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Abstract

The serum assay for 25-Hydroxyvitamin D [25(OH)D] is used to determine the vitamin D status of patients. The serum 25(OH)D assay measures both 25(OH)D$_2$ and 25(OH)D$_3$. The automated Nichols Advantage® 25(OH)D assay is based on chemiluminescence detection and vitamin D binding protein (DBP) for competitive displacement. To determine how efficient and accurate the Nichols Advantage chemiluminescence assay was for detecting both 25(OH)D$_2$ and 25(OH)D$_3$, as compared to other methods, serum samples were obtained from healthy adults and from patients who were treated for several months with pharmacologic doses of vitamin D$_3$. The serum was divided into aliquots and numbered. Frozen samples were provided in a blinded fashion for analysis by four methods: (1) high performance liquid chromatography (HPLC), (2) manual RIA (3) D-binding protein isotopic assay and (4) the Nichols Advantage System. The results were compared to that from liquid chromatography-mass spectrometry (LC-MS/MS). These samples were tested at independent laboratories. A comparison of all the assays suggests that there are differences among the methods. The Nichols Advantage assay was able to detect and quantify 25(OH)D levels in samples containing 25(OH)D$_2$ and 25(OH)D$_3$.

Methods for 25(OH)D

- **LC-MS/MS**
  Sample preparation: For each sample, three 90 microliter aliquots were spiked with 20 ng of internal standard [²H$_2$-25(OH)D$_3$], diluted to incubate at 37°C for 30 mins and then run through a C-18 cartridge chromatography (Hollis BW, Clin Chem 32, 2060-2063, 1986), and finally, normal phase chromatography. Analysis Detection is by TSQ Quantum Ultra LC-MS/MS system. The results were compared to that from liquid chromatography-mass spectrometry (LC-MS/MS). These samples were tested at independent laboratories. A comparison of all the assays suggests that there are differences among the methods. The Nichols Advantage assay was able to detect and quantify 25(OH)D levels in samples containing 25(OH)D$_2$ and 25(OH)D$_3$.

- **HPLC**
  Detection of 25(OH)D$_2$ and 25(OH)D$_3$. First, plasma or serum sample was spiked with 800 cpm [³H$_2$-25(OH)D$_3$] diluted to 2 volumes of acetonitrile, followed by C-18 cartridge chromatography (Hollis BW, Clin Chem 32, 2060-2063, 1986), and finally, normal phase chromatography to separate 25(OH)D$_2$ and 25(OH)D$_3$.

- **Manual Radioimmunoassay (RIA)**
  Automated antibody based assay using radio-label and performed by manual extraction procedure (Diasorin).

- **Nichols Advantage® Automated Chemiluminescence Assay**
  The assay uses a human DBP-containing reagent, labeled with the DBP-detection (25(OH)D$_2$ and 25(OH)D$_3$) labeled with acridinium ester and 25(OH)D$_2$ binding chain bound to magnetic particles. The 25(OH)D$_2$ is released from the binding protein by a releasing agent and the competitive assay is completed automatically by the Nichols Advantage System.

Results vs. LC-MS/MS in samples predominantly with 25(OH)D$_2$.

**Manual D-Binding Protein Competitive Isotopic Assay**

Protein Binding assay was performed according to the method described previously (Chen TC, Turner AK, Holick MF. J Nutr Biochem, 1:315-319, 1990). Serum or plasma was first extracted with 100% ethanol, followed by a protein-binding assay.

**Nichols Advantage® Automated Chemiluminescence Assay**

The assay is based on human DBP binding protein, anti-human DBP-labeled with acridinium ester and 25(OH)D$_2$ binding chain bound to magnetic particles. The 25(OH)D$_2$ is released from the binding protein by a releasing agent and the competitive assay is completed automatically by the Nichols Advantage System.

* Results for the different methods were obtained from several laboratories.

Conclusions

1. HPLC and the manual D-binding protein methods compared favorably with LC-MS/MS.
2. The RIA method shows over estimation.
3. Based on LC-MS/MS results, Nichols Advantage® assay can detect and quantify 25(OH)D$_2$. Some samples show under estimation.
4. Nichols Advantage® method can determine the increase in serum 25(OH)D levels in vitamin D deficient patients who were treated with pharmacologic doses of vitamin D$_3$. 

**Response after treatment with Vitamin D$_2$ (50,000 IU) in vitamin D deficient patients**

- **Nichols Advantage®**
  After treatment with pharmacologic doses of vitamin D$_2$, serum obtained from blood samples were made into several aliquots and were frozen for shipment and testing by different methods.