Etienne Cavalier*, Pierre Lukas, Anne-Catherine Bekaert, Stéphanie Peeters, Caroline Le Goff, Eric Yayo, Pierre Delanaye and Jean-Claude Souberbielle

Analytical and clinical evaluation of the new Fujirebio Lumipulse®G non-competitive assay for 25(OH)-vitamin D and three immunoassays for 25(OH)D in healthy subjects, osteoporotic patients, third trimester pregnant women, healthy African subjects, hemodialyzed and intensive care patients

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Abstract

Background: In this study, we provide a short analytical evaluation of the new Fujirebio Lumipulse®G non-competitive immunoassay for 25(OH)D. Clinical performance was compared with three commercial competitive automated immunoassays against a Vitamin D Standardization Program (VDSP)-traceable liquid chromatography-tandem mass spectrometry (LC-MS/MS) in six different clinically relevant populations.

Methods: Lumipulse®G 25(OH)D precision, measurement uncertainty, recovery, limit of quantification were assessed, as well as 25(OH)D2 and C3-epimer recovery. For method comparison, 250 serum samples obtained in healthy Caucasians and Africans, osteoporotic, hemodialyzed and intensive care patients and 3rd trimester pregnant women were analyzed by all methods. Correlation was studied using Passing-Bablok and Bland-Altman analysis. Concordance correlation coefficient (CCC) was calculated to evaluate agreement between immunoassays and the LC-MS/MS.

Results: The Lumipulse®G 25(OH)D assay presented interesting analytical features and showed excellent correlation to the LC-MS/MS results (y=1.00×–1.35 ng/mL), as obtained in healthy Caucasian individuals. In the other special populations, Lumipulse®G presented a concordance with LC-MS/MS which was generally higher than competitors, even if all methods significantly underrecovered 25(OH)D in hemodialyzed patients. Intra-assay CV ranged from 12.1% at 9.6 ng/mL to 2.1% at 103.7 ng/mL and inter-assay CV ranged from 16.2 to 3.7% at the same concentrations, respectively. Measurement uncertainty, with a probability of 95%, were respectively 33.1 and 7.6% at these concentrations. LOQ was found to be at 4.6 ng/mL. Mean (95% CI) 25(OH)D2 revovery was 77% (74–81) and no cross-reactivity was observed with C3-epimer.

Conclusions: Fujirebio Lumipulse®G 25-OH Vitamin D Total assay is therefore considered suitable for assessment of vitamin D status in clinical routine.

Keywords: assay performance; concordance; immunoassay; LC-MS/MS; vitamin D.

***Corresponding author: Pr. Etienne Cavalier,** Department of Clinical Chemistry, University of Liège, CHU Sart-Tilman, Domaine du Sart-Tilman, 4000 Liège, Belgium, Phone: +32 4 3667692, Fax: +32 4 3667691, E-mail: Etienne.cavalier@chu.ulg.ac.be
Pierre Lukas, Anne-Catherine Bekaert, Stéphanie Peeters and Caroline Le Goff: Department of Clinical Chemistry, University of Liège, CHU Sart-Tilman, Domaine du Sart-Tilman, Liège, Belgium
Eric Yayo: Department of Clinical Chemistry, University of Liège, CHU Sart-Tilman, Domaine du Sart-Tilman, Liège, Belgium; and Laboratoire du Centre d'Hémodialyse, Université Félix Houphouët Boigny, Abidjan, Côte d'Ivoire
Pierre Delanaye: Department of Nephrology, Dialysis and Transplantation, University of Liège, CHU Sart-Tilman, Domaine du Sart-Tilman, Liège, Belgium
Jean-Claude Souberbielle: Laboratoire d'exploration fonctionelles, Hôpital Necker-Enfants malades, Paris, France

Introduction

The analytical determination of 25-hydroxyvitamin D (25(OH)D) is far from an easy task [1, 2]. Indeed, several important problems have to be overcome to correctly assess this variable. Among them, the very high lipophilic nature of the molecule and its strong association with vitamin D

binding protein (VDBP) necessitates a thorough separation step and, for the one-phase immunoassays, a good equilibrium between the analyte and the antibodies used in the kits [3]. VDBP can be present at different concentrations according to some physiological or pathological conditions, like race [4], pregnancy or chronic kidney disease [5–7], which could influence the dissociation kinetics of the molecule. Vitamin D can be found as vitamin D2 or D3 and the assay should ideally measure equally 25(OH)D2 and 25(OH)D3 [8]. Different other metabolites of vitamin D, such as C3-epimer or 24,25(OH)2-vitamin D can be present in patients' serum at different concentrations, possibly interfering with immunoassays or liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods [9]. As any other immunoassays, vitamin D assays are prone to heterophilic antibodies interference, leading to potential spurious results [10]. Last but not least, the standardization of the different assays remains a major problem. Hopefully, we have now a commonly accepted reference method and an ongoing worldwide standardization program, the Vitamin D Standardization Program (VDSP) coordinated by the CDC, the NIST and the University of Ghent [11]. These efforts have globally improved the global concordance of different assays for 25(OH)D determination in the "normal healthy" population, but some issues are still remaining, notably in "special" populations, such as pregnant women and African (or Asian) subjects because of their, respectively high and low concentrations of VDBP [7].

Due to potential actions of vitamin D on different diseases [12], the number of 25(OH)D determinations has dramatically increased over the last 10 years. This increasing number of requests have led most of the clinical laboratories to move from the DiaSorin RIA, the most widely used method in the 1990s and early 2000s, to methods presenting a larger throughput, i.e. automated immunoassays or liquid chromatographs coupled with two mass spectrometers in tandem (LC-MS/MS). All immunoassays were yet designed as competitive methods. Very recently, Fujirebio (Tokyo, Japan) was the first company to launch on the Lumipulse®G instrument a non-competitive (sandwich) method for 25(OH)D determination based on antimetatype monoclonal antibodies against a hapten-antibody immunocomplex using an ex vivo antibody development system, namely the Autonomously Diversifying Library system, a process which has recently been validated [13]. The aim of this study is first to provide a short analytical evaluation of the new sandwich immunoassay for 25(OH)D and also to compare the results obtained with this method and three other commercial competitive automated immunoassays to a VDSP-traceable LC-MS/MS in different clinical situations.

Materials and methods

Analytical validation

Assay precision was determined across the dynamic range using serum samples according to a modified version from CLSI protocol EP05-A2 [14]. Six human serums were run in duplicate, in the morning and in the afternoon, for 5 days (n=20 per sample). The "true" value of each of these samples was determined with our VDSP-traceable LC-MS/MS (see [7] for details of the LC-MS/MS method), which allowed to calculate the measurement uncertainty, as requested by the ISO 15189, on two different lots. Variability was expressed in standard deviation (SD) and percent coefficient of variation (CV). Recovery was obtained by mixing three different high and low level samples ¾; ¼, ½; ½ and ¼; ¾ dilutions. Limit of quantification (LOQ) was obtained from six samples with low values, run in duplicate, in the morning and in the afternoon, for 5 days. Two different lots (5045 and 5066) were used for the evaluation, except for the LOQ, for which lot 5117 was used .

The 25(OH)D2 recovery was obtained with 20 native samples containing high 25(OH)D2 concentrations according to the method we previously published [8]. We also used the same method to calculate the percentage of cross-reactivity with the C3-epimer in 20 vitamin D3 supplemented subjects presenting high 25(OH)D values which were thus prone to present detectable C3-epimer concentrations by LC-MS/MS.

Clinical validation

As all the results were compared to our VDSP traceable LC-MS/MS, we confirmed the accuracy of this method with the Labquality panel. Next to LC-MS/MS, Lumipulse®G 25(OH)D was also compared to three commercial immunoassays for 25(OH)D, namely DiaSorin Liaison XL (lot 13181), IDS iSYS (lot 2175) and Roche Elecsys (lot 00180293). For this latter method, values >70 ng/mL were censored and could not thus be used for comparison purposes.

The first comparison was achieved in 100 young healthy subjects spanning the measuring range that had been included in a supplementation trial. The second comparison was performed with samples obtained from 30 women referred to a specialized osteoporosis clinic. We also compared the methods in populations who are known to present different VDBP concentrations: the first one was 3rd trimester pregnant women (n=30; high VDBP values) and the second one was African healthy subjects from the area of Abidjan, Côte d'Ivoire (n=30; low VDBP values). We also compared the methods in a population of 30 stable hemodialyzed (HD) patients and in 30 patients from the general intensive care unit of our hospital. Indeed, these subjects can have a serum matrix which is very different from healthy individuals due to nitrogen products accumulation or proteins carbamylation or fluid shifts [5–7, 15]. Finally, we compared the assays in subjects presenting high C3-epimer concentrations and endogenous high 25(OH)D2 levels, as determined by our LC-MS/MS.

All the samples used in this study were leftover samples kept at −80 °C that underwent one cycle of freeze/thawing, which does not alter 25(OH)D values [16]. All the comparisons have been performed in parallel on all the instruments by our experienced R&D team, in

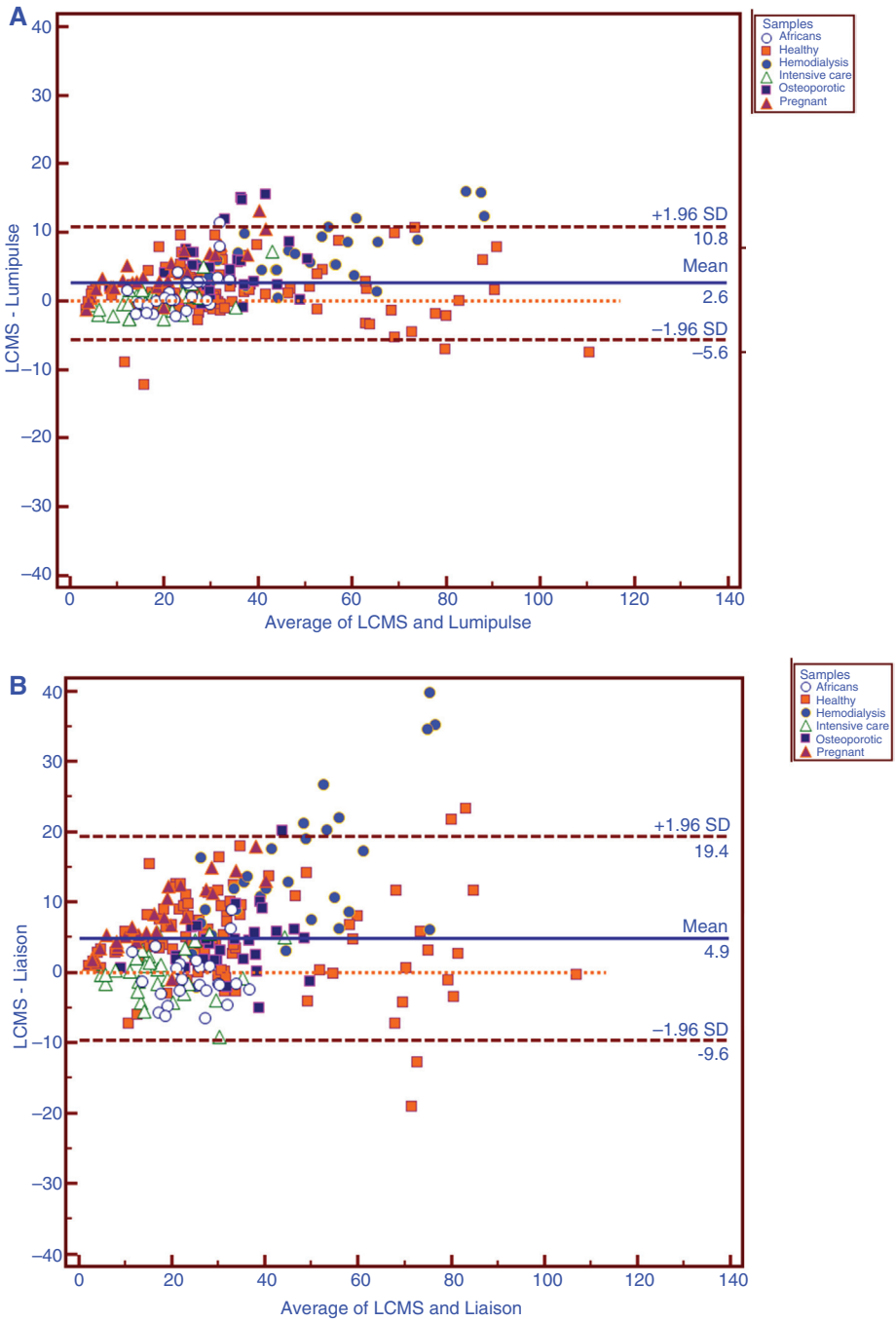

Figure 1: Bland-Altman plot of Fujirebio Lumipulse (A), DiaSorin Liaison (B), IDS iSYS (1C) and Roche Elecsys (D) against a VDSP-traceable LC-MS/MS method in healthy Caucasian subjects, osteoporotic patients, third trimester pregnant women, healthy African subjects, hemodialyzed and intensive care patients.

our ISO 15189 accreditated laboratory. The Ethics Committee of the CHU de Liege has been informed of the study and has accepted its modality.

MedCalc software (Oostende, Belgium) was used for the statistical comparisons and allowed to perform the Passing-Bablock regressions and concordance correlation coefficient (CCC). The CCC evaluates the degree to which pairs of observations fall on the 45° line through the origin [17]. It contains a measurement of precision "r" and accuracy C_b and is calculated as CCC=r $C_{b,}$ where r is the Pearson correlation coefficient (which measures how far each observation deviates from the best-fit line and thus the precision), and C_b is a bias correction factor that measures how far the best-fit line deviates from the 45° line through the origin, and is thus a measure of accuracy. CCC result can be interpreted as follows: poor (<0.90), moderate (0.90–0.95), substantial (0.95–0.99) and almost perfect (>0.99) [18].

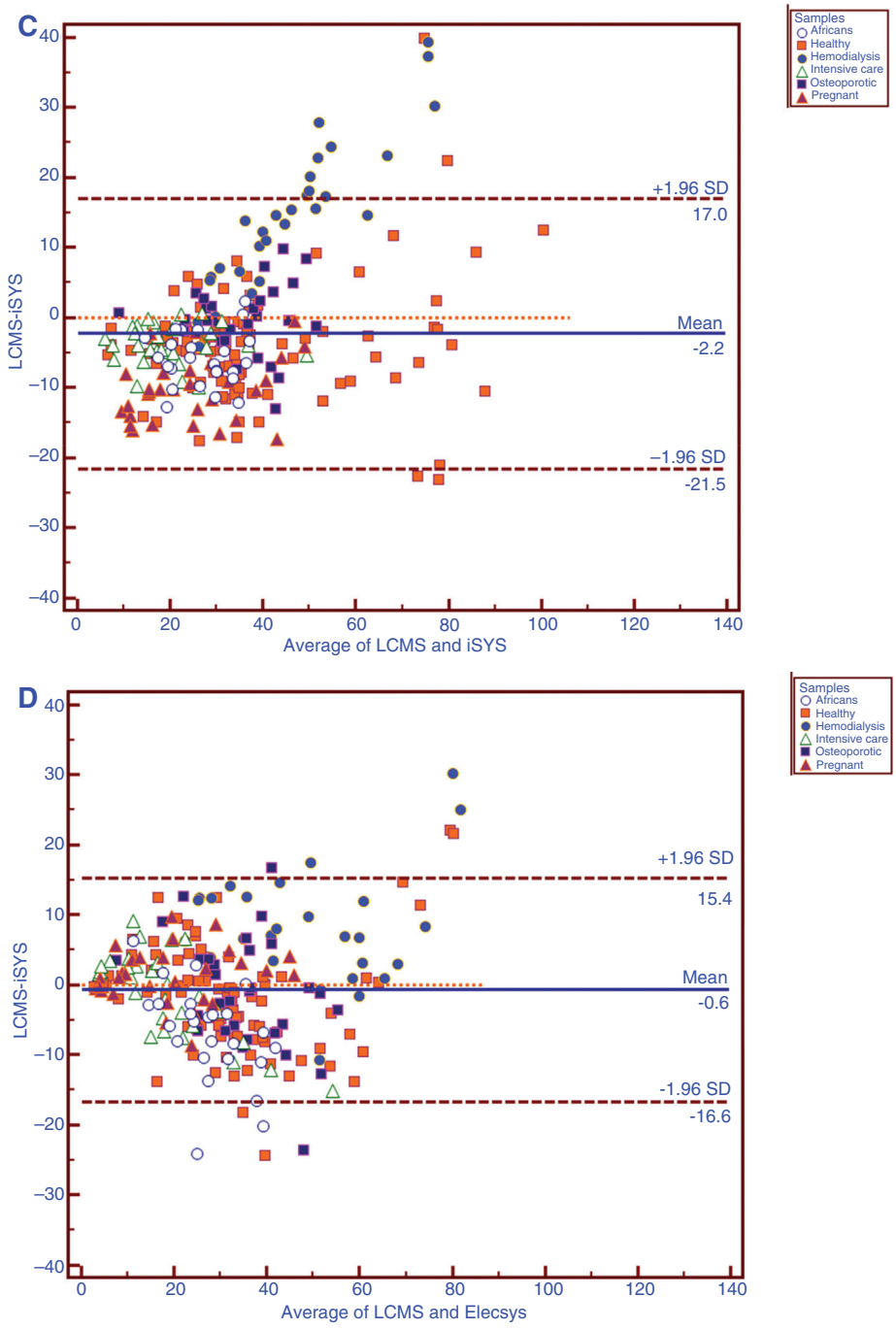

Figure 1: (continued)

Results

Analytical validation of the Lumipulse®G 25(OH)D assay

The intra-assay CV ranged from 12.1% at 9.6 ng/mL to 2.1% at 103.7 ng/mL and the inter-assay CV ranged from 16.2 to 3.7% at the same concentrations, respectively. The measurement uncertainty, with a probability of 95% ranged from 33.1 to 7.6% for these two concentrations. In other words, with an uncertainty of 33.1%, there is 95% of chance that the "true" value of i.e. sample 1, which presents a value of 9.6 ng/mL with our reference method, is comprised between 6.4 and 12.8 ng/mL. The mean recovery was 101.2±2.4%. The LOQ was found to be at 4.6 ng/mL.

The samples obtained in healthy subjects supplemented with vitamin D2 contained a mean concentration

of 25(OH)D3 of 14.0±6.8 ng/mL (range: 6.3–29.5 ng/mL) and a mean 25(OH)D2 concentration of 52.8±15.8 ng/mL (range: 18.6–80.1 ng/mL) as determined by the LC-MS/MS. The mean (95% CI) 25(OH)D2 revovery was 77% (74–81) for Fujirebio Lumipulse®G, 89% (82–96) for DiaSorin Liaison, 86% (80–92) for IDS iSYS and 61% (56–66) for Roche Elecsys.

The mean 25(OH)D values observed in the vitamin D3 supplemented subjects selected to study the C3-epimer cross-reactivity was 58.9±14.9 ng/mL (range: 32.1–90.1 ng/mL). The mean concentration of C3-epimer as measure by LC-MS/MS was 7.5±9.6 ng/mL (range: 3.1–47.4 ng/mL). None of the immunoassays tested in this study presented a significant cross-reactivity with the C3-epimer as the 95% confidence interval of the mean of cross-reactivity encompassed the zero value.

Clinical validation of the Lumipulse®G 25(OH)D assay

On the Labquality panel, the regression equation we obtained for our LC-MS/MS method was Reference method=−1.37+1.05 Liege LC-MS/MS. This slope of 1.05 is in accordance with the VDSP recommendations that accept a bias of ±5% [19]. The Bland-Altman plots of the different immunoassays and the LC-MS/MS are presented in Figure 1. The Passing-Bablock slopes and intercepts, as well as the CCC and quality of the agreement between immunoassays and LC-MS/MS, for the global population, but also for all the sub-populations are presented in Table 1. To summarize, the agreement was substantial with Lumipulse®G, moderate with Liaison and poor with iSYS and Elecsys on the overall population (n=250). This agreement was much better in the healthy population (substantial/substantial/moderate/poor for Lumipulse, Liaison, iSYS and Elecsys, respectively) compared to the subpopulations. Indeed, the agreement was poor for four methods in osteoporotic patients and healthy Africans, moderate for Lumipulse but poor for the three other immunoassays in hemodialyzed patients, poor for Liaison and iSYS but moderate for Lumipulse and substantial for Elecsys in pregnant women and was substantial for Lumipulse, moderate for Liaison and poor for both iSYS and Elecsys for intensive care patients.

Discussion

In this study, we validated the new Fujirebio Lumipulse®G 25(OH)D assay, which is the first non-competitive method for 25(OH)D determination. This method presents interesting analytical features, even if the CV in the lower range could be improved. The limit of quantification at 4.6 ng/mL is however, acceptable for clinical purposes. The Fujirebio Lumipulse®G assay partially recognizes the 25(OH)D2 form and this could be a limitation for the accurate follow-up of subjects supplemented with vitamin D2. This study does not totally confirm the results we obtained in a previous study [20] as none of the assays encompass the 100%. If we remain close to 100% for DiaSorin Liaison and IDS iSYS, the cross-reactivity for Roche Elecsys that we found here was much lower than previously shown. There are multiple reasons that could explain this discrepancy. The first one could be the concentrations observed in this study vs. the previous one. Indeed, we have here much higher 25(OH)D2 (mean: 52.8 ng/mL) and much lower 25(OH)D3 (14.0 ng/mL) than in the other study (means of 40.3 ng/mL for 25(OH)D3 and 14.3 ng/mL for 25(OH)D2). Another explanation may also be the huge difference between the Passing-Bablock equation observed between the LC-MS/MS and the Roche Elecsys assay in the healthy patients of this study for 25(OH)D3 (Roche Elecsys=1.23×LC-MS/MS−3.35) and the one we observed in a comparable healthy population in our previous study (Roche Elecsys=0.80×LC-MS/MS+3.41) whereas the other assays tested in both studies present similar equations. It is not clear why we observed such discrepancies for this assay, but there was no reason to reject the series performed on Elecsys as the internal QC fitted perfectly in the range and the instrument is handled and maintained according to the manufacturer's specifications. In any case, these results warrant further investigation.

We also confirmed in this study that the immunoassays (at least those that have been tested here) do not cross-react with the C3-epimer form. This is also the case for the Roche Elecsys assay and we confirm here the results obtained by van den Ouweland et al. on native samples [21].

One of the major findings in this paper is that, if there is a relatively good concordance between the assays in the overall and in the healthy population, this is absolutely not the case anymore if we look closely at the subgroups. Even if the problem had already been evoked by different authors in hemodialyzed and pregnant patients [5, 6], this is the first study, to the best of our knowledge, that also describes this problem to other subpopulations of diseased or healthy African subjects in which the sample matrix and/or VDBP polymorphism and concentration may differ from healthy Caucasians. On top of that, these results may suggest that, during the life of a kit on the market, different things can happen (like changes in the process, new suppliers, etc.) that affect the diseased, but not the healthy population. Clinical chemists must be aware that some minor changes that might not have any

Table 1: Passing-Bablok regression statistics and concordance correlation analysis of immunoassays against the VDSP traceable LC-MS/MS method in different populations: healthy subjects, osteoporotic, hemodialyzed and intensive care patients, healthy African subjects and 3rd trimester pregnant women.

Population	Method	Slope	95% CI	Intercept, ng/mL	95% CI, ng/mL	CCC	95% (CI)	*r* precision	C_b accuracy	Agreement
Overall n=250	Fujirebio	0.91	0.88;	0.53	−0.20;	0.97	0.96;	0.98	0.99	Substantial
	Lumipulse		0.94		1.1		0.97			
Median (95% CI) (ng/mL)	DiaSorin	0.88	0.83;	−0.95	−2.2;	0.90	0.87;	0.93	0.96	Moderate
28.6 (27.0–30.1)	Liaison		0.93		0.39		0.92			
Range (ng/mL):	IDS iSYS	0.84	0.77;	7.96	6.2;	0.85	0.81;	0.87	0.97	Poor
2.70–106.7			0.90		9.4		0.88			
	Roche	1.09	1.01;	−1.82	−3.2;	0.89	0.85;	0.89	1.0	Poor
	Elecsys		1.18		0.0		0.91			
Healthy n=100	Fujirebio	1.00	0.95;	−1.35	−2.44;	0.98	0.97;	0.98	1.00	Substantial
	Lumipulse		1.03		0.02		0.99			
Median (95% CI) (ng/mL)	DiaSorin	1.00	0.92;	−4.32	−6.32;	0.96	0.95;	0.97	0.99	Substantial
29.8 (27.6–32.7)	Liaison		1.06		−2.27		0.97			
Range (ng/mL):	IDS	0.98	0.90;	4.54	2.11;	0.90	0.86;	0.94	0.96	Moderate
2.70–106.7	iSYS		1.06		6.81		0.93			
	Roche	1.23	1.10;	−3.35	−7.75;	0.87	0.82;	0.90	0.98	Poor
	Elecsys		1.36		−0.84		0.92			
Osteoporotic n=30	Fujirebio	0.93	0.75;	−2.53	−8.83;	0.77	0.61;	0.89	0.87	Poor
	Lumipulse		1.13		3.85		0.87			
Median (95% CI) (ng/mL)	DiaSorin	0.89	0.77;	0.26	−4.53;	0.81	0.66;	0.89	0.91	Poor
34.1 (29.8–38.9)	Liaison		1.04		4.19		0.90			
Range (ng/mL):	IDS	0.98	0.77;	1.21	−5.95;	0.87	0.75;	0.87	1.00	Poor
9.4–53.7	iSYS		1.20		7.20		0.93			
	Roche	1.32	0.99;	−10.89	−25.82;	0.74	0.55;	0.77	0.96	Poor
	Elecsys		1.78		1.34		0.86			
3rd trimester pregnant n=30	Fujirebio	0.78	0.73;	0.44	−0.24;	0.91	0.86;	0.99	0.92	Moderate
	Lumipulse		0.83		1.17		0.94			
Median (95% CI) (ng/mL)	DiaSorin	0.67	0.61;	−0.53	−1.31;	0.75	0.62;	0.97	0.77	Poor
18.4 (10.7–24.1)	Liaison		0.72		0.04		0.83			
Range (ng/mL):	IDS	0.88	0.75;	12.23	9.89;	0.68	0.52;	0.94	0.72	Poor
2.8–46.9	iSYS		1.02		14.73		0.79			
	Roche	0.93	0.82;	−0.12	−2.25;	0.95	0.89;	0.95	0.99	Substantial
	Elecsys		1.07		1.21		0.97			
Healthy Africans n=30	Fujirebio	0.77	0.65;	4.20	1.48;	0.85	0.74;	0.92	0.93	Poor
	Lumipulse		0.91		7.06		0.92			
Median (95% CI) (ng/mL)	DiaSorin	0.97	0.74;	2.32	−5.94;	0.85	0.70;	0.86	0.99	Poor
24.0 (20.5–28.2)	Liaison		1.25		7.26		0.93			
Range (ng/mL):	IDS	1.00	0.73;	6.5	−1.37;	0.62	0.41;	0.85	0.73	Poor
12.9–37.4	iSYS		1.32		12.49		0.77			
	Roche	1.48	1.11;	−5.35	−19.66;	0.54	0.31;	0.76	0.72	Poor

Table 1 (continued)

Population	Method	Slope	95% CI	Intercept, ng/mL	95% CI, ng/mL	CCC	95% (CI)	*r* precision	C_b accuracy	Agreement
	Elecsys		2.10		2.65		0.71			
Hemodialyzed patients n=30	Fujirebio Lumipulse	0.85	0.80; 0.92	1.27	−2.93; 3.56	0.91	0.85; 0.95	0.99	0.92	Moderate
Median (95% CI) (ng/mL) 51.3 (42.7–60.7)	DiaSorin Liaison	0.63	0.52; 0.84	3.77	−4.80; 9.52	0.60	0.42; 0.73	0.88	0.67	Poor
Range (ng/mL): 24.1–95.3	IDS iSYS	0.47	0.41; 0.55	13.03	8.46; 16.11	0.53	0.38; 0.65	0.95	0.55	Poor
	Roche Elecsys	1.00	0.78; 1.21	−7.21	−18.66; 2.21	0.80	0.65; 0.89	0.89	0.91	Poor
Intensive care unit patients n=30	Fujirebio Lumipulse	0.90	0.80; 0.98	1.66	0.42; 2.99	0.97	0.95; 0.98	0.98	0.99	Substantial
Median (95% CI) (ng/mL) 16.1 (12.4–19.5)	DiaSorin Liaison	0.98	0.84; 1.17	0.25	−2.14; 2.04	0.94	0.87; 0.97	0.94	1.00	Moderate
Range (ng/mL): 3.7–46.7	IDS iSYS	1.01	0.88; 1.14	3.83	0.53; 5.32	0.88	0.79; 0.94	0.95	0.92	Poor
	Roche Elecsys	1.42	1.21; 1.64	−5.09	−9.29; −2.70	0.87	0.79; 0.92	0.94	0.93	Poor

impact on the healthy population or internal QC can dramatically affect different categories of patients.

Osteoporotic women are probably the patients for whom vitamin D supplementation and 25(OH)D monitoring is the most relevant. Unfortunately, all the immunoassays tested here present a poor concordance with our LC-MS/MS. Yet, this population is not homogenous and the samples have been selected according to the fact that patients had undergone a blood sampling in the frame of a specialized osteoporosis consultation. These subjects are mainly post-menopausal women treated (or not) for osteoporosis but cannot be considered sensu strictu as "healthy". We have unfortunately no clear explanation for these observed discrepancies and this deserves further investigations. As already mentioned, previous authors had already shown important discrepancies when 25(OH)D was measured by immunoassays or LC-MS/MS in third trimester pregnant women or hemodialyzed patients [5, 6]. The immunoassays tested here differ from those previously tested but the conclusion remains the same: they remain very inaccurate in these patients and a thorough improvement is mandatory. Indeed, this lack of precision can have clinical consequences in the sense that under-recovery of some immunoassays may lead clinicians to increase the supplementation doses to reach a target of 30 ng/mL. As higher doses of vitamin D can potentially have toxic effects [22, 23] this is not free from consequences. We also observed an under-recovery in healthy Black African subjects, probably depending on the lower concentrations of VDBP of these patients, compared to Caucasians [4] and the immunoassays tested here and in another study [24] are poorly in agreement with the LC-MS/MS for this population. Recent papers have shown that the "bioavailable" vitamin D [i.e. the free 25(OH)D and the 25(OH)D bound to albumin] was probably a better indicator of the vitamin D status for Black Americans and have led researchers to revisit the free-vitamin D hormone hypothesis [25], even if this concept is far from being new [26, 27].

The only "special" population that provided acceptable results was the patients from the general intensive care units. We had previously shown that immunoassays were inaccurate in severe burn patients [15], but this was not specially the case here. The explanation may be due to the fact that the patients of this study were chosen among the general ICU ward, and not the burn unit. It is not clear however, why the results observed in ICU patients are more in agreement with the LC-MS/MS than those observed in ambulatory osteoporotic patients.

The most important limitation of this study is the relatively small number of subjects included in each subgroups (n=30) which could perhaps partially explain

some situations of discrepancies, but our study has also some strengths, like the use of a VDSP-traceable LC-MS/MS, re-validated on the Labquality panel, the ISO 15189 accreditation of our laboratory and, of course, the important sub-populations that we studied.

Finally, an important conclusion regarding the standardization of the assays can be drawn from this study. Indeed, our results show that the different immunoassays presented here are not far from being correctly "standardized" compared to our VDSP-traceable LC-MS/MS in a general healthy population. Having a correct "standardization" in diseased patients whom 25(OH)D is requested seems much more complicated and this should be the challenge of both manufacturers and VDSP consortium to achieve this goal.

In conclusion, the new sandwich assay for 25(OH)D determination provided by Fujirebio on the Lumipulse®G presents interesting analytical results and better clinical results compared to classical competition assays, but we have to unfortunately acknowledge a poor concordance of the immunoassays with LC-MS/MS in patients for whom the test is important. It is clear that the "vitamin D assays market" is now saturated, but efforts in improving the assays are mandatory and collaboration between IVD manufacturers and clinical laboratories is of highest importance to improve the assays.

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