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Automated measurement of 25-OH Vitamin D on the LUMIPULSE® *G*1200: analytical verification and method comparison

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ABSTRACT

Background: Because of its potential value in several pathologies, clinical interest in 25-hydroxy Vitamin D (25OH-D) is increasing. However, the standardisation of assays remains a significant problem. Our aim was to evaluate the performance of the novel Lumipulse *G* 25-OH Vitamin D assay (Fujirebio), comparing results with the Liaison (Diasorin) method.

Methods: Analytical verification of the Lumipulse *G* 25-OH Vitamin D assay was performed. Both methods were compared using sera from 226 patients, including 111 patients with chronic renal failure (39 on haemodialysis) and 115 patients without renal failure. In addition, clinical concordance between assays was assessed.

Results: For Lumipulse *G* 25-OH Vitamin D assay, the limit of detection was 0.3 ng/mL, and the limit of quantification was 2.5 ng/mL with a 9.7% of coefficient of variation. Intra-and inter-assay coefficients of variation were <2.3 and <1.8% (25.4–50.0 ng/mL), respectively. Dilution linearity was in the range of 4.5–144.5 ng/mL. Method comparison resulted in a mean difference of −6.5% (95% CI from −8.8 to −4.1) for all samples between Liaison and Lumipulse *G*. Clinical concordance assessed by Kappa Index was 0.66.

Conclusions: Lumipulse *G* 25-OH Vitamin D showed a good clinical concordance with the Liaison assay, although overall results measured in Lumipulse were higher by an average of 6.5%.

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Introduction

During the last decade, the demand for Vitamin D testing has increased exponentially. The causes for this are mainly linked to a recognised clinical interest to detect and to treat Vitamin D deficiency, which is highly prevalent, and in view of its potential relevance for osteoporosis, malignancies, and cardiovascular, infectious and autoimmune diseases.[1,2] However, the lack of assay standardisation remains a significant problem. The measurement of 25-hydroxy Vitamin D (25OH-D) can be performed by immunoassay, HPLC and liquid chromatography–tandem mass spectrometry (LC–MS/MS).[3–5] The golden standard is the LC–MS/MS method, although it was not harmonised until the introduction of a standard reference material (SRM) by the National Institute for Standards and Technology (NIST, www.nist.gov), specifically NIST SRM 972, and the application of reference measurement procedures (RMPs) validated by NIST and Ghent University.[6–8] Immunoassays are also a useful tool to measure 25OH-D, with the ability to detect both 25OH-D2 and 25OH-D3 in an equimolar manner, and so the opportunity to report total 25OH-D.[6]

As a result of the increased Vitamin D testing, there has been a raised interest in automated Vitamin D immunoassays. Several manufacturers have launched automated 25OH-D immunoassays, such as Abbott (Chicago, IL, USA), Diasorin (Saluggia, Italy), IDS (Boldon, United Kingdom), Roche (Basel, Switzerland), Siemens (Munich, Germany) and, more recently, Fujirebio (Ghent, Belgium). However, there is a great deal of variation between performances of these automated 25OH-D immunoassays.[3,9–12] This assay variation makes it difficult for clinicians to compare results from different studies,[9,13] despite the similar cut points established by The Endocrine Society (www.endocrine.org) and World Health Organization.[14,15] There is still no consensus on appropriate reference range for deficiency and insufficiency, but there is agreement that 30 ng/mL 25OH-D level is the cut-off to define an optimal Vitamin D status. For this reason, in 2009, the NIST, in collaboration with the National Institutes of Health Office of Dietary Supplements (NIH-ODS; Bethesda, MD, USA.), established the first accuracy-based programme with the goal of improving the comparability of laboratory 25OH-D assay, which was called the NIST/NIH Vitamin D Metabolites Quality Assurance Program (VitDQAP). They concluded that there is a large intra- and inter-laboratory variability (≥5 and 7–28%, respectively) that hinders definitive assessment of bias and accuracy.[16] Besides the VitDQAP, they collaboratively support Vitamin D metabolite metrology through SRM; for instance, SRM 972 Vitamin D in human serum, which

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was the first certified reference material for Vitamin D metabolites.[16] With the aim of correcting this variation problem, the NIH-ODS has also established the Vitamin D Standardization Program in November 2010, leading to a Vitamin D Standardization Certification.[13]

Our goals were to evaluate the limit of detection (LoD) and quantification (LoQ), reproducibility and linearity from Lumipulse *G* (Fujirebio Europe, Ghent, Belgium) and to study the agreement between this automated immunoassay and the Liaison 25 OH Vitamin D assay (Diasorin, Saluggia, Italy), assessing its clinical concordance.

Materials and methods

Analytical performance parameters and study population

Assay performance characteristics of LoD, LoQ, reproducibility and dilution linearity were studied to conduct an analytical verification of the novel Lumipulse *G* 25-OH Vitamin D assay. LoD was determined as the concentration corresponding to the signal obtained at two standard deviations from the mean of the signal of zero-concentration samples. Twenty replicates of the zero-concentration sample were assayed and used to determine the mean and standard deviation. The apparent concentration at two standard deviations from this mean was extrapolated. LoQ was determined as the mean analyte concentration at which the mean imprecision, expressed as coefficient of variation (CV), was <10%. Four samples in the range of 2 to 8 ng/mL were analysed in replicates of 10.

Inter-assay reproducibility was determined in two different ways. The first was to determine three quality controls (Vitamin D Control, Fujirebio Diagnostics Inc, Malvern, PA, USA), with a 25OH-D concentration of 10.8, 32.6 and 73.9 ng/mL, during ten operator days. The second was to analyse four replicates each of five serum pools (25.4–50.0 ng/mL) per run, two runs per day, for three days. Intra-assay reproducibility corresponding to serum pools was determined for each day. Dilution linearity (recovery) was assessed by serial dilution of five high-concentration patient samples (>100 ng/mL). Dilutions were carried out with Lumipulse *G* Specimen Diluent 1 (Fujirebio). The results were then plotted and the expected vs. observed values were analysed by linear regression.

For the method comparison, 25OH-D was measured by Lumipulse *G* and Liaison in a clinical panel, which included 226 serum samples from subjects aged 18–79 years over the measuring range of 4.0–150.0 ng/mL. This panel was divided into two groups: routine samples without renal failure (115 samples; 31% of male patients and average age of 60) and another group of chronic renal failure patients (111 samples; 72% of male patients and average age of 64), 39 of them with hemodialysis. After venous blood collection, serum samples were automatically aliquoted by Genesis FE500 Workcell (Tecan, Männedorf, Switzerland). Subsequently, serum aliquots were frozen at –20 °C within 4 h. They were thawed only once before the 25OH-D analysis. Samples were analysed by both automated immunoassays on the same day.

Clinical concordance was assessed according to Endocrine Society's Guideline [14] 25OH-D serum levels: deficiency <20 ng/mL; insufficiency 20–29 ng/mL; sufficiency 20–100 ng/mL and potential toxicity >100 ng/mL. The study was specifically approved by the hospital Ethics Committee.

Instruments and assays

In our laboratory, routine 25OH-D measurement is performed by Liaison (Diasorin, Saluggia, Italy). This assay is a chemiluminescent immunoassay method which consists of a direct competitive immunoassay using magnetic microparticles, coated with specific antibody to Vitamin D (solid phase), while the latter is linked to an isoluminol derivative. The Lumipulse *G* assay is based on a chemiluminescent enzyme immunoassay (CLEIA) technology, which uses a two-step sandwich immunoassay method. In both assays, there is first a dissociation of 25OH-D from Vitamin D binding protein (VDBP). However, Lumipulse *G* new assay is different from the current ones available at present because is the only method using a sandwich immunoassay instead of a competitive assay and due to its unique VDBP release method (substitution instead of pre-treatment method).

Statistics

The 25OH-D results obtained by Lumipulse *G* and Liaison were analysed by Bland–Altman plots.[17] Differences between groups are presented as mean with 95% confidence intervals and *p*-values take into account a significance level of 5% and an allowable difference of 10%. Dilution linearity was determined by regression analysis and correlation. Clinical concordance was assessed by overall concordance (%) and Kappa Index.[18]

Results

Assay verification

The Lumipulse *G* LoD was determined as a concentration of 0.3 ng/mL. To evaluate LoQ, four samples with concentrations of 2.5, 4.8, 6.3 and 7.3 ng/mL were analysed in replicates of ten. LoQ was 2.5 ng/mL at 9.7% of CV. Intra- and inter-assay reproducibility was tested with five serum pools (Table 1). Intra-assay reproducibility, determined for three days, with two runs per day and four replicates of each pool was <2.3%. Inter-assay reproducibility of these pools ranged from 1.3 to 1.8%. The analysis of three quality controls during ten operator days leads an inter-assay

Table 1. Intra- and inter-assay reproducibility analysis for the Lumipulse G 25-OH Vitamin D assay.

			Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Intra-assay	Day 1	Mean	26.0	30.1	37.6	44.8	48.0
		SD	0.32	0.63	0.66	0.74	0.84
		CV (%)	**1.22**	**2.08**	**1.75**	**1.66**	**1.75**
	Day 2	Mean	26.0	30.6	38.0	44.5	47.9
		SD	0.26	0.44	0.42	0.56	1.11
		CV (%)	**1.02**	**1.43**	**1.11**	**1.26**	**2.31**
	Day 3	Mean	26.3	30.4	38.0	45.4	48.2
		SD	0.39	0.33	0.48	0.60	0.76
		CV (%)	**1.49**	**1.08**	**1.26**	**1.31**	**1.58**
Inter-assay	Total	CV (%)	**1.29**	**1.62**	**1.45**	**1.63**	**1.83**
			QC1	**QC2**	**QC3**		
		Mean	11.6	31.7	72.7		
		SD	0.6	2.4	1.6		
		CV (%)	**5.2**	**7.4**	**2.2**		

Notes. SD – standard deviation; CV – coefficient of variation; QC – Vitamin D Quality Control.

Table 2. Dilution linearity analysis of five high-concentration patient samples (110.1–144.5 ng/mL) of the Lumipulse G 25-OH Vitamin D assay.

Sample	*r*	Slope	Intercept
1	0.998	0.99 (0.92–1.06)	1.98 (–1.94–5.91)
2	0.994	0.99 (0.89–1.09)	3.74 (–2.06–9.54)
3	0.996	0.96 (0.77–1.14)	7.79 (–7.46–23.05)
4	0.996	0.97 (0.89–1.06)	6.29 (0.85–11.73)
5	0.991	0.97 (0.84–1.10)	6.15 (–0.51–12.82)

Note. Data expressed as slope (confidence interval) and intercept (confidence interval). r coefficient of correlation.

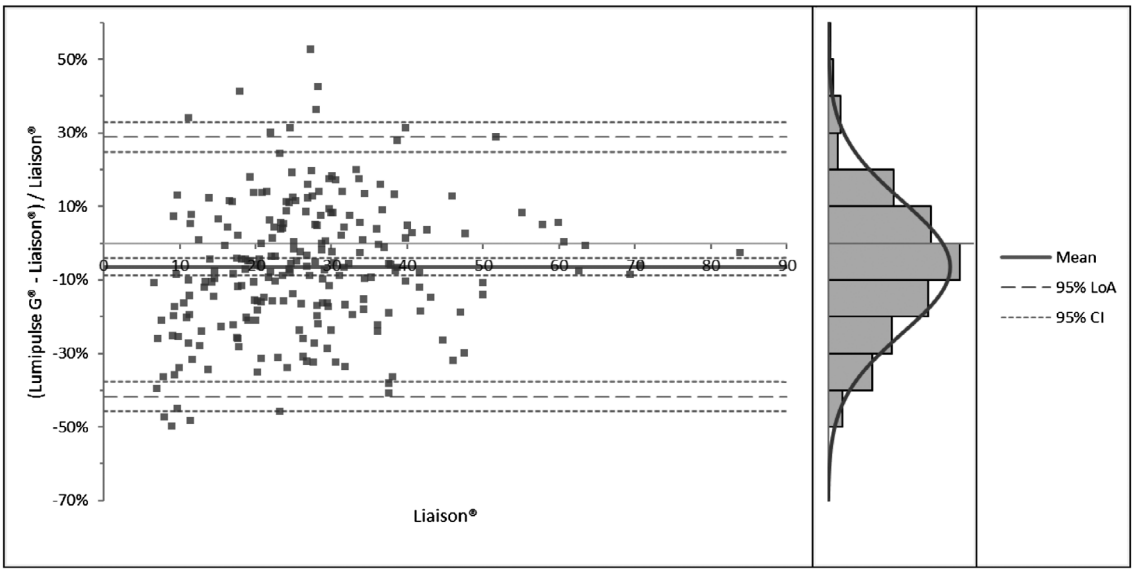

Figure 1. Bland–altman plot for 25OH-D obtained in 226 serum samples with the Liaison and Lumipulse ***G***.
LoA limits of agreement, CI confidence interval. Mean bias of –6.5% (95% CI from –8.8 to –4.1), standard deviation (SD) of 18.0% and LoA from –41.8 to 28.8%. On the horizontal axis, 25OH-D serum levels measured by Liaison are expressed from 0 to 90 ng/mL.

reproducibility ranging from 2.2 to 7.4%. Dilution linearity was determined by serial dilutions (from 1:2 to 1:32) of five high-concentration patient samples. The equations obtained by linear regression showed a correlation coefficient (*r*) from 0.991 to 0.998 (Table 2).

Method comparison

The method comparison, with Liaison assay as the reference method, was done for all samples and individually for samples of patients with normal creatinine, with high creatinine without hemodialysis and with hemodialysis. Bland–Altman analysis for all samples showed a mean difference of –6.5% (95% CI from –8.8 to –4.1, $p = 0.002$), standard deviation (SD) of 18.0% and limits of agreement (LoA) from –41.8 to 28.8% (Figure 1). Mean difference was –8.4% (95% CI from –11.4 to –5.5, $p = 0.148$; SD of 15.9%) for normal creatinine samples, –5.5% (95% CI from –10.1 to –0.9, $p = 0.026$; SD of 19.4%) for high creatinine samples and –2.5% (95% CI from –9.1 to 4.2, $p = 0.014$; SD of 20.6%) for dialysis samples, as shown in Table 3.

Clinical concordance, according to the cut-offs indicated by the Endocrine Society's Guideline[14], led to an

Table 3. Bland–Altman plots for 25OH-D between Liaison and Lumipulse G.

	SD (%)	Mean % difference	95%CI	*p*-value*	95%LL LoA	95%UL LoA
All samples (*n* = 226)	18.0%	−6.5%	(−8.8)–−4.1)	0.002	−41.8	28.8
Normal creatinine (*n* = 115) [73; 43–114 µmol/L]	15.9%	−8.4%	(−11.4)–(−5.5)	0.148	−39.7	22.8
High creatinine (*n* = 72) [209; 116–557 µmol/L]	19.4%	−5.5%	(−10.1)–(−0.9)	0.026	−43.6	32.6
Dialysis (*n* = 39) [606; 184–1028 µmol/L]	20.6%	−2.5%	(−9.1)–4.2	0.014	−42.7	37.8

Notes. SD – standard deviation; CI – confidence interval; LoA – limits of agreement; LL – lower limit; UL – upper limit. The creatinine concentration of each group is expressed in terms of mean and range.
*Statistically significant differences when *p*-value is less than 0.05 (significance level of 5%).

Table 4. Clinical concordance and kappa index between Liaison and Lumipulse G.

25OH-D concentration	<20 ng/mL	20–29 ng/mL		30–100 ng/mL
Liaison	67	88		71
Lumipulse *G*	84	73		69
Overall concordance (%)	79.8	82.9		97.2
	Value	Standard Error (a)	T approx (b)	*p*-value
Kappa Index	0.66	0.041	14.201	<0.01
Number of valid cases	226			

overall concordance between both methodologies from 79.8% (<20 ng/mL) to 97.2% (30–100 ng/mL), as shown in Table 4. Kappa Index was 0.66, suggesting a substantial agreement between them.

Discussion

Analytical and clinical verification of new assays for the measurement of 25OH-D is an important requirement as there will inevitably be differences between manufacturers, technology involved in the assay and analytical difficulties related to 25OH-D itself. Characteristics of 25OH-D molecule that can interfere with assays include the lipophilic nature of Vitamin D, high levels of 25OH-D2, C3-epimers, heterophilic antibodies, strong affinity to VDBP and association with human serum albumin. [6] Furthermore, because of treatments with Vitamin D2 supplementation, it is also important that Vitamin D assays measures in an equimolar fashion both 25-OHD2 and 25-OHD3 and reports the total 25-OHD result. For these reasons, differences between assays are often remarkable and the latter studies about the performance of 25OH-D assays stressed the need of standardisation and harmonisation of 25OH-D measurements.[4,10,11]

The new fully automated Vitamin D assay from Fujirebio has unique methodological features. It is a non-competitive sandwich immunoassay, which permits the reaction of the analytical target with excess amount of antibodies and the double recognition of the target with the primary and labelled antibodies. This feature, compared to conventional competitive immunoassays, leads to a better sensitivity and specificity.[19] The second one is the unique VDBP release method, which consists in a substitution instead of a pre-treatment method.

With the recent availability of the Lumipulse ***G*** 25-OH Vitamin D assay and knowing the analytical and clinical aspects that impact 25OH-D assays performance, we investigated its reproducibility, sensitivity and linearity. Lumipulse ***G*** 25-OH Vitamin D assay proved to be a solid test with a LoD of 0.3 ng/mL and LoQ of 2.5 ng/mL. It is important to take into account the analysis of low 25OH-D concentrations, since that will define the lower limit to which laboratories report. It has been commented upon that most immunoassays have difficulties measuring low concentrations and, specifically, some automated immunoassays showed excessive difference bellow 8 ng/mL.[3] With regard to inter-assay reproducibility assessment, Lumipulse ***G*** 25-OH Vitamin D assay demonstrated a total CV from 1.3 to 1.8% over the clinically significant assay ranging from 25.4 to 50.0 ng/mL (Table 1). Comparing the previous results with the inter-assay CV obtained with the Liaison (CV of 4.8 and 4.9%; quality controls of 15 and 53 ng/mL 25OH-D concentrations, respectively), better inter-assay reproducibility is achieved with Lumipulse assay. Our intra-assay reproducibility results were extremely similar to those results obtained by Omi et al.[19], researchers who had developed the new non-competitive immunoassay based on antibodies for Lumipulse ***G***.

In other studies that have compared different immunoassays with LC-MS/MS, Liaison has shown a good agreement with the LC-MS/MS method with the lowest mean difference and good intra-assay (≤10%) and inter-assay (≤15%) reproducibility.[3,20] Other immunoassays from Abbott, Roche and Siemens validated in similar studies showed generally good performance, but had some limitations when their performance was challenged with samples with low and high 25OH-D concentrations, heterophilic antibodies or high 25OH-D2 concentrations.[9] Due to the above, the Diasorin Liaison test is an adequate method in order to compare the performance of Fujirebio's Lumipulse ***G*** 25-OH Vitamin D assay.

This study demonstrated concordance of both automated immunoassays Lumipulse ***G*** and Liaison for the measurement of 25OH-D concentrations. According to

Table 5. Summary.

What is known about this subject	What this paper adds
• Due to the increased Vitamin D testing, several manufacturers have launched automated 25-hydroxy Vitamin D (25OH-D) immunoassays • Vitamin D deficiency has been associated with numerous health outcomes • There is a lack of equivalency between 25OH-D assays, and thus the absence of standardisation remains a significant problem	• The novel automated 25OH-D assay from Fujirebio has unique methodological features • Analytical verification of the Lumipulse *G* 25OH-D assay (Fujirebio) showed overall acceptable analytical performance • Method comparison with Liaison (Diasorin) showed good agreement and clinical concordance, although overall results measured in Lumipulse were higher by an average of 6.5%

Bland–Altman plots (Figure 1), the Lumipulse ***G*** 25-OH Vitamin D assay correlates well with the Liaison assay. Selecting those samples with normal creatinine, the mean difference is worse (−8.4%) than comparing to all samples (−6.5%). However, if we focus on the samples with high creatinine, mean difference was similar (−5.5%) to the agreement with all samples. Surprisingly, the group of patients under dialysis has the lowest mean difference (−2.5%). We have no clear explanation for these low differences, but this point has not been assessed in other studies.

It is important that all 25OH-D assays clearly distinguish between different categories of clinical classification routinely used by clinicians, especially when involves the initiation of a pharmacology therapy or a treatment change. We classified samples included in our study according to Endocrine Society's Guideline[14], showing that for 25OH-D concentrations higher than 30 ng/mL, both Lumipulse ***G*** and Liaison 25-OH Vitamin D assays gave a high overall clinical concordance (Table 4). In addition, for 25OH-D concentrations lower than 30 ng/mL, the overall concordance was approximately 80%. Kappa Index (0.66) is considered as substantial agreement according to the score described by Viera and Garrett[18]; therefore, good clinical concordance between methods was achieved.

To sum up, this work demonstrates that the new automated 25OH-D immunoassay shows an appropriate analytical performance as well as good agreement and clinical concordance with Liaison (Table 5). Interestingly, Lumipulse *G* 25-OH Vitamin D demonstrated a good clinical concordance with the Liaison assay, although overall results measured in Lumipulse were higher by an average of 6.5%. Finally, our study highlights the importance of analytical and clinical verification of 25OH-D immunoassays due to the lack of equivalency between 25OH-D methodologies.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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