Simultaneous determination of twelve constituents of I-tzu-tang, a Chinese herbal preparation, by high-performance liquid chromatography and capillary electrophoresis

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Abstract

A high-performance liquid chromatographic method and a capillary electrophoretic method for the separation of four anthraquinoids, five flavones, two carboxylic acids and one saponin in I-tzu-tang were developed. Detection at 254 nm with a linear gradient elution system of tetrabutylammonium bromide and dihydrogenphosphate buffer or with micellar electrokinetic chromatography with sodium dodecyl sulphate and sodium cholate buffer was found to be the most suitable approach for this analysis. The contents of these components in an unpretreated I-tzu-tang extract could be easily determined within 50 min by HPLC or 14 min by CE. The effect of buffers on this separation and the validation of these methods are discussed.

1. Introduction

Chinese herbal preparations are widely used in eastern Asia and suitable assay methods are therefore needed urgently for quality control purposes. Recently, high-performance liquid chromatography (HPLC) [1-4] and capillary electrophoresis (CE) [5-8] have been employed to establish the optimum conditions for examining two or three constituents of the preparations. However, as our knowledge of the effective components of Chinese herbal preparations is still limited and their chemical compositions are very complicated, to determine accurately the contents of Chinese herbal preparations is very difficult. Efforts to develop simpler and more

rapid methods that can assay as many bioactive constituents as possible are therefore necessary.

I-tzu-tang (Cimicifuga Combination) is a herbal prescription often used to treat those having constipation with bleeding and severe local pain, and is composed of six herbs, Cimicifugae Rhizoma, Bupleuri Radix, Angelicae Radix, Glycyrrhizae Radix, Scutellariae Radix and Rhei Rhizoma [9]. In this study, twelve bioactive components, sennoside A (SA), sennoside B (SB), emodin (E) and aloe-emodin (AE) in Rhei Rhizoma, baicalin (BG), baicalein (B), wogonin (W), oroxylin A 7-O-glucuronide (OG) and wogonin 7-O-glucuronide (WG) in Scutellariae Radix, glycyrrhizin (GZ) in Glycyrrhizae Radix, ferulic acid (FA) in Angelicae Radix and caffeic acid (CA) in Cimicifugae Rhizoma [10] were selected for analysis; their structural formulae are shown in Fig. 1. Using these compounds,
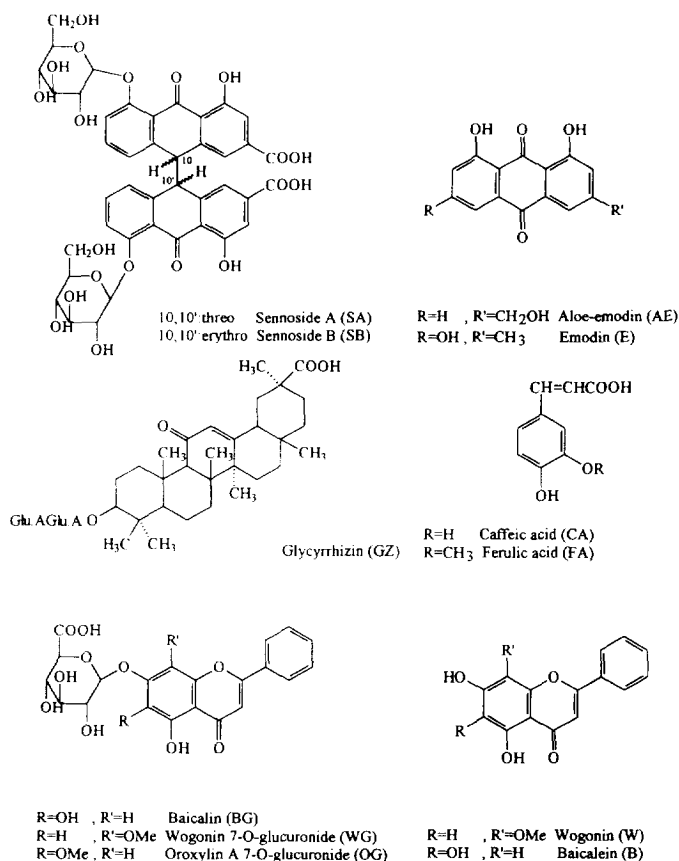
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Fig. 1. Structures of the twelve marker substances.

consisting of four anthraquinoids, five flavones, two carboxylic acids and one saponin as marker substances, an HPLC and a CE method for determining the quality of I-tzu-tang were developed. The suitabilities of these two methods were compared and are discussed.

2. Experimental

2.1. Reagents and materials

Baicalin, baicalein, wogonin, sennoside A and sennoside B were purchased from Yoneyama (Osaka, Japan), tetrabutylammonium bromide (TBA), potassium dihydrogenphosphate, sodium dihydrogenphosphate, sodium borate and salicylic acid from Kanto (Tokyo, Japan), glycyrrhizin,

tetralin (tetrahydronaphthalene), ferulic acid and caffeic acid from Tokyo Kasei (Tokyo, Japan), emodin and aloe-emodin from Extrasynthese (Genay, France) and sodium dodecyl sulphate (SDS) and sodium cholate (SC) from Sigma (St. Louis, MO, USA). Methanol and acetonitrile were of the LC grade (Mallinkrodt, St. Louis, MO, USA) and phosphoric acid was of analytical-reagent grade (Merck, Darmstadt, Germany). Oroxylin A 7-O-glucuronide and wogonin 7-O-glucuronide were isolated from Scutellariae Radix [11–14]. Three I-tzu-tang samples were purchased from two Chinese pharmaceutical companies in Taiwan. Peak identification and purity checking of all marker substances and test samples were done with a photodiode-array detector or a variable wavelength UV detector.

2.2. Preparation of Chinese herbal preparation extracts

A 1.0-g sample of I-tzu-tang was extracted with 70% methanol (7.5 ml) by stirring at room temperature for 15 min, then centrifuged at 1500 g for 5 min. Extraction was repeated three times. The extracts were combined and filtered through a No. 1 filter-paper. After addition of internal standard solution (for HPLC, 0.5 mg of tetralin in 1 ml of 70% methanol was prepared and 1.25 ml were taken each time; for CE, 1 mg of salicyclic acid in 1 ml of 70% methanol was prepared and 2.5 ml were taken each time), the Chinese herbal preparation extract was diluted to 25 ml with 70% methanol. This solution was passed through a 0.45-μm filter and the filtrate was injected into the HPLC or CE systems.

2.3. Apparatus and conditions

HPLC system

HPLC was performed on a Shimadzu LC-6AD apparatus equipped with a Shimadzu SCL-6B system controller and a Shimadzu SPD-M6A photodiode-array detector (254 nm). Satisfactory separation of the marker substances was obtained with a reversed-phase column (Cosmosil 5C$_{18}$-AR, 5 μm, 25 cm × 4.6 mm I.D.) (Nacalai Tesque, Kyoto, Japan) eluted at a flow rate of 0.8 ml/min with a linear solvent gradient of A–B [A = buffer–CH$_3$OH–CH$_3$CN (8:1:1, v/v), adjusted to pH 3.5 with phosphoric acid; B = buffer–CH$_3$OH–CH$_3$CN (1:2:2, v/v), adjusted to pH 4.2 with phosphoric acid; buffer = aqueous solution consisting of 3.0 mM TBA and 7.3 mM KH$_2$PO$_4$ (0.1 g of KH$_2$PO$_4$ was made into a 100 ml aqueous solution)] varying as follows: 0 min, 95:5; 5 min, 85:15; 10 min, 80:20; 20 min, 60:40; 35 min, 25:75; and 40–50 min, 0:100.

CE system

The electrophoretic experiments were carried out on a Spectra Phoresis 1000 capillary electrophoresis system equipped with a UV detector set at 254 nm and a 70 cm × 75 μm I.D. fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ, USA) with the detection window

placed at 62.5 cm. The conditions were as follows: injection mode, 2 psi s (hydrodynamic); cartridge temperature, 30°C; run time, 15 min; applied voltage, 20 kV (constant voltage, positive to negative polarity). The electrolyte was a buffer solution consisting of 18 mM SDS, 2 mM SC, 12.5 mM Na$_2$B$_4$O$_7$ and 10 mM NaH$_2$PO$_4$.

3. Results and discussion

3.1. Analytical conditions for HPLC method

All twelve marker substances contain either carboxylic or phenolic groups and are able to be separated by optimizing the pH and concentrations of tetrabutylammonium and dihydrogenphosphate in the eluent. Buffers of pH 3.5 and 4.2 in the two solvent reservoirs of a high-pressure gradient system were chosen, because with a pH higher or lower than these values, acceptable separation was not obtained.

A primary experiment was first conducted at 7.3 mM KH$_2$PO$_4$ without TBA. In this case, E, WG, OG and BG could be separated, but other compounds were partially or completely overlapped. With TBA, however, the formation of an ion pair not only enhanced the separation of the sample components but also shortened the running time of the whole analysis.

Dihydrogenphosphate concentration

Several KH$_2$PO$_4$ buffer solutions with pH 3.5 and 4.2 at different concentrations ranging from 0 to 15 mM were used in order to study the effect of dihydrogenphosphate on the separability. In Fig. 2, the capacity factors for the marker substances obtained at different KH$_2$PO$_4$ concentrations are shown. From the results it can be seen that by using the mobile phase without KH$_2$PO$_4$, a good resolution was obtained only for E, WG, OG and BG. With the addition of KH$_2$PO$_4$ to the mobile phase, a much better separation was obtained, especially for highly polar compounds such as CA, SB, SA, BG, OG, WG, FA and GZ. Increasing the buffer concentration from 0 to 15 mM not only varies the retention time but also narrows peaks of the
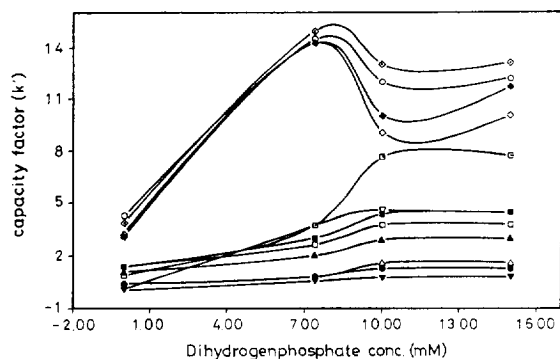
Fig. 2. Effect of KH_2PO_4 concentration on capacity factor (k'). (▼) CA = caffeic acid; (□) OG = oroxylin A 7-O-glucuronide; (◇) B = baicalein; (●) SB = sennoside B; (■) WG = wogonin 7-O-glucuronide; (◆) W = wogonin; (△) SA = sennoside A; (▽) FA = ferulic acid; (○) AE = aloe-emodin; (▲) BG = baicalin; (⊡) GZ = glycyrrhizin; (◇) E = emodin.

components in I-tzu-tang. As shown in Fig. 2, satisfactory resolution was obtained at 10 mM KH_2PO_4. However, when the chromatogram was closely examined, it was found that the separation of FA from WG and SA from SB was somewhat incomplete, and the peak of AE was too broad.

TBA concentration

Three buffer systems (prepared by mixing 7.3 mM KH_2PO_4 with 0, 3 and 6 mM TBA) were used in order to study the effect of TBA on the separability. The results obtained are shown in Fig. 3, where the capacity factors are plotted
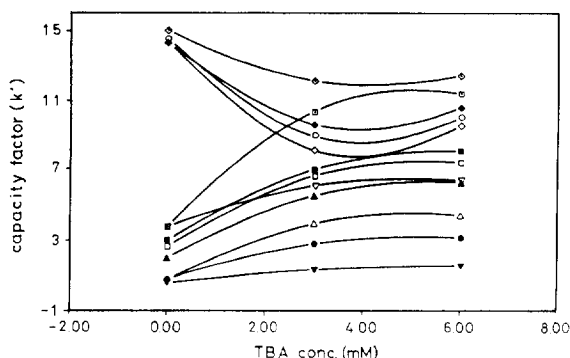
against TBA concentration. There was an increase in the retention times of carboxylic acids and glycosides but a decrease in those of the less polar compounds when the TBA concentration in the buffer solution increased. From the results, a buffer solution of 7.3 mM KH_2PO_4 and 3 mM TBA was found to produce the best resolution.

The running time for the separation of all twelve authentic compounds was about 50 min (Fig. 4). As a methanol–water extract of the I-tzu-tang sample was injected directly and analysed, the results were as good as those obtained with pure chemical samples, except for SB, FA, CA and AE, the concentrations of which were either to low to be detected or to be subject to serious interference by other components, as shown in Fig. 5.

3.2. Analytical conditions for CE method

According as the method for determining anthraquinones [15] (a buffer solution consisting of 10 mM SDS, 12.5 mM NaH_2PO_4 and 15 mM $Na_2B_4O_7$ and acetonitrile in the ratio 3:1), we used a buffer solution of 20 mM SDS, 12.5 mM NaH_2PO_4 and 15 mM $Na_2B_4O_7$ in our primary study and found that there was a well separated electropherogram for eight of the twelve components, but it was difficult to separate B from

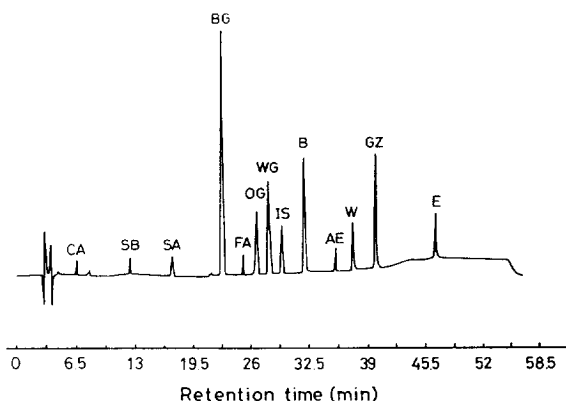Fig. 3. Effect of TBA concentration on capacity factor (k'). Symbols as in Fig. 2.

Fig. 4. HPLC of the twelve authentic compounds of marker substances. IS = internal standard (tetralin); other symbols as in Fig. 1.
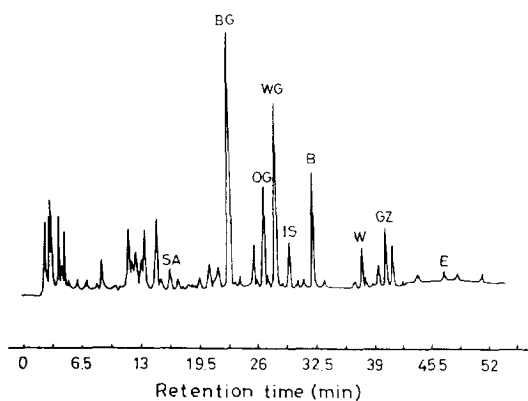
Fig. 5. HPLC of I-tzu-tang.

WG and BG from FA. Substituting sodium cholate for SDS, it was found that the separation of B from WG and BG from FA was complete, but the separation of OG from WG and W from BG failed. Therefore, a buffer system consisting of SDS, SC, borate and dihydrogenphosphate was chosen.

Effect of SDS and SC

Seven electrolyte systems containing different molar ratios of SDS to SC ranging from 1:0 to 0:1 (A:B = 1:0, 9:1, 5:1, 2:1, 1:2, 1:5 and 0:1; A = buffer solution of 20 mM SDS, 15 mM Na$_2$B$_4$O$_7$ and 12.5 mM NaH$_2$PO$_4$; B = buffer solution of 20 mM SC, 15 mM Na$_2$B$_4$O$_7$ and 12.5 mM NaH$_2$PO$_4$) were used to study the effect of SDS to SC ratio on the separability. The results obtained are shown in Fig. 6, where
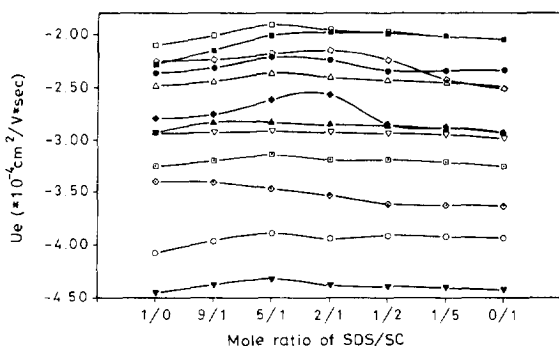
the effective mobilities (U_e) are plotted against mole-ratio of SDS to SC. There was first a decrease and then a gradual increase in the effective mobilities of the marker substances, especially those of W, B and WG, when the SDS to SC ratio in the electrophoretic solution decreased. This change can be explained by the fact that there was great difference in the critical micelle concentration between SDS and SC. From Fig. 6, it is clear that analysis carried out at a buffer solution consisting of 18 mM SDS, 2 mM SC, 15 mM Na$_2$B$_4$O$_7$ and 12.5 mM NaH$_2$PO$_4$ could offer a good separation.

Effect of pH

Seven electrolyte systems containing 18 mM SDS and 2 mM SC at different pH values ranging from 8.14 to 9.74 (prepared by mixing 10 mM NaH$_2$PO$_4$ with different concentrations of Na$_2$B$_4$O$_7$) were used in order to study the effect of pH on the separability. In Fig. 7, the effective mobilities for the components obtained at different pH values are shown. The effective mobilities of these twelve compounds varied with the pH of the buffer, especially those of W, B, E and AE. From the results, a buffer solution of pH 9.35 (12.5 mM Na$_2$B$_4$O$_7$) was found to produce the best resolution.

The running time for the separation of the twelve marker substances was 14 min (Fig. 8) when using an electrolyte of 18 mM SDS, 2 mM SC, 12.5 mM Na$_2$B$_4$O$_7$ and 10 mM NaH$_2$PO$_4$. As a methanol–water extract of the I-tzu-tang

Fig. 6. Effect of SDS-to-SC mole-ratio on effective mobility. Symbols as in Fig. 2.
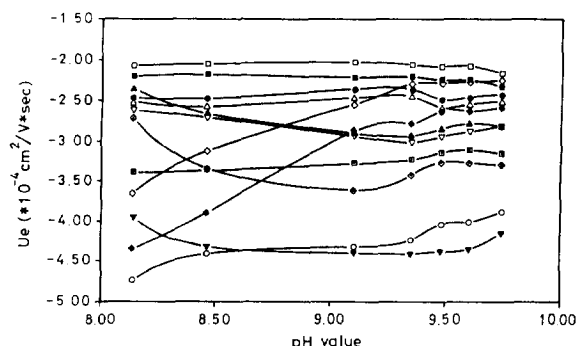

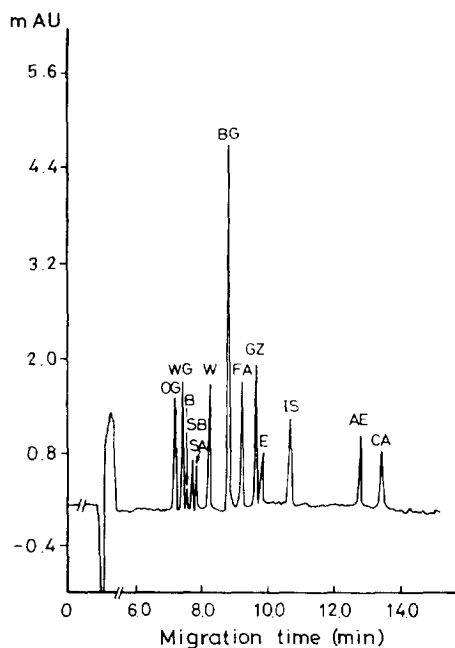Fig. 7. Effect of pH on effective mobility. Symbols as in Fig. 2.

Fig. 8. CE of the twelve authentic compounds. IS = internal standard (salicylic acid); other symbols as in Fig. 1.
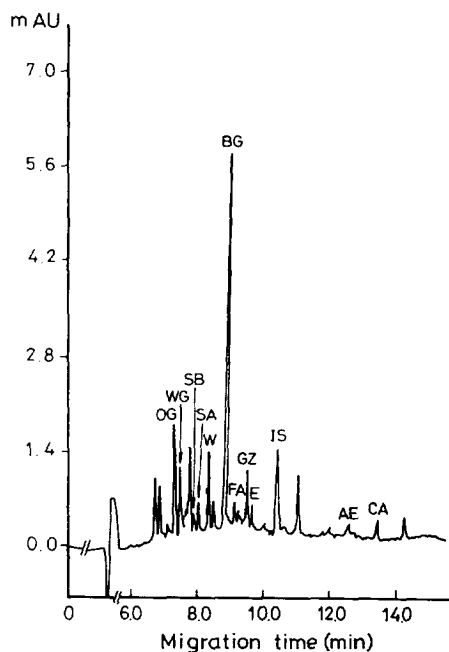

Fig. 9. CE of I-tzu-tang.

sample was injected directly and analysed, the results were as good as those obtained with authentic compounds, except for B, which suffered serious interference by the components of rhubarb, as shown in Fig. 9.

3.4. Method validation

The marker substances in the samples were identified by comparing the retention times or migration times of authentic standards with those obtained in the sample chromatograms or electropherograms and the UV spectra obtained from the photodiode-array detector, and also were checked with the blank test solutions. The detection limit ($S/N = 3$) of each marker substances in HPLC varied from 0.08 to 0.50 ng (0.008–0.050 μg/ml, flow cell length 10 mm) and in CE from $2.25 \cdot 10^{-3}$ to $8.00 \cdot 10^{-3}$ ng (0.45–1.60 μg/ml, column I.D. 75 μm).

Linearity

The linearity of the plot of peak-area ratio (X) vs. concentration (Y, mg/ml) for each of the

marker substances in HPLC and CE was investigated. The regression equations of the graphs and the correlation coefficients for these compounds are given in Table 1.

Precision

The reproducibility (relative standard deviation) of the proposed methods, on the basis of peak-area ratios for six replicate injections, was 0.84–1.20% for HPLC and 0.94–2.01% for CE. The relative standard deviation of the retention time or migration time of each peak for six replicate injections was less than 0.45% (HPLC) or 2.64% (CE). The detailed data for individual constituents are given in Table 2.

Accuracy

Suitable amounts (0.10–0.20 mg) of the marker substances were added to a sample of I-tzu-tang of known component contents and analysed using the two proposed procedures. The recoveries of all constituents determined by either method were around 98–103%. The tailing factors of all peaks were very close to unity.

Table 1
Data for linear ranges and correlation coefficients

Constituent	HPLC				CE			
	Linear range (μg/ml)	Slope	Intercept	r	Linear range (μg/ml)	Slope	Intercept	r
SA	1.0–18.0	0.283	$1.50 \cdot 10^{-3}$	0.9995	1.0–18.0	0.569	$2.60 \cdot 10^{-3}$	0.9996
BG	12.6–226.8	0.245	$4.57 \cdot 10^{-2}$	0.9997	7.8–140.4	0.405	$1.71 \cdot 10^{-2}$	0.9996
OG	1.6–28.3	0.182	$7.91 \cdot 10^{-3}$	0.9992	1.6–28.3	0.259	$4.40 \cdot 10^{-3}$	0.9994
WG	2.8–50.0	0.182	$1.37 \cdot 10^{-2}$	0.9994	2.8–50.0	0.384	$1.16 \cdot 10^{-2}$	0.9994
B	1.2–21.6	0.134	$7.40 \cdot 10^{-3}$	0.9996				
W	0.8–13.7	0.113	$3.90 \cdot 10^{-3}$	0.9994	1.3–22.5	0.151	$1.50 \cdot 10^{-3}$	0.9995
GZ	8.6–154.1	0.294	$1.71 \cdot 10^{-2}$	0.9999	5.1–91.8	0.597	$2.93 \cdot 10^{-2}$	0.9998
E	0.5–9.0	0.095	$1.18 \cdot 10^{-3}$	0.9995	0.6–10.8	0.132	$1.80 \cdot 10^{-3}$	0.9991
SB					0.8–13.7	0.527	$4.30 \cdot 10^{-3}$	0.9992
AE					0.8–13.7	0.235	$1.75 \cdot 10^{-2}$	0.9993
FA					1.3–22.5	0.119	$1.00 \cdot 10^{-3}$	0.9994
CA					2.5–45.0	0.190	$1.48 \cdot 10^{-2}$	0.9995

3.5. Determination of marker substances in I-tzu-tang

When the test solution of I-tzu-tang was analysed by HPLC and CE under the selected conditions, the calculated contents of marker substances given in Table 3 were obtained. These data indicate that both of the proposed methods are relatively suitable for the determination of a large number of anthraquinone, flavone, carboxylic acid and saponin type compounds in a complicated system.

In conclusion, by optimizing the pH, buffer composition and buffer concentration of the eluent or carrier, eight components of I-tzu-tang extract could be determined by HPLC within 50 min and eleven of the twelve marker substances could be determined by CE within 14 min. Although the HPLC method had difficulty in separating FA, CA and SA owing to serious

Table 2
Reproducibility of separation of marker substances

Constituent	HPLC		CE	
	R.S.D. (%) ($n = 6$)		R.S.D. (%) ($n = 6$)	
	Retention time	Amount measured	Migration time	Amount measured
SA	0.08	0.92	1.54	1.82
BG	0.09	1.01	2.15	1.09
OG	0.11	1.10	1.31	1.45
WG	0.17	0.87	1.32	1.56
B	0.20	0.94		
W	0.24	0.84	2.45	1.14
GZ	0.40	0.95	2.08	0.94
E	0.45	1.20	2.51	1.73
SB			1.67	2.01
FA			1.95	1.56
AE			2.64	1.72
CA			2.45	1.24

Table 3
Contents of marker substances in commercial l-tzu-tang[a]
(mg/g)

Constituent	Sample I		Sample II		Sample III	
	HPLC	CE	HPLC	CE	HPLC	CE
SA	0.62	0.60	0.79	0.74	0.68	0.64
BG	5.23	5.54	8.06	8.21	7.14	7.48
OG	0.81	0.86	1.83	1.95	1.50	1.62
WG	1.04	1.10	2.24	2.24	2.13	2.25
B	0.76		1.15		0.98	
W	0.20	0.21	0.28	0.29	0.26	0.27
GZ	0.54	0.55	0.77	0.78	0.99	0.97
E	0.09	0.08	0.11	0.10	0.12	0.11
SB		0.47		0.57		0.55
AE		0.03		0.09		0.10
FA		0.05		0.07		0.07
CA		0.07		0.08		0.09

[a] Composition of l-tzu-tang (amount in grams) Cimicifugae
Rhizoma, 1.5; Bupleuri Radix, 5.0; Angelicae Radix, 6.0;
Glycyrrhizae Radix, 2.0; Scutellariae Radix, 3.0; and Rhei
Rhizoma, 1.0.

interference from some impurities and the CE
method failed to determine B owing to interfer-
ence from the components of rhubarb, the two
proposed methods showed good linear relation-
ships between the peak-area ratio and concen-
tration, acceptable reproducibilities and high
recoveries, and should therefore be useful for
the analysis of herbal preparations. Compared
with the HPLC method, the CE method is more
attractive, especially owing to its shorter running
time, and therefore should be useful for large
numbers of samples and for quality control in
pharmaceutical plants.

Acknowledgement

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