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Effects of Ougan juice on P450 activities using a cocktail method

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Ougan (*Citrus suavissima Hort. ex Tanaka*) is an important domesticated fruit which is used medicinally in China. To date, a number of methods for its identification and chemical analysis have been studied. However, the effects of Ougan juice on CYP isozymes have not been reported. Therefore, the objective of our study was to evaluate the potential effects of Ougan juice on the CYP isozymes CYP1A2, CYP2C9, CYP2C19 and CYP2D6 in rats using a cocktail approach involving the probe drugs phenacetin, tolbutamide, omeprazole and dextromethorphan. These four probe drugs were simultaneously administered to rats after single and multiple dosing of Ougan juice by gastric irrigation. The pharmacokinetics of the probes in the plasma were simultaneously determined by HPLC-MS. The main pharmacokinetic parameters of the four probe drugs were not significantly different in rats after single dose of Ougan juice. The $t_{1/2}$ and $AUC_{(0-\infty)}$ of phenacetin and omeprazole increased significantly and their CL_z decreased markedly after multiple dosing of Ougan juice. However, the $t_{1/2}$ of tolbutamide decreased notably, while the $t_{1/2}$ of dextromethorphan was not changed. The findings of this study suggest that a single administration of Ougan juice had little effect on P450 activities while multiple administration of Ougan juice tended to inhibit CYP1A2 and CYP2C19 and induce CYP2C9, but did not influence CYP2D6.

1. Introduction

The cytochrome P450 (CYP) system comprises primarily drug-metabolizing enzymes in humans (Rendic and Di Carlo 1997) and plays an important role in the biotransformation of numerous endogenous and exogenous compounds and xenobiotics (Nebert and Russell 2002). In humans, the major CYP isozymes found in connection with the metabolism of more than 90% of marketed drugs are CYP1A2, CYP2A9, CYP2A19, CYP2D6 and CYP3A (Chang and Kam 1999; Rodrigues 1999). Specific probe drugs have been widely used to determine the real-time activity of the CYP isozymes (Streetman et al. 2000a). Recently, a cocktail approach, which detects the activity of several CYP isozymes simultaneously after the concurrent administration of several CYP-specific probes, has shown great advantages in assessing the activity of various drug-metabolizing enzymes in one study (Chainuvati et al. 2003; Christensen et al. 2003; Frye et al. 1997; Streetman et al. 2000a, b; Tanaka et al. 2003; Zhu et al. 2001). As a result, the cocktails developed have been suggested for predicting the effect of a drug on the activity of CYP isozymes prior to more definitive single-probe substrate studies.
Ougan (*Citrus suavissima Hort. ex Tanaka*), a mandarin cultivar, belongs to the rutaceae family of citrus plants and is a traditional fruit, characteristic of Wenzhou city, Zhejiang province, China. Ougan has a history of cultivation of at least 1,000 years since the Song Dynasty (Lin and Xu 2007). Due to the high contents of metals and essential amino acids in Ougan (Chen et al. 2009), it has a good effect in the treatment of cough, measles, fever and other febrile diseases. However, no research into the influence of Ougan on the activity of the principal cytochrome P450 enzymes involved in drug metabolism has not been done so far. In this present study, we use a established probe cocktail consisting of phenacetin, tolbutamide, omeprazole and dextromethorphan to investigate the effects of Ougan on cytochrome P450 activities, including CYP1A2, CYP2C9, CYP2C19 and CYP2D6, in rats.

2. Investigations and results

2.1. Validation of the cocktail method

Under the conditions described in the experimental section, a reliable liquid chromatography-mass spectrometry method has been developed for the simultaneous evaluation of the activities of four CYP isozymes (CYP1A2, CYP2C9, CYP2C19 and CYP2D6) in rats. The calibration curves were linear over the concentration range 5.0–1000 ng/mL in rat plasma (Table 1). The results of linear regression analysis show that the correlation coefficients of the calibration curves for all sample types were above 0.996. The limit of quantitation (LOQ) was defined as the lowest concentration of the sample resulting in a signal-to-noise ratio of 10:1. Intra-day and inter-day precision and accuracy were determined by analyzing quality control (QC) samples ($n = 6$) on three different days. Recoveries at three QC levels (10, 80, and 800 ng/mL) were determined by comparing the peak areas of extracted plasma standards with the peak areas of post-extraction plasma blanks spiked with equivalent concentrations using six replicates. Assay performance data are presented in Table 2. The result of the chromatographic validation showed that the assay methods were suitable for this study.

Table 1: Regression equation, correlation coefficient and LOQ for probe drugs (Mean ± SD, n = 3)

Probe Drug	Regression Equation	Correlation Coefficient (ng/mL)	LOQ (ng/mL)
Phenacetin	$Y = (0.00335 \pm 0.000342)C + (0.027775 \pm 0.016827)$	$r = 0.99775 \pm 0.00136$	5.0
Tolbutamide	$Y = (0.0035 \pm 0.000432)C + (0.11405 \pm 0.179651)$	$r = 0.99625 \pm 0.002583$	5.0
Omeprazole	$Y = (0.00395 \pm 0.000569)C + (0.06663 \pm 0.028635)$	$r = 0.99795 \pm 0.001823$	5.0
Dextromethorphan	$Y = (0.023575 \pm 0.002629)C + (0.035475 \pm 0.029559)$	$r = 0.99755 \pm 0.002704$	5.0

(y = peak area ratio of each probe drug versus IS; c = concentration of each probe drug)

2.2. In vivo studies

After introducing Ougan juice into the stomach, the pharmacokinetics of the four probe drugs were determined simultaneously by HPLC-MS. The mean time-concentration curves of the four probe drugs are shown in Fig. 1 and the pharmacokinetic parameters are given in Table 3.

Single doses of Ougan juice did not influence the plasma concentrations and the corresponding pharmacokinetic parameters of the four probe drugs. In multiple doses, however, Ougan juice increased the $t_{1/2}$ and area under the curve of phenacetin, and the corresponding decrease in CL_z suggests that Ougan juice could inhibit CYP1A2. Similar results were found with the probe drug omeprazole. The $t_{1/2}$ and AUC of omeprazole were significantly increased but its CL_z was markedly decreased, demonstrating that Ougan juice can also inhibit the activity of CYP2C19. In contrast, the $t_{1/2z}$ of tolbutamide notably decreased, which is accounted for by the induction of CYP2C9 by Ougan juice. The pharmacokinetics of dextromethorphan showed no statistically significant difference from control rats. This means the activity of CYP2D6 is little altered by Ougan juice.

3. Discussion

The cocktail approach involving multiple probe drugs has been widely used for phenotyping various individual cytochrome P450 activities. A number of phenotyping cocktails have previously been proposed and evaluated (Liu et al. 2009; Ryu et al. 2007; Yin et al. 2004; Zhang et al. 2008). The use of phenotyping cocktails for the simultaneous administration of multiple probes of drug-metabolizing enzymes offers several important advantages and this approach has been applied extensively to the study of drug influences on P450 activities and potential drug-drug interactions (Bruce et al. 2001; Chow et al. 2006; Gao et al. 2007; Krosser et al. 2006; Smith et al. 2007; Tang et al. 2008; Tomalik-Scharte et al. 2005; Yao et al. 2007; Zhang et al. 2011). A new four-drug cocktail including phenacetin, tolbutamide, omeprazole and dextromethorphan has previously been established in our laboratory to study the influence of natural products and new chemical drugs on the metabolizing activity of CYP1A2, CYP2C9, CYP2C19 and CYP2D6, respectively. I has been demonstrated that the ability of the four probe drugs to determine the activity of each enzyme is not affected by potential analytical interference from the co-administered probe drugs or their metabolites.

The present study represents the first attempt to investigate the possible effects of single and multiple doses of Ougan juice on the induction or inhibition of cytochrome P450 activities in rats. This study also provides an opportunity to help find the metabolites of Ougan juice. Pharmacokinetic parameters determined *in vivo* reflect the hepatic metabolism of Ougan juice more reliably than an *in vitro* study. Through this *in vivo* study, we found that single doses of Ougan juice influenced neither the plasma concentration nor the associated pharmacokinetic parameters of the four probe drugs, and had very little or no affect on P450 activities. But an obvious difference was found with multiple doses, in that Ougan juice decreased the activity of CYP 1A2 and CYP2C19, prolonging the $t_{1/2z}$ of phenacetin and omeprazole. On the other hand, Ougan juice increased the activity of CYP2C9 and had no obvious effect on the activity of CYP2D6 in rats. The mechanism of these effects of Ougan juice on the activity of P450 isozymes warrants further research.

These regulating effects might have promising clinical significance, since CYP1A2 accounts for approximately 13% of the total CYP content in human liver (Shimada et al. 1994). This enzyme is involved in the metabolic transformation of a number of clinically important drugs and, especially, procarcinogens including aromatics, heterocyclic amines and PAHs (Eaton et al. 1995). Like CYP1A2, CYP2C19 is also responsible for the metabolism of approximately 10% of compounds, including omeprazole, lansoprazole, imipramine and S-mephenytoin (Zhou et al. 2009a). The inhibition of CYP1A2 and 2C19 must be paid due attention on account of changes in the pharmacokinetics of co-administered drugs, especially substrates of CYP1A2 or 2C19. Due to its inhibitory effects, it is most important to evaluate the clinical efficacy and safety of Ougan juice. CYP2C9

Table 2: Precision, accuracy and recovery of probe drugs in rat plasma (Mean ± SD, n = 6)

Probe Drug	Concentration (ng/mL)	RSD (%)		RE (%)		Recovery (%)
		Intra-day	Inter-day	Intra-day	Inter-day	
Phenacetin	10	9.60	9.05	11.60	12.60	93.23 ± 3.33
	100	7.81	8.79	8.36	3.86	85.13 ± 2.08
	800	6.09	6.64	5.04	2.46	93.67 ± 1.02
Tolbutamide	10	7.50	8.26	8.70	9.21	85.95 ± 3.84
	100	5.39	6.94	6.53	8.74	82.84 ± 7.56
	800	6.53	7.66	3.69	5.68	83.74 ± 1.12
Omeprazole	10	7.68	8.50	7.30	10.09	89.87 ± 9.02
	100	6.93	7.03	−4.68	−5.89	90.42 ± 2.89
	800	4.75	5.07	−5.84	−3.34	99.34 ± 0.03
Dextromethorphan	10	8.36	9.46	7.60	9.86	84.34 ± 3.21
	100	5.61	8.90	4.64	6.76	80.65 ± 3.12
	800	4.53	6.36	−2.3	2.93	99.01 ± 3.01

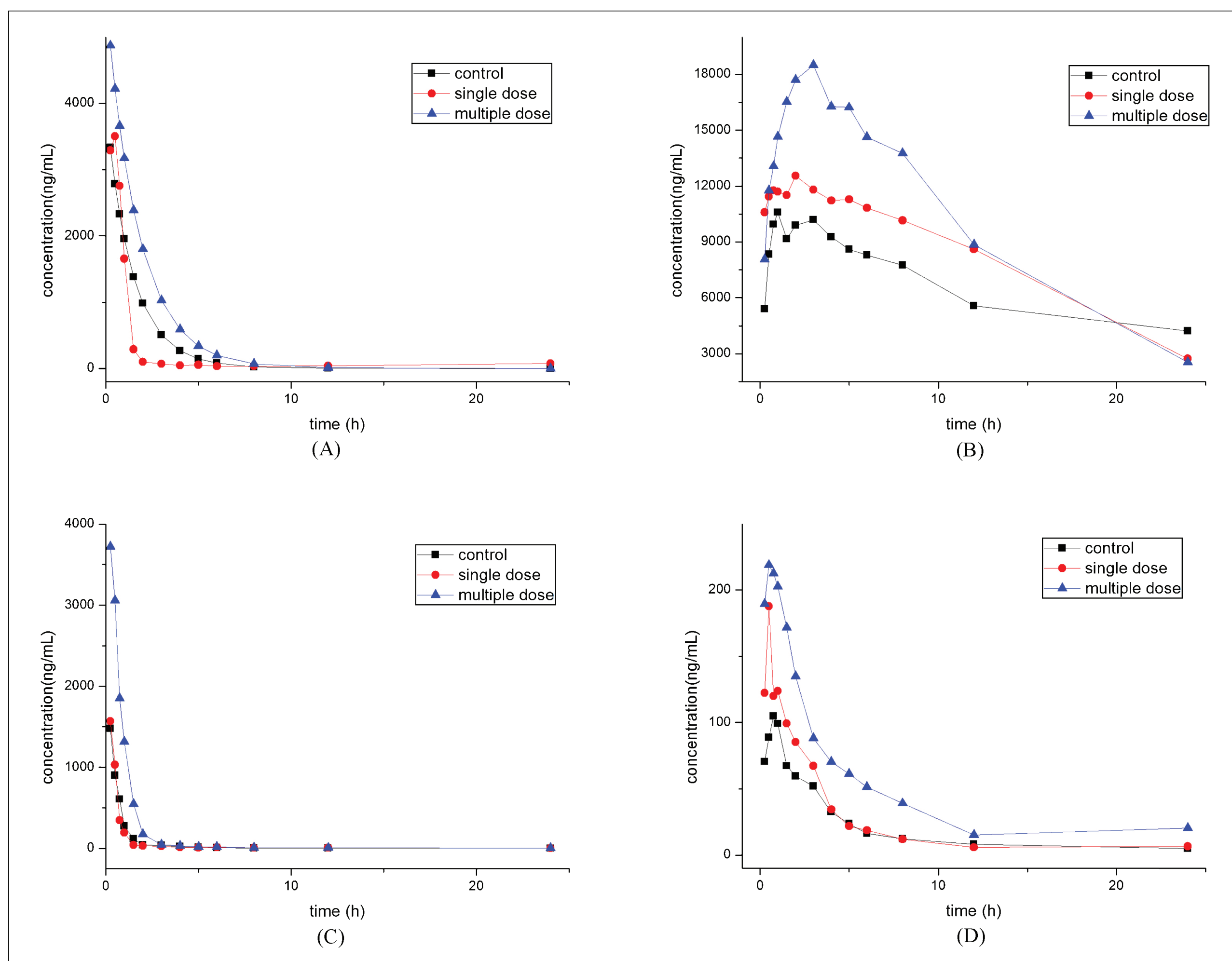

Fig. 1: Time concentration curves of probe drugs after single and multiple dose of Ougan juice. A: Phenacetin; B: Tolbutamide; C: Omeprazole; D: Dextromethorphan

is one of the most abundant CYP enzymes in the human liver, forming 20% of the total hepatic CYP content and metabolizes about 15% of clinical drugs (Zhou et al. 2009a). Ougan juice can increase the activity of CYP2C9 in multiple doses, showing why Ougan juice improves liver metabolism of drugs, benefits the body by promoting excretion of endogenous substances and

Table 3: Effects of Ougan juice on pharmacokinetic parameters of four probe drugs (Mean ± SD, n = 6)

Probe Drug	Parameter	Control	Single dose	Multiple dose
Phenacetin	$t_{1/2}$ (h)	5.20 ± 2.72	6.06 ± 1.50	18.03 ± 8.27 [a]
	C_{max} (ng/mL)	4618.1 ± 1521.0	3502.0 ± 971.4	5691.6 ± 1279.1
	$AUC_{(0-\infty)}$ (min ng/mL)	4570.7 ± 938.2	4751.0 ± 1519.7	7337.5 ± 2011.7[a]
	V_z (L/kg)	25.09 ± 13.73	29.23 ± 8.86	52.45 ± 15.44 [b]
	CL (L/h/kg)	3.38 ± 0.63	3.45 ± 1.23	2.16 ± 0.55[a]
Tolbutamide	$t_{1/2}$ (h)	21.99 ± 8.14	7.10 ± 0.95[a]	7.26 ± 1.91[a]
	C_{max} (ng/mL)	11506.2 ± 3080.3	12284.8 ± 1745.2	19026.5 ± 6633.6 [a]
	$AUC_{(0-\infty)}$ (min ng/mL)	309549.6 ± 116449.9	190579.7 ± 27612.7	261997.5 ± 68263.0
	V_z (L/kg)	0.55 ± 0.13	0.27 ± 0.05[a]	0.23 ± 0.15[a]
	CL (L/h/kg)	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01
Omeprazole	$t_{1/2}$ (h)	4.44 ± 2.60	10.24 ± 5.10	9.12 ± 3.68 [a]
	C_{max} (ng/mL)	1548.8 ± 486.9	1566.5 ± 356.4	3720.4 ± 859.3 [a]
	$AUC_{(0-\infty)}$ (min ng/mL)	1064.3 ± 116.8	1079.0 ± 311.0	3274.2 ± 882.0 [a]
	V_z (L/kg)	91.86 ± 56.50	221.04 ± 133.73	65.01 ± 32.97
	CL (L/h/kg)	14.23 ± 1.64	14.78 ± 4.07	4.79 ± 1.04 [b]
Dextromethorphan	$t_{1/2}$ (h)	10.96 ± 5.24	8.02 ± 5.51	4.82 ± 0.81
	C_{max} (ng/mL)	116.3 ± 42.5	187.7 ± 11.6	232.0 ± 137.3
	$AUC_{(0-\infty)}$ (min ng/mL)	508.1 ± 116.8	548.5 ± 277.9	1107.8 ± 738.6
	V_z (L/kg)	519.2 ± 365.6	429.16 ± 369.80	122.33 ± 70.77
	CL (L/h/kg)	30.94 ± 8.37	36.06 ± 25.79	17.80 ± 9.43

[a] $P < 0.05$; [b] $P < 0.01$ vs control

exogenous compounds, and this also helps to explain its antitoxicity mechanism. In the present research, Ougan juice was not found to influence the activity of CYP2D6 which is the major isoenzyme in the liver (Zhou et al. 2009b).

In summary, the current pharmacokinetic approach of a cocktail of probe drugs allowed the effects of Ougan juice on P450 activities in rats to be systemically investigated. The results suggested that single doses of Ougan juice did not affect the plasma concentrations of the four probe drugs and their corresponding pharmacokinetic parameters. But in multiple doses Ougan juice could inhibit CYP1A2 and CYP2C19, and induce CYP2C9 although it had no effect on CYP2D6. The results will help our understanding the induction or inhibition effects of Ougan juice on CYP1A2, CYP2C9, CYP2C19 and CYP2D6, as well as evaluating the drug-drug interactions of Ougan juice with other drugs.

4. Experimental

4.1. *Chemicals and reagents*

Phenacetin, tolbutamide, omeprazole, dextromethorphan and the internal standard carbamazepine (IS) were purchased from Sigma-Aldrich Company (St. Louis, USA). HPLC grade acetonitrile and methanol were from Merck Company (Darmstadt, Germany). All other chemicals were of analytical grade. Ultra-pure water (resistance > 18 mΩ) was prepared by a Millipore Milli-Q purification system (Bedford, USA).

Stock solutions of 1.0 mg/mL each of phenacetin, tolbutamide, omeprazole, dextromethorphan and IS were prepared in methanol. The working standard solutions of each analyte were prepared by serial dilution of the stock solution with methanol.

4.2. *Animals*

Wistar rats with body weights of 180 ± 30 g were provided by the Animal Care and Use Committee of Wenzhou Medical College. They were housed in cages at 20–24 °C and allowed free access to regular rodent diet and water.

4.3. *Apparatus and chromatographic conditions*

All analyses were performed with a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump, a degasser, an autosampler, a thermostatted column compartment, and a Bruker Esquire HCT mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ion source and controlled by ChemStation software.

Chromatographic separation was achieved on a 150 mm × 2.1 mm, 3.5 μm particle, Agilent Zorbax SB-C18 column at 30 °C. A gradient elution programme was conducted for chromatographic separation with mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile) as follows: 0–1.5 min (10–85% B), 1.5–6.0 min (85–85% B), 6.0–7.0 min (85–10% B), 7.0–10.0 min (10–10% B). The flow rate was 0.4 mL/min.

The quantification was performed by the peak-area method. The determination of target ions was performed in SIM mode (m/z 180 for phenacetin, m/z 271 for tolbutamide, m/z 346 for omeprazole, m/z 272 for dextromethorphan and m/z 237 for IS) with a positive ion electrospray ionization interface. Drying gas flow was set to 6 L/min and temperature to 350 °C. Nebuliser pressure and capillary voltage of the system were adjusted to 20 psi and 3,500 V, respectively.

4.4. *Preparation of calibration standards and limit of quantitation*

The calibration standards were prepared by spiking blank rat plasma with appropriate amounts of phenacetin, tolbutamide, omeprazole and dextromethorphan. Calibration plots for each probe drug were constructed in the range 5–1,000 ng/mL for plasma (5, 10, 20, 50, 100, 200, 500, 1,000 ng/mL).

4.5. *Sample preparation*

In a 1.5 mL centrifuge tube, an aliquot of 10 μL of the internal standard working solution (1.0 μg/mL) was added to 0.1 mL of collected plasma sample followed by the addition of 0.2 mL of acetonitrile. After the tube was vortex-mixed for 1.0 min, the sample was centrifuged at 15,000 rpm for 10 min. The supernatant (10 μL) was injected into the LC-MS system for analysis. The standards were prepared in the same way.

4.6. *Effects of Ougan juice on P450 activities*

Twelve male Wistar rats (180 ± 30 g) were randomly divided into two groups, a control group and a test group. Ougan juice was administered orally at a dose of 0.5 mg/kg for the test group and sodium chloride was administered in the same way. Three minutes after administration of Ougan juice, rats of the two groups were administered by gastric irrigation doses of 15, 5, 15 and 15 mg/kg of phenacetin, tolbutamide, omeprazole and dextromethorphan, respectively.

Blood samples of 0.3 mL were obtained through the tail vein into heparinized 1.5 mL polythene tubes at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, 12 and 24 h after the administration of the probe drugs. The samples were immediately centrifuged at 5,000 rpm for 10 min, and then 100 μL plasma were transferred to another tube and stored at −20 °C until analysis.

4.6.2 Effects of multiple dosage of Ougan juice on P450 activities

In this procedure, six male Wistar rats (180 ± 30 g) were selected for 14-day oral administration of Ougan juice (0.5 mg/kg, twice daily). Dosage of the probe drugs was the same as for the single dose.

4.7. *Statistical analysis*

The concentration-time profile of each probe drug was analyzed by DAS software (Version 2.0, Wenzhou Medical College, China) and statistical analyses were tested by ANOVA using SPSS (Version 13.0, Wenzhou Medical College, China). A value of $P < 0.05$ was considered to be statistically significant.

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