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Stereospecific analysis of omeprazole in human plasma as a probe for CYP2C19 phenotype

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Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

Omeprazole is a class referred to as proton pump inhibitor; it acts to regulate acid production in the stomach and is used to treat various acid-related gastrointestinal disorders. In the liver, it is metabolized to varying degrees by several cytochrome P-450 (CYP) isoenzymes which are further categorized into subfamilies of related polymorphic gene products. The metabolism of omeprazole is to a large extent dependent on CYP3A4 and CYP2C19. Omeprazole is metabolized to two major metabolites, 5-hydroxyomeprazole (CYP2C19) and omeprazole sulfone (CYP3A4). Minor mutations in CYP2C19 affect its activity in the liver and, in turn, the metabolic and pharmacokinetic profiles of omeprazole. The frequency of CYP2C19 poor metabolizers in population of Asian descent has been reported to range from 10 to 20%. Accordingly, results from population studies indicate that omeprazole can be used as a probe drug for phenotyping CYP2C19. The optical isomers of omeprazole show a clear difference in their metabolism by human liver microsomes. This study demonstrates the stereospecific analysis of omeprazole in human plasma as a probe drug of CYP2C19 phenotyping. The chiral separation of omeprazole was achieved on a chiral column with circular dichroism (CD) detection and LC/MS. A good resolution of enantiomers was obtained. The column used for chiral separation was CHIRALPAK AD-RH column (4.6 × 150 mm) using phosphate buffer and (or ammonium acetate) acetonitrile as an eluent. After a single oral dose of omeprazole (20 mg), the plasma concentrations of the separate enantiomers of omeprazole were determined for 3.5 h after drug intake. The present study is useful because of the part polymorphism plays in the therapeutic effectiveness of proton pump inhibitors during the treatment of acid-related diseases.
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Keywords: Omeprazole; Proton pump inhibitor; Cytochrome P-450; CYP2C19; Stereospecific analysis; Circular dichroism detection; LC/MS

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1. Introduction

Omeprazole (5-methoxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl) methyl] sulphinyl]-1*H*-benzimidazole) is a pyridinylsulfinylbenzimidazole compound, a class referred to as proton pump inhibitor. It acts to regulate acid production in the stomach and is used to treat various acid-related gastrointestinal disorders.

The polymorphic oxidative metabolism of *S*-mephenytoin 4′-hydroxylation via cytochrome P-450 (CYP) 2C19 is well known [1]. This genetic polymorphism shows a cosegregation with the oxidative metabolism of several clinically important drugs such as diazepam, imipramine, omeprazole, propranolol, and selective serotonin reuptake inhibitors. Furthermore, this pharmacogenetic entity has shown a marked interethnic difference in the incidence of the poor metabolizer (PM) phenotype. The frequency of PM phenotype of the *S*-mephenytoin oxidation is approximately 2–6% of Caucasians, whereas the frequency of PMs in Japanese (19–23%) is much higher. Thus, if these pharmacogenetic determinants could have clinical implications, a drug whose metabolism is mediated via CYP2C19 might be of more clinical concern among Oriental patients than among Caucasian patients. The primary defect in PMs is a single base-pair mutation in exon 5 of *CYP2C19*, resulting in an aberrant splice site [2,3]. This defect (called *CYP2C19*2*; old nomenclature $CYP2C19_{m1}$) is common in both Asian and Caucasian populations. A second mutation, in exon 4 (*CYP2C19*3*; $CYP2C19_{m2}$), appears to be present only in Asians. According to a genotyping analysis of *CYP2C19*, PMs consist of three genotypes (i.e. *CYP2C19**2/*2, *3/*3 or *2/*3), while extensive metabolizer (EM) include two genotypes, homozygous (i.e. *2C19**1/*1) and heterozygous (i.e. *2C19**1/*2, *1/*3) EMs in Japanese subjects [4].

The metabolism of omeprazole is to a large extent dependent on CYP3A4 and CYP2C19. Omeprazole is metabolized to two major metabolites, 5-hydroxyomeprazole (CYP2C19) and omeprazole sulfone (CYP3A4), as shown in Fig. 1. In EMs of mephenytoin, hydroxylation by CYP2C19 is the principal route of elimination for omeprazole. Moreover, CYP2C19 also catalyzes the hydroxylation of omeprazole sulfone. The genetic polymorphism of CYP2C19 could be of clinical concern in the treatment of acid-related diseases with proton-pump inhibitors. Omeprazole is used for the treatment of infection caused by *Helicobacter pylori*. Recently, CYP2C19 genotype-related anti-*H. pylori* efficacy by the combination of omeprazole and antibiotics was reported [5,6].

We have been interested in determining the stereochemical composition of the drug in plasma after racemate administration and stereospecific analysis of chiral drugs such as benzodiazepines and 2-arylpropionic acids on a chiral stationary phase by CD detection [7–13]. Recently, we reported the determination of omeprazole and its metabolites in human plasma by LC/MS [14].

The stereoselective metabolism of the optical isomers omeprazole in human is mediated primarily by cytochrome CYP2C19, as indicated by studies using cDNA-expressed enzymes [15]. This study demonstrates the stereospecific analysis of omeprazole in human plasma as a probe drug of CYP2C19 phenotyping.

2. Experimental

2.1. Chemicals

Omeprazole and β-diphosphopyridine nucleotide disodium salt reduced form (NADPH) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Recombinant *CYP2C19* in yeast microsomes was purchased from Sumitomo Chemical Co. (Osaka, Japan). All other reagents and solvents were of analytical grade and purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. LC/MS conditions

The assay was developed using a model M-8000 LC/MS system (Hitachi, Tokyo, Japan). The absorbance was monitored at 302 nm. The analytical column was a Chiralpak AD-RH column (4.6 × 150 mm, Daicel Chemical Industries, Tokyo, Japan) operated at 40 °C. The mobile phase

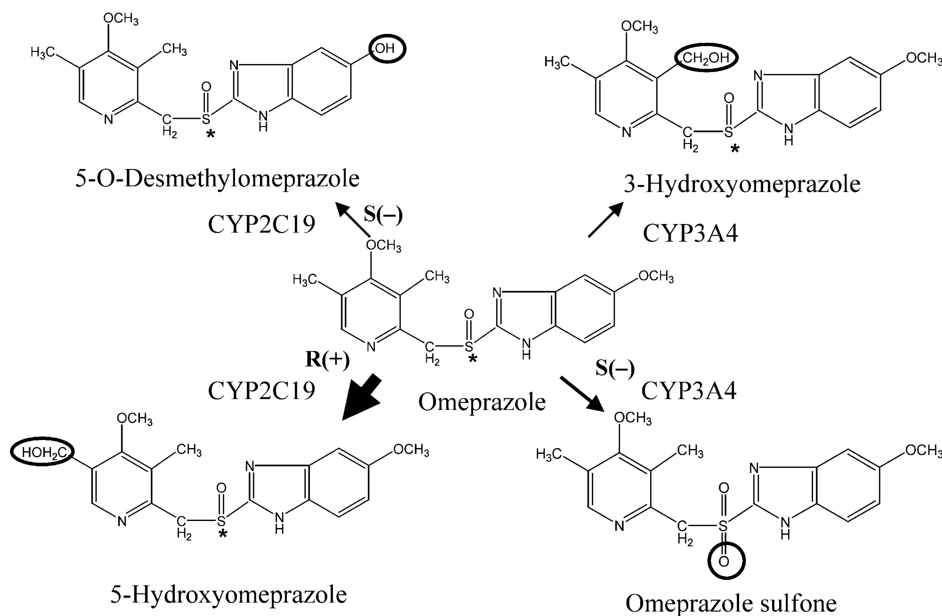

Fig. 1. Metabolic pathways of omeprazole.

was acetonitrile–50 mM ammonium acetate (pH 4.65) (35:65) at a flow-rate of 0.2 ml/min. The drift voltage was 30 V. The sampling aperture was heated at 110 °C and the shield temperature was 230 °C.

2.3. Chiral separation

High-performance liquid chromatography (HPLC) was performed using an L-6200 pump, D-2500 integrators (Hitachi, Tokyo, Japan). The column effluent was introduced to a CD-1595 circular dichroism detector (JASCO Corporation, Tokyo, Japan). The column used for chiral separation was Chiralpak AD-RH column (4.6×150 mm) using phosphate buffer/acetonitrile as an eluent and operated at 40 °C. The flow rate was 0.5 ml/min and the detection was 302 nm.

2.4. Sample preparation

Standard samples for calibration were prepared as follows: after a series of calibration standards were prepared by 10 μl omeprazole (the concentration range 0.125, 0.25, 0.50, 0.75, 1.00 mg/ml in methanol) spiked with 90 μl of drug-free human plasma, calibration curves were constructed by plotting the peak area against the concentration of the omeprazole, the data were then subjected to a linear-regression analysis.

Plasma samples were collected from healthy adults 3.5 h after a single oral dose of 20 mg omeprazole (Omepral® tablets; AstraZeneca Co. Ltd, Osaka, Japan) following an overnight fast. A 2 ml volume of the plasma was loaded into a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) after conditioning the cartridge with methanol, water and 50 mM ammonium acetate, a 5 ml volume of 50 mM ammonium acetate as a washing solution was passed through the cartridge. The sample fraction was then obtained by elution with 5 ml of methanol–50mM ammonium acetate (4:1). After evaporation under reduced pressure, the residue was dissolved in 100 μl of the eluent.

2.5. Genotyping procedures for CYP2C19

Blood samples were obtained from two Japanese subjects (female), and genomic deoxyribonucleic acid (DNA) was isolated from peripheral lymphocytes with an extraction kit (*SepaGene*®, Sanko Pure Chemicals, Tokyo, Japan). The

*CYP2C19*1* (*wt*) gene and two mutated alleles associated with deficient (*S*)-mephenytoin hydroxylation, *CYP2C19*2* (*m1*) and *CYP2C19*3* (*m2*), were identified by PCR amplification using allele-specific primers according to the methods of de Morais et al. [2,3].

2.6. In vitro experiment by recombinant CYP2C19

The basic incubation medium contained 100 mM potassium phosphate buffer (pH 7.4), 3 mM NADPH and 2.9 mM omeprazole in a final volume of 0.5 ml. After preincubation at 37 °C for 5 min, 50 pmol of recombinant CYP2C19 was added to the mixture and incubation was carried out at 37 °C for 60 min. Adding 3 ml of cold acetonitrile stopped the reaction. After terminating the incubation, the mixture was centrifuged at 3000 r.p.m. for 10 min and 3 ml of the supernatant was loaded into a Sep-Pak C_{18} cartridge. After the same treatments as described above, the residue was dissolved in 50 µl of the eluent, and a 3 µl volume of the sample was injected into a HPLC system.

3. Results and discussion

3.1. In vitro experiment by recombinant CYP2C19

Omeprazole (2.9 mM concentration) was incubated in vitro with recombinant CYP2C19 isoform. The incubation mixture and other conditions are described in Section 2. The LC-3DQMS with an SSI interface was used to analyze omeprazole and its metabolites. Following recombinant CYP2C19 incubations, chromatographic peaks were detected with retention times corresponding to those of *S*(−)5-*O*-desmethylomeprazole (18.8 min), *R*(+)5-hydroxyomeprazole (21.6 min), *S*(−)omeprazole (55.0 min) and *R*(+) omeprazole (75.7 min). Fig. 2 shows TIC, mass chromatograms and mass spectra of omeprazole and its metabolites. There was no interference from extracted components of the incubation system. The well resolved chromatograms were obtained with acetonitrile and ammonium acetate as the eluent at a flow rate of 0.2 ml/min. The mass spectrum of omeprazole is almost the same as that obtained by direct analysis. The protonated molecular ions $[M+H]^+$ of *S*(−)5-*O*-desmethylomeprazole, *R*(+)5-hydroxyomeprazole, *S*(−)omeprazole, and *R*(+)omeprazole were clearly observed at *m*/*z* 331, 361, 345 and 345, respectively. The fragment ions of each peak were observed at *m*/*z* 214 and 198, respectively. And the peaks of $[M+Na]^+$ of *S*(−)5-*O*-desmethylomeprazole, *R*(+)5-hydroxyomeprazole, *S*(−)omeprazole, and *R*(+)omeprazole were also observed at *m*/*z* 354, 384, 368 and 368, respectively.

Fig. 3 shows UV and CD chromatograms of omeprazole and its metabolites. The peaks of *S*(−)5-*O*-desmethylomeprazole, *S*(−)5-hydroxyomeprazole, *R*(+)5-hydroxyomeprazole, *S*(−) omeprazole, and *R*(+)omeprazole were observed. Recently Äbelö et al. reported that CYP2C19 favors 5-hydroxylation of the pyridine group of *R*-omeprazole, whereas the same enzyme mainly 5-*O*-demethylates *S*-omeprazole in the benzimidazole group. Sulfoxidation mediated by CYP3A4 highly favors the *S*-form [15]. A similar result was obtained in our study; omeprazole metabolized mainly *R*(+)5-hydroxyomeprazole and *S*(−)5-*O*-desmethylomeprazole by CYP2C19. A small peak of *S*(−)5-hydroxyomeprazole was observed and *R*(+)5-*O*-desmethylomeprazole were not observed with in vitro experiment by recombinant CYP2C19.

3.2. Calibration curves and precision

The linear relationship was calculated between the peak-area (y) on CD chromatogram and the concentration (x µg/ml) of omeprazole in plasma, with conditions described in Section 2. The correlation coefficients (r) were as follows:

$$S(-)\text{omeprazole}: y = 3.36x + 0.48 \ (r^2 = 0.990)$$

$$R(+)\text{omeprazole}: y = 3.73x + 0.31 \ (r^2 = 0.992)$$

The linearity between the peak-area on UV chromatogram and the concentration of omeprazole in plasma was also obtained. The lower limit of quantification for omeprazole was 2.7 ng by UV detection and 60 ng by CD detection, respectively,

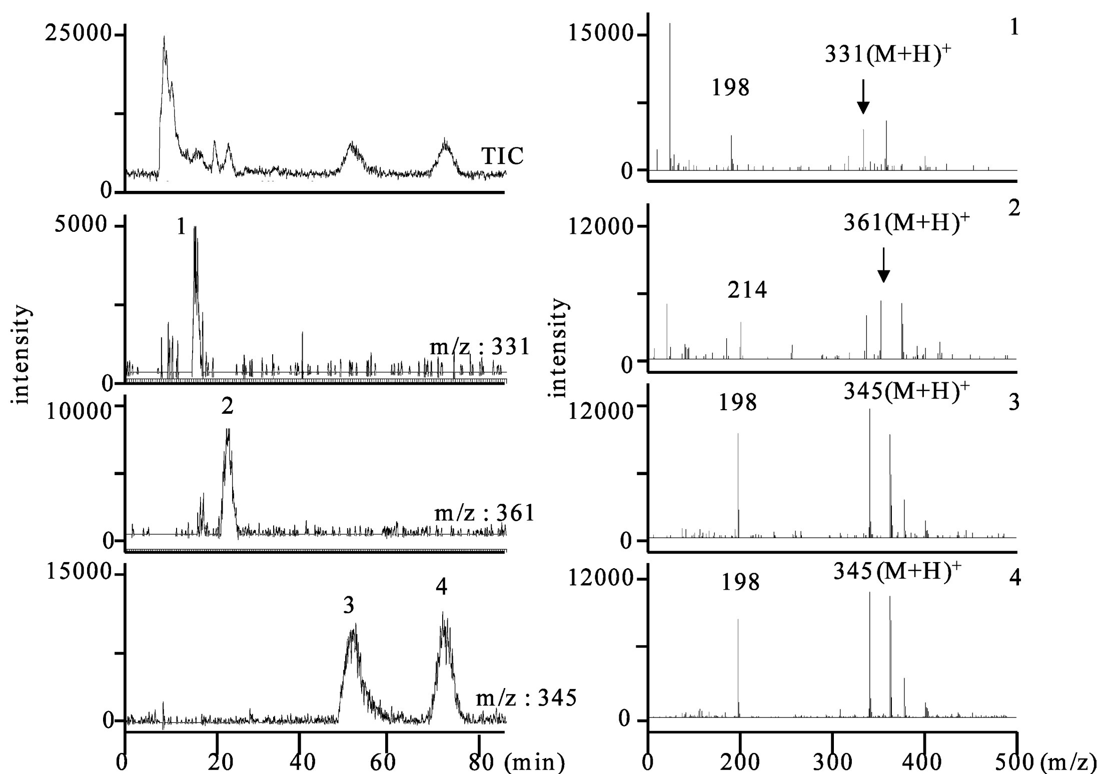

Fig. 2. TIC, mass chromatograms and mass spectra of omeprazole and its metabolites. Peaks: 1: *S*(−)5-*O*-desmethylomeprazole, 2: *R*(+)5-hydroxyomeprazole, 3: *S*(−)omeprazole, 4: *R*(+)omeprazole, column: Chiralpak AD-RH (150 × 4.6 mm I.D.), Eluent: 20 mM CH_3COONH_4 (pH 4.65)/CH_3CN = 65/35, Flow rate: 0.2 ml/min, column temperature: 40 °C, wavelength: 302 nm, Inj. vol.: 25 μl.

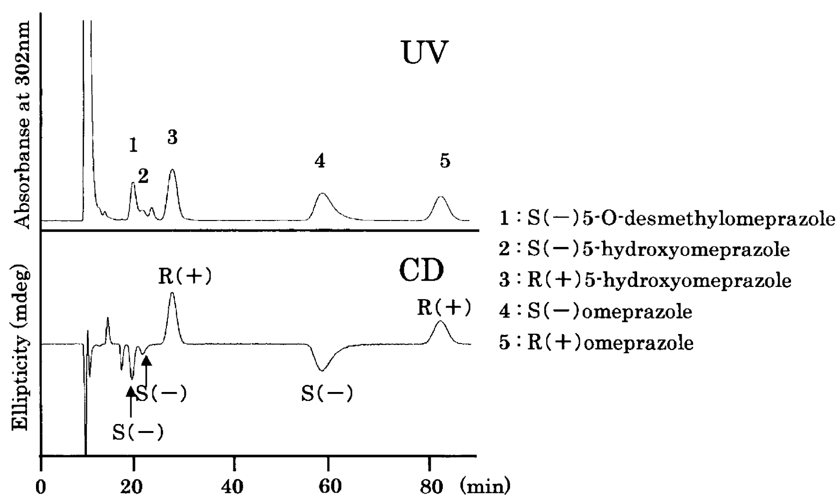

Fig. 3. UV and CD chromatograms of omeprazole and its metabolites. Column: Chiralpak AD-RH (150 × 4.6 mm I.D.), Eluent: 20 mM CH_3COONH_4 (pH 4.65)/CH_3CN = 65/35, flow rate: 0.2 ml/min, column temperature: 40 °C, wavelength: 302 nm, Inj. vol.: 20 μl.

at a signal-to-noise ratio of 3. The recoveries of the analytical procedure *S*(−)omeprazole and *R*(+) omeprazole from plasma were 91.1 and 93.5%, respectively. As shown in Table 1, the precision of the method was established from five assays. The C.V. values of the retention times were less than 0.3% and those of the peak areas on CD chromatograms were less than 3%. The present method is sufficiently sensitive and accurate to measure pharmacokinetic parameters.

3.3. Determination of plasma levels of omeprazole and its metabolites

The allele-specific polymerase chain reaction (PCR) based method allows genetic determinations, thus predicting their phenotype. Therefore, we examined the pharmacokinetic profile of omeprazole as a probe drug in relation to genotyping for two known mutations, *CYP2C19*2* and *CYP2C19*3*.

Two different human samples, which were genotyped for the *CYP2C19* gene, were used for the analysis. Stereoselective disposition of omeprazole and its formed 5-hydroxy metabolite were studied in PM and EM.

Fig. 4 shows UV and CD chromatograms of an extract of plasma sample obtained after a single dose of 20 mg omeprazole in healthy subject with heterozygous EM (subject 1, *CYP2C19*1/*2*). Well-resolved chromatograms were obtained without any influence of endogenous compounds in plasma with acetonitrile–20 mM phosphate buffer (pH 4.65) (3:7) as an eluent at a flow-rate of 0.5 ml/min. The peaks of *R*(+)5-hydroxyomeprazole, omeprazole sulfone, *S*(−)omeprazole and *R*(+) omeprazole were observed on UV chromatogram with retention times of 12.90, 21.54, 34.10 and

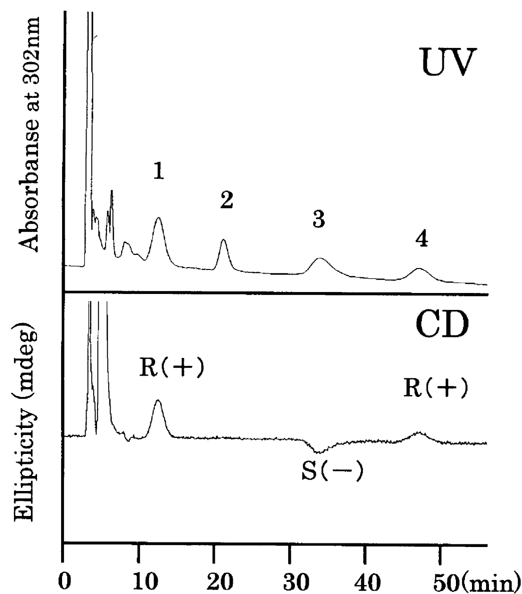

Fig. 4. UV and CD chromatograms of an extract of plasma sample obtained after a single dose of 20 mg omeprazole in healthy subject with heterozygous EM (subject 1, *CYP2C19*1/*2*). Peaks: 1: *R*(+)5-hydroxyomeprazole, 2: omeprazole sulfone, 3: *S*(−)omeprazole, 4: *R*(+)omeprazole, column: Chiralpak AD-RH (150 × 4.6 mm I.D.), eluent: 20 mM KH_2PO_4 (pH 4.65)/CH_3CN = 70/30, flow rate:0.5 ml/min, column temperature: 40 °C, wavelength: 302 nm, Inj. vol.: 60 µl.

46.93 min, respectively. The peaks of *R*(+)5-hydroxyomeprazole, *S*(−)omeprazole and *R*(+) omeprazole were also observed clearly on CD chromatogram without omeprazole sulfone, which is not optically active. It is very useful to have a dual detector, specifically to record simultaneously UV and CD. In UV detection, two enantiomers give rise to peaks characterized by identical response factors, since they have one and the same absorption spectrum. In CD detection, information on the stereochemistry of the elutes

Table 1
Precision of the enantiomer composition of racemic omeprazole

	Retention time (min)		Peak area ($\times 10^5$)	
	Mean ± S.D.	C.V. (%)	Mean ± S.D.	C.V. (%)
S(−)omeprazole	34.81 ± 0.08	0.08	2.92 ± 0.08	2.70
R(+)omeprazole	50.31 ± 0.69	0.24	3.02 ± 0.05	1.52

can be obtained. HPLC-CD analysis offers enantiomeric excess, elution order, and measurement of the CD spectrum of a product in a mixture.

Fig. 5 shows UV and CD chromatograms of an extract of plasma sample obtained after a single dose of 20 mg omeprazole in healthy subject with PM (subject 2, *CYP2C19*2/*3*). The peaks of omeprazole sulfone and $R(+)$omeprazole were greater than that in subject 1 with hetero EM. Although the peak of $R(+)$5-hydroxyomeprazole was observed, the peak was smaller than that in subject with EM. The peaks of $S(-)$- and $R(+)$omeprazole were clearly observed on both UV and CD chromatograms. The peak of omeprazole sulfone was strongly observed on UV chromatogram, as shown in Fig. 5. CD detection is inherently sensitive only to chiral nonracemic molecules. Therefore, it operates as a filter, which cuts off signals arising from achiral components. Comparison between CD and UV detection provides relevant analytical information. Although the sensitivity for omeprazole using CD detection is less than that of the method using UV detection, we determined the plasma concentration of $S(-)$ and $R(+)$omeprazole from CD chromatogram. The hydroxy metabolite from $S(-)$-omeprazole was not observed significantly in subject 1 (EM) and subject 2 (PM).

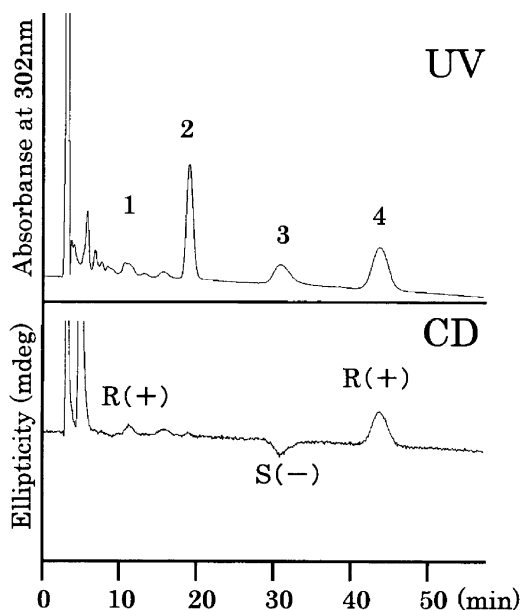

Fig. 5. UV and CD chromatograms of an extract of plasma sample obtained after a single dose of 20 mg omeprazole in healthy subject with PM (subject 2, *CYP2C19*2/*3*). Peaks: 1: $R(+)$5-hydroxyomeprazole, 2: omeprazole sulfone, 3: $S(-)$omeprazole, 4: $R(+)$omeprazole, column: Chiralpak AD-RH (150 × 4.6 mm I.D.), eluent: 20 mM KH_2PO_4 (pH 4.65)/CH_3CN = 70/30, flow rate: 0.5 ml/min, column temperature: 40 °C, wavelength: 302 nm, Inj. vol.: 60 µl.

Table 2
Plasma concentration (ng/ml) and hydroxylation index of omeprazole

	Subject 1	Subject 2
CYP2C19 genotype	**1/*2 (wt/m1)*	**2/*3 (m1/m2)*
$S(-)$omeprazole	182.2	165.4
$R(+)$omeprazole	79.4	520.8
OPZ-HI	2.18	19.28
(R)-OPZ-HI	0.23	7.62

Table 2 shows the concentrations of omeprazole determined by the present method. The plasma concentration ratio of omeprazole to 5-hydroxyomeprazole (omeprazole hydroxylation index; OPZ-HI) obtained 2–4 h after the drug intake has been used to distinguish between extensive and PM phenotypes. Since $R(+)$-omeprazole is to a major extent hydroxylated by CYP2C19, we determined $R(+)$-OPZ-HI (the relative concentration ratios of $R(+)$omeprazole to $R(+)$5-hydroxyomeprazole). The concentration of $R(+)$ omeprazole was greater in PM than in EM. The $R(+)$-OPZ-HI was higher in PM than in EM, compared with OPZ-HI.

These results indicate that a correlation exists between the rate of stereoselective metabolism of omeprazole and the genotype, and that significant differences exist in the stereoselective disposition kinetics of omeprazole among subjects with different genotype patterns.

4. Conclusion

In this study, two different human samples, which were genotyped for CYP2C19 gene, were used for analysis. The significant differences in the stereoselective metabolism rate of omeprazole among subjects with different genotype are clearly

observed. With use of the ratio between the $R(+)$-enantiomers of omeprazole and its metabolite, a better discrimination between phenotypes was obtained.

Omeprazole even has some advantages as a probe drug for CYP2C19 because of its more favorable safety profile, higher intraindividual reproducibility of phenotyping, and potential to differentiate between EM and PM.

The present method is sufficiently sensitive and accurate for a study of phenotyping of CYP2C19.

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