Assay of 1,25-Dihydroxy Vitamin D₃ by Isotope Dilution–Mass Fragmentography

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We describe a specific assay of 1,25-dihydroxy vitamin D₃ in human serum, based on isotope dilution–mass fragmentography. We added [26-²H₃]-1,25-dihydroxy vitamin D₃ to a fixed amount of serum. The steroids were extracted with chloroform/methanol and purified by liquid chromatography. The purified material was converted into the trimethylsilyl ether and analyzed. Unlabeled 1,25-dihydroxy vitamin D₃ was quantitated from the ratio between the tracings at m/e 452 and 455. The two ions used correspond to loss of two trimethylsilyloxy functions from the molecular ion. Essentially the same results, with some problems of interference, were obtained when we used the more intense ions at m/e 131 and 134 (corresponding to cleavage between C-24 and C-25). The detection limit was about 5 ng/L of serum; the coefficient of variation was about 6%. The accuracy of the method was assessed by recovery experiments. 1,25-Dihydroxy vitamin D₃ in sera from 15 healthy subjects was found to average 55 ± 10 ng/L (±SD). We believe this represents the first determination of 1,25-dihydroxy vitamin D₃ in serum by use of a method not based on radioimmunoassay or receptor assay. Slightly lower values have been reported by those latter techniques.

Additional Keyphrases: “high-performance” liquid chromatography • normal values • gas chromatography/mass spectrometry • potential reference method

Clinical applications in the rapidly expanding field of vitamin D research have to a great extent been limited by the methodologies used. Moreover, the different metabolites of vitamin D present in plasma are unstable, occur in small quantities, and are difficult to assay with sufficient accuracy. In general, 25-hydroxy vitamin D₃ (1–5) and 1,25-dihydroxy vitamin D₃ (6–10) are assayed in serum by use of competitive binding techniques. 25-Hydroxy vitamin D₃ in serum can also be assayed by “high-performance” liquid chromatography (HPLC) (11–13). The specificity of competitive binding assay and HPLC may vary considerably, however. Preferably such techniques should be evaluated with more accurate methods before general use. When there is a demand for high specificity and accuracy, isotope dilution–mass fragmentography seems to be the method of choice (14).

Such a technique was recently developed for assay of 25-hydroxy vitamin D₃, with [26-²H₃]-labeled 25-hydroxy vitamin D₃ as internal standard (15–17). The internal standard was added to serum before extraction and chromatographies, and it is assumed that the degradation or decomposition of this labeled standard was the same as the degradation of unlabeled 25-hydroxy vitamin D₃. In the final mass-spectrometric step, the ratio between unlabeled and labeled molecules was determined with high accuracy. Such a specific method also is needed for 1,25-dihydroxy vitamin D₃.

Here, we report a mass-fragmentographic assay for 1,25-dihydroxy vitamin D₃ in human serum, with [26-²H₃]-1,25-dihydroxy vitamin D₃ used as internal standard.

Materials and Methods

Materials

[26-²H₃]-1,25-Dihydroxy vitamin D₃. 26-²H₃-labeled 25-hydroxy vitamin D₃ was synthesized as described previously (15), and [26-²H₃]-1,25-dihydroxy vitamin D₃ was biosynthetically produced from this material by incubation with kidney homogenate from rachitic chicks (18, 19). Kidneys from five chicks were homogenized (1 g/10 mL) in a medium consisting of, per liter, 0.25 mol of sucrose, 15 mmol of HEPES buffer (pH 7.4), and 1 mmol of EGTA. The nuclear fraction was removed by centrifugation (1000 × g, 10 min). The supernate, 10 mL, was incubated under oxygen together with 144 μg of [26-²H₃]-25-hydroxy vitamin D₃ for 30 min at 37°C in the presence of 15 mmol of malate per liter and 2.5 mmol of MgCl₂ per liter. The reaction was stopped by adding 20 mL of chloroform/methanol (1/2 by vol). The 1,25-dihydroxy vitamin D₃ obtained in the chloroform phase was purified by chromatography on Sephadex LH-20 and HPLC on Spherisorb silica and Spherisorb ODS-silica as described below for the serum samples. The ultraviolet spectrum disclosed that the final preparation was not entirely pure. Thus the trough normally seen at 228 nm was absent. The exact yield could not be determined, but was estimated to be about 2.5 μg. No interfering compounds were seen, however, in the mass-fragmentographic assay (Figure 3B). According to the mass-fragmentographic analysis, the material contained 1.4% unlabeled 1,25-dihydroxy vitamin D₃.

Unlabeled 1,25-dihydroxy vitamin D₃. This compound, a gift, was at least 95% pure as judged by thin-layer chromatography with ethyl acetate as the developing solvent and by gas–liquid chromatography of the trimethylsilyl ether (cf. below). The final quantitation of this compound before gas chromatography–mass spectrometry was performed with

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Received Nov. 20, 1978; accepted Jan. 30, 1979.

Nonstandard abbreviations used: EGTA, [ethylenedia(oxyethylenenitrito)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; and HPLC, “high-performance” liquid chromatography.
1,25-dihydroxy vitamin D₃ (as determined in separate runs, with biosynthetically produced radiolabeled 1,25-dihydroxy vitamin D₃ as marker) were collected. The solvent was evaporated under nitrogen and the residue was dissolved in 100 μL of isopropanol/hexane (7.5/92.5 by vol). The entire sample was then chromatographed in a Spectra Physics 3500 B chromatographic system equipped with a Rheodyne Model 7120 injector, a 100-μL sample loop, and a prepacked 4.6 × 250 mm column of Spherisorb 5-μm (particle diameter) silica Phase Separations, Ltd., Queensferry, England. A fixed-wavelength (254-nm) ultraviolet detector, Spectra Physics Model 8200, was used to monitor eluted 1,25-Dihydroxy vitamin D₃ was eluted at a flow rate of 2 mL/min with the isopropanol/hexane mixture. The appropriate fractions were collected, the solvent was evaporated under nitrogen, and the residue was dissolved in the solvent used for reversed-phase HPLC, for which we used a Spherisorb ODS-silica column and methanol/water (75/25 by vol) at a flow rate of 2.4 mL/min. One-minute fractions were collected, and two fractions containing 1,25-dihydroxy vitamin D₃ were collected in each chromatographic run. After removal of the solvent, the material was converted into the trimethylsilyl ether (21) and dissolved in 25 μL of hexane.

Combined gas chromatography–mass spectrometry. An LKB 2091 instrument equipped with a multiple ion detector was used. The gas chromatography was performed with a 1.5% SE-30 column (on Chromosorb W, 80–100 mesh, 2 mm × 1.5 m). The carrier gas was helium and the flow rate about 15 mL/min. The temperature of the column was about 250 °C and that of the ion source about 270 °C. The electron energy was set at 32 eV. The electron multiplier sensitivity was set to 500–500. The first channel of the multiple ion detector was focused on the ion at m/e 452, the second at m/e 455. In general, the amplification used for the first channel was 500X and for the second channel 200X. The peak heights of the multiple ion recordings were measured because we found this gave more reproducible results than measurement of peak area. In some experiments, designed to test the specificity of the assay, the two channels were focused on the ions at m/e 131 and 134.

**Results**

Figure 1 shows typical HPLC chromatograms for [26,24-³H]1,25-dihydroxy vitamin D₃, unlabeled 1,25-dihydroxy vitamin D₃, and 1,25-dihydroxy vitamin D₃ isolated from serum, to which deuterium-labeled 1,25-dihydroxy vitamin D₃ had been added. As seen in the chromatogram shown in Figure 1C, the fractions obtained in the isolation of material from serum, contained some unidentified ultraviolet-absorbing compound(s) in addition to 1,25-dihydroxy vitamin D₃. The purity was satisfactory, however, for the further analysis by gas chromatography–mass spectrometry. Figure 2 shows a mass spectrum of trimethylsilyl ether of 1,25-dihydroxy vitamin D₃. There were small peaks at m/e 632 (M) and at m/e 617 (M-15) and more prominent peaks at m/e 542 (M-90), 452 (M-2X90), and 362 (M-3X90). The base peak in the mass spectrum was at m/e 131, corresponding to cleavage between C-24 and C-25 (15, 22, 23).

With respect to sensitivity, the ion at m/e 131 should be preferred. The specificity of a mass-fragmentographic assay, however, in general decreases with decreasing relative molecular mass of the fragment chosen for assay. Thus it was shown that use of the ion at m/e 131 was sometimes complicated by interference (cf. below). Therefore we chose the ion at m/e 452 for assay of 1,25-dihydroxy vitamin D₃ in the standard procedure.

Typical multiple-ion detector recordings of the trimethylsilyl derivative of unlabeled and deuterium-labeled 1,25-dihydroxy vitamin D₃ are shown in Figure 3A and B, respec-

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**Figure 1.** "High-performance" liquid chromatography on Spherisorb ODS-silica of biosynthetically produced [26,24-³H]1,25-dihydroxy vitamin D₃ (A), authentic 1,25-dihydroxy vitamin D₃ (B) and prepurified extract of serum to which 1,25-dihydroxy 25-³H vitamin D₃ had been added (C).

The experimental procedure is given in Materials and Methods. The arrows indicate the fraction collected for gas chromatography–mass spectrometry.
Fig. 2. Mass spectrum of the tri-trimethylsilyl derivative of unlabeled 1,25-dihydroxy vitamin D₃ (pyro-form)
Except for the M and M-15 peaks, all peaks with intensity less than 5% of that of the base peak have been deleted.

In accordance with previous work, two peaks were obtained, corresponding to the pyro and isopyro form, respectively (15, 24). Only the pyro forms were used in the quantitations. No interfering compounds were seen in the chromatograms, in spite of the fact that the 26-²H₃-labeled 1,25-dihydroxy vitamin D₃ was not entirely pure (cf. Methods).

Figure 3C shows typical recordings of a purified extract from serum, to which [26-²H₃]-1,25-dihydroxy vitamin D₃ (about 10 ng) was added. No interfering peaks appeared in the chromatogram. When we used the ions at m/e 131 and 134, similar chromatograms were obtained (Figure 5). Sometimes interfering peaks were seen when using the ions at m/e 131 and 134, and the baseline was often difficult to define in the tracing at m/e 131, but in most cases similar or slightly higher values.
were obtained when the ions at m/e 131 and 134 were used. Also the analytical variation was greater with these ions than with the ions at m/e 452 and 455 (about twofold higher coefficient of variation).

Under the conditions finally adopted, the coefficient of variation, as calculated from five replicates of the same serum, was 6%.

The accuracy of the technique was tested by assay of 1,25-dihydroxy vitamin D3 in sera to which a known amount of unlabeled 1,25-dihydroxy vitamin D3 had been added. As seen in Table 1, there was a good agreement between expected and found value.

The concentration of 1,25-dihydroxy vitamin D3 in sera from 15 healthy subjects was 55 ± 10 ng/L (mean ± SD).

**Discussion**

To our knowledge, the concentration of 1,25-dihydroxy vitamin D3 in serum has not previously been determined with other methods than those based on receptor binding. Thus it is of interest to compare the results obtained by the present method with those obtained previously (Table 2). In theory, nonspecific techniques are likely to give higher values than are more specific techniques. Surprisingly, however, slightly lower values for 1,25-dihydroxy vitamin D3 have been obtained in the previous studies with receptor assays. This may be attributable to the age distribution in the populations studied; the mean age of our population was considerably lower than in at least one of the previous studies (8). Another explanation may be that the present method compensates more efficiently than previous methods for losses of 1,25-

![Graph](Image)

**Fig. 4.** Standard curve for determination of 1,25-dihydroxy vitamin D3 in serum, 0–5 ng/20 mL (corresponding to 0–250 ng/L)

![Graph](Image)

**Fig. 5.** Multiple-ion detector recording of trimethylsilyl derivative of material isolated from a serum to which 26-3H5-labeled 1,25-dihydroxy vitamin D3 had been added, with use of the ions at m/e 131 and 134

<table>
<thead>
<tr>
<th>Sample</th>
<th>1,25-Dihydroxy vitamin D3 ng/L</th>
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<tbody>
<tr>
<td>Serum</td>
<td>48.5 ± 1.3</td>
</tr>
<tr>
<td>Serum + 50 pg 1,25-dihydroxy vitamin D3/mL</td>
<td>99.8 ± 0.3</td>
</tr>
<tr>
<td>Serum + 100 pg 1,25-dihydroxy vitamin D3/mL</td>
<td>146.8 ± 1.8</td>
</tr>
</tbody>
</table>

*Mean ± SEM.*
dihydroxy vitamin D₃ owing to degradation or decomposition. Another reason for differences in results obtained in different laboratories may be differences in purity of the unlabeled 1,25-dihydroxy vitamin D₃ used as standard. This compound is unstable, especially in high dilution, so there is always some uncertainty with respect to concentration of a specific standard solution.

It may be pointed out that the present method is completely specific for the D₃-form of the hormone. In most of the previous assays (6, 8, 9), both 1,25-dihydroxy vitamin D₃ and 1,25-dihydroxy vitamin D₂ are determined simultaneously. It has been shown, however, that normally more than 90% of the circulating form of the hormone is 1,25-dihydroxy vitamin D₃ (7).

A serious drawback of the present method is the great amount of serum needed, 20 mL. Under optimal conditions, with the instrumentation used in the present work, it should be possible to reduce the amount of serum to about 5 mL. By use of capillary gas chromatography–mass spectrometry, the volume of serum may be reduced even more. Attempts further to improve the method with respect to volume of serum are in progress.

The skillful technical assistance of Trond Bodvar and Catharina Nyström is gratefully acknowledged. This work was supported by the Swedish Medical Research Council (Project 03X-3141) and by the Norwegian Research Council for Science and the Humanities. We are grateful to Dr. Uskovskoe (Hoffmann-La Roche, Nutley, NJ) for a generous gift of unlabeled 1,25-dihydroxy vitamin D₃.

References