RESEARCH ARTICLE

A Stability Indicating RP-HPLC Method for the Estimation of Nebivolol Hydrochloride in Human Plasma

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ABSTRACT

Nebivolol hydrochloride is used as a β1 receptor and calcium channel blocker to treat hypertension. This research aims to provide novel stability indicating reverse phase high-performance liquid chromatography (RP-HPLC) approach for estimating nebivolol hydrochloride in human plasma. The protein precipitation technique used 5% formic acid and methanol to extract the plasma. The separation was accomplished by using Inertsil ODS-3V (150 mm X 4.6 mm, 5μm) column, using acetonitrile: 0.1% trifluoroacetic acid in water (40:60% v/v) as a mobile phase, at flow rate of 1.0 mL/min and detection was done on a photodiode array detector at 282 nm. The retention time was found to be 5.55 minutes. Linearity was observed between 4.50 to 180 μg/mL with R^2 values of 0.999. No interference was detected in the drug retention times in selectivity and sensitivity. Accuracy as well as precision were observed within limit. All the analytical validation parameters were determined as per the USFDA guidelines. The stability study was conducted on NBH containing human plasma using two levels, LQC and HQC, exhibiting that the drug was stable under different conditions. The conveyed RP-HPLC method for estimation of NBH was simple, precise, and accurate; therefore, it can be used in bioavailability and bioequivalence studies, pharmacokinetics and toxicology studies of NBH in human plasma.

Keywords: Nebivolol hydrochloride, Method validation, Human plasma, Protein precipitation.

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INTRODUCTION

Chronically high blood pressure (BP) is the hallmark of hypertension, an asymptomatic disease affecting billions worldwide. Heart failure, stroke, renal illness, and myocardial infarction (MI) are some other conditions and occurrences that have been related to it.[1-3] One of the most commonly prescribed classes of cardiovascular medicines areβ-blockers. As beta-adrenergic antagonists, they are utilized in treating BP.[4] Nebivolol HCl (NBH) is a β1 receptor blocker. It is chemically (1R)-1-[(2R)-6-fluoro-3,4-dihydro-2H-1-benzopyran-2-yl-2-{[(2R)-2-[(2S)-6-fluoro-3,4dihydro-2H-1benzopyran-2-yl]-hydroxyethyl] amino} ethan-1-ol hydrochloride (Figure 1). It is used alone or in conjunction with other medications to lower BP. Adrenergic agonist action at the beta-3 adrenergic receptor activates the endothelial nitric oxide synthase, which results in vasodilation and reduces BP.[5] The cytochrome P-450 2D6 enzyme metabolizes it in the first pass.[6] NBH is a lipophilic beta-blocker utilized to rehabilitate BP by elevating peripheral vascular resistance and improving stroke volume while keeping cardiovascular output constant. Figure 1 shows the NBH structure.

Previously reported data represent several chromatographic methods such HPLC, tandem mass spectrometry and UPLC for estimation of NBH were used.[7] These methods have been reported to include maximum retention time for individuals and simultaneous estimation of NBH. Hence, this work aims to develop and validate simple, accurate, precise and less time-consuming new bioanalytical technique for assessing NBH in human plasma using the RP- HPLC method. The materials and procedures used to estimate drug in human plasma are covered in the next section.

MATERIALS AND METHODS

Chemicals

Mylan Laboratory Pvt. Ltd. in Nasik, India, provided the NBH as a gift sample. Analytical HPLC grade methanol, acetonitrile, orthophosphoric acid, ammonium acetate and trifluoroacetic acid were purchased from Qualigens (Thermo Fisher Scientific). The ultra-pure water of HPLC grade was procured from Moreshwar Enterprises, India.

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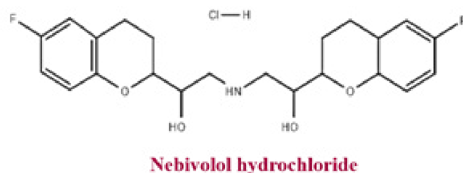

Figure 1: Chemical structure of NBH

Chromatographic Conditions

The RP-HPLC system deployed for the development of method and validation comprised 1260 Infinity II with a G7111A pump, degasse, G7129A autoinjector. Analyses and separations have been performed using an Inertsil ODS-3V column (150 mm x 4.6 mm, 5 m). Acetonitrile: 0.1% Trifluoroacetic acid in water (ACN:0.1%TFA water) (40:60 v/v) as mobile phase. A 20 µL sample was injected during analysis and the flow rate was maintained at 1.0 mL/min. Photodiode array detector was used and absorbance at 282 nm was measured.

Development of Quality Control (QC) and Calibration Standards for Extraction

A 40.2 mg of NBH in 20 mL of methanol was dissolved to prepare a stock solution which was used to make the calibration and quality control samples. In 1-mL was pipetted out from this solution and diluted with human plasma. Six calibration standards were prepared at 1.51, 10.05, 20.10, 30.15, 45.23 and 60.30 µg/mL.

Extraction and Preparation of Plasma

Processes for extraction and preparation of plasma samples were described in the following sections.[8]

Plasma sample Extraction

The plasma sample was extracted using the protein precipitation technique. Polypropylene disposable tips were used to separate the plasma from the blood, which was subsequently maintained in the freezer at -20°C 2 until further examination.

Blank Plasma Preparation

The human plasma and 5% formic acid 475 and 50 µL for each and 1-mL methanol were combined and kept 1-minutes for sonication. Samples were centrifuged at 4000 rpm for 3 minutes after being maintained in a vortex for 30 seconds. To analyze the supernatant sample, 0.22 µm filter was used to filter the sample and pipetted out from this 0.5 mL of supernatant.

Spiked Plasma Preparation

The human plasma and 5% formic acid 475 and 50 µL for each, 25 µL NBH in methanol solution were mixed and kept 30 seconds to sonicat at room temperature, then centrifuged at 4000 rpm for 3 minutes. An eppendorf tube containing the supernatant (550 µL) was placed in a mild stream of nitrogen at 42°C to evaporate the supernatant after that 1-mL of mobile phase was added to the residue to reconstitute it. The sample was filtered using a 0.22 µm filter, and 0.5 mL of supernatant was pipette out and subjected to chromatographic analysis.

Development of RP-HPLC Method

Various RP-HPLC chromatographic conditions were devised to estimate NBH in human plasma. Porous membranes with pore sizes of 0.45 µm were used to filter the ACN: 0.1%TFA water (40:60 v/v) for method development. A flow rate was 1-mL/min with isocratic mode. The ODS-3V column was used. PDA detector at 282 nm was used to detect the sample 20 µL in the RP-HPLC system.[9]

Extraction Recovery

The word "recovery" indicates the extraction effectiveness of an analytical procedure in terms of a percentage.[10] Analyzing the response of QC samples to after extraction of spiked QC samples at the same concentration allows for determining the extraction recovery of NBH. It was calculated at 550 µg/mL.

Method Validation

The validation of the bioanalytical technique for estimating NBH was performed in accordance with USFDA guidelines.[11]

Linearity

The linearity studies were performed at various concentrations in the range (4.50–180 µg/mL). The calibration curve was plotted between area and concentration. The slope and regression coefficient were calculated.

Selectivity and sensitivity

to illustrate the insufficiency of chromatographic interference at the retention time of NBH six batches of human blank plasma, lipemic plasma and hemolyzed plasma were examined. The acceptance conditions were found less than 20% of the LLoQ response.

Accuracy and precision

Six replicates of NBH QC test samples at four distinct levels (0.15–5.0 g/mL) were analysed on three different days to

Table 1: Extraction recovery data for NBH

QC	*Recovery vial*	*Extracted*	*%Recovery*	*Mean Recovery*	*%CV*	*Overall Recovery*	*Overall %CV*
	1194384	1189875	99.62				
LQC	1201337	1218678	101.44	100.58	0.908		
	1186728	1194658	100.67			99.76	1.20
	12894718	12697312	98.47				
HQC	12803046	12795867	99.94	98.94	0.873		
	12935425	12731482	98.42				

determine interday, intraday, precision, and accuracy. %RSD for precision and relative error (RE) for accuracy were calculated.

Stability studies

The method's stability was evaluated using two-level LQC and HQC at (13.52 and 150.19 µg/mL) concentrations of NBH in the plasma sample. The bench-top stability at (25°C, 6 h), freeze-thaw at RT for 2 hours and refreeze at 30°C for 24 hours, 24 hours for autosampler, stock solution and process sample for (24 hours) were performed. The percent recovery against a freshly prepared standard solution of NBH was calculated.[12,13]

RESULT AND DISCUSSION

The study aimed to establish a method suitable for estimating NBH from human plasma with reasonable accuracy and reliability in the shortest possible run time. The plasma precipitation procedure was used to extract the material with 5% formic acid and methanol. The process initiated with the selection of an appropriate mobile phase. Acetonitrile %Trifluoroacetic acid in water (40:60 v/v) runs on an inertsil ODS-3V column, allowing rapid peak retention and resolution in a short period of 5.5 minutes. Method validation and stability studies were conducted in accordance with USFDA guidelines. Validation parameters, including linearity, precision, accuracy, selectivity, and sensitivity, were within the acceptable limit and exploring that method was precise and accurate. Numerous stability studies were performed to assess NBH stability in human plasma at different storage conditions such as room temperature or under refrigeration. There was no significant change in the results of NBH in human plasma, whether stored at ambient temperature or under refrigeration; hence the delay in the sample analysis will not have a major effect.

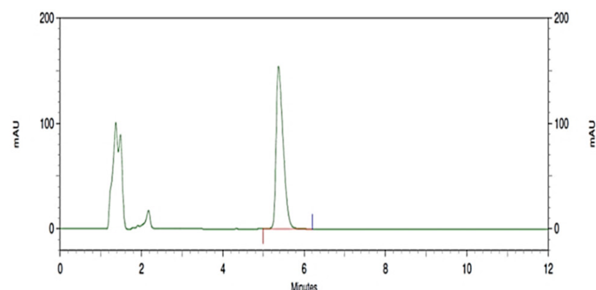

Figure 2: Optimized chromatogram of NBH

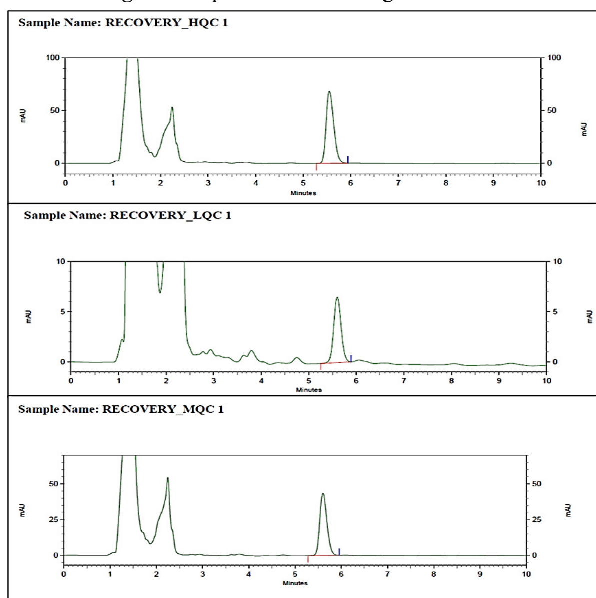

Figure 3: Chromatogram of extraction recovery

Table 2: The summary of the linearity study

Concentration (µg/mL) of NBH	*Peak area*
4.50	1022637
32.02	5719635
64.05	10952634
90.07	15462186
130.10	22064518
180.14	30954857

Method Optimization

The development of the method and analysis conditions were optimized considering the physicochemical characteristics of NBH. The ODS-3V column, mobile phase ACN:0.1%TFA water (40:60 v/v) with flow rate 1-mL/min were demonstrated to optimize chromatographic variables. The retention time of NBH was observed at 5.5 minutes, respectively. Better separation is shown by the solvent used in method development. Hence this column and mobile phase were used for further analysis. The optimized chromatogram of NBH is shown in Figure 2.

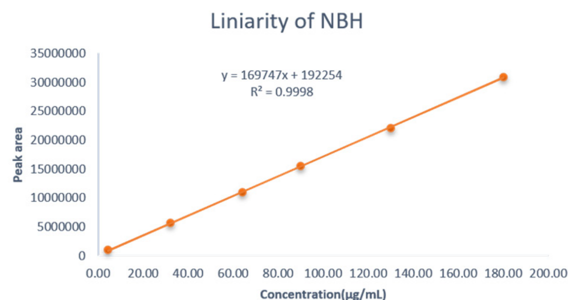

Figure 4: Calibration graph of linearity study

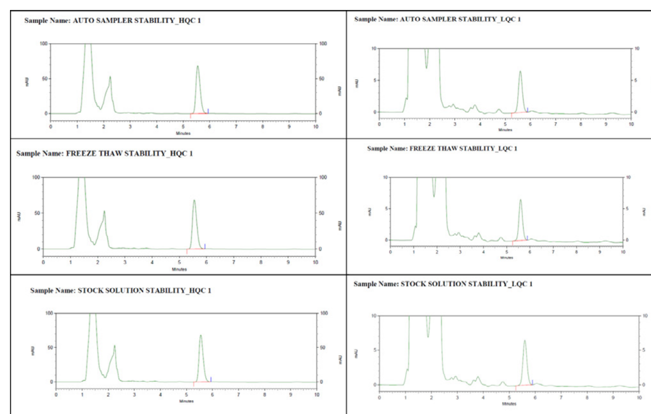

Figure 5: The chromatograms of stability study

Table 3: Summary of selectivity blank

Plasma Lot	*Area of blank plasma at R.T. of NBH*	*Area of LLoQ*	*%Interference*
LOT 1	0	456859	0.00
LOT 2	0	446284	0.00
LOT 3	0	453981	0.00
LOT 4	0	455184	0.00
LOT 5	0	453941	0.00
LOT 6	0	457319	0.00
Hemolyzed	0	453182	0.00
Lipemic	0	457564	0.00

Table 4: Summary of sensitivity for NBH

LLoQ Levels	*Area of LLoQ*
1	454675
2	456183
3	454931
4	453128
5	452791
6	453861
Mean	454262
STDV	1258.488578
CV	0.28

Table 5: Summary of accuracy

SETS	*QC*	*Area*	*Recovered conc (µg/mL)*	*%Accuracy*
1	LLoQ 1	458641	1.37	91.33
	LQC 1	1189875	4.26	94.46
	MQC 1	7825843	30.47	101.30
	HQC 1	12697312	49.72	99.18
2	LLoQ 2	466458	1.40	93.33
	LQC 2	1218678	4.37	96.90
	MQC 2	7802846	30.38	101.00
	HQC 2	12795867	50.11	99.96
3	LLoQ 3	463864	1.39	92.67
	LQC 3	1194658	4.27	94.68
	MQC 3	7761857	30.22	100.47
	HQC 3	12731482	49.85	99.44
4	LLoQ 4	453185	1.35	90.00
	LQC 4	1197581	4.29	95.12
	MQC 4	7836078	30.51	101.43
	HQC 4	12648749	49.52	98.78
5	LLoQ 5	461051	1.38	92.00
	LQC 5	1190764	4.26	94.46
	MQC 5	7831846	30.50	101.40
	HQC 5	12317491	48.22	96.19
6	LLOQ 6	460371	1.37	91.33
	LQC 6	1231589	4.42	98.00
	MQC 6	7931461	30.89	102.69
	HQC 6	12647183	49.52	98.78

Table 6: Summary of the precision

LEVEL	*QC*	*Recovered concentration*	*Mean Recovered conc. (µg/mL)*	*%CV*
LLoQ	1	1.37	1.38	1.272
	2	1.40		
	3	1.39		
	4	1.35		
	5	1.38		
	6	1.37		
LQC	1	4.26	4.31	1.562
	2	4.37		
	3	4.27		
	4	4.29		
	5	4.26		
	6	4.42		
MQC	1	30.47	30.50	0.727
	2	30.38		
	3	30.22		
	4	30.51		
	5	30.50		
	6	30.89		
HQC	1	49.72	49.49	1.335
	2	50.11		
	3	49.85		
	4	49.52		
	5	48.22		
	6	49.52		

Extraction Recovery

The extraction recovery of QC samples was found to be 99.76% at LQC and HQC levels, as shown in Table 1 and retention time was found to be 5.55 minutes for both levels, as shown in Figure 3. The final extraction method was simple, rapid and accurate; thus, it was applicable for the analysis of large number of samples in a standard laboratory.

Method Validation

The newly developed method was found simple, rapid, sensitive and reproducible with NBH. The next section includes the outcomes of several validation parameters.

Linearity

Linear calibration curve was found for NBH in the specified ranges, marked by the similarity of the correlation coefficient R^2=0.999. The calibration graph shows that the optimized method obeys the peak area of each concentration. The summary of the linearity study is given in Table 2 and the calibration graph of linearity is shown in Figure 4.

Selectivity and sensitivity

The method's selectivity and sensitivity were evaluated by analyzing blank plasma, lipemic, and hemolyzed samples to demonstrate the absence of chromatographic interference during NBH analysis. Regarding selectivity, no interference

Table 7: Summary of stability studies

Nominal concentration (NBH)	*Bench-top Stability (%)*	*Processed Extracted*	*Auto-sampler stability (%)*	*Freeze-thaw stability (%)*	*Stock solution stability (%)*
LQC (13.52)	95.35	95.86	92.39	98.08	0.37
HQC (150.19)	99.53	97.92	99.81	98.82	0.37

was detected in the drug retention times. The selectivity summary of NBH is indicated in Table 3. In the sensitivity study, the optimized method was found to be sensitive, and the coefficient of variation for NBH was found to be 0.28. The summary of sensitivity for NBH is shown in Table 4.

Precision and accuracy

The accuracy of NBH in sample solutions was analyzed at four levels (LLoQ, LQC, MQC, and HQC) in the range of 90 to 102%; this demonstrates that the devised method was accurate. The results of the accuracy study for six sets are summarized in Table 5. In accordance with FDA requirements, NBH precision was below 15% for interday and intraday samples, indicating that the method was precise, reproducible and repeatable. Summary of study for precision is shown in Table 6.

Stability studies

NBH stability in human plasma was studied under various conditions (bench top, freeze-thaw cycle, process extract, and autosampler). Under these conditions, NBH was found to be stable. The recovery values and chromatograms for two levels of stability study are shown in Table 7 and Figure.5. There were no significant changes in the NBH human plasma sample whether stored at ambient temperature or under refrigeration hence the delay in the sample analysis will not have a major effect.

CONCLUSION

In conclusion, it was revealed that the established RP-HPLC technique for NBH quantification in human plasma employing protein precipitation for sample preparation was a sensitive, linear, accurate, selective, precise and reliable analytical method. It also discovered that the NBH in human plasma was stable at ambient temperature and refrigeration. As a result, this technique is well-suited for routine quantitative investigation of the pharmaceutical dosage form. As a therapeutic drug monitoring unit, pharmacokinetic and toxicological investigations of NBH in human plasma may also be performed using the proposed method.

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