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Original Article

Biochemistry Section

# Analytical Performance of Vitamin D Immunoassays Compared with Electrochemiluminescence Immunoassay: A Cross-sectional Study

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## ABSTRACT

**Introduction:** Vitamin D, the fat-soluble steroid hormone, is important in maintaining homeostasis of calcium and phosphorus. The diagnosis of Vitamin D deficiency has become considerably more frequent due to its importance in skeletal and non skeletal disorders. Comparison among immunoassay methods is essential as methodological differences can lead to variations in 25-Hydroxyvitamin D (25-OH Vitamin D) results, potentially affecting the clinical interpretation.

**Aim:** To evaluate the analytical agreement and assay trueness of Chemiluminescence Immunoassay (CLIA) (Alkaline Phosphatase- and Acridinium Ester-based) and Latex-Enhanced Immunoturbidimetric Technique (LEIT) methods for 25-OH Vitamin D quantification using Roche e411 Electrochemiluminescence Immunoassay (ECLIA) as the reference platform.

**Materials and Methods:** This cross-sectional study was conducted at the Department of Research and Development, Agappe Diagnostics Limited, Cochin, Kerala, India, from April 2025 to June 2025. A total of 153 randomly selected participants were included in the study. 25-OH Vitamin D quantification was performed using CLIA cartridge analyser Microprocessor-based Immunoassay Platform Analyser-i60 (MISPA-i60), CLIA cassette analyser MISPA-i180, LEIT cartridge analyser MISPA-i3, and Roche Cobas e411 ECLIA analyser as the reference platform. The strength of linear association between methods was evaluated using the Pearson's correlation coefficient (r). Agreement between methods, systematic trend or heteroscedasticity across the measurement range were assessed using Bland–Altman analysis.

**Results:** The cartridge-based CLEIA 25-OH Vitamin D reagent in MISPA-i60 had the best correlation with Roche e411, having a Pearson's correlation coefficient of r=0.9612. The other two platforms, cassette-based 25-OH Vit D CLIA reagent in MISPA-i180 and LEIT reagent in MISPA-i3, had good correlation regressions of r=0.9409 and r=0.9246, respectively, with the Roche e411 results. Chemiluminescence immunoassay-based platforms are more or less equal in performance, while the LEIT deviates more among the methods.

**Conclusion:** The study observed acceptable limits of deviation among the methods of CLIA and LEIT. While all evaluated methods show strong correlation, systematic bias limits their interchangeability. It is always better to stick to the same method or even the same reagent-analyser combination for the follow-up studies of 25-OH Vitamin D quantification.

**Keywords:** Assay trueness, Cholecalciferol, Homeostasis, Vit D quantification

## INTRODUCTION

Vitamin D exists in the human body in two forms, namely Vitamin D2 (ergocalciferol) and Vitamin D3 (cholecalciferol), collectively known as calciferols. The structural difference between the two forms is the double bond between the 22nd and 23rd carbon and a methyl group on carbon 24 of the four-ringed cholesterol backbone on Vitamin D3 [1]. The biologically inactive form of Vitamin D undergoes a hydroxylation reaction first in the liver and then in the kidney, forming 25–OH Vitamin D and 1,25 – dihydroxy Vitamin D, respectively. Upon considering the concentration level in serum and the half-life of hydroxylated Vitamin D forms, 25OH Vitamin D is the best analyte for accurately assessing the Vitamin D status. About 90% of Vitamin D is obtained from a reactive process converting cholesterol into Vitamin D3 after UVB exposure of wavelength 290-320 nm in the epidermis of skin. The active form of Vitamin D stimulates the absorption of calcium in the duodenum and increases calcium influx in distal tubules of the kidney through nuclear Vitamin D receptor (VDR); the latter is specifically regulated by parathyroid hormone level [2-5].

There are many common consequences of Vitamin D deficiency, like rickets in children due to lower amounts of calcium and phosphorus in the blood [6,7]. Vitamin D has a protective role in cancer by promoting apoptosis and decreasing cell proliferation [8]. Vitamin D has shown a correlation with the pathogenesis and prevention of Diabetes mellitus, a metabolic disease that affects nearly every organ system in the body [9]. Vitamin D may contribute to improved insulin secretion through maintenance of calcium homeostasis Vitamin D helps in increasing the production and secretion of insulin, thereby reducing insulin resistance [10-12]. A negative correlation has been found between Vitamin D levels and levels of Anti-thyroid peroxidase (Anti-TPO), emphasising the role of Vitamin D deficiency in the development of Hashimoto thyroiditis [13,14].

The analytical method used for the quantification of 25-OH Vit D in human serum ranges from LEIT, Enzyme-Linked Fluorescent Assay (ELFA), Enzyme-Linked Immunosorbent Assay (ELISA), CLIA and Immunofluorescent Assay (IFA) [15]. Competitive immunoassay is the basic principle used in immunoassay standardisation, considering the limitation in epitope availability for monoclonal antibody development [16]. New strategies on using inter-species specific 25-OH Vit D monoclonal antibodies having variation in epitope antigenicity provided the option for sandwich-based immunoassay standardisation in Vitamin D quantitation.

Method-to-method variation in the results is a major concern on 25-OH Vit D immunoassays [17]. This study focussed on analysing

the method-to-method variations in 25-OH Vit D quantitation in comparison to Electrochemiluminescence (ECLIA). The Roche e411 ECLIA 25-OH Vit D reagent is considered as the reference method and compared with CLIA (Alkaline phosphatase based and Acridinium Ester based), and LEIT in terms of assay trueness.

Hence, the study aimed to evaluate the analytical performance and method-to-method variability of 25-OH Vitamin D quantification across different immunoassay platforms, using ECLIA as the reference method.

## MATERIALS AND METHODS

This cross-sectional study was conducted at the Department of Research and Development, Agappe Diagnostics Limited, Cochin, Kerala, India, from April 2025 to June 2025, and all participants provided informed consent before sample collection.

**Sample size:** A total of 153 randomly selected participants were included in the study. As this was an analytical method-comparison study intended to evaluate agreement across multiple platforms rather than estimate population parameters, all eligible samples available during the study period were included. Therefore, a formal sample size calculation was not performed. Samples were selected using simple random sampling from eligible participants.

**Inclusion criteria:** Subjects aged >18 years who underwent Vitamin D analysis, were included in the study.

**Exclusion criteria:** Subjects with chronic kidney and liver disorders were excluded to minimise potential biological interference in Vitamin D metabolism.

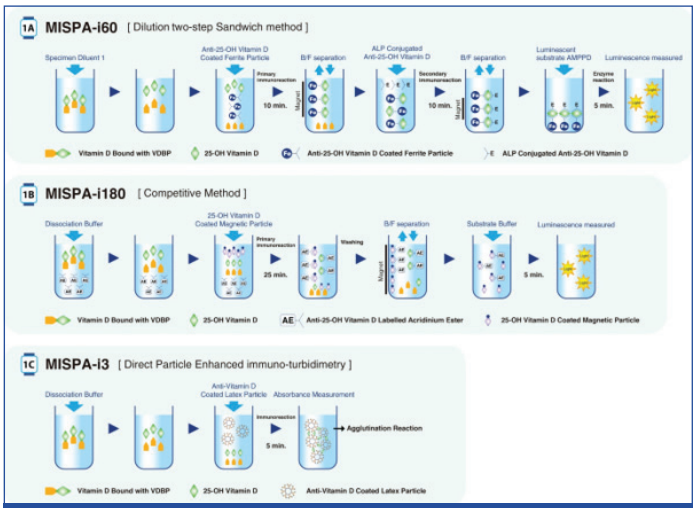
### Study Procedure

Random venous blood samples (~5 mL) were collected, observing standard aseptic procedures and transferred to a plain clot tube. The blood collected in the plain tube was centrifuged at 3000 rpm for 10 minutes, and the separated serum was aliquoted and stored at -20- until used for CLIA or LEIT testing.

**Assay methods:** 25-OH Vit D in the serum was quantified using CLIA cartridge analyser MISPA-i60, CLIA cassette reagent analyser MISPA-i180, LEIT cartridge analyser MISPA-i3, and ECLIA analyser Roche Cobas e411. All the instruments used were closed system auto-analysers.

**MISPA-i60 CLEIA analyser:** Measurement of 25-OH Vit D in MISPA-i60 was based on Chemiluminescence Enzyme Immunoassay (CLEIA) technology by a two-step dilution sandwich immunoassay method [Table/Fig-1a]. Initially, the sample was auto-diluted with the diluent and the 25-OH Vit D in specimens was separated from vitamin D-binding protein and specifically bound to anti-25-OH Vit D monoclonal antibody (sheep) on the particles, resulting in the formation of antigen-antibody immunocomplexes. In the second reaction, the alkaline phosphatase-labelled anti-recombinant chicken monoclonal antibody was added, which specifically binds to 25-OH Vit D immunocomplexes on the particles, and additional immunocomplexes were formed. Then the substrate solution was added and mixed with the immunocomplexes. 3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)phenyl-1,2-dioxetane Disodium Salt (AMPPD) contained in the Substrate Solution was dephosphorylated by the catalysis of ALP conjugated to the secondary antibody. Luminescence (at a maximum wavelength of 477 nm) was generated by the cleavage reaction of dephosphorylated AMPPD. The luminescent signal directly reflects the amount of 25-OH Vit D in the specimen [18,19].

- **MISPA-i180 CLIA analyser:** The 25-OH Vit D reagent in MISPA-i180 follows competitive chemiluminescence microparticle immunoassay [Table/Fig-1b]. In the first step, the specimen was mixed with mouse 25-OH Vit D monoclonal antibody-acridinium-labelled conjugate and assay-specific buffer (with dissociation agent). 25-OH Vit D in the specimen was separated from binding protein under the action of the dissociation agent and combined with the mouse anti-25-OH Vit D monoclonal antibody labelled with acridinium ester. Then 25-OH Vit D-coated magnetic microparticles were added. 25-OH Vit D in the specimen and 25-OH Vit D-coated magnetic microparticles competitively bind to the acridinium ester-labelled mouse anti-25-OH Vit D monoclonal antibody. After washing, substrate buffer was added; the resulting chemiluminescent reaction was quantified in Relative Light Unit (RLU). The value of RLU is inversely proportional to the concentrations of 25-OH Vit D in the specimen [20].
- **MISPA-i3 protein analyser:** Turbidimetry, a technique that measures the attenuation of transmitted light caused by particle-induced scattering, is the principle behind the MISPA-i3 analyser [Table/Fig-1c]. 25-OH Vit D Assay in MISPA-i3 is a direct latex particle-enhanced immunoturbidimetric assay. In the first step, the dissociation reagent broke apart the complex between 25-OH Vit D and its binding proteins in the blood sample. Then the particles coated with antibodies specific to 25-OH Vit D were added to the sample. These antibodies bind to the 25-OH Vit D molecules released in the previous step. When the antibodies bind to multiple Vit D molecules, the particles clump together, forming larger aggregates. The aggregation was detected as an absorbance change, with the magnitude of the change being proportional to the quantity of 25-OH Vit D in the sample [21].




**[Table/Fig-1]:** a) Depicts the two-step dilution sandwich method adopted in MISPA-i60 analyser for 25-OH Vit D quantitation. b) Depicts the competitive method adopted in the MISPA-i180 analyser for 25-OH Vit D quantitation. c). Depicts the latex-enhanced immunoturbidimetry method adopted in the MISPA-i3 analyser for 25-OH Vit D quantitation.

- **Roche Cobas e411 ECLIA analyser:** 25-OH Vit D ECLIA in the Roche e411 analyser is a competitive immunoassay. In the first step, the sample was incubated with pretreatment solutions so that the bound 25-OH Vit D was released from the Vit D Binding Protein (VDBP). In the next step, the pretreated sample was incubated with the ruthenium-labelled vitamin D binding protein, and a complex between the 25-OH Vit D and the ruthenylated VDBP is formed. A specific unlabelled antibody binds to 24,25-dihydroxyvitamin D present in the sample and inhibits cross-reactivity to this vitamin D metabolite. In the final step, streptavidin-coated microparticles and 25-OH Vit D labelled with biotin were added to it, and the unbound ruthenylated labelled Vit D binding proteins became occupied. A complex consisting of the ruthenylated Vit D binding protein and the biotinylated 25-OH Vit D was formed and bound to the solid phase via interaction of biotin and streptavidin. Unbound substances were then removed by washing. Application of a voltage to the electrode then induced chemiluminescent emission, which was measured by a photomultiplier [22].

## STATISTICAL ANALYSIS

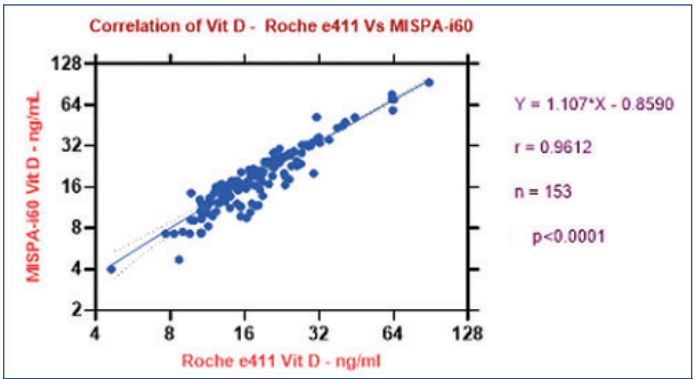
GraphPad Prism 10.4.2 was used for statistical analysis of data correlation. The continuous variable results were expressed as mean with range format. For all experiments, two-tailed p-values were calculated, and $p<0.05$ was considered significant. Method comparison between the test assays and the reference method was performed using multiple complementary statistical approaches. The strength of linear association was evaluated using the Pearson's correlation coefficient. Agreement between methods was assessed using Bland–Altman analysis. The mean difference (bias) between methods was calculated along with the 95% Limits of Agreement (LoA). The presence of any systematic trend or heteroscedasticity across the measurement range was evaluated from the Bland–Altman plots.

## RESULTS

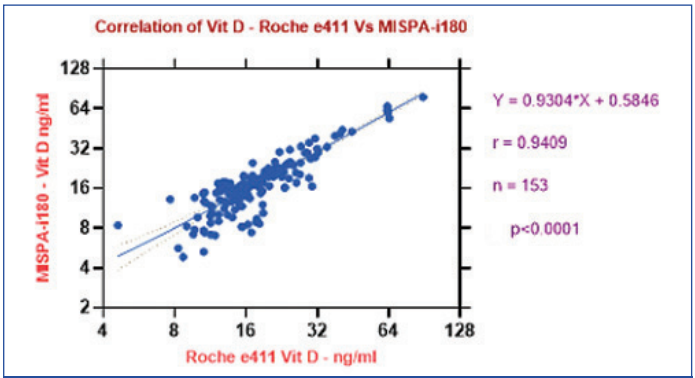
A method-to-method correlation study for Vit D quantification was conducted on a total of 153 randomly selected participants, comprising 89 males and 64 females. The age of the study population ranged from 23 to 58 years.

**Correlation among different immunoassays of 25-OH Vit D:** The cartridge-based CLEIA 25-OH Vit D assay on MISPA-i60 demonstrated the strongest linear association with the reference Roche e411, with a Pearson correlation coefficient of r=0.9612 [Table/Fig-2a]. The cassette-based CLIA assay on MISPA-i180 and the LEIT reagent on MISPA-i3 also showed strong correlations with r=0.9409 and r=0.9246, respectively [Table/Fig-2b,c].

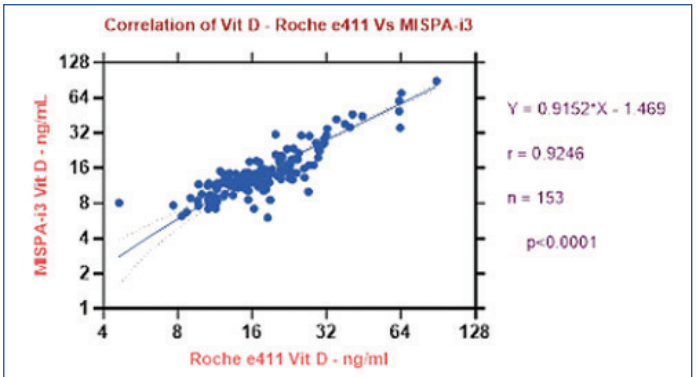
The agreement between methods was further evaluated using Bland–Altman analysis [Table/Fig-3a,b,c]. The Bland–Altman analysis revealed measurable systematic bias between the methods: MISPA-i60 vs Roche e411 demonstrated a positive mean bias of 1.247 ng/mL, indicating a slight overestimation relative to the reference method, with limits of agreement ranging from –6.321 to 8.815 ng/mL. MISPA-i180 vs Roche e411 showed a negative mean bias of -0.7833 ng/mL, suggesting a minor underestimation, with limits of agreement from -8.495 to 6.929 ng/mL. MISPA-i3 vs Roche e411 exhibited the highest deviation, with a negative mean bias of -3.136 ng/mL, indicating consistent underestimation, and wider limits of agreement (-11.85 to 5.582 ng/mL). Visual inspection of the Bland–Altman plots indicated a relatively uniform scatter of differences across the measurement range, with no strong evidence of proportional bias, although increased dispersion at higher concentrations was observed in MISPA-i3.



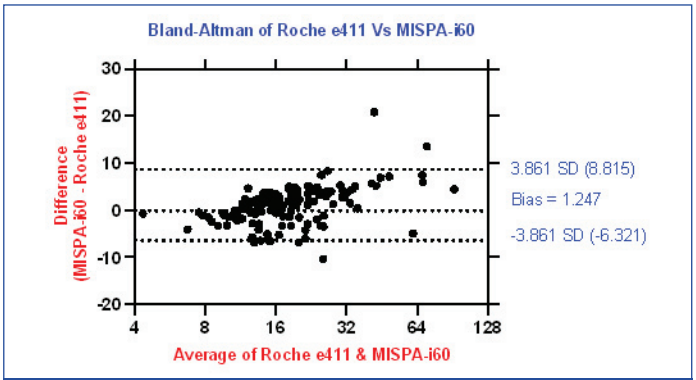

**[Table/Fig-2a]:** Accuracy in terms of trueness between Roche e411 and MISPA-i60 for 25-OH Vit D.



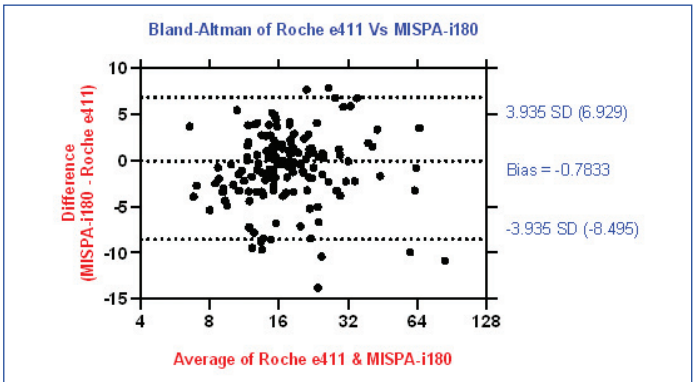

**[Table/Fig-2b]:** Accuracy in terms of trueness between Roche e411 and MISPA-i180 for 25-OH Vit D.



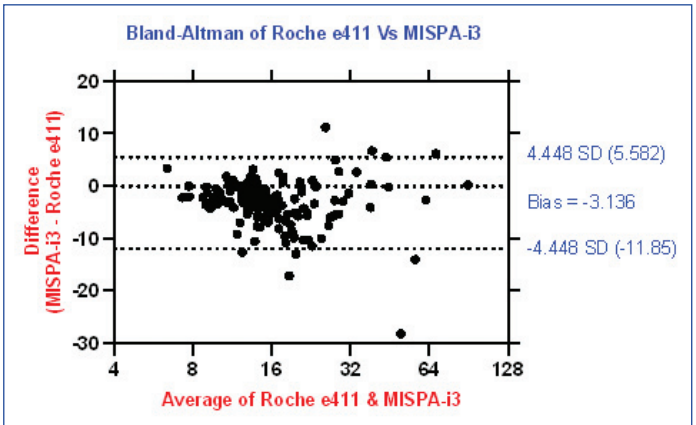

**[Table/Fig-2c]:** Accuracy in terms of trueness between Roche e411 and MISPA-i3 for 25-OH Vit D.




**[Table/Fig-3a]:** Bland-Altman analysis between Roche e411 and MISPA-i60 resulted in a bias of 1.247.



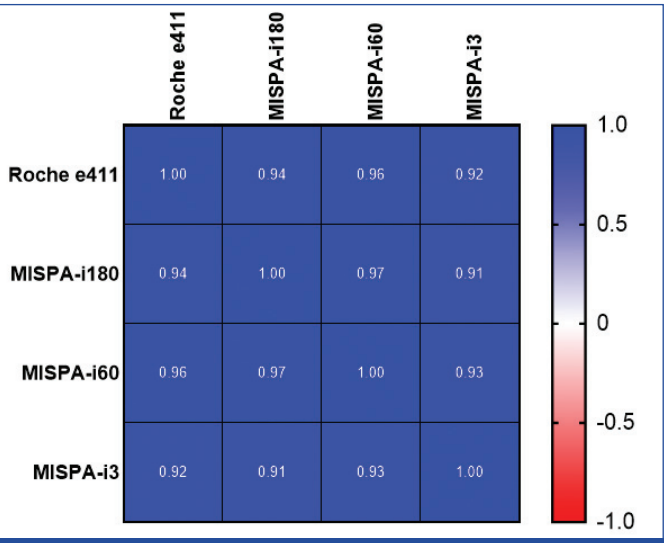

**[Table/Fig-3b]:** Bland-Altman analysis between Roche e411 and MISPA-i180 resulted in a bias of -0.7833.




**[Table/Fig-3c]:** Bland-Altman analysis between Roche e411 and MISPA-i3 resulted in a bias of -3.136.

The statistical findings indicate that while all three methods show strong correlation with the reference method, systematic bias exists across platforms, with MISPA-i60 and MISPA-i180 demonstrating closer agreements and MISPA-i3 showing comparatively higher deviation. Considering the World Health Organisation reference cut-off of 20 ng/mL for Vit D deficiency, the observed biases are unlikely to significantly impact clinical interpretation at the population level. However, individual-level misclassification near the decision threshold cannot be excluded, particularly for assays with higher bias, and should be interpreted with caution.

Method-to-method correlation among the 25-OH Vit D reagents depicted through the Pearson's correlation matrix [Table/Fig-4].




**[Table/Fig-4]:** Correlation among the 25-OH Vit D reagents in Roche e411, MISPA-i60, MISPA-i180 and MISPA-i3.

Descriptive statistical analysis was performed for Vit D quantification using the reference method, Roche e411, and the three MISPA platforms: MISPA-i180, MISPA-i60, and MISPA-i3. The Vit D concentrations measured by the Roche e411 assay ranged from 4.63 to 89.28, with a median value of 16.71 and a mean of 19.65±11.51. The MISPA-i180 assay demonstrated comparable results, with values ranging from 4.85 to 78.48, a median of 16.58, and a mean of 18.87±11.38. Similarly, the MISPA-i60 assay showed a measurement range of 4.00 to 93.80, with a median of 17.20 and a mean of 20.90±13.26. In contrast, the MISPA-i3 assay yielded comparatively lower central tendency values, with a range of 6.01 to 89.53, a median of 13.36, and a mean of 16.51±11.39 [Table/Fig-5].

| Descriptive statistics | Roche e411 | MISPA-i180 | MISPA-i60 | MISPA-i3 |
|---|---|---|---|---|
| Number of values | 153 | 153 | 153 | 153 |
| Minimum | 4.63 | 4.85 | 4 | 6.01 |
| 25% percentile | 13.23 | 12.59 | 13.50 | 11.20 |
| Median | 16.71 | 16.58 | 17.20 | 13.36 |
| 75% percentile | 22.11 | 20.80 | 23.65 | 16.97 |
| Maximum | 89.28 | 78.48 | 93.8 | 89.53 |
| **95% CI of median** | | | | |
| Actual confidence level | 96.48% | 96.48% | 96.48% | 96.48% |
| Mean | 19.65 | 18.87 | 20.90 | 16.51 |
| Std. Deviation | 11.51 | 11.38 | 13.26 | 11.39 |
| Std. Error of mean | 0.9304 | 0.9199 | 1.072 | 0.9209 |

**[Table/Fig-5]:** Descriptive statistics of 25-OH Vit D level in Roche e411, MISPA-i60, MISPA-i180 and MISPA-i3 was measured in ng/mL.

## DISCUSSION

The present study evaluated the analytical performance and inter-method variability of 25-OH Vit D across multiple immunoassay platforms, considering Roche Cobas e411 as the reference method. Although strong linear associations were observed between all methods ($r>0.92$), the findings demonstrate that correlation alone is insufficient to establish agreement or interchangeability.

Assessment using Bland–Altman analysis revealed the presence of systematic bias across all platforms. MISPA-i180 CLIA showed the least bias (–0.7833 ng/mL), followed by MISPA-i60 (1.247 ng/mL), whereas MISPA-i3 (LEIT) demonstrated the highest positive bias (3.136 ng/mL) and wider limits of agreement. These results indicate that while MISPA-i60 and MISPA-i180 exhibit acceptable agreement with the reference method, MISPA-i3 shows comparatively greater deviation. The observed variability can be attributed to differences in assay principles, including antibody specificity, detection chemistry, and efficiency of dissociation of 25-OHD from Vit D binding protein. Competitive immunoassays may be particularly influenced by incomplete dissociation and cross-reactivity, while turbidimetric methods are more susceptible to matrix interference [22,23].

The observations of the present study are consistent with previous reports describing inter-assay variability in Vit D measurement. Several comparative studies have demonstrated that even assays exhibiting excellent correlation coefficients may still present clinically significant proportional or constant bias. It was reported that differences in calibration traceability, antibody affinity, and assay standardisation contribute significantly to variability among commercial immunoassays. Similar to the present findings, automated chemiluminescence-based assays generally demonstrated better analytical agreement with reference methods compared to alternative immunoassay formats [24,25].

Previous investigations comparing CLIA/CLEIA systems with Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) reference methods have also reported biases ranging from approximately -5 ng/mL to +5 ng/mL depending on the platform and patient population. The comparatively lower bias observed with MISPA-i60 and MISPA-i180 in the present study supports the improved analytical performance associated with chemiluminescent enzyme immunoassays. In contrast, studies evaluating latex-enhanced immunoturbidimetric assays have frequently reported wider variability and susceptibility to matrix-related interference, particularly at lower Vit D concentrations [25,26].

Similar prevalence rates have been reported in several Indian and Asian population-based studies despite abundant sunlight exposure, emphasising the growing public health importance of accurate 25(OH)D assessment. Vit D insufficiency has been associated with skeletal disorders such as osteomalacia and osteoporosis, as well as non skeletal complications including metabolic disorders, cardiovascular disease, and impaired immune function [27-29].

### Limitation(s)

The study is limited by the absence of a reference measurement procedure such as LC-MS/MS and reliance on a single immunoassay as the reference method. Additionally, the study population was relatively homogeneous, and advanced regression methods were not applied.

## CONCLUSION(S)

The present study observed acceptable limits of deviations among the methods of CLIA and LEIT. While all evaluated methods show strong correlation, systematic bias limits their interchangeability. It is preferable to stick to the same method or even the same reagent-analyser combination for the follow-up studies of 25-OH Vit D quantification. Transforming the test principle from competitive to sandwich method in CLIA improves the accuracy and sensitivity of the results while considering the calibration linearity in the sandwich assay.

Larger multicentric studies involving diverse patient populations, including individuals with severe Vit D deficiency, chronic kidney

disease, liver disorders, and altered Vit D-binding protein concentrations, are recommended to assess assay performance under varying clinical conditions. Standardisation efforts should be strengthened through alignment with internationally recognised reference materials and participation in external quality assessment programs to reduce inter-assay variability.

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**PLAGIARISM CHECKING METHODS:** [Jain H et al.]
- Plagiarism X-checker: Mar 04, 2026
- Manual Googling: Jul 11, 2026
- iThenticate Software: Jul 14, 2026 (2%)

**ETYMOLOGY:** Author Origin

**EMENDATIONS:** 8

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- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

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