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Construction of a model to estimate the CYP3A inhibitory effect of grapefruit juice

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Grapefruit juice (GFJ) is known to affect the pharmacokinetics of a variety of drugs administered concomitantly and this is due to inhibition of intestinal CYP3A, a barrier protein for drug absorption. Some compounds such as furanocoumarin derivatives have been reported as inhibitors of the enzyme. On the other hand, inhibitory potentials of GFJ on CYP3A-oxidation activities differ widely between brands of juices. Information on the percentage contributed by ingredients in GFJ is also limited. Therefore, construction of prediction models for the CYP3A inhibitory potentials of GFJ brands was attempted by using concentrations of ingredients in GFJ. Concentrations of bergaptol, bergamottin, 6', 7'-dihydroxybergamottin, naringin, and naringenin in 23 kinds of GFJ were determined with high-performance liquid chromatography (HPLC). Furthermore, inhibitory effects on CYP3A activity were measured based on the initial rate for testosterone 6β-hydroxylation in the presence of each GFJ. Results of multi-regression analyses between the ingredients and the enzymatic inhibitory effects revealed that concentrations of bergamottin, 6', 7'-dihydroxybergamottin, and naringin were significant variables for CYP3A inhibition of GFJ. According to the standard partial regression coefficient for each explanatory variable, bergamottin and 6',7'-dihydroxybergamottin are the most important factors for inhibition. The multiple correlation coefficient (R) and the multiple correlation coefficient with leave-one-out cross validation (Q) of the model equation were 0.94 and 0.91, respectively. These results suggest that the concentrations of ingredients can explain most variances of inhibitory effects among brands. This model may be a useful method for the prediction of the GFJ interaction potential.

1. Introduction

Grapefruit juice (GFJ) has been reported to cause pharmaceutical interactions that lead to significant increases in drug concentrations in the systemic circulation (Bailey 1989, 1991). The interactions are able to trigger adverse effects of the drugs as their potency is made higher than that of the normal prescription (Lundahl 1995). The inhibitory effects of GFJ on intestinal CYP3A enzymes (Lown 1997) are related to the barrier function for the absorption of low-molecular compounds such as drugs (Obach 2001). When first discovered, naringin (NG), a high concentration ingredient in GFJ (Kane and Lipsky 2000), and naringenin, an aglycone of naringin, were considered as the candidates that are most responsible for the interactions (Guengerich and Kim 1990; Fuhr 1993). The discovery of these components indicated CYP3A inhibition in *in vitro* experiments (Guengerich and Kim 1990). However, studies on treatments with drugs combined with NG revealed that it does not contribute to the interactions (Bailey 1993). Currently, furanocoumarin derivatives such as bergamottin (BG) (He 1998; Eagling 1999; Malhotra 2001; Mohri and Uesawa 2001; Goosen 2004; Paine 2004; Girennavar 2006), 6',7'-dihydroxybergamottin (DHB) (Eagling et al. 1999; Malhotra et al. 2001; Paine et al. 2004; Girennavar et al. 2006), and paradisins (Tassaneeyakul 2000; Girennavar et al. 2006) are putative ingredients implicated in the interactions. The enzymatic inhibitory effects of these ingredients have been studied. However, the contributing rate of each derivative in the pharmaceutical interaction or CYP3A inhibitory effect is still being debated. Estimation of the amount of contribution to the inhibitory effect by the purified ingredients might be difficult because concentrations of the furanocoumarins in GFJ vary with the brand of juice (Uesawa 2008b; Uesawa and Mohri 2008). While it is considered that estimation of the interaction potential on each GFJ brands is useful to select drinkable brands for patients with pharmaceutical treatments, complexity of interaction mechanisms with plural causative ingredients makes the estimation difficult. Therefore, we investigated the relationships between CYP3A inhibitory effects in a variety of GFJs and the concentrations of the ingredients to construct a prediction model for the interaction potentials of GFJ.

2. Investigations and results

The concentrations of bergaptol, DHB, BG, NG, and naringenin in GFJ used in this study were 31.6 ± 26.5, 4.97 ± 4.48, 10.4 ± 7.4, 364 ± 97, and 1.37 ± 1.05 μM (mean ± S.D.),

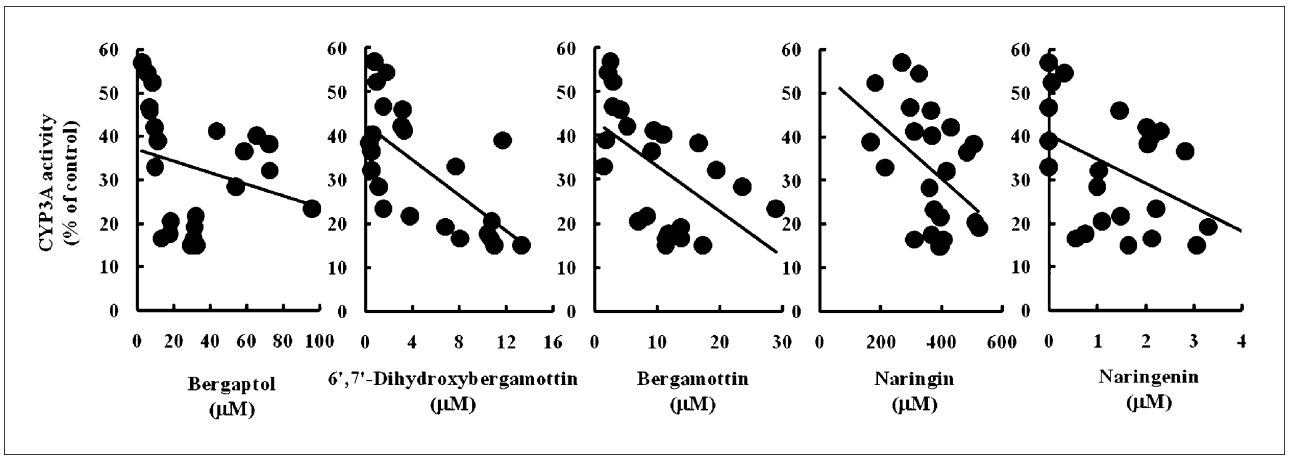

Fig. 1: Plots of concentrations of ingredients vs. observed CYP3A activities (% of control) with 23 kinds of GFJ.

Table: Simple linear regression between concentrations of ingredients vs. observed CYP3A activities (% of control) with 23 kinds of GFJ and their respective statistical parameters

Component	n	r	Regression coefficient	Intercept	F	RMSE	p
Bergaptol	23	−2.262	−0.134 ± 0.108	36.9 ± 4.4	1.55	13.4	0.226
6',7'-Dihydroxybergamottin	23	−0.670	−2.03 ± 0.49	42.8 ± 3.2	17.1	10.3	0.001
Bergamottin	23	−0.561	−1.03 ± 0.33	43.4 ± 4.2	9.65	11.5	0.005
Naringin	23	−0.441	−0.0617 ± 0.0274	55.2 ± 10.3	5.07	12.4	0.035
Naringenin	23	−0.429	−5.55 ± 2.55	40.3 ± 4.4	4.73	12.5	0.041

respectively. Addition of the juice to the reaction mixtures reduced human liver microsomal CYP3A activities to 32.7% of the control. The residual activities underwent a lot of changes (from 14.9% to 56.9% of the control, CV value: 41.6%) depending on the GFJ sample. The relationship between the concentration of 5 kinds of ingredients present in GFJ and their residual activities on CYP3A were studied to investigate the cause of the variability. Fig. 1 shows stacker plots between bergaptol, DHB, BG, NG, and naringenin concentrations and the remaining CYP3A activities in the 23 kinds of GFJ. All the ingredients except bergaptol showed significant negative relationships for the residual activities (Table). Thus, multiple linear regression analysis was performed to estimate the contribution ratios of the GFJ-ingredients on the inhibitory effects on CYP3A activity. Concentrations of the 5 ingredients as well as CYP3A activities were used in the analysis as the explanatory and an objective variable, respectively. A multiple regression model where concentrations of DHB, BG, and NG were used as the significant variables was constructed (Fig. 2). The model was validated for its normality and robustness by leave-one-out cross validation and regression diagnostics with a normal residual plot and sufficiently-low values of Variance Inflation Factor (VIF) (under 1.3). In other words, the multiple regression model was as follows:

$$\text{Activity} = (65.5 \pm 4.5) - (2.18 \pm 0.25)\text{DHB} - (0.936 \pm 0.167)\text{BG} - (0.0338 \pm 0.0127)\text{NG}$$

($n = 23$, $R^2 = 0.875$, Q^2 (leave-one-out) $= 0.830$, $F = 44.2$, RMSE $= 5.16$, $p < 0.0001$)

In the equation, "activity" indicates the remaining CYP3A activity in the reaction mixture added to each GFJ. "DHB", "BG", and "NG" indicate the respective concentrations (μM) in each GFJ.

3. Discussion

The inhibitory effects of GFJ on CYP3A activities could be attributed to almost all these ingredients because the contribution ratio in the above equation was 88% (Fig. 2). On the other hand, for 23 GFJ samples used in present study including a semi-outlier sample, the experimental and predicted inhibitory effects were 22% and 36%, respectively. That is, this GFJ sample showed stronger inhibition compared with the calculated effect from the equation. If this point is excluded, the contribution ratio would reach 92.5%. This finding suggests that some other ingredients might be involved in the inhibition of CYP3A in small number of GFJ brands. It was reported that furanocoumarin dimers such as paradisin A, B, and C are present in GFJ in low concentrations but could be more effective components in the GFJ interaction (Fukuda 2000; Guo 2000). The paradisins, furanocoumarin-dimers, may have contributed to the

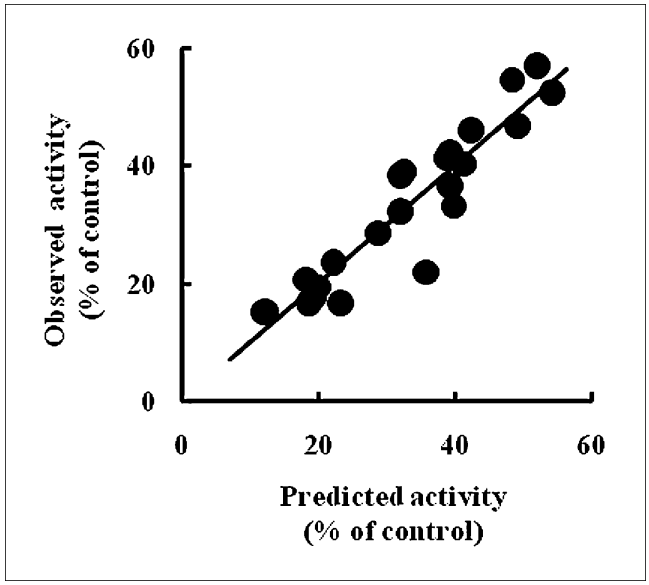

Fig. 2: Multiple linear regression between predicted and observed CYP3A activities (% of control) with 23 kinds of GFJ.

CYP3A-inhibition. However, they are only present in probably the minority among the commercially-supplied GFJs because only one of the 23 GFJ samples was underestimated by the model equation that did not include the concentrations of dimers in the explanatory variables. Dimer concentrations may be too low to exert any inhibitory effect on CYP ability. In fact, one brand of GFJ with active interaction ability *in vivo* did not contain any dimer (Mohri and Uesawa 2001).

Standardized partial regression coefficients of DHB, BG, and NG in the model were –0.720, –0.509, and –0.241, respectively. This result suggests that the order of contribution of the ingredients in the whole juice to the CYP3A inhibition is in the order DHB>BG»NG. We believe that the furanocoumarins including DHB and BG were important factors in the GFJ interactions compared with the other components such as flavonoids. This conclusion is in agreement with many reports in which furanocoumarins were found to be responsible factors in the interactions (Uesawa 2008a). On the other hand, there is a report that indicates a weak inhibitory effect of BG on CYP3A activity (Guo et al. 2000). Recently, we found that the inhibitory effects of furanocoumarin derivatives depend on the lipophilicity of the molecules (Uesawa and Mohri 2010). That is, logarithmic IC_{50} values of a variety of furanocoumarin derivatives were explained with a quadratic model consisting of logP values as the explanatory variables ($R^2 = 0.812$). In the QSAR model, the inhibitory effect of BG was comparable with that of DHB. In fact, it became evident that BG is one component that is involved in the GFJ-drug interaction, which results in inhibition of the CYP3A enzyme in the intestinal lumen *in vivo* (Uesawa and Mohri 2008). Goosen et al. (2004) reported a significant increase in felodipine AUC when subjects were co-administered BG at the same concentration of GFJ. Results in the present study also supported the importance of BG in CYP3A inhibition as well as DHB. Our findings suggest that a quantitative determination of DHB and BG is a useful method for brief assessment of GFJ brands in pharmaceutical interactions.

4. Experimental

4.1. Materials

Anthracene [internal standard (IS)] was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Bergamottin (BG) and 6',7'-dihydrobergamottin (DHB) were purchased from Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan). Bergaptol was obtained from Funakoshi Co. Ltd. (Tokyo, Japan). Naringin (NG) and naringenin were obtained from Sigma Chemical co. (St Louis, MO, USA). Testosterone was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). 6β-Hydroxytestosterone and corticosterone [internal standard (IS2)] were purchased from Daiichi Pure Chemicals and Wako Pure Chemical Industries, respectively. Pooled human hepatic microsomes were obtained from BD Biosciences (San Jose, CA, USA). Methanol, acetonitrile, and phosphoric acid of HPLC grade were used (Wako). All other chemicals were of reagent grade (Wako). Different GFJ including 17 commercialized products were purchased from local markets in Japan. Furthermore, six kinds of hand squeezed juice were made from pulps of grapefruits purchased from separate local markets in Japan.

4.2. Assay of testosterone 6β-oxidation activities

An assay of testosterone 6β-oxidation activity with human liver microsomes was performed according to the manual of the NADPH Regenerating System (BD Biosciences, Inc., CA, USA). Briefly, microsomes (150 μg) were incubated in 10% of neutralized GFJ samples in 50 mM sodium phosphate buffer (pH 7.4) with 1.3 mM NADP, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride, and 0.2 mM testosterone (final incubation volume, 100 μL) at 37 °C. After 30 min of incubation, 1 mL of IS solution (2 μg/mL corticosterone in acetonitrile) was added to the reaction mixture. The sample was mixed vigorously for 20 s and centrifuged at 16,000 × g for 5 min at 4 °C; then the supernatant (5 μL) was injected into LC/ESI/MS. ESI mass spectra were obtained using a Shimadzu LCMS-2010EV LCMS system with an ESI probe (Shimadzu Co. Ltd.) equipped with a reversed-phase analytical Capcell Pak MGII-ODS column [2.0 mm (inside diameter) × 15 cm; particle size 5 μm (Shiseido Co. Ltd., Kyoto, Japan)]. The flow rate was set at 0.2 mL/min. [M - H]$^-$ ions at m/z 303.2 and 345.2 for 6β-hydroxytestosterone and corticosterone, respectively, and monitored for negative ions; the interface voltage was 4.5 kV, and the detector voltage was 1.5 kV. The heat block and CDL temperatures were 200 °C and 250 °C, respectively. Nitrogen was used as the nebulization gas at flow rates of 1.5 L/min. A mobile phase consisting of water and acetonitrile was pumped through the column at 0.2 mL/min using a gradient ranging from 10% to 100% acetonitrile in 8 min and subsequently 100% for 12 min. Calibration curves (1 to 60 μM of 6β-hydroxytestosterone) were constructed using linear regression analysis. Testosterone 6β-oxidation activities with GFJ expressed as percentages of the control velocity in a 6β - hydroxytestosterone - production reaction without GFJ.

4.3. Detection of naringin and naringenin in GFJ

Acetonitrile (400 μL) was added to 100 μL of the GFJ in a 2-mL plastic tube. After being shaken vigorously, the sample was centrifuged at 16,000 × g and 4 °C for 10 min; 5 μL of the supernatant was injected directly into LC/ESI/MS. ESI mass spectra were obtained using a Shimadzu LCMS-2010EV LCMS system with an ESI probe (Shimadzu Co. Ltd.) equipped with a reversed-phase analytical Capcell Pak MGII-ODS column [2.0 mm (inside diameter) × 15 cm; particle size 5 μm (Shiseido Co. Ltd., Kyoto, Japan)]. The flow rate was set at 0.2 mL/min. [M - H]$^-$ ions at m/z 271.1 and 579.2 for naringenin and naringin, respectively, and monitored for negative ions; the interface voltage was 4.5 kV, and the detector voltage was 1.5 kV. The heat block and CDL temperatures were 200 °C and 250 °C, respectively. Nitrogen was used as the nebulization gas at flow rates of 1.5 L/min. A mobile phase consisting of water and acetonitrile was pumped through the column at 0.2 mL/min using a gradient ranging from 10% to 100% acetonitrile in 9 min and subsequently 100% for 12 min. Calibration curves (0.1 to 10 μM of naringenin and 10 to 500 μM of NG) were constructed using linear regression analysis.

4.4. Detection of furanocoumarin derivatives in GFJ

The detection of furanocoumarin derivatives in GFJ was performed as previously described (Uesawa and Mohri 2005). Briefly, juice samples were analyzed using HPLC equipped with a reverse-phase analytical Capcell Pak SG-Phenyl column [4.6 mm (inside diameter) × 25 cm; particle size 5 μm; Shiseido Co. Ltd., Tokyo, Japan]. A photodiode array detector (MD-910, JASCO Corp. Ltd., Tokyo, Japan) was used because it reveals the characteristic UV-absorption spectrum of the furanocoumarin derivatives was obtained commonly at a maximum wavelength of 311 nm (Uesawa and Mohri 2005). The mobile phase consisting of 0.1% phosphoric acid and acetonitrile was pumped through the column at a rate of 1.0 mL/min with a gradient of 0 to 5 min, followed by 40% acetonitrile, and subsequently from 40% to 100% acetonitrile in 30 min. IS solution (10 μg/mL anthracene in acetonitrile, 400 μL) was added to 100 μL of the GFJ in a 2-mL plastic tube. After being shaken vigorously, the sample was centrifuged at 16,000 × g and 4 °C for 10 min; 50 μL of the supernatant was injected directly into the HPLC system. Calibration curves (1 to 50 μg/mL of BG, DHB, and bergaptol) were drawn by linear least-squares regression analysis.

4.5. Data analysis

The relation between the objective variables and the explanatory variables was investigated using statistical techniques including simple and multiple regression analyses by JMP version 8.0.2 (SAS Institute Inc., Cary, NC, U.S.A.). Stepwise procedure with the forward selection method was adopted in the variable selection in the multiple regressions. To verify the validity and stability of the model obtained, the leave-one-out cross-validation test (Cruciani 1992) by MobyDigs version 1.0 (Talete srl, Pisani, Milano, Italy) and regression diagnostics using Variance Inflation Factor (VIF), Mahalanobis distance, and Lack-of-Fit (LOF) test by JMP were performed.

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