DEVELOPMENT AND VALIDATION OF LC-MS/MS METHOD FOR THE ESTIMATION OF NEBIVOLOL IN HUMAN PLASMA

Praveen Kumar Dasari* and Srinivasa Rao Dorapaneedi


ABSTRACT

In the present work, determination of Nebivolol in human plasma was developed and validated with a large calibration curve range (10 - 4000 pg/mL) by specific, sensitive, an accurate liquid chromatography-tandem mass spectrometry method. The analyte from the human plasma was extracted from Liquid-liquid extraction method. The separation was achieved using Xbridge Zorbax Eclipse XDB - C18 (150 x 4.6 mm, 5 µ) column with Acetonitrile: 20mM Ammonium formate (pH-3.0) (50: 50, v/v) as a mobile phase. A flow rate of 1.0 mL/min and run time 10.0 min was used for the chromatographic analysis of Nebivolol. Sensitivity of this method was found to be 10pg/mL. The analyte was analyzed by mass spectrometry in the multiple reaction monitoring modes. The precursor-product ion m/z was 406.2 → 151.0 m/z and Nebivolol-D4 was 410.2 → 151.0 m/z were used for quantification of an analyte and its IS. The method was validated in terms of accuracy, precision, selectivity, recovery, freeze-thaw stability, bench-top stability, stock solution stability and re-injection reproducibility. Within- and between-batch precision was obtained within the range 0.7 to 8.2. The mean recovery for drug was obtained 87.71% where as the mean recovery of IS was 84.97%. The % RSD value at higher concentration and lower concentration in all stability experiments was within 15%. This method is free from ion suppression, ion enhancement and any type of abnormal ionization.

KEYWORDS: Nebivolol, LC-MS/MS, Human Plasma.

INTRODUCTION

Nebivolol is chemically known as 1-(6-fluoro-3, 4- dihydro-2H-1-benzopyran-2-yl)-2-[[2-(6-fluoro- 3, 4-dihydro-2H-1-benzopyran-2-yl)-2-hydroxyethyl] amino} ethan-1-ol. It is a highly
cardio selective vasodilatory beta1 receptor blocker used in treatment of hypertension. Nebivolol is a selective β1-receptor antagonist. Activation of β1-receptors by epinephrine increases the heart rate, blood pressure and the heart consumes more oxygen. Nebivolol blocks these receptors which reverses the effects of epinephrine, lowering the heart rate and blood pressure. In addition, beta blockers prevent the release of renin, which is a hormone produced by the kidneys which leads to constriction of blood vessels. At high enough concentrations, this drug may also bind beta 2 receptors.

Different analytical methods have been reported in the literature for the assay of nebivolol in pharmaceuticals and include spectrophotometry, UV\cite{1, 2}, HPLC\cite{3}, HPTLC\cite{4, 5}, RP-HPLC\cite{6-9}, LC-MS\cite{10}. Sample pre-treatment has also been an item of interest for the analysis of Nebivolol in biological samples. Previous methods included solid-phase extraction (SPE), protein precipitation and Liquid-liquid extraction (LLE). SPE is relatively expensive, time-consuming and complex for a large number of samples collected and treated. LLE with diethylether/hexane (80:20, v/v), methyl tert-butyl ether and diethylether has been reported, but hexane and methyl tert-butyl ether were toxic and expensive, whereas diethylether were easy to evaporate. The present investigation reports a simple a simple, sensitive, precise, economical and less toxic LC-MS/MS method for the analysis of nebivolol in plasma based on the LLE with ethyl acetate. The developed method was validated as per FDA guidelines\cite{11}.

MATERIAL AND METHODS

Instrumentation

The Agilent 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany) connected to the API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) with turbo electrospray interface in positive ionization mode was used. Data processing was performed on Analyst 1.4.1 software package (SCIEX).

Reagents / Materials

Nebivolol (NB) (fig. 1A) was obtained from Hetero Pharmaceuticals, Hyderabad, India. Nebivolol-D4 (NBD4) (fig. 1B) was procured from AI-BCD labs, India. Water (HPLC Grade), Ammonium formate, Formic Acid (analytical grade) were purchased from Merck, Mumbai, India. Acetonitrile (HPLC Grade), Diethyl ether and dichloromethane (HPLC grade) were obtained from J.T. Baker, USA. Human plasma was procured from Navjeevan Blood Blank, Hyderabad. Milli Q water was taken from the in-house Milli-Q system.
Detection

Detection was done by turboionspray (API) positive mode with unit resolution. Quantification was by MRM, where the acquired masses for Nebivolol were 406.2 → 151.0 m/z (fig. 2) and Nebivolol-D4 was 410.2 → 151.0 m/z (fig. 3).

Fig. 1: Chemical Structure 1A) Nebivolol (NB) 1B) Nebivolol-D4 (NBD4)

Fig. 2: Mass scan spectrum of Nebivolol

Fig. 3: Mass scan spectrum of Nebivolol-D4
Chromatographic conditions
Chromatographic separation was performed using an Xbridge Zorbax Eclipse XDB - C18 (150 x 4.6 mm, 5 µ) at a temperature of 40°C. The mobile phase was composed of Acetonitrile: 20mM Ammonium formate (pH-3.0) (50: 50, v/v) at a flow rate of 1.0 mL/min. Deuterated NBD4 (Internal standard-IS) was used as the appropriate IS in terms of chromatography and extractability. NB and NBD4 were eluted at 5.09 and 5.25 min, approximately, with a total run time of 10 min for each sample.

Preparation of standards and quality control samples
Standard stock solutions of NB (1.0 mg/mL) and NBD4 (1.0 mg/mL) were prepared in Methanol. The IS spiking solution (25.0 pg/mL) was prepared in mobile phase solution (Acetonitrile: 20 mM Ammonium formate (pH-3.0) (50: 50, v/v) from NBD4 stock solution. Standard stock solutions and IS spiking solutions were stored in refrigerator conditions of 2–8 ºC until analysis. Standard stock solutions of NB (1.0 mg/mL) were added to drug-free screened human plasma to obtain concentration levels of 10, 20, 50, 100, 400, 800, 1600, 2400, 3200 and 4000 pg/mL for analytical standards, and 10 (LLOQ), 30 (LQC), 1500 (MQC) and 3000 pg/mL (HQC) for quality control (QC) standards, and stored in the freezer at 30 ºC until analysis. The aqueous standards were prepared in a mobile phase solution (Acetonitrile: 20 mM Ammonium formate (pH-3.0) (50: 50, v/v) and stored in the refrigerator at 2–8 ºC until analysis.

Biological Matrix
Human plasma containing K2EDA as anticoagulant was used as a biological matrix during method validation. Selectivity and sensitivity tests were performed before bulk spiking.

Sample preparation
The LLE method was used to isolate NB and NBD4 from human plasma. For this purpose, 50 µL of NBD4 (10 pg/mL) and 100µL of plasma sample were added to the labelled polypropylene tubes and vortex briefly for about 5 min. Thereafter 3 mL of extraction solvent (in the ratio of diethyl ether: dichloromethane, 50:50, v/v) were added and vortex for about 10 min. Next, the samples were centrifuged at 4000 rpm for approximately 5 min at ambient temperature. From each, a supernatant sample was transferred into labelled polypropylene tubes and evaporated to a dryness of 40 ºC briefly, and then reconstituted with a mobile phase solution (Acetonitrile: 20 mM Ammonium formate (pH-3.0) (50: 50, v/v), and the sample was transferred into autosampler vials and injected into the LC-MS for study.
Method Validation
The validation was performed as per FDA guidelines to evaluate the method in terms of linearity response, sensitivity, selectivity, precision and accuracy (within-batch and between-batch/inter-day), stabilities (freeze-thaw, bench top, short-term and long-term stock solutions, working solutions and long term stability in matrix), carryover effects, recovery, dilution integrity, matrix effect, matrix factor, autosampler re-injection reproducibility and ruggedness experiment.

System suitability
System suitability experiment was performed by injecting six consecutive injections at least once in a day with using aqueous MQC solution. System performance experiment was performed by injecting sequence of injections at the beginning of analytical batch and % CV was calculated.

Selectivity and sensitivity
Selectivity was performed by analyzing human blank plasma samples from six different sources (donors) with an additional hemolyzed group and lipidemic group to test for interference at the retention times of analytes. The sensitivity was compared with the lower limit of quantification (LLOQ) of the analyte with its blank plasma sample. The peak area of blank samples should not be more than 20% of the mean peak area of the limit of quantification (LOQ) of NB and 5% of the mean peak area of NBD4.

Calibration of standard curve (Linearity and range)
The linearity of the method was determined by using standard plots associated with nine point standard curve including LLOQ and ULOQ. Concentration of calibration curve standards was calculated against the calibration curve and the linearity of the method was evaluated by ensuring the acceptance of precision and accuracy of calibration curve standards. Two consecutive calibration curve standards should not be beyond the acceptance criteria. The LLOQ was the lowest concentration at which the precision expressed by relative standard deviations (RSD, CV %) is better than 20% and the accuracy (bias) expressed by relative difference of the measured and true value was also lower than 20 %.

Precision and accuracy
The within-run and between-run percentage mean of precision and accuracy of the NB were measured by the percent coefficient by using six replicate samples of variation over the
concentration range of LLOQ (Low limit), LQC (Low), MQC (Middle) and HQC (high) quality control samples for the three precision and accuracy batches to their nominal values. The acceptable % coefficient of precision and accuracy should be less than 15 %. Between and within batch % mean precision and accuracy for LQC, MQC and HQC samples were within the range of 85.00-115.00 % and for the LLOQ within the range of 80.00-120.00 % respectively.

**Recovery**
The % mean recoveries was determined by comparing the mean peak area of the 6 replicates of extracted plasma quality control samples at high, middle 1&2 and low concentrations against respective mean peak area of the six replicates of un-extracted quality control samples at high, middle and low concentrations. A recovery of more than 50 % was considered adequate to obtain required sensitivity. The % mean internal standard recovery was determined by comparing the mean peak area of internal standard in the extracted plasma quality control samples at MQC concentration against the mean peak area of internal standard in the un-extracted quality control samples at MQC concentration.

**Ruggedness**
Ruggedness of the method was evaluated by using different analyst and different column of the same make and model or different equipment of the same make and model. The ruggedness experiment should meet the acceptance criteria for linearity and intra-batch accuracy & precision.

**Matrix effect**
To predict the variability of matrix effects in samples from individual subjects, matrix effect was quantified by determining the matrix factor, which was calculated as follows.

\[
\text{Matrix Factor} = \frac{\text{Peak response ratio in presence of extracted matrix (post extracted)}}{\text{Peak response ratio in aqueous standards}}
\]

Six lots of blank biological matrices were extracted each in triplicates and post spiked with the aqueous standard at the mid QC level, and compared with aqueous standards of same concentration. The overall precision of the matrix factor is expressed as coefficient of variation (CV %) and % CV should be < 15 %.
Stability of Nebivolol (NB) and Nebivolol-D4 (NBD4)

Long term stock solution stability
Long term stock solution stability for NB and NBD4 were performed at the stock concentration by using six consecutive injections of aqueous standard equivalent to ULOQ concentration and working concentration respectively after storage of at least 4 days in the refrigerator at 2-8 °C. Stability was assessed by comparing the stock dilutions of NB and NBD4 prepared from the freshly prepared stock solutions (comparison) against stock dilutions of NB and NBD4 prepared from the stock solutions stored at 2-8 °C (stability). Long term stock solution stability was evaluated by comparing the mean response of stability samples against mean response ratios of comparison samples.

Stability of Drug in Biological Matrix
Perform the matrix stability experiment by using freshly prepared calibration curve standard and three replicates of freshly prepared batch qualifying quality control samples at HQC and LQC levels. The precision and accuracy for the stability samples must be within ±15 and ±15 %, respectively, of their nominal concentrations. Stability studies in biological matrix were conducted in the various conditions at LQC and HQC levels.

Freeze thaw stability
Freeze thaw stability of the spiked quality control samples were determined after 1 st and 3 rd freeze thaw cycles stored at -20±5 °C. Six replicates of each HQC and LQC samples were used for assessing each freeze thaw experiment (for first and third cycle at both the freezing temperatures). The first freeze-thaw cycle was of at least 24 hours followed by minimum of 12 hours for subsequent cycles. Process and analyze freeze thaw stability samples along with freshly spiked calibration curve and comparison samples (6 replicates of each LQC and HQC) in screened biological matrix. Evaluate the freeze thaw stability on the basis of % change of LQC and HQC samples. The % Accuracy and % CV of LQC and HQC should be within ± 15.00 and ≤ 15.00 respectively.

Bench top stability
Spiked quality controlled samples (6 replicates of each LQC and HQC) were stored in deep freezer at temperature -20±5 °C, which was retrieved after minimum 12 hours of freezing and was kept at ambient temperature on working bench for recommended period of at least 21 hours. Six replicates of each HQC and LQC samples were used for assessing the
bench top stability experiment. Upon the completion of recommended period, process and analyze bench top stability samples along with freshly spiked calibration curve and comparison samples (6 replicates of each LQC and HQC) in screened biological matrix. Evaluate the bench top stability on the basis of % Accuracy and % CV of LQC and HQC samples.

**Autosampler re-injection reproducibility**

Autosampler re-injection reproducibility was evaluated by re-injecting accepted precision & accuracy batch, which were stored preferably in either autosampler or in refrigerator for at least 71 hours or as per requirement. Autosampler re-injection reproducibility was evaluated by % Accuracy and % CV of LQC and HQC samples.

**Long Term Stability in Biological Matrix**

The long-term stability samples of LQC, MQC and HQC samples were kept frozen in vials at -20±5 °C for 99 days were assessed along with freshly processed calibration and comparison samples (six samples each of LQC, MQC, HQC). The initial NB concentration freshly after sample treatment preparation was assumed to be 100 %. The selection of the stability duration is on the basis of the characteristic of the analyte(s).

**RESULTS AND DISCUSSION**

**Method Validation**

**System Suitability**

System performance experiment was performed by injecting six consecutive injections at the beginning of analytical batch. % CV was 1.34.

**Carryover Test**

For carryover test two samples of ULOQ and 4 samples of blank plasma were processed. These samples were injected in the following sequence. a) 2 blank samples b) 2 ULOQ samples c) 2 blank samples. The step (b) and (c) were repeated 2 times. The results demonstrate that there was no interference from the previous injection.

**Selectivity and specificity**

The analysis of NB and NBD4 using MRM (Multiple reaction monitoring) function was highly selective with no interfering compounds. Chromatograms obtained from plasma spiked with NB and NBD4 (fig. 4A & 4B).
Limit of Detection (LOD) and Quantification (LOQ)

The limit of detection was used to determine the instrument detection levels for NB even at low concentrations. 30 μL of a 1.0 pg/mL solution was injected to give an on-column mass of 0.03 pg/ml. The limit of quantification for this method was proved as lowest concentration of the calibration curve which was proved as 10.0 pg/ml.

Calibration curve standards, Precision and Accuracy

Calibration curves were plotted as the peak area ratio (NB/NBD4) versus (NB) concentration. Calibration was found to be linear over the concentration range of 10.0- 4000.0 pg/mL. The determination coefficients ($r^2$) were greater than 0.9997 for all curves (fig. 5). The intra-batch CV % was 0.7 to 8.2 and inter-batch CV % was 4.5 to 6.1 %. These results indicate the adequate reliability and reproducibility of this method within the analytical range.
Recovery
The recovery following the sample preparation using Liquid-liquid extraction with Methyl tertiary butyl ether was calculated by comparing the peak area of NB in plasma samples with the peak area of solvent samples and was estimated at control levels of NB. The overall average recovery NB and NBD4 were found to be 87.71 and 84.97 % respectively (Table. 1).

Table.1: Validation parameters

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Nebivolol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal Standard (IS)</td>
<td>Nebivolol D4</td>
</tr>
<tr>
<td>Method description</td>
<td>Liquid-liquid extraction with LC-MS/MS technique</td>
</tr>
<tr>
<td>Selectivity</td>
<td>No known, endogenous plasma components, common drugs and commonly used female contraceptives interfere with the analytical assay</td>
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<tr>
<td>Limit of quantitation</td>
<td>10.0 pg/mL</td>
</tr>
<tr>
<td>Limit of Detection</td>
<td>0.03 pg/ml</td>
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<tr>
<td>Average recovery of drug</td>
<td>87.71 %</td>
</tr>
<tr>
<td>Average recovery of IS</td>
<td>84.97 %</td>
</tr>
<tr>
<td>Standard curve Linearity &amp; Regression</td>
<td>10.0 to 4000.0 pg/mL &amp; 0.9997</td>
</tr>
<tr>
<td>QC concentrations</td>
<td>QC A: 29.9 pg/mL</td>
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<tr>
<td></td>
<td>QC B: 1495.8 pg/mL</td>
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<tr>
<td></td>
<td>QC C: 2991.7 pg/mL</td>
</tr>
<tr>
<td>QC Intraday precision range</td>
<td>0.7 to 8.2</td>
</tr>
<tr>
<td>QC Inter day accuracy range</td>
<td>4.5 to 6.1%</td>
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<tr>
<td>Matrix factor</td>
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<tr>
<td>Bench-top stability</td>
<td>21 hours @ room temperature &amp; 1.2 to 1.5</td>
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<tr>
<td>Master Stock stability &amp; %Accuracy</td>
<td>56 days @ refrigerated condition &amp; 91.65 and 87.81%</td>
</tr>
<tr>
<td>Processed stability</td>
<td>71 hours @ refrigerated condition &amp; 1.1 to 1.7%</td>
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<tr>
<td>Freeze-thaw stability</td>
<td>3 cycles &amp; 1.3 to 1.7%</td>
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<tr>
<td>Long-term storage stability</td>
<td>99 days @ -20°C set point freezer &amp; 0.9 to 1.2</td>
</tr>
<tr>
<td>Regression model</td>
<td>Weighted (1/conc²) linear</td>
</tr>
<tr>
<td>Analysis method</td>
<td>Peak area ratio (PAR)</td>
</tr>
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</table>

Matrix effect
Six lots of blank biological matrices were extracted each in triplicates and post spiked with the aqueous standard at the mid QC level, and compared with neat standards of same concentration in alternate injections. The overall precision of the matrix factor is 5.11 for NB. There was no ion- suppression and ion- Enhancement effect observed due to IS and analyte at respective retention time.
Stock Solution Stability

Stock solution stability at refrigerator (2-8°C)

Stock solution each of NB and internal standard were stable after approximately 56 days and at refrigerated temperature 2-8 °C. For NB and NBD4 the % Accuracy was 91.65 and 87.81 respectively.

Stability of NB in plasma samples (Freeze - thaw, Auto sampler, Bench top, Long term)

Quantification of the NB in plasma subjected to 3 freeze-thaw (-30 °C to room temperature) cycles show the stability of the analyte and % CV was in between 1.3 to 1.7. No significant degradation of the NB was observed even after 71 hr storage period in the autosampler tray and the % CV was in between 1.1 to 1.7. No significant degradation of the NB was observed even after 21 hr storage period in the room temperature and % CV was in between 1.2 to 1.5 of the theoretical values. In addition, the long-term stability of NB in QC samples after 99 days of storage at -20 °C was also evaluated and % CV was ranged from 0.9 to 1.2. These results confirmed the stability of NB in human plasma for at least 99 days at -20 °C (Table-1).

CONCLUSION

The LC–MS/MS validated method has proved to be very simple, sensitive and reliable and successfully applied for the pharmacokinetic study in human plasma. The assay method is specific due to the inherent selectivity of tandem mass spectrometry. The major advantage of this method is the use of deuterated Nevibolol-D4 as an internal standard. The run time is within 10 min and only 0.200 mL of plasma was required for each determination of Nevibolol, and thus the stress to volunteers or patients in clinical studies was greatly reduced. This method is very suitable and convenient for pharmacokinetics and bioavailability study of the drug Nevibolol.

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