DETERMINATION OF VITAMIN-D LEVEL IN FORTIFIED MILK BY ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A reliable ultra-performance liquid chromatography (UPLC) assay was developed for simultaneous quantification of vitamin D-2 (VD-2) and vitamin D-3 (VD-3) levels in VD-fortified milk, using laurophenone as an internal standard (IS). Milk samples were prepared by overnight incubation with a mixture of ethanol and 50% potassium hydroxide, extracted with hexane, and cleaned-up with ethanol and 5% potassium hydroxide. After evaporation, the residue was dissolved in methanol, centrifuged, and 5.0 µl of the clear solution was injected into Acquity UPLC BEH C18 column. The mobile phase, composed of methanol, acetonitrile, and water (80:10:10, v:v:v), was used at a flow rate of 0.35 ml/min and a run time of 15 minutes. Eluents were monitored by photodiode array detector, with the wavelength set at 265 nm. The relationship between the concentration of VD-2 and VD-3 and their corresponding peak area ratios to the IS was linear over the range of 2.5 – 60 ng/ml. Mean extraction recovery for VD-2, VD-3, and IS was 93%, 94% and 70%, respectively. Stability of VD-2 and VD-3 before and after extraction was studied under various laboratory conditions. Further, the method was successfully used to measure vitamin D levels in milk samples from the local market.

KEYWORDS: Laurophenone, VD-fortified milk.

INTRODUCTION

Adequate level of vitamin D plays an important role in protection against metabolic bone disease and possibly other chronic diseases.[1-2] Vitamin-D deficiency is recognized as
worldwide human health problem; recent studies suggest that 30-50% of children and adult have vitamin-D levels below the normal range.\textsuperscript{[3-4]} Various steps have been taken, at population level to minimize the extent of vitamin D deficiency including vitamin D fortification of milk and beverages.\textsuperscript{[5-6]} The US Food and Drug Authorities (FDA) recommends that vitamin D level in milk should between 10 - 15 ng/ml (400-600 IU/L, 26 – 39 nmol/L).\textsuperscript{[7]}

Vitamin D-2 (VD-2, ergocalciferol) and Vitamin D-3 (VD-3, cholecalciferol) have been interchangeably used as milk and beverages supplements. In general, ±20% of the label claim is considered acceptable for quality assurance purposes.\textsuperscript{[8]} However, the level may vary more widely due to manufacturing and storage reasons; indicating the importance of surveillance programs. The complexity of determination of fat soluble vitamins is related to the presence of endogenous interfering substances and to the reagents used in sample processing. A thorough literature search revealed a number of HPLC methods to determine VD-2 and VD-3 in milk that involved alkaline hydrolysis or dervatization followed by extraction and chromatographic purification.\textsuperscript{[9-15]} Some of these methods used VD-2 as an internal standard\textsuperscript{[13-15]} precluding simultaneous measurement of VD-2 and VD-3. An HPLC method with electrochemical detection has also been described.\textsuperscript{[16]} Further, a liquid chromatographic mass spectrometric (LCMS)- based method using isotope-labeled VD-2 and VD-3 as IS has been reported for simultaneous measurement of VD-2 and VD-3 in milk.\textsuperscript{[17]} Nevertheless, a recent methodology survey indicated that there is a need of simpler methods.\textsuperscript{[18]} Further, literature review indicate that little data are available on VD stability in milk.\textsuperscript{[19-20]}

The objective of the current study was first to develop and validate, a simple and rapid ultra-performance liquid chromatography (UPLC) method for simultaneous quantification of VD-2 and VD-3 in milk samples using a readily available internal standard, and second to study VD-2 and VD-3 stability in milk under various laboratory conditions.

**EXPERIMENTAL**

**Apparatus**

Chromatography was performed on Acquity Ultra Performance System (UPLC) (Waters Associates Inc, Milford, MA, USA) composed of quaternary pump, autosampler, column thermostat, and photodiode array detector set at 265 nm. A reversed-phase Acquity UPLC BEH C\textsubscript{18} (2.1 x 100 mm, 1.7-µm) column protected by a guard pre-column BEH C\textsubscript{18} (2.1 x
10 mm, 1.7-μm) was used for separation. The data were collected with a Pentium IV computer using Empower Chromatography Manager Software.

**Material And Reagents**

Vitamin D-2 and Vitamin D-3 (purity > 98.0%) were purchased from Sigma-Aldrich, Taufkirchen, Germany and laurophenone from Acros Organics, New Jersey, USA. Potassium hydroxide, ethanol, methanol, and acetonitrile were purchased from Fisher Scientific, Fairlawn, NJ, USA. Water for HPLC was prepared by reverse osmosis and further purified by passing through a synergy water purification system (Millipore, Bedford, MA, USA). All chemicals were of analytical grade unless stated otherwise. Cow milk was purchased from the local market in Riyadh, Saudi Arabia.

**Chromatographic conditions**

The mobile phase was composed of a mixture of methanol, acetonitrile, and water (80:10:10, v:v:v) and was used in isocratic condition at a flow rate 0.35 ml/min. The column and autosampler temperatures were maintained at 30 ºC and 4 ºC, respectively. The run time was 15 minutes.

**Calibration standards / Quality control samples**

10 mg of VD-2, VD-3, or IS was dissolved in 100 ml methanol by vigorous shaking to produce a concentration of 100 μg/ml. IS working solution (0.5 μg/ml) was prepared in methanol biweekly. VD-2 and VD-3 eight calibration solutions (2.5 to 60 ng/ml) and three quality control (QC) solutions (7.5, 30, and 54 ng/ml) were prepared in milk weekly. The calibration and QC solutions were vortexed and 20 ml aliquots were transferred into 50 ml polypropylene centrifuge tubes and stored at 4°C until used.

**Sample preparation**

100 μl of the IS working solution was added to 20 ml milk samples, calibration standards, or QC samples in a 50 ml centrifuge tubes. 25 ml of a mixture of 50% potassium hydroxide and ethanol (2:8, v:v) was added to each tube. Samples were mixed thoroughly over night on nutating mixer plate at room temperature. 8.0 ml hexane was added to the mixture and vortexed for 30 seconds, then centrifuged at 1200 rpm (8°C) for 10 minute. The supernatant organic layer was collected and washed with 5 ml mixture of 5% potassium hydroxide and ethanol (95:5, v:v). After another centrifugation for five minutes at 1200 rpm (8°C), the clear
supernatant layer was transferred to a clean culture tube and dried under a gentle steam of nitrogen at 40°C. The residue was reconstituted in 100 μl methanol, centrifuged, and 5.0 μl of the clear solution was injected into the UPLC system.

METHOD VALIDATION
We followed FDA guidelines for method validation.\textsuperscript{[21]} Validation criteria included: specificity, accuracy, precision, and linearity.

Calculations
In order to correct for VD-3 level in “blank” milk, we used the difference in peak area ratios between each consecutive concentration (rather than the observed peak area ratio) as the response in constructing the VD-3 standard curve. For VD-2, the peak area ratios was directly used. Bias (%) was calculated as the difference between measured and nominal concentration divided by nominal level times 100, whereas coefficient of variation (CV, %) was calculated as standard deviation divided by mean concentration times 100.

Extraction recovery: To calculate extraction recovery of VD-2 and VD-3, five replicates of three concentrations (7.5, 30 and 54 ng/ml) were prepared in milk or in mobile phase and peak areas were compared. Similarly, the recovery of the IS was determined by comparing peak areas of the IS in five replicates of IS spiked milk samples with peak areas of equivalent samples prepared in mobile phase.

Stability
The stability of VD-2 and VD-3 in milk was investigated under various analytical laboratory conditions.

Freeze and thaw stability: Stability of VD-2 and VD-3 was determined over three freeze and thaw cycles. Fifteen aliquots of each of two QC samples (7.5 and 54 ng/ml) were stored at \(-20\) °C. After 24 hours, all aliquots were left to thaw unassisted at room temperature. When completely thawed, 5 aliquots of each QC sample were analyzed. The other aliquots were returned to \(-20\) °C and kept for another 24 hours. The procedure was repeated three times. The concentrations in freeze-thaw samples were compared with the concentration of freshly prepared and analyzed samples.
Processed sample stability: Fifteen aliquots of each of two QC samples (7.5 and 54 ng/ml) were processed. Five aliquots of each QC sample were analyzed immediately. The other aliquots were analyzed after being stored at room temperature for 24 hours or at 4 °C for 48 hours.

Long term stability (unprocessed sample): Thirty aliquots of each of two QC samples (7.5 and 54 ng/ml) were prepared. Five aliquots of each QC sample were analyzed immediately. Five aliquots of each QC sample were allowed to stand on the bench-top for 24 hours at room temperature before extraction. Five aliquots of each QC sample were stored at 4 °C for 1, 3, 5, or 8 weeks before analysis.

RESULTS AND DISCUSSION

Optimization of chromatographic condition

Figure 1 depicts the structures of the compounds used in the study. We varied the relative proportion of the component of the mobile phase and found that methanol, water, and acetonitrile (80:10:10, v:v:v) in isocratic elution mode provided the best reproducible separation on Acquity UPLC BEH C\textsubscript{18} column at 30°C. The compounds of interest were detected using a photodiode array detector set at 265 nm. The retention times (RT) of the IS, VD-2, and VD-3 were around 3.14, 11.97, and 12.58 minutes, respectively (Figure 2).

Selection of internal standard and sample preparation

Some published methods\cite{15-16} used VD-2 as IS for measuring VD-3 and thus the method was not applicable for simultaneous measurement of VD-2 and VD-3 concentrations. An LCMS method\cite{17} was able to simultaneously measure VD-2 and VD-3 but used as IS isotope-labeled VD-2 and VD-3. These are regulated products and are not readily available for commercial use. We found that benzopyrene\cite{22} is not suitable as IS due to its partial co-elution with milk endogenous components. We used laurophenone as IS with satisfactory results. Figure 3 shows an overlay chromatogram of milk samples spiked with IS and VD-2 and VD-3 at 7.5, 30, and 54 ng/ml.

Specificity

Potential interfering substances in milk samples includes endogenous components, supplements, and additives. In order to assess for possible the interference, commonly used additive namely: vitamin-A (retinol), vitamin B-1 (thiamin), vitamin B-2 (riboflavin), vitamin B-9 (folic acid), vitamin C (L-ascorbic acid), vitamin E (α-tocopherol), and vitamin K
(phytonadione) were studied. None co-eluted with VD-2, VD-3, or the IS. The relative retention times (RT of analyte/RT of IS) are given in Table 1. Further, we analyzed five different brands of low fat milk purchased from local market and found no interference with the IS or VD-2 peak.

**Recovery**

In order to maximize the extraction recovery of VD-2, VD-3, and the IS, we evaluated the effect of alkaline hydrolysis using different strength of potassium hydroxide (40-60%) and proportion of ethanol (60-90%). Optimal extraction recovery was obtained using 50% potassium hydroxide and ethanol in 2:8 (v:v) proportion. The results are presented in Table 2. Mean extraction recovery was 93%, 94%, and 70% for VD-2, VD-3, and IS, respectively. The recovery of IS from milk was low compare to VD-2 and VD-3. However, it was consistent (± 4%) under the applied conditions.

**Linearity, Precision and Accuracy**

Linearity was evaluated by analyzing a series of calibration standards over the range 2.5-60 ng/ml. Peak area ratios (directly observed for VD-2 and calculated for VD-3, see methods) were subjected to regression analysis against spiked concentrations. Mean (SD) R², slope, and intercept were 0.9979 (0.001), 0.0065 (0.006), and 0.0142 (0.001) for VD-2 and 0.9981 (0.001), -0.0038 (0.008), and 0.0132 (0.002) for VD-3. Precision and bias were determined in five replicates of three concentrations (7.5, 30 and 54 ng/ml). The results are presented in Table 3. Maximum coefficients of variation was ≤ 7.0% and bias were in range of -8.0 to 3.5% for VD-2, similarly 8.7% and bias were in rage of -8.6 to 1.3 % for VD-3, respectively.

**Stability Studies**

In previous reports\(^{[19-20]}\) stability of VD-3 but not VD-2 in milk was studied. It is generally assumed that VD-2 has a shorter shelf life than VD-3.\(^{[20]}\) We assessed the stability of VD-2 and VD-3 in processed and unprocessed milk samples and in stock solutions under various conditions. No major change in chromatographic patterns or calculated concentrations in unprocessed samples was observed. Table 4, indicates that VD-2 and VD-3 were stable in unprocessed samples after at least three cycles of freeze and thaw, 24 hours at room temperature, or 8 weeks at 4 °C. In processed samples, they were stable for at least 24 hours at room temperature or 48 hours at 4 °C. Moreover, VD-2 and VD-3 in stock solutions (100 μg/ml in methanol) were also stable for at least 24 hours at room temperature or 8 weeks at -20 °C.
Application of Method

Figure 4 depicts a chromatogram of representative milk sample purchased from the local market. Measured level of VD-3 was 10.5 ng/ml (427 IU/L), which is 107% of the label claim (400 IU/L).

Table 1: Specificity of Vitamin D2 and D3 Assay

<table>
<thead>
<tr>
<th>Component</th>
<th>Relative Retention Timea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin – Vit D2</td>
<td>3.43</td>
</tr>
<tr>
<td>Vitamin – Vit D3</td>
<td>3.60</td>
</tr>
<tr>
<td>Laurophenone (IS)</td>
<td>1.00</td>
</tr>
<tr>
<td>Retinol – Vit A</td>
<td>0.81</td>
</tr>
<tr>
<td>Thiamin – Vit B1</td>
<td>0.90</td>
</tr>
<tr>
<td>Riboflavin – Vit B2</td>
<td>0.16</td>
</tr>
<tr>
<td>Folic acid – Vit B9</td>
<td>0.19</td>
</tr>
<tr>
<td>L- Ascorbic acid – Vit C</td>
<td>0.22</td>
</tr>
<tr>
<td>Tocopherol – Vit E</td>
<td>5.47</td>
</tr>
<tr>
<td>Phytonadione – Vit K1</td>
<td>3.22</td>
</tr>
</tbody>
</table>

*The relative retention time was calculated with respect to IS retention time (Vitamin/IS). The retention time of the IS was 3.1 minutes.

Table 2: Recovery of vitamin D-2, D-3, and Internal Standard from Cow Milk

<table>
<thead>
<tr>
<th>Nominal Level</th>
<th>Milk</th>
<th>Mobile Phase</th>
<th>** Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Peak Area</td>
<td>Mean Peak Area</td>
<td></td>
</tr>
<tr>
<td>VD-2 (ng/ml)</td>
<td>3.2072 770</td>
<td>3.4442 291</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>104085 2123</td>
<td>109786 444</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>166795 10684</td>
<td>182745 5438</td>
<td>91</td>
</tr>
<tr>
<td>VD-3 (ng/ml)</td>
<td>3.0603 837</td>
<td>3.2925 473</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>104099 2081</td>
<td>108505 525</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>168744 10944</td>
<td>1800637 6046</td>
<td>93</td>
</tr>
<tr>
<td>IS: 0.5 (ng/ml)</td>
<td>196475 2203</td>
<td>279413 3673</td>
<td>70</td>
</tr>
</tbody>
</table>

*Mean of 5 replicates. ** Mean peak area of spiked milk samples divided by mean peak area of spiked mobile phase X 100. SD: standard deviation.
### Table 3: Intra- and Inter-run Precision and Bias of Vitamin D-2 and D-3 Assay

<table>
<thead>
<tr>
<th>Nominal level (ng/ml)</th>
<th>Mean† measured level (ng/ml)</th>
<th>SD</th>
<th>Precision (%)*</th>
<th>Bias (%)**</th>
<th>Intra-run</th>
<th>Mean† measured level (ng/ml)</th>
<th>SD</th>
<th>Precision (%)*</th>
<th>Bias (%)**</th>
<th>Inter-run</th>
</tr>
</thead>
<tbody>
<tr>
<td>VD-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>7.2</td>
<td>0.17</td>
<td>2.3</td>
<td>-4.0</td>
<td>7.6</td>
<td>0.47</td>
<td>6.2</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>31.8</td>
<td>2.22</td>
<td>7.0</td>
<td>-6.0</td>
<td>30.2</td>
<td>2.61</td>
<td>8.6</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>55.9</td>
<td>2.71</td>
<td>4.8</td>
<td>3.5</td>
<td>51.2</td>
<td>4.47</td>
<td>8.7</td>
<td>-5.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VD-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>6.9</td>
<td>0.23</td>
<td>3.4</td>
<td>-8.0</td>
<td>6.9</td>
<td>0.34</td>
<td>4.9</td>
<td>-8.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>27.6</td>
<td>0.64</td>
<td>2.3</td>
<td>-8.0</td>
<td>28.9</td>
<td>1.65</td>
<td>5.7</td>
<td>-3.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>51.8</td>
<td>1.11</td>
<td>2.1</td>
<td>-4.1</td>
<td>52.3</td>
<td>3.50</td>
<td>6.7</td>
<td>-3.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

†Mean of 5 replicate analysis. *Precision as coefficient of variation (CV, %) = standard deviation divided by mean measured level X 100. **Bias (%) = difference between nominal and mean measured level divided by nominal level X 100.

### Table 4: Stability of Vitamin D-2 and D-3

<table>
<thead>
<tr>
<th>Nominal Level (ng/ml)</th>
<th>Unprocessed</th>
<th>Processed</th>
<th>Freeze-Thaw</th>
<th>**Stock Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hrs RT</td>
<td>8 wks 4 °C</td>
<td>24 hrs RT</td>
<td>48 hrs 4 °C Cycle</td>
</tr>
<tr>
<td>VD-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>96</td>
<td>97</td>
<td>102</td>
<td>96</td>
</tr>
<tr>
<td>54</td>
<td>97</td>
<td>108</td>
<td>107</td>
<td>107</td>
</tr>
<tr>
<td>VD-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>94</td>
<td>103</td>
<td>92</td>
<td>94</td>
</tr>
<tr>
<td>54</td>
<td>102</td>
<td>100</td>
<td>102</td>
<td>98</td>
</tr>
</tbody>
</table>

*Stability (%) = mean measured concentration (n=5) at the indicated time/condition divided by mean measured level (n=5) at baseline X 100. **Stock solution 0.1 mg/ml in methanol.
Fig: 1. Chemical structures of vitamin D-2, vitamin D-3, and the internal standard laurophenone (IS).

Fig: 2. Representative chromatogram of VD-2, VD-3, and IS in methanol.
CONCLUSIONS
The described UPLC method uses alkaline hydrolysis followed by hexane extraction and laurophenone as IS, provides sensitive, accurate, and precise simultaneous quantification of VD-2 and VD-3 levels in cow milk. The method was successfully applied to measure VD
level in commercially available milk sample and to study stability of VD under various laboratory conditions.

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