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Pharmacological properties of galenical preparations (XVII)[1]: Active compounds in blood and bile of rats after oral administrations of extracts of Polygalae Radix

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Abstract

We made a trial of searching for the bioactive substances from Polygalae Radix by a new efficient method. The main constituents in blood and bile sample of rats after oral administration of the extracts of Polygalae Radix were analyzed by three dimensional high-performance liquid chromatography (3D-HPLC). In blood samples, 3,4,5-trimethoxycinnamic acid (TMCA) and methyl 3,4,5-trimethoxycinnamate (M-TMCA) were identified after oral administration of a water extract of Polygalae Radix, and these compounds and p-methoxycinnamic acid (PMCA) were also identified after oral administration of a chloroform extract. In bile samples, M-TMCA was identified after oral administration of water and chloroform extracts of Polygalae Radix. TMCA, M-TMCA and PMCA induced the prolongation of hexobarbital sleeping time in mice at doses of 100 mg/kg, 100 mg/kg and 150 mg/kg, respectively. We concluded that TMCA, M-TMCA and PMCA were the bioactive substances derived from Polygalae Radix ; and then these results will provide certain support for the sedative use of this crude drug in oriental medicine. This experimental method can be considered to be useful and applicable to other crude drugs.

Key words *Polygala tenuifolia,* 3,4,5-trimethoxycinnamic acid, methyl 3,4,5-trimethoxycinnamate, p-methoxycinnamic acid, absorption, hexobarbital sleeping, sedative agent.

Abbreviations CE, chloroform extract of Polygalae Radix ; CPZ, chlorpromazine ; 3D-HPLC, three dimensional high-performance liquid chromatography ; M-TMCA, methyl 3,4,5-trimethoxycinnamate ; RT, retention time ; TMCA, 3,4,5-trimethoxycinnamic acid ; PMCA, p-methoxycinnamic acid ; WE, water extracts of Polygalae Radix.

Introduction

Onji (遠志), Polygalae Radix, *Polygala tenuifolia* WILLDENOW (Yuan zhi in China), is a well known Chinese traditional medicine used as a sedative, expectorant and tonic agent. The studies on the chemical components of this crude drug have proved the presence of polygalitol,[2] N-acetyl-D-glucosamine,[2] onjisaponins A-G,[3,4] various xanthones,[5,6] 3,4,5-trimethoxycinnamic acid,[5] tenuifolioses A-P,[7,8] tenuifolisides A-D[9] and sucrose derivatives.[10] It has been reported that the extracts of Polygalae Radix and onjisaponins have some pharmacological activities.[11-15] Since there are few studies on the active compounds in Polygalae Radix, we were interested in researching this crude drug for bioactive substances. We focused on the fact that a drug needs to be absorbed into the body for showing its physiological or pharmacological effects. Now, as a part of our studies on bioactive compounds in Polygalae Radix, we wish to report the bioactive compounds detected in the blood and bile of the rats after oral administration of its extracts.

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Materials and Methods

Crude drug : Polygalae Radix, the dried root of *Polygala tenuifolia* WILLD., was commercially obtained from the Japanese market, Mikuni Co., Ltd. in Osaka. Polygalae Radix was cut into small pieces and used for this experiment.

Chemicals : 3,4,5-Trimethoxycinnamic acid (TMCA), p-methoxycinnamic acid (PMCA), chlorpromazine (CPZ) and hexobarbital (Teikoku Kagaku Sangyo Ltd.) were purchased from Wako Pure Chemical Industries Ltd. in Japan. Methyl 3,4,5-trimethoxycinnamate (M-TMCA) was isolated from the bile of rats after administration of TMCA by means of separative HPLC and was synthesized by methylation of TMCA with diazomethane in our laboratory.

Animals : Male Wistar/ST rats, weighing 190-210 g, and male ddY mice, weighing 24-28 g, used in this experiment were purchased from Nihon SLC Co., Ltd. in Hamamatsu, Japan. They were housed under conditions of 24±1 °C and 12 h light (from 6 a.m. to 6 p.m.) and fed a commercial diet (MF, Oriental Yeast Co., Tokyo) and allowed tap water *ad libitum* before the experiments.

Preparation of extracts of Polygalae Radix : The procedures used for the preparations of two kinds of extracts were as follows. 1) Water Extract (WE) : 100 g of Polygalae Radix 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 4 g of crude drug) was kept in a refrigerator. 2) Chloroform Extract (CE) : Polygalae Radix was extracted with boiling water and the filtered decoction was condensed into a concentration corresponding to 1 g crude drug per ml. The solution was extracted three times with chloroform : methanol (4 : 1). The chloroform phase was concentrated to dryness, and kept in a refrigerator. A 1 g of the chloroform extract corresponds to 113 g of crude drug. The WE and the CE were dissolved and/or suspended in water just before the oral administration to rats respectively.

Biological experiment

Analysis of constituents in blood and bile : The above-mentioned two kinds of extracts of Polygalae Radix were orally administered to rats at a dose of 1.5 g of WE/rat or 177 mg of CE/rat in a form of aqueous solution or suspension, respectively. 1) Preparation of blood sample for HPLC analysis : A blood sample was collected from a portal vein at 30 min after administrations of the extracts. Serum was immediately separated from the blood sample by centrifugation. Twelve ml of methanol was added to 2 ml of the serum and vortexed. 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 chloroform : methanol (4 : 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 0.2 ml of acetonitrile : water (4 : 1) and filtered through a 0.45 μm filter for 3D-HPLC analysis. 2) Preparation of bile sample for HPLC analysis : Bile duct cannulation was performed to anesthetized rat and bile sample was collected from the duct for one hour after administration of the extracts. The bile sample was treated with the same methods as the blood sample for 3D-HPLC analysis.

Test for prolongation of hexobarbital sleeping time in mice : TMCA, PMCA and M-TMCA 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 control group. Chlorpromazine (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 991 J 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 200-400 nm. Mobile phase was a gradient solvent system starting from 20 % (v/v) of acetonitrile/80 % (v/v) of 0.1 % aqueous acetic acid and going to 60 % (v/v) of acetonitrile/40 % (v/v) of 0.1 % aqueous acetic acid for 55 min. Injection volumes were 50 μl and 20 μl for the test solutions of blood sample and bile sample, respectively.

Results

Constituents of extracts from Polygalae Radix

Fig. 1 shows the 3D-HPLC profile for WE administered to rats, and Fig. 2 shows for CE. It was observed that the WE contained onjisaponins A-G (Retention time, RT, 40-55 min in Fig. 1) and non-identified others. The CE contained TMCA (RT 24 min in Fig. 2) as a main constituent and non-identified others. These results were confirmed by direct comparisons of the retention times and the spectral features with authentic samples (refer to Fig. 3).

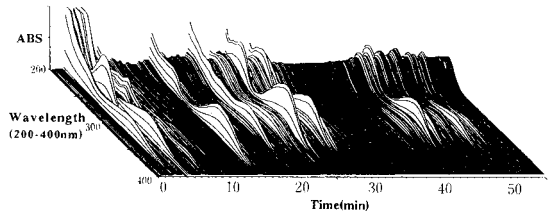

Fig. 1 3D-HPLC profile of water extract of Polygalae Radix.
Analytical conditions : A Waters 600 multisolvent delivery system equipped with a Waters 991 J photodiode array detector and its Data processor. Column : Inertsil ODS-2 (5 μm, 4.6 i.d.×250 mm, GL Science Inc.). Column temperature: 40 °C. Detection wavelength : 200-400 nm. Mobile phase : water (X)-acetonitril (Y) gradient ; (X)/(Y)=80/20 →(X)/(Y)=40/60 for 55 min. Flow rate : 1 ml/min.

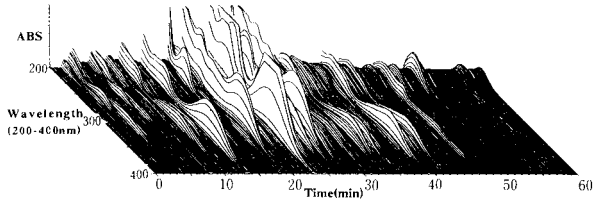

Fig. 2 3D-HPLC profile of chloroform extract of Polygalae Radix.
Analytical conditions, see Fig. 1.

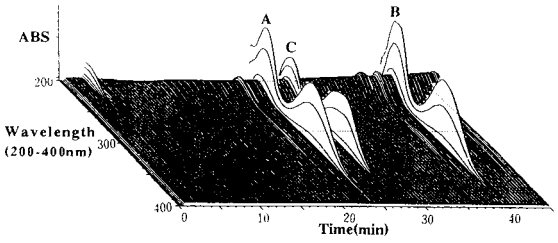

Fig. 3 3D-HPLC profile of authentic samples.
Analytical conditions, see Fig. 1..
A (RT, 24 min) : TMCA ; **B** (RT, 39 min) : M-TMCA ; **C** (RT, 27.5 min) : PMCA.

Constituents in blood samples after oral administration of extracts from Polygalae Radix

Fig. 4, 5 and 6 show representative chromatograms for blood samples obtained from the rats which were given tapwater, WE and CE, respectively. 3D-HPLC profile of the blood sample collected from the rat after oral administration of WE revealed the presence of two distinct peaks and these peaks were tentatively named **A** (RT 24 min) and **B** (RT 39 min) in the decreasing order of polarity, as shown in Fig. 5.

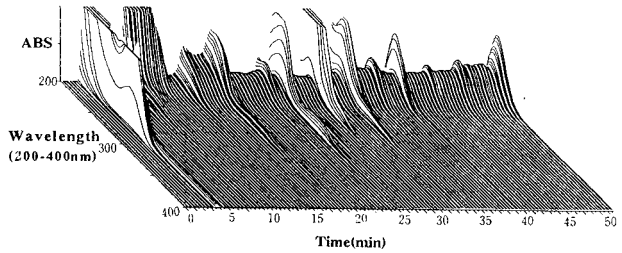

Fig. 4 3D-HPLC profile of serum of control rats.
Analytical conditions, see Fig. 1.

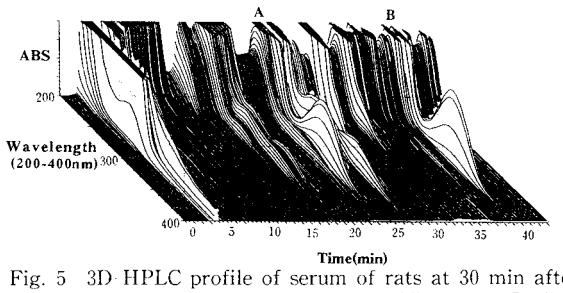

Fig. 5 3D-HPLC profile of serum of rats at 30 min after oral administration of water extract of Polygalae Radix.
Analytical conditions, see Fig. 1.
A (RT, 24 min) : TMCA ; **B** (RT, 39 min) : M-TMCA.

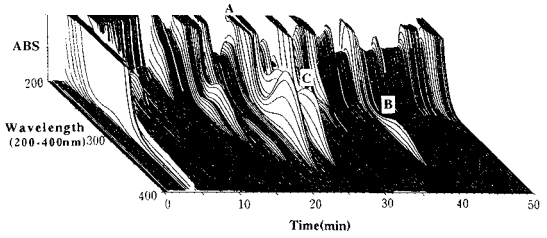

Fig. 6 3D-HPLC profile of serum of rats at 30 min after oral administration of chloroform extract of Polygalae Radix.
Analytical conditions, see Fig.1.
A (RT, 24 min) : TMCA ; **B** (RT, 39 min) : M-TMCA ; **C** (RT, 27.5 min) : PMCA.

The UV spectral feature of published data[4] suggested to us that **A** was TMCA, which was confirmed by direct comparisons of the UV spectrum and the retention time with an authentic sample in 3D-HPLC (Fig. 3). **B** was present only in small amounts in the serum of extracts-dosed rat. However, when TMCA was given orally or intraperitoneally, it was found that **B** was excreted in appreciable amounts in the bile of rat. Then **B** was isolated from the bile of TMCA-dosed rat and was identified as M-TMCA by direct comparisons with the synthesized authentic compound mentioned above. 3D-HPLC profile of the blood sample collected from the rat after oral administration of CE revealed the presence of three distinct peaks, and these peaks were tentatively named **A** (RT 24 min), **C** (RT 27.5 min) and **B** (RT 39 min), as shown in Fig. 6. **A** and **B** were the same compounds as **A** and **B** in Fig. 5, respectively. **C** was identified as PMCA by the comparisons with an authentic sample in 3D-HPLC (Fig. 3).

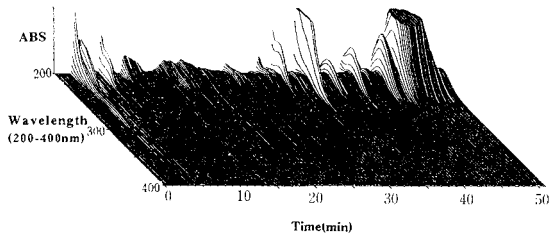

Fig. 7 3D-HPLC profile of bile of control rats.
Analytical conditions, see Fig. 1.

Constituents in bile samples after oral administration of extracts from Polygalae Radix

Fig. 7, 8 and 9 show representative chromatograms for bile samples obtained from the rats which were given tapwater, WE and CE, respectively. Fig. 8 and 9 showed the presence of one distinct peak and

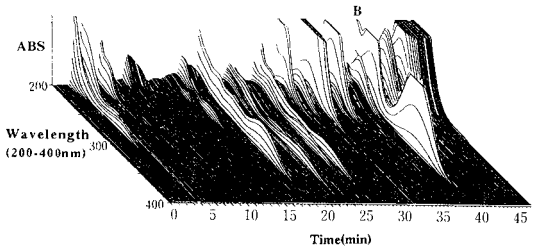

Fig. 8 3D-HPLC profile of bile of rats after oral administration of water extract of Polygalae Radix.
Analytical conditions, see Fig. 1. **B** (RT, 39 min): M-TMCA.

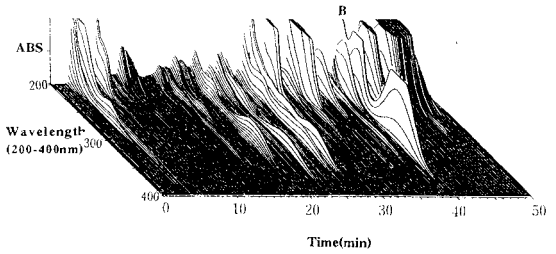

Fig. 9 3D-HPLC profile of bile of rats after oral administration of chloroform extract of Polygalae Radix.
Analytical conditions, see Fig. 1. **B** (RT, 39 min): M-TMCA.

Table I Effects of TMCA, M TMCA and PMCA on the duration of hexobarbital sleeping time in mice.

Drug	Dose	Control		Test		P[2]
	(mg/kg, i.p.)	n	mean±S.E.[1]	n	mean±S.E.[1]	
TMCA	100	9	26.3±2.3	8	33.6±1.0	<0.05
	200	10	28.4±2.1	10	40.1±2.5	<0.01
	400	10	19.9±1.9	11	43.0±3.8	<0.01
M-TMCA	50	8	28.0±1.6	9	30.3±0.9	
	100	10	24.3±1.6	8	33.4±2.2	<0.01
	200	10	24.3±1.6	10	32.0±1.7	<0.01
PMCA	75	7	24.1±3.3	10	27.7±1.8	
	150	18	25.1±2.3	8	38.6±2.1	<0.01
	300	18	25.1±2.3	8	48.7±3.4	<0.01
CPZ	3	8	28.0±1.6	8	61.3±5.0	<0.001

1)All figures are sleeping time (min). 2)Significant difference from control.
TMCA : 3,4,5-trimethoxycinnamic acid. PMCA : p-methoxycinnamic acid.
M-TMCA : methyl 3,4,5-trimethoxycinnamate. CPZ : chlorpromazine.

this peak was tentatively named **B** (RT 39 min). **B** was identified as M-TMCA by the method mentioned above. Other peaks were present only in small amounts in the bile of extracts-dosed rats and they were non-identified compounds in this experiment.

Prolongation of hexobarbital sleeping time in mice

The results are shown in Table I. The duration of sleeping of the mice treated with TMCA, M-TMCA and PMCA at doses of 100 mg/kg, 100 mg/kg and 150 mg/kg, respectively, was significantly longer than that of untreated mice. We also found the decrease of spontaneous locomotive movement and the relaxation of skeletal muscle in the mice treated with these compounds.

Discussion

The purpose of our study was to find the bioactive compounds in Polygalae Radix. There are two usual ways of searching for bioactive substances from crude drugs. In one method, the peculiar chemical substances isolated from a crude drug are tested for the screening of their pharmacological activity ; in the other, the extracts of a crude drug are tested for the screening of their pharmacological activity, and the extracts that show activity are further fractionated, and the fractions are monitored by measurement of their activity, and then as a result of repeating the same work, the bioactive substances are isolated and determined. In this experiment, we made a trial of searching for the bioactive substances from Polygalae Radix by a new efficient method which differs from the two general ways mentioned above. First of all, we analyzed the ingredients attributed to Polygalae Radix in blood samples collected from the rats after oral administration of WE and CE ; and then TMCA, M-TMCA and PMCA were detected. Polygalae Radix has been used as a sedative agent in Chinese medicine ; therefore the next experiment was designed to clarify if these three compounds have pharmacological potential. These three compounds induced the prolongation of hexobarbital sleeping time in mice respectively. It is suggested that the concentration of TMCA and M-TMCA in blood after oral administration of WE has a close relationship with the sedative effect of Polygalae Radix in traditional Chinese medicine. These results will provide certain support for the sedative use of Polygalae Radix in oriental medicine, and it is suggested that TMCA, M-TMCA and PMCA are the bioactive substances derived from this crude drug. Many substances, such as onjisaponins, sucrose derivatives and others, were contained in the WE, as shown in Fig. 1 ; however they were difficult to be detected in unchanged forms of themselves in blood sample after oral administration of the WE. TMCA and M-TMCA were detected in the blood sample after oral administration of the WE, as shown in Fig. 4 ; however they were difficult to be detected in the WE. These results suggested that TMCA and M-TMCA were the metabolites of some constituents in Polygalae Radix. TMCA and M-TMCA were found to be detected in the blood and bile of rats which were given only TMCA ; therefore this result showed one of the fact that M-TMCA was the metabolite of TMCA. Now, the original substances of TMCA and M-TMCA detected in the blood after oral administration of the WE are not clear because TMCA and M-TMCA were hardly contained in the WE. We consider that the original substances of TMCA and M-TMCA in the blood will be attributed to the constituents (so-called prodrug) in Polygalae Radix, such as onjisaponin E,F,G,[3,4] sucrose derivatives[10] and others which contain a 3,4,5-trimethoxycinnamoyl moiety within their chemical structures and which may be hydrolyzed in rat gastrointestinal tract and methylated in liver. The studies on absorption, metabolism and excretion of these constituents in Polygalae Radix will provide the biopharmaceutical information for the studies on the activity and toxicity of this crude drug.

We achieved our purpose of searching for the bioactive substances ascribed to Polygalae Radix by the new efficient method described above, which can be considered to be useful and applicable to other crude drugs.

Acknowledgement

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cal Science Program" of the Public Health Bureau of the Tokyo Metropolitan Government.

和文抄録

我々は新たに効率的な方法によって，"遠志"から生物活性物質の検索を試みた。"遠志"エキスをラットに経口投与後，血液および胆汁試料中に含まれる主要成分を三次元高速液体クロマトグラムを用いて分析した。血液試料中に於て，"遠志" 水エキスの経口投与後に 3,4,5-trimethoxycinnamic acid (TMCA) と Methyl 3,4,5-trimethoxycinnamate (M-TMCA) を確認し，"遠志" クロロホルムエキスの経口投与後には TMCA, M-TMCA, p-methoxycinnamic acid (PMCA) を確認した。胆汁試料中に於ては，"遠志"の水およびクロロホルムエキスの経口投与後に M-TMCA を確認した。TMCA, M-TMCA, PMCA は各々 100 mg/kg, 100 mg/kg, 150 mg/kg の投与で，マウスによるヘキソバルビタール睡眠時間を延長させた。我々は TMCA, M-TMCA, PMCA は "遠志" に由来する生物活性成分であると判断した。また，さらにこれらの結果は "遠志" が東洋医学において鎮静薬として用いられることへの，ある一つの証拠となるであろう。今回の実験方法は他の生薬においても有用で応用可能な方法であると考察される。

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