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Short communication

Determination of eight constituents of Hsiao-cheng-chi-tang by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method for the simultaneous determination of eight constituents (gallic acid, sennoside B, sennoside A, naringin, hesperidin, honokiol, magnolol and emodin) of the Chinese herbal formula Hsiao-cheng-chi-tang was established. Various samples of the formula were separated using a Cosmosil $5C_{18}$ column with a linear gradient elution system consisting of acetate buffer as mobile phase. Contents of these marker substances in an unpretreated Hsiao-cheng-chi-tang extract could be easily determined within 60 min. The effects of pH, buffer concentration and column selectivity for this method are described.

1. Introduction

Chinese herbal preparations have been used for several hundred years and are still widely used, especially in China, Japan and Taiwan. Therefore, suitable assay methods are urgently needed for quality control purposes. Recently, high-performance liquid chromatography (HPLC) [1–5] and capillary electrophoresis (CE) [6–10] have been employed to establish the optimum conditions for examining two or three constituents of the preparations. However, as our knowledge of the effective components of Chinese herbal preparations is still limited and their chemical compositions are very complicated, the exact determination of the quality of a Chinese herbal preparation is very difficult. Efforts to develop simpler and more rapid methods that can assay all the bioactive constituents known are therefore necessary.

Hsiao-cheng-chi-tang (Minor Rhubarb Combination) is a herbal prescription often used for treating patients with bloating, constipation, moist fever and a sinking pulse, and is composed of Rhei Rhizoma, Aurantii Fructus Immaturus and Magnoliae Cortex [11]. It is well known that Aurantii Fructus Immaturus and Magnoliae Cortex can dispel distention and contain a number of bioactive components such as naringin (**4**) and hesperidin (**5**) in the former and magnolol (**7**) and honokiol (**6**) in the latter. Rhei Rhizoma is an antipyretic and has gallic acid (**1**), sennoside A (**3**), sennoside B (**2**) and emodin (**8**) as its major bioactive components [12]. Using these eight compounds (as shown in Fig. 1) as marker substances, an HPLC method was developed. The difference in column selectivity and the suitability of this method are also discussed.

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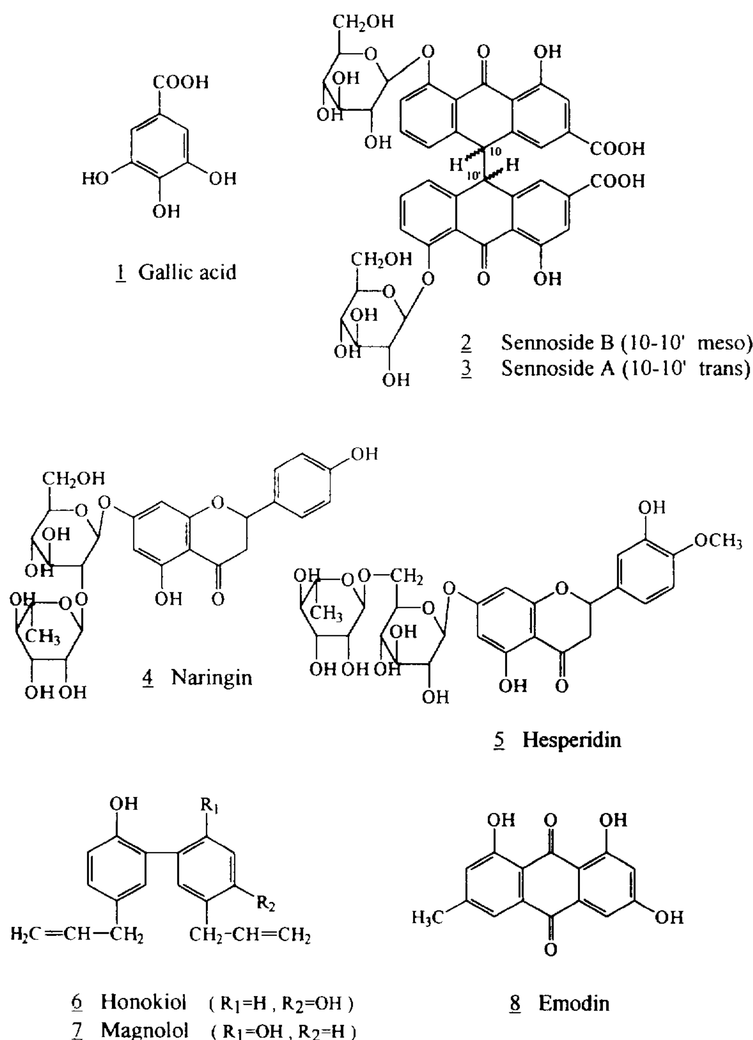

Fig. 1. Structures of the eight marker substances.

2. Experimental

2.1. Reagents and materials

Sennoside A and B were purchased from Yoneyama (Osaka, Japan), emodin and hesperidin from Sigma (St. Louis, MO, USA), naringin from Aldrich (St. Louis, MO, USA), *p-tert.*-octylphenol from Nacalai Tesque (Kyoto, Japan), acetic acid and gallic acid from Merck (Darmstadt, Germany) and sodium acetate from Kanto (Tokyo, Japan). Acetonitrile and methanol were of LC grade (Mallinckrodt, St. Louis, MO, USA). Magnolol and honokiol were isolated from Magnoliae Cortex [13]. Hsiao-cheng-chi-tang was provided by a Chinese pharmaceutical company in Taipei (Taiwan). Peak identification and purity checking of all the marker substances and test samples were done with a photodiode-array detector.

2.2. Preparation of Chinese herbal preparation extract

A 0.5170-g sample of Hsiao-cheng-chi-tang was extracted with 70% methanol (7 ml) by

stirring at room temperature for 30 min, then centrifuged at 1500 *g* for 5 min. Extraction was repeated three times. The extracts were combined and filtered through a No. 1 filter-paper. After adding 2.5 ml of internal standard solution (0.1172 g of *p-tert.*-octylphenol in 100 ml of methanol), the herbal preparation extract was diluted to 25 ml with 70% methanol. A 5-μl volume of this solution was injected directly into the HPLC system.

2.3. Apparatus and conditions

The HPLC system consisted of two Waters Model 510 pumps, a Waters Model 680 automated gradient controller and a Waters Model 991 photodiode-array detector (280 nm). The separations were obtained with a reversed-phase column (Cosmosil $5C_{18}$, 5 μm, 25 cm × 4.6 mm I.D.) (Nacalai Tesque) and by linear gradient elution, using eluents A and B [A = buffer–CH_3CN (9:1), the buffer being an aqueous solution consisting of 0.02 *M* sodium acetate and 0.4197 *M* acetic acid (24 ml of acetic acid was made to a 1000 ml aqueous solution); B = CH_3OH–CH_3CN–1% acetic acid (9:9:2)] according to the following A–B profile: 0 min, 90:10; 14–22 min, 75:25; 30 min, 70:30; 35 min, 20:80; 45–55 min, 0:100; 60 min, 90:10. The flow-rate was kept constant at 0.8 ml/min. A precolumn of μBondapak C_{18} (Millipore, Milford, MA, USA) was used to protect the column.

3. Results and discussion

3.1. Analytical conditions

Two methods for the determination of naringin and hesperidin in Aurantii Fructus Immaturus or herbal preparations containing it have been developed by Ishihara et al. [14] and Wu and Sheu [15]. The former used acetonitrile–water containing 0.05 *M* phosphate as the mobile phase whereas the latter used a mixture of acetonitrile and 0.5% acetic acid. However, with these solvent systems, analysis for the eight marker substances did not afford a satisfactory result. The HPLC separation of Hsiao-cheng-chi-tang extract was conducted by addition of CH_3COONa and CH_3COOH to the mobile phase.

After a series of experiments, it was found that a pH 3.56 solution (mixture of 0.02 *M* sodium acetate and 0.4197 *M* acetic acid) could separate all the constituents well. At lower pH (3.40), the peaks of sennoside A and naringin could not be separated. At higher pH (3.86, adjusted by either increasing the acetate concentration or decreasing the acetic acid concentration), the separation was satisfactory for the eight authentic marker substances but it was unsuitable for the analysis of the extract owing to the overlapping of the sennoside A and B peaks with those of some unknown components of the drug.

Acetic acid (1%) was added to the mixture of the methanol and acetonitrile in reservoir B of the gradient system in order to obtain an ideal chromatogram with a much smoother baseline.

The mobile phase and stationary phase have mutual influences on each other. We therefore fixed the concentration of buffer at 0.02 *M* CH_3COONa and 0.4197 *M* CH_3COOH as discussed above and compared the selectivities of nine commercial C_{18} columns (all 25 cm in length, except column IX, which was 30 cm, as shown in Table 1). After a series of experiments, the capacity factors (k') of the constituents were as shown in Fig. 2.

The data in Fig. 2 show that all the columns except I, V and VIII failed to separate the eight compounds completely. Among columns I, V and VIII, which can give a fairly good separation of all the constituents, column I was found to be the best as it gave a higher resolution in the separation of the crude extract. There was seriously overlapping between the trace sennoside B and some unknown components of the extract when columns V and VIII were used.

From the above results, the best resolution was obtained with a buffer containing 0.02 *M* CH_3COONa and 0.4197 *M* CH_3COOH and with the reversed-phase Cosmosil $5C_{18}$ column. Fig. 3 presents a chromatogram showing the separation

Table 1
Columns used

No.	Column	Producer and location	Column length (cm)	Column I.D. (mm)
I	Cosmosil $5C_{18}$	Nacalai Tesque (Kyoto, Japan)	25	4.6
II	Cosmosil $5C_{18}$-MS	Nacalai Tesque	25	4.6
III	Cosmosil $5C_{18}$-AR	Nacalai Tesque	25	4.6
IV	Intersil ODS-2	GL Science (Tokyo, Japan)	25	4.6
V	LiChrosorb RP-18	Merck (Darmstadt, Germany)	25	4.0
VI	LiChrospher 60RP-Select B	Merck	25	4.0
VII	Purosphere RP-18	Merck	25	4.0
VIII	Nucleosil 100 $5C_{18}$	Macherey–Nagel (Düren, Germany)	25	4.0
IX	Novapak C_{18}	Millipore (Milford, MA, USA)	30	3.9

Particle size = 5 μm for all columns.

of the eight marker substances in a sample of Hsiao-chen-chi-tang extract. The tailing factors (half the peak width at 5% of the peak height divided by the distance between the peak front and the peak maximum measured at 5% of the peak height) of all the peaks to be determined are very close to unity.

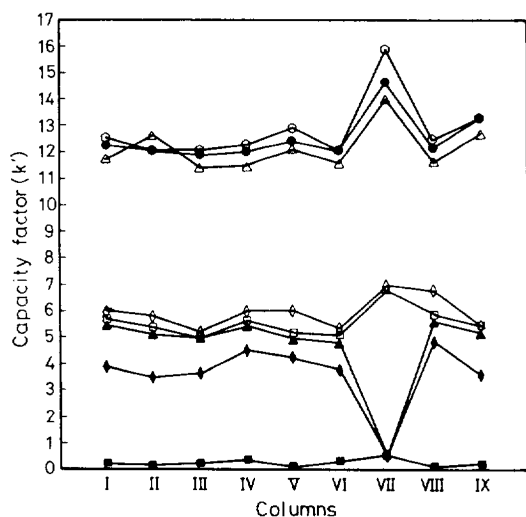

Fig. 2. Effect of column selectivity on capacity factor (k'). (■) 1 = gallic acid; (◆) 2 = sennoside B; (▲) 3 = sennoside A; (□) 4 = naringin; (◇) 5 = hesperidin; (△) 6 = honokiol; (●) 7 = magnolol; (○) 8 = emodin.

3.2. Calibration graphs for marker substances

Calibration graphs (peak-area ratio, y, vs. concentration, x, in $\mu g\ ml^{-1}$) were constructed

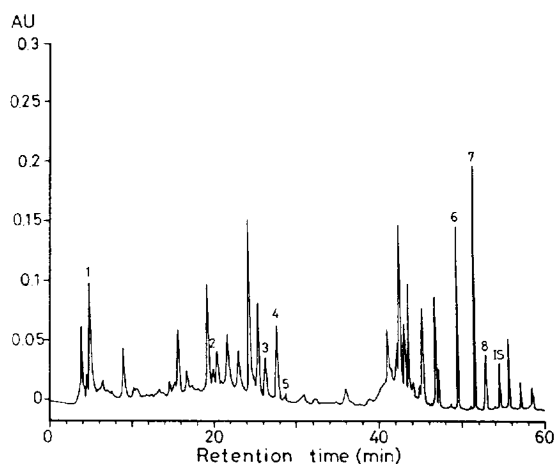

Fig. 3. Chromatogram of Hsiao-cheng-chi-tang. IS = internal standard (p-$tert$.-octylphenol); other peaks as in Fig. 1.

in the range 0.0282–0.2112 mg ml^{-1} for gallic acid, 0.0026–0.0528 mg ml^{-1} for sennoside B, 0.0046–0.0159 mg ml^{-1} for sennoside A, 0.0089–0.1680 mg ml^{-1} for naringin, 0.0010–0.0204 mg ml^{-1} for hesperidin, 0.0089–0.4480 mg ml^{-1} for honokiol, 0.0385–0.7236 mg ml^{-1} for magnolol and 0.0022–0.0426 mg ml^{-1} for emodin. The retention times of the marker substances and the regression equations of these graphs and their correlation coefficients were as follows: gallic acid, 5.0 min, $y = 0.0512x + 1.0448$ ($r = 0.9997$); sennoside B, 19.8 min, $y = 0.0141x + 3.4872$ ($r = 0.9997$); sennoside A, 25.8 min, $y = 0.0159x + 2.6812$ ($r = 0.9997$); naringin, 27.8 min, $y = 0.0260x + 9.3242$ ($r = 0.9997$); hesperidin, 28.2 min, $y = 0.0365x + 0.1172$ ($r = 0.9997$); honokiol, 49.5 min, $y = 0.0335x + 2.3599$ ($r = 0.9999$); magnolol, 51.5 min, $y = 0.0213x + 18.3838$ ($r = 0.9996$); emodin, 53.0 min, $y = 0.0735x + 0.8804$ ($r = 0.9997$); and *p-tert.*-octylphenol (internal standard), 54.2 min.

3.3. Determination of the constituents of Hsiao-cheng-chi-tang sample

When the test solution of Hsiao-cheng-chi-tang extract was analysed by HPLC under the selected conditions, the graph shown in Fig. 3 was obtained. The calculated contents of the individual constituents of two commercial Hsiao-cheng-chi-tang samples are given in Table 2. The data in Table 2 show that the quality of the formula in different commercial products varied.

Table 2
Contents of marker substances in two commercial concentrated herbal preparations of Hsiao-cheng-chi-tang (mg/g)

Constituent	Sample 1	Sample 2
Gallic acid	1.72	1.79
Sennoside B	0.60	0.64
Sennoside A	2.49	3.27
Naringin	2.73	3.05
Hesperidin	0.13	0.99
Honokiol	1.84	0.62
Magnolol	5.02	3.72
Emodin	0.37	0.69

The relative standard deviations of the proposed method, on the basis of peak-area ratios for six replicate injections, were 1.26% (intra-day) and 2.09% (inter-day) for gallic acid, 2.26 and 2.51% for sennoside B, 1.86 and 1.40% for sennoside A, 1.11 and 1.61% for naringin, 1.54 and 1.56% for hesperidin, 1.37 and 1.94% for honokiol, 1.24 and 1.67% for magnolol and 1.05 and 1.32% for emodin, respectively. Suitable amounts (2.33–69.29 mg) of the eight marker substances were added to a sample of Hsiao-cheng-chi-tang of known content and the mixture was extracted and analysed using the proposed procedure. The recoveries were 99.04% for gallic acid, 94.70% for sennoside B, 92.55% for sennoside A, 99.56% for naringin, 97.94% for hesperidin, 94.43% for honokiol, 97.89% for magnolol and 99.54% for emodin.

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