ORIGINAL ARTICLE

New Bioanalytical Method Development and Validation for Extraction of Mirabegron in Human Plasma by using Quechers Method and Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

Aim: To develop and validate a sensitive, robust liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for quantifying Mirabegron in K2EDTA human plasma, ensuring suitability for routine analytical applications. **Materials and Methods:** Employing a C18 column and an isocratic mobile phase, achieving excellent chromatographic resolution of mirabegron was achieved. Plasma samples were prepared using the QuEChERs extraction technique, ensuring efficient sample cleanup and consistent recovery. Mirabegron D5 served as the internal standard, closely matching the analyte in terms of extraction efficiency and chromatographic behavior. The autosampler temperature was set at 5°C with a total run duration of 4.5 min. Mirabegron and its internal standard exhibited a retention time of 2.05 min. **Results and Discussion:** The QuEChERs extraction technique met all validation criteria, demonstrating a mean recovery of 79.44% for mirabegron and 78.74% for the internal standard. The calibration curve was linear over 0.201–100.677 ng/mL ($r^2 = 0.9976$), with a lower limit of quantification of 0.201 ng/mL, confirming high sensitivity. The method's simplicity, robustness, and rapid analysis time underscored its applicability for high-throughput studies. **Conclusion:** The validated LC-MS/MS method proved effective, simple, and robust, making it well-suited for routine pharmacokinetic and bioequivalence studies of mirabegron in human plasma.

Key words: Mirabegron, human plasma, QuEChERS, validation, liquid chromatography-tandem mass spectrometry

INTRODUCTION

Chemically, mirabegron (MBG) is 2-(2-amino-1, 3-thiazol-4-yl)-N-C4-(2-[(2R)-2-hydroxy 2-phenylethyl]aminoethyl)-phenyl] [Figure 1].[1] The molecular weight of the molecular formula $C_{21}H_{24}N_4O_2S$ is 396.506 g/moL.[2] Overactive bladder presents a clinical challenge, often managed with MBG, a selective β3 adrenergic receptor agonist serving as a substitute for antimuscarinic medications. In healthy volunteers, oral administration of MBG aims to optimize absorption, maintaining therapeutic focus for approximately 3.5 h post-dose. Notably, a dosage of 25 mg initiates this effect, while escalating to 50 mg enhances total bioavailability to 35%, driven by a remarkable absolute increase of 29%. Metabolism of MBG involves various pathways, including dealkylation, oxidation, direct glucuronidation, and amidation, forming several metabolites, with glucuronide as the predominant form in human plasma.[3] Pharmacologically, these metabolites exhibit inactivity towards β3 adrenergic receptors. In addition, MBG undergoes active renal elimination via glomerular filtration and tubular secretion. The literature review identifies two robust analytical methods, liquid chromatography-tandem mass spectrometry (LC-MS/MS)

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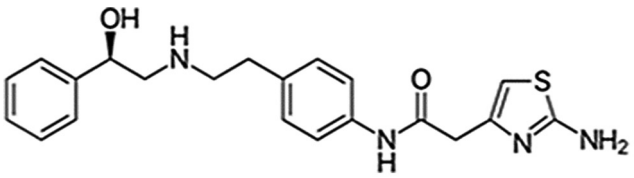

Figure 1: Mirabegron chemical structure

and high-performance liquid chromatography (HPLC), as efficient tools for MBG quantification in routine laboratory settings, thus facilitating comprehensive pharmacokinetic (PK) evaluations and therapeutic monitoring.[4,5]

The current study's targets were to create and evaluate parameters for MBG and to establish a validated LC-MS/MS assay to quantify MBG in human plasma as a different biological matrix to the drug.

MATERIALS AND METHODS

Reagents and chemicals

All reagents and solvents were HPLC, or analytical grade, were used in the study: Acetonitrile, water, methanol, ammonium acetate, magnesium sulfate, and purchase and sale agreement (PSA), which are purchased from SD fine chemicals, Mumbai, India. Standards MBG and MBG-D5 were obtained by Vivan Life Sciences Pvt. Limited.

Methodology

Several trials were carried out for method optimization, such as in chromatographic conditions like mobile phase, we performed different compositions of acetonitrile and ammonium acetate, reduced peak noise threshold, and columns (Thermo Hypersil BDS C_{18} 100 mm × 4.6 mm, 5 µm) for peak optimization. In the extraction procedure, we used different salts (NaCl and $MgSo_4$) with different proportions to increase the recovery.

Instrumentation

This study's LC-MS/MS system comprised an Agilent 1200 series coupled with a Tandem mass spectrometer of API 3200 by AB Sciex. Chromatographic separation was facilitated using a Discovery® C18 column, measuring 100 mm in length and 4.6 mm in diameter, with a particle size of 5 µm.[7]

Conditions of LC-MS/MS

The chromatographic conditions are described in Table 1.

Table 1: Chromatographic and mass spectroscopic details

Chromatographic conditions	
Mobile phase	(90:10) (Acetonitrile: 10 mM Ammonium Acetate pH 4.00)
Diluents	50% Methanol
Column	Analytical column (Discovery® C18 100 cm×4.6 mm, 5 µm)
Column oven temperature	30°C
Auto sampler temperature	5°C
Run time	4.05 min
Retention time	Analyte 2.05±0.60 min and ISTD 2.05±0.6 min
Mass spectrometer parameters	
Mirabegron	Q1/Q3–397.200/260.200 m/z
Mirabegron d5	Q1/Q3–402.200/260.200 m/z
Ionization mode	Turbo ion spray (TIS/ESI)
Polarity	Positive
Acquisition mode	Multiple reaction monitoring

Stock solution preparation

MBG stock solution (100.000 µg/mL)

To acquire a known concentration of 100.000 µg/mL of MBG, weigh approximately 1.000 mg and dissolve in 10.00 mL of methanol. Place the stock solution in the refrigerator (2–8°C).[8]

MBG D5 stock solution (100.000 µg/mL)

A known concentration of 100.000 µg/mL can be obtained by dissolving approximately 1.000 mg of MBG D5 in 10.000 mL of methanol. Put a label on the stock solution and store it in the refrigerator (2–8°C).[9-11]

Preparation of internal standard (ISTD) dilution

Aliquot 50.000 µL from a stock solution of MBG D5 and dilute into 50.000 mL with 50% Methanol to obtain a 100.000 ng/mL concentration. Label the ISTD dilution and store it in the refrigerator (2–8°C).[13]

Preparation of calibration curve (CC) spiking solutions

CC spiking solutions were prepared from the analyte stock solution in the range 10.016 ng/mL–5067.405 ng/mL for MBG using 50% Methanol.[13-15]

Preparation of QC spiking solutions

The quality control (QC) spiking solutions were prepared by diluting the analyte stock solution to achieve the desired concentrations. The concentrations of the QC spiking solutions are as follows:

- High QC (HQC): 3828.045 ng/mL
- Medium QC 1 (MQC 1): 1749.417 ng/mL
- MQC 2: 250.167 ng/mL
- Low QC (LQC): 29.019 ng/mL
- Lower limit of quantification (LLOQ): 10.099 ng/mL.

These QC spiking solutions serve as reference standards for assessing the precision, accuracy, and linearity of the analytical method across a range of concentrations, ensuring reliable quantification of the analyte in samples.

Preparation of spiked plasma samples

Retrieved the screened K_2EDTA Human Plasma blank plasma lots from deep freeze (−70°C) and spiking 2% of CC spiking solution and QC spiking solution into that. To achieve final concentrations of CC standards, concentrations ranging from 0.201 to 100.677 ng/mL, and QC sample concentrations ranging from 76.561 to 0.201 ng/mL were stored in a deep freeze for subsequent use.

Sample preparation (extraction procedure)

50.000 µL of ISTD dilution (About 100.000 ng/mL) was added into pre-labeled polypropylene tubes (except blanks). 50.000 µL of 50% methanol (diluent) was added for blank samples. Aliquot 100.000 µL of the sample (above spiked plasma CC standard/QC samples) to each tube and vortexed for a few seconds. 1 mL of methanol was added to the vortex for 5 min, followed by 300 mg of QuEChERS salting out and purification ($MgSO_4$) and 40 mg of PSA into the samples. Samples were subjected to centrifugation at 15,000 revolutions/min for 15 min at 5°C. Collect the supernatant and subject it to nitrogen at 40°C until the dryness is complete. The dried residue samples should be reconstituted with 0.200 mL of mobile phase, quickly vortexed, and then transferred into the appropriate labeled autosampler vials.[16-19]

Method development

The goal of the current work was to create and validate the analyte through QuEChERS extraction process for the analyte using ISTD effectively and straightforwardly. The MS/MS instrument's tuning parameters are listed below to create a dependable approach that may be used for PK investigations.[20-23]

System dependent parameters

The system-oriented constraints are elaborated in Table 2.[24,25]

Compound dependent parameters

These are illustrated in Table 3.[26,27]

Method validation

According to the US Food and Drug Administration (FDA) guidelines,[28] the established LC-MS/MS assay was validated for carryover, selectivity, matrix factor, linearity, sensitivity, accuracy and precision, recovery, dilution integrity, run size evaluation, reinjection reproducibility, ruggedness, and stability. More information on the LC-MS/MS assay's validation parameters, which were created to measure digital mobile broadcasting, was previously provided.[29-31] These parameters were calculated using the CC equations ($y = ax+b$) and the least squares statistical approach. The r^2 value was used to confirm the linear fit.

Carryover was assessed by injecting the following sequence of samples: Extracted blank plasma, upper limit of quantitation (ULOQ) sample, double extracted blank plasma, LLOQ, and the same order follows for aqueous samples.

Selectivity

The purpose of this test is to evaluate the selectivity of the bioanalytical method for the analyte(s) and ISTD(s) of interest from the naturally occurring variation of endogenous matrix components among individuals for various separate matrix lots.[3] Ten different plasma lots (Screened), two Lipemic (L), and two Haemolyzed (H) blanks, as well as the LLOQ standard for the relevant lots, were extracted.[12]

Table 2: The system-dependent parameters used in the study

ESI source parameter	Settings
CUR (curtain gas) (Psi)	30
CAD (collision gas) (Psi)	10
IS (ion spray voltage) (V)	2500 (2.5×10^3)
TEMP (°C)	600 (6.0×10^2)
GS1 (Psi)	50 (5.0×10^1)
GS2 (Psi)	60 (6.0×10^1)
Ihe (interface heater)	ON

Table 3: Compound dependent on parameters used in the study

Parameter	Analyte	ISTD
DP (declustering potential) (V)	50	50
EP (entrance potential) (V)	10	10
CE (collision energy) (V)	26	26
CXP (collision cell exit potential) (V)	10	10
Dwell time (msec)	400	400

Matrix factor

In this parameter, ten different blank matrix lots in which two lipemic (L) and two Heamolyzed (H) samples were processed post-extraction, each in triplicates after reconstitution with aqueous dilutions at LQC and HQC Levels. These samples were then compared to aqueous samples of the same concentration. The post-extracted LQC and HQC samples and 10 replicate injections of aqueous samples of LQC and HQC.[4]

Linearity

Calibration standards added 20.000 µL in blank plasma samples containing K_2EDTA as an anticoagulant. The 10-point CC was magnetically linear from 0.201 to 100.677 ng/mL for MBG.[6,28,29] The concentration of calibration standards was analyzed for three replicates, and goodness-of-fit was determined through a linear least squares regression with a weight factor of $1/\times^2$.

Precision and accuracy

For this assessment, matrix samples from A&P run with various doses of MBG were analyzed. The CC (LLOQ to ULOQ) and six replicates of each of the QC samples HQC, MQC1, MQC2, LQC, and LLOQ QC were required for each run, and three precision and accuracy runs were processed and examined using a freshly spiked CC.[26,30] The assay's quantitative precision and accuracy were assessed within and between runs.

Recovery

The recovery of the analyte at different concentration levels such as HQC, MQC, and LQC (six replicates of extracted QCs) was evaluated relative to the six replicates of post-extracted HQC, MQC, and LQC (at equivalent concentrations) for both analyte (MBG) and ISTD (MBG D5).[7,21,24]

Stability

Stability tests were conducted to assess the samples' stability under the conditions that will be present during handling, storing, processing, and analyzing the subject samples. At room temperature and 5°C, the analyte and ISTD stock solutions' short- and long-term stability were investigated. For benchtop experiments, extracted sample stability was done using freshly spiked CC standards, and QC samples were used. Processed samples were stable at room temperature (24 ± 4°C), under refrigeration (2–8°C), and through freeze-thaw cycles.[8,10,25,27]

RESULTS AND DISCUSSION

Our bioanalytical method has been thoroughly validated following the US-FDA and ICH guidelines for bioanalytical method validation. This validation encompassed selectivity, sensitivity, accuracy, precision, recovery, and stability, all assessed using human plasma, a widely accepted matrix for bioanalytical method development. Human plasma provides a relevant biological environment to evaluate method performance. This study aimed to develop and validate a sensitive and selective LC-MS/MS method for quantifying MBG in human plasma. The objectives were achieved by optimizing the extraction procedure, mass spectrometry parameters, and chromatographic conditions.

The results were evaluated for carryover in the run for blanks, which were injected following a ULOQ standard. It was shown to be a negligible response and no enhancement response in blank samples after subsequent injection of ULOQ standard at their retention times of both analyte and ISTD.

Selectivity

The analysis of all samples revealed the absence of significant interference from endogenous matrix components for both the analyte(s) and ISTD(s). This observation, illustrated in [Figure 2a-e], underscores the method's selectivity, particularly at the LLOQ, affirming the accuracy of the analytical approach. The test implies that predose samples analysis because we are collecting blood samples at 0 h.

The lack of interference from endogenous matrix components is crucial for ensuring the reliability and accuracy of quantitative analysis. By demonstrating selectivity at the LLOQ, the method can accurately detect and quantify the target analyte(s) even at low concentrations within complex biological matrices.

This robust selectivity is a testament to the effectiveness of the sample preparation and chromatographic separation techniques employed in the method. The QuEChERS extraction process, coupled with chromatographic resolution on the Discovery® C18 column, ensures efficient removal of interfering substances and precise quantification of the analyte(s) of interest.

Matrix factor

In this extraction procedure, negligible ion suppression or enhancement in the signal was observed, with coefficient of variation (%CV) values of 0.86% and 1.55% at the LQC and HQC levels, respectively, in comparison to the signal obtained from aqueous samples.

These low %CV values indicate minimal ionization efficiency variability between the extracted and aqueous samples, suggesting consistent and reliable performance of the extraction method.

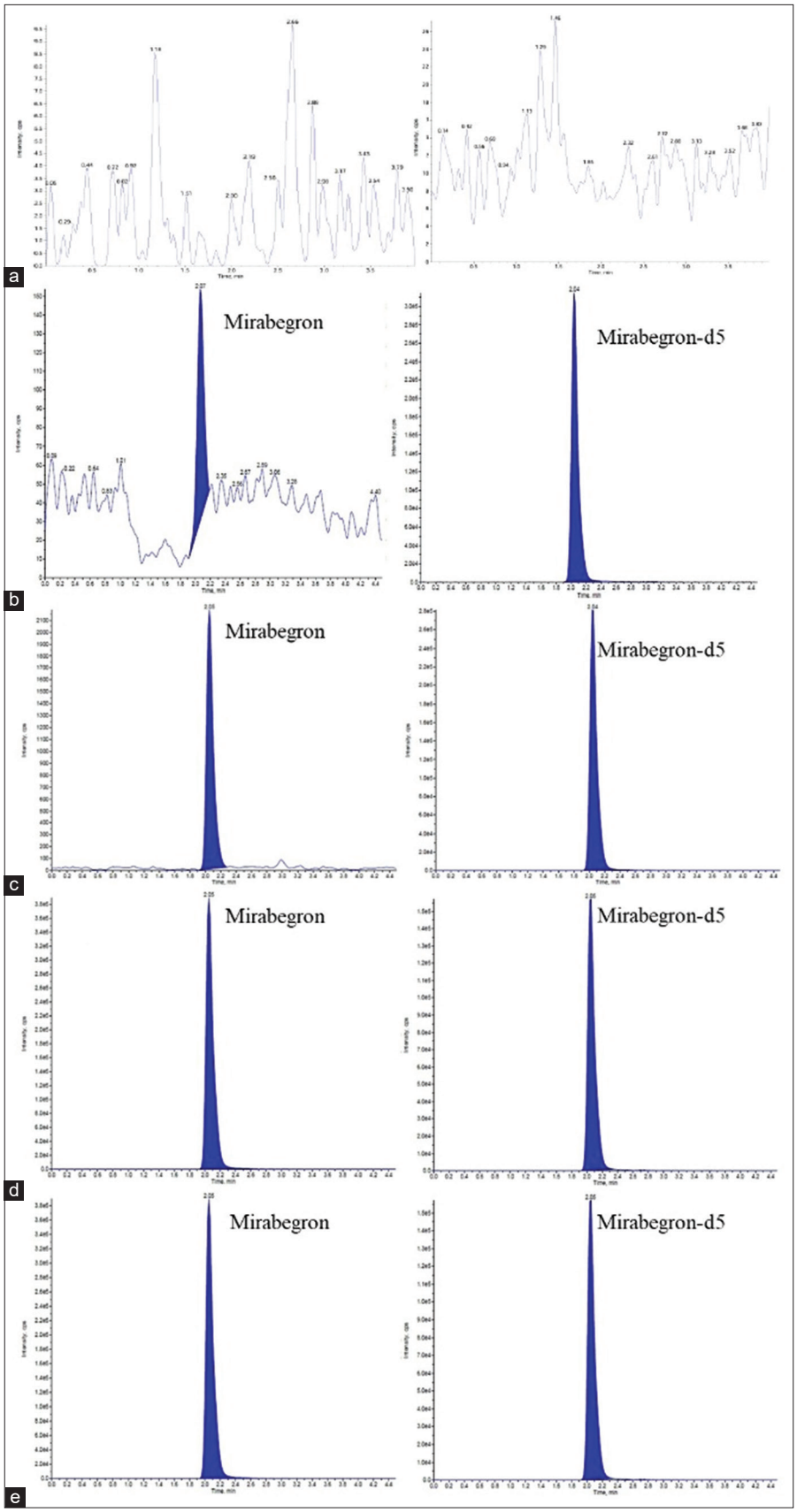

Figure 2: Multiple reaction monitoring ion–chromatograms of Mirabegron, Mirabegron d5, in (a) blank plasma, (b) blank plasma spiked with internal standard, (c) Extracted lower limit of quantification, (d and e) Extracted upper limit of quantitation

Table 4: Back-calculated concentrations of calibration curve samples for mirabegron in human plasma

CC ID	Nominal concentration (ng/mL)								
STDs	0.2	0.401	1.203	4.01	16.041	40.103	60.178	80.237	100.548
1	0.194	0.421	1.139	3.963	15.652	40.656	55.201	75.858	99.836
2	0.202	0.385	1.179	4.09	15.195	40.39	59.999	78.151	96.926
3	0.201	0.388	1.175	4.121	15.855	40.253	60.316	76.201	98.381
Mean	0.199	0.398	1.16433	4.058	15.5673	40.433	58.5053	76.7367	98.381
Standard deviation	0.00436	0.01997	0.02203	0.08372	0.33805	0.20491	2.86602	1.2368	1.455
%CV	2.2	5	1.9	2.1	2.2	0.5	4.9	1.6	1.5
% Accuracy	99.5	99.3	96.8	101.2	97	100.8	97.2	95.6	97.8

The absence of significant ion suppression or enhancement further validates the robustness of the extraction procedure and underscores its suitability for accurate and reproducible quantification of the analyte(s) in complex biological matrices.

Linearity

All CCs demonstrated excellent linearity across the concentration range 0.201–100.677 ng/mL. The mean correlation coefficient exceeded 0.999 consistently across all three cases. This exceptional linearity indicates that the relationship between the analyte(s) concentration and the corresponding response signal is highly predictable and reliable throughout the specified concentration range. The consistently high correlation coefficient further reinforces the robustness and accuracy of the calibration method.

Establishing linear CCs with such high correlation coefficients is essential for ensuring accurate quantification of the analyte(s) in samples of varying concentrations. These results validate the method's suitability for precisely determining analyte concentrations in complex biological matrices. These results validate the suitability of the method for PK studies and therapeutic drug monitoring [Tables 4 and 5].

Precision and accuracy

The method's intra- and inter-day precision and accuracy were evaluated across different concentration levels, yielding (%CV values ranging from 1.74% to 5.77% and 2.24% to 5.05%, respectively. The accuracy ranged from 95.28% to 100.90% and 93.73% to 100.20%.

These results demonstrate excellent precision and accuracy of the method across various concentrations. The low %CV values indicate minimal variability in replicate measurements within the same day (intra-day) and between different days (inter-day), ensuring reliable and consistent results. The high accuracy values also indicate that the measured concentrations closely match the true concentrations, further validating the method's reliability [Table 6].

Table 5: Linearity parameters summary for mirabegron in human plasma

Calibration curve	Slope	y-intercept	Correlation coefficient (r^2)
1	0.0737	−0.00015	0.9958
2	0.0706	−0.00464	0.9968
3	0.0678	−0.000325	0.9976

Recovery

The global recovery of MBG across three concentration levels was determined to be 79.44%, while for the ISTD, it was found to be 78.74%. These recovery values reflect the efficiency of the extraction process in recovering MBG from the sample matrix. The high global recovery percentage indicates that a significant proportion of the analyte was successfully extracted and recovered from the sample matrix during sample preparation.

Similarly, the recovery of the ISTD is crucial for ensuring accurate quantification of the analyte, as it serves as a reference for correcting variations in the extraction efficiency and instrument response [Table 7].

Stability

Two concentration levels, HQC and LQC, were utilized to assess the stability parameters under various conditions, including freeze/thaw cycles, processed sample stability, and benchtop stability, with samples freshly prepared for comparison. As detailed in Table 5, the results indicate that no stability-related issues were encountered, suggesting the assay's suitability for PK studies.

Furthermore, additional metrics were evaluated using criteria established by the USFDA. These encompassed robustness, reinjection reproducibility, post-injection delay impact, dilution integrity, prolonged processing and analysis batch, and robustness and stability of aqueous solutions. Notably,

Table 6: Precision and accuracy (intra-batch and inter-bach) of mirabegron

QC level (nominal concentration, ng/mL)	Intra batch (*n*=6; single batch)			Inter batch (*n*=18; 6 from each batch)		
	Mean conc. found (ng/mL)	Accuracy (%)	%CV	Mean conc. Found	Accuracy (%)	%CV
HQC (76.561)	72.332	94.48	1.4	71.266	93.08	0.6
MQC–1 (34.988)	34.133	97.56	4.2	33.331	95.27	0.9
MQC–2 (5.003)	5.199	103.91	3.5	5.278	105.51	5.6
LQC (0.580)	0.543	93.56	4.6	0.529	91.12	0.9
LLOQQC (0.202)	0.203	100.33	0.1	0.201	99.34	1.3

Table 7: Extraction recovery for mirabegron

QC level (nominal concentration, ng/mL)	Mean area response (n=6)				Recovery (B/A%)		Global % recovery	
	A (Post extracted area) Analyte	B (Extracted Area) Analyte	A (Post extracted area) IS	B (Extracted Area) IS	Analyte	IS	Analyte	IS
HQC (76.561)	3348881.0	2721398.8	632299.2	505182.0	81.26	79.90	79.44	78.74
MQC-1 (34.988)	2116745.5	1695248.0	656645.2	518977.0	80.09	79.03		
LQC (0.580)	29354.5	22594.8	717059.7	554322.0	76.97	77.30		

Table 8: Stability of mirabegron in human plasma at various conditions (*n*=6)

Stability	Storage condition	Duration	Level	Nominal concentration (ng/mL)	Mean Stability sample (mean±SD, ng/mL)	%CV	Accuracy
Benchtop	Room temperature	8 h	HQC	76.561	74.7535±3.13	4.2	98.79
			LQC	0.580	0.5640±0.008	3.4	97.84
Processed sample stability	Refrigerated (2–8°C)	3 days	HQC	76.561	74.1577±2.96	4.0	98.00
			LQC	0.580	0.5450±0.021	3.21	96.63
Processed sample stability	Room temperature (24±4°C)	28 h	HQC	76.561	74.7535±3.13	4.0	99.20
			LQC	0.580	0.5518±0.018	3.4	98.76
Freeze/thaw	−70±10°C	6 cycles	HQC	76.561	75.6707±0.40	1.11	98.51
			LQC	0.580	0.5468±0.021	3.96	98.92

all findings fell within the acceptable range prescribed by regulatory guidelines, reinforcing the reliability and suitability of the assay for precise and consistent quantification of analytes in complex biological matrices [Table 8].

CONCLUSION

The validation results obtained across MBG concentrations ranging from 0.201 ng/mL to 100.677 ng/mL in human plasma confirm the precision and reliability of the analytical instrument and the assay methodology. The utilization of the QuEChERS extraction technique, recognized for its advanced capabilities in solid-phase extraction, yields favorable recovery rates. Notably, the absence of anticipated interferences underscores the accuracy of the analytical approach. Furthermore, the demonstrated stability of the examined analytes under challenging conditions bolsters confidence in the method's robustness. Overall, the proposed analytical method provides a dependable means for routine analysis and presents opportunities for diverse PK and bioequivalence investigations, contributing significantly to advancements in therapeutic research and clinical practice.

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