Stability-Indicating HPLC–UV Method for Vitamin D₃ Determination in Solutions, Nutritional Supplements and Pharmaceuticals

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Received 2 April 2015; Revised 23 February 2016

Abstract

A simple and fast high-performance liquid chromatography method with UV detection for determination of vitamin D₃ in stability studies as well as in solutions, nutritional supplements and pharmaceuticals was developed. Successful separation of vitamin D₃ from its degradation products was achieved on a Gemini C18 100 × 3.0 mm column using a mixture of acetonitrile and water (99:1, v/v) as a mobile phase. The method was successfully validated according to the ICH guidelines. The described reversed-phase HPLC method is favorable compared with other published HPLC–UV methods because of its stability-indicating nature, short run time (3.3 min) and wide analytical range with outstanding linearity, accuracy and precision. The method was further applied for quantification of vitamin D₃ in selected liquid and solid nutritional supplements and prescription medicines, confirming its suitability for routine analysis. Degradation products, formed under stress conditions (hydrolysis, oxidation, photolysis and thermal degradation), were additionally elucidated by suitable equipment (LC–DAD–MS) to confirm the stability-indicating nature of the developed method.

Introduction

Vitamin D₃ (cholecalciferol) is a unique fat-soluble vitamin because it can be obtained by endogenous synthesis. Its bioactive form calcitriol (1,25(OH)₂-D₃) plays an essential role in bone development and the homeostasis of calcium and phosphorus. Severe vitamin D deficiency manifests as rickets in infants and children, and osteomalacia in the elderly (1). Less severe deficiencies have been implicated in a wide variety of diseases, including some types of cancer, allergic diseases, cardiovascular diseases, diabetes, etc. (2). In medical practice, vitamin D₃ is typically used from 400 to 1,000 IU/day (0.010–0.025 mg/day). Low vitamin D₃ concentrations in nutrition supplements and prescription medicines, the presence of excipients that might interfere with its determination and its lipophilic nature can lead to various analytical problems in the identification and quantification of the vitamin.

HPLC methods offer the best approach to accurate content determination of vitamin D₃ in foods and pharmaceuticals, as well as stability testing. In the last decade, high-performance liquid chromatography coupled to mass spectrometry has become the technique of choice for vitamin D₃ determination in foods, feeds and pharmaceuticals. This technique is especially convenient for quantification of the vitamin D₃ in various complex matrices and multivitamin mixtures (3–6). The published HPLC–UV methods for quality control of pharmaceuticals and nutritional supplements containing vitamin D₃ are quite limited regarding the stability-indicating capabilities (7–15), often have run time longer than 10 min (7, 8, 10–12, 14) and are generally preceded by highly complicated and time-consuming sample preparation such as solid-phase or supercritical fluid extraction (8, 9, 12, 13).

The aim of the present report was to develop a simple and fast stability-indicating method for the analysis of vitamin D₃ in stability studies as well as in pharmaceutical preparations using a reversed-phase HPLC method with UV detection. The method that covers the determination of vitamin D₃ in various matrices was successfully validated according to the International Conference on Harmonization (ICH) guidelines. In addition, liquid and solid nutritional supplements and prescription medicines were analyzed to confirm the adequacy of the method. Degradation products, formed under stress conditions (hydrolysis, oxidation, photolysis and thermal degradation), were additionally elucidated by suitable equipment (LC–DAD–MS) to confirm the stability-indicating nature of the developed method.
Experimental
Reagents and chemicals
Vitamin D₃ (98%, CAS No. 67-97-0) and 30% solution of hydrogen peroxide (H₂O₂) were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium hydroxide (NaOH), hydrochloric acid (HCl), formic acid (HCOOH) and orthophosphoric acid (H₃PO₄) were obtained from Merck (Darmstadt, Germany). Both acetonitrile and methanol of HPLC grade were purchased from Sigma-Aldrich (Steinheim, Germany). Ultra-pure water was obtained through a Milli-Q water purification system A10 Advantage (Millipore Corporation, Bedford, MA, USA).

HPLC analysis
A high-performance liquid chromatograph Series 1100/1200 (Agilent Technologies, USA), equipped with a diode array detector and a ChemStation data acquisition system, was used. The chromatographic separation was performed on a reversed-phase Gemini C18 100 × 3.0 mm, 3 µm particle size column (Phenomenex, USA) at 40°C using acetonitrile–water (99:1, v/v) as a mobile phase at a flow rate of 1 mL/min. The injection volume was set between 3 and 50 µL, depending on the type of the preparation. The detection was carried out at 265 nm.

LC–MS analysis
Identification of vitamin D₃ degradation products was carried out on an Agilent Infinity 1290 LC (Agilent Technologies) attached to a 6460 QQQ mass spectrometer (Agilent Technologies) using electrospray ionization (ESI) in positive scan mode from m/z 200 to 500 to confirm the stability-indicative nature of the developed HPLC–UV method. In addition, MS–MS product ion scans for precursor ions were performed at a collision energy of 15 eV. The operating conditions for MS were as follows: drying gas temperature 275°C, drying gas flow 5 L/min, nebulizer 45 PSI, sheath gas temperature 320°C, sheath gas flow 11 L/min, capillary entrance voltage 4,000 V, nozzle voltage 1,000 V and delta EMV 200 V. The chromatographic conditions were the same as for HPLC analysis, except for the mobile phase. The mixture of methanol and 0.1% formic acid (93:7, v/v) was used instead, as it provides better ionization of vitamin D₃ and its related compounds at comparable retention times. Additionally, a diode array detector was coupled to the LC–MS system to obtain support information about the degradation products.

Procedure for forced degradation study of vitamin D₃
Forced degradation was performed according to the ICH guidelines Q1A (R2) (16). Each sample was prepared in triplicate. Degradation was initiated by dissolving 50.0 mg of vitamin D₃ in 10.0 mL of methanol and by further dilution (c = 200 mg/L) with various solvents: water (thermal degradation), 0.1 M hydrochloric acid (acidic degradation), 0.1 M sodium hydroxide (basic degradation) and 0.3% and 15% H₂O₂ (oxidation). All samples were exposed to selected stress conditions for 24 h at 25 and 60°C. Water samples were also exposed to light at room temperature (photodegradation). After the degradation treatments, the samples were cooled to room temperature, neutralized with NaOH or HCl (if required) and analyzed. Because of the fast degradation in water, stress tests were also conducted using methanol solutions of vitamin D₃.

Preparation of solutions for method validation
The working standard solution (c = 100 mg/L) was prepared by dissolving accurately weighed 10.0 mg of vitamin D₃ into 100.0 mL methanol in a volumetric flask, each day of the validation. Eight calibration standards were prepared for each standard calibration curve. Appropriate volumes of the working standard solution were pipetted into separate amber vials, diluted to a volume of 1,000 µL with methanol and mixed, resulting in the following concentrations: 0.5, 1, 2, 5, 10, 20, 50 and 100 mg/L. Three quality control samples at 1.5 (low), 7.5 (medium) and 75 mg/L (high) were prepared by diluting fresh working standard solution with methanol.

Assay of commercially available nutritional supplements and prescription medicines
Liquid dosage forms
The developed method was applied to assay the content of vitamin D₃ in commercial liquid prescription medicines A, B and C (4,000, 20,000 and 2,000 IU/mL, respectively) and one liquid nutrition supplement D (4,000 IU/mL). Three different batches of each preparation were analyzed at least in triplicate. Liquid preparations were directly analyzed, without any sample pretreatment. The injection volume was adjusted according to the content of vitamin D₃: 5 µL for preparations A, C and D and 3 µL for preparation B. The recovery of the method for liquid forms was determined by spiking the original preparations with approximately the same amount of vitamin D₃, as contained in the preparations, in triplicate (total amount found). Unspiked preparations (original amount) and the methanol standard solution of vitamin D₃ containing the added amount (amount spiked) were separately analyzed. The average recoveries were calculated by the formula: recovery (%) = (total amount found – original amount)/amount spiked × 100%.

Solid dosage forms
Three batches of commercially available nutritional supplements from two different producers (preparations E and F) prepared in triplicate were analyzed. The labeled content of vitamin D₃ in both preparations was 400 IU per tablet. The extraction procedure was adapted from Kucukkolbasti et al. (8). Two weighed tablets were added to 2.0 mL of 0.1% orthophosphoric acid into a centrifuge tube and vortexed for 2 min. Subsequently, 8.0 mL of methanol was added. The samples were sonicated for 10 min and again vortexed for 2 min. The samples were then centrifuged for 10 min at 4°C and 5,000 rpm. The clear supernatant was filtered through a 0.45-µm filter prior to HPLC analysis. The injection volume was 50 µL. The recovery studies were performed in the same way as for the liquid preparations. A known amount of vitamin D₃ was added to 2.0 mL of 0.1% orthophosphoric acid, into which two tablets of preparation E or F were further added and subjected to the extraction procedure, as previously described. Unspiked preparations and methanol standard solution of vitamin D₃ containing the added amount were separately extracted and analyzed. Recoveries were calculated using the same formula as for liquid preparations.

Results
Forced degradation studies
The forced degradation study showed that vitamin D₃ is liable to hydrolytic, oxidative, thermal and photolytic conditions. Its degradation was observed at all stress conditions in water samples after 24 h. Thermal degradation of vitamin D₃ resulted in the formation of more degradation products (Figure 1). UV and oxidative degradation resulted in the formation of the same degradation products as after thermal degradation while the alkaline hydrolytic degradation resulted in one additional peak (at 0.8 min). The rate of acid hydrolysis was very rapid...
and resulted in complete degradation of vitamin D₃ within 1 h. It was observed that in 0.1 M HCl there was no degradation peak product eluted in the chromatogram.

Identification of obtained degradation products was performed using a LC-MS system coupled with diode array detector. A chromatogram after thermal degradation is shown in Figure 1. Vitamin D₃ eluting at a retention time of 2.6 min has a typical molecular ion [M+H]⁺ at m/z 385.4. The first eluting peak (rt 0.4) belongs to the compound with m/z 325.3 (loss of water and an isopropyl group from vitamin D₃). More degradation products can be seen in Figure 1, at retention times from 0.9 to 1.6 min, which result from the oxidation of vitamin D₃ to various hydroxy vitamin D₃ degradation products (OH-vit. D₃) with a mass peak at m/z 401.4 [(OH-vit. D₃)+H]⁺ (increase in molecular weight of 16 compared with vitamin D₃) or m/z 383.4, which corresponds to further fragmentation of OH-vit. D₃ to [(OH-vit. D₃)-H₂O+H]⁺. Additionally, a product ion scan of m/z 401.4 confirms the tentative structure of hydroxylated vitamin D₃ degradation products (Table I).

Because vitamin D₃ undergoes extensive degradation to secondary degradation products in water stress samples, stress samples in methanol were used to obtain relevant degradation products (<15% degradation of the principal peak). Typical chromatograms obtained with the proposed method after thermal, oxidative, acidic and alkaline hydrolysis and photodegradation of vitamin D₃ are shown in Figure 2. Stress samples after thermal and alkaline degradation show comparable chromatograms (Figure 2B), with a single peak for vitamin D₃. Oxidative degradation resulted in complete degradation of vitamin D₃ without any degradation product peak in the chromatogram (Figure 2C). UV and acidic degradation resulted in the formation of degradation products (Figures 2A and D), which were identified as vitamin D₃ isomers, as they have the same mass ions as vitamin D₃ ([M+H]⁺ = 385.4). Additional confirmation that these degradation products are vitamin D₃ isomers was performed by product ion scan mode, which showed the same fragmentation profiles (Table I). Diode array detection was used to further identify individual isomers. UV spectra of vitamin D₃ and its isomers were recorded from 190 to 400 nm (Figure 3) and found to be consistent with the literature UV spectra of particular isomers (17, 18). The identified degradation products, their elution order, retention times, characteristic absorption maxima, molecular mass information and five most abundant product ions of each particular molecular ion are shown in Table I. Chromatographic peaks were observed for tachysterol D₃, trans-vitamin D₃ and vitamin D₃, while pre-vitamin D₃ and lumisterol D₃ were identified only after MS analysis. Pre-vitamin D₃ was found in stress samples after thermal degradation, while lumisterol D₃ after photodegradation and acidic hydrolysis.

**Analytical method validation**

The method was validated according to the ICH guidelines Q2(R1) in terms of selectivity, linearity, repeatability, precision, accuracy, detection limit (LOD), quantification limit (LOQ) and sample stability (19).

Method selectivity was assessed by comparing the chromatograms of vitamin D₃ standard solution and potential solvents, several common excipients found in commercial preparations, such as citric acid, EDTA, sodium hydrogen phosphate, glycerol, propylene glycol, sodium benzoate, methyl- and propylparaben and butylhydroxytoluene as well as some active ingredients such as vitamins A, C, E, ascorbyl palmitate and coenzyme Q10. The selectivity of the method was confirmed (Figure 4) as no interfering peaks were found at the retention time of vitamin D₃. The method was also found selective in the presence of the formed degradation products during the forced degradation study. In addition, the peak purity analysis was determined to confirm that there was no co-elution of any degradation product with the principal peak of vitamin D₃. The peak purity test complied for the peak of vitamin D₃ in all stressed samples and indicated no co-elution of degradation products.

Linearity was evaluated based on eight calibration standards in the concentration range from 0.5 to 100 mg/L. The procedure was repeated three times, using different working standard solution, for three consecutive days of the validation. The injection volume during validation was 20 µL. The calibration standards from the second validation day were reanalyzed with variations of the injection volume (5 and 50 µL). Slopes from the obtained calibration lines were comparable with the original slope (99.70 and 100.11%) and between

**Figure 1.** A chromatogram of the vitamin D₃ sample after thermal degradation in water: (a) fragment of vitamin D₃ and (b–d) hydroxy vitamin D₂.

**Table I.** Identified Vitamin D₃-Related Compounds

<table>
<thead>
<tr>
<th>Abbreviationsᵃ</th>
<th>Compound</th>
<th>Retention time (min)</th>
<th>UV absorption maxima (nm)</th>
<th>[M+H]⁺ (m/z)</th>
<th>Product ions (m/z)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>tachy.</td>
<td>Tachysterol D₃</td>
<td>2.28</td>
<td>272, 280, 291</td>
<td>385.4</td>
<td>107.1, 133.1, 159.1, 259.3, 367.4</td>
</tr>
<tr>
<td>pre-vit. D₃</td>
<td>Pre-vitamin D₃</td>
<td>2.39</td>
<td>260</td>
<td>385.4</td>
<td>107.1, 133.1, 159.1, 259.3, 367.4</td>
</tr>
<tr>
<td>trans.</td>
<td>Trans-vitamin D₃</td>
<td>2.43</td>
<td>273</td>
<td>385.4</td>
<td>107.1, 133.1, 159.1, 259.3, 367.4</td>
</tr>
<tr>
<td>vit. D₃</td>
<td>Vitamin D₃</td>
<td>~2.60</td>
<td>265</td>
<td>385.4</td>
<td>107.1, 133.1, 159.1, 259.3, 367.4</td>
</tr>
<tr>
<td>lum.</td>
<td>Lumisterol D₃</td>
<td>3.05</td>
<td>276, 286, 298</td>
<td>385.4</td>
<td>107.1, 135.1, 159.1, 259.3, 367.4</td>
</tr>
<tr>
<td>b–d</td>
<td>Hydroxy vitamin D₃</td>
<td>0.9–1.6</td>
<td>250</td>
<td>401.4</td>
<td>109.1, 175.1, 247.1, 365.3, 383.1</td>
</tr>
</tbody>
</table>

ᵃAbbreviations refer to the peaks as labeled in Figures 1 and 2.

ᵇMost abundant product ions.
themselves (99.81%). Linearity was determined based on the least-square linear regression. The acceptance criterion for the determination coefficient was $R^2 > 0.999$. The results for linearity of the method, including determination coefficients, are summarized in Table II. These results indicate that the presented method has excellent linearity.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the regression lines, using the following equations: $LOQ = (10 \times \sigma)/S$ and $LOD = (3.3 \times \sigma)/S$, where $\sigma$ is the standard deviation of the intercepts and $S$ is the average slope of the calibration curve. The determined LOD and LOQ values were 0.019 and 0.057 mg/L, respectively.

Method precision and accuracy were examined in terms of repeatability, intermediate precision and accuracy (intra- and inter-day) during three validation days, based on three different quality control samples covering the whole analytical range. The quality control samples were prepared in triplicate, each day of the validation. Injection repeatability was evaluated on three concentration levels by re-injecting the same quality control sample six times. Method precision, expressed as relative standard deviation (RSD), should be <5% (2% for injection repeatability). Accuracy was presented as ratio (%) between the calculated and the theoretical concentration of vitamin D$_3$ in samples. The acceptance criterion for accuracy was the interval 95–105%. As shown in Table III, the results are in accordance with the defined acceptance criteria.

Sample stability was evaluated for three consecutive days of the validation. The quality control samples were kept in the autosampler at 25°C and analyzed at 0, 6, 24 and 48 h. The presented results (% ± standard error of the mean) are expressed as a ratio between the response at a certain time and the response at time 0. The

**Figure 2.** Chromatograms of vitamin D$_3$ stress samples in methanol after (A) UV degradation, (B) thermal degradation, same as alkaline, (C) oxidative degradation and (D) acidic degradation.

**Figure 3.** UV spectra of vitamin D$_3$ and its related compounds: tachysterol D$_3$, pre-vitamin D$_3$, trans-vitamin D$_3$, lumisterol D$_3$ and hydroxy vitamin D$_3$. 

Stability-Indicating HPLC–UV Method for Vitamin D$_3$ Determination
The acceptance limit was 100 ± 5%. The stability of vitamin D3 after 6, 24 and 48 h in the autosampler at 25°C was 100.07 ± 0.09; 100.02 ± 0.14 and 99.35 ± 0.16%, respectively.

Method application: assay of vitamin D3 in commercial nutrition supplements and prescription medicines

The validated method was applied to assay the content of vitamin D3 in commercial liquid prescription medicines A, B and C, liquid nutrition supplement D and solid nutrition supplements E and F. The results for the average content of vitamin D3 and the calculated recoveries are presented in Table IV.

Discussion

The main objective of the study was to establish a simple and fast stability-indicating HPLC–UV method, which accurately measures the changes in vitamin D3 concentration without interference from other degradation products, impurities and excipients. In addition to quantitative analysis of vitamin D3 in pharmaceutical preparations, such method would be also suitable in stability studies of vitamin D3. The development of the method was quite challenging due to the highly lipophilic nature of vitamin D3, which required a unique approach. Several reversed-phase analytical columns, mobile phase compositions (mixtures of methanol and/or acetonitrile with water), injection volumes (3–50 μL), flow rates (0.5–2.0 mL/min) and column temperatures (25–40°C) were tested to optimize the chromatographic peak shape, peak width and separation of vitamin D3 from its degradation products. Optimal conditions were obtained on a Gemini C18 100 × 3.0 mm column with a mobile phase consisted of water and acetonitrile in ratio 1:99 (v/v), which resulted in short retention time of vitamin D3 (2.6 min) and satisfying separation from its nearby eluting acidic and photodegradation products (Figure 2). The obtained retention time was favorable in comparison to other published HPLC–UV methods for determination of vitamin D3, where only one has a comparable retention time (13), two have it up to 10 min (9, 15), while the rest have even longer retention time (7, 8, 10–12, 14).

According to the results of forced degradation study, vitamin D3 was found to be the most prone to degradation under acidic and oxidizing conditions. Methanol solutions of vitamin D3 were generally more stable than water solutions. Vitamin D3 was converted to various isomers in a lesser extent: pre-vitamin D3 by heat, trans-vitamin D3 and lumisterol D3 by light and tachysterol D3 and lumisterol D3 under acidic conditions. The absence of a peak corresponding to vitamin D3 after exposure to stress conditions shows that potential degradation products do not interfere with the determination of vitamin D3. Moreover, the stability-indicating ability of the method was proven as the peak purity test successfully passed the analysis of vitamin D3 in all stressed samples, confirming the spectral similarity of the principal peak (match factor >0.999). On the basis of the obtained data including also LC–MS analyses, it can be concluded that the herein described method is stability-indicating and suitable for the analysis of vitamin D3 stability in simple nonfood matrices.

The optimized method was successfully validated, as all tested parameters met the defined acceptance criteria. Selectivity as one of the crucial validation parameters was extensively evaluated during the

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**Table II. Results Obtained for the Linearity Study**

<table>
<thead>
<tr>
<th>Calibration line</th>
<th>Injection volume (μL)</th>
<th>Slope</th>
<th>Intercept</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>51,663</td>
<td>1.8376</td>
<td>0.9999</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>49,301</td>
<td>−2.7535</td>
<td>1.0000</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>50,938</td>
<td>−5.6904</td>
<td>1.0000</td>
</tr>
<tr>
<td>A</td>
<td>5</td>
<td>50,641</td>
<td>−2.2021</td>
<td>1.0000</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>12,288(49,152)a</td>
<td>−0.9142</td>
<td>1.0000</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>123,177(49,271)a</td>
<td>−0.4534</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

*aCalculated to an injection volume of 20 μL.*
The validated method was further applied to assay the content of vitamin D3 in commercially available pharmaceuticals. Good accuracy in terms of high recoveries and precision (Table IV) confirmed the suitability of the established method for the quantification of vitamin D3 in liquid (A, B, C and D) and in solid preparations (E and F). The proposed method enables direct determination of vitamin D3 in various liquid pharmaceutical preparations and nutritional supplements without any pretreatment. It is also applicable for the quantification of vitamin D3 in solid dosage forms after a simple and rapid pretreatment.

**Conclusion**

A fast and simple stability-indicating HPLC–UV method for quantification of vitamin D3 in the presence of its degradation products was developed and validated according to the ICH guidelines. The described reversed-phase HPLC method is favorable compared with other published HPLC–UV methods because of its stability-indicating nature, short run time and wide analytical range with outstanding linearity, accuracy and precision. The proposed method allows the determination of vitamin D3 and its related compounds in a single chromatographic run and is suitable for the analysis of the stability of vitamin D3. The obtained results from the assay of vitamin D3 in commercial nutrition supplements and prescription medicines confirmed that the method is appropriate for the routine analysis of various pharmaceuticals.

**References**

fat-soluble vitamins in food and pharmaceutical supplement; *Talanta*, (2008); 75(1): 141–146.


