J. Toxicol. Pub. Health
Vol. 17, Supplement, pp. 127-133, 2001

Phenotyping of Flavin-Containing Monooxygenase (FMO) Activity and Factors Affecting FMO Activity in Korean

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ABTRACT : *Together with cytochrome P450 (CYP), flavin-containing monooxygenase (FMO) present in liver microsomes oxidizes various endogenous and exogenous chemicals. In an effort to determine the human FMO activity, we have developed two non-invasive urine analysis methods using caffeine (CA) and ranitidine (RA) as the probe compounds. As the production of theobromine (TB) and ranitidine N-oxide (RANO) from CA and RA is catalyzed primarily by the hepatic FMO, we have assigned the urinary molar ratios of TB/CA and RA/RANO as the in vivo FMO activity. In 200 age-matched Korean volunteers, the obtained TB/CA ratio ranged from 0.4 to 15.2 (38-fold difference) and the RA/RANO ratio from 5.7 to 27.2 (4.8-fold). The FMO activity of 20's, determined by caffeine metabolism, was the highest (2.5±1.9) and those of 30's, 40's, 50's, 60's and 70's were 40%, 50%, 24%, 39% and 36% of the 20's, respectively. Intake of grapefruit juice, known to contain flavonoids, inhibited the in vivo FMO (TB/CA) activity by 79%. Addition of the flavonoids like naringin, quercitrin and kaempferol, present in grapefruit juice, to the in vitro microsomal FMO assay, thiobenzamide S-oxidation, produced 75%, 70% and 60% inhibition, respectively. Obtained Ki values of quercitrin, kaempferol and naringin on the in vitro FMO activity were 6.2, 12.0 and 13.9 μM, respectively. This suggested that the dose of drug should need to be adjusted to suit the individual FMO activities when the drugs metabolized by FMO are given to patients. As the intake of grapefruit juice has been identified to inhibit the FMO as well as CYP3A4 and 1A2 activities, patients taking drugs metabolized by these enzymes should not drink grapefruit juice as the carrier.*

Key Words : *Caffeine, Ranitidine, FMO, In vivo probe, Phenotyping, Grapefruit juice, Flavonoids*

I. INTRODUCTION

Flavin-containing monooxygenase (FMO) is known to catalyze the oxidative metabolism of nitrogen-, sulfur- and phosphorus-containing compounds including many clinically useful drugs, plant alkaloids and endogeneous chemicals (Ziegler, 1988, 1993; Cashman, 1995, Hodgson *et al.*, 1995). FMO in human is known to be responsible for the oxidation of trimethylamine, and subjects who lack FMO may suffer from trimethylaminuria and "fish-odor" syndrome (Al-Waiz *et al.*, 1987a, b).

FMO3 among five isoforms (FMO1-5) found in human is known to be the major form present in adult liver (Lomri *et al.*, 1992; Dolphin *et al.*, 1996; Overby *et al.*, 1997). FMO3 is thought to catalyze most of the FMO medicated drug oxidation *in vivo*. Oxidative metabolism of some clinically useful drugs (i.e. tamoxifen, olanzapine, clozapine, cimetidine, ranitidine, ketoconazole and methimazole) as well as some food-borne secondary and tertiary amines such as trimethylamine has been reported to be catalyzed by FMO3. As these drugs are used clinically and the pharmacokinetics of these drugs are dependent on the activities of hepatic FMO3, a non-invasive means to determine FMO activity *in vivo* is needed for the safe use of these drugs in humans.

While some compounds like nicotine contained in cigarette are available for a non-invasive determination of FMO activity in human (Cashman *et al.*, 1992), non-toxic probe drugs which are used commonly by human and whose metabolites are easily detected in urine have not available. A recent study conducted in our laboratory has shown that production of theobromine from caffeine is catalyzed primarily by the FMO present in adult liver microsomes (Chung and Cha,

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1997; Chung *et al.*, 1998). Utilizing this information, FMO activity *in vivo* was determined non-invasively by taking urinary molar concentration ratio of theobromine/caffeine in Korean populations (Chung *et al.*, 1999).

In a continued effort to expand a more suitable probe than caffeine, which was metabolized to theobromine minorly by CYP1A2, we have considered several drug candidates for the phenotyping of FMO activity. Ranitidine is a potent non-imidazole type of H_2-receptor antagonist used widely as a leading anti-ulcer drug. It has been known to be metabolized to its *N*-oxide almost exclusively by hepatic FMO3 (Overby *et al.*, 1997). We have developed another phenotyping method using ranitidine by comparing the amounts of ranitidine and its *N*-oxide excreted in urine (Kang *et al.*, 2000).

We have determined human FMO activities using caffeine and ranitidine as *in vivo* probes in Koreans. Also the factors affecting FMO activity such as smoking habits, age and intakes of grapefruit juice have been described in this paper.

II. METHODS

1. Volunteers

For the determination of basal level of FMO activity, the study protocol was followed as described (Chung *et al.*, 1999). Briefly, 133 age-matched healthy Korean volunteers were recruited from students at Inha Medical colleges and elderly people living near the Inha University located at Inchon. They are 84 men (39 cigarette smokers and 45 non-smokers) and 49 women (all non-smokers). Their ages ranged from 21 to 78 years (mean±SD, 35±16 years).

To measure effect of intake of grapefruit juice on the FMO activity, 47 Korean volunteers were participated. They were 38 men (18 smokers and 20 non-smokers) and 9 women (all non-smokers). Their ages ranged from 21 to 30 years (mean±SD, 23±2 years). All procedures were the same as described by Kang *et al.* (1997).

For the determination of FMO activity using ranitidine as probe, 200 unrelated healthy medical students of Inha University were participated as volunteers. They are 163 men and 37 women. Ages of the 200 volunteers ranged from 19 to 36 years with mean of 23 years.

All volunteers participated in this study provided their written informed consent. None of the subjects were using any concurrent medications. All volunteers were asked not to take any methylxanthine-containing drinks, foods, or drugs for 2 days before and during the study. Compliance was confirmed by detection of no caffeine in the control baseline urine.

2. Urine collection

The procedures for the determination of basal FMO activity were basically the same with the study of Chung *et al.* (1999). Briefly, after an overnight fast (10 hours), each volunteers voided a control baseline urine sample and was given a cup of coffee (200 ml) prepared from two packs of instants coffee (Taster's Choice, 12 g, Nestle Vevey, Switzerland) containing 110 mg caffeine. An 1 hour urine sample was collected between 4 and 5 hours after the coffee intake and immediately thereafter, the pH of all urine samples was adjusted to 3.5 with hydrochloic acid as described by Nakajima *et al.* (1994).

To determine the effect of intake of grapefruit juice on the FMO activity in human, 47 young and healthy Korean medical students at Inha University participated. After one week of washout period for the ordinary caffeine challenge with additional drinking of water, each volunteer voided control urine early in the morning of the test day. They were given 500 ml of grapefruit juice (Pink Grapefruit Cocktail, Ocean Spray, USA). One hour after the oral administration of grapefruit juice, volunteers were given a cup of coffee (200 ml) prepared from 2 packs of instant coffee (Tasters Choice, 110 mg caffeine). One and 3 hr after the coffee intake, volunteers drank 500 ml of grapefruit juice again. One hour urine (between 4 and 5 hr after the coffee intake) was collected, volume was measured, pH was adjusted and an aliquot was stored as before.

For the determination of the FMO activity using ranitidine, each volunteer voided control urine after overnight fasting (10 hr). They were given a single tablet of Zantac® (GlaxoWellcome Korea Ltd.) containing 168 mg ranitidine hydrochloride (equivalent to 150 mg ranitidine base). All urine produced by each volunteer

for 8hr after the ranitidine administration was bulked and the volume was measured as described by Kang *et al.* (2000). A 10 m*l* aliquot was saved for storage at -70°C for later high performance liquid chromatography (HPLC) analysis.

3. HPLC analysis of urine sample

Caffeine and its metabolites were analyzed by use of an HPLC system with detection at 280 nm according to the method described by Chung and Cha (1997). Amounts of ranitidine and its *N*-oxide, *S*-oxide and desmethylranitidne metabolites present in urine were determined using HPLC with detection at 320 nm as described by Chung *et al.* (2000).

4. Data analysis

The FMO activity from a challenge of caffeine was assessed by the ratio of theobromine to caffeine (TB/CA) as described previously (Chung *et al.*, 1999; Park *et al.*, 1999). The FMO activity from a challenge of ranitidine was assessed by the ratio of ranitidine to ranitidine *N*-oxide (RA/RANO) as described by Kang *et al.* (2000).

The difference between two groups of sex and smoking in FMO activity was determined by Mann-Whitney test. To determine whether the activities of FMO activity are affected by grapefruit juice, the obtained urinary caffeine metabolite ratios were compared by the Wilcoxon signed rank test.

5. Inhibitory effects of flavonoids on the rat liver microsomal FMO activity

Liver microsomes were prepared from the male Sprague-Dawley rats weighing 150~180 g which have been obtained from the Animal Breeding Laboratory of Inha University according to the method described by Chung and Buhler (1994). The liver microsomal FMO activity was determined by using thiobenzamide *S*-oxidation assay described by Cashman and Hanzlik (1981). Typical incubation mixture contained 0.5 mg/m*l* liver microsomes, 0.125 mM NADPH (instead of NADP for constant provision of NADPH), 2.5 mM glucose-6-phophate, 0.1 unit glucose-6-phophate dehydrogenase, various flavonoid inhibitors (1 mM naringin, 1 mM naringenin, 0.7 mM quercetin, or 0.25 mM kaempferol), 1 mM EDTA in 25 mM potassium phosphate buffer (pH 8.4). The mixture was preincubated for 15 min at 37°C prior to the addition of 0.75 mM thiobenzamide, the substrate of FMO. For the determination of inhibition constant (*Ki*) of flavonoids, 0.1, 0.25, 0.5, 1 and 2 mM thiobenzamide were used in the presence of various flavonid inhibitors (0.063 mM naringin, 0.1 mM quercetin, or 0.025 mM kaempferol).

The rate of thiobenzamide S-oxide production was monitored at 370 nm for 5 min. The FMO activity was calculated using the molar extinction coefficient of 2930 cmM^{-1}. *Ki* values were calculated from Lineweaver-Burke equation and the double reciprocal plot.

III. RESULTS

1. Variations of FMO activities in koreans

The molar concentration ratio of urinary TB over CA (TB/CA) after intake of a coffee was assigned as the FMO activity. The lowest TB/CA ratio observed was 0.4 and the highest ratio was 15.2, showing 38-fold differences (Fig. 1). The median ratio for the total volunteers was 1.93 and the statistical mean was 2.23 with S.D. of 2.10. The molar concentration ratio of urinary ranitidine over ranitidine *N*-oxide (RA/RANO) after intake of Zantac® was assigned as the

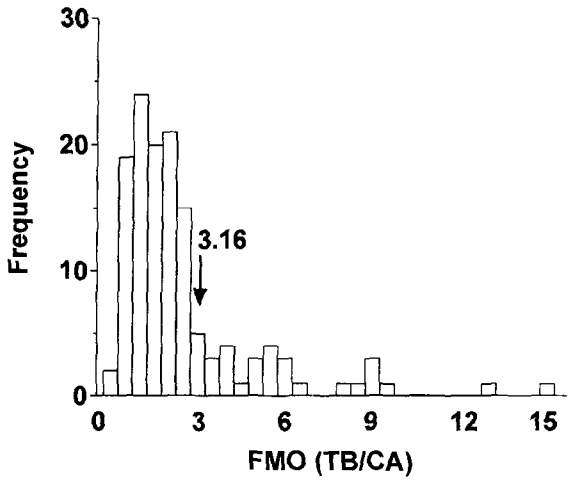

Fig. 1. Frequency distribution of the FMO activity obtained from the ratio of urinary theobromine/caffeine (TB/CA) for Korean volunteers. One hour urine between 4 and 5 hrs after taking a cup of coffee containing 110 mg caffeine was collected and analyzed described in Methods. Arrow marks the potential modal division points.

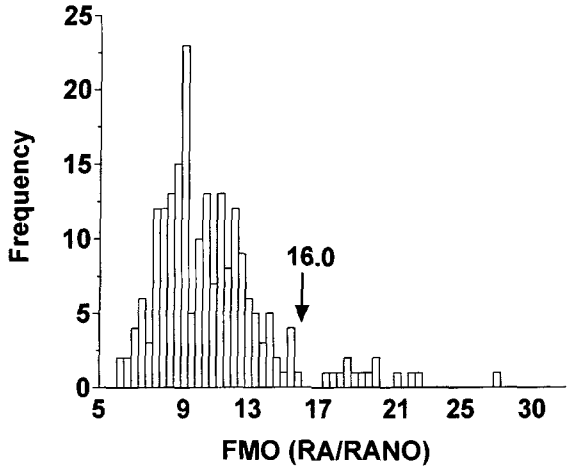

Fig. 2. Frequency distribution of the FMO activity obtained from the ratio of urinary ranitidine to ranitidine *N*-oxide (RA/RANO) for Korean volunteers. All urine 8 hour urine produced by each volunteer for 8 hr after taking Zantac® (GlaxoWellcome Korea Ltd.) containing 150 mg ranitidine base was bulked and analyzed described in Methods. Arrow marks the potential modal division points.

FMO activity. The lowest RA/RANO ratio was 5.7 and the highest ratio was to 27.2, showing 4.8-fold differences (Fig. 2). The median ratio for the total volunteers was 10.33 and the statistical mean was 10.91 with S.D. of 3.27.

2. No gender differences in FMO activities

To compare gender differences of FMO activity, results obtained only from the non-smoking male and female subjects were compared. The range of FMO activities (urinary TB/CA) for all ages of non-smoking male volunteers was between 0.23 and 8.59, showing 37-fold maximal individual differences. For the non-smoking female volunteers, the lowest FMO activity was 0.21 and the highest was 8.76, showing 42-fold maximal individual differences. The mean FMO activity for the males was 2.00±1.83 and for the females, it was 1.53±1.47. However, they were not significantly different ($p=0.17$). Therefore, gender does not have any influence on the activities of FMO in a Korean population.

3. Effect of smoking on the FMO activity

FMO activities (TB/CA) of 95 non-smokers and 38 smokers were compared. Mean of the ratios in the non-smokers was 1.76±1.66 and in the smokers, it

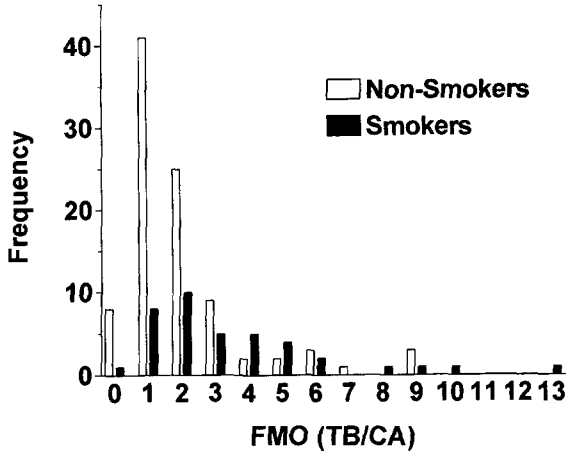

Fig. 3. Effect of smoking on the distribution of FMO activity obtained from the ratio of urinary theobromine/caffeine (TB/CA) for Korean volunteers. The mean of TB/CA ratio was 1.76±1.66 in 95 nonsmokers and 3.40±2.72 in 38 smokers ($p<0.001$ with Mann-Whitney test).

was 3.40±2.72 and they were significantly different ($P<0.001$). Similarly, when the individual FMO activities of smokers and non-smokers were compared in the frequency distribution plot (Fig. 3), again, the distribution of smokers was shifted towards higher ratios indicating that the FMO activity was increased.

4. Effect of age on the FMO activity

The mean of FMO activities in the non-smoking subjects (males and females) belonging to the 20's

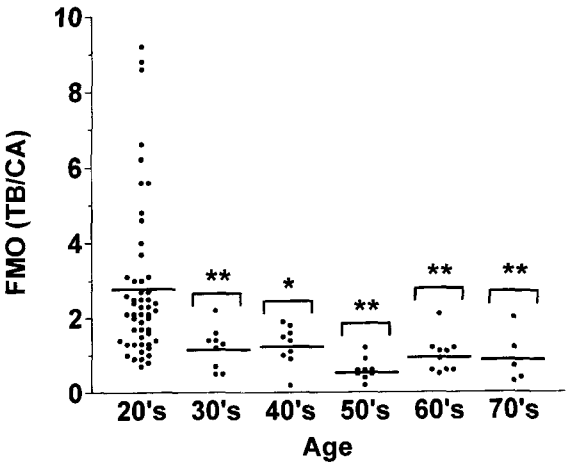

Fig. 4. Effect of age on the FMO activity obtained from the ratio of urinary theobromine/caffeine (TB/CA) for Korean non-smoking volunteers. The mean FMO activity in the 20's was the highest and was higher than those of the subjects in their 30's, 40's, 50's, 60's and 70's (*$p<0.05$, **$p<0.01$).

decennial group was 2.5±1.9. The FMO activity observed in the 20's group was the highest among all decennial age groups (30's to 70's) (Fig. 4). While data are not shown, the FMO activities of non-smoking male and female subjects in each of the decennial age groups were not significantly different. The mean of FMO activities in the 20's was 2.5±1.9 and the mean of the 30's was 0.99±0.6 which was only 40% ($P< 0.01$) of the activity found in the 20's. The enzyme activities of the 40's, 50's, 60's and 70's were 50% ($P<0.05$), 24% ($P<0.01$), 39% ($P<0.01$) and 36% ($P<0.01$) of that found in the 20s, respectively (Fig. 4). Thus, it appeared that the FMO activity declines significantly at the 30's.

5. Effect of grapefruit juice intake on FMO activity

Prior to drinking grapefruit juice, the range of TB/CA ratio obtained from 47 volunteers was between 0.64 and 11.9, showing 18-fold difference among volunteers. The median was 1.92 and the mean ratio was 2.91±2.41. After grapefruit juice intake, the range for TB/CA for the same volunteers was between 0.19 and 2.54, showing only 13-fold difference among volunteers. The median was 0.49 and the mean ratio was 0.61±0.43. As the result shown in Fig. 5, FMO activity in all volunteers, except for 1 subject, was decreased by the grapefruit juice. There was a 79% reduction on the average of FMO activity ($p<0.0001$, Wilcoxon signed rank test). In 46 among 47 volunteers, the decrease of TB/CA ratio ranged from 28% to 97% with 76% decrease in the median value. In 1 exceptional person, the TB/CA ratio increased by 45% upon grapefruit juice intake.

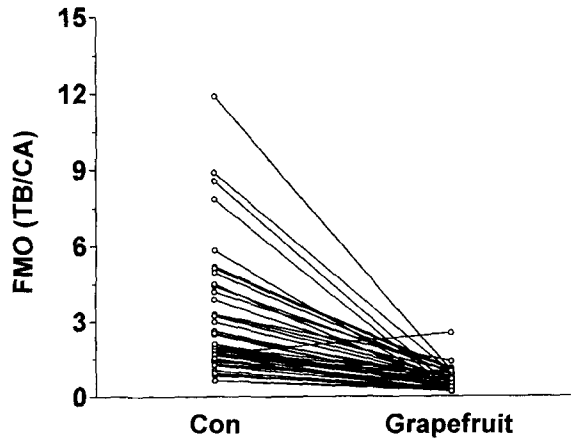

Fig. 5. Inhibition of the FMO activity from the urinary ratio of theobromine/caffeine (TB/CA) by taking grapefruit juice. *CON* : taking water as vehicle, Grapefruit: taking grapefruit juice as mentioned in Methods. After taking grapefruit juice, the average of decrease in the FMO activity was 79% ($p< 0.0001$, Wilcoxon signed rank test).

6. Inhibition of hepatic microsomal FMO activity by flavonoids known to be present in grapefruit juice

The glycoside naringin (naringenin 7-rhamno-glucoside) inhibited the FMO activity in a dose dependent (up to 1 mM) as well as time dependent (up to 15 min of pre incubation) manner upto by 75%. The aglycoside naringin, however, did not inhibit the FMO activity. Again, the glycoside quercitrin (quercetin 3-rhamnoside) inhibited the enzyme activity by more than 70%, while the aglycoside quercetin inhibited only by 48%. Kaempferol inhibited the FMO activity by 60%. Thus, the flavonoid glycosides such as naringin and quercitrin were more potent than the flavonoid aglycas such as naringenin and quercetin. Obtained *Ki* values of quercitrin, kaempferol and naringin on the *in vitro* FMO activity were 6.2, 12.0 and 13.9 μM, respectively. Thus, several flavonoids known to be present in the grapefruit juice were found to be potent inhibitors of hepatic microsomal FMO activity. This indicated that not one but several gradients in grapefruit juice is expected to be responsible for the inhibition of FMO activity in human.

IV. DISCUSSION

While trimethylamine (TMA) is available for the non-invasive determination of FMO activity in human (Al-Waiz *et al.*, 1987a, b), it is contained in many foods such as fish, cheese, eggs and liver in the form of choline derivatives (De la Hurga and Popper, 1951). Thus, TMA is not suitable as a sensitive and specific probe compound to phenotype the FMO activity. Another available probe, nicotine which contained in cigarette and are available for a non-invasive determination of FMO activity in human (Cashman *et al.*, 1992) is toxic to be accepted generally by volunteers.

In an effort to develop new satisfactory probes for the determination of FMO activity, we have developed two methods using caffeine and ranitidine with several

merits. Caffeine which contained in coffee and teas is relatively innocuous. Ranitidine is a safe and well-tolerated H_2 receptor antagonist and is used widely as a leading anti-ulcer agent (Tougas and Armstrong, 1997) and has fewer side effects (Shinn, 1992). Therefore, both caffeine and ranitidine are more easily acceptable to volunteers with a great safety than nicotine. Caffeine is contained only in coffee, teas and drugs, and ranitidine is a drug. Thus, they are more easily controllable than trimethylamine, which is contained in various foods such as fish, cheese, soybeans, peas and eggs.

Another merit of these substrates as probes for the FMO activity is related to the Km values. FMO is known to be present in a reactive state and ready to oxidized available substrates (Ziegler, 1990). *In vivo* oxidation of substrate with low Km value, such as trimethylamine or methimazole, is so large by the mass transfer rate (Ziegelr, 1988). Thus, 5-10 fold changes of FMO activity created by genetic differences would not produce recognizable differences in the metabolic rate for these low Km substrates. Therefore, rather poor substrate of FMO, such as ranitidine with 2 mM of high Km value (Overby *et al.*, 1997) and caffeine (higher than 2 mM, Chung *et al.*, unpublished data) would serve as more sensitive indicator of changes in FMO activity.

Smoking is known to increase CYP1A2 activity (Schrenk *et al.*, 1998). Results obtained from the TB/CA ratio after taking caffeine indicated that the FMO activity was higher in smokers than in non-smokers. At this moment, it is not clear that higher FMO activity in smokers is due to the true enhancement of the enzyme with the increased transcription or stability of mRNA of FMO enzyme.

Results of the coffee test in age and gender-matched non-smoking Korean volunteers indicated that the FMO activity was the same between men and women. However, the activity was high in younger adults but declined with old age. To our knowledge, there was no report on the changes of *in vivo* FMO activity in human. In relation to cytochrome P450 (CYP), a non-specific nonspecific CYP substrate, antipyrine clearance rate (46.4±18.5 ml/min) remained in young adults (20's and 30's groups) but declined linearly after 40 years by 0.34 ml/min/year to the decrease by 29% after 70 years of age (Sotaniemi *et al.*, 1997). Thus, the age is another factor to be considered when prescribed the drugs metabolized by FMO as well as CYPs.

Grapefruit juice is known to inhibit CYP3A4 activity (Bailey *et al.*, 1991; Ameer and Weintraub, 1997). Naringenin, kaempferol, quercetin and fucocoumarins are known as the active ingredients in grapefruit juice for inhibition of CYP3A4 (Guenergich and Kim, 1990; Ha *et al.*, 1995; Fukuda *et al.*, 1997). Taking grapefruit juice also inhibited *in vivo* FMO activity by 79% in this study. Thus, naringenin, kaempferol and quercetin were employed to test whether they also inhibit the liver microsomal FMO activity. Naringin, quercitrin and kaempferol inhibited the *in vitro* microsomal FMO assay, thiobenzamide S-oxidation, by 75%, 70% and 60%, respectively. Obtained Ki values of quercitrin, kaempferol and naringin on the *in vitro* FMO activity were 6.2, 12.0 and 13.9 μM, respectively. Thus, these flavonoids together are expected to be responsible for the inhibition of *in vivo* FMO activity by intake of grapefruit juice in human.

In summary, we have developed two novel non-invasive methods of determining the *in vivo* FMO activity using caffeine and ranitidine in human. FMO activity showed marked individual variations among Koreans and decreased with age. This suggested that the dose of drug should need to be adjusted to suit the individual FMO activities when the drugs metabolized by FMO are given to patients. As the intake of grapefruit juice has been identified to inhibit the FMO as well as CYP3A4 and 1A2 activities, patients taking drugs metabolized by these enzymes should not drink grapefruit juice as the carrier.

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