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Full Length Research Paper

Comparative excretion of vitexin-2"-*O*-rhamnoside in mice after oral and intravenous administration

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The aim of the present study was to characterize comparative excretion of pure vitexin-2"-*O*-rhamnoside (VR) in mice following oral and intravenous administration at dose of 30 mg/kg, therefore, a sensitive and specific high-performance liquid chromatography (HPLC) method using vitexin-4"-*O*-glucoside as internal standard developed and validated for quantitative analysis of VR. The results of elimination of VR in urinary and fecal excretion following oral and intravenous dosing indicated that VR was mainly excreted as prototype for both routes of administration, and biliary and renal excretions are two major ways of elimination of VR.

Key words: Excretion, high-performance liquid chromatography (HPLC), Vitexin-2"-*O*-rhamnoside.

INTRODUCTION

Vitexin-2"-*O*-rhamnoside (VR) abundantly exists in fruits and leaves of *Crataegus pinnatifida* Beg. var *major* (hawthorn and hawthorn leaves), both of which are very popular herbal materials in traditional Chinese medicine (TCM) and are well used in treating cardiovascular diseases (PRC, 2010). As VR is one of the main components of flavonoid of hawthorn leaves (Ding et al., 1990) and also a bioactive constituent on cardiovascular system in hawthorn (Liang et al., 2007), many pharmacological studies of VR have been reported until now, such as protective effect on the injured cardiac myocytes and endothelial cells (Zhu et al., 2003, 2006) and strongly inhibiting deoxyribonucleic acid (DNA) synthesis in MCF-7 human breast cancer cells (Ninfali et al., 2007). In recent years, many articles focus on *in vitro* study and pharmacokinetic studies (Cheng et al., 2007; Ying et al., 2007, Du et al., 2011). However, there is little research on the comparative excretion of pure VR isolated from hawthorn leaves following oral and intravenous routes of administration. In our study, a sensitive and specific high-performance liquid chromatography (HPLC) method using vitexin-4"-*O*-glucoside (VG) as internal standard thereby was first established to fully evaluate the urinary and fecal excretion content of VR following oral and intravenous route of administration. In addition, the differences of excretion after two forms of administration can be identified.

MATERIALS AND METHODS

Reagents and chemicals

The water used in all experiments was purified by a Milli-Q® Biocel Ultrapure Water System (Millipore, Bedford, MA, USA). Methanol, acetonitrile and tetrahydrofuran were all of HPLC grade and purchased from Xinxing Chemical Reagent Plant (Shanghai, China). All other chemicals of analytical grade were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

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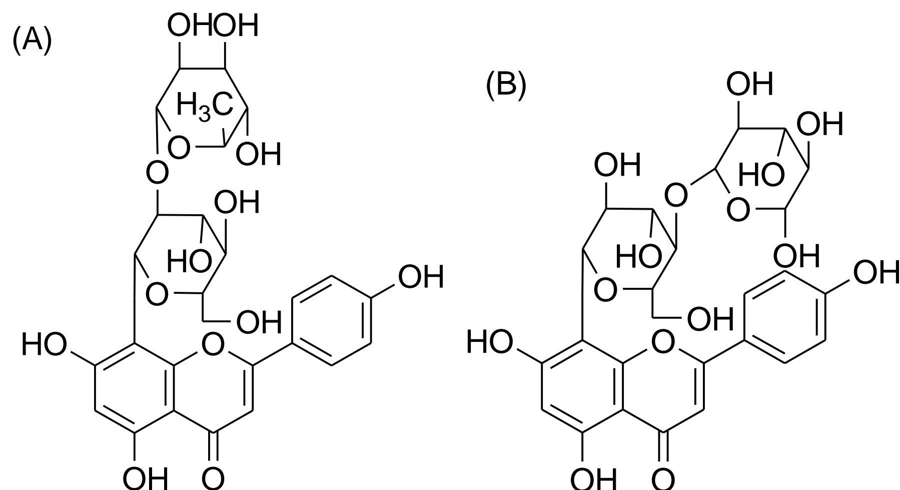

Figure 1. (A) Chemical structure of vitexin-2"-O-rhamnoside. (B) Chemical structure of vitexin-4"-O-glucoside (I.S.).

Plant

Leaves of *C. pinnatifida* Bge.var *major* were collected in Shenyang, Liaoning Province, China and identified by Prof. Ting-Guo Kang. A voucher specimen (20110921) was deposited in Liaoning University of Traditional Chinese Medicine.

Extraction and isolation

A sample of the leaves (2 kg) of *C. pinnatifida* Bge. var. *major* was cut in small pieces and refluxed with 60% aqueous ethanol for two times, each for 2 h. The crude extract after concentrated under reduced pressure was then adsorbed on a porous-polymer resin (AB-8, Tianjin, China) column, removed impurity with water and eluted with a gradient of 30, 50 and 70% ethanol. The fraction eluted with 30% ethanol was evaporated under reduced pressure to obtain extract, which was then chromatographed on silica gel column and eluted with ethyl acetate/butanone/formic acid/water 3:3:1:1. Fractions of similar composition were pooled on the basis of TLC analysis (UV monitoring at 365 nm) were repeatedly subjected to silica gel column chromatography and eluted with ethyl acetate/butanone/formic acid/water 5:3:1:1 to directly obtain crystal of VR (1 g) and VG (0.2 g) internal standard (I.S.), and purities of them were both over 99% by HPLC analysis. The chemical structures of VR and VG, confirmed by ^{1}HNMR, ^{13}CNMR and MS data, are shown in Figure 1.

Chromatographic system

The analysis was carried out on an Agilent 1100 series HPLC system (Agilent technology, Palo Alto, CA, USA) which consisted of a quaternary Pump (G1310A), a vacuum degasser (G1322A), a UV-VIS spectrophotometric detector (G1314A) and Chemstation software (Agilent). The analytical column was a Diamonsil C18 column (150 mm × 4.6 mm i.d., 5 μm, Diamonsil, USA) protected by a KR C18 guard column (35 mm × 8.0 mm, i.d., 5 μm, Dalian Create Science and Technology Co., Ltd., China). The optimal mobile phase used for separation is a mixture consisting of methanol-acetonitrile-tetrahydrofuran-1% glacial acetic acid (6:2:18:74, v/v/v/v). All the chromatographic measurements were performed at room temperature and a flow rate of 1 ml/min with the detection wavelength of 330 nm.

Preparation of standards and quality control samples

Standard stock solutions of VR and I.S. were both prepared in methanol to yield the concentrations of 4000 μg/ml and 276 μg/ml, respectively. Stock solution of VR was serially diluted with methanol to desired concentrations over the range of 0.16 to 3200 μg/ml. All the solutions were stored at 4°C before use. The calibration samples were prepared by spiking 200 μl blank mouse urine or 500 μl blank feces homogenates successively with appropriate amount of working solutions of VR (50 μl), acetic acid (20 μl) and I.S.. The volume of I.S. is 40 μl for urine samples and 80 μl for feces samples. The quality control samples of VR in the method validation were similarly prepared to the standard calibration samples.

Animals and dosing

SPF male Kunming strain mice (20 ± 2 g) were obtained from the Experimental Animal Center of Liaoning University of Traditional Chinese Medicine (Shenyang, China) and housed in an air-controlled breeding room for a week. Before starting the experiment, all the mice formerly free access to standard laboratory food as well as water ad libitum were fasted for more than 12 h. All experiments involving animals were approved by the animal ethics committee of Liaoning University of traditional Chinese medicine and performed according to the Guidelines for Animal Experimentation of this institution. For excretion studies, ten mice were randomly divided into two groups to be administrated VR at dose of 30 mg/kg. Among the two groups, one was orally administrated and the other intravenously administrated. All the mice were respectively housed in stainless-steel metabolism cages to collect urine and feces at different time. Two hours after administration, water and standard laboratory food were offered. Urine and feces samples were collected at 0 to 2, 2 to 4, 4 to 6, 6 to 8, 8 to 12 and 12 to 24 h post-dosing. The volume of each collected urine sample and the weight of each collected feces sample were separately recorded. All the sample s were stored at -20°C until analysis.

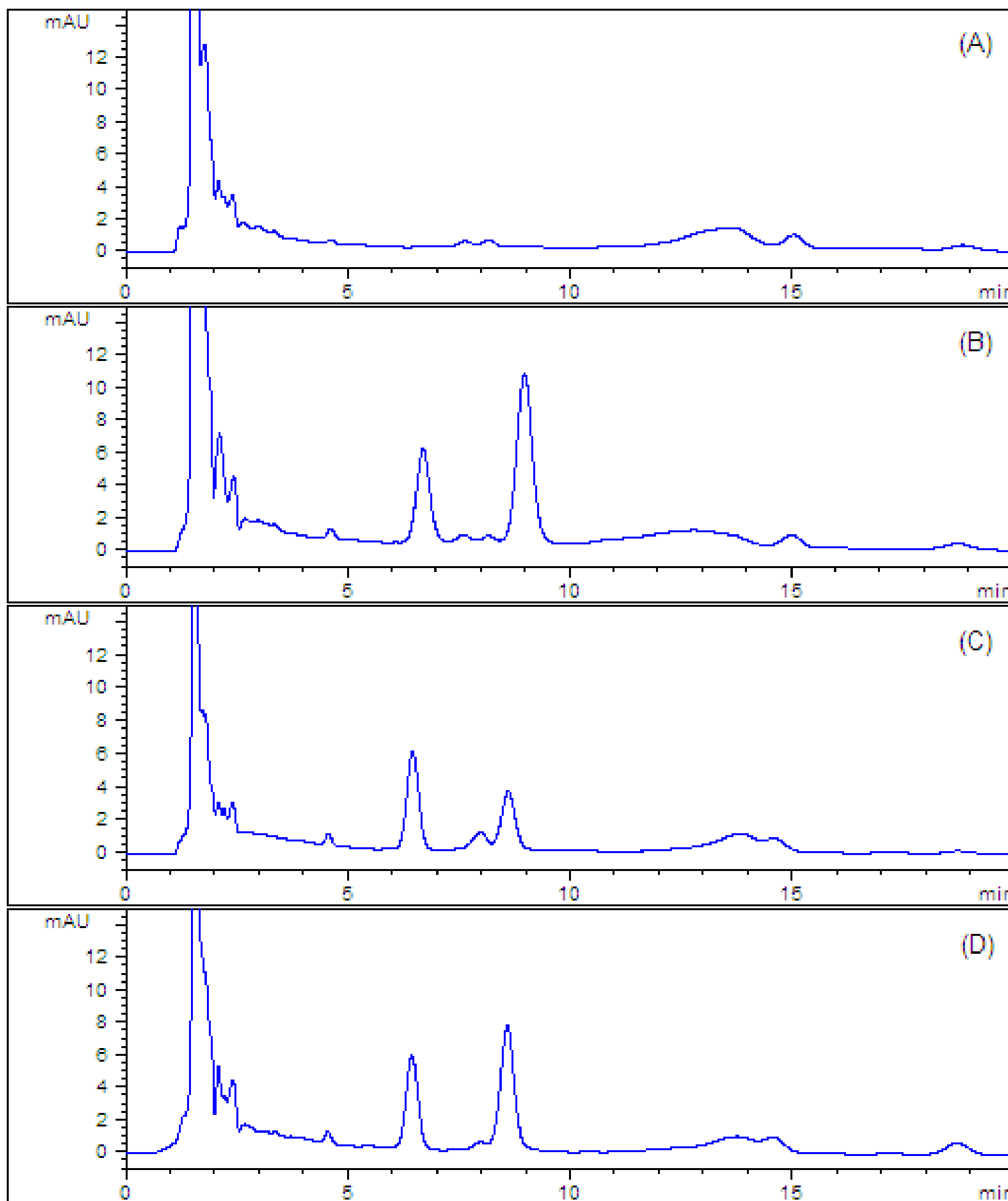

Figure 2. Typical chromatograms of urine excretion study (A-D) respectively obtained from blank urine sample, blank urine sample spiked with standard analyte and I.S., and urine samples collected from 4 to 6 h following oral and intravenous routes of administration of VR at dose of 30 mg/kg.

Sample processing

To 200 µl urine samples, 20 µl of acetic acid, 40 µl of I.S. and 1 ml of methanol were successively pipetted, followed by vortex mixing for 1 min. After samples being centrifuged at 890 g for 15 min, the supernatant was separated and evaporated to dryness under a gentle stream of nitrogen at 50°C. The residue was diluted in 200 µl of mobile phase and again centrifuged at 15,092 g for 10 min. Then an aliquot (20 µl) of clean supernatant was injected into HPLC column for analysis. Feces (0.2 g) were homogenized in 0.5 ml of saline solution. The homogenate was successively added 20 µl of acetic acid, 80 µl of I.S. and 2 ml of methanol and then treated as urine samples.

RESULTS AND DISCUSSION

HPLC chromatograms

Typical chromatograms of urinary and fecal excretion studies are shown in Figures 2 and 3, respectively. The total run time was no more than 20 min and the retention times of I.S. and VR were approximately 6.6 and 8.7 min, with no interfering peaks detected at the retention times of VR or I.S.

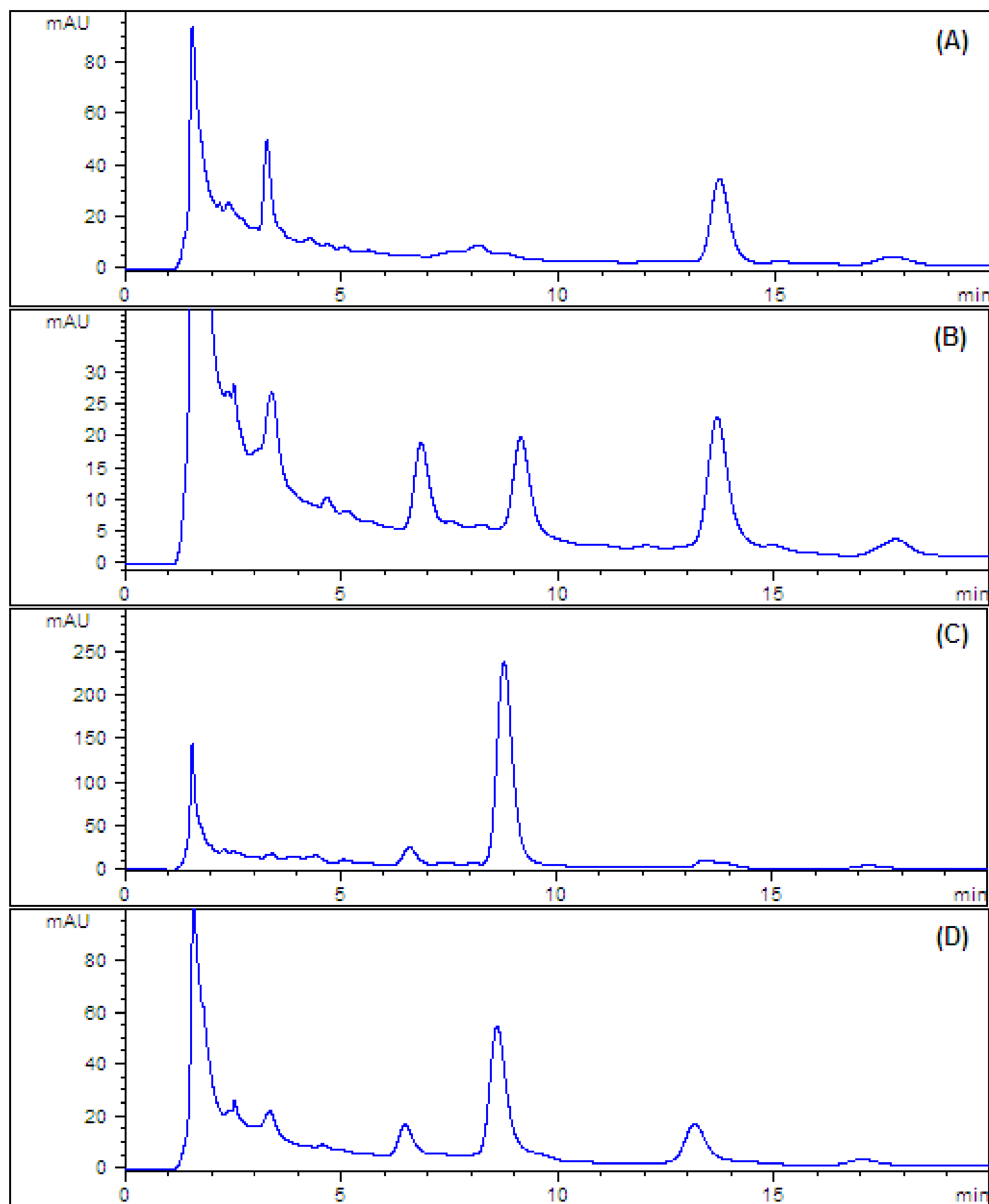

Figure 3. Typical chromatograms of fecal excretion study (A-D) respectively obtained from blank feces sample, blank feces sample spiked with standard analyte and I.S., and feces samples collected from 6 to 8 h following oral and intravenous routes of administration of VR at dose of 30 mg/kg.

Method validation

Method validation involves linearity, precision, limit of detection (LOD), limit of quantification (LOQ), recovery and stability. The linear range for urine and feces were within 0.4 to 400 μg/ml with $R^2 > 0.99$. The LOD (*S/N* > 3) and the LOQ (*S/N*> 10) were respectively 0.121 and 0.363 μg/ml in urine and 0.121 and 0.363 μg/g in feces. Both of the precision (RSD%) and accuracy (RE%) were below 15%, conforming to the criteria for the analysis of biological sample according to guidance of USFDA. The extraction recoveries of VR in urine and feces ranged from 82.36 ± 5.82% to 108.9 ± 8.47%. The results of short-term stability, long-term stability and freeze-thaw stability indicated that no remarkable degradation occurred during chromatography, extraction and sample storage processes for excreta samples.

Excretion studies

Figure 4 shows the urinary and fecal excretion time profiles

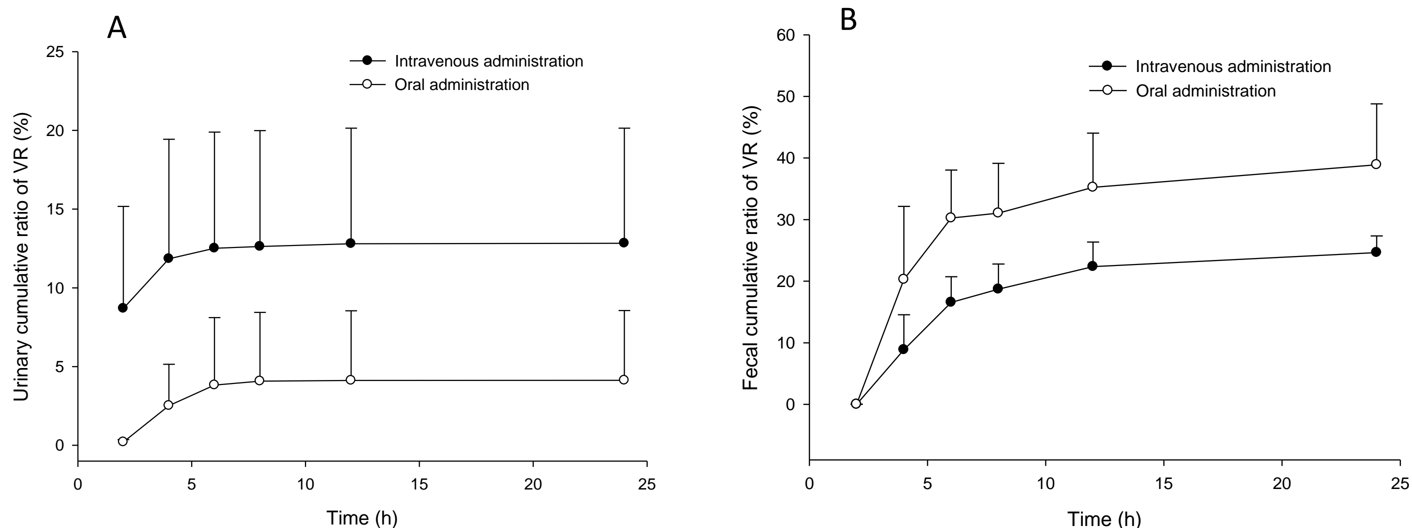

Figure 4. (A) Urinary cumulative ratio of vitexin-2"-*O*-rhamnoside in mice (mean ± S.D., *n* = 5) following oral and intravenous routes of administration at dose of 30 mg/kg. (B) Fecal cumulative ratio of vitexin-2"-*O*-rhamnoside in mice (mean ± S.D., *n* = 5) following oral and intravenous routes of administration at dose of 30 mg/kg.

of VR following oral and intravenous administration at dose 30 mg/kg. The urinary and fecal cumulative ratios of unchanged VR amounted to 4.13 ± 0.01% and 38.89 ± 3.04% after oral administration, and amounted to 12.83 ± 0.03% and 24.65 ± 1.75% after intravenous administration. VR as prototype was detected in excreta by comparing HPLC chromatogram of blank biological samples with the tested one. The total VR recoveries of excreta were 37.48 ± 1.78% (12.83 ± 0.03% in urine; 24.65 ± 1.75% in feces) following intravenous dosing and 43.02 ± 3.05% (4.13 ± 0.01% in urine; 38.89 ± 3.04% in feces) following oral dosing, demonstrating that biliary excretion and renal excretion are two major ways of elimination of VR and VR underwent extensive first-pass effect after oral administration and that not being absorbed was mainly excreted as feces. Recently, Ma et al. (2010) studied the excretions of VR after orally administrated hawthorn leaves extract and found that the total recovery of the dose in 24 h was 89.01% (0.72% in urine; 88.29% in feces) for VR (Ma et al., 2010), which is different from the results of monomer administration of VR were 43.02 ± 3.05% (4.13 ± 0.01% in urinary excretion; 38.89 ± 3.04% in fecal excretion) following oral dosing, suggesting that various kinds of components in hawthorn leaves extract affect the concentration and duration of VR *in vivo*.

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