Comparison of Commercially Available $^{125}$I-based RIA Methods for the Determination of Circulating 25-Hydroxyvitamin D

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**Background:** Measurement of circulating 25-hydroxyvitamin D [25(OH)D] is important in the management of metabolic bone disease. The aim of this study was to compare two widely used methods for the quantification of circulating 25(OH)D with attention to their abilities to measure 25-hydroxylated ergocalciferol (vitamin D$_2$) [25(OH)D$_2$] and cholecalciferol (vitamin D$_3$) [25(OH)D$_3$].

**Methods:** We used two commercially available, Food and Drug Administration-approved, radioiodine ($^{125}$I)-based RIA kits for the detection of 25(OH)D (DiaSorin, Stillwater, MN and IDS Ltd, Tyne and Wear, United Kingdom). These methods were tested for general assay performance, including antibody specificity. Results were compared with those of an HPLC-based direct ultraviolet detection method.

**Results:** Within- and between-run CVs were $\leq 10\%$. Both methods quantitatively recovered 25(OH)D$_3$ added to serum, but only the DiaSorin kit quantitatively recovered 25(OH)D$_2$. The primary antibody in the IDS kit had unequal reactivities with pure 25(OH)D$_2$ and 25(OH)D$_3$, whereas the DiaSorin primary antibody reacted with them equally. In 50 patient samples assayed by HPLC, the IDS method, but not the DiaSorin method, underestimated total circulating 25(OH)D when significant circulating 25(OH)D$_2$ was present in patient samples.

**Conclusions:** Some immunoassays may underestimate total 25(OH)D when 25(OH)D$_2$ constitutes an appreciable part of the total.

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25(OH)D (8). This assay has become the method of choice for assessing 25(OH)D status and was the first test for vitamin D approved for clinical diagnosis by the Food and Drug Administration (FDA) and available through DiaSorin Corporation (Stillwater, MN). Recently, another manufacturer (IDS Ltd, Tyne and Wear, United Kingdom) received FDA approval for a similar device. This report compares the performance of these two methods.

Materials and Methods

Reagents

Pure 25(OH)D$_3$ and 25(OH)D$_2$ were obtained from Hoffmann-La Roche. Reagents for the $^{125}$I-based RIAs were obtained from DiaSorin and IDS Ltd. HPLC-grade hexane, isopropanol, methylene chloride, and acetonitrile were from Fisher. Unless otherwise noted, all other reagents were reagent grade.

Clinical samples

Serum or plasma samples from 50 apparently healthy subjects from previous studies in our laboratory were used for this comparative study. These subjects ranged in age from 4 months to 70 years. Some subjects received daily supplements of vitamin D$_2$ or vitamin D$_3$ (400–1200 IU/day) for various periods of time. Other subjects received no known supplemental vitamin D.

Methods

Spectroscopy. Concentrations of 25(OH)D$_2$ and 25(OH)D$_3$ were determined by UV spectroscopy with molar absorptivity ($\varepsilon_{264}$) values of 19 400 and 18 300 mol$^{-1}$ cm$^{-1}$, respectively.

RIA. 25(OH)D was measured as directed by the manufacturers’ product inserts. To perform the reactivity studies, pure 25(OH)D$_2$ and 25(OH)D$_3$ were dissolved in acetonitrile to concentrations of 0, 2, 4, 10, 20, 40, 100, 200, and 400 pg in 40  $\mu$L for the IDS RIA and the same concentrations in 23 $\mu$L for the DiaSorin RIA. The respective RIAs were performed by placing these volumes of each concentration in 12 $\times$ 75 mm borosilicate culture tubes. From this point, the methods were performed as directed by the manufacturers’ product inserts. Each concentration was measured in triplicate. Radioactivity was determined with a gamma well-counting system.

Direct UV detection of 25(OH)D$_2$ and 25(OH)D$_3$ in plasma or serum after HPLC. 25(OH)D$_2$ and 25(OH)D$_3$ were determined in samples by direct quantification by UV absorbance after HPLC purification as described previously (9).

Analytical recovery. Twenty-five microliters of acetonitrile containing 0, 50, 100, or 200 ng of pure 25(OH)D$_2$ or 25(OH)D$_3$ was added to a 5-mL pooled serum sample from five individuals. This provided addition concentrations of 0, 10, 20, and 40 $\mu$g/L for each metabolite. The samples, which were in 16 $\times$ 100 mm borosilicate glass culture tubes, were vortex-mixed and incubated for 30 min at room temperature to equilibrate. The samples were then assayed as directed by the manufacturers’ product inserts.

Results

Specificity of the RIAs

The reactivities of 25(OH)D$_2$ and 25(OH)D$_3$ with the primary antisera from the DiaSorin and IDS RIAs are shown in Fig. 1. The antibody from the DiaSorin method reacted equally with 25(OH)D$_2$ and 25(OH)D$_3$. The detection limits of the DiaSorin method were 0.30 and 0.31 pg/tube for 25(OH)D$_2$ and 25(OH)D$_3$, respectively. The median effective doses (ED$_{50}$s) in this assay were 14.6 and 15.5 pg/tube for 25(OH)D$_2$ and 25(OH)D$_3$, respectively. Conversely, the antibody from the IDS method did not react equally with 25(OH)D$_2$ and 25(OH)D$_3$. The detection limits for the IDS method were 5.0 and 1.0 pg/tube for 25(OH)D$_2$ and 25(OH)D$_3$, respectively. The ED$_{50}$s also were markedly different: 23.6 pg/tube for 25(OH)D$_3$ and 50.3 pg/tube for 25(OH)D$_2$.

Detection limit, analytical recovery, and precision

The detection limit, defined as 3 SD from the mean for the zero calibrator, was 1 $\mu$g/L for both the DiaSorin and the IDS RIA. The ED$_{50}$s for 25(OH)D$_3$ were 24.0 and 15.3 $\mu$g/L for the DiaSorin and IDS methods, respectively.

The analytical recovery data for both methods, using human serum, are shown in Table 1. Recovery for the DiaSorin method was 91–100% for both 25(OH)D$_2$ and 25(OH)D$_3$. In comparison, the IDS method recovered...
92–95% of 25(OH)D₃ added to serum samples. However, recovery of 25(OH)D₂ was only 21–29% and was uniform at all concentrations of 25(OH)D₂ tested (Table 1).

Imprecision (CV), both within and between assays, was determined with serum samples at various points on the calibration curves. The within- and between-assay CVs were 2.2–8.6% for the DiaSorin method and 2.1–10% for the IDS method. These values are similar to those reported in the respective product inserts.

Separate quantification of 25(OH)D₂ and 25(OH)D₃ by direct UV detection after HPLC allowed the human serum samples to be divided into two groups. The first group had no detectable circulating 25(OH)D₂ (<2 μg/L), but all had detectable 25(OH)D₃. This group will be referred to as having “minimal circulating 25(OH)D₂”. In the second group, both 25(OH)D₂ and 25(OH)D₃ were detectable, 18.3 ± 5.3 and 9.0 ± 5.2 (mean ± SD) μg/L, respectively. This group will be referred to as having “significant circulating 25(OH)D₂”. For the patient population with minimal circulating 25(OH)D₂, linear regression analysis comparing the two RIAs displayed excellent results (Fig. 2A). The mean circulating 25(OH)D concentrations for this group were 20.3 ± 10.5, 20.3 ± 8.2, and 20.3 ± 9.8 μg/L as measured by the DiaSorin and IDS methods, respectively. In the group with significant circulating 25(OH)D₂, the relationship between the two RIA methods was poor (Fig. 2B). The mean circulating 25(OH)D concentrations in this group were 26.3 ± 4.8 and 18.6 ± 4.0 μg/L as measured by the DiaSorin and IDS methods, respectively.

The relationships between direct UV quantification of 25(OH)D and the two RIA methods are displayed on Figs. 3 and 4. The methods, as compared by linear regression analysis, were in good agreement for subjects with minimal circulating 25(OH)D₂ (Fig. 3). The mean concentrations of circulating 25(OH)D in this comparison were 20.3 ± 10.5, 20.3 ± 8.2, and 20.3 ± 9.8 μg/L for HPLC, IDS, and DiaSorin, respectively. In subjects with significant circulating 25(OH)D₂, the DiaSorin RIA and HPLC method exhibited excellent agreement (Fig. 4A), whereas the IDS RIA and HPLC did not (Fig. 4B). The mean circulating 25(OH)D concentrations, as determined by each method, in this comparison were 27.3 ± 4.4, 26.2 ± 4.8, and 18.6 ± 4.0 μg/L for HPLC, DiaSorin, and IDS, respectively.

**Discussion**

During the past 30 years, many methods have been developed for determining vitamin D status in humans (5–9). These techniques have involved competitive protein-binding assays (5), direct UV detection after HPLC (6, 9), and RIA (7, 8). The ¹²⁵I-based RIA has emerged as the method of choice for the determination of circulating 25(OH)D. The clinical use and utility of this marker for calcium homeostasis greatly increased in 1992 after a report that demonstrated a relationship between circulating concentrations of 25(OH)D and hip fracture incidence.
Today, the determination of circulating 25(OH)D is a common clinical event. To that end, there currently are two FDA-approved devices in use clinically for the detection of circulating 25(OH)D in the United States. These methods were both evaluated in this study.

In the determination of nutritional vitamin D status, it is imperative that the method of choice measure circulating 25(OH)D2 and 25(OH)D3 equally to provide total circulating 25(OH)D. Both of the methods evaluated in this study claim to measure total 25(OH)D. The reactivities of both RIAs were first investigated by the use of pure 25(OH)D2 and 25(OH)D3 to generate competition curves with the primary antibody from each RIA. These data are displayed in Fig. 1, which demonstrates that the primary antibody in the DiaSorin RIA recognizes both 25(OH)D2 and 25(OH)D3 equally, whereas the antibody used in the IDS method does not. Data from the calibration curves suggest that the IDS method will underestimate total 25(OH)D when significant 25(OH)D2 is present in the circulation. Underestimation of circulating 25(OH)D by the IDS RIA was confirmed when the analytical recoveries of 25(OH)D2 and 25(OH)D3 were assessed. Table 1 shows that both the IDS and DiaSorin methods quantitatively recovered 25(OH)D3 from human serum, whereas only the DiaSorin method quantitatively recovered 25(OH)D2. In fact, the recovery of 25(OH)D2 from human samples was poor at all concentrations tested when the IDS method was used (Table 1). This is because the calibration curves for the IDS method, like the DiaSorin method, are constructed using 25(OH)D3. The IDS method does appear to be valid when 25(OH)D3 is the only circulating 25(OH)D species. Figs. 2, 3, and 4 show some disparity among the DiaSorin, IDS, and HPLC methods for samples containing primarily 25(OH)D3. These differences are most likely attributable to the different calibrators used in the DiaSorin and IDS methods and exemplify the point that every laboratory should establish its own range of values for whatever method is chosen.

The in vitro data suggest that the DiaSorin method will accurately estimate total 25(OH)D in the presence of significant circulating 25(OH)D2, whereas the IDS method will underestimate total 25(OH)D under the same conditions. Figs. 2, 3, and 4 exemplify this point. When human samples containing minimal circulating 25(OH)D2 were
analyzed by the IDS and DiaSorin methods, the results were in good agreement (Fig. 2A). However, in samples containing significant circulating 25(OH)D₂, the IDS method underestimated total circulating 25(OH)D by an average of 30% (Fig. 2B). These data are confirmed in Figs. 3 and 4, which show that the DiaSorin method agreed with UV-HPLC analysis for samples with significant or minimal circulating 25(OH)D₂, whereas the IDS method underestimated 25(OH)D, on average, by ≥30% in samples containing significant circulating 25(OH)D₂ (Fig. 4B).

Vitamins D₂ and D₃ are both widely utilized in the food supply and are interchangeably supplemented in the milk supply in the United States (10–12). Furthermore, vitamin D₂ is widely used in pharmaceutical preparations worldwide, including the United States, Europe, and Japan. Hence, it is very important to select an analytical method that will accurately estimate total circulating 25(OH)D independent of the circulating concentrations of 25(OH)D₂ and 25(OH)D₃. From the data generated in this study, it is clear that the DiaSorin and HPLC-based methods fill this purpose, whereas the IDS method does not.

Dr. Hollis is a paid consultant of DiaSorin Corporation.

References