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AN INNOVATIVE BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF TRIMETAZIDINE FROM ITS API AND BULK DOSAGE FORM IN HUMAN PLASMA BY RP-HPLC WITH LLE AND ITS CURRENT APPLICATION IN BIOEQUIVALENCE STUDY

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ABSTRACT

Trimetazidine hydrochloride **[1]** (TMZ) is an antianginal drug (BCS Class I) used to treat angina pectoris. A simple, rapid, sensitive and economical RP-HPLC method were developed and validate (ICH M10) for quantification of trimetazidine in human plasma by using internal standard as caffeine. Liquid-Liquid extraction (LLE) method was developed for the optimum extraction of trimetazidine and caffeine from biological matrix. For that, Aliquots (450µL) of plasma were spiked with known amount of trimetazidine and caffeine. The separation was accomplished with the help of column Nucleosil ODS (15cm×4.6 mm, 5µ) at 25°C and an Agilent HPLC with control panel software. An isobestic wavelength set at 210 nm on advanced PDA detector and the mobile phase was composed of Methanol: Water: Acetonitrile: Sodium Hept-Sulfonate Buffer (40:60:10:03%, v/v/v/v). The injection volume was 5µL and the flow rate was 1.3 ml/min. According to bioanalytical ICH M10 guidelines, the bioanalytical method

was validated and found to be selective and linear (r^2 0.997, n = 8) after the method was tested across a linear concentration range of 5.04 - 201.60 ng/ml. The obtained result showed that high precision and accuracy. The recovery of TMZ in human blood plasma was found to be 95.80 % w/v. The developed and validated method by using C_{18} column as per the ICH guidelines can be utilised for routine preliminary pharmacokinetic investigation as well as therapeutic medication monitoring.

Keywords: Trimetazidine Hydrochloride, Caffeine, LLE, human plasma, RP-HPLC

1. INTRODUCTION:

Trimetazidine hydrochloride is antianginal agent and it is used to treat angina pectoris. Trimetazidine enhances cardiac metabolism and shields the heart from the damaging effects of ischemia by suppressing the metabolism of fatty acids and thereby increasing the metabolism of glucose **[2]** According to recent research, the enzyme involved in beta-oxidation, consist of long chain 3-ketoacyl coenzyme-A thiolase, is selectively blocked, which causes this result **[3]**. As a result of this inhibition, the myocardium uses glucose oxidation as a fuel source more frequently instead of free fatty acids as a source of energy. Chemically, trimetazidine is denoted by the name 2 [1-(2, 3, 4- trimethoxy benzyl)-piperazine dihydrochloride **[4]**. Trimetazidine promotes cell tolerance to myocardial ischemia by preserving cellular homeostasis TMZ is regarded as an oral modified release dosage form due to its excellent solubility as well as short half-life, and therapeutic utility in treating chronic disorders **[5]**.

Caffeine is used as an internal standard during development on human plasma **[6]**.

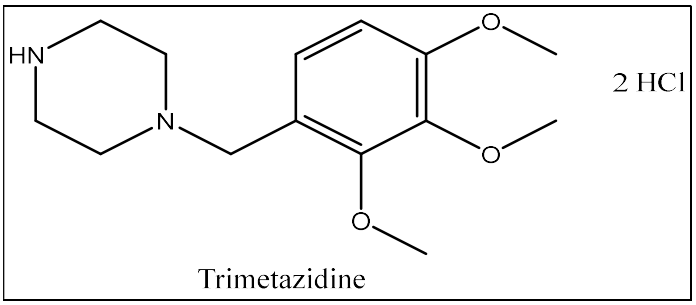

Figure 1

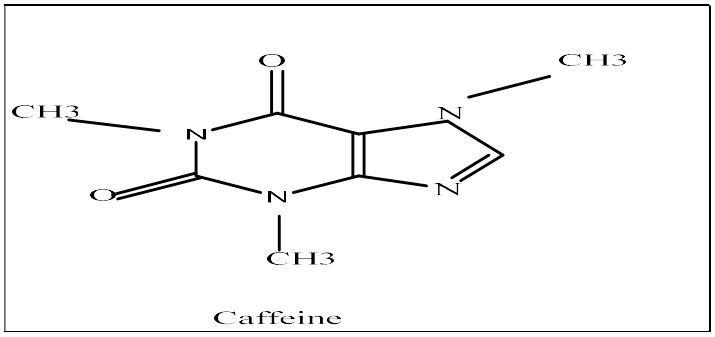

Figure 2

The purpose of this work was to designed and validated a quick, easy, and accurate RP-HPLC technique for the detection of trimetazidine in human plasma samples with the desired sensitivity. As a result, the main goal of this study was to create a novel, straight forward, affordable, selective, reproducible, and reliable HPLC method with a broad linear range and good sensitivity for the assay of trimetazidine hydrochloride **[7]** .

Most previous methods rely on sophisticated instruments such as liquid chromatography-mass spectrometry (LC-MS) **[8]** (LC-ESI-MS) **[9]** & UPLC-MS, which are not commonly found in academic laboratories so that this method was selected.

2. MATERIAL AND METHOD:

Trimetazidine hydrochloride was received as a gift sample from Servier India Private Limited, Goa, Caffeine used as an internal standard were provided by Kapynag Pharma PVT Ltd Talegaon Dindori, Dr. Vasantrao Pawar Medical College, Hospital and Research Centre, Nashik, India, supplied blank human plasma as a gift sample. All the solvents are of AR Grade, Acetonitrile, methanol, and triethyl amine were provided by Merck, Mumbai (India). The membrane filter papers Durapore, 0.45 mm x 47 mm, were acquired from Millipore (India) PVT. Ltd. in Bengaluru, India.

EQUIPMENT'S:

Chromatographic analysis was performed using an C_{18} HPLC system (Infinity 1260, Agilent Technology) with a quaternary pump. An UV detector with a 100 µL injection volume capacity, detection was carried out using Open Lab control panel software. For detection isobestic wavelength was set at 210 nm UV visible spectroscopy.

PREPARATION OF MOBILE PHASE BUFFER (SHS BUFFER):

5.05 gm of 1-heptanesulfonic acid sodium salt anhydrous in volumetric flask of 100 mL. Dissolve it with 50 mL of water. Addition of OPA and make up the volume up to the mark with the help of water. The buffer was filtered and kept at room temperature and 0.22 m membrane filter was used.

PREPARATION OF MOBILE PHASE

The correct amounts of SHS buffer, acetonitrile, and methanol and water were added to a reagent bottle, properly mixed, sonicated for five minutes. The mobile phase was then filtered using a 0.22 m membrane filter. The HPLC experiment was conducted out on an RP-HPLC system with isocratic elution mode Buffer and Methanol as the mobile phase: Water: Acetonitrile: Sodium Hept-Sulfonate Buffer (40:60:10:03% v/v/v/v) on an Interchim) Nucleosil C_{18} column (150 mm 4.6 mm, 5 µm particle size) with 1.3 mL/min flow rate at 210 nm using a PDA detector.

PREPARATION OF DILUENT:

For the preparation of further solution from stock solution for that methanol and water (40:60 v/v) were employed as diluents to get the volume up to the required level.

PREPARATION OF STOCK SOLUTION:

To prepare the Trimetazidine stock solution, 10 mg of Trimetazidine was put into a 10 mL volumetric flask, make up the volume with diluent, and the volume was increased to the appropriate level. In order to create a Caffeine stock solution, a precise 10 mg of Caffeine (IS) were weighed, transferred to a 10 mL volumetric flask, and make up the volume with diluent

PREPARATION OF WORKING STANDARD SOLUTION FOR TRIMETAZIDINE AND CAFFEINE

Stock solution of Trimetazidine and internal standard 1000µg/mL were prepared in diluent and serial dilution of stock solution were prepared and used as a working standard for analysis.

PREPARATION OF CALIBRATION CURVE (CC) STANDARDS AND QC SAMPLES

Add 450 µL of blank plasma were taken in glass tube to this add 25 µL of each working standard solution of TMZ and 25 µL of working standard of caffeine (IS) was added. By reviewing the literature C max was found to be 91.2 From that linearity range was selected from 5 to 200 ng/mL and were vortex it for 1 min to get CC standards respectively. The QC samples was prepared with consist of 5 concentration level as LLOQQC, LQC, MQC, HQC and ULOQQC containing 5.04, 12.7, 100.8, 173.38 & 201.6 respectively.

SAMPLE PREPARATION USING LLE PROCEDURE

There are various extraction techniques available like protein precipitation extraction, solid phase extraction, and Liquid Liquid Extraction (LLE) **[10]**. From that, we have chosen the LLE extraction method because of higher recovery, minimum cross contamination and clean extract of sample can be obtained with good selectivity and reproducible **[11]**. Take 450µL blank plasma +25 µLTMZ + 25µL IS into a small centrifuge tube. adjusted pH in

the sample with a buffer solution for acidic solution to ensure efficient extraction. DCM as extraction solvent is added to the tube Then, the tube is vortex for 1min to mix the sample and the extraction solvent. Centrifuge of the mixture for 3 min Then tube is allowed to stand for the two phases separation and evaporate the solvent, dried and reconstitute with mobile phase and inject to C_{18} HPLC system for analysis.

3. BIOANALYTICAL METHOD DEVELOPMENT:

On unextracted samples trials on different mobile phases, Injection volume, Flow rate, Column temperature were performed. Various mobile phases were tested, including water, methanol, acetonitrile, buffer at various ratio was calculated and changes in flow rate and column oven temperature for eluting analyte, also changes in injection volume trials are taken **[12]**.

CHROMATOGRAPHIC CONDITION:

Chromatographic separation was obtained a separation of TMZ and IS with good peak shape, stable baseline and good resolution occurs by using Column Nucleosil C_{18} (150×4.6mm,5µm) with mobile phase water: methanol: acetonitrile: SHS buffer (60:40:10:03 %v/v/v/v) at flow rate 1.3 mL/min with 25^0C as column oven temperature. Injection volume was 5µL. quantitation was obtained with UV detection 210nm **(Table 1)**.

Table 1: Optimize Chromatographic condition

Drug	Mobile phase	Column used	Flow rate mL/min	Retention time	Peak shape
TMZ	Methanol: Water: Acetonitrile: SHS buffer (40:60:10:03 %v/v/v/v)	Nucleosil C_{18} (150×4.6mm,5µm)	1.3	13.06	Narrow and sharp peak
IS	Methanol: Water: Acetonitrile: SHS buffer (40:60:10:03 %v/v/v/v)	Nucleosil C_{18} (150×4.6mm,5µm)	1.3	3.96	Narrow and sharp peak

4. METHOD VALIDATION:

The bioanalytical technique was validated in accordance with the US-FDA ICH M10 and EMA criteria in accordance with "Guidance for Industry: Bioanalytical Method Validation." with respect to the parameters as follow **[12-16]**.

SYSTEM SUITABILITY TEST (SST):

The SST was done before each batch of sample analysis to confirm the repeatability of the chromatographic apparatus. The C_{18} HPLC SST was carried out by injecting five diluted drugs and IS into the CC's linear regression and calculating the %RSD.

Acceptance Criteria: Percentage RSD should NMT 2.0% for five replicate injections of standard. USP tailing factor is NMT 2.0. The column efficiency, as assessed by theoretical plate count, should be NLT 2000.

SELECTIVITY:

The selectivity parameter was demonstrated to ensure analyte separation and to distinguish the analyte from other interfering of the human plasma. The LLE procedure was used to extracting sample from plasma. Eight separate batches of blank human plasma (6 distinct sources of blank human plasma, 2 haemolyzed plasma, and 2 lipemic plasma) were used for selectivity.

SENSITIVITY:

Analysing repeated samples (n = 6) yielded the lower limit of quantification (LLOQ), or the lowest concentration of CCs that can be quantified with acceptable intraday precision and accuracy. The acceptance value used to define sensitivity of method were a %RSD is NMT 20%.

CALIBRATION CURVES AND RANGES:

Two standard curves of 8 different CC standards, as well as 2 blank samples, were analysed. Calibration curves constructed using 8 CC ranging from 5.04 to 201.6 ng/ml revealed a linear relationship for plasma matrix, observed between peak area ratios and concentrations. uses of blank sample are to check for interference and contamination any endogenous substances. CC acceptance criteria for each CC: %RSD from the LLOQ standard should NMT 20% and all other standards should NMT 15%, and at least 67% of standards should passed and out of 8 concentrations minimum 6 concentration meet the above criteria.no LLOQ and ULOQ should be passed. Ranges consisting the difference in between the highest and lowest concentrations. The method was linear, with a good correlation value, and it worked well for estimating TMZ in human plasma at concentrations ranging from 5.04 to 201.6 ng/mL.

PRECISION AND ACCURACY:

Six spiked samples were tested for TMZ concentrations ranging from 5.04 to 201.60ng/mL. The intraday %RSD and %recovery values in plasma estimated using 5 quality control samples (LLOQ QC, LQC, MQC, HQC, ULOQ QC) were 5.04, 12.70, 100.80, 173.38, 201.60 ng/mL, respectively. Six spiked TMZ samples at each QC level were analysed to assess precision and accuracy within each batch. For research were conducted to determine intraday precession and accuracy 5 replicates of each concentration per day on the same day, standard curves were generated and analysed to determine the concentration of each sample. Acceptance criteria for %RSD for LLOQ standard should NMT 20% and all other standards should NMT 15%. And % recovery for LLOQ standard should between 80-120% and for others should be 85-115%.

RECOVERY:

This procedure was carried out for three different quality control concentration levels

of analyte of 6 replicate (LQC, MQC, and HQC)12.70, 100.80, 173.38ng/mL, final recovery value called as global recovery is the mean of the 6 different replicates for each concentration.

$$\% \text{ Absolute Recovery} = \frac{\text{response of analyte spiked into blood plasma (Aqueous mixture)}}{\text{response of analyte without spiked in blood plasma (extracted mixture)}} \times 100$$

STABILITY:

To investigate the stability of analyte in blood plasma, LQC and HQC samples were analysed in duplicate after being subjected to various experimental conditions that simulate sample storage and handling procedures. After three freeze-thaw cycles at -20°C, sample of LQC and HQC samples were stored at -20°C for 12hr, thawed sample at room temperature for 30 min, and refrozen for 12hr under the same conditions until the three cycles were completed. for bench top spiked plasma sample was removed from the freezer and placed at room temperature for 3hr. The auto sampler stability of TMZ was observed in processed samples kept in the auto sampler for 24 hr at room temperature.

5.RESULTS AND DISCUSSION:

OPTIMIZATION CONDITION

On unextracted samples, trials with various mobile phases, flow rate, injection volume, and column oven temperature were carried out. Various mobile phases, such as methanol, water, and acetonitrile, buffer was tested, and buffer at various ratios were calculated, as well as changes in flow rate, injection volume and column oven temperature for eluting analyte. the HPLC analysis was carried out using Methanol: Water: Acetonitrile: SHS buffer (40:60:10:03 %v/v/v/v), as the mobile phase, which was found to provide good resolution and separation. The wavelength was determined by scanning the solution with a UV spectrophotometer and confirming isobestic at 210 nm. The injection volume was fixed at 5µL.The temperature of the column oven was kept constant at 25°C (ambient). Flow rate was fixed at 1.3 mL/min, retention time of TMZ were of 13.06 ± 0.02 minutes, and for Caffeine were 3.96 ± 0.02 minutes (IS). **Figure 4** shows a representative chromatogram. During the method development process, we found that LLE produced better results, good separation, recovery, and efficiency than other extraction methods such as PP and SPE.

SYSTEM SUITABILITY TEST:

Number of area ratio, RT, and peak areas were also determined as a means of

validation parameter. The values are shown in **Table 2**. The % CV of the RT calculated for the method was found to be 0.04% (≤2%) and the % CV of the peak area was found to be 0.07%, theoretical plate count was found to be 0.39% which is required ≤5.00% These values met the requirements of USP24/NF19 46 and were, therefore, found to be satisfactory. From all five samples of TMZ and IS, the parameters of system suitability are within limit and hence it is clear that the system is suitable for given method.

SELECTIVITY AND SENSITIVITY:

There is no chemical interaction between the TMZ and IS, and both the TMZ and IS were fully resolved. At the retention durations of TMZ and IS, no interfering peak/s were seen in the chromatogram produced from the blank plasma. **Figure 3** shows method is selective for analysis there is no interference any other peak other than TMZ and IS **Figure 4**. Sensitivity was recognised when the Lower limit of quantification (LLOQ) value of 5.04 ng/mL were set. That demonstrated acceptable precision and accuracy: **Table 3** shows that the overall precision value is 4.89 % and the accuracy between 80% and 120%. As a result, with the goal of providing a simple and quick analysis capability, the obtained LLOQ value is sufficiently low to allow the application of this bioanalytical method.

CALIBRATION CURVE:

Calibration samples were created and confirmed by dissolving Trimetazidine and an internal standard in plasma at concentrations ranging from 5.04 to 201.6 ng/mL, and the CC was plotted as peak area ratio against concentration **(Figure 5)**. It was denoted by the coefficient of determination (r^2) 0.995 and it was found to be linear. Linearity concentration was calculated using a regression equation Y = 0.0203x + 0.0019.

PRECISION AND ACCURACY

The QC samples were subjected to accuracy and precision tests. The precision of the approach was determined during validation by the percentage Coefficient of variation (% CV) over the concentration range of LLOQ QC, LQC, MQC, HQC, and ULOQ QC drug samples. The percentage mean accuracy was determined to be in the 99.04 to 102.45% range **(Table 5)**.

EXTRACTION RECOVERY

Recovery was calculated by comparing the peak areas of the analyte in the unextracted sample to those peak areas of the analyte in the extracted sample of TMZ, which were found to be 100.32, 100.87, and 97.60, respectively, and it was shown to be consistent, precise, and reproducible at low, medium, and high concentrations **(Table 6)**.

STABILITY:

Fresh sample stability, bench top stability at room temperature for 12 hours, autosampler

stability freeze-thaw stability (three cycles), and TMZ stability data. All of the findings demonstrated stable behaviour during these tests, and no stability-related issues arose during the validation and stability testing. The stability trials are summarised in **Table 7**.

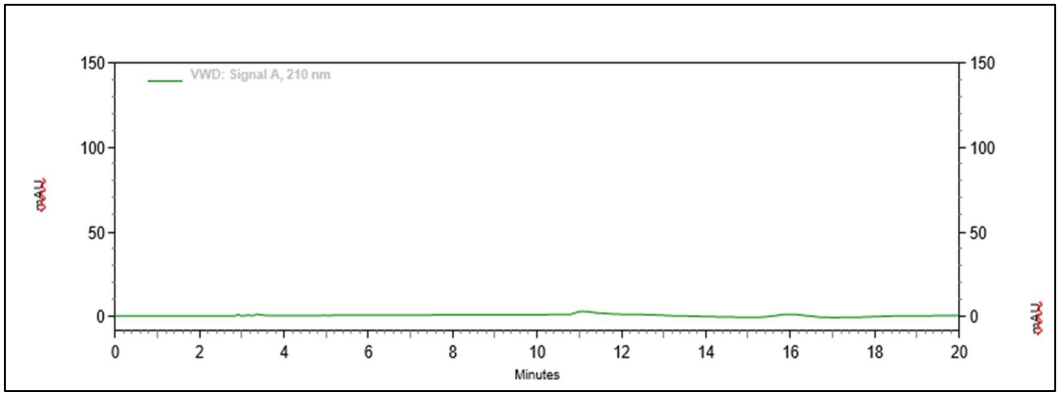

Figure 3: Chromatogram of blank plasm

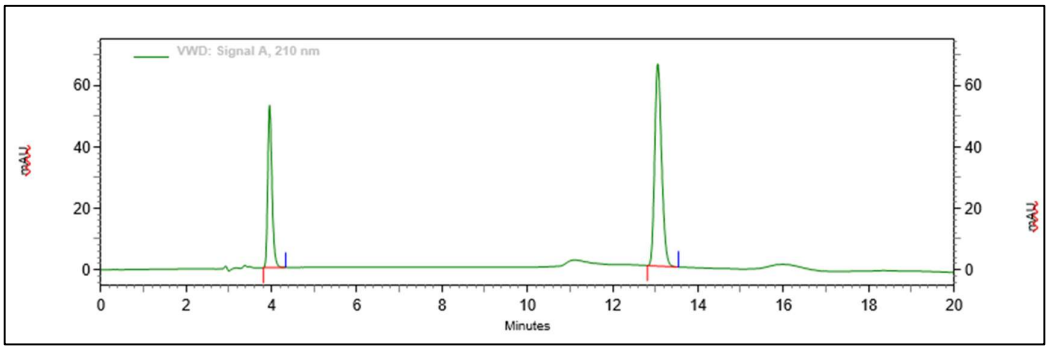

Figure 4: Chromatogram of Trimetazidine and IS

Table 2: System suitability test for the proposed HPLC method

Parameters	TMZ ±%CV	IS±%CV
Retention time (RT)	13.06	3.96
Tailing factor (TF)	1.23	1.28
Theoretical plates (TP)	29737	7778

Table 3: Results of LLOQ sample for TMZ determination for Sensitivity

Sr No.	TC	Area of Drug	Area of IS	PC	Accuracy	Limit for Accuracy	Precision RSD	Limit for Precision
LLOQQC1	5.01	661116	6190762	5.17	102.58	80-120	4.89	<20 %
LLOQQC2		610605	6189730	4.76	94.44			
LLOQQC3		641738	6191494	5.01	99.4			
LLOQQC4		600282	6189580	4.68	92.86			
LLOQQC5		680793	6190531	5.33	105.75			
LLOQQC6		641003	6191480	5	99.21			

Table 4: Results of five calibration curves for TMZ determination for the intraday validation.

Cc Standard	T Conc (ng/ml)	P Conc. (ng/ml)	Peak Area Ratio	Y-C	% Accuracy	Limit (%)	Slope	Inter cept	Correlation Coefficient
STD A	5.04	4.81	0.10	0.098	95.44	80-120	0.0203	0.0019	0.9995
STD B	10.08	10.22	0.21	0.207	101.39	85-115			
STD C	20.16	20.4	0.42	0.414	101.19				
STD D	40.32	39.71	0.81	0.806	98.49				
STD E	60.48	63.36	1.29	1.286	104.76				
STD F	100.8	98.37	2.00	1.997	97.59				
STD G	151.2	149.84	3.04	3.042	99.1				
STD H	201.6	203.28	4.13	4.127	100.83				

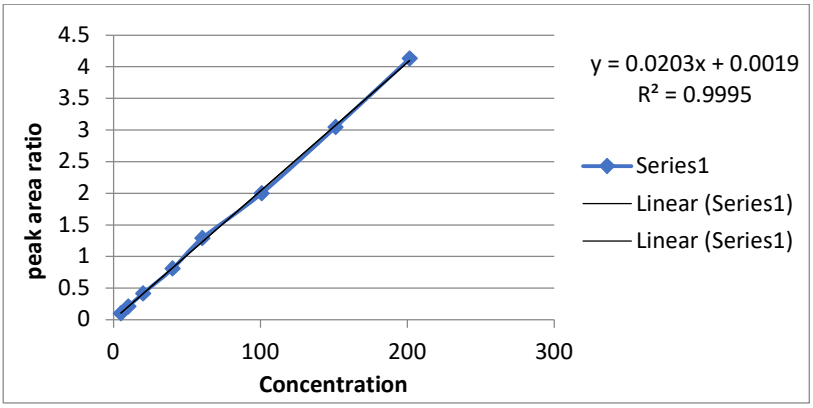

Figure 5: Calibration curve for intraday validation

Table 5: The result of intraday precision (% RSD) and accuracy (%) values obtained for TMZ in plasma at the five quality control sample concentration levels representative of the calibration ranges

Sr. No.	C nominal ng/ml	Mean Area ratio	Measured Conc. ng/ml	Precision %RSD	% Accuracy
1	5.04	0.10	4.99	4.89	99.04
2	12.7	0.27	13.01	4.76	102.45
3	100.8	2.06	101.45	4.9	100.65
4	173.38	3.52	173.06	4.33	99.82
5	201.6	4.10	202.04	3.11	100.22

Table 6: Result of Recovery studies for determination of TMZ from spiked human plasma

Sr. No.	QC samples	Area of Mean Standard	Area of Mean Sample	% Recovery	Global recovery
1	LQC	1597722	242891	97.60	95.8
2	MQC	13106946	2859411	100.87	
3	HQC	23684563	3838581	100.32	
4	IS	6483806	6101720	94.1	

Table 7: Result of Stability of TMZ in plasma at low (LQC) and high (HQC) concentrations of the calibration range in unprocessed and processed samples under difference storage conditions

Sr. No.	Types of	Stabilities	% Stability	
			LQC	HQC
1	Unprocessed sample	Stock solution stability	98.92	99 .10
		Freeze-thaw stability	98.69	99.73
		Bench top stability	100.29	100.44
2	Processed sample	Autosampler stability	98.77	99.86

6. CONCLUSION:

In these studies, a simple, precise, selective, and sensitive validated bioanalytical approach was established, as well as a method that is relatively easy and economical for the detection of trimetazidine in human blood plasma using RP-HPLC. The validation results demonstrated high accuracy and reliability. This method has been used effectively in preclinical pharmacokinetic studies as well as therapeutic drug monitoring.

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CONFLICT OF INTEREST

No any Conflict of Interest.

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