SENSITIVE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF BISOPROLOL AND TRIAMTERENE IN HUMAN PLASMA

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ABSTRACT

Objective: A highly specific, sensitive, and rapid liquid chromatography tandem mass spectrometric method has been developed and validated for the simultaneous quantification of bisoprolol and triamterene in human plasma using metoprolol as an internal standard (IS).

Methods: Both the analytes and IS were extracted from plasma using a protein precipitation extraction method. Chromatography was achieved on Welchrom XB C18, 50 × 4.6 mm0, 5 μm column using an isocratic mobile phase (2 mM ammonium formate: acetonitrile, 70:30 v/v) at a flow rate of 0.60 ml/min.

Results: The total chromatographic run time was 3.5 minutes and the elution of bisoprolol, triamterene, and IS occurred at ~2.57, 1.30, and 1.57 minutes, respectively. A linear response function was established at 2.04-210 ng/ml for both the analytes in human plasma. The intraday and interday accuracy and precisions were in the range of 1.12-7.87% and 1.26-6.36% and 1.46-6.13% and 1.65-7.34% for bisoprolol and triamterene, respectively.

Conclusion: A new robust method was developed for simultaneous determination of bisoprolol and triamterene in human plasma. The method was strictly validated according to the ICH guidelines. The information thus obtained from the study can be used for the full pharmacokinetic profiling in individuals.

Keywords: Bisoprolol, Triamterene, Liquid chromatography tandem mass spectrometric, Method validation, Human plasma, Pharmacokinetics.

INTRODUCTION

Bisoprolol (Fig. 1), chemically is 1-{4-[(2-isopropoxyethoxy) methyl] phenoxy}-3-(isopropyl amino) propan-2-ol and belongs to the group of beta-blockers. It is a β_1 adrenergic receptor blocker which is used to treat several cardiovascular diseases such as hypertension, arrhythmia coronary heart disease, ischemic heart disease, and myocardial infarction as monotherapy and/or in combination with other agents.

Triamterene (Fig. 2) chemically is 6-phenylpteridine-2,4,7-triamine. It is a potassium-sparing diuretic which is used in combination with thiazide diuretics for the treatment of edema and hypertension. The epithelial sodium channel present on the lumen side of the kidney collecting tubule is blocked by triamterene.

In the contemporary literature, few bioanalytical methods were reported for quantification of bisoprolol. Dinga *et al.* reported an LC-electrospray ionization (ESI)-MS for determination of bisoprolol in human plasma. Plasma aliquots were processed using ethyl acetate liquid–liquid extraction method. Chromatographic separation of bisoprolol was achieved on ZORBAX SB-C_{18} column using gradient elution. The linearity range for bisoprolol in plasma was found to be 0.05-120 ng/ml. Total run time for this analysis was 5.0 minutes [1]. Similarly, Bhatt *et al.* reported the determination of bisoprolol in human plasma by an LC-ESI-MS method. Plasma aliquots were processed using solid phase extraction methodology. Chromatographic separation of bisoprolol was achieved on BetaBasic 8 column using an isocratic elution. The linearity range was 0.5-70 ng/ml for bisoprolol in plasma [2]. Recently, Liu *et al.* reported a liquid chromatography-tandem mass spectrometric (LC-MS/MS) method for the quantitation of bisoprolol in human plasma with a linearity range of 0.5-100 ng/ml. Plasma aliquots spiked with bisoprolol were processed using simple protein precipitation extraction method [3] followed by

chromatographic separation of bisoprolol achieved on Capcell Pak C_{18} MG III column (100 mm × 2.0 mm, 5 μ), using gradient elution.

Bisoprolol fumarate can be determined by various methods such as ultraviolet, reverse phase high-performance LC (HPLC), LC-MS/MS, and high-performance thin-layer chromatography. Methods have been reported for analysis of bisoprolol fumarate either alone or in combination with other drugs in pharmaceutical formulations [4-8].

Metoprolol (Fig. 3) is structurally similar to bisoprolol. On the benzene ring, it has two substituents present in the paraposition [9]. Hence, metoprolol was selected as the internal standard (IS) for the simultaneous estimation.

In the contemporary literature, few bioanalytical methods were also reported for quantification of triamterene. Hsu *et al.* reported an LC-MS/MS for determination of triamterene in urine samples. Aliquots of urine sample spiked with triamterene were processed using solid phase extraction method. Chromatographic separation of triamterene was achieved on HS-C_{18} column using a gradient elution with a linearity range of 25-500 ng/ml. Total run time for this analysis was 15.0 minutes [10].

Most of these published methods were suitable for only single analyte determination and utilized more than 200 μl of plasma volume. In this paper, the development and validation of a simple, specific, sensitive, and high-throughput LC-MS/MS method for the simultaneous quantitation of bisoprolol and triamterene in a small volume (100 μl) human plasma is being reported. The main advantages of this method are higher sensitivity over the reported lower limit of quantitation (LLOQ) (for triamterene), isocratic mobile phase elution, and shorter run time (3.5 minutes). This method can be successfully applied to quantitate levels of bisoprolol and triamterene in human pharmacokinetic studies.
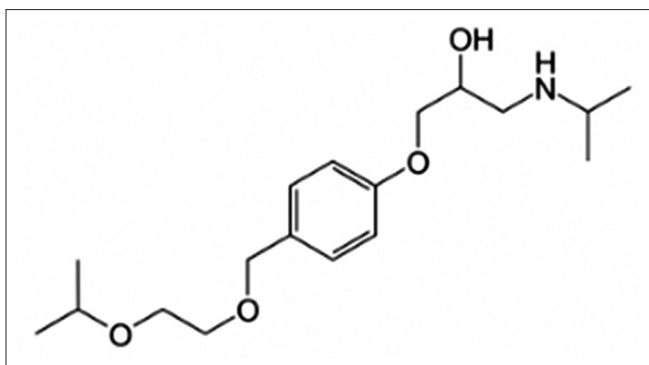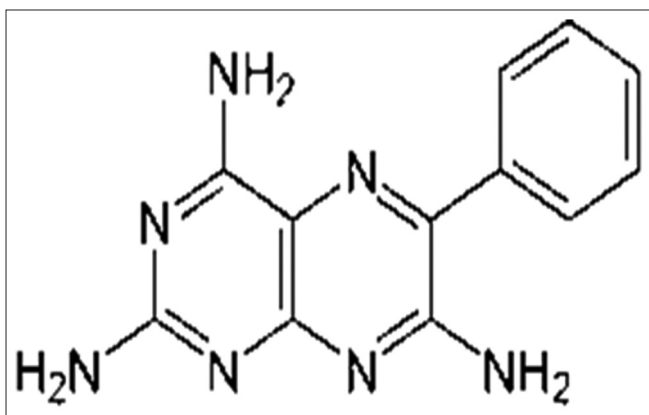
Fig. 1: Structural representation of bisoprolol

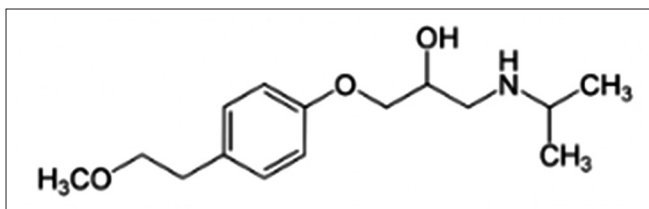**Fig. 2: Structural representation of triamterene**

Fig. 3: Structural representation of metoprolol (internal standard)

From the literature survey, it is very clear that no method has been reported for simultaneous determination of bisoprolol and triamterene by LC-MS/MS. As for quantification, LC-MS/MS seems to be more sensitive and precise for the simultaneous determination of bisoprolol and triamterene in human plasma. The proposed method is validated as per guidelines [11].

METHODS

Chemicals and reagents

Bisoprolol (purity 99.3%), triamterene sodium (purity 99.7%), and metoprolol succinate (purity 99.9%) were purchased from Vivan Life Sciences Pvt. Ltd., India. HPLC grade acetonitrile and methanol were purchased from J T Baker, India. Analytical grade formic acid and ethyl acetate were purchased from Spectrochem and Merck India, respectively. Dimethyl sulfoxide and Phenacetin were procured from Sigma-Aldrich, India. All other chemicals and reagents were of analytical grade. Microcaps® disposable micropipettes (50 μl, catalog number: 1-000-0500) were purchased from Drummond Scientific Company, USA. The control human dipotassium ethylenediaminetetraacetic acid (K_2EDTA) plasma sample was procured from Red Cross Society, Bengaluru.

Instrumentation and chromatographic conditions

A Shimadzu HT (Shimadzu, Japan) LC system equipped with degasser (DGU-20A5), binary pump (LC-20AD) along with auto-sampler (SIL-HTC) was used to inject 10 μl aliquots of the processed samples on Welchrom XB C_{18} column (50 mm × 4.6 mm, 5 μm) maintained at 40±1°C. The isocratic mobile phase consisted of a mixture of 2 mM ammonium formate and acetonitrile mixture (70:30 v/v) which was filtered through a 0.45 μm membrane filter (XI5522050) (Millipore, USA or equivalent) and then degassed ultrasonically for 5 minutes. The mobile phase was delivered at a flow rate of 0.600 ml/min into the mass spectrometer ESI chamber.

Quantitation was achieved by MS/MS detection in positive ion mode for bisoprolol, triamterene, and IS using a MDS Sciex (Foster City, CA, USA) API-4000 mass spectrometer, equipped with a Turbo ion spray™ interface at 450°C temperature and 4500 V ion spray voltage. The source parameters namely, curtain gas; GS1 and GS2 were set at 30, 55 and 65 psi. The compound parameters namely, declustering potential, entrance potential, collision energy, and collision cell exit potential were 70, 10, 50, and 15 V for bisoprolol; 50, 10, 35, and 18 V for triamterene; and 75, 10, 30 and 8 V for IS, respectively. Detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of the m/z (mass-to-charge ratio) 326 precursor ion to the m/z 116 product ion for bisoprolol; m/z 254 precursor ion to the m/z 237 product ion for triamterene; and m/z 268 precursor ion to the m/z 116 product ion for IS (metoprolol). Quadrupole Q1 and Q3 were set on unit resolution. The dwell time was 200 ms. The analytical data were processed by Analyst Software (version 1.5.2).

Standard solutions

Bisoprolol, triamterene, and IS were weighed accurately into volumetric flasks using an analytical microbalance. Approximately 1 mg/ml primary stock solutions of bisoprolol, triamterene, and IS solutions were prepared in methanol. The stock solutions of bisoprolol, triamterene, and IS were stored at −20°C, which were found to be stable for 1 month. The stock solutions of bisoprolol and triamterene were successively diluted with methanol: water (50:50 v/v) to prepare secondary stocks and working solutions. Secondary stock solutions and working solutions were used to prepare calibration curve (CC) and quality control (QC) samples. Working stock solutions were stored at 4°C for a week. Working stocks were used to prepare plasma calibration standards. A working IS solution (150 ng/ml) was prepared in methanol: water (50:50 v/v). Blank human plasma was screened before spiking to ensure that it was free from endogenous interference at retention times of bisoprolol, triamterene, and IS, respectively. Eight-point calibration standards' samples (2.02-210 ng/ml for both bisoprolol and triamterene) were prepared by spiking the blank human K_2EDTA plasma with appropriate concentration of bisoprolol and triamterene. Samples for the determination of precision and accuracy were prepared by spiking control human plasma in bulk with bisoprolol and triamterene at appropriate concentrations (2.02 ng/ml LLOQ, 8.42 ng/ml low QC [LQC], 84.22 ng/ml medium QC [MQC], and 168.45 ng/ml high QC [HQC]) and 120 μL plasma aliquots were distributed into different tubes. All the samples were stored at −80°C±10°C.

Sample preparation

A simple protein precipitation extraction method was followed for extraction of bisoprolol and triamterene from human plasma. From the deep freezer, the required quantities of CC standards and QC samples were withdrawn. The samples were thawed at room temperature. To an aliquot of 100 μl plasma, 50 μl of IS was added. To this mixture, 400 μl of acetonitrile was added and vortexed for 2 minutes, followed by centrifugation at 4000 rpm for 5 minutes at 4°C. After centrifugation, approximately 200 μl supernatant was aliquoted into, respectively, labeled autosampler vials, which were later placed in the autosampler at 10°C±4°C. 10 μl of the sample was injected onto LC-MS/MS system for analysis.

Method validation

A full validation according to the ICH guidelines [11] was performed for the assay in K_2EDTA human plasma.

Specificity and selectivity

The specificity of the method was evaluated by analyzing human plasma samples from at least six different lots to investigate the potential interferences at the chromatographic peak region for analytes and IS. The acceptance criterion for the experiment was that at least four out of six lots should have <20% area response to that of the LLOQ level response in the same matrix. Two lots of hemolyzed plasma samples were also analyzed to ensure specificity against potential biological interferences.

Recovery

The efficiency of bisoprolol, triamterene, and IS extraction from human plasma was determined by comparing the responses of the analytes extracted from replicate QC samples (n=6) with those of neat standard solutions spiked in post-extracted plasma blank sample at equivalent concentrations by protein precipitation extraction method. Recovery of bisoprolol and triamterene was determined at LQC (8.42 ng/ml) and HQC (168.45 ng/ml) concentrations, whereas the recovery of IS was determined at a single concentration of 150 ng/ml.

Matrix effect

The effect of human plasma constituents over the ionization of bisoprolol, triamterene, and IS was determined by post-column infusion method to evaluate matrix effect [11]. Briefly, an infusion pump delivers a constant amount of analyte into LC system outlet entering to mass spectrometer inlet. To follow the analyte signal, the mass spectrometer was operated in MRM mode. The human plasma constituent sample extract was injected on LC column. A steady ion response was obtained as a function of time since the analyte was infused at a constant rate. Any endogenous compound that elutes from the column which causes a variation in ESI response of the infused analyte was seen as a suppression or enhancement in the response of the infused analyte. A separate experiment was performed with bisoprolol, triamterene, and IS solutions, which were infused at a constant rate, and blank matrix sample injected through the LC. To evaluate matrix effect, six different lots of human plasma were spiked with analyte concentration levels at LQC and HQC levels. According to guidelines, the acceptance criterion for each back-calculated concentration was ±15% deviation from the nominal value [11].

CC

The eight-point CC for bisoprolol and triamterene (2.02-210 ng/ml) was constructed by plotting the peak area ratio of each analyte: IS against the nominal concentration of calibration standards in K_2EDTA human plasma. Following the evaluation of different weighing factors, the results were fit into linear regression analysis using $1/X^2$ (X: Concentration) weighing factor. The CC should have a correlation coefficient (r) of 0.99 or better. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value except at LLOQ, which was set at ±20% [11].

Precision and accuracy

The intra-assay precision and accuracy were estimated by analyzing six replicates containing bisoprolol and triamterene at four different QC levels (2.10 ng/ml [LLOQ], 8.42 ng/ml [LQC], 84.22 ng/ml [MQC], and 168.45 ng/ml [HQC]) in human plasma. The four-level QC samples on four different runs were performed to assess the interassay precision. The acceptance criteria for each back-calculated standard concentration were 85-115% accuracy from the nominal value except at LLOQ, which was set at 80-120% [11].

Stability experiments

Stability tests were conducted to evaluate the stability of bisoprolol and triamterene in plasma samples under different conditions. 8 hrs

bench top stability, processed samples stability (autosampler stability for 26 hrs at 10°C), three cycles of freeze-thaw stability, 30 days of long-term stability at −80±10°C were performed at LQC and HQC levels using six replicates at each level. Samples were considered stable if assay values' acceptance criterion was of accuracy (i.e., 85-115% from fresh samples) and precision (i.e., ±15% relative standard deviation [RSD]).

Dilution integrity

Dilution integrity was investigated to ensure that samples could be diluted with blank matrix without affecting the final concentration. Dilution integrity experiment was performed for study sample concentrations crossing the upper limit of quantitation (ULOQ). Bisoprolol and triamterene spiked human plasma samples were prepared at two concentrations (168 and 336 ng/ml) of bisoprolol and triamterene and were diluted with pooled human blank plasma at dilution factors of 20 and 40 in six replicates and analyzed. The back-calculated standard concentrations had to comply to have both precision of <15% and accuracy of 100±15% similar to other QC samples. The criteria for acceptance of the analytical runs encompassed the following: (i) 67% of the QC samples accuracy must be within 85-115% of the nominal concentration and (ii) not <50% at each QC concentration level must meet the acceptance criteria [11].

RESULTS

Bisoprolol, triamterene, and IS eluted at ~2.57, 1.31, and 1.57 minutes, respectively. During a direct infusion experiment, the mass spectra for bisoprolol, triamterene, and IS revealed peaks at m/z 326, 254, and 268, respectively as protonated molecular ions, $[M+H]^+$. Following detailed optimization of mass spectrometry conditions (provided in mass spectrometry operating conditions section), MRM reaction pair of m/z 326 precursor ion to the m/z 116 was used for quantification for bisoprolol and m/z 254 precursor ion to the m/z 237 was used for quantification for triamterene. Similarly, for IS MRM reaction, pair of m/z 268 precursor ion to the m/z 116 was used for quantification purpose. The fragmentation pattern of bisoprolol, triamterene and IS are shown in Figs. 4-6.

Table 1 shows the mean recovery for bisoprolol, triamterene (at LQC and HQC level), and IS including matrix effect.

Fig. 7 shows chromatograms for the blank human plasma (free of analytes and IS) for bisoprolol. Fig. 8 shows blank human plasma spiked with IS without analyte for bisoprolol. Fig. 9 shows bisoprolol at LLOQ concentration along with IS. Fig. 10 shows bisoprolol at ULOQ concentration along with IS. Similarly, Fig. 11 shows chromatograms for the blank human plasma (free of analytes and IS) for triamterene. Fig. 12 shows blank human plasma spiked with IS without analyte for triamterene. Fig. 13 shows triamterene at LLOQ concentration along with IS. Fig. 14 shows triamterene at ULOQ concentration along with IS.

Table 1: Recovery and matrix data for bisoprolol and triamterene

Concentration (ng/ml)	Mean±SD (n=6)	
	Mean recovery (%)	Mean absolute matrix effect
LQC		
Bisoprolol – 8.42	51.8±1.43	99.6±3.35
Triamterene – 8.17	50.8±1.96	101±3.05
HQC		
Bisoprolol – 168.45	53.4±0.54	102±1.41
Triamterene – 163.49	52.3±0.68	102±0.28
Metoprolol (IS) - 150	61.9	

The results have shown that the precision and accuracy for analyzed samples were within acceptance range. LQC: Low-quality control, HQC: High-quality control, IS: Internal standard, SD: Standard deviation
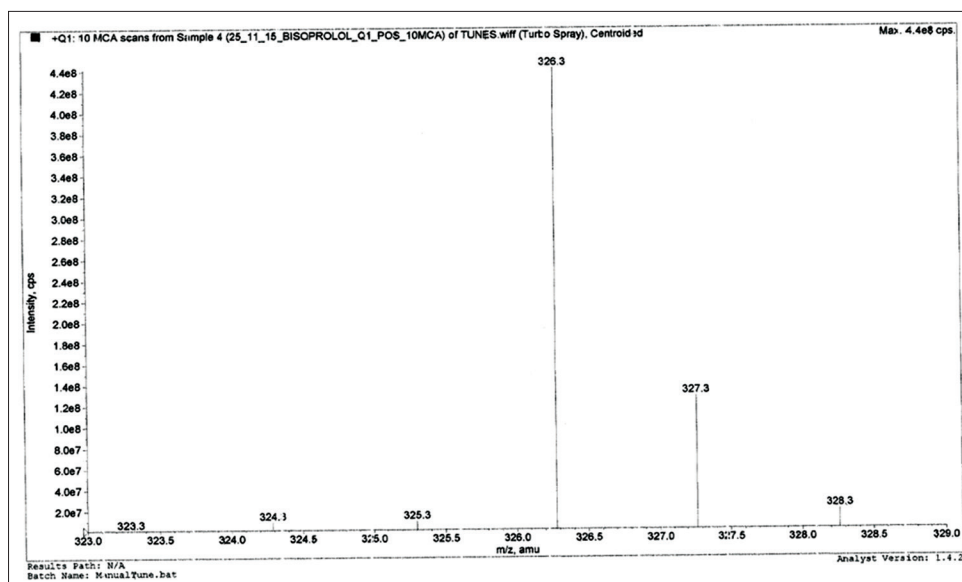
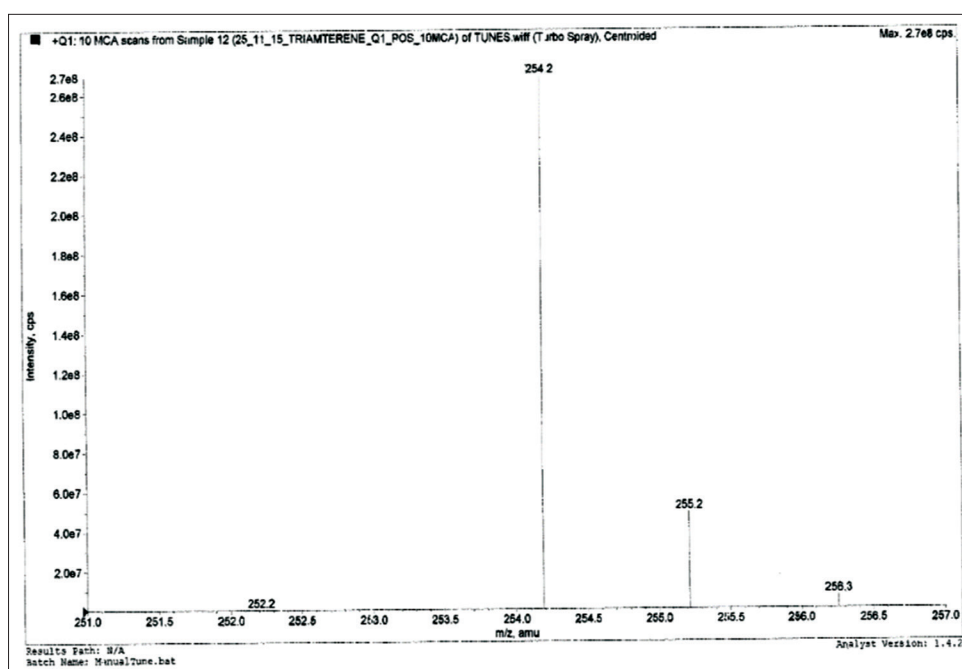**Fig. 4: Mass fragmentation of bisoprolol**

Fig. 5: Mass fragmentation of triamterene

The retention time of bisoprolol, triamterene, and IS was ~2.57, 1.31, and 1.57 minutes, respectively. The total chromatographic run time was 3.5 minutes.

The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The average regression (n=3) was found to be >0.997 for both bisoprolol and triamterene. The lowest concentration with the RSD <20% was taken as LLOQand was found to be 2.10 ng/ml for both bisoprolol and triamterene. The percentage accuracy observed for the mean of back-calculated concentrations for three CCs for bisoprolol and triamterene was within 87.0-114, whereas the precision (% CV) values ranged from 0.99 to 7.56 and 1.19 to 7.98, respectively.

Accuracy and precision data for intra- and inter-day plasma samples for bisoprolol and triamterene are presented in Table 2.

The predicted concentrations for bisoprolol and triamterene at 8.17 and 163 ng/ml deviated within ±15% of the fresh sample concentrations in a battery of stability tests namely, in-injector (22 hrs), bench-top (7 hrs), and repeated four freeze/thaw cycles stability (Table 3).

DISCUSSION

LC
LC separation of bisoprolol and IS has been carried out using various mobile phases consisting of different aqueous solutions and methanol or acetonitrile as the organic phase. Hernando *et al.* [12] used acetonitrile as organic mobile phase to achieve shorter retention times and better resolution of bisoprolol and IS. Formic acid solution as an additive in water was used by Li *et al.* [13] to improve the sensitivity of MS detection.

In the present study, optimization and critical evaluation of buffer, mobile phase composition, flow rate, and analytical column were
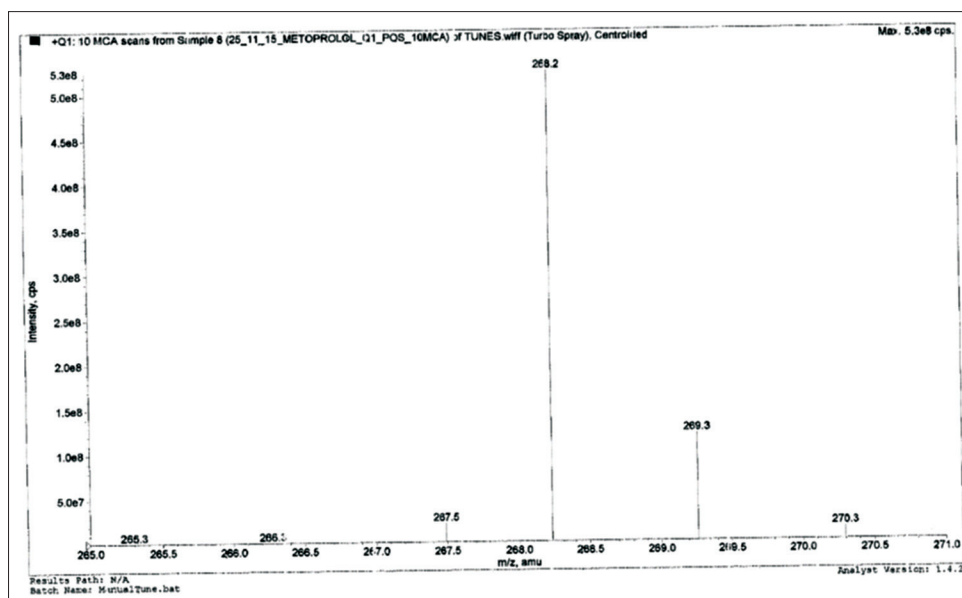
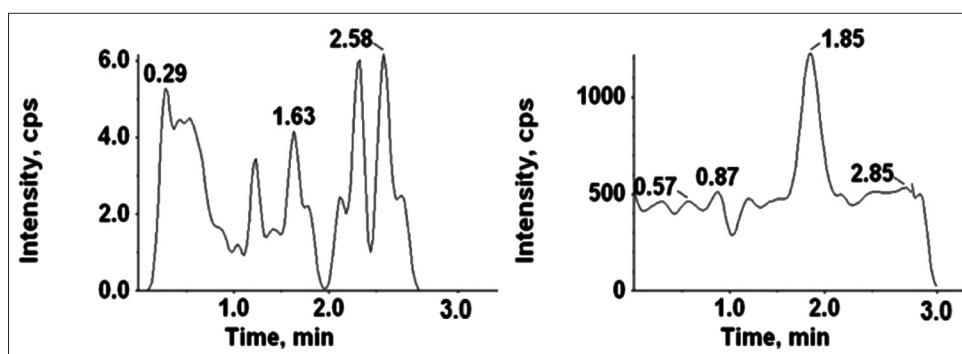Fig. 6: Mass fragmentation of internal standard (metoprolol)

Fig. 7: Typical multiple reaction monitoring chromatograms of bisoprolol (left panel) and internal standard (right panel) in dipotassium ethylenediaminetetraacetic acid human blank plasma
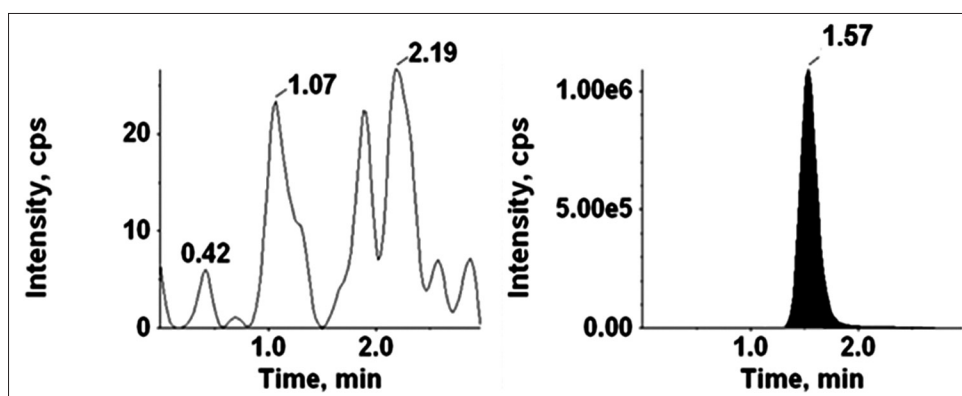

Fig. 8: Typical multiple reaction monitoring chromatograms of bisoprolol (left panel) and internal standard (IS) (right panel) in dipotassium ethylenediaminetetraacetic acid human blank plasma spiked with IS

important to obtain good resolution of peaks of interest from the endogenous components, which in turn affect reproducibility and sensitivity of the method. Selection of chromatographic conditions for the proposed method was optimized to suit the preclinical pharmacokinetic studies. To ease the sample preparation in microtubes and to reduce the usage of solvent, the plasma volume was kept low. Initial feasibility experiments of a various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate, and formic acid along with altered flow rates (in the range of 0.1-0.6 ml/min) were performed to optimize an effective chromatographic resolution of bisoprolol, triamterene, and IS. Various analytical columns such as Zorbax, Inertsil, Prodigy, Kromasil, Atlantis, and Hypersil were tested to obtained good and reproducible response within short run time. The resolution of peaks was best achieved with an isocratic mobile phase comprising acetonitrile and 2 mM ammonium formate in water (30:70, v/v) delivered at a
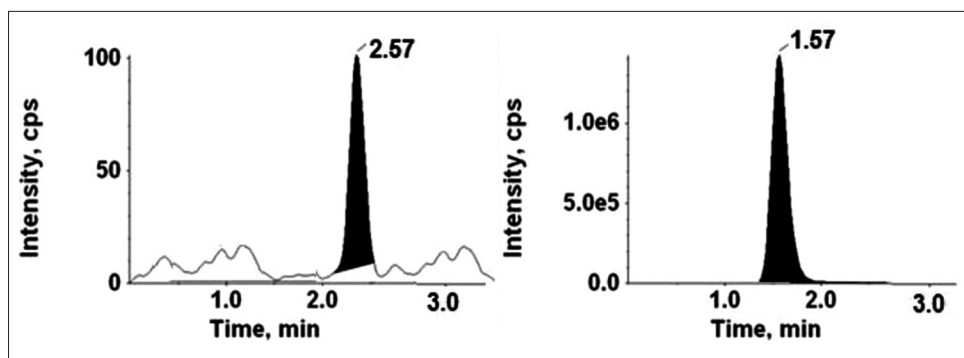
Fig. 9: Typical multiple reaction monitoring chromatograms of bisoprolol (left panel) and internal standard (IS) (right panel) in dipotassium ethylenediaminetetraacetic acid human blank plasma spiked bisoprolol at lower limit of quantitation (2.10 ng/ml) and IS
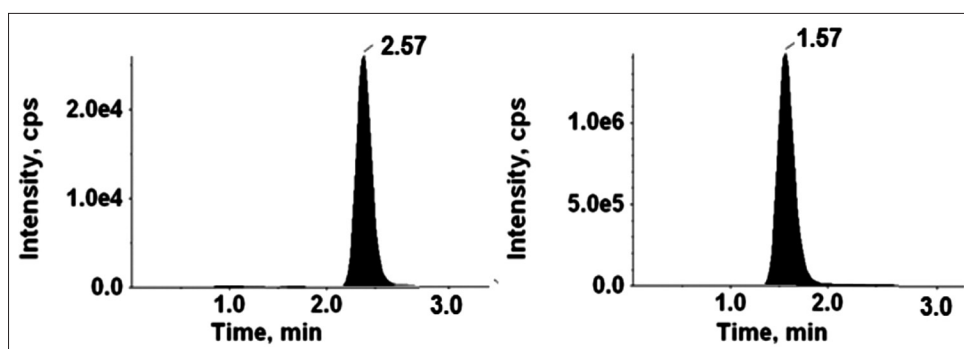

Fig. 10: Typical multiple reaction monitoring chromatograms of bisoprolol (left panel) and internal standard (IS) (right panel) in dipotassium ethylenediaminetetraacetic acid human blank plasma spiked bisoprolol at upper limit of quantitation (208.091 ng/ml) and IS

Fig. 11: Typical multiple reaction monitoring chromatograms of triamterene (left panel) and internal standard (right panel) in human blank plasma

Fig. 12: Typical multiple reaction monitoring chromatograms of triamterene (left panel) and internal standard (IS) (right panel) in human blank plasma spiked with IS

Fig. 13: Typical multiple reaction monitoring chromatograms of triamterene (left panel) and internal standard (IS) (right panel) in human blank plasma spiked with triamterene at lower limit of quantitation (2.10 ng/ml) and IS
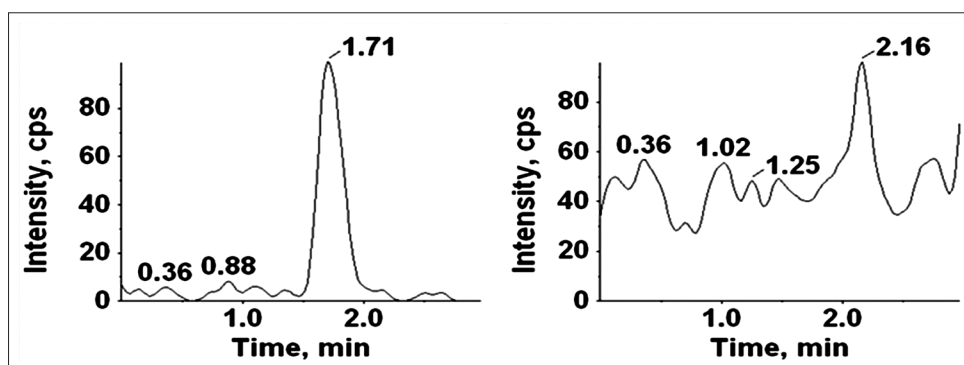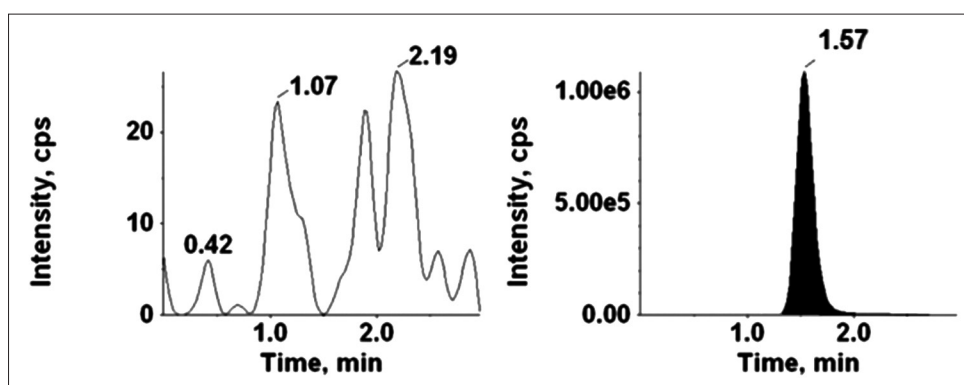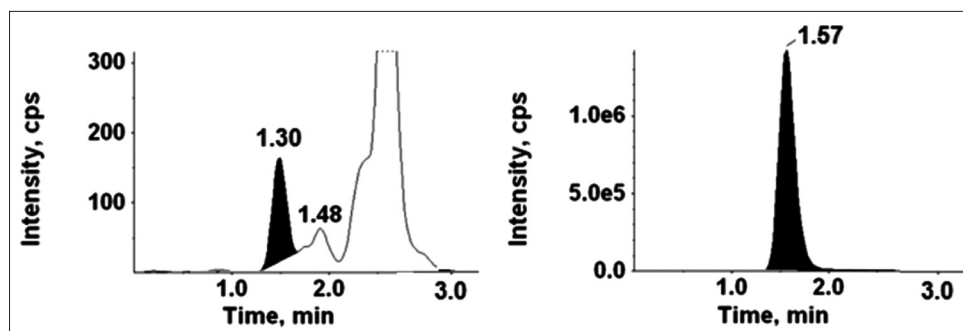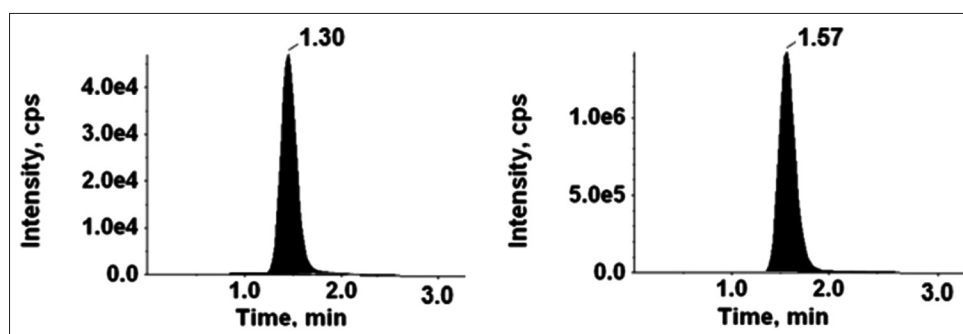

Fig. 14: Typical multiple reaction monitoring chromatograms of triamterene (left panel) and internal standard (IS) right panel) in human blank plasma spiked with triamterene at upper limit of quantitation (210 ng/ml) and IS

Table 2: Intra- and inter-day precision and accuracy determination of bisoprolol and triamterene quality controls in human plasma

Nominal concentration (ng/ml)	Mean±SD	RSD	Accuracy (%)
Intraday variation (6 replicates at each concentration)			
Bisoprolol - 2.10	2.02±0.15	7.87	96.1
Triamterene - 2.12	1.89±0.12	6.13	89.1
Bisoprolol - 8.42	8.31±0.13	1.60	98.7
Triamterene - 8.17	7.81±0.26	3.28	95.6
Bisoprolol - 84.22	82.8±0.92	1.12	98.2
Triamterene - 81.74	82.9±1.21	1.46	101
Bisoprolol - 168.45	166±2.59	1.56	98.7
Triamterene - 163.49	167±2.56	2.51	102
Inter-day variation (18 replicates at each concentration)			
Bisoprolol - 2.10	2.078±0.13	6.36	92.3
Triamterene - 2.12	2.05±0.15	7.34	93.1
Bisoprolol - 8.42	8.39±0.18	2.09	97.3
Triamterene - 8.17	8.05±0.25	3.13	95.8
Bisoprolol - 84.22	83.7±1.05	1.26	97.5
Triamterene - 81.74	82.1±1.35	1.65	99.1
Bisoprolol - 168.45	168±2.56	1.52	98.3
Triamterene - 163.49	165±2.80	1.70	101

RSD: Relative standard deviation (SD×100/mean). SD: Standard deviation

flow rate of 0.6 ml/min. Welchrom XB C$_{18}$ column (50 mm × 4.6 mm, 5 μm) was found to be suitable for sharp and symmetric peak shapes among few other columns tested in the method optimization process. The injection volume was set at 10 μl since low injection volume was expected to result in increased ionization and decreased possible chemical noise.

The purpose of sample extraction optimization is mainly to achieve high extraction recovery with negligible or low matrix effects to improve sensitivity and reliability of LC-MS/MS analysis. A poor extraction procedure decreases method robustness due to the presence of endogenous interference in the sample extracts, which are not efficiently cleaned up due to poor extraction procedure decreases the method robustness due to the endogenous interference in the sample extracts. With time-saving advantage and simplicity, the protein precipitation extraction method was chosen as an extraction method. The attained LLOQ (2.11 ng/ml) was sufficient to quantify bisoprolol and triamterene in low-dose pharmacokinetic studies.

Mass spectroscopy

To optimize ESI conditions for bisoprolol, triamterene, and IS, quadrupole full scans were carried out both in positive and negative ion detection mode [14]. A good response was achieved in positive ionization mode. During a direct infusion experiment, the mass spectra for bisoprolol, triamterene, and IS revealed peaks at m/z 326, 254, and 268, respectively, as protonated molecular ions [M+H]$^+$. Following detailed optimization of mass spectrometry conditions (provided in mass spectrometry operating conditions section), MRM reaction pair of m/z 326 precursor ion to the m/z 116 was used for quantification for bisoprolol and m/z 254 precursor ion to the m/z 237 was used for quantification for triamterene. Similarly, for IS MRM reaction, pair of m/z 268 precursor ion to the m/z 116 was used for quantification purpose.

Recovery

One step protein precipitation extraction process proved to be robust and provided clean samples. The comparisons of plasma-extracted standards versus the neat solution spiked into post-extracted blank sample at equivalent concentration were estimated for bisoprolol and triamterene and IS.

Matrix effect

No significant signal suppression or enhancement was observed in the region of elution of bisoprolol and triamterene and IS. The results have shown that the precision and accuracy for analyzed samples were within acceptance range. Overall, it was found that there was no impact on the ionization of analyte and IS.

Table 3: Stability data of bisoprolol and triamterene QCs in human plasma

Nominal concentration (ng/ml)	Stability	Bisoprolol			Triamterene		
		Mean±SD$^{\varrho}$ (n=6)	Accuracy (%)•	Precision (% CV)	Mean±SD$^{\varrho}$ (n=6)	Accuracy (%)•	Precision (% CV)
Bisoprolol – 8.42 Triamterene – 8.17	0 h	8.20±0.35	97.3	4.29	7.92±0.48	96.9	6.07
	7 h (bench-Top)	8.24±0.19	97.9	2.36	8.15±0.34	99.7	4.20
	22 h (in-injector)	8.37±0.22	99.3	2.60	8.06±0.33	98.6	4.14
	3 FT cycles	8.34±0.31	98.9	3.73	8.02±0.30	98.1	3.69
Bisoprolol – 168.45 Triamterene – 163.49	0 h	164±2.17	97.4	1.32	162±3.90	99.2	2.40
	7 h (bench-top)	159±12.5	94.5	1.38	163±4.28	99.9	2.62
	22 h (in-injector)	163±2.25	96.8	7.87	163±1.71	99.5	1.05
	3 FT cycles	163±1.94	96.9	1.19	162±3.53	98.9	2.18

$^{\varrho}$Back-calculated plasma concentrations; •Mean assayed concentration/mean assayed concentration at 0 hrs × 100. FT: Freeze-thaw, SD: Standard deviation, QC: Quality control

Specificity and selectivity

No interfering peaks from endogenous compounds were observed at the retention times of bisoprolol, triamterene, and IS in the matrix. The specificity of the method was evaluated by analyzing human plasma samples from six different lots to investigate the potential interferences at the LC peak region for analytes and IS. Six replicates of LLOQ samples were prepared from the clean blank samples and analyzed and were found to be acceptable with precision (% CV) <5%.

CC

The plasma CC was constructed in the linear range using eight calibration standards (namely, 2.02, 4.08, 22.7, 37.8, 75.7, 126, 168, and 210 ng/ml for bisoprolol and triamterene). The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range.

Accuracy and precision

The assay values on both the occasions (intra- and inter-day) were found to be within the accepted limits.

Stability

The predicted concentrations for bisoprolol and triamterene at 8.17 and 163 ng/ml deviated within ± 15% of the fresh sample concentrations in a battery of stability tests namely, in-injector (22 hrs), bench-top (7 hrs), and repeated four freeze/thaw cycles stability (Table 3). The results were found to be within the assay variability limits during the entire process.

Dilution effect

The dilution integrity was confirmed for QC samples that exceeded the upper limit of the standard CC (up to 7513 ng/ml). The results have shown that the precision and accuracy for 20 and 40 times diluted test samples were within the acceptance range (% CV values were between 5.23 and 2.35 for both the dilutions).

CONCLUSION

In summary, a highly sensitive, specific, reproducible, and high-throughput LC-MS/MS assay was developed and validated to quantify bisoprolol and triamterene in human plasma as per the regulatory guidelines. The present method involved a simple precipitation method of sample preparation, which gave consistent and reproducible recoveries. The sample volume requirement supports the possibility to study the full pharmacokinetic profile in individuals. Furthermore, the rationale for selecting the combination of bisoprolol and triamterene is justifiable. The use of beta-blockers and diuretics is well-established in the management of hypertension, and their combination leads to an additive blood pressure-lowering effect. Beta-blockers blunt increase in plasma renin level induced by diuretics, and diuretics decrease sodium and water retention caused by beta-blockers. Hence, the combination

was taken up for developing a bioanalytical method development and validation so that further it would be useful for performing pharmacokinetic studies.

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