Development of a Simple HPLC Method for Determination of Paeoniflorin-Metabolizing Activity of Intestinal Bacteria in Rat Feces

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Received April 15, 2002; accepted May 27, 2002

A simple and reproducible HPLC method for the determination of paeoniflorin (PF)-metabolizing activity of intestinal bacteria in rat feces was developed and validated. Orally administered PF, a major active constituent of Paeoniae Radix, is metabolized into a bioactive compound, paeonimetabolin I (PM-I) by intestinal bacteria. Direct determination of the PF-metabolizing rate into PM-I is hard to achieve by HPLC due to the lack of intense chromophore in PM-I. However, when PF was incubated with *Lactobacillus brevis*, an intestinal bacterium, in the presence of phenylmercaptan, the metabolizing rate of PF into 8-phenylthio-paeonimetabolin I (PT-PM-I) was found to be equivalent to that of PF into PM-I. Thus, the PF-metabolizing activity of intestinal bacteria in rat feces was determined by measuring the rate of biotransformation of PF into PT-PM-I, which was detected by HPLC at 255 nm. This method can be utilized in the biopharmaceutical study of traditional Chinese formulations containing Paeoniae Radix.

Key words paeoniflorin; intestinal bacteria; HPLC; biotransformation; Paeoniae Radix; *Lactobacillus brevis*

Orally administered glycoside constituents of herbal drugs are biotransformed into various metabolites by intestinal bacteria.[1] The metabolizing activity of intestinal bacteria is affected by concurrent administration of other drugs such as antibiotics.[2] In our biopharmaceutical study, we wanted to determine the paeoniflorin (PF, **1**)-metabolizing activity of intestinal bacteria of rats, when Shaoyao-Gancao-tang (Shakuyaku-Kanzo-to in Japanese) was administered together with certain other synthetic drugs including antibiotics.[3]

PF is one of the active constituent of Shaoyao (Paeoniae Radix) and is biotransformed into a bioactive compound, paeonimetabolin I (PM-I, **2**) by intestinal bacteria.[4] The PF-metabolizing rate into **2** by rat intestinal bacteria can be analogized with the rate determined from the incubation of **1** with fecal suspension.[5] However, **2** thus formed is hard to detect by HPLC using an UV detector, due to the lack of chromophore in its structure. Although an enzyme immunoassay method for the determination of **2** has been established,[6] it is a time consuming procedure. Thus, a simple and efficient method for determining the PF-metabolizing activity of the intestinal bacteria is highly desired.

Here, we attempted to determine the PF-metabolizing activity through some other metabolites detectable by HPLC with an UV detector. We first prepared four thiopaeonimetabolin-I (thio-PM-I) derivatives (**3**—**6**) by incubating **1** with *Lactobacillus brevis* in the presence of four mercaptan compounds as previously reported.[7,8] Among them, the metabolizing rate of **1** into 8-phenylthio-paeonimetabolin-I (PT-PM-I, **3**) was found to be equivalent to that of **1** into **2**. Therefore, the PF-metabolizing activity in rat fecal suspension was determined by measuring the amount of **3** in the reaction mixture using HPLC at 255 nm. We herein report this new HPLC technique and its statistical validation as well as the spectroscopic data of the new thio-PM-I derivatives obtained during the biotransformation reactions.

Experimental

Chemicals PF (**1**) and benzyl mercaptan (α-toluenethiol) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phenylmercaptan (thiophenol, **7**) and thiobenzoic acid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). 3-Phenylpropyl mercaptan (3-phenyl-1-propanethiol) was purchased from Acros Organics (New Jersey, U.S.A.). General anaerobic medium (GAM) broth was a product of Nissui Seiyaku Co., Ltd. (Tokyo, Japan). Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals and solvents used were of analytical and/or HPLC grade.

General Experiments and Apparatus Optical rotations were recorded on a JASCO DIP-360 automatic polarimeter. UV spectra were measured with a Shimadzu UV-2200 UV-VIS spectrophotometer. IR spectra were measured with a Hitachi 260-01 spectrometer in KBr disks. NMR spectra were taken on a JEOL JNM-GX 400 spectrometer with tetramethylsilane (TMS) as an internal standard, and chemical shifts are expressed in δ values. High resolution (HR)-EI-MS measurements were carried out on a JEOL JMS-AX 505 spectrometer, and TLC-densitometric scannings in a Shimadzu CS-9000 dual-wavelength flying-spot scanner. Analytical and preparative TLC was carried out on pre-coated Merck silica gel 60 F_{254} plates (0.25 or 1.0 mm).

The HPLC system used consisted of a Jasco DG-980-50 three-line degasser, a Jasco PU-980 pump, a Jasco LG-1580-02 ternary gradient unit, a Jasco MD-910 multiwavelength detector (Tokyo), and a Rheodyne 7725 injector fitted with a 100 μl loop (U.S.A.) with a Jasco-Borwin chromatography data treatment system. The column was a YMC-Pack ODS-A-303 column (250×4.6 mm I.D., 5 mm, YMC, Inc., U.S.A.) with a Develosil packed guard column (ODS-MG-5, Nomura Chemical Co., LTD., Japan) housed in a Tosoh CO-8020 oven (Tokyo) set at 40 °C.

***L. brevis* and Experimental Animals** *L. brevis* was kindly provided by Prof. T. Mitsuoka when he was in the Institute of Physical and Chemical Research. Wistar male rats (8 weeks old) were purchased from Japan SLC Inc. (Hamamatsu, Japan) and maintained on a 12-h light–dark cycle at 21—24 °C with free access to water and standard laboratory chow. All animal experiments were carried out in accordance with the Guidelines of the Animal Care and Use Committee of Toyama Medical and Pharmaceutical University approved by the Japanese Association of Laboratory Animal Care.

Preparation of Thio-PM-I Derivatives with *L. brevis* Four thio-PM-I derivatives were prepared as reported previously.[7,8] Briefly, a suspension (250 ml) of the overnight-precultured original *L. brevis* was added to GAM broth (2.25 l) and cultivated for 6 h at 37 °C under anaerobic conditions. The culture was centrifuged (5000 rpm, 20 min) and the precipitate was suspended in 50 mM Na-phosphate buffer (pH 7.2, 50 ml). A mixture containing *L. brevis* suspension (50 ml), **1** (at a final concentration of 2.5 mM) and the same buffer (40 ml) was incubated at 37 °C, in the presence of four mercaptan compounds (at a final concentration of 5.0 mM): phenylmercaptan (**7**, for PT-PM-I, **3**[8]), phenylpropyl mercaptan (for 8-phenylpropylthio-paeonimetabolin-I: PPT-PM-I, **4**), benzyl mercaptan (for 8-benzylthio-paeonimetabolin-I[8]: BT-PM-I, **5**) and thiobenzoic acid (for 8-benzoylthio-paeonimetabolin-I[7]: BzT-PM-I, **6**), respectively.

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After 1.5 h of incubation, the reaction mixture was extracted with AcOEt and the soluble fraction was concentrated and directly applied to the preparative TLC plates, followed by developing in a $CHCl_3$–MeOH–benzene (4 : 1: 1.7) solvent system. The bands of the target compounds were detected under 254 nm UV light and scraped off. Elution with the 30% MeOH–$CHCl_3$ solvent afforded four thio-PM-I derivatives, **3** (18.8 mg, 24.5%), **4** (15.0 mg, 17.2%), **5** (8.0 mg, 10.0%) and **6** (8.4 mg, 9.6%).

2 (6 mg, 12.1%)[9] was prepared as above in the absence of mercaptans and the target zone in the preparative TLC plates was detected by spraying 5% anisaldehyde–H_2SO_4 reagent in a small piece cut from the developed preparative TLC.

Separation and Structure Elucidation of Thio-PM-I Derivatives The products obtained were the epimeric mixtures of isomers at C-7 (Chart 1), which showed a single spot on TLC in the solvent systems used. These epimeric mixtures (3.0 mg each) were separated into individual pure isomers (7*S* and 7*R*) of **3** (**3a**, 2.0 mg and **3b**, 0.9 mg) and **4** (**4a**, 1.9 mg and **4b**, 0.9 mg) by HPLC using the mobile phase H_2O–CH_3CN (60 : 40).

The physical and spectroscopic properties of the new 7*S*- and 7*R*-isomers of **4** as well as those of **3**[10] are as follows.

7*S*-PPT-PM-I (**4a**): Colorless oil. $[\alpha]_D^{25}$: +13.2° (*c*=0.04, MeOH). UV (MeOH) λ_{max} nm (log ε): 215 (3.92). IR (KBr) ν_{max} cm^{-1}: 3350 (br, OH), 1710 (C=O). Electron impact (EI)-MS *m/z* (rel. int.): 348 ($[M]^+$, 17), 252 (24), 152 (38), 119 (16), 118 (97), 117 (73), 105 (23), 91 (100), 77 (27), 69 (24). HR-EI-MS *m/z*: 348.1401 ($[M]^+$, Calcd for $C_{19}H_{24}O_4S$, 348.1395). ^{1}H-NMR (CD_3OD, 400 MHz) δ: 1.22 (3H, s, 10-H_3), 1.70 (1H, br t, *J*=7.8 Hz, 7-H), 1.89 (2H, qn, *J*=7.6 Hz, 8′-H_2), 2.09 and 2.29 (each 1H, dd, *J*=13.8, 2.2 Hz; dd, *J*=13.8, 3.4 Hz, 5-H_2), 2.49 and 2.85 (each 1H, d, *J*=16.9 Hz; d, *J*=16.9 Hz, 2-H_2), 2.51 (2H, t, *J*=7.6 Hz, 9′-H_2), 2.54 and 2.75 (each 1H, dd, *J*=14.8, 7.8 Hz; dd, *J*=14.8, 7.8 Hz, 8-H_2), 2.72 (2H, dd, *J*=13.9, 7.6 Hz, 7′-H_2), 2.77 (1H, m, 4-H), 5.31 (1H, s, 9-H), 7.15 (1H, tt, *J*=7.3, 1.9 Hz, 4′-H), 7.18 (2H, td, *J*=7.3, 1.9 Hz, 3′-H, 5′-H), 7.24 (2H, dd, *J*=7.3, 1.9 Hz, 2′-H, 6′-H). ^{13}C-NMR: see Table 1.

7*R*-PPT-PM-I (**4b**): Colorless oil. $[\alpha]_D^{25}$: +29.1° (*c*=0.04, MeOH). UV (MeOH) λ_{max} nm (log ε): 215 (3.91). IR (KBr) ν_{max} cm^{-1}: 3350 (br, OH), 1710 (C=O). EI-MS *m/z* (rel. int.): 348 ($[M]^+$, 16), 252 (21), 152 (39), 119 (14), 118 (95), 117 (70), 105 (22), 91 (100), 77 (23), 69 (17). HR-EI-MS *m/z*: 348.1368 ($[M]^+$, Calcd for $C_{19}H_{24}O_4S$, 348.1395). ^{1}H-NMR (CD_3OD, 400 MHz) δ: 1.86 (1H, br t, *J*=7.6 Hz, 7-H), 1.22 (3H, s, 10-H_3), 1.89 (2H, m, 8′-H_2), 2.22 and 2.24 (each 1H, br d, *J*=13.6 Hz; dd, *J*=13.6, 3.4 Hz, 5-H_2), 2.48 and 2.72 (each 1H, dd, *J*=14.6, 7.1 Hz; dd, *J*=14.6, 7.5 Hz, 8-H_2), 2.50 and 2.77 (each 1H, d, *J*=17.2 Hz; d, *J*=17.2 Hz, 2-H_2), 2.51 (2H, t, *J*=7.6 Hz, 9′-H_2), 2.68 (2H, dd, *J*=13.9, 7.6 Hz, 7′-H_2), 2.78 (1H, m, 4-H), 5.38 (1H, s, 9-H), 7.15 (1H, tt, *J*=8.3, 1.4 Hz, 4′-H), 7.17 (2H, td, *J*=8.3, 1.4 Hz, 3′-H, 5′-H), 7.24 (2H, dd, *J*=8.3, 1.4 Hz, 2′-H, 6′-H). ^{13}C-NMR: see Table 1.

7*S*-PT-PM-I (**3a**): Colorless oil. $[\alpha]_D^{25}$: −9.6° (*c*=0.2, MeOH). UV (MeOH) λ_{max} nm (log ε): 225 (4.04), 255 (4.08). IR (KBr) ν_{max} cm^{-1}: 3375 (br, OH), 1710 (C=O). EI-MS *m/z* (rel. int.): 306 ($[M]^+$, 79), 197 (6), 151 (60), 123 (33), 110 (100), 109 (66), 77 (55), 69 (96). HR-EI-MS *m/z*: 306.0928 ($[M]^+$, Calcd for $C_{16}H_{18}O_4S$, 306.0926). ^{1}H-NMR (CD_3OD, 400 MHz) δ: 1.21 (3H, s, 10-H_3), 1.70 (1H, t, *J*=7.3 Hz, 7-H), 2.09 and 2.32 (each 1H, dd, *J*=13.9, 2.4 Hz; dd, *J*=13.9, 3.4 Hz, 5-H_2), 2.50 and 2.83 (each 1H, d, *J*=16.9 Hz; d, *J*=16.9 Hz, 2-H_2), 2.80 (1H , br s, 4-H), 2.95 and 3.15 (each 1H, dd, *J*=14.1, 8.3 Hz; dd, *J*=14.1, 7.3 Hz, 8-H_2), 5.34 (1H, s, 9-H), 7.22 (1H, tt, *J*=8.7, 1.2 Hz, 4′-H), 7.30 (2H, td, *J*=8.7, 1.2 Hz, 3′-H, 5′-H), 7.39 (2H, dd, *J*=8.7, 1.2 Hz, 2′-H, 6′-H). ^{13}C-NMR: see Table 1.

7*R*-PT-PM-I (**3b**): Colorless oil. $[\alpha]_D^{25}$: −5.4° (*c*=0.08, MeOH). UV (MeOH) λ_{max} nm (log ε): 225 (4.03), 255 (4.07). IR (KBr) ν_{max} cm^{-1}: 3375 (br, OH), 1710 (C=O). EI-MS *m/z* (rel. int.): 306 ($[M]^+$, 65), 197 (7), 151 (46), 123 (27), 110 (100), 109 (61), 77 (50), 69 (71). HR-EI-MS *m/z*: 306.0928 ($[M]^+$, Calcd for $C_{16}H_{18}O_4S$, 306.0926). ^{1}H-NMR (CD_3OD, 400 MHz) δ: 1.23 (3H, s, 10-H_3), 2.02 (1H, m, 7-H), 2.18 and 2.24 (each 1H, dd, *J*=13.7, 2.7 Hz; dd, *J*=13.7, 3.4 Hz, 5-H_2), 2.50 and 2.71 (each 1H, d, *J*=17.4 Hz; d, *J*=17.4 Hz, 2-H_2), 2.79 (1H, m, 4-H), 2.63 and 2.89 (each 1H, dd, *J*=13.9, 9.5 Hz; dd, *J*=13.9, 5.9 Hz, 8-H_2), 5.41 (1H, s, 9-H), 7.19 (1H, tt, *J*=7.6, 1.4 Hz, 4′-H), 7.30 (2H, td, *J*=7.6, 1.4 Hz, 3′-H, 5′-H), 7.35 (2H, dd, *J*=7.6, 1.4 Hz, 2′-H, 6′-H). ^{13}C-NMR: see Table 1.

Quantitative Analysis by HPLC After the incubation of **1** and the four mercaptan compounds with *L. brevis* suspension, an aliquot (200 μl) of the reaction mixture was mixed with 200 μl MeOH containing IS (internal standard: **4**, 4.5 μg/ml), followed by vortex-agitation and centrifugation (10000 rpm, 10 min). The supernatant was filtered (0.45 μm), and an aliquot (100 μl) of the filtrate was analyzed by HPLC under the conditions described in the legend of Fig. 1.

Quantitative Analysis by TLC Densitometry An aliquot (90 μl) of the reaction mixture was extracted with *n*-BuOH (180 μl), an aliquot (30 μl) of the *n*-BuOH layer as well as the standard solutions was directly applied to the silica gel TLC plates, and the plates were developed by $CHCl_3$–MeOH–benzene (4 : 1 : 1.7). The developed plates were air-dried and sprayed with 5% anisaldehyde–H_2SO_4 reagent, followed by heating at 120 °C for 10 min. The spots were evaluated by TLC-densitometric scanning at 560 nm and 785 nm (reference wavelength) in reflection mode using a linear scan mode and 10.0×0.4 mm (length×width) light beam. Regression equation for **2**: Y=0.44X−225.46, r=0.9956 (Y: **2** *ng*, X: spot area). Regression equation for **3**: Y=0.35X+257.93, r=0.9967 (Y: **3** *ng*, X: spot area).

Comparison of Biotransformation Rate between PM-I (2) and Thio-PM-I Derivatives PF (**1**) was incubated with an aliquot (0.2 ml) of the *L. brevis* suspension, prepared as above, under the conditions described in the legend of Fig. 2. At time intervals of 30, 60 and 90 min, 90 μl of the reaction mixture was withdrawn. The amounts of the **2** and thio-PM-I derivatives formed as well as the **1** left were determined by TLC densitometry or/and HPLC as described above, respectively.

Optimization of the Biotransforming Reaction Conditions 1) Amounts of Rats Feces and Incubation Time: Fresh rat feces (4 g) were suspended in 50 mM Na-phosphate buffer (pH 7.2, 20 ml) by gentle homogenization. The biotransforming reactions in various amounts of rat fecal suspension (0.1—0.5 ml) for different incubation periods (5—40 min) were investigated under the conditions described in the legend of Table 2. Protein in the fresh rat fecal suspension was determined by the Lowry method with BSA as the standard sample[11] to express the PF-metabolizing activity as activity per unit weight of protein.

2) PF (**1**) and Phenylmercaptan (**7**) Concentrations: The biotransforming reactions using various concentrations of **1** or **7** were examined under the conditions described in the legend of Figs. 3 and 4, respectively.

Validation of HPLC Analysis of PT-PM-I (3) in Fecal Suspension The fresh fecal suspension prepared as above was divided into several aliquots (4 ml each) and stored at −20 °C. Intra-day precision (repeatability) of the method was studied by analyzing the biotransformation rate of **1** into **3** in the same aliquot of the fecal suspension, based on five replicate reactions on the same day. Inter-day precision (reproducibility) was assessed by analyzing the formation rate of **3** in the aliquots of the stored suspension (always first thawed) based on repeated reactions on four different days over two weeks under the same conditions.

Accuracy of the technique used for the determination of the **3** converted in the reaction mixture was evaluated by the calibration solutions, which were prepared from a sterilized fecal suspension spiked with the standard solutions of **3** (in MeOH) at 0.5, 5.0, 10, 50 and 500 μg/ml.

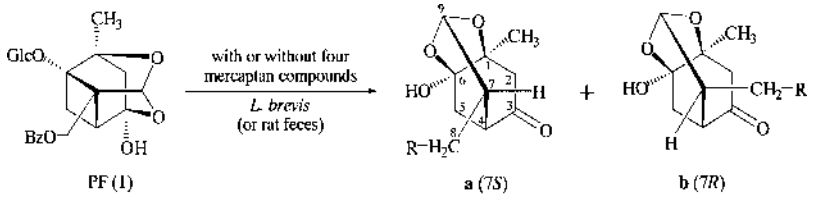
Efficiency of the extraction of **3** by MeOH from the reaction mixture was assessed by comparing the peak areas of **3** obtained from the calibration solutions (0.5, 5.0, 10, 50, 500 μg/ml) following extraction, to those obtained from the standard solutions of **3** of the same concentration directly injected into HPLC. Similar evaluation was performed on IS (**4** at 4.5 μg/ml) to study the efficiency of distribution of the IS into the MeOH soluble portion when the IS-containing MeOH was mixed with the reaction mixture.

The extracts of the reaction mixture were stored at −20 °C and the stability of the **3** was assessed by comparing the peak area of **3** obtained from the stored and the fresh extracts. This experiment was performed on four different days over two weeks under the same conditions.

Results and Discussion

Thio-PM-I Derivatives Transformed from PF (1) (Chart 1, Table 1) Four thio-PM-I derivatives possessing UV absorbing chromophores were prepared by the incubation of **1** with *L. brevis* in the presence of four mercaptan compounds. These thio-PM-I derivatives are believed to be formed by non-enzymatic Michael-type reactions during the metabolism of **1**.[7] They were obtained as epimeric mixtures at C-7 (Chart 1), and were separated into individual 7*S*- and 7*R*-isomers by HPLC using H_2O–CH_3CN (60 : 40) as a mobile phase.

The structures of the thio-PM-I derivatives, including those of the two new isomers of **4**, were confirmed by spectroscopic means including ^{1}H–^{1}H correlation spectroscopy (COSY), ^{1}H detected heteronuclear multiple quantum coher-



PM-I (**2**: **a** and **b**) : R = H
PT-PM-I (**3**: **a** and **b**) : R = S-Ph
PPT-PM-I (**4**: **a** and **b**) : R = S-$CH_2CH_2CH_2$Ph
BT-PM-I (**5**: **a** and **b**) : R = S-CH_2Ph
BzT-PM-I (**6**: **a** and **b**) : R = S-CH_2COPh

Chart 1. Structures of PF (**1**), PM-I (**2**) and Four Thio-PM-I Derivatives

Table 1. ^{13}C-NMR Data of PT-PM-I (**3**) and PPT-PM-I (**4**)

	PT-PM-I (**3**)		PPT-PM-I (**4**)	
	7*S* (**3a**)	7*R* (**3b**)	7*S* (**4a**)	7*R* (**4b**)
1	79.4	80.0	79.4	79.9
2	48.3	48.1	48.3	48.3
3	213.3	212.6	213.7	212.7
4	48.5	49.3	48.5	48.5
5	31.5	35.6	32.2	35.7
6	102.9	102.7	102.9	102.5
7	44.2	44.7	44.5	45.1
8	34.3	33.4	31.9	31.5
9	102.3	101.8	102.5	101.9
10	21.8	21.8	21.7	21.8
1′	136.4	136.9	142.8	142.8
2′, 6′	131.4	130.8	129.4	129.5
3′, 5′	130.1	130.2	129.5	129.4
4′	127.7	127.5	126.9	126.9
7′			35.5	35.5
8′			32.3	32.6
9′			32.1	31.9

CD_3OD, 100 MHz, δ.

ence (HMQC) and heteronuclear multiple bond connectivity (HMBC) experiments. The absolute configuration of 7*S*- and 7*R*-isomers were determined based on the steric interactions between C-5 and C-8 units and the ^{13}C-NMR chemical shift data.[7,12] In both **3** and **4**, the C-5 of the 7*S*-isomers resonated at upfield (δ_C 31.5 and δ_C 32.2) while those of the 7*R*-isomers resonated at downfield (δ_C 35.6 and δ_C 35.7), respectively (Table 1).

Quantitative Analysis of Thio-PM-I Derivatives by HPLC (Fig. 1) As exemplified in the HPLC profile (Fig. 1), the thio-PM-I derivatives were eluted as double peaks indicating the 7*S*- and 7*R*-isomers. The retention time of 7*S*-isomer was found to be shorter than that of 7*R*-isomer for all of the thio-PM-I products. The peak area ratio (7*S* : 7*R*) was 2.2 : 1.0 for **3** and 2.1 : 1.0 for **4**, respectively. The ratios for the other compounds were also in agreement with those in the previous report.[9]

The calibration curves developed by plotting the peak area (sum of two isomers) ratios (*e.g.*, **3** *vs.* IS: **4**) against the amount of the analyte injected showed good linearity at the range of 0.1 μg/ml to 500 μg/ml with the regression coefficients in the range of 0.9991—0.9999.

Formation Rate of PM-I (2) and Thio-PM-I Derivatives (Fig. 2) The time courses of bioformation of the four thio-

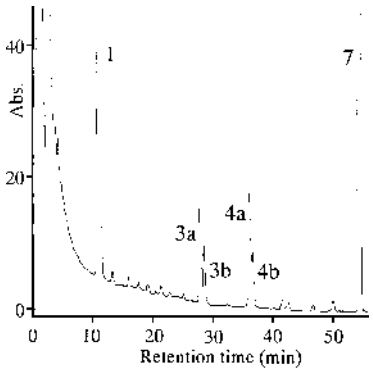


Fig. 1. HPLC Profile of MeOH Soluble Portion of Incubation Mixture of Fecal Suspension with PF (**1**) and Phenylmercaptan (**7**)

HPLC conditions: column, YMC-Pack ODS-A-303 (250×4.6 mm I.D., 5 μm); gradient mobile phase, H_2O–CH_3CN (90 : 10→0 : 100, v/v, in 70 min); flow-rate, 1.0 ml/min; temperature, 40 °C; detection, UV at 255 nm; injection volume, 100 μl. Peaks **1**: PF; **3a** and **3b**: 7*S*- and 7*R*-PT-PM-I (**3**); **4a** and **4b**: 7*S*- and 7*R*-PPT-PM-I (**4**) (IS); **7**: phenylmercaptan (thiophenol).

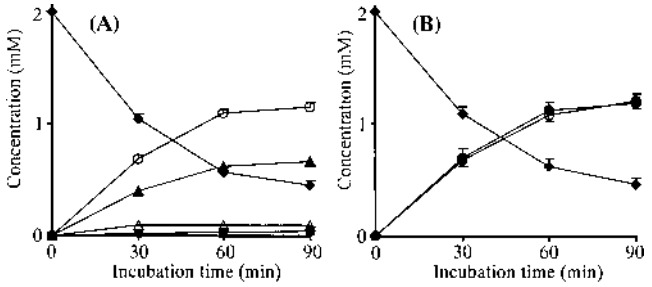


Fig. 2. Formation Rate of PM-I (**2**) and Thio-PM-I Derivatives Obtained by Incubating *L. brevis* and PF (**1**) without or with Mercaptan Compounds

A: determined by HPLC; B: determined by TLC densitometry. Each piece of data represents mean±S.D. (n=3). A mixture containing *L. brevis* suspension (0.2 ml, 1.43 mg protein), PF (final 2.0 mM) without or with four mercaptan compounds (final 5.0 mM) in 50 mM Na-phosphate buffer (pH 7.2), total 0.4 ml, was incubated at 37 °C. ◆: **1** left during its transformation into **3**. ●: **2** obtained in the absence of mercaptan compound. ○: **3**; ▲: **5**; △: **6**; ■: **4**, obtained in the presence of mercaptan compounds. Mercaptan compounds: phenylmercaptan (**7**, for **3**), benzyl mercaptan (for **5**), thiobenzoic acid (for **6**) and phenylpropyl mercaptan (for **4**). The formation rate of **3** determined by HPLC was equivalent to that determined by TLC densitometry.

PM-I derivatives from **1** and the four mercaptan compounds by *L. brevis* were compared with that of **2** from **1** alone. **3** exhibited higher bioformation rate than the three other adducts, **4**, **5** and **6** (Fig. 2A: determined by HPLC). This bioformation rate of **3** was found to be equivalent to that of **2** (Fig. 2B: determined by TLC densitometry). Thus, the PF-metabolizing activity of intestinal bacteria could also be determined

through the rate of bioformation of **3** from **1** and **7**, not only through that of **2** from **1** alone.

Moreover, the yields of **3** converted from **1** (0.38 mg) after incubation with *L. brevis* suspension (0.2 ml, 1.43 mg protein) and rat fecal suspension (0.4 ml, 4.68 mg protein) for 15 min was 42.3 μg and 2.5 μg, respectively. This proved that the PF-metabolizing activity of rat feces was much lower than that of *L. brevis*. Since the **2** formed by *L. brevis* was undetectable by HPLC (data not shown) due to the lack of chromophore in its structure, that produced by the rat feces was even more difficult to determine. However, the **3** formed could be easily detected by HPLC at 255 nm (Fig. 1). The bioformation rate of **3** determined by HPLC was equivalent to that determined by TLC densitometry (Fig. 2). Therefore, instead of **2**, **3** can be utilized as a marker substance for the determination of PF-metabolizing activity of intestinal bacteria using HPLC.

Optimization of Feces Amounts and Incubation Time As shown in Table 2, when the amounts of the fecal protein were in the range of 1.17—4.68 mg, and incubated with **1** (2.0 mM) and **7** (5.0 mM) for 5—25 min, good linearities were observed between the concentration of the **3** formed and the incubation time. Based on these results, the incubation conditions using fecal suspension (0.4 ml, 4.68 mg protein) and incubation time (15 min) were proved to be the optimal conditions for determining the PF-metabolizing activity of intestinal bacteria in rat feces.

Optimization of PF (1) and Phenylmercaptan (7) Amounts (Figs. 3, 4) The bioformation rates of **3** obtained while maintaining **7** at 5.0 mM in the fecal suspension (0.4 ml, 4.68 mg protein) were directly plotted against different concentrations of **1** (Fig. 3A). The effects of the concentrations of **1** on the biotransformation rates were evaluated using the Michaelis–Menten method (steady-state method). The achieved curve was in good agreement with the Michaelis–Menten model (a rectangular hyperbola), and approached the steady kinetic state (asymptote) reflecting the substrate saturation and the maximal transformation rate when **1** was 2.0 mM. The apparent Michaelis–Menten constant (K_m, a measure of enzyme-substrate affinity) of the feces for **1** obtained from a double reciprocal plot (a Lineweaver–Burke plot) of the same data was 0.12 mM (Fig. 3B). Moreover, the K_m of *L. brevis* (0.2 ml, 1.43 mg protein) for **1**, which was evaluated under the same conditions, was 0.14 mM, similar to that of the feces. This proved that the feces and *L. brevis* have similar utilization of **1**.

A similar investigation was performed on **7** to find its optimal concentration, with **1** at 2.0 mM in the same amount of fecal suspension. As shown in Fig. 4, the bioformation rate of **3** increased with increasing concentrations of **7** and reached maximum when **7** was 5.0 mM or more. A similar result was obtained for *L. brevis*. Based on the above results, 2.0 mM of **1** and 5.0 mM of **7** were selected as the proper concentrations for determining the bioformation rate of **3** from **1** in fecal suspension.

Validation of HPLC Analysis of PT-PM-I (3) in Fecal Suspension (Fig. 5) From the above results, the optimal reaction conditions for the bioformation of **3** from **1** and **7** by rat fecal suspension were established (Fig. 5). The concentration of **3** in the reaction mixture was determined under the HPLC conditions described in the legend of Fig. 1.

The mean intra-day precision of the method for determination of the rat feces activity was 2.6% (coefficient of variation, CV%, $n=5$). The inter-day precision was not evaluated since the activity was greatly decreased (37% of the original, $n=4$) after being stored frozen for 1 d. However, after the fecal suspensions were processed with reaction and treated with methanol, the extracts were stable when stored at −20 °C for at least two weeks (4.3% change from the fresh extracts, $n=4$). The mean accuracy for the determination of the **3** formed in the extracts was 103% (percentage of nominal concentrations, $n=5$). The mean efficiency of the extraction was 94% ($n=5$) and 93% ($n=3$) for **3** and the internal standard (**4**), respectively. These data were acceptable in accordance with the generally accepted guidelines for assay validation.[13]

Table 2. Time Course of PT-PM-I (**3**) Formation in Rat Fecal Suspension

Incubation time (min)	Rat fecal suspension		
	0.1 ml (1.17 mg)	0.2 ml (2.34 mg)	0.4 ml (4.68 mg)
5	1.13±0.04	2.36±0.09	4.53±0.15
10	2.30±0.07	4.70±0.17	9.12±0.31
15	3.40±0.09	7.05±0.16	13.61±0.23
20	4.62±0.08	9.34±0.29	18.03±0.29
25	5.63±0.07	11.42±0.31	22.15±0.49

Each value represents mean±S.D. ($n=3$) of the **3** formed (μM). Values in parenthesis represent protein contents in the fecal suspension. Transforming reaction: the rat fecal suspension (fresh feces 0.2 g/ml in 50 mM Na-phosphate buffer, pH 7.2) was mixed with **1** (final 2.0 mM) and **7** (final 5.0 mM), total 0.6 ml, and incubated at 37 °C. At the indicated time, 70 μl of the reaction mixture was withdrawn and immediately mixed with 70 μl of IS-containing MeOH. After filtration (0.45 μm), 100 μl of the filtrate was subjected to HPLC for the quantitative analysis of the **3** formed.

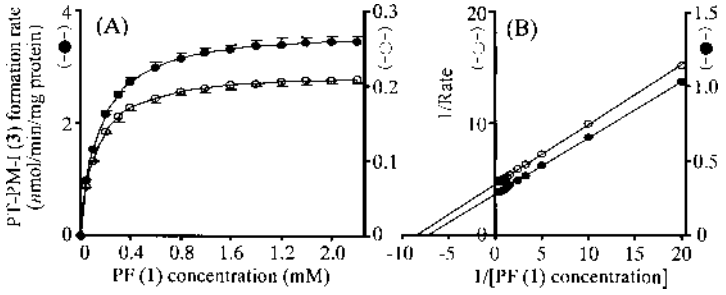


Fig. 3. Effect of PF (**1**) Concentration on PT-PM-I (**3**) Formation Rate

A: formation rate; B: Lineweaver–Burk Plots (inverse of formation rate *vs.* inverse of PF concentration). Each piece of data represents mean±S.D. ($n=3$). ●: incubation with *L. brevis* suspension; ○: incubation with fecal suspension. A mixture containing 0.4 ml fecal suspension (or 0.2 ml *L. brevis*), various concentrations of **1** (final 0.05—2.2 mM), **7** (final 5.0 mM) and 50 mM Na-phosphate buffer (pH 7.2), total 0.6 ml (or 0.4 ml), was incubated at 37 °C. After 15 min, 200 μl of the reaction mixture was withdrawn and immediately extracted with 200 μl MeOH, and the formation rate of **3** was determined by HPLC as described in the legend of Fig. 1.

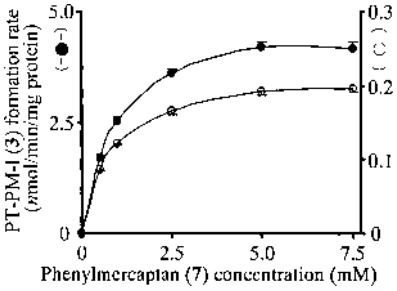


Fig. 4. Effect of Phenylmercaptan (**7**) Concentration on PT-PM-I (**3**) Formation Rate

Each piece of data represents mean±S.D. (n=3). ●: incubation with *L. brevis* suspension; ○: incubation with fecal suspension. A mixture containing 0.4 ml fecal suspension (or 0.2 ml *L. brevis*), various concentrations of **7** (final 0.5—7.5 mM), **1** (final 2.0 mM) and 50 mM Na-phosphate buffer (pH 7.2), total 0.6 ml (or 0.4 ml), was incubated at 37 °C. After 15 min, 200 μl of the reaction mixture was withdrawn, and the formation rate of **3** was determined by HPLC as described in the legend of Fig. 1.

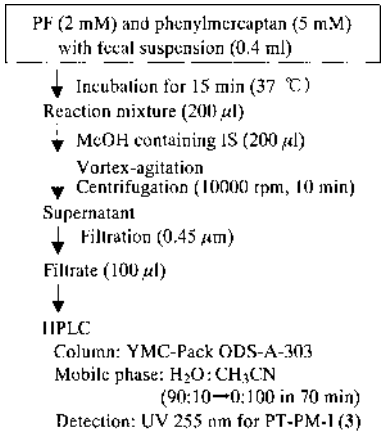


Fig. 5. Procedure Used for the Determination of PF-Metabolizing Activity of Intestinal Bacteria in Rat Feces

Fecal suspension: fresh feces 0.2 g/ml in 50 mM Na-phosphate buffer (pH 7.2). IS: **4** (4.5 μg/ml). HPLC conditions: see legend to Fig. 1. The standard curve for determination of the concentration of **3**: Y=0.56X (r=0.9999), Y: **3** (μg) and X: A/B (A and B are the sum of the peak area of the two isomers of **3** and **4**, respectively). Detection limit: 0.05 μg /ml. Recovery rate: 94%.

In summary, the purpose of this research was to develop a rapid and efficient HPLC analytical method for measuring the PF-metabolizing activity of rat intestinal bacteria. In this regard, the rate of **1** into **2** was found to be equivalent to that of **1** into **3**, which was detected by HPLC at 255 nm. Thus, **3** was used as a marker substance in this HPLC method to determine the PF-metabolizing activity of intestinal bacteria. The optimal conditions for the incubation and HPLC determination for **3** are shown in Fig. 5 and Fig. 1, respectively. Since this method was proven to be a simple and reproducible analytical method for the determination of PF-metabolizing activity of rat intestinal bacteria, it can be used for the biopharmaceutical study of traditional Chinese formulations containing Paeoniae Radix. We are now using this method to investigate the influence of some synthetic drugs on the pharmacokinetic fate of **1** from Shaoyao-Gancao-tang.

Acknowledgments This work was supported in part by a grant from Toyama Prefecture. The authors are grateful to Prof. Masao Hattori and visiting Prof. M. R. Meselhy of the Institute of Natural Medicine, Toyama Medical and Pharmaceutical University for their helpful discussions, and also to Prof. T. Mitsuoka for his generous provision of *L. brevis* when he was in the Institute of Physical and Chemical Research.

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