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Note

Quantitative analysis of the metabolites of saikosaponin a using high-performance liquid chromatography

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Kampo-Hozai have been used clinically in China for 2000 years and their efficacy was clinically established. Among various Kampo-Hozai, Saiko-zai and Kuoketsu-zai have been used for chronic diseases, and, above all, their therapeutic effects on chronic hepatitis and chronic nephritis have well been recognized; their frequency of application in these chronic diseases is increasing rapidly in Japan and China.

The pharmacological effects of Saiko-zai may be due to that of the main crude drug, Bupleuri Radix, and several reports have been published concerning the chemical structure of the main constituent, sakosaponin [1—6]. Some pharmacological effects of saikosaponins concerning the anti-inflammatory action have also been reported [7, 8]. But there is no report about the actual active constituents — whether they are the glycosides themselves or aglycones. Thus, we have examined the absorption of the glycosides, saikosaponin a and saikosaponin b_1, and of their aglycone (non-sugar part), saikogenin A, in blood after oral administration, using high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Standard and reagents

Saikosaponin a, saikosaponin b_1 and saikogenin A were extracted and identified with authentic samples in our laboratory. Oleana-11,13(18)-diene-3β-21α-28-triol (ODT), internal standard, was synthesized in our laboratory [9, 10].

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Animals

Male ddY strain mice of 25—35 g body weight were used. The animals were housed in an air-conditioned room for a week after purchasing.

Apparatus

The apparatus used in the present study was a Shimadzu Model 4A chromatograph with a Shimadzu Model SPD 2A ultraviolet detector. A stainless-steel column (25 cm × 4 mm I.D.), packed with reversed-phase Hypersil ODS (5 μm; Erma Optical Works, Tokyo, Japan) was used. The mobile phase was acetonitrile—water (46:54). The column temperature was 45°C, the flow-rate was 1.0 ml/min. Detection wavelength was 210 nm for the saponins and 254 nm for the aglycone. The sensitivity of the detector was set at 0.005 a.u.f.s. The peak area was measured using a Shimadzu C-R2A computing integrator.

Sample preparation

To 1.9 ml of artificial gastric juice (hydrochloric acid solution, pH 1.2) were added 50 μg of saikosaponin a dissolved in 100 μl of water. The mixture was incubated at 37°C, and a 50-μl aliquot of the incubation mixture was withdrawn after 5, 10, 20, 30, 60, 90, 120, 180, 240 and 300 min. After adding 200 ng of ODT, as the internal standard, in 50 μl of 1% ethanol solution, the aliquot was injected into the C_{18} cartridge column (Waters Assoc., Milford, MA, U.S.A.). After washing with 15% of methanol, elution was carried out with 3 ml of methanol. The eluate was concentrated to dryness under reduced pressure, and the residue was dissolved in 100 μl of methanol. After filtering the solution through a 0.45-μm membrane filter (Toyo Kagaku Sangyo, Osaka, Japan), 40 μl of filtrate were subjected to HPLC analysis.

At 15 and 30 min, and 1, 2, 3 and 5 h after oral administration of 40 mg/kg saikosaponin b_1, or 20 mg/kg saikogenin A, blood specimens (100 μl) were collected from the veniplex of the fundus oculi, and the collected blood was allowed to stand at room temperature for 30 min. Then it was centrifuged at 3000 rpm (6000 *g*) for 10 min and 30 μl of serum were obtained. To 30 μl of serum thus obtained, 25.0 ng of ODT were added as the internal standard, and 50 μl of methanol were added. The mixture was centrifuged at 11,000 rpm (22,000 *g*) to remove protein. The supernatant was injected into the C_{18} cartridge column and eluted with 3 ml of methanol. The eluate was evaporated to dryness, the residue was dissolved in 50 μl of methanol, and the suspension was filtered through a 0.45-μm membrane filter; 30 μl of the aliquot were subjected to HPLC analysis.

Calibration curve

A calibration graph was obtained with standard material ranging from 2 to 50 μg of saikosaponin a and saikosaponin b_1 using 200 ng of ODT as the internal standard according to the extraction procedure for the artificial gastric juice.

For the analysis of saikogenin A in blood, a calibration curve was prepared with the standard material, saikogenin A, ranging from 2 to 50 μg using 25.0 μg of ODT as the internal standard according to the extraction procedure for blood specimens.

RESULTS AND DISCUSSION

Saikosaponin a was incubated in artificial gastric juice, and an aliquot of the mixture was subjected to HPLC. The chromatogram obtained after 20 min of

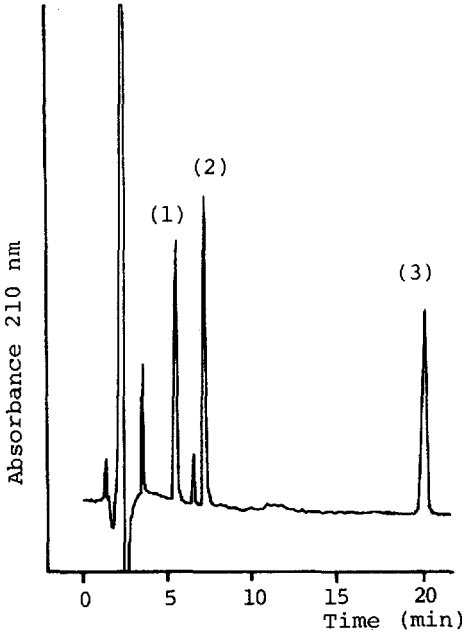

Fig. 1. Chromatogram of the artificial gastric juice 20 min after the addition of saikosaponin a. Peaks: 1 = *saikosaponin* a, 2 = *saikosaponin* b_1, 3 = ODT (internal standard).

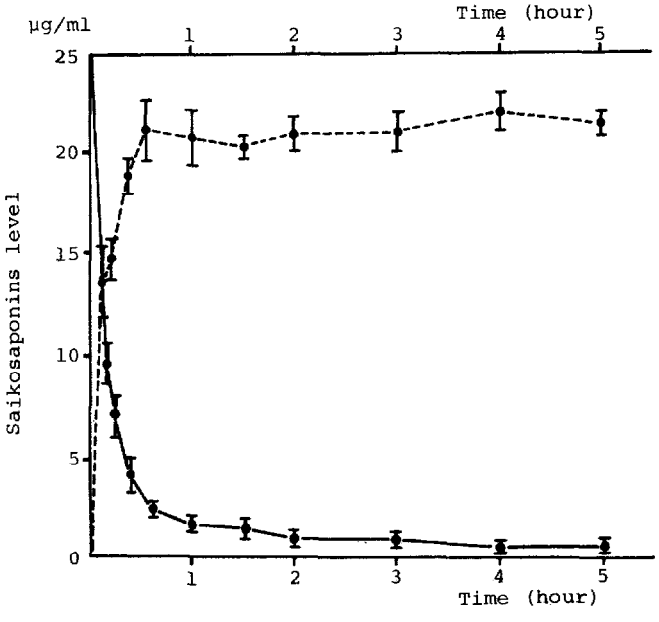

Fig. 2. Time course change of saikosaponin a to saikosaponin b_1 in the artificial gastric juice. The level of saikosaponin a is represented by a solid line and that of saikosaponin b_1 by a dotted line. Each point indicates the mean of four samples, vertical bars indicate S.E.

incubation is shown in Fig. 1. The peak of saikosaponin a corresponds to the peak appearing at a retention time of 5.8 min. A new peak, corresponding to saikosaponin b_1, appeared at 7.0 min with an unknown peak, 15—20% of the amount of saikosaponin b_1, at 6.3 min. The time course of the change of saikosaponin a to saikosaponin b_1 in the artificial gastric juice is shown in Fig. 2. The amount of saikosaponin a was rapidly decreased in acidic conditions (pH 1.2) to give saikosaponin b_1 which has a heteroannulardiene structure (see Fig. 5). The amount of saikosaponin b_1 was not decreased in acidic medium after 5 h, showing that no hydrolysis takes place at 37°C to yield sugar and saikogenin A (Fig. 5).

Then, uptake of saikosaponins into blood was examined. It may be well assumed that orally administered saikosaponin a would be changed in vivo to saikosaponin b_1 in the stomach by the action of acidic gastric juice, and the saikosaponin b_1 thus formed might be moved to the intestine. For such a reason, saikosaponin b_1 was administered to mice orally to examine its uptake into the blood stream from the intestine. A chromatogram of serum obtained 2 h after the oral administration of saikosaponin b_1 is shown in Fig. 3. Saikogenin A, the aglycone of saikosaponin b_1, was identified as the main peak at 2 h after administration. The time course of change of blood saikogenin A is shown in Fig. 4.

When saikogenin A was orally administered instead of saikosaponin b_1, the blood level of saikogenin A reached a maximum in 15 min, and then declined reaching half level at 40 min. The time lag may be interpreted as follows: saikogenin A, an aglycone of saikosaponin b_1, can be absorbed in the intestine and taken up rapidly into the blood stream. On the ohter hand, saikosaponin b_1, a glycoside, might be hydrolysed by the intestinal bacteria in the blind gut and in the large intestine to sugar and saikogenin A.

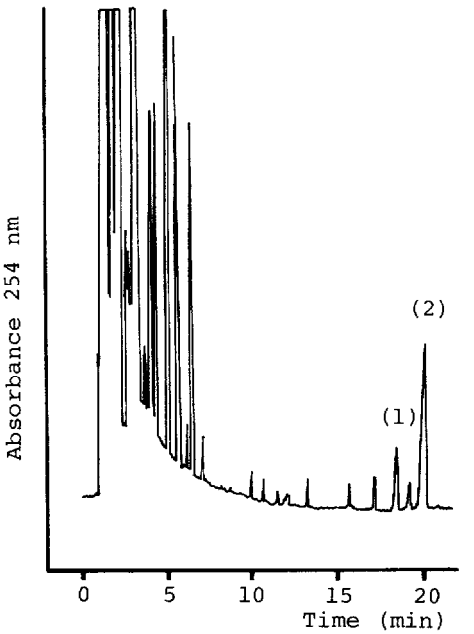

Fig. 3. Chromatogram of saikogenin A in blood 2 h after the oral administration of saikosaponin b_1. Peaks: 1 = saikogenin A, 2 = ODT (internal standard).

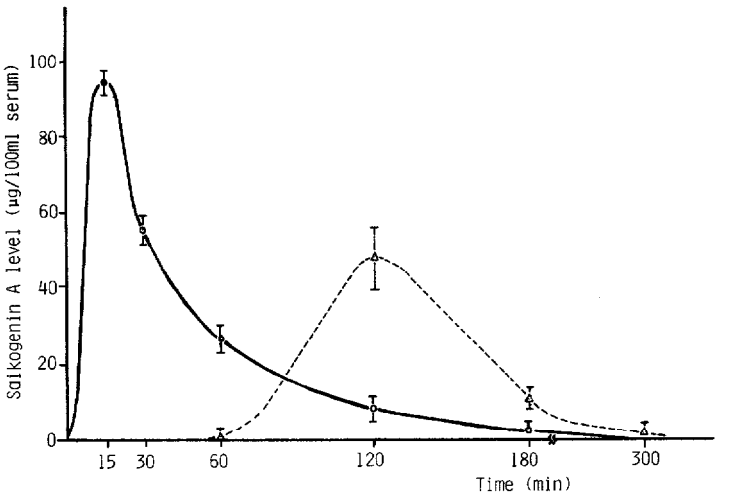

Fig. 4. Time course change of blood level of saikogenin A after oral administration of saikosaponin b_1 and saikogenin A. The blood level of saikogenin A after oral administration of saikogenin A is represented by a solid line and the blood level of saikogenin A after oral administration of saikosaponin b_1 by a dotted line. Each point indicates the mean of four samples, vertical bars indicate S.E.

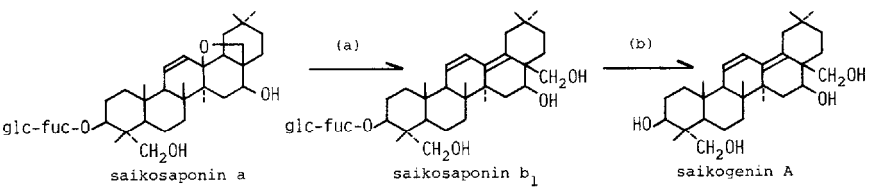

Fig. 5. Mechanism of saikosaponin b_1 and saikogenin A formation from saikosaponin a: (a) under acidic conditions in the artificial gastric juice; (b) hydrolysis of saponin by the intestinal bacteria.

The recovery of saikogenin A from serum ranged from 84.4% to 91.2% with an average of 88.0% for four determinations.

The change in chemical structure from saikosaponin a to saikogenin A, observed in the present study, is shown in Fig. 5.

It may be strongly suggested that the anti-inflammatory effect and other pharmacological effects observed by the oral administration of saikosaponin a would be the effect of its aglycone, saikogenin A.

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