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BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR OMEPRAZOLE USING LC-MS/MS

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LC- MS (Liquid Chromatography- Mass spectroscopy),
(BMV) Bio-analytical method validation, FDA (Food and Drug Administration USA), CDER (Centre for Drug Evaluation and Research - FDA),
ICH (International Conference on Harmonization),
LLOQ (Lower limit of quantification,
MQC (Middle Quality Control),
HQC (High Quality Control),
LQC (Low Quality Control),
IS (Internal Standard),
GLP (Good Laboratory Practice),
SOP (Standard Operation Procedure),
RSD Relative Standard Deviation

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ABSTRACT

Analytical methods employed for the determination of drugs and metabolites in biological matrices such as urine, plasma and serum are essential throughout drug discovery and development. It is well-known that, analytical techniques are constantly undergoing change and improvements and each analytical method has its own characteristics which may vary from analyte to analyte at different conditions. As the drug continues through development, the decisions become more critical; therefore, the bio-analytical methods that produce the data should be accurate. In the present study, we have developed a simple, precise and reproducible liquid chromatography tandem mass spectrometry method and validated according to FDA-GLP guidelines for quantification of Omeprazole in human plasma using Lansoprazole as internal standard utilizing LC-MS/MS incorporated with quadrapole mass spectrometer utilizing electrospray ionization technique. Samples were analyzed for Accuracy, Precision, Sensitivity, Selectivity, Recovery, stability and accuracy of method by injecting spiked matrix (human plasma) in to LC-MS/MS. The method specificity was determined by analyzing six different batches of human plasma to check the chromatographic conditions from endogenous plasma components. Sample recovery was determined at LLQQ, HQC, MQC and LQC levels using internal standard at single concentration. The objective of the present study is to determine the appropriateness of this method to omeprazole at different quantification level and keeping different criteria such as instrument stability, precision and accuracy, sample preparation strategies, calibration of instrument, recovery and matrix effect using omeprazole and the internal standard.

INTRODUCTION: Bio-analytical methods involving rapid and sensitive analysis of drug substances in the matrix (body fluids) serve as an important tool in the evaluation, interpretation of clinical trial study results, drug discovery process [1, 2]. According to FDA, the methods used in such analytical method should follow standard regulatory guidelines and the measurement of analyte in the biological matrix using a sophisticated instrument need to be validated and the procedure used for quantitative measurement of a analyte should be reliable and reproducible [3]. Liquid chromatography-mass spectrometry (LC-MS) in an analytical chemistry technique that combines the physical separation of a analyte (drug) and with the help of the mass analysis of drug (using mass spectrometry) is a very high

sensitivity and specific tool for bio-analytical requirements in clinical trial research [4, 5].

LC-MS is frequently used in drug development at many different stages including peptide mapping, glyco-protein mapping, natural products, bio-affinity screening, in vivo drug screening, metabolic stability screening, metabolite identification, impurity identification, degradant identification, quantitative bio-analysis, and quality control [6, 7]. LC-MS is also used in the study of proteomics where samples of complex biological fluids like human serum may be run and result in over 1000 proteins being identified very fast.

MS instruments consist of three modules: an ion source, which can convert gas phase sample molecules into ions (or, in the case of electro spray ionization, move ions that exist in solution into the gas phase); a mass analyzer, which sorts the ions by their masses by applying electromagnetic fields; and a detector, which measures the value of an indicator quantity and thus provides data for calculating the abundances of each ion present [8]. Mass analyzers separate the ions according to their mass-to-charge ratio. The major advantage MS has is the use of tandem MS-MS. The detector may be programmed to select certain ions to fragment.

The process is essentially a selection technique, but is in fact more complex. The measured quantity is the sum of molecule fragments chosen by the operator. As long as there are no interferences or ion suppression, the LC separation can be quite quick. It is common now to have analysis times of 1 minute or less by MS-MS detection, compared to over 10 minutes with UV detection. There are many types of mass analyzers, using either static or dynamic fields, or magnetic or electric fields, but all operate according to the above differential equation. Each analyzer type has its strengths and weaknesses [9, 10].

Quadrupole mass analyzers use oscillating electrical fields to selectively stabilize or destabilize the paths of ions passing through a radio frequency (RF) quadrupole field. Only a single mass/charge ratio is passed through the system at any time, but changes to the potentials on magnetic lenses allows a wide range of m/z values to be swept rapidly, either continuously or in a succession of discrete hops [11, 12]. A linear quadrupole ion trap is similar to a quadrupole ion trap, but it traps ions in a two dimensional quadrupole field, instead of a three dimensional quadrupole field as in a quadrupole ion trap. Thermo Fisher's LTQ (Linear trap quadrupole) is an example of the linear ion trap [13, 14].

In the present study, we have developed a method validation for Omeprazole. Omeprazole (C_{17}, H_{19}, N_3 O_3 S) a proton pump inhibitor belongs to the class of anti-secretary compound used in the treatment of peptic ulcer, gastro oesophageal reflux, dyspepsia. Omeprazole is a white crystalline powder that melts with decomposition about 155^{o}C and it is a weak base freely soluble in ethanol and methanol and slightly soluble in acetone and isopropanol and very slightly soluble in water. This drug has a half-life of 1 to 2 hours and protein binding capacity is 95%, molecular mass is 345.4 g/mol the bio-availability is 35 to 60%, excretion is 80% renal and 20% fecal [15].

It was first marketed in the US in 1989 by Astra Zeneca as the magnesium salt omeprazole magnesium under the brand names Losec and Prilosec, and is now also available from generic manufacturers under various brand names. Omeprazole is one of the most widely prescribed drugs internationally and is available over the counter in some countries. The chemical name for omeprazole magnesium is 6-methoxy-2-4-methoxy-3, 5- dimethylpyridin- 2- yl) methylsulfinyl- 1*H*-b enzo[*d*] imidazole. Its structural formula is (**Fig. 1**).

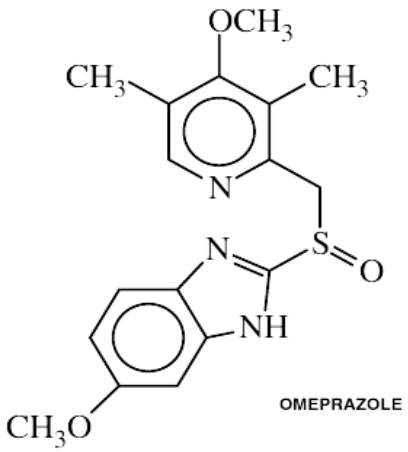

FIG. 1: STRUCTURE OF OMEPRAZOLE

Methodology and Experimental conditions:

Instrumentation and chromatographic conditions: TSQ Quantum Ultra LC-MS/MS interfaced with Surveyor LC pump and Surveyor auto sampler was

used to inject 25µl aliquots of the processed samples on a Cosmosil, RP-C8 (50 X 4.6mm, 5µm particle size) column kept at room temperature.

The isocratic mobile phase, a mixture of methanol: water (containing 0.5% formic acid) 8:2 was delivered at 0.6 mL min-1 into the mass spectrometer's electrospray ionization chamber. Quantization was achieved by MS-MS detection in positive ion modes for all the analytes. HPLC grade and high purity chemicals and reagents are used as per GLP (Good Laboratory Practice) regulations mentioned in the SOP (Standard Operation Procedure) as this technique is a precision method requiring high degree of purity and specific methodology [16, 17].

Solution preparation: Primary stock solution of Omeprazole and Lansoprazole were prepared by weighing separately. Omeprazole stock solution was prepared by dissolving 10 to 12mg of the drug in methanol and the volume was made up to 10 ml. Lansoprazole stock solution was prepared by dissolving 10.06mg of the drug in methanol and the final volume was made upto 10 ml. Both the stock solutions were sonicated and labeled for further use. Standard Stock dilutions of Omeprazole have been prepared in the concentration range of 256.0000ng/ml to 1000000.0000ng/ml by using equal volume mixture of Methanol and Water (50:50v/v). Just prior to spiking Lansoprazole standard stock dilution of 10ug/m was prepared using Carbinol and Water (50:50 v/v)

Standard preparation:

Stock Standard: 10mg of omeprazole working standard was weighed and transferred into 10 ml volumetric flask. To this, 5 ml methanol was added and dissolved to make up the final volume to obtain 1mg/ml solution. And a batch number was given and stored in the refrigerator at 2 to 8^{o}C.

Concentration of stock in ng/ml =

$$\frac{\text{Omeprazole wt (mg) x \% purity x Mol wt x 1000 x1000}}{\text{10 (final volume x 100 x Formula wt)}}$$

Molecular wt- 345.2; Formula wt- 345.2; Purity- 99.65 %

Preparation of Stock Dilution: Just prior to spiking, stock dilution of Omeprazole was prepared in the concentration rage of 256.0000ng/ml to 1000000.0000 ng/ml using equal volume of mixture of methanol and water (50: 50 v/v).

Preparation of internal standard (Stock solution w/v): 10 mg of Lansoprazole was weighed and transferred into a 10 ml volumetric flask and dissolved in methanol and a final volume was made up to 1mg/ml strength of Lansoprazole. Just prior to spiking, w/v stock solution of Lansoprazole internal standard is prepared by diluting 500 µg/ml using methanol.

Calibration and Quality control of samples: Calibration samples were prepared by adding 500µl of plasma with 200µl of each analyte and 50µl of internal standard on the day of analysis. Samples for the determination of precision and accuracy were prepared by spiking plasma with the analyte at Lower limit of Quantification(LLOQ), High Quality control(HQC), Middle Quality control(MQC) and Low Quality control(LQC). For checking the stabilities of the sample Quality control samples were bulk spiked and stores in deep freezer (-80°C) until analysis.

Sample Preparation: The spiked plasma samples were withdrawn from deep freezer and were allowed to thaw at room temperature. 200µl of plasma was aliquot into a clean RIA vial and 50µl of Lansoprazole (Internal Standard) were added and vortex well. 600µl of Carbinol (Extraction solvent) was added to the vial and the samples were mixed well at 2500 rpm for 10 minutes in vibromax shaker. The samples were then centrifuged at 4000rpm for 10 minutes at 4°C. 100µl of the supernatant was reconstituted with 900µl of mobile phase 10µl were injected into the LC-MS-MS system.

Method Validation: The method was validated to meet the acceptance criteria of industrial guidance for the bio-analytical method validation.

Specificity and Selectivity: The specificity of the method was determined by analyzing six different batches of human plasma as is, to demonstrate the lack of chromatographic interference from endogenous plasma components.

Recovery: The recovery of Omeprazole and Lansoprazole were determined by comparing the responses of the analytes extracted from six replicate

QC samples with the response of analytes from post extracted plasma standard sample at equivalent concentrations. Recoveries was determined at low, mid and high quality control concentrations, whereas the recovery of the IS was determined at a single concentration

Matrix Effect: The effect of plasma constituents over the ionization of analytes and IS was determined by comparing the responses of the six post extracted plasma standard QC samples with the response of analytes from neat samples at equivalent concentrations. Matrix effect was determined at same concentration of analyte and IS as in recovery experiment

Calibration Curve: Calibration curves were acquired by plotting the peak-area ratio of the transition pair of analytes to that of IS against the nominal concentration of calibration standards. The results were fitted to linear regression analysis. The acceptance criterion for each back-calculated standard concentration was ±15% deviation from the nominal value except at LLOQ, which was set at ± 20%.

Precision and Accuracy: Inter- and intra-assay precision and accuracy was determined by analyzing six replicates at four different QC levels (LLOQ, LQC, MQC and HQC). The criteria for acceptability of the data included accuracy within ±15% deviation (SD) from the nominal values and a precision of within ±15% relative standard deviation (RSD), except for LLOQ, where it should not exceed ±20% of Standard deviation.

Stabilities: Six different stability studies have been performed with a freshly prepared Calibration curve sample and Quality control sample. The stability of analytes and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples up to 24 h (in Auto sampler stability at 10°C) after the initial injection. The peak-areas of the analytes and IS obtained at initial cycle were used as the reference to determine the relative stability of the analytes at subsequent points.

Stability of analytes in the bio-matrix after 6 h exposure in bench top was determined at two concentrations (HQC and LQC) in six replicates. Freezer stability of the analytes in bio-matrix was assessed by analyzing the QC samples stored at –80°C. The stability of analytes in bio-matrix following repeated three freeze-thaw cycles (stored at –80°C between cycles) was assessed using QC samples spiked with analytes.

Samples were processed as described under as above. Samples were considered to be stable if assay values were within the acceptable limits of accuracy and precision.

RESULTS:

Specificity and Selectivity: No significant interfering peaks from endogenous compounds were observed at the retention times of analyte and IS which mean the retention times of analyte and IS were 2.0+0.5 mins respectively. The total chromatographic run time was 3 min.

Recovery: Peak-area ratios (analyte /IS) were used for the calculations of recovery. Recovery was not concentration dependent and the mean extraction recovery was greater than 80.1% for all analytes. The extraction recovery of IS was 88.8%.

Matrix effect: Matrix effect was evaluated by analyzing the samples from six different lots. Blank and HQC were processed from all the six lots and the samples were injected. Average matrix effect values obtained were <10% for all analyte and IS. No significant peak-area differences were observed.

Calibration curve: The plasma calibration curves were quadratic (r2 > 0.99) over the concentration range of 5.224ng/ml to 2040.8320ng/ml for Omeprazole. The standard curve had a reliable reproducibility over the standard concentrations across the calibration range. Calibration curve was prepared by determining the best fit of peak-area ratios (peak-area analyte/peak-area IS) versus concentration, and fitted to the $y = ax^2 + bx + c$.

The average regression was found to be greater than 0.99.The lowest concentration with the RSD < 20% was taken as LLOQ and was found to be 6.5508. The % accuracy observed for the mean of back-calculated concentration for four linearity was within 93.71 to 113.15%. The precision (% CV) values ranged from 2.383 to 14.991%. Representative chromatogram of omeprazole calibration curve is given represented in

Fig. 2. Representative chromatogram for HQC (**Fig. 3**), MQC (**Fig. 4**), LQC (**Fig. 5**) and LLOQ (**Fig. 6**) is represented below.

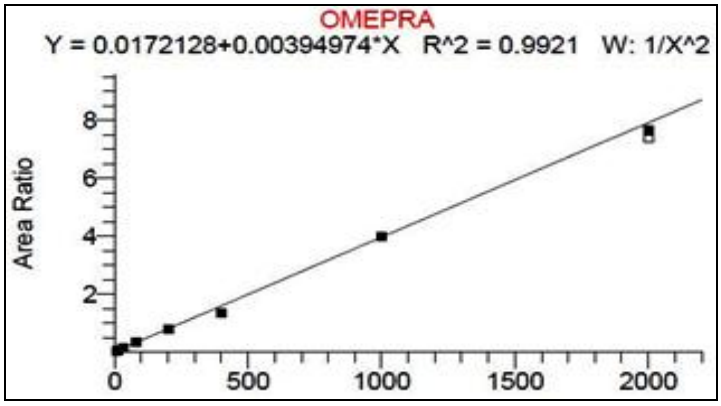

FIG. 2: REPRESENTATIVE CHROMATOGRAM OF OMEPRAZOLE CALIBRATION CURVE)

Precision and Accuracy: The accuracy, intra-and inter-assay precision which were determined by analyzing six replicates of QC samples are represented in **Table 1**.

TABLE 1: INTERDAY AND INTRADAY P & A

QC ID	LLOQ	LQC	MQC	HQC
Actual (ng/ml)	5.290	14.6940	734.700	1836.749
P&A-01	5.987	13.062	688.475	1708.585
P&A-02	6.011	15.262	704.788	1657.558
P&A-03	5.979	15.713	666.221	1769.037
P&A-04	5.735	14.268	688.084	1761.012
Mean	5.928	14.57625	686.892	1724.048
SD	0.12938	1.17621	15.82702	51.80210
% Cv	2.18	8.07	2.30	3.00
%Nominal	112.06	99.20	93.49	93.86

Stability: The predicted concentrations for each analyte at LQC and HQC samples deviated within ±15% of the nominal concentrations in a batter of stability tests viz., autosampler (24 h), bench top (6 h), repeated three freeze/thaw cycles and at –80°C. The results were found to be within the assay variability limits during the entire process.

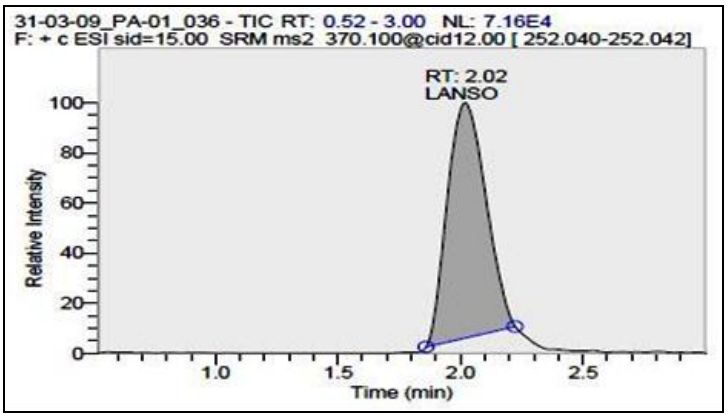

FIG. 3: REPRESENTATIVE CHROMATOGRAM OF OMEPRAZOLE HIGH QUALITY CONTROL

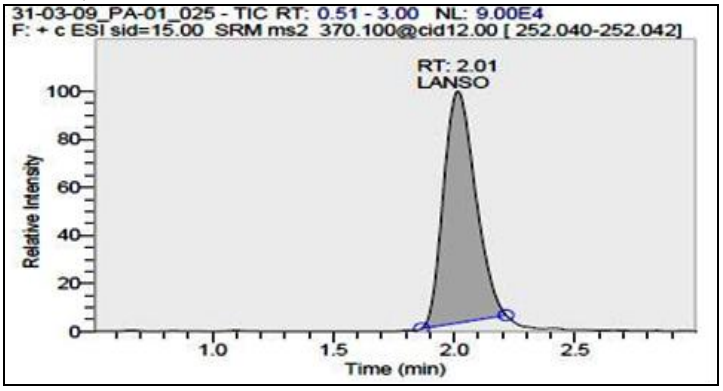

FIG. 4: REPRESENTATIVE CHROMATOGRAM OF OMEPRAZOLE LOWER QUALITY CONTROL

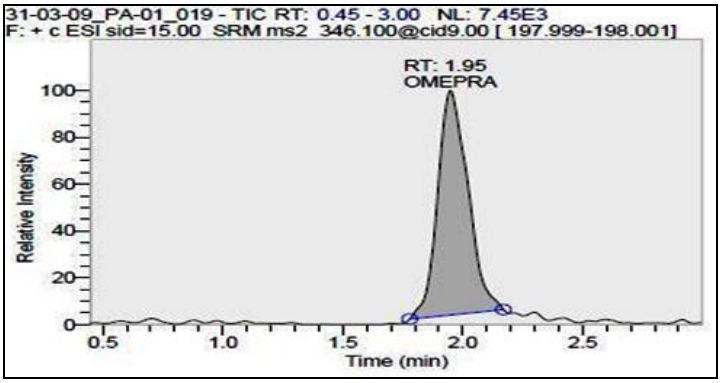

FIG. 5: REPRESENTATIVE CHROMATOGRAM OF OMEPRAZOLE LOWER QUALITY CONTROL

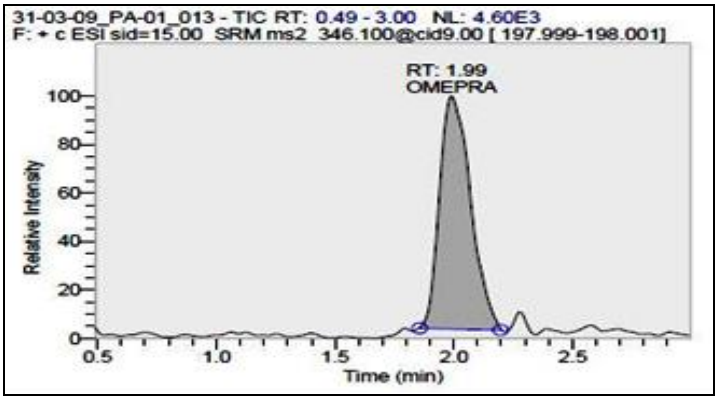

FIG. 6: REPRESENTATIVE CHROMATOGRAM OF OMEPRAZOLE LOWER LIMIT OF QUANTIFICATION

Discussion: Bio-analytical method validation (BMV) employed for the quantitative determination of drugs and their metabolites in biological fluids (matrix) provides a significant interpretation of Bio-availability, Bio-equivalence, pharmacokinetic and toxicokinetic data of the investigational new drug (IND). According to FDA analytical method employed in the quantitative determination of drugs and their metabolites must generate reproducible and reliable data. It is essential to employ well characterized and fully validated analytical methods to yield reliable results that can be satisfactorily interpreted.

LC-MS is used in pharmacokinetic studies of pharmaceuticals and is thus the most frequently used technique in the field of bio-analysis. These studies give information about how quickly a drug will be cleared from the hepatic blood flow, and organs of the body. MS is used for this due to high sensitivity and exceptional specificity compared to UV (as long as the analyte can be suitably ionized), and short analysis time. Bio-analytical method validation consists of procedures to determine if a specific method is accurate and precise for the quantitative determination of an analyte in a particular matrix.

Ultimately, the validated procedure will be used to determine the concentrations of analyte in real study samples. Thus all parameters affecting sample analysis, from collection of samples to actual time for analysis, need to be investigated. In addition to the typical GLP documentation procedures including CFR 21 Part 11, the basic parameters to consider for bio-analytical method validation include:

- Accuracy,
- Precision,
- Sensitivity,
- Selectivity,
- Recovery and
- Stability

The validation criteria used in the present study is instrument stability, sample preparation strategy, calibration, precision and accuracy, specificity and selectivity at different concentrations of drug. Upon injection of spiked plasma (matrix) after a valid extraction procedure in to the LC-MS/MS instrument at LLOQ the selectivity and specificity hold good which means the instrument at this lowest concentration could able to give a reproducible result. There was no significant peaks from the endogenous compounds are observed at retention times of analyte and the internal standard which mean the chromatographic conditions were quite perfect for omeprazole and the standard analyte within the chromatographic run time (3 minutes).

Further we noticed a good result in plasma calibration curves. The average matrix effect value obtained from all the six lot and the injected samples were <10% for all the analyte including the IS and we did not notice any peak-area differences which means the matrix effect does not affect this test procedure, which mean the sample preparation strategy used in this method is valid one.

The results obtained from inter-and intra assay precision and accuracy was determined using six sample replicates at four different concentration QC levels (LLOQ, LQC, MQC and HQC) reveals the acceptability of the data included accuracy within ±15% deviation (SD) from the nominal values and a precision of within ±15% relative standard deviation (RSD), except for LLOQ, where it should not exceed ±20% of Standard deviation which shows that the method was validated to meet the acceptance criteria of industrial guidance for the bio-analytical method validation.

Hence we conclude that after determining the standard and the omeprazole at different limit of quantification using proper regulatory guidelines and organized standard operation procedures keeping the criteria viz the accuracy, intra and inter-assay precision, calibration and sensitivity of the LC-MS/MS it is understood that the method used for preparation and detection of omeprazole is valid and this technique can be employed further for detection of omeprazole. We strongly recommend this validated method for Bio-analytical and Bio-equivalent studies used in clinical trials and since the technique can be employed well even at LLOQ this method can be applied in phase zero (phase 0 trials) which require micro-dosing in study subjects.

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