Vitamin D toxicity? A case study

Vichet Khieng, BMLSc, Medical Laboratory Scientist; Catherine Stevens, NZCS, Senior Medical Laboratory Scientist

Department of Biochemistry, Southern Community Laboratories, Dunedin City Hospital

Abstract

One of vitamin D’s important roles is body calcium regulation. Vitamin D deficiency can lead to hypocalcaemia, and consequently cause bone related disorders. On the other hand, prolonged excessive vitamin D can lead to hypercalcaemia, and consequently cause renal stones. Compared to vitamin D deficiency, vitamin D toxicity is very rare. We report a case where the patient was initially thought to be hypervitaminosis D. In our laboratory, we use the ECLIA method from Roche Diagnostic to measure serum vitamin D. The patient had vitamin D levels of >250 nmol/L on more than one occasion whilst appearing to be clinically vitamin D deficient. Furthermore, results for vitamin D levels on the same samples by another laboratory with a different assay method came back as low normal. We carried out investigations for possible immunoassay interference. We found that an interfering factor caused falsely high results in competitive assays and falsely low results in a sandwich assay. The sample was sent to Roche Diagnostics in Germany for further investigation. Roche Diagnostics was able to show that the interfering factor was to the solid phase, but did not identify the interfering factor.

Key words: vitamin D, 25-hydroxyvitamin D, Elecsys Vitamin D3, interfering factor

Introduction

Clinicians have used the media on numerous occasions to express their concerns regarding vitamin D deficiency in the general population, particularly among the elderly. Never has there been concern about vitamin D toxicity, also known as hypervitaminosis D. This is because an important role of vitamin D is in body calcium regulation. Vitamin D deficiency is known to be associated with rickets among children and osteomalacia among adults (1). However, in rare cases, highly elevated vitamin D levels can cause hypercalcaemia and, in the long term, can lead renal stones (2-4).

Vitamin D is categorized into two main forms: vitamin D3 and vitamin D2. Vitamin D3 is produced in the skin by exposure to sunlight, whilst vitamin D2, from dietary sources such as fish oil, egg yolk, liver and certain plants. Both forms of vitamin D are metabolically inert. They become active through hydroxylation processes first in the liver, and subsequently in the kidney. In the liver, vitamin D3 is hydroxylated to 25-hydroxyvitamin D3 and vitamin D2 to 25-hydroxyvitamin D2. In the kidney, both 25-hydroxyvitamin D are hydroxylated to 1,25-dihydroxyvitamin D, the active form (4).

Only the measurements of 25-hydroxyvitamin D and 1,25-hydroxyvitamin D have been proven to provide clinical value (5-7). In our laboratory we use the Elecsys Vitamin D3 (25-OH) assay from Roche Diagnostics. The assay only measures 25-hydroxyvitamin D3. The 25-hydroxyvitamin D is the main form of vitamin D in circulation, and 25-hydroxyvitamin D form accounts for more than 95% of the 25-hydroxyvitamin D. The 25-hydroxyvitamin D only becomes detectable when taking vitamin D supplements. Furthermore, the 25-hydroxyvitamin D has a longer half-life compare to the more labile 1,25-hydroxyvitamin D, and it also shows better correlation with the nutritional status of vitamin D (4).

Case report

A 36-year-old female patient first presented to her clinician complaining of bouts of fatigue and weight loss. The clinician requested routine haematology and biochemistry tests including a vitamin D level, and queried iron or vitamin D deficiency. The haematology and biochemistry results were normal. The iron study results also indicated adequate iron stores. The vitamin D level, however, was unexpectedly high at >250 nmol/L (reference range: 50 - 150 nmol/L).

Laboratory investigations

The Elecsys Vitamin D3 (25-OH) assay has only recently been introduced to our laboratory. Since its introduction, we have been closely monitoring the assay performance including assay calibrations, quality controls and patient results. Vitamin D levels above the detectable limit (>250 nmol/L) are not common. In this case, to ensure the validity of our result, we sent the patient’s sample from her first visit to two laboratories for vitamin D. Laboratory A uses the same Elecsys technology to measure vitamin D, plus an in-house dilution method. The vitamin D result from laboratory A was 413 nmol/L, which agreed with our result. Laboratory B uses Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). The vitamin D level from laboratory B was 101 nmol/L, which disagreed with our laboratory and laboratory A results (Table 1). The method uses by laboratory B is considered the gold standard method for measuring vitamin D (8). Furthermore, the result from laboratory B correlated well with the patient’s clinical picture.

The vitamin D level was repeated on a subsequent sample requested by the clinician. In addition, calcium and parathyroid levels were also requested to assess the parathyroid status. The vitamin D level remained high at >250 nmol/L. The parathyroid level, on the other hand, was exceedingly low at 0.1 pmol/L (reference range: 1.6 - 7.0 pmol/L). These findings are consistent with secondary hypothyroidism along with elevated calcium levels (4). However, the patient’s calcium was well within the normal range at 2.20 mmol/L (reference range: 2.05 - 2.60 mmol/L). The patient had no particular medical history that could explain the results obtained, nor was she on any supplement or medication that contained vitamin D.

For further investigation we sent the subsequent sample on the patient to laboratory B for vitamin D level and laboratory C for parathyroid hormone (PTH). Vitamin D result from laboratory B was 43 nmol/L. Laboratory C measures PTH on an Abbott Architect analyser using chemiluminescent microparticle immunoassay (CMIA). The parathyroid hormone was 1.7 pmol/L. Table 2 compares our laboratory results to laboratories B and C.

In addition, we ran the subsequent patient’s sample for thyroid stimulating hormone and free T4 on the E170 and the Advia Centaur analyser in our laboratory. The Advia Centaur uses by laboratory B is considered the gold standard method for measuring vitamin D (8). Furthermore, the result from laboratory B correlated well with the patient’s clinical picture.
chemiluminescent immunoassay technology. The results obtained were significantly different between the two analysers as shown in Table 3.

Interference in the Elecsys assays was suspected after thorough examinations of all the results and discussions with our pathologists. We noted that all falsely elevated results used competitive assay principle whilst all falsely low results used sandwich assay principle. Table 4 summarises all results performed on the E170 analyser.

Further tests were performed to exclude some common immunoassay interferences. Heterophilic antibody-blocking tube was used to adsorb heterophilic antibodies prior to vitamin D assay. The vitamin D result after heterophilic antibody blocking was still >250 nmol/L. The sample showed no visible haemolysis. Bilirubin level was within the normal reference range. This excluded the possibility of interference due to icterus. Lipid levels were also normal. Interference due to rheumatoid factors was also excluded. The patient was not noted to be on biotin therapy, which can potentially interfere with Elecsys assays.

In addition, we attempted serial dilutions on the patient sample to determine the actual concentration of vitamin D in the sample. The sample was manually diluted with a low vitamin D serum level available as recommended by the manufacturer. Linearity is normally observed in normal serial dilutions. In this case, however, the serial dilution was non-linear. This also supported the presence of interfering factor in the sample.

We referred the sample to Roche Diagnostics in Germany for further investigation. Roche Diagnostics screened the sample for the possibility of interference due to heterophilic antibodies. The sample was adsorbed on uncoated microparticles and microparticles coated with streptavidin prior to vitamin D assay. The vitamin D level was significantly lower compared to the untreated sample.

Table 1. Comparisons of vitamin D results from different laboratories

<table>
<thead>
<tr>
<th></th>
<th>Our Laboratory E170 (nmol/L)</th>
<th>Laboratory A E170 (In-house Dilution) (nmol/L)</th>
<th>Laboratory B LC-MS/MS (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D</td>
<td>&gt;250</td>
<td>431</td>
<td>101</td>
</tr>
</tbody>
</table>

Table 2. Comparison of vitamin D and parathyroid hormone results

<table>
<thead>
<tr>
<th>Result</th>
<th>Our Laboratory (E170)</th>
<th>Laboratory B (LC-MS/MS)</th>
<th>Laboratory C (Abbot Architect)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D (nmol/L)</td>
<td>&gt;250</td>
<td>43</td>
<td>1.7</td>
</tr>
<tr>
<td>PTH (pmol/L)</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Comparisons of thyroid stimulating hormone and free T4 results

<table>
<thead>
<tr>
<th>Result</th>
<th>E170</th>
<th>Advia Centaur</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSH (mIU/L)</td>
<td>0.35</td>
<td>0.79</td>
</tr>
<tr>
<td>Free T4 (pmol/L)</td>
<td>27</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 4: Elecsys assay results and assay principles

<table>
<thead>
<tr>
<th>Result</th>
<th>Reference Range</th>
<th>Assay Principles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D (nmol/L)</td>
<td>&gt;250</td>
<td>50 – 150</td>
</tr>
<tr>
<td>Free T4 (pmol/L)</td>
<td>27</td>
<td>10 – 23</td>
</tr>
<tr>
<td>PTH (pmol/L)</td>
<td>0.1</td>
<td>1.6 – 7.0</td>
</tr>
<tr>
<td>TSH (mIU/L)</td>
<td>0.35</td>
<td>0.3 – 4.0</td>
</tr>
</tbody>
</table>

Theory to the interference

Figure 1 illustrates the ECLIA competitive principle. This principle is applied to analytes with low molecular weight, such as vitamin D. The first reaction involves interaction between vitamin D in the sample to vitamin D-specific ruthenium labelled antibody to form an immunocomplex. Biotinylated vitamin D and streptavidin-coated microparticles are added in the second reaction. Biotinylated vitamin D occupies the still free binding sites of the labelled antibody. The biotinylated immunocomplex subsequently binds to the microparticle via interaction of biotin and streptavidin. The microparticle is then magnetically captured onto the surface of an electrode. This follows by a washing step to removed unbound substances. A voltage is then applied to the electrode to induce chemiluminescent emission. The emission is measured by a photomultiplier. The amount of emission produced is inversely proportional to the concentration of vitamin D in the sample (9).

Figure 2 illustrates the ECLIA sandwich principle. This principle is applied to analytes with high molecular weight, such as PTH. The first reaction involves interaction between PTH in the sample, a biotinylated PTH-specific antibody, and PTH specific ruthenium labelled antibody to from a sandwich immunocomplex. The immunocomplex subsequently binds to streptavidin-coated microparticle in the second reaction via interaction of biotin and streptavidin. The microparticle is then magnetically captured onto the surface of an electrode. This follows by a washing step to remove unbound substances. A voltage is then applied to the electrode to induce chemiluminescent emission. The emission is measured by photomultiplier. The amount of emission produced is directly proportional to the concentration of PTH in the sample (9).

In the case of our patient, interference to streptavidin would cause falsely high results in competitive assays, as seen in with vitamin D and free T4 levels, and falsely low results in sandwich assays, as seen with PTH and TSH levels. The interfering factor competes with biotin for streptavidin binding. This reduces the interaction between biotin and streptavidin. Reduction in biotin and streptavidin interaction consequently leads to decreased chemiluminescent emission. In competitive assay, reduced chemiluminescent emission means high concentration of analyte in the sample because of the inverse relationship. Whereas in the sandwich assay, reduced chemiluminescent emission means low concentration of analyte in the sample because of a direct relationship (9). Figure 3 demonstrates the interference in the competitive assay while Figure 4 demonstrates the interference in the sandwich assay.
Figure 1. Competitive ECLIA principle (modified from Modular Analytics SWA: E Module - Immunology Principle; chapter 17, page 112).
Figure 2. Sandwich ECLIA principle (modified from Modular Analytics SWA: E Module - Immunology Principle; chapter 17, page 114).
Figure 3. Interference to streptavidin in competitive ECLIA
Figure 4. Interference to streptavidin in sandwich ECLIA
Roche Diagnostics in Germany concludes that an interfering factor to the solid phase, which most likely has caused the high Vitamin D3 value, was present in the sample. This finding is supported by all results obtained with different Elecsys assays and other immunoassay techniques.

Discussion
The introduction of automated immunoassay for vitamin D has provided a relatively easy and cost effective alternative to the standard method. Since the introduction of vitamin D assay into our laboratory, the number of request from clinicians has increased exponentially. This is the first confirmed case of vitamin D assay interference in our laboratory.

The Elecsys Vitamin D (25-OH) reagent package insert states that interference may cause by visible signs of haemolysis, grossly lipaemic, gross icteric and highly elevated rheumatoid factors sample, and high doses of biotin. These interfering factors were excluded during our preliminary investigation and the patient was not on biotin therapy.

Heterophilic antibodies are anti-animal antibodies present in human serum. Such antibodies are also a well known cause of interference in immunoassay (10, 11). The Elecsys Vitamin D (25-OH) assay uses polyclonal sheep antibodies. Therefore, the presence of anti-sheep antibodies in the patient's serum may be a cause for interference. The assay has suitable additives included to minimize such interference. These additives, however, do not completely eliminate the risk of interference due to heterophilic antibodies. Unfortunately, the use of heterophilic antibody-blocking tube also had no effect on the vitamin D level compared to the untreated sample.

The Elecsys Vitamin D (25-OH) reagent package insert also states that, in rare cases, interference caused by extremely high titres of antibodies to streptavidin and ruthenium may occur. Roche Diagnostics in Germany uses an in-house method to successfully adsorb the interfering factor. They were able to successfully absorb the interfering factor and the vitamin D result obtained was comparable to the standard method. However, they did not include a detailed method in their report. Furthermore, the identity of the interfering factor remains unknown only that it is interfering in the solid phase of the assay. After further research we came to the conclusion that there are two possible interfering factors: anti-streptavidin antibodies and endogenous biotin, both of which can cause the same interfering outcome. Anti-streptavidin antibodies are the most likely in this case because of repeated incidences of elevated vitamin D levels, indicating a constant present of the interfering factor in the body. However, we cannot exclude the other possibility as interference can occur in individuals with extremely high levels of endogenous biotin (12).

Roche Diagnostics in Germany did not provide the details on their in-house method. Therefore, we currently do not have a resolution to this problem should it occur again. So, our concern remains.

In the case of erroneously high vitamin D results, consequences are less likely to be serious. The patient in this case was referred to an endocrinologist. Fortunately, no further interventions were undertaken because of good communication between the laboratory and the clinicians. However, because of the nature of the interference it could affect any of the Elecsys assays. It could be troponin T, beta HCG or tumour markers where the consequences of erroneous results could be more serious. Therefore, it is very important for laboratory personnel and clinicians to be aware of such interference. More importantly, as recommended by the manufacturer, for diagnostic purposes, results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

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References

Author contributions
Vichet Khieng was the primary author, conceived the study and carried out the laboratory testing. Catherine Stevens substantially rewrote parts of the manuscript for intellectual content. The authors have no conflicts of interest to declare.

Address for correspondence: Vichet Khieng, Department of Biochemistry, Southern Community Laboratories, Dunedin City Hospital, Dunedin, New Zealand. Email: vichetkh@hotmail.com