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Full Length Research Paper

Simultaneous Ultra Performance Liquid Chromatography (UPLC) analysis of five components in *Fructus aurantii*-type formulae

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A simple, rapid and sensitive liquid chromatographic method has been established for simultaneous analysis of five compounds (narirutin, hesperidin, naringin, neohesperidin and meranzin hydrate) in *Fructus aurantii*-type preparations. The compounds were separated in less than 12 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 *F. aurantii* -type preparations.

Key words: *Fructus* aurantii, formulae, chemical compounds, UPLC-PDA.

INTRODUCTION

In China, *Fructus aurantii* (FA) has been used for more than 2000 years (Peng et al., 2006). Clinical and experimental studies indicated that FA has prokinetic (Guan et al., 2002), anti-dyspepsia, antioxidative and anti-inflammatory effects (Moonkyu et al., 2007). FA mainly contains naringin, hesperidin, neohesperidin, narirutin and meranzin hydrate (Figure 1). The latter, a novel enterokinetic compound, was first separated and identified by UV, RI, MS and NMR from FA in our recently unpublished work.

Among these compounds, hesperidin stimulates the gastrointestinal movement (Fang et al., 2009). Naringin, narirutin, hesperidin and its derivative, neohesperidin, possess antioxidative and/or anti-inflammatory effects (Kanno et al., 2003; Funaguchi et al., 2007; Jagetia et al., 2003; Lee et al., 2009), targeting the pathogenesis of functional gastrointestinal disorders (FGID) (Ren et al., 2006). Interestingly, their parent herb, FA, also has the similar therapeutic target. FA is the main component in some traditional Chinese medicine (TCM) formulae, such as FM (*F. aurantii* and *Magnolia* bark) (Ding, 2005) and Xiaoyao-San-Jiawei (XSJ) (Ren et al., 2006; Li, 2004). Traditionally, FA plays an important role of activating qi (prokinetic effect) in treating gastroptosis (Guan et al., 2002). FA and magnolia bark in combination, that is FM, can synergistically increase gut motor (Ding, 2005). XSJ is an effective formula in treating functional dyspepsia (Ren et al., 2006; Li, 2004). The prokinetic action is in the order FM > FA > XSJ (Guan et al., 2002; Ding, 2005; Luo et al., 2006). Yet, the association of this magnitude and

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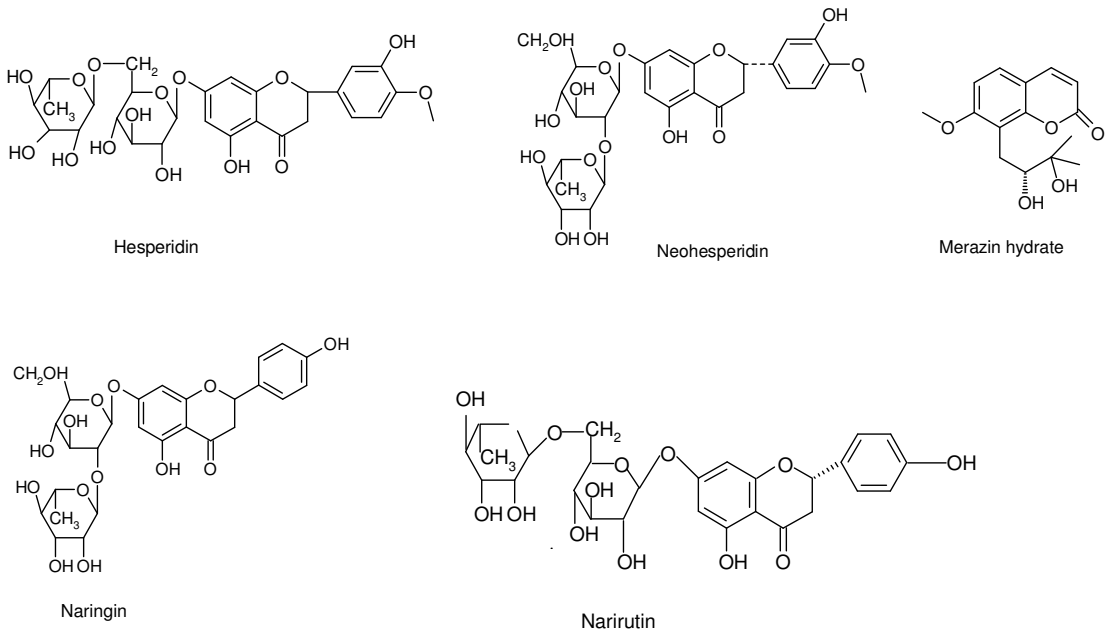

Figure 1. The structures of five constituents in FA.

compound contents are largely unclear. So, we are eager to analyze the contents of narirutin, hesperidin, naringin, neohesperidin, and meranzin hydrate as shown in Figure 1.

Many flavonoids in FA have been analyzed (Peng et al., 2006; Zhou et al., 2009; Qin et al., 2009). However, the quantitative comparisons of the 4 flavonoids plus meranzin hydrate among FA-type formulae have never been reported before. We aim to develop a quantitative UPLC method in comparing their contents for quality control and clinical monitor. For these and the therapeutic mechanism, anyway, it is necessary.

EXPERIMENTAL

Material and reagents

Materials

The two preparations consist of fourteen crude drugs: *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*. FM contains the first two, XSJ contains all. All were purchased from LBX pharmacy (Changsha, China) and identifiied. Voucher specimens (No.20090601) were deposited at the Laboratory of Ethnopharmacology in Xiangya Hospital (Changsha, China).

Reagents

The reference compounds are naringin, hesperidin, neohesperidin, narirutin and meranzin hydrate. Naringin and hesperidin were purchased from Organic Herb Company (Changsha, China), narirutin was purchased from Sikehua Bio-Tech. Company (Chengdu, China), meranzin hydrate was purchased from DIAO Company (Chengdu, China), neohesperidin was purchased from Fukete Biochemical Technology Company (Changsha, 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.

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 was kept constant at 0.5% during the entire method) with gradient elution (0 to 10 min, 13 to 18%A; 10 to 20 min, 18 to 25%A; 20 to 25 min. 25 to 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.

Preparation of standard solutions

A standard stock solution of each of the 5 components were directly prepared in methanol. Working standard solutions containing the 5 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.

Preparation of sample solutions

In decoction FM and XSJ, *Frucus aurantii* and magnolia bark at the ratio of 1:1. FA, FM, and XSJ were heating under reflux with water for 1 h, and the extracts were concentrated and lyophilized. The dried powder was stored at 4℃ before use. For LC analysis the lyophilized powder was dissolved in distilled water, the amount dissolved being equivalent to 15 mg FA ml-1 water. All solutions were filtered through a 0.45 µm pore size filter before LC analysis. The injection volume was 3 µL, the concentration of FA in each decoction before injection is 0.31 mg/ml.

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℃ for 0, 24 and 48 h.

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.

RESULTS AND DISCUSSION

Chromatography

In the present study, an UPLC method was used in detecting five components, in 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 FA, FM and XSJ were recorded at 280 nm, respectively, in Figures 2a, b, c and d.

Validation of the method

Table 1 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 9.71% for the five analytes, indicating that the method is reproducible with good precision. For all five compounds recovery was within the range 91.37 to 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. 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.

Application to FA-type preparations

The present study provides a simple, reliable and rapid method for simultaneously quantitating the five compounds in FA-type decoction. The amounts of merazin hydrate, narirutin and neohesperidin were in the order FM>XSJ>FA, hesperidin and naringin were XSJ>FM>FA. Due to the above five compounds` effects are similar to XSJ`s, the amounts and the related orders of the formulae in the present study were quality criteria for the reproducible effects induced by these formulae. However, clinical prokinetic order of FM, FA and XSJ is unavailable because it is based on different studies (Guan et al., 2002; Ding, 2005; Luo et al., 2006) and the phytochemical contents of these formulae were not detected. So, it is necessary for these formulae with consistent compound contents, as shown in the present study, to induce the reproducible prokinetics in unified protocol.

Multi-herbal formulae are great complex mixtures; usually containing hundreds of compounds ranging in concentration from mg/g to ng/g. Herb–herb interactions could affect amounts of the components (Qin et al., 2009). There are many factors that could affect the therapeutic effects of herbal preparations such as herbal compatibility, decocting time, water volume, and the presence of the other herbs (Yang et al., 2004). Compared with FA, the content amounts of merazin hydrate, narirutin, neohesperidin (FM>XSJ>FA), hesperidin and naringin (XSJ>FM>FA) increased significantly in FM and XSJ. Apparently, herb–herb interactions occurred when boiled together, it often happened in multi-herbal formulae. But, how did these phytochemicals interact each other? It needs further study.

Conclusion

A simple, specific, sensitive, accurate and rapid UPLC method has been developed for simultaneously quantitating narirutin, hesperidin, naringin, neohesperidin and merazin hydrate in FA-type formulae for quality

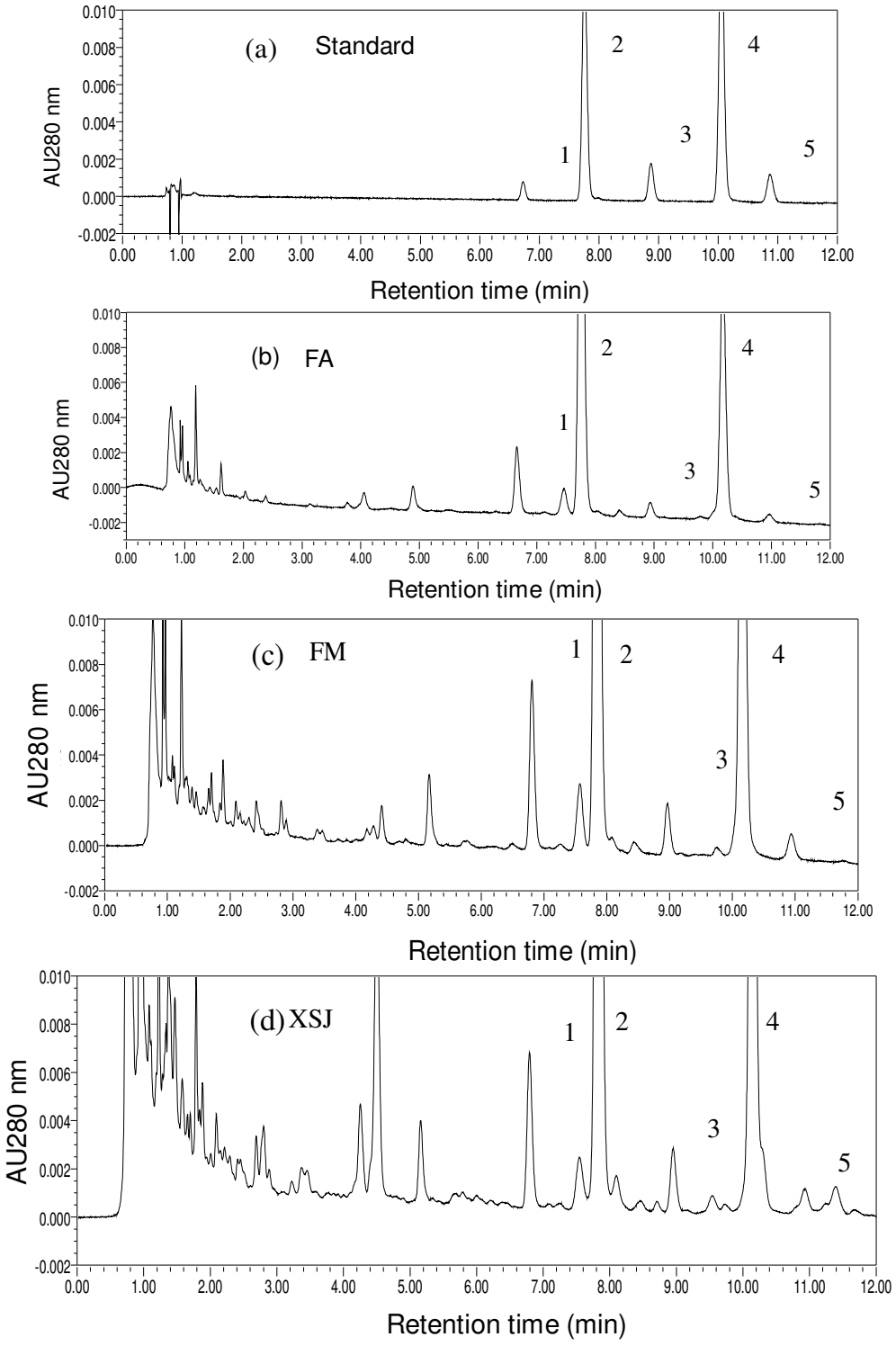

Figure 2. Chromatograms obtained, at 280 nm, from (a) a mixed standard solution, (b) FA, (c) FM and (d) XSJ: 1 = narirutin; 2 = hesperidin; 3 = naringin; 4 = neohesperidin; 5 = meranzin hydrate.

control. It is highlighted in elucidating Chinese herbal compatibilities and therapeutic mechanism for 100,000 formulae.

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Table 1. Regression data and LODs for the 5 components determined (n = 6).

Components	Regression equation	Correlation coefficient(r^2)	Linear range (μg/ml)	LOD (μg/ml)
Narirutin	y = 16355.83x + 272.38	0.9994	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
Neohesperidin	y = 23497.33x + 8581.13	0.9999	1.33-192	0.0480
Merazin hydrate	y = 46566.5x + 4314.77	0.9998	0.33-48	0.0300

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