Bioavailability of Vitexin-2"-*O*-rhamnoside after Oral Co-administration with Ketoconazole, Verapamil and Bile Salts

Dong-rui LU [1], Wen-jie ZHANG [1], Hong-jun ZOU [2], He-fei XUE [1], Jing-jing YIN [1], Ying-hui CHEN [1], Xi-xing YING [1]*, Ting-guo KANG [1]

[1] *School of Pharmacy, Liaoning University of Traditional Chinese Medicine, Dalian 116600, China*
[2] *Shenyang Yongda Pharmaceutical Co., Ltd., Shenyang 110027, China*

SUMMARY. A sensitive and specific HPLC method with internal standard was developed and validated to determine vitexin-2"-*O*-rhamnoside (VOR) in rat plasma after oral and intravenous administration. VOR presented a very low bioavailability, the P-glycoprotein inhibitors such as verapamil and ketoconazole and absorption promoting agent, *i.e.* bile salts were therefore respectively applied to investigate the reasons leading to the low oral bioavailability. The results indicated that the oral bioavailability increased about 1.77 and 3.15-fold after co-administration of VOR with verapamil and high concentration of bile salts, respectively, but only a little increased after co-administration with ketoconazole and low concentrations of bile salts.

INTRODUCTION

The leaves of *Crataegus pinnatifida* Bge. var. *major,* a famous traditional Chinese medicine (TCM) was used for the treatment of qi-stagnancy and blood stasis, chest distress, palpitation, loss of memory, dizziness and tinnitus [1], which mainly contain flavonoids compounds including vitexin-4"-*O*-glucoside, vitexin-2"-*O*-rhamnoside (VOR), vitexin, rutin, hyperoside, quercitrin, etc. [2]. In recent years, more scholars have paid more attention on the pharmacodynamic [3] and the pharmacokinetic [4,5] studies of the hawthorn leaf extract and the pure compounds. As VOR is a main component of the hawthorn leaves [6] and has many pharmacological activities such as protective effect on the injured cardiac myocytes and endothelial cells [7] and strongly inhibiting deoxyribonucleic acid (DNA) synthesis in MCF-7 human breast cancer cells [8], so many studies also concentrated on its pharmacokinetics and found it presenting a poor oral bioavailability [9]. The aim of this study is to investigate whether the metabolic enzymes, efflux transporter and absorption promoting agent have a significant impact on the oral bioavailability of VOR, in other words, to elucidate the reasons leading to the low oral bioavailability via comparing the absorption differences of the oral absorptions of VOR after co-administration with ketoconazole, verapamil (the metabolic enzymes, efflux transporter) and bile salts (the absorption enhancers), respectively.

## MATERIALS AND METHODS
Plant material

The leaves of *C. pinnatifida* Bge. var. *major* were collected in Shenyang, China and identified by Prof. Tingguo Kang. Voucher specimens (20110921) were maintained at the Liaoning University of Traditional Chinese Medicine.

Reagent and chemicals

VOR (Fig. 1A) was isolated from the hawthorn leaves in our laboratory, with purity over 99% by HPLC analysis. The internal standard, hesperidin (Fig. 1B) was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Verapamil hydrochloride (Fig. 1C) was obtained from the Central Pharmaceutical Co. Ltd. (Tianjin, China), Ketoconazole tablets (Fig. 1D) from Xian-Janssen Pharmaceutical Co., Ltd. (Xian, China), Bile salts (Fig. 1E) from Shanghai Hengdailao biological Co., Ltd. (Shanghai, China). The water used in all experiments was purified

* Author to whom correspondence should be addressed. *E-mail:* yingxixiang@163.com
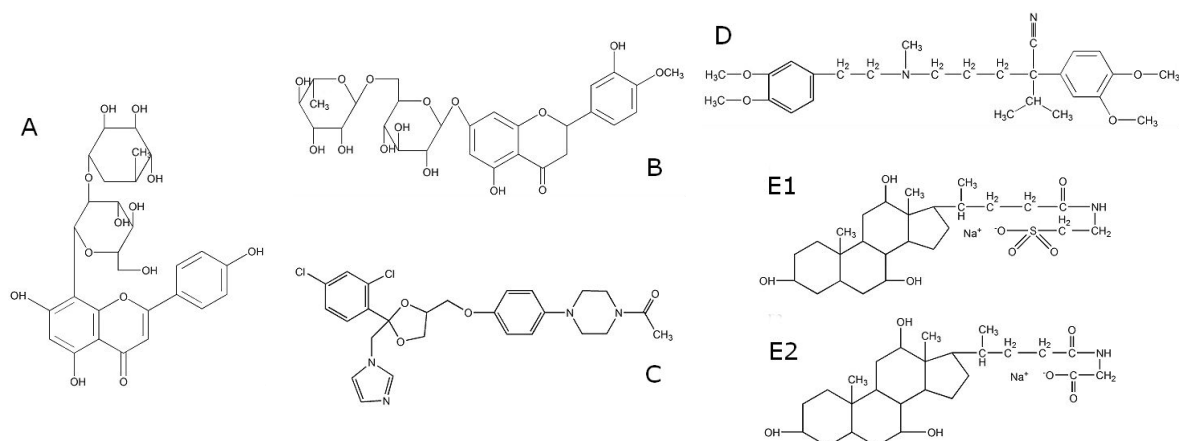
Figure 1. Chemical structures of vitexin-2"-*O*-rhamnoside (**A**), hesperidin (I.S.) (**B**), ketoconazole (**C**), verapamil (**D**) and Bile salts (**E**): sodium taurocholate (**E₁**) and sodium glycocholic (**E₂**).

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

Chromatographic system

The experiment was performed on an Agilent 1100 series HPLC system (Agilent technology, Palo Alto, CA, USA) equipped with a quaternary Pump (G1310A), a vacuum degasser (G1322A), a UV-VIS spectrophotometric detector (G1314A) and Chemstation software (Agilent). The analytes were determined at room temperature on an analytical Diamonsil (ODS, 150 × 4.6 mm i.d., 5 µm, Dikma Technologies, Beijing, China) protected by a KR C_{18} guard column (35 × 8.0 mm, 5 µm, Dalian Create Science and Technology Co., Ltd., China). The mobile phase, which consisted of the solvent (A), acetonitrile-tetrahydrofuran (95:5, v/v); solvent (B), 0.1% aqueous formic acid (v/v), was filtered and degassed under reduced pressure prior to use; the elution profile was 0-10 min, 12-17% (A); 10-20 min, 17-20% (A); 20-35 min, 20-23% (A) and then returned to initial condition for a 5 min re-equilibration, with total run time 35 min. The analysis was carried out at a flow rate of 1 mL/min with the detection wavelength of 330 nm.

Preparation of standards and quality control samples

Standard stock solution of VOR and I.S. were both prepared in methanol to yield the concentrations of 4000 µg/mL and 216 µg/mL, respectively. Standard stock solution of VOR was serially diluted with methanol to desired concentrations over the range of 0.16-80 µg/mL. Stock solution of I.S. was diluted with methanol to 43.2 µg/mL. All working solutions were stored at 4 °C before use. The quality control (QC) samples were prepared at 0.16, 5 and 64 µg/mL of VOR in bulk, and aliquots were stored frozen before use.

Plasma sample preparation

Twenty µL of acetic acid, 30 µL of I.S. (43.2 µg/mL), and 1 mL of acetonitrile were successively pipetted into the 200 µL plasma samples, followed by vortex mixing for 1 min and centrifuged at 3500 rpm for 15 min. The supernatant was collected and evaporated to dryness under a gentle stream of nitrogen at 50 °C. The dried residue was then reconstituted in 200 µL of mobile phase, and centrifuged at 10000 rpm for 10 min. A 20 µL aliquot of each supernatant was injected into the HPLC system for analysis.

Method validation
Selectivity

Selectivity was shown by comparing chromatograms of blank plasma obtained from rats prior to dosing with those of corresponding standard plasma samples spiked with VOR and I.S., and plasma samples from rats after the intravenous and oral administration of VOR.
Linearity, LOD and LOQ

The linearity was evaluated over the concentration range of 0.16-80 µg/mL for VOR. The calibration curves were linear in the determined concentration ranges. The calibration curve for each analyte in plasma was generated by plot-

ting the peak area ratio of them to I.S. *versus* the nominal concentrations in the standard plasma samples. The regression equation was obtained by weighted $(1/C^2)$ least square linear regression. LOD and LOQ were determined by stepwise dilution of the QC sample at low concentration level using a signal-to-noise ratio of 3 and 10, respectively, giving an acceptable accuracy (RE) within ± 20% and a precision (RSD) that did not exceed 20%.

Precision and Accuracy

The intra- and inter-assay precisions were assessed by determination of QC samples (n = 6) at three concentration levels of each compound (0.16, 5, and 64 µg/mL). For the intra-day validation, five replicates of the QC plasma samples were analyzed on the same day, while the inter-day values were carried out over three consecutive days. The accepted criteria for each QC sample were that the precision and accuracy should not exceed 15%, except at the LOQ where it should not exceed 20%. The precision was expressed as the relative standard deviation (RSD) and the accuracy as the relative error (RE).

Extraction Recovery

Recoveries in the plasma of VOR were calculated by comparing chromatographic peak areas of the extracted quality control samples to that of the unextracted standard solutions containing the equivalent amount of analytes (n = 6).

Stability

Stability was investigated at three quality control levels (low, medium and high). The short-term stability and long-term stability were determined by analyzing QC samples kept at ambient temperature (25 °C) for 4 h and stored at -20 °C for one month, respectively. The freeze-thaw stability was investigated after three freeze (-20 °C)-thaw (room temperature) cycles. Then, the samples were processed and analyzed. The concentrations obtained were compared with the nominal values of QC samples.

Animals and dosing

SPF male Wister rats (180 ± 20 g) were obtained from the Experimental Animal Center of Liaoning University of Traditional Chinese Medicine (Shenyang, China). Rats fed on a standard laboratory diet and allowed tap water *ad libitum* were used for at least 7 days, and then fasted for more than 12 h prior to each experiment. 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. They were divided randomly into six groups (n = 6), two groups were received VOR intravenously (10 mg/kg) and orally (30 mg/kg); two groups received VOR orally (30 mg/kg) with co-administered ketoconazole (20.83 mg/kg) and verapamil (31.25 mg/kg), respectively. The rest two groups were, respectively, combined different concentrations of bile salts (1 g/kg, 0.5 g/kg orally) with VOR (30 mg/kg). Blood samples were collected into heparinized tubes from the vena orbitalis at times of 3, 5, 10, 20, 30, 45, 60, 90, 120, and 240 min after administration of each group and then centrifuged at 3500 rpm for 15 min. The obtained plasma was stored at -20 °C until analysis.

Data analysis

All data were subsequently processed with the computer program Microsoft Office Excel 2003 and 3P97 (Practical Pharmacokinetic Program, 1997, China) to determine the compartment models and pharmacokinetic parameters.

RESULTS AND DISCUSSION
Method validation

To determine the selectivity of this method, blank rat plasma, plasma spiked with known amounts of VOR and hesperidin, and plasma samples from rats after oral and intravenous administration of VOR were analyzed, respectively. Fig. 2 respectively show the selectivity of plasma indicating that there were no interfering substances in the region of the peaks of the analyte and I.S.. The retention times of VOR and I.S. were approximately 18.46 and 27.83 min, respectively. The total run time was 35 min.

The linear range of VOR plasma was within 0.16-80 µg/mL. The regression equation and coefficients were: $y = 0.3656 x + 0.0118$, $r^2 = 0.9998$. The LOD ($S/N > 3$) and the LOQ ($S/N > 10$) were respectively 0.048 and 0.16 µg/mL in plasma. Both of the precision (RSD%) and accuracy (RE%) were below 15%, conforming to the criteria for the analysis of biological samples according to the USFDA [10]. The extraction recoveries of VOR in plasma ranged from 89.36 ± 0.227 to 93.71 ± 0.350% (Table 1). The result of short-term stability, long-term stability and freeze-thaw stability, indicating that no remarkable depredation occurred during chromatography, extraction and sample storage processes for the samples.
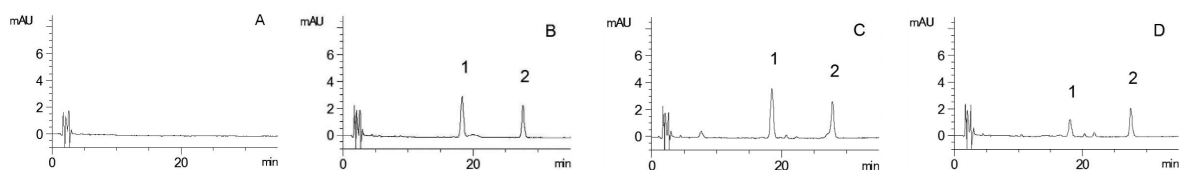
Figure 2. Representative chromatograms of blank plasma (**A**), plasma spiked with vitexin-2"-*O*-rhamnoside and hesperidin (**B**), plasma sample at 30 min after the intravenous administration of vitexin-2"-*O*-rhamnoside at a dose of 10 mg/kg (**C**) and plasma sample at 20 min after the oral administration of vitexin-2"-*O*-rhamnoside at a dose of 30mg/kg (**D**). Peak 1: vitexin-2"-*O*-rhamnoside; Peak 2: hesperidin.

Added Conc. (µg/mL)	Intra-day			Inter-day			Recovery (%)	RSD (%)
	Conc. (µg/mL)	RSD (%)	RE (%)	Conc. (µg/mL)	RSD (%)	RE (%)		
0.4	0.39 ± 0.06	4.0	-3.6	0.39 ± 0.08	4.24	-2.7	89.36 ± 0.227	5.3
5	4.86 ± 0.27	5.8	2.4	4.82 ± 0.25	5.16	3.4	93.71 ± 0.350	3.8
64	63.69 ± 0.13	3.6	-3.2	63.58 ± 0.42	3.79	-2.2	90.54 ± 0.182	4.0

**Table 1**. Precision and accuracy of vitexin-2"-*O*-rhamnoside in rat plasma ($n = 6$, mean ± S.D.).

Pharmacokinetic studies

Plasma concentration-time curves for VOR in rats following oral and intravenous administration are shown in Fig. 3. The significant differences of pharmacokinetic parameters calculated from these data are given in Table 2. The area under the plasma concentration-time curve from zero to infinity ($AUC_{0\to\infty}$) was calculated by means of the trapezoidal rate constant. The peak plasma concentration (C_{max}) and the peak time directly obtained from the drug plasma concentration-time profiles. The absolute bioavailability (F) was calculated as $(AUC_{oral}/AUC_{i.v.}) \times (dose_{i.v.}/dose_{oral})$. The data were statistically analyzed (ANOVA). The difference were considered significant when $P < 0.05$. All data are presented as means ± standard errors.

Oral bioavailability

To improve the oral bioavailability, a number of researchers focus on the reasons leading to the poor bioavailability. As drug metabolizing enzymes and intestinal drug transporter have become the two main factors that affect drug metabolism and oral absorption [11,12], the aim of this study was to investigate whether the metabolic enzymes, efflux transporter and absorption promoting agent have a significant impact on the oral bioavailability of VOR.

Ketoconazole being a known selective inhibitor of CYP3A had a greater impact on oral bioavailability such as 2 and 2.5-fold increase in oral bioavailability of tacrolimus [13] and cyclosporine [14], respectively, after co-administra-
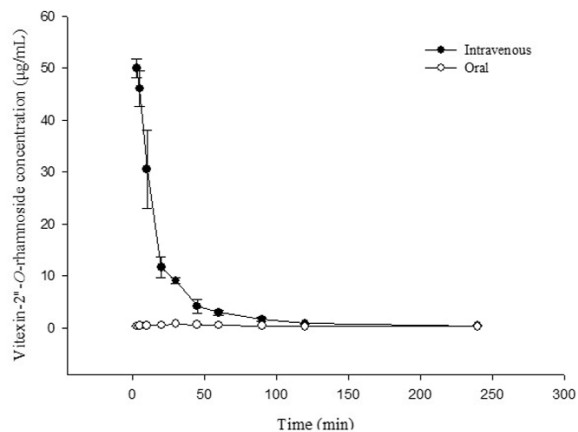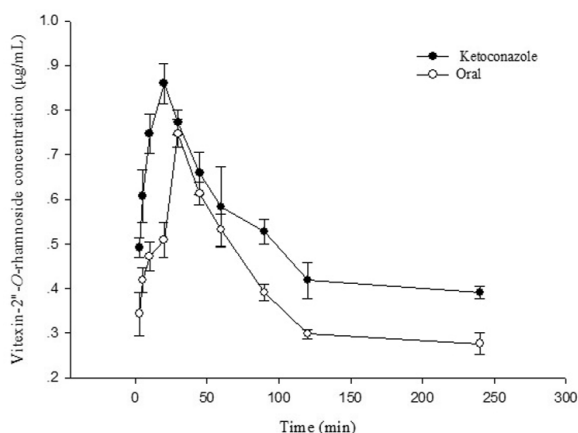

Figure 3. Plasma concentration-time curves of vitexin-2"-*O*-rhamnoside in rats after oral and intravenous administration.

tion of ketoconazole. Therefore, in this study, co-administration VOR with ketoconazole (20.83 mg/kg) was first practiced to study the oral bioavailability, and the AUC was up to 164.65 ± 13.26 µg/mL slightly higher compared to that of the control (146.73 ± 23.07). CYP3A, the major phase I drug metabolic enzyme presenting in human hepatocytes and intestinal enterocytes is implicated in the metabolic elimination of many drugs [15]. The small intestinal cells possess a glucoside-hydrolyzing activity and their glucose transport system is capable of participating in the glucoside absorption [16], and the hydrolysis process is part of the phase I metabolic processes, by the close involvement of the cytochrome

Administration		CL (L·min/kg)	$t_{1/2\beta}$ (min)	$AUC_{0\to\infty}$ (µg·min/mL)	C_{max} (µg/mL)	T_{max} (min)	F (%)
				Parameters			
Intravenous (VOR, 10 mg/kg)		0.0028	24.04	867.27	-	-	-
VOR (30 mg/kg)		0.065	60.70	146.73	0.82	13.67	5.63
Oral	+ ketoconazole (20.83 mg/kg)	0.29	421.92	164.65	0.83	30.27	6.328
	+ verapamil (31.25 mg/kg)	0.11	591.71	260.17	1.27	15.64	9.99
(VOR,	+ bile salts (0.5 g/kg)	0.0043	17568.92	196.19	0.88	16.81	7.54
30 mg/kg)	+ bile salts (1 g/kg)	0.0057	6624.61	460.91	1.68	23.02	17.71

Table 2. Pharmacokinetic parameters following intravenous and oral administration of vitexin-2"-O-rhamnoside in rats (mean ± SD, *n* = 5).

Figure 4. Plasma concentration-time curves of vitexin-2"-O-rhamnoside in rats after oral co-administration of vitexin-2"-O-rhamnoside (30 mg/kg) with ketoconazole (20.83 mg/kg).
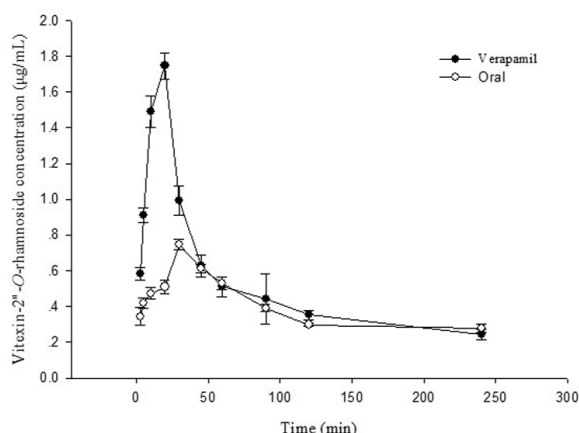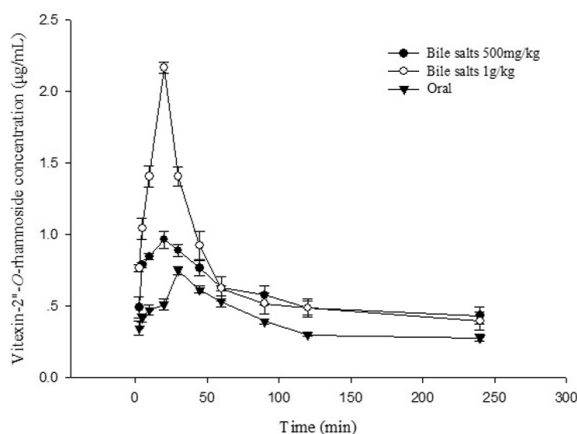

Figure 5. Plasma concentration-time curves of vitexin-2"-O-rhamnoside in rats after oral co-administration of vitexin-2"-O-rhamnoside (30 mg/kg) with verapamil (31.25 mg/kg).

P450. As shown in Fig. 4, although the concentration of VOR in plasma rats increased after oral co-administration with ketoconazole, the oral bioavailability of VOR has no significant changes, meaning that ketoconazole, a potent inhibitor of CYP3A enzymes, unremarkably decreased the dose into the epithelial cells of the small intestine to affect the oral bioavailability of VOR, and the quantity of CYP3A enzyme in the intestine is less than in the liver [17]. Leading to this result may be due to some other complex processes *in vivo*.

Najafzadeh *et al.* [18] demonstrated that the serum concentration of salinomycin was significantly enhanced in diabetic rats when received verapamil together. The P-glycoprotein inhibitor, verapamil (31.25 mg/kg), was also co-administrated with VOR (30 mg/kg), the AUC increased from 146.73 ± 23.07 (control) to 260.17 ± 23.14 µg/mL (p < 0.05), approximately 1.77-fold increased in AUC and 1.5-fold higher in C_{max}. P-glycoprotein as an energy-dependent pump is expressed mainly in the liver, kidneys

and intestines. Drug is absorbed into the mesenteric capillaries can be subjected to metabolic transformation by the metabolic enzymes, particularly phase II conjugation enzymes such as the glucuronosyltransferases and sulfotransferases [19]. These metabolites were extruded to the gut lumen by P-glycoprotein that prevents the cellular accumulation of some xenobiotics and endogenous compounds [20]. P-glycoprotein inhibitor could reduce the dose extruded into the gut lumen to improve oral uptake of drugs, thereby reducing dosing requirements [21]. Verapamil being the typical of P-glycoprotein inhibitors can competitively inhibit p-glycoprotein to increase the intracellular drug concentration. As shown in Fig. 5, the plasma concentration of VOR after oral co-administration of verapamil remarkably increased compared with administrated of VOR alone, and the absolute bioavailability of VOR increased to 10%, suggesting that P-glycoprotein inhibitor affects the uptake of VOR and increases the oral bioavailability of VOR.

Figure 6. Plasma concentration-time curves of vitexin-2"-*O*-rhamnoside in rats after oral co-administration of vitexin-2"-*O*-rhamnoside (30 mg/kg) with bile salts (0.5 g/kg and 1 g/kg).

Bile salts as the absorption enhancers were applied to improve the oral bioavailability [22]. Plasma concentration-time curves for VOR administered alone and co-administered with different concentration of bile salts are shown in the Fig. 6. The absolute bioavailability of VOR increased to 17.71% following combined treatment with bile salts (1 g/kg), and the AUC increased from 146.73 ± 23.07 to 460.91 ± 16.73 µg/mL (P < 0.05), approximately 3-fold increased in AUC and 2.1-fold higher in C_{max}. However, only a little difference of the AUC happened after co-administration low concentration of bile salts (146.73 ± 23.07 vs 196.19 ± 26.7 µg/mL). The results indicated that the high concentration of the bile salts as the absorption enhancers had a very significant effect on the oral bioavailability for VOR.

CONCLUSION

A sensitive and specific HPLC method was established to investigate the oral bioavailability of VOR via determining VOR in rat plasma after the oral and intravenous administration, and the highest absolute bioavailability of VOR was obtained after co-administration with bile salts (1 g/ml). Verapamil being the inhibitor of P-glycoprotein also play an important role in improving the bioavailability of VOR. However, only a little increased after co-administration with ketoconazole and the low concentration of bile salt (0.5 g/ml), respectively, and the reasons will be investigated in the further study in the future.

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