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Enzyme kinetics study and metabolites identification of paeonol in rat liver homogenate

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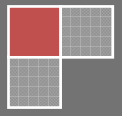
ABSTRACT

Objective to study the enzyme kinetics of paeonol in homogenized liver of rats, and to evaluate the effect of the CYP450 enzyme on the metabolism of paeonol. The metabolites of paeonol were also identified. Methods hplc method was established for quantitative analysis of remaining concentration of paeonol in liver homogenate, and enzyme kinetic parameters were calculated. The metabolites of paeonol in liver homogenate were identified with HPLC/MS/MS. Results The metabolism of paeonol was significantly promoted by the enzymes subtypes of CYP450(CYP4503A) which was induced by dexamethasone, and there was no change in the metabolism of paeonol in liver homogenate pretreated by the enzymes subtypes of CYP450 (CYP4502B) which was induced by phenobarbital. The metabolites of paeonol in liver homogenate were 4-methoxyacetophenone-2-O-glucuronide and 2,4-dihydroxyacetophenone-5-O-sulphate. Conclusion CYP3A may be mainly involved in the metabolism of paeonol, and then to provide scientific evidence for rational administration and metabolic mechanism.

KEYWORDS

Paeonol; Liver homogenate; Enzyme kinetics; HPLC/MS/MS; CYP450; Metabolites.



INTRODUCTION

Hepatic cytochrome P450 is responsible for the metabolism of the majority of xenobiotics (including drugs). Using in vitro experimental system[1], such as homogenate, microsomes, tissue slice, etc., the methods for characterizing the metabolic stability, metabolite profile, metabolic enzyme profile, inhibition or induction of metabolic enzymes have been well established. Hepatic metabolism in vitro is shown to be a good model for drug biotransformation study.

Paeonol, a major phenolic component of Moutan Cortex, is known to have antitumor effects, antibacterial, antitumor, anti-atherosclerosis, against ischemia-reperfusion injury, neuroprotective effect, antiallergic, anti-inflammatory[2-10]. There were fewer papers about the metabolism of Paeonol. In particular, metabolism studies include the determination of the specific enzymes responsible for breaking down the components, the kinetic parameters of enzyme interactions, and the products of the reactions were unknwon. It is important to understand the metabolism of Paeonol to identify the roles of CYP isoforms and metabolites of Paeonol. The metabolizing method was established based on the in vitro metabolism by rat liver homogenate. which isoform of CYPs involved in the Paeonol metabolism were determined. Metabolites of Paeonol were detected and characterized by LC-MS analysis.

MATERIALS AND METHODS

Animals

Female Wister rats weighing (180 ± 20) g were purchased from the Laboratory Animal Center of Changchun National Biomedical Industrial Base in Changchun Province in China, Lot: SCXK-(Kyrgyzstan) 2003-2004.

Instrumentation

Waters HPLC (W2695 pump, W2996DAD detector, automatic injector, Empower). Agilent 1100 Liquid Chromatography/VL-type ion trap LEDs Imaging Power Supplies (equip with atmospheric pressure electric spray source ESI); Instrument operating and data processing system by using HPLC-3D ChemStation; TDL80-2B type high speed centrifuges; DKZ-2 Shaking Water Bath ; sartorius PB-10 Standard pH meter; XW-80A-type vortex mixer; Low temperature-high-speed centrifuge (Beckman Coulter Allegra 64R Centrifuge).

Chemicals

Paeonol (110708-200505) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); Reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH) was obtained from Roche (Basel, Switzerland). acetonitrile (chromatography pure, Shandong Yu Wang), methanol (chromatography pure, Shandong Yu Wang), glacial acetic acid (chromatography pure, Shanghai Career), ultra pure water. Krebs-Henseleit buffer solution (3.435gNaCl+0.2gKCl+0.061g$MgSO_4$+0.065gKH_2PO_4+1.1g$NaHCO_3$+ 0.125g $CaCl_2$+2.5g glucose, then adding water to reach 500 ml, PH7.4, keep at 4℃).

HPLC-UV analysis conditions

Column: Symmetry ShieldTM RP18 (5μm, 3.9 mm ×150 mm); Mobile phase: methanol-1 % glacial acetic acid solution;Column temperature: 35 °C; flow rate: 0.8 mL min-1, detection wavelength: 274 nm. (Figure 1,2).

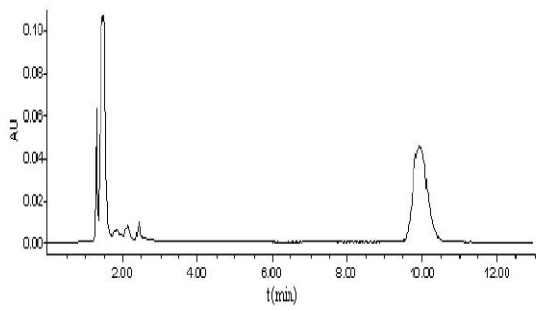


Figure 1: Chromatograms of paeonol incubated for 0 min

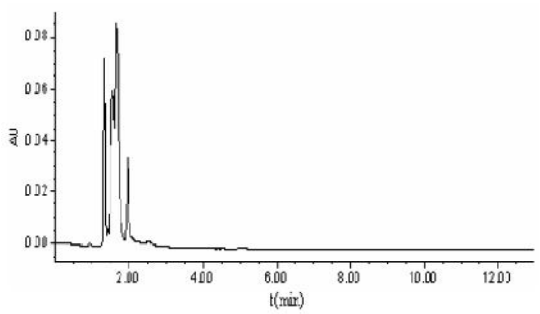


Figure 2: Chromatograms of blank liver homogenate

HPLC- ESI/MS analysis conditions

Column: Symmetry ShieldTM RP18 (5μm, 4.6 mm ×250 mm); Mobile phase: Acetonitrile-0.2% formic acid solution;Column temperature: 30 °C; flow rate: 1.0 mL/min, detection wavelength: 254 nm. Mass spectrometer ion source: atmospheric pressure electrospray ionization (ESI); atomizing air pressure: 0.17MPa; drying gas flow: 10L/min; capillary voltage: 4KV; transmission voltage:70V. Select the negative ion scan mode, scan range: *m/z* 50-1000. Gradient elution procedure were shown in TABLE 1. The chromatograms is present in Figure 3.

TABLE 1: Linearity of mobile system in fingerprints assay

Time (min)	Flow (mL/min)	Acetonitrile (%)	0.2 % formic acid solution (%)
0	1.0	2	98
35	1.0	10	90
75	1.0	20	80
115	1.0	45	55
135	1.0	45	55

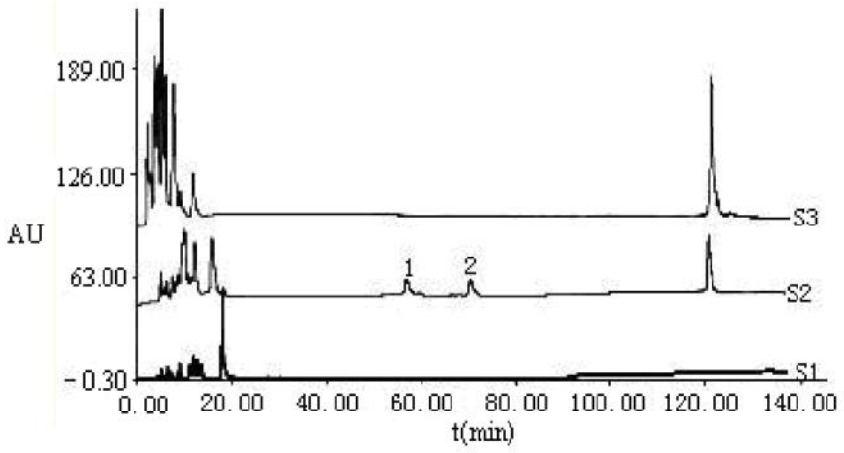


Figure 3: Chromatograms of blank liver homogenate (S1)、paeonol was incubated for 60 min (S2) and blank rat liver homogenate + paeonol (S3)

Preparation of rat liver homogenizations

20 Wistar rats (male, 180±20g) were divided into four groups. One group received daily intraperitoneal injections of 15ml·kg^{-1} Physiological saline for seven days, the second group received phenobarbital (0.8ml·kg^{-1}, ip×7d), the third group received dexamethasone (15ml·kg^{-1}, ip×4d) and the fourth group received quercetin (15mL·kg^{-1}, ip×7d). About 24h after the last treatment and with no food supplied for 12 h, the rats were sacrificed by decapitation. livers were rapidly excised and then were washed with buffer solution in ice bath. Liver homogenates were prepared in the Krebs-Henseleit buffer, (pH 7.4, 1 g liver/5 mL buffer). Homogenates were centrifuged at 2,000 r·min^{-1} for 3 minutes.

Enzymatic saturation kinetics

Mixing 1mL homogenate, 20μL NADPH, with various concentrations (1.6、3.3、5、6.7、8.4、10.1mg·mL^{-1}) of Paeonol. Mixture were incubated at 37 ℃ for 60 min, The reaction was terminated by adding 1 mL of methanol, then vortexed and centrifuged at 3000 rpm for 20 min to remove precipitated protein. 20 μL of supernatant solution was used for analysis.

Enzyme kinetic analysis in vitro

To conduct the enzyme kinetic study, various concentrations of Paeonol (ranging from 1.6 to 8.4 mg·mL^{-1}) were incubated with 1mL homogenate and 20μL NADPH at 37°C for 1 h. The maximum

velocity (V_{max}) and Michaelis–Menten constant (K_m) values for zolmitriptan were determined by regression analysis of Lineweaver–Burk plots. Intrinsic metabolic clearance (CL_{int}) was calculated by the following equation $CL_{int} = V_{max}/K_m$.

Induction and inhibition experiment

The metabolic character of Paeonol was studied in vitro to identify which isoforms of cytochrome P450 were responsible for Paeonol metabolism in rats. The inducers studied were phenobarbital (CYP2B) and dexamethasone (CYP3A), the inhibitors studied was quercitin (CYP3A). Phenobarbital, dexamethasone induced group, quercitin inhibition group, control group were conducted under the same condition. V_{max}, K_m, C_{lint} were calculated.

Metabolites of Paeonol in vitro

10mg·mL^{-1} Paeonol were incubated with the above condition. Metabolites found in incubation systems were identified by HPLC-MS.

RESULTSAND DISCUSSION

Results of enzymatic saturation kinetics

In the range of 1.6 to 8.4 mg·mL^{-1}, the amount of metabolic Paeonol is proportional to the concentration. Over 8.4 mg·mL^{-1}, the amount of metabolic Paeonol is constant. The results are shown inTABLE 2 and Figure 4.

TABLE 2: Datas of paeonol in enzyme kinetics saturated experiment (n=5)

concentration (mg/mL)	1.6	3.3	5.0	6.7	8.4	10.1
amount of metabolic Paeonol (mg)	0.0513±0.0121	0.289±0.011	0.398±0.005	0.465±0.023	0.51±0.03	0.5102±0.0006

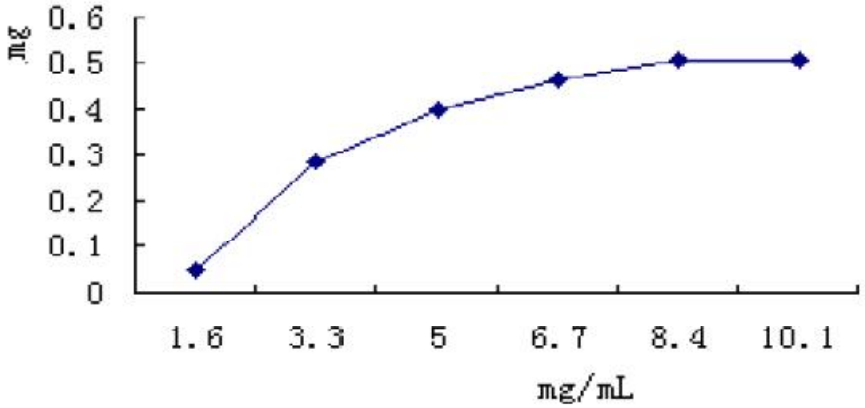


Figure 4: Influences of paeonol concentrations on reaction velocity

Data analysis

The kinetic parameter of Paeonol were shown in TABLE 3 and Figure 5.

TABLE 3: Enzyme kinetics datas of paeonol (n=5)

S (mg/mL)	1.0	0.8	0.6	0.4	0.2
1/[S]	0.90	1.25	1.67	2.50	5.00
1/V	75.00046±1.64453	102.3044±1.68002	130.4336±1.79491	180±2	350.5221±1.63903
Vmax	(0.049450±0.002334) μmoL·mL^{-1}·min^{-1}				
Km	(3.459944±0.004960) μmoL·mL^{-1}				
C_{lint}	(0.014294±0.000657) min^{-1}				

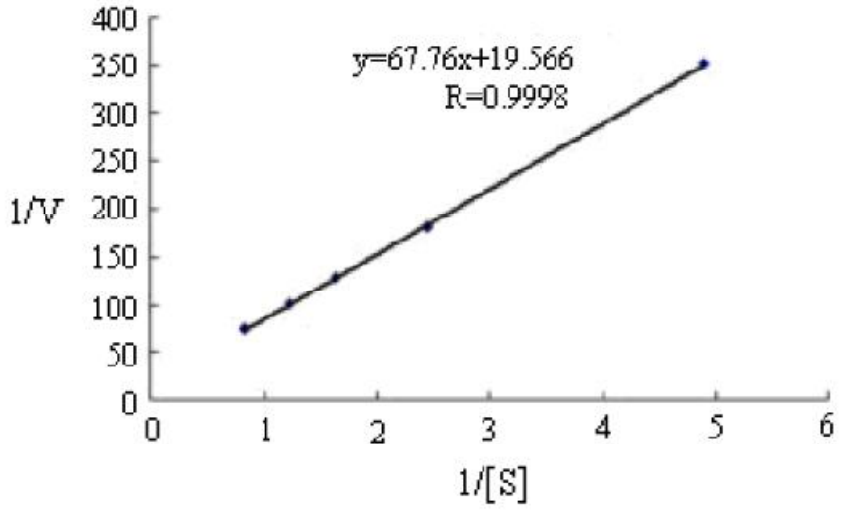


Figure 5: Enzyme dynamics curve of paeonol

Results of iduction and inhibition experiment

Lineweaver-Burk plot of phenobarbital induced group was y=67.463x+24.272(r=0.9992, n=5), in dexamethasone induced group was y=60.379x+11.737(r=0.9998, n=5), in control group was y=72.375x+20.939(r=0.9997, n=5). Mabolism rate were 67.68%, 75.32% and 67.65% separately. Dxamethasone, an inducer of CYP3A, can speed-up paeonol's metabolism speed in vitro. But phenobarbital had no obvious effect on the metabolism of paeonol. To further verification, quercitin (inhibitor of CYP3A) was examined whether or not show inhibitor effect on the metabolism of paeonol. ineweaver-Burk plot of quercitin inhibition group was y=541.29. x+236.910(r=0.9999, n=5), metabolism rate were 20.64%. It was suggested that CYP3A is a major CYP450 isoform involved in the metabolism of paeonol. The results are shown in TABLE 4.

TABLE 4: Enzyme kinetics parameters of each group compared with the control group (n=5)

	Vmax	Km	C_{lint}
control group	0.049450±0.002334	3.459944±0.004960	0.014294±0.000657
dexamethasone induced group	0.085550±0.000495*	5.052041±0.127387*	0.016937±0.000530*
phenobarbital induced group	0.078750±0.031749	5.407854±0.007298	0.014476±0.000491
quercitin inhibition group	0.004830±0.000863**	2.701049±0.588685	0.001796±0.000072**

Identification of metabolites

Following incubation of paeonol in vitro, paeonol was rapidly metabolized and two metabolites were identified, they are 4-methoxyacetophenone-2-O- glucuronide and 2,4-dihydroxyacetophenone -5-O-sulphate. The results are shown in TABLE 5.

TABLE 5: MS and MS/MS spectra for the identification of metabolites

NO of characteristic peaks	Retention time (min)	MS (m/z)	M.W.	Compound presumed
		MS MS/MS		
1	57.36	341[M-H]$^-$ 165	342	4-methoxyacetophenone-2-O-glucuronide
2	70.23	247[M-H]$^-$ 167	248	2,4-dihydroxyacetophenone-5-O-sulphate

In summary, characterization of metabolites and cytochrome P450 isoforms involved in the metabolism of paeonol were investigated in vitro metabolic experiment. The results showed that CYP3A

may be mainly involved in the metabolism of paeonol in vitro. It provided information for the interactions between paeonol and drugs co-administrated in clinic, and it is helpful to explain the drug–drug interactions of clinical relevance on enzyme level. Two metabolites were identified in the incubation mixture. All of the above results contribute to our understanding of the metabolism pathway of paeonol in rats.

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