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Short communication

Using LC/MS/MS to determine matrine, oxymatrine, ferulic acid, mangiferin, and glycyrrhizin in the Chinese medicinal preparations Shiau-feng-saan and Dang-guei-nian-tong-tang

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Abstract

We have developed a simple, rapid, selective, and reproducible method for the quality control of traditional Chinese medicinal preparations. In this study, we used LC/MS/MS to simultaneously identify and quantify five marker compounds – matrine, oxymatrine, ferulic acid, mangiferin, and glycyrrhizin – in preparations of Shiau-feng-saan and Dang-guei-nian-tong-tang. The calibration curves for the five marker compounds were linear over the concentration range 50–2500 ng/mL ($R^2 > 0.9971$). The matrix effect was minimized and the recoveries of the five marker compounds were >90% at a concentration of 1 μg/mL. Our experimental data reveal that significant differences exist between samples obtained from different sources.

Keywords: Matrine; Oxymatrine; Ferulic acid; Mangiferin; Glycyrrhizin; Shiau-feng-saan; Dang-guei-nian-tong-tang; *Sophorae* Radix; *Angelicae sinensis* Radix; *Anemarrhena* Rhizoma; *Glycyrrhizae* Radix

1. Introduction

1.1. Chinese medicinal preparations

Chinese herbal medicines (CHMs) and Chinese proprietary medicines (CPMs) are used widely throughout the world. In recent years, decoction dosage forms have gradually been replaced by the concentrated dosage forms, which are adapted widely for clinical treatment. Quality control, however, of those concentrated forms is difficult because the materials are derived from many different sources. Hence, to improve the quality of CPMs, specific components have been selected as markers for the analysis of Chinese medicines; this approach is highly promising [1–3].

A number of LC/MS methods have been developed for the determination of one or two constituents in crude drugs [4–6]. There have been few reports, however, on the simultaneous determination of multiple constituents in preparations containing very complicated matrices. The LC/MS technique has attracted a great deal of attention because of the fact that it does not require sample derivatization and because it allows the simultaneous determination of non-volatile and thermally unstable compounds. Consequently, liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) has become the important technique for the analysis of Chinese herbal medicines.

In this study, we examined two concentrated preparation dosages: Shiau-feng-saan and Dang-guei-nian-tong-tang. They both contain the same four herbs – *Sophorae* Radix, *Angelicae sinensis* Radix, *Anemarrhenae* Rhizoma, and *Glycyrrhizae* Radix – and we selected five marker substances for their analysis: matrine, oxymatrine, ferulic acid, mangiferin, and glycyrrhizin. We used liquid chromatogra-

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phy coupled with an ion trap mass spectrometer to verify the presence of these five marker substances. Next, we examined the feasibility of applying this method to determine the five markers in real samples by analyzing commercial Chinese herbal formulations. Our long-term efforts are focused on developing methods for determining every representative marker of each herb in traditional Chinese medicines (TCMs).

1.2. Marker compounds in herbs

Fig. 1 presents the structures of the five marker compounds we chose for the four target herbs. Matrine and oxymatrine are the main active components of *Sophorae* Radix. Matrine displays antibacterial and antitumor activity; oxymatrine has been used as an efficient antitumor treatment [7–9]. These compounds are, however, toxic and may paralyze the respiratory system. The LD_{50} of matrine i.v. to mice is 72.1 mg/kg [10]. Ferulic acid, an aromatic acid, is one of the target compounds of *Angelicae sinensis* Radix. It is the bioactive compound that is used for its anti-inflammatory and anti-asthmatic effects [11]. Mangiferin (MA) is one of the main active compounds in *Anemarrhenae* Rhizoma. It is a natural glucosyl xanthone that is used to treat skin diseases (dermatosis). Recent studies have indicated that MA is an active agent for antitumor, anti-HIV, antiviral, and anticancer treatments [12,13]. It does, however, cause diarrhea when used in overdose amounts. Glycyrrhizin (GL), a triterpene saponin, and a principal active component of *Glycyrrhizae* Radix, has been used to cure Addison's disease, although it may induce heart disease after long-term use. It has also been used as an internal standard in LC/MS for the analysis of soy saponins [14]; other studies of GL through the use of LC/MS are rare. Saikosaponin-a, which we used as the internal standard in this study, is one of the major compounds found in the herbal medicine *Bupleuri* Radix.

2. Experimental

2.1. Materials and reagents

Matrine and oxymatrine were obtained from Chuang Song Zong Pharmaceutical Company (Pingtung, Taiwan). Mangiferin was purchased from Sigma (St. Louis, MO, USA). Ferulic acid was obtained from Aldrich (USA). Glycyrrhizin was purchased from Nacalai Tesque (Japan).

Shiau-feng-saan and Dang-guei-nian-tong-tang were purchased from the Chuang Song Zong Pharmaceutical Company (Ligang, Pingtung; samples S-01 and D-01), Sun Ten Pharmaceutical Company (Taichung; S-02 and D-02), and Sheng Foong Pharmaceutical Company (Taipei; S-03 and D-03) of Taiwan.

Shiau-feng-saan is used to cure eczema, urticaria, rubeola, and medica mentosa dermatitis. Shiau-feng-saan contains 13 herbs, including *Sophorae* Radix, *Angelicae sinensis* Radix, *Anemarrhena* Rhizoma, *Glycyrrhizae* Radix, *Soposhinkoviae* Radix, *Atractylodis* Rhizoma, *Akebiae* Caulis, *Gypsum* Fibrosum, *Arctii* Fructus, *Rehmanniae* Radixet Rhizom, *Cicadae* Periostracum, and *Schizonepetae* Herba.

Dang-guei-nian-tong-tang is used as a remedy for waist soft-tissue strain, rheumatic arthritis, sciatic neuralgia, and rheumatoid arthritis. Dang-guei-nian-tong-tang contains 15 herbs, including *Sophorae* Radix, *Angelicae sinensis* Radix, *Anemarrhena* Rhizoma, *Glycyrrhizae* Radix, *Artemisiae capillaris* Herb, *Notopterygii* Rhizoma, *Cimicifugae* Rhizoma, *Puerariae* Radix, *Atractylodis* Rhizoma, *Scutellariae* Radix, *Ginseng* Radix, *Polyporus*, *Alismatis* Rhizoma, and *Atractylodis* Rhizoma.

2.2. Preparation of standard solutions

Matrine, oxymatrine, ferulic acid, mangiferin, and glycyrrhizin were dissolved in 70% methanol and in matrix solution (in the extract of Dang-guei-nian-tong-tang) to provide a series of concentrations in the range 50–2500 ng/mL, respectively. The internal standard, saikosaponin-a, was added to each vial at a concentration of 500 ng/mL. Calibration curves were plotted after linear regression of the ratios of the peak areas of the analytes to those of the internal standard.

2.3. Extraction and preparation of samples

The powder form of the crude drug was accurately weighed (0.5 g), washed with *n*-hexane, and then a 10-fold mass of a methanol–water mixture (7:3, v/v) was added. The solutions were extracted for 30 min in an ultrasonic bath and then were centrifuged for 10 min at 5000 rpm. The supernatant was collected and 70% methanol was added up to a total of 10 mL to provide the real sample stock solution. This solution was diluted 20-fold. An appropriate amount of the internal standard was spiked. The sample solution for analysis was obtained after filtering through a 0.22-μm membrane filter.

2.4. Liquid chromatography conditions

LC analyses were performed using a Surveyor liquid chromatography system (Thermo Finnigan, San Jose, CA, USA). The five marker substances and the internal standard were separated on a Phenomenex 5-μm Luna C18 column (150 × 2.0 mm ID) using an injection volume of 5 μL. Gradient elution was achieved using two solvents – (A) 0.005% (v/v) trifluoroacetic acid buffer (pH 3) and (B) acetonitrile – at a flow rate of 200 μL/min. The linear gradient program that gave the optimal sensitivity was the following: a linear increase from 5% solution B to 10% over 1.5 min, increased to 40% solution B over 8.5 min, maintained isocratically for 5 min, increased to 50% solution B over 7 min, increased to 90% solution B over 5 min, increased to 100% B over 3 min, and then maintained at that level for a further 8 min.

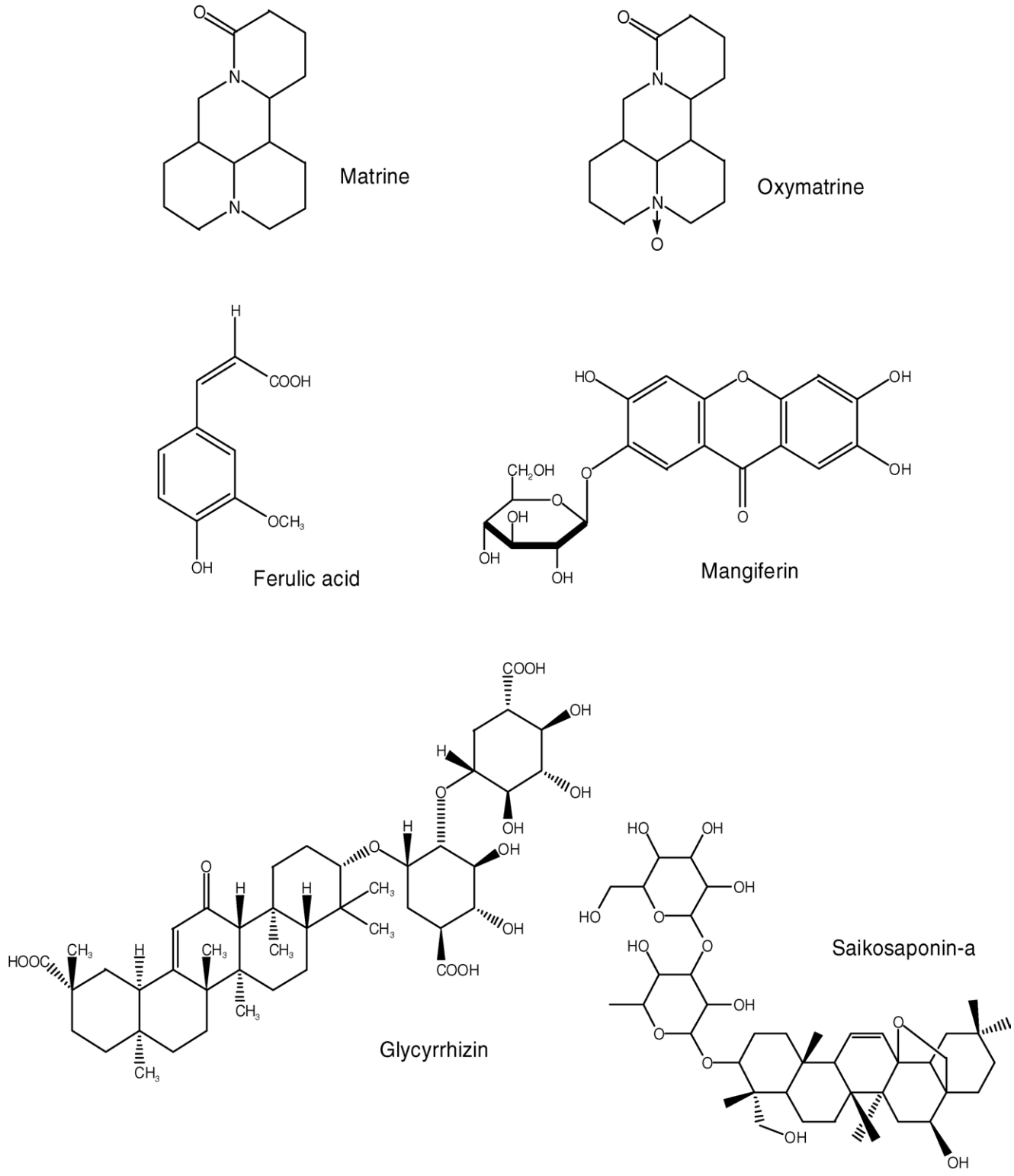

Fig. 1. Structures of the five marker compounds.

2.5. Optimized mass spectrometry conditions

Mass spectra were obtained using an electrospray ionization (ESI) source on a quadrupole ion trap instrument (Thermo Finnigan LCQ). For high sensitivity, the parameters of the electrospray were tuned to obtain the best ionization efficiency. The voltages of the first multipole offset, second multipole offset, inter-multipole offset, API heated capillary, and tube lens offset were autotuned using infusion injection. The sheath gas flow rate, auxiliary gas flow rate, spray volt-

age, heated capillary temperature, and injection time were tuned manually through flow injection analysis. The sheath gas flow rates were set from 1 to 1.5 L/min and the auxiliary gas flow rates were set between 3 and 7.5 L/min for the four different segments. The injection time was set at 250 ms and the capillary temperature at 230 °C for all of the targets. To obtain optimum sensitivity, four segments and a total of six scan events were performed in each run.

- Segment 1 (2.5–6.5 min for detecting matrine and oxymatrine), including scan event 1 (MS^2 scan, m/z 249 →) and scan event 2 (MS^2 scan 265 →).
- Segment 2 (6.5–14.5 min for detecting ferulic acid and mangiferin), including scan event 1 (MS^2 scan m/z 195 →) and scan event 2 (MS^2 scan m/z 423 →).
- Segment 3 (14.5–19 min for detecting the internal standard saikosaponin-a), one event (MS^2 scan m/z 803 →).
- Segment 4 (19–30 min for detecting glycyrrhizin), one event (MS^2 scan m/z 845 →).

3. Results and discussion

Mass spectrometry is used in many laboratories to elucidate the presence of marker compounds. The five target molecules in this study belong to different classes of compounds, including alkaloids, aromatic acids, quinones, and triterpene saponins. We found that the best conditions for ionizing all five markers in the mass spectrometer occurred when using the ESI (+) ionization mode and adding 0.005% trifluoroacetic acid.

In the positive-ion-mode ESI mass spectra of marker compounds, the protonated molecules were the most abundant ions for all of the markers, except for glycyrrhizin, for which the sodium adduct ion, $[M + Na]^+$ was the most abundant. From MS and MS/MS analyses, the mass spectra of matrine and oxymatrine, which are lupinane alkaloids, displayed protonated molecular ions ($[M + H]^+$) at m/z 249 and 265, respectively, and major daughter ions at m/z 148 and 247 $[M + H - H_2O]^+$, respectively, as reported by Wong [4]. Ferulic acid displayed its protonated molecular ion at m/z 195 ($[M + H]^+$) and lost one water ($[M + H - H_2O]^+$) to obtain its major daughter ion at m/z 177. The mass spectra of mangiferin, which is a glycosyl polyhydroxyquinone, displayed its protonated molecular ion at m/z 423 ($[M + H]^+$) and its sodium adduct molecular ion ($[M + Na]^+$) at a high capillary temperature. Because the protonated molecular ion was more stable in acidic solution at lower temperature and revealed an intense and stable daughter ion at m/z 405, which results from the loss of water from $[M + H]^+$. We chose this ion for quantitation. We did not observe the characteristic loss of a glucose moiety ($[M + H - Glc]^+$) from the precursor ion in the spectrum. Glycyrrhizin, which is a triterpene saponin, displayed its protonated molecular ion at m/z 823 ($[M + H]^+$) and sodium adduct ion at m/z 845 ($[M + Na]^+$); the sodium adduct ion was more stable under MS/MS fragmentation and provided a daughter ion at m/z 669 arising from the characteristic loss of a glucuronic acid group.

Trace analysis of a complex matrix is difficult especially for Chinese medicinal preparations when using a combination only of LC and UV spectroscopy. In such a case, it is preferable to employ the high selectivity of a mass spectrometer. In particular, the selected reaction mode (SRM) of MS/MS can reduce the background noise, and hence, improve the signal-to-noise ratio. The ability to improve the signal-to-noise ratio is very important when determining the analytes present in a complicated matrix.

In this study, we employed the SRM mode of MS/MS for the quantitative measurement of marker compounds in Chinese medicinal preparations. Saikosaponin-a was used as an internal standard. The precursor and product ions selected to monitor matrine, oxymatrine, ferulic acid, mangiferin, saikosaponin-a, and glycyrrhizin in MS/MS transitions were those at m/z 249 → 148, 265 → 247, 195 → 177, 423 → 405, 803 → 371, and 845 → 669, respectively. To demonstrate the reliability of our method, we evaluated the correlation coefficients, linear ranges, and detection limits by spiking these compounds into a 70% standard methanol solution and into the Dang-quei-nian-tong-tang samples. We obtained the linearity and limits of detection under the optimum conditions that we had established for the LC/MS/MS procedure; each experiment was performed in triplicate. The linear regression analyses of matrine, oxymatrine, ferulic acid, mangiferin, and glycyrrhizin displayed good linear relationships between the ratios of the peak areas of the analytes to the internal standard over the range of concentrations studied. The calibration graphs for matrine, oxymatrine, ferulic acid, mangiferin, and glycyrrhizin in 70% standard methanol solution and in matrix solution (in the extract of Dang-guei-nian-tong-tang) were linear over the ranges 50–1500, 50–1500, 50–1500, 50–2000, and 75–2500 ng/mL, respectively (Table 1). The squares of the linear correlation coefficients were all over 0.9971.

Table 1
Linear ranges, calibration curves, correlation coefficients (R^2), and detection limits for matrine, oxymatrine, ferulic acid, mangiferin, and glycyrrhizin, as obtained by LC/(+)ESI/MS/MS of Dang-guei-nian-tong-tang matrix solution

Sample	Linear range (ng/ml)	Calibration curve	R^2	LOD (S/N = 3) (ng/ml)
Matrine	50–1500	$y = 0.0044x + 0.4188$	0.9987	20
Oxymatrine	50–1500	$y = 0.0028x + 0.1373$	0.9987	20
Ferulic acid	50–1500	$y = 0.0023x + 0.0455$	0.9977	20
Mangiferin	50–2000	$y = 0.0026x + 0.054$	0.9975	20
Glycyrrhizin	75–2500	$y = 0.0009x + 0.3138$	0.9971	60

Table 2
Relative standard deviations and recoveries (%) of the five marker compounds*

Concentration (ng/ml)	Matrine		Oxymatrine		Ferulic acid		Mangiferin		Glycyrrhizin	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
100	88	12.3	85	14.5	89	10.8	81	14.3	73	15.7
500	93	3.5	92	3.8	92	5.1	93	4.3	89	10.2
1000	95	2.7	91	4.3	96	3.2	91	6.7	90	7.5

* $n = 5$.

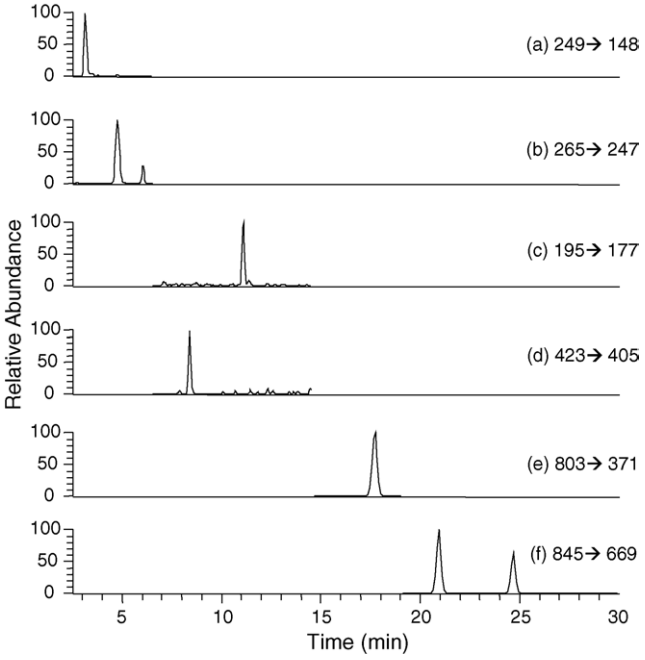

Fig. 2. SRM chromatograms of: (a) matrine, (b) oxymatrine (Rt = 4.8 min), (c) ferulic acid, (d) mangiferin, (e) saikosaponin-a (internal standard), and (f) glycyrrhizin (Rt = 20.8 min) produced by LC/(+)ESI/MS/MS from an extract of Dang-guei-nian-tong-tang sample D-02.

The limits of detection (LOD) evaluated were based on the lowest detectable peak having a signal-to-noise ratio of 3. Under the SRM experimental conditions, listed in Table 1, we found that the LODs were all 20 ng/mL, except that for glycyrrhizin, which was 60 ng/mL.

Fig. 2 displays the selected reaction monitoring chromatograms of five markers obtained from the extract of a sample of Dang-guei-nian-tong-tang (D-02). The peaks obtained for all of the analytes had good shapes and were separated well when with using the LC/(+)ESI/MS/MS technique. The five markers eluted at 3.3 (matrine), 4.8 (oxymatrine), 11.3 (ferulic acid), 8.6 (mangiferin), and 20.8 min (glycyrrhizin); saikosaponin-a (the internal standard) eluted after 17.7 min.

We pretreated the Dang-guei-nian-tong-tang sample using the method described above. The preparative matrix solution was spiked with three different amounts (concentrations of 100, 500, and 1000 ng/mL) of the five marker compounds and then the samples were injected into the LC/MS/MS for analysis. We express the reproducibility as the relative standard deviation (R.S.D.), which varied between 2.7 and 15.7%. We calculated the recoveries of the five marker compounds by comparing the peak areas obtained from the extract of the spiked Dang-quei-nian-tong-tang samples with those obtained by direct injection of the standard solution. All of the recoveries were >90% at a concentration of 1 μg/mL (Table 2).

We tested the effectiveness of this method for simultaneously determining the amounts of matrine, oxymatrin, ferulic acid, mangiferin, and glycyrrhizin in actual samples by analyzing samples of each preparation (Shiau-feng-saan: S-01, S-02, and S-03; Dang-guei-nian-tong-tang: D-01, D-02, and D-03) obtained from three different Pharmaceutical Companies. Three injections of each sample were performed to obtain the mean values and R.S.D.s; Table 3 presents the analytical data. Our results indicate that all five of the marker compounds were detectable in each of the samples we studied. Matrine was detected over the range from 103.7 to 503.6 μg/g; oxymatrine from 81.9 to 315.8 μg/g; ferulic acid from 79.6 to 217.9 μg/g; mangiferin from 77.4 to 113.8 μg/g;

Table 3
Concentrations (μg/g) of the five marker components in three different samples of Shiau-feng-saan and Dang-guei-nian-tong-tang*

	Matrine (R.S.D.%)	Oxymatrine (R.S.D.%)	Ferulic acid (R.S.D.%)	Mangiferin (R.S.D.%)	Glycyrrhizin (R.S.D.%)
Shiau-feng-saan					
S-01	503.6 (7.5)	315.8 (4.8)	217.9 (9.8)	113.8 (5.9)	109.4 (6.2)
S-02	349.1 (4.2)	167.5 (3.3)	109.4 (7.7)	107.3 (5.8)	91.3 (5.7)
S-03	198.5 (5.1)	84.6 (7.0)	80.8 (5.2)	97.4 (7.4)	75.2 (10.3)
Dang-guei-nian-tong-tang					
D-01	121.3 (3.8)	91.4 (6.9)	81.4 (6.1)	93.7 (6.3)	180.6 (8.1)
D-02	103.7 (4.9)	81.9 (7.5)	79.6 (5.5)	88.9 (6.8)	110.2 (5.2)
D-03	215.2 (3.3)	127.3 (3.2)	90.1 (7.2)	77.4 (10.5)	137.5 (4.8)

* Sample weight was 0.5 g. Triplet analysis.

glycyrrhizin from 75.2 to180.6 μg/g. These results obtained using real samples clearly indicate that our method is a very useful one for the practical determination of traces of marker compounds. Furthermore, our results indicate that there is a great variety in the amounts of each compound in the different samples. For example, the matrine and oxymatrine contents in sample S-01 were three times as large as those in sample S-03.

4. Conclusions

For Chinese medicines, the simultaneous determination of formative crude drugs is generally a difficult task because of the complexity of the matrix and differences in the formulations of the various specific components. In this paper, we have proposed a simple and efficient method that uses LC/MS/MS to detect five different herbal target components in the Chinese medicine preparations Shiau-feng-saan and Dang-guei-nian-tong-tang. This approach can be used to establish quality control standards for Chinese medicinal preparations. In comparison with traditional TLC and conventional HPLC methods, the LC/MS/MS technique exhibits a greater selectivity for determining each herb's marker component present in the preparation. In addition, from the study of contents of matrine and oxymatrine in various preparations of Shiau-feng-saan samples, we confirmed that significant differences exist between samples obtained from different sources.

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