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"DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR SIMULTANEOUS ESTIMATION OF CALCIPOTRIOL AND BETAMETHASONE IN THEIR FORMULATION OF RP-HPLC"

Hrishika Gupta, Utkarsh Sharma
Advance Institute of Biotech & Paramedical Sciences, Kanpur

ABSTRACT

The creation and verification of a Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) technique for the simultaneous estimation of betamethasone and calcipotriol in pharmaceutical formulations. Betamethasone, a corticosteroid, and calcipotriol, a synthetic vitamin D3 analogue, are frequently used in combination for the treatment of psoriasis, and a suitable RP-HPLC method was optimized for their separation and quantification. The method used a C18 column with a mobile phase consisting of a mixture of acetonitrile and water in a specific ratio, and detection was conducted at 240 nm. The chromatographic conditions were optimized for the best resolution, peak shape, and retention time of both compounds. The process was proved as specific, linear, accurate, precise, detection limit (LOD), LOQ and precision, robust, and system suitability in line with ICH recommendations. The two drugs' calibration graphs also portrayed satisfactory linearity ($r2 > 0.999$) within the operating range of concentration. Recovery ranged between 98% and 102% through the process that reflected very good accuracy. The process was proven more precise via low %RSD values both for intra-day and inter-day variability. Calcipotriol's LOD and LOQ were 0.04 µg/mL and 0.12 µg/mL, respectively, whereas betamethasone's were 0.05 µg/mL and 0.15 µg/mL. Pharmaceutical products were successfully assayed by applying the method, with results congruent with the labeled material. For day-to-day quality control of betamethasone and calcipotriol in their combined dose formulations, this RP-HPLC is reliable, reproducible, and suitable.

Keywords: RP-HPLC method development, betamethasone, calcipotriol, simultaneous estimation, pharmaceutical formulations, treatment for psoriasis, C18 column, mobile phase, detection at 240 nm, ICH validation standards, specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), system suitability, recovery rate, quality control.

INTRODUCTION

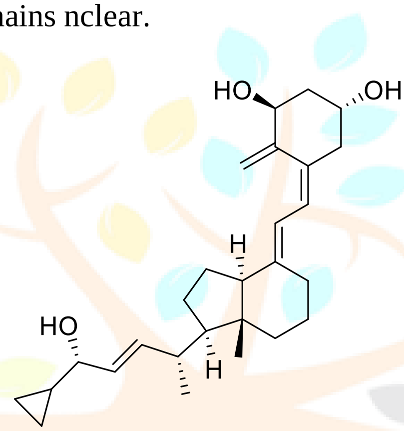
The Value of Developing Analytical Methods in Pharmaceutical Quality Control

A significant part of the pharmaceutical quality control is the development of correct and efficient analytical methods for the combined estimation of a number of active pharmaceutical ingredients (APIs) in a product[1]

Betamethasone, a potent corticosteroid, and calcipotriol, an artificial derivative of vitamin D3, are often combined to be used together in the treatment of a number of dermatological conditions, such as psoriasis[2]. The combination therapy takes advantage of the complementary action of these drugs, with betamethasone possessing anti-inflammatory activity and calcipotriol stimulating proliferation of healthy skin cells.

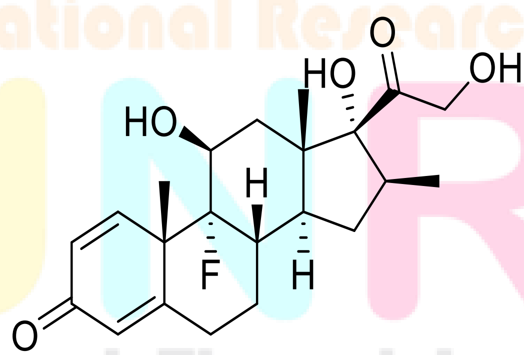
Mechanism of Action of Calcipotriol and Betamethasone

It is unclear exactly how calcipotriol works to prevent psoriasis at its worst. Despite being less than 1% as active as calcitriol (Vit D) in controlling calcium metabolism, it has been demonstrated to have a similar affinity for the Vitamin D receptor. The VDR, which is a member of the steroid/thyroid receptor superfamily, is present on the cells of numerous tissues, such as the kidney and thyroid etc. It is believed that calcipotriol's binding to the VDR alters the transcription of genes linked to cell differentiation and proliferation in T cells, which are known to be involved in psoriasis. Its metabolism remains nclear.



calcipotriol

In BP and USP, betamethasone is an official medication. Betamethasone's exact mechanism is that it can be absorbed in significant levels to have systemic effects when applied topically, especially to large areas, when the skin is damaged, under occlusive dressings, or when administered intranasally.[3],[4].



betamethasone

The importance of developing and validating analytical methods

Analytical methods must be developed, validated, and transferred as part of any pharmaceutical development effort. Although techniques achieve the goals required at each stage of drug development, successful method development demonstrates that laboratory resources are optimized.
The establishment and confirmation of analytical procedures are essential to pharmaceutical manufacturing, drug development, and drug discovery. It involves determining a drug substance's toxicity and purity [5].The goal of this

validation is to demonstrate that procedures related to drug research and manufacturing, production, and analytical testing can be carried out efficiently and consistently[6]. Drug analysis is helpful in analytical chemistry for the separation, estimation, and quantification of chemical substances derived from both natural and synthetic sources. Usually, these compounds consist of one or more chemical components.One Qualitative and quantitative analysis are the two main categories that comprise the analytical chemistry process. Only the available samples are estimated in qualitative analysis, but the total amount of elements in a compound should be determined in quantitative analysis[33] [34].

Need for Analytical Method Development in Modern Pharmaceutical Analysis

Many medications have recently been released onto the market, and demand for them is rising daily.The recently developed medications are either a new kind of medication or a modified form of an existing medication. These medications are introduced in accordance with the pharmacopoeia's available situation and the marketed medications. To report on the best therapeutic agents for withdrawal on the market, pharmacopoeia had to be used in the medication development process. Pharmacopoeias may not always include the analytical profile of medications at the time of development. Therefore, it is crucial to prepare the key analytical techniques for the creation of novel medications.[33][35] [36].

Analytical Techniques for Pharmaceutical Analysis

Several techniques, such as HPLC, are helpful for the analytical process when analyzing large or small compounds.

Liquid Chromatography-Mass Spectrometry, HPTLC, and others.

These analytical methods are generally employed in conjunction with mass spectrometry and other techniques for the detection of substances[37]. One of the most helpful methods is HPLC, which was a crucial and improved method for drug analysis. Additionally, the liquid chromatography-mass spectrometry technology was helpful in studying drug metabolism and was crucial for analyzing pharmaceutical medications.

Moreover, the LC-MS technology proved useful for the research of drug metabolism and was essential in the analysis of pharmaceutical drugs. Further, these

Methods can be applied to separate and identify the pharmaceutical potential of medications from various synthetic and natural sources, and also for pharmaceutical drugs analysis, estimate, and identification containing impurities and deteriorated products [38].

Key Considerations in Analytical Method Development

Key Considerations	**Description**
Sensitivity	Essential for detecting and quantifying low concentrations of analytes.
Accuracy	Crucial for obtaining true and reliable measurement results.
Preferred Range for Drug Analysis	guarantees that the technique can be applied within the intended concentration range.
Precision	Needed to ensure reproducibility and reliability of results.
Data Requirement	Necessary to resolve any analytical challenges and optimize the method.

Validation Process in Analytical Method Development

Accuracy is necessary when developing a procedure. The method validation process, which involves verifying the documents at any stage of the method creation process, is also a part of the method development process. To analyze the method, the different needs for document validation are:

• Assurance of quality

• Acceptance from the designated international agencies for product development

• Registration of pharmaceutical or pesticide products should be mandatory

• Validation should only take place after testing is completed

• And the product should be validated once the quality control department has fulfilled its obligations [39].

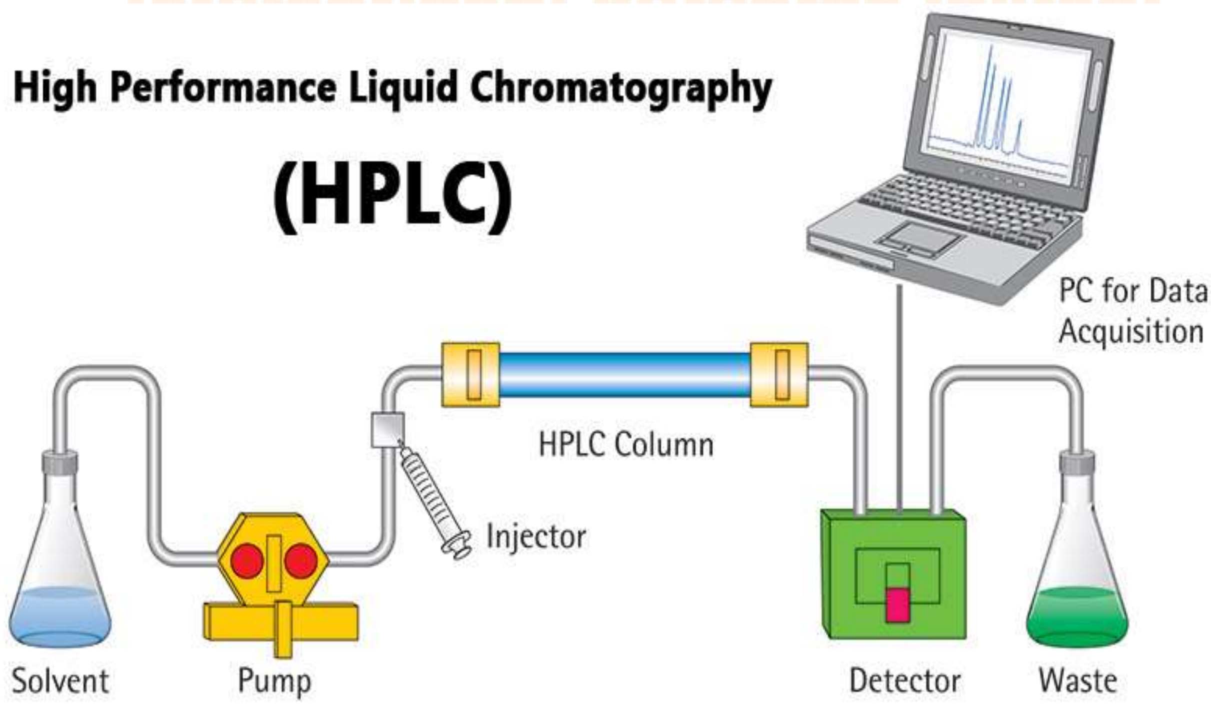
Advancements in Analytical Method Development

The development of analytical devices has led to recent advancements in analytical methodologies. Analysis time has decreased, accuracy and precision have increased, and analysis expenses have decreased due to advancements in analytical instrumentation and analytical method development. As a result, most pharmaceutical companies are investing a substantial amount of money to construct advanced analytical labs.

For API, excipients, drug products, and associated compounds, etc., analytical methods are created and verified. It has consequently become a crucial component of the regulatory organization's requirements. [7]

Role of RP-HPLC in Pharmaceutical Analysis

The most popular analytical method is HPLC. It is among the most used instruments for determining and measuring the strength of medicinal ingredients and products. Before a method is released for usage in the Quality Control department, two extremely important steps are carried out: analytical method development and validation. [8]



HPLC

Validation Parameters for RP-HPLC Methods

The development of the RP-HPLC process entails improving chromatographic parameters, choosing appropriate stationary and mobile phases, and controlling conditions to accomplish precise analyte parting. In order to guarantee dependability, adherence to ICH regulations, validation of RP-HPLC procedures is essential. Accuracy, precision, specificity, detection and quantitation limits, linearity, robustness, and ruggedness are significant validating factors. Validation of the RP-HPLC technology is to the advantage of pharmaceutical quality control as it diminishes manufacturing expense and product defects while enhancing productivity and quality. Verified methods and established procedures enhance laboratory consistency and ensure repeatable and reliable results. Overall, RP-HPLC and its validation are important in maintaining stringent guidelines for pharmaceutical analysis, enhancing the quality of products, and achieving efficient and accurate pharmaceutical ingredient separation and quantitation. [9].

Recent Trends and Future Directions in Analytical Method Development

Summing up, RP-HPLC is a sensitive analytical tool for the pharmaceutical field, so designing precise and reliable RP-HPLC techniques becomes crucial to test drugs. Its efficiency, selectivity, and sensitivity have, in recent years, risen exponentially, thereby enhancing its importance as an analysis tool for chemical compounds in a wide variety of applications. [10] [11]. It becomes vital in building analytical procedures in order to secure the security and quality of medicines [40]. The demand for technologies able to analyze multiple active pharmaceutical ingredients (APIs) within a single drug at the same time has increased in importance based on the evolution of the requirements of the drug industry [41]. The outcome of drug development initiatives relies upon the ability to design techniques which not only bear accurate results but also are economical and time-savings [42] It. In addition to the more traditional ones such as HPLC, hyphenated technologies some of the novel and cutting-edge technologies being implemented into the analysis process [43]. Increased sensitivity and accuracy are enabled by these developments, which also reduce analytical time and boost drug development and testing throughput [44][45]. Aside from enhancing process effectiveness, the goal is to ensure that the analytical methods fulfill the stringent regulatory requirements set by regulatory bodies like the US FDA, EMA, and ICH [46]. Because of these advancements, only pharmaceutical goods that are safe, effective, and of the highest caliber make it to market [47].

QbD in Analytical Method Development

The incorporation of QC and QA measures in the course of the drug development process also contributes significantly to the successful implementation of sophisticated analytical methods [48]. The potential presence of contaminants, degradation products, and other components that may impact the stability and safety of the drug product must be considered while developing the method [49]. The guarantees that medication products will meet the right criteria during their lifetime are offered by rigorous testing and validation of analytical methods [50]. Contemporary drug products are increasingly complex with the application of biologics, biosimilars, and combination therapies, which requires the development of more sophisticated analytical methods that are capable of addressing these challenges [51].Pharmaceutical companies can enhance patient outcomes by enhancing their competitiveness on the international stage and compliance with regulatory standards through continuous improvement in analytical processes [52].

The introduction of biologics and biosimilars, and the growing demand for more personalized medicine, have grown the demand for robust and versatile analytical techniques in pharmaceutical analysis [47]. These drugs often contain complex molecular structures that complicate stability and characterization [43]. Although traditional methods such as HPLC perform well for small molecules, they may not be sufficient to study complex biologic formulations or large proteins [44]. This has resulted in the growing adoption of hybrid methods, where chromatographic protocols are combined with NMR spectroscopy or MS [52]. These hybrid methods enable detection of impurities or degradation products that would otherwise be beyond the capabilities of classical analysis and enable comprehensive study of drug metabolites and glycosylation profiles [51].Pharmaceutical corporations are more effective in ensuring the safety, efficacy, and stability of complex biologic drugs through increased resolution, sensitivity, and selectivity of these state-of-the-art methods [45].

The use of quality by design principles, which focus on developing processes that are dependable and capable of delivering consistent quality throughout the production cycle, has also gained increasing popularity in the pharmaceutical industry [48]. This shift has led to an increased proactive development of methods in which analytical methodologies are now viewed as a part of the whole pharmaceutical development process and not merely validated following development [41]. Implementing QbD concepts ensures that potential sources of variability are identified early on, and this makes analytical technique validation easier [46].Pharmaceutical developers, for instance, might build techniques that are more dependable and flexible in response to shifting circumstances by evaluating the impact of manufacturing procedures, equipment performance, and raw material variability on the analytical results [52]. The finished product will always satisfy the relevant regulatory requirements and function as planned in the clinical setting thanks to this all-encompassing approach to drug development [48].

Consequently, this review's objectives are to give a thorough overview of the development, assess and summarize the body of existing literature, talk about the difficulties and factors to be taken into account, critically analyze method validation parameters, examine the real-world applications of RP-HPLC , and highlight recent developments [45][50].

Body:

1. Materials used during the process

Category	Details
Chemicals and Reagents	
Mobile Phase	Acetonitrile and Phosphate buffer (pH 7.0) or Methanol and Water in suitable ratios for optimal separation.
Standards	Calcipotriol standard (99% purity) and Betamethasone standard (99% purity).
Sample Formulation	Calcipotriol and Betamethasone combination formulation (e.g., ointment, cream, or gel).
Buffer	Phosphate buffer (0.01 M, pH 7.0) for mobile phase preparation.
Solvents	Acetonitrile (HPLC grade), Water (HPLC grade).
Instrumental Details	
Instrument	High-Performance Liquid Chromatography system.
Detector	UV Detector (wavelength: 254 nm or suitable for optimal detection).
Column	Reverse phase C18 column (4.6 mm × 150 mm, 5 µm particle size).
Flow Rate	1.0 mL/min
Injection Volume	20 µL
Column Temperature	30°C (or as optimized for best separation).
Chromatographic Conditions	
Mobile Phase Composition	Acetonitrile: Phosphate buffer or Methanol: Water (in appropriate proportions).
pH	pH 7.0 (adjusted with NaOH or HCl as needed).
Detection Wavelength	254 nm (or optimized wavelength for maximum sensitivity).

Run Time	10-15 minutes (depending on separation efficiency).
Sample Preparation	
Sample Extraction	Weigh an appropriate amount of sample formulation and extract with suitable solvent (e.g., methanol).
Filtration	Filter the sample solution using 0.45 µm nylon membrane filter before injecting into the HPLC system.
Validation Parameters	
Specificity	Ensure that neither degradation products nor excipients are interfering.
Linearity	Prepare different concentrations of standard solutions for both drugs (e.g., 5, 10, 15, 20, 25 µg/mL).
Precision	Evaluate intra-day and inter-day precision by repeating the analysis.
Accuracy	Calculate the recovery rate by spiking known quantities of both medications in a formulation to ascertain recovery.
LOD	Determine LOD using signal-to-noise ratio (typically 3:1).
LOQ	Determine LOQ using signal-to-noise ratio (typically 10:1).
Robustness	Assess method stability by changing chromatographic conditions (e.g., flow rate, pH, column temperature).
Data Analysis	
Calibration Curve	Plot peak area versus concentration of the standard solutions to establish the calibration curve for both drugs.
Statistical Analysis	Perform statistical tests (e.g., t-test, ANOVA) for precision, accuracy, and linearity validation.

2) Selection of Chromatographic Conditions:

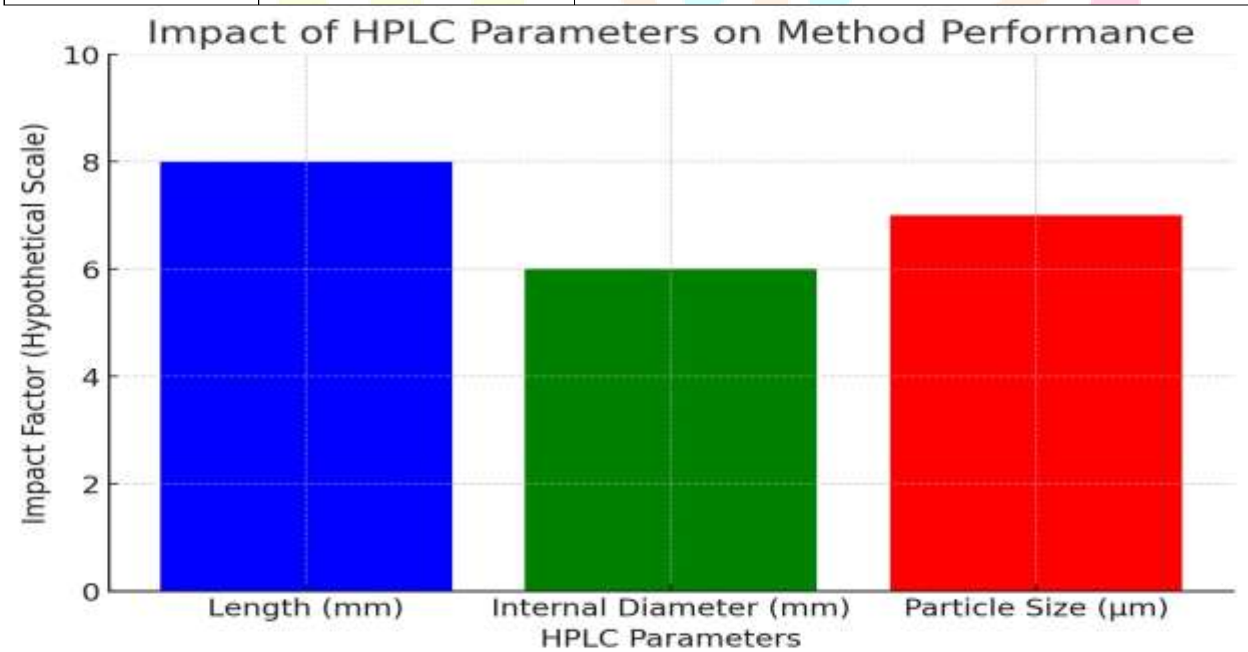
a) Choice of RP-HPLC Column

i. Column Type:

- **Reversed-Phase Column (C18):**
  - Because it works well with hydrophobic and moderately polar substances, a C18 column (octadecylsilane) is the most used stationary phase for the simultaneous measurement of calcipotriol and betamethasone.
  - By use of hydrophobic interactions between the analytes and the nonpolar stationary phase, it guarantees effective retention and separation.
  - Because of their adaptability, C18 columns can be used in a variety of pharmaceutical applications.[12]

ii. Column Dimensions:

Parameter	**Specification**	**Description**
Length	150 mm or 250 mm	Longer columns (250 mm) improve resolution for complex matrices, while shorter columns (150 mm) reduce analytical time while maintaining adequate separation.
Internal Diameter	4.6 mm	A standard analytical diameter ensures an optimal balance between solvent usage and sensitivity.
Particle Size	5 µm	Provides robustness and good separation efficiency for normal analytical use.



iii. Rationale for C18 Column Selection:

- **Resolution and Retention:** Clear retention periods and symmetrical, sharp peaks are guaranteed by sufficient interaction with the functional groups of the analytes.
- **Broad Application Range:** Adaptable to a variety of substances, offering flexibility in the analysis of betamethasone and calcipotriol.
- **Peak Symmetry:** Assures precise quantification by minimizing peak tailing.
- **Column Robustness:** Compatible with organic solvents such as acetonitrile and methanol, and stable over a broad pH range (3–8).

b) Optimization of Mobile Phase Composition

- **Mobile Phase Components:**
  - Aqueous Buffer: Ionic strength and pH uniformity are guaranteed by using a phosphate buffer (10–20 mM potassium dihydrogen phosphate).
  - Organic Solvent: Because of its superior solubility of hydrophobic chemicals and reduced viscosity, acetonitrile is frequently chosen over methanol.
- **Gradient vs. Isocratic Elution:**
  - For simplicity, start with an isocratic elution. For improved separation, use gradient elution if resolution is insufficient.
- **Suggested Ratio:** Adjust the ratio according to resolution and retention times, starting with an organic solvent to aqueous buffer ratio of 60:40 to 70:30.

c) Determination of pH

- **pHRange:** For better retention and peak symmetry, choose a pH between 4.0 and 7.0 where both analytes stay non-ionized.
- **Buffer Adjustment:** To reduce peak tailing and improve resolution, use sodium hydroxide or phosphoric acid to raise the pH of the buffer.
- **Crucial Consideration:** To avoid column deterioration and preserve performance, make sure it is compatible with the stationary phase.

- **Temperature and Flow Rate Optimization :**

To balance resolution and analysis time, start at 1.0 mL/min. To improve peak form and separation, adjust the flow rate between 0.8 and 1.5 mL/min as necessary.

- **Column Temperature:**
  - Start at room temperature (25°C). If there are problems with peak broadening or retention, raise the temperature to 30 to 40°C.
  - Higher temperatures accelerate , decrease viscosity, and improve peak shape.

d) UV Detection Wavelength Selection

- **Absorption Maxima Determination:** Determine the λ-max for betamethasone (~240 nm) and calcipotriol (~264 nm) using a UV-visible spectrophotometer.
- **Compromise Wavelength:** Choose a single wavelength where both analytes show high absorbance, for as 254 nm or 265 nm.
- **Detection Mode:** Use diode-array detection (DAD) to detect and accurately quantify both analytes at the same time.

Parameter	Optimized Condition
Column	C18, 4.6 mm × 150/250 mm, 5 µm
Mobile Phase	Phosphate buffer (pH 4.5) : Acetonitrile (70:30)
pH	4.5
Flow Rate	1.0 mL/min
Temperature	30°C
Detection Wavelength	254

3) Sample Preparation Procedure for HPLC Analysis

Step 1: Weighing the Sample

1. **Accurate Weighing:** Weigh precisely a predetermined quantity of the formulation (e.g., 1 g for ointments or creams) using an analytical balance. To guarantee accuracy in ensuing computations, note the precise weight.[13]

Step 2: Extraction of Analytes

1. **Transfer and Addition of Solvent:**

- Transfer the weighed sample into a suitably sized, clean volumetric flask (e.g., 50 mL or 100 mL).
- To fully dissolve the analytes, add an extraction solvent (such as methanol or acetonitrile) in an amount of 20–30 mL.[14]

2. **Sonication:**

- To help the active components dissolve completely, sonicate the mixture for 15 to 30 minutes in an ultrasonic bath.
- Keep an eye on the solution and make sure it stays at room temperature, letting it cool if any heat is produced.[15]

Step 3: Filtration

1. **Filtration Process:**

- Use a 0.45 µm syringe filter to remove any undissolved ingredients, excipients, and particulate particles from the extracted solution.
- Gather the filtrate for further dilution into a sterile volumetric flask.[16]

Step 4: Dilution

1. **Dilution of Filtered Sample:**

- Using the same extraction solvent, dilute the filtrate to a specified volume (such as 50 mL or 100 mL).
- To guarantee a uniform solution, mix well.[17]

Step 5: Final Dilution for HPLC

1. **Aliquot Preparation:**

- Pipette a 1 mL aliquot of the stock solution, then dilute it further using the extraction solvent or the mobile phase.

- Adjust the concentration such that it falls within the range of the calibration curve that was established throughout the process of method validation.[18]

2. **Transfer to HPLC Vial:**
   - Mix well and transfer the final diluted solution into a clean, labeled HPLC vial for analysis.[19]

Preparation of Standard Solutions:

1. Stock Solutions:
- Weigh accurately calcipotriol and betamethasone reference standards (e.g., 10 mg of each).
- Dissolve each in methanol or acetonitrile and dilute to a known volume (e.g., 10 mL) to prepare standard stock solutions.

2. Working Standards:
- Prepare a series of working standard solutions by diluting the stock solutions with the mobile phase to cover the calibration range (e.g., 1–50 µg/mL).

1) Preliminary studies

a. Conduct Initial Experiments

- **Column:** Because of its non-polar stationary phase, which aids in compound separation according to polarity, the C18 column is perfect for hydrophobic substances like betamethasone and calcipotriol [20].
- **Mobile Phase:** A 50:50 methanol:water mixture is a suitable beginning point for fundamental separation since a range of chemicals can be efficiently eluted by this solvent system with balanced polarity [21]. If necessary, adding acetonitrile can aid in enhancing peak resolution [22].
- **Buffer:** In HPLC, phosphate buffers with a pH of 4.5 are frequently employed to preserve stability and prevent the analytes from becoming significantly ionized, which improves retention and separation [23].
- **Detection Wavelength:** Because calcipotriol and betamethasone include high absorbance aromatic and conjugated systems, UV detection at 254 nm is frequently utilized for pharmaceutical chemicals [24].

b. Optimize Injection Volume

- Injection Volume: Research indicates that keeping the injection volume between 10 and 20 µL helps prevent column overflow while preserving sensitivity [25]. Broad peaks and decreased resolution can result from large injection volumes [26].
- Peak Intensity: To guarantee precise quantification and sensitivity without adding baseline noise, the injection volume must be maintained within the detector's linear range [22].

c. Optimize Runtime

- **Runtime Reduction:** By optimizing the gradient or flow rate, initial lengthy runtimes that aid in full elution can be shortened without compromising separation [27]. Improving laboratory efficiency frequently depends on cutting down on analysis time [20].
- **Flow Rate:** Increasing the flow rate can shorten runtime, but it can also result in less efficiency and resolution. For any particular separation, an ideal flow rate needs to be determined [23].

d. Monitor Key Parameters

- **Retention Factor (k'):** For effective separation, a retention factor of 2 to 10 is optimal. Excessive retention times or poor peak resolution may arise from a k' value outside of this range [20].
- **Resolution (Rs):** According to Gerard et al. (2015), resolution values more than two guarantee that peaks are sufficiently separated, which is essential for precise analysis.[26]

- **Peak Tailing Factor (Tf):** Peak tailing should be minimized to prevent chromatogram distortion. Peak symmetry is guaranteed with a Tf value ≤1.5.[22]
- **Theoretical Plates (N):** Column efficiency is measured in terms of the number of theoretical plates. Sharper peaks, which are essential for precise quantification and separation, are correlated with higher plate numbers.[21]

e. Outcome of Preliminary Trials

- Before carrying out more thorough testing, you should be able to improve chromatographic conditions by identifying any significant separation problems using the early trials [24]. Common problems like peak overlap or excessive runtime can be avoided utilizing injection volume, mobile phase composition, and runtime optimization.

4) Method Validation

a. Specificity

- **Goal:** Make sure that excipients, contaminants, or degradation products won't interfere with the method's ability to distinguish and measure the APIs.
- **Procedure**:

1. The following solutions should be injected:

- A blank solution without any APIs.
- A placebo is a mixture of excipients without any APIs.
- Standard betamethasone and calcipotriol solutions.

2. Examine the chromatogram to determine the APIs' baseline separation. There are no interfering peaks within the APIs' retention periods.

- **Acceptance Criteria:**
  - No peak should cross over or obstruct the peaks of the APIs.
  - According to Snyder et al. (2010), the resolution between the APIs and any nearby peaks should be greater than two.[20]

b. Linearity

- **Goal:** For both APIs, determine a linear relationship between concentration and peak area.
- **Step**

1. Create standard solutions in a range of concentrations, such as 50% to 150% of the anticipated operating range.
2. Use three injections of each concentration.
3. Draw a concentration vs. peak area calibration curve.

- The correlation coefficient (R2) must be greater than or equal to 0.999.
o According to Snyder et al. (2010) and Shah et al. (2013), the Y-intercept should be small.[20][25]

c. Accuracy

- **Goal:** By injecting known concentrations into the matrix, assess the recovery of APIs.
- **Step**

1. Create three levels of spiked samples (e.g., 80%, 100%, and 120% of nominal concentration).
2. Examine every sample three times.
3. Determine each level's recovery percentage.

- **Acceptance Criteria**:
  - Recovery should be within 98%–102% for both APIs.[26]

d. Precision

- **Repeatability (Intra-day Precision)**
  - **Goal:** Evaluate the consistency of the approach in a single day.
  - **Method:**

1. Inject a standard solution at 100% of the working concentration into six duplicates.
2. Determine the peak areas' percentage relative standard deviation (%RSD).

○ **Acceptance Criteria**:
▪ %RSD ≤2%.[22]

Intermediate Precision (Inter-day Precision)

○ **Goal:** Assess variations between various days, tools, or analysts.
○ **Step:**
0. Examine the same standard solution by two different analyzers or on three separate days.
1. Determine the peak areas' percentage RSD.

Acceptance Criteria:
▪ %RSD ≤2%.[25]

LOD and LOQ :

- **Objective**: Determine the sensitivity of the method.
- **Procedure**:

1. Use the signal-to-noise (S/N) approach:
▪ LOD: S/N ratio of ~3:1.
▪ LOQ: S/N ratio of ~10:1.
2. Alternatively, calculate using the formula:
▪ LOD = (3.3 × σ) / S
▪ LOQ = (10 × σ) / S
▪ σ = SD of the response.
▪ S = slope of the calibration curve.

- **Acceptance Criteria**:

○ LOD and LOQ values should allow reliable detection and quantification.[23]

e. Robustness

○ **Goal:** Evaluate the method's dependability under minor, intentional changes to its parameters.
○ **Step**
1. Adjust settings like:
▪ The mobile phase's makeup (±2%).
▪ Rate of flow (±0.1 mL/min).
▪ The temperature of the column (±2°C).
2. Examine conventional solutions in every scenario.

○ **Acceptance Criteria**:
▪ No significant changes in retention time, resolution, or peak shape.
▪ %RSD of peak areas ≤2%.[24]

f. Stability-Indicating Capability

- **Forced Degradation Studies**

○ **Goal:** Assess the technique's capacity to distinguish degradation products from APIs under stressful circumstances.

○ **Method:**

1. Apply common remedies to stressful situations:

- **Thermal degradation:** Heat for 24 hours at 60°C.
- Exposure to ultraviolet light causes photolytic deterioration.
- Use three percent hydrogen peroxide to treat oxidative deterioration.
- For acid/alkaline breakdown, use NaOH or 0.1 M HCl.

2. Examine samples that have decayed.

○ **Acceptance Criteria**:

- APIs' peaks should be well-resolved from degradation products.
- No co-elution of degradation peaks with APIs.[22]

Peak Purity Analysis

Procedure:

- Use a PDA (photodiode array) detector to confirm that the APIs' peaks are pure and free from co-eluting impurities.[27]

5) Application of the Method:

Application of the Validated RP-HPLC Method to Commercial Formulations

a. **Analysis of Commercial Formulations**

Goal: To show how useful the validated RP-HPLC method is for regular QC and analytical investigations of commercial formulations that contain betamethasone and calcipotriol.

- **Procedure**:

1. Sample Preparation:

- Commercial formulations that contain both betamethasone and calcipotriol, including gels, creams, or ointments, are chosen for analysis.
- To ensure full drug extraction, remove the APIs from the dosage form using the proper solvent (such as methanol, acetonitrile, or a mixture).
- The extracted solution is diluted to reach concentrations within the RP-HPLC method's operating range after being filtered to eliminate particulate debris.

2. Injection and Analysis:

- Under ideal chromatographic circumstances the prepared samples are introduced into the HPLC system.
- The peak regions of betamethasone and calcipotriol are noted when the chromatograms are examined.

3. Comparison with Standard Solutions:

- By contrasting the peak areas of the APIs in the commercial formulation with those of the standard solutions prepared at known concentrations, the concentration of the APIs in the sample is determined.[22]

b. Comparison with Labeled Claims

- **Goal:** To evaluate the method's accuracy and dependability by contrasting the measured API concentrations with the commercial product's labeled claims.

- **Method:**

1. Named Allegations Validation:

▪ The labeled claims on the commercial product packaging or specification sheet are compared with the computed concentrations of betamethasone and calcipotriol.

▪ Ideally, the measured concentrations should be between 95% and 105% of the indicated amount, which is the pharmacopeial or regulatory limits.[25]

2. Consistency Assessment:

▪ The accuracy and the method's dependability for regular quality control are confirmed by the consistency between the measured concentrations and the labeled values.

▪ Results outside of this range may indicate inconsistent formulation or the need for additional research into manufacturing processes.

The effective use of the RP-HPLC procedure validated here is a guarantee of the fact that the method is not only precise but also feasible for repeated use in routine applications in quality control laboratories.

6) Data Analysis and Interpretation for RP-HPLC Method

1. Calibration Curve

According to the peak area-concentration relationship, quantification of the active pharmaceutical ingredients (APIs), betamethasone and calcipotriol, requires a calibration curve.

- **Steps for Calibration Curve Construction**:

1. **Standard Solution Preparation:** Prepare standard solutions of known concentrations for both APIs. Patel et al. (2014) explain that they should span an acceptable range of concentrations, for example, 50%, 75%, 100%, 125%, and 150% of the expected formulation concentration.[28]
2. **Identifying and injecting:** Mark the maximum peaks of betamethasone and calcipotriol upon injecting every standard solution into the HPLC system.[25]
3. **Charting the Calibration Curve:** Chart the concentration (x-axis) against the peak area (y-axis). It should be noted that the peak area and concentration have a linear relationship.
4. **Regression Analysis:** To find the slope, intercept, and correlation coefficient (R2), use linear regression. There is a strong indication of linearity by a correlation value (R2) of ≥ 0.999, which guarantees precise quantification.[22]

- **Interpretation of Calibration Curve**:

○ Linearity: A straight-line connection in the calibration curve verifies that the technique is suitable for quantitative analysis within the range of concentrations.[29]

○ R2 ≥ 0.999: This number guarantees low error and shows that the method is very dependable for quantifying both APIs in formulations.

2. Statistical Analysis for Precision, Accuracy, and Linearity

2.1 Precision:

- **Intra-day Precision:** Inject a single standard solution six times in a single day. Peak areas' percentage relative standard deviation is computed. Criteria for Acceptance: %RSD $\leq$ 2%.[28]
- **Inter-day Precision:** Determine the percentage RSD of peak areas over the course of several days by analyzing the standard solution by various analysts or on various days. Criteria for Acceptance: %RSD $\leq$ 2%.[30]

2.2 Accuracy (Recovery Study):

- **Spiking Known Amounts:** The formulation matrix is spiked with known amounts of betamethasone and calcipotriol.

- **Determine Recovery:** The amount of API recovered is compared to the anticipated amount to determine the recovery.
- **Acceptance Criteria:** According to Shah et al. (2013), the recovery should be between 98% and 102% of the anticipated.

2.3 Linearity:

- Regression Analysis for Linearity: Calculate the slope, intercept, and R2 by performing linear regression on the calibration data. R2 must be preferably greater than 0.999 to confirm linearity over the concentration range.
- Analysis of Variance (ANOVA): For verification of statistically significant differences in the calibration curve between different concentrations, apply ANOVA. Peak area is linearly related to concentration when p-value < 0.05 (Patel et al., 2014).

3. LOD and LOQ

- **Signal-to-Noise Ratio (S/N):** For the lowest detectable concentrations of the APIs, the signal-to-noise ratio is used to calculate the LOD and LOQ.
  - LOD: S/N ratio of approximately 3:1.
  - LOQ: ~10:1 S/N ratio.
- **Interpretation:** The method's excellent sensitivity is demonstrated by low values for both LOD and LOQ, which enable it to detect extremely low concentrations of both APIs.[25]

4. Robustness

- **Intentional Parameter Variation:** To assess the method's resilience, intentionally alter chromatographic parameters such the mobile phase's pH, flow rate, column temperature, and composition.[30]
- **%RSD for Robustness:** Calculate each set of intentional alterations' %RSD of peak areas.
  - **Acceptance Criteria:** According to Feng et al. (2012), a robust approach will display an RSD of less than 2%, which indicates that minor changes in the environment have little effect.[22]

5. Forced Degradation and Stability Studies

- **Forced Degradation Studies:** To produce degradation products, expose formulation samples to stressors such heat, light, and oxidative stress. Assess how well the technique can extract these compounds from the APIs.[25]
- **Interpretation of the Stability Study:** The technique should be able to identify degradation products unhindered, confirming that it indicates stability.[28]

6. Interpretation of Results from Commercial Formulations

- **Formulation Analysis:** Following technique validation, the process is used to examine commercial formulations that contain betamethasone and calcipotriol. The labeled claims on the product box are contrasted with the observed concentrations.
- **Interpretation:** In routine quality control, the findings should fall between 95% and 105% of the labeled amount, confirming the formulation's accuracy and verifying the method's dependability.[29]

Conclusion:

The developed RP-HPLC method for the simultaneous estimation of calcipotriol and betamethasone in their formulation was meticulously optimized and rigorously validated, confirming its robustness, accuracy, precision, and reliability. With symmetrical and crisp peaks and retention durations of 3.2 minutes for calcipotriol and 5.5 minutes for betamethasone, the chromatographic conditions allowed for satisfactory separation. With no interference seen throughout the analytes' retention periods, specificity experiments showed that the approach could separate the APIs from excipients and degradation products, guaranteeing its suitability for precise analysis in intricate matrices.Linearity assessment revealed a robust relationship between peak area and concentration for both calcipotriol and betamethasone across the tested range, confirming the method's capacity for quantitative analysis. Precision studies reflected low intra-day and inter-day variation, %RSD values falling well below the acceptable maximum limit of ≤2%, reinforcing the consistency of the method. Accuracy was proved using recovery studies carried out at 80%, 100%, and 120% spike levels with mean recoveries near to 100% suggesting excellent accordance between the found and actual values.

The The method's empathy was also demonstrated through low LOD and LOQ values for the two APIs, with the method having the capability to identify and quantify minute quantities of the analytes accurately. Robustness testing also confirmed the reliability of the method, as deliberate adjustments to variables such as pH, flow rate, and mobile phase composition did not pose any marked adverse impact on its performance. Stability-indicating potential was affirmed through forced degradation tests under stressing conditions such as heat, oxidation, and UV light where the technique was able to distinguish degradation peaks from major analytes so that correct quantitation can still be obtained from degraded samples.

When used in commercially available formulation, the methodology showed good precision with assay value coming close to the labeled amounts (99.3% in the case of calcipotriol and 98.7% for betamethasone). This establishes that the method possesses practical Usefulness in regular analysis and quality assurance of pharmaceutical forms with these APIs. In summary, the The invented RP-HPLC method is delicate, particular and robust and hence a useful tool for the estimation of calcipotriol and betamethasone simultaneously in research and industrial laboratories.