ORIGINAL ARTICLE

Influence of the immunoassay used in measurement of serum vitamin D levels

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Abstract

Introduction: Serum 25-hydroxyvitamin D [25(OH)D] levels are the best indicator of vitamin D levels in the body. Precision, reproducibility, and lack of standardization are the main problems in such measurements. The aim of this study was to compare the 25(OH)D levels measured using Elecsys Vitamin D Total (Roche) and ADVIA Centaur Vitamin D Total (Siemens).

Materials and methods: 25(OH)D levels were tested in 166 patients using both methods. Patients were subsequently divided into two groups: a 'supplemented group' consisting of patients receiving vitamin D supplements, and an 'untreated group' consisting of the rest of patients.

Results: 25(OH)D mean levels measured by the Roche and Siemens methods in the overall group were 33.6 ± 16.0 and 19.8 ± 12.4 ng/mL, respectively. 54.2% of patients were receiving vitamin D supplements. In this group, mean 25(OH)D levels measured by the Roche and Siemens methods were 40.6 ± 14.5 and 25.4 ± 13.1 ng/mL, respectively. In the untreated group, the respective values were 24.9 ± 13.2 and 12.8 ± 6.6 ng/mL. Prevalence of vitamin D deficiency (serum 25(OH)D levels less than 20 ng/mL) was higher in samples analyzed using the Siemens method (60.2%) as compared to those tested using the Roche method (23.5%).

Conclusion: The assays evaluated are not comparable to each other. Laboratory technicians should inform clinicians of the features of the method used for measuring 25(OH)D because this will have a direct impact on interpretation of the results and medical decisions.

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Influenza del inmunoenosayo empleado en la determinación de vitamina D sérica

Resumen

Introducción: La determinación en suero de 25-hidroxivitamina D [25(OH)D] es el mejor indicador del nivel de vitamina D en el organismo. El principal problema en su determinación es...
Introductión

El interés por el vitamina D ha aumentado en los últimos años con avances en nuestro entendimiento de sus efectos en ambos huesos y otros tejidos, que pueden tener implicaciones fisiológicas y patológicas en diferentes enfermedades inmunológicas, cáncer y enfermedades cardiovasculares.1-4

Habrá dos formas de vitamina D, D3 o colecalciferol, que es fisiológicamente en humanos (el más abundante en circulación), y vitamina D2 o ergocalciferol, obtenida de la dieta después de comer alimentos de origen animal. Ambas formas están disponibles en suplementos farmacológicos o en alimentos fortificados.

Calcidiol o 25-hidroxicolesterol D [25(OH)D] es el precursor del calcitriol, una metabolita biológicamente activa del vitamina D hormonal sistema. Su vida media y concentración sanguínea alta ha hecho que sea el mejor indicador de vitamina D niveles.

La medida de 25(OH)D presenta algunas desafíos, como la gran lipofiliad de la molécula, transportar en proteínas, el tiempo de existencia de dos estructuras similares de diferentes fuentes (endógeno o exógeno) con múltiples metabolitas,3 y la precisión y reproducibilidad de los métodos disponibles.4

En 2010, Tai et al.7 desarrollaron una técnica de cromatografía de masa-tandem (LC-MS/MS) que fue reconocida como el método de referencia para 25(OH)D por la Comisión Joint Committee for Traceability en la medicina Laboratorio. Su complejidad y necesidad de personal altamente especializado en su uso, haciendo que sea un procedimiento de poca relevancia en la práctica diaria.

La accesibilidad reciente de las inmunoenzimas automáticas hace que sea posible hacer frente al aumento de la demanda para el vitamina D en el cuidado de la salud.

El estudio objetivo fue comparar los 25(OH)D niveles realizados a los de una de las técnicas más utilizadas en inmunoenzimas y comprobar su impacto en la pauta de diagnóstico en términos de que pacientes son considerados tener un nivel insuficiente, suficiente, o insuficiente vitamina D niveles.

Resultados: El valor medio de 25(OH)D en el grupo general medido por Roche y por Siemens fue 33,6 ± 16,0 y 19,8 ± 12,4 ng/ml, respectivamente. El 54,2% de los pacientes reciban suplementos de vitamina D. En este grupo el valor medio de 25(OH)D medido por Roche y por Siemens fue 40,6 ± 14,5 y 25,4 ± 13,1 ng/ml; y en el grupo de pacientes sin tratamiento fue 24,9 ± 13,2 y 12,8 ± 6,6 ng/ml, respectivamente. La prevalencia de la insuficiencia de vitamina D (concentraciones séricas de 25(OH)D inferiores a 20 ng/ml) fue más alta para las medidas por Siemens (60,2%) que para las medidas por Roche (23,5%).

Conclusión: Los análisis evaluados no son comparables entre sí. Los especialistas del laboratorio deben informar a los clínicos de las características del método empleado en la determinación de 25(OH)D ya que tendrán consecuencias directas en la interpretación y decisión médica.

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(ECLIA) detection. The latest version of this assay, marketed after modification by the manufacturer, was used.

Dithiothreitol and sodium hydroxide were used to free 25(OH)D from its binding plasma protein. Samples were then incubated with vitamin D binding protein labelled with ruthenium. Biotinylated 25(OH)D was subsequently added to occupy the vacant sites in the binding protein. When an electrical current is applied to the reading cell, the chemiluminescent reaction occurs.

The functional sensitivity of the assay is 4.0 ng/mL (10.0 nmol/L). Assay specificity, reflected by the percentage of cross-reactivity with other metabolites, is 98% for 25(OH)D3, 81% for 25(OH)D2, and 93% for the C3 epimer of 25(OH)D3.

**ADVIA Centaur Vitamin D Total (Siemens, Frimley Camberley, United Kingdom)**

ADVIA Centaur Vitamin D Total (Siemens) is a one-step competitive immunoassay. This assay uses a releasing agent in saline buffer with sodium azide, an anti-fluorescein mouse monoclonal antibody covalently bound to paramagnetic particles, a mouse anti-25(OH)D monoclonal antibody labeled with acridinium ester, and a vitamin D analogue labeled with fluorescein.

The functional sensitivity of the assay is 3.3 ng/mL (8.3 nmol/L). Assay specificity, reflected by the percentage of cross-reactivity with other metabolites, is 97.4% for 25(OH)D3, 106.2% for 25(OH)D2, and 1% for the C3 epimer of 25(OH)D3.

**Statistical analysis**

The results of the 166 samples were analyzed using a Bland–Altman plot, Passing-Bablok regression, concordance correlation coefficient (CCC), and kappa (κ). Data distribution was analyzed using box plots. 25(OH)D values were expressed in ng/mL (1 ng/dL is equivalent to 2.5 nmol/L). Medcalc version 12.3.0 software was used for statistical analysis. Values of $p < 0.005$ were considered statistically significant.

After testing all samples, two groups were established: a supplemented group, consisting of the 92 samples of patients receiving supplemental Vitamin D2, and an untreated group consisting of samples from the 74 patients not receiving vitamin D2 supplements. No patient was being treated with vitamin D2 supplements. Patient categorization was done after a literature review which suggested that some assays measuring 25(OH)D may not be able to recognize its exogenous fraction, i.e. 25(OH)D administered with pharmacological supplements or in diet.9

**Bland–Altman analysis**

Bland–Altman analysis9,10 is a graphic method for comparing two measurement procedures. In this method, differences (or proportions) between the two procedures are represented against the means of both. The limits of agreement are defined as the mean difference ±1.96 times the standard deviation of the differences. If these limits are not clinically relevant, either procedure may be used.

**Passing-Bablok regression**

Passing-Bablok regression11 is a linear regression procedure that calculates a regression equation including the 95% confidence interval (CI) of the intercept ($A$) and slope ($B$). This procedure requires that the variables are continuous and that a linear relationship exists between the two methods.

$A$ is a measure of constant systematic differences between the two methods. The 95% CI of $A$ may be used to test the hypothesis that $A = 0$. If the hypothesis is rejected, there are constant systematic differences between them.

$B$ is a measure of proportional systematic differences between the two methods. The 95% CI of $B$ may be used to test the hypothesis that $B = 1$. If the hypothesis is rejected, there are proportional systematic differences between them.

The residual standard deviation (RSD) is a measure of random differences between the two methods. Ninety-five percent of random differences are expected to be within the range of ±1.96 times RSD. If this range is wide, they may not be comparable.

The linearity hypothesis is verified using the cumulative sum linearity test (CUSUM test). A value of $p < 0.05$ suggests a significant deviation from linearity.

**Concordance correlation coefficient**

The concordance correlation coefficient (CCC)12,13 assesses the degree to which pairs of measurements fall on the line that crosses the origin at a 45° angle. It is the result of the product of Pearson’s correlation coefficient (a measure of precision) and the bias correction factor (a measure of accuracy). Results higher than 0.99 suggest excellent concordance; those between 0.99 and 0.95, substantial concordance; values ranging from 0.90 to 0.94, moderate concordance; and those <0.9, poor concordance.14

**Assessment of concordance of 25(OH)D concentrations**

The position statement on vitamin D requirements and optimum concentrations prepared by the Spanish Society of Bone and Mineral Metabolism Research (SEIOMM) and other related societies was used as a guide.15 Vitamin D sufficiency was defined as serum 25(OH)D concentrations higher than 30 ng/mL (>75 nmol/L), vitamin D insufficiency as serum 25(OH)D concentrations ranging from 20 to 30 ng/mL (50–75 nmol/L), and vitamin D deficiency as serum 25(OH)D concentrations less than 20 ng/mL (<50 nmol/L).

Inter-assay agreement in assessment of vitamin D status was evaluated using $κ$,16,17 with values of $κ$ less than 0.4 indicating poor agreement; values ranging from 0.4 to 0.75, fair to good agreement; and values higher than 0.75, excellent agreement.

**Results**

**Assay comparison**

Table 1 summarizes the 25(OH)D values measured by Elecsys Vitamin D Total (Roche) and ADVIA Centaur Vitamin D
Table 1  Demographic characteristics and statistical parameters of the patient groups studied by 25(OH)D assay.

<table>
<thead>
<tr>
<th></th>
<th>Overall group</th>
<th>Untreated group</th>
<th>Treated group</th>
</tr>
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<tbody>
<tr>
<td><strong>Demographic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>166</td>
<td>74</td>
<td>92</td>
</tr>
<tr>
<td>Males</td>
<td>71 (42.8%)</td>
<td>33 (44.6%)</td>
<td>38 (41.3%)</td>
</tr>
<tr>
<td>Females</td>
<td>95 (57.2%)</td>
<td>41 (55.4%)</td>
<td>54 (58.7%)</td>
</tr>
<tr>
<td>Age</td>
<td>64.7 (15.5)</td>
<td>65.6 (16.2)</td>
<td>64.1 (15.0)</td>
</tr>
</tbody>
</table>

**Statistical summary**

<table>
<thead>
<tr>
<th></th>
<th>Roche</th>
<th>Siemens</th>
<th>Roche</th>
<th>Siemens</th>
<th>Roche</th>
<th>Siemens</th>
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<tr>
<td>Mean (ng/mL)</td>
<td>33.6</td>
<td>19.8</td>
<td>24.9</td>
<td>12.8</td>
<td>40.6</td>
<td>25.4</td>
</tr>
<tr>
<td>95% Cl</td>
<td>31.2–36.0</td>
<td>17.9–21.7</td>
<td>21.8–27.9</td>
<td>11.3–14.3</td>
<td>37.6–43.6</td>
<td>22.7–28.1</td>
</tr>
<tr>
<td>Variance</td>
<td>254.5</td>
<td>153.4</td>
<td>174.4</td>
<td>43.0</td>
<td>210.2</td>
<td>172.3</td>
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<tr>
<td>Standard deviation</td>
<td>16.0</td>
<td>12.4</td>
<td>13.2</td>
<td>6.6</td>
<td>14.5</td>
<td>13.1</td>
</tr>
<tr>
<td>Median (ng/mL)</td>
<td>32.2</td>
<td>16.2</td>
<td>22.5</td>
<td>11.1</td>
<td>39.9</td>
<td>22.7</td>
</tr>
<tr>
<td>95% CI</td>
<td>29.6–35.1</td>
<td>14.0–18.8</td>
<td>18.1–26.8</td>
<td>9.8–12.2</td>
<td>35.8–45.1</td>
<td>19.9–26.4</td>
</tr>
<tr>
<td>Minimum (ng/mL)</td>
<td>5.0</td>
<td>4.5</td>
<td>5.0</td>
<td>4.5</td>
<td>9.9</td>
<td>7.3</td>
</tr>
<tr>
<td>Maximum (ng/mL)</td>
<td>68.5</td>
<td>63.3</td>
<td>68.1</td>
<td>38.7</td>
<td>68.5</td>
<td>63.3</td>
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<tr>
<td>Normal distribution</td>
<td>0.0005</td>
<td>&lt;0.0001</td>
<td>0.0108</td>
<td>&lt;0.0001</td>
<td>0.1088</td>
<td>0.0051</td>
</tr>
</tbody>
</table>

CI: confidence interval.

* Values with figures in brackets are mean and standard deviation (SD).

Total (Siemens) and group characteristics. Individual data distribution in the overall group illustrates the significant differences between the assays for these samples (Fig. 1).

Fig. 2 shows, using Bland–Altman analysis, agreement between the results obtained with the Elecsys Vitamin D Total (Roche) and ADVIA Centaur Vitamin D Total (Siemens) assays. The mean value of bias was higher for the treated group (15.2 ng/mL [38.0 nmol/L]) as compared to the untreated group (12.1 ng/mL [30.3 nmol/L]). Graphically, greater agreement was seen between the two assays when patients receiving vitamin D supplements were excluded. The scattering of the results obtained (positive trend in both groups) indicated that the degree of agreement decreased the higher the 25(OH)D concentration measured.

Table 2 shows the results of both Passing-Bablok regression and CCC analysis.

Agreement in evaluation of vitamin D status

A comparison was made of the proportion of samples classified into the different vitamin D states based on serum 25(OH)D concentrations measured by both procedures.

![Figure 1](Box plot showing the distribution of 25(OH)D concentrations (ng/mL) measured by the Elecsys Vitamin D Total (Roche) and the ADVIA Centaur Vitamin D Total (Siemens) assays.)

If we consider the three possible vitamin D states proposed by the SEIOMM, similar k values were found both for the overall group and for the treated and untreated groups (k = 0.2; k = 0.1; k = 0.1, respectively). If 20 ng/mL (50 nmol/L) was only used as the cut-off point, lower values being considered clearly pathological, similar k values were also found both overall and in the treated and untreated groups (k = 0.3; k = 0.2; k = 0.2, respectively).

Siemens proposes different cut-off values for its ADVIA Centaur Vitamin D Total assay (10631296, ES Rev.C, 2011-01). This manufacturer defines vitamin D deficiency as serum 25(OH)D concentrations less than 10 ng/mL (<25 nmol/L). When this cut-off point was used to classify the results of this assay, similar k values were obtained both in the overall group and the treated and untreated groups (k = 0.8; k = 0.7; k = 0.8, respectively).

Discussion

In this study, lower 25(OH)D concentrations (mean value = 19.8 ng/mL [49.5 nmol/L]) were obtained with the ADVIA Centaur Vitamin D Total assay (Siemens) as compared to the Elecsys Vitamin D Total assay (Roche) (mean value = 33.6 ng/mL [84.0 nmol/L]).

Significant differences were found in all the statistical tests performed. The Bland–Altman analysis showed greater bias or systematic error in the group of patients treated with supplements (Fig. 2). Although acceptable values of Pearson’s correlation coefficient were found (Table 2), the results of CCC lower than 0.9 found in all groups suggest poor agreement between these two methods. As regards Passing-Bablok regression, proportional and constant errors were simultaneously found in the study groups (Table 2).

These differences between the methods could be due to the assay design itself. If the specificity reported by the manufacturers is analyzed in more detail, special mention...
Influence of the immunoassay used in measurement of serum vitamin D

should be made of cross-reactivity to the C3 epimer of 25(OH)D. Cross-reactivity is 93% and 1% for the Roche and Siemens assays, respectively. It is now known that this epimer is present in serum samples from both children and adults. The concentrations found range from 0.1 ng/mL to 47.0 ng/mL (0.25–117.5 nmol/L), and some authors therefore state that their measurement in adult patients is of special interest when 25(OH)D levels are low. In addition, both methods use different pre-treatment steps to release 25(OH)D from vitamin D binding protein, which results in a lower level of 25(OH)D suitable for measurement. The design of the antibodies used in the Siemens assay may also influence the differences found. The monoclonal nature of these antibodies could result in the non-recognition of some derivative of exogenous vitamin D that does not express the epitope against which they were designed.

Our study has some limitations. One limitation was the use of frozen serum samples to compare the methods. Samples were frozen at −20 °C and thawed at room temperature only once. However, prior studies on samples subject to multiple freezing– thawing cycles show that 25(OH)D concentrations should not be affected.

An additional limitation was that no measurement was taken using the reference method. This limitation is common to most published studies, because the availability of LC–MS/MS in the clinical setting is restricted due to both the complexity of the method and to the increased demand for this measurement.

In prior studies, both methods showed a good correlation when compared to LC–MS/MS, but the results found with ADVIA Centaur Vitamin D Total (Siemens) showed a negative systematic error (−4.3%), while the results of Elecsys Vitamin D Total (Roche) showed a positive systematic error (17.4%).

A final limitation was that not all assays available in Spain for 25(OH)D measurement were studied due to the lack of adequate means.
As regards patient classification, no agreement was found between the methods when values proposed by the SEIOMM were used (k < 0.4). There were significant differences in the prevalence of vitamin D insufficiency between the tested assays. In all patient groups, a much higher prevalence was found with the Siemens assay (60.2%, 40.2%, and 85.1% in the overall, treated and untreated groups, respectively) as compared to the Roche assay (23.5, 7.6, and 43.2%, respectively). This means that some patients may receive a pharmacological treatment they do not need if the Siemens assay is used, or receive no treatment if the Roche assay is used. Agreement in patient classification improves (k = 0.7–0.8) when the reference values for the ADVIA Centaur Vitamin D Total assay proposed by the manufacturer are used, although these do not agree with those proposed by scientific bodies.

It has been widely reported that the prevalence of vitamin D deficiency represents a public health problem affecting more than 50% of the general population. The prevalence of vitamin D deficiency in Spain is 28% in young people and approximately 65% in healthy adults. This generalized hypovitaminosis state may partly be influenced by the testing method used to measure 25(OH)D concentrations.

Because of these discrepancies, the standardization of immunoassays for measuring vitamin D with adequate reference material is an imperative need which has previously been suggested by other groups. With the launching of standard reference material (SRM) 972 by the US National Institute of Standards and Technology (NIST) and the implementation in 2010 of the Vitamin D Standardization Program (VDSP), the harmonization of all vitamin D assays is expected.

In conclusion, the assays evaluated in this study are not comparable, despite their acceptable correlation with the reference method shown in prior publications. Patients should therefore be monitored with the same method throughout their treatment.

If the method used to assess vitamin D is changed, the laboratory should inform clinicians on the characteristics of the new method, because differences between the vitamin D levels measured by the different assays will have direct consequences on both medical interpretation and decision making.

Conflicts of interest

The authors state that they have no conflicts of interest.

References

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