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HPLC Assays of Naringin and Hesperidin in Chinese Herbs and Serum

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ABSTRACT

High performance liquid chromatographic methods for simultaneous determination of naringin and hesperidin in Chinese herbs and serum were established. By using a LiChrospher RP-18 column, the herbal extracts were separated by gradient elution with water-acetonitrile(0-10 min 80:20, 14-25 min 63:37) and the serum sample was separated by isocratic elution with water-acetonitrile(80:20) as the mobile phase. Both methods employed a flow rate of 1.0 ml/min with detection at 270 nm. Ethylparaben and vanillin were used as the internal standards in herbal extracts and in serum, respectively. The precision and accuracy of these methods were satisfactory for intraday and interday assays. The recoveries of naringin and hesperidin were almost quantitative from serum and from herbs including Fructus Aurantii Immaturus, Fructus Citri Immaturus Exsiccatus and Pericarpium Citri Chachiensis.

Key words: **naringin, hesperidin, HPLC, herb, serum.**

INTRODUCTION

Fructus Aurantii Immaturus (枳實), Fructus Citri Immaturus Exsiccatus (枳 殼) and Pericarpium Citri Chachiensis (陳皮) are dried peels or small fruits of several *Citrus* species, including *C. wilsonii* Tanaka, *C. aurantium* Linne., *C. sinensis* Osbeck, *C. chachiensis* Hortorum and *C. reticulata* Blanco. They are widely used in clinical Chinese medicine for regulating "qi" and enabling "qi" to flow smoothly in the human body [1].

Fructus Aurantii Immaturus (FAI) and Fructus Citri Immaturus Exsiccatus (FCIE) contain naringin and hesperidin [2,3]. Pericarpium Citri Chachiensis (PCC) contains hesperidin [4]. Naringin and hesperidin are flavanone glycosides, also found in orange juice, grapefruit juice and in many commercial health foods, too[5,6,7,8,9]. The structures of naringin and hesperidin are shown in Fig 1. Both compounds were found to show superoxide scavenger activity [10]. Although many HPLC methods have been reported for the analysis of naringin and hesperidin in juice, young leaves and immature fruit of *Citrus*, all of these previous methods used acid, other additives or a quaternary mixture in their mobile phases to separate a large number of flavonoids at one time

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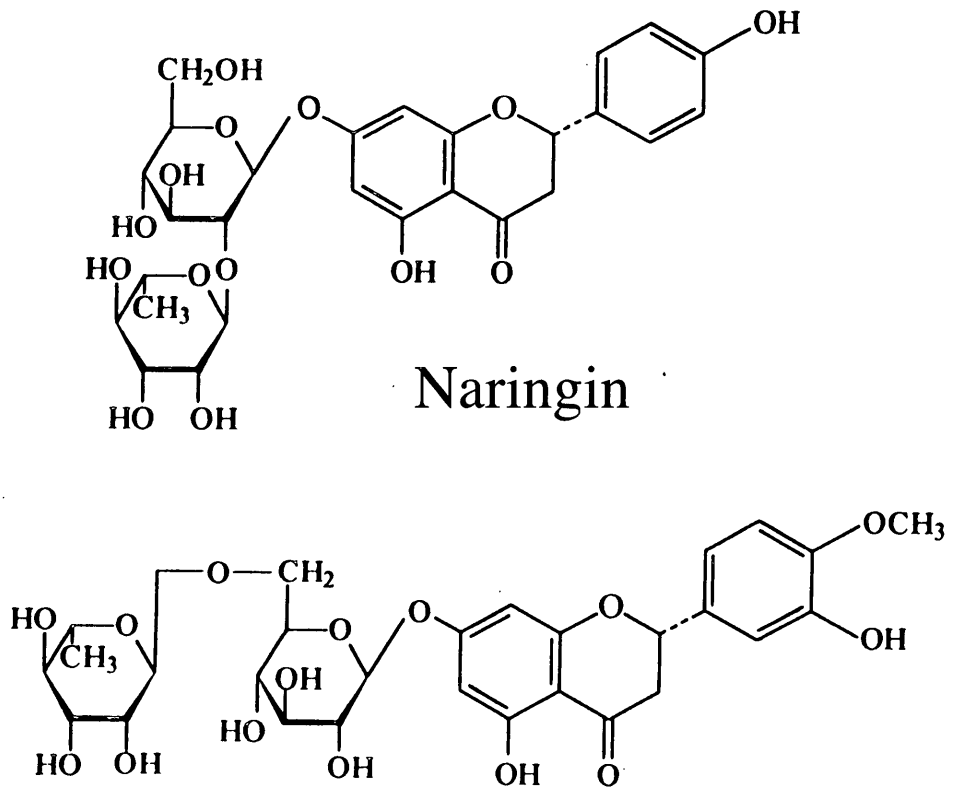

Figure 1. Structures of naringin and hesperidin.

[5,6,7,8]. Moreover, an HPLC method for the analysis of naringin and hesperidin in serum has not been found in the literature. This study is to establish simpler, efficient and reliable methods for the simultaneous quantification of naringin and hesperidin in Chinese herbs and in serum.

MATERIALS AND METHODS

I. *Materials*

FAI, FCIE and PCC were purchased from three Chinese herb stores at Taichung, Yuan-Lin and Chia-Yee in Taiwan.

Blank serum was obtained from volunteers after overnight fasting.

Ⅱ. *Chemicals and Reagents*

Naringin and hesperidin were purchased from Aldrich (97%, Milwaukee, WI, U.S.A.) and ethylparaben and vanillin from Sigma (St. Louis, MO, U.S.A.). Acetonitrile, methanol and water (HPLC grade) were purchased from ALPS Chem. Co. (Taiwan). DMSO (dimethylsulfoxide) was purchased from Merck (Darmstadt, Germany).

Ⅲ. *Instrumentation*

The HPLC apparatus was equipped with two pumps (LC-6AD, Shimadzu, Japan), monitored by an SLC-6B controller, a photodiode array detector (SPD-M6A, Shimadzu, Japan) or an UV spectrophotometric detector (SPD-6A, Shimadzu, Japan) and a data processor (Acer 1116 SX) with a NEC color printer or a chromatopac (C-R6A, Shimadzu, Japan). The RP-18 column (LiChrospher 100, 5μm, 250×4 mm) was equipped with a guard column (LiChrospher 100,

5μm).

Ⅳ. *Liquid Chromatography*

A water-acetonitrile mixture was used as the mobile phase. For the separation of herbal water extracts, the elution consisted of three stages: the first, with isocratic elution for 10 min, used 80:20 water-acetonitrile; then a gradient elution began at 10 min, which gradually changed the water-acetonitrile ratio to 63:37 at 14 min; in the third and final stage, this ratio was maintained until the end of the run at 25 min. It then took 5 min to return to the initial state before starting a second run. For the analysis of serum, isocratic elution was carried out using water-acetonitrile (80:20). For all analysis, the flow rates were 1.0 ml/min and detection was at 270 nm.

Ⅴ. *Preparation of Standard Solutions*

(I) For herbal analysis: Naringin or hesperidin was accurately weighed and dissolved in methanol (two drops of dimethylsulfoxide were added to help hesperidin dissolve in the methanol) to give various concentrations within the ranges of 10.0-400.0 μg/ml and 5.0-200.0 μg/ml, respectively. A suitable amount of internal standard (ethylparaben in methanol, 50 μg/ml) solution was added to give a concentration of 25.0 μg/ml in each calibrator.

(Ⅱ) For serum analysis: Naringin and hesperidin solutions of appropriate concentrations (20 μl) were mixed with blank serum (180 μl) and internal standard solution (vanillin in acetonitrile, 6 μg/ml, 600 μl) to afford calibrators in concentrations of 50.0, 25.0, 12.5, 5.0, 2.5 and 1.25 μg/ml of naringin and hesperidin. The concentration of vanillin in each calibrator was 4.5 μg/ml.

Calibration graphs were plotted after linear regression of the peak-area ratios with concentrations.

Ⅵ. *Preparation of Samples*

(I) For the preparation of water extract, each herb (1.0 g) was extracted twice with water (35 ml) in a water bath at 100°C for 15 min and filtered into an 100 ml volumetric flask to which water was then added to the meniscus. The herbal sample solution (500 μl) was mixed with the internal standard solution (50.0 μg/ml, 500 μl), vortexed for 1 min and then centrifuged at 15,000 rpm for 5 min. The supernatant was filtered through a 0.45 μm membrane before being used for HPLC analysis.

(Ⅱ) For the assay of serum, 200 μl of serum sample was mixed with internal standard solution (6.0 μg/ml, 600 μl), vortexed for 1 min and then centrifuged at 15,000 rpm for 5 min. The supernatant was decanted and evaporated by blowing N_2 gas, then reconstituted with 200 μl of acetonitrile for HPLC analysis.

Ⅶ. *Recovery Study*

(I) For herbal extracts: After analysis of naringin and hesperidin content, the herbal sample(250 μl) was mixed with standard solution (250 μl) of appropriate concentrations and internal standard solution (500 μl), vortexed for 1 min and then centrifuged at 15,000 rpm for 5 min. The supernatant was used immediately for HPLC analysis. The recoveries were calculated based on the detected amount compared to the amount of added standard. Each analysis was replicated three times.

(Ⅱ) For serum: 180 μl of blank serum or water was mixed with naringin and hesperidin solutions (20 μl) in concentrations of 500.0, 125.0 and 12.5 μg/ml, as well as internal standard solution (600 μl), vortexed for 1 min and then centrifuged at 15,000 rpm for 5 min. The supernatant was used immediately for HPLC analysis. The recoveries were calculated based on the detected concentration in serum compared to that in water. All procedures were performed in triplicate.

RESULTS AND DISCUSSION

Naringin and hesperidin are bioflavonoids with similar structures and properties. Both compounds are present in *Citrus* fruits. It was not easy to separate or distinguish them in HPLC when eluted in a short period. Previous studies

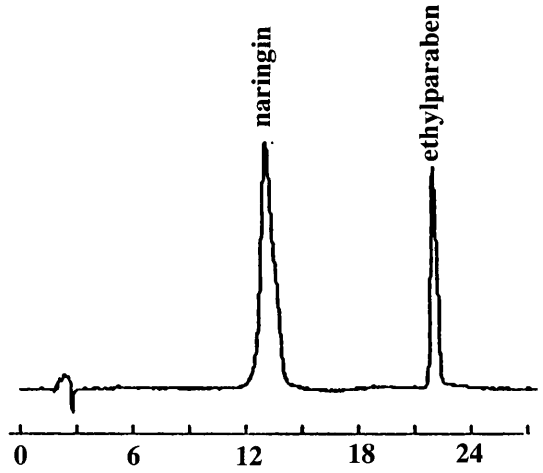

Figure 2. Chromatogram of naringin with internal standard (ethylparaben).

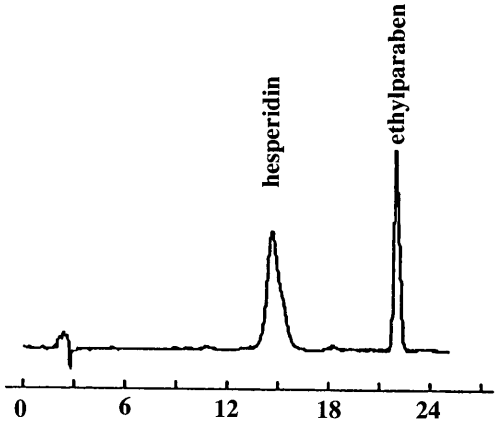

Figure 3. Chromatogram of hesperidin with internal standard (ethylparaben).

Table 1. Intraday and interday analytical precisions for naringin (n=3)

conc. (μg/ml)	Intraday mean ± S.D. (R.S.D.)	Interday mean ± S.D. (R.S.D.)
400	395.2 ± 2.7 (0.7 %)	396.3 ± 3.6 (0.9 %)
300	303.3 ± 2.8 (0.9 %)	296.8 ± 5.5 (1.9 %)
150	152.0 ± 0.8 (0.5 %)	149.3 ± 3.3 (2.2 %)
50	48.0 ± 0.3 (0.6 %)	45.6 ± 1.6 (3.5 %)
25	24.9 ± 0.3 (1.2 %)	23.5 ± 0.9 (3.8 %)
10	10.4 ± 0.2 (1.9 %)	10.2 ± 0.5 (4.9 %)

Table 2. Intraday and interday analytical precisions for hesperidin (n=3)

conc. (μg/ml)	Intraday mean ± S.D. (R.S.D.)	Interday mean ± S.D. (R.S.D.)
200	202.7 ± 2.3 (1.1 %)	203.7 ± 3.3 (1.6 %)
100	102.3 ± 0.7 (0.6 %)	104.4 ± 2.1 (2.0 %)
50	51.1 ± 1.5 (2.9 %)	52.0 ± 2.0 (3.8 %)
25	25.4 ± 0.4 (1.6 %)	26.1 ± 0.8 (3.1 %)
10	10.5 ± 0.3 (2.9 %)	10.7 ± 0.4 (3.7 %)
5	4.7 ± 0.2 (4.3 %)	4.9 ± 0.2 (4.1 %)

used acidic mobile phase and took more than 30 min for the simultaneous determination of naringin and hesperidin in FAI, and FCIE[2,3]. Rutin has been used as an internal standard in the study of FCIE [3]. No internal standard was reported in the studies of FAI or PCC [2,4]. This study has found ethylparaben to be a suitable internal standard for the assays of these three crude drugs (FAI, FCIE, PCC) and water-acetonitrile mixture to be a good mobile phase without

Table 3. The amount of naringin and hesperidin in water extracts from each gm of crude drug

Crude drug samples [a]		Naringin (mg)	Hesperidin (mg)
FAI	A	10.83	–
	B	15.32	–
	C	26.60	–
FCIE	A	50.81	2.40
	B	49.23	–
	C	58.61	–
PCC	A	–	8.38
	B	–	15.63
	C	–	18.63

FAI: Fructus Aurantii Immaturus.
FCIE: Fructus Citri Immaturus Exsiccatus.
PCC: Pericarpium Citri Chachiensis.
[a]A, B and C represent samples purchased from different stores.

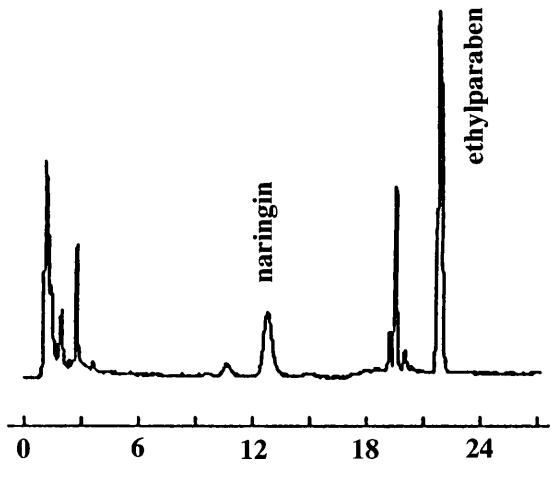

Figure 4. Chromatogram of FAI extracted by H_2O.

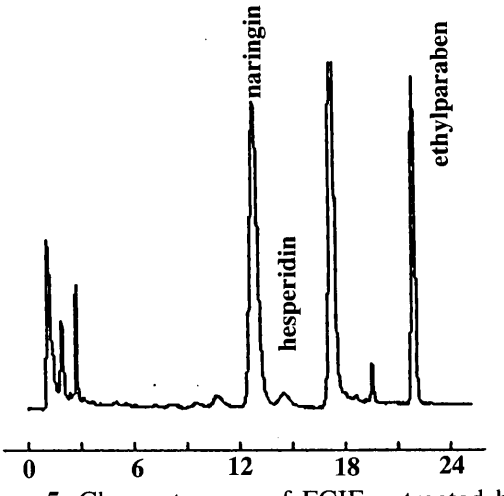

Figure 5. Chromatogram of FCIE extracted by H_2O.

acidification. It took only 25 min for a complete run. Compared to previous studies, this method is simpler and more rapid. The chromatograms of naringin and hesperidin with internal standard are shown in Fig. 2 and Fig. 3, respectively. The detection limits of this method were 0.5 μg/ml for naringin and hesperidin (S/N > 3).

Calibration graphs for naringin and hesperidin were obtained over the ranges 10.0-400.0 and 5.0-200.0 μg/ml, respectively. The regression equations were $y = 8.619 \times 10^{-3}x - 2.179 \times 10^{-3}$ ($r = 0.9997$) for naringin and $y = 7.545 \times 10^{-3} x + 1.993 \times 10^{-3}$ ($r = 0.9999$) for hesperidin, where y is the peak-area ratio of naringin or hesperidin to the internal standard and x is the concentration of naringin or hesperidin. These results show good linear relationships between peak-area ratios and concentrations within the above ranges.

To evaluate the precision of the system, standard solutions of naringin at concentrations of 400.0, 300.0, 150.0, 50.0, 25.0 and 10.0 μg/ml were injected into the system three times in one day and three times on three consecutive days. The results are shown in Table 1. The intraday relative standard deviations (R.S.D.) were 0.7, 0.9, 0.5, 0.6, 1.2 and 1.9%, respectively. The interday R.S.D. were 0.9, 1.9, 2.2, 3.5, 3.8 and 4.9%, respectively. Standard solutions of hesperidin at concentrations of 200.0, 100.0, 50.0, 25.0, 10.0 and 5.0 μg/ml were injected into the system three times in one day and three times on three consecutive days. The results are shown in Table 2. The intraday R.S.D. were 1.1, 0.6, 2.9, 1.6, 2.9 and 4.3%, respectively. The interday R.S.D. were 1.6, 2.0, 3.8, 3.1, 3.7 and 4.1%, respectively. These results indicated that the intraday and interday analytical precisions for naringin and hesperidin were satisfactory. Application of the developed method to herbs provided the results given in Table 3. Figures 4, 5 and 6 showed chromatograms of the water extracts of FAI, FCIE and PCC, respectively. The peak purities of naringin and hesperidin were monitored with a photodiode-array detector and showed no interference within the peak. The accuracy of this method was determined by recovery tests which indicated both naringin and hesperidin were almost quantitatively recovered from herbal water extracts (Table 4).

The quantitative results indicated that both FAI and FCIE contained significant amounts of naringin although there was less in FAI. No hesperidin was found in FAI and only a trace amount of hesperidin was detected in one of the three samples of FCIE. PCC contained significant amount of hesperidin, but no naringin was detected. Previous studies reported that FAI contained more naringin and little hesperidin[2], but that FCIE contained more hesperidin and little naringin[3]. This latter result is in apparent contradiction to the findings of the present study in terms of the content of hesperidin in FCIE. FAI comes from the same fruit as FCIE, but FAI is

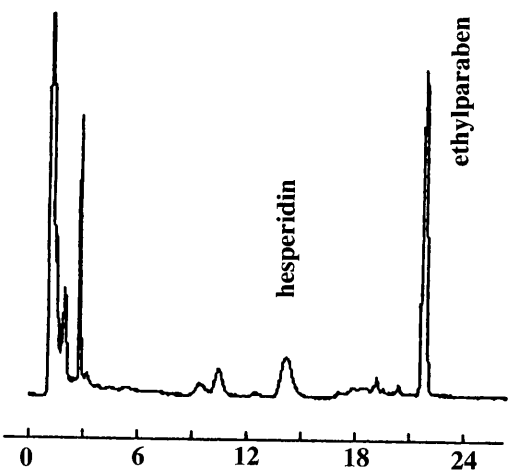

Figure 6. Chromatogram of PCC extracted by H_2O.

Table 4. Recovery (%) of naringin and hesperidin from water extracts of crude drug

Constituent	naringin	naringin	hesperidin
Crude drug	FAI	FCIE	PCC
1	103.8	103.1	99.5
2	102.5	98.2	98.9
3	100.9	105.5	103.2
mean±S.D.	102.4±1.2	102.3±3.0	100.5±1.9

FAI: Fructus Aurantii Immaturus.
FCIE: Fructus Citri Immaturus Exsiccatus.
PCC: Pericarpium Citri Chachiensis.

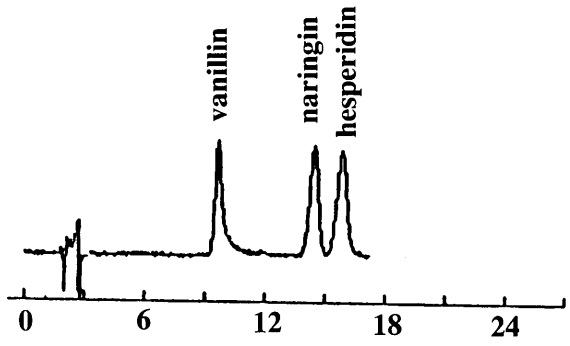

Figure 7. Chromatogram of naringin and hesperidin with internal standard (vanillin) in mobile phase.

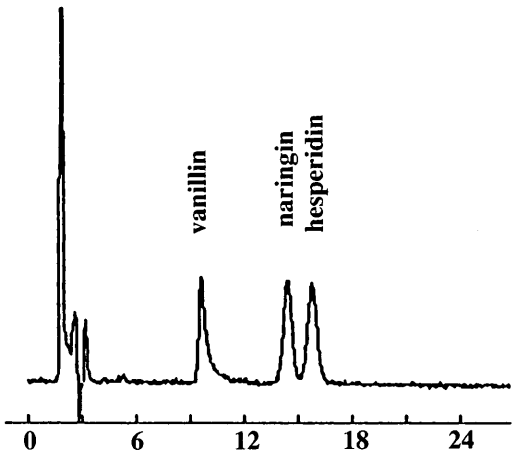

Figure 8. Chromatogram of naringin and hesperidin with internal standard (vanillin) in serum.

Table 5. Intraday and interday analytical precisions of naringin in serum (n=3)

conc. (μg/ml)	Intraday mean ± S.D. (R.S.D.)	Interday mean ± S.D. (R.S.D.)
50.0	49.40 ± 0.63 (1.28 %)	49.34 ± 1.0 (2.03 %)
25.0	25.15 ± 0.13 (0.52 %)	24.97 ± 0.27 (1.08 %)
12.5	12.76 ± 0.17 (1.33 %)	12.82 ± 0.22 (1.65 %)
5.0	5.10 ± 0.12 (2.35 %)	5.23 ± 0.22 (4.21 %)
2.5	2.30 ± 0.06 (2.61 %)	2.48 ± 0.10 (4.03 %)
1.25	1.22 ± 0.02 (1.64 %)	1.18 ± 0.04 (3.39 %)

harvested two months earlier than FCIE [11]. Thus although we cannot account for the findings of the previous report[3], we consider our result to be more reasonable from the biosynthetic point of view.

Figures 7 and 8 show the chromatograms of naringin and hesperidin with vanillin as the internal standard spiked in mobile phase and serum, respectively. Calibration curves for naringin and hesperidin in serum were obtained over the range 1.25-50.0 μg/ml. The regression equations were $y = 2.27 \times 10^{-2}x - 9.901 \times 10^{-5}$ for naringin ($r = 0.9999$) and $y = 2.569 \times 10^{-2}x - 6.269 \times 10^{-3}$ ($r = 0.9998$) for hesperidin, where y is the peak-area ratio of naringin or hesperidin to the internal standard in serum and x is the concentration of naringin or hesperidin. These results showed good linear relationships between peak-area ratios and concentrations within the range 1.25-50.0 μg/ml.

To evaluate the precision of the system, serum concentrations of naringin and hesperidin at 50.0, 25.0, 12.5, 5.0, 2.5,1.25 μg/ml were injected into the system three times in one day and three times on three consecutive days. The results are shown in Tables 5 and 6. The R.S.D.of intraday and interday assays were satisfactory. Recovery tests indicated that hesperidin were quantitatively recovered from serum as shown in Table 7. This method is simple and efficient for serum assay.

In summary, the HPLC methods established

Table 6. Intraday and interday analytical precisions of hesperidin in serum (n=3)

conc. (μg/ml)	Intraday mean ± S.D. (R.S.D.)	Interday mean ± S.D. (R.S.D.)
50.0	49.46 ± 0.97 (1.96 %)	49.83 ± 1.48 (2.97 %)
25.0	24.88 ± 0.36 (1.45 %)	24.36 ± 0.93 (3.82 %)
12.5	12.36 ± 0.09 (0.73 %)	12.58 ± 0.27 (2.15 %)
5.0	4.79 ± 0.08 (1.67 %)	5.04 ± 0.26 (5.16 %)
2.5	2.46 ± 0.10 (4.07 %)	2.49 ± 0.13 (5.22 %)
1.25	1.41 ± 0.05 (3.55 %)	1.49 ± 0.07 (4.73 %)

Table 7. Recovery (%) of naringin and hesperidin from serum

Constituent	naringin			hesperidin		
conc. (μg/ml)	50.0	12.5	1.25	50.0	12.5	1.25
1	101.2	103.5	104.6	100.0	98.9	106.1
2	97.1	101.7	99.0	94.7	106.4	102.1
3	99.9	98.4	102.7	99.3	104.4	97.8
mean±S.D.	99.4±1.7	101.2±2.1	102.1±2.3	98.0±2.3	103.2±3.2	102.0±3.4

in this study could be applied to the quantification of naringin and hesperidin in FAI, FCIE and PCC or for pharmacokinetic studies of naringin and hesperidin in animals or in man.

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中藥材及血清中柚皮苷及柑果苷之高效層析定量

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摘 要

本研究建立高效液相層析法分析並定量中藥材枳實、枳殼和陳皮中以及人體血清中的柚皮苷（naringin）及柑果苷（hesperidin）兩成分。利用 LiChrospher RP-18 管柱為固定相，中藥材之水萃取物以水和氰甲烷之混合液為移動相進行梯度沖提（0-10 分鐘 80：20，14-25 分鐘 63：37），ethylparaben 為內標準；血清檢品則以水和氰甲烷之混合液為移動相進行等壓沖提（80：20），vanillin 為內標準。兩種方法流速皆採 1 ml/min，檢測波長皆以紫外光 270 nm。同日內及異日間之測試結果顯示此二方法之精確度良好。回收率試驗顯示 naringin 和 hesperidin 皆可從血清或中藥材枳實、枳殼和陳皮中定量回收。

關鍵詞：柚皮苷，柑果苷，高效液相層析法，中藥材，血清。