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Histochemistry. X.¹⁾ Distributions of Aluminum, Phosphorus, and Other Elements in *Bupleurum falcatum* Root Cultivated in Japan

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The concentrations and distributions of nine elements and three saikosaponins in *Bupleurum falcatum* root cultivated in Japan were histochemically examined by means of an electron microprobe X-ray microanalyzer (EXMA), inductively coupled plasma spectrometry (ICP) and high-performance liquid chromatography. Point and line analyses by semi-quantitative EXMA coupled with quantitative ICP proved to be a convenient and selective method for histochemical analysis of the tissue-specific distributions of the elements in plant roots. The present histochemical examination showed that aluminum and manganese along with three saikosaponins a, c and d occur exclusively in the outermost peripheral layer, consisting of a cork layer and pericycle, of the root, and that phosphorus occurs in the innermost xylem layer at a high concentration.

Keywords—X-ray microanalyzer; plasma spectrometer; HPLC; histochemistry; *Bupleurum falcatum*; saikosaponin; aluminum; phosphorus; Chinese crude drug

Bupleuri Radix (saiko in Japanese) is a Chinese crude drug widely used in Kampo prescriptions such as sho-saiko-to, kami-shoyo-san and hocyu-ekki-to. According to the XIth Japanese Pharmacopoeia, the crude drug is a dried root of some *Bupleurum* species (Umbelliferae). In the course of our histochemical analyses of crude drugs, we have examined the distribution of the saikosaponins, the active constituents of the drug, in the root of *B. falcatum*.¹⁾

To further clarify the nature of Bupleuri Radix, we examined the distribution profiles of various elements, including aluminum, potassium, calcium and phosphorus, in the *B. falcatum* root in relation to those of saikosaponins. Although these elements have not been studied in connection with the biological activities of the plant, they are considered to be related to the growth conditions, which influence the quality of the crude drug prepared from the plant.

Experimental

Material—Fresh roots of 1-year-old *B. falcatum* plants cultivated in Kumamoto (harvested in December 1982) and Ibaraki (in November 1982) Prefectures were used.

Sample Preparation—Fresh main roots of the plant washed free of soil particles with deionized and distilled water obtained from an automatic still (AS-20S, Iwaki-garasu) were cross-sectioned, as described previously¹⁾ to obtain thin slices for observation with a microscope (Vanox, Olympus) equipped with a microphotographic camera (PM-10, Olympus). Relatively thick (about 2 mm) cross-sections were also prepared and subjected to both line and point analyses by the use of an electron microprobe X-ray microanalyzer (EXMA, an energy-dispersive type, 8500E, Horiba).

For semi-quantitative analysis with EXMA and quantitative histochemical analyses of elements and saikosaponins, fresh main roots were cut into about 3 cm pieces, which were divided concentrically into three tissues: the outermost peripheral tissue (ph2) consisting of cork layer (kl) and pericycle (pr), the inner phloem tissue (ph1) consisting of parenchyma cells, secretory canals (sec) and phloem (ph) between the pericycle and cambium (c), and the innermost xylem tissue (xy) consisting of vessels (v), wood fibers (wf) and medullary rays. To calculate the weight ratio of the three tissues, they were dried naturally at room temperature and weighed using an electronic balance (ED-200, Shimadzu).

The dividing procedures to obtain the three tissues were repeated three times using five roots, and three quantitative analyses were carried out to obtain the means and the standard deviations (mean $\pm$ S.D.) of the observed values.

Qualitative Analysis of Elements—Point analysis at five points in the cross-section of a main root as shown in Fig. 1 was performed by means of EXMA at an accelerating voltage of 30 kV, a sample current of 3×10^{-11} A, a vertical scale of 512 counts and an integrated pulse counting time of 100 s. The electron beam scanning image at each point showing the distributions of nine elements: magnesium (Mg), aluminum (Al), silicon (Si), phosphorus (P), sulfur (S), chlorine (Cl), potassium (K), calcium (Ca) and iron (Fe) was analyzed (Fig. 1).

Line profile analysis using the same slice by EXMA progressing along the line in the scanning transmission image (Fig. 1) was also carried out to evaluate the aluminum distribution in the slices. The operating conditions were as follows: energy range of 1.48 keV (Al K_{α}), a vertical full scale of 500 counts and a counting time constant of 0.3 s.

Furthermore, the three dried tissues separated as above were pulverized in an agate mortar and each powder (50 mg each) was formed into a pellet 13 mm in diameter under a pressure of 560 kg/cm² by means of an evacuable KBr die (Shimadzu, for infrared spectroscopy). The point analysis using three pellets was performed by EXMA under the following conditions: vertical scale of 1000 counts, a counting time of 200 s and an electron beam spot of 5.4×4 mm² of the scanning transmission image at a magnification of 25 times.

Quantitative Analysis of Elements—One hundred milligrams of each of the dried and pulverized tissues was accurately weighed into a quartz dish and treated for 24 h to obtain a dry ash in a plasma asher (ASH-302, Hitachi) at a high-frequency power of 150 W and oxygen gas flow rate of 50 ml/min. The ash thus obtained was dissolved in an acid mixture (3 ml) of concentrated HCl and HNO₃ (3:1) and then made up to 100 ml with deionized water.

The sample solutions were diluted to suitable concentrations as follows: for Ca, 100 to 500 times; Mg and Al, 100 times; and K, P, Fe, manganese (Mn) and strontium (Sr), 5 times. Standard element solutions were also prepared for CaCl₂, KCl, Na₂HPO₄, MgCl₂, AlCl₃, FeCl₃, MnCl₂, and Sr(NO₃)₂. The sample solution was mixed with two concentrations of the standard solutions: Ca, Al, Mn and Sr, 0.3 and 0.6 ppm; K and P, 10 and 20 ppm; Fe 0.1 and 0.2 ppm; and Mg, 1 and 2 ppm. The blank solution (100 ml) contained 3 ml of the concentrated HCl-HNO₃ mixture and water.

The four solutions obtained above were analyzed with an inductively coupled plasma spectrometer (ICP, Hitachi 300). The operating conditions of ICP were as follows: photomultiplier voltage, 610 V; anode current, 220 mA; anode voltage, 2.0 kV; sheath gas, argon 0.78 kg/cm², 3.5 l/min; plasma gas, argon 1.28 kg/cm², 3.5 l/min; slit width (μ m) (entrance 30, exit 30). The detecting wavelengths were: P, 2553.2 Å; Mn, 2576.1 Å; Mg, 2795.5 Å; Fe, 3719.9 Å; Ca, 3933.6 Å; Al, 3961.5 Å; K, 4044.1 Å; and Sr, 4215.5 Å.

The peak heights of the eight elements were corrected against the blank solution. The concentrations of the eight elements in the samples were determined by the standard addition method using the regression equation obtained from the corrected values of three sample solutions and two solutions to which two concentrations of the standard solution had been added.

All chemicals were of an atomic absorption spectral grade and were purchased from Kanto Chemical Co., Inc.

Quantitative Analysis of Saikosaponins—The methanol extracts of the dried and powdered three tissues (ph1 and xy, 250 mg; ph2, 50 mg) obtained from five roots were treated with a Sep-Pak C₁₈ cartridge (Waters) as reported previously.¹⁾ Concentrations of saikosaponins a, c, and d in the samples were determined by high-performance liquid chromatography (HPLC) on a LC-3A apparatus (Shimadzu) equipped with a NQVA-PAK C₁₈ stainless-steel column (15 cm $\times$ 3.9 mm, Waters), an ultraviolet (UV) detector (SPD-6A, Shimadzu) and a computing integrator (C-R1A, Shimadzu). The mobile phase, flow rate, monitoring wavelength, and other detection conditions were the same as reported previously.¹⁾

Elution times of the three saikosaponins in the samples were comparable to those of the authentic saikosaponins (kindly supplied by the Shionogi Research Laboratory²⁾) under the same HPLC conditions.

Statistical Analysis—To compare the twelve mean values (x_i ; $i = 1-12$), i.e., concentrations of Ca, K, P, Mg, Mn, Al, Sr, Fe, saikosaponins a (ss-a), c (ss-c), d (ss-d) and total saikosaponins (total-ss), each variable was standardized according to the equation $z_i = (x_i - \bar{X})/SD$, where $\bar{X}$ represents the twelve grand means and SD is a standard deviation obtained for all three tissues. The twelve standardized values (z_i ; $i = 1-12$) of each tissue were calculated on a computer (PC-9801Vm2, NEC) with a multivariate analysis program written by Nippon Maikon Gakuin. The data were analyzed by using a radar chart (Fig. 4) to determine the tissue-specific distribution patterns of elements and saikosaponins.

Results and Discussion

Direct point analysis by EXMA at five spots of various tissues in the cross-section of a main root of *B. falcatum* was first carried out to clarify the distribution of the elements. The nine elements Mg, Al, Si, P, S, Cl, Ca, K and Fe were detected simultaneously. Although copper (Cu), zinc (Zn) and Mn in *Bupleuri Radix* have been determined by quantitative X-ray fluorescence spectrometry after several pretreatments (ignition at 500 °C, digestion with 6 N HCl and complex formation with pyrrolidine dithiocarbamate³⁾), these three elements were not measured in the present non-destructive EXMA examination. However, Mn in the samples was determined by the ICP method as described later.

A semi-quantitative comparison of the elements was made with the intensity along the vertical scale as shown on the right side of Fig. 1. The distribution of elements was not uniform throughout the root tissues as judged from the peak heights obtained at the five different points.

The two high peaks at 1.49 and 1.74 keV observed in the outer point (A) in the pericycle were identified as being due to Al and Si. This seems to be the first report of the occurrence of Al in *B. falcatum* root. The site of accumulation of Al was also identified by line analysis of EXMA along the electron scanning line. It starts in the cork layer, passes the pericycle, parenchyma, phloem, cambium and ends in the xylem of the cross-sectioned slice as shown in Fig. 1. The Al concentration along the line represents the intensity of the horizontal scale on the left side of Fig. 1. This is a direct proof of the high concentration of Al in the outermost layer of the root, and is in agreement with the finding⁴⁾ of Al in the epidermal cells of the root of *Zea mays*.

In the direct point analysis, sizable peaks of K at 3.31 keV and of Ca at 3.69 keV were also found in point (A), although K and Ca were present in all tissue types of the root. Crude

Fig. 1. Line (a) and Point (b) Analyses of *Bupleurum falcatum* Root (Cultivated in Ibaraki) with an Electron Microprobe X-Ray Microanalyzer (EXMA)

kl, cork layer; pr, pericycle; p, parenchyma; ph, phloem; c, cambium; xy, xylem.

Fig. 2. Point Analysis Using Pellets of Three Tissues Divided from *Bupleurum falcatum* Root (Cultivated in Kumamoto) by EXMA

sec, secretory canal; v, vessel; wf, wood fiber; others are given in the legend to Fig. 1. Three tissues: ph2, ph1 and xy (see text).

drugs and their decoctions generally contain K and Ca.⁵⁾

A small peak of Fe at 6.40 keV was detected only in point (A). *Bupleuri Radix* characteristically contains Fe in a high concentration, like *Carthami Flos*, *Schizonepetae Herba* and *Asiasari Radix*.^{3, 5, 6)}

All the above findings were supported by further EXMA analysis using the pellets prepared from the three tissues divided from the roots. Three electron beam scanning images (Fig. 2) showing the distributions of the nine elements in the three tissues were obtained. The principal feature was the high concentrations of Si and Al in the outermost layer (ph2), where Fe was also detected. A marked peak of P at 2.01 keV was detected in the two inner tissues (ph1 and xy).

The two EXMA scanning images of the pellets prepared by the same procedures using different samples harvested in Kumamoto and Ibaraki Prefectures were found to be similar. The EXMA methods described in this paper provide a qualitative and non-destructive means⁷⁾ for determining the tissue-specific distribution of elements in plants.

The patterns of elements in the three types of tissues examined by qualitative EXMA analyses were confirmed quantitatively by an ICP determination. The EXMA analyses showed that the outermost tissue (ph2) had higher contents of K, Al, Ca and Fe than the inner two tissues. Markedly low concentrations of K, Al and Ca were observed in the two inner tissues, as shown in Fig. 3. On the other hand, the main sites of concentration of P were the two inner tissues, and Mg was uniformly distributed within the three root tissues.

Mn and Sr were contained at $\mu\text{g/g}$ concentrations, whereas the other seven elements were contained at mg/g levels, and they were also distributed mainly in the outermost tissues (ph2: Mn, $390.0 \pm 10.8 \mu\text{g/g}$; Sr, $134.1 \pm 40.0 \mu\text{g/g}$) and decreased toward the innermost xylem layer (xy: Mn, $21.0 \pm 3.8 \mu\text{g/g}$; Sr, $23.5 \pm 5.2 \mu\text{g/g}$).

Based on the weight ratios of tissues, element concentrations in the three tissues obtained by the ICP method were converted into the contents relative to the whole root weight: Ca, 2.10 mg/g; K, 5.85 mg/g; P, 4.13 mg/g; Mg, 2.00 mg/g; Al, 0.34 mg/g; Fe, 0.62 mg/g; Mn, 0.047 mg/g; Sr, 0.035 mg/g. Most of the quantitative data from the present ICP method are similar to the reported profiles^{5,6)} of mineral contents in crude drugs found by using atomic absorption spectrometry.

By combining the direct measurements using semi-quantitative EXMA and the quanti-

Fig. 3. Concentrations of Elements and Saikosaponins in Three Tissues Divided from *Bupleurum falcatum* Root (Cultivated in Kumamoto)

ph1, ph2, and xy are as shown in Fig. 2. ss-a, ss-c, and ss-d represent saikosaponins a, c, and d.

Fig. 4. Tissue-Specific Distribution Profiles of Elements and Saikosaponins of *Bupleurum falcatum* Root (Cultivated in Kumamoto)

●—●, ph1; ■—■, ph2; ▲—▲, xy. Each value of the twelve variables represents the standardized mean. ss-a, ss-c, ss-d and total ss are saikosaponins a, c, d and total saikosaponin.

tative ICP analyses, the tissue-specific occurrence of elements in crude drugs could be determined.

Previously,¹⁾ we found that the saikosaponins in *B. falcatum* root were localized mainly in the outer peripheral tissues consisting of pericycle and parenchyma, containing many secretory canals. In the present sample, the outermost tissue (ph2), whose weight ratio on a whole root weight basis is 3.6%, was also found to contain the highest concentration ($2.51 \pm 0.29\%$) of saikosaponins a, c and d, amounting to 43% of the total saikosaponin content of the whole root. The inner phloem layer (ph1: weight ratio, 53.5%) had a lower concentration ($0.20 \pm 0.05\%$) of the three saikosaponins and only trace amounts of saikosaponins were found in the innermost xylem layer (xy: weight ratio, 43.0%) in the root. Thus, the outermost tissue (ph2) of the *B. falcatum* root contains a considerable part of the whole content of saikosaponins and elements such as Al, Si, Ca, K, Fe, Mn and Sr.

The radar graph summarizes the tissue-specific distribution patterns of saikosaponins and elements found by the HPLC and ICP analyses (Fig. 4). The comparison is simplified by the use of standardized mean values of the three tissues. It is noteworthy that the highest concentration of saikosaponins and elements except P and Mg are found in the outermost peripheral layer (ph2). Si, Al and Mn, along with the saikosaponins, occur exclusively in ph2, where minimal amounts of P are detected. A slightly higher content of P is observed in the innermost layer (xy), where the lowest concentrations of the three saikosaponins and other elements are found. Mg as well as P are present at the highest concentrations in the second phloem layer (ph1).

The physiological significance of the fact that saikosaponins and some elements coexist together in a specific tissue is a subject for future study. Further histochemical experiments on the effects of conditions of soil and manure and external root structures on the distribution of these components in *B. falcatum* root are necessary.

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