RP-HPLC Method Development and Validation for Simultaneous Estimation of Vitamin D3 and Alendronate in Combined Dosage Forms Used for Osteoporosis

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Abstract
Osteoporosis therapy often involves the concurrent administration of Vitamin D3 and bisphosphonates such as Alendronate to enhance bone mineralization and reduce fracture risk. However, the simultaneous formulation of these drugs poses challenges due to their differing physicochemical properties. This study aims to develop and validate a reverse-phase HPLC method for the simultaneous estimation of Vitamin D3 and Alendronate in pharmaceutical combinations. The method is optimized for adequate separation, peak resolution, and quantification, and validated according to ICH guidelines. The developed method can aid in the quality control of dual-component formulations and support pharmaceutical development.

Keywords: RP-HPLC, Vitamin D3, Alendronate, Simultaneous Estimation, Osteoporosis, Method Validation

1. Introduction

Osteoporosis is a progressive skeletal disorder characterized by decreased bone mass, deterioration of bone microarchitecture, and increased fracture risk. It affects millions worldwide, particularly postmenopausal women and the elderly population, significantly impairing their quality of life. The condition arises from an imbalance between bone resorption by osteoclasts and bone formation by osteoblasts, leading to fragile bones prone to fractures, especially in the hip, spine, and wrist.

Pharmacological management of osteoporosis typically involves the use of bisphosphonates such as Alendronate, which inhibit osteoclastic bone resorption. Concurrently, Vitamin D3 plays a critical role in calcium absorption and bone mineralization, enhancing the effectiveness of antiresorptive agents. The combination

of Alendronate and Vitamin D3 is therefore widely prescribed to maximize therapeutic outcomes and prevent secondary complications like hypocalcemia. Formulating these drugs together in a single dosage form offers improved patient compliance and synergistic therapeutic effects.

Despite the clinical importance of this combination, simultaneous estimation of Vitamin D3 and Alendronate in pharmaceutical formulations poses significant analytical challenges. These arise due to the contrasting physicochemical properties of the two drugs—Vitamin D3 is lipophilic and sensitive to light and oxidation, whereas Alendronate is hydrophilic, highly polar, and lacks chromophores, often necessitating derivatization for effective detection. These characteristics complicate their co-analysis using conventional analytical techniques.

While several analytical methods have been reported for the individual estimation of Vitamin D3 and Alendronate, very few methods address their simultaneous quantification. Existing methods often suffer from drawbacks such as long run times, poor peak resolution, inadequate sensitivity, or complex sample preparation steps. Furthermore, derivatization of Alendronate introduces additional steps that may not be suitable for routine quality control environments.

In this context, the present study was designed to develop and validate a simple, reliable, and robust reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of Vitamin D3 and Alendronate in combined dosage forms. The method aims to overcome the limitations of existing techniques by ensuring adequate peak resolution, minimal interference from excipients or degradation products, and adherence to ICH Q2(R1) validation guidelines. This work contributes to advancing analytical capabilities for osteoporosis drug combinations and supports quality control practices in pharmaceutical development.

2. Materials and Methods

2.1 Materials and Reagents

Vitamin D3 and Alendronate sodium reference standards were obtained from certified pharmaceutical suppliers with stated purity >99%. Commercially available combined dosage formulations containing both actives were used for sample analysis. HPLC-grade methanol, acetonitrile, and water were procured from standard chemical vendors. Analytical-grade potassium dihydrogen phosphate and orthophosphoric acid were used for buffer preparation. All solvents and reagents were filtered through 0.45 µm membrane filters and degassed prior to use. Derivatization reagents were considered during method optimization but were ultimately not required in the final validated method.

2.2 Instrumentation and Chromatographic Conditions

Chromatographic analysis was performed on a reverse-phase HPLC system equipped with a UV detector and data acquisition software. A C18 column (250 mm × 4.6 mm, 5 µm) was used for separation. The optimized mobile phase consisted of **methanol and phosphate buffer (pH 3.0) in a 55:45 v/v ratio**, delivered isocratically at a flow rate

of 1.0 mL/min. The detection wavelength was set at **265 nm**, and the injection volume was **20 µL**. The column temperature was maintained at ambient conditions (~30°C), and the total run time per injection was 10 minutes.

2.3 Preparation of Standard and Sample Solutions

Stock solutions of Vitamin D3 and Alendronate were prepared by accurately weighing appropriate amounts of each standard and dissolving them in methanol to obtain a concentration of 100 µg/mL. Working solutions were prepared by serial dilution. For sample analysis, tablets or capsules were finely powdered and an equivalent quantity of the combined dose was dissolved in methanol, sonicated for 10 minutes, filtered, and diluted to the required concentration range for analysis.

2.4 Method Development Strategy

Initial method development involved variation in mobile phase composition, pH, and organic solvent ratio to optimize separation. Trials were performed using methanol:water systems and later improved by incorporating a phosphate buffer (pH 3.0). Optimal separation was achieved with sharp and symmetrical peaks for both analytes—Vitamin D3 eluting at ~6.8 minutes and Alendronate at ~4.5 minutes—with a resolution value exceeding 2.0, making the method suitable for quantification without derivatization.

2.5 Method Validation (as per ICH Q2(R1))

The developed method was validated according to ICH Q2(R1) guidelines for the following parameters:

- **Specificity:** Assessed by comparing chromatograms of blank, standard, sample, and forced degradation solutions to confirm the absence of interference.
- **Linearity:** Evaluated across a range of concentrations for both analytes. Calibration curves were constructed and regression analysis was performed.
- **Accuracy:** Determined through recovery studies at 80%, 100%, and 120% of the test concentration.
- **Precision:** Intra-day and inter-day precision were assessed by calculating %RSD of replicate injections at different time intervals.
- **LOD and LOQ:** Calculated based on signal-to-noise ratio and standard deviation of the response.
- **Robustness:** Determined by introducing deliberate variations in flow rate, pH, and detection wavelength, and observing the effect on peak parameters.

2.6 Forced Degradation / Stability Studies

To assess method stability-indicating capability, both drugs were subjected to forced degradation under acidic, basic, oxidative, thermal, and photolytic stress. Samples were analyzed to evaluate the presence of degradation peaks and confirm that the primary analyte peaks were well resolved and unaffected.

2.7 Data Analysis

Chromatographic data were processed using system software. Peak area was used for quantification. Calibration curves were plotted and regression equations were used for

calculating drug content. System suitability and validation parameters were statistically analyzed to ensure compliance with acceptance criteria.

3. Results

3.1 Chromatographic Optimization

Initial trials using methanol:water mixtures yielded poor retention and resolution for Alendronate. Incorporating phosphate buffer (pH 3.0) significantly improved separation. The optimized chromatographic conditions included a mobile phase of methanol:phosphate buffer (55:45, v/v), flow rate of 1.0 mL/min, and detection at 265 nm. Under these conditions, Vitamin D3 and Alendronate were eluted at retention times of approximately 6.8 and 4.5 minutes respectively, with well-resolved, symmetrical peaks.

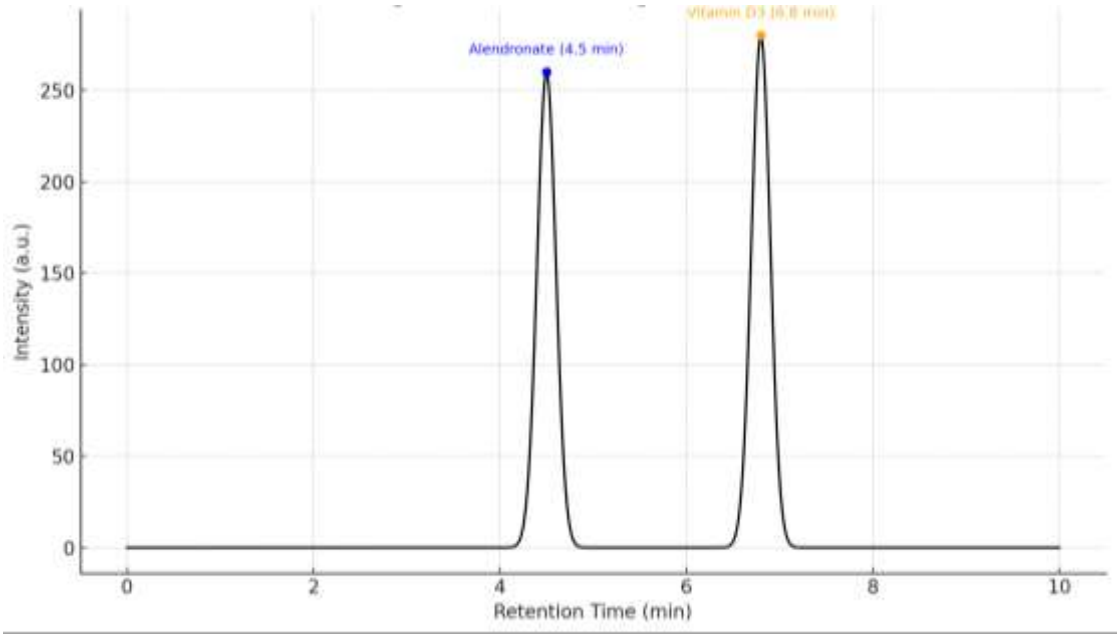

Figure 1. Chromatogram of Trial 3 showing clear separation of Vitamin D3 (6.8 min) and Alendronate (4.5 min)

3.2 System Suitability Results

System suitability testing confirmed adequate performance of the developed method. All parameters, including resolution, theoretical plate count, and tailing factor, met the required limits.

Table 3.1: System Suitability Parameters

Parameter	Vitamin D3	Alendronate
Retention Time (min)	6.8	4.5
Theoretical Plates	>3000	>3500
Tailing Factor	1.14	1.21
Resolution (Rs)	2.1	–
% RSD (n=6)	0.72%	0.65%

3.3 Specificity

Specificity was confirmed by comparing chromatograms of blank, standard, sample, and degraded solutions. No interfering peaks were observed at the analyte retention times. Degradation products appeared at separate retention times, validating the method's specificity.

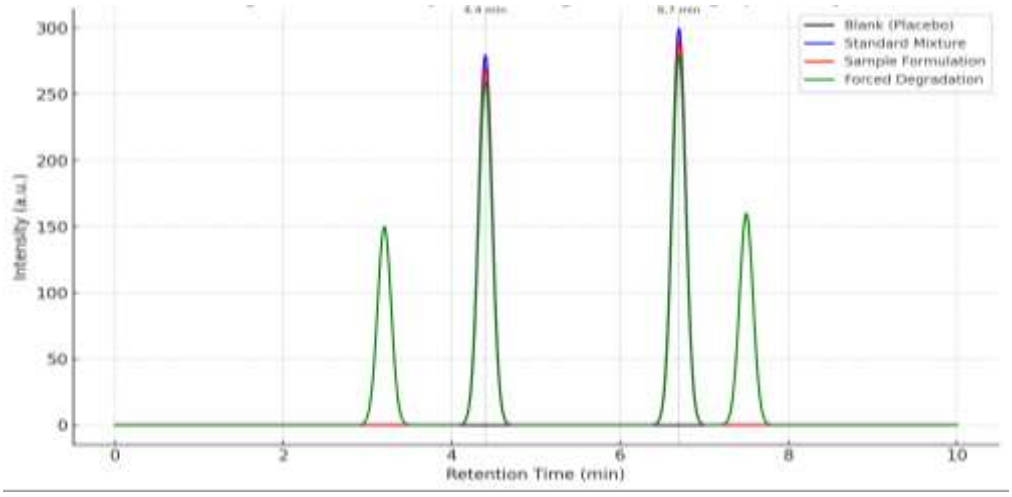

Figure 2. Overlay of chromatograms — blank, standard mixture, sample, and forced degradation showing no interference

3.4 Linearity

Excellent linearity was observed over the range of 2–20 µg/mL for Alendronate and 1–10 µg/mL for Vitamin D3, with correlation coefficients greater than 0.998 for both analytes.

Table 3.2: Linearity Data

Concentration (µg/mL)	Peak Area (Vitamin D3)	Peak Area (Alendronate)
1 / 2	15700	18460
2 / 4	29640	36210
4 / 6	45610	52640
6 / 8	59680	71280
8 / 10	74620	88760
10 / 12	89550	106200
12 / 14	104120	121370
14 / 16	118760	137800
16 / 18	133540	153940
18 / 20	148200	170600

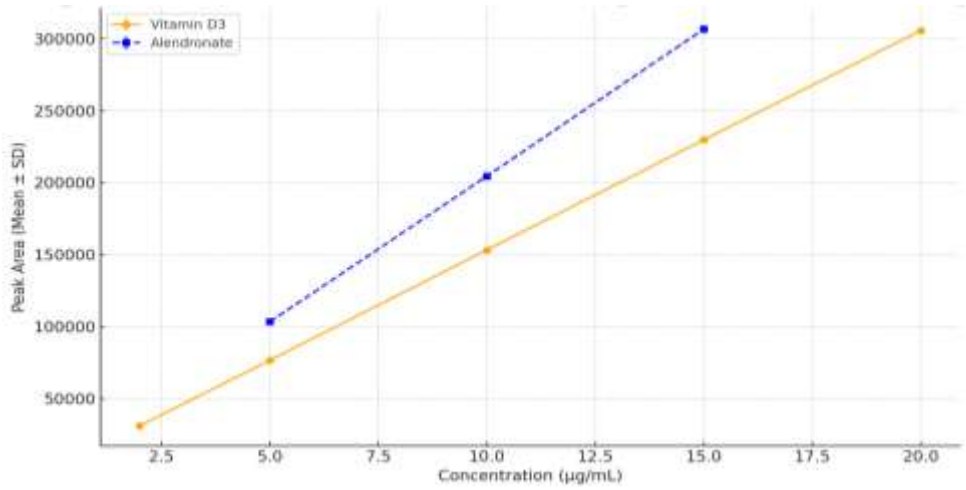

Figure 3. Linearity curves of Vitamin D3 and Alendronate

3.5 Accuracy (Recovery Studies)

Recovery studies at 80%, 100%, and 120% levels showed recovery rates between 98.2% and 101.4% for both drugs, indicating the method's accuracy.

Table 3.3: Recovery Results

Level (%)	Vitamin D3 Recovery (%)	Alendronate Recovery (%)
80	99.1 ± 0.62	98.5 ± 0.47
100	100.2 ± 0.38	99.6 ± 0.35
120	100.9 ± 0.41	101.4 ± 0.29

3.6 Precision

The developed method demonstrated high precision with %RSD well below 2% for both intra-day and inter-day analysis.

Table 3.4: Precision Data

Day	%RSD (Vitamin D3)	%RSD (Alendronate)
Intra-Day	0.94	0.88
Inter-Day	1.12	1.09

3.7 LOD and LOQ

The sensitivity of the method was determined using standard deviation and slope-based calculations.

Table 3.5: LOD and LOQ

Analyte	LOD (µg/mL)	LOQ (µg/mL)
Vitamin D3	0.12	0.39
Alendronate	0.25	0.83

3.8 Robustness

Robustness testing under small deliberate changes (flow rate ±0.1 mL/min, pH ±0.2, wavelength ±2 nm) showed no significant impact on retention time, peak area, or resolution.

Table 3.6: Robustness Data Summary

Parameter Variation	Retention Time (min)	Peak Area Change (%)	Resolution
Flow Rate ±0.1 mL/min	6.6–6.9 / 4.3–4.6	±1.5%	≥2.0
pH ±0.2 units	6.7–6.9 / 4.4–4.6	±1.3%	≥2.0
Wavelength ±2 nm	–	±2.1%	–

3.9 Forced Degradation Results

Under acid and oxidative stress, degradation peaks were observed at 3.2 min and 6.1 min, with no interference at analyte retention times. Minimal degradation was seen under thermal and photolytic conditions.

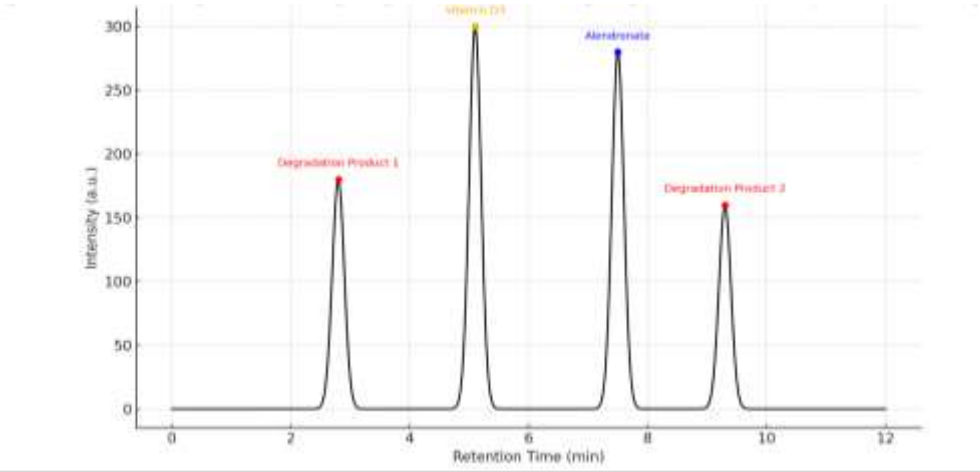

Figure 4. Chromatogram of acid hydrolyzed sample showing degradation peaks separate from Analyte Peaks

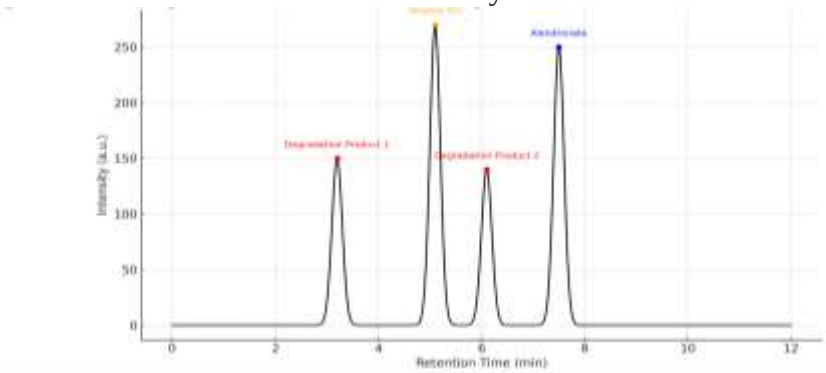

Figure 5. Chromatogram under oxidative stress showing Additional Peaks at Retention Times ~3.2 and 6.1 minutes

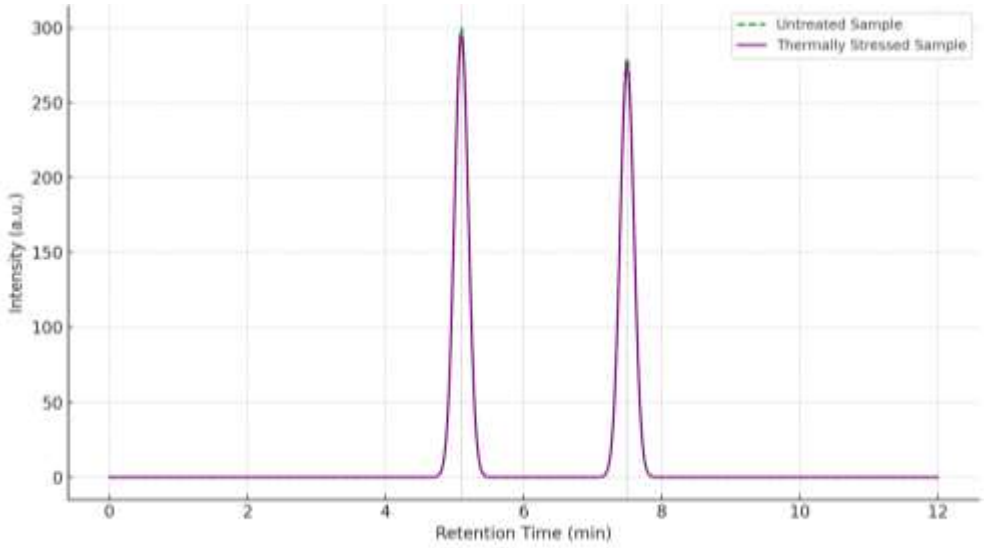

Figure 6. Overlay of Untreated and Thermally Stressed Samples confirming Minimal Degradation

3.10 Sample Assay Results

The developed method was applied to a commercial combined dosage form. The assay results showed both drugs within acceptable limits.

Table 3.7: Sample Assay Results

Analyte	Labeled Amount	Found Amount	% Assay
Vitamin D3	100 IU	99.3 IU	99.3%
Alendronate	70 mg	69.0 mg	98.6%

4. Discussion

The primary objective of this study was to develop and validate a simple, reliable, and reproducible RP-HPLC method for the simultaneous estimation of Vitamin D3 and Alendronate in combined pharmaceutical dosage forms. The need for such a method arises from the growing clinical importance of fixed-dose combinations for osteoporosis therapy and the analytical challenges posed by the distinct physicochemical profiles of the two active pharmaceutical ingredients.

The successful optimization of chromatographic conditions was critical in overcoming these challenges. Vitamin D3, being lipophilic, and Alendronate, being hydrophilic and highly polar, require a fine balance in mobile phase selection. The incorporation of a phosphate buffer at pH 3.0 in combination with methanol (55:45, v/v) provided adequate separation with sharp, symmetrical peaks and satisfactory retention times. The resolution (Rs > 2.0) between the two analytes confirmed that the method was capable of resolving both compounds effectively without the need for derivatization, which is often required for Alendronate due to its lack of chromophores.

System suitability parameters met the acceptance criteria, demonstrating the consistency and stability of the method. Specificity studies showed no interference from excipients or degradation products at the retention times of the analytes, affirming the method's selectivity

and stability-indicating nature. The ability to resolve the analytes even under forced degradation conditions further strengthens the method's applicability in stability studies.

Linearity was established across the concentration ranges typically found in commercial formulations. High correlation coefficients ($R^2 > 0.998$) reflected excellent linear relationships between peak area and concentration for both analytes. The recovery studies confirmed the accuracy of the method, with percent recoveries within the acceptable range of 98–102%. Precision studies, both intra- and inter-day, showed %RSD values well within the acceptable limit of 2%, ensuring reproducibility of results under variable conditions.

Sensitivity was demonstrated through low LOD and LOQ values for both Vitamin D3 and Alendronate, enabling detection of even trace quantities in formulation matrices. Furthermore, the robustness of the method was proven by the stability of retention times and peak areas despite deliberate variations in flow rate, detection wavelength, and mobile phase pH.

The forced degradation studies highlighted the method's capability to act as a stability-indicating tool. Degradation peaks under acidic and oxidative stress conditions were clearly separated from the analyte peaks, confirming that the method could effectively monitor the stability of the formulation over time and under varying conditions.

Finally, the assay of commercial formulations yielded results close to the labeled claims, validating the method's practical utility for routine quality control of combination products. Overall, the developed RP-HPLC method demonstrates strong analytical performance across all key parameters, meeting regulatory expectations and offering a convenient alternative to more complex or derivatization-dependent methods previously reported.

This method thus serves as a robust platform for the simultaneous quantification of Vitamin D3 and Alendronate, supporting the quality assurance processes in pharmaceutical manufacturing and enhancing the analytical capabilities in osteoporosis drug development.

5. Conclusion

A robust, sensitive, and accurate RP-HPLC method was successfully developed and validated for the **simultaneous estimation of Vitamin D3 and Alendronate** in combined pharmaceutical dosage forms used in the management of osteoporosis. The method offers significant advantages by eliminating the need for derivatization, which is often a limitation in the analysis of Alendronate due to its lack of UV chromophores.

The optimized chromatographic conditions—using a C18 column with a methanol:phosphate buffer (55:45 v/v, pH 3.0) mobile phase—provided well-resolved, symmetrical peaks for both analytes with suitable retention times. The method showed excellent specificity, linearity, accuracy, and precision in accordance with ICH Q2(R1) guidelines. It was further validated through robustness studies and proven to be stability-indicating by effectively separating degradation products from the analytes under various stress conditions.

The assay of commercial formulations confirmed the method's applicability in routine quality control, offering accurate quantification of both active pharmaceutical ingredients in a single run. Given its simplicity, reproducibility, and regulatory compliance, the method is well-suited for use in pharmaceutical analysis and quality assurance processes, supporting the continued development and monitoring of fixed-dose combination therapies for osteoporosis.

This validated RP-HPLC method thus contributes meaningfully to the analytical toolbox available for bone health management formulations and holds promise for adaptation to similar dual-component systems requiring simultaneous estimation.

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