Title: 25-HYDROXYVITAMIN D ASSAYS: POTENTIAL INTERFERENCE FROM
OTHER CIRCULATING VITAMIN D METABOLITES.

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Abstract

The Vitamin D External Quality Assessment Scheme (DEQAS) for 25-hydroxyvitamin D (25-OHD) has approximately 1100 participants in 53 countries using 26 different methods or variants of methods (October 2014). In April 2015, the scheme was extended to cover 24,25-dihydroxyvitamin D (24,25(OH)₂D). Since 2013, the 25-OHD scheme has been accuracy-based with values assigned by the NIST Reference Measurement Procedure (RMP). DEQAS is uniquely placed to assess the accuracy (bias) and specificity of 25-OHD methods in a routine laboratory setting. Other vitamin D metabolites are known to interfere in 25-OHD assays and DEQAS has distributed samples spiked with 3-epi-25-OHD₃ (52.4 nmol/L), 24R,25(OH)₂D₃ (14.4 nmol/L) and 24S,25(OH)₂D₃ (57.9 nmol/L). The 3-epimer showed a cross reactivity of 56% in a competitive protein binding assay but was not detected in any antibody-based methods. Not all HPLC/UV or LC-MS/MS methods were able to resolve 3-epi-25-OHD₃ from 25-OHD₃ and thus overestimated total 25-OHD. The cross reactivity of 24R,25(OH)₂D₃ (24S,25(OH)₂D₃) ranged from <5% (<5%) to 548% (643%) in ligand binding assays. Both 24-hydroxylated metabolites were resolved by HPLC/UV and LC-MS/MS methods and thus caused no complications in the measurement of 25-OHD. Most antibodies to 25-OHD are known to cross-react with dihydroxylated metabolites but interference in some assays was far greater than expected. This may be related to the anomalous behaviour of exogenously added metabolites in these 25-OHD methods.

1.Introduction

The Vitamin D External Quality Assessment Scheme (DEQAS) is an international external quality assessment scheme which has been assessing the performance of 25-hydroxyvitamin D (25-OHD) assays since 1989 and 1,25-dihydroxyvitamin D (1,25(OH)₂D) assays since 1997. The scheme was extended in April 2015 to cover 24,25-dihydroxyvitamin D (24,25(OH)₂D). Performance assessment is achieved by the quarterly distribution of 5 samples of human serum to the approximately 1100 laboratories participating in the 25-OHD scheme. DEQAS has participants in 53 countries (October 2014). Samples are prepared from blood donated locally by polycythaemic or haemochromatosis patients undergoing therapeutic venesection and, since April 2013, from serum purchased from the USA (Solomon Park, Kirkland, WA, USA).

Since April 2013, as a result of support from the NIH Office of Dietary Supplements, the 25-OHD scheme has been accuracy based with values assigned to each sample by the NIST Reference Measurement Procedure (RMP) (1). Thus DEQAS is well placed to assess the accuracy (bias) and specificity of 25-OHD methods in a routine laboratory setting. The vitamin D metabolite 24,25(OH)₂D, concentrations of which can range from 2% to 20% of total 25-OHD (2), is known to cross react in several ligand binding assays and might account for their variable bias, particularly in samples with high 25-OHD. Many routine HPLC /UV and LC-MS/MS assays fail to resolve 3-epi-25-OHD₃ from 25-OHD₃ which might contribute to the persistent positive bias seen in these methods (3).

The objectives of this study were twofold:

- 1) To measure the concentration of 3-epi-25-OHD₃ and 24R,25(OH)₂D₃ in a series of DEQAS samples and determine the relationship of each metabolite to 25-OHD₃.
- 2) To calculate the cross reactivity of 24R,25(OH)₂D₃ and 24S,25(OH)₂D₃ in commonly used ligand binding assays for 25-OHD and the degree of interference in HPLC/UV and LC-MS/MS methods.

2. Materials and Methods

2.1 Assignment of values to DEQAS samples

The concentrations of 25-OHD₃ and 25-OHD₂ were determined by NIST using their LC-MS/MS Reference Measurement Procedure (RMP) (1). A non-RMP, but similar method was employed for 3-epi-25OHD₃ determination. The concentrations of 24,25(OH)₂D₃ were determined by LC- MS/MS using the method of Kaufmann et al. (4).

2.2 Spiking of pools with 3-epi-25-OHD₃, 24S,25(OH)₂D₃ and 24R,25(OH)₂D₃

Synthetic vitamin D metabolites: 3-epi-25-OHD₃, 24S,25(OH)₂D₃ and 24R,25(OH)₂D₃ were obtained from Sigma- Aldrich (Gillingham, Dorset, UK) and used without further purification. The concentration of ethanol solutions of each metabolite was checked by UV spectrophotometry using a molar absorption coefficient of 18200.

Two serum pools were prepared from blood donated by haemochromatosis or polycythaemic patients. The first pool was divided equally and one half spiked with an ethanol solution of the 3-epi-25-OHD₃ to give a final concentration of 52.4 nmol/L. A similar approach was used for the second pool which was spiked with 24S,25(OH)₂D₃ to give a final concentration 57.9 nmol/L. A third pool was prepared from serum obtained from a commercial source (Solomon Park, Kirkland, WA, USA). This was equally divided and one half spiked with an ethanol solution of 24R,25(OH)₂D₃ to give a final concentration 14.4 nmol/L.

An equal volume of ethanol was added to the unspiked sera; the final concentration of ethanol was <0.1%. All pools were equilibrated overnight by mixing on a magnetic stirrer at room temperature. Samples of each pool were dispatched as part of a normal DEQAS distribution; in October 2011 (3-epi-25-OHD₃), April 2012 (24S,25(OH)₂D₃) and October 2014 (24R,25(OH)₂D₃).

3. Results

3-epi-25-OHD₃ and 24,25(OH)₂D₃ both showed strong positive correlations with 25-OHD₃ (Figs.1 and 2) with the regression lines crossing the 25-OHD axis at approximately 20 and 15 nmol/L respectively.

The Roche Total 25-OHD competitive protein binding assay showed a cross reactivity* of 56% with 3-epi-25-OHD₃ but no significant interference was observed in any of the immunoassays. In general, HPLC/UV and LC-MS/MS methods did not resolve the 3-epimer from 25-OHD₃ (Fig.3).

In samples spiked with exogenous 24,25(OH)₂D₃, cross reactivity* of 24R,25(OH)₂D₃ (24S,25(OH)₂D₃) in ligand binding assays ranged from <5% (<5%) to 548% (643%). Both 24-hydroxylated metabolites were easily resolved from 25-OHD₃ by HPLC/UV and LC-MS/MS methods (Fig.4).

Cross reactivity (CR) of the 3-epimer and 24,25(OH)₂D₃ was calculated using the formula $\% \text{ CR} = \{ (S - U)/C \} \times 100$ where S = observed 25-OHD concentration in spiked serum pools, U = 25-OHD concentration in unspiked pool and C= concentration of added metabolite. Example: For a serum pool with a total 25-OHD concentration of 64.7 nmol/L (U), the addition of 14.4 nmol/L of 24R,25(OH)₂D₃ (C) increased the apparent concentration of 25-OHD to 118.3 nmol/L (S). Cross reactivity (CR) = $\{ (118.3 - 64.7)/14.4 \} \times 100 = 372.2\%$.

Fig.1 Relationship between 3-epi-25-OHD₃ and 25-OHD₃ in a series of DEQAS samples distributed between October 2012 and October 2014.

Fig.2 Relationship between 24,25(OH)₂D₃ and 25-OHD₃ in a series of DEQAS samples distributed between October 2012 and October 2014.

Fig.3 Mean and standard deviation of Total 25-OHD concentrations given by commonly used methods on a sample containing exogenous 3-epi-25OHD₃. (ALTM; All Laboratory Trimmed Mean).

Fig.4 Cross Reactivity of exogenous 24R,25(OH)₂D₃ and 24S,25(OH)₂D₃ in commonly used Ligand Binding assays and their influence on HPLC/UV and LC-MS/MS 25-OHD methods (user numbers in parenthesis refer to the sample containing 24R,25(OH)₂D₃).

Table 1 shows 25-OHD results and %bias of 2 unspiked DEQAS samples (467 and 470) from the same distribution (January 2015). In general, results for sample 470 with its high concentration of 25-OHD showed a greater bias than sample 467, possibly due to the higher concentration of endogenous 24,25(OH)₂D₃; the Abbott Architect and the Roche Total 25-OHD methods showed the biggest difference.

If it were assumed that the positive bias of, say, the Abbott Architect result in the unspiked sample 470 was wholly accounted for by the presence of endogenous 24,25(OH)₂D₃, (11.04 nmol/L), then application of the previously established cross reactivity of exogenous 24R,25(OH)₂D₃ (548%; Fig. 4)) would give a result of 178.8 nmol/L [118.4 + (11.04 x 5.48)], not the observed 145.7 nmol/L. Similar reasoning can be applied to the other 25-OHD methods.

Table 1. Total 25-OHD Concentrations and % Bias (from NIST RMP) of 2 unspiked samples (467 and 470) distributed in January 2015.

* Total 25-OHD concentrations predicted from the measured concentration of endogenous 24,25(OH)₂D₃ (11.04 nmol/L) and the cross reactivity of exogenous 24R,25(OH)₂D₃ (Fig.4).

Sample Number	467		470		470*
Method	nmol/L	%Bias	nmol/L	%Bias	nmol/L (predicted)
Abbott Architect (22)	40.7	-8.3	145.7	23.1	178.8
DiaSorin RIA (25)	44.6	0.5	109.4	-7.6	131.5
DiaSorin Liaison (371)	38.2	-14.0	115.6	-2.4	153.1
IDS RIA (10)	55.3	24.5	138.8	17.2	150.4
IDS EIA (A) (35)	47.8	7.7	137.2	15.9	158.0
IDS Octeia (65)	45.3	2.0	129.7	9.5	154.3
IDS iSYS (137)	49.8	12.2	134.7	13.8	159.5
Roche Total 25OHD (81)	37.8	-14.9	145.5	22.9	129.3
Siemens Advia Centaur (57)	46.9	5.6	118.6	0.2	139.0
HPLC (32)	46.1	3.8	121.1	2.3	
LC-MS/MS (155)	45.3	2.0	121.6	2.7	
NIST Target	44.4		118.4		

4. Discussion

3-epi-25-OHD₃ can be present in very high concentrations in neonates of up to one year of age (≈50% of 25-OHD₃) and the metabolite is now known to be present in most adult sera (5,6,7). In a study of 216 random clinical samples (211 from patients >20 years of age), mean (SD) 3-epi-25-OHD₃ concentration was 3.8 (4.8) nmol/L (range 0.25 – 59.3 nmol/L); 92% of results were below 7.5 nmol/L (7). Many routine HPLC/UV and LC-MS/MS assays fail to resolve the 3-epimer from 25-OHD₃ in the chromatography stage (3) and, because the masses are identical, this leads to an over-estimation of 25-OHD₃ (Fig. 3). Because of the positive correlation between the 3-epi-25-OHD₃ and 25-OHD₃ (Fig. 1), the error will increase with increasing concentration of 25-OHD₃. Of the ligand binding assays, only the Roche competitive protein binding assay showed cross reactivity with the 3-epimer. However, there is evidence that this might be due to the anomalous behaviour of exogenously added metabolite and that no cross reactivity occurs with endogenous 3-epi-25-OHD₃ in neonatal sera (8). The biological importance of the 3-epimer is uncertain but current thinking is that it should not be included in 'total 25-OHD' when this is used to assess vitamin D nutrition (9). It is therefore important that laboratories are aware of the specificity of their assays and that neonatal samples are analyzed by a method specific for 25-OHD.

1 Analysis of 40 DEQAS samples showed a strong positive correlation between
2 24,25(OH)₂D₃ and 25-OHD₃ with the expression of CYP24A1 (24-hydroxylase) being first
3 apparent at a 25-OHD₃ concentration of approximately 15 nmol/L, the point at which the
4 regression line crosses the 25-OHD₃ axis (Fig.2). Similar values have been reported
5 elsewhere (2,4) and are consistent with the physiological necessity of maximizing the
6 conversion of 25-OHD to 1,25(OH)₂D during vitamin D deficiency

7
8 Most 25-OHD immunoassays are known to cross - react with dihydroxylated metabolites of
9 vitamin D but not to the degree suggested by the results of the current investigation.
10 Evidence that the cross reactivity of exogenous 24,25(OH)₂D is spuriously high is provided
11 by results for sample 470 which contained only endogenous 24,25(OH)₂D (11.04 nmol/L);
12 with the exception of the Roche assay, measured total 25-OHD concentrations were lower
13 than would have been predicted from the cross reactivity of exogenous 24R,25(OH)₂D₃
14 (table 1). This appears to be another example of the anomalous behaviour of exogenously
15 added vitamin D metabolites, first observed with 25-OHD and so far unexplained (10).
16 Nevertheless, interference by 24,25(OH)₂D₃ in some methods is thought to contribute to the
17 greater positive (or less negative) bias found in samples containing high concentrations of
18 25-OHD, as exemplified by the results of sample 470 (table 1).

19
20 In summary; HPLC/UV and LC-MS/MS methods for 25-OHD may not resolve 3-epi-25-
21 OHD₃ from 25-OHD₃ and that can lead to an over-estimation of 25-OHD in these assays.
22 Immunoassays for 25-OHD do not show interference by 3-epi-25-OHD₃ and the apparent
23 cross reactivity of this metabolite in the Roche competitive protein binding assay may be an
24 anomaly.

25 Expression of CYP24A1 occurs at a 25-OHD concentration of about 15 nmol/L; above this
26 the cross reactivity of 24,25(OH)₂D₃ in some immunoassays is likely to inflate 25-OHD
27 results but by substantially less than indicated by spiking experiments.

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