Determination of Vitamins D by Gas-Liquid Chromatography

I. Differentiation and Assay of Vitamins D₂ and D₃

KIYOSHI TSUKIDA AND KAYOKO SAIKI

Kobe Women's College of Pharmacy, Higashinada-Ku, Kobe
(Post No. 658)

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The gas chromatographic conditions for the assay of vitamins D were described. The analysis was performed on Shimazu Gas Chromatograph GC-4AP equipped with a hydrogen flame ionization detector. Glass columns (4 i.d. × 1000 mm) packed with 1.5% OV-17 on Shimalite W, 80-100 mesh, silanized, were operated at 240°C and a flow rate of 40 ml/min (N₂) was maintained. Adoption of "unmodified" D as an analytical form and good GLC resolution under the operated condition made the procedure more simple and time-saving. Employment of 7-dehydrocholesterol acetate as an internal standard enhanced the accuracy of the analysis. Satisfactory result was obtained in the determination of vitamins D₂ and D₃ in mixtures.

The determination of vitamins D by GLC has been the subject of several investigations (1, 2), initiated by H. Ziffer et al. (3) in 1960. However, the majority of reports in the literature emphasized the qualitative microdetection of vitamins D and no satisfactory method has been developed as a routine quantification of vitamins D.

As is well known, vitamins D are cyclized thermally during GLC to give twin peaks of pyro- and isopyro-D₂ or D₃ in a definite ratio, the larger "pyro" peak being eluted earlier. However, complete gas chromatographic resolution of these peaks is not always observed on the reported columns. The trimethylsilyl ethers of vitamins D give also similar twin peaks and an excellent qualitative separation is obtained in our experiment, using OV-17 column operated at 230°C (Fig. 1). For quantitative purpose, however, several analytical problems such as silylation yield and appearance or overlap of minor peaks are brought about simultaneously.

It is a recent idea to analyse vitamins D by modifying to their appropriate derivatives which are expected to give rise to a single peak on GLC. Examples are demonstrated in conversions to isovitamins D (4) and to isotac-hysterols (5) including their derivatives such as hepta-fluorobutyrates (6), catioles (7) and so forth. These attempts may offer a technique of potential value in the determination of vitamins D, but are always hampered by additional factors such as reaction conditions to be carried out, yields, or heat-stabilities of the compounds.

In the present paper we reinvestigate primarily on the basic condition of GLC for "unmodified" pure vitamins D standards and present data on a simultaneous determination of vitamins D.

1 Following abbreviations are used: GLC, gas-liquid chromatography; RRT, relative retention time; i.d., internal diameter

2 月田 満, 畑木加代子.
EXPERIMENTAL AND RESULTS

Materials
Crystalline vitamins D$_2$ and D$_3$ were purchased from Philips-Duphar Co., Holland. Lumisterol$_2$ and 7-dehydrocholesteryl acetate were kindly provided by Dr. T. Kobayashi. The acetate and the butyrate (mp 103°C) of cholesterol were synthesized in the usual manner from cholesterol, pyridine, and acetic or butyric anhydride.

Table 1
Relative retention data for vitamins D and related compounds on OV-17 column

<table>
<thead>
<tr>
<th>Compound</th>
<th>RRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D$_2$*</td>
<td>0.57/0.68</td>
</tr>
<tr>
<td>Vitamin D$_3$*</td>
<td>0.66/0.78</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.64</td>
</tr>
<tr>
<td>Cholesteryl acetate</td>
<td>0.87</td>
</tr>
<tr>
<td>Cholesteryl butyrate</td>
<td>1.69</td>
</tr>
<tr>
<td>7-Dehydrocholesteryl acetate</td>
<td>1.00 (23.4 min)</td>
</tr>
<tr>
<td>Ergosterol</td>
<td>0.87</td>
</tr>
<tr>
<td>Lumisterol$_2$</td>
<td>0.68</td>
</tr>
</tbody>
</table>

* D$_2$ and D$_3$ give twin peaks, the larger “pyro” peak being eluted earlier.

RRT of vitamins D and related compounds are given in Table 1.

NMR (δ in CDCl$_3$): Vitamin D$_3$: 9.45 (s, 18-Me), 9.13 (d, J 5.5, 26-Me+27-Me), 9.07 (d, J 4.5, 21-Me), 8.36 (s, OHi), 6.06 (m, 3-CH), ca. 5.17 (d)+4.95 (m) (19=CH$_2$), 4.02+3.66 (dd of AB type, J 11.2, 6-7-CH=CH=), 7.0-8.8 (equiv. to 22 H). Vitamin D$_2$: 9.45 (s, 18-Me), 9.18 (d, J 5.5, 26-Me+27-Me), 9.08 (d, J 5.7)+8.98 (d, J 6.1) (28-Me+21-Me), 6.07 (m, 3-CH), ca. 5.18 (d)+4.96 (m) (19=CH$_2$), ca. 4.8 (m, 22, 23-CH=CH=), 4.04+3.68 (dd of AB type, J 11.2, 6-7-CH=CH=), 7.0-8.8 (equiv. to 22 H). 7-Dehydrocholesteryl acetate: 9.38 (s, 18-Me), 9.13 (d, J 5.4, 26-Me+27-Me), 9.06 (d?, 21-Me), 9.05 (s, 19-Me), 7.98 (s, Ac), 5.34 (m, 3-CH), 4.56 (d, J 12)+4.47 (d, J 12) (6,7-CH=CH=), 7.3-8.9 (equiv. to 28 H).

Apparatus
Gas Chromatograph GC-4AP (Shimazu Seisakusho) equipped with a hydrogen flame ionization detector was used for GLC, and Varian A 60-D for NMR measurements.

Operational Parameters
The glass column, 1 m long and 4 mm i.d., was
packed with either 1% NGS, 1% XE-60*1, 1.5% SE-30, 1.5% SE-52, 1.5% OV-1, 1.5% QF-1*1, 1.5% OV-17, or 3% OV-101, and was maintained at 240°. Considering the gas chromatographic resolution obtained on each column as well as polarities or heat-stabilities of the stationary phases, the best result was observed with 1.5% OV-17 column (Fig. 2). On this column the twin peaks of vitamins D were not accompanied with any other peaks which might derive from possible degradation products. No significant difference was observed on further examination of the effect on retention times by other parameters, i.e., length or i.d. of a column, temperature operated (220-300°) as well as a flow rate of the carrier gas (30-80ml/min). On the basis of the experimental evidence, the sample solution*2 was applied in the instrument by on-column injection and operated under the following optimum conditions. All injections were made using a 10 µl microsyringe (Terumo) with the injection volume being 1 µl equivalent to 1-2 µg of vitamins D.

Stationary phase: 1.5% OV-17 on Shimalite W, 80-100 mesh, silanized.

Temperatures: column, 240°; injector port, 250°; detector, 300°.

Flow rates: carrier gas, nitrogen, 40 ml/min; detector gas, hydrogen, 0.55 kg/cm²; air, 0.8 kg/cm².

Detector sensitivity: sens. 10⁴, range 6.4 V.

Chart speed: 10 mm/min.

Gas Chromatograms and Internal Standard

The gas chromatograms of vitamins D₂ and D₃ obtained under the operating condition were illustrated in Fig. 2, and the relative retention data were given in Table 1. As a suitable internal standard in this study, 7-dehydrocholesterol acetate was newly selected among several related compounds. Although vitamin D₂ and lumisterol² were reported to have similar RRT and were difficult to separate each other on SE-30 column [2], our own finding with the present column indicated that they were able to be differentiated qualitatively.

Standard Calibrations

From three 0.4% (w/v)-standard stock solutions in acetone of vitamins D₂, D₃, or 7-dehydrocholesterol acetate (the internal standard), fourteen standard working solutions in acetone of vitamins D₂ and D₃ were prepared. The ratio of D₃ to the internal standard in each solution was adjusted to 0.5:1, 1:1, 1.5:1, 2.0:1, 2.5:1, 3.0:1, or 4.0:1, respectively.

These standard working solutions were alternately injected into the instrument with the described operational parameters and the gas chromatographic results were calibrated between the peak area ratio vs. the weight ratio (vitamin D/internal standard) (Fig. 3). Each peak area was determined by the height multiplied by the peak width at half height on the “pyro”, the “isopyro”, or the internal standard peak. All calibration curves exhibited excellent lineairities, penetrating through the origin. When the weight ratio of D₂ to the internal standard was 2:1, average x and standard deviation for pyro- and isopyro-D₂ were 1.250, 0.011 and 0.741, 0.006, respectively.

Determination of Vitamins D₂ and D₃ in Mixtures

Six working solutions containing vitamins D₂ and D₃ in the ratio*3 of 0:5, 1:4, 2:3, 3:2, 4:1, and

<p>| Table 2 | Determination of vitamins D₂ and D₃ in mixtures by gas chromatography on OV-17 |
|----|----|----|----|----|----|</p>
<table>
<thead>
<tr>
<th>Mixture</th>
<th>Vitamin D₂</th>
<th>Present</th>
<th>Found</th>
<th>Recovery</th>
<th>Present</th>
<th>Found</th>
<th>Recovery</th>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
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<td>4.90</td>
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<tr>
<td>2</td>
<td>1.00</td>
<td>0.99</td>
<td>99.0</td>
<td>4.00</td>
<td>3.98</td>
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</tr>
<tr>
<td>3</td>
<td>2.00</td>
<td>1.96</td>
<td>98.0</td>
<td>3.00</td>
<td>3.15</td>
<td>105.0</td>
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<tr>
<td>4</td>
<td>3.00</td>
<td>2.89</td>
<td>96.3</td>
<td>2.00</td>
<td>2.15</td>
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<tr>
<td>5</td>
<td>4.00</td>
<td>3.84</td>
<td>96.0</td>
<td>1.00</td>
<td>1.30</td>
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<td>6</td>
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<td>4.93</td>
<td>98.6</td>
<td>0</td>
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</tr>
</tbody>
</table>

Vitamins D₃ and D₂ are determined by GLC using the pyro-D₃ and the isopyro-D₂ peaks, respectively. The ratio of total D to the internal standard is 5:2.
DISCUSSION

Chemical methods heretofore proposed for microdetermination of vitamins D commonly suffer primarily from lack of specificity, sensitivity, and stability. Furthermore, need of purification techniques designed to remove interfering substances presents considerable analytical problems. Consequently, specific method of microdetermination by GLC or fluorometry has been desired, but there exist no successful method as a routine quantification of vitamins D. Several gas chromatographic attempts have been proposed to determine vitamins D by modifying to their appropriate derivatives. In fact, excellent gas chromatographic tracing was obtained for the trimethylsilyl ethers of vitamins D in our experiment (Fig. 1). For quantitative purpose, however, special considerations should be carefully paid as described in the introductory section.

Although unmodified vitamins D cyclized thermally to give twin peaks on GLC, good resolution was easily obtained under the present operated conditions. Then it would be possible to estimate vitamins D by taking the "pyro" peak (a or a’ in Fig. 2) for a sample containing either D₂ or D₃, and the pyro-D₃ peak as well as the isopyro-D₂ peak (a” in Fig. 2) for a sample containing both of them. In order to compensate for column characteristics, instrumental variations, and sample introduction technique, 7-dehydrocholesteryl acetate was newly employed as a suitable internal standard. All calibration curves obtained indicated linear responses to the weight ratio of vitamin D to the internal standard, penetrating through the origin (Fig. 3). The optimum weight ratio of vitamin D to the internal standard was around 2:1. Examination of samples containing both D₂ and D₃ in mixture revealed that good analysis was obtained on vitamin D₃ content within an experimental error, while positive errors were affected on the analysis of vitamin D₂ when its content was too low compared with that of vitamin D₃ (Table 2). In the latter case, however, the disadvantage could be easily eliminated with an addition of a definite amount of vitamin D₂ in advance and no trouble was encountered in overall ranges for determination of vitamins D₂ and D₃ in mixture.

Investigation on the determination of vitamins D in the presence of interfering substances is proceeding and will be discussed in the following paper.

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REFERENCES


7. Panalaks, T., addressed at the Research Seminar [Kobe Women’s College of Pharmacy, Apr. 1970].