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Original Article

DEVELOPMENT AND VALIDATION OF THE RP-HPLC METHOD FOR ESTIMATION OF CILNIDIPINE IN RAT PLASMA

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ABSTRACT

Objective: A simple, precise, accurate, robust, and reliable RP-HPLC technique for detecting cilnidipine in Wistar rat plasma was developed and validated.

Methods: The chromatographic separation was performed using the C-18 reverse phase column Oyster BDS Premium C18 column (4.6 × 150 mm, 5 µm particle size). The flow rate was set to 1.2 ml/min, and the chromatogram was recorded at 240 nm. The proposed method was validated in compliance with the International Conference on Harmonization (ICH) guidelines.

Results: The calibration curves plotted were found to be linear over the concentration range of 40-200 ng/ml in Wistar rat plasma, with a regression coefficient of R^2>0.999, suggesting that the linearity was within limits. The LOD and LOQ of the present method were found to be 2.184 and 4.518 ng/ml, respectively. Drug extraction recoveries from Wistar rat plasma were found to be >98%.

Conclusion: Using Wistar rat plasma, a simple, fast, specific, accurate, and precise analytical approach was developed and validated. The technique was validated according to the ICH guidelines. The obtained results show that the suggested technique can be simply and effectively utilized for routine cilnidipine analysis in Wistar rat plasma.

Keywords: RP-HPLC, Cilnidipine, Rat plasma, Development, Validation

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INTRODUCTION

Cilnidipine (CLN) [2-methoxyethyl-(E)-3-phenyl-2-propen-1-yl(±)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl) pyridine-3,5-dicarboxylate] [1], is a newly developed dihydropyridine calcium antagonist with a prolonged duration of action and a gradual onset [2-4]. It can modulate the catecholamine release, which is directly related to intracellular Ca2+levels. CLN has a slow-onset, long-lasting anti-hypertensive activity and distinct inhibitory activities on sympathetic neurotransmission when compared to other calcium antagonists [5, 6]. CLN is a BCS Class II drug with poor water solubility and a slow rate of dissolution. With a log P value of 4.7, it is extremely lipophilic and so has good intestinal permeability. However, CLN oral bioavailability in humans is quite low (~13%) [7, 8]. It is soluble in methanol and partially soluble in ethanol [9].

The therapeutic usage of CLN is limited due to its low water solubility, poor dissolution and high hepatic first-pass metabolism in the liver, which are the primary reasons for poor oral bioavailability [10]. CLN has a relatively rapid absorption with a peak concentration after 2 h [11]. CLN is an antihypertensive, vasodilator dual blocker of L-type voltage-gated Ca2+channels in vascular smooth muscle and N-type Ca2+channels in sympathetic nerve terminals that supply the blood vessels [12, 13]. Because there are so many distinct drug compounds, it is vital to ensure their safety before these treatments are marketed. As a result, new analytical techniques for analyzing novel entities in pharmaceutical formulations and biological materials are developed. Furthermore, technique validation will provide insight into the identification, characterization, and estimate of the novel therapeutic drug molecule. Several analytical methods for CLN analysis have been established and validated [14, 15]. These methods include HPLC, HPTLC, UV/Vis-Spectrophotometry, electrophoresis, hyphenated techniques, FT-IR, capillary zone, voltammetry, and fluorescence measurement [16]. However, they are time-consuming and require the use of sophisticated equipment. As a result, there is a need for a unique approach that can increase column life while also improving effectiveness. However, most existing technologies have disadvantages such as time-consuming sample preparation, higher solvent consumption, long-run durations for biological samples, limited sensitivity, uneconomical and poor symmetry. An efficient approach is one that is cost-effective, sensitive, and uses simple sample preparation processes. Bioanalytical technique development often includes monitoring several technical factors such as volume of solvent used, selection of solvents for sample extraction, centrifugation speed and time, chromatographic conditions (organic solvents, buffer pH, ratios employed), etc. [17]. Thus, the main objective of this work was to develop a simple, affordable, yet precise bioanalytical RP-HPLC technique for estimating CLN in rat plasma and then validate it using ICH guidelines.

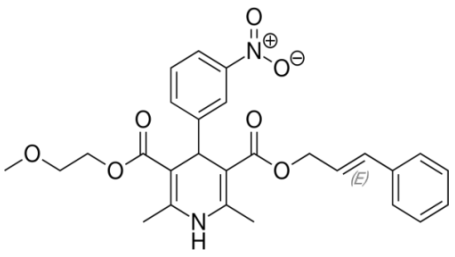

Fig. 1: Structure of cilnidipine [18]

MATERIALS AND METHODS

Materials

Chemicals

Cilnidipine was supplied as a gift sample from Prayosha Healthcare, Ankleshwar (Gujarat, India). HPLC grade methanol (MeOH) was procured from Merck, Mumbai, India. Orthophosphoric acid (KH2PO4)

was purchased from Fisher Scientific, Mumbai, India. High-quality HPLC grade water obtained from our laboratory's Milli-Q water purification equipment (Millipore®, MA, USA) was utilized throughout the study. All other chemicals were HPLC analytical grade.

Methods

Chromatographic conditions

The HPLC system (LC-2010HT, Shimadzu Corporation, Japan) had two pumps, a high-efficiency 5-line degasser, a block heating-type column oven, a sample cooler (LC-2010CHT), an intelligent autosampler and a dual-wavelength UV-visible detector. The entire system was controlled by the LC solution software. A C-18 reverse phase column Oyster BDS Premium C18 column (4.6 × 150 mm, 5 µm particle size, Merck India) was used for chromatographic separations. The mobile phase was made up of methanol and 0.05 % orthophosphoric acid in a 30:70 v/v ratio. A 20 µl sample was injected into the column, which was kept at 35 °C. The column was equilibrated for at least 30 min with the mobile phase running through the instrument before injecting the drug solution. The flow rate was set to 1.2 ml/min and the chromatogram was recorded at 240 nm.

Preparation of stock and standard working solutions

In a volumetric flask, all standard stock solutions and standard working solutions were prepared. To begin, 100 mg CLN was dissolved in the mobile phase to yield a standard stock solution of 100 mg/ml. Following that, the working standard solutions were formed by serial dilution of the stock solutions with the mobile phase mixture. Calibration standards of 40, 80, 120, 160 and 200 ng/ml were prepared for control Wistar rat plasma samples by combining with 10 µl of CLN working stock solutions. Before use, all solutions were stored in amber-colored bottles at-4 °C.

Preparation of sample solution

Male Wistar rats (200-250 g) were procured from the University Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur and were approved by the Institutional Animal Ethics Committee. Before beginning the studies, they were maintained in an environmentally controlled room for at least one week and provided standard laboratory food and water. The rats were fasted for 12 h before each study, with free access to water. Intraperitoneal xylazine and ketamine were used to anesthetize rats [19]. The blood samples were obtained from the retro-orbital plexus, about 200 µl aliquot of the Wistar rat plasma sample. The sample was then extracted with 1.5 ml of tertiary-butyl methyl ether (TBME). The mixture was vortexed for 15 min before being centrifuged at 3,000 rpm for 10 min. Following centrifugation, the organic phase was transferred to glass tubes and evaporated to dryness for 5 min using a TurboVap LV Evaporator at 50 °C under a nitrogen stream. Upon drying, the residue was reconstituted with 150 µl of mobile phase mixture and vortexed for 1 minute. The HPLC system was then injected with a volume of 10 µl [20].

Method validation

The CLN analysis technique was developed and validated in accordance with ICH guidelines [21, 22]. Samples were evaluated for selectivity, sensitivity, method linearity, accuracy, precision and recovery in accordance with the U. S. Food and Drug Administration's bioanalytical method validation standards [23, 24].

Specificity study

The specificity was determined by screening six different sources of blank Wistar rat plasma. Samples were processed and injected into the HPLC to see how many endogenous constituents interfered with CLN retention time. If an interfering peak is present at the drug retention time, its response should be less than 20% of the mean response of six extracted Wistar rat plasma samples [25].

Linearity and range

The peak-area ratio versus nominal analyte concentration was used to assess the linearity of a plasma sample. Calibration curves in plasma were prepared in the concentration range of 40-200 ng/ml to develop the linearity range of the proposed method.

Selectivity

The selectivity was originally explored by comparing the chromatograms. The pooled blank rat plasma and the chromatogram from the same samples were spiked using CLN and standard internal amlodipine (AML).

Accuracy

Acquisition studies were carried out using the standard addition method, in which a known amount of CLN was added to pre-analyzed samples at 80, 100, and 120 % levels of labeled claim before being subjected to the proposed analytical process. For each concentration, the percent recovery and relative standard deviation (% RSD) were measured. Accuracy was calculated using the formula

$$\% \text{ RE} = (\text{E} - \text{T}) \times \frac{100}{\text{T}}$$

Where, E is the experimentally determined concentration, and T is the theoretical concentration [26].

Recovery

Wistar rat plasma was used for the recovery trials. The percentage recovery was calculated utilizing accuracy samples at 80%, 100%, and 120% concentrations of 96, 120, and 144 ng/ml and single concentration injection volume (10 µg/ml). The acceptable percentage recovery criteria should be between 98 and 102% [27].

Precision

The precision was measured using quality control samples (n = 3) with CLN concentrations of 40, 120 and 200 ng/ml in Wistar rat plasma at low, medium, and high levels. The precision of the test was determined by measuring the inter-day and intra-day variability of quality control samples. Inter-day precision data were collected by assessing three sets of quality control samples on three successive days of an assay, whereas intra-day precision data were obtained by examining three sets of quality control samples on a single day. The sample precision was calculated using the formula [28].

$$\% \text{ RSD} = \frac{\text{SD}}{\text{M}} \times 100,$$

Where M is the mean of the experimentally determined concentrations and SD is the standard deviation of M.

Robustness

The robustness of the developed method was investigated by examining the effect of a minor but deliberate changes in chromatographic conditions. Flow rate and mobile phase composition were the parameters investigated [29].

Ruggedness

The method's robustness was investigated by adjusting the experimental conditions with precision and accuracy in batches. Ruggedness was evaluated in the same laboratory by separate analyzers.

Limit of detection (LOD) and limits of quantification (LOQ)

The lowest concentration of analyte in a sample that can be detected but not necessarily measured under defined experimental circumstances (LOD) was determined using the y-intercepts of linearity curve regression lines.

$$\text{LOD} = 3.3\frac{\sigma}{\text{s}}$$

Where σ is the standard deviation of response; s is the slope of the calibration curve

Limits of quantification (LOQ), or the lowest concentration of analyte in a sample that can be quantitatively measured within an acceptable level of accuracy and precision within specified operating parameters of the technique, varies depending on the kind of method used and the working conditions. LOQ was determined using the y-intercepts of the linearity curve's regression lines [30].

$$\text{LOQ} = 10\frac{\sigma}{\text{s}}$$

Where σ is the standard deviation of response; s is the slope of the calibration curve.

Statistical treatment of data

All experimental results are given as mean, standard deviation (SD). The mean, standard deviation and % relative standard deviation were determined using an MS Excel worksheet.

RESULTS AND DISCUSSION

Method development

The key analytical challenge in developing a new method is separating active pharmaceutical components [31]. The chromatographic conditions were optimised to give good performance. CLN is a weakly water-soluble and extremely lipophilic drug. It was required to adjust parameters such as the organic solvent selection, pH, mobile phase composition, flow rate and temperature conditions during method development. 240 nm was discovered to be the optimal wavelength for detecting CLN with appropriate sensitivity. For the separation experiments, potassium dihydrogen orthophosphate buffer and methanol at 0.05 M were used once again. CLN was resolved with excellent peak shape at pH 4.2, while peak tailing of CLN was detected at pH>4.2. Hence For the preliminary separation investigations, potassium dihydrogen orthophosphate buffer (0.05 M; pH 4.2) and methanol were used.

Studies were carried out using various methanol compositions in the mobile phase to improve CLN resolution. Because of its poor aqueous solubility, the quantity of organic composition in the mobile phase greatly influenced CLN retention time. The best peak shape was observed with the mobile phase consisting of Methanol: 0.05 M potassium dihydrogen orthophosphate buffer pH 4.2 in the ratio of 30:70 (v/v); small variations in the mobile phase composition caused slight variations in the retention time of CLN without altering its peak response.

Flow rates of 1.0, 1.2, and 1.4 ml/min with optimal mobile phase composition were evaluated. The peak width of CLN decreased as the flow rate progressed; however, there was no significant drop in the peak width at flow rates of 1.2 and 1.4 ml/min. As a result, a flow rate of 1.2 ml/min was chosen as the best flow rate because it produced acceptable peak shapes with no endogenous peak interference during the analyte retention period.

The influence of column temperature was investigated at both ambient and higher temperatures. The peak width CLN had no significant influence, while increasing the oven temperature produced a drop in the peak width CLN. Thus, the ideal oven temperature with the optimized chromatographic condition was 25 °C (Room Temperature). CLN's average retention time was found to be 7.029 min.

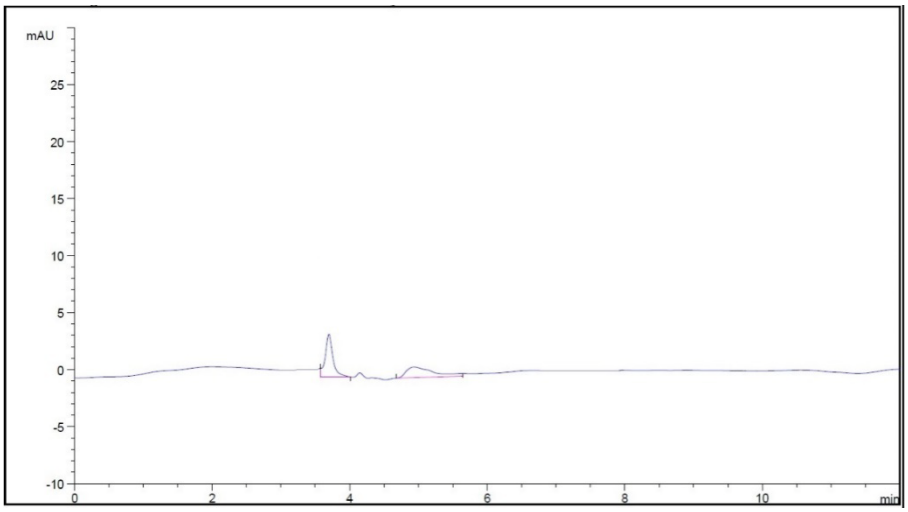

Fig. 2: Chromatogram of blank wistar rat plasma

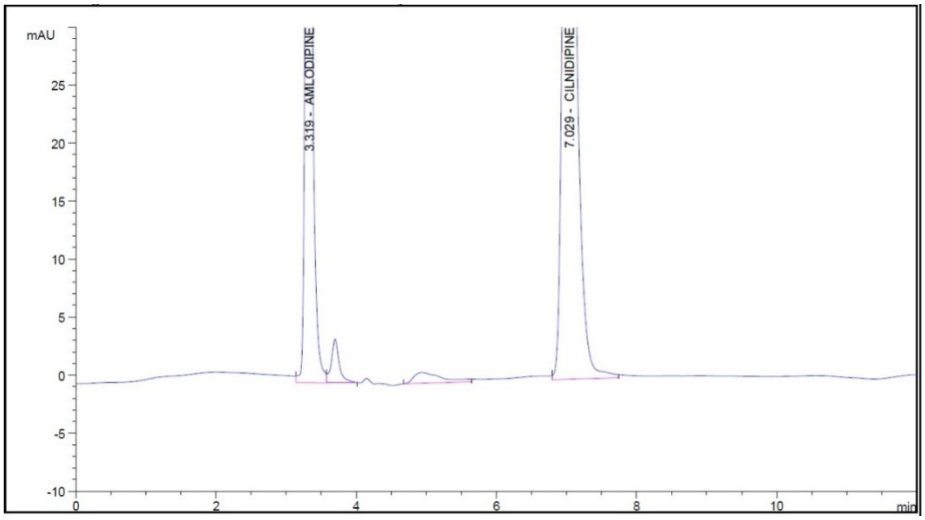

Fig. 3: Chromatogram of CLN and AML from plasma

Specificity

The established method was found to be drug molecule specific, as no other peak was noticed in the chromatogram of the blank plasma sample at the drug retention time and λ max. As a result, it was concluded that the chosen method was drug-specific [32].

Six distinct blank Wistar rat plasma samples were used to assess specificity. Fig. 2 and 3 depicts chromatograms generated from Wistar rat plasma samples. At the CLN retention period, no interference peaks of endogenous substances were identified. CLN was eluted after 7.029 min. However, the sample runtime was optimized to 7 min.

Linearity

CLN was assigned a five-point linearity curve. The peak area of CLN was used to evaluate the samples. The peak area of CLN concentration in plasma samples was plotted to develop standard calibration curves. To estimate the slope, intercept and coefficient of correlation, the least squares linear regression analysis y = mx+c was used. CLN's calibration curves have a correlation value of 0.999. The mean (standard deviation) slope and intercept values for the CLN calibration curve were 1049.9 and 437.5, respectively. The results are shown in fig. 4.

Accuracy

The method's accuracy was determined by the recovery of CLN through a 10 µg/ml standard solution spiked with 80, 100 and 120 % extra CLN. The method revealed that the percentage recovery ranged between 99.12-99.86. The results are given in table 1.

Precision

Precision was measured intra-day (repeatability) by assaying three different concentrations of CLN (40, 120 and 200 ng/ml) at different time intervals on the same day and inter-day (intermediate) precision by repetition for three days as per ICH. The method demonstrated good precision and RSD (%) for repeatability and intermediate precision, which were within the NMT % limit of 0.19-45 % and 0.26-0.58 %, respectively (table 2).

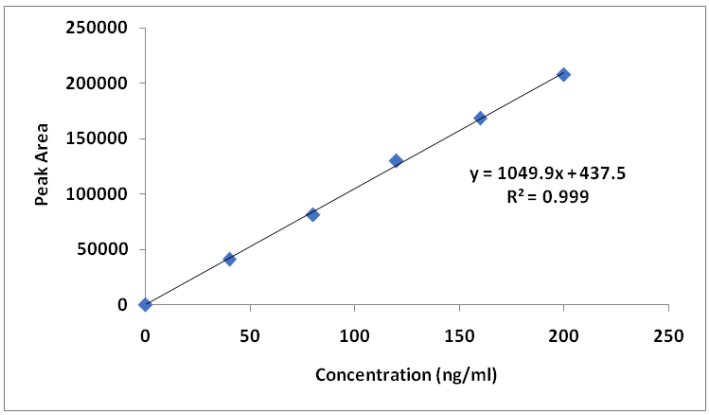

Fig. 4: Linearity curve of CLN in plasma at 240 nm RP-HPLC method

Table 1: CLN accuracy study results

S. No.	% Level	Cilnidipine	
		Mean % recovery	Standard deviation (SD)
1	80	99.12	0.542
2	100	99.86	0.298
3	120	99.59	0.691

(n=3)

Table 2: The precision of CLN in wistar rat plasma by RP-HPLC method

Concentration (ng/ml)	Intra-day		Inter-day	
	Concentration found (mean±SD) (n = 3)	% RSD	Concentration found (mean±SD) (n=3)	% RSD
40	39.28±0.32	0.45	39.86±0.14	0.58
120	119.13±0.21	0.29	119.87±0.29	0.42
200	199.67±0.26	0.19	199.92±0.24	0.26

Table 3: Robustness of CLN in plasma by RP-HPLC method

Flow rate (ml/min)	Retention time		Theoretical plates	
	(mean±SD) (n=3)	%RSD	(mean±SD) (n=3)	% RSD
1.0	7.238±0.024	0.489	6281±28.38	0.25
1.2	7.029±0.018	0.540	6356±44.52	0.53
1.4	7.149±0.013	0.236	6521±18.27	0.46

Recovery

In order to determine recovery, CLN in Wistar rat plasma was separated into 3 replicates at 80, 100 and 120 % quality control concentrations. The mean recovery of CLN in Wistar rat plasma was 99.12 %, 99.86 % and 99.59 %, respectively.

Robustness

The low percent RSD values for the drug suggested that during all planned variations, the assay result of test preparation was unaffected and it was by actual. This suggests that the developed method was robust under the given test conditions [33] (table 3).

Ruggedness

A separate analyst was utilized to assess the method's ruggedness. The % RSD values were less than 2, indicating the robustness of the proposed analytical method. The results are summarised in table 4.

Table 4: Ruggedness of CLN in wistar rat plasma by RP-HPLC method

Analyst	CLN concentration (ng/ml)*	% RSD
1	91.02±0.25	0.261
2	90.52±0.16	0.185

*mean±SD (n =3)

Limits of detection (LOD) and quantitation (LOQ)

The LOD and LOQ were determined to be 2.184 and 4.518 ng/ml, respectively. These low values indicated the developed method's high sensitivity. The method has been validated in compliance with ICH guidelines [34].

CONCLUSION

For the quantitative determination of CLN in small volumes of rat plasma, a simple, rapid, specific, sensitive and reproducible bioanalytical RP-HPLC method has been developed and validated. In addition, the method demonstrated better drug recovery from plasma samples, lower linearity and range, and lower LOQ and LOD values when compared to existing methods for estimating CLN in biological samples. The developed method can be used to conduct pharmacokinetic and biodistribution studies on drugs in bulk or CLN present in various pharmaceutical formulations.

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Nil

AUTHORS CONTRIBUTIONS

Ramankit Jaiswal performed experiments, analyzed data and drafted a manuscript. Dr. Rita Wadetwar critically reviewed the data and revised the manuscript.

CONFLICTS OF INTERESTS

The authors report no conflicts of interest

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