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Evaluation of two fully automated immunoassay based tests for the measurement of 1α,25-dihydroxyvitamin D in human serum and comparison with LC-MS/MS

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Abstract

Background: 1α,25-Dihydroxyvitamin D [1,25$(OH)_2$ vitD] is the bioactive form of vitamin D. Due to the very low concentrations of 1,25$(OH)_2$ vitD in the blood and its lipophilic character, measurement of this parameter is analytically challenging. Requiring preceding manual extraction steps before analysis, previous assays have been laborious.

Methods: In the presented study, we evaluated the performance of two immunoassays from DiaSorin and from Immunodiagnostic Systems (IDS) which combine fully automated extraction and measurement of 1,25$(OH)_2$ vitD. Imprecision and linearity were verified according to Clinical and Laboratory Standards Institute EP15-A3 and EP6-A guidelines, respectively. Ninety-three patient serum samples sent to our institute for determination of 1,25$(OH)_2$ vitD, as well as 20 Vitamin D External Quality Assessment Scheme (DEQAS) samples, were used to evaluate correlation and agreement of 1,25$(OH)_2$ vitD measurements between the two immunoassays and with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Results: Total imprecision was 5.2% or less for the DiaSorin test but reached 20.1% for the IDS iSYS test. 1,25$(OH)_2$ vitD concentrations measured with the DiaSorin assay showed a strong correlation with 1,25$(OH)_2$ vitD levels measured by LC-MS/MS and a good agreement with method specific means of DEQAS samples. By contrast, the IDS iSYS test overestimated 1,25$(OH)_2$ vitD concentrations in human serum, particularly at higher concentrations.

Conclusions: Due to its high sensitivity, low imprecision, broad measurement range, and good agreement with 1,25$(OH)_2$ vitD concentrations measured by LC-MS/MS, the DiaSorin test is a valuable analytical option for the determination of 1,25$(OH)_2$ vitD.

Keywords: 1α,25-dihydroxyvitamin D; immunoassay; method comparison; serum.

Introduction

1α,25-Dihydroxyvitamin D [1,25$(OH)_2$ vitD], the active form of vitamin D, is synthesized by mitochondrial 25-hydroxyvitamin D-1α-hydroxylase in the kidney and in extrarenal tissues. In addition to the kidney, particularly disease-activated tissue macrophages and the placenta may contribute to circulating concentrations of 1,25$(OH)_2$ vitD [1]. 1,25$(OH)_2$ vitD mediates actions generally ascribed to vitamin D including the absorption of calcium and phosphorus from the intestine, the retention of calcium from the kidney and bone mineralization, thereby preventing rickets and osteomalacia. Recently observed associations of low vitamin D levels with increased risks of cardiovascular diseases [2–4], type 2 diabetes [5], autoimmune diseases [6], infections of the upper respiratory tract [7], neurodegenerative disease [8], as well as breast or colorectal cancer [9, 10] point to extraskeletal actions of vitamin D. The expanded knowledge of the relevance of sufficient vitamin D levels is reflected by an experienced increased demand for the determination of vitamin D status in patients.

Although 1,25$(OH)_2$ vitD is less suitable than 25-hydroxyvitamin D [25(OH) vitD] to assess vitamin D status, there are some indications to specifically determine 1,25$(OH)_2$ vitD serum levels. This mainly includes differential diagnosis of hypercalcemia in patients with sarcoidosis and other granulomatous diseases. Determination of 1,25$(OH)_2$ vitD can also be useful in patients with unexplained hyperparathyroidism who have adequate 25(OH) vitD levels. 1,25$(OH)_2$ vitD is particularly

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suitable to differentiate between two hereditary defects namely vitamin D-dependent rickets (VDDR) type I, caused by vitamin D 1α-hydroxylase deficiency, and VDDR type II, caused by mutation of the vitamin D receptor gene leading to end-organ resistance to vitamin D. The growing knowledge of the prognostic significance of $1,25(OH)_2$ vitD after cardiac surgery and in sepsis and heart failure [11–13] will further increase the medical need to analyze $1,25(OH)_2$ vitD.

Due to its low concentration (pmol/L) and lipophilic nature, the quantification of $1,25(OH)_2$ vitD in serum is analytically challenging and requires extraction and separation steps prior to measurement. So far, mainly radioimmunoassays have been used for the measurement of $1,25(OH)_2$ vitD. They measure this parameter with sufficient sensitivity but are laborious, time consuming and prone to laboratory error associated with manual handling of the probes. The first automated assay including manual extraction had been developed by Immunodiagnostic Systems (IDS) Ltd., using the IDS iSYS immunoanalyzer. Recently, two assays became commercially available, which include not only the automated measurement but also an automated extraction of $1,25(OH)_2$ vitD prior to quantification. In this study, we evaluated and compared the performance of these two fully automated assays with each other and with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

The measurement of $1,25(OH)_2$ vitD is poorly standardized. Whereas for the standardization of 25(OH) vitD measurements, a reference measurement procedure as well as National Institute of Standards and Technology standard reference material is available [14, 15], for $1,25(OH)_2$ vitD, a lot of work remains to be done to render the results from different laboratories and methods comparable. The Vitamin D External Quality Assessment Scheme (DEQAS) supports this standardization process by providing external quality control material not only for 25(OH) vitD but also for $1,25(OH)_2$ vitD. We therefore compared the values measured using the fully automated assays with the results obtained by DEQAS.

Materials and methods

Samples

Remaining material of 142 patient serum samples submitted to the Institute of Clinical Chemistry of the University Hospital Zurich for routine measurement of $1,25(OH)_2$ vitD was aliquoted and stored at −80 °C until analysis. All samples with extensive hemolysis, bilirubinemia or lipemia were excluded from the study. For analysis with the different tests, serum samples were thawed and one aliquot each was analyzed according to the manufacturer's instructions. Of 93 samples, sufficient serum material was available for measurement of $1,25(OH)_2$ vitD concentrations using a LC-MS/MS based method.

Only samples of patients aged 18 years or older were included in the study. The study has been approved by the Zurich Cantonal Ethical Committee.

Immunoassays

Serum samples were measured with the LIAISON XL 1,25 Dihydroxyvitamin D assay (DiaSorin, Stillwater, MN, USA) on the LIAISON XL platform and with the IDS-iSYS 1,25 VitD^{Xp} assay (IDS, Boldon, UK) on the IDS-iSYS Multi-Discipline Automated System. Both tests are chemiluminescence-immunoassays and both tests capture the analyte from the probe prior to measurement of analyte concentration. Whereas the DiaSorin test uses a recombinant fusion protein to capture $1,25(OH)_2$ vitD from the probe [16], the IDS test uses anti-$1,25(OH)_2$ vitD antibody coated magnetic particles for immunopurification. After several washing steps, the purified analyte is measured by an immunoassay. The DiaSorin test determines the $1,25(OH)_2$ vitD concentration directly after addition of the conjugate and a starter reagent to induce the light reaction [16]. The resulting signal is directly proportional to the analyte concentration. The IDS test, in contrast, uses a competitive immunoassay for detection of $1,25(OH)_2$ vitD concentration, where a certain concentration of an $1,25(OH)_2$ vitD acridinium conjugate competes for the binding sites of a biotinylated sheep anti-$1,25(OH)_2$ vitD antibody. Thus, the resulting signal is inversely proportional to the amount of the analyte in the probe.

The performance characteristics of the immunoassays are outlined in Table 1.

Mass spectrometry

For measurement of $1,25(OH)_2$ vitD with LC-MS/MS, aliquots were shipped on dry ice to the laboratory of Prof. Hoofnagle at the University of Washington (Seattle, WA, USA). $1,25(OH)_2$ vitD2 and $1,25(OH)_2$ vitD3 serum concentrations were measured there as previously described [17].

Imprecision and accuracy

Assay imprecision has been estimated according to Clinical and Laboratory Standards Institute (CLSI) EP15-A3 guidelines [18]. Manufacturers' quality control material as well as patient serum samples have been analyzed over 5 days with five measurements a day. From the obtained 25 measurements, within-run, between-run, and total imprecision have been estimated.

Linearity

Serial dilutions of a sample with high $1,25(OH)_2$ vitD concentration have been prepared using serum with very low or even non-measureable low levels as diluent in order to cover the measurement range of the tests. $1,25(OH)_2$ vitD concentrations were determined in

Table 1: Assay specifications of the two commercial assays for determination of $1,25(OH)_2$ vitD used in this study.

Assay	LIAISON XL 1,25 Dihydroxyvitamin D assay	IDS-iSYS 1,25 VitD^{Xp} assay
Analyzer	LIAISON XL	IDS iSYS
Principle	CLIA with prior extraction	CLIA with prior extraction
Extraction of $1,25(OH)_2$ vitD	Recombinant fusion protein	Anti-$1,25(OH)_2$ vitD antibody
Antibody used for $1,25(OH)_2$ vitD determination	Mouse, monoclonal	Sheep, monoclonal
Label	Luminol	Acridinium
Measuring range	5.0–200.0 ng/L	7.5–210.0 ng/L
LoQ	5.0 ng/L	Not given
Needed amount of serum	225 µL (75 µL + 150 µL dead volume)	400 µL (220 µL + 180 µL dead volume)
Cross reactivity		
$1,25(OH)_2$ vitD3	100% (100 ng/L)	98% (conc. not given)
$1,25(OH)_2$ vitD2	104% (100 ng/L)	57% (conc. not given)
25(OH) vitD3	< 0.1% (100,000 ng/L)	0.0014% (conc. not given)
25(OH) vitD2	< 0.1% (50,000 ng/L)	0.0005% (conc. not given)
$24,25(OH)_2$ vitD3	< 0.1% (50,000 ng/L)	0.0005% (conc. not given)
$24,25(OH)_2$ vitD2	< 0.1% (50,000 ng/L)	0.041% (conc. not given)
3-epi25(OH) vitD3	< 0.1% (50,000 ng/L)	0.0005% (conc. not given)

Data derived from package inserts provided by the manufacturers. CLIA, chemiluminescence immunoassay; LoQ, limit of quantification.

duplicates and measured serum concentrations were compared to expected concentrations. Polynomial regression analysis has been used for the assessment of linearity according to the National Committee on Clinical Laboratory Standards (NCCLS) EP6-A evaluation protocol [19]. A linear or non-linear coefficient was assumed to be statistically significant if p was < 0.05.

Method comparison

Frozen aliquots were thawed and equilibrated to room temperature. Samples were mixed gently and analyzed in batches on the IDS iSYS and the LIAISON XL immunoanalyzer. Quality control samples were analyzed together with each batch of samples to monitor the performance of the assays and the instruments. Correlation and agreement between the tested methods were assessed by means of Passing-Bablok regression analysis [20], Bland-Altman difference plots [21], and determination of Pearson's coefficient of correlation. For correlation analysis, samples below the lower limit of the measurement range were assigned the value of the lower limit (7.5 and 5.0 ng/L for the IDS test and the DiaSorin test, respectively).

Statistical analysis

All statistical analyses were performed using Analyse-it, a statistical software for Microsoft Excel (Analyse-it Software Ltd.)

Results

Imprecision and accuracy

For the determination of imprecision, quality control material available from the respective manufacturer as well as human serum pools were analyzed according to the CLSI EP15-A3 guidelines. Imprecision results from a total of 25 measurements of each probe are shown in Table 2.

The DiaSorin test determined $1,25(OH)_2$ vitD very precisely, with total imprecision ranging between 3.1% and 5.2% and hence within the range of 3.6%–6.6% reported by the manufacturer. In contrast, the total imprecision of the IDS test ranged between 7.1% and 20.1%. Intra- and inter-assay imprecisions were between 5.6% and 19.8% and between 3.5% and 15.3%, respectively, as compared to intra-assay and inter-assay imprecisions reported by the manufacturer, being 6.4%–12.1% and 4.6%–9.6%, respectively. However, for the imprecision determined by the manufacturer, only serum samples with concentrations of 25.3 ng/L and above have been investigated. Furthermore, we found that the DiaSorin test measured $1,25(OH)_2$ vitD concentrations with better accuracy than the IDS test, namely with a bias of 2.5% and – 0.1% as compared to 21.3% and 14.1%.

Linearity

A series of dilutions was prepared by adding different amounts of serum with very low or non-measurable low $1,25(OH)_2$ vitD concentrations to a serum sample with high $1,25(OH)_2$ vitD concentrations. Within the measurement ranges either test showed good correlations between measured and expected $1,25(OH)_2$ vitD concentrations (Figure 1). Statistical analysis revealed that none of the non-linear coefficients was statistically significant. Thus,

Table 2: Imprecision and inaccuracy of the IDS iSYS and the DiaSorin LIAISON XL- 1,25(OH)$_2$ vitD assays.

Sample	Human serum				Control 1	Control 2
	Pool 1	Pool 2	Pool 3	Pool 4		
IDS iSYS						
Mean concentration, ng/L	14.4	27.3	108.1		43.2	88.6
Target concentration, ng/L (range)					35.6 (18.5–52.7)	77.7 (49.7–105.7)
Bias, ng/L					7.6	10.9
Bias, %					21.3	14.1
Imprecision, %						
Total (within laboratory)	20.1	18.9	7.1		15.9	8.9
Within run (repeatability)	19.8	11.1	5.8		7.5	5.6
Between run	3.5	15.3	4.1		14.0	7.0
DiaSorin LIAISON XL						
Mean concentration, ng/L	8.5	13.0	29.8	91.9	31.2	121.9
Target concentration, ng/L (range)					30.4 (21.9–38.9)	122.0 (92.8–151)
Bias, ng/L					0.8	– 0.1
Bias, %					2.5	– 0.1
Imprecision, %						
Total (within laboratory)	4.4	5.2	4.1	3.1	4.5	3.3
Within run (repeatability)	2.5	4.4	3.7	1.5	3.5	2.5
Between run	3.6	2.8	1.8	2.7	2.9	2.1

For measurement of imprecision in human serum human serum pools were used. Controls 1+2: quality control material level 1 and 2 of the respective test. There were no statistical outliers as tested by Grubbs' test.

for the IDS test as well as the DiaSorin test a linear equation models the data best. Reflecting the high imprecision, the IDS test showed poorer agreement between duplicate measurements.

Method comparisons

Correlation and agreement between the immunoassays

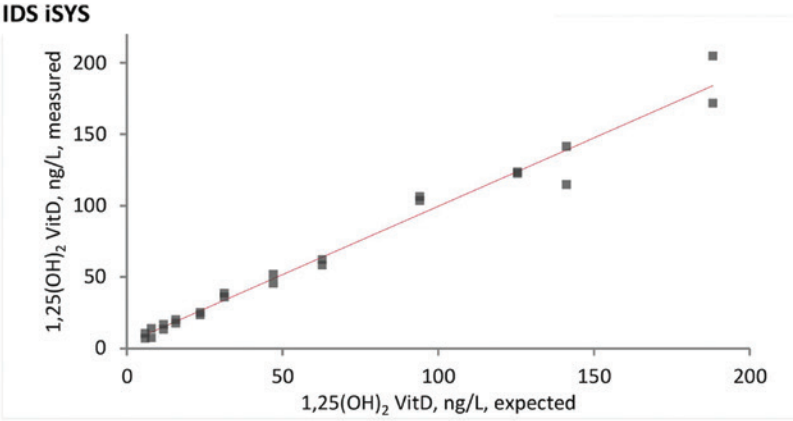
One hundred forty-two serum samples, sent for routine analysis of 1,25(OH)$_2$ vitD to our institute, were measured with both, the LIAISON XL 1,25 Dihydroxyvitamin D assay and the IDS-iSYS 1,25 VitD^{Xp} assay (for correlation see Supplementary Figure 1). In the 93 patient samples with sufficient material for measurement with either method, the immunoassays and LC-MS/MS, 1,25(OH)$_2$ vitD concentrations ranged from < 7.5 to 202.5 ng/L if measured with the IDS test, from < 5.0 to 126.0 ng/L if measured with the DiaSorin test, and from 3.0 to 118.6 ng/L if measured by LC-MS/MS. 1,25(OH)$_2$ vitD concentrations measured with the IDS test tended to be higher than those measured with the DiaSorin assay (Figure 2A). This was most pronounced in the higher concentration range (above about 40 ng/L) as indicated by the respective Bland-Altman-Plot (Figure 2B). Over the whole measurement range, the scattering of the values was high as reflected by the 95% limits of agreement of the bias (±1.96*standard deviation [SD]): Bland-Altman analysis revealed a mean bias of 4.9%, the lower and upper limits of agreement were – 67.5% and 77.4%, respectively.

Correlation and agreement of the immunoassays with the LC-MS/MS method

To check for accuracy, results obtained by the two immunoassays on 93 patient samples were compared with the results obtained by LC-MS/MS, a method propagated as the reference method for 1,25(OH)$_2$ vitD measurement. Compared to the IDS test, the DiaSorin measurement results showed stronger correlations with the LC-MS/MS results (r = 0.852, Figure 2C vs. r = 0.967, Figure 2E). Moreover, the IDS test overestimated 1,25(OH)$_2$ vitD serum concentrations especially in the higher concentration range, whereas the DiaSorin test showed good agreement. In the Bland-Altman analysis mean bias was 7.0% (95% LoA, – 69.8% and 83.9%) for the IDS test but only 2.3% (95% LoA, – 29.2% and 33.7%) for the DiaSorin test (Figure 2D, F).

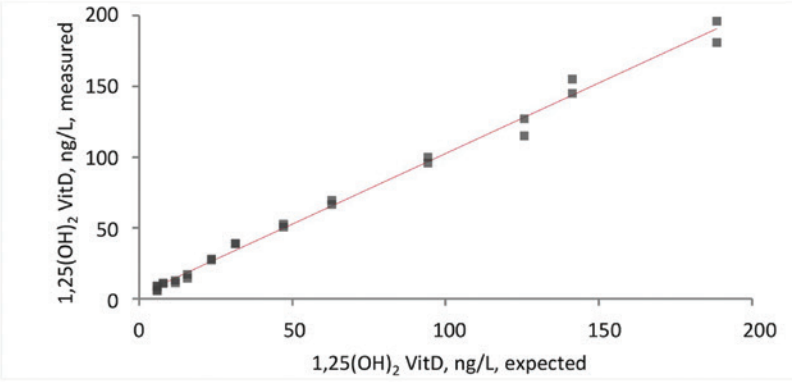
Unlike the immunoassays, LC-MS/MS can distinguish between 1,25(OH)$_2$ vitD2 and 1,25(OH)$_2$ vitD3. The majority of the samples had very low 1,25(OH)$_2$ vitD2 levels, only 6 of the 93 samples with total 1,25(OH)$_2$ vitD concentrations between 7.8 and 85.1 ng/L measured with LC-MS/MS contained 5% or more 1,25(OH)$_2$ vitD2. One of these

IDS iSYS



Order	Coeff. Symbol	Coeff. value	p-Value
first	b0	3.860	0.1401
	b1	0.9568	< 0.0001
second	b0	4.020	0.2584
	b1	0.9492	< 0.0001
	b2	4.311×10^{-5}	0.9453
third	b0	0.6700	0.8887
	b1	1.215	< 0.0001
	b2	– 0.003631	0.3245
	b3	1.286×10^{-5}	0.3118

DiaSorin LIAISON XL



Order	Coeff. Symbol	Coeff. value	p-Value
first	b0	2.897	0.0415
	b1	0.9964	< 0.0001
second	b0	1.644	0.3546
	b1	1.061	< 0.0001
	b2	-3.766×10^{-4}	0.2720
third	b0	0.3292	0.8886
	b1	1.177	< 0.0001
	b2	– 0.002005	0.3084
	b3	5.757×10^{-6}	0.3990

Figure 1: Assessment of method linearity for the IDS iSYS and the DiaSorin $1,25(OH)_2$ vitD assay.
For evaluation of the IDS test a serum sample with a mean concentration of 188.2 ng/L $1,25(OH)_2$ vitD was diluted with a serum sample with a $1,25(OH)_2$ vitD concentration below the detection limit of the test. For the DiaSorin test a serum probe containing 188.5 ng/L $1,25(OH)_2$ vitD was diluted with serum with a $1,25(OH)_2$ vitD concentration of 5.8 ng/L. Statistical analysis was performed according to the NCCLS EP6-A evaluation protocol.

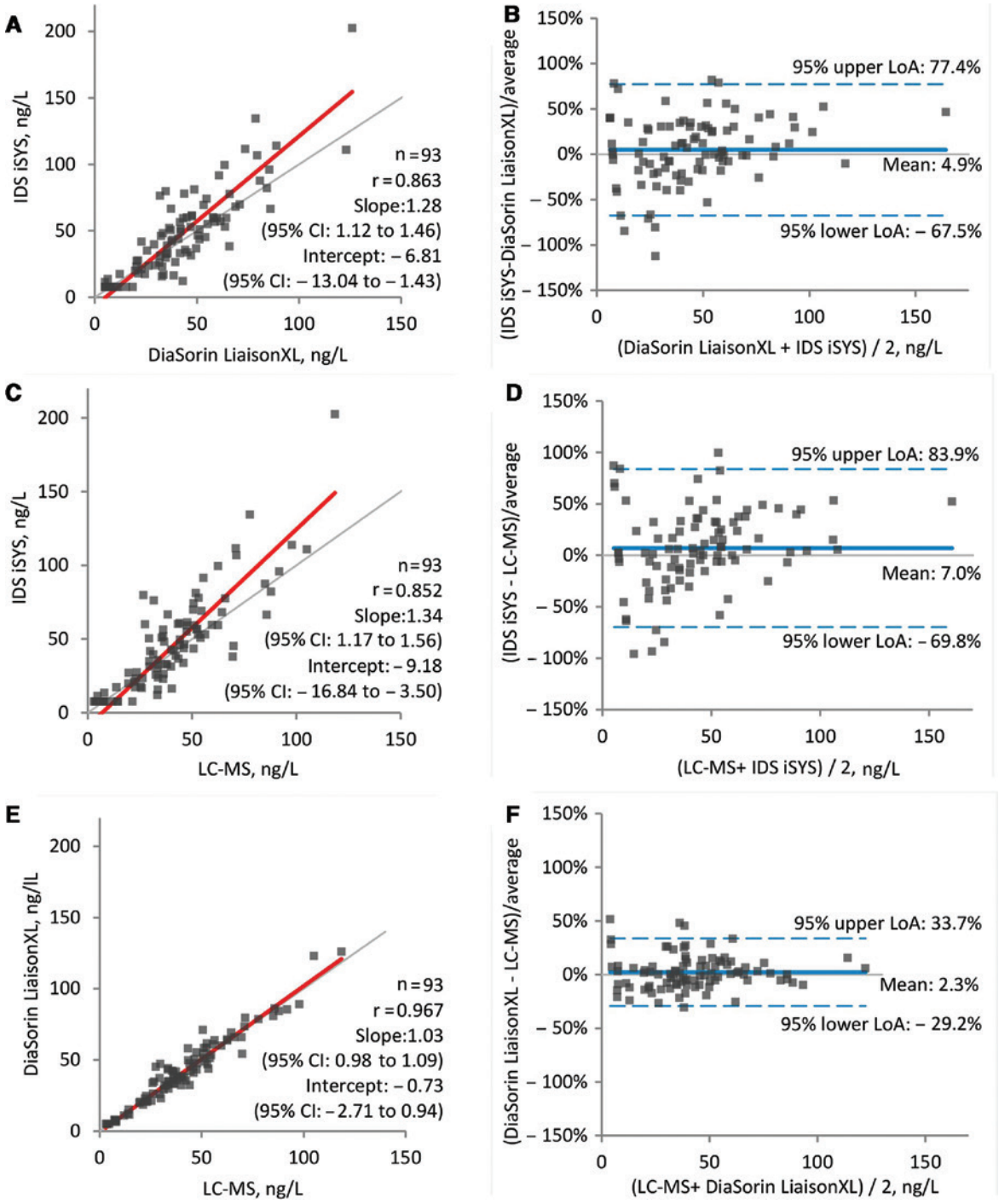


Figure 2: Method comparison of the two fully automated immunoassays and a LC-MS based method for measurement of $1,25(OH)_2$ vitD. Left panel: Passing-Bablok regression analysis of the IDS with the DiaSorin test (A) and the IDS (C) and the DiaSorin test (E), respectively, with LC-MS as a reference method. The bold red solid line represents the regression line, the fine grey line represents the line of equality. Right panel: Bland-Altman plots for comparison of the IDS with the DiaSorin assay (B) and the IDS (D) and the DiaSorin assay (F), respectively, with LC-MS. The bold blue solid line indicates the relative mean bias, the dashed blue lines represent the upper and lower limit of agreement, respectively.

samples had a $1,25(OH)_2$ vitD concentration below the detection limit when measured with the IDS test and was therefore excluded from statistical analysis for this test. The $1,25(OH)_2$ vitD concentrations of the samples with $1,25(OH)_2$ vitD2 concentrations between 5.8% and 76.6% of total $1,25(OH)_2$ vitD showed a mean deviation of 86.7

(IDS) and 98.2% (DiaSorin) from $1,25(OH)_2$ vitD3 measured by LC-MS/MS, but of only 3.7 (IDS) and 1.2% (DiaSorin), respectively, when compared to the sum of $1,25(OH)_2$ vitD2 and $1,25(OH)_2$ vitD3. This suggests nearly complete cross-reactivity of either immunoassay with $1,25(OH)_2$ vitD2.

Performance in external quality control measurements

To date, no international standard is available for the measurement of $1,25(OH)_2$ vitD. To compensate for this lack, we measured 20 samples from DEQAS, the largest vitamin D quality assessment program worldwide, with the two novel immunoassays to compare our measurements with the results of other laboratories (Table 3). Seventy-five percent and 95% of results measured by the IDS test and the DiaSorin test, respectively, are within 1 SD of the method specific mean. None of our results showed deviations of 2 or more SDs from the method mean. Thus, measurements in our laboratory are comparable to the results obtained by other laboratories using the IDS iSYS or the DiaSorin test.

Both immunoassays showed a good agreement of the quality control sample measurements with LC-MS/MS. Mean deviations of the IDS and DiaSorin measurement results from the mean of LC-MS/MS measurements were 2.1% and 1.8%, respectively. However, individual measurements with the IDS test deviated more strongly from the LC-MS/MS method (range, – 51% to 50%) than measurements with the DiaSorin test (– 20% to 26%), reflecting the higher imprecision of the IDS test as seen in the method comparison and the imprecision study (Figure 3).

Discussion

In the presented study, we evaluated the analytical performance of two new commercially available fully automated assays for the measurement of $1,25(OH)_2$ vitD. Both immunoassays allow for determination of $1,25(OH)_2$ vitD within a shorter time frame compared to radioimmunoassays and other tests with manual sample pretreatment. Overall, the DiaSorin test performed better in terms of accuracy, sensitivity and imprecision compared to the IDS iSYS test.

The DiaSorin test running on the LIAISON XL analyzer measured $1,25(OH)_2$ vitD with good sensitivity and high reliability. Precision was very good even in the low concentration range and comparable to precision values previously described. Van Helden and Weiskirchen

Table 3: Immunoassay measurement results and method means and standard deviations of DEQAS samples.

DEQAS sample ID	IDS iSYS			DiaSorin			LC-MS/MS	
	Measurement Value, ng/L	Method mean (SD)[b], ng/L	n[a]	Measurement Value, ng/L	Method mean (SD)[b], ng/L	n[a]	Method mean (SD)[b], ng/L	n[a]
351	19.3	22.6 (6.2)	32	21.0	22.8 (4.6)	17	25.1 (7.0)	13
352	43.5	42.6 (7.3)	32	38.8	37.3 (7.7)	17	41.6 (13.1)	13
353	70.8	64.6 (10.4)	32	52.7	52.6 (9.7)	17	55.4 (22.0)	13
354	69.8	61.8 (9.7)	32	49.7	48.1 (8.0)	17	46.3 (13.1)	13
355	58.8	58.7 (7.7)	32	57.3	53.5 (9.0)	17	49.9 (15.5)	13
361	37.8	37.3 (8.9)	35	37.8	35.7 (4.8)	47	38.0 (8.2)	13
362	28.9	25.8 (6.3)	35	30.8	24.9 (3.3)	47	29.6 (7.5)	13
363	55.2	48.6 (9.2)	35	43.5	43.3 (4.8)	47	45.0 (8.0)	13
364	65.8	52.8 (9.3)	35	51.6	47.2 (5.0)	47	44.5 (8.1)	13
365	37.1	32.3 (6.5)	35	37.2	34.6 (3.8)	47	32.7 (6.0)	13
371	24.2	32.5 (7.3)	37	34.2	29.8 (5.0)	74	38.0 (5.5)	14
372	60.7	60.9 (10.1)	37	55.2	52.6 (8.1)	74	52.0 (9.8)	14
373	29.7	37.2 (8.9)	37	37.1	36.0 (5.2)	74	36.0 (7.2)	14
374	58.8	67.1 (11.5)	37	55.4	51.5 (7.4)	74	59.4 (18.8)	14
375	45.0	47.9 (9.2)	37	58.0	60.0 (7.9)	74	46.0 (19.1)	14
376	47.9	64.6 (13.5)	36	64.6	62.6 (6.7)	70	54.2 (6.3)	14
377	44.9	53.2 (10.3)	36	48.3	48.4 (5.5)	70	49.4 (7.2)	14
378	11.5	18.5 (5.7)	36	18.4	17.7 (2.8)	70	23.1 (9.8)	14
379	42.5	47.4 (9.6)	36	45.4	47.3 (5.3)	70	41.5 (7.3)	14
380	31.7	43.0 (9.2)	36	29.9	31.4 (4.2)	70	34.5 (9.9)	14

[a]n, number of participants; [b]SD, standard deviation.

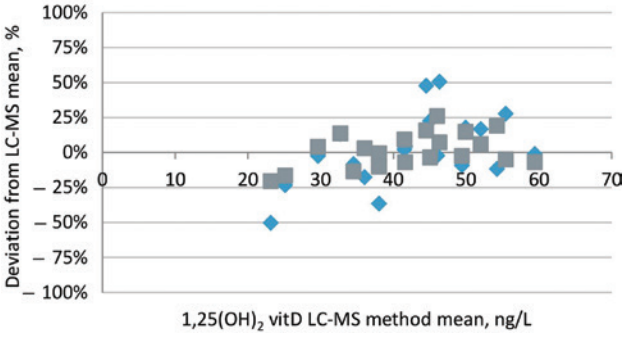


Figure 3: Relative deviation of $1,25(OH)_2$ vitD concentrations measured with the IDS iSYS and the DiaSorin $1,25(OH)_2$ vitD assay, respectively, from LC-MS/MS method mean in DEQAS samples. The blue rhombs represent IDS values, grey squares represent the concentrations measured with the DiaSorin test.

measured $1,25(OH)_2$ vitD in control material and serum pools with concentrations between 22.1 and 184.7 ng/L and found a repeatability and intermediate precision between 1.0%–5.0% and 3.8%–7.1%, respectively [16]. In a different study, intra- and inter-assay imprecision ranged from 1.1% to 4.7% and from 3.4% to 7.2%, respectively, in controls and serum pools with concentrations between 25.5 and 180.4 ng/L [22]. As we demonstrate here, the DiaSorin test measures $1,25(OH)_2$ vitD very precisely even at concentrations <10 ng/L. Furthermore, $1,25(OH)_2$ vitD concentrations measured with this test showed a very good correlation and agreement with $1,25(OH)_2$ vitD levels determined with the LC-MS/MS reference method.

The IDS assay with automated extraction running on the IDS iSYS immunoanalyzer measured $1,25(OH)_2$ vitD less precisely compared to the DiaSorin test. At concentrations of 14.4 ng/L total imprecision amounted to 20%. The limit of detection as indicated by the manufacturer is 7.5 ng/L for the IDS test. Even the limit of quantification of 5.0 ng/L for the DiaSorin test is lower than the IDS detection limit, reflecting the high imprecision of the IDS test.

Furthermore, compared to the DiaSorin test, $1,25(OH)_2$ vitD concentrations measured with the IDS test showed weaker correlations and poorer agreement with those measured by LC-MS/MS. Both the comparison with LC-MS/MS measurements and the measurement of DEQAS quality controls revealed that the IDS test underestimates $1,25(OH)_2$ vitD concentrations below 40 ng/L and overestimates higher concentrations. IDS test results deviated from the LC-MS/MS method mean of DEQAS by – 50% to + 51% compared to – 20% to + 26% deviations of the DiaSorin test.

The two methods apply different fully automated extraction procedures prior to the measurement of $1,25(OH)_2$ vitD. Whereas the DiaSorin test uses a recombinant fusion protein to capture $1,25(OH)_2$ vitD, the IDS test purifies the analyte from the serum using an anti-$1,25(OH)_2$ vitD antibody. One might argue that the fusion protein binds to $1,25(OH)_2$ vitD with higher specificity than the antibody, leading to the lower imprecision and the better correlation with LC-MS/MS of the DiaSorin test. However, our currently used IDS test with manual extraction of $1,25(OH)_2$ vitD shows significantly stronger correlation with the LC-MS/MS measurements than the fully automated IDS test (r = 0.908 vs. r = 0.852, Figure 4), although this was still weaker than that of the DiaSorin test (r = 0.967). Assuming that the same antibody is used for the manual and automated extraction prior to

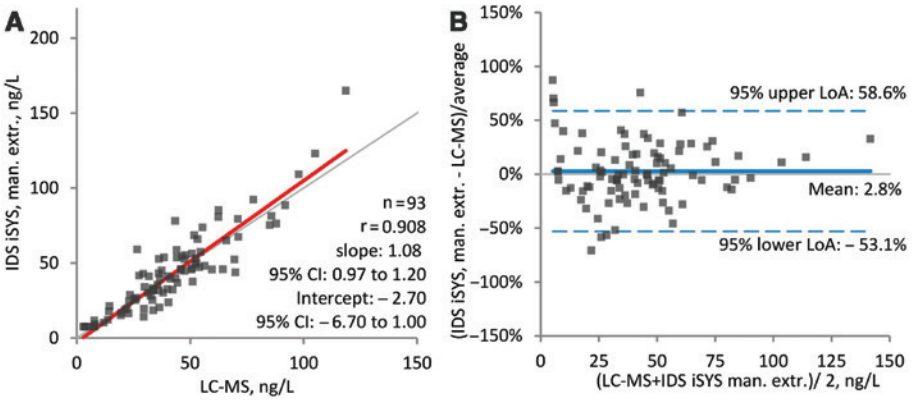


Figure 4: Method comparison of the IDS test using manual immunopurification of $1,25(OH)_2$ vitD with LC-MS analysis.
Left panel (A): Passing-Bablok regression analysis. The bold red solid line represents the regression line, the fine grey line represents the line of equality. Right panel (B): Bland-Altman plot. The bold blue solid line indicates the relative mean bias, the dashed blue lines represent the upper and lower limit of agreement, respectively.

measurement of $1,25(OH)_2$ vitD, the automated extraction steps of the IDS test need to be optimized.

1,25-Dihydroxyvitamin D2 is derived from dietary ergocalciferol, which is subsequently 25-hydroxylated in the liver and 1α-hydroxylated in the kidney. Both, vitamins D2 and D3 are comparably biologically active [23]. In order to correctly represent the biologically relevant amounts, the tests used for the determination of $1,25(OH)_2$ vitD should measure either form. The LC-MS/MS method used in our study differentiates between D2 and D3. Of the six serum samples with $1,25(OH)_2$ vitD2 concentrations above 5% of total $1,25(OH)_2$ vitD, five samples contained amounts between 1.3 and 8.3 ng/L, most likely due to a high content of vegetable food and/or due to substitution. In one patient with a total $1,25(OH)_2$ vitD concentration of 29.7 ng/L, the $1,25(OH)_2$ vitD2 proportion amounted to 76.6%. Since we did not have any information about lifestyle or medication, the reason for the high $1,25(OH)_2$ vitD2 level in this particular patient remains unclear. When we compared the $1,25(OH)_2$ vitD concentrations measured with the immunoassays versus LC-MS/MS results in samples with significant amounts of $1,25(OH)_2$ vitD2, we found a mean deviation of 3.7% and 1.2% for the IDS test and the DiaSorin test, respectively. Thus, we assume that both tests almost completely cross-react with $1,25(OH)_2$ vitD2. This is in accordance with the manufacturer's data for the DiaSorin test. For the IDS test, a cross-reactivity of 57% is indicated by the manufacturer, but the respective tested concentration is not indicated. In our study, we only had six samples with $1,25(OH)_2$ vitD2 concentrations above 5% of total $1,25(OH)_2$ vitD. Further studies involving more samples with significantly elevated $1,25(OH)_2$ vitD2 levels are necessary to precisely evaluate the cross-reactivity of the tests with $1,25(OH)_2$ vitD2. No or only very low cross-reactivity is indicated by either manufacturer for vitamin D metabolites with less biological activity.

Measurement of $1,25(OH)_2$ vitD using fully automated tests is of significant advantage, particularly as the number of samples submitted for the determination of this parameter has increased over the recent years. The implementation of such tests allows for higher throughput and less hands-on time compared to the manual tests used so far. Together with the high sensitivity, low imprecision, the broad measurement range and the good agreement with $1,25(OH)_2$ vitD concentrations measured with LC-MS/MS, the DiaSorin test improves $1,25(OH)_2$ vitD diagnostics. The fully automated test performed on the IDS iSYS has a comparable broad measurement range but needs further improvement with respect to sensitivity, precision and standardization. The respective limitations have most recently been addressed by the supplier by a further development of the test, for which the presented study will serve as an ideal starting point and framework for the evaluation of critical performance factors.

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