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Short communication

Phenotype of CYP2C19 and CYP3A4 by determination of omeprazole and its two main metabolites in plasma using liquid chromatography with liquid–liquid extraction

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Abstract

We present a new simple and reliable HPLC method for measuring omeprazole and its two main metabolites in plasma. This can be used for studying CYP2C19 and CYP3A4 genetic polymorphisms using omeprazole as the probe drug. Omeprazole, hydroxyomeprazole and omeprazole sulfone were extracted from plasma samples with phosphate buffer and dichloromethane–ether (95:5). HPLC separation was achieved using an Ultrasphere ODS C_{18} (Beckman) column. The mobile phase was acetonitrile–phosphate buffer (24:76, pH 8), containing nonylamine at 0.015%. Retention times were 9.5 min for omeprazole, 3.25 min for hydroxyomeprazole, 7.4 min for omeprazole sulfone and 6.27 min for internal standard (phenacetine). Detection (UV at 302 nm) of analytes was linear in the range from 96 to 864 ng/ml. This is useful for calculating metabolic index for CYP2C19 and CYP3A4 in adults and children. This method is stable, reproducible, improves resolution and has practical advantages such as low cost.
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1. Introduction

Omeprazole (OME, Fig. 1) a substituted benzimidazole (5-methoxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl) methyl] sulfinyl]-1H-benzimidazole) is used in the treatment of gastric acid related disorders [1,2]. The drug inhibits the (H^+, K^+)–ATPase in the gastric parietal cells, resulting in a diminution of gastric acid secretion [3,4]. OME is effective in the control of gastric acidity of patients with Zollinger–Ellison syndrome, as well as, in patients that do not respond well to histamine H_2 receptor antagonists [3,4].

In the liver, OME is extensively metabolized to several metabolites (Fig. 1): Omeprazole sulfone (OMES), hydroxyomeprazole (HOME) and omeprazole sulfide [5–7]. In the plasma, OMES is the major metabolite while the major metabolite in the plasma and urine is HOME [5–7]. Concentrations of

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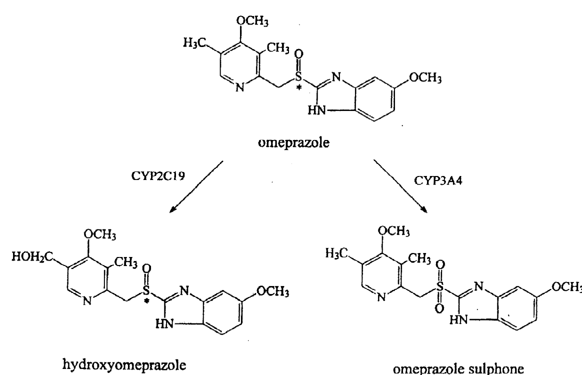

Fig. 1. Metabolism of Omeprazole showing structures of hydroxyomeprazole and omeprazole sulfone. The chiral sulfurs are pointed out by an asterisk.

omeprazole sulfide are usually too low to be determined in plasma or urine. OME concentrations are also negligible in urine [8]. HOME and OMES are produced by the Cytochrome P-450 (CYP) isoforms 2C19 and 3A4 (Fig. 1). CYP2C19 presents a genetic polymorphism, individuals can be either extensive (EM) or poor metabolizers (PM). The characterization of the polymorphic CYP2C19 phenotype is commonly performed by measuring the metabolic ratio [OME]/[HOME], after oral administration of the 20 mg dose of OME. The frequencies in the phenotypes vary between different populations. There are several reports indicating the frequencies of CYP2C19 phenotypes in Caucasian [9], Oriental [10] and African populations [11]. CYP3A4 protein has also a genetic polymorphism [12]. Midazolam [13] is being used for evaluating its phenotypes. But a method allowing the evaluation of both polymorphisms with just one probe drug would be very useful. Actually this approach was proposed by the Swedish group [9], but has not been fully exploited.

A review of the published literature (see Table 1) revealed few methods for OME, HOME and OMES. Two HPLC methods [9,10] are capable of measuring OME, OMES and omeprazole sulfide, in plasma and urine samples, but they do not mention HOME. For OME, most methods use UV detection at 302 nm, while others use tandem mass spectrum [10] or an enantioselective column [11]. An HPLC method [9] is problematic; it involves two separate HPLC systems: normal-phase for OME and OMES and reversed-phase for HOME. In some reports [14,15], either the chromatographic resolution or the analyte extraction need to be improved. A recent work by Dubuc and coworkers [16] described a method using solid-phase separation of OME and the two metabolites. This method was aimed to measure OME only. Its resolution and quantification range is good but a diode array detector is used. Another method [17], for OME and OMES, includes an automatic setup but it does not apply to HOME. For pharmaceutical samples an electrochemical detection method [18] has been described; it measures OME from 10 to 10 000 ng/ml by using a C_8 column.

The method we present here is reliable, robust, simple and practical. It permits the simultaneous determination of omeprazole, hydroxiomeprazole and sulfone metabolites in plasma. Phenotype of CYP2C19 is determined by OME/HOME concentration ratio and CYP3A4 by OME/OMES.

2. Experimental

2.1. Reagents and materials

OME, OMES and HOME were generous gifts from AstraZeneca, Hassle AB, Sweden. Phenacetin was obtained from Collins Laboratories SA (Mexico). HPLC-grade methanol and acetonitrile were purchased from Mallinckrodt. The water was obtained using a Milli-Q system from Waters. All other reagents and buffer solutions were prepared with analytical grade chemicals (Sigma, Merck and KEM, Mexico).

2.2. Instrumentation and chromatographic conditions

HPLC analyses were performed with a Beckman System (Palo Alto, California) which consists of a pump (mod. 126), a detector (mod. 167), an autosampler (mod. 507) and system Gold software. Columns were Ultrasphere ODS C_{18} (15 cm×4.6 mm, with a particle size of 5 μm, Beckman) and a precolumn Ultraspher C_{18} ODS (4 cm×4.5 mm, particle size of 5 μm). Columns were kept at room temperature.

The mobile phase, running at 1.5 ml/min, was acetonitrile–0.025 *M* phosphate buffer (24:76, pH

Table 1
Advantages of this method and comparison with previous methods

	This method	Langerström, 1984	Amantea, 1988	Kobayashi,1992	Cairns, 1994	Tybring, 1984	Woolf, 1998	Yim, 2001	Dubuc, 2001
Solvent extraction	Dichloromethane:ether	●	●	●		●	●		
Solid phase extraction					●			●	●
Normal phase column		●							
Reverse phase column	Ultrasphere ODS C_{18}	●	●	●				●	●
Column chiral phase					●	●	●		
Resolution	Excellent (due to nonylamine)			●	●	●	●	●	●
UV detection	At 302 nm	●	●	●	●	●		●	●
Mass spectrum							●		
Detects HOME	●	●		●			●		●
Detects OMES	●	●	●	●				●	●

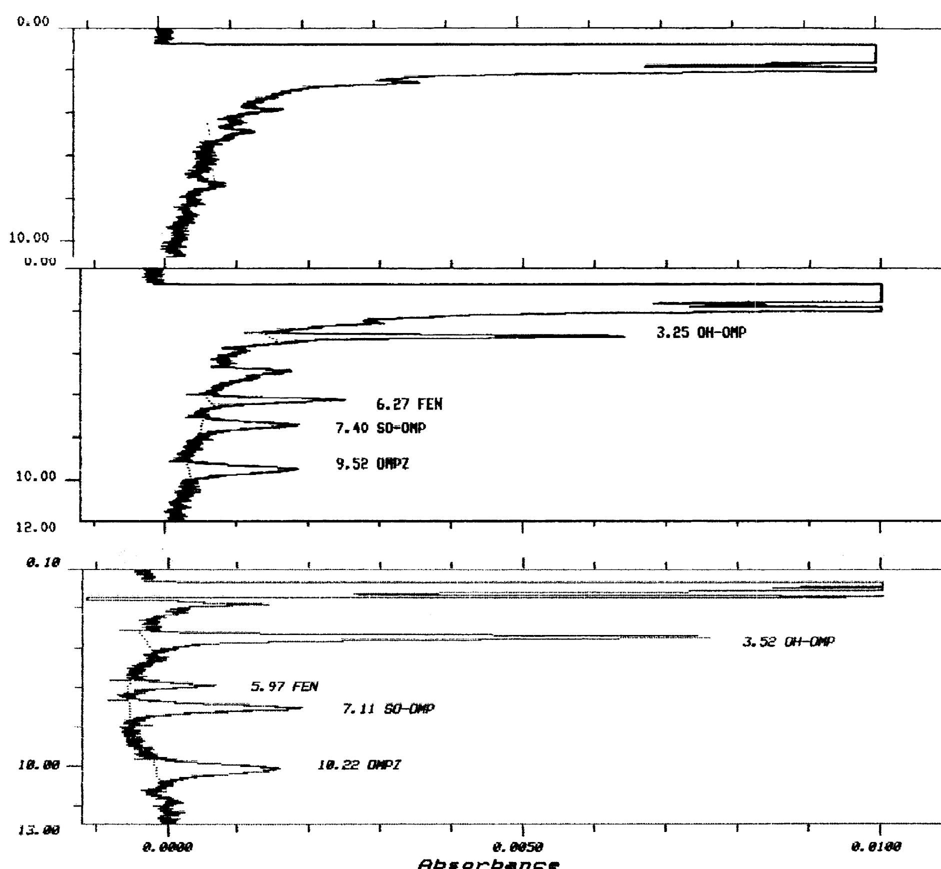

Fig. 2. Chromatograms of (A) blank plasma, (B) standards and (C) plasma from a volunteer. Peaks are well resolved, time of run is convenient and the system is practical. Abr.: OME, omeprazole; HOME, hydroxyomeprazole, OMES, omeprazole sulfone and FEN, phenacetin. Concentrations in (B) are: HOME 620, OMES 20 and OME 50 ng/ml. In (C): HOME 1790, OMES 400 ng/ml and OME 228. Volunteer is a CYP2C19 extensive metabolizer as its metabolic index is 0.127 and also extensive metabolizer for CYP3A4 with a metabolic index of 0.57.

8.0) containing 0.015% of nonylamine. The UV detection was done at a wavelength of 302 nm.

2.3. Extraction of omeprazole and metabolites

Plasma samples (0.5 ml) were spiked with 20 μl of phenacetine (80 μg/ml), used as internal standard. Variable concentrations of OME, HOME and OMES standards in volumes of 100 μl (each) were added. Finally, 100 μl of phosphate buffer (pH 8.7±0.01) and 50 mg of NaCl were dispensed. For extraction, 2.5 ml of dichloromethane–ether (95:5) were added. The tubes were gently shaken for 10 min and then centrifuged at 2500 rpm (1580 *g*) for 5 min. The organic phase was separated using Pasteur pipettes and evaporated to dryness under nitrogen. The dried residue was resuspended in 400 μl of the mobile phase. Following a filtration with 0.22 μm Millipore membrane 100 μl aliquots were injected to the HPLC.

3. Results and discussion

3.1. Chromatography, recovery and linearity

Chromatography conditions were optimized for performance and feasibility to clinical studies in

Table 2
Recovery (%±SEM) after six repetitions

Concentration (ng/ml)	OME		HOME		OMES	
60	104.4	2.1	76.9	1.5	105.5	3.8
180	96.3	5.3	79.6	4.9	91.3	4.5
960	97.8	2.1	74.8	2.3	99.0	3.3

children and adults. Chromatograms are shown in Fig. 2. The quality and resolution of the peaks was due to the use of nonylamine as phase modifier. Nonylamine has an affinity for silane groups of the column. Since it anchors by van der Waals forces with the aliphatic chain to the C_{18} it increases the retention of compounds in a differential way. OMES has the greater change, while the tailing of OME is reduced with a change on acetonitrile composition. All this together increases the resolution (see Fig. 3). The maximum working resolution is achieved at 0.015% of nonylamine with a pH of 8.0.

Several pH were tested to improve the resolution between OME and OMES: 6.5, 7.0, 7.5, 8.0 and 8.5. A pH of 8.5 and a nonylamine concentration of 0.010% were the optimum conditions, but to increase the life of the column pH of 8.0 and a nonylamine concentration of 0.015% were selected (see Fig. 3). Using a pH of 8.5 allows about 150 injections, but using pH at 8.0 allows about 350 injections.

The retention times of HOME, FEN, OMES and OME were 3.2, 6.3, 7.4 and 9.5 min, respectively. HPLC run duration was 12 min. The detection limit was 30 ng/ml for each analyte.

Validation parameters were evaluated as described by Causon [19]. Recovery of internal standard was 102%. Average recovery of OME, HOME and OMES were 99, 77 and 99% in average, as shown on Table 2.

Area under the peak for compounds and internal standard was chosen as response function. Linearity of the system was evaluated by six repetitions on six

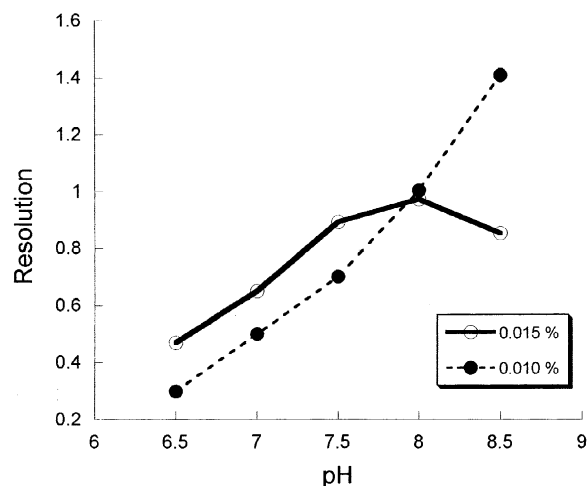

Fig. 3. Effect of nonylamine concentration and pH on resolution of omeprazole and its metabolites. Because the duration of the column is improved using a pH of 8.0 the concentration of 0.015% was chosen. Resolution was measured as (Retention time of OME−Retention time of OMES) (Peakwide of OME−Peakwide of OMES). OME and OMES were the peaks more difficult to separate.

Table 3
Linearity obtained after regression analysis

		Coefficients	Standard error	r^2
OME	Intercept	−0.168	0.034	0.9999
	Slope	0.026	0.000	
HOME	Intercept	−0.150	0.072	0.9994
	Slope	0.026	0.000	
OMES	Intercept	−0.128	0.072	0.9993
	Slope	0.024	0.000	

Table 4
Mean of 4 days accuracy and precision

Accuracy	OME	HOME	OMES
	−0.5%	1.3%	0.6%
Precision			
Concentration (ng/ml)			
96	10.5%	11.0%	14.0%
202	11.3%	8.0%	10.3%
398	10.8%	11.5%	10.8%
864	10.8%	8.3%	11.3%

concentrations, (Table 3). It was calculated after regression analysis. The linearity range for the three compounds was between 60 and 960 ng/ml, i.e. 174 to 2778 n*M* for OME. Slopes for OME, HOME and OMES were similar (Table 3). These indicate good sensitivity as a small change in the concentration was reflected in a proportional change on the response function. The lowest limits for quantification were 60 ng/ml for every analyte. The highest limit of detection was 960 ng/ml.

3.2. Precision and accuracy

Precision was evaluated during 4 days with four concentrations (see Table 4) and with six repetitions. An analysis of variance evaluated the robustness of the system by comparing variability on 4 days and concentration. There were no differences between days but only due to concentrations, the lack of interaction demonstrated the parallelism between the curves on different days (data not shown). Variability during these days is displayed in Table 4. Coefficients of variability were between 3 and 15%.

Accuracy is displayed in Table 4, were bias function is in function of day. Data from the same evaluation for precision were used, that is six repetitions on 4 days for four concentrations. The method was demonstrated to be accurate for the three analytes.

3.3. Selectivity and applications

This method is designed to be used for pharmacogenetic studies. Its performance reflects an improvement compared with traditional methods such as that described in Table 1. The cost reductions are also an advantage especially when a high number of samples is required.

Some previous methods are expensive, because they use chiral columns [20] or mass-spectrum detection [21] (Table 1). We tested unsuccessfully three of them [14,15,22]. Resolution of some of them was not satisfactory.

Pharmacogenetic studies usually report average levels around 500 ng/ml, for OME, HOME and OMES. Lower levels of OME have been observed in children due to a faster metabolism. For concentrations below 60 ng/ml, volumes of 1 ml can be taken, so up to 30 ng/ml can be measured. Concentrations greater than 960 ng/ml can be measured by an adequate dilution.

We have been using the present method for analyzing metabolism dependent on CYP2C19 and CYP3A4 on 70 adults plus 30 children. A chromatogram of a volunteer is shown in Fig. 2.

This method is compatible with a cocktail approach for pharmacokinetic studies. Caffeine (CYP1A2 substrate) and its main metabolites, as well as dextrometorphan (CYP2D6 substrate) and its metabolite dextrorphan did not interfere with the test.

Our method is convenient for pharmacogenetics studies. In general, as a large number of samples have to be analyzed; this method has a performance/cost advantage. For this approach chiral columns are unnecessary. Although, OME is metabolized in a enantioselective form [20], the characterization of the CYP2C19 and CYP3A4 phenotypes can be done without considering the enantiomers.

In our experience precolumns support 150 injections, while columns support 500 injections. The filtration with the Millipore filter prevents the accumulation of debris in the columns.

4. Conclusion

We propose an alternative liquid chromatography method for determining OME, HOME and OMES in plasma samples. This method can be used to evaluate the phenotype for CYP2C19 and CYP3A4 polymorphisms. This method is sensitive, precise, accurate and selective. The recovery and the linearity were good. It is a practical method for pharmacogenetic studies involving the evaluation of phenotype of CYP2C19 and CYP3A4 using omeprazole as probe drug.

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