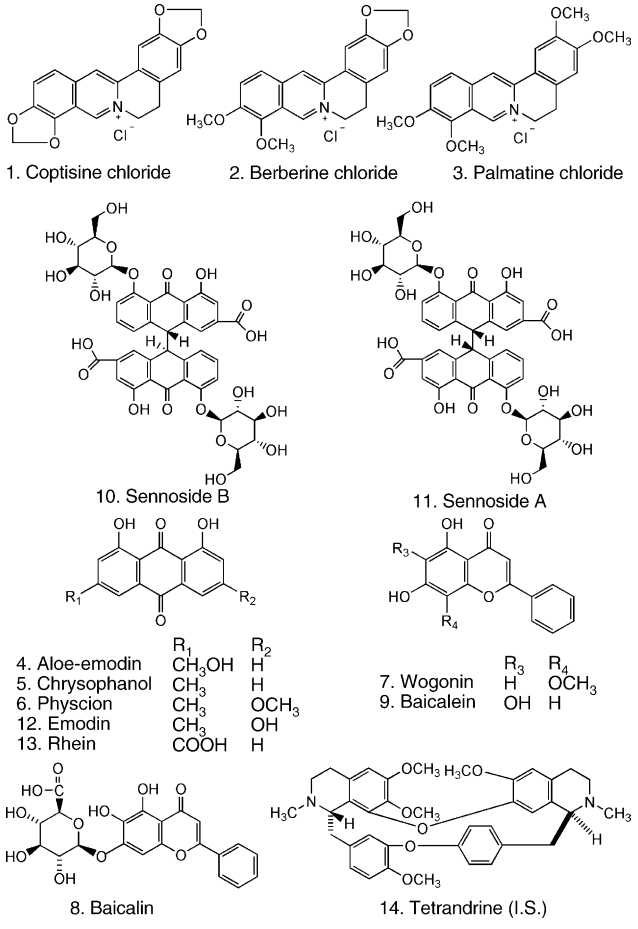

Fig. 1. Structures of 13 compounds analyzed and tetrandrine (I.S.).

3.1.1. Influence of buffer pH

Separations in the pH range 7.3–8.0 were investigated since sodium deoxycholate tended to precipitate easily under more acidic environment while the peak shape of rhein was greatly distorted at more basic pH value. The results are shown in Fig. 2. The pH effect on coptisine, berberine, palmatine, aloe-emodin, chrysophanol, and physcion was not obvious within this range while migration times of other compounds appeared to have an overall pattern of increasing gradually to different extent with increasing pH, except for some slight decrease at pH 8.0 occurring in some compounds. The subtle difference accounted for each separation pattern at respective pH value. As mentioned above, rhein tended to have tailing shape at higher pH values. Therefore, 13 peaks could not be separated simultaneously except at pH

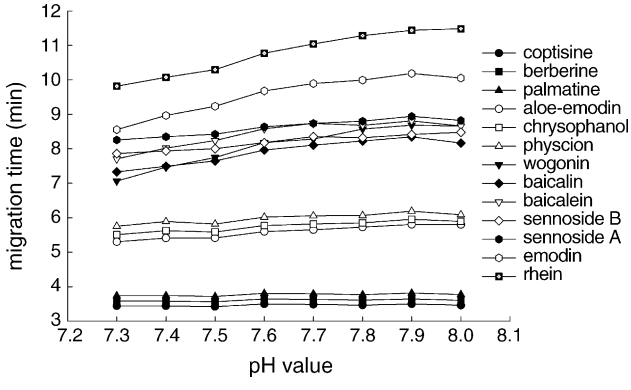

Fig. 2. Effect of buffer pH on migration time. Buffer composition: 3 mM di-sodium tetraborate, 10 mM sodium dihydrogen phosphate, and 50 mM sodium deoxycholate with pH 7.3–8.0; 30% acetonitrile.

7.3 and 7.4. Since the distance between baicalein and sennoside B was larger at pH 7.3 so as to make the nearby peaks to be more evenly separated, 7.3 was chosen to be the optimized buffer pH value.

3.1.2. Influence of sodium deoxycholate concentration

Based on linear solvation energy relationships (LSER), surfactant concentration could influence retention behavior of uncharged solutes by changing the phase ratio without significantly affecting selectivity [19–21]. Generally, the increase of surfactant concentration would lead to increased migration time [10,22,23], which can also be seen in Fig. 3, while the mixtures contained both ionic and non-ionic compounds. The elution order changed while the concentration was increased from 20 to 30 mM, since the retention of emodin was greatly increased. It was probably due to its high hydrophobicity and the stronger interaction with increased amount of the surfactant added. As the elution window was extended with the increment of surfactant concentration, the resolution was also improved, especially for aloe-emodin, chrysophanol, and physcion. A concentration of 50 mM was chosen as the optimized condition, since both resolution and peak shape were acceptable. Further increase of the concentration could only extend migration times without significant improvement on separation.

3.1.3. Influence of ACN percentage

Through the addition of organic modifier, separation condition could be improved by the expansion of elution window, decrease of analyte retention, change of selectivity and so on [24]. Since the unique helical structure of sodium deoxycholate micelle [25] was found to be more tolerant to the addition of organic solvents [26], a range from 0 to 40% ACN (v/v) was studied and the results are shown in Fig. 4. It was found that the percentage of ACN to be the most influential of the three parameters optimized, not only on selectivity but also on peak shapes. Complicated variation in elution order and broad peaks were observed from 0 to 25% ACN. Without the addition of ACN, there were serious overlaps between peaks.

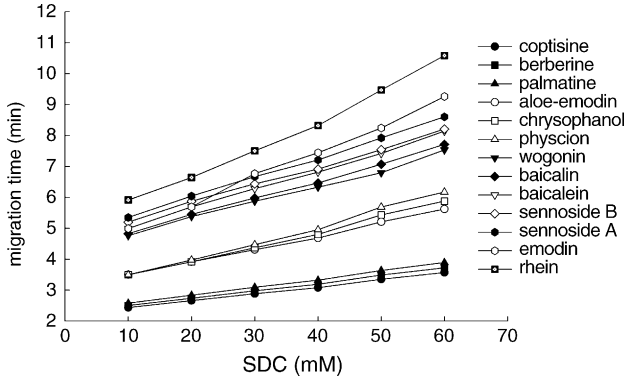

Fig. 3. Effect of sodium deoxycholate (SDC) concentration on migration time. Buffer composition: 3 mM di-sodium tetraborate, 10 mM sodium dihydrogen phosphate, and 10–60 mM sodium deoxycholate; pH 7.3; 30% acetonitrile.

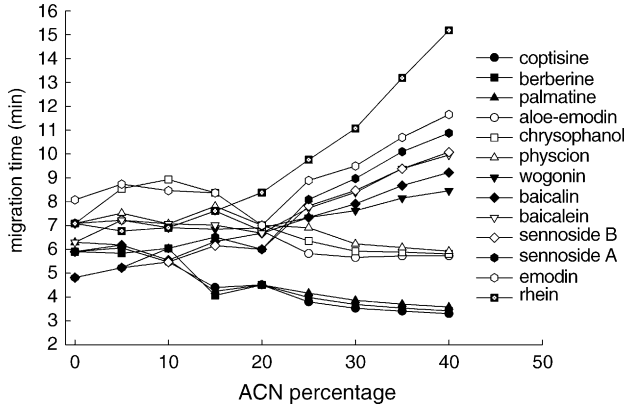

Fig. 4. Effect of acetonitrile (ACN) percentage on migration time. Buffer composition: 3 mM di-sodium tetraborate, 10 mM sodium dihydrogen phosphate, and 50 mM sodium deoxycholate; pH 7.3; 0–40% acetonitrile.

Three alkaloids migrated after electroosmotic flow, possibly due to the electrostatic interaction with the negative charges of the micelles in addition to the hydrophobic interaction. Upon the addition of ACN, these three alkaloids migrated faster and finally appeared in front of electroosmotic flow at 25% ACN as three well-separated peaks. Aloe-emodin, chrysophanol, and physcion, possessing large hydrophobicity, migrated faster while adding ACN, probably due to less solubilization by the micelles. Emodin had similar migration behavior as the above three anthraquinones. However, while the ACN percentage was above 20%, the effect of ACN on decreasing the velocity of electroosmotic flow seemed to be dominant in affecting the migration of emodin. The same effect was observed at much earlier start, i.e., 5% addition of ACN, in the migration of rhein, possibly due to its less hydrophobicity, and so were sennoside A and sennoside B. Although flavonoids showed much more complex behavior while adding ACN, the migration pattern became stable at the ACN percentage above 25%. Since all the 13 compounds could be separated at 30% ACN and further increase would only prolong the migration time or even distorted some peaks at 40% ACN, 30% were chosen as the optimized percentage.

According to the above results, the optimized separation was found to be achieved at pH 7.3 using a buffer mixture of 3 mM di-sodium tetraborate, 10 mM sodium dihydrogen phosphate, and 50 mM sodium deoxycholate with 30% acetonitrile. The electropherogram of 13 standard substances is shown in Fig. 5. While applying the method to analyze the model preparation, tetrandrine was found to be the most suitable internal standard without interfering those complex components existing in San-huang-xie-xin-tang extract after testing many other compounds. The electropherogram of the San-huang-xie-xin-tang extract together with tetrandrine is displayed in Fig. 6.

3.2. Method validation

For entire 13 components, run-to-run repeatability ($n = 10$) and day-to-day reproducibility ($n = 3$) of the method

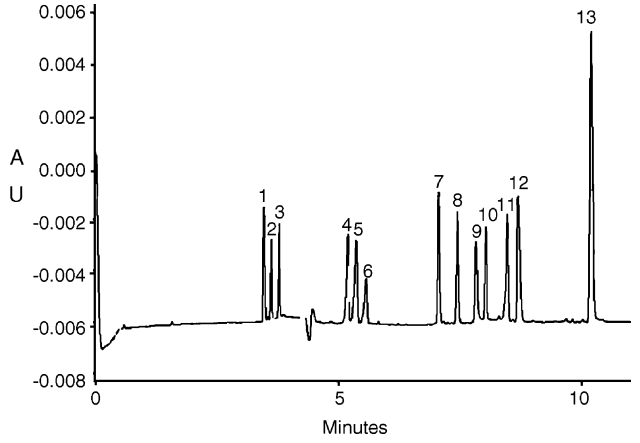

Fig. 5. Electropherogram of 13 analytes under optimum conditions as described in text.

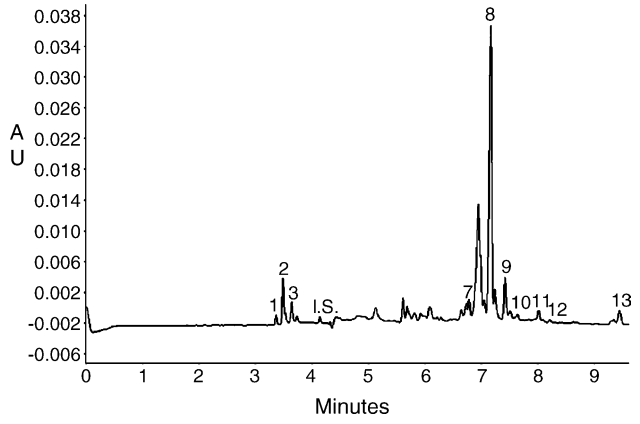

Fig. 6. Electropherogram of San-huang-xie-xin-tang extract under optimum conditions as described in text.

in terms of migration times were less than 0.88 and 2.16% relative standard deviation (R.S.D.), respectively. However, the capillary in our condition must be carefully pre-conditioned to achieve the equilibrium so as to attain acceptable results. While the capillary was challenged by a series of different compositions to observe the effect of a certain parameter, the migration times could be varied (Figs. 2–4). The limits of detection (LOD, S/N = 3), determined by diluting the standard solutions, are listed in Table 1. For eight components to be determined quantitatively, run-to-run repeatability ($n = 6$) and day-to-day reproducibility ($n = 3$) of peak-area-ratios (with respect to I.S.) of the method were less than 4.77 and 9.79% (R.S.D.), respectively. The concentrations of the standard solutions used to assess repeatability and reproducibility were at the lowest level of each calibration curve. The acceptable but relatively large R.S.D. values may be attributed to high proportion (30%, v/v) of organic modifier

Table 1
Limits of detection of 13 components

Compounds	LOD (μg/ml)
Coptisine	0.65
Berberine	0.40
Palmatine	0.48
Aloe-emodin	1.11
Chrysophanol	2.06
Physcion	1.41
Wogonin	0.17
Baicalin	0.52
Baicalein	0.45
Sennoside B	0.64
Sennoside A	0.18
Emodin	0.47
Rhein	0.68

Table 2
Linear relationships between peak-area-ratios and concentrations (μg/ml) for eight components

Compounds	Linear range (μg/ml)	Intercept	Slope	r^a
Coptisine	10–30	−0.0156	0.0436	0.9999
Berberine	50–150	−0.3797	0.0419	0.9997
Palmatine	20–60	0.2167	0.0465	0.9998
Baicalin	60–180	−4.5482	0.1964	0.9983
Sennoside B	20–60	−0.8711	0.1012	0.9981
Sennoside A	2.5–20	−0.0407	0.0618	0.9986
Emodin	2.5–20	−0.0902	0.2055	0.9997
Rhein	2.5–20	−0.1928	0.2900	0.9990

[a] Correlation coefficient.

Table 3
Intra-assay ($n = 3$) and inter-assay ($n = 9$) determinations of eight components in spiked San-huang-xie-xin-tang

Compounds Concentration added (μg/ml)	Intra-assay precision (R.S.D. (%))	Inter-assay precision (R.S.D. (%))	Analytical recovery (%)
Coptisine			
5	2.53	3.32	100.79
10	2.82	2.83	98.17
Berberine			
10	2.67	5.21	101.14
25	2.69	3.09	97.24
40	3.60	3.60	98.12
Palmatine			
15	2.77	2.77	101.50
30	2.82	2.84	102.30
Baicalin			
55	6.61	6.61	100.27
75	5.08	5.08	105.08
95	3.48	4.77	107.58
Sennoside B			
15	2.74	2.74	102.13
30	2.82	2.82	100.34
Sennoside A			
3	5.21	5.21	104.38
6	3.48	5.18	105.70
Emodin			
5	3.57	3.57	103.90
10	3.58	5.59	104.39
Rhein			
3	3.32	3.32	100.79
6	3.48	4.52	100.70

Table 4
Contents of eight components in San-huang-xie-xin-tang ($n = 3$)

	Content (mg/g) S.D.	S.D.
Coptisine	1.55	0.02
Berberine	7.83	0.11
Palmatine	2.55	0.04
Baicalin	30.81	0.59
Sennoside B	0.99	0.01
Sennoside A	1.48	0.04
Emodin	0.14	0.01
Rhein	0.77	0.04

added in buffer, which may be susceptible to evaporation loss. Linearity was evaluated using five different concentrations and measuring the relative responses with respect to I.S. The linearity range and the characteristics of the regression lines are listed in Table 2. The accuracy was determined by adding a suitable amount of standard to sample solutions and assessed at different concentration levels three times a day on three different days. Intra-assay precision (overall repeatability, expressed in R.S.D.), inter-assay precision (intermediate precision/reproducibility, expressed in R.S.D.) were summarized in Table 3. The calculations of intra- and inter-assay precision were based on reference [27].

3.3. Analysis of the model preparation

While analyzing the model preparation, 10 components were identified by the additions of respective standards. Among 10 components identified in the extract, baicalein was susceptible to decomposition in the analyzed condition. Besides, wogonin could not be well-separated from other constituents of the extract. Therefore, the remaining eight bioactive components were determined under optimized separation condition. The results are listed in Table 4.

4. Conclusion

In order to analyze common herbal combinations of Coptidis Rhizoma, Scutellariae Radix, and Rhei Rhizoma, a MEKC method for separation of 13 bioactive components was developed. Buffer pH was found to affect the resolution of 13 components. The elution window was extended by the increment of sodium deoxycholate concentration and the resolution was also improved. Among the factors investigated, ACN percentage was the most influential one, which significantly alter the resolution, peak shape, and elution window. When applying the optimized condition on the model preparation containing all three herbs, eight components were determined. The present study proposed a suitable method to assess San-huang-xie-xin-tang within short analysis time and also provided a possible starting point to evaluate related herbal preparations containing these herbs mentioned above.

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