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Development and Validation of Bioanalytical Method for Simultaneous Estimation of Nebivolol Enantiomers in Human Plasma Using Liquid Chromatography-tandem Mass Spectrometry

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The present study describes a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous determination of S-RRR and R-SSS nebivolol (nebivolol enantiomers) in human plasma using solid phase extraction technique. Method of both S-RRR and R-SSS nebivolol (nebivolol enantiomers) has been developed and validated using racemic nebivolol D4 as an internal standard. Analytes from human plasma were extracted by ion exchange cartridges and subsequently separated on chiral column using acetonitrile: ammonium carbonate in water, 158 mg l^{-1} 80:20% v/v as a mobile phase, at a flow rate of 0.9 ml min^{-1}. Quantification of S-RRR and R-SSS nebivolol and R-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 20.0-6000 pg ml^{-1} 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 current regulatory requirement (US FDA, EU) such as selectivity, matrix effect, recovery and stability (in solution and in matrix stability). Overall the present study revealed the selectivity and sensitivity of this method for the simultaneous estimation of S-RRR and R-SSS nebivolol (nebivolol enantiomers) in human plasma.

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

INTRODUCTION

Essential hypertension, a condition associated with endothelial dysfunction, is caused by production of oxygen free radicals that destroy nitric oxide and impair its beneficial and protective actions on vessel wall. Beta blockers for many years have been established as first line therapy in management of hypertension. Nebivolol, a third generation highly selective β adrenoceptor antagonist, is indicated for the treatment of essential hypertension. In addition to its beta blocking effects, nebivolol has an endothelium dependent vasodilator property which is mediated *via* L-arginine/NO pathway [1-4].

Nebivolol is administered as a racemic mixture of equal proportions of "d" and "l" enantiomers. Nebivolol is 1-(6-Fluorochroman-2-yl)-{[2-(6-fluorochroman-2-yl)-2-hydroxy-ethyl] amino} ethanol having four asymmetric centres where d- isomer refers to (S,R,R,R)-nebivolol and l-isomer to (R,S,S,S)-nebivolol and are pharmacologically active at clinical dosages. However, the enantiomers show unequal clinical potency with regard to β-receptor blocking activity and nitric oxide mediated vasodilation [1].

Qualitative and quantitative determination of nebivolol enantiomers and racemic nebivolol in the biological fluids is studied extensively using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and high performance liquid chromatography (HPLC). Ramakrishna *et al.* [5] employed high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) to quantify racemic nebivolol in the range 50.0 to 10,000 pg ml^{-1}. Samples were

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Table 1. Calibration Standard Data Detailing Slope, Intercept, Correlation-coefficient (R^2) for Drugs (n = 15)

Analyte		Slope	Intercept	R^2
S-RRR nebivolol	Mean	1.1400	0.0001	0.9974
	%CV	10.04	41.14	0.0018
R-SSS nebivolol	Mean	0.6843	0.0001	0.9984
	%CV	7.61	38.45	0.1000

extracted through Liquid-Liquid extraction method. This method was also employed to the determination of the same sample by Nandania *et al.* [6] with lower limit of quantification 50 pg ml^{-1} and by Lindamood *et al.* [7] in the range of 20-1500 pg of each isomer/ml of plasma; however details of the extraction and chromatographic procedures largely remained out of focus. Other previous studies described the use of LC-MS/MS in the analysis of isomeric mixture of nebivolol in plasma, employing non-chiral columns, in lower limit of quantification of 10-50 pg of nebivolol/ml of plasma utilizing 200-500 µl of plasma. Daniel Valente Neves *et al.* [8] quantified nebivolol enantiomers in the range of 25 - 2500 pg ml^{-1} using volume 2 ml of plasma and run time of this method was 17 min. M for determination of ethod nebivolol and other 30 compounds has been developed and reported by Mario Thevis *et al.* [9]. Stability indicating method has been developed using high performance liquid chromatography and was reported by kachhadia *et al.* [10] for tablet formulation. For detection of hydroxyl functions in hydroxylated metabolites of nebivolol, method has been developed and reported by hendrickx *et al.* [11]. Comparison of nebivolol reported methods has been presented in Table 1.

In the light of above background, the present study was planned to develop and validate a stereoselective and run time efficient analytical method of nebivolol enantiomers in human plasma using a chiral column and minimum sample volume. Further, the method proposed is sensitive enough to quantify samples from a single-dose pharmacokinetic study.

MATERIALS AND METHODS

Chemicals and Reagents

S-RRR and R-SSS nebivolol were purchased from Clearsynth Labs, Mumbai, India. R-nebivolol D4 was purchased from Bioorganics, Mumbai, India. Human plasma was purchased from Supratech Laboratory, Ahmedabad, Gujarat, India. Methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from Spectrochem Pvt. Ltd., Mumbai, India. Formic acid (Emparta grade), ammonium carbonate (Emparta grade) and ammonia solution (Emparta grade) were purchased from Merck Millipore, Mumbai, India. Waters MCX 30 mg ml^{-1} extraction cartridges were purchased from Waters Corporation, India. HPLC grade water was obtained using a Milli-Q water purification system, Millipore Pvt. Ltd., Ahmedabad, Gujarat, India.

Instrumentation

For chromatographic separation, a Shimadzu UFLC system (Shimadzu Corporation, Japan) equipped with a LC-30AD solvent delivery system, a DGU-20A5R vacuum degasser, a CTO-20AC thermostated column oven, a SIL-20AC autosampler, and a triple quadrupole mass spectrometer LCMSMS-API 5500 (AB Sciex, USA) was used. Data acquisition and processing were performed using analyst software (version 1.6.3; AB Sciex, USA) and Watson LIMS (version 7.3; Thermo, USA).

Chromatographic Conditions

Chromatographic separation was achieved on CHIRALPACK AY-RH 5 µm, 150 mm × 4.6 mm column placed at 35 °C in thermostated column oven by passing mobile phase consisting of acetonitrile: ammonium carbonate in water 158 mg l^{-1}, 80:20% v/v at a flow rate of 0.9 ml min^{-1}. 10% mobile phase was introduced in mass spectrometer by diverting 90% to waste using T-splitter. Analytical run time was 9.0 min.

Mass Spectrometric Conditions

The determination of S-RRR, R-SSS nebivolol and racemic 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 (S-RRR & R-SSs) nebivolol and R-nebivolol-D4 were m/z 406.300 > 151.100 and 411.300 > 151.100 respectively. The source dependant parameters were Gas 1 (Nebuliser Gas): 60 psig; Gas 2 (Heater Gas): 70 psig; ion spray voltage (ISV): 5500 V, turbo heater temperature (TEM): 500 °C; interface heater (Ihe): ON; collision activation dissociation (CAD): 6 psig and curtain gas (CUR): nitrogen: 30 psig. The dwell time of 500 ms per transition and collision energy (CE) of 40 were used. Mass spectra of product ions are presented in Fig. 1.

Preparation of Standard and Quality Control (QC) Samples

The standard stock solutions of S-RRR nebivolol, R-SSS nebivolol and R-nebivolol D4 were prepared at 100 µg ml^{-1} of each sample in methanol. The analyte stock solutions were further diluted with methanol to give final concentration of 2.00 µg ml^{-1} of mixed S-RRR, R-SSS nebivolol. The working solution of mixed analyte (S-RRR and R-SSS nebivolol) at the concentrations of 1.0, 2.0, 5.0, 10.0, 25.0, 75.0, 150.0 and 300.0 ng ml^{-1} of each sample were prepared by serial dilution from 2.00 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-nebivolol D4 in methanol:water (50:50) to achieve a final concentration of 40.0 ng ml^{-1}. The mixed analyte working solutions (20 µl) were used to spike blank human plasma sample (980 µl) to prepare the calibration curve standards of both the analytes at concentration of 20.0, 40.0, 100, 200, 500, 1500, 3000 and 6000 pg ml^{-1} in validation study [12]. The plasma concentrations of QC samples were prepared at 20.0, 60.0, 300, 2400 and 4800 pg ml^{-1} for S-RRR and R-SSS nebivolol. All working solutions were stored at 2-8 °C until analysis.

Plasma Sample Preparation

Plasma samples were taken in 5 ml eppendorf centrifuge tubes, 50 µl of IS solution was added and vortexed. 300 µl of formic acid in water, 2% v/v, was added to it and vortexed. The resultant sample was loaded on to Oasis MCX 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 formic acid in water, 2% v/v, and 1.0 ml of formic acid in Methanol, 2% v/v, followed by two times 1 ml of acetonitrile. Washed cartridges were dried with nitrogen gas by positive pressure at maximum flow rate for 5 min on Ezypress 48 positive pressure processor. Contents were eluted from the cartridges with 1.0 ml ammonia solution in methanol, 2% v/v. 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 80 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 R-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 [12] and European Medicines Agency (EMEA) guidelines [13].

Selectivity. The selectivity of method was assessed by comparing the interfering signals in nine different lots of plasma (six lots of plasma with K_3EDTA anticoagulant, one

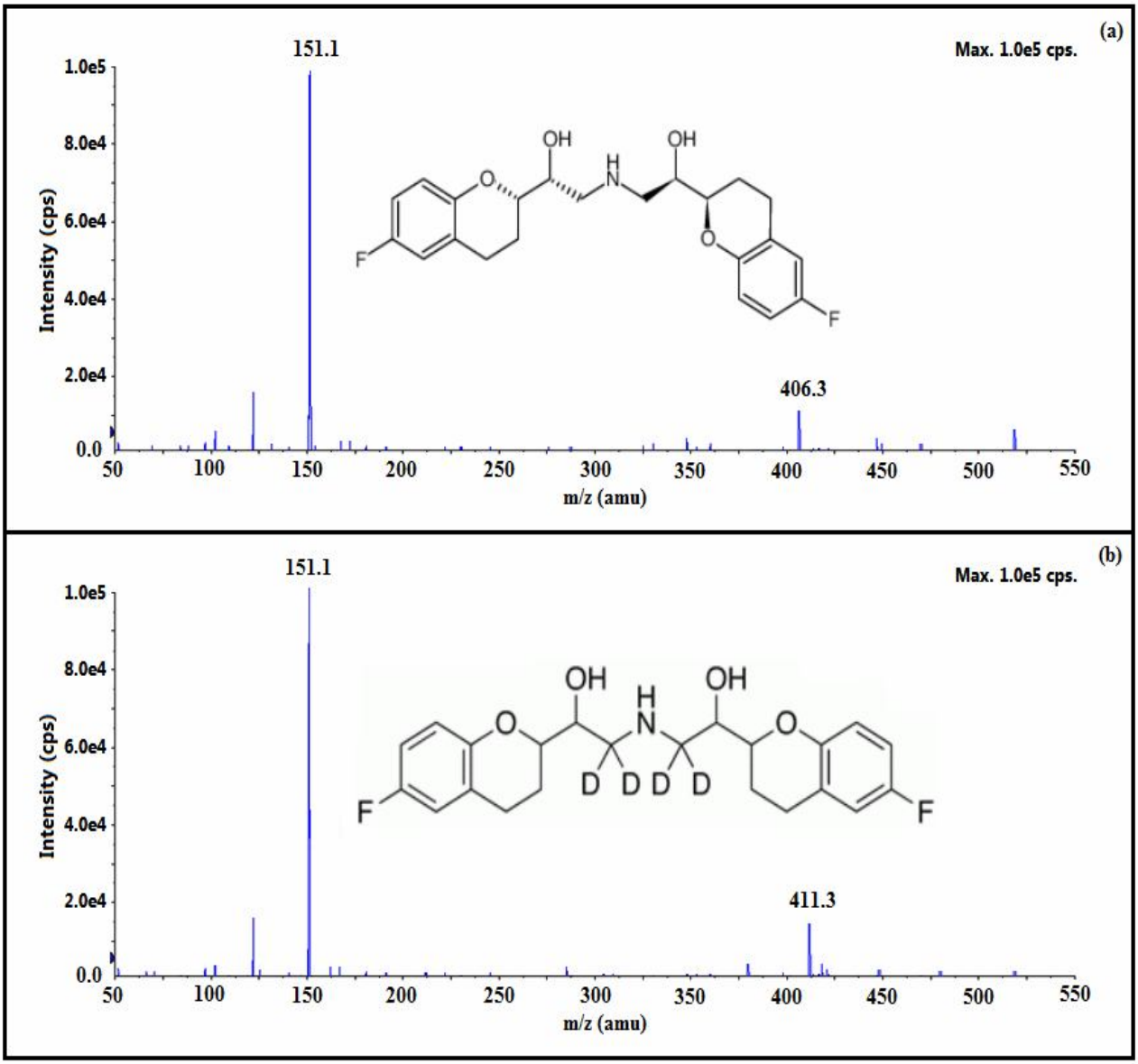

Fig. 1. Spectrum of product ion scans of Nebivolol (a) and Nebivolol D4 (b).

lot each of lipidemic K_3EDTA plasma, haemolysed K_3EDTA plasma and heparinized plasma) with the signals of analytes and IS in LLOQ 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 100% recovery.

Matrix effect. Matrix effect was evaluated by comparing the mean peak area of analytes in presence of matrix ions (A) (by post spiking analyte and IS in extracted blank plasma samples) and in the absence of matrix ions (B) (neat analyte and IS solutions). Post spiked analyte and IS in blank plasma samples were prepared in six different human blank plasma lots.

$$\text{Matrix effect} = (A/B) \times 100$$

IS normalized factor was calculated by ration of matrix factor of analyte and matrix factor of IS. In order to get free the method from relative matrix effect, the percentage of coefficient of variance (CV) of IS normalized matrix factor should be less than 15% [13].

Stability. The present study evaluated the stability studies of S-RRR and R-SSS nebivolol in plasma samples. The bench top (9 h at ambient temperature), freeze-thaw (at -20 °C), processed samples stability (14 h at room temperature and 104 h at 2-8 °C), dry extract stability (102 h at -20 °C) and long term storage in freezer stability (145 days at -20 °C) 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 [12,13]. Freeze-thaw cycle included thawing of samples at room temperature and then refreezing at -20 °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

For optimum detection and simultaneous quantification of S-RRR and R-SSS nebivolol with IS in human plasma, it was necessary to adjust chromatographic conditions and mass spectrometric parameters as well. The mass spectrometric parameters were tuned in both the positive and negative ionization modes for all analytes. S-RRR, R-SSS nebivolol and IS showed prominent peak in the positive ionization mode.

Optimization of ionization voltage, interface temperature, curtain gas, GS1, GS2, and CAD gas flow are of utmost importance in order to minimize ion suppression and to increase sensitivity. The results of the present study showed ionization voltage, interface temperature, curtain gas, GS1, GS2 and CAD gas flow above 5500, 500 °C, 30.0, 60.0, 70.0 and 6.00, respectively, and augmented the intensity of the analyte. A dwell time of 500 ms for S-RRR, R-SSS nebivolol and IS was sufficient and no cross talk was found between all multiple reaction monitoring.

Optimization of the Sample Preparation and Chromatographic Conditions

One of the key fundamental steps in the development of an analytical method is sample preparation. Sample preparation procedure should be quick, and easy to proceed and should require the least amount of reagents with maximum recovery of analytes. In this regards, literature review revealed the use of liquid-liquid extraction technique for the extraction of S-RRR and R-SSS nebivolol [8]. However, we employed solid phase extraction as the sample preparation method and it yielded cleaner sample, least matrix effect and more recoveries of the analytes compared to liquid-liquid extraction. Some reported methods also employed the higher plasma volume (2 ml) for sample preparation and injection volume for the chromatographic development [8]. Interestingly, the proposed method was developed with less plasma (0.3 ml) and injection volume, which may lead to better acceptability of the method. To develop rugged solid phase extraction method, several trials of different SPE cartridges were evaluated with changes made in buffer, washing and elution solution. Better response, least matrix effect, less interference and highest

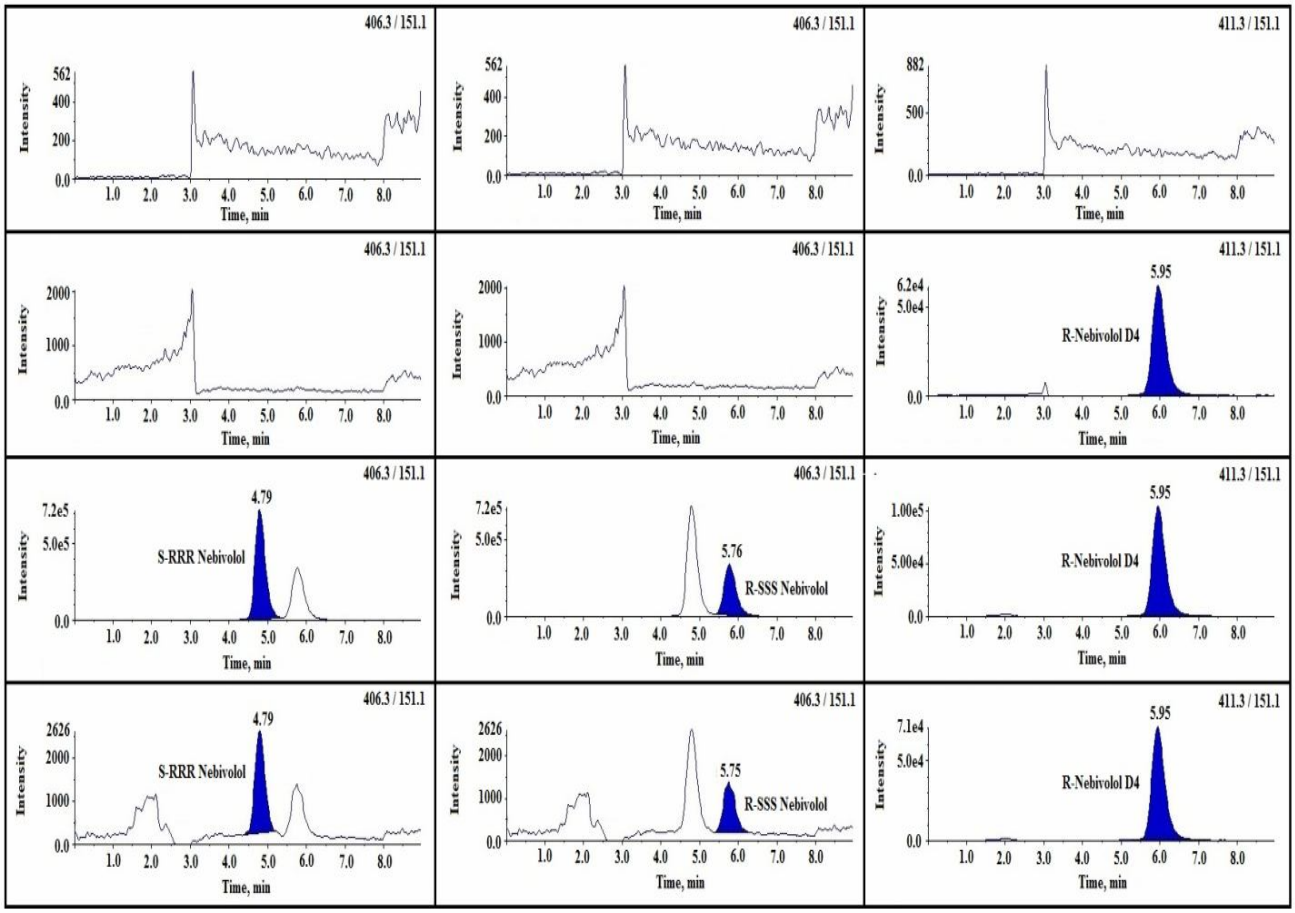

Fig. 2. Representative MRM Chromatogram STD BL, STS Zero, ULOQ LLOQ of S-RRR nebivolol and STD BL, STS Zero, ULOQ, LLOQ of R-SSS nebivolol and STD BL, STS Zero, ULOQ, LLOQ of R-Nebivolol D4 in human plasma.

recovery were obtained using oasis MCX cartridges 1 cc (30 mg) [14]. Nebivolol contained iminodiethanol group (cationic group) which could specifically bind to the sulfuric acid group present on mixed mode cation exchange cartridge. Different cation exchange cartridges of waters, phenomenex and agila were checked during method development. Better results were observed with waters oasis MCX cartridges.

R-nebivolol D4 was chosen as the IS because of its same physicohemical properties in comparison to analyte which compensate easily any extraction loss and equipment response variation, thereby, area ratio remains unchanged. Nowadays, isotopes labelled IS are recommended by regulatory agency [13] preferably to use as IS. Chromatographic conditions were optimized to achieve desired sensitivity and peak shape for both analytes and IS, as well as a short chromatographic run time with proper separation of both peaks. In this study, we tried CHIRALPACK AY-RH and Lux Amyllose-2 columns with various mobile phases such as methanol, acetonitrile, isopropyl alcohol, ammonium acetate and ammonium carbonate. The CHIRALPACK AY-RH 5 µm 150 mm × 4.6 mm column was selected as it gave better sensitivity and separation between S-RRR and R-SSS nebivolol with mobile phase acetonitrile:ammonium carbonate in water, 158 mg l^{-1} 80:20% v/v [15-17]. Low injection volume of 10

Table 2. Accuracy and Precision Performance Data for the Quantification of S-RRR Nebivolol and R-SSS Nebivolol in Human Plasma

Analyte	Nominal conc. (ng ml^{-1})	Intra batch precision (n = 6) (%CV)	Inter batch precision (n = 18) (%CV)	Intra batch accuracy (n = 6) (%)	Inter batch accuracy (n = 18) (%)
S-RRR nebivolol	0.0200	5.26	5.00	95.23	99.54
	0.0600	3.51	3.39	97.54	98.25
	0.300	3.07	3.51	100.87	99.87
	2.400	6.63	3.93	96.54	97.23
	4.800	6.42	3.83	98.78	98.56
R-SSS nebivolol	0.0200	10.00	10.53	94.24	99.27
	0.0600	5.17	5.04	95.87	97.54
	0.300	2.75	2.78	101.24	98.61
	2.400	2.97	1.92	98.29	97.98
	4.800	2.75	1.69	95.87	99.67

µl reduced overloading of column with analytes which ensured more numbers of analyses on the same column. Chromatograms of STD BL, STD ZERO, STD1 and LLOQ of S-RRR and R-SSS nebivolol and R-nebivolol D4 are presented in Fig. 2.

Selectivity

Figure 2 depicts that there was no interfering peaks observed from endogenous compounds at retention time in any of the samples of S-RRR, R-SSS nebivolol and R-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 4.80 and 5.70 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 6000 pg ml^{-1} for S-RRR and R-SSS nebivolol (Dynamic Linearity range of 300 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 [$1/x^2$] (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 of the calibration curve, c = y-axis intercept of the calibration curve), on a validation batch was: y = 1.1400x + 0.0001 (r^2 = 0.9974) for S-RRR nebivolol and y = 0.6843x + 0.0001 (r^2 = 0.9984) for R-SSS nebivolol (Table 2). The proposed method can detect lower concentration up to 5% of C_{max} of both analytes and upper

Table 3. Recovery Performance Data of S-RRR Nebivolol, R-SSS Nebivolol (at Three QC Level) and Racemic Nebivolol d4

Analyte (pg ml^{-1})	Mean peak area (n = 6)		Mean recovery (%)	CV (%)
	Post-spiked extracted samples[a]	Extracted samples[b]		
		S-RRR nebivolol		
60	129931.2	102563.2	78.94	
2400	5525997.8	4474704.8	80.98	5.87
4800	11918912.5	8616510.5	72.29	
		R-SSS nebivolol		
60	77726.7	62862.5	80.88	
2400	3164092.3	2613460.8	82.60	5.49
4800	6652257.0	4945688.2	74.35	
		R-nebivolol D4		
40.000	2144296.5	1848575.5	86.21	

[a]Peak area of analytes solution spiked in mobile phase. [b]Peak area of analytes extracted from spiked human plasma. [c]Recovery was expressed as the percentage of the mean peak area of the analytes prepared in the mobile phase relative to that of analytes extracted from human plasma.

concentration was more than two times of the C_{max} of analytes. Good linearity was obtained with aforementioned concentration ranges with a regression coefficient (r^2) greater than 0.995. Table 3 summarizes the results for intra- and inter-day precision and accuracy for S-RRR and R-SSS nebivolol measured by QCs. To validate the accuracy and precision of the developed method, five level concentrations of QCs in six replicates were analysed in a single batch. The results showed that the intra- and inter-day accuracy ranged from 95.23-100.87% for S-RRR and 94.24-101.24% 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 [12,13].

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 78.94%, 80.98% and 72.29% at low, middle and high concentration levels, respectively. The recovery of R-SSS nebivolol was 80.88%, 82.60%, and 74.35% at low, middle and high concentration levels, respectively. The recovery of R-nebivolol D4 was 86.21% (Table 4). In bioanalytical method development the extent of recovery is not considered but it should be precise and consistent [12,13].

Matrix Effect

Two QC concentrations of each tested analyte and IS were utilized in the test with six different sources of human plasma. Table 5 depicts that there was no significant ion suppression or enhancement observed for all the analytes and IS under the present experimental conditions.

Table 4. Assessment of Matrix Effect of S-RRR Nebivolol, R-SSS Nebivolol and R-nebivolol D4

Analyte (pg ml^{-1})	Mean peak area		Matrix effect[c]	CV (%)
	Neat solution[a]	Post-spiked extracted samples[b]		
		S-RRR nebivolol		
4800	28024514.3	27453056.5	0.980	5.51
60	359024.3	362453.1	1.009	5.21
		R-SSS nebivolol		
4800	15216507.1	15428965.2	1.014	1.19
60	196294.3	189865.2	0.967	1.70
		R-nebivolol D4		
40000				

[a]Peak area of analytes solution without plasma extracts. [b]Peak area of analytes spiked in plasma extracts. [c]Matrix effect was expressed as the percentage of the mean peak area of the analytes spiked in blank extracted plasma samples relative to that of analytes prepared in the mobile phase.

Stability

The stability studies of S-RRR and R-SSS nebivolol in plasma has been evaluated. The bench top (9 h at ambient temperature), freeze-thaw (at -20 °C), processed samples stability (14 h at room temperature and 104 h at 2-8 °C), dry extract stability (102 h at -20 °C) and long term storage in freezer stability (at -20 °C) study 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 [12] and EMEA [13] 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 -20 °C, such five cycles were completed for freeze-thaw stability. Long term storage in freezer stability was performed by storing the stability samples for 145 days at -20 °C in freezer. In all the stability experiments, stability samples were quantified *versus* freshly prepared calibration standards per regulatory requirement [12,13]. Results of stability experiments were given in Table 6.

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 5mg tablets in healthy human volunteers. Incurred sample reanalysis results were within acceptance criteria (Fig. 3). This sample was also analysed with achiral method for determination of racemic nebivolol. Results of this racemic nebivolol were compared with sum of S-RRR nebivolol and R-SSS nebivolol. Comparison of concentration-time profile of racemic nebivolol by chiral method (through summation of concentration of S-RRR & R-SSS nebivolol) and achiral method (direct estimation of racemic nebivolol) revealed similar results. A graphical comparison of racemic nebivolol concentration results is presented in Fig. 4.

Table 5. Stability Data of S-RRR Nebivolol and R-SSS Nebivolol at Low and High QC Level (n = 6)

Stability study	S-RRR nebivolol		l Nebivolol	
	Concentration (pg ml^{-1})	Mean stability (%)[a]	Concentration (pg ml^{-1})	Mean stability (%)[a]
Bench top stability (09 h at ambient temperature)	4800	6.50	4800	-1.67
	60	0.00	60	-1.21
Process stability (Storage of 104 h at 5 ± 3 °C)	4800	5.44	4800	-1.54
	60	-1.67	60	-0.33
Process stability (Storage of 14 h at ambient temperature)	4800	3.77	4800	-0.21
	60	-3.33	60	-1.67
freeze-thaw stability (Five cycle)	4800	2.75	4800	0.12
	60	-1.85	60	-0.44
Dry extract stability	4800	2.35	4800	0.23
	60	-6.67	60	0.21
Long term stability (145 days)	4800	-4.23	4800	-1.20
	60	-2.13	60	2.43

[a]%Mean stability = %Mean change in the concentration of the stability samples when compare to the freshly spiked samples.

CONCLUSIONS

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 R-nebivolol D4 as an internal standard. The present method has advantage of low processing volume (300 µl), shorter run time (9 min) and minimum matrix effect in comparison to existing chiral method [8]. Comparison of validation results has been given in Table 7. To the best of our knowledge, the findings of the present study provide 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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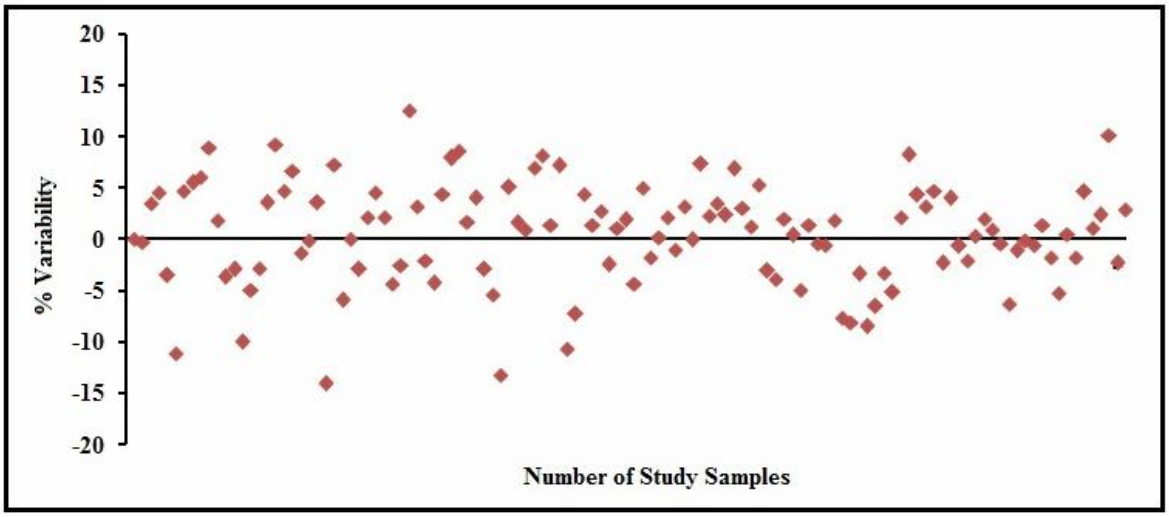

Fig. 3. Incurred sample reanalysis results.

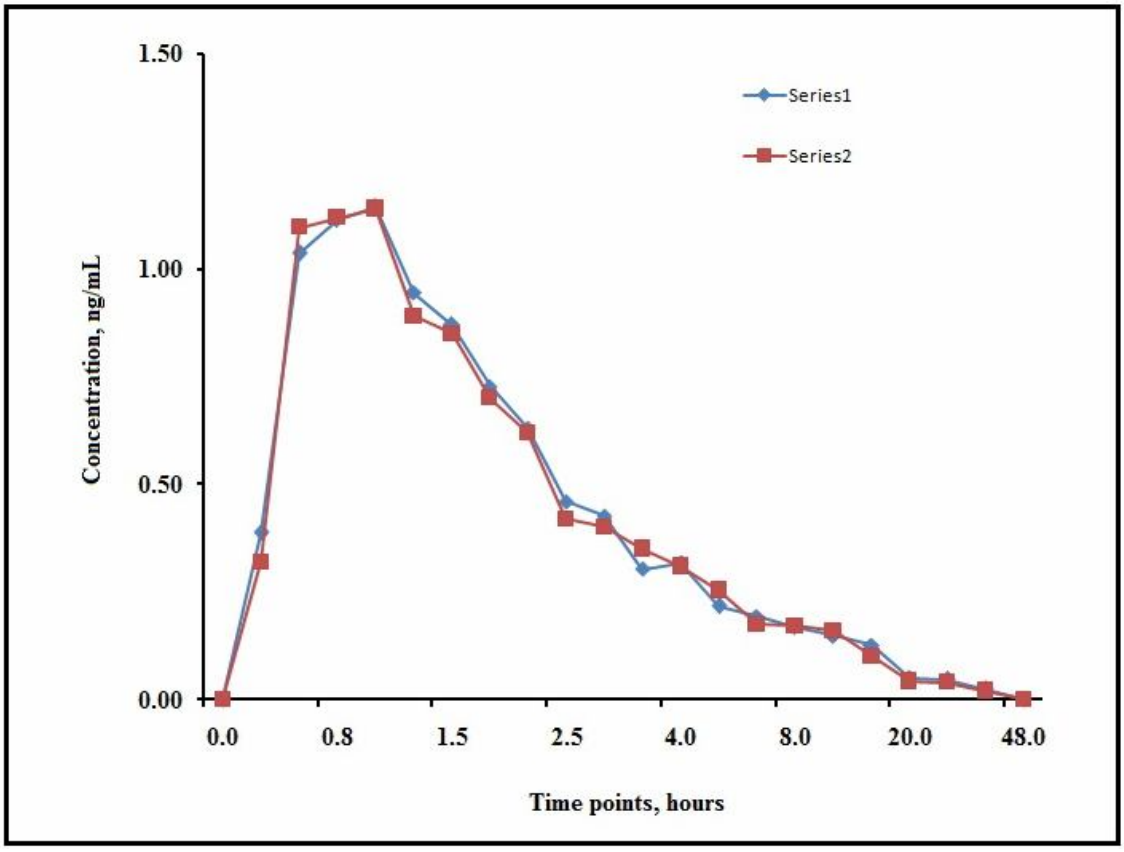

Fig. 4. Comparison of S-RRR and R-SSS nebivolol results with racemic nebivolol method.

REFERENCES

[1] G.N. Sahana, N. Sarala, T.N. Kumar, Int. J. Biol. Med. Res. (2011) 577.

[2] J. Fongemie, E. Felix-Getzik, Drugs 7512 (2015) 1349.

[3] J.G. Howlett, Can. J. Cardiol. 30 (2014) S29.

[4] S.F. Ang, S.M. Moochhala, M. Bhatia, C.C. Bauer, J.P. Boyle, K.E. Porter, C. Peers, *et al*. Biol. 30 (2010) 1998.

[5] N.V.S. Ramakrishna, K.N. Vishwottam, M. Koteshwara, S. Manoj, M. Santosh, D.P. Varma, 39 (2005) 1006.

[6] J. Nandania, S.J. Rajput, P. Contractor, P. Vasava, B. Solanki, M. Vohra. J. Chromatogr. B 923 (2013) 110.

[7] C. Lindamood, S. Ortiz, A. Shaw, R.S. Rackley, J. Christopher Gorski, J. Clin. Pharmacol. 514 (2011) 575.

[8] D.V. Neves, C.P. Vieira, E.B. Coelho, M.P. Marques, V.L. Lanchote, J. Chromatogr. B 940 (2013) 47.

[9] M. Thevis, W. Schänzer, J. Chromatogr. Sci. 43 (2005) 22.

[10] P.K. Kachhadia, A.S. Doshi, H.S. Joshi, J. AOAC Int. 91 (2008) 557.

[11] J. Hendrickx, M. Bockx, C. Zwijsen, C. Borgmans, G. Mannens, W. Meuldermans, J. Heykants, J. Chromatogr. A 729 (1996) 341.

[12] US Food and Drug Administration. Guidance for Industry, Bioanalytical Method Validation. http://www.fda.Gov/cder/guidance/4252fnl.htm, 2001.

[13] Committee for Medicinal Products for Human Use. Guideline on Bioanalytical Method Validation. European Medicines Agency, 2011.

[14] M. Caban, P. Stepnowski, M. Kwiatkowski, J. Maszkowska, M. Wagil, J. Kumirska, J. Chem. 2015.

[15] A.A. Younes, D. Mangelings, Y.V. Heyden, J. Chromatogr. A 1269 (2012) 154.

[16] K. Tachibana, A. Ohnishi, J. Chromatogr. A 906 (2001) 127.

[17] B. Chankvetadze, Enantioseparations by High-Performance Liquid Chromatography Using Polysaccharide-based Chiral Stationary Phases: An Overview, In Chiral Separations: Methods and Protocols, 2013, Vol 970. Humana Press, Totowa, NJ, pp. 81-111.