Original Article

Performance evaluation of two immunoassays for 25-hydroxyvitamin D

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Although immunoassays in measuring 25-hydroxyvitamin D [25(OH)D] have been improved recently, relatively large differences are still seen between results of 25(OH)D measured by immunoassays and by liquid chromatography-tandem mass spectrometry (LC-MS/MS). In the present studies, we compared two immunoassays with LC-MS/MS for measuring 25(OH)D concentrations. Concentrations of 25-hydroxyvitamin D_2 [25(OH)D_2] and 25-hydroxyvitamin D_3 [25(OH)D_3] in serum samples from 59 healthy subjects were measured by two immunoassays including Siemens ADVIA Centaur Vitamin D Total (Centaur) and Roche Elecsys Vitamin D Total (Elecsys) and LC-MS/MS. To determine the cross reactivity of Elecsys and Centaur toward 25(OH)D_2, a dosage of 200,000 IU vitamin D_2 was given after first sampling. Serum samples were obtained 30 days later and concentrations of 25(OH)D_2 and 25(OH)D_3 were measured again. The results showed poor agreement between the immunoassays and LC-MS/MS in 25(OH)D_2 and 25(OH)D_3 measurements. The percentage of 25(OH)D_2 cross-reactivity was 45.3% for Centaur and 41.2% for Elecsys and there was no significant difference between Centaur and Elecsys. In conclusion, Centaur and Elecsys perform unsatisfactorily in measuring 25(OH)D levels, especially for 25(OH)D_2 cross-reactivity. Therefore, clinicians need to be aware of the underestimation of vitamin D status when using these immunoassays for measuring individuals supplemented with vitamin D_2.

Key Words: **25(OH)D, immunoassay, LC-MS/MS, Centaur, Elecsys**

The active metabolite of vitamin D is widely known to play a critical role in maintaining calcium and phosphorus homeostasis. Severe vitamin D deficiency manifests as rickets in children and osteomalacia in adults.[1] Recent studies have indicated that physiological roles which vitamin D played are not only in the bone but also in a wide variety of tissues.[2,3] Vitamin D deficiency has been shown to be associated with increased risk of many diseases, such as cardiovascular diseases, certain types of cancer, diabetes, and immune diseases.[4–7]

There are two forms of vitamin D in the circulation: vitamin D_3 or cholecalciferol and vitamin D_2 or ergocalciferol. Vitamin D_3 is derived endogenously from skin exposure to the sunshine and exogenously from vitamin D_3 supplements and certain animal foods such as fatty fish. Vitamin D_2 is derived mainly from vitamin D_2 supplements and certain plant foods such as irradiated mushrooms. Vitamin D_2 and vitamin D_3 are converted to 25(OH)D_2 and 25(OH)D_3, respectively, in the liver and then to the active form 1.25-dihydroxyvitamin D_2 [1,25$(OH)_2D_2$] and 1.25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] mainly in the kidney. Among all the related metabolites, 25(OH)D is regarded as the best indicator of vitamin D nutritional status.[8]

Vitamin D deficiency has become a worldwide issue.[9] Studies have shown that vitamin D deficiency or insufficiency is prevalent in almost all age groups if individuals are not taking vitamin D supplements or are lacking sufficient sunshine exposure.[10,11]

There has been a technical improvement in measuring 25(OH)D and a massive rise in 25(OH)D testing all over the world. In the competitive market, new methods emerge in an endless stream and typical ones of noted brands have been committed them to modification. Current methods for measuring 25(OH)D includes immunoassay that use antibodies or vitamin D binding protein (DBP) against the D_2 and/or D_3 form of 25(OH)D and chromatographic assay [including high performance liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC-MS/MS)] that separate 25(OH)D_2 and 25(OH)D_3 based on their respective chemical properties.[12] This means higher precision for chromatographic methods and makes them as the gold standard for measuring 25(OH)D. However, high cost of instrument, large sample volume and lack of operating expertise restrict their use in hospitals and laboratories. High-throughput, easy practicality and reduced operator error contribute to the immunoassays popularity in markets. However, immunoassays which do not separate 25(OH)D from DBP thoroughly are affected by competition between the analyte capture antibodies or DBP and are more flexible to matrix effects. Inability of fully recovering 25(OH)D_2 is a major cause for the variation between immunoassays and chromatography methods according to previous reports.[13]

Recently several diagnostic manufacturers have claimed to be able to align their assays to a truth in 25(OH)D testing. To determine whether manufacturers' efforts in modifying technical issues in recent years exhibiting actual effects in improving the performance of 25(OH)D_2 immunoassays, we compared two automated immunoassays including Simens ADVIA Centaur Vitamin D Total assay (Siemens Healthcare Diagnostics Inc, Tarrytown, NY) and Roche Elecsys Vitamin D Total assay (Roche Diagnostics, Mannheim, Germany) with LC-MS/MS which is regarded as the gold-standard reference for their precision of detecting the total level of 25(OH)D and the ability of recovering 25(OH)D_2.

Materials and Methods

Study design. Fifty nine healthy adults worked in the same corporation were recruited in May, 2014. Blood were collected by routine venipuncture and then centrifuged at 3,000 rotate per minute for 5 min. No visible sign of hemolysis or lipemia was observed in these samples. Serum was isolated and divided into 3 aliquots and frozen at –20°C until transported on dry ice to designated laboratories. Storage time for analysis did not exceed 3 weeks and all aliquots thawed only once. All subjects received a single dosage of 200,000 IU vitamin D_2 by intramuscular route immediately after the first sampling. Six subjects were withdrawn from the study during the interval. Fifty three subjects had

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repeated serum samples collected 30 days later. The samples were processed as described above. During the interval, none of the subjects had taken any foods or supplements containing vitamin D. Measurements were performed in the same analytical run as singletons in a blinded fashion in 3 different laboratories. The Centaur measurements were performed in the laboratory of Institute of Metabolism and Endocrinology, the Second Xiangya Hospital of Central South University (Changsha, Hunan, China). The Elecsys measurements were performed in the laboratory of Chengdu Division of KingMed Diagnostics (Chengdu, Sichuan, China). The LC-MS/MS measurements were performed in the laboratory of Guangzhou Division of KingMed Diagnostics (Guangzhou, Guangdong, China). The LC-MS/MS was used as a gold-standard reference. The ethics committee of the Second Xiangya Hospital approved this study (project number MK0217A-356) and written, full-informed consent was obtained from all trial subjects before participating in the study.

The LC-MS/MS analysis. The LC-MS/MS analysis was performed on an API 4000 ABSCIEX triple quadrupole tandem mass analyzer (ABSciex, Framingham, MA) with an atmospheric pressure chemical ionization (APCI) source at KingMed Diagnostics (Guangzhou, Guangdong, China). Briefly, 200 µl serum was mixed with protein precipitant and internal standard (IS)[25(OH)D_3-d6] (Sigma Aldrich, St. Louis, MO). Liquid-liquid extraction was used to separate analytes from its binding proteins. After centrifugation, the supernatant was pipetted into glass vials and dried under nitrogen, and redissolved. The analyte was separated thoroughly from interfering components in the liquid chromatography system with a quaternary gradient pump. The quantification of 25(OH)D_2, 25(OH)D_3 and IS, a stable vitamin D derivative, was performed on the tandem mass spectrometer. The LC-MS/MS method was calibrated using commercially available calibrators traceable to standard reference materials (SRM) 972 from National Institute of Standards and Technology (NIST).

Siemens ADVIA Centaur Vitamin D total assay. Siemens ADVIA Centaur Vitamin D Total assay (Centaur) is a one-step, 18-min competitive chemiluminescent immunoassay. The assay employs an anti-fluorescein monoclonal mouse antibody covalently bound to paramagnetic particles, an anti-25(OH)D monoclonal mouse antibody labeled with acridinium ester, and a vitamin D analog labeled with fluorescein. There was an inverse relationship between the resulting chemiluminescent signal detected by the system and the amount of vitamin D present in the patient sample. The measurements with Siemens ADVIA Centaur Vitamin D Total kits were performed on the ADVIA Centaur XP Immunoassay System according to the manufacturer's protocol.

Roche Elecsys Vitamin D total assay. Roche Elecsys Vitamin D Total assay (Elecsys) is a competitive, 27-min electrochemiluminescence binding assay. The assay uses a ruthenium labeled vitamin D binding protein as capture agent. Similar to Centaur, there was an inverse relationship between the resulting chemiluminescent signal detected by the Roche system and the amount of vitamin D present in the patient sample. The measurements with Roche Elecsys Vitamin D Total kits were performed on the Modular Cobas E601 Analyzer according to the manufacturer's protocol.

Precision. Precision studies were performed at three different laboratories. Between-run CVs were obtained by measuring two separate pools of the control sample (BIO-RAD, Hercules, CA) once every day for 7 consecutive days. Within-run CVs were determined by measuring 7 replicates of two separate pools of the control sample.

Accuracy. Accuracy studies for LC-MS/MS were performed by using calibrators traceable to SRM 972 from NIST to ensure its correctness (data not provided). In addition, accuracy studies for LC-MS/MS were evaluated and validated by participating in Endocrinology Proficiency Testing Program of New York State Department of Health. The same recovery test was used to evaluate the accuracy of the LC-MS/MS, Centaur and Elecsys. Briefly, the standard serum containing a low or high concentration of 25(OH)D was added to the serum sample from a healthy subject at a dilution of 1:9 and then the background and spikes were measured twice to calculate their recovery rates.

25(OH)D_3 cross-reactivity. 25(OH)D_3 is the predominant form in circulation to evaluate vitamin D nutritional status. The concentration of 25(OH)D_2 is usually minimal unless vitamin D_2 containing supplements are taken. Therefore, the analysis of 25(OH)D_3 cross-reactivity was achieved by directly measuring the total 25(OH)D in serum samples before treatment as the samples contained almost exclusively 25(OH)D_3. The levels of 25(OH)D_3 were measured with Siemens' kits, Roche's kits and LC-MS/MS.

25(OH)D_2 cross-reactivity. Separate quantification of 25(OH)D_2 and 25(OH)D_3 can be acquired by LC-MS/MS, but not immunoassays, which can not differentiate 25(OH)D_2 from 25(OH)D_3. For this reason, the analysis of 25(OH)D_2 cross-reactivity was performed indirectly by calculating 25(OH)D_2 concentrations from the total concentration of 25(OH)D measured by the two immunoassays. The concentrations of 25(OH)D_2 was calculated from results of the total 25(OH)D obtained from each immunoassay using equations as previously described.[14–16] Firstly, we established the linear regression equation for samples containing exclusively 25(OH)D_3 before treatment: $Y = a + bX_3$, where Y was values measured by immunoassays before treatment and X_3 was values measured by LC-MS/MS before treatment. Then, we used the following equation to calculate 25(OH)D_3 after treatment: $Y' = a + bX_3'$, where Y' was the estimated 25(OH)D_3 for immunoassays and X_3' was the 25(OH)D_3 concentrations measured by LC-MS/MS after treatment. The concentration of 25(OH)D_2 for an immunoassay was calculated by subtracting the estimated 25(OH)D_3 value from the total 25(OH)D value after treatment. The percentage 25(OH)D_2 cross-reactivity for each immunoassay was computed by dividing the 25(OH)D_2 values from immunoassays by the 25(OH)D_2 values from LC-MS/MS after treatment.

Statistical analysis. The non-parametric Friedman *M* test was used to compare the results of the three methods before or after treatment and paired Student-test was used to compare the results of each method before and after treatment. Serum 25(OH)D concentrations determined by LC-MS/MS were arbitrarily defined as a reference to which the other results were compared using Passing–Bablok regression, concordance correlation coefficient and Bland–Altman plots analysis. The slope and intercept of the Passing–Bablok regression were referred to in the text as systemic and constant bias, respectively. The cumulative sum linearity test (cusum linearity test) was used to confirm the linear relationship between two methods. The Concordance Correlation Coefficient (CCC) was used to evaluate the degree to which pairs of observations fall on the 45° line through the origin and contains a measurement of precision (Pearson correlation coefficient r) and accuracy (bias correction factor Cb). Interpretation of CCC results was as follows: >0.99, excellent agreement; 0.99–0.95, substantial agreement; 0.90–0.94, moderate agreement; <0.9, poor agreement. The former methods were applied to determine agreement of all the commercial assays against LC-MS/MS. Assay biases were calculated by the Bland–Altman plot. In addition, the Pearson regression equation was applied for estimated 25(OH)D_2 calculation. The analyses were performed using SPSS19.0 and Medcalc ver. 11.4.2.0. A p value <0.05 was considered statistically significant.

Results

The results of precision studies for LC-MS/MS and the two immunoassays were shown in Table 1. The LC-MS/MS method showed that within-run and between-run CVs were less than 5% for both 25(OH)D_2 and 25(OH)D_3 at each of two different levels.

Table 1. Precision studies for LC-MS/MS and two immunoassays performed using same two serum pools with different 25(OH)D concentrations

		Within-run		Between-run	
		Mean ± SD (nmol/L)	CV (%)	Mean ± SD (nmol/L)	CV (%)
Centaur	pool 1	132.0 ± 13.6	10.3	130.4 ± 14.3	11
	pool 2	338.0 ± 31.1	9.2	340.2 ± 17.3	5.1
Elecsys	pool 1	14.0 ± 0.2	1.4	14.3 ± 3.6	25.2
	pool 2	33.6 ± 0.5	1.5	36.2 ± 7.1	19.6
LC-MS/MS 25(OH)D_2	pool 1	18.7 ± 0.5	2.7	19.3 ± 1.1	5.7
	pool 2	44.4 ± 1.0	2.3	46.5 ± 2.4	5.2
LC-MS/MS 25(OH)D_3	pool 1	17.9 ± 0.4	2.2	18.3 ± 0.8	4.4
	pool 2	38.9 ± 1.3	3.3	37.9 ± 1.7	4.5

The Centaur assay showed that within-run and between-run CVs were 10.3% and 11.0% at one level, and 9.2% and 5.1% at the other level. The Elecsys assay showed that within-run CVs were less than 5%, but between-run CVs were more than 10% at two different levels.

The accuracy of LC-MS/MS was validated by participating in Endocrinology Proficiency Testing Program of New York State Department of Health. The results were shown in Supplemental Table 1*. In our laboratory, the accuracy of LC-MS/MS, Centaur and Elecsys was tested by the recovery test. The results showed a good recovery efficiency for LC-MS/MS (124.1% for low level of additive 25(OH)D and 93.6% for the high level), but a modest recovery efficiency for Centaur (75.9% and 51.7%) and Elecsys (82.4% and 62.9%) (Supplemental Table 2*).

The mean (±SD) total 25(OH) D concentrations measured with Centaur kits, Elecsys kits and LC-MS/MS before and after vitamin D_2 supplement are shown in Table 2. Friedman *M* test analysis revealed that the concentrations of 25(OH)D_3 obtained from three different methods before treatment were not significantly different from each other. The results showed after treatment the highest values of total 25(OH)D measured by LC-MS/MS (56.0 nmol/L), followed by Elecsys (46.7 nmol/L) and then Centaur (43.9 nmol/L). There was an apparent elevation in total 25(OH)D concentration 30 days after vitamin D_2 treatment. As expected, the LC-MS/MS method revealed a marked increase in 25(OH)D_2 concentrations from 0.6 nmol/L to 15.6 nmol/L in average after 25(OH)D_2 treatment, and a subtle but statistically significant increase in 25(OH)D_3 concentrations from 37.8 nmol/L to 40.6 nmol/L after treatment probably due to the slightly increased sun exposure.

Table 2. 25(OH)D values in nmol/L by each immunoassay and LC-MS/MS before and 30 days after a single dosage of 200,000 IU vitamin D_2

	Before treatment (nmol/L)	After treatment (nmol/L)
Centaur	35.4 ± 6.2	43.9 ± 8.7†,‡
Elecsys	37.6 ± 11.8	46.7 ± 13.4†,‡
LC-MS/MS 25(OH)D_2	0.6 ± 2.2§	15.6 ± 7.6‡
LC-MS/MS 25(OH)D_3	37.8 ± 10.2	40.6 ± 12.7‡
LC-MS/MS total	38.3 ± 10.7	56.0 ± 14.7†,‡

$^{\dagger}p<0.05$, comparing the three assays in the same before or after treatment group, using Friedman *M* test; $^{\ddagger}p<0.05$, comparing the corresponding before and after treatment value, using paired *t* test; §most 25(OH)D_2 levels before treatment were undetectable (<5.5 nmol/L), which we regarded as 0.

Having shown that there was no difference in the mean values of 25(OH)D measured by three different methods before treatment, we wanted to know whether the results of immunoassays correlated with that of LC-MS/MS. To address this issue, we did Passing–Bablok regression coeffecient and CCC analyses. Table 3 shows the results of the Passing–Bablok regression coeffecients, CCCs, and their 95% confidence intervals. The scatters (Passing–Bablok regression) and Bland–Altman plots for the total 25(OH)D before and after treatment are shown in Fig. 1. Although Elecsys demonstrated better CCCs than Centaur before and after treatment, CCCs of the two immunoassays against LC-MS/MS, varying from 0.39 to 0.69, were all below 0.9. The Passing–Bablok regression analysis revealed the slopes for Centaur were 0.48 (95% CI of 0.34 to 0.65) before treatment and 0.53 (95% CI of 0.42 to 0.73) after treatment. However, the slopes for Elecsys were 1 before and after treatment. The intercepts for Centaur were 16.9 (95% CI of 10.8 to 22.1) before treatment and 13.8 (95% CI of 4.4 to 19.8) after treatment. The intercepts for Elecsys before and after treatment were 0. In addition, the best fit lines for Centaur before and after treatment crossed the line of identity, suggesting a low bias at the concentration above 35 nmol/L and a high bias below this value. These data indicate a poor agreement

Table 3. Passing–Bablok regression and concordance correlation analysis for each immunoassay against LC-MS/MS total or 25(OH)D_2

	Passing–Bablok regression					Concordance correlation			
	Intercept	95%CI	Slope	95%CI	Cusum test	CCC	95%CI	r	Cb
Before-Centaur	16.9	10.8 to 22.1	0.48	0.34 to 0.65	$p>0.05$	0.43	0.25 to 0.58	0.52	0.83
Before-Elecsys	−5.3	−19.4 to 3.6	1.12	0.87 to 1.47	$p>0.05$	0.69	0.52 to 0.80	0.69	0.99
After-Centaur	−13.8	4.4 to 19.8	0.53	0.42 to 0.73	$p>0.05$	0.39	0.25 to 0.51	0.68	0.58
After-Elecsys	13.8	−22.9 to 1.9	1	0.78 to 1.22	$p>0.05$	0.61	0.45 to 0.73	0.75	0.81
Adjusted 25(OH)D_2									
Centaur	−8.4	−13.1 to −3.7	1.05	0.73 to 1.50	$p>0.05$	0.34	0.17 to 0.49	0.54	0.63
Elecsys	−19.1	−30.8 to 10.9	1.54	1.07 to 2.50	$p>0.05$	0.26	0.09 to 0.42	0.41	0.64

CCC, concordance correlation coefficient; CI, confidence interval; r, Pearson's correlation coefficient; Cb, bias correction factor; Before-Centaur, Centaur values before treatment; Before-Elecsys, Elecsys values before treatment; After-Centaur, Centaur values after treatment; After-Elecsys, Elecsys values after treatment.

*See online. https://www.jstage.jst.go.jp/article/jcbn/58/3/58_15-61/_article/supplement

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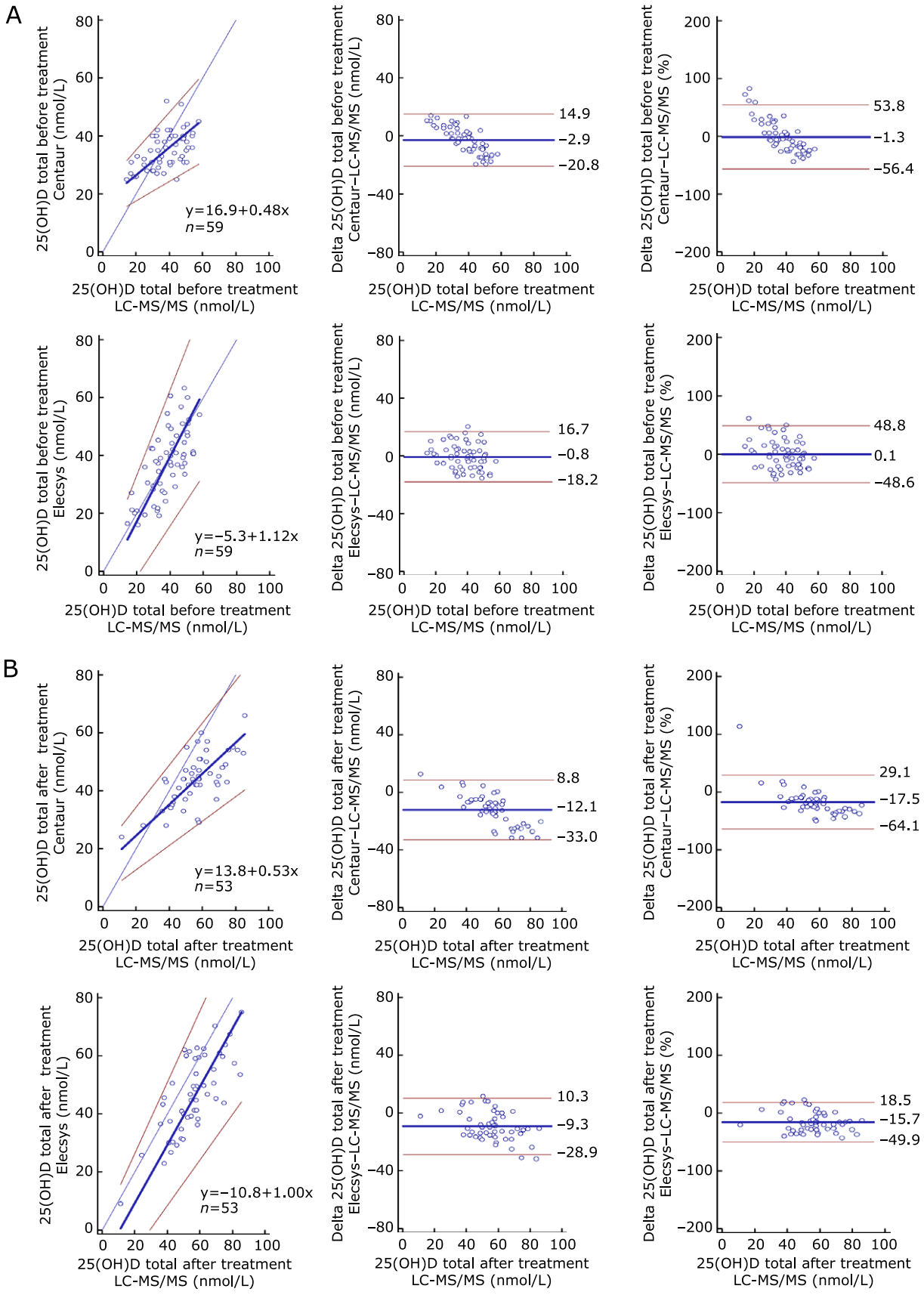

Fig. 1. Comparison of 25(OH)D total levels for the two immunoassays against LC-MS/MS total by Passing–Bablok regression analysis (left panels) and Bland–Altman plots [middle (nmol/L) and right panels (%)], for before treatment (A) and after treatment (B). Left panels: thin black lines denote lines of identity; thick blue lines denote regression lines; red lines denote 95% confidence intervals; blue circles denote individual samples. Middle and right panels: thick blue lines denote mean biases; red lines denote 95% confidence intervals; blue circles denote individual samples.

Table 4. Estimated $25(OH)D_2$ concentrations (nmol/L) and percentage $25(OH)D_2$ cross-reactivity (%) for each immunoassay compared to LC-MS/MS $25(OH)D_2$

	Estimated $25(OH)D_2$ concentration mean ± SD (nmol/L)	Percentage $25(OH)D_2$ cross-reactivity mean ± SD (%)	Commercial claim for $25(OH)D_2$ cross-reactivity (%)
Centaur	7.5 ± 7.6	45.3 ± 54.2	105
Elecsys	6.8 ± 9.8	41.2 ± 76.4	92

SD: standard deviation.

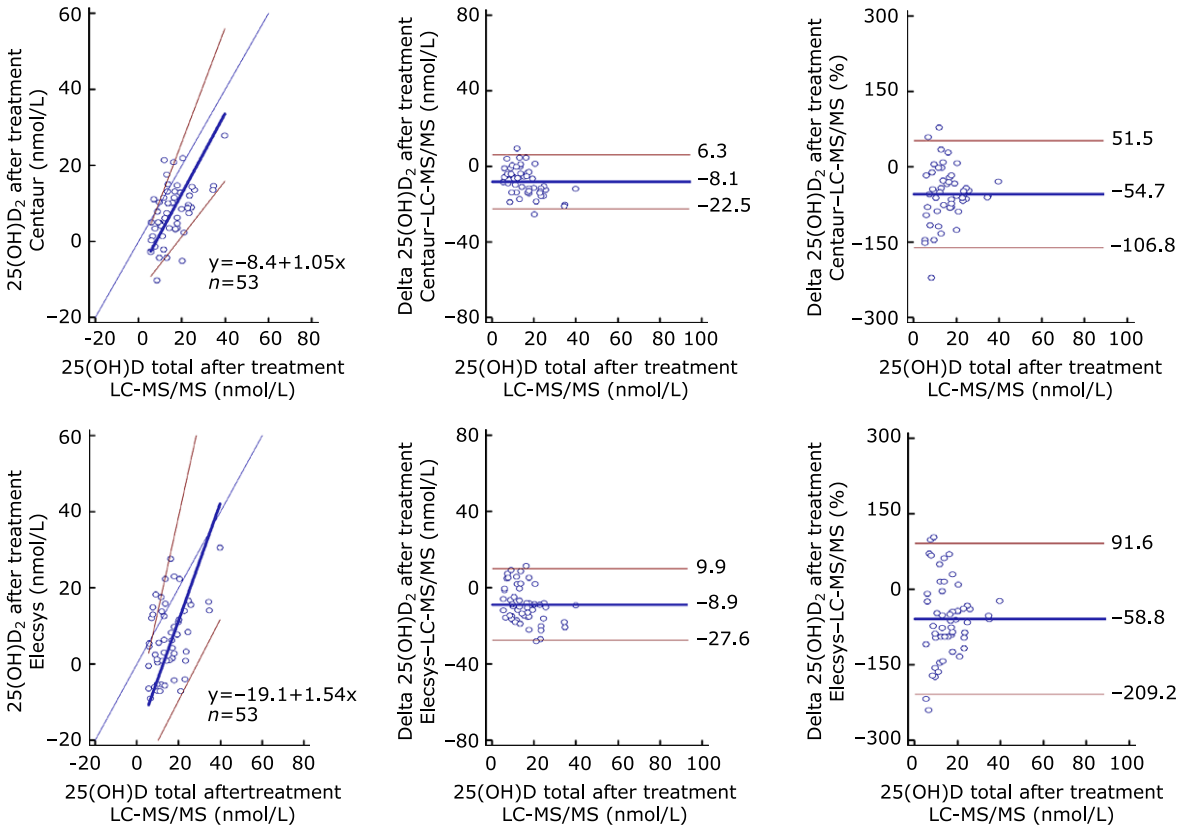

Fig. 2. Comparison of the adjusted $25(OH)D_2$ levels for the two immunoassays against LC-MS/MS $25(OH)D_2$ by Passing–Bablok regression analysis (left panels) and Bland–Altman plots [middle (nmol/L) and right panels (%)]. Left panels: thin black lines denote lines of identity; thick blue lines denote regression lines; red lines denote 95% confidence intervals; blue circles denote individual samples. Middle and right panels: thick blue lines denote mean biases; red lines denote 95% confidence intervals; blue circles denote individual samples.

between either of immunoassay and LC-MS/MS although there was no significant difference in the mean values of $25(OH)D_3$ among three different methods before treatment and both agreement coefficients and mean values of immunoassays against that of LC-MS/MS are not satisfactory after treatment.

According to the Bland–Altman plots for analyzing assay bias, the mean biases for Elecsys (before/after treatment, –0.8 nmol/L/–9.3 nmol/L) were not different from those for Centaur (before/after treatment, –2.9 nmol/L/–12.1 nmol/L), with striking bias increases after ergocalciferol treatment for both assays. By expressing the bias in percentage, the data showed a similar tendency as described above.

To determine whether there was a difference between Centaur and Elecsys in the recovery of $25(OH)D_2$, we calculated $25(OH)D_2$ concentrations of each immunoassay. The values and percentages of $25(OH)D_2$ for immunoassays are shown in Table 4. The paired *t* test of the values of $25(OH)D_2$ showed no significant difference between Centaur and Elecsys ($p>0.05$). So did the paired *t* test of percentage $25(OH)D_2$ for Centaur and Elecsys. The results indicate comparable $25(OH)D_2$ cross-reactivities between Centaur and Elecsys assays.

The results of CCC, Passing–Bablok regression analysis and Bland–Altman plots for $25(OH)D_2$ of immunoassays against LC-MS/MS $25(OH)D_2$ are shown in Table 2 and Fig. 2. In regard to the $25(OH)D_2$ recovery, both assays showed poor agreement against LC-MS/MS, with CCC = 0.34 for Centaur and 0.26 for Elecsys. The slope for Centaur, 1.05 (95% CI of 0.73 to 1.50), was not different from 1, but its intercept, –8.4 (95% CI of –13.1 to –3.73) was still evident. The slope for Elecsys, 1.54 (95% CI of 1.07 to 2.50) and its intercept, –19.1 (95% CI of –30.79 to –10.92) were both statistically significant, indicating a positive systemic bias and negative constant bias for this assay against LC-MS/MS. The Bland–Altman plots showed comparable mean bias [–8.1 nmol/L (–54.7%) for Centaur and –8.9 nmol/L (–58.8%) for Elecsys], but Elecsys had a broader bias distribution than Centaur in $25(OH)D_2$ recovery. The concentration of $25(OH)D_2$ in serum before vitamin D treatment was too low to detect and not shown in Table 3.

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Discussion

Although there is no doubt that levels of 25(OH)D in serum is a marker of vitamin D nutritional status, the assay of serum 25(OH)D is not standardized. LC-MS/MS is now widely accepted as the gold standard for 25(OH)D analysis, but they have not been offered by most laboratories. Immunoassays is widely used to measure serum 25(OH)D concentrations. This situation raised a concern about the accuracy of test results for 25(OH)D. Although several diagnostic companies recently claimed that the 25(OH)D immunoassays had been largely improved, we found a poor agreement between immunoassays and the LC-MS/MS assay in measuring 25(OH)D concentrations in serum in the present studies. More importantly, the immunoassays markedly underestimate $25(OH)D_2$ levels in serum when compared with LC-MS/MS.

The obvious disagreement, demonstrated in slope, intercept and CCC, between immunoassays and LC-MS/MS in $25(OH)D_3$ cross-reactivity has also been reported by several investigators previously.[18–20] Because of the lipophilic nature of 25(OH)D, the immunoassays, without extraction step, are more vulnerable to non-specific matrix effects, which are caused by mainly liquids and lately claimed any substances in plasma or serum samples for direct assays.[13] Matrix effects are unpredictable in individual samples for different assays, therefore contributing to measuring variation.[21] On the other hand, the immunoassays are designed to displace the analyte from its binding protein, which involves disrupting DBP. The percentages of free 25(OH)D based on their own protocols and the interaction with other less known factors such as DBP are other explanations for their variable performances against the gold reference LC-MS/MS.[22] The narrow distribution of values (mostly <50 nmol/L) in our study might also play a role in the unsatisfactory performance for immunoassays.

Another potential problem is the assay sensitivity in detecting $25(OH)D_2$ for immunoassays. Due to its solvent extraction step to separate $25(OH)D_2$ and $25(OH)D_3$, LC-MS/MS is regard as the gold reference for high specificity and accuracy. As for immunoassays, those unable to release 25(OH)D from DBP in the same step as capture of the analyte, tend to show spurious $25(OH)D_2$ results.[23] With 25(OH)D released in the absence of capture moiety, it is not surprising that Centaur and Elecsys exhibit underrecovery of $25(OH)D_2$. The analyte captures employed for Centaur and Elecsys are specific antibodies and DBP, respectively. As DBP showing a low affinity for $25(OH)D_2$, Elecsys and Centaur may need to pay more attention in this respect, which can also be ascertained from our data that the intercept, slope and CCC of Elecsys showed poor performance in $25(OH)D_2$ cross-reactivity.[24]

The analysis of percentage $25(OH)D_2$ cross-reactivity demonstrated a comparable performance for Centaur and Elecsys, but was significantly lower than what Le Goff *et al.*[14] previously reported and what Centaur (105%) and Elecsys (92%) claimed. Le Goff *et al.*[14] treated subjects with larger dosage of vitamin D_2 for longer duration, thus resulting markedly higher $25(OH)D_2$ levels than ours.[25] Previous studies reported that most immunoassays had difficulties in measuring low concentrations of 25(OH)D total, so our lower $25(OH)D_2$ concentration range may account for poor performance in detecting $25(OH)D_2$ by the immunoassays in our study.[26] Therefore, we deduced that low $25(OH)D_2$ cross-reactivity for the two immunoassays may be related to their difficulties in detecting low $25(OH)D_2$ concentrations. The differences between our results and commercial claims might be due to the source of $25(OH)D_2$ used. In studies by Cavalier *et al.*[15] and studies by Horst *et al.*,[26] the recovery of $25(OH)D_2$ for immunoassays kits was performed by adding exogenous $25(OH)D_2$ to the human serum samples, unlike our present studies using samples containing endogenous $25(OH)D_2$ after vitamin D_2 treatment. Spiking exogenous 25(OH)D metabolites have been well known to affect analytical cross-reactivity with vitamin D compounds when using the immunoassays. One study reported an around 50% of $25(OH)D_2$ cross-reactivity for Elecsys although the sample size was relatively small and vitamin D was not supplemented to these subjects.[27] In the present study, we used larger sample size and fixed dosage of vitamin D_2 and demonstrated 41.2% and 45.3% of $25(OH)D_2$ cross-reactivity for Elecsys and Centaur.

There are some limitations in our study. One problem was that our samples were collected in the summer. Different amount of sun exposure may result in variability of the 25(OH)D concentrations. During the one month interval between May and June, for sample collection, the sun exposure was gradually increased, which might have resulted in the elevation of $25(OH)D_3$ concentrations, thus might have affected the accuracy of $25(OH)D_2$ measuring concentration. Another limitation is that the LC-MS/MS method were arbitrarily regarded as a gold reference with 100% recovery of $25(OH)D_2$ and $25(OH)D_3$, which was a theoretical assumption. One limitation for LC-MS/MS is its inability to discriminate between 25(OH)D and its inactive isomer C-3 epimers, thus overestimate 25(OH)D concentrations when the C-3 epimers account for a considerable proportion of the circulating $25(OH)D_3$ concentration.[28]

Carter reported that the results of the LC-MS/MS assay developed as a candidate reference measurement procedure by NIST were on average 11.2% lower than those given by routine LC-MS/MS methods.[13] These data indicate that most routine LC-MS/MS assays may have overestimated 25(OH)D by failing to resolve a molecule having the same mass as 25(OH)D and a similar fragmentation pattern. However, the LC-MS/MS method employed in the present study uses calibrators traceable to SRM 972 from NIST to ensure its correctness. Its correctness was also qualified by Endocrinology Proficiency Testing Program of New York State Department of Health. Given the discrepancies found among different sorts of assays, it is important to standardize all assays, including LC-MS/MS methods, to achieve comparable accuracy.

Taken together, Centaur and Elecsys lack agreement with LC-MS/MS both in $25(OH)D_3$ and $25(OH)D_2$ measurement. Moreover, both immunoassays has cross-reactivity lower than 50% for $25(OH)D_2$, which is much lower than what has been previously reported and commercially claimed. Thus, clinicians should carefully interpret the results when using Centaur or Elecsys immunoassay to measure serum 25(OH)D concentrations in individuals who have received vitamin D_2 supplements to avoid overestimating vitamin D deficiency.

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Abbreviations

CDC	the Centers for Disease Control and Prevention
CI	confidence interval
DBP	vitamin D binding protein
HPLC	high performance liquid chromatography
IS	internal standards
LC-MS/MS	liquid chromatography-tandem mass spectrometry
NIST	the National Institute for Standards and Technology
$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D

$25(OH)D_2$	25-hydroxyvitamin D_2
$25(OH)D_3$	25-hydroxyvitamin D_3
SD	standard deviation
SRM	standard reference materials

Conflict of Interest

No potential conflicts of interest were disclosed.

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