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SEPARATION AND QUANTITATIVE ANALYSIS OF SAIKOSAPONINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

High-performance liquid chromatographic analysis of saikosaponin a, b_1, b_2 and d is described. A satisfactory separation and determination were achieved using a reversed-phase column. The application of this method to the evaluation of Bupleuri Radix and Shōsaiko-Tō is also reported.

INTRODUCTION

Bupleuri Radix (the root of *Bupleurum falcatum* L.) is a well known crude drug which has been used for the treatment of disease in Chinese traditional medicine and contains important saponins[1–6] named saikosaponin a, b_1, b_2, and d. In Japan, Bupleuri Radix (Japanese name: Saiko) is used as an important component of Kampō-Hōzai, which is a mixture of several crude drugs. Shibata[7] described the use of thin-layer chromatography (TLC) for the identification of the constituents of Bupleuri Radix. The determination of the saponins is necessary in order to clarify their important physiological actions. Akabori and co-workers[8–10] reported the assay of saikosaponins by preparative TLC. As this method is complicated, we have developed a more rapid and satisfactory analysis by high-performance liquid chromatography (HPLC). This paper describes the determination of saikosaponins by HPLC and the application of the procedure to extracts of Bupleuri Radix and Shōsaiko-Tō, which is a mixture of seven crude drugs.

EXPERIMENTAL

Apparatus

A Shimadzu Model LC-4A high-performance liquid chromatograph equipped with an SPD-2AF spectrometric detector (Shimadzu, Kyoto, Japan) was used with a stainless-steel column (50 cm × 4 mm I.D.) packed with Develosil-ODS (5 μm) (Nomura Chemical, Aichi, Japan). The mobile phase was acetonitrile–water (47:53). Other conditions were column temperature 45°C, flow-rate 0.7 ml/min, wavelength 210 nm, sensitivity 0.04 a.u.f.s. and chart speed 2.5 mm/min.

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Plant materials

An 8-g amount of either Mishima-Saiko (Bupleuri Radix produced in Japan), Kara-Saiko (Bupleuri Radix imported from China) or Shoku-Saiko (Bupleuri Radix imported from Korea) was extracted with 120 ml of 3% pyridine–methanol or 120 ml of water under reflux for 1 h. Shōsaiko-Tō, a mixture of Glycyrrhizae Radix 3.0, Zizyphi Fructus 3.0, Zingiberis Rhizoma 1.0, Ginseng Radix 3.0, Pinelliae Tuber 8.0, Scutellariae Radix 3.0 and Bupleuri Radix 8.0 (the numbers indicate the proportions of the crude drugs by dry weight) was prepared by extraction with 120 ml of water.

Preparation of HPLC samples

After centrifugation of the 3% pyridine–methanol or water extract (120 ml) of Bupleuri Radix (8 g) or the water extract of Shōsaiko-Tō, the supernatant solution (2 ml) was injected in a C_{18} cartridge column (Waters Assoc., Milford, MA, U.S.A.), washed with 15% methanol (4 ml) and eluted with methanol (4 ml), and the total volume was adjusted to 4 ml with methanol and filtered using a 0.45-μm membrane filter (Toyo Kagaku Sangyo, Tokyo, Japan). The filtrate was then subjected to HPLC analysis.

Isolation of standard saikosaponins

Bupleuri Radix (1.85 kg) was extracted with 3% pyridine–methanol under reflux for 1 h. This procedure was repeated three times. The extracts were filtered and evaporated *in vacuo*. The residue was dissolved in water and extracted with diethyl ether. The aqueous solution was extracted with *n*-butanol three times. The organic layer was evaporated to obtain a crude glycoside fraction (54.3 g), 20 g of which were chromatographed on silica gel with chloroform–methanol–water (65:30:10) to yield a mixture (8 g) of saikosaponin a and d. The mixture (1.5 g) was subjected to droplet counter-current chromatography[11,12] [the mobile phase was the upper layer and the stationary phase was lower layer of the solvent system chloroform–methanol–water–benzene–ethyl acetate (45:60:40:2:3)] to afford saikosaponin a (743 mg) and saikosaponin d (292 mg).

Saikosaponin a (100 mg) was dissolved in a mixture of 1% sulphuric acid and dioxane (1:1) and allowed to stand at 70°C for 80 min. The reaction mixture was extracted with *n*-butanol saturated with water, washed with water three times and evaporated *in vacuo* to give a crude saikosaponin b_1 fraction (51 mg), which was purified by preparative HPLC using acetonitrile–water (47:53) and with detection at 210 nm.

Saikosaponin d (100 mg) was dissolved in a mixture of 1% sulphuric acid solution and dioxane (1:1) and allowed to stand at 70°C for 20 min. The reaction mixture was treated as mentioned above. A crude saikosaponin b_2 fraction (53 mg) was obtained and purified by preparative HPLC.

All saikosaponins were identified by comparison with authentic samples using ^1H and ^{13}C NMR spectra.

RESULTS

Separation of saikosaponins

Standard saikosaponin a, b_1, b_2 and d were investigated by HPLC. Saiko-

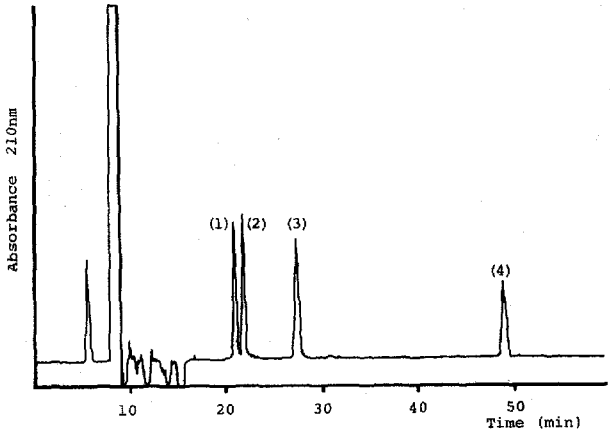

Fig. 1. Separation of standard saikosaponins by reversed-phase HPLC. Peaks: 1, saikosaponin b_2; 2, saikosaponin a; 3, saikosaponin b_1; 4, saikosaponin d.

saponin a and b_2 were separated completely by reversed-phase chromatography on Develosil ODS (5 μm), at a flow-rate of 0.7 ml/min using 47% acetonitrile–water. Saikosaponin b_1 and d were easily separated from saikosaponin a and b_2 under the same conditions. The results are shown in Fig. 1. The retention times were 21 min (saikosaponin b_2), 22 min (saikosaponin a), 27 min (saikosaponin b_1) and 49 min (saikosaponin d).

Calibration graph for saikosaponins

Saikosaponin a, b_1, b_2 and d at concentrations varying from 0.1 to 2.5 μg were

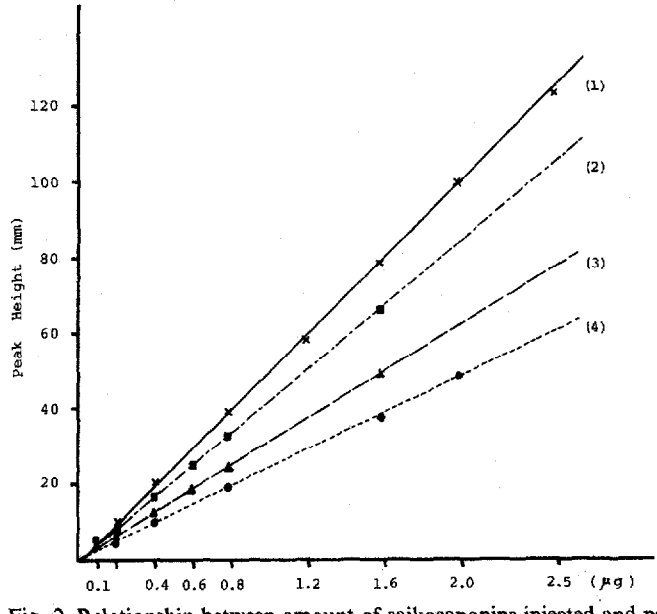

Fig. 2. Relationship between amount of saikosaponins injected and peak height.

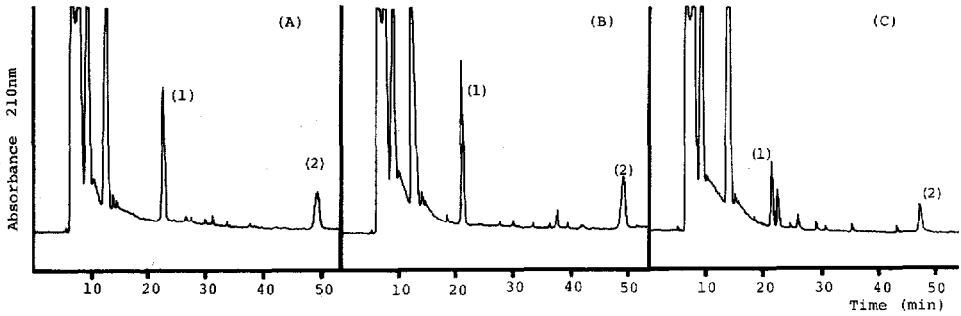

Fig. 3. Chromatogram of the methanol extracts of Bupleuri Radix. (A) Mishima-Saiko; (B) Shoku-Saiko; (C) Kara-Saiko. Peaks: 1, saikosaponin a; 2, saikosaponin d.

dissolved in methanol and the calibration graphs were obtained by using the procedure described above. The results are shown in Fig. 2. The graphs were linear in the range studied and the minimum concentration determinable was 100 ng in each instance.

Determination of saikosaponins in Bupleuri Radix

Fig. 3 and Table I show the chromatogram and the content of saikosaponins in 3% pyridine–methanol extracts of Bupleuri Radix, respectively. The samples of Bupleuri Radix used were Mishima-Saiko (lot A was cultivated for 3 years and lot B for 2 years), Kara-Saiko (lots A and B) and Shoku-Saiko (lots A and B). The contents of saikosaponins were highest in Shoku-Saiko. The contents in Mishima-Saiko cultivated for 3 years were three to five times greater than those cultivated for 2 years. In three samples, saikosaponin a and d were the main saponins. Trace amounts of saikosaponin b_1 which was derived from saikosaponin a and saikosaponin b_2 which was derived from saikosaponin d were determined in Kara-Saiko. However, with Mishima-Saiko and Shoku-Saiko, quantitative analysis was impossible, as these saponins were present only in trace amounts.

Fig. 4 and Table II show the chromatogram and the content of saikosaponins in aqueous extracts of Bupleuri Radix, respectively. In all samples, saikosaponin a and b_2 were the main components. In half the samples, trace amounts of saikosaponin b_1 were found. No sample gave a peak of saikosaponin d.

TABLE I

CONTENTS OF SAIKOSAPONINS IN THE METHANOL EXTRACTS OF BUPLEURI RADIX (1 g)

Each value is the mean ± standard error of six determinations.

Source	*Lot*	*Saikosaponin a (mg)*	*Saikosaponin d (mg)*	*Saikosaponin b_1 (mg)*	*Saikosaponin b_2 (mg)*
Mishima-Saiko	A	2.46 ± 0.04	1.96 ± 0.03	0	0
	B	0.71 ± 0.01	0.41 ± 0.004	0	0
Shoku-Saiko	A	3.07 ± 0.05	2.21 ± 0.05	0	0
	B	3.35 ± 0.03	2.43 ± 0.07	0	0
Kara-Saiko	A	1.06 ± 0.01	1.05 ± 0.06	0	0
	B	1.09 ± 0.02	1.04 ± 0.03	0	0

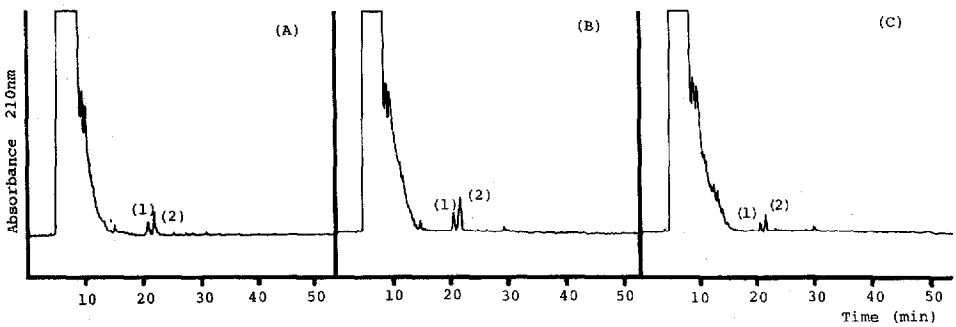

Fig. 4. Chromatogram of aqueous extracts of Bupleuri Radix. (A) Mishima-Saiko; (B) Shoku-Saiko; (C) Kara-Saiko. Peaks: 1, saikosaponin b_2; 2, saikosaponin a.

Determination of saikosaponins in Shōsaiko-Tō

Fig. 5 and Table III show the chromatogram and the contents of saikosaponins in Shōsaiko-Tō, respectively. Saikosaponin a and b_2 show sharp peaks. There are no overlapping peaks of saikosaponins with unknown components observed in Shōsaiko-Tō. Saikosaponin b_1 and d were not found in Shōsaiko-Tō.

DISCUSSION

In recent years, the evaluation of crude drugs has become increasingly important owing to the variety of producers, different seasons and different conditions of cultivation. Nowadays, gas–liquid chromatography (GLC) or HPLC is used widely to determine the main components of crude drugs, but with Bupleuri Radix, the main components of which are saikosaponins, there have been no reports of GLC or HPLC analysis; this is related to the difficult separation of saikosaponin a and b_2.

In this study, we have succeeded in the complete separation of saikosaponin a and b_2 by reversed-phase chromatography. Six different kinds of carriers have been investigated, and saikosaponin a and b_2 were difficult to separate with five of them. However, using Develosil ODS (C_{18}, 5 μm), both saponins were separated efficiently.

In HPLC analysis, an acidic buffer is commonly used to achieve good separa-

TABLE II

CONTENTS OF SAIKOSAPONINS IN THE WATER EXTRACTS OF BUPLEURI RADIX (1 g)

Each value is the mean ± standard error of six determinations.

Source	*Lot*	*Saikosaponin a (mg)*	*Saikosaponin d (mg)*	*Saikosaponin b_1 (mg)*	*Saikosaponin b_2 (mg)*
Mishima-Saiko	A	0.52 ± 0.01	0	0	0.30 ± 0.003
	B	0.45 ± 0.02	0	0	0.33 ± 0.04
Shoku-Saiko	A	0.61 ± 0.05	0	0	0.31 ± 0.01
	B	0.90 ± 0.02	0	0	0.54 ± 0.03
Kara-Saiko	A	0.34 ± 0.01	0	0	0.38 ± 0.005
	B	0.35 ± 0.01	0	0	0.32 ± 0.01

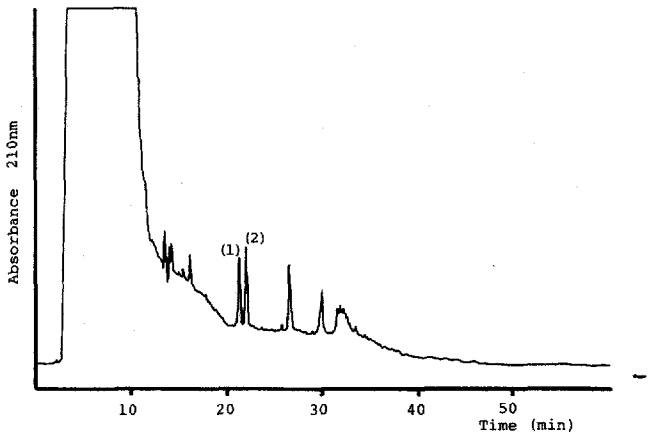

Fig. 5. Chromatogram of Shōsaiko-Tō. Peaks: 1, saikosaponin b_2; 2, saikosaponin a.

TABLE III

CONTENTS OF SAIKOSAPONINS IN SHŌSAIKO-TŌ

The amount analysed was one daily human dose, which contains 8 g of Bupleuri Radix. The Bupleuri Radix used was Shoku-Saiko. Each value is the mean ± standard error of six determinations.

Lot	*Saikosaponin a (mg)*	*Saikosaponin d (mg)*	*Saikosaponin b_1 (mg)*	*Saikosaponin b_2 (mg)*
A	2.28 ± 0.23	0	0	2.09 ± 0.14
B	2.14 ± 0.19	0	0	1.57 ± 0.19

tions. As saikosaponin a and d, but not saikosaponin b_1 and b_2, are unstable under acidic conditions, a neutral buffer was used in our experiments. Standard samples of saikosaponin a, b_1, b_2 and d showed complete separations using acetonitrile–water as the solvent system.

Three samples of Bupleuri Radix produced in Japan (Mishima-Saiko), China (Kara-Saiko) and Korea (Shoku-Saiko) were investigated. Aqueous extracts of Bupleuri Radix contained no saikosaponin d, because of the instability of its ether ring under the mild acidic conditions which arose during the extraction, which led to its conversion into saikosaponin b_2. This result suggests that the existence of an α-axial hydroxyl function at C_{18} of saikosaponin d gives rise to greater sensitivity to acids than the β-equatorial hydroxyl function at C_{18} of saikosaponin a. In Shōsaiko-Tō, no peaks of saikosaponins overlapped with those of unknown compounds.

The results indicate that the application of HPLC to the evaluation of Bupleuri Radix is a useful method which is rapid and accurate. Experiments are now in progress to determine saikosaponins in other sources of Kampō-Hōzai which contain Bupleuri Radix.

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