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Review

A novel, fully-automated, chemiluminescent assay for the detection of 1,25-dihydroxyvitamin D in biological samples

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ABSTRACT

Background: 1,25-Dihydroxyvitamin D (1,25-$(OH)_2$D), the hormonal form of vitamin D, is difficult to measure because of its low circulating levels (pg/mL), and similarity to more abundant metabolites. Here a fully-automated chemiluminescent assay that accurately and precisely measures 1,25-$(OH)_2$D is described.

Method: The novel 1,25-$(OH)_2$D assay was conceived based on four pillars: (1) the VDR's ligand binding domain (LBD) as a capture molecule; (2) reaction conditions wherein 1,25-$(OH)_2$D favors binding to LBD vs. the vitamin D binding protein; (3) exploitation of liganded-LBD's conformational change; (4) a monoclonal antibody specific to liganded-LBD. This specific, conformational, sandwich approach, unique for automated measurement of haptens, is superior to more cumbersome, conventional competitive formats.

Results: Accuracy of the 1,25-$(OH)_2$D assay was corroborated by its alignment against LC–MS/MS with fit Deming regression equations of $y = 0.98x + 1.93$ ($r = 0.92$), and $y = 1.07x + 3.77$ ($r = 0.94$) for different methods from Endocrine Sciences, Laboratory Corporation of America® and the University of Washington, respectively. Good analytical precision was manifested by its low estimated limit of quantitation (1.57 pg/mL), average intra-assay imprecision (3.5%CV; range 1.1–4.7%), and average inter-assay imprecision (4.5%CV; range 3.4–7.2%). Expected and measured recovery values were congruent (93.4% mean).

Conclusions: The novel 1,25-$(OH)_2$D method exhibited excellent correlation with well validated LC–MS/MS assays from two laboratories. Significantly, its 65 min turn-around time is quicker, and sample volume smaller (75 μl) than current methods.

Contents

Abbreviations: LBD, ligand binding domain; VDBP, vitamin D binding protein; 25-(OH)D, 25 hydroxyvitamin D; 1,25-$(OH)_2$D, 25-dihydroxyvitamin D; VDR, vitamin D receptor; VDRE, vitamin D response element; LOQ, limit of quantitation; LOB, limit of blank; Mab, monoclonal antibodies; NMR, nuclear magnetic resonance; DPI, Dual Polarization Interferometry; DEQAS, vitamin D External Quality Assessment Scheme.

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Introduction

The synthesis of vitamin D_3 in skin is a two-step process: 7-dehydrocholesterol is converted to previtamin D_3 upon exposure to UV light; previtamin D_3 isomerizes to vitamin D [1]. Vitamin D_3 and vitamin D_2 (that originates from irradiation of ergosterol) may also be obtained in the diet. [1]. Upon entry into the circulation, vitamin D is readily hydroxylated in the liver at carbon-25 to produce 25-hydroxyvitamin D (25-(OH)D), the major circulating metabolite [2]. Vitamin D metabolites generally associate with the vitamin D binding protein (VDBP) to be carried through the bloodstream [3]. Although 25-(OH)D is inactive itself, its serum level affords the commonly accepted method for assessing vitamin D status of patients.

The classical role of vitamin D is the maintenance of plasma calcium and phosphate at near constant levels for proper neuromuscular function, bone mineralization, and the prevention of hypocalcemic tetany [4]. Under physiological conditions where calcium and phosphate homeostasis is disturbed, 25-(OH)D is converted in the kidney by 1α-hydroxylase to produce 1α,25-dihydroxyvitamin D (1,25-$(OH)_2$D), which acts on target organs to normalize plasma calcium and phosphate [4]. Most, if not all, of circulating 1,25-$(OH)_2$D is produced in the kidney as revealed by the extremely low circulating levels in nephrectomized rats [5] or patients [6].

The functions of 1,25-$(OH)_2$D are mediated through a nuclear receptor that binds to vitamin D response elements (VDREs) in target genes to regulate their transcription. The vitamin D receptor (VDR) is a member of the steroid/thyroid hormone nuclear receptor superfamily and contains characteristic functional domains including the DNA binding domain and the ligand binding domain (LBD) [7]. The structure of the LBD was recently solved by NMR studies, which depict a distinct, ligand-specific conformational change upon binding of 1,25-$(OH)_2$D [8].

It has long been established that vitamin D deficiency causes bone diseases such as rickets, osteomalacia, and secondary hyperparathyroidism, but with the discovery of VDR in non-classical target tissues, 1,25-$(OH)_2$D is now known to have a broader spectrum of actions, and has been associated with increased risks for various chronic diseases including infectious and autoimmune, diabetes, cancer, cardiovascular ailments, hypertension and complications during pregnancy [9]. Clinical studies implicating vitamin D in the forgoing conditions have been based predominantly upon measurement of 25-(OH)D. Since 1,25-$(OH)_2$D is the active hormone and does not correlate with 25-(OH)D status, important relationships have remained unexplored. Facile measurement of 1,25-$(OH)_2$D has been difficult due to its extremely low circulating levels (pg/mL) [10]. Current methods, requiring laborious, sample consumptive, pre-analytical steps are significantly compromised at the low end of their measuring range because of their intrinsic imprecision [10]. In this manuscript, a new, fully-automated method for measurement of 1,25-$(OH)_2$D in biological fluids is presented. The novel assay is accurate, precise throughout the entire assay range, fast, and requires only 75 μl of sample unencumbered by the need for pre-analytical enrichment through precipitation and subsequent reconstitution. The exquisite specificity and avidity of the VDR for its substrate was used to produce the first sandwich, automated chemiluminescent assay for a small molecule hapten like 1,25-$(OH)_2$D.

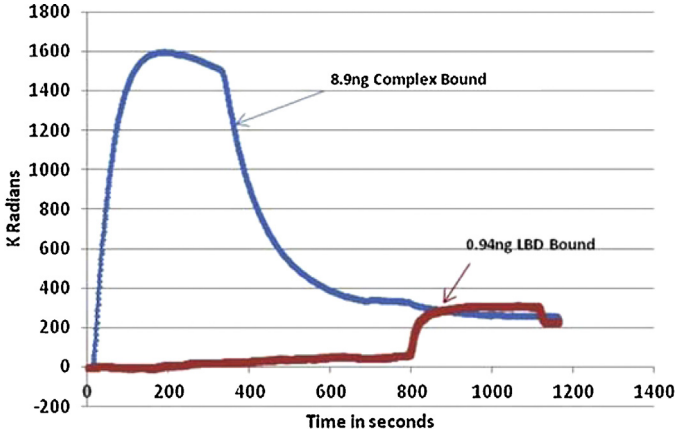

Fig. 1. Specificity of the 11B4 antibody used in the novel 1,25-$(OH)_2$D assay. A Dual Polarization Interferometry experiment was performed by coating a 2-channel chip with the monoclonal antibody 11B4 raised against the liganded LBD. Channel 1 was injected with LBD-1,25-$(OH)_2$D complex (blue), and channel 2 was injected with apo-LBD (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Specimens

To evaluate the LIAISON® XL 1,25-Dihydroxyvitamin D assay in biological fluids, 78 human serum samples routinely submitted to Laboratory Corporation of America for 1,25-$(OH)_2$D testing were anonymized and used to make pools with concentrations covering the analytical measuring range of the assay and parsed into multiple aliquots for analysis by multiple methods. The protocol for this study was determined to be exempt under existing regulations by the Institutional Review Board.

2.2. Method comparison

Method comparison of 1,25-$(OH)_2$D measurement included two different LC–MS/MS methods using immune-extraction to enrich for 1,25-$(OH)_2$D as a pre-analytical step; the first method was from Endocrine Sciences, Laboratory Corporation of America (Calabasas Hills, CA), and the second LC–MS/MS analysis was performed at the University of Washington and is outlined by Strathmann et al. [11]. A commercially available immunoassay (Method A) was also compared to the LIAISON® XL 1,25-dihydroxyvitamin D assay (DiaSorin, Stillwater, MN).

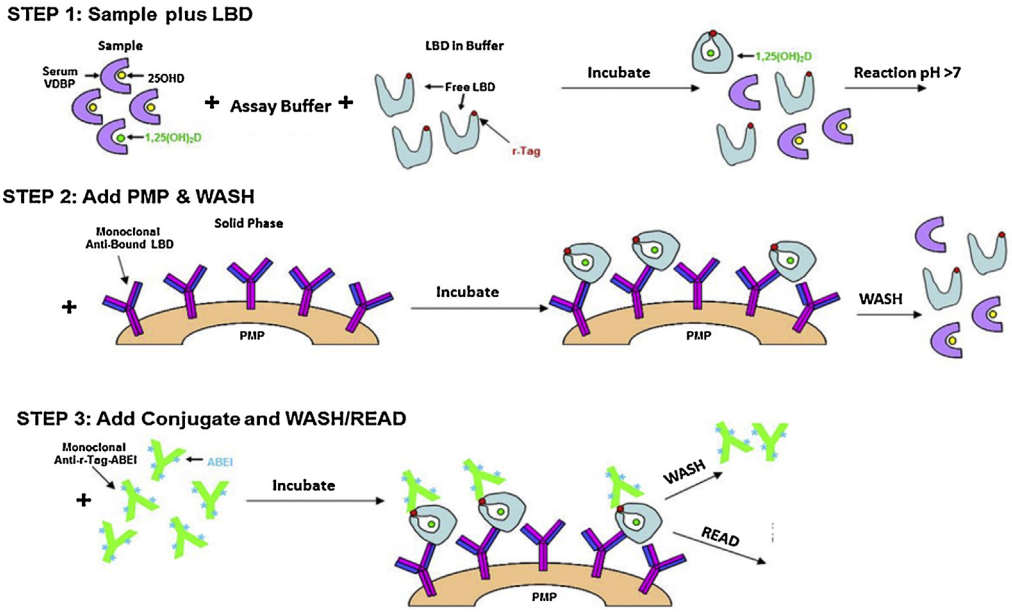

Fig. 2. Schematic of the novel 1,25-$(OH)_2$D assay. An overview of the components, incubations [3], and wash steps [2] of the fully-automated assay to be run on a LIAISON® XL instrument. Sample size required is 75 μl, and time to first result is 65 min. PMP: paramagnetic particles; ABEI: isoluminol derivative.

2.3. Performance evaluation

Imprecision, recovery, limit of quantitation (LOQ), limit of blank (LOB), linearity, and analytical specificity were evaluated according to CLSI guidelines. The reference range was verified on a subset of 41 apparently healthy blood donor volunteers.

2.4. Statistical analysis

All data were analyzed using EP Evaluator® (Data Innovations, LLC). Linear regressions were performed by regular and Deming methods using *Analyse-it*™ for Microsoft Excel.

2.5. The novel 1,25-dihydroxyvitamin D assay

Specimens were tested to evaluate the novel 1,25-$(OH)_2$D method using the CE marked, Investigational Use Only DiaSorin LIAISON® XL 1,25-Dihydroxyvitamin D assay (Stillwater, MN). The novelty and efficacy of this 1,25-$(OH)_2$D assay depend upon four key design pillars: (**1**) use of the ligand binding domain (LBD) of the VDR as a capture molecule; (**2**) use of reaction conditions where binding of 1,25-$(OH)_2$D to the LBD is favored over binding to the VDBP by ~200-fold. At those same conditions, inactive metabolites of vitamin D, including 25(OH)D, 24,25-dihydroxyvitamin D, and 25,26-dihydroxyvitamin D, preferentially bind to the VDBP by 10-100-fold creating a binding differential of 2000-20,000-fold (which greatly minimizes interference from the 100-1000-fold higher physiological concentrations of inactive metabolites compared to 1,25$(OH)_2$D); (**3**) exploitation of the LBD conformational change induced upon 1,25-$(OH)_2$D binding as delineated by NMR analysis [8]; (**4**) the use of a monoclonal antibody that selectively recognizes the unique LBD conformation produced when 1,25-$(OH)_2$D is bound to the LBD.

Monoclonal antibodies (Mab) against the LBD-1,25-$(OH)_2$D complex screened by ELISA were characterized by Dual Polarization Interferometry, DPI (AnaLight 4D, Farfield Group, UK). DPI measures real time binding events on the face of a biosensor chip. The selected clone 11B4 accumulated 8.9 ng of the LBD-1,25-$(OH)_2$D complex within the first 400 sec as opposed to a minimal amount of unbound, control apo-LBD during the same time frame (Fig. 1), showing the high specificity of the 11B4 Mab for the LBD-1,25-$(OH)_2$D complex.

Fig. 2 depicts the DiaSorin LIAISON® XL 1,25-Dihydroxyvitamin D assay's mechanics. First, 75 μl of sample are incubated with the ligand binding domain (LBD) of the VDR. Under incubation conditions, the transfer of 1,25-$(OH)_2$D from VDBP to the LBD is greatly favored. Second, coated magnetic beads with Mab 11B4, specific to the LBD-1,25-$(OH)_2$D complex conformation, are added to the reaction mixture. Following incubation, unbound materials (unbound-LBD, VDPB, inactive vitamin D metabolites) are removed with a wash cycle. Finally in step 3, conjugate (Mab with a chemiluminescent label) specific to a distal epitope of the LBD is added, and upon binding to the solid phase bound LBD-1,25-$(OH)_2$D complex a sandwich is completed.

Following a second wash step to remove unbound conjugate, starter reagents are added triggering a chemiluminescent reaction. The light flash emitted, measured by a photomultiplier tube, affords a signal directly proportional to the amount of 1,25-$(OH)_2$D present in the sample.

3. Results

3.1. Accuracy

A method comparison was performed on 78 patient samples. Fig. 3 shows a Deming regression fit (left) and an Altman–Bland difference plot (right) for each method pairing. In Fig. 3A, excellent agreement is observed for the LIAISON® XL 1,25-Dihydroxyvitamin D assay against the Endocrine Sciences, Laboratory Corporation of America reference LC–MS/MS, with correlation coefficient $R = 0.92$, slope of 0.98 (0.90–1.06 95% CI) and an intercept of 1.93 (−1.81–5.67, 95% CI). The mean bias derived from the Altman–Bland difference plot was 2.4% (−0.5–5.2%, 95% CI) with 95% limits of agreement between −22.5% and 27.3%. Fig. 3B shows good alignment against the University of Washington LC–MS/MS that

uses immuno-enrichment of 1,25-$(OH)_2$D as part of its procedure with correlation coefficient $R = 0.94$, slope of 1.07 (1.00–1.15, 95% CI) and an intercept of 3.77 (0.41–7.13, 95% CI). The mean bias was 15.5% (12.8-18.3% 95% CI) with 95% limits of agreement between −8.1% and 39.2%.

The above comparisons indicate that the LIAISON® XL 1,25-Dihydroxyvitamin D assay is comparable to LC–MS/MS, which is considered a higher order method with respect to accuracy.

The LIAISON XL 1,25-Dihydroxyvitamin D assay was also compared to a commercially available assay (Method A) and resulted in less than optimal alignment with correlation $R = 0.90$, slope of 0.78 (0.69-0.88, 95% CI) and an intercept of 11.38 (6.79–15.98, 95% CI). The mean bias was 3.1% (−0.6%–6.9%, 95% CI) with 95% limits of agreement between −29.4% and 35.7% (Fig. 4). As shown in Fig. 3C&D, Method A aligned poorly with LC–MS/MS with Deming regression fit equations of $y = 1.26x - 12.42$ and $y = 1.37x - 9.75$ when compared to Endocrine Sciences, Laboratory Corporation of America and University of Washington LC–MS/MS methods, respectively.

Recovery studies were performed to determine agreement between expected and measured amounts. Three sets of low and high samples were mixed at different ratios (3:1, 1:1 and 1:3) and tested. Table 1 depicts neat, expected, and measured concentrations, as well as the percent recovery which was calculated from the measured and expected values. The assay had a mean recovery of 93.4%.

3.2. Imprecision and sensitivity

The LIAISON® XL 1,25-Dihydroxyvitamin D assay was evaluated for intra-assay imprecision using 2 levels of control and 4 patient sample pools. Twenty replicates of each of the controls and 4 patient pools were assayed. Inter-assay precision was performed in 5 assay runs with at least 4 replicates of each of the 2 levels of control and 4 sample pools. The average intra-assay imprecision was 3.5%CV (range 1.1-4.7%CV), and inter-assay imprecision average was 4.5%CV (range 3.4–7.2%CV) (Table 2).

One of the beneficial characteristics of this assay, is its extremely low imprecision, even at low 1,25-$(OH)_2$D concentrations (<5 pg/mL). A LOQ study was performed by measuring samples with concentrations ranging from 0.342 to 16.5 pg/mL for 10 days. In Fig. 5 EP Evaluator was used to derive the estimated LOQ (1.57 pg/mL) based on a fitted curve. As shown %CV remain relatively constant down to sample concentrations as low as 1–2 pg/mL, while other methods routinely exhibit higher imprecision especially at concentrations below 15 pg/mL [11–15].

The limit of blank was determined with a total of 10 replicates of the zero standard. The lowest non-zero concentration that could be differentiated from zero was calculated as 0.071 pg/mL (95% confidence) using EP Evaluator®, which is well below the manufacturer's claim of 0.35 pg/mL.

3.3. Linearity

One serum sample with a concentration of 217 pg/mL was chosen. Sample dilutions of 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% and 0% (neat diluent) were assayed. Diluted sample concentrations were measured and compared against calculated values. The slope of the determined linear regression was 1.02 with an intercept of 3.779. Average recovery was 107.8% (range 103.2–110.9%)

3.4. Specificity

Consistent with the design elements enumerated above in Section 2, the LIAISON XL 1,25-Dihydroxyvitamin D assay exhibits high specificity for 1,25-$(OH)_2$D with minimal cross-reactivity against other vitamin D metabolites (25-$(OH)D_{2+3}$, 24,25-dihydroxyvitamin D_3, 25,26-dihydroxyvitamin D3, 3-epi-25(OH)D3, vitamin D_{2+3}). The lack of correlation between levels of 25-(OH)D and levels of 1,25-$(OH)_2$D in the samples (Pearson's correlation $R = 0.15$, $P = 0.18$) supports the specificity of this assay (Fig. 6).

Interfering substances were evaluated for the LIAISON XL 1,25-dihydroxyvitamin D assay using the Assurance Interference Test Kit from Sun Diagnostics New Gloucester, ME. The effect of hemolysis and icteric and lipemic matrix constituents on measurement were evaluated based on CLSI EP7-A. No significant interference was observed for triglycerides (3000 mg/dL), hemolysate (500 mg/dL), conjugated bilirubin (20 mg/dL) and unconjugated bilirubin (20 mg/mL).

3.5. Reference range

Forty-one ostensibly "normal" patient samples (samples from healthy volunteers) were assayed to verify the reference range for serum samples as stated by the manufacturer per package insert (25.0–86.5 pg/mL). The central 95% interval revealed by the data for the 41 points assayed was 29.7–88.8 pg/mL. The mean of the samples tested was 54.4 pg/mL.

4. Conclusions

A novel method was developed for the measurement of 1,25-$(OH)_2$D in biological samples. The unique design of this assay makes it the first fully-automated, small-molecule, sandwich chemiluminescent assay available and allows for the measurement of a molecule that is present at 1000x lower concentrations than other quite similar metabolites. The assay is extremely accurate when compared to LC–MS/MS and has low imprecision, especially near its limit of quantitation. The assay has a rapid time to first result (65 min) with a throughput of 50–80 tests/hr thereafter, requires significantly smaller sample volume (75 μl), and performs well in terms of accuracy, imprecision, sensitivity, linearity and specificity. The novel assay does not require any pre-analytical extraction or purification steps, which greatly diminishes its imprecision which was shown to be very low in this evaluation. Gold-standard methods like LC–MS/MS, however, still require pre-analytical processing steps which can contribute to higher imprecision, attributable to extraction in addition to measurement, rather than just direct measurement.

The assay is robust, and its close alignment with LC–MS/MS standardization is supported by its performance in the vitamin D External Quality Assessment Scheme (DEQAS), where multiple labs perform very well against the target value and with very small variation between participants (data not shown). van Helden and Weiskirchen also reported on their evaluation of the assay and concluded that the novel 1,25-$(OH)_2$D assay is highly robust [16].

The simplicity of use, and its ability to accurately measure even small concentrations of 1,25-dihydroxyvitamin D make this assay a valuable tool for future prospective and retrospective studies in patients with not only bone and mineral metabolism diseases, but also in other disease states such as infectious and autoimmune diseases, diabetes, cancer, cardiovascular ailments, hypertension and complications during pregnancy, where associations to 25(OH)D have been previously shown, but may need to be revisited to determine the role of the active form of vitamin D, 1,25-$(OH)_2$D in subjects/patients with low circulating 25(OH)D.

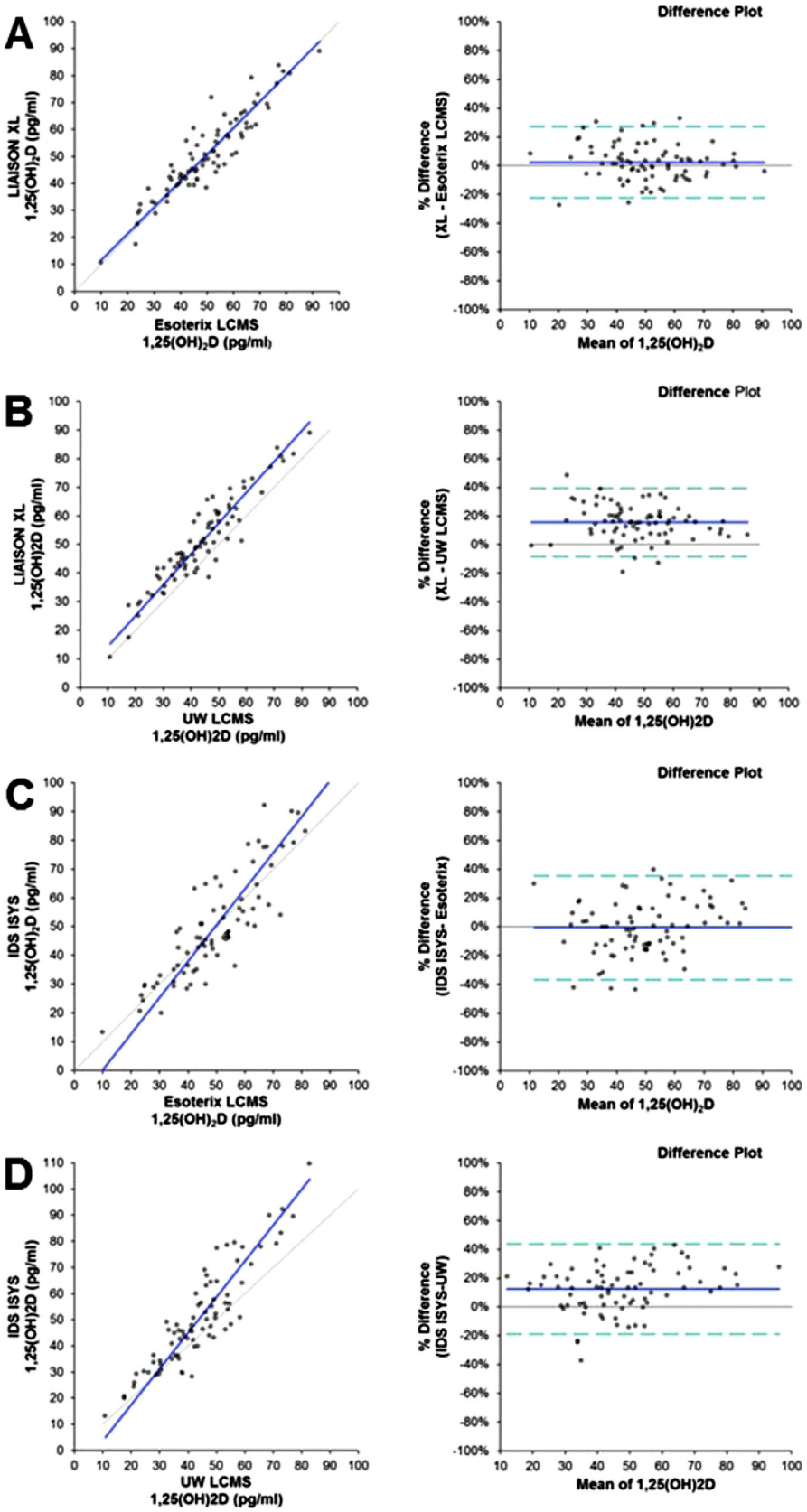

Fig. 3. 1,25-$(OH)_2$D method comparisons. 78 patient samples were analyzed by 4 different methods: Endocrine Sciences, Laboratory Corporation of America LC–MS/MS, University of Washington LC–MS/MS, commercially available Method A, and LIAISON® XL immunoassays. Deming regression (left) and Altman–Bland difference plots with mean bias and 95% limits of agreement (right) were generated for each method comparison shown.
(A) Total 1,25-$(OH)_2$D was measured with Endocrine Sciences, Laboratory Corporation of America LC–MS/MS as a reference method compared to the LIAISON XL novel assay. The blue line is the Deming regression with a slope of 0.98 (0.90–1.06, 95% CI) and an intercept of 1.93 (−1.81–5.67, 95% CI). Correlation coefficient R =0 .92. The mean bias is 2.4% (−0.5–5.2%, 95% CI).
(B) Total 1,25-$(OH)_2$D was measured with University of Washington LC–MS/MS as a reference method compared to the LIAISON XL novel assay. The blue line is the Deming regression with a slope of 1.07 (1.00–1.15, 95% CI) and an intercept of 3.77 (0.41-7.13, 95% CI). Correlation coefficient R = 0.94. The mean bias is 15.5% (12.8–18.3%, 95% CI).

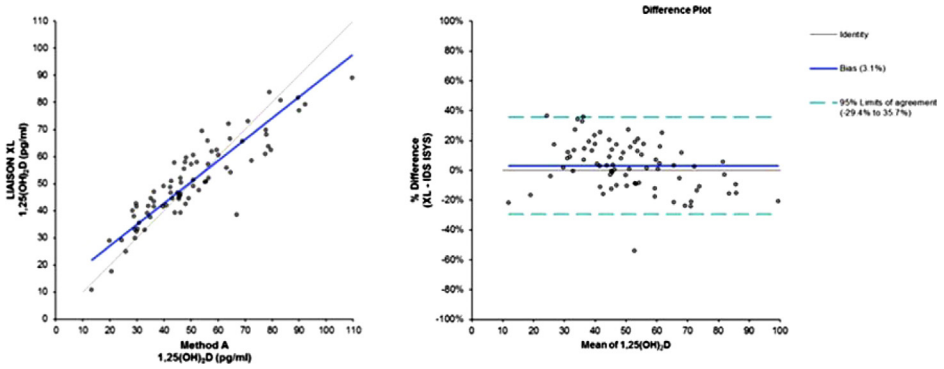

Fig. 4. Total 1,25-$(OH)_2$D was measured with Method A and compared to the LIAISON XL novel assay. The blue line is the Deming regression with a slope of 0.78 (0.69–0.88, 95% CI) and an intercept of 11.38 (6.79–15.98, 95% CI). Correlation coefficient $R = 0.90$. The mean bias is 3.1% (−29.4–35.7%, 95% CI). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Recovery of 1,25-$(OH)_2$D in serum using the novel 1,25-$(OH)_2$D assay. Three separate specimens (low samples) were assayed to determine circulating levels of 1,25-$(OH)_2$D using the novel assay. Each sample was mixed with different concentrations of another patient sample (high sample) through sample mixing at ratios of 3:1, 1:1 and 1:3 (low sample: high sample) and assayed. The high samples were also assayed neat. Percent recoveries were calculated from measured and expected values.

	Defined Concentration	Expected pg/mL	Measured pg/mL	Recovery %
Low Sample 1 (LS1)	**17.4**		17.4	
0.75 LS1:0.25 HS1		54.6	51.4	94.2%
0.50 LS1:0.50 HS1		91.7	86.3	94.1%
0.25 LS1:0.75 HS1		128.9	122.3	94.9%
High Sample 1 (HS1)	**166.0**		166.0	
Low Sample 2 (LS2)	**17.8**		17.8	
0.75 LS2:0.25 HS2		62.4	58.4	93.7%
0.50 LS2:0.50 HS2		106.9	95.7	89.5%
0.25 LS2:0.75 HS2		151.5	139.7	92.2%
High Sample 2 (HS2)	**196.0**		196.0	
Low Sample 3 (LS3)	**21.8**		21.8	
0.75 LS3:0.25 HS3		59.1	54.7	92.6%
0.50 LS3:0.50 HS3		96.4	91.9	95.3%
0.25 LS3:0.75 HS3		133.7	126.3	94.5%
High Sample 3 (HS3)	**171.0**		171.0	

Table 2
Intra—and Inter-assay imprecision for the novel 1,25-$(OH)_2$D assay. The 1,25-$(OH)_2$D assay was evaluated for intra-assay imprecision using 2 levels of control and 4 patient sample pools. Twenty replicates of each of the controls and 4 patient pools were assayed. The inter-assay imprecision was performed in 5 assay runs on different days with at least 4 replicates of each of the 2 levels of control and 4 sample pools.

		Intra-assay			Inter-assay		
Sample	N	Mean (pg/mL)	SD (pg/mL)	CV %	Mean (pg/mL)	SD (pg/mL)	CV %
Control 1	20	25.5	1.06	4.2	23.2	0.78	3.4
Control 2	20	94.9	4.48	4.7	105.7	3.99	3.8
Patient Pool 1	20	25.8	1.06	4.1	27.6	1.05	3.8
Patient Pool 2	20	42.3	0.45	1.1	44.5	2.00	4.5
Patient Pool 4	20	91.8	3.86	4.2	89.0	6.37	7.2
Patient Pool 5	20	180.4	4.50	2.5	178.5	8.10	4.5

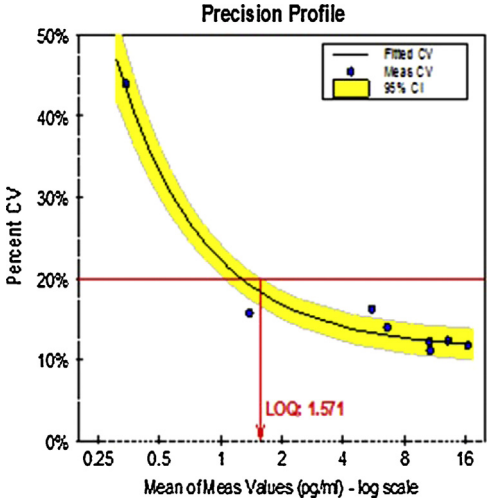

Fig. 5. Sensitivity: Limit of Quantitation (LOQ). Eight samples around the limit of quantitation indicated by the manufacturer (5 pg/mL) were measured for 10 days and %CVs were determined. EP Evaluator® was used to estimate the LOQ, the point at which the fitted curve crosses the 20% CV line. The estimated LOQ is 1.57 pg/mL.

(C) Total 1,25-$(OH)_2$D was measured with Endocrine Sciences, Laboratory Corporation of America LC–MS/MS as a reference method compared to Method A. The blue line is the Deming regression with a slope of 1.26 (1.11–1.41, 95% CI) and an intercept of −12.42 (−20.01 to −4.83, 95% CI). Correlation coefficient is $R = 0.87$. The mean bias is −0.7% (−4.9–3.4%, 95% CI)
(D) Total 1,25-$(OH)_2$D was measured with University of Washington LC–MS/MS as a reference method compared to Method A. The blue line is the Deming regression with a slope of 1.37 (1.24–1.50, 95% CI) and an intercept of −9.75 (−15.58 to -3.92 95% CI). Correlation coefficient is $R = 0.90$. The mean bias is 12.4% (8.8–16.0%, 95% CI).

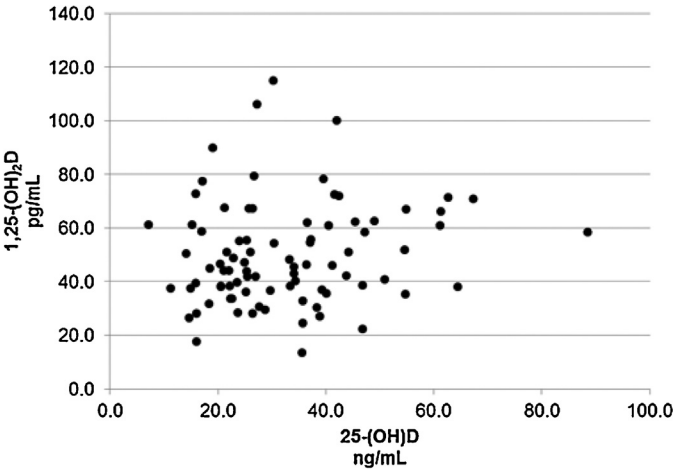

Fig. 6. Measurements of 25-(OH)D and 1,25-$(OH)_2$D for each sample are displayed by scatter plot. Pearson's correlation analysis showed no significant correlation between levels of 25-(OH)D and 1,25-$(OH)_2$D ($R = 0.15$, $P = 0.18$).

Previous presentation

The method was presented at the 2014 IFCC World Lab in Instanbul, Turkey.

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