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Simultaneous Estimation of S-RRR Nebivolol and R-SSS Nebivolol in Human Plasma by Using Liquid Chromatography-tandem Mass Spectrometry

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Abstract

The present study describes a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous estimation of S-RRR and R-SSS nebivolol in human plasma using solid phase extraction technique. Method of both S-RRR and R-SSS nebivolol has been developed, validated by using S-RRR nebivolol D4 and R-SSS nebivolol D4 as an internal standard. Analytes from human plasma were extracted by ion exchange cartridges and subsequently separated on chiral column using acetonitrile: 1.5 mM ammonium carbonate in water, 70:30% v/v as a mobile phase, at a flow rate of 1.0 ml min^{-1}. Quantification of S- RRR and R-SSS nebivolol and nebivolol D4 was performed using multi-reaction monitoring mode (MRM) in positive mode. The calibration curve was linear (r^2 > 0.99) over the concentration range of 20.0-20000.0 pg ml for S-RRR and R-SSS nebivolol. The intra-day and inter-day assay precision revealed within ±15% (at LLOQ level ±20%) with accuracy within 85-115% (at LLOQ level 80-120%). The LC-MS/MS method was fully validated for all the validation parameters as per M10 and current regulatory requirement such as selectivity, matrix effect, recovery and stability. Overall the present study revealed the selectivity and sensitivity of this method for the simultaneous estimation of S-RRR and R-SSS nebivolol in human plasma.

Keywords: Bioanalytical method, Nebivolol, Human plasma, LC-MS/MS.

Introduction

Clinical Pharmacology: Pharmacodynamic Properties: Mechanism of action: The exact mechanism by which Nebivolol 20 mg tablets exert their antihypertensive effect has not been fully established. However, several mechanisms are thought to contribute, including: (1) a reduction in heart rate, (2) decreased myocardial contractility, (3) attenuation of tonic sympathetic outflow to the peripheral circulation via cerebral vasomotor centers, (4) inhibition of renin release, and (5) vasodilation leading to reduced peripheral vascular resistance.

Pharmacokinetics Properties: Absorption: Absorption of Nebivolol 20mg tablets is similar to an oral solution. The absolute bioavailability has not been determined. Mean peak plasma nebivolol concentrations occur approximately 1.5 to 4 hours post-dosing in EMs and PMs. Food does not alter the pharmacokinetics of nebivolol. Under fed conditions, nebivolol glucuronides are slightly reduced. Nebivolol 20mg tablets may be administered without regard to meals.

Distribution: Nebivolol exhibits approximately 98% binding to human plasma proteins in vitro, primarily to albumin. This binding is not influenced by the concentration of nebivolol.

Metabolism: Nebivolol is primarily metabolized through direct glucuronidation of the parent compound. To a lesser extent, it also undergoes N-dealkylation and oxidative metabolism via the cytochrome P450 2D6 (CYP2D6) enzyme. Its stereospecific metabolites contribute to its overall pharmacologic effects.

Elimination: Following a single oral dose of radiolabeled (^14C)-nebivolol, excretion patterns differ based on metabolic status: in extensive metabolizers (EMs), approximately 38% of the dose is recovered in urine and 44% in feces; in poor metabolizers (PMs), around 67% is recovered in urine and 13% in feces. Nearly all of the administered nebivolol is eliminated as multiple oxidative metabolites or their glucuronide conjugates.

Essential hypertension, often associated with endothelial dysfunction, is partly driven by oxidative stress that depletes nitric oxide (NO), impairing vascular function. Beta-blockers have long served as first-line treatment for hypertension. Nebivolol, a third-generation, highly selective β1-adrenoceptor antagonist, is indicated for the treatment of essential hypertension. Beyond beta-blockade, nebivolol promotes endothelium-dependent vasodilation via the L-arginine/NO pathway.

Nebivolol is administered as a racemic mixture containing equal proportions of the “d” and “l” enantiomers. Structurally, it is 1-(6-fluorochroman-2-yl)-{[2-(6-fluorochroman-2-yl)-2-

hydroxyethyl] amino} ethanol, featuring four asymmetric centers. The "d"-isomer corresponds to (S, R, R, R)-nebivolol and the "l"-isomer to (R, S, S, S)-nebivolol, both of which are pharmacologically active. However, the enantiomers differ in their potency regarding β1-receptor antagonism and NO-mediated vasodilatory effects[1-9].

Table-1: Summary of Calibration Standards and Summary of calibration Curve Parameter.

Analyte	Summary of Calibration Standards and Summary of calibration Curve Parameter	
S-RRR nebivolol	% Accuracy	96.47 to 104.62%
	% CV	0.31 to 2.86%
	r	r ≥ 0.9993
R-SSS nebivolol	% Accuracy	97.50 to 102.65%
	% CV	0.88 to 2.88%
	r	r ≥ 0.9996

Materials and Methods

Chemicals and Reagents: S-RRR and R-SSS nebivolol were purchased from Shubham Biopharma, Navi Mumbai, India. S-RRR nebivolol D4 and R-SSS nebivolol D4 was purchased from daicel chiral technologies, Hyderabad, India. Human plasma was purchased from kiln health bio research, Pune, India. Methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from Thermo Fisher Scientific. Formic acid purchased from Biosolve chimie, ammonium carbonate purchased from Loba chimie. Phenomenex strata X33µm polymeric reverse, 30 mg cartridges were purchased from Strata. HPLC grade water was obtained using a Milli-Q EXL water purification system, Mumbai, India[10-12].

Instrumentation: For chromatographic separation, a Exion LC equipped with a triple quard mass spectrometer LCMSMS-API 6500 (Sciex, USA) was used. Data acquisition and processing were performed using analyst software (version 1.7.1; Sciex, USA.

Chromatographic Conditions: Chromatographic separation was achieved on CHIRALPACK AY-RH 5 µm, 150 mm × 4.6 mm column placed at 35°C in thermostat column oven by passing mobile phase consisting of acetonitrile: 1.5 mM ammonium carbonate in water, 70:30% v/v at a flow rate of 1.0 ml min^{-1}. Analytical run time was 8.0 min[13].

Mass Spectrometric Conditions: The determination of RSSS Nebivolol, SRRR Nebivolol, RSSS Nebivolol D4 and SRRR Nebivolol D4 was performed with tandem mass spectrometer operated in the positive ion electrospray ionisation (ESI+) and multiple reaction monitoring (MRM) mode. MRM of the ions for RSSS Nebivolol, SRRR Nebivolol, RSSS Nebivolol D4 and SRRR Nebivolol D4 were m/z 406.200 > 151.00, 410.200 > 151.100, 406.200 > 151.100, 410.200 > 151.100 respectively. The source dependent parameters were Gas 1 (Nebulizer Gas): 40 psig; Gas 2 (Heater Gas): 70 psig; ion spray voltage (ISV): 5500 V, turbo heater temperature (TEM): 550°C; interface heater (Ihe): ON; collision activation dissociation (CAD): 7 psig and curtain gas (CUR): nitrogen: 35 psig. The dwell time of 300 ms per transition and collision energy (CE) of 40 were used.

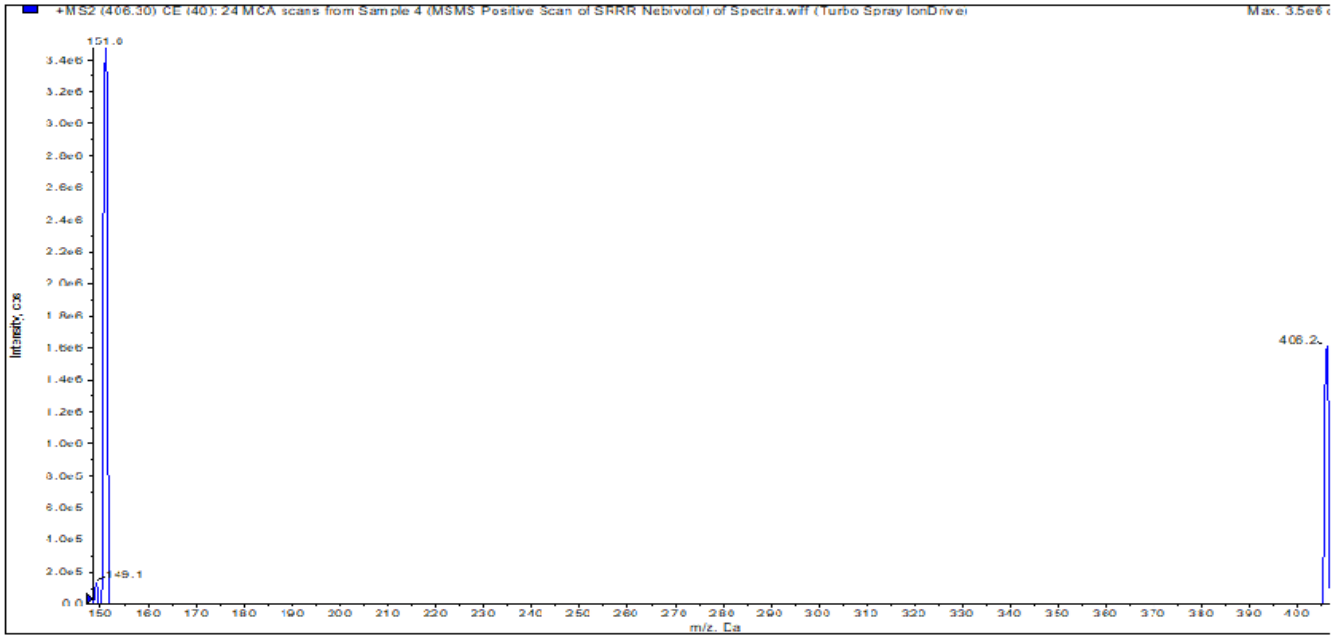

Figure-1: Spectrum of product ion scans of RSSS Nebivolol.

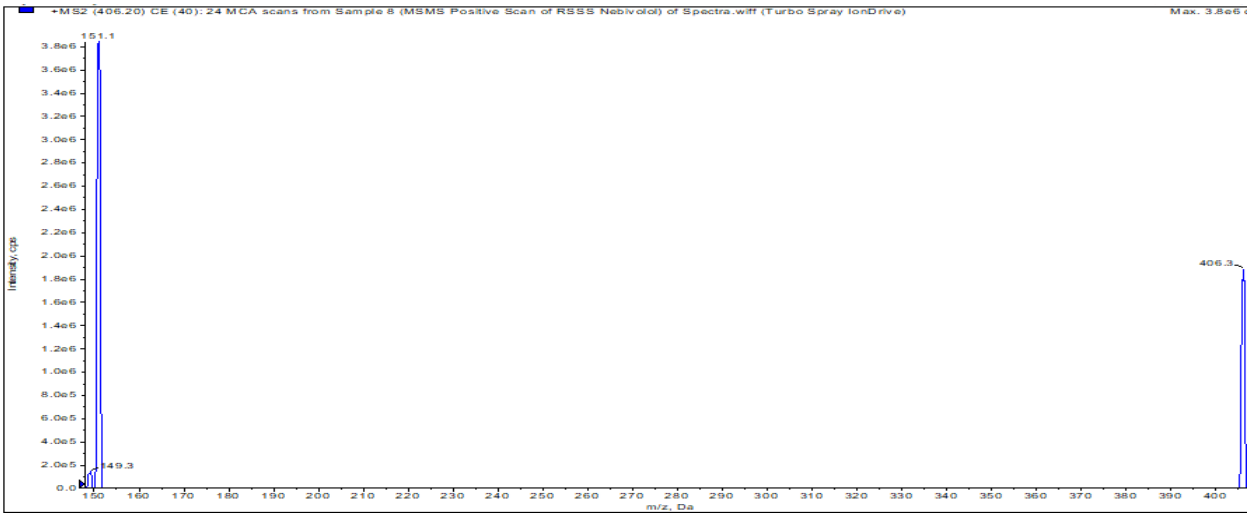

Figure-2: Spectrum of product ion scans of SRRR Nebivolol.

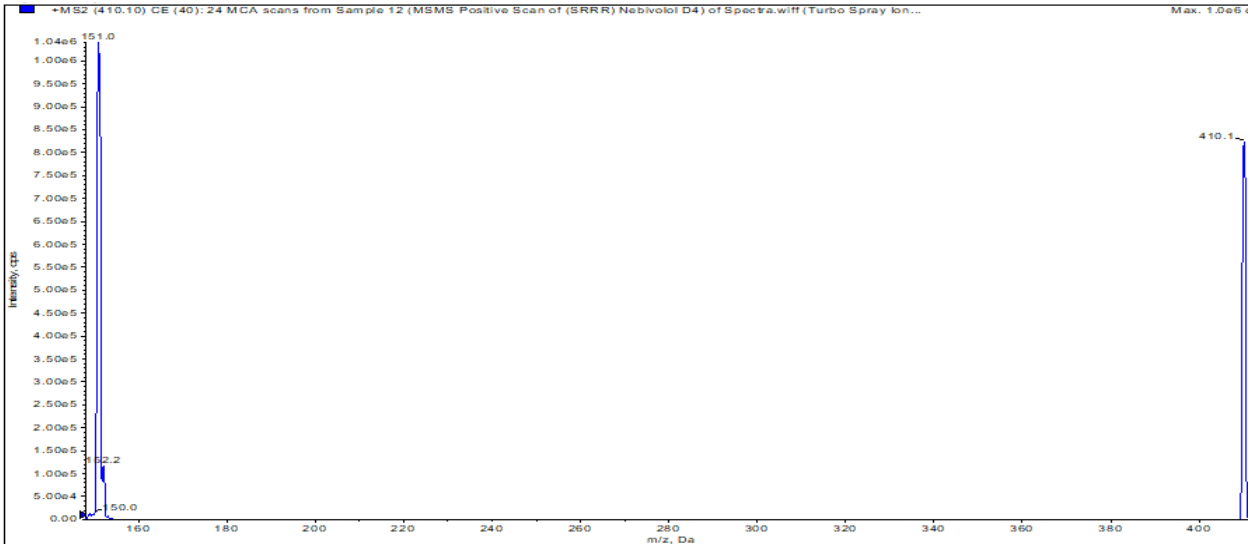

Figure-3: Spectrum of product ion scans of RSSS Nebivolol D4.

Figure-4: Spectrum of product ion scans of SRRR Nebivolol D4.

Preparation of Standard and Quality Control (QC) Samples: The standard stock solutions of S-RRR nebivolol, R-SSS nebivolol, S-RRR-nebivolol D4 and R-SSS-nebivolol D4 were prepared at 200 µg ml^{-1} of each sample in methanol (DMSO?). The working solution of mixed analyte (S-RRR and R-SSS nebivolol) at the concentrations of 0.400, 0.800, 40.000, 80.000, 160.000, 240.000, 340.000 and 400.000 ng ml^{-1} of each sample were prepared by Parallel dilution from 200.00 µg ml^{-1} mixed solution with methanol: water (50:50). A working solution of IS was prepared by diluting the standard stock solution of R-SSS nebivolol D4 and S-RRR nebivolol in methanol: water (50:50) to achieve a final concentration of 10000.000 pg ml^{-1}. The mixed analyte working solutions (50 µl) were used to spike blank human plasma sample (950 µl) to prepare the calibration curve standards of both the analytes at concentration of 20.000, 40.000, 2000.000, 4000.000, 8000.000, 12000.000, 17000.000 and 20000.000 pg ml^{-1} in validation study. The plasma concentrations of QC samples were prepared at 20.000, 56.000, 1600.000, 8000.000 and 16000.000 pg ml^{-1} for S-RRR and R-SSS nebivolol. All working solutions were stored at 2-8°C until analysis.

Plasma Sample Preparation: 300 µl Plasma samples were taken in 5 ml RIA vial, 50 µl of IS solution was added and vortexed. 300 µl of formic acid in water, 0.2% v/v, was added to it and vortexed. The resultant sample was loaded on Strata X cartridges 1 cc (30 mg) preconditioned with 1 ml of methanol and 1 ml of water. The loaded cartridges were washed with 1.0 ml of water, and 1.0 ml of Methanol in water, 10% v/v, followed by 1 ml of Methanol. Washed cartridges were dried with nitrogen gas by positive pressure at maximum flow rate for 5 min on Athena 144 positive pressure processor. Contents were eluted from the cartridges with 1.0 ml methanol. Eluate was evaporated to dryness in an evaporator at 40 °C under the gentle stream of nitrogen. The dried samples were reconstituted by addition of 150 µl of mobile phase, loaded into autosampler, and 10 µl of reconstituted samples were injected into LC-MS/MS system.

Quantification: Quantitative analysis of S-RRR and R-SSS nebivolol was performed using S-RRR nebivolol D4 and R-SSS nebivolol D4 as an internal standard. Calibration curves were established with standards prepared in plasma. Eight-point standards calibration curve was constructed using peak area ratio of analyte area to IS area. Concentration of analytes in QCs and unknown samples were calculated by interpolation from the calibration curves.

Method Validation: Method validation protocol was based on the recommendations of the United States Food and Drugs Administration (USFDA) guidelines and European Medicines Agency (EMEA) guidelines[14-17].

Selectivity: The selectivity of method was assessed by comparing the interfering signals in nine different lots of plasma (six lots of plasma with K_2EDTA anticoagulant, one lot each of lipidemic K_3EDTA plasma, haemolysed K_3EDTA plasma and heparinized plasma) with the signals of analytes and IS in LLOQ sample (06 Normal, 01 Hemolyzed and 01 Lipemic plasma lot) with 01 set of calibration curve and 02 set of quality control sample.

Linearity, accuracy and precision: The linearity of the method was assessed by an eight-point calibration curve over the concentration range of 20.0 to 6000 pg ml^{-1} for S- RRR and R-SSS nebivolol in three consecutive runs. Calibration curves were constructed by fitting the analyte concentrations of the calibrators versus the peak area ratios of the analyte area to IS area. Each calibration curve was analyzed individually by least square weighted ($1/x^2$) linear regression. The inter- or intra-batch accuracy and precision were evaluated using six replicates of QC samples at LLOQ, lower (LQC), middle-2 (MQC-2), middle-1 (MQC-1) and higher (HQC) concentration levels for three separate runs. The criteria for acceptability of the data included precision within 15% coefficient of variance (%CV) and an accuracy within ±15% relative error (%RE) of the nominal values. Limit of quantification was established by six replicates at 20 pg ml-1 in each three separate runs.

Recovery: Recovery of the analytes at low, middle and high concentration levels and IS through solid phase extraction was assessed by comparing mean peak-area of the extracted samples with mean peak-area of post-spiked extracted samples, which represent ~90-100% recovery.

Matrix effect: Matrix effect was evaluated by acquiring 3 LQC and 3 HQC from eight plasma lot (06 Normal, 01 Hemolyzed and 01 Lipemic plasma lot) with 01 set of calibration curve and 02 set of quality control sample.

Stability: The present study evaluated the stability studies of S-RRR and R-SSS nebivolol in plasma samples. The bench top (17 hrs 57 Min at ambient temperature), freeze-thaw (at -70 °C), processed samples stability (03 hrs and 47 min at room temperature and 94 hrs and 38 min at 2-8°C) and dry extract stability (04 hrs and 17 min at room temperature) of each analyte was evaluated at LQC and HQC concentration level using six replicates at each concentration. Analyte was considered stable if the percentage of changes is less than or equal to 15%, according to the USFDA and EMEA guidelines. Freeze-thaw cycle included thawing of samples at room temperature and then refreezing at -70°C. Concentrations of stability samples were calculated and stability was shown as the percentage of mean changes in the calculated concentration.

Results and Discussion

Optimization of the Mass Spectrometric Condition: Mass spectrometric parameters were optimized in both positive and negative ionization modes for all analytes. The enantiomers S-RRR- and R-SSS-nebivolol, along with the internal standard (IS), exhibited prominent peaks in the positive ionization mode.

To enhance sensitivity and reduce ion suppression, key parameters such as ionization voltage, interface temperature, curtain gas, GS1, GS2, and collision-activated dissociation (CAD) gas flow were carefully optimized. The study identified the optimal values as follows: ionization voltage > 5500 V, interface temperature at 550°C, curtain gas at 35.0, GS1 at 40.0, GS2 at 70.0, and CAD gas flow at 7.0. These settings significantly improved analyte signal intensity.

A dwell time of 300 ms for both S-RRR- and R-SSS-nebivolol, as well as the IS, was sufficient to ensure accurate quantification. No cross-talk was observed between transitions during multiple reactions monitoring (MRM), confirming the method's specificity.

Optimization of the Sample Preparation and Chromatographic Conditions: Sample preparation is a critical step in analytical method development, aiming to be rapid, simple, reagent-efficient, and capable of yielding high analyte recovery. A review of the literature highlighted the use of liquid-liquid extraction (LLE) for isolating S-RRR- and R-SSS-nebivolol. However, in the present study, solid-phase extraction (SPE) was employed as a superior alternative, providing cleaner samples, minimal matrix effects, and improved analyte recovery compared to LLE.

While some previously reported methods required large plasma volumes (up to 2 mL) for extraction and higher injection volume, the proposed method was optimized using only 0.3 mL of plasma and a low injection volume, enhancing efficiency and acceptability in routine analysis.

To develop a rugged SPE procedure, multiple cartridge types were evaluated by varying buffer composition, wash solutions, and elution conditions. The Phenomenex Strata-X (1 cc, 30 mg) cartridge delivered optimal results, with the highest recovery, minimal interference, and negligible matrix effects.

Given that nebivolol contains an iminodiethanol group (a cationic moiety), cation exchange cartridges were also explored. Among various options from Waters, Phenomenex, and Agilent, Waters Oasis MCX cartridges provided the best performance.

Deuterated internal standards R-SSS-nebivolol-D4 and S-RRR-nebivolol-D4 were selected due to their similar physicochemical properties to the analytes, ensuring consistent area ratios by compensating for extraction losses and instrument variability. Use of isotopically labeled internal standards is currently recommended by regulatory agencies.

Chromatographic conditions were further optimized to achieve excellent sensitivity, sharp peak shapes, and effective separation of both enantiomers and IS within a short run time. Several chiral columns, including CHIRALPAK AY-RH and Lux Amylose-2, were evaluated using various mobile phases (e.g., methanol, acetonitrile, isopropyl alcohol, ammonium acetate, and ammonium carbonate). The CHIRALPAK AY-RH (5 µm, 150 × 4.6 mm) column was ultimately selected for its superior resolution and sensitivity.

The optimal mobile phase consisted of acetonitrile and 1.5 mM ammonium carbonate in water (70:30, v/v). A low injection volume of 10 µL was employed to prevent column overloading and extend column life, enabling a higher number of analyses per column.

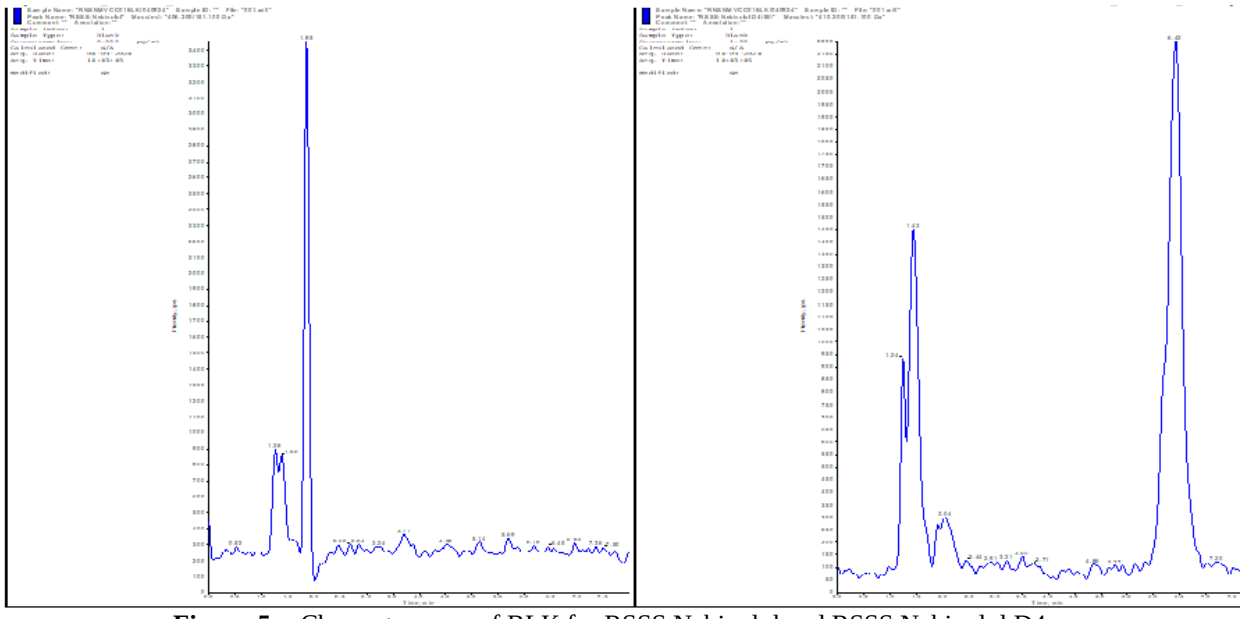

Figure-5a: Chromatograms of BLK for RSSS Nebivolol and RSSS Nebivolol D4.

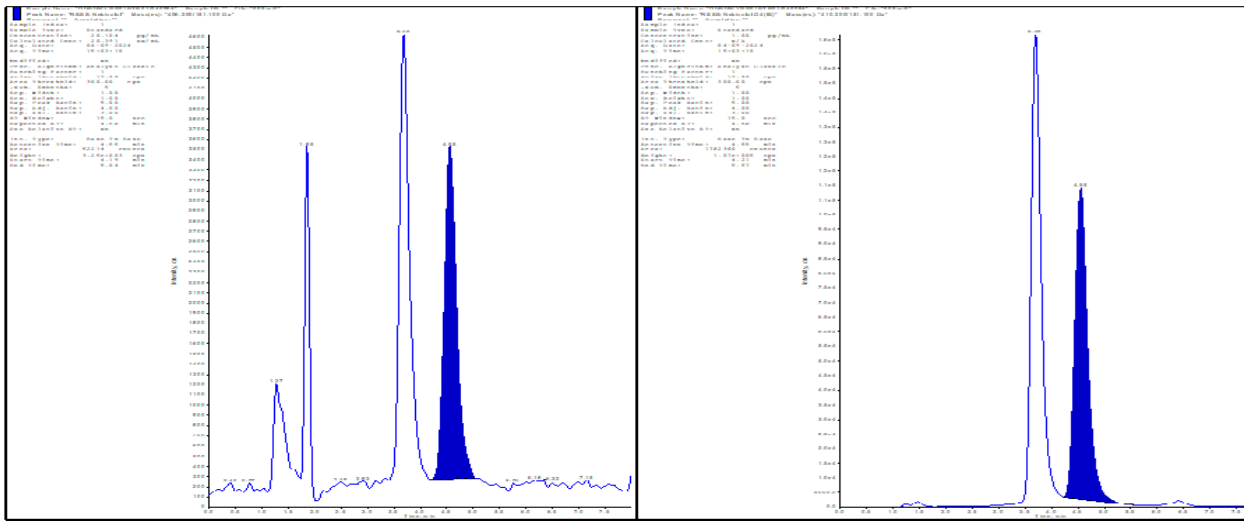

Figure-5b: Chromatograms of STD1 for RSSS Nebivolol and RSSS Nebivolol D4.

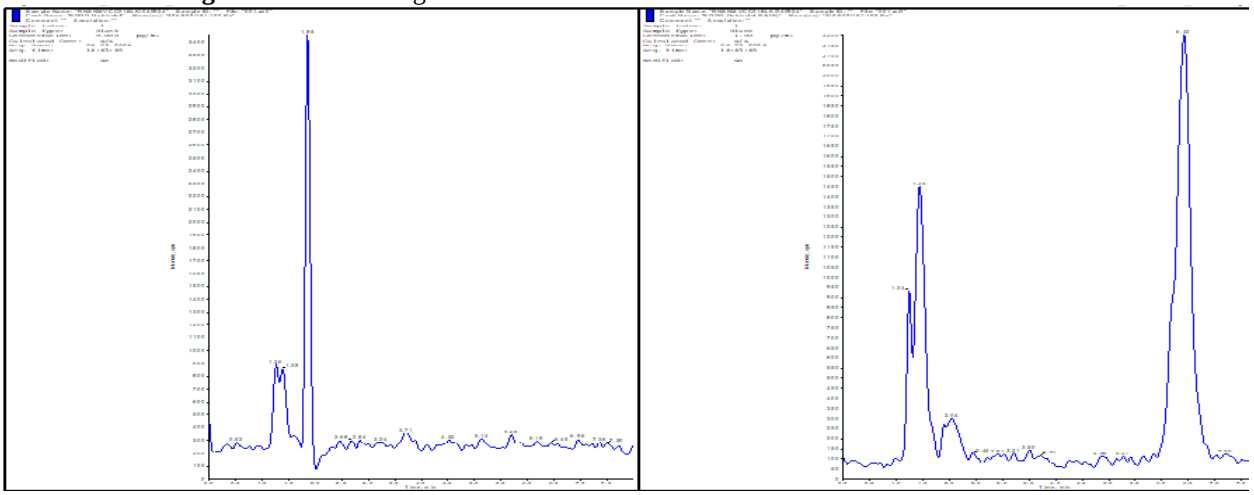

Figure-5c: Chromatograms of BLK for SRRR Nebivolol and SRRR Nebivolol D4

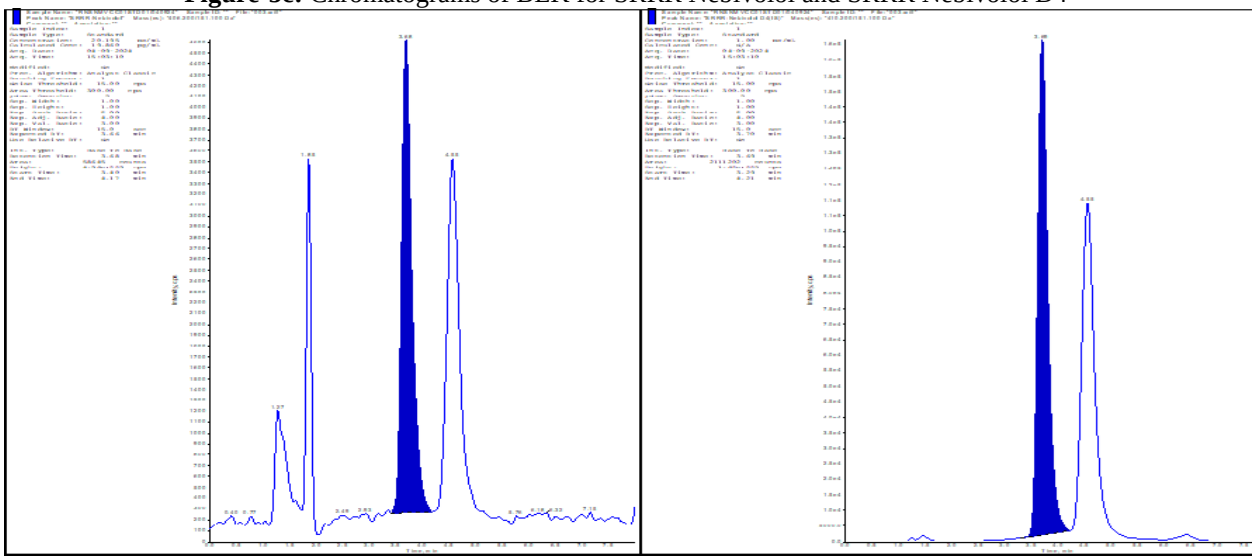

Figure-5d: Chromatograms of STD1 for SRRR Nebivolol and SRRR Nebivolol D4.

Figure-5: Chromatograms of BLK, STD1 for RSSS Nebivolol, SRRR Nebivolol, RSSS Nebivolol D4 and SRRR Nebivolol D4 are presented.

Selectivity: Figure-5a and 5c depicts that there were no interfering peaks observed from endogenous compounds at retention time in any of the samples of S-RRR, R-SSS nebivolol and S-RRR, R-SSS nebivolol D4 extracted from plasma. The responses of interfering peaks at the retention time of analytes in blank plasma were less than 1.03% and 1.55% for S-RRR nebivolol and R- SSS nebivolol, respectively, at LLOQ of 20.0 pg ml^{-1}. Typical retention time of S-RRR nebivolol and R-SSS nebivolol was 3.68 and 4.57 min, respectively.

Linearity, Accuracy and Precision: Usually, the least square method can create relatively large errors at the levels with low concentrations, as in case with the proposed developed method. The proposed method utilized relatively lower LLOQ of 20.0 pg ml-1 to very high ULOQ of 20000 pg ml-1 for S-RRR and R-SSS nebivolol (Dynamic Linearity range of 1000 times ratio of ULOQ to LLOQ concentration). To overcome this error, the concept of weighted calibration curves was applied and calculation was constructed by applying weighting factor [1/x2] (where X stands for concentration). The results indicated that the weighted data for calibration curve was more accurate in the quantification and the application of weighting factor was the best choice for the proposed method. A regression equation of the calibration curve (y = mX + c, where y = peak area ratio, m = slope, c = y- axis intercept of the calibration curve).

The proposed method can detect lower concentration up to 5% of C_{max} of both analytes and upper concentration was more than two times of the C_{max} of analytes. Good linearity was obtained with aforementioned concentration ranges. To validate the accuracy and precision of the developed method, five level concentrations of QCs in six replicates were analyzed in a single batch. The results showed that the intra- and inter-day accuracy ranged from 96.47-104.62% for S-RRR and 97.50-102.65% for R-SSS nebivolol. In context to this, the present LC-MS/MS method for simultaneous assessment of S-RRR and R-SSS nebivolol was found to meet the predefined criteria of accuracy and precision experiments.

Matrix Effect: Two QC concentrations of each tested analyte and internal standard (IS) were evaluated using six different sources of human plasma. The results indicated that no significant ion suppression or enhancement was observed for any of the analytes or IS under the experimental conditions employed.

Recovery: The recovery was determined by comparing the mean peak area of the extracted samples versus mean peak area of un-extracted samples at three different concentrations. The recovery of S-RRR nebivolol was 91.46%, 84.84% and 92.10% at low, middle and high concentration levels, respectively. The recovery of R-SSS nebivolol was 86.31%, 82.30%, and 84.62% at low, middle and high concentration levels, respectively. The recovery of S-RRR nebivolol D4 was 95.83%, 93.74% and 100.01% at low, middle and high concentration levels, respectively. The recovery of R-SSS nebivolol D4 was 93.39%, 91.68%, and 93.89% at low, middle and high concentration levels, respectively.

Table-2: Recovery SRRR Nebivolol and For SRRR Nebivolol D4.

Analyte			
QC level Parameter	LQC	MQC	HQC
% Recovery	91.46	84.84	92.10
Mean % Recovery	89.47		
Mean % CV	4.49		
Internal Standard			
QC level Parameter	LQC	MQC	HQC
% Recovery	95.83	93.74	100.01
Mean % Recovery	96.53		
Mean % CV	3.31		

Table-3: Recovery RSSS Nebivolol and RSSS Nebivolol D4

Analyte			
QC level Parameter	LQC	MQC	HQC
% Recovery	86.31	82.30	84.62
Mean % Recovery	84.41		
Mean % CV	2.38		
Internal Standard			
QC level Parameter	LQC	MQC	HQC
% Recovery	93.39	91.68	93.89
Mean % Recovery	92.99		
Mean % CV	1.25		

Stability: The stability studies of S-RRR and R-SSS nebivolol in plasma has been evaluated. The bench top (17 hrs and 57 min at ambient temperature), freeze-thaw (at -70°C), processed samples stability (03 hrs and 47 min at room temperature), dry extract stability (04 hrs and 17 min at room temperature) of each analyte were evaluated at LQC and HQC concentration level using six replicates of each. Analyte was considered stable if the percentage of changes is less than 15% per regulatory guidance

of USFDA and EMEA for bioanalytical method validation. The bench top stability, processed samples stability and freeze-thaw stability for S-RRR and R-SSS Nebivolol were assessed at different conditions of temperature and time. Frozen samples were allowed to thaw at room temperature and then refrozen at -70°C, such five cycles were completed for freeze-thaw stability. In all the stability experiments, stability samples were quantified versus freshly prepared calibration standards per regulatory requirement.

Table-4: Stability details of RSSS Nebivolol and RSSS Nebivolol D4.

Experiments for RSSS Nebivolol	Results Summary		
Freeze and Thaw Stability at -70°C ±10°C after 5th Cycles	QC level Parameter	LQC	HQC
	% CV	1.90	0.82
	% Accuracy	96.22	101.49
Auto sampler Stability at 10°C after 94 Hours 38 Minutes	QC level Parameter	LQC	HQC
	% CV	4.12	1.83
	% Accuracy	100.65	100.49
Bench Top Stability at RT after 17 Hours 57 Minutes	QC level Parameter	LQC	HQC
	% CV	6.39	0.87
	% Accuracy	96.76	100.83
Wet Extract Stability at RT after 03 Hours 47 Minutes	QC level Parameter	LQC	HQC
	% CV	1.47	1.19
	% Accuracy	97.56	101.75
Dry Extract Stability at RT after 04 Hours 17 Minutes	QC level Parameter	LQC	HQC
	% CV	3.30	1.40
	% Accuracy	98.90	102.44
Whole Blood Stability (02 Hours 02 Minutes at RT)	QC level Parameter	LQC	HQC
	% Difference	1.92	2.77

Table-5: Stability details of SRRR Nebivolol and SRRR Nebivolol D4.

Experiments for SRRR Nebivolol	Results Summary		
Freeze and Thaw Stability at -70°C ±10°C after 5th Cycles	QC level Parameter	LQC	HQC
	% CV	2.71	2.34
	% Accuracy	101.01	98.75
Autosampler Stability at 10°C after 94 Hours 38 Minutes	QC level Parameter	LQC	HQC
	% CV	4.67	2.18
	% Accuracy	104.06	99.42
Bench Top Stability at RT after 17 Hours 57 Minutes	QC level Parameter	LQC	HQC
	% CV	2.03	1.08
	% Accuracy	101.15	99.05
Wet Extract Stability at RT after 03 Hours 47 Minutes	QC level Parameter	LQC	HQC
	% CV	1.36	2.48
	% Accuracy	100.84	101.75
Dry Extract Stability at RT after 04 Hours 17 Minutes	QC level Parameter	LQC	HQC
	% CV	2.79	1.68
	% Accuracy	101.56	101.72
Whole Blood Stability (02 Hours 02 Minutes at RT)	QC level Parameter	LQC	HQC
	% Difference	0.00	0.80

Study Sample Analysis: This method has been successfully applied for the estimation of S-RRR nebivolol and R-SSS nebivolol from plasma samples of bioequivalence study of nebivolol 20mg tablets in healthy human volunteers

Pharmacokinetics (PK) and Statistical Analysis: SAS (SAS Institute Inc. U.S.A.) was used to calculate the pharmacokinetic (PK) parameters from human plasma samples. Visual inspection of the plasma of S-RRR and R-SSS nebivolol concentration-time profiles was done in order to determine the Cmax and Tmax values. For S-RRR and R-SSS nebivolol, the primary pharmacokinetic parameters were Cmax, AUC0-t and AUC0-∞. Using the linear trapezoidal method, the AUC0–t was determined. The AUC0-∞ was determined up to the final concentration that could be measured. Based on this concentration and the terminal elimination rate constant (Kel),

which was estimated using the linear regression method on the slope of the plasma concentration-time curve of S-RRR and R-SSS nebivolol, extrapolations were obtained. Next, 0.693/Kel was determined to be the terminal elimination half-life, or t1/2.

Statistical analysis of the pharmacokinetic parameters was performed using SAS (SAS Institute Inc., U.S.A.). For the pharmacokinetic parameters, descriptive statistics were calculated and presented. The natural log-transformed Primary Pharmacokinetic parameters Cmax, AUC0-t and AUC0-∞ for S-RRR and R-SSS nebivolol will be subjected to an ANOVA (Analysis of Variance) in accordance with the two-sided test procedure for bioequivalence.

Results and Discussion

Data collected from 64 participants and 62 (2 subjects were dropouts) who finished the study were subjected to pharmacokinetics (PK) and statistical analysis using SAS. Graphical representation of Mean Plasma Concentrations versus Time in gives following figures.

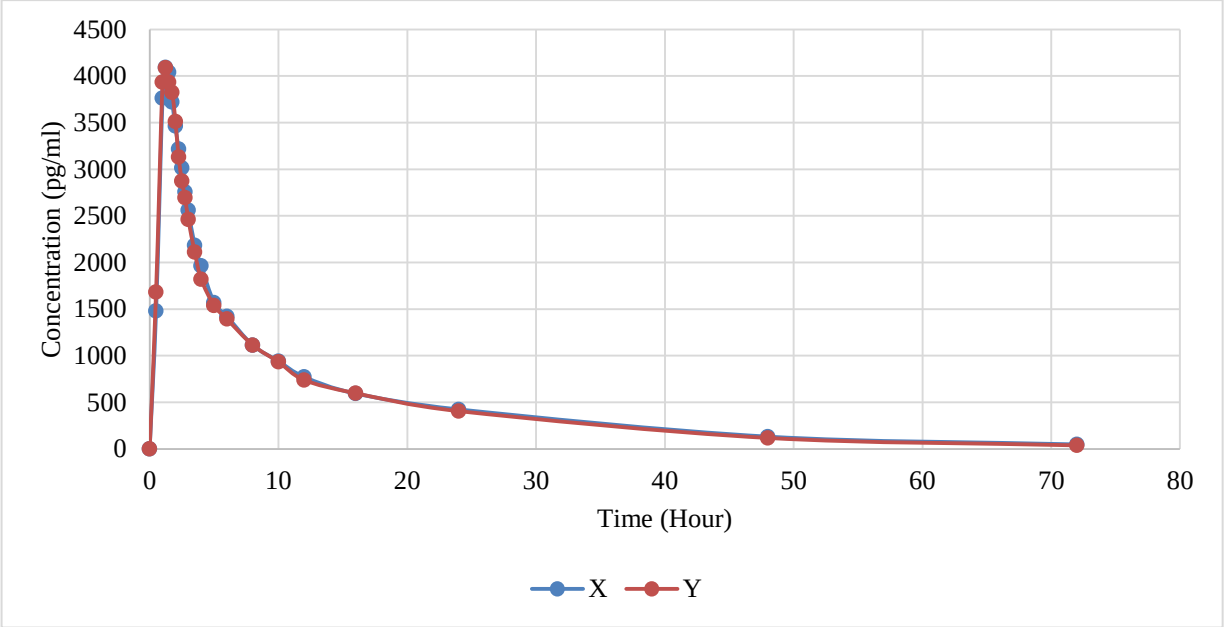

Figure-6: Linear Plot of Mean Plasma Concentrations (pg/ml) of S-RRR nebivolol vs. Time (hr.).

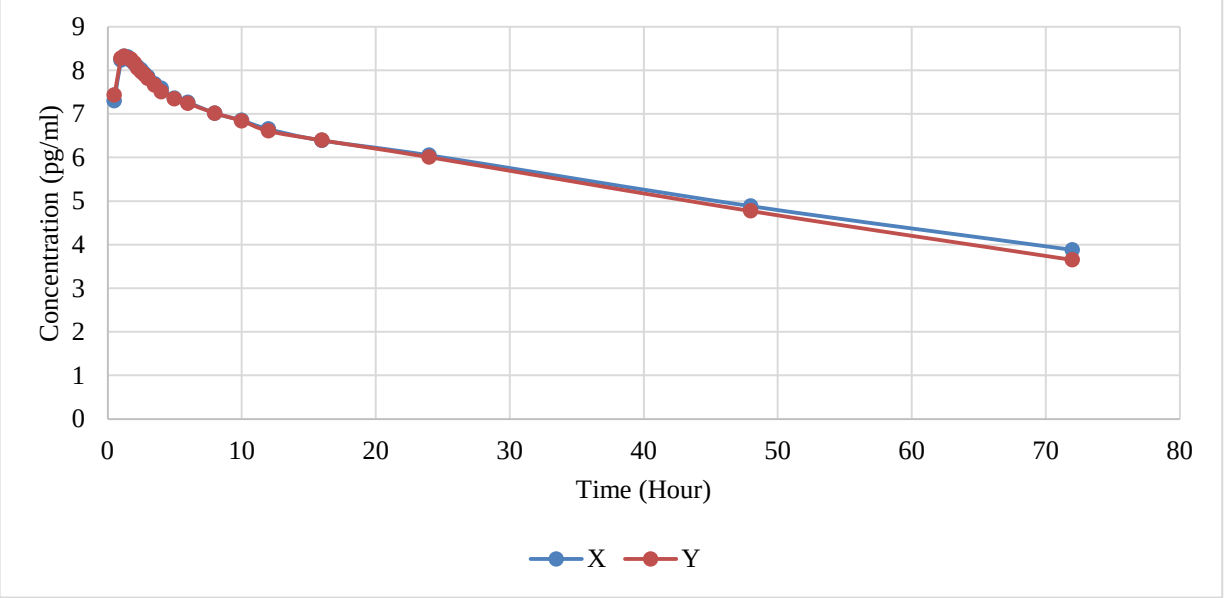

Figure-7: Semi Log-Linear Plot of Mean Plasma Concentrations (pg/ml) of S-RRR nebivolol vs. Time (hr.).

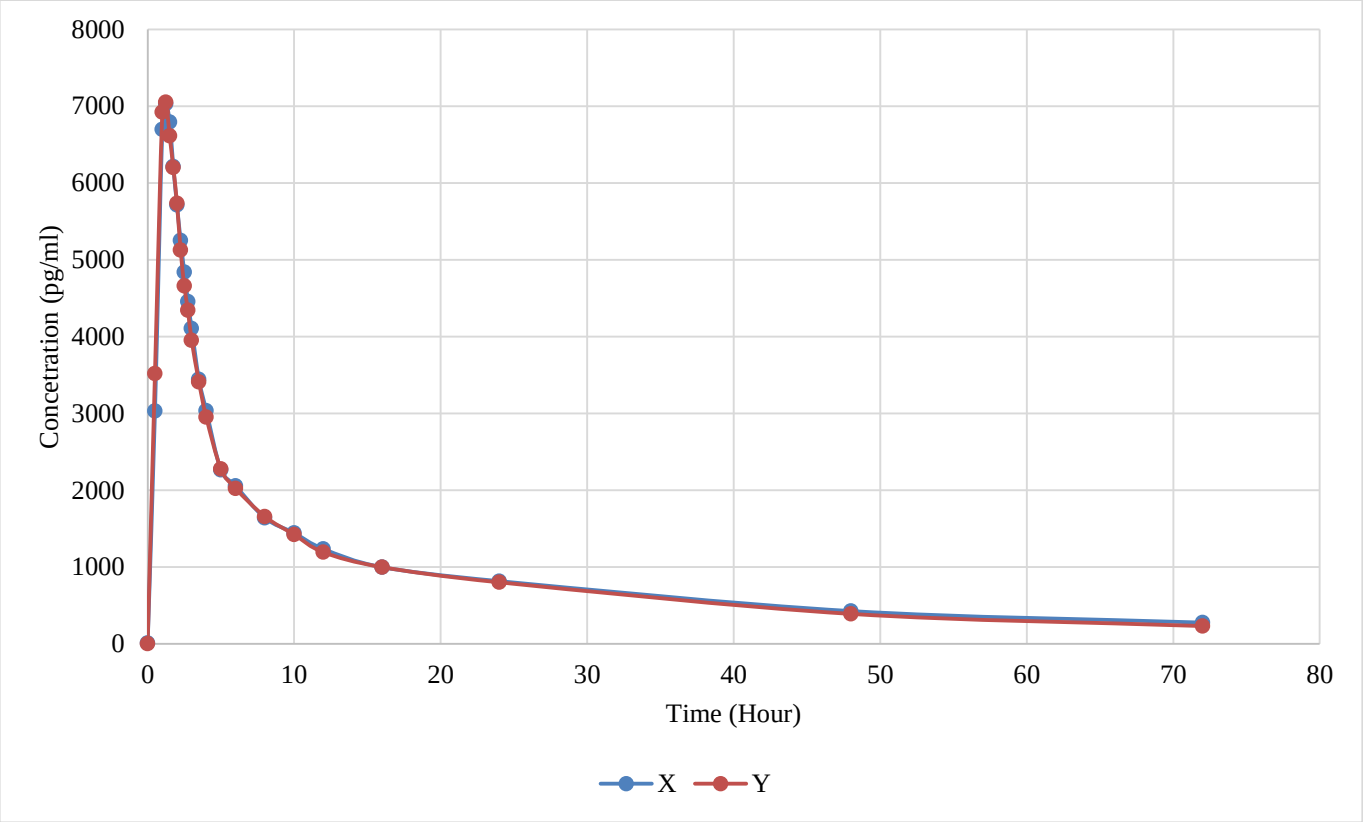

Figure-8: Linear Plot of Mean Plasma Concentrations (pg/ml) of R-SSS nebivolol vs. Time (hr.).

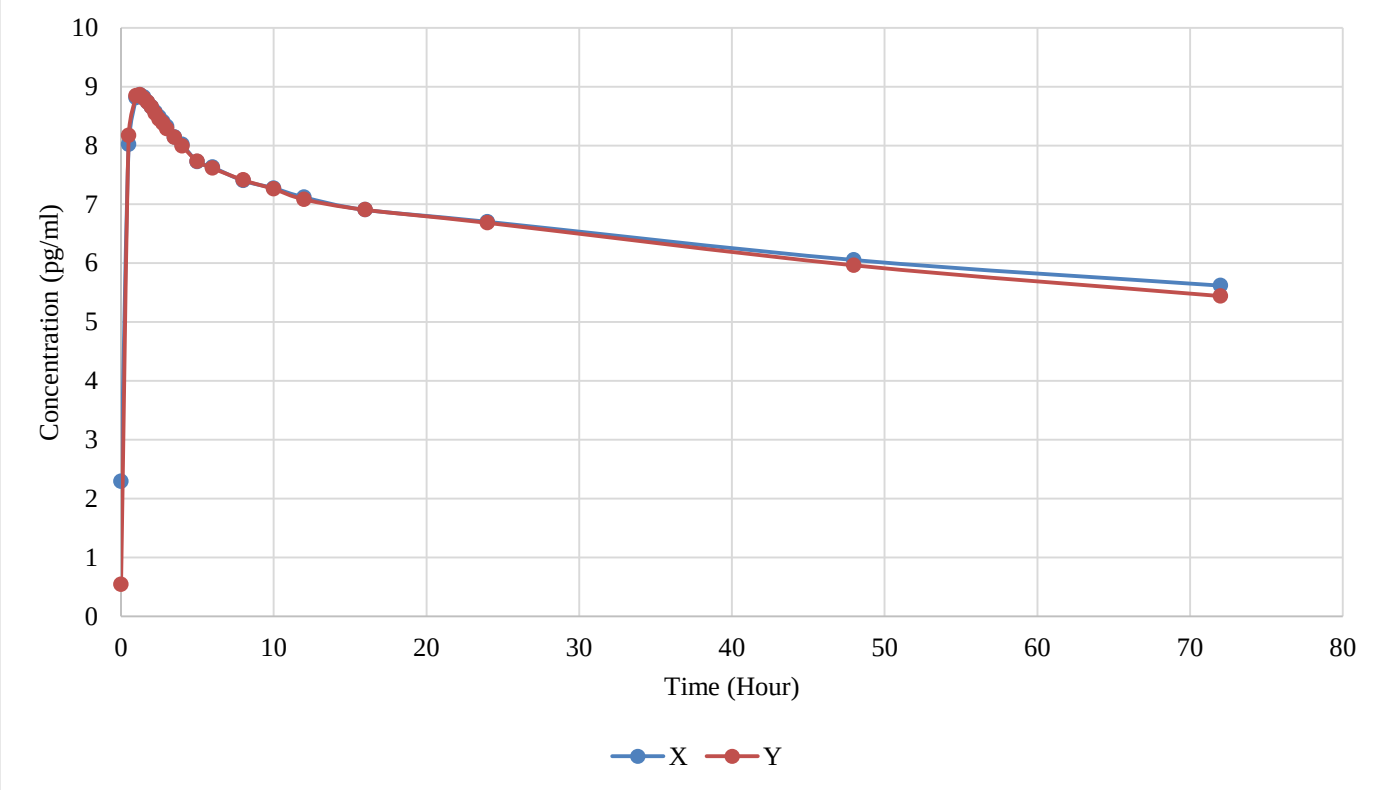

Figure-9: Semi Log-Linear Plot of Mean Plasma Concentrations (pg/ml) of R-SSS nebivolol vs. Time (hr.).

Table-6: Descriptive Statistics of Pharmacokinetic (PK) Parameters of Test Product (T) and Reference Product (R) for S-RRR nebivolol.

Form	Variable	Mean	SD	Minimum	Median	Maximum	CV%
R	C_{max} (pg/mL)	4902.6193	2248.9249	1660.7300	4472.0270	12297.4620	45.8719
	AUC_{0-t} (pg.hr/mL)	35394.7239	53233.5333	8786.0530	18292.2430	306948.819	150.3996
	$AUC_{0-\infty}$ (pg.hr/mL	37629.9308	59720.5150	9315.4630	18631.1965	368257.236	158.7048
	T_{max} (hr)	1.4806	0.7775	0.5000	1.2500	6.0000	52.5114
	K_{el} (hr^{-1})	0.0689	0.0224	0.0250	0.0650	0.1770	32.5671
	$t_{1/2}$ (hr)	10.9187	3.2860	3.9200	10.7350	27.7600	30.0954
	$AUC_{\_\% \text{ Extra pobs}}$	5.1539	3.9474	0.7800	3.6800	16.6500	76.5908
	$AUC_{\% \text{ Ratio}}$	94.8461	3.9474	83.3500	96.3200	99.2200	4.1619
T	C_{max} (pg/mL)	4793.9154	2303.2054	1921.2450	4253.8650	13801.2960	48.0443
	AUC_{0-t} (pg.hr/mL)	34425.8872	51949.5288	8487.2190	18143.6760	269242.332	150.9025
	$AUC_{0-\infty}$ (pg.hr/mL	36181.0576	55663.7803	9266.3740	18879.9415	306451.509	153.8479
	T_{max} (hr)	1.3924	0.7431	0.5000	1.2500	6.0000	53.3660
	K_{el} (hr^{-1})	0.0655	0.0156	0.0280	0.0630	0.1170	23.8156
	$t_{1/2}$ (hr)	11.1994	2.9179	5.9100	10.9700	24.5500	26.0543
	$AUC_{\_\% \text{ Extra pobs}}$	5.1500	3.7701	1.1700	3.4600	15.9800	73.2056
	$AUC_{\_\% \text{ Ratio}}$	94.8500	3.7701	84.0200	96.5400	98.8300	3.9748

Table-7: Descriptive Statistics of Pharmacokinetic (PK) Parameters of Test Product (T) and Reference Product (R) for R-SSS nebivolol.

Form	Variable	Mean	SD	Minimum	Median	Maximum	CV%
R	C_{max} (pg/mL)	8358.8507	3085.5349	2858.5960	7922.6335	17582.8680	36.9134
	AUC_{0-t} (pg.hr/mL)	67342.6977	128404.148	19708.4450	35409.3785	851043.702	190.6727
	$AUC_{0-\infty}$ (pg.hr/mL	105323.741	374203.302	20266.3250	37228.5600	2880342.46	355.2887
	T_{max} (hr)	1.3955	0.7541	0.5000	1.2500	6.0000	54.0382
	K_{el} (hr^{-1})	0.0494	0.0103	0.0050	0.0500	0.0690	20.9155
	$t_{1/2}$ (hr)	16.5300	17.0329	10.0600	13.9100	144.9200	103.0426
	$AUC_{\_\% \text{ Extra pobs}}$	4.1385	9.4717	0.9100	2.1050	70.4500	228.8657

	$AUC_{\_\% Ratio}$	95.8615	9.4717	29.5500	97.8950	99.0900	9.8806
T	C_{max} (pg/mL)	8175.5354	3015.8915	3773.7750	7466.7475	19536.5880	36.8892
	AUC_{0-t} (pg.hr/mL)	65512.9853	119380.131	20492.0430	35666.6540	742461.701	182.2236
	$AUC_{0-\infty}$ (pg.hr/mL	83023.4634	218521.087	21447.5030	36608.2860	1590434.38	263.2040
	T_{max} (hr)	1.4448	1.0002	0.5000	1.0300	8.0000	69.2252
	K_{el} (hr^{-1})	0.0483	0.0090	0.0090	0.0490	0.0640	18.5628
	$t_{1/2}$ (hr)	15.5926	8.4835	10.9100	14.1550	77.1500	54.4073
	$AUC_{\_\%Extra\ pobs}$	3.7097	6.9180	1.1100	2.4450	53.3200	186.4849
	$AUC_{\_\% Ratio}$	96.2903	6.9180	46.6800	97.5550	98.8900	7.1845

Statistical Result of analyzed formulations: For the Ln transformed pharmacokinetic parameters Cmax, AUC0-t and AUC0-∞ of S-RRR and R-SSS nebivolol, the ratios (T/R) of geometric least squares means (and confidence intervals) of the test product (T) and reference product (R) were determined to be 97.36 (92.77% - 102.17%), 96.66 (93.04% - 100.41%) and 96.64 (93.24%-100.17%) and 98.09 (93.51% - 102.88%), 98.17 (94.99%- 101.47%) and 97.24 (93.89% - 100.70%) respectively.

The test product (T) and reference product (R) had Intra-subject CV% of 16.18%, 12.74%, 11.99% and 16.01%, 11.03%, 11.71% respectively, for the Ln-transformed pharmacokinetic parameters Cmax, AUC0-t and AUC0-∞ of S-RRR and R-SSS nebivolol respectively.

Bioequivalence Conclusion: The bioequivalence acceptance limits of 80.00%–125.00% are met by the 90% confidence intervals of the ratio of geometric least squares means for the Ln-transformed pharmacokinetic parameters Cmax, AUC0-t and AUC0-∞ of S-RRR and R-SSS nebivolol. Therefore, it can be concluded that the test product (T) and reference product (R) are bioequivalent in terms of absorption rate and extent.

Conclusion

A simple, rapid, sensitive and selective LC-MS/MS method was developed and validated for the simultaneous estimation of S-RRR and R-SSS nebivolol in human plasma using S-RRR nebivolol D4 and R-SSS nebivolol D4 as an internal standard. The present method has advantage of low processing volume (300 µl), shorter run time (8 min) and minimum matrix effect in comparison to existing chiral method. Strong scientific evidence for accuracy and precision of quantification of S-RRR and R-SSS nebivolol in human plasma. This method may have application to characterize the clinical pharmacokinetic studies of S-RRR and R-SSS nebivolol in human.

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