International Journal of Preventive Medicine

Original Article Open Access

Competitive Protein-binding assay-based Enzyme-immunoassay Method, Compared to High-pressure Liquid Chromatography, Has a Very Lower Diagnostic Value to Detect Vitamin D Deficiency in 9–12 Years Children

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How to cite this article: Zahedi Rad M, Neyestani TR, Nikooyeh B, Shariatzadeh N, Kalayi A, Khalaji N, *et al*. Competitive protein-binding assay-based enzyme-immunoassay method, compared to high-pressure liquid chromatography, has a very lower diagnostic value to detect vitamin d deficiency in 9-12 years children. Int J Prev Med 2015;6:67.

ABSTRACT

Background: The most reliable indicator of Vitamin D status is circulating concentration of 25-hydroxycalciferol (25(OH) D) routinely determined by enzyme-immunoassays (EIA) methods. This study was performed to compare commonly used competitive protein-binding assays (CPBA)-based EIA with the gold standard, high-pressure liquid chromatography (HPLC).

Methods: Concentrations of 25(OH) D in sera from 257 randomly selected school children aged 9–11 years were determined by two methods of CPBA and HPLC.

Results: Mean 25(OH) D concentration was 22 ± 18.8 and 21.9 ± 15.6 nmol/L by CPBA and HPLC, respectively. However, mean 25(OH) D concentrations of the two methods became different after excluding undetectable samples (25.1 ± 18.9 vs. 29 ± 14.5 nmol/L, respectively; $P = 0.04$). Based on predefined Vitamin D deficiency as 25(OH) D < 12.5 nmol/L, CPBA sensitivity and specificity were 44.2% and 60.6%, respectively, compared to HPLC. In receiver operating characteristic curve analysis, the best cut-offs for CPBA was 5.8 nmol/L, which gave 82% sensitivity, but specificity was 17%.

Conclusions: Though CPBA may be used as a screening tool, more reliable methods are needed for diagnostic purposes.

Keywords: Competitive protein-binding assays, high-pressure liquid chromatography, Vitamin D deficiency, Vitamin D measurement

Access this article online	
Quick Response Code:	Website: www.ijpvmjournal.net/www.ijpm.ir
	DOI: 10.4103/2008-7802.161069

INTRODUCTION

Vitamin D deficiency is a global health problem. The importance of this is heightened when considering the myriad functions of the vitamin. Poor Vitamin D status may, therefore, have a role in many human pathologies including musculoskeletal disorders,[1,2] cancers,[3] cardiovascular disease,[4] both types of diabetes[5,6] and

overall mortality.[7] Elderly, dark-skinned and obese persons, as well as inhabitants in northern latitudes where sun exposure is inefficient especially during winter are particularly at risk of deficiency.[8-11] Accurate determination of Vitamin D status is, therefore, crucial for both clinicians and public health policy makers for appropriate intervention.[4,6,12,13] The most reliable, widely used and most suitable indicator of Vitamin D status is measurement of 25-hydroxycalciferol (25(OH) D) in serum or plasma.[12-14] The measurement of 25(OH) D is challenging because circulating 25(OH) D is highly lipophilic, bound strongly to protein, presents in low (nanomolar) concentrations and exists in two structurally similar forms, 25(OH) D3 and 25(OH) D2.[3] The methods usually used to measure 25(OH) D are high-pressure liquid chromatography (HPLC) and mass spectrometry, radioimmunoassay (RIA), enzyme-immunoassays (EIA), competitive protein-binding assays (CPBA), automated chemiluminescence protein-binding assays and chemiluminescent immunoassays.[14] Several studies have reported inconsistency and variability in 25(OH) D measurement among methods and laboratories, which calls the ability of 25(OH) D assays for accurate reflection of individuals' Vitamin D status into question. It seems necessary to determine the advantages and limitations of different methods comparing to a standard method.[4,6,12] Despite escalating number of physician orders for 25(OH) D assay,[15] external quality control program for 25(OH) D test results of diagnostic laboratories is not currently implemented by the Reference Health Laboratories of the Iran Ministry of Health. EIA-based methods are commonly used in diagnostic laboratories. However, the precision of these methods are questionable. This study was, therefore, performed to evaluate the CPBA-based EIA method as compared to HPLC, the gold standard for 25(OH) D assay.[13]

METHODS

Subjects

In this study we used the information and serum samples of 257 randomly selected children out of 1111 children of a huge study "Vitamin D and calcium deficiency prevalence of Tehran's elementary school children (VDPT)" performed in fall and winter 2008. This study was conducted by National Nutrition and Food Technology Research Institute (NNFTRI) in cooperation with Iran Ministry of Education in Tehran. An informed consent was sent to parents and they were asked to announce if their child had history of diabetes, allergy or autoimmune disease and if has taken calcium, Vitamin D and fish oil supplements during 3 months ago. The inclusion criteria used in VDPT were age nine to 12 years, having no clinical disease including diabetes, allergy or autoimmune disorders and not taking calcium, Vitamin D and fish oil supplements since 3 months prior to the study.

Blood sampling and handling

Venous blood samples collected in glass tubes were transported to the Laboratory of Nutrition Research, NNFTRI, in <2 h. Sera were separated, aliquoted and stored at −80°C for further analyses, as previously described.[16]

Serum concentration of 25(OH) D was determined by two methods: High-performance liquid chromatography HPLC and 25-OH Vitamin D EIA kit based on CPBA.

High-pressure liquid chromatography analysis

Equipment

High-pressure liquid chromatography system equipped with UV detector (Young Lin, Seoul, South Korea). HPLC column was C18 Tracer Excel 120 ODS 15 × 0.4, 3 μm (Teknokroma, Spain).

Solvents

All solvents (methanol, acetonitrile, hexane, propanol, and ethanol) were HPLC grade and purchased from Romil, England.

25-hydroxycalciferol D3 standard was purchased from Sigma-Aldrich. A 1 mg/ml standard of 25(OH) D was prepared from stock standard, and then 10, 25, 50, 75 and 100 nmol/L were prepared from this one.

Procedure

The procedure has been fully described elsewhere.[7] Sera were melted by keeping at room temperature for 30–45 min, then 500 μl of serum was transferred to a clean glass tube, ethanol was added and let it stay for 10 min till proteins were completely precipitated. Then methanol: Isopropanol was added and shaken for 20 s. Hexane was added to the extract. The extraction procedure was repeated again, and the supernatant was collected and evaporated under nitrogen flow. Reconstitution was done by adding methanol which was then filtered using 0.25 μm syringe filter. Finally filtrate was injected to the column [Figure 1]. The intra- and inter-assay variations were 8.1% and 12.6%, respectively, and the recovery percent was 100% ± 5%. The detection limit was 12.5 nmol/L. In this study total, 25(OH) D was measured and considered as the indicator of Vitamin D status.

Competitive protein-binding assay-based enzyme-immunoassay

This method was performed using 25(OH) D EIA kit (Immundiagnostik AG, Austria, Wien). This measurement is based on competition of 25(OH) D present in the sample with 25(OH) D tracer for binding the pocket of Vitamin D-binding protein (VDBP). Since all circulating 25(OH) D is bound to VDBP *in vivo*,

samples have to be precipitated with precipitation reagent to extract the analyte.

Procedure

Competitive protein-binding assays procedure was done according to kit manual. Microplates were read at 450 nm and 630 nm by Microplate ELISA Reader StatFax 3200 (Awareness, USA). According to the manufacturer, the performance characteristics were: Intra- and inter-assay variations 10.7% and 11.8–13.2%, respectively, recovery percent 94% and detection limit 5.6 nmol/L.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). The normality of data distribution was checked using Kolmogorov–Smirnov. Between-group comparison of values was performed by Student's *t*-test (for data with the normal distribution) or Mann–Whitney U-test (for data with nonnormal distribution). Correlations between variables were evaluated by either Pearson (r) (for data with the normal distribution) or Spearman (r_s) (for data with nonnormal distribution). Differences in proportions were evaluated using Chi-square test. The usefulness of CPBA for evaluating Vitamin D status was analyzed using a receiver operating characteristic curve.

All statistical analyses were done by Statistical Package for Social Sciences (SPSS version 16; SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered significant.

RESULTS

25-hydroxycalciferol concentration measured by HPLC and CPBA, and sun exposure time did not have a normal distribution. Children comprised 138 girls (53.7%) and 119 boys (46.3%) from 3 different economically different regions (poor, middle, rich) of Tehran. The mean age was 10.1 ± 0.7 years and the mean duration of sun exposure was 41.2 ± 34.6 min/day (36.0 ± 24.7 min/day and 47.1 ± 42.6 min/day for girls and boys, respectively; $P = 0.059$).

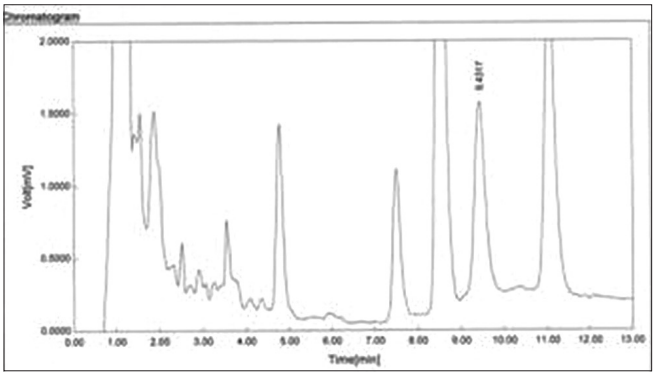


Figure 1: High-pressure liquid chromatography chromatogram of 25-hydroxycalciferol (25(OH)D); 25(OH)D RT is 9.4317 min

Serum 25(OH) D concentration was 22 ± 18.8 and 21.9 ± 15.6 nmol/L by CPBA and HPLC, respectively ($P = 0.369$). However, the difference between two methods became different after excluding nondetectable samples ($n = 40$ from CPBA and $n = 77$ from HPLC; 25.1 ± 18.9 vs. 29 ± 14.5 nmol/L, respectively, $P = 0.044$).

Results of these two methods were classified according to two usual cut-offs for 25(OH) D. The first set of cut-offs was: Sever deficiency <12.5 nmol/L, intermediate deficiency 12.5–25 nmol/L, and mild deficiency 25–37, sufficient >37 nmol/L.[8-10] The second set was: Sever deficiency <37 nmol/L, intermediate deficiency 37–50 nmol/L, and mild deficiency 50–75 nmol/L, sufficient >75 nmol/L [Table 1].[8,11,17]

The distribution of Vitamin D status in the subjects

Vitamin D status was determined by CPBA and HPLC methods. Comparison did not show any significant difference between two methods based on either first (χ^2, $P = 0.92$) or second set of definitions [Table 2a,2b,2c,2d].

Competitive protein-binding assays sensitivity, specificity, positive predictive value, negative predictive value and accuracy were compared to HPLC according to different cut-offs for Vitamin D status [Table 3].

In CPBA, the increment in sensitivity was accompanied by a decrement in validity. Despite similar mean values of 25(OH) D in CPBA and HPLC, we found no significant correlation between the values of the two methods ($P = 0.145$, $r_s = 0.091$).

Bland–Altman plot

We used Bland–Altman plot for analyzing CPBA agreement with HPLC.[18] In this method mean difference of one sample in two methods (CPBA, HPLC) is plotted across mean results of two methods for that sample. As it is shown in Figure 2, dot lines show the mean difference

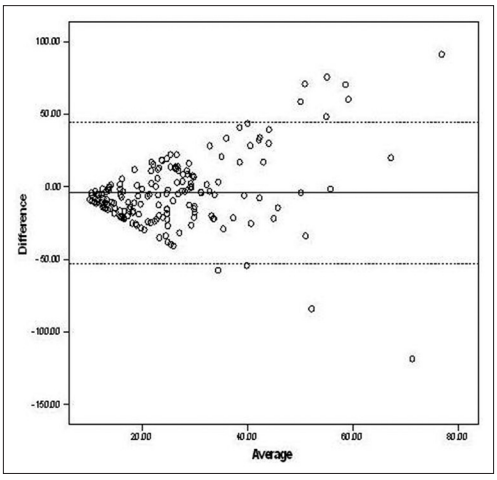


Figure 2: Competitive protein-binding assays compared to high-pressure liquid chromatography by Bland–Altman plot

of two methods concentrations ± 2 SD. Divergence of the diagram shows that there was not a good agreement between CPBA and HPLC for measuring 25(OH) D in serum.

Receiver operating characteristic curve analysis (ROC curve analysis)

ROC curve is a graphical plot which illustrates the performance of a binary classifier system as its discrimination threshold is varied. It is created by plotting the fraction of true positives out of the total actual positives (true positive rate) versus the fraction of false positives out of the total actual negatives (false positive rate), at various threshold settings. By drawing this curve, we can determine lower and upper limit of a test, and we can find points with accurate sensitivity and validity. In this study the best cut-offs for CPBA was 5.8 nmol/L which gave us 82% sensitivity, but at this point specificity was 17%, indicating the failure of this method to distinguish Vitamin D insufficient samples from

Table 1: Comparison of vitamin D status measured by HPLC and CPBA according to two usual cut off points (no (%))

Method	No.(%)	
Vitamin D status	HPLC	CPBA
Deficiency according to first category		
Severe	77 (30)	105 (40.9)
Intermediate	86 (33.5)	62 (24.1)
Mild	65 (25.3)	57 (22.2)
Sufficient	29 (11.3)	33 (12.8)
Deficiency according to second category		
Severe	228 (88.7)	223 (86.8)
Intermediate	18 (7)	13 (5.1)
Mild	9 (3.5)	13 (5.1)
Sufficient	2 (0.8)	8 (3.1)

P>0.05

Table 2a: Comparison of vitamin D status of the participants based on the results of CPBA-EIA and HPLC methods according to the first category (no (%))

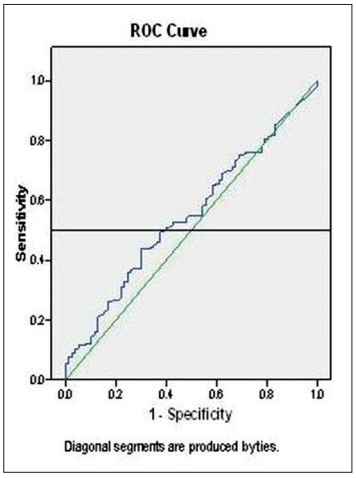
CPBA HPLC	Severe deficiency	Intermediate deficiency	Mild deficiency	Normal	total
Severe deficiency	34 (13.2)	20 (7.8)	15 (5.8)	8 (3.1)	77
Intermediate deficiency	37 (14.4)	18 (7.0)	20 (7.8)	11 (4.3)	86
Mild deficiency	24 (9.3)	15 (5.8)	17 (6.6)	9 (3.5)	65
Sufficiency	10 (3.9)	9 (3.5)	5 (1.9)	5 (1.9)	29
Total	105	62	57	33	257

P>0.05

Table 2b: Comparison of the occurrence of severe vitamin D deficiency and vitamin D insufficiency/sufficiency among the participants based on the results of CPBA-EIA and HPLC methods according to the first category (no (%))

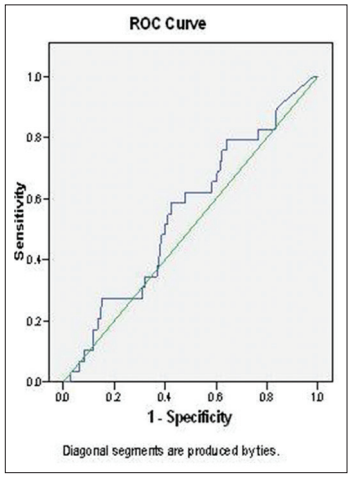
CPBA HPLC	Severe deficiency	Insufficiency/ sufficiency	Total
Severe deficiency	34 (13.2)	43 (16.7)	77
Insufficiency/sufficiency	71 (27.6)	109 (42.3)	180
Total	105	152	257

P>0.05



Area	Standard deviation	P	Asymptotic 95% confidence interval	
			Lower bound	Lower bound
0.548	0.038	0.219	0.474	0.623

Figure 3: Receiver operating characteristic curve of comparing competitive protein-binding assays versus high-pressure liquid chromatography in first category



Area	Standard deviation	P	Asymptotic 95% confidence interval	
			Lower bound	Upper bound
0.554	0.055	0.344	0.446	0.661

Figure 4: Receiver operating characteristic curve of comparing competitive protein-binding assays versus high-pressure liquid chromatography in second category

Table 2c: Comparison of vitamin D status of the participants based on the results of CPBA-EIA and HPLC methods according to the second category (no (%))

CPBA / HPLC	Severe deficiency	Intermediate deficiency	Mild deficiency	Sufficiency	total
Severe deficiency	199 (77.4)	17 (6.6)	5 (1.9)	2 (0.8)	223
Intermediate deficiency	10 (3.9)	1 (7.4)	2 (0.8)	0	13
Mild deficiency	12 (4.7)	0	1 (0.4)	0	13
Sufficiency	7 (2.7)	0	1 (0.4)	0	8
Total	228	18	9	2	257

P>0.05

Table 2d: Comparison of the occurrence of severe vitamin D deficiency and vitamin D insufficiency/sufficiency among the participants based on the results of CPBA-EIA and HPLC methods according to the second category (no (%))

CPBA HPLC	Severe deficiency	Insufficiency/ sufficiency	Total
Severe deficiency	199	29	228
Insufficiency/sufficiency	24	5	29
Total	223	34	257

P>0.05

Table 3: CPBA sensitivity, specificity, accuracy, positive and negative predictive values compared to HPLC in two set of cut-offs

CPBA compared to HPLC (%)	First set of cut-offs	Second set of cut-offs
Sensitivity	44.2	88.7
Specificity	60.6	17.2
Accuracy	55.6	79.7
Positive predictive value	32.4	89.3
Negative predictive value	71.7	15.2

sufficient ones. In other words, CPBA could diagnose really deficient samples but by this test a considerable proportion of sufficient samples may be considered as deficient (high rate of false positive) [Figures 3 and 4]. The method might, therefore, be a good test for screening.

DISCUSSION

Competitive protein-binding assays, compared to HPLC, may over-read or under-read 25(OH) D concentrations though the mean concentrations may show no significant levels at the population level. Studies showed that if 25(OH) D3 is the dominant form, CPBA kit will 58% overestimate 25(OH) D concentration and if 25(OH) D2 is the dominant one, CPBA will 27% underestimate concentrations.[19] One of the noticeable limitations of CPBA method is measuring 25(OH) D in serum where there are other Vitamin D metabolites such as 24, 25-dihydroxyvitamin D, 26, 25-dihydroxyvitamin D, 26, 25-dihydroxyvitamin D-26, 23-lactone. This polar metabolites concentration is 10–15% of 25(OH) D concentration, and D-binding protein recognizes them in some degrees, and this may result in 10–20% overestimation.[20] Studies showed that CPBA and RIA kits will overestimate 25(OH) D3 and underestimate 25(OH) D2 compare to HPLC method.[21,22]

Another study used cartridge/CPBA and RIA and showed that the cartridge extracts more lipids, and, therefore, more Vitamin D. Results of cartridge/CPBA were same as HPLC/CPBA. RIA kit was more accurate than CPBA, but its sensitivity and specificity was low in a deficiency range or around it so that it was not capable of determining 25(OH) D in the samples determined by CPBA.[23]

Several studies have documented very alarming rates of Vitamin D deficiency/insufficiency in different subgroups of the Iranian population.[16,24,25] On the other hand, assessment of Vitamin D status has recently become a routine diagnostic as well as checkup test. As different laboratories use various methods, the results of 25(OH) D assays can be misleading to both policymakers and practitioners.

CONCLUSIONS

CPBA-based EIA, as one of the mostly used method, has the advantages of high throughput and the performance simplicity. However, though it may give a rather good view of the Vitamin D status at the population level, its diagnostic value is questionable. Further research is needed to develop a less expensive, user-friendly and high-throughput method with acceptable precision and accuracy. Moreover, quality control of the laboratories results for 25(OH) D by a reference laboratory using a standard method of HPLC is recommended.

ACKNOWLEDGEMENTS

This research, funded by NNFTRI (No. *p*/25/47/2027) was part of a M.Z.'s dissertation for M.Sc. of biochemistry under the supervision of T.R.N. Statistical analyses were done under the guidance of B.N. A.K., N.S., N.K. and A.G. were contributed in the field and laboratory bench works. All laboratory works were

done at the Laboratory of Nutrition Research, NNFTRI. We do appreciate all children who participated in this study and also their parents who let their children to do so.

Received: 02 Aug 14 **Accepted:** 02 Feb 15
Published: 27 Jul 15

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Source of Support: National Research Institute, Faculty of Nutritional Science and Food Technology, **Conflict of Interest:** None declared.