Simplified Assay of Vitamin D₂ in Fortified Dried Milk by Using Two Steps of High-Performance Liquid Chromatography

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(Received June 26, 1981)

Summary A simplified method for the determination of vitamin D₂ in fortified dried milk was established by using two steps of high-performance liquid chromatography (HPLC). About 1g of fortified dried milk was accurately weighed and directly saponified. The extracted unsaponifiable matter was first subjected to preparative HPLC using a Nucleosil 5C₁₈ column (reversed-phase type) with acetonitrile–methanol (1:1) as a mobile phase and a fraction containing vitamin D₂ was separated. The separated fraction was subsequently subjected to analytical HPLC using a Zorbax SIL column (straight-phase type) with 0.4% isopropanol in n-hexane as a mobile phase. Since the peak corresponding to vitamin D₂ was clearly observed with separation from other concomitants on the chromatogram of the analytical HPLC, the vitamin was assayed by estimating the peak height. The overall recovery of vitamin D₂ by the proposed method was 94.3 ± 2.3% (mean ± SD). Naturally occurring vitamin D₃ derived from cow’s milk was negligible in commercial fortified dried milk sold in Japan. When the proposed method was applied to 5 kinds of commercial fortified dried milk, satisfactory results were obtained.

Key Words ergocalciferol, fortified dried milk, high-performance liquid chromatography, vitamin D₂, vitamin D₃

Vitamin D₂ which shows practically the same physiological effects as vitamin D₃ in humans has been predominantly added to fortified dried milk sold in Japan because of its lower price than vitamin D₃. In a previous paper (1), the levels of 25-
OH-D₂ and 25-OH-D₃ as major circulating metabolites of vitamin D₂ and D₃ were determined in the plasma samples of mothers, cords and neonates. The results strongly suggest that the vitamin D nutrition in early periods of bottle-fed infants is mainly affected by daily intake of exogenous vitamin D₂ from fortified dried milk rather than endogenous vitamin D₃ or 25-OH-D₃ obtained from their mothers. Therefore, it is very important that the contents of vitamin D₂ enriched in commercial dried milk should always be kept near the respective indicated values and the stability of vitamin D₂ should be assured during storage. In order to perform such quantitative control and stability tests, a simplified method for determination of vitamin D₂ in dried milk should be established.

Since the contents of vitamin D₂ enriched in commercial fortified dried milk are usually very low (usually 320–500 I.U./100 g i.e., 80–125 ng/g), microdetermination with elimination of large amounts of lipophilic concomitants (vitamin A and E, sterols and others) is necessary. Recently, progress in HPLC has permitted the microdetermination of vitamin D and its metabolites. Some research groups applied HPLC to the determination of vitamin D in dried milk (2, 3), fortified milk (4, 5) and feeds (6, 7) but the clean-up procedures used were rather complicated. In the previous report (11), 25-OH-D₂ and 25-OH-D₃ in human plasma were simultaneously determined by two steps of HPLC using different columns. The first preparative HPLC using a Nucleosil 5C₁₈ column (reversed-phase type) was for the purpose of clean-up, and the second analytical HPLC using a Zorbax SIL column (straight-phase type) was for assaying the metabolites. Since the method was very simple and gave exact results, a similar procedure was applied to the determination of vitamin D₂ in fortified dried milk. When the same columns as in the previous report and modified solvent systems were used, good results were obtained. The proposed procedure and the results obtained by applying it to fortified dried milk are described in this paper.

EXPERIMENTAL

1. Materials and reagents

   Commercial grades (Philips-Duphar Co., The Netherlands) of vitamin D₂ and D₃ were used as the respective standard compounds.

   Organic solvents were purified according to the usual methods and distilled before use. Other guaranteed reagents were used.

2. Samples of fortified dried milk

   Five kinds of fortified dried milk sold in Japan were used. Their indicated contents of vitamin D₂ are within 332 and 420 I.U./100 g of dried milk (83–105 ng/g).

3. Instrumentation

   1) First preparative HPLC. The first preparative HPLC to isolate a vitamin D₂ fraction from the unsaponifiable matter of a sample was performed on a Shimadzu-Dupont 841 high-performance liquid chromatograph equipped with a
UVD-1 detector (fixed at 254 nm, AUFS 0.01). Separation was carried out in a stainless steel tube (300 × 7.5 mm i.d.) packed with Nucleosil 5C18 (reversed-phase type, Nishio Industrial Co., Japan) using 50% methanol in acetonitrile as the mobile phase. Elution was performed at a constant flow rate of 2.0 ml/min (column pressure: 55 kg/cm²) and the eluate between 2,500 and 2,850 drops (the drops corresponded to the eluate between about 16 and 19 min of retention time) was collected as a vitamin D₂ fraction by a mini-fraction collector with a drop counter (Gilson Co., USA).

2) Second analytical HPLC. The second analytical HPLC was performed on a Shimadzu LC-3A high-performance liquid chromatograph equipped with a Shimadzu UVD-2 detector (fixed at 254 nm, AUFS 0.001). Separation was carried out in a stainless steel tube (250 × 4.6 mm i.d.) packed with Zorbax SIL (straight-phase type, DuPont Co., USA) using 0.4% isopropanol in n-hexane as the mobile phase. Flow rate was 1.6 ml/min (column pressure: 40 kg/cm²).

4. Procedure for the determination of vitamin D₂ in fortified dried milk

1) Saponification and extraction of unsaponifiable matter. About 1 g of fortified dried milk was accurately weighed in a saponification flask and then suspended with 40 ml of 10% pyrogallol-ethanol solution. After adding 10 ml of 90% (w/v) KOH solution, the solution was saponified by refluxing for 30 min at 80°C under infusion of argon gas. It was cooled immediately and exactly 100 ml of benzene was added to isolate the unsaponifiable matter according to Mulder's benzene washing method (8). It was well shaken and poured into a separation funnel without rinsing. After adding 50 ml of 1 N KOH solution and shaking, it was allowed to stand to separate the two layers. The lower turbid aqueous layer was discarded and the upper benzene layer was washed with 50 ml of 0.5 N KOH solution. The aqueous layer was again discarded and the benzene layer was repeatedly washed with water until the washed water became neutral. The separated benzene layer was filtered through a Whatman 1PS filter paper.

2) Isolation of the vitamin D₂ fraction by the first preparative HPLC. Exactly 80 ml of the filtrate was placed in a round bottom flask and evaporated under reduced pressure below 40°C. The resulting residue was dissolved in 5.0 ml of n-hexane. Exactly 4.5 ml of the n-hexane solution was placed in a test tube and evaporated under reduced pressure. The resulting residue was dissolved accurately in 500 µl of 50% methanol in acetonitrile and exactly 200 µl of the solution was subjected to the first preparative HPLC described in Section 3-1). The vitamin D₂ fraction was isolated and evaporated under reduced pressure. The resulting residue was dissolved accurately in 200 µl of 0.4% isopropanol in n-hexane and the solution was denoted as a sample solution for the second analytical HPLC.

3) Determination of vitamin D₂ by the second analytical HPLC. Exactly 100 µl of the sample solution was subjected to the second analytical HPLC described in Section 3-2) and the peak height corresponding to vitamin D₂ was estimated on the chromatogram of HPLC. Exactly 1 ml of a vitamin D₂ standard solution (100 ng/ml of authentic vitamin D₂ in ethanol) was treated according to the whole procedure.
the same as a sample and the peak height corresponding to vitamin D₂ was also estimated on the second analytical HPL-chromatogram. The content of vitamin D₂ in a sample of fortified dried milk can be calculated by the following formula:

\[
\text{Content of vitamin D}_2 \text{ (ng/g)} = \frac{P_{sa}}{P_{st}} \times \frac{S}{W}
\]

- \(P_{sa}\): Peak height of vitamin D₂ on the chromatogram of HPLC obtained from a sample.
- \(P_{st}\): Peak height of vitamin D₂ on the chromatogram of HPLC obtained from a vitamin D₂ standard solution.
- \(S\): Quantity of vitamin D₂ (ng) in the standard solution applied to the procedure. This is 100 in the above case.
- \(W\): Weight of a sample (g) applied to the procedure.

The obtained value (ng/g) can be calculated into I.U./100 g as the usual indicated value of commercial fortified dried milk by the calculation (a value of ng/ml × 4).

RESULTS AND DISCUSSION

1. Profiles of the preparative HPLC

Profiles of the preparative HPLC on authentic vitamin D₂ and the unsaponifiable matter of a sample of fortified dried milk are shown in Figs. 1a and 1b. The retention time of authentic vitamin D₂ was 17.8 min and the vitamin D₂ fraction to isolate for the following analytical HPLC was decided to be the eluate between 2,500 and 2,850 drops (about 16 and 19 min of retention time) from the HPLC chromatogram (Fig. 1a). The recovery of vitamin D₂ by the HPLC alone was 98.6% and good reproducibility was obtained. Many large irrelevant peaks which might be due to unknown concomitants were observed in the chromatogram of the sample (Fig. 1b), but most of them were eluted before the vitamin D₂ fraction. A peak corresponding to vitamin D₂ was observed on the chromatogram of the sample, but it was disturbed by an unknown peak as shown in Fig. 1b. Therefore, the determination of vitamin D₂ was impossible by preparative HPLC alone and the isolated fraction was necessary to subject to the following second analytical HPLC.

2. Profiles of the analytical HPLC

Profiles of the analytical HPLC on authentic vitamin D₂ and the vitamin D₂ fraction isolated from preparative HPLC are shown in Figs. 2a and 2b. The retention time of authentic vitamin D₂ was 22.8 min (Fig. 2a). The chromatogram of the sample solution also gave a single peak whose retention time agreed with authentic vitamin D₂. Since no other peak interfering with the peak was observed on the chromatogram, we considered determination of vitamin D₂ was possible by the HPLC (Fig. 2b).
Fig. 1. Profiles of the first preparative HPLC of authentic vitamin D$_2$ and the unsaponifiable matter obtained from a sample of fortified dried milk. Authentic vitamin D$_2$ or the unsaponifiable matter was applied to the preparative HPLC described in EXPERIMENTAL. (a) Authentic vitamin D$_2$, (b) the unsaponifiable matter.

Fig. 2. Profiles of the second analytical HPLC of authentic vitamin D$_2$ and the vitamin D$_2$ fraction isolated from the preparative HPLC of a sample of fortified dried milk. Authentic vitamin D$_2$ or the vitamin D$_2$ fraction was applied to the analytical HPLC described in EXPERIMENTAL. (a) Authentic vitamin D$_2$, (b) vitamin D$_2$ fraction.
3. **Identification of the peak as vitamin D$_2$**

The peak observed in the analytical HPLC (Fig. 2b) was confirmed to be due to vitamin D$_2$ by the following methods.

1) **UV spectrum.** The unsaponifiable matter of a sample of fortified dried milk was applied to the whole procedure and the peak corresponding to vitamin D$_2$ on the analytical HPLC was isolated. The same procedure was repeatedly applied to other parts of the unsaponifiable matter and the isolated fractions were joined. After evaporating under reduced pressure and dissolving in ethanol, the UV spectrum was estimated with a Hitachi automatic spectrophotometer (Japan). As shown in Fig. 3, a maximum at 265 nm and a minimum at 229 nm showing the typical triene structure of vitamin D$_2$ were observed.

2) **Co-chromatography.** Co-chromatography was performed by mixing the sample solution isolated from the analytical HPLC with authentic vitamin D$_2$. As shown in Fig. 4b, the peak corresponding to vitamin D$_2$ was detected as a single one with an increase of peak height by addition of authentic vitamin D$_2$. 

3) **Appearance of the peak corresponding to pre-D$_2$ by thermal isomerization.** It is well documented that vitamin D$_2$ is thermally isomerized into pre-D$_2$ to reach an equilibrium between the two compounds (9) and the reaction is typical for the molecule of vitamin D. Therefore, if the appearance of pre-D$_2$ is observed in a refluxed solution of vitamin D$_2$ fraction obtained from the analytical HPLC of the sample solution, it must give evidence to identify the existence of vitamin D$_2$. The peak corresponding to vitamin D$_2$ on the analytical HPLC of the sample solution

![UV spectrum of the vitamin D$_2$ fraction purified from fortified dried milk. Ethanol was used as a solvent.](image-url)

*Fig. 3. UV spectrum of the vitamin D$_2$ fraction purified from fortified dried milk. Ethanol was used as a solvent.*

Fig. 4. Profiles of the co-chromatography on the analytical HPLC. (a) Vitamin D₂ fraction isolated from the analytical HPLC of a sample of fortified dried milk, (b) the fraction with addition of authentic vitamin D₂.

Fig. 5. Profiles of the analytical HPLC of authentic pre-D₂ and vitamin D₂ and the vitamin D₂ fraction of a sample of fortified dried milk after thermal isomerization. (a) Authentic pre-D₂ and vitamin D₂, (b) vitamin D₂ fraction isolated from the analytical HPLC of a sample of fortified dried milk before heating, (c) the fraction after heating at 80°C for 2 hr.

was collected and further purified by re-chromatography. After evaporating under reduced pressure and dissolving in ethanol, the resulting solution was refluxed for 2 hr at 80°C. After evaporating under reduced pressure, the residue was dissolved in Vol. 27, No. 6, 1981
0.4% isopropanol in n-hexane and then applied to the analytical HPLC. As shown in Fig. 5c, the peak whose retention time agreed with that of authentic pre-D\textsubscript{2} appeared by refluxing the sample solution.

All results by UV spectrum, co-chromatography and thermal isomerization supported that the peak on the HPL-chromatogram (Fig. 2b) was due to vitamin D\textsubscript{2}. However, since vitamin D\textsubscript{3} showed in a similar manner to vitamin D\textsubscript{2} on the above experiments, distinction between vitamin D\textsubscript{2} and D\textsubscript{3} was carried out as shown in the following.

4. **Investigation on naturally occurring vitamin D\textsubscript{3}**

For economic reasons, vitamin D\textsubscript{2} is predominantly enriched in commercial fortified dried milk, whereas naturally occurring vitamin D in cow's milk was

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**Fig. 6.** Purification steps of the vitamin D fraction including vitamin D\textsubscript{2} and D\textsubscript{3} from the unsaponifiable matter of a sample of fortified dried milk. (a)–(c) Profiles of the three steps of HPLC of authentic vitamin D\textsubscript{2} and D\textsubscript{3} (columns and mobile phases are given on the upper sides of the figures), (a')–(c') profiles of the unsaponifiable matter of a sample of fortified dried milk obtained by the purification steps using HPLC.

identified as vitamin D₃ (10). Therefore, it is possible that fortified dried milk contains both vitamin D₂ and D₃. In order to confirm if fortified dried milk contains vitamin D₃ besides vitamin D₂, the following experiment was carried out.

The unsaponifiable matter obtained from about 1 g of fortified dried milk was purified by applying successively to preparative HPLC using two different sizes of Zorbax SIL columns (straight-phase type) and a vitamin D fraction was isolated. The column sizes and conditions for the HPLC are given in Fig. 6. Since separation between vitamin D₂ and D₃ was impossible by HPLC using Zorbax SIL as shown in the results performed on the authentic vitamins, the isolated vitamin D fraction must include both vitamins (Figs. 6a and 6b). The vitamin D fraction obtained above was successively subjected to HPLC using a Nucleosil 5C₁₈ column (reversed-phase type) and 20% methanol in acetonitrile as a mobile phase. Since vitamin D₂ and D₃ could be separated from one another by the HPLC as shown in Fig. 6c, the two peaks must be observed if fortified dried milk contains both vitamin D₂ and D₃. The profile of HPLC (Fig. 6c') showed that the peak corresponding to vitamin D₂ appeared as a large one while that corresponding to vitamin D₃ was observed only as a trace one. Since similar results were also observed on 4 other samples of dried milk, we concluded that the vitamin D in commercial fortified dried milk mainly originated from enriched vitamin D₂ while trace amounts of naturally occurring vitamin D₃ from cow’s milk could be neglected in the determination of vitamin D.

5. Calibration curve of vitamin D₂

Authentic vitamin D₂ was dissolved in ethanol to concentrations of 50, 100, 150 and 200 ng/ml. Exactly 1.0 ml of the standard solutions was applied to the whole procedure in order to obtain a calibration curve. As shown in Fig. 7, a straight line passing through the origin was observed between the peak heights and the amounts of vitamin D₂ (0–200 ng).

Since the proposed method includes a saponification procedure by heating at 80°C, thermal isomerization of vitamin D₂ into pre-D₂ during saponification is a problem in the determination of vitamin D₂. Keverling Buismann et al. (9) reported

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![Fig. 7. Calibration curve of vitamin D₂ on the analytical HPLC.](image-url)
that the thermal isomerization is reversible and the isomerization ratio depends on
temperature alone. We solved this problem by applying the whole procedure to both
a sample and authentic vitamin D2 simultaneously. As shown in Fig. 7, a good
calibration curve with good reproducibility was obtained by applying the whole
procedure to the vitamin D2 standard solutions. Therefore, we concluded that an
assayed value of a sample was exactly corrected by comparing the peak height with
that obtained from a vitamin D2 standard solution.

6. Recovery of vitamin D2 for the whole procedure

One g of a sample of fortified dried milk was treated according to the whole
procedure with or without addition of 100 ng of authentic vitamin D2 in order to
carry out a recovery test. As shown in Table 1, the overall recovery was 94.3 ± 2.3% (mean ± SD), which was satisfactory.

Table 1. Recovery test of vitamin D2 in commercial dried milk by the proposed
method.

<table>
<thead>
<tr>
<th>Vitamin D2</th>
<th>Number of experiments</th>
<th>Sample weights (g)</th>
<th>Added value (ng)</th>
<th>Found value (ng)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without addition</td>
<td>6</td>
<td>1.0</td>
<td>—</td>
<td>111.2 ± 10.8*</td>
<td>—</td>
</tr>
<tr>
<td>With addition</td>
<td>6</td>
<td>1.0</td>
<td>100.0</td>
<td>205.5 ± 14.2*</td>
<td>94.3 ± 2.3*</td>
</tr>
</tbody>
</table>

* Mean ± SD, ** Indicated value of vitamin D2 for the sample was 100 ng per g of milk powder.

7. Assayed values of vitamin D2 in fortified dried milk

Five kinds of fortified dried milk sold in Japan were subjected to the whole
procedure and the assayed values of vitamin D2 are shown in Table 2. The
percentages of the values to the respective indicated values were within the range of
about 90–130%, which suggested that the vitamin D2 enriched in commercial
fortified dried milk was kept stable during storage. All of the profiles of analytical
HPLC obtained from the samples showed that the peak due to vitamin D2 was
clearly separated from other concomitants.

Some research groups applied HPLC to the determination of vitamin D in
dried milk (2,3), fortified milk (4,5) or feeds (6,7). However, since their used UV
detectors for the analytical HPLC had lower sensitivity than the present one, rather
large quantities of a sample (10–50 g for dried milk) should be taken for one assay.
Therefore, rather complicated procedures, e.g., some steps of open column chroma-
tography or preparative thin-layer chromatography, were necessary to eliminate
large amounts of lipophilic concomitants from vitamin D. On the other hand, the
presently proposed method using a high sensitivity UV detector (AUFS 0.001) for
the analytical HPLC needed a small sampling scale (about 1 g) and therefore the
preparative HPLC using a reversed-phase column was enough for the clean-up use.
Table 2. Determination of vitamin D$_2$ in commercial dried milk by the proposed method.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Number of trial</th>
<th>Indicated value of D$_2$ (ng/g*)</th>
<th>Assayed value of D$_2$ (ng/g*)</th>
<th>Percent for indicated value (%)</th>
<th>CV value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>100</td>
<td>111.2±10.8**</td>
<td>111.2±10.8**</td>
<td>9.7</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>100</td>
<td>91.1±7.5**</td>
<td>91.1±7.5**</td>
<td>8.2</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>105</td>
<td>107.3±6.6**</td>
<td>102.1±6.3**</td>
<td>6.2</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>100</td>
<td>135.8±9.6**</td>
<td>135.8±9.6**</td>
<td>7.1</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>83</td>
<td>113.1±5.8**</td>
<td>136.3±7.0**</td>
<td>5.1</td>
</tr>
</tbody>
</table>

* Milk powder, ** mean±SD.

As mentioned in the previous report (11), when an expensive reversed-phase column was used for analytical HPLC, deterioration of the column soon occurred and the width of peaks soon became broad without changes of retention times. This might be due to the overload on the column. However, when reversed-phase columns were used for preparative purposes, deterioration of the columns gave no trouble even with repeated use. For these reasons we used a reversed-phase column for preparative HPLC.

The determination of vitamin D$_2$ in 10 samples by the proposed method is possible as one day’s work for a laboratory worker. We concluded from these results that the proposed method is suitable as a simplified routine procedure.

REFERENCES


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