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Determination of rabeprazole enantiomers and their metabolites by high-performance liquid chromatography with solid-phase extraction

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Abstract

Here, we describe the development of a rapid, simple and sensitive high-performance liquid chromatography (HPLC) method for the simultaneous quantitative determination of rabeprazole enantiomers (**1a**,**b**) and their metabolites, rabeprazole-thioether (**2**) and rabeprazole sulfone (**3**), in human plasma. Analytes and the internal standard (omeprazole-thioether) were separated using a mobile phase of 0.5 M $NaClO_4$–acetonitrile (6:4, v/v) over a Chiral CD-Ph column. Analysis required only 100 μl of plasma and involved solid-phase extraction with an Oasis HLB cartridge, which gave high recovery (>91.8%) with good selectivity for all analytes. The lower limit of quantification was 5 ng/ml for analytes **1a**, **1b** and **3** and 10 ng/ml for **2**. Linearity of this assay was determined to lie between 5 and 1000 ng/ml for **1a**, **1b** and **3** and 10 and 1000 ng/ml for **2** ($r^2 > 0.982$ of the regression line). Inter- and intra-day coefficients of variation were less than 7.8% and accuracies were within 8.4% over the linear range for all analytes. Our results indicate that this method is applicable to the simultaneous monitoring of plasma levels of rabeprazole enantiomers and associated metabolites in human plasma.

Keywords: Rabeprazole; Rabeprazole-thioether; Rabeprazole sulfone; Enantiomer; HPLC

1. Introduction

Rabeprazole [2-[[4-(3-methoxypropoxy)-3-methyl-2-pyridinyl]-methylsulfinyl]-1*H*-benzimidazole] is a proton pump inhibitor (PPI) that inhibits gastric acid secretion via interaction with (H^+/K^+)-ATPase in gastric parietal cells [1–3]. This drug contains an asymmetric sulfur in its chemical structure and is clinically administered as a racemic mixture of *R*- and *S*-enantiomers (**1a** and **1b**, respectively) (Fig. 1). While rabeprazole is primarily converted non-enzymatically to rabeprazole-thioether (**2**) (Fig. 1), some is oxidized to desmethylrabeprazole by CYP2C19, which is hardly detectable in human plasma, and some to rabeprazole sulfone (**3**) by CYP3A4 (Fig. 1) [4–7].

However, to date there has been no published high-performance liquid chromatography (HPLC)-based method for the simultaneous determination of rabeprazole enantiomers and associated metabolites in human plasma. While an HPLC method for the simultaneous determination of rabeprazole enantiomers and four metabolites has been reported for dog plasma, this assay required a 1 ml plasma sample and had a quantification limit of 30 ng/ml for each compound [8].

The method presented here is rapid and simple, and consists of a solid-phase extraction followed by selective HPLC allowing the simultaneous determination of **1a**, **1b**, **2** and **3** in human plasma. The extraction procedure used for the plasma sample pre-treatment ensures high recovery from a relatively small amount of plasma (100 μl) for complete analysis. To validate this method, we used it to investigate the pharmacokinetics of rabeprazole enantiomers and their metabolites in humans [9]. The method is already being used to measure plasma concentrations of rabeprazole in renal transplant recipients.

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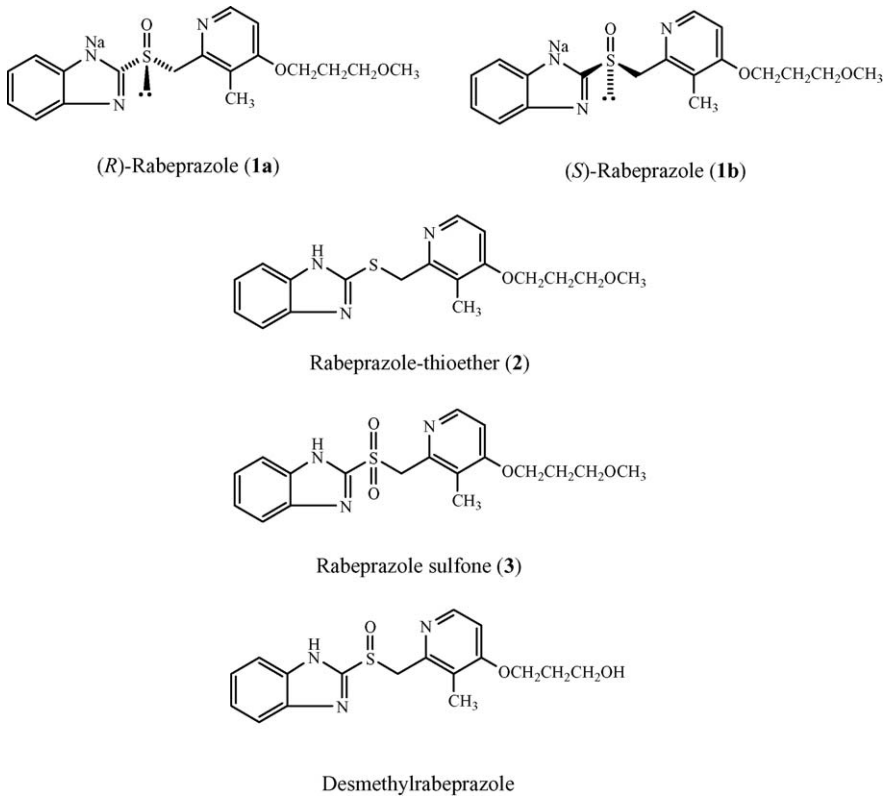

Fig. 1. Chemical structures of (*R*)-rabeprazole, (*S*)-rabeprazole, rabeprazole-thioether, rabeprazole sulfone and desmethylrabeprazole.

2. Experimental

2.1. Chemicals and reagents

Rabeprazole enantiomers, rabeprazole-thioether, rabeprazole sulfone, desmethylrabeprazole and omeprazole-thioether were donated by Eisai Co. Ltd. (Tokyo, Japan). The Oasis HLB extraction cartridge was purchased from Waters (Milford, MA, USA). All other reagents and chemicals were purchased from Wako Chemical Industries or Nacalai Tesque (Kyoto, Japan). All solvents were of HPLC grade.

2.2. Chromatographic conditions

A Model 510 chromatography pump (Waters) equipped with a Waters 486 ultraviolet detector was used. The HPLC column used was a Chiral CD-Ph (250 mm × 4.6 mm i.d., Shiseido, Tokyo, Japan) with a mobile phase consisting of 0.5 M $NaClO_4$–acetonitrile (6:4, v/v), which was degassed in an ultrasonic bath prior to use. A flow-rate of 0.5 ml/min was used at ambient temperature and sample detection was carried out at 285 nm.

2.3. Extraction method

Following the addition of omeprazole-thioether (20 ng) in methanol (10 μl) to 100 μl plasma samples as an internal standard, plasma samples were diluted with 1.0 ml water, and vortexed for 30 s. This mixture was applied to an Oasis HLB extraction cartridge that had been activated previously with methanol and water (1.0 ml each). Cartridges were then washed with 1.0 ml 60% methanol in water, and eluted with 1.0 ml 100% methanol. Eluates were evaporated to dryness under vacuum at 40 °C using a rotary evaporator (Iwaki, Tokyo, Japan). The residues were then dissolved in 50 μl methanol, vortex-mixed for 30 s, 50 μl mobile phase was added and samples vortex-mixed for a further 30 s. Aliquots of 50 μl were then injected into the HPLC apparatus.

2.4. Identification of elution orders of desmethylrabeprazole enantiomers

The elution order of desmethylrabeprazole enantiomers in the HPLC chromatogram was identified by the in vitro metabolism of (*R*)- or (*S*)-rabeprazole using human CYP2C19 expressed in a cell line (Gentest Corporation, Woburn, MA, USA). Incubations were carried out with reconstituted human liver microsomes in 5 ml test tubes using a shaking water bath for 30 min at 37 °C. A typical incubation mixture consisted of a cofactor solution (100 μl), microsomal CYP2C19 preparation (50 μl, 0.5 mg protein) and substrate (5 μl, 131 μM for (*R*)- or (*S*)-rabeprazole) in a total volume of 0.2 ml. The cofactor solution consisted of $NADP^+$ (1.3 mM), glucose-6-phosphate (3.3 mM), glucose-6-phosphate dehydrogenase (0.4 units) and magnesium chloride (3.3 mM) in sodium phosphate buffer (0.1 M, pH 7.4). The metabolic reaction was initiated by the addition of cofactor solution and terminated by immersion in an ice bath. Before the extraction, omeprazole-thioether (20 ng) in methanol (10 μl)

was added to the incubation mixture as an internal standard. Each mixture was applied to an Oasis HLB® extraction cartridge as described above.

2.5. Calibration graphs

Calibration curves were obtained for spiked blank plasma samples in concentration ranges of 5–1000 ng/ml for **1a**, **1b** and **3** and 10–1000 ng/ml for **2**. Blank plasma samples were treated as described above. Calibration graphs were constructed as peak-height ratios of each analyte to the omeprazole-thioether internal standard from the HPLC chromatograms and then plotted against the nominal concentration of each analyte.

2.6. Recovery

Recovery following the extraction procedure was determined by comparing the peak areas of blank plasma samples extracted according to the above procedure with those of non-extracted control samples. Control samples were prepared by mixing solutions containing the same amount of compound as spiked into blank plasma but directly evaporated to dryness rather than extracted, and then reconstituted in methanol.

2.7. Assay validation

Inter-day precision and accuracy were evaluated from the analysis of control samples measured on five different days, whereas intra-day precision and accuracy were evaluated by analyzing spiked controls five times over the course of 1 day in random order. The precision of the method at each concentration was evaluated by comparing the coefficient of variation (C.V.) obtained by calculating the standard deviation (S.D.) as a percentage of the mean calculated concentration, with the accuracy estimated for each spiked control by comparing the nominal concentration with the assayed concentration. Limits of quantification (LOQ) were determined as the lowest non-zero concentration measured with an intra-day C.V. of <20% and an accuracy of <±20% [10], and limits of detection (LOD) determined as the lowest concentration with a signal to noise ratio of 3.

2.8. Application to pharmacokinetics studies

The method was used to quantitate the plasma concentrations of rabeprazole enantiomers and rabeprazole-thioether in renal transplant recipients that were either extensive metabolizers (EMs) or poor metabolizers (PMs) of CYP2C19. This study was approved by the Ethics Committee of Akita University Hospital, and all patients gave written informed consent. Renal transplant recipients that were receiving combination immunosuppressive therapy consisting of tacrolimus and mycophenolate mofetil as equally divided doses every 12 h at a designated time (9 a.m. and 9 p.m.), were given 20 mg of the Pariet® brand of rabeprazole (Eisai) at 8 a.m. (30 min after breakfast). Meals were served at 7:30 a.m., 12:30 p.m. and 6 p.m. daily. While meal content (Japanese food) varied each day for each patient, energy, fat, protein and water contents were standardized (energy: 1700–2400 kcal, protein: 70–90 g, fat: 40–50 g and water: 1600–2000 ml) according to body weight. On day 28 after renal transplantation, whole blood samples (2 ml) were collected by venipuncture at: 1, 2, 3, 4, 5, 7, 10, 13 and 24 h after oral racemic rabeprazole administration. Plasma was isolated by centrifugation at 1900 × *g* for 15 min and stored at −30 °C until analysis, which was usually carried out within 1 week of collection [11]. Patient plasma samples (100 μl) were then extracted as described above and injected into the HPLC system. Pharmacokinetic analysis of rabeprazole enantiomers and rabeprazole-thioether were carried out according to a standard non-compartmental method using WinNonlin software (Pharsight Co., CA, Version 3.1). Total area under the observed plasma concentration–time curves (AUC) was calculated using the linear trapezoidal rule. Values for the maximum plasma level (C_{max}) and time to reach the peak (t_{max}) were obtained directly from the profile.

3. Results and discussion

3.1. Chromatograms

In the present study, we describe a sensitive and specific HPLC method for the simultaneous determination of concentrations of rabeprazole enantiomers, rabeprazole sulfone and rabeprazole-thioether in human plasma.

Typical chromatograms obtained for blank plasma and for spiked plasma samples spiked with racemic desmethylrabeprazole and rabeprazole (50 ng/ml each), and metabolites **2** and **3** (50 ng/ml each) are shown in Fig. 2. The enantiomers of both desmethylrabeprazole and rabeprazole were well separated. By in vitro analysis using recombinant CYP2C19 (*R*)- and (*S*)-desmethylrabeprazole resolved as peaks **4a** and **4b**, respectively, in Fig. 2. In addition, the separation of **1a**, **1b**, **2**, **3** desmethylrabeprazole, and omeprazole-thioether was also satisfactory and free of interfering peaks in the biological matrix using our extraction method and chromatographic system. Although the total HPLC run time was relatively long (about 60 min), to our knowledge the method described in this paper is the first suitable for the enantioselective determination of rabeprazole and three rabeprazole metabolites in human plasma. Desmethylrabeprazole could not be detected in human plasma [5,12], and we therefore did not carry out precision and accuracy studies of (*R*)- and (*S*)-desmethylrabeprazole herein. However, all other analyte peaks were clearly separated following chiral separation using a Chiral CD-Ph analytical column (Shiseido).

3.2. Calibration curves

Calibration curves for all analytes in plasma were found to be linear over the concentration ranges of 5–1000 ng/ml for **1a**, **1b** and **3** and 10–1000 ng/ml for **2**. The calibration curves (obtained using the least-squares method) for **1a**, **1b**, **2** and **3** could be expressed as the equations: $Y = 0.007X - 0.061$ ($r^2 = 0.982$); $Y = 0.006X - 0.016$ ($r^2 = 0.983$); $Y = 0.004X + 0.016$ ($r^2 = 0.993$)

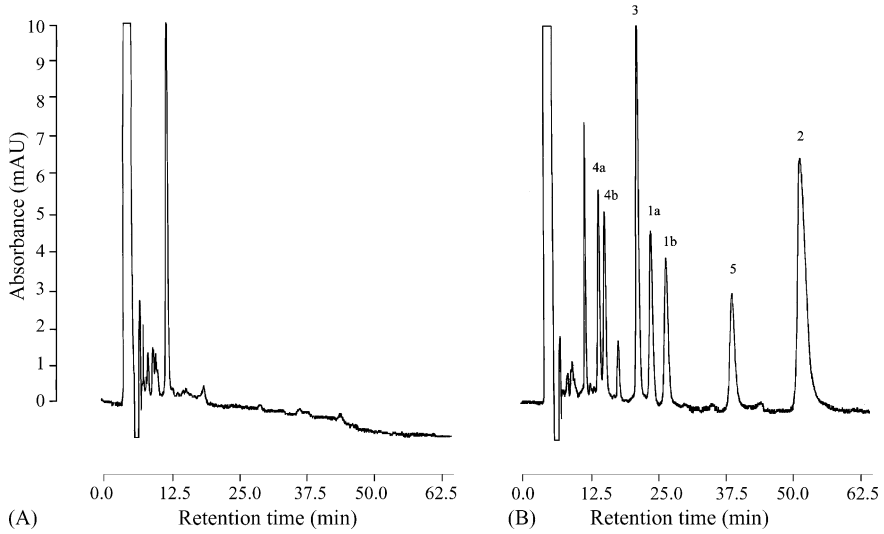

Fig. 2. Typical chromatograms of (A) plasma blank, (B) 100 μl plasma spiked with racemic rabeprazole (**1ab**) (50 ng), rabeprazole-thioether (**2**) (50 ng), rabeprazole sulfone (**3**) (50 ng), racemic desmethylrabeprazole (**4ab**) (50 ng) or omeprazole-thioether (**5**) (20 ng). Peaks: **1a**, (*R*)-rabeprazole; **1b**, (*S*)-rabeprazole; **2**, rabeprazole-thioether; **3**, rabeprazole sulfone; **4a**, (*R*)-desmethylrabeprazole; **4b**, (*S*)-desmethylrabeprazole and **5**, omeprazole-thioether (IS).

and $Y = 0.011X + 0.006$ ($r^2 = 0.996$), respectively, with Y being the peak height ratio and X being the concentration in ng/ml.

3.3. Recovery

The results of recovery studies from human plasma are shown in Table 1, with the recovery of **1a** and **1b** determined by adding five known racemic rabeprazole concentrations (10, 100, 500, 1000 and 2000 ng/ml), and the recovery of **2** and **3** determined by adding known concentrations (10, 50, 100, 500, 1000 ng/ml and 5, 50, 100, 500 and 1000 ng/ml, respectively) to drug-free plasma sample. Mean extraction recovery values for **1a**, **1b** and **3** were 95.2–97.8, 94.1–97.3 and 96.2–97.8%, respectively, within concentration ranges of 5–1000 ng/ml, while recovery of **2** was 91.8–93.2% within the concentration range of 10–1000 ng/ml.

Assays that require small sample volumes are very useful for routine drug monitoring of patients. Previously published extraction procedures developed for the analysis of rabeprazole in human plasma have been based on liquid–liquid extraction, which require plasma volumes of at least 1 ml [11,13–15]. In

Table 1
Accuracy and precision of the determination of rabeprazole enantiomers and their metabolites in human plasma ($n = 5$)

Compounds	Added (ng/ml)	Intra-day			Inter-day			Recovery (%)
		Found mean ± S.D.	C.V. (%)	Accuracy (%)	Found mean ± S.D.	C.V. (%)	Accuracy (%)	
(*R*)-Rabeprazole	5.0	5.2 ± 0.2	3.9	4.4	5.2 ± 0.3	6.0	4.8	95.2
	50	49 ± 1.8	3.7	−1.2	49 ± 1.6	3.4	−2.4	95.8
	250	247 ± 11	4.3	−1.0	248 ± 12	4.9	−1.0	96.0
	500	497 ± 8.5	1.7	−0.5	494 ± 13	2.6	−1.2	96.4
	1000	1004 ± 14	1.4	0.4	1001 ± 16	1.6	0.1	97.8
(*S*)-Rabeprazole	5.0	5.1 ± 0.3	5.3	2.8	5.0 ± 0.4	7.8	0.8	94.1
	50	48 ± 1.7	3.4	−3.3	48 ± 1.5	3.1	−3.2	96.2
	250	245 ± 11	4.4	−2.2	243 ± 10	4.3	−2.9	95.8
	500	492 ± 6.9	1.4	−1.6	487 ± 11	2.4	−2.6	96.8
	1000	997 ± 15	1.5	−0.3	994 ± 16	1.6	−0.6	97.3
Rabeprazole-thioether	10	9.4 ± 0.3	3.1	−6.4	9.2 ± 0.3	3.6	−8.4	91.8
	50	47 ± 1.2	2.6	−6.0	46 ± 1.1	2.5	−7.2	92.4
	100	96 ± 1.1	1.2	−3.6	96 ± 1.1	1.2	−4.4	92.2
	500	481 ± 7.9	1.7	−3.8	479 ± 7.5	1.6	−4.2	92.8
	1000	975 ± 8.6	0.9	−2.5	972 ± 7.1	0.7	−2.8	93.2
Rabeprazole sulfone	5.0	5.0 ± 0.3	5.7	0.4	4.9 ± 0.2	4.2	−1.2	96.2
	50	50 ± 2.1	4.1	0.8	50 ± 2.1	4.1	0.8	96.6
	100	102 ± 3.0	3.0	1.6	100 ± 2.6	2.6	0.4	97.4
	500	499 ± 9.6	1.9	−0.2	495 ± 8.9	1.8	−1.0	97.8
	1000	1005 ± 15	1.5	0.5	998 ± 13	1.3	−0.2	97.2

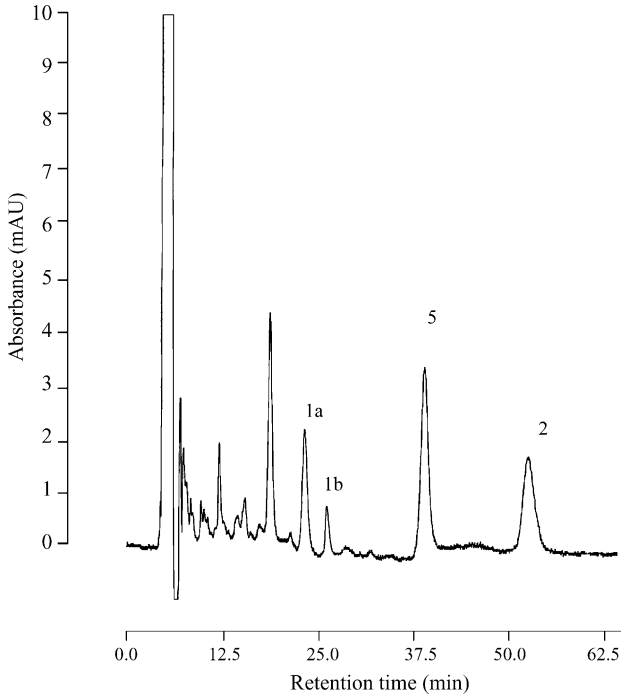

Fig. 3. Typical chromatograms of a plasma sample taken 5 h after oral administration of 20 mg racemic rabeprazole to a patient with CYP2C19 EM. Calculated concentrations of (*R*)-rabeprazole (**1a**), 105 ng/ml; (*S*)-rabeprazole (**1b**), 45 ng/ml; rabeprazole-thioether (**2**), 125 ng/ml and omeprazole-thioether (**5**), 200 ng/ml.

contrast, the solid-phase extraction procedure described here needs only a small amount of plasma (100 μl) for one complete analysis while providing high extraction recovery (>91.8% for all compounds) and good selectivity.

3.4. Precision and accuracy

The coefficients of variation and accuracy for intra- and inter-day assays were determined at concentrations of 5–1000 ng/ml for **1a**, **1b** and **3** and 10–1000 ng/ml for **2**. C.V. values for intra- and inter-day assays were less than 7.8% for all analytes (Table 1). Accuracies for intra- and inter-day assays were within 4.8% for **1a** and 3.3% for **1b**. The percentage error values for the metabolites were less than 8.4% for **2** and 1.6% for **3**.

3.5. Sensitivity

Values for the lower limit of quantification and limit of detection from 100 μl of plasma were 5.0 and 1.0 ng/ml, respectively, for **1a**, **1b** and **3**, and 10 and 5.0 ng/ml, respectively, for **2**.

3.6. Application

To test our new HPLC method, we measured the concentration of (*R*)- and (*S*)-rabeprazole in plasma samples obtained from renal transplant recipients. Chromatograms of a plasma sample collected from a CYP2C19 EM patient 5 h after administration of a 20 mg oral dose of racemic rabeprazole are shown in Fig. 3. The peaks corresponding to **1a**, **1b** and **3** were well separated, with no interfering peaks detected at the retention of each analyte.

Time courses of the mean concentrations of rabeprazole enantiomers and rabeprazole-thioether in plasma samples from three CYP2C19 EM and three CYP2C19 PM renal transplant recipients receiving 20 mg rabeprazole daily are shown in Fig. 4, and the pharmacokinetic parameters for each compound given in Table 2. The mean maximum concentration (C_{max}) for (*R*)- and (*S*)-rabeprazole in the CYP2C19 EM patients were 219 and 186 ng/ml, respectively, whereas the mean C_{max} of (*R*)- and (*S*)-rabeprazole in the CYP2C19 PM patients were 399 and 423 ng/ml, respectively. For the CYP2C19 EM patients, the half-life ($t_{1/2}$) values for (*R*)- and (*S*)-rabeprazole were 1.7 and 0.9 h, respectively, whereas the mean $AUC_{(0-\infty)}$ values were 754 and 528 ng h/ml, respectively. However, for the CYP2C19 PM patients the half-life ($t_{1/2}$) values for (*R*)- and (*S*)-rabeprazole were 2.1 and 1.2 h, with mean $AUC_{(0-\infty)}$ values of 1207 and 954 ng h/ml, respectively. Our HPLC method was applied to the

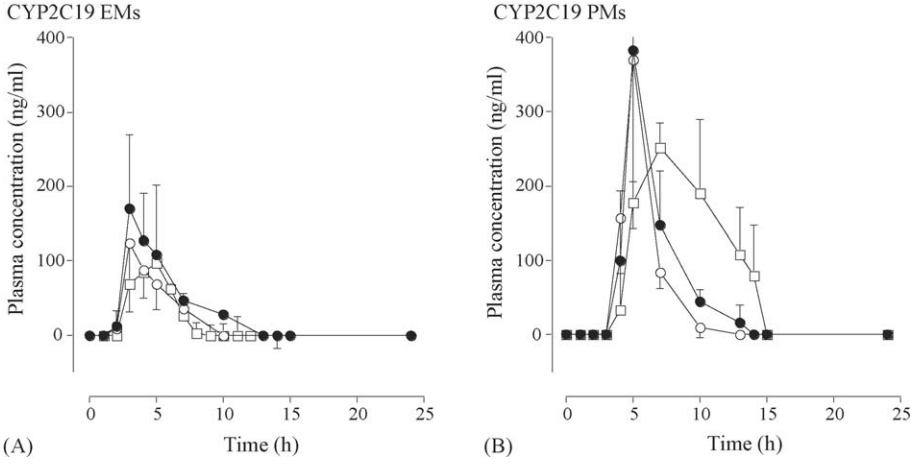

Fig. 4. Plasma concentration–time profiles of (*R*)-rabeprazole (solid circles), (*S*)-rabeprazole (open circles) and rabeprazole-thioether (open squares) after oral administration of 20 mg racemic rabeprazole to renal transplant recipients with CYP2C19 EM (A) or PM (B) ($n = 3$ each).

Table 2
Pharmacokinetic parameters of rabeprazole enantiomers and rabeprazole-thioether in renal transplant recipients

Parameter	CYP2C19 EMs			CYP2C19 PMs		
	(*R*)-Rabeprazole	(*S*)-Rabeprazole	Rabeprazole-thioether	(*R*)-Rabeprazole	(*S*)-Rabeprazole	Rabeprazole-thioether
C_{max} (ng/ml)	219 ± 79	186 ± 68	138 ± 74	399 ± 279	423 ± 153	252 ± 33
t_{max} (h)	4.5 ± 1.5	4.5 ± 1.5	5.2 ± 1.1	4.5 ± 0.7	4.5 ± 0.7	7.0 ± 0.4
Half-life (h)	1.7 ± 0.6*	0.9 ± 1.1	2.7 ± 1.4	2.1 ± 0.2*	1.2 ± 0.2	4.2 ± 2.5
$AUC_{0–24}$ (ng h/ml)	754 ± 445	528 ± 413	814 ± 112	1207 ± 591	954 ± 216	2182 ± 1044

Values are presented as the mean ± S.D. of three patients. C_{max}, maximum plasma concentration; t_{max}, time to reach C_{max}; $AUC_{0–24}$, area under the plasma concentration–time curve from 0 to 24 h.
* $P < 0.05$ compared with the (*S*)-enantiomer.

pharmacokinetic study of rabeprazole in renal transplant recipients that also received tacrolimus and mycophenolate mofetil for immunosuppression [9], and no interference between these compounds and the analytes was observed (Fig. 3).

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