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Variability in the analysis of 25-hydroxyvitamin D by liquid chromatography–tandem mass spectrometry: The devil is in the detail

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ABSTRACT

Background: Liquid chromatography–tandem mass spectrometry (LC–MS/MS) is increasingly used in clinical laboratories for the analysis of 25-hydroxyvitamin D (25OHD), but measurement is not straightforward. Importantly, LC–MS/MS is not a single technique: variables in sample preparation, chromatography and ionisation/fragmentation should each be considered.
Methods: We analysed results from a survey organised by the international Vitamin D External Quality Assessment Scheme (DEQAS), to determine the influence of such variables on the results for two DEQAS distributions.
Results: 65 laboratories returned questionnaires. 346 (57%) individual results were from laboratories using electrospray ionisation (ESI), and 259 (43%) from laboratories using atmospheric pressure chemical ionisation (APCI). Although the mean ratio of results was not significantly different between ESI and APCI ($P = 0.5828$), there was greater variation ($P < 0.0001$) in results obtained by laboratories using ESI. Greater variation ($P < 0.05$) was also observed between results from laboratories monitoring non-specific water-loss transitions. Only 3 laboratories (5%) could resolve the isobaric metabolite 3-*epi*-25OHD$_3$ from 25OHD$_3$.
Conclusions: There are many variables to consider when using LC–MS/MS, including assay standardisation/calibration, chromatography and MS conditions. MS/MS alone cannot distinguish isobaric metabolites such as 3-*epi*-25OHD$_3$. Interference can also occur if non-specific transitions are used. Laboratories should always subscribe to an EQA scheme for 25OHD analysis.

1. Introduction

'Vitamin D' is a term used to describe a *group* of seco-steroid compounds, the most important of which are cholecalciferol (vitamin D$_3$) and ergocalciferol (vitamin D$_2$). Often, the term is incorrectly used to incorporate a number of related vitamin D metabolites, including 25-hydroxyvitamin D (25OHD) and the active hormone 1α,25-dihydroxyvitamin D amongst others. Measurement of total serum 25OHD (i.e. 25-hydroxyvitamin D$_2$ and 25-hydroxyvitamin D$_3$ — 25OHD$_2$, 25OHD$_3$) is universally considered a reliable and robust marker of vitamin D status and for monitoring supplementation in vitamin D deficient subjects, since this concentration reflects both dietary and/or supplementary intake and dermal production [1].

It is known that severe deficiency (total serum 25OHD < 10 ng/mL) leads to rickets in children and osteomalacia in adults [2]. The concentrations of total 25OHD that relate to adequacy and sufficiency are less well defined, and are a subject of ongoing debate, although current opinion is that the optimum concentration of 25OHD$_3$ should be greater than 30 ng/mL [3,4]. Vitamin D status has been the focus of much recent literature attention and has now been linked to a range of pathologies, including heart disease, hypertension, diabetes, cancer and autoimmune diseases [5]. As a consequence, there has been a dramatic increase in the number of requests for the measurement of serum 25OHD [6]. In order to meet demands, many laboratories considered high-throughput, automated immunoassays to replace more laborious solvent extraction methods, such as the initial competitive protein binding assay reported by Haddad and Chyu [7]. However, method differences between laboratories soon became apparent [8–12], as was shown by the results of the Vitamin D External Quality Assessment Scheme (DEQAS), which was established in 1989. Immunoassays for 25OHD have an inherent problem of inability to differentiate between a myriad of polar metabolites and vitamin D-like seco-steroids which contribute to the

Abbreviations: 25OHD, 25-hydroxyvitamin D; 25OHD$_2$, 25-hydroxyvitamin D$_2$; 25OHD$_3$, 25-hydroxyvitamin D$_3$; DEQAS, Vitamin D External Quality Assessment Scheme; LC–MS/MS, liquid chromatography–tandem mass spectrometry; *m/z*, mass-to-charge ratio; ALTM, all laboratory trimmed mean; CV, coefficient of variation; ESI, electrospray ionisation; APCI, atmospheric pressure chemical ionisation; APPI, atmospheric pressure photoionisation; PTAD, 4-phenyl-1,2,4-triazoline-3,5-dione; Q1, first quadrupole (in triple quadrupole LC–MS/MS instruments); SRM, selected reaction monitoring; MRM, multiple reaction monitoring; PFP, pentafluorophenyl; HPLC, high-performance liquid chromatography.

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total 25OHD assay. In addition, analytical methodology details, including assay calibration, are not always disclosed because of commercial sensitivities.

There has also been a move in the last decade towards clinical laboratories using liquid chromatography–tandem mass spectrometry (LC–MS/MS) for a number of analyses which were previously performed using immunometric techniques [13]. LC–MS/MS can distinguish and independently quantify, for example, $25OHD_2$ from $25OHD_3$ by mass-to-charge ratio (m/z) alone. Since the first reported use of LC–MS/MS for the analysis of 25OHD by Watson et al. [14], improvements in the automation of sample preparation, the speed of chromatographic steps, and the sensitivity of MS instrumentation have meant that LC–MS/MS assays with higher sample throughput are increasingly being adopted, and the technique now accounts for over 10% of all DEQAS returns (October 2010). LC–MS/MS is considered by some the future 'gold standard' for analysis of 25OHD [15–18].

However, LC–MS/MS for the analysis of serum 25OHD is not straightforward. Initially, inter-laboratory (% CV) agreement between LC–MS/MS users was poor. Some of the variation could be explained by the use of 'in-house' calibration standards, the preparation of which varied between laboratories. Indeed, in a DEQAS study reported by Carter and Jones [19], it was found that use of a common standard (from Chromsystems® — now traceable to the National Institute of Standards and Technology reference material, NIST SRM 972) improved the mean inter-laboratory imprecision for total 25OHD from 16.4% to 10.4%. Better insight into the importance of assay standardisation and other factors affecting LC–MS/MS methods, such as the tubes used for sample collection and preparation [6,20] and interference from other vitamin D metabolites has further improved inter-laboratory agreement, but variability between laboratories does still exist and requires investigation. Importantly, LC–MS/MS is not a single, 'off-the-shelf' technique: variability in sample preparation, chromatographic separation and finally ionisation/fragmentation should each be considered. That said, the flexibility of LC–MS/MS systems may be beneficial, since there is the opportunity to adapt, individualise and standardise methods.

We analysed the results from a survey of LC–MS/MS users organised by the international Vitamin D External Quality Assessment Scheme, to determine the influence of such variables on the reported results for two DEQAS distributions (10 samples, July and October 2010).

2. Materials and methods

2.1. Questionnaires

Questionnaires designed and organised by DEQAS were distributed in September 2010 to all DEQAS participants reporting results using LC–MS/MS. Laboratories were asked to voluntarily complete the questions, and identify themselves by DEQAS laboratory number when returning results. The questions asked were as follows:

- Which LC–MS instrument manufacturer/model do you use?
- Which LC system/column(s) do you use?
- Which method of sample preparation do you use?
- Do you use a commercially available assay kit?
- Do you use commercially available standards to calibrate your assay? If so, is this standard used as the primary standard in each batch analysis?
- Which internal standard(s) do you use?
- Which ionisation method do you use?
- Which m/z transition(s) do you use for 25-hydroxyvitamin D_2/D_3 and internal standards?
- Are chromatograms/results visually inspected prior to reporting?
- Does your method resolve 3-*epi*-25-hydroxyvitamin D_3 from 25-hydroxyvitamin D_3?

2.2. Statistical analysis

The responses were collated, along with individual laboratory returns for two DEQAS distributions (10 samples, July and October, 2010). For statistical analysis, individual results for each of the 10 samples were divided by the corresponding LC–MS method mean concentration for that sample. It was assumed that no changes were made to analytical methods used between the time of completing the questionnaires and analysis of the two sample distributions (i.e. the answers submitted for each question were applied to all 10 reported results for further analysis). Statistical analysis and production of box and whisker plots were carried out using GraphPad Prism for Windows (version 5.04). All calculations were carried out using reported results for total 25OHD. All box and whisker plots are presented showing the median, interquartile range, and 5th and 95th percentile data with individually plotted outliers.

2.3. DEQAS samples

Details of the scheme have been described previously [10], and details are available on the DEQAS website (http://www.deqas.org). Five liquid samples of human serum are distributed quarterly at ambient temperature. Returned results are statistically analysed using the method described by Healy [21].

3. Results

3.1. LC–MS vs. all methods

The results for the 10 samples (sample numbers 376–385, July and October 2010) are summarised in Table 1. Shown are the all

Table 1
Summary results for DEQAS Samples 376–385.

Sample no. [total no. of reports from all methods (number LC–MS)]	All methods			LC–MS		
	ALTM [nmol/L, (ng/mL)]	SD [nmol/L, (ng/mL)]	CV (%)	Method mean [nmol/L (ng/mL)]	SD [nmol/L, ng/mL)]	CV (%)
376 [815 (94)]	47.0 (18.8)	8.1 (3.2)	17.2	58.8 (23.5)	7.6 (3.1)	13.0
377 [795 (91)]	19.3 (7.7)	5.0 (2.0)	26.1	17.9 (7.2)	2.6 (1.1)	14.8
378 [815 (94)]	68.8 (27.5)	12.9 (5.2)	18.7	82.2 (32.9)	9.2 (3.7)	11.2
379 [812 (93)]	45.1 (18.0)	5.8 (2.3)	13.0	44.6 (17.8)	4.4 (1.7)	9.8
380 [814 (94)]	60.6 (24.3)	8.2 (3.3)	13.5	64.5 (25.8)	6.8 (2.7)	10.6
381 [875 (98)]	85.2 (34.1)	13.0 (5.2)	15.2	100.8 (40.3)	12.7 (5.1)	12.6
382 [875 (98)]	37.8 (15.1)	5.5 (2.2)	14.6	40.2 (16.1)	5.3 (2.1)	13.1
383 [874 (98)]	73.1 (29.2)	10.4 (4.2)	14.2	84.3 (33.7)	9.0 (3.6)	10.7
384 [873 (98)]	28.7 (11.5)	4.1 (1.6)	14.3	29.6 (11.8)	3.9 (1.5)	13.0
385 [874 (97)]	56.3 (22.5)	8.0 (3.2)	14.2	66.1 (26.4)	7.4 (3.0)	11.2