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# Analytical Quality Goals for 25-Vitamin D Based on Biological Variation

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*Background*: Measurement of 25-hydroxyvitamin D, (25D) is central in the investigation of pathologies of bone and mineral ion metabolism and in determining a patient's vitamin D status. More recently much research interest has lead to investigating the role it can play in decreasing the risk of many chronic illnesses, including common cancers, autoimmune diseases, infectious diseases, and cardiovascular disease. Knowledge of the biological variation of an analyte forms an essential part of evaluating a new analyte enabling the objective assessment of the changes in serial results, the utility of reference intervals as well as establishing laboratory quality specifications. *Methods*: This study determined the biological variation of 25D in 20 healthy individuals that was calculated according to the familiar methods outlined by Fraser and Harris. *Results*: The within-subject variation was 12.1% and the between subject variation was 40.3%. The critical difference for sequential values significant at $P<0.05$ was calculated as 38.4%. The within-subject variation forms a relatively small part of the reference interval shown by the low index of individuality of 0.3. Objective analytical quality goals have also been established which have shown achievable minimum performance for imprecision of ~6%. The desirable analytical bias goal was ~10%. *Conclusion*: This study has objectively shown that the analytical precision of current instruments is being achieved contrary to the known problems surrounding the analytical bias for 25D assays. The limitations of using reference intervals for 25D, both in diagnoses and monitoring are shown. J. Clin. Lab. Anal. 25:130–133, 2011. © 2011 Wiley-Liss, Inc.

**Key words:** biological variation; laboratory quality specifications; 25-hydroxyvitamin D; reference change value

The vitamin D endocrine system plays an essential role in calcium homeostasis and bone metabolism, but research during the past two decades has revealed a diverse range of biological actions that include induction of cell differentiation, inhibition of cell growth, immunomodulation, and control of other hormonal systems (1). Vitamin D from the skin and diet is metabolized in the liver to 25-hydroxyvitamin D (25D) which can be measured in serum to determine a patient's vitamin D status (2). Measurement of 25D is also central in the investigation of pathologies of bone, calcium, and phosphate metabolism.

25D is metabolized in the kidneys by the enzyme 25-hydroxyvitamin D-1α-hydroxylase to its active form, 1,25-dihydroxyvitamin D (1,25D). Its major effects on bone and mineral ion metabolism are through the nuclear receptors in the intestine and osteoblasts which alters the transcription rates of target genes. The accurate assessment of vitamin D status has challenged laboratorians for more than 60 years (3,4).

Data on biological variation forms part of the comprehensive evaluation required for analytes that are measured in the clinical laboratory and is an essential prerequisite to the introduction of new analytes (5). Biological variation data have several important clinical and laboratory applications that include: setting analytical quality specifications, evaluating the significance of changes in serial results (the reference change

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value (RCV) or "critical difference"), assessing the utility of population-based reference intervals and calculating the number of specimens required to estimate the homeostatic set point (6,7). A comprehensive biological variation database of all known analytes which is frequently updated contains more than 300 analytes and references more than 200 publications and serves as a useful reference for many clinical laboratories (8). Interestingly, no data are available for the biological variation of 25D, despite it being measured more frequently as opposed to many other more esoteric tests. Without information on the biological variation it remains difficult to objectively assess serial patient results as well as data on internal control and quality assessment. We subsequently set out to generate this data.

20 apparently healthy individuals who reside in Hertfordshire, in the United Kingdom (approximate latitude 51 north) participated in the study. This included 10 men (2 Black and 8 Caucasian) and 10 women (1 Black, 1 Asian and 8 Caucasian). The median age was 37 years (range 19–60 years). We previously described this cohort elsewhere (9). All subjects were consented before the study, which had been approved by the local Research and Ethics Committee. None of the subjects received any prescription medication, had any recent illnesses (previous 3 months) or during the study period that required at least general practitioner consultation, and none had a history of current or previous pathology related bone and/or mineral ion metabolism. No abnormalities in the relevant routine biochemistry investigations were detected on any of the subjects. Venous blood was collected between 08:45 and 09:30 on the same day of the week, weekly for 5 weeks, during the months of October and November. All the samples were collected by one person. Samples were separated by centrifugation at 4,500*g* for 10 min and the serum was stored immediately at −80°C.

Routine biochemistry investigations were performed on the Olympus AU 2700 (Olympus, Watford, UK) according to manufacturer specifications. 25D analyses were performed on the automated enzyme-linked immunosorbent assay analyzer, Triturus® (Grifols, Cambridge, UK). The assay principle is based on competitive protein binding using reagent from Immundiagnostik (Bensheim, Germany). The samples were analyzed once before being randomized, and then reanalyzed. One analyst performed all the analyses, employing the same batches of reagent, quality control material and calibrators as suggested by Fraser and Harris (7).

Biovariability data was analyzed using Excel (Microsoft Corporation®, Microsoft Limited, Microsoft CampusThames Valley Park Reading Berkshire, RG6 1WG, United Kingdom). The respective analytical, within-subject (or intraindividual), and between-subject (or interindividual) variances ($SD_A^2$, $SD_I^2$, $SD_G^2$) were calculated as described by Fraser and Harris (7). By this technique, analytical variance ($SD_A^2$) was calculated from the difference between duplicate results for each specimen ($SD_A^2 = \Sigma d^2/2N$, where $d$ is the difference between duplicates, and $N$ is the number of paired results). The variance of the first set of results for each subject was used to calculate the average biological intra-individual variance ($SD_I^2$) by subtraction of $SD_A^2$ from the observed dispersion (equal to $SD_I^2+SD_A^2$). Subtracting ($SD_I^2+SD_A^2$) from the overall variance of the set of first results determined the between-subject variance ($SD_G^2$).

As presented previously, Table 1 gives the details of the 20 subjects who participated in the study (9). Figure 1 shows the median and range of the 25D concentrations. Of the total test variance, analytical variance contributed 3%, within-subject variance contributed 8%, and between-subject variance contributed 89%. The respective analytical coefficient of variation ($CV_A$), within-subject coefficient of variation ($CV_I$), and between-subject coefficients of variation ($CV_G$) were 6.7, 12.1, and 40.3%.

With knowledge of biological variation and analytical imprecision for the assay, it is possible to calculate a RCV for serial results to be significantly different, using the following equation (7,10):

$$RCV = 2^{1/2} XZX(CV_A^2 + CV_I^2)^{1/2}$$

$CV_A$ is analytical imprecision and $CV_I$ is intraindividual variation. A change with 95% probability is regarded as significant and 99% as highly significant. The corresponding $Z$ scores are 2.58 and 1.96, respectively (for 25D bidirectional $Z$ scores would be applicable). The respective 95 and 99% significant changes were 38.4 and 50.6%. What does this imply for serial results? For an initial 25D concentration of 69 nmol/l (the median value of all the results), a follow-up result needs to be lower than 42 nmol/l or 33 nmol/l to be regarded as "critically different" at the respective 95 and 99% levels. This shows that it is possible to misclassify individuals to be vitamin D deficient [generally taken to be apparent at concentrations below 50 nmol/l (2)] without considering within-subject variation.

The usefulness of reference intervals has been addressed by the concept of biological individuality also referred to as the "index of individuality" (IoI) (11,12). This is expressed as the ratio of $CV_I/CV_G$, which is the ratio of within-subject to between-subject variation (11,12). When the index is low, particularly when it is $<0.6$, the dispersion of values for any individual will span only a small part of the reference interval. Reference values will thus be of little use; in particular, for deciding whether a significant change has occurred. Conversely, when the index is high, particularly when it

**TABLE 1. Characteristics of the Individual Patients That Took Part in This Study With the Respective Reference Intervals in Brackets (9)**

| Subject | Age | Sex | Adjusted calcium (2.2–2.65 mmol/l) | Creatinine (45–125 μmol/l) | Phosphate (0.75–1.36 mmol/l) | Magnesium (0.74–1.0 mmol/l) | Alkaline phosphatase (30–115 IU/l) |
|---|---|---|---|---|---|---|---|
| 1 | 23 | Male | 2.43 | 103 | 1.15 | 0.83 | 77 |
| 2 | 38 | Female | 2.32 | 77 | 0.96 | 0.81 | 62 |
| 3 | 39 | Male | 2.17 | 88 | 1.13 | 0.78 | 80 |
| 4 | 49 | Male | 2.4 | 87 | 1.17 | 0.89 | 80 |
| 5 | 45 | Male | 2.41 | 94 | 0.57 | 0.89 | 89 |
| 6 | 37 | Male | 2.29 | 78 | 1.05 | 0.81 | 50 |
| 7 | 43 | Female | 2.33 | 87 | 0.99 | 0.87 | 47 |
| 8 | 21 | Male | 2.38 | 104 | 1.36 | 0.87 | 84 |
| 9 | 40 | Female | 2.37 | 85 | 1.1 | 0.77 | 55 |
| 10 | 43 | Male | 2.24 | 95 | 0.96 | 0.74 | 64 |
| 11 | 44 | Female | 2.31 | 80 | 1.21 | 0.8 | 45 |
| 12 | 44 | Male | 2.5 | 106 | 0.83 | 0.78 | 74 |
| 13 | 49 | Male | 2.34 | 75 | 1.07 | 0.84 | 90 |
| 14 | 24 | Female | 2.35 | 77 | 0.96 | 0.81 | 98 |
| 15 | 22 | Female | 2.46 | 85 | 1.15 | 0.76 | 51 |
| 16 | 60 | Female | 2.4 | 78 | 1.38 | 0.85 | 97 |
| 17 | 19 | Female | 2.25 | 82 | 1.11 | 0.71 | 70 |
| 18 | 27 | Female | 2.44 | 92 | 1.38 | 0.82 | 71 |
| 19 | 22 | Female | 2.26 | 90 | 1.21 | 0.77 | 61 |
| 20 | 47 | Male | 2.29 | 102 | 1.24 | 0.69 | 80 |

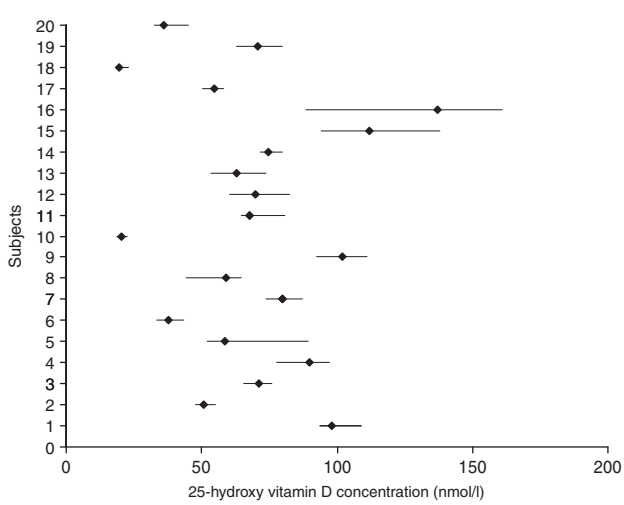


**Fig. 1.** The median (diamond) and range of the 25-hydroxyvitamin D concentrations of study subjects are shown.

is $>1.4$, values from a single individual will cover much of the entire distribution of the reference interval. In this context, reference values will be of significant value for clinical interpretation. The IoI of 0.3 emphasizes the limitation of using population based reference intervals for 25D.

It is widely accepted that analytical quality specification might best be based on the components of biological variation (namely $CV_I$ and $CV_G$) (6). Desirable performance for analytical imprecision ($CV_A$) is defined as $CV_A<0.5CV_I$. Minimum performance is further defined as $CV_A<0.75CV_I$ and optimum performance as $CV_A<0.25CV_I$ (13,14). Analogously to imprecision, the quality specification for bias ($B_A$) defines desirable performance as $B_A<0.250\ (CV_I^2+CV_G^2)^{1/2}$, optimum performance as $B_A<0.125\ (CV_I^2+CV_G^2)^{1/2}$, and minimum performance as $B_A<0.375\ (CV_I^2+CV_G^2)^{1/2}$ (12,13). From this the total allowable error (TE) may be calculated, where $TE<k.\ CV_A+B_A$ (where $k=1.65$ at $\alpha=0.05$) (15).

The respective desirable, optimal and minimum analytical quality goals for 25D are: $CV_A<6$, 3, and 9%; $B_A<10.5$, 5.3, and 21%; $TE<21.7$, 16.4, and 32.2%.

This is the first formal report on the biological variation of 25D in a healthy population. A previous study assessed the within- and between-subject variations of 25D using data from four different dietary intervention studies in postmenopausal women. The authors neither elaborated on their methodology nor provided specific percentages of $CV_I$ and $CV_G$ (16). A recent paper which provided specifications for trueness and precision of a reference measurement system for 25D extrapolated the $CV_I$ and $CV_G$ to be 8 and 21%, respectively (17). Our data generated respective values of 12 and 40%. The differences can be explained by the more heterogeneous population in our study which included males and premenopausal females as opposed to postmenopausal females only. Furthermore, we do not have specific details on how the data were generated from this study. The specifications suggested

for trueness and precision for routine methods range between 3 and 22% for $CV_A$ and between 3 and 10% for $B_A$ (17). This is in accordance with our data which suggested respective desirable $CV_A$ of 6% and $B_A$ of 10%. The fact that the biological variation of 25D has not been formally assessed before this study is rather surprising, taken that 25D analysis forms part of the investigation of hypocalcaemia and hypophosphataemia and in the diagnosis and management of rickets, granulomatous disorders, hyperparathyroidism, vitamin D deficiency, and osteoporosis.

Contrary to this, the seasonal variations in 25D have long been known (18) and levels can fluctuate from nadir to zenith by as much as 20% (19).

We have also been able to set analytical quality specifications based on these data. Most current assays are able to achieve desirable analytical goals for imprecision. Goals for analytical bias have also been established. Data from the international Vitamin D Quality Assessment Scheme shows that despite the fact that most commercial assays are able to provide results close to the target value, results span a substantial range and are also highly operator dependent (20). For example, a recent study, which included 564 samples, demonstrated that results span 30–70 nmol/l for a target value of 50 nmol/l with most laboratories achieving the desirable bias goal of ~10%. The recent public interrogation of the quality of vitamin D assay performance has exposed the scientific community (21,22) and we believe that these new data will be able to provide guidance on the quality goals and thus continue our focus on all aspects of analytical quality. Further quality improvements are expected with the development of standard reference material (23). Even though the difficulties surrounding the analytical bias have long been known these data have now made the analytical quality goals more tangible.

Importantly these data have also highlighted the often-overlooked limitations of using reference intervals when diagnosing disease as well as the limitations of monitoring serial results.

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