Development of a Reversed-Phase High-Performance Liquid Chromatographic Method for Analyzing Furanocoumarin Components in Citrus Fruit Juices and Chinese Herbal Medicines

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Abstract

A rapid and sensitive reversed-phase high performance liquid chromatographic method for the quantitation of five furanocoumarins (bergaptol, psoralen, bergapten, 6',7'-dihydroxybergamottin, and bergamottin) is developed and validated. HPLC analysis of these five furanocoumarins is performed on a reversed-phase Inertsil ODS-2 column with a particle size of 5 µm. Using only water and acetonitrile as solvents, good separation, good precision, and high accuracy are obtained for the analysis of furanocoumarin components. This method is validated and applied to analyze the composition of five furanocoumarins in four citrus fruit juices (grapefruit, pomelo I, pomelo II, and shaddock) and ten Chinese herbal medicines (Bai-Zhi, Qiang-Huo, Du-Huo, Fang-Feng, Dang-Gui, Huang-Qin, Gan-Cao, Chen-Pi, Ge-Gen, and Yin-Chen-Hao) prepared by water decoction or an alcohol infusion. Results show that four of the five furanocoumarins (but not bergapten) are detected in grapefruit, pomelo I, and pomelo II, and the highest amount of these components is found in grapefruit juice. In the ten Chinese herbal medicines, the five furanocoumarins are not detected in Ge-Gen or Yin-Chen-Hao. The remaining herbs contain various compositions and amounts of furanocoumarins. In general, Chinese herbal medicines prepared by the 40% ethanol infusion contain larger amounts of furanocoumarins than those prepared by hot water decoction.

Introduction

Grapefruit juice has been reported to increase the oral availability of many clinically important drugs (1). The interaction is caused by the inhibition of their first pass metabolism, mainly catalyzed by intestinal cytochrome P450 (CYP) 3A4. Recently, six furanocoumarin derivatives, bergamottin, 6',7'-epoxybergamottin, 6',7'-dihydroxybergamottin (DHBG), and three furanocoumarin dimers isolated from grapefruit juice were determined to be inhibitors of CYP3A4 and are now suggested to be clinically active constituents (2). However, two minor components, GF-I-1 (FC726) and GF-I-4, are reported to be over 100 times stronger inhibitors of microsomal CYP3A4 activities than are bergamottin or DHBG (3,4). Therefore, the exact role of each component in grapefruit juice/drug interactions remains unclear (5). Although further studies are required to confirm the effects of these furanocoumarins on in vivo drug metabolism, these chemicals are reasonable candidates as causative components in grapefruit juice because of their inhibitory and inactivating potencies on CYP3A from in vitro studies.

Pomelo (*citrus grandis*), a citrus fruit of Asian origin, is popular in Japan and Taiwan. Pomelo is botanically close to grapefruit (*C. paradise*) and contains furanocoumarins, which have been identified as inhibitors of both CYP3A4 and P-glycopotein (6). Besides the Rutaceae family (like grapefruit), many plants of the other families such as the Umbelliferae, Leguminosae, and Moraceae also contain furanocoumarin components (7). Many of these plants are used as common vegetables or traditional medicines. Thus it is possible that furanocoumarin components contained in these fruit or crude drugs also change the pharmacokinetics when a drug is co-administered. Ultimately, identification of the causative component(s) is one of the keys to understanding their clinical significances in herb (fruit)/drug interactions.

Relative proportions of different furanocoumarins might also vary from one species to another and probably are not even consistent in different preparations made from the same species. It was necessary to develop a method for the quantitation of as many furanocoumarin components as possible. The screening of furanocoumarin components in citrus fruits by an enzyme-linked immunosorbent assay

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(ELISA) was developed by Saita et al. (8). The ELISA is capable of detecting DHBG and bergamottin. It is also possible to analyze furanocoumarins by gas chromatography–mass spectroscopy (GC–MS) (9), but some products need to be derivatized to enhance their volatility. This explains why high-performance liquid chromatography (HPLC) has always been the technique of choice for the analysis of furanocoumarins in citrus oils. LC–MS with electrospray ionization or atmospheric pressure chemical ionization (APCI) has been used to detect and quantitate major coumarins, psoralens, and polymethoxyflavones in various citrus oils (10). LC–nuclear magnetic resonance (NMR) has been applied to identify these products in lemon peel oil (11). However, normal laboratories with limited financial resources are less likely to have access to special techniques and complicated procedures, so a simpler assay method must be developed.

Normal-phase HPLC was first used to characterize oils from different species. Different gradient programs with hexane, ethyl acetate, and ethanol were necessary to achieve acceptable separations for each type of oil (12). The normal phase is still used (e.g., for authenticating lemon oils from different geographical origins). Reversed-phase (RP) HPLC is also used to separate closely related furanocoumarins. Brown and Thompson compared normal and reverse phases for the separation of 19 furanocomarins (13). Their conclusion was that the two techniques were complementary. Kaminski et al. described in detail the quantitation of selected coumarins in plant extracts (14). They used RP-HPLC to obtain good resolution with ternary gradients of water, THF, and methanol. They also used a time-programmed wavelength with a diode array detector (DAD) at 340, 325, and 300 nm. Unfortunately, only three furanocoumarins (psoralen, bergapten, and xanthotoxin) were studied (15). Therefore, there is still a demand for the development of a simpler and more sensitive HPLC method for the analysis of additional furanocomarin components in plants belonging to the families Rutaceae, Umbelliferae, Leguminosae, and Moraceae.

The purpose of the present study was to develop a simple and rapid quantitative method for five important furanocoumarin components (bergaptol, psoralen, bergapten, DHBG, and bergamottin) using an RP-HPLC system. This validated HPLC method was then applied to the qualitative and quantitative analysis of five furanocoumarin components in citrus fruits (grapefruit and different species of pomelo) and ten Chinese herbal medicines. Different preparations of Chinese herbal medicines by either hot water decoction or a 40% alcoholic infusion made from the same species were also examined.

Materials and Methods

Drugs and chemicals

Bergamottin and psoralen were obtained from Fluka Chemice (Steinheim, Germany). DHBG, bergaptol, and bergapten were purchased from Ultrafine (Manchester, UK) and Sigma-Aldrich Chemie (Steinheim, Germany), respectively. HPLC-grade acetonitrile for LC was obtained from Merck (Darmstadt, Germany). All other reagents used were reagent-grade or better.

Samples of juices and Chinese herbal medicines

Juice samples of grapefruit (Rutaceae, *C. paradise* MacFaden), pomelo I (Rutaceae, *C. grandis* Osbeck var. Xi-Shi-Yu), pomelo II (Rutaceae, *C. grandis* Osbeck var. Pei-Yu), and shaddock (Rutaceae, *C. grandis* Osbeck var. Ma-Tou Wentan) were obtained from local commercial sources (Taipei, Taiwan). All juice samples obtained from the corresponding fruits were kept at 4ºC soon after being squeezed. Ten Chinese herbal medicines were purchased from a local drug store (Taipei, Taiwan). Available information about these crude drugs is listed in Table I. All Chinese herbal medicines were cut into small pieces and prepared as water decoctions and 40% ethanol infusions. In brief, 50 g of sample was boiled in water (500 mL) for 60 min to reduce the volume by half to make a water extract (250 mL), mimicking a typical decoction procedure for a traditional minimum daily dosage. For the 40% ethanol infusion, samples were immersed in 250 mL of 40% ethanol at room temperature for 45 days. Furthermore, all samples were filtered, and filtrates were stored at 4ºC until being analyzed.

Chromatography

An HPLC system equipped with two pumps (Jasco PU-980 Intelligent HPLC pump) and an autosampler (Jasco AS-950-10 Intelligent Sampler) was used. A 15 cm × 4.6 mm i.d. RP Inertsil ODS-2 column (Verpcopak, Taipei, Taiwan) with a particle size of 5 µm was employed. A model Super Co-150 column oven (Enshine, Taipei, Taiwan) was used to keep the column temperature constant at 40ºC. The mobile phase consisting of a gradient of solvent A (acetonitrile) and solvent B (deionized water containing 1% acetic acid) was eluted as follows: solvent A, 20% at 0 min, 40% at 2 min, 40% at 14 min, 90% at 17 min, 90% at 20 min, and 20% at 23 min. The flow rate was set to 1.0 mL/min. The elute was detected with a Jasco UV-975 UV-VIS detector at a wavelength of 310 nm. The HPLC system was controlled by a PC workstation using Chromatography Data Station software (SISC, Taipei Taiwan).

Table I. Available Information on the Crude Chinese Drugs Tested in this Study

Crude drug	Source	Family	Species
Bai-Zhi	uncertain*, China	Umbelliferae	*Angelicae dahuricae* radix
Qiang-Huo	Guangdong, China	Umbelliferae	*Notopterygium forbesii* root
Du-Huo	Guangdong, China	Umbelliferae	*Angelicae pubescens* radix
Fang-Feng	Inner Mongolia, China	Umbelliferae	*Saposhnikoviae divaricata* radix
Dang-Gui	Gansu, China	Umbelliferae	*Chinese Angelicae sinensis* radix
Huang-Qin	uncertain*, China	Labiatae	*Scutellaria baicalensis* radix
Gan-Cao	Inner Mongolia, China	Leguminosae	*Glycyrrhiza uralensis* radix
Ge-Gen	uncertain*, Thailand	Leguminosae	*Pueraria lobata* radix
Yin-Chen-Hao	Guangdong, China	Compositae	*Artemisiae capillaris* herbs
Chen-Pi	Guangdong, China	Rutaceae	*Aurantii pericarpium*

* Obtained at a local drug store in Taiwan without knowledge of the origin of production.

Quantitation and calibration curve

To examine the linearity of the assay, calibration curves for the five furanocoumarins at concentrations ranging from 31 to 10,000 ng/mL were prepared. The preparation of sample solutions is described as follows. First, each compound was diluted to 0.5 mg/mL in dimethyl sulfoxide. The stock solution (50 µg/mL) was prepared by mixing 0.1 mL of each solution and bringing it to 1 mL with acetonitrile. Then the stock solution was diluted in acetonitrile to produce calibration solutions of 10,000, 5000, 2500, 1000, 500, 250, 125, 63, and 31 ng/mL. The peak areas of these compounds were measured and calibration curves were obtained from the least-squares linear regression of the peak area versus spiked concentrations. The regression lines were used to calculate unknown concentrations of furanocoumarins in citrus fruit juices and Chinese herbal medicines.

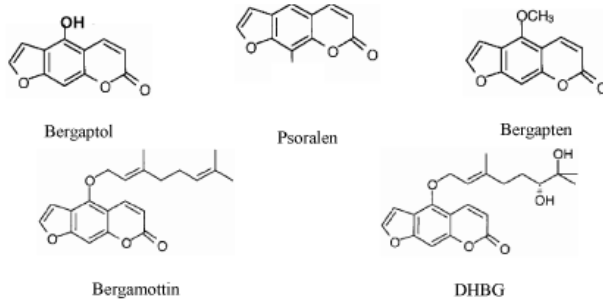

Figure 1. Structures of the five furanocoumarins.

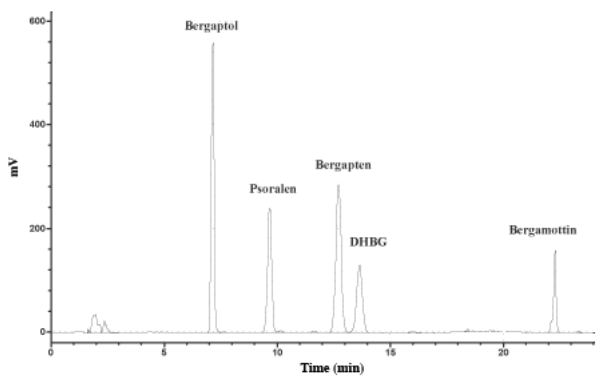

Figure 2. HPLC chromatogram of the five standard furanocoumarins.

Validation of the assay method

The coefficients of variation (CVs) and relative errors of the mean (REMs) were used to validate the precision and accuracy of the intra- and inter-day assays. For the inter-day validation, five sets of control samples at nine different concentrations (31~10,000 ng/mL) were evaluated on five different days. For the intra-day validation, five sets of controls at nine different drug concentrations were assayed with one standard curve on the sample run.

Results and Discussion

Using only water and acetonitrile as solvents, a good separation was obtained for the analysis of bergaptol, psoralen, bergapten, DHBG, and bergamottin (Figure 1). This demonstrates that the RP ODS column system is suitable for analyzing furanocoumarin components from citrus fruit juices and Chinese herbal medicines because of its excellent resolution, appropriate retention times, and good sensitivity. A typical HPLC chromatogram for the five furanocoumarin components is shown in Figure 2. The retention times of bergaptol, psoralen, bergapten, DHBG, and bergamottin were around 7.13, 9.65, 12.70, 13.63, and 22.26 min, respectively. Good separation and baselines with low background noise were observed. Also, the symmetry of all peaks was clearly indicated. The calibration curves of furanocoumarin components that were applied for quantitation based on the HPLC method were constructed. The regression equation, the limit of quantitation (LOQ), and the limit of detection (LOD) for the five furanocoumarin components using the HPLC analysis are listed in Table II. The linearity of the calibration curve of the five furanocoumarins was well correlated ($r^2 > 0.999$) within a range of 31.3~10,000.0 ng/mL for the intra- and inter-day assays. For the five furanocoumarins, the LOQ and LOD were found to be 31.3 and 0.09~0.13 ng/mL, respectively. All data showed the excellent reproducibility of the sample analysis.

This HPLC method for assaying the five furanocoumarin components was validated with precisions (CV) for the inter- and intra-day runs of 0.28%~16.52% and 1.26%~18.52%, and accuracies (REM) of –2.89%~2.79% and –6.21%~4.44%, respectively. The intra- and inter-day validations for assaying the furanocoumarin components showed good precision and high accuracy for the analysis. The method can be applied as a

Table II. The Regression Equation, LOQ, and LOD for Five Furanocoumarins Using HPLC Analysis

	Intra-day		Inter-day		LOQ	LOD
	Equation*	r^2	Equation	r^2	(ng/mL)	(ng/mL)
Bergaptol	$y = 0.998x - 18.455$	0.999	$y = 0.932x - 15.802$	0.999	31.3	0.1
Psoralen	$y = 0.978x + 14.865$	0.999	$y = 1.033x - 30.891$	0.999	31.3	0.1
Bergapten	$y = 0.991x + 6.093$	0.997	$y = 0.984x + 8.671$	0.999	31.3	0.1
DHBG	$y = 0.986x + 8.296$	0.998	$y = 0.945x + 25.660$	0.999	31.3	0.1
Bergamottin	$y = 0.997x + 2.551$	0.999	$y = 0.988x - 2.814$	0.999	31.3	0.1

* y: experimental concentration (ng/mL), x: spiked concentration (ng/mL).

simple and practical way to analyze the contents of bergaptol, psoralen, bergapten, DHBG, and bergamottin in citrus fruit juices and Chinese herbal medicines.

The suitability was further confirmed in an analysis of the five furanocoumarin components from four citrus fruit juices and ten Chinese herbal medicines prepared from a hot water decoction and 40% ethanol infusion. In general, the main difficulty with the analysis of furanocoumarins is the presence of numerous components in Chinese herbal medicines. Even the best HPLC method cannot always avoid co-elution problems. Figures 3A and 3B show HPLC chromatograms of furanocoumarin components extracted from citrus fruit juices (grapefruit juice is used as a typical example) and from Chinese herbal medicines (a 40% ethanol infusion of Qiang-Huo is used as a typical example), respectively. No interfering peaks were observed. Tables III and IV list the contents of the five furanocoumarin components in four citrus fruit juices and ten Chinese herbal medicines prepared with hot water decoctions and 40% ethanol infusions. These results show that the compositions of the five furanocoumarins greatly differed among the four citrus fruit juices and ten Chinese herbal medicines prepared with hot water decoctions and 40% ethanol infusions.

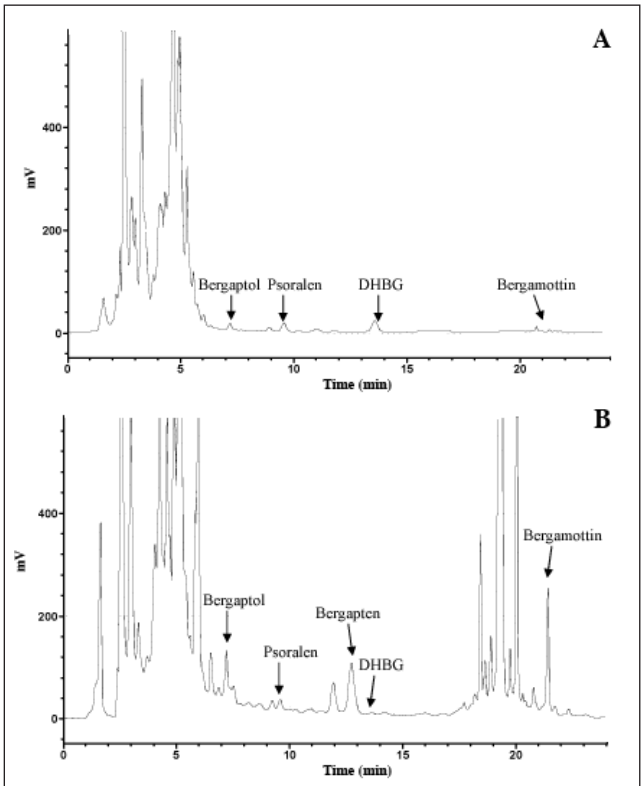

Figure 3. HPLC chromatograms of the furanocoumarin components extracted from grapefruit juice (A) and Qiang-Huo prepared with a 40% ethanol infusion (B).

The content of bergapten was almost negligible in grapefruit, pomelo I, and pomelo II, but was quite high in shaddock. Bergaptol, psoralen, DHBG, and bergamottin were present in grapefruit, pomelo I, and pomelo II, but not in shaddock. These results suggest that citrus fruits (not only grapefruit) contain furanocoumarins and are related to the CYP3A inhibitory properties of grapefruit juice. Furthermore, the contents and types of the five furanocoumarin components in the ten Chinese herbal medicines prepared with hot water decoctions and 40% ethanol infusions greatly differed from each other. In general, the Chinese herbal medicines prepared by the 40% ethanol infusion contained larger amounts of furanocoumarins than those prepared by the hot water decoction. Differences in the chemical compositions of different preparations made from the same species will affect the inhibition of CYP3A4 activity. Bergapten was the major furanocoumarin in some of the ten Chinese herbal medicines, but varied from one species to another and probably would not even be consistent for different preparations made from the same species. DHBG and bergamottin were almost negligible in Fan-Feng, Dang-Gui, Huang-Qin, Gan-Cao, and Chen-Pi. In the ten Chinese herbal medicines, the five furanocoumarins were not detected in Ge-Gen or Yin-Chen-Hao, while the rest of the herbal preparations contained different compositions and amounts of these furanocoumarins. Dang-Gui and Gan-Cao prepared with water and 40% ethanol contained only one of the five referenced furanocoumarins. Qiang-Huo prepared with a 40% ethanol infusion contained all five furanocoumarins. However, there is no clear evidence that any one furanocoumarin component is primarily responsible for inhibiting CYP3A4, and it is possible that the interaction arises from the cumulative effect of a number of furanocoumarin constituents present in citrus fruit juices and Chinese herbal medicines. Guo et al. showed that all major furanocoumarins are necessary for the maximal inhibition of CYP3A activity observed in grapefruit juice, and no clear correlation existed with the specific contents of any one of the chemicals. The simultaneous addition of all experimental furanocoumarin components resulted in stronger CYP3A inhibitory potencies than when they were separately tested at the highest levels of these furanocoumarins. Thus the contents of total furanocoumarins contained in these foods or herbal medicines also affect CYP3A activity and change the pharmacokinetics of drugs. In the present study, the total furanocoumarin contents of Bai-Zhi, Qiang-Huo, and Du-Huo were abundant. Actually, Bai-Zhi, Qiang-Huo, and Du-Huo also showed significant inhibitory potencies on CYP3A activity in our preliminary experiments (data not shown).

Table III. Contents of Furanocoumarins in Citrus Fruit Juices

Citrus fruit	Concentration (ng/mL) (mean ± SD)*				
	Bergaptol	Psoralen	Bergapten	DHBG	Bergamottin
Grapefruit	356 ± 17	624 ± 15	–†	1623 ± 19	110 ± 3
Pomelo I	55 ± 6	158 ± 3	–	166 ± 4	78 ± 2
Pomelo II	28 ± 19	114 ±1	–	146 ± 3	79 ± 3
Shaddock	–	–	522 ± 24	–	–

* Data are shown as the mean ± S.D. (n = 3).
† –: Not detected.

Table IV. Contents (µg/g) of Furanocoumarins in Chinese Herbal Medicines Prepared with Water and Alcohol Extracts*

	Bergaptol		Psoralen		Bergapten		DHBG		Bergamottin	
Crude drug	Water	Alcohol	Water	Alcohol	Water	Alcohol	Water	Alcohol	Water	Alcohol
Bai-Zhi	34.7 ± 0.1	43.3 ± 0.1	–†	2.0 ± 0.0	12.4 ± 0.1	34.1 ± 0.2	–	–	0.9 ± 11.8	–
Qiang-Huo	27.1 ± 0.2	190.1 ± 0.1	–	69.3 ± 0.1	10.1 ± 0.2	197.6 ± 0.1	–	16.0 ± 0.2	5.5 ± 0.1	43.9 ± 0.1
Du-Huo	9.9 ± 0.1	1.5 ± 0.0	–	–	7.4 ± 0.1	3.8 ± 0.1	9.7 ± 0.2	21.7 ± 0.2	–	–
Fang-Feng	–	–	3.8 ± 0.1	1.7 ± 0.2	1.5 ± 0.1	21.1 ± 0.2	–	–	–	–
Dang-Gui	–	–	–	–	10.1 ± 0.2	5.1 ± 0.1	–	–	–	–
Huang-Qin	–	–	–	7235.5 ± 0.1	6.4 ± 0.2	–	–	–	–	–
Gan-Cao	19.0 ± 0.2	31.4 ± 0.1	–	–	–	–	–	–	–	–
Chen-Pi	–	–	52.6 ± 0.1	110.7 ± 0.2	110.2 ± 0.2	240.3 ± 0.1	–	–	–	–

* Data are shown as the mean ± S.D. (n = 3).
† –: Not detected.

Conclusion

We have developed a simple and rapid method for the calibration and quantitation of five different furanocoumarins by RP-HPLC coupled with a UV detector. The method allows for the quantitation of citrus fruits (grapefruit and different species of pomelo) and ten Chinese herbal medicines with excellent precision and accuracy.

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