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Short communication

Determination of hesperidin in Pericarpium Citri Reticulatae by semi-micro HPLC with electrochemical detection

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Abstract

Determination of hesperidin contents in Pericarpium Citri Reticulatae was performed by a simple extraction with methanol and semi-micro high-performance liquid chromatography with electrochemical detection (μHPLC–ECD). Chromatography was performed using a microbore octadecylsilica (ODS) column, methanol–water–phosphoric acid (40:60:0.5, v/v/v), as a mobile phase and applied potential at +0.9 V versus Ag/AgCl. Peak heights were found linearly related to the concentrations of hesperidin injected 9.16 ng/ml to 3.06 μg/ml ($r > 0.999$). The detection limit ($S/N = 3$) was 3.06 ng/ml (15.3 pg). Hesperidin of 305 ng/ml was detected with a relative standard deviation (R.S.D.) of 0.79% ($n = 5$). Hesperidin in Pericarpium Citri Reticulatae was extracted with methanol, diluted with the mobile phase, and injected into the μHPLC–ECD for determination. The hesperidin content of Pericarpium Citri Reticulatae from four different districts in China were determined with R.S.D. of 3.59%, 2.29%, 2.36%, and 2.32% ($n = 5$), respectively. Recoveries of hesperidin from the four Pericarpium Citri Reticulatae sources were 100.3%, 99.83%, 100.7%, and 100.6%, respectively. This method is useful for the determination of hesperidin in Pericarpium Citri Reticulatae, and especially so for instances when samples are sparse.

Keywords: Hesperidin; Pericarpium Citri Reticulatae; Chinpi; HPLC with electrochemical detection

1. Introduction

Pericarpium Citri Reticulatae is the dried pericarp of the ripe fruit of *Citrus reticulata* Blanco or its cultivars (Fam. Rutaceae). The main cultivars are Citrus reticulate "Chachi" (Guang Chenpi), Citrus reticulate "Dohongpao", Citrus reticulate "Unshiu" and Citrus reticulate "Tangerina". The pericarp is peeled off when the fruit is ripe and dried in the sun or at a low temperature. It is generally known that there are four kinds of Pericarpium Citri Reticulatae (Chinpi, Chenpi, Chachi, and Dahongpao). Pericarpium Citri Reticulatae has been acknowledged by the People's Republic of China pharmacopoeia, and has been used in traditional Chinese herbal medicines for a long time. The indications of Pericarpium Citri Reticulatae are distension and epigastrium with anorexia, vomiting, diarrhea, and a cough with copious phlegm [1]. Pericarpium Citri Reticulatae has been extensively applied in food industries because of its smell, flavor, and curative effects. It is usually added to foods as a condiment or sometimes used to regulate the taste of Chinese medicines. Thus the quality control of Pericarpium Citri Reticulatae is important for pharmaceutical and food companies in the production of traditional Chinese herbal medicines and foods. It has been reported that Pericarpium Citri Reticulatae contains many flavonoids, for instance, tangeretin (4′,5,6,7,8-pentamethoxyflavone); hesperidin (hesperetin-7-O-rutinoside); citromitin (3′,4′,5,6,7,8-hexamethoxyflavanone); nobiletin (3′,4′,5,6,7,8-hexamethoxyflavone); neohesperidin (hesperetin-7-neohesperidoside) and *d*-limonene. Hesperidin is a major flavonoid in Pericarpium Citri Reticulatae and its properties are very stable [2–5]. So hesperidin is an ideal stable marker for the quality control of Pericarpium Citri Reticulatae. Thus, the development of a simple quantitative method with high sensitivity and high accuracy has been desired to sustain green-chemistry.

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HPLC with UV detection (HPLC–UV) [2,3,6–9], capillary electrophoresis with electrochemical detection (CE–ECD) [10], CE with UV detection (CE–UV) [11,12], LC with mass spectrometry (LC–MS) [13–16], and spectrophotometry [17] have been used for determining hesperidin in pharmaceutical formulations, citrus juices, and rat plasma and urine. However, these methods require complicated pretreatments of the samples and most of the methods lack sensitivity for determining hesperidin content in instances when samples are sparse. HPLC–ECD is sensitive, inexpensive, and works without complex and time-consuming sample preparations, as reported by Careri et al. [18], Sontag et al. [19], and Jin et al. [20]. In our previous reports, we successfully developed a more than 30-fold sensitive HPLC–ECD method for determining catechins, quercetin, and *ortho*-phenylphenol [21–23] by using a microbore octadecylsilica (ODS) column. When compared to a reported HPLC–ECD method that used a conventional ODS column [24–26], our method would be useful for the quality control of Pericarpium Citri Reticulatae using hesperidin content as a marker. In the present study, we applied the present μHPLC–ECD method for the determination of hesperidin contents in Pericarpium Citri Reticulatae from four different districts in China.

2. Experimental

2.1. Materials and reagents

Hesperidin (94.5%, Lot: 31k2628) and myricetin (99.8%, Lot: 092k2517) were obtained from Sigma Chemical Co. (St. Louis, MO), and their structures are shown in Fig. 1. Methanol (>99.8%, HPLC grade) and phosphoric acid (>85%, reagent grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents were of reagent grade available from commercial sources.

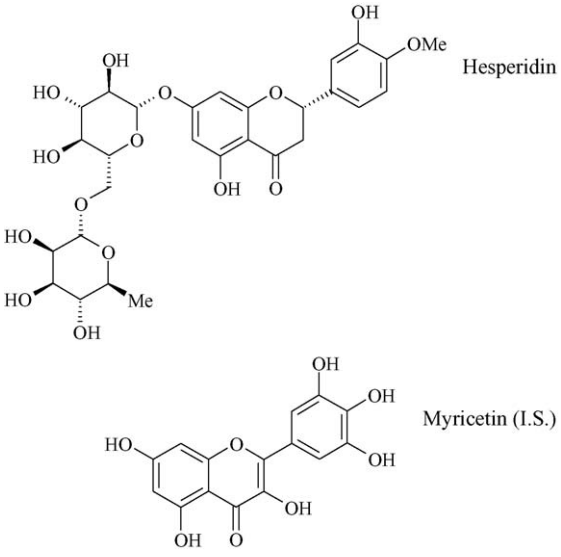

Fig. 1. Structures of hesperidin and myricetin.

2.2. Pericarpium Citri Reticulatae of the People's Republic of China pharmacopoeia

Chachi (Zhejiang), Chenpi (Zhejiang), Chenpi (Guangdong), and Dahongpao (Shandong) were obtained from Kangqiao Yinpian Factory (Shanghai, China), Dehua Yinpian Factory (Shanghai, China), Leiyunshang Yinpian Factory (Shanghai, China), and Lunan Herb Station (Shandong, China), respectively.

2.3. μHPLC–ECD system and conditions

The μHPLC–ECD was comprised of an LC-26A vacuum degasser (BAS, Tokyo, Japan), an LC-100 pump (BAS), a 7125 injector fitted with a 5 μl injection loop (Reodyne, CA, USA), a Capcell Pak C18 UG 120 microbore ODS column (150 mm × 1.0 mm i.d., 3 μm, Shiseido, Tokyo, Japan), an FT-1 column oven (BAS), and an LC-4C electrochemical detector (BAS). The commercially available electrochemical cell (Radial flow cell, BAS) was constructed from a glassy carbon working electrode, an Ag/AgCl reference electrode, and a stainless steel auxiliary electrode. Methanol–water–phosphoric acid (40:60:0.5, v/v/v) was used as mobile phase. The applied potential was +0.9 V versus Ag/AgCl, the flow rate was 30 μl/min, and the column temperature was 30 °C. An internal standard (IS) method was used for the determination of hesperidin amounts in the sample solution, and myricetin was used as the IS.

2.4. Sample preparation

An accurately weighted amount of thin powder of Pericarpium Citri Reticulatae (0.1 g) was added to 90 ml of methanol and ultrasonicated for 30 min. The solution was diluted with methanol to 100 ml and filtered. One milliliter of the filtrate was diluted with methanol to 10 ml. One milliliter of the diluted filtrate was added to 2 ml of 2.5×10^{-3} M IS solution, and diluted with methanol–water–phosphoric acid (40:60:0.5, v/v/v) to 10 ml. This solution was passed through a membrane filter (pore size, 0.45 μm) and used as the test solution. A 5 μl volume of the test solution was injected into the μHPLC–ECD system.

3. Results and discussion

3.1. Optimization of μHPLC–ECD conditions

A hydrodynamic voltammogram (Fig. 2) was measured in order to determine the optimal detection potentials of hesperidin and myricetin. Hesperidin and myricetin were oxidized at potentials more positive than +0.6 and +0.3 V versus Ag/AgCl, respectively. Two oxidation waves of hesperidin, the half wave potentials of +0.75 and more positive than +1.2 V versus Ag/AgCl, were observed in the hydrodynamic voltammogram. For potentials more positive than +1.1 V versus Ag/AgCl, sensitivity was higher, but reproducibility was less, possibly due to the roughness of the electrode surface caused by oxidation of the glassy carbon working electrode surface. For highly sensitive determination without loss of selectivity and reproducibility, the

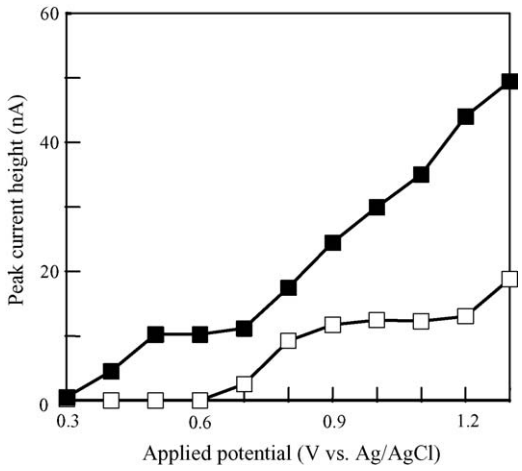

Fig. 2. Hydrodynamic voltammograms of hesperidin (□) and myricetin (■). HPLC conditions used were: column, microbore ODS column (150 mm × 1.0 mm i.d., 3 μm); column temperature, 30 °C; mobile phase, methanol–water–phosphoric acid (40:60:0.5, v/v/v); flow rate, 25 μl/min.

potential value +0.9 V versus Ag/AgCl was adopted for the present study.

An examination was made of how the ratio of water to methanol in the mobile phase influenced the separation for the determinations of hesperidin and myricetin. The larger the content of water, the greater was the separation of these peaks. To determine hesperidin in Pericarpium Citri Reticulatae with adequate resolution, within a short time, a mixture of methanol–water (40:60) was chosen for the most suitable mobile phase, and the column temperature, during separation, was maintained at 30 °C. Flow rate was selected at 30 μl/min. Under these conditions, the chromatographic peaks of hesperidin and myricetin (IS) were obtained within 25 min, where the resolution (Rs) of hesperidin and myricetin was 8.26. To shorten the measurement time, a column temperature of 40 °C was selected. However, the chromatographic peak of hesperidin was affected by the solvent peak and myricetin (IS). Although it was obtained within 20 min, and Rs of hesperidin and myricetin was 5.31. In addition, when both flow rate and column temperature were made faster and higher, the hesperidin peak was completely affected by the solvent peak.

Thus, the optimal HPLC conditions in this study were: methanol–water–phosphoric acid (40:60:0.5, v/v/v); flow rate, 30 μl/min; column temperature, 30 °C; and applied potential, +0.9 V versus Ag/AgCl.

3.2. Linear range and detection limit for determining hesperidin

Fig. 3A shows a chromatogram of hesperidin of 305 ng/ml, and myricetin (IS). The retention times of hesperidin and myricetin were 15.5 and 22.5 min, respectively. Peak height was found to be linearly related to the concentration of hesperidin in the standard solution from 9.16 ng/ml to 3.06 μg/ml ($r > 0.999$), i.e., the amount of hesperidin from 45.8 pg to 15.3 ng ($r > 0.999$). The accuracy of the present method is listed in Table 1. The accuracy of the calculated data, as relative error (R.E.) at low, medium, and high concentrations, was within the range of −4.6 to 5.7%. In addition, the regression equation with regression coefficients showed good linearity. Hesperidin, 305 ng/ml, was detected with a relative standard deviation (R.S.D.) of 0.79% ($n = 5$). The R.S.Ds. of the intra-day and inter-day precisions for 305 ng/ml hesperidin were less than 3.0%. The detection limit ($S/N = 3$) for hesperidin was 3.06 ng/ml (15.3 pg). The sensitivity was compared with other available methods. As shown in Table 2, the present method is the most sensitive technique

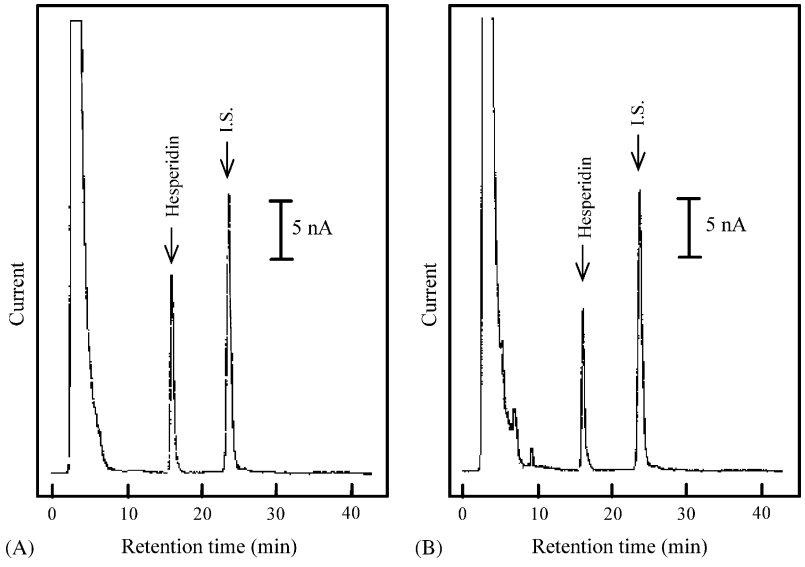

Fig. 3. Chromatograms of (A) standard solution of hesperidin and (B) Pericarpium Citri Reticulatae. (A) Hesperidin was dissolved in methanol–water–phosphoric acid (40:60:0.5, v/v/v), and was injected into HPLC at a concentration of 305 ng/ml. (B) The sample solution was prepared from Pericarpium Citri Reticulatae (Chachi, Zhejiang) of the People's Republic of China Pharmacopoeia as described in the Section 2. HPLC conditions used were: column, microbore ODS column (150 mm × 1.0 mm i.d., 3 μm); column temperature, 30 °C; mobile phase, methanol–water–phosphoric acid (40:60:0.5, v/v/v); flow rate, 30 μl/min, applied potential, +0.9 V vs. Ag/AgCl.

Table 1
Intra-day accuracy and calibration curve linearity for the measurement of hesperidin peaks

Nominal concentration (μg/ml)	Determined concentration[a] (μg/ml)	Accuracy[a] (%, RE)	Linearity		
			Regression equation[b,c]	r[c]	Range[c]
3.05	3.04	−0.5	$y = 43.9x - 0.0464$	0.999	9.16 ng/ml–3.05 μg/ml
1.22	1.27	3.8			
0.305	0.291	−4.6			
0.122	0.124	1.6			
0.0305	0.0318	4.0			
0.0122	0.0129	5.7			

[a] Determined concentration and accuracy were obtained in duplicate.
[b] x and y indicate hesperidin concentration (μg/mL) and peak current height (nA), respectively.
[c] The number of plots used to show linearity in the concentration range was 11.

Table 2
Detection limit of hesperidin by various methods

Method	Column		Injection volume (μl)	Detection limit (ng/ml)	Lower quantification limit (ng/ml)	Reference
	Internal diameter (mm)	Length (mm)				
μHPLC–ECD	1.0	150	5	3.1	9.2	Present method
HPLC–ECD[a]	4.6	250	5	1600	50000	[19]
HPLC–ECD[b]	4.6	150	1	500	2000	[18]
HPLC–UV	2.0	150	1	300	1000	[18]
LC–MS/MS	4.6	250	25	0.7	2.0	[15]
CE–UV	0.075	600		1000	3333	[11]
CE–ECD	0.025	400		400	3053	[10]

[a] Amperometric detection was used.
[b] Coulometric detection was used.

among common methods such as: HPLC–ECD with conventional column, HPLC–UV, CE–UV, and CE–ECD. The high sensitivity of our method is very probably due to the fact that it uses a microbore column that avoids diffusing samples and makes the flow rate slow, thereby increasing the electrolytic efficiency of samples on the working electrode. Although the detection limit "concentration" of hesperidin in LC–MS/MS appears to be more sensitive than the present method, the absolute "amount" of detection limits in μHPLC–ECD and LC–MS/MS were 15.3 pg and 17.5 pg, respectively. Therefore, the sensitivity of the present μHPLC–ECD method is comparable to that of LC–MS/MS.

3.3. Determination of hesperidin in Pericarpium Citri Reticulatae

For extracting hesperidin from the sample, water, 50% methanol, mobile phase and methanol were compared. Hesperidin easily dissolves in methanol and thus methanol extraction of the sample by ultrasonication was conducted for this study. A single extraction (30 min ultrasonication × 1 time) was compared with repeated extraction (10 min ultrasonication × several times). The results demonstrated that the content of hesperidin no longer increased when the extraction procedure was repeated more than two times.

Hesperidin in Pericarpium Citri Reticulatae was determined by μHPLC–ECD method. A typical chromatogram for Pericarpium Citri Reticulatae (Chachi, Zhejiang) is shown in Fig. 3B. There was no interference with the peak of hesperidin and myricetin (IS). Hesperidin in Pericarpium Citri Reticulatae from different districts in China were analysed and the results are listed with their recovery data in Table 3. R.S.D. ($n = 5$) in all cases were less than 2.95%. Recovery of hesperidin from the spiked test solutions were more than 99.83% and R.S.D. ($n = 5$) was less than 3.59%. The results demonstrate that the

Table 3
Content and recovery of hesperidin in Pericarpium Citri Reticulatae from different districts in China

Sample			Content ($n = 5$)		Recovery[a] ($n = 5$)		
Name	District	Lot	Amount (mg/g)	R.S.D. (%)	Spiked amount (mg/g)	Recovery (%)	R.S.D. (%)
Chachi (Guang Chenpi)	Zhejiang	20040218	31.40	1.29	32.81	100.3	3.59
Chenpi	Zhejiang	2004010261	30.18	2.06	33.87	99.83	2.29
Chenpi	Guangdong	2005030215	32.95	1.98	33.87	100.7	2.36
Dahongpao (Guang Chenpi)	Shandong	20040328	23.91	2.95	33.87	100.6	2.32

[a] For recovery test, the hesperidin standard at each amount was directly spiked to each Pericarpium Citri Reticulatae. Hesperidin derived from both Pericarpium Citri Reticulatae and the standard was extracted, and a test solution was injected into μHPLC–ECD to obtain a chromatogram. The recovery was 100% when the hesperidin content in the recovery test was equal to the sum of hesperidin in Pericarpium Citri Reticulatae and the spiked standard.

μHPLC–ECD method is characterized by higher reproducibility, indicating the present μHPLC–ECD method provides quite accurate measurements of hesperidin in Pericarpium Citri Reticulatae. The content of hesperidin in Dahongpao was lower than in other samples. Chachi and Dahongpao are all *C. reticulata* Blanco's cultivars, but the content of hesperidin in Chachi was mostly the same as in *C. reticulata* Blanco. The different source of *C. reticulata* Blanco and its cultivars, and different processing, can affect the quantity of Pericarpium Citri Reticulatae.

There are two reasons why such a simple methanol extraction was enough to determine hesperidin in Pericarpium Citri Reticulatae. First, hesperidin is an electrochemically oxidizable compound. It means that hesperidin is selectively detected by ECD. Second, the HPLC–ECD method presented is sensitive, enabling highly sensitive measurement. In this study, 0.1 g Pericarpium Citri Reticulatae were used for the determination of hesperidin content because our goal was the quality control of Pericarpium Citri Reticulatae. However, even if the sample amount for an assay were 1/10 000, the present methods would be carried out for the determination of hesperidin in Pericarpium Citri Reticulatae because the minimum amount of Pericarpium Citri Reticulatae necessary for determining hesperidin contents is only 10 μg.

4. Conclusions

In this study, the μHPLC–ECD method has been established as a sensitive, selective, rapid and accurate method for the determination of hesperidin with simple sample preparation. This method, using small sample amounts, would be useful for instances when samples are sparse. For example, one can predict the quality of *C. reticulata* Blanco or its cultivars before harvesting using sparse amounts of peels as a sample. Therefore, the present method would be a convenient application in the quality control of Pericarpium Citri Reticulatae and the harvesting and processing of traditional Chinese herbal medicines.

The sensitivity of the present μHPLC–ECD method is superior to HPLC–ECD methods with a conventional column. Additionally, because the downsizing of HPLC–ECD was performed by microbore column, the flow rate of the μHPLC–ECD was about 1/50 compared with conventional HPLC, thereby decreasing the volumes of running solvents and chemicals required for one μHPLC assay. Thus the development of the present method for the analysis of quality control of Pericarpium Citri Reticulatae will reduce the environmental impact of chemical enterprises.

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