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Harmonization of serum 25-hydroxycalciferol assay results from high-performance liquid chromatography, enzyme immunoassay, radioimmunoassay, and immunochemiluminescence systems: A multicenter study

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Background: Remarkable disagreement among different systems of 25-hydroxy vitamin D 25(OH)D assay makes decision making for both clinical and community interventions very difficult. This study aimed to harmonize the results obtained from different 25(OH)D assay systems.

Methods: A total of 275 serum samples were analyzed for 25(OH)D using DIAsource-enzyme immunoassay (EIA), DIAsource-radioimmunoassay (RIA), Roche-electrochemiluminescence (ECL), Diasorin-chemiluminescent immunoassay (CLIA), and high-performance liquid chromatography (HPLC), as the reference method. Serum intact parathyroid hormone (iPTH) was also measured in all samples. Between-system agreement and harmonization were evaluated using Bland–Altman analysis, receiver operating characteristic (ROC), and regression analysis.

Results: Mean serum 25(OH)D concentrations and frequency distribution of vitamin D status showed a significant difference among the studied systems ($P<.001$ for both). Serum 25(OH)D assay results from all systems correlated with those from HPLC. As compared with HPLC, ECL showed a positive bias (+3.8 nmol/L), whereas CLIA had a negative bias (−11.9 nmol/L). Both EIA and RIA showed a more or less similar positive bias (8.0 and 8.1 nmol/L, respectively). Using serum iPTH-based 25(OH)D cutoff points, only ECL results became comparable to and without significant difference with HPLC. However, when system-specific cutoffs were defined based on HPLC results using regression equations, mean 25(OH)D and frequency distribution of vitamin D status were more harmonized compared with the other methods.

Conclusion: Our findings showed that with adjustment of circulating 25(OH)D based on HPLC, frequency distribution of vitamin D status, as judged by different methods, can be well harmonized with no statistically significant inter-system difference.

Abbreviations AUC, area under the ROC curve; CI, confidence interval; CLIA, chemiluminescent immunoassay; CLSI, Clinical and Laboratory Standards Institute; DBP, vitamin D-binding protein; DEQAS, Vitamin D External Quality Assurance Scheme; ECL, electrochemiluminescence; EIA, enzyme immunoassay; FDA, Food and Drug Administration; GC, gas chromatography; HOPRM, high-order primary reference material; HRL, Health Reference Laboratories; HPLC, high-performance liquid chromatography; iPTH, intact parathyroid hormone; LC, liquid chromatography; MS, mass spectrometry; NNFTRI, National Nutrition and Food Technology Research Institute; NPV, negative predictive value; PPV, positive predictive value; RAM, reference assay method; RIA, radioimmunoassay; RM-ANOVA, repeated-measures analysis of variance; RT, room temperature; SD, standard deviation; UVB, ultra violet beam; VDR, vitamin D receptor; VUS, volume under the ROC surface.

KEYWORDS
25-hydroxycalciferol, harmonization, high-performance liquid chromatography, immunoassay, vitamin D

1 | INTRODUCTION

Vitamin D has two major vitamers, D_2 or ergocalciferol and D_3 or cholecalciferol. Ergocalciferol has a plant origin, whereas cholecalciferol is a secosteroid hormone synthesized in the skin following direct exposure to the solar ultra violet beam (UVB) in the spectrum of 290-315 nm and consequent activation of the precursor 7-dehydrocholesterol.[1] Dietary and endogenous D vitamers have a similar fate in the body. Upon two steps activation in liver and kidney, 25-hydroxycalciferol (25(OH)D or calcidiol) and then 1, 25-dihydroxycalciferol (1, 25$(OH)_2$D or calcitriol) are formed, respectively. It is believed that calcidiol is the major circulating form reflecting the body storage, while calcitriol is the functional isoform of the vitamin.[2]

Despite the presence of an eternal source of natural vitamin D, i.e., solar beam, vitamin D deficiency has become a global health problem for many sociocultural and environmental reasons.[3] Poor vitamin D status is accompanied by higher circulating parathyroid hormone (PTH) concentrations and consequent lesser bone mass and muscular weakness.[4] Detection of vitamin D receptor (VDR) in many tissues and cells led to definition of many so-called noncalcemic functions of this vitamin.[5] As a result, contribution of vitamin D deficiency has been documented in many human disorders, such as multiple sclerosis, diabetes, and various types of malignancies.[6] Early detection and proper treatment of vitamin D deficiency has, therefore, attracted a great deal of concern both in clinical practice and at the community level.[7,8] However, there is no general agreement on definition of vitamin D deficiency at the time. While 25(OH)D concentrations above 50 nmol/L (20 ng/mL) are considered sufficient by some scientific bodies,[9] it is debated by some experts.[10,11] The other important issue is determination of circulating calcidiol itself. Though many commercial kits using various analytical techniques have been introduced to the market, measurement of serum 25(OH)D is still not as easy as many other serum analytes.[12] Remarkable disagreement among different systems of 25(OH)D assay has been shown by several studies.[13-15] Large variances among the results obtained from different assay systems make clinical follow-up of patient's status and also national and international comparisons of the prevalence of vitamin D deficiency very problematic, if not impossible.[16,17] For this reason, definition of method-specific cutoff points[18] and standardization of assay results based on a reference method[19] have been both suggested.

Lack of standard assay method for 25(OH)D is another issue.[20] Methods employing gas chromatography (GC) in conjunction with mass spectrometry detection and recently liquid chromatography (LC)-tandem mass spectrometry with on-line solid phase extraction have been proposed as reference methods.[21,22] Despite their high accuracy and precision, these methods cannot be routinely used in either diagnostic or community research laboratories because of their complexity and low throughput.[23] Harmonization of 25(OH)D assay results may potentially improve the agreement among different methods and laboratories.[24]

The term "standardization" denotes to traceability of the results obtained from different systems of assaying an analyte by using a high-order primary reference material (HOPRM) and/or a reference assay method (RAM).[25] In this study, we used HPLC as a reference method. When HOPRM or RAM is not available, harmonization may be employed.[25] The Clinical and Laboratory Standards Institute (CLSI) has defined "harmonization" as "the process of recognizing, understanding, and explaining differences while taking steps to achieve uniformity of results, or at minimum, a means of conversion of results such that different groups can use the data obtained from assays interchangeably."[26]

In this study, we attempted to harmonize the results obtained from different assay systems of serum calcidiol. To do this, we tried several methods including adjustment of different assay results according to HPLC, as a reference method, and definition of method-specific cutoff points using either serum iPTH changes or regression analysis model. Finally, the results of all harmonization methods were evaluated and the most proper one was proposed.

2 | MATERIALS AND METHODS

A total of 275 adult subjects aged 20-60 years were enrolled in the study. Before blood sampling, the aims of the study were fully described for the subjects and then an informed written consent was signed by the participants. This study was approved by the Ethics Committee of the National Nutrition and Food Technology Research Institute (NNFTRI).

2.1 | Blood sampling and handling

Ten milliliters fasting blood sample was drawn from antecubital vein. Following centrifugation at 800 *g* at room temperature (RT) for 15 minutes, sera were recovered and aliquoted in several microtubes which were then kept at −80°C until the day of analysis. Replicates of the serum samples in cryo-boxes were transferred to both Health Reference Laboratories (HRL) and Laboratory of Day Hospital, accredited by HRL, while preserving the cold chain.

2.2 | Determination of circulating 25(OH)D

Serum 25(OH)D concentration of each sample was determined using enzyme immunoassay (EIA), radioimmunoassay (RIA),

chemiluminescence, and high-performance liquid chromatography (HPLC), as the reference method.

2.2.1 | EIA and RIA

We used DIAsource kits (Louvain-la-Neuve, Belgium) for both EIA and RIA. These kits are HRL approved. EIA tests were performed at HRL, whereas RIA assay was done at the Laboratory of Nutrition Research, NNFTRI. As claimed by the manufacturers of EIA and RIA kits, limits of detection (LOD) were 3.75 and 1 nmol/L, intra-assay variations were <7.8% and <5.2%, and inter-assay variations were <9.2% and <9.8%, respectively.

2.2.2 | HPLC

Concentrations of 25(OH)D in serum samples were determined by the method described elsewhere[27] at the Laboratory of Nutrition Research, NNFTRI. In our hands, LOD was 10 nmol/L and intra- and inter-assay variations were 8.1% and 12.6%, respectively. This laboratory has been participating in Vitamin D External Quality Assurance Scheme (DEQAS) since 2012.

2.2.3 | Chemiluminescence

Two chemiluminescence systems were applied for 25(OH)D assay including Elecsys electrochemiluminescence (ECL, Roche, Basel, Switzerland) and Liaison chemiluminescent immunoassay (CLIA, Diasorin, Stillwater, MN, USA). According to the manufacturers, for Elecsys-ECL (Roche), LOD was 10 nmol/L and intra- and inter-assay variations were <5.7% and <9.9%, respectively. As for Liaison-CLIA (Diasorin), LOD was 10 nmol/L and intra- and inter-assay variations were <4.8% and <12.2%, respectively. All 25(OH)D assays using these two systems were done at the Laboratory of Day Hospital.

2.3 | Intact parathyroid hormone (iPTH) assay

Determination of serum iPTH was performed using Elecsys-ECL system at the Laboratory of Day Hospital. According to the manufacturer, limit of detection was 1.20 pg/mL. In a multicenter study, intra- and inter-assay variations were 3.1%-6.6% and 3.4%-15.6%, respectively. The analytical sensitivity was below 2.70 pg/mL.[28]

2.4 | Statistical analyses

Quantitative or qualitative data were expressed as mean±standard deviation (SD) or absolute and proportional frequencies, respectively. Means were compared using repeated-measures analysis of variance (RM-ANOVA) and variances were evaluated by Levene's test. To evaluate agreement among the methods, sensitivity, specificity, and positive and negative predictive values were calculated and Bland–Altman analysis was also used.[29,30] Correlations between continuous data were evaluated using Pearson's or Spearman's correlation coefficient. To compare correlation coefficients, Fisher *r*-to-*z* test was employed. To adjust different analytical methods based on HPLC, single-variable regression analysis was used.

Receiver operating characteristic (ROC) analysis was used to determine method-based cutoff points for 25(OH)D according to serum iPTH concentrations. First, the area under the ROC curve (AUC) was used to evaluate the markers. The ROC curve analysis was then generalized to allow the tests to have more than two classes. The generalized ROC curve leads to a surface. In this case, the AUC changes to the volume under the ROC surface (VUS). In this study, we used the ROC surface analysis to determine two cutoff points, simultaneously. Vitamin D sufficiency was defined as a concentration of circulating 25(OH)D, wherein iPTH concentration attains a plateau.[31] Maximum Youden index was considered to set proper cutoff points.[32] In this study, $P<.05$ was considered as significant. All statistical tests were performed using Statistical Package for Social Sciences (SPSS, version 21; SPSS Inc., Chicago, IL, USA).

3 | RESULTS

A total of 275 blood samples were taken from the subjects (129 males and 146 females) aged 40.6±10.7 years (males 41.5±10.0 years, females 40.2±10.4 years). Comparison of mean serum 25(OH)D concentrations obtained by HPLC, ECL, CLIA, EIA, and RIA showed a significant difference (43.6±25.8, 46.3±28.5, 34.1±24.4, 51.2±26.5, and 54.8±37.7, respectively, $P<.001$). Further analysis using paired *t*-test followed by Bonferroni correction revealed a significant difference in 25(OH)D concentrations obtained from HPLC with those from ECL, CLIA, EIA, and RIA ($P<.001$ for all). Comparison of the variance of 25(OH)D concentrations using Levene's test showed a significant difference between HPLC and RIA ($P<.001$), but HPLC did not differ significantly with ECL ($P=.802$), CLIA ($P=.052$), or EIA ($P=.605$).

Frequency distribution of vitamin D status based on HPLC results significantly differed with the results obtained from other systems with the exception of RIA which surprisingly showed no significant difference ($P=.760$). Overall, ECL had the most acceptable sensitivity and specificity. CLIA showed highest sensitivity (99.3%) at the cost of its very poor specificity (47.3%).

Serum 25(OH)D assay results from all systems correlated well with those from HPLC, with the strongest correlation with CLIA ($r=.883$, $P<.001$), followed by ECL ($r=.855$, $P<.001$), EIA ($r=.799$, $P<.001$), and RIA ($r=.739$, $P<.001$). The correlation coefficient of CLIA was significantly bigger than EIA and RIA as judged by Fisher *r*-to-*z* test ($P<.001$ for both). A significant inverse correlation was observed between serum iPTH and 25(OH)D concentrations obtained from HPLC ($r=-.221$, $P<.001$), ECL ($r=-.289$, $P<.001$), CLIA ($r=-.201$, $P<.001$), EIA ($r=-.278$, $P<.001$), and RIA ($r=-.227$, $P<.001$). Fisher *r*-to-*z* test showed no significant difference among these correlation coefficients.

To evaluate agreement between assay systems, Bland–Altman analysis was employed. As compared with HPLC, ECL showed a positive bias (+3.8 nmol/L), whereas CLIA had a negative bias (−11.9 nmol/L). Both EIA and RIA showed almost similar positive bias (8.0 and

8.1 nmol/L, respectively), but especially for RIA, several samples demonstrated huge differences.

Using regression analysis, an equation was developed to adjust the results from each method according to HPLC as follows:

1. ECL-Roche: $25(OH)D^{Adj}$ nmol/L=(ECL-3.55)/0.99; 95% CI: 3.08-3.15
2. CLIA-Diasorin: $25(OH)D^{Adj}$ nmol/L=(CLIA+4.55)/0.84; 95% CI: −12.36 to −11.27
3. EIA-DIAsource: $25(OH)D^{Adj}$ nmol/L=(EIA-14.12)/0.856; 95% CI: 7.44-8.47
4. RIA-DIAsource: $25(OH)D^{Adj}$ nmol/L=(RIA+2.81)/1.219; 95% CI: 6.65-8.44

wherein $25(OH)D^{Adj}$ is adjusted serum calcidiol concentration.

RM-ANOVA revealed no statistical significant difference in mean harmonized circulating 25(OH)D concentrations among HPLC, ECL, CLIA, EIA, and RIA (43.6±25.8, 43.2±28.8, 46.0±29.1, 42.7±29.6, and 42.7±29.6 nmol/L, respectively, *P*=.514). Comparison of HPLC and other systems using paired *t* test followed by Bonferroni adjustment revealed no significant difference between HPLC results and those of adjusted values obtained from equations for ECL (*P*=.219), CLIA (*P*=.219), EIA (*P*=.249), or RIA (*P*=.248). When occurrence of vitamin D deficiency and insufficiency was calculated using adjusted values, no significant difference was observed between HPLC and other systems except for RIA. Table 1 shows vitamin D status of the studied population based on predefined cutoff points (deficiency <27.5, insufficiency: 27.5-50, and sufficiency >50 nmol/L) before and after harmonization. Table 2 demonstrates pre- and post-harmonization sensitivity, specificity, and positive and negative predictive values of different methods as compared with the reference method, i.e., HPLC, to diagnose undesirable vitamin D status (<50 nmol/L).

In another attempt to harmonize calcidiol assay results, serum concentrations of 25(OH)D and iPTH were applied in ROC analysis model. By considering circulating 25(OH)D concentrations wherein serum concentration of iPTH attains a plateau (28 ng/L) and the proposed serum level of PTH indicating hyperparathyroidism (>65 ng/L),[33] new serum 25(OH)D cutoff points were determined for the reference HPLC method. Thus, the cutoff points for vitamin D deficiency, insufficiency, and sufficiency were then defined based on serum 25(OH)D concentrations (nmol/L) as follows:

HPLC: <19.0; 19.0-38.6; >38.6
ECL-Roche: <19.5; 19.5-43.5; >43.5
CLIA-Diasorin: <13.4; 13.4-30.0; >30.0
EIA-DIAsource: <34.2; 34.2-51.2; >51.2
RIA-DIAsource: <22.6; 22.6-51.4; >51.4

By using these system-specific cutoff points, proportion of the subjects diagnosed as deficient decreased dramatically, while proportion of insufficient subjects increased (Table 3). This was especially the case with HPLC, ECL, and CLIA. However, only ECL results became comparable to and without significant difference with HPLC results. Again here, both EIA and RIA showed less specificity than other systems (Table 4).

Since vitamin D functions are by no means confined just to the bones and hard tissues, determination of proper cutoff points for circulating 25(OH)D has been extremely challenging and controversial. With the assumption that IOM proposed cutoff points are based on a reference method, another set of system-specific cutoffs were defined using the above regression equations. Based on serum concentrations of 25(OH)D (nmol/L), the cutoff points for vitamin D deficiency, insufficiency, and sufficiency were then defined as follows:

HPLC: <27.5; 27.5-50; >50.0
ECL-Roche: <30.8; 30.8-53.0; >53.0
CLIA-Diasorin: <18.6; 18.6-37.4; >37.4
EIA-DIAsource: <37.7; 37.7-59.9; >59.9
RIA-DIAsource: <30.7; 30.7-58.1; >58.1

Using these cutoff points, distribution of vitamin D status in our subjects and diagnostic characteristics of the systems were the same as in Table 1, after harmonization (data not shown). To further evaluate this method actually, 25(OH)D concentration in 50 additional serum samples obtained from Day Hospital was determined using the studied systems. Mean 25(OH)D concentrations showed a significant between-system difference (*P*<.001). Post hoc comparisons of each system with HPLC revealed that, except for ECL-Roche, mean calcidiol concentration from

TABLE 1 Vitamin D status of the studied subjects based on different systems of 25(OH)D analysis [n (%)] before and after harmonization based on HPLC results

	Before harmonization				After harmonization			
System	Deficient	Insufficient	Sufficient	*P* value*	Deficient	Insufficient	Sufficient	*P* value*
HPLC	97 (35.8)	81 (29.9)	93 (34.3)	—	97 (35.8)	81 (29.9)	93 (34.3)	—
ECL-Roche	87 (32.7)	77 (28.9)	104 (38.3)	.016	106 (39.8)	69 (25.9)	91 (34.2)	.368
CLIA-Diasorin	120 (48.4)	83 (33.5)	45 (18.1)	<.001	76 (30.6)	89 (35.9)	83 (33.5)	.172
EIA-DIAsource	40 (15.0)	110 (41.4)	116 (43.6)	<.001	79 (29.7)	109 (41.0)	78 (29.3)	.614
RIA-DIAsource	68 (30.4)	59 (26.3)	97 (43.3)	.760	87 (38.8)	54 (24.1)	83 (37.1)	.010

Vitamin D status definitions based on serum 25(OH)D concentration (nmol/L): deficiency: <27.5; insufficiency: 27.6-50.0; sufficiency: >50.
*Comparison of each method with HPLC was done by Wilcoxon's test.

TABLE 2 Sensitivity, specificity, and positive and negative predictive values of different systems compared with HPLC before and after harmonization

	Before harmonization				After harmonization			
System	ECL-Roche	CLIA-Diasorin	EIA-DIAsource	RIA-DIAsource	ECL-Roche	CLIA-Diasorin	EIA-DIAsource	RIA-DIAsource
Sensitivity	86.4 CI: 80.2-90.9	99.3 CI: 95.9-99.9	78.1 CI: 71.1-83.7	82.7 CI: 74.9-88.5	90.3 CI: 84.8-94.1	93.5 CI: 88.1-96.6	91.4 CI:86.0-94.9	87.2 CI: 80.0-92.1
Specificity	87.9 CI: 78.9-93.5	47.3 CI: 36.9-57.8	87.9 CI: 78.9-93.5	81.7 CI: 72.0-88.6	83.1 CI: 73.4-89.9	78.4 CI: 68.5-86.0	69.2 CI: 58.5-78.2	72.5 CI: 62.0-81.1
PPV	93.2 CI: 88.0-96.4	75.8 CI: 69.2-81.4	92.6 CI: 86.9-96.0	86.6 CI: 79.1-91.7	91.4 CI: 86.0-94.9	87.8 CI: 81.6-92.2	85.1 CI: 79.0-89.7	82.2 CI: 74.7-87.9
NPV	76.9 CI: 67.4-84.3	97.7 CI: 86.7-99.8	67.2 CI: 57.9-75.3	76.7 CI: 67.0-84.4	81.3 CI: 71.4-88.4	87.9 CI: 78.5-93.7	80.7 CI: 69.9-88.4	79.5 CI: 68.9-87.2

NPV, negative predictive value; PPV, positive predictive value.
Vitamin D status was defined based on serum 25(OH)D concentrations (nmol/L): desirable >50, undesirable <50.

all other systems differed significantly from that of HPLC. However, this between-system difference disappeared after standardization (P=.624). The significant difference in distribution of vitamin D status according to different systems also removed following standardization (data not shown).

4 | DISCUSSION

We found that harmonization of the results of different assay systems caused an agreement in means of serum 25(OH)D concentrations and also in occurrence rates of vitamin D deficiency/insufficiency. The only exception was RIA based on which distribution of vitamin D status in our population following harmonization differed significantly with that of HPLC. Re-evaluation of data revealed that the major difference in vitamin D status categories between HPLC and RIA was in deficiency and insufficiency. In other words, RIA had more efficiency in discrimination of undesirable from desirable status (i.e., calcidiol concentrations below and above 50 nmol/L, respectively). Harmonization caused a relative improvement of RIA sensitivity at the cost of a more decrease in its specificity.

Wide agreement range of RIA and HPLC, as judged by Bland–Altman analysis, indicates a wide scattering of RIA results around real values; thus, actually no acceptable agreement could be made between RIA and HPLC by any methods.

It has been proposed that for serum 25(OH)D, like other blood analytes, there must be just a normal range of 32-100 ng/mL (80-250 nmol/L) and categorization of vitamin D status to insufficiency and deficiency is unnecessary and confusing.[34] Obviously, raising desirable limit of serum calcidiol to this level will inevitably lead to a dramatic increase in prevalence rates of vitamin D deficiency and also to changes of sensitivity and specificity of assay systems. Notwithstanding, there is no general agreement on this proposed normal range.[35,36] Classification of undesirable vitamin D status to "deficiency" and "insufficiency" bears a clinical implication. Vitamin D deficiency is accompanied by a high risk of bone problems, whereas vitamin D insufficiency is associated with an increased risk of noncalcemic complications.[36] Consequently, identification of different states of undesirable vitamin D status and further prediction of its potential outcomes can have a determining role in management of the problem at both clinical and community settings.

In this study, cutoff points for circulating 25(OH)D concentrations were determined according to serum iPTH concentrations. Based on these cutoffs, only ECL results (including frequency distribution of vitamin D status) became almost similar to those of HPLC. In a study on 214 serum samples collected between February 2005 and December 2011 from children aged 0.1-19.2 years, vitamin D deficiency, based on elevation of serum PTH concentrations above 50 ng/L, was defined as circulating 25(OH)D <34 nmol/L. However, in each time period during the study and in accord with introduction of new systems to the market, one type of assay system for 25(OH)D was employed. Consequently, during 6 years of serum sample collection, three

TABLE 3 Vitamin D status of the studied subjects according to serum iPTH-based system-specific cutoff points for 25(OH)D assay [n (%)]

System	Deficient	Insufficient	Sufficient	*P* value
HPLC	37 (13.7)	105 (38.7)	129 (47.6)	—
ECL-Roche	39 (14.7)	110 (41.4)	117 (44.0)	.174
CLIA-Diasorin	30 (12.1)	101 (40.7)	117 (47.2)	.012
EIA-Diasource	58 (21.8)	98 (36.8)	110 (41.4)	<.001
RIA-Diasource	44 (19.6)	87 (38.8)	93 (41.5)	<.001

TABLE 4 Sensitivity, specificity, and positive and negative predictive values of different systems compared with HPLC based on system-specific cutoff points

	ECL-Roche	CLIA-Diasorin	EIA-DIAsource	RIA-DIAsource
Sensitivity	92.1 CI: 86.1-95.8	92.4 CI: 85.7-96.2	90.1 CI: 83.7-94.3	91.8 CI: 84.0-96.1
Specificity	84.6 CI: 76.8-90.2	83.5 CI: 75.7-89.3	77.2 CI: 68.6-84.1	67.2 CI: 58.1-75.1
PPV	87.2 CI: 80.5-91.9	83.9 CI: 76.3-89.5	82.0 CI: 74.9-87.5	68.7 CI: 59.9-76.3
NPV	90.5 CI: 83.2-94.9	92.2 CI: 85.3-96.1	87.1 CI: 79.0-92.5	91.3 CI: 83.1-95.8

Vitamin D status based on circulating 25(OH)D concentration (nmol/L) for HPLC: sufficiency >38.55, insufficiency ≤38.55; ECL-Roche: sufficiency >43.5, insufficiency ≤43.5; CLIA-Diasorin: Sufficiency >30.0; insufficiency ≤30.0; EIA-Diasource: sufficiency >51.2; insufficiency ≤51.2; RIA-Diasource: Sufficiency >51.4; insufficiency ≤51.4.
To develop HPLC-specific cutoff points, changes of serum iPTH in different concentrations of serum 25(OH)D were used. Using ROC analysis, these cutoffs were then employed to develop cutoff points for other systems.

different systems, including Nichols-chemiluminescence (7 months), IDS-RIA (~2.5 years) and finally LC-tandem MS (~3 years), with different performance characteristics were used to measure circulating calcidiol.[37] The major critique of PTH-based serum 25(OH)D cutoff points is that only calcemic effects of vitamin D are considered in this way. While a desirable level of circulating calcidiol is a concentration at which both calcemic and noncalcemic effects of vitamin D are exerted for a long-term health. In British National Diet and Nutrition Survey, some elderly subjects (85 years plus) had high serum PTH concentrations despite having desirable vitamin D status,[38] indicating that PTH-based serum calcidiol diagnostic limits may not be applicable for some subpopulations.

To harmonize 25(OH)D assay results from various systems, we proposed system-specific cutoff points by using regression analysis. In this case, diagnostic characteristics of the systems actually did not differ with those from standardization of the results. Notwithstanding, using defined cutoff points is much easier for both medical and research laboratories.

During the recent decades, a remarkable improvement has been made in measurement of circulating 25(OH)D. According to DEQAS report, inter-laboratory variations have decreased from 32% in 1994 to 15.3% in 2009.[14] Nevertheless, high inter-method and inter-laboratory variations are still challenging. There are several reasons for these variations. Vitamin D is a hydrophobic and matrix-sensitive compound. However, vitamin D analytes are stable for 2 weeks at 30°C and for 1 year (and even longer) at −20°C and are not affected by up to four freeze-thaw cycles of serum samples.[23] Ultra violet beam also does not influence calcidiol content of a serum sample.[34] Both D2 and D3 isoforms may be found in the body. Binding molecules (antibodies or vitamin D-binding protein [DBP]) commonly used in immunoassays have usually more affinity to D3 isoform.[20] However, this issue does not seem to be problematic in our study. Because of very limited food sources, ergocalciferol is actually undetectable in the circulation of most, if not all, Iranians.[27]

Some limitations of this study are acknowledged. Results from a commercial assay kit of a certain method cannot necessarily be extended to all kits of that method. Though we used HRL-approved kits, performance characteristics of different manufacturers may vary. Immunoassay kits from Diasorin and IDS both have FDA approval, but performance characteristics of Diasorin were shown to be actually more satisfactory.[39] Harmonization, by any method, is mostly applicable to minimize the systematic errors. Assay systems with inherent possibility of random errors may not be harmonized efficiently. In previous generation of commercial immunoassay kits, for instance, there was one step of solvent extraction for DBP removal and releasing calcidiol. This step was highly prone to random errors. In newer generations, this step is performed in situ using a denaturing agent which is the manufacturer's secret so there is no available data on its compound and efficacy.[39] Finally, though the results of our evaluation study on additional serum samples seem promising, the number of the tested samples was limited and the analyses were performed in the same laboratory in which the other tests were done. Therefore, further studies on a large number of serum samples in different laboratories are needed.

5 | CONCLUSION

Our findings showed that with adjustment of circulating 25(OH)D based on HPLC, frequency distribution of deficiency, insufficiency, and sufficiency, as judged by different methods, can be well harmonized with no statistically significant inter-system difference. We

were unable to harmonize RIA-DIAsource results by any methods because of its very wide agreement range with HPLC. Further researches are needed to evaluate this method in large population-based studies.

AUTHORS CONTRIBUTIONS

This study was designed and supervised by TRN with intellectual assistance of BN. All statistical analyses were done by BN, HA, and NB. SMS and MRF supervised the tests performed at HRL and Day Laboratory. MZ performed HPLC tests. Other laboratory bench works were performed by AK, EG, YG, NS, BN, and TRN. The manuscript was written by TRN and BN.

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