Clinical Chemistry 58:3
486–488 (2012)

25-Hydroxyvitamin D: A Difficult Analyte

Graham D. Carter[1*]

In their article in the current issue of *Clinical Chemistry*, Farrell et al. *(1)* describe the latest in a number of studies *(2, 3)* highlighting the method-related variability in serum 25-hydroxyvitamin D (25-OHD)[2] results. There is common agreement that 25-OHD is a "difficult" analyte. That quality is generally ascribed to its hydrophobic nature, its existence in several different molecular forms, its tight binding to vitamin D–binding protein (VDBP) and, until recently, the absence of reference materials or a reference measurement procedure (RMP) against which assays could be standardized. This last problem was mitigated to some extent with the introduction in 2009 of the NIST Standard Reference Materials (SRM 972 and SRM 2972) and the acceptance of the NIST and University of Ghent liquid chromatography–tandem mass spectrometry (LC-MS/MS) assays as RMPs *(4)*. Unfortunately, 3 of the 4 SRM 972 reference materials are either spiked with exogenous metabolites or diluted with equine serum, characteristics that render these reference materials unsuitable for many immunoassays *(5, 6)*.

The history of 25-OHD methodology could serve as a case study of the consequences of transferring a rigorous but labor-intensive method from the unhurried atmosphere of the research laboratory to the bustle of a routine clinical laboratory having to meet tight deadlines. The pioneering competitive protein-binding (CPB) method of Haddad and Chyu *(7)* involved solvent extraction and chromatography, with the results of every sample corrected for procedural losses. Subsequent attempts at simplifying a CPB method by omitting the chromatography stage proved unsuccessful *(8)*, and an automated version (Nichols Advantage) introduced in 2004 was withdrawn in 2006. The successive abandonment of sample extraction, chromatography, and correction for procedural losses in immunoassays has undoubtedly contributed to the inconsistencies reported by Farrell et al., as have differences in assay standardization. Nevertheless, results submitted to the international Vitamin D External Quality Assessment Scheme (DEQAS) have shown a gradual reduction in interlaboratory imprecision (CV) in recent years—from >30% in 1995 to 15% in 2011. This reduction may be due to the increasing dominance of automated immunoassays, in particular the DiaSorin LIAISON Total method and, more recently, greater use of the NIST SRMs in standardization. Whatever the explanation, it appears that there is currently reasonable agreement, with 1 or 2 notable exceptions, between most 25-OHD methods *(9)*. Nevertheless, the fact that a change of method can affect the continuity of results has implications for the monitoring of patients on vitamin D supplements and for long-term epidemiologic studies. Even within the same method, the consistency of results can be compromised by subtle lot-to-lot changes in reagent kits or more overtly by a change of antibody or in a reformulation of reagents.

With the introduction of the RMPs and the expected release in 2012 of a new generation of human serum–based SRMs, assay standardization should become less of an issue, and attention is likely to focus on the reliability of individual results, a far more intractable problem. The use of summary statistics in method comparisons, particularly the correlation coefficient, and linear regression equations can disguise a marked variance in the results given by 2 methods for the same sample. Method differences are best revealed by the use of Bland–Altman plots, which allow discrepancies between results for individual samples to be seen at a glance. These apparently random errors undermine the confidence in the veracity of all 25-OHD results and suggest that some methods are subject to interference from other components of the sample matrix. The study reported by Heijboer et al. *(10)* (also in the current issue) not only identifies a potential culprit (VDBP) but quantifies its effect. The automated 25-OHD methods, which have necessarily abandoned solvent extraction or protein precipitation to free 25-OHD from binding proteins, are likely to be more

[1] Imperial College Healthcare NHS Trust, Clinical Biochemistry Department, Charing Cross Hospital, London, UK.
* Address correspondence to the author at: DEQAS Organizer, Imperial College Healthcare NHS Trust, Clinical Biochemistry Department, Charing Cross Hospital, Fulham Palace Rd., London, W6 8RF, UK. Fax +44-20-331-17007; e-mail b.carter1@which.net.
Received December 29, 2011; accepted December 30, 2011.
Previously published online at DOI: 10.1373/clinchem.2011.180562
[2] Nonstandard abbreviations: 25-OHD, 25-hydroxyvitamin D; VDBP, vitamin D–binding protein; RMP, reference measurement procedure; SRM, Standard Reference Material; LC-MS/MS, liquid chromatography–tandem mass spectrometry; CPB, competitive protein-binding (method); DEQAS, Vitamin D External Quality Assessment Scheme; 24,25-$(OH)_2$D, 24,25-dihydroxyvitamin D; 3-epi-25-OHD_3, 3-epi-25-hydroxyvitamin D_3.

susceptible to variations in VDBP concentration. Manufacturers have been coy about disclosing details of the separation step used in these assays, but most appear to involve the denaturation of VDBP via a pH change. Whatever the detail, there is an assumption—with little or no published evidence—that 100% of 25-OHD is displaced from VDBP or, if not, that the percentage displaced in different samples is consistent. The data presented in the report of Heijboer et al. suggest this is not the case. Four of the 5 nonextraction immunoassays compared with the reference LC-MS/MS method had a highly significant correlation of bias to the VDBP concentration. Moreover, the scatter of results indicates that the bias was far from consistent; this finding offers one explanation for the arbitrary nature of method differences with individual samples.

A recent and surprising twist in the debate on matrix effects was the report by Cavalier et al. *(11)* of interference in the DiaSorin LIAISON method by human antianimal antibodies. Interference from heterophilic antibodies is a problem more generally associated with noncompetitive immunoassays than with the competitive assays used to measure 25-OHD. When the affected samples were reassayed by the DiaSorin RIA method, the problem largely disappeared, presumably because of the initial protein-precipitation step used in this method. DiaSorin subsequently produced a modified version of the LIAISON method, which was apparently unaffected by heterophilic antibodies.

A limitation of the studies of Farrell et al. and Heijboer et al., which is explained by local prescribing conventions, is their failure to recruit more than a few individuals supplemented with vitamin D_2. Heijboer et al. acknowledge that the detection of 25-OHD_2 is method dependent [the Abbott ARCHITECT assay has only a 52% cross-reactivity with 25-OHD_2 *(12)*], and it is interesting to speculate whether VDBP, to which 25-OHD_2 binds less strongly than 25-OHD_3 *(13)*, influences the detection of this metabolite in nonextraction assays.

Neither of these reports addresses the effects of interference from other hydroxylated vitamin D metabolites, such as 24,25-dihydroxyvitamin D [24,25-$(OH)_2D$] or 3-epi-25-hydroxyvitamin D_3 (3-epi-25-OHD_3). All immunoassays for 25-OHD show high cross-reactivity with 24,25-$(OH)_2D$, which can be present in serum at concentrations of up to about 12 nmol/L *(14)*. A recent DEQAS investigation *(9)* has confirmed manufacturers' assertions that 3-epi-25-OHD_3 is not detected by any of the widely used immunoassays, including the ones used in the Farrell et al. study. Interestingly, the Roche CPB method for total 25-OHD used in the investigations of Heijboer et al. did show significant cross-reactivity (approximately 57%) with the 3-epimer. Most LC-MS/MS and HPLC/UV assays did not resolve the 3-epimer. Although the amounts in adult serum are generally small, very high concentrations have been reported in some individuals *(15)*. No mention is made of the 3-epimer in the report by Heijboer et al.

With the introduction of LC-MS/MS assays for 25-OHD came the opportunity for routine clinical laboratories to return to a more rigorous technique in which sample extraction, chromatography, and correction for procedural losses were restored and in which specificity was enhanced by the addition of mass spectrometry. LC-MS/MS is now widely used as the reference method in comparison studies such as the ones published in the current issue of this journal. Manufacturers are also beginning to use LC-MS/MS to calibrate their assay standards, and the perceived virtues of this technique are in danger of being exaggerated. In practice, the supposed specificity conferred by the MS/MS component is limited by its being an achiral technique, which by definition cannot distinguish between 25-OHD_3 and its epimer, 3-epi-25-OHD_3. Shah et al. *(16)* have demonstrated the need to chromatographically separate other isobaric substances, in particular 7-α-hydroxy-4-cholesten-3-one, an endogenous precursor of bile acids found in 4 of their 5 healthy volunteers. The authors claim that this substance would have inflated 25-OHD results by as much as 39% if not separated chromatographically, although there is no evidence of this degree of interference in routine LC-MS/MS assays. Failure to resolve the 3-epimer and other isobars, however, probably accounts for much of the persistent positive bias shown by "routine" LC-MS/MS methods in DEQAS and other method comparisons. Unfortunately, increasing the specificity of LC-MS/MS will necessitate longer chromatography run times, making the technique less suitable for the busy routine clinical laboratory. Nevertheless, LC-MS/MS is currently used by approximately 11% of DEQAS participants, and its popularity is likely to increase as the specificity problems are addressed.

The consequences of systemic differences between 25-OHD assays are not confined to clinical laboratories. Epidemiologic studies are an important source of normative 25-OHD data, but differences in methods used by national surveys have limited the extent to which results could be pooled. Recognizing the advantages to be gained from creating an international database, the US Office of Dietary Supplements (NIH) has organized a project (the Vitamin D Standardization Program), the goal of which is to standardize measurements done in national health surveys to those of the RMPs developed by NIST and the University of Ghent. It also allows for participation of clinical, public health, research, and commercial organizations in the standardization effort *(17)*.

In summary, the studies of Farrell et al. and Heijboer et al. *(1, 10)* have identified general and specific problems

in measuring 25-OHD. The standardization of 25-OHD assays is slowly being addressed, but the elimination of matrix effects, of which VDBP is an example, will require the continual vigilance of analysts and clinicians and an ongoing dialogue with manufacturers.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *No authors declared any potential conflicts of interest.*

References

1. Farrell CJL, Martin S, McWhinney B, Straub I, Williams P, Herrmann M. State-of-the-art vitamin D assays: a comparison of automated immunoassays with liquid chromatography–tandem mass spectrometry methods. Clin Chem 2012;58:531–43.
2. Binkley N, Krueger D, Cowgill CS, Plum L, Lake E, Hansen KE, et al. Assay variation confounds the diagnosis of hypovitaminosis D: a call for standardization. J Clin Endocrinol Metab 2004;89:3152–7.
3. Roth HJ, Schmidt-Gayk H, Weber H, Niederau C. Accuracy and clinical implications of seven 25-hydroxyvitamin D methods compared with liquid chromatography–tandem mass spectrometry as a reference. Ann Clin Biochem 2008;45:153–9.
4. Bureau International des Poids et Mesures. Database of higher-order reference materials, measurement methods/procedures and services. JCTLM database: laboratory medicine and in vitro diagnostics. http://www.bipm.org/jctlm/ (Accessed December 2011).
5. Carter GD, Jones JC, Berry JL. The anomalous behaviour of exogenous 25-hydroxyvitamin D in competitive binding assays. J Steroid Biochem Mol Biol 2007;103:480–2.
6. Horst RL. Exogenous versus endogenous recovery of 25-hydroxyvitamins D_2 and D_3 in human samples using high-performance liquid chromatography and the DiaSorin LIAISON Total-D assay. J Steroid Biochem Mol Biol 2010; 121:180–2.
7. Haddad JG, Chyu KJ. Competitive protein-binding radioassay for 25-hydroxycholecalciferol. J Clin Endocrinol Metab 1971;33:554–7.
8. Dorantes LM, Arnaud SB, Arnaud CD, Kilgust KA. Importance of the isolation of 25-hydroxyvitamin D before assay. J Lab Clin Med 1978;91:791–6.
9. Vitamin D External Quality Assessment Scheme (DEQAS) 25(OH)D report, October 2011 distribution. London: Charing Cross Hospital; 2011.
10. Heijboer AC, Blankenstein MA, Kema IP, Buijs MM. Accuracy of 6 routine 25-hydroxyvitamin D assays: influence of vitamin D binding protein concentration. Clin Chem 2012;58:543–8.
11. Cavalier E, Carlisi A, Bekaert A-C, Rouselle O, Chapelle J-P. Human anti-animal interference in DiaSorin Liaison total 25(OH)-vitamin D assay: towards the end of a strange story? Clin Chim Acta 2012;413:527–8.
12. Abbott Diagnostics. ARCHITECT 25-OH Vitamin D [product insert]. Abbott Park (IL): Abbott Diagnostics; 2011.
13. Hollis BW. Comparison of equilibrium and disequilibrium assay conditions for ergocalciferol, cholecalciferol and their major metabolites. J Steroid Biochem 1984;21:81–6.
14. Coldwell RD, Trafford DJ, Makin HL, Varley MJ, Kirk DN. Specific estimation of 24,25-dihydroxyvitamin D in plasma by gas chromatography-mass spectrometry. Clin Chem 1984;30:1193–8.
15. Lensmeyer G, Poquette M, Wiebe D, Binkley N. The C-3 epimer of 25-hydroxyvitamin D_3 is present in adult serum. J Clin Endocrinol Metab 2012;97:163–8.
16. Shah I, James R, Barker J, Petroczi A, Naughton DP. Misleading measures in vitamin D analysis: a novel LC-MS/MS assay to account for epimers and isobars. Nutr J 2011;10:46.
17. Notice of vitamin D standardization program. Federal Register 2011;76: 11502.