

# Simultaneous determination of five marker compounds in Xuanfu Daizhe Tang by high-performance liquid chromatography coupled with diode array detection for quality control

Kunming Qin<sup>1,2</sup>, Bin Wang<sup>2</sup>, Hao Cai<sup>1</sup>, Weidong Li<sup>1</sup>, Zhongqing Yao<sup>2</sup>, Xingde Zhang<sup>1</sup>, Tulin Lu<sup>1</sup>, Baochang Cai<sup>1,2</sup>

<sup>1</sup>Engineering Research Center of State Ministry of Education for Standardization of Chinese Medicine Processing, Nanjing University of Chinese Medicine, Nanjing 210029, <sup>2</sup>Nanjing Haichang Chinese Medicine Group Corporation, Nanjing 210061, PR China

Submitted: 27-12-2011

Revised: 26-02-2012

Published: 22-11-2012

## ABSTRACT

**Background:** Xuanfu Daizhe Tang (XDT) is a classical traditional Chinese medicinal prescription that has been widely used for treating digestive system illnesses for hundreds of years. **Materials and Methods:** In this study, a simple and sensitive high-performance liquid chromatography coupled with diode array detection (HPLC-DAD) method was established for the simultaneous determination of five marker compounds in XDT including chlorogenic acid, glycyrrhizic acid, ginsenoside Rg1, ginsenoside Rb1 and ginsenoside Re, for quality control of this well-known traditional Chinese medicine (TCM). **Results:** These compounds were separated in less than 130 min using a YMC C18 column with a gradient elution system of acetonitrile and 0.1% phosphoric acid water solution at a flow rate of 1 ml/min. All calibration curves of standard components showed good linearity with  $R^2 > 0.9991$ . Limit of detection and limit of quantification varied from 0.11 to 4.3  $\mu\text{g/ml}$  and 0.20 to 11.6  $\mu\text{g/ml}$ , respectively. The relative standard deviations (RSDs) of the intra-day and inter-day experiments were less than 4.72 and 5.48%, respectively. The accuracy of recovery test ranged from 95.0 to 105.0% with RSD values 1.28- 4.32%. **Conclusion:** The validated method is simple, reliable, and successfully applied to determine the contents of the selected compounds in XDT for quality control.

**Key words:** High-performance liquid chromatography coupled with diode array detection, quality control, traditional Chinese medicine, Xuanfu Daizhe Tang

## INTRODUCTION

Traditional Chinese medicine (TCM) has been widely used because of its high effectiveness against many diseases with low toxicity.<sup>[1]</sup> TCM prescription is a formula of several single herbs combined at an intrinsic mass ratio. Combining the herbs together and boiled in water makes the decoction. Each herb has its own bioactivities, but when multiple herbs are combined and decocted, there maybe chemical changes of active components, resulting in new bioactivities for new clinical indications. Qualitative evaluation of TCM prescription is often challenging because the active compounds maybe originally from single herbs and also be resulted from the decocting process.<sup>[2]</sup>

**Address for correspondence:** Dr. Kunming Qin, Nanjing University of Chinese Medicine Mailbox8, 282, Hanzhong Road, Nanjing, Jiangsu, 210029 People's Republic of China Nanjing China. E-mail: qinkm123@yahoo.com.cn

In recent years, many analytical techniques have been developed for evaluating the quality of herbs or TCM prescriptions. These include determination of single compound or multiple constituents, as well as fingerprint analysis. Of these, single marker compound determination is simple, but it can not afford sufficient quantitative information for other active components in complex TCM.<sup>[3]</sup> Fingerprint analysis can evaluate the quality consistency and stability of herbal products, but cannot enable accurate quantification of analytes.<sup>[4-6]</sup> Many pure standards are required and suitable chromatographic conditions are difficult to optimize, but multi-constituent determination is widely used to control the quality of TCM<sup>[7-9]</sup> because of the advantage of simultaneous determination of many markers from different herbs for evaluation of total quality. In the process, technologies such as high-performance liquid chromatography (HPLC), high-performance capillary electrophoresis (HPCE), and

### Access this article online

Website:  
[www.phcog.com](http://www.phcog.com)

DOI:  
10.4103/0973-1296.103647

### Quick Response Code:



liquid chromatography–mass spectrometry (LC–MS) are often used.<sup>[10]</sup> HPLC is simple, sensitive and inexpensive, and has been widely used in the pharmaceutical field.

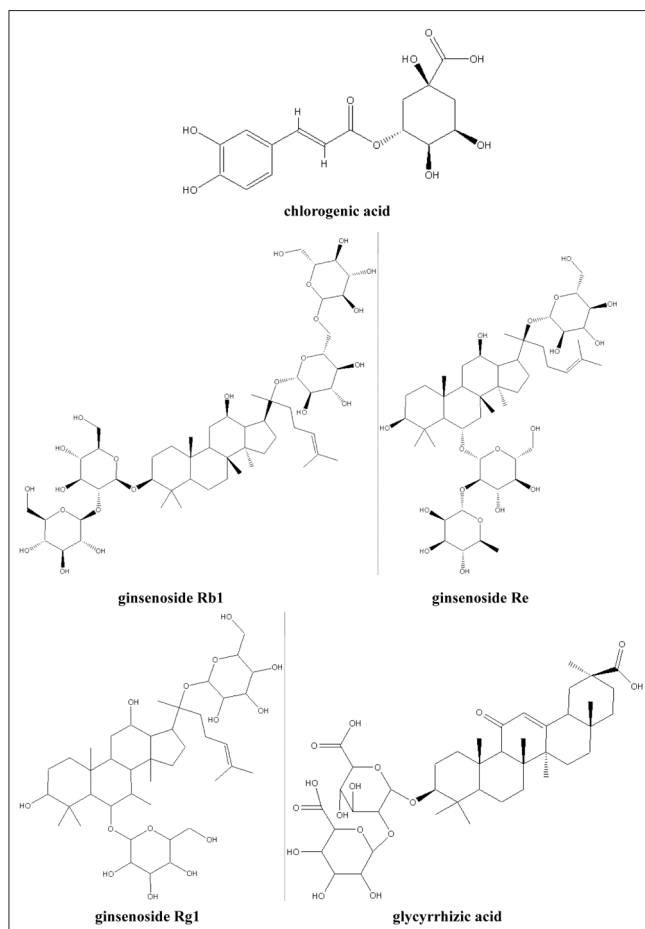
Xuanfu Daizhe Tang (XDT) is originally from the Shang Han Za Bing Lun, which is a famous clinical medical book on traditional Chinese medicine, written by Zhang Zhong Jing around 200BC. This famous formula has been widely used in China for the treatment of digestive system diseases, such as chronic gastritis, stomach neurosis, reflux esophagitis, and so on.<sup>[11–13]</sup> XDT is composed of seven medicinal herbs: *Inulae Flos* (*xuanfubua*); *Haematitum* (*daizheshi*); *Ginseng Radix Et Rhizoma* (*renshen*); *Glycyrrhizae Radix Et Rhizoma* (*gancao*); *Pinelliae Rhizoma Praeparatum* (*fabanxia*); *Jujubae Fructus* (*daqiao*) and *Zingiberis Rhizoma* (*shengjiang*). Chemical and pharmacological studies have shown that chlorogenic acid from *Inulae Flos*,<sup>[14]</sup> ginsenosides including ginsenoside Rg1, ginsenoside Rb1 and ginsenoside Re from *Ginseng Radix Et Rhizoma*,<sup>[15,16]</sup> and glycyrrhizic acid from *Glycyrrhizae Radix Et Rhizoma*,<sup>[17,18]</sup> are considered to be the active compounds in XDT. These components are usually regarded as the markers of quality control and evaluation only by consideration of their actions, contents and suitable UV absorptions. However, the simultaneous determination of multiple constituents in XDT for quality control has not been reported.

In this study, a convenient, reliable and sensitive HPLC method for simultaneous determination of chlorogenic acid, glycyrrhizic acid, ginsenoside Rg1, ginsenoside Rb1 and ginsenoside Re [Figure 1] in XDT was developed. This is the first report for the simultaneous determination of the 5 compounds in XDT.

## EXPERIMENTAL

### Reagents and Materials

All the five standard compounds, chlorogenic acid, ammonium glycyrrhizinate, ginsenoside Rg1, ginsenoside Rb1 and ginsenoside Re, were purchased from Chinese National Institute of Control of Pharmaceutical and Biological Products (Beijing, China). The batch numbers were 110753, 110731, 110703, 110724 and 110754, respectively. The purity of all five marker constituents was more than 98%. All the medicinal herbs were purchased from Nanjing Haiyuan Chinese Prepared Slices Co. Ltd (Jiangsu, China) and authenticated by Dr. Jianwei Chen (Nanjing University of Chinese Medicine, Nanjing, China). Acetonitrile was of HPLC grade (Tedia Company Inc., Fairfield, USA). Phosphoric acid and other reagents were of analytical grade and purchased from Nanjing Wanqing Chemical Factory (Jiangsu, China). Reverse osmosis Milli-Q water (18M; Millipore, USA) was used for all solutions and dilutions.



**Figure 1:** The chemical structures of the tested components in Xuanfu Daizhe Tang

### Instrument and chromatographic conditions

Analysis was performed on the Shimadzu LC-20 system (Shimadzu, Kyoto, Japan) equipped with a pump (LC-20AD), auto sampler (SIL-20A), and column oven and diode array detector (SPD-M20A). The output signal of the detector was recorded using LC Solution software. The separation was executed on a YMC-Pack ODS-A C18 (250 mm×4.6 mm, 5 μm). The mobile phase was composed of acetonitrile (A) and 0.1% phosphoric acid water solution (B) with gradient elution system (0–10 min, 5%–10% A; 10–28 min, 10–15% A; 28–58 min, 15–18% A; 58–75 min, 18%–25% A; 75–125 min, 25%–50% A; 125–130 min, 50%–5% A) at a flow rate of 1.0 ml/min. The injection volume was 10 μl. The detection UV wavelength was set at 203nm. The column temperature was maintained at 35°C.

### Preparation of standard solutions

Each standard stock solution was prepared by dissolving each marker components in methanol at a concentration of 1 mg/ml. They were then diluted to five concentrations for construction of calibration plots in the ranges of 39.1–391

(chlorogenic acid), 25.6–256.5 (glycyrrhizic acid), 51.3–513 (ginsenoside Rg1), 66–660 (ginsenoside Rb1), 51.5–515 (ginsenoside Re)  $\mu\text{g}/\text{ml}$ . Further dilution with the lowest concentrations in the calibration curves were carried out to afford a series of standard solutions for evaluating the limits of detection (LOD) and the limits of quantity (LOQ) of the compounds. The stock and working solutions were stored at 4°C.

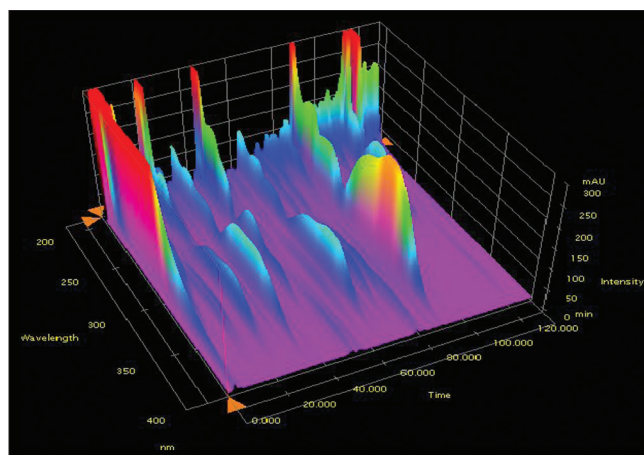
### Preparation of sample solutions

After drying, both herbs (containing *Inulae Flos* 9g; *Haematitum* 9g; *Ginseng Radix Et Rhizoma* 6g; *Glycyrrhizae Radix Et Rhizoma* 6g; *Pinelliae Rhizoma Praeparatum* 9g; *Jujubae Fructus* 10g and *Zingiberis Rhizoma* 10g) were mixed together in distilled water (ranging from 360ml to 840ml) and soaked for a few time (ranging from 0 min to 60 min), then decocted by boiling for a few time (ranging from 30 min to 150 min). The operation was repeated ranging from 1 to 3 times with different volume of water, respectively. The extracts were combined and added to the same volume with water. An aliquot of 100 ml of the extract was concentrated to dryness by rotary vaporization at 80°C under reduced pressure. The residues were extracted with 80% methanol for 0.5h under ultrasonic condition, and then the extract was transferred into a 10 mL volumetric flask with 80% methanol. After centrifugation at 12,000 rpm for 10 min, an aliquot of 10  $\mu\text{l}$  sample solution was injected into the HPLC system.

## RESULTS AND DISCUSSION

### Optimization of HPLC conditions

In general, a suitable chromatographic column, mobile phase, elution mode and detection wavelength are critically important for good separation. In the present study, different columns, different mobile phases and elution modes were tested. The columns Kromasil C 18 (250 mm $\times$ 4.6 mm, 5  $\mu\text{m}$ ), Lichrospher C 18 (250 mm $\times$ 4.6 mm, 5  $\mu\text{m}$ ), Zorbax SB C 18 (250 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ ), Hypersil C 18 (150 mm  $\times$  4.6mm, 5  $\mu\text{m}$ ), Lichrosorb C 18 (150 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ ) and YMC-Pack ODS-A C18 (250 mm  $\times$  4.6 mm, 5 mm) were employed. Different mobile phases consisting of acetonitrile–water and methanol–water with some modifiers including acetic acid, formic acid and phosphoric acid with different pH values were investigated under different gradient elution modes. The detection wavelength was selected according to the maximum adsorption wavelengths of chlorogenic acid, glycyrrhizic acid, ginsenoside Rg1, ginsenoside Rb1 and ginsenoside Re at 244, 254, 203, 205 and 205nm, respectively, shown in UV spectra with three dimension chromatograms of DAD [Figure 2]. The flow rate was also optimized. After many tests, excellent separations were achieved and the chromatograms are shown in Figure 3, in which chromatograms A and B correspond to mixed



**Figure 2:** Three dimensional chromatogram of Xuanfu Daizhe Tang

standards and XDT. The peaks 1–5 represent chlorogenic acid, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1 and glycyrrhizic acid, respectively.

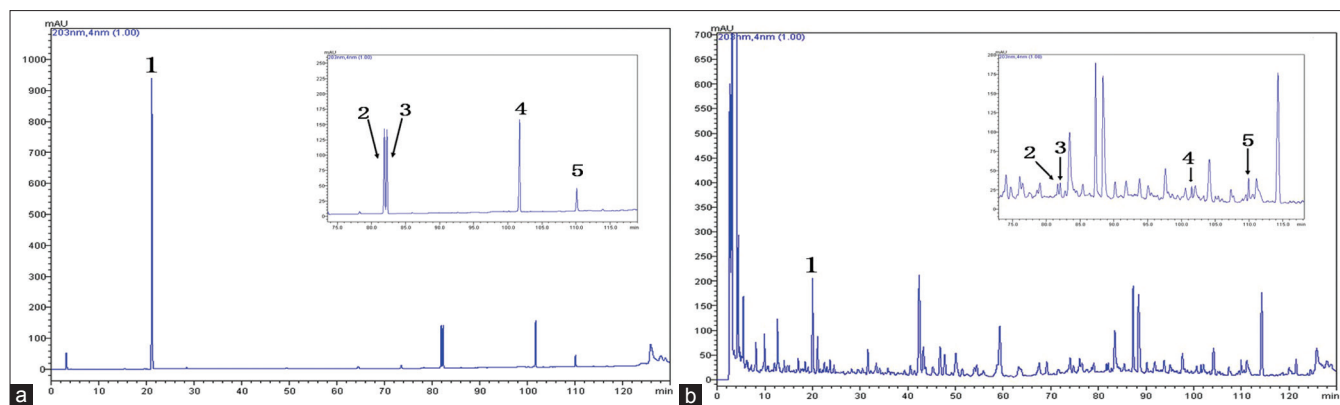
### Optimization of extraction conditions

Boiling is often used to extract the components from traditional Chinese medicinal formulas. In the extraction process, soaking time, extraction times, sample–solvent ratio and extraction time are critical for high extraction efficiency. In the present study, different soaking time (0 min, 15 min, 30 min, 45 min and 60 min) and extraction time (30 min, 60 min, 90 min, 120 min and 150 min) were examined to extract the targets from XDT. The results shown in Figure 4a and b indicated that the extraction values of most targets gradually increased with increase of the soaking time and extraction time when the soaking time was <30 min and the extraction time was <60 min. Long soaking time and extraction time did not benefit efficient extraction. Soaking for 30 min and extraction for 60 min had better extraction values. Thus, soaking for 30 min and extraction for 60 min were selected as the extraction parameters. Second, a suitable sample–solvent ratio was investigated and five ratios (1:6, 1:8, 1:10, 1:12, 1:14, w/v) were tested. The sample–solvent ratio controlled at 1:10 was the best [Figure 4c]. Different extraction times (1, 2 and 3) were also optimized; the extraction times controlled at 3 was better [Figure 4d].

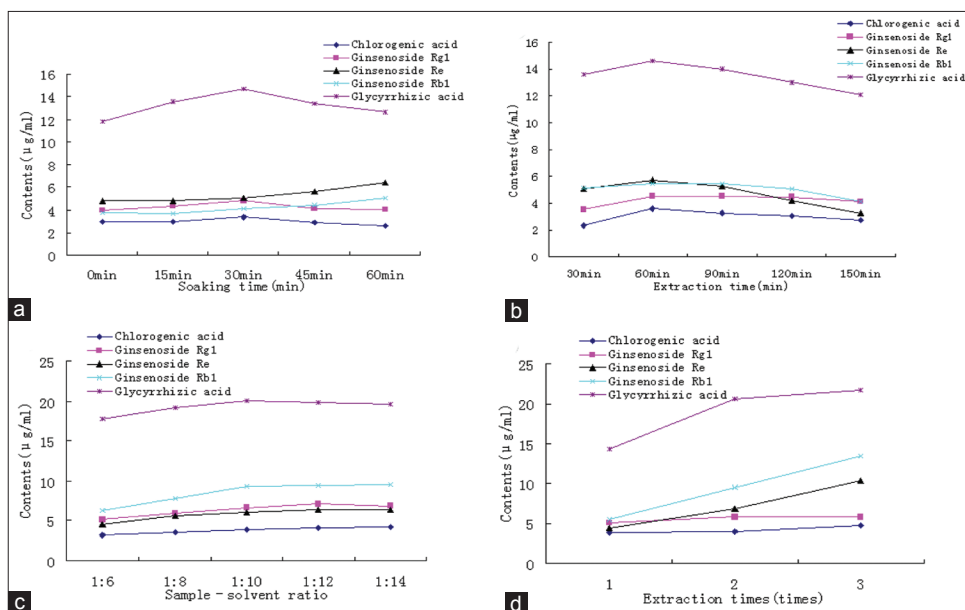
### Method validation

#### Specificity

Specificity was confirmed by the purity of peaks detected by the diode array detector. The absorption spectrum of a single component remained little variable at each time point in one peak, which supported the specificity of each peak. Our results clearly showed the specificity of each peak for five marker compounds by comparing the retention times with the standards were noted.



**Figure 3:** Typical chromatograms for determination of 5 active compounds in Xuanfu Daizhe Tang. (a) mixed standards; (b) sample solution; Peak 1: chlorogenic acid, 2: ginsenoside Rg1, 3: ginsenoside Re, 4: ginsenoside Rb1, 5: glycyrrhizic acid



**Figure 4:** The results of optimization suitable extraction conditions. (a) the influence of different soaking times; (b) the influence of different extraction time; (c) the influence of different sample-solvent ratio; (d) the influence of different extraction times

### Calibration curves, limits of detection and limits of quantity

The calibration curves were plotted with a series of concentrations of standard solutions. The regression equations were calculated in the form of  $Y = aX + b$ , where X and Y are the concentration of the standard solution (µg/ml) and the corresponding peak area, and a and b are the slope and the intercept, respectively. Good calibration curves of chlorogenic acid, glycyrrhizic acid, ginsenoside Rg1, ginsenoside Rb1 and ginsenoside Re were obtained. High correlation coefficient values ( $R^2 > 0.9991$ ) showed good linearity at a relatively wide range of concentration. LOD and LOQ expressed by 3- and 10- fold of the ratio of the signal-to-noise (S/N) were also acquired. LOD and LOQ of five marker compounds were within a range of 0.115 - 4.3 µg/ml and 0.201 - 11.6 µg/ml, respectively, which showed a high sensitivity at this chromatographic condition. Detailed

information regarding calibration curves, linear ranges, LOD and LOQ are listed in Table 1.

### Precision

Instrument precision was evaluated by carrying out intra- and inter-day assays. Intra-day precision was validated with three concentrations of mixed standard solutions under the optimized conditions for five times in 1 day. Inter-day precision was validated with the mixed standard solutions used above for once a day on 5 consecutive days. Inter- and intra- day precisions for all investigated components expressed as relative standard deviation (RSD) were 1.59 - 5.48% and 1.05 - 4.72%, respectively. These results indicated that this method exerted good precision [Table 2].

### Repeatability and stability

Six independent sample solutions of XDT in parallel were prepared and analyzed for evaluation of repeatability. RSD

**Table 1: Linear relationships between peak area and sample concentration**

Compounds	Regression equation (Y=aX+b)	R <sup>2</sup>	Linear range (µg /mL)	LOD (µg/mL)	LOQ (µg/mL)
Chlorogenic acid	Y=1 000 000X+21 600	0.999 8	39.1~391	0.115	0.201
Ginsenoside Rg1	Y=283 788X+9 604.2	0.999 9	51.3~513	2.5	4.7
Ginsenoside Re	Y=275 188X+869.28	0.999 9	51.5~515	3.1	7.2
Ginsenoside Rb1	Y=225 878X+8 560.5	0.999 9	66.0~660	1.9	5.7
Glycyrrhizic acid	Y=753 880X-64 440	0.999 1	25.6~256.5	4.3	11.6

**Table 2: Analytical results of intra- and inter-day test**

Compounds	Concentration (µg /ml)	Intra-day (n=5)		Inter-day (n=5)	
		Mean (µg /ml)	RSD(%)	Mean (µg /ml)	RSD(%)
Chlorogenic acid	312.8	314.55	1.05	311.98	1.59
	78.2	79.43	3.32	77.95	3.83
	39.1	39.92	3.98	38.67	4.26
Ginsenoside Rg1	410.4	412.22	1.83	410.69	2.75
	102.6	105.31	2.09	103.85	2.14
	51.3	52.29	3.55	50.51	3.17
Ginsenoside Re	412	413.01	2.17	411.82	2.93
	103	104.19	2.59	102.63	3.49
	51.5	52.9	3.46	50.38	4.12
Ginsenoside Rb1	528	529.73	1.93	525.69	2.63
	132	133.85	1.45	130.14	2.79
	66.0	67.50	3.76	65.07	4.35
Glycyrrhizic acid	204.8	205.67	2.55	201.33	3.42
	51.2	53.56	3.19	53.73	3.55
	25.6	26.09	4.72	24.91	5.48

**Table 3: Analytical results of recovery test**

Compounds	Added amount (µg)	Measured amount (µg)	RSD (%)	Recovery (%)
Chlorogenic acid	273.7	271.8	1.28	99.37
	234.6	239.3	2.93	102.15
	195.5	193.1	3.96	98.76
Ginsenoside Rg1	1128.6	1173.7	2.69	104.42
	1026	1056.8	2.35	103.35
	923.4	932.6	3.48	101.13
Ginsenoside Re	1390.5	1383.5	1.97	99.52
	1287.5	1226.9	2.11	95.39
	1184.5	1185.1	3.42	100.48
Ginsenoside Rb1	1254	1237.7	1.79	98.71
	1056	1077.1	2.73	102.34
	858	892.3	4.18	104.15
Glycyrrhizic acid	1282.5	1294.0	2.71	100.91
	1154.2	1211.9	3.02	105.33
	1026	1056.8	4.32	103.28

of retention times and peak areas for the 5 compounds were between 0.13% and 1.47%, and 3.08% and 4.90%, respectively. Stability was also tested at room temperature, and samples were analyzed in triplicate every 8h within 48h RSD values were not more than 4.55% for all components.

### Recovery test

Three quantities (low, medium and high) of the authentic standards were added to the known XDT sample. Resultants were extracted and analyzed. The quantity of each compound was realized from the corresponding

calibration curve. Average recoveries of investigated targets ranged from 95.0 to 105.0%, and RSD values were all <5% (n=3). It was clear that the developed method was reliable and accurate for the measurement [Table 3].

### Sample analysis

The developed method was used to determine the compounds in XDT (3 batches). Contents of the 5 components in the samples are listed in Table 4. Of these, ginsenoside Re was the main component (>25 µg/ml) in XDT. The second was glycyrrhizic acid (>23 µg/ml). The

**Table 4: Determination of the 5 marker components in Xuanfu Daizhe Tang by the developed high-performance liquid chromatography method**

No.	Contents (µg /ml)				
	Chlorogenic acid	Ginsenoside Rg1	Ginsenoside Re	Ginsenoside Rb1	Glycyrrhizic acid
Batch 1	4.71	22.30	25.31	22.23	23.79
Batch 2	4.60	21.95	25.50	22.12	24.94
Batch 3	4.63	22.18	25.62	21.18	24.11

contents of these components maybe considered for quality control of XDT. The quality evaluation regarding XDT was that the main 5 compounds could be detected, and the contents of chlorogenic acid, glycyrrhizic acid, ginsenoside Rg1, ginsenoside Rb1 and ginsenoside Re were >4.0, 18.0, 20.0, 18.0 and 20.0µg /ml, respectively. Our HPLC system maybe used as a tool to evaluate the quality of natural products.

## CONCLUSION

Increasing numbers of traditional Chinese medicines are being used worldwide. Efficient protocols to evaluate and control the quality of herbal products are urgently needed. This is the first report for simultaneous determination of the 5 marker compounds in XDT. The established HPLC method has the advantages of simplicity, precision, accuracy and sensitivity, and is suitable to control the quality of XDT. Therefore, the results suggest that this analysis method can be successfully applied for the quantification of marker compounds in XDT for quality control.

## ACKNOWLEDGEMENTS

This research was partially supported by Jiangsu Province Natural Science Foundation (BK2011135), Jiangsu Province Science and technology Achievements Transformation Project (BA2010024) and public welfare industry research project of State Administration of Traditional Chinese Medicine (201007010).

## REFERENCES

- Jia W, Gao WY, Yan YQ, Wang J, Xu ZH, Zheng WJ, et al. The rediscovery of ancient Chinese herbal formulas. *Phytother Res* 2004;18:681-6.
- Hayashi K, Shimura K, Makino T, Mizukami H. Comparison of the contents of kampo decoctions containing ephedra herb when prepared simply or by re-boiling according to the traditional theory. *J Nat Med* 2010;64:70-4.
- Tian S, Shi Y, Yu Q, Upur H. Determination of oleanolic acid and ursolic acid contents in *Ziziphora clinopodioides* Lam. by HPLC method. *Pharmacogn Mag* 2010;6:116-9.
- Wang L, Yuan K, Yu W. Studies of UPLC fingerprint for the identification of *Magnoliae officinalis* cortex processed. *Pharmacogn Mag* 2010;6:83-8.
- Fan XH, Cheng YY, Ye ZL, Lin RC, Qian ZZ. Multiple chromatographic fingerprinting and its application to the quality control of herbal medicines. *Anal Chim Acta* 2006;555:217-24.
- Zhong XK, Li DC, Jiang JG. Identification and quality control of Chinese medicine based on the fingerprint techniques. *Curr Med Chem* 2009;16:3064-75.
- El Sohafy SM, Metwalli AM, Harraz FM, Omar AA. Quantification of flavonoids of *Psidium guajava* L. preparations by Planar Chromatography (HPTLC). *Pharmacogn Mag* 2009;5:61-6.
- Chen J, Wang GY, Shi YP. Method development and validation for simultaneous HPLC analysis of six active components of the Chinese medicine Qin-Bao-Hong antitussive tablet. *Acta Chromatogr* 2009;21:341-54.
- Weon JB, Yang HJ, Ma JY, Ma CJ. A HPLC-DAD method for the simultaneous determination of five marker components in the traditional herbal medicine Bangpungtongsung-san. *Pharmacogn Mag* 2011;7:60-4.
- Qin KM, Cai H, Zhang L, Shi Y, Li P, Cai BC. Chemical constituents and effective substances of traditional Chinese medicinal formula. *Prog Chem* 2010;22:2436-49.
- Yang YX, Yuan HX, Han SP, Ma Y, Cao LX, Wang ZG. Effects of Xuanfu Daizhe Decoction and its decomposed prescription on COX 2 expression in rats of mixed reflux esophagitis. *Shanghai J Tradit Chin Med* 2011;45:63-5.
- Shui DK, Xie S. Effect of Xuanfudaizhe decoction on 5-HT APUD cells in gastric antrum tissues of low gastric motility rats. *Chin J Integr Trad West Med Dig* 2010;18:312-5.
- Shi SH, Lin LL, Tao CH, Shi T, Wang XN, Wei XM. Effects of xuanfu daizhe decoction on contraction induced and the pathways of signal transduction in gastric smooth muscle cells in rats. *Chin J Integr Trad West Med Dig* 2010;18:43-6.
- Wen DW, Liu YP, Li W, Liu HW. Separation methods for antibacterial and antirheumatism agents in plant medicines. *J Chromatogr B* 2004;812:101-17.
- Chung IM, Kim JW, Seguin P, Jun YM, Kim SH. Ginsenosides and phenolics in fresh and processed Korean ginseng (*Panax ginseng* C.A. Meyer): Effects of cultivation location, year, and storage period. *Food Chem* 2012;130:73-83.
- King ML, Murphy LL. Role of cyclin inhibitor protein p21 in the inhibition of HCT116 human colon cancer cell proliferation by American ginseng (*Panax quinquefolius*) and its constituents. *Phytomedicine* 2010;17:261-8.
- Hennell JR, Lee S, Khoo CS, Gray MJ, Bensoussan A. The determination of glycyrrhizic acid in *Glycyrrhiza uralensis* Fisch. ex DC. (ZhiGanCao) root and the dried aqueous extract by LC-DAD. *J Pharm Biomed Anal* 2008;47:494-500.
- Arjumand W, Sultana S. Glycyrrhizic acid: A phytochemical with a protective role against cisplatin-induced genotoxicity and nephrotoxicity. *Life Sci* 2011;89:422-9.

**Cite this article as:** Qin K, Wang B, Cai H, Li W, Yao Z, Zhang X, et al. Simultaneous determination of five marker compounds in Xuanfu Daizhe Tang by high-performance liquid chromatography coupled with diode array detection for quality control. *Phcog Mag* 2012;8:250-5.

**Source of Support:** Research was partially supported by Jiangsu Province Natural Science Foundation (BK2011135), Jiangsu Province Science and technology Achievements Transformation Project (BA2010024) and public welfare industry research project of State Administration of Traditional Chinese Medicine (201007010), **Conflict of Interest:** None declared.