Enzyme immunoassay for paeonimetabolin I, a major metabolite of paeoniflorin by intestinal bacteria

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Abstract

For quantitative determination of paeonimetabolin I (PM-I), a mixture of *(7R)*- and *(7S)*-isomers transformed from paeoniflorin (PF) by intestinal bacteria, we established an enzyme immunoassay (EIA) method as follows.

As a hapten, a mixture of *(7R)*- and *(7S)*-8-(2-carboxyethylthio)paeonimetabolin I (CEPM) was prepared by anaerobic incubation of PF with *Lactobacillus brevis* in the presence of 3-mercaptopropionic acid. Without separation of both isomers, CEPM was coupled with β-galactosidase (β-Gal) and bovine serum albumin (BSA) *via* an *N*-hydroxysuccinimide ester method to give CEPM-β-Gal (enzyme-labeled antigen) and CEPM-BSA (immunogen), respectively. The anti-PM-I antiserum, which had been elicited in rabbits by immunization with CEPM-BSA, was specific to *(7R)*- and *(7S)*-PM-I and their 1 : 1 mixture but not to other related compounds, when the assay was carried out by the double antibody technique. Satisfactory standard curves of (*7R*)-, (*7S*)-PM-I and both isomers were obtained in a range of 0.1–500 ng/tube but appreciably interfered by addition of serum or urine to an assay mixture. By using the newly developed method, quantitative determination of the PM-I [*(7R)*-and *(7S)*-isomers] contents was possible in several fractions taken during the incubation of PF with *L. brevis*.

Key words enzyme immunoassay, human intestinal bacteria, paeoniflorin, paeonimetabolin, peony root.

Abbreviations BSA, bovine serum albumin; CEPM, 8-(2-carboxyethylthio)paeonimetabolin I; EIA, enzyme immunoassay; β-Gal, β-galactosidase; PF, paeoniflorin; PM-1, paeonimetabolin I.

Introduction

Paeoniae Radix, the dried roots of *Paeonia albiflora* PALL., is one of the most important crude drugs in traditional Chinese medicine. The drug has been used for treatment of abdominal pain and syndromes such as stiffness of abdominal muscles.[1] Various monoterpenoids, such as paeoniflorin (PF),[2-4] oxypaeoniflorin, benzoylpaeoniflorin, albiflorin,[5] paeoniflorigenone,[6,7] have been isolated from Paeoniae Radix. PF was shown to have hypotensive, vasodilative, anti-oxytocic actions, as well as relaxative and inhibitory actions on the movement and tonus of smooth muscle.[8-11] Recently, Watanabe *et al.*[12-16] reported that PF significantly attenuated aging-induced and nucleus basalis magnocellularis lesion-induced learning deficits and a spatial working memory deficit produced by scopolamine.

In our preceding papers, we have reported that

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PF is metabolized to paeonimetabolins I-III by human intestinal bacteria[17-19] and found that major metabolites, *(7R)*-and *(7S)*-paeonimetabolin I(PM-I), had anticonvulsant actions in El mice, a model animal of heredity epilepsy, and in rats given pentyleneterazol.[20] The anticonvulsant potency of PM-I was stronger than that of PF, suggesting the metabolic activation of PF by intestinal bacteria. However, it is not yet clear whether orally administered PF is converted to these metabolites in the gastrointestinal tract and absorbed into the body fluid or not, due to the major difficulty in quantitative determination of the metabolites having no intense chromophores in the molecules. As reported previously,[21] we prepared a series of PM-I derivatives having a variety of side chains at the 8-position by anaerobic incubation of PF with intestinal bacteria in the presence of 3-mercaptopropionic acid, 2-mercaptoethanol and thiobenzoic acid. These compounds were considered to be useful intermediates for preparation of haptens in EIA for PM-I, which enables us to determine PM-I concentrations in the body fluid with high sensitivity, specificity and quantification.

The present paper describes the preparation of a hapten and an antiserum specific to *(7R)*-and *(7S)*-PM-I, which includes the biotransformation of PF into *(7R)*- and *(7S)*-8-(carboxyethylthio)paeonimetabolin I (CEPM) followed by condensation of it with β-D-galactosidase (β-Gal) and bovine serum albumin (BSA). Furthermore, we investigated the newly developed EIA method of PM-I with respect to sensitivity, quantification and applicability.

Materials and Methods

Apparatus : Ultraviolet (UV) spectra were measured with a Shimadzu UV-260 spectrophotometer (Kyoto, Japan). Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured with a JEOL FX-270 NMR spectrometer (270 MHz ; Akishima, Japan). Fluorometry was performed on a Shimadzu RF-5000 spectrofluorometer.

Chromatography : Thin-layer chromatography was performed on silica gel plates (0.25 mm thickness, Kieselgel F_{254}, Merck, Darmstadt, FRG) and spots were detected under UV light or by spraying with H_2SO_4-anisaldehyde reagent followed by heating.

Chemicals : A goat antiserum to rabbit IgG was purchased from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan) and Sepharose 6B from Pharmacia Biotech (Uppsala, Sweden). BSA and γ-globulin were purchased from Sigma Chemicals (St. Louis, USA). General anaerobic medium (GAM) was a product of Nissui Seiyaku Co. Ltd. (Tokyo, Japan). Complete Freund's adjuvant was a product of Difco Co. (Detroit, USA). 3-Mercaptopropionic acid and *N*-hydroxysuccinimide were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan). β-Galactosidase (EC 3.2.1.23) from *Escherichia coli* was obtained from Boehringer Co. (Mannheim, Germany). Paeoniflorin (PF), benzoylpaeoniflorin, paeoniflorigenone, oxypaeoniflorin and albiflorin were isolated from the dried roots of *Paeonia albiflora* PALL.[2,5,6] Product F was synthesized by the method of Shibata *et al.*[2] and Aimi *et al.*[4] *(7R)*-and *(7S)*-PM-I were prepared as reported previously.[18] Buffer A was 20 mM phosphate buffered saline (pH 7.0) containing 0.1 % BSA, 0.1 % NaN_3 and 0.001 % $MgCl_2$, buffer B, 20 mM phosphate buffered saline (pH 7.0) containing 0.1 % γ-globulin, 0.1 % NaN_3 and 0.001 % $MgCl_2$, and buffer C, 20 mM phosphate buffered saline (pH 7.0) containing 1 % NaN_3 and 0.001 % $MgCl_2$.

Preparation of (7R)-and (7S)-8-(2-carboxyethylthio)paeonimetabolin-I (CEPM) : According to the previously reported method,[21] a precultured bacterial suspension (600 ml) of *Lactobacillus brevis* provided by T. Mitsuoka, the University of Tokyo, was added to GAM broth (6 ℓ) and anaerobically cultured for 12 hours at 37°C. After the culture was centrifuged at 7500×g for 10 min, the precipitates were washed with a saline solution, centrifuged and suspended in 50 mM phosphate buffer, pH 7.3 (900 ml). PF (1.2 g) and 3-mercaptopropionic acid (500 mg) were added to the suspension and the mixture was anaerobically incubated at 37°C. After 4 hours incubation, the mixture was adjusted to pH *ca.* 4 with a dilute HCl solution and extracted three times with ethylacetate (200 ml each). The organic layer was washed with an NaCl-saturated aqueous solution and evaporated *in vacuo* to give an oily residue. The residue was applied to a column of silica gel (60 g, 24 i.d.×240 mm). The column was thoroughly washed with $CHCl_3$ and eluted

with $CHCl_3$-MeOH (100 : 2) to give a mixture of *(7R)*- and *(7S)*-8-(2-carboxyethylthio)paeonimetabolin-I (CEPM). The products were chromatographically identical with authentic samples prepared previously.[21]

Preparation of succinimidyl CEPM : A solution of CEPM (289 mg, 0.96 mmol) as a mixture of *(7R)*-and *(7S)*-isomers and *N*-hydroxysuccinimide (138 mg 1.2 mmol) in dimethyl formamide (0.5 ml) was stirred for 30 min on an ice bath. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC ; 230 mg, 1.2 mol) was then added and the reaction mixture was stirred for 24 hours at room temperature. The mixture was poured into cold water saturated with NaCl (15 ml) and extracted with $CHCl_3$ (40 ml×3). The $CHCl_3$ solution was washed with water saturated with NaCl, dried over $MgSO_4$ and evaporated *in vacuo* to give succinimidyl CEPM (352 mg, *ca.* 90 % in yield) as an oily residue.

Preparation of a CEPM-BSA conjugate : A solution of BSA (34.9 mg, 0.5 μmol) in 50 mM phosphate buffer (pH 7.4, 2 ml) was added to a solution of succinimidyl CEPM (6 mg, 15 μmol) in pyridine (0.2 ml) stirring at 3°C. After being stirred for 24 hours at the same temperature, the mixture was successively dialyzed for 10 days against 10, 5 and 2.5 % pyridine-H_2O, and H_2O, and then lyophilized to give a CEPM-BSA conjugate.

Determination of the number of CEPM residues linked to a BSA molecule : A number of CEPM residues linked to the CEPM-BSA conjugate was estimated according to the method as described by Inoue.[22]

Preparation of a CEPM-β-Gal conjugate : Twenty-nine microliters of a solution of succinimidyl CEPM (2 mg of succinimidyl CEPM had been dissolved in 1 ml of dioxne) were added to a solution of β-D-galactosidase (5 μg, 9.7 nmol) in 50 mM phosphate buffer (pH 7.4, 1 ml) and the mixture was stirred at 4°C for 2 days and then chromatographed on a Sepharose 6B column (2 i.d.×31.5 cm) with a buffer solution (3.6 mM Na_2HPO_4, 1.6 mM NaH_2PO_4, 20 mM NaCl, 0.2 mM $MgCl_2$ and 3.2 mM NaN_3, pH7.0). Fractions having β-galactosidase activity were pooled and kept at 4°C until use. The fractions were used as a CEPM-β-Gal conjugate.

Preparation of anti PM-I antiserum : A CEPM-BSA conjugate (2 mg) was dissolved in a sterile saline (2 ml) and emulsified with a complete Freund's adjuvant (3 ml). The emulsion was injected into domestic albino female rabbits subcutaneously and intramuscularly at multiple sites on the back and legs. A half of the initial dose of immunogen was used as a booster once in two weeks for two months and monthly thereafter. The blood was collected from an ear vein 10 to 14 days after the injection of the last booster. The sera were obtained by centrifugation at 1550×g for 15 min and were stored at -20°C until use.

Antiserum dilution curve : Unless otherwise stated, dilution was carried out with buffer A. A mixture of 400-fold diluted CEPM-β-Gal conjugate (50 μl) and 100-fold or more diluted anti-PM-I antiserum (100 μl) was incubated in the presence or absence of PM-I (100 ng in 100 μl of buffer C) at room temperature for 2 hours. Ten-fold diluted goat antiserum to rabbit IgG (50 μl) and 100-fold diluted normal rabbit serum (20 μl) were added to the incubation mixture, and the mixture was allowed to stand at 3°C overnight. After addition of buffer A (1 ml), the resulting mixture was centrifuged at 700×g for 20 min. The supernatant was removed, and the immunoprecipitate was washed with buffer A (1 ml) and centrifuged. The precipitate was used for measurement of enzyme activity.

Assay procedure : A sample or a standard solution of PM-I (100 μl) was added to a mixture of 500-fold diluted anti-PM-I antiserum (100 μl) and a 400-fold diluted CEPM-β-Gal conjugate (50 μl). After the mixture was incubated at room temperature for 2 hours, 100-fold diluted normal rabbit serum (20 μl) and 10-fold diluted goat antiserum to rabbit IgG (50 μl) were added. Then, the mixture was kept overnight at 4°C and the resulting immunoprecipitate was treated according to the same procedure as described above.

Measurement of β-D-galactosidase activity : The immunoprecipitate was incubated with 150 μl of 0.1 mM 4-methylumbelliferyl β-D-galactoside at 30°C for 30 min. The reaction was stopped by the addition of 2 ml of 100 mM glycine-NaOH buffer (pH 10.3), and 7-hydroxy-4-methylumbelliferone released was measured by spectrofluorometry at wavelengths of 365 nm for excitation and 448 nm for emission.

Influence of serum and urine on EIA for PM-I: Serum and urine collected from humans were diluted with the same volume of buffer B. Each aliquot (100 μl) was mixed with a solution (100 μl) containing various amounts of PM-I as *(7R)*-and *(7S)*-isomers (0.1-400 ng). The mixtures were assayed by EIA under the above conditions.

Time course of the PM-I formation by intestinal bacteria: A precultured bacterial suspension of *L. brevis* was incubated in GAM broth (200 ml) for 24 hours at 37°C under anaerobic conditions. The culture was centrifuged at 7500×g for 10 min. The precipitate was washed with saline and phosphate buffer (pH 7.3) and suspended in the same buffer (20 ml) which was divided into 10 tubes. PF (1 mg) was added to each tube and the mixture was incubated at several time-intervals at 37°C under anaerobic conditions. The mixture in each tube was extracted with ethylacetate (2 ml), vortexed for 1 min, and then centrifuged at 300 ×g for 5 min. The organic layer was separated and evaporated to dryness *in vacuo*. The residue was dissolved in buffer C (1 ml), and serial dilutions were prepared for quantitative analysis of *(7R)*-and *(7S)*-PM-I by EIA.

Results and Discussion

For the purpose of establishing an EIA method for quantitative analysis of PM-I, bovine serum albumin (BSA) and β-galactosidase (β-Gal) were used as a carrier protein and a labeled enzyme, respectively. As a hapten which is linked to BSA or β-Gal, 8-(2-carboxyethylthio)paeonimetabolin-I (CEPM) was prepared by anaerobic incubation of paeoniflorin (PF) with *Lactobacillus brevis* in the presence of 3-mercaptopropionic acid.[21] This compound was a mixture of *(7R)*- and *(7S)*- isomers, whose ratio was determined to be approximately 1 : 1 by ^{1}H-NMR.[19] Without separating the two isomes, CEPM was treated with *N*-hydroxysuccinimide in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide to give succinimidyl CEPM, which was subsequently coupled with BSA and β-Gal to afford CEPM-BSA and CEPM-β-Gal conjugates, respectively (Fig. 1).

According to the method of Inoue *et al.*,[22] it was determined that 34 molecules of CEPM were incorporated into a BSA molecule. The anti-PM-I antiserum was elicited in rabbits by immunization with the CEPM-BSA conjugate. The EIA was performed by mixing the antiserum and the enzyme-labeled antigen

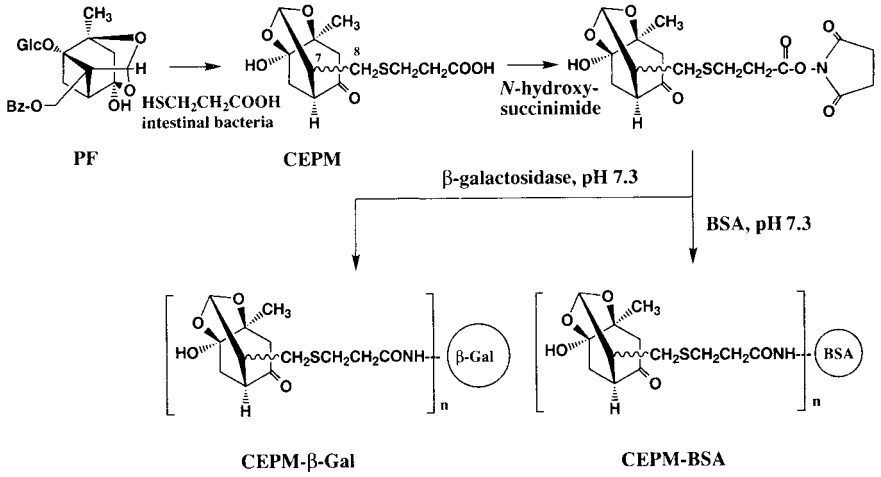


Fig. 1 Synthetic scheme for a hapten CEPM, and CEPM-BSA and CEPM β Gal conjugates.

(CEPM β-Gal) in the presence of a test sample or unlabeled antigen (PM-I), followed by adding a goat antiserum to rabbit IgG as the second antibody. The β-Gal activity in the immunoprecipitate was fluorometrically determined with 4-methylumbelliferyl β-D-galactoside as a substrate.

Fig. 2 shows dilution curves of the anti-PM-I antiserum. The maximal difference in fluorescence intensity was observed for a 500-fold dilution of antiserum in the presence and the absence of 100 ng PM-I [1 : 1 mixture of *(7R)*-and *(7S)*-PM-I] per tube. Therefore, all the experiments described below were performed by using this dilution of the antiserum.

Fig. 3 shows typical standard curves of *(7R)*-, *(7S)*-PM-I and a mixture of both isomers. The anti-PM-I antiserum prepared by immunization of an approximately 1 : 1 mixture of the *(7R)*- and *(7S)*-

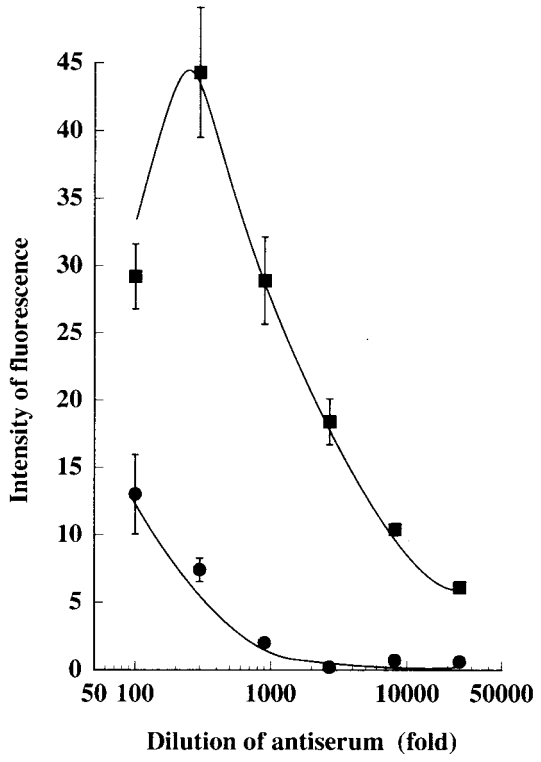


Fig. 2 Binding curves of a CEPM β Gal conjugate with various dilutions of an anti-PM I antiserum in the presence (●) and absence (■) of an unlabeled antigen PM-I [*(7R)*and *(7S)*-PM-I] (100 ng/tube).
Each point represents the mean ± S.E. (n=3)

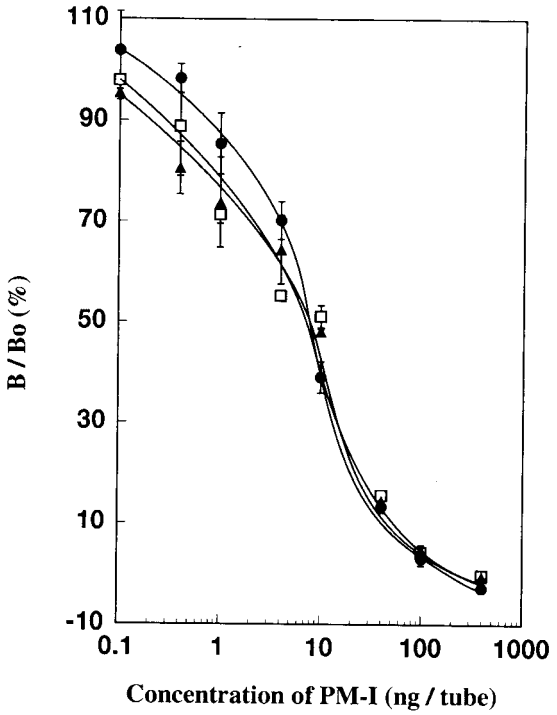
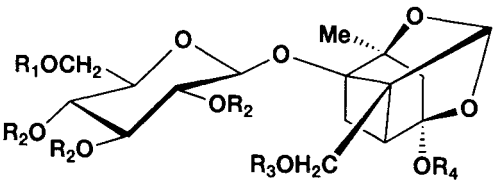


Fig. 3 Standard curves for EIA of *(7R)*, *(7S)*-PM I and their 1 : 1 mixture
B/B_0 represents the percentage of a CEPM-β Gal conjugate bound to the anti-PM-I antiserum in the presence of various concentrations of unlabeled antigens. Each point represents the mean ± S.E. (n=3). *(7R)*-PM I (●), *(7S)* PM-I (□), a 1 : 1 mixture of *(7R)*-and *(7S)* PM-I (▲)

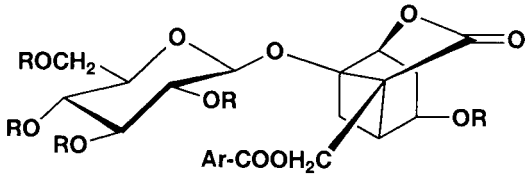
isomers in rabbit was equally reactive with the respective antigens [*(7R)*-and *(7S)*-PM-I and a mixture of both forms], giving similar standard curves.

The cross-reactivity of several analogous compounds shown in Fig. 4 were investigated against a mixture of *(7R)*- and *(7S)*-PM-I. The respective isomers, *(7R)*- and *(7S)*-PM-I, were more specific to the anti-PM-I antiserum than the mixture of them, their cross-reactivities being 123.0 and 145.0 %, respectively (Table I). Other PF-related compounds were not cross-reactive except for paeoniflorigenone. Paeoniflorigenone was reactive with the antiserum with a cross-reactivity of 3 %, due to its close similarity in structure to PM-I.

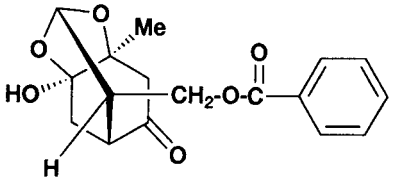
Next, we investigated the influence of serum and urine on the EIA of PM-I. In the presence of 10 % v/v serum or urine from humans as a final concentration, the standard curves of a mixture of *(7R)*-and *(7S)*-



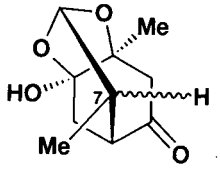
Paeoniflorin (PF) $R_1=R_2=R_4=H$, $R_3=Bz$
Tetraacetylpaeoniflorin $R_1=R_2=Ac$, $R_3=Bz$, $R_4=H$
Pentaacetylpaeoniflorin $R_1=R_3=Ac$, $R_3=Bz$, $R_4=Ac$
Benzoylpaeoniflorin $R_1=R_3=Bz$, $R_2=R_4=H$
Oxypaeoniflorin $R_1=R_2=R_4=H$, $R_3=p$-HO-Phe
Desbenzoylpaeoniflorin $R_1=R_2=R_3=R_4=H$
Product F $R_1=R_2=R_3=H$, $R_4=Me$



Albiflorin R=H
Pentaacetylalbiflorin R=Ac



Paeoniflorigenone



***(7R)- and (7S)*-Paeonimetabolin I (PM-I)**

Fig. 4 Structures of paeonimetabolin I (PM-I), paeoniflorin (PF) and related compounds.

Table I Specificity of the anti-PM-I antiserum.

Compound	Cross reaction (%)	Compound	Cross reaction (%)
(7R)- and *(7S)*-PM-I[a]	100.0	Oxypaeoniflorin	0.02
(7R)-PM-I	123.0	Paeoniflorin (PF)	0.02
(7S)-PM-I	145.0	Pentaacetylalbiflorin	0.02
Paeoniflorigenone	3.0	Pentaacetylpaeoniflorin	0.02
Albiflorin	0.02	Product F	0.02
Benzoylpaeoniflorin	0.02	Tetraacetylpaeoniflorin	0.02

Cross-reaction ratio (%) represents a relative concentration of a mixture of *(7R)*-and *(7S)*-PM-I, which is required to induce 50 % inhibition of the binding between the above compound and the antiserum.
[a] a 1 : 1 mixture of *7R* and *7S*-isomers.

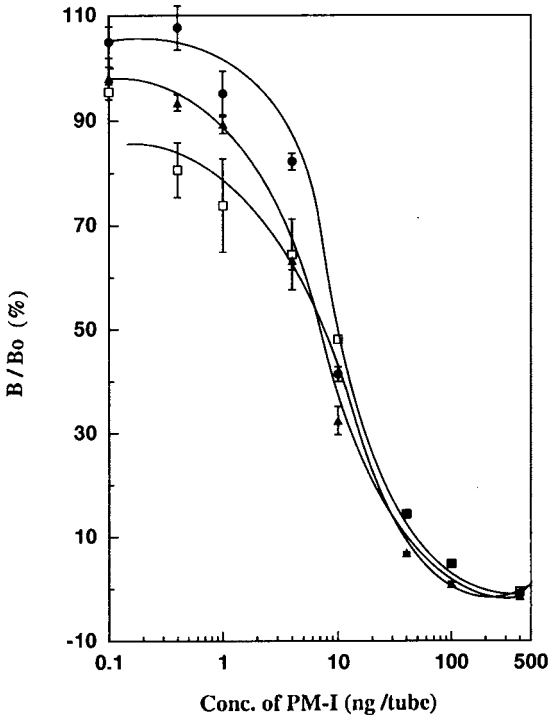


Fig. 5 Standard curves for EIA of a 1 : 1 mixture of *(7R)*- and *(7S)*-PM-I in the presence of urine and serum. Each point represents the mean± S.E. (n=3) (●), in the presence of 10 % human urine as a final concentration (v/v) ; (▲), in the presence of 10 % human serum ; (□), without urine and serum.

PM-I were appreciably changed, especially in concentrations less than 10 ng PM-I/tube, those prepared in the absence of these fluids, due to the interference by some components present in the serum and the urine (Fig. 5).

For demonstrating the applicability of the above EIA method, we examined the quantitative analysis of PM-I formed by the anaerobic incubation of PF with *Lactobacillus brevis*. The incubation mixture was taken out at several hours intervals and the amount of a mixture of *(7R)*-and *(7S)*-PM-I produced was calculated from the standard curve. Fig. 6 shows that PF was abruptly converted to PM-I at the beginning of incubation and the maximal concentration of the metabolite observed at 10 hours (135 μg/ml of culture medium), corresponding to 67 % in yield. Then, PM-I decreased in concentration after prolonged incubation up to 24 hours, indicating that PM-I is transformed to other metabolites or utilized as a carbon source by

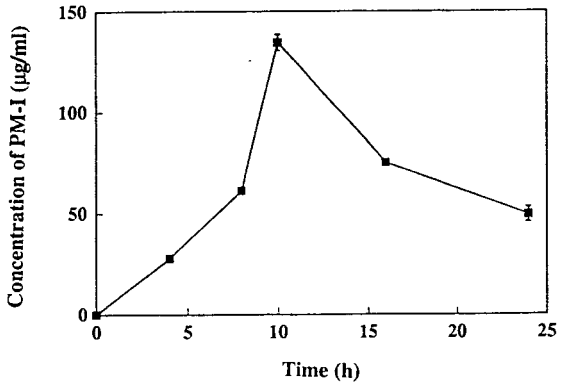


Fig. 6 Time course of the formation of a mixture of *(7S)*- and *(7R)*-PM-I from PF by *Lactobacillus brevis*. After anaerobic incubation of PF with *L. brevis* and extraction of the metabolites with an organic solvent, the amount of PM-I was quantitatively determined by EIA.

intestinal bacteria.

The proposed EIA method showed a high sensitivity in detection of PM-I, which will be applied to the determination of PM-I concentrations in the blood, urine and tissues after administration of PF. The pharmacokinetic study on PF and PM-I is now under investigation in rats and human subjects.

和文抄録

腸内細菌による paeoniflorin (PF) の主代謝物 paeonimetabolin I (PM-I) の定量を行う目的で以下の酵素免疫分析法を開発した。すなわち，ハプテンとして 3-mercaptopropionic acid の存在下，PF を *Lactobacillus brevis* と共に嫌気培養を行い 8-(2-carboxyethylthio) paeonimetabolin I (CEPM) を得た。これを *N*-hydroxysuccinimide エステル法で bovine serum albumin (BSA), β-galactosidase (β-Gal) と反応させ，抗原および酵素標識抗原を作製した。CEPM-BSA を兎に接種することにより生成した抗 PM-I 抗血清を二抗体法で検定したところ，本抗血清は *(7R)*-, *(7S)*-PM-I およびそれらの混合物に特異的であり，paeoniflorigenone (交叉活性 3 %) を除く他の類似化合物と反応しなかった (交叉活性 0.02 % 以下)。PM-I は 0.1-500 ng/tube の範囲で良好な EIA 標準曲線を得ることができたが血清や尿を添加すると検量線は妨害された。この酵素免疫分析法を応用して，*Lactobacillus brevis* による PF の代謝において生成する PM-I [*(7S)*-, *(7R)*-体の混合物] を定量的に測定することができた。

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