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Research Article

Determination of Metoprolol in Human Plasma by Liquid Chromatography Tandem-Mass Spectrometry

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Article info	Abstract
	A high performance liquid chromatography-tandem mass spectrometry method was developed for the determination of Metoprolol in human plasma. Liquid-liquid extraction technique was employed to extract Metoprolol and the internal standard (IS) from the plasma. The separation was achieved on a unison US C18 column (50 x 4.6mm and 3.5µm) with isocratic elution by using a mobile phase consisting of 10mM ammonium acetate and acetonitrile. Electro spray ionization in multiple reactions monitoring mode was used to monitor the parent and the daughter ions of the analyte and the internal standard. The response was linear over a range of 5 to 500 ng/ml concentration with a correlation coefficient (r^2) greater than 0.996. The validated method can be applied to pharmacokinetic and bioequivalence studies.

1. INTRODUCTION

Metoprolol is a selective β_1 receptor blocker used in treatment of several diseases of the cardiovascular system, especially hypertension. Due to its selectivity in blocking the beta$_1$ receptors in the heart, Metoprolol is also used to treat performance anxiety, social anxiety disorder, and other anxiety disorders. Metoprolol has a short half life and is taken twice a day as slow release preparation.

Quantification of Metoprolol by HPLC with UV detection [1-2], HPLC with flouroscence detection [3-11], gas chromatography with electron capture detection [12] and HPLC with mass detection [13-18] were reported. The sample preparation for the extraction of metaprolol from the human plasma was based on expensive solid phase extraction and liquid-liquid extraction using ethylacetate, dichloromethane and a mixture of diethylether and dichloromethane. We report here a simple, sensitive and cost effective method for the determination of Metoprolol in human plasma. Electro spray ionization with multiple reaction monitoring was adopted to increase the sensitivity and selectivity. Methyl tertiary butyl ether was used as the extraction solvent for the extraction of the drug and the internal standard from the plasma. The extraction efficiencies were found to be greater compared to other solvents reported in the literature. The method required very low volumes of plasma of 100 µl with a short run time to analyze large number of samples. The developed method was validated as per FDA guidelines [19].

2. MATERIALS AND METHODS

2.1 Reagents and Chemicals

The reference standards of Metoprolol and bisoprolol were obtained from Natco Pharma Limited, India. Ammonium acetate from Fluka, analytical grade acetonitrile and methyl tertiary butyl ether were purchased from JT Bakers and water used was of Milli Q grade.

2.2 Instrumentation

Waters Quattro-Micro API mass spectrometer equipped with 2695 LC separation module connected to a triple quadrupole analyzer was used for LC/MS/MS analysis. The separation was carried out using unison US C18 column (50 x 4.6mm and 3.5µm) equipped with an ODS guard column. An isocratic elution of 10 mM ammonium acetate - acetonitrile (10:90 v/v) with a run time of 6.0 min and an injection volume of 10 µl were used. The flow rate was fixed at 0.8 ml/min with a split ratio of 7:3. Electron spray ionization in positive ion detection mode with MRM using parent-daughter transition of 268.71>116.2 for Metoprolol and 326.76>116.2 for bisoprolol was adopted.

2.3 Preparation of Stock Solutions

Stock solutions of Metoprolol and bisoprolol standards were prepared by dissolving appropriate amounts in acetonitrile. The working standard solutions for calibration curve and quality control and internal standard were prepared by dilution of the above stock solutions.

2.4 Preparation of Calibration Curve and Quality Control Samples

Bulk spiking of the calibration and quality control samples was done by adding required amounts of working standard solutions to drug-free human plasma. The calibration samples at concentrations of 5.0, 10.0, 20.0, 50.0, 100.0, 200.0 and 500.0 ng/ml and quality control samples at concentrations of 5.0, 15.0, 150.0 and 350.0ng/ml were spiked. After spiking aliquots each of 100 µl were transferred into eppendorff tubes and were stored at -70°C until further analysis. Three successive validation batches were done and on each validation the aliquots were thawed, 20 µl of internal standard were added and extracted by liquid-liquid extraction method.

2.5 Plasma Sample Extraction Procedure

To 100µl plasma, 20µl of internal standard (0.25µg/ml) were added and vortexed for about 30 sec. To this, 4ml of methyl tertiary butyl ether extraction solvent were added, vortexed for 5 min using

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Multipulse vortexer (Glas-Col) then 2 ml of the supernatant clear organic layer were transferred to a 7.5 ml test tube and evaporated to dryness using Speedovap at 40°C under a stream of nitrogen. Then the dried extract was reconstituted with 100μl of diluent (Water: Acetonitrile- 1:1) and a 10μl aliquot was injected into the chromatographic system. During each run a plasma blank sample and a zero standard sample (IS) were also analyzed.

3. RESULTS AND DISCUSSION

3.1 Optimization of Tandem Mass Spectrometry Conditions

The optimization of the source parameters was carried out by infusing 0.1mg/ml aqueous samples of Metoprolol and bisoprolol. In positive ionization mode the source parameters such as cone voltage, capillary voltage, desolvation gas temperature, desolvation gas flow and cone flow were optimized. Then the daughter ion was optimized using daughter ion scan by using collision gas. The collision energy and collision gas flow was optimized by using the MRM scan mode. All these parameters were optimized to produce maximum intensity of the ions. The optimized source parameters of the mass spectrometry are summarized in the table.1.

Table 1: LC/MS/MS source parameters

Source parameters	Value
Capillary voltage (KV)	3.6
Cone voltage (V)	35
Extractor voltage (V)	2
RF voltage(V)	0.2
Source temperature ($^\circ$C)	120
Desolvation temperature ($^\circ$C)	350
Desolvation gas flow (l/hr)	650
Cone gas flow (l/hr)	50
Resolution	12
Collision energy (eV)	20

3.2 Optimization of Chromatographic Conditions

Several trials were made by changing the pH of the buffer, solvent ratios of acetonitrile and methanol and by using different columns. Finally the optimized chromatographic conditions were set to obtain sharp peak shapes and short run time.

3.3 Method Validation

The method was validated for linearity, selectivity, sensitivity, accuracy, precision, recovery, autosampler stability, bench top stability and freeze thaw stability.

3.4 Linearity

The linearity was fixed from 5.0 to 500.0ng/ml based on the available clinical data and the response was found to be linear over this concentration range. The best linear fit and least square linear regression analysis for the curves was achieved with a weighing factor of 1/x to determine the intercept, slope and the correlation coefficient. Five different calibration curves were analyzed and the correlation coefficients were found to be greater than 0.995. The calibration curve is represented in the Fig.1 and the curve parameter summary of five calibration curves is given in the Table-2.
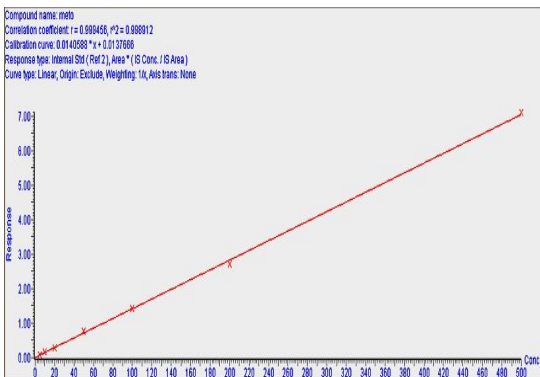

Fig.1: Calibration curve

Table 2: Curve parameter summary of five calibration curves

Curve code	Slope(a)	y-intercept (b)	Correlation coefficient (r^2)
1	0.0100	0.0066	0.997
2	0.0116	0.0050	0.998
3	0.0073	0.0106	0.997
4	0.0101	0.0033	0.996
5	0.0118	0.0113	0.998

3.5 Selectivity

The selectivity was established by analyzing blank human plasma extracts to check for potential interferences at the retention times of the analyte and the internal standard. The plasma samples analyzed showed that there is no interference due to any endogeneous components. A representative chromatogram of blank plasma, blank plasma spiked with IS and analyte at LLOQ concentration are shown in Fig.2, Fig.3 and Fig.4 respectively.
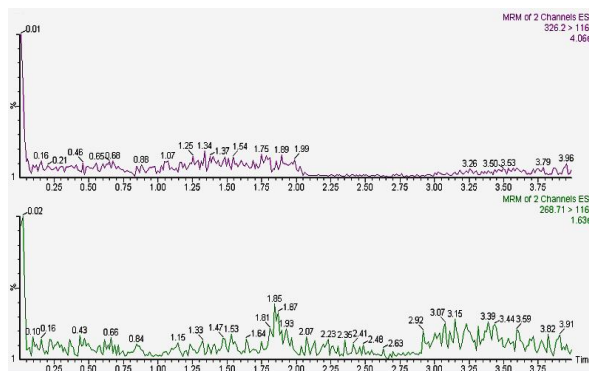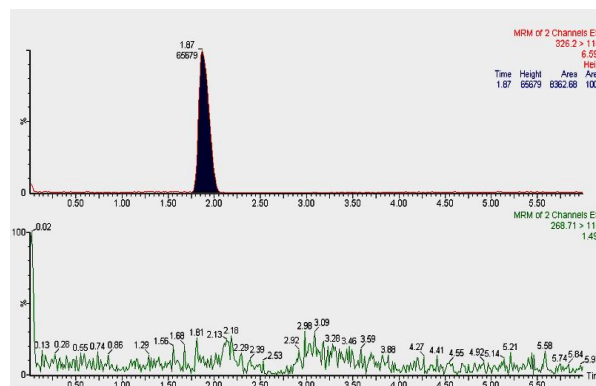

Fig.2: MRM chromatogram of blank human plasma

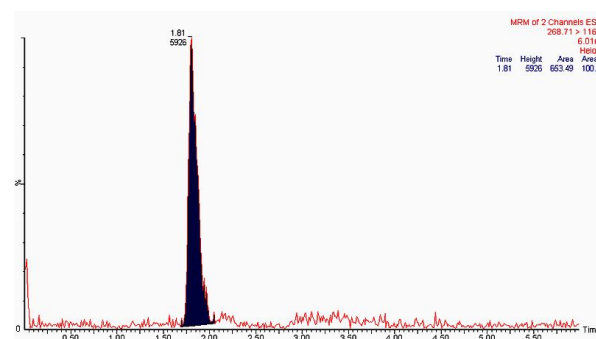**Fig 3:** MRM chromatogram of blank plasma spiked with internal standard

Fig 4: MRM chromatogram of LLOQ spiked plasma sample

3.6 Lower Limit of Quantification

Limit of quantification is the concentration at which the response is greater than 5 times compared to the response of blank plasma sample. A lower limit of quantification of 5ng/ml was established with an accuracy of 101.9% and a precision of 5.2%.

3.7 Accuracy and Precision

The accuracy of the assay is defined as the ratio of the mean of the assay values to the actual values expressed in percentage. The accuracy and precision were checked by analyzing six replicates of all the three quality control samples (15, 150.0 and 350.0ng/ml) against a single linearity curve on three different days. The intra-day/within run accuracy ranged from 92.7% to104.9% and the intra-day/within run precision ranged from 4.4% to 9.9%. The inter-day/between run accuracy ranged from 94.1% to 105.7% and the inter-day/between run precision ranged from 5.8% to 9.7%. The intra-day and inter-day accuracy and precision are given in Table-3

Table 3: Intra-day and inter-day accuracy and precision

Spiked concentration (ng/ml)	Intra-day (n=6)			Inter-day (n=18)		
	Determined concentration	Accuracy (%)	Precision (%)	Determined concentration	Accuracy (%)	Precision (%)
15.0	13.9	92.7	9.9	14.1	94.1	9.7
150.0	153.5	102.3	4.4	147.4	98.2	7.9
350.0	366.9	104.9	4.6	370.1	105.7	5.8

3.8 Recovery (Extraction Efficiency)

The extraction efficiency was determined by comparing the peak area of the extracted plasma samples of the quality controls to the peak area of extracted blank plasma spiked with standards containing the same concentrations. The extraction efficiency or the recovery of the analyte and the internal standard were consistently greater than 90% (95.2% for Metoprolol and 91.8% for bisoprolol).

3.9 Stability

Autosampler stability was determined for ~24 hrs to cover the anticipated run time for analytical batch and also to allow for delayed injection owing to unforeseen circumstances like instrument malfunction. The concentrations of the stability samples kept at autosampler temperature of 5°C for ~24 hrs were compared with the concentrations of the freshly prepared samples. The accuracy ranged from 89.5% to 98.1% and precision of the stability samples ranged from 5.5 to 6.4%. Bench-top stability was checked for six replicate QC samples at three different concentrations kept for 6 hrs at ambient temperature and processed thereafter. The concentration of the stability samples were compared against freshly spiked and processed standards. Metoprolol was found to be stable even after 6 hrs with accuracy ranged from 94.2% and 98.1% and precision ranged from 5.6 and 10.9%. Freeze thaw stability was determined during three freeze thaw cycles of six replicate QC samples at three different concentrations. The percentage degradation was determined by comparing the concentration of Metoprolol from the freshly prepared plasma validation samples at the same concentrations. After three successive freeze thaw cycles the concentrations of Metoprolol were nearly the same with the original concentrations and the percentage remaining at 95.3% to 109.2%.

3.10 Application of the Method

The validated bioanalytical method was successfully applied to quantify the concentrations of Metoprolol in plasma samples collected periodically up to 48 hrs after oral administration of 100 mg tablet to 12 healthy adult male volunteers during the development of a conventional formulation. The mean plasma concentration curves obtained for test and reference are represented in Fig.5. The 90% confidence interval of individual ratio geometric mean for test/reference was within 85–120% for $AUC0_t$, $AUC0_{inf}$, and C_{max}.
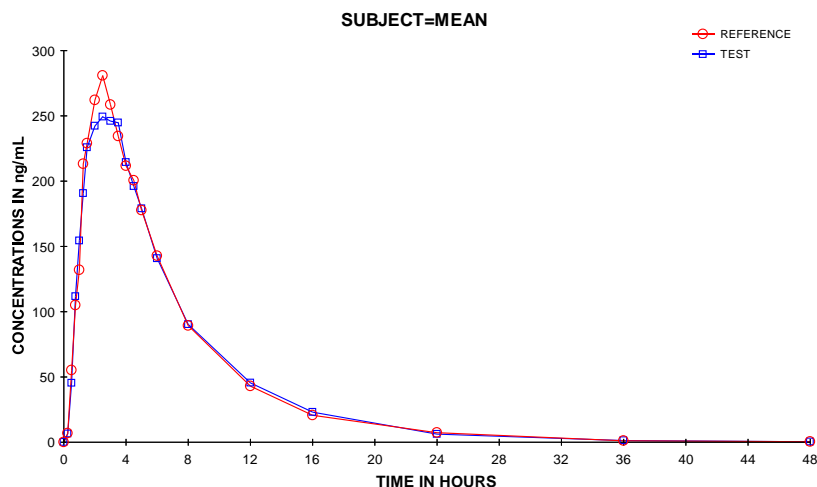

Fig 5: Mean concentration curves for test and reference

4. CONCLUSION

The proposed bioanalytical LC/MS/MS method for Metoprolol is simple, economical, sensitive and accurate to quantify the concentrations in human plasma in a small volume of 100 ul. A simple liquid-liquid extraction procedure which is cheaper compared to the solid phase extraction procedure and with a short run time is very useful to analyze large number of samples. This method is suitable for pharmacokinetic and bioequivalence studies in human plasma.

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