Indonesian J. Pharm. Vol. 27 No. 2 : 80 – 91
ISSN-p : 2338-9427
DOI: 10.14499/indonesianjpharm27iss2pp80

Research Article

AN LC- MS/MS METHOD FOR THE DETERMINATION OF OMEPRAZOLE ON PROTON PUMP INHIBITOR IN HUMAN PLASMA

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Submitted: 09-02-2016
Revised: 13-03-2016
Accepted: 02-04-2016

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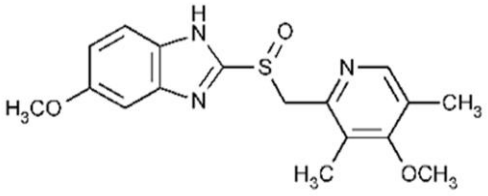
ABSTRACT

A sensitive and selective liquid chromatographic method coupled with tandem mass spectroscopy (LC-MS/MS) was developed for the quantification of omeprazole in human plasma. Lansoprazole was used as internal standard with plasma samples, extracted using 10mM ammonium acetate. A centrifuged upper layer was then evaporated and reconstituted with Acetonitrile: mobile phase buffer 70:30%v/v. The reconstructed samples were injected into a C_{18} column purospher star 5µ. The mobile phase was composed of ACN: mobile phase buffer (5mm ammonium bicarbonate buffer) in the ratio of 70:30%v/v with flow rate 1.0mL/min. The mass spectrometer was operated using positive ion mode and turbo electro spray ionisation. Nitrogen was used as the nebulizer, curtain, collision and auxiliary gases. Using MS/MS with multiple reactions monitoring (MRM) mode, omeprazole was detected without severe interferences from plasma matrix. Detection of omeprazole in human plasma was accurate and precision. This method has been successfully applied to the study of omeprazole in human specimens

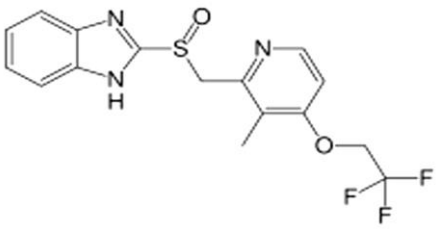
Keywords: Proton pump inhibitor, omeprazole, lansoprazole, LC-MS/MS, liquid liquid extraction

INTRODUCTION

Omeprazole is chemically known as 1*H*-Benzimidazole,5-methoxy-2-[[(4-methoxy-3,5-dimethyl-2pyridinyl)methyl]sulfinyl]-.5-Methoxy-2-[[(4-methoxy-3,5-dimethyl-2pyridinyl)methyl] sulfinyl] benzimidazole. Lansoprazole (internal standard) is chemically known as (Figure-1) (RS) -2- ([3 – methyl-4-(2, 2, 2 – trifluoromethoxy) pyridine-2-yl] methyl sulfinyl) -1 H- benzo[d] imidazole. Omeprazole is a selective and irreversible proton pump inhibitor. Omeprazole suppresses gastric acid secretion by specific inhibition of the hydrogen–potassium adenosine triphosphatase (H^+K^+-ATPase) enzyme system found at the secretory surface of parietal cells. It inhibits the final transport of hydrogen ions (via exchange with potassium ions) into the gastric lumen. Since the H^+K^+-ATPase enzyme system is regarded as the acid (proton) pump of the gastric mucosa. Omeprazole is known as a gastric acid pump inhibitor. Omeprazole is also used to treat infection caused by Helicobacter pylori (H. pylori). Recently, cytochrome p-450 2C19 genotype related anti–H. pylori efficacy by combining omeprazole and antibiotics were reported (Rang *et al.,* 2007 and Furuta *et al.,* 2007). Determination of the omeprazole concentration in body fluids including serum, plasma and cerebrospinal fluid is of importance in conducting clinical studies of this drug with regard to efficacy, toxicity and dose ranging.



Omeprazole



Lansoprazole

Figure 1. Structure for omeprazole and lansoprazole

Modern pharmacokinetic studies require parts per billion characterizations and quantification as well as the ability to provide analytical results with rapid turnaround from large batches of samples. However, in recent years the use of high performance liquid chromatography (HPLC) with ultra violet (UV) detection, mass spectrometric detection (MSD) and tandem mass spectrometry (MS-MS) have been found to be ideally suited for the determination of analyte in diverse biometrics. Determination of omeprazole and its main metabolites by liquid chromatography using hybrid miceller mobile phase (Rambala, *et al.,* 2009). Enantiomers of omeprazole in blood plasma were determined by normal-phase liquid chromatography and detected using atmospheric pressure ionization tandem mass spectrometry (Stenhoff *et al.,* 1999). Chiral assay of omeprazole and metabolites and its application to a pharmaco-kinetics related to CYP2C19 genotypes (Shiohira *et al.,* 2011). Assay of omeprazole and omeprazole sulfone has been reported using semi-micro column liquid chromatography with mixed-function precolumn (Yim *et al*., 2001). Several HPLC methods have been published for quantitatively determining omeprazole levels in human plasma (Vittal *et al*, 2009; DeSmet *et al*., 2010; TsingHua 2004; Bharathi *et al*., 2009; Kanazawa *et al*., 2004; Wang *et al*., 2004 and Kang *et al*., 1999). The methods are isocratic and gradient HPLC (or) HPLC with column switching liquid chromatography mass spectrometry (LC-MS/MS). Some of these assay methods are relatively time consuming while others have poor lower limit of quantitation. Sample preparation by liquid – liquid extraction was another time consuming step in these methods. Therefore, our goal is to develop a relatively rapid, high sensitivity method for omeprazole in human plasma with very low limit of quantification using LC-MS/MS instrument. This paper discusses the method validation of rapid, liquid chromato-graphy tandem mass spectrometric (LC-MS/MS) assay for omeprazole in human plasma using lansoprazole as internal standard.

The sample preparation is simple and consists of precipitating plasma with 10mm extraction buffer ammonium acetate (pH 10.0) and 2mL TBME solvent by vortex mixing and spinning down the protein into a pellet. The supernatant is injected into a LC column and eluted with isocratic of 5mm ammonium bicarbonate buffer (pH 8.0) and Acetonitrile. A board calibration curve range from 5.016 to 4013.028ng/mL was adequate to handle most pharmacokinetic samples. Some samples were obtained at extended sampling intervals beyond the 12h and also to measure samples from peak concentrations without dilution. However, these published methods are not ideal for pharmacokinetic work, because they are time consuming i.e., derivatization step, arduous sample preparation and long chromatographic run time. Likewise, they need a relatively large amount of sample to reach a low quantification limit. In addition detection of omeprazole using LC-MS/MS has to be reported.

Therefore, this study established a novel quantification method for detecting omeprazole in human plasma using liquid chromatography electron spray tandem mass spectroscopy. This method has been successfully applied to pharmacokinetic studies to determine the concentration of omeprazole in human plasma.

MATERIAL AND METHODS

Chemicals and reagents

All solvents including HPLC grade methanol, acetonitrile and tertiary butyl methyl ether were obtained from Rankem, Mumbai. Ammonia solution (SQ grade 25%), sodium bicarbonate (GR grade 99.5%), ammonium bicarbonate (HR grade 99%), ammonium acetate (GR grade 98%) were also obtained from Rankem Mumbai. Aurobindho Pharmaceuticals provided both Omeprazole, Internal standard (Lansoprazole) and ethylene diamine tetra acetic acid (EDTA) treated plasma sample. A Millipore Milli-Q Plus was used to generate deionized water in-house.

Instrumentation

Water 2695 HPLC coupled with a Quattro premix XE mass spectrometer was utilized for the separation and detection of Omeprazole. Both HPLC and mass spectrometer were controlled remotely using Mass Lynx software V 4.1. Data analysis was performed with the QuanLynx module that accompanies Mass Lynx.

Calibration standard and quality control preparation

A 1mg/mL master stock solution of Omeprazole was prepared in 7:3v/v ACN: mobile phase buffer (5Mm ammonium bicarbonate buffer 8.0). This solution was diluted to make a series of standard working solutions at 5,10,40,80,200,400,1000,2000,3200 and 4000ng/mL. A 100µg/mL stock solution of internal standard (Lansoprazole) was prepared in 7:3v/v ACN: mobile phase buffer and diluted to 1µg/mL. For preparation of calibration curves, 10µL of appropriate levels of calibration standard and 10µL of Lansoprazole (IS) was added to 200µL of freshly thawed EDTA plasma in a borosilicate glass culture tube, and mixed. A separate weighing was used in order to prepare 1mg/mL quality control master stock solution in 7:3-ACN: mobile phase buffer. The quality control master stock solution was diluted to 256.43, 732.672, 6912.00, 17280.00, 86400.00 and 160000.00ng/mL for preparing lower limit, lower, intermediate, medium$_1$, medium$_2$ and high –level quality control working solutions. Plasma quality controls were prepared by addition of 0.2mL of the appropriate level of quality control working solution to 9.8mL of EDTA plasma, for final concentrations of 5, 14.653, 345.600, 1728.00, 3200.00ng/mL. All master solutions, working standards and quality control standards were stored in refrigerator at 2-8°C.

Sample preparation

Calibration standards were prepared as described above. Additional samples consisted of 200µL of unknown or quality control plasma. Internal standard and 7:3-ACN: mobile phase buffer were added to all unknown and quality control samples. Blank (plasma only with 7:3 ACN: mobile phase buffer, no internal standard) and 100µL of plasma sample with internal standard and 20µL ACN: mobile phase. Samples were included in every run. An adaptation of a previously published liquid – liquid extraction was employed in order to separate Omeprazole from plasma components (Wang *et al.*, 2004). All calibration standards, quality control samples, and unknown samples were alkalised by the addition of an equal volume of 10mM ammonium acetate buffer pH 10.0, and then mixed by vortexing in a glass culture tubes. Omeprazole was extracted by an addition of 2mL of tertiary butyl methyl ether and samples were thoroughly mixed by vortexing for a period of 15min in rotospin at 40rpm. Then centrifugation was done for 4500rpm at 40°C for 5min. Following centrifugation, the aqueous component was frozen in a dry ice isopropanol bath and the organic component was decanted into a separate 2.0mL micro centrifuge tube. Organic phase was evaporated under a stream of nitrogen in 30±5°C water bath using a TurboVap LV concentration work station. Dried samples were reconstituted to 500µL using reconstituted solution and mixed by vortexing for about 1min. 10µL sample solution were then transferred to low volume inserts in HPLC vials and loaded into the autosampler.

HPLC separation and MS-MS condition

All samples were subjected to separation using a Water 2695 HPLC with a Purospher Star C_{18} column (5µ, 100X4.6mm). Separation was achieved with an isocratic run using mobile phase consisted of a mixture of acetonitrile with 5mM ammonium bicarbonate buffer (7:3%v/v). The pH of the mobile phase was then adjusted to 8.0 with formic acid. Injection consisted of 10µL of each sample, the flow was set at 1.0mL/min and the overall run time was 2.0min. Omeprazole and internal standard (lansoprazole) were detected as they eluted from the column using MS-MS detection in the electrospray positive (ES^+) mode. The first Quadra pole Q_1 was set to monitor the protonated molecule (m+1) at m/z 346.18 and 369.97 for omeprazole and internal standard respectively. Product ions resulting from collision induced fragmentation at Q_2 were monitored via Q_3 at m/z 198.00 and 250.00 for omeprazole and internal standard respectively (Table I).

Bioanalytical method validation

A through and complete method validation in human plasma was done following the U.S.FDA guidelines.

Validation runs containing the full calibration curve, blank samples, six replicates each of the lower limit of quantification

(LLOQ 5ng/mL) low, mid and high level quality control samples were run on six different days. Six different lots of EDTA – treated human plasma were used during the validation process.

The carryover effect of the auto sampler was evaluated by sequentially injecting solutions of analytes (aqueous standard), reconstitution solution, standard blank and extracted standards of analytes, equivalent to highest standard in the calibration range.

The calibration procedure is based on the use of various standards, often prepared and measured in duplicate, for the assessment of the calibration model. The use of pure synthetic solutions that can be used for the preparation of standards is limited to those methods without matrix effects (Antonio Checa *et al*). Calibration models are relying on linear relationship between responses and concentration. Calibration standards in plasma sample containing 5.0 to 4000.00ng/mL were used to establish a single calibration curve with $1/X^2$weighed linear equation. Lowest limit of quantification (LLOQ) of drug as the minimal concentration in the samples could be determined with a deviation lower than ±20%. Concentrations of LLOQ and quality control samples were determined from calibration curves created with each run and at least four out of six of these had be within 15% of nominal value.

The selectivity / specificity of the method towards endogenous plasma matrix components, metabolites and concomitant medications was assessed in ten batches (7 normal of K_2EDTA, 1 heamolyzed, 1 lipidemic and 1 heparinized) of blank human plasma. Cross talk of MRM channels for analyte and IS were checked using the highest concentration from the calibration curve and the working solution of IS. The effect of potential interfering drugs like paracetamol, ibuprofen, caffeine, diphenyl hydramine hydrochloride, diclofenac sodium and chlopheniramine maleate was studied under the same condition , and their possible interference at the elution time of analyte and IS was observed.

The precision and accuracy are evaluated as the relative standard deviation of within or run to run (intra) assay as well as between or day to day (inter) assays. Within and between batch precision were evaluated using previously frozen quality control samples at four different concentrations of 14, 345,1728, 3200ng/mL, designated as Low, medium$_1$, medium$_2$ and high concentrations. For between precision, was analysed using six samples of each concentrations for a total of 24 assays on 3 different days using six sets of standard curves. Mean and standard deviation were obtained for the calculated drug concentration over all 3 days and coefficient of variation (%CV) for all four different levels (n = 18 for each) were determined. For within precision 6 samples from each of four concentrations were assayed with a single calibration curve and coefficient of variation for the calculated drug concentrations were determined.

The Recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical within the limits of variability. Recovery of omeprazole from plasma following sample preparation was assessed by comparing the concentration of drug from extracted plasma quality control samples against unextracted quality control samples at different levels. Percentage mean recovery and %CV were calculated.

The matrix effect is defined as the combined effect of all components of the sample other than the analyte on the measurement of the quantity. The evaluation of matrix effect is another issue that cannot be underestimated as the calibration strategy to be followed depends on this. It has been proved that cleaner extract such as those resulting from LLE are less affected by matrix effects. Besides the influence of the sample matrix composition on MS and MS/MS responses has often been found to be significant. As a result, strategies based on post extraction and post column infusion have been utilized for assessing matrix effect (Taylor, 2005). To study the effect of matrix on analyte quantification with respect to consistency in signal enhancement / suppression, it was checked in screened 6 different batches (4 for normal plasma, 1 haemolytic and 1lipidemic). Each lot of plasma

having concentration equivalent to HQC and LQC were prepared in triplicate and injected. Then it was checked for % accuracy and precision (%CV) at HQC and LQC level. This was assessed by comparing back calculated value from the QC's nominal concentration. The deviation of standard should not be more than ±15%, and at least 90% of the lots at each QC level should be within the acceptance criteria.

Stability experiments were carried out to examine the analyte stability in stock solution and in human plasma samples under different conditions. The long term stability of human plasma was assessed based on the analysis of QC (HQC and LQC) samples that had been prepared and stored at 2-8°C for a storage period of 6days and 21h. The short term stability studies were determined by the QC samples was prepared, stored at 7h and 12min. To assess the room temperature stability of omeprazole in human plasma, quality control samples were allowed to remain at room temperature for 4.30h before analysis. Stability was assessed by comparing against the freshly prepared sample with stability sample. Autosampler stability, bench top stability, dry extract stability and freeze extract stability were performed at HQC and LQC using three replicates at each level.

To authenticate the ruggedness of the proposed method, it was done on two precision and accuracy batches. The first batch was analysed by different analysed by different analysts, while the second batch was analysed on two different columns.

Dilution integrity experiment was evaluated by diluting the stock solution prepared as spiked standard at concentration of 9880.73ng/mL in the screened plasma for analyte. The precision and accuracy for dilution integrity standards at $1/5^{th}$ (1976.14ng/mL) and $1/10^{th}$ (988.074) were determined by analysing the sample against calibration curve standards.

Bioequivalence study design

This validated method applied to measure the omeprazole concentration in serum samples to evaluate the bioavailability of the single dose of omeprazole 20 mg capsule in 24 healthy volunteers (Kamrun *et al.,* 2009). The procedures followed while dealing with human subjects were based on International conference on Harmonization, E6 Good Clinical Practice (ICH, E6 GCP) guidelines. The medication was administered under fasting conditions with 250ml of water. Blood samples were collected at 0.0 (pre dose) 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 8.0, 10.0, 12.0, 24h in labelled K2 EDTA vacuettes. After each blood sampling plasma was separated by centrifugation at 3000 rpm for 15min and stored at -80°C until further analysis. Serum samples were prepared by liquid -liquid extraction technique.

RESULTS AND DISCUSSION

Representative chromatograms and mass spectrum of omeprazole and internal standard, extracted under the condition of the assay and of the LOQ (5ng/mL) (Figure 2) and demonstrate excellent separation of the omeprazole and internal standard with short run times around 1.25 and 1.35min respectively. Peak area ratios of omeprazole to IS for the calibration standards were proportional to the concentration of Omeprazole in plasma over the range tested. As the variance increased in proportion to the concentration, the best weighting was $1/x^2$. The ten standard curves were linear from 5.016 to 4013.02ng/mL, with a mean equation 0.004749+0.002984 and an average coefficient of variation were found to be 0.9984. The selectivity of the method towards endogenous plasma matrix was ascertained in ten batches of human plasma by analysing blanks and spiked plasma samples at LLOQ concentration. No endogenous peaks were observed at the retention time of the analytes for any of the batches.

Recovery

Six replicates at LQC, MQC and HQC levels (extracted and unextracted) were prepared for recovery determination. The mean percentage recovery at HQC, MQC and LQC levels for omeprazole and IS were 87.54, 86.34, 84.27% and 86.81, 87.24, 83.62 respectively. The %CV at all QC levels for omeprazole and internal standard were 6.10 and 4.14% respectively.

Table I. Settings for MS – MS detection of omeprazole

Source (ES^+)	Settings	Analyzer	Settings
Capillary (kV)	3.50	Low mass I resolution	13.0
Extractor (V)	4.00	High mass I resolution	13.0
RF lens (V)	0.0	Ion energy I	1.0
Source temperature (0°C)	120	Entrance	2.0
Desolvation temperature (0°C)	400	Exit	2.0
Cone gas flow (L/h)	50	Low mass II resolution	13.0
Desolvation gas flow (L/h)	800	High mass II resolution	13.0
		Ion energy II	1.5
		Multiplier (V)	650
		Collision gas pressure (mbar)	$3.5e^{-3}$

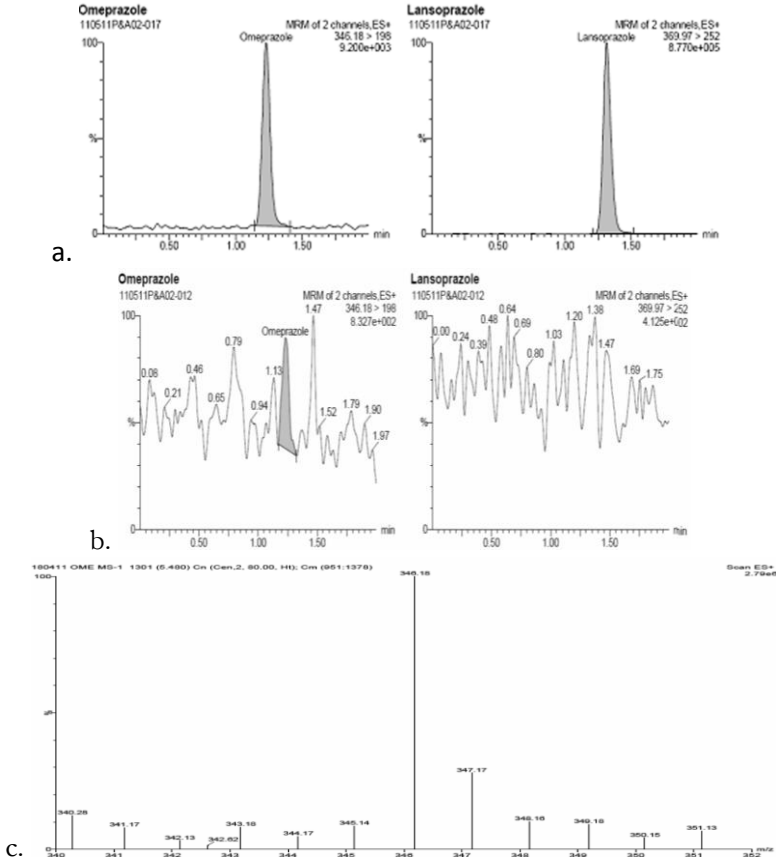


Figure 2. a Mass chromatograms of omeprazole and lansoprazole, b Mass Spectrum of omeprazole and lansoprazole, c Mass spectrum of omeprazole

Within and between batch accuracy and precision

The within and between batch accuracy and precision was determined in four batches at LQC, MQC1, MQC2 and HQC levels with six replicates for each batch. Precision (%CV) for within batch and between batches ranged from 0.59 to 8.92% and 2.49 to 5.17% for analyte. The accuracy results for within batch and between batches were ranged from 91.56% to 99.01% and 93.32 to 98.0% for analyte respectively, at all quality control levels (Table II).

Table II. Within- and between batch Precision/Accuracy for omeprazole

QC ID	Nominal Conc (ng/mL)	n	Mean Observed Conc (ng/mL)	% CV	% Accuracy
Within batch					
HQC	3266.69	6	3213.45	2.13	98.37
MQC1	1764.04	6	1746.56	0.59	99.01
MQC2	352.80	6	346.05	2.63	98.09
LQC	14.95	6	13.69	2.50	91.56
LLOQ	5.14	6	4.70	5.71	91.34
Between Batch					
HQC	3266.69	18	3183.52	2.97	97.45
MQC1	1764.04	18	1725.63	2.49	97.82
MQC2	352.80	18	345.93	5.17	98.05
LQC	14.95	18	13.95	3.42	93.32
LLOQ	5.14	18	4.78	10.24	92.93

Table III. Matrix Effect Data of Omeprazole in Six Different Lots of Human Plasma at HQC and LQC Level

Lot no	HQC 3266.7*		LQC 14.95*	
	Calculated Conc. (ng/mL)	% Accuracy	Calculated Conc. (ng/mL)	% Accuracy
1	3025.19	92.60	13.62	91.10
2	3224.07	99.30	14.69	98.26
3	3133.16	95.91	13.04	87.22
4	3398.86	104.04	14.20	94.98
5	3308.31	101.27	14.47	96.89
6	3130.99	95.84	12.47	83.41
Mean		98.16		91.97
SD		4.17		5.82
%CV		5.62		6.32

*Nominal concentration (ng/mL)

Ion suppression, matrix effect

Matrix effect may arise due to co-elution of some unintended components present in biological samples or which are added as a part of analysis. These components may result in ion suppression/enhancement, decrease/ increase in sensitivity of analyte over a period of time, increase baseline, imprecision of data, drift in retention time and distortion or tailing of a chromatographic peaks. (Matuszewski *et al.*, 2003; Dams *et al.*, 2003) The precision (%CV) values for HQC and LQC samples were observed were 4.32 and 5.46%, while the accuracy found at this levels were 96.46 and 91.18% for analyte (Table III).

Stock solutions for long- term and short – term stability of the analyte and internal standard were stable at room temperature for minimum period of 21h between 2-8°C for 6 days, respectively. Analyte in control human plasma (bench top) at room temperature were stable at least 7hr at 25°C and for a minimum of four freeze – thaw- cycles. Spiked plasma samples stored at 2-8°C for long stability experiment were stable for a minimum of 6day. Dry extract stability of the spiked quality control samples stored at -28°C was determined up to 66h. Auto sampler stability of the spiked quality control samples maintained at 5°C was determined up to 67h. Different stability experiments in plasma and the values for the precision and percentage change (Table IV).

Table IV. Stability Data of omeprazole in Human plasma under various storage conditions (n=6)

Storage Conditions	Mean comparison samples	Calculated Concentration (ng/mL)	
		Mean stability	% mean sample stability
Auto sampler stability			
HQC	3153.69	3403.28	107.91
LQC	15.03	15.96	106.19
Bench Top Stability			
HQC	3208.09	3202.75	99.83
LQC	14.47	15.17	104.87
Freeze thaw stability			
HQC	3153.69	3346.69	105.81
LQC	15.03	16.13	98.43
Wet extract stability			
HQC	3208.09	3125.64	97.43
LQC	14.47	13.89	96.03
Dry extract stability			
HQC	3153.69	3368.50	106.81
LQC	15.03	15.89	105.74

Table V. Mean pharmacokinetic parameter following Oral administration of 20mg tablet formulation (Test and Reference) of Omeprazole in 20 healthy human subjects

Parameter	Test Formulation	Reference formulation
T_{max} (h)	2.60	2.08
C_{max} (ng /ml)	1011.53	1335.46
$t_{1/2}$(h)	1.27	1.05
$AUC_{0-\infty}$	3240.01	3480.69
AUC_{0-t}	3252.80	3456.04

T_{max}- Time of the C_{max}, C_{max}-Maximum observed concentration, $AUC_{0-\infty}$ - area under the concentration time curve extrapolated to infinity, AUC_{0-t}-area under the concentration curve from time zero to the last measurable concentration, $t_{1/2}$- elimination half –time.

For ruggedness study, the precision and accuracy of calibration curve standards and LLOQ were between 1.43-5.25% and 96.14-109.45% for analyte, which is within the acceptance criteria. For both experiments the precision and accuracy for LLOQ, all QC samples ranged from 1.98- 11.53% and 97.89-102.73 for analyte, which are within the acceptance limit of 15% in precision and 85.0-115.0% in mean accuracy.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out analyte concentration above the upper limit of quantification (ULOQ), which may be encountered during real subject sample analysis. The precision of dilution integrity of $1/5^{th}$ and $1/10^{th}$ dilution were found to be 0.75 and 4.17% respectively for analyte, while the accuracy results were found to be 98.08 and 94.49% respectively for analyte. The results were within the acceptance limit of 15% for precision (%CV) and 85.0-115.0% for accuracy.

Application of the method in Human subjects

The analytical method had been applied successfully for the analysis of samples from several pharmacokinetic studies. The assay of omeprazole in healthy volunteers in the age group of 20-45 years. Figure 3 shows the mean plasma concentration time profile following 20mg of dosing to 30 human subjects under fasting conditions up to 24h. In all samples

including the calibration and quality control samples with volunteers samples were run and analysed over a period of 10days. The precision and accuracy for calibration and QC samples were well within acceptable limits. The pharmacokinetic parameters like plasma concentration maximum (C_{max}), area under the plasma concentration –time curve from zero hour to infinity ($AUC_{0\text{-}inf}$), ($AUC_{0\text{-}t}$) area under the concentration time curve extrapolated to infinity, time point of plasma concentration maximum (T_{max}) were calculated. The pharmacokinetic parameters obtained for the test and reference formulation (Table V).

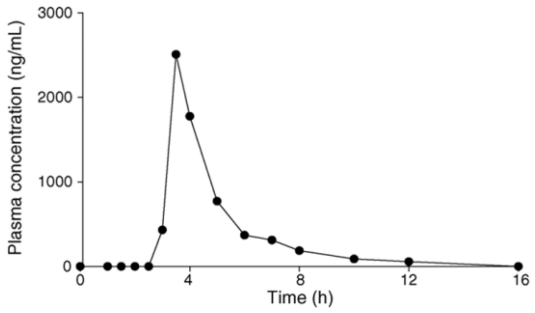


Figure 3. The mean plasma concentration time curve

The aim of the work was to develop and validate an assay for omeprazole in human plasma, which will be suitable for clinical pharmacology purposes. For clinical pharmacology studies, a simple and rapid extraction step is necessary, which is why liquid-liquid extraction was chosen. This extraction method was also used by Kang *et al.*, 1999; Wang *et al.*, 2004; Desmet *et al.*, 2010; but with higher limit of quantification by Bharathi *et al.*, 2009A solid phase extraction of 90% efficiency was proposed by Woolf *et al.*, 1998; Kanazawa *et al.*, 2004 but it is more expensive than liquid-liquid extraction. The simple protein precipitation extraction procedure was investigated but proved to be unacceptable due to interference and low sensitivity. Liquid-liquid extraction was then investigated using different extraction buffers like, 0.5% NH_3, 10mM ammonium acetate buffer pH 8.0, ammonium acetate pH 10.0 and 0.1% formic acid. Among these buffers 10mM ammonium acetate (pH10.0) buffer was found to be showing reproducible results. 50µL instead of 100µL extraction buffer and 2mL TMBE were used. In general HPLC coupled to MS/MS instruments is used to monitor specific mass transitions so analytes can be quantified free of interferences even in poorly resolved chromatographic peaks. Compounds are usually detected in positive mode from their $[M+H]^+$ peaks. Electrospray is the ionization source to be combined with triple quadrupole analyser. Full-scan positive–ion spectra of omeprazole and internal standard predominantly the protonated molecular ion at m/z 346.18 and 369.97 respectively. The product ion mass spectra of these protonated molecular ions (Figure 2) show the presence of one predominant ion for each compound at m/z and for omeprazole and internal standard respectively. Several columns were tested to assay omeprazole, such as Kromosil luna (100X 4.6mm, 5µ, C_{18}), Grace smith (150 X 4.6mm, 5µ, C_{18}). In these two columns response peak shape was good, column pressure was more and lower level repeatability was bad. Phospher star (100X 4.6mm, 5µ, C_{18}) column at pH 8.0 was evaluated and found to provide adequate peak shape and resolution for the analysis of omeprazole. Omeprazole when prepared in solution with a pH of less than 8 has been reported to degrade rapidly. Therefore, in order to maximize analyte stability, a chromatographic system that utilized a mobile phase with a pH greater than 8 was required. Several mobile phases were tested with the chosen column methanol/ammonium acetate (pH 8.0) mixture that was associated with a bad peak resolution, ammonium bicarbonate and ammonium formate buffer / methanol in different proportions that provided a retention time superior to 10 minutes. Ammonium bicarbonate 5mM buffer pH (8.0)/ acetonitrile are needed to maintain omeprazole (without peak tailing factor) and ensure symmetry and thickness of Omeprazole peak. Therefore bicarbonate buffer was selected rather than the acetate and formate buffers used by other authors authors (Vittal *et al.*, 2009; TsingHua, 2004 Kanazawa *et al.*, 2004) since the possibility of precipitation in HPLC system was less likely. The LLOQ found with our method (5ng/mL) is lower than the LOQ of other quantification LC-MS/MS methods (Vittal *et al.*, 2009; Desmet *et al.*, 2010 Wang *et al.*, 2004) but greater than the HPLC quantification methods

(Bharathi *et al*., 2009; Zaraghi *et al*., 2006) . Limit of quantification of the methods using HPLC 20.6ng/mL with plasma sample and a liquid/liquid step for (Bharathi *et al*., 2009) 10ng/mL with plasma sample and a liquid /liquid extraction step for Zarghi *et al.* (2006) However, the LOQ of 5ng/mL is sufficient to quantify plasma omeprazole concentration in healthy volunteers according to previously published papers (Kamrun *et al*., 2009; Liu *et al*., 2012).

Carryover evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and precision of the proposed method. There was no carry over observed during autosampler carryover experiment. Also no enhancement in the response was observed in the double blank after subsequent injection of highest calibration standards at the retention time of omeprazole and internal standard respectively. Moreover, no ghost peaks appeared during the analysis of blank samples. The retention time for omeprazole and internal standard were at 1.25 and 1.35min respectively. None of the concomitant medications considered showed interfering signals at the retention time of omeprazole or internal standard. This demonstrates that the method is highly selective and free from interference due to matrix components and other prescribed medications. The assessment of matrix effect constitutes an important an integral part of validation for quantitative LC-MS-MS there, the matrix effect for the intended method was assessed by using chromatographically screened human plasma. From the result of analysis (Table III) it indicated thus the method was rugged and gave accurate and consistent results when applied to subject sample analysis. In comparison with the previously developed methods, the present method offers an undoubted advantage in terms of overall analytical performance.

CONCLUSION

A simple and sensitive rapid LC-MS/MS assay for the quantitation of omeprazole in small volume of plasma was developed and validated. The method was shown to be specific, accurate, precision and reproducible. The method can be applied to the pharmacokinetic evaluation and also can be applied for further pharmacokinetic characterization of Omeprazole.

ACKNOWLEDGMENT

The authors wish to thank for the support received from The Erode college of Pharmacy, Erode, Tamilnadu, India, for providing literature survey, The Dr.M.G.R. Medical University, Chennai, India. Authors are grateful to Aurbindo Pharmaceutical Pvt, Ltd, Hyderabad, India for providing clinical samples and support from Pharmacokinetic data.

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Figure 3 The mean plasma concentration time curve