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An evaluation of automated methods for measurement of serum 25-hydroxyvitamin D

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Abstract

Objectives: To compare two new automated assays with the well-established reference method, DiaSorin radioimmunoassay (RIA), for quantitation of serum total 25-hydroxyvitamin D [25(OH)D].

Methods: 25(OH)D from human sera ($n=158$) was measured using DiaSorin RIA and two automated platforms, DiaSorin "LIAISON 25 OH Vitamin D TOTAL", and Roche Modular "Vitamin D3 (25-OH)". Methods were compared by regression and Bland–Altman analyses.

Results: DiaSorin LIAISON demonstrated a stronger correlation ($r=0.918$) and better agreement (bias=−0.88 nmol/L) with DiaSorin RIA than the Roche Modular assay ($r=0.871$, bias=−2.55 nmol/L). Precision ranges (CV%) for the RIA, LIAISON, and Roche Modular assays, respectively, were: within run (6.8–12.9%, 2.8–8.1%, and 1.9–5.5%), and total precision (7.4–14.5%, 7.3–17.5%, and 7.6–14.5%).

Conclusion: DiaSorin LIAISON displayed the best correlation and agreement with DiaSorin RIA. The DiaSorin LIAISON 25 OH Vitamin D TOTAL assay is an accurate and precise automated tool for serum total 25(OH)D determination.

Keywords: Vitamin D; 25-hydroxyvitamin D; 25(OH)D; Automation; RIA; LIAISON; Roche; Methods evaluation

Introduction

The most reliable indicator of vitamin D status is measurement of 25-hydroxyvitamin D [25(OH)D] in serum or plasma. 25(OH)D, the major circulating metabolite of vitamin D, is produced in the liver by a hydroxylation of vitamin D at carbon 25. Two distinct forms of 25(OH)D exist: 25(OH)D_3, formed from vitamin D_3 (cholecalciferol), and 25(OH)D_2, produced from vitamin D_2 (ergocalciferol). Vitamin D_3 is synthesized naturally in skin exposed to UV radiation and also found in fatty fish. Vitamin D_2 is generated by UV irradiation of the plant sterol, ergosterol, and is less potent than vitamin D_3 [1–3]. Low circulating 25(OH)D concentrations have been associated with increased risk and progression of several diseases, including osteoporosis [4,5], cancers [6–8], multiple sclerosis [9,10], and cardiovascular disease [11,12]. Such research into the role of vitamin D beyond calcium homeostasis has substantially increased clinical interest in vitamin D.

The measurement of 25(OH)D is challenging because circulating 25(OH)D is highly lipophilic, bound strongly to protein, present in low (nanomolar) concentrations, and exists in two structurally similar forms, 25(OH)D_3 and 25(OH)D_2 [13]. Several published methods exist for determining 25(OH)D concentrations, including competitive protein-binding assays, radioimmunoassay (RIA), high performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC-MS), and the more recent automated immunoassays. In 1989, the International External Quality Assessment Scheme for Vitamin D metabolites (DEQAS, Northwest Thames, United Kingdom) was established to monitor the analytical reliability of 25(OH)D assays [14]. However, several reports have

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demonstrated large inconsistency and variability in 25(OH)D measurements between methods and laboratories [15–17]. As a result, some groups have emphasized a need for appropriate reference materials and standardization of 25(OH)D assays [15,16].

The rising clinical demand for assessment of vitamin D status has increased the need for simple, high-throughput methods for measuring 25(OH)D in patient samples. Protein binding assays, HPLC, and LC-MS are manual methods that can be time and labour intensive, technique and operator dependent, and require costly equipment and large sample volumes. The DiaSorin RIA was the first vitamin D test approved for clinical diagnosis by the US Food and Drug Administration (FDA) and has been the most widely used method since. However, being a manual method, the RIA has been challenged by the rapidly increasing demand for 25(OH)D testing. Recently, automated chemiluminescence-based immunoassays have become available which offer higher-throughput capacity, lower sample volume requirement, and reduced operator error. In 2007, DiaSorin received FDA approval for clinical use of its second-generation automated "LIAISON 25 OH Vitamin D TOTAL" chemiluminescent immunoassay (CLIA). More recently, Roche Diagnostics released an automated electrochemiluminescence immunoassay (ECLIA) called "Vitamin D3 (25-OH)" that can be performed on their Elecsys, Modular Analytics, and Cobas analyzers. The objective of the present study was to compare the analytical performance of these two new automated assays (LIAISON and Roche) with the reference method (DiaSorin RIA) for the determination of serum 25(OH)D.

Materials and methods

Samples

Human serum samples ($n=400$) were obtained from a clinical trial in Toronto, Canada (latitude 43°N) in which healthy adults received either 28,000 IU vitamin D_3/week or a placebo for 8 weeks [18]. Serum aliquots were stored at −80 °C until analysis. Under these storage conditions, 25(OH)D is very stable in serum or plasma over a prolonged time and repeated freeze–thaw cycles [13,19,20]. Quantitative determination of serum 25(OH)D was performed in singleton by: DiaSorin "25-hydroxyvitamin D ^{125}I RIA" in April 2007 ($n=390$), DiaSorin "LIAISON 25 OH Vitamin D TOTAL" CLIA in September 2007 ($n=390$), and Roche Modular "Vitamin D3 (25-OH)" ECLIA in October 2007 ($n=158$). The DiaSorin 25(OH)D RIA served as the reference method. Out of the 400 serum samples acquired, 390 samples were analyzed by DiaSorin RIA and DiaSorin LIAISON TOTAL (10 samples had insufficient volume) and 158 samples were analyzed by the Roche Modular assay because there were not enough reagent kits to analyze the full 400 samples. Therefore, direct method comparisons were limited to those samples that were measured by all three assays ($n=158$). The 158 samples pertained to baseline and end-of-study (week 8) measurements of 20 subjects taking placebo and 59 subjects taking vitamin D_3 [18].

25(OH)D assays

DiaSorin 25(OH)D ^{125}I RIA

The DiaSorin 25(OH)D RIA method is based on a competitive principle with a goat antibody against 25(OH)D, an iodinated (^{125}I) 25(OH)D_3 tracer, and donkey anti-goat precipitating complex as secondary antibody. The first part of the assay involves a rapid extraction of 25(OH)D and other hydroxylated metabolites from serum or plasma with acetonitrile. Following extraction, the sample, antibody, and tracer are incubated for 90 min at 20–25 °C. Phase separation is accomplished after a 20 min incubation at 20–25 °C with the secondary antibody. A buffer is then added prior to centrifugation to reduce non-specific binding. Radioactivity is measured by a gamma counter and is inversely proportional to the concentration of 25(OH)D in the sample.

DiaSorin LIAISON 25(OH)D TOTAL CLIA

The LIAISON 25 OH Vitamin D TOTAL Assay is a direct competitive chemiluminescence immunoassay for human serum or plasma intended for use on the DiaSorin LIAISON automated analyzer. The assay uses magnetic particles (solid phase) coated with antibody against 25(OH)D and 25(OH)D conjugated to an isoluminol derivative (tracer). During the first incubation phase (10 min), 25(OH)D is dissociated from binding protein by buffer containing 10% ethanol and then binds to the anti-25(OH)D antibody on the solid phase. After a second 10 min incubation with the tracer, the unbound material is washed off and starter reagents are added to generate a flash chemiluminescent signal which is measured by a photomultiplier and is inversely related to 25(OH)D concentration.

This assay differs from its older version, "LIAISON 25 OH Vitamin D", due to alterations in the on-board extraction procedure, the addition of a second incubation step, and the use of human serum-based calibrators instead of horse serum.

Roche Modular 25(OH)D ECLIA

The Roche Vitamin D3 (25-OH) assay is a direct competitive electrochemiluminescence immunoassay for human serum or plasma intended for use on Roche automated immunoassay analyzers. In this study, the Modular Analytics analyzer was used. The assay employs microparticles coated with streptavidin and a polyclonal sheep antibody against 25(OH)D, which is labeled with ruthenium. In the first incubation, 25(OH)D_3 in the sample competes with biotin labelled 25(OH)D for binding with the anti-25(OH)D antibody. In the second incubation, the biotin-25(OH)D/anti-25(OH)D antibody immunocomplex becomes bound to the microparticles via interaction of biotin and streptavidin. The microparticles are then magnetically captured onto the surface of an electrode. A voltage is applied to the electrode to produce a chemiluminescent emission, which is measured by a photomultiplier and is inversely proportional to 25(OH)D concentration.

Specifications for the three assays, as stated by the manufacturer, are listed in Table 1. According to the product inserts, none of the analytical methods are significantly affected by

Table 1
Assay specifications, as stated in the manufacturer's product insert.

	DiaSorin 25-Hydroxyvitamin D ^{125}I RIA	DiaSorin LIAISON 25 OH Vitamin D TOTAL	Roche Vitamin D3 (25-OH)
Assay format	Extraction, equilibrium RIA	Direct, competitive, CLIA	Direct, competitive, ECLIA
Platform	Manual	Automated	Automated
Analyzer(s)	N/A	LIAISON	Elecsys, Modular Analytics, or Cobas
Sample volume	50 μL	25 μL	35 μL
Sample type	Serum or plasma (EDTA, Hep)	Serum or plasma (EDTA, Hep)	Serum or plasma (EDTA, Hep)
Assay time	110 min	20 min	18 min
Analytical sensitivity	3.75-NR	10–375 nmol/L	10–250 nmol/L
Analytical specificity	% Cross-reactivity	% Cross-reactivity	% Cross-reactivity
Vitamin D3	0.8	<1	<1
Vitamin D2	0.8	<1	<1
25(OH)D3	100	100	100
25(OH)D2	100	104	<10
1,25(OH)2D3	11	17	Up to 100
1,25(OH)2D2	11	40	NR
Precision	% CV	% CV	% CV
Within-run	8.6–12.5	2.9–5.5	3.5–4.9
Total	8.2–11.0	6.3–12.9	4.2–7.8
Method comparison	NR DiaSorin RIA is usually the reference method	n=155, against RIA LIAISON=0.99 (RIA)+2.4 r=0.97	n=291, against automated assay Roche=1.272 (other)−0.045 r=0.912 n=771 against LC-MS-MS Roche=1.008 (LC-MS-MS)+0.045 r=0.902

NR=not reported.

levels of hemolysis or lipemia typically encountered in conventionally collected and prepared samples.

Quality assessment

All assays were performed in accordance with the manufacturer's instructions and complied with our standard operating procedures for good laboratory practice. DiaSorin RIA and LIAISON 25(OH)D results from our laboratory consistently fall within one standard deviation of the group mean in the international DEQAS proficiency surveys. In the January 2009 DEQAS results, the "all methods" mean±SD (CV%) for a test sample was 47.2±6.1 nmol/L (12.9%), compared to 46.7±7.7 nmol (16.5%) for DiaSorin RIA, 46.6±6.0 nmol/L (12.9%) for DiaSorin LIAISON TOTAL, and 52.1±5.9 nmol/L (11.3%) for the Roche assay.

Statistical analyses

Concentrations of 25(OH)D are given in nmol/L units. All data were analyzed with SPSS software (version 13.0) and Analyse-it for Microsoft Excel. The criterion for significance was set at $P<0.05$.

Results

Samples

Overall, 158 complete cases were evaluated. None of the tested samples showed visible signs of hemolysis or lipemia. Descriptive statistics of the 25(OH)D concentrations measured by the DiaSorin RIA, DiaSorin LIAISON, and Roche Modular assays, respectively, were: mean±SD (76.4±39.5, 75.5±39.3, and 73.8±31.2 nmol/L; $P>0.05$), median (66.0, 67.0, and 68.4 nmol/L), 95% CI (70.2–82.6, 69.3–81.7, and 68.9–78.7 nmol/L), and range (16.0–183.0, 17.1–176.0, and 16.7–189.6 nmol/L). Table 2 compares the assays based on the proportion of samples fulfilling commonly-used decision criteria for 25(OH)D status.

Precision

The precision of the RIA, LIAISON, and Roche Modular assays was determined by using 5 human serum-based quality controls (kit, in-house pooled serum, and patient samples), spanning a 25(OH)D range of 35–180 nmol/L. Each control sample was assayed in 2–6 replicates per run for 3–5 runs. Precision values are shown in Table 3. Precision ranges (CV%) for the RIA, LIAISON, and Roche Modular assays, respectively, were: within run (6.8–12.9%, 2.8–8.1%, and 1.9–5.5%), and total precision (7.4–14.5%, 7.3–17.5%, and 7.6–14.5%). These precision values fall within the CV ranges

Table 2
Concordance of assays to 25(OH)D decision criteria.

Serum 25(OH)D	RIA	LIAISON	Roche
N	158	158	158
<40 nmol/L, % (n)	21 (33)	24 (38)	11 (18)
<75 nmol/L, % (n)	54 (85)	55 (87)	58 (91)

Table 3
Within-run and total precision of the 25(OH)D assays evaluated.

Assay/sample	*n*	Mean (nmol/L)	Within-run precision (%CV)	Total precision (%CV)
RIA				
Kit control 1	6	36.8	10.6	12.2
Kit control 2	5	164.6	6.8	7.4
In-house Level 1	15	41.2	9.9	10.2
In-house Level 2	15	88.3	9.2	10.9
In-house Level 3	15	176.9	12.9	14.5
LIAISON				
Kit control 1	16	40.5	7.3	11.3
Kit control 2	18	125.5	5.7	12.8
In-house Level 1	6	57.5	8.1	15.9
In-house Level 2	7	93.0	7.8	17.5
In-house Level 3	7	152.9	2.8	7.3
Roche				
Kit control 1	7	71.9	5.5	10.0
Kit control 2	7	93.0	5.0	9.5
Kit control 3	7	147.2	2.8	8.5
Sample A	4	37.3	1.9	14.5
Sample B	4	106.9	2.2	7.6

typically encountered with 25(OH)D methods in DEQAS (10–20%).

Method correlations

The three methods were compared by both linear and Deming regression. Regression parameters are shown in Table 4. Deming regression plots of the 25(OH)D assays are presented in Fig. 1. Based on the regression analysis, the DiaSorin LIAISON platform correlated best with DiaSorin RIA ($r=0.918$, $n=158$). Furthermore, this correlation, based on samples measured by all 3 assays ($n=158$), was equivalent to the correlation between DiaSorin LIAISON and RIA 25(OH)D assays in the larger trial cohort ($r=0.917$, $n=390$). The Roche Modular method correlated reasonably well with DiaSorin RIA ($r=0.871$, $n=158$) and LIAISON ($r=0.862$, $n=158$).

Method agreement

The agreement among 25(OH)D assays was analyzed by the mean, difference method of Bland and Altman [21]. Bland–Altman analyses of the 25(OH)D methods are presented in Fig. 2. LIAISON showed little bias when compared to DiaSorin RIA [bias±SD (95% CI)=−0.88±15.95 (−3.38 to 1.63) nmol/L]. Roche Modular demonstrated higher bias compared to DiaSorin RIA [−2.55±19.67 (−5.64 to 0.54) nmol/L] than to LIAISON [−1.67±20.14 (−4.83 to 1.50) nmol/L].

Discussion

The assessment of vitamin D status, through the measurement of 25(OH)D in serum or plasma, has received considerable attention in the last decade. A growing number of studies have reported widespread vitamin D deficiency in apparently healthy populations worldwide [22–26]. Low vitamin D status, defined by low circulating 25(OH)D, has been associated with several diseases, including osteoporosis [4,5], cancers [6–8], multiple sclerosis [9,10], cardiovascular disease [11,12], diabetes [27,28], and microbial infections [29,30]. A recent meta-analysis demonstrated that vitamin D supplementation was associated with a 7% reduction in total mortality [31]. The latest consensus indicated that a serum 25(OH)D concentration of 75 nmol/L or greater is sufficient or optimal for health [32], putting the majority of the North American and European population at varying levels of deficiency. Therefore, the measurement of circulating 25(OH)D is becoming increasingly important and the clinical demand for 25(OH)D assays has risen substantially.

The DiaSorin ^{125}I-based RIA has been the method of choice for measuring 25(OH)D concentrations. However, the RIA is time-consuming, labour intensive, and employs radioactive compounds, which pose a health hazard and limit automation. The increasing demand for non-radioactive, high-throughput 25(OH)D measurement has led to the development of several automated platforms. DiaSorin and Roche Diagnostics have recently introduced fully automated immunoassay systems employing chemiluminescent technology for 25(OH)D determination in serum or plasma. Here, we have compared these automated methods with the reference method, DiaSorin RIA. To our knowledge, the comparison of DiaSorin LIAISON Total, Roche Modular, and DiaSorin RIA methods for 25(OH)D has never been reported in the literature. We found that DiaSorin LIAISON demonstrated a stronger correlation ($r=0.918$) and better agreement (bias=−0.88 nmol/L) with the DiaSorin RIA reference method than the Roche Modular assay ($r=0.871$, bias=−2.55 nmol/L).

Table 4
Regression (linear, Deming) and correlation parameters of the methods being compared.

Comparison	*n*	Linear regression	Deming regression	Correlation
LIAISON vs. RIA	158	LIAISON=0.91 (RIA)+5.75	LIAISON=0.99 (RIA)−0.42	$r=0.918$
		Sy|x=15.625	Sy|x=15.951	*rho*=0.938
	390	LIAISON=0.91 (RIA)+5.80	LIAISON=0.99 (RIA)−0.90	$r=0.917$
		Sy|x=14.02	Sy|x=14.32	*rho*=0.923
Roche vs. RIA	158	Roche=0.69 (RIA)+21.30	Roche=0.76 (RIA)+15.57	$r=0.871$
		Sy|x=15.365	Sy|x=15.65	*rho*=0.895
Roche vs. LIAISON	158	Roche=0.68 (LIAISON)+22.20	Roche=0.77 (LIAISON)+16.04	$r=0.862$
		Sy|x=15.894	Sy|x=16.217	*rho*=0.894

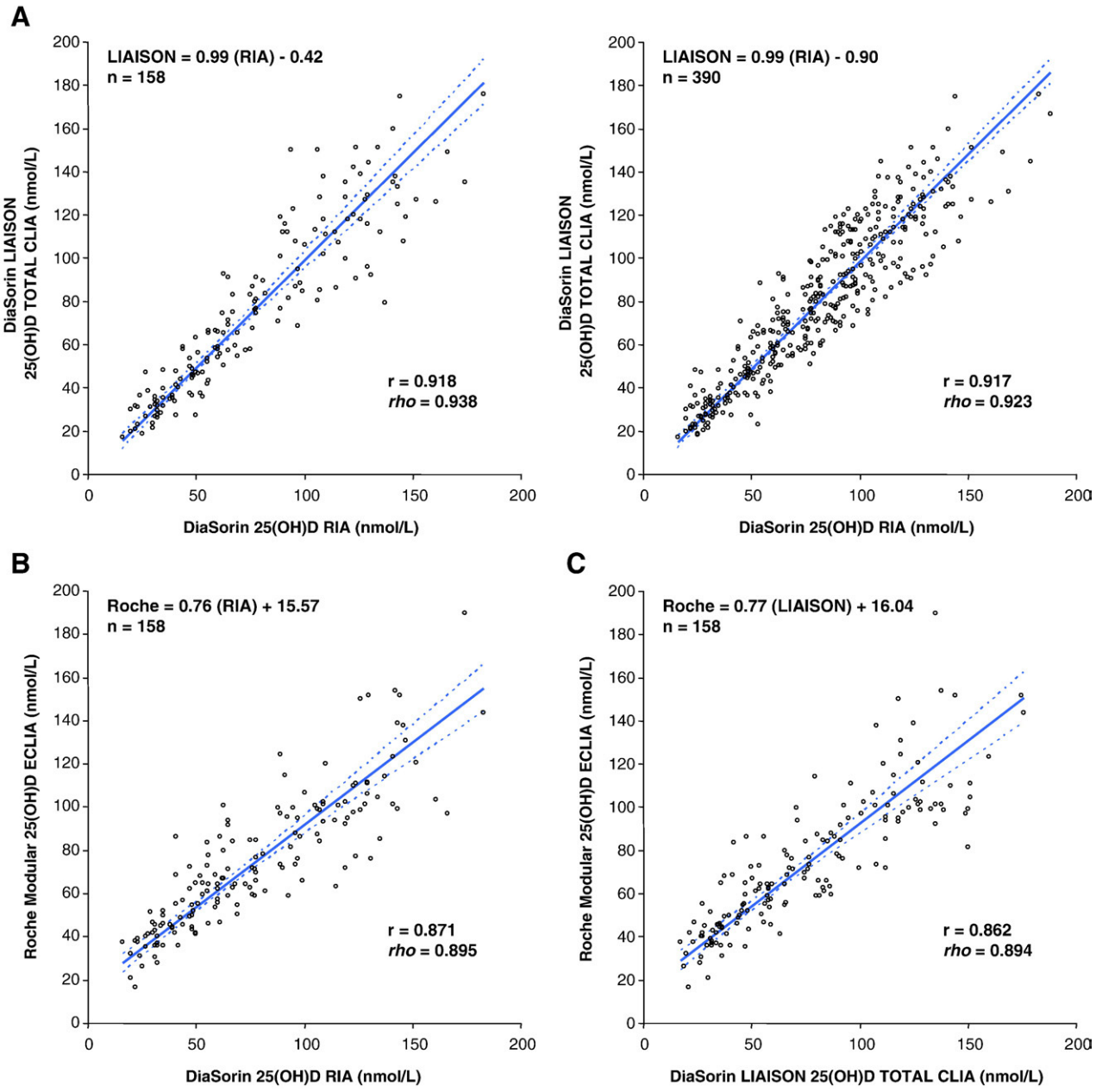

Fig. 1. Deming regression of 25(OH)D quantitation comparisons: DiaSorin RIA reference method with DiaSorin LIAISON Total and Roche Modular automated platforms. (A) DiaSorin RIA vs. DiaSorin LIAISON Total (left: $n=158$; right: $n=390$, full cohort). (B) DiaSorin RIA vs. Roche Modular ($n=158$). (C) DiaSorin LIAISON Total vs. Roche Modular ($n=158$). Both the Pearson and the non-parametric Spearman correlation coefficients are indicated. Dotted lines indicate 95% confidence intervals.

This study employed the second-generation and most recent version of the LIAISON assay, "LIAISON 25 OH Vitamin D TOTAL". Our results indicate that this modified version showed a higher correlation with DiaSorin RIA than reported previously with the older version of the assay, "LIAISON 25 OH Vitamin D" [17,33]. Recently, Roth et al. [34] evaluated the accuracy of several 25(OH)D methods, including the LIAISON and Roche assays presented here. However, they chose LC-MS/MS as their reference method and made no direct comparisons between the automated methods and RIA, making it difficult to compare data. DiaSorin RIA is the more appropriate reference method because its use is the basis of virtually all the research linking circulating 25(OH)D to health and disease outcomes and reference values [32,35]. In contrast, there have been no large clinical trials demonstrating decision-based reference values for 25(OH)D based upon clinical data using LC-MS methods. Nonetheless, the LIAISON and Roche assays demonstrated good correlation and agreement with LC-MS/MS. Leino et al. [36] recently showed that the Roche 25(OH)D assay performed similarly against DiaSorin RIA ($r=0.836$, $n=163$) to what we report here. Our investigation had the advantage of having compared two new automated assays with a method (DiaSorin RIA) that has set the standard for clinical diagnosis of vitamin D deficiency [13]. Furthermore, we analyzed a large number of

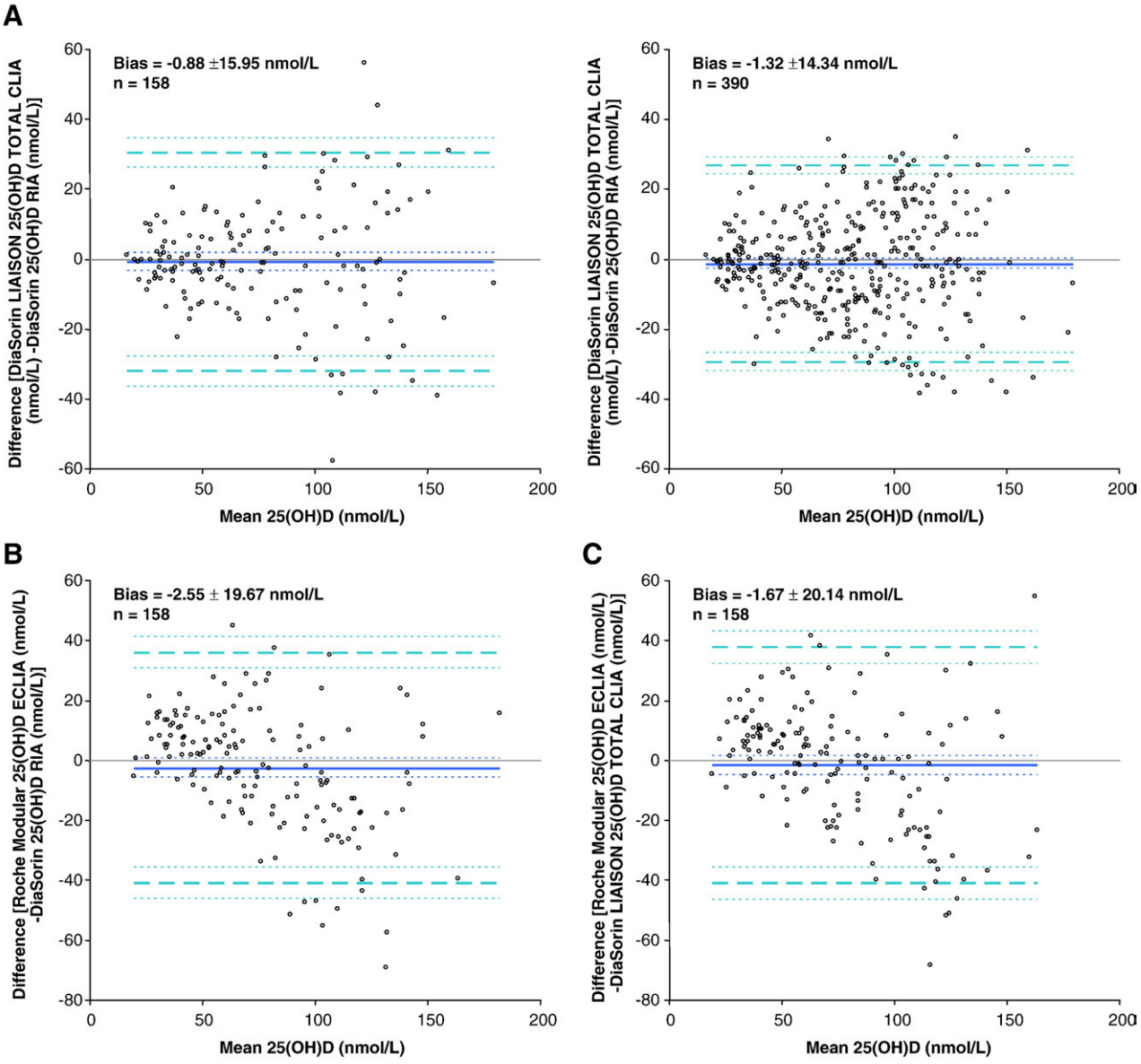

Fig. 2. Bland–Altman plots, showing means of paired differences between 25(OH)D quantitation comparisons: DiaSorin RIA reference method with DiaSorin LIAISON Total and Roche Modular automated platforms. (A) DiaSorin RIA vs. DiaSorin LIAISON Total (left: $n=158$; right: $n=390$, full cohort). (B) DiaSorin RIA vs. Roche Modular ($n=158$). (C) DiaSorin LIAISON Total vs. Roche Modular ($n=158$). Thick solid lines show bias (means of paired differences), denoted numerically as bias±SD. Thin solid lines represent lines of identity. Dashed lines show 95% limits of agreement (bias±1.96*SD). Dotted lines indicate 95% confidence intervals.

samples with a wider range of 25(OH)D concentrations than previously reported because we used the baseline and final serum samples from a placebo-controlled, vitamin D dosing clinical trial [18]. An appropriate methods evaluation should test a broad range of analyte concentrations. This is particularly relevant for vitamin D because there is a wide distribution of 25(OH)D in the general population.

Clinical diagnosis and treatment decisions related to vitamin D are based on assessment of total 25(OH)D concentration. Therefore, the analytical method of choice should detect 25$(OH)D_3$ and 25$(OH)D_2$ equally to report an accurate total 25(OH)D value. The major limitation of the Roche assay is its inability to detect 25$(OH)D_2$. As stated in the product insert (Table 1), the Roche assay has <10% cross-reactivity with 25$(OH)D_2$. In contrast, the DiaSorin RIA and LIAISON assays claim 100% cross-reactivity with 25$(OH)D_2$ and 25$(OH)D_3$ on an equimolar basis. Consumption of supplements or foods containing vitamin D_2 (e.g. mushrooms) will contribute to total 25(OH)D concentrations; however, this contribution would be underestimated by the Roche assay. It is unlikely that vitamin D_2 significantly affected the total 25(OH)D concentrations measured in our Canadian subjects because vitamin D_3 is more commonly used in Canada. However, this problem would be more pronounced in the US, where vitamin D_2 is commonly used, and also in patients receiving pharmaceutical preparations of high-dose vitamin D, which only exist as vitamin D_2 (e.g.

Calciferol or Drisdol). It is more likely that the lower correlation and agreement of the Roche assay with the reference method is related to the assay itself. As shown in Table 2 and Fig. 2B, the Roche Modular assay tended to overestimate 25(OH)D at low concentrations (<40–50 nmol/L) and underestimate 25(OH)D at high concentrations (>75–100 nmol/L). For example, when DiaSorin RIA reference values of 25 nmol/L and 150 nmol/L are applied to the Deming regression equations, the corresponding DiaSorin LIAISON concentrations are 24.3 nmol/L and 148.1 nmol/L, and the Roche Modular values are 34.6 nmol/L and 129.6 nmol/L, respectively. Furthermore, following our analysis, we noticed the same discrepancy in the January 2009 DEQAS results. For example, the DiaSorin RIA ($n=16$ laboratories) mean±SD for a "low" DEQAS test sample was 26.5±4.2 nmol, compared to 22.1±3.5 nmol/L for DiaSorin LIAISON TOTAL ($n=99$), and 44.8±9.9 nmol/L for the Roche assay ($n=15$). In contrast, the DiaSorin RIA ($n=16$) mean±SD for a "high" DEQAS test sample was 79.3±13.8 nmol, compared to 73.3±8.8 nmol/L for DiaSorin LIAISON TOTAL ($n=100$), and 53.0±6.1 nmol/L for the Roche assay ($n=15$). The discordant sensitivity at the lower and upper end of the measuring range may be related to the extraction procedure, the antibody used, or matrix effects that lead to variability in individual patient samples [36]. Furthermore, this analytical issue with the Roche assay could have negative implications in the clinical assessment of vitamin D status. However, we believe that the performance and validity of the Roche assay can be improved by modifying the method to correct the problems outlined.

Within-run precision was generally higher in the automated LIAISON and Roche Modular assays compared to the manual RIA. The Roche method displayed the best within-run precision, however, its quality controls cover a relatively high 25(OH)D range (~60–180 nmol/L), compared to the ranges encompassed by the DiaSorin RIA and LIAISON quality controls (~30–180 nmol/L) which are more representative of values observed in the general population. Total precision was not substantially different among the three assays. Of note, our precision values were slightly lower than those reported in the product inserts (Table 1). A more comprehensive evaluation of precision performance would have used a greater number of replicates and runs than those used in the present study.

The variation among 25(OH)D methods observed in the present study was smaller than previously reported, but still illustrates the need for standardization of 25(OH)D assays. We conclude that the DiaSorin LIAISON 25 OH Vitamin D TOTAL assay is an accurate and precise tool for the determination of 25 (OH)D. The LIAISON assay exhibited better correlation and agreement with the reference RIA method than the recently introduced Roche assay. Automated, accurate 25(OH)D methods provide greater speed and convenience, and improve work flow and efficiency in the high-throughput clinical laboratory as it continues to meet the increasing demand for 25(OH)D testing. The diagnostic field awaits an automated assay for determination of 1,25-dihydroxyvitamin D [1,25(OH)2D], the active hormonal metabolite of vitamin D.

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