International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.10 No.10, pp 752-761, 2017

Bioanalytical Method Validation for determination of Macitentan in K_2EDTA Human plasma by LC-MS/MS

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Abstract: A simple reverse phase liquid chromatographic and mass spectroscopic analytical method has been developed and validated for estimation of Macitentan in plasma. The separation was carried out on Accucore AQ 100 X 2.1 mm, 2.6 µm as Stationary phase, Mobile Phase: 0.1% Formic acid: Acetonitrile Elution mode : Isocratic A: B= 20:80% v/v Flow rate: 350 µL/min. Losartan was used as internal standard. The Macitentan and Losartan showed retention factor of 1.01 min ± 0.5 minand 0.9 min ± 0.5 minrespectively. The injection volume was 5µL and the total run time was 3 min. The method shows selectivity and linearity. The described LC-MS/MS method was linear over a concentration range of 0.997 to 1020.793 ng/mL.The extraction recoveries for Macitentan and Losartan were found to be between 101.12 and 96.29%. The method shows to be stable for the studied parameters. The stability of the drug spiked human plasma samples during three freeze thaw cycles were stable in plasma for about one month when stored at frozen state. The results of the study showed that the proposed LC-MS/MS method is simple, rapid, precise and accurate, which is useful for the estimation of Macitentan in bulk fluids and biological plasma sample analyte with accuracy and reproducibility.
Keywords: Macitentan, LC MS method, Losartan and Freeze thaw cycles.

Introduction:

Macitentan,{[5-(4-bromophenyl)-6-{2-[(5-bromopyrimidin-2-yl)oxy]ethoxy}pyrimidin-4-yl]sulfamoyl }(propyl)amine is indicated for patients with pulmonary arterial hypertension, and is marketed under the brand name Opsumit. Macitentan is an antagonist/blocker of endothelin receptors on blood vessels and smooth muscle, and, thus, blocks the stimulation of vasculature hypertrophy, inflammation, fibrosis, proliferation, and vasoconstriction. Similar to all drugs acting on the renin-angiotensin system, macitentan is associated with embryo and fetal toxicity, so it should not be used in pregnancy and has special precautions that must be followed for all females of child-bearing age.Macitentan is an antagonist/blocker of endothelin receptors. Endothelin receptors are found in the endothelial cells of blood vessels and smooth muscle. Macitentan binds to the receptors, endothelin A and B (ETA and ETB), which prevents the agonist endothelin -1 (ET-1) from binding and stimulating the ETA and ETB receptors. [1-10].

Literaturesurvey revealed that Macitentan is estimated by High-performance Liquid Chromatography-tandem Mass Spectrometry (HPLC-MS/MS), high-performance liquid chromatography coupled to tandem mass spectrometry, Spectrophotometric, Spectrofluorometric, High-performance liquid chromatography with amperometric detection, HPLC and Chemometrically-Assisted Spectrophotometric Estimation, liquid chromatography-tandem mass spectrometry, liquid chromatography/UV diode array detection/atmospheric pressure chemical ionization mass spectrometry, Several methods have been reported for quantification of

Macitentan in plasma as mentioned above. The present investigation reports a simple, rapid, sensitive, and reproducible LC MS method for analysis of Macitentan in plasma, using Losartan as internal standard (IS) [11-15].

The Plan of the present study is as follows: Optimization of chromatographic conditions were proposed to be developed and optimized like selection of Ionization, selection of initial separation conditions, nature of the stationary phase, nature of the mobile phase (pH, peak modifier, solvent strength, ratio and flow rate) and Selection of internal standard. The developed method were also proposed to be validated using the various validation parameters such as, Accuracy, Precision, Linearity and Range, Limit of detection (LOD) / Limit of quantitation (LOQ), Selectivity / specificity, Stability and System suitability as per ICH guidelines [16, 17]. The Macitentan present in the biological fluid was proposed to be estimated.

Methodology:

Samples were separated on a reversed phase Accucore AQ 100 X 2.1 mm, 2.6 µm in isocratic mode. Mobile phase was 0.1% Formic acid: Acetonitrile (20/80, v/v) at a constant flow rate of 350 µL/min. The column temperature was kept constant at 40˚C. The injection volume was 5µL and the total run time was 3 min. Macitentan and Losartan were ionized via electrospray ionization (ESI) in positive ion mode. The electrospray source parameters were fixed as follows: electrospray capillary voltage 3.5 kV, source temperature 100˚C and desolvation temperature 300˚C. Nitrogen was used in the electrospray ionization source. The cone and desolvation gas flows were 50 and 600 L·h−1, respectively. The detection of the ions was performed in the selected reaction monitoring (SRM) mode, monitoring the transition of the precursor ion at m/z 589 to the product ion at m/z 203 for Macitentan, and the transition of the precursor ion at m/z 423 to the product ion at m/z 207 for Losartan.

Solution Preparation:

Mobile Phase A: [0.1% Formic acid in Water]

Added 1 mL of formic acid to 1000 ml HPLC grade water in a 1000 ml measuring cylinder and mixed well. The resulting solution was transferred to 1000ml reagent bottle, sonicated and labelled with three days of expiry from date of preparation.

Mobile Phase B: [Acetonitrile]

Acetonitrile was used as Mobile phase B. A volume of 500ml of Acetonitrile was transferred to 500ml reagent bottle and labelled with three days of expiry from date of preparation.

Diluent: [Methanol: Water (50:50 V/V)]

Added 500 mL of methanol and 500 mL of water in a 1000 mL reagent bottle,mixed well and labelled with three days of expiry from date of preparation.

Preparation of calibration standards and spiked calibration standards in plasma

5.102 mg of Macitentan was weighed and transfered into a pre-labeled clean and dry 5 ml volumetric flask. Dissolved the contents with 0.5 ml of methanol and made up to 5.0 ml with methanol. The ccstock and working solutions were stored at 2°c to 8°c. The final concentration was achieved upon purity and salt correction was 1016.727 µg/ml

Preparation of Quality Control Samples and Spiked Quality Control Samples:

5.103 mg of Macitentan was weighed and transferred into a pre-labeled clean and dry 5 mL volumetric flask. Dissolved the contents with 0.5 mL of methanol and made up to 5.0 mL with methanol. The QCstock and working solutions were stored at 2°C to 8°C. The final concentration achieved upon purity and salt correction was 1016.926 µg/mL.

Preparation of Internal Standard Stock and Working Solution:

10.117mg of Losartan was weighed and transfered into a pre-labeled clean and dry 10 mL volumetric flask. Dissolved the contents with 0.5 mL of methanol and made up to 10.0 mL with methanol. The QCstock and working solutions were stored at 2°C to 8°C. The final concentration achieved upon purity & salt correction was 1008.463 µg/mL.

Sample Preparation

The frozen QC samples and Plasma were retrieved from deep freezer and thawed at room temperature. The STD blank and STD zero were prepared by adding 20µL of diluent and 980 µL of blank plasma. All (CC, QC & STD Blank) samples were vortexed for homogeneity. Into a prelabelled poly propylene vial 250 µL of sample was aliquoted and added with 50.0 µl of ISTD (0.500 µg/mL) other than STD Blank sample and mixed well. The mixture was processed using solid phase extraction technique with Sola SCX 10 MG/1Ml Cartridge following protocol, Conditioning stage: 1000 µL methanol then 1000 µL water, Application stage: load pre-treated sample, Washing stage1: 1000 µL 95:5 (v/v) water / methanol (twice), Washing stage2: 1000 µL 80:20 (v/v) water / methanol, Elution stage: 1000 µL methanol and Additional stage: Dry down under a stream of nitrogen at 50 °C. Reconstituted in 250 µL of Mobile Phase transferred approximately 0.200mL of supernatant to pre-labeled HPLC vials and analysed in LC-MS/MS.

Data processing and calculations

Chromatograms acquired using the Thermo LCQuan 3.0 software version supplied by thermo. The calibration curve was constructed by using a suitable linear regression analysis of the peak area ratio (Drug/ISTD) vs. the concentration of drug. The concentration of the Quality control samples were calculated from following equation using regression analysis of spiked plasma calibration curve standard.

$Y = m X + C,$
X = Concentration in µg/mL
Y= Peak area ratio of drug to ISTD
m = Slope
C= Intercept.

Method Validation:

The method was validated for system suitability, auto sampler carryover test, selectivity, matrix effect, linearity, accuracy, precision, recovery, stability according to the principles of the FDA industry guidance

System suitability:

System suitability of the instrument for analysis was performed by injecting six replicates of neat MQC concentration samples of Macitentan with internal standard (Losartan) in mobile phase.

Autosampler carryover test

Autosampler carryover test was performed by injecting the processed blank sample following the highest calibration standard (STD-11). No significant interference at the retention time of analyte or internal standard was observed during the period of validation.

Selectivity:

The selectivity of this method was performed by analyzing blank plasma samples obtained from 6 healthy subjects, a lipid sample and a hemolyzed sample. In order to test the interference at the retention time of Macitentan at quantification limit and Losartan (IS) at working concentration, the blank plasma samples, a human plasma sample spiked with Macitentan and a human plasma sample spiked with Losartan were analyzed according to the methodology.

Matrix Effect

Matrix Factor was established in six individual plasma lots obtained from individual donors. Each lot was spiked with LQC and HQC samples and analysed under the calibration curve.

Linearity:

The linearity of calibration curve for Macitentan was assessed at ten concentration levels in the range of 0.997 to 1020.793 ng/mL in plasma samples. Peak area ratios for each solution against its corresponding concentration were measured and the calibration curve was obtained from the least-squares linear regression presented with their correlation coefficient.

Extraction Recovery:

The extraction recovery of analyte at three QC samples was determined by measuring the peak area responses from plasma samples spiked with analyte before extraction with those from drug-free plasma samples extracted and spiked with same concentration of analyte after extraction. The recovery of Macitentan and Losartan were determined using six replicates. The extraction recovery at low, medium and high levels of QC samples was obtained according Equation:

$$R(\%) = (PSbe/PSae) \times 100\%$$

where: R is extraction recovery, PSbe is the mean value of the peak area responses obtained from plasma samples spiked with analyte before extraction and PSae is the mean value of the peak area responses obtained from plasma samples spiked with analyte after extraction.

Accuracy and Precision

The intra-day data reflects the precision and accuracy of the method under the same conditions within one day. Intra-day accuracy and precision were obtained by analyzing six replicates of three QC samples (low, medium and high levels). Accuracy was determined by the regressed (measured) concentration represented as a percentage of the target (nominal) concentration. The percent relative standard deviation (% RSD) of the regressed (measured) concentrations was used to report precision. The inter-day precision and accuracy were verified by repeating the above procedure at three different occasions.

Stability:

Stability of Macitentan in plasma was performed using six replicates of two QC samples at low and high levels. Samples were prepared by spiking drug-free plasma with appropriate volumes of Macitentan standard solutions. The stability was evaluated with six studies; stability in bench top stability, freeze-thaw, autosampler, short-term and long-term stability as well as standard solution stability, according to described in subsequent sections.

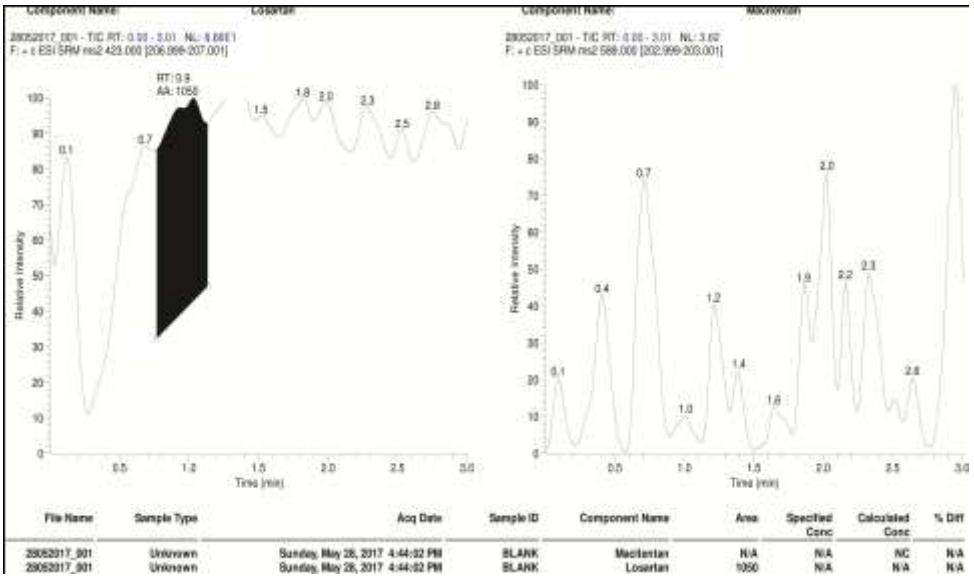

Figure no 1: chromatogram for blank

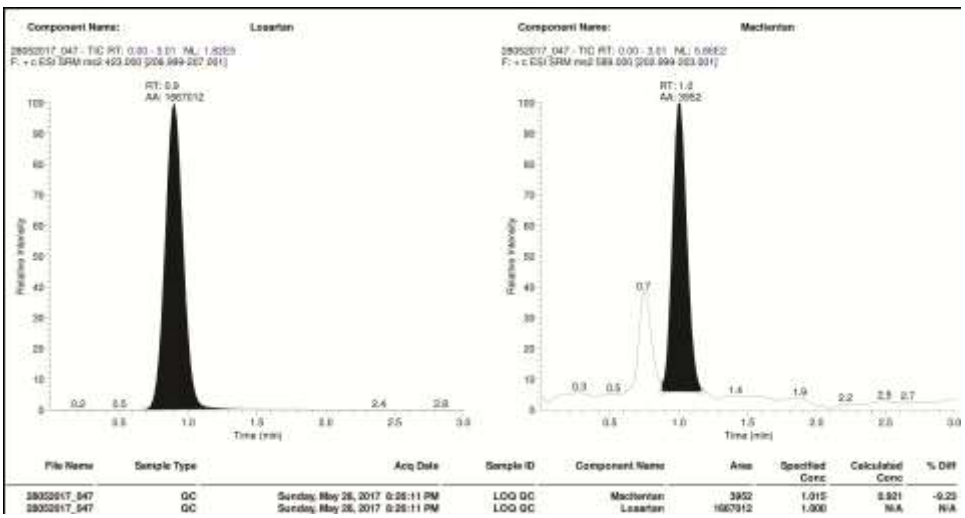

Figure no 2: chromatogram for LQC QC of Macitentan and Losartan

Results and Discussion:

Sample Preparation and LC–MS/MS Analysis

The main aim of this work was to develop a rapid, selective and sensitive analytical method including an efficient and reproducible sample clean-up step for quantitative analysis of Macitentan in human plasma. Based on our previous experience on optimization of analyses in plasma, sodium hydroxide was added to plasma samples in order to increase extraction efficiency, because weak bases as Macitentan and Losartanare in an undissociated form at neutral or alkaline pH values, resulting in higher extraction efficiency. Subsequently, a simple and inexpensive extraction procedure that could be implemented in monitoring laboratories provided an assay well suited for real time analyses. In optimizing the chromatographic conditions, the ammonium acetate buffer solution was adopted in the mobile phase of the HPLC in order to suppress the tailing phenomena of chromatographic peaks of Macitentan and Losartan. Besides, the concentration of formic acid was investigated and the concentration of 0.1% formic acid made the chromatographic peaks sharp and symmetric.

The acceptable retention and separation of Macitentan and Losartan was obtained by using an elution system of 0.1% formic acid/Acetonitrile (20/80v/v) as the mobile phase. The LC/MS/ MS method described here satisfies the requirement of routine analyses since it has a short run time (3 min), which has advantages

over other methods described in the literature. The MS optimization was performed by direct injection of Macitentan and Losartan into the mass spectrometer. The mass parameters were optimized to obtain better ionization of Macitentan and Losartan molecules. The full scan spectrum was dominated by protonated molecules [M+H]+ m/z589 and 423 for Macitentan and Losartan molecules, and the major fragment ions observed in each product spectrum were at m/z 203 and 207 respectively.

System suitability:

System suitability of the instrument for analysis was performed by injecting six replicates of neat MQC concentration samples of Macitentan with internal standard (Losartan) in mobile phase. The CV% for area ratio of Analyte /Internal standard during system suitability for the method validation period was < 2.60%. The system suitability was performed prior to initiating any experiment on daily basis and found satisfactory. The data for one validation day is represented below as an example

Autosampler carryover test

Autosampler carryover test was performed by injecting the processed blank sample following the highest calibration standard (STD-11). No significant interference at the retention time of analyte or internal standard was observed during the period of validation. Thus the method has no carry over related issues and the rinsing solution cleans the injector appropriately.

Selectivity & Sensitivity

Selectivity was established by using six plasma lots obtained from individual donors. Each individual plasma lot was analyzed as Blank, Blank+ISTD and LLOQ+ISTD. All lots met the acceptance and no significant interference was observed in the any of the individual lots.

Matrix Effect

Matrix Factor was established in six individual plasma lots obtained from individual donors. Each lot was spiked with LQC and HQC samples and analysed under the calibration curve. All lots met the acceptance of ±15% to the nominal concentration. Hence the method does not have any matrix interferences using the method designed.

Recovery

The recovery of Macitentan from matrix (at low, middle and high QC concentrations) was evaluated by comparison of area with extracted plasma samples to that of the neat samples prepared at the same quality control level concentration.

Linearity

The linearity of the method was established by analyzing three calibration curve of the validation runs. The method was linear through the range of 0.997 to 1020.793 ng/mL.

The r^2 value was above 0.98 for all the calibration curve analyzed in the validation.

Precision and Accuracy

The Precision and Accuracy of the QC samples were analysed from 3 PA runs. The inter and intra run precision (%CV) and Accuracy (% Bias) of the QC's were calculated within the batch and between the batch. All samples met the acceptance of ± 20% (%CV & % Bias) for LLOQ and ± 15% (%CV & % Bias) for LQC,MQC and HQC.

Stabilities

Stability of Macitentan was established under the below categories, which involved preparation of quality control samples LQC and HQC and analysed as per the analytical method.

I) Pre – processing stability

a) **Bench top stability**

Quality control samples in K_2EDTA human plasma (n = 6 at low and high QC concentrations) were thawed on a bench at room temperature for 6 h 17 min prior to sample preparation. Macitentan was found to be stable in human plasma for at least 6 h 17 min on a bench at room temperaturebefore analysis. Results of the analysis are presented below and met acceptance criteria

b. Freeze thaw stability

Quality control samples (n = 6 at low and high QC concentrations) in K2EDTA Human plasma were subjected to three freeze-thaw cycles consisting of thawing on a bench at room temperature for at least 60 minutes, vortexing, and then refreezing(-60^{o}C to -80^{o}C) for at least 12 h. After three freeze-thaw cycles the samples were analyzed using freshly spiked calibration standards. Results of the analysis are presented in table and met acceptance criteria

c. Long term stability

Quality control samples LQC and HQC were stored frozen for 24 days -70±10°C prior to bioanalysis. Acceptable stability for Macitentan was demonstrated in K_2EDTA human plasma for 24 days . Results of the analysis are presented belowand met acceptance criteria.

II) Post - processing stability

a. Auto sampler stability

Quality control samples LQC and HQC were processed and stored in auto sampler for 19 hrs at 10°C and analysed under the CC. Acceptable stability for macitentan was demonstrated in K_2EDTA human plasma for 19hrs. Results of the analysis are presented belowand met acceptance criteria.

b. Stock and working solution stabilities

i) Stock solution short term

The stock solution (0.200 mL) of Analyte and ISTD was kept on bench for 6 hrs 50mins at room temperature and compared with the same stock stored at 2-8°C.The MQC level concentration for analyte and working concentration of ISTD was used to compare stability of the samples. The samples were within the acceptance criteria of ±10%.

ii) Stock solution longterm

The stock solutions of Analyte and ISTD were stored at 2-8°C for 24 days and compared with fresh stock.The MQC level concentration for analyte and working concentration of ISTD was used to compare stability of the samples. The samples were within the acceptance criteria of ±10%. The stability was corrected using the correction factor for the difference between the fresh and the stored stock.

iii) Working solution short term

The working solution (0.200 mL) of Analyte at MQC and ISTD50 µg/mL was kept on bench for 6 hrs 45mins at room temperature and compared with the same working solutions stored at 2-8°C.The MQC level concentration for analyte and working concentration of ISTD was used to compare stability of the samples. The samples were within the acceptance criteria of ±10%.

iv) Working solution longterm

The working solution of Analyte and ISTD were stored at 2-8°C for 24 days and compared with fresh working solutions.The MQC level concentration for analyte and working concentration of ISTD was used to

compare stability of the samples. The samples were within the acceptance criteria of ±10%. The stability was corrected using the correction factor for new stock and stability stock used for preparing the working solutions.

Table no 1: Data of validation parameters for Macitentan

PARAMETERS	Macitentan
System Suitability	
Analyte	3028412
Internal Standard	1646295
Area ratio	1.84
Auto Sampler Carryover test	No significant interference at the retention time of analyte or internal standard was observed during the period of validation
Selectivity and Specificity	All lots met the acceptance and no significant interference was observed in the any of the individual lots
Matrix effect	The method does not have any matrix interferences
Recovery studies	
LQC	101.12%
MQC	102.63%
HQC	100.37%
ISTD	96.29%
Linearity and Range	0.997 to 1020.793 ng/mL
Slope	0.000198
Standard deviation	0.000549
Correlation co-efficient	0.9945
Precision and Accuracy	
LLQC	0.996 ng/mL
LQC	2.879 ng/mL
MQC	473.977 ng/mL
HQC	786.426 ng/mL
Stability	
Bench top stability	
LQC (% RE)	0.72%
HQC (% RE)	3.43%
Freeze thaw stability	
LQC (% RE)	-5.48%
HQC (% RE)	6.21%
Long term stability	
LQC (% RE)	-1.00%
HQC (% RE)	7.43%
Auto sampler stability	
LQC (% RE)	5.90%
HQC (% RE)	-0.36%
Solution stability	
Stock solution	
Short term (analyte, ISTD in %)	103.57%, 102.47%
Long term (analyte, ISTD in %)	97.34%, 100.02%
Working solution	
Short term (analyte, ISTD in %)	96.17%, 99.42%
Long term (analyte, ISTD in %)	100.74%, 100.86%

Summary

An alternative LC-MS/MS method for quantification of Macitentan in human plasma has been successfully developed and validated. A simple and inexpensive precipitation extraction procedure and an isocratic chromatography condition using a reversed-phase column provided an assay well suited for real time analyses. The method exhibited excellent performance in terms of system suitability, selectivity, matrix effect, linearity, accuracy, precision, recovery and stability. In addition, the reported method has a short analysis run time, an advantage over previously reported methods. Therefore, this method is suitable for therapeutic drug monitoring of Macitentan and can be used in pharmacokinetic or bioequivalence studies of this drug.

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