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Received September 11, 2009
Revised October 27, 2009
Accepted October 27, 2009

Research Article

A bio-analytical hydrophilic interaction LC-MS/MS method for the simultaneous quantification of omeprazole and lansoprazole in human plasma in support of a pharmacokinetic omeprazole study in children

A hydrophilic interaction LC method with MS/MS was developed and validated for the simultaneous quantification of omeprazole and lansoprazole in human plasma. Chromatographic separation was achieved on a Betasil silica column using a high organic mobile phase (eluent A: ACN/formic acid 997.5:2.5 v/v; eluent B: water/formic acid 997.5:2.5 v/v) and gradient elution. The mass spectrometer was operated in the Multiple Reaction Monitoring mode. Prior to chromatography, liquid–liquid extraction with ethyl acetate was used and the organic layer was diluted with ACN, allowing direct injection on column. The method showed acceptable linearity, high precision (RSD% < 10.5%), accuracy (88.9–109.3%) and selectivity in the two concentration ranges studied: 1.5–100 and 5–2000 ng/mL. The LOQ was established at 1.5 and 5 ng/mL for the two concentration ranges. Lack of variability in matrix effects was demonstrated and mean extraction recovery for omeprazole and lansoprazole was determined in the low (56.3–67.7%) and high (45.3–44.3%) concentration range, respectively. Additionally, plasma samples were found to be stable after three freeze–thaw cycles and for at least 15 h after extraction. This assay was successfully applied to a pharmacokinetic omeprazole study in children with cerebral palsy and mental retardation.

Keywords: Hydrophilic interaction LC / Lansoprazole / Omeprazole / Proton pump inhibitor / Quantification
DOI 10.1002/jssc.200900590

1 Introduction

Omeprazole and lansoprazole are proton pump inhibitors that reduce the acid secretion in the stomach and are therefore often prescribed for treating various acid-related gastrointestinal disorders [1–4]. The pharmacokinetic properties of these two proton pump inhibitors are well characterized in healthy adults [5–10]. However, pathological conditions can lead to physiological alterations that may change the pharmacokinetics of omeprazole and lansoprazole. In addition, the efficacy of the treatment may be affected, requiring adjustment of the dosage. Investigation of the particular pharmacokinetics of proton pump inhibitors is of vital importance in specific patient populations, such as patients with cerebral palsy and mental retardation. Consequently, there has been growing interest in a single bio-analytical method for the quantification of omeprazole and lansoprazole in human plasma, enabling routine analysis using the same method irrespective of which of the two proton pump inhibitors is the subject for a particular pharmacokinetic study.

Omeprazole and lansoprazole have been determined in human plasma by different methods, using HPLC with UV detection [11–14] or HPLC coupled to MS/MS [15, 16]. These particular assays describe the quantification of a single proton pump inhibitor, albeit often in combination with its metabolites. Lansoprazole and five metabolites were analyzed by HPLC with UV detection at 285 nm [14, 17]. In addition, omeprazole and its major metabolites were determined using HPLC with UV detection at 302 nm [18–22] or HPLC with MS/MS [23, 24]. Because the metabolites of either omeprazole or lansoprazole are inactive metabolites, their analysis is of limited importance in our pharmacokinetical context.

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Abbreviations: FA, formic acid; **HILIC,** hydrophilic interaction LC; **IS,** internal standard; **LLE,** liquid-liquid extraction; **MF,** matrix factor; **MRM,** multiple reaction monitoring; **QC,** quality control

Finally, enantioselective determination of omeprazole and lansoprazole has been reported [23–30]. Only a few methods describe the simultaneous quantification of lansoprazole and omeprazole [31–33]. Bharathi *et al.* recently reported a HPLC-UV method for the simultaneous estimation of lansoprazole, omeprazole, pantoprazole and rabeprazole in human plasma [33]. However, the LOQ of their method was established at 20.61 ng/mL using 500 μL plasma sample. The method we aimed for not only has to be sensitive enough for the pharmacokinetic studies we intend to set up, but also simple and fast allowing high throughput analysis. Most bio-analytical methods use liquid–liquid extraction (LLE) for sample pretreatment requiring evaporation of the organic extracts and subsequently reconstitution in eluent A, allowing injection onto a reversed-phase HPLC system. These time-consuming steps can be eliminated by using hydrophilic interaction LC (HILIC) as high organic content extracts can directly be injected. Separation of analytes is performed on a polar stationary phase and the mobile phase typically consists of a high organic phase with a small amount of water [34, 35]. Only one assay reported the use of HILIC-MS/MS for the determination of omeprazole and its metabolite 5-OH omeprazole in human plasma [23]. In a 96-well plate, these two analytes were extracted from plasma with ethyl acetate. After dilution of this organic phase, the sample was directly injected onto the chromatographic system. Lansoprazole, on the contrary, has never been quantified using HILIC-MS/MS.

The aim of this study was to develop and validate a single method for the quantification of omeprazole and lansoprazole in human plasma with minimal sample pretreatment and a short analysis time, allowing routine analysis.

2 Materials and methods

2.1 Chemicals

Omeprazole and lansoprazole were obtained from Sigma-Aldrich (St. Louis, MO, USA). The internal standard (IS), 5-methoxy-2-((3,5-dimethyl-2-pyridinyl)methylsulfinyl)-benzimidazole, was purchased from Ramidus AB (Lund, Sweden). Methanol and ACN (both HPLC grade) were from Biosolve (Valkenswaard, The Netherlands) and HPLC-grade water was prepared using a Millipore Synergy 185 water purification system (Millipore, Billerica, MA, USA). Ethyl acetate and acetic acid were obtained from Sigma-Aldrich, ammonium hydroxide (25%) from Merck (Darmstadt, Germany) and formic acid (FA) from Acros Organics USA (Morris Plains, NJ, USA).

2.2 Standard solutions

Primary stock standards of omeprazole, lansoprazole and IS were prepared in methanol at a concentration of 1 mg/mL. These standards were appropriately diluted to working standards used for calibration purposes, and stored at 4°C for maximum 3 months. In addition, plasma calibration standards were prepared by adding 250 μL of working standard to 10 mL drug-free plasma, and stored at −20°C for 3 months. There were two different sets of calibration standards; each set consisted of ten standards with the first set (also called ''high set'') ranging from 5 to 2000 ng/mL and the second set (also called ''low set'') from 1.5 to 100 ng/mL.

2.3 Sample pretreatment

Depending on the expected concentration of omeprazole or lansoprazole, there were two extraction procedure variants. In general, 100 μL of a plasma sample was used and fortified with 50 μL IS (1 μg/mL). By adding 20 μL of a 2% NH_4OH solution, a basic pH was created, allowing extraction of the proton pump inhibitors into the organic phase (300 μL ethyl acetate). After extraction (10 min) and centrifugation (5 min, 55 × *g*, Eppendorf, VWR, West Chester, USA), 200 μL of the upper organic phase was transferred into an autosampler vial containing 400 μL ACN. After vortex mixing for a few seconds, an aliquot of 20 μL was injected onto the column.

For samples with an exceedingly low concentration, 300 μL plasma was needed for extraction. In this case, 50 μL IS (0.1 μg/mL) and 20 μl of a 6% NH_4OH solution were added, and the proton pump inhibitors were extracted with 400 μl ethyl acetate. Extraction and centrifugation was followed by a transfer of 100 μL of the upper organic layer into a vial containing 200 μl ACN. An aliquot of also 20 μL was injected.

2.4 Chromatographic conditions

LC was performed on a Thermo Betasil silica-100 column (50 × 3.0 mm, 5 μm particle size) (Interscience, Breda, The Netherlands) using an Agilent HP 1100 Series HPLC system (Palo Alto, CA, USA) equipped with a degasser, binary gradient pump, column oven (25°C) and autosampler (sample compartment protected from ambient light). The LC system was controlled by ChemStation A.10.02 [1757] software (Agilent Technologies, Santa Clara, CA, USA). Eluents A and B consisted of 0.25% v/v FA in ACN and 0.25% FA in water, respectively. After an isocratic 1.5 min plateau (6.5% eluent B) at a flow of 800 μL/min, linear gradient elution was used from 6.5 to 26.5% eluent B within 0.5 min. After regaining initial conditions within 0.1 min, the column was re-equilibrated for 2.9 min, yielding a total run time of 5 min. The needle was rinsed after each extraction with 10 μL wash solution (methanol containing 1% acetic acid).

2.5 MS conditions

The HPLC system was directly interfaced with a Waters Quattro Ultima triple quadrupole system (Micromass

Waters, Manchester, UK), equipped with an orthogonal electrospray source (Z-spray®) operated in the positive ion mode. A standard 120 μm capillary was used in the ESI interface. The following ionization parameters were optimized for omeprazole, lansoprazole and IS. The source was operated at 135°C and the desolvation temperature was 450°C. Electrospray voltage and cone voltage were optimized up to 3.8 kV and 40 V, respectively. Nitrogen served both as nebulizer (154 L/h) and desolvation gas (584 L/h), and argon was used as collision gas.

Time dependent Multiple Reaction Monitoring (MRM) transitions were used for the quantification of the proton pump inhibitors (Table 1). Data were collected and processed using the Masslynx® and Quanlynx® software (Micromass Waters).

2.6 Method validation

The validation requirements as described in the current FDA Guidance [36] and in the subsequent 2006 Bioanalytical Methods Validation Workshop white paper [37] were used.

2.6.1 Linearity

Calibration curves of the high set plasma standards were constructed over a range of 5–2000 ng/mL (5; 10; 30; 50; 150; 250; 600; 1000; 1500; 2000 ng/mL). Those of the low set standards ranged from 1.5 to 100 ng/mL (1.5; 2; 2.5; 3; 4; 5; 10; 20; 50; 100 ng/mL). Quality control (QC) samples at three different concentration levels ($QC_1/QC_2/QC_3$ 7.5/180/1800 and 3.5/7/70 ng/mL for the high and low set, respectively) were used to accept or reject the analytical run. Analyte-to-IS peak area ratios were plotted against the corresponding concentrations. The simplest model, which adequately describes the relation between ratio and concentration, was used. The calibration curve was also evaluated by its correlation coefficient, slope and intercept.

2.6.2 Precision, accuracy and LOQ

To determine within-day precision and accuracy, six extracts of each QC sample were analyzed on the same day. Between-day precision was evaluated by analyzing the QC samples in duplicate for several days. Between- and within-day precision were expressed as the RSD% of the measured QC samples. Accuracy was calculated as a trueness and the LOQ was defined as the lowest concentration on the calibration curve, which could be measured ($n = 5$) with a precision (RSD%) not exceeding 20% and with an accuracy between 80 and 120%.

2.6.3 Matrix effect and recovery

Matrix effect was expressed as the IS-normalized matrix factor (MF), defined as the ratio of the peak area (analyte/IS) in presence of matrix ions (= post-extraction sample) to the peak area ratio in absence of matrix ions (= pure sample) [37]. The post-extraction samples were prepared by spiking a quantity of the proton pump inhibitors into a blank plasma extract, while the pure samples were prepared by spiking an equivalent quantity of proton pump inhibitor into neat extraction reagent (ethyl acetate/ACN 1:2 v/v). IS was added post-extraction. The IS-normalized MF was determined for six different lots of plasma, allowing the evaluation of the variability in MFs, measured by the RSD%, which should be less than 15% [37]. These experiments were performed at all three of the QC concentration levels.

The recovery of an analyte extracted from plasma was determined by comparing the responses (peak area ratios analyte/IS) of the proton pump inhibitor spiked before extraction (pre-extraction sample) into plasma with those spiked after extraction of a blank plasma sample (post-extraction sample). The IS was added post-extraction. The matrix effects are assumed to be similar for pre- and post-extraction samples, since both samples have the plasma ingredients present. Consequently, any difference in response may be considered as caused by extraction recovery. At the three QC concentration levels, the recovery was evaluated by analysis in sixfold in a single lot of plasma. Subsequently, extraction mean recovery was calculated.

2.6.4 Selectivity and stability

Selectivity was assessed by examining peak interference from five independent sources of blank plasma. The interference of co-medication was additionally tested; alizapride, carbamazepine, cisapride, clonazepam, diazepam, phenobarbital, lamotrigine, lynestrenol, oxcarbazepine, tetrazepam and topiramate were added to blank

Table 1. MRM transitions and MS operational parameters

		Precursor-ion (*m/z*)	Product-ion (*m/z*)	Dwell time (s)	Collision energy (eV)	Start time (min)	End time (min)
Lansoprazole	Quantifier	370.3	251.9	0.15	9	0.0	1.6
	Qualifier	370.3	119.0	0.15	9	0.0	1.6
IS	Quantifier	316.2	167.8	0.15	9	0.0	1.6
	Qualifier	316.2	148.9	0.15	9	0.0	1.6
Omeprazole	Quantifier	346.1	197.8	0.30	9	1.6	3.0
	Qualifier	346.1	167.8	0.30	9	1.6	3.0

plasma samples at a concentration of fivefold the C_{max} of each drug. These drugs were chosen on the basis of their frequent use in the typical patient population targeted by our pharmacokinetic studies.

Short-term stability of the proton pump inhibitors in plasma was studied under two experimental conditions: after storage in autosampler (20°C) for 15 h and after three freeze–thaw cycles. For both high and low set samples, the autosampler tray stability was tested according to the following procedure: six extracts of a middle standard were made of which three were analyzed immediately, while the other three were analyzed 15 h later. Stability was then determined by comparing the concentration of the first three analyzed samples with the last three, applying statistical data analysis. Furthermore, freeze–thaw stability was determined using the commonly accepted procedure [36]. After three freeze–thaw cycles, the samples were compared with regularly analyzed samples having the same amount of analyte.

3 Results

3.1 Method development

3.1.1 Optimization of the chromatographic separation

A HILIC-MS/MS method has been developed based on the method described by Song and Naidong [23]. Using HILIC for the separation of omeprazole and lansoprazole has several advantages; due to the high organic concentration of the mobile phase, high flow rates can be used and a mass spectrometer can easily be coupled to the LC system.

In our study, linear gradient elution was carried out at a flow of 800 μL/min. The mobile phase typically consisted of ACN with a small amount of water, which is considered to be necessary for the chromatographic separation in a HILIC system. To improve protonation and ionization of the components, promoting detection, different percentages (0.10, 0.25 and 0.50%) of FA were added to the mobile phase. These alterations also affected the retention and separation of omeprazole and lansoprazole on the HILIC-column. The best peak shapes were obtained with 0.25% FA, instead of 0.10% FA, used by Song and Naidong [23]. In addition, as we considered chromatographic separation of the compounds desirable (albeit not vital), several methods for gradient elution have been investigated. Different combinations starting between 2.5 and 10% eluent B (water containing 0.25% FA) and subsequently linearly raising eluent B up to somewhere between 22.5 and 28.5%, at different gradient rates, were examined. Under our optimized conditions (as described in Section 2), the two proton pump inhibitors and IS were well separated (Fig. 1.). An equilibration time of 2.9 min showed to be sufficient to re-equilibrate the column and to obtain stable inter-run retention times.

3.1.2 Optimization of the detection

In this assay, MS/MS was applied. The product ion spectra are shown in Fig. 2. The two most important transitions were monitored for each component. One product ion served as a quantifier, the other as a qualifier. The mass spectrometer was therefore operated in the MRM mode to quantify the proton pump inhibitors.

3.2 Extraction procedure

The choice of HILIC as a separating technique was also inspired by its impact on the extraction procedure; the high organic mobile phase facilitated direct injection of organic extracts and, moreover, eliminated the need for time-consuming and error-prone evaporation and reconstitution steps.

In initial experiments, the LLE method described by Song and Naidong [23] was used. Although Song and Naidong [23] obtained an LOQ of 2.5 ng/mL starting from 50 μL plasma, our instrumental set-up failed to achieve this. By increasing the amount of plasma to 100 μL and changing the ratio aqueous/organic phase, (validated) sensitivity was initially improved down to an LOQ of 5 ng/mL. This final procedure, summarized in Section 2, was applied to analyze patient plasma samples for a pharmacokinetic study on omeprazole.

In spite of data from literature and modeling indicating that a LOQ of 5 ng/mL would be more than suitable, a large part of the samples from our first clinical study had omeprazole concentrations below this LOQ. This necessitated the optimization of the extraction procedure with an LOQ of 1.5 ng/mL as main purpose. However, difficulties were met in obtaining a more concentrated extract because of the many dilution steps involved in our extraction procedure and the absence of evaporation and reconstitution steps. Different possibilities were evaluated to alleviate this problem such as (i) direct injection of the undiluted ethyl acetate phase, (ii) reduction of the volume ethyl acetate used for extraction and (iii) increase in amount of plasma. Sensitivity improvement of the analytical method was most suitably obtained by extracting distinctly more (300 μL) plasma with only limitedly more (400 μL) ethyl acetate, resulting in an LOQ of 1.5 ng/mL. A detailed description of this procedure can be found in Section 2.

3.3 Method validation

3.3.1 Linearity

Statistical analysis of the concentration-peak area ratios (analyte/IS) proved in all cases that linear correlation was the best model in the two concentration ranges studied (5–2000 ng/mL and 1.5–100 ng/mL, high and low set, respectively) (Table 2). This was also confirmed by the

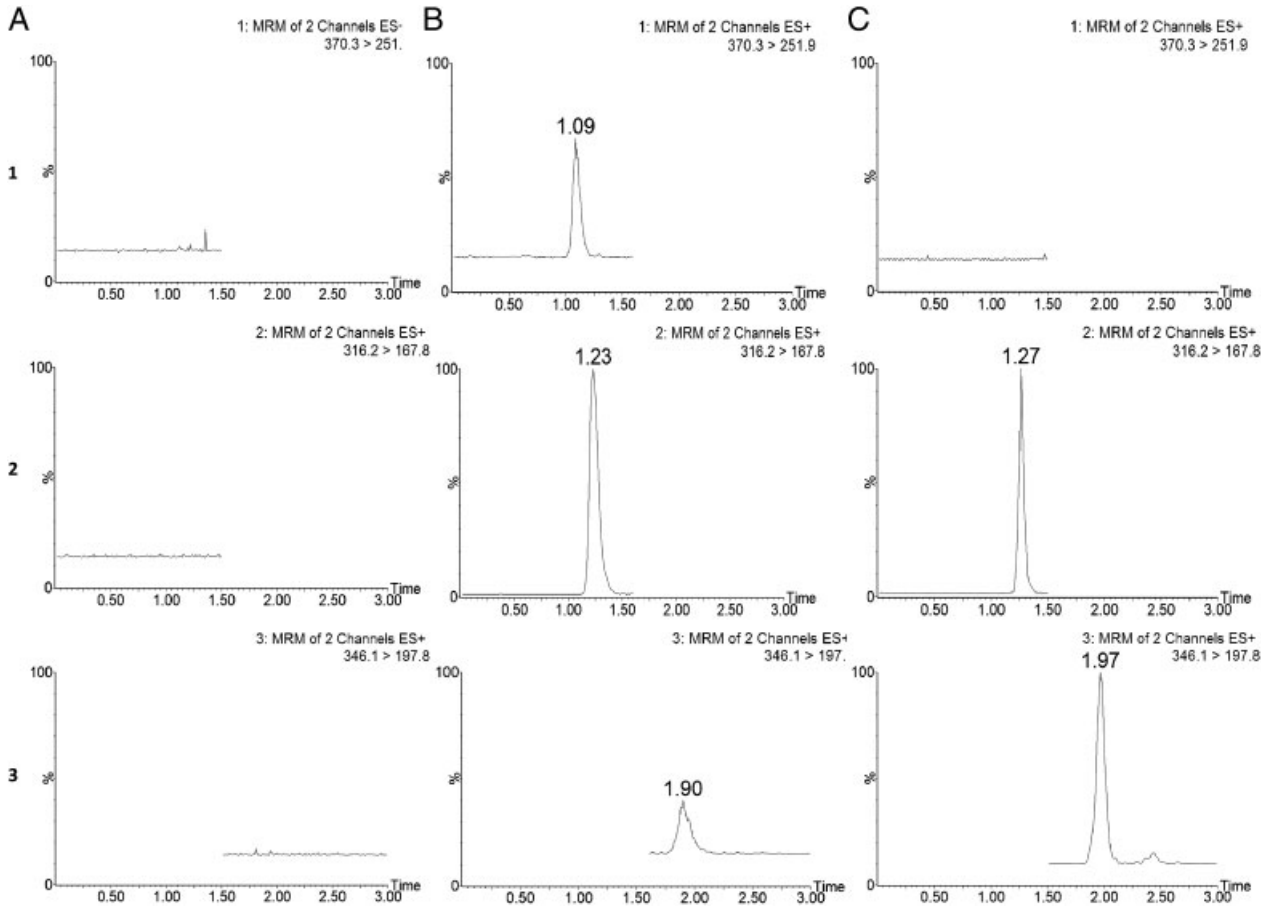

Figure 1. Mass chromatograms of the extracts from (A) blank human plasma; (B) spiked (10 ng/mL) human plasma with (1) lansoprazole, (2) IS and (3) omeprazole and (C) plasma from a patient 2.5 h after a multiple dose of 20 mg of omeprazole.

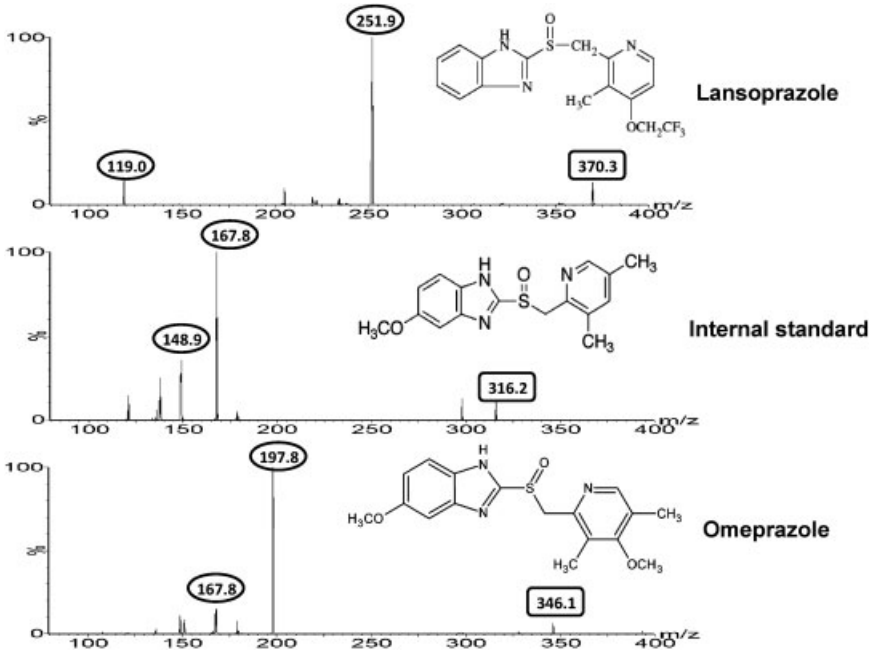

Figure 2. Product ion spectra of lansoprazole, IS and omeprazole.

back-calculated calibrator concentrations. In the regression analysis, a $1/x^2$ weighting factor was used to obtain the best residuals and consequently the best accuracy.

3.3.2 Precision, accuracy and LOQ

At all three QC concentration levels for both sets, between- and within-day precision was better than 10.5% for the two proton pump inhibitors and accuracy ranged from 88.9 to 109.3% (Table 3). The method therefore proved to be precise and accurate. Furthermore, the criteria for the LOQ described in Section 2.6.2 were entirely met and the lowest standard on the calibration curve was consequently accepted as LOQ, namely 5 and 1.5 ng/mL for the high and low set, respectively.

3.3.3 Matrix effect and recovery

The IS-normalized MFs of six different lots of plasma are summarized in Table 4, indicating that the matrix effect is negligible. This is also confirmed by the variability measured in MF: 3.39% at 7.5 ng/mL, 3.07% at 180 ng/mL and 1.02% at 1800 ng/mL for omeprazole; 4.86% at 7.5 ng/mL, 3.82% at 180 ng/mL and 4.65% at 1800 ng/mL for lansoprazole. Clearly, the variability in MF is within generally accepted limits [37].

Furthermore, omeprazole was extracted with a mean recovery of 45.3% for the high set samples and 56.3% for the low set samples. Extraction mean recovery for lansoprazole was 44.3% and 67.68% for the high and low set samples, respectively.

3.3.4 Selectivity and stability

The analysis of five independent sources of blank plasma samples showed no interference at the retention times of lansoprazole, omeprazole or IS. In addition, the interference of co-medication was tested. Again, no interference was observed.

Statistical data analysis was applied to determine short-term stability. For both extraction procedures, autosampler tray stability was tested. In the high set samples, a statistically significant difference in mean was observed (for a standard with a concentration of 500 ng/mL): 540.83 ± 3.19 after 15 h *versus* 514.66 ± 10.23 for omeprazole ($p = 0.01$); 515.59 ± 13.67 after 15 h *versus* 549.57 ± 5.45 for lansoprazole ($p = 0.02$) (*t*-test). However, these differences are insignificant to this analytical method since the mean of the measured concentration of the samples after 15 h still lies within 15% of its respective nominal value (according to the internationally accepted criteria [36]). Moreover, no significant difference was found in the low set samples (for a standard with a concentration of 10 ng/mL): 8.91 ± 0.55 for omeprazole; 9.21 ± 0.56 for lansoprazole; $p > 0.05$. Extracted samples from both sets could therefore be left in the autosampler tray for 15 h prior to analysis.

In three freeze–thaw cycles, omeprazole and lansoprazole were stable in plasma, indicating no significant loss during repeated thawing and freezing at all three QC concentration levels (data not shown). Freeze–thaw stability is independent of the analytical method used so the results for the high set samples are also valid for the low set samples.

4 Discussion

Although in most assays RP-HPLC is used for the separation of proton pump inhibitors, our method involves HILIC as separation technique. The use of underivatized silica columns coupled with MS/MS detection in bio-analysis has been reviewed by Naidong and showed to be a valuable alternative to RP columns [38]. This approach, compared with RP-HPLC, offers several advantages, which facilitate high throughput analysis. Due to its low aqueous/high organic mobile phase, HILIC can be easily coupled to electrospray MS/MS. In water-miscible polar organic solvents such as ACN, ionization will be easier achieved and the sensitivity will be improved. In addition, the low viscosity of these organic solvents allows higher flow rates compared with RP-HPLC and thus results in faster separation. Furthermore, using water as the stronger eluent makes direct injection of the organic phase after LLE possible. This also contributes to a high throughput analysis since evaporation and reconstitution steps can be eliminated [34, 38].

Based on a report by Song and Naidong [23], a HILIC-MS/MS method has been developed for the simultaneous

Table 2. Statistical data of the calibration curves

	Slope Mean ± SD	Intercept Mean ± SD	Linearity (r^2) Mean ± SD	S.E.[a)] of estimate Mean ± SD
High set ($n = 5$)				
Omeprazole	0.0028 (±0.0002)	−0.0009 (±0.0011)	0.9985 (±0.0008)	0.0001 (±0.0000)
Lansoprazole	0.0040 (±0.0003)	−0.0025 (±0.0022)	0.9967 (±0.0015)	0.0002 (±0.0000)
Low set ($n = 4$)				
Omeprazole	0.0868 (±0.0041)	−0.0111 (±0.0042)	0.9938 (±0.0022)	0.0050 (±0.0008)
Lansoprazole	0.1071 (±0.0049)	−0.0025 (±0.0110)	0.9945 (±0.0022)	0.0053 (±0.0010)

a) S.E. = standard error.

Table 3. Between- and within-day precisions (RSD%) and accuracies (as trueness) of omepazole and lansoprazole for the two concentration ranges

High set Added concentration (ng/mL)	Within-day assays ($n = 6$)			Between-day assays ($n = 14$)	
	Measured concentration (mean ± SD) (ng/mL)	RSD%	Accuracy (%)	Measured concentration (mean ± SD) (ng/mL)	RSD%
Omeprazole					
7.50	6.86 ± 0.30	4.40	91.60	6.90 ± 0.40	5.80
180.00	189.79 ± 10.60	5.58	105.54	179.19 ± 14.05	7.84
1800.00	1964.96 ± 77.35	3.94	109.27	1849.69 ± 136.42	7.38
Lansoprazole					
7.50	7.31 ± 0.67	9.22	98.42	7.13 ± 0.59	8.23
180.00	176.69 ± 16.19	9.16	99.05	171.95 ± 13.86	8.06
1800.00	1812.57 ± 51.30	2.83	101.61	1799.91 ± 98.62	5.48
Low set Added concentration (ng/mL)	Within-day assays ($n = 6$)			Between-day assays ($n = 12$)	
	Measured concentration (mean ± SD) (ng/mL)	RSD%	Accuracy (%)	Measured concentration (mean ± SD) (ng/mL)	RSD%
Omeprazole					
3.50	3.21 ± 0.17	5.17	91.77	3.13 ± 0.26	8.43
7.00	6.24 ± 0.20	3.13	89.29	6.19 ± 0.27	4.36
70.00	69.61 ± 3.52	5.05	99.54	68.47 ± 5.40	7.89
Lansoprazole					
3.50	3.08 ± 0.24	7.86	88.85	3.18 ± 0.32	10.16
7.00	6.31 ± 0.30	4.72	90.94	6.43 ± 0.47	7.33
70.00	68.64 ± 3.31	4.82	98.95	67.98 ± 6.11	8.99

Table 4. IS-normalized matrix factors of omeprazole and lansoprazole

IS-normalized MF ($n = 2$)	Omeprazole			Lansoprazole		
	7.50 ng/mL	180 ng/mL	1800 ng/mL	7.50 ng/mL	180 ng/mL	1800 ng/mL
Plasma A	0.98	1.05	0.95	1.06	1.02	1.05
Plasma B	0.97	0.97	0.93	1.13	0.94	0.95
Plasma C	0.98	0.99	0.94	1.16	1.01	0.94
Plasma D	0.98	1.04	0.95	1.06	0.99	0.92
Plasma E	0.94	1.01	0.95	1.02	1.05	0.96
Plasma F	0.90	1.04	0.96	1.07	1.03	0.93

quantification of omeprazole and lansoprazole in human plasma. A short analysis time, a simple sample pretreatment with a small sample volume and a sensitive analytical method were considered to be critical aspects.

Not only a high flow rate of 800 μL/min, but also fast gradient elution accelerated the chromatographic process, resulting in a total run time of 5 min. Lansoprazole and omeprazole were nevertheless well separated and time dependent MRM could therefore be used for their quantification, reducing the risk of mass spectrometric cross-talk to a minimum. Since the application field of this assay was pharmacokinetics, a wide concentration range was favored. Our initial method was successfully validated in a range from 5 to 2000 ng/mL. Literature and modeling taken into account, this concentration range was considered to be more than adequate for the quantification of patient plasma samples of a first pharmacokinetic study on omeprazole. However, in a substantial number of patients, very low omeprazole plasma levels were obtained of which several were below the LOQ. This consequently complicated the construction and interpretation of the corresponding pharmacokinetic profiles, more specifically the determination of the terminal elimination phase. In order to fully suit our purposes, an in-depth optimization of analytical method sensitivity was undertaken. From the initial development stages, it was clear that sensitivity enhancement of the LC-MS/MS part was only achievable at the cost of selectivity. In view of our limited sample clean-up, this was undesirable, and in addition, the LC-MS/MS part was actually only really meant to be discriminating between compounds with

largely different characteristics. Therefore, we focused on the sample preparation itself. However, the use of HILIC-MS/MS as analytical technique leads to a restricted choice of possible manners to optimize the extraction. Clearly, the advantage of the simple extraction procedure permitted by our HILIC approach now somewhat turned against us. The limited number of procedural steps combined with the fact that HILIC separations are much more sensitive to the deleterious effects of injecting solvent mixtures of different composition to the starting chromatographic eluent, offers few degrees of freedom to fine-tune a certain method. A few possible manners to optimize the extraction were studied. Initially, the necessity of diluting the ethyl acetate phase with ACN prior to injection has been evaluated. Direct injection of the undiluted ethyl acetate phase, however, demonstrated an interference with the chromatography. Therefore, diluting the extract with ACN in a ratio of ethyl acetate/ACN of 1:2 was considered to be essential, once more proving the somewhat lower robustness of HILIC chromatography, compared with RP-LC. Reducing the number of dilution steps during extraction proved not possible. On the contrary, adjusting the dilution ratios could offer a solution. In our initial approach, the proton pump inhibitors were extracted from 100 μL plasma with 300 μL ethyl acetate. A reduction of this volume to 200 μL ethyl acetate, however, did not fulfill our expectations, most probably due to an unfavorable ratio between the aqueous and organic phase. One of the possible options left was increasing the amount of plasma. Even though sensitivity was significantly improved by using 300 μL plasma compared with the previous method, it was found to be necessary to additionally increase the volume of ethyl acetate to 400 μL, allowing the formation of 100 μL supernatant, which was considered to be sufficient for further processing. Furthermore, good results were obtained using this method and sensitivity was greatly improved resulting in a LOQ of 1.5 ng/mL.

As a result, we now had two possible options, (i) enlarging the calibration range downwards or (ii) developing a derivative method especially for samples with a low concentration. One should note that a concentration range from 5 to 2000 ng/mL is already a wide range. Considering that these extracts will be more concentrated (to gain measurement sensitivity), detection saturation at the upper calibration end is likely to occur. In addition, it is well known that the more calibration points used in a small range, the more accurate the concentrations measured will be. From that point of view, developing a derivative analytical method with a smaller concentration range proved to be a better option for the analysis of samples with an exceedingly low concentration, also taking into consideration that a re-validation was necessary anyhow. Consequently, a dedicated ''low set'' samples procedure with minor changes as detailed above, was validated (fully, according to the FDA Guidance [36]) and implemented in parallel.

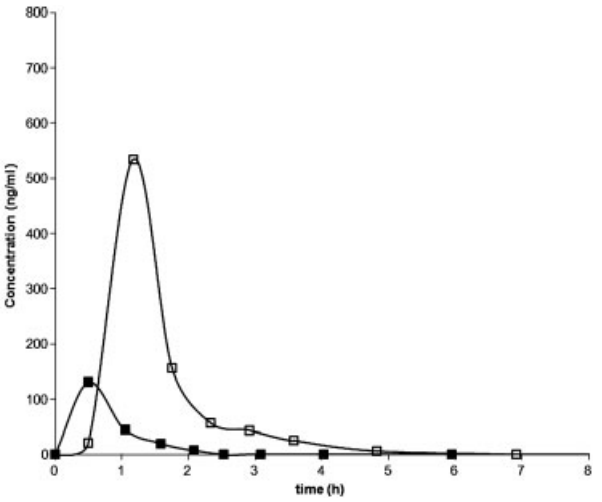

Figure 3. Plasma concentration over time profile for omeprazole administered as Losec MUPS® (□) and as suspension in 8.4% bicarbonate (■).

As already referred to, we used this procedure in the bio-analytical support of a first pharmacokinetic study dealing with omeprazole administration in children. The resulting full pharmacokinetic data and interpretation supersede the scope of this manuscript but a single patient pharmacokinetic profile of omeprazole administrated as Losec MUPS® and as a suspension in 8.4% bicarbonate solution (in cross-over) is shown in Fig. 3., illustrating the practical applicability of our analytical method.

5 Concluding remarks

This paper presents the first validated method for the simultaneous determination of omeprazole and lansoprazole in human plasma by HILIC-MS/MS. The method complies with pre-set requirements of high sensitivity (LOQ 1.5 ng/mL), specificity and rapid sample throughput. HILIC based chromatography coupled to MS/MS was essential in fulfilling these goals resulting in a fast and easily applicable routine method. Furthermore, the simultaneous omeprazole/lansoprazole approach provides us with one single validated method that can be used at any time, regardless of which proton pump inhibitor is being studied. The assay has already been successfully applied to analyze patient plasma samples for a pharmacokinetic study on omeprazole. Follow-up clinical studies on omeprazole, esomeprazole (the omeprazole *S*-enantiomer) or lansoprazole are now all analytically within reach, some of which are already forthcoming.

Part of the research presented here was supported by grants G.0320.02 by the FWO-Vlaanderen and 01B.05305 (Ghent University BOF).

The authors have declared no conflict of interest.

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