Current Trends in Biotechnology and Pharmacy
Vol. 16 (Supplementry Issue 2) 87 - 95, October 2022, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2022.3s.67

Development and Validation of a Sensitive and Rapid Bioanalytical RP-HPLC Method for the Quantification of Nebivolol Hydrochloride in Rat Plasma

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Abstract

A simple and specific bioanalytical reverse phase high performance liquid chromatography (HPLC) assay method was established and validated for estimation nebivolol HCl in rat plasma using amlodipine besylate as internal standard. The chromatographic separation was achieved by reversed phase Knauer C18 column and mobile phase composition of acetonitrile, methanol and 0.1N orthophosphoric acid (80:20:10) with ultraviolet detector at the wavelength of 272 nm. The flow rate was maintained at 0.5 mL/min. System suitability parameters of mobile phase composition, flow rate, and wavelength were optimized to provide highly sensitive peaks for the drug samples. The calibration curve was found linear in the range of 400 ng/mL to 1800 ng/mL with a retention time of 5.48 minutes. LOD and LOQ were found at 40.24 ng/mL and 121.94 ng/mL respectively. The developed method was validated in terms of linearity, recovery, precision, robustness, ruggedness, and stability (short term & long-term stabilities and freeze & thaw stability). All the experiments were done as per ICH guidelines and the outcomes were well inside the limits determined.

Keywords: Bioanalytical, Reversed Phase HPLC, Nebivolol HCl, ICH

Introduction

Bioanalytical method is used to determine the concentration of drug and metabolites present in the biological matrix like serum, plasma, cerebrospinal fluid etc. The developed method can be used to detect the content of drug quantitatively. Validation of an analytical method involve in documentation of laboratory investigations to estimate the performance characteristics of procedures meeting required specifications and proposed applications. Validation of bioanalytical method demonstrate that the method developed is sensitive, accurate, precise and suitable for the intended purpose.

Nebivolol hydrochloride is a cardio selective adrenergic beta-1 blocker used in the treatment of hypertension and chronic heart failure in elderly patients. The drug also has vasodilation property acting through L-arginine/nitric oxide pathway. It is chemically 1-(6-fluoro-3,4-dihydro-2H-chromen-2-yl)-2-((2-(6-fluoro-3,4-dihydro-2H-chromen-2-yl)-2-hydroxyethyl)amino) ethanol;hydrochloride. It is generally prescribed for elderly patients with a starting dose of 2.5 mg in tablet dosage form (1). The chemical structure of drug was depicted in (Figure 1). Several methods were developed and reported for the estimation of NBH till date including RP-HPLC (2,3), RP-HPTLC (4) and bioanalytical method development in human plasma were reported (5). In the current study, a sensitive bioanalytical method was developed for the estimation of nebivolol HCl in rat plasma using HPLC equipped with absorbance detector as per the FDA guidance (6). The method involved liquid-liquid extraction of NBH from the plasma using acetonitrile, methanol, and

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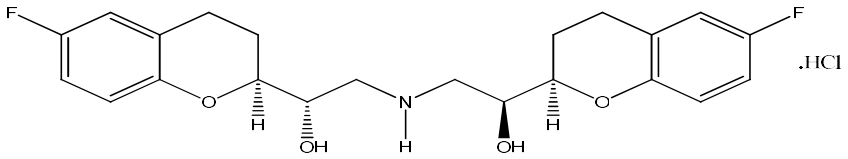

Fig 1. Chemical Structure of Nebivolol Hydrochloride

buffer mixture. The developed method was validated using ICH Q2 (R1) guidelines (7).

Materials and Methods

***Materials and Reagents*:** Waters HPLC system equipped with Knauer C_{18} (250 X 4.6 mm, 5µ), column, solvents, and buffers of HPLC grade purchased from Merck Ltd. Nebivolol HCl (NBH) was purchased from Yarrow Chem. Products, Mumbai.

***Instruments*:** Waters HPLC 2695 alliance system with 2487 UV detector, Empower-2 software, Remi vortex shaker.

***Plasma Samples*:** Plasma samples for the method development were collected from animal house facility, Bapatla College of Pharmacy, Bapatla, Guntur with Regd. No. 1032/PO/Re/S/07/CPCSEA. Blood samples were collected from Albino Wistar rats using retro orbital puncture without sacrificing animals. Blood samples were collected in sample tubes with anticoagulant (EDTA), centrifuged at 400 rpm for 20 minutes. The supernatant plasma was collected and stored at -20°C in deep freezer until further use.

***Preparation of Standard Solutions*:** Stock solutions of nebivolol HCl (1 mg/mL) and amlodipine besylate (1 mg/mL) were prepared with methanol-acetonitrile mixture (5:5 v/v) using standard powders. Working standard solution of NBH was prepared by diluting stock solution with above solvent mixture to get 0.4 to 1.8 µg/mL solution. The same method was used to prepare 1000 ng/mL working standard solution of amlodipine besylate.

***Preparation of Plasma Samples*:** Plasma samples of drug and IS mixture were prepared using 100 µL of blank plasma, 100 µL of NBH standard solution and 100 µL of IS in an Eppendorf tube. The sample extraction was conducted using liquid-liquid extraction technique (LLE) with 100 µL of acetonitrile & methanol solution (1:1) to the above drug containing plasma mixture, vortexed for 2 minutes and then centrifuged at 15000 rpm at 4°C for 5 minutes. 100 µL of supernatant was transferred to the sample tube vial and 20 micro liters solution was then injected into the chromatographic system.

The blank plasma solution was prepared by spiking 0.1 mL of plasma into extraction solution (acetonitrile-methanol mixture), vortexed for 1 minute and further centrifuged at 4°C at 15000 rpm for 10 minutes. The supernatant was collected, filtered, and used as blank solution for analysis.

***Method Development*:** The validation parameters like specificity, linearity, sensitivity, accuracy, precision, and recovery in rat plasma, were determined according to the US-FDA guidance for industry, bio-analytical method development and validation document 2018.

***Linearity*:** The linearity of the proposed method was established from the standard calibration curve constructed at six concentrations (0.4 to1.8 µg/mL) of nebivolol HCl with constant concentration of IS (Amlodipine besylate at 1 µg/mL), on six consecutive days in order to confirm reproducibility of results. Calibration curve was constructed for the drug in the spiked plasma samples by plotting the relative peak area (ratio of peak area of drug to peak area of IS) against their respective concentrations using a linear least squares regression analysis.

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***Specificity/Selectivity*:** The specificity/selectivity of the analytical method was investigated by confirming the complete separation and resolution of the required peak area of nebivolol HCl and the IS in plasma samples, spiked with appropriate concentration of these compounds. The tests were accomplished to verify the potential interferences of endogenous plasma components co-eluting with drug and IS.

Accuracy and Precision (8)

***Intra-day Accuracy and Precision*:** The intra-day precision and accuracy of the method were determined by analyzing six spiked samples of nebivolol HCl at three different concentrations LQC, MQC and HQC (0.4, 1.2 and 1.8 µg/mL). The deviation of the mean from the true value serves as the measure of accuracy. The statistical evaluation includes calculation of parameters such as mean, standard deviation, coefficient of variation, and percentage relative error. Accuracy and Precision were determined using % relative error and %RSD respectively.

***Inter-day Accuracy and Precision*:** The inter-day accuracy and precision was determined for six replicates at three different concentrations 0.4, 1.2 and 1.8 µg/mL over three days. The statistical evaluation includes calculation of parameters such as mean, standard deviation, coefficient of variation, accuracy, and percentage relative error. Accuracy was determined using % relative error and precision was determined by calculating %RSD.

***Sensitivity*:** The sensitivity of the method detected using limit of detection (LOD) and limit of quantification (LOQ) concentrations of drug samples. LOD is a parameter used to detect the lowest amount of analyte that can be detected from background noise and cannot be quantitated and LOQ stands for the lowest concentration of analyte that can be detected and quantitated. LOD and LOQ can be measured using signal to noise ratio of 3:1 and 10:1. They can be determined based on the standard deviation of the response (σ) and the slope of the calibration curve (s) using known concentrations of analyte with those of blank samples. The standard deviation of the response can be determined based on standard deviation of Y–intercepts of regression lines. LOD & LOQ are calculated based on the following formulae (9):

$$Limit\ of\ Detection\ (LOD) = 3.3\frac{\sigma}{s}$$

$$Limit\ of\ Quantification\ (LOQ) = 10\frac{\sigma}{s}$$

Where,

'σ' is regression standard deviation of intercept.

's' is the slope.

***Recovery*:** Recovery of drug sample can be calculated using analyte response to that of maximum concentration of pure sample. During the bioanalytical development, nebivolol HCl was determined for absolute and relative recoveries of drug and IS from rat plasma using quality control samples. The absolute recovery of drug and IS were calculated using measured values of extracted samples compared with un-extracted pure authentic samples whereas; relative recovery of drug and IS were computed using peak areas (detector response) obtained from the sample injections of the prepared plasma standards compared to the nominal concentration prepared. The standard used was prepared to contain a drug concentration assuming 100% recovery. Recovery of nebivolol HCl was measured at three concentration levels (0.4, 1.2 and 1.8 µg/mL).

***Robustness*:** Robustness of the method developed for the drug was evaluated by changing small but deliberate variations in chromatographic conditions. The parameters studied in this study were flow rate and mobile phase composition.

Stability Study (10)

LOQ, MOQ and HOQ samples of six replicates were retrieved from deep freezer

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after three freeze thaw cycles. Samples were frozen at -20°C followed by thawing at the time of experiment. Short term and long-term stabilities of samples were conducted by preserving samples at room temperature for 24 hours and -20°C deep freezer for 45 days respectively. The precision and accuracy of stability samples must be within 15% of nominal concentrations.

Results and Discussion

Selection of Chromatographic Conditions: The chromatographic conditions for the method validation were selected based on the peak resolutions, day-to-day reproducibility of the retention times, peak shapes and back pressure. Selection of mobile phase is the key for reproducible elution of characteristic peaks and different combination of solvents were tested starting with acetonitrile-methanol mixture (80:20, %v/v) where, merging of peaks observed. The mobile phase composition was changed with addition of water to the above composition at acetonitrile-methanol-water (65:35:5, %v/v) that resulted in proximity of two peaks of drug analyte and IS. Finally, the composition was selected with replacing orthophosphoric acid instead of water with a composition of acetonitrile: methanol: 0.1% Orthophosphoric acid (70:20:10) produced sharp reproducible peaks with system suitability conditions at 2.6 and 5.4 minutes for nebivolol HCl and IS respectively.

Linearity: The analytical curves were constructed with standard concentrations ranging 0.4 µg/mL to 1.8 µg/mL of NBH along with constant concentration of 1.0 µg/mL of IS in rat plasma. All the samples were extracted for the drug and IS contents using the above-mentioned procedure. The baseline was monitored and 20 µL of these solutions were injected into the system. Calibration curves were constructed using ratio of peak areas of drug to IS on Y-axis and concentration of pure drug on X-axis for replicated samples and correlation coefficient was calculated (Table 1). The curve showed enough linearity with 'r' value of 0.998 and regression equation of $Y = 0.8639X (\pm 0.0053) + 0.218(\pm 0.0059)$ measured using simple regression model as showed in (Figure. 2).

Specificity/Selectivity:
Representative chromatogram of blank plasma was confirmed the presence of very little interference from the endogenous component. Chromatograms of spiked plasma samples of nebivolol HCl at 0.4 µg/mL concentration along with IS at a constant concentration (1.0 µg/mL) confirmed that drug and IS peaks were well resolved and completely separated at retention times of 2.60 and 5.48 minutes respectively.

Accuracy and Precision

Intra-day Accuracy and Precision: Intra-day accuracy of the method for drug ranged from 99.010% to 99.481%, while the intra-day precision ranged from 0.219% to 0.372% at concentrations of 0.4 µg/mL (LQC) 1.2 µg/mL (MQC) and 1.8 µg/mL (HQC).

Inter-day Accuracy and Precision: Inter-day accuracy of the method for drug ranged from 98.361%to 99.083%, while precision of the method for the same ranged

Table 1. Concentration Data for NBH in Wistar Rat Plasma

Concentration (µg/mL)	Peak Area (n=3)		Peak Area Ratio (D/IS)
	Drug	IS	
0	0	0	0
0.4	29547.2±230.49	52649.1±210.42	0.566±0.0044
0.8	48120.4±291.41	52168.7±97.50	0.909±0.0070
1.0	56403.8±70.86	52981.6±127.28	1.064±0.0029
1.2	67217.5±249.53	53012.1±109.27	1.281±0.0041

RP-HPLC Method for the Quantification

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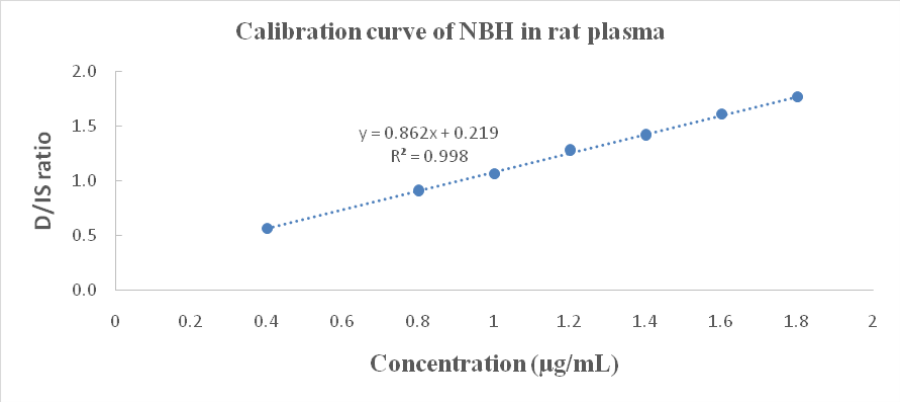

Fig 2. Calibration Curve for the Estimation of Nebivolol HCl in Plasma using HPLC Method

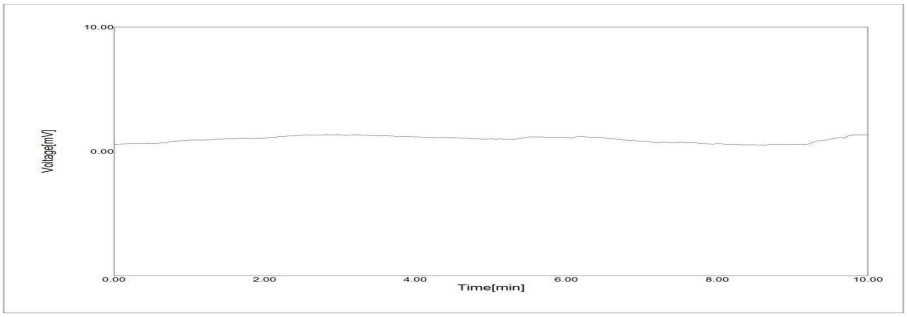

Fig 3. HPLC Chromatogram of Blank Plasma

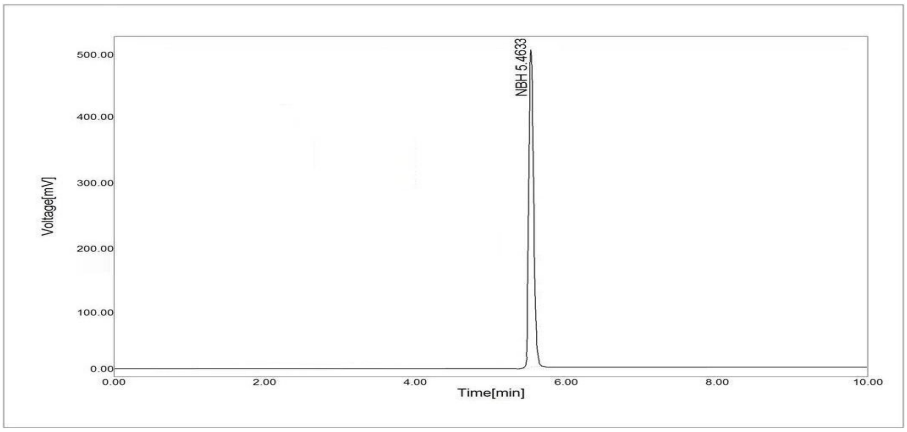

Fig 4. HPLC Chromatogram of Pure Nebivololl HCL

RP-HPLC Method for the Quantification

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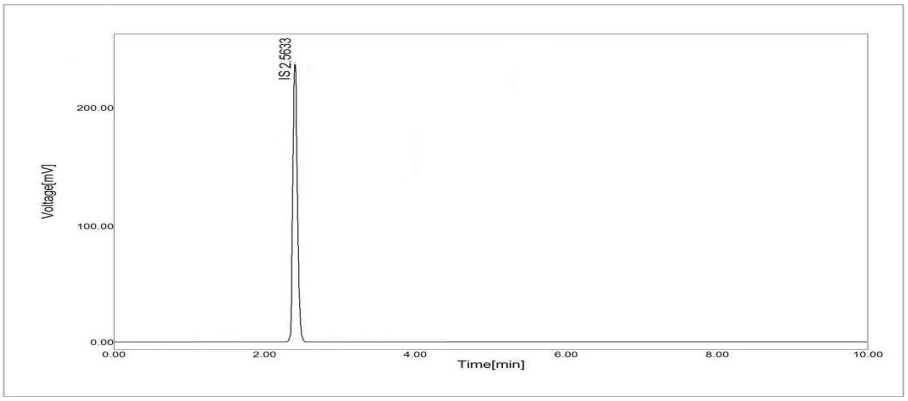

Fig 5. HPLC Chromatogram of Internal Standard (Amlodipine Besylate)

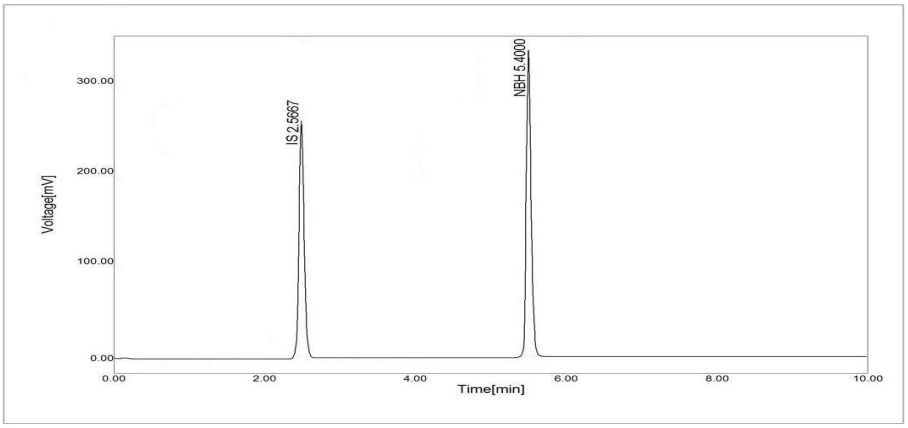

Fig 6. HPLC Chromatogram of Nebivolol Hydrochloride+ Amlodipine Besylate (Drug+IS)

from 0.322% to 0.547% at concentrations 0.4, 1.2 and 1.8 µg/mL.

The results were depicted in (Table 2 and 3) for intra and inter day accuracy and precision. The coefficient of variation% for all the levels of nebivolol HCl in the plasma samples were found to be within acceptable limits indicating a reasonable intermediate precision (intra and inter-day) of the proposed method.

***Recovery*:** The absolute and relative recovery was determined for nebivolol HCl and represented in (Table 4). The values showed to be consistent, precise and reproducible at the three levels 0.4, 1.2 and 1.8 ng/ml. The absolute analytical recovery of internal standard was found to be 95.11%.

By verifying the data for accuracy, precision and linearity it was found that the method developed was justified within the range.

***Sensitivity (LOD & LOQ)*:** The LOD and LOQ were defined as the lowest concentration giving a signal-to-noise (S/N)

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Table 2. Intra-day Precision, Accuracy, and Relative Error for Nebivolol HCl Measured in Spiked Rat Plasma Samples

Intraday (n=6)			
	0.4 µg/mL	1.2 µg/mL	1.8 µg/mL
Mean	0.397	1.188	1.790
S.D.	0.0013	0.0044	0.0039
Precision as %CV	0.346	0.372	0.219
Accuracy %	99.375	99.010	99.481
Relative error %	0.625	0.990	0.519

Table 3. Inter-day precision, accuracy, and relative error for nebivolol HCl measured in spiked rat plasma samples

Inter day (n=6)			
	0.4 µg/mL	1.2 µg/mL	1.8 µg/mL
Mean	0.396	1.187	1.770
S.D.	0.0019	0.0038	0.0096
Precision as %CV	0.496	0.322	0.547
Accuracy %	99.083	98.972	98.361
Relative error %	0.916	1.028	1.63

ratio of at least 3:1 and 10:1, respectively. In the estimation of lower limit samples of pure drug, sensitivity was established using LOD and LOQ that were considered to be 40.24 ng/mL and 121.94 ng/mL for LOD & LOQ respectively.

***Robustness*:** The developed method for estimation of NBH was tested for robustness using small variations in mobile phase composition (65:25:10), (70:20:10) and (75:15:10) and flow rate (0.9, 1.0, 1.1). The %RSD results of these changes were in the range of 1.5-3.0% which were in accordance with the actual values shown in calibration data. The tailing factor for the drug (nebivolol HCl) was always less than 1.5 and the components were well separated under all the changes carried out (mobile phase composition and flow rate). Considering the factors like modification in the system suitability parameters, specificity of the method and conducting the experiment at room temperature it may be indicated that the method was found to be robust. Hence, the newly developed method was considered robust.

Table 4. Percent Recovery of Nebivolol HCl in Spiked Rat Plasma Samples

Concentration (ng/mL)	Extraction Recovery (%)	Relative Recovery (%)
0.4	93.57	100.90
1.2	96.42	100.86
1.8	92.27	99.49

***Ruggedness*:** Ruggedness was studied along with precision and accuracy of batches where the effect of the column, and analyst change was observed. The observed value for analyst variation and results obtained for precision and accuracy were within the acceptance criteria (there were no changes in

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Table 5. Stability Study of Nebivolol HCl Analytes Stored at Different Conditions

Nominal Concentration (µg/mL)	Fresh Sample Concentration Determined (µg/mL)	Sample Concentration after Storage (µg/mL)	%RSD	% Mean Deviation
Short Term (24h)				
0.4	0.396	0.393	1.47	0.63
1.2	1.202	1.194	0.47	0.77
1.8	1.790	1.729	0.70	3.40
Freeze-thaw Cycles (n=3)				
0.4	0.400	0.397	0.94	0.75
1.2	1.194	1.191	0.44	0.22
1.8	1.791	1.733	0.47	3.21
Long Term (45 days)				
0.4	0.394	0.384	1.63	2.51
1.2	1.198	1.189	0.48	0.75
1.8	1.788	1.702	1.0	4.70

the retention time, recovery, and precision of the drug) according to guidelines.

Stability

The results of stability testing done at various conditions showed within the limits described by standards as depicted in (Table 5). Nebivolol HCl was found to be stable at room temperature for 24 hours and after three freeze thaw cycles. All analytes were stable at room temperature showing consistent results. The long-term storage conditions in freezer for 45 days also showed stability with no large deviations in mean of replicates. Thus, the analytes stored at prescribed conditions showed no degradation and remains stable in plasma samples which was seen with excellent recoveries at different storage conditions.

Conclusion

A validated RP-HPLC method was developed for nebivolol HCl in rat plasma according to the ICH guidelines. The method was optimized with different mobile composition and flow rate for sensitive and rapid detection of drug in the plasma samples. The method was validated as per the ICH Q2A (R1) guidelines. It consists of a mobile phase composition of acetonitrile, methanol and 0.1N orthophosphoric acid (70:20:10 %v/v) with Knauer C_{18} column with amlodipine besylate as internal standard. The retention time of NBH was found at 5.48 minutes. The calibration curve for the concentration range of 0.4-1.8 µg/mL was found linear with correlation coefficient of 0.9988. The percentage recoveries for three levels LQC, MQC and HQC were found within the range of 92.27% to 96.42%. All the analytes showed good precision and accuracy with excellent stability at various conditions. Therefore, the developed method was deemed to be accurate, precise, simple, sensitive, and reliable to estimate the drug content in rat plasma. This method can therefore be used in computation of pharmacokinetic parameters of nebivolol HCl in pharmaceutical dosage forms.

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