Analytical Method Development And Validation Of Alfacalcidol By Hplc Method

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ABSTRACT

The analytical method development and validation was simple, efficient, and less time-consuming for simultaneous estimation of Alfacalcidol capsules form by HPLC. Chromatographic separation was accomplished in the isocratic mode Mix 920 Volumes of n-Hexane, 40 Volumes of IPA, 40 Volumes of Tetrahydrofuran and 2 Volumes of Acetic acid (920:40:40:2) in 1000 mL beaker and Mixed well then sonicate for 5 minutes as the mobile phase by using the column Thermo scientific silica, 250 X 4.6mm, 5µm or equivalent as the stationary phase with a flow rate of 2.0 ml/min using an UV detector maintained at 265nm. The reported periods of retention time of alfacalcidol were found to be 10.9 min . As per ICH guidelines, this procedure has been validated for Specificity, Linearity and Range, Accuracy, System Precision, Method Precision, Intermediate Precision, Robustness, Solution stability and Filter Integrity. Our research focusses on reducing the complexity and duration of time required for analysing alfacalcidol capsules. The results obtained were found to be good and within the limit, proving that the developed method can be used for determination of alfacalcidol.

Keywords: HPLC, Alfacalcidol, ICH guidelines. Method Development, Validation

INTRODUCTION

The early 1970s saw the introduction of alfacalcidol (1-hydroxyvitamin D3), a synthetic vitamin D analogue. Calcitriol (1,25-dihydroxyvitamin D3), a prodrug, is one of the most effective and swiftly acting drugs now used in the prevention and treatment of vitamin D deficiency illnesses and hypocalcemia. The condition and parameter that should be followed in the creation and validation of a particular active component will be improved by establishing an appropriate analytical approach in a more focused, accurate, and exact manner..[1]

Alfacalcidol has been used as a vitamin supplement due to its lengthy half-life.[2] It is a vitamin D active metabolite that is crucial for regulating calcium metabolism and equilibrium.[3-4] It is a vitamin D active metabolite that plays a significant role in controlling the calcium balance and bone metabolism. Early injection of alfacalcidol can safely and beneficially change the normal course of renal bone disease in patients with mild to severe renal failure.[5]

Alfacalcidol Structure

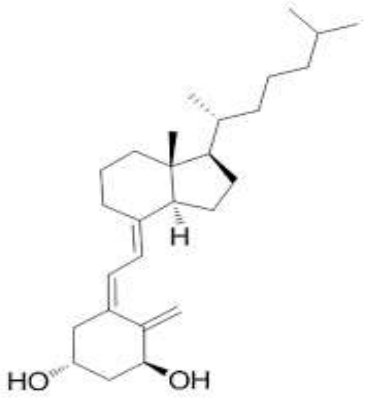


Figure 1: Alfacalcidol

Table 1: DRUG PROFILE

Comments	Hydroxylated analog of vitamin D
Chemical Formula	C27 H44 O2
Properties Physical Properties	Colorless to white, odorless crystalline compound; sensitive to heat, light and air; store at 2–8C under nitrogen depending on temperature and time, reversible isomerization to pre-alfacalcidol occurs in solution.
Molecular Weight	400.643
Solubility	Freely soluble in alcohol; soluble in fatty acids; insoluble in water

METHOD DESCRIPTION:

Principle:

Separation and quantification based on the isocratic reverse phase chromatography with UV detection.

INSTRUMENTATION, CHROMATOGRAPHC CONDITION

HPLC with UV/PDA detector analysis was carried out on Thermoscientific silica, 250 X 4.6mm, 5µm or equivalent, normal phase column. The mobile phase consisting of Mix 920 Volumes of n-Hexane, 40 Volumes of IPA, 40 Volumes of Tetrahydrofuran and 2 Volumes of Acetic acid in 1000 mL beaker and Mixed well then sonicate for 5 minutes. The flow rate of mobile phase was 2.0 mL/min with the injection volume of 100µL.The detection was carried out by at 265 nm. All analysis was carried out at a column oven temperature of 37^0C, sample temperature 15^0 C under isocratic condition.

Preparation of diluent :

Mobile phase used as the diluent.

Standard preparation:

weigh about 5 mg of alfacalcidol rs/ws into a 100 ml volumetric flask, add 50 ml of diluent, shake well to dissolve and make up to its volume with diluent. further dilute 5ml of the above solution to 250 ml volumetric flask and make up to 250 ml with diluent.

Check standard preparation:

Weigh about 5 mg of alfacalcidol rs/ws into a 100 ml volumetric flask, add 50 ml of diluent, shake well to dissolve and make up to its volume with diluent. further dilute 5ml of the above solution to 250 ml volumetric flask and make up to 250 ml with diluent.

Sample preparation:

cut and open. collect the medicament in a clean petridish. weigh about 1000 mg of medicament (equivalent to 10 mcg of alfacalcidol) in a 10 ml volumetric flask and make up the volume to 10 ml with diluent and shake well.

Sample preparation-Test

Procedure:

Separately inject 100 µl of diluent as blank, six replicate injections of standard preparation, two injection of check standard preparation and two fresh injection of each test preparation and one standard preparation as bracketing standard into the chromatograph. record the chromatograms and measure the responses for the major peaks.

Instrumentation & material:

Instrument

Equipment and instruments used in validation has been calibrated and maintained and will be used within their calibrated period. the weighing operations for this study were performed on the shimadzu analytical balance.the samples were ultrasonically processed using an ultrasonicator from chem54/chemlabs. chromatographic separation was performed using HPLC with pda detector lab solution by using the thermo scientific 250 x 4.6mm, 5µm or

equivalent chromatograms were recorded using the lab solution version installed on a personal computer.

Reagents and chemicals

alfacalcidol was obtained from n-hexane (HPLC grade), tetrahydrofuran (HPLC grade), isopropyl alcohol (HPLC grade), glacial acetic acid (laboratory reagent), sodium hydroxide (analytical grade), hydrochloric acid , hydrogen peroxide(solution qualigens)

Results

Primarily numerous trials for optimization of method were performed using different phase composition, different organic solvent,different ratio of organic to buffer ,different stationary phase and different internal standard chromatographic settings to achieve the finest peak resolution of alfacalcidol.

Analytical method validation

The analytical method was optimized and validated in accordance with the current ich guidelines and to accomplish the vision of specificity, accuracy, linearity, precision, robustness, filter validation, solution stability.

System suitability:

As per protocol standard solution of alfacalcidol is prepared and injected in six replicates into HPLC system.

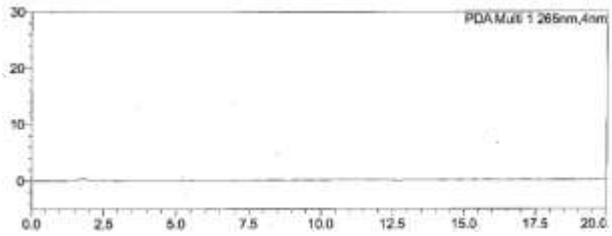
Acceptance criteria :

acceptance criteria for tailing factor nmt 2.0 and observed value 1.2 which is with in limit .acceptance criteria for theoretical plate count nlt 2000 and observed value 44710 which is with in limit. acceptance criteria for % rsd for rt alfacalcidolpeak is nmt 1.0 and observed value 0.0 which is within limit . acceptance criteria for % rsd for peak responses is nmt 2.0 and observed value is 0.6.

Specificity:

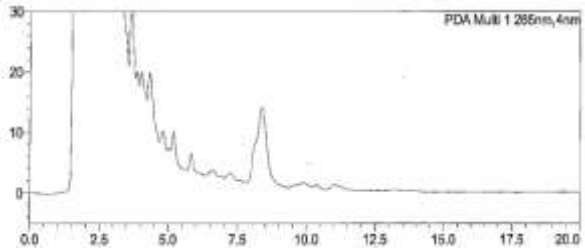
Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present for specificity evaluation: blank solution, placebo solution, sample solution and standard solution were injected into the hplc system.

Peak	RT	Area
Alfacalcidol (std)	10.8	119809
Alfacalcidol (sample)	10.8	118576



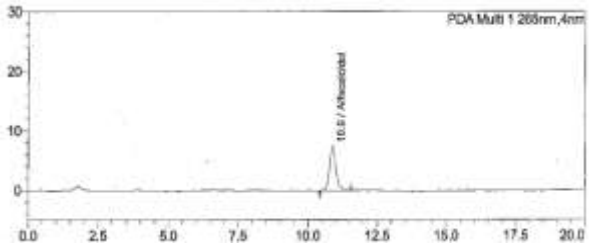
Specificity Blank:

Figure 2: Blank



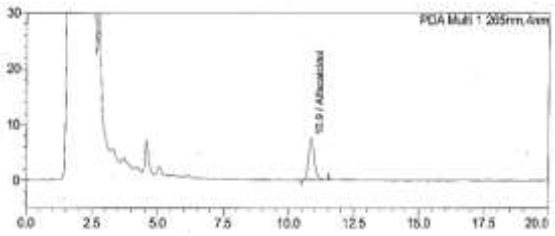
Specificity Placebo:

Figure 3:Placebo



Specificity Standard:

Figure 4:Standard



Specificity Sample:

Figure 5: Sample

FORCED DEGRADATION STUDY:

Forced degradation studies include the degradation of new drug substance and drug product at conditions more severe than accelerated conditions. These studies illustrate the chemical stability of the molecule which further facilitates the development of stable formulation with suitable storage conditions. ICH guidelines demonstrate certain degradation conditions like light, oxidation, dry heat, acidic, basic, hydrolysis etc. ICH Q1A, QIB and Q2B exemplify the forced degradation studies.

System Suitability:

As per protocol Standard Solution of Alfacalcidol is prepared and injected in six replicates into HPLC System. The system suitability parameters were calculated as per protocol and the average area of the injection are 130631. The standard deviation area was found to be 484 and the % RSD area was found to be 0.3.

Acceptance Criteria:

Acceptance criteria for tailing factor NMT 2.0 and observed value 1.3 which is with in limit. Acceptance criteria for theoretical plate count NLT 2000 and observed value 85013 which is with in limit. Acceptance criteria for % RSD for RT of alfacalcidol peak is NMT 1.0 and observed value 0.0 which is with in limit. Acceptance criteria for % RSD for peak responses is NMT 2.0 and observed value is 0.6.

Table 3 : force degradation studies.

Stressed Conditions	Content in %	% Degradation	Peak purity index
Unstressed sample	101.7	NA	1.000
Acid Hydrolysis Sample	93.6	8.0	1.000
Base Hydrolysis Sample	92.9	8.7	1.000
Peroxide Oxidation Sample	96.3	5.4	1.000
UV Light Sample	99.7	2.0	0.999

Forced Degradation Acid sample

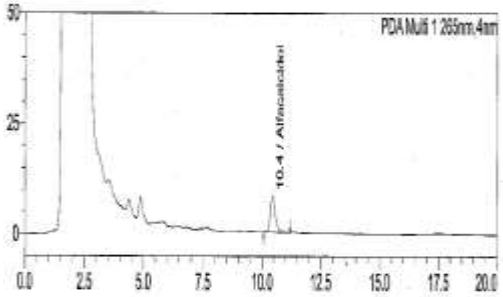


Figure 6: Acid sample

Forced Degradation Oxidation sample

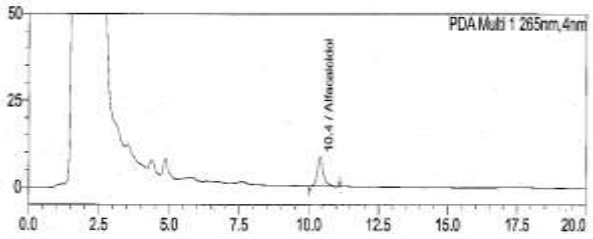


Figure 7: Oxidation sample

Forced Degradation Base sample

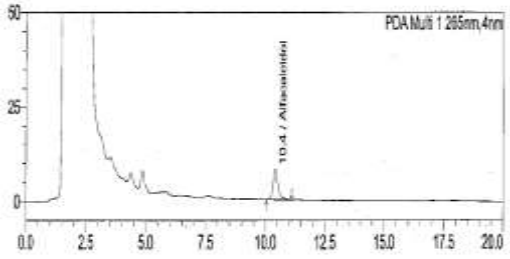


Figure 8: Base sample

Forced Degradation UV Light sample

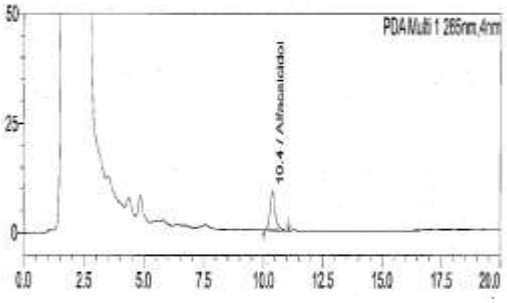


Figure 9: UV Light sample

Interference: Blank and Placebo does not show any interference at main peak RT.

LINEARITY AND RANGE:

The linearity of the method is established by performing six test concentrations from 25.0% to 150% of working concentrations as per protocol. The standard solutions were prepared with the concentrations of 25%, 50%, 75%, 100%, 125% and 150% with respect to 100% working concentration. For each concentration 3 replicate injections are given into HPLC system. Based on the average area obtained with each concentration, a graph is plotted between Area and Concentration. The details are given below.

For Alfacalcidol:

Table 4 : Linearity And Range .

S.NO	% Concentration	Average Peak area of Alfacalcidol
1	**25**	32922
2	**50**	62252
3	**75**	104352
4	**100**	142216
5	**125**	177246
6	**150**	212963

Linearity curve

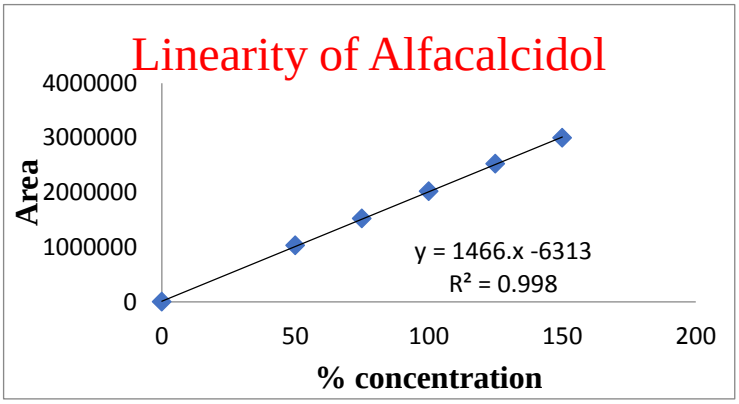


Figure 10: Linearity curve

ACCURACY:

Accuracy is the closeness of the test results obtained by the method with the true value. Accuracy may often be expressed as percent recovery by the Assay of known, added amounts of analyte. Accuracy is a measure of the exactness of analytical method. Accuracy was assessed using '3' concentration (50.0%, 100.0% and 150%). As per the protocol Standard and spiked sample solutions are prepared with concentrations of 50%, 100% and 150%. Based on the area obtained for each concentration, % of Recovery is calculated. The details are given below.

Test Solution:

Table 5: Test solution of Accuracy .

S.NO	Average peak Area	Amount of alfacalcidol added (ppm)	Amount of alfacalcidol recovered (ppm)	Average	%RSD
50% -1	66282	0.5105	0.51213	100.3199	0.5
50% -2					
50% -3					
100% -1	132676	1.0210	1.02516	100.4080	0.6
100% -2					
100% -3					
150% - 1	200277	1.5315	3.61146	101.0468	0.0
150% - 2					
150% - 3					

Result:

The recovery obtained with each concentration level, % RSD of recovery was within the acceptance criteria. Hence it is concluded that the method is accurate and precise in the range of 50% to 150% with respect to 100% working concentration.

Precision

System Precision

As per protocol Standard Solution of Alfacalcidol was prepared and injected in six replicates into HPLC System. The system suitability parameters were calculated as per protocol and the average area of six injection are 117598 . Standard deviation response for six injection are 642 and the %RSD response for six injection are 0.6.

Result:

The system suitability parameters are within the acceptance criteria. Hence the system is suitable to carry out the analysis for estimation of Content of Alfacalcidol in "Alfacalcidol 1.0 mcg capsules".

Method precision:

As per protocol 6 replicate Sample Solution of "Alfacalcidol 1.0 mcg capsules" were prepared and injected into HPLC System. The Method precision parameters are calculated as per protocol and the Average and the average area of the injection are 101.4. The standard deviation area was found to be 0.9881 and the % RSD area was found to be 0.9.

Result:

The Assay values of 6 replicate preparations and % RSD show that the method is precise and repeatable.

Intermediate Precision (Reproducibility):

Intermediate precision is demonstrated by analyzing the same batch of "Alfacalcidol 1.0 mcg capsules" as in precision with 6 replicate samples, in the different lab and a different Analyst, using a different Instrument and column on a different day. Average of the Alfacalcidol sample are 99.20, Standard deviation of the Alfacalcidol sample are 0.6998 and % RSD of the s Alfacalcidol sample are 0.7.

Table 7: Intermediate Precision (Reproducibility).

Content	**ALFACALCIDOL**	
Preparation	**METHOD PRECISION**	**INTERMEDIATE PRECISION**
	1.0 mcg	**1.0 mcg**
1	101.47	99.41
2	102.97	100.17

3	102.06	98.69
4	101.33	98.40
5	100.22	98.82
6	100.63	98.85
Average	**101.4**	**99.20**
SD	**0.9881**	**0.6998**
%RSD	**0.9**	**0.7**
Confidence limit	**0.7**	**0.5**

Summary Results :

Acceptance criteria for tailing factor for Alfacalcidol peak from first standard injection NMT 2.0 and observed value for method precision are 1.2 and intermediate precision are 1.4 which is with in limit. Acceptance criteria for theoretical plate count for Alfacalcidol peak from standard injection NLT 2000 and observed value for method precision are 44710and intermediate precision are 31045 which is with in limit. Acceptance criteria for % RSD for RT of alfacalcidol peak is NMT 1.0 and observed value 0.0 which is with in limit .Acceptance criteria for % RSD for peak responses is NMT 2.0 and observed value for is 0.6.

FILTER VALIDATION:

Filter validation was demonstrated by assaying the sample without filtration by centrifuging the sample solution, filtering through 0.45 µm Nylon and through 0.45µm PVDF.

The average of % Content of Alfacalcidol when centrifuged 101.3 , filtered through 0.45µm nylon is 101.0 and through 0.45 µm PVDF filter 98.6. the limit of acceptance cirteria is 90.0 to 110.0.

STABILITY OF ANALYTICAL SOLUTIONS

The solution stability is demonstrated by injecting standard and sample solution up to 48 hours. The % RSD of area of standard and sample solutions is calculated as shown below.

Table 8: stability of analytical solution.

Time Intervals	Standard Area	Sample Area
Initial	119809	118576
12th Hour	119898	121708

24th Hour	123339	122916
36th Hour	123699	124510
Average	**121686**	**121928**
Std dev	**2121.6857**	**2511.7836**
% RSD	**1.7**	**2.0**

Acceptance criteria:

Acceptance criteria for % RSD of the peak area of Alfacalcidol obtained from standard solution at different time interval NMT 2.0 and observed value 1.7 which is with in limit. Acceptance criteria for % RSD of the peak area of Alfacalcidol obtained from sample solution at different time interval NMT 2.0 and observed value 2.0 which is with in limit.

ROBUSTNESS

The robustness of the analytical method for Alfacalcidol content in Alfacalcidol 1.0 mcg Capsules is demonstrated with small but deliberate variations in Column oven temperature, Flow rate and Wavelength.

Effect of variation in Flow rate (±0.2mL/minute) for Alfacalcidol:

The robustness of the analytical method is demonstrated by small variations in the Flow rate (1.8 mL/min, 2.0 mL/min and 2.2 mL/min) as per protocol. The standard and sample solutions were prepared and injected into HPLC as per the method. The results obtained are listed below

Effect of variation in Column Oven Temperature (±2ºC) for Alfacalcidol:

The robustness of the analytical method is demonstrated by small variations in the column oven Temperatures 35^0C, 37^0C and 39^0C as per protocol. The standard and sample solutions were prepared and injected into HPLC as per the method. The results obtained are listed below

Effect of variation in wavelength for Alfacalcidol:

The robustness of the analytical method is demonstrated by small variations in the wavelength 265nm, 267nm, 269nm as per protocol. The standard and sample solutions were prepared and injected into HPLC as per the method. The results obtained are listed below.

Table 9: Robustness

Parameters	Variation	RSD	Tailing factor	RT
Acceptance criteria		NMT 2.0	NMT 2.0	NMT 1.0
Change in flow rate	1.8 mL/min	1.1	1.2	0.4
	2.0 mL/min	0.6	1.2	0.0
	2.2 mL/min	1.4	1.2	0.6
Change in column temperature	35^0C	0.8	1.2	0.8
	37^0C	0.6	1.2	0.0
	39^0C	0.4	1.0	0.4
Change in wavelength	265nm	0.6	1.2	0.0
	267nm	0.6	1.2	0.0
	269nm	0.5	1.2	0.0

Discussion

A straightforward HPLC test technique was created and used to analyse the alfacalcidol capsule . The next section presents the assay parameters after the technique underwent thorough validation. Chromatograms produced during the analysis of blank , standard, sample, and placebo solutions show the method's specificity (Figures 2, 3 , 4 and 5). No peak with a similar retention time to the major curcumin peak existed. Furthermore, peak purity information (not displayed) supports the suggested method's specificity. According to the results of the calibration curve in the concentration range of 20 g/mL to 60 g/mL, the suggested technique is linear. There is a linear relationship between the concentration that was injected and its peak area, with a r2 value of more than 0.9980 and a line equation of y = 1466.x 6313. By using the suggested approach, the RSD values for the intra- and inter-day analysis of alfacalcidol were less than 2 %. The suggested approach (Tables 7) has great accuracy . The average recovery of the standard from the sample matrix was between 98.0 and 102.0 %, indicating a procedure with acceptable accuracy. Additionally, (Table 5) shows that the standard deviation of the outcomes was less than 2 %. The findings of the robustness assessment (Table 9) revealed that the alfacalcidol chromatograms are not significantly affected by minor changes in the mobile phase's chemical makeup, pH, flow rate, column temperature, or C18 column packing. Syringe filters were used to filter the samples for the

Qc tests, and the filtrate was then collected and subjected to HPLC analysis for API quantification. Standard and sample solutions are stable for up to 24 hours on a work surface and in a refrigerator (2-8 °C). The suggested procedures were verified in accordance with the ICH criteria, which are clear, easy to follow, and effective for quantifying curcumin without excipient influence.

Conclusion

The developed method was validated for various parameters as per ICH guidelines like accuracy, precision, linearity, specificity, system suitability, solution stability and robustness. The results obtained were within the acceptance criteria. So, it can be concluded that the developed method is simple, precise, cost-effective, eco-friendly, safe and can be successfully employed for the routine analysis of curcumin in bulk and pharmaceutical dosage forms.

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