Simultaneous Determination of Omeprazole, Hydroxyomeprazole and Omeprazole Sulphone in Human Plasma by Isocratic HPLC-DAD: Application to the Phenotyping of CYP2C19 and CYP3A4 in Brazilian Volunteers

Rafael Linden,*a,b Ana Luiza Ziulkoski,a Maína Wingert,a Paula Tonelloa and André A. Soutob

a Centro Universitário Feevale, Rodovia RS 239, 2755, 93352-000 Novo Hamburgo-RS, Brazil
b Pontifícia Universidade Católica do Rio Grande do Sul, Av. Ipiranga, 6681, 90619-900 Porto Alegre-RS, Brazil

Foi desenvolvido um método simples para a determinação simultânea de omeprazol (OME), 5-hidroxiomeprazol (HOME) e omeprazol sulfona (OMES) por meio de CLAE-DAD, utilizando coluna de fase reversa e eluição isocrática. O método proposto avaliou polimorfismos genéticos de CYP2C19 e CYP3A4 utilizando omeprazol como fármaco sonda em um grupo de voluntários brasileiros. OME, HOME e OMES foram extraídos de amostras de plasma com tampão Tris pH 9,5 (0,2 mol L-1) e acetato de etila. A separação por Cromatografia Líquida de Alta Eficiência (CLAE) foi realizada com uma coluna Shim-Pack RP-18e (150 × 4,6 mm d.i., 5 μm), com acetonitrila-tampão fosfato pHe 7,6 (24:76, v/v) como fase móvel e tempo total da corrida de 15 min. Os tempos de retenção foram 2,7 min para o padrão interno (sulpirida), 4,1 min para HOME, 11,6 minutos para OME e 12,6 min para OMES. A detecção dos analitos (UV a 302 nm) foi linear na faixa de 25 a 1000 ng mL-1. As recuperações absolutas variaram de 64,3 a 73,2% para todos os analitos. Um grupo de 38 voluntários sadios brasileiros foi fenotipado com esse método, após a ingestão de uma dose oral única de 20 mg de omeprazol. O método apresentou precisão e exatidão adequadas, com limite de quantificação de 25 ng mL-1 para OME e seus metabolitos, o que permitiu a identificação de metabolizadores ultra-rápidos tanto para CYP2C19 quanto para CYP3A4, aproveitando as vantagens inerentes à identificação seletiva oferecida pelos detectores de arranjo de diodos.

A simple HPLC-DAD method using a reverse phase column and isocratic elution for the simultaneous determination of omeprazole (OME), 5-hydroxyomeprazole (HOME) and omeprazole sulphone (OMES) was developed. The proposed method was used to study CYP2C19 and CYP3A4 genetic polymorphisms using OME as the probe drug in a group of Brazilian volunteers. OME, HOME and OMES were extracted from plasma samples with Tris buffer pH 9.5 (0.2 mol L-1) and ethyl acetate. HPLC separation was achieved using a Shim-Pack RP-18e (150 × 4.6 mm i.d., 5 μm) column, with acetonitrile phosphate buffer pH 7.6 (24:76) as mobile phase and total run time of 15 min. Retention times were 2.7 min for internal standard (sulpiride), 4.1 min for HOME, 11.6 min for OME and 12.6 min for OMES. Detection (UV at 302 nm) was linear in the range from 25 to 1000 ng mL-1. Extraction recoveries were in the range of 64.3 to 73.2% for all analytes. A group of 38 healthy Brazilian volunteers was phenotyped with this method, after a single oral dose of 20 mg omeprazole. The method presented adequate accuracy and precision, with limit of quantification of 25 ng mL-1 for omeprazole and metabolites, which allowed the identification of ultra-rapid metabolizers for both CYP2C19 and CYP3A4 and took advantage of the selective identification offered by diode-array detectors.

Keywords: omeprazole, phenotyping, CYP2C19, CYP3A4, HPLC-DAD

Introduction

Omeprazole (OME), a substituted α-pyridylmethylsulfinylbenzimidazole, is a gastric H⁺, K⁺, ATPase inhibitor. OME has been widely used as a potent inhibitor of gastric acid secretion for the treatment of peptic ulcer, refractory gastroesophageal reflux disease and Zollinger–Ellison syndrome. OME undergoes extensive hepatic metabolism by the cytochrome P450 system, especially by the isoforms CYP2C19 and CYP3A4. Its main metabolites are 5-hydroxyomeprazole (HOME) and...
Omeprazole is administered as a racemic mixture and presents enantioselective metabolism, with the (R)-(+) -omeprazole being mainly hydroxylated by CYP2C19 and (S)-(−)-omeprazole being preferentially metabolized to the sulfone by the CYP3A4. OME is rapidly removed from plasma, with an estimated half-life of about 2 h.

OME hydroxylation is well correlated with S-mephenytoin 4-hydroxylation, which has been widely used as a phenotypic evaluation of CYP2C19 polymorphism. This genetic polymorphism shows a cosegregation with the oxidative metabolism of several clinically important drugs such as amitriptyline, nortriptyline, diazepam, imipramine, omeprazole, propranolol and selective serotonin reuptake inhibitors.

Several studies had used OME as a phenotyping probe for CYP2C19 and CYP3A4. Three major phenotypes have been reported: poor metabolizer (PM), extensive metabolizer (EM) and ultra rapid metabolizer (UM). These phenotypes can be classified using metabolic ratios of omeprazole after oral administration. Usually, an oral dose of 20 to 40 mg of omeprazole is orally administered and a blood sample is collected after 3 h and metabolic ratios are calculated based on HOME, OME and OMES plasma concentrations.

Knowledge of an individual phenotype for metabolizing enzymes can be useful in the adjustment of dosage regimen in pharmacotherapy. Van der Weide et al. have observed consistent relationship between metabolic ratios of amitriptyline with CYP2C19 genotype. Kirchheiner and coworkers have recently published dose recommendations for antidepressant drug based on CYP2C19 and CYP2D6 genotypes. It was expected that PM would require lower doses of drugs, increasing the toxicity risk under standard dose regimens, and UM would need increased doses, hence standard posology can lead to poor clinical response. Considering the high correlation of metabolic ratios for omeprazole and the CYP2C19 and CYP3A4 genotypes, the use of metabolic phenotypes to dose adjustment can also be further explored.

Several high-performance liquid chromatographic (HPLC) methods either with tandem mass spectrometry or photometric detection systems have been described to determine OME, HOME and OMES in biological fluids, but none of them used diode array detectors (DAD). The use of DAD allows comparison of ultraviolet absorption spectra of sample peaks with reference spectra on a computer library and the determination of spectral purity of the peaks, greatly improving the specificity of the analysis. Despite the great improvement in specificity brought to HPLC analyses by DAD, only Duboc et al. used a HPLC-DAD for OME determination in human plasma samples. To date, there is no report of an HPLC-DAD method for simultaneous quantification of OME, HOME and OMES in human plasma samples.

The aim of the present work is to develop a simple HPLC-DAD method for simultaneous determination of OME, HOME and OMES in human plasma, using liquid-liquid extraction with a non halogenated solvent and isocratic elution and allowing rapid processing of a high number of samples. An additional goal of this work is to apply the developed method in the phenotype determination for CYP2C19 and CYP3A4 in a group of Brazilian volunteers.

**Experimental**

**Chemicals**

Omeprazole (99%) was purchased from Sigma Chemical Company (St. Louis, USA). Omeprazole sulphone (98%)

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**Figure 1.** Chemical structures of omeprazole, 5-hydroxyomeprazole and omeprazole sulphone showing main metabolic pathways.
and 5-hydroxyomeprazole (95%) were supplied by Astra Zeneca (Mölndal, Sweden). Sulpiride (99%) was obtained from Purifarma Química e Farmacêutica (São Paulo, Brazil). HPLC grade acetonitrile, methanol and all other chemicals were obtained from Merck (Darmstadt, Germany). Water was obtained by distillation and deionization and additionally purified with an Elga Purelab Ultra SC system purchased from Analítica (São Paulo, Brazil). Phosphate buffer pH 7.6 was prepared dissolving 0.73 g of monobasic sodium phosphate and 4.47 g of anhydrous dibasic sodium phosphate in 1000 mL of water. Final pH was adjusted with phosphoric acid 85% (v/v). Before mobile phase preparation, phosphate buffer was filtered through a 0.45 μm cellulose acetate membrane (Sartorius, Germany). Tris buffer pH 9.5 (0.2 mol L⁻¹) was prepared dissolving 23.4 g of tris-hydroxy-methyl-aminomethane in 1000 mL of water. Generic 20 mg omeprazole capsules were obtained from Medley S/A Indústria Farmacêutica (São Paulo, Brazil).

**Equipment**

The chromatographic apparatus consisted of a binary pump LC-10AT, an on line degasser DGU-14, a column oven CTO 10AS, an automatic injector SIL-10AF and a diode array detector SPD-M10A. The system was controlled by the software Class VP 6.13 SP2. The complete system was from Shimadzu (Kyoto, Japan).

**Chromatographic conditions**

The separation was performed on a Shim-Pack RP-18e (150 × 4.6 mm i.d., 5 μm) column with a guard column Shim-Pack G-ODS (10 × 4.0 mm i.d., 5 μm), both from Shimadzu (Kyoto, Japan). The integration wavelength was set at 302 nm, with diode array spectral acquisition in the range of 200 to 380 nm. The mobile phase consisted of phosphate buffer pH 7.6 and acetonitrile (76:24, v/v) at a flow rate of 1.2 mL min⁻¹. The column was kept at 30 °C during the analyses. The mobile phase was prepared daily and degassed by ultrasonication before use.

**Standard solutions**

Stock solutions of HOME, OME and OMES were prepared in methanol at the concentration of 0.5 mg mL⁻¹. Working solutions of HOME, OME and OME were prepared from stock solutions by dilution with methanol in order to obtain concentrations of 50, 25, 12.5, 6.5, 2.5 and 1.25 μg mL⁻¹. The stock solution of sulpiride (internal standard) was prepared in methanol at a concentration of 2 mg mL⁻¹. The working solution of sulpiride (20 μg mL⁻¹) was obtained by dilution of the stock solution with methanol. The standard solutions were stored at −20 °C.

**Sample preparation**

To 1000 μL of plasma in a glass-stoppered 10 mL centrifuge tube were added 50 μL of sulpiride as internal standard (2 μg mL⁻¹), 500 μL of Tris buffer pH 9.5 and 6 mL of ethyl acetate. After mixing (15 min), the mixture was centrifuged for another 15 min at 3500 g and then maintained at -20 °C for 40 min. Then, 5 mL of the upper organic layer were transferred to clean evaporation tubes and concentrated to dryness at 40 °C under a gentle stream of nitrogen. The dried extract was recovered with 100 μL of mobile phase and 20 μL were injected into the liquid chromatograph.

**Biological samples**

Blank plasma used to obtain the calibration samples was obtained from a local blood bank. These samples were tested previously with the proposed method in order to avoid interferences. Blank plasma for specificity testing was obtained from healthy volunteers. All blank plasma samples were stored at -20 °C, being thawed just before use. Patient samples were obtained as detailed in the section pharmacogenetic analysis. Concentrated samples were diluted with blank plasma in order to obtain values within the calibration curve range.

**Stability**

The stability of HOME, OME and OMES was assessed for spiked plasma samples stored at -20 °C and +4 °C for one month, with weekly analysis. Stability was also tested at room temperature for 24 h at the chromatograph autosampler. The stability of stock solutions stored at above mentioned temperatures was determined by injecting appropriate dilutions of stocks in methanol at different days and comparing their peak areas with fresh stock prepared on the day of analysis. Samples were considered stable if the assay values were within the acceptable limits of accuracy and precision, defined as a mean value of 80-120% of theoretical value and R.S.D. < 15%, according to Shah et al.²⁴

**Plasma calibration curves and quantitation**

To 940 μL of blank plasma, 20 μL of working standards of each of the three analytes were added, yielding final concentrations of HOME, OME and OMES of 25, 50,
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125, 250, 500 and 1000 ng mL\(^{-1}\). To this mixture, 50 μL of internal standard working solution were added to yield a IS concentration of 1000 ng mL\(^{-1}\). Calibration samples were prepared for analysis as described above. Calibration curves were constructed by plotting peak area ratio (y) of HOME, OME and OMES to the internal standard versus HOME, OME and OMES concentrations (x). A linear regression was used for quantitation, with a weighting factor of \(1/concentration^2\).

Selectivity

Control human plasma, obtained from 6 healthy volunteers, was assessed by the procedure as described above and compared with respective plasma samples to evaluate selectivity of the method. Reference samples of metoprolol and hydroxymetoprolol were also analyzed with the method, once they can be co-administered with omeprazole in cocktails for simultaneous phenotyping of CYP2C19, CYP3A4 and CYP2D6. In all runs, peaks related to OME, HOME and OMES were evaluated with respect to their spectral purity.

Precision and accuracy

The precision and accuracy of the method were examined by adding known amounts of OME, HOME and OMES to blank plasma, yielding final concentrations of 50, 200, 600 and 900 ng mL\(^{-1}\) of each analyte. For intra-day precision and accuracy, six replicate quality control samples at each concentration were assayed on the same day. The inter-day precision and accuracy were evaluated on six different days, with three samples being assayed each day. Precision was evaluated as percentage relative standard deviation (R.S.D. %) and accuracy was evaluated as percentage of the spiked concentration.

Lower Limit of Quantitation

The Lower Limit of Quantitation (LLOQ) was established as the lowest concentration on the calibration yielding acceptable precision (R.S.D. < 20%) and accuracy (80-120% of theoretical value), according to Shah and et al.\(^{24}\)

Extraction yield

The extraction yield for plasma at four different concentrations of OME, HOME and OMES (50, 200, 600 and 900 ng mL\(^{-1}\)) was determined. Known amounts of analytes were added to drug-free plasma and submitted to the analysis procedure previously described. The extraction yield was calculated by comparing the peak areas for extracted HOME, OME and OMES from spiked plasma and a standard solution of the analytes in methanol containing internal standard with the same initial concentration (six samples for each concentration level).

Pharmacogenetic analysis

The pharmacogenetic study protocol was approved by the Ethics Committees of Centro Universitário Feevale and of Pontifícia Universidade Católica do Rio Grande do Sul, and a written informed consent was obtained from the volunteers. Before inclusion on the study, hepatic and renal function of all volunteers were evaluated by biochemical assays. Inclusion criteria included serum glutamic oxalacetic aminotransferase (SGOT) in the range of 12-46 U L\(^{-1}\), serum glutamic pyruvic transaminase (SGPT) in the range of 3-50 U L\(^{-1}\) and serum creatinine below 1 mg dL\(^{-1}\). Subjects were excluded if they were under any drug or herbal treatment, had the presence of any factor affecting OME pharmacokinetics or had a reported allergy to drugs. Special instructions were given to all subjects regarding the avoidance of coffee, alcohol, and herbal products 48 h before the OME administration. OME was administered in a single dose of 20 mg to the volunteers after an 8 h fasting. Blood samples were collected 3 h after OME administration. Plasma was immediately separated and transferred to polypropylene tubes and frozen at -20 °C until assayed. Plasma concentrations of OME, HOME and OMES were determined and metabolic ratios were calculated as log ([OME]/[HOME]) and log ([OME]/[OMES]) for the phenotypic classification for CYP2C19 and CYP3A4, respectively. Thirty-eight individuals participated in the study.

Results and Discussion

Under the chromatographic conditions described, HOME, OME and OMES and the internal standard peaks were well resolved. Endogenous plasma components did not give any interfering peaks. Separation was performed on a common reversed-phase column, with isocratic elution. Several binary mobile phase compositions of phosphate buffer pH 7.6 and acetonitrile were tested, with phosphate buffer proportions ranging from 85 to 70%. The mobile phase composed of phosphate buffer pH 7.6 and acetonitrile in the ratio of 74:26 (v/v) was the best compromise between resolution of internal standard, HOME, OME and OMES and analysis time. Increased
The average retention times of sulpiride, HOME, OME and OMES were 2.7, 4.1, 11.6 and 12.6 min, respectively. In all validation runs, the peaks were checked for spectral purity, without identification of any endogenous interferents. The retention times of hydroxymetoprolol and metoprolol were also tested, once metoprolol can be co-administered with OME to simultaneously phenotype CYP2C19, CYP3A4 and CYP2D6. Hydroxymetoprolol and metoprolol had average retention times of 1.9 and 5.4 min, respectively, showing no interference with internal standard, HOME, OME and OMES. A typical chromatogram of a calibration plasma sample is shown in Figure 3.

Several sample preparation methods have been used for analysis of OME and its metabolites in biological samples, including protein precipitation, liquid-liquid and solid phase extraction. For studies where large number of samples must be processed, solid phase extraction still remains as an expensive alternative. We tested protein precipitation with diluted perchloric acid, after which no analytes were detected at a concentration level of 250 ng mL⁻¹. González et al. used liquid-liquid extraction with a mixture containing halogenated solvent. Ethyl acetate was chosen as an alternative solvent due to moderate polarity and favorable safety characteristics. After homogenization, the samples were kept for 40 min in a freezer in order to facilitate the organic phase separation, forming a solid aqueous phase and increasing ethyl acetate viscosity.

Spiked samples were found to be stable for at least one month at −20 °C, after four freeze and thaw cycles, with HOME, OME and OMES concentration values found to be 97.2 ± 6.7 % of the initial values. At room temperature (20-30 °C) no significant difference in concentrations was found after 24 h.

The calibration curve for the determination of HOME, OME and OMES in plasma was linear over the range 25-1000 ng mL⁻¹. A weighted least squares model, with a weighting factor of 1/concentration², adequately compensate for heteroscedasticity of the data. The determination coefficients (r²) for calibration curves were either equal to or better than 0.995 (Table 1). The goodness of fit was highly significant and no significant lack-of-fit was observed in any of the calibration curves.

In order to determine the extraction yield of HOME, OME and OMES, known amounts of HOME, OMES and OME were added to drug-free plasma in concentrations ranging from 50 to 900 ng mL⁻¹ (50, 200, 600 and 900 ng mL⁻¹). Internal standard was added and the extraction yield was calculated by comparing the peak areas for extracted HOME, OME and OMES from spiked plasma with a standard solution of the same analytes in methanol containing internal standard with the same initial concentration. The average recoveries were 64.3 ± 7.5% for HOME, 73.2 ± 6.3% for OME and 71.2 ± 5.9% for OMES (Table 2). The obtained recoveries were acceptable for CYP2C19 and CYP3A4 phenotyping under the applied procedure, allowing the identification of OME and its metabolites even in ultra-rapid metabolizers. The lowest limit of quantification, calculated as the lowest concentration on the calibration curve yielding acceptable precision and accuracy, according to Shah et al., were 25 ng mL⁻¹ for HOME, OME and OMES. The analysis of 6 independent blank plasma samples did not presented any interfering peaks at the eluting times of IS, HOME, OME and OMES. In all processed samples, the use of a diode array detector allowed the verification of the spectral purity of all peaks related to the analytes, as well as the comparison of the obtained spectra with those in the equipment library. No interferences were detected in the performed analysis.
The precision of the method was assessed by the repeated analysis of plasma specimens containing known concentrations of HOME, OME and OMES. As shown in Table 3, intra-day R.S.D. % were in the range of 3.1 to 7.3 % and inter-day R.S.D. % were in the range of 4.4 to 7.9. Accuracy was in the range of 96.3 to 101.4 %. Both precision and accuracy are acceptable for a bioanalytical method, according to the Conference Report on Bioanalytical Method Validation.24

A group of 38 healthy Brazilian volunteers were phenotyped after an oral dose of 20 mg omeprazole. A chromatogram of a calibration plasma sample is presented in Figure 3. Figure 4 presents a chromatogram obtained from a volunteer presenting an ultra-rapid (UM) phenotype for CYP2C19.

Omeprazole was well tolerated by all subjects, without any report of adverse reactions. No secondary effects were reported in any volunteer. Mean concentrations of 5-hydroxyomeprazole, omeprazole and omeprazole sulfone were 225 ng mL\(^{-1}\) (range 79-2103), 287 ng mL\(^{-1}\) (range 52-750) and 509 ng mL\(^{-1}\) (range 33-9917), respectively. The wide dispersion of the results can be credited to the very extreme values of ultra-rapid metabolizers for CYP2C19 and CYP3A4.

All subjects presented detectable amounts of HOME, OME and OMES.

The frequency of the different metabolic ratios for CYP2C19 and CYP3A4 are presented in Figures 5 and 6.
The antimode for the frequency of the metabolic ratios, calculated as log ([OME]/[HOME]), is usually used to classify individuals as PM, EM or UM for CYP2C19. Between PM and EM, this value has been found to vary considerably among different populations, being 0.84 in Korean, 0.60 in West Mexicans, and 1.16 in South Indians. The prevalence of PMs in the South Indian population is 14%, being 12-23% in Orientals and 1% in Caucasians. Considering the small population tested in our study, it is not possible to determine the antimode in Brazilian population. Besides that, we found individuals with markedly different metabolic activities for both CYP2C19 and CYP3A4, which denotes the wide diversity of metabolic profiles that may be considered in clinical pharmacology.

One subject presented the ultra-rapid phenotype for CYP2C19, particularly with very high levels of 5-hydroxyomeprazole (2103 ng mL⁻¹). In addition, one subject presented the ultra-rapid phenotype for CYP3A4, with a surprisingly elevated concentration of OMES in the order of 9917 ng mL⁻¹. We found one individual with metabolic ratios with respect to CYP2C19 of 0.99, denoting OME concentrations about 10 times higher than HOME. EM had average OME concentrations about 1.5 times higher than HOME concentrations.

Individuals with elevated metabolic ratios are much prone to having adverse reactions when submitted to standard doses of drugs which are substrates for the specific enzyme tested. These individuals would require much lower doses of these drugs. In addition, we found two individuals with very low metabolic ratios denoting a very fast metabolism. These patients would require higher doses to achieve plasma therapeutic concentrations of drug metabolized by CYP2C19 and CYP3A4.

Considering the cost associated with genotyping procedures, an interesting alternative could be the establishment of correlations of metabolic indexes of usual drugs with those from probe drugs as omeprazole. This approach, already evaluated for the demethylation of amitriptyline by Van der Weide et al., can permit dose adjustments based on usual therapeutic drug monitoring procedures.

**Conclusions**

A rapid and simple HPLC method using diode array detector has been described for analysis of omeprazole, 5-hydroxyomeprazole and omeprazole sulphone in human plasma. Using reversed-phase column, the chromatographic elution step was undertaken in a short time with adequate resolution. Sample preparation involved liquid-liquid extraction with a single component solvent. To the best of our knowledge, this is the first reported HPLC-DAD method for simultaneous determination of HOME, OME and OMES. The use of diode array detection provided high specificity. Moreover, due to low LLOQ, good accuracy and precision this method is suitable for pharmacogenetics studies, allowing
the identification of PM, EM and UM with regards to CYP2C19 and CYP3A4. Despite the small population of phenotyped individuals, a wide variability in the metabolic ratios for CYP2C19 and CYP3A4 was observed, which has important consequences on dose adjustment for drugs that are substrates for these CYP enzymes.

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References