Determination of 25-Hydroxyvitamin D in Serum by HPLC and Immunoassay, Ursula Turpeinen, Ulla Höenthal, and Ulf-Håkan Stenman (Helsinki University Central Hospital, Laboratory, Haartmaninkatu 2, 00290 Helsinki, Finland; * author for correspondence: fax 358-9-4717-4945, e-mail ursula.turpeinen@hus.fi)

Vitamin D status is usually assessed by measuring the serum concentration of 25-hydroxyvitamin D [25(OH)D]. Its measurement is important as a clinical indicator of nutritional vitamin D deficiency, which is one of the causes of osteoporosis (1). Vitamin D exists in two forms: cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2). Vitamin D2 is further metabolized to 25(OH)D2. Vitamin D3 is formed in the skin from its precursor 7-dehydrocholesterol after ultraviolet irradiation or is absorbed from the diet (2). It is further hydroxylated in the liver to 25(OH)D3, as the first step of its conversion in the kidney to 1,25-dihydroxyvitamin D3, which is the biologically active form. 25(OH)D3 is the main circulating form of vitamin D. Clinically it is important to measure both forms of 25-hydroxyvitamin D to monitor the effect of vitamin D3 supplementation on total vitamin D status.

The first routine methods for measurement of 25(OH)D concentrations in human plasma were based on competitive protein binding and using vitamin D-binding protein and a tritium-labeled tracer (3). These methods were replaced by a simpler, rapid RIA (4), and a radioiodinated tracer was incorporated into the RIA in 1993 (5). This assay principle is the basis of several commercially available methods. Quantitative HPLC assays have been developed based on ultraviolet detection and normal-phase separation (6), combined use of normal- and reversed-phase separations (7), or reversed-phase separation alone (8). Recently, reversed-phase HPLC methods for 25(OH)D3 in human plasma have been developed with normal-phase preparivification of the sample (9) or liquid extraction only (10).

Earlier HPLC methods for 25(OH)D3 in serum were designed mainly for research purposes and were therefore too complicated for routine use. The present method was designed to be easy to use, sensitive, and rapid with simple sample preparation. Separation and quantification of 25(OH)D3 from 25(OH)D2 are achieved with an isocratic elution.

To 0.5 mL of serum, we added 350 μL of methanol–2-propanol (80:20 by volume). The tubes were mixed in a Multitube vortex mixer for 30 s. 25(OH)D was extracted by mixing three times (60 s each time) with 2 mL of hexane. The phases were separated by centrifugation, and the upper organic phase was transferred to a conical tube and dried under nitrogen. The residue was dissolved in 100 μL of mobile phase. Calibration curves were constructed using four concentrations of 25(OH)D3 (15–120 nmol/L; cat. no. H-4014; Sigma Chemical Co.) and human serum albumin (50 g/L; The Finnish Red Cross).

For chromatography we used an Agilent series 1100 HPLC system with a quaternary pump. Separation was performed on a LiChrospher 60 RP select B column (4 × 250 mm; 5 μm bead size; Merck) maintained at 40 °C. The mobile phase was 760 mL/L methanol in water, and the flow rate was 1 mL/min. Detection was at 265 nm, and the injected volume was 50 μL. The chromatographic separations obtained with calibrators and human sera are shown in Fig. 1. A patient sample containing 25(OH)D2 is shown in Fig. 1C. The 25(OH)D3 and 25(OH)D2 peaks are completely resolved with retention times of 20.8–21.1 min and 23.1 min, respectively. The prominent peak at 18.1–18.4 min is retinol. Our HPLC assay is based on that of Aksnes (8), but we used isocratic rather than gradient elution to separate 25(OH)D3 from 25(OH)D2 and retinol. The percentage of methanol in the mobile phase is critical for separation of these analytes. Extraction of the serum samples with hexane before HPLC analysis was simple and fast (30 min), and it gave high and reproducible recoveries of 25(OH)D3. Total recoveries of 15, 30, 60 and 120 nmol/L 25(OH)D3 added to five different sera were 85–105%. The peak areas of the endogenous analyte were subtracted from the supplemented sera before comparison. 25(OH)D2 and 25(OH)D3 (Fig. 1C) can be separately quantified, and there were no interfering peaks, although in some samples an extra peak appeared between that of retinol and 25(OH)D3. To clearly separate all of the peaks with the mobile phase used, a column 250 mm in length was necessary. With a 150-mm column, the peaks partially overlapped. The sensitivity of the ultraviolet detector is also critical, e.g., the HP 1100 diode array and variable wavelength detectors (Agilent Technologies) provided reliable results, but the HP 1090 did not. Samples containing up to 5 g/L hemoglobin or 100 μmol/L bilirubin did not interfere with the quantification of 25(OH)D3.

Our assay was linear at 15–200 nmol/L. The mean slope, intercept, and correlation coefficient (r) for the calibration curve were 0.222 (95% confidence interval, 0.212–0.231), 2.2 nmol/L (0.41–4.0 nmol/L), and 0.9993, respectively. The lower limit of detection, defined as the lowest concentration with a minimum signal-to-noise ratio of at least 3:1, was 3 nmol/L. The limit of quantification, defined as the lowest concentration with a signal-to-noise ratio of 10:1, was 10 nmol/L. A serum concentration of <30–37 nmol/L is considered indicative of vitamin D deficiency (11, 12). Our lower limit of quantification, 10 nmol/L, is sufficient to detect subnormal serum concentrations. If 1 mL of serum is used, the lower limit of quantification can be lowered to 5 nmol/L. These limits of quantification are comparable to those reported earlier for other HPLC methods (8, 10).

The within-assay and total CVs calculated from 10–15 replicates of samples containing 21.6–167 nmol/L are shown in Table 1. The within-assay CVs of the RIA were 3–10%, and the total CVs were 4–17%, calculated from the two controls of the assay reagent set.

The 25-hydroxyvitamin D RIAs were from DiaSorin. The assays were performed according to the manufacturer's instructions. 25(OH)D was also measured on the Liaison analyzer (Byk-Sangtec) which uses a competitive chemiluminescence (LIA) format with one incubation (DiaSorin). The antibody used is the same as in the RIA.
The regression lines between each pair of assay methods were calculated by the Deming method (13, 14). This method allows for variation in both the x and y axes at the same time. The correlation by the Deming method was: 

\[ \text{RIA} = 1.02(\text{HPLC}) - 1.02 \text{ nmol/L} \quad (r = 0.829; n = 301). \]

The overall correlation was fairly acceptable, and the slope was close to unity; several samples, however, displayed very large differences. This is in agreement with a previous study in which the correlation was 0.86 with only 25 samples (15). The correlation between the HPLC (x) and the LIA method (y) was: 

\[ \text{LIA} = 1.05(\text{HPLC}) - 4.84 \text{ nmol/L} \quad (r = 0.735; n = 203), \]

and that between Liaison and RIA was: 

\[ \text{Liaison} = 1.03(\text{RIA}) - 3.78 \text{ nmol/L} \quad (r = 0.595; n = 203). \]

The correlation between HPLC and the Liaison

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**Fig. 1.** Chromatograms of a calibrator in human albumin containing 60 nmol/L 25(OH)D3 (A), a patient sample containing 72 nmol/L 25(OH)D3 (B), and a patient sample containing both 25(OH)D3 and 25(OH)D2 (C).
assay was poor, and that between RIA and LIA still worse. The poor correlation between RIA and LIA is surprising because both assays use the same antibody. This may be explained by the use of a separate precipitation/extraction step before the immunoassay in the RIA.

We estimated the costs (in US dollars) of the tests based on 1000, 2000, 3000, and 4000 samples/year. The instrument costs for the HPLC method were calculated on the basis of a leasing fee of 30% of the price of the automated HPLC instrument (US $38 000). The cost for the HPLC column was US $360. The cost for the DiaSorin RIA reagents (US $383) was calculated on the basis of optimal usage, i.e., with 100 tubes, 6 calibrators, 42 samples, and 2 controls analyzed in duplicate. Labor costs were estimated according to our local expenses. In our setting, the costs of the HPLC method (US $10 per sample) were clearly lower than that of the RIA (US $16–18 per sample), irrespective of the number of samples analyzed per year. We did not calculate the costs for the automated method because they would have been competitive only if the analyzer was used mainly for other purposes.

The finding (1) of lowered hip fracture incidence and higher circulating concentrations of 25(OH)D has greatly increased the use of vitamin D assays. The use of vitamin D supplementation makes reliable measurement of 25(OH)D important. Results of an international comparison of serum 25-hydroxyvitamin D measurements, however, show that 25(OH)D values from different laboratories are often not comparable, with interlaboratory differences being up to 38% (16). More accurate assays are therefore needed.

To determine nutritional vitamin D status, it is important that the method used measures circulating 25(OH)D and 25(OH)D$_2$ equally to provide total circulating 25(OH)D. The primary antibody in the DiaSorin RIA is claimed to recognize both forms of vitamin D equally, although the calibration curves are constructed with 25(OH)D$_3$ (15). That makes it possible to monitor the effect of supplemented vitamin D$_2$ on total vitamin D status. Of the many protein-binding methods developed for the determination of vitamin D status during the past 30 years, 125I-based RIAs have become most widely used methods for determining circulating 25(OH)D. HPLC methods (6, 7) that use ultraviolet detection are much less common, apparently because of their laborious prepurification steps and the use of normal-phase chromatography. Recently, relatively simple and sensitive methods based on reversed-phase chromatography have been developed (8–10).

In Finland, vitamin D deficiency is quite common during winter time, and recognition of low values is important (12). On the basis of the results obtained in the present study, we have decided to switch to the HPLC method. Ideally, a mass spectrometric method should be used to validate the HPLC method, but in earlier studies a similar HPLC method was shown to correlate strongly with isotope-dilution mass spectrometry (17). The good recovery and precision also indicate that the HPLC assay provides more correct results than the immunologic methods. In our setting, the lower cost also favors use of the HPLC method. The automated method is potentially more economical if the analyzer is used mainly for other assays, but further development of this assay is necessary to make it a viable alternative. We could not explain the reasons for the poor correlation with the immunoassays, but based on results from earlier studies, immunoassays are affected by nonspecific interference (17).

In conclusion, our HPLC method with ultraviolet detection enables reliable quantification of 25(OH)D$_2$ and, if present, 25(OH)D$_3$. The short and relatively simple sample preparation and ease of use make it useful for routine determinations.

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**Table 1. Within-run and total precision for 25(OH)D$_3$ in serum.**

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<thead>
<tr>
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<th>Within-run</th>
<th>Total</th>
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<tbody>
<tr>
<td>Mean, nmol/L</td>
<td>21.6</td>
<td>63.8</td>
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<tr>
<td>SD, nmol/L</td>
<td>5.6</td>
<td>3.7</td>
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<tr>
<td>CV, %</td>
<td>26.4</td>
<td>58.6</td>
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<td>n</td>
<td>14</td>
<td>12</td>
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### References

Antibody Phenotyping Test for the Human Apolipoprotein E2 Isoform, Robert L. Raffai,1* Ruth McPherson,1 Karl H. Weisgraber,2 Thomas L. Innerarity,2 Eric Rassart,3 Thomas P. Bersot,2 and Ross W. Milne1 (1 Lipoprotein and Atherosclerosis Group, University of Ottawa Heart Institute, Ottawa Civic Hospital, Ottawa, Ontario, K1Y 4E9 Canada; 2 The Gladstone Institute of Cardiovascular Disease, Cardiovascular Research Institute, University of California, San Francisco, CA 94141-9100; 3 Département des Sciences Biologiques, Université du Québec, Montréal, CP 8888, Québec, H3C 3P8 Canada; * author for correspondence: fax 415-285-5632, e-mail rraffai@gladstone.ucsf.edu)

Numerous methods have been described to determine the apolipoprotein E (apoE) phenotype or genotype of individuals attending the University of Ottawa Heart Institute Lipid Clinic and the San Francisco General Hospital Lipid Clinic. In all cases, the apoE genotype of all individuals was determined by standard PCR amplification followed by digestion with HhaI (data not shown) (2). Initially, the antibody test was developed as a sandwich RIA. In all cases, the concentration of total apoE in donor plasma was determined by a sandwich RIA in which immobilized mAb 6C5 was the capture antibody and 125I-labeled 3H1 was the detection antibody.

For the determination of apoE in plasma, the assay was performed by coating Immunolon II Removawells (Dynatech) with mAb 6C5 overnight at a concentration of 2 mg/L in phosphate-buffered saline (PBS), pH 7.5. Once coated, the wells can be stored for at least 1 week at 4 °C. Before use, the wells were washed with PBS containing 0.25 mL/L Tween 20 (PBS-Tween) and blocked for 1 h with PBS containing 10 g/L bovine serum albumin (PBS-BSA). The wells were then filled and serially diluted with 100 μL of sample plasma previously diluted 1:20 in PBS containing 10 g/L BSA and 0.1 mL/L Tween 20 (PBS-BSA-Tween). After incubation for 1 h at room temperature, the wells were emptied and washed three times with PBS-Tween. The wells were then filled with 100 μL of 125I-labeled 3H1, which corresponded to 100 000 cpm, diluted in PBS-BSA-Tween and were incubated for 1 h at room temperature. The wells were then emptied, washed three times as before, and counted in a gamma counter. The bound radiolabeled antibody counts were plotted as a function of the plasma dilution to quantify plasma apoE concentrations (Fig. 1B). Determination of the E2 phenotypic isoform in plasma was performed in parallel, using the same format but with 125I-labeled 2E8 as the identification antibody (Fig. 1C). Ten confirmed unrelated E2 homozygotes and 2 unrelated E2/E3 heterozygotes were unambiguously ascribed the correct phenotype, and 8 individuals were correctly identified as not having inherited an APOE2 allele.

The three antibodies recognized lipid-free and lipid-associated apoE with the same affinity and isoform specificity (5), and the test worked well with both fresh plasma and plasma frozen at −20 °C. The test has been adapted to an ELISA format using 3H1-HRP and 2E8-HRP conjugates as detection antibodies for quantification and isoform identification, respectively (Fig. 1E; conjugation performed by Bethyl Inc.). The basic experimental method of the ELISA format is identical to the RIA format described above. However, the bound conjugated antibodies were detected by incubating the washed wells with 100 μL of hydrogen peroxide and o-phenylenediamine (Sigma), and the color was allowed to develop for 3–5 min before the reaction was quenched with 100 μL of 2.5 mol/L sulfuric acid. The absorbance of the reaction mixture at 490 nm was determined in a Spectra MAX 250 ELISA reader (Molecular Devices).

Using the ELISA format, we have correctly identified APOE2 inheritance for the 10 apoE2 homozygotes, 10 E2/E3 heterozygotes, and 2 E4/E2 heterozygotes who were tested. All E3/E3, E4/E3, and E4/E4 individuals who were tested were also correctly categorized as having not inherited an APOE2 allele. With the ELISA format, plasma from individuals known to lack an APOE2 allele...