SU-585

Evaluation of Precision and Accuracy of Nichols Advantage® 25-Hydroxyvitamin D Assay for 25(OH)D_2 and 25(OH)D_3: Comparison of Four Methods with LC-Mass Spectrometry.*

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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_2. 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.

Introduction

25-Hydroxyvitamin D measurement is useful in determining the status of vitamin D levels. Deficiency of vitamin D directly affects bone health and a variety of other cellular functions. Vitamin D deficiency is treated either with the natural vitamin D_3 or the synthetic vitamin D_2. Both are converted to the active 1,25-Dihydroxyvitamin D via first to 25(OH)D. Successful treatment with vitamin D_2 or vitamin D_3 can be determined by the increase in the levels of 25(OH)D. Therefore methods used for determining 25(OH)D need to detect both 25(OH)D_2 and 25(OH)D_3.

LC-MS/MS system

Objective

Compare the 25(OH)D results of four different methods with that of LC-MS/MS on patient samples before and after treatment with pharmacologic doses of Vitamin D_2.

Methods for 25(OH)D

- **LC-MS/MS**

  Sample preparation: For each sample, three 50 microliter aliquots were spiked with 20 ng of internal standard [^{2}H]$_2$-25(OH)D_3, allowed to incubate at 37°C for 30 mins and then run through a Cohesive Technologies turbulent flow system followed by laminar flow chromatography. *Analysis:* Detection is by TSQ Quantum Ultra mass spectrometer (Thermo Finnigan Corp.). MS is focused on the transition for 25(OH)D_3 and 25(OH)D_2. The efficiency of assay for each analyte was corrected by use of an internal standard, [^{2}H]$_2$-25(OH)D_3. Concentration and calibration curves were generated by LC-Quan (Thermo Electron Corp.). *Calibration:* Calibration curve was generated by analyzing the eight calibration standards in five replicates. *Internal Standard:* [^{2}H]$_2$-25(OH)D_3 was synthesized in house from pure 25(OH)D_3 (Calbiochem) with purity >92%.

- **HPLC**

  Determination of 25(OH)D_2 and 25(OH)D_3. First, plasma or serum spiked with 8000 cpm [^{3}H]-25(OH)D3 was extracted with 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)**

  A commercially available antibody based assay using radio-label and performed by manual extraction procedure (Diasorin).

- **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 D-binding protein, anti-human DBP labeled with acridinium ester and 25(OH)vitamin D3 bound to magnetic particles. The 25(OH)D 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.*

Subjects

Inclusion criteria: Adults 18 or older, able and willing to give informed consent and comply with study protocol.

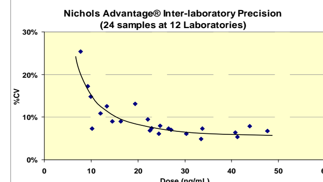
Exclusion criteria: People who are taking medications that can affect vitamin D metabolism.

Treatment: Patients who were vitamin D deficient were treated with 50,000 IU vitaminD_2 once a week for 8 weeks followed by 50,000 IU vitamin D_2 once every 2-4 weeks for a minimum of 8 weeks.

Samples: Serum obtained from blood samples were made into several aliquots and were frozen for shipment and testing by different methods.

Inter-assay precision

Nichols Advantage®



Results vs. LC-MS/MS in samples predominantly with 25(OH)D_2

Manual D-Binding Protein assay

D-Binding Protein Assay (ng/mL)	Total LC-MS/MS (ng/mL)	D-Binding Protein Assay vs. LC-MS/MS
53	48	110%
63	40	158%
35	33	106%
46	49	94%
39	40	98%
18	24	75%
(n=6)	Mean + SD Range	107 + 28% 78 to 158%

Nichols Advantage®

Nichols Advantage (ng/mL)	Total LC-MS/MS (ng/mL)	Nichols Advantage vs. LC-MS/MS
45	48	94%
27	40	68%
43	50	86%
30	58	52%
30	33	91%
29	49	59%
19	40	48%
14	24	58%
20	40	50%
(n=9)	Mean + SD Range	67 + 18% 48 to 94%

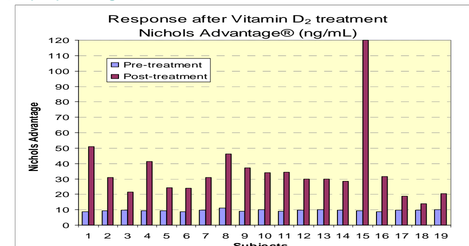
HPLC

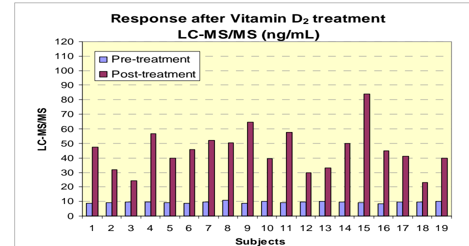
HPLC (ng/mL)	LC-MS/MS 25-OH-D_2	LC-MS/MS 25-OH-D_3	Total LC-MS/MS (ng/mL)	HPLC vs. LC-MS/MS
62	37	11	48	129%
43	34	6	40	108%
47	47	3	50	94%
87	53	5	58	150%
28	24	9	33	85%
47	42	7	49	96%
38	36	4	40	95%
18	20	4	24	75%
37	40	0	40	93%
(n=9)			**Mean + SD** **Range**	103 + 23% 75 to 150%

RIA

RIA (ng/mL)	Total LC-MS/MS (ng/mL)	RIA vs. LC-MS/MS
59	48	123%
48	40	120%
85	50	170%
71	58	122%
37	33	112%
56	49	114%
47	40	118%
29	24	121%
39	40	98%
(n=9)	Mean + SD Range	122 + 20% 98 to 170%

Response after treatment with Vitamin D_2 [50,000 IU] in vitamin D deficient patients [25(OH)D<10ng/ml]





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_2.