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A high throughput and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the estimation of bisoprolol in human plasma using multiplexing technique

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Abstract

A high throughput and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed and validated for the estimation of bisoprolol in human plasma using multiplexing technique (two HPLC units connected to one MS). Bisoprolol was extracted from human plasma using solid-phase extraction technique using metoprolol as internal standard. A Betabasic 8 column provided chromatographic separation of analytes followed by detection with mass spectrometry. The mass transition ion-pair was followed as *m/z* 326.2 → 116.1 for bisoprolol and *m/z* 268.2 → 191.0 for metoprolol. The method involves a simple multiplexing, rapid solid-phase extraction, simple isocratic chromatography conditions and mass spectrometric detection which enable detection at sub-nanogram levels. The proposed method has been validated for a linear range of 0.5–70.0 ng/mL with correlation coefficient ≥0.9991. The precision and accuracy were within 10% for intra-HPLC runs and inter-HPLC runs. The overall recoveries for bisoprolol and metoprolol were 93.89% and 77.65%, respectively. Total MS run time was 0.90 min only. The developed method was applied for the determination of pharmacokinetic parameters of bisoprolol following a single oral administration of a 10 mg bisoprolol tablet in 18 healthy male volunteers.

Keywords: Bisoprolol; LC–MS/MS; Human plasma; β-Blockers; Multiplexing

1. Introduction

Bisoprolol, 1-[4-[[2-(1-methylethoxy)ethoxyl-methyl]phenoxy]-3-[1-methylethyl)amino]-2-propanol, is a highly selective β1 receptor antagonist [1,2]. Bisoprolol is almost completely absorbed after oral administration and undergoes only minimal first-pass metabolism. Peak plasma concentration is reached 2–4 h after oral administration. It is moderately lipid-soluble and rapidly distributed in the body. Its plasma protein binding is 30–35% with bioavailability 90% [3].

Only few analytical methods have been reported in literature for monitoring plasma, serum and urine levels of bisoprolol [4–7]. The techniques used in the reported methods include use of a HPLC method with fluorescence detection [4–6], and a capillary isotachophoresis method [7]. The HPLC methods has limit of quantification ≥5 ng/mL for estimation of bisoprolol in plasma and ≥10 ng/mL for urine [4–6]. The capillary isotachophoresis method has a limit of quantification of 40 μg/mL and 3 μg/mL for estimation of bisoprolol in serum and urine, respectively [7]. The reported methods are not sensitive enough to detect sub-nanogram levels of bisoprolol in human plasma [4–7]. The reported HPLC methods follow time consuming and error prone liquid–liquid extraction procedure involving drying and reconstitution [4–6]. Also these methods have long run time, low recovery and large plasma processing volume [4–6]. The capillary isotachophoresis method additionally involves solid-phase extraction which makes the method lengthy [7]. As the use of liquid chromatography–tandem mass spectrometry (LC–MS/MS) for analysis of drug candidates in biological matrices has grown exponentially in the last decade, due to its un-matched sensitivity, extraordinary selectivity and rapid rate of analysis, it was necessary to develop a sensitive, selective and rapid LC–MS/MS analytical method for the quantification of bisoprolol in human plasma.

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A critical review is reported for published work in optimization of method development strategy, sample preparation techniques, chromatography and parallel analysis to achieve high throughput using LC–MS/MS methods [8]. Parallel analysis approach for achieving high throughput, either using multiple inlets in the mass spectrometer source or multiplexing LC units into one MS is well established. While the multiple inlets in the mass spectrometer allow four-fold increase in throughput, sensitivity loss and potential cross-inlets contamination are major drawbacks [9]. Multiplexing LC units set-up enables to overcome the two shortcomings and demonstrates that when two LC units feed one MS, the run time is reduced by 50% [10–12]. Therefore, in present work, multiplexing LC units set-up is utilized to achieve high throughput.

This paper describes development and validation of a high throughput and sensitive LC–MS/MS method for the quantification of the bisoprolol in human plasma having reduced sample preparation and analysis time relative to other commonly employed techniques with a limit of quantification (LOQ) 0.5 ng/mL. Metoprolol was used as an internal standard.

2. Experimental

2.1. Chemicals and reagents

The working standards of bisoprolol fumarate and metoprolol succinate were obtained from Torrent Research Centre (Ahmedabad, India). High purity water was prepared in-house using a Milli-Q water purification system obtained from Millipore (Bangalore, India). HPLC grade methanol and acetonitrile were purchased from Ranbaxy (New Delhi, India). Suprapure ammonium formate, sodium hydroxide and formic acid were purchased from Merck (Darmstadt, Germany). Drug free (blank) buffered human plasma was obtained from Green Cross Laboratory (Ahmedabad, India) and stored at −20 °C prior to use.

2.2. Calibration curves

The stock solutions of bisoprolol and metoprolol were prepared in methanol at free base concentration of 1 mg/mL. Secondary standard solutions for bisoprolol, 10.0 μg/ml, was prepared from stock solution using water:methanol (80:20, % v/v) as diluent. Secondary standard solutions for metoprolol, 100 μg/ml, was prepared from stock solution. Working standard solutions of bisoprolol calibration standards 1400 ng/mL, 1260 ng/mL, 840 ng/mL, 560 ng/mL, 280 ng/mL, 140 ng/mL, 70 ng/mL, 20 ng/mL and 10 ng/mL were prepared from secondary standard solutions by dilution with water:methanol (80:20, % v/v). Working standard solutions of bisoprolol quality control samples 30 ng/mL, 610 ng/mL and 1200 ng/mL were prepared from secondary standard solutions by dilution with water:methanol (80:20, % v/v). Working standard solution 10 ng/mL of bisoprolol was used for LOQ quality control samples. Metoprolol Working standard solution of metoprolol 2 μg/ml was prepared from secondary standard solutions by dilution with water. These diluted working standard solutions were used to prepare the calibration curve standards and quality control samples.

A nine-point standard calibration curve for bisoprolol was prepared by spiking the blank plasma with appropriate amount of bisoprolol working standard solutions. The calibration curve ranged from 0.5 ng/mL to 70.0 ng/mL with calibration standards at 0.5 ng/mL, 1.0 ng/mL, 3.5 ng/mL, 7.0 ng/mL, 14.0 ng/mL, 28.0 ng/mL, 42.0 ng/mL, 63.0 ng/mL and 70.0 ng/mL. Quality control samples were prepared at four concentration levels of 0.5 ng/mL (LOQ), 1.5 ng/mL (LQC), 30.5 ng/mL (MQC) and 60.0 ng/mL (HQC) for bisoprolol in a manner similar to the calibration standard from the working standard solutions.

2.3. Sample preparation

A 0.5 mL aliquot of human plasma sample was mixed with 25 μl of internal standard working solution (2.0 μg/mL of metoprolol). Added 0.5 mL of 100 mM sodium hydroxide and mixed well. The sample mixture was loaded onto an Oasis HLB (1 cm^3/30 mg) extraction cartridge that was pre-conditioned with 1.0 mL of methanol followed by 1.0 mL of water. The extraction cartridge was washed with 1.0 mL of water followed by 1.0 mL of 5% methanol. Bisoprolol and metoprolol were eluted with 1.0 mL of acetonitrile; 10.0 μl of the extract was injected into the LC–MS/MS system. The vacuum applied to each step of solid phase extraction was 15 bar for 30 s. All the plasma samples including blanks, calibration standards, quality control samples and subject samples were treated with the same sample preparation.

2.4. Instrumentation

Multiplexing set-up was achieved with two Shimadzu Prominence HPLC having Shimadzu SIL-HTc autosampler and one flow change over valve FCV-AH12. Chromatographic separation was carried out with Betabasic 8 (5.0 μm, 100 mm × 4.6 mm) purchased from Thermo Electron Corporation, UK. A mobile phase consisting of acetonitrile and ammonium formate (pH 2.80, 5.0 mM) in ratio of 80:20 (v/v) was delivered using low pressure quaternary gradient HPLC pump with a flow rate of 1.0 mL/min (flow was splitted 30% to the source and 70% to waste). The column oven temperature was kept 40 °C. The total run time for each sample analysis was 2.0 min for HPLC and 0.90 min for MS. Mass spectra were obtained using a TSQ Quantum mass spectrometer, a triple stage quadrupole mass analyzer with electron multiplier detector, equipped with electrospray ionization (ESI) source (Thermo Finnigan Ltd., UK). The mass spectrometer was operated in the selected reaction-monitoring (SRM) mode. The HPLC and MS parameters for analytes are listed in Tables 1 and 2, respectively. Class-VP software (version 6.14 SP2) was used for data acquisition on HPLC where as MS data acquisition was ascertained by Xcalibur LCquan 2.5 SUR1 software. The HPLC and MS data were synchronized by contact closure using a RS232 (recommended standard 232) communication and triggering cable. For quantification the peak area ratios of the target ions of the drug to those of the internal standard were compared with weighted (1/c) least

Table 1
Ion source and analyte-dependent parameters

Ion source		
Spray voltage	3500 V	
Capillary temperature	350 °C	
Sheath gas	40 (arbitrary)	
Auxillary gas	20 (arbitrary)	
Polarity mode	Positive	
Analyte dependent	**Bisoprolol**	**Metoprolol**
Precursor ion (*m/z*)	326.2	268.2
Product ion (*m/z*)	116.1	191.0
Tube lens off set (V)	72	79
Q1 Pw[a] (amu)	0.70	0.70
Q3 Pw[b] (amu)	0.70	0.70
Collision energy (eV)	19	18

[a] Quadrupole 1 peak width.
[b] Quadrupole 3 peak width.

squares calibration curves in which the peak area ratios of the calibration standards were plotted versus their concentrations.

2.5. Validation

The method has been validated for selectivity, specificity, sensitivity, linearity, precision, accuracy, recovery, stability, matrix effect and carry over check. Selectivity and specificity was performed by analyzing and comparing the blank plasma samples and spiked LOQ samples from different sources (or donors) to ensure absence of any possible interference at the retention time of bisoprolol and metoprolol. Sensitivity was determined by analyzing six replicates of spiked plasma with the analyte at the lowest level of the calibration curve. The intrarun accuracy and precision was determined by replicate ($n = 6$) analysis of quality control samples and at LOQ that were extracted from a single sample batch. The interrun accuracy and precision was determined by replicate ($n = 6$) analysis of quality control samples and at LOQ that were extracted from three different batches. Interrun precision and accuracy of the calibration standards was assessed using the five calibration curves used for assay validation.

Table 2
Shimadzu LC method parameters (time program) for multiplexing system

Time (min)	Module	Event	Value
Time program for HPLC-1			
1.00	Subcontroller vp	RV.B (2.Pos.)	1
1.01	SCL-10Avp	Event	1
1.10	SCL-10Avp	Event	0
1.20	SCL-10Avp	Event	3
1.30	SCL-10Avp	Event	0
2.00	SCL-10Avp	Stop	0
Time program for HPLC-2			
1.01	SCL-10Avp	Event	1
1.10	SCL-10Avp	Event	0
1.20	SCL-10Avp	Event	3
1.30	SCL-10Avp	Event	0
2.00	SCL-10Avp	Stop	0

Event value 0 = all events off; event value 1 = giving signal to the MS to start acquisition; event value 3 = giving signal to the next HPLC to start run.

Accuracy is defined as the percent relative error (%RE) and was calculated using the formula $\%RE = (E - T)(100/T)$, where E is the experimentally determined concentration and T is theoretical concentration. Assay precision was calculated by using the formula $\%R.S.D. = (S.D./M)(100)$, where M is the mean of the experimentally determined concentrations and S.D. is the standard deviation of M.

Five replicates at low, medium and high quality control concentration for the bisoprolol were prepared for recovery determination. The extraction efficiencies of bisoprolol and metoprolol were determined by comparing the peak area of extracted analytes to the peak area of equivalent non-extracted standards (drug spiked in extracted blank plasma).

The processed sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0), with the samples that were re-injected after keeping in the auto sampler at 5 °C for 24.0 h. The stability of spiked human plasma stored at room temperature (bench-top stability) was evaluated for 6 h and compared with freshly prepared extracted samples. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen and thawed three times, with freshly spiked quality control samples. The stability of spiked human plasma stored at −70 °C (long-term stability) was evaluated by analyzing low, medium and high quality control samples that were stored at −70 °C for 48 days together with freshly spiked calibration standard and quality control samples. All stability evaluations were based on back-calculated concentrations. Analytes were considered stable if the deviation of the mean test responses were within 15% of freshly prepared or comparison sample.

Matrix effect was checked with total six different lots of plasma. Three samples each of LQC and HQC were prepared from different lots of plasma (in total 36 QC samples) and checked for the accuracy in all the QC samples. This was performed with the aim to see the matrix effect of these different lots of plasma on the back-calculated value of QCs nominal concentration. If the deviation of the mean test responses is within 15% of nominal concentration it was concluded that there is no effect of matrix on analyte assay.

Carry over check of autosampler and system was also performed by sequential injections of extracted LOQ, extracted LOQ, extracted blank plasma, extracted ULOQ, extracted blank plasma, and extracted ULOQ, extracted blank plasma. These sequential runs were performed parallel on both the HPLC system to ensure absence of any carry over from individual system as well as multiplexed system. If any peak is present at the retention time of analyte, its area response should be ≤20% of the mean response of an extracted LOQ and any peak is present at the retention time of an internal standard, its area response should be ≤5% of the mean response of an internal standard.

3. Results and discussion

3.1. Method development

To develop a rapid, sensitive and simple assay method for the extraction and quantification of bisoprolol during method

development different options were evaluated to optimize detection and chromatography parameters. Bisoprolol accepts the proton in an acidic mobile phase and produced a protonated precursor ion ($[M+H]^+$) at *m/z* 326.2. The mass spectra of precursor ions of bisoprolol and metoprolol are presented in Fig. 1. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were evaluated to get better response of analytes. It was found that the best signal was achieved with ESI positive ion mode. One product ions *m/z* 116.1 of bisoprolol monitored which gives better sensitivity and selectivity.

Further optimization in chromatography conditions increased signal of analytes. A mobile phase containing 5 mM ammonium formate (pH 2.80) buffer in combination with acetonitrile resulted in improved signal. Use of Betabasic 8 (100 mm × 4.6 mm id, 5 μm) column resulted in HPLC run time of 2.0 min and a reduced MS acquisition time as low as 0.90 min with multiplexing. The optimized chromatography and detection parameters enabled to eliminate the laborious extraction steps of evaporation and reconstitution, which further resulted in reduced processing and analysis time.

3.2. Selectivity

Utilization of predominant product ions for each compound enhanced mass spectrometric selectivity. The mass transition

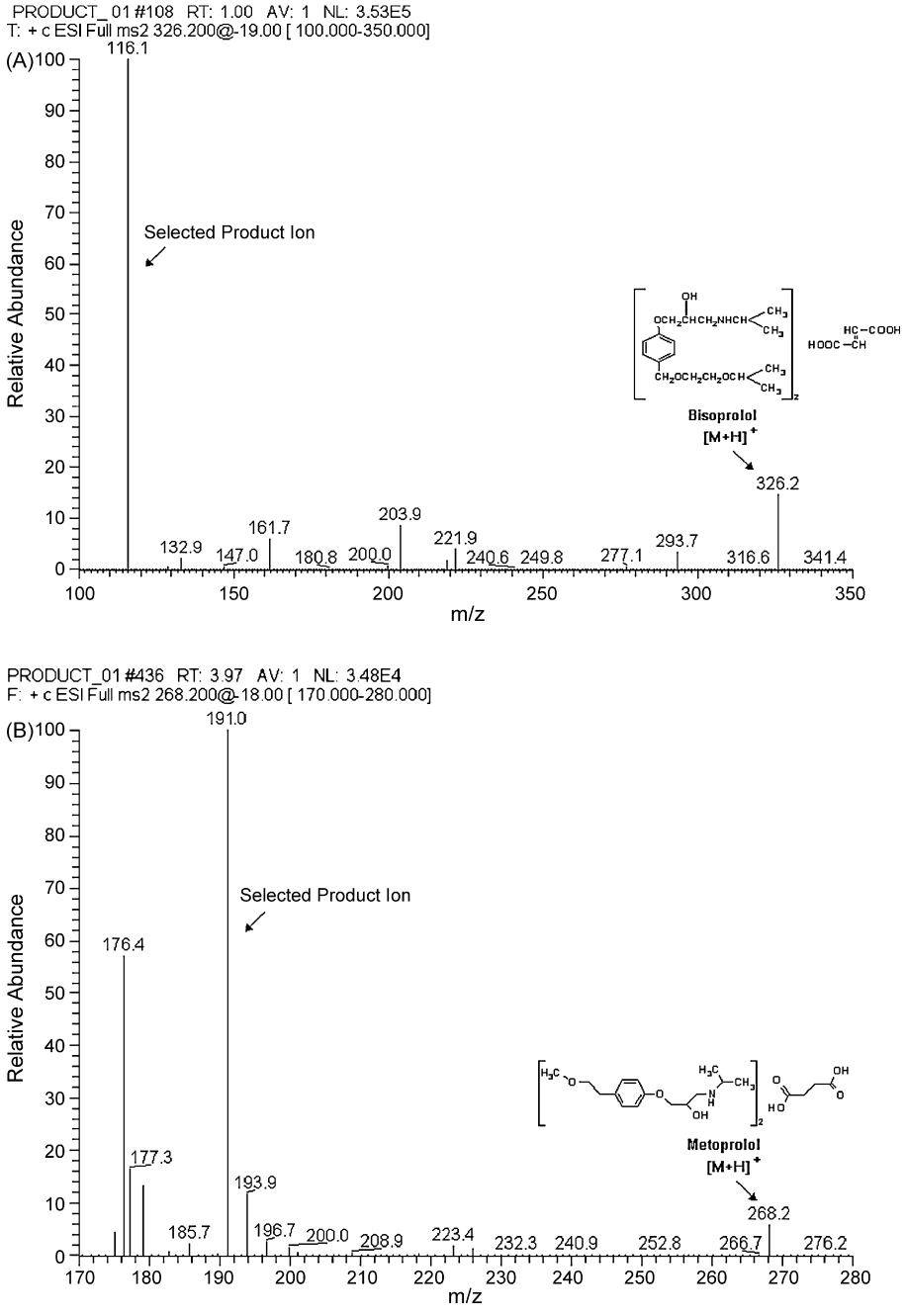

Fig. 1. ESI product ion mass spectra for the precursor ions of (A) bisoprolol and (B) metoprolol.

ion-pair was selected as, 326.2 → 116.1 for bisoprolol and 268.2 → 191.0 for metoprolol. The predominant product ion of *m/z* 116.1 was specific for bisoprolol and *m/z* 191.0 was specific for metoprolol.

Chromatographic selectivity of the method was demonstrated by the absence of endogenous interfering peaks at the retention times for bisoprolol and metoprolol in six different lots of extracted blank plasma. Representative chromatograms of extracted blank plasma, extracted plasma samples containing 0.5 ng/mL bisoprolol (low standard) and plasma samples from subjects are presented in Fig. 2a–c, respectively.

3.3. Linearity

The peak area ratios of calibration standards were proportional to the concentration of analytes in each assay over the nominal concentration range of 0.5–70.0 ng/mL for bisoprolol. The calibration curves appeared linear and were well described by least squares lines. A weighting factor of 1/concentration was chosen to achieve homogeneity of variance. The correlation coefficients were ≥0.9991 ($n = 5$) for bisoprolol. The mean (±S.D.) slopes of the calibration curves ($n = 5$) for bisoprolol were 0.052507 (±0.000501) for HPLC-1 and HPLC-2.

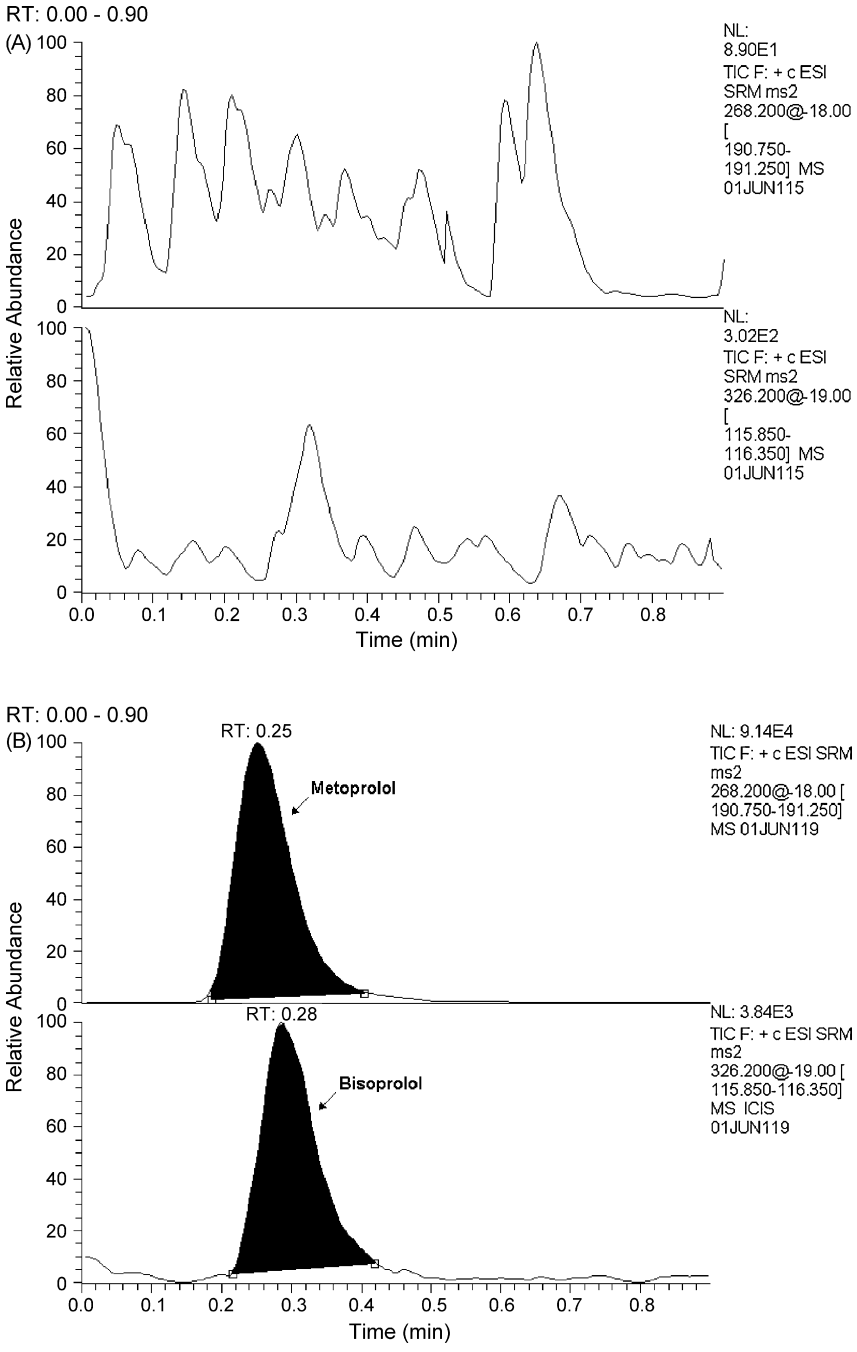

Fig. 2. (a) Representative chromatogram of extracted blank plasma sample. (b) Representative chromatogram of extracted plasma LOQ sample. (c) Representative chromatogram of extracted subject sample.

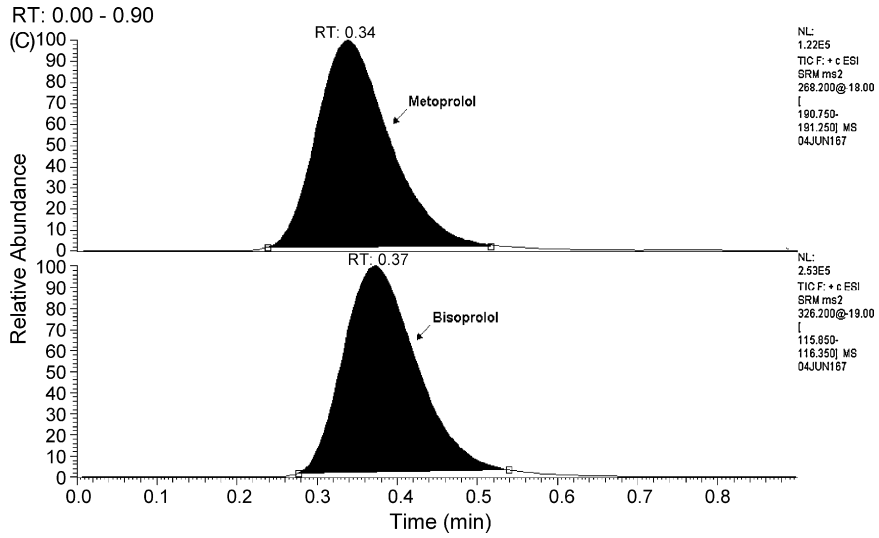

Fig. 2. (*Continued*).

Table 3
Intrarun or within-batch ($n = 6$) precision and accuracy of bisoprolol in human plasma in HPLC-1 and HPLC-2

Analyte	Spiked conc. (ng/ml)	Mean calculated conc. (ng/ml)	%R.S.D.	%RE
Bisoprolol (HPLC-1)	0.500	0.454	2.05	−9.20
	1.500	1.423	2.42	−5.13
	30.500	31.625	1.61	3.69
	60.000	62.880	4.06	4.80
Bisoprolol (HPLC-2)	0.500	0.461	2.22	−7.80
	1.500	1.445	4.47	−3.67
	30.500	31.288	1.79	2.58
	60.000	60.817	4.27	1.36

3.4. Sensitivity (lower limit of quantification)

The LOQ is defined as the lowest concentration of the calibration standard yielding accuracy ±20% and precision of ≤20%. The LOQ for bisoprolol was 0.5 ng/mL. The intrarun precision of the LOQ plasma samples containing bisoprolol were 2.05% and 2.22% for HPLC-1 and HPLC-2, respectively. The mean intrarun accuracy of the LOQ plasma samples containing bisoprolol were 9.20% and 7.80% for HPLC-1 and HPLC-2, respectively (Table 3).

3.5. Precision and accuracy

The intrarun precision was ≤4.06% for HPLC-1 and ≤4.47% for HPLC-2. The intrarun accuracy was ≤9.20% for HPLC-1 and ≤7.80% for HPLC-2 (Table 3). The interrun precision and accuracy were determined by pooling all individual assay results of replicate ($n = 6$) QC samples over the five separate batch runs. The interrun precision was ≤9.51%. The interrun accuracy was ≤3.12% for bisoprolol (Table 4).

3.6. Recovery

The mean recovery for bisoprolol was 93.89% with precision of 4.46%. The mean recovery for metoprolol was 77.65%.

3.7. Stability

The results of the stability studies are enumerated in Table 5. The bench-top stability, process stability and freeze and thaw

Table 4
Interrun or between-batch ($n = 5$) precision and accuracy of bisoprolol in human plasma in HPLC-1 and HPLC-2

Analyte	n	Spiked conc. (ng/ml)	Mean calculated conc. (ng/ml)	%R.S.D.	%RE
Bisoprolol	30	0.500	0.502	9.51	0.40
(HPLC 1) and (HPLC-2)	30	1.500	1.480	5.23	−1.33
	30	30.500	31.310	2.10	2.66
	30	60.000	61.872	3.92	3.12

Table 5
Stability results for bisoprolol ($n = 6$)

Stability	Spiked conc. (ng/ml)	Mean (±S.D.) obtained conc. (ng/ml)	%RE
Process[a]	1.500	1.482 (±0.085)	−1.20
	60.000	62.040 (±1.737)	3.40
Bench-top[b]	1.500	1.576 (±0.036)	5.07
	60.000	59.309 (±0.890)	−1.15
Freeze/thaw[c]	1.500	1.613 (±0.052)	7.53
	60.000	60.080 (±1.863)	0.13
Long term[d]	1.500	1.494 (±0.035)	−0.40
	30.500	30.491 (±0.185)	−0.03
	60.000	59.391 (±0.526)	−1.02

[a] After 24 h in autosampler at 5 °C.
[b] After 6 h at room temperature.
[c] After three freeze/thaw cycles at −70 °C.
[d] −70 °C for 48 days.

stability of bisoprolol in plasma were investigated by analyzing quality control samples in replicates ($n = 6$) at LQC and HQC level. Process stability, the results indicated that the difference in the back-calculated concentration from time 0 to 24 h is ≤3.40%, which allowed to conclude that processed samples are stable at least for 24 h at 5 °C in autosampler. Bench-top stability results allowed us to conclude that bisoprolol is stable for at least 6 h at room temperature in plasma samples. Freeze and thaw stability results indicated that the repeated freeze and thawing (three cycles) did not affect the stability of bisoprolol. Long-term stability of bisoprolol in plasma at −70 °C was performed at LQC, MQC and HQC level, it was found to be stable for at least 48 days at −70 °C.

3.8. Matrix effect

Three quality control samples at each level along with the set of calibration standards were analyzed and the %bias of the samples analyzed was found within ±15 for each QC level for bisoprolol (Table 6).

Hence this clearly proves that the elution of endogenous matrix peaks during the run has no affect on the quantification of bisoprolol. Therefore, the method of extraction of bisoprolol from plasma was rugged enough and gave accurate and consistent results when applied to real patient samples.

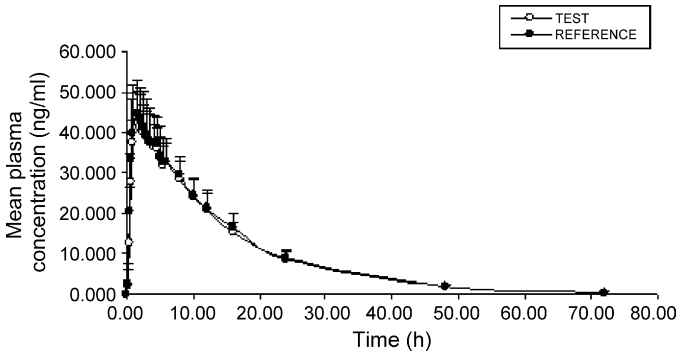

Fig. 3. Mean bisoprolol plasma concentration–time profile following a 10 mg oral dose of bisoprolol to human subjects.

3.9. Carry over check

No carry over for analyte and internal standard was observed after sequential injections of blank plasma and extracted standards as per validation section.

3.10. Application of method

The proposed method was applied for the determination of bisoprolol in plasma samples from ongoing projects for the development of an immediate release formulation. Plasma samples were periodically collected up to 72 h after a single oral dose administration of a 10 mg tablet to 18 healthy male volunteers in each phase. The time periods at which the plasma samples were drawn at 0.00, 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00, 8.00, 10.00, 12.00, 16.00, 24.00, 48.00 and 72.00 h after a single oral dose administration of a 10 mg tablet. A total of 792 human plasma samples from 18 male volunteers were analyzed along with calibration standards and QC samples in HPLC-1 and HPLC-2. Two calibration curves were made for sample quantification on each HPLC-1 and HPLC-2. Total 400 samples were analyzed per day. No interference peak was found in pre-dose samples for all volunteers. The mean (±S.D.) plasma maximum concentrations obtained for the bisoprolol test and reference formulations were 41.391(±8.141) ng/mL and 44.694 (±8.345) ng/mL, respectively. The mean bisoprolol plasma concentration–time profile following a 10 mg oral dose of bisoprolol to human subjects is shown in Fig. 3. The area under the curve (AUC) measured from

Table 6
Matrix effect ($n = 3$) for bisoprolol

S. no.	Plasma lot no.	LQC (1.5 ng/ml)			HQC (60.0 ng/ml)		
		Mean calculated conc.	%R.S.D.	%RE	Mean calculated conc.	%R.S.D.	%RE
1	LOT-A1	1.478	3.28	−1.47	65.327	4.67	8.88
2	LOT-A2	1.417	3.65	−5.53	61.985	2.82	3.31
3	LOT-A3	1.410	2.91	−6.00	63.496	4.38	5.83
4	LOT-A4	1.390	2.75	−7.33	62.399	0.65	4.00
5	LOT-B	1.449	1.94	−3.40	64.847	4.35	8.08
6	LOT-C	1.388	3.67	−7.47	62.175	3.70	3.62

A: Normal citrate dextrose phosphate plasma; B: lipimic plasma; C: haemolysed plasma.

0 h to the last sampling point was higher than 90% of the value of AUC extrapolated from 0 to infinity, which indicates the suitability of the LOQ of the analytical method for pharmacokinetic study.

4. Conclusions

A simple, specific, rapid and sensitive LC–MS/MS method has been developed for the determination of bisoprolol in human plasma. The proposed method provided excellent specificity and reproducibility with a limit of quantification of 0.5 ng/mL for bisoprolol.

It is concluded that this sensitive and specific method is applicable for the quantitative determination of bisoprolol in human plasma in pharmacokinetic and bioavailability studies of bisoprolol.

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