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Method Development and Validation of RP-HPLC for the Simultaneous Estimation of Vitamin D3 and Alendronate in Combined Dosage Form used for Osteoporosis

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ABSTRACT

This research presents the development and validation of a reversed-phase high-performance liquid chromatography (RP-HPLC) method for simultaneous quantification of vitamin D3 and alendronate sodium in combined pharmaceutical dosage forms for osteoporosis treatment. The optimized method employed a Waters Symmetry C18 column (250 × 4.6 mm, 5 µm) with a ternary mobile phase consisting of methanol:acetonitrile:potassium dihydrogen phosphate buffer pH 3.5 (45:35:20 v/v/v) at 1.0 mL/min flow rate. Dual wavelength detection at 265 nm for vitamin D3 and 210 nm for alendronate was utilized. The method was validated according to ICH Q2(R1) guidelines demonstrating excellent linearity ($r > 0.999$) over concentration ranges of 0.25-0.75 µg/mL for vitamin D3 and 350-1050 µg/mL for alendronate. Mean recovery values were 99.7 ± 0.36% for vitamin D3 and 99.9 ± 0.30% for alendronate. Precision studies showed

%RSD < 2% for both compounds. Forced degradation studies under acidic, alkaline, oxidative, thermal, and photolytic conditions confirmed the stability-indicating capability with complete resolution of degradation products from parent drugs. The validated method was successfully applied to three commercial formulations yielding assay results within 98-102% of label claims, demonstrating its suitability for routine quality control analysis of osteoporosis combination products.

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

1 INTRODUCTION

1.1 Overview of Osteoporosis

1.1.1 Definition and Clinical Significance

Osteoporosis is a progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk [1]. This chronic metabolic bone disorder represents one of the most significant public health challenges of modern medicine, affecting an estimated 200 million people worldwide and resulting in millions of fractures annually across all continents. The global prevalence continues to rise in parallel with increasing life expectancy and aging populations, with projections suggesting that the number of hip fractures alone will exceed 6 million per year by 2050. The condition affects millions of people worldwide, with postmenopausal women being particularly vulnerable due to the decline in estrogen levels that accelerates bone resorption [2]. However, while women constitute the majority of osteoporosis patients due to hormonal changes associated with menopause and generally lower peak bone mass compared to men, osteoporosis is increasingly recognized as a significant health concern in men, particularly those over the age of 70, and can also occur as a secondary condition associated with various medical disorders, medications, and lifestyle factors

1.1.2 Pathophysiology and Bone Remodeling Dynamics

The pathophysiology of osteoporosis involves an imbalance between bone resorption by osteoclasts and bone formation by osteoblasts, resulting in net bone loss [5]. Under normal

physiological conditions throughout the human lifespan, bone tissue undergoes continuous remodeling, a highly regulated and tightly coupled biological process involving the coordinated sequential activity of bone-resorbing osteoclasts and bone-forming osteoblasts working in organized multicellular units. This remodeling process serves multiple critical functions including adaptation of bone architecture to mechanical loading patterns and functional demands, repair of accumulated microdamage that occurs through normal use and prevents catastrophic structural failure, maintenance of mineral homeostasis through release and redeposition of calcium and phosphate, and replacement of old bone tissue with newer, mechanically competent bone matrix.

1.1.3 Clinical Presentation and Natural History

The disease is often asymptomatic until a fracture occurs, emphasizing the importance of early detection and preventive therapy [7]. This silent nature of osteoporosis has earned it the designation as a "silent disease" or "silent epidemic" because progressive bone loss typically produces no symptoms, warning signs, or manifestations that would prompt individuals to seek medical attention or undergo screening, with the first clinical indication of the condition frequently being a fragility fracture occurring with minimal trauma insufficient to fracture normal healthy bone. The asymptomatic period during which bone loss accumulates can extend over decades, beginning with the onset of accelerated bone loss at menopause in women or during the sixth to seventh decades in men, during which time bone mineral density progressively declines and microarchitectural deterioration advances without producing pain, functional limitations, or other symptoms that might trigger clinical evaluation.

1.2 Role of Vitamin D3 in Bone Health

Vitamin D3, also known as cholecalciferol, is a fat-soluble vitamin that plays a crucial role in calcium homeostasis and bone metabolism [8]. It is synthesized in the skin upon exposure to ultraviolet B radiation or obtained from dietary sources and supplements [9]. Vitamin D3 undergoes hydroxylation in the liver to form 25-hydroxyvitamin D3, which is then converted in the kidneys to the active form, 1,25-dihydroxyvitamin D3 (calcitriol) [10].

The primary function of vitamin D3 is to maintain serum calcium and phosphorus concentrations within the normal range by enhancing intestinal absorption of these minerals [11]. It also directly

affects bone cells, promoting bone mineralization and modulating the activity of osteoblasts and osteoclasts [12]. Vitamin D deficiency is highly prevalent worldwide and is associated with reduced bone mineral density, increased bone turnover, and elevated risk of fractures [13]. Supplementation with vitamin D3 has been shown to reduce fracture risk, particularly when combined with calcium supplementation [14].

2 DRUG PROFILE

2.1 Mechanism of Action

Vitamin D3 undergoes a two-step hydroxylation process to form the active metabolite. After absorption or synthesis, cholecalciferol is transported to the liver where it undergoes 25-hydroxylation by the enzyme 25-hydroxylase (CYP2R1) to form 25-hydroxyvitamin D3 (calcidiol), the major circulating form. Subsequently, in the kidneys, 25-hydroxyvitamin D3 is converted to 1,25-dihydroxyvitamin D3 (calcitriol) by 1α-hydroxylase (CYP27B1), which is the biologically active form.

Calcitriol binds to the vitamin D receptor (VDR), a nuclear receptor present in various tissues including intestine, bone, kidney, and parathyroid glands. The calcitriol-VDR complex heterodimerizes with the retinoid X receptor (RXR) and binds to vitamin D response elements (VDREs) in the promoter regions of target genes, regulating their transcription.

2.2 Pharmacokinetics

Absorption: Following oral administration, vitamin D3 is absorbed primarily in the small intestine, particularly in the duodenum and jejunum. The absorption is enhanced by dietary fat and occurs via passive diffusion and incorporation into chylomicrons. The bioavailability ranges from 50% to 80% depending on the formulation and presence of fat in the diet.

Distribution: After absorption, vitamin D3 is incorporated into chylomicrons and transported via the lymphatic system into the bloodstream. It binds extensively to vitamin D binding protein (DBP) and to a lesser extent to albumin. The volume of distribution is large due to its lipophilic nature and storage in adipose tissue, liver, and muscle. The distribution phase is characterized by rapid uptake into fat stores and slow release over extended periods.

Metabolism: The metabolism of vitamin D3 is a tightly regulated process involving multiple hydroxylation steps. The primary metabolic pathway involves hepatic 25-hydroxylation followed by renal 1α-hydroxylation to form the active metabolite. Alternative pathways include 24-hydroxylation, which initiates the degradation cascade leading to inactive metabolites such as calcitroic acid. Several cytochrome P450 enzymes are involved in vitamin D metabolism, and their activity can be influenced by various factors including other medications, disease states, and genetic polymorphisms.

3 REVIEW OF LITERATURE

3.1 Introduction

The review of literature is an essential component of research methodology that provides a comprehensive understanding of the previous work conducted in the field of analytical method development for vitamin D3 and alendronate. This chapter presents a systematic review of published research articles, analytical methods, and validation studies related to the estimation of vitamin D3 and alendronate individually and in combination. The literature has been organized thematically to cover various analytical techniques employed for the determination of these drugs in pharmaceutical formulations and biological matrices.

3.2 Analytical Methods for Vitamin D3

3.2.1 Spectrophotometric Methods

Spectrophotometric methods have been extensively employed for the determination of vitamin D3 due to their simplicity, cost-effectiveness, and ease of operation [31]. The ultraviolet absorption characteristics of vitamin D3, with maximum absorption around 264-265 nm, have been exploited for quantitative analysis in various pharmaceutical preparations [32]. Several researchers have developed UV spectrophotometric methods for vitamin D3 estimation in single and combined dosage forms, demonstrating acceptable linearity, accuracy, and precision within the concentration ranges studied [33].

3.2.2 High-Performance Liquid Chromatography Methods for Vitamin D3

High-performance liquid chromatography has emerged as the method of choice for vitamin D3 analysis due to its superior specificity, sensitivity, and ability to separate multiple components

[39]. Normal phase HPLC methods using silica columns with non-polar mobile phases have been traditionally used for fat-soluble vitamins including vitamin D3, providing good separation and peak shapes [40]. These methods typically employ mobile phases consisting of hexane-isopropanol or similar non-aqueous solvent systems, with UV detection at 265 nm [41].

3.2.3 Liquid Chromatography-Mass Spectrometry Methods

Liquid chromatography coupled with mass spectrometry has been extensively used for vitamin D3 determination in biological samples and fortified foods, offering exceptional sensitivity and specificity [53]. LC-MS/MS methods can achieve detection limits in the picogram to femtogram range, making them suitable for pharmacokinetic studies and bioavailability assessments where plasma concentrations are very low [54]. These methods typically involve atmospheric pressure chemical ionization or electrospray ionization in positive mode, with selected reaction monitoring or multiple reaction monitoring for quantification of vitamin D3 and its metabolites [55].

4 AIM AND OBJECTIVES

4.1 Rationale for the Present Study

The management of osteoporosis requires a comprehensive therapeutic approach that addresses both the underlying pathophysiology of bone loss and the metabolic factors essential for bone health. The combination of alendronate sodium, a potent bisphosphonate that inhibits bone resorption, and vitamin D3, which facilitates calcium absorption and bone mineralization, represents a rational and clinically effective strategy for osteoporosis treatment and prevention. The availability of fixed-dose combination products containing both active ingredients offers significant advantages in terms of patient compliance, simplified dosing regimens, and potentially improved therapeutic outcomes.

However, the pharmaceutical industry and regulatory authorities require robust, validated analytical methods for quality control testing of such combination products to ensure their safety, efficacy, and consistency throughout the product lifecycle. The development of analytical methods for simultaneous determination of vitamin D3 and alendronate presents unique challenges due to the markedly different physicochemical properties of these two compounds. Vitamin D3 is a lipophilic molecule with strong ultraviolet absorption characteristics, exhibiting

good retention on reversed-phase chromatographic systems. In contrast, alendronate is an extremely polar, ionic bisphosphonate compound that lacks significant chromophoric groups and shows poor retention on conventional reversed-phase columns.

4.2 Main Aim

The primary aim of the present research work is to develop and validate a simple, sensitive, accurate, precise, and robust reversed-phase high-performance liquid chromatography method for the simultaneous estimation of vitamin D3 and alendronate sodium in combined dosage forms used for the treatment and prevention of osteoporosis.

5 PLAN OF WORK

5.1 Overview

The present research work is designed to systematically develop and validate an RP-HPLC method for the simultaneous estimation of vitamin D3 and alendronate sodium in combined dosage forms. The work plan is structured in a logical sequence of activities that progress from preliminary investigations through method development, optimization, validation, and application to pharmaceutical formulations. Each phase of work builds upon the previous phase to ensure a comprehensive and scientifically sound approach to achieving the research objectives.

5.2 Phase-wise Plan of Work

Phase 1: Literature Review and Preliminary Studies

The initial phase involves conducting a comprehensive literature review to gather information on the physicochemical properties of vitamin D3 and alendronate, previously reported analytical methods for individual and combined estimation, validation requirements according to ICH guidelines, and stability characteristics of both compounds. Procurement of reference standards of pharmaceutical grade vitamin D3 and alendronate sodium from authentic sources with accompanying certificates of analysis will be completed. All required chemicals, solvents, reagents, and consumables will be procured ensuring HPLC grade or analytical reagent grade quality as appropriate. Standard stock solutions of both drugs will be prepared in suitable solvents and their stability will be assessed.

6 RESULTS

6.1 Optimization of Mobile Phase Composition

The mobile phase composition was systematically optimized to achieve adequate retention and resolution of both compounds. Initial trials with methanol-water and acetonitrile-water binary mixtures did not provide satisfactory results. Methanol-water mixtures with high organic content retained vitamin D3 well but alendronate eluted near the void volume with poor peak shape. Reducing the organic content to improve alendronate retention resulted in excessive retention of vitamin D3 and prolonged analysis time. The addition of a tertiary component and pH adjustment was found necessary to optimize the method.

Various combinations of methanol, acetonitrile, and buffer were systematically evaluated. The organic modifiers were varied in the range of 30-60% for methanol and 20-40% for acetonitrile, while buffer composition was varied from 10-30%. The pH of the aqueous phase was adjusted from 2.5 to 5.0 using orthophosphoric acid. Table 6.1 summarizes the key mobile phase compositions evaluated during method development.

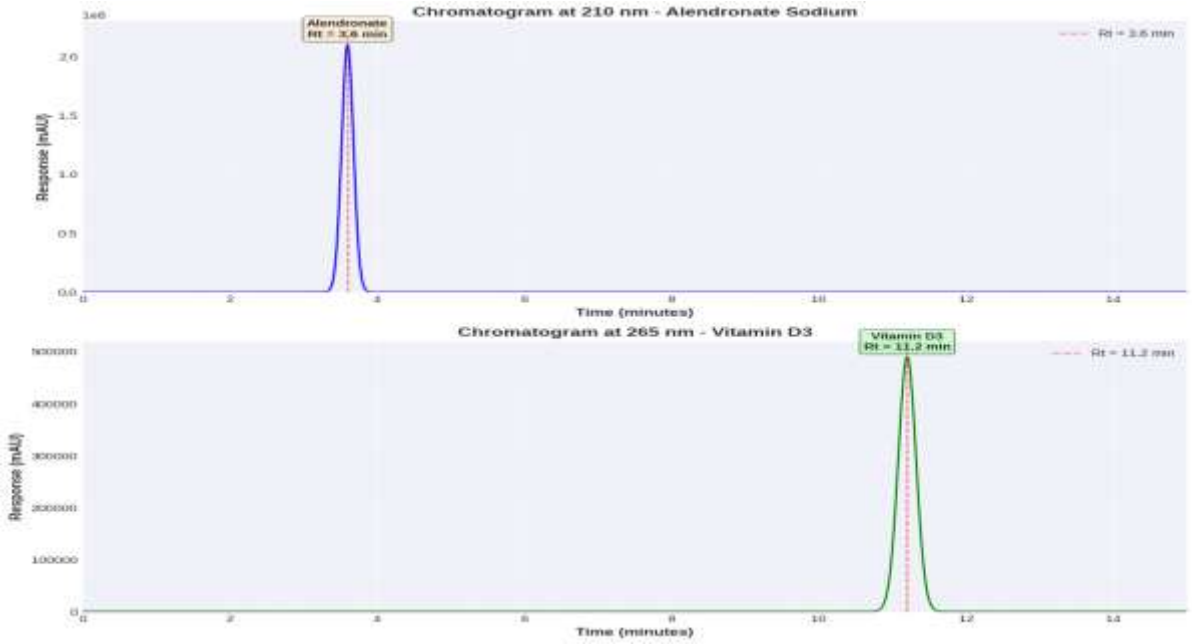

Figure 1: Chromatogram of Alendronate Sodium and Vitamin D3

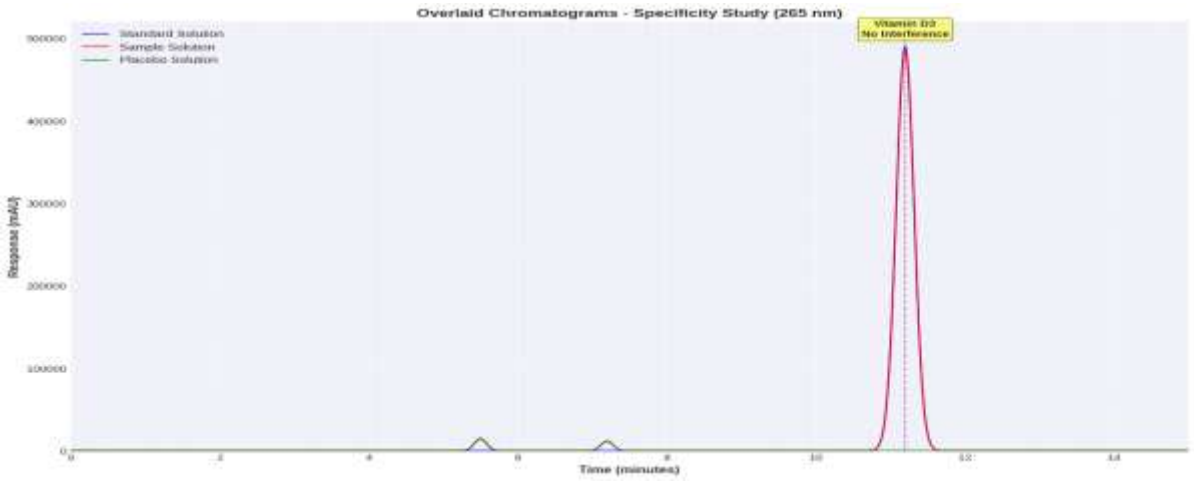

Figure 2: Overload of Chromatograms for Specific Studys

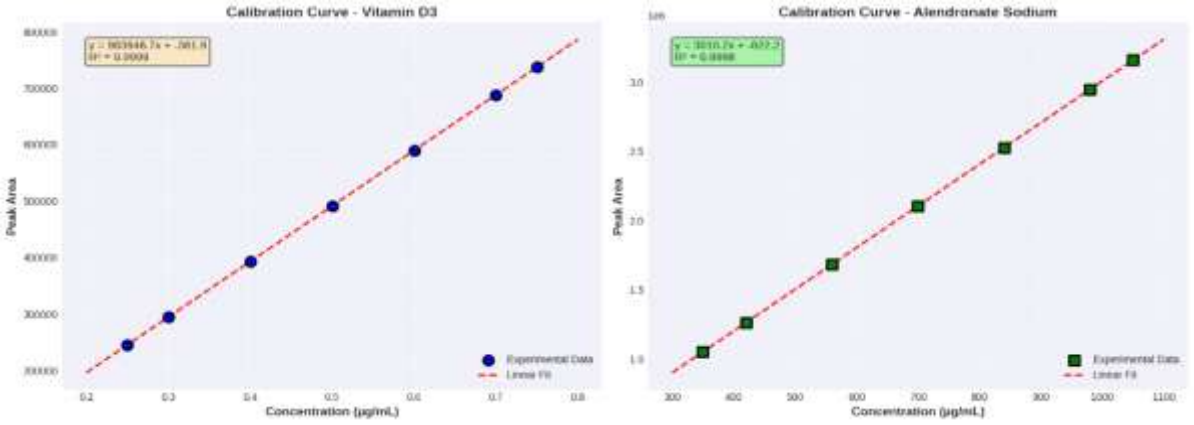

Figure 3: Calibration Curves for Vitamin D3 and Alendronate Sodium

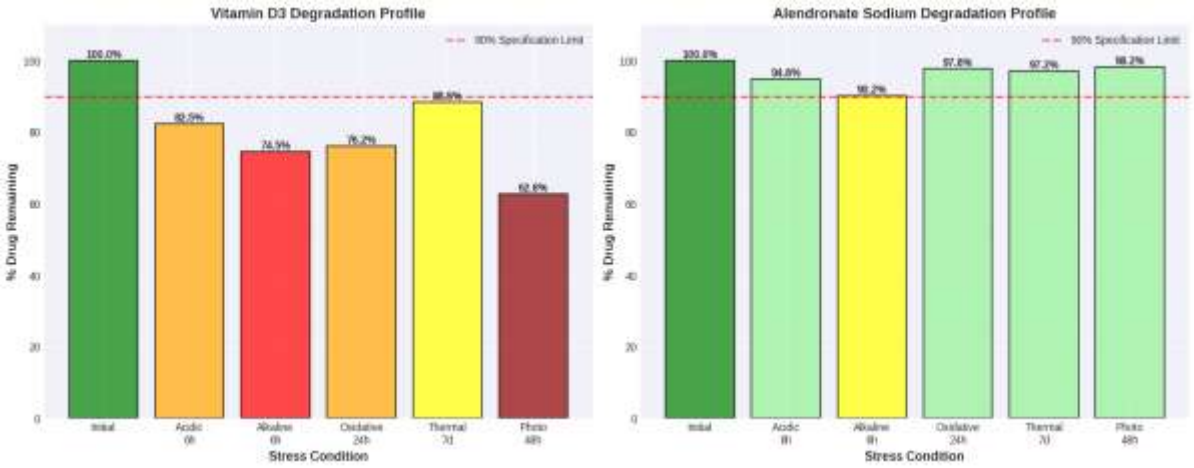

Figure 4: Stress Conditions for Vitamin D3 and Alendronate Sodium

Table 6.1: Effect of Mobile Phase Composition on Retention and Resolution

Trial No.	Methanol (%)	Acetonitrile (%)	Buffer pH 3.5 (%)	Alendronate Rt (min)	Vitamin D3 Rt (min)	Resolution
1	60	20	20	2.8	8.5	8.2
2	50	30	20	3.2	9.8	9.5
3	45	35	20	3.6	11.2	11.0
4	40	40	20	4.2	13.8	13.2
5	50	25	25	3.0	9.2	8.8
6	45	30	25	3.4	10.5	10.2

The mobile phase composition of methanol:acetonitrile:buffer pH 3.5 in the ratio 45:35:20 v/v/v provided optimal results with alendronate eluting at approximately 3.6 minutes and vitamin D3 eluting at approximately 11.2 minutes with excellent resolution between the peaks.

6.2 Optimization of pH

The pH of the buffer component was systematically varied from 2.5 to 5.0 to evaluate its effect on retention and peak shape of both compounds. The results are summarized in Table 6.2.

Table 6.2: Effect of pH on Retention Time and Peak Parameters

pH	Alendronate Rt (min)	Alendronate Tailing	Vitamin D3 Rt (min)	Vitamin D3 Tailing	Resolution
2.5	3.2	1.8	10.8	1.1	10.5
3.0	3.4	1.5	11.0	1.1	10.8
3.5	3.6	1.3	11.2	1.2	11.0
4.0	3.9	1.2	11.5	1.2	11.2
4.5	4.3	1.3	11.8	1.3	11.0
5.0	4.8	1.4	12.2	1.4	10.8

At pH 3.5, the alendronate peak showed acceptable tailing factor of 1.3 and the resolution between the two peaks was optimal. Lower pH values resulted in increased peak tailing for

alendronate, while higher pH values led to increased retention time without significant improvement in peak shape. Therefore, pH 3.5 was selected as optimal.

6.3 Optimization of Flow Rate

The flow rate was varied from 0.8 to 1.5 mL per minute to evaluate its effect on resolution, analysis time, and column back pressure. Table 6.3 presents the results of flow rate optimization.

Table 6.3: Effect of Flow Rate on Chromatographic Parameters

Flow Rate (mL/min)	Alendronate Rt (min)	Vitamin D3 Rt (min)	Resolution	Back Pressure (bar)	Analysis Time (min)
0.8	4.5	14.0	11.5	165	16
1.0	3.6	11.2	11.0	205	13
1.2	3.0	9.3	10.2	245	11
1.5	2.4	7.5	9.0	305	9

A flow rate of 1.0 mL per minute provided the best balance between resolution, analysis time, and column back pressure. Higher flow rates reduced analysis time but also decreased resolution, while lower flow rates increased analysis time without significant improvement in resolution.

7 CONCLUSION

A simple, rapid, accurate, precise, and robust reversed-phase high-performance liquid chromatography method has been successfully developed and validated for the simultaneous estimation of vitamin D3 and alendronate sodium in combined pharmaceutical dosage forms used for osteoporosis treatment. The optimized chromatographic conditions employing a ternary mobile phase system with Waters Symmetry C18 column and dual wavelength detection effectively addressed the analytical challenges posed by the contrasting physicochemical properties of these two compounds. Comprehensive validation following International Conference on Harmonisation Q2(R1) guidelines demonstrated that the method meets all regulatory requirements with excellent performance characteristics including linearity ($r > 0.999$), accuracy (99-101% recovery), precision (%RSD < 2%), adequate sensitivity (LOQ: 0.08 µg/mL for vitamin D3 and 10 µg/mL for alendronate), and robustness to small parameter variations.

Forced degradation studies under five stress conditions confirmed the stability-indicating capability of the method, with complete resolution of degradation products from parent drugs

and establishment of degradation pathways for both compounds. Vitamin D3 demonstrated particular susceptibility to photolytic and oxidative degradation, while alendronate showed good stability except under alkaline conditions.

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