Pharmacological Properties of Traditional Medicines. XXIII.[1] Searching for Active Compounds in the Blood and Bile of Rats after Oral Administrations of Extracts of Sansohnin (酸棗仁)

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We made a trial of searching for the bioactive substances from Sansohnin (酸棗仁). The blood and bile of rats after the oral administration of extracts of Sansohnin were analyzed by three-dimensional high-performance liquid chromatography (3D-HPLC). In blood, spinosin and feruloyl spinosin were found after the oral administration of a butanol extract of Sansohnin. In bile, spinosin and feruloyl spinosin were also identified after the oral administration of a water extract of Sansohnin. Spinosin and feruloyl spinosin induced the prolongation of hexobarbital sleeping time in mice at doses of 50 and 100 mg/kg, respectively. We concluded that spinosin and feruloyl spinosin were the bioactive constituents of Sansohnin. It was assumed that spinosin and feruloyl spinosin circulated through the intestine and liver, therefore, these results will provide support for the sedative and hypnotic use of this crude drug in Oriental medicine.

Key words *Zizyphus spinosa* HU.; spinosin; feruloyl spinosin; sedative; hypnotic

It is reported that one out of 4—5 people suffer from sleeping disorders,[2] and insomnia has become one of the most important problems in healthy living. Chemically produced drugs have been developed and used in order to treat sleeping disorders. However, synthetic drugs not only induce unnatural sleep but also cause side effects and addiction.

Knowing these problems of synthetic drugs, we have been seeking hypnotic drugs from among traditional Sino-Japanese herbal medicines. Approximately sixty of the traditional formulae are used to treat sleep disorders, among then, Sansohnin-to (酸棗仁湯).[3]

Sansohnin-to, as a typical combination for insomnia, helps patients with weakness and fatigue, annoyance, insomnia, physiological weaknesses in sick, elderly patients, lethargy, neurasthenia, night sweats, amnesia, fright, rapid heart palpitations, vertigo, excessive dreaming and neurotic symptoms.[4]

Sansohnin (酸棗仁), the main component of Sansohnin-to, is prepared from the seeds of *Zizyphus spinosa* HU. (Rhamnaceae), and has been prescribed for sedation and to treat insomnia in China.[5] Betulin,[6] jujuboside A and B,[7] swertisin,[8] spinosin,[9] feruloyl spinosin[10] and alkaloids[11] have been isolated from Sansohnin. Few studies on the effect of these components, however, have been sufficiently done.

We have succeeded in using the following method in the identification of active components of other herbal medicines such as the roots of *Polygala tenuifolia* WILL.,[12] the rhizome of *Atractylodes japonica* KOIZUMI et KITAMURA,[13] the root bark of *Morus alba* L.[1] and so on.[14]

This new method differs from others by the procedure described below: In order for pharmacological effects to appear, active components have to be absorbed into body. Thus, first we have to identify the heterogeneous compounds in blood, bile and urine of the experimental animals that have been orally administrated crude drugs, and secondly, these compounds found in the blood should be pharmacologically examined.

In this report, studies of the active components of Sansohnin by this new method will be discussed.

MATERIALS AND METHODS

Crude Drug Sansohnin was commercially obtained from the Japanese market, Mikuni Co., Ltd. in Osaka. Powdered Sansohnin was used for this experiment.

Chemicals Chlorpromazine (CPZ) and hexobarbital were purchased from Wako Pure Chemical Industries, Ltd., in Japan. Spinosin and feruloyl spinosin were isolated from Sansohnin by means of silica gel column chromatography[9,10] and preparative HPLC.

Animals Male Wistar/ST rats (8 weeks old, 260—300 g) and male ddY mice (4 weeks old, 25—30 g) used in this experiment were purchased from Nihon S.L.C. Co., Ltd., in Hamamatsu, Japan. They were housed under conditions of 24±1 °C and a 12-h dark-light cycle, and were fed a commercial diet (MF, Oriental Yeast Co., Tokyo) and allowed tap water *ad libitum* before the experiments.

Preparation of Extracts of Sansohnin The procedures used for the preparations of two kinds of extracts were as follows. 1) Water extract (WE): 100 g of Sansohnin was mixed with 2000 ml of distilled water, and the whole was boiled until the volume was reduced to 1000 ml. The filtered decoction was freeze-dried and the obtained powder (1 g corresponds to 7 g crude drug) was kept in a refrigerator. 2) Butanol extract (BE): 100 g of Sansohnin was extracted with 2000 ml of boiling water and the filtered decoction was extracted two times with 1500 ml of *n*-butanol. The butanol phase was concentrated to dryness and kept in a refrigerator. A 1 g sample of the butanol extract obtained corresponds to 25 g of crude drug. The WE and the BE were dissolved and/or suspended in water

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(1 g/ml) just before the oral administration to rats, respectively.

Analysis of Constituents in Blood and Bile The two above-mentioned extracts of Sansohnin, WE and BE, were orally administered to rats at a dose of 3 g/rat in the form of either aqueous solution or suspension, respectively. 1) Preparation of the Blood Sample for HPLC Analysis: Blood sample was collected from a portal vein into a heparinized-tube 30 min after the administrations of BE. Plasma was immediately separated from the blood sample by centrifugation. Eight ml of methanol was added to 2 ml of the plasma and stirred. The mixture was centrifuged at 3000 rpm for 10 min at room temperature and the supernatant solution was evaporated to dryness below 40 °C under reduced pressure. One ml of methanol : water (1 : 1) was added to the residue and mixed. The mixture was centrifuged at 3000 rpm for 10 min at room temperature and the supernatant solution obtained was evaporated to dryness below 40 °C under reduced pressure. The residue was dissolved in 400 μl of methanol : water (1 : 1) and filtered through a 0.45 μm filter for three-dimensional-HPLC (3D-HPLC) analysis. 2) Preparation of the Bile Sample for HPLC Analysis: Bile duct cannulation was performed on anesthetized rats and bile samples were collected from the duct at 30 min after the oral administration of WE. The bile sample was treated in the same way as the blood sample for 3D-HPLC analysis.

Test for Prolongation of Hexobarbital Sleeping Time in Mice Spinosin and feruloyl spinosin were injected intraperitoneally to mice, and 15 min later 70 mg/kg of sodium hexobarbiturate was injected *via* the same route. The duration of loss of the righting reflex was measured and compared with that of the control group. CPZ was used as a reference agent. Assays were repeated several times and the data were statistically analyzed by Student's *t*-test.

HPLC Analysis 3D-HPLC was carried out on a Waters 600 gradient system equipped with a Waters 991J Photodiode array detector and its data processor. The column was Inertsil ODS-2 (4.6 × 250 mm, GL Science, Inc.). Column temperature was 40 °C. Flow rate was 1 ml/min. Wavelength was 230—400 nm. Mobile phase was a mixed gradient solvent system of 0.1% acetic acid in water (X) and 0.1% acetic acid in acetonitrile (Y). The gradient mixing system for the samples was from X/Y = 85/15 to X/Y = 50/50 in 50 min. Injection volumes were 20 μl for the test solutions.

RESULTS

Constituents of Sansohnin Extracts Figure 1 shows the 3D-HPLC profile of WE, and Fig. 2 shows that of BE. The UV spectral feature of the published data[9] suggested to us that A was spinosin and B was feruloyl spinosin. These were confirmed by direct comparisons of the retention times (RT) and the spectral features with authentic samples (refer to Fig. 3), respectively.

Constituents in Blood Samples after The Oral Administration of Extracts from Sansohnin The peaks in a chromatogram of blood obtained from WE-dosed rats were very small and their UV spectral features were not identified. Next, BE was examined in the same way. Figures 4 and 5 show representative chromatograms for blood obtained from the rats which were given tapwater and BE, respectively. Figure 5 shows the presence of two distinct peaks other than the compounds which always exist in blood, and these peaks were tentatively named A (RT, 12.5 min) and B (RT, 17.5 min) in the decreasing order of polarity.

Constituents in Bile Samples after the Oral Administra-

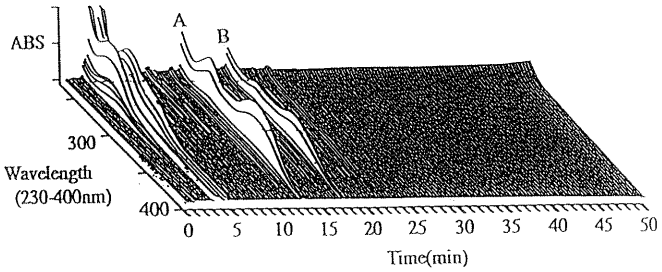

Fig. 1. 3D-HPLC Profile of WE of Sansohnin

Analytical conditions: a Waters 600 multisolvent delivery system equipped with a Waters 991J photodiode array detector and its data processor. Column: Inertsil ODS-2 (5 μm, 4.6 i.d. × 250 mm, GL Science Inc.). Column temperature: 40 °C. Detection wavelength: 230—400 nm. Mobile phase: water (X) acetonitrile (Y) gradient: X/Y = 85/15 → X/Y = 50/50 for 50 min. Flow rate: 1 ml/min.

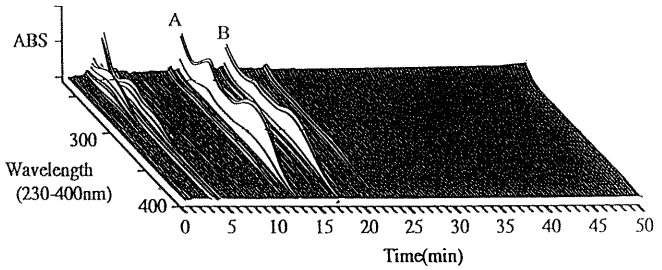

Fig. 2. 3D-HPLC Profile of BE of Sansohnin

Analytical conditions, see Fig. 1.

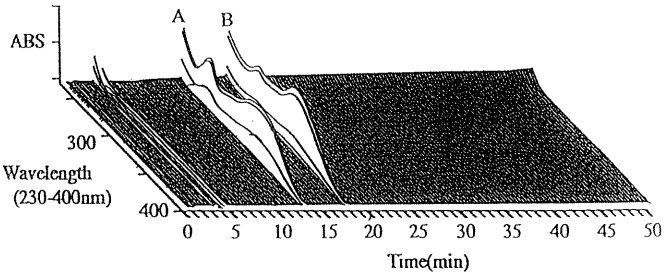

Fig. 3. 3D-HPLC Profile of Authentic Samples

Analytical conditions, see Fig. 1. A (RT, 12.5 min): spinosin; B (RT, 17.5 min): feruloyl spinosin.

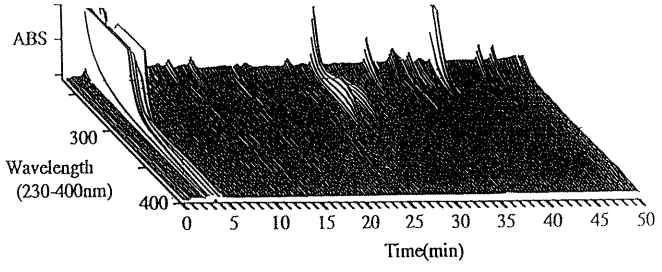

Fig. 4. 3D-HPLC Profile of Plasma of Control Rats

Analytical conditions, see Fig. 1.

tion of Extracts from Sansohnin Figures 6 and 7 show representative chromatograms of the bile obtained from the rats which were given tap water and WE, respectively. Figure 7 shows the presence of two distinct peaks, which represeted the same compounds as A and B in Fig. 5, respectively. Other peaks derived from WE were present only in small amounts in the bile of WE-dosed rats, and they were non-identified compounds in this experiment.

Prolongation of Hexobarbital Sleeping Time in Mice The results are shown in Table 1. The duration of sleeping of the mice treated with spinosin and feruloyl spinosin at doses of 50 and 100 mg/kg, respectively, was significantly longer than that of the untreated mice.

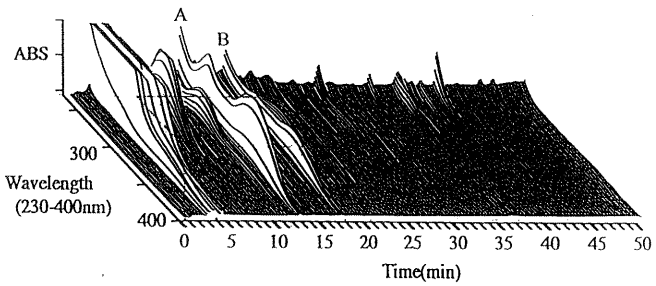

Fig. 5. 3D-HPLC Profile of Plasma of Rats at 30 min after Oral Administration of BE of Sansohnin

A (RT, 12.5 min): spinosin; **B** (RT, 17.5 min): feruloyl spinosin.

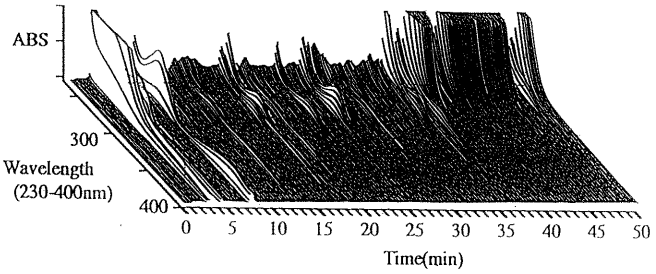

Fig. 6. 3D-HPLC Profile of Bile of Control Rats

Analytical conditions, see Fig. 1.

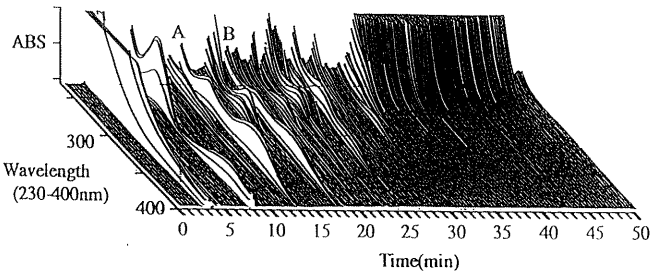

Fig. 7. 3D-HPLC Profile of Bile of Rats after Oral Administration of WE of Sansohnin

Analytical conditions, see Fig. 1.

DISCUSSION

We undertook this study for the purpose of determining the bioactive compounds in Sansohnin. There are two usual ways of searching for bioactive substances from crude drugs. In one way, the peculiar chemical substances isolated from a crude drug are tested to screen their pharmacological activity; in the other, the extracts of a crude drug are tested to screen their pharmacological activity, the extracts which show activity are further fractionated, and the fractions are monitored by measurement of their activity; then, as a result of repeating the same work, the bioactive substances are isolated and determined. In this experiment, we searched for the bioactive substances from Sansohnin by a method which differs from two general methods mentioned above.

Spinosin, feruloyl spinosin and others are contained in BE. As a result, spinosin and feruloyl spinosin were detected in blood. Spinosin, feruloyl spinosin and others were also contained in WE. Therefore, spinosin and feruloyl spinosin were also detected in bile. Sansohnin has been used as a sedative and hypnotic agent in Chinese medicine; thus the next experiment was designed to clarify whether these two compounds have pharmacological potential. These two compounds each induced the prolongation of hexobarbital sleeping time in mice. It is suggested then, that spinosin and feruloyl spinosin are closely related to the sedative and hypnotic effect of Sansohnin in traditional Chinese medicine. These results provide support for the sedative and hypnotic use of Sansohnin in Oriental medicine, and it is suggested that spinosin and feruloyl spinosin are the main bioactive substances in this crude drug.

It was found that spinosin and feruloyl spinosin were absorbed in blood, then excreted in unchanged forms of themselves in bile. Therefore, we consider that spinosin and feruloyl spinosin circulated through the intestines and liver. Further studies on the absorption and excretion of these constituents in Sansohnin will provide biopharmaceutical information about for the activity and toxity of this crude drug.

In our previous paper,[12] we made a trial search for bioactive substances in the blood of rats after the oral

Table 1. Effects of Spinosin and Feruloyl Spinosin on the Duration of Hexobarbital Sleeping Time in Mice

Drug	Dose (mg/kg, i.p.)	Control		Test		$p^{b)}$
		n	Mean ± S.E.[a)]	n	Mean ± S.E.[a)]	
Spinosin	25	14	23.2 ± 1.7	10	24.7 ± 1.3	
	50	15	23.9 ± 1.1	8	30.9 ± 1.1	<0.01
	100	13	24.0 ± 1.5	8	32.8 ± 1.1	<0.01
Feruloyl spinosin	50	15	22.9 ± 1.5	8	23.3 ± 2.0	
	100	9	23.9 ± 2.4	6	34.3 ± 2.2	<0.01
	200	12	25.4 ± 2.0	8	35.5 ± 2.7	<0.01
CPZ	3	8	28.0 ± 1.6	8	61.3 ± 5.0	<0.001

CPZ: chlorpromazine. *a*) All figures are sleeping time (min). *b*) Significant difference from control.

administration of the extracts of Onji (遠志), roots of *Polygala tenuifolia* WILL., and finally found 3,4,5-trimethoxycinnamic acid (TMCA), which induced the prolongation of hexobarbital sleeping time in mice. The interaction of spinosin, feruloyl spinosin and TMCA may thus be expected in the case of combining Sansohnin and Onji in a Kampo formulation.

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