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Research Article

Pharmaceutical Analysis

Stability Indicating Method for Known and Unknown Impurities Profiling for Cholecalciferol Tablets

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Abstract: The main objective of this research work was to develop and validate, a new gradient, highly sensitive, specific and stability indicating Reverse Phase HPLC method for quantitative determination of known, unknown impurities and degradant impurities profiling for Cholecalciferol tablets. No Pharmacopoeial method is available to quantify known, unknown impurities and degradants profiling for Cholecalciferol tablets. The impurities were separated on the Hypersil BDS column (150 mm x 4.6mm, 3µm) with a mobile phase of mixture of Trifluoroacetic acid buffer and acetonitrile with flow rate of 1.5 mL minute$^{-1.}$ The column compartment was maintained at 40°C and the detection wavelength was at 265nm. Cholecalciferol, its known impurity and unknown impurities have been well resolved from each other. Recovery of the known and unknown impurities found between 80% to 120% as per ICH guideline. Method found linearity over the working concentration range with acceptance criteria of correlation coefficient greater than 0.99. Method precision and intermediate precision results found with percentage relative standard deviation of impurity content less than 10% for replicated analysis of test samples. A stress study was conducted with a drug product that was exposed to different conditions of acid, base, oxidation, heat, humidity and photolytic degradation. Cholecalciferol was found to degrade significantly under Photolytic, Thermal and Alkaline stress conditions. The degradation products were well resolved from Cholecalciferol and its impurities. For each stress condition, peak purity of Cholecalciferol was assessed using the Photodiode Array detector and found homogeneous in nature. The mass balance for stress study was found in between 95% and 105%. Thus, proving the stability indicating nature of the analytical method. The developed method was validated as per ICH guidelines. The method found accurate, precise, linear, robust, rugged, specific and stability indicating in nature.

Keyword: Cholecalciferol, Stability Indicating, Analytical Method Validation, Impurities profiling, ICH guidelines.

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1. INTRODUCTION

The main objective of this research study is to develop a new gradient, highly sensitive, specific and stability indicating Reverse Phase HPLC method for quantitative determination of known, unknown impurities and degradant impurities profiling for Cholecalciferol tablets which is not earlier reported. The degradation kinetics with respect to known and unknown impurities of the drug products also not discussed and reported in any research journal. Cholecalciferol is also known as Vitamin D_3 Cholecalciferol is synthesized endogenously from 7-dehydrocholesterol after ultraviolet irradiation or absorbed from the diet[1-2]. Cholecalciferol is a type of Vitamin D. Vitamin D is also found in few foods and can be taken as a dietary supplement[3]. It is a unique fat soluble vitamin. Vitamin D is not a single compound but is a family of compounds that exhibit Vitamin D activity. Clinically its measurement in the body is very important because it is a nutritional indicator which decides its deficiency, which is one of the causes of osteoporosis. Vitamin D plays an important role to fight against a broader range of various diseases like cancer, cardiovascular disease, autoimmune disease, hypertension, diabetes mellitus, viral respiratory infection etc. [4-11] The Literature survey revealed that the several RP-HPLC method were reported for the determination of Cholecalciferol alone[12-17] and in combination with other drugs or components[18-22]. No Pharmacopoeial method (USP/BP/EP/JP/IP) is available to quantify known, unknown impurities and degradants profiling for Cholecalciferol tablets. For Active pharmaceutical ingredient (API) the method for determination of the related substances (impurities) is available in British Pharmacopeia and European Pharmacopeia, which involves chromatography in the normal phase, but the method suffer various disadvantages with involving complicated, tedious, thermal reflux procedure and not economically feasible. However, the comprehensive literature review found that very few methods were reported for the determination of related substances[23-26], degradation products[27-28] and metabolites[29-32] of cholecalciferol. The methods that have been reported for related substances involve normal phase chromatography [23], qualitative and comparative study using two new stationary phases[24], Estimation of Impurities of Vitamin D3 Analogue and not impurities of Vitamin D3[25] and related substances by LC-MS method [26]. Degradation & metabolites studies reported by LC-MS methods[27-32]. None of the above methods discussed pertaining to the stability indicating nature, the degradation kinetics of the product and degradant impurities profiling which is the essential regulatory pharmaceutical requirement of the various countries for the therapeutic efficacy and safety of the pharmaceutical dosage form. The LC-MS methods involve a higher cost per sample analysis than the reverse-phase chromatography method. Therefore it is essential to develop a reverse phase liquid chromatographic procedure that will serve as a reliable, accurate, sensitive, rugged, robust and cost effective for monitoring the impurities and degradation product of Cholecalciferol in Cholecalciferol tablets. The chemical name & structure of Cholecalciferol and its impurity are shown in Table-1 and Figure-1 and Figure-2.

Table 1: Chemical name of Cholecalciferol and its impurity

Compound Name	Chemical Name
Cholecalciferol	(3β, 5Z,7E)-9,10-secocholesta-5,7,10(19)-trien-3-ol
Cholecalciferol Impurity-A	(5E,7E)-9,10-secocholesta-5,7,10(19)-trien-3β-ol

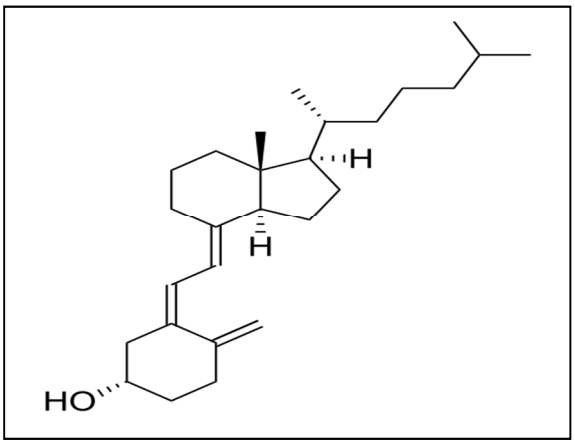

Fig 1. Chemical structure of Cholecalciferol

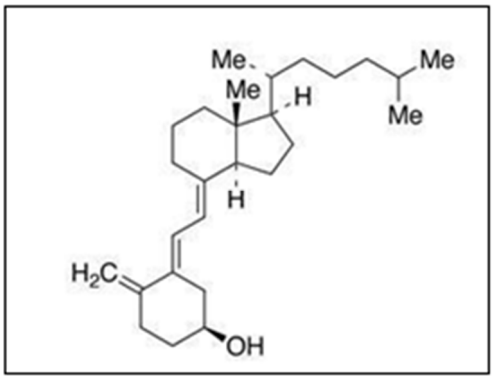

Fig 2. Chemical structure of Cholecalciferol impurity-A

The therapeutic efficacy and safety of drug products will depend on the presence of unknown or degrading impurities if they permit within the maximum limits set by the ICH guideline[33-34]. Therefore, the drug regulations require that the stability study of the drug substance and drug product for the impurity profile study be conducted using a stability indicating validated analytical method. [35-39] To develop and prove the stability indicating natue of the analytical method it is essential to conduct a stress study on drug substances and drug products, in order to produce the potential degrading impurities and to monitor the increase in known impurities under stress conditions. Environmental factors such as sun light, Heat and presence of moisture (hydrolysis) or air (oxidation) can lead to generation of different potential degradants impurities[40-41]. The stress study will help to identify unknown potential degradants, and provide information necessary for the stability of drug substances, and pharmaceutical products. Therefore, the results of the cholecalciferol degradation study, which will provide information on the intrinsic stability of the drug product, are reported here.

2. MATERIALS AND METHODS

2.1 Reagents and Materials

Marketed samples of Cholecalciferol tablets were used for analytical method development and validation. The related substances (Impurity-A) of Cholecalciferol were procured from Analytica Chemie Inc., Bangalore (India). Trifluoroacetic acid, methanol & acetonitrile were obtained from spectrochem Limited, HPLC grade and water was obtained from Milli-Q purification system.

2.2 Instrumentation

HPLC system (Make: Waters,) equipped with an auto sampler and quaternary gradient pump was used. The column compartment having temperature controlled and photodiode array detector (PDA) was used throughout the analysis. Chromatographic data was acquired using empower software.

2.3 Chromatographic conditions

Hypersil BDS (150 x 4.6) mm, 3µm (Thermo scientific) column was used as stationary phase and maintained at 40°C. The mobile phase involved a variable composition of buffer and organic solvents, Mobile phase-A (mixture of 0.1% Trifluoroacetic acid Buffer pH 3.0 and Acetonitrile in the ratio of -10:90v/v respectively), and mobile phase-B (mixture of 0.1% Trifluoroacetic acid Buffer pH 3.0 and Acetonitrile in the ratio of -90:10 v/v respectively). HPLC gradient programme run mentioned in Table-2.

2.4 Diluent

Mixture of acetonitrile and water in the ratio 80:20 %v/v. used as a diluent.

Table 2: The mobile phase programme for gradient elution

Time (minute)	Flow (ml min^{-1})	Mobile phase-A (%)	Mobile phase-B (%)
0	1.5	100	0
20	1.5	100	0
30	1.5	80	20
45	1.5	80	20
50	1.5	100	0
60	1.5	100	0

2.5 Solution preparations

2.5.1 Standard solution

Solution containing 0.2µg mL^{-1} of Cholecalciferol working standard prepared in diluent.

2.5.2 Impurity-A stock solution

Solution containing 20µg mL^{-1} of Cholecalciferol impurity-A standard prepared in acetonitrile.

2.5.3 Standard stock solution

Solution containing 200µg mL^{-1} of Cholecalciferol standard prepared in acetonitrile.

2.5.4 System suitability solution

Transfer 10mL of standard stock solution (200µg mL^{-1} of

Cholecalciferol) into 100mL volumetric flask. Add 75mL diluent and heat in a water bath at 70°C for 45minutes, and then cool the solution. Add 1.0mL of Impurity-A stock solution (20µg mL^{-1}) in it. Dilute the solution up to the mark with diluent.

2.5.5 Sample solution: (Strength: 0.05mg/tablet)

Transfer 10 tablets into a 25mL volumetric flask. Add 5mL of water, sonicated to completely disperse the tablets, then add 15mL acetonitrile, sonicated for 30 minutes with intermittent shaking then dilute with acetonitrile. Filtered through 0.45µm PVDF filter. (Concentration: 20µg mL^{-1}).

2.6 Forced degradation study

Multiple stressed samples were prepared as indicated below. They were chromatographed along with a non-stressed sample. %Degradation was calculated in terms of % total impurities (known and unknown impurities), and % degraded Cholecalciferol peak along with mass balance reported in Table-3.

2.6.1 Acid Degradation

Solution containing 20mg mL^{-1} of Cholecalciferol was treated with 0.5N HCl in a water bath maintained at 50°C for 1hour. The solution was neutralized as needed with 0.5N NaOH.

2.6.2 Base Degradation

Solution containing 20mg mL^{-1} of Cholecalciferol was treated with 0.5N NaOH in a water bath maintained at 50°C for 1hour. The solution was neutralized as needed with 0.5N HCl.

2.6.3 Peroxide Degradation

Solution containing 20mg mL^{-1} of Cholecalciferol was treated with 30% w/v H_2O_2. This treated sample solution was kept for 24 hours at room temperature.

2.6.4 Thermal degradation

Transfer 10 tablets into dry 25mL volumetric flasks, these tablets were exposed to 60 °C in the oven for 24hours.

2.6.5 Photolytic degradation

Tablets are directly exposed in photo jilytic chamber for 1.2million Lux hours with energy of not less than 200Wh/m2

2.6.6 Humidity degradation

Transfer 10 tablets into dry 25mL volumetric flasks, these tablets were exposed to 40°C /75%RH for 5days in the humidity chamber.

Table 3: Degradation study data for Cholecalciferol tablets

Stress conditions	% Total Impurity	% Area of Cholecalciferol	Purity Angle	Purity Threshold	Purity flag	% Mass Balance
Base	12.94	89.94	0.274	1.364	No	102.9
Peroxide	5.10	94.41	0.525	1.262	No	99.5
Humidity	6.96	93.49	0.376	1.272	No	100.5
Thermal	8.37	88.48	0.396	1.276	No	96.9
Photolytic	11.58	87.7	0.429	1.269	No	99.3
Acid	5.89	94.76	0.380	1.282	No	100.7

3. RESULTS

3.1 Method validation

The developed Reverse Phase HPLC analytical method validated according to ICH guidelines[38] with respect to specificity, accuracy, precision (method precision and intermediate precision), linearity, range and robustness.

3.2 System suitability

The system suitability of the test method was evaluated [43, 44] by injecting system suitability solution once, and standard solution six times. Resolution between peaks due to Impurity-A and Pre-cholecalciferol from system suitability solution and USP plate count, USP tailing, and %RSD of Cholecalciferol peak area from six replicate injections of standard solution were evaluated. The acceptance criteria defined for system suitability test was resolution between peak due to Impurity-A and pre-cholecalciferol should not be less than 1.5 and %RSD of Cholecalciferol peak area in six replicate injections should not be more than 5.0%, USP tailing for Cholecalciferol peak should not be more than 2.0 and USP plate count for Cholecalciferol peak should not be less than 5000.

3.3 Specificity

Peak purity results for the analyte in forced degradation studies were determined with the PDA detector[45-47] under optimised chromatographic conditions considered homogeneous (purity angle < purity threshold) indicating that no additional peaks were co-eluting with the analyte (Cholecalciferol & it's known impurity) and which indicate the specificity of the method. Resolution was achieved for all known, unknown impurities and degradants.

3.4 Stability of drug substance in analytical solution

The stability of the drug in the analytical solution was verified by preparing the sample solution from drug product and injected at regular time intervals into the HPLC at 5°C and at room temperature. On verifying the formation of additional peaks, it was found that no additional peaks were formed and no increase of present known and unknown impurities by 0.04% level from its initial level to till 42hours, indicating that the sample solution is stable for about 42 hours at room temperature, as well as at 5°C. The stability of the standard solution is evaluated by injecting the standard solution at various time intervals up to 43 hours into the HPLC at 5°C and at room temperature. %RSD of the peak area was monitored from its initial level to 43 hours and found less than 5.0% from its initial level, indicating that the standard

solution is stable for about 43 hours at room temperature, as well as at 5°C.

3.5 Linearity and range [38]

The test concentration for Cholecalciferol is 20.0mg mL^{-1}. Considering the impurity tolerance limit level 1.0% with respect to test concentration of Cholecalciferol. The Response function was determined by preparing a standard solution of Cholecalciferol and impurity-A at different concentration levels ranging from the lower limit of quantification to 150% of the impurity tolerance level. The graph of peak responses of the analytes relative to their corresponding concentrations were determined and found that they fitted straight lines responding to equations. The y-intercepts were close to zero with their confidence intervals containing the origin. The correlation coefficient (r) found less than 0.99 the acceptance threshold suggested for linearity of procedures for the determination of impurity content in bulk drugs. Furthermore, the graph of residuals exhibited random patterns with the residuals passing the normal distribution test ($p < 0.05$), all of which evidenced that the method is linear in the tested range. The Linear graph for cholecalciferol and impurity-A are shown below along with Regression statistics of linearity experimental results are shown in Table-4.

Table 4: Regression statistics of linearity experimental results

Compound	Concentration µg mL^{-1}	Multiple R	Regression equation	F-value	P-Value
Impurity-A	0.02 to 0.30	0.999	y=104478x-13.078	4.60	0.0016
Cholecalciferol	0.02 to 0.30	0.999	y=194233x+206.75	4.60	0.0015

3.6 Determination of limit of quantification and detection (LOQ and LOD[38]

The linearity performed as mentioned above, used for the determination of Limit of Quantification (LOQ) and Limit of Detection (LOD). Residual standard deviation (σ) method was used, and the LOQ and LOD values were predicted using the following formulas and established the precision at these predicted levels. The visuals method also considered assessing the signal to noise ratio of impurities and analyst peak.[36-38] The LOQ and LOD results are tabulated in Table-5.

$$LOQ = \frac{10 \times \sigma}{S}$$

$$LOQ = \frac{3.3 \times \sigma}{S}$$

σ = residual standard deviation of response
S = slope of the calibration curve

3.7 Determination of response factor (RF) with linear calibration curve [38]

Calibration curves for all components were constructed using the peak areas and analyte concentrations in the range reported in Table-4 by linear regression analysis. The linear regression equation containing the slope for all components was summarized in Table-4. The Response Factor (RF) was determined, as the ratio of slope of the regression line of the main drug component (Cholecalciferol) to that for impurity, and is listed in Table-5.

Table 5: Limit of quantification, detection, response factor (LOQ, LOD and RF)

Compound	LOQ		LOD		RF
	µg mL^{-1}	%w/w*	µg mL^{-1}	%w/w*	
Impurity-A	0.02	0.10	0.01	0.05	1.86
Cholecalciferol	0.02	0.10	0.01	0.05	1.00

Note:* %w/w calculated w.r.t. sample concentration (20 µg mL^{-1})

3.8 Accuracy[38]

Accuracy was evaluated by the simultaneous determination of analytes in solution prepared by standard addition method. The experiment was conducted by adding the known amount of impurity (Impurity-A) in the test sample, and cholecalciferol in the placebo (excipient) corresponding to four concentration levels at LOQ, 50%, 100% and 150% by considering the tolerance level i.e.1.0%w/w with respect to test sample concentration. The samples were prepared in triplicate at each level. The quantification of added analyte i.e. impurity-A and Cholecalciferol (%weight/weight) was calculated as per methodology by applying RF (response factor) of impurity. The experimental results revealed that approximately 80% to 120% of the recovery was obtained for the related compounds studied, and %RSD for triplicate preparation of recovery results found less than 10%. Therefore, based on the recovery data (Table-6 and Table-7) the estimation of related compounds that are prescribed in this report was shown to be accurate for its intended purpose, and is adequate for routine analysis.

Table-6: Recovery for impurity-A

Impurity-A	Preparation	% Recovery	Mean	%RSD
LOQ	1	100.9	101.2	0.6
	2	101.9		
	3	100.9		
50%	1	102.8	102.2	0.7
	2	101.5		
	3	102.4		
100%	1	105.9	107.3	1.1
	2	107.6		
	3	108.3		
150%	1	111.5	111.9	0.5
	2	111.7		
	3	112.6		

Table-7: Recovery for Cholecalciferol

Cholecalciferol	Preparation	% Recovery	Mean	%RSD
LOQ	1	108.3	108.0	2.1
	2	110.2		
	3	105.6		
50%	1	107.9	107.4	0.8
	2	106.4		
	3	107.9		
100%	1	103.9	104.8	0.8
	2	105.4		
	3	105.1		
150%	1	106.0	104.7	1.1
	2	104.4		
	3	103.7		

3.9 Method precision and ruggedness[38]

According to the ICH (International Conference on Harmonization of technical Requirements for Registration of Pharmaceuticals for Human Use) ruggedness is considered as the method reproducibility and intermediate precision. Method precision was evaluated by preparing six spike samples by spiking the known impurity (Impurity-A) at 1.0%w/w level with respective of test concentration 20.0 mg mL^{-1}. Intermediate precision was evaluated by different analysts on different HPLC systems, on different columns, and on different days. Experiment was conducted the same as the method precision by spiking the known impurities (Impurity-A) at 1.0%w/w level with respect to test concentration. Results (%w/w) were calculated for known, unknown and total impurities for method precision and intermediate precision experiment. %RSD was calculated for (%w/w) known, unknown and total impurities and found less than 10%. Overall %RSD for (%w/w) known, unknown and total impurities was calculated for method precision and intermediate precision experiment results (n=12 results, six from method precision, and six from intermediate precision) and found less than10.0%. The results for method precisions and intermediate precision were listed in Table-8 and Table-9 revealed that the method has good reproducibility with acceptable precision.

Table-8: Comparison of method precisions and intermediate precision results.

Name	MP*Impurity-A	IP*Impurity-A	MP*SM* unknown	IP*SM* unknown
Spike sample-1	1.13	1.12	0.92	0.97
Spike sample-2	1.15	1.14	0.93	0.98
Spike sample-3	1.16	1.20	0.91	0.95
Spike sampel-4	1.16	1.15	0.91	0.96
Spike sample-5	1.17	1.12	0.91	0.99
Spike sample-6	1.17	1.18	0.91	0.95
Mean	1.16	1.15	0.92	0.97
%RSD	1.30	2.82	0.91	1.69
Overall Mean	1.15		0.94	
Overall %RSD	2.10		3.15	

MP*: Method precision IP*: Intermediate precision. SM*: Single max

Table 9: Comparison of method precisions and intermediate precision results

MP* Name	IP* Total Impurities	Total Impurities
Spike sample-1	2.75	2.74
Spike sample-2	2.79	2.88
Spike sample-3	2.76	2.81
Spike sample-4	2.76	2.75
Spike sample-5	2.77	2.78
Spike sample-6	2.77	2.88
Mean	2.77	2.81
%RSD	0.49	2.20
Overall Mean	2.79	
Overall %RSD	1.71	

MP*: Method precision IP*: Intermediate precision.

3.10 Robustness

The robustness of the method has demonstrated by establishing the system suitability parameter with change in flow rate by ± 0.1 mL min^{-1}, change in column oven temperature by ±5°C, change in organic composition of mobile phase by ± 2% absolute and change in wavelength by ± 5nm. The method was found robust by deliberate change in chromatographic conditions as mentioned above.

4. DISCUSSION

The probable impurities of Cholecalciferol are very similar to the respective structure of drug substances[23]. Cholecalciferol is practically insoluble in water and freely soluble in acetonitrile, methanol and ethanol. The molecular structure (Figure-1 and Figure-2) shows that the related compounds of Cholecalciferol (Impurity-A) and Cholecalciferol are nonpolar in nature. But the presence of Hydroxyl group (-OH) adds some polarity extent in nature[36]. The objective of the method development is to separate all known, unknown impurities and degradants from each other, as well as from the main drug substance with short run time of analysis. Different columns consisting of different stationary phases (RP-C_8 and RP-C_{18}), and different particle sizes of the column (3 µm and 5 µm) were also tested. Considering Cholecalciferol and their known related impurities with nonpolar features, the following mobile phases with gradient elution attempts were used to separate all the impurities which are present and generated during stress studies. a) Orthophosphoric acid buffer with methanol, b) Orthophosphoric acid buffer with acetonitrile, c) Perchloric acid buffer with methanol, d) Perchloric acid buffer with acetonitrile, e) Trifluoroacetic acid buffer with methanol and f)Trifluoroacetic acid buffer with acetonitrile.

4.1 Selection of stationary phase[36,39]

The poor resolution between Cholecalciferol and Cholecalciferol impurity-A, as well as broad peak shape for Cholecalciferol observed, early elution (in void volume) of impurities implies that C_8 stationary phase is not suitable for this application. Hence C_{18} stationary phase was chosen to improve resolution among the peaks and peak shape for Cholecalciferol. The peak shape for Cholecalciferol and resolution among all components improved with Hypersil BDS C18, 150 mm × 4.6mm, 3 µm columns. But the stationary phase is not only the parameter which can give better separation among all impurities. Mobile phase, additives and organic modifiers also play a very important role which leads to the best separation.

4.2 Selection of mobile phase [36,39]

Resolution among the known related impurities, and unknown impurities of Cholecalciferol was found poor using mobile phase with orthophosphoric acid, and perchloric acid buffer. Mobile phase containing Trifluoroacetic acid buffer gives the better resolution among the impurities, but having peak tailing with mobile phase pH more than 3.5.

4.3 Influence of organic modifier and additives [36, 39]

Initial method development started with methanol as an organic modifier. With the use of methanol, disturbed, and noisy baseline was observed throughout the development paths and peak response for related known impurity was low and not precise for accurate quantification. The retention behaviour for all known and unknown impurities was higher, and poor resolution among the unknown impurities was noted. Therefore to improve the response of known and unknown impurities and their resolution among them, acetonitrile has been tried as an organic modifier. With the use of acetonitrile response for all known and unknown impurities was improved and no noisy baseline line was observed. Therefore to increase the response of impurities, acetonitrile was considered. Consequently, it was decided to use a mixture of trifluoroacetic acid and acetonitrile for further method development. After an extensive study, the method has been finalized on Hypersil BDS, 150 mm 4.6 mm, 3µm using variable composition of mobile phase-A (mixture of 0.1% Trifluoroacetic acid Buffer pH 3.0, and acetonitrile in the ratio of 100:900 v/v respectively), and mobile phase-B (mixture of 0.1% Trifluoroacetic acid Buffer pH 3.0, and acetonitrile in the ratio of 900:100 v/v respectively). The mobile phase at a flow rate of 1.5 mL min^{-1} and column compartment temperature kept at 40°C. The detector response for all the components found maximum at 265 nm; hence the typical chromatogram was recorded at this wavelength. The typical HPLC chromatograms for Blank, Placebo, Spike sample, and individual Impurity-A represent in Figure-3, Figure-4, Figure-5 and Figure-6, respectively.

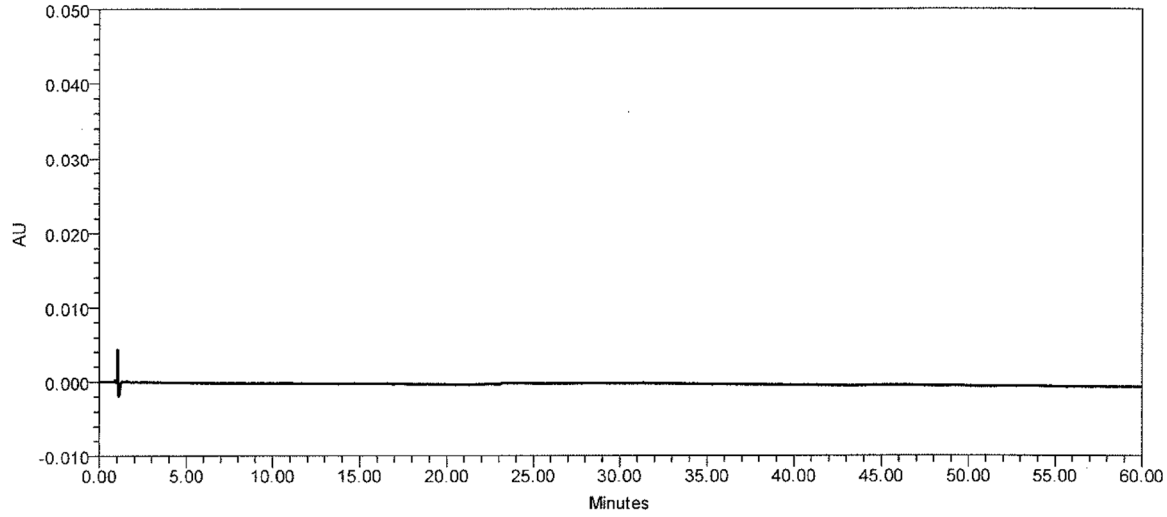

Fig 3. Typical HPLC chromatogram of Blank

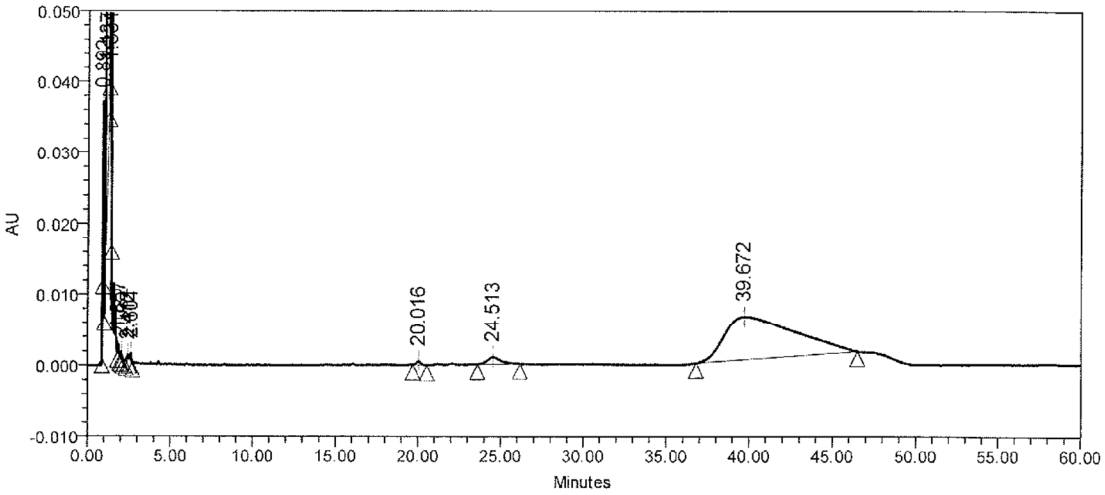

Fig 4. Typical HPLC chromatogram of Placebo

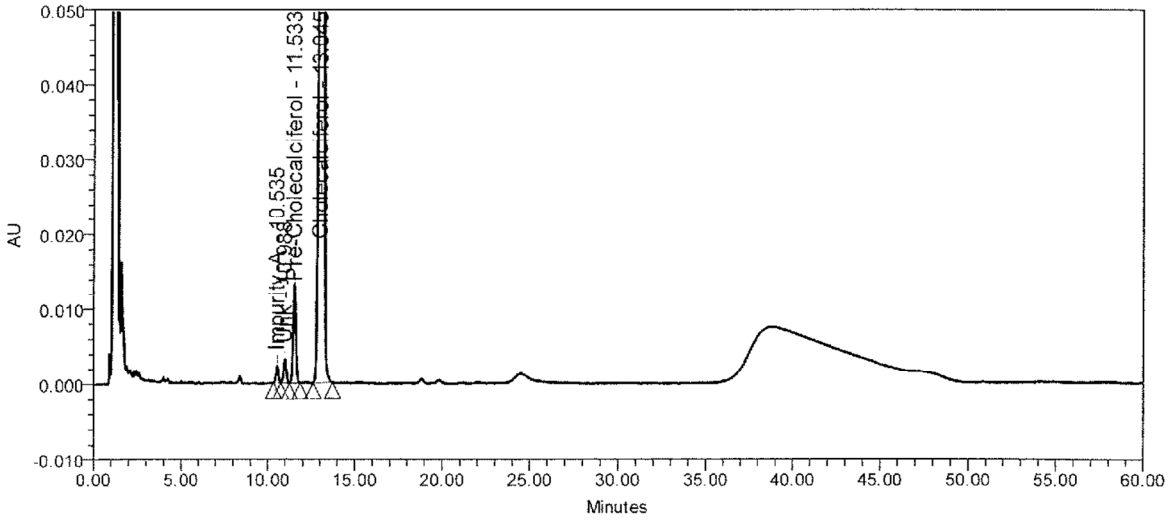

Fig 5. Typical HPLC chromatogram of spike sample

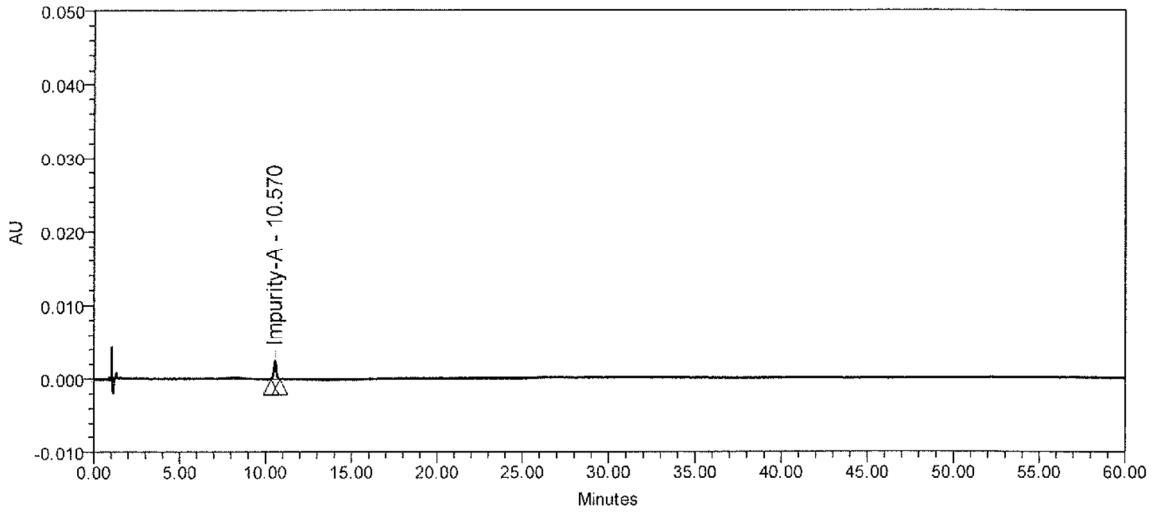

Fig 6. Typical HPLC chromatogram of Individual Impurity-A

The typical HPLC chromatograms of photolytic, thermal and alkaline stressed conditions (force degradation) sample represented in Figure-7, Figure-8 and Figure-9, respectively. In Figure-7 photolytic degradation, it was observed that the unknown impurity at 0.44 RRT found major degradation impurity, and known impurity-A was not increased significantly from its not detected level in the initial sample. In Figure-8 Thermal degradation, it was observed that pre cholecalciferol found major degradation impurity and known impurity-A was not increased significantly from its not detected level in the initial sample. In Figure-9, alkali degradation, it was observed that in unknown impurity at 0.84 RRT found major degradation impurity.

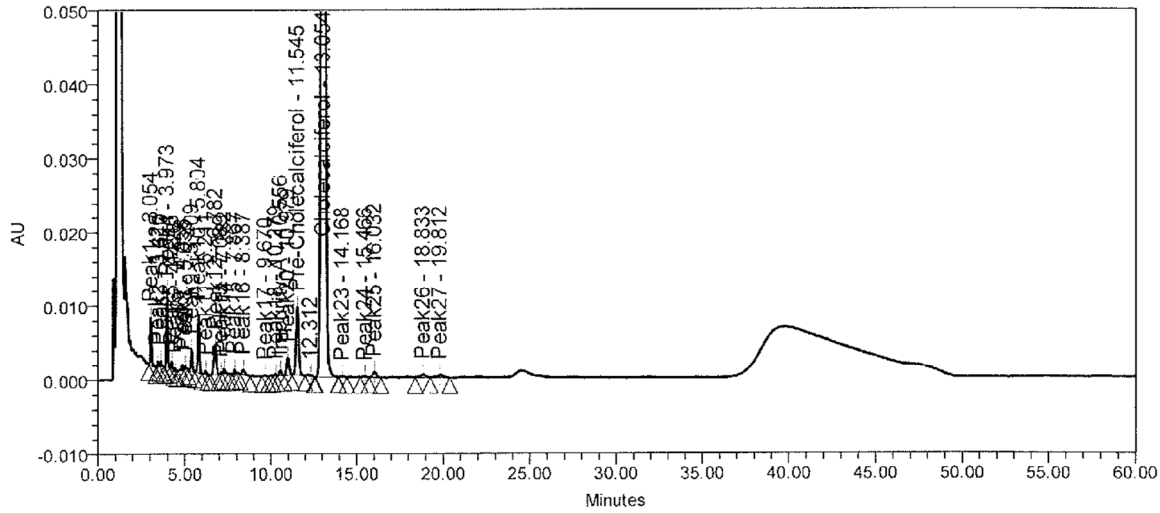

Fig 7. Typical HPLC chromatogram of photolytic degradation

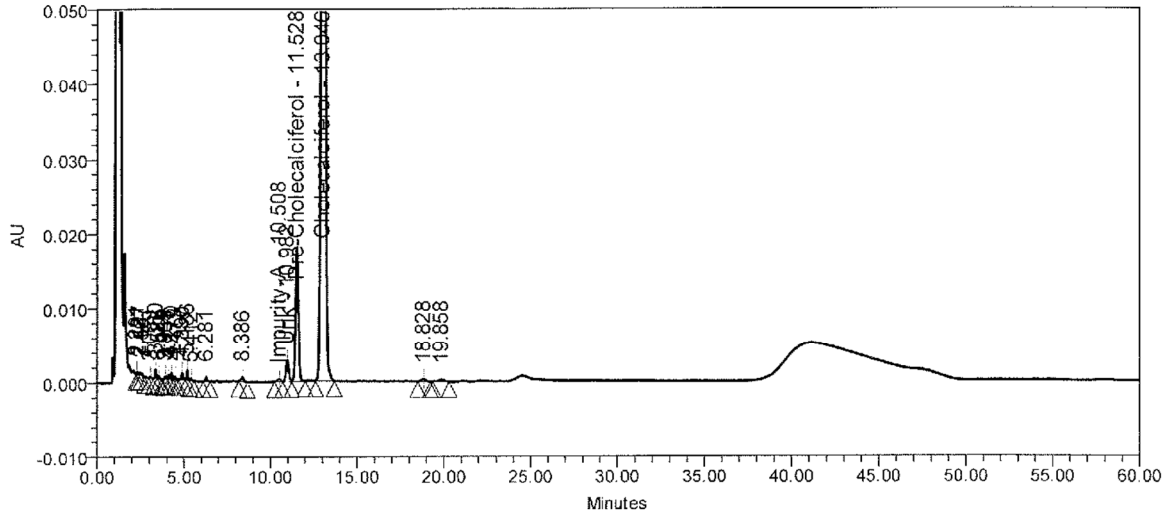

Fig 8. Typical HPLC chromatogram of thermal degradation.

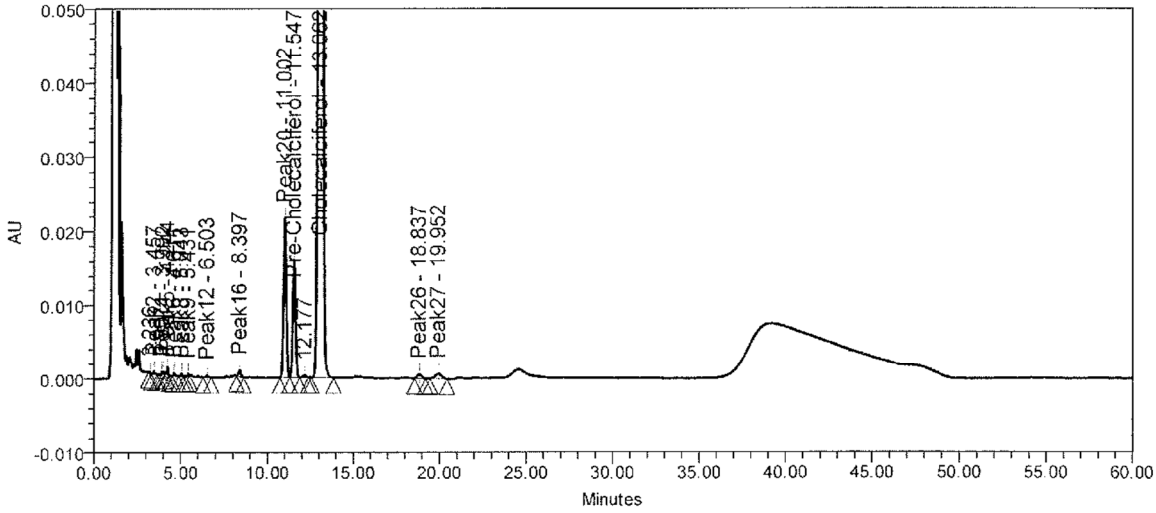

Fig 9. Typical HPLC chromatogram of alkaline degradation

5. CONCLUSION

Analytical method validation experiment results revealed that the newly developed analytical method is linear, accurate, specific and precise in the proposed working range. Forced degradation and specificity experiment results with mass balance prove the stability indicating nature of the method and separate all known, unknown impurities and degradants from each other, as well as from the main drug component (Cholecalciferol). The method is robust to change in flow rate, column oven temperature, change in wavelength and change in organic composition of mobile phase. Hence this cost-effective analytical method can be used for routine analysis as well as to monitor the known, unknown and degradation profile of Cholecalciferol in Cholecalciferol pharmaceutical dosage (tablets).

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7. AUTHORS CONTRIBUTION STATEMENT

Mr. Nitin Mahajan conceptualized, designed and executed the current research work. Dr. Suparna Deshmukh and Dr. Mazahar Farooqui have supervised the work. All Authors have read and agreed to the published version of the manuscript.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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