GAS-LIQUID CHROMATOGRAPHIC DETERMINATION OF VITAMIN D IN MULTIVITAMIN PREPARATIONS CONTAINING EXCESS AMOUNTS OF VITAMIN E1,2

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Summary Gas-liquid chromatographic (GLC) determination of vitamin D in multivitamin preparations containing excess amounts of vitamin E (a more than 2,500 weight ratio of dl-α-tocopheryl acetate to vitamin D) was investigated and a simplified routine method was established because the method reported previously (1) could not be applied to such special preparations. After applying the unsaponifiable matters of a sample to a phosphate-treated alumina column chromatography prepared according to MULDER et al. (2), the eluate was evaporated and the residue was subjected to thin-layer chromatography (TLC) and GLC. When this method was applied to model preparations made by mixing vitamin D2 and dl-α-tocopheryl acetate (excess amounts), good results were obtained. Since the results on a commercial multivitamin preparation containing excess amounts of vitamin E were also satisfactory, it was confirmed that the proposed method could be used for simplified routine determinations.

It was reported in a previous paper (1) that a simplified routine method for the GLC determination of vitamin D in multivitamin preparations had been established. The proposed method includes alkali-saponification of samples, extraction of the unsaponifiable matters, TLC and GLC analysis. It was confirmed that the proposed method could be used for preparations within the 104 (I.U.

1 Studies on the Gas-Liquid Chromatographic Determination of Vitamin D. Part II. Part 1, see Ref. 1).
2 Following Abbreviations are used: pre-D and D2, precalciferol and preergocalciferol; pyro-D2, pyroergocalciferol; isopyro-D2, isopyroergocalciferol; SA, stigmasteryl acetate; GLC, gas-liquid chromatography; TLC, thin-layer chromatography; A/D, a content ratio of vitamin A to vitamin D; E/D, a content ratio of vitamin E to vitamin D; I.U., international unit.
3 小林正, 足立昌子
4 Vitamin D usually means the sum of vitamin D and pre-D.
ratio) A/D and 2,500 (weight ratio) E/D. When the ratios of 36 kinds of commercial multivitamin preparations sold in Japan were studied, the A/D of all of them and E/D of most of them were within the limitations while the E/D of two exceptions were over the limitation. Therefore, most of preparations except the two can be determined by the proposed method. However, a routine method for the exceptions should also be established because they are presently on sale in Japan.

When phosphate-treated alumina column chromatography prepared according to Mulder et al. (2) was used for the preparations containing excess amounts of vitamin E as a pre-treatment to the TLC in the proposed method, the influences were successfully eliminated and satisfactory results were obtained. In this paper, the determination of vitamin D in such multivitamin preparations is described.

EXPERIMENTAL

1. Materials and reagents

Phosphate-treated alumina. This was prepared according to Mulder et al. (2). After adding 1,600 ml of distilled water and 20 g of disodium hydrogen phosphate (Na₂HPO₄·2H₂O) to 250 g of alumina (type 1097, E. Merck Co.), it was heated for 30 min on a water bath. The mixture was cooled with gentle swirling and the upper aqueous layer was decanted. After filtering the residue by suction on a Büchner funnel, the alumina was activated for 3 hr at 130°C. The cooled alumina was stored in a rubber-stoppered container which was kept in a desiccator until use.

Petroleum ether. Refluxed over KOH pellets and distill to collect the fraction between 40 and 60°C.

Ethyl ether. Peroxide-free ethyl ether was used.

Other materials and reagents were used according to a previous paper (1).

2. Multivitamin preparation sample

A commercial multivitamin capsule containing vitamin D₃ with vitamin A, E, B₁, B₂, B₆, B₁₂, C, nicotinamide and calcium lactate was used as a sample. Since one capsule of this preparation contains 400 I.U. of vitamin D₃, 2,000 I.U. of vitamin A palmitate and 50 mg of dl-α-tocopherol calcium succinate, the A/D and E/D are 5 and 4,310, respectively.

5 A/D usually means a I.U. ratio of vitamin A to vitamin D. A weight ratio of vitamin A to vitamin D can be calculated by A/D (I.U. ratio) times either 12.0 for vitamin A alcohol, 13.8 for vitamin A acetate or 20.8 for vitamin A palmitate.

6 E/D usually means a weight ratio of dl-α-tocopherol acetate to vitamin D. The weight ratios of dl-α-tocopherol, its acid succinate and its calcium succinate can be calculated by the E/D times 0.88, 1.12 and 1.16, respectively.

7 The E/D shows as the ratio of dl-α-tocopherol acetate to vitamin D₂ (weight ratio) calculated from the ratio of dl-α-tocopherol calcium succinate to vitamin D.
3. **Procedure**

1) Deactivation of phosphate-treated alumina and preparation of column. According to Mulder et al. (2), weigh 30 g of phosphate-treated alumina in a 100 ml erlenmeyer flask and add 1.5 ml of water using a pipet. Close the flask with a rubber stopper, shake vigorously and then let stand for 15 min. Transfer the deactivated alumina gradually to a glass tube (2.0 × 30 cm) containing 50 ml of petroleum ether and let settle. The height of alumina becomes about 12 cm.

2) Saponification and isolation of the unsaponifiable matters. Treat according to a previous paper (1).

3) Phosphate-treated alumina column chromatography. Accurately take 50.0 ml of the benzene solution obtained by the procedure 2) and evaporate the solvent under a reduced pressure (lower than 40°C). After dissolving the residue in 5 ml of petroleum ether, the solution is transferred to the phosphate-treated alumina column with the aid of 15 ml of petroleum ether. Let elution start at a flow rate of 4 to 5 ml/min with 200 ml of 8% ethyl ether in petroleum ether followed by 100 ml of 30% ethyl ether in petroleum ether. Discard the former eluate and collect the latter eluate in a round-bottom flask. Evaporate the solvent under reduced pressure and dissolved the residue in 1.0 ml of acetone. This acetone solution is used in the following procedure.

4) TLC and GLC. The acetone solution is applied to the TLC followed by the GLC according to the previous paper (1).

5) Calculation. The content of vitamin D in a sample is calculated according to the previous paper (1).

### RESULTS

1. **Recovery test for vitamin D₂ by phosphate-treated alumina column chromatography**

   After applying 2,000 I.U. of vitamin D₂ to the phosphate-treated alumina column chromatography described in the procedure and collecting the eluate of 30% ethyl ether in petroleum ether, the solvent was evaporated under reduced pressure. The residue was dissolved in 0.5 ml internal standard solution (50 μg/ml of SA solution in acetone) and then applied to the GLC. When the recovery was compared with the results obtained without the use of column chromatography, calculations showed it to be 99.2±0.2 (Mean±S.D.) which is quite satisfactory.

2. **Determination of vitamin D₂ in model preparations**

   Model preparations were made by mixing vitamin D₂ (8,000 I.U.) with either 1,000 or 2,000 mg of vitamin E (dl-α-tocopheryl acetate). The E/D are 5,000 and 10,000, respectively. The determination of vitamin D₂ in the preparations was carried out according to the whole procedure, and satisfactory results were obtained as shown in Table 1. As shown in Fig. 1 (the upper side), the gas chromatogram of a model preparation showed no interference to pyro-D₂ peak.
Table 1. Recovery of vitamin D$_2$ in model preparations.

<table>
<thead>
<tr>
<th>Model preparation</th>
<th>Trial</th>
<th>Vitamin D$_2$</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Added value (I.U.)</td>
<td>Estimated value (I.U.)</td>
</tr>
<tr>
<td>No. 1 (E/D = 5,000)</td>
<td>1</td>
<td>8,000</td>
<td>7,936</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8,000</td>
<td>7,968</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>7,952</td>
<td>99.4</td>
</tr>
<tr>
<td>No. 2 (E/D = 10,000)</td>
<td>1</td>
<td>8,000</td>
<td>7,913</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8,000</td>
<td>7,800</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>7,857</td>
<td>98.7</td>
</tr>
</tbody>
</table>

Note: The model preparations No. 1 and 2 were made by mixing 8,000 I.U. (0.2 mg) of vitamin D$_2$ with 1,000 and 2,000 mg of vitamin E (dl-$\alpha$-tocopheryl acetate), respectively.

Fig. 1. Gas chromatograms of a model preparation made by mixing vitamin D$_2$ (8,000 I.U.) and dl-$\alpha$-tocopheryl acetate (1,000 mg) and a commercial multivitamin capsule after treating according to the whole procedure. 1, pyro-D$_2$; 2, isopyro-D$_2$; SA, stigmasteryl acetate; U, unknown peak.

Table 2. Determination of vitamin D$_2$ in a commercial multivitamin preparation containing excess amounts of vitamin E.

<table>
<thead>
<tr>
<th>Sample (Indicated value of vitamin D$_2$)</th>
<th>Trial</th>
<th>Estimated value of vitamin D$_2$ (I.U./cap.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multivitamin capsule</td>
<td>1</td>
<td>444</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>436</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>412</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>408</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td></td>
<td>425 ± 15</td>
</tr>
</tbody>
</table>
3. Determination of vitamin D$_2$ in a multivitamin preparation

When vitamin D$_2$ in a commercial multivitamin capsule containing excess amounts of vitamin E (refer to EXPERIMENTAL) was repeatedly determined according to the whole procedure, satisfactory results were obtained as shown in Table 2. As shown in Fig. 1 (the lower side), no interference was not observed in the gas chromatogram.

DISCUSSION

Because excess amounts of vitamin E (more than 2,500 of E/D) interfere with the determination of vitamin D by the method reported previously (1), phosphate-treated alumina column chromatography was used to eliminate the interference. As shown in Tables 1 and 2, the results on both the model and commercial multivitamin preparations were quite satisfactory. Since no interference to the pyro-D$_2$ peaks could not be observed in the gas chromatograms, the elimination of influences by excess amounts of vitamin E was confirmed to be performed by the procedure. From these results, we concluded that the proposed method was suitable for preparations containing excess amount of vitamin E as a routine method. It takes about 4 hr to perform the whole procedure.

REFERENCES