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Chemistry

DEVELOPMENT AND VALIDATION OF AN ACCURATE, SENSITIVE AND REPRODUCIBLE RP-HPLC METHOD FOR THE ESTIMATION OF VITAMIN D3 IN PHARMACEUTICAL DRUGS AND CONFIRMATION OF THE RESULTS BY UV/VIS-SPECTROPHOTOMETRY

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ABSTRACT A rapid, simple, sensitive isocratic reproducible reversed phase HPLC technique has been developed for the quantitative assay evaluation of Vitamin D3 in various pharmaceutical drugs. The Chromatographic separation of Cholecalciferol samples were performed on a C18-column by isocratic elution at the column at 30 °C. The Mobile Phase used was methanol. The flow rate was 1ml/min. The maximum area was resolved at 250.0 nm by using UV/Vis photometric detector. The calibration curve was found to be in the range 10 ppm to 50 ppm with regression coefficient 0.9804. The % recovery for D3 MUST tablet was 105% respectively by HPLC technique. The above results have also been verified by UV/Vis- spectrophotometry technique within limits of ±10% error. The method was validated to determine the accuracy and precision by performing recovery studies. The proposed method can be successfully used to quantify the amount of Vitamin D3 in formulated drugs.

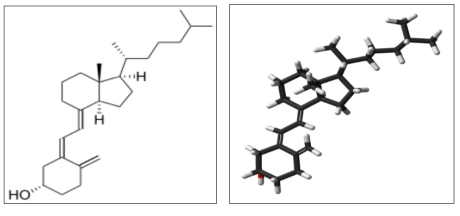
KEYWORDS : Cholecalciferol, RP- HPLC, Double Beam Spectrophotometry, Method Validation.

INTRODUCTION

Cholecalciferol is a "*vitamer*" of **vitamin D_3** is one of the five forms of vitamin D.[1][2] Cholecalciferol is white, needle-like crystals. Practically insoluble in water, freely soluble in Abs. Ethanol, Methanol and some other organic solvents and slightly soluble in vegetable oils. It is a secosteroid, that is, a steroid molecule with one ring open. This medication is an analogue of vitamin D, prescribed for hypocalcaemia, hypoparathyroidism, hypophosphataemia, renal osteodystrophy, and osteomalacia. Since it does not require any activation process by kidneys like other vitamin D supplements, more useful for people who have kidney problems. This and all forms of vitamin D are misnamed: vitamins by definition are essential organic compounds which cannot be synthesized by the body and must be ingested; cholecalciferol is synthesized by the body, and functions as a prehormone. Cholecalciferol is inactive: it is converted to its active form by two hydroxylations: the first in the liver, the second in the kidney, to form calcitriol, whose action is mediated by the vitamin D receptor, a nuclear receptor which regulates the synthesis of hundreds of enzymes and is present in virtually every cell in the body.

IUPAC name of Cholecalciferol is (3β, 5Z, 7E)-9, 10-secocholesta-5, 7, 10(19)-trien-3-ol and other names vitamin D_3, activated 7-dehydrocholesterol. Commercially Cholecalciferol in various formulations is available such as tablets, capsules, injections and syrups etc.

Quantitative assay is an important method for checking the commercially formulated product.



Vitamin D3
Cholecalciferol (Molecular Formula, $C_{27}H_{44}O$)
(Molecular weight, 384.64 g/mol)

The main aim of this study is to develop and validate a new quantitative assay method for checking quality and quantity of Cholecalciferol from formulated drug products by HPLC and UV/Vis-Spectrophotometry techniques.

MATERIALS AND METHODS

Materials

Pharmaceutical products (D_3 MUST tablet) used for this project was obtained from local market of areas in Pune. The 98% pure Cholecalciferol drug was obtained from Research-Lab fine Chemical Industries, Mumbai. HPLC grade methanol, water and 0.45µm nylon filter membranes were purchased from Merck India Ltd., Mumbai.

Instrumentation

The analysis was carried out on a HPLC system (Shimadzu-LC 20AD) equipped LC system with UV/Vis detector (SPD-20AD) at 250 nm and UV/Vis spectrophotometer (HITACHI µ2000 Double Beam Spectrophotometer) with Photodiodes detectors were used for lmax detection. C_{18} column ($ST_5C_{18}G_{120}$) was used for separation. The flow rate of elution was 1.0 ml/min at 30 °C. An ultrasonic sonicator was used for the sonication of mobile phase, standard solution and sample solution. The injection volume was 20 µL.

Chromatographic Conditions

C_{18} column ($ST_5C_{18}G_{120}$) was used for separation. The flow rate of elution was 1.0 ml/min at 30 °C. An ultrasonic sonicator was used for the sonication of mobile phase, standard solution and sample solution. The injection volume was 20 µL.

Preparation of Mobile Phase

A methanol was used as mobile phase. The mobile phase was filtered through 0.45µm nylon membrane and degasses by ultrasonic sonication.

Preparation of Standard Stock solution-(I)

A 1mg/ml 100m stock solution was prepared by dissolving accurately weighed 20.4mg Cholecalciferol in little amount of methanol(mobile phase) and make it to 100 ml volume in a volumetric flask. It covers the 200 ppm concentration range.

Preparation of stock solution-(II)

From the above solution (stock solution-(I)) 25 ml volume has taken and diluted it to 50 ml volumetric flask. It covers the concentration 100 ppm.

Preparation of Calibration Curve

A calibration curve was constructed by injecting the different concentration of serial dilutions (standard drug) in range 12.5, 25, 50, 75 and 100ppm in trice replication.

The calibration curve was obtained by plotting the average peak areas against these different known concentrations.

Preparation of sample solution (I)

Two tablets of pharmaceutical drug (D_3 MUST 60K, label claim: 1500 µg (60,000 IU) Cholecalciferol per tablet), was weighed and crushed. The crushed tablet was mixed well and then equivalent amount of 1.212 mg was transferred into small conical flask and extract with 30

ml mobile phase. The extract covers the 100 ppm concentration range.

Preparation of sample solution (II)

From the above sample solution (100 ppm, working concentration range) 1 mL was taken out and make a volume to 10ml in a volumetric flask by methanol (mobile phase).

Detection of wavelength

In the present study individual drug solutions of 100 ppm was prepared in different solvent mixtures of HPLC Grade organic and inorganic solvents at methanol as mobile phase. This drug solution was then scanned in the UV region of 200-400 nm and the spectrum was recorded to get λ_{max} of analyte in mobile phase shown in table 1. Wavelength selected for the estimation of this combination was 250 nm.

RESULT AND DISCUSSION

The mobile phase was selected under reversed phase partition chromatographic condition. The mobile phase developed was studied in order to achieve suitable system stability. The different rations of (0:100, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10 and 100:0) mobile phase's compositions were tested at ambient temperature 25ºC. The mobile phase methanol/water on the ratio of 100:0 (v/v) i.e. 100% Methanol was given suitable time and better resolution without any interference.

Method Validation

According to International Conference Harmonization (ICH) guide lines [2-4] there were several parameters of method validation studied such as: - accuracy, precision, linearity, system suitability test, reproducibility.

System Suitability Test

System suitability was checked to ensure that, the system was working correctly. The system suitability parameters peak area, retention time, resolution factor and flow rate were checked according to international conference harmonization (ICH) guide lines [2-4]. This test was performed during development of the method. The test was performed by injecting the standard mixture in n=2 replicates.

Accuracy/recovery and Precision

"*Accuracy is the degree of agreement between the measured value and the true value.*"

Accuracy/recovery was calculated for the three runs of each solution. Performance at validated method is confirmed by the performing inter-day recovery study at different concentration levels 10, 20, 30, 40 and 50 ppm. The five different concentration diluted from the sock solution were added to an extract with known content of Cholecalciferol and the percentage recovery of the respected constituents was calculated by

$$R\% = \frac{\text{(Peak area of the drug in the sample x 100)}}{\text{Peak area of the drug in the standard}}$$

(Where R% is the percentage recovery)

Precision

Precision is related to reproducibility of the measurement. The results of accuracy/recovery and precision experiments are recorded in table and table. The data indicate an adequate percentage of accuracy/ recovery for the HPLC method for the quantification of Cholecalciferol in the pharmaceutical preparations.

Range

Range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The concentration range 10 mg/L to 50mg/L range has been used in the present study.

System suitability parameters

System suitability parameters can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The system suitability parameters like theoretical plates (N), resolution (R), and tailing factor (T) were calculated and compared with the standard values to ascertain whether the proposed RP-HPLC method for the estimation of vitamin D_3 in pharmaceutical formulations were validated or not. System suitability is usually developed after method development and validation has been completed. In both the methods standard deviation and recovery study were found within the limits of ± 10% error. Hence, both methods are suitable for the quantitative estimation.

Conclusions

The system suitability study indicates that the applied method was suitable for the analysis. Wavelength is the primary need for the chromatographic analysis. To select the wavelength for cholecalciferol were investigated in order to determine a suitable wavelength for the assay evaluation. The suitable wavelength was found to be 264 nm. The selection of mobile phase is an important secondary basic need for the chromatographic analysis. The mobile phase was selected was under reversed phase- partition chromatographic conditions. The recovery results were showed good accuracy with ±10 coefficient variation percentage in both methods. Hence, the HPLC and UV-Spectrophotometer results were significant.

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TABLE 1: Average determination of peak area, retention time and accuracy for standard solutions and sample of D_3 MUST 60K tablet (Marketed by MANKIND PHARMA Ltd.) by using HPLC technique.

Concentration of Cholecalciferol/ ppm	Retention Time/ min	Peak Area/cm²	Recovery (%)	X ± SD	RSD (%)
10	3.656	77637	101.74% 101.47%	101.605 ± 0.191	0.188%
20	3.662	129735	60.92% 60.69%	60.805 ± 0.163	0.268%
30	3.641	227525	34.71% 34.63%	34.67 ± 0.056	0.161%
40	3.675	253601.5	31.14% 31.07%	31.105 ± 0.049	0.157%
50	3.682	315515.5	25.02% 24.98%	25.0 ± 0.0282	0.113%
D_3 MUST 60K tablet	**3.419**	**78885**			

TABLE 2: Summary of linearity (n = 2) correction range, regression equation and regression coefficient data for D_3 MUST 60K Tablets.

Parameters	D_3 MUST Tablets	
	HPLC method	Double Beam Spectrophotometer
Correction range (ppm)	10 to 50	10 to 50
Regression Equation	y = 6574.465x + 0	y = 0.02368x + 0
Regression coefficient (R^2)	0.9804	0.9993

TABLE 3: Assay results for the determination of Cholecalciferol in for D_3 MUST 60K Tablets.

		By HPLC		By Double Beam Spectrophotometer	
Preparation	Label Content (mg)	Actual content ± SD(mg)	Recovery (%)	Actual content ± SD (mg)	Recovery (%)
D3 MUST 60K Tablets	1.5	1.575 ± 0.212	105%	1.554 ± 0.198	103.6%

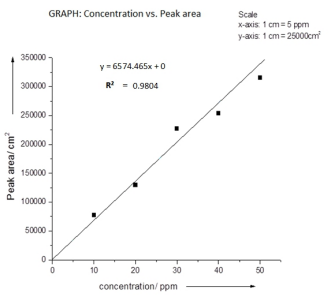

Fig.1 Calibration curve of peak area vs. concentration for D_3 MUST Tablets by HPLC technique

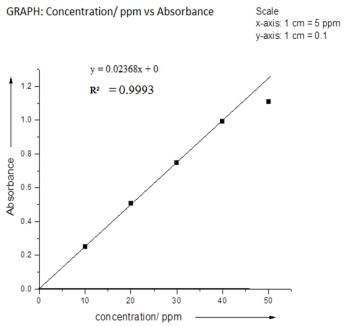

Fig.2 Calibration curve of absorbance vs. concentration for D3 MUST \ 60K Tablets UV/VIS Spectrophotometry technique

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