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Editorial

The measurement of 25-hydroxy vitamin D – an analytical challenge

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Over the last decade requests for the measurement of 25-hydroxy vitamin D (25-OHD) have risen exponentially. For example, statistics from the Australian government provides evidence for a 100-fold increase between 2000 and 2010 (1). This burst of vitamin D requests has several causes including an increased awareness of the medical community and the general population to the high prevalence of vitamin D deficiency and its relevance for osteoporosis, cardiovascular disease, malignancies, infectious and autoimmune disease. The massively increased demand for 25-OHD testing has important implications for healthcare systems and clinical laboratories. Whereas healthcare systems worldwide face an explosion of costs for 25-OHD testing, laboratories have to adopt methods that can cope with the elevated workload. External quality control programs show that most laboratories use automated immunoassays. Diagnostic manufacturers have identified 25-OHD as a highly profitable and strategic test, which has lead to the commercialization of new automated immunoassays during recent years. However, it has to be acknowledged that 25-OHD is a challenging analyte to accurately measure in human blood (2). Analytical difficulties are related to its lipohilic nature, strong affinity to vitamin D binding protein (VDBP) and association with human serum albumin, as well as the presence of very low to high levels of 25-OH vitamin D_3 (25-OHD_3) and/or 25-OH vitamin D_2 (25-OHD_2), multiple 25-OHD metabolites (e.g., 24,25-dihydroxy vitamin D_3), C3-epimer of 25-OHD_3 and 25-OHD_2 (25-OHD_3-epi and 25-OHD_2-epi), and other sources of assay interference, such as heterophilic antibodies. Therefore, 25-OH vitamin D immunoassays are required to detect 25-OHD_2 and 25-OHD_3 in an equimolar fashion and report a total 25-OHD result.

Previous automated immunoassay comparison studies have demonstrated significant analytical limitations for some of these assays (2, 3). External quality assurance programs, such as DEQAS, or the Quality Assurance Program of the Royal Australian College of Pathologists, also show a wide spread of reported results for the same sample, which in some cases vary from deficient (<25 nmol/L) to sufficient (>75 nmol/L). Analytical problems of 25-OHD immunoassays have also been noted by clinicians who often question the laboratory about results that do not correspond to the clinical picture of their patients. The apparent issues of automated immunoassays have resulted in the worldwide withdrawal of the Roche 25-OHD_3 assay in 2010 and to the modification of the assay conditions of several other commercial immunoassays, such as SIEMENS Centaur, Abbott ARCHITECT and DiaSorin LIAISON. Regulatory bodies, such as the FDA have also become aware of analytical issues with some of the recently launched assays. For example, the FDA has imposed certain conditions on some of the recently cleared 510(k) assays, such as limiting the measuring range of the Abbott ARCHITECT assay from 13 ng/mL (32.5 nmol/<L) to 96 ng/mL (240 nmol/L).

The first automated vitamin D immunoassay was a vitamin D binding protein (VDBP) based test developed for the Nichols Advantage analyzer (4). This assay underestimated 25-OHD at low levels and overestimated 25-OHD at high levels resulting in a substantial misclassification of patients (5). A comparison of the Nichols Advantage assay with liquid chromatography tandem mass spectrometry (LC-MS/MS) also revealed problems with assay linearity (6) and individual patient results were highly variable. Due to these and other issues the Nichols binding protein assay was discontinued and companies like DiaSorin and Roche developed assays that employ specific antibodies to capture 25-OHD. While the DiaSorin LIAISON test used a polyclonal antibody with 100% and 104% cross-reactivity to 25-OHD_3 and 25-OHD_2, respectively, the Roche assay employed an antibody that cross-reacted 100% with 25-OHD_3 with no cross-reactivity to 25-OHD_2. Methods like the Abbott ARCHITECT, DiaSorin LIAISON, IDS iSYS and Siemens Centaur have a competitive format where analyte concentration and signal intensity are inversely related, but they have different mechanisms for signal generation (e.g., vitamin D analogue and conjugate capture approach). Recently, Roche Diagnostics launched a new total vitamin D assay, which uses recombinant VDBP instead of an antibody to capture 25-OHD. Unlike the previous 25-OHD_3 specific assay this approach allows the detection of both 25-OHD_3 and 25-OHD_2. Importantly, the test is standardized against LC-MS/MS with traceability to the NIST 972 standard reference material. In this issue of Clinical Chemistry and Laboratory Medicine several comparison and evaluation studies assessed the performance of the new Roche total and other latest generation automated 25-OHD immunoassays (7–12). Virtually all of these studies concluded that the performance of most assays was acceptable and that they were suitable for routine use. Although the analytical performance of most 25-OHD immunoassays has improved, significant issues remain. For example, it is questionable if a correlation of r=0.90 between two methods which are supposedly measuring the same analyte can be regarded as ‘adequate for

the purpose', and the studies by Emmen et al. (7) or Franken et al. (12) highlight this. Furthermore, a narrow linear range of 7.5–175 nmol/L, such as the one of the Roche assay, requires dilution to identify samples with toxic 25-OHD levels (>250 nmol/L) making the diagnosis of intoxication cumbersome.

The 25-OHD assay comparison studies in this issue of CCLM unequivocally demonstrate that most automated immunoassays are able to detect 25-OHD_3 with reasonable precision and accuracy (7–12). Only the SIEMENS Centaur assay appears to not achieve satisfactory performance (2, 11). If read uncritically one may conclude that latest generation assays are fit for purpose. However, the design of most of these studies does not address the following analytical and clinical aspects that impact assay performance under routine conditions, and do not truly establish if a given immunoassay is clinically fit for use:

1) Most of the studies published do not adequately challenge claimed immunoassay measuring ranges and linearity. It is best practice to ensure the method comparison samples have doses assigned by the reference method (i.e., LC-MS/MS) that span the measuring range(s) of the test method(s). There should also be a sufficient number of specimens (i.e., ≥80 samples) evenly spanning the test method(s) measuring range for a statistically sound, non-biased, regression and bias plot analysis. Moreover, since immunoassays will invariably under- or over-recover exogenous 25-OHD spikes, it is very important that all method comparison samples consist of 100% endogenous 25-OHD. Serum is the preferred sample type for all commercial vitamin D immunoassays, HPLC and LC-MS/MS.
2) Most of the studies published here do not permit a judgment on cross-reactivity or recovery with 25-OHD_2 as only a very few samples contained significant levels of endogenous 25-OHD_2. This was because they were performed in Europe where vitamin D_2 containing supplements were rare. However, in other parts of the world, such as the USA and the UK vitamin D_2 containing supplements are commonly used. In these countries knowledge about the detection of 25-OHD_2 is very relevant. The studies by Le Goff et al. (9) and a previous study by Cavalier et al. (13) nicely illustrate this. Le Goff et al. found that the Abbott Architect assay grossly underestimates 25-OHD_2 while the SIEMENS Centaur overestimates 25-OHD_2 (9). Such limitations can only be detected with an appropriate study design.
3) The presence of the 25-OHD_3-epimer, a metabolite with reported reduced biological activity, also affects assay performance (15). The study by Farrell et al. demonstrated the presence of 25-OHD_3-epimer in about 40% of the study population. 25-OHD_3-epi levels ranged between 5 and 14 nmol/L. While most of the automated immunoassays claim that they do not cross-react with 25-OHD_3-epi the new Roche assay does capture this metabolite. Therefore, to properly interpret the performance of an immunoassay it is important to know if this assay and the reference method used for comparison detect 25-OHD_3-epi (11).
4) Another major problem is that the LC-MS/MS reference methods that were used were not harmonized. They were all different and were unaligned to any of the recently endorsed standard reference methods (15, 16). Although the introduction of the NIST 972 reference material has improved the comparability of LC-MS/MS methods, proficiency testing programs around the world shows that there is still substantial variation between these methods.
5) Heterophilic antibodies may affect immunoassay performance and are typically found at rates of <1 in 1000. In a routine setting where a given test is used to measure tens of thousands of samples such interference can cause substantial problems. Therefore, in addition to the samples selected to span the assay measuring range, method comparison studies should also challenge the test methods with a set of samples known to contain heterophilic antibodies. As it is very difficult to identify and source samples with interfering heterophilic antibodies, it is more practical to question assay results that do not fit the clinical picture and reflex test these samples by a heterophilic antibody-free reference method, such as LC-MS/MS. For example, samples with doses outside the normal/reference range of a given assay are likely candidates for heterophilic interference if the LC-MS/MS results are significantly different from the test method.

How these aspects affect assay performance is nicely shown in the study Farrell et al. where automated 25-OHD immunoassays were specifically challenged with high and low samples as well as samples containing 25-OHD_2 and heterophilic antibodies (11).

In conclusion, assay comparison studies should specifically challenge the assays to report a true and robust total 25-OHD value across the claimed measuring range of a given assay. This includes the measurement of serum samples containing endogenous 25-OHD_2, 25-OHD_3- and 25-OHD epi, as well as selected samples with interfering heterophilic antibodies. Furthermore, LC-MS/MS methods that are used for comparison should be aligned to one of the recently endorsed standard reference methods (15, 16). And last but not least, there is a need to adopt robust criteria for the decision if the performance of an assay is acceptable for routine use.

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