

## Determination of Liquiritin, Naringin, Hesperidin, Thymol, Imperatorin, Honokiol, Isoimperatorin, and Magnolol in the Traditional Chinese Medicinal Preparation Huoxiang-zhengqi Liquid Using High-performance Liquid Chromatography

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High-performance liquid chromatography was employed to determine the contents of the eight marker components liquiritin, naringin, hesperidin, thymol, imperatorin, honokiol, isoimperatorin, and magnolol in the traditional Chinese medicinal preparation Huoxiang-zhengqi liquid. The separation was performed on a C<sub>18</sub> column by stepwise gradient elution with water-methanol-acetonitrile (0.01 min, 68 : 30 : 2; 20 min, 60 : 38 : 2; 50 min, 34 : 64 : 2; 65 min, 34 : 64 : 2; 75 min, 28 : 70 : 2; 85 min, 68 : 30 : 2) as the mobile phase at a flow rate of 1 ml/min, with UV detection at 283 nm. Eight regression equations showed good linear relationships between the peak area ratio of each marker to internal standard and amounts. The recoveries of the markers listed above were 97.4, 98.5, 97.4, 98.6, 97.8, 99.2, 97.0, and 97.5 %, respectively. The repeatability and reproducibility (relative standard deviation) of the method were less than 2.2 and 3.0%, respectively.

**Key words**—Huoxiang-zhengqi liquid; hesperidin; imperatorin; honokiol; isoimperatorin; magnolol

### INTRODUCTION

Traditional Chinese medicinal (TCM) prescriptions have been used for more than 1000 years. Most are composed of many herbs that contain complicated chemical constituents. Because of the complexity and interference, the effectiveness and safety of TCM remains to be established. Appropriate methods for quality control are also needed.

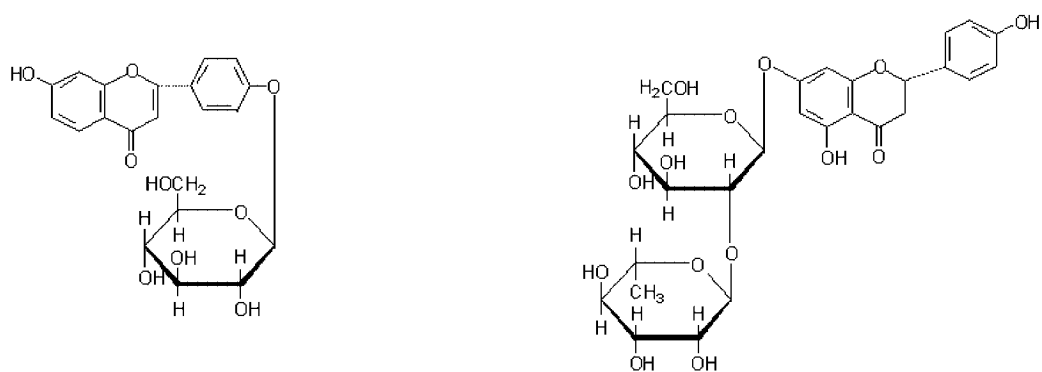
Huoxiang-zhengqi liquid,<sup>1)</sup> which was recorded originally in Formularies of the Bureau of People's Welfare Pharmacies (Tai Ping Huiming He Ji Ju Fang) in the Song Dynasty of China, consists of 10 common crude drugs and contains flavonoids, phenolics, coumarins, triterpenes, volatile oil, alkaloids, etc. *Pericarpium Citri Reticulatae* and *Cortex Magnoliae Officinalis* are the main crude drugs in the formula<sup>2)</sup> and it contains the chemical constituents of naringin, hesperidin, thymol, honokiol, and magnolol. The formula is used for treating colds, enteritis, ringworm, and vomiting diseases and inhibits bacterial growth, relieves pain and spasm and decreases vomiting frequency and incidence in phar-

macologic experiments.<sup>3)</sup> Although many high-performance liquid chromatographic (HPLC) methods have been developed for the determination of one or two constituents in crude drugs or preparations,<sup>4–6)</sup> there have been few reports on the simultaneous determination of multiple constituents in preparations. To promote Good Manufacturing Practice (GMP) of Chinese medicinal prescriptions and establish rapid and simple HPLC methods for routine quantitative analysis, we have tried to develop a method to assay multiple constituents in this preparation simultaneously. Among them, the eight marker components liquiritin (present in *Extractum Glycyrrhizae*), naringin, hesperidin, and thymol (in *Pericarpium Citri Reticulatae*), imperatorin and isoimperatorin (in *Radix Angelicae Dahuricae*), and honokiol and magnolol (in *Cortex Magnoliae Officinalis*) were selected for analysis<sup>7–10)</sup> (Fig. 1). An HPLC method was developed for the simultaneous determination of the contents of the eight markers using water-methanol-acetonitrile as the eluant, and the method was validated.

### EXPERIMENTAL

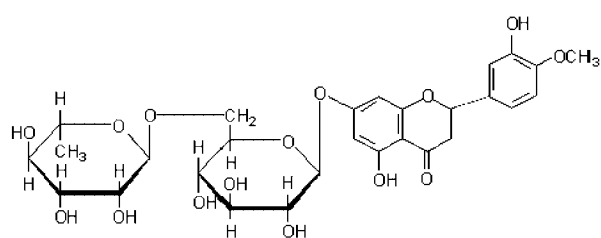
**Materials and Reagents** Huoxiang-zhengqi liq-

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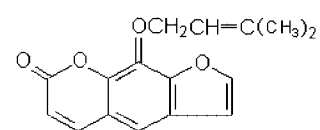


Liquiritin

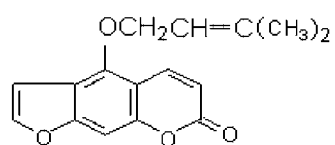
Naringin



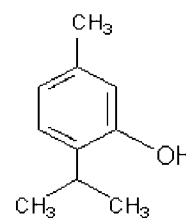
Hesperidin



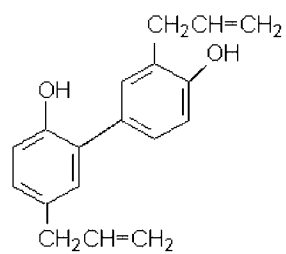
Imperatorin



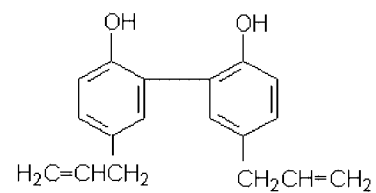
Isoimperatorin



Thymol



Honokiol



Magnolol

Fig. 1. Structures of Marker Components

uid (lot nos. 4144658, 4144670, 5141152, Tongrentong Technology Development Pharmaceutical Co., Ltd., Beijing) were purchased at NEP-Star Drugstore, Shenzhen, China. Huoxiang-zhengqi liquid (lot nos. 0504013, 0504020, 0507059, Huiren Pharmaceutical Co., Ltd., Jiangxi) were purchased at Accord Pharmacies, Shenzhen, China. Liquiritin, naringin, hesperidin, thymol, imperatorin, honokiol, isoimperatorin, magnolol, and methyl hesperidin (internal standard) were all ordered from National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Methanol and acetonitrile were of chromatographic grade. Water was doubly distilled.

**Chromatographic System** An Agilent 1100LC composed of a 4 quaternary gradient system, high-speed autosampler, column oven, and UV detector was used with an Agilent 1100 chemical station. The 250×4.6 mm *i.d.* column had Zorbax C<sub>18</sub> (5 μm particle size) as the stationary phase, and the 50×4.6 mm *i.d.* guard column had Shim-pack VP-C<sub>18</sub> (5-μm particle size) as the stationary phase. The mobile phase was a stepwise gradient of water-methanol-acetonitrile (0.01 min, 68 : 30 : 2; 20 min, 60 : 38 : 2; 50 min, 34 : 64 : 2; 65 min, 34 : 64 : 2; 75 min, 28 : 70 : 2; 85 min, 68 : 30 : 2). The analysis were carried out at a flow rate of 1 ml/min with UV detection at 283 nm. The operation temperature was 27°C.

**Preparation of Standard Solutions** To prepare a standard solution containing liquiritin, naringin, hesperidin, thymol, imperatorin, honokiol, isoimperatorin, and magnolol, accurately weighed amounts of each compound were dissolved in methanol to give serial amounts with the ranges 0.198–0.858, 0.026–0.208, 4.200–9.600, 0.060–18.000, 0.358–0.780, 0.782–5.980, 0.098–0.588, and 0.820–2.050 μg, respectively. Calibration graphs were plotted after linear regression of the peak area ratio of each marker to internal standard methyl hesperidin (1.05 mg/ml) with amounts of each marker.

**Preparation of Sample Solutions** About 10 ml of Huoxiang-zhengqi liquid was removed with reduced-pressure evaporation. The residue was dissolved with methanol in a 10-ml volumetric flask. One milliliter of sample solutions and 0.2 ml of internal standard were mixed before injection. All samples were filtered through a 0.45 μm Millipore filter and 10 μl was injected for HPLC analysis.

**Interference Test** An appropriate amount of

crude drugs of Huoxiang-zhengqi liquid with one crude drug (*Cortex Magnoliae Officinalis*, *Pericarpium Citri Reticulatae*, *Extractum Glycyrrhizae*, *Radix Angelicae Dahuricae*) subtracted each time was weighed. *Rhizoma Atractylodis*, *Cortex Magnoliae Officinalis*, *Pericarpium Citri Reticulatae*, and *Radix Angelicae Dahuricae* were dipped in a 10-fold amount of 60% ethanol solution at room temperature for 24 h and then filtered. *Poria* was dipped in a 10-fold amount of water at 80°C for 3 h after boiling. *Pericarpium Arecae*, *Rhizoma Pinelliae* (processed with ginger) and *Extractum Glycyrrhizae* were boiled with a 10-fold amount of water for 3 h. The above extractions were repeated twice. *Oleum Pogostemonis* and *Oleum Perillae* were dissolved with 60% ethanol. The above extraction solvents were combined and concentrated with reduced-pressure evaporation. Ethanol and water were removed, and the residue was dissolved in methanol. All samples were filtered through a 0.45-μm Millipore filter and used for analysis.

**Recovery Tests** An appropriate amount of crude drugs of Huoxiang-zhengqi liquid was weighed accurately and extracted as above. The filtrate was divided into four portions (one as a control group), and each portion was spiked with different microliter volumes of standard solution to add various amounts of liquiritin (0.198, 0.264, 0.396 μg), naringin (0.026, 0.052, 0.078 μg), hesperidin (1.200, 2.400, 3.600 μg), thymol (3.000, 6.000, 12.000 μg), imperatorin (0.130, 0.260, 0.390 μg), honokiol (0.460, 0.920, 1.380 μg), isoimperatorin (0.098, 0.196, 0.294 μg), and magnolol (0.410, 0.820, 1.230 μg). Specific amounts of internal standard were spiked into these portions. All samples were filtered through a 0.45-μm Millipore filter and injected for HPLC analysis to calculate the recovery rates.

## RESULTS AND DISCUSSION

Calibration graphs for liquiritin, naringin, hesperidin, thymol, imperatorin, honokiol, isoimperatorin, and magnolol were obtained over the ranges 0.198–0.858, 0.026–0.208, 4.200–9.600, 0.060–18.000, 0.358–0.780, 0.782–5.980, 0.098–0.588, and 0.820–2.050 μg, respectively. These results showed good linear relationships between peak area ratio and amounts.

To check the precision of this method, we injected standard solutions of liquiritin, naringin, hesperidin, thymol, imperatorin, honokiol, isoimperatorin, and

magnolol with internal standard at the concentrations of 0.22, 0.13, 0.12, 0.60, 0.13, 0.23, 0.49, and 0.41 mg/ml, respectively, five times on the same day. The intraday relative standard deviations (R.S.D.s) were 1.6, 1.4, 1.2, 1.8, 1.7, 2.2, 1.6, and 1.8%, respectively. The interday R.S.D.s obtained for a 5-day period were 2.1, 1.7, 2.3, 2.4, 1.5, 3.0, 2.6, and 2.2%, respectively. The recovery rates of liquiritin, naringin, hesperidin, thymol, imperatorin, honokiol, isoimperatorin, and magnolol were 97.4, 98.5, 97.4, 98.6, 97.8, 99.2, 97.0, and 97.5%, respectively. For herbal analysis, the values mentioned above indicated acceptable precision and accuracy.

To ensure the specificity and selectivity of the method, we prepared four blank samples for comparison. They were combined excluding, one at a time, *Cortex Magnoliae Officinalis*, *Pericarpium Citri Reticulatae*, *Extractum Glycyrrhizae*, and *Radix Angelicae Dahuricae*. The chromatograms are shown in Fig. 2. The retention times of the marker components, liquiritin, naringin, hesperidin, thymol, imperatorin, honokiol, isoimperatorin, and magnolol, were 12.5, 18.1, 20.1, 50.7, 53.5, 63.7, 66.3, and 75.3 min, respectively. On inspection of the chromatograms, these constituents all showed good purity. There was no peak found at their retention times in the blank sample.

In this study, the eight marker components of Huoxiang-zhengqi liquid could not be separated effectively using isocratic mobile solvents because of the

difference in their chemical properties. To find an easy way to analyze the specimens, we employed a gradient solvent system (water, methanol, acetonitrile) that can effectively separate the eight markers simultaneously. Different combinations of the three-solvent gradient were investigated. An increase in the aqueous component solved the problem of the short retention time of liquiritin, naringin, and hesperidin. Five types of chromatographic column, Zorbax C<sub>18</sub> (250×4.6 mm, 5 μm), Kromasil C<sub>18</sub> (250×4.6 mm, 5 μm), Polaris C<sub>18</sub> (250×4.6 mm, 5 μm), Nucleosil C<sub>18</sub> (250×4.6 mm, 5 μm), and Shimadzu C<sub>18</sub> (250×4.6 mm, 5 μm), were investigated. The Zorbax C<sub>18</sub> column, which provided sufficient resolution and good peak sharpness, was satisfactory.

The UV absorption maxima of liquiritin and thymol were around 276 nm, those of naringin and hesperidin around 283 nm, those of honokiol and magnolol around 294 nm, and those of imperatorin and isoimperatorin around 254 nm. The monitoring wavelength for quantitative determination at 283 nm was used for the determination of naringin with selectivity, since its content was lower in this preparation.

Determination results of samples are given in Table 1. The chromatograms of standard mixture and samples are shown in Figs. 3 to 5. Naringin can be determined in Huiren samples, but not in Tongrentang samples, which indicates that their crude drugs sources were different. The content changes in each compound between lots from the same pharmaceuti-

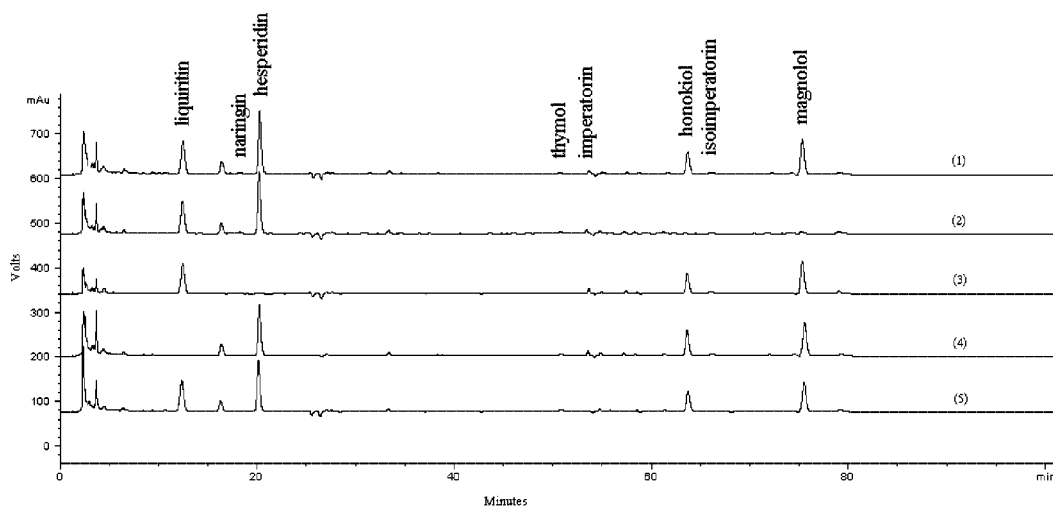


Fig. 2. Chromatograms of Liquiritin, Naringin, Hesperidin, Thymol, Imperatorin, Honokiol, Isoimperatorin, and magnolol in Huoxiang-zhengqi Liquid

(1) Test sample, (2) Test sample without *Cortex Magnoliae Officinalis*, (3) Test sample without *Pericarpium Citri Reticulatae*, (4) Test sample without *Extractum Glycyrrhizae*, (5) Test sample without *Radix Angelicae Dahuricae*.

Table 1. Determination Results of Samples

Marker component	Contents ( $\mu\text{g/ml}$ )					
	Tongrentang			Huiren		
	4144658	4144670	5141152	0504013	0504020	0507059
Liquiritin	52.5	53.4	41.6	31.8	45.4	67.4
Naringin	—	—	—	14.8	16.6	4.4
Hesperidin	1640.1	1237.7	1256.3	636.5	819.8	1298.1
Thymol	—	1588.9	1102.3	103.8	62.1	386.0
Imperatorin	43.2	61.7	58.3	43.6	41.8	53.7
Honokiol	285.8	316.1	526.4	77.3	113.3	194.6
Isoimperatorin	19.3	13.7	12.2	15.1	22.4	43.9
Magnolol	173.3	144.0	164.2	109.2	117.7	129.3

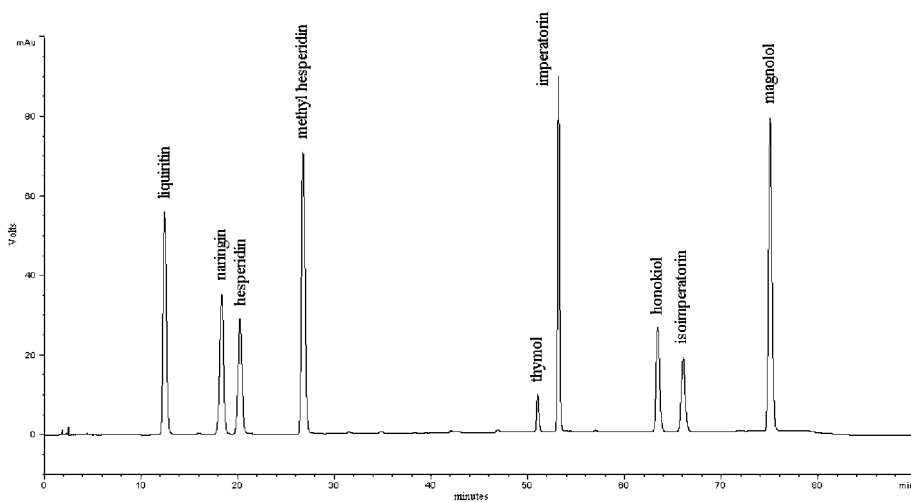


Fig. 3. Chromatogram of Standard Mixture

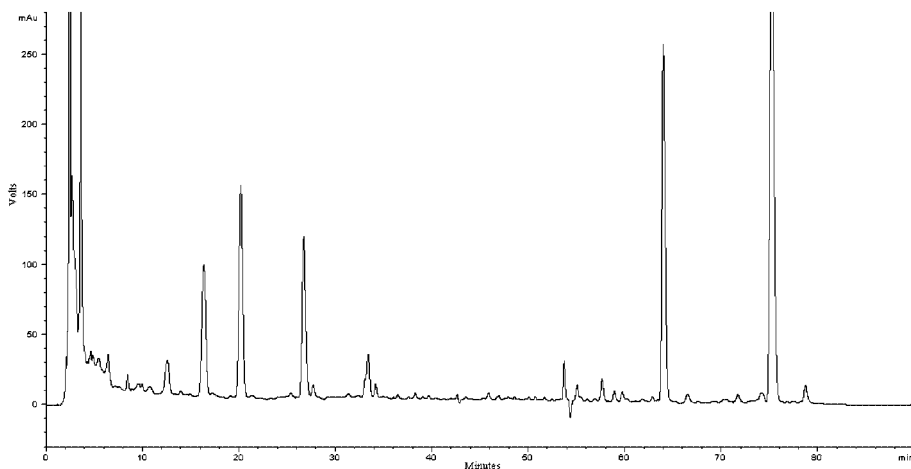


Fig. 4. Chromatogram of Tongrentang Sample (Lot No. 4144658)

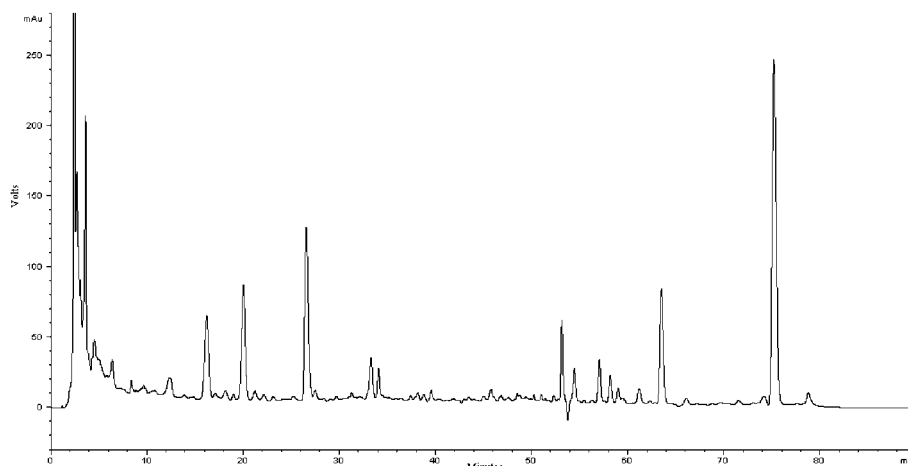


Fig. 5. Chromatogram of Hui ren Sample (Lot No. 0504013)

cal factory indicates that product quality is unstable.

### CONCLUSION

The method described is rapid, linear, accurate, reproducible, and capable of simultaneously quantitating the eight marker components in Huoxiang-zhengqi liquid. It can thus be used for the routine analysis of sample stability and the quality control of products.

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