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Development and Validation of Bioanalytical Method for Simultaneous Quantification of Anti-Hypertensive Drugs by Using HPLC

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Abstract

The chromatographic separation was achieved by using eclipse plus C_8 (250mm x 4.6mm, 5µm) column by applying isocratic elution using the mobile phase I consist of (methanol + acetonitrile 60:40 v/v) and mobile phase II consist of (water with 0.01% of diethyl amine) in the ratio 80: 20 (v/v) detection was carried at the 225 nm by employing 1ml/min flow rate. The separation of drugs from the plasma was achieved using protein precipitation technique by acetonitrile. The retention time was found to be 6.573 min and 5.421 min for metoprolol succinate and cilnidipine respectively. The developed method has the linearity range was obtained for RP-UFLC method was 0.5 -16 µg/ml and 1-32 µg/ml for cilnidipine and metoprolol succinate respectively and the regression co-efficient (r^2) is 0.999 for the both drugs. Accuracy & precision is evaluated for the method and found be within the limit and the results were reproducible.

Keywords: Cilnidipine, Metoprolol Succinate, Human plasma, Anti-Hypertensive Drugs, HPLC.

Introduction

Cilnidipine is an antihypertensive agent used as dual blocker of L-type voltage-gated calcium channels in vascular smooth muscle and N-type calcium channels in sympathetic nerve terminals that supply blood vessels. Chemically it is: 3-O-(2-methoxyethyl) 5-O-[(E)-3-phenylprop-2-enyl] 2, 6- dimethyl-4-(3-nitrophenyl)-1, 4-dihydropyridine-3, 5-dicarboxylate. Literature survey revealed that few spectroscopic methods, RP-HPLC and LC-MS method have been reported for the estimation of Cilnidipine with other drugs [1,2]. Metoprolol succinate is a anti-arrhythmic agent used hypertension, angina pectoris, cardiac arrhythmias, migraine prophylaxis and hyperthyroidism. Chemically it is (RS)-1-(Isopropylamino)-3- [4-(2 methoxyethyl) phenoxy] propan-2-ol. Literature survey revealed that few spectrophotometric methods and RP-HPLC, LC-MS/MS method and UPLC have been reported for the estimation of Metoprolol succinate with other drugs [3,4]. From the literature survey, it was found that many methods have been reported for estimation of Cilnidipine and Metoprolol succinate individually and in combination with other drugs and no HPLC method for simultaneous estimation of Cilnidipine and Metoprolol succinate has been reported so far. Hence an attempt has been made to develop new HPLC method which is simple, rapid, reproducible and economical method for simultaneous

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estimation of Cilnidipine and Metoprolol succinate in Human Plasma. (The description of Cilnidipine and Metoprolol Succinate shown in Table No 1 and Table No 2)

Table 1:

a) Cilnidipine

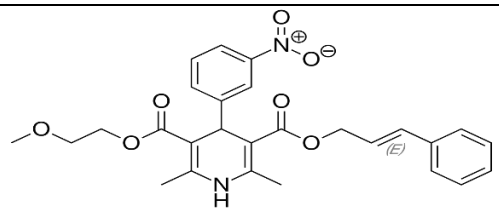
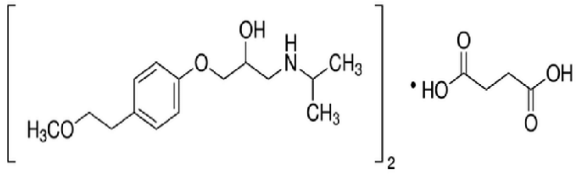
Structure	
Chemical name	3-(2-methoxyethyl) 5-(2E)-3-phenylprop-2-en-1-yl 2,6-dimethyl-4-(3-nitrophenyl)-1,4- dihydropyridine-3,5-dicarboxylate
Category	Anti-Hypertensive drug
Molecular weight	492.52 g/mol
Molecular formula	$C_{27}H_{28}N_2O_7$
Melting point	108°C-113°C
Solubility	Very Soluble in-N Dimethyl acetamide; sparingly soluble in methanol; freely soluble in an acetonitrile, practically insoluble in water.
pKa (Strong Acid)	19.46
Pka (Stron Basic)	-4.1

Table 2:

b) Metoprolol Succinate

Structure	
Chemical name	1-(isopropyl amino)-3-[4-(2-methoxyethyl) phenoxy] propan-2-ol
Category	Anti-Hypertensive drug
Molecular weight	652.8 g/mol
Molecular formula	$C_{34}H_{56}N_2O_{10}$
Melting point	108°C-113°C
Solubility	Very Soluble in Methanol.
pKa (Strong Acid)	14.09
Pka (Stron Basic)	9.67

Materials and Methods

Reagents and Chemicals

Metoprolol Succinate standard drug was obtained as a gift sample from Reine life science, Gujarat and Cilnidipine was Purchased from Yarrow chemicals Pvt. Ltd., Mumbai. Acetonitrile (HPLC grade), Methanol (HPLC grade) and Diethyl amine from S D Fine chem and Millipore water from inhouse Mysuru.

Criteria for Chromatography

The Analysis was carried out with a mobile phase I consist of (methanol + acetonitrile 60:40 v/v) and mobile phase II consist of (water with 0.01% of diethyl amine) in the ratio 80: 20, (v/v), on a column of Eclipse plus C_8 (4.6 mm×250 mm, 5.0 µm). With the help of membrane filter (0.45 mm) mobile phase was degassed and

filtered, then pumped at a flow rate of 1 mL min^{-1}. The run time was 10 min under these conditions. Chromatogram was shown (Fig 1 and 2)

Preparation of mobile phase and standard solution

Acetonitrile and methanol were mixed in the proportion of 40:60 v/v considered as mobile phase I and mobile phase II consist of water (0.01% of diethyl amine). It was then filtered through 0.45 µm membrane filter. Finally, the mobile phase was sonicated for 20 min to degas it.

Preparation of diluents

Acetonitrile and Methanol were mixed in the Proportion of 40: 60 (% v/v).

Standard preparation Stock solution of Cilnidipine

By dissolving 0.01g of pure Cilnidipine drug in 10ml of diluents (10mg/ml), the stock solution was prepared and sonicated for 10 mins. A series of standard solutions were prepared to prepare the working standard solution (1µg/ml) at a concentration of 0.5 - 16 ng/ml with the required dilution of the stock standard solution of Cilnidipine with Diluents.

Stock solution of Metoprolol succinate

By dissolving 0.01g of pure Metoprolol Succinate drug in 10ml of diluents (10mg/ml), the stock solution was prepared and sonicated for 10 mins. A series of standard solutions were prepared to prepare the working standard solution (1µg/ml) at a concentration of 1 - 32 ng/ml with the required dilution of the stock standard solution of Metoprolol Succinate with Diluents.

Method validation

Analytical system validation was conducted in accordance with USFDA Guidelines Validation parameters, such as system suitability, linearity, accuracy, specificity, precision, Selectivity, recovery studies and Stability studies were discussed.

Linearity and Range

Std calibration curve using 6 std solutions (nanogram) in the range of concentration 0.5-16 ng ml^{-1} and 1- 32 ng ml^{-1} ng/mL were prepared using Chromatography was conducted for each standard solution for three times in the run time of 10 minute under optimized chromatographic conditions. To evaluate the method's linearity, the analysis of the average peak area versus concentration data was used for Least Squares linear regression.

Specificity/Selectivity

Selectivity is the capability of the analytical method in the presence of other interference to elicit a response for the analyte. In contrast to the chromatograms obtained for std, tablet, and blank solutions of Cilnidipine and Metoprolol succinate, the selectivity of the method was checked. The retention time and parameters of the tailing factor were measured to show that the selected approach was peculiar.

Precision

Inter-day and intra-day (replicability performed by std solution analysis on the same day) process variations were analyzed by measuring accuracy (repeatability conducted on three different days by analyzing the standard solution). By using a standard solution, the precision study was conducted six times at three separate concentrations.

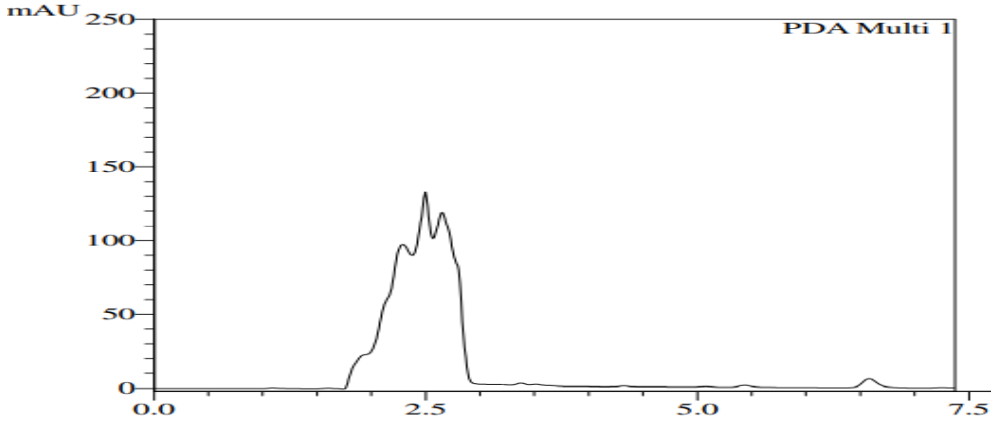
Accuracy

Recovery studies were conducted to confirm the accuracy of the proposed method via the traditional addition technique. The recovery of Cilnidipine and Metoprolol succinate was measured for each concentration by taking 50, 100 and 150 percent of the sample solutions which were previously studied was added to three distinct levels of pure drugs.

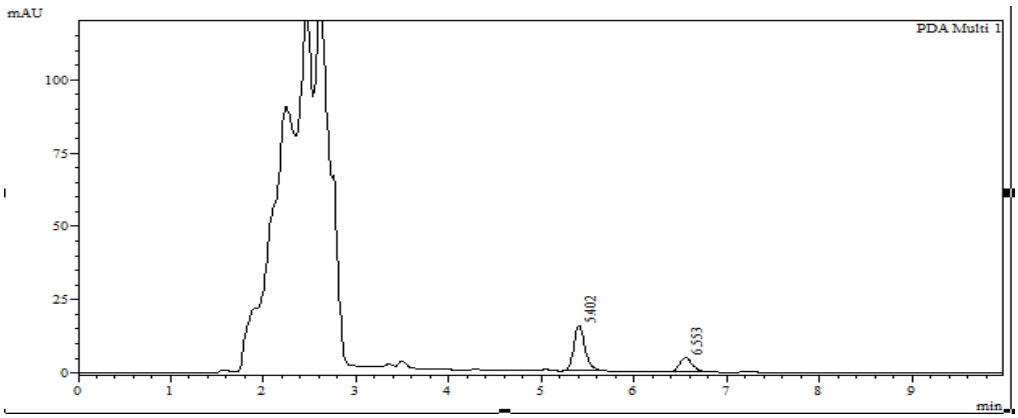
Result and Discussion

Method development

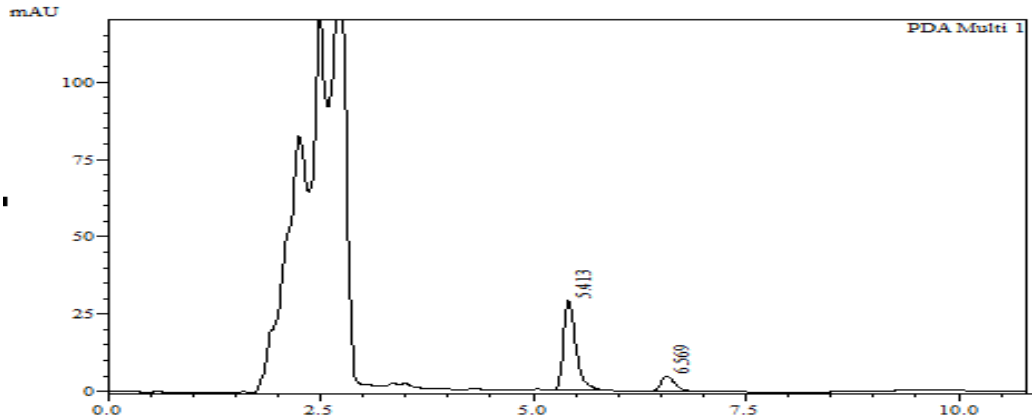
The simultaneous estimation of Cilnidipine and Metoprolol succinate was done by RP-HPLC and mobile phase consists of (800 volumes of (ACN + Methanol) and 200 volumes of Water with 0.01% of diethyl amine. Then finally filtered using 0.45µ membrane filter paper and degassed in sonicator for 15 minutes. The detection is carried out using PDA detector at 225nm. The solutions are following at the constant flow rate of 1.0 ml/min. The retention time for CLN and MTS was 5.421 and 6.573 minutes respectively. Linearity ranges for CLN and MTS were 0.5-16 ng/mL and 1-32 ng/mL respectively and the results were found for in the acceptable as (R^2) = 0.9983 and 0.9975 for Cilnidipine and Metoprolol Succinate respectively. The all parameters value of RSD is less than 2.0% indicating the accuracy and precision of the method. The percentage recoveries were found 99.4- 100.3% and 99.8-100.4% for CLN and MTS respectively.



Chromatogram of Blank Plasma



Chromatogram of CIL and MTS with Plasma (50/100) ng/ml [LLOQ]



Chromatogram of CIL and METS with Plasma (800/1600) ng/ml

Linearity

In order to achieve Std solutions within the range of concentration 0.5-16 ng mL^{-1} of Cilnidipine and 1-32 ng mL^{-1} of Metoprolol succinate the stock standard solution was correctly diluted. Into the UFLC system each Std solution was being injected three times under the above-mentioned conditions of chromatography. The linearity of the proposed method was estimated by regression analysis at concentration levels of 5 in the range of 0.5-32 and 1-32 ngmL-. By plotting the average peak area versus concentration, the calibration curve was developed. (shown in fig 3 and 4)

Figure 4

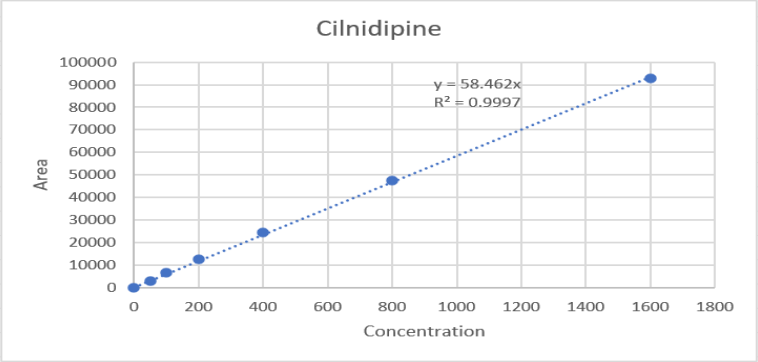
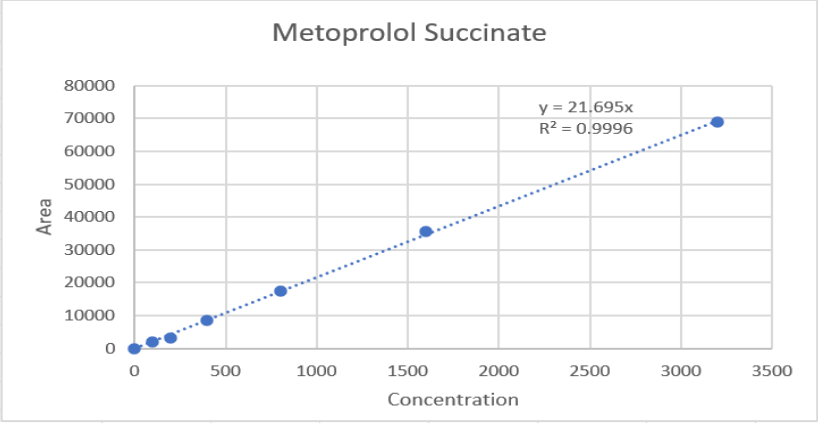
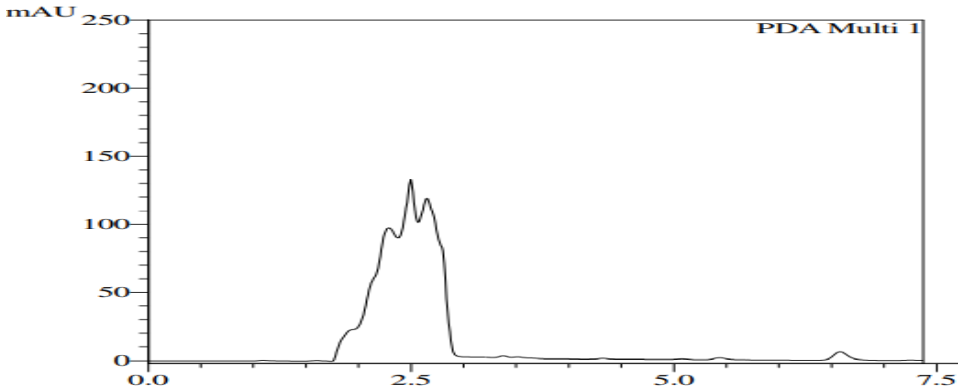


Figure 5

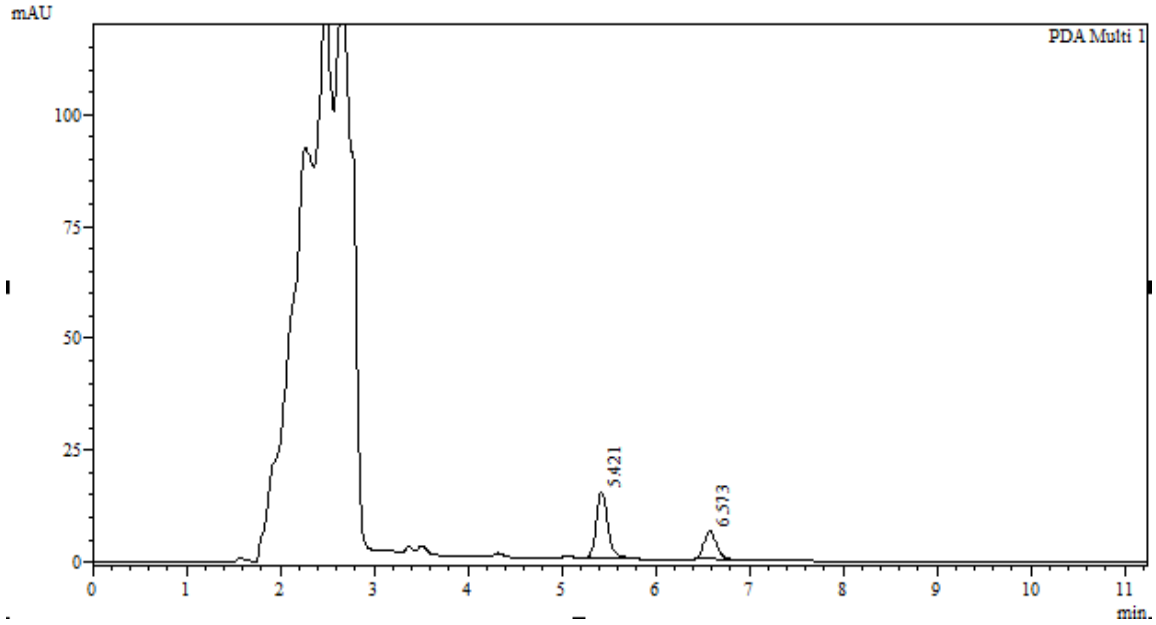


Specificity/selectivity

The Standard solution chromatogram of Favipiravir was given in (A). In this chromatogram having retention time of 5.421 min and 6.573 min having peak. In this chromatogram, no other peaks caused by impurities are observed. No other peaks in this chromatogram are induced by the content of the mobile phase (Figure 5). This indicates that it is unique to the analytical method.



Selectivity Chromatogram of Blank Plasma



Selectivity Chromatogram of CIL and METS with Plasma

Precision

In the precise study, three consecutive days as well as the same day three different concentrations, 200, 400, 800 ng mL^{-1} of Cilnidipine and 400, 800, 1600 ng mL^{-1} of Metoprolol succinate (std solution) was injected six times. Data of Precision has been mentioned in Table 3. Relative standard deviation values of all were less than 0.5 and 2.0 % for peak area and retention for selected concentrations. The method is effective in this condition and can be utilized for our studies. (Table 3)

Table No 3:

SL.No	Sample Name	Intra day Precision		Inter day Precision	
		Conc(ng/ml)	%RSD	Conc(ng/ml)	%RSD
01	Cilnidipine	200	1.16	200	1.18
		400	1.52	400	1.51
		800	1.02	800	1.01
02	Metoprolol succinate	400	1.03	400	1.02
		800	1.14	800	1.13
		1600	0.51	1600	0.52

Accuracy study

To a Std solution of known quantity (50 %, 100 % and 150 %) the sample solutions which are earlier examined (at three different levels) have been added. Three concentrations have been evaluated for the recovered amount of Cilnidipine and Metoprolol succinate. (Table 4 And Table No 5). For both studies, percentage RSD values were less than 2 percent, demonstrating that excipients present in Cilnidipine and Metoprolol succinate will not affect and that the analytical technique is also very accurate.

Table No 4

Accuracy for cilnidipine

Sl.No	% of drug added	Amount of drug taken (ng/ml) (STD)	Amount of drug added (ng/ml) (sample)	Total amount of drug(n=3)	Total amount of drug found	% Recovery	Mean	%SD	%RSD
1	50%	200	100	300	300	100	99.6	0.089	0.089
					299	99.2			
					300	100			
2	100%	200	200	400	400	100	99.1	0.67	0.77
					402	100.25			
					388	98.5			
3	150%	200	300	500	500	100	98.7	0.057	0.057
					497	98.9			
					500	100			

Table No 5:

Accuracy for Metoprolol Succinate

Sl.No	% of drug added	Amount of drug taken (ng/ml) (STD)	Amount of drug added (ng/ml) (sample)	Total amount of drug(n=3)	Total amount of drug found	% Recovery	Mean	%SD	%RSD
1	**50%**	400	200	600	599	99.8	99.9	0.094	0.094
					600	100			
					600	100			
2	**100%**	400	400	800	788	98.5	99.5	0.77	0.77
					802	100.25			
					800	100			
3	**150%**	400	600	1000	1000	100	99.9	0.047	0.047
					999	99.9			
					1000	100			

Stability Studies

The stability study was investigated for the plasma sample under various stability periods and storage conditions. They were carried out at three concentration levels (low, medium and high) at six replicates. The percentage stability was estimated by comparing the mean of back calculated concentrations of all analytes from the stored stability samples with that of freshly spiked QC samples (Table 6 and 7).

Stability Studies for Cilnidipine

Table No 6:

Stability	Spiked QC sample Conc. (ng/ml)	Mean ± SD (ng/ml)	Recovery (%)	RSD (%)
Long-term (30 days)	200	209.8±12.2	103.19	1.97
	400	403.8±15.3	102.40	2.26
	800	814.1±13.3	104.3	3.86
Short-term (8 hours)	200	209.5±14.6	104.1	1.95
	400	408.8±23.1	105.5	4.9
	800	811.6±21.5	109.1	4.3
Auto-sampler (24 hours)	200	215.5±14.2	105.0	3.0
	400	413.6±25.4	109.9	5.48
	800	819.3±30.2	108.3	6.2
Freeze-Thaw	200	214.3±29.2	108.9	6.3
	400	418.6±17.5	107.4	3.7
	800	819±29.7	109.7	6.1

Stability Studies for Metoprolol Succinate

Table No 7:

Stability	Spiked QC sample Conc. (ng/ml)	Mean ± SD (ng/ml)	Recovery (%)	RSD (%)
Long-term (30 days)	400	411.8±12.2	104.19	2.67
	800	813.8±15.3	106.40	3.26
	1600	1616.1±13.3	105.3	2.86
Short-term (8 hours)	400	409.5±14.6	105.1	1.9
	800	811.8±23.1	107.5	4.9
	1600	1611.6±21.5	109.1	4.3
Auto-sampler (24 hours)	400	415.5±14.2	105.0	3.0
	800	813.6±25.4	109.9	5.48
	1600	1619.3±30.2	108.3	6.2
Freeze-Thaw	400	414.3±29.2	108.9	6.3
	800	818.6±17.5	107.4	3.7
	1600	1619±29.7	109.7	6.1

Recovery of drugs from plasma

A protein precipitation method was found to be successful in the extraction of CIL and MTS drug from plasma, and the recovery was determined by comparing peak areas of spiked plasma extracts with those of unextracted neat standards freshly prepared in ACN and MeOH. Plasma samples spiked with the analytes at their respective LQC, MQC and HQC levels were analysed. The area ratios of the targeted drugs were compared with those obtained from blank extracts spiked with the target drug after extraction (taken as 100% recovery of the drug from that particular matrix). Recoveries of the drugs are summarized in **Table 9 and 10**.

Table 9: Extraction Recovery and Matrix Effect of Cilnidipine

QC Levels	In Plasma	Averge In Plasma	In ACN+MeOH and Water	Average in ACN+MeOH and Water	Recovery %
LQC (200 ng/ml)	12456 11998 12117	12190.33	12786 13024 12878	12896	94.52
MQC (400 ng/ml)	24357 24121 24579	24352.33	24757 24986 24893	24878.67	97.88
HQC (800 ng/ml)	47574 47368 47534	47492	47697 47829 47999	47841.67	91.26

Table 10: Extraction Recovery and Matrix Effect of Metoprolol Succinate

QC Levels	In Plasma	Averge In Plasma	In ACN+MeOH and Water	Average in ACN+MeOH and Water	Recovery %
LQC (400 ng/ml)	8674 8687 8754	8705	8854 8957 8798	8866.6	98.17
MQC (800 ng/ml)	17578 17476 17567	17540.3	17986 18456 18214	18218.6	96.2
HQC (1600 ng/ml)	35654 35620 35529	35599	35745 35799 35846	35796	93.4

LQC- Lower quality control sample, MQC- Medium quality control sample , HQC- Higher quality control sample.

Conclusions

A very quick, cost-effective, precise and accurate UFLC method for the determination of simultaneous estimation for Cilnidipine and metoprolol succinate has been developed and validated in compliance with ICH guidance Q2. Besides the short run time (10 min), retention time For Cilnidipine and Metoprolol Succinate was found to be 5.421 and 6.573 minutes respectively and flow rate of mobile phase (1 $mLmin^{-1}$) made the method attractive because these features save analysis time and cost. In short, this method is sensitive, selective, reproducible and rapid for cilnidipine and metoprolol succinate in API. The accuracy and precision are within reasonable limits and finally Bioanalytical method is reliable, Precise and Accurate.

Acknowledgment

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