

RESEARCH ARTICLE

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Method for simultaneous analysis of eight analogues of vitamin D using liquid chromatography tandem mass spectrometry

Iltaf Shah¹, Andrea Petroczi¹ and Declan P Naughton^{1,2*}

Abstract

Background: Despite considerable global investigation over several decades, the roles of vitamin D in health and disease development remains convoluted. One recognised issue is the difficulty of accurately measuring the active forms of vitamin D. Advances made include some new methods addressing the potential interference by excluding epimers and isobars. However, there is no evidence that epimers are without function. Therefore, the aim of this study was to develop and validate, for the first time, a new assay to simultaneously measure levels of six forms of vitamin D along with two epimers. The assay was applied to multilevel certified reference material (CRM) and 25 pooled human sera samples, obtained from the Vitamin D External Quality Assessment Scheme (DEQAS), to demonstrate its efficiency.

Results: The assay is capable of simultaneously measuring eight vitamin D analogues over the calibration ranges and LODs (in nmol/L) of: 1 α 25(OH)₂D₂ [0.015-1; 0.01], 1 α 25(OH)₂D₃ [0.1-100; 0.01], 25OHD₃ [0.5-100, 0.025], 3-epi-25OHD₃ [0.1-100, 0.05], 25OHD₂ [0.5-100, 0.025], 3-epi-25OHD₂ [0.1-100, 0.05], vitamin D₃ [0.5-100, 0.05] and vitamin D₂ [0.5-100, 0.05], using stanozolol-d₃ as internal standard. Certified reference material and external quality control samples (DEQAS) were analysed to meet the standards outlined by National Institute of Standards and Technology (NIST). Validation steps included recovery and both precision and accuracy under inter- and intra-day variation limit of detection, and analysis of each analyte over a linear range. All validation parameters were in line with acceptable Food and Drug Administration (FDA) guidelines. All eight analogues were quantified with the 25OHD levels being commensurate with DEQAS data.

Conclusions: This report details the application of a new LC-MS/MS based assay for the efficient analysis of eight analogues of vitamin D over a range of samples, which is a significant advance over the existing methods. Simultaneous measure of eight vitamin D analogues does not compromise the analytical capability of the assay to quantify the commonly used biomarker (25OHD) for vitamin D status. The results demonstrate the feasibility of applying the assay in research and clinical practice that i) excludes misleading measures owing to epimers and isobars and ii) is able to quantify the excluded component to facilitate further in vivo investigation into the roles of ubiquitous epimers.

Keywords: Vitamin D, Epimer, Analyses, LC-MS/MS, 25-hydroxyvitamin-D₃, 25-hydroxyvitamin-D₂, 3-epi-25OHD₃, 3-epi-25OHD₂

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Background

Many studies have demonstrated that hypovitaminosis D is widespread through the Western world leading to postulations that it is associated with the development and/or progression of a number of conditions ranging from neurological disorders through to hypertension, type 1 diabetes, kidney disease and cystic fibrosis [1]. In consequence, many supplemental studies have been conducted with very few successfully altering disease outcomes [1]. Beyond rickets disease, clinical research on the putative roles of vitamin D in health and disease has proven difficult for a number of reasons including: i) the vitamin exists in multiple forms that are not facile to measure accurately, with considerable questions about several methods [2], ii) sources are varied with sunlight generating the primary form of vitamin D₃ in the skin and both vitamin D₃ and vitamin D₂ are available through diet, making supplemental studies very difficult to control, and iii) although studies are conducted globally, there is no worldwide consensus on key quantitative measures of deficiency, insufficiency and sufficiency of vitamin D in blood samples [1,3].

Vitamin D exists in several forms ranging from the dietary derived D₂ and photo-activated D₃ analogues, although a fraction of vitamin D₃ also arises from the diet. Hydroxylation in the liver affords the circulating 25-hydroxyvitamin-D₃ (25OHD₃) and 25-hydroxyvitamin-D₂ (25OHD₂) forms, frequently measured collectively as 25OHD. Additional metabolism in the kidney generates the active metabolites 1- α ,25-dihydroxyvitamin-D₃, (1 α 25(OH)₂D₃) and 1- α ,25-dihydroxyvitamin-D₂ (1 α 25(OH)₂D₂) along with the minor metabolite 24,25(OH)₂D₃ [4-6]. Recent efforts have resulted in significant improvements in the understanding of limitation and strengths of various assays for vitamin D forms [3,7-10].

Further confounding factors arise as recent studies have revealed the presence of the C-epimer form of 25OHD₃ in adult blood samples [11], which was subsequently quantified in further studies [3,12,13]. The role of vitamin D epimers is yet to be elucidated but their presence is likely to have an associated function [14]. Given that the epimers can hamper current assays by cross-reacting, and that they may have biological functions, it is timely to develop an accurate method to analyse all forms of vitamin D, including accurate levels of the epimers. Similar efforts to date resulted in assays quantifying up to five analogues in various combinations [15-18] but none included the primary forms of vitamin D, 3-epi-25OHD₂ or 1 α 25(OH)₂D₂.

This paucity of advances, despite the existence of numerous reports, led us to develop a new assay for the simultaneous analysis of eight key analogues of vitamin D including two epimers. The aim of this paper is to report the validated assay along with its application to

reference samples to quantify the levels of various analogues of vitamin D.

Materials and methods

Samples

Samples were obtained from the Vitamin D External Quality Assessment Scheme (DEQAS). All the samples are pooled sera prepared from donations from patients undergoing therapeutic venesection for haemochromatosis and occasionally polycythaemia.

Standards and reagents

All chemicals and reagents were of high purity grade. Stanazolol-D₃ (internal standard) and 7- α -hydroxy-4-cholesten-3-one (7 α C₄) were obtained from LGC standards (Teddington, UK). Vitamin D₃, vitamin D₂, 25OHD₃, 25OHD₂, 1 α 25(OH)₂D₃, 1 α 25(OH)₂D₂, 3-epi-25OHD₃, 3-epi-25OHD₂, isopropanol, dichloromethane, methanol, hexane, deionised water, formic acid, ammonium hydroxide, acetonitrile, ether and hexane were purchased from Sigma Aldrich (Poole, UK).

Preparation of standards and samples

Methanolic stock solutions of all analytes were prepared at a concentration of 1 mg/mL and stored in amber glass vials at -80°C in the dark. All methanolic working solutions of analytes and the internal standard were prepared by serial dilution of stock solutions. A standard working solution mixture of analytes and the standalone internal standard was prepared at a concentration of 1 μ g/mL.

Sample pre-treatment

Sera samples (1 mL) were thawed equilibrated at room temperature for 15 minutes and vortex mixed. Except for the blank, 25 μ L of working solution of stanazolol-d₃ (internal standard) was added to all samples. 50 μ L of 2 M formic acid were added, followed by 3 mL of isopropanol/methanol/ (1:1, v/v) mixture and vortex mixed to release the protein bound analyte and to promote protein precipitation during a 15 minutes incubation at 4°C. The resulting mixture was centrifuged at 3500 g for 5 minutes at 4°C to remove the suspended matter, with supernatants transferred to clean amber glass tubes for liquid-liquid extraction.

Liquid-liquid extraction (LLE)

A 3 mL dichloromethane/hexane/(1:1, v/v) mixture was added to the remaining solution after protein precipitation and the solution was vortex mixed for 1 minute and then centrifuged at 3500 g for 5 minutes at 4°C. The upper clear layer was transferred to a new set of glass tubes with a further two extractions conducted on the residual lower layer. The extracts were pooled and dried under a gentle stream of nitrogen at room temperature

prior to reconstitution in 200 μ L of LC-MS grade methanol/water (1:1, v/v) solution.

LC-MS/MS procedure

A Sciex API-3000 LC-MS/MS instrument (Applied biosystems division of MDS health group Ltd, Canada) was used for analysis together with an autosampler (PAL-CTC Analytics, Switzerland), and turbomolecular pump (1100 series, Agilent Technologies, USA). Analyst software version 1.4.2 (AB Sciex) was used for data analyses. A 10 μ L aliquot of the sample was injected into the LC-MS/MS system for analysis in low light conditions. An Ultrason ES-OVM chiral column (2 mm \times 150 mm, 5 μ m) was used in tandem with an Agilent rapid resolution microbore Zorbax SB-C18 RRHD column (2.1 mm \times 100 mm, 1.8 μ m) with corresponding inlet filters for guarding the columns.

The column oven temperature was maintained at 40°C. However, the column switching programme was set in a way so that the column temperature switch from right to left after 8 minutes and then switch back to right after 13 minutes with a temperature tolerance of $\pm 1^\circ\text{C}$. The use of switching temperatures technique resulted in good separation, better peak shape and reproducibility. Various mobile phases and differing gradient compositions were explored for optimal results with the final selected parameters as follows. Mobile phases A consisted of acetonitrile (100%) with 0.3% (v/v) formic

acid and B containing 0.1% (v/v) formic acid and ammonium acetate (2 mM) with a flow rate in all steps at 0.2 mL/min. The gradient program was as follows: initial: 35% B; 0–4.0 min: a gradient change to 98% A; from 4.0–10 min: kept at 98% A; from 10–14 min: reversion of the mobile phase to 35% B; 14.1–20.0 min; 35% B.

The mass spectrometer was auto-equilibrated for 0.5 min. The channel electron multiplier (CEM) detector was set to 3290 eV with a deflection of 400. The mass spectrometer parameters like deflection potential, focusing potential, energy potential and collision cell exit potential was optimised for each analyte. The mass spectrometer was set to run three periods sequentially with optimised conditions for selected analytes. The total run time for mass spectrometer was 20 minutes. First period was run for 8 minutes with optimum conditions for $1\alpha 25(\text{OH})_2\text{D}_3$ and $1\alpha 25(\text{OH})_2\text{D}_2$ and stanzolol-d3 internal standard followed by second period for 5 minutes with optimised conditions for 25OHD3, 25OHD2, 3-epi-25OHD3, 3-epi-25OHD2 and $7\alpha\text{C}_4$ and a 3rd period was employed for a final 7 minutes for determination of vitamin D3 and vitamin D2. The mass spectrometer was operated in positive electrospray ionisation (ESI) mode at an average spray voltage of 4500 V and average capillary temperature of 450°C between the three periods. The generated protonated molecules of all analytes were used as precursor ions for collision activated dissociation (CAD) into product ions in MS-MS analysis.

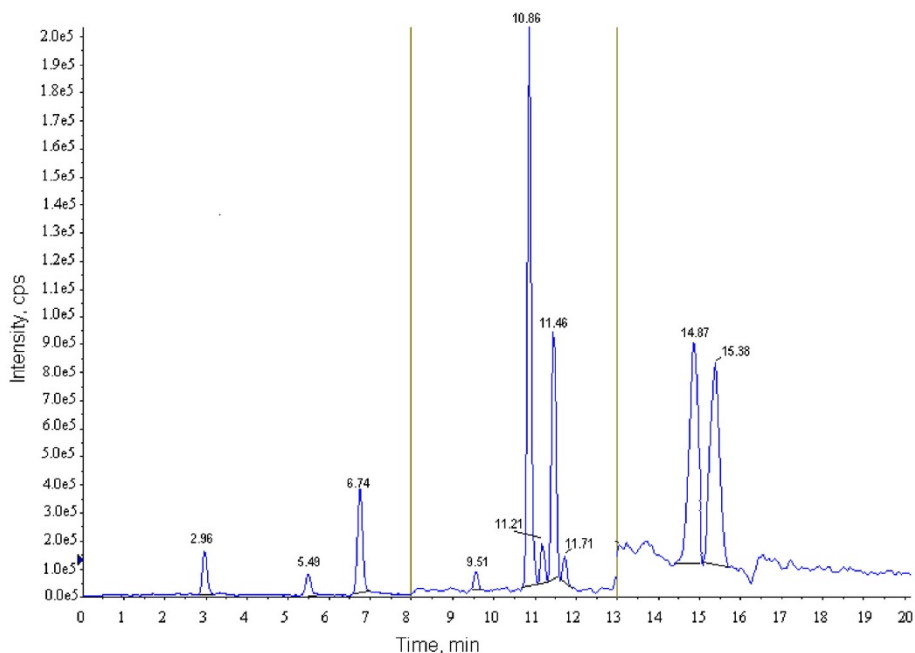


Figure 1 Typical chromatogram showing vitamin D analogues [1ST period analytes: Stanzolol-d3-RT = 2.96, $1\alpha 25(\text{OH})_2\text{D}_2$ -RT = 5.49, $1\alpha 25(\text{OH})_2\text{D}_3$ -RT = 6.74; 2nd period analytes: $7\alpha\text{C}_4$ -RT = 9.51, 25OHD3-RT = 10.86, 3-epi-25OHD3-RT = 11.21, 25OHD2-RT = 11.46 and 3-epi-25OHD2-RT = 11.71; 3rd period analytes: vitamin D3-RT = 14.87 and vitamin D2-RT = 15.38].

Data analysis

Results from the new assay was compared to the DEQAS mean using Bland–Altman plot to allow showing discrepancies for individual samples. Coefficient of variation (CV) was calculated as standard deviation/mean and expressed as percentage. Data analysis was performed using Microsoft Excel 2007.

Results and discussion

Method validation

A typical chromatogram is shown in Figure 1 illustrating the separation of eight vitamin D analogues plus the internal standard (stanozolol-d3) and isobaric component 7- α -hydroxy-4-cholesten-3-one (7 α C4) in a spiked extracted serum sample. The calibration curves and quality controls were prepared in vitamin d free phosphate-buffered saline with 60 g/L human serum albumin [19]. The sample pre-treatment and extraction

method was followed with minor changes [19]. The accuracy of the assay was determined as the ratio of the compound added to the measured (mean value/nominal value) \times 100. The extraction efficiency of the compounds was determined by spiking blank serum samples prepared in human serum albumin. Absolute recoveries were determined by comparing the concentration of the detector response obtained from peak heights ratios of extracted serum samples with actually added concentrations of un-extracted standard response for the detector. Relative recoveries were determined by comparing the % of drug recovered from serum matrix against detector response for extracted standards. Three different concentrations of quality controls were utilised in this assay.

To check for the assay variations for 25OHD3 and 25OHD2, a multilevel certified reference material (CRM) serum calibrator set (Chromsystems, Germany) was also used (based on human serum) and handled in the same

Table 1 Validation results for vitamin D assay

Analytes in (nmol/L)	Concn. QC's	Linear range	LOD	r ²	Intra-day		Inter-day	
					Precision % CV	Accuracy%	Precision % CV	Accuracy%
Vitamin D3	20	0.5-100	0.05	0.9997	4.13	87.1	5.32	89.8
	40				2.86	99.4	2.33	89.4
	80				1.31	98.9	2.89	90.2
Vitamin D2	20	0.5-100	0.05	0.9996	6.3	99.2	11.3	93.2
	40				11.3	97.5	13.3	94.8
	80				9.4	89.4	9.6	89.3
25OHD3	20	0.5-100	0.025	0.9999	6.1	99.9	7.4	98.1
	40				2.7	97.3	3.7	99.8
	80				3.5	99.1	2.9	99.2
25OHD2	20	0.5-100	0.025	0.9998	2.8	99.8	3.4	101.2
	40				2.7	99.9	3.9	98.9
	80				3.3	88.7	3.8	89.3
1 α 25(OH) ₂ D3	0.2	0.015-1	0.010	0.9998	4.4	89.2	5.6	88.5
	0.4				4.2	86.4	4.4	87.3
	0.8				3.6	99.7	2.6	88.9
1 α 25(OH) ₂ D2	0.2	0.015-1	0.010	0.9998	3.2	98.9	5.2	89.8
	0.4				4.4	99.4	6.3	98.9
	0.8				3.9	99.9	4.3	87.8
3-epi-25OHD3	20	0.1-100	0.05	0.9997	3.5	89.2	4.3	98.5
	40				3.1	87.3	10.9	87.6
	80				3.3	89.1	8.9	99.9
3-epi-25OHD2	20	0.1-100	0.05	0.9998	7.1	85.8	3.5	88.5
	40				4.9	84.6	2.7	87.1
	80				3.2	89.9	2.2	88.3
7 α C4	20	0.5-100	0.1	0.9999	3.2	98.3	5.4	86.2
	40				2.3	88.6	3.1	85.3
	80				4.1	79.9	3.1	88.1

Table 2 MRM transitions of vitamin D analogues and internal standard

Compounds	Precursor	Product	CE
Vitamin D3	385.3	159.2	40
Vitamin D2	397.4	159.2	43
25OHD3	401.3	383.1	41
	401.3	365.1	41
	401.3	159.2	45
25OHD2	413.3	377.2	51
	413.3	355.5	51
	413.3	383.1	35
1 α 25(OH) ₂ D3	399.1	135.1	61
	399.1	381.1	41
1 α 25(OH) ₂ D2	411.5	151.1	55
	401.3	383.1	41
3-epi-25OHD3	401.3	383.1	41
	401.3	365.1	41
	401.3	159.2	45
3-epi-25OHD2	413.3	377.2	51
	413.3	355.5	51
	413.3	383.1	35
7 α C4	401.3	383.1	41
	401.3	365.1	41
	401.3	159.2	45
Stanozolol-D3	332.2	81.2	45

manner as volunteer specimens [3]. The CRM calibrators were analysed along with routine samples to meet the standards outlined by National Institute of Standards and Technology (NIST). External quality control samples from the International Vitamin D External Quality Assessment Scheme (DEQAS) were analysed for comparison with other participating laboratories already using LC-MS/MS for 25OHD3 and 25OHD2 analysis.

Validation results

Intra-day and inter-day precision and accuracy are shown in Table 1. The absolute recoveries of the analytes range from 88.2 to 97.8% and internal standard 89.6%. Relative recoveries for analytes ranged from 89.8 to 98.2% and internal standard 89.9%. The r^2 in Table 1 represents linear regression, where all the results are presented in units of nmol/L.

Under- or over-estimation of actual 25OHD3 concentrations may occur owing to co-eluting epimers (e.g. 3-epi-25OHD3) and isobars (e.g. 7 α C4). The endogenous bile acid precursor 7 α -hydroxy-4-cholestene-3-one (7 α C4) may cause isobaric inference in MS assays [3]. The use of high resolution microbore tandem column technology not only chromatographically separated all isobars and epimers from 25OHD co-eluting peaks but also facilitated accurate and sensitive determination of the analyte ions by sequential application of three

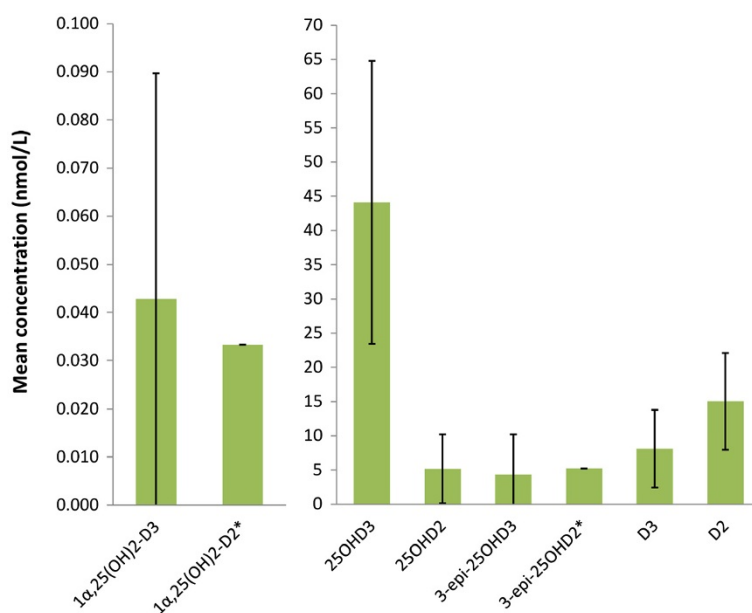


Figure 2 Analytical data for DEQAS samples using the new assay. * indicates that single value is presented as no mean value is available only one positive sample was recorded. The error bars represent standard deviations for the 25 samples.

periods during an MS/MS run. The protonated molecules, $[M + H]^+$, the precursor ions and the diagnostic product ions were monitored in the multiple reactions monitoring (MRM) mode. The MRM parameters were optimised for each analyte using a direct infusion of 0.1 $\mu\text{g/mL}$ individual solutions and of standard compounds mixture. The MRM sequence consisted of three periods executed sequentially to monitor different transition pairs using parameters optimised for each period. The most abundant MRM transitions for each analyte were acquired using the conditions given in Table 2.

Profiles of vitamin D analogues

The results from the pooled DEQAS samples are given in Figure 2 revealing that all analogues were quantifiable. As expected, the highest level was recorded for 25OHD3 with 25OHD2 present at a considerable level in all samples. The C-3 epimer of 25OHD3 and $1\alpha,25(\text{OH})_2\text{D}_2$ were quantified in most samples, whereas the $1\alpha,25(\text{OH})_2\text{D}_2$ D2 and 3-epi-25OHD2 were only present in one sample (410), which contained a significant amount of endogenous 25OHD2. It should be noted, however, that these are pooled samples from donors undergoing treatment and thus are used for the purposes of assay comparisons.

The Bland-Altman and correlation plots (Figure 3) show the level of agreement and relationship between the new method and the All Laboratory Trimmed Mean (ALTM) data provided with the DEQAS sample. The analytical data 25OHD for the individual samples are presented in Figure 4 with detailed results given in Additional file 1. As the Bland-Altman plot showed no proportional bias ($r^2 = 0.0341$, $p = 0.376$), the classic 96% confidence interval approach was applied using the Bland-Altman formula (mean difference $\pm 1.96\text{SD}$) to set the limits of agreement. Of the total 25 samples, 15 were within the limits of agreement by Bland-Altman limit of agreement (Figure 3A); and 18 were within the DEQAS CV = 30.0 limit (Figure 4). Generally, the 25OHD concentration level measures falling outside the Bland-Altman and DEQAS limits were recorded at a lower level than the DEQAS' ALTM means and confidence intervals. A strong positive relationship was evidenced between the new assay and the ALTM ($r = 0.72$, $p < 0.001$). Our data were within the limit of tolerance of the subset of 5 pooled DEQAS samples (for samples 396–400) which were analysed by the Centers for Disease Control and Prevention (Atlanta) with known 3-epi-25OHD3 levels.

Our results for the serum pools averaged -11.8% from the overall DEQAS-ALTM for the participating laboratories

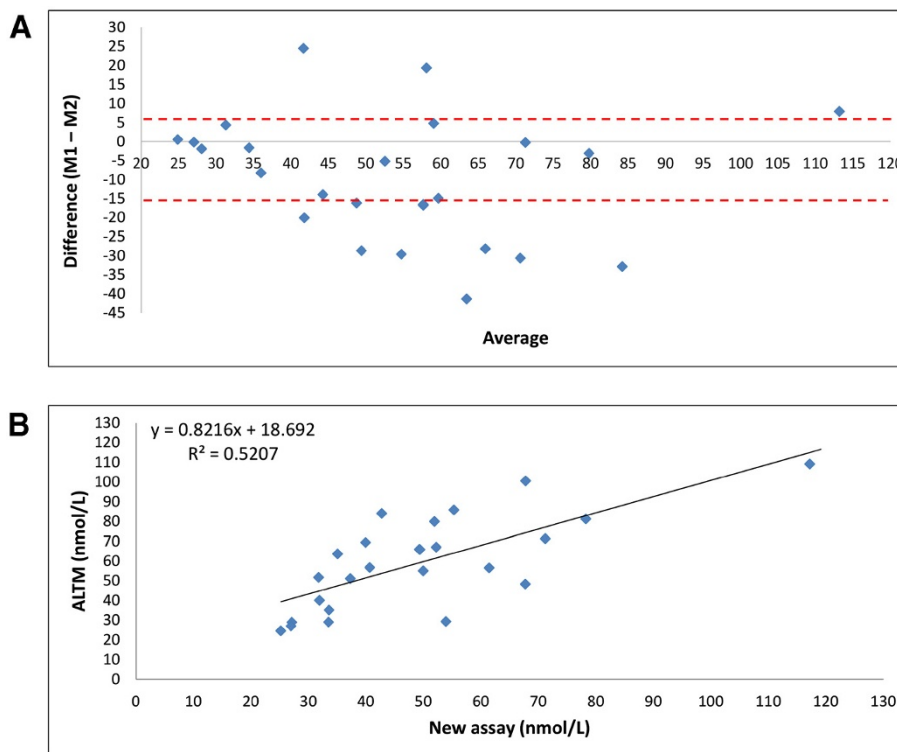


Figure 3 A: Bland-Altman plot comparing the 25OHD concentrations obtained with the new assay (M1) to DEQAS. Lines represent limits of agreement. The mean difference is -5.329 , the lower limit line is at -15.774 and the upper limit is at 5.116 . **B:** Correlation between the 25OHD concentrations obtained using the new assay and the ALTM.

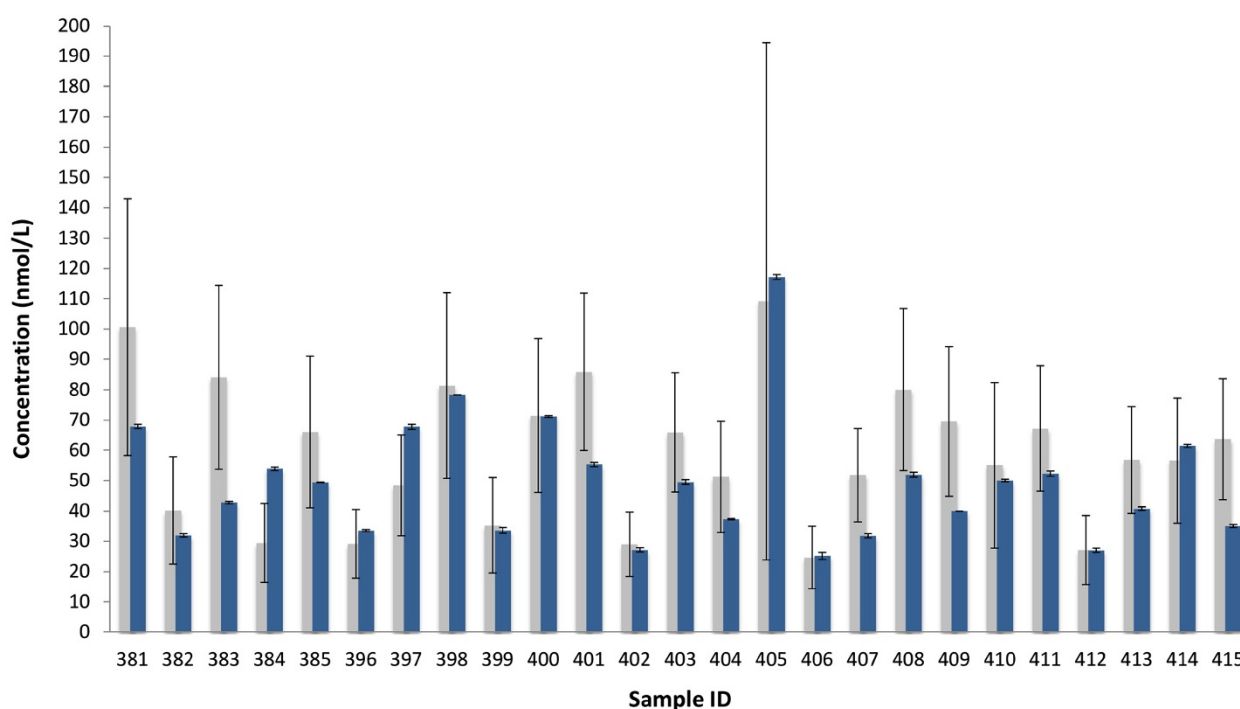


Figure 4 Comparison of 25OHD concentration levels obtained using the new assay (blue) and ALT M (grey). Bars for new assay represent SEM of duplicate measures. Bars on ALT M represent CV=30.0 and denote the lower and upper limit of satisfactory performance [http://www.deqas.org].

using LC-MS/MS methods. However, we did see a diversification in some samples that afforded higher concentrations of the 25OHD. Thus, as seen in Figure 4, five results for the new assay lie below the range of the DEQAS data (383, 401, 407, 409, 415), with two samples being above the DEQAS (384, 397).

Conclusion

In conjunction with the ability to separate $7\alpha C_4$, the assay is capable of distinguishing between every major form of vitamin D reported to date. The results show that the new assay's ability to quantify 25OHD levels is comparable to other LC-MS/MS methods represented in the DEQAS data, thus the simultaneous measures of eight analogues does not compromise its analytical capability for assessing the commonly used biomarker (25OHD) for vitamin D status. In contrast, it adds a significant capability to identify potential issues such as conversion of primary forms to circulating or highly active forms [19]. However, the additional capability of the new assay, namely the ability of not only excluding but also quantifying 3-epimers of D2 and D3, along with the primary forms (D2 and D3), $1,25\alpha(OH)_2D_3$ and $1,25\alpha(OH)_2D_2$, makes this assay instrumental in research and clinical practice where specific and accurate measurement of the different forms is required.

Additional file

Additional file 1: The following additional data are available with the online version of this paper. Additional data file 1 is a table listing the concentrations \pm SEM of the 8 vitamin D forms (nmol/L) in DEQAS pooled samples.

Competing interests

The authors declare that they have no conflicts of interest.

Authors' contributions

DPN and AP initiated the study. The method development, validation and sample analyses were conducted by IS with contributions from DPN. AP conducted the data analyses. All authors contributed to the study design, interpretation of the results and preparation of the manuscript and have read and approved the final version.

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