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Improvement and validation of a high performance liquid chromatography in tandem mass spectrometry method for plasma level monitoring of omeprazole

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Abstract:	Omeprazole (OME) is a proton pump inhibitor (PPI) with 58% bioavailability after single oral dose, presenting large inter-individual variations and significant drug-drug interactions. A simple and rapid liquid chromatography in tandem with mass spectrometry (LC/MS-MS) with solid phase extraction (SPE) and isotope-labelled internal standard (IS) method was developed to monitor the plasma levels of OME for application in pharmacokinetics and drug-drug interactions studies. OME and its IS (OME-D3), were eluted with Zorbax extend C-18 rapid resolution (4.6 mm x 50 mm, 3.5 µm) at 25ºC, under isocratic conditions through a mobile phase consisting of 1 mM ammonium acetate, pH 8.5 (55%), and acetonitrile (ACN, 45%). The flow rate was 0.8 mL/min and the run time of chromatogram was 1.2 min. OME was detected and quantified by LC-MS/MS with positive electrospray ionization (ESI) that operates in multiple-reaction monitoring (MRM) mode. The method was linear in the range of 1.5-2000 ng/mL for OME. The validation assays of accuracy and precision, matrix effect, extraction recovery and stability of the samples for OME did not deviate more than 20% for the lower limit of quantification (LLOQ) and no more than 15% for other quality controls (QCs), according to regulatory agencies.

Improvement and validation of a high-performance liquid chromatography in tandem mass spectrometry method for monitoring of omeprazole in plasma

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Abstract

Omeprazole (OME) is a proton pump inhibitor (PPI) with 58% bioavailability after a single oral dose. It is subject to marked inter-individual variations and significant drug-drug interactions. The authors developed a simple and rapid method based on liquid chromatography in tandem with mass spectrometry (LC/MS-MS) with solid phase extraction (SPE) and isotope-labelled internal standard (IS) to monitor plasma levels of OME in pharmacokinetics and drug-drug interaction studies. OME and its IS (OME-D3) were eluted with the Zorbax Extend C-18 rapid resolution column (4.6 mm x 50 mm, 3.5 μm) at 25ºC, under isocratic conditions through a mobile phase consisting of 1 mM ammonium acetate, pH 8.5 (55%), and acetonitrile (ACN, 45%). The flow rate was 0.8 mL/min, and the chromatogram run time was 1.2 min. OME was detected and quantified by LC-MS/MS with positive electrospray ionization (ESI), which operates in multiple-reaction monitoring (MRM) mode. The method was linear in the range of 1.5-2000 ng/mL for OME. The validation assays for accuracy and precision, matrix effect, extraction recovery, and stability of the samples for OME did not deviate more than 20% for the lower limit of quantification (LLOQ) and no more than 15% for other quality controls (QCs). These findings are consistent with the requirements of regulatory agencies.

Keywords: Omeprazole; liquid chromatography in tandem with mass spectrometry; solid phase extraction; proton pump inhibitor; peptic ulcer.

Abbreviations used:

ACN: Acetonitrile

CAL: Calibration standard

C_{max}: Maximum plasma concentration

AUC: Area under curve

CV: Coefficient of variation

EDTA: Ethylenediaminetetraacetic acid

EIC: Extraction ion chromatogram

EMA: European Medicines Agency

ESI: Electrospray ionization

FDA: Food and Drug Administration

HPLC: High-performance liquid chromatography

IS: Internal standard

LC-MS/MS: Liquid chromatography in tandem with mass spectrometry

LLE: Liquid-liquid extraction

LLOQ: Lower limit of quantification

MeOH: Methanol

MRM: Multiple-reaction monitoring

OME: Omeprazole

PPI: Proton pump inhibitor

PPT: Protein precipitation

QC: Quality control

R^2: Correlation coefficient

RT: Retention time

SD: Standard deviation

SPE: Solid phase extraction

T_{max}: Time of occurrence of C_{max}

$T_{1/2}$: Half-life

TIC: Total ion chromatogram

UV: Ultraviolet

1. Introduction

The prodrug **omeprazole** (OME) is a substituted benzimidazole (6-methoxy-2-[(4-methoxy-3,5- dimethyl-2-pyridinyl)methylsulfinyl]-1*H*-benzimidazole) that acts as a specific PPI by reducing the amount of gastric acid produced by parietal cells. OME was the first PPI used to treat stomach ulcers, gastroesophageal reflux disease, Zollinger-Ellison syndrome, and infection by *Helicobacter pylori* [1-3]. The bioavailability after a single oral dose of 40 mg OME is approximately 58% owing to first-pass liver metabolism [1-3]

Drug-drug interactions with OME result from increased gastric pH or inhibition of the metabolism of some drugs [4, 5]. For instance, phenytoin, warfarin, diazepam, and citalopram decreased clearance [6-9]; digoxin increased and clopidogrel decreased absorption [10, 11]. Therefore, the extensive pharmacokinetic variability of OME and its interactions with other drugs—few are clinically significant—mean that there are situations in which OME must be monitored. Dosage would therefore have to be tailored to individual patient requirements, and pharmacokinetic studies would be necessary to clarify drug-drug interactions, particularly in the case of polymedicated elderly patients or those receiving drugs with a narrow therapeutic index.

To date, plasma OME levels have been analyzed using high-performance liquid chromatography (HPLC) with coulometric detection [12] and ultraviolet detection (UV) [13-19]. However, the lower limit of quantification (LLOQ) in this technique is higher or the run time longer than in methods based on **liquid chromatography in tandem with mass spectrometry** (LC-MS/MS) and **electrospray ionization** (ESI) [20-25]. Although LC-MS/MS improves sensitivity and selectivity, ESI is hampered by ion suppression or

by enhancement from the sample matrix [26] and interference from metabolites [27]. **Solid phase extraction** (SPE) and application of an isotope-labeled **internal standard** (IS) reduced the matrix effect. Matuszewski [28] showed that the use of stable isotope-labelled IS eliminated the relative matrix effect due to similarities between physical-chemical properties, thus reducing variability during sample preparation and ionization.

However, most LC-MS/MS methods with ESI are based on protein precipitation (PPT) [22] or liquid-liquid extraction (LLE) [21, 23, 24], or non-isotopic labelled IS instead of SPE and stable isotope-labelled IS. Although SPE has been carried out using HPLC with UV detection [14] and LC-MS/MS [25, 29], performance times were longer, evaporation and reconstitution steps were necessary, and neither technique was based on isotope-labelled IS. Dodgen and coworkers [20] used LC-MS/MS with automated online SPE and column switching; however, this technology is available in very few laboratories and is difficult to adapt to routine therapeutic drug monitoring.

Therefore, the aim of the present study was to develop a simple, sensitive, and reproducible LC-MS/MS method based on SPE and isotope-labelled IS (OME-D3) to monitor plasma levels of OME in pharmacokinetics and drug-drug interaction studies using enhanced speed of analysis and optimized chromatographic conditions.

2. Materials and Methods

2.1 Chemicals and reagents

All chemicals were analytical or LC-MS grade. OME (6-methoxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulfinyl]-1*H*-benzimidazole) and OME-D3 (6-(methoxy-d3)-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulfinyl]-1*H*-benzimidazole) were supplied by Toronto Research Chemicals Inc. (North York, Canada) and acetic acid by Panreac Quimica (Madrid, Spain). Methanol (MeOH), **acetonitrile** (ACN) **and ammonium acetate** were purchased from LAB-SCAN Analytical Science (SYMTA, Madrid, Spain). The water for preparing the mobile phase was obtained using a Milli-Q system (Millipore-Ibérica, Madrid, Spain). Blank human plasma samples were from 2 sources: the Blood Donation Unit of Hospital Universitario de la Princesa, Madrid, Spain and the Transfusion Center of the Autonomous Community of Madrid.

2.2 Stock solutions, calibration standards (CALs), and quality controls (QCs)

Two separate stock solutions of OME, one for CALs and another for QCs were prepared by dissolving an accurately weighed quantity in MeOH to obtain a concentration of 1 mg/mL. Both stock solutions were diluted independently to obtain several secondary and working solutions for the preparation of CALs and QCs. The IS (OME-D3) stock solution was prepared by dissolving an exact amount in MeOH to obtain a concentration of 1 mg/mL and diluted 40 times to give a working solution of 25 µg/mL. CALs were prepared by independent dilution, in which a specific volume of secondary CAL solutions (0.15 µg/mL, 1 mg/mL, 5 µg/mL, 20 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL, and 200 µg/mL) was added to blank plasma to obtain concentrations of 1.5, 10, 50, 200, 500, 1000, 1500, and 2000 ng/mL, respectively. A calibration curve (1.5 to 2000 ng/mL) was thus generated according to the

recommendations of the United States Food and Drug Administration (FDA) [30] and the European Medicines Agency (EMA) [31] for bioanalytical method validation. QC samples were also made by independent dilution, in which a specific volume of QC secondary solutions (0.15 μg/mL, 0.45 μg/mL, 900 μg/mL, and 1600 μg/mL, respectively) was added to blank plasma to obtain concentrations of 1.5 ng/mL (LLOQ), 4.5 (QC_{Low}), 900 ng/mL (QC_{Medium}), and 1600 ng/mL (QC_{High}), respectively. A drug-free blank plasma sample and drug-free zero plasma sample (processed with IS) were included. All CAL and QC solutions were stored at –80°C until analysis to avoid more than 3 cycles of freezing. Storage was no longer than 2 months.

2.3 Chromatographic conditions

The HPLC system consisted of a 1200 Series separation module (Agilent Technologies, Madrid, Spain) combined with Agilent MassHunter Workstation Data Acquisition software for programming samples and controlling chromatographic conditions. Separations were carried out at 25ºC in a Zorbax Extend C18 Rapid Resolution column (4.6 mm x 50 mm [particle size 3.5 μm] Agilent Technologies, Madrid, Spain). The mobile phase consisted of a combination of ammonium acetate 1 mM in water (pH = 8.5, solution A) and ACN (solution B). It is important to maintain this pH during recording, because OME is very sensitive to acidic pH. The chromatographic run was performed under isocratic conditions at a flow rate of 0.8 mL/min with 55% solution A and 45% solution B. The elution time of each sample was 0.889 min for OME and 0.884 min for OME D3. The total run time was 1.2 min, and a re-equilibration time was not required owing to the isocratic conditions used. At the end of every day, the column

was washed by increasing the percentage of ACN to 100% at a 0.8-mL/min flow rate for 20 min and then returning to the initial conditions within 5 min. Washing was then continued for a further 10 min.

2.4 Mass spectrometry

The mass spectrometry detection system consisted of an Agilent Technologies 6410 triple quadrupole mass spectrometer with ESI in positive ion mode. Mass spectrometry was performed in multiple-reaction monitoring (MRM) mode. Desolvation gas (N_2) and flow were set at 310°C and 8.5 L/min, respectively. Thus, the acetic acid of the mobile phase was easily volatilized at this temperature. The nebulizer pressure was 40 psi, which assured good nebulization efficiency for the chromatographic conditions; the capillary voltage was 4 kV. The mass spectrometry collision gas was high-purity N_2 (> 99.9995). The fragmentor voltage was 75 V and dwell time 200 ms for all compounds. The collision energy was set at 5 eV for OME (quantifier ion) and OME-D3, and at 15 eV for OME (qualifier ion). After separation with HPLC, the peak area corresponding to the transition m/z 346.2 → 198.1 for OME (quantifier ion) was measured relative to that of the transition m/z 349.2 → 198 for its IS (OME-D3). For identification of OME, the m/z 346.2 → 151.1 reaction for the qualifier ion was also monitored to add specificity (**Supplementary Table 1** and **Fig. 1A-B**). The integration peak area of the MRM transitions of each analyte was calculated using MassHunter Workstation Quantitative Analysis software (Agilent Technologies, Madrid, Spain).

2.5 Sample preparation

Samples were prepared by SPE using Nexus Versaplate Bond Elute C18 and 30 mg of polymeric sorbent (Agilent Technologies, Madrid, Spain) with a vacuum pressure of about 3-5 mmHg. The sample was applied after pre-conditioning of the cartridges with 1000 μL MeOH followed by 1000 μL Milli-Q water. This procedure was carried out by spiking 200 μL of plasma with 10 μL of IS (25 μg/mL) and 790 μL of ammonium acetate 1 mM (pH 8.5) for one sample, although the IS was calculated for more samples and pre-mixed with ammonium acetate for the general procedure. Next, a washing step was performed with 1000 μL of 95% ammonium acetate 1 mM (pH 8.5) in 5% MeOH. Elution was performed with 1000 μL of 90% MeOH and 10% ACN plus 1% ammonium acetate 1 mM in water (pH 8.5), which was collected on a 96-well (1 mL) plate. After extraction, samples were transferred to vials or they were directly read from the collection plate. Only 1 μL of eluate was directly injected into the LC-MS/MS. The whole sample preparation procedure was carried out at pH 8.5 to ensure the stability of OME, which degrades rapidly under acidic solutions [32].

2.6 Assay validation procedures

The method was validated in order to demonstrate the reliability of OME in plasma, its biological matrix. Accordingly, the authors followed the recommendations published online by the FDA [30] and the EMA [31].

2.6.1 Calibration curve and LLOQ

Quantitative analysis of OME in plasma was performed using OME D3 as the IS. Eight calibration standards—1.5, 10, 50, 200, 500, 1000, 1500, 2000 ng/mL—were used for validation. A weighted least-square linear regression model was used to calculate the equation relating the ratio of the area of OME to the area of IS and the concentration of OME in the calibration standards. The inverse of the concentration ($1/X^2$) was used as a weighting factor. Six standard curves were analyzed. The standard curve was chosen to cover the range of clinically relevant concentrations expected in most patients. To validate the curve, at least 6 of 8 calibration standards should be less than 15% of the coefficient of variation (CV). For each point of the calibration curve, the error of accuracy and CV should be less than 15% for all calibration standards, except for the LLOQ, which was less than 20%. The LLOQ response of the analyte should be at least 5 times higher than the blank response.

2.6.2 Precision and accuracy

The precision, repeatability, and accuracy of the assay (ie, the closeness of the determined value to the true value) are critical factors when measuring reproducibility. **"The authors assessed the precision and accuracy of the method by analyzing replicate QC samples of 1.5 ng/mL (LLOQ), 10 ng/mL (QC_{Low}), 900 ng/mL (QC_{Medium}), and 1600 ng/mL (QC_{High}) of OME. The intra-day precision and accuracy were evaluated by analyzing 5 samples of each QC on a single day. The inter-day variation was evaluated by injecting a further 5 samples of each QC**

sample (LLOQ, QC_{Low}, QC_{Medium}, and QC_{High}) on 3 consecutive days. In all the samples the pre-treatment procedure was performed before the injection". Precision is defined as a coefficient of variation (%). Accuracy was measured as the percentage difference between the theoretical and the measured value according to the following equation:

$$\text{Accuracy (\%)} = \frac{(concentration_{measured} - concentration_{theoretical})}{(concentration_{theoretical})} \times 100\%$$

To verify precision and accuracy, error must be less than 15% for all samples except the LLOQ (<20%).

2.6.3 Selectivity

The selectivity of the method was examined by analyzing 6 different lots from human blank plasma, with the IS (zero plasma) or without the IS. Each blank or zero sample was tested for interference. The method is considered selective when the response is less than 5 times the LLOQ for OME and less than 20 times for the IS.

2.6.4 Extraction recovery and matrix effect

Recovery is measured as the ratio of the compound concentrations in plasma following SPE to the same concentration dissolved directly in elution solution. Three repetitions of the QCs for OME (4.5 ng/mL [QC_{Low}], 900 ng/mL [QC_{Medium}], and 1600 ng/mL [QC_{High}] ng/mL) were analyzed in 3 different lots of human plasma. To be adequate, recovery of the analyte did not need to be 100%, but the extent of recovery of QC samples had to be precise, reliable, and reproducible.

The matrix effect of plasma was investigated by addition of a known concentration of analyte with its IS to a human blank plasma sample that had undergone SPE. The response was compared with the addition of the same amount of analyte and IS to the final elution solution. This time, 6 repetitions per concentration were analyzed in 6 different lots of human plasma at 4.5 ng/mL (QC_{Low}) and 1600 ng/mL (QC_{High}) for OME. To validate the matrix effect, the coefficient of variation (CV) could not be larger than 15% for all the QCs.

2.6.5 Stability

For OME to guarantee the storage conditions and each step taken during sample preparation and analysis, the authors conducted the following stability assays at 4.5 ng/mL (QC_{Low}) and 1600 ng/mL (QC_{High}):

- after 3 cycles of freeze-thaw in the freezer at –80ºC
- after 24 h at room temperature (short-term stability)
- after 7 h at 23°C in the autosampler
- after 72 h at 4ºC in the fridge
- after 2 months at –80ºC in the freezer (long-term stability)

For all studies, 3 replicates of QC_{Low} and QC_{High} for OME were performed and analyzed according to requirements. Analyte stability had to be less than 15% for all the QCs used.

2.2.7 Carry-over

During validation, carry-over was assessed by injecting blank samples after a high concentration sample or CAL at the upper limit of quantification (2000 ng/mL). Carry-over in the blank sample following the high concentration standard could not be greater than 20% of the LLOQ. The needle was washed between injections with water (55%) and ACN solution (45%) to prevent carry-over.

2.2.8 Preliminary human experiments

The proposed method was applied to determine the plasma profile of OME after a standard oral dose (40 mg single dose) of OME (Losec®, AstraZeneca) from 6 healthy volunteers under fasting conditions. Blood samples were taken at the following time intervals after dosing: 0, 0.66, 1, 1.33, 1.66, 2, 2.33, 2.66, 3, 3.33, 3.66, 4, 4.5, 5, 5.5, 6, 7, 8, 10, and 12 h. Blood samples were collected in ethylenediaminetetra-acetic acid dipotassium dihydrate (EDTA K_2) tubes (Vacuette®) and centrifuged at 3000 rpm for 10 min at 4ºC. The plasma was separated and stored at –20ºC. The study was approved by the local ethics committee (Clinical Research Ethics Committee of "Hospital Universitario de la Princesa", Madrid, Spain), and informed consent was obtained from

healthy volunteers. The pharmacokinetic analysis was carried out by means of a model-independent method with WinNonLin Professional Edition, version 2.0 (Scientific Consulting, Inc, Cary, USA). Maximum plasma concentration (C_{max}) and time of occurrence (T_{max}) were determined directly from plasma concentration data. The area under the plasma concentration-time curve from time zero to the time of the last measurable concentration (AUC_{0-t}) was calculated using the trapezoidal method. The $AUC_{0-\infty}$ was calculated as AUC_{0-t} + C_t/k ratio, with C_t as the last detectable concentration and k the slope of the line obtained by linear regression from the points corresponding to the elimination phase. The half-life ($T_{1/2}$) was calculated as ln2/k.

3. Results

3.1. Optimization of MS/MS conditions and chromatography

The ESI in positive mode and full scan spectra of all compounds indicated that the most abundant ions were the protonated molecules ($[MH]^+$), which were therefore selected to detect the most abundant products. The percent abundance of the precursor and product ions of the quantifier, qualifier, or IS versus mass to charge (m/z) are shown in **Fig. 1A-B** under product ion mode. The fragmentation patterns are also shown. Optimized mass spectrometer parameters such as scan time, fragmentor voltage, and collision energy for OME (quantifier and qualifier ion) and for its IS in MRM mode are summarized in **Supplementary Table 1**.

Fig. 1C shows a typical extraction ion chromatogram (EIC) of plasma spiked with QC_{Medium} (900 ng/mL) and its IS (**1190 ng/mL**) in MRM mode. The areas and

retention times (RT) are shown. Although the RT of OME (0.889 min) and its IS (0.884 min) are very close, they can be separated by the analysis of EIC based on reconstructed ion currents. In addition, total recording time was too short (1.2 min) and did not require a post-time for re-equilibration owing to isocratic elution.

3.2. Calibration curve and LLOQ

The calibration curves with the 8 CALs of OME were linear in the range from 1.5 ng/mL to 2000 ng/mL, with lines of regression forced through the origin. The slope and correlation coefficient (r^2) values were 0.1152 ± 0.0039 and 0.9967 ± 0.0014, respectively, for the average of 6 calibration curves.

The LLOQ at 1.5 ng/mL (Fig. 2) showed an identifiable and reproducible response with an intra-day accuracy of 4.3% and CV of 2.7% and inter-day accuracy of 13.0% and CV of 8.7% (Table 2). The response was more than 5 times higher than that of any target plasma (364 ± 16.65 signal/blank signal). In all CALs, the accuracy did not exceed ±15% of the theoretical value, including the LLOQ.

Supplementary Fig. 1A shows a representative total ion chromatogram (TIC) of a blank plasma sample without IS. **Supplementary Fig. 1B** and **1C** display a typical EIC of blank plasma with IS (zero plasma) and plasma spiked with 1.5 ng/mL (LLOQ) of OME, respectively. The area values of LLOQ of OME (523) are higher than those found for this compound in a human blank plasma sample, taking into account that all chromatograms were normalized to the largest peak.

3.3. Precision and accuracy

The precision and accuracy of the method were assessed by analyzing replicates of 5 samples of 1.5 ng/mL (LLOQ), 4.5 ng/mL (QC_{Low}), 900 ng/mL (QC_{Medium}), and 1600 ng/mL (QC_{High}) for OME. The standard deviation (SD), CV, and accuracy were calculated for each sample. **Table 1** summarizes the results for precision and accuracy of the validation method. The intra-day precision was optimal, with CVs of between 1% and 6%, and accuracy was optimal between 4% and 8%. Variations in inter-day precision were less than 15%, even for the LLOQ (1.5 ng/mL), with CVs of between 5% and 13% and accuracy of between 7% and 13%. We can conclude that the method is reproducible and accurate for OME.

3.4. Selectivity

The present method was selective, as no interference was found in the detection of OME in the absence or presence of IS in 6 different blank samples. **Supplementary Fig. 1** shows the lack of interference of a representative total ion chromatogram (TIC) of blank plasma samples with IS (**Supplementary Fig. 1A**) or without IS (**Supplementary Fig. 1B**). The area of the zero plasma with IS is much more than 20 times the area of the blank plasma of OME (209,901 ± 16,589). Similarly, the EIC showed no interference at the LLOQ of OME (**Supplementary Fig. 1C**).

3.5. Extraction recovery and matrix effect

The extraction recovery of human plasma was determined at 4.5 ng/mL (QC_{Low}), 900 ng/mL (QC_{Medium}), and 1600 ng/mL (QC_{High}) of OME compared with the blank plasma samples spiked with a known concentration of OME after SPE and immediately before injection. The mean recoveries were 103.8%, 87.0%, and 85.9%, and the CVs were in the range of 3% to 12% at the 3 given concentrations, respectively. Therefore, the extraction recoveries were higher than 86% and the bias less than 12%; the extraction was accurate and reproducible, as the recommendations suggest (**Table 2**).

The matrix effect was carried out at 4.5 ng/mL (QC_{Low}) and 1600 ng/mL (QC_{High}) in 6 different human plasma samples. The mean matrix effect (relative extraction) was more than 94%, and the CVs were in the range of 6% to 8%. No matrix effect was observed after the tests were performed; consequently, the response of OME was not significantly reduced by ion suppression (**Table 2**).

3.6. Stability

The stability assays at 4.5 ng/mL (QC_{Low}) and 1600 ng/mL (QC_{High}) of OME are summarized in **Table 2**. Stability tests after 3 freeze-thaw cycles in the freezer at –80ºC showed no degradation, with a mean of more than 95% and CV in the range of 2% to 4%. The short-term stability test after 24 h at room temperature revealed a mean of more than 99% and CV in the range of 4% to 5%. After 7 h at 23ºC in the autosampler, the stability test revealed a mean of more than 98% and CV in the range of 1% to 6%. The stability test after 72 h at 4ºC in the fridge also showed a mean of more than 98% and CV in the range of 1 to 2. Moreover, the extracts were stable after 2 months at –

80ºC (long-term), with a mean of more than 93% and CV in the range of 3% to 8%. The CV was less than 9% in all cases, and the mean was close to 100%.

3.7. Carry-over

The carry-over in the blank sample following the high-concentration standard calibration was 7.2 ± 4.76% of the LLOQ, i.e., less than 20%. Thus, in line with EMA regulations, OME did not present carry-over [31].

3.8. Application of the method

The method the authors developed was successfully applied to pharmacokinetic analysis of 240 samples from 6 healthy volunteers who received an oral dose of OME (Losec®, 40 mg). **Fig. 2** shows OME plasma concentration versus time acquired after administration of OME (0 h to 12 h) in the 6 healthy volunteers. The average maximum plasma concentration (C_{max}) was 1,395.18 ± 814.67 ng/mL at 1.61 **(1.00-3.00)** h (T_{max}) after administration of OME. **Table 3** shows the mean pharmacokinetic parameters of OME after oral administration of 40 mg of OME under fasting conditions. All the results were within the range of 1.5-2000 ng/mL in the calibration curves. **"Our approach was similar to that of clinical practice, since 3 of the 6 healthy volunteers were taking concomitant therapy: norgestimate-ethinyl estradiol) 1 tablet/24 h, acetaminophen 1 g, and ibuprofen 600 mg. Even though these very common over-**

the-counter drugs were taken by our patients, the selectivity of our assay remained unaltered. No interference was observed with any of them during the study".

4. Discussion

PPIs are among the most consumed over-the-counter drugs. OME is widely used and has marked inter-subject variability owing to the different activities of the cytochrome P_{450} system [1, 22, 33]. Drug monitoring should take account of broad pharmacokinetic variability, which makes the relationship between dose and plasma concentration and therapeutic effect unpredictable. Administration of OME can become problematic because of drug-drug interactions, which are especially relevant in patients with chronic diseases and those taking multiple drugs concomitantly or drugs with a narrow therapeutic index [2, 5]. Therefore, we developed an LC-MS/MS–based analytical method with SPE and isotope-labelled IS (OME-D3) to determine OME in human plasma, unravel new drug-drug interactions for pharmacokinetic studies, and thus improve clinical practice.

"The method was linear within a wide range of concentrations (1.5 to 2000 ng/mL), which facilitated measurement of the high inter-subject variability between samples". The LC-MS/MS the authors used had an LLOQ of 1.5 ng/mL for OME and higher sensitivity and selectivity than authors who used HPLC with UV detection between 3-96 ng/mL [13-19] . The most relevant LC-MS/MS–based methods have sensitivities of 0.05 ng/mL [23, 24], 0.4 ng/mL [21], and 1.2 ng/mL[22], which were slightly lower than those used by the authors of the present study. In all of these studies, the run

times were longer, and samples were prepared using LLE or PPT. Even in SPE, which better eliminates the interference of the matrix effect, the run times were too long [25, 29]. Dodgen and coworkers [20] recorded short run times, although they used automated online SPE, which is only available in selected laboratories and difficult to obtain for routine application in hospitals.

Our chromatographic run lasted 1.2 min, which is shorter than times recorded elsewhere (>1.3 min [13, 15, 17, 21-24] or even >16 min [17]). The authors used a Zorbax Extend C-18 high-resolution column, which enabled us to work with a high flow of 0.8 mL min and provided a short analysis time.

The mass spectrometer was operated with the ESI source, which can produce matrix effects that alter ionization efficiency owing to the presence of co-eluting substances such as phospholipids, mobile phase modifiers, and formulation agents [34, 35]. **"One of the advantages was the use of isotope-labeled IS and a more selective sample preparation procedure such as SPE, which helps to eliminate the matrix effect and improves assay selectivity [28], as in the present study".** The authors' approach achieved good recoveries, with average values of 86% to 104%, which are similar to those reported by Dodgen and coworkers [20] and Macek and coworkers [22] or better than those reported elsewhere (>63% [23], 71% to 74% [24], and 83% to 87% [17]). These recoveries were achieved thanks to an ultraclean polymeric sorbent of SPE, which has bimodal porosity and a high surface area. Both the mobile phase and the whole sample preparation procedure were conducted using basic solutions (pH 8.5) so as not to degrade OME, as reported elsewhere [32]. The ratio of MeOH/ammonium acetate buffer at the washing step was critical to extraction yield, as reported by Martens-Lobenhoffer

and coworkers [36]. The authors obtained the best result with 5% MeOH and 95% ammonium acetate, since a higher percentage of MeOH led to a loss of analyte. Other pH values (e.g., 9.5) during sample preparation, or decreasing percentages of ammonium acetate (e.g., 0.5 or 0.1) at the elution step did not increase the efficacy of recovery. **"Another advantage of this procedure was that it did not require evaporation and subsequent reconstitution".** The authors also studied the effect of carry-over, which is not investigated by most authors, and recorded a value of 7.2%, which is lower than that allowed by the EMA [31], thus showing the quality of the method presented here.

We also used small injection volumes (1 μL) to minimize interference and matrix effect, since desorption is easier to perform. The method used only 200 μL of plasma, which is less than that reported elsewhere (250 to 450 μL [21-24] or even 1000 μL [17]). Hence, the quantity of plasma required to validate the bioanalytical methods is reduced.

Finally, this procedure was successfully applied in the analysis of 240 samples from 6 healthy volunteers with a mean (± SD) C_{max} of 1395.18 ± 814.67 ng/mL. The mean plasma concentration was higher under fasting conditions than with food [37]. These results are in line with those of previous reports on the pharmacokinetics of OME by Liu and coworkers (C_{max} of 1330.46 ± 758.07 ng/mL) [38]. Other authors reported lower C_{max} owing to lower doses of OME [24, 39]. The high inter-subject variability indicated by the error bars in **Fig 2** is similar to the findings reported by other authors [22, 38], thus making them suitable for monitoring.

5. Conclusion

"The advantages of the method presented here over other methods are as follows: 1) the technique is linear in the range of 1.5 to 2000 ng/mL and does not require a dilution factor in most cases; 2) shorter run times (1.2 min); 3) good reproducibility, selectivity, recovery efficiencies (86% to 104%) and minimum matrix effect thanks to SPE, isotope-labelled IS, and a 1-μL injection volume; 4) small plasma volume needed (200 μL); 5) no carry-over effect; 6) no evaporation and reconstitution, since direct injection simplifies sample preparation. In summary, the LC-MS/MS method is consistent with the recommendations of the FDA [30] and EMA [31]. It enables fast quantification of OME levels and is easily adapted to pharmacokinetic and drug-drug interaction studies".

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Figure Legends

Fig. 1: Product ion mass spectra and chemical structure of $[M+H]^+$ for omeprazole (OME, A) and its **internal standard** (IS), OME-D3 (B). The fragmentation pattern of OME and its IS are indicated by an arrow on the chemical structure of each compound. The precursor ion and the product ions are shown in the figure. The typical extraction ion chromatogram (EIC) of plasma spiked with 900 ng/mL (QC_{Medium}) of OME and its IS under multiple reaction monitoring (MRM) mode. Retention times (RT) and height and area values are given for OME and OME-D3. These values were calculated from the EIC. All chromatograms have been normalized to the largest peak.

Fig. 2: Mean plasma concentration of omeprazole (OME) versus time (A)

Table 1. Intra-day and inter-day precision and accuracy. Data were obtained from 4 quality controls (QCs) of **omeprazole (OME**; 1.5, 4.50, 900, and 1600 ng/mL) repeated 5 times on the same day for precision and accuracy intra-day and on 3 consecutive days for inter-day assays. The lower limit of quantification (LLOQ) was 1.5 ng/mL. The mean ± SD of the number of total experiments is shown in parenthesis.

Table 2. Extraction recoveries, matrix effect, **and stability tests**. Averaged data for extraction recoveries are the mean ± SD of 3 different blank human plasma samples spiked with 3 quality controls (QCs) of omeprazole (OME; 4.5, 900, and 1600 ng/mL) after **solid phase extraction (SPE)** and compared to 6 blank plasma samples under SPE and spiked with the same known concentrations of 3 QCs immediately before injection

in **high-performance liquid chromatography (HPLC)**. Data are presented as a percentage of recovery. Average data for the matrix effect are the mean ± SD of 6 different blank human plasma samples spiked with 2 QCs of OME (4.5 and 1600 ng/mL) after SPE and compared to QCs without SPE. Data are presented as a percentage of recovery. The number of the total experiments is shown in parenthesis with each QC**.** The storage stability of OME (4.5 and 1600 mg/mL) in the human plasma sample after 3 freeze–thaw cycles (−80°C for 24 h at room temperature, 7 h at 23°C in the autosampler, 72 h at 4ºC in the fridge, and 2 months at –80ºC in the freezer). Data are presented as a percentage of mean ± SD. The total number of experiments is shown in parenthesis with each QC.

Table 3. Main pharmacokinetic parameters obtained after oral administration of **omeprazole (OME** 40 mg, **Losec®**) to 6 healthy volunteers. The pharmacokinetic parameters were maximum plasma concentration (C_{max}), time to C_{max} (T_{max}), area under the plasma concentration-time curve (AUC_{0-t} and $AUC_{0-\infty}$), and half-life ($T_{1/2}$). All pharmacokinetic parameters were expressed as mean ± SD except T_{max}, which is expressed as median with minimum and maximum values.

Supplementary Fig. 1: Representative total ion chromatograms (TIC) of a blank human plasma sample **without** internal standard (IS) (A) and extraction ion chromatogram **(EIC)** of blank human plasma **with** IS (**zero plasma**, B). EIC of plasma spiked with the lower limit of quantification (LLOQ) at 1.5 ng/mL of omeprazole (OME, C). Retention times (RT) and height and area values are given for OME and IS. All chromatograms have been normalized to the largest peak.

Supplementary Table 1. Ions and fragmentation conditions used for multiple reaction monitoring (MRM) for omeprazole (OME) and **its internal standard (IS),** OME-D3.

Figure 1
Click here to download Figure: Fig 1 _Ome_TDM_270814.ppt

Fig. 1

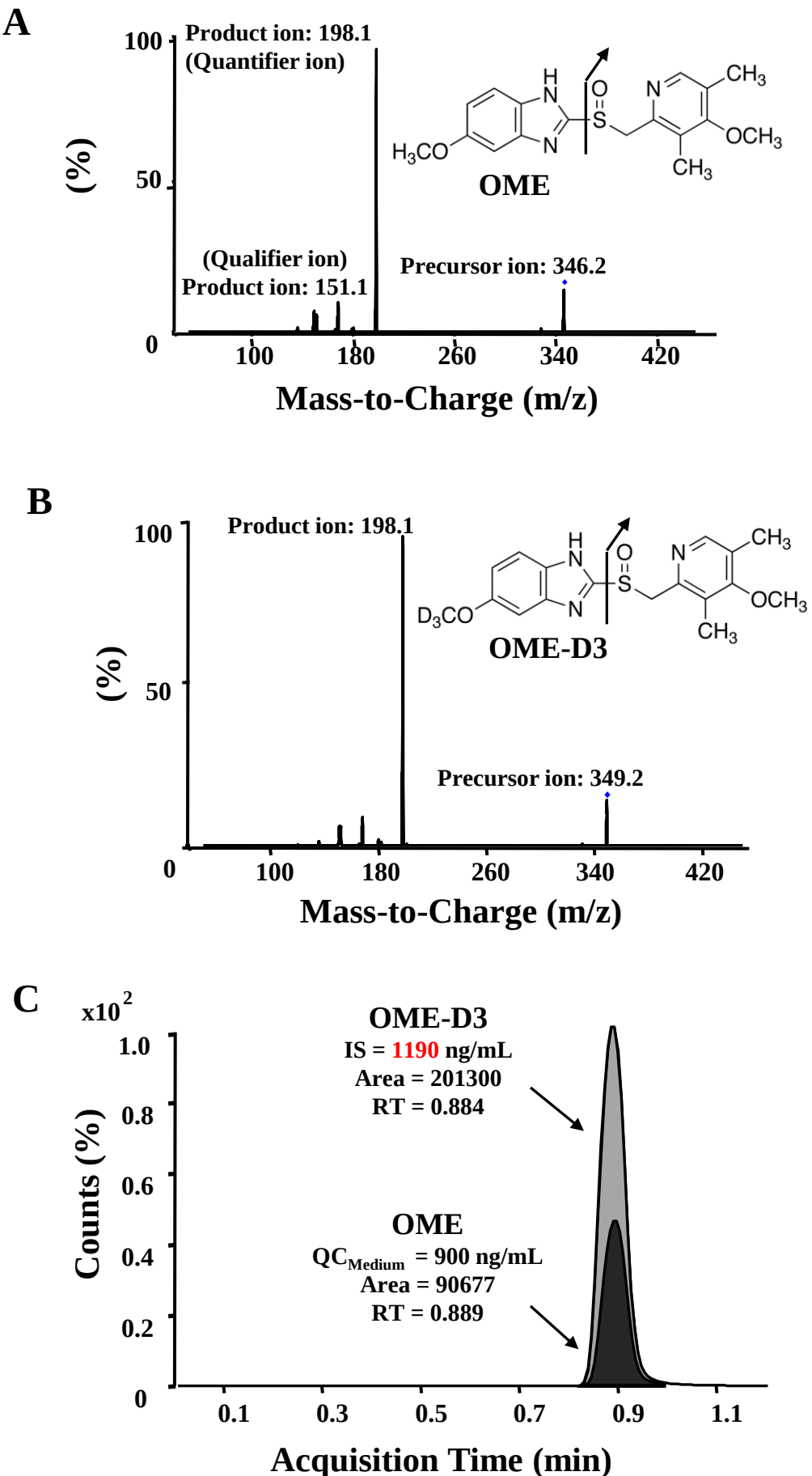
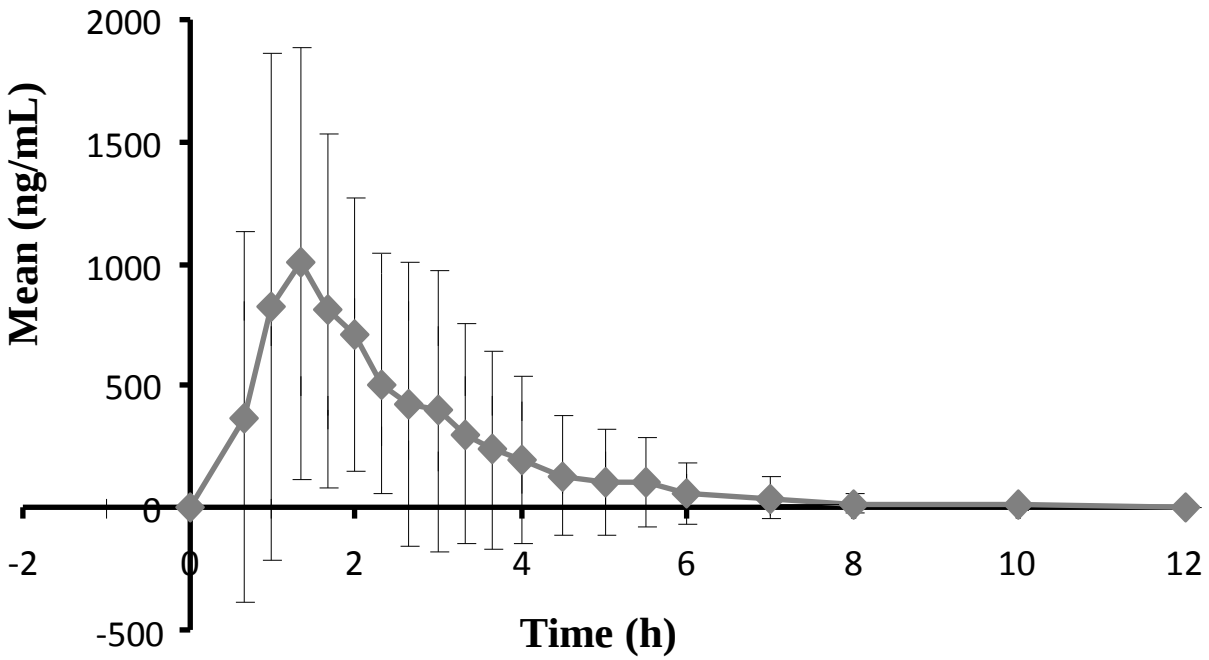


Figure 2

Click here to download Figure: Fig 2 _Ome_TDM_270814.ppt

Fig. 2



Supplementary Fig. 1

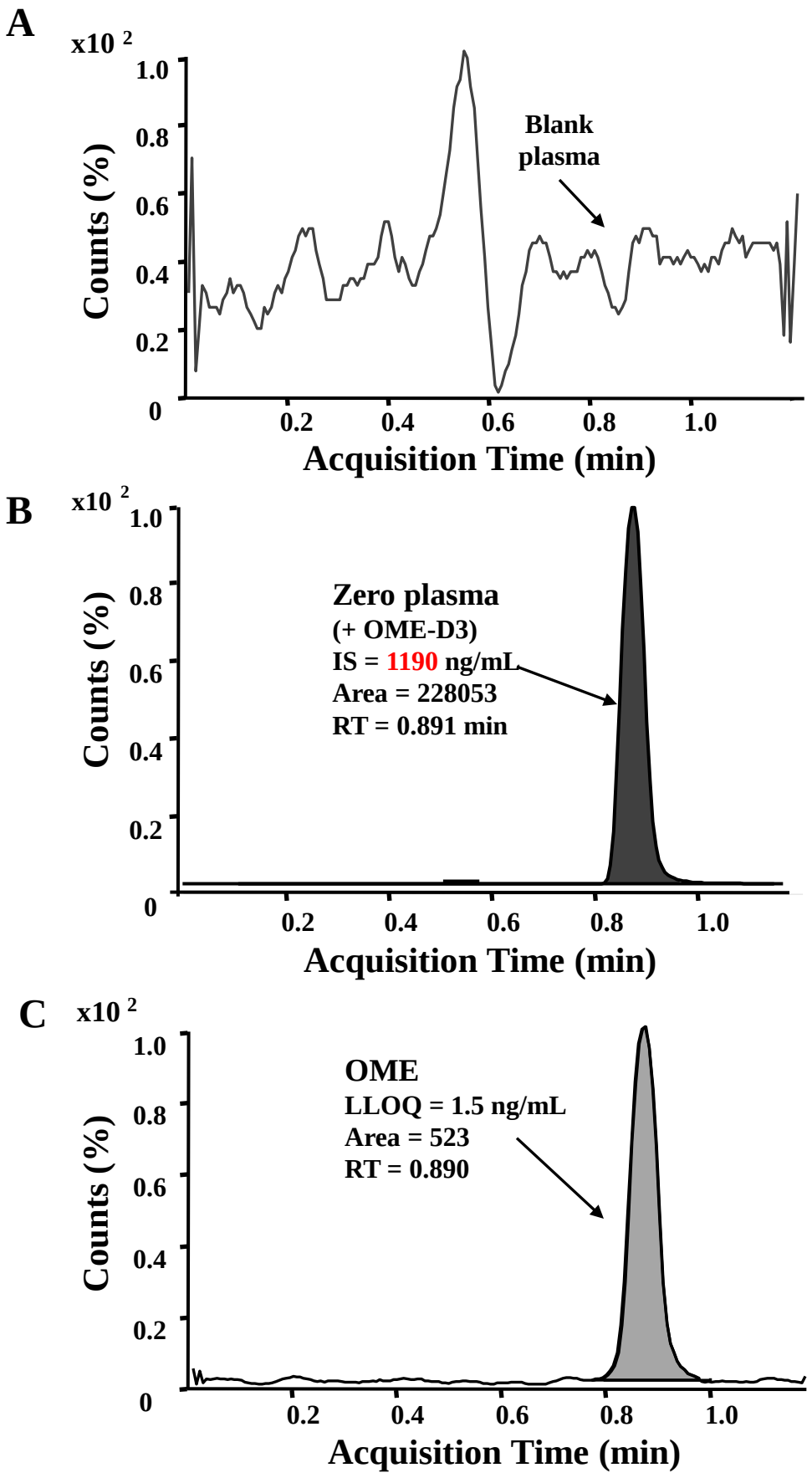


Table 1.

	Concentration (ng/mL)	Mean ± SD (ng/mL)	CV (%)	Accuracy (%)
Intra-day run (n = 5)	1.5 (LLOQ)	1.56 ± 0.04	2.70	4.32
	4.5	4.67 ± 0.27	5.87	3.75
	900	956.39 ± 16.36	1.71	6.27
	1600	1733.12 ± 11.43	0.66	8.32
Inter-day run (n = 15)	1.5 (LLOQ)	1.70 ± 0.15	8.70	13.01
	4.5	5.16 ± 0.67	12.89	14.67
	900	965.05 ± 56.41	5.84	7.23
	1600	1777.87 ± 83.30	4.69	11.12

Table 2

Table. 2

	Concentration (ng/mL)	Mean ± SD (%)	CV (%)
Extraction recovery (n = 3)	4.5	103.82 ± 3.45	3.32
	900	87.01 ± 8.92	10.25
	1600	85.94 ± 10.14	11.80
Matrix effect (relative extraction) (n = 6)	4.5	94.54 ± 7.73	8.17
	1600	100.74 ± 6.26	6.21
Freeze-thaw (3nd cycle) (n = 3)	4.5	95.27 ± 4.09	4.29
	1600	103.99 ± 2.17	2.09
Short-term for 24 h (n = 3)	4.5	99.66 ± 4.48	4.50
	1600	103.98 ± 4.89	4.70
Autosampler after 7 h at 23°C (n = 3)	4.5	98.97 ± 5.57	5.62
	1600	100.45 ± 0.76	0.76
Fridge after 72 h at 4°C (n = 3)	4.5	98.68 ± 1.93	1.95
	1600	99.34 ± 1.36	1.37
Long-term 2 months at 80°C (n = 3)	4.5	118.56 ± 9.81	8.28
	1600	93.48 ± 3.13	3.35

Table. 3

Pharmacokinetic parameters of OME (mean ± SD)

Parameter	OME (Losec®)
*C*max (ng/mL)	1395.18 ± 814.67
*T*max (h)	1.61 (1.00-3.00)
$T_{1/2}$ (h)	0.84 ± 0.44
AUC_{0-t} (ng x h/mL)	2312.44 ± 2783.21
$AUC_{0-\infty}$ (ng x h/mL)	2322.88 ± 2800.39