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Development And Validation of Rp-HPLC Method for The Estimation of Calcipotriene in Tablet Dosage Form

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ABSTRACT
This study presents the development and validation of a Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method for the quantitative determination of Calcipotriene, an anti-cancer agent. The chromatographic separation was achieved using an Agilent C18 column (250 mm × 4.6 mm, 5 µm) with methanol and water (0.05% OPA, pH 3.5) in the ratio of 65:35 v/v as mobile phase at a flow rate of 0.7 ml/min. Detection was carried out at 264 nm with a retention time of approximately 6.8 minutes. The method was validated according to ICH guidelines, demonstrating linearity in the concentration range of 10-50 µg/ml with a correlation coefficient (r^2) of 0.999 and linear regression equation y = 22.17x + 21.41. The developed method showed good resolution, symmetrical peak shape (symmetry factor 0.85), and theoretical plates (11465), indicating method efficiency. This simple, precise, and accurate analytical method can be effectively applied for routine analysis of Calcipotriene in pharmaceutical formulations.

KEYWORDS: High-Performance Liquid Chromatography, anti-cancer agent, Calcipotriene, ICH guidelines, linearity.

INTRODUCTION

ANALYTICAL CHEMISTRY [1-2]

Analytical Chemistry is a measurement of science consisting of a set of powerful ideas and methods that are useful in all fields of science and medicine. It seeks ever improved means of measuring the chemical composition of natural and artificial materials. This branch of chemistry, which is both theoretical, and a practical science, is practiced in a large number of laboratories in many diverse ways while analytical method, is a specific application of a technique to solve an analytical problem. Methods of analysis are routinely developed, improved, validated, collaboratively studied and applied. The discipline of analytical chemistry consists of qualitative and quantitative analysis

1.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY [3]

The term 'Chromatography' covers those processes aimed at the separation of the various species of a mixture on the basis of their distribution characteristics between a stationary and a mobile phase.

1.2 MODES OF CHROMATOGRAPHY [4]

Modes of chromatography are defined essentially according to the nature of the interactions between the solute and the stationary phase, which may arise from hydrogen bonding, Vander walls forces, electrostatic forces or hydrophobic forces are based on the size of the particles (e.g. Size exclusion chromatography)
Different modes of chromatography are as follows -

- Normal Phase Chromatography
- Reverse Phase Chromatography
- Reverse Phase – ion pair Chromatography
- Ion Chromatography
- Ion-Exchange Chromatography
- Affinity Chromatography
- Size Exclusion Chromatography

1.3 STRATEGY FOR METHOD DEVELOPMENT OF HPLC

Selection of suitable chromatography for organic compounds,

- First reverse phase should be tried.
- If not successful, then, normal phase should be taken into consideration. Before making experimentation with ion-exchange or ion-pair chromatography, ion suppression by pH controls and reverse phase chromatography should be

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tried for ion forming organic compounds. Ion-pair chromatography should be preferred to Ion Exchange chromatography.

1.4 METHOD DEVELOPMENT AND OPTIMIZATION [5-10]

During the optimization stage, the initial sets of conditions that have evolved from the first stages of development are improved or maximized in terms of resolution and peak shape, plate counts asymmetry, capacity, elution time, detection limits, limit of quantitation, and overall ability to quantify the specific analyte of interest.

Optimization of a method can follow either of two general approaches -

1. Manual
2. Computer driven

The manual approach involves varying one experimental variable at a time, while holding all others constant, and recording changes in response. The variables might include flow rates, mobile or stationary phase composition, temperature, detection wavelength, and pH this univariate approach to system optimization is slow, time consuming and potentially expensive. However, it may provide a much better understanding of the principles and theory involved and of interactions of the variables.

In the second approach, computer driven automated methods development, efficiency is optimized while experimental input is minimized. Computer driven automated approaches can be applied to many applications. In addition, they are capable of significantly reducing the time, energy and cost of virtually all- instrumental methods development.

The various parameters that include to be optimized during method development,

1. Mode of separation
2. Selection of stationary phase
3. Selection of mobile phase
4. Selection of detector

1.5 METHOD VALIDATION [6-7]

Method validation can be defined as establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics.

Method validation is an integral part of the method development; it is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, and potency of the drug substances and drug products. Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose.

Method Validation, however, is generally a one-time process performed after the method has been developed to demonstrate that the method is scientifically sound and that it serves the intended analytical purpose.

All the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, and detection and data evaluation. For chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters including:

- Specificity /Selectivity
- System suitability
- Precision
  - Repeatability
  - Intermediate precision
  - Reproducibility
- Accuracy
- Linearity
- Range
- Limit Of Detection
- Limit Of Quantitation
- Robustness

EXPERIMENTAL PART AND RESULT

- **DRUG PROFILES:**

"Calcipotriene" [11-13]

Structure:

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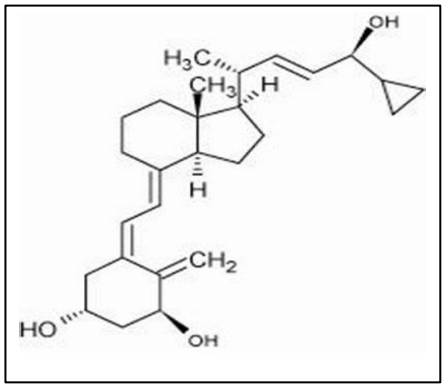

Fig No.6: Structure of Calcipotriene.

IUPAC Name : (1R,3S,5E)-5-{2-[(1R,3aS,4Z,7aR)-1-[(2R,3E)-5-cyclopropyl-5- hydroxypent-3-en-2-yl]-7a-methyl-octahydro-1H-inden-4-ylidene] ethylidene}-4-methylidenecyclohexane-1,3-diol
Molecular Formula: $C_{27}H_{40}O_3$
Molecular Weight: 412.6047 g/mol
Appearance: White fine powder
Solubility: soluble in, acetonitrile, sparingly soluble in ethanol
Category: Anti- cancer agents

a) Chromatographic conditions :

The following chromatographic conditions were established by trial and error and were kept constant throughout the experimentation.

1.	HPLC	Agilent (Auto sampler)Gradient SystemDAD
2.	Software	Chemstation
3.	Column	(Agilent) C_{18} column (4.6mm x 250mm)
4.	Particle size packing	5 □m
5.	Stationary phase	C-18 (Agilent)
6.	Mobile Phase	MEOH: Water (0.05% with OPA pH 3.3) 65 : 35
7.	Detection Wavelength	264 nm
8.	Flow rate	0.7ml/min
9.	Temperature	33° C (Ambient)
10.	Sample size	20 □l
11.	pH	3.0
12.	Run Time	15 min
13.	Filter paper	0.45cm

Selection of mobile Phase

Sr.No.	Mobile Phase
1.	80% MEOH +20% Water (pH 3.5 adjust with 0.05 % OPA) Flow 0.7 ml/min abs at 264 nm 50 mcg(250 X 4.6 X5)
2.	70% MEOH +30 Water (pH 3.5 adjust with 0.05 % OPA) Flow 0.8 ml/min abs at 264 nm 50 mcg(250 X 4.6 X5)
3	60% MEOH +40% Water (pH 3.5 adjust with 0.05% OPA) Flow 0.9 ml/min abs at 264 nm 50 mcg(250 X 4.6 X5)
4	50% MEOH +50% Water (pH 3.5 adjust with 0.05% OPA) Flow 1 ml/min abs at 264 nm 50 mcg(250 X 4.6 X5)
5	70% MEOH +30%Water (pH 3.5 adjust with 0.05% OPA) Flow 0.7 ml/min at 264 nm 50 mcg(250 X 4.6 X5)
6	65% MEOH +35%Water (pH 3.5 adjust with 0.05% OPA) Flow 0.7 ml/min at 264 nm 50 mcg(250 X 4.6 X5)

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Chromatographic conditions applied on the Preparation of standard solution:
Preparation of std. Calcipotriene solution: (Stock I)
An accurately weighed quantity, 10 mg of Calcipotriene was dissolved in methanol in a 10 ml volumetric flask and volume made up to 10.0 ml to produce a solution of 1000 ug/ml.

Selection of mobile phase:
Each mobile phase was vacuum degassed and filtered through 0.45µ membrane filter. The mobile phase was allowed to equilibrate until mobile phase by baseline was obtained. The standard solution containing solution of Calcipotriene was run with different individual solvents were tried to get a good and stable peak. From the various mobile phases tried, mobile phase containing MEOH and Water (0.05%OPA) was selected since it gave sharp, well resolved peaks with symmetry within the limits and significant reproducible retention time for Calcipotriene.

Studies of Calibration plot: -
Optimization of Chromatographic condition:
The following chromatographic conditions were established by trial and error and were kept constant throughout the analysis.
Column : C18 (250 mm× 4.6 mm) Particle size packing : 5µm
Detection wavelength : 264 nm Flow rate : 0.7 ml/min
Temperature : Ambient
Sample size : 20 µl
Mobile phase : MEOH: Water (0.05%OPA) (65:35)

Procedure for calibration curve of Calcipotriene
The mobile phase was allowed to equilibrate with stationary phase until OPA by baseline was obtained. From the freshly prepared standard stock solution, pipette out 10 mg Calcipotriene in 10 ml of volumetric flask and diluted with mobile phase. From it 0.1, 0.2, 0.3, 0.4 and 0.5 of solution were pipette out in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 10, 20, 30, 40 and 50µg/ml of Calcipotriene. Samples were injected and peaks were recorded at 264 nm as the graph plotted as concentration of drug verses peak area is depicted .

Calibration Experiment:
HPLC Method :
a) Preparation of Calibration curve standard:
The above standard stock solution (1000 µg/ml) of Calcipotriene was diluted with mobile phase to yield five calibration curve (cc) standards with concentrations of 10, 20, 30, 40 and 50 µg/ml of Calcipotriene.
b) Selection of detection Wavelength:
Standard solutions were scanned in the range of 200-400nm, against 10 ml MEOH and volume make with Methanol solvent system as reference Calcipotriene were showed absorbance maxima (lambda max) at 264 nm respectively.
c) Calibration standard drug and regression equation data:
From the standard stock solution of Calcipotriene, different concentration were prepared respectively in the range of 10-50 µg/ml for Calcipotriene measured at 264 nm. The calibration curves were plotted and Regression equation data presented.
d) Calibration runs and regression analysis:
These calibration standard solutions were analyzed in three replicates using the under mentioned chromatographic conditions.

- Analytical column: AgilentC18 Column (100mm x 4.6mm, 2.5µm particle size).
- Injection volume : 20µl.
- Flow rate : 0.7 ml/min.
- Mobile phase : MEOH: Water (0.05%OPA) (65:35 % V/V).
- Detection : 264 nm.

Validation of method for analysis of Calcipotriene
The developed method was validated as per ICH guidelines.
Linearity:
Linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.
Determination:
The linearity of the analytical method is determined by mathematical treatment of test results obtained by analysis of samples with analyte concentrations across the claimed range. Area is plotted graphically as a function of analyte

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concentration.Percentage curve fittings are calculated**.**

Acceptance Criteria:

The plot should be linear passing through the origin.

Correlation Coefficient should not be less than 0.999.

Preparation of standard stock solution for linearity:

Average weight of marketed formulation (equivalent to 10 mg of Calcipotriene) were weighed and transferred to 10 mL volumetric flask & diluent was added to make up the volume. Sonicated for 10 min with occasional swirling. 0.1 ml of this solution diluted up to 10 ml volumetric flask with diluents was added to make up the volume.

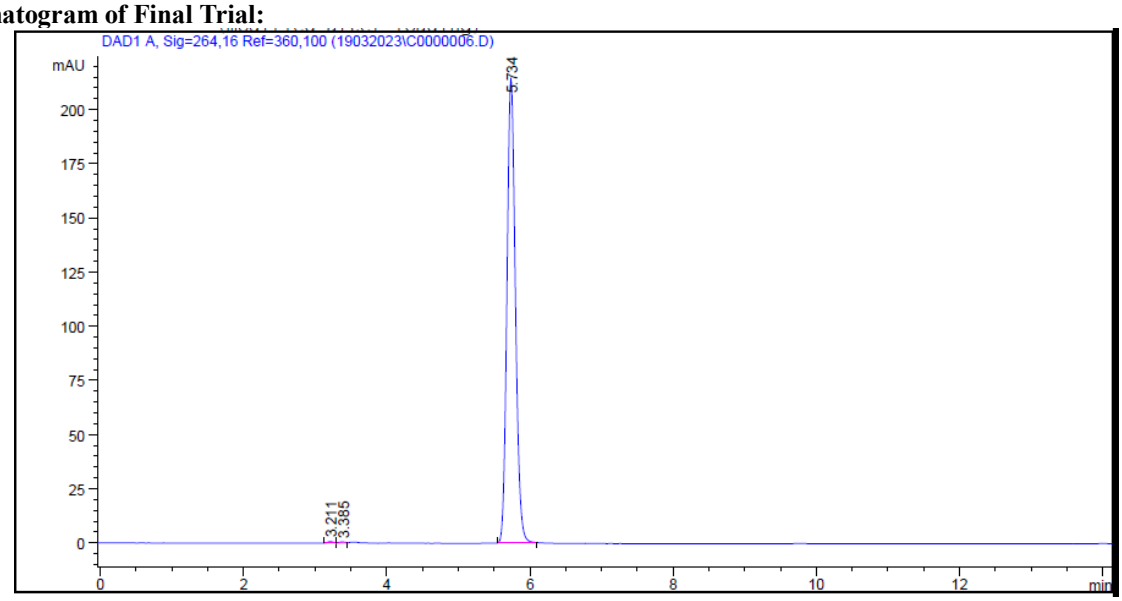
Preparation of linearity solution:

A series of standard preparations of working standard of were prepared.

Table of linearity for RP -HPLC Method

Concentration (µg/mL)
Calcipotriene
10
20
30
40
50

Chromatogram of Final Trial:



Representative Chromatogram of Calcipotriene on 65 % MEOH +35% Water (pH 3.5 adjust with 0.05 % OPA) Flow 0.7 ml/min abs at 264 nm 50 mcg (250 X 4.6 X 5)

Chromatogram result of Calcipotriene on 65% MEOH +35% Water (pH 3.5 adjust with 0.05% OPA) Flow 0.7 ml/min abs at 264 nm 50 mcg (250 X 4.6 X5)

Drug name	**R. T**	**AREA**	**TH. PLATES**	**SYMM**
Calcipotriene	6.824	1751.0327	11465	0.85

Calibration experiment

- **RP-HPLC Method:**

The absorption spectra were recorded in the wavelength region of 200 - 400 nm in UV-Spectrophotometric methods. Beer-Lambert's law was followed in the conc. range of **10-50** µg/ml for Calcipotriene. Linearity was observed with correlation co- efficient (r2) values **0.999 with linear equation for y = 22.17x+ 21.41** .

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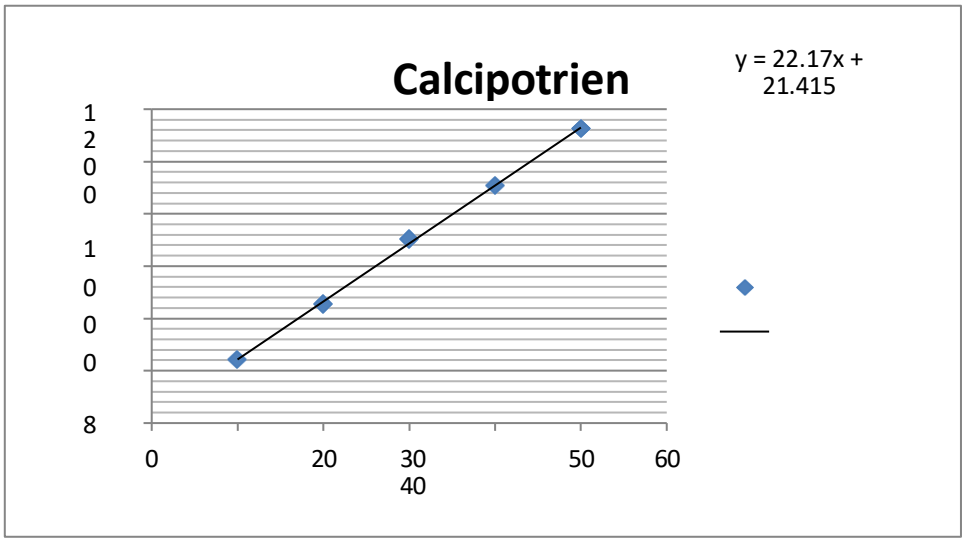
Linearity data for Calcipotriene

Method	Conc µg/ml	Peak area(µV.sec)		Average peak area (µV.sec)	S.D. of Peak Area	% RSD of Peak Area
		1	2			
HPLC Method	10	241.9301	244.12	9.99	243.03	1.56
	20	453.8302	453.82	19.50	453.83	0.00
	30	701.2855	705.73	30.76	703.51	3.15
	40	904.8309	910.24	39.96	907.54	3.83
	50	1123.475	1125.8	49.76	1124.68	1.70
	Equation		y = 22.17x – 21.41			
	R^2		0.999			

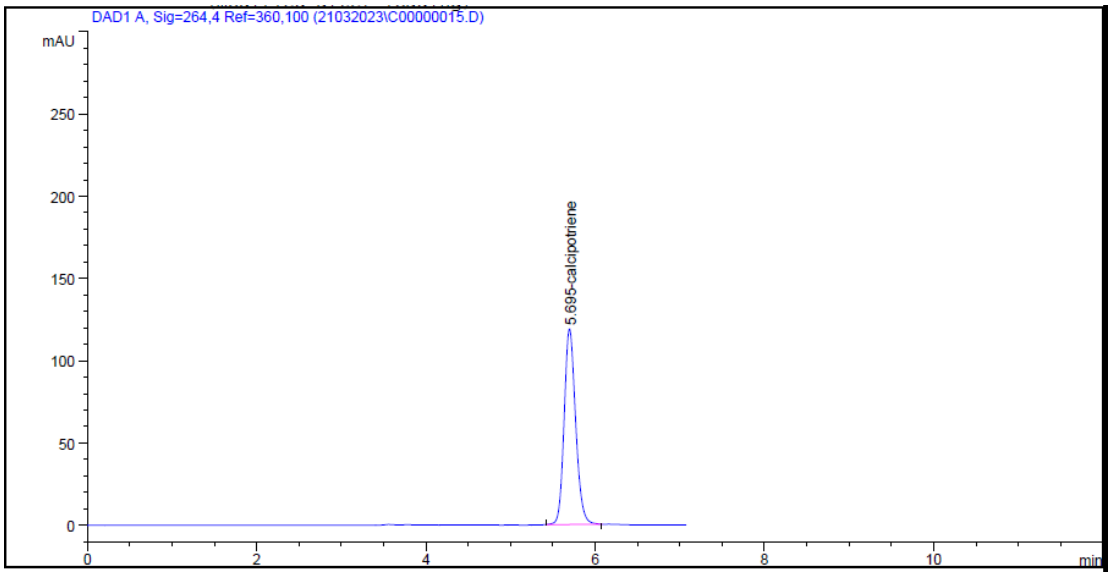


Calibration curve of Calcipotriene

Method Validation:

Linearity:

From Calcipotriene standard stock solution, different working standard solution (10- 50 µg/ml) were prepared were prepared in mobile phase 20 µl of sample solution was injected into the chromatographic system using fixed volume loop injector. Chromatogram was recorded.



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Chromatogram of linearity LIN 50 mcg microgram/ml-2

DRUG NAME	R.T	AREA	T.P	SYMM.
Calcipotriene	5.695	1125.88062	8951	0.82

Linearity of Calcipotriene

Concentration ug/ml	**Area Calcipotriene**
Method	**HPLC**
10	243.03
20	453.83
30	703.51
40	907.54
50	1124.68

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