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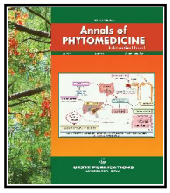

Review Article : Open Access

A review of the development of analytical methods by RP-HPLC for vitamin D_3

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Abstract

An analytical approach for quantifying vitamin D_3 was developed using high-performance liquid chromatography (HPLC). The vitamin D_3 was separated using a C-18 column. In the mobile phase, a methanol-to-water ratio of 97:3 was used. The wavelength of the vitamin D_3 chromatogram was 264 nm, and the flow rate was 1.2 ml/min. In this study, analytical quality by design (AQbD) approaches were used to optimize chromatographic parameters for routine cholecalciferol (CHL) assays. Key technique parameters were screened and optimized using Taguchi orthogonal array design and Box-Behnken design to enhance the approach's performance. Vitamin D_3 insufficiency can lead to illnesses and lowered immunity, if one lives in a northern climate and eats an insufficient diet. To compensate for vitamin D insufficiency, prescription drugs that include cholecalciferol, often known as vitamin D_3, one of the active forms of vitamin D, are utilized. Make an effort to determine the cholecalciferol content of certain pharmaceuticals and dietary supplements that are sold in the Russian Federation, as well as to develop and validate an HPLC technique for detecting vitamin D_3 in vitamin drugs. The robustness of the method was demonstrated by the fact that the findings did not significantly change when the mobile phase concentration and flow rate were adjusted. The proposed method is suitable for bulk vitamin D_3 measurement in a variety of pharmaceutical formulations and passed all validation tests.

1. Introduction

Ergocalciferol (vitamin D_2) is generated exogenously by irradiating ergosterol; vitamin D_3 (cholecalciferol) is synthesized endogenously from 7-dehydrocholesterol following UV irradiation or is absorbed from the diet. Calcium and phosphorus levels in the blood are maintained at adequate levels by vitamin D, which is essential for bone development and maintenance. According to scientific research, it is not only linked to skeletal disorders but also has a significant impact on cancer, heart disease, autoimmune diseases, hypertension, diabetes mellitus, and other conditions (Glendenning *et al*., 2006). Vitamin D is a collection of molecules with vitamin D action rather than a single substance. Its assessment is crucial as a clinical marker of dietary vitamin D insufficiency, which is among the reasons for osteoporosis. Through the synthesis of cathelicidin, a crucial host defense peptide that supports both innate and adaptive immunity, vitamin D_3 promotes wound healing by eliminating bacteria that are present in the vicinity of wounds, regulating the immune system, and promoting wound healing. All types of skin conditions can be improved with cathelicidin, including rosacea, psoriasis, and atopic dermatitis (Divya Singh, 2021).

Numerous analyses suggest that vitamin D_3 is superior to vitamin D_2 in terms of its ability to raise blood concentrations of the active form of the vitamin. As a result, vitamin D_3 may end up being the recommended option for future formulation development. Additionally, it stays in the bloodstream longer than vitamin D_2. Calcitriol, the most active form of vitamin D_3 is produced in the kidney and liver after hydroxylation (Harinarayan and Joshi, 2007). Supplementation with vitamin D_3 is recommended to restore calcitriol (CT) concentrations. An international unit dose of up to 4000 international units (IU) per day is considered safe. Vitamin D_3 supplements can be found at pharmacies and supermarkets in the form of pills or liquid drops containing 400 to 2000 IU. Vitamin D supplements and fortified foods are indispensable sources of vitamin D, although high doses might be toxic. Consequently, vitamin D testing accuracy is important for clinical research, food businesses, and nutritional chemistry.

Vitamins K_2 and D_3 have been shown to assist in balancing calcium levels for cardiovascular health and bone, but recent research suggests vitamin D increases bone formation, increases vitamin K-dependent bone protein concentration, and stimulates osteoblastic gene expression (Kennel *et al.,* 2010). The multivitamin formulation includes the vitamins D_3 and K_2 in addition to calcium because they promote bone and cardiovascular health. Therefore, it is necessary and important to determine them in dietary supplements; however, there is currently no approved, validated method for measuring vitamins D_3 and K_2 at the same time. The primary obstacle in clinical research, as opposed to other fields such as food analysis, is sample size constraints. Despite this, the majority of the approaches for estimating vitamin D_3 that are now accessible were created using LC-MS/MS, which raises the analysis cost per sample (Ahmad *et al*., 2016).

As a result, we must create an affordable, straightforward, and sensitive RP-HPLC technique for estimating vitamin D_3. Moreover,

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this kind of method development only offers a partial understanding of a technique's capabilities and resilience. Using QbD principles to develop an analytical method may avoid this problem since it employs a statistical experimental design to create an operational design space for an analytical method (Vimal Raj and Sumithra, 2023).

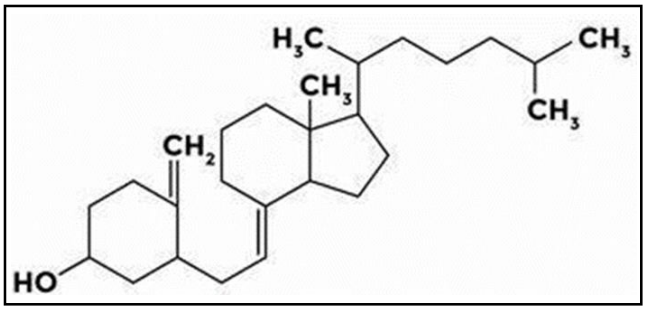

Figure 1: Structure of vitamin D_3.

Nonetheless, several studies and data from the published literature indicate that vitamin D insufficiency is widespread in India, affecting people of all ages and genders. Because vitamin D status screening makes it possible to track a patient's reaction to vitamin D medication and assess the effectiveness of treatment, samples that yield fast and accurate findings are highly valued (Marwaha and Sripathy, 2008). It is an essential fat-soluble vitamin for both humans and animals to get enough vitamin D in their diets. Vitamin D_2 and vitamin D_3 are the two types. By irradiating ergosterol, vitamin D_2 (ergocalciferol) is synthesized exogenously; vitamin D_3 (cholecalciferol) is synthesized endogenously from 7-dehydrocholesterol, following UV irradiation or absorbed from the diet (Pradeep Singh *et al*., 2022).

The significance that vitamin D plays in the preservation of appropriate blood levels of calcium and phosphorus, which are necessary for healthy bone growth and maintenance. According to scientific research, it is not only linked to skeletal disorders but also has a significant impact on cancer, heart disease, autoimmune diseases, hypertension, diabetes mellitus, and other conditions. Vitamin D is a collection of molecules with vitamin D action rather than a single substance. As a clinical biomarker of dietary vitamin D insufficiency, which is one of the causes of osteoporosis, its assessment is significant. Here, we have created and presented a comparison analysis between two HPLC techniques for measuring vitamin D_3. The goal of the current study was to create a vitamin D assessment procedure that was simple to use, sensitive, quick, and affordable on a lab Scale (Sivakumar *et al*., 2022).

2. Drug profile

Table 1: Drug profile of Vitamin D_3

IUPAC name	(3β, 5z, 7e) -9, 10- secocholesta-5,7,10 (19)- trien-3-ol
Chemical name	Cholecalciferol
Molecular formula	$C_{27}H_{44}O$
Molecular weight	384.6377
Pka	2.0, 4.85
Solubility	<0.1g/l (20°c)
Melting point	84°c – 85°c

2.1 Mechanism of action

The majority of people get enough vitamin D from their diets and from sunshine exposure, which naturally converts the vitamin D_3 precursor 7-dehydrocholesterol in the skin. Examples of these foods include eggs, salmon, and cheese. Sunlight exposure, diet low in vitamin D, genetic abnormalities with the endogenous vitamin D receptor, or even serious liver or kidney disease can all contribute to a vitamin D shortage (Beg *et al*., 2012). Such a deficiency is known to cause diseases such as osteomalacia or rickets, which are all associated with increased parathyroid hormone production and secretion2, decreased blood concentrations of calcium ions, and insufficient bone mineralization (Blessy *et al*., 2014). Increases in parathyroid hormone cause the skeletal muscle to mobilize, and calcium and phosphorus are excreted by the kidneys. Porotic bone diseases are the result of this increased skeletal calcium mobilization. As a dietary supplement, vitamin D_3 may also be obtained from a variety of food and pharmaceutical products. Vitamin D_3 is normally produced in the skin by photochemical reactions **(**Aaisha Ansari *et al*., 2020).

Vitamin D primarily maintains appropriate levels of serum calcium and phosphorus by improving the small intestine's ability to absorb these minerals from a meal (Borman *et al*., 2010). A hydroxylation of vitamin D_3 or D_2 in the liver produces 25-hydroxyvitamin D, which is then further hydroxylated in the kidney to produce 1, 25-dihydroxyvitamin D, which is the primary active metabolite of vitamin D (Sengottuvelu *et al*., 2021). This final metabolite acts as a regulator after binding to endogenous vitamin D receptors. It maintains calcium balance, controls parathyroid hormone, encourages renal calcium reabsorption, increases calcium and phosphorus absorption in the intestinal tract, and mobilizes calcium and phosphorus from bones to plasma to maintain balanced levels.

3. Method development

3.1 Method 1

Using UV detection at a wavelength of 266 nm, HPLC was used to determine the amount of vitamin D_3. The preparation of vitamin drug samples involved extracting liquid dosage forms from aqueous or triglyceride solutions using methanol, and extracting solid dosage forms from water-soluble substances containing vitamin D_3 from aqueous-methanol solutions in a 2 to 8 ratio. Throughout this investigation, an HPLC system with a quaternary pump was employed (Ahmad *et al*., 2016).

3.2 Method 2

Using a vanquish UHPLC system (Thermo Fisher Scientific, Germering, Germany) for ultra-high-pressure liquid chromatography along with a DAD HL diode array detector (Dionex/Thermo Fisher Scientific, germering, Germany), the analysis was conducted at 30°C on an Acclaim C30 150 x 46 m, 5 m (Thermo Scientific, Sunnyvale, CA, USA). Methanol (B) and ultrapure water (A) made up the mobile phase. 98% B was used in the isocratic elution, which was carried out at a rate of 1 ml/min. The autosampler was set at 10°C and the injection volume was 10 liters to avoid sample degradation due to temperature. At 265 nm, the DAD detector was calibrated. Validation of the method by evaluating the primary parameters, the method was validated (Blessy *et al.,* 2014).

3.3 Method 3

Examination of nutritional supplements. The suggested procedure was used to extract and analyze two synthetic pharmaceuticals in commercial capsule form as well as six distinct nutritional supplements. The concentrations that were achieved with the verified method and those listed on the product labels were contrasted. To get a linear graph or straight line equation for the method validation of vitamin D_3, methanol, and HPLC grade water were used in varied ratios for the mobile phase, and a C18 column was chosen as the stationary phase (Kennel *et al*., 2010).

3.4 Method 4

This study used the RP-HPLC technique to analyze cholecalciferol 60,000 IU chewable tablets and vitamin D_3 mouth-dissolving tablets. The HPLC technique for CHL was developed using Jasco AS-2055 plus (Tokyo, Japan) equipment. This includes a system controller, quaternary gradient pump, mobile phase degasser, autoinjector (with a 5 to 100 ml injection volume), and photodiode array detector PDA (Weaver and Fleet, 2004).

3.5 Method 5

Methodological, scientific components of ATP are defined and suggested by AQbD-based methodology for methodical, scientific development of analytical procedures. It is possible to summarize the quality aspects of the analytical technique in the method goals. Important components of the ATP framework are shown in Table 1 to produce an effective HPLC technique for CHL. Qualities of critical analysis (CAAs): Different CAAs were identified and investigated to get the expected ATP. Peak area, peak tailing factor, theoretical plates, and retention time are these (Venkatachalam *et al*., 2021).

A simultaneous measurement of cholecalciferol and calcitriol in dietary supplements containing vitamin D_3. A single injection can quickly quantify and separate a wide variety of target analytes (Akella Anuradha *et al*., 2023). This validated technique demonstrated good linearity, good recovery, intra-day and inter-day precision, high selectivity, specificity, and a low level of detection and limit of quantification (D_3) for each of the target nutritional substances. (Dhritimoni Devi *et al.,* 2023). Using HPLC chromatography for CHL analysis, this article demonstrates how to improve HPLC through a better understanding of the factor-response connection, which is vital to quality by design. As a result of CHL's AQbD-driven approach to method development, the robustness of the analytical technique was guaranteed before validation trials were conducted. This new technique aids in the analyst's creation of control measures to lessen these CMVs' undesired impact on method performance. High degrees of precision, accuracy, and linearity were validated by the validation investigations (Subhamalar *et al*., 2023; Holick, 2004).

4. Conclusion

The majority of them are unable to pay the high cost of the existing vitamin D diagnostic procedures. As a result, we must create a straightforward, trustworthy, and affordable technique for estimating vitamin D. Analytical techniques are more sensitive and economical than RIA- or ELISA-based procedures, as is widely proven. Vitamin D_2 has been separated using a variety of chromatographic settings, but the best separation was eventually accomplished. In this study, the quality-by-design approach is effectively used to improve the efficiency of the HPLC chromatographic technique by providing a better understanding of the important factor-response relationship. The robustness of the analytical technique was verified before validation investigations by the development of an A QbD-driven HPLC method by CHL.

This new technique aids in the analyst's creation of control measures to lessen these CMVs' undesired impact on method performance. High degrees of precision, accuracy, and linearity were validated by the validation investigations. Because methanol and water are utilized as solvents in this procedure, it is both unique and accurate. Comparing this approach to other developed methods, it was discovered that the least quantity of vitamin D_3 could be computed since the detection limit was determined to be 0.0005 µg. The method for estimating vitamin D_3 that has been developed is simple, affordable, robust, sensitive, and repeatable.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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