Clinical importance of "Bioavailable" Vitamin D: Development and analytical validation of Bioavailable 25 Hydroxy Vitamin D assay

AACC 2013
Houston, TX

Raj Pandian, Jothi Pandian and Alan Elias,
Pan Laboratories, Irvine, CA.

Abstract # A-342

Objective: To develop a reproducible assay for quantitation of "bioavailable" Vitamin D (Bio D) in human serum samples.

Relevance: Vitamin D deficiency is determined by measuring circulating 25 hydroxy Vitamin D (25(OH) D). Over 85 % of circulating 25(OH) D is tightly bound to a specific vitamin D binding protein (DBP). A lesser amount is bound loosely with albumin. Less than 1% is free Vitamin D (Free D). The free fraction along with the albumin bound fraction, called Bioavailable Vitamin D, is readily available for metabolic function.

Recent studies indicate that bioavailable, and not total 25(OH) D, correlate well with serum calcium. There has been poor correlation between 25(OH) D levels with bone mineral density in studies that examined this relationship. However, the correlation between Bio D and bone mineral density was good. Similarly, measurement of Bio D in hemodialysis patients showed better correlation in terms of mineral metabolism and PTH levels than total Vitamin D measurement. It is therefore important to measure Bio D in some of the clinical conditions associated with potential mineral metabolic changes.

Methodology: Bioavailable 25(OH) D is vitamin D (25 OH) not bound to DBP. To obtain the bioavailable fraction, total vitamin D was quantitated using an immunoassay with equal cross reactivity with D2 and D3 (Calbiotech). DBP was quantitated by an immunoassay using reagents from R & D systems. Albumin was quantitated by a calorimetric method. Using the affinity constant of 25(OH)D for DBP (Ka = 7 X 108 M-1) and albumin (Ka = 6 X105M-1), Bio D, DBP bound 25(OH)D, albumin bound 25(OH)D and free 25(OH)D were calculated . Bioavailable is the combination of albumin bound 25(OH) D + free 25(OH)D.

Results: The assays used in this study are 25(OH) D, DBP and albumin. All the assays are all very reproducible, individually and in combination, for calculations of Bio D with a CV of less than 13 %. Sensitivity, specificity, and interference studies met the acceptability criteria .All the three assays were run in normal samples and calculated Bioavailable vitamin D (3.87 ± 2.0 ng/ml), calculated free D (9.94 ± 5.47 pg/ml)and DBP bound 25(OH)D (28.14 ± 15.2 ng/ml). Correlation of Bio D with calculated free D in these normal samples was good (r2 = 0.97) whereas correlation with Bio D with total 25(OH) D was poor (r2 =0.366).

Conclusion: We have developed a reproducible bioavailable 25(OH) Vitamin D assay, useful for routine testing in a clinical lab. The availability of "Bioavailable " vitamin D may be useful to elucidate accurately the nature of relationship between Vitamin D and wide range of disorders including fracture , infection, cancer and cardiovascular diseases .

Introduction

Vitamin D is determined by measuring circulating 25 hydroxy Vitamin D (25(OH)D). Over 85 % of circulating 25(OH) D is tightly bound to a specific vitamin D binding protein (DBP). A lesser amount is bound loosely with albumin. Less than 1% is free Vitamin D (Free D). DBP levels are high during pregnancy and individuals with estrogen treatment. The free fraction along with the albumin bound fraction, called Bioavailable Vitamin D, is readily available for metabolic function.
Recent studies indicate that bioavailable, and not total 25(OH) D, correlate with serum calcium. There is a poor correlation between 25(OH) D levels with bone mineral density. However, the correlation between Bioavailable Vitamin D and bone mineral density is better. Similarly, measurement of Bioavailable D in hemodialysis patients shows better correlation in terms of mineral metabolism and PTH levels than total Vitamin D measurement. It is therefore important to measure Bioavailable D in clinical conditions associated with potential mineral metabolic changes.

We have developed a reproducible bioavailable vitamin D method using a lab developed vitamin D binding protein (DBP) assay. The bioavailable D can be determined whether the total vitamin D is measured by immunoassay or by LC MS MS. Bioavailable vitamin D2 as well as bioavailable vitamin D3 can be determined if the total vitamin D is analyzed as D2 and D3 by LC MS MS.

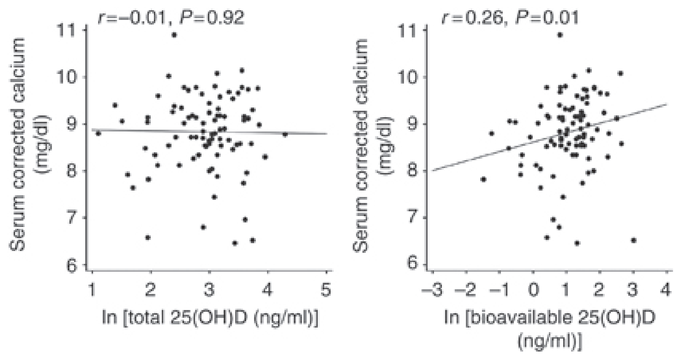

Fig 1 (Source: Ref 1)

Method

Vitamin D 25 hydroxy assay: Total vitamin D is performed by immunoassays (ELISA) using reagents from Calbiotech or IDS (performance details in Table 1) or by LC MS MS (PAML, Spokane, WA). **Albumin** is determined in COBAS Mira plus instrument using bromocresol green. **Vitamin D binding protein (DBP) assay**: Immunometric assay (sandwich ELISA) using DBP antibody coated microtiter plates and biotin labeled anti-DBP .The assay performance for DBP is in Table 1.

Bioavailable and free vitamin D is calculated using the affinity constants of 25 hydroxy Vitamin D to DBP and albumin. Bioavailable 25 (hydroxy) D is 25 hydroxy vitamin D not bound to DBP. To obtain the bioavailable fraction, total vitamin D, DBP and albumin are quantitated .Using affinity constant of 25(OH) D for DBP (7 X 10 8 M) and albumin (6 X 10 5 M), bioavailable Vitamin D and free vitamin D are calculated. The results include the concentration of Bioavailable Vitamin D, Calculated Free Vitamin D, Vitamin D binding protein (DBP) bound vitamin D, albumin bound vitamin D , concentration of DBP and Total 25 hydroxy Vitamin D .

Vitamin D 25 Hydroxy Fractions: Assay Characteristics				
ASSAYS	Vitamin D 25 hydroxy by immunoassay		Vitamin D Binding Protein by immunoassay	Bioavailable Vitamin D
Method	Calbiotech	IDS	Lab Developed test (LDT)	LDT
Instra assay Variation	< 6%	< 7%	< 4.1%	< 8%
Total Imprecision (%CV)	< 8%	< 9%	< 5.8%	< 14%
Limit of Quantitation	1.25 ng/mL	2.5 ng/mL	15 ng/mL (30µg/mL for serum-1:2000 dilution)	0.1 ng/mL
Spike & Recovery	98.70%	ND	106.90%	NA
Dilution Studies	98.40%	ND	101.20%	NA
Specificity:				
25 Hydroxy Vitamin D3	100%	100%	Less than 0.1% crossreactivity with serum albumin, gamma globulins	NA
25 Hydroxy Vitamin D2	122%	75%		
Vitamin D2 or D3	< 0.1%	< 0.3%		

Table 1

Results

Bioavailable vitamin D in normal serum samples (n=55) correlated with total 25 hydroxy vitamin D. The correlations are poor. As shown below, the bioavailable D correlates well with calculated free vitamin D.

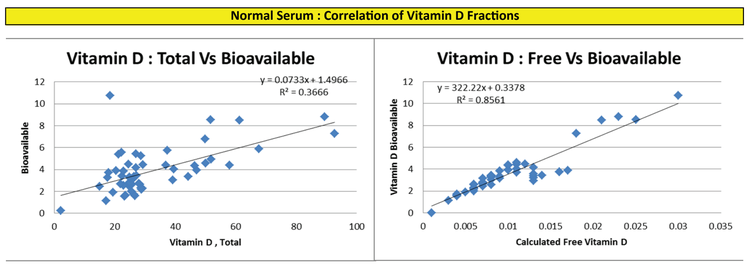

Figure 2

The correlation of bioavailable vitamin D fraction with total 25 hydroxy vitamin D, is not different when the assay for vitamin D is by Immunoassay or by LC MS MS method. Bioavailable D correlates well with calculated free vitamin D.

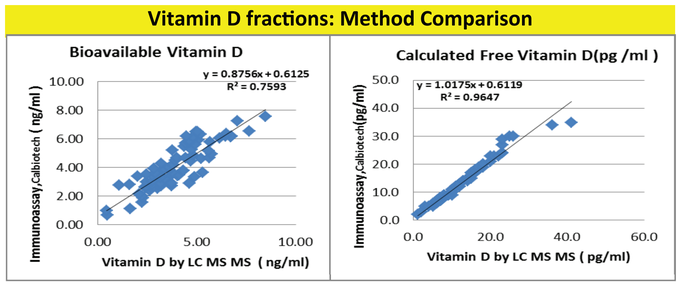

Figure 3

Results (cont.)

The figure below indicates the level of Vitamin D binding protein in normals (n = 55) and in pregnancy (3 rd trimester, n = 54). Vitamin D binding protein is increased during pregnancy.

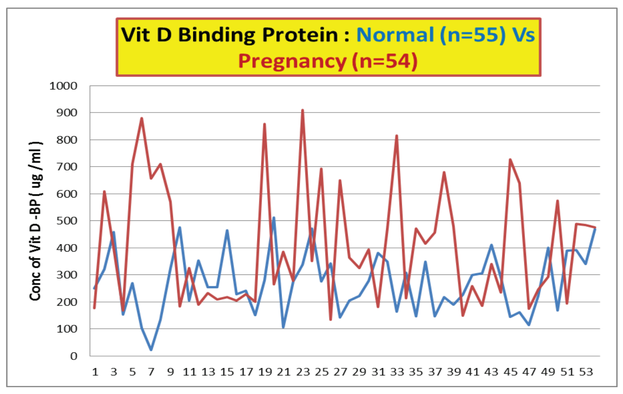

Figure 4

Correlation of vitamin D 25 hydroxy with bioavailable vitamin D in pregnancy serum is also poor . But the correlation of bioavailable D with calculated free D is excellent.

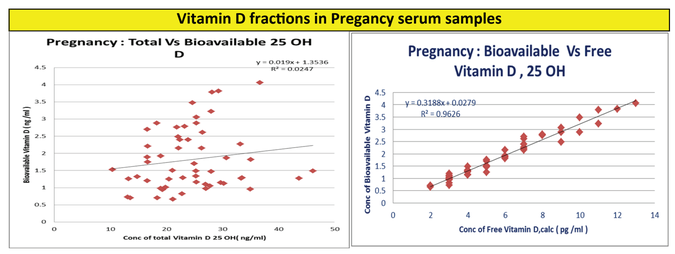

Figure 5

Comparison of reference range for various 25 hydroxy Vitamin D fractions in normal and pregnancy samples:

Comparison of Norman and Pregnancy Samples (25 Hydroxy D Fractions)		
	Reference range (Mean ± 2 SD)	
	Normal (n =55)	Pregnancy (n=54)
Vitamin D, Total (25 OH D)	15 -60 ng /ml	13.1 - 46 .2 ng /ml
Bioavailable Vitamin D(25OH D)	1.92 - 8.82 ng /ml	0.66 - 4.06 ng /ml
Calculated Free Vitamin D(25 OH D)	5.0 - 18 .0 pg /ml	2.0 - 13.0 pg /ml
Vitamin D Binding Protein	104 -477 ug /ml	169 - 910 ug/ml
Albumin, serum	3.2 - 4.5 g /dL	2.8 - 4.5 g / dL

Table 2

Conclusions

1. Bioavailable vitamin D is calculated using binding affinity and concentration of albumin and vitamin D binding protein (DBP) to vitamin D.
2. Bioavailable vitamin D measurement is better than total vitamin D as bioavailable Vitamin D correlates better with calculated free vitamin D.
3. Bioavailable vitamin D can be determined using total vitamin D measured, either by immunoassay or by LC MS MS.
4. Vitamin D binding protein is high in pregnancy. Bioavailable appears to be the better marker than total vitamin D during pregnancy since it correlates well with calculated free vitamin D.

References

1. Bhan I et al (2012) Bioavailable vitamin D is more tightly linked to mineral metabolism than total vitamin D in incident hemodialysis patients . Kidney International 82: 84 – 89.
2. Powe CE et al (2011) Vitamin D-Binding protein modifies the Vitamin D – Bone mineral density relationship. J of Bone Mineral Research 26: 1609 – 1616.
3. Powe CE et al (2010) First trimester Vitamin D, Vitamin D binding protein and subsequent Preeclampsia. Hypertension 56: 758 – 763.

Acknowledgement:
Samples with LC MS MS results were kindly provided by Dr. Carmen Wiley, PAML , Spokane, WA and pregnancy samples were provided by Dr. Martha Garity, Immunodiagnostics system, UK.