Laboratory of Ethnopharmacology[1], Institute of Integrated Traditional Medicine and Western Medicine; National Key Clinical Specialist vocational school of TCM encephalopathy[2], Xiangya Hospital, Central South University; TCM Pharmacogenetics Laboratory[3], Central South University, Changsha; Jiangxi Qingfeng Pharmaceutical Company Ltd[4], Ganzhou, China

Simultaneous UPLC analysis of three major flavonoids in granule decoctions of *Fructus Aurantii*-type formulae

Wei Huang[1,2,3], Zhi-hua Xiong[1,2], Xi Huang[1*,2,3], Xiao Chen[4], Wei-ping Liu[1,2], Yang Wang[1,2,3], Ping Ren[1,2]

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Xi Huang, No. 87 of Xiangya Road, Changsha, Hunan Province, 410008, People's Republic of China
tcmhuangx59@163.com

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A simple, rapid, and sensitive liquid chromatographic method has been established for the simultaneous analysis of three compounds (narirutin, hesperidin and naringin), in granule decoctions of *Fructus Aurantii*-type formulae. The compounds were separated in less than 10 min using a C18 column with gradient elution using (A) acetonitrile, (B) water, and (C) acetic acid at a flow rate of 0.3 mL/min, and with a PDA detector. The method was validated for specificity, accuracy, precision, and limits of detection. Good linear regression data (r2 > 0.9980) were obtained for all the calibration plots within the ranges tested. The method is an attractive alternative for quality control and clinical monitor of granule decoctions of *Fructus Aurantii*-type formulae.

1. Introduction

Traditional Chinese herbal medicine is the oldest and most comprehensive form of herbal medicine in the world. Chinese herbs are almost always prescribed as a formula, consisting of multiple herbs. Composite formula of Chinese Medicine (CFCM) have a long therapeutic history of over thousands of years (Normile 2003; Bent et al. 2004). The three methods of administering Chinese herbal medicine are granule herbs (granule decoctions), tablets and decoctions (traditional herb decoctions). Granule herbs are a most modern way to dispense and administer herbal medicinals (Song et al. 2009). They represent a perfect balance between convenience, quality, potency, and cost. Raw herbs are selected, harvested, and tested for heavy metals and pesticides. The herbs are then cooked and strained to form a extract (decoction). The extract then goes through a low heat, high pressure process to remove the water. What is left over is a highly concentrated granule powder that contains the active components of the herbs (Jia et al. 2007). However, crude drugs with different origins, sources, cultural manner, harvest time, pretreatment processes, and manufacturing processes will be of different quality, which will result in significant differences in the same formula when supplied by different factories or even by the same factory (Shi et al. 2005). Therefore, quality control of aqueous extract granules of herbal medicines is required (Jing et al. 2010).

In China, *Fructus aurantii* (FA) is one of the most popular traditional Chinese herbal medicines which has been used for more than 2000 years (Peng et al. 2006). FA is widely used in clinic and it was often used in compatibility with *Magnolia bark* such as FM (*Fructus aurantii* and *Magnolia bark*) (Ding 2005) and Xiaoyao-San-Jiawei (XSJ) (Ren et al. 2006; Li 2004). Clinical and experimental studies indicated that FA has prokinetic (Guan et al. 2002), anti-dyspepsia, antioxidative and anti-inflammartory effects (Moonkyu et al. 2007). The major flavonoids in FA are naringin, hesperidin and narirutin (Fig. 1). These compounds possess antioxidative and/or anti-inflammatory effects (Kanno et al. 2003; Funaguchi et al. 2007; Jagetia et al. 2003; Lee et al. 2009), hesperidin can stimulate the gastrointestinal movement (Fang et al. 2009). The three major flavonoids have similar effects to FA to some extent and all of them are thought to be biologically active.

The three flavonoids in FA have been analyzed (Peng et al. 2006; Zhou et al. 2009; Qin et al. 2009). However, the quantitative comparisons of the three major flavonoids among granule decoctions of FA-type formulae have never been reported before. In order to control the quality and ensure the security and efficiency in clinical use of FA granule powder and study the influence of compatibility with other herbs on the three major flavonoids of herb FA, we will analysis the three major flavonoids and compare their contents in granule decoctions of *Fructus Aurantii*-Type formulae through UPLC method in this paper.

2. Investigations, results and discussion

2.1. Chromatography

In the present study, an UPLC method in detecting three components in granule decoctions of FA-type formulae was successfully established. These were identified by comparison of retention times and UV spectrum with those of the authentic standards. Chromatograms obtained from the authentic standards and from granule decoction of FA, granule decoction of FM and granule decoction of XSJ granules were recorded at 280 nm, respectively, in Fig. 2 a, b, c, d.

2.2. Validation of the Method

The Table shows the regression data and LODs of the components analyzed. For all calibration plots good linear regression ($r^2 > 0.9980$) was achieved within the test ranges. Intra-day and inter-day variation was less than 9.75% for the five

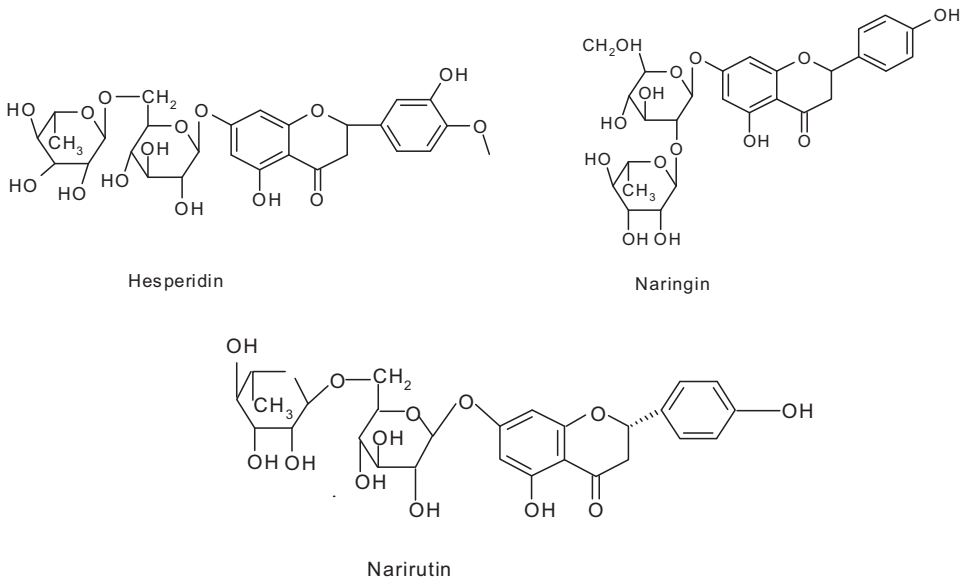

Fig. 1: Chemical structures of the three investigated compounds in *Fructus Aurantii*

analytes, indicating the method is reproducible with good precision. For all five compounds recovery was within the range 91.37–99.70%, indicating the accuracy of the method is acceptable. The standard deviations of peak areas obtained from stability testing were no more than 9.59%. Solutions were therefore regarded as stable for at least 48 h.

2.3. Application to granule decoctions of FA-Type formulae

The present study provides a simple, reliable and rapid method for simultaneously quantitating three compounds in granule decoctions of FA-type formulae. These compounds were separated in less than 10 min. The results suggested that the amounts of narirutin, hesperidin and naringin were in the order granule decoction of XSJ□granule decoction of FM□granule decoction of FA. Compared with granule of FA, the content amounts of narirutin, hesperidin and naringin increased significantly in granule decoctions of FM and XSJ. The study proved that when used in compatibility with other herbs, the contents of the three major flavonoids in FA could be affected.

Composite formulae of Chinese Medicine, presenting as a collection of crude herbs, are a complex mixture containing hundreds of constituents. It is well known that herb–herb interactions could affect amounts of the components when boiled together, it often happened in multi-herbal formulae (Qin et al. 2009). From this experiment we can see that herb–herb interactions could also occur when individual granule herbs were mixed into a customized formula by warm water. This phenomenon was in accordance with Chinese formula compatibility principles. But, how did these phytochemicals interact with each other? This needs further study.

Traditional Chinese Medicine (TCM) is an integral part of Chinese culture, making great contribution to the prosperity of chinese civilization and guaranteeing the life and health of chinese people. Granule is one of the modern dosage forms of Chinese medicine preparations. The extract granules have been a substitution and supplement of traditional herbal drugs. The use of extract granules brings dosage convenience, high efficiency, stability and other advantage to the practice of TCM. Extraction solvents, manufacturing temperature and heating time for the preparation of granule herbs should be taken into account as the major parameters that affect its quality. Therefore, it is important to formulate the regulatory requirements and implement measures to carry out large scale evaluation program to standardize the quality control methods of granules in future.

2.4. Conclusion

A simple, specific□sensitive, accurate and rapid UPLC method has been developed for simultaneously quantitating narirutin, hesperidin and naringin in granule decoctions of FA-type formulae for quality control. At the same time, we also proved that herb–herb interactions could also occur in granule decoctions of chinese medicine formulae just like it occurs in a traditional decoction.

3. Experimental

3.1. Materials

The three preparations consist of fourteen kinds of granules: *Cortex Magnoliae officinalis* granule, *Fructus Aurantii* granule, *Radix Bupleuri* granule, *Radix Angelicae sinensis* granule, *Radix Paeoniae alba* granule, *Rhizoma Atractylodis macrocephalae* granule, *Poria* granule, *Rhizoma Zingiberis recens* granule, *Radix Glycyrrhizae* granule, *Herba Menthae* granule, *Cortex Moutan* granule, *Fructus Gardeniae* granule, *Radix Puerariae* granule and *Fructus Jujubae* granule. FM contains the first two, XSJ contains all. All were purchased from SANJIU MEDICAL & PHARMACEUTICAL CO., LTD (shenzhen, China) and identifiied. Voucher specimens (No.20090901)

Table: Regression data and LODs for the 3 components determined (n = 6)

Constituents	Regression equation	Correlation coefficient(r^2)	Linear range (μg/ml)	LOD (μg/ml)
Narirutin	y = 16355.83x + 272.38	0.9996	0.1−14.4	0.0090
Hesperidin	y = 127409.5x + 8195.57	0.9998	0.2−28.8	0.0072
Naringin	y = 2901.47x + 1561.74	0.9997	1.28−184	0.1150

In the regression equation y = ax + b, x refers to the concentration (μg/ml), y indicates the peak area, and r^2 is the correlation coefficient of the equation. LOD, limit of detection.

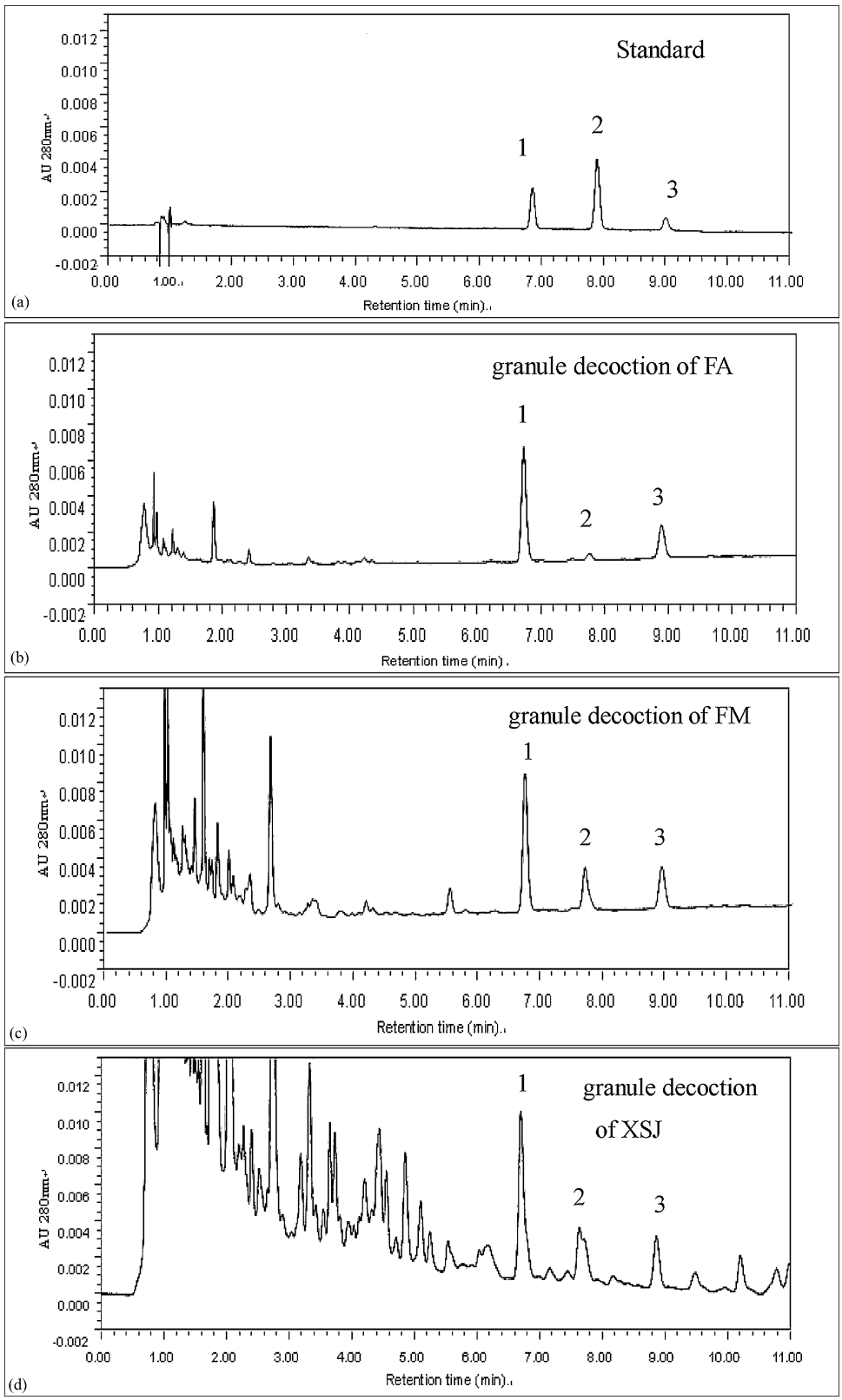

Fig. 2: Chromatograms obtained, at 280 nm, from (a) a mixed standard solution, (b) granule decoction of FA, (c) granule decoction of FM and (d) granule decoction of XSJ; 1 = narirutin; 2 = hesperidin; 3 = naringin

were deposited at the Laboratory of Ethnopharmacology in Xiangya Hospital (Changsha, China).

3.2. Reagents

The reference compounds are naringin, hesperidin and narirutin. Naringin and hesperidin were purchased from Organic herb company (Changsha, China), narirutin was purchased from Sikehua Bio-Tech. Company (Chengdu, China). Methanol was LC-grade (Tedia, USA), acetic acid was from Sinopharm Chemical Reagent Co.Ltd (Shanghai, China), and all water was triple-distilled water from silica glass equipment in this laboratory.

3.3. Apparatus and chromatographic conditions

Analysis was performed using a Waters Acquity UPLC BEH 2.1 × 100 mm, 1.7 μm C18 column system (Waters Corporation, Milford, USA), consisting of a quaternary pump solvent management system, an on-line degasser, and an autosampler. The raw data were detected, acquired, and processed

with Empower Software. The mobile phase was composed of A-acetonitrile, B-water, and C-acetic acid (the amount of acetic acid is kept constant at 0.5% during the entire method) with gradient elution (0–10 min, 13–18%A; 10–20 min, 18–25%A; 20–25 min. 25–60%). The flow rate of the mobile phase was 0.3 mL/min, and the temperature was maintained at 25 °C. The components were quantified based on peak areas at the maximum wavelength in their UV spectrum.

3.4. Preparation of standard solutions

A standard stock solution of each of the three components was directly prepared in methanol. Working standard solutions containing the three compounds were prepared and diluted with methanol to appropriate concentrations for establishment of calibration curves. The standard stock solutions and working solutions were all prepared in dark brown calibrated flasks and stored at 4 °C. The linearity of the responses was determined for seven concentrations. Empower software was used to prepare the standard curves from the peak area of each compound. The contents of these constituents in the test samples were calculated using the regression parameters obtained from the standard curves.

3.5. Preparation of sample solutions

In decoction FA, pulverized sample of *Fructus Aurantii* was accurately weighed and dissolved in warm distilled water and then ultrasonicated. For FM decoction, pulverized samples of *Fructus Aurantii* and *Cortex Magnoliae officinalis* (w:w = 1:1) were accurately weighed, mixed and dissolved in warm distilled water and then ultrasonicated. For XSJ decoction, powdered samples of Cortex *Magnoliae officinalis, Fructus Aurantii, Radix Bupleuri, Radix Angelicae sinensis, Radix Paeoniae alba, Rhizoma Atractylodis macrocephalae, Poria, Rhizoma Zingiberis recens, Radix Glycyrrhizae, Herba Menthae, Cortex Moutan, Fructus Gardeniae, Radix Puerariae* and *Fructus Jujubae* at the weight ratio of 1.2:1.2:1:1:1:0.6:1:1:0.6:0.6:0.6:0.6:1.6:0.6, were accurately weighed, mixed and dissolved in warm distilled water and then ultrasonicated. In decoction FM and XSJ, *Frucus aurantii* and *Magnolia bark* at the weight ratio of 1:1. All solutions were filtered through a 0.45-μm pore size filter before LC analysis. The injection volume was 3 μL, the concentrarion of FA in each decoction before injection is 0.26 mg/ml.

3.6. Validation of the method

The method was validated by investigating its specificity, linearity, precision, accuracy, and stability in accordance with criteria for analytical methods proposed by the US Food and Drug Administration. Linearity was determined by analysis of standard solutions at seven different concentrations. LOD was determined as the concentration resulting in a peak height greater than three times the baseline noise level (S/N = 3). Intra-day and inter-day precision were determined by assay of standard solutions at three concentrations on a single day and on five different days, respectively. Accuracy was determined by measurement of recovery. Stability was tested by analysis of sample solutions stored at 4 °C for 0, 24, and 48 h.

3.7. Statistical analysis

All data are expressed as mean ± standard deviation and were processed by use of SPSS (Chicago, USA)15.0 software. Differences between two groups were analyzed by one-way ANOVA. A probability of less than 0.05 was considered to be indicative of statistical significance.

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