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Development and validation of a bioanalytical method for the quantification of Alosetron in human plasma using LC–MS/MS

Abstract

A sensitive, cost effective bioanalytical method for Alosetron using LC-MS/MS involving simpler extraction procedure and short run time. A new LC/MS/MS has been developed and validated as per regulatory requirements with a linearity range between 25-18000pg/ml. Total run time in this method is 3.00 mins and the retention time for the analyte is 1.78 and for ISTD 1.79. The chromatography was performed on C_{18} (4.6×100mm, 2.7µm) column was used as stationary phase and Acetonitrile : 2mm ammonium acetate pH-7.20 adjusted with ammonia (85:15%v/v) was used as the mobile phase. For quantitative determination Ion transitions monitoring in the positive ionization mode were m/z 295.18 for Alosetron and m/z 299.330 for Alosetron 13C D3. Recovery for both analyte and internal standard was found to be good and there was no interference at retention time of analyte.

Introduction

Bioanalytical Method Development and Validation

Bioanalysis is an essential part in drug discovery and development. It is related to the analysis of drugs, metabolites, biomarkers in biological samples. Bioanalysis involves various steps starting from sample collection to sample analysis and data reporting. The method adopted for bioanalysis must be validated. The purpose of bioanalytical method development is to define the design, operating conditions, limitations, and suitability of the method for its intended purpose and to ensure that the method is optimized for validation.

A sensitive, specific bioanalytical method is very much critical for a reliable analysis of drugs, metabolites, biomarkers in biological samples. It is a challenging task for a analyst to to provide an accurate and reliable bioanalytical data. . The development of a bio-analytical method requires knowledge about the analyte and the biological matrix from which the analyte is being extracted. This includes the study of physico-chemical properties, stability in the matrix, type of the biological matrix and thorough knowledge of physico-chemical interactions of molecules in a solvent system, which helps in establishing appropriate extraction techniques along with analytical methods.

The chromatographic techniques, LC-MS/MS or High performance Liquid Chromatography coupled (HPLC) with different detection systems are the predominant techniques, routinely employed in bioanalytical labs as compared to any other method of analysis owing to their precision, accuracy, reliability and applicability to the large-scale analysis. Scientific validation of a developed bioanalytical method is a prerequisite in bio-analysis to assure the reliability of the data. The guidelines by regulatory agencies (eg: USFDA, EMEA) and current industry practices play an important role in establishing various validation parameters to ensure the reliability of the bioanalytical data generated as a support for new drug or generic drug approval process. The guidance document by USFDA systematically describes the validation experiments and procedures required to demonstrate the reliability and reproducibility of the bioanalytical method. As per the regulatory guidelines, the fundamental parameters of method validation include accuracy, precision, selectivity, sensitivity, reproducibility and stability. The process of method validation involves laboratory investigations, documentation and assessment of performance characteristics (average, % CV, % nominal concentration) and their conformity to the acceptance criteria. Considering variability due to laboratory, instrument, assay-conditions, analyst and solvent, it is not necessary that the reported methods can directly be applied in a given laboratory. That would often require modification to suit the laboratory. These modifications call for method validation. The extent of validation can vary with respect to the changes done to the original method ranging from partial validation involving just one validation test or complete validation for all the parameters. Cross validation becomes essential when a study requires more than one laboratory. All the bioanalytical validation experiments are carried out using samples spiked with reference standards of the analyte (drug). Calibration standards, generally nine-point calibration and quality control, generally at four different levels (LLOQC, LQC, MQC and HQC) are prepared with certified reference standards so that the spiked samples do not affect the study data.

Chemicals and Materials

Reference standards of Alosetron and Alosetron 13C D3 were used from authenticated producer. HPLC grade Methanol, Acetonitrile, Tertiary butyl methyl ether and Dichloromethane were obtained from RCI labscan. Ammonia, Sodium hydrogen carbonate and ammonium acetate were purchased from Merck. Water was prepared from Milli-Q water purification system from Millipore.

Chromatographic and Mass detection conditions

The chromatographic system consisted of Shimadzu HPLC Ascentis express C18 4.6×100mm, 2.7µm column used. The analytes were eluted under isocratic mode using mixture of Acetonitrile and 2mm Ammonium acetate (pH-7.2) adjusted with ammonia (85:15%v/v) as mobile phase and was delivered at a flow rate of 0.500ml/min. quantitative determination was performed on triple quadrupole mass spectrometer from thermo scientific technologies, with Heated electron spray ionization interference in multiple reaction monitoring (MRM) mode. Ion transitions monitoring in the positive ionization mode were m/z 295.18 for Alosetron and m/z 299.330 for Alosetron 13C D3. LC QUAN 4.5.6 was used for data collection and to control all parameters of HPLC and mass spectrometer.

Calibration standards and quality control samples

The stock solution of Alosetron(0.1000mg/ml) was prepared by using methanol. Intermediate stock solution(500000pg/ml) was prepared by using water : methanol (60:40%v/v). The calibration standards were prepared by using intermediate stock solution, the concentration of calibration standards was 25.000, 50.000, 1000.000, 2000.000, 4000.000, 8000.000, 12000.000, 16000.000 and 20000.000 pg/ml for Alosetron. Quality control samples were prepared separately concentration of LLOQC- 25.000, LQC- 62.500, MQC-10000.000 and HQC- 18000.000 pg/ml. Stock solution of internal standard(0.1000mg/ml) was prepared by using appropriate amount of Alosetron 13C D3 was dissolved in methanol. Working solution(50.000ng/ml) was prepared by using stock solution in water-methanol(60:40%v/v). Standard and working stock solutions were used for spiking and stored at 5-8˚C.

Extraction procedure

Samples were retrieved from deep freezer and spiking stock, quality samples from the refrigerator. Samples allowed to thaw in normal light to room temperature. Samples were vortexed and 0.300ml transferred to prelabeled Ria vials and 0.015ml of spiking stock solution and 0.015ml of internal standard were added except in blank sample and then vortexed. 0.300ml of 50mm sodium hydrogen carbonate was added and 3.00ml of extraction solution (TBME:DCM) (70:30%V/V) was added and vortexed for mixing for 5mins at 2500 rpm. After vertexing the samples were centrifuged for 5mins at 4500rpm at 4˚C. Supernatant was separated by flash freezing and kept the samples in evaporator for drying at 40˚C. Sample residue is

reconstituted with 0.600ml of reconstitute solution(ACN:WATER) (90:10%V/V). Injected 10.00µl in to LC/MS-MS.

Method validation parameters

System Suitability

System suitability test was performed to ensure that the complete testing system is suitable for the intended application. System suitability test was done before every sequence acquisition. Six-replicate injections of aqueous MQC solution mixed with IS were injected. % CV of peak area ratio and retention time of analyte and IS were examined.

Specificity

Selectivity: In which method can be determine a compound in examined matrices without interference from matrix components.

Specificity: It is the ability of the method to evaluate the analyte in the presence of other components that are expected to be present (impurities, degradation products, matrix components).

Selectivity can be analyzed by using six samples of blank samples from different biological lots can be ensured with lower limit of quantification(LLOQ).

Specificity is to be ensured at lower limit of quantification (LLOQ) for which human plasma is obtained from six different subjects. These lots of plasma were extracted with and without internal standard and tested for interference at the retention time of analyte or IS.

Sensitivity

Sensitivity is determined at LLOQ level, where blank plasma sample along with six replicates of the LLOQ samples with IS were extracted and analyzed with proposed chromatographic conditions. These blanks samples were compared with extracted LLOQ samples for interference at analyte and IS response area.

Linearity (Calibration range)

It includes Blank sample, Zero sample and Nine non-zero standards of calibration rangeThese sample were extracted and analyzed using proposed chromatographic conditions. % nominal

concentration and % CV were calculated for at each calibration level to determine precision at each calibration level.

Accuracy & Precision

It includes Blank sample, zero sample and Nine non-zero standards of calibration range, Bulk spiked six sets of quality control samples (LLOQ, LQC, MQC, HQC). Three batches of precision and accuracy were performed throughout validation. These processed samples were tested for % nominal concentration and % CV at each level of quality control samples. Precision and accuracy include intra-day and inter-day batches.

Matrix effect

It is tested over six different lots of biological matrix. These blank samples were extracted using proposed extraction method. These samples were analyzed and compared with freshly spiked aqueous solution of standards at same concentration.

Recovery

Recovery was determined by comparing mean area response at LQC, MQC and HQC level of quality control with mean area response freshly spiked aqueous samples.

Recovery for IS was determined by comparing mean area response of extracted samples with that of freshly prepared sample. Variability across QC levels was determined.

Dilution integrity

Dilution integrity was determined through preparing sample with twice concentration of ULOQ level. These samples were diluted to half fold and one fourth fold of original concentration. Calibration standards and diluted samples were analyzed in single run and % nominal concentration and % CV were calculated.

Carry-over

Carry over is assessed by injecting blank sample, two repeated injection of ULOQ and three injections of LLOQ to check if auto sampler retains some amount of ULOQ concentration and affects accuracy of measurement.

Stability Studies

Stock solution stability studies were performed at room temperature for 13 hours 20 and refrigerated conditions at 2 to 8˚C for 28 days for analyte and internal standard. After completion of the period, instrument response of stability sample was compared with that of the freshly spiked samples. % mean change in concentration was calculated for Alosetron and Alosetron 13C D3.

Bench top stability

Bench top stability was assessed using six sets of LQC and HQC samples at room temperature for 6 hrs 20 mins. Those samples were compared with freshly spiked calibration range and quality control samples at LQC and HQC level. Mean % change at LQC and HQC was determined.

In-injection stability

It was assessed using six sets of LQC and HQC samples at 10˚C in autosampler for 70 hrs. Those samples were compared with freshly spiked calibration range and quality control samples at LQC and HQC level. Mean % change at LQC and HQC was calculated.

Freeze and thaw stability

One set of QC samples were refrigerated for 24 hours and thawed at room temperature at least for 3 hours. After first cycle, same procedure was repeated for two more times. After fourth cycle, samples were assessed using six sets of LQC and HQC samples previously frozen and thawed at room temperature for three cycles. Mean % change at LQC and HQC was calculated.

Dry-extract stability

Dry-extract stability was assessed using six sets of LQC and HQC samples. Samples were pretreated up to evaporation step and stored for 4hrs 50mins at RT. Those samples were reconstituted and analyzed with freshly spiked calibration range and quality control samples at LQC and HQC level. Mean % change at LQC and HQC was calculated.

Long Term Stability

LT stability was assessed using six sets of LQC and HQC samples by processing and analyzing stored for 28 days at 2-8˚C. Those samples were reconstituted and analyzed with freshly spiked

calibration range and quality control samples at LQC and HQC level. Stability and freshly prepared samples were analyzed in single run. Mean % change at LQC and HQC was calculated.

Re-injection Reproducibility

The same batch of LQC and HQC samples were re-injected to calculate mean % change after period of 24 hours. The mean % change in low- and high-quality control sample was calculated.

Results and discussion

System suitability test:

System suitability was done before starting every batch separately. Solution containing sample (Aqueous MQC) and internal standard was injected 5 times. % CV was found to be 0.70, 1.43 for area ratio of analyte and ISTD. % CV of retention time was found to be for analyte 0.00 to 0.22 and for ISTD 0.00%.

Specificity

Specificity was evaluated using different lots of biological matrix obtained from different source for interference of matrix components. Sample injections at LLOQC level to be injected was prepared from different lots and verified for interference. There was no interference of endogenous components at retention time of analyte and IS respectively which was within acceptance criteria ≤5%.

Sensitivity

This was done by injecting six LLOQC samples. The results were obtained with in acceptance criteria. The %CV was 13.82 and mean % nominal concentration was 82.46%.

Linearity

Linearity was evaluated by injecting samples thrice with concentrations across calibration curves range. The results were found to be with in acceptance criteria. r^2 and mean % nominal concentration was 99.19-100.06%.

Accuracy and Precision

Intraday and inter day precision and accuracy was determined by injecting six replicates of each QC level in three sets of QC samples.

Intra batch or within batch

% nominal concentration of LLOQC, LQC, MQC and HQC samples were found to be 87.9, 95.53, 101.35 and 99.94 respectively. These results are within acceptance criteria for LQC, MQC and HQC 85-115% for LLOQC 80-120%.

%CV was found to be 3.68, 5.79, 0.85 and 2.37 for LLOQC, LQC, MQC and HQC respectively and these are in with in acceptance criteria ≤15% for LQC, MQC and HQC for LLOQC ≤20%.

Matrix effect

Matrix effect was studied using six lots of biological matrix from different source and tested for the interference. It was done by comparing the instrument response of the extracted sample with post spiked samples at LQC and HQC level. % CV at LQC and HQC level were 11.26 and 8.11 respectively and for ISTD it was found to be 6.24 % and were within the range ≤ ±15%.

Recovery

It was done by comparing the extracted samples at LQC, MQC, and HQC concentrations with extracts of blank samples spiked with the analyte post extraction at LQC, MQC, and HQC. Mean % recovery for aqueous versus extracted LQC, MQC and HQC was 82.33, 82.87 and 88.05 respectively. For post extracted samples versus extracted samples 79.07, 87.62 and 79.28 for LQC, MQC and HQC. % recovery was consistent and reproducible.

Dilution integrity

It was performed by evaluating six replicates of each dilution that should be two times of ULOQ. One by two and one fourth dilutions were made and analyzed against fresh calibration curve. The % nominal concentration was 96.08 and 95.22% and %CV was 3.52 and 3.43%.

Carry over

Sample carry over was tested initially at highest concentration injections followed by injecting blank sample and three lowest concentrations to check if the autosampler retains some amount of

ULOQC and affects accuracy and precision of the method. % CV for analyte and IS was found to be 4.17, 2.02 and was well within the limit of 20% analyte response at LLOQC and 5% of the IS response.

Bench top stability

These was assessed for six sets of the fresh spiking samples kept on bench top for 6hrs 20mins at RT. % mean change of LQC and HQC was found to be 0.38 and 2.06 respectively.

In injector stability

It was performed by using six sets of LQC and HQC samples at 10^0 C in autosampler for 70hrs 15mins. Those samples were compared with freshly spiked calibration range and quality control samples at LQC and HQC level. Mean % change at LQC and HQC was found to be -1.11 and 2.66 respectively. Mean % change was within limit of ±15%.

Freeze thaw cycle stability

These was assessed for six sets of the fresh spiking samples with freeze thaw samples after 4^{th} cycle at -70±5˚C was assessed. % mean change of LQC and HQC was found to be 0.52 and 2.09 respectively.

Dry extract stability

This was estimated by %mean change for six sets of the fresh spiking samples with dry extracted samples 4 hrs 50mins at RT. Mean % change for LQC and HQC were found to be 1.14 and 2.03 respectively.

Long term Stability studies

Long term stability studies were done by comparing the stability of stock solution at 2-8˚C for 28 days 04 hrs with freshly prepared samples. The %mean change of LLOQ and ULOQ was -4.49 and -1.74. for ISTD %mean change was found to be -3.74. These are done by comparing spiking stock solutions. The main stock solutions %mean change was found to be -3.39 at ULOQ level of Alosetron for ISTD % mean change was -2.99.

Reinjection reproducibility

The same batch of LQC and HQC samples were re-injected to calculate mean % change after period of 24 hours. The mean % change LLOQC, LQC, MQC and HQC samples was found to be 6.77, 4.12, -1.36, -0.85% respectively.

Conclusion

A new LC/MS/MS has been developed and validated as per regulatory requirements with a linearity range between 25-18000pg/ml. Total run time in this method is 3.00 mins and the retention time for the analyte is 1.78 and for ISTD 1.79. C18 column was used as stationary phase and acetonitrile : 2mm ammonium acetate pH-7.20 (85:15%v/v) was used as the mobile phase. Liquid-liquid extraction method was used for extraction of analyte, which is economical compared to the reported methods where solid phase extraction procedure has been used for the extraction which is more costlier. No significant interference from the blank plasma was observed at the retention times of both analyte and internal standard. The current method is more sensitive than the reported method. The mobile phase composition used in the present study is preferred over the mobile phase and the pH of the mobile phase used in the reported methods since they may have effect on the life time of the detector. Thus the present method is cost effective, more sensitive compared to the reported method.

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***Graphical Abstract**

Abstract

A sensitive, cost effective bioanalytical method for Alosetron using LC-MS/MS involving simpler extraction procedure and short run time. A new LC/MS/MS has been developed and validated as per regulatory requirements with a linearity range between 25-18000pg/ml. Total run time in this method is 3.00 mins and the retention time for the analyte is 1.78 and for ISTD 1.79. The chromatography was performed on C_{18} (4.6×100mm, 2.7µm) column was used as stationary phase and Acetonitrile : 2mm ammonium acetate pH-7.20 adjusted with ammonia (85:15%v/v) was used as the mobile phase. For quantitative determination Ion transitions monitoring in the positive ionization mode were m/z 295.18 for Alosetron and m/z 299.330 for Alosetron 13C D3. Recovery for both analyte and internal standard was found to be good and there was no interference at retention time of analyte.

Cover Letter

V. Mahesh Reddy

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Dear Editor,

I am pleased to submit an original research article entitled Development and validation of a bioanalytical method for the quantification of Alosetron in human plasma using LC-MS/MS for consideration for publication in the journal of food and drug analysis. In this manuscript we show that method development and validation parameters for drug Alosetron.

We believe that this manuscript is appropriate for publication by the journal of food and drug analysis. Our manuscript is useful for future studies of Alosetron in human plasma.

This manuscript has not been published and is not under consideration for publication elsewhere.

Thank you for consideration,

Sincerely,

Mahesh Reddy (M. Pharm),

Pharmaceutical Analysis,

Manipal college of pharmaceutical sciences,

Manipal,

Karnataka-576104.

***Highlights (for review)**

A new LC/MS/MS has been developed and validated as per regulatory requirements with a linearity range between 25-18000pg/ml. Total run time in this method is 3.00 mins and the retention time for the analyte is 1.78 and for ISTD 1.79. C18 column was used as stationary phase and acetonitrile : 2mm ammonium acetate pH-7.20 (85:15%v/v) was used as the mobile phase. Liquid-liquid extraction method was used for extraction of analyte, which is economical compared to the reported methods where solid phase extraction procedure has been used for the extraction which is more costlier. No significant interference from the blank plasma was observed at the retention times of both analyte and internal standard. The current method is more sensitive than the reported method. The mobile phase composition used in the present study is preferred over the mobile phase and the pH of the mobile phase used in the reported methods since they may have effect on the life time of the detector. Thus the present method is cost effective, more sensitive compared to the reported method.

***Authorship & Conflict of Interest Statement**

Manuscript title: Development and validation of a bioanalytical method for the quantification of Alosetron in human plasma using LC–MS/MS

AUTHORSHIP

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication. Indicate the specific contributions made by each author (list the authors' initials followed by their surnames, e.g., Y.L. Chang). The name of each author must appear at least once in each of the three categories below.

Conception and design of study, Acquisition of data and Analysis /or interpretation of data: V. Mahesh Reddy

Drafting the manuscript: V. Mahesh Reddy

Revising the manuscript: G. Gautham shenoy

Approval of the version of manuscript to be published: Dr. Raghavendra Shetty, G. Gautham shenoy, V. Mahesh Reddy.

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