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Determination of Metoprolol in human blood plasma by LC-MS/MS

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ABSTRACT

A high performance LC-MS/MS method for determination of Metoprolol in human plasma was developed and successfully validated. Metoprolol is a β1-selective aryloxy propanolamine used in the treatment of cardiovascular disorders such as hypertension, arrhythmia and heart failure. Metoprolol (analyte) and the Metoprolol d7 (Internal Standard) were extracted from K3EDTA based Human Plasma samples by Liquid-liquid extraction method. Chromatographic separation was achieved on Gemini 5µm C18 110Å (150x4.6mm) column using Methanol: 10mM Ammonium Formate containing 0.1% Formic Acid Solution (95:5 v/v) as a mobile Phase at a flow rate of 0.800 mL/min with 80% flow splitting. The retention time of Metoprolol and Metoprolol d7 was found to be 1.49 minute and 1.49 minute respectively. The standard curve was linear (R2 > 0.99) over the concentration range of 0.68 ng/mL to 399.22 ng/mL. All the bioanalytical validation parameters were determined as per US FDA guidelines. The developed bioanalytical method was sensitive with a limit of detection below 1 ng/mL, with good linearity (r2 >0.99). The peaks obtained for the Metoprolol and Metoprolol d7 were symmetrical in nature and well resolved from each other without any interferences from human plasma.All the validation data, such as accuracy, precision, and percent recovery, lipemic effect, haemolysis effect, matrix factor, etc. were within the required limits as stated by guidelines. The method can be used for pharmacokinetic as well as Bioavailability, and bioequivalence studies.

Keywords: LC-MS/MS, Metoprolol, Liquid-liquid extraction, Human plasma, etc.

1. INTRODUCTION

Metoprolol, sold under the brand name Lopressor, among others, is a selective β_1 receptor blocker medication. It is used to treat high blood pressure, chest pain due to poor blood flow to the heart, and a number of conditions involving an abnormally fast heart rate. It is also used to prevent further heart problems after myocardial infarction and to prevent headaches in those with migraines. Metoprolol belongs to a class of drugs known as beta blockers. It works by blocking the action of certain natural chemicals in your body, such as epinephrine, on the heart and blood vessels. This effect lowers the heart rate, blood pressure, and strain on the heart. Therefore, to promote further

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understanding of Metorpolol plasma pharmacokinetics, we have developed a method for metoprolol using liquid liquid extraction and liquid chromatography coupled with tandem mass spectrometry detection in order to measure concentration of drug in human blood plasma.

2. EXPERIMENTAL

2.1 Principle of the method

Metoprolol and the Internal Standard Metoprolol-d7 were extracted from K_3EDTA based Human Plasma samples by Liquid-Liquid Extraction procedure and processed samples were then subjected to analysis using Methanol: 10mM Ammonium Formate containing 0.1% Formic Acid (95:5 v/v) as Mobile Phase. Quantitation was done by ***'Peak area ratio method'***. All regression and values in the validation procedure was generated by ***'Analyst Software'***.

2.2 Metabolite Information:

Quantification of Metoprolol from plasma samples was done using LC/MS/MS instrument. In this LC/MS/MS method, Multiple Reaction Monitoring (MRM) mode was used for the analysis. This MRM mode of analysis is very specific to molecule and analyzes the compound on the basis of fragmentation pattern i.e., parent ion (Q1) to daughter ion/s (Q3). In our analytical method, MRMs used were 268.200 / 116.100 amu for Metoprolol in positive polarity mode.

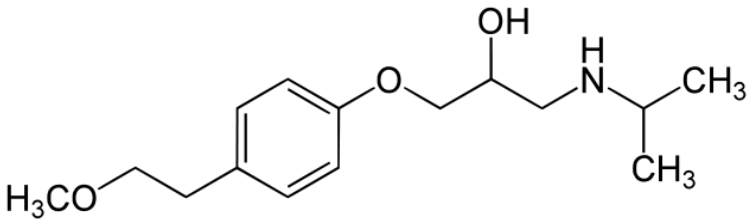

Fig. 1 Metoprolol (Molecular weight: 267.364 g/mol)

2.3 Reagents and Preparation of solutions

All the solvents, including LC-MS grade methanol and acetonitrile were obtained from Fisher Scientific. Formic acid (92%) was also obtained from Fisher Scientific. Vivan Life science provided both Metoprolol and Metoprolol d7 which serve as the internal standard (DTG-IS). Multiple lots of K3EDTA plasma were obtained from Biological specialities corporation. Water was purified on-site using Milli-Q water purification system from Millipore Corporation.

2.4 Biological matrix:

Biological matrix was K3EDTA based Human Plasma which was procured from M/s Srinivasa clinical Lab, Hyderabad. Whole Blood used for stability experiment was collected in In-house Clinical Laboratory. Metoprolol is known to be light sensitive molecule and hence all the activities were done under Sodium Vapour lamp.

2.5 Instrumentation and software:

Shimadzu Integrated UFLC-XR system (consisting of a CMB-20A Lite controller, LC-30AD pump, a DGU-20A_{5R} degasser, a SIL-30AC_{MP} autosampler, and a CTO-20AC column oven) coupled with an AB Sciex Linear Ion Trap Quadrupole 5500 were utilized for the separation and detection of Dolutegravir and Dolutegravir internal standard. Both the UFLC and mass spectrometer were

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controlled remotely using Analyst software v. 1.6.3. All statistical calculations were performed using Microsoft Excel capabilities.

Table 2.5.1. Summary of MS/MS parameters optimized for Metoprolol detection

<table>
<tr><th>Sr. No.</th><th>Parameter</th><th>Details</th></tr>
<tr><td>1</td><td>MS Transition</td><td>Metoprolol : Q1 mass 268.200 amu, Q3 mass 116.100 amu.
Metoprolol-d7: Q1 mass 275.060 amu, Q3 mass 122.900 amu.
<table>
<tr><th>Parameters</th><th>Metoprolol</th><th>Metoprolol-d7</th></tr>
<tr><td>Polarity</td><td>+ve</td><td>+ve</td></tr>
<tr><td>DP (volts)</td><td>23.00</td><td>23.00</td></tr>
<tr><td>EP (volts)</td><td>6.00</td><td>6.00</td></tr>
<tr><td>CEP(volts)</td><td>14.75</td><td>14.75</td></tr>
<tr><td>CE (volts)</td><td>28.00</td><td>28.00</td></tr>
<tr><td>CXP (volts)</td><td>3.00</td><td>3.00</td></tr>
<tr><td>FP (volts)</td><td>380.00</td><td>380.00</td></tr>
<tr><td>Dwell Time (msec)</td><td>200.00</td><td>200.00</td></tr>
</table></td></tr>
<tr><td>2</td><td>Source Dependent Parameters</td><td>CUR : 20.00
CAD : 6.00
IS (volts) : 5000.00
TEM(^{o}C) : 380.00
GS1 : 30.00
GS2 : 70.00</td></tr>
</table>

2.6 UPLC separation and MS-MS detection

All samples were subjected to chromatographic separation using a Shimadzu integrated UFLC-XR system with Gemini 5µm C18 110Å (150x4.6mm) column. Chromatographic analyses were performed isocratically at 30^0C and at a flow rate of 0.800 mL/min, with an overall run time of 2.40 minute. The mobile phase was composed of Methanol: 10mM Ammonium Formate containing 0.1% Formic Acid Solution (95:5 v/v). Samples are maintained at 5^0C in the autosampler with a 5 µL aliquot of each sample being injected onto the column. The needle was washed before sample aspiration with Methanol: Water (50:50 v/v), to minimize carryover between injections. Metoprolol and Metoprolol -IS eluted from the column at approximately 1.50 min, and were detected using multi reaction monitoring (MRM). The API 4500 instrument was used in positive TurbolonSpray mode and the following transitions for protonated products $[M+H]^+$ were monitored and acquired: m/z 268.200 / 116.100 amu for Metoprolol; m/z Metoprolol -IS 275.060 →122.900. Settings for the individual mass spectrometer parameters are listed in Table 2.5.1. Traces correlating to the above transitions were integrated using the MultiQuant software and concentration values were obtained using Metoprolol to Metoprolol -IS peak area ratio.

2.7 Solution preparation

Table 2.7.1: Preparation of Spiking solution for Calibration Curve Standards

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Parent Solution ID	Concentration of Parent Solution (ng/mL)	Volume of Parent Solution (mL)	Volume of Diluent (mL)	Final Volume (mL)	Concentration of Spiking Solution (ng/mL)	Spiking Solution ID
Metoprolol Intermediate Solution (CC)	75040.99	1.064	8.936	10.000	7984.36	**SS CS 8**
SS CS 8	7984.36	8.000	2.000	10.000	6387.49	**SS CS 7**
SS CS 7	6387.49	7.500	2.500	10.000	4790.62	**SS CS 6**
SS CS 6	4790.62	6.800	3.200	10.000	3257.62	**SS CS 5**
SS CS 5	3257.62	5.000	5.000	10.000	1628.81	**SS CS 4**
SS CS 4	1628.81	5.000	5.000	10.000	814.41	**SS CS 3**
SS CS 3	814.41	0.330	9.670	10.000	26.88	**SS CS 2**
SS CS 2	26.88	5.050	4.950	10.000	13.57	**SS CS 1**

Table 2.7.2: Preparation of Spiking Solutions for Quality Control Samples

Parent Solution ID	Concentration of Parent Solution (ng/mL)	Volume of Parent Solution (mL)	Volume of Diluent (mL)	Final Volume (mL)	Concentration of Spiking Solution (ng/mL)	Spiking Solution ID
Metoprolol Intermediate Solution (QC)	75169.69	0.820	9.180	10.000	6163.91	**SS HQC**
SS HQC	6163.91	4.000	6.000	10.000	2465.56	**SS MQC**
SS MQC	2465.56	0.165	9.835	10.000	40.68	**SS LQC**
SS LQC	40.68	3.350	6.650	10.000	13.63	**SS LLOQ**

Table 2.7.3: Preparation of Bulk Spiked Calibration Curve Standards

Spiking Solution ID	Concentration of Spiking solution of Analyte (ng/mL)	Volume of Spiking solution of Analyte (mL)	Volume of Plasma (mL)	Final Volume (mL)	Final Concentration (ng/mL)	Calibration Curve Standards ID
SS CS 8	7984.36	0.050	0.950	1.000	399.22	**CS 8**
SS CS 7	6387.49	0.050	0.950	1.000	319.37	**CS 7**
SS CS 6	4790.62	0.050	0.950	1.000	239.53	**CS 6**
SS CS 5	3257.62	0.050	0.950	1.000	162.88	**CS 5**
SS CS 4	1628.81	0.050	0.950	1.000	81.44	**CS 4**
SS CS 3	814.41	0.050	0.950	1.000	40.72	**CS 3**
SS CS 2	26.88	0.050	0.950	1.000	1.34	**CS 2**
SS CS 1	13.57	0.050	0.950	1.000	0.68	**CS 1**
Diluent	0.00	0.100	1.900	2.000	0.00	**Blank & Blank+IS**

Table 2.7.4: Preparation of Freshly Spiked Quality Control Samples

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Spiking Solution ID	Concentration of Spiking solution (ng/mL)	Volume of Spiking solution (mL)	Volume of Plasma (mL)	Final Volume (mL)	Final Concentration (ng/mL)	Quality Control Samples ID
SS HQC	6163.91	0.050	0.950	1.000	308.20	**HQC**
SS MQC	2465.56	0.050	0.950	1.000	123.28	**MQC**
SS LQC	40.68	0.050	0.950	1.000	2.03	**LQC**
SS	13.63	0.050	0.950	1.000	0.68	**LLOQ**

Table 2.7.5: Preparation of Bulk Spiked Quality Control Samples for Metoprolol

Spiking Solution ID	Concentration of Spiking Solution (ng/mL)	Volume of Spiking Solution (mL)	Volume of plasma (mL)	Final Volume (mL)	Final Concentration of (ng/mL)	Quality Control Samples ID
SS HQC	6163.91	3.000	57.000	60.000	308.20	**HQC**
SS LQC	40.68	3.000	57.000	60.000	2.03	**LQC**

2.8 Sample preparation:

The extraction of metoprolol was carried out by using liquid liquid extraction technique.

2.9 Data Calculations:

Chromatograms were acquired using the Data acquisition software. The concentrations of the samples were calculated using linear regression equation ($y = ax + b$) with $1/x^2$ weighing factor by Analyst software 1.6.3. The final print of the data was taken using Cred-Bio Application, version 1.0.2.215, Scientific Data Management System (SDMS) and these electronically generated printouts were used for further activities. Final concentration values for Analyte were reported in ng/mL. The Precision (% RSD), % Accuracy and % Bias were rounded to the 2nd decimal place.

2.10 Method validation:

Method validation is a part of GLP study and it is to ensure the quality, reproducibility and reliability of the method. Developed method was validated as per US FDA guidelines for bioanalytical method validation.

2.10.1 Precision and Accuracy:

Precision and Accuracy was performed at LLOQ QC, LQC, MQC and HQC levels which were extracted as per extraction procedure. Inter batch and Intra-batch Precision and Accuracy experiments were done as a part of this validation exercise. A single Precision and Accuracy batch consisted of one set of Calibration Curve Standards and six replicates of extracted samples for each concentration of LLOQ QC, LQC, MQC and HQC.

% RSD and % Nominal of back calculated concentration of analyte was calculated.

2.10.1.1 Intra-Day Precision and Accuracy

Intra-Day Precision and Accuracy experiment should be evaluated with Precision and Accuracy batches, which were processed separately on same analytical day.

2.10.1.2 Inter-Day Precision and Accuracy

Inter-Day Precision and Accuracy experiment should be evaluated with Precision and Accuracy batches, which were processed on different analytical days.

2.10.2 Sensitivity:

Sensitivity was evaluated by injecting extracted samples in K3EDTA based Human Plasma from six different lots. The samples were spiked with spiking solution of STD A (LLOQ) and processed as per extraction procedure along with one Precision and Accuracy batch in K3EDTA based human plasma. Back calculated concentration was obtained from the software.

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Signal to Noise ratio (S/N) was calculated for each sensitivity sample. The % RSD and % Nominal of back calculated concentration of Analyte at LLOQ level was calculated.

2.10.3 Calibration Curve Standards:

Calibration Curve Standards were prepared using pooled K3EDTA based Human Plasma. Calibration Curve Standards consisted of Blank sample (matrix sample processed without analyte and IS), Blank + IS (matrix sample processed with IS) and eight non-zero standards in the concentration range of 0.68 ng/mL to 399.22 ng/mL.

Table 2.10.3.1: Range of Calibration Curve Standards

STD ID	Concentration in Plasma (ng/mL)
Blank	0.00
Blank + IS	0.00
CS 1	0.68
CS 2	1.34
CS 3	40.72
CS 4	81.44
CS 5	162.88
CS 6	239.53
CS 7	319.37
CS 8	399.22

2.10.4 Haemolysis effect

This exercise should be done to assess the Haemolysis effect throughout the application of this method. Haemolyzed matrix has a lot of inherent variability and can affect the response of Analyte during the method validation and subsequently in subject sample analysis. The quantification of Analyte from plasma can be grossly affected by Haemolysis of samples.

For Haemolysis effect experiment, LQC and HQC concentration levels were spiked in Haemolyzed plasma (six individual sample preparations). These samples were extracted as per the extraction procedure along with Precision and Accuracy batch prepared in Non-Haemolyzed plasma. Back calculated concentration should be obtained from the software.

2.10.5 Lipemic effect

This exercise will be done to assess the Lipemic effect throughout the application of this method. Lipemic matrix has a lot of inherent variability and can affect the response of Analyte during the method validation and subsequently in subject analysis. The quantification of Analyte from plasma can be grossly affected by a significant Lipid content in sample. For Lipemic effect experiment, LQC and HQC concentration levels were spiked in Lipemic plasma (six individual sample preparations). These samples were extracted as per procedure along with Precision and Accuracy batch prepared in non Lipemic plasma.

2.10.6 Matrix factor

Matrix Factor should be performed at Low Quality Control (LQC) and High Quality Control (HQC) concentration in at least eight different lots of same type of matrix, out of which 06 should be normal buffered / heparinised / EDTA matrix, and out of other two, one Lipemic matrix and one Haemolysed matrix or other method specific anticoagulant and if applicable, sample matrix from special populations, such as renally or hepatically impaired populations. This is to ensure that there is no impact / effect of different matrix lots or matrix composition (Lipemic matrix, Haemolysed matrix, etc.) on the methods reproducibility with respect to selectivity, precision and accuracy of results.

To evaluate Matrix Factor process two samples from each of the above mentioned eight matrix lots, as blank, as per the procedure. These final elute / reconstituted solution samples are considered as extracted blank with matrix ions.

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Table 2.10.6.1: Sample Preparation for Matrix Factor

Volume of SS LQC/ SS HQC	Volume of Internal Standard Spiking Solution	Volume of Mobile phase	Final volume
100 µL	200µL	700 µL	1000 µL

2.10.7 Percent Recovery

Preparation of Unextracted Samples:

Eighteen blank plasma samples (six samples each for concentration at LQC, MQC and HQC level) were processed upto Step No. 11 as described in procedure. After drying all the samples were reconstituted with 200 µL of respective solution prepared as per Table 7.5.8.1. All pp tubes were vortexed for about 30 seconds. These processed samples were then subjected to LC/MS/MS analysis.

Table 2.10.7.1: Sample Preparation for Percent Recovery

Volume of SS LQC/ SS MQC/ SS HQC	Volume of Internal Standard Spiking Solution	Volume of Mobile phase	Final volume
100 µL	200µL	700 µL	1000 µL

Preparation of Extracted Samples:

The recovery batch was consisting of one set of Calibration Curve Standards and Quality Control Samples spiked with analyte at 3 different concentration levels i.e. LQC, MQC and HQC. These samples were processed as per extraction procedure and processed samples were then subjected to LC/MS/MS analysis.

2.10.8 Dilution Integrity

Dilution integrity should be performed to quantify values (concentrations) which are above the Upper Limit of Quantification (ULOQ). These samples cannot be accurately quantified using the calibration curve as per the validation data used in this Bioanalytical method.

Dilution Integrity Spiking Solution (DISS) for DI standard (1/2) and DI standard (1/10) was prepared as per Table 7.5.9.1. DI standard (1/2) and DI standard (1/10) having concentration of 2 times and 10 times of spiking solution of HQC concentration level was prepared as per Table 2.10.8.1. The DI 1/2 and DI 1/10 sample was prepared from Dilution Integrity standards by diluting the samples to 2 and 10 times using screened K_3EDTA based Human Plasma (Six individual preparation at each dilution level). These samples were extracted as per procedure along with Precision and Accuracy batch. Back calculated concentrations were obtained from the software.

Table 2.10.8.1 Preparation of Spiking Solutions for Dilution Integrity Standards

Parent Solution ID	Concentration of Parent Solution (ng/mL)	Volume of Parent Solution (mL)	Volume of Diluent (mL)	Final Volume (mL)	Concentration of spiking Solution (ng/mL)	Spiking Solution ID
Metoprolol Intermediate Solution	75040.99	0.822	4.178	5.000	12336.74	SS DI (1/2)
		0.822	0.178	1.000	61683.69	SS DI

Table 7.5.9.2: Preparation of Dilution Integrity Standard Samples

Spiking Solution ID	Concentration of Spiking solution (ng/mL)	Volume of Spiking solution (mL)	Volume of Plasma (mL)	Final Volume (mL)	Final Concentration (ng/mL)	STD ID

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SS DI (1/2)	12336.74	0.250	4.750	5.000	616.84	**DI STANDARD (1/2)**
SS DI (1/10)	61683.69	0.250	4.750	5.000	3084.18	**DI STANDARD (1/10)**
STD ID	**Concentration DI Standard (ng/mL)**	**Volume of DI Standard (mL)**	**Volume of Plasma (mL)**	**Final Volume (mL)**	**Final Concentration (ng/mL)**	**Sample ID**
DI STANDARD (1/2)	616.84	0.250	0.250	0.500	308.42	**DI (1/2)**
DI STANDARD (1/10)	3084.18	0.050	0.450	0.500	308.42	**DI (1/10)**

3. RESULT AND DISCUSSION:

3.1 Chromatography, detection and quantitation of Metoprolol

Initial optimization of the triple quadrupole mass spectrometer for the detection of an dolutegravir begins with optimizing for the parent compound, in this case dolutegravir, in the single-quadrupole mode. Plasma samples were then spiked with DTG, extracted and reconstitution volume and product ions were optimized further. Ultimately, only a single channel each for Metoprolol and Metoprolol - IS (m/z 268.200→116.100) and (m/z 275.060 →122.900.) was chosen and used for quantitation.

3.2 Method Validation

Validation parameters were performed on different days. Each validation run contained Blank and Blank + IS samples, a full 9-point curve, plus six replicates each of LLOQ, LQC, MQC and HQC respectively.

3.2.1 Precision and Accuracy

Table 3.2.1.1: Observations of Inter-batch Precision and Accuracy

Global Calculation for Inter-batch Precision and Accuracy				
QC ID	**LLOQ QC**	**LQC**	**MQC**	**HQC**
Nominal Concentration (ng/mL)	**0.68**	**2.03**	**123.28**	**308.20**
Acceptance Limit (ng/mL)	**0.54 to 0.82**	**1.73 to 2.33**	**104.79 to 141.77**	**261.97 to 354.43**
N	**30**	**30**	**30**	**30**
Mean	0.70	2.19	125.81	316.88
SD	0.0676	0.1990	2.8976	6.5771
% RSD	9.66	9.09	2.30	2.08
% Mean Accuracy	102.94	107.88	102.05	102.82

Table 3.2.1.2: Observations of Intra-batch Precision and Accuracy

Global Calculation for Intra-batch Precision and Accuracy				
QC ID	**LLOQ QC**	**LQC**	**MQC**	**HQC**

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Nominal Concentration (ng/mL)	**0.68**	**2.03**	**123.90**	**302.21**
Acceptance Limit (ng/mL)	**0.54 to 0.82**	**1.73 to 2.33**	**105.32 to 142.49**	**256.88 to 347.54**
N	**63**	**63**	**63**	**63**
Mean	0.65	1.97	123.26	300.54
SD	0.0694	0.1419	3.2999	11.8967
% RSD	10.68	7.20	2.68	3.96
% Mean Accuracy	95.59	97.04	99.48	99.45

Acceptance Criteria

Parameter	Limit
% RSD for LLOQ QC level	NMT 2.00%
% RSD for samples other than LLOQ QC	NMT 15.00%
% Bias for LLOQ QC level	± 20.00%
% Bias for samples other than LLOQ QC	± 15.00%
% Nominal for LLOQ QC level	80.00% to 120.00%
% Nominal for samples other than LLOQ QC	85.00% to 115.00%
At least 67% of total QC samples and 50% at each concentration level must comply with above mentioned criteria of % Nominal.	

Result: Both Intra and Inter day Precision and Accuracy batch found within the acceptance criteria.

3.2.2 Sensitivity

Table 3.2.2.1: Observations of Sensitivity

Batch ID	**300519PA02,SEN02-01**	**Signal to noise ratio (S/N)**	**310519PA03,SEN03,HE01,LP01-01**	**Signal to noise ratio (S/N)**	**010619PA04,SEN04,DI01**	**Signal to noise ratio (S/N)**
Sample ID	**LLOQ**		**LLOQ**		**LLOQ**	
Nominal Concentration (ng/mL)	**0.68**		**0.68**		**0.68**	
Acceptance Limit (ng/mL)	**0.54 to 0.82**		**0.54 to 0.82**		**0.54 to 0.82**	
Sr. No.	**Back Calculated Concentration (ng/mL)**		**Back Calculated Concentration (ng/mL)**		**Back Calculated Concentration (ng/mL)**	
1	0.71	50.63	0.79	84.25	0.67	24.53
2	0.68	24.61	0.63	89.49	0.74	39.95
3	0.74	51.54	0.61	98.14	0.72	31.28
4	0.77	54.11	0.73	103.33	0.91	6.29
5	0.69	36.89	0.66	112.23	0.71	30.09
6	0.64	64.37	0.67	87.15	0.76	28.43
Mean	0.71		0.68		0.75	
SD	0.0459		0.0671		0.0833	
% RSD	6.46		9.87		11.11	
% Mean Accuracy	104.41		100.00		110.29	

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Acceptance Criteria:
Precision and Accuracy batch acceptance criteria must be met. S/N Ratio of Sensitivity samples should not less than 5.00, %RSD should not more than 20.00% and %Nominal should be in between 80.00 to 120.00%
Result*:* The acceptance criteria were met.

3.2.3 Calibration curve standards

Table 3.2.3.1: Observation of Freshly Spiked Calibration Curve Standards (0.68 ng/mL to 399.22 ng/mL)

STD ID	CS 1	CS 2	CS 3	CS 4	CS 5	CS 6	CS 7	CS 8
Nominal Concentration (ng/mL)	**0.68**	**1.34**	**40.72**	**81.44**	**162.88**	**239.53**	**319.37**	**399.22**
Acceptance Limit (ng/mL)	**0.54 to 0.82**	**1.14 to 1.54**	**34.61 to 46.83**	**69.22 to 93.66**	**138.45 to 187.31**	**203.60 to 275.46**	**271.46 to 367.28**	**339.34 to 459.10**
Batch ID	**Back Calculated Concentration (ng/mL)**							
280519SEL02	0.68	1.33	39.52	81.69	162.73	237.65	319.95	414.12
300519PA02,SEN02-01	0.65	1.46	38.59	78.82	159.93	241.92	316.42	422.23
310519PA03,SEN03,HE01,LP01-01	0.68	0.85*	38.79	80.32	159.60	234.48	318.21	441.17
310519RUGG(DA,DC)01	0.66	1.40	40.34	82.79	156.71	240.37	323.46	395.36
010619PA04,SEN04,DI01	0.67	1.40	39.32	77.75	170.65	242.83	325.61	390.19
010619RUGG(DE)01	0.66	1.42	40.62	82.57	164.24	241.31	313.34	384.89
Mean	0.67	1.40	39.53	80.66	162.31	239.76	319.50	407.99
SD	0.0121	0.0471	0.8148	2.0599	4.8544	3.1367	4.5235	21.6852
% RSD	1.81	3.36	2.06	2.55	2.99	1.31	1.42	5.32
% Mean Accuracy	98.53	104.48	97.08	99.04	99.65	100.10	100.04	102.20

Acceptance Criteria:
Correlation Coefficient should be ≥ 0.99, %Nominal at STD A level should be in between 80.00 to 120.00% and for standards other than STD A should be 85.00 to 115.00%. At least seven out of nine non-zero standards must meet the above mentioned criteria. For Blank and Blank + IS sample: If any peak area is present at the retention time of Analyte in Blank or Blank+IS sample, its area response must be < 20.00% of Analyte area response of LLOQ standard. If any peak area is present at the retention time of internal standard in Blank sample, its area response must be < 5.00% of the IS area response of LLOQ standard. Both Blank & Blank+IS samples must meet above mentioned acceptance criteria.
Result: Calibration Curve Standards were found within the acceptance criteria.

3.2.4 Haemolysis Effect

Table 3.2.4.1 Observations of Haemolysis effect

Sample ID	HE LQC	HE HQC
Nominal Concentration (ng/mL)	**2.03**	**308.20**
Acceptance Limit (ng/mL)	**1.73 to 2.33**	**261.97 to 354.43**
Sr. No.	**Back Calculated Concentration (ng/mL)**	
QC No.	**HE LQC (01 to 06)**	**HE HQC (01 to 06)**
1	1.86	333.73
2	2.00	325.55
3	2.08	320.19

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4	1.85	323.50
5	1.75	319.93
6	1.93	332.80
Mean	1.91	325.95
SD	0.1175	6.0503
% RSD	6.15	1.86
% Mean Accuracy	94.09	105.76

Acceptance criteria:
% RSD should not more than 15.00%, % Nominal should in between 85.00 to 115.00%
At least 67 % of total QC samples and 50 % at each concentration level must comply with above mentioned criteria of % Nominal.
Result: The acceptance criteria were met.
Conclusion: Quantitation of Metoprolol was not affected by Haemolysis of samples.

3.2.5 Lipemic Effect

Table 3.2.5.1: Observations of Lipemic effect

Sample ID	**LP LQC**	**LP HQC**
Nominal Concentration (ng/mL)	**2.03**	**308.20**
Acceptance Limit (ng/mL)	**1.73 to 2.33**	**261.97 to 354.43**
Sr. No.	**Back Calculated Concentration (ng/mL)**	
QC No.	**LP LQC (01 to 06)**	**LP HQC (01 to 06)**
1	1.97	324.89
2	1.88	332.77
3	1.77	318.96
4	1.69	327.30
5	1.84	327.69
6	2.11	328.32
Mean	1.88	326.66
SD	0.1488	4.5603
% RSD	7.91	1.40
% Mean Accuracy	92.61	105.99

Acceptance criteria:
% RSD should not more than 15.00%, % Nominal should in between 85.00 to 115.00%
At least 67 % of total QC samples and 50 % at each concentration level must comply with above mentioned criteria of % Nominal.
Result: The acceptance criteria were met.
Conclusion: Quantitation of Metoprolol was not affected by Haemolysis of samples.

3.2.6 Matrix factor

Table 3.2.6.1: Observation of Matrix effect for LQC Concentration

Sr. No.	**Lot No.**	**Unextracted Analyte Area**	**Aqueous Analyte Area**	**Matrix Factor For Analyte**	**Unextracted Internal Standard Area**	**Aqueous Internal Standard Area**	**Matrix Factor for Internal Standard**	**IS Normalized Matrix Factor**
1.	P7912	9404	11922	0.79	299126	375732	0.80	0.99
2.	P8230	7590	12173	0.62	242031	375237	0.65	0.95
3.	P8329	8491	11997	0.71	259593	365151	0.71	1.00

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4.	P8338	8214	11786	0.70	253553	373997	0.68	1.03
5.	P8339	8109	12379	0.66	251461	383415	0.66	1.00
6.	P8341	7462	11465	0.65	238220	353267	0.67	0.97
7.	H7941	7993	11677	0.68	252458	380185	0.66	1.03
8.	L7938	8915	11708	0.76	266697	383706	0.70	1.09
Average								1.01
SD								0.0430
% RSD								4.26

Table 3.2.6.2: Observation of Matrix effect for HQC Concentration

Sr. No.	Lot No.	Unextracted Analyte Area	Aqueous Analyte Area	Matrix Factor For Analyte	Unextracted Internal Standard Area	Aqueous Internal Standard Area	Matrix Factor for Internal Standard	IS Normalized Matrix Factor
1	P7912	833889	1214291	0.69	178578	250407	0.71	0.97
2	P8230	868277	1230499	0.71	184410	255299	0.72	0.99
3	P8329	894875	1217452	0.74	188377	252219	0.75	0.99
4	P8338	898963	1266104	0.71	191280	266584	0.72	0.99
5	P8339	917339	1281453	0.72	197798	267690	0.74	0.97
6	P8341	983738	1272708	0.77	211280	265164	0.80	0.96
7	H7941	963852	1236854	0.78	200320	259650	0.77	1.01
8	L7938	991820	1249325	0.79	207309	265559	0.78	1.01
Average								0.99
SD								0.0185
% RSD								1.87

Acceptance criteria: % Nominal for LQC & HQC samples should in between 85.00 to 115.00% and % RSD of back calculated concentration of six samples should not more than 15.00%. At least 67% of total QC samples & 50% at each concentration level must comply with above mentioned criteria of % Nominal.

Result: The acceptance criteria were met.

Conclusion: Quantitation of Metoprolol was not affected by Matrix Effect.

3.2.7 Percent Recovery

Table 3.2.7.1: Observations for Recovery of Metoprolol

QC ID	Peak area for Analyte					
	LQC		MQC		HQC	
Sr. No.	**Extracted**	**Unextracted**	**Extracted**	**Unextracted**	**Extracted**	**Unextracted**
1	6414	8932	373761	485928	782196	1089772
2	6904	9237	371451	511272	790996	1077573
3	6503	8859	378779	473381	791876	1049844
4	6110	9443	369816	497335	773225	1051602
5	6812	9252	371969	494556	783865	1088546
6	6424	9547	383592	511648	804671	1044549
Mean	6527.83	9211.67	374894.67	495686.67	787804.83	1066981.00
% Recovery	70.86		75.63		73.83	

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Table 3.2.7.2: Observations for Recovery of Metoprolol-d7

QC ID	Peak area for Internal standard					
	LQC		MQC		HQC	
Sr. No.	Extracted	Unextracted	Extracted	Unextracted	Extracted	Unextracted
1	189169	264635	170868	223972	148148	210086
2	191735	252537	173023	234543	153576	203618
3	188792	241738	174216	219110	152839	198457
4	184719	267979	175008	225507	144601	195293
5	189470	274506	174114	231555	147125	206578
6	186465	269155	178770	243916	153215	205852
Mean	188391.67	261758.33	174333.17	229767.17	149917.33	203314.00
% Recovery	71.97		75.87		73.74	

Table 3.2.7.3: Observations for Average Recovery of Metoprolol and Metoprolol d7

Sr. No.	QC Level	% Recovery of Metoprolol	% Recovery Metoprolol-d7
1	LQC	70.86	71.97
2	MQC	75.63	75.87
3	HQC	73.83	73.74
Average Recovery		73.44	73.86
SD		2.4088	1.9528
% Mean RSD		3.28	2.64

Acceptance Criteria:
Precision (%RSD) of overall (%) Recovery must be within 15% and (%) Recovery must be ≥ 40.00% at individual quality control level (i.e. LQC, MQC and HQC). % RSD of Analyte and internal standard area response must be within 15% at individual quality control level for extracted and Unextracted/Aqueous samples.
Result: The acceptance criteria were met.

3.2.8 Dilution Integrity

Table 3.2.8.1: Observations of Dilution Integrity

Sample ID	DI 1:2	DI 1:10
Nominal Concentration (ng/mL)	616.84	3084.18
Acceptance Limit (ng/mL)	524.31 to 709.37	2621.55 to 3546.81
Sr. No.	Back Calculated Concentration (ng/mL)	
QC No.	DI 1/2 (01 to 06)	DI 1/10 (01 to 06)
1.	645.25	3251.80
2.	641.77	3215.85
3.	653.18	3263.19
4.	662.22	3284.63
5.	662.12	3279.48
6.	656.73	3240.96
Mean	653.55	3255.99
SD	8.5618	25.6068
% RSD	1.31	0.79
% Mean Accuracy	105.95	105.57

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Acceptance criteria:
% RSD should not more than 15.00% and % Nominal should in between 85.00 to 115.00%. At least 67 % of DI samples at each concentration level must comply with above mentioned criteria.
Result: The % RSD and % Nominal of back calculated concentration of Dilution Integrity samples were within the acceptance criteria.
Conclusion: Samples having concentration above ULOQ can be analyzed by diluting the sample with maximum concentration of 3084.18 ng/mL.

4. CONCLUSION

A Sensitive, precise, and rapid LC-MS/MS method was developed and validated for determination of Metoprolol in human plasma with investigated concentration range 0.68 ng/mL to 399.22 ng/mL. Upon injection of spiked plasma after a valid extraction procedure into LC-MS/MS instrument at LLOQ, sensitivity and specificity shows acceptable results which means that instrument at this lowest concentration is capable to give a reproducible results. The method has excellent accuracy, and precision which enables detection of Metoprolol. There were no significant haemolysed and lipemic effects were observed by analysing the plasma samples on LC-MS/MS. Further good results were obtained in plasma calibration curve. Stability data of Metoprolol found to be satisfactory. This liquid-liquid extraction method has several advantages including good recovery and accuracy, easier collection of total fraction of analyte, etc. Hence, it can be concluded that the Sensitive, rapid LC-MS/MS method was developed and validated for determination of Metoprolol in Human plasma and can be successfully applied for bioequivalence studies in human subjects.

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