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Advanced LC-MS/MS Analysis for Concurrent Quantification of Metoprolol Succinate and Hydrochlorothiazide in Human Plasma

ASHVINI TRAMBAK VARUNGASE[1,2]* and NIRMALA VIKRAM SHINDE[1]

[1]Department of Pharmaceutical Chemistry, S.M.B.T.College of Pharmacy, Nandi Hills, Dhamangaon, Igatpuri, Nashik, Affiliated by Savitribai Phule Pune University, Pune, Maharashtra, India.
[2]Department of Pharmaceutical Chemistry,Amrutvahini College of Pharmacy, Sangamner, Ahilyanagar, Affiliated by Savitribai Phule Pune University, Pune, Maharashtra, India.
*Corresponding author E-mail: atvarungase@gmail.com

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ABSTRACT

A sensitive and selective liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method was developed and validated for the simultaneous quantification of metoprolol succinate (MPL) and hydrochlorothiazide (HCTZ) in human plasma. Sample preparation involved a simple liquid-liquid extraction procedure using dichloromethane:tert-butyl ether (85:15% v/v). Chromatographic separation was achieved on an ACE column using a mobile phase consisting of methanol and water with 0.1% formic acid (70:30, v/v). The analytes were detected using electrospray ionization in negative ion mode and quantified using multiple reaction monitoring. MPL D4 and HCTZ 13C15N2 D2 were used as internal standards. The method was validated over concentration ranges of 10-5000 ng/mL for MPL and 1-500 ng/mL for HCTZ. The intra- and inter-day precision and accuracy were within acceptable limits. The method demonstrated excellent selectivity, recovery, and stability. Matrix effects were minimal, and no significant ion suppression or enhancement was observed. The validated method was successfully applied to a pharmacokinetic study in human subjects. The proposed LC-MS/MS method offers a rapid, sensitive, and reliable approach for the simultaneous determination of MPL and HCTZ in human plasma, suitable for clinical pharmacokinetic studies and therapeutic drug monitoring.

Keywords: Metoprolol succinate (MPL), Hydrochlorothiazide (HCTZ), Bioanalytical method, Antihypertensive Agents, Dried Blood Spot (DBS), Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

INTRODUCTION

Excessive blood pressure in the arteries is a persistent medical condition known as hypertension, or high blood pressure.[1] It can be classified as primary (important) or secondary.[1] In 90-95% of cases, elevated blood pressure without a known medical cause is referred to as "primary hypertension".[1] The residual 5-10% of instances, referred to as secondary hypertension, are caused

by other circumstances affecting the different systems of the body.[1] The maximum and minimum range of the blood pressure should be maintained as per standard.[1] 1-[4-(2-methoxyethyl)phenoxy]-3-[(1-methylethyl)amino]-2-propanol succinate (Metoprolol succinate) is a beta-adrenergic blocking drug that decreases elevated blood pressure and relieves chest pain.[1] Official reports on Metoprolol succinate can be found in BP 2009 and USP 2007, whereas USP 2007 includes reports on liquid chromatography techniques.[2] Hepatically, Metoprolol succinate is metabolized mostly by CYP2D6.2 Urine contains about 95% of the dose that can be retrieved.[2] Fewer than 5-6% of oral dosages and fewer than 10-11% of IV doses are eliminated as unaltered medication in the waste of the majority of participants.[2] Metoprolol succinate is given orally; peak plasma concentration is reached after two to three hours, and the half-life is three to seven days.[2]

Hydrochlorothiazide (HCTZ) is chemically designated as 2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide, 6-chloro-3,4-dihydro-2H-1, and is among the first thiazide diuretics employed in the management of hypertension.[3]

Numerous studies have proposed various methods for the analysis of MPL and HCTZ in biological solutions using HPLC and LC-MS/MS techniques. Moreover, the stability evaluation of MPL and HCTZ in formulations has been examined using HPTLC.[4-5]

Pridhvi Krishna Gaddey *et al.,* (2024) developed and validated a LC-MS/MS method for measuring amivantamab levels in rat plasma. This study was employed the liquid-liquid extraction. The method was characterized to be simple, accurate, precise, and reproducible.[6]

C. Gupta *et al.,* (2023) conducted a review on bioanalytical method development and validation for Ramipril and its metabolite Ramiprilat using LC-MS/MS. The study introduced an innovative approach to quantify Ramipril and Ramiprilat in plasma, employing Enalapril and Enalaprilat as internal standards. The method was validated per ICH guidelines and was found to be specific, accurate, and reliable for pharmacokinetic and bioequivalence studies.[7]

Vineeta V. Khanvilkar *et al.,* (2022) devised and confirmed the effectiveness of an HPTLC bioanalytical method for the simultaneous estimation of Empagliflozin and Telmisartan in human plasma. This approach was found suitable for pharmacokinetic and toxicokinetic studies of these drugs.[8]

Pradeep Singh Rawat *et al.,* (2020) developed and validated an LC-MS/MS method for the simultaneous quantification of Nebivolol and Labetalol in aqueous humor and plasma. The method used nebivolol-d4 and metoprolol as internal standards. The method was found satisfactory as per ICH guidelines.[9]

Mohammad Abdul-Azim Mohammad *et al.,* (2020) developed and validated an LC-MS/MS method for the simultaneous quantification of enalapril maleate, enalaprilat, nitrendipine, dehydronitrendipine, and hydrochlorothiazide (in human plasma. The method used felodipine as an internal standard and achieved good chromatographic separation on a Symmetry C18 column. Detection was performed in MRM mode with ESI.The method, validated according to U.S. FDA guidelines, showed outstanding sensitivity and selectivity, making it apt for therapeutic drug monitoring and bioequivalence studies.[10]

Whilst MPL and HCTZ have been thoroughly investigated in human bodily fluids and numerous formulations using various analytical methods, little research has focused on the simultaneous assessment of these two compounds. This investigation was conceived and authenticated to concurrently measure MPL and HCTZ in plasma usingLC-MS/MS.

MATERIALS AND METHODS

Chemicals

MPL was procured from LGC Promochem, positioned in Bangalore, India. Hydrochlorothiazide 13C 15N2 D2 was obtainedfrom Clearsynth Labs in Mumbai, India. MPL D4 and HCTZ have been procured from Vivan Life Sciences positioned in Mumbai, India. Human plasma obtained from Supratech Laboratory, Ahmedabad, India. The issuer of HPLC-grade methanol was Spectrochem Pvt. Ltd., Mumbai, India. Hydrochloric acid and formic acid have been procured from Merck, Mumbai, India.

HPLC-grade dichloromethane and diethyl ether have been obtained from Spectrochem, Mumbai, India. The Milli-Q water purification gadget was procured from Millipore Pvt. Ltd., Ahmedabad, India.

Instrumentation

A triple quadrupole MS, liquid chromatography mass spectrometer, API 3000 (MDS Sciex, USA), connected to a Shimadzu LC system (Shimadzu Corporation), equipped with an solvent system, degasser and a autosampler. The system also included a column thermostat oven. Watson LIMS was used for laboratory information management. Analyst software, MDS Sciex, USA was used for data evaluation.

Chromatographic conditions

The separation was performed using a mobile phase of methanol and water with formic acid at a concentration of 0.1% in the ratio of 70:30 (v/v), housed in a thermostated column oven at 42°C. The injection volume was five µL and the analysis time was three minutes.

Mass spectrometric conditions

Electrospray ionization (ESI) was used as the ion source for the mass spectrometer operating in negative ion mode. To optimize the tuning parameters of MPL, HCTZ, and internal standards (IS), solutions at concentrations of 260 nanograms per milliliter of each analyte were injected. The parameters dependent on the source for both MPL and HCTZ were: nebulizer gas (12 pounds per square inch gauge), ion spray voltage (ISV) (-4500V), heater temperature (400°C), interface heater (Ihe) (ON), collisionally activated dissociation (CAD) (7 pounds per square inch gauge), and curtain gas (CUR) (nitrogen: 12 pounds per square inch gauge).[11]

Analytes were quantified via multiple reaction monitoring (MRM), with a dwell duration of 220 ms for each transition:

- Mass/charge 427.61 → mass/charge 192.0 for MPL.
- Mass/charge 292.8 transitions to mass/charge 262.0 for HCTZ.
- Mass/charge 432.1 → mass/charge 192.0 for MPL D4.
- Mass/charge 302.9 → mass/charge 271.2 for HCTZ 13C15N2 D2.

Optimized collision energies of -25 were utilized for MPL and its internal standard, whereas fixed collision energies of -26 were applied for HCTZ and its internal standard.[12]

To prepare quality control (QC) and standard samples

The optimal concentration of each analyte, 1100 µg/mL, was attained by dissolving 5 milligram of the analyte in methanol to prepare stock solutions for MPL, MPL D4, HCTZ, and HCTZ 13C15N2 D2. The solutions had been finally diluted with methanol to reap very last concentrations of 500 microgram per millilitre for MPL and 50 microgram per millilitrefor HCTZ. Working standard solutions were obtained by further thinning these solutions with methanol to the following concentrations:

- For HCTZ: 0.05, 0.1, 0.2, 0.4, 0.8, 2.0, 5.0, 12.5, and 25.0 microgram per millilitre.
- For MPL: 0.5, 1.0, 2.0, 4.0, 8.0, 20.0, 50.0, 125.0, and 250.0 microgram per millilitre.

The standard stock solutions of MPL D4 and HCTZ 13C15N2 D2 were diluted in methanol to achieve concentrations of tenmicrogram per millilitre for MPL D4 and fourmicrogram per millilitrefor HCTZ 13C15N2 D2, resulting in a working solution of the internal standard (IS).[13]

Blank human plasma samples (980 microliters) were spiked with 20 microliters of working standard solution to generate a calibration curve to verify quality control (QC) during validation experiments. The ultimate concentrations were:

- For HCTZ: 1.0, 2.0, 4.0, 8.0, 16.0, 40.0, 100, 250, and 500 nanogramper millilitre.
- For MPL: 10.0, 20.0, 40.0, 80.0, 160, 400, 1000, 2500, and 5000 nanogramper millilitre

The calibration and QC samples were prepared in a similar manner, resulting in plasma concentrations of:

- For HCTZ: 1.0, 3.0, 200, and 400 nanogramper millilitre.
- For MPL: 10.0, 30.0, 2000, and 4000 nanogramper millilitre.

All samples were kept at temperatures ranging from 2 to 8 degree celcius until they were analyzed.[13]

Sample Preparation

A 6 mm diameter round punch was taken from each sample on the DBS card. Each punched sample was placed in a Cryotube, and 210 microlitre of sample solution was added. The Cryotube was then mixed and placed in a sonication bath at 42±1degree celcius for 20±5 minutes. The sample was centrifuged at 4000±100 rpm for 5-6 min, and 50±1 microlitre of the supernatant was collected from the vial. Then, 160±10 microlitre of Milli-Q water was added. The samples were thoroughly mixed to ensure homogenization before analysis. The collected volume for analysis was 10±1 microlitre per sample.

Method Validation

The validation was done according to international regulatory guidelines.

Preparation of plasma sample

In 5 millilitre Eppendorf centrifuge tubes, samples of 500 ± 10 microlitres were prepared. These samples included blanks, calibration standards (CC), unidentified plasma, and quality control (QC) specimens. Each tube contained 50 microliters of buffer (0.5 N HCl) and 50 ± 5 microliters of internal standard solution (IS). Samples were mixed in the extractor at 40 rpm for 15 min, accompanied by the incorporation of 2.5 millilitre of liquid extraction mixture. The solutions were then centrifuged at 4000±100 rpm at 15 degree celcius for 5-6 minutes.

The resulting 2.0 milliliter supernatant was transferred to vials and evaporated under a light nitrogen stream at 40degree celcius. After evaporation, 100 microliters of mobile phase was added to the dried residues to reconstitute the samples. The reconstituted samples were loaded into the autosampler, and 5 µL of each sample was introduced into the LC-MS/MS system.[14]

Quantification

The internal standards (IS), MPL D4 and HCTZ 13C15N2 D2, had been applied for the quantitative evaluation of MPL and HCTZ, respectively. A calibration curve (CC) changed into built the use of CC samples organized in plasma. Peak region ratios of the analytes had been plotted in opposition to their respective IS to generate a nine-factor calibration curve. The concentrations of the analytes withinside the QC and unknown samples had been decided with the aid of using interpolating the values from those calibration curves.[15]

Method validation

The validation protocol was doneinline with ICH and other regulatory guidelines.

Selectivity

The selection of the plasma matrix was aimed at contrasting the signals of analytes with interfering signals in 10 distinct batches of plasma. These included 7 K3EDTA, one hemolyzed, and one heparinized for lipidemic plasma. Blank and LOQ samples had been organized using aliquots of the to be had plasma samples. The analyte response at the LOQ level was required to remain below 20%.[16-18]

Linearity, Precision and Accuracy

Method linearity was evaluated through three consecutive tests using nine-point calibration data within concentration ranges of 5–5000 nanogram per millilitre for MPL and 1–500 nanogram per millilitrefor HCTZ. The internal standard (IS) was utilized to correlate the calibrators' analyte concentrations with the analytes' peak area ratios, thereby generating the calibration. Least-squares linear regression ($1/x^2$) was used to analyze each calibration curve. To assess accuracy and precision for inter- and intra-lot comparisons, six duplicate QC samples were used at LOQ, low, mid, and high concentration levels on three consecutive days. The data obtained had to meet standard criteria of precision within 15% coefficient of variation (%CV) and ±15% relative error of the nominal value (%RE).[16-18]

The LOQ was demonstrated to have satisfactory precision and accuracy across three consecutive validation tests, with six replicates showing an RSD of less than 20 ± 1% and accuracy within ±20-22%.[16-18]

Recovery

The average peak response of test samples was compared with post-extraction standard samples to assess analyte recovery during liquid-liquid extraction across low, intermediate, and high concentrations, all demonstrating 100% recovery. In general, the internal standard recovery was estimated using all quality control samples at the median concentration level, following the same procedure.[16-18]

Matrix effect

The analyte spiked into test blank plasma samples was extracted and compared to the analyte added during the reconstitution stage (A). The average peak area of the analytes was used as a benchmark for comparison with this value (B), which was used to assess the interference of the analyte solutions in the plasma samples. Calculation of the matrix effect was done using following formula:

Matrix effect = (A/B) × 100

The coefficient of variation (CV) for the normal matrix effect should be less than 15% for the processed matrix system.[16-18]

Stability

The stability testswere done using following solutions: 1 milligram per millilitre of MPL, 1 milligram per millilitreof HCTZ, 1 milligram per millilitreof MPL D4, and 1 milligram per millilitreof HCTZ 13C15N2 D2. The solutions were stored at two to eight degree celcius for 10±1 days and at ambient temperature for twenty two hours. The average peak area of the fresh solution was compared to that of the stable solution and expressed as a percentage change in the mean value. In-process stability was measured at two to eight degree celcius for 48±2 h, long-term stability at negative twentydegree celcius and negative seventy eightdegree celcius ±10, and freeze-thaw cycles at negative twentydegree celcius and negative seventy eightdegree celcius. Six replicates of each concentration were performed. Results were obtained at LQC and HQC concentrations. Solutions were considered stable if the percentage change was less than 15 percentage. Different temperatures and time constraints were used to evaluate the freeze/thaw stability, process stability, and bench-top stability of the pharmaceutical product. Frozen samples were thawed at room temperature for sixty minutes and then refrozen at negative twentydegree celcius and negative seventy eightdegree celcius ±10degree celcius. After determining the concentrations of the freshly prepared samples and the stability samples, stability was assessed using the average percentage change in calculated concentration. Long-term stability of hundred and eighteen days was achieved. Samples were processed and compared to freshly prepared solutions after hundred and eighteendays of storage at negative twentydegree celcius and negative seventy eightdegree celcius.[16-18]

RESULTS

Development of the MS spectrometric conditions

To achieve optimal detection and concomitant evaluation of MPL, HCTZ, and the internal standard in plasma, it was essential to fine-tune the MS parameters and chromatographic conditions. The mass parameters of each analyte were adjusted in both positive and negative ionization modes. MPL, HCTZ, and the internal standard showed distinct peaks in negative ionization mode. It was imperative to optimize all conditions so as to increase sensitivity by mitigating suppression of ions. The findings of this study indicated that the intensity of the analyte rose as temperatures increased. This trend was observed across various temperature parameters, including block, interface, and DL temperatures, as well as diverse flow temperatures spanning from 200 to 400 degrees Celsius, when the flow rate was maintained at one millilitre per minute. Furthermore, signal intensity was not significantly affected by small adjustments in the nebulizer gas flow rate (4 liters per minute) and ion spray voltage (4.5 kV). A residence time of 0.201 seconds was sufficient for MPL, HCTZ, and IS, and no crosstalk was observed in any of the multiple reaction monitoring with this configuration.

Optimization of chromatographic conditions

Sample preparation is a crucial step in the analytical process, and it should be straightforward, quick, and involve minimal chemical usage while ensuring optimal analyte recovery. Literature reviews indicate that MPL and HCTZ are commonly extracted using the solid-phase extraction (SPE) method. However, compared to liquid-liquid extraction (LLE), the SPE technique requires more costly components and a longer processing time. To reduce processing time and achieve satisfactory analyte recoveries, we utilized the LLE method for sample preparation. Moreover, several documented procedures have employed larger plasma volumes for chromatographic development and sample preparation.

Nevertheless, the method's acceptability was improved by the development of the proposed approach, which included reduced injection and plasma volumes. In order to establish a liquid-liquid extraction process that is both rapid and efficient, a variety of extraction solvents were evaluated, such as n-hexane, diethyl ether,ethyl acetate, methyl tert-butyl ether and dichloromethane. The optimal responsiveness and recovery were achieved by

employing dichloromethane:tert-butyl ether (85:15% v/v) as the extraction solvent. The internal standard did not influence ion suppression, sensitivity,analyte recovery and no endogenous or external plasma matrix interference was observed.

The ISs used for MPL and HCTZ were MPL D4 and HCTZ 13C15N2 D2, respectively. To achieve a rapid chromatographic run with good peak shape and sensitivity for both analytes and the IS, chromatographic conditions were optimized. Various mobile phases, including acetonitrile, acetic acid, methanol, formic acid, aqueous ammonia and ammonium acetatewere tested. The acidic mobile phase system, formic acid in watercombined with methanol provided the highest sensitivity. The ACE column was selected for its superior separation of MPL and HCTZ. Injection volume of 5±1 microlitre prevented column overloading and allowed for multiple tests on the same column. LC-MS/MS was used to simultaneously quantify MPL and HCTZ. Fig. 1, 2 and 3 demonstrate Standardblank, Standard zero, standard-1and limit of quantification for MPL and HCTZ, respectively and scan for range of samples ion.

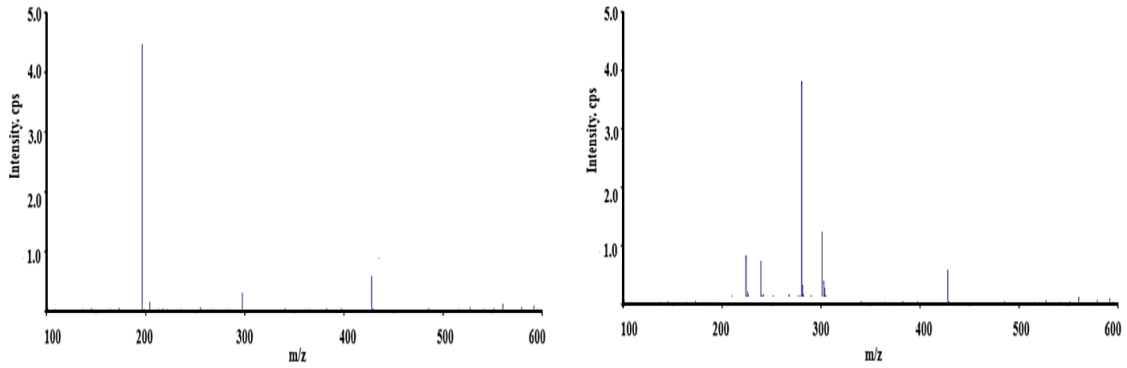
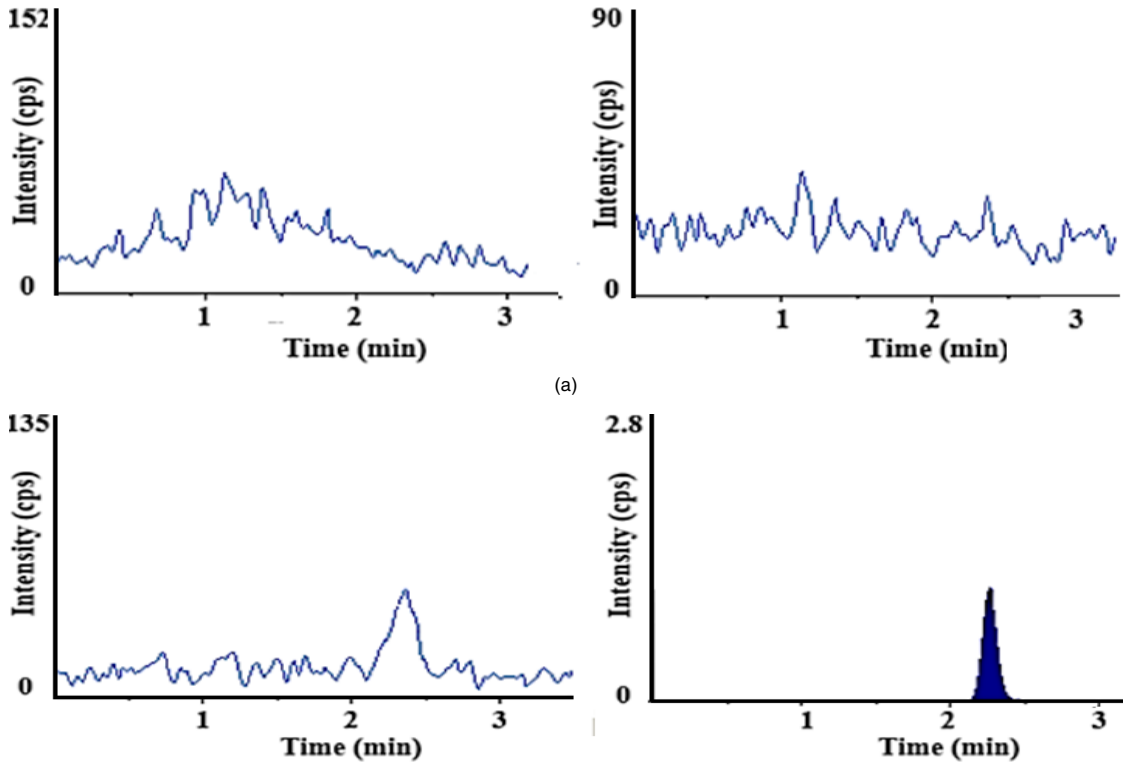
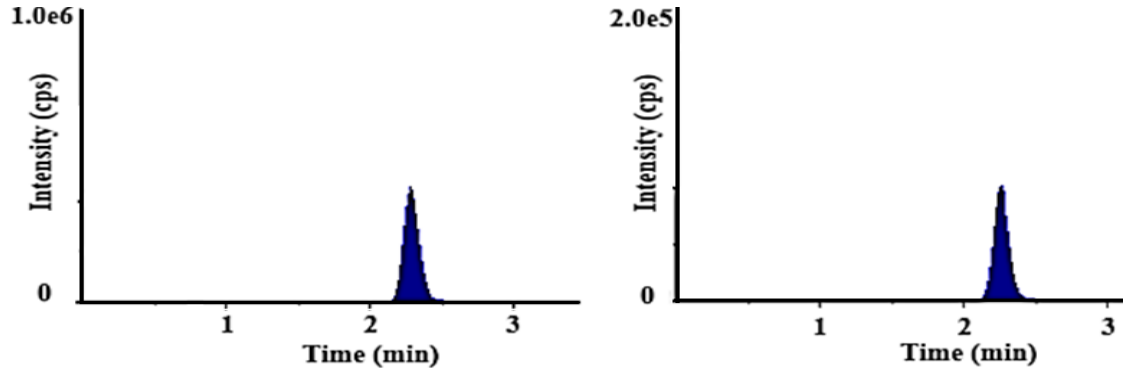
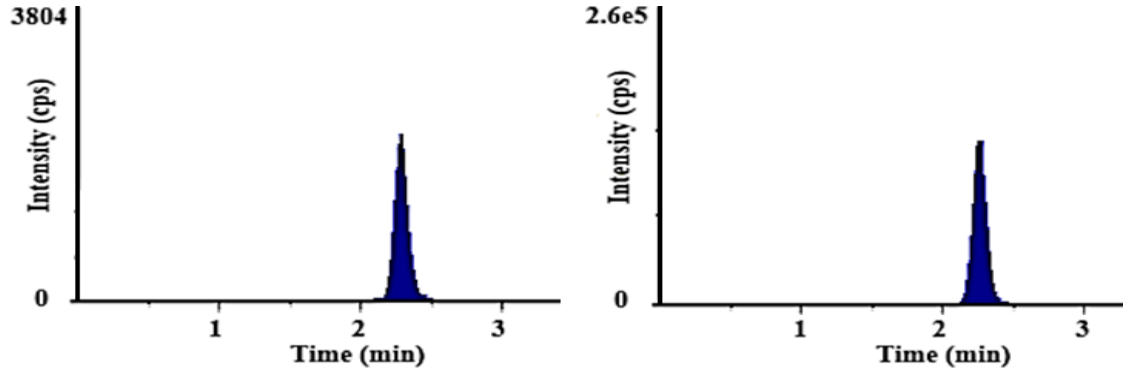


Fig. 1. Ion scan ranges for Metoprolol Succinate (MPL) and Hydrochlorothiazide (HCTZ): (a). Ion scan range for Metoprolol Succinate (MPL), (b). Ion scan range for Hydrochlorothiazide (HCTZ)




1.0e6
Intensity (cps)
0
1
2
3
Time (min)
2.0e5
Intensity (cps)
0
1
2
3
Time (min)

(c)


3804
Intensity (cps)
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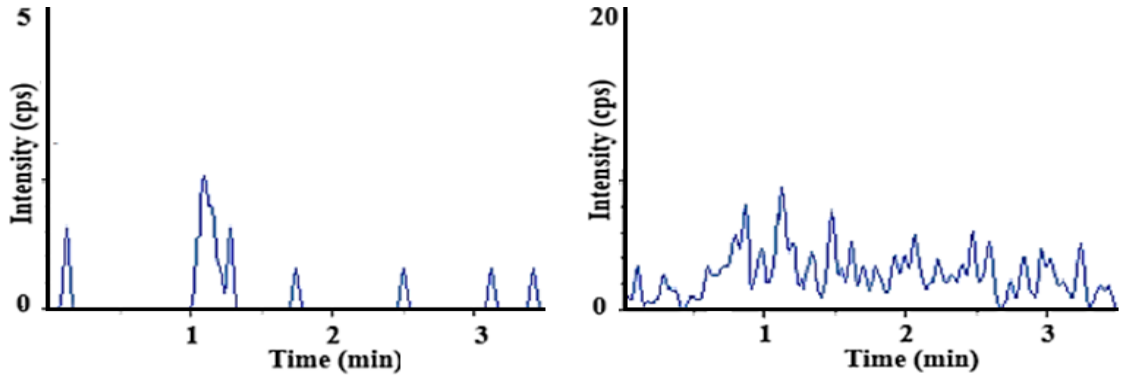
1
2
3
Time (min)
2.6e5
Intensity (cps)
0
1
2
3
Time (min)

(d)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Intensity (cps)
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3
Time (min)
20
Intensity (cps)
0
1
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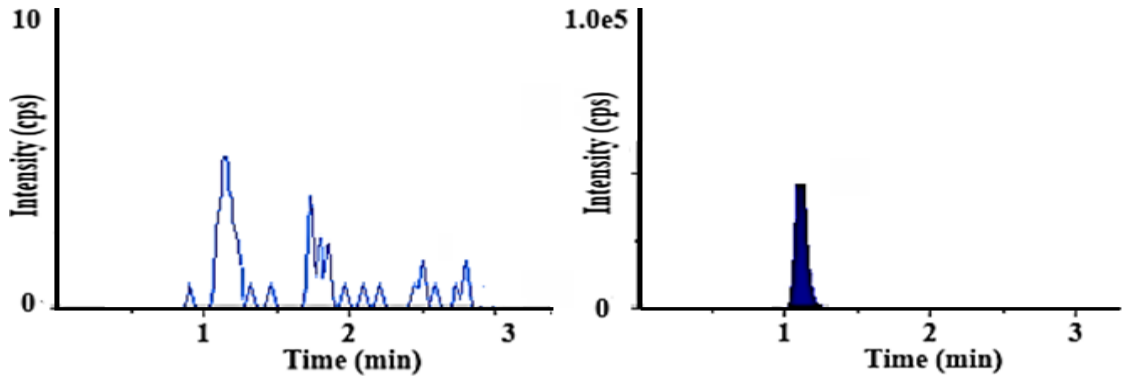
3
Time (min)

(e)


10
Intensity (cps)
0
1
2
3
Time (min)
1.0e5
Intensity (cps)
0
1
2
3
Time (min)

(f)

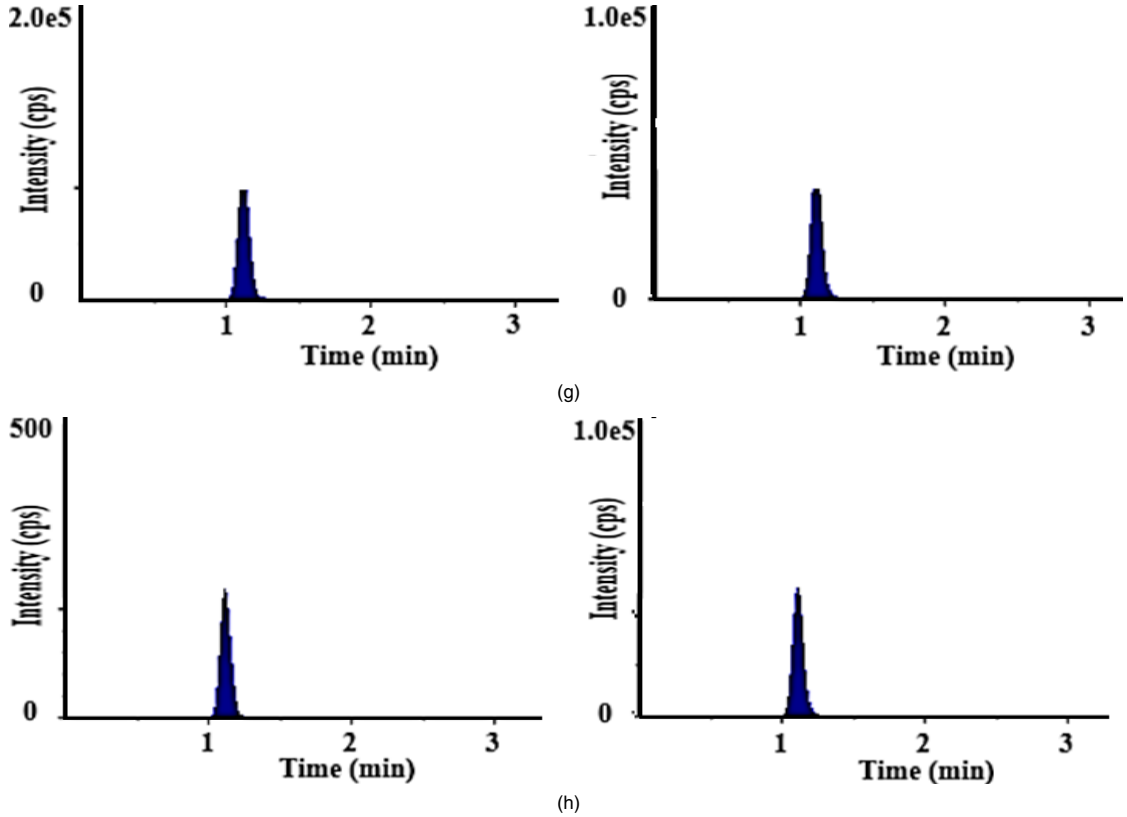


Fig. 2. Selective MRM chromatograms for Metoprolol Succinate (MPL) and Hydrochlorothiazide (HCTZ) under various conditions: (a). Standard Blank (STD BL) for Metoprolol Succinate, (b). Standard Zero (STS Zero) for Metoprolol Succinate (c). Upper Limit of Quantification (ULOQ) for Metoprolol Succinate, (d). Limit of Quantification (LOQ) for Metoprolol Succinate, (e). Standard Blank (STD BL) for Hydrochlorothiazide, (f). Standard Zero (STS Zero) for Hydrochlorothiazide (g). Upper Limit of Quantification (ULOQ) for Hydrochlorothiazide, (h) Limit of Quantification (LOQ) for Hydrochlorothiazide

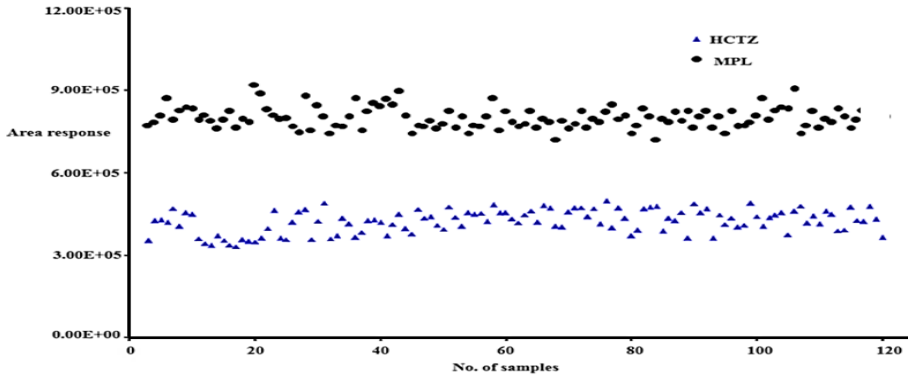


Fig. 3. Internal Standard (IS) response areas for Metoprolol Succinate D4 and Hydrochlorothiazide 13C15N2 D2 under various conditions

Selectivity

No interference peaks from endogenous substances were reported during analysis of MPL, HCTZ, MPL D4, and HCTZ 13C15N2 D2 isolated from plasma. At LOQ, MPL at 10±1 nanograms per milliliter and HCTZ at 1 nanogram per milliliter gave blank plasma drug responses of less than 2.95% and 5.21%, respectively. Typical retention times for MPL,HCTZ, MPL D4, and HCTZ 13C15N2 D2 were 3.21, 2.11, 3.21, and 2.11 min, respectively.

Linearity, Accuracy and Precision

Similar to the established method, the least squares approach often results in significant

errors at low concentration levels. The method uses lower concentration ranges of 1-500 nanogram per millilitre for HCTZ and 10-5000 nanogram per millilitrefor MPL. To address this issue, weighted calibration curves were employed. The calibration curve was computed using weighting parameters [none (unweighted) and $1/x^2$]. The results proves that the data offered improved precision, making the use of a weighted factor the most effective choice for the proposed method. A validation batch's calibration curve equation can be illustrated as follows:

y=0.0013x +0.0018 (r^2=0.9980) for MPL y=0.0038 x+0.0006 (r^2=0.9980) for HCTZ (Refer Table 1).

Table 1: Calibration data

Drug		Slope	Intercept	r^2
MPL	Average	0.0013	0.0018	0.9980
	% coefficient of variation	9.34	42.19	0.0012
HCTZ	Average	0.0038	0.0006	0.9980
	% coefficient of variation	9.12	41.01	0.0012

The current bioanalytical approach demonstrated a wide range of linearity and a low limit of quantitation. This method can detect drug concentrations as low as 5% of C_max and as high as more than twice the C_max. Excellent linearity was achieved within the specified concentration ranges, with an r^2 value greater than 0.996. The results for MPL and HCTZ, as measured by QC samples, in terms of both intraday and interday precision and accuracy, are summarized in Table 2.

Table 2: Quantification of MPL and HCTZ in human plasma

Drug	Normal Conc µg/mL	Intra-batchn=6	Inter Batchn=12	Intra-Batch accuracy	Inter-Batch accuracy
MPL	10	5.37	4.59	97.31	99.51
	30	2.94	5.26	96.34	100.69
	2000	2.43	3.08	97.51	99.00
	4000	2.49	3.45	97.26	100.00
HCTZ	1	7.60	10.68	88.21	95.61
	3	4.70	5.54	96.68	99.68
	200	2.94	3.36	98.00	100.51
	400	2.48	3.87	106.76	103.26

To verify the precision and accuracy of the devised method, six replicates of three different concentrations of QC samples were used. The results showed that for MPL, intraday and interday accuracy ranged from 96.34% to 100.00%, while for HCTZ, it ranged from 88.21% to 106.76%. These findings indicate that the current LC-MS/MS approach for the concomitant estimation of MPL and HCTZ meets the established criteria for precision and accuracy in both experiments.

Recovery

To assess the percentage recovery, the average peak area of extracted specimens was contrasted with that of newly prepared, unextracted samples at three separate concentrations.

MPL and HCTZ exhibited recovery percentages of 60.3%, 66.3%, and 68.6% and 36.8%, 37.6%, and 40.2%, respectively. MPL D4 had a mean recovery of 65.5%, while HCTZ 13C15N2 D2 had a mean recovery of 36.8% (refer Table 3).

Table 3: Details about Recovery of MPL and HCTZ

Analytes (ng/mL)	Mean Peak Post spiked	(% Mean R) Exp	%CV Extract of sample
MPL			
30	13.681	8.505	60.3
7.72			
2000	759.390	530.397	66.3
4000	1304.112	912.914	68.6
HCTZ			
3	2.951	1.701	36.8
5.57			
200	223.398	45.676	37.6
400	351.591	98.934	40.2
Metoprolol succinate D4 10000	374.064	176.742	65.5
Hydrochlorothiazide 13C15N2D2 5000	250.162	54.526	36.8

In analytical procedures, it is documented that the recovery percentage should ideally be 80%. However, in the development of bioanalytical methods, as long as the method produces results that are sensitive, accurate, and precise, the recovery percentage is not always a critical factor.

Matrix effect

The assay employed six distinct sources of human plasma, with 2 concentrations for each analyte and internal standard. No major ion enhancement or suppression was observed for any of the analytes or internal standards under the current experimental conditions, as demonstrated in Table 4.

Table 4: Calculations of different conditions at 3 QC level

Analytes (ng/mL)	Mean Peak Area		Matrix sample	% CV
	Reg Sol	Post spiked samples		
Metoprolol succinate				
30	13.575	13.952	2.04	3.70
4000	1298.542	1274.591	1.99	4.11
Hydrochlorothiazide				
3	2.932	2.971	2.03	4.02
400	350.347	350.672	2.01	4.06
Metoprolol succinate D4 10000	377.783	375.015	1.98	3.55
HCTZ 13C 15N2 D2 5000	246.797	248.255	2.02	2.98

Stability

The solution stability was evaluated over 10 days at 2-8 degree C and for 24 h at room temperature for the drugs and internal standard solutions. Methanol was determined to be the optimal solvent, demonstrating superior stability.

The stability of MPL and HCTZ in plasma was evaluated under a variety of conditions, such as benchtop, normal, freeze-thaw, and autosampler conditions. The experiment was conducted at both low and high QC levels (n=6) and at varying temperatures and storage conditions. The QC samples had been analyzed after seventeen hours of room temperature storage (benchtop), two hours of room temperature processing, and forty-eight hours at 2-8 degree celcius (process stability). Additionally, the QC samples had been saved at -20 degree celcius and -78 degree celcius for hundred and eighteen days (long-time period stability) and subjected to 5 cycles of chilling (-20 degree celcius and -78 degree celcius) and thawing (room temperature). Table five suggests the stability statistics.

DISSCUSION

The optimisation of mass spectrometry parameters and chromatographic conditions was essential for the effective detection and concurrent quantification of MPL and HCTZ with IS in human plasma samples.Guidance for sample preparation in analytical approaches is essential and ought to be simple, rapid, and require minimum chemical compounds whilst maximizing analyte recovery. Literature review suggests that MPL and HCTZ are typically extracted using solid phase extraction. This current study used liquid-liquid extraction (LLE) which was found to be more cost and time efficient and had better recovery rates. These finds are correlated with some of the previous studies like that of Lara *et al.,* (2010) and Ramkumar *et al.,* (2015).[11,13]

In our tests, MPL, HCTZ, MPL D4, and HCTZ13C15N2 D2 isolated from plasma showed no interference peaks from endogenous substances at their retention times. These results are in-line with previous studies like Bharathi *et al.,* (2012) and Hari Krishan *et al.,* (2013) that have suggested minimal matrix effects using the LC-MS/MS technique for quantification of antihypertensive drugs.[14,15] The absence of notable ion suppression or enhancement further validates the method for its robustness.

The least squares approach, commonly used, often results in significant errors at low concentration levels. To address this issue, weighted calibration curves with concentration ranges of 1–500 nanograms/mL for HCTZ and 10–5000 nanograms/mL for MPL were implemented in method. The approach is supported in previous approaches of Ganesan *et al.,* (2010) and Anand *et al.,* (2012).[12,16]

The mean peak area of extracted specimenswere contrasted to that of newly prepared, nonextractedspecimens at three distinct concentrations to determine the percentage recovery. The recovery percentages of MPL and HCTZ were 60.3%, 66.3%, and 68.6%, and 36.8%, 37.6%, and 40.2%, respectively. These recoveries are lower than ideal values but are consistent with findings of other bioanalytical methods including Wellington *et al.,* (2002) and Amol *et al.,* (2010) and the recovery rates are dependent on the extraction technique and matrix complexity. The method showed excellent precision and accuracy which validates the approach and the method.[10,17]

Two QC concentrations had been evaluated for every analyte and IS in six different sources of human plasma. Under the current experimental conditions, none of the analytes or internal standards exhibited notable ion suppression or enhancement effects, as illustrated in Table 4.These findings are consistent with FDA guidelines on bioanalytical method validation.[18]

The described method demonstrates accurate results with both AHDs and DBS. It provides reliable quantitative values, confirming patient non-adherence. The use of DBS simplifies sampling and contributes positively to personalized treatment, highlighting the validation's balance between analytical standards and clinical needs. This approach is useful in large-scale clinical studies.

MPL D4 and HCTZ13C15N2 D2 were used as internal standards to create and confirm a precise, accurate, quick and straightforward LC-MS/MS method for the concomitant evaluation of Metoprolol Succinate and HCTZ in plasm a samples. This methodology presents advantages including uncomplicated specimen preparation, minimal sample quantity requirements, and uniform recovery of both target compounds and internal standards with reduced matrix effects. The findings provide robust scientific support for the precision and accuracy in quantifying metoprolol succinate and hydrochlorothiazide within human plasma samples. This analytical technique is suitable for examining the pharmacokinetics of MPL and HCTZ in human subjects. These are demonstrated in some of the previous pharmacokinetic studies including that of Burnier *et al.,* (2000) and Aulakh *et al.,* (2007).[3,4]

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Conflicting Interests

The authors declare that there is no conflict of interest.

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