The Use of Heat Treatment to Eliminate Drug Interactions Due to Grapefruit Juice

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Grapefruit juice (GJ) contains components that may increase the bioavailability of drugs; however, approaches to the removal of these components have been little investigated. It is known that furanocoumarin derivatives (FCs), such as bergamottin (BG) and 6',7'-dihydroxybergamottin (DHB) in GJ, induce such drug interactions. In the present study, it was found that the heat treatment of grapefruit juice decreases concentrations of BG and DHB as well as their interactions both in vitro and in vivo. We incubated GJ for 10, 20, 30, 40, 50, and 60 min at 37, 62, 72, and 95 °C; FCs in each sample were then measured, using high-performance liquid chromatography (HPLC). The concentrations of BG and DHB were decreased in a time- and temperature-dependent manner, by 82.5 and 97.9% respectively, after incubation for 1 h at 95 °C. In contrast, the concentration of bergaptrol (BT) increased in a time- and temperature-dependent manner (27.7% after 60 min at 95 °C). In addition, the effect of each GJ sample on testosterone 6β-oxidation in human liver microsomes was observed. The inhibitory effects of GJ heated to 95 °C were decreased in a time-dependent manner, as in the case of BG and DHB concentrations. Furthermore, 2 ml of GJ treated for 60 min at 95 °C was administered into the rat duodenum. After 30 min, nifedipine (NFP) was administered intraduodenally at a dose of 3 mg/kg body weight. The concentrations of NFP in the plasma samples were determined by HPLC. No significant increase in the AUC of NFP was observed in the rats given heat-treated GJ. These results suggest that the heat treatment of GJ reduces the concentrations of FCs, thus eliminating the potential for drug interactions.

Key words grapefruit juice; furanocoumarin; pharmacokinetic interaction; nifedipine; heat treatment

It has been reported that when many types of drugs, including dihydropyridine calcium antagonists, are taken with grapefruit juice (GJ), their concentrations in plasma increase due to their increased bioavailabilities.1,2) Serious adverse effects have been known to result.3,4) Many patients being treated for chronic diseases are forbidden to drink GJ for the rest of their lives because of the risk of such drug interactions. The drugs interacting with GJ are metabolized by CYP3A enzymes in the small intestine. Furanocoumarin derivatives (FCs), such as bergamottin (BG) and 6',7'-dihydroxybergamottin (DHB), in GJ inhibit this process.5—11) Therefore, in order to produce GJ that vulnerable patients may drink, it would be useful to remove the interactive components of GJ. This possibility has been little investigated.

We recently reported that ultraviolet (UV) irradiation eliminates FCs in GJ.12) Furthermore, GJ that was UV-irradiated for 6 h lost the ability to interact with intraduodenal nifedipine (NFP) in rats. However, this method has the drawback that, over the long term, toxic substances might be formed in the small intestine. We incubated GJ for 10, 20, 30, 40, 50, and 60 min at 37, 62, 72, and 95 °C; FCs in each sample were then measured, using high-performance liquid chromatography (HPLC). The concentrations of BG and DHB were decreased in a time- and temperature-dependent manner, by 82.5 and 97.9% respectively, after incubation for 1 h at 95 °C. In contrast, the concentration of bergaptrol (BT) increased in a time- and temperature-dependent manner (27.7% after 60 min at 95 °C). In addition, the effect of each GJ sample on testosterone 6β-oxidation in human liver microsomes was observed. The inhibitory effects of GJ heated to 95 °C were decreased in a time-dependent manner, as in the case of BG and DHB concentrations. Furthermore, 2 ml of GJ treated for 60 min at 95 °C was administered into the rat duodenum. After 30 min, nifedipine (NFP) was administered intraduodenally at a dose of 3 mg/kg body weight. The concentrations of NFP in the plasma samples were determined by HPLC. No significant increase in the AUC of NFP was observed in the rats given heat-treated GJ. These results suggest that the heat treatment of GJ reduces the concentrations of FCs, thus eliminating the potential for drug interactions.

MATERIALS AND METHODS

All handling procedures involving NFP and nitrendipine were performed under subdued light.

Materials Anthracene [internal standard (IS)] was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). BG and DHB were purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Bergaptol (BT) was obtained from Funakoshi Co., Ltd. (Tokyo, Japan). NFP was purchased from Wako Pure Chemical Industries. Nitrendipine [internal standard (IS2)] was obtained from Yoshitomi Pharmaceutical Industries, Ltd. (Osaka, Japan). Testosterone was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). 6β-Hydroxytestosterone and corticosterone [internal standard (IS3)] were purchased from Daiichi Pure Chemicals and Wako Pure Chemical Industries, respectively. Pooled human hepatic microsomes were obtained from BD Biosciences (San Jose, CA, U.S.A.). Methanol, acetonitrile, and phosphoric acid of HPLC grade were used (Wako). All other chemicals were of reagent grade (Wako). GJ was obtained from Kanda Foods Laboratory Co., Ltd. (Tokyo, Japan).

Treatment of GJ at Each Temperature One milliliter each of the GJ Samples (pH 3.9) in 1.5-ml microtubes were...
treated at 4, 37, 62, 72, and 95 °C for 10, 20, 30, 40, 50, and 60 min, respectively. Crushed ice and TAITEC Dry-Thermo Unit TAH-1G (Taitec Co., Ltd., Saitama, Japan) were used in setting the temperatures. FC concentrations were then measured in each GJ sample. The GJ samples treated at 95 °C were used to measure the inhibition of micromosal oxidation in vitro and pharmacokinetic experiments in vivo.

Detection of FCs The detection of FCs in GJ was performed according to a previously described method with minor modifications.14) The GJ 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] and a guard column Nova-Pak C18; particle size 5 μm (Waters Co., Ltd., MA, U.S.A.). A photodiode array detector (MD-910, JASCO Corp. Ltd., Tokyo, Japan) was used because it reveals the characteristic UV-absorption spectrum of FCs, commonly with 311 nm as the maximum wavelength. A mobile phase consisting of 0.1% phosphoric acid and acetonitrile was pumped through the column at a speed of 1.0 ml/min with a gradient of 0 to 5 min, using 40% acetonitrile and subsequently from 40 to 100% acetonitrile in 30 min. Then 400 μl of IS2 solution (10 μg/ml anhydroacene in acetonitrile) was added to 100 μl of each GJ sample in a 2-ml plastic tube. After being shaken vigorously, the sample was centrifuged at 16000 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 BT) were drawn by linear least-squares regression analysis.

Assay of Testosterone 6β-Oxidation Activities An assay of testosterone 6β-oxidation activity with microsomes from human liver was performed according to a manual of NADPH Regenerating System (BD Biosciences, Inc., CA, U.S.A.). Briefly, the microsomes (150 μg) were incubated in 10% GJ samples in 50 mM sodium phosphate buffer (pH 7.4) with 1.3 mM NADP, 3.3 mM glucose-6-phosphate, 0.4 μM 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 IS3 solution (2 μg/ml corticosterone in acetonitrile) was added to the reaction mixture. The sample was mixed vigorously for 20 s and centrifuged at 16000 g for 5 min at 4 °C; then the supernatant (5 μl) was injected into LC/ESI/MS. ESI mass spectra were obtained using 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 were 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 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 a speed of 0.2 ml/min with 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.

Animals and Drug Administration Nine-week-old male Wistar-ST rats (Japan SLC Inc., Shizuoka, Japan) weighing 280 to 300 g were used throughout the study. The rats were housed in stainless steel cages with three animals per cage in a temperature-controlled (24 to 26 °C) room under a 12 h light/dark cycle. The rats were allowed free access to standard rat chow (Sankyo Labo Service Co., Ltd., Tokyo, Japan) and water for 1 week before the experiments. The rats were fasted overnight before the experiments. Each animal was anesthetized with 20% w/v urethane [1 g/kg body weight, intraperitoneally (IP)]. The femoral vein was cannulated with PE-10 tubing (Clay Adams, Parsippany, NJ, U.S.A.) for saline infusion and drug administration. The femoral artery was cannulated with PE-50 tubing (Clay Adams) to collect blood samples over time, with an established heparin lock using 100 U/ml heparin in saline. During the experiment, body temperature was kept at 38±0.5 °C. An NFP solution for injection was prepared by dissolving 50 mg of NFP in a mixture of polyethylene glycol 400 (5 ml), ethanol (5 ml), and saline (10 ml). Then 2 ml of GJ, GJ heat-treated at 95 °C for 60 min (HJG), and saline was directly injected into the duodenum using a syringe with a needle. After 30 min, NFP was administered at a dose of 3 mg/kg body weight in the same procedure [intraduodenal (i.d.) administration]. Blood samples (each approximately 0.2 ml) were collected via the femoral artery at 0, 5, 10, 15, 20, 30, and 45 min and at 1.0, 1.5, 2.0, 3.0, and 4.0 h. The samples were immediately centrifuged at 16000 g for 15 min at 4 °C, and the plasma was separated. Each rat was also given saline via the femoral cannula in a volume equivalent to the volume of blood collected. At this point 180 μl of IS solution (1 μg/ml NTP in acetonitrile) was added to 20 μl of the plasma in a 2-ml plastic tube. After being shaken vigorously, the sample was centrifuged at 16000 g and 4 °C for 10 min; 25 μl of the supernatant was injected directly into the HPLC system as previously described.12) Briefly, the HPLC system was equipped with a UV/VIS detector (UV-970, JASCO Corp. Ltd., Tokyo, Japan) and a reverse-phase analytical Capcell Pak UG-ODS column [4.6 mm (inside diameter)×25 cm; particle size 5 μm (Shiseido Co., Ltd., Tokyo, Japan)]. A mobile phase consisting of 0.085% of phosphoric acid/acetonitrile (55:45 vol/vol) was pumped through the column at a speed of 1.0 ml/min. NFP was quantified at a wavelength of 260 nm. Calibration curves (0.63 to 20 μg/ml of NFP) were drawn by linear least-squares regression analysis. The plasma concentration/time data from each rat were analyzed with a model-independent method using the WinNonlin computer program.

Data Analysis In the inhibition and the pharmacokinetic experiments, all data were expressed as mean±S.D. An unpaired Student’s t-test and one-way ANOVA, followed by least-significant-difference analysis, were used to test for significant differences in mean values. The significance level was set at p<0.05.

RESULTS

Change of the FC Concentration in GJ at Each Temperature HPLC chromatograms of FCs in the GJ samples are shown in Fig. 2. BG, DHB, and BT (Fig. 1) were identified as major FCs in the GJ used in this study (17.9, 7.85, 50.7 μM, respectively; Fig. 2, left). BG and DHB showed a...
consistent decrease during treatment at 95 °C for 1 h (Fig. 2, right; Fig. 3). Interestingly, the concentration of BT in GJ was reversely increased in this condition. The increment of BT in GJ rose to 14.1 μM after 60 min of treatment. At 37 °C, each FC concentration did not almost change during incubation for 60 min (Fig. 3). Figure 4 shows the concentrations of BG, DHB, and BT after incubation at each temperature for 60 min. Concentrations of all FCs were altered very little at 4 and 37 °C. At 62, 72, and 95 °C, concentrations of BG and DHB decreased in a temperature-dependent manner. The remainders of BG and DHB at 95 °C for 60 min were 3.14 and 0.163 μM, respectively. On the other hand, BT increased in a temperature-dependent manner. The concentration of BT at 95 °C for 60 min was 64.8 μM.

Inhibition of CYP3A Activities The testosterone 6β-hydroxylation rate was 2.14 nmol/min/mg protein in human liver microsomes; 10% of GJ (adjusted at pH 7.4) in the reaction mixture decreased the oxidation activity to 0.303 nmol/min/mg protein (14.1% of the control, Fig. 5). Treatment of GJ at 95 °C invalidated the inhibitory effect of GJ by heat treatment in a time-dependent manner. The testosterone 6β-hydroxylation rate with GJ heat-treated at 95 °C for 60 min (HGJ) was 0.617 nmol/min/mg protein (28.8% of the control).

Effects of HGJ on NFP Pharmacokinetics The NFP plasma concentration/time profiles after saline, GJ, and HGJ administration to rats are shown in Fig. 7. After intraduodenal administration, the mean AUC values of the rats receiving GJ were approximately 1.95 times greater and the mean Cmax values were approximately 1.61 times greater, respectively, than those of the rats receiving HGJ (Fig. 7, Table 1). On the other hand, the mean AUC and the mean Cmax values were not significantly different between saline and HGJ administration.

DISCUSSION

It has been reported that GJ intake causes pharmacokinetic
interactions with many drugs.1,2) Some FCs in GJ, including BG and DHB, were established as substances that cause GJ-drug interactions in vitro and in vivo.5–11) Therefore FCs in GJ used in this investigation were determined comprehensively by the HPLC system with a photodiode array detector.

As a result, three kinds of FCs—BG, DHB, and BT—were detected: 17.9, 7.85, and 50.7 μM, respectively (Fig. 2). BG and DHB have structures constructed with BT, the simplest FC in citrus fruits, and isoprene side chains combined through the fifth oxygen atom of BT (Fig. 1). It was reported that BG and DHB decrease oxidation for the drugs with mechanism-based inhibition of CYP3A expressed in small intestinal epithelial cells.15) On the other hand, BT did not inhibit CYP3A activities in microsomes from humans and rats.5,16) Furthermore, the uptake experiment of vinblastine by Caco-2 cells revealed that BT has little inhibitory effect on P-glycoprotein unlike BG and DHB.17) The pharmacological effect of BT has hardly been reported.

**Measurement of FCs in GJ** Concentrations of the major FCs—BG, DHB, and BT—were measured in GJ treated at 4, 37, 62, 72, and 95 °C. The results showed that BG and DHB in GJ were unstable at high-temperature conditions. That is, both compounds showed decreases in dependence on temperature and treatment time (Figs. 3, 4). On the other hand, the concentrations of BT in GJ were increased by heat treatment. Because BG and DHB are composed of BT and isoprene residues, the FCs may be the source of BT in the heated GJ (Fig. 1). The comprehensive determination of FCs indicated that, except for BG and DHB, the source compound of BT hardly exists in GJ. However, the isoprene residues bind with FC skeletons in derivatives of the ether bond, which are difficult to be hydrolyzed. BT generation reaction from BG or other FC analogues has been reported; these were anhydrous reactions under strongly acidic conditions involving sulfuric or hydrochloric acid.18–20) Because the scission processes of ary1 ethers under slightly acidic conditions, as in the case of GJ (pH 3.9) are not general, the peculiar structure of the FC skeleton may contribute to the decomposition reactions.

**The Inhibition of Testosterone 6β-Oxidation** The remaining concentrations of BG and DHB, the important constituents in GJ for drug interactions, in HGJ were 3.14 and 0.16 μM, respectively. On the other hand, BT, which does not contribute to CYP3A inhibition in GJ, was increased to 64.8 μM in HGJ (Figs. 3, 4). It was expected from these results that the CYP3A inhibitory effect and the pharmacokinetic interactions of GJ would disappear as a result of heat treatment. Then, testosterone 6β-hydroxylation with human liver microsomes and GJ treated at 95 °C were measured in order to investigate the effect of the heating on CYP3A oxidation. As a result, the turnover rate of 6β-hydroxylation of testosterone decreased as the duration of heat treatment increased (Fig. 5). The testosterone 6β-hydroxylation rates were negatively related to the concentrations of BG and DHB in GJ samples treated at 95 °C (Fig. 6, correlation coefficients: r = -0.994 and -0.951, respectively). These observations suggest that the lower amounts of BG and DHB in GJ due to heating at 95 °C controlled the inhibition of testosterone 6β-hydroxylation with GJ. No study of inhibition of the CYP3A metabolism with FC-free GJ has been reported. In the present study, the remaining activities of CYP3A in the microsomal reactions with GJ and HGJ were 14.1% and 28.8%, respectively, compared with the reaction without GJ. It is believed that the 14.7% difference between the results with HGJ and GI stem from the net inhibition with BM and DHB in this condition. On the other hand, it is suggested that the 71.2% difference between the control and HGJ represents net inhibition with contents in GJ other than FCs. This finding indicates that the FC was responsible for only part of the inhibition with whole GJ in testosterone 6β-hydroxylation with human liver microsomes. Other GJ constituent such as naringin and naringenin may be responsible.21)
Pharmacokinetic Interactions  It was shown that HGJ produced a CYP3A-inhibitory effect of 71.2% in comparison with untreated GJ. Therefore the effects of HGJ on NFP pharmacokinetics in rats were evaluated in vivo. We have shown, in earlier studies, that the AUC of NFP is significantly increased by the intraduodenal administration of GJ but not of orange juice, sweetie juice, or saline.5,22,23) These results suggest that GJ caused increased gastrointestinal absorption of NFP in rats. It was thought that NFP oxidation by CYP3A in the intestinal mucosa was inhibited by GJ administration. Actually, our rat studies with small intestinal microsomes24) indicated that BG and DHB contribute to the inhibition of NFP oxidation in rat small intestine.5) These observations in rats are very similar to those in humans.25) These observations suggest that evaluation using rats is useful for predicting drug–food interactions. Therefore the effect of HGJ administration on NFP pharmacokinetics using rats was investigated in the present study. Injection of HGJ into the duodenum 30 min before NFP administration did not affect the plasma concentration/time profile of NFP (Fig. 7, Table 1). On the other hand, the AUC and Cmax were significantly increased in the HGJ-preadministered group compared with the HGJ-administered group (1.95 and 1.61 times, respectively; Table 1). These observations show that the administration of HGJ, unlike that of GJ, probably does not increase the small intestinal absorption of NFP. The results also suggest that inhibitory contents of CYP3A in HGJ, as observed in the in vitro experiments, because of the disappearance of BG and DHB in HGJ, do not contribute pharmacokinetic interactions between NFP and GJ. Mechanism-based inhibitors such as BG and DHB may be able to reduce the activity of CYP3A in the intestinal tract effectively.15) In fact, unlike GJ, naringin, a potent, but not mechanism-based inhibitor of CYP3A,21) has been reported not to increase the availability of nisoldipine in humans.26)

The present investigations clearly showed the contributions of FCs on drug interactions with GJ in in vitro and in vivo experiments using GJ samples, eliminated BG and DHB following high temperature treatments. Furthermore, these observations may develop as fundamental knowledge to create “drinkable GJ” for patients receiving medications that induce interactions with GJ. In the previous report, we showed that sweetie juice did not have a significant effect on NFP pharmacokinetics in rats.22) The concentrations of BG and DHB in the sweetie juice used in the study were 1.6 and 0.51 μM, respectively. In other words, low FC concentrations such as that in sweetie juice and HGJ hardly relate to drug bioavailability. Examination of the respective threshold concentrations of the FCs is important in terms of pharmacokinetics in order to ensure the quality of the GJ from which FCs have been removed. On the other hand, heat treatment at 95°C in the present basic study seems to have a detrimental effect on the taste, flavor and nutrients of GJ. However, it might be possible to develop GJ processing methods with lower temperatures thereby avoiding these problems, as, the concentrations of BG and DHB in GJ are low at 62°C. In addition, understanding the thermal decomposition mechanism of FCs may enable the selection of effective low-temperature catalysts. Although it is necessary to examine the heating condition, we presumed that the results of the heat treatment of GJ will contribute to the development of practical research on the prevention of drug interactions, and may contribute to resolving this problem in clinical settings. This study offers a new method that is applicable in research on drug interaction with various food and drinks.

Acknowledgments  We thank Dr. Hitoshi Kondo of Dainippon Ink and Chemicals, Inc. for his advice and suggestions about reactions of FCs.

This study was supported in part by a research grant for the Open Research Center Project from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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