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Rapid quantification of nebivolol in human plasma by liquid chromatography coupled with electrospray ionization tandem mass spectrometry

N.V.S. Ramakrishna *, K.N. Vishwottam, M. Koteshwara, S. Manoj, M. Santosh, D.P. Varma

Biopharmaceutical Research, Suven Life Sciences Ltd., Serene Chambers, Road #7, Banjara Hills, Hyderabad 500034, India

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Abstract

A simple, sensitive and rapid liquid chromatographic/electrospray ionization tandem mass spectrometric method was developed and validated for the quantitation of nebivolol in human plasma. The method involved a simple single-step liquid–liquid extraction with diethyl ether/dichloromethane (70/30). The analyte was chromatographed on Waters symmetry® C_{18} reversed-phase chromatographic column by isocratic elution with water:acetonitrile:formic acid (30:70:0.03, v/v) and analyzed by mass spectrometry in the multiple reaction monitoring mode. The precursor to product ion transitions of *m/z* 406.4–151.5 and *m/z* 409.1–228.1 were used to measure the analyte and the internal standard (I.S.), respectively. The chromatographic runtime was 2 min and the weighted ($1/x^2$) calibration curves were linear over the range 50–10,000 pg/mL. The method was validated in terms of accuracy, precision, absolute recovery, freeze-thaw stability, bench-top stability and re-injection reproducibility. The limit of detection and lower limit of quantification in human plasma were 10 and 50 pg/mL, respectively. The within- and between-batch accuracy and precision were found to be well within acceptable limits (<10%). The analyte was stable after three freeze-thaw cycles (deviation <10%). The average absolute recoveries of nebivolol and tamsulosin, used as an internal standard, from spiked plasma samples were 73.4 ± 3.7 and 72.1 ± 2.0%, respectively. The assay method described here was applied to study the pharmacokinetics of nebivolol.

Keywords: Nebivolol; Reversed-phase chromatography; Tandem mass spectrometry; Multiple reaction monitoring; Human plasma; Pharmacokinetic study

1. Introduction

Nebivolol hydrochloride, (±)-[2*R**[*R**[*R**(*S**)]]]-α,α′-[iminobis(methylene)]bis-[6-fluoro-3,4-dihydro-2H-1-benzopyran-2-methanol] hydrochloride, the most selective β_1 receptor antagonist currently available for clinical use, is a racemate of two enantiomers with four chiral centers (Fig. 1) [1]. The *SRRR*-enantiomer (D-nebivolol) is a potent and cardioselective β1-adrenergic blocker. The *RSSS*-enantiomer (L-nebivolol) has a favourable hemodynamic profile, in that normal energy supply during exercise is not affected [2–4]. It is a vasodilating β-blocker, which can be distinguished from other β-blockers by its haemodynamic profile [5]. It combined β-adrenergic blocking activity with a vasodilating effect mediated by the endothelial L-arginine nitric oxide (NO) pathway [6]. To date this has been demonstrated in volunteers and small numbers of patients. If this mechanism is shown to result in improved clinical outcomes, nebivolol could be of value in managing hypertensive patients with endothelial dysfunction e.g. those with diabetes mellitus or hypercholesterolaemia and in patients with ischaemic heart disease [7]. The blood pressure lowering effect of nebivolol

* Corresponding author. Tel.: +91 40 23556038/41142; fax: +91 40 23541152.
E-mail address: nvsrk@suven.com (N.V.S. Ramakrishna).

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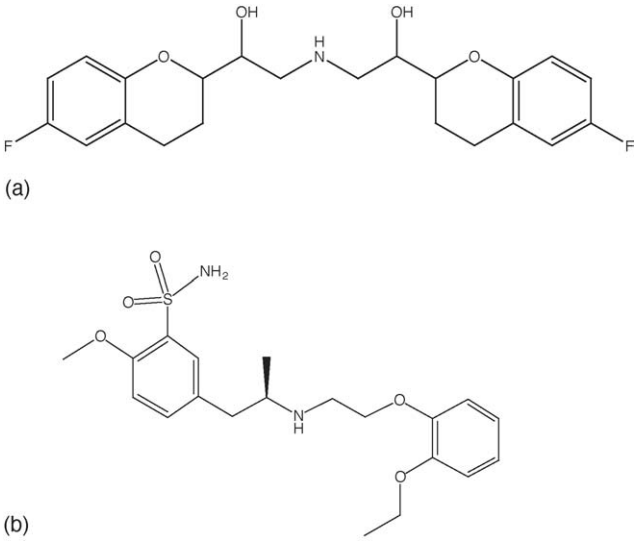

Fig. 1. Chemical structures of (a) nebivolol and (b) tamsulosin (I.S.).

is linked to a reduction in peripheral resistance and an increase in stroke volume and preservation of cardiac output. It is devoid of any intrinsic sympathomimetic activity (ISA) at therapeutic doses [7]. Nebivolol is rapidly absorbed after oral administration: time to peak plasma drug concentration (C_{max}) for the racemic mixture is reported to be about 0.5–2.0 h and is not significantly affected by the presence of food [7,8]. After single dose oral nebivolol 5 mg, the mean C_{max} for unchanged DL-nebivolol was 1.48 ng/mL in healthy volunteers [5]. After oral administration nebivolol undergoes extensive first-pass metabolism, which produces active β-blocking hydroxy-metabolites. Elimination half-lives of DL-, D- and L-nebivolol averaged about 10 h [7–9]. For the particular pharmacokinetic profile and the dual mechanism of action, nebivolol is used in the management of patients affected by mild to moderate uncomplicated essential hypertension with highly favorable tolerance profile [7].

Stereoselective radioimmunoassay methodology has been used to measure active fraction of D- and L-nebivolol and hydroxylated metabolites [7,10,11]. The detection limit of the radioimmunoassay procedure was 0.5 ng/mL. Radioimmunoassays often lack specificity, especially for drugs that are extensively metabolized. To date, there is only one published chromatographic technique on nebivolol determination in human plasma [12]. HPLC-fluorescence method has been explored to quantify nebivolol in human plasma with a limit of detection of 0.1 ng/mL [12]. However, the shortcomings of this method seemed to be unreliability with regard to sensitivity and specificity, especially in the pg/mL range. In addition, the extraction method is time-consuming due to multiple step procedures. In this reported method, nebivolol was extracted from alkalinized human plasma with heptane–isoamyl alcohol (95:5, v/v), back-extracted with dilute sulphuric acid and re-extracted after alkalinization [12].

The advent of the atmospheric pressure ionization (API) source was a breakthrough that allowed the efficient coupling of liquid chromatography (LC) and mass spectrometry (MS). The usefulness of LC/electrospray (ESI) MS has been demonstrated for a wide range of applications in the bioanalytical, environmental and pharmaceutical fields [13–20]. This powerful separation and detection technique is widely used for the determination of drugs in biological fluids. This paper presents, for the first time, the development and validation of a sensitive and specific LC–MS/MS method in the multiple reaction monitoring (MRM) mode for the quantification of nebivolol in human plasma. It was essential to establish an assay capable of quantifying nebivolol at concentration down to 50 pg/mL. At the same time, it was expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of nebivolol. The procedure consisted of a liquid–liquid extraction of nebivolol and tamsulosin (internal standard, I.S.) from 500-μL human plasma with diethyl ether/dichloromethane (70/30, v/v). After extraction, the samples were injected onto a Waters Symmetry® C18 reversed-phase chromatographic column for separation. The analyte was detected by tandem mass spectrometry using positive electrospray ionization in MRM mode. The concentration of the analyte was calculated by peak area ratios of the analyte to the I.S. using standard curves generated with weighted linear regression analysis. Acceptable precision and accuracy of the method were achieved for quality control, freeze-thaw stability and long-term storage stability samples. The runtime of the present method is 2.0 min, which assures for high throughput. The method was validated over the concentration range 50–10,000 pg/mL. The method can be applied to generate pharmacokinetic data following the administration of nebivolol as a part of its pharmacokinetic studies.

2. Experimental

2.1. Chemicals

Nebivolol was obtained from Lotus Labs (Bangalore, India). Tamsulosin, used as internal standard, was obtained from our R&D (Hyderabad, India). Chemical structures are presented in Fig. 1. HPLC-grade LiChrosolv methanol and LiChrosolv acetonitrile were purchased from Merck (Darmstadt, Germany). Dichloromethane, diethyl ether, formic acid and sodium hydroxide pellets were purchased from Merck (Worli, Mumbai, India). HPLC Type I water from Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

2.2. Calibration and quality control samples

Standard stock solutions of nebivolol (1 mg/mL) and the I.S. (1 mg/mL) were separately prepared in 10 mL volumet-

ric flasks with methanol. Working solutions for calibration and controls were prepared from the stock solution by adequate dilution using diluent (water/methanol, 50/50, v/v). The I.S. working solution (20 ng/mL) was prepared by diluting its stock solution with diluent. Twenty-five microliters working solutions were added to 475-μL drug-free human plasma to obtain nebivolol concentration levels of 50, 100, 200, 500, 1000, 2000, 5000 and 10,000 pg/mL. Quality control (QC) samples were prepared separately as a bulk, at four different concentration levels (50, 150, 4000 and 8000 pg/mL as lower limit of quantification (LLOQ), low, medium and high, respectively), as a single batch at each concentration and then divided in aliquots that were stored in the freezer at below −50 °C until analysis. A calibration curve was constructed from a blank sample (a plasma sample processed without the I.S.), a zero sample (a plasma processed with the I.S.) and eight non-zero samples covering the total range (50–10,000 pg/mL), including LLOQ. Such calibration curves were generated using the analyte to I.S. peak area ratios by weighted ($1/x^2$) least-squares linear regression on 6 consecutive days.

2.3. Sample preparation

Sample preparation involved a simple single-step liquid–liquid extraction with diethyl ether/dichloromethane (70/30, v/v) (4 mL). The processing volume of plasma was fixed as 500-μL. The 50 μL of I.S. solution (20 ng/mL) and 50 μL of 0.1N NaOH solution were added into such aliquots and vortex mixed for 30 s prior to the addition of the extraction solvent. After mixing thoroughly with a Multi-pulse vortex mixer (Glas-col, Terre Haute, USA) for 3 min, the supernatant organic layer (3.5 mL) was transferred into another set of clean glass tubes. Then, the organic phase was evaporated to dryness using a TurboVap LV Evaporator (Zymark, Hopkinton, MA, USA) at 40 °C under a stream of nitrogen. The dried extract was reconstituted in 250 μL of diluent and transferred into injector vials. From these, a 25-μL aliquot was injected into chromatographic system.

2.4. LC–MS/MS instrument and conditions

The HPLC Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany) is equipped with G1312A binary pump, G1379A degasser, G1367A autosampler equipped with a G1330B thermostat, G1316A thermostatted column compartment and G1323B control module. The chromatography was on Waters symmetry C_{18} column (5 μm, 150 mm × 4.6 mm i.d.) at 30 °C temperature. The mobile phase composition was a mixture of water:acetonitrile:formic acid (30:70:0.03, v/v), which was pumped at a flow-rate of 1.0 mL/min.

Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using multiple reaction monitoring (MRM). A turbo electrospray interface in positive ionization mode was used.

Table 1
Tandem mass-spectrometer main working parameters

Parameter	Value
Source temperature (°C)	250
Dwell time per transition (ms)	200
Ion source gas (gas 1) (psi)	20
Ion source gas (gas 2) (psi)	20
Curtain gas (psi)	25
Collision gas (psi)	5
Ion spray voltage (V)	5700
Entrance potential (V)	10
Declustering potential, DP (V)	85 (analyte) and 100 (I.S.)
Collision energy (V)	30 (analyte) and 45 (I.S.)
Collision cell exit potential (V)	5.50 (analyte) and 10 (I.S.)
Mode of analysis	Positive
Ion transition for nebivolol (*m*/*z*)	406.4/151.5
Ion transition for tamsulosin (*m*/*z*)	409.1/228.1

The main working parameters of the mass spectrometer are summarized in Table 1. Data processing was performed on Analyst 1.4 software package (SCIEX).

2.5. Validation

The method was validated on accuracy, precision, specificity, sensitivity, linearity, recovery and stability. On day 1 the linearity of the calibration curves and the stability in the autosampler were determined. On the days 2–6 accuracy and precision, sensitivity, recovery and freeze-thaw stability were tested. During validation six blank human plasma samples obtained from six different subjects were tested to demonstrate that there were no interfering components. The results of the tests were evaluated against internationally used acceptance criteria described by Shah et al. [21].

3. Results and discussion

3.1. Method development

Electrospray MS–MS was used to analyze nebivolol, as it is beneficial in developing a selective and sensitive method. The positive ion TurboIonspray Q1 mass spectrum and product ion mass spectrum of nebivolol are shown in Fig. 2a and b, respectively. The positive ion TurboIonspray Q1 mass spectrum and product ion mass spectrum of the I.S. are shown in Fig. 3a and b, respectively. $[M + H]^+$ was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain product ion spectra. The most sensitive mass transition was from *m*/*z* 406.4 to 151.5 for nebivolol and from *m*/*z* 409.1 to 228.1 for the I.S. LC-MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity and specificity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. The MRM state file parameters were optimized to maximize the response for the analyte. The parameters presented in Table 1 are the result of this optimization.

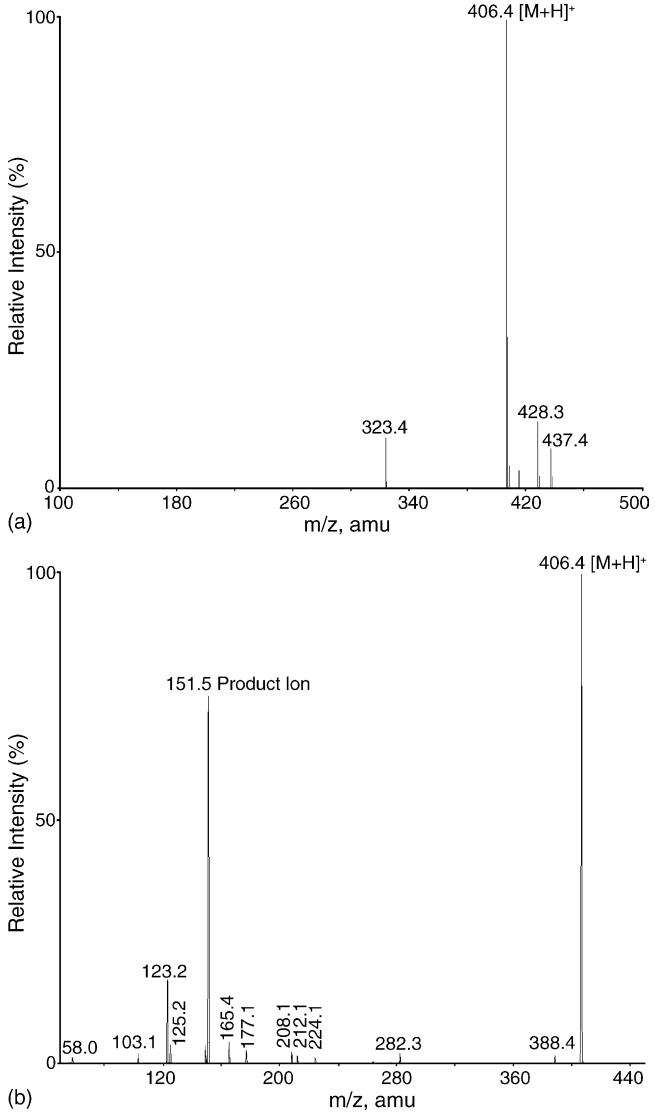

Fig. 2. Full scan positive ion TurboIonspray (a) Q1 mass spectra and (b) product ion mass spectra of nebivolol.

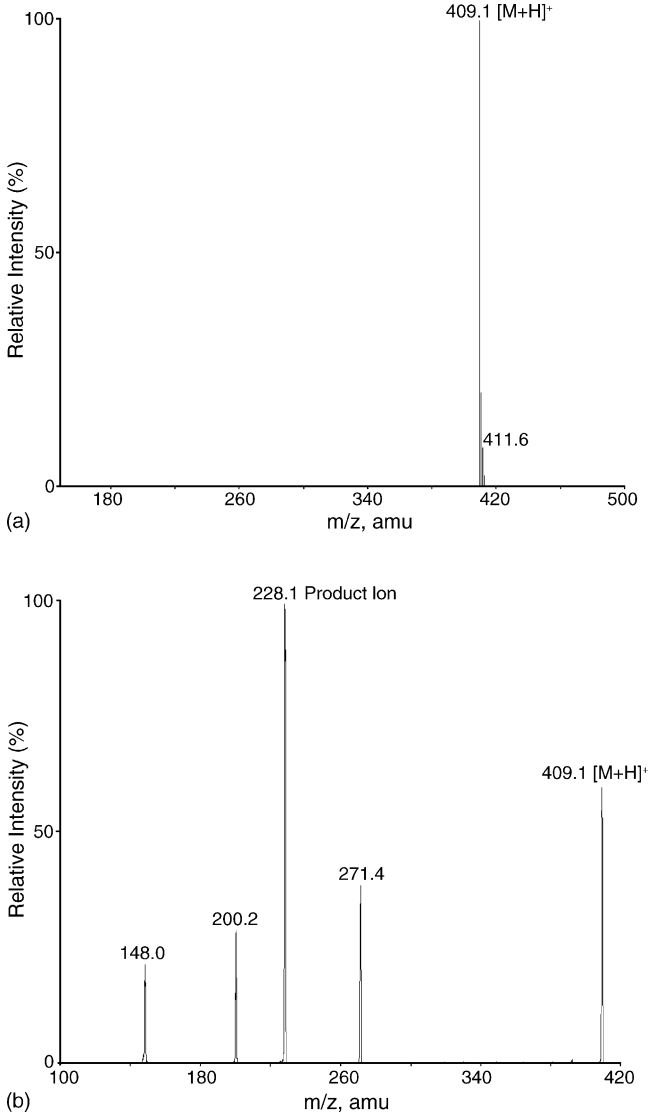

Fig. 3. Full scan positive ion TurboIonspray (a) Q1 mass spectra and (b) product ion mass spectra of I.S.

Different mobile phases consisting of water–methanol or water–acetonitrile were evaluated to improve HPLC separation and enhance sensitivity in MS. Buffers such as formic acid and ammonium formate alone or in combination in different concentrations were added. The best signal was achieved using water:acetonitrile:formic acid (30:70:0.03, v/v). The formic acid was found to be necessary in order to lower the pH to protonate the nebivolol, and thus deliver good peak shape. The percentage of formic acid was optimized to maintain this peak shape whilst being consistent with good ionization and fragmentation in the mass spectrometer.

The tandem mass spectrometer allows the selective detection of substances with varying masses or fragments without chromatographic separation. The development of the chromatographic system was focused on short retention times in order to assure high throughput, paying attention to matrix effects as well as good peak shapes. A high proportion of organic solvent (water:acetonitrile:formic acid (30:70:0.03, v/v)) was used to co elute both substances at retention times of 0.95 min. Flow-rate of 1 mL/min produced a good peak shape and brought the runtime to 2 min.

The best way to cope with sample matrix effects is to use a stable isotope labeled analyte as internal standard. Since such internal standard is not commercially available, an alternative approach has been used. Internal standard substance should match the chromatographic retention, recovery and ionization properties with the matrix of nebivolol. Tamsulosin (Fig. 1) was found to fulfill these criteria sufficiently. The matrix effects were similar to the matrix effects for nebivolol. Hence tamsulosin has been chosen as internal standard in the quantitative assay for nebivolol from plasma.

3.2. Assay performance and validation

Accuracy, precision, specificity, sensitivity, linearity and stability were measured and used as the parameters to assess the assay performance. The peak area ratio of nebivolol to I.S. in human plasma was linear with respect to the analyte concentration over the range 50–10,000 pg/mL. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors ($1/x$, $1/x^2$ and $1/\sqrt{x}$). The residuals improved by weighted ($1/x^2$) least-squares linear regression. The best fit for the calibration curve could be achieved with the linear equation $y = mx + c$ with a $1/x^2$ weighing factor. The mean linear regression equation of calibration curve for the analyte was $y = 0.0001x - 0.0028$, where y was the peak area ratio of the analyte to the I.S. and x was the concentration of the analyte. The correlation coefficient (r) for nebivolol was above 0.999 over the concentration range used. Table 2 summarizes the calibration curve results for the analyte. These calibration curves were suitable for generation of acceptable data for the concentrations of the analyte in the samples during between-batch and within-batch validations.

LC–MS/MS analysis of the blank human plasma samples showed no interference with the quantification of nebivolol and the I.S. The specificity of the method was established with pooled and individual plasma samples from six different sources. Representative chromatograms of extracted blank plasma, blank plasma fortified with nebivolol (LLOQ) and drug-free plasma fortified with I.S. (Fig. 4), demonstrating the specificity of the method. Fig. 4c shows the absence of interference from the I.S. to the MRM channels of the analyte. The retention times of the analyte and the I.S. showed less variability with a relative standard deviation (R.S.D.) well within the acceptable limit of 5%.

The product ion-chromatogram obtained from an extracted plasma sample of a healthy volunteer who participated in a bioequivalence study conducted on 24 persons, is depicted in Fig. 5. Nebivolol was unambiguously identified and was quantified as 636 pg/mL. Fig. 6 represents plasma concentration versus time curve obtained from a subject after the oral administration of 5 mg of nebivolol.

Due to the components of the sample matrix, signal suppression or enhancement may occur. These matrix effects in the LC–MS/MS method were evaluated by spiking blank plasma extracts with low and high QC samples. The resulting chromatograms were compared with chromatograms of pure samples equally concentrated. Six independent plasma lots were used with six samples from each lot. The results (data was not shown) showed that there was no significant difference for peak responses between these samples.

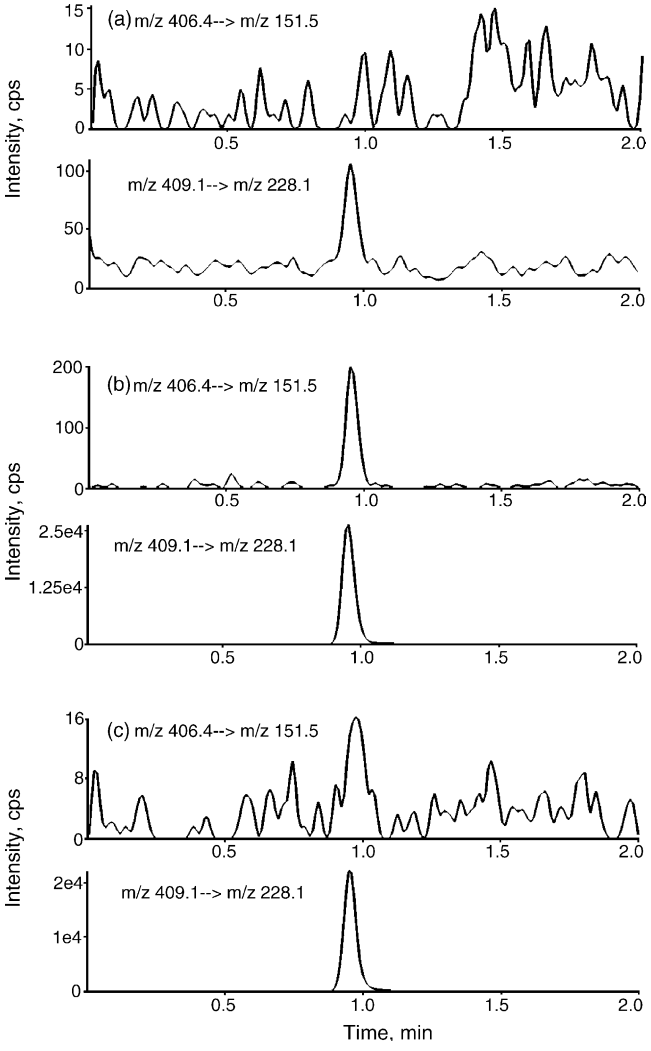

Fig. 4. MRM ion-chromatograms resulting from the analysis of (a) blank (drug and internal standard free) human plasma for nebivolol and I.S., (b) 50 pg/mL (LLOQ) of nebivolol spiked with I.S., and (c) blank (drug-free spiked with I.S.) human plasma for nebivolol and I.S.

Table 2
Precision and accuracy data of back-calculated concentrations of calibration samples for nebivolol in human plasma

Concentration added (pg/mL)	Concentration found (mean ± S.D., $n = 6$) (pg/mL)	Precision (% R.S.D.)	Accuracy (% bias)
50	54.3 ± 4.1	7.5	7.5
100	101.3 ± 8.4	8.3	0.3
200	205.3 ± 11.4	5.6	1.6
500	491.4 ± 33.4	6.8	−2.7
1000	1003.5 ± 59.1	5.9	−0.7
2000	1866.9 ± 110.3	5.9	−7.6
5000	5055.7 ± 82.0	1.6	0.1
10000	10269.9 ± 136.2	1.3	1.6

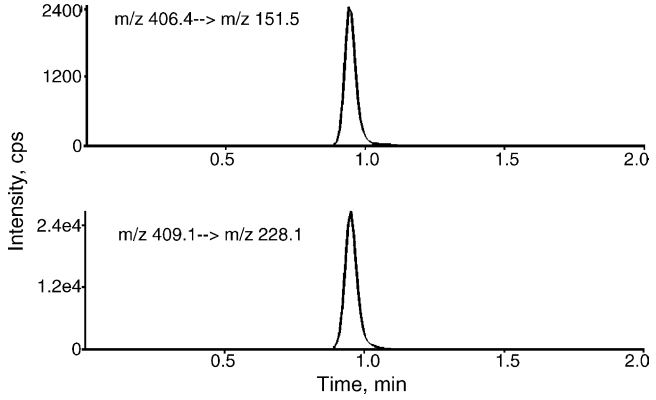

Fig. 5. MRM ion-chromatograms resulting from the analysis of a volunteer plasma sample after the administration of an oral single dose of 5 mg of nebivolol. The sample concentration was 636 pg/mL.

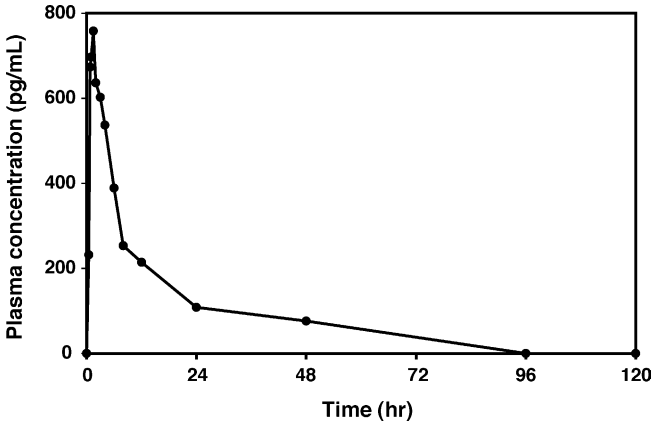

Fig. 6. Representative plasma concentration vs. time curve obtained from a subject after the oral administration of 5 mg of nebivolol.

The limit of detection (LOD) demonstrated that the analyte gave a signal-to-noise ratio (S/N) of ≥3 for 10 pg/mL extracted/injected. The lower limit of quantification, the lowest concentration in the standard curve, which can be measured with acceptable accuracy and precision for the analyte from normal human plasma was established as 50 pg/mL. The mean response for the analyte peak at the assay sensitivity limit (50 pg/mL) was ≈12.5-folds greater than the mean response for the peak in six blank human plasma samples at the retention time of the analyte. Excellent sensitivity was observed for 25-μL injection volume corresponding to 1250 fg on-column.

Accuracy and precision (within- and between-batch) were calculated with six (excluding blank) determinations per concentration level on 3 days and are presented in Table 3. The precision was determined as the within- and between-assay R.S.D. and accuracy was expressed as percentage bias. The results show that the method is accurate, as the bias is within the acceptable limits of ±20% of the theoretical value at the LLOQ and ±15% at all other concentration levels. The precision around the mean value never exceeded 12% at any of the concentrations studied.

The recovery of the I.S. from the extracted calibration standard and QC samples during validation was 72.1 ± 2.0%. The recovery of nebivolol was 73.4 ± 3.7% on average and the dependence on concentration is negligible. The recoveries were calculated from the average peak areas of extracted fortified plasma samples read against that of reference solutions in diluent (analytical standards) of the same concentration, as there was no appreciable matrix suppression. The validity of this approach was verified by comparing the absolute recoveries obtained here with that from the classical approach, in which the mean peak areas from spiked samples were compared with those of reference solutions spiked in extracted drug-free plasma samples. The deviations observed (R.S.D.) between the mean recoveries calculated by the two approaches were less than 5% at the concentration levels studied. This showed that this approach could be successfully applied in recovery determination of the analyte, thus reducing the biomatrix requirements for a validation programme, if there is no appreciable matrix effect. The recovery of the analyte and the I.S. were high and also the extent of recovery of the analyte and of the I.S. were consistent, precise and reproducible.

These results show that the method is accurate and precise over the concentration range 50–10,000 pg/mL.

3.3. Stability studies

QC samples were subjected to short-term room temperature, long-term storage conditions (below −50 °C) and freeze-thaw stability studies. All the stability studies were carried out at two concentration levels (150 and 8000 pg/mL as low and high, respectively) with six determinations.

There was no significant difference between the responses of spiked standards at time zero and after 24 h for nebivolol, indicating the stability of analyte at room temperature over

Table 3
Precision and accuracy of the method for determining nebivolol concentrations in plasma samples

Concentration added (pg/mL)	Within-batch precision ($n = 6$)			Between-batch precision ($n = 3$)		
	Concentration found (mean ± S.D.) (pg/mL)	Precision (% R.S.D.)	Accuracy (% bias)	Concentration found (mean ± S.D.) (pg/mL)	Precision (% R.S.D.)	Accuracy (% bias)
50	56.3 ± 2.3	4.1	11.5	53.6 ± 2.4	4.5	6.2
150	161.9 ± 3.3	2.1	6.8	162.8 ± 2.6	1.6	7.5
4000	4090.2 ± 252.5	6.1	1.2	4243.6 ± 171.8	4.0	5.0
8000	8158.1 ± 441.6	5.4	0.9	8471.5 ± 301.4	3.5	4.7

Table 4
Stability data for nebivolol in spiked plasma ($n = 6$)

Storage conditions	Nominal concentration (pg/mL)	Mean concentration at $t = 0$ (pg/mL)	Mean concentration recovered (pg/mL)	S.D. (pg/mL)	R.S.D. (%)
24 h at RT[a]	150	158.5	166.7	17.6	10.5
	8000	8088.7	8055.5	247.2	3.1
30 days at <−50 °C	150	159.2	167.4	7.9	4.7
	8000	8116.0	8094.5	196.1	2.4

[a] Room temperature.

24 h. Moreover, the analyte was found to be stable after reconstitution in diluent for at least 12 h at 4 °C. The re-injection reproducibility was established to determine if an analytical run could be reanalyzed in case of an unexpected delay in analysis. The same set of QC samples were repeated after the analysis with a 3 h gap between, during which the samples were stored at 4 °C, and in all cases the deviations were less than 15%. On similar lines, stability of the extracted dry residue was also established to be over 24 h (deviations observed <10%). In addition, the stock solutions of nebivolol and I.S. were also found to be stable for at least 3 months at 4 °C.

The deviations observed after the first, second and third freeze-thaw cycles were within ±10%, at the concentration levels used for nebivolol, indicating adequate freeze-thaw stability. Spiked QC samples ($n = 6$), which were extracted and analyzed immediately, were used as the reference point to calculate the percentage deviations after the first, second and third freeze-thaw cycles. Also, the QC samples stored at below −50 °C were analyzed after 30 days and there were no significant deviations with respect to the immediately analyzed samples. The long-term stability data at below −50 °C are shown in Table 4. On similar lines, stability of the extracted dry residue and stability of the processed sample staying in the autosampler were also established to be over 24 h (deviations observed < 10%).

4. Conclusions

The first LC–MS/MS method for the quantitation of nebivolol in human plasma was developed and fully validated as per FDA guidelines [22]. This method offers significant advantages over those previously reported, in terms of improved sensitivity and selectivity and faster runtime (2 min). Acceptable data were generated for nebivolol using weighted linear regression (1/concentration^2) and full calibration curves for human plasma samples. The desired sensitivity of nebivolol was achieved with an LLOQ of 50 pg/mL, which has a within- and between-batch CV of 4.1 and 4.5%, respectively. Acceptable accuracy and precision were obtained for concentrations above the sensitivity limit and within the standard curve range of 50–10,000 pg/mL. Nebivolol was shown to be stable in routine analysis conditions and in human plasma for up to 30 days when stored at below −50 °C. The simplicity, liquid–liquid extraction and sample turnover rate of 2 min per sample make it an attractive procedure in high-throughput bioanalysis of nebivolol. Hence the method can be considered suitable for application to pharmacokinetic studies of nebivolol.

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