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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINATION OF CHLORTHALIDONE AND CILNIDIPINE DRUGS IN HUMAN PLASMA BY RP-HPLC

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ABSTRACT

A simple, rapid, sensitive, precise and accurate high performance liquid chromatography method was developed for simultaneous determination of Chlorthalidone and Cilnidipine in human plasma using Azilsartan as internal standard (ISTD). The analytes were extracted from 500 µL aliquots of human plasma sample by direct protein precipitation technique using acetonitrile. Evaluation of content of the drugs were done by employing a mixture of acetonitrile and 0.1% orthophosporic acid (OPA) buffer in the ratio of 35:65 v/v as the mobile phase with a flow rate of 1ml/mL and injection volume of 10µL. Chromatographic separation was accomplished using Inertsil C18, (150×4.6 mm; 5µm) analytical column and the effluents were monitored at 248 nm with photo diode array (PDA) detector. The total run time was 8 min with retention time of Chlorthalidone, Cilnidipine and Azilsartan 3.516 min, 3.518 min and 2.308 min respectively. Linearity was established at a concentration range of 0.05-5.00 µg/mL for Chlorthalidone and 0.025-2.5 µg/mL for Cilnidipine. The method was validated as per the US-FDA guidelines and the results were within the acceptance criteria. And proposed method was successfully applied for the simultaneous determination of Chlorthalidone and Cilnidipine in human plasma.

Keywords: Chlorthalidone, Cilnidipine, Protein precipitation, Human plasma and RP-HPLC.

INTRODUCTION

Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites (analytes) are critical for the successful evaluation of preclinical, biopharmaceutical and clinical pharmacological studies. Bioanalytical method validation includes all of the procedures which demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, these methods are reliable and reproducible[1].

Chlorthalidone is a diuretic drug used in treatment of hypertension. Chemically it is (*RS*)-2-Chloro-5-(1-hydroxy-3-oxo-2,3–dihydro-1*H*-isoindol-1-yl)benzene-1-sulfonamide [Fig. 1]. The molecular formula is $C_{14}H_{11}ClN_2O_4S$ and molecular weight is 338.766 g/mol. It inhibits sodium ion transport across the renal tubular epithelium in the cortical diluting segment of the ascending limb of the loop of henle. By increasing the delivery of sodium to the distal renal tubule, Chlorthalidone indirectly increases potassium excretion via the sodium-potassium exchange mechanism[2-4]. It is official in IP, BP and USP and estimated by Potentiometric titration as per IP and Liquid Chromatography as per BP and USP [5-8].

Cilnidipine is a novel dihydro pyridine calcium-channel blocker drug. It is used in treatment of

high blood pressure. Chemically it is 3-(E)-3-Phenyl-2-propenyl 5-2 Methoxy ethyl 2, 6-dimethyl-4-(m-nitro phenyl) 1, 4-dihydropyridine-3, 5-dicarboxylate) [Fig. 2]. The molecular formula and molecular weight is $C_{27}H_{28}N_2O_7$ and 492.52 g/mol respectively. It is an L-type and N-type calcium channel blocking function. It inhibits cellular calcium influx, thus causing vasodilatation. It has greater selectivity for vascular smooth muscle. Cilnidipine and its formulations are not official in any pharmacopoeias [9-11].

Azilsartan is a novel nonpeptide angiotensin II type 1 (AT1) receptor blocker that was recently approved for treatment of hypertension. It is chemically designated as ((5-methyl-2-oxo-1,3-dioxol-4yl) methyl 2-ethoxy1-((2′-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl) biphenyl4-yl) methyl)-1*H*-benzimidazole-7-carboxylate) [Fig. 3]. The molecular formula is $C_{30}H_{23}KN_4O_8$ and molecular weight is 568.542 g/mol. Azilsartan, the active metabolite of AZL; it has a superior ability to control systolic blood pressure relative to other widely used ARBs. Greater antihypertensive effects of AZL might be due in part to its unusually potent and persistent ability to inhibit binding of angiotensin II to AT1 receptors. Because AZL is a new product and was recently introduced into the market, it is not yet official in any of the pharmacopoeias[12]. Fixed dose combination therapy of Chlorthalidone and Cilnidipine is used in the treatment of high blood pressure effectively. Recent studies reveal that shows significantly better symptom relief when compared with each of the treatments alone.

Literature survey revealed that few analytical methods have been reported for estimation of Chlorthalidone and Cilnidipine individually or in combination with other drugs. The reported methods include Spectrophotometric[13-15], RP-HPLC[16-23], Stability indicating RP-HPLC[24-33], RP-ULPC[34-35], Stability-indicating HPTLC[36-37], Bioanalytical HPLC[38-39], Simultaneous estimation of Chlorthalidone and Cilnidipine in combined pharmaceutical formulations by RP-HPLC[40-41]. There are no reports as per our knowledge that methods developed for the analysis of these two drugs in combination in blood plasma. The present study was aimed to develop a simple, sensitive, rapid, precise, accurate, and validated the bioanalytical method for the simultaneous estimation of Chlorthalidone and Cilnidipine in human plasma. The developed method was validated according to US-FDA[42] guidelines by using high performance liquid chromatography.

MATERIAL AND METHODS

Chemicals and Reagents

Blank human plasma, pure samples including Chlorthalidone, Cilnidipine and Azilsartan were obtained from Spectrum Pharma Research Solutions, Hyderabad, India. HPLC grade acetonitrile was obtained from Merck Chemical Division, Mumbai. Analytical grade of orthophosporic acid purchased from SD Fine Chemicals Ltd., Mumbai, India. The double distillation and purification with Milli-Q water purification system of purified water helped to prepare HPLC grade water.

Instrumentation

The analysis was performed by using Waters 2695 series HPLC comprised of vacuum degas, auto injector, and dual gradient pump with photo diode array detector. The HPLC system was equipped with Empower 2 software.

Chromatographic Conditions

Drug samples were analysed with Inertsil, C18 (150 x 4.6 mm, 5 µm) column as stationary phase and was maintained at 30^0 C. The mobile phase was a mixture of acetonitrile and orthophosporic acid buffer (0.1%) in the ratio of 35:65 v/v. The flow rate of the mobile phase was 1.0 mL/min and sample Injection volume 10 µL. The detection of the effluents was carried out at 248 nm with PDA detector. Samples of Chlorthalidone and Cilnidipine were prepared using water and acetonitrile diluent in 50:50 ratios.

Buffer preparation

(0.1% OPA) 1 mL of orthophosporic acid was transferred into 1000 mL volumetric flask and volume was made up to produce 1000 mL with milli-Q water.

Preparation of stock solutions of analytes and internal standard

Primary stock solutions of Chlorthalidone, Cilnidipine and Azilsartan were prepared individually by dissolving 10 mg, 5 mg and 10 mg of each pure drug in 10 mL volumetric flasks in diluent to produce final concentrations of Chlorthalidone, Cilnidipine and Azilsartan 1 mg/mL, 0.5 mg/mL and 1 mg/mL respectively.

Preparation of Chlorthalidone Spiking Solutions (0.05 µg/mL to 5.00 µg/mL)

From the above Chlorthalidone stock solution 0.01 mL, 0.02 mL, 0.03 mL, 0.2 mL, 0.5 mL, 0.6 mL, 0.8 mL and 1.0 mL was pipette and transferred in to 8 individual 10 mL volumetric flasks and volume made up to the mark with diluent to produce 1 µg/mL, 2 µg/mL, 3 µg/mL, 20 µg/mL, 50 µg/mL, 60 µg/mL, 80 µg/mL and 100 µg/mL. Calibration standards and quality control (QC) samples were prepared by

spiking blank plasma with working stock dilutions of analytes to produce 0.05 μg/mL, 0.1μg/mL, 0.15 μg/mL, 1 μg/mL, 2.5 μg/mL, 3.0 μg/mL, 4.0μg/mL and 5.0 μg/mL.

Preparation of Cilnidipine Spiking Solutions (0.025 μg/mL to 2.5 μg/mL)

From the above Chlorthalidone stock solution 0.01 mL, 0.02 mL, 0.03 mL, 0.2 mL, 0.5 mL, 0.6 mL, 0.8 mL and 1.0 mL was pipette and transferred to 8 individual 10 mL volumetric flasks and made up the volume up to the mark with diluent to produce 0.5 μg/mL, 1.0 μg/mL, 1.5 μg/mL, 10 μg/mL, 25 μg/mL, 30 μg/mL, 40 μg/mL and 50 μg/mL.

Calibration standards and quality control (QC) samples were prepared by spiking blank plasma with working stock dilutions of analytes to produce 0.25 μg/mL, 0.5 μg/mL, 0.75 μg/mL, 5.0 μg/mL, 1.25 μg/mL, 1.50 μg/mL, 2.0 μg/mL and 2.5 μg/mL.

Preparation of spiked plasma sample

500 µL of human plasma, 400 µL of internal standard, 0.01 µL of Chlorthalidone and 0.01 µL of Cilnidipine were pipette and transferred into 10 mL centrifuge tube and to it 2 mL of acetonitrile was added. The mixture was mixed with cyclomixer for 15 sec and then vortexed for 2 min and finally the mixture was centrifuged at 3200 rpm for 5 min. After the centrifugation, 10 µL of the supernatant layer was collected and injected into RP-HPLC injection port.

Method validation

The analytical method was validated according to US-FDA guidelines with respect to the following parameters:

System suitability test

The system suitability test was performed before analysis of every batch of sample to ensure the reproducibility of the chromatographic system[43].The HPLC system suitability test was performed by running six injections of diluted drugs and ISTD in the linear region of the calibration curve and measuring the percentage coefficient of variance (% CV).

Linearity

The Linearity experiment was performed six times to check the detector response to the drug to be linear in function with various concentrations of Chlorthalidone and Cilnidipine 0.05-5.0 µg/mL and 0.025-2.5 µg/ mL respectively. The working standards were prepared by adding different concentrations of Chlorthalidone and Cilnidipine and fixed concentration of Azilsartan (10 µg/mL) solution spiked in plasma to obtain the required concentration range. Samples were extracted and injected into the HPLC system. The drug /ISTD peak area ratio was plotted against the concentration of the drug and expressed in terms of coefficient of determination (r^2).

LLOQ (Sensitivity)

The lower limit of quantification (LLOQ) is the lowest concentration of analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision. The LLOQ is considered being the lowest calibration standard. In addition, the analyte signal of the LLOQ sample should be at least 5 times the signal of a blank sample[1, 44].

Precision and Accuracy

Precision and accuracy of the developed method was determined by analysis of quality control (QC) samples at three different concentrations covering the low, medium and higher ranges of the calibrations curve. Intra-day variation of the assay was done by injecting six samples for each concentration on the same day. Interday variation was assessed by injecting nine samples of each concentration (on 15 days) over a period of two weeks. The precision of the method is expressed in terms of percent coefficient of variance (% CV), and accuracy was expressed as a percentage of the theoretical concentration (Observed concentration / Theoretical concentration × 100) [45].

Recovery

The recoveries for the Chlorthalidone , Cilnidipine and Internal standard were determined by spiking known amount of drugs into drug free human plasma to obtain three different concentration covering the low, medium and higher ranges of the calibration curves. Recoveries were determined by comparing the peak area of extracted QC samples with the peak area of recovery standards at the same nominal concentrations [46].

Specificity/Selectivity

The specificity was verified by checking the interference of endogenous compound in human plasma at the retention time of the Chlorthalidone, Cilnidipine and ISTD by evaluating six lots of plasma.

Ruggedness: The ruggedness was done by changing the person to person for linearity, precision and accuracy in the levels of ULOQ, LQC, MQC and HQC.

Stability

Stability studies were performed as zero hours, long term at -28 ºC and long term at -80 ºC. Day zero having two samples with six replicates of high quality control (HQC) and lower quality control (LQC) levels. Long term at -28ºC and long term at -80ºC have HQC and LQC level with % Stability finding by comparison sample and stability sample.

RESULTS AND DISCUSSION

In the present study, acetonitrile was the solvent of choice, in order to obtain satisfactory values for recovery of Chlorthalidone and Cilnidipine which showed good resolutions with no interferences peak. Hence, extraction with acetonitrile was optimized as the sample treatment procedure[47]. The mobile phase was optimized to provide sufficient selectivity towards the drugs. Orthophosporic acids contribute high sensitivity and selectivity when compared with other buffers. The optimized mobile phase consisted acetonitrile and 0.1% OPA buffer in the ratio of 35:65 v/v. Injection volume was optimized to 10 µL. The column temperature was maintained at 30°C (ambient). Retention times were 3.516 ± 0.05 min for Chlorthalidone, 3.518 ± 0.05 min for Cilnidipine and 2.308 ± 0.05 min for Azilsartan (ISTD).The representative chromatogram of blank human plasma with internal standard shown in Fig. 4.

System Suitability Test

Number of area ratio, retention time and peak areas were also determined as a means of validation parameter. The values obtained are listed in Table 1. The % CV calculated for the method was found to be less than 2%, which revealed the suitability of the developed method and the optimized chromatographic conditions. These values met the requirements of USP24/ NF19 [46] and were therefore found to be satisfactory and results of the study were shown in Table 1.

Specificity/Selectivity

Representative chromatogram of blank plasma confirmed there is no significant interference from the endogenous component as shown in Fig. 5. Chromatograms of spiked plasma samples of Chlorthalidone and Cilnidipine at a concentration of 4.00 µg/mL, 2.00 µg/mL, respectively with the internal standard at a constant concentration 10 µg/mL conforming that Chlorthalidone and Cilnidipine and internal standard were well resolved and completely separated at retention time of 3.537, 4.359 and 2.312 min respectively, The resulted chromatogram was shown in Fig. 6.

LLOQ (Sensitivity)

The sensitivity of the method was determined at 0.05 µg/mL of Chlorthalidone with % CV of 4.44 and 0.025 µg/mL of Cilnidipine with % CV of 4.70. The data for LLOQ was presented in Table 2. The chromatogram of an extracted plasma sample spiked with 0.05 µg/mL and 0.025 µg/mL of Chlorthalidone and Cilnidipine were revealed in Fig. 7.

Linearity

The linearity of the method was evaluated from the calibration curve of spiked plasma samples at several concentration levels of Chlorthalidone and Cilnidipine (constructed for six consecutive days). The mean area ratio of peak area of the drug to the peak area of the internal standard yielded a linear correlation over a concentration ranges from 0.05 - 5.0 µg/mL and 0.025 - 2.5 µg/mL respectively. The method exhibited excellent linearity for this range. A typical calibration curve of spiked plasma samples with the regression equation and the respective correlation coefficient (r^2) of Chlorthalidone and Cilnidipine were shown in Fig. 8, Fig. 9 and Table 3.

Precision and Accuracy

The precision of the method was measured by the percentage Coefficient of variance (% CV) over the concentration range of LQC, MQC and HQC samples respectively of drug during course of validation. Intra-day precision of the method ranged from 2.51 to 9.13 % CV. Inter-days precision of the method was found to be 2.55 to 9.45 % CV. Nominal values (%) for recovery of Chlorthalidone and Cilnidipine from QC samples were tested of intra-day and inter-days. Intra-day accuracy ranges from 79.71 to 101.71% whereas Inter-days accuracy ranges from 79.71 to 101.70%. Result from determination of intra-day and inter-day, accuracy and precision are given in Table 4. Reproducibility of developed assay method was observed on same day and at different days. Percentage coefficient of variance (% CV) was found to be less than 15% for both samples over the concentration range assayed.

Recovery

The recovery for the Chlorthalidone, Cilnidipine and internal standard were determined by spiking known amount of drugs into drug free human plasma to obtain three different concentrations covering the low, medium and higher ranges of the calibration curve. The samples were then extracted and analyzed as described earlier. The recovery was calculated by comparing the peak areas of the drugs with those obtained from pure standards in mobile phase and ISTD in mobile

phase at the same concentration[48]. The recovery of Chlorthalidone and Cilnidipine ranges from 94.11 ± 7767.22 % to 98.05 ±277.82 %, while the absolute recovery for ISTD was 92.79 ±13831.16%, the results of recovery studies were shown in Table 5.

Stability studies

Stability studies were performed to evaluate the stability of Chlorthalidone and Cilnidipine both in aqueous solution and in plasma after exposing to various stress conditions. Chlorthalidone and Cilnidipine were stable did not show any degradation when stored at different conditions. Low value of percentage difference (<15) between area ratio for stability test samples and fresh QC samples confirm the stability of drugs on Zero hours, long term at -28 ºC and long term at -80 ºC, results of LQC, MQC, and HQC were more than 85% which are within acceptance limits. The results of stability study were given in Table 6.

CONCLUSION

The developed RP-HPLC Bioanalytical method is an accurate, specific and simple method for simultaneous determination of Chlorthalidone and Cilnidipine. The method involves simple extraction procedure, separation on a reversed phase column with an internal standard and PDA detector. The validation data demonstrated good precision and accuracy, which proves the reliability of proposed method. Thus the method suits for routine therapeutic drug monitoring (TDM), specializes in the measurement of medication concentrations in blood for Chlorthalidone and Cilnidipine. It is also helpful in pharmacogenetic, demographic and clinical information, and/or on the posterior measurement of blood concentrations of drugs (pharmacokinetic monitoring) of Chlorthalidone and Cilnidipine in human plasma. The present developed method could be adapted for the determination of bioavailability and bioequivalence required for filing NDA and ANDA.

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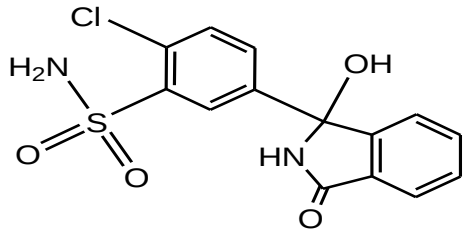

Fig. 1: Chemical Structure of Chlorthalidone

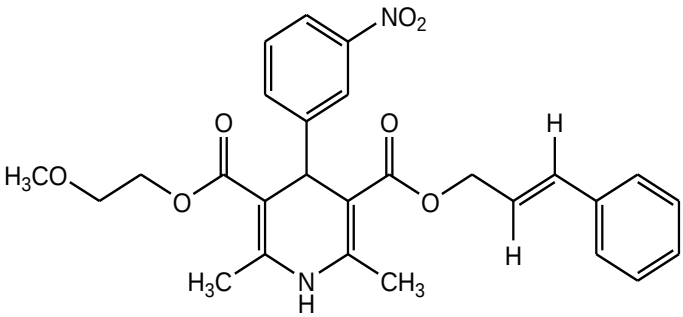

Fig. 2: Chemical Structure of Cilnidipine

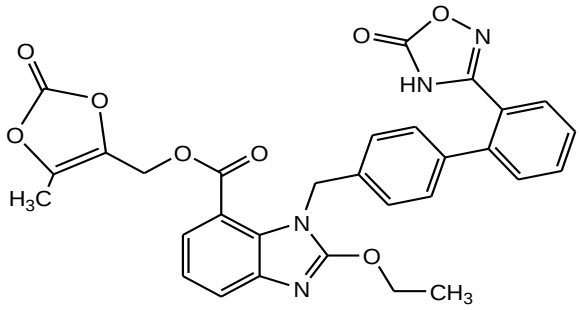

Fig. 3: Chemical Structure of Azilsartan Medoxomil

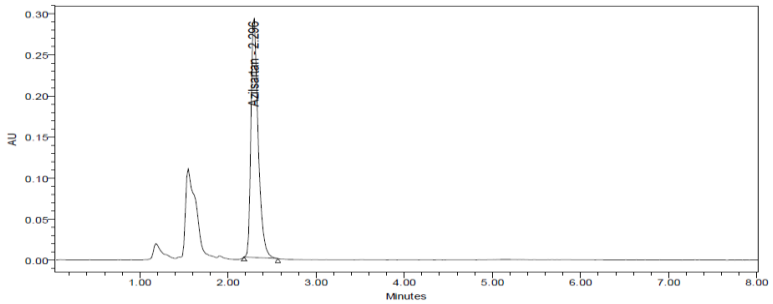

Fig. 4: Representative Chromatogram Human Plasma with Azilsartan (ISTD)

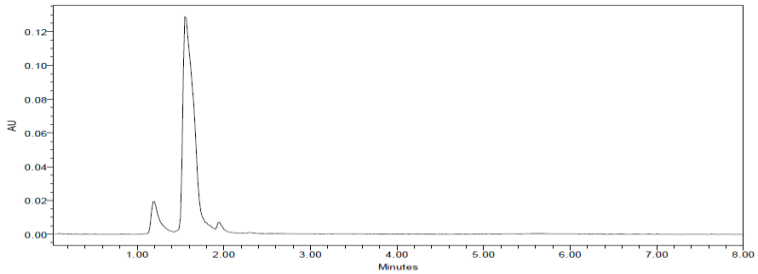

Fig. 5: A Representative Chromatogram of Human Blank Plasma

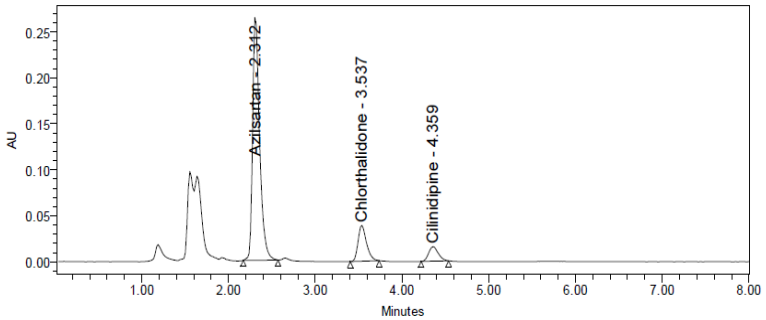

Fig. 6: A Representative Chromatogram of Spiked Plasma Sample (HQC) of analytes

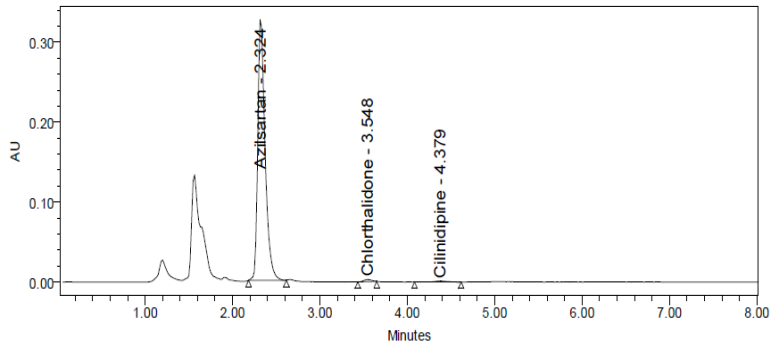

Fig. 7: A Representative Chromatogram of Spiked Plasma Sample (LLOQ) of analytes

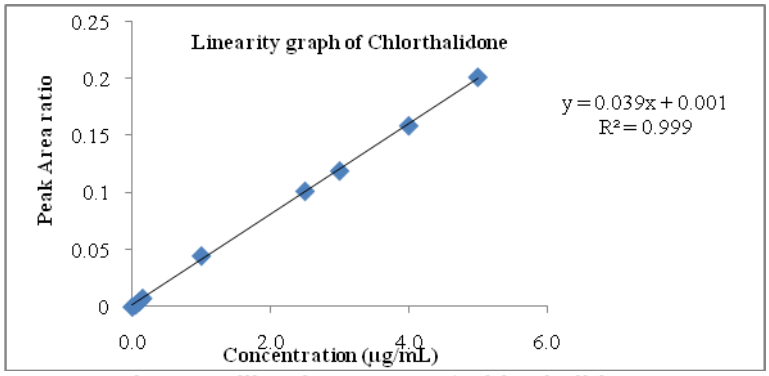

Fig. 8: Calibration Curve of Chlorthalidone

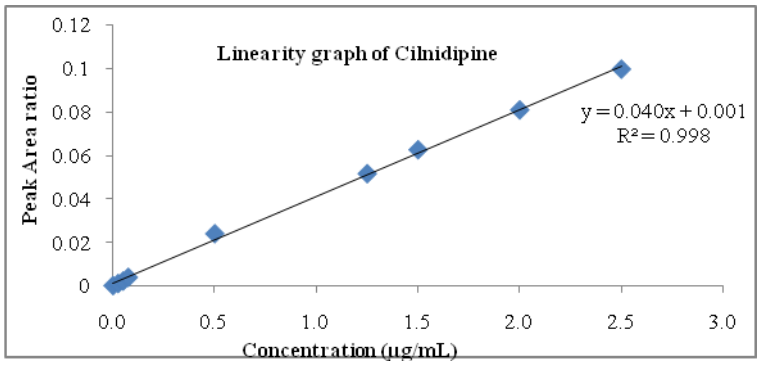

Fig. 9: Calibaration Curve of Cilnidipine

Table 1: System Suitability Data of Chlorthalidone and Cilnidipine

S. No.	Chlorthalidone		Internal standard		Peak Area ratio	Cilnidipine		Internal standard		Peak Area ratio
Sample Name	RT (Min)	Peak Area	RT (Min)	Peak Area		RT (Min)	Peak Area	RT (Min)	Peak Area	
AQMQC	3.47	188203	2.35	1865224	0.1009	4.33	96528	2.35	1870782	0.0516
	3.48	190728	2.37	1880236	0.1014	4.34	96299	2.37	1815184	0.0531
	3.46	191657	2.38	1870120	0.1025	4.32	95524	2.38	1867518	0.0512
	3.47	188531	2.38	1862320	0.1012	4.33	95440	2.38	1878918	0.0512
	3.48	186702	2.38	1856494	0.1006	4.32	95429	2.38	1862490	0.0512
	3.41	189559	2.35	1856494	0.1010	4.33	96236	2.35	1837654	0.0519
Mean	3.46	189203	2.363	1865148	0.10127	4.327	95909.33	2.363	1855484	0.05170
SD	0.026	1798.7044	0.015	9057.100	0.00066	0.0042	498.151	0.0151	24162.201	0.0007
% CV	0.75	0.95	0.64	0.48	0.66	0.10	0.51	0.64	1.30	1.40

Acceptance Criteria: The % CV of the retention time (RT) should be ≤2.00 %.
The % CV of the area ratio should be ≤ 5.00%

Table 2: Sensitivity Results of Lower Limit of Quantitation (LLOQ)

Sample /Parameter	Chlorthalidone Nominal Concentration 0.050 µg/mL		Cilnidipine Nominal Concentration 0.025 µg/mL	
	Cal. Conc. (µg/mL)	% Nominal Conc. (µg/mL)	Cal. Conc. (µg/mL)	% Nominal Conc. (µg/mL)
LLOQ-1	0.048	96	0.025	100
LLOQ-2	0.045	90	0.027	108
LLOQ-3	0.048	96	0.026	104
LLOQ-4	0.051	102	0.025	100
LLOQ-5	0.047	94	0.028	112
LLOQ-6	0.050	100	0.024	96
Mean Cal. Conc.	0.0482 µg/mL		0.0259 µg/mL	
SD	0.00214		0.00121	
% CV	4.44		4.70	
% Mean Accuracy	96.33		103.33	

Acceptance Criteria: At least 67% (4 out of 6) of samples should be within 80.00-120.00%.
%Mean accuracy should be within 80.00-120.00%. % CV accuracy should be ≤ 20.00%.

Table 3: Calibration Curve (Linearity) Data of Chlorthalidone and Cilnidipine

Nominal Concentrations (µg/mL)	Chlorthalidone			Nominal Concentrations (µg/mL)	Cilnidipine		
	*Mean	% CV	% Mean Accuracy		*Mean	% CV	% Mean Accuracy
0.05	0.0487	3.14	97.33	0.025	0.0257	5.95	102.67
0.100	0.0980	1.77	98.00	0.050	0.0757	4.08	98.00
0.150	0.147	1.18	98.00	0.075	0.4933	6.52	100.89
1.000	0.9970	0.30	98.00	0.500	1.210	7.12	98.67
2.500	2.4633	1.29	99.70	1.250	1.51	2.91	96.83
3.000	2.996	0.12	99.89	1.500	2.0233	2.39	100.67
4.000	3.996	0.09	99.92	2.000	2.4860	3.02	101.17
5.000	4.9950	0.06	99.90	2.500	2.4860	4.99	99.44
*Mean values represent three different samples for each concentration							

Acceptance Criteria: The regression coefficient should be R^2 = 0.0999.

Table 4: Precision and Accuracy Data of Chlorthalidone and Cilnidipine

Drugs	Concentration added (µg/mL)	Recovery (% Mean±S.D)	Intra Day # % CV	Accuracy (%)	Recovery (%Mean±S.D)	Inter Days $ % CV	Accuracy (%)
Chlorthalidone	0.150	0.1504 ± 0.004	3.09	100.26	0.150 ± 0.0048	4.26	100.25
	3.000	3.051 ± 0.145	4.76	101.71	3.051 ± 0.1504	4.92	101.70
	4.000	4.0629 ± 0.155	3.83	101.57	4.062 ± 0.1568	3.85	108.50
Cilnidipine	0.075	0.0514 ± 0.021	41.11	68.53	0.051 ± 0.0126	7.50	68.52
	1.250	1.230 ± 0.035	2.84	98.47	1.230 ± 0.0361	2.93	76.59
	2.000	2.003 ± 0.050	2.51	100.01	2.000 ± 0.0510	2.55	100.01

Mean values represent six different plasma samples for each concentration.
$Interday was determined from nine different runs over two-week period.
The concentration of each run was determined from a single calibration curve run on the first day of the study.
Acceptance Criteria: The within and between batch precision for LQC, MQC and HQC
samples should be ≤15.00% and for the LLOQ QC, It should be ≤ 20.00%.

Table 5: Recovery Study Data of Chlorthalidone, Cilnidipine and Internal Standard Drugs from Human Plasma

Drugs	Concentration added (µg/mL)	Recovery (% Mean ± S.D)	% CV	Overall % CV
Chlorthalidone	0.150	108.17 ± 75.26	0.52	1.31
	3.000	95.87 ± 1402.65	0.75	
	4.000	94.11 ± 7767.22	2.66	
Cilnidipine	0.075	95.22 ± 107.64	1.42	1.18
	1.250	98.05 ±277.82	0.29	
	2.000	95.13 ±2814.03	1.85	
Internal Standard	10.00	92.79 ±13831.16	0.74	-

Acceptance Criteria: The % CV of recovery at each QC level and for ISTD should be ≤ 15.00%. The overall mean recovery % CV for all QC levels should be ≤ 20.00%.

Table 6: Stability Study Data of Chlorthalidone and Cilnidipine

	Chlorthalidone				Cilnidipine			
Sample	Nominal Concentration (µg/mL)	Mean calculated Conc. ± S.D (µg/mL)(n=6)	%CV	% Mean Accuracy	Nominal Concentration (µg/mL)	Mean calculated Conc. ± S.D (µg/mL) (n=6)	% CV	% Mean Accuracy
Stability at day Zero								
HQC	4.000	4.0232 ±0.0036	2.82	100.58	2.000	2.0120±0.027	1.36	100.60
LQC	0.150	0.1518 ±0.004	2.66	101.17	0.075	0.0745±0.005	7.53	99.33
Long term at -28ºC								
HQC	4.000	3.9488 ±0.1060	2.69	98.72	2.000	1.9985±0.005	0.28	99.93
LQC	0.150	0.1511 ±0.002	1.34	99.42	0.075	0.0735±0.005	7.64	98.00
Long term at -80ºC								
HQC	4.000	3.9643±0.098	2.48	99.11	2.000	2.0450±0.058	2.84	102.25
LQC	0.150	0.1502±0.002	1.54	100.33	0.075	0.0735±0.005	7.64	98.00

Acceptance Criteria: At least 67% (8 out of 12) of total QC samples and 50% (3 out of 6) at each level should be within 85.00 - 115.00%. The %Mean accuracy of LQC and HQC should be within 85.00-115.00%. The % CV of LQC and HQC samples should be ≤ 15.00%.

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