An Anti-aldosteronic Diuretic Component (Drain dampness) in Polyporus Sclerotium (猪苓)

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Polyporus Sclerotium (猪苓), botanically from the *Polyporus umbellatus* (PERS.) FRIES, was traditionally used for the purpose of promoting diuresis. The present study investigated the diuretic effect of ergosta-4,6,8(14),22-tetraen-3-one (ergone) which is a maker component according to the chemical assay for its quality standardization. It resulted in a reversion to ordinary value of the urinary ratio of Na/K in deoxycoricosterone acetate (DOCA)-treated and adrenalectomized rats, although it had no this effect on the Na or K contents as well as Na/K value both in normal rats and in adrenalectomized rats without DOCA. These data indicate that ergone possesses an anti-aldosteronic diuretic effect. Moreover, it was identified in the blood and bile of rats after its administration to the gastrointestinal tract. The above results demonstrate that it is an active component of Polyporus Sclerotium.

Key words ergosta-4,6,8(14),22-tetraen-3-one (ergone); Polyporus Sclerotium; diuresis; marker component

Polyporus Sclerotium (猪苓) botanically from the sclerotium of *Polyporus umbellatus* (PERS.) FRIES was an important crude drug that was often combined in such traditional formulas, traditionally used to promote urination (diuresis), as Gorei-san (五苓散, Wuling-san, Five-ingredient Powder with Poria), Chorei-to (猪苓湯, Zhuling-tang, Polyporus Decoction), Irei-to (胃苓湯, Weiling-tang, Calm the stomach and Polyporus Decoction), Bunsyou-to (分消湯, Fenxiao-to, Separation and Reduce Decoction), Inchingorei-san (茵陳五苓散, Yinchenwuling-san, Artemisia Yinchenhao and Five-ingredient), *etc.* The previous reports[1,2] showed that its water extract resulted in an increase in urine production and a promotion of electrolyte excretion, however the active components were unknown.

Ergosta-4,6,8(14),22-tetraen-3-one (ergone, Fig. 1) was investigated as a marker component in our previous paper about the chemical assay for its quality standardization, in which its suitability to a marker component were discussed on the basis of the studies on its analytical methods and the comparative study on the similitudes to Polyporus Sclerotium.[3] It is of interest, therefore, to undertake the further study on the relationship between ergone and the diuretic effect of Polyporus Sclerotium in the present study.

MATERIALS AND METHODS

Materials The reagents and solvents of special grade including ergosterol, toluene, desoxycoricosterone acetate (DOCA), *p*-benzoquinone, sodium hydroxide, aluminium *tert*-butoxide, calcium chloride, benzene and ethylether, as well as activated alumina for column chromatography (about 75 mm), sodium standard solution, potassium standard solution, soybean oil were purchased from Wako Pure Chemical Industries, Co., Ltd. (Osaka). Spironolactone was obtained from Fujisawa Pharmaceutical, Co., Ltd. (Osaka).

Synthesis of Ergone Ergone was synthesized following J. Elks' method.[4] Briefly, aluminium *tert*-butoxide (10 g) was added to a solution of ergosterol (10 g) and *p*-benzoquinone (20 g) in toluene (220 ml), and then the mixture was boiled under reflux for 1 h. After being cooled, the solution was filtered, and the dark solid on the filter was washed with warmed benzene (50 °C). The filtrate was washed with 0.1 N sodium hydroxide, and the organic layer was further washed with H_2O. The final organic layer was dried with calcium chloride, and evaporated to dryness under reduced pressure. The obtained red residue was dissolved in benzene, and then chromatographed on alumina. After red benzene eluate was removed, subsequent yellowish benzene/ether (1 : 1) eluate was collected and evaporated to dryness under reduced pressure. Crystallization of the residues from light petroleum (bp 40—60 °C) gave 1.1 g of yellow plates (mp 112—114 °C). After further recrystallization from methanol, a yellow squamous crystal melted at 113—114 °C was obtained, which is identified as ergone with UV spectra, ^{1}H- and ^{13}C-NMR spectroscopy.

Instrumentals Hitachi 180-80 polarized Zeeman atomic absorption spectrophotometer equipped with Hitachi hollow cathode lamp (Hitachi Ltd., Tokyo) was used to analyze the sodium and potassium concentration. HPLC was performed using Shimadsu LC-6AD pump, Shimadsu CTO-10AS column oven (Shimadsu Co., Kyoto) and Waters 2996 Photodiode Array Detector (Waters Co., Milford, MA, U.S.A.).

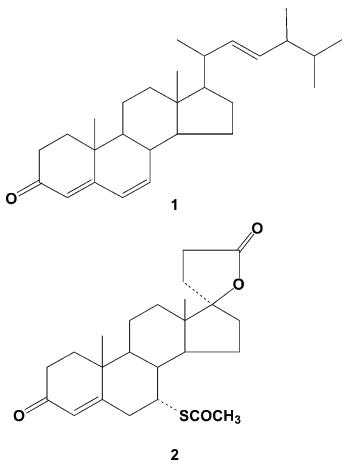

Fig. 1. Structure of Ergone (**1**) and Spironolactone (**2**)

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Animals Male Wistar rats, 5-week age with the body weight of 150—200 g and 7-week age with the body weight of 250—300 g were purchased from Japan SLC (Hamamatsu). They were used in the experiments after 5 d of acclimatization. All experimental animals were handled following the Guiding Principles for the Care and Use of Experimental Animals from Hokkaido College of Pharmacy. The adrenalectomized rat models were performed by Tsuji's method.[5] In brief, the rats under light ether anesthesia were dorsally fixed, cut open at about 2 cm above the lumbar vertebra, and then adrenalectomized using the forceps.

Animal Experiments (1) For the experiments of diuretic effects of ergone in normal rats, they were fed with sucrose cubes instead of the diets for 48 h before the experiments and were allowed access to tap water. The following experiments are carried out according to Kagawa's method.[6] They were treated with subcutaneous injection of 2.5 ml saline, subsequently, injection of ergone dissolved in soybean oil. After that, they were placed in metabolic cages, and kept for 4 h. Urine samples and washings of metabolic cages with *ca.* 10 ml water were collected, and the total volume was adjusted into 50 ml by addition of methanol. (2) For the experiments of diuretic effects of ergone in DOCA-treated adrenalectomized rats, the adrenalectomized rats were conditioned similarly to normal rat models at once after the operation. Forty-eight hours later, they were treated with subcutaneous injection of 2.5 ml saline, and then with subcutaneous injection of 12 μg/rat of DOCA in soybean oil. Subsequently, ergone in soybean oil was subcutaneously injected. Four hours urine sample was collected following the same procedures as the experiment in normal rat model. (3) For the experiments of diuretic effects of ergone in DOCA-untreated adrenalectomized rats, the adrenalectomized rats were conditioned similarly to normal rat models at once after the operation. Forty-eight hours later, they were treated with subcutaneous injection of 2.5 ml saline, subsequently with subcutaneous injection of ergone in soybean oil. Four hours urine sample was collected following the same procedures as the experiment in normal rat model.

Measurement of Sodium and Potassium Each 50 ml pooled specimen was filtrated through 0.45 μm membrane filter. The three mixtures of 400 μl of 1 M HCl and 200 μl of the pooled specimen separately containing 0, 100 μl and 200 μl of sodium standard solution (100 ppm) were prepared for the measurement of sodium. Similarly, three mixtures of 400 μl of 1 M HCl and 400 μl of the pooled specimen separately containing 0, 200 μl and 400 μl of potassium standard solution (100 ppm) were prepared for the measurement of potassium. The concentration values (mM) representing measures of total sodium and potassium were computed from the calibration curves generated by using the absorbance detected by atomic absorption spectrophotometer and the 3 corresponding concentrations of both standard solution.

Analysis for the Absorption of Ergon in Rats The dosing solution was prepared by dissolving 10 mg of ergone in 325 μl soybean oil, adding 200 μl of bile and 300 μl of water, and thoroughly stirring. The abdomen of Wistar rat (10 weeks old) was cut open under light anesthesia with ether. The upper part of small intestine was cannulated with polyethylene tubing. Dosing solutions were, then, orally given slowly. Bile samples were collected for 2 h after the drug administration, subsequently, and then, blood samples were collected from cervical vein. Heparinized blood and bile samples of 10 rats were stored at −80 °C until analysis. Fifteen milliliters of bile samples were diluted with the same amounts of saline, and then were extracted with 60 ml of ether. The ether layer was evaporated to dryness under the normal pressure. The residue was dissolved with 1 ml of methanol, and filtered through a 0.45 μm membrane filter. Twenty microliters of the methanol solution was applied to HPLC analysis as mentioning below. Forty-five milliliters of saline was added to 45 ml of blood samples, and mixed in an ultrasonic bath. The solution was extracted with about 150 ml of ether after adjusting its pH to 4.5 with 1 M hydrochloric acid. The ether layer was separated and evaporated to dryness under the normal pressure. The residue was dissolved in 1 ml of MeOH/H_2O 90 : 10, and then centrifuged (3000 rpm, 15 min). The supernatant was loaded onto a disposable Sep-pack C_{18} cartridges (Waters), subsequently washed with 1 ml of MeOH/H_2O 90 : 10. The eluate from 3 to 10 ml was pooled and evaporated to dryness under vacuum, and the residue was dissolved in 1 ml of methanol, filtered through a 0.45 μm membrane filter. Twenty microliters of methanol solution was, then, applied to HPLC analysis as follows: column, Inertsil ODS-3 (5 μm, 150 mm×4.6 mm i.d., GL Science Inc., Tokyo); temperature, 40 °C; mobile phase, MeOH/H_2O 90 : 10; flow rate, 1.0 ml/min.

Statistical Analysis The results are expressed as means±S.D. Statistical significance of differences was evaluated by one-way analysis of variance (ANOVA) followed by multiple test of Bonferroni/Dunnet. A value of $p<0.05$ was considered as statistically significant.

RESULT

Diuretic Effect of Ergone in Normal Rats Ergone did not show significant effect on the excretion of both urinary sodium and potassium in normal rats (Table 1).

Diuretic Effect of Ergone in DOCA-Treated Adrenalectomized Rats In the positive experiment, spironolactone dose-dependently increased both the urinary sodium excretion and the ratio of Na/K in DOCA-treated adrenalectomized rats, which data was in good agreement with previous report.[6] Ergone at a dose of 10 mg/rat significantly elevated the urinary ratio of Na/K as well in spite of no such effect shown at a dose of 1.0 mg/rat (Table 2).

Diuretic Effect of Ergone in DOCA-Untreated Adrenalectomized Rats There is no significant difference in the effect on the urinary sodium excretion and the ratio of Na/K between the administrations of either ergone or spironolactone and no administration of both in DOCA-untreated

Table 1. Effect of Ergone in Normal Rats

Treatment/rat		Values (mM)		Ratio
DOCA (μg)	Ergone (mg)	Na	K	(Na×10)/K
—	—	3.31±2.14	1.24±0.52	32.0±22.6
—	1.0	3.02±1.59	1.18±0.22	24.9±10.0
—	10.0	3.10±1.70	1.54±0.58	19.2±5.7

Values are cation concentrations in 4 h urine of the rat diluted to 50 ml and expressed as mean±S.D. (n=5—6).

Table 2. Effect of Ergone in DOCA-Treated Adrenalectomized Rats

Treatment/rat		Values (mM)		Ratio
DOCA (μg)	Spironolactone (mg)	Na	K	(Na×10)/K
—	—	1.95±0.85	0.75±0.38	27.0±5.8
12	—	0.77±0.36†††	0.96±0.59	9.5±4.4†††
12	0.3	1.31±0.47	1.32±0.67	11.0±4.9
12	3.0	1.85±0.90*	0.80±0.18	24.8±13.0**

Treatment/rat		Values (mM)		Ratio
DOCA (μg)	Ergone (mg)	Na	K	(Na×10)/K
—	—	1.57±1.02	0.83±0.40	18.6±8.5
12	—	0.99±0.63	1.23±0.56†	8.7±4.3††
12	1.0	0.87±0.50	0.92±0.44	10.8±5.7
12	10.0	1.42±0.65	0.95±0.49	16.0±6.6**

Values are cation concentrations in 4 h urine of the rat diluted to 50 ml and expressed as mean±S.D. (n=4—16). $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ *vs.* normal (without DOCA and Ergone) group, $*p<0.05$, $**p<0.01$ *vs.* control (with DOCA without Ergone) group.

Table 3. Effect of Ergone in DOCA-Untreated Adrenalectomized Rats

Treatment/rat		Values (mM)		Ratio
DOCA (μg)	Spironolactone (mg)	Na	K	(Na×10)/K
—	—	2.11±0.89	1.18±0.36	17.9±4.8
—	3.0	1.81±0.77	0.86±0.40	27.5±21.2

Treatment/rat		Values (mM)		Ratio
DOCA (μg)	Ergone (mg)	Na	K	(Na×10)/K
—	—	1.69±0.71	0.95±0.29	17.9±5.8
—	10.0	1.80±0.83	0.96±0.31	18.2±5.1

Values are cation concentrations in 4 h urine of the rat diluted to 50 ml and expressed as mean±S.D. (n=8—9).

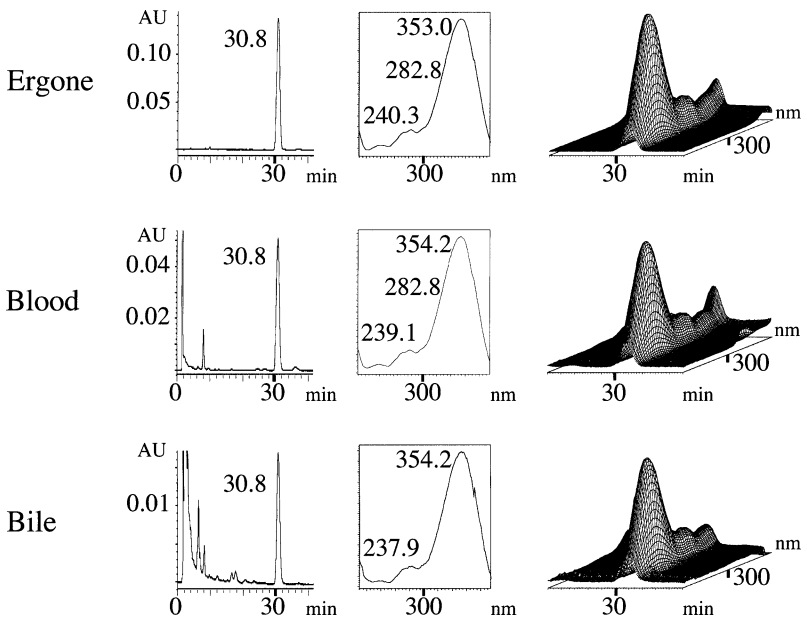

Fig. 2. HPLC Profiles (left) of Ergone Standard (upper), Blood of Rats (Holizonally Middle) and Bile of Rats (Lower) as Well as UV Spectra (Vertially Middle) and 3D-HPLC Profiles (Right) after Oral Administration of Ergone

For sample preparation and analytical conditions, see the Materials and Methods section. The peak (30.8 min) refers to ergone. UV Spectra were obtained from Waters 2996-Diode Array Detector.

adrenalectomized rats (Table 3).

Absorption of Ergone The characteristic peaks of ergone at the retention time of 30.8 min in blood and bile specimens were identified according to the same retention time and UV spectra as those of ergone standard (Fig. 2).

DISCUSSION

It is thought that Polyporus Sclerotium, among the crude drugs, possesses a relative diuretic effect and the ability to reinforce the functioning of water pathways capable of drain dampness, which is described in such an ancient work as *Shennong's Herbal Classics* (神農本草経), Shen nong ben cao jing). It was also described that it promotes urination and leaves out dampness, the problems caused by stagnance of dampness such as edema, scanty urine, vaginal discharge, cloudy painful urinary dysfunction, as well as jaundice and diarrhea.[7] As to its diuretic effect reported in early literatures, the active components were not related although its decoction showed the ability to block the absorption of urinary electrolyte and water.[7] It is reported as well that in normal dosage, it has shown no significant the diuretic effect in humans or animals, while in slight higher dosage, an increase of urine production of up to 62% has been demonstrated.[7] This herb does not contain high level of potassium, and it is thought that its diuretic effect takes place at the level of the glomeruli.[8]

Our previous study[3] suggested that ergone may be used as a marker component for the chemical standardization of Polyporus Sclerotium, which has a great similarity to spinorolactone-like diuretics (Fig. 1), anti-aldosteronic agents, from the chemically structural standpoint. Thus, it is investigated whether it is associated with the anti-aldosteronic diuretic effects in this paper. Ergone did not show a significant effect on urinary excretion of sodium and potassium in normal rats at the dose of 1.0 mg or 10 mg per rat. In such an experiment as a rat was loaded 2.5 ml of saline, the functioning the urinary water excretion, caused by elevation of circulatory plasma volume, may result in a negative feedback to the mechanism of water re-absorption. In this instance, it may be expected that the anti-aldosteronic diuretic effect of ergone does not appear because of a decrease of aldosterone. Spinorolactone as an anti-aldosteronic agent, in fact, exerts a significant diuresis in the aldosterone-increased states without effectiveness in the states of aldosterone at normal level.[9] From this view, it is considered that ergone possesses a similar effect to spinorolactone. The above results also agree with the experimental data that Polyporus Sclerotium shows no significant diuresis in normal animals in the previous studies.[7]

However, the comparative experiment between in DOCA-treated adrenalectomized rats and in normal ones produced a significant low urinary ratio of Na/K. Thus, the effect of DOCA on the enhancement of urinary re-absorption of sodium was confirmed. In this rat models, both ergone and spironolactone showed a dose-dependent blocking activity to the effect of DOCA, which is an effect of the sodium loss and potassium retention. The effect of ergone on the variation in the urinary ratio of Na/K may deduce two possibilities of ergone having aldosterone-blocking activity and having a direct effect on Na/K ion channel. The following tests in DOCA-untreated adrenalectomized rats was undertaken based on such an consideration as ergone's effect was not associated with DOCA if it directly affected on Na/K ion channel. The experimental data showed that the significantly different effect was not confirmed in comparison the ergone-treated group with the control group. Thus, it is suggested that ergone is an antagonism to aldosterone without a direct effect on Na/K ion channel. All above-mentioned results make clear that ergone may increases urinary volume in the states of water accumulation or in the stimulated states of water re-absorption caused by mineralocorticoids.

Study on the absorption of ergone in the gastrointestinal tract is important for the traditional Chinese medicine since Polyporus Sclerotium is a crude drug combined in the traditional prescriptions. The attempt to identify ergone from blood and bile specimens was made by administrating it to the gastrointestinal tract. The elucidation of HPLC analytical data confirmed it both in blood and in bile for by the same retention time and UV spectra from both specimens. It demonstrates that ergone is absorbed by oral route. However, the content of ergone in Polyporus Sclerotium is about 0.003 (w/w) %,[3] and the dosage in this experiment is too higher than the human dosage. Since this herbal medicine showed diuretic effects in clinical trials,[7,8] there would be other active constituents in this herb, and further studies to isolate other diuretic constituents are needed.

CONCLUSIONS

1. Ergone, one of components in Polyporus Sclerotium, possesses a diuretic effect by blocking mineralcorticoids.
2. Ergone can be absorbed by oral route.

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