IDENTIFICATION OF VITAMIN D₃ AND 7-DEHYDROCHOLESTEROL IN COW'S MILK BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY AND THEIR QUANTITATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY¹

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Summary Identification of vitamin D₃ and 7-dehydrocholesterol (7-DHC) in cow's milk by gas chromatography-mass spectrometry (GC-MS) and their quantitation by high-performance liquid chromatography (HPLC) were investigated. When vitamin D and provitamin D fractions purified from a sample of commercial cow's milk were applied to GC-MS, the results showed that the fractions contained vitamin D₃ and 7-DHC, respectively, while neither vitamin D₂ nor ergosterol could be detected in the milk. HPLC methods for the determination of vitamin D₃ and 7-DHC in cow's milk were then proposed as routine methods. The method for assaying vitamin D₃ included the isolation of lipids from milk according to the directions of BELL and CHRISTIE (5), saponification, isolation of unsaponifiable matter, digitonin-Celite column chromatography, preparative thin-layer chromatography (TLC) and application to HPLC. On the other hand, 7-DHC in milk could be simultaneously determined without the purification by digitonin-Celite column chromatography. The peak corresponding to either vitamin D₃ or 7-DHC in the respective HPL-chromatograms was clearly separated from possible interfering substances and the recovery experiments for both vitamin D₃ and 7-DHC gave satisfactory results. When the proposed methods were applied to 10 samples of commercial cow's milk, the assayed values of vitamin D₃ and 7-DHC were 19–79 I.U./liter and 14–56 µg/liter, respectively.

Keywords cow's milk, 7-dehydrocholesterol, gas chromatography-mass spectrometry, high-performance liquid chromatography, vitamin D₃

¹ Following abbreviations are used: 7-DHC, 7-dehydrocholesterol; pre-D₃, precholecalciferol; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; UV, ultraviolet.

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It is very important from the viewpoint of nutritional sciences to establish a simple routine method for the determination of vitamin D in milk. However, no satisfactory method has been reported, because the content of the vitamin in milk is too small to be quantified and large amounts of interfering substances usually coexist with the vitamin.

Colorimetric methods for the purpose have been reported by several research groups (1-3), but the procedures are very complicated and time-consuming because the complete removal of interfering substances is essential for the colorimetric methods. On the other hand, GLC methods were also used for the purpose (4, 5), but the application was restricted to milk containing comparatively large amounts of vitamin D, *e.g.* fortified milk or condensed milk, because the sensitivity of GLC using an ordinary hydrogen flame ionization detector is not enough to determine such micro-quantities of vitamin D in ordinary human and cow's milk.

Recently, HPLC has shown remarkable progress in the field of microassay of various compounds because it gives not only good separation but also high sensitivity. The chromatography also has been applied to the determination of vitamin D in various preparations (6-9) and of 25-hydroxyvitamin D in tissues (10-14). THOMPSON *et al.* (15) and HENDERSON and WICKROSKI (16) applied HPLC to the determination of vitamin D in milk, but their methods were restricted to fortified milk containing comparatively large amounts of the vitamin. We tried to find a simple, routine HPLC method useful for ordinary cow's milk or human milk having a very low concentration of the vitamin. When the clean-up procedures previously reported for the GLC determination of vitamin D in various preparations (17-19) were used before applying to HPLC, satisfactory results were obtained. The established method for assaying vitamin D₃ in cow's milk is described in this paper.

Although the existence of vitamin D in cow's milk has been known for a long time, the form of the vitamin (vitamin D₂ or D₃) had not been confirmed. We applied the technique of GC-MS for this purpose. The results showed that vitamin D₃ not vitamin D₂ exists in the milk. Furthermore, we identified the existence of 7-DHC in the milk by GC-MS and quantified the amount by HPLC. These results are also described in this paper.

EXPERIMENTAL

1. General

1) *Materials and reagents.* Vitamin D₃. Commercial grades, recrystallized from acetone-water (4:1), Philips-Duphar Co., mp 85-86°C.

7-Dehydrocholesterol (7-DHC). Commercial grades, recrystallized from diethyl ether, Fluka, Co., mp 150-151°C.

Alpha-naphthol, sodium hexametaphosphate and Triton X-100. Guaranteed reagents of Nakarai Chem. Ind. Co. were used.

Triton X-100 solution. This was prepared according to the direction of BELL

and CHRISTIE (5). A solution of sodium hexametaphosphate (200 g/800 ml of water) was mixed with a solution of Triton X-100 (100 g/800 ml of water) and then water was added to make 2 liters of total volume. The solution was shaken well and allowed to stand overnight before use.

Internal standard solution. Alpha-naphthol was dissolved in *n*-pentane to obtain a concentration of 20 µg/ml.

Vitamin D₃ standard solution. Vitamin D₃ and α-naphthol were dissolved in *n*-pentane to obtain a concentration of 200 I.U./ml for vitamin D₃ and 20 µg/ml for α-naphthol, respectively.

7-DHC standard solution. 7-Dehydrocholesterol and α-naphthol were dissolved in *n*-pentane to obtain a concentration of each of 20 µg/ml.

Organic solvents were purified according to conventional methods and distilled before use. Other materials and reagents were used according to previous papers (17-19).

2) *Samples of cow's milk.* Ten samples of commercial cow's milk (sample no. 1-10) including two samples of processed milk (sample no. 9 and 10) were used. Sample no. 3 was kindly given by Yukijirushi Milk Products Co., while the others were purchased from a market.

3) *Gas chromatography-mass spectrometry (GC-MS).* Gas chromatography-mass spectrometry (GC-MS) was performed on a Shimadzu GC-MS 9000 gas chromatograph-mass spectrometer system. A glass column (100 cm × 0.3 cm *i.d.*) packed with 3% OV-17 on Gas Chrom Q (80-100 mesh) was run at 240°C with a helium flow of 35 ml/min and the mass spectra were estimated when the peaks were obtained from the gas chromatography.

4) *High-performance liquid chromatography (HPLC).* High-performance liquid chromatography (HPLC) was performed on a Shimadzu-DuPont 841 high-performance liquid chromatograph equipped with a UV detector (254 nm). Separation was carried out in a stainless steel tube (25 cm × 2.1 mm *i.d.*) packed with "Zorbax Sil" (DuPont Co.). A solvent system of 0.15% methanol and 2% diethyl ether in *n*-pentane was used as the mobile phase. A suitable volume of a sample solution taken with a microsyringe was injected into a high pressure line of 60-100 kg/cm² (this pressure, which gave a flow rate of 0.6 ml/min, depended on the column used) to the separation tube by a stop-flow technique. Alpha-naphthol was used as an internal standard.

2. Determination of vitamin D₃ in milk

1) *Extraction of lipids.* Five hundred ml of a milk sample was mixed with 50 ml of Triton X-100 solution and then warmed in a steam bath (higher than 80°C) for 10 min. After cooling, the solution was mixed with 200 ml of ethanol and then cooled again. The lipids were extracted twice from the solution with each 250 ml of petroleum ether-diethyl ether (1:1) by vigorous shaking. The solvent was evaporated to dryness (evaporation of solvents in this work was always carried out under reduced pressure below 40°C).

2) *Saponification and isolation of unsaponifiable matter.* The residue obtained above was saponified by refluxing for 30 min with 100 ml of ethanol, 40 ml of 20% pyrogallol solution in ethanol and 25 ml of 90% (w/v) KOH solution. After cooling, the unsaponifiable matter was extracted with exactly 200 ml of benzene according to the previous paper (17).

3) *Digitonin-Celite column chromatography.* Exactly 150 ml of the benzene layer obtained above was placed in a round bottom flask and evaporated. After dissolving the residue in 5 ml of *n*-hexane, the solution was transferred onto a digitonin-Celite column prepared according to the previous paper (18) with the aid of 5 ml of *n*-hexane. The elution was started with *n*-hexane at a flow rate of 1–2 ml/min and the first 50 ml of the eluate was collected in a round bottomed flask. The solvent was evaporated to dryness and the resulting residue was dissolved in 0.5 ml of acetone.

4) *TLC.* Using a micropipette, 0.4 ml of the acetone solution obtained above was taken and applied as a zone onto a Kieselgel GF₂₅₄ (E. Merck Co.) plate. The TLC was developed with a mixed solvent of *n*-hexane–ethyl acetate (4:1) and the zones corresponding to vitamin D₃ and pre-D₃ were scraped according to the previous paper (17). After extracting the two combined zones with 30 ml of acetone and then filtering, the remaining powder on the filter paper was repeatedly washed with small quantities of acetone. The washed solutions were combined with the filtrate and the solvent was evaporated to dryness.

5) *HPLC.* The residue obtained above was dissolved in 0.5 ml of the internal standard solution. Ten μ l of the sample solution obtained was applied to the HPLC described in Section 1.4. Ten μ l of the vitamin D₃ standard solution was similarly applied to the HPLC.

6) *Calculation.* The peak area ratios of vitamin D₃ to α -naphthol were estimated on the HPLC chromatograms obtained from the sample and vitamin D₃ standard solutions, respectively. The content of vitamin D₃ in a milk sample was calculated by the following formula:

$$\text{Content of vitamin D}_3 \text{ (I.U./liter)} = \frac{R_{sa}}{R_{st}} \times \frac{S}{2} \times \frac{V}{W}$$

R_{sa} : Peak area ratio of vitamin D₃ to α -naphthol on the HPL-chromatogram obtained from a sample solution.

R_{st} : Peak area ratio of vitamin D₃ to α -naphthol on the HPL-chromatogram obtained from the vitamin D₃ standard solution.

S : Concentration of vitamin D₃ in the vitamin D₃ standard solution (I.U./ml). This is 200 I.U./ml in the above case.

V : Multiple for dilution ratio. This is $\frac{5}{3} \left(= \frac{200}{150} \times \frac{0.5}{0.4} \right)$ in the above case.

W: Volume of a milk sample taken for the determination (liter). This is 0.5 liter in the above case.

3. Determination of 7-DHC in milk

1) *TLC*. Thirty ml of the benzene solution including the unsaponifiable matter obtained in Section 2.2 was taken in a round bottomed flask and evaporated to dryness. The resulting residue was dissolved in 0.5 ml of acetone and 0.4 ml of the solution was applied to the TLC described in Section 2.4. The zone corresponding to 7-DHC was scraped and extracted according to Section 2.4. The solution obtained was evaporated to dryness.

2) *HPLC*. The residue obtained above was dissolved in 0.5 ml of the internal standard solution and 10 μ l of the solution was applied to the HPLC described in Section 1.4. Ten μ l of the 7-DHC standard solution was similarly applied to the HPLC.

3) *Calculation*. The peak area ratios of 7-DHC to α -naphthol were estimated on the HPLC chromatograms obtained from the sample and 7-DHC standard solutions, respectively. The content of 7-DHC in a milk sample was calculated by the following formula:

$$\text{Content of 7-DHC } (\mu\text{g/liter}) = \frac{R'_{sa}}{R'_{st}} \times \frac{S'}{2} \times \frac{V'}{W'}$$

R'_{sa}: Peak area ratio of 7-DHC to α -naphthol on the HPL-chromatogram obtained from a sample solution.

R'_{st}: Peak area ratio of 7-DHC to α -naphthol on the HPL-chromatogram obtained from the 7-DHC standard solution.

S': Concentration of 7-DHC in the 7-DHC standard solution (μ g/ml). This is 20 μ g/ml in the above case.

V': Multiple for dilution ratio. This is $\frac{25}{3} \left(= \frac{200}{30} \times \frac{0.5}{0.4} \right)$ in the above case.

W': Volume of a milk sample taken for the determination (liter). This is 0.5 liter in the above case.

4. Identification of vitamin D₃ and 7-DHC in milk by GC-MS

Twenty-five liters of a sample of commercial cow's milk (sample no. 3) were divided into 25 parts each of 1 liter. The lipids extracted from one part of the milk were saponified and the unsaponifiable matter was isolated according to Section 2.1 and 2.2, but a double quantity of each reagent was used in this case. The same procedure was also applied to the other 24 parts of the milk. The resulting unsaponifiable matter was combined with that of the first part and 44.8 g of oily residue was obtained. The combined residue was dissolved in 200 ml of methanol and the solution was cooled overnight in a freezer in order to crystallize the sterols.

The crystalline precipitates formed were separated by filtration and the filtrate was evaporated to dryness. The resulting residue was dissolved in 100 ml of 72% ethanol. A digitonin solution in 72% ethanol (0.1 g/ml) was added to the solution until the remaining sterols were no longer precipitated as digitonides. The precipitates formed were separated by filtration and the filtrate was evaporated to dryness. The resulting residue was purified by the TLC described in Section 2.4 and the vitamin D fraction was obtained.

On the other hand, the separated precipitates of digitonides were heated with about 100 ml of absolute ethanol to loose the free sterols and then extracted with diethyl ether. After combining the crystalline precipitates obtained by the methanol cooling procedure with the ether extracts, the solvent was evaporated to dryness. The resulting residue was purified by the TLC described in Section 2.4 and the provitamin D fraction was obtained.

The vitamin D and provitamin D fractions obtained were individually applied to the GC-MS described in Section 1.3.

5. *Irradiation of milk with UV light*

About 500 ml of a sample of commercial milk (sample no. 3) was placed in a photochemical reaction apparatus (Taika Ind., Co.). Irradiation was carried out with a high-pressure mercury lamp with continuous stirring by bubbles of nitrogen gas.

RESULTS AND DISCUSSION

1. *Identification of vitamin D₃ and 7-DHC in cow's milk*

The vitamin D and provitamin D fractions isolated from a sample of commercial cow's milk (sample no. 3) were applied to GC-MS according to Section 4 of the Experimental part. The results are shown in Fig. 1. Although vitamin D was thermally isomerized into the two cyclized products "pyro- and isopyro-vitamin D" at the temperature of GLC analysis, the gas chromatogram of the vitamin D fraction gave only a single peak as shown in the right side of no. 1 of Fig. 1. The peak was derived from a mixture of the two cyclized products which were not separated from one another by GLC because the column temperature was too high. However, since the mass spectra of pyro- and isopyro-vitamin D are known to be practically identical (20), there is no trouble in identifying the existence of vitamin D by the GC-MS. The mass spectrum (no. 1 of Fig. 1) which gave a molecular ion at m/e 384 and fragment ions at m/e 351, 325, 271 and 253 completely agreed with that derived from authentic vitamin D₃. The results showed no information on the existence of vitamin D₂.

On the other hand, a main peak corresponding to 7-DHC, and some unknown minor peaks, were observed in the gas chromatogram of the provitamin D fraction, as shown in the right side of no. 2 of Fig. 1. The mass spectrum of the main peak (no. 2 of Fig. 1) which gave a molecular ion at m/e 384 and fragment ions at m/e 351, 325,

Fig. 1. Analysis of the vitamin D and provitamin D fractions isolated from a sample of commercial cow's milk by GC-MS. The profiles shown in each right side of the mass spectra are the GLC chromatograms of the vitamin D or provitamin D fraction.

Table 1. Recovery of vitamin D₃ from cow's milk (Sample no. 2).

Assayed value of vitamin D ₃ (I.U./liter)				
Added value	With addition of vitamin D ₃ (a)	Without addition of vitamin D ₃ (b)	a - b	Recovery (%)
80	129	50	79	98.8
80	131	49	82	102.5
80	128	42	86	107.5
80	123	44	79	98.8
M ± SD		46 ± 3		101.9 ± 3.5

271 and 253 completely agreed with that derived from authentic 7-DHC. The results gave no information on the existence of ergosterol.

It was concluded from the results that vitamin D₃ and 7-DHC existed in the milk while vitamin D₂ and ergosterol were not detected.

2. Recovery experiments

Since the vitamin D₃ concentration in ordinary cow's milk is extremely low, a large volume of a sample (more than 500 ml) should be taken for the determination

Table 2. Recovery of 7-DHC from cow's milk (Sample no. 2)

Added value	Assayed value of 7-DHC ($\mu\text{g/liter}$)		a - b	Recovery (%)
	With addition of 7-DHC (a)	Without addition of 7-DHC (b)		
20	36	17	19	95.0
20	37	19	18	90.0
20	39	20	19	95.0
20	38	19	19	95.0
M $\pm$ S.D.		19 $\pm$ 1		93.8 $\pm$ 2.1

even though the HPLC method having a high sensitivity is proposed. The direct saponification procedure could not be applied to such a large volume of the sample and preliminary extraction of lipids before saponification was essential. The method using Triton X-100 solution according to BELL and CHRISTIE (5) was proposed for the lipid extraction and good results were obtained. The other clean-up procedures were practically identical with those used previously for the GLC determination of vitamin D in various preparations (17-19).

In order to obtain the critical proof that vitamin D₃ and 7-DHC in milk can be determined by the proposed methods, recovery experiments were carried out. The whole procedure for assaying vitamin D₃ or 7-DHC (Sections 2 or 3 of the Experimental part) was applied to a sample of commercial cow's milk (sample no. 2) with or without the addition of a known amount of vitamin D₃ or 7-DHC. As shown in Tables 1 and 2, the results showing recovery of greater than 90% were satisfactory.

3. Determination of vitamin D₃ and 7-DHC in commercial cow's milk

Vitamin D₃ and 7-DHC in 10 samples of commercial cow's milk (sample no. 1-10) were determined by the proposed methods. Figures 2 and 3 show the HPL-chromatograms used for the final calculation for assaying vitamin D₃ and 7-DHC, respectively. The peak corresponding to either vitamin D₃ or 7-DHC which was clearly separated from other interfering substances was observed in all chromatograms. As shown in Table 3, the assayed values of vitamin D₃ and 7-DHC in the samples were within the ranges of 19-79 I.U./liter and of 14-56 $\mu\text{g/liter}$, respectively. There was a significant correlation between the respective data of vitamin D₃ and 7-DHC (correlation coefficient: 0.808).

By combining the results of the recovery experiments and the HPL-chromatograms, we concluded that the proposed methods were useful as routine methods for the determination of vitamin D₃ and 7-DHC in milk. Vitamin D₃ in a sample of milk can be measured by the proposed method within 5 hr. When determination of vitamin D₃ and 7-DHC in a sample of milk is simultaneously

Fig. 2. HPLC behavior of cow's milk (vitamin D₃ fraction). The asterisk (*) in each chromatogram means the eluate corresponding to vitamin D₃.

performed, 7-DHC can be determined within 1 hr by the procedure after the isolation of unsaponifiable matter.

4. Irradiation of cow's milk with ultraviolet light

As it was expected that 7-DHC existing in cow's milk could be photochemically converted to vitamin D₃, experiments on UV irradiation were performed. A sample of commercial cow's milk (sample no. 3) was irradiated according to the procedure described in Section 5 of the Experimental part. The contents of vitamin D₃ and 7-DHC in the milk were determined by the proposed methods (Sections 2 and 3 of the Experimental part) with the lapse of irradiation times. The results are shown in Fig. 4. 7-Dehydrocholesterol in the milk rapidly decreased from the start of irradiation and the sterol could not be detected after 45 min of irradiation. On the other hand, the content of vitamin D₃ was increased until a maximum yield was obtained after 45 min of irradiation. The maximum value obtained was about 6 times as much as that at the initial stage. However, because an undesirable smell was produced from the first stage, we thought that the technique of UV irradiation might be useless for the purpose of vitamin D fortification of cow's milk.

Fig. 3. HPLC behavior of cow's milk (7-DHC fraction). The asterisk (*) in each chromatogram means the eluate corresponding to 7-DHC.

Table 3. Assayed values of vitamin D₃ and 7-DHC in cow's milk.

Sample	Specific gravity	Milk fat (%)	Assayed value	
			Vitamin D ₃ (I.U./liter)	7-DHC (μg/liter)
Sample no. 1	1.028	3.0	19	14
no. 2	1.031	3.0	46	19
no. 3	1.031	3.0	66	56
no. 4	1.029	3.0	57	37
no. 5	1.030	3.5	63	33
no. 6	1.029	4.0	79	47
no. 7	1.031	3.3	48	44
no. 8	1.030	3.0	39	37
no. 9	1.031	3.3	23	18
no. 10	1.032	3.1	24	21

All values are the mean of triplicate determinations.

Fig. 4. Assayed values of vitamin D₃ and 7-DHC in UV-irradiated cow's milk.

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