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Original Article

# A SENSITIVE AND A SIMPLE RP-HPLC METHOD DEVELOPMENT AND VERIFICATION FOR THE QUANTITATIVE ESTIMATION OF CHOLECALCIFEROLIN TABLETS

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**ABSTRACT**

**Objective:** To develop a sensitive, simple, accurate, precise and linear Reverse Phase High-Performance Liquid Chromatographic (RP-HPLC) method and verify for the quantitative estimation (Assay) of Cholecalciferol in tablets.

**Methods:** The optimized method uses a reverse phase column, Waters X-Bridge C8 (150 X 4.6 mm; 3.5µ), a mobile phase of Methanol: Acetonitrile: HPLC grade water/Milli-Q water in the proportion of 60:30:10 v/v/v, flow rate of 0.8 ml/min, injection volume of 100 µl, and detection wavelength of 265 nm using a UV/PDA detector.

**Results:** The developed method gave Cholecalciferol eluting at about 9 min. Cholecalciferol exhibited linearity in the range 0.058-0.466 µg/ml. The precision is exemplified by a relative standard deviation of 1.40%. Percentage of individual recovery was found to be in the range of 97.0 and 103.0 during accuracy studies.

**Conclusion:** A sensitive, simple, accurate, precise and linear RP-HPLC method was developed and verified for the quantitative estimation (Assay) of Cholecalciferol in tablets and hence this method can be explored for the analysis of Cholecalciferol in tablets/various dosage forms in various pharmaceutical industries.

**Keywords:** RP-HPLC, Cholecalciferol, Analytical Method development, Tablets


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## INTRODUCTION

Cholecalciferol, also known as Vitamin $D_3$, is widely prescribed for the treatment osteomalacia and osteoporosis [1]. It also plays a key role in calcium and phosphorus homeostasis and skeletal mineralization [2]. IUPAC name of Cholecalciferol is (3β, 5Z, 7E)-9,10-secocholesta-5,7,10(19)-trien-3-ol, whose molecular weight is 384.64 g/mol and its molecular formula is C27H44O. The chemical structure of Cholecalciferol is given in fig. 1.

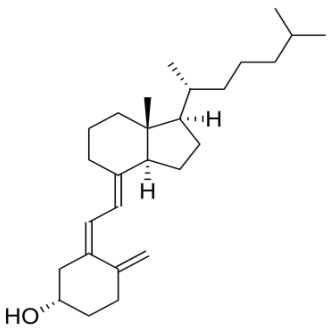


**Fig. 1: Structure of cholecalciferol**

Various analytical methods are reported in literature by UV or HPLC, either in single or in combination with other drugs in various dosage forms [3-8]. In this research work, we focussed on using a simple isocratic elution technique against gradient mode, mobile phase and diluent in a better combination and a simple sample extraction procedure for acheiving reproducible assay results. In this research article, we present purely only the analytical work on developing and verifying a simple and sensitive RP-HPLC method for the quantitative estimation (Assay) of Cholecalciferol in tablets, whose label claim is 6.25µg.

## MATERIALS AND METHODS

### Chemicals and reagents

Analytically pure sample of Cholecalciferol with purities greater than 95% was obtained from Sigma Aldrich and tablet formulation was prepared in our Formulation R and D laboratory, with a labelled amount 6.25 µg of Cholecalciferol. Acetonitrile and Methanol (HPLC grade of Standard make) and water (MilliQ) were used for the analysis.

### Instrument

HPLC analysis was performed on Agilent and Waters makes HPLC' shaving UV detector capable of setting a detection wavelength of 265 nm. Are verse phase C8 column, Waters X-Bridge(150 X4.6 mm; 3.5µ), part number 186003055 was used. The HPLC system was controlled with "EMPOWER" software. An electronic analytical weighing balance (0.001 mg sensitivity, Sartorius make, ME5 model), and a sonicator (H washin Make, Power sonic 420 model) were used for the analysis.

### Selection of wavelength

Suitable wavelength for the HPLC analysis for Cholecalciferol was determined by recording UV spectrum in the range of 200-400 nm. Suitable wavelength selected was 265 nm, considering the maximum absorbance at this wavelength (fig. 2).

### Chromatographic conditions

The developed method uses a reverse phase C8 column of Waters make, X-Bridge C8 (150 X 4.6 mm; 3.5µ) bearing a Part number of 186003055, mobile phase of Methanol: Acetonitrile: HPLC grade water/Milli-Q water in the proportion of 60:30:10 v/v/v respectively setting a flow rate of 0.8 ml/min, injection volume as 100µl, detection wavelength as 265 nm, setting column temperature and sample compartment temperature of 30°C and 5°C respectively and run time as 15 min.

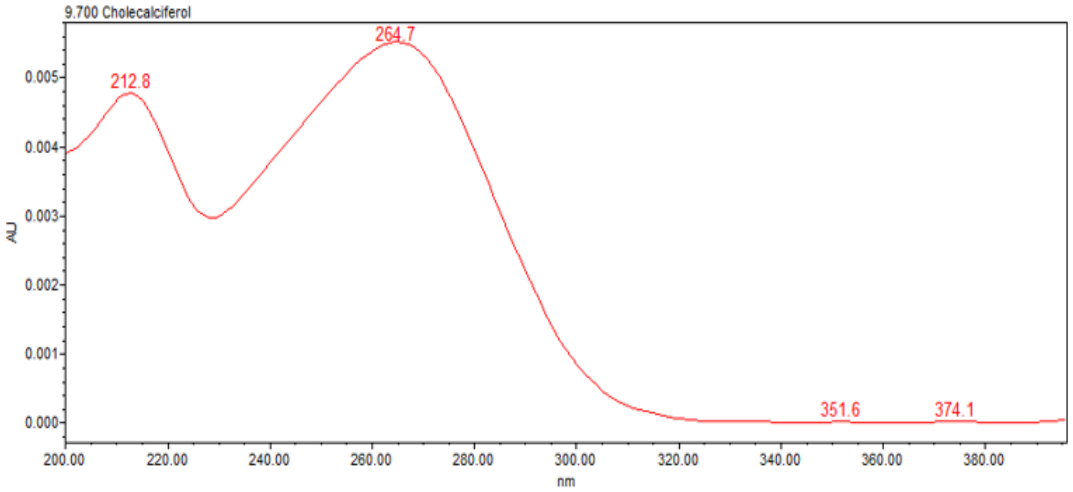


**Fig. 2: UV spectrum of cholecalciferol**

**Mobile phase preparation**

The mobile phase was prepared by mixing Methanol: Acetonitrile: HPLC grade water/Milli-Q water in the proportion of 60:30:10 v/v/v respectively, followed by degassing in a sonicator for 10 min.

**Diluent preparation**

The diluent solution was prepared by mixing Methanol and HPLC grade water/MilliQ in the ratio of 90:10 v/v, respectively, followed by degassing in a sonicator for 10 min.

**Preparation of stock and working standard solution**

Weighed accurately about 50 mg of Cholecalciferol standard into a clean and dried amber coloured200 ml volumetric flask containing 10 ml of Methanol and then sonicated for 10 min to dissolve. Later the solution was made up to the mark using the diluent. This is considered as stock standard solution of a concentration of about 250µg/ml. From the stock solution, 5.0 ml was pipetted out and diluted to 250 ml using the diluent to get a concentration of about 5µg/ml. From this standard solution, 5.0 ml was pipetted out and diluted to 100 ml using the diluent to get a working standard concentration of about 0.25µg/ml.

**Preparation of system suitability solution**

Transfer about 30 ml of the above working standard solution into a clean and dried 100 ml amber-coloured volumetric flask and then heat the solution at 60 °C for 1 and half hour.

**Preparation of stock and working sample solution**

Not less than 20 doses of tablets were weighed, taken in a mortar and pestle and crushed to get a powder carefully to avoid any losses. Immediately transferred the complete powder into a clean and dried 500 ml amber-colored volumetric flask. Added about 350 ml of diluent and then swirled the flask to ensure complete wetting of the tablets and later sonicated for about 5 min with intermittent shaking. Later, shaken the volumetric flask on a rotary shaker at 200 rpm for 15 min and then diluted to volume with diluent and mixed well. Centrifuged a portion of the sample solution at 3000 rpm for 10 min and taken an aliquot of the supernatant for analysis.

**RESULTS AND DISCUSSION**

**Method development**

A Reverse phase HPLC method chromatopgraphic conditions were developed keeping in mind the system suitability parameters *i.e.,* Tailing factor (T), % RSD from six replicate injections of standard and Blank interference along with simple extraction procedure, reproducible mobile phase and diluent, sensitivity and runtime. The optimized method developed resulted in the elution of Cholecalciferol at about 9.0 min. Fig. 3-6 represent specimen chromatograms of blank, placebo, standard and sample solutions. The total run time is 15 min. System suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system, whose details are summarized in tables 1.

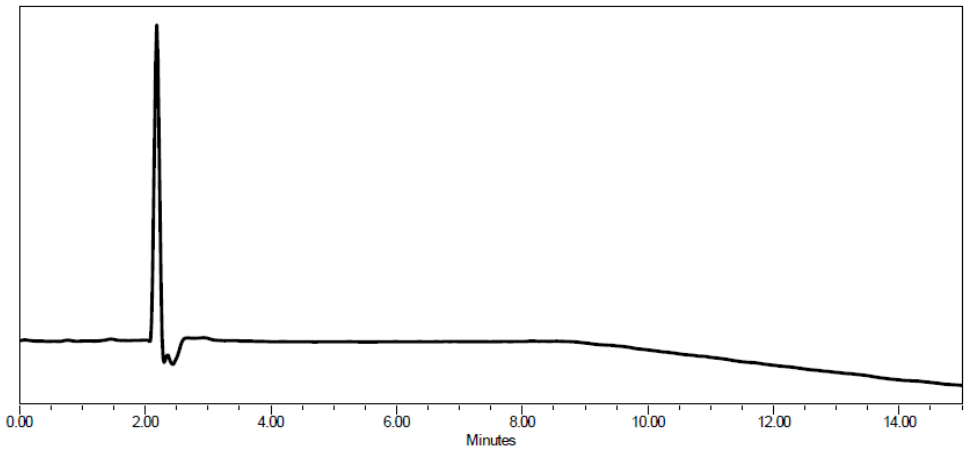


**Fig. 3: Typical chromatogram of blank solution**

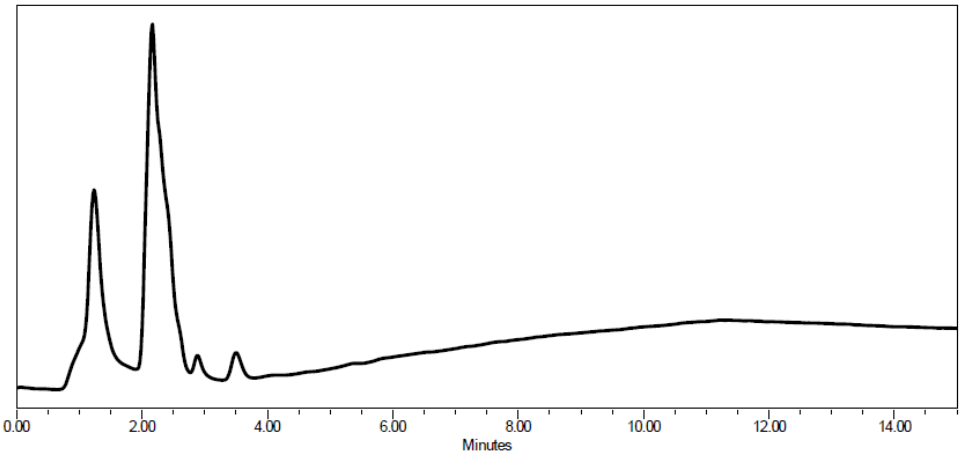


**Fig. 4: Typical chromatogram of the Placebo solution**

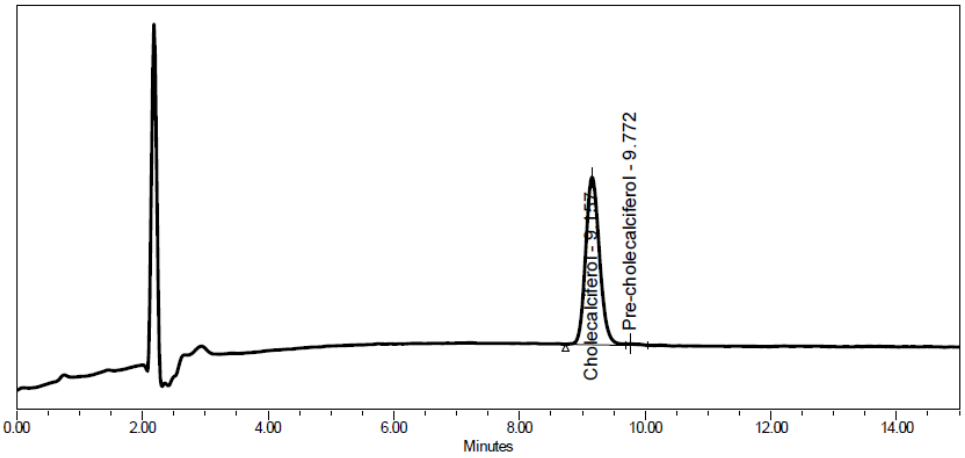


**Fig. 5: Typical chromatogram of the standard solution**

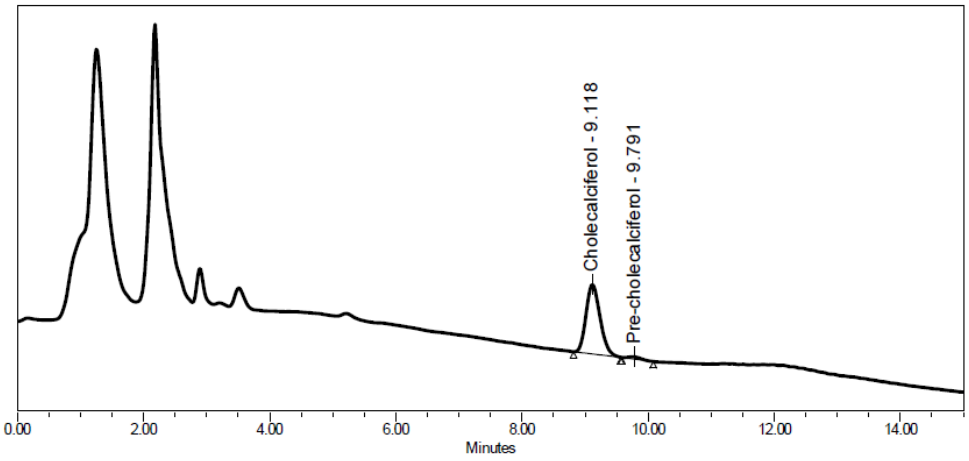


**Fig. 6: Typical chromatogram of the sample solution**

**Table 1: System suitability**

| Parameters | Acceptance criteria | Results |
|---|---|---|
| Blank interference | No peak shall be detected in the blank at the retention time of Pre-Cholecalciferol and Cholecalciferol peaks. | Nil |
| Tailing factor (T) | The Tailing factor for Cholecalciferol peak shall not be more than 2.0 from first injection of Standard solution. | 1.16 |
| % RSD (Relative standard deviation) | The percent relative standard deviation of the average peak area response from 6 injections of Cholecalciferol shall be NMT 3.0%. | 1.04 |

In order to test the applicability of the developed method to a formulation, Cholecalciferol tablets were chromatographed at a concentration of about 0.25µg/ml and it is shown in fig. 5. The sample peak was identified by comparing the retention time with the standard drug fig. 4. To ensure the method developed meets the requirements of verification parameters, verification was employed whose details are mentioned in the below section.

**Calculations**

% Assay of Cholecalciferol in tablets/tablets blend was calculated by using the below formula.

$$\text{Tablets Assay (\% Percent Label claim)} = \frac{A_T \times Ws \times 5 \times 5x \times 500 \times P \times 100}{As \times 200 \times 250 \times 100 \times N \times 100 \times L}$$

Where,

(i) Peak area of Cholecalciferol = Peak area of Cholecalciferol+Peak area of pre-

Cholecalciferol in the standard as well as sample chromatogram.

(ii) Relative retention time (RRT) for Pre-Cholecalciferol is about 1.08 with respect to Cholecalciferol.

$A_T$ is the peak area of Cholecalciferol in the sample solution.

$A_S$ is the average peak area of Cholecalciferol from 6 replicate injections of Standard solution A injected under system suitability.

$W_S$ is the weight of Cholecalciferol standard taken, in mg for the preparation of Standard solution

N is the number of doses 20 used during the sample solution preparation

P is the potency of Cholecalciferol standard

L is the label claim of Cholecalciferol in mg, 0.00625 mg

**Method verification**

Assay method verification of the analytical method was done for the parameters like system suitability, specificity, linearity, accuracy and precision.

**Specificity (Placebo interference)**

To establish non-interference, blank and placebo solutions were prepared and injected into HPLC along with standard and sample solutions (Refer fig. 3-6).

**Acceptance criteria**

No peak shall be detected in the blank and placebo chromatograms at the retention time of Cholecalciferol and Pre-cholecalciferol peaks.

**Results and conclusions**

No peak was observed in the blank and placebo chromatograms at the retention time of Cholecalciferol peak when compared to the standard and sample chromatograms. Accordingly, it is concluded that, the method developed is said to be specific.

**Method precision**

Label claim is 6.25 µg of Cholecalciferol per tablet.

Method precision was determined by performing % Assay or % Label claim of the tablet blend, which is prepared by spiking API (at 100% level) to the placebo equivalent to the tablet weight and extracting as per the sample preparation procedure in triplicate. The percentage assay of each replicate, average of three replicates and % RSD were calculated.

**Acceptance criteria**

1. The % Assay shall be between 97.0 and 103.0.

2. The relative standard deviation of three replicate assay results shall be not more than 2.0%.

Results and conclusions:

The results were found to be within the acceptance criteria and summarized in table 2. Hence, the method is precise.

**Table 2: Method precision results**

| Sample no. (n) | % Assay OR % label claim |
|---|---|
| 1 | 97.81 |
| 2 | 100.33 |
| 3 | 100.07 |
| Average | 99.40 |
| %RSD | 1.40 |

**Accuracy**

Accuracy was determined by means of recovery experiments, by the determination of % mean recovery of the sample at two different levels (100 and 150%). At each level, three determinations were performed. Individual and percent mean recovery was calculated as shown in table 3**.**

**Acceptance criteria**

The accepted limits of recovery are 97.0%-103.0%.

**Results and conclusions**

All observed data are within the required range which indicates good recovery values and hence the accuracy of the method developed.

**Linearity**

Standard solutions of Cholecalciferol at different concentrations level (25%, 50%, 80%, 100%, 125%, 150% and 200%) were prepared. Calibration curve was constructed by plotting the concentration level of the drug versus the corresponding peak area.

**Acceptance criteria**

1. The correlation coefficient shall be not less than 0.999.

2. Bias-Y-Intercept shall not be more than±2% of the response at 100% level.

**Results and conclusions**

The results show an excellent correlation between peak area and concentration level of the drug within the concentration range (0.058-0.466 µg/ml) for the drug and the results; linearity graph are given in table 4**.** The correlation coefficient of Cholecalciferol is 0.99962. The results reveal that method is linear for the quantification Cholecalciferol in the proposed range.

**Table 3: Results of accuracy studies**

| % Level | % Individual recovery | % Mean recovery |
|---|---|---|
| 100 | 97.81 | 99.40 |
| | 100.33 | |
| | 100.07 | |
| 150 | 100.66 | 101.16 |
| | 101.12 | |
| | 101.69 | |

**Table 4: Linearity data and linearity graph**

| % Level | Concentration (µg/ml) | Peak area |
|---|---|---|
| 25 | 0.058 | 20146 |
| 50 | 0.116 | 41320 |
| 80 | 0.186 | 64988 |
| 100 | 0.233 | 82715 |
| 125 | 0.291 | 101210 |
| 150 | 0.349 | 121595 |
| 200 | 0.466 | 166913 |
| Regression/Correlation coefficient | | 0.99962 |
| Bias | | -1.18 |
| Regression equation | | y= 356318x-978.33 |

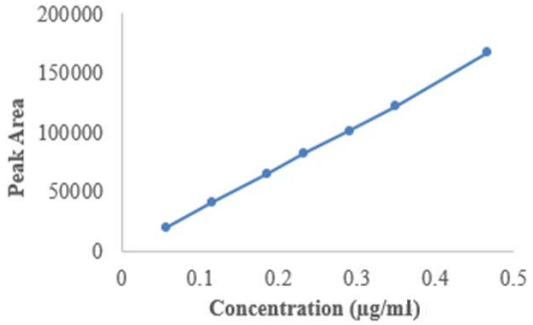


**Fig. 7: Linearity graph**

## CONCLUSION

A reverse phase HPLC isocratic method developed has been verified in terms of specificity, accuracy, precision and linearity for the quantitative estimation (Assay) of Cholecalciferolin tablets. The precision is exemplified by a relative standard deviation of 1.40%. A good linear relationship was observed for the drug between concentration ranges of 0.058 and 0.466µg/ml. Accuracy studies revealed that mean recoveries were between 97.0 and 103.0%, an indicative of accurate method. Accordingly, it can be concluded that the developed reverse phase isocratic HPLC method is specific, accurate, precise and linear and therefore, the method can be explored for the routine analysis of Cholecalciferolin tablets.

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Nil

## AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

## CONFLICTS OF INTERESTS

Declared none

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