Quantitative determination of paeoniflorin and its major metabolite, paeonimetabolin I, in the rat plasma by enzyme immunoassay

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Abstract

After oral administration of paeoniflorin (PF) at a dose of 20 mg/kg in rats, the plasma concentrations of PF and its major metabolite paeonimetabolin I (PM-I) were determined by using the enzyme immunoassay method. The plasma concentration of PF rapidly reached a C_{max} (95 ng/ml) at 30 min, then decreased to 0.2 ng/ml at 240 min after administration of PF. On the other hand, the plasma concentration of PM-I reached a C_{max} (400 ng/ml) at 140±24.7 min, then decreased to 0.5 ng/ml at 480 min. The *AUCs* were 7700±900 and 52400±17500 ng min・ml^{-1}, for PF and PM-I, respectively, indicating that the latter was a major compound present in the plasma. These findings suggest that orally administered PF is scarcely absorbed from the gastrointestinal tract (GIT), while the unabsorbed one is transformed to PM-I by intestinal bacteria, which is subsequently absorbed from GIT. This is the first report showing the presence of PM-I in the plasma after oral administration of PF.

Key words enzyme immunoassay, paeoniflorin, paeonimetabolin, peony root, metabolism.

Abbreviations PF, paeoniflorin ; PM-I, paeonimetabolin I ; EIA, enzyme immunoassay ; BSA, bovine serum albumin ; CEP, 8-(2′-carboxyethylthio) paeonimetabolin ; CMP, 8-(carboxymethylthio)-paeonimetabolin ; *AUC*, area under the plasma concentration ; C_{max}, maximal plasma concentration.

Introduction

Paeoniflorin (PF ; **1**) is a characteristic monoterpene glucoside present in peony roots and one of the important crude drugs in traditional Chinese medicine. The therapeutic effects of peony roots have been explained by the pharmacological actions of PF (**1**).[1,2] However, recent studies showed the extremely poor absorption and low bioavailability of orally administered PF (**1**),[3,4] suggesting the bacterial degradation of PF (**1**) in the gastrointestinal tract and the role of metabolites in the therapeutic effects of peony roots. These are also supported by the evidence that PF (**1**) is readily metabolized to a series of metabolites named paeonimetabolins I, II and III by human intestinal bacteria *in vitro*.[5-7] In the previous paper, we reported the development of an enzyme immunoassay (EIA) method capable of determining the major metabolite, paeonimetabolin I (PM-I, **2**), sensitively and specifically.[8]

For the purpose of finding the significance of these metabolites in manifestation of pharmacological effects, we determined the plasma concentrations of PF (**1**) and its major metabolite PM-I (**2**), and their pharmacokinetic parameters after oral administration of PF (**1**) in rats by applying the respective EIA methods for PM-I (**2**)[8] and PF (**1**).[9]

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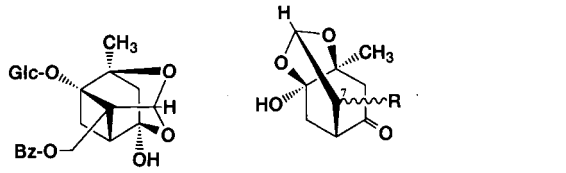

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2 : R = CH_3
3 : R = CH_2-S-CH_2-COOH
4 : R = CH_2-S-CH_2-CH_2-COOH

Fig. 1 Structures of paeoniflorin, paeonimetabolin and related compounds

Materials and Methods

Chemicals : The goat antiserum to rabbit IgG and *N*-hydroxysuccinimide were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Bovine serum albumin (BSA) was purchased from Sigma Chemicals (St. Louis, USA). Freund's complete adjuvant was a product of Difco Co. (Detroit, USA). β-Galactosidase (EC 3.2.1.23) from *Escherichia coli* was obtained from Boehringer Co. (Mannheim, Germany). 4-Methylumbelliferyl β-D-galactoside was purchased from Nacalai Tesque (Tokyo, Japan). PF (**1**) was isolated from the dried roots of *Paeonia alibiflora* PALL.[10, 11] A mixture of (*7R*)- and (*7S*)-PM-I (**2**) was prepared as reported previously,[12] their ratio being approximately 1 : 1 on the basis of ^{1}H-NMR spectral analysis.[7] The respective mixtures of (*7R*)- and (*7S*)-8-(2′-carboxyethylthio)paeonimetabolin I (CEP ; **4**) and (*7R*)- and (*7S*)- 8 -(carboxymethylthio)paeonimetabolin I (CMP ; **3**) were also prepared according to the previously reported methods.[8] Buffer A was 20 mM phosphate buffered saline (pH 7.3) containing 0.1 % BSA, 0.1 % NaN_3 and 0.001 % $MgCl_2$, and buffer B was 20 mM phosphate buffered saline (pH 7.3) containing 0.1 % NaN_3 and 0.001 % $MgCl_2$.

Animals : Male Wistar rats (7-8 weeks old, SLC Co., Hamamatsu, Japan) were used. Animals were fed standard laboratory chow with water *ad libitum* and fasted overnight before experiments.

Administration of PF to rats : A dose of 20 mg/kg PF (**1**) dissolved in distilled water was orally administered to four rats anaesthetized with ether, and blood samples (1 ml each) were taken from the tail vein with a 1 ml syringe, which had been washed with heparin at a concentration of 100 int. unit/ml. The samples were taken at 0.25, 0.5, 1, 2, 3, 4, 6 and 8 hour(s)(rats, n=3-4 at each point) and immediately centrifuged to obtain the plasma, which was directly used for measuring the concentrations of PF (**1**) and PM-I (**2**) by the respective EIA methods.

Determination of PF : Concentrations of PF (**1**) in the rat plasma were determined according to the EIA method of Kanaoka *et al.*[9]

Preparation of an antiserum to PM-I : A mixture of (*7R*)- and (*7S*)-8-(2′-carboxyethylthio)paeonimetabolin I (CEP ; **4**) was coupled with BSA by the *N*-hydroxysuccinimide ester method to give a CEP-immunogen as reported previously.[8] The immunogen was dissolved in saline and emulsified with Freund's complete adjuvant. The emulsion was subcutaneously injected into domestic albino female rabbits (n=3) at multiple sites on the back.[8] After several booster injections, the blood was withdrawn and separated by centrifugation. The sera were stored at -80°C until use.

Preparation of a labeled antigen : A mixture of (*7R*)- and (*7S*)-8-(carboxymethylthio) paeonimetabolin I (CMP ; **3**) was coupled with β-galactosidase by the *N*-hydroxysuccinimide ester method to give a CMP-labeled antigen.[8]

Determination of PM-I : Concentrations of PM-I (**2**) in the plasma were determined according to the method of Hattori *et al.*[8] The antiserum and labeled antigen were diluted with buffers A and B, respectively, to appropriate concentrations. A sample or a standard solution (50 μl) containing various amounts of PM-I (**2**) was added to the 10000-fold diluted antiserum (50 μl) and 1000-fold diluted labeled antigen (25 μl). The mixture was kept for 2 hours at room temperature, then 10-fold diluted goat anti-rabbit IgG (50 μl) and 100-fold diluted normal rabbit serum (20 μl) were added, and the mixture was incubated at 4°C overnight. After the incubation, 1 ml of buffer A was added and the solution was centrifuged at 3000×g for 25 min at 4°C. The supernatant was removed and the immunoprecipitate was washed with buffer A, followed by recentrifugation. The resulting immunoprecipitate was incubated with 0.1 mM 4-methylumbelliferyl β-D-galactoside (150 μl) at 30°C for 30 min.

Then, 3 ml of 0.1 M glycine-NaOH buffer (pH 10.3) were added to the reaction mixture, and the fluorescence intensity of 7-hydroxy-4-methylumbelliferon formed was measured spectrofluorometrically at wavelengths of 364 nm and 448 nm for excitation and emission, respectively.

Calibration curves for PF and PM-I : The plasma samples were used without dilution. The calibration curves were prepared for PF (**1**) and PM-I (**2**) in the presence of plasma from normal rats and constructed as the linearized logit-log plot. The calibration curves ranged from 0.5-500 ng/tube for both PF (**1**) and PM-I (**2**).

Pharmacokinetic analysis : The maximal plasma concentration (C_{max}) and the time at C_{max} (T_{max}) were determined from the individual profile. Area under the plasma concentration (AUC) in the time-course curve was calculated from zero to 480 min after administration by the trapezoidal rule.

Results and Discussion

As regards the pharmacological effects on the peony root and its constituents, most studies have been focused on the characteristic monoterpene glucosides such as PF (**1**) and albiflorin. However, the former showed appreciable pharmacological effects only at relatively high dosage in experimental animals,[1,2] except for the recent studies by Ohta *et al.*[13-16] and Nishi *et al.*[17] who showed that PF (**1**) significantly attenuated aging-induced and nucleus basalis magnocellularis lesion-induced learning deficits and a spatial working memory deficit produced by scopolamine, at quite low dosages. On the other hand, we previously found that PF (**1**) was metabolized to three metabolites by human intestinal bacteria[5-7] and the major metabolite PM-I (**2**) had anticonvulsant actions in rats given pentilenetetrazole and in El mice, a model animals of heredity epilepsy.[18] In this case, anticonvulsant potency of PM-I (**2**) was greater than that of PF (**1**), suggesting the metabolic activation of PF (**1**) by intestinal bacteria. These findings had stimulated us to develop a sensitive EIA method for PM-I (**2**), and we succeeded to prepare anti-PM-I antiserum which enables us to investigate the metabolism of PF (**1**) and the disposition of PM-I (**2**).[8]

After oral administration of PF (**1**) at a dose of 20 mg/kg in rats, the plasma concentrations of PF (**1**) and PM-I (**2**) were monitored by the respective EIA methods. Fig. 2 shows the time course of both compounds after the administration. The plasma concentration of PF (**1**) rapidly reached a C_{max} (95 ng/ml) at 30 min (T_{max}), and decreased to 0.2 ng/ml within 240 min, while the plasma concentration of PM-I (**2**) reached a C_{max} (400 ng/ml) later at 140 ± 24.7 min (T_{max}), then decreased to 0.5 ng/ml within 480 min. The $AUCs$ were 7700±900 and 52400±17500 ng min·ml^{-1}, for PF (**1**) and PM-I (**2**), respectively (Table I). The AUC and C_{max} values of PM-I (**2**) were 7 and 4 times greater than those of PF (**1**), respectively, indicating that PM-I (**2**) is a major compound in the plasma rather than PF (**1**) after oral administration of PF (**1**).

These findings revealed that an only small amount of orally administered PF (**1**) was immediately absorbed from the gastrointestinal tract (GIT) and rapidly eliminated from the plasma within a short period of time (T_{max}=30 min, disappearance after 240 min), which is in accordance with the previous observation that PF (**1**) was poorly absorbed from the intestinal wall and its bioavailability after oral administration was quite low (0.03-0.04) from detailed pharmacokinetic analysis of PF (**1**) in rats.[3,4] In contrast, PM-I (**2**) was absorbed later than PF (**1**) from the GIT and retained in the plasma for a relatively longer period (T_{max}=140±24.7 min, disappearance after 480 min). Moreover, it was suggested that a large amount of PM-I (**2**) was absorbed in comparison with the absorption of a small amount of PF (**1**), though pharmacokinetic parameters (Vd and AUC) of PM-I (**2**) after its intravenous injection are necessary for the validity.

Since PF (**1**) was not converted to any metabolite by incubation with a liver homogenate of rats and no PM-I (**2**) was detected in the plasma after intravenous administration of PF (**1**) in rats (unpublished results), the liver and other organs seem to have no metabolic enzymes capable of transforming PF (**1**) to PM-I (**2**). Therefore, the transformation of PF (**1**) to PM-I (**2**) was concluded to be performed by intestinal flora in rats, as demonstrated by the *in vitro* experiments using human intestinal bacteria[5,6] and by the recent

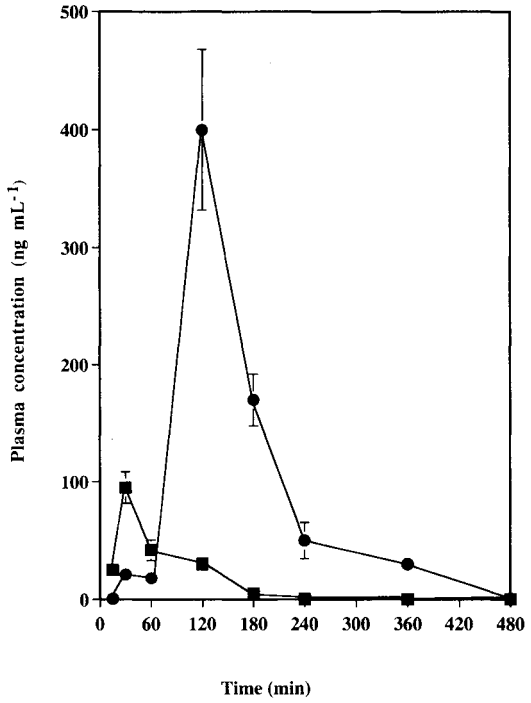

Fig. 2 Plasma concentrations of PF (**1**) (■) and PM-I (**2**) (●) after oral administration of PF (**1**) at a dose of 20 mg/kg body weight in rats.
Each point represents mean±S.E. of 3-4 rats.

Table I Pharmacokinetic data of PF (**1**) and PM-I (**2**) after oral administration of PF (**1**) at a dose of 20 mg/kg in rats

Compound	C_{max} *(ng ml^{-1})*	T_{max} *(min)*	AUC_{0-480} *(ng min ml^{-1})*
PF (**1**)	95±13.3	30±0.00	7700±900
PM-I (**2**)	400±68	140±24.7	52400±17500

observation that the plasma concentration of PF (**1**) was two-fold higher in germ free rats than in conventional rats, due to the degradation of PF (**1**) by intestinal flora in the latter.[19] These results clearly indicate the importance of metabolites rather than PF (**1**) for understanding the efficacy of the peony roots used in traditional Chinese medicine. This is the first report on PM-I (**2**) detected in the plasma after oral administration of PF (**1**) in rats, using a sensitive and specific EIA method,[8] and further pharmacokinetic experiments on PM-I (**2**) are in progress in our laboratory to elucidate the role of PM-I (**2**) in manifestation of pharmacological effects after oral administration of peony roots.

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和文抄録

ラットに 20 mg/kg のペオニフロリンを経口投与後，血漿中のペオニフロリン（PF）およびその主代謝物ペオニメタボリン-I（PM-I）の濃度をエンザイムイムノアッセイ法を用いて測定した。血漿中の PF 濃度は 30 分で急激に最高濃度（C_{max} 95 ng/ml）に達し，240 min で 0.2 ng/ml に減少した。一方，PM-I の血漿中の濃度は 140±24.7 min で最高濃度（400 ng/ml）に達し，480 min 後に 0.5 ng/ml まで減少した。PF および PM-I の AUC はそれぞれ 7700±900，524000±17500 ng/ml^{-1} であり，後者の代謝物が前者より長期間高い濃度で血漿中に存在することを示した。経口投与した配糖体の PF は消化管（GIT）から吸収されにくく，未吸収体が腸内細菌により代謝物に変換され，次いで GIT から吸収されることを示している。これは PF を経口投与した後，血漿中に PM-I が高濃度に存在することを示した最初の報告であり，PF の薬効を研究する場合，その代謝物の薬理作用を検討する必要があることを強く示唆するものである。

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