Dissolution Profiles of Principal Ingredients in Kampo Medicinal Powders by High-Performance Liquid Chromatography

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We describe here a method using HPLC for the simultaneous determination of albiflorin, paeoniflorin, glycyrrhizin and six flavanone glycosides (liquiritin, liquiritin apioside, naringin, neohesperidin, hesperidin and narirutin) in the Kampo medicines, Shigyaku-san and Haino-san. All nine components were separated in less than 40 min by linear gradient elution using a mobile phase containing aqueous phosphoric acid and acetonitrile. The dissolution of these components from powders of Shigyaku-san in aqueous solution at pH 1.80, 4.08 and 6.89 was examined. All of the components except glycyrrhizin were dissolved entirely within 5 min regardless of pH. Dissolution of glycyrrhizin was dependent on the pH of the aqueous solution, and increased with increasing pH.

Key words Kampo medicine; dissolution; albiflorin; paeoniflorin; glycyrrhizin; flavanone glycoside

Most Kampo medicines are usually prescribed as decoctions, but powdered formulations of crude drugs are also used. These different dosage forms have been developed on the basis of clinical experience. Components of the decoction present in solution or suspension in water are believed to be well dispersed in the alimentary canal. Dissolution of components in powders is related to their absorption in the alimentary canal. However, there have been few studies of dissolution of components in such powders with gastric and intestinal juice, and it is important to clarify the relationship between dosage form and dissolution.

Shigyaku-san and Haino-san are used for the treatment of cholecystitis, cholelithiasis and gastric ulcer, and acute purulent dermatitis, respectively.[1] These formulations consist of peony root and immature orange, and also include licorice root and bupleurum root, and platycodon root, respectively. Peony root and licorice root contain albiflorin (**1**) and paeoniflorin (**2**), and liquiritin (**3**), liquiritin apioside (**4**) and glycyrrhizin (**5**) as characteristic components, respectively. Immature orange from the immature fruit of *Citrus aurantium* L. var. *daidai* MAKINO and *C. natsudaidai* HAYATA, contains naringin (**6**) and neohesperidin (**7**) as major components, both of which are flavanone neohesperidosides. Narirutin (**8**) and hesperidin (**9**), both flavanone rutinosides, are present as minor components in the immature fruit of *C. aurantium* L. var. *daidai* MAKINO and *C. natsudaidai* HAYATA.[2]

Shigyaku-san and Haino-san have the name "san" indicating that they are powders (SS-P and HS-P, respectively), and they have been traditionally used as powdery dosage forms of crude drugs. They are often administered in the form of decoctions prepared by boiling crude drugs, which are called Shigyaku-san-ryo (SS-D) and Haino-san-ryo (HS-D), respectively.

The determination of **2**, **6** and **7** in methanol extract of HS-P was accomplished using a dual wavelength TLC-scanner.[3] In a recent study using HPLC, **2**, **5**, **9** and saikosaponin a in SS-D were determined by an ion-pair technique.[4] Since Kampo medicines are combinations of crude drugs, it is important for both quality control and scientific evaluation of the formulations to analyze more of the characteristic components specific for crude drugs. In the present study, we examined dissolution profiles of nine marker substances in SS-P by HPLC.

Experimental

Materials Commercial powders of SS-P and HS-P were obtained from Uchida Wakanyaku. The cut crude drugs were purchased from Tochimoto-Tenkaido. Compounds **1**, **2**, **3**, **5** and **9** were purchased from Wako Pure Chemical Industries (Wako). Compounds **6**, **7** and **8** were purchased from Funakoshi. Compound **4** was kindly provided by Prof. M. Kobayashi of Osaka University. HPLC grade acetonitrile and analytical reagent grade phosphoric acid (85%) were purchased from Wako. Water was purified by a Millipore Milli-Q water system.

Preparation of Decoction Daily dosage of SS-D (immature orange 2 g, peony root 4 g, licorice root 1.5 g, bupleurum root 5 g)[1] and HS-D (immature orange 3 g, peony root 3 g, platycodon root 1 g)[1] were prepared as follows: daily dosage of cut crude drugs was boiled with 500 ml water on an electric heater for 40 min, halving the original volume. The decoction was filtered through a colander while hot, and after cooling the volume was adjusted to 250 ml with water. Aliquots of the adjusted decoction were diluted two-fold (SS-D) or four-fold (HS-D) with water, and the mixtures were filtered through a Sartorius syringe filter (Minisart-RC 15, 0.45 μm) and analyzed.

Instrumentation and HPLC Analysis The HPLC apparatus was a Hewlett-Packard system composed of an HP1100 Series Binary Pump and an HP1100 Series Autosampler (set at 5 μl). The detection wavelength was set at 230 nm for determination and in the range of 200 to 500 nm for validation of peak purity. An HP1100 Series 3D ChemStation was used for data acquisition and integration. Separation was achieved on a Wako Wakosil-II 5C18 AR reversed-phase semi-micro column (particle size 5 μm, 150×2.0 mm i.d.). The mobile phase consisted of a mixture of (A) water containing 200 $\mu l \cdot l^{-1}$ (*ca.* 0.017%) phosphoric acid and (B) acetonitrile (linear gradient program: 0—10 min, 9% B; 15 min, 13% B; 17 min, 18% B; 27 min, 23% B; 32 min, 46% B; 40 min, 46% B), and was degassed in an ultrasonic bath prior to use. A re-equilibration period of 15 min was used between individual runs. Chromatography was performed at 45 °C with a flow rate of 0.2 $ml \cdot min^{-1}$. Chromatographic peaks were identified and purity was estimated with an HP1100 Series Photodiode Array Detector.

Standard Curve Preparation Compounds **1**—**9** were dissolved in 10% methanol to give concentrations within the range 0.125 to 4986 $\mu g \cdot ml^{-1}$. The standard curves were prepared using the peak areas of ten different concentrations in triplicate assays.

Interference Trial and Peak Purity Variant decoctions (blank decoction), in which one crude drug in each Kampo medicine was excluded, were also prepared by the procedures described above. The purity of the peaks was checked by diode-array detection. The UV spectra of peaks at three different points (upslope, top and downslope) were compared with those of authentic specimens.

Recovery A mixture of **1**—**9** dissolved in methanol was used as a standard solution. The test solutions were prepared by addition of SS-D, HS-D

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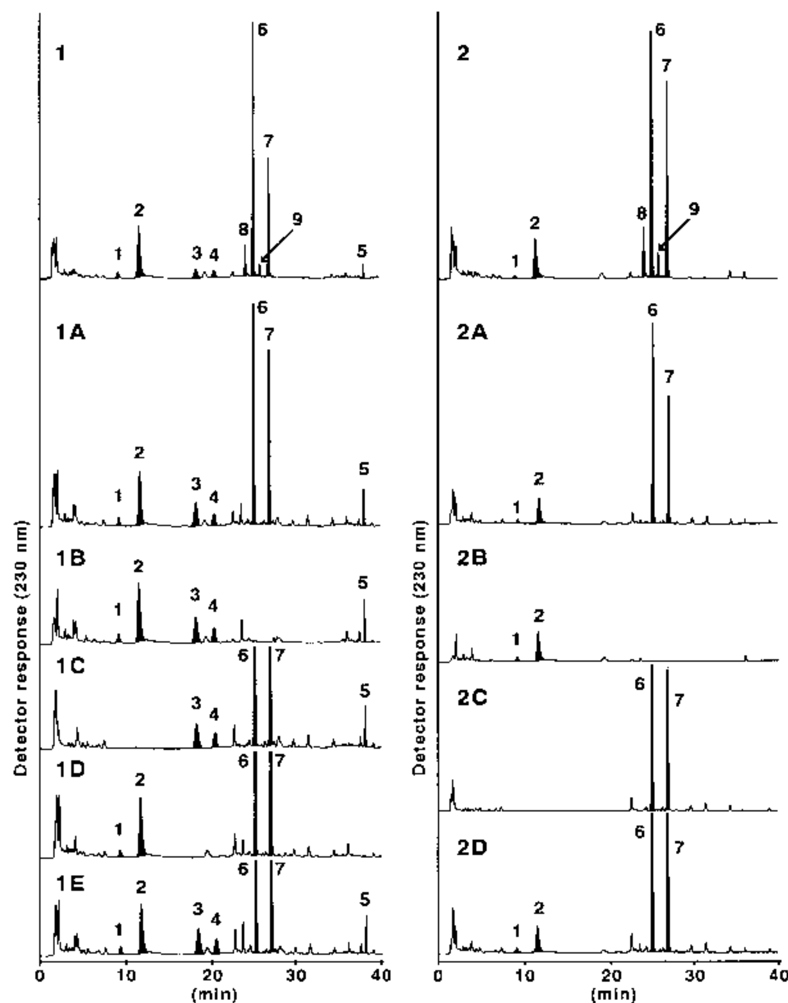

Fig. 1. Chromatograms of Shigyaku-san and Haino-san and Their Blank Decoctions

1 = extract of commercial SS-P, 1A = SS-D, 1B = 1A without immature orange, 1C = 1A without peony root, 1D = 1A without licorice root, 1E = 1A without bupleurum root, 2 = extract of commercial HS-P, 2A = HS-D, 2B = 2A without immature orange, 2C = 2A without peony root, 2D = 2A without platycodon root. For peak numbers, see text. Commercial powders (2 g) of SS-P and HS-P were refluxed for 60 min with 50 ml of water.

or water (0.9 ml) to standard solution (0.1 ml), followed by 2-, 4-, 8- and 16-fold dilution with SS-D–methanol (9 : 1), HS-D–methanol (9 : 1) or water–methanol (9 : 1). All test solutions were filtered through a Minisart-RC 15 filter and subjected to HPLC. The recoveries were determined from the slope ratios of regression equations with/without the decoction.

Dissolution of Marker Substances from Powders SS-P was prepared by mixing with powdered crude drugs (80 mesh). A daily dosage of SS-P (immature orange 2 g, peony root 4 g, licorice root 1.5 g, bupleurum root 5 g)[1] was shaken with 250 ml of three aqueous solutions (pH 1.80: 34.5 mM NaCl, 84 mM HCl, pH 4.08: 0.2 M acetate buffer, pH 6.89: 50 mM KH_2PO_3, 23.6 mM NaOH) on a water bath shaker (120 times $\cdot$ min^{-1}) at 37 °C. After 5, 60, 120 and 240 min, the aqueous solution (1 ml) was filtered with a Millex-GP filter. The filtrate was diluted two-fold with water, and the mixture was analyzed.

Results and Discussion

To select the best detection conditions, the UV spectra of marker substances were recorded by three-dimensional chromatography. The spectra of flavanone glycosides **3**, **4**, **6**, **7**, **8** and **9** exhibited major absorption maxima in the regions of 210 to 230 and 280 nm. Absorption maxima of **1** and **2**, and **5** were observed around 230 and 250 nm, respectively. While their maxima belonged to three distinct UV regions, at 200 to 230, 250 and 280 nm, the optimum monitoring wavelength for quantification was fixed at 230 nm, where the appreciable absorbance shown by these marker substances secured a suitable analytical response.

The retention times (and capacity factors, k') were 9.12 (**1**, k'=4.44), 11.50 (**2**, k'=5.87), 18.14 (**3**, k'=9.83), 20.28 (**4**, k'=11.11), 24.19 (**8**, k'=13.42), 25.20 (**6**, k'=14.09), 26.01 (**9**, k'=14.58), 27.04 (**7**, k'=15.19) and 38.07 min (**5**, k'=21.73). The calibration curves of **1**—**9** were linear in the ranges of 0.51 to 526, 4.83 to 10388, 2.58 to 1399, 0.34 to 1490, 2.32 to 5003, 4.21 to 618, 0.52 to 558, 0.49 to 319 and 0.21 to 502 μM, respectively. The standard curve showed good linearity, as indicated by linear least-squares regression analysis, and the method permitted determination of these components in Kampo medicines over a wide range of concentrations. The detection limits (signal-to-noise ratio = 3) of **1**—**9** were 1.4, 2.1, 0.5, 0.5, 0.4, 0.1, 0.2, 0.1 and 0.2 pmol per injection (5 μl), respectively.

Figure 1 shows typical chromatograms obtained from commercial powders, decoctions and blank decoctions of Shigyaku-san and Haino-san. Nine marker components were clearly separated without any prepurification, and determined within 40 min without any pre-cleaning of the column. Distinct elution profiles of **1**—**9** were obtained without any interfering peaks from the decoctions, prepared by excluding

immature orange, peony root, licorice root, bupleurum root or platycodon root from SS-D and HS-D. The commercial SS-P and HS-P showed peaks of **8** and **9**, which were attributed to different sources of immature orange. Saikosaponin d (t_R: 42.10 min, k': 24.13) and b2 (t_R: 39.03 min, k': 22.30) contained in bupleurum root were not detected in the present decoctions. Synephrine contained in citrus plants was eluted within 2 min, and neoeriocitrin (t_R: 22.65 min, k': 12.50), a flavanone neohesperidoside in immature orange, could not be separated from peaks of the component of peony root. A peak of isoliquiritin (t_R: 27.83 min, k': 15.61) in licorice root overlapped with peaks of other components of licorice root.

Table 1. Within-Day and Day-to-Day Relative Standard Deviations (RSD) for Marker Substances in Shigyaku-san-ryo (SS-D) and Haino-san-ryo (HS-D)

Marker substance	RSD (%)	
	Within-day[a)]	Day-to-day[b)]
Shigyaku-san-ryo (SS-D)		
Albiflorin (**1**)	0.56	0.79
Paeoniflorin (**2**)	0.68	0.57
Liquiritin (**3**)	0.73	0.59
Liquiritin apioside (**4**)	0.60	0.63
Naringin (**6**)	0.41	0.48
Neohesperidin (**7**)	0.36	0.49
Glycyrrhizin (**5**)	0.76	0.26
Haino-san-ryo (HS-D)		
Albiflorin (**1**)	1.43	1.35
Paeoniflorin (**2**)	0.95	0.81
Naringin (**6**)	0.19	0.27
Neohesperidin (**7**)	0.20	0.45

a) $n=10$. *b)* $n=5$.

The within-day and the day-to-day precision of the method for detection of each marker substance were evaluated using SS-D and HS-D. The within-day precision was examined with ten replicate assays per day and the day-to-day precision by assays on five different days. As shown in Table 1, the within-day and the day-to-day RSDs were 0.19 to 1.43% and 0.26 to 1.35%, respectively. Recovery was investigated using SS-D and HS-D spiked with a standard mixture of the marker substances described above. The slopes of regression equations of standards and the recoveries by this method are shown in Table 2. The recoveries of marker substances were 99.0 to 103%.

Kampo medicines are generally administered in the form of decoctions prepared by boiling crude drugs, but SS-P and HS-P are traditionally used as powdered dosage forms of crude drugs. To examine dissolution of components in the powders, we analyzed **1**—**7** in SS-P dissolved in aqueous solutions at pH 1.80, 4.08 and 6.89 for 5, 60, 120 and 240 min. With the exception of **5**, marker components were dissolved entirely within 5 min regardless of the pH. Table 3 shows the quantitative results between SS-D and the aqueous solution of SS-P at different pH at the first sampling time point for 5

Table 2. Slopes of Regression Equations of Marker Substances with and without Shigyaku-san-ryo (SS-D) and Haino-san-ryo (HS-D)

Marker substance	Initial amount of decoction (μM)	Slope of standard solution[a)]		Recovery[b)] (%)
		With decoction	Without decoction	
Shigyaku-san-ryo (SS-D)				
Albiflorin (**1**)	44.9	10.22	9.906	103
Paeoniflorin (**2**)	348	8.826	8.520	104
Liquiritin (**3**)	126	16.10	16.08	100
Liquiritin apioside (**4**)	65.4	13.05	12.60	104
Naringin (**6**)	279	20.23	19.83	102
Neohesperidin (**7**)	172	22.80	22.05	103
Glycyrrhizin (**5**)	183	3.765	3.652	103
Haino-san-ryo (HS-D)				
Albiflorin (**1**)	20.6	10.19	9.906	103
Paeoniflorin (**2**)	148	8.846	8.520	104
Naringin (**6**)	191	19.63	19.83	99.0
Neohesperidin (**7**)	120	22.11	22.05	100

a) Concentration of standards (μM): SS-D: **1** = 115, **2** = 923, **3** = 237, **4** = 127, **6** = 226, **7** = 215, **5** = 322; HS-D: **1** = 81, **2** = 672, **6** = 291, **7** = 215. *b)* Values are means of five experiments.

Table 3. Contents of Marker Substances in Decoction and Dissolution of Shigyaku-san

Marker substance	Contents (mg/g crude drug)			
	Shigyaku-san-ryo (SS-D)	Shigyaku-san (SS-P)		
		pH 1.80	pH 4.08	pH 6.89
Albiflorin (**1**)	2.38±0.34	2.20±0.05	2.15±0.10	2.00±0.18
Paeoniflorin (**2**)	22.1±1.15	28.8±0.40	29.1±0.43	29.6±0.89
Liquiritin (**3**)	17.5±0.97	18.0±0.23	19.5±0.17	21.3±0.85
Liquiritin apioside (**4**)	12.5±0.54	12.9±0.28	14.2±0.24	14.8±0.26
Naringin (**6**)	59.4±5.46	74.1±1.78	77.9±0.94	80.8±1.82
Neohesperidin (**7**)	37.3±3.70	47.0±1.19	49.8±0.60	52.0±1.30
Glycyrrhizin (**5**)	46.7±1.69	3.82±0.91	21.3±0.14	71.9±2.20

Results are means ± standard deviations from five experiments.

min. The concentrations of **1**—**4**, **6** and **7** in the aqueous solution were 84 to 139% of those in SS-D. The dissolution amounts of **5** at pH 1.08 and 4.08 were 8 and 46% of that in SS-D, respectively, but the amount at pH 6.89 was significantly higher (154%). The solubility of **5** was thought to be enhanced by increase in the pH in aqueous solution because **5** has a glucuronic acid moiety. Compound **5** used for the treatment of gastric ulcers[5] has been reported to be hydrolyzed to the active metabolic glycyrrhetinic acid (**5a**) by the action of intestinal bacteria in the large intestine.[6] Thus, the presence of a considerable amount of **5** in the aqueous solution at pH 6.89 may enhance the efficacy due to **5a** obtained by the microbial metabolism of **5** dissolved in intestinal juice.

Although **3** was stable in aqueous solution at pH 1.80 for at least 4 h, the quantities of **3** at pH 4.08 and 6.89 were decreased to 63 and 53%, respectively, during a period of about 240 min. Consequently, a new peak (t_R: 28.12 min, k': 15.76) corresponding to liquiritigenin (**3a**), an aglycone of **3**, appeared associated with a corresponding decrease in the peak of **3**. Since the hydrolysis of **3** to **3a** at pH 4.08 and 6.89 was inhibited by heating at 90 °C for 10 min and by dilution with methanol (not shown), the reduction of **3** might be mediated by enzymes.

An effective HPLC method using a semi-micro reversed-phase column was developed to analyze Kampo medicines containing **1**—**9**. The concentrations of components dissolved from SS-P with the exception of **5** were similar to those in SS-D. Dissolution of **5** was dependent on the pH of the aqueous solution, and increased with increasing pH. These findings suggested the possible availability of **5** by oral administration in the form of SS-P, because the dissolution of **5** would be significantly increased with the elevated pH in the large intestine.

Acknowledgments We are grateful to Professor Motomasa Kobayashi (Osaka University) for providing an authentic sample of liquiritin apioside, and Tochimoto-Tenkaido for supplying plant material.

References and Notes

1) Supervision by the Pharmaceutical and Supply Bureau of Health and Welfare Ministry of Japan (ed.), Ippan-yo Kampo Shoho No Tebiki, Yakugyo Jihosha, Tokyo, 1976.
2) Hosoda K., Noguchi M., *Yakugaku Zasshi*, **108**, 1008—1011 (1988).
3) Saito K., Konoshima M., Kano Y., *Shoyakugaku Zasshi*, **39**, 126—130 (1985).
4) Ueda J., Ohsawa K., *Natural Medicines*, **51**, 186—189 (1997).
5) Takagi K., Okabe S., Sasaki R., *J. Pharmacol.*, **19**, 418—426 (1969).
6) Hattori M., Sakamoto T., Kobashi K., Namba T., *Planta Medica*, **48**, 38—42 (1983).