[Chem. Pharm. Bull. 27(8)1836—1841(1979)]

UDC 547.918.08 : 543.544.2.061

Application of High Performance Liquid Chromatography to the Analysis of Crude Drugs: Separatory Determination of Saponins of Bupleuri Radix

Hiroko Kimata, Chizuko Hiyama, Shoji Yahara, Osamu Tanaka,[1a] Osamu Ishikawa, and Makoto Aiura[1b]

Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine[1a] *and Institute of Toyo Soda Manufacturing Co., Ltd.*[1b]

(Received January 20, 1979)

The optimal conditions for conversion of saikosaponins-a (**1**) and -d (**3**), the pharmacologically active major saponins of roots of *Bupleurum falcatum*, into diene-saponins (saikosaponins-b_1 (**4**) and -b_2 (**6**)) was investigated. By means of high performance liquid chromatography (HPLC) on an octadecylsilylated silica gel column, complete separation and highly sensitive quantitative analysis of these diene-saponins, which exhibit characteristic ultraviolet maxima, were possible, offering a rapid and selective procedure for the determination of **1** and **3** in commercial samples of Bupleuri radix. Some of the crude drugs on the Japanese market were evaluated by this procedure and it was found that the HPLC pattern of "Chiku-Saiko" imported from Korea was different from those of other crude drugs and cultivated *B. falcatum*, but similar to that of roots of *B. longeradiatum*, supporting the morphological comparison of Konoshima *et al.*

Keywords——saikosaponins; Bupleuri radix; *Bupleurum falcatum*; high performance liquid chromatography of saponins; quantitative analysis of saponins; *Bupleurum longeradiatum*; Umbelliferae; ODS column

Bupleuri radix (Chaihu) is a well-known and very important crude drug in traditional Oriental medicine. In Japan, the roots of *Bupleurum falcatum* L. (Japanese name, Mishima-saiko; Umbelliferae) have been used as a source of this crude drug. Three major oleanane-saponins named saikosaponins-a (**1**), -c (**2**), and-d (**3**) and many other minor saponins were isolated from the roots of this plant and their structures have been elucidated.[2a,b] Anti-inflammatory action,[3a] plasma-cholesterol lowering action,[3b] hemolytic activity,[4a] effects on membrane fluidity,[4b] and protective action against hepatic damage by D-galactosamine[5] were reported for **1** and **3**, whereas such biological activities were not observed for **2**.

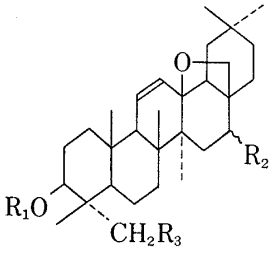
Recently, because of the increasing demand for this crude drug as well as a shortage of domestic supply, many kinds of Chaihu, the roots of related *Bupleurum* spp., have been imported from China and Korea.[6] As for the chemical evaluation of this crude drug, separatory analysis of its major saponins by thin-layer chromatography (TLC)[7] and by

1) Location: *a) 1-2-3 Kasumi, Hiroshima-shi, 734, Japan.* Correspondence should be addressed to O. Tanaka; *b) 4560, Tonda, Shin-Nanyo-shi, 746, Japan.*
2) *a)* A. Shimaoka, S. Seo, and H. Minato, *J.C.S. Perkin I*, **1975**, 2043; and references cited therein; *b)* H. Ishii, S. Seo, K. Tori, T. Tozyo, and Y. Yoshimura, *Tetrahedron Lett.*, **1977**, 1227; K. Yamasaki, R. Kasai, Y. Masaki, M. Okihara, O. Tanaka, H. Ohshio, S. Takagi, M. Yamaki, K. Masuda, G. Nonaka, M. Tsuboi, and I. Nishioka, *ibid.*, **1977**, 1231.
3) *a)* M. Yamamoto, A. Kumagai, and Y. Yamamura, *Arzneim.-Forsch.*, **25**, 1021 (1975); *b) Idem, ibid.*, **25**, 1240 (1975).
4) *a)* H. Abe, M. Sakaguchi, H. Konishi, T. Tani, and S. Arichi, *Planta Medica*, **34**, 160 (1978); *b)* H. Abe, S. Odashima, and S. Arichi, *ibid.*, **34**, 287 (1978).
5) M. Sakaguchi, H. Abe, and S. Arichi, Annual Meeting of the Japanese Society of Pharmacognosy (Fukuoka, October, 1978).
6) M. Konoshima, T. Miyagawa, and M. Takigami, *Shoyakugaku Zasshi*, **28**, 161 (1974).
7) A. Akabori, K. Kagawa, and A. Shimaoka, *Shoyakugaku Zasshi*, **29**, 99 (1975); ref. S. Hiai and H. Oura, *Proc. Sym. Wakan-yaku*, **9**, 77 (1975).

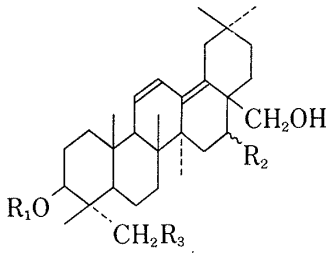
droplet counter-current chromatography (DCCC)[8] have been reported. However, it was demonstrated that the complete separation of saikosaponins could not be achieved by TLC, and the separation process by DCCC required a long period. In a continuation of studies on crude drug analysis by high performance liquid chromatography (HPLC),[9] the present authors have investigated the rapid, selective and highly sensitive determination of the pharmacologically important saponins **1** and **3** in this crude drug by means of HPLC.

It was reported that most of the genuine saikosaponins contain the very unstable allyl-oxide linkage, and are converted into artificial diene-saponins by mild acid treatment[2a]; saikosaponin-b_1 (**4**) from **1**, the saponin (**5**) from **2**, and saikosaponin-b_2 (**6**) from **3**. The genuine saponins, **1**, **2**, and **3** do not exhibit any absorption maximum in the ultraviolet (UV) region above 220 nm, while these unnatural diene-saponins show three characteristic and strong absorption maxima (*e.g.*, **4**: λ_{max}^{EtOH} 242.5 (ε 23700), 251 (ε 27600), and 260.5 nm (ε 17400)),[2a] being easily detectable with high sensitivity by HPLC with a UV-monitor (at 254 nm). For the quantitative analysis of **1** and **3** as their diene-saponins, **4** and **6**, separation of these diene-saponins by HPLC using a variety of stationary phases and mobile phases was tested, and the best separation of **4**, **5**, and **6** was obtained by reverse-phase chromatography on a column of octadecylsilylated (ODS) silica gel LS-410 with a mixture of methanol, water, acetic acid, and triethylamine as a mobile phase (Fig. 1). It was also found that anthracene could be used as an appropriate internal standard for quantitative analysis.

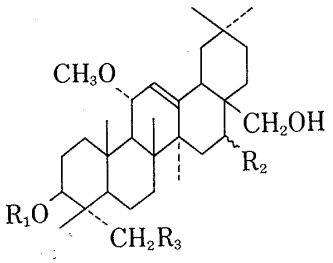
Next, the optimal conditions for the conversion of **1** and **3** into the corresponding diene-saponins, **4** and **6**, for quantitative analysis were investigated. The saponins, **1** and **3** were



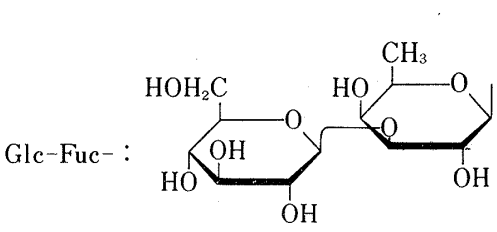
	R_1	R_2	R_3
1	Glc-Fuc-	β-OH	OH
2	Glc-Glc(Rham)-	β-OH	H
3	Glc-Fuc-	α-OH	OH



	R_1	R_2	R_3
4	Glc-Fuc-	β-OH	OH
5	Glc-Glc(Rham)-	β-OH	H
6	Glc-Fuc-	α-OH	OH



	R_1	R_2	R_3
7	Glc-Fuc-	β-OH	OH
8	Glc-Fuc-	α-OH	OH



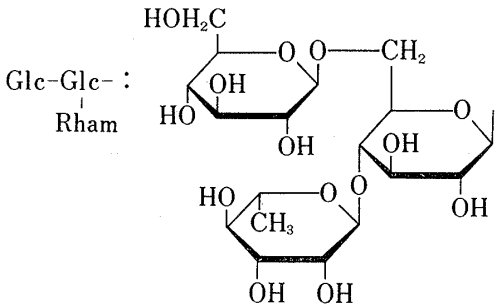


Chart 1. Structures of Saikosaponins

8) H. Otsuka, S. Kobayashi, and S. Shibata, *Planta Medica*, **33**, 152 (1978).
9) C. Hiyama, S. Miyai, H. Yoshida, K. Yamasaki, and O. Tanaka, *Yakugaku Zasshi*, **98**, 1132 (1978); O. Ishikawa, T. Hashimoto, T. Nakajima, O. Tanaka, and H. Itokawa, *ibid.*, **98**, 976 (1978).

treated with 2% HCl in 50% methanol at 25° and the reaction time course was followed by HPLC. As shown in Fig. 2, a constant yield of the diene-saponins was obtained between 6 and 20 hr for **1** and between 3 and 20 hr for **3**. TLC of the reaction mixture confirmed that this reaction proceeded almost quantitatively, and no hydrolysis of the sugar moiety took place under these conditions, whereas the formation of the prosapogenins or diene-sapogenins was observed at elevated temperature (over 30°) or under more acidic conditions.

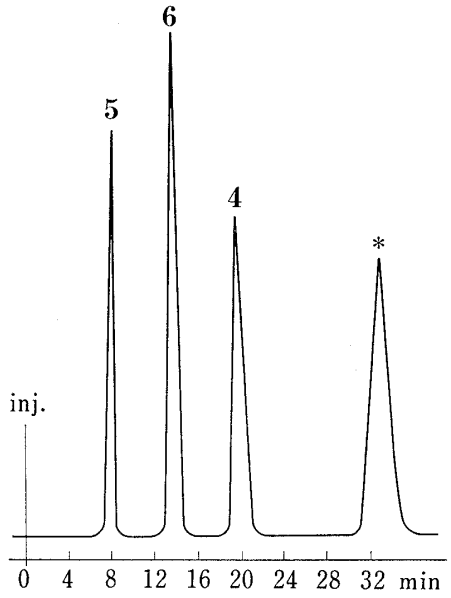


Fig. 1. High Performance Liquid Chromatogram of the Diene-Saponins **4**, **5** and **6**

HLC-802UR
Column: TSK-Gel LS-410 (4 mm i.d. × 30 cm).
Mobile Phase: MeOH: H_2O: AcOH: $(Et)_3N$
75 25 0.2 0.2
Flow rate: 1.2 ml/min.
Temp.: room temperature.
Detector: UV 254 nm.
5: diene-saponin from Saikosaponin-c (**2**)
6: Saikosaponin-b_2 from Saikosaponin-d (**3**)
4: Saikosaponin-b_1 from Saikosaponin-a (**1**)
*: anthracene (internal standard).

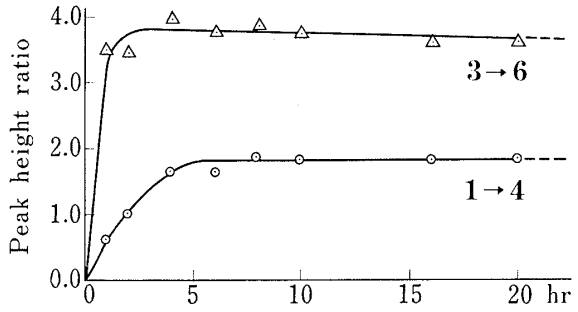


Fig. 2. Time Courses of Formation of Saikosaponins -b_1 (**4**) and -b_2 (**6**) from Saikosaponins-a (**1**) and -d (**3**), respectively: 2% HCl/50% MeOH, 25°

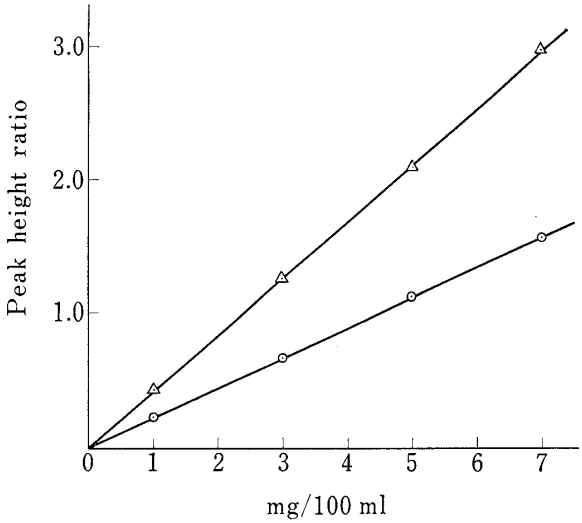


Fig. 3. Calibration Curves

○: saikosaponin-b_1 (**4**) from saikosaponin-a (**1**).
△: saikosaponin-b_2 (**6**) from saikosaponin-d (**3**).
Column: TSK-Gel LS-410 (4 mm i.d × 30 cm)
Mobile phase: MeOH: H_2O: AcOH: $(Et)_3N$
75 25 0.2 0.2
Flow rate: 1.2 ml/min.
Temp.: room temperature.
Detector: UV 254 nm.
Injection vol.: 20 μl.
Internal standard: anthracene 0.02 mM.

Authentic samples of **1** and **3** were treated with acid under the above conditions for 16 hr (overnight) and the resulting diene-saponins were subjected to HPLC analysis using anthracene as an internal standard. As shown in Fig. 3, calibration plots for peak height ratio *vs.* concentration were found to be linear for both saponins up to a concentration of 1.4 μg per injection and the curves could be extrapolated through zero.

As mentioned above, **1**, **2**, and **3** are extremely unstable, being partly converted into the diene-saponins along with other secondary products such as **7** and **8** even during the process of the extraction of the crude drug with boiling methanol. For this reason, refluxing in methanol containing a base such as pyridine has been used for their extraction.[2a] On the other hand, extraction by standing in methanol at room temperature, which could

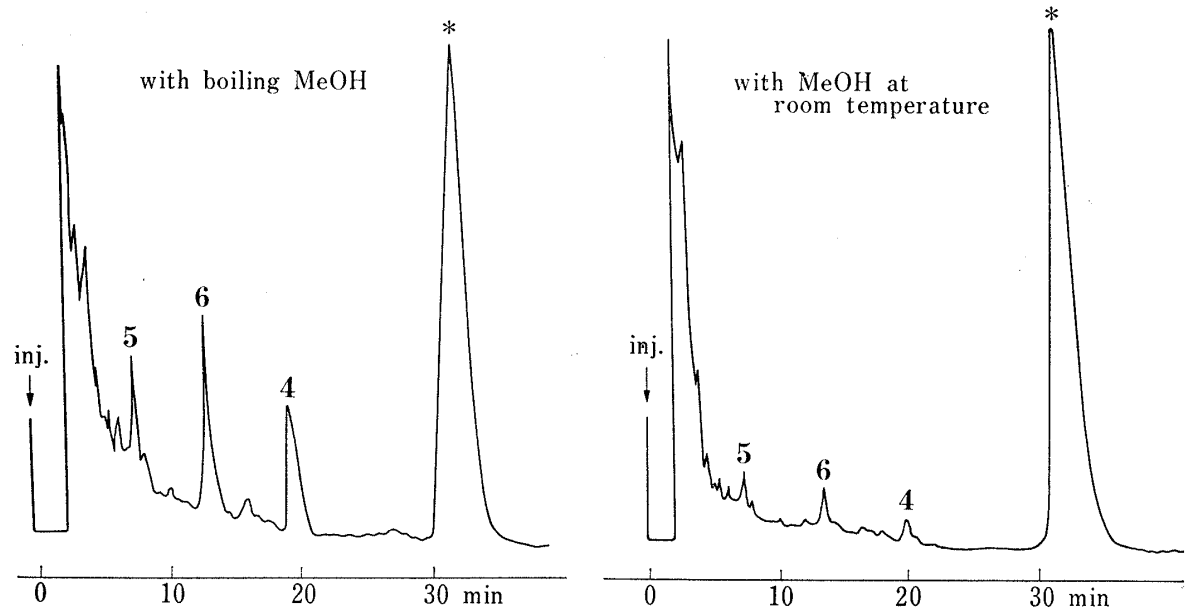


Fig. 4. High Performance Liquid Chromatogram of Extracts of Roots of *Bupleurum falcatum* L.

Column: TSK-Gel LS-410 (4 mm i.d. × 30 cm).
Mobile phase: MeOH: H_2O: AcOH: $(Et)_3N$
75 25 0.2 0.2
Flow rate: 1.2 ml/min.
Temp.: room temperature.
Detection: UV 254 nm.
*: anthracene (internal standard)

minimize the formation of the diene-saponins, requires a long time for complete extraction (more than three days). We have examined extraction procedures for analysis and found that complete and rapid extraction of the saponins with neglegible formation of the diene-saponins could be achieved with methanol at room temperature with the aid of a blender (3 min, three times) (Fig. 4). This extraction procedure is also useful for preparative scale experiments.

On the basis of these findings, a procedure for the analysis of **1** and **3** in the crude drug was established as described in the experimental section, affording acceptable reproducibility (see Table I). By this procedure, the saponin contents of roots of cultivated *B. falcatum* and several commercial Bupleuri radix were determined. The results are shown in Table I and Figs. 5 and 6. It was found that the saponin contents were not always parallel with the yields of methanolic extract, but the HPLC patterns of most of the commercial crude drugs in the present study were found to be similar to that of roots of cultivated *B. falcatum*, except for "Chiku-Saiko" imported from Korea (Fig. 6). The contents of **1** and **3** in "Chiku-Saiko" were found to be

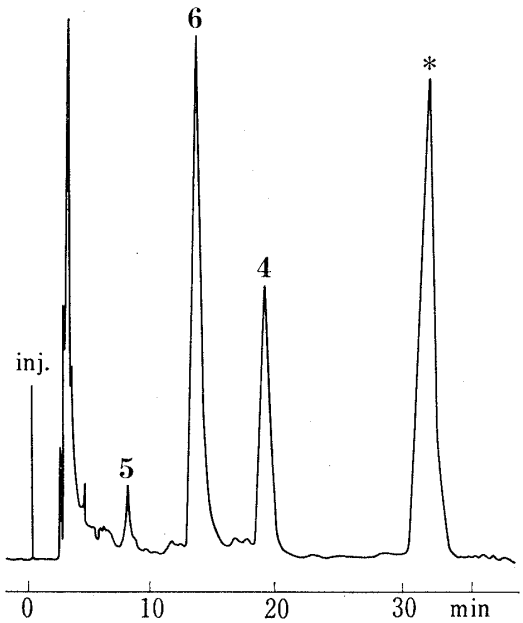


Fig. 5. High Performance Liquid Chromatogram of Acid Treated Saponins of "Kara-Saiko" imported from China

Column: TSK-Gel LS-410 (4 mm i.d. × 30 cm).
Mobile phase: MeOH: H_2O: AcOH: $(Et)_3N$
75 25 0.2 0.2
Flow rate: 1.2 ml/min.
Temp.: room temperature.
Detection: UV 254 nm.
6: saikosaponin-b_2 from saikosaponin-d (**3**).
4: saikosaponin-b_1 from saikosaponin-a (**1**).
*: anthracene (internal standard).

significantly lower than those in other crude drugs and its HPLC pattern was quite similar to that of roots of *B. longeradiatum* Turcz. collected in Nagano prefecture, supporting the morphological identification by Konoshima *et al.*[6] Although the TLC pattern of the saponin

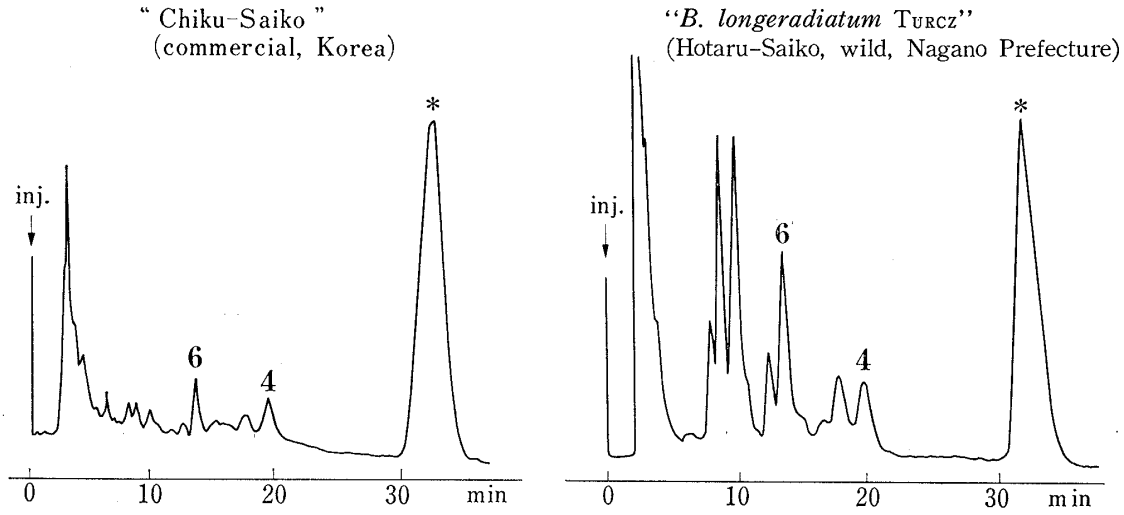


Fig. 6. High Performance Liquid Chromatogram of Acid Treated Saponins of "Chiku-Saiko" imported from Korea

Column: TSK-Gel LS-410 (4 mm i.d. × 30 cm).
Mobile phase: MeOH: H_2O: AcOH: $(Et)_3N$
75 25 0.2 0.2
Flow rate: 1.2 ml/min.
Temp.: room temperature.
Detection: UV 254 nm.
*: anthracene (internal standard).

Table I. Contents of Saikosaponins (% in crude drugs)

No.	Bupleuri radix			MeOH ext. (%)	a (%)	a Average	b (%)	b Average
1	Japan-1	Mishima-Saiko	Cultivated	30.7	0.38	0.38	0.50	0.52
					0.38		0.52	
					0.38		0.54	
2	Japan-2	Wa-Saiko	Commercial	24.0	0.52	0.52	0.62	0.63
					0.52		0.64	
					0.52		0.62	
3	Japan-3	Wa-Saiko	Commercial	19.7	0.24	0.24	0.24	0.25
					0.24		0.24	
					0.24		0.26	
4	China	Kara-Saiko	Commercial	8.6	0.52	0.51	0.57	0.54
					0.50		0.53	
					0.50		0.52	
5	Korea-1	Shoku-Saiko	Commercial	13.0	0.62	0.60	0.46	0.45
					0.60		0.46	
					0.58		0.44	
6	Korea-2	Chiku-Saiko	Commercial	12.0	0.12	0.11	0.08	0.08
					0.10		0.08	
					0.12		0.08	
7	Korea-3	Yama-Saiko	Commercial	9.8	0.58	0.58	0.62	0.62
					0.58		0.62	
					0.58		0.62	
8	Korea-4		Commercial	16.0	0.82	0.81	0.62	0.61
					0.82		0·62	
					0.80		0.60	

fraction of roots of *B. longeradiatum* seems to indicate that the main saponins of this plant are **1**, **2**, **3**, and an unidentified saponin,[10] the present HPLC results show the need for definite identification of the saponins. Isolation and structure studies of saponins of both "Chiku-Saiko" and roots of *B. longeradiatum* are in progress.

Experimental

Standard Samples of Saponins——Saikosaponins **1**, **2**, **3**, **4**, and **6** were isolated from roots of *B. falcatum* cultivated at the Medicinal Plant Farm (Kyoto) of Takeda Pharmaceutical Industry Co. by a combination of the procedures reported by Shimaoka *et al.*[2a] and Otsuka *et al.*[8] The formation of **5** from **2** was also demonstrated by HPLC, though it has not yet been isolated. The purity of each isolated saponin was confirmed by TLC (on silica gel HF_{254} (Merck) with the following solvent systems: EtOAc–EtOH–H_2O (8: 2: 1, homogeneous) and $CHCl_3$–MeOH–H_2O (30: 10: 1, homogeneous); detection: UV at 254 nm or H_2SO_4), HPLC, optical rotation, UV, and ^1H and ^{13}C NMR spectroscopy in C_5D_5N.[11]

HPLC Conditions——A Toyo Soda HLC-802 UR liquid chromatograph equipped with UV and RI monitors was used. Column: a stainless steel column (4 mm I.D. × 30 cm) packed with Toyo Soda TSK-gel LS-410 (P-5). Temperature: room temperature. Mobile phase: MeOH–H_2O–AcOH–Et_3N (75: 25: 0.2: 0.2). Flow rate: 1.2 ml/min. Monitored at 254 nm. Internal standard solution: a conc. solution of anthracene (analytica grade) in MeOH was diluted to 0.2 mM with the mobile phase in a calibrated flask.

Optimal Conditions for Conversion of 1 and 3 into 4 and 6——A mixture of **1** and **3** (1 mg each) was dissolved in 2% HCl/50% MeOH (0.5 ml) and the solution was allowed to stand at 25°. The time courses of formation of **4** and **6** were followed by HPLC. Aliquots of the mixture were neutralized with 2% NaOH. After addition of the internal standard solution (0.2 mM, 1 ml), the mixture was diluted to 10 ml with the mobile phase and an aliquot of the solution (20 μl) was subjected to HPLC analysis. The results are shown in Fig. 2.

Calibration Curves——Solutions of mixtures of **1** and **3** (1, 3, 5, and 7 mg each) in 2% HCl/50% MeOH (0.5 ml) were allowed to stand at 25° for 16 hr (overnight). Each reaction mixture was neutralized with 2% aqueous NaOH and diluted with the mobile phase to 10 ml. The internal standard solution (0.2 mM, 1 ml) was added to 1 ml of each solution and the solution was further diluted to 10 ml with the mobile phase in a calibration flask, then 20 μl of the solution was subjected to HPLC. The calibration curves are shown in Fig. 3.

Extraction of Saponins from the Crude Drug——Roots of the cultivated *B. falcatum* were extracted by the following procedures and the formation of the diene-saponins was monitored by HPLC. a) The sliced roots were extracted with boiling MeOH for 6.5 hr and the solution was concentrated to dryness *in vacuo*. A solution of 25 mg of the resulting MeOH extract in the mobile phase (10 ml) containing 0.02 mmol of the internal standard was subjected to HPLC analysis (injection: 20 μl), giving the chromatogram shown in Fig. 4. b) Sliced roots of the cultivated *B. falcatum* (10 g) were extracted with MeOH (100 ml) at room temperature for 3 min in a blender under vigorous stirring. The powdered roots were further extracted twice in the same way and the combined solutions were concentrated to dryness *in vacuo* to give the MeOH extract in a yield of 24.5%. Further extraction of the roots by the same procedure afforded only a trace of material, demonstrating that three extractions were sufficient. A solution of 25 mg of this extract in the mobile phase (10 ml) containing 0.02 mmol of the internal standard was subjected to HPLC analysis (injection: 20 μl) to give the chromatogram shown in Fig. 4.

Quantitative Analysis of 1 and 3 in Bupleuri Radix——The sliced crude drug (500 mg) was dried at 40° for 1 hr then extracted three times with MeOH (25 ml) at room temperature as described above. The combined solution was concentrated and adjusted to 50 ml in a calibrated flask, then 2—6 ml of this sample solution was accurately measured so as to contain about 5 mg of the MeOH extract and evaporated to dryness. A solution of the residue in 2% HCl/50% MeOH (0.5 ml) was allowed to stand at 25° for 16 hr (overnight). The reaction mixture was neutralized with 2% aqueous NaOH. After addition of the 0.2 mM internal standard solution (1 ml), the mixture was diluted with the mobile phase to a volume of 10 ml in a calibrated flask, and 20 μl of the solution was subjected to HPLC analysis. The results are shown in Table I and Figs. 5 and 6.

Acknowledgement We are grateful to Dr. M. Goto and Dr. T. Matsuoka, Takeda Pharmaceutical Industry Co. Ltd., for providing roots of cultivated *B. falcatum* and to Mr. Z. Nagao and Nihon-Funmatsu Co. Ltd., Osaka for providing commercial samples of Bupleuri radix. Thanks are also due to Drs. R. Kasai, H. Yoshida, and K. Yamasaki of this Institute for their valuable advice and to Dr. T. Hashimoto, Institute of Toyo Soda Manufacturing Co. Ltd., for his encouragement.

10) S. Shibata, I. Kitagawa, R. Takahashi, and H. Fujimoto, *Yakugaku Zasshi*, **86**, 1132 (1966).
11) K. Tori, S. Seo, Y. Yoshimura, M. Nakamura, Y. Tomita, and H. Ishii, *Tetrahedron Lett.*, **1976**, 4167.