ANALYTICAL METHOD VALIDATION FOR IDENTIFICATION AND ASSAY OF ALFACALCIDOL IN ALFACALCIDOL SOFT GELATIN CAPSULES BY HPLC

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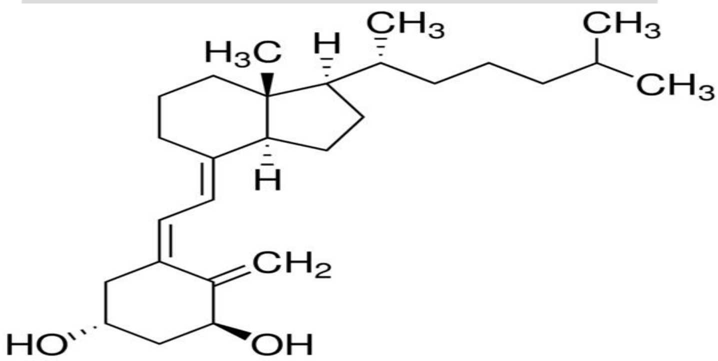
Abstract: The analytical method validation was simple, efficient, and less time-consuming for simultaneous estimation of alpha-calcidol capsules by HPLC. Chromatographic separation was accomplished in the isocratic mode. Mix 920 volumes of n-Hexane, 40 volumes of IPA, 40 volumes of tetrahydrofuran, and 2 volumes of acetic acid (920:40:40:2) in a 1000-mL beaker and mix well, then sonicate for 5 minutes as the mobile phase by using the column Thermo Scientific silica, 250 x 4.6 mm, 3µm, or equivalent as the stationary phase with a flow rate of 2.0 ml/min using an UV detector maintained at 265 nm. The reported periods of retention time of alfacalcidol were found to be 9.4 minutes. 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.

Keywords: HPLC, Alfacalcidol, ICH guidelines. Method Validation

INTRODUCTION:

Alfacalcidol, or 1-alpha-hydroxycholecalciferol or 1-alpha-hydroxyvitamin D3, is a non-endogenous analogue of vitamin D. It plays an essential function in calcium homeostasis and bone metabolism. Alfacaldisol is activated by the enzyme 25-hydroxylase in the liver to mediate its effects in the body, or most importantly, the kidneys and bones[1,2]. The pharmacological actions of alfacalcidol are prolonged than vitamin D because a negative feedback mechanism regulates the final activation step of vitamin D in the kidneys. Alfacalcidol has a weaker impact on calcium metabolism[3] and parathyroid hormone levels[4] than calcitriol; but significant effects on the immune system, including regulatory T cells.[5] It is considered to be a more useful form of vitamin D supplementation, mostly due to much longer half-life and lower kidney load .

STRUCTURE :



DRUG PROFILE :

Synonyms : 1-Hydroxycholecalciferol

Molecular Formula : $C_{27}H_{44}O_2$

Molecular weight : 400.64 g/mol

Dose : Capsules, 1 and 0.25 mcg

Category : Osteomalacia (ricketts), Vitamin D deficiency

Apperance : Alfacalcidol is a colorless crystalline compound .

Solubility : It is sensitive to light and very soluble in methanol, ethanol and Chloroform,soluble in ether ,sparingly soluble in methylformate and acetonitrile

Half Life : The half-life of alfacalcidol ranges from three to four hours

Melting point : 136°C -144°C.

pKa : 14.43

Storage : Store it at room temperature (25°C), and in an airtight container.

METHOD DESCRIPTION :

INSTRUMENTATION, CHROMATOGRAPHC CONDITION

HPLC with UV/PDA detector analysis was carried out on Thermoscientific silica, 250 X 4.6mm, 3µm 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 15^0C 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 30 ml of diluent, shake well to dissolve and make up to its volume with diluent. further dilute 3ml 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 30 ml of diluent, shake well to dissolve and make up to its volume with diluent. further dilute 3ml 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 100 ml volumetric flask and make up the volume to 100 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.

MATERIALS :

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)

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.

TABLE :01

No of Injection	RT (Minute)	Alfacalcidol peak area
01	9.4	71047
02	9.4	71411
03	9.4	70905
04	9.4	71361
05	9.4	70963
06	9.4	71174
Average	**9.4**	**71143**
SD	**0.0**	**209**
%RSD	**0.0**	**0.2**

Conclusion:

The system suitability parameters are within the acceptance criteria. Hence the system is suitable to carry out the analysis for estimation of Assay in Alfacalcidol capsules 0.25mcg and 1.0mcg. During regular analysis, these system suitability parameters must be ensured before starting sample analysis.

SPECIFICITY:

"Specificity is the ability of the method to measure the analyte in the presence of matrix components.

Prepare the following solutions for Specificity :Prepare mobile phase , standard solution , placebo preparation , Sample preparation , medicament preparation of 0.25 &1.0mcg capsules.

For Alfacalcidol capsules 0.25 mcg: (Finished product & medicament)

TABLE :02

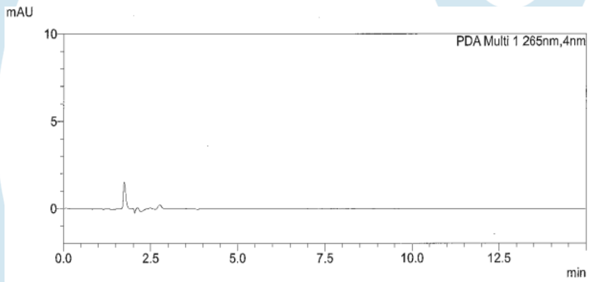
System Suitability Parameters	Observed Value					Acceptance criteria
	Blank	Placebo 0.25 mcg	Standard	Finished product 0.25 mcg	Medicament 0.25 mcg	
Interference	NA	NA	NI	NI	NI	**Blank and Placebo peak should not show interference with main analyte**
Retention time	NA	NA	9.4	9.4	9.4	
Area response	NA	NA	70844	73187	74049	**NA**
Peak Purity Index	NA	NA	1.000	1.000	1.00	**NLT 0.99**

For Alfacalcidol capsules 1.0 mcg:(Finished product & medicament)

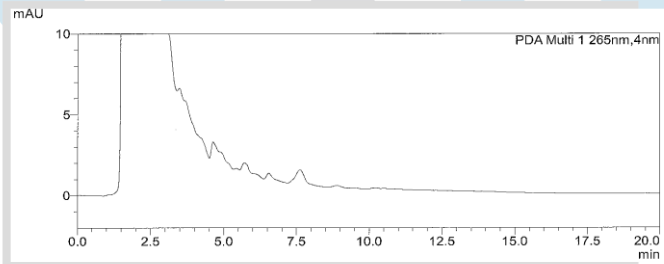
TABLE :03

System Suitability Parameters	Observed Value					Acceptance criteria
	Blank	Placebo 1.0 mcg	Standard	Finished product 1.0 mcg	Medicament 1.0 mcg	
Interference	NA	NA	NI	NI	NI	**Blank and Placebo peak should not show interference with main analyte**
Retention time	NA	NA	9.4	9.4	9.4	
Area response	NA	NA	70844	72878	74394	**NA**
Peak Purity Index	NA	NA	1.000	1.000	1.00	**NLT 0.99**

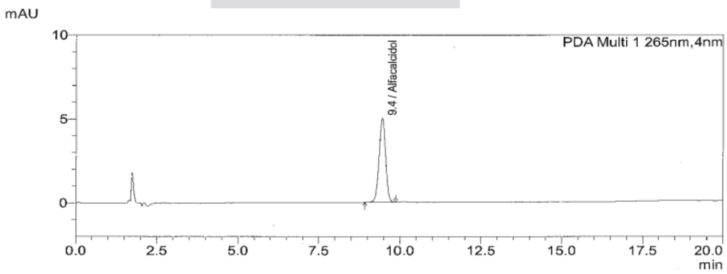
SPECIFICITY BLANK FIGURE :01



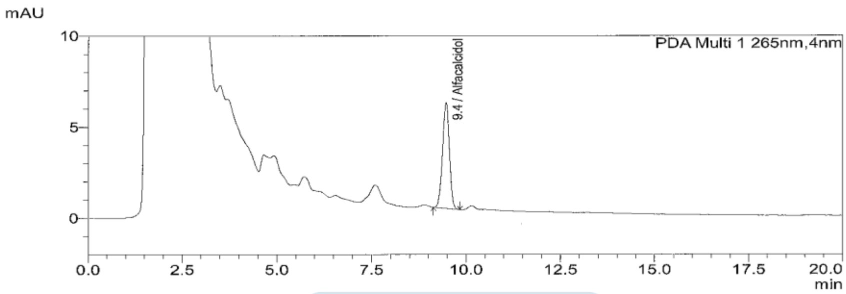
PLACEBO FIGURE :02



STANDARD FIGURE :03



SAMPLE : FIGURE :04



ACCEPTANCE CRITERIA

The chromatograms of Blank, Placebo and stressed sample solutions shows that there is no interference of Blank ,placebo and degradant peaks at the retention time of Alfacalcidol. The Peak Purity index of sample should not be less than 0.99 .

Identification of Alfacalcidol by Retention Time:

Peak identification solution: (Alfacalcidol):

Weigh accurately about 5 mg of Alfacalcidol WS/BPCRS into a 100mL volumetric flask, add 30 mL of diluent shake to dissolve and make up the volume with diluent. Dilute 3 mL of above solution to 250mL with diluent.

Procedure:

For Chromatographic condition, diluent, standard preparation, Check standard preparation, Sample preparation and mobile phase preparation refer above in assay by HPLC.

Record the retention time of Standard and Sample and compare the assessment.

Identification by retention time:

Retention time for Alfacalcidol from Sample preparation for Alfacalcidol capsules 0.25&1.0 mcg (Finished Product, medicament) is **9.4**

Identification of Alfacalcidol by UV spectrum

Procedure:

For Chromatographic condition, diluent, standard preparation, Check standard preparation, Sample preparation and Mobile phase preparation refer above in assay by HPLC.

Spectrum maxima for Alfacalcidol from Sample for Alfacalcidol capsules 0.25&1.0 mcg Finished Product **263**, Medicament **264 .**

FORCED DEGARATATION STUDY:

Forced degradation is a process that involves degradation of drug products and drug substance deliberately at conditions more severe than accelerated conditions and thus generates degradation products that can be studied to determine the stability of the molecule. These studies are used to evaluate an analytical method’s ability to measure an active ingredient and its degradation products without interference.

Samples or drug product (spiked placebo) and drug substance are exposed for extended periods of time, to extremes of pH (aqueous, hydrochloric acid or sodium hydroxide solutions) at elevated temperatures, to hydrogen peroxide at room temperature or to UV light and to dry heat (in an oven) while adopting trial and error approach to select the strength, temperature and time of exposure to the stress conditions so as to achieve degradation to an extent of 10-20%.

For Alfacalcidol capsules 0.25 mcg:

TABLE :04

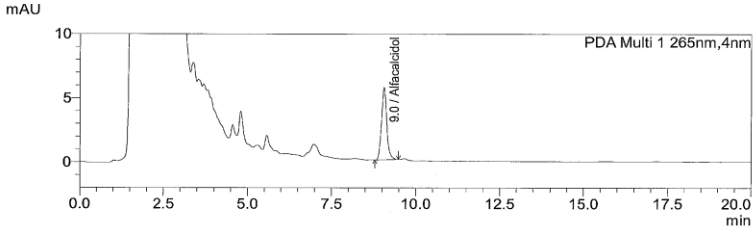
Stressed conditions	Content in %	% Degradation	Peak purity index
Unstressed sample	102.7	**NA**	**NA**
Acid (1 hour)	85.0	17.2	1.000
Base (1 hour)	83.4	18.8	1.000
Oxidation (1 hour)	96.6	5.9	1.000
Humidity	100.8	1.9	1.000
UV	103.2	-0.5	1.000
Light	98.8	3.8	1.000
Thermal	102.6	0.1	1.000

For Alfacalcidol capsules 1.0 mcg:

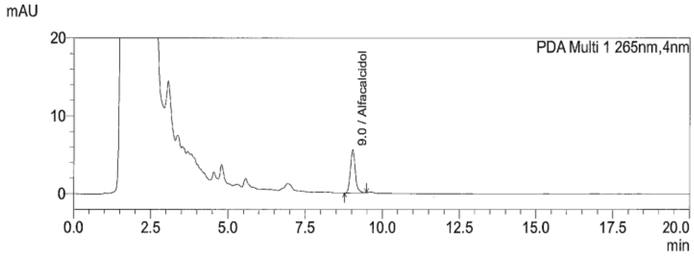
TABLE :05

Stressed conditions	Content in %	% Degradation	Peak purity index
Unstressed sample	102.0	**NA**	**NA**
Acid (1 hour)	87.0	14.7	1.000
Base (1 hour)	85.6	16.1	1.000
Oxidation (1 hour)	85.5	16.2	1.000
Humidity	101.7	0.3	1.000
UV	101.9	0.1	1.000
Light	100.8	1.2	1.000
Thermal	101.3	0.7	1.000

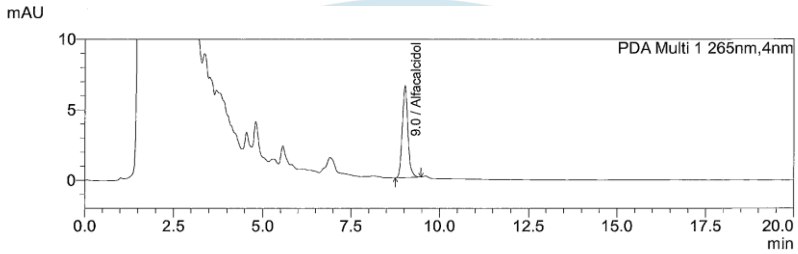
Acid 1 Hr sample : FIGURE :05



Base 1 Hr sample: FIGURE :06



Oxidation 1 Hr sample :FIGURE :07



LINEARITY AND RANGE:

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

Preparation of Linearity Standard stock Solution: (30mcg/mL of Alfacalcidol)

Weigh accurately about 6 mg of Alfacalcidol WS/BPCRS into a 200mL volumetric flask, add 30 mL of diluent shake to dissolve and make up the volume with diluent.

Preparation of Linearity Solutions:

50 % solution: (0.3mcg/mL of Alfacalcidol)

Pipette out 2 mL of the above solution into a 200 mL volumetric flask, dilute to volume with diluent.

75 % solution: (0.45mcg/mL of Alfacalcidol)

Pipette out 3 mL of the above solution into a 200 mL volumetric flask, dilute to volume with diluent.

100 % solution: (0.6mcg/mL of Alfacalcidol)

Pipette out 4 mL of the above solution into a 200 mL volumetric flask, dilute to volume with diluent.

125 % solution: (0.75mcg/mL of Alfacalcidol)

Pipette out 5 mL of the above solution into a 200 mL volumetric flask, dilute to volume with diluent.

150 % solution: (0.9mcg/mL of Alfacalcidol)

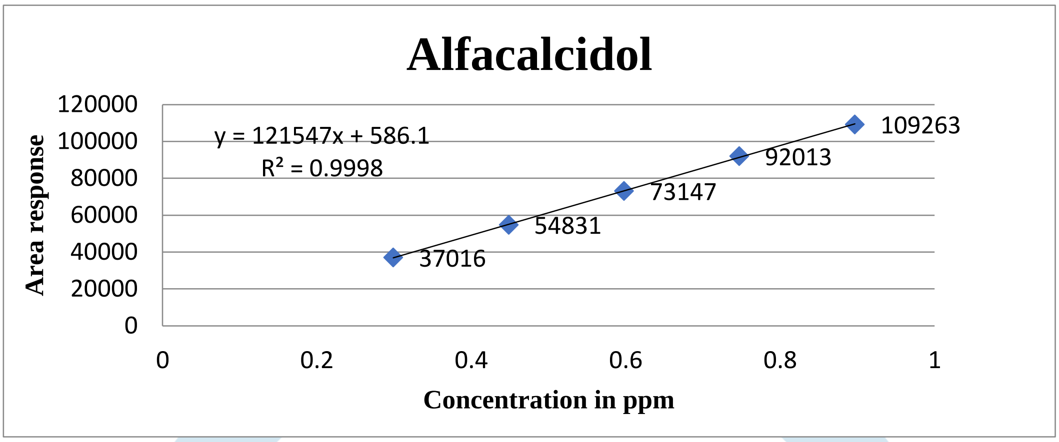
Pipette out 6 mL of the above solution into a 200 mL volumetric flask, dilute to volume with diluent.

TABLE :06

Conc. in (%)	AREA
50	37016
75	54831
100	73147
125	92013

150	**109263**

FIGURE : 08



ACCURACY:

Accuracy is the closeness of the test results obtained by the method with the true value. Accuracy shall be assessed using '3' concentrations (50%, 100% and 150%).

TABLE :07

Accuracy conc.	Area	Amount added (ppm)	Amount recovered (ppm)	% Recovery	Average	%RSD
50% -1	37039		0.3075	100.6217		
50% -2	37229	0.3056	0.3090	101.1125	100.9	0.2
50% -3	37193		0.3087	101.0143		
100% -1	72567		0.6024	98.5602		
100% -2	73266	0.6112	0.6082	99.5091	99.1	0.4
100% -3	73005		0.6061	99.1655		
150% - 1	110751		0.9194	100.2835		
150% - 2	111180	0.9168	0.9230	100.6762	100.7	0.4
150% - 3	111761		0.9278	101.1998		

ACCEPTANCE CRITERIA :

The Correlation coefficient and y-Intercept of the plot for Alfacalcidol are well within the acceptance criteria. Hence it is concluded that the method is linear within the concentration of 50% to 150% with respect 100 % working concentration. Correlation coefficient. (R^2) of Alfacalcidol NLT 0.99 , % RSD for lower Concentration and higher concentration NMT 2.0.

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 71143 . Standard deviation response for six injection are 209 and the %RSD response for six injection are 0.2.

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 :

TABLE :08

Preparation	% Assay of Alfacalcidol			
	% Assay of 0.25 mcg		% Assay of 1.0mcg	
	Finished product	Medicament	Finished product	Medicament
1	101.8000	103.2400	101.4800	103.2800
2	102.4000	104.0800	101.8500	102.4300
3	102.7200	103.1200	101.6100	102.2300
4	103.0800	103.0800	100.7100	102.4800
5	101.6400	104.8400	101.1900	101.4100
6	103.0400	103.1600	102.5800	101.8900
Average	**102.4**	**103.6**	**101.6**	**102.3**
SD	**0.6162**	**0.7198**	**0.6312**	**0.6282**
%RSD	**0.6**	**0.6**	**0.6**	**0.6**
Confidence limit	**0.4**	**0.5**	**0.5**	**0.5**

Intermediate Precision (Reproducibility):

Intermediate precision will be demonstrated by analyzing same batch as analyzed as part of Method precision in the different lab by a different Analyst, using a different Instrument, different column and on a different day.

For Blank, Standard, Check Standard and Sample preparations:

% of Alfacalcidol capsules 0.25&1.0 mcg(Finished product)

TABLE :09

% of Alfacalcidol capsules 0.25 mcg(Finished product)			**% of Alfacalcidol capsules 1.0mcg(Finished product)**	
PREPARATION	METHOD PRECISION	INTERMEDIATE PRECISION	METHOD PRECISION	INTERMEDIATE PRECISION
1	101.8000	104.1600	101.4800	101.5300
2	102.4000	104.0000	101.8500	101.6600
3	102.7200	103.9200	101.6100	102.6100

4	103.0800	104.1200	100.7100	101.1500
5	101.6400	104.6800	101.1900	101.5200
6	103.0400	104.2400	102.5800	100.7400
AVERAGE	102.4	104.2	**101.6**	**101.5**
Average from 12 samples	104.2		**101.6**	
%RSD	**0.6**		**0.6**	

% of Alfacalcidol capsules 0.25&1.0 mcg(Medicament)

TABLE :10

% of Alfacalcidol capsules 0.25 mcg(MEDICAMENT)			**% of Alfacalcidol capsules 1.0mcg(MEDICAMENT)**	
PREPARATION	METHOD PRECISION	INTERMEDIATE PRECISION	METHOD PRECISION	INTERMEDIATE PRECISION
1	103.2400	103.7200	103.2800	100.8900
2	104.0800	103.4800	102.4300	101.0200
3	103.1200	103.9600	102.2300	102.2500
4	103.0800	104.0400	102.4800	101.1800
5	104.8400	104.3600	101.4100	101.1600
6	103.1600	103.6000	101.8900	101.5300
AVERAGE	**103.6**	**103.9**	**102.3**	**101.3**
Average from 12 samples	**103.7**		**101.8**	
%RSD	**0.5**		**0.7**	

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 101.4 , 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.

ACCEPTANCE CRITERIA :

The filtration by using 0.45µm Nylon filter and 0.45µm PVDF(with discarding volumes of 1mL, 2mL and 3mL) and Centrifugation are found satisfactory. Hence, it is recommended to use Centrifuge/0.45µm Nylon filter/0.45µm PVDFwith discarding volume of 1mL for regular usage.

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 :11

Time interval	Area		
	Standard	**Sample (Finished product)**	**Sample (Medicament)**
Initial	74373	79554	77027
12th Hours	74289	78082	78253
24th Hours	74284	79919	78785
36th Hours	74234	79650	78746
48th Hours	74446	80144	78532
Average	**74325**	**79470**	**78269**
SD	**83.9684**	**809.6105**	**725.5641**
%RSD	**0.1**	**1.0**	**0.9**

ACCEPTANCE CRITERIA

The Cumulative % RSD of peak area for Standard and sample solution obtained with different time intervals and % RSD of assay results from initial with different time intervals shows that both standard and sample solutions are stable up to 48 hours at room temperature. NMT 2.0

ROBUSTNESS :

The robustness of the analytical method will be demonstrated by performing analysis with small but deliberate variations in the method

Effect of variation in flow rate (mL/minute) forAlfacalcidol:

TABLE :12

Preparation	Low-1.8 mL/min		Actual-2.0 mL/min		High-2.2 mL/min	
	FP	**Medicament**	**FP**	**Medicament**	**FP**	**Medicament**
1	105.3600	105.4800	104.2800	104.3600	102.1600	103.5200
2	104.0800	104.9200	103.2400	103.7600	102.8800	102.4800
Average	**104.7**	**105.2**	**103.8**	**104.1**	**102.5**	**103.0**
SD	**0.9050**	**0.3959**	**0.7353**	**0.4242**	**0.5091**	**0.7353**
%RSD	**0.8**	**0.3**	**0.7**	**0.4**	**0.4**	**0.7**

Effect of variation in Column OvenTemperature (°C) for Alfacalcidol

TABLE :13

Preparation	Low-35°C		Actual-37°C		High-39°C	
	FP	**Medicament**	**FP**	**Medicament**	**FP**	**Medicament**
1	102.9200	103.3600	103.2000	104.0800	105.2400	105.8000
2	103.6400	103.6400	104.000	103.6800	105.6400	105.7200
Average	**103.3**	**103.5**	**103.6**	**103.9**	**105.4**	**105.8**
SD	**0.5091**	**0.1979**	**0.5656**	**0.2828**	**0.2828**	**0.0565**

Preparation	Low-35°C		Actual-37°C		High-39°C	
	FP	Medicament	FP	Medicament	FP	Medicament
%RSD	0.4	0.1	0.5	0.2	0.2	0.0

Effect of variation in Mobile phase composition:

TABLE :14

Preparation	Alfacalcidol Capsules 0.25 mcg (Finished product) Results in %				
	Actual	Variation-1	Variation-2	Variation-3	Variation-4
1	104.2800	104.3600	104.3600	103.9600	105.0000
2	103.2400	104.0000	103.2000	103.0400	104.1200
Average	103.8	104.2	103.8	103.5	104.6
SD	0.7353	0.2545	0.8202	0.6505	0.6222
%RSD	0.7	0.2	0.7	0.6	0.5

Preparation	Alfacalcidol Capsules 0.25 mcg (Medicament) Results in %				
	Actual	Variation-1	Variation-2	Variation-3	Variation-4
1	104.3600	104.2000	103.8800	104.4000	105.3200
2	103.7600	104.5200	102.9600	104.4800	104.6400
Average	**104.1**	**104.4**	**103.4**	**104.4**	**105.0**
SD	**0.4242**	**0.2262**	**0.6505**	**0.0565**	**0.4808**
%RSD	**0.4**	**0.2**	**0.6**	**0.0**	**0.4**

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 1 2, 3 and 4). 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 0.9998 and a line equation of $y = 121547x + 586.1$. 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 6,7) shows that the standard deviation of the outcomes was less than 2 %. The findings of the robustness assessment (Table 12,13,14) 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. 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.

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