High-Performance Liquid Chromatographic Determination of Vitamin D₃ in Fish Liver Oils and Eel Body Oils

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(Received February 27, 1984)

Summary Identification and determination of vitamin D₃ (or D₂) and 25-OH-D₃ in fish liver oils and eel body oils were carried out. By co-chromatography on HPLC, UV spectra and/or GC-MS, vitamin D₃ was identified in naturally occurring fish liver oils and eel body oils, whereas a drop of fish liver oil contained supplemented vitamin D₂. 25-OH-D₃ was identified only in skipjack liver oil. The HPLC method proposed in a previous report (Takeuchi, A. et al. (1984): J. Nutr. Sci. Vitaminol., 30, 11–25) was confirmed to also be useful for determination of vitamin D₃ (or D₂) in fish liver oils and eel body oils. The assayed values of vitamin D₃ in skipjack and tuna liver oils were 57,760 and 16,200 IU/g, respectively, which were much higher than those in cod and pollack liver oils. The assayed values of vitamin D₃ in eel body oils were very low (16–43 IU/g) and showed no appreciable change despite differences in the farming conditions. Determination of 25-OH-D₃ in skipjack oil was performed by using HPLC, and the assayed value was 1.8 µg/g. This was about 1/800 lower than that of vitamin D₃.

Key Words cod, determination of vitamin D, eel body oil, fish liver oil, high-performance liquid chromatography, 25-hydroxyvitamin D₃, pollack, skipjack, tuna, vitamin D₃

It is well-known that fish liver oils contain vitamin D₃. Colorimetric or gas-liquid chromatographic methods for determination of the vitamin in liver oils have been used previously (1–5). However, the clean-up procedures are very complicated and the sensitivity is not sufficient for application to some kinds of liver oils with a

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Abbreviations: D₂/D₃, vitamin D₂/D₃ (ergocalciferol/cholecalciferol); 25-OH-D₂/25-OH-D₃, 25-hydroxyvitamin D₂/D₃; GC-MS, gas-liquid chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; AUFS, absorbance unit as full scale; UV, ultraviolet; M, mean; SD, standard deviation; CV, coefficient variation.
low concentration of vitamin D₃. On the other hand, recent progress in HPLC has permitted micro-determination of vitamin D and its metabolites in various samples, because the development of microparticle columns and UV detectors included in HPLC analysis gives good separation with good reproducibility and extremely high sensitivity. This technique has been applied to determine the vitamin by several research groups (6–8). In previous reports (9, 10), we established an HPLC method for the determination of vitamin D in fortified dried milk, foods, feeds and pharmaceuticals by using two different kinds of column. The same method was confirmed to be useful also for assaying vitamin D₃ in fish liver oils and eel body oils. The results are described in this paper.

Identification of vitamin D₃ and 25-OH-D₃ in the oils was also carried out. 25-Hydroxyvitamin D₃ was detected only in skipjack liver oil and the concentration was also determined by an HPLC method.

EXPERIMENTAL

1. Materials and reagents

Commercial grades (Philips-Duphar Co., The Netherlands) of 25-OH-D₃ and the in vivo generated and purified 25-OH-D₂, as reported in a previous paper (11), were used as the respective standard compounds.

The other materials and reagents used were as described in a previous paper (10).

2. Samples

1) Fish liver oils. Skipjack, tuna, cod and pollack liver oils were used. These oils were kindly supplied by Tokai Regional Fisheries Research Laboratory and Riken Vitamin Oil Co., Tokyo.

2) Commercially available fish liver oil capsule and drop. Commercially available fish liver oil capsules and drops were used. The two samples consist of pollack liver oil, but the former includes the oil alone while the latter is supplemented by synthetic vitamin D₂ (indicated value: vitamin D₂, 200 IU/drop; vitamin A, 2,000 IU/drop).

3) Eel body oils. Four kinds of eel body oils were used. Three were produced from eels farmed in Japan, respectively: indoor with a mixed feeds diet (no. 1), and outdoor with a raw fishes diet (no. 2) or with a mixed feeds diet (no. 3). The fourth oil was produced in Formosa from eels farmed outdoors on raw fishes and mixed feed diets (no. 4). The oils were kindly supplied by Tokai Regional Fisheries Research Laboratory.

3. Instrumentation for GC-MS

The GC-MS was performed on a Hitachi M-80 gas chromatograph-double focusing mass spectrometer (Hitachi, Japan) with a unit of electron impact as an energy source. A glass column (0.3 \text{i.d.} \times 150\text{cm}) packed with 1.5\% OV-17 on
Shimalite W (60-80 mesh) was run at 280°C with a helium flow of 30 ml/min (pressure: 0.3 kg/cm²). The temperature of separator and injection port was controlled at 300°C (for vitamin D₃ analysis) or 320°C (for 25-OH-D₃ analysis). Ionizing voltage was kept at 20 eV.

4. Procedure for identification of vitamin D₃ and 25-OH-D₃

An adequate amount of fish liver oil (2–10 g) was weighed and treated according to the procedure as summarized in Fig. 1. Saponification and extraction of unsaponifiable matter were performed according to a previous report (10). The two types of high-performance liquid chromatographs as described in the previous paper (10) were used. The analytical conditions of each HPLC in Fig. 1 are as follows:

**Reversed-phase HPLC I and III**
- **Column**: Nucleosil 5C₁₈ (7.5 i.d. × 300 mm, Nagel Co., West Germany).
- **Mobile phase**: 50% methanol in acetonitrile.
- **Flow rate**: 2.0 ml/min (50 kg/cm²).
- **Retention time**: 25-OH-D₃, 8.8 min; 25-OH-D₂, 9.2 min; vitamin D₂, 21.0 min; vitamin D₃, 22.7 min.

**Straight-phase HPLC II for vitamin D analysis**
- **Column**: Zorbax SIL (4.6 i.d. × 250 mm, DuPont Co., USA).
- **Mobile phase**: 0.4% isopropanol in n-hexane.
- **Flow rate**: 1.8 ml/min (60 kg/cm²).

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Retention time: 21.1 min for vitamin D₂ and D₃.

Straight-phase HPLC IV for 25-OH-D analysis
Column: Zorbax SIL (4.6 i.d. × 250 mm).
Mobile phase: 5.5% isopropanol in n-hexane.
Flow rate: 0.8 ml/min (20 kg/cm²).
Retention time: 25-OH-D₂, 13.0 min; 25-OH-D₃, 14.4 min.

Reversed-phase HPLC V for 25-OH-D analysis
Column: Nucleosil SC18 (7.5 i.d. × 300 mm).
Mobile phase: 20% methanol in acetonitrile.
Flow rate: 1.7 ml/min (40 kg/cm²).
Retention time: 25-OH-D₂, 13.0 min; 25-OH-D₃, 14.8 min.

Straight-phase HPLC VI (for 25-OH-D₃ analysis)
Column: Zorbax SIL (4.6 i.d. × 250 mm).
Mobile phase: 2.2% isopropanol in n-hexane.
Flow rate: 1.7 ml/min (45 kg/cm²).
Retention time: 25-OH-D₂, 16.5 min; 25-OH-D₃, 21.5 min.

5. Procedure for determination of vitamin D₃ or D₂

The same procedure as described in a previous paper (10) was also applied to the samples in this report for determination of vitamin D₃ or D₂, but the clean-up procedure with a SEP-PAK silica cartridge could be omitted for skipjack and tuna liver oils and a commercially available fish liver oil drop. Authentic vitamin D₃ was generally used as a reference standard, but authentic vitamin D₂ was used for the liver oil drop including supplemented vitamin D₂. Since the contents of vitamin D₃ in skipjack and tuna liver oils were so high, a small volume of the benzene solution of unsaponifiable matter (10.0 ml) was sufficient for use in the following procedure.

6. Procedure for determination of 25-OH-D₃

About 0.5 g of fish liver oils was accurately weighed, saponified and the unsaponifiable matter was extracted with benzene according to the procedure for vitamin D₃ or D₂. Exactly 10.0 ml of the benzene solution was taken and evaporated under reduced pressure. The resulting residue was subsequently applied to the reversed-phase HPLC I, the reversed-phase HPLC V and the straight-phase HPLC VI as described in Fig. 1. The peak height corresponding to 25-OH-D₃ on the final HPLC was compared with the reference standard of 25-OH-D₃.

RESULTS AND DISCUSSION

1. Identification of vitamin D₃ in fish liver oils

Skipjack and tuna liver oils were subjected to the purification procedure described in Fig. 1. The profiles of HPLC performed in steps are shown in Fig. 2. Each of the single peaks corresponding to vitamin D₃ was observed in the both chromatograms of the reversed-phase HPLC III. The peaks co-migrated with
authentic vitamin D₃ by co-chromatography on the HPLC. Each purified D fraction was used to the estimation of UV spectra and GC-MS. The results completely agreed with the respective data for authentic compounds and the existence of vitamin D₃ in skipjack and tuna liver oils was detected.

Other samples were also subjected to the purification steps as described in Fig. 1. It was confirmed from the retention times and co-chromatography on the reversed-phase HPLC III that the fish liver drop contained vitamin D₂ (supplemented) while the other samples contained vitamin D₃.

2. Identification of 25-OH-D₃ in fish liver oils

Skipjack, tuna and pollack liver oils were subjected to the purification procedure described in Fig. 1 to obtain purified 25-OH-D fractions. Figure 3 shows the profiles of the straight-phase HPLC VI on the purified fractions. A peak corresponding to authentic 25-OH-D₃ was observed in the chromatogram of skipjack liver oil, but it could not be detected in the chromatograms of tuna and pollack liver oils. No peak corresponding to authentic 25-OH-D₂ could be observed in the three chromatograms.

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When the purified 25-OH-D fraction obtained from the skipjack liver oil was subjected to GC-MS, the pattern of mass chromatograms and the fragmentation patterns completely agreed with those of authentic 25-OH-D3. Moreover, the peak corresponding to 25-OH-D3 in the purified fraction co-migrated with the authentic compound on the co-chromatography using the straight-phase HPLC VI. From these results, we concluded that the skipjack liver oil contained 25-OH-D3. However, the tuna and pollack liver oils did not reveal the existence of 25-OH-D3 when using the same identification method mentioned above.

3. Recovery tests

Each 0.5g sample of fish liver oils was treated according to the whole procedure for determination of vitamin D3 as described in EXPERIMENTAL with or without addition of authentic vitamin D3 in order to perform recovery tests. As shown in Table 1, the overall recovery rate was higher than 95%, which was satisfactory.

4. Determination of vitamin D3 or D2 in samples

Determination of vitamin D3 in fish liver oils, capsule and eel body oils or of vitamin D2 in fish liver oil drop (vitamin D2 supplemented) was performed according to the proposed procedure. As shown in Fig. 4, all the peaks due to vitamin D3 or D2 in the chromatograms of the second analytical HPLC were clearly separated from interfering substances, showing that the proposed method was very useful. The assayed values are shown in Table 2. Vitamin A was also determined by the spectrophotometric method described in Japan Pharmacopeia X (12). The concentrations of vitamin D3 in skipjack and tuna liver oils were 10–1,500 times higher than those in cod and pollack liver oils. The reason why the two liver oils contain such higher levels of vitamin D3 is unclear at the present time.
Table 1. Recovery tests of vitamin D₃ in fish liver oils.

| Sample             | Trial | Sample weight (g) | Added value | Recovery (%) (M ± SD) | CV (%) |
|--------------------|-------|-------------------|-------------|-----------------------|--------|
| Tuna liver oil     | 6     | 0.5               | 200 µg      | 99.7 ± 9.8            | 9.9    |
| Cod liver oil      | 6     | 0.5               | 2 µg        | 99.8 ± 2.8            | 2.8    |
| Pollack liver oil  | 6     | 0.5               | 500 ng      | 95.0 ± 6.8            | 7.2    |
| Fish liver oil capsule | 6   | 0.5               | 200 ng      | 96.0 ± 7.8            | 8.1    |

Note: The added values mean the amounts of vitamin D₃ added to a sample.

Fig. 4. Profiles of the second analytical HPLC on the samples. (a) skipjack liver oil, (b) tuna liver oil, (c) cod liver oil (from Norway), (d) pollack liver oil, (e) eel body oil (no. 2), (f) eel body oil (no. 4), (g) fish liver oil capsule, (h) fish liver oil drop.

The assayed values of vitamin D₃ in eel body oils were very low and showed no change appreciable despite differences in the farming conditions (farming districts or diets). Since the CV values lower than 10% were obtained even for the oils containing low levels of vitamin D₃, the proposed method was confirmed to be both sensitive and accurate.

The content of vitamin D₃ in a commercially available fish liver oil capsule was very low, because pollack liver oil containing low levels of the vitamin was used without any supplementation of vitamin D. On the other hand, since a commercially available fish liver oil drop was supplemented by synthetic vitamin D₂, the
Table 2. Assayed values of vitamin D$_3$ or D$_2$ in fish liver oils, eel body oils and fish liver oil preparations (capsule and drop).

| Kind of vitamin D | Sample                      | $n$ | Assayed value of vitamin D M±SD (IU/g) | CV (%) | Assayed value of vitamin A (IU/g) | IU ratio (A/D) |
|-------------------|-----------------------------|-----|----------------------------------------|--------|----------------------------------|----------------|
| D$_3$             | Skipjack liver oil          | 6   | 57,760±110                             | 1.9    | 24,000                           | 0.4            |
|                   | Tuna liver oil              | 6   | 16,200±593                             | 3.7    | 93,200                           | 5.8            |
|                   | Cod liver oil               | 6   | 163±5                                  | 3.0    | 1,140                            | 7.0            |
|                   | Cod liver oil (from Norway) | 6   | 55±1                                   | 2.3    | 1,290                            | 23.5           |
|                   | Pollack liver oil           | 6   | 38±2                                   | 4.9    | 4,530                            | 120.1          |
|                   | Eel body oil no. 1$^a$      | 6   | 37±3                                   | 7.9    | 278                              | 7.5            |
|                   | Eel body oil no. 2$^a$      | 6   | 16±1                                   | 6.3    | 142                              | 8.8            |
|                   | Eel body oil no. 3$^a$      | 6   | 37±3                                   | 7.0    | 188                              | 5.0            |
|                   | Eel body oil no. 4$^a$      | 6   | 43±2                                   | 4.6    | 202                              | 4.7            |
|                   | Fish liver oil capsule      | 6   | 12±1                                   | 11.6   | 4,200                            | 334.9          |
| D$_2$             | Fish liver oil drop         | 6   | 264±7$^b$                              | 2.8    | 2,300                            | 8.7            |

$^a$For kinds of eel body oils were used, refer to EXPERIMENTAL. $^b$The assayed values as IU/drop were 247±7, 124±3% of the indicated value (200IU/drop).

Table 3. Comparison of vitamin D$_3$ concentrations in fish liver oils reported in the literature.

| Reference                  | Fish liver oil | Assayed value or range of vitamin D$_3$ (IU/g) | Method          |
|----------------------------|----------------|-----------------------------------------------|-----------------|
| This study                 | Skipjack       | 57,760                                        | HPLC            |
| Yamakawa et al. (2)        | Skipjack       | 25,000–250,000                                | Colorimetric    |
| This study                 | Tuna           | 16,200                                        | HPLC            |
| Kobayashi et al. (5)       | Tuna           | 10,800                                        | GLC             |
| Yamakawa et al. (2)        | Tuna           | 10,000–45,000                                 | Colorimetric    |
| This study                 | Pollack        | 38                                            | HPLC            |
| Yamakawa et al. (2)        | Pollack        | 20                                            | Colorimetric    |
| This study                 | Cod$^a$        | 163                                           | HPLC            |
| Stancher et al. (13)       | Cod            | 377                                           | HPLC            |
| Egaas et al. (7)           | Cod            | 207                                           | HPLC            |
| Ali (6)                    | Cod            | 101                                           | HPLC            |
| Pask-Hughes et al. (8)     | Cod            | 34–172                                        | HPLC            |
| Hommes et al. (4)          | Cod            | 147                                           | GLC             |
| Bell et al. (3)            | Cod$^b$        | 49–410                                        | GLC             |
| Ali (1)                    | Cod            | 104                                           | Colorimetric    |
| Yamakawa et al. (2)        | Cod            | 85–500                                        | Colorimetric    |

$^a$From Norway. $^b$Type of oil: high potency.
assayed value of the vitamin was 264 IU/g, which was much higher than that of the above sample. These results suggest that supplementation of vitamin D (D$_2$ or D$_3$) or use of fish liver oils (e.g., skipjack or tuna liver oils) is necessary to supply the dietary allowance of vitamin D (400 IU/day for children and 100 IU/day for adults) with such capsule or drop preparations.

5. Comparison of our assayed values of vitamin D$_3$ with those in the literature

Our assayed values of vitamin D$_3$ in fish liver oils were compared with those in the literature (1–8, 13) as shown in Table 3. Because concentrations of naturally occurring compounds are usually variable, approximate values were taken for the respective fish liver oils.

6. Determination of 25-OH-D$_3$ in skipjack liver oil

Since 25-OH-D$_3$ was identified in a skipjack liver oil, determination of the metabolite was performed according to the method described in EXPERIMENTAL. The assayed value (an average of two trials) was 1.8 µg/g. This was much lower than that of vitamin D$_3$ (about 1/800) and the reason is under investigation.

The authors wish to thank Dr. T. Yamakawa, Dr. Y. Fujii, Dr. T. Kinumaki, Dr. K. Sugii and Dr. M. Takeuchi of Tokai Regional Fisheries Laboratory and Riken Vitamin Oil Co. for their helpful discussions and for donating the samples of fish liver oils and eel body oils. The authors are also grateful to Dr. K. Saiki and Miss T. Sai of the College, for measuring the GC-MS.

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