An immunoassay for Free 25-Hydroxy vitamin D

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Abstract

For the last three decades, no one knowledgeable in vitro diagnostics would have considered only a total thyroid (T3 or T4) hormone measurement without some understanding the thyroid hormone binding protein status. That could be as simple as a T3 Uptake or T Uptake assay to help interpret the total thyroid measurement in the context of binding protein status. Free T4 measurements, which reflect both total T4 levels and binding protein capacity, have become the recognized standard for determining thyroid status in conjunction with a TSH measurement.

Binding proteins for 25 Hydroxy Vitamin D2 and D3 are altered by some of the same factors that control thyroid hormone binding protein levels. Pregnancy and oral contraceptive use increase both thyroid and 25 Hydroxy Vitamin D binding proteins and the corresponding total analyte concentrations may give misleading results. Liver disease decreases both thyroid and 25 hydroxy vitamin D binding protein levels and total 25 hydroxy vitamin D. Total 25 hydroxy vitamin D levels may underestimate vitamin levels. End stage renal disease and dialysis also represent interesting challenges for measurement of free versus total 25 Hydroxy Vitamin D.

Binding protein levels may play an important role in 25 Hydroxy Vitamin D bioavailability, and current assays destroy that information with sample pretreatment steps before the actual assay begins.

Our novel Free 25 Hydroxy Vitamin D assay captures information on both the total 25 Hydroxy Vitamin D levels and the serum binding protein status in a simple, easily automatable format. Antibodies reactive with 25 hydroxy vitamin D are immobilized on traditional solid phases such as microtiter wells or superparamagnetic particles. Standards, controls and patient samples are exposed to these antibodies in a simple buffer and allowed to react. Free 25 hydroxy vitamin D is captured on the antibody and accumulated during a 90-minute incubation. The solid phase is then washed and a labeled analog of 25 hydroxy vitamin D is allowed to react with the remaining antibody in second incubation. This incubation is followed by another wash and quantitation of the signal, which is inversely proportional to the level of free 25 hydroxy vitamin D in the sample.

Other vitamins such as Vitamin B12 and Folic Acid may also benefit from a measurement that reflects both total vitamin levels and their respective serum binding protein levels.

Materials and Methods

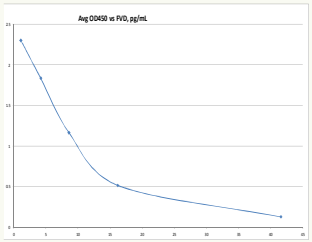
Nunc Maxisorb plates were coated with Goat Anti-mouse antibody by standard methods. 2ng of a commercially available antibody specific for 25 Hydroxy Vitamin D were added to each well. The following assay protocol was used.

Free Vitamin D standards were made in Matribase supplemented by human Vitamin D Binding Protein (affinity purified on an actin support) at 200 ug/mL and spiked with 25 Hydroxy Vitamin D3. The standards were calibrated by symmetric dialysis[1] Total 25 Hydroxy Vitamin D was measured by immunoassay using a Diasorin kit or by LC-MS. Human VDBP was measured with a kit from R&D Systems.

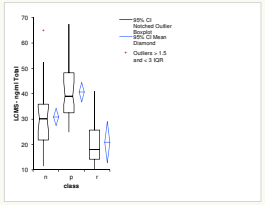
Assay Protocol

Add 90 µl sample diluent to the wells.
Add 10 µl sample to the wells.
Incubate for 90 minutes at 37°C on a shaker.
Wash 3 times with 350 µl wash buffer.
Add 100 µl biotinylated 25 OH VitD analog solution to the wells
Incubate for 30 minutes at 37°C on a shaker.
Wash 3 times with 350 µl wash buffer.
Add 100 µl Streptavidin HRP solution to the wells.
Incubate for 20 minutes at 37°C on a shaker.
Wash 3 times with 350 µl wash buffer.
Add 100 µl TMB solution to the wells.
Incubate at RT and stop the reaction after 20 minutes with 100 µl Stop solution (1 M H2SO4)
Measure the optical density at 450 nm.

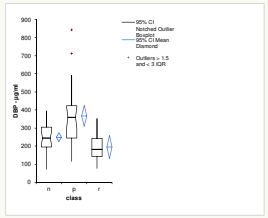
Typical Standard Curve



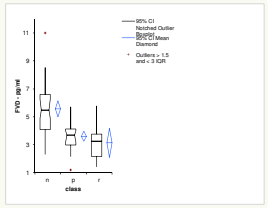
Box and Whisker Plot of Total 25 OH Vit D by LC-MS in Normal, Pregnant and Renal Failure Patients



Box and Whisker Plots of VDBP (ug/mL) in Normal, Pregnant and Renal Failure Patients



Box and Whisker Plots of FVD (pg/mL) in Normal, Pregnant and Renal Failure Patients



Effect of Repeated Immuno-Extractions on FVD Values

First pass	Second pass	Third pass
pg/ml Free	pg/ml Free	pg/ml Free
7.0	7.4	7.9
4.7	5.1	5.8
9.3	10.6	11.1
4.7	4.9	5.3
8.3	7.6	7.4
5.3	6.1	6.6
5.4	6.2	6.7
3.6	3.8	4.4
3.9	4.5	4.9
2.1	3.0	3.1

Results

We have demonstrated a practical, automatable immunoassay for the measurement of Free 25 OH Vitamin D (FVD). This assay helps to normalize FVD levels despite the effects of variations in VDBP that occur in pregnancy and liver disease. Conventional total 25 OH Vitamin D assays by LC-MS or by immunoassay can give misleading results in these physiological states. That is because conventional 25 OH Vitamin D assays begin with a sample pre-treatment step that destroys the VDBP. This new assay works with both the total 25 OH Vitamin D levels and the levels of the VDBP to give a more accurate assessment of the bioactive FVD in the patient sample.

Some of the Free Vitamin D must be sequestered on the antibody in order to measure the parameter. The unique 2 step format of this assay allows us to assess the effect that this immunoextraction has on the FVD value. Repeated immunoextractions of the samples in different antibody coated wells followed by the rest of the assay show little change in the FVD levels even after 2 or 3 passes. In the actual assay, only one immunoextraction would be done.

Discussion

The levels of FVD measured in this assay are in the range of 1.1 pg/mL to 40 pg/mL with a 10 uL serum sample. We add 14 pg of 25 OH Vit D biotinylated analog as a tracer. Obviously this is not a typical immunoassay such as Digoxin or Total T4 where the vast majority of the analyte is captured by the antibody. Binding protein have the function in the body of buffering the concentrations of the molecules they carry so that the level of the free analyte stays as constant as possible every where blood flows. They do this by rapidly releasing the protein bound analyte. The free analyte then can either be rebound or be taken up by the target cell. In a sense the antibody replaces the target cell and integrates the FVD concentration over time during the first or immunoextraction step. The amount of FVD accumulated on the antibody is proportional to the instantaneous concentration of FVD in the reaction. The repeated immunoextraction experiment demonstrates that this immunoextraction has little or no effect on the ability of the system to buffer FVD concentrations

Conclusions

We have shown that practical immunoassay for Free 25 OH Vitamin D is feasible and that it can help normalize distortions in Total 25 OH Vitamin D levels caused by variations in VDBP

References

Ref 1. van Hoof HJ, Swinkels LM, Ross HA, Sweep CG and Benraad TJ, Determination of non-protein-bound plasma 1,25-dihydroxyvitamin D by symmetric (rate) dialysis. Anal Biochem 1998 May 1;258(2):176-83.

Immunoassay for 25 hydroxy vitamin D

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Abstract

We present a method for the measurement of 25(OH)Vitamin D [25(OH)Vit D] in serum or plasma including a novel approach for the release of 25(OH)Vitamin D bound to Vitamin D binding Protein (DBP).

Introduction

About 85% of the circulating 25(OH)VitD is bound to Vitamin D binding Protein (DBP) with a relatively high affinity (1).

The accuracy of 25(OH)Vit D measurement in serum by immunoassay depends on the efficiency of the release of endogenous 25(OH)Vit D from DBP.
There are several approaches to eliminate the effect of DBP in the assay for 25(OH)Vit D. Organic solvents may be used to solubilize and separate 25(OH)Vit D from its binding protein. For this purpose ethanol can be used, but this method requires centrifugation of the denatured protein and therefore is not suitable for automation. Other methods rely on competitive displacement using compounds such as Anilino Sulfonic Acid (ANS), or on displacement of bound 25(OH)Vit D by lowering the pH.

Methods

We propose an alternative method for the release of 25(OH)Vit D using FD Release reagent. A specific, commercially available antibody directed against 25(OH)Vit D was used. The assay was performed on microtiterplates but can easily be adapted to an automated platform.

The assay protocol is depicted below:

Add 90 µL assay diluent to the wells
Add 10 µL of sample or calibrator to the wells.
Incubate for 30 minutes at 37°C on a shaker
Add 50 µL Biotin-D3 solution to the wells.
Incubate for 30 minutes at 37°C on a shaker.
Wash 3 times with 350 µL wash buffer.
Add 100 µL streptavidin HRP solution to the wells.
Incubate for 20 minutes at 37°C on a shaker.
Wash 3 times with 350 µL wash buffer.
Add 100 µL TMB solution to the wells.
Incubate at RT for 20 minutes.
Stop the reaction with 100 µL stop solution.
Measure the plate at 450 nm.

Results

The specific FD release reagent enhances the solubility of 25(OH)Vit D. The efficacy of release from DBP was compared with the efficiency of displacement using a combination of methanol and ANS. The displacement was monitored using ^{3}H-25(OH)Vit D.

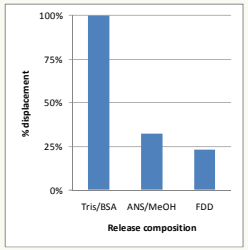
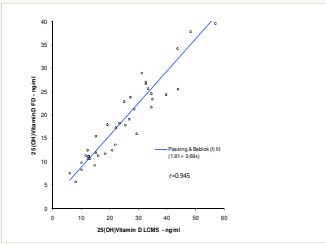


Figure 1. *Percent displacement in serum observed with different release compositions. The displacement was compared with the binding observed in TRIS buffer with 0.05% BSA. A specific surfactant (FDD) effectively releases 25(OH)Vit D from serum proteins.*

A typical standard curve for the assay is depicted in the table below:

Standard curve	St 0	St A	St B	St C	St D	St E
dose (ng/mL)	0	4.1	13.5	24.9	52.1	136
Abs. 450 nm (1)	2.337	2.233	1.717	1.114	0.568	0.138
Abs. 450 nm (2)	2.308	2.095	1.569	1.029	0.540	0.116
A450 mean	2.322	2.164	1.643	1.071	0.554	0.127
A450 SD	0.021	0.098	0.105	0.060	0.020	0.016
A450 %CV	0.9%	4.5%	6.4%	5.6%	3.6%	12.3%
Binding %	**100.0%**	**93.2%**	**70.7%**	**46.1%**	**23.8%**	**5.4%**

The concentration of 25(OH)Vit D was measured in 40 serum samples obtained from normal controls.
The results obtained with the immunoassay and novel displacement were compared with results obtained with LCMS. A good agreement was observed (r=0.945) with a small intercept (1.9 ng/ml). Values measured with the ELISA were systematically lower than those obtained with LCMS (slope 0.69), indicating that the calibrator matrix needs optimization.



Conclusion

We conclude that this alternative release approach is an effective, easily automatable method for the assay of 25(OH)Vit D.

Literature

Haddad JG. The Vitamin D binding protein and its clinical significance. In: Vitamin D. Physiology, Molecular Biology and Clinical Applications. 1999, 101-107. Edited by MF Holick.