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Application of Liquid Chromatography/Mass Spectrometry to the Qualitative Analysis of Saponins. II[1)]

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Qualitative analysis of crude saponin fractions from *Panax ginseng*, *Panax japonicus* and *Bupleurum falcatum*, as well as authentic saponins, was performed with a new type of liquid chromatography/mass spectrometry (LC/MS) system equipped with a frit-fast atom bombardment (FRIT-FAB) interface. The separation of saponins was achieved on a semi-micro column by gradient high performance liquid chromatography using an acetonitrile and water system as a mobile phase, and the elution profile was monitored by fast atom bombardment mass spectrometry (FAB-MS). The peaks were readily identified from their retention times and negative ion FAB spectra. Thus, LC/MS in the gradient-elution mode was demonstrated to be useful for qualitative analysis of saponins in crude drugs.

Keywords——ginsenoside; chikusetsusaponin; LC/MS; saikosaponin

The combination of high-performance liquid chromatography (HPLC) and mass spectrometry (MS) has been attempted ever since the early 1970s[2)] and liquid chromatography/mass spectrometry (LC/MS) systems with several types of interfaces for direct and indirect introduction of the column effluent have been developed. However, LC/MS is not yet as widely used as gas chromatography/mass spectrometry, because of some limitations of these interfaces. A new system capable of direct coupling of micro HPLC with fast atom bombardment mass spectrometry (FAB-MS) has recently been developed by Ito *et al.*[3)] and application of this system to the analysis of a bile acid mixture has been reported.[4)]

In the preceding papers,[1,5)] we have also reported the qualitative analysis of saponins with the LC/MS system, in which the microbore column was replaced by a semi-microbore column and the effluent was split prior to introduction into the ion source of the mass spectrometer.

In the present paper, we report the qualitative analysis of crude saponin fractions from the roots of *Panax ginseng* and *Bupleurum falcatum*, and from the rhizomes of *Panax japonicus*, which are widely used in traditional Chinese medicine.

Materials and Methods

Chemicals——Saikosaponins (a, c and d) and ginsenosides (Rb_1 and Rg_1) were purchased from Wako Pure Chemicals Co. (Osaka). Ginsenosides (Rc, Rd and Re) and chikusetsusaponins (III, IV and V) were purchased from Funakoshi Chemical Co. (Tokyo). Kizuta saponins K_{10} and K_{12} were isolated from *Hedera rhombea* BEAN[6a)] and cauloside F from *Caulophyllum thalictoroides* MICHX subsp. *robustum* KITAM.[6b)]

Plant Materials——The roots of *Panax ginseng* C. A. MEYER and *Bupleurum falcatum* L. and the rhizomes of *Panax japonicus* C. A. MEYER were purchased from Tochimoto Tenkaido Co., (Osaka).

Instruments——LC/MS was carried out on a JMS-DX 303 mass spectrometer (JEOL, Tokyo) equipped with a FRIT-FAB LC/MS interface (JEOL) and a TRI ROTAR-V HPLC system (JASCO, Tokyo). Gradient elution for semi-micro HPLC was performed with a micro gradient system (Brownlee Labs, U.S.A.). The LC/MS system was a minor improvement over that developed by Ishii *et al.*[3]; the effluent (*ca.* 100 μl) from a semi-micro column (25 cm × 1.5 mm, i.d.) was split to reduce the volume to *ca.* 5 μl before being led to the FRIT.

Preparation of Crude Saponin Fractions——a) Saponin Fraction from the Rhizomes of *Panax japonicus*: Pulverized rhizomes of *P. japonicus* (1 kg) were extracted five times with MeOH (2 l each) at 65 °C. The combined solutions were evaporated to give a residue. The residue was suspended in water (1000 ml) and extracted with ether, followed by water-saturated *n*-BuOH (1000 ml × 3). The *n*-BuOH solution was evaporated to give a crude saponin fraction (134 g). A portion of this fraction was analyzed by LC/MS.

b) Saponin Fraction from the Roots of *Panax ginseng*: Pulverized roots of *P. ginseng* (20 g) were extracted with MeOH (70 ml) under reflux for 2 h. The solution was filtered and the residue was extracted three times in a similar fashion. The combined solutions were concentrated and water (50 ml) was added. The suspension was extracted with ether (50 ml × 3) and *n*-BuOH (30 ml × 3). The *n*-BuOH layer was evaporated *in vacuo* to give a crude saponin fraction (175 mg).

c) Saponin Fraction from the Roots of *Bupleurum falcatum*: Pulverized roots (20 g) of *B. falcatum* were allowed to stand in contact with MeOH (150 ml) for 1 d at room temperature. After filtration, the residue was reextracted in the same fashion. The combined solutions were evaporated to give a residue. The residue was suspended in water (100 ml) and successively extracted with ether (50 ml × 5) and water-saturated *n*-BuOH (50 ml × 4). The combined *n*-BuOH solutions were evaporated to give a crude saponin fraction.

LC/MS Measurement——Samples were dissolved in methanol (4—10 μg/μl), and 5 μl of the solution was injected into a semi-micro column (25 cm × 1.5 mm i.d.) of μS-Finepak SIL NH_2 (JASCO) attached to the HPLC system. The column was eluted with a linear gradient of CH_3CN and water, containing 1% glycerol. The effluent was split in a ratio of 1 : 20 and passed to a FRIT (*ca.* 5 μl/min). Mass detection was carried out by repeated scanning (3 s/scan) in the negative FAB mode from *m/z* 100 to 1500 under the following conditions: FAB energy, 4 kV; emission current, 10—20 mA; neutral gas, Xe; matrix, glycerol.

Gradient Operation——(i) Gradient-Elution Program A: The gradient was from 10% solvent B (90% solvent A) to 30% solvent B (70% solvent A) in 15 min in a linear mode at the flow rate of 100 μl/ml, and the final condition (30% solvent B) maintained for 5 min. Solvent A consisted of CH_3CN–H_2O–glycerol (100 : 10 : 1) and solvent B, H_2O–glycerol (100 : 1).

(ii) Gradient-Elution Program B: The linear gradient was started with 30% solvent A (70% solvent B) and ended at 70% solvent A (30% solvent B) within 15 min.

Results

HPLC was carried out with a semi-micro column of μS Finepak SIL NH_2 by using a

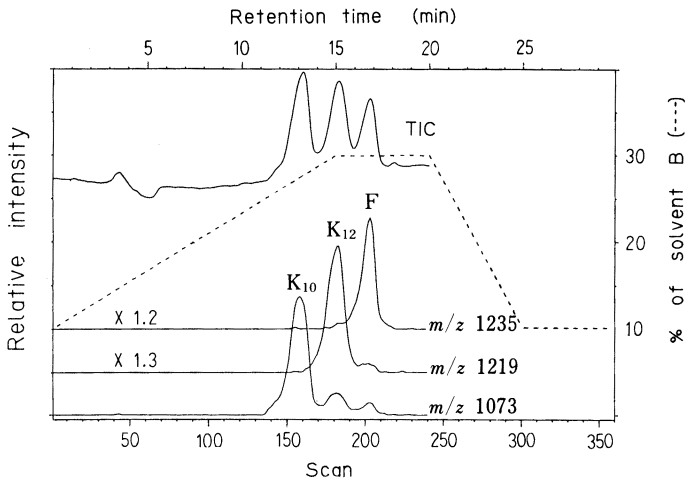

Fig. 1. Total Ion Current and Mass Chromatograms of Kizuta Saponin K_{10} (K_{10}), Kizuta Saponin K_{12} (K_{12}), and Cauloside F (F)

Column, μS Finepak SIL NH_2 (25 cm × 1.5 mm i.d.); sample injected, 5 μl (10 μg each in MeOH); gradient program A; flow rate, 100 μl/min.

gradient-elution technique and the eluate was continuously monitored by FAB. Since FAB spectra of saponins showed abundant quasi-molecular ions in general when operated in the negative ion mode rather than in the positive ion mode,[1] all mass spectrometric measurements were performed in the former mode.

Analysis of Kizuta Saponin K_{10}, Kizuta Saponin K_{12} and Cauloside F

Figure 1 shows the total ion current chromatogram (TIC) and the mass chromatogram (MC) traced at *m/z* 1073, 1219 and 1235 corresponding to the respective deprotonated molecular ions. The peaks were well separated on both chromatograms and their FAB spectra showed appreciable $[M-1]^-$ ions as well as fragment ions ascribed to the elimination of sugar

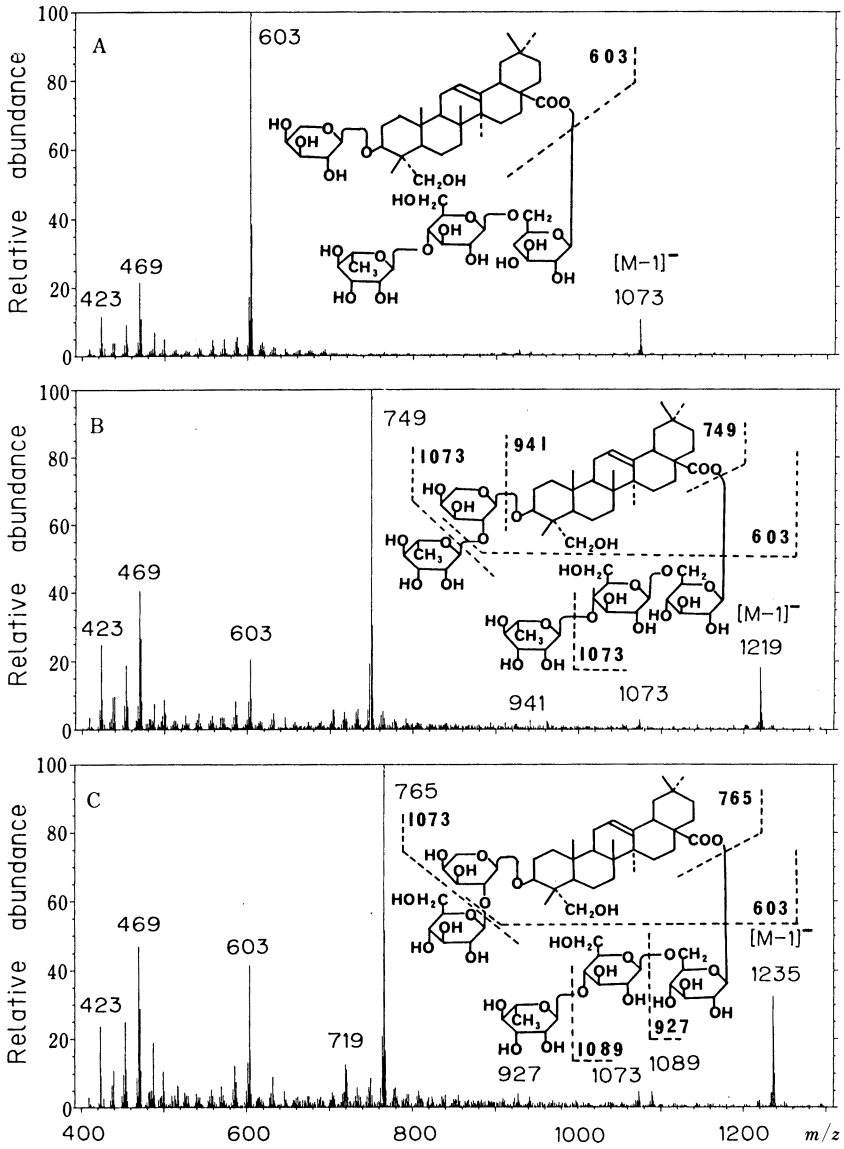

Fig. 2. LC/MS Negative Ion FAB Spectra of Kizuta Saponin K_{10} (A), Kizuta Saponin K_{12} (B) and Cauloside F (C)

The MS were obtained from the corresponding peaks in Fig. 1.

moieties (Fig. 2). The abundant ions at *m/z* 603, 749 and 765 were derived from facile loss of the sugar chains attached to the aglycone with an ester linkage, and other fragment ions resulted from the successive losses of sugar units from the $[M-1]^-$ ions of each saponin molecule. These data can be helpful for characterization of either known or unknown compounds. By using gradient elution, the peak sharpness was appreciably improved when compared to that obtained with single solvent-system elution.

Analysis of Saponins from *Panax ginseng*

Figure 3 shows the TIC and MC of a crude saponin fraction obtained from the roots of *Panax ginseng*. The elution profile was quite similar to that of an artificial mixture of

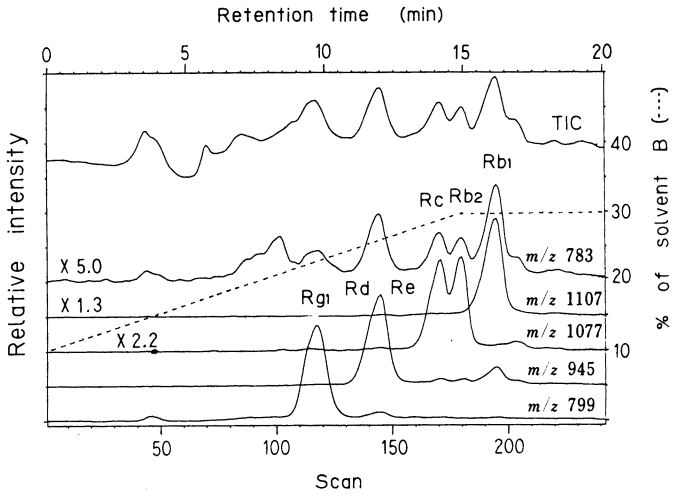

Fig. 3. Total Ion Current and Mass Chromatograms of a Crude Saponin Fraction from *Panax ginseng*

The analytical conditions were identical with those described in the legend to Fig. 1, except for the amount of sample injected (20 μg).

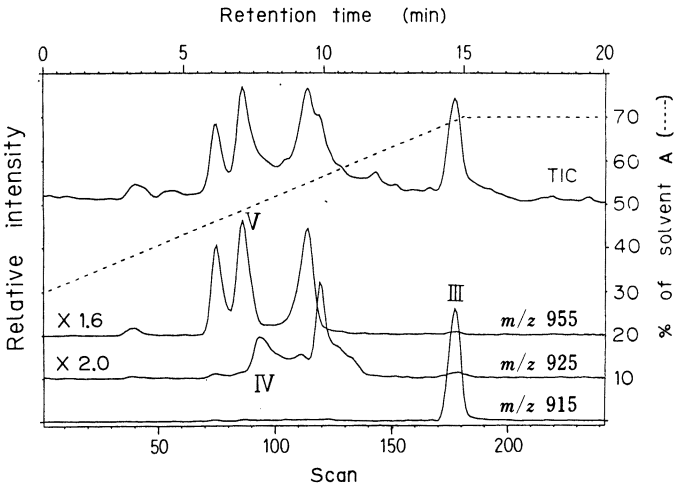

Fig. 4. Total Ion Current and Mass Chromatograms of a Crude Saponin Fraction from *Panax japonicus*

Sample injected, 20 μg; gradient program, B.

authentic samples (ginsenosides Rb_1, Rb_2, Rc, Rd, Re and Rg_1); most of the saponins except ginsenosides Rd and Re were well separated and the MC traced at *m/z* 799, 945, 1077 and 1107, which correspond to the deprotonated molecular ions of the saponins, enabled us to assign all of the peaks. The respective FAB spectra were not only in accordance with those measured with an individual sample under the same conditions but also with those obtained with the non-gradient elution device.[1] A combination of the FAB spectra and the retention times allowed us to identify readily the respective peaks. The semi-micro column of SIL NH_2 was better for the separation of saponins except for ginsenosides Rd and Re than the corresponding column of octadecyl silica (ODS). In the former column, ginsenosides were eluted in the order of their molecular weights.

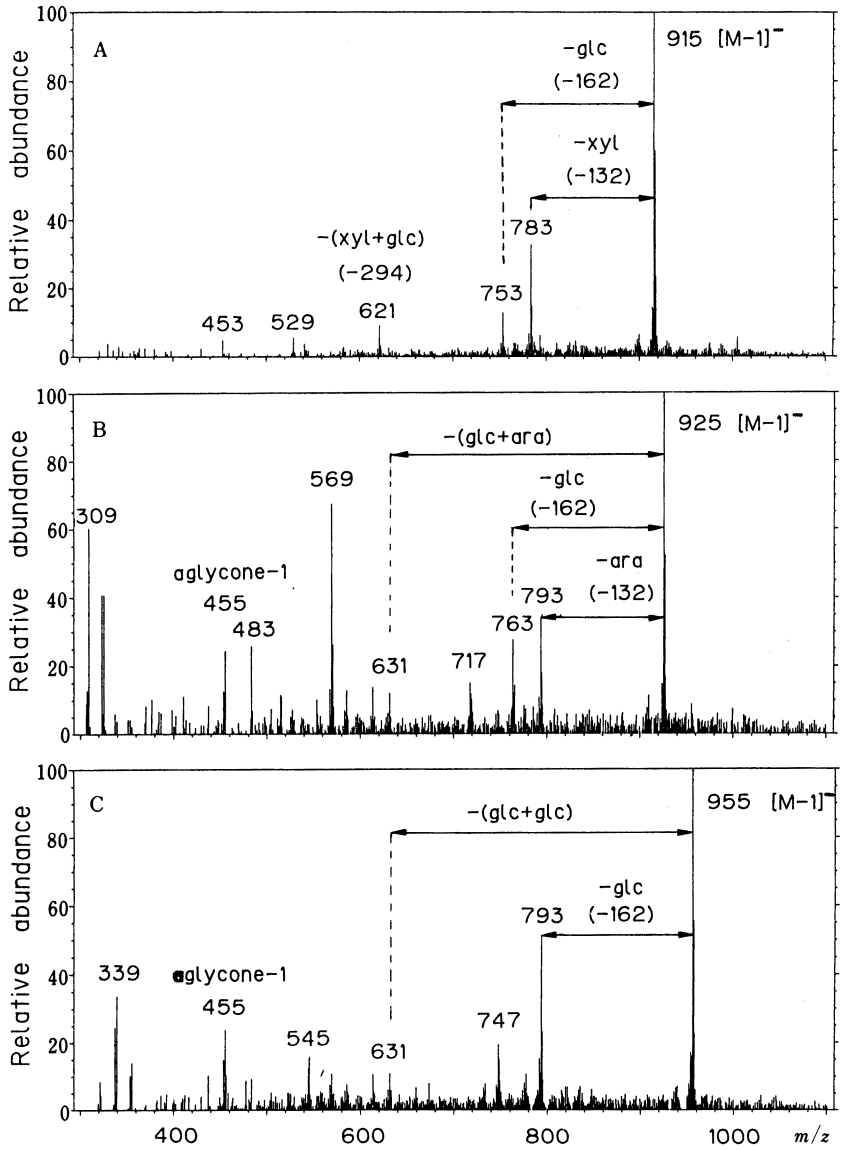

Fig. 5. LC/MS Negative Ion FAB Spectra of Chikusetsusaponins III (A), IV (B) and V (C)

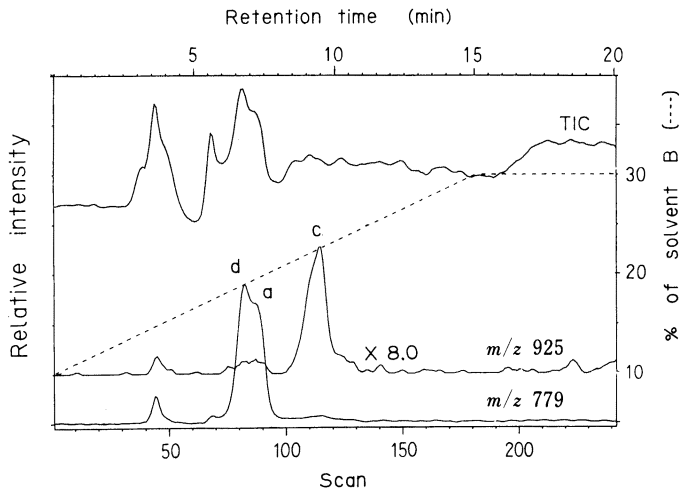

Fig. 6. Total Ion Current and Mass Chromatograms of a Crude Saponin Fraction from *Buplerum falcatum*

The analytical conditions were identical with those shown in Fig. 4.

Analysis of Saponins from *Panax japonicus*

Figure 4 shows the TIC of a crude saponin fraction from *Panax japonicus* and the MC traced at *m/z* 915, 925 and 955 due to deprotonated molecular ions of chikusetsusaponins III, IV and V. The FAB spectra corresponding to the saponin peaks agreed with those of authentic samples; the abundant $[M-1]^-$ ions and fragment ions due to successive elimination of sugar units supported the identifications (Fig. 5). In addition, a common peak at *m/z* 455 in the spectra of chikusetsusaponins IV and V was ascribed to an [oleanolic acid $-1]^-$ ion.

Analysis of Saponins from *Bupleurum falcatum*

Similarly, a crude saponin fraction from *Bupleurum falcatum* was analyzed by LC/MS (Fig. 6). The presence of saikosaponins a, c and d was confirmed on the basis of their retention times and FAB spectra,[1] but epimeric saikosaponins a and d were not well separated under the conditions used.

Discussion

Extensive studies on the biological effects of saponins from a variety of medicinal sources such as *Panax ginseng*, *Panax japonicus* and *Bupleurum falcatum* have been conducted in recent years[7,8] and their saponin contents and compositions have been analyzed for the evaluation of these crude drugs by various analytical means[9,10]; thin-layer chromatography (TLC) using a flame ionization detector,[9a] TLC-densitometry,[9e,f,10b] HPLC using a ultraviolet (UV) detector at short wavelength (203 nm),[9b,c] and TLC and HPLC after suitable derivatization.[10g] However, the identities of peak components could be confirmed only by either *Rf* values or retention times. Application of MS as a tool for the definitive identification of compounds in the peaks is therefore desirable. In the preceding paper,[1] we reported the preliminary application of LC/MS to the qualitative analysis of saponins; the HPLC-elution profiles of saponins were well traced by TIC and MS, and the positive or negative FAB spectra allowed identification of each peak. However, there still remained some problems such as poor peak sharpness, peak tailing, lack of sensitivity due to the ef-

fluent splitting device, *etc.*

In an attempt to improve the chromatogram, we tried gradient-elution HPLC/MS for qualitative analysis of crude saponin fractions. Gradient-elution HPLC is not particularly recommended for routine analysis and is troublesome with a UV detector at a short wavelength (203—205 nm) due to an appreciable drift of the base line. Nevertheless, this technique has been found to be versatile and convenient for FRIT-FAB LC/MS analysis of saponin fractions from crude drugs, which contain various types of saponins that are difficult to analyze simultaneously by HPLC with a fixed mobile phase. The peak sharpness was significantly improved and the peak tailing was also reduced, giving the adequate resolution. In addition, no appreciable drift of the base line was observed. Some saponins such as ginsenosides Rd and Re and saikosaponins a and d could not be well separated but this problem should be soluble by optimization of the HPLC conditions.

These results indicate the good potential of the newly developed FRIT-FAB LC/MS system with a gradient-elution device for the qualitative analysis of non-volatile, thermally labile molecules including saponins in crude drugs.

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