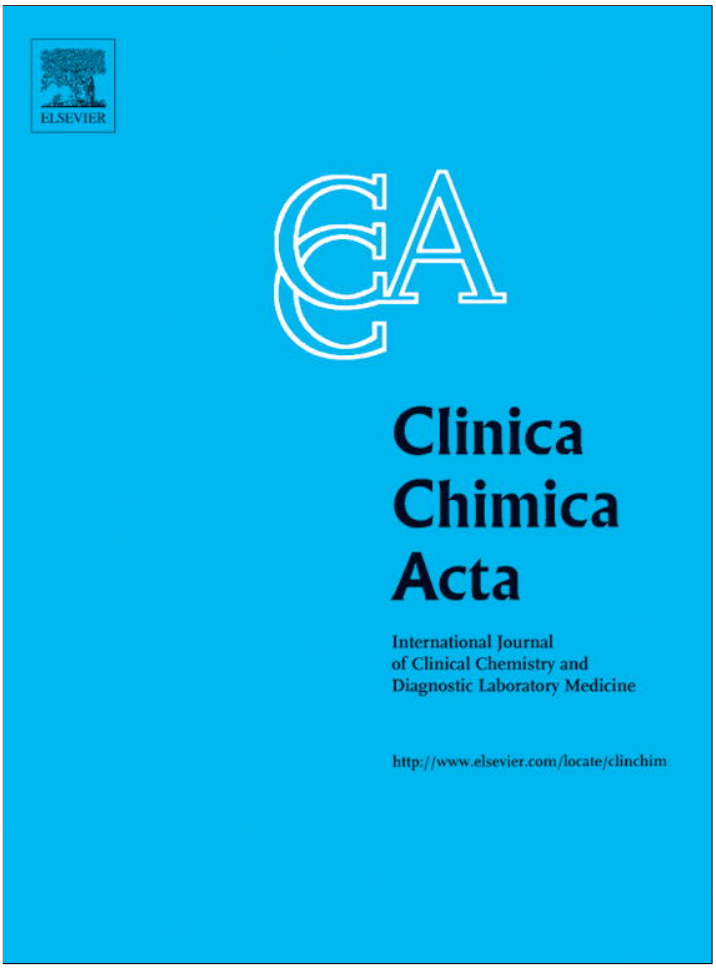

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Accuracy of three automated 25-hydroxyvitamin D assays in hemodialysis patients

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ABSTRACT

Introduction: We evaluated the accuracy of three automated assays for 25(OH)D measurement in comparison to ID-XLC-MS/MS in hemodialysis patients, considering the importance of their vitamin D status and reported discrepant results obtained with automated assays.
Methods: All three assays were heterogeneous, competitive immunoassays or vitamin D binding protein assays on Architect (Abbott), Modular E170 (Roche) and iSYS (IDS). Measurements were performed in serum of 99 hemodialysis patients and 50 healthy subjects, double blind with a different operator and aliquot for each method.
Results: Architect showed the highest deviation for hemodialysis (slope 0.3864, intercept 8.7409) and healthy subjects (slope 0.5024, intercept 6.8426) and reported significant lower results. Considering 30 ng/ml as cut-off for optimal 25(OH)D concentration, Architect falsely assigned 48.5% of the hemodialysis and 6% of the healthy subgroup a suboptimal vitamin D status. iSYS results of hemodialysis patients also deviated (slope 0.6136, intercept 8.6604) but showed less discordant values than Modular E170 in patients with 25(OH)D concentrations between 10 and 40 ng/ml.
Conclusion: We conclude that not all automated 25(OH)D assays may be considered equally accurate in samples from hemodialysis patients compared to samples from healthy subjects. We found most deviating results with Abbott (Architect) measurements compared to ID-XLC-MS/MS in hemodialysis patients as well as in healthy subjects. We suggest a possible role of matrix effects like elevated urea or other retained metabolites in hemodialysis sera, causing incomplete binding disruption between 25(OH)D and DBP, in the poor assay accuracy.

1. Introduction

The increased scientific and public attention to the prevalence and clinical consequences of vitamin D deficiency has enormously elevated laboratory test frequency and stimulated manufacturers' development of automated immunoassays for the past decade. Patients with chronic kidney disease (CKD) stages 3–5 (NKF KDOQI guidelines) and those who follow dialysis therapy form an important subgroup for vitamin D measurements with high needs for accurate results and monitoring. Renal dysfunction and diminished active vitamin D production combined with nephrotic damage increases the risk for vitamin D deficiency. Proteinuria and proximal tubular cell injury, associated with the clinical course of kidney disease, are responsible for significant urinary losses of 25(OH)D (25-hydroxyvitamin D) and its binding protein DBP [1]. Vitamin D deficiency is a major direct stimulus for PTH secretion, thereby increasing the risk for secondary hyperparathyroidism, a hallmark of CKD with several deleterious consequences [2]. Therefore, KDOQI guidelines for bone metabolism and disease in CKD stipulate measurement of serum 25(OH)D if PTH is above target range for the stage of CKD. Also the KDIGO guidelines recommend measurement of 25(OH)D and treatment of vitamin D deficiency or insufficiency [3]. Supplementation of hemodialysis patients with vitamin D is recommended by the KDOQI guidelines if 25(OH)D<30 ng/ml [4]. Consequently, 25(OH)D measurement is a necessary practice for management of CKD patients.

Current assays for 25(OH)D measurement are radio- and chemiluminescence immunoassays, DBP assays, high-performance liquid chromatography and liquid chromatography–tandem mass spectrometry (LC–MS/MS). Advantages of chromatographic analyses are the high accuracy and possibility to distinguish cross-reactants like 25(OH)D_2 and 3-epi-25(OH)D. High throughput automated assays without manual sample preparation try to fulfill the increasing clinical demand and laboratory workload of 25(OH)D analyses. These assays are issued by three challenges for development: Firstly, the Food and Drug Administration (FDA) stipulates concomitant measurement of both 25(OH)D_3 (cholecalciferol, the predominant endogenous circulating form) and 25(OH)D_2 (ergocalciferol) besides the already difficult measurement of the single molecule with small immunogenic character. In the absence of vitamin D_2 supplementation, the ratio of 25(OH)D_3/25(OH)D_2 lies around

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95/5 [5–8]. Secondly, the strong binding between highly hydrophobic 25(OH)D and DBP creates competition with the capturing antibodies or DBP. Thirdly, the low 25(OH)D circulating serum concentration (10% of total body presence) requires highly sensitive assays [9–11]. For clinical decisions, the limit of quantification (LoQ) for any 25(OH)D assay should be <10 ng/ml [12].

The National Institute of Standards and Technology (NIST) released in 2008 Standard Reference Material (SRM) to improve the analytical performance and standardization of 25(OH)D assays [13]. This material has recently (2012) been modified to obtain human-based serum instead of spiked equine serum [14]. SRM material is certified and assigned reference values for 25(OH)D_2, 25(OH)D_3, and 3-epi-25(OH)D_3 by three isotope-dilution mass spectrometry approaches performed at NIST and at the Centers for Disease Control and Prevention (CDC). Recently, two candidate reference methods have been developed by Tai et al. [15] and Stepman et al. [16] that can be used by manufacturers and laboratories to calibrate and validate their methods.

Despite standardization efforts, unresolved discrepancies and unacceptable bias persist among immunoassays compared to the reference methods, particularly in samples from diseased patients with atypical plasma or serum matrix composition such as hemodialysis patients. As CKD patients represent an important target population for vitamin D testing, often forgotten in method validation studies, we specifically evaluated the performance of three automated assays in hemodialysis patients. To exclude sample type variability as a cause for inter-method differences by not using the same matrix for each method, as occurred in a previous study [17], we wanted to confirm these data by using only serum.

The aim of this study was to evaluate the accuracy of three different assays on Architect i2000sr (Abbott), Modular E170 (Roche), and iSYS (IDS) analyzers for 25(OH)D measurement in comparison to a higher reference isotope dilution/online solid-phase extraction liquid chromatography tandem mass spectrometry (ID-XLC-MS/MS) method [17], in serum from hemodialysis patients.

2. Materials and methods

2.1. Samples

We studied 99 hemodialysis patients (47 women, 52 men, age 24–94 years) and a healthy control group of 50 blood donors (34 women, 16 men, age 20–65 years). During the periodic control of hemodialysis patients (2 days) in our hospital, blood samples were collected just before start of the regular dialysis run, allowed to clot, and centrifuged (10 min at 4000 rpm), and serum was divided automatically (RSA Pro, Roche Diagnostics) into 4 aliquots (minimum 350 μl) and frozen (−20 °C). Serum samples of healthy blood donors were obtained similarly over a period of 10 days. All measurements were performed double blind with a different operator and aliquot for each method. Measurements on Architect and Modular E170 were performed in the laboratory of St Jan Hospital (Bruges, Belgium), analyses with iSYS and ID-XLC-MS/MS required ice-cooled transport to Immunodiagnostic Systems (IDS) S.A. (Liège, Belgium) and the VU University Medical Center (Amsterdam, The Netherlands), respectively. Storage time for analysis did not exceed 26 days and none of the aliquots were thawed and refrozen during storage and transport. In this way, maximum one freeze–thaw cyclus was performed for all frozen aliquots, although multiple freeze–thaw cycli do not affect serum 25(OH)D concentration [18].

2.2. Automated assays

All three automated assays are heterogeneous, competitive assays measuring both 25(OH)D_2 and 25(OH)D_3. A pre-treatment step to release bound 25(OH)D from DBP is included. The 25(OH)D assays on Architect i2000sr (Abbott; lot no. 00612B000 S/N 03527) and iSYS (IDS; lot no.1072 S/N B300B0203) are immunoassays based on chemiluminescence using acridinium-conjugated antibodies. The 25(OH)D assay on Modular E170 (Roche; kit 05894913190 lot 167728) is an electrochemiluminescent assay using ruthenium-labeled DBP, biotin-labeled vitamin D and streptavidin-coated microparticles. The signals produced are all inversely proportional to the 25(OH)D concentrations in the original sample.

Intra- and inter-run coefficients of variation (CV) were evaluated by ten times repeated measurement of 2 aliquoted and stored (−20 °C) serum pools with low (17.94 ng/ml) and high (63.92 ng/ml) 25(OH)D concentrations. The intra-assay CVs (<3.5%, <5.5% and <2.0%) and interassay CVs (<6.5%, <16.9% and <5.12%) on Architect, iSYS, and Modular E170, respectively were satisfactory according to the performance goals based on biological variation for routine testing (CV≤10%, bias≤5%) as described by Stöckl et al. [19]. Inter-assay CV on iSYS (16.9%) however did not agree.

2.3. ID-XLC-MS/MS

All 25(OH)D measurements by ID-XLC-MS/MS were performed at the Endocrine Laboratory of the VU University Medical Center (Amsterdam, the Netherlands) as described before [17], with only minor adjustments. For calibration, an in-house prepared solution of vitamin D standard (Sigma Aldrich, St Louis, MO, USA) was used. The candidate reference method by the group of Thienpont [16] was used to check standardization of ID-XLC-MS/MS. In short, deuterated internal standard (IS) [25(OH)D3-d6] (Synthetica, Oslo, Norway) was added to the samples and 25(OH)D was released from its binding proteins with acetonitrile. Samples were extracted and analyzed by XLC-MS/MS [a Symbiosis online SPE system (Spark Holland, Emmen, the Netherlands)] coupled to a Quattro Premier XE tandem mass spectrometer (Waters Corp., Milford, MA, USA). The limit of quantitation (LOQ) was 1.6 ng/ml; intra-assay CV was <6%, and interassay CV was <8% for concentrations between 10 and 72 ng/ml. 25(OH)D_2 and 25(OH)D_3 were measured separately without distinguishing for 3-epi-25(OH)D forms. Measuring results were expressed in nmol/l. We took into account the different molecular weights of D_2 (413.2 g/mol) and D_3 (400.64 g/mol) and divided by respectively factor 2.42 and 2.5 to express the results in ng/ml. In case of presence of 25(OH)D_2, 25(OH)D_2 and 25(OH)D_3 concentrations were added to reflect the total 25(OH)D concentration, which was then used for comparison to the 3 automated assays. Comparison to SRM and DEQAS reference material showed that measurement of 25(OH)D_2 and 25(OH)D_3 deviated at most ±1 SD from the overall method mean of the DEQAS participants, and reached the certified SRM 972 levels (including 25(OH)D_2).

2.4. Statistics

Assay precision was assessed by calculating the CV. We followed performance goals based on biological variation for routine testing (CV≤10%, bias≤5%) as described by Stöckl et al. [19]. All results of the hemodialysis (n=99) and donor serum samples (n=50) were analyzed by Weighted Deming regression (Table 1). This way, we considered a possible error on the X-values and a non constant SD but constant CV, suited for larger datasets. By using Weighted Deming regression, we also tested the assumption of linearity and calculated 95% confidence intervals (CI) for the slope (b), intercept (a_0) and the magnitude of the random error ($SD_{y/x}$). Since a correlation coefficient is related to the slope and $SD_{y/x}$, factors that depend on the regression model, we preferred interpretating the Weighted correlation coefficient instead of Pearson coefficients. The two sample *T*-test with equal (hemodialysis) and unequal (healthy subjects) variances was performed to highlight the magnitude of measurement differences between Architect and ID-XLC-MS/MS. The Pearson correlation coefficient was however used to investigate a trend

Table 1
Weighted Deming regression data of each immunoassay compared to ID-XLC-MS/MS for hemodialysis patients (n = 99) and healthy blood donors (n = 50).

	Weighted Deming regression				Weighted correlation coefficient	
Assay compared to ID-XLC-MS/MS	Slope	95% CI	Intercept	95% CI	$SD_{y/x}$	(p<0.001)
Architect (Abbott)						
Hemodialysis patients	**0.3864**	0.3138–0.4591	**8.7409**	5.58–11.9017	0.1181	**0.8688**
Healthy subjects	**0.5024**	0.3737–0.6312	**6.8426**	4.7845–8.9008	0.153	**0.8059**
Modular E170 (Roche)						
Hemodialysis patients	**0.9552**	0.8379–1.0726	**−5.4077**	−10.0758, −0.7397	0.2597	**0.8824**
Healthy subjects	**0.9200**	0.7037–1.1363	**2.2602**	−1.1798, −5.7002	0.2306	0.8207
iSYS (IDS)						
Hemodialysis patients	0.6136	0.4397–0.7875	8.6604	0.5038–16.817	0.1604	0.871
Healthy subjects	0.8062	0.6527–0.9598	1.2848	−0.9746, −3.5441	0.2046	**0.8554**

between serum urea concentrations and the percent deviation of 25(OH)D immunoassays from ID-XLC-MS/MS.

3. Results

3.1. Vitamin D concentrations

ID-XLC-MS/MS measured 25(OH)D concentrations between 11.6–86.0 ng/ml in the hemodialysis patients and 7.2–47.2 ng/ml in the healthy subjects. Measuring ranges of Architect, Modular E170 and iSYS were 10.0–52.0 ng/ml, 4.0–70.0 ng/ml, and 10.1–64.1 ng/ml for the hemodialysis patients and 10.6–39.3 ng/ml, 10.4–51.4 ng/ml, and 7.1–47.4 ng/ml for the healthy subjects, respectively. Box and Whisker plots illustrate the differences between 25(OH)D immunoassays and ID-XLC-MS/MS for hemodialysis patients (Fig. 1A) and healthy subjects (Fig. 1B). For each method, the test for deviation from linearity with Weighted Deming regression was not significant. ID-XLC-MS/MS detected 25(OH)D_2 within 10 of the hemodialysis (9.9%) and 4 of the donor samples (8.0%).

3.2. Comparison with ID-XLC-MS/MS in hemodialysis patients

Weighted Deming regression (Fig. 2, A–C) showed a proportional deviation for all assays compared to ID-XLC-MS/MS. Architect (Abbott) (Fig. 2A) showed the largest deviation with a slope significance (p<0.01) differing from 1 (0.3864), an intercept of 8.7409 and a correlation coefficient of 0.8688. Over the whole measuring range, Architect reported lower values than ID-XLC-MS/MS (one exception). A 2-sample *T*-test with equal variances (one sample F-test) showed significant difference between Architect and ID-XLC-MS/MS (p-value<0.0001). Box and Whisker plots (Fig. 1, A–B) illustrate this extreme deviation.

Comparison of iSYS with ID-XLC-MS/MS showed a slope of 0.6136, an intercept of 8.6604 and a correlation coefficient of 0.871. Modular E170 showed the best fit that crossed the line of identity (slope 0.9552, intercept −5.4077) and the highest correlation coefficient (0.8824) with ID-XLC-MS/MS. Below 40 ng/ml, however, iSYS reported less discordant 25(OH)D values (Fig. 2C) than Modular E170 (Fig. 2B) compared to ID-XLC-MS/MS. Modular E170 reported 2 patients <10 ng/ml, 4 <20 ng/ml, and 6 <30 ng/ml with ID-XLC-MS/MS values of >10, >20, and >30 ng/ml, respectively (12 underestimated results), while iSYS reported no patients with values <10 or <20 ng/ml and only 1 patient <30 ng/ml with an ID-XLC-MS/MS values of >10, >20, and >30 ng/ml, respectively. On the other hand, both methods almost equally yield the same number of overestimations in the 25(OH)D range 10–40 ng/ml. Modular E170 reported 3 patients >30 ng/ml and 2 >40 ng/ml with ID-XLC-MS/MS values of <30 and <40 ng/ml, respectively (5 discordances), while iSYS reported 1 patient >20 ng/ml, 1 >30 ng/ml and 2 >40 ng/ml with ID-XLC-MS/MS values of <20, <30 and <40 ng/ml, respectively (4 discordances). In the area >40 ng/ml (measured with ID-XLC-MS/MS), both Modular E170 and iSYS reported approximately the same number of discordant results (12 versus 13, respectively).

Serum urea concentrations were elevated in hemodialysis patients, except one, ranging from 62 mg/dl to 251 mg/dl. Pearson correlation analysis of the percentage deviation (compared to ID-XLC-MS/MS) of negatively biased Architect results showed a weak but significant correlation with the serum urea concentration (r = 0.2381, p-value = 0.019). This trend was not observed for analyses on iSYS and Modular E170.

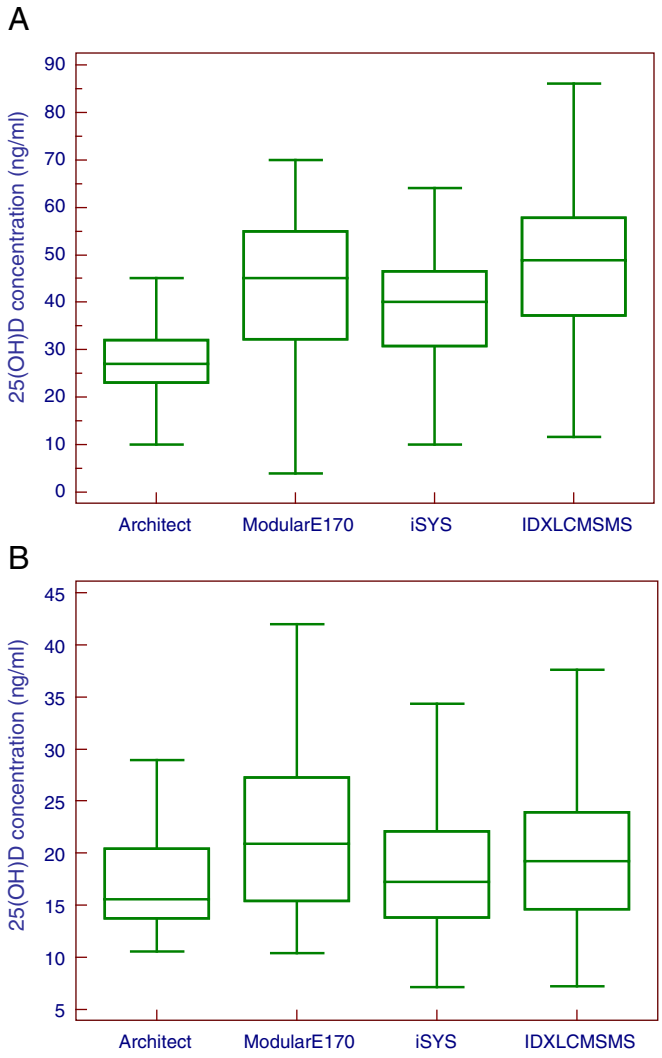

Fig. 1. Box and Whisker plots showing distributions of 25(OH)D in 99 hemodialysis patients (A) and 50 healthy subjects (B) measured with Architect i2000sr (Abbott), Modular E170 (Roche), iSYS (IDS) and ID-XLC-MS/MS.

3.3. Comparison with ID-XLC-MS/MS in healthy subjects

Weighted Deming regression (Fig. 2, D–F) showed a proportional deviation for all assays, with Architect (Abbott) (Fig. 2D) having the highest deviation with a slope (0.5024) significance differing ($p<0.001$) from 1, an intercept of 6.8426 and the lowest weighted correlation coefficient (0.8059). A 2-sample *T*-test with unequal variances (one sample F-test) showed significant difference between Architect and ID-XLC-MS/MS results (p-value<0.05). We report higher values in the very low concentration area until 25(OH)D equals 13.6 ng/ml (10 samples). From here, Architect reports lower vitamin D concentrations than ID-XLC-MS/MS (2 exceptions). Again, Modular E170 (Fig. 2E) shows the best fit that crossed the line of identity (slope 0.9200, intercept 2.2602). Nevertheless, the highest correlation coefficient was obtained with iSYS (0.8554), which showed a comparable performance to Modular E170.

3.4. Diagnostic performance

If we consider 30 ng/ml as the cut-off for optimal vitamin D status [20,21] and ID-XLC-MS/MS as gold standard, then Architect falsely assigned 48.5% of the dialysis patients as suboptimal, of which 36.4% were assigned as sufficient (20–30 ng/ml) (Table 2). The magnitude of this error was much less severe with Modular E170 (7.1% of dialysis patients) and iSYS (5.1% of dialysis patients). Architect falsely assigned 12.2% of dialysis patients and 18.0% of healthy subjects as vitamin D insufficient (<20 ng/ml) while other assays categorized essentially similarly to ID-XLC-MS/MS, although more patients were apparently insufficient with Modular E170 than with iSYS (Table 2).

4. Discussion

We report that distribution of measured 25(OH)D concentrations showed differences between automated assays and ID-XLC-MS/MS, with greater deviations in sera from hemodialysis patients than from healthy subjects. Compared to ID-XLC-MS/MS, Modular E170 measurements showed the best fit that crossed the line of identity for dialysis patients (slope 0.9552, intercept −5.4077) and healthy subjects (slope 0.9200, intercept 2.2602) and the highest Weighted correlation coefficient for the dialysis patients (0.8842). iSYS showed the best correlation coefficient and a comparable performance to Modular E170 among the healthy subjects, but results deviated from ID-XLC-MS/MS (slope 0.6136, intercept 8.6604) among hemodialysis patients although less discordant values were obtained with iSYS than with Modular E170 at 25(OH)D concentrations below 40 ng/ml. Architect showed the highest deviation from ID-XLC-MS/MS for dialysis patients (slope 0.3864, intercept 8.7409) and healthy subjects (slope 0.5024, intercept 6.8426).

Recent studies have reported discrepant results and significant deviations of the Abbott assay compared to higher reference methods. Farrel et al. [22] showed that the Abbott assay had the greatest overall deviation (+28%) and highest bias compared to LC–MS/MS. Confirmation was followed by the study of Heijboer et al. [17], who reported a concentration dependent difference for Architect (Abbott) measurements in hemodialysis patients: Architect reported higher than ID-XLC-MS/MS in the area 8.0–37.6 ng/ml, a trend that inversed when the concentrations exceeded 37.6 ng/ml. In our study, however, this inverse trend was not present. We found Architect consistently producing lower results for hemodialysis patients (one exception) as well as for healthy subjects (two exceptions). Also this latter finding is in contrast to Heijboer et al. [17], who consistently reported

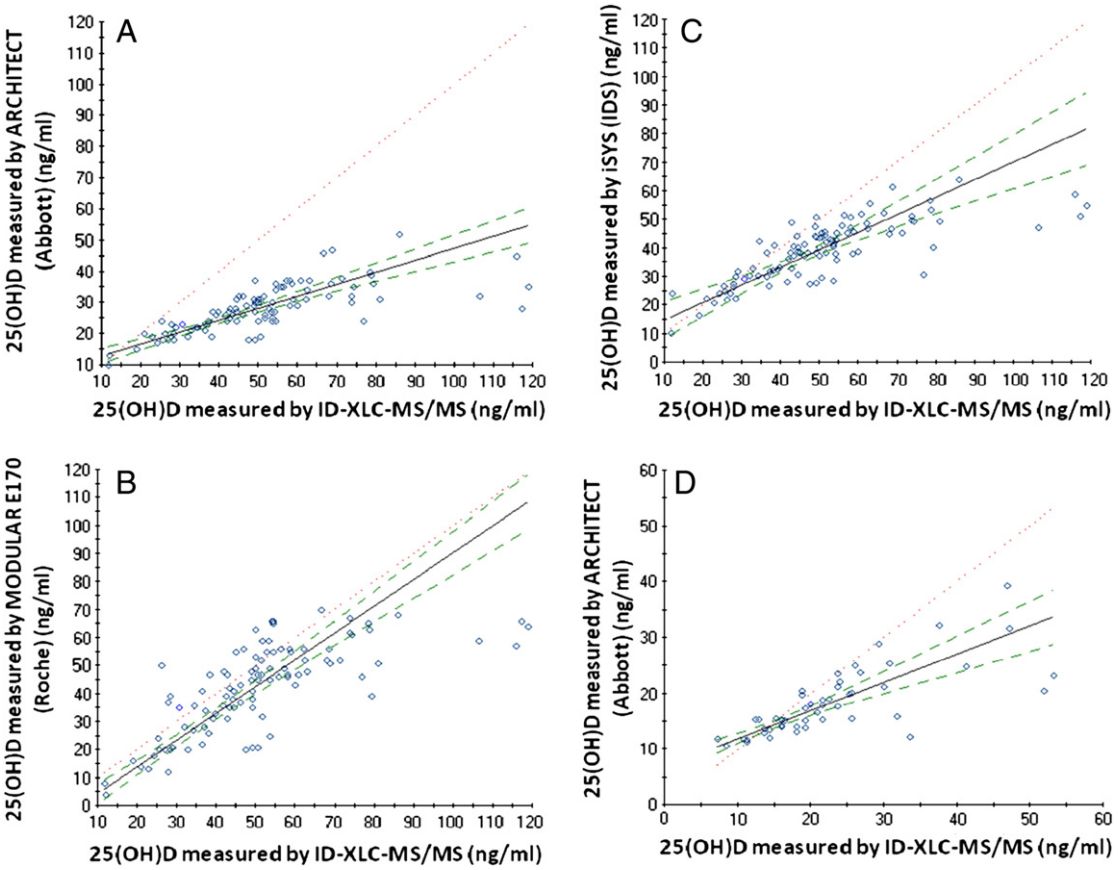

Fig. 2. Weighted Deming regressions for 25(OH)D measurements with Architect i2000sr (Abbott), Modular E170 (Roche) and iSYS (IDS) compared to ID-XLC-MS/MS in hemodialysis patients (A, B, C) and healthy subjects (D, E, F).

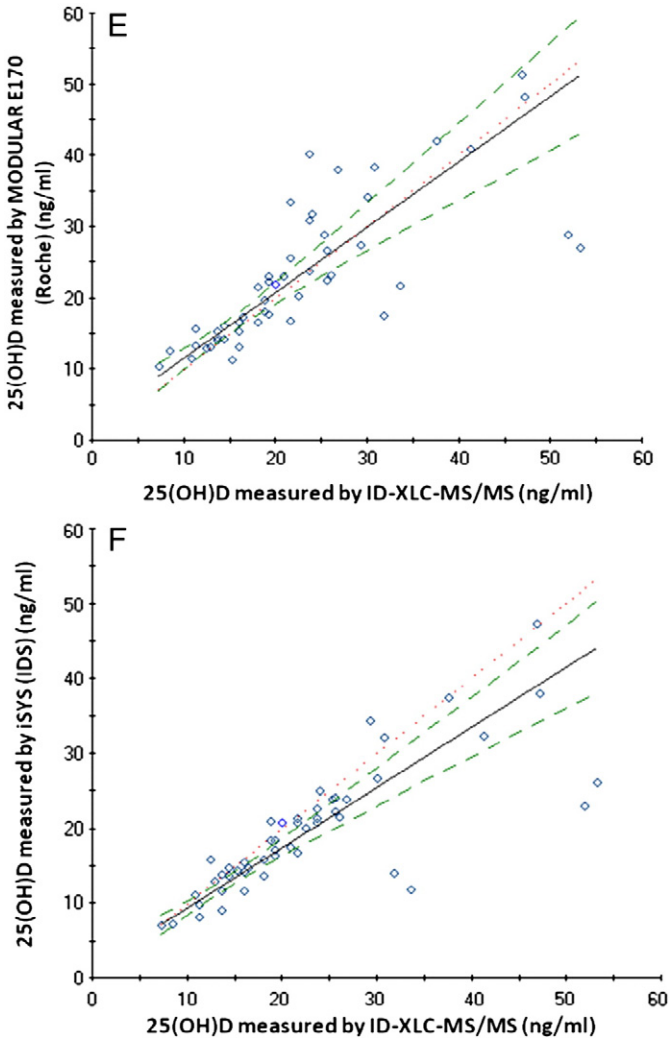

Fig. 2 (*continued*).

higher values for healthy subjects by Architect compared to ID-XLC-MS/MS. No changes were made by Abbott, IDS or Roche concerning the reagents composition or calibration procedures between the study of Heijboer et al. [17] and our study. A possible explanation for the differing trends observed by comparing Architect with ID-XLC-MS/MS could be the use of EDTA-plasma by Heijboer et al. [17] compared to the use of serum in our study.

We observed that the 25(OH)D assay on Architect leads to unjustified classifications of the vitamin D status. Considering a cut-off of 30 ng/ml for optimal vitamin D concentrations [20,21], Architect falsely assigned 48.5% of the hemodialysis as having suboptimal vitamin D concentrations and 12.2% of patients as insufficient (<20 ng/ml). As supplementation of hemodialysis patients with vitamin D is recommended when 25(OH)D<30 ng/ml [4], falsely lowered vitamin D values can lead to unnecessary concerns and therapies, increasing the cost–benefit ratio of patient management.

Table 2
Numbers of patients classified according to vitamin D status with the measured 25(OH)D concentrations (ng/ml) by each method in hemodialysis patients and healthy subjects.

Assay and sample group	Deficient <10 ng/ml	Insufficient <20 ng/ml	Sufficient 20–30 ng/ml	Optimal >30 ng/ml
Hemodialysis patients (n=99)				
Architect (Abbott)	0	15	48	36
Modular E170(Roche)	2	5	15	77
iSYS (IDS)	0	2	18	79
ID-XLC-MS/MS	0	3	12	84
Healthy subjects (n=50)				
Architect (Abbott)	0	33	14	3
Modular E170(Roche)	0	23	16	11
iSYS (IDS)	5	22	17	6
ID-XLC-MS/MS	2	24	18	6

There may be several explanations for the observed inter-method differences between the automated assays and ID-XLC-MS/MS.

First of all, incomplete disruption of 25(OH)D from DBP leading to minor separated or residual active DBP is a well-known problem for heterogeneous immunoassays. 25(OH)D circulates bound to DBP for more than 99%, with serum DBP concentrations 100- to 1000-fold higher than 25(OH)D [23,24]. DBP is a polymorphic protein with 3 alleles and more than 120 variants, all exhibiting different affinities for D_2 and D_3. The strong binding between the highly hydrophobic 25(OH)D and DBP creates competition with the capturing antibodies. DBP has to be inactivated or completely removed from the sample prior to signal generation, since residual active DBP of as little as 4 nmol/l (0.5% of total DBP) may already interfere [24]. ID-XLC-MS/MS utilizes a proprietary protein precipitation (ACN) in a manual extraction procedure followed by centrifugation to overcome this problem. This manual pre-analytical procedure is hard to automate, forcing routine assays to use less-effective combinations of organic solvents for binding disruption and DBP inactivation often based on pH changes [25]. We assume that elevated concentrations of several serum compounds, such as urea or other retained metabolites in hemodialysis patients, may interfere in immunoassays by causing difficulties in extracting 25(OH)D from DBP and producing falsely low vitamin D measurements. Indeed, we found that the percentage deviation from ID-XLC-MS/MS of negatively biased Architect results correlated with elevated serum urea concentrations in hemodialysis patients ($r=0.2381$, p-value$=0.019$), although this does not prove a direct causal relationship. Moreover, differing serum DBP concentrations may exist among various clinical subgroups. However, we exclude this issue as a possible confounding factor, since DBP concentrations don't seem to be significantly different between hemodialysis patients and healthy subjects [17,26].

Secondly, the concomitant measurement of 25(OH)D_2 and 25(OH)D_3 for vitamin D status evaluation, as recommended by the 2011 JCBN clinical guidelines [27], should be taken into consideration. Separate quantification of 25(OH)D_2 (MW 412.65 g/mol) and 25(OH)D_3 (MW 400.64 g/mol) is only possible with chromatographic procedures. ID-XLC-MS/MS detected 25(OH)D_2 in 9.9% of our hemodialysis patient samples. These findings were somewhat higher than expected, since the literature reports that 25(OH)D_2 averagely accounts for only 5% of the total 25(OH)D. The recent studies performed by Farrel [22] and Heijboer et al. [17] also reported only very few samples positive for 25(OH)D_2. However, 25(OH)D_2 can rise among patients depending on nutrition habits and supplementation combinations. As only minor 25(OH)D_2 concentrations (1.7–4.1 ng/ml) were measured, this can so forth explain slightly higher incidence of detectable 25(OH)D_2 of our hemodialysis group. Immunoassays, on the other hand, exhibit unequal detection of 25(OH)D_2 and 25(OH)D_3 due to different cross-reactivities. Hypothetically, this factor could partly be responsible for inter-method differences, seen the significant presence of 25(OH)D_2 among the dialysis samples and strongly differing specificity for 25(OH)D_2 (Architect 52%, Modular E170 81%, iSYS 100%) as reported by the manufacturers. However, since the relative contribution of 25(OH)D_2 to total 25(OH)D is minor (2.59–11.49%), we consider the influence of this fraction on the observed inter-method differences to be minimal.

The different cross-reactivities for 3-epi-25(OH)D3 reported by manufacturers (Architect 2.7%, Modular E170 92%, iSYS undefined) could also hypothetically aggravate inter-method differences. Kamao et al. [28] stated that C-3 epimerization is a common pathway for all major D_3 metabolites, mainly occurring in infants<1 year [29]. Separation is recommended, since an elevated fraction can overestimate serum 25(OH)D levels [30]. Nevertheless, Stepman et al. [31] observed

C3-epi-25(OH)D_3 to 25(OH)D_3 ratios between 2.5 and 17% in an adult population, also confirmed by NIST [14]. A limitation of our study was the lack of investigation for influence of C3-epi-25(OH)D. We justify this lacking because our study population did not include infants, the most important group for epimerization and our reference method did not distinguish C3-epimer forms.

Thirdly, all assays are known to show difficulties with accurate measuring in the low concentration area [22,25]. For clinical decisions, the LoQ for any 25(OH)D automated assay should be <10 ng/ml [12]. In our study, ID-XLC-MS/MS measured 5 donor samples with a concentration in this problematic area (7.2–11.2 ng/ml) and 1 hemodialysis patient sample (11.6 ng/ml). Given this low relative frequency in our sample cohort, we can neglect the possible impact on the observed inter-method differences. The broader measuring range of hemodialysis patients (11.6–86.0 ng/ml) compared to healthy subjects (7.2–47.2 ng/ml), and the more deviating results within the dialysis subgroup, could lead to the assumption that immunoassays also show difficulties in measuring very high results besides the known problematic low area.

5. Conclusion

We conclude that not all automated 25(OH)D assays may be considered equally accurate in samples from hemodialysis patients compared to samples from healthy subjects. We found most deviating results with Abbott (Architect) measurements compared to ID-XLC-MS/MS in hemodialysis patients as well as for healthy subjects. In the hemodialysis groups, we did not observe the previously reported [17] concentration-dependent difference with ID-XLC-MS/MS (possibly due to the use of EDTA-plasma instead of serum), but found Architect almost consistently (99%) yielding lower results in serum.

iSYS (IDS) shows better performance than Modular E170 (Roche) for the hemodialysis patients in the 25(OH)D range 10–40 ng/ml, due to a higher number of apparently underestimated concentrations by Modular E170. At this clinically important concentration range, iSYS can be considered a reliable instrument. For 25(OH)D concentrations > 40 ng/ml, Modular E170 and iSYS report approximately the same number of discordant results.

The reason why these immunoassays deviate more from ID-XLC-MS/MS for hemodialysis patients than for healthy subjects, remains unexplained. A hypothesis is that sample matrix effects, such as elevated urea or other retained metabolites in hemodialysis sera, could interfere with the release of 25(OH)D from DBP or with the assay reagents. As the number of patients recommended to take vitamin D supplementation depends on the current available assay used to determine 25(OH)D, further investigations with the in-vitro diagnostic manufacturers are necessary to clarify and solve this issue. Also possible, seen the higher upper limit of dialysis sample results versus healthy subjects, is that immunoassays show difficulties in yielding accurate results for the high concentration area.

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