Stability indicating Analytical method Development and validation of calcitriol by using NP - HPLC

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Abstract

A NP-HPLC is a simple, accurate, and rapid with reliable UV/PDA detector for the simultaneous estimation and validation of calcitriol showed the maximum absorbance at 265 nm. The separation was done by using the column zodiac silica, 4.6 × 250 mm, 5µm. The mobile phase used consisted of Mix 1 volume of propan-1-ol, 2 volumes of methanol, 40 volumes of n-hexane and 60 volumes of ethyl acetate).The chromatographic condition were performed at the flow rate of 1.2ml/min by isocratic elution mode. According to ICH guidelines, the method is validated for accuracy, linearity, precision, intermediate precision, robustness, specificity system suitability, forced degradation study, effect of variation in flow rate, stability of analytical solution and filter validation .The calibration curve were linear at the concentration of 50–150µg/ml for calcitriol were 0.9978 respectively. The percentage recovery of calcitriol were found to be 99.68% to 101.08 % respectively. The developed method was applied successfully for the HPLC analysis of simultaneous estimation and validation of calcitriol combined dosage form.

Key words: Calcitriol, HPLC, Method development, Validation, ICH Guidelines

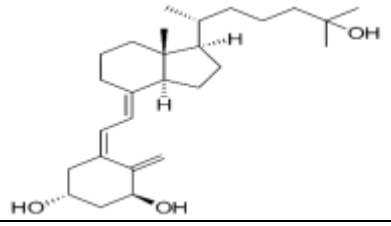
Introduction

Calcitriol is a hormonally-active, synthetic vitamin D analog prescribed to treat hypocalcemia, osteoporosis, and the prevention of corticosteroid-induced osteoporosis. Systemic calcitriol is FDA indicated to control hypocalcemia in patients on chronic renal dialysis, secondary hyperparathyroidism in those with chronic kidney disease not yet on dialysis, and hypocalcemia in patients with hypoparathyroidism and pseudohypoparathyroidism. There are also other approved indications. This activity reviews the mechanism of action, adverse event profile, toxicity, dosing, pharmacodynamics, and

monitoring of calcitonin, pertinent for clinicians and all inter professional team members to ensure proper utilization of calcitriol therapy.

According to the literature review[1-9].There is no analytical methods was developed and validated in HPLC

Table 1 Description of calcitrol

Description	**Calcitriol**
Molecular formula	$C_{27}H_{44}O_3$
Molecular weight	416.6 gm/ml
Solubility	Insoluble in water, Soluble in ethanol, methanol ethyl acetate, THF.
Chemical structure	

Materials and Methods

Chemicals & Reagents:

Propan-1-ol, Methanol, n-Hexane and Ethyl acetate are HPLC Grade. Sodium hydroxide, Hydrochloric acid, Hydrogen peroxide and Sodium sulfate are Analytical Research Grad.

Instruments and Chromatographic Conditions

High Performance Liquid Chromatography system (Agilent) with UV detector, Analytical Balance, Ultra sonicator and Labouratory balance were used. The HPLC (Agilent) system equipped with UV/ PDA detector, Silica, (250 X 4.6mm, 5µm) was used to achieve chromatographic separation. Mobile phase was composed of 1 volume of propan-1-ol, 2 volumes of methanol, 40 volumes of n-hexane and 60 volumes of ethyl acetate v/v ratio. The mobile phase was filtered through 0.45 µ membrane filter, degassed and injected onto the column at 1.2 ml/min flow rate. Load volume of the drug solution was 100 µl, and the detection was recorded at 265 nm.

Preparation of mobile phase:
Mix 1 volume of propan-1-ol, 2 volumes of methanol, 40 volumes of n-hexane and 60 volumes of ethyl acetate.

Diluent: Mobile phase.

Peak identification solution: (Pre-Calcitriol)
Weigh accurately about 5 mg of calcitriol WS/BPCRS into a 100ml volumetric flask, add 30 ml of diluent shake to dissolve and make up the volume with diluent. Pipette out transfer 10ml of above solution into 20ml volumetric flask and keep in the water bath at the temperature of 80°C for 30 minutes. Cool and inject.

Standard Preparation: (0.25mcg/ml of calcitriol)
Weigh accurately about 5 mg of calcitriol WS/BPCRS into a 100ml volumetric flask, add 30 ml of diluent shake to dissolve and make up the volume with diluent. Dilute 5 ml of above solution to 100ml with diluent. Further, dilute 5 ml of above solution into 50ml volumetric flask dilute to volume with diluent.

Check standard Preparation: (0.25mcg/ml of calcitriol)
Weigh accurately about 5 mg of calcitriol WS/BPCRS into a 100ml volumetric flask, add 30 ml of diluent shake to dissolve and make up the volume with diluent. Dilute 5 ml of above solution to 100mlwith diluent. Further, dilute 5 ml of above solution into 50ml volumetric flask dilute to volume with diluent.

Finished Product:

Sample preparation for Calcitriol capsules 0.25 mcg: (0.25mcg/ml of Calcitriol)

Stage 1:
Take 100 ml amber colour volumetric flask and place glass funnel on top of the flask. Select and weigh accurately about 25 capsules, cut each capsule with help of sharp cutter or blade by holding each capsule by using SS forceps. Cutting of capsules process should be done above the glass funnel to avoid any medicament loss. After cutting, place each capsules with medicine on the glass funnel. Rinse the forceps, cutter, capsule shell and sides of the funnel with at least 3 X 3ml of mobile phase until capsule shell is free from medicament. Use maximum 15 ml of mobile phase for rinsing. After washing, transfer all empty capsule shells into the same 100ml volumetric flask using forceps. Shake the volumetric flask by hand for about 3 minutes.

Stage 2:
Transfer the solution and capsule shell from the 100ml volumetric flask into a 25ml amber volumetric flask with the help of glass funnel. Rinse the 100 ml volumetric flask with 2 ml of mobile phase and transfer the rinsing solution into the same 25ml amber volumetric flask by using funnel. Then, remove each capsule shell by using forceps from the glass funnel, rinse the funnel with 2ml of mobile phase and make up to the volume with mobile phase.

Sample preparation for calcitriol capsules: (0.25mcg/ml of calcitriol)

Stage 1:

Take 100 ml amber colour volumetric flask and place glass funnel on top of the flask. Select and weigh accurately about 25 capsules, cut each capsule with help of sharp cutter or blade by holding each capsule by using SS forceps. Cutting of capsules process should be done above the glass funnel to avoid any medicament loss. After cutting, place each capsules with medicine on the glass funnel. Rinse the forceps, cutter, capsule shell and sides of the funnel with at least 3 X 3ml of mobile phase until capsule shell is free from medicament. Use maximum 15 ml of mobile phase for rinsing. After washing, transfer all empty capsule shells into the same 100ml volumetric flask using forceps. Shake the volumetric flask by hand for about 3 minutes.

Stage 2:

Transfer the solution and capsule shell from the 100ml volumetric flask into a 50ml amber volumetric flask with the help of glass funnel. Rinse the 100 ml volumetric flask with 2 ml of mobile phase and transfer the rinsing solution into the same 50ml amber volumetric flask by using funnel. Then, remove each capsule shell by using forceps from the glass funnel, rinse the funnel with 2ml of mobile phase and make up to the volume with mobile phase.

Medicament:
Sample preparation for calcitriol capsules 0.25 mcg: (0.25mcg/ml of calcitriol)

Weigh about 4000mg of medicament (Equivalent to 6.25mcg of calcitriol) into a 25ml volumetric flask, add 15ml of diluent shake through mechanical shaker for 15 minutes and sonicate for 5 minutes. Make up the volume to 25ml with diluent.
Procedure:

- Inject the blank, peak identification, standard, check standard and sample preparation as per following sequence.

- Make entry in instrument and column usage log book and calculate from the values obtain.

Results & Discussion

The analytical method was optimized and validated in accordance with the current ICH guidelines and to accomplish the vision of accuracy, linearity, precision, intermediate precision, robustness, specificity system suitability, forced degradation study, effect of variation in flow rate, stability of analytical solution and filter validation**.**

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present

For evaluation: Blank solution, Placebo solution, Sample solution and standard solution were injected into the HPLC system In below the chromatograms obtained in fig 1,2,3,4,5

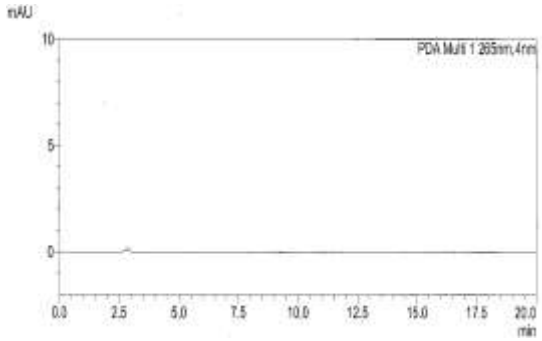


Fig :1 Blank

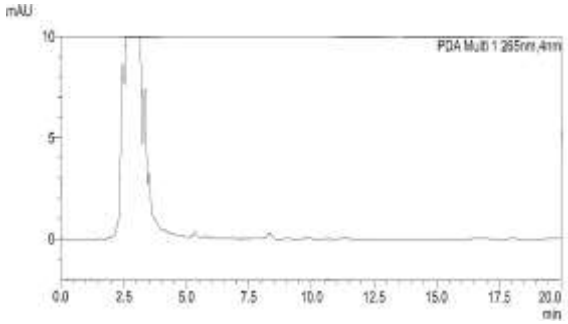


Fig :2 Placebo

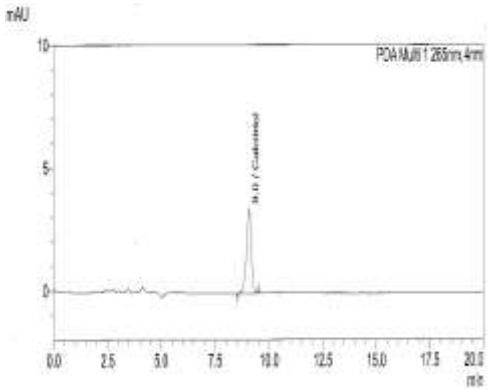


Fig 3: Standard

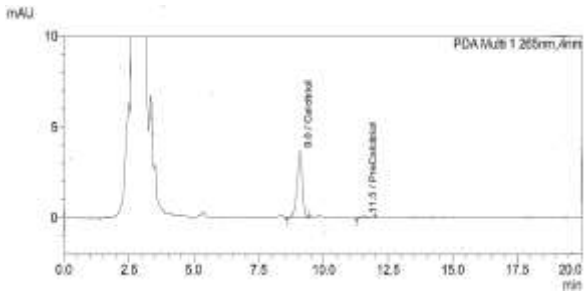


Fig 4:Sample for 0.25mcg: (Finished product)

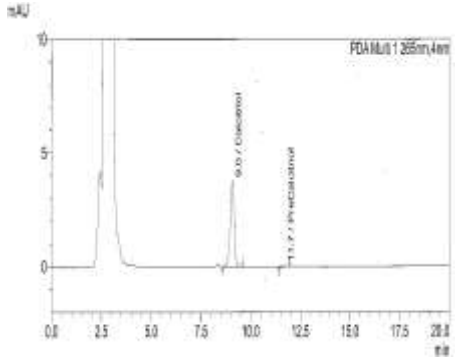


Fig 5: Sample for 0.25mcg: (Finished product)

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 content 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 150.0%).

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.

Calcitriol:

Accuracy concentration	**Area**	**Amount added (ppm)**	**Amount recovered (ppm)**	**% Recovery**	**Average**	**% RSD**
50% -1	24376	0.1261	0.1257	99.6827	99.2	0.5
50% -2	24095		0.1243	98.5725		
50% -3	24254		0.1251	99.2069		
100% -1	49444	0.2523	0.2551	101.1097	100.6	0.8

100% -2	49399		0.2549	101.0305		
100% -3	48704		0.2513	99.6036		
150% - 1	74313		0.3834	101.2945		
150% - 2	73481	0.3785	0.3791	100.1585	100.8	0.5
150% - 3	74145		0.3826	101.0832		

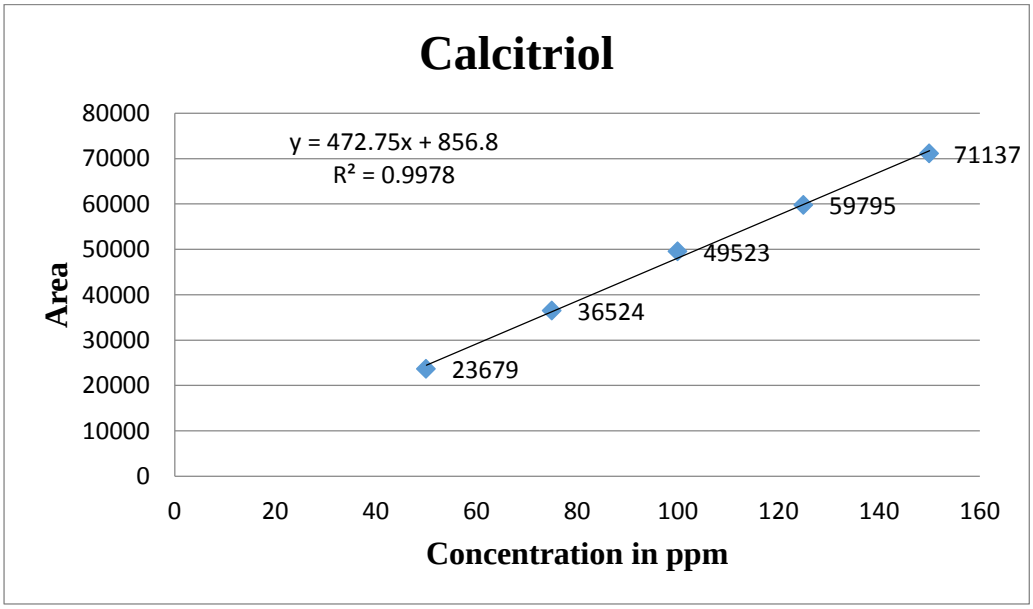
Linearity and range:

The linearity of the method is established by performing five test concentrations from 80.0% to 120% 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.

The details are given below.

For calcitriol:

Linearity curve for calcitriol:



Graphical representation

Linearity and Range parameter		
Correlation coefficient. (r) of sum of Calcitriol and pre calcitriol	0.998	NLT 0.99
% of y-Intercept of sum of Calcitriol and pre calcitriol	1.7	± 5.0

% RSD for lower Concentration and higher concentration	Low (50%)	0.6	NMT 2.0
	High (150%)	1.6	

Precision:

a) System Precision:

As per protocol Standard Solution of Calcitriol was prepared and injected in six replicates into HPLC System. The system suitability parameters were calculated as per protocol and the results are tabulated as shown below.

b) Method precision:

As per protocol 6 replicate sample solution of calcitriol capsules 0.25mcg were prepared and injected into HPLC System. The method precision parameters are calculated as per protocol and the results are tabulated as shown below.

C) Intermediate precision (Reproducibility):

Intermediate precision is demonstrated by analysing the same batch of Calcitriol capsules 0.25mcg as in precision with 6 replicate samples, in the different lab and a different Analyst, using different Instrument and different column on a different day.

Preparation	**% of Calcitriol capsules 0.25 mcg (Medicament)**	
	Method Precision	**Intermediate Precision**
1	103.8800	105.4400
2	103.8800	105.6800
3	104.0400	104.9200
4	104.4800	105.3200
5	103.9600	104.5200
6	103.6000	104.9200
Average	104.0	105.1
Average from 12 samples	104.6	
SD	0.2891	0.4230
SD from 12 samples	0.6973	
%RSD	0.2	0.4
%RSD from 12 samples	0.6	
Confidence limit	0.2	0.3
Confidence limit from 12 samples	0.5	

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

a) Mobile phase variation (± 10 % organic content)

System suitability criteria should pass as per system suitability requirements in in all altered conditions. The % assay difference between altered conditions to initial condition should not be more than ± 2.0%.

Preparation	Variation-1		Actual		Variation-2	
	FP	Medicament	FP	Medicament	FP	Medicament
1	103.1200	105.0400	103.0000	104.7600	104.3600	102.1600
2	102.1200	103.1600	102.2400	103.6000	103.2400	101.1600
Average	102.6	104.1	102.6	104.2	103.8	101.7
SD	0.7071	1.3293	0.5374	0.8202	0.7919	0.7071
%RSD	0.6	1.2	0.5	0.7	0.7	0.6

b) Flow rate (± 0.2 ml/min)

System suitability criteria should pass as per system suitability requirements in in all altered conditions. The % assay difference between altered conditions to initial condition should not be more than ± 2.0%.

Preparation	Low-1.0 ml/min		Low-1.2 ml/min		Low-1.4 ml/min	
	FP	Medicament	FP	Medicament	FP	Medicament
1	102.0000	103.8800	103.0000	104.7600	102.6400	103.7200
2	104.0000	102.9200	102.2400	103.6000	103.3200	103.8000
Average	**103.0**	**103.4**	**102.6**	**104.2**	**103.0**	**103.8**
SD	**1.4142**	**0.6788**	**0.5374**	**0.8202**	**0.4808**	**0.0565**
%RSD	**1.3**	**0.6**	**0.5**	**0.7**	**0.4**	**0.0**

c) Column Temperature (± 5°C)

System suitability criteria should pass as per system suitability requirements in in all altered conditions. The % assay difference between altered conditions to initial condition should not be more than ± 2.0%

Preparation	Low-28°C		Actual-30°C		High-32°C	
	FP	Medicament	FP	Medicament	FP	Medicament
1	102.5600	103.4400	103.0000	104.7600	103.9600	104.4800

2	102.0800	103.8000	102.2400	103.6000	103.4000	104.4000
Average	**102.3**	**103.6**	**102.6**	**104.2**	**103.7**	**104.4**
SD	**0.3394**	**0.2545**	**0.5374**	**0.8202**	**0.3959**	**0.0565**
%RSD	**0.3**	**0.2**	**0.5**	**0.7**	**0.3**	**0.0**

Forced degradation study:

Study – 01:
For calcitriol capsules 0.25 mcg:
Acid stress:

Concentration of degradant solution : 0.1 N methanolic HCl
Concentration of neutralizing solution : 0.1 N methanolic NaOH
Degradant added in ml : 2 ml
Neutralizing solution added in ml : 2 ml
Exposure Time : 6 hrs in bench top
Temperature : 25°C

Base Stress:

Concentration of degradant solution : 0.1 N methanolic NaOH
Concentration of neutralizing solution : 0.1 N methanolic HCl
Degradant added in ml : 2 ml
Neutralizing solution added in ml : 2 ml
Exposure Time : 6 hrs in bench top
Temperature : 25°C

Oxidation:

Concentration of degradant solution : 1%
Degradant added in ml : 2 ml
Exposure time : 6 Hrs in bench top
Temperature : 25°C

Thermal degradation:

Exposure time : 24 Hrs
Temperature : 80°C

(Photolytic degradation) UV:

Exposure : 200 watts hour/m^2 in photo stability chamber.

As per protocol standard solution of calcitriol is prepared and injected in six replicates into HPLC system. The system suitability parameters were calculated as per protocol and the results are tabulated as shown below.

For calcitriol:

No of Injection	**RT(Minutes)**	**Area**
01	7.6	50731
02	7.6	50709

03	7.6	50951
04	7.6	50426
05	7.6	50637
06	7.6	50669
Average	7.6	50687
SD	0.0	169
%RSD	0.0	0.3

For calcitriol capsules 0.25 mcg:

Stressed conditions	**Content in percentage**	**Calcitriol**	
		percentage Degradation	**Peak purity index**
Unstressed sample	100.4	NA	NA
Acid	71.9	28.4	1.000
Base	72.4	27.9	1.000
Oxidation	100.6	-0.2	0.999
Heat	104.2	-3.8	1.000
UV	101.0	-0.6	1.000

Humidity degradation:

Humidity : 90% RH
Temperature : NMT 25°C
Duration : 7 days

Area : Desiccator

For Calcitriol:

No of Injection	**RT(Minutes)**	**Area**
01	8.8	48516
02	8.8	47464
03	8.8	47471
04	8.8	47394
05	8.8	47601
06	8.8	47579
Average	8.8	47671
SD	0.0	421
%RSD	0.0	0.8

For Calcitriol capsules 0.25 mcg:

Stressed conditions	**Content in %**	**Calcitriol**	
		% Degradation	**Peak purity index**
Unstressed sample	101.6	NA	NA

Acid	95.5	6.0	1.000
Base	95.1	6.4	1.000
Humidity	102.1	-0.5	1.000
Light	102.0	-0.4	1.000

Stability of analytical solution:

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

Time interval	**Area**		
	Standard	**Sample (Finished product)**	**Sample (Medicament)**
Initial	48464	50351	51516
12^{th} Hours	48405	50355	51646
24^{th} Hours	48397	50503	51207
36^{th} Hours	48113	50489	51046
48^{th} Hours	48548	50760	51026
Average	48385	50492	51288
SD	163.8117	166.2852	280.1413
%RSD	0.3	0.3	0.5

Time interval	**Sample (Finished product)**		**Sample (Medicament)**	
	Results in %	**% RSD**	**Results in %**	**% RSD**
Initial	102.5600	NA	104.3200	NA
12th Hours	102.7200	0.1	104.7200	0.2
24th Hours	103.0400	0.3	103.8400	0.3
36th Hours	103.6000	0.7	104.1200	0.1
48th Hours	103.2400	0.4	103.1600	0.7
Average	103.0	NA	104.0	NA
SD	0.4141		0.5833	
%RSD	0.4		0.5	

Discussion The present work is based on the quantification of calcitriol dosage by using HPLC with UV/ PDA detector. The physical and chemical parameters were chosen based on calcitriol. Based on the system suitability the stationary phase was chosen. zodiac silica, 4.6 X 250mm, 5µm was used for separation of analytes for evaluating the parameters. The optimization of mobile phase done on the different preliminary trials. Mobile phase containing Mix 1 volume of Propan-1-ol, 2 volumes of methanol, 40 volumes of n-hexane and 60 volumes of ethyl acetate were found to ideal combination for the system suitability parameters. The mobile phase ratio, flow rate, wavelength, column temperature was done with different preliminary trials for evaluation of chromatographic peak resolution with minimum solvent consumption. The filter validation studies is the critical part of qc tests samples were filtered by using 0.45 µm PVDF filters and filtrate were collected and analyzed by HPLC for the quantification of API. Centrifuged samples were used as controls for recovery of 100% and to calculate the analyte binding in a 0.45 µm PVDF filter. The solution stability studies were also done by storing the sample and standard solution in the proper room temperature for determining the stability of samples and standard solution. The developed methods were validated as per ICH guidelines which specific, simple and reliable which successfully applied for the quantification of calcitriol without any excipient interference.

Conclusion:

The cumulative percentage RSD of peak area for Standard and sample solution obtained with different time intervals and percentage 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.

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