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A simple and sensitive bioanalytical assay for simultaneous determination of omeprazole and its three major metabolites in human blood plasma using RP-HPLC after a simple liquid–liquid extraction procedure

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Abstract

A simple, sensitive and specific reverse-phase high-performance liquid chromatography (HPLC) assay for the simultaneous quantitative determination of omeprazole and its three metabolites in human plasma was developed and validated. This method provides excellent chromatographic resolution and peak shape for the four components and the internal standard within a 17 min run time. The simple extraction method results in a clean base line and relatively high extraction efficiency. The method was validated over the range of 2–2000 ng/mL, with 2.0 ng/mL as the lower limit of quantification. Within- and between-day accuracies for five different concentrations ranged from 95 to 102%, and 95 to 114%, respectively. Within- and between-day precision ranged from 1.1 to 6.3% and 0.5 to 6.2%, respectively. Simplicity and high throughput make this method suitable for clinical pharmacokinetic studies.

Keywords: Chromatography; HPLC; Omeprazole; 5-Hydroxyomeprazole; Omeprazole sulfone; Omeprazole sulfide

1. Introduction

Omeprazole, a substituted benzimidazole (5-hydroxy-2-[[(4-methoxy-3, 5-dimethy-2-pyridinyl)methyl]sulfonyl]-1H-bezimidazole) (Fig. 1) is a proton pump inhibitor. It decreases acid production in the stomach and is used to treat various acid-related gastrointestinal disorders [1]. In the liver, omeprazole is extensively metabolized to three primary metabolites: omeprazole sulfone, 5-hydroxyomeprazole, and omeprazole sulfide [2–4]. Omeprazole sulphone and 5-hydroxyomeprazole are the major metabolites in plasma, while 5-hydroxyomeprazole is the major metabolite in urine [2–4].

Omeprazole is metabolized by the cytochrome P-450 (CYP) 2C19 enzyme to 5-hydroxyomeprazole. CYP2C19 exhibits genetic polymorphisms [5–8], and characterization of the polymorphic CYP2C19 phenotype can be performed by measuring a plasma metabolic ratio (omeprazole/5-hydroxyomeprazole), after oral administration of a 40 mg dose of omeprazole [9]. Omeprazole is also metabolized to omeprazole sulfone by CYP3A isozymes. Although CYP3A activity can be measured by other established biomarkers such as midazolam or erythromycin [10,11], a method allowing the evaluation of both enzyme activities with the administration of just one drug would be very useful.

Methods that simultaneously measure omeprazole and 5-hydroxyomeprazole and omeprazole sulfone have been published [12,13]. Other published methods measure omeprazole, omeprazole sulfone, and omeprazole sulfide [6,10].

In this manuscript, we summarize the development and validation of the simultaneous determination of omeprazole and its three metabolites in small sample volumes after a simple and highly reproducible liquid–liquid extraction procedure.

2. Experimental

2.1. Chemicals

Omeprazole was purchased from the Sigma Chemical Company (St. Louis, MO, USA). Phenacetin, manufactured by

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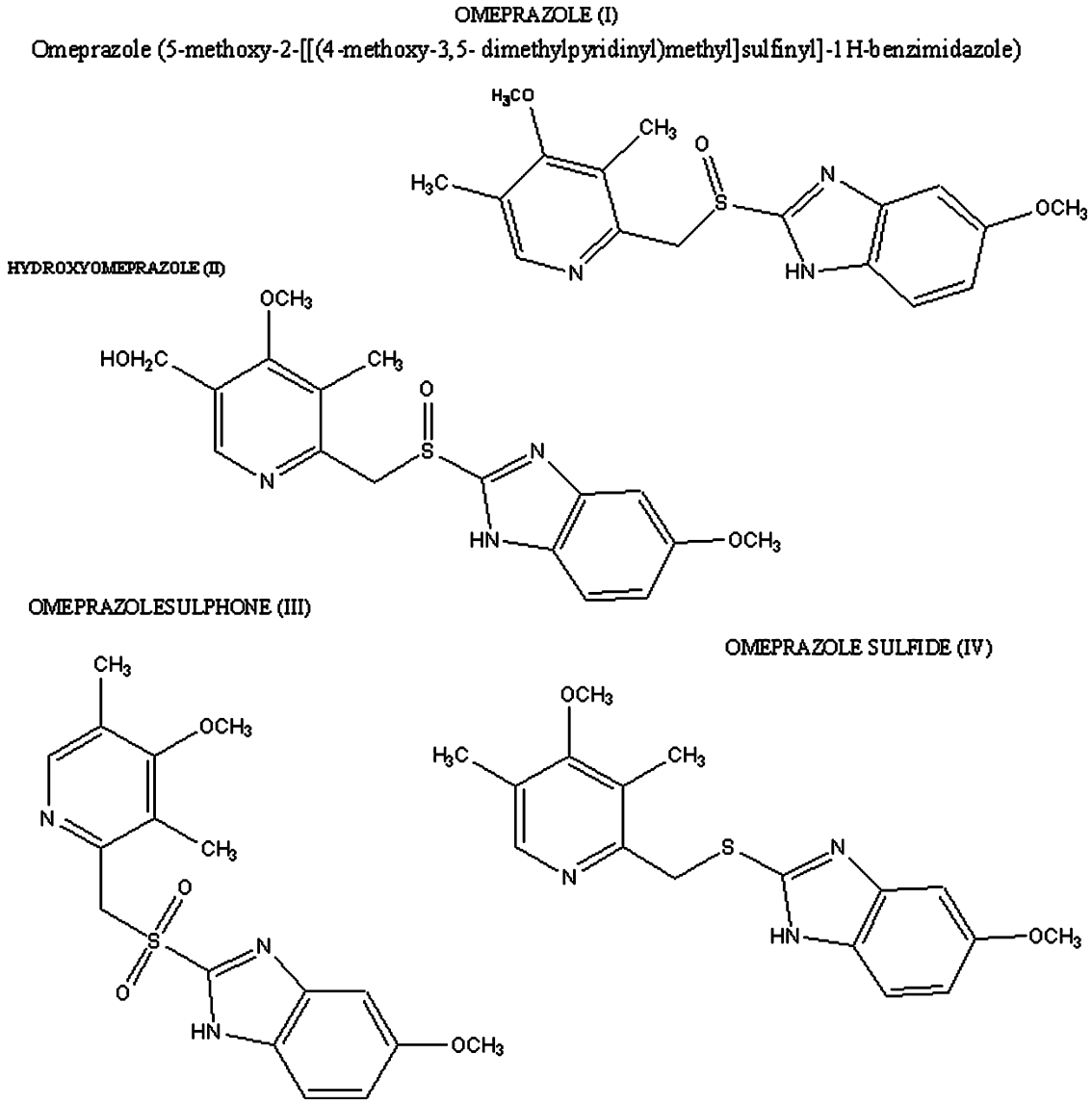

Fig. 1. Chemical structure of omeprazole and its metabolites 5-hydroxyomeprazole, omeprazole sulphone, and omeprazole sulfide.

US Pharmacopeia, was purchased from Fisher Scientific (Norcross, GA, USA). Hydroxyomeprazole, omeprazole sulphone and omeprazole sulfide were a generous donation from Dr. Kjell Andersson, Ph.D., AstraZeneca (Molndal, Sweden). HPLC grade chemicals were purchased from Fisher Scientific (Norcross, GA, USA). Purified compressed nitrogen gas used was obtained from National Welders Supply (Charlotte, NC, USA).

2.2. Equipment

A high-performance liquid chromatography (HPLC) system consisting of an Agilent Technologies (Wilmington, DE, USA) Model HP1100 binary pump, an HP1100 degasser, an HP1100 thermostated autosampler, an HP1100 UV-DAD-detector, and HP ChemStation software (Version A.09.03) run on a IBM computer (operated by Windows XP professional), was used for this method.

2.3. Preparation of standards

Preparing standard master stock solution: Individual clear stock solutions of omeprazole, 5-hydroxyomeprazole, omeprazole sulphone and omeprazole sulfide were prepared at a 1 mg/mL concentration. Five milligrams of each analyte were accurately weighed and dissolved in alkalinized 50% methanol in water (50 μL of diethylamine to 10 mL HPLC water and methanol, pH 10). The master stock solution was prepared as a composite of all four compounds (1.0 ml each), adjusted to a final concentration of 100 μg/mL by 6.0 mL of alkalinized HPLC-grade water (50 μL of diethylamine to 10 mL HPLC water, pH 10). This 100 μg/mL standard was used to prepare a 20 μg/mL composite in alkalinized HPLC water. From this intermediate, seven solutions (10, 5, 1, 0.5, 0.1, 0.05 and 0.02 μg/mL) in alkalinized HPLC water were made. Plasma working calibration solutions at 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, and 0.002 μg/mL for all four compounds were prepared by diluting the interme-

diate solutions in human drug-free plasma in a ratio of 1:9. From another 10 μg/mL intermediate stock solution, concentrations of 15.0, 7.5, 0.75, 0.25 and 0.075 μg/mL were prepared in alkalinized HPLC water. Working quality control samples of 1.5, 0.75, 0.075, 0.025, and 0.0075 μg/mL were prepared by diluting the quality control intermediate solutions in human drug-free plasma in a ratio of 1:9. The plasma used as the matrix was obtained from whole blood anticoagulated with sodium EDTA (Biological Specialty Corporation, Colmar, PA, USA).

2.4. Internal standard (IS) preparation

Phenacetin (5 mg) was weighed and dissolved in 50% methanol in water to achieve a final concentration of 1.0 mg/mL (stock solution). The internal standard working solution was prepared by diluting 20 μL of this solution in 9.98 mL of alkalinized water to achieve a final concentration of 2.0 μg/mL.

2.5. Samples and pre-treatment

This method was used to measure omeprazole and its metabolites in clinical samples. These clinical samples were obtained from healthy volunteer subjects administered a phenotyping cocktail. Omeprazole was used to phenotype CYP2C19 activity. Each study subject received a single oral dose of omeprazole 40 mg. Blood samples were collected at multiple time points following omeprazole administration.

Blood samples were collected in 3.0 mL Vacutainer tubes containing 8.55 mg K3EDTA (15% additive solution) as an anticoagulant and kept on ice after collection for a maximum of 15 min. Blood plasma was separated by centrifugation at 2800 rpm for 15 min at 4 °C. Plasma samples were aliquotted and transferred to a −80 °C temperature-monitored freezer for storage until analysis. Prior to extraction, all plasma samples were brought to room temperature then gently mixed.

2.6. The extraction procedure

On the day of analysis, 50 μL of the internal standard was placed into a 2.0 mL labeled conical plastic Eppendorf tube, followed by 200 μL of plasma (either for blank samples, calibrators, quality control samples or patient samples). To each tube, 1.5 mL of methyl tertiary butyl ether was added. The solutions were vortex-mixed for 30 s, then vertically vortexed for 10 min and centrifuged at 12 000 rpm for 1.0 min. Sample separation was performed by quick-freezing the aqueous part of the solution in a dry ice-acetone bath. The organic portion was transferred to another labeled 1.5 mL eppendorf tube.

The organic portion was evaporated to dryness under a gentle nitrogen stream at 25 °C, and the residue was reconstituted with 50 μL of a 19:1 alkalinized HPLC water:methanol solution. These reconstituted solutions were carefully vortexed for 30 s and centrifuged at 12 000 rpm for 10 min. The supernatants were transferred to 100 μL HPLC microvials (Agilent Technologies) and 40 μL was injected onto the column.

Table 1
The elution gradient delivery system of mobile phases A and B over 17 min of run time

Time (min)	Mobile phase		Flow rate (mL/min)
	A%	B%	
0.00	74	26	0.55
13	34	66	0.56
14	4	96	0.70
15	4	96	0.70
17	74	26	0.55

2.7. High performance liquid chromatographic conditions

Chromatographic separation of analytes was performed using gradient elution. The mobile phase gradient and flow rate gradient are shown in Table 1. The detection wavelength was adjusted to 302 nm. The resolution and analysis of four analytes and the IS utilized a Zorbax® C-18 (150 mm × 3.0 mm, 3.5 μm particle size, Agilent, Wilmington, DE, USA) analytical column, with a Zorbax® C-18 (12.5 mm × 4.6 mm, Agilent, Wilmington, DE, USA) guard column.

The mobile phase consisted of two components. Mobile phase A was 22.0 mM phosphate mono basic, adjusted to a pH of 6.0 with diluted sodium hydroxide. This solution was filtered through a 0.45 μm membrane filter (Millipore, Milford, MA, USA) then mixed as 900 mL buffer to 100 mL methanol. Mobile phase B was composed of 100 mL of the phosphate buffer as mobile phase A, mixed with 800 mL of acetonitrile, 100 mL of methanol, and 100 μL TFA.

Each injection required 20 min of analysis time, including 3 min for re-equilibration. The autosampler temperature was set to 8 (±2) °C. The analysis was performed at 30 °C, with an initial mobile phase flow rate of 0.55 mL/min.

2.8. Specificity and selectivity

Interference from endogenous compounds was investigated by analysis of blank plasma samples from six different male and female volunteers. Interference from other medications used for cytochrome P450 and p-glycoprotein activity phenotyping was also investigated. These compounds included: caffeine, paraxanthene, midazolam, 1- and 4-hydroxymidazolam, digoxin, dextromethorphan, and dextrophan.

2.9. Linearity, limit of quantification and limit of detection

Linearity was assessed using three calibration curves analyzed on separate days. For validation, each point on the calibration curve was run in duplicate (two separate extractions), and the curves were constructed by calculating the peak area ratios of each compound to the internal standard and plotting these against the nominal concentration of the sample. Standard curve equations for each analyte were derived using weighted ($1/Y^2$) linear least-squares regression analyses.

The upper limit of quantitation (ULQ) was defined as the concentration for which both the relative standard deviation and

the percent deviation from the nominal concentration were less than 15% [14]. The lower limit of quantification (LLOQ) was defined as the concentration for which both the relative standard deviation (R.S.D.) and the percent deviation from the nominal concentration were less than 20% though the linear range 2–2000 ng/mL. The detection limit was defined as signal to noise ratio of 3:1.

2.10. Accuracy, precision and recovery (extraction efficiency)

Accuracy and precision of the analytical method was quantified using five concentrations of quality control samples: 1.5, 0.75, 0.075, 0.025, and 0.0075 μg/mL. Additionally, within-day variability was quantified at the upper limit (ULQ) and the lower limit of quantification (LLOQ) run in triplicate. The calculated concentrations were compared to the nominal concentrations.

Recovery (extraction efficiency) of the four analytes after the liquid–liquid extraction procedure was determined by comparing peak areas of each compound in extracted plasma to those of non-processed standard solutions. The quality control aliquots of 0.025, 0.075, and 0.75 μg/mL were used for recovery evaluation.

2.11. Stability

To test stability, samples were left at room temperature for 24 h prior to analysis. Stability during sample handling was also verified by subjecting samples to three freeze-thaw cycles, and storage for 7 days in the refrigerator at 4 °C prior to analysis. Quality controls (QC) samples at the three concentrations of 0.025, and 0.075, and 0.75 μg/mL were utilized for this purpose. Also, stability in the reconstitution solution was tested to assure the stability of samples waiting in the autosampler at 8 °C for 24 h.

3. Results

3.1. Linearity

The calibration curve was calculated using peak area ratio values at eight standard concentrations. The peak area ratios were proportional to the concentration of analyte in plasma over the range tested. The data for the calibration curves ($n = 3$) are shown in Table 2, along with the mean ± S.D. of three standard curve slopes and intercepts, and correlation coefficients (r^2). The concentration range was linear from 2 to 2 000 ng/mL for all analytes. The regression coefficient (r^2) for all calibration curves was greater than 0.999.

3.2. Selectivity

A representative chromatogram of the extracted IS in blank plasma is illustrated in Fig. 2. The approximate retention times for all four compounds and internal standard are listed in Table 2. No endogenous substances interfered with any of the ana-

Table 2
Summary of linearity (range, slope, r^2, and intercept values), analyte retention times, and extraction efficiency (%)

Analyte	Linearity				Retention time (min)	Extraction efficiency (%)
	Range (ng/mL)	r^2	Slope ± S.D.	Intercept ± S.D.		
5-Hydroxyomeprazole	2–2000	0.999	0.14 ± 0.01	0.01 ± 0.61	4.3	103
Phenacetin (IS)					5.4	ND
Omeprazole sulphone	2–2000	0.999	0.43 ± 0.02	−0.16 ± 1.84	7.4	81
Omeprazole	2–2000	0.999	0.39 ± 0.02	0.82 ± 1.66	7.9	84
Omeprazole sulfide	2–2000	0.999	0.50 ± 0.01	−7.06 ± 4.71	10.4	89

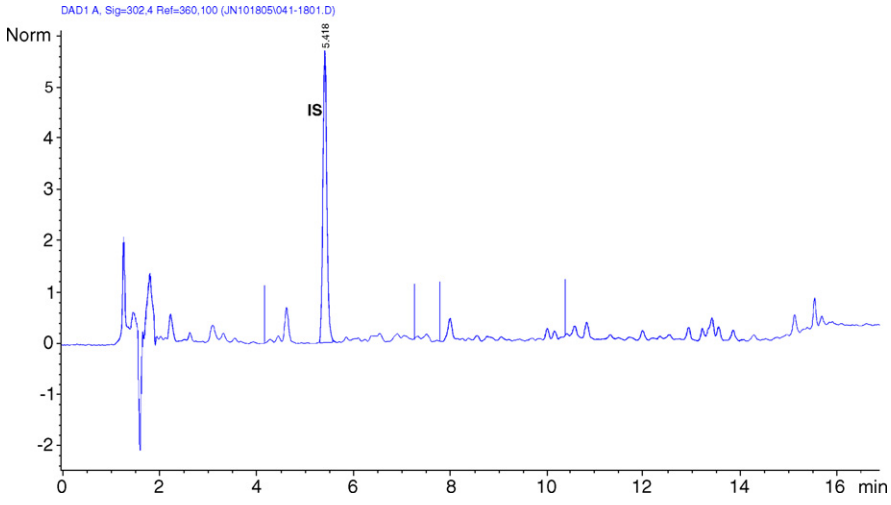

Fig. 2. Sample chromatogram of blank pooled human plasma, extracted with internal standard. Vertical lines indicate analyte elution times.

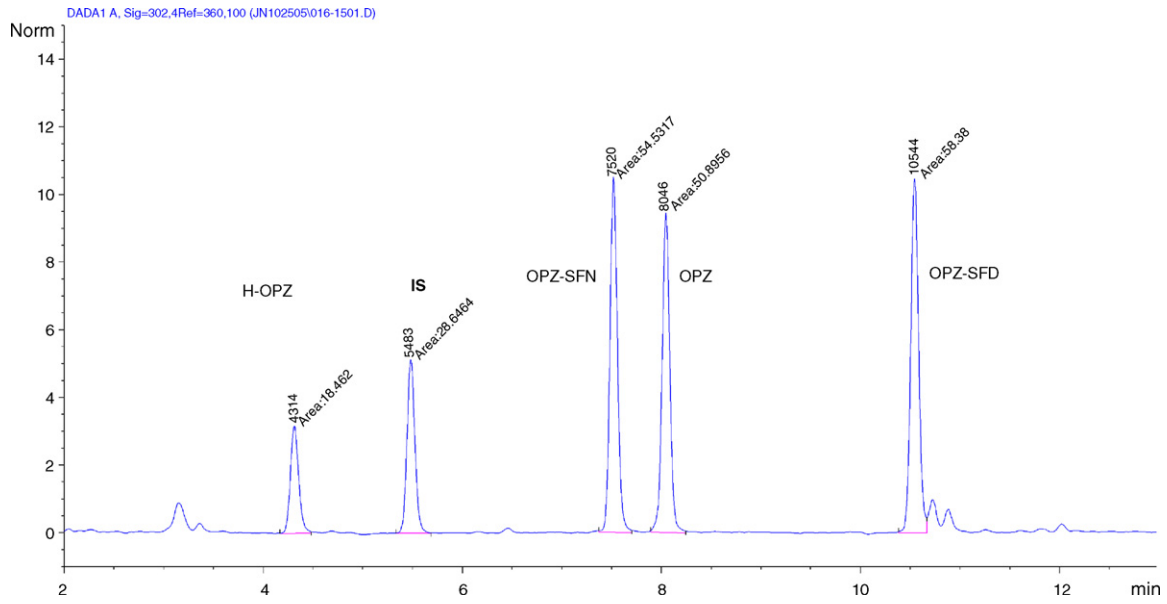

Fig. 3. Chromatogram of 100 ng/mL of omeprazole (OPZ), 5-hydroxyomeprazole (H-OPZ), omeprazole sulphone (OPZ-SFN), omeprazole sulfide (OPZ-SFD), and the internal standard phenacetin (IS).

lytes in blank plasma from six different replicates. There was no interference of other compounds used in cytochrome P450 and g-glycoprotein activity phenotyping. The retention times of these compounds were either in the wash-out region of the chromatogram, or did not interfere with any of the analytes of interest.

3.3. The limit of quantification

The low limit of quantification for all analyte compounds was 2 ng/mL, and the upper limit of quantification was 2 000 ng/mL. Chromatograms from the standard curves at 2 ng/mL and 100 ng/mL are shown in Figs. 3 and 4.

3.4. Accuracy, precision, and recovery

The results of our accuracy and precision experiments throughout the concentration range are shown in Table 3. Within-day accuracy of all analytes ranged from 95 to 110%, with a mean of 101.4%. Between-day accuracy of all analytes ranged from 95 to 114%, with a mean of 101.6%. Within- and between-day precision varied from 1.1 to 3.6%, and 0.5 to 6.2%, respectively.

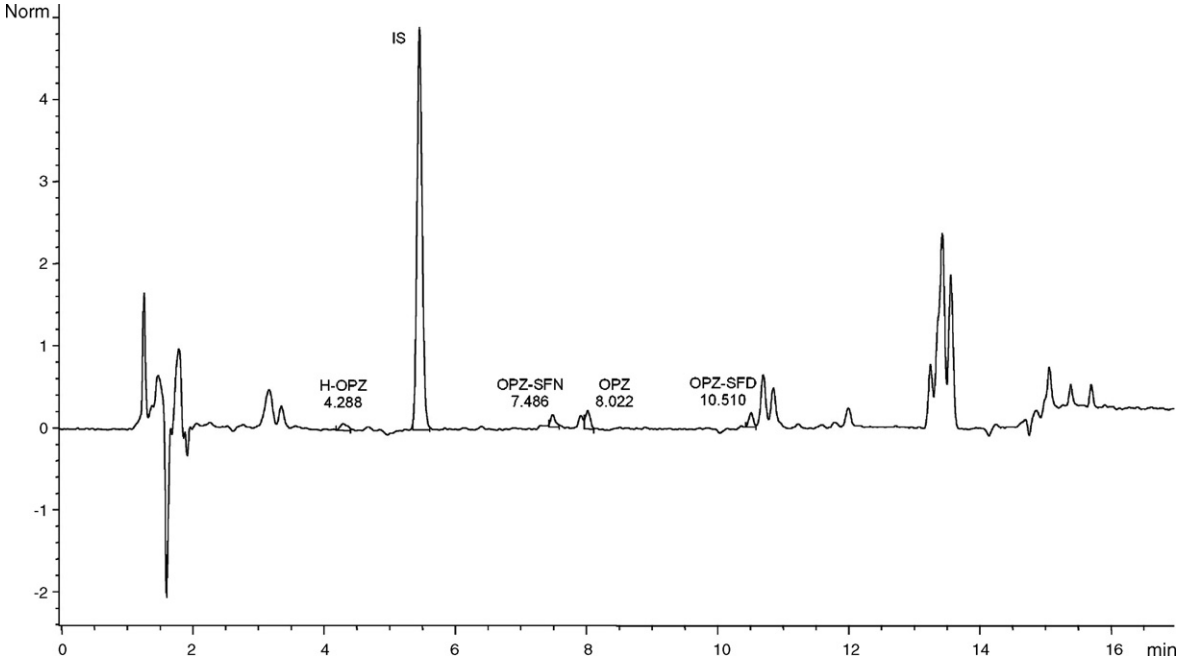

Fig. 4. Chromatogram of 2 ng/mL of omeprazole (OPZ), 5-hydroxyomeprazole (H-OPZ), omeprazole sulphone (OPZ-SFN), omeprazole sulfide (OPZ-SFD), and the internal standard phenacetin (IS).

Table 3
Summary of accuracy and precision during method validation at seven concentrations (CV = coefficient of variation)

Compound	Concentration (ng/mL)	Within-day		Between-day	
		Accuracy (%), $N = 5$	CV (%), $N = 5$	Accuracy (%), $N = 15$	CV (%), $N = 15$
5-Hydroxyomeprazole	2.0	103	4.6	96	5.8
	7.5	108	1.7	100	1.8
	25	99	5.1	102	3.9
	75	105	3.3	99	3.3
	750	101	1.4	101	1.4
	1500	101	3.5	100	3.5
	2000	96	1.1	102	1.3
Omeprazole sulphone	2.0	98	6.3	95	6.2
	7.5	106	4.1	101	3.7
	25	95	3.2	99	3.1
	75	100	3.1	99	1.1
	750	101	1.0	101	1.4
	1500	100	2.8	100	2.8
	2000	96	1.7	98	0.5
Omeprazole	2.0	105	5.9	114	5.8
	7.5	101	3.8	113	2.1
	25	98	2.3	101	4.8
	75	101	3.8	100	5.8
	750	99	2.6	99	4.8
	1500	100	5.3	98	4.6
	2000	98	2.1	96	0.7
Omeprazole sulfide	2.0	97	6.2	103	5.2
	7.5	107	3.5	105	3.4
	25	98	5.9	100	3.1
	75	96	3.0	96	1.5
	750	100	3.3	99	5.2
	1500	110	3.6	104	3.1
	2000	99	3.2	98	2.1

The mean within-day precision was always less than 6.7%. Overall, our results indicate that the method was accurate and precise in the calibration range for each analyte.

The absolute recovery of omeprazole and its three metabolites after the optimized liquid–liquid extraction procedure was greater than 81%. This extraction method reliably eliminated interfering material from plasma, with good recovery for 5-hydroxyomeprazole, omeprazole sulphone, omeprazole, and omeprazole sulfide, as shown in Table 2.

3.5. Stability

The parent drug and metabolites were stable under all tested conditions. After 24 h at room temperature, the four compounds were stable in plasma, being at least 87% of the initial concentration. In three freeze-thaw cycles, the four compounds were stable in plasma with concentrations of at least 92% of the initial concentration. Also, all four compounds proved to be stable in the reconstitution solution for 24 h at 8 °C, with final concentrations of at least 93% that of the initial concentration.

3.6. Analysis of patient samples

We examined the applicability of the described method by analyzing plasma samples collected from healthy volunteers given single 40 mg doses of omeprazole. Omperazole and its three metabolites were found in all samples. A representative volunteer sample chromatogram is shown in Fig. 5. This plasma sample was obtained 3 h after the omeprazole dose, and the calculated concentrations for this sample were 230.5 ng/mL (5-hydroxyomeprazole), 171.5 ng/mL (omeprazole sulphone), 51.2 ng/mL (omeprazole), and 4.9 ng/mL (omeprazole sulfide). Fig. 6, derived from three subjects, shows a combined concentration-time profile for omeprazole and its three metabolites over 24 h. In addition to omeprazole, these subjects also took a cytochrome P450 and p-glycoprotein phenotyping cocktail consisting of caffeine, dextromethorphan, warfarin, Vitamin K, midazolam, and digoxin.

4. Discussion

To our knowledge, there is no published analytical method that simultaneously measures omeprazole and its three major metabolites in plasma samples. Previous published methods focused only on analyzing the parent drug and one or two metabolites. Our current method is the first to quantify the complete metabolic profile for omeprazole.

Our low detection limit is partially attributed to the mobile phase with a pH of 6.0. This allowed for a short run time and sharp peaks. Due to omeprazole's poor stability under acidic

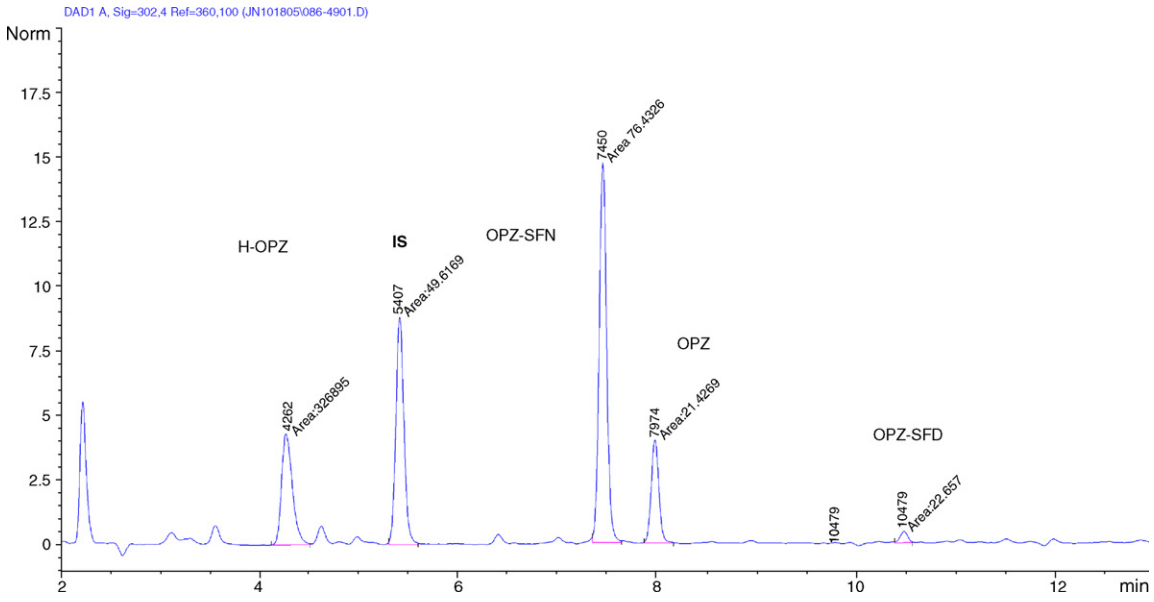

Fig. 5. A chromatogram of a representative volunteer sample (3 h post-dose) with calculated concentrations of 230.5 ng/mL for 5-hydroxyomeprazole, 171.5 ng/mL for omeprazole sulphone, 51.2 ng/mL for omeprazole, and 4.9 ng/mL for omeprazole sulfide).

conditions, previous methods have performed chromatographic separation at a pH of 7.0 or greater. However, we were able to overcome this limitation using a short run time, a final sample matrix extract with a pH of 10.0, and thermostated (8 °C ± 2) autosampler. Using these conditions, no degradation was observed.

The chosen column was a Zorbax® C-18 (150 mm × 3.0 mm, 3.5 μm particle size). The lower internal diameter improves the method sensitivity while injecting a sample volume of 40 μL. The smaller 3.5 μm particle size in a 150 mm length column was crucial for a high theoretical plate number, and optimal peak resolution. Additionally the mobile phase and flow rate gradients, in which the column was washed after each injection, were designed to optimize the chromatography. In this way, we were able to avoid the use of expensive chiral columns similar to the method by Tybring et al. [15].

Recently, Salama et al. [16] reacted omeprazole with iron(III), forming a sharp peak of orange colored chelate at a wavelength of 411 nm. However, this chelate, which is stable in a spectrophotometric method, is not sensitive for biometric applications. Therefore, we monitored omeprazole at a wavelength of 302 nm. This wavelength proved to be specific for all analytes.

A recently published LC–MS quadrupole linear iontrap method [17] achieved a lower limit of quantitation for omeprazole of 0.5 ng/mL, using 500 μL of plasma In the analysis of clinical pharmacokinetic studies, low sample volume can be critically important. In this method, we were able to achieve a lower limit of detection of 2 ng/mL for all analytes using only 200 μL of human plasma.

Finally, we are the first to design a method to measure three omeprazole metabolites. This not only allows for a more comprehensive profile of omeprazole metabolism but also, as Sluggett et al. determined [18], omeprazole sulfide is an acidic degrade, and it's measurement may be helpful in evaluation sample handling.

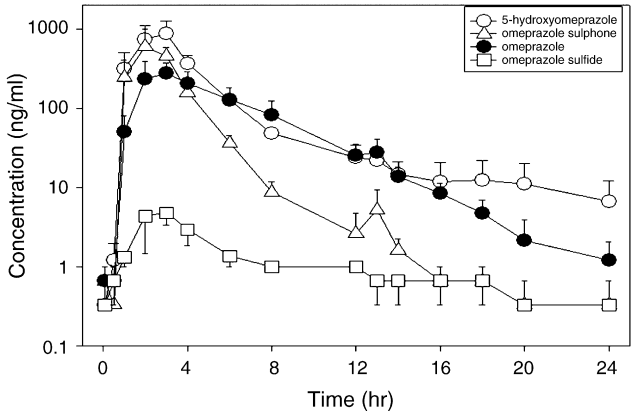

Fig. 6. Concentration of omebrazol (OPZ), 5-hydroxyomeprazole (H-OPZ), omeprazole sulphone (OPZ-SFN) and omeprazole sulfide (OPZ-SFD) in plasma vs. time after administration.

5. Conclusion

We successfully developed a simple HPLC method for simultaneously assaying omeprazole and its three major metabolites in human plasma. The assay has been validated with respect to accuracy, precision, linearity and limit of detection, recovery and stability. It has been successfully applied to clinical samples from healthy volunteer subjects. This method has many clinical applications, as it is simple, highly sensitive, and inexpensive.

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