行政院國家科學委員會專題研究計畫　期中進度報告

配醣體之生可用率、代謝與動力學(1/3)

計畫類別：個別型計畫
計畫編號：NSC93-2320-B-039-028-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：中國醫藥大學藥學系

計畫主持人：李珮端
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報告類型：精簡報告
報告附件：出席國際會議研究心得報告及發表論文
處理方式：本計畫可公開查詢

中　華　民　國 94 年 4 月 29 日

A Deglucosylated Metabolite of Paeoniflorin of the Root of *Paeonia Lactiflora* and Its Pharmacokinetics in Rats

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Abstract

Paeoniflorin is a bioactive monoterpene glucoside in Paeoniae Radix (PR), the roots of *Paeonia lactiflora* (Ranunculaceae). By oral administration to rats with the decoction of PR, the metabolism and pharmacokinetics of paeoniflorin was investigated in this study. A deglucosylated metabolite of paeoniflorin, paeoniflorgenin (PG), in serum was identified based on HPLC/MS and NMR spectral data. HPLC/UV methods were developed for determining PG in serum and feces suspension. Noncompartment model was used for the calculation of pharmacokinetic parameters. Moreover, the metabolism of paeoniflorin by various types of feces was investigated as well.

The paeoniflorin levels in serum were below the detection limit throughout the study. The C_{max}, t_{max}, and AUC_{0-t} of PG were 8.0 μg/ml, 10 min and 487.0 μg min/ml, respectively. Paeoniflorin was found hydrolyzed into PG through incubation with feces of rabbit, rat, pig or human. Similar profiles of PG were shown for various types of feces except for rabbit.

In conclusion, paeoniflorin was not absorbed *per se*, whereas its aglycon paeoniflorgenin was absorbable and circulating in the bloodstream. Rat and pig are appropriate models for investigating the metabolism and pharmacokinetics of paeoniflorin.

Key words: *Paeonia lactiflora*; Ranunculaceae; paeoniflorin; paeoniflorgenin; pharmacokinetics.

Introduction

Paeoniflorin (Fig. **1**) is a bioactive monoterpene glucoside in Paeoniae Radix (PR), the roots of *Paeonia lactiflora* PALL (Ranunculaceae), which has been widely used in clinical Chinese medicine. Paeoniflorin possesses several pharmacological activities, such as analgesia [1], anti-inflammation, anti-allergy [2], muscle relaxation [3], cognitive enhancement [4], and inhibition of steroid protein binding [5]. In addition, anti-hyperglycemia [6], anti-hypotension [7], activation of adenosine A-1 receptor [8] and prolongation of thrombosis time [9] have been reported in recent studies.

Previous studies also reported extremely poor absorption and low bioavailability of orally administered paeoniflorin [10], [11], which was readily metabolized into metabolites named paeonimetabolins I and II (Fig. **1**) by human intestinal bacteria *in vitro* [12], [13]. Heikal et. al. found that blood concentrations of paeonimetabolin I after oral administration of paeoniflorin was relatively higher than paeoniflorin [14], [15]. Employing various types of feces e.g. rabbit, rat, pig and human in this study, we successfully identified a new metabolite of paeoniflorin and the pharmacokinetics of this metabolite was then investigated in rats.

Materials and Methods

Chemicals

Paeoniflorin (purity 98 %) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Methylparaben and β-glucosidase (HP-2, from *Helix pomotia*) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Potassium dihydrogen phosphate was obtained from Wako (Osaka, Japan). Sodium hydroxide was purchased from Merck (Darmstadt, Germany). All other chemicals and solvents used were of analytical grade or HPLC quality. Milli-Q plus water (Millipore, Bedford, MA, U.S.A.) was used for all preparations.

Instrumentation

Bruker Advance DPX-200 FT-NMR Spectrometer was used. The LC/MS spectrometer used was Micromass Quattro Ultima equipped with a Waters 2690 Alliance LC and 996 PDA automatic liquid sampler and injector. The HPLC/UV system consisted of a Hitachi Model L-6200 intelligent pump and an L-3000 photo diode array detector, equipped with a Shimadzu SIL-9A autosampler. A Cosmosil 5C18-AR II column, 5 μm, 150 × 4.6 m.m. with a guard column (MetaGuard 4.6 mm Polaris 5 μ C18-A, MetaChem, Torrance) was employed.

Preparation of paeoniflorgenin (PG) from paeoniflorin

Paeoniflorin (50.0 mg) was dissolved in 5 drops dimethyl sulfoxide, to which 2.5 ml of β-glucosidase solution (15.0 mg/ml, 14 units/mg) was added with shaking in water bath (37 ℃, 100 rpm). The reaction was continued for 5 h, then partitioned two times with ethyl acetate. The ethyl acetate layers were combined, and evaporated to dryness under vacuo. The residue was recrystallized from methanol to afford PG. $[\alpha]_D$ = - 37°(c 1.0 mg/ml, MeOH). UV λ_{max} (MeOH): 203 (log ε 4.25), 227 (log ε 4.33) nm. LC/MS *m/z*: 317 (M-1, ESI^-). ^{13}C-NMR (CD_3OD, 50 MHz) δ: 100.9 (C1), 52.2 (C2), 111.5 (C4), 78.3 (C6), 35.7 (C7), 60.0 (C8), 101.4 (C9), 20.5 (C10), 130.2 (C1'), 129.1 (C2', 6'), 127.9 (C3', 5'), 132.5 (C4'), 168.4 (C7'). ^{1}H-NMR (CD_3OD, 200 MHz) δ: 8.05 (2H, d, J = 5.9 Hz, H-2', 6'), 7.63 (1H, m, H-4'), 7.51 (2H, m, H-3', 5'), 5.41 (1H, s, H-9), 1.26 (3H, s, H-10).

LC/MS conditions

A column of LiChrospher^R 100, RP-18e, 5 μm, 250 × 4.6 m.m. was employed. Mobile phase consisted of acetonitrile and 0.1 % phosphoric acid (15:85, v/v). Capillary voltage: 3 kV; cone voltage: 80 V; collision energy: 25 eV; source temperature: 100℃; desolvation

temperature: 250℃. The UV detector was set at 230 nm and the flow rate was 0.5 ml/min.

Preparation of PR decoction and quantitation of paeoniflorin

Seventy-five grams of PR was soaked in 1.5 l water for 30 min, and then boiled for longer than 2 h to a reduced volume of about 50 ml. After filtration while hot, sufficient water was added to 50 ml. This solution was stored at -30℃ before use.

Paeoniflorin was accurately weighed and dissolved in methanol to afford a series concentrations within the range of 25.0 ~ 500.0 μg/ml. An appropriate amount of internal standard solution of methylparaben in methanol was added to each solution to afford a final concentration of 103.0 μg/ml. Calibration curve was plotted after linear regression of the peak area ratios (paeoniflorin to internal standard) against the concentrations of paeoniflorin.

Half ml of PR decoction was added with suitable amount of methylparaben and sufficient 70 % methanol to make 5 ml to afford a final concentration of 103.0 μg/ml methylparaben. The solutions were filtered through 0.45 μm syringe filter before HPLC analysis. Mobile phase consisted of acetonitrile and 0.1 % phosphoric acid (15:85, v/v). The UV detector was set at 230 nm and the flow rate was 1.0 ml/min.

Acid hydrolysis of PR decoction

One ml of PR decoction was diluted with 9 ml methanol. To 1.5 ml of the methanolic solution, 0.5 ml of 2.4 N HCl was added and incubated in a water bath at 80 ℃ for 2 h. The solution was filtered through a 0.45 μm syringe filter before HPLC analysis. The analysis conditions are identical to those for paeoniflorin.

Animals and drug administration

Six Sprague-Dawley rats, weighing 250 ~ 350 g, were housed in a 12-h light-dark, constant temperature environment prior to study. All rats were fasted 1 day before drug administration and the fasting continued for 4 h thereafter. Water was supplied *ad libitum*. PR decoction was given orally at a dose of 110 mg kg^{-1} paeoniflorin. Drug administration was carried out via gastric gavage. The animal study adhered to "The Guidebook for the Care And Use of Laboratory Animals (2002)" (Published by The Chinese Society for the Laboratory Animal Science, Taiwan, R.O.C.).

Blood samples (0.5 ml) were withdrawn via cardiopuncture at 5, 10, 30, 60, 180, 300, 420 and 540 min after drug administration. All blood samples were centrifuged at 9,860 g for 15 min and the serum stored at -30℃ until analysis.

Calibration curve for quantitation of PG in serum and method validation

Twenty μl of PG standard solution was spiked into 180 μl serum to afford serum standards with concentrations of 0.20, 0.39, 0.78, 1.56, 3.12, 6.25 and 12.50 μg ml^{-1}. To 200 μl of serum standard, 800 μl of methanol containing 1.0 μg ml^{-1} of methylparaben as internal standard was added for deprotenization. The mixture was vortexed for 10 sec and centrifuged at 9,860 g for 15 min, the supernatant was removed and evaporated to dryness by blowing nitrogen. The residue was reconstituted with 50 μl methanol, of which 20 μl was subjected to HPLC analysis. The calibration curve was drawn by linear regression of the peak-area ratios (PG to methylparaben) against concentrations of PG.

The precision and accuracy of the analysis method was evaluated by intra-day and inter-day analysis of triplicate serum standards within one day and over a period of three days. By spiking PG into blank serum and water in triplicates to afford concentrations of 0.78, 3.12 and 12.50 μg ml^{-1}, respectively, a recovery study was done to further assess the accuracy of

this method. The concentrations obtained in blank serum to the corresponding ones in water were compared. LLOQ (Lower Limit of Quantitation) represents the lowest concentration of analyte that can be determined with acceptable precision and accuracy, whereas LOD (Limit of Detection) represents the lowest concentration of analyte that can be detected (with S/N＞3).

Quantitation of PG in serum

Two hundreds μl of serum sample was added with 1,800 μl of methanol containing 1.0 μg ml^{-1} of methylparaben as internal standard. The remaining procedures followed those for calibrators. Mobile phase consisted of acetonitrile and 0.1 % phosphoric acid (25:75, v/v). The UV detector was set at 230 nm and the flow rate was 1.0 ml/min.

Data analysis

The pharmacokinetic parameters of paeoniflorgenin were calculated using noncompartment model with the aid of WINNONLIN (version 1.1, SCI software, Statistical Consulting, Inc., Apex, NC). The peak plasma concentration (C_{max}) and the time to peak concentration (T_{max}) were obtained from experimental observations. The area under the serum concentration-time curve (AUC_{0-t}) was calculated using trapezoidal rule to the last point.

Fermentation of paeoniflorin and quantitation of PG in feces

The fresh feces of New Zealand white rabbits and Sprague-Dawley rats were collected from the animal center of China Medical University. Yorkshire pig feces were obtained from Animal Technology Institute, Miaoli, Taiwan. Human feces was obtained form a healthy child. Twenty grams of feces was accurately weighed, and 60 ml artificial intestinal juice (pH 7.5) was added. The mixture became a homogenate by using an agitator, and the

homogenate was filtered through gauze.

Fecal suspensions of rabbit, rat, pig and human (12.42 ml) were spiked with paeoniflorin standard solutions (1.38 ml, 1.0 mg/ml) and mixed well with stirring in beakers individually. Each aliquot of 600 μl was placed in a dark brown glass tube, sealed with a septum and air was removed with syringe. All samples were prepared in triplicates and kept on ice before incubation. The tubes were then incubated in a shaking water bath (100 rpm) at 37℃ for 0, 1, 2, 4, 8, 12 and 24 h, and then samples were stored at –30℃ until analysis.

Feces samples were added with 600 μl ethyl acetate (containing 25.0 μg/ml methylparaben as the internal standard), and vortexed for 10 sec. The mixture was centrifuged at 9,860 g for 15 min and the supernatant was blown with N_2 gas until dry. The residue was reconstituted with 50 μl methanol, and 20 μl was subjected to HPLC analysis. The HPLC system was identical to that used for PR decoction as described above. Metabolic profiles were drawn by plotting the peak area ratios (PG to internal standard) against incubation time of paeoniflorin with various types of feces.

Results

Figure 2 is the chromatogram of PR decoction, showing that paeoniflorin and the internal standard were well resolved within half an hour without any interference. A good linear relationship existed for paeoniflorin over the concentration range of 25.0 ~ 500.0 μg/ml. The content of paeoniflorin in the decoction was 8.1 mg from each gram of PR. Acid hydrolysis of PR decoction resulted in the transformation of paeoniflorin to PG which existed only in very low concentration before hydrolysis.

A good linear relationship existed over the concentration range of 0.20 ~ 12.50 μg/ml for PG in serum. The coefficients of variation were below 6.0 % and the relative errors were

between + 12.3 and – 4.1 % for intra-day and inter-day assays. These indicate that the precision and accuracy were satisfactory. The LLOQ and LOD of PG were 0.20 μg/ml and 0.10 μg/ml, respectively. The recoveries of PG from serum were 82.5, 81.9 and 88.7 % at concentrations of 0.78, 3.12 and 12.50 μg/ml, respectively. Throughout this study, only PG was determined in serum, whereas paeoniflorin was not detected with a photo diode array detector. Fig. 3 depicts the profile of mean serum concentrations of PG in rats after administration of PR decoction, and the pharmacokinetic parameters of PG are listed in Table 1.

A high performance liquid chromatographic method was developed for determining paeoniflorgenin in rabbit, rat, pig and human feces suspensions. The respective retention times of PG and methylparaben (I.S.) were 21.1 and 24.6 min as shown in Fig. 4. Figure 5 depicts the metabolic profiles of paeoniflorin to form PG during incubation with rabbit, rat, pig and human feces. At the beginning of fermentation, PG was not detected in various types of feces. One h after incubation, PG emerged in the feces of rabbit, rat and humans, but still missing in pig feces. At 8 h, PG almost remained constant concentration in various feces.

Discussion

The aglycone of paeoniflorin PG was prepared by hydrolysis of paeoniflorin with β-glucosidase and its structure could be determined by comparing the NMR spectral data with those of paeoniflorin [17]. The ^{1}H-NMR and ^{13}C-NMR spectra of PG revealed the presence of a benzene ring moiety, indicating that PG was different from those metabolites reported previously [11-15]. The ^{13}C-NMR spectra of PG did not reveal the absorptions of all carbons, probably due to the fact that C3 and C5 were hidden under the solvent peak at δ 46.1~48.7. The LC/MS result shows the molecular weight of PG is 318, corresponding to

the deglucosylated derivative of paeoniflorin whose molecular weight is 480.

Each rat received PR decoction at a dose of 13.5 g/kg of crude drug containing 110 mg/kg of paeoniflorin, that was similar to the usual daily dose 15 g for clinical use. After oral administration of PR decoction to rats, a metabolite with identical retention time and UV spectrum with PG was detected in the serum of all rats. Accordingly, this compound can be inferred as the deglusosylated metabolite of paeoniflorin. Moreover, acid hydrolysis of PR decoction yielded a new component that also showed identical retention time and UV spectrum with PG, that gave more proof to the finding. This fact further confirmed that hydrolysis was a major metabolic fate of paeoniflorin. No conjugation metabolism was found for PG, that was in agreement with the biological fate of glycyrrhetic acid, a triterpene aglycone of glycyrrhizin [18]. In contrast to these alcoholic aglycones, phenolic aglycones were generally further metabolized to conjugated metabolites, suggesting that the activities of phase II metabolic enzymes toward phenols were significantly higher than alcohols [19].

Heikal et. al. [15] reported pharmacokinetic parameters of paeoniflorin and paeonimetabolin I after oral administration of 5 mg/kg paeoniflorin to rats, the C_{max} and AUC of paeoniflorin and paeonimetabolin I were 20.3, 101.7 ng/ml and 1,174, 12,358 ng min/ml, respectively. However, the parent compound paeoniflorin was not detected in rat serum in the present study. That EIA method was more sensitive than UV method [20] could be an explanation for this discrepancy. In regard to the other metabolites reported in the above literature e.g. paeonimetabolins I and II, they were not detected in this study due to the absence of the benzene ring chromophore in their structure to absorb UV.

Our study demonstrated that paeoniflorin was readily metabolized into PG and T_{max} was the first sampling time at 10 min after drug administration. The C_{max} of PG was 8.0 μg/ml and its elimination half-life was 126 min. As compared to the pharmacokinetics of paeoniflorin and

paeonimetabolin I reported by previous study [15], PG showed much higher C_{max} and AUC based on dose correction. In spite of being a major metabolite of paeoniflorin, to our great surprise PG has never been reported before in literature. The bioactivity investigations on PG by *in vitro* models could partially account for the *in vivo* efficacy of paeoniflorin or PR decoction. However, the information of PG bioactivity is still lacking in literature and is worthy of more investigations.

The metabolic profiles of paeoniflorin in various types of feces showed that a common metabolite was gradually generated in the presence of fecal bacteria. This metabolite was found identical with that in serum after oral administration of PR decoction by HPLC/UV analysis and accordingly identified as PG. The profiles indicated that PG was not further degraded by fecal flora. This phenomenon is very different from flavonoid aglycones which were markedly degraded by fecal flora [21], suggesting that terpenes are more resistant to enterobacterial degradation than heterocyclic aromatic compounds. The metabolic pattern of paeoniflorin was similar among rats, pigs and humans, whereas rabbit feces hydrolyzed paeoniflorin to a lesser extent.

In summary, PG is a major metabolite of paeoniflorin *in vivo*. It is suggested that rat and pig are appropriate animal models for the pharmacokinetic study of paeoniflorin or PR decoction.

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Table **1** Mean (±S.E.) pharmacokinetic parameters of paeoniflorgenin after oral administration of Paeoniae Radix decoction in 6 rats.

Parameters	Mean ± S.E.
T_{max} [a] (min)	10.0 ± 4.1
C_{max} [b] (μg/ml)	8.0 ± 0.6
$T_{1/2}$ [c] (min)	126.2 ± 4.0
AUC [d] (μg min/ml)	487.0 ± 95.6
MRT [e] (min)	135.5 ± 2.4

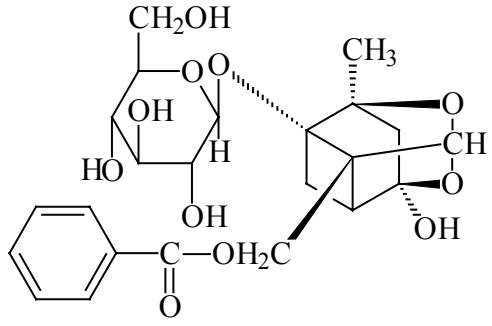
[a] time of peak serum level

[b] concentration of peak serum level

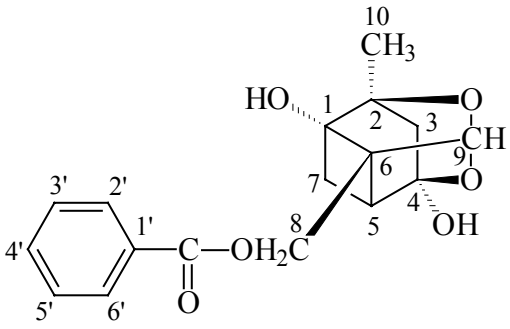
[c] elimination half life

[d] area under serum concentration – time curve to the last point

[e] mean residence time



(a)



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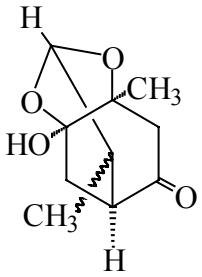

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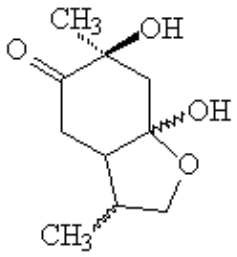

(d)

Fig. **1** Structures of (a) : paeoniflorin ; (b) paeoniflorgenin ; (c) : 7S- or 7R-paeonimetabolin I and (d) : paeonimetabolin II.

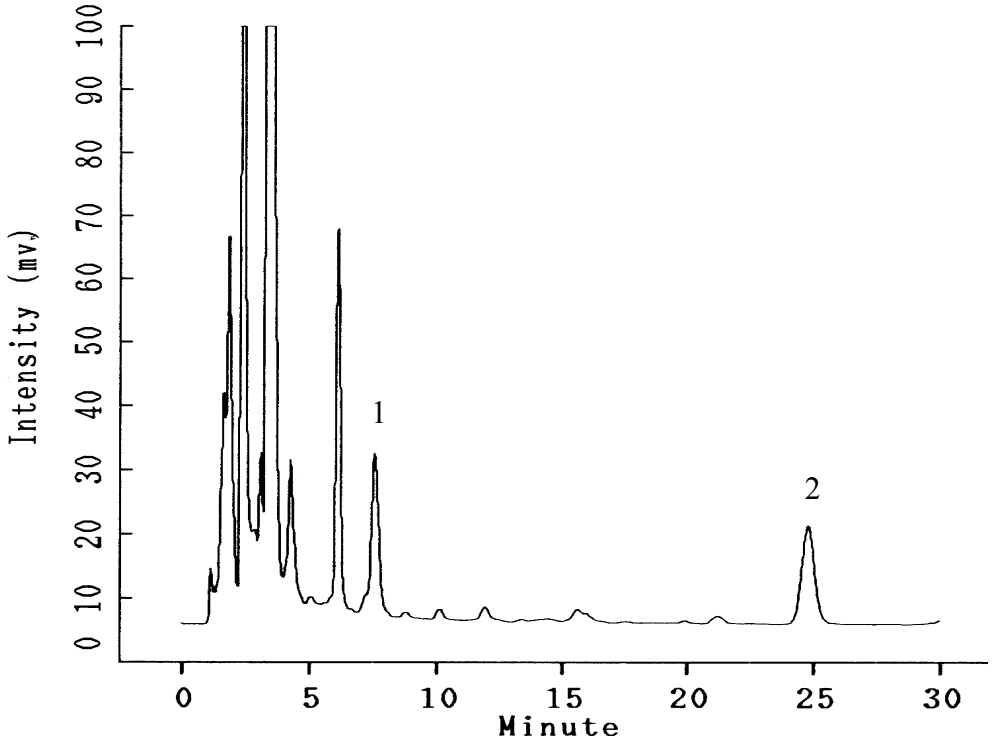

Fig. **2** HPLC chromatogram of Paeoniae Radix decoction, 1: paeoniflorin; 2: methylparaben (internal standard).

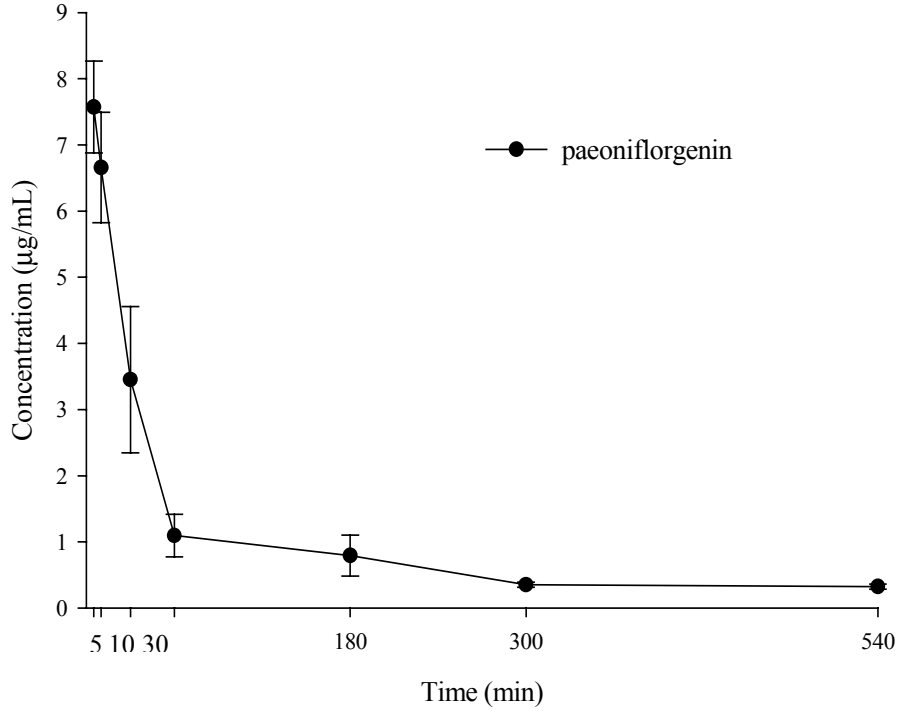

Fig. **3** Mean (± S.E.) serum concentration-time profile of paeoniflorgenin after oral administration of Paeoniae Radix decoction to six rats.

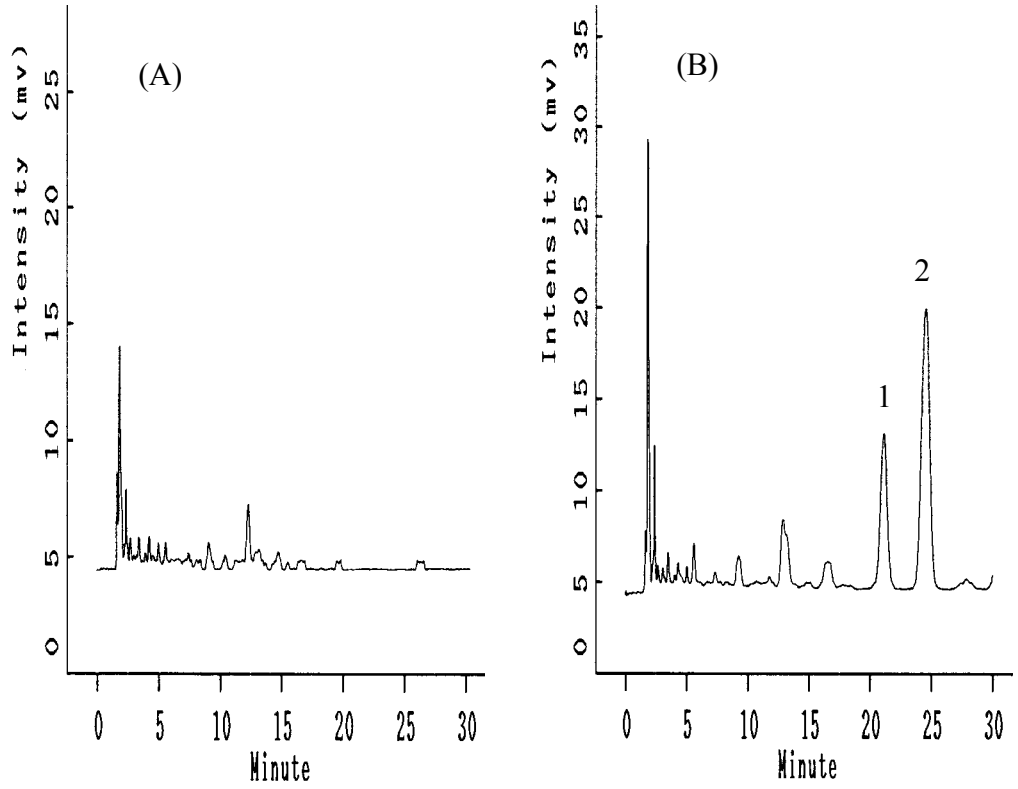

Fig. **4** HPLC chromatograms of (A): blank human feces; (B): human feces with Paeoniae Radix decoction. 1: paeoniflorgenin; 2: methylparaben (internal standard).

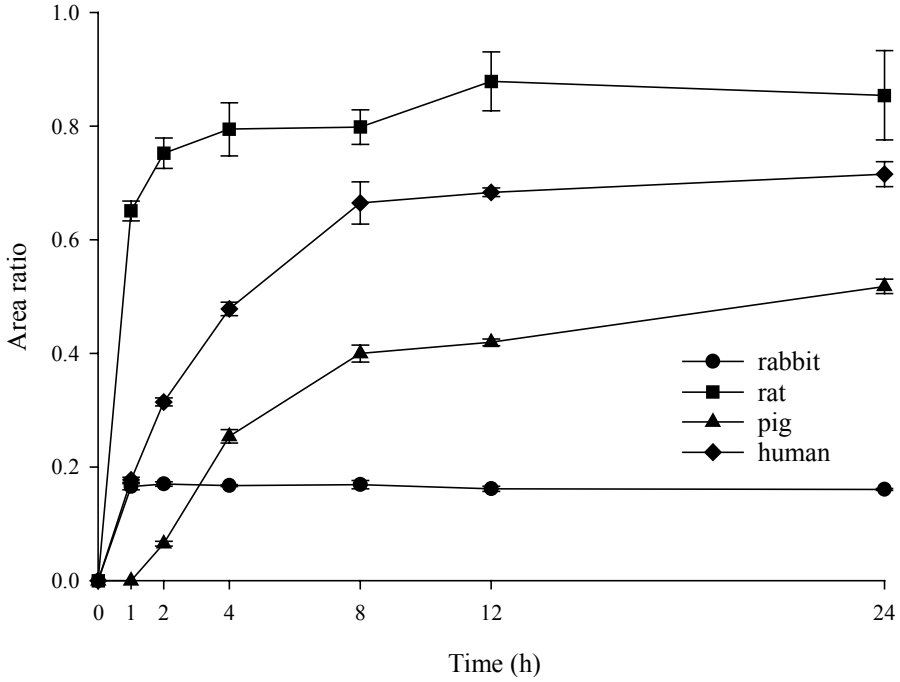

Fig. **5** Mean (± S.D.) area ratio-time profiles of paeoniflorgenin during incubation of paeoniflorin in rabbit, rat, pig and human feces (n = 3).