Comparison of Three Methods for 25 Hydroxyvitamin D Measurement in Patients with no Supplementation or Supplemented with Ergocalciferol, Cholecalciferol or Both.

Basilotta N1, Insúa A2, Reverendo A1, Andrada R1, Guerrero L1, García L2, Quiroga S1.

1 Endocrine Laboratory, Department of Laboratory Tests, 2 Endocrine Division, Department of Internal Medicine. Centro de Educación Médica e Investigaciones Clínicas (CEMIC). Av. Galván 4102, 1431, CABA, Argentina.

ABSTRACT

Introduction: There are several methodological options for 25 hydroxyvitamin D (25OHD) measurement. The lack of standardization across methods can lead to discrepant results, which could be accentuated in the case of patients supplemented with different forms of vitamin D.

Objective: To compare three methods for 25OHD quantification and to compare the 25OHD results from untreated subjects with those obtained from subjects receiving ergocalciferol (D2), cholecalciferol (D3), or both.

Materials and Methods: We analyzed 82 samples by CLIA of Abbott Diagnostics (Architect i1000), ECLIA Roche Diagnostics (Cobas 601) and RIA DiaSorin. Samples were divided into four groups: G1: untreated; G2: treated with D2, G3 treated with D3 and G4: treated with D2 + D3.

Results: Considering all samples, there was a significant difference between mean 25OHD results obtained by the three methods (F: 14.80, p <0.0001), being similar with RIA and ECLIA but lower with CLIA (p <0.05). In the four groups studied, RIA and ECLIA results were similar in the presence or absence of treatment. In G2, there was a significant trend to lower levels with CLIA, compared to the other two methods (p = 0.0003), and the same trend was observed in G4 (p <0.02). This difference in G3, albeit significant (p <0.05), was less marked. Bland and Altman showed that CLIA underestimated the measured concentrations compared with EQLIA, average Δ RIA: - 5.69 to – 14 ng/mL). This was not observed when comparing ECLIA vs. RIA (Δ: - 3.45 to 0.47 ng/mL).

Conclusions: There are methodological differences in the design and specificity of immunoassays, which recognize 25OHD and its metabolites in different proportions. Therefore, patients might be classified as 25OHD sufficient or insufficient depending on the methodology used. Results suggest that RIA and ECLIA measurements are comparable in untreated patients and in patients treated with vitamin D2, D3 or both.

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Keywords: vitamin D, 25 (OH) cholecalciferol, immunoassays

INTRODUCTION

Vitamin D status assessment by measuring serum 25 (OH) Vitamin D (25OHD) levels has gained growing importance in clinical endocrinology practice, both in the general population (1) and in patients with osteopenia(2), allowing for the detection of Vitamin D deficiency or insufficiency, so that subjects eligible for replacement therapy can be identified(3). Measurement of 25OHD is also useful for differential diagnosis of secondary hyperparathyroidism, rickets and osteomalacia, among other disorders of mineral metabolism(4). In addition, decreased vitamin D levels have been epidemiologically linked to an increased risk of certain cancers(4-8) and cardiovascular diseases(9). While there is no universally accepted consensus to define 25 hydroxyvitamin D (25OHD) status in a specific population(10), vitamin D deficiency is
usually defined as <20 ng/ml (50 nmol/L), and insufficiency as 20 to 29 ng/ml\(^5\). Most authors define vitamin D adequacy as levels above 30 ng/mL (75 nmol/L)\(^2,3\).

There is enough clinical evidence to affirm that, in patients with osteopenia or osteoporosis, therapy with daily doses of vitamin D equal to or above 700 IU reduces bone mass loss and the incidence of fractures\(^11-16\). Thus, monitoring of treatment with vitamin D2 (ergocalciferol) or D3 (cholecalciferol) may also require 25OHD measurement.

Vitamin D measurement may be influenced by both pre-analytical\(^17\) and analytical variables that may affect the interpretation of results\(^18,19\). Despite recent advances in this field, there are several conceptual and methodological aspects to be defined:

- The use of different laboratory methodologies, since in the last decade, several automated immunoassays have been developed for measuring vitamin D in a more rapid and economical manner than the traditional RIA. Like RIA, these novel immunoassays pose methodological challenges still unresolved, i.e., reference method, international standards, traceability, specificity, etc.\(^19\)

- Differences in the affinity of the binding proteins of the assays for serum vitamin D metabolites, with dissimilar biological activity and reactivity, which generates differences in specificity across methods.

- The availability of multiple therapeutic formulae (D3, D2, or both) further complicates the situation, based on the differences mentioned above.

Given the availability of various immunoassays for 25OHD measurement, it is appropriate to know the potential methodological differences so that they can be efficiently used, in an adequate clinical setting, in untreated patients as well as in patients treated with D2, D3 or both.

**OBJECTIVES**

The objective is to compare three methods for 25OHD measurement in untreated subjects and in subjects treated with ergocalciferol (D2), cholecalciferol (D3) or both.

**MATERIALS AND METHODS**

Eighty-two samples were analyzed by three methodologies: 25OH Vitamin D by the Abbott Diagnostics (Architect i1000) Chemiluminescence immunoassay (CLIA) revised in June 2011, Roche Diagnostics vitamin D total electrochemiluminescence assay (ECLIA) (Cobas 601) and DiaSorin manual radioimmunoassay (RIA), considered as reference method because it is the standard method used in our setting from which the currently proposed cutoff values have been obtained. Automated Abbot and Roche Total methods have recently become commercially available. Vitamin D total ECLIA formulation is different from the 2007 design, which only recognized 25OHD using polyclonal antibodies with no preceding extraction. RIA samples were measure in duplicate.

All three methods have an extraction step prior to measurement, different antibody specificity and design (Tables I and II).

Untreated patients and patients under treatment (for at least two months) with vitamin D were enrolled and divided into four groups:

- Group 1 (G1): patients with no previous treatment (n = 19)
- Group 2 (G2): patients supplemented with D2 (n = 21)
- Group 3 (G3): patients supplemented with D3 (n = 21)
- Group 4 (G4): patients supplemented with D2 + D3 (n = 21).
Samples, once collected in gel tubes, were centrifuged within one hour and stored until assayed at –20 °C.

Data were analyzed by a variance analysis (ANOVA) with Bonferroni adjustment\(^{(20)}\). The ANOVA determines if differences in means among three or more groups are statistically significant. If differences are found by ANOVA, the Bonferroni adjustment for multiple comparisons determines which groups are homogeneous to each other with a certain statistical significance. Correlation was obtained by Passing-Bablok\(^{(21)}\) regression analysis, with no special requirements regarding the distribution of the samples and the measurement errors. The result does not depend on the assignment of the methods to X and Y. The slope and intercept are calculated with a 95% confidence interval.

Averages versus differences were obtained by the Bland and Altman method\(^{(22)}\), which assesses agreement between two methods of measurement. This approach plots differences between a pair of values against their mean. If there is no systematic error, the points will be distributed randomly on either side of the line corresponding to 0 on the axis representing the difference in the means. Statistical analyses were performed using the Method Validator Software: comparison plots and Statistix Analytical Software 1985-2000.

### Table I. Main characteristics of three 25 (OH) vitamin D assays according to manufacturers

<table>
<thead>
<tr>
<th>Design</th>
<th>RIA</th>
<th>CLIA</th>
<th>ECLIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previous extraction</td>
<td>Acetonitrile</td>
<td>Methanol-TEA-ANSA</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>Capture</td>
<td>Polyclonal</td>
<td>Polyclonal</td>
<td>Vitamin D binding protein</td>
</tr>
<tr>
<td>Separation</td>
<td>Secondary antibody</td>
<td>Anti-biotin monoclonal antibody</td>
<td>Streptavidin microparticles</td>
</tr>
<tr>
<td>Brand</td>
<td>(^{125})I Vitamin D</td>
<td>Acridine Vitamin D</td>
<td>Ruthenylated Vitamin D binding protein + biotinylated Vitamin D</td>
</tr>
<tr>
<td>Measuring range</td>
<td>5-100 ng/mL</td>
<td>8-160 ng/mL</td>
<td>3-70 ng/mL</td>
</tr>
<tr>
<td>Inter-assay CV% according to manufacturer</td>
<td>8.2 % (22.7 ng/mL)</td>
<td>4.6 % (19.5 ng/mL)</td>
<td>8.5 % (17.1 ng/mL)</td>
</tr>
<tr>
<td></td>
<td>11.0 % (49.0 ng/mL)</td>
<td>2.8 % (38.0 ng/mL)</td>
<td>5.8 % (29.4 ng/mL)</td>
</tr>
</tbody>
</table>
**Table II.** Specificity of all three assays for the various forms of vitamin D according to manufacturer’s instructions

<table>
<thead>
<tr>
<th>Specificity</th>
<th>RIA %</th>
<th>CLIA %</th>
<th>ECLIA %</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 OH Vitamin D3</td>
<td>100</td>
<td>105</td>
<td>98</td>
</tr>
<tr>
<td>25 OH Vitamin D2</td>
<td>100</td>
<td>82 *</td>
<td>81</td>
</tr>
<tr>
<td>24,25 (OH)2 Vitamin D3</td>
<td>100</td>
<td>112</td>
<td>121</td>
</tr>
<tr>
<td>24,25 (OH)2 Vitamin D2</td>
<td>100</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1,25 (OH)2 Vitamin D3</td>
<td>11</td>
<td>12.6</td>
<td>5</td>
</tr>
<tr>
<td>1,25 (OH)2 Vitamin D2</td>
<td>11</td>
<td>--</td>
<td>6</td>
</tr>
<tr>
<td>Cholecalciferol (Vitamin D3)</td>
<td>0.8</td>
<td>0.3</td>
<td>5</td>
</tr>
<tr>
<td>Ergocalciferol (Vitamin D2)</td>
<td>0.8</td>
<td>0.1</td>
<td>6</td>
</tr>
<tr>
<td>C3-epimer of 25 (OH) vitamin D</td>
<td>--</td>
<td>2.7</td>
<td>93</td>
</tr>
</tbody>
</table>

Ref.: *82% estimated by non-standard method at low doses.

Specificity is usually calculated as:

\[
\text{Cross-reactivity} \% = \frac{\text{Spiked sample value (ng/mL)} - \text{unspiked sample value (ng/mL)}}{\text{Concentration spiked (ng/mL)}} \times 100
\]

In the specific case of CLIA methodology (*) the calculation was performed differently: unspiked samples were used with a ratio of 25-OH vitamin D3 /25-OH vitamin D2 > 10.0 and a concentration of 25-OH vitamin D3 < 5.0 ng/mL.

**Table III.** Results of all patients by all three methodologies:

<table>
<thead>
<tr>
<th></th>
<th>Mean (ng/mL)</th>
<th>Standard deviation (ng/mL)</th>
<th>Min. (ng/mL)</th>
<th>Max. (ng/mL)</th>
<th>p 25 (ng/mL)</th>
<th>p 50 (ng/mL)</th>
<th>p 75 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA</td>
<td>N=82</td>
<td>34.1</td>
<td>12.1</td>
<td>8.9</td>
<td>67.9</td>
<td>24.1</td>
<td>32.8</td>
</tr>
<tr>
<td>CLIA</td>
<td>N=82</td>
<td>25.9</td>
<td>7.7</td>
<td>11.1</td>
<td>48.9</td>
<td>20.7</td>
<td>24.4</td>
</tr>
<tr>
<td>ECLIA</td>
<td>N=82</td>
<td>33.1</td>
<td>11.2</td>
<td>3.6</td>
<td>53.3</td>
<td>24.4</td>
<td>32.9</td>
</tr>
</tbody>
</table>

Ref.: ANOVA: F =14.80; p < 0.0001; Bonferroni p < 0.05

**Table IV.** Parameters of the comparison curve for CLIA and ECLIA against RIA

<table>
<thead>
<tr>
<th></th>
<th>SLOPE</th>
<th>INTERCEPT (ng/mL)</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLIA</td>
<td>0.59</td>
<td>4.93</td>
<td>0.692</td>
</tr>
<tr>
<td>ECLIA</td>
<td>0.92</td>
<td>2.09</td>
<td>0.704</td>
</tr>
</tbody>
</table>
RESULTS

Results of overall samples
Table III shows results of overall patients (from group 1 to 4), regardless of the type of therapy received, as this is the standard sample receipt procedure.

A significant difference was found among 25OHD means obtained by all three methods (F: 14.80, p < 0.0001). Means and percentiles of RIA and ECLIA did not significantly differ, but both differed from CLIA measurement (p < 0.05). Data obtained by CLIA and ECLIA as compared to RIA showed that, even if the correlation is similar (r: 0.692 and 0.704, respectively), the slopes significantly differed. ECLIA demonstrated higher similarity with RIA, as shown in Table IV and Figure 1. The mean difference of CLIA vs. RIA by the Bland and Altman method was -8.64 (-1.06 to -6.72) ng/ml, while for ECLIA vs. RIA, the mean difference was -0.987 (-2.94 to 0.963) ng/mL, confirming this difference between the two methods.

Results of groups according to treatment
Results of 25OHD (ng/ml) for all 4 groups are shown in Table V for untreated patients, G1; patients treated with D2, G2; patients treated with D3, G3 and patients treated with D2 + D3, G4. Mean 25OHD values obtained by RIA and ECLIA were similar, regardless of the presence or absence of treatment. In G1, there was a non-significant tendency to lower values with CLIA (p = 0.2175), compared to the remaining two methods. The difference was more marked in G2 (p = 0.0003), and somewhat lower in G4 (p < 0.02). In G3, such difference, albeit significant (p < 0.05), was less marked.

The agreement between CLIA and RIA was similar to that between ECLIA and RIA (Table VI) (r: >0.67) in Groups G1 to G3, but decreased in G4 (r: ~0.5). Groups 2 and 4 showed a smaller slope for both methods, which is more marked in CLIA (Figures 2 to 5).

The analysis of differences by the Bland and Altman approach showed that CLIA would underestimate the measured concentrations as compared to ECLIA, mainly in patients treated with D2 (G2 and G4) (mean difference between CLIA and RIA = -5.69 to -14.0 ng/ml according to the group). Thus, CLIA showed that in G1, 71% of patients had values below the RIA mean; this difference increased to 90.5% in G2. Instead, the mean difference between ECLIA and RIA was -3.45 to 0.47 ng/ml according to the group (Figures 2 to 5).
### Table V. Results of 25(OH) D (ng/ml) for all groups:

<table>
<thead>
<tr>
<th></th>
<th>RIA</th>
<th>CLIA</th>
<th>ECLIA</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>Mean</td>
<td>26.1</td>
<td>20.9</td>
<td>24.8</td>
<td>1.57</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>8.5</td>
<td>7.3</td>
<td>11.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>16.4-45.2</td>
<td>11.1-38.5</td>
<td>3.6-45.9</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>Mean</td>
<td>37.9</td>
<td>25.1</td>
<td>34.9</td>
<td>9.08</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>13.5</td>
<td>6.6</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>10.3-67.0</td>
<td>14.5-39.6</td>
<td>20.7-52.9</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>Mean</td>
<td>36.2</td>
<td>29.8</td>
<td>36.7</td>
<td>3.28</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>10</td>
<td>7.8</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>18.3-57.6</td>
<td>18.5-48.9</td>
<td>15.7-53.3</td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>Mean</td>
<td>35.1</td>
<td>26.9</td>
<td>34.9</td>
<td>4.55</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>12.5</td>
<td>6.6</td>
<td>13.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>8.9-56.4</td>
<td>13.7-38.0</td>
<td>18.7-48.6</td>
<td></td>
</tr>
</tbody>
</table>

Ref.: F: ANOVA statistic. p: p value.

**Figure 1. Results of overall samples.** Comparison of CLIA and ECLIA vs. RIA. a) Bland and Altman plot. The solid line represents the mean difference between the two methods. Dotted lines represent the 95% confidence interval of the mean difference between the two methods.
B) Passing-Bablok plot. Regression analysis: dotted line: theoretical straight line; solid line: straight line obtained from comparison of methods.

Referencias: CAMBIAR COMAS POR PUNTOS EN TODOS LOS DECIMALES
QLIA vs. RIA: CLIA vs. RIA
Diferencia: difference
Diferencia de valores de 25OHD QLIA-RIA (ng/mL): CLIA-RIA difference in 25OHD values (ng/mL)
Media: mean
Promedio de valores 25OHD (ng/ml): Average 25OHD values (ng/ml)

QLIA: CLIA
25OHD (ng/ml) QLIA: 25OHD (ng/ml) CLIA
25OHD (ng/ml) RIA: 25OHD (ng/ml) RIA
Pendiente 0.594 (0.477-0.731); Slope 0.594 (0.477-0.731)
Ord. Origen: 4.93 (0.69-9.19); y-intercept: 4.93 (0.69-9.19)

EQLIA vs. RIA: ECLIA vs. RIA
Diferencia: difference
Diferencia de valores de 25OHD EQLIA-RIA (ng/mL): ECLIA-RIA difference in 25OHD values (ng/mL)
Media: mean
Promedio de valores 25OHD (ng/ml): Average 25OHD values (ng/ml)

EQLIA: ECLIA
25OHD (ng/ml) EQLIA: 25OHD (ng/ml) ECLIA
25OHD (ng/ml) RIA: 25OHD (ng/ml) RIA
Pendiente 0.920 (0.767-1.092); Slope 0.920 (0.767-1.092)
Ord. Origen: 2.092 (-3.399-6.523); y-intercept: 2.092 (-3.399-6.523)
Figure 2. Results of Group 1 (G1). Comparison of CLIA and ECLIA vs. RIA. a) Bland and Altman plot. The solid line represents the mean difference between the two methods. Dotted lines represent the 95% confidence interval of the mean difference between the two methods.

Referencias
QLIA vs. RIA: CLIA vs. RIA
Diferencia: difference
Diferencia de valores de 25OHD QLIA-RIA (ng/mL): CLIA-RIA difference in 25OHD values (ng/mL)
Media: mean
Promedio de valores 25OHD (ng/ml): Average 25OHD values (ng/ml)

QLIA: CLIA
25OHD (ng/ml) QLIA: 25OHD (ng/ml) CLIA
25OHD (ng/ml) RIA: 25OHD (ng/ml) RIA
Pendiente 0.92 (0.59-1.6): Slope 0.92 (0.59-1.6)
Ord. Origen: -4.51 (-20.46-4.66); y-intercept: -4.51 (-20.46-4.66)

EQLIA vs. RIA: ECLIA vs. RIA
Diferencia: difference
Diferencia de valores de 25OHD EQLIA-RIA (ng/mL): ECLIA-RIA difference in 25OHD values (ng/mL)
Media: mean
Promedio de valores 25OHD (ng/ml): Average 25OHD values (ng/ml)

EQLIA: ECLIA
25OHD (ng/ml) EQLIA: 25OHD (ng/ml) ECLIA
25OHD (ng/ml) RIA: 25OHD (ng/ml) RIA
Pendiente 1.33 (0.99-2.02): Slope 1.33 (0.99-2.02)
Ord. Origen: -8.62 (-27.44-0.72): y-intercept: -8.62 (-27.44-0.72)

![Diagram of Group 2 (G2) results comparing CLIA and ECLIA vs. RIA.](image)

**Figure 3. Results of Group 2 (G2).** Comparison of CLIA and ECLIA vs. RIA. a) Bland and Altman plot. The solid line represents the mean difference between the two methods. Dotted lines represent the 95% confidence interval of the mean difference between the two methods.

**Referencias**
QLIA vs. RIA: CLIA vs. RIA
Diferencia: difference
Diferencia de valores de 25OHD QLIA-RIA (ng/mL): CLIA-RIA difference in 25OHD values (ng/mL)
Media: mean
Promedio de valores 25OHD (ng/ml): Average 25OHD values (ng/ml)

**QLIA:**
25OHD (ng/ml) QLIA: 25OHD (ng/ml) CLIA
25OHD (ng/mL) RIA: 25OHD (ng/ml) RIA
Pendiente 0.48 (0.29-0.73): Slope 0.48 (0.29-0.73)

**EQLIA vs. RIA:**
ECLIA vs. RIA
Diferencia: difference
Diferencia de valores de 25OHD EQLIA-RIA (ng/mL): ECLIA-RIA difference in 25OHD values (ng/mL)
Media: mean
Promedio de valores 25OHD (ng/ml); Average 25OHD values (ng/ml)

EQLIA vs. ECLIA
25OHD (ng/ml) EQLIA: 25OHD (ng/ml) ECLIA
25OHD (ng/ml) RIA: 25OHD (ng/ml) RIA
Pendiente 0.66 (0.40-1.01); Slope 0.66 (0.40-1.01)
Ord. Origen: 10.11 (-4.75-20.83); y-intercept: 10.11 (-4.75-20.83)

Figure 4. Results of Group 3 (G3). Comparison of CLIA and ECLIA vs. RIA. a) Bland and Altman plot. The solid line represents the mean difference between the two methods. Dotted lines represent the 95% confidence interval of the mean difference between the two methods.

Referencias
QLIA vs. RIA: CLIA vs. RIA
Diferencia: difference
Diferencia de valores de 25OHD QLIA-RIA (ng/mL): CLIA-RIA difference in 25OHD values (ng/mL)
Media: mean
Promedio de valores 25OHD (ng/ml): Average 25OHD values (ng/ml)

QLIA: CLIA
25OHD (ng/ml) QLIA: 25OHD (ng/ml) CLIA
25OHD (ng/ml) RIA: 25OHD (ng/ml) RIA
Pendiente 0.76 (0.57-1.01); Slope 0.76 (0.57-1.01)
Ord. Origen: 2.68 (-7.43-8.10); y-intercept: 2.68 (-7.43-8.10)

EQLIA vs. RIA: ECLIA vs. RIA
Diferencia: difference
**Diferencia de valores de 25OHD EQLIA-RIA (ng/mL):** ECLIA-RIA difference in 25OHD values (ng/mL)

**Media:** mean

**Promedio de valores 25OHD (ng/ml):** Average 25OHD values (ng/ml)

**EQLIA vs. ECLIA**

<table>
<thead>
<tr>
<th>25OHD (ng/ml) EQLIA</th>
<th>25OHD (ng/ml) ECLIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>25OHD (ng/ml) RIA</td>
<td>25OHD (ng/ml) RIA</td>
</tr>
</tbody>
</table>

Pendiente 1.11 (0.73-1.61): Slope 1.11 (0.73-1.61)


**EQLIA vs. RIA**

<table>
<thead>
<tr>
<th>25OHD (ng/ml) QLIA</th>
<th>25OHD (ng/ml) CLIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>25OHD (ng/ml) RIA</td>
<td>25OHD (ng/ml) RIA</td>
</tr>
</tbody>
</table>

Pendiente 0.44 (0.22-0.72): Slope 0.44 (0.22-0.72)


**Referencias**

QLIA vs. RIA: CLIA vs. RIA

Diferencia: difference

Diferencia de valores 25OHD QLIA-RIA (ng/mL): CLIA-RIA difference in 25OHD values (ng/mL)

**Media:** mean

**Promedio de valores 25OHD (ng/ml):** Average 25OHD values (ng/ml)

QLIA: CLIA

<table>
<thead>
<tr>
<th>25OHD (ng/ml) QLIA</th>
<th>25OHD (ng/ml) CLIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>25OHD (ng/ml) RIA</td>
<td>25OHD (ng/ml) RIA</td>
</tr>
</tbody>
</table>

Pendiente 0.44 (0.22-0.72): Slope 0.44 (0.22-0.72)


**EQLIA vs. RIA:** ECLIA vs. RIA

**Figure 5. Results of Group 4 (G4).** Comparison of CLIA and ECLIA vs. RIA. a) Bland and Altman plot. The solid line represents the mean difference between the two methods. Dotted lines represent the 95% confidence interval of the mean difference between the two methods.
Table VI. Correlation results of CLIA versus RIA and ECLIA versus RIA

<table>
<thead>
<tr>
<th></th>
<th>CLIA</th>
<th></th>
<th>ECLIA</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope</td>
<td>intercept</td>
<td>r</td>
<td>Bland and Altmann</td>
</tr>
<tr>
<td>G1</td>
<td>0.92</td>
<td>-4.5</td>
<td>0.77</td>
<td>-5.69</td>
</tr>
<tr>
<td>G2</td>
<td>0.48</td>
<td>6.31</td>
<td>0.73</td>
<td>-14.0</td>
</tr>
<tr>
<td>G3</td>
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<td>2.68</td>
<td>0.85</td>
<td>-6.45</td>
</tr>
<tr>
<td>G4</td>
<td>0.44</td>
<td>10.24</td>
<td>0.54</td>
<td>-8.28</td>
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</tbody>
</table>

DISCUSSION

In recent years, measurement of serum 25OHD levels has become more frequent as a result of the knowledge gained about the classical and non-classical effects of this hormone in relation to various conditions. Traditionally, the most widely used assay was the DiaSorin RIA, but this assay is operator-dependent and requires prolonged processing times. In order to achieve faster time-to-results, a number of methods have been developed that employ automated platforms.

The designs of the 25OHD assays considered in this study have similarities and differences that should be known at the time of assessing the performance of each method. In order to interpret results in the overall sample, it is important to analyze the features shown in Table 1.

The vitamin D binding protein (VDBP)\(^{(23)}\) binds 85 to 88% of 25OHD and its serum metabolites while 12 to 15% of 25OHD is bound to albumin and only 0.03% is free. In order to be able to measure vitamin D, it is essential to dissociate it from binding proteins by means of some serum pretreatment. The three assays discussed in this study use different extraction reagents; all three are supposed to achieve complete extraction \(^{(24-26)}\).

In addition to 25OHD, other metabolites such as cholecalciferol (vitamin D3), calcitriol and 24-hydroxylated metabolites circulate in plasma, as well as vitamin D (ergocalciferol) should the patient be receiving this vitamin. The capture reagent is the most important component for immunoassay specificity, which is defined by cross-reactivity. Two of the immunoassays evaluated in this study use polyclonal antibodies (of different specificity) while in the ECLIA assay, the reagent is the binding protein (VDBP) itself (Table 2).

According to manufacturers, all three methods have similar (though not identical) specificities. Based on our results, specificities might not be comparable, as also
suggested by others (27). When analyzing the samples as a whole, CLIA yields significantly lower values (ANOVA) throughout all the concentration range (Table III). CLIA is shown to be significantly different from the remaining assays when the Bonferroni adjustment is applied. This finding is confirmed by the Bland and Altman and Passing-Bablok methods. It would be interesting to replicate and further extend these observations in a larger number of samples.

To date, the definition of a population’s vitamin D status is universal and independent of the methodology used (2,5). Based on the differences observed in our study, difficulties might arise at the time of evaluating patients, who might be classified as vitamin D-insufficient by one method and as vitamin D-sufficient by another, particularly when levels are close to cutoff values.

When samples were divided into four groups based on the supplementation received, these inter-assay differences became enhanced (Table V). In the group of untreated patients (G1), the same trend as that observed in the whole samples was documented. The absence of statistical significance is likely to be due to the number of samples. In the group of patients treated with vitamin D3 (G3), the tendency to lower values by CLIA reached statistical significance. In patients treated with D2 (G2 and G4), the apparent underestimation of 25OHD levels was even greater (Table VI).

In our opinion, findings as a whole might be attributed to differences in the specificity of assays greater than those reported by the manufactures in the package insert.

These concepts are also relevant for replacement therapy monitoring, as the true change in vitamin D status might not be reflected by the CLIA considered in this study.

In our setting there are other immunoassays in which detection is performed by CLIA. For example, when comparing the two DiaSorin assays, RIA vs. CLIA (LIAISON) (28), even if there was a good inter-assay agreement, a positive bias was observed in the latter in the population studied.

Finally, it is important that the laboratory, when reporting 25OHD results, should state the specificity of the method used for ergocalciferol and cholecalciferol so that the treating physician may appropriately monitor replacement therapy.

REFERENCES


24. Inserto 25-hydroxyvitamina D \textsuperscript{125}I RIA kit. Stillwater, Minnesota: DiaSorin Corporation.


26. Inserto Vitamina D Total Roche Diagnostics, Indianápolis, USA, 2011
