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**Original Research Article**

# Development and validation of spectrophotometric and spectrofluorimetric methods for determination of cilnidipine

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## Abstract

***Purpose:*** *To develop simple and reliable quantitative methods for the determination of cilnidipine (CLD) in pharmaceutical tablets.*
***Methods:*** *Two simple and sensitive methods (spectrophotometric and spectrofluorimetric) were developed for the determination of cilnidipine (CLD) in pure form and in a pharmaceutical preparation. Spectrophotometric method (A) is based on oxidation of CLD with a known excess amount of N-bromosuccinamide (NBS) in acidic medium, followed by addition of methyl orange indicator and absorbance measurement at 510 nm. The spectrofluorimetric method (B) is based on oxidation of CLD to cerium (IV), followed by measurement of fluorescence emission of Ce (III) at 350 nm. Factors that affect the performance of the two methods were studied and optimized.*
***Results:*** *The spectrophotometric and spectrofluorimetric procedures were successfully used for measuring CLD levels in pharmaceutical dosage form, in the ranges of 2.0 - 25.0 and 0.25 - 11.2 µg/mL, at detection limits of 1.05 and 0.13 µg/mL, respectively. There were no significant differences between the proposed methods and a standard reference method ($p < 0.05$).*
***Conclusion:*** *The developed methods provide simple and reliable procedures for quantitative measurement of CLD in bulk and tablet forms.*

***Keywords:*** *Cilnidipine, Oxidation, Spectrophotometric, Spectrofluorimetric, Drug formulation*

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## INTRODUCTION

Cilnidipine (CLD) (Figure 1) is a unique 1,4-dihydropyridine derivative and $Ca^{2+}$ channel blocking agent with potent inhibitory effects on L-type and N-type voltage-dependent calcium channels [1]. Cilnidipine (CLD) is a fourth-generation 1,4-dihydropyridine derivative used in the treatment of hypertension. It depresses sympathetic nervous system activity and reduces the associated adverse effects, with good therapeutic outcome. It has some advantages over old generation treatments [2].

There are limited analytical procedures for quantification of CLD in its pure and tablet forms. These methods are UV-VIS spectrophotometry [3-6], HPLC [7-10], and electrochemistry [11].

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Spectrophotometric methods have many advantages over other methods, in terms of simplicity, fair sensitivity and relative cheapness. This research was designed to evolve easy and selective procedures based on spectrophotometry and spectrofluorimetry for the determination of CLD in drug preparations without laborious procedures such as extraction and derivatization steps.

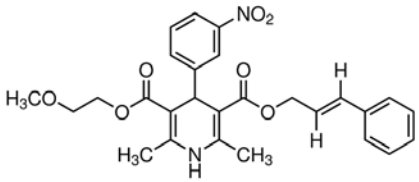

**Figure 1:** Cilnidipine structure

To the knowledge of the authors, there are no reported spectrofluorimetric methods for the determination of CLD in pure and pharmaceutical preparations. The first method was an indirect spectrophotometric procedure in which CLD was oxidized with excess amount of N-bromosuccinamide (NBS) in acidic medium. Then, methyl orange indicator was used to determine the amount of unreacted NBS by measuring the absorbance of residual dye at 510 nm, which is directly proportional to the original amount of the drug. In the spectrofluorimetric method, the drug was oxidized with cerium (IV) in acidic medium, and the resultant Ce (III) was monitored by measuring its emission fluorescence at 350 nm following excitation at 254 nm. Both methods were utilized for construction of standard calibration curves for the determination of the concentration of CLD either in pure or pharmaceutical tablets. The oxidation reaction conditions were optimized and validated for sensitivity, precision, and accuracy. The proposed methods can be readily used without the need for expensive apparatus and complicated steps.

## EXPERIMENTAL

### Materials

Cilnidipine hydrochloride (CLD) in its pure form and pharmaceutical preparation (Cilicar) containing 20 mg of active drug were purchased from J.B. Pharmaceutical and Chemicals Ltd. Analytical-grade reagents and chemicals were used, and were procured from Scharalu.

### Preparation of solutions

*Cilnidipine*: A 100 µg/mL solution of CLD was made in acetonitrile. Using micropipettes, working solutions were prepared via appropriate dilutions in 10-mL calibrated flasks.

*N-Bromosuccinamide*: A 0.20-mg/mL solution of N-bromosuccinamide was freshly prepared in double-distilled water.

*Methyl orange:* A 0.2-mg/mL solution of methyl orange solution was prepared in double-distilled water.

*Cerium ammonium sulfate:* A 20-mg/mL solution of Ce (IV) was made by dissolving 2.0 grams of cerium ammonium sulfate in 500 mL of 0.25 M sulfuric acid.

Hydrochloric acid stock solution (5.0 M) was used.

### Instrumentation and techniques

Absorbance measurements were made using CARY UV-VIS spectrophotometer (CARY model) and 1-cm glass cells. Fluorescence spectra measurements were done on an Agilent Technology, Cary Eclipse, G9800AA model Luminescence spectrometer (Australia) equipped with a xenon arc lamp. The slit width for excitation and emission measurements was set at 5.0 nm, and readings were taken with a 1.0-cm internal diameter quartz cell at 25.0 °C.

#### *General procedures*

***Method A*** (spectrophotometric method): Aliquots of standard CLD solution (100 µg/mL) were accurately transferred to a series of 10-mL volumetric flasks and made up to final concentrations in the range of 1 - 50 µg/mL. Then, 1.0 mL of NBS and 2.0 mL of 5 M HCl solutions were added to each flask, shaken and left to stand at room temperature for 40 min. Thereafter, 1.0 mL of 0.2 mg/mL MO dye solution was added to each flask, and the resulting solution were shaken, followed by absorbance reading at 510 nm.

***Method B*** (Spectrofluorimetric method): Aliquots of standard CLD solution (100 µg/mL) were transferred into a series of 10-mL volumetric flasks and made up to final concentrations ranging from 0.10 to 25 µg/mL. This was followed by the addition of 1.0 mL of Ce (IV) reagent solution to each flask. Then, the volume of liquid in each flask was adjusted to 5.0 mL with water, and each flask was kept in a shaking water bath at 40º C for 1 h. Thereafter, the solutions were cooled to room temperature (25º C), and their fluorescence emission intensities

was read at excitation and emission wavelengths of 254 nm and 350 nm, respectfully.

**Preparation of tablet**

Ten 20-mg CLD tablets were accurately weighed and finely powdered. Then, an amount of powder equivalent to 10 mg CLD was subjected to dissolution in acetonitrile (10 mL), and the solution was filtered. Aliquots of the clear filtrate were analyzed using each of the developed methods.

## RESULTS

For both methods being developed, optimization of reaction conditions including reagent concentrations, diluents, temperature and time was done. Method validation in terms of ISH guidelines including accuracy, precision, sensitivity, selectivity, range and linearity was performed. The reaction conditions for the two developed methods were optimized with respect to reagent concentration, diluent, temperature and reaction duration (time). In addition, method validation was carried out in line with ISH guidelines for accuracy, precision, sensitivity, selectivity, range and linearity.

**Optimized reaction conditions**

Factors that affect oxidation reactions were studied and optimized. These include reagent concentration and duration of reaction.

**Spectrophotometric method**

The effect of different variables that affect oxidation reactions were studied carefully, to obtain maximum absorbance at low drug concentration. These conditions included:

***(i) Effect of NBS volume***

Different volumes of NBS solution (0.20 mg/mL) were used, while keeping the concentration of CLD at 5.0 µg/mL, in addition to maintaining constant, 1 mL of MO (0.20 mg/mL) and 1.0 mL of 5M HCl. As shown in Figure 2, it was found that 1.5 mL of NBS resulted in maximum absorbance.

***(ii) Type and concentration of acid***

Different acids (HCl, $H_2SO_4$ and $H_3PO_4$) were used at same concentrations. It was found that HCl resulted in faster color development than any of the other acids used. The optimum amount of 5 M HCl used was 1.0 mL.

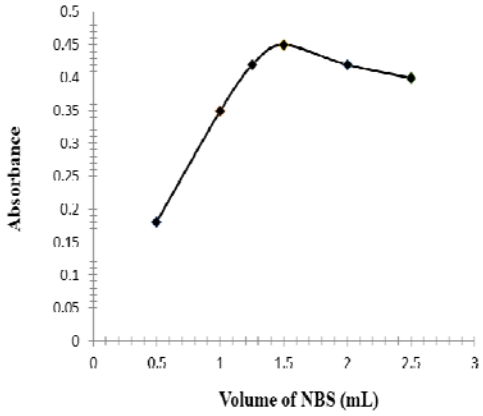


**Figure 2:** Effect of volume of NBS (0.2 mg/mL) added to CLD (5 µg/mL) on absorbance

***(iii) Effect of volume of methyl orange***

The optimum volume of MO was determined by varying its volume from 0.5 to 2.5 mL, while using fixed amount of CLD at 5.0 µg/mL, 1.0 mL of NBS solution and 1mL of 5M HCl.

***(iv) Temperature and time***

The progress of reaction was monitored by measuring absorbance at 510 nm between 5 and 60 min. at 25 ºC. It was discovered that absorbance became stable after about 30 min at 25 ºC. Therefore, 30-min standing time was selected for all measurements. Color was stable for about 180 min.

**Spectrofluorimetric method**

The excitation and emission spectrum shown in Figure 3 was used to select suitable wavelengths for the determination of CLD from the emission wavelength at 354 nm.

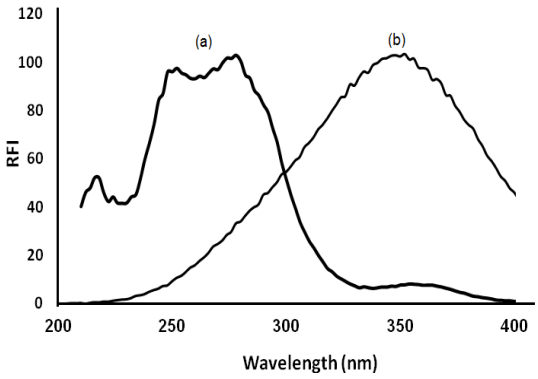


**Figure 3:** Excitation (a) and emission (b) spectra of 1.0 µg/mL CLD after oxidation with Ce (IV) according to calibration curve procedure for method B

### *(i) Effect of Ce (IV) volume*

An optimum volume of 6.3x10$^{-3}$ M cerium (IV) solution was obtained by increasing the volume of Ce (IV) solution used from 0.2 to 2.0 mL, as shown in Figure 4. It is clear that peak fluorescence intensity was reached using about 1.0 mL of the oxidant, with fixed amount of CLD.

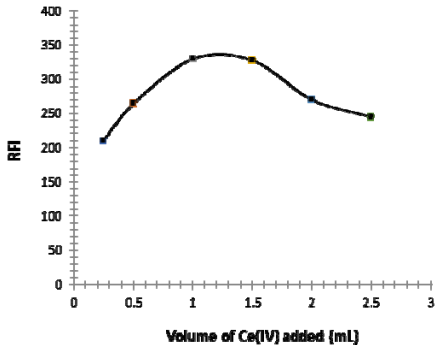


**Figure 4:** Effect of added volume of 6.3X10$^{-3}$ M Ce (IV) to CLD (5.0 µg/mL) solution

### *(ii) Temperature and time*

The reaction was monitored at different temperatures (25, 40 and 60 ºC) at different durations (5 - 40 min), after which the solutions were cooled to 25 before measuring the fluorescence intensities. It was found that heating temperature of 40 ºC after 60 min gave the highest response values and stability.

### *(iii) Diluting solvent*

Different diluting solvents (water, methanol, ethanol, acetonitrile, dioxan and cyclohexane) were used. As shown in Figure 5, acetonitrile provided the maximum fluorescence intensity and the highest stability. It was therefore chosen as the optimum solvent.

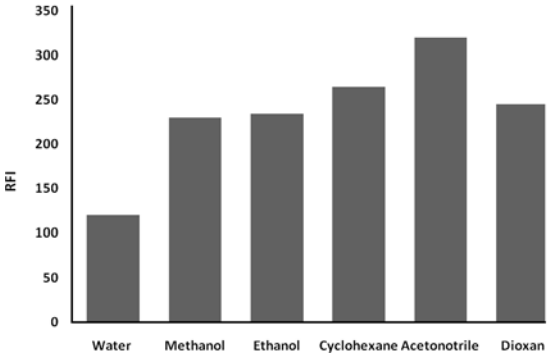


**Figure 5:** Influence of diluting solvents on RFI of CLD (5.0 µg/mL) solution

## Validated methods

### *Linearity, range and sensitivity*

Different CLD concentrations were prepared and applied in both methods. For the spectrophotometric method, a linear plot of CLD concentration against absorbance of MO dye at 510 nm produced a correlation coefficient ($R^2$) of 0.992). For the spectrofluorimetric method, a plot of CLD concentration against relative fluorescence intensities (RFI) was linear within the range of 0.25 - 11.2 µg/mL, with a correlation coefficient ($R^2$) of 0.994). The calibration plots of the two methods are shown in Figure 6.

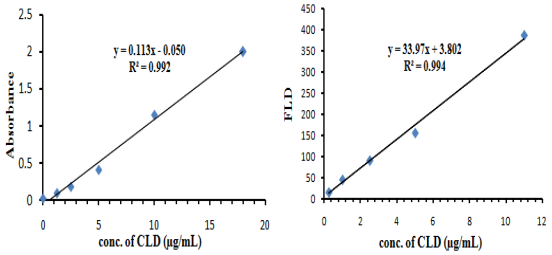


**Figure 6:** Standard curves. Spectrophotometric procedure (left), and spectrofluorimetric procedure (right)

From the data shown in Table 1, it is clear that both methods have excellent correlations and good concentration ranges.

The sensitivities were measured in terms of limit of detection (LOD) and limit of Quantitation (LOQ) which were determined in line with ICH guidelines as in Eqs 1 and 2 [16] (Table 1).

$$LOD = \frac{3.3\,X\sigma}{S} \quad \ldots\ldots\ldots (1)$$

and

$$LOQ = \frac{10X\sigma}{S} \quad \ldots\ldots\ldots (2)$$

where $\sigma$ represents SD of y-intercept of regression lines, and $S$ is gradient of standard curve.

### *Accuracy and precision*

Determinations were done with 3 concentrations of CLD to measure inter-day precision of five replicates for six days using the developed procedures. The RSDs were between 0.87 -1.4 %, with high % recovery for inter-day analysis, indicating high precision and accuracy of the procedures. These results are presented in Table 2.

**Table 1:** Data showing analytical performance of the methods

| Parameter | Method A | Method B |
|---|---|---|
| Linear range (µg/mL) | 2.0-25.0 | 0.25-11.2 |
| Wavelength (nm) | 510 | λ(excitation) 250 |
| Correlation coefficient | 0.992 | 0.994 |
| Slope (b) | 0.113 | 39. 97 |
| Intercept (a) | 0.05 | 3.9 |
| SD of the intercept (SDa) | 0.021 | 0.3 |
| LOD[a] (µg/mL) | 1.05 | 0.13 |
| LOQ[b] (µg/mL) | 3.17 | 0.41 |

[a]Limit of detection, [b]Limit of quantitation, LOD = 3.3SDa/b; LOQ = 10SDa/b, where SD[b] refers to the intercept, and b is gradient

### Selectivity studies

The selectivity of each developed method was measured through analyses of placebo and synthetic admixture. The blank was prepared from acacia, starch, OH-cellulose and Na alginate which were thoroughly mixed and made into tablets which were then analyzed. Signals obtained from the two methods with respect to the blank were nearly equivalent to those of the reagent blank, indicating that there were no appreciable interferences due to excipients.

**Table 2:** Recovery data for inter-day precision and accuracy

| Method | CLD taken (µg/mL) | CLD found (µg/mL) | RSD (%) | Recovery (%) |
|---|---|---|---|---|
| A | 4.0 | 3.9 | 1.35 | 97.5 |
| | 8.0 | 7.6 | 1.24 | 95.0 |
| | 12.0 | 12.1 | 1.65 | 100.8 |
| B | 1.0 | 1.0 | 0.87 | 100.5 |
| | 3.0 | 2.9 | 1.12 | 96.7 |
| | 8.0 | 7.9 | 1.45 | 98.8 |

### Application to drug formulation

The proposed methods were applied successfully to only one marketed product (Cilicar tablet) due to the difficulties of obtaining other brands of CLD because the drug is not approved in many countries. The results, which showed high accuracy and precision using three different concentration levels of CLD, are summarized in Table 3.

**Table 3:** Precision and accuracy of each method

| Method | Conc. of CLD analyzed (µg/mL) | Conc. of CLD found[a] (µg/mL) | RSD (%)[b] | Recovery (%) |
|---|---|---|---|---|
| ***A*** | | | | |
| Level 1 | 5.1 | 5.2 | 2.6 | 98.1 |
| Level 2 | 7.4 | 7.2 | 3.98 | 97.3 |
| Level 3 | 11.3 | 11.2 | 1.2 | 99.1 |
| ***B*** | | | | |
| Level 1 | 1.4 | 1.5 | 2.4 | 94.6 |
| Level 2 | 3.7 | 3.5 | 3.3 | 94.6 |
| Level 3 | 8.2 | 8.1 | 1.5 | 98.7 |

[a] Values are mean (n = 5)

### Statistical evaluation of the two methods

Statistical evaluation of was carried out by applying the two methods and the reference method [17] for analysis of 20 mg tablets, with five determinations per method. The results obtained from the proposed methods were compared to those obtained from a standard reference method with respect to accuracy (*t*-test) and precision (*F*-test).

The calculated values of student *t*-test and the variance *F*-test (Table 4) were less than the critical values, indicating that there was no significant difference in accuracy and precision between any of the proposed methods and the reference method.

## DISCUSSION

The first developed method (spectrophotometric) was based on selective oxidation of CLD using NBS and methyl orange as chromogenic agents. Studies have shown that NBS is used as a strong bromination agent for alkenes and other organic compounds [14,15].

**Table 4:** Statistical comparison of results obtained in analysis of 20 mg tablet using the proposed methods, and a reference method

| Parameter | Spectrophotometric method* | Spectrofluorimetric method* | Standard reference method |
|---|---|---|---|
| Recovery (%) | 96 ± 1.7 | 97 ± 1.0 | 99 .1 ± 1.1 |
| *t* | 2.6 | 2.4 | |
| *F* | 1.4 | 1.2 | |

*Mean value of five determinations as percent recovery ± SD. Tabulated values: t = 2.77, F = 6.39 at 95 % confidences level and four degrees of freedom

In this study, NBS was used as a bromination agent for the studied drug, with the bromination taking place on the double bond attached to the phenyl group of CLD. The principle of the proposed method involves indirect spectrophotometric analytical method based on the oxidation of CLD with a slight excess of NBS in acidic medium. At the end of the oxidation reaction, a fixed amount of methyl orange dye is added, which is easily bleached by the residual NBS oxidant [16]. As a result, the absorbance of MO dye is directly proportional to the concentration of CLD in the original solution (Figure 7).

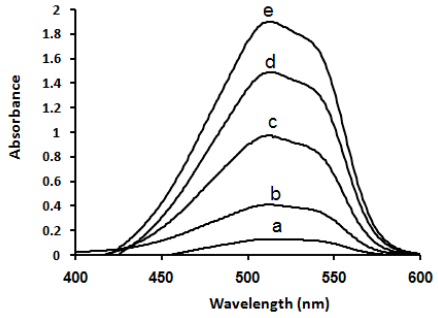


**Figure 7:** Absorption spectra of CLD. a: 2.5, b: 5.0, c: 7.5, d: 12, and e: 18 µg/mL, based on calibration curve procedure for method A

An alternative method was developed based on oxidation of CLD with Ce (IV) in sulfuric acid, producing highly native fluorescent Ce (III) which exhibits a maximum emission light at 350 nm after excitation at 245 nm. Similar spectrofluorimetric methods have been widely applied in pharmaceutical analysis due to their high sensitivity, selectivity and stability [17]. As shown in Figure 8, the emission fluorescence intensities were proportional to the concentration of CLD.

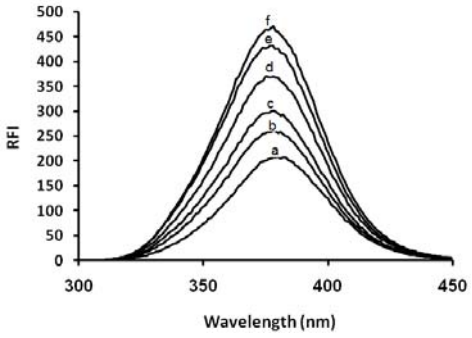


**Figure 8:** Correlation of the emission spectra of different concentrations of CLD within the linear range of the calibration. a: 0.25, b: 1.25, c: 5.0, d: 7.0, e: 10.0, and f: 11.2 µg/mL, for method B

## CONCLUSION

New, simple and sensitive spectrophotometric and spectrofluorimetric methods based on the oxidation of cilnidipine have been developed. These methods used N-bromosuccinimide and Cerium (IV) as oxidants. Both methods have been validated and successfully applied for the determination of CLD as a pure powder and as a single pharmaceutical preparation. Therefore, the new procedures are adequate for routine determination of cilnidipine in bulk and as a drug tablet.

## DECLARATIONS

### *Acknowledgement*

The authors are thankful to Ruba Zaloum, Amina Nyazi, Maysoun Keswani and Alae Abu Jabal for their assistance in carrying out some measurements.

### *Conflict of interest*

No conflict of interest is associated with this work.

### *Contribution of authors*

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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