Journal of Chromatography B, 923–924 (2013) 110–119

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Quantitative determination of nebivolol from human plasma using liquid chromatography–tandem mass spectrometry

Jatin Nandania[a,*], S.J. Rajput[a], Pritesh Contractor[b], Pragnesh Vasava[a], Bhavik Solanki[b], Mohsin Vohra[a]

[a] Pharmacy Department, Faculty of Technology and Engineering, The M.S. University of Baroda, Gujarat, India
[b] Veeda Clinical Research Pvt. Ltd., Near IIM, Ahmedabad, Gujarat, India

ARTICLE INFO

Article history:
Received 2 September 2012
Accepted 31 January 2013
Available online 18 February 2013

Keywords:
Nebivolol
Liquid–liquid extraction
Pharmacokinetic profile
LC/MSMS
Bioavailability
Bioequivalence

ABSTRACT

In the present work, a rapid, sensitive, specific, precise and accurate liquid chromatography–tandem mass spectrometry method for determination of nebivolol in human plasma was developed and validated with a large calibration curve range (50–5000 pg/mL) which can be used for routine drug analysis and bioequivalence studies. Liquid–liquid extraction method was used to extract the analyte from the human plasma. The separation was achieved using Waters symmetry, C18, 4.6 × 150 mm, 5 μm column with formic acid in water, 0.01%, v/v: Acetonitrile (40:60) as a mobile phase. A flow rate of 0.8 mL/min, no splitting and run time 2.00 min was used for the chromatographic analysis of nebivolol. Sensitivity of this method was found to be 30 pg/mL. The analyte was analyzed by mass spectrometry in the multiple reaction monitoring mode. A Turbo-Ion spray source was interfaced between the HPLC and triple quadrupole mass spectrometer (MDS Sciex API 4000). The precursor-product ion *m/z* 406.00–151.00 for nebivolol and *m/z* 410.20–151.00 for nebivolol-D4 were used for quantification of an analyte and its IS. The method was validated in terms of accuracy, precision, selectivity, absolute recovery, freeze-thaw stability, bench-top stability, dry extract stability, short and long term stock solution stability, wet extract stability and re-injection reproducibility. The within- and between-batch accuracy was found to lie within the range of 87.00–100.40% and within- and between-batch precision was obtained within the range 0.33–8.67%. The mean recovery of all three concentration levels for drug was obtained 67.67% where as the mean recovery of IS was 68.74%. The %RSD value at higher concentration and lower concentration in all stability experiments was within 15%. This method is free from ion suppression, ion enhancement and any type of abnormal ionization.

1. Introduction

Nebivolol is widely used for the treatment of hypertension. It is a competitive and cardioselective beta-blocker with mild vasodilating properties, possibly due to an interaction with the L-arginine/nitric oxide pathway [1]. Chemically it is 1-(6-fluorochroman-2-yl)-{[2-(6-fluorochroman-2-yl)-2-hydroxy-ethyl] amino} ethanol [2]. Chemical structure of Nebivolol is shown in Fig. 1. It is evidently a racemate of D-Nebivolol and L-Nebivolol with the stereochemical designations of [SRRR]-Nebivolol and [RSSS]-nebivolol [3]. Nebivolol is soluble in N,N-dimethylformamide, methanol, dimethylsulfoxide; sparingly soluble in polypropylene glycol, polyethylene glycol and ethanol; and very slightly soluble in dichloromethane, hexane and methylbenzene [3]. Comparison between the safety of Nebivolol and other beta-blockers shows that nebivolol could be worth in the management of hypertensive patients with endothelial dysfunction e.g. those with diabetes mellitus or hypercholesterolemia and in patients with ischemic heart disease with less side effects [4–8]. Human studies in a small number of healthy volunteers confirmed that nebivolol has nitric oxide mediated venodilator effect [9]. Most of the generic companies have conducted bioavailability/bioequivalence (BA/BE) studies for ANDA application as nebivolol is one of the most effective drug for the treatment of hypertension. So, it is important to have an effective analytical method to quantify nebivolol in the biological matrix which can be also operative to generate pharmacokinetic profile. Number of published methods is available for the estimation of nebivolol. Srinivasulu et al. performed reverse phase HPLC method for the analysis of nebivolol in pharmaceutical dosage forms over the linearity range from 5 to 100 μg/mL [10]. Levesque et al. developed

* Corresponding author at: Veeda Clinical Research Pvt. Ltd., Near IIM, Ahmedabad, Gujarat, India. Tel.: +91 9979783582.
E-mail address: jatinnandania@gmail.com (J. Nandania).

1570-0232/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2013.01.034

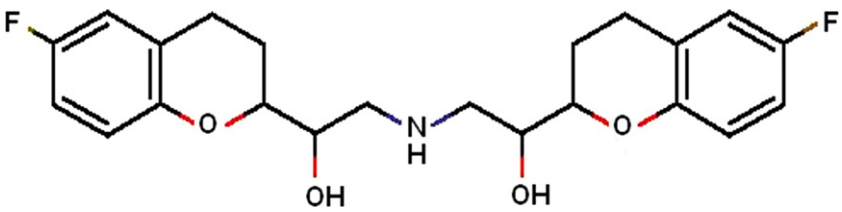

Fig. 1. Chemical structure of Nebivolol.

method for nebivolol in Human EDTA K2 Plasma by LC/MS/MS using automated SPE extraction on Oasis MCX extraction plates on MultiPROBE II system [11]. Senthamil et al. performed simultaneous determination of fixed dose combination of nebivolol and valsartan in human plasma by liquid chromatographic–tandem mass spectrometry. The linearity was over the concentration range of 0.01–50.0 ng/mL and 1.0–2000.0 ng/mL and the lower limits of quantitation were 0.01 ng/mL and 1.0 ng/mL for nebivolol and valsartan, respectively [12]. Kokil and Bhatia determined Simultaneous estimation of nebivolol hydrochloride and valsartan using RP HPLC [13]. Aim of the present work was to develop a simple, sensitive, accurate, precise, rapid and selective bioanalytical method for the determination of nebivolol in human plasma by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The proposed method would be useful for routine drug analysis and bioequivalence studies. The purpose of this method development was to analyze nebivolol in human plasma samples obtained from clinical study (An Open Label, Balanced, Randomized, Two-Treatment, Two-Sequence, Two-Period, Single-Dose, Crossover Oral Bioequivalence Study of Nebivolol Tablet 20 mg and Bystolic® (Nebivolol) tablets 20 mg of Forest Pharmaceuticals, Inc. in Normal, Healthy, Adult, Human Male and Female Subjects under Fasting and Fed Condition). By now, it seems that most analytical challenges are identified in quadrupole LC–MS/MS or LC–MS using the ESI or APCI interface [14]. LC–MS/MS provides a powerful separation and detection technique for the determination of drugs in biological fluids [14,15]. This method was developed to quantify nebivolol from human plasma at low concentration (30 pg/mL). However, this method was validated over a wide range of 50–5000 pg/mL. Further, this method with the shorter analysis run time can be used for analysis of more than 300 samples per day. The validated high through put method can be employed to analyze a large number of subject samples from BA/BE studies to generate pharmacokinetic profile after therapeutic doses of nebivolol. This method involves liquid–liquid extraction (LLE) of nebivolol from human plasma. After extraction, the samples were injected in HPLC and final detection was done by tandem mass spectrometry. Many analytical challenges were considered during the method development and its validation [16]. Matrix effect was not observed in this method and additionally nebivolol-d4 was used as an internal standard (IS) to reduce impact of matrix on the quantification of nebivolol [17,18]. Post Column Infusion (PCI) experiment was performed to check ion suppression at retention time (RT) of the nebivolol and the method was found to be free from ion suppression/enhancement [19]. Drifting in response of drug and IS was not observed as batch size experiment which was performed for 130 samples was found to be acceptable. It confirms that this method could be effective for analyzing large number of subject sample at a time. This method was validated in terms of accuracy, precision, sensitivity, selectivity, absolute recovery, freeze-thaw stability, bench-top stability, dry extract stability, short and long term stock solution stability, wet extract stability and re-injection reproducibility as per USFDA guidelines [20]. Hence, the method can be applied to generate pharmacokinetic profile during the bioequivalence study.

2. Experiments

2.1. Chemicals and reagents

Nebivolol with 99.00% purity and water content 0.14% was used for analysis. It was supplied by Samex Overseas (India). Nebivolol-D4 was used as an internal standard (IS). %purity and water content for Nebivolol-D4 were 99.34% and 0.07% respectively and it was supplied by Samex Overseas (India). HPLC grade methanol and acetonotrile were obtained from Mallinckrodt Baker (S.A. de C.V. Mexico). Other chemicals like ammonium formate (AR grade), formic acid (GR grade), ammonia solution, ethyl acetate and n-hexane were obtained from Merck Specialties Pvt. Ltd. (India). Water used in the entire analysis was prepared from Milli-Q water purification system procured from Millipore (Bangalore, India). Drug free K_3EDTA human plasma was procured from Supratech Micropath Laboratory and Research Institute, Ahmedabad and stored in to $-70 \pm 5\,^{\circ}C$ until use.

2.2. Solutions prepared

Nebivolol stock solution was prepared in methanol to give a final concentration of 0.250 mg/mL, which was further diluted in methanol to attain the final concentration 1,250,000 pg/mL for the nebivolol intermediate stock solution. Calibration Curve (CC) spiking solutions and Quality Control (QC) spiking solution were prepared in diluent (formic acid in acetonitrile, 0.1% v/v) using nebivolol intermediate stock solution by serial dilution to give final range of 250,000–2500 pg/mL for CC spiking solutions and 225,000 pg/mL, 27,000 pg/mL, 7492.50 pg/mL and 2500 pg/mL for QC spiking solutions viz. SS HQC, SS MQC, SS LQC and SS LLOQ QC respectively. Nebivolol-D4 working solution (75 ng/mL) was prepared by diluting its stock solution with diluent. All this stock solutions, intermediate solutions, CC spiking solutions and QC spiking solutions were stored at 2–8 °C. These solutions were found to be stable and used for complete method validation. Spiked CC standards were prepared by spiking the respective CC spiking solutions in drug free K_3 EDTA human plasma to establish final range of 5000 pg/mL to 50.0 pg/mL. Similarly, spiked QC samples with four different levels like lower (LLOQ QC), low (LQC), middle (MQC) and higher (HQC) having concentrations 50 pg/mL, 150 pg/mL, 540 pg/mL and 4500 pg/mL respectively were also prepared.

2.3. Apparatus and software

A Shimadzu LC-VP HPLC system (Kyoto, Japan) consisting of LC-20AD prominence pump, SIL-HTc autosampler, CTO 10 ASvp column oven and DGU-20A3 degasser was used for setting the reverse phase liquid chromatography. Ionization and detection of analyte and its IS was carried out on a triple quadrupole mass spectrometer, MDS SCIEX API 4000 (Toronto, Canada), equipped with electro spray ionization and operating in positive mode. The chromatographic integration was performed by Analyst software

(version: 1.4.2; Applied Biosystems). The data was generated using Watson LIMS software, version: 7.3.

2.4. Chromatographic condition

Chromatographic separation was done using Waters symmetry, C18, 4.6 × 150 mm, 5 μm, analytical column and the mobile phase was a mixture of formic acid in water, 0.01% to acetonitrile at a ratio of 40:60% v/v. Injection volume was 5 μL. The flow rate was 0.800 mL/min, no splitting. Total analysis time of single injection was 2.00 min. Column oven temperature and auto sampler temperature was set to 40 ± 3 °C and 5 ± 3 °C respectively. Other parameters like rinsing volume, rinsing speed, needle stroke, sampling speed, purge time and rinse dip time were set to 500 μL, 35 μL/s, 52 mm, 5.0 μL/s, 1.000 min and 2 s respectively. Rinse mode was set to before and after aspiration.

2.5. Mass spectrometric conditions

The plasma concentrations of nebivolol were quantified using API 4000 LC–MS/MS system (MDS Sciex, Canada). The instrument was set to LC Sync synchronization mode. Turbo ion spray (TIS) probe was used for positive ion generation $[M+H]^+$. Scan type was a Multiple Reactions Monitoring (MRM). Tuning of instrument was done in positive mode to set various compound dependent mass parameters and source dependent mass parameters. The optimized source dependent parameters like Curtain gas (CUR), Ion spray voltage (IS), Temperature (TEM), Nebulizer gas (GS1), Heater gas (GS2) and collision gas (CAD) were set at 20, 5500 V, 500 °C, 40, 60 and 7 respectively. The optimized compounded dependent parameters like declustering potential, collision energy, entrance potential, and collision cell exit potential were 90, 40, 10 and 12 V for nebivolol and 100, 45, 10 and 12 V for the IS respectively. Nitrogen gas was used as Gas 1, Gas 2, Curtain gas and Collision-Activated Dissociation (CAD) gas. Quantification was done with a peak area ratio (area of nebivolol/area of IS) and a linear least-squares regression curve with weighting factor of $1/x^2$. The mass transitions used for nebivolol and the IS were m/z 406.00 → 151.00 and m/z 410.20 → 151.00 respectively, with a dwell time of 200 ms per transition. The analytical data were processed by Analyst software (Version 1.4.2; Applied Biosystems).

2.6. Sample preparation

Nebivolol and its IS were extracted using one step LLE method. Though different methods like Protein Precipitation (PPT) and Solid Phase Extraction (SPE) were also tried to increase sensitivity and chromatography, acceptable chromatography and sensitivity were obtained by using LLE method only. In 0.500 mL of spiked plasma sample, 50 μL of ISTD Dilution (nebivolol-D4 dilution, 75 ng/mL) was added except STD BL sample. After vortexing for 20 s, 50 μL of 0.1 N sodium hydroxide was added and vortexed again for about 20 s. Followed by this, 2.5 mL of extraction solvent (diethyl ether: dichloromethane; 50:50 v/v) was added and extracted on extractor (ROTISPIN) for 20 min at 40 rpm followed by centrifugation at 4000 rpm for 5 min at 10 °C. Then, the organic phase (2.0 mL) was evaporated to dryness using an evaporator (Caliper Turbovap LV) at 40 °C under a stream of nitrogen. The dried extract was reconstituted in 100 μL of reconstitution solution (Methanol:Water; 50:50 v/v) and transferred into pre-labeled auto-sampler vials. From these, a 5 μL volume was injected into the chromatographic system.

3. Results and discussion

3.1. Method development

LC–MS/MS method with electron spray ionization was used to analyze nebivolol as it is advantageous to develop very selective, sensitive and reproducible method [21]. Tuning was done in positive mode to find m/z of Q1 ion (parent ion) and m/z of Q3 ion (daughter ion) to increase selectivity and sensitivity of the method. The positive ion TurboIonspray Q1 mass spectrum and product ion mass spectrum of nebivolol and its IS are shown in Figs. 2–5. $[M+H]^+$ ion was considered as predominant ion as it was obtained with high intensity in spectrum during tuning and was used as a precursor ion to obtain product ion. Hence, 406.00–151.00 mass transition for nebivolol and 410.20–151.00 mass transition for IS were found appropriate to develop selective method for nebivolol estimation. Various mass parameters were optimized to increase sensitivity of present method. Similarly, various HPLC parameters were also optimized to increase response (in terms of peak area). A variety of reverse phase HPLC columns range from 50 mm to 150 mm in length and a different mobile phase composition were tried to get better separation and peak shape. Acceptable chromatography was obtained in Waters symmetry, C18, 4.6 × 150 mm, 5 μm column with formic acid in water, 0.01% v/v: acetonitrile (40:60) mobile

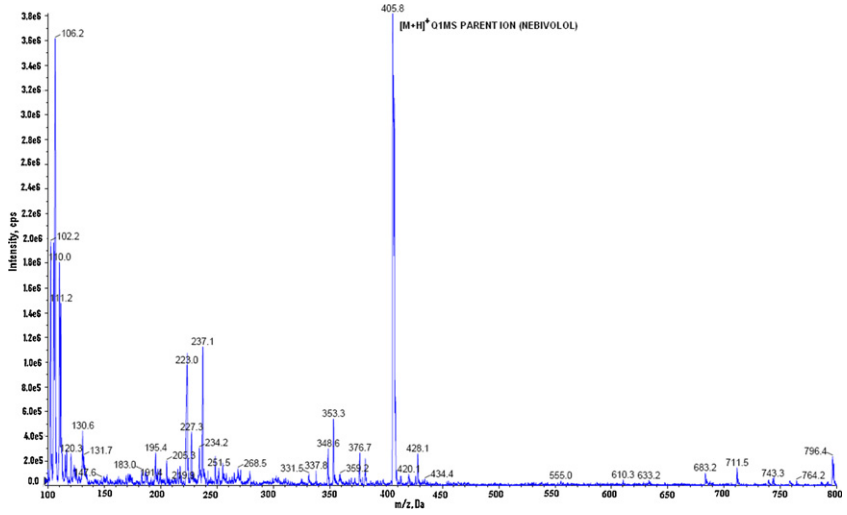

Fig. 2. Q1 mass spectrum of Nebivolol.

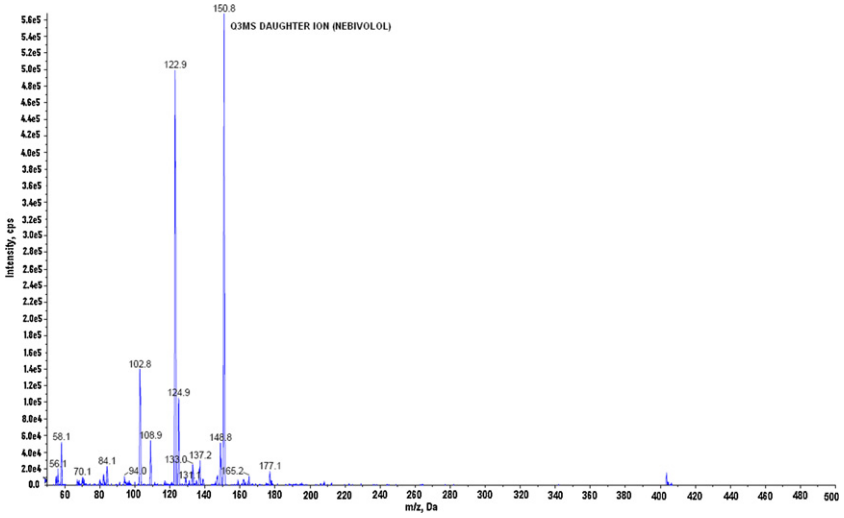

Fig. 3. Q3 mass spectrum of Nebivolol.

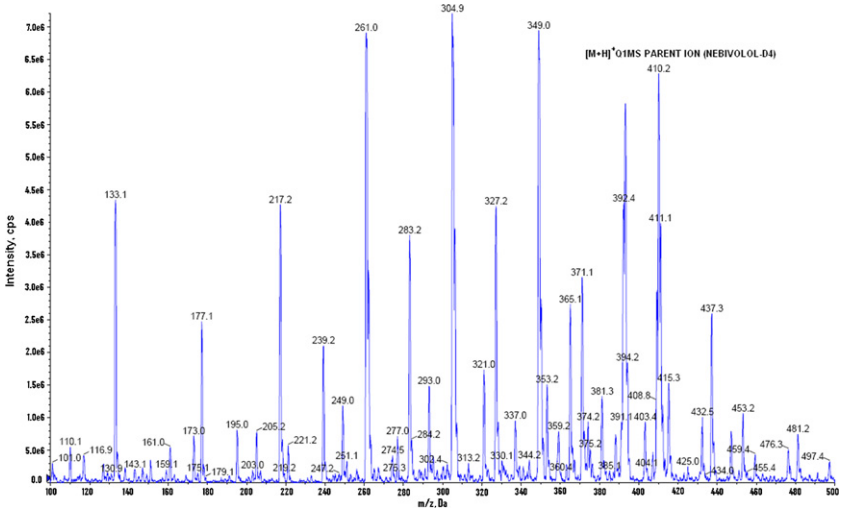

Fig. 4. Q1 mass spectrum of Nebivolol-D4.

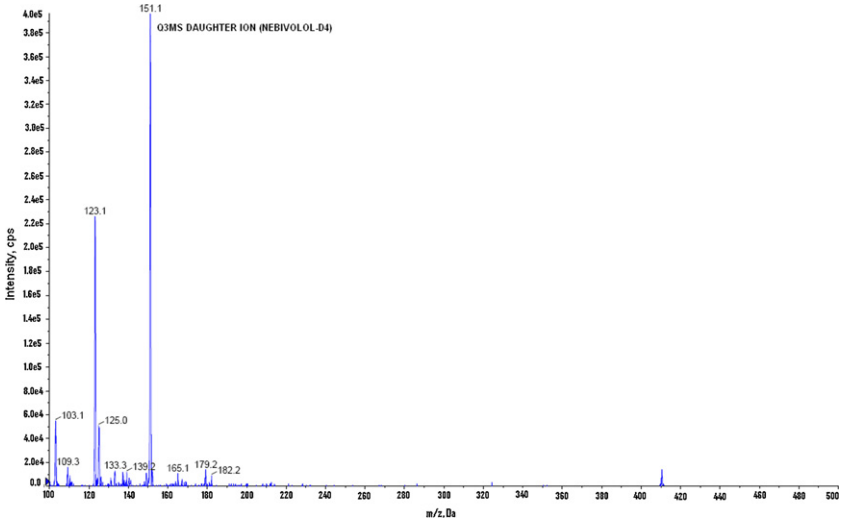

Fig. 5. Q3 mass spectrum of Nebivolol-D4.

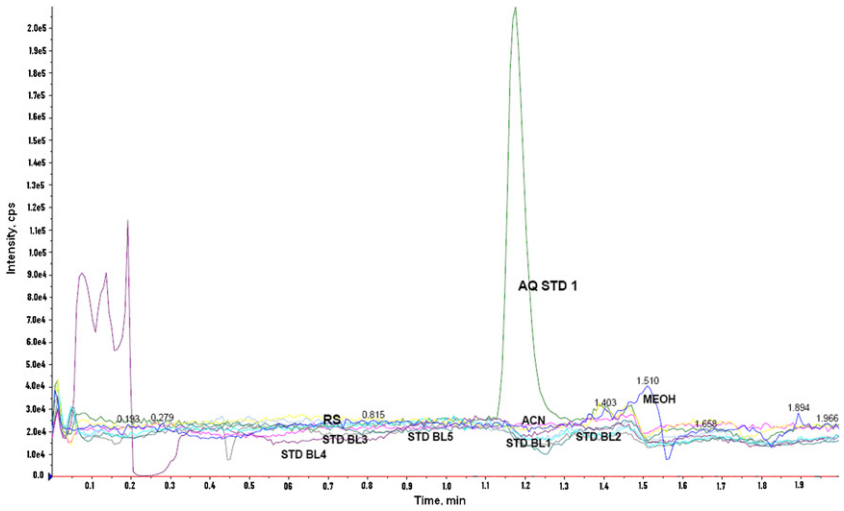

Fig. 6. Evaluation of ion suppression/enhancement during PCI experiment.

phase. LLE method was selected for the extraction of analyte from the human plasma. In SPE method, inconsistency in the drug and IS extraction were found and samples were not clear. In LLE methods, matrix effect problem was not found and consistency in the recovery of IS and drug were observed. So, it was decided to optimize the LLE method by changing the various parameters like extraction solvent, extraction buffer, mobile phase and reconstitution solution for better quantification of analyte from human plasma by reducing matrix effect. One of the best approaches to reduce problem of matrix effect is to use stable isotope labeled analyte as an internal standard. Nebivolo-D4 is a radio labeled isotope of nebivolol having same chromatographic retention, recovery and ionization properties like nebivolol. The matrix effect was similar to that of the nebivolol. Hence, nebivolol-D4 has been opted as an internal standard for the quantification of nebivolol from human plasma. PCI experiment was also done to check ion suppression at RT of analyte and IS and the developed method was found to be free from Ion suppression at RT of the analyte and IS. Fig. 6 illustrates that there is no ion suppression/enhancement at RT of Nebivolol and IS. This method is also free from bioanalytical risk associated with plasma phospholipids [22]. Effect of different fragments with different m/z of phospholipids during long run of extracted STD BL sample is shown in Fig. 7. Three precision and accuracy batches (P&A) include STD BL, STD ZERO, STD 1–8 and 6 replicates of HQC to LLOQ QC were performed before starting method validation. The peak area ratio was found to be liner with respect to

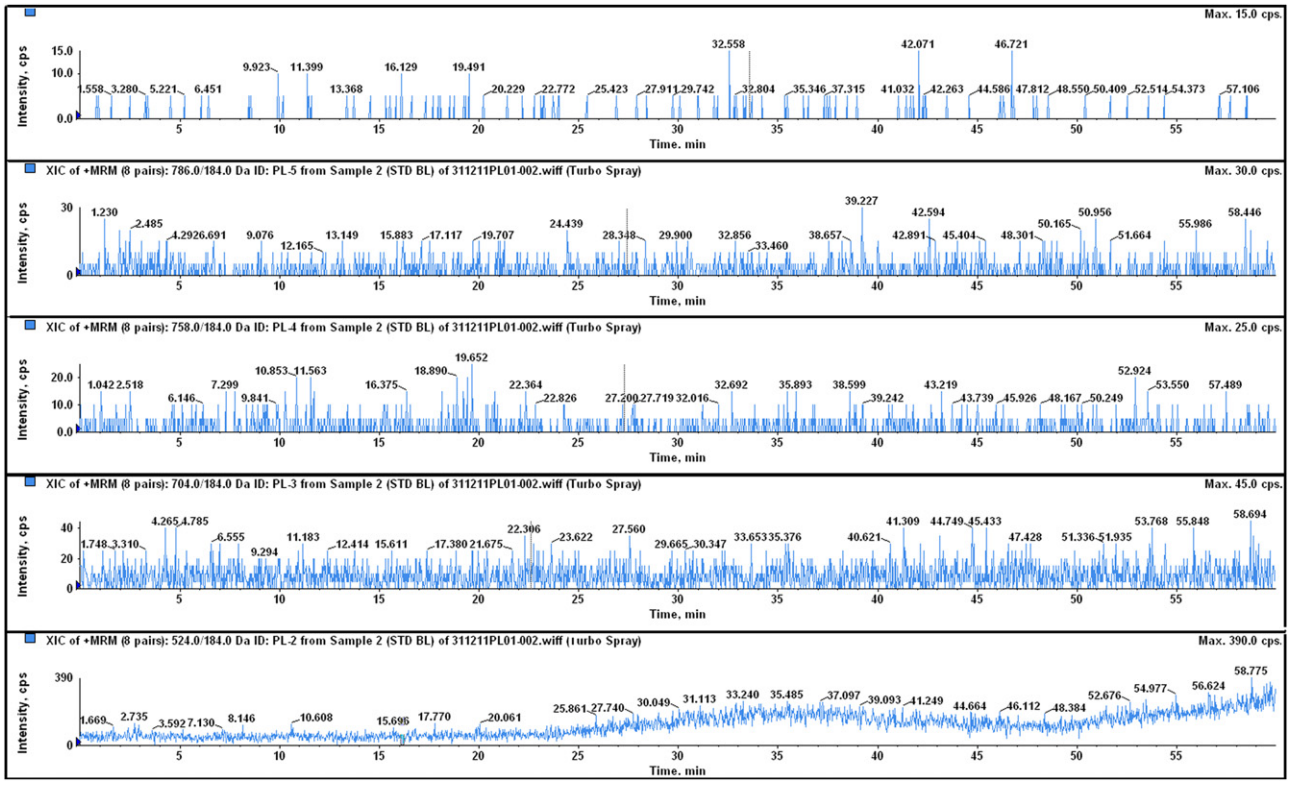

Fig. 7. No interferences of different fragments with different m/z of phospholipids during estimation of Nebivolol.

Table 1
Summary of stability experiments for nebivolol.

Stability	Nominal concentration (pg/mL)	Mean concentration of comparison samples (pg/mL)	Mean concentration of stability samples (pg/mL)	S.D.	% R.S.D.
Dry extract stability	4500.00	4460.00	4520.00	42.426	0.94
	150.00	144.00	142.67	0.943	0.66
Wet extract stability	4500.00	4460.00	4330.00	91.924	2.12
	150.00	144.00	148.00	2.828	1.91
Bench-top stability	4500.00	4460.00	4146.67	221.560	5.34
	150.00	144.00	147.00	2.121	1.44
Freeze-thaw stability at −20 ± 5 °C	4500.00	4460.00	4326.67	94.281	2.18
	150.00	144.00	139.00	3.536	2.54
Freeze-thaw stability at −78 ± 8 °C	4500.00	4460.00	4280.00	127.279	2.97
	150.00	144.00	138.33	4.007	2.90
Long term stability at −20 ± 5 °C	4500.00	4360.00	4290.00	325.000	7.58
	150.00	141.00	145.00	12.500	8.62
Long term stability at −78 ± 8 °C	4500.00	4360.00	4110.00	62.600	1.52
	150.00	141.00	135.00	9.980	7.39

Stock solution stability	Nominal concentration (pg/mL)	Mean area ratio of comparison samples	Mean area ratio of stability samples	S.D.	% R.S.D.
Short term stability of drug	5000.00	0.948	0.941	0.005	0.53
Short term stability of ISTD	75.00	1.055	1.035	0.014	1.35
Long term stability of Drug	5000.00	0.968	0.987	0.014	1.37
Long term stability of ISTD	75.00	1.034	0.987	0.033	3.39

concentration over the range of 50–5000 pg/mL. Different weighing factors like no weighing factor, $1/x$ and $1/x^2$ were applied to get best linear regression. Minimum absolute error was found with $1/x^2$.

3.2. Method validation

Developed LLE method for nebivolol was validated according to USFDA guidelines. To determine various validation parameters during the method validation process, procedure of extracted sample preparation was followed to prepare all samples for each P&A batch and other experiments such as matrix effect, specificity, auto-sampler carryover, recovery, ruggedness and stability. Stability of nebivolol and IS were assessed in different condition like in plasma during storage, during processing, in dry extract, after 5 freeze-thaw cycles and in stock & working solutions. Stability samples were compared with freshly processed calibration standards and QC samples. Stability studies were carried out at higher and lower concentration level (HQC (4500 pg/mL) and LQC (150.00 pg/mL)) respectively. Summary of results of stability experiments is given in Table 1. System suitability experiment was performed by injecting six consecutive injections using aqueous MQC vial at the start of the method validation and on each day. The % CV of system suitability was observed in the range of 0.05–0.17% for RT of drug, 0.05–0.17% for RT of ISTD and area ratio in the range of 0.63–2.12% during different days of method validation. Auto-sampler carryover experiment was also performed at the start of method validation to check carryover of ULOQ (STD 1) in STD BL sample at RT of analyte and IS. There was no significant carry over observed in reconstitution solution (RS) and in STD BL during this experiment. Following experiments were performed for the method validation.

3.2.1. Selectivity

Selectivity was proved by determining two different parameters:

A Specificity: The specificity of the intended method was established by screening the standard blank with different batches/lots of commercially available human plasma. Seven different lots of plasma (K_3EDTA) and three different lots of plasma (one lipidemic, one hemolytic and one heparinised) were screened and found free from endogenous significant interferences (i.e. area of the peak at the RT of drug in standard blank samples was ≤20.00% of the area of the drug in the extracted LLOQ sample; area of the peak at the RT of IS in standard blank samples was ≤5.00% of the area of the ISTD in the extracted LLOQ sample). Refer Figs. 8 and 9 for representative chromatogram of STD BL and LLOQ.

B Matrix effect: Matrix effect was evaluated by calculating matrix factor. Six different screened blank plasma lots including hemolytic and lipidemic were processed as per extraction procedure. After extraction, the dry extract was spiked with analyte at concentration equivalent to those in the low, middle and high QC extracted samples ($n = 2$ at each level for each lot of biological matrix) and internal standard at its working concentration. Aqueous vials of neat solutions of analyte and internal standard with the concentrations equivalent to those in low, middle and high QC extracted samples ($n = 6$ at each level) were also prepared. Mean peak area in presence of matrix ions by considering the mean of peak area of the post extraction spiked samples at low, middle and high QC level and mean peak area in absence of matrix ions by considering the mean of the peak area of the neat solutions of analyte and IS prepared at levels equivalent to low, middle and high QC extracted samples were calculated. Matrix factor was calculated by dividing mean peak area in the presence of matrix ions with mean peak area in the absence of matrix ions. % variability in the matrix factor at each level as measured by the coefficient of variation and overall coefficient of variation for all three levels was found less than 15%.

3.2.2. Linearity, accuracy, and precision of calibration curve standards

For linearity, accuracy and precision three precision and accuracy batches (P&A) were performed by following the procedure of extracted sample preparation. Each P&A batch consists of one STD BL, one STD zero (Blank + ISTD), STD 1–8, six sets of each HQC, MQC, LQC, LLOQ QC. The linearity of the method was determined by using a $1/x^2$ weighted least square regression analysis of standard plots associated with an eight point standard curve. All the three calibration curves analyzed on different days during the course of validation were linear for the standards ranging from 50 to

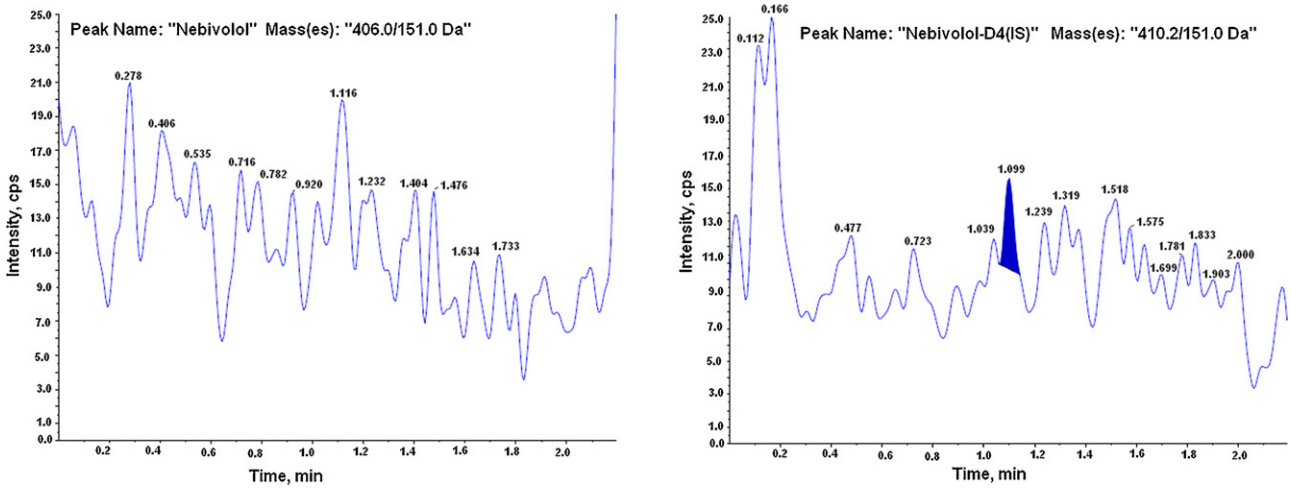

Fig. 8. Representative chromatograms of STD BL.

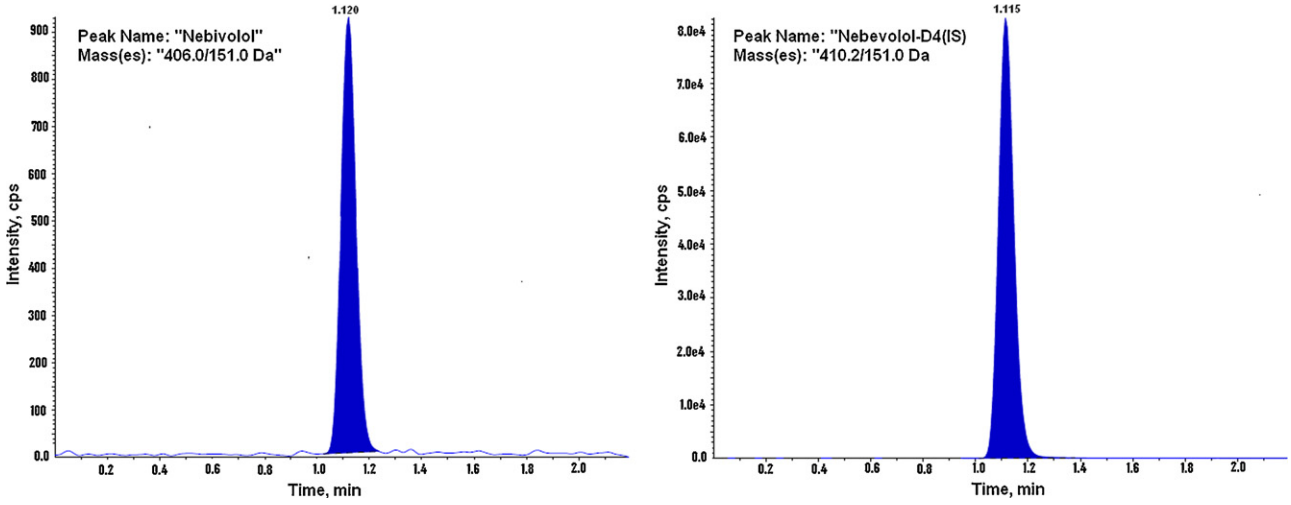

Fig. 9. Representative chromatograms of LLOQ.

5000 pg/mL. Linear calibration curve with eight point standard is shown in Fig. 10. The regression coefficient observed more than 0.99 during the course of validation. The mean accuracy observed for the CC standards were ranged from 85.00 to 115.00% except of LLOQ standard where it was 80.00–120.00%. The % mean precision observed for the all CC standards were found within the acceptance limit of 15.00% except of LLOQ standard where it was 20.00%. Refer Table 2.

3.2.3. Accuracy and precision of quality control samples

Accuracy was evaluated by measuring % mean accuracy at each concentration level of QC and precision was calculated by measuring %CV at each concentration level of QC. The result showed that the analytical method was accurate, as the accuracy of QCs (within- and between-batch) were within the acceptance limits of 85.00–115.00% at their respective concentration levels. The precision of QCs (within- and between-batch) around the mean value was never greater than 15% at any of the concentrations level. Refer Table 3.

3.2.4. Ruggedness

Ruggedness was performed by using three precision and accuracy batches. One batch was analyzed by using different column, second batch was analyzed by using different analyst and third batch was analyzed by using different equipment. In all the three cases the % accuracy and precision for all CC standards and QC samples were found within the acceptance criteria. Refer Table 4.

Table 2
Back calculated concentrations for calibration curve standards.

STD ID	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8
Nominal concentration (pg/mL)	5000.00	3000.00	1500.00	750.00	400.00	200.00	100.00	50.00
P&A I	5110.00	3010.00	1510.00	755.00	380.00	199.00	103.00	49.60
P&A II	5200.00	3040.00	1520.00	741.00	397.00	201.00	90.00	52.50
P&A III	4910.00	3060.00	1480.00	761.00	403.00	199.00	98.90	50.30
Mean	5073.33	3036.67	1503.33	752.33	393.33	199.67	97.30	50.80
SD	148.44	25.17	20.82	10.26	11.93	1.15	6.65	1.51
% CV	2.93	0.83	1.38	1.36	3.03	0.58	6.83	2.98
% Mean accuracy	101.47	101.22	100.22	100.31	98.33	99.83	97.30	101.60

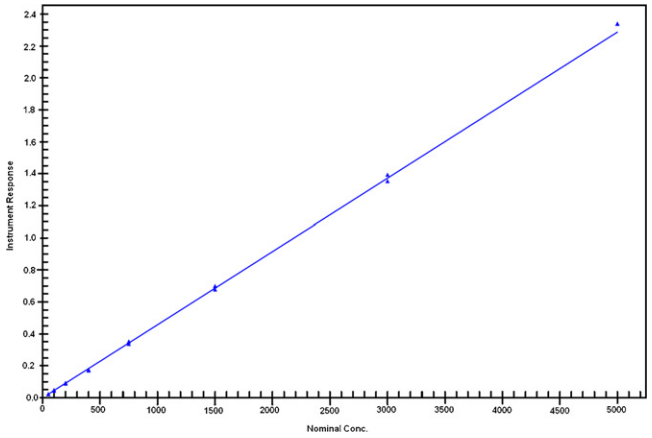

Fig. 10. Liner calibration curve of calibration standards for Nebivolol with weighting factor $1/x^2$, slope = 0.0005, intercept = −0.0008, r^2 = 0.9984 and response = slope × conc. + intercept.

Table 3
Within and between accuracy and precision for QC samples.

Within batch accuracy and precision				
	HQC	MQC	LQC	LLOQ QC
Nominal concentration (pg/mL)	4500.00	540.00	150.00	50.00
P&A01				
Mean	4458.00	537.80	144.20	43.92
SD	38.341	1.789	4.147	0.683
% CV	0.86	0.33	2.88	1.56
% Mean accuracy	99.07	99.59	96.13	87.84
% RE	−0.93	−0.41	−3.87	−12.16
P&A02				
Mean	4434.00	538.20	143.00	44.36
SD	71.624	12.755	4.301	2.688
% CV	1.62	2.37	3.01	6.06
% Mean accuracy	98.53	99.67	95.33	88.72
% RE	−1.47	−0.33	−4.67	−11.28
P&A03				
Mean	4420.00	522.40	150.60	47.30
SD	82.158	15.010	11.238	4.099
% CV	1.86	2.87	7.46	8.67
% Mean accuracy	98.22	96.74	100.40	94.59
% RE	−1.78	−3.26	0.40	−5.41
Between-batch accuracy and precision				
Mean	4437.33	532.80	145.93	45.19
SD	63.860	13.029	7.630	3.067
% CV	1.44	2.45	5.23	6.79
% Mean accuracy	98.61	98.67	97.29	90.38
% RE	−1.39	−1.33	−2.71	−9.62

3.2.5. Recovery

The % mean recovery was determined by measuring the response of the extracted plasma quality control samples at HQC, MQC and LQC against un-extracted quality control samples at HQC, MQC and LQC. The % mean recovery for nebivolol in HQC, MQC and LQC was 74.71, 63.96, and 64.33% respectively. The mean recovery of all three QC levels was 67.67% where as the mean recovery of IS was 68.74%.

3.2.6. Bench-top stability

Bench-top stability was determined for 6 h 6 min at ambient temperature by keeping the spiked quality control samples at room temperature and then analyzed with the fresh comparison samples.

3.2.7. Freeze-thaw stability

Freeze-thaw stability of the spiked quality control samples was carried out for 5 cycles at −20 ± 5 °C and at −78 ± 8 °C. The deviations observed after the fifth freeze-thaw cycle were within ±10%, at the concentration levels used for nebivolol, indicating adequate freeze-thaw stability.

3.2.8. Auto-sampler stability (wet extract stability)

After a full P&A batch was run, six replicates of HQC and LQC vials were kept in auto-sampler to prove stability and maintained at 5 °C. After 28 h 49 min, the same samples in the same sequence were re-injected for analysis and the results were compared with that of the comparison samples. Wet extract stability was carried out at 5 °C for 28 h.

3.2.9. Dry extract stability

One set of six replicates of HQC and LQC were processed (up to drying) by using the procedure for extracted sample preparation. Dried samples (before reconstitution) in ria vials were capped by white cap and stored in deep freezer below −20 °C. After about 24 h 20 min dried samples were reconstituted with reconstitution solution and injected for analysis. Dry Extract stability was carried out below −20 °C for 24 h 20 min.

3.2.10. Short term and long term stock solution stability

Short term and long term stock solution stabilities for drug at concentration 0.250 mg/mL were determined using aqueous standards approximately equivalent to SS STD1 concentration (250,000 pg/mL) after the storage for 9 h at ambient temperature for STSS and 28 days 19 h at 5 ± 3 °C for LTSS. Stability was assessed by comparing against the freshly prepared Drug stock solutions which were diluted approximately equivalent to SS STD1 concentration 250,000 pg/mL. STSS and LTSS for Nebivolol and ISTD were proved for 9 h at ambient temperature and 28 days at 5 ± 3 °C respectively.

Table 4
Ruggedness for drug-mean calculated concentrations for CC standards and QC samples.

Sample ID	Nominal concentration (pg/mL)	Mean calculated concentration (pg/mL)	% CV	% Mean accuracy
STD 1	5000.00	4913.33	2.24	98.27
STD 2	3000.00	3000.00	0.33	100.00
STD 3	1500.00	1480.00	0.00	98.67
STD 4	750.00	758.00	0.48	101.07
STD 5	400.00	401.67	0.72	100.42
STD 6	200.00	203.67	1.24	101.83
STD 7	100.00	101.57	4.59	101.57
STD 8	50.00	49.40	2.13	98.80
HQC	4500.00	4420.00	1.91	98.22
MQC	540.00	528.00	1.00	97.78
LQC	150.00	149.00	5.20	99.33
LLOQ QC	50.00	48.60	3.74	97.20

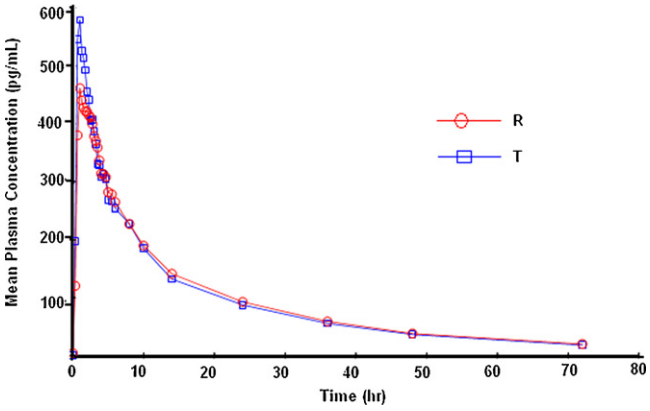

Fig. 11. Linear plot of mean plasma concentrations of racemic nebivolol after administration of reference (R) and test (T) formulation in normal, healthy, adult, human male and female subjects under fasting condition.

3.2.11. Long term stability at −20 ± 5 °C and −78 ± 8 °C

Long term stability of the spiked quality control samples was determined by using six replicates of HQC and LQC stored at −78 ± 8 °C and −20 ± 5 °C for at least a period of 30 days. The stability samples analyzed along with freshly spiked CC and six replicates of freshly prepared LQC and HQC samples. long term stability of analytes will be assessed by comparing the stability samples against six replicates of freshly prepared LQC and HQC samples (as comparison/recently prepared samples). Long term stability at −20 ± 5 °C and −78 ± 8 °C for Nebivolol and IS was proved for 63 days.

3.2.12. Concomitant drug experiment (CDE)

To evaluate the selectivity of the method for nebivolol in presence of over the counter (OTC) like acetaminophen, nimesulide, cetirizine, domperidone, rantidine, diclofenac and ibuprofen at concentration 4 μg/mL, 9 μg/mL, 400 ng/mL, 20 ng/mL, 545 ng/mL, 2 μg/mL and 45 μg/mL respectively, one STD BL and one sample equivalent to LLOQ with spiked concomitant drugs were prepared separately using at least 6 different lots of plasma. CDE experiment was found within the acceptance criteria as response of interfering peaks at RT of the drug was less than 20% of the response of respective LLOQ and response of interfering peaks at RT of the IS was less than 5% of the response of IS of the respective LLOQ.

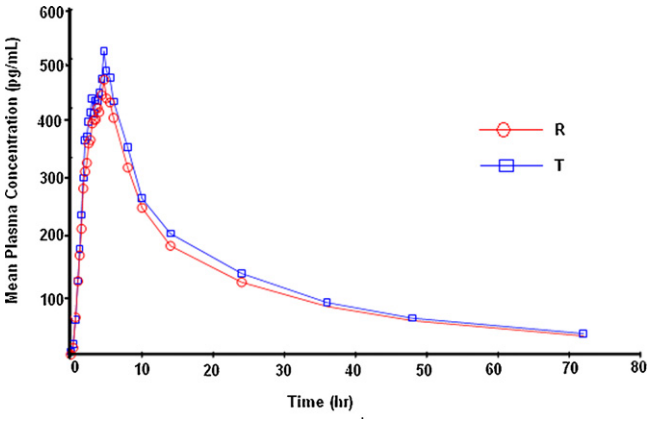

Fig. 12. Linear plot of mean plasma concentrations of racemic nebivolol after administration of reference (R) and test (T) formulation in normal, healthy, adult, human male and female subjects under fed condition.

3.2.13. Batch size experiment

Batch size experiment was performed to evaluate the maximum number of samples that can be processed and analyzed in a single batch during subject sample analysis. Experiment was performed by processing STD BL, STD ZERO, two replicate of CC standards and 38 replicates of each of HQC, MQC and LQC. Experiment was passed as all CC standards and QC samples were found within the acceptance criteria. No drifting was observed during long batch run.

3.3. Application

The validated bioanalytical method was successfully used to quantify racemic nebivolol in human plasma after administering 20 mg of nebivolol in 48 human subjects in both fast and fed condition. The analysis of subject's samples will be done using a calibration curve with quality control samples will be equally distributed throughout the analytical batch. Pharmacokinetic parameters, T_{max}, C_{max}, AUC_{0-t}, $AUC_{0-\infty}$, $t_{1/2}$ and K_{el} can be calculated for racemic nebivolol using the plasma concentration vs time profile (actual time of sample collection) data of both investigational products in individual subjects using WinNonlin® Professional Software Version 5.3 or higher (Pharsight Corporation, USA). All concentration values below the Limit of Quantification (LOQ) will be set to "zero" for all pharmacokinetic and statistical calculations. Profile of the mean plasma concentration of nebivolol versus time after administering 20 mg dose of nebivolol under fasted and fed condition are shown in Figs. 11 and 12 respectively.

4. Conclusion

A rapid, sensitive, specific, precise and accurate Bioanalytical method for Nebivolol in human plasma has been developed and validated with a larger calibration curve range (i.e. 50–5000 pg/mL) using liquid chromatography–tandem mass spectrometry which can be used for routine drug analysis and bioequivalence studies. Moreover, developed bioanalytical method for nevibolol is a highly sensitive (lower limit of quantification 30 pg/mL) which is significant advantages over the other technique and can be employed for the routine quality control analysis. LLE method was performed on 500 μL buffered plasma. High accuracy and good precision obtained from the statistical analysis in the proposed method shows adequate reliability. This method was productively applied to generate pharmacokinetic profile for nebivolol after administrating 20 mg dose of nebivolol in 48 human subjects in both fast and fed condition.

Acknowledgements

The authors acknowledge the support provided by Veeda Clinical Research Pvt. Ltd. for performing the aforementioned studies. The authors thank Mr. Primal Sharma for his constructive comments regarding the project and Mr. Hardik Gandhi for providing help in preparing the manuscript.

References

[1] J.R. Cockcroft, et al., J. Pharmacol. Exp. Ther. 274 (1995) 1067.
[2] Drug bank open data drug & drug target data base. http://www.drugbank.ca/drugs/DB04861 (last accessed 4.08. 12.).
[3] Bystolic™ Prescribing information, Forest Pharmaceuticals Inc., 12, 2007.
[4] R. Fogari, et al., J. Hum. Hypertens. 11 (1997) 753.
[5] N.L. Van, et al., Am. J. Ther. 5 (1998) 237.
[6] N.L. Van, et al., J. Hum. Hypertens. 11 (1997) 813.
[7] N.L. Van, et al., J. Hum. Hypertens. 12 (1998) 135.
[8] I. Czuriga, et al., Cardiovasc. Drugs Ther. 17 (2003) 257.

[9] Vascular Health and Risk Management (2006) PubMed Central, Dovepress. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1993984/ (last accessed 4.08.12).
[10] B.S. Sastry, D. Srinivasulu, H. Ramana, J. Pharm. Res. Health Care 1 (2009) 25.
[11] M. Levesque, M. Coulombe, J. Couture, F. Vallee, AAPS J. 8 (2006) S2.
[12] P.S. Senthamil, V.K. Gowda, U. Mandal, W.D. Sam Solomon, T.K. Pal, J. Chromatogr. 585 (2007) 143.
[13] S.U. Kokil, M.S. Bhatia, Indian J. Pharm. Sci. 71 (2010) 111.
[14] T.R. Covey, E.D. Lee, J.D. Henion, Anal. Chem. 58 (1986) 2453.
[15] T.R. Covey, Anal. Chem. 57 (1985) 474.
[16] G. Evans, A Handbook of Bioanalysis and Drug Metabolism, Crc Press, Boca Raton, USA, 1996.
[17] M. Ivano, V. Veronique, B. Flavia, F. Marc, S. Martial, R. Serge, V. Jean-Luc, J. Chromatogr. A 1217 (2010) 4071.
[18] V.E. Ann, L. Katrien, S. Sophie, S. Ilse, M. Yuette, J. Chromatogr. B 877 (2009) 2198.
[19] G. Fabio, M. Eleonora, Z. Davide, C.G. Maria, J. Chromatogr. A 1217 (2010) 3929.
[20] Guidance for Industry Bioanalytical Method Validation, US Department of Health and Human services, Food and Drug Administration, Center for Drug Evaluation and Research Rockville. http://www.fda.gov/cder/guideline.html, 2001 (last accessed 4.08.12).
[21] E.D. Hoffmann, V. Stroobant, Mass Spectrometry Principles and Applications, 3rd ed., Wiley, New York, 1991.
[22] J. Mohammed, O. Zheng, X. Yuan-Qing, Biomed. Chromatogr. 24 (2010) 2.