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Accuracy of 6 Routine 25-Hydroxyvitamin D Assays: Influence of Vitamin D Binding Protein Concentration

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BACKGROUND: Recent recognition of its broad pathophysiological importance has triggered an increased interest in 25-hydroxyvitamin D [25(OH)D]. By consequence, throughput in 25(OH)D testing has become an issue for clinical laboratories, and several automated assays for measurement of 25(OH)D are now available. The aim of this study was to test the accuracy and robustness of these assays by comparing their results to those of an isotope dilution/online solid-phase extraction liquid chromatography/tandem mass spectrometry (ID-XLC-MS/MS) method. We put specific focus on the influence of vitamin D–binding protein (DBP) by using samples with various concentrations of DBP.

METHODS: We used 5 automated assays (Architect, Centaur, iSYS, Liaison, and Elecsys), 1 RIA (Diasorin) preceded by extraction, and an ID-XLC-MS/MS method to measure 25(OH)D concentrations in plasma samples of 51 healthy individuals, 52 pregnant women, 50 hemodialysis patients, and 50 intensive care patients. Using ELISA, we also measured DBP concentrations in these samples.

RESULTS: Most of the examined 25(OH)D assays showed significant deviations in 25(OH)D concentrations from those of the ID-XLC-MS/MS method. As expected, DBP concentrations were higher in samples of pregnant women and lower in samples of IC patients compared to healthy controls. In 4 of the 5 fully automated 25(OH)D assays, we observed an inverse relationship between DBP concentrations and deviations from the ID-XLC-MS/MS results.

CONCLUSIONS: 25(OH)D measurements performed with most immunoassays suffer from inaccuracies that are DBP concentration dependent. Therefore, when interpreting results of 25(OH)D measurements, careful consideration of the measurement method is necessary.

Vitamin D sufficiency plays an important role not only in the prevention of osteoporosis or osteomalacia, but also, as suggested recently, in the prevention of other diseases such as cancer and autoimmune diseases *(1–3)*. Consequently, clinical laboratories receive numerous requests for measuring 25-hydroxyvitamin D [25(OH)D][4] concentrations. Traditionally, assays for 25(OH)D have involved extraction with organic solvents, reconstitution of the specimen in a suitable matrix, and quantification by immunoassay *(4, 5)*. Alternative methods include HPLC *(6)*. Because such methods are rather laborious, automation of 25(OH)D measurements is desirable, and many automated methods have been developed recently.

A major challenge in measuring 25(OH)D is the displacement of 25(OH)D from vitamin D–binding protein (DBP). The organic solvents used to release 25(OH)D from its binding protein are not compatible with most immunoassays or protein-binding assays. Alternative methods for displacement have been devised, but it remains unclear whether the automated methods are sufficiently effective in liberating 25(OH)D from DBP *(7–9)*.

The aim of this study was to test the accuracy of the currently available 25(OH)D assays by comparing 6 available routine vitamin D assays with an isotope dilution/online solid-phase extraction liquid chromatography/tandem mass spectrometry (ID-XLC-MS/MS) method, using plasma not only from healthy individuals, but also from patients with a broad range of DBP concentrations, to assess the sensitivity of the various assays to differences in circulating DBP concentrations.

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[4] Nonstandard abbreviations: 25(OH)D, 25-hydroxyvitamin D; DBP, vitamin D–binding protein; IC, intensive care; ID-XLC-MS/MS, isotope dilution/online solid-phase extraction liquid chromatography/tandem mass spectrometry; IC, intensive care; IS, internal standard; LOQ, limit of quantitation.

Materials and Methods

SAMPLES

We obtained EDTA-plasma and serum from leftover material. Blood for plasma and serum was drawn from 51 healthy individuals, 52 pregnant women, 50 hemodialysis patients, and 50 intensive care (IC) patients. The male/female ratios and age ranges of these 4 participant groups were 23/28 and 20–64 years, 0/52 and 19–40 years, 24/26 and 33–89 years, 28/22 and 18–89 years, respectively. All samples were handled identically. After centrifugation, plasma and serum were aliquoted and frozen at −20 °C until blinded analysis took place. Storage time of specimens did not exceed 2 months. Only EDTA-blood was drawn from IC patients. Plasma and serum from pregnant women came from prenatal screening (9–14 weeks of pregnancy). Blood from hemodialysis patients was drawn just before the start of their regular dialysis turn.

METHODS

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. First, 25(OH)D was released from its binding proteins with a proprietary protein disruption buffer. Completion of release was verified by dialysis experiments. Deuterated internal standard (IS) [25(OH)D_3-d6] was added and samples were mixed. Samples were extracted and analyzed by XLC-MS/MS [a Symbiosis online SPE system (Spark Holland)] coupled to a Quattro Premier XE tandem mass spectrometer (Waters Corp.). We quantified plasma 25(OH)D by relating analyte/IS peak area ratios in patient plasma to analyte/IS peak area ratios in BSA-PBS buffer spiked with 25(OH)D_2 and 25(OH)D_3 at concentrations ranging from 0–100 μg/L (0–400 nmol/L) and IS at a fixed concentration. Limit of quantitation (LOQ) was 1.6 μg/L (4.0 nmol/L), intraassay CV was <6%, and interassay CV was <8% for 3 concentrations between 10 and 72 μg/L (25 and 180 nmol/L). We established the accuracy of 25-hydroxyvitamin D results by measuring the standard and a control with a reference method *(10)*. 25(OH)D_2 and 25(OH)D_3 were measured separately.

Routine 25(OH)D assays. We measured concentrations of 25(OH)D by 6 routine assays. Five of these were automated assays, performed on Architect i2000SR (Abbott Diagnostics; lot no. 01511A000), Centaur XP (Siemens Diagnostics; lot nos. 66758002 and 10491994), iSYS (25-Hydroxy Vitamin D; IDS; lot no. 727A), Liaison (25-OH-Vitamin D Total; Diasorin; lot no. 125651B), and Modular Analytics E170 (Elecsys Vitamin D total; Roche Diagnostics; lot no. 270910MP). One assay was a RIA preceded by extraction (Diasorin; lot no. 125783C). Analyses on iSYS, Liaison, and Modular were performed at Medial Diagnostic Centers, and analyses on Architect, Centaur, and the RIA were performed at the Endocrine Laboratory of the VU University Medical Center.

All analyses were performed in EDTA plasma except the analyses on the iSYS, which were performed in serum, as the assay is not appropriate for plasma, according to the manufacturers.

Interassay CVs were <8%, <10%, <8%, <8%, <13%, and <11% for 3 concentrations between 10 and 44 μg/L (25 and 110 nmol/L) using the Architect, Centaur, iSYS, Liaison, Modular, and RIA, respectively.

All 6 routine assays are competitive assays, where 25(OH)D in the sample competes with labeled 25(OH)D for binding to assay specific antibodies or—in the case of the Elecsys Vitamin D total assay—to recombinant DBP.

DBP assay. We measured DBP concentrations in EDTA-plasma using ELISA (R&D Systems). Intraassay variation was 4.3% at 249 μg/mL.

Statistics. All 25(OH)D concentrations measured by routine assays were, within patient groups, compared to concentrations measured by ID-XLC-MS/MS. We calculated correlation coefficients (Pearson) and Passing–Bablok regression. 25(OH)D and DBP concentrations were nonparametrically compared between patient groups. All statistical analyses were performed using MedCalc. P values ≤0.05 were considered to reflect statistical significance.

Results

In only 11 of the 203 study participants (5 healthy individuals, 1 IC patient, 2 pregnant women, and 3 dialysis patients) was 25(OH)D_2 detectable by ID-XLC-MS/MS. Concentrations of 25(OH)D_2 in these individuals were between 1.2 and 6 μg/L (3 and 15 nmol/L). In these cases, 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 6 25(OH)D assays.

Table 1 shows the slopes and intercepts obtained from the Passing–Bablok regression equations and correlation coefficients for each of the routine 25(OH)D assays compared to the ID-XLC-MS/MS method per patient study group. Figures of the respective Passing–Bablok regression graphs for each patient group and each 25(OH)D assay are provided as Supplemental Data, which accompanies the online version of this article at http://www.clinchem.org/content/vol58/issue3.

Table 1. Correlation coefficient, slope, and intercept according to Passing–Bablok regression for each 25(OH)D assay compared with ID-XLC-MS/MS in each clinical subject group.

Assay and patient group	Slope	Intercept, nmol/L[a]	r
Architect			
Healthy individuals	1.09	4.8[b]	0.94
Pregnant women	0.89	7.6[b]	0.96
Dialysis patients	0.50[b]	18.8[b]	0.84
IC patients	1.00	10.0[b]	0.82
Centaur			
Healthy individuals	0.55[b]	16.7[b]	0.94
Pregnant women	0.45[b]	14.0[b]	0.88
Dialysis patients	0.37[b]	21.3[b]	0.86
IC patients	0.62[b]	18.3[b]	0.83
iSys			
Healthy individuals	0.83[b]	0.33	0.97
Pregnant women	0.76[b]	−3.6	0.92
Dialysis patients	0.60[b]	5.0[b]	0.97
IC patients	ND[c]	ND	ND
Liaison			
Healthy individuals	1.07	2.8	0.93
Pregnant women	0.94	2.6	0.92
Dialysis patients	0.72[b]	9.9[b]	0.93
IC patients	1.29	9.2	0.81
Elecsys			
Healthy individuals	1.00	0.0	0.92
Pregnant women	0.98	−3.5	0.92
Dialysis patients	0.80[b]	−1.6	0.93
IC patients	0.91	−6.2[b]	0.91
RIA			
Healthy individuals	0.89	6.13[b]	0.97
Pregnant women	0.98	2.43	0.91
Dialysis patients	0.82[b]	6.8[b]	0.92
IC patients	0.98	4.9[b]	0.94

[a] To convert 25(OH)D concentrations to ng/mL, multiply by 0.4.
[b] Significantly different from 1.00 (slope) and 0.00 (intercept) ($P < 0.05$).
[c] ND, not determined.

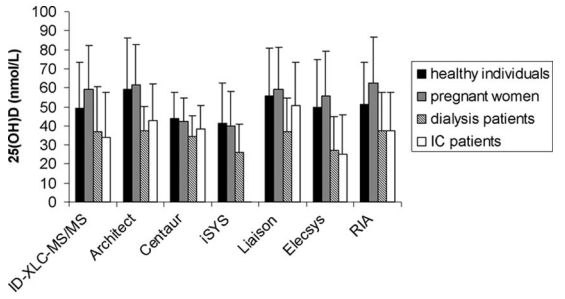

Fig. 1. 25(OH)D concentrations [mean (SD)] measured by different methods in healthy individuals (n = 51), pregnant women (n = 52), hemodialysis patients (n = 50), and IC patients (n = 50). 25(OH)D was not measured in plasma from IC patients using the iSYS.

To convert 25(OH)D concentrations to ng/mL, multiply by 0.4.

Mean concentrations of 25(OH)D determined by all 25(OH)D methods investigated in this study in the group of healthy individuals, pregnant women, and both patient groups are shown in Fig. 1.

Pregnant women showed significantly higher DBP concentrations [358 (143) μg/mL] and IC patients significantly lower DBP concentrations [155 (67) μg/mL] compared to healthy individuals [275 (89) μg/mL], $P < 0.001$, whereas DBP concentrations in hemodialysis patients [241 (103) μg/mL] did not show a significant difference from the healthy individuals (Fig. 2). The correlation and correlation coefficient between the DBP concentration and the deviation of each method from the ID-XLC-MS/MS method are shown in Fig. 3.

Discussion and Conclusion

Major differences were found between 25(OH)D concentrations as measured by the various 25(OH)D assays tested in this study.

The differences observed suggest that there might be a standardization problem for some of the 25(OH)D assays. Recently, the group of Thienpont published a candidate reference method that allows manufacturers and laboratories to calibrate their method *(10)* and was used to check standardization of the ID-XLC-MS/MS method used in this study.

In the Centaur assay, a concentration-dependent difference (measuring falsely high in the low concentration range and falsely low in the high concentration range) is seen in all patient groups. As the Centaur assay was introduced very recently, no data were available about this assay in the literature. A subset of the measurements were repeated with the help of Siemens Diagnostics, which confirmed the data. Other assays also showed such a concentration-dependent difference in the dialysis patient group. According to Siemens Diagnostics, new Centaur 25(OH)D reagents with an adjustment to the low end will be released shortly.

Regression analysis also revealed significant differences in results between the various patient groups. We hypothesized that variations in DBP concentrations between these patient groups were the cause for the observed differences.

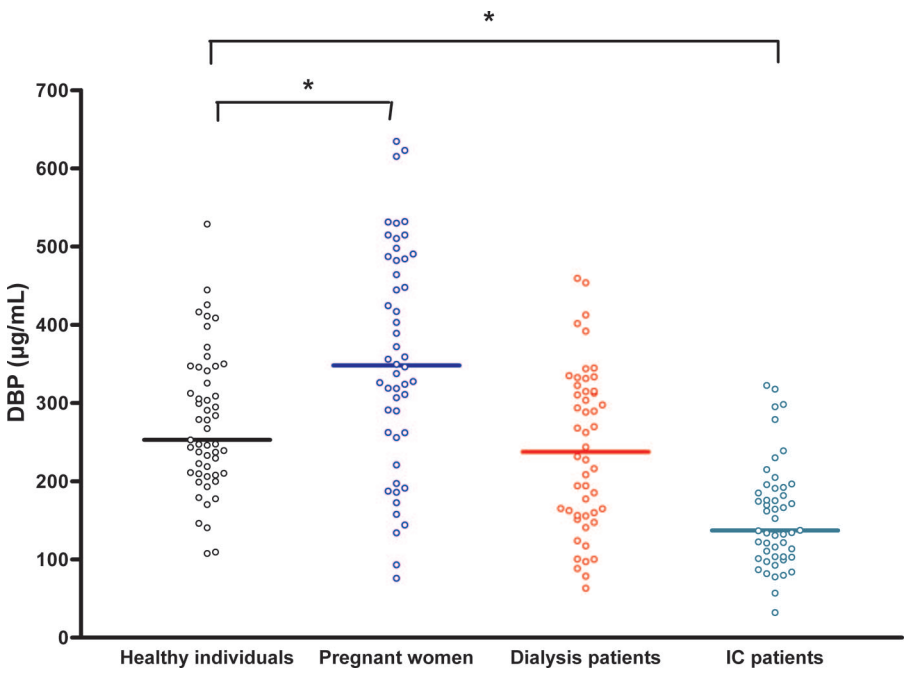

Fig. 2. DBP concentrations in the circulation of healthy individuals, pregnant women, dialysis patients, and IC patients.

*$P \leq 0.001$.

Pregnant women, because of the estrogen-dependent production of DBP, have increased DBP concentrations *(11–13)*, a finding that was reconfirmed in our study. Pregnant women also showed, on average, higher 25(OH)D concentrations than our healthy controls, as measured by ID-XLC-MS/MS. These higher concentrations might be caused by the higher concentrations of DBP in pregnant women, but could also be due to vitamin D supplementation that is advised for pregnant women *(14)*.

Critically ill patients with sepsis and organ dysfunction have lower DBP concentrations *(15, 16)*, which was in agreement with our data. These decreased DBP concentrations might be a factor causing a significantly decreased mean 25(OH)D concentration in IC patients (measured with ID-XLC-MS/MS).

DBP is a highly polymorphic serum protein with 3 common alleles and >120 rare variants. We measured DBP concentration and did not make a distinction between the different forms of DBP. The various forms of DBP might have different affinity for 25(OH)D, resulting in variation in binding affinity in the plasma samples studied, that in theory might lead to the more extreme differences in people with variants. However, whether the binding affinity is different among the DBP variants is still a matter of debate *(17–20)*.

Although the possibility that variations in DBP concentration might be problematic for automated 25(OH)D assays has been discussed, this is the first study that shows an inverse relationship between DBP concentrations and deviations of measured 25(OH)D concentrations from ID-XLC-MS/MS results for all 4 fully automated immunoassays tested. It appears that in these fully automated assays, not all 25(OH)D is extracted from the DBP in sera that have a relatively high DBP concentration. Such incomplete extraction leads to falsely low 25(OH)D concentration results. The fully automated Roche assay and the RIA that were also investigated in this study did not show such DBP concentration-dependent differences compared to the ID-XLC-MS/MS results. In the case of the RIA, this might be explained by the thorough extraction procedure preceding the RIA.

Taken together, our data suggest that not all assays are suitable for measuring 25(OH)D in all patient groups.

The DBP concentrations in our hemodialysis patient group did not differ from those in the group with healthy individuals, which is in accord with other data in the literature *(21)*. Despite DBP concentrations within the reference interval, however, most routine assays yielded 25(OH)D concentrations differing from

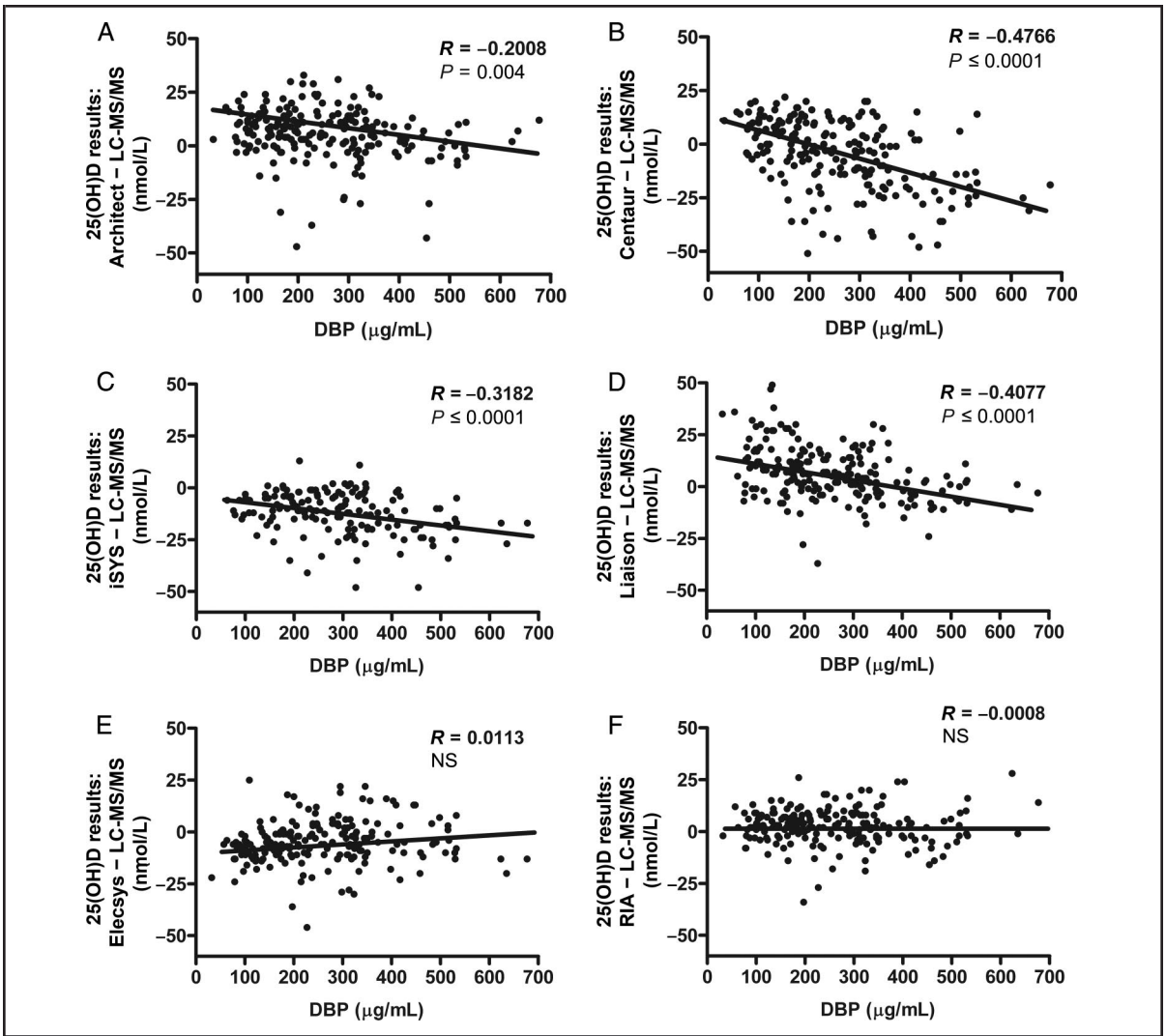

Fig. 3. Correlation of the DBP concentration and the difference between the results of each of 6 routine 25(OH)D assays and the results of the ID-XLC-MS/MS (LC-MS/MS) assay.

For the Architect ($P \leq 0.005$), Centaur, iSYS, and Liaison ($P \leq 0.0001$) assays, the correlation (R) was statistically significant. To convert 25(OH)D concentrations to nanograms per milliliter, multiply by 0.4. NS, not significant.

those obtained with the ID-XLC-MS/MS in this hemodialysis patient group. Noteworthy is that the regression analysis of data in this patient group for all investigated assays had slopes that significantly differed from 1.00. We can only speculate about the cause of these deviations. Urea, for instance, could interfere with the release of 25(OH)D from its binding protein, with the equilibrium of 25(OH)D binding or with the reagents of the assay. Further studies have to be performed to clarify this issue.

In addition to the issue of releasing 25(OH)D from its binding protein, the differential cross-reactivity of $25(OH)D_3$ and $25(OH)D_2$ in immunoassays and protein-binding assays is a potential problem *(7, 9)*. However, as $25(OH)D_2$ is not commonly used for dietary supplementation in our region, only very few of the tested samples contained $25(OH)D_2$. This potential problem therefore did not play a role in the differences that were observed between the assays in our study.

The question remains whether the observed differences in 25(OH)D measurements among different 25(OH)D assays are of clinical significance. Our study points to the importance of the 25(OH)D assay when choosing a cutoff for deficiency or insufficiency, as the percentage of vitamin D–sufficient and –insufficient people is critically dependent on the assay. For in-

stance, 20% of the IC patients were found to be vitamin D–sufficient when measured with ID-XLC-MS/MS, whereas 52% were sufficient when measured with the Liaison assay. Of the pregnant women, 67% were vitamin D–sufficient when measured with ID-XLC-MS/MS, whereas only 24% and 25% were sufficient when measured with the Centaur and iSYS assays, respectively. Further results on the percentage of subjects with deficiency [25(OH)D <12 μg/L (<30 nmol/L)], insufficiency [25(OH)D 12–20 μg/L (30–50 nmol/L)], and sufficiency [25(OH)D ≥ 20 μg/L (≥ 50 nmol/L)] per patient study group and per 25(OH)D method are shown in the online Supplemental Figure. The differences between the tested 25(OH)D methods are substantial, so at the moment, the number of patients recommended to take 25(OH)D supplementation depends on the assay used to determine 25(OH)D, and this is undesirable.

Clinical chemistry laboratories should be aware of the analytical problems in currently available 25(OH)D assays and be critical when introducing a new assay. Also physicians, researchers, reviewers, and authorities who provide advice on cutoffs for sufficiency and supplementation of 25(OH)D should carefully consider the 25(OH)D assay used in studies, before any conclusions are drawn or decisions made.

In conclusion, some of the assays used to measure 25(OH)D are not well standardized and report significantly different results from measurements performed with ID-XLC-MS/MS. The deviations, which are sometimes serious, are different in various patient groups and are dependent on the concentration of DBP and other still-unknown interfering factors.

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