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Rapid Identification of the Hepatic Cytochrome P450 2C19 Activity Using a Novel and Noninvasive [^{13}C]Pantoprazole Breath Test

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ABSTRACT

We tested the hypothesis that the stable isotope [^{13}C]pantoprazole is *O*-demethylated by cytochrome P450 CYP2C19 and that the $^{13}CO_2$ produced and exhaled in breath as a result can serve as a safe, rapid, and noninvasive phenotyping marker of CYP2C19 activity in vivo. Healthy volunteers who had been genotyped for the *CYP2C19*2*, *CYP2C19*3*, and *CYP2C19*17* alleles were administered a single oral dose of [^{13}C]pantoprazole sodium-sesquihydrate (100 mg) with 2.1 g of sodium bicarbonate. Exhaled $^{13}CO_2$ and $^{12}CO_2$ were measured by IR spectroscopy before (baseline) and 2.5 to 120 min after dosing. Ratios of $^{13}CO_2/^{12}CO_2$ after [^{13}C]pantoprazole relative to $^{13}CO_2/^{12}CO_2$ at baseline were expressed as change over baseline (DOB). Maximal DOB, DOB_{15} to DOB_{120}, and area under the DOB versus time curve (AUC_{0-120} and $AUC_{0-\infty}$) were significantly different among three genotype groups (*CYP2C19*1/*1*, $n = 10$; *CYP2C19*1/*2* or *CYP2C19*1/*3*, $n = 10$; and *CYP2C19*2/*2*, $n = 5$) with predicted extensive metabolizers (EMs), intermediate metabolizers (IMs), and poor metabolizers (PMs) of CYP2C19, respectively (Kruskal-Wallis test, $p < 0.01$); linear regression analysis indicated a gene-dose effect relationship (r^2 ranged between 0.236 and 0.522; all $p < 0.05$). These breath test indices were significantly lower in PMs than IMs ($p < 0.05$) or EMs ($p < 0.01$) of CYP2C19. [^{13}C]Pantoprazole plasma exposure showed significant inverse correlation with breath test indices in the respective subjects (Pearson $r = -0.74$; $p = 0.038$). These feasibility data suggest that the [^{13}C]pantoprazole breath test is a reliable, rapid, and noninvasive probe of CYP2C19 and seems to be a useful tool to optimize drug therapy metabolized by CYP2C19.

Human cytochrome P450 CYP2C19 is important in the metabolism of several drugs, including proton pump inhibitors (e.g., omeprazole, lansoprazole, and pantoprazole), antidepressants, diazepam, carisoprodol, nelfinavir, clopidogrel, voriconazole, thalidomide, clonazepam, and cyclophosphamide (Ando et al., 2002; Desta et al., 2002; Takada et al., 2004; Hulot et al., 2006). The clearance of drugs metabolized by CYP2C19 varies 5- to 20-fold among individuals and ethnic groups primarily because of effects of genetic polymorphisms (Goldstein, 2001; Desta et al., 2002) but also as a result of nongenetic factors [(e.g., drug interactions) (Desta et al., 2002), age (Ishizawa et al., 2005), pregnancy (McGready et al., 2003), and disease state (Desta et al., 2002; Frye et al., 2006)].

S-Mephenytoin hydroxylase, which was later purified as CYP2C19 (Wrighton et al., 1993), was first reported in 1979 (Kupfer et al., 1979). The molecular basis of this polymorphism was identified later with the cloning of the gene (de Morais et al., 1994; Goldstein and de Morais, 1994). Twenty-one alleles associated with complete loss of enzyme activity (e.g., CYP2C19*2 to *8) (Goldstein, 2001; Desta et al., 2002), decreased activity (e.g., *CYP2C19*9, CYP2C19*11, and*

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Z.D., A.M., Y.K., and D.A.F. are co-owners of a patent on a clinical test designed to determine CYP2C19 activity using the [^{13}C]pantoprazole breath test (http://www.wipo.int/pctdb/en/wo.jsp?WO=2008028116&IA=US2007077362&DISPLAY=STATUS).

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ABBREVIATIONS: PM, poor metabolizer; IM, intermediate metabolizer; EM, extensive metabolizer; UM, ultrarapid metabolizer; GCRC, General Clinical Research Center; DOB, delta over baseline; PDR, percentage dose recovered; DOB_{max}, maximal DOB; T_{max}, time to maximal concentration or DOB; AUC, area under the concentration-time curve or area under DOB-time curve; C_{max}, maximal plasma concentration.

*CYP2C19*13*) (Blaisdell et al., 2002), or increased activity (*CYP2C19*17*) (Sim et al., 2006) have been reported currently (http://www.cypalleles.ki.se/cyp2C19.htm). Based on the ability to metabolize probe drugs, individuals can be categorized as CYP2C19 poor metabolizers (PMs), intermediate metabolizers (IMs), extensive metabolizers (EMs), and ultrarapid metabolizers (UMs) (Desta et al., 2002; Sim et al., 2006). The most common loss-of-function alleles accounting for the majority of PMs are *CYP2C19*2* and *CYP2C19*3* (Goldstein et al., 1997; Xie et al., 1999; Goldstein, 2001; Desta et al., 2002; Hamdy et al., 2002). The allelic frequency of the *CYP2C19*2* allele is 23 to 39%, 11 to 16%, and 13 to 25% in Asian, white, and black subjects, respectively. The frequency of the *CYP2C19*3* allele is 5 to 12% in Asians and <2% in white and black subjects. Thus, considerable interethnic differences in the distribution of PMs have been observed: e.g., 2 to 5% in whites, 4 to 7.5% in blacks, 13 to 20% in east Asians, and 38 to 79% in Pacific Islanders (Xie et al., 1999; Goldstein, 2001; Desta et al., 2002). The allelic frequency of the *CYP2C19*17* allele (associated with gain in function) ranges from 18 to 32.9% in whites, 4% in Ethiopians, and 1.3% in Japanese (Kurzawski et al., 2006; Rudberg et al., 2008; Sugimoto et al., 2008).

A growing body of evidence suggests that altered CYP2C19 activity is clinically important. Compared with EMs, PMs of CYP2C19: 1) are at increased risk for diazepam and clonazepam adverse effects (Desta et al., 2002), 2) achieve greater exposure of proton pump inhibitors, gastric acid suppression, and eradication of *Helicobacter pylori* infection (Furuta et al., 1998, 1999, 2005, 2007); and 3) show markedly less clinical response to prodrugs that requires metabolic activation by CYP2C19 (e.g., clopidogrel, cyclophosphamide, and thalidomide) (Takada et al., 2004; Hulot et al., 2006; Li et al., 2007; Gilard et al., 2008). Thus, knowledge of CYP2C19 activity may help optimize therapy and avoid adverse effects of drugs metabolized by this enzyme.

CYP2C19 metabolic status in vivo can be inferred from genotype or by measuring the metabolism of a probe substrate (Desta et al., 2002). Reliable genotyping platforms are currently available, although accurate prediction of phenotype from genotype seems difficult in some cases for similar reasons outlined for CYP2D6 recently (Gaedigk et al., 2008): uncertainty of the functional consequences of certain variants, inability to capture changes in activity caused by nongenetic factors, and the need to genotype for large number of (rare) variants and their combinations. Conventional in vivo CYP2C19 phenotyping tests (e.g., *S*-mephenytoin 4-hydroxylation or omeprazole 5-hydroxylation) are attractive tools because they can capture changes in CYP2C19 activity caused by both genetic and nongenetic factors (Desta et al., 2002). However, their routine clinical use has been limited because these procedures are time and resource intensive and often invasive. A phenotyping test that overcomes deficiencies of existing approaches would be of great value.

Stable isotope ^{13}C-labeled compounds have been increasingly used as diagnostic probes in a variety of settings (Modak, 2005), including assessment of drug metabolism (Mattison et al., 2004; Leeder et al., 2008). The main purpose of the present study was to determine whether stable isotope [^{13}C]pantoprazole is *O*-demethylated by CYP2C19, and the $^{13}CO_2$ produced and exhaled in breath as a result (Fig. 1) can serve as a safe, rapid, and noninvasive phenotyping marker of CYP2C19 activity in vivo. Pantoprazole was selected for study because of its wider clinical use, wide safety margin, extensive metabolism in the liver primarily through CYP2C19-mediated *O*-demethylation (Fig. 1) (Andersson, 1996; Tanaka et al., 1997a, 2001), and a favorable structural feature for ^{13}C stable-isotope labeling (Fig. 1).

Materials and Methods

Study Subjects. A total of 25 healthy female and male volunteers, mainly of Asian origin (18–49 years old with body weight of at least 110 pounds and body mass index ≤ 30) and pregenotyped for CYP2C19*2, *3, and *17 alleles, were studied at the outpatient clinic of the Indiana University School of the Medicine General Clinical Research Center (GCRC). This study was approved by the Institu-

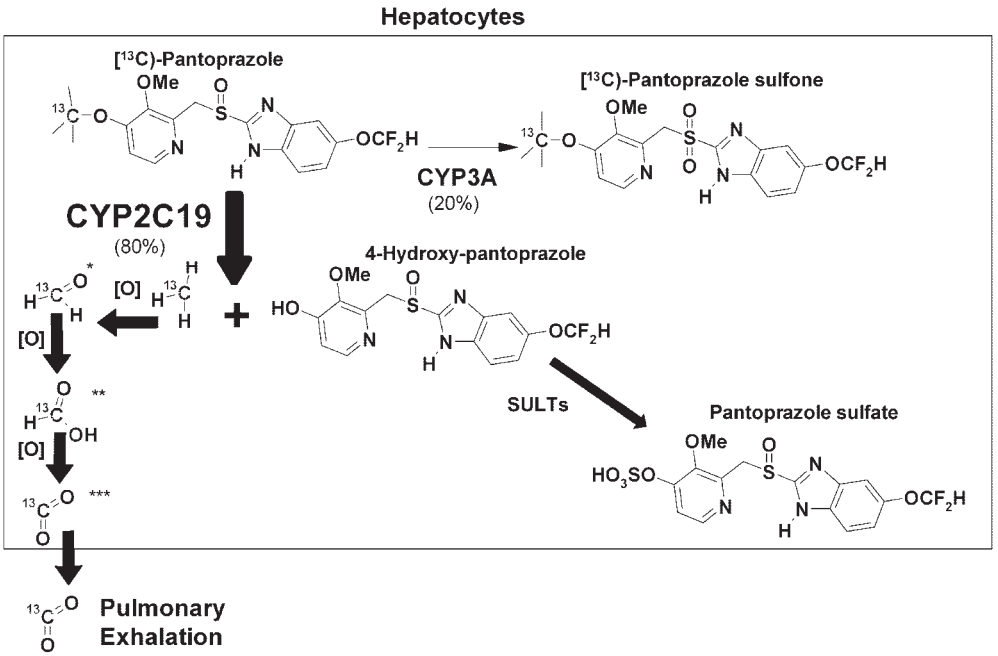

Fig. 1. Proposed human metabolism of [^{13}C]pantoprazole and production of $^{3}CO_2$ in breath. This metabolic pathway of ^{13}C labeling is inferred from known metabolism of unlabeled pantoprazole (Tanaka et al., 1997a), assuming that both have similar metabolic pathways. *, formaldehyde; **, formic acid; ***, carbon dioxide.

tional Review Board of the Indiana University. Investigative Device Exemption application G070004 to conduct the study was also approved by the Food and Drug Administration. This trial is registered at http://www.ClinicalTrials.gov (identifier: NCT00668902). All study subjects provided written informed consent before participation. Subjects were screened for any medical abnormalities within 6 weeks before initiating the breath test study and judged healthy on the basis of medical history, physical examination, vital signs, and standard laboratory tests. Blood samples (approximately 10 ml) were obtained during the screening for DNA analysis. Subjects were asked to refrain from taking any prescription, over-the-counter, or herbal medications and from alcohol consumption 1 week before the start of the study and during the study period. Excluded from the study were those who were tobacco smokers, had a history of intolerance or allergy to pantoprazole or sodium bicarbonate, had donated blood within the last 60 days of the screening visit or had planned to donate blood during the course of the study, had treatment with any investigational drug within the past 30 days, had used illegal drugs within 3 months before enrollment, were females pregnant or lactating, were females taking oral contraceptive birth control pills and who were unwilling or unable to stop oral contraceptives and use a barrier contraceptive method starting from the time of screening phase to the completion of the study, and were unreliable in the opinion of the study physician.

Study Design. This was an open-label, single-dose clinical trial. Eligible subjects were admitted to the GCRC at approximately 7:00 AM after an overnight fast. For female subjects, a urine pregnancy test was conducted before administration of study medications.

One hundred milligrams of [^{13}C]pantoprazole sodium-sesquihydrate (4-*O*-[*methyl*-^{13}C]pantoprazole, 99%; CLM-7831-SP; lot no. PR-17177), which was synthesized and supplied by Cambridge Isotope Laboratories, Inc. (Andover, MA) a powder meeting chemical purity specification (>98%), was weighed and placed into a snap-seal plastic container provided by Cambridge Isotope Laboratories, Inc. Because the pharmacokinetics of pantoprazole are linear with dose, and pantoprazole has a wide safety margin, an oral dose of 100 mg of [^{13}C]pantoprazole that represents a higher therapeutic dose range was used in this proof-of-concept study to maximize production and quantification of $^{13}CO_2$. Sodium bicarbonate (2.1 g) was weighed and transferred into the same snap-seal plastic container that contained [^{13}C]pantoprazole and dissolved with water. Because uncoated pantoprazole is acid labile, sodium bicarbonate was concomitantly dispensed with [^{13}C]pantoprazole to alkalinize the pH and facilitate absorption by preventing its degradation in the gut. This approach has been used successfully previously to prevent degradation of omeprazole (Howden, 2005) and pantoprazole (Ferron et al., 2003) by acid in the stomach. After baseline, breath samples were collected in 1.2-liter aluminum-lined bags (Otsuka Pharmaceuticals, Tokushima, Japan), and the solution containing [^{13}C]pantoprazole and 2.1 g of sodium bicarbonate was then orally administered to each subject. The caps were rinsed three times with water and administered to the subjects. Breath samples were collected at 2.5, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, and 120 min after dosing. Venous blood samples (10 ml each) were collected before (predose) and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, and 12 h after [^{13}C]pantoprazole administration. Plasma was separated by centrifugation and stored frozen at −80°C until use. All subjects were allowed to eat regular meals after the last breath test was obtained, which was 120 min (2 h) after [^{13}C]pantoprazole dosing. The subjects were allowed to drink water freely.

***CYP2C19* Genotyping.** Genomic DNA was extracted at the Indiana University GCRC Biochemistry Core laboratory from human whole blood with the QIAGEN DNA MiniKit (QIAGEN, Valencia, CA). Genotyping for *CYP2C19*2* (rs4244285), *CYP2C19*3* (rs4986893), and *CYP2C19*17* (rs12248560) was performed by use of the predeveloped TaqMan Assay-Reagents Allelic Discrimination Kits (Applied Biosystems, Foster City, CA) according to the supplier's instructions. Their assay identifications were C__ _25986767_70, C__ _27861809_10, and C__ _469857_10, respectively. Three groups of genotypes were identified: homozygous wild type (*CYP2C19*1/*1*), heterozygous (*CYP2C19*1/*2 or CYP2C19*1/*3*), and homozygous variant (*CYP2C19*2/*2*).

Quantitation of $^{13}CO_2$. The [^{13}C]pantoprazole breath test exploited the use of the ^{13}C label that is incorporated at the 4-*O*-methyl site of pantoprazole, which specifically was designed for the hepatic CYP2C19-mediated *O*-demethylation. The assumption was that because isotope-unlabeled pantoprazole is mainly cleared by CYP2C19-mediated *O*-demethylation (Tanaka et al., 1997a, 2001), [^{13}C]pantoprazole is *O*-demethylated by the same enzyme, resulting in stepwise release of $^{13}CO_2$ and ultimately elimination from the body via pulmonary exhalation (Fig. 1) and that quantification of $^{13}CO_2$ exhaled in breath will serve as a measure of in vivo hepatic CYP2C19 activity. To test this possibility, we measured the concentrations of $^{13}CO_2$ and $^{12}CO_2$ in exhaled breath samples at baseline and after dosing using the UBiT-IR300 IR spectrometry (Meretek Diagnostics, Rockville, MD) equipped with interference filters that are wavelength-selective for the absorbance of $^{13}CO_2$ and $^{12}CO_2$ (Fig. 2). The assay was conducted within 3 days of sample collection. The $^{13}CO_2$ content in breath collection bags stored at room temperature has been shown to be stable up to 210 days (Mattison et al., 2004). Enrichment of $^{13}CO_2$ in expired air was calculated at each sampling point. The delta over baseline (DOB) in the $^{13}CO_2/^{12}CO_2$ ratio after [^{13}C]pantoprazole relative to predose (baseline) $^{13}CO_2/^{12}CO_2$ ratio was calculated as follows (Mattison et al., 2004; Leeder et al., 2008):

$$\mathrm{DOB} = 1000^{*}[(^{13}CO_2/^{12}CO_2)\ \mathrm{post-dose} - (^{13}CO_2/^{12}CO_2)\ \mathrm{baseline}] / R_{PDB} \quad (1)$$

where DOB was expressed as change per milliliter (0/00) and R_{PDB} = 0.0112372 = $^{13}CO_2/^{12}CO_2$ in the international standard Pee Dee Belemnite. The relative amount of [^{13}C]pantoprazole metabolized and released into the breath as $^{13}CO_2$ at each sampling time was calculated using the equation described elsewhere (Mattison et al., 2004) and expressed as the cumulative percentage of dose recovered (PDR).

Measurement of [^{13}C]Pantoprazole. Plasma concentrations of [^{13}C]pantoprazole were measured using a previously described method (Tanaka and Yamazaki, 1996; Tanaka et al., 1997a), with slight modification. To 100 μl of plasma, internal standard (50 μg/ml phenacetin) was added and deproteinized with 200 μl of acetonitrile. After centrifugation at 3000*g* for 10 min, the supernatant was evaporated to dryness, the residue was reconstituted in 50 μl of 50 mM sodium perchlorate and acetonitrile [80:20 (v/v)] (solvent A), and 25 μl was injected into a high-performance liquid chromatography system. Separation was performed by a Chiralcel OJ column (5.0 × 150 mm, 5 μm) (Chiral Technologies, Inc., Exton, PA), and a mobile

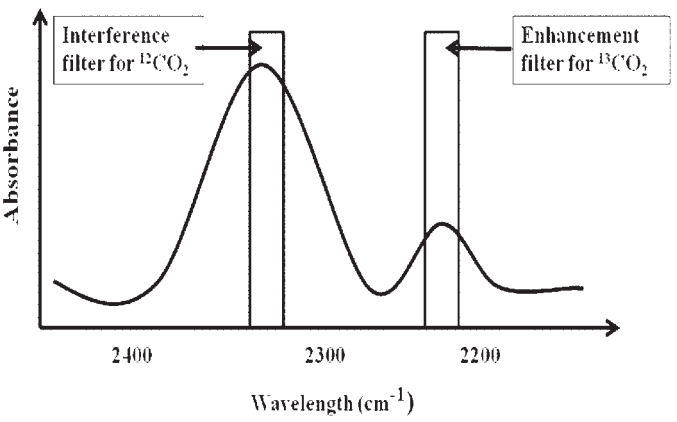

Fig. 2. Quantitation of $^{13}CO_2$ and $^{12}CO_2$ by IR spectroscopy.

phase was delivered by a gradient pump: 0 to 10 min, 100% solvent A (flow rate, 1.0 ml/min); 10 to 25 min, 100% solvent B [50 μl of 50 mM sodium perchlorate and 70:30 (v/v) acetonitrile]; and 25 to 35 min, 0% solvent A. The UV detector was set at 290 nm.

Analysis of Breath Test Indices and Pharmacokinetics. Breath test indices and pharmacokinetic parameters were determined by fitting the DOB data or plasma concentration data to a standard noncompartmental analysis using WinNonlin professional software (version 5.01; Pharsight, Mountain View, CA).

Statistical Analysis. Continuous variables were summarized by groups using descriptive statistics. Differences in pharmacokinetic parameters and breath test indices [maximal DOB (DOB_{max}), T_{max}, AUC, DOB_{30min}, and PDR] among different genotypes of CYP2C19 were analyzed by the nonparametric Kruskal Wallis test with Dunnett's multiple comparison post test. Linear regression analysis was implemented to determine gene-dose effects. Pearson's correlation analysis was performed to determine relationships between breath indices. All statistical tests were conducted using GraphPad Prism version 5.00 for Windows (GraphPad Software Inc., San Diego, CA). $p < 0.05$ was considered statistically significant.

Results

A total of 25 subjects of mainly Asian origin [eight Chinese, four Vietnamese, three Taiwanese, two Koreans, two Filipinos, one Japanese, one Indian, and four others (one Japanese/African, one Korean/white, one Chinese/Vietnamese/white, one Filipino/white] genotyped for the *CYP2C19*2*, **3*, and **17* alleles, were studied (Table 1). Ten subjects were carriers of two functional alleles (*CYP2C19*1/*1* genotype, $n = 9$; and *CYP2C19*1/*17* genotype, $n = 1$); 10 carried one loss-of function allele (*CYP2C19*1/*2* genotype, $n = 9$; and *CYP2C19*1/*3* genotype, $n = 1$), and five carried two loss-of function alleles (*CYP2C19*2/*2* genotype). The *CYP2C19*17* alleles was rare in this population, consistent with the literature (Sim et al., 2006; Sugimoto et al., 2008). However, the frequency of the *CYP2C19*3* allele was lower than what would be expected in an Asian population (Desta et al., 2002; Hamdy et al., 2002), probably because of the heterogeneity of the Asian populations studied. A subject with *CYP2C19*1/*17* genotype was analyzed, together with the *CYP2C19*1/*1* genotype, based on the inferred phenotype. CYP2C19 EMs, IMs, and PMs were inferred from *CYP2C19*1/*1* and *CYP2C19*1/*17*, *CYP2C191/*2* and *CYP2C19*1/*3*, and *CYP2C19*2/*2*, respectively. In the subsequent texts, EM, IMs, and PMs are used to reflect these specific genotype groups. There was no statistically significant difference in the distribution of demographic characteristics among the three genotype groups, except that female subjects dominated over male in EMs (6:4) and IMs (7:3) of CYP2C19 (Table 1).

Enrichment of $^{13}CO_2$ in expired air, expressed as the change or DOB in the $^{13}CO_2/^{12}CO_2$ ratio after [^{13}C]pantoprazole relative to predose (baseline) $^{13}CO_2/^{12}CO_2$ ratio, was determined as a marker of [^{13}C]pantoprazole *O*-demethylation. When data from all 25 subjects who completed the study were analyzed together, DOB values progressively increased with time, reached a DOB_{max} value (3.36 ± 1.86%) at 33.8 ± 12.36 min after [^{13}C]pantoprazole dosing, and then declined thereafter (DOB, 2.20 ± 1.2 at $t = 120$ min after dosing) (Fig. 3A). The relative amount of [^{13}C]pantoprazole metabolized and released into the breath as $^{13}CO_2$ (expressed as PDR) also progressively increased over time (cumulative PDR at $t = 120$ min; 10.43 ± 5.42) (Fig. 3B).

To test the influence of *CYP2C19* genetic polymorphisms on [^{13}C]pantoprazole *O*-demethylation, as measured by $^{13}CO_2$ in expired air, DOB values were determined in EMs, IMs, and PMs of CYP2C19 at different times (2.5–120 min) after the administration of a fixed dose of [^{13}C]pantoprazole (Fig. 4A). Upon visual inspection, DOB values were lower in PMs than IMs and EMs of CYP2C19. However, previous studies have suggested that body weight might influence breath test indices when fixed doses of phenotyping probes such as [^{13}C]uracil (Mattison et al., 2004) and dextromethorphan (Leeder et al., 2008) were administered. To test this possibility, DOB values at each time point were multiplied by body weight and plotted against time (Fig. 4B), from which DOB_{max} and area under DOB versus time curve (AUC_{0-120}) were calculated. DOB_{max} or AUC_{0-120} values after correcting for body weight significantly correlated with those without correction for body weight (Pearson $r = 0.9$; $p < 0.0001$). Therefore, data uncorrected for body weight were used for subsequent analysis. The corresponding breath test parameters are listed in Table 2. DOB_{max} and AUC_{0-120} showed a statistically significant difference among the three groups ($p < 0.01$, Kruskal-Wallis test) (Table 2) and with a gene-dose effect ($r^2 = 0.455–0.485$, $p < 0.001$). The $AUC_{0-\infty}$ showed a statistically significant difference among the three groups ($p = 0.0028$, Kruskal-Wallis test) (Table 2) and with a gene-dose effect ($r^2 = 0.225$, $p = 0.017$). T_{max} remained comparable between the genotypes ($p = 0.51$). Post hoc analysis (Dunn's multiple comparison test) revealed that PMs had significantly lower DOB_{max} and AUC_{0-120} compared with IMs ($p < 0.05$) or EMs ($p < 0.01$) of CYP2C19 (Table 2); the AUC_{0-120} in PMs (*CYP2C19*2/*2* genotype) was ~5.3-fold lower than in IMs (*CYP2C19*1/*2/*1/*3* genotypes) and

TABLE 1

Subject demographics in *CYP2C19*1/*1* (EMs), *CYP2C19*1/*2* or *CYP2C19*1/*3* (IMs), and *CYP2C19*2/*2* (PMs) genotypes

Data are presented as mean ± S.D. Note: all subjects were mainly Asian.

	CYP2C19 Metabolic Status			*p* Value
	EM ($n = 10$)	IM ($n = 10$)	PM ($n = 5$)	
Age (years)	26.2 ± 5.3	26.4 ± 6.1	24.3 ± 3.3	0.86
Height (cm)	164.4 ± 7.0	165.9 ± 9.9	163.4 ± 7.1	0.97
Weight (kg)	67.2 ± 11.3	70.0 ± 16.1	61.5 ± 5.3	0.62
BMI	24.8 ± 3.6	24.8 ± 3.5	23.0 ± 1.3	0.71
Female/male ratio	6:4	7:3	2:3	

BMI, body mass index.

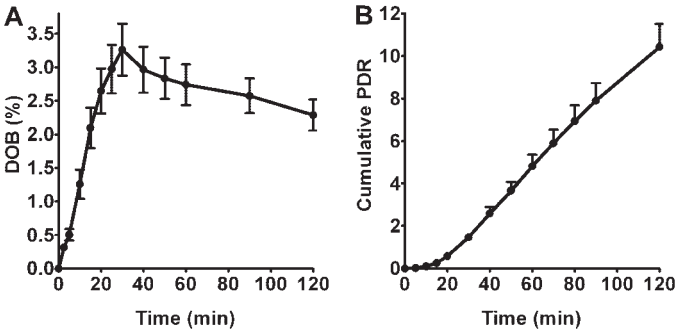

Fig. 3. $^{13}CO_2$ breath patterns (mean ± S.E.) versus time in all healthy volunteers ($n = 25$) after oral administration of a solution consisting of 100 mg of [^{13}C]pantoprazole sodium-sesquihydrate and 2.1 g of sodium bicarbonate. Breath samples were collected at baseline (before) and up to 120 min after pantoprazole dosing. The amounts of $^{13}CO_2$ in breath (expressed as DOB) versus time (A) and the cumulative PDR versus time (B) are presented as mean ± S.E.

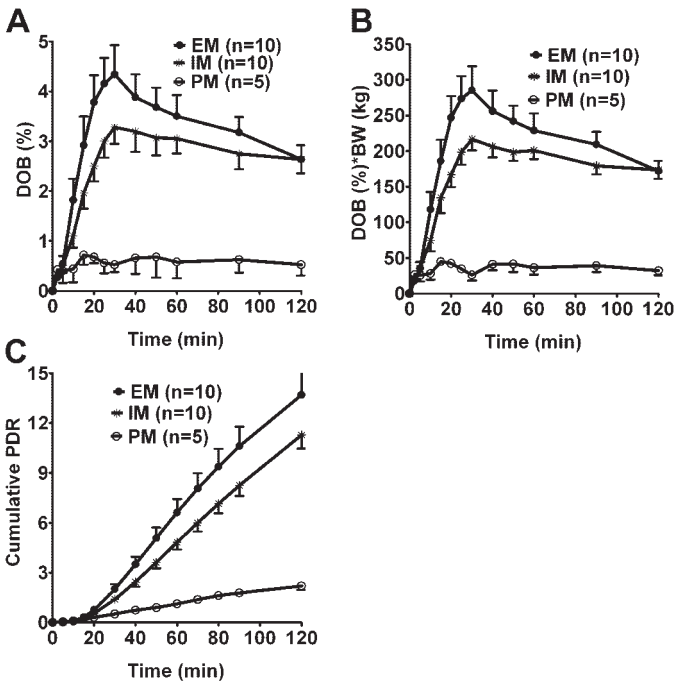

Fig. 4. $^{13}CO_2$ breath patterns (mean ± S.E.) versus time in healthy volunteers with *CYP2C19*1/*1* (EMs), *CYP2C19*1/*2* or *CYP2C19*1/*3* (IMs), and *CYP2C19*2/*2* (PMs) genotypes after oral administration of a solution consisting of 100 mg of [^{13}C]pantoprazole sodium-sesquihydrate and 2.1 g of sodium bicarbonate. Breath samples were collected at baseline (before drug administration) and up to 120 min after pantoprazole dosing. The amounts of $^{13}CO_2$ in breath (expressed as DOB) were multiplied by body weight versus time (A), body weight uncorrected DOB versus time (B), and cumulative PDR (C). Breath test indices calculated from the data in B and C are presented in Table 2.

~6.4-fold lower than in EMs (*CYP2C19*1/*1* genotype) of CYP2C19. The same trend was observed among the three genotype groups with regard to $AUC_{0-\infty}$. Although the $^{13}CO_2$ exhaled in breath was lower in genotypes that are associated with partially reduced function (IMs) than those with two fully functional alleles (EMs), this difference did not reach a statistically significant level (Table 2).

We also calculated the relative amount of [^{13}C]pantoprazole metabolized and recovered in the breath as $^{13}CO_2$ at each sampling time from which the cumulative PDR in breath could be estimated in the different genotypes (Fig. 4C). The cumulative PDR values (up to t = 120 min) are shown in Table 2. Consistent with the changes observed with DOB and AUC values, there was a statistically significant difference (Kruskal-Wallis test) in cumulative PDR values among the three genotype groups ($p < 0.01$). PMs had significantly lower cumulative PDR values (post hoc) compared with IMs ($p < 0.05$) or EMs ($p < 0.01$) of CYP2C19 (Table 2), and the effect seen was consistent with a gene-dose effect ($r^2 = 0.55$, $p < 0.0001$). Differences in cumulative PDR values among IMs and EMs did not reach a statistically significant level.

The differences in $^{13}CO_2$ breath indices among the different genotypes are more apparent when the individual values are displayed (Fig. 5, A–D). PMs of CYP2C19 had not only significantly lower $^{13}CO_2$ breath indices, but the indices in PMs did not overlap and were clearly separated from IMs and EMs of CYP2C19. Although the mean (Table 2) and median (Fig. 5, A–D) values were lower in IMs than EMs of CYP2C19, none of the $^{13}CO_2$ breath indices could segregate the two genotype groups with certainty because the values in the two groups show substantial overlap.

To determine whether [^{13}C]pantoprazole breath test serves as a rapid phenotype marker of CYP2C19 activity in the different genotypes, DOB values at each sampling point (2.5–120 min) were compared among EMs, IMs, and PMs of CYP2C19 by nonparametric analysis of variance and for gene-dose effect relationship by linear regression analysis (Table 3). A statistically significant difference among the three genotype groups was observed as early as 10 min after [^{13}C]pantoprazole dosing, but more robust relationships were seen starting 15 min (peaked at approximately 20–25 min), and this robustness continued until the last breath sampling (t = 120 min), with a significant gene-dose effect (Table 3). A multiple regression analysis was also performed in an attempt to relate $AUC_{0-\infty}$ with DOB values (10–120 min) in all subjects without considering the genotypes. Although a significant association was observed between $AUC_{0-\infty}$ and DOB at the different time points (10–120 min), model-derived r^2 was improved from $r^2 = 0.297$ for 20 min or less to $r^2 = 0.886$ for 120 min or less sampling time, suggesting that the last sampling time is more predictive of $AUC_{0-\infty}$.

The pharmacokinetic profiles of [^{13}C]pantoprazole from nine subjects (n = 3 in each genotype) are shown in Fig. 6, and the pharmacokinetic parameters derived are listed in Table 4. Significant differences in terminal elimination half-life, AUC_{0-120}, $AUC_{0-\infty}$, and apparent oral clearance (body weight adjusted) were observed among the three genotypes (all p values < 0.05, Kruskal-Wallis test) (Table 4), with post hoc analysis showing that PMs had significantly ($p < 0.05$) longer $t_{1/2}$, higher AUC_{0-12}, $AUC_{0-\infty}$, and lower clearance compared with EMs of CYP2C19. The $AUC_{0-\infty}$ of [^{13}C]pantoprazole was inversely correlated with the $AUC_{0-\infty}$ obtained from the breath test, and this was statistically significant (Pearson $r = 0.74$; $p = 0.038$).

Discussion

In the present study, we have shown for the first time that: 1) [^{13}C]pantoprazole is effectively *O*-demethylated in humans, as shown by an increase in $^{13}CO_2$ in breath; 2) $^{13}CO_2$ production was dependent on CYP2C19; and 3) enrichment of exhaled $^{13}CO_2$ through the lung was inversely related to [^{13}C]pantoprazole exposure. These data support the idea that $^{13}CO_2$ exhaled via the lung as a result of *O*-demethylation of ^{13}C-labeled pantoprazole is a reliable phenotype probe to identify PM individuals from IMs and EMs of CYP2C19.

Nonisotope-labeled pantoprazole is predominantly cleared by hepatic CYP2C19-mediated *O*-demethylation and, to some extent, through CYP3A-mediated sulfone formation (Andersson, 1996; Tanaka et al., 1997a, 2001; Furuta et al., 2005). The selection of ^{13}C-labeled pantoprazole for the study was based on the assumption that incorporation of the ^{13}C label at the 4-*O*-methyl site of the pyridine ring of pantoprazole would not influence the pattern of metabolism. Consistent with this suggestion, we have shown that [^{13}C]pantoprazole is effectively *O*-demethylated in humans, as shown by a substantial increase in the enrichment of $^{13}CO_2$ in breath over time (Fig. 3). We also have shown that [^{13}C]pantoprazole *O*-demethylation is mainly mediated by CYP2C19; the amount of $^{13}CO_2$ in breath and the cumulative percentage of

TABLE 2

[^{13}C]Pantoprazole breath test indices in healthy volunteers with *CYP2C19*1/*1* (EMs), *CYP2C19*1/*2* or *CYP2C19*1/*3* (IMs), and *CYP2C19*2/*2* (PMs) genotypes after oral administration of a solution consisting of 100 mg of [^{13}C]pantoprazole sodium-sesquihydrate and 2.1 g of sodium bicarbonate

Breath test indices are presented as mean ± S.D.

Parameters	EM (n = 10)	IM (n = 10)	PM (n = 5)	p Value[a]
T_{max} (min)[b]	30 (20–50)	30 (25–60)	40 (10–50)	0.51
DOB_{max}	4.44 ± 1.79**	3.49 ± 1.18*	0.92 ± 0.18	0.0017
AUC_{0-120}	378.9 ± 133.8**	315.8 ± 101.6*	59.6 ± 18.0	0.0019
$AUC_{0-\infty}$	744.2 ± 212.8*	852.7 ± 378.8**	163.2 ± 75.8	0.0028
PDR	13.7 ± 4.62**	11.28 ± 2.58*	2.21 ± 0.55	0.0021

* $p < 0.05$, PMs versus IMs; ** $p < 0.01$, PMs versus EMs; no statistically significant difference between IMs and EMs.
[a] Comparison between the three genotypes was made by Kruskal-Wallis statistic with post hoc analysis using Dunn's multiple comparison test.
[b] Median (minimum to maximum).

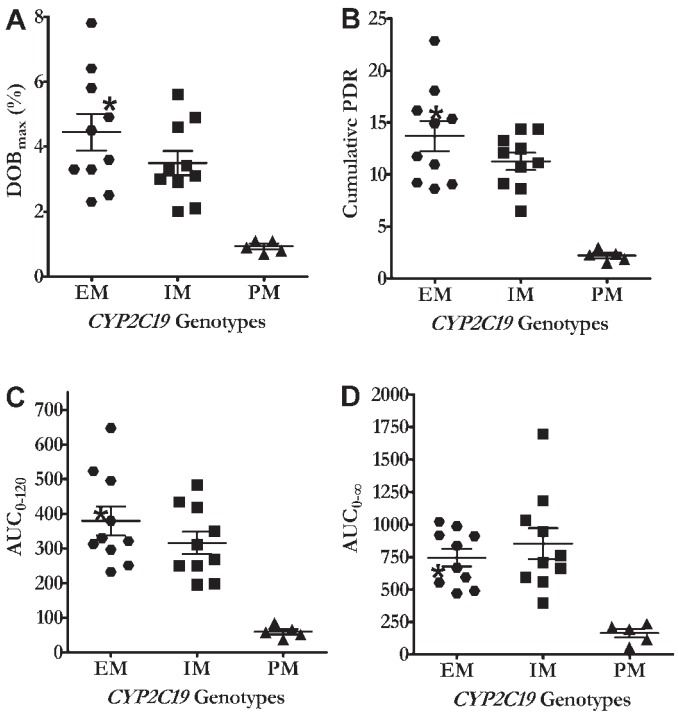

Fig. 5. Individual values of [^{13}C]pantoprazole breath test indices in healthy volunteers with *CYP2C19*1/*1* (EMs), *CYP2C19*1/*2* or *CYP2C19 *1/*3* (IMs), and *CYP2C19*2/*2* (PMs) genotypes after oral administration of a solution consisting of 100 mg of [^{13}C]pantoprazole sodium-sesquihydrate and 2.1 g of sodium bicarbonate. *, data from a subject who carried the *CYP2C19*1/*17* genotype. PDR, recovered as $^{13}CO_2$.

dose recovered as $^{13}CO_2$ in breath was significantly lower in subjects being PMs than IMs or EMs with respect to CYP2C19 (Figs. 4 and 5; Tables 2 and 3); and [^{13}C]pantoprazole exposure in plasma (Table 4) was significantly higher in PM than EM subjects. These data suggest that the differences in breath test indices among the genotypes were due to marked reduction in [^{13}C]pantoprazole *O*-demethylation in PMs and are consistent with previous data that the systemic exposure of nonisotope-labeled pantoprazole is approximately 6-fold higher in PM than in EM subjects (Tanaka et al., 1997a). Therefore, CYP2C19-mediated *O*-demethylation seems to be the major route of metabolism of both ^{13}C-labeled and -unlabeled pantoprazole; incorporation of the ^{13}C label at the 4-*O*-methyl site of the pyridine ring does not seem to alter the pattern of pantoprazole metabolism.

The time to DOB_{max} and C_{max} of [^{13}C]pantoprazole was shorter (on the average, 33.8 and 30 min, respectively) as opposed to the time to C_{max} of the unlabeled drug, which is ~2.4 h in pharmacokinetic studies (Tanaka et al., 1997a, 2001). Like other proton pump inhibitors, pantoprazole is acid labile, and the oral formulation of pantoprazole is often administered as an enteric-coated tablet to avoid degradation by gastric acid. In the present study, sodium bicarbonate was used to transiently neutralize the gastric acid and to prevent degradation of pantoprazole. This and the administration of the test compounds as a solution might have enhanced its rapid absorption, allowing a rapid and early time point breath test measurement to effectively distinguish PMs from IMs and EMs of CYP2C19. As shown in Table 3, a statistically significant difference in DOB values among the genotypes was observed as early as 10 min after [^{13}C]pantoprazole administration, and a robust difference was seen at 15 min and thereafter. This test seems to be as reliable as the established phenotyping approaches (Desta et al., 2002). Our feasibility study suggests that the [^{13}C]pantoprazole breath test offers advantages over existing genotype and phenotype approaches in predicting or assessing CYP2C19 activity in vivo, particularly in effectively distinguishing PMs from IMs and EMs of CYP2C19 (Fig. 5, A–D), because it can be performed rapidly and possibly at a single time point in a noninvasive manner. However, the optimal time breath test measurement that effectively distinguishes PMs from IMs and EMs of CYP2C19 awaits further investigation and should take into account factors such as absorption lag time. In addition, a relatively higher dose of [^{13}C]pantoprazole was used in this feasibility study to maximize the $^{13}CO_2$ signal in expired air, but the utility of the lowest doses of this probe that is appropriate for the phenotyping purpose and avoids any potential adverse effects associated with the use of high dose should be explored in the future.

Although the [^{13}C]pantoprazole breath test is expected to discriminate PMs from UMs and probably IMs from UMs, our sample size (only one subject with the *CYP2C19*1/*17* genotype) did not allow us to properly evaluate the performance of this test with respect to UMs versus other phenotype groups. The ability of the [^{13}C]pantoprazole breath test to discriminate IMs from EMs of CYP2C19 with certainty seems to be weak. Although a gene-dose effect relationship was noted regarding the influence of CYP2C19 genotype on the inferred phenotypes (Figs. 4 and 5; Tables 2 and 3), no statistically significant difference in phenotype was observed among those that were predicted to be CYP2C19 IMs and EMs. It is noteworthy that conventional probes such as omeprazole and *S*-mephenytoin also accurately discriminate PMs from IMs or EMs, but there is often uncertainty in their

TABLE 3
Analysis of DOB values at each sampling time in healthy volunteers with *CYP2C19*1/*1* (EMs), *CYP2C19*1/*2* or *CYP2C19*1/*3* (IMs), and *CYP2C19*2/*2* (PMs) genotypes after oral administration of a solution consisting of 100 mg of [^{13}C]pantoprazole sodium-sesquihydrate and 2.1 g of sodium bicarbonate

DOB Values at Each Sampling Time	Differences among Genotypes[a] (p Values)	Differences between Genotypes[b]		Gene-Dose Effect[c]	
		PMs versus IMs	PMs versus EMs	r^2	p Values
$DOB_{2.5}$	0.0455		*	0.18	0.038
DOB_{5}	0.99			0.01	0.72
DOB_{10}	0.017		*	0.236	0.014
DOB_{15}	0.0017	*	**	0.299	0.0048
DOB_{20}	0.0008	*	***	0.474	0.0001
DOB_{25}	0.0016	*	**	0.522	<0.0001
DOB_{30}	0.0044	*	**	0.459	0.0003
DOB_{40}	0.002	*	**	0.430	0.0004
DOB_{50}	0.0019	*	**	0.464	0.0002
DOB_{60}	0.0023	*	**	0.438	0.0003
DOB_{90}	0.0022	*	**	0.478	0.0001
DOB_{120}	0.003	**	**	0.366	0.0014

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. No statistically significant difference between IM and EM subjects at any of the time points.
[a] Comparison of DOB values at each sampling time among the three genotype groups (EM, IM, and PM subjects) was performed by Kruskal-Wallis test.
[b] Post hoc analysis was performed using Dunn's multiple comparison test.
[c] Gene-dose effect among the three genotypes was determined by linear regression.

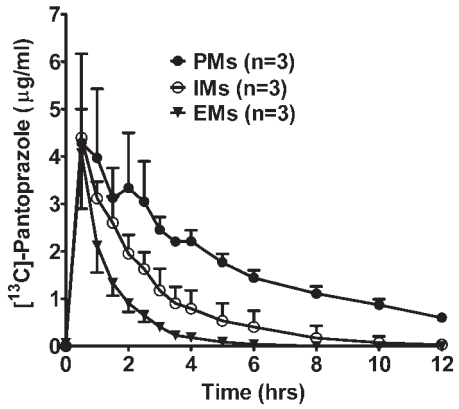

Fig. 6. [^{13}C]Pantoprazole plasma concentrations (mean ± S.D.) versus time curves in healthy volunteers with *CYP2C19*1/*1* (EMs), *CYP2C19*1/*2* or *CYP2C19*1/*3* (IMs) and *CYP2C19*2/*2* (PMs) genotypes after oral administration of a solution consisting of 100 mg of [^{13}C]pantoprazole sodium-sesquihydrate and 2.1 g of sodium bicarbonate.

ability to effectively distinguish subjects with IMs from those with EMs of CYP2C19, despite the fact that a statistically significant difference between the two groups has been reported (Yin et al., 2004; Furuta et al., 2005). Our findings suggest a better separation of IMs from EMs when [^{13}C]pantoprazole exposure is considered relative to the breath test indices. The reasons for this observation are not fully known. The methyl group formed from CYP2C19-mediated [^{13}C]pantoprazole *O*-demethylation passes through the carbon pool ($^{13}CH_3$ to ^{13}CHO to ^{13}COO and $^{13}CO_2$) before it eventually traverses to the lung and is exhaled. Differential handlings during these processes might influence the breath test indices. Although we attempted to minimize the impact of baseline $^{13}CO_2$ on the calculation of enriched $^{13}CO_2$ by requesting subjects to refrain from activities that increase ^{13}C in the body before and during the study (e.g., eating foods enriched with ^{13}C, exercise, alcohol and cigarette consumption, and nonfasting overnight before the study), the level of compliance was difficult to assess. The influence of baseline $^{13}CO_2$ level was partially corrected because the $^{13}CO_2$ measured after the administration of [^{13}C]pantoprazole was corrected for baseline values. However, given the small difference and overlapping DOB values among the IM and EM groups, the possibility that baseline $^{13}CO_2$ might influence the calculation of $^{13}CO_2$ enrichment cannot be ruled out. Third, pantoprazole is also metabolized to pantoprazole sulfone, which could be *O*-demethylated and contribute to $^{13}CO_2$ production. Although factors independent of hepatic CYP2C19 activity could potentially affect $^{13}CO_2$ measurement, this test still seems to be a noninvasive, safe, and rapid indirect surrogate marker of CYP2C19 activity in vivo.

The *CYP2C19*2/*2* genotype associated with PM status is expected to produce no $^{13}CO_2$ in breath because this genotype is expected to produce nonfunctional CYP2C19 enzymatic activity. Assuming that pantoprazole *O*-demethylation is exclusively catalyzed by CYP2C19, no production of $^{13}CO_2$ should have been expected in PMs of CYP2C19. However, as shown in Figs. 3 and 4 and Table 2, small but appreciable enrichment of $^{13}CO_2$ in breath in PM subjects was observed. These data suggest that, although CYP2C19 is the major enzyme catalyzing *O*-demethylation of pantoprazole, the contribution of other enzymes cannot be ruled out. Pantoprazole is a chiral drug that is clinically administered as a racemic mixture. In vivo, (+)-pantoprazole has been shown to be more dependent on CYP2C19 than (−)-pantoprazole (Tanaka et al., 1997b, 2001), which seems to be a characteristic of omeprazole and lansoprazole (Andersson and Weidolf, 2008). Thus, it is likely that *O*-demethylation of (−)-pantoprazole is catalyzed by cytochromes P450 other than CYP2C19, particularly when the activity of CYP2C19 is substantially diminished. The [^{13}C]pantoprazole used in our study is a racemic mixture (approximately 50:50). Because the metabolic profiles of stable isotope-labeled and -unlabeled [^{13}C]pantoprazole seem similar, it is logical to suggest that (+)-[^{13}C]pantoprazole is more dependent on CYP2C19 and that enzymes other than CYP2C19 that are involved in the *O*-demethylation of (−)-[^{13}C]pantoprazole might have contributed to the residual $^{13}CO_2$ in the breath of PMs of CYP2C19. The possibility that the sulfone metabolite of pantoprazole might also be *O*-demethylated to release $^{13}CO_2$ cannot be excluded.

In summary, given the salient features of nonradioactive ^{13}C labeling, the wide margin of pantoprazole safety, and the noninvasive, inexpensive, and rapid procedures involved, the [^{13}C]pantoprazole breath test seems to offer a useful screening method that can be applied in most clinical settings (e.g.,

TABLE 4

Pharmacokinetic parameters of [^{13}C]pantoprazole (mean ± S.D.) in healthy volunteers with *CYP2C19*1/*1* (EMs), *CYP2C19*1/*2 or CYP2C19*1/*3* (IMs), and *CYP2C19*2/*2* (PMs) genotypes after oral administration of a solution consisting of 100 mg of [^{13}C]pantoprazole sodium-sesquihydrate and 2.1 g of sodium bicarbonate

Pharmacokinetic parameters are presented as mean ± S.D.

Parameters	EM (n = 3)	IM (n = 3)	PM (n = 3)	p Value[a]
T_{max} (h)	0.5	0.5	0.5	
Half-life (h)	0.88 ± 0.14*	1.60 ± 0.61	4.23 ± 0.57	0.0439
C_{max} (μg/ml)	4.06 ± 1.16	4.39 ± 0.61	4.29 ± 1.88	0.88
AUC_{0-120} (h/μg/ml)	4.99 ± 1.08*	9.96 ± 3.43	21.19 ± 4.25	0.0439
$AUC_{0-\infty}$ (h/μg/ml)	5.09 ± 1.08*	10.35 ± 3.69	24.94 ± 4.08	0.0439
Vz/F (liters)	26.20 ± 8.35	22.20 ± 1.48	25.10 ± 7.35	0.88
Cl (ml/h/kg)	326.9 ± 97.00*	159.30 ± 52.00	67.78 ± 7.60	0.0439

AUC, area under the concentration versus time curve, 0 to 120 min or 0 to infinity; Vz/F, apparent volume of distribution; Cl, apparent oral clearance corrected for body weight.

* $p < 0.05$, PM versus EM.

[a] Comparison between the three genotypes was compared by Kruskal-Wallis statistic with post-hoc analysis using Dunn's Multiple Comparison Test.

hospitals and physicians' offices) to identify CYP2C19 function before dosing with CYP2C19 substrates. Furthermore, this novel tool should facilitate research and screening of subjects in clinical trials involving CYP2C19. Emerging evidence suggests that interindividual and interethnic differences in CYP2C19 activity influence therapeutic response of drugs, such as proton pump inhibitors, clopidogrel, cyclophosphamide, and thalidomide (Furuta et al., 1998, 2005, 2007; Desta et al., 2002; Takada et al., 2004; Gilard et al., 2008; Hulot et al., 2006; Li et al., 2007). A rapid phenotype test that captures variability of CYP2C19 enzyme activity because of genetic and nongenetic factors and potentially offers greater practical clinical utility than the existing approaches, such as the [^{13}C]pantoprazole breath test described herein, should be an important step to optimize therapy with CYP2C19 substrates or select alternative drugs for the individual patient.

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