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Original Article

Effects of crude and vinegar-processed Curcumae Rhizoma on the activities of CYP450 enzymes in rats

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Abstract: Curcumae Rhizoma (CR) has been used in traditional Chinese medicine for thousands of years as an anti-blood stasis and pain-alleviating agent. However, the effects of crude and vinegar-processed Curcumae Rhizoma (VCR) on CYP450 activities are poorly understood. In this study, a "cocktail" method was designed to assess CYP1A2, CYP3A1 and CYP2E1 enzymatic activities *in vivo* using theophylline, dapsone and chlorzoxazone as probe substrates, based on a developed and validated HPLC method. In addition, real-time RT-PCR was performed to assess CYP1A2, CYP3A1 and CYP2E1 mRNA expression in the rat liver. The results indicated that long-term treatment with the CR extract inhibited CYP1A2 enzymatic activity and induced CYP3A1 activity. Further, VCR inhibited CYP1A2 and CYP2E1 enzymatic activities and induced CYP3A1 activity after administration of both a single dose and multiple doses, and these effects occurred in a time-dependent manner. VCR had greater inhibitory and inducing effects than CR. The mRNA expression results were consistent with the pharmacokinetic results. These results provide a scientific basis for the safe clinical application of CR and VCR in combination with other drugs, potentially preventing possible side effects induced by herb-drug interactions.

Keywords: Curcumae Rhizoma, vinegar-processed, CYP450, cocktail, pharmacokinetic, mRNA expression

Introduction

Curcumae Rhizoma (CR), or EZhu in Chinese, is derived from the dried rhizomes of three species of *Curcuma* L. of the Zingiberaceae family, including *Curcuma phaeocaulis* Val., *Curcuma kwangsiensis* S.G. Lee et C.F. Liang and *Curcuma wenyujin* Y.H. Chen et C. Ling, as described in the current Chinese Pharmacopoeia [1]. CR has been used in traditional Chinese medicine for thousands of years as an anti-blood stasis and pain-alleviating agent for the treatment of blood-stasis syndrome, menstrual disorders, epilepsy, etc., both alone and in combination with other herbs [1]. Pharmacological studies have reported that CR, including the essential oil and curcuminoids isolated from CR, has a broad spectrum of biological activities, including anti-tumor, anti-inflammatory, anti-angiogenic and hepatoprotective activities [2-5].

In recent years, studies have shown that CR extract and its four selected constituents, including zedoary oil, curcumol, cincole and isoborneol, increase CYP3A enzymatic activity and induce expression of the CYP3A4 gene through activation of PXR [6]. Curcumol, a bioactive compound of CR, has been demonstrated to have an inhibitory effect on CYP2C19 enzymatic activity using omeprazole as a probe substrate [7]. Curcumin, a major component of CR, has been reported to cause a dose-dependent decrease in carbon monoxide binding to rat liver microsomes and to promote the dose-dependent inhibition of CYP1A1, 1A2 and 2B1 activities, as indicated by decreased formation of resorufin using biochemical probes [8]. Inhibition of cytochrome P450 (CYP450) enzymatic activity can significantly increase exposure of co-administered drugs that are metabolized by the same CYP enzyme, which can result in significant adverse events [9, 10]. Howev-

er, no single component of CR is completely responsible for the effects of the whole CR extract. In addition, the effects of vinegar-processed CR, which is often used in the clinical setting, on CYP450 activities are poorly understood. The purpose of the present study was to evaluate the effects of crude CR and CR processed with vinegar (VCR) on CYP450 activities.

CYP450 enzymes comprise a superfamily of hemoproteins that play important roles in the biotransformation of many endogenous and exogenous substances, including drugs. The activities of CYP450 isozymes can be inhibited or induced by exogenous substances; these effects are among the major causes of clinical drug-drug interactions (DDIs) and herb-drug interactions [11]. It is well known that the liver is responsible for the majority of drug metabolism and that CYP1A2, CYP3A4 and CYP2E1 are the three major enzyme subtypes of the CYP450 system in the liver [12]. CYP1A2 is one of the major CYPs in the liver, and it metabolizes approximately 20% of clinically used drugs [13]. CYP3A4 has been reported to contribute to the metabolism of the vast majority of drugs compared with other Phase I oxidative enzymes. Human CYP3A subfamily enzymes include CYP3A4, 3A5, 3A7 and 3A43, and CYP3A4 is the most abundant in the liver and small intestine and metabolizes more than 50% of clinically used drugs [14]. CYP2E1, an ethanol-inducible enzyme, is involved in the metbolic activation of many low-molecular-weight copounds, such as N-nitrosamines, aniline, vinyl chloride, and urethane [15, 16]. CYP2E1 has also been shown to play key roles in the hepatotoxicity of ethanol and other xenobiotics. With the increasing use of herbal medicines in combination with drugs, many metabolism-based side effects caused by their interactions have been reported [17, 18]. While rats do have the CYP1A2 and 2E1 sub-families, they do not have the CYP3A4 sub-family. In the CYP3A family, the rat enzymes with the highest protein homology to human CYP3A4 are CYP3A1 (73% identical) and CYP3A2 (72% identical), with CYP3A1 and CYP3A2 being 88% identical to each other [19]. To avoid possible toxic adverse reactions induced by herb-drug interactions, we evaluated the effects of CR and VCR on the activities and mRNA expression of the CYP1A2, CYP3A1 and CYP2E1 enzymes in rats.

The activities of drug-metabolizing enzymes can be assessed *in vivo* using specific probe drugs [20]. For example, caffeine and theophylline are often used as probe substrates of CYP1A2, chlorzoxazone is used for CYP2E1, and dapsone is often used for CYP3A4 [21]. In this study, a "cocktail" method was designed to assess CYP activities *in vivo* using theophylline, dapsone and chlorzoxazone as probe substrates of the CYP1A2, CYP3A1 and CYP2E1 enzymes, respectively. We developed and validated this high-performance liquid chromatography method for simultaneous detection of the three probe drugs and successfully applied it to assess their pharmacokinetics. Further, real-time RT-PCR was performed to determine the CYP1A2, CYP3A1 and CYP2E1 mRNA expression levels in the rat liver. The results of this study provide a basis for establishing the safety of the clinical use of CR and VCR.

Material and methods

Chemicals and reagents

Theophylline (purity > 99%), chlorzoxazone (purity > 99%) and antipyrine (purity > 99%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Nanjing, China). Dapsone (purity > 99%) was obtained from Sigma Chemicals (St. Louis, MO, USA). HPLC-grade acetonitrile was purchased from Merck (Merck, Darmstadt, Germany). Ultra-pure water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

Plant materials and extract preparation

The crude herb of CR was purchased from Yueqing, Zhejiang Province, China. All of the raw materials were identified by Professor Tu-lin Lu of Nanjing University of Chinese Medicine. The voucher specimens (No. 110602) of CR were deposited at the College of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, China. To prepare the VCR, 100 g CR and 20 mL vingar (Shanxi Mature Vinegar Group, China; total acid 4.5 g/100 ml) were mixed well in a suitable airtight container. Then, the sample was moisturized for 20 min and subsequently stir-fried at 100°C for 5 min, removed from the container and cut into slices.

The CR and VCR extracts were prepared as follows: pieces of CR and VCR (100 g) were immersed in 90% ethanol for 1 h and were then boiled with a reflux condenser 2 times for 1.5 h each time. The extracts were then pooled and

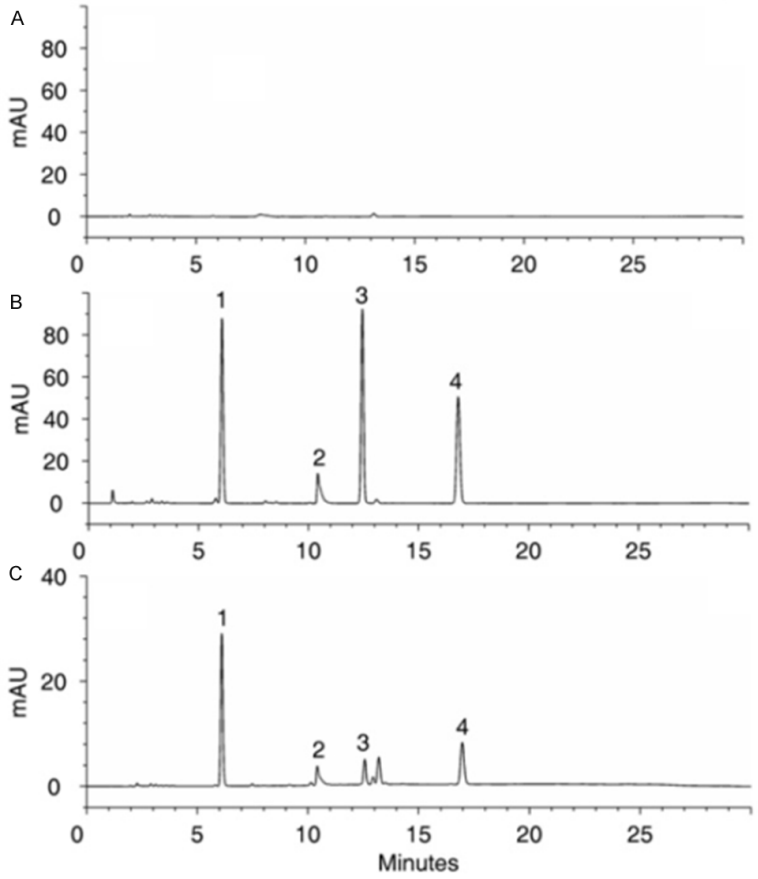

Figure 1. Typical chromatograms of (A) blank plasma; (B) plasma spiked with probe drugs and the internal standard; and (C) rat plasma at 3 h after administration of CR extract. Peaks: 1: theophylline; 2: antipyrine; 3: dapsone; and 4: chlorzoxazone.

evaporated under vacuum at 40°C. Finally, the fraction was dissolved in 0.5% sodium carboxymethyl cellulose (CMC-Na) at a concentration of 1.0 g/mL.

Animals

Male Sprague-Dawley rats weighing 230 ± 20 g were purchased from Shanghai Sipper-BK Lab Animal Co. Ltd. The experiments were carried out following an approved protocol from the Nanjing University of Chinese Medicine Animal Care and Use Committee. The animals were housed with unlimited access to food and water, except when they were fasted for 12 h before initiation of the experiments.

Instrumentation

An Agilent 1100 HPLC system (Agilent Technologies, CA, USA) equipped with a solvent degasser, quatpump, autosampler, and ultraviolet detector was used in the study. Data were acquired and processed using Agilent Chemstation software. A New Classic MS e-lectronic analytical balance (Metter-Toledo Instruments Co., Ltd., Shanghai, China), SpectraMax M5 multifunctional microplate reader (Molecular Devices Spectra Max Plus 384, Sunnyvale, CA, USA) and Mini-Opticon™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) were also used.

Effects of CR and VCR on CYP1A2, CYP2E1 and CYP3A1 activities in rats: a cocktail method

Plasma collection (single dose): Eighteen rats were randomly assigned to the following groups and orally administered a single 9 g/kg dose of CR, VCR or 0.5% CMC-Na, respectively: a CR group, VCR group and control group (n=6/group). After 5 min, a probe solution mixed with theophylline (30 mg/kg), dapsone (20 mg/kg) and chlorzoxazone (50 mg/kg) was orally administered to the rats. Blood samples (0.5 mL) were collected in heparinized tubes pre-dose (0 h) and at 0.05, 0.15, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 10, 12 and 24 h post-dose. In addition, plasma samples were collected after centrifugation at 1000×g for 10 min and stored at -80°C.

Plasma collection (multiple doses): Eighteen rats were randomly assigned to the following groups and administered a single 9 g/kg dose of CR, VCR or 0.5% CMC-Na, respectively: a CR group, VCR group and control group (n=6/group). After oral administration for 7 consecutive days, each group was orally administered a probe solution mixed with theophylline (30 mg/kg), dapsone (20 mg/kg) and chlorzoxazone (50 mg/kg) on the eighth day. Then, blood samples (0.5 mL) were collected into heparinized tubes pre-dose (0 h) and at 0.05, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 10, 12, and 24 h post-dose. In addition, plasma samples were

Table 1. Precision and accuracy of analytes in rat plasma (mean ± SE, n=6)

Analytes	Concentration (μg/mL)	Intra-day assay		Inter-day assay		Accuracy (%)
		Measured quantity (μg/mL)	RSD (%)	Measured quantity (μg/mL)	RSD (%)	
Theophylline	1.306	1.67 ± 0.08	4.51	1.67 ± 0.01	0.10	113.0 ± 0.08
	6.528	5.69 ± 0.42	7.42	5.69 ± 0.02	0.41	87.45 ± 0.42
	26.11	23.72 ± 1.00	4.30	23.84 ± 0.10	0.60	91.93 ± 0.99
Dapsone	1.304	1.53 ± 0.20	1.38	1.54 ± 0.02	1.20	111.4 ± 0.07
	6.522	5.72 ± 0.44	7.64	5.77 ± 0.05	0.88	89.33 ± 0.37
	26.09	27.52 ± 2.50	9.10	27.49 ± 0.03	0.10	105.3 ± 2.60
Chlorzoxazone	0.678	0.75 ± 0.10	1.36	0.74 ± 0.01	1.00	104.7 ± 0.09
	3.392	2.91 ± 0.23	7.72	2.87 ± 0.03	0.99	84.18 ± 0.22
	13.57	13.42 ± 0.73	5.51	13.30 ± 0.11	0.81	97.40 ± 0.74

Table 2. Extraction recoveries of analytes in rat plasma (mean ± SE, n=6)

Analytes	Concentration (μg/mL)	Recovery (%)	RSD (%)
Theophylline	1.306	72.06 ± 0.01	5.9
	6.528	83.36 ± 0 .08	7.9
	26.11	79.26 ± 0.18	4.2
Dapsone	1.304	79.52 ± 0.03	14
	6.522	89.03 ± 0.07	6.8
	26.09	92.44 ± 0.53	9.7
Chlorzoxazone	0.678	91.69 ± 0.03	14
	3.392	97.23 ± 0.06	7.8
	13.57	96.30 ± 0.21	5.6

collected after centrifugation at 1000×g for 10 min and stored at -80°C.

Preparation of plasma: Twenty microliters of a reference substance and 20 μL of an internal standard solution (antipyrine) were added to a 100 μL plasma sample. Then, the plasma sample was spiked with 800 μL ethyl acetate and vortexed for 2 min. The sample was centrifuged at 10,000×g for 10 min at 4°C. Next, the organic layer (700 μL) was transferred to another tube and dried under nitrogen. For HPLC analysis, the residue was dissolved in 100 μL acetonitrile-water (30:70, v/v), and a 10 μL aliquot was injected into the column. The standards were prepared in the same manner.

Chromatographic conditions: HPLC separation was performed on a Kromasil C18 column (250 mm × 4.6 mm, i.d., 5 μm). The mobile phase consisted of a combination of the solvent (A) acetonitrile and (B) water, and the following gradient program was used: 12%-40% A (from 0-10 min); 40% A (from 10-22 min); 40%-12% A (from 22-24 min); and 12% A (from 24-30 min). The detection wavelength was 280 nm, and the column oven temperature was held at 30°C. The flow rate was set at 1.0 mL/min, and the injection volume was 10 μL.

Preparation of standard solutions and QC samples: A stock solution was prepared by dissolving specific amounts of theophylline, dapsone and chlorzoxazone in methanol and then diluting the solutions with methanol to obtain the following target concentrations: 326.4 μg/mL for theophylline, 326.1 μg/mL for dapsone and 169.6 μg/mL for chlorzoxazone. Antipyrine (IS) was also prepared in methanol to obtain a working solution of 200.96 μg/mL. This working solution was diluted with methanol to prepare a series of working solutions at the appropriate ratios to obtain the desired concentrations for use as calibration standard samples. Low-, medium- and high-level QC samples of theophylline, dapsone and chlorzoxazone were also prepared with methanol.

Method validation: The method validation procedures were performed according to the FDA guidelines and as described previously [22, 23] to evaluate the suitability of the method for the quantitative determination of theophylline, dapsone and chlorzoxazone. Specificity was assessed by analysis of five independent drug-free human plasma samples supplemented only with the internal standard to ensure for the absence of endogenous compounds with the same retention times as the analytes of interest. The linearity of the method was evaluated by generating six-point calibration curves with ranges of 0.6528~32.64, 0.6522~32.61 and 0.3392~16.96 ng/mL for theophylline, dap-

Table 3. Stability of the analytes (mean ± SE, n=6)

Analytes	Concentration (μg/mL)	At room temperature, 24 h		Freeze-thaw stability		At -80°C, 30 d	
		Measured quantity (μg/mL)	RSD (%)	Measured quantity (μg/mL)	RSD (%)	Measured quantity (μg/mL)	RSD (%)
Theophylline	1.306	1.89 ± 0.00	0.19	1.77 ± 0.15	8.21	1.58 ± 0.00	0.47
	6.528	6.00 ± 0.02	0.41	5.84 ± 0.25	4.32	5.44 ± 0.08	3.26
	26.11	28.61 ± 0.21	0.75	26.16 ± 3.30	1.26	27.71 ± 0.21	2.58
Dapsone	1.304	1.27 ± 0.04	3.20	1.42 ± 0.17	1.25	1.56 ± 0.02	0.45
	6.522	5.77 ± 0.05	0.78	5.80 ± 0.06	0.96	5.62 ± 0.10	3.21
	26.09	27.00 ± 0.23	0.86	27.32 ± 0.22	0.81	26.88 ± 0.13	4.39
Chlorzoxazone	0.678	0.91 ± 0.00	0.19	0.81 ± 0.11	1.34	0.81 ± 0.04	1.20
	3.392	3.15 ± 0.02	0.68	3.01 ± 0.21	7.02	2.67 ± 0.03	6.84
	13.57	14.84 ± 0.12	0.84	14.01 ± 1.10	7.51	14.25 ± 0.15	1.11

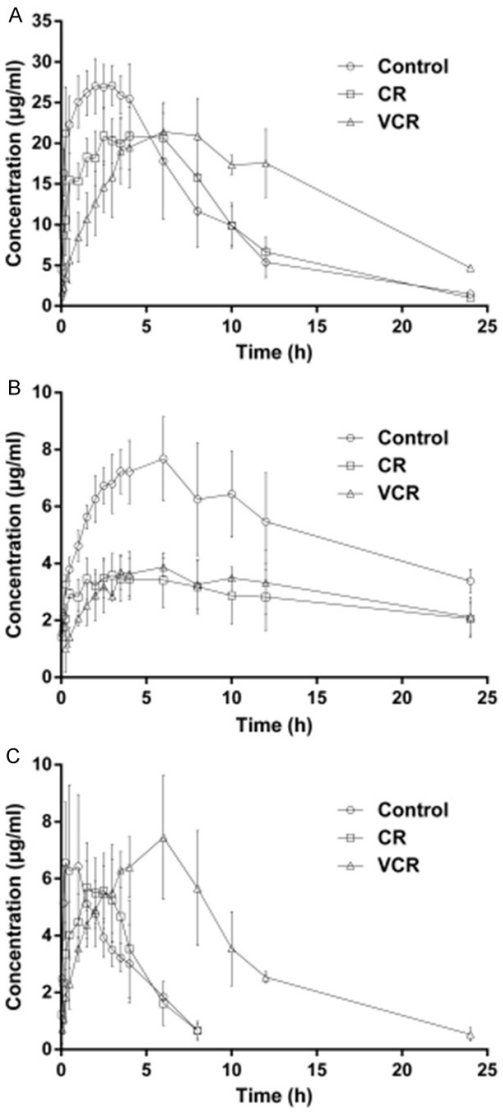

Figure 2. Time-concentration curves for theophylline (A), dapsone (B) and chlorzoxazone (C) after administration of a single dose of CR or VCR (mean ± SE, n=6).

sone and chlorzoxazone, respectively. The calibration curves were constructed by plotting the peak area ratio of antipyrine/IS versus the concentrations of theophylline, dapsone and chlorzoxazone using linear regression. Linear least-squares regression analysis was conducted to determine the slope, intercept and coefficient of determination (r) to demonstrate the linearity of the method. The LLOD was defined as a signal-to-noise (S/N) ratio of 3:1, and the LLOQ was defined as an S/N ratio of 10. Intra- and inter-day precision were evaluated by replicative analysis of five sets of samples spiked with QC plasma samples with three concentrations of theophylline, dapsone and chlorzoxazone on the same day and on five consecutive days. Precision was denoted by the RSD. Accuracy was calculated by comparing the average measured concentration (Cmea) to the nominal concentration (Cnom) using the QC plasma samples with the three concentrations of theophylline, dapsone and chlorzoxazone and five replicates, and it was expressed as a percentage. The extraction recoveries of theophylline, dapsone and chlorzoxazone from plasma were determined using the QC samples with the three different concentrations of the probe drugs as described above. Twenty microliters of an internal standard was added to 100 μL blank plasma, and then the samples were extracted and analyzed according to the aforementioned procedure. For the reference material, the same concentrations of the standard solutions were injected directly into the HPLC system. The extraction recoveries of theophylline, dapsone and chlorzoxazone were calculated by comparing the mean peak areas (n=6 at each concentration) of the extracted QC samples with that of the unextracted standard solution containing equivalent amounts of ana-

Table 4. The statistical results for the pharmacokinetic parameters after administration of a single dose of CR or VCR (mean ± SE, n=6)

Analytes	Parameters	Control Group	CR Group	VCR Group
Theophylline	$T_{1/2}$ (h)	3.636 ± 1.00	3.956 ± 1.12	10.83 ± 5.66**
	CL/F (L/kg/min)	0.682 ± 0.26	0.650 ± 0.10	0.383 ± 0.11*
	AUC_{0-t} (μg·h/mL)	211.8 ± 54.65	216.0 ± 57.13	321.0 ± 101.5**
	$AUC_{0-\infty}$ (μg·h/mL)	225.7 ± 53.91	236.9 ± 46.02	437.4 ± 138.4**
	T_{max} (h)	2.208 ± 1.25	3.167 ± 1.84	5.667 ± 1.51**
	C_{max} (h)	30.02 ± 1.43	23.35 ± 2.40*	23.44 ± 4.77*
Dapsone	$T_{1/2}$ (h)	14.65 ± 5.51	13.44 ± 18.00	12.52 ± 4.20
	CL/F (L/kg/min)	0.765 ± 0.14	1.012 ± 0.29	1.479 ± 0.53*
	AUC_{0-t} (μg·h/mL)	128.6 ± 25.67	96.93 ± 18.65	66.06 ± 31.39**
	$AUC_{0-\infty}$ (μg·h/mL)	201.3 ± 34.50	179.2 ± 45.81	111.4 ± 35.52**
	T_{max} (h)	4.833 ± 2.21	3.917 ± 1.88	5.667 ± 2.00
	C_{max} (h)	8.008 ± 1.21	6.365 ± 1.05	4.481 ± 0.79
Chlorzoxazone	$T_{1/2}$ (h)	1.934 ± 0.58	1.531 ± 0.49	3.823 ± 1.28*
	CL/F (L/kg/min)	5.758 ± 1.26	5.606 ± 1.79	2.087 ± 0.76*
	AUC_{0-t} (μg·h/mL)	24.48 ± 6.90	25.72 ± 6.13	76.31 ± 29.07**
	$AUC_{0-\infty}$ (μg·h/mL)	27.23 ± 6.49	27.52 ± 6.10	80.16 ± 29.11**
	T_{max} (h)	0.833 ± 0.74	2.083 ± 0.49	5.167 ± 1.33**
	C_{max} (h)	7.434 ± 2.34	6.141 ± 1.32	7.852 ± 1.74

*P < 0.05 compared with the control group; **P < 0.01 compared with the control group.

lytes. The stabilities of theophylline, dapsone and chlorzoxazone were evaluated under conditions mimicking those of sample storage, with assessment of three replicate QC samples in each analysis. Freeze-thaw stability was determined after three freeze and thaw cycles. The QC samples were stored at -80°C for 30 d and were thawed unassisted at room temperature for 24 h.

Effects of CR and VCR on CYP1A2, CYP3A1 and CYP2E1 mRNA expression in rat liver

Sample collection (single dose): Nine rats were randomly divided into a CR group, VCR group and control group and orally administered a single 9 g/kg dose of CR, VCR and 0.5% CMC Na, respectively. The rats were sacrificed at 2.5 h after treatment. Then, liver samples were collected, immediately frozen and stored at -80°C.

Sample collection (multiple doses): Nine rats were randomly divided into a CR group, VCR group and control group and orally administered a single 9 g/kg dose of CR, VCR and 0.5% CMC-Na, respectively. After treatment for 7 consecutive days, the rats were sacrificed on the eighth day. Then, liver samples were collected, immediately frozen and stored at -80°C.

Total RNA isolation: Total RNA was isolated using SunShineBio™ Total RNA Extraction Reagent (Nanjing, Jiangsu, China) according to the manufacturer's instructions. The RNA concentration was determined, and the quality of the isolated RNA was assessed according to the 260/280 nm absorbance ratio (1.8-2.0 indicates a highly pure sample) [24].

Synthesis of cDNA: One microliter of RNA was added to a 20 μL reaction mixture for cDNA synthesis using RevertAid™ M-MuLV RT (Fermentas, Hanover, MD, USA) according to the manufacturer's instructions.

Polymerase chain reaction: Reactions were performed in a final volume of 20 μL containing IQ SYBR Green Supermix (10 μL), cDNA (2 μL), forward primers (2 μL), reverse primers (2 μL) and DEPC-treated water (4 μL). The PCR conditions were as follows: 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. The sequences of the primers used in the experiment are as follows:

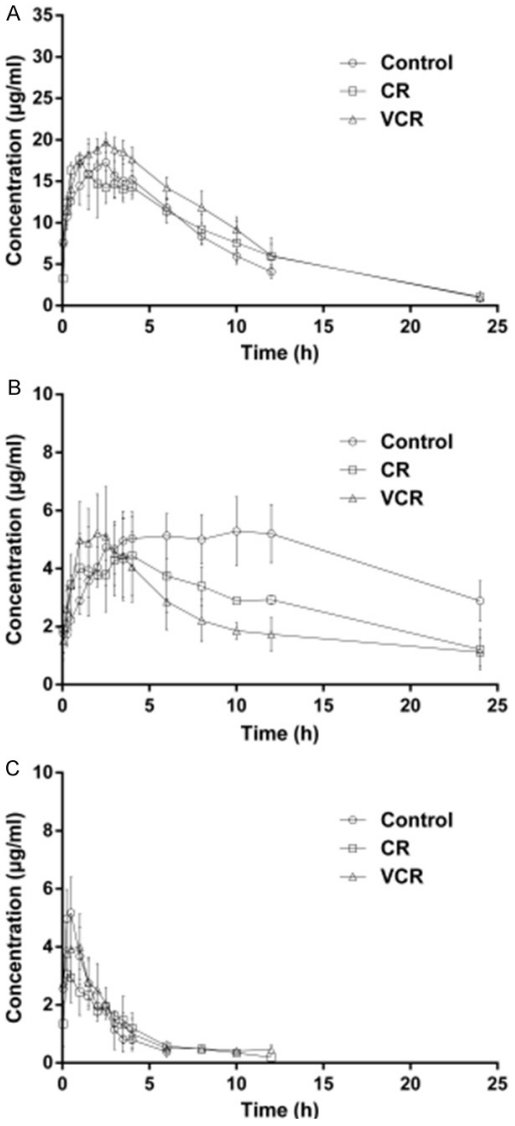

Figure 3. Time-concentration curves for theophylline (A), dapsone (B) and chlorzoxazone (C) after administration of multiple doses of CR or VCR (mean ± SE, n=6).

P450	5' Sense primer	3' Antisense primer	Fragment Size (bp)
CYP1A2	5'-GTCACCTCAGGGAATGCTGTG-3'	5'-GTTGACAATCTTCTCCTGAGG-3'	236
CYP3A1	5'-ATCCGATATGGAGATCAC-3'	5'-GAAGAAGTCCTTGTCTGC-3'	579
CYP2E1	5'-CTCCTCGTCATATCCATCTG-3'	5'-GCAGCCAATCAGAAATGTGG-3'	473

Statistical analysis

The concentrations of theophylline, dapsone and chlorzoxazone in all samples were calculated using the calibration curve. Pharmacokinetic parameters were computed using the DAS 2.0 software package (Mathematical Pharmacology Professional Committee of China, Shanghai, China). All data are presented as the mean ± SE.

Microsoft Excel was used for analyses conducted using the T-test, and a $P < 0.05$ was used to determine significant differences between two groups.

Results

Effects of CR and VCR on CYP1A2, CYP2E1 and CYP3A1 activities in rats

Under the chromatographic conditions of the cocktail experiment, three probe drugs, theophylline, dapsone and chlorzoxazone, and an internal standard, antipyrine, separated well, with retention times of 6.089, 12.532, 16.938 and 10.405 min, respectively (**Figure 1**). The results showed good specificity.

Each calibration curve was constructed by linear fitting with 6 non-zero concentration points with quantification ranges of 0.6528~32.64, 0.6522~32.61 and 0.3392~16.96 ng/mL for theophylline, dapsone and chlorzoxazone, respectively. The regression equations were A=0.1781 C-0.0676 (r=0.9952) for theophylline, A=0.2008 C-0.0736 (r=0.9958) for dapsone, and A=0.2777C-0.0243 (r=0.9985) for chlorzoxazone, where C is the analyte concentration in rat plasma, and A is the ratio of the analyte peak area to the internal standard peak area. The calibration curves showed good linear relationships for the three probe drugs. The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) for theophylline, dapsone and chlorzoxazone were 0.27, 0.10, and 0.14 μg/mL and 0.82, 0.41, and 0.42 μg/mL, respectively.

The precision of theophylline, dapsone and chlorzoxazone was calculated as the relative standard deviations (RSDs) at three concentrations, and it was lower than 15% for the intra-day and inter-day assays. In addition, the accuracy was within 15% of that of the quality control (QC) samples. The results demonstrated that the precision and accuracy of this method were acceptable. The details are provided in **Table 1**.

As shown in **Table 2**, the mean extraction recoveries of the three analytes at low, medium and high concentrations were between 72.06% and

Table 5. The statistical results for the pharmacokinetic parameters after administration of multiple doses of CR or VCR (mean ± SE, n=6)

Analytes	Parameters	Control Group	CR Group	VCR Group
Theophylline	$T_{1/2}$ (h)	4.086 ± 0.97	5.971 ± 2.73	5.285 ± 1.99
	CL/F (L/kg/min)	0.970 ± 0.08	0.815 ± 0.11*	0.742 ± 0.09*
	AUC_{0-t} (μg·h/mL)	130.6 ± 11.40	177.1 ± 17.80*	204.8 ± 10.11**,a
	$AUC_{0-\infty}$ (μg·h/mL)	155.5 ± 12.25	186.7 ± 23.70*	212.5 ± 12.85**,a
	T_{max} (h)	2.000 ± 0.32	2.000 ± 1.58	2.250 ± 0.61
	C_{max} (μg/mL)	17.02 ± 2.67	19.77 ± 6.64	19.67 ± 0.88
Dapsone	$T_{1/2}$ (h)	20.34 ± 10.21	11.62 ± 5.68	13.42 ± 6.28
	CL/F (L/kg/min)	0.921 ± 0.44	1.935 ± 0.88*	2.081 ± 0.30**
	AUC_{0-t} (μg·h/mL)	104.5 ± 26.33	67.44 ± 22.40**	50.34 ± 7.80**,a
	$AUC_{0-\infty}$ (μg·h/mL)	201.0 ± 101.3	90.52 ± 35.13**	73.38 ± 11.33**,a
	T_{max} (h)	8.000 ± 3.10	4.250 ± 4.05*	1.833 ± 0.61**
	C_{max} (μg/mL)	5.726 ± 1.53	5.604 ± 1.49	5.448 ± 1.58
Chlorzoxazone	$T_{1/2}$ (h)	1.946 ± 1.56	3.366 ± 2.69	3.850 ± 2.10
	CL/F (L/kg/min)	13.48 ± 2.86	12.04 ± 2.77	9.695 ± 1.74*
	AUC_{0-t} (μg·h/mL)	9.943 ± 2.26	11.09 ± 3.92	13.07 ± 3.38
	$AUC_{0-\infty}$ (μg·h/mL)	11.68 ± 3.16	13.07 ± 3.28	15.87 ± 2.71*
	T_{max} (h)	0.417 ± 0.13	1.083 ± 1.01	0.750 ± 0.27*
	C_{max} (μg/mL)	5.290 ± 1.12	3.362 ± 0.71	4.156 ± 1.16

*P < 0.05 compared with the control group; **P < 0.01 compared with the control group; aP < 0.05 compared with the CR group.

83.63%, 79.52% and 92.44%, and 91.69% and 97.23%, respectively.

The experiment investigated the stabilities of short-term, long-term and freeze-thaw samples. The results demonstrated that no significant degradation of theophylline, dapsone or chlorzoxazone occurred in the plasma under our experimental conditions (**Table 3**).

A developed and validated HPLC method was used to determine the levels of the three probe drugs (theophylline, dapsone and chlorzoxazone) in rat plasma after administration of single (**Figure 2**; **Table 4**) and multiple (**Figure 3**; **Table 5**) doses of the CR and VCR extracts.

Table 4 shows that after the single-dose treatment, the pharmacokinetic parameters did not significantly differ between the CR and control groups for theophylline, dapsone or chlorzoxazone. The $T_{1/2}$, T_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values for theophylline and chlorzoxazone were increased and the CL/F values for theophylline and chlorzoxazone were decreased in the VCR group compared with those in the control group. In addition, the AUC_{0-t} and $AUC_{0-\infty}$ values for dapsone were decreased, and the CL/F value was increased.

These findings suggest that a single dose of CR has no significant effects on the CYP1A2, CYP3A1 or CYP2E1 CYP450 isoform. After administration of a single dose of VCR, CYP1A2 and CYP2E1 enzymatic activities were inhibited, and CYP3A1 activity was induced.

Table 5 shows that after treatment with multiple doses, the AUC_{0-t} and $AUC_{0-\infty}$ values for theophylline were increased in the CR and VCR groups and the CL/F value for theophylline was decreased compared with those in the control group. In addition, the AUC_{0-t} and $AUC_{0-\infty}$ values for dapsone were decreased in the CR and VCR groups, and the CL/F value for dapsone was increased. Further, the $AUC_{0-\infty}$ value for chlorzoxazone was increased in the VCR group, and the CL/F value for chlorzoxazone was decreased. No significant differences in these values for chlorzoxazone were detected between the CR and control groups. Finally, the VCR group had higher AUC_{0-t} and $AUC_{0-\infty}$ values for theophylline and lower AUC_{0-t} and $AUC_{0-\infty}$ values for dapsone compared with the CR group.

These results suggest that both CR and VCR inhibit CYP1A2 enzymatic activity and induce CYP3A1 activity. In addition, they indicate that the inhibitory and inducing effects of VCR are

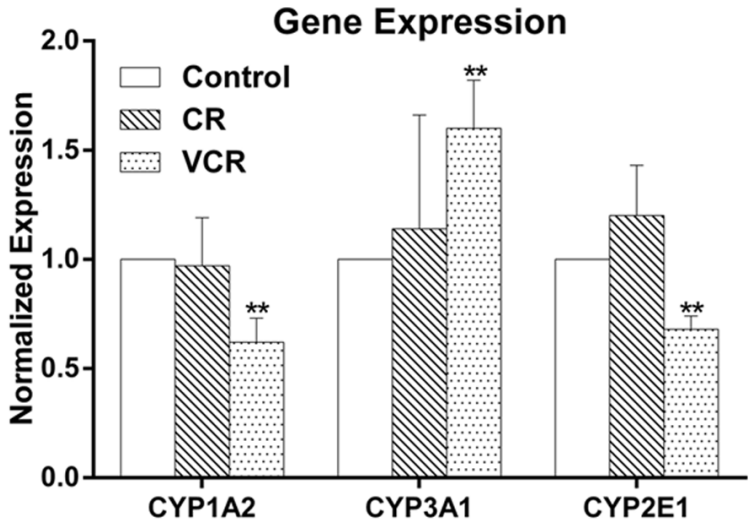

Figure 4. Effects of CR or VCR on CYP1A2, CYP3A1 and CYP2E1 mRNA expression in rat liver after administration of a single dose (mean ± SE, n=3). *P < 0.05 compared with the control group; **P < 0.01 compared with the control group.

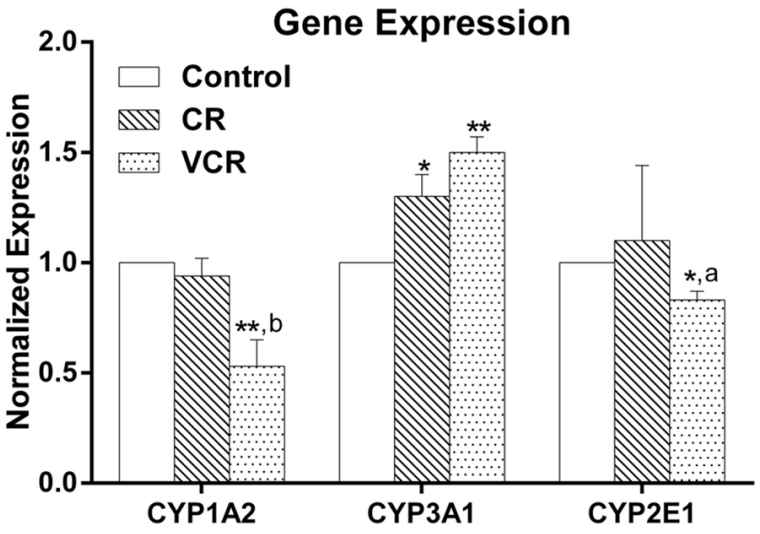

Figure 5. Effects of CR or VCR on CYP1A2, CYP3A1 and CYP2E1 mRNA expression in rat liver after administration of multiple doses (mean ± SE, n=3). *P < 0.05 compared with the control group; **P < 0.01 compared with the control group; [a]P < 0.05 compared with the CR group; [b]P <0.01 compared with the CR group.

stronger than those of CR and that VCR inhibits CYP2E1 enzymatic activity, while CR has no effect on this activity.

Effects of CR and VCR on CYP1A2, CYP3A1 and CYP2E1 mRNA expression in rat liver

Figures 4 and **5** show the effects of CR and VCR on CYP1A2, CYP3A1 and CYP2E1 mRNA expression in the rat liver following administration of a single dose or multiple doses.

Figure 4 shows that after treatment with a single dose, no significant differences in CYP1A2, CYP2E1 or CYP3A1 mRNA expression in the rat liver were detected between the CR and control groups (P > 0.05). Further, the expression of CYP1A2 and CYP2E1 was significantly lower (P < 0.05) and that of CYP3A1 was significantly higher (P < 0.05) in the VCR group than in the control group.

In general, CR did not significantly affect CYP1A2, CYP3A1 or CYP2E1 mRNA expression. However, administration of a single dose of the VCR extract decreased CYP1A2 and CYP2E1 mRNA expression and increased CYP3A1 mRNA expression.

Figure 5 shows that after treatment with multiple doses, no changes in CYP1A2 or CYP2E1 mRNA expression were detected (P > 0.05) and that CYP3A1 mRNA expression was significantly increased (P < 0.05) in the CR group compared with the control group. Further, the mRNA expression of CYP1A2 and CYP2E1 was significantly decreased (P < 0.05) and that of CYP3A1 was significantly increased (P < 0.05) in the VCR group compared with the control group; in addition, CYP1A2 and CYP2E1 mRNA expression was significantly decreased (P < 0.05).

In general, administration of multiple doses of the CR extract induced CYP3A1 enzymatic activity. In addition, administration of multiple doses of the CR and VCR extracts resulted in decreased CYP1A2 and CYP2E1 mRNA expre-

ssion and increased CYP3A1 enzymatic activity. The mRNA expression results were consistent with the pharmacokinetic results.

Conclusions

As DDIs are closely associated with the safety of patients and effectiveness of drugs, they have always been a major concern of clinicians and patients in the clinical setting. With the increasingly widespread use of herbal medicines in combination with synthetic drugs, the potential for clinical herb-drug interactions is becoming a common problem [25, 26]. Inhibition and induction of CYP450 enzymes are likely the most common causes of documented interactions [27]. Several herbs, including garlic, milk thistle, Ginkgo biloba, and St. John's wort, have the potential to inhibit or induce human drug-metabolizing enzymes, especially CYP450 enzymes [28]. Inhibition of drug metabolism by competition for the same enzyme may result in undesirable elevations in plasma concentrations of drugs, which can lead to serious adverse effects and toxicity. Europe and the United States require the inclusion of drug screening and metabolic research based on the CYP450 system in new drug evaluations [29]

CR and VCR extracts are two clinically used forms of Curcumae Rhizoma recorded in the Chinese Pharmacopoeia [1]. According to the traditional theory of Chinese medicine, the vinegar-processing method alleviates the effects of the drug properties and components with direct actions on the liver; in addition, the pain-alleviating, blood-activating and stasis-eliminating activities of CR can be enhanced after processing with rice vinegar [30]. Modern pharmacological studies published to date have demonstrated that CR and VCR increase the pain threshold of mice and decrease the thrombocyte adherence rate in blood stasis model animals and that VCR has higher activity than CR [31]. These variations in the pharmacological effects may be attributed to differences in the chemical compositions. Studies have shown that the total volatile oil content is lower in VCR than in CR samples. These studies reported the detection of two constituents, 4-methylethyl-benzoic acid and 2-methyl-5-(1-methylethenyl)-cyclohexanone, and the disappearance of other components following processing with vinegar. The total curcumin concentration in CR does not substantially change after processing with vinegar [32]. However, no reports concerning the possible differential effects of CR and VCR on CYP450 activities are currently available. In view of the well-known effects of CR and VCR, we aimed to investigate their influences on metabolism and mRNA expression of the CYP1A2, CYP3A1 and CYP2E1 enzymes in rats. We used probe cocktail and real-time RT-PCR methods to predict interactions between the herb and drugs.

The results of this study have indicated that long-term treatment with CR extract inhibits CYP1A2 enzymatic activity and induces CYP3A1 activity. In addition, VCR inhibits CYP1A2 and CYP2E1 activities and induces CYP3A1 activity after administration of both a single dose and multiple doses, and these effects occur in a time-dependent manner. This is the first study conducted using the cocktail method and RT-PCR to assesses the activities and mRNA expression of the CYP1A2, CYP3A1 and CYP2E1 enzymes after oral administration of CR and VCR to rats. The results provide a scientific basis for the safe clinical application of CR or VCR in combination with other drugs, potentially preventing possible side effects induced by herb-drug interactions. Further study is required to determine whether and how CR and VCR affect the activities of CYP450 enzymes.

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Disclosure of conflict of interest

None.

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