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Note

Simple method for the determination of paeonol in human and rabbit plasma by high-performance liquid chromatography using solid-phase extraction and ultraviolet detection

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Paeonol (2-hydroxy-4-methoxyacetophenone, Fig. 1) is a natural product which is isolated from the herb, *Pycnostelma paniculatum* (*Bunge*) *K.S.*, and the root of the plant, *Paeonol suffruticola*. It is a white needle crystal with a relatively low melting point of 51–52°C. It has analgesic, antipyretic and anti-inflammatory properties and may find use in the treatment of arthritis [1]. There are two metabolites of paeonol, 2,5-dihydroxy-4-methoxyacetophenone and 2,6-dihydroxy-4-methoxyacetophenone [2]. These metabolites have been determined in the urine of rabbits by quantitative thin-layer chromatography and in human urine by gas chromatography–mass spectrometry (GC–MS) [2,3] and there has been one report of the determination of the parent compound in plant material, Moutan Cortex (*Paeonia mouton*), using high-performance liquid chromatography (HPLC) [4]. However, there have been no reports to date on the determination of paeonol in plasma. The present study describes a simple, sensitive and rapid

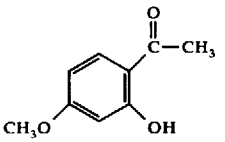

Fig. 1. Structure of paeonol (2-hydroxy-4-methoxyacetophenone).

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method for determining paeonol in plasma which should be useful in animal studies and in the routine monitoring of paeonol in the clinic.

EXPERIMENTAL

Chemicals and reagents

Paeonol was obtained from the Liu Zhon Pharmaceutical Factory (Guong Xi Province, People's Republic of China). HPLC-grade acetonitrile and methanol were obtained from Fisher Scientific (St. Louis, MO, U.S.A.) and GC-grade acetophenone was obtained from Aldrich (Milwaukee, WI, U.S.A.). All the other chemicals were at least reagent grade and were obtained from various sources.

Chromatography

The chromatographic system consisted of a Model M6000 Waters pump, a Model 440 Waters UV detector (280 nm) set at 0.02 a.u.f.s., a U6K Waters injector (Waters Assoc., Woburn, MA, U.S.A.) and Fisher Recordall 5000 chart recorder. The CPS Hypersil column (5 μm particle size, 15 cm $\times$ 4.6 mm I.D.) (Keystone Scientific, State College, PA, U.S.A.) was packed in the upward direction using methanol as the slurry solvent. The column was eluted with methanol–water (60:40, v/v) at flow-rate of 1.2 ml min^{-1}.

Standard curves and sample preparation

Standard curves of paeonol were prepared by spiking rabbit plasma. Aliquots (0, 5, 10, 20, 40, 80 and 120 μl) of a 160 μg ml^{-1} stock solution of paeonol in methanol were pipetted into 15-ml glass centrifuge tubes containing plasma (1 ml), acetophenone in methanol (3 μl ml^{-1}; 15 μl) and acetonitrile (5 ml). This was adjusted with distilled water to a final volume of 8 ml, mixed for 20 s and then centrifuged for 10 min at 2000 *g* at ambient temperature (22–23°C). The clear supernatant was passed through a solid-phase extraction C_{18} cartridge (J.T. Baker, Phillipsburg, NJ, U.S.A.) and 30 μl of the eluent were injected into the HPLC column. (The latter step removed late eluting peaks from the HPLC system.) The peak-height ratio of paeonol to acetophenone was plotted against the concentration of paeonol injected to obtain a standard curve. The samples for analysis were prepared in a similar manner. Calibration curves in spiked human plasma were prepared in a similar manner with the exception that the stock solution of paeonol was 200 μg ml^{-1} and the aliquots added to the plasma were 0, 2.5, 5, 10, 20, 40 and 80 μl.

Pharmacokinetics in rabbits

A sterile solution of 15 mg ml^{-1} paeonol was prepared as follows. Paeonol (750 mg) was dissolved in 95% (v/v) ethanol (20 ml) and propylene glycol (5 ml), adjusted to 50 ml with sterile water and heated at 100°C for 40 min. A dose of 30 mg kg^{-1} was administered to a 4.1-kg New Zealand white rabbit via the medial vein of one ear. Blood samples (2 ml) were collected from the opposite ear at 0, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240 and 360 min after dosing. The blood samples were centrifuged (500 *g*, 10 min) immediately after collection and the

plasma stored over ice water (0°C) until analysis. Plasma samples (1 ml) were taken for analysis. The oral bioavailability of paeonol was assessed by administering an identical dose to the same animal by oral gavage.

RESULTS AND DISCUSSION

The rapid separation of paeonol and acetophenone (internal standard) from endogenous substances in plasma was achieved within 6 min using a cyanopropyl bonded phase (CPS Hypersil) and a mobile phase of methanol–water (60:40, v/v) (Fig. 2). The retention times of acetophenone and paeonol were 4 and 5 min, respectively. Initially, the extraction of paeonol and acetophenone from plasma and aqueous solutions with ethyl acetate and diethyl ether was investigated. Analysis of the aqueous phases after extraction with these solvents indicated that the extraction efficiencies for paeonol were close to 100%. Furthermore drug-free plasma extracted in the same manner was free of interferences after evaporation

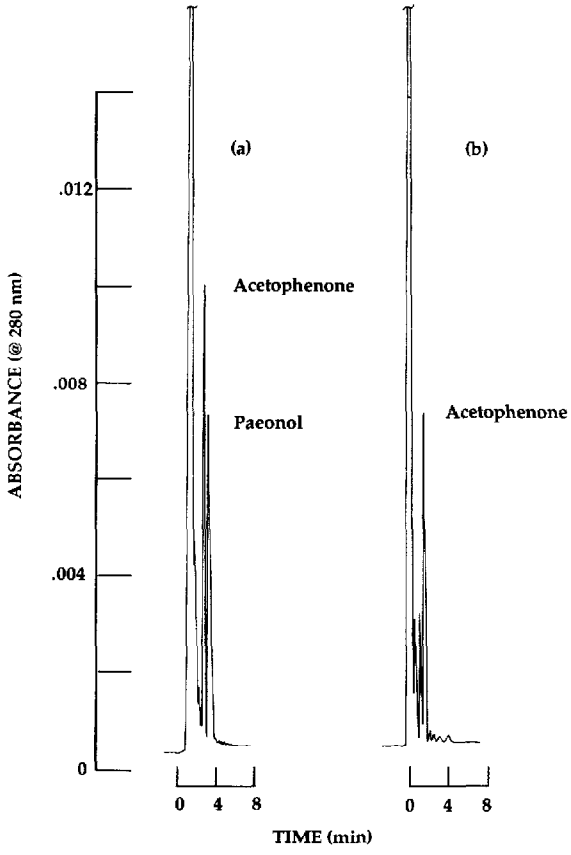

Fig. 2. Chromatogram of rabbit plasma (a) 10 min after administration of a 30 mg kg^{-1} intravenous dose of paeonol and (b) 60 min after administration of a 30 mg kg^{-1} oral dose of paeonol, showing complete absence of the drug. Stationary phase: CPS Hypersil. Mobile phase: methanol–water (60 40, v/v). Flow-rate: 1.2 ml min^{-1}. Injection volume: 30 μl. Internal standard: acetophenone.

and reconstitution with mobile phase. However, the absolute recoveries of paeonol and acetophenone from human plasma were only 50 and 5%, respectively. The low recoveries were attributed to loss of the volatile solutes during the evaporation step and alternative strategies for the isolation of paeonol were pursued.

Plasma proteins were precipitated with acetonitrile and late eluting peaks were adsorbed onto a C_{18} solid-phase extraction cartridge. Since this method of sample preparation was successful, further methods of protein precipitation such as addition of methanol or perchloric acid were not studied. The recoveries of acetophenone and paeonol were both 100%. The paeonol and the internal standard were not retained by the solid-phase extraction cartridge and the eluent from the cartridge was injected directly onto the HPLC column. The mean accuracies for the determination of paeonol in rabbit and human plasma were 94.2 and 94.4%, respectively (Table I).

The limit of detection at a signal-to-noise ratio of 3:1 of paeonol in plasma was 3 ng ml^{-1}. The relationships between the peak-height ratios (A, paeonol/acetophenone) and concentrations (in $\mu g\ ml^{-1}$) of paeonol in rabbit (C_r) and human plasma (C_h) were described by virtually identical linear relationships (eqns. 1 and 2, respectively).

$$A_r = 15.2\ C_r - 0.0038\ (n=6, r=0.997) \quad (1)$$

$$A_h = 13.9\ C_h + 0.029\ (n=6, r=0.999) \quad (2)$$

The coefficients of variation (C.V.) of the slopes of these lines obtained on four separate days were less than 5% for each (eqns. 1 and 2). The C.V. for triplicate

TABLE I

VALIDATION DATA FOR THE ANALYSIS OF PAEONOL IN HUMAN AND RABBIT PLASMA

Paeonol concentration ($\mu g\ ml^{-1}$)		Accuracy[b] (%)
Added	Found[a]	
Rabbit		
9.00	9.16	101.0
18.0	16 7	92.4
36.0	33.9	94.1
54.0	50.7	93.9
66.0	59.9	90.8
Human		
6.00	5.59	93.2
11.3	11.1	98.6
22.5	21.2	94.2
45.0	42.0	93.3
90.0	84.0	93.3

[a]Mean of two solutions.
[b](Found/added)·100.

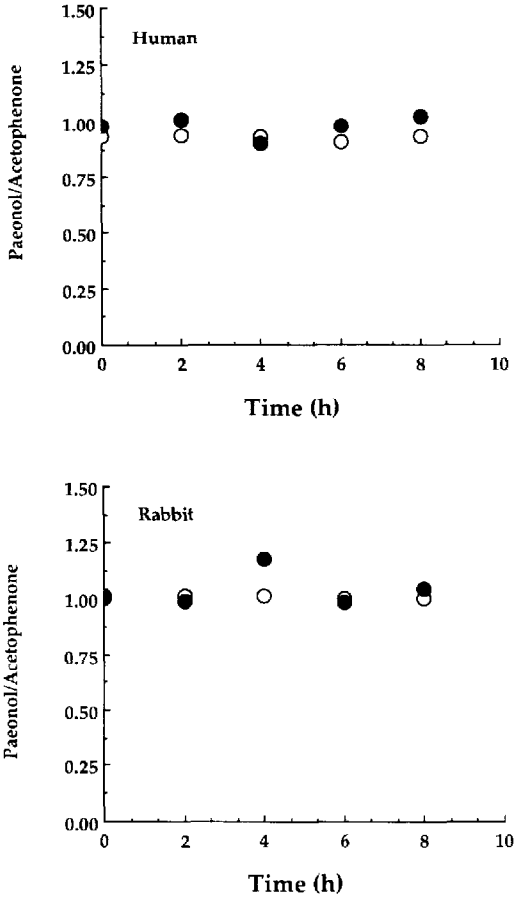

Fig. 3 Stability of paeonol in human and rabbit plasma at 25 °C (open symbols) and 37 °C (closed symbols).

determinations of 37.5 μg ml^{-1} paeonol in rabbit plasma and 48 μg ml^{-1} paeonol in human plasma were 4.1 and 2.3%, respectively. To ensure that the paeonol was not lost during sample handling as a result of chemical or enzymatic degradation, its stability in human and rabbit plasma was studied at 25 and 37 °C. Fig. 3 shows that paeonol was stable for at least 8 h in plasma under these conditions and the drug was always analyzed within this time period.

Fig. 4 shows the time course of paeonol in plasma following bolus intravenous injection of the drug at a dose of 30 mg kg^{-1}. No paenol was detected in the plasma of the rabbit after oral administration. Since the metabolites of paenol have been detected in the urine of rabits following oral administration [2,3] it seems likely that the poor oral bioavailability of paeonol seen here was due to rapid and complete first-pass metabolism of the drug. No attempts in this study were made to analyze for the metabolites, however, a method which is capable of determining both paeonol and its metabolites seems warranted. Additionally, it has not yet been determined what clinical use is to be made of this drug; therefore it was not possible to predict other agents that might interfere with this assay.

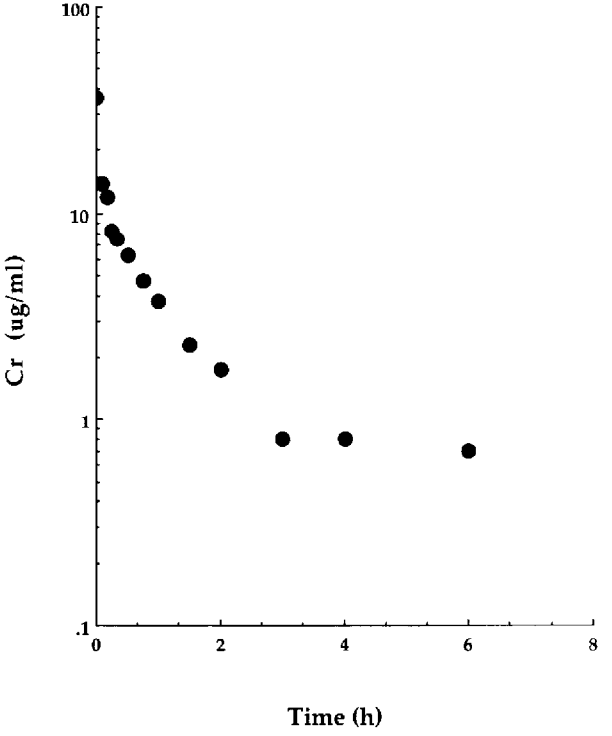

Fig. 4. Plasma concentrations (C_r) of paeonol following intravenous bolus administration of a 30 mg kg^{-1} dose to a 4.1-kg New Zealand white rabbit.

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