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Application of High Performance Liquid Chromatography and Field Desorption Mass Spectroscopy to Separative Analysis of Bitter Secoiridoid Glucosides of Swertiae Herba

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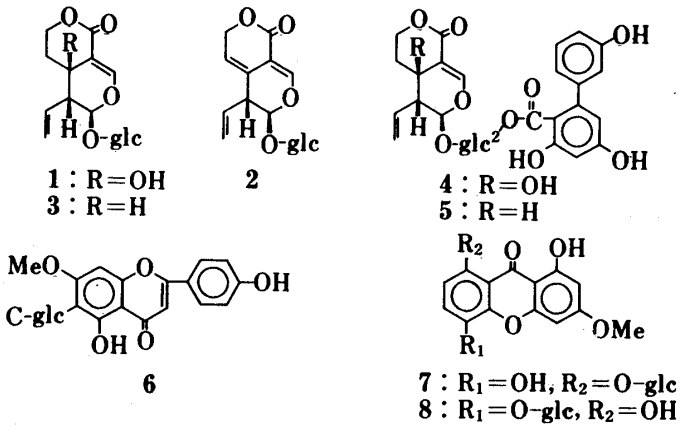
The clear-cut separation of all of the secoiridoid glucosides of Swertiae Herba into acylated and unacylated glucosides fractions was achieved by solvent extractions of an aqueous suspension of the methanolic extract. The separation of all of the secoiridoid glucosides in both fractions was accomplished by high performance liquid chromatography (HPLC) on a reverse phase column, and the homogeneity of each peak was examined by means of field desorption mass spectrometry.

The conditions for the quantitative analysis of these glucosides by HPLC and the application of HPLC to the analysis of the constituents of *Swertia japonica* and *S. pseudochinensis* are reported.

Keywords——Swertiae Herba; *Swertia japonica; Swertia pseudochinensis;* bitter principle; secoiridoid glucoside; high performance liquid chromatography; field desorption mass spectroscopy; quantitative analysis; chemical evaluation

Swertiae Herba (herb of *Swertia japonica* MAKINO, Japanese name: Senburi or Toh-yaku; Gentianaceae) is a well-known Japanese folk medicine and has been used as a bitter stomachic.

From the glycoside fraction of this crude drug, five bitter secoiridoid glucosides, swertiamarin(**1**), gentiopicroside(**2**), sweroside(**3**),[1] amaroswerin(**4**) and amarogentin(**5**)[2] have been isolated along with a flavone *C*-glucoside, swertisin(**6**)[3] and a xanthone glucoside, swertianolin (**7**).[4] Recently, we reported the isolation and the structure elucidation of a new minor xanthone glucoside named isoswertianolin(**8**) and in connection with this study, the structure of **7** was revised on the basis of nuclear magnetic resonance (NMR) spectroscopic results as shown in Chart 1.[5] For the purpose of the chemical evaluation of this crude drug, several



glc : β-glucopyranosyl

Chart 1

studies on the analysis of the secoiridoid glucosids have been reported; thin-layer chromatographic (TLC) analysis of **1**,[6] high performance liquid chromatographic analysis (HPLC) for **1** and **2**[7] and for **2**, **4** and **5**.[8] However,the serial analysis of all of the above glucosids has not appeared in the literature. The present paper deals with the systematic separation of the glucosids **1**—**5** by means of solvent partition followed by HPLC and its application to the quantitative analysis of the glucosides in this crude drug. In order to check the identification and the purity of each peak in HPLC, field desorption mass spectroscopy (FD-MS) of these glucosides was also investigated.

Experimental

Plant Materials——Commercial Swertiae Herba (samples 1—3) was purchased in Hiroshima market in 1981. *Swertia japonica* MAKINO: sample 4 was collected at Yasufuruichi, Hiroshima on October 21, 1976; sample 5 was colleted at Kaiken-zan, Hiroshima on October 28, 1973; sample 6 was collected at Kurose-cho, Hiroshima on October 3, 1973; sample 7 was collected at Yoshiwa-son, Hiroshima on October 9, 1981. *S. pseudochinenisis* HARA: sample 8 was collected Hiba-san, Hiroshima on October 11, 1971; sample 9 was collected at Akiyoshi-dai, Yamaguchi on November 2, 1977.

Authentic Samples——Authentic samples of **1**, **2** and **3** were extracted and purified through their acetates[9] and those of **4** and **5** were obtained according to the previous report.[1] Authentic samples of **6** and **7** were supplied by Prof. M. Komatsu, Josai University and Prof. T. Tomimori, Hokuriku University, respectively.

Extraction and Separation——Powdered Swertiae Herba (or other plant materials) (2 g) was extracted with hot MeOH (30 ml) for 5 min three times to ensure the complete extraction of the glucosides. Prolonged extraction was found to result in some decomposition of the glucosides. The combined MeOH extracts were concentrated to dryness *in vacuo*. A suspension of the residue in H_2O (20 ml) was washed with Et_2O (15 ml) three times and then extracted with EtOAc (15 ml) three times. The combined EtOAc layers were concentrated *in vacuo* to 5.0 ml to give the "EtOAc fraction." The aqueous layer was further extracted with 1-BuOH saturated with H_2O (15 ml) three times and the combined BuOH layers were concentrated to 5.0 ml to give the "BuOH fraction."

For quantitative analysis, 1.0 ml of the above EtOAc fraction was concentrated to dryness. The residue was dissolved in an MeOH solution containing methyl acetylsalicylate as an internal standard (2.5 mg/ml) (1.0 ml) and subjected to HPLC analysis. The BuOH fraction (1.0 ml) was treated in the same way. The residue was dissolved in an MeOH solution containing acetoaminophen as an internal standard (0.1 mg/ml) (1.0 ml) and subjected to HPLC analysis.

HPLC——A model M-6000A pump (Waters), a model U6K sample injection valve (Waters) and a variable-wavelength UV detector (JASCO) were used. Column: A prepacked μBondapak C_{18} (30 cm×4 mm). Injection volume: 5 μl of each fraction. Mobile phase and flow rate: CH_3CN–H_2O (1:9) at 0.7 ml/min for BuOH fraction and CH_3CN–H_2O (2:8) at 0.8 ml/min for EtOAc fraction. Peaks were monitored at 254 nm. Semi-preparative HPLC for FD-MS: 50 μl/injection (three times) with reduced detector sensitivity; other conditions were similar to those described above. The combined eluents of each peak were concentrated to dryness and the residue was subjected to FD-MS.

FD-MS——Determined on a JEOL D-300 mass spectrometer equipped with a combined electron impact/field ionization/FD ion source. All spectra were recorded electrically and data acquisition and processing were performed by a JEOL JMA-2000 data system. For calibration, the electron impact mass spectra of perfluorokerosene and tris(perfluoroheptyl)-*s*-triazine were used. Silicone emitters (JEOL) were used and spectra were produced at +3 or +4 kV ion source potential for the the field anode, at −6 kV for the slotted cathode plate, and at 3×10^{-7} Torr ion source pressure. Each sample was desorped by direct heating at 0—29 mA supplied emitter heating current with rough control of the emission of FD ions by the total ion monitor.

Results and Discussion

A suspension of methanolic extract of the crude drug was extracted with ethyl acetate (EtOAc fraction) and then extracted with 1-butanol saturated with water (BuOH fraction).

HPLC of the EtOAc fraction on a reverse phase column of octadecylsilylated silica gel using CH_3CN–H_2O (2:8) as a mobile phase, as shown in Fig. 1, gave excellent separation of two acylated secoiridoid glucosides (**4** and **5**) and two phenolic glucosides (**6** and **7**), each of which was identified by HPLC and FD-MS (*vide infra*) comparison with authentic samples. The minor glucoside (**8**) could not be clearly detected at this concentration, and it is

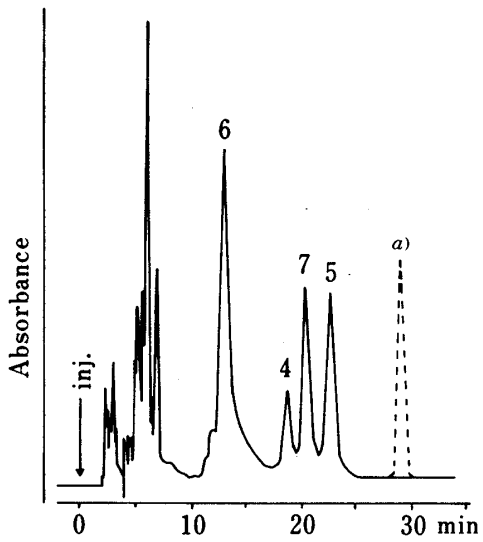


Fig. 1. High Performance Liquid Chromatogram of EtOAc Fraction of Swertiae Herba

Column: μBondapak C_{18} (4 mm i.d. × 30 cm). Mobile phase: CH_3CN–H_2O (2 : 8). Flow rate: 0.8 ml/min. Temp.: room temperature. Detector: UV 254 nm (range 0.16).
4: amaroswerin(**4**), 5: amarogentin(**5**), 6: swertisin(**6**), 7: swertianolin(**7**).
a) Methyl acetylsalicylate(internal standard).

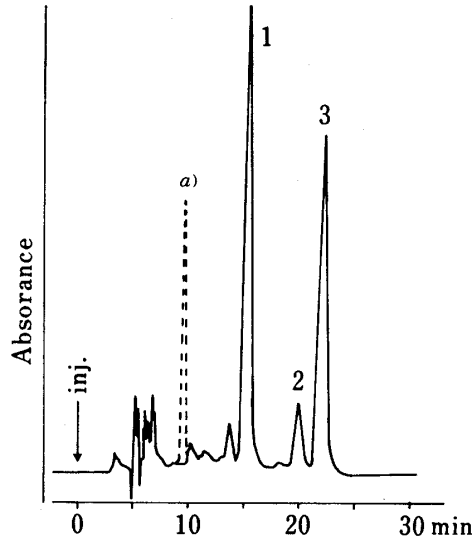
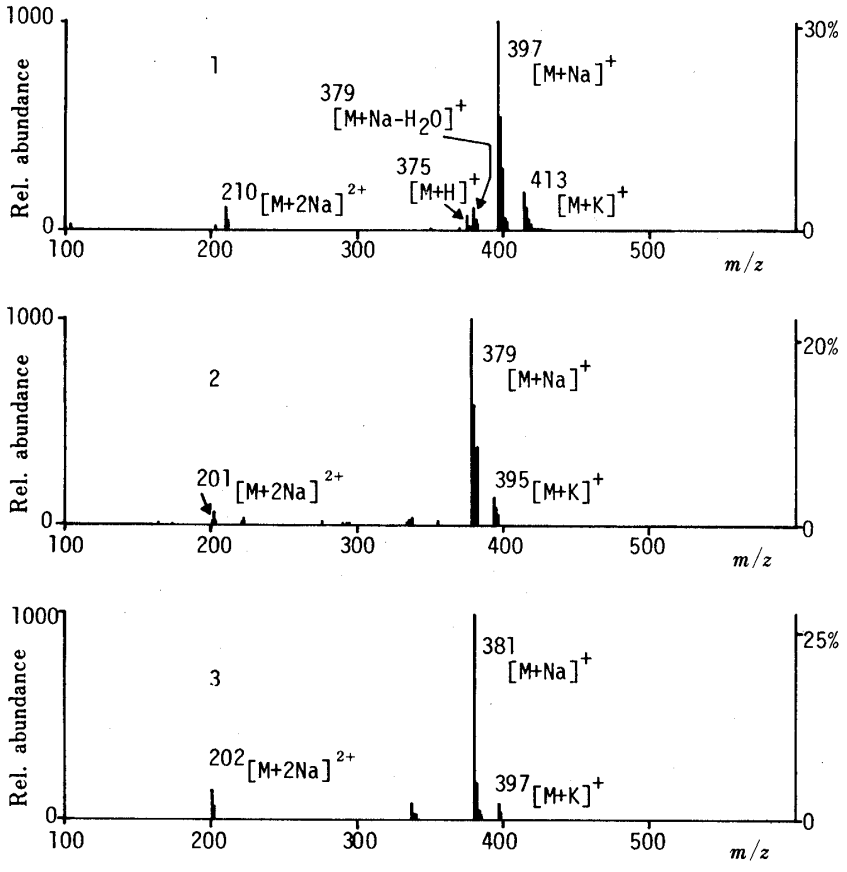


Fig. 2. High Performance Liquid Chromatogram of BuOH Fraction of Swertiae Herba

Column: μBondapak C_{18} (4mm i.d. × 30 cm). Mobile phase: CH_3CN–H_2O (1 : 9). Flow rate: 0.7 ml/min. Temp.: room temperature. Detector: UV 254 nm (range 0.16).
1: swertiamarin(**1**), 2: gentiopicroside(**2**), 3: sweroside(**3**). *a*) Acetaminophen (internal standard).



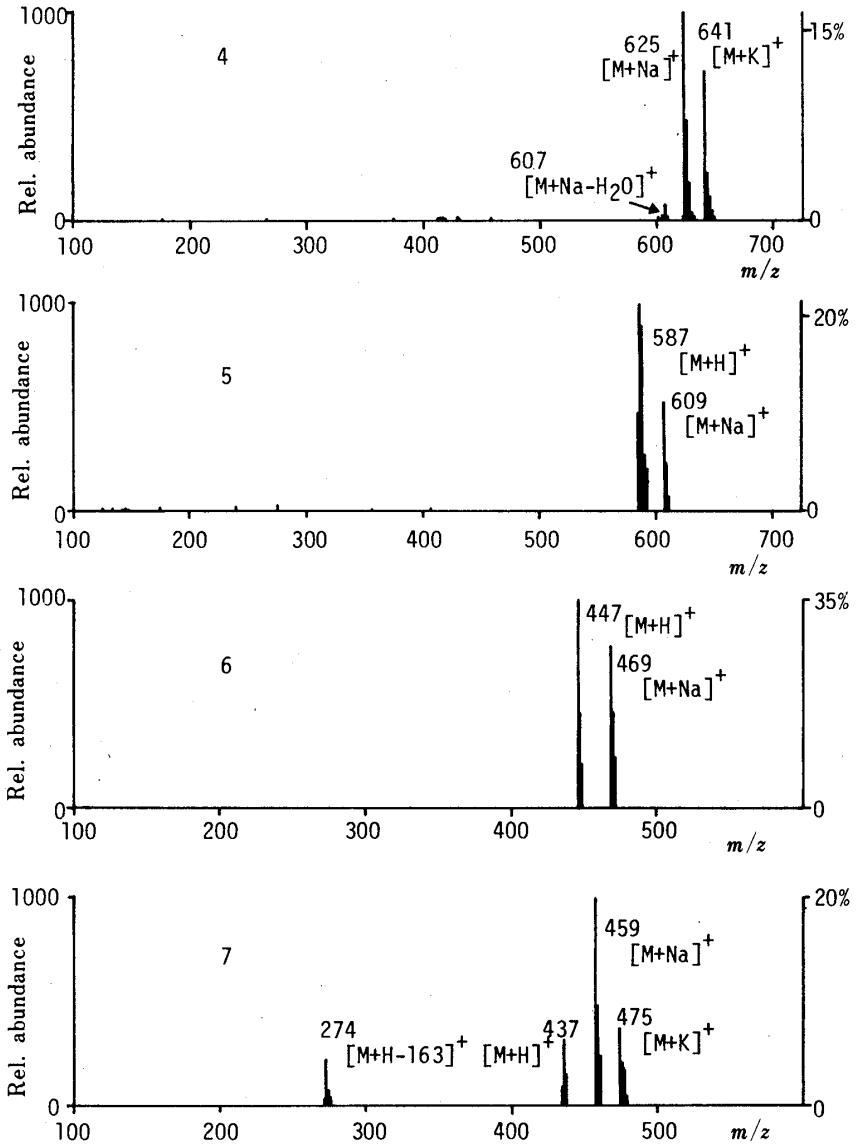


Fig. 3. Field Desorption Mass Spectra
1: swertiamarin(**1**), 2: gentiopicroside(**2**), 3: sweroside(**3**), 4: amaroswerin(**4**), 5: amarogentin(**5**), 6: swertisin(**6**), 7: swertianolin(**7**).

significant that no peak of unacylated secoiridoid glucosides (**1, 2** and **3**) was observed in the HPLC of this fraction even under the conditions used for the BuOH fraction (*vide infra*).

Fig. 2 shows HPLC of the BuOH fraction on the same column as above (mobile phase: CH_3CN–H_2O (1:9)), demonstrating the complete separation of **1, 2** and **3**. The identification of each peak was confirmed by FD-MS (*vide infra*) as well as HPLC comparison with authentic samples. It was confirmed that the acylated secoiridoid glucosides (**4** and **5**) were not detectable in this fraction. Further, the peaks of the phenolic glucosides (**6** and **7**), which were also present in this fraction, did not disturb the analysis of **1, 2** and **3** because of their very much slower t_R values in HPLC as compared with those of **1,2** and **3**. This indicates that the solvent partition in the present study (extraction with EtOAc followed by BuOH extraction) is quite effective for the quantitative separation of the glucosides into two groups *i.e.*, A) acylated secoiridoid glucosides and B) unacylated secoiridoid glucoside group.

In order to examine the identity and the purity of each HPLC peak in the present study, semi-preparative HPLC followed by FD-MS was investigated. As shown in Fig. 3, a strong $(M+Na)^+$ peak accompanied by $(M+K)^+$ and/or $(M+H)^+$ was observed in the spectra of **1**—**6**

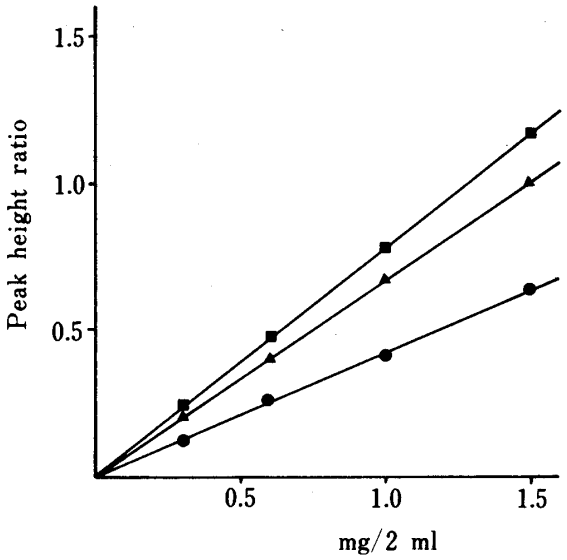


Fig. 4. Calibration Curves
●—●: swertiamarin(**1**), ▲—▲: gentiopicroside(**2**), ■—■: sweroside(**3**).
Column: μBondapak C_{18} (4 mm i.d. ×30 cm). Mobile phase: CH_3CN-H_2O(1:9). Flow rate: 0.7 ml/min. Temp.: room temperature. Detector: UV 254 nm(range 0.16). Injection vol.: 5 μl. Internal standard: acetaminophen 0.1 mg/ml.

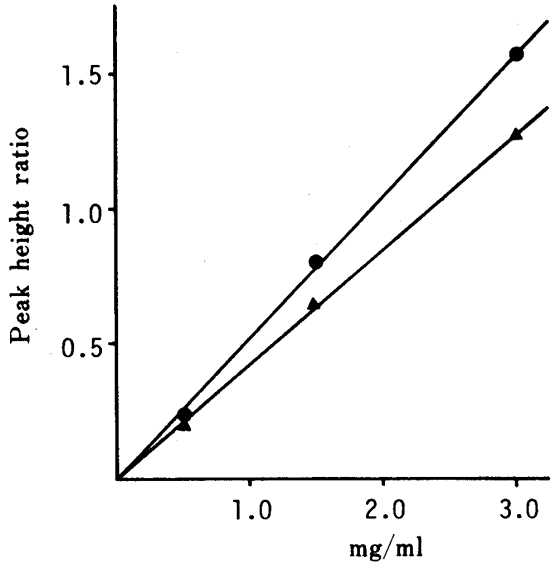


Fig. 5. Calibration Curves
▲—▲: amaroswerin(**4**), ●—●: amarogentin(**5**). Column: μBondapak C_{18} (4 mm i.d. × 30 cm). Mobile phase: CH_3CN-H_2O (2:8). Flow rate: 0.8 ml/min. Temp.: room temperature. Detector: UV 254 nm (range 0.16).
Injection vol.: 5 μl. Internal standard: methyl acetylsalicylate 2.5 mg/ml.

under the conditions described in the experimental section, and the spectra of all the HPLC peaks were found to be identical with those of corresponding authentic samples. These results showed that FD-MS must be useful even for studies of highly unstable secoiridoid glycosides such as bitter principles of Swertiae Herba. It was also confirmed that each HPLC peak of the glucosides (**1**—**7**) in the present study did not contain any impurity with a different molecular weight.

Finally, for quantitative analysis of the secoiridoid glucosides, **1**—**5** in Swertiae Herba and related plants by HPLC, the following commercially available compounds were found to be appropriate for use as internal standards; acetaminophen for analysis of **1**, **2** and **3** in the BuOH fraction and methyl acetylsalicylate for that of **4** and **5** in the EtOAc fraction. As illustrated in Figs. 4 and 5, calibration plots for peak height ratio *vs.* concentration were found to be linear for **1**, **2** and **3** up to concentrationa of 3.75 μg/injection and for **4** and **5** up to 15.0 μg/injection. Calibration curves of all the compounds could be extrapolated through zero. By means of the present procedure, quantitative analyses of **1**—**5** in commercial Swer-

TABLE I. Contents of Bitter Secoiridoids (%)

Sample No.		1	2	3	4	5
Swertiae Herba	1 (Commercial)	0.45	0.35	5.88	0.13	0.60
	2 (Commercial)	2.57	0.09	0.18	0.55	0.26
	3 (Commercial)	0.17	0.03	0.48	0.35	0.50
S. japonica	4 (Yasufuruichi)	0.45	0.06	1.60	0.38	0.55
	5 (Kaiken-zan)	—	0.06	2.20	—	0.40
	6 (Kurose-cho)	2.45	0.10	0.93	0.35	0.20
	7 (Yoshiwa-son)	—	0.16	3.40	—	0.85
S. pseudochinensis	8 (Hiba-san)	1.35	0.02	0.14	0.80	0.25
	9 (Akiyoshi-dai)	0.01	0.01	1.10	—	—

—: <0.01%.
1: Swertiamarin, **2**: Gentiopicroside, **3**: Sweroside, **4**: Amaroswerin, **5**: Amarogentin.

tiae Herba, collected *Swertia japonica* and a related plant, *Swertia pseudochinensis* HARA (Japanese name: Murasaki Senburi), were conducted. As shown in Table I, the contents of the glucosides were found to be remarkably variable from specimen to specimen.

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