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Determination of Hesperetin and Its Conjugate Metabolites in Serum and Urine

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ABSTRACT

Hesperidin and hesperetin are flavanones in many Citrus fruits with antiulcer, antioxidation and anticancer activities. Hesperetin is the aglycone and a metabolite of hesperidin. HPLC analysis was developed for the determination of hesperetin and its conjugate metabolites in serum and urine prior to and after hydrolysis following oral administrations of hesperetin, hesperidin, or decoction of Pericarpium Citri Reticulatae (Chenpi). The hydrolysis was conducted away from light by using glucuronidase/sulfatase in the presence of ascorbic acid at 37°C. The HPLC methods employed a LiChrospher 100 RP-18e column, using acetonitrile/1% acetic acid (36/65) and methanol/acetonitrile/1% acetic acid (17/26/57) as mobile phases for serum and for urine, respectively. For internal standard, 5,7-dimethoxycoumarin was used. The present methods are rapid, sensitive, reproducibility, precise, and accurate with good recoveries. They are applicable for the pharmacokinetic studies of hesperidin and hesperetin as well as Citrus foods or herbs containing them.

Key words: hesperidin; hesperetin ; serum; urine; HPLC.

INTRODUCTION

In recent years, flavonoids are receiving growing interest due to their various beneficial pharmacological activities. The most widely consumed flavanone glycoside is hesperidin (hesperetin-7-rhamnoglucoside) which is abundant in *Citrus* fruits like oranges and Chinese herb Pericarpium Citri Reticulatae (陳皮) which is used in clinical Chinese medicine to treat fullness, poor appetite. It has been reported that hesperidin shows pharmacological effects including anti-inflammatory[1,2], hypolipidemic[3], anti-hypertensive[3], anti-oxidative[4], anticarcinogenic effects[5,6] and the like, whereas its aglycone, hesperetin, has many beneficial effects including antioxidation[7], antivirus[8] and cholesterol-lowering[9].

Although there are several reports concerning quantitation of hesperidin in orange juice[10] or Chinese herbal medicine[11] or formula[12], limited information about quantitation of metabolites of hesperidin and hesperetin after their intake was available for pharmacokinetic study. Flavonoid glycosides are metabolized to their aglycones by human intestinal microflora[13,14]. Growing evidence showed that glucuronidation is central to flavonoid metabolism and absorption[15]. Our previous studies on oral flavonoid glycosides and their aglycones[16,17] also support that hesperidin and hesperetin may be metabolized into hesperetin glucuronides/sulfates after oral administration. In this study, we established methods employing enzymatic treatment of serum or urine in post oral dosing of hesperetin, hesperidin

or the decoction of Pericarpium Citri Reticulatae to release hesperetin from its conjugate metabolites with a strategy to protect from decomposition. HPLC assays were also developed for the determination of the total hesperetin including parent form and those from conjugates.

MATERIALS AND METHODS

I. *Chemicals*

Hesperidin, hesperetin, β-glucuronidase (HP-2, containing 110,305 units/ml β-glucuronidase and 4,194 units/ml of sulfatase) and glacial acetic acid were purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A.). Acetonitrile (LC grade) and ethyl acetate (LC grade) were obtained from Alps (Taipei, Taiwan) and Mallinckrodt Baker, Inc. (U.S.A.). 5,7-Dimethoxycoumarin (99%) was purchased from Aldrich (Milwaukee, WI, U.S.A). Dimethylacetamide was obtained from Wako (Osaka, Japan). PEG 400 and hydrochloric acid were purchased from Merck (Germany). Potassium biphthalate was available from Katayama Chemical (Osaka, Japan). Milli-Q plus water (Millipore, Bedford, MA, U.S.A.) was used for all preparation. Chenpi (Pericarpium Citric Retculate) was purchased from a herbal store in Taichung, Taiwan.

II. *Instrumentation and HPLC conditions*

The HPLC apparatus included two pumps (LC-6AD, Shimadzu, Japan), an SLC-6B controller, an UV spectrophotometric detector (SPD-6A, Shimadzu, Japan) and a chro-

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matopac (C-R6A, Shimadzu, Japan). The RP-18e column (LiChrospher 100, 5 µm, 250 × 4 mm) was equipped with a guard column (LiChrospher 100, 5 μ m). The mobile phase was acetonitrile/1% acetic acid (36/65) for serum and methanol/acetonitrile/1% acetic acid (17/26/57) for urine, respectively. The flow rate was 1.0 ml/min with the detection wavelength set at 287 nm. The injection volume of HPLC sample was 20 µL. Shaker ZC4000 was provided by Deng Ying Co. (Taichung, Taiwan). Nitrogen evaporator (N-EVAP112) was supplied by Organomation Associates, Inc. (U.S.A.)

III. *Preparation of calibration curves*

Calibration curves were established for the quantitation of hesperetin in serum and urine samples.

a. For serum assay

Hesperetin stock solutions (in CH_3OH) in various concentrations were spiked into blank serum to afford a series of serum standards consisting of 25.0, 12.5, 6.3, 3.1, 1.6, 0.8, and 0.4 µg/mL. To 300 µL serum standard, 200 µL buffer solution (pH 5.0), 90 µL ascorbic acid (75 mg/mL), and 90 µL 0.1 N HCl were added, followed by partitioning with 680 µL ethyl acetate (containing 5, 7- dimethoxycoumarin, 2.5 µg/mL). The ethyl acetate layer was evaporated under N_2 , reconstituted with 50 µL acetonitrile and then 20 µL was subjected to HPLC analysis.

b. For urine assay

Hesperetin stock solution (in CH_3OH) in various concentrations were spiked into blank urine to afford urine standards consisting of 50.0, 25.0, 12.5, 6.3, 3.1 and 1.6 µg/ml. To 100 µL urine standard, 70 µL buffer solution (pH 5.0), 30 µL ascorbic acid (75 mg/mL), and 30 µL 0.1 N HCl were added, followed by partitioning with 230 µL ethyl acetate (containing 5, 7-dimethoxycoumarin, 10.0 µg/mL). The subsequent procedures were the same as those for serum standards.

The peak-area ratios (hesperetin to 5, 7-dimethoxycoumarin) of serum standards and urine standards at various concentrations were determined in duplicates, respectively. Calibration curves were drawn for serum and urine after linear regressions of the peak-area ratios against the concentrations of hesperetin.

IV. *Validation of assay method*

The precision of the system was evaluated using the intra-day and inter-day assays of six serum calibrators and five urine calibrators over three consecutive days in triplicates. The accuracy of the system was expressed by the relative error of the mean calculated concentration from the concentration of each calibrator.

The accuracy of the method was further assessed with recovery studies by spiking hesperetin stock solution into blank serum, blank urine and water, in triplicates to afford 0.4, 25.0, 100.0 µg/mL for serum and 1.6, 12.5, 50.0 µg/mL for urine. The concentrations of hesperetin obtained in blank serum or blank urine to the corresponding ones in water were compared to calculate the recovery.

V. *Drug administration*

Male New Zealand white rabbits, weighing 2-3 kg, were fasted for 24 hr before drug administration. Hesperetin was freshly dissolved in a solvent mixture containing dimethylacetamide, PEG 400 and water (1: 5: 4), whereas hesperidin was suspended in the same solvent mixture.

An IV bolus of hesperetin was given to rabbits via the left ear vein at a dose of 20 mg/kg. The solution was filtered through a 0.2 µm membrane before administration. On the other hand, oral doses of 20 mg/kg hesperetin and 100 mg/kg hesperidin were given to rabbits with gastric gavage.

Decoction of Pericarpium Citri Reticulatae was prepared by boiling 10 g crude drug of Pericarpium Citri Reticulatae with water (200 mL) until the volume reduced to less than 100 mL, filtered while hot and then added with adequate water to 100 mL. A male, 22 years old, weighing 60 kg, was fasted overnight and received a dose of 100 mL warm decoction which was quantitated by HPLC method to contain 37.8 mg hesperidin.

VI. *Blood and urine collection*

After administration, blood samples of rabbits were withdrawn from left ear vein at specific time points and allowed to clot and then centrifuged at 9860 g for 15 min to obtain serum which was stored at -30°C until analysis. Several urine samples of the male volunteer were collected after specific time intervals. Then an aliquot of each sample was stored at -30°C until analysis.

VII. *Determination of hesperetin and its glucuronides/sulfates in serum and urine*

For the determination of the parent form hesperetin in serum, 300 µL sample was processed as described for calibrators. For the determination of total hesperetin including parent form and its glucuronides/sulfates, serum sample (300 µL) was added with 200 µL β-glucuronidase (β-glucuronidase 110.4 units/mL, sulfatase 4.2 units/mL in pH 5 buffer) and 90 µL ascorbic acid (75 mg/mL). The vial was wrapped with aluminum foil. Incubation was conducted in a water bath shaker at 37°C for 4 hr. After incubation, each sample was treated as calibrators prior to HPLC analysis. The concentration of hesperetin glucuronides/sulfates in each serum sample was calculated by subtracting the parent form concentration from the total hesperetin concentration after enzymatic hydrolysis.

For the determination of hesperetin glucuronides/sulfates in urine, 100 µL sample was added with 70 µL β-glu-

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curonidase (β-glucuronidase 552 units/ml, containing sulfatase 21 units/mL in pH 5 buffer) and 30 µL ascorbic acid (75 mg/mL). The subsequent treatments followed the same procedures for serum samples.

RESULTS AND DISCUSSION

Because the standards of hesperetin glucuronides/sulfates were not available, an alternative method for determination of hesperetin conjugate metabolites in serum or urine was established using glucuronidase/sulfatase to release hesperetin which was then assayed by HPLC. In our preliminary study, it was found that the total hesperetin, including parent form and those hydrolyzed from its conjugated metabolites, was significantly lower than the parent form determined prior to enzymatic treatment. This was a clear indication that hesperetin might decompose during the incubation for enzymatic hydrolysis. In order to prevent the potential oxidation of hesperetin, ascorbic acid was added to the serum and the incubation was conducted away from light with reference to our previous method for protection of naringenin and quercetin[16,18]. A time study determined the optimal hydrolysis time for the conjugated metabolites of hesperetin to be 4 hrs while incubated with glucuronidase/sulfatase. Our hydrolysis time was quite different from a previous study[19] which conducted the hydrolysis for 17 hrs. This might be due to the difference in the amount of

enzymes used. Moreover, the liquid-liquid partition using ethyl acetate for the extraction of hesperetin adapted in the present method was simply carried out in a microvial which was much easier and cheaper than a previous report using solid phase extraction method[19].

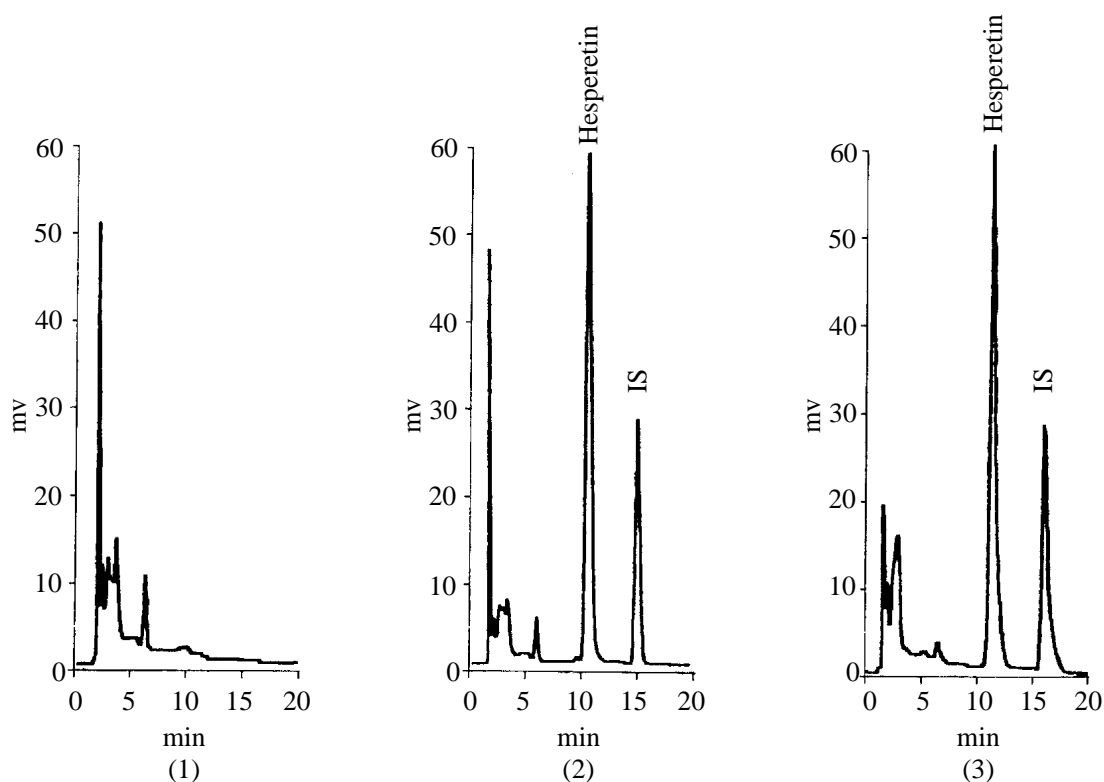As shown in Figures 1 and 2, hesperetin was well resolved using acetonitrile/1% acetic acid (36/65) and
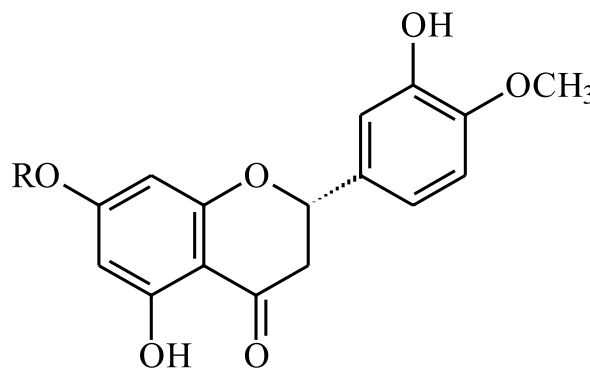
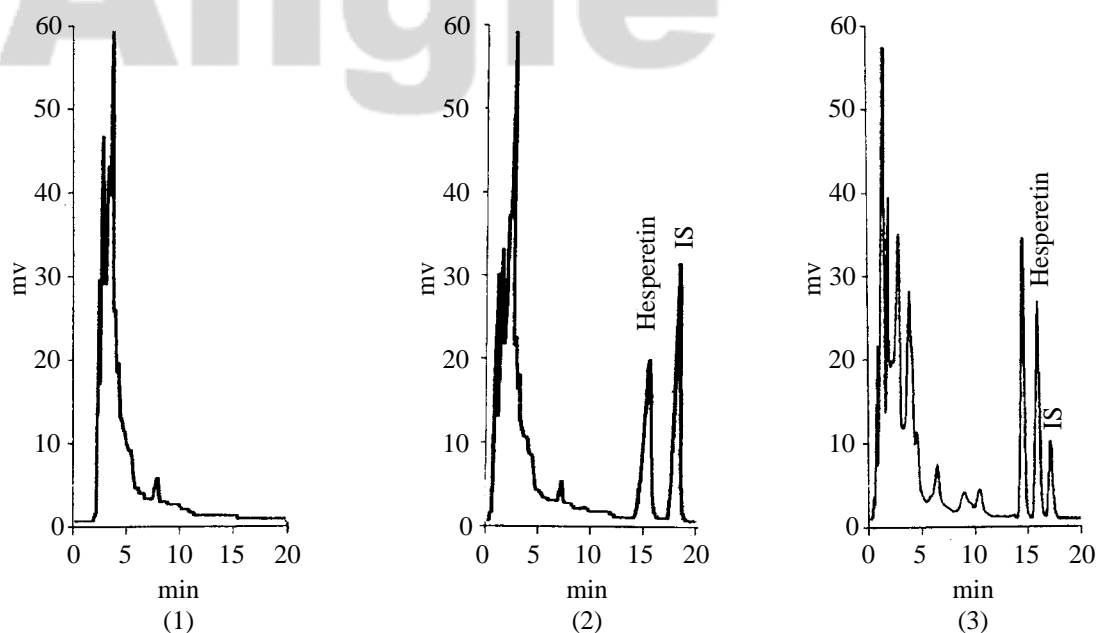|	R
Hesperetin :	H
Hesperidin :	Rhamnoglucose

Structures of hesperetin and hesperidin

Figure 1. Chromatograms of hesperetin with internal standard (IS: 5,7-dimethoxycoumarin) in rabbit serum: (1) blank serum; (2) hesperetin (11 mim, 12.5 µg/mL) and internal standard spiked in serum; (3) serum sample (9.6 µg/mL of hesperetin) obtained 30 min after oral administration of hesperetin.

Figure 2. Chromatograms of hesperetin with internal standard (IS: 5,7-dimethoxycoumarin) in urine: (1) blank urine; (2) hesperetin (14 min, 3.1 µg/mL) and internal standard spiked in blank urine; (3) urine sample (5.6 µg/mL of hesperetin) of the volunteer collected during 2-4 hr after oral administration of decoction of Pericarpium Citri Reticulatae.

Table 1. Intra-day and inter-day analytical precision and accuracy of hesperetin in rabbit serum.

Conc. (µg/mL)	Intraday		Interday	
	Precision Mean ± S.D.(C.V.%)	Accuracy (%)	Precision Mean ± S.D.(C.V.%)	Accuracy (%)
25.0	24.5 ± 0.1 (0.2)	-1.9	24.3 ± 0.3 (1.2)	-3.0
12.5	12.3 ± 0.1 (0.9)	-1.3	12.6 ± 0.4 (3.4)	0.4
6.3	6.4 ± 0.1 (2.3)	2.8	6.3 ± 0.02 (0.3)	0.1
3.1	3.2 ± 0.03 (0.8)	1.0	3.1 ± 0.04 (1.4)	0.7
1.6	1.6 ± 0.00 (0.2)	3.8	1.6 ± 0.1 (3.4)	4.7
0.8	0.7 ± 0.00 (0.04)	-7.2	0.8 ± 0.02 (2.8)	-8.2
0.4	0.3 ± 0.01 (3.4)	-14.7	0.4 ± 0.03 (7.1)	-7.1

n=3

Table 2. Intra-day and inter-day analytical precision and accuracy of hesperetin in rabbit urine.

Conc. (µg/mL)	Intraday		Interday	
	Precision Mean ± S.D.(C.V.%)	Accuracy (%)	Precision Mean ± S.D.(C.V.%)	Accuracy (%)
50.0	49.8 ± 1.1 (2.1)	-0.4	50.0 ± 1.2 (2.4)	0.04
25.0	24.1 ± 0.5 (2.2)	-3.7	24.3 ± 0.3 (1.4)	-2.8
12.5	13.5 ± 0.1 (0.7)	-7.8	13.5 ± 0.1 (0.6)	-8.0
6.3	6.3 ± 0.1 (1.5)	-1.0	6.4 ± 0.03 (0.5)	-1.7
3.1	3.1 ± 0.02 (0.6)	-0.3	3.2 ± 0.03 (0.8)	-0.9
1.6	-1.5 ± 0.04 (2.8)	-2.5	1.5 ± 0.1 (4.1)	-3.8

n=3

methanol/acetonitrile/1% acetic acid (17/26/57) as the mobile phases for serum and urine assays, respectively. Regarding urine analysis, a complex mobile phase system including three solvents was used due to the overlap among the internal standard, hesperetin, and naringenin. The latter two were hydrolyzed from the metabolites of hesperidin and narirutin in Pericarpium Citri Reticulatae, respectively. Hesperetin in both serum and urine were eluted within 20 min. Good linearity (r = 0.999) were shown in the ranges of 0.4~25.0 µg/mL (Y= 0.36 x + 0.09) and 1.6~50.0 µg/mL (Y= 0.11 x + 0.08) in serum and urine, respectively. The intraday and interday coefficients of variation for hesperetin assay in serum were 0.0~3.4% and 0.3~7.1%, respectively, as shown in Table 1, while those for urine assay were 0.6~2.8% and 0.5~4.1%, respectively, as shown in Table 2. The accuracy was satisfactory for both serum and urine assays. The limits of quantitation (LOQ) representing the lowest concentration of analyte in any sample that can be

Table 3. Recovery (%) of hesperetin from rabbit serum.

Conc. spiked	10.0 µg/mL	25.0 µg/mL	0.4 µg/mL
1	102.1	95.8	95.2
2	105.9	89.1	93.2
3	105.7	94.3	98.9
Mean ± S.D.	101.1 ± 0.6	106.0 ± 2.5	105.7 ± 4.1

n=3

Table 4. Recovery (%) of hesperetin from the volunteer's urine.

Conc. spiked	50.0 µg/mL	12.5 µg/mL	1.6 µg/mL
1	100.5	104.1	105.2
2	101.1	105.1	101.8
3	101.7	108.9	110.0
Mean ± S.D.	101.1 ± 0.6	106.0 ± 2.5	105.7 ± 4.1

n=3

determined with acceptable precision and accuracy were 0.4 µg/mL and 1.6 µg/mL for serum and urine, respectively. The limits of detection (LOD) representing the lowest concentration of analyte in any sample that can be detected were both 0.06 µg/mL in serum and urine. Good recoveries obtained from serum and urine were 93.1~104.5 and 101.1~105.7%, as shown in Table 3 and Table 4, respectively, indicating almostly quantitative recoveries.

Using our HPLC method prior to and after enzymatic pretreatment on serum samples after oral administration of hesperetin, hesperetin and its conjugate metabolites were quantitated. While after oral administration of hesperidin, neither hesperidin nor its conjugates was detected in serum. Also, neither hesperetin nor its conjugate metabolites was detected. It might be attributable to the poor dissolution and thus poor absorption of hesperidin in gastrointestinal tract. However, hesperetin conjugate metabolites were detected in the urine of the volunteer after intake of decoction of Pericarpium Citri Reticulatae. This might be attributable to the solubilization effect on hesperidin by other component(s) present in the decoction.

In summary, according to our present method, hesperetin was well protected from decomposition when serum or urine samples were incubated with enzymes. Furthermore, the HPLC methods developed in this study were rapid and good in precision, accuracy, selectivity, sensitivity, and recovery and thus are applicable in pharmacokinetic studies of hesperidin and hesperetin as well as fruits, juices and Chinese herbs containing them.

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血清與尿液中橙皮素及其結合態代謝物之定量

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摘　要

　　橙皮苷與橙皮素為雙氫黃酮類，具抗胃潰瘍、抗氧化及抗癌等作用，存在於許多柑橘屬果實中。橙皮素為橙皮苷之苷元代謝物。本研究開發高效液相層析法檢測口服橙皮素、橙皮苷與陳皮水煎劑後之血清或尿液於酶水解前後之橙皮素含量，以分別定量其中之橙皮素及其結合態代謝物。酶解使用 β-葡萄糖醛酸酶／硫酸酶之混合酶於37°C下進行，採避光並添加抗壞血酸。高效液相層析法採用LiChrospher 100 RP-18e column，以5,7-dimethoxycoumarin為內標準，血清分析之移動相組成為乙腈／1% 醋酸水溶液（36/65, v/v），尿液分析之移動相組成為甲醇／乙腈／1% 醋酸水溶液（17/26/57, v/v）。此等分析方法快速、靈敏、再現性、精密度、準確度俱佳且回收率高。因此，本方法可提供橙皮苷、橙皮素或含彼等成分之柑橘屬食品或中草藥之藥物動力學研究。

關鍵詞：橙皮苷，橙皮素，結合態代謝物，高效液相層析法，酶解