### Title

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# Utilizing cooled liquid chromatography and chemical derivatization to separate and quantify C3-epimers of 25-hydroxy vitamin D and low abundant 1α,25(OH)$_2$D3: application in a pediatric population

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## Abstract

There is need for a single assay able to quantify the most biologically active metabolite, 1α,25-dihydroxy-vitamin-D3, and the recently discovered biologically distinct C3-epimers of 25OHD, in addition to traditional vitamin D metabolites. We developed a method of chromatographic separation and absolute quantification of the following ten forms of vitamin D: 3-epi-25OHD3, 25OHD3, 3-epi-25OHD2, 25OHD2, 1α,25(OH)$_2$D3, 24R,25(OH)$_2$D3, 23R,25(OH)$_2$D3, 1α,25(OH)$_2$D2, D3, and D2 by single extraction and injection. Chemical derivatization followed by liquid chromatography using a charged surface hybrid C18 column and subsequent tandem mass spectrometry was utilized to detect and quantify each metabolite. This method is remarkable as a cooled column was required to achieve chromatographic resolution of epimers. Validation of each metabolite was performed at four concentrations and revealed inter- and intraday precision and accuracy below 15% across three consecutive days of analysis.

After validation, this method was applied to analyze the blood plasma from 739 samples from 352 subjects (8mo to 20yr), 79 pooled plasma samples, and 10 NIST SRM972a samples. Healthy control samples (n =357) were used to investigate developmentally associated changes in vitamin D metabolite concentrations during early life. This method yields excellent linearity ($R^2 \geq 0.99$) across concentrations encompassing the biological range of many metabolites including 1α,25(OH)$_2$D3. Concentrations of 25OHD2 and 24R,25(OH)$_2$D3 were significantly ($q \leq 0.05$) lower in infants compared to both children and adolescents. The percentage of 3-epi-25OHD3 in

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total 25OHD3 was significantly lower ($q \geq 0.009$) in post-puberty subjects. Here we present a single assay capable of separating and quantifying ten vitamin D metabolites including C3-epimers of 25OHD, and quantifying 1α,25-dihydroxy-vitamin-D3 at and below concentrations observed in human plasma (LLOQ <10 pM).

**Keywords**

1α,25-dihydroxy-vitamin-D3; C3-epimers; LC-MS/MS; Quantitation; Vitamin D; Cooled-chromatography

## 1. Introduction

Increased interest in studying the entire vitamin D metabolism pathway and the emergence of recently discovered, biologically distinct epimers is causing the field of vitamin D analysis to evolve. Historically, the majority of epidemiological studies investigating vitamin D in humans focused solely on circulating levels of 25OHD by radioimmunoassay (RIA) and competitive protein binding. These assays lacked specificity[1], and were known to bind other forms of vitamin D, leading to overestimation of 25OHD levels[2]. Furthermore, these methods either could not bind 3-epi-25OHD3, or could not differentiate 3-epi-25OHD3 from 25OHD3[3]. In the early 2000's liquid chromatography-tandem mass spectrometry (LC-MS/MS) began to replace RIA and competitive binding as the standard in many research laboratories. In the last few years, the discovery of 3-epi-25OHD3 in human blood plasma[4] has forced the development of more sophisticated methodologies able to resolve this biologically relevant epimer[5–8].

Vitamin D comes from two sources; diet and sunlight catalyzed synthesis of D3 in the epidermis. Vitamin D3, and its biologically similar relative, vitamin D2, are rapidly converted in the liver to 25-Hydroxy-vitamin D3 (25OHD3) and 25OHD2 respectively. 25OHD3 and 25OHD2 are the major circulating forms of vitamin D; combined they are the metabolites assayed to determine the clinical vitamin D status of a person[9]. The hormonally active vitamin D metabolite with the greatest biological activity is 1α,25-dihydroxy-vitamin D3 (1α,25$(OH)_2$D3), formed by la-hydroxylase (CYP27B1) catalysis of 25OHD3 (Figure 1). Its metabolic activation and subsequent inactivation, via 24-hydroxylase, is highly regulated and results in circulating levels of 1α,25$(OH)_2$D3 in the low picomolar range making quantification in blood plasma difficult.

In recent years the discovery of the biologically relevant epimers of vitamin D[3–4], characterized by the modification of a hydroxyl group from alpha to beta orientation at C-3 position of the A-ring of vitamin D[3], has led to speculation regarding the role of this class of epimers. The C-3 epimer of 25OHD3, (3-epi-25OHD3), has been observed in blood of healthy infants, children, adolescents, and adults. The discovery of this class of vitamin D metabolites has forced the field of vitamin D quantitation to re-evaluate its methodology[10,11]. New LC-MS/MS methods are specific, accurate, and precise at quantifying 25OHD and its epimers[5], but they are unable to simultaneously detect and quantify the picomolar concentrations of the active 1α,25$(OH)_2$D3 in blood. Chemical derivatization is often performed to increase the signal generated from 1α,25$(OH)_2$D3. 4-

phenyl-1,2,4-triazoline-3,5-dione (PTAD) derivatization is a widely used derivatization agent in vitamin D analysis. PTAD is inexpensive, simple to execute, and yields near complete (>99%) product[12]. However, when previously published methods of separating epimers of 25OHD[5] are applied to PTAD-derivatized vitamin D epimers are not chromatographically separated.

Vitamin D metabolism[9] and its canonical role in bone metabolism is well characterized[9,13]. Increased vitamin D levels correlate with decreased rates of cancer[14–17], type-2-diabetes[18,19], asthma[20], cardiovascular disease[21], multiple sclerosis[22,23], and arthritic diseases[24,25]. Vitamin D is also a potent immune modulator[26,27] affecting a wide range of cells and tissues[9]. The classical role of vitamin D, and particularly 1α,25$(OH)_2$D3, in calcium homeostasis points to the importance of vitamin D in developing humans. However, the majority of studies investigating vitamin D and its effects on human health have been conducted in mature adults. A thorough review, summarizing a number of studies of the C-3 epimers of vitamin D, strongly indicates 3-epi-25OHD3 decreases from infant to pediatric populations[3]. The specific role of 3-epi-25OHD3 is still unknown, but its prevalence in young children and its distinct biological differences from 25OHD3 suggest quantitation of this epimer should be performed in all epidemiological studies. LC-MS/MS has become the gold-standard in quantification of vitamin D metabolites due to its superior selectivity, precision, and accuracy and avoidance of high cross-reactivity[28] suffered by immunoassays. Despite the many advantages of LC-MS/MS, quantification of biologically active 1α,25$(OH)_2$D3 remains difficult due to its very low concentration. There are numerous chemical derivatization techniques[12,29,30] to increase ionization potential of 1α,25$(OH)_2$D3 and other vitamin D metabolites to allow quantitation by LC-MS/MS. There have also been many published LC-MS/MS methods that can separate 25OHD3 from 3-epi-25OHD3[4–7], however, none of these assays have the sensitivity to quantify 1α,25$(OH)_2$D3 at the range found in human blood. Here we present a single assay to quantify 10 vitamin D metabolites, including 1α,25$(OH)_2$D3 (lower limit of quantitation (LLOQ) below biological range) and chromatographic separation of 25OHD and 3-epi-25OHD. We applied this new method to quantify vitamin D metabolites in 739 blood plasma samples from 352 subjects ranging 0 to 20 years of age. The 357 samples from 177 healthy control subjects were then analyzed to investigate the age related changes in vitamin D status across three developmental checkpoints (infancy, childhood, and adolescence). The developmentally related changes observed in this cohort were then further investigated in a subset of subjects where multiple samples were collected spanning at least two age groups, to better understand how vitamin D levels change in healthy children.

## 2. Materials and methods

### 2.1. Chemicals

Reference standards and deuterium labeled internal standards for vitamin D metabolites were purchased from Medical Isotopes Inc. (Pelham, NH). An ACQUITY Charged Surface Hybrid (CSH) C18 (2.1 mm, 150mm, 1.7μm) and 96-well Ostro™ plates (25mg) were purchased from Waters (Milford, MA). Derivatization reagent 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) was purchased from Sigma-Aldrich (St. Louis, MO). 12-

[[(cyclohexylamino)carbonyl]-amino]-dodecanoic acid (CUDA) was purchased from Cayman Chemical (Ann Arbor, MI). Formic acid, ammonium acetate, LC-MS grade water, LC-MS grade methanol, and LC-MS grade acetonitrile were purchased from Fisher Scientific (Hampton, NH). Pooled human plasma collected from at least 10 healthy drug free individuals was purchased from BioIVT (Westbury, NY). Standard reference material (SRM) 972a was purchased from the National Institute of Standards and Technology (NIST) (Gaithersburg, MD).

### 2.2. Preparation of standard solution

A working solution of deuterated internal standards ranging from 20-500 nM was prepared in methanol containing; 1α25$(OH)_2$D3 d6, 1α25$(OH)_2$D2 d6, 24R,25$(OH)_2$D3 d6, 3-epi-25OHD3 d3, 25OHD3 d6, and 25OHD2 d6, of which 10 μL was added to each sample during extraction. Additionally, a 12-point, 4x dilution, calibration curve containing standards for all analytes was constructed, all sample data points were contained within this curve. A 24-point, 2x dilution, calibration curve was built to find the linear range and LLOQ. LLOQ was defined as signal fivefold greater than the blank injection.

### 2.3. Sample collection and ethical approval

Samples were collected, with appropriate investigational review board approval, and analyzed as part of a larger study coordinated by the Diabetes Auto Immunity Study in the Young (DAISY), described at ClinicalTrials.gov (NCT03205865), and in previous publications[31,32]. Pubertal progression was assessed by subject self-report using Tanner staging[33]. Children reporting tanner stage <2 were categorized as pre-pubertal (child grouping) and those reporting stages >2 were categorized as pubertal (adolescent grouping).

### 2.4. Plasma extraction

All sample preparation work was performed in a fume hood equipped with yellow/red spectrum lights and in a room with no natural light to minimize UV degradation of 1α,25$(OH)_2$D3. Samples were kept in amber polypropylene Eppendorf tubes prior to extraction. Samples were thawed on wet ice. Once thawed, 200 μL of sample plasma was transferred into 96-well Ostro™ plates for pass-through phospholipid and protein removal. All subsequent work was done in 96-well plates using a multichannel pipette. Deuterated internal standard mix (10 μL) were added to the samples and allowed to equilibrate at room temperature for 30 min, this equilibration allowed the labeled standards to interact with plasma proteins to account for loss of analyte during sample preparation. 600 μL of acetonitrile with 1% formic acid was added to the wells, samples were aspirated 5 times, and then plates were centrifuged in a Genevac EZ-2 plus centrivap (Ipswich, UK) for 5 min. Plate eluant was dried down in the same centrivap.

### 2.5. Chemical derivatization and resuspension for analysis

Samples were derivatized with the reagent PTAD via Diels-Alder reaction described previously.[12] Briefly, 100 μL of acetonitrile containing PTAD (0.75 mg/mL) was added to each well containing dried blood plasma extract. Plates were vortexed for 5 min at speed 6 with a VWR VX-2500 multi-tube vortexer (Radnor, PA). Sample plates were kept at 4°C

overnight (12+ hours) to ensure complete derivatization. The derivatized extracts were then dried and re-suspended in 60 μL 78% methanol in water, containing 5 ng/mL CUDA (Cayman Chemical, Ann Arbor, MI) as an injection standard. After another 5 min vortex, re-suspended samples were passed through a 0.2 μm PVDF filter (Agilent Tech., Santa Clara, CA) to remove insoluble particles, sealed in a 96-well plate, and stored at 4°C until analyzed. Subject samples were analyzed within one week of derivatization, PTAD derivatized vitamin D metabolites has been shown previously to be stable at 4°C for at least one week[12].

## 2.6. LC-MS MS analysis

An ACQUITY i-class fixed loop ultra-performance liquid chromatography (UPLC; Waters, Milford, MA) was coupled to a Sciex 6500+ QTRAP (Redwood City, CA) mass spectrometer. The mass spectrometer was operated with electrospray ionization in positive ionization mode (ESI+). Multiple reaction monitoring (MRM) mode was used to scan for ten vitamin D metabolites and seven standards. Ion spray voltage was 5500 volts, source temperature was 325°C, and collision gas was set to “medium”. A complete list of transitions, collision energies (CE), declustering potentials (DP), entrance potentials (EP), and cell exit potentials (CXP) can be found in Supplemental Table 1

Separation of vitamin D metabolites was achieved using an ACQUITY C18 CSH (1.7 μm 2.1 mm x 150mm) UPLC column, which was cooled and maintained at 15°C to accomplish separation of C-3 epimers from 25OHD. Equation 1 was used to calculate chromatographic resolution of C-3 epimers, where $t_R$ is the retention time of the apex of the peak and $W$ is the width of the peak.

$$\mathrm{R}_s = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(W_1 + W_2)} \quad \text{Eq. 1}$$

Mobile phase “B” consisted of methanol with 0.3% formic acid, mobile phase “A” was 2 mM ammonium acetate and 0.1% formic acid in water. Flow rate of 0.2 mL/min was maintained throughout analysis time. Initial conditions were 78% mobile phase B for 11 min, from 11 to 12 min the percent B was increased to 98%. Flow rate was increased to 0.4 mL/min for 5 min to clean the column; total run time was 25 min.

## 2.7. Method validation

A 4% bovine serum albumin in phosphate-buffered saline (BSA) solution was used as a blank matrix to test method accuracy and precision. Vitamin D standards were spiked into the BSA solution at four concentrations and allowed to equilibrate for 30 min at room temperature prior to extraction. BSA samples were extracted and analyzed by the same methods described for plasma samples. Five replicates were extracted and analyzed per concentration. This procedure was repeated over three consecutive days to test inter-day precision and accuracy. Percent relative standard deviation (%RSD), a measure of precision, was calculated as shown in Equation 2, where σ is the standard deviation and μ is the mean.

$$\%\text{RSD} = \frac{\sigma}{\mu} * 100\% \quad \text{Eq. 2}$$

Accuracy was calculated as shown in Equation 3,

$$\text{accuracy} = \left[\frac{\#\text{ experimental} - \#\text{ actual}}{\#\text{ actual}}\right] * 100\% \quad \text{Eq. 3}$$

Extraction recovery was originally tested by spiking pooled human plasma with deuterium labeled pure vitamin D standards at five different concentrations, but it was discovered there were low-intensity interfering signals in the pooled plasma obscuring the deuterium labeled standards, specifically at the lowest concentration of 1α,25$(OH)_2$D3 d6. When pooled human plasma was replaced with 4% BSA matrix, we were able to include our non-deuterated standards and test the recovery of all 10 target analytes. Standards were added to six replicates prior to extraction and another six replicates after extraction, at five concentrations per standard. We acknowledge that BSA cannot perfectly mimic human blood plasma, however the use of isotope labeled internal standards allows for sample specific normalization to account for differences interaction between vitamin D metabolites and sample protein.

### 2.8. Analysis of cohort samples and quality control

A total of 739 blood plasma samples from 352 subjects enrolled in the DAISY study were analyzed for vitamin D metabolites as part of a case-control study to determine the role, if any, that vitamin D plays in the prevention of type 1 diabetes (T1D). All children enrolled in DAISY either had high-risk HLA genotypes, or a first degree relative with T1D. Full details of enrollment have been published previously[31,32] A subset of 357 healthy control samples belonging to 177 subjects under the age of 18 who did not develop preclinical diabetes-related autoimmunity nor T1D were then selected from this group to investigate the changes in vitamin D metabolites during the early years of life. Samples were divided into three age groups; infant (8 to 12mo; n=41), child (1yr old to puberty; n=255), and adolescent (post-puberty to 18yr, n=61). Pubertal progression was assessed by subject self-report using Tanner staging[33]. Each metabolite and age group was tested for significant differences based on gender.

Plasma samples were collected from 32 subjects at both the infant and child ages, allowing for longitudinal analysis. Similarly, longitudinal samples were collected from all 23 subjects at at time points corresponding to both the child and adolescent groups. Additional analysis was performed on the groups of paired-longitudinal samples as a means of confirming findings from the larger cohort. Mean values were used in the paired-longitudinal analysis for cases where a single subject had multiple samples in the same age group.

Samples were prepared in 96-well plates. Each plate contained one NIST SRM 972a to control for accuracy. Additionally, pooled human EDTA blood plasma from at least 10 healthy, drug-free adults was purchased from BioIVT (Westbury, NY) and analyzed every 10 samples as a precision control. Furthermore, there were 22 blinded pairs of samples included

in the 739 samples. After the data were delivered to the collaborator the 22 sample pairs were unblinded and compared to further assess method precision.

### 2.9. Statistics

Quartile-quartile plots revealed all metabolites to have a non-normal distribution. The Kruskal-Wallis rank sum test was used to compare differences in vitamin D metabolites between the three age groups. Post-hoc analysis of age data was done using Wilcoxon rank sum test and Holm-Bonferroni correction. The Wilcoxon rank sum test and Holm-Bonferroni correction were also used to determine metabolite differences by sex within each age group. Metabolites that were found to have significant differences by gender, in any age group, were then separated by sex and re-analyzed by Kruskal-Wallis rank sum and Wilcoxon rank sum with Holm-Bonferroni correction. Paired samples were analyzed by Wilcoxon signed rank test. Statistical processing described above was performed in R studio. Also in R, ggplots2 package[34] was used to generate box and whisker plots. Correlation analysis, including Pearson's correlation and linear trendline fitting and slope generation was performed in Excel 2016 (Microsoft Corporation, Redmond WA). Time-series plots were generated using Prism 8 (GraphPad Software, San Diego CA).

## 3 Results

### 3.1 Method development

Chemical derivatization of blood plasma samples was required to achieve a 1α,25$(OH)_2$D3 LLOQ below the biological range. PTAD was selected as a derivatization reagent, because it has been shown to increase the signal of 1α,25$(OH)_2$D3 by approximately 100-fold and yields a near complete (>99%) derivatization.[12] When optimizing ion-source parameters, we found source temperature to be vital in maximizing sensitivity. At low source temperatures (200°C) two primary 1α,25$(OH)_2$D3 precursor ions were observed at relatively equal intensity, $[M+H]^+$ and $[M-H_2O+H]^+$. Repeat injections of 1α,25$(OH)_2$D3 standard while changing the source temperature by 25°C increments resulted in an optimal source temperature of 325°C (Supplemental Figure 1). Despite derivatization 1α,25$(OH)_2$D3 signal remains low compared to other vitamin D metabolites, furthermore the MRM transition yielding the greatest signal is shared by other compounds in blood plasma resulting in a chromatogram with numerous peaks at close retention times. To confirm the correct peak is quantified we spiked an authentic 1α,25$(OH)_2$D3 standard into BioIVT plasma at a number of concentrations (Supplemental Figure 2), furthermore a mix of standards including 1α,25$(OH)_2$D3 was injected every ten samples during analysis to account for any in-run retention time shifting and ensure the correct metabolite was quantified.

Derivatization greatly improved the signal of vitamin D metabolites, but it also hindered chromatographic resolution of the C3-epimer of 25OHD from its canonical isobars. A previously published method[10] used a Waters ACQUITY UPLC HSS pentafluorophenyl (PFP) column (1.8 pm particle size, 2.1 mm x 100 mm) to separate native, underivatized, C3-epimers from 25OHD. When the previous method[10] was duplicated, chromatographic resolution of PTAD derivatized 3-epi-25OHD3 from 25OHD3 was not achieved. Ultimately, a 150 mm C18 column, maintained at precisely 15°C was required for baseline separation of

3-epi25OHD3 from 25OHD3 ($R_s$ ≥1.5) and 3-epi-25OHD2 from 25OHD2 ($R_s$ ≥1.0) (Figure 2). This fully optimized method quantified ten vitamin D metabolites, while utilizing six deuterium labeled internal standards.

When derivatized by PTAD, vitamin D and its metabolites, form both 6*S* and 6*R* epimers[12] In many of our target metabolites we observed these epimers as two separate chromatographic peaks. There were three exceptions, which did not yield two separated peaks: 3-epi-25OHD3, 23R,25$(OH)_2$D3 and D3 (Supplemental Figure 3).

### 3.2 Method validation

After optimizing separation of 3-epi-25OHD3, 25OHD3, 1α,25$(OH)_2$D3 and other Vitamin D metabolites, we showed LLOQ values were below the biological range typical in blood plasma. All ten metabolites exhibited exceptional linearity ($R^2$ >0.99) over a broad range of concentrations (Table 1). We found accuracy and precision consistent in all metabolites tested and across a wide range of concentrations. Method validation yielded inter- and intra-day accuracy and precision below 15% (Table 2).

High-throughput extraction was performed by pass-through phospholipid removal 96-well plate. Hydroxylation was a major factor in the degree to which vitamin D metabolites were recovered (Table 3). Recoveries of four dihydroxy-vitamin D metabolites were between 68% and 84% across all tested concentrations. Monohydroxy-vitamin D metabolites had lower recoveries, ranging from 49% to 67% at all concentrations. Vitamin D2 and D3 are the most lipophilic of the metabolites assayed, and as such were largely retained by the Ostro™ plate, resulting in 2% to 5% recovery.

### 3.3 Quality control samples

Seven forms of vitamin D were detected and quantified from 739 EDTA blood plasma samples in this case-control study. 1α,25$(OH)_2$D3, 25OHD3, 3-epi25OHD3, 24R,25(OH)2D3, and 25OHD2 were quantified in all 739 samples. Vitamin D3 and D2 were quantified in 94% and 9% of the samples respectively. Three metabolite targets, 23R,25$(OH)_2$D3, 1a,25$(OH)_2$D2, and 3-epi-25OHD2, were not observed in blood plasma extracts from any subjects or control samples.

Commercially available EDTA plasma purchased from BioIVT was extracted in-parallel, with every ten patient samples, and examined throughout the run to monitor assay precision. There were 79 BioIVT samples analyzed and five hydroxylated vitamin D metabolites were quantified in those samples, %RSD ranged from 10% to 15.8% for those five metabolites across the 21 day analysis (Table 4). NIST offers a commercially available vitamin D reference blood plasma, sold as SRM 972a. The SRM 972a package comes with four vials; each vial contains plasma with different concentrations of the same four vitamin D metabolites. We quantified ten SRM 972a samples, one per 96-well plate, three SRM 972a concentrations were used over the course of data collection. The accuracy of 25OHD3, 3-epi25OHD3, and 24R,25$(OH)_2$D3 were within 15% (Table 4).

Included in the 739 samples were 22 blinded, nested-pairs of samples used as external controls. After the data was quantified and shared with our collaborator, all samples were

subsequently unblinded and the 22 sample pairs were compared for method precision. Metabolite concentrations from each sample were plotted against its replicate pair sample for all 22 samples. A linear trendline was applied to each plot yielding slopes between 0.85 and 1.33, a Pearson's correlation yielded R values greater than or equal to 0.75 (Table 4) indicating a strong reproducibility in replicate samples. Individual plots of the 22 pairs of replicate samples can be found in Supplemental Figure 4 Vitamin D2 was only detected in three of the 22 paired samples, and was excluded from slope and Pearson's correlation testing.

### 3.4 Sample analysis

Healthy control samples (n=357) from our 739 sample case-control study were further investigated to determine vitamin D status and metabolic changes across major developmental points in early life. Metabolites 25OHD3 and 3-epi-25OHD3 were found to have significant ($q$ <0.05) gender differences in the child group. Neither 3-epi-25OHD3 nor 25OHD3 were significantly different across the three developmental stages investigated, in females or males. Percentage of 3-epi-25OHD3 in total 25OHD3 (%3-epi), commonly used to asses 3-epi-25OHD3 levels in subjects of all ages[4,5,7], did not have significant differences based on gender. No other age groups or metabolites had significant differences based on gender.

A decrease in 3-epi-25OHD3, relative to total 25OHD3, correlating with increasing age has been reported in a number of studies and ages[4,5,7]. Similarly, when %3-epi in this study was reviewed we found a significant decrease in adolescents compared to both children and infant populations. Our results show a decrease ($q \leq 0.009$) in %3-epi, correlating with puberty (Figure 3A). Adolescents had the lowest %3-epi, mean 7.1%, significantly below pre-pubescent groups; infants and children were 8.5% and 8.0% respectively. Twenty-three subjects had at least one sample collected in both the child and adolescent groups. In those paired-samples we observed decreased %3-epi ($q$ =0.001) in 17/23 (74%) of subjects (Figure 3B).

The metabolite with the greatest concentration change between age groups was 25OHD2. The median concentration was nearly 3-fold higher in children compared to infants (Figure 4A). In fact, significantly ($q \geq 1e^{-6}$) higher concentrations were observed in both children and adolescents compared to infants. Furthermore, the concentration of 25OHD2 increased in 24/32 (75%) of subjects with samples collected in both infant and child groups ($q$ =0.0002) (Figure 4B).

The effect of childhood aging on 1α,25$(OH)_2$D3 concentration has not been well studied, in part due to the difficulty of quantifying 1α,25$(OH)_2$D3 in blood. We observed significant decreases in 1α,25$(OH)_2$D3 concentration in each subsequent age group (Figure 5A). While these decreases were statistically significant, the fold-change was modest. We found 22/32 subjects (69%) had lower concentrations of 1α,25$(OH)_2$D3 as children compared to their infant sample ($q$ =0.02; Figure 5B). There was no significant association exhibited in paired subjects with both childhood and adolescent samples 12/23 (52%).

Previous reports[35,36] found concentrations of 24R,25$(OH)_2$D3 in neonates significantly lower than children and adolescents. We observed 24R,25$(OH)_2$D3 remains significantly lower in 8-12mo olds, compared with children and adolescents (Figure 6A). Twenty-four of 32 subjects (75%) had higher concentrations of 24R,25$(OH)_2$D3 as children compared to their infant sample ($q$=0.0006; Figure 6B). Vitamin D concentrations for all 357 control samples is provided in Supplemental Table 2, summary statistics of subjects examined in this study are provided in Supplemental Table 3.

# 4 Discussion

## 4.1 Method development

Curiously, PTAD derivatized 3-epi-25OHD3 and 25OHD3 were not separated by published chromatography methods[10] despite the epimeric hydroxyl group not being affected by the derivatization reaction. Using a 150mm CSH C18 column, an isocratic solvent mixture, maintained at 40°C, we were able to improve upon the PFP method, achieving non-baseline separation of the PTAD-25OHD epimers. While not fully separated, this column was a promising improvement and we attempted further optimization with the hope of reaching baseline separation. Increasing the column temperature (60°C) was counterproductive, yielding closer epimer peaks. Near baseline separation was achieved when column temperature was maintained at 25°C, leading us to investigate the effects of a cooled column. After testing a number of temperatures from 4°C to 25°C, we found that 15°C provided the optimal separation while still maintaining sharp, symmetrical peak shape (Figure 7). This temperature-dependence was remarkable as most other chromatographic optimizations use temperatures higher than room temperature, not lower. When considering the Van Deemter equation elevated temperature is associated with increased rate of solute transfer from mobile phase to stationary phase[37]. Hence comparing the chromatographic resolution at the experimentally derived optimal temperature (15°C) (Figure 7B) to a typical column temperature (60°C) (Figure 7D) it is plausible the lower temperature increases the interaction of vitamin D metabolites with the stationary phase resulting in later eluting, better resolved peaks. Interestingly, at temperatures lower than 15°C we observed deterioration of the peak shape characterized by increased peak width and fronting (Figure 7A). The peak fronting observed at lower temperatures further highlights the effects of temperature on the equilibrium of metabolites between mobile and stationary phases.

## 4.2 Method validation

After optimizing the separation of 3-epi-25OHD3, 25OHD3, 1α,25$(OH)_2$D3 and other Vitamin D metabolites, we showed that lower limits of quantification values were below the biological range typically found in human blood plasma. We found the accuracy and precision to be consistent in all metabolites tested and across a wide range of concentrations. 3-epi-25OHD2 was also successfully separated from 25OHD2. 3-epi-25OHD2 produced two distinct chromatographic peaks, both eluting between the 6$S$ and 6$R$ epimers of 25OHD2 (Figure 2B). Extraction was performed using a pass-through phospholipid removal 96-well plate. The level of hydroxylation played a major role in the degree to which vitamin D metabolites were recovered (Table 3). Recoveries of the four dihydroxy-vitamin D metabolites were between 68% and 84% at all tested concentrations. Monohydroxy-vitamin

D metabolites had lower recoveries, ranging from 49% to 67% at all concentrations. Vitamin D2 and D3 are the most lipophilic of the metabolites screened for in this assay, and as such were largely retained by the Ostro plate, resulting in 2% to 5% recovery. Despite the low recoveries D3 was quantified in 94% of samples analyzed.

### 4.3 Quality control samples

The assay's precision over the course of our 21-day period of data collection was excellent. It was not surprising that the least abundant analyte, 1α,25(OH)$_2$D3, had the highest variability (15.8% RSD). The concentration of 1α,25(OH)$_2$D3 in BioIVT plasma was approximately half of the mean from the patient samples, and only 3-fold higher than our LLOQ. Those factors, and the extraordinarily long analysis time, may have contributed to an elevated %RSD.

A surprising finding was the accuracy discrepancy when we calculated the concentration of 25OHD2 in NIST SRM972a plasma. NIST SRM972a gives known concentrations of four metabolites (25OHD2, 25OHD3, 3-epi25OHD3, and 24R,25(OH)2D3). We found the accuracy of three metabolites, 25OHD3, 3-epi25OHD3, and 24R,25(OH)2D3, to be within 15% of the values provided by NIST. Yet, for the fourth compound, 25OHD2, we found concentrations that were two-fold higher than the values NIST provided. This discrepancy in accuracy was observed at all three 25OHD2 concentrations of the NIST SRM972a samples that we assayed.

We did not observe this discrepancy during method validation. Unlike earlier eluting compounds, 25OHD2 elutes after the isocratic portion of the run, just as analytes eluted by the LC-gradient are reaching the detector. In this compound rich area of the chromatogram, it is possible interfering signals from the matrix are leading to the over estimation of 25OHD2 in subject and quality control samples. In future versions of this method it may be advantatgous to slow the gradient at this portion of the chromatogram to remove the interfering signals and improve the accuracy of our 25OHD2 quantification in blood plasma. Despite this difference in measured and certified concentrations, the precision of 25OHD2 in the assay, as highlighted in Supplemental Figure 4, and Tables 2 & 4, showed that the method was valid and that the differences observed between children's age groups were true.

### 4.4 Pediatric sample analysis

A decrease in 3-epi-25OHD3, relative to total 25OHD3, correlating with increasing age has been reported in a number of studies, and has been observed at all ages from the very young[4,7] to adult populations[5,7]. Previous publications have shown the highest levels of %3-epi occur in the first one-hundred days of life[4,7]. We did not observe significant differences in %3-epi between infants and children, likely due to the limited age range (8–12mo) of our infant group. While others have reported the decrease in %3-epi associated with aging, our study is the first to investigate 3-epi-25OHD3 in relation to puberty. Using the onset of puberty as an aging landmark we found a significant ($q \leq 0.009$) decrease in %3-epi, correlating with puberty. Adolescent subjects had the lowest percent of 3-epi-25OHD3, mean 7.1%, significantly lower than pre-pubescent groups, infant and children are 8.5% and 8.0% respectively. An earlier publication[7] presented data showing the %3-epi to be

significantly decreased with age in people age 1 to 94 years old, but this cohort was grouped by age-decades which likely obscured the effect puberty might have on 3-epi-25OHD3 levels.

The exact mechanism and enzyme responsible for vitamin D epimerization at the C-3 location has yet to be discovered in humans. It is known that epimerization of all major vitamin D metabolites can occur in human keratinocytes[38] and is likely unidirectional[39]. It is likely the observed decrease in %3-epi correlating with age is due to a decrease in epimerization activity. Furthermore, based on the observations in this study it is possible puberty is a checkpoint responsible for decreased epimerization activity. More studies are needed to better understand the mechanism of epimerization and clarify the role puberty has in its down-regulation.

We observed a significantly lower concentration of 25OHD2 (mean 2.0 nM) in infants compared to both children (mean 5.7 nM, $q=3e^{-7}$) and adolescents (4.3 nM, $q=1e^{-6}$). Compared to 25OHD3, relatively little is known about 25OHD2. 25OHD2 has a reduced affinity for vitamin D binding protein resulting in a shorter biological half-life,[40] supplementation studies have shown it clears the system faster than 25OHD3[41]. We were able to find only one published value for 25OHD2 in children; Geigy Scientific Tables[42] reveal a 10-fold increase from newborns (0-30 days) to children (1-13 yr) Unfortunately, the Geigy data cannot be directly compared with our data because our infant group does not overlap with their newborn age-range, but the increased concentration they observed is similar to the nearly 3-fold increase we observed, where very young children have much lower concentrations of 25OHD2.

The changes in concentration of 1α,25$(OH)_2$D3 associated with childhood aging have not been thoroughly studied, in-part due to the difficult nature of 1α,25$(OH)_2$D3 quantitation in human blood. We observed a significant decrease in 1α,25$(OH)_2$D3 concentration in each subsequent age group. While these decreases were statistically significant, the fold-change in concentration was modest. It is known that 1α,25$(OH)_2$D3 increases intestinal absorption of calcium and phosphorus, and works with parathyroid hormone to increase bone reabsorption. It is feasible that the decrease in circulating 1α,25$(OH)_2$D3 concentration is simply the product of an overall decrease in growth rate in children as they age, however the additional information needed to definitely confirm the association of growth rate to 1α,25$(OH)_2$D3, such as additional hormone levels and subject growth data were not collected in this study.

Previous reports[35,36] found concentrations of 24R,25$(OH)_2$D3 in neonates (≤1 wk) significantly lower than older children and adolescents. In this study we observe in-fact 24R,25$(OH)_2$D3 remains significantly lower in 8-12 month olds, compared with children and adolescents. For many years 24R,25$(OH)_2$D3 was believed to be merely the first metabolite in the excretion pathway of vitamin D. Recent studies have shown 24R,25$(OH)_2$D3 binds to membrane receptors on the growth plate resting zone chondrocyte, and effects both cartilage and bone formation[43]. Further study will be needed to determine if the changes in 24R,25$(OH)_2$D3 concentration associated with childhood development are

due to the biological activity of 24R,25$(OH)_2$D3 or if the observed changes are simply the result of decreased 1α,25$(OH)_2$D3.

Vitamin D deficiency, defined as total 25OHD <20 ng/mL[9], has been linked to a number of diseases; we found minimal incidence of deficiency in our cohort, only 6 in 357 (1.7%) were vitamin D deficient. Insufficiency (20 ng/mL <total 25OHD <29.99 ng/mL) was observed in 17% of the cohort; 12%, 16%, and 23% in infants, children and adolescents respectively. 25OHD2 is not a major determining factor of vitamin D status, in our data 25OHD2 makes up just 5% of total 25OHD. Not surprisingly, concentrations of 25OHD2 were not correlated with total 25OHD in our subjects ($R^2$ <0.01), nor were they correlated in just the infant population ($R^2$ <0.01). 3-epi-25OHD3 makes up nearly 8% of total 25OHD, we found 3-epi-25OHD3 is correlated to total 25OHD ($R^2$ =0.61), and is most highly correlated in adolescents ($R^2$ =0.69) (Supplemental Figure 5).

## 5 Conclusion

Here we present a method for separation and quantification of 10 vitamin D metabolites, including C-3 epimers, in human blood plasma. This method has been shown to be accurate and precise in measuring metabolomic targets, including low abundance 1α,25$(OH)_2$D3. Furthermore, this method was applied to analyze 739 patient samples yielding excellent reproducibility over a 21-day analysis. Metabolites of vitamin D were examined in the context of aging in a cohort of infants, children, and adolescents and illustrated the changes of metabolites associated with age, including the increase in 25OHD2 concentration correlating with age and the decrease in percent 3-epi-25OHD3 as a percent of total 25OHD3 correlating with onset of puberty.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations:

| | |
|---|---|
| **25OHD3** | 25-hydroxy-vitamin-D3 |
| **25OHD2** | 25-hydroxy-vitamin-D2 |
| **1α,25$(OH)_2$D3** | 1α,25-dihydroxy-vitamin-D3 |
| **3-epi-25OHD3** | 3-epi-25-hydroxy-vitamin-D3 |
| **LC-MS/MS** | liquid chromatography coupled to tandem mass spectrometry |

| | |
|---|---|
| **PTAD** | 4-phenyl-1,2,4-triazoline-3,5-dione |
| **CUDA** | 12-[[(cyclohexylamino)carbonyl]amino] dodecanoic acid |
| **UPLC** | ultra-performance liquid chromatography |
| **CSH** | Charged Surface Hybrid |
| **BSA** | bovine serum albumin in phosphate-buffered saline |
| **%RSD** | Percent relative standard deviation |
| **BioIVT** | pooled plasma from healthy adults |
| **DAISY** | Diabetes Auto Immunity Study in the Young |
| **T1D** | type-1-diabetes |
| **SRM972a** | NIST standard reference material 972a |
| **LLOQ** | lower-limit of quantification |
| **24R,25(OH)$_2$D3** | 24.25-dihydroxy-vitamin-D3 |
| **23R,25(OH)$_2$D3** | 23.25-dihydroxy-vitamin-D3 |
| **3-epi-25OHD2** | 3-epi-25-hydroxy-vitamin-D2 |
| **%3-epi** | Percentage of 3-epi-25OHD3 in total 25OHD3 |

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## Highlights

- New method to separate 10 Vitamin D epimers using derivatization and column cooling.
- Quantification in blood validated accuracy at better than 15% RSD and 10 pM quantification limits.
- In a pediatric cohort of 739 samples, differences in plasma concentrations of Vitamin D epimers were established between infants, children and adolescents.

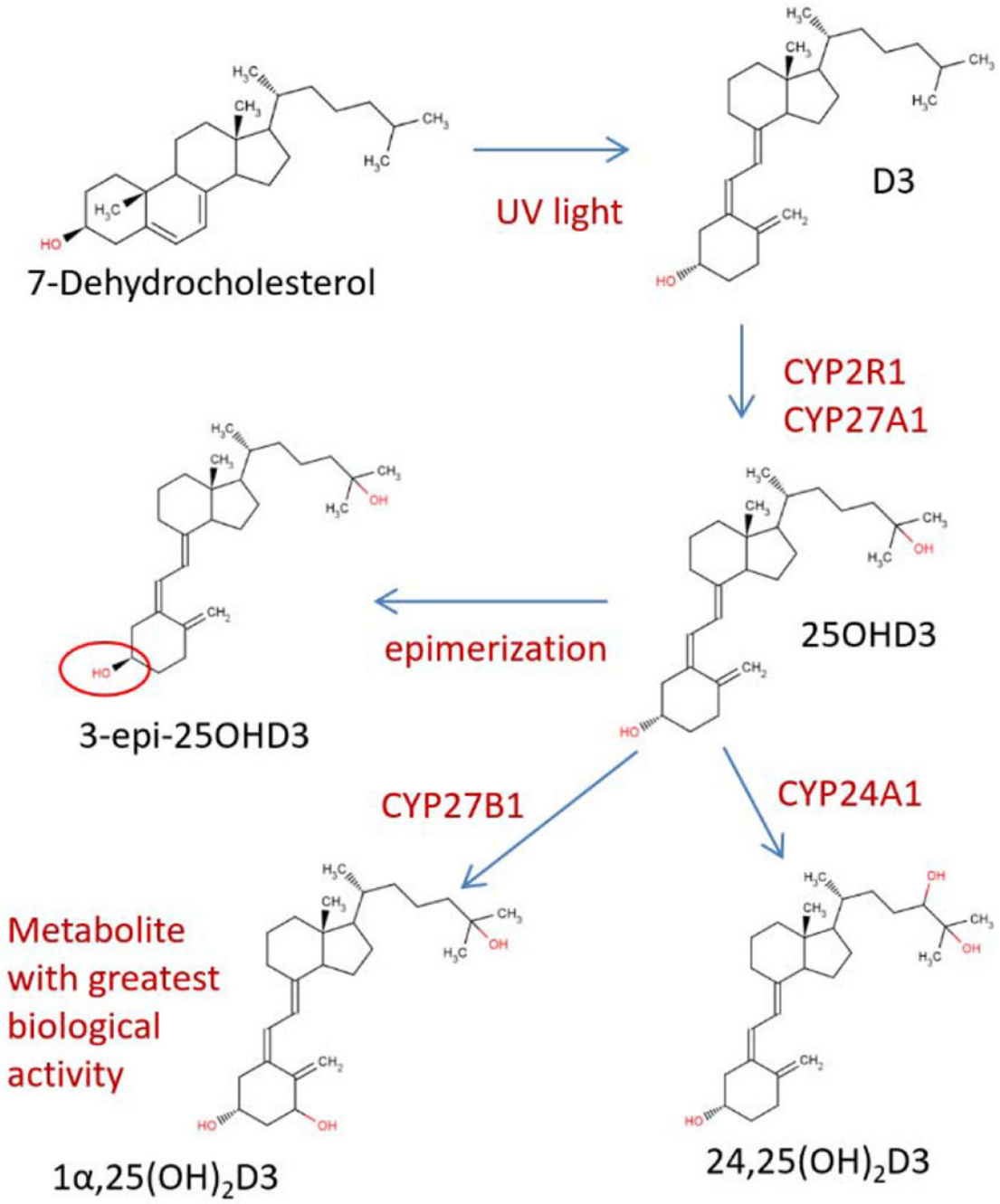


**Figure 1). Major intermediates of the vitamin D3 metabolic pathway.**

25OHD3 is the predominate circulating form of vitamin D3. Exact mechanism of epimerization has yet to be discovered, but 3-epi-25OHD3 has been observed in people of all ages. $1\alpha,25(OH)_2D3$ is highly regulated and is commonly observed in the low picomolar range in blood plasma.

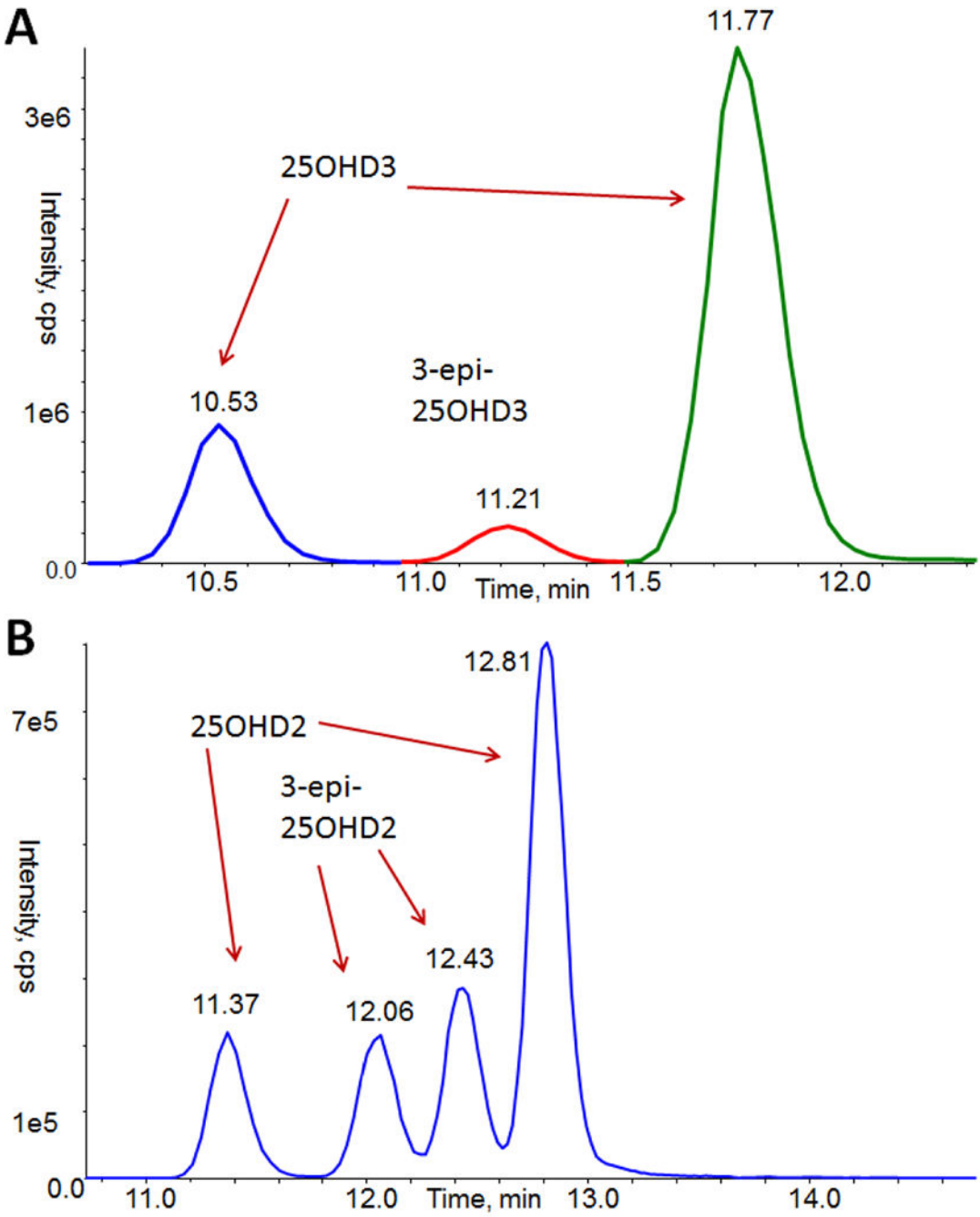


**Figure 2). Extracted ion chromatograms for transitions of 25OHD and their C3-epimers.**
**A)** Baseline separation ($R_s$ = 2.1 and 1.5) of endogenous 3-epi-25OHD3 from 25OHD3. Derivatization by PTAD leads to (*R*) and (*S*) isomers, however only one peak was observed for 3-epi-25OHD3. **B)** Separation of 25OHD2 from 3-epi-25OHD2, $R_s \geq 1$ was observed for all peaks. ($R_s$ = 1.7, 1, and 1).

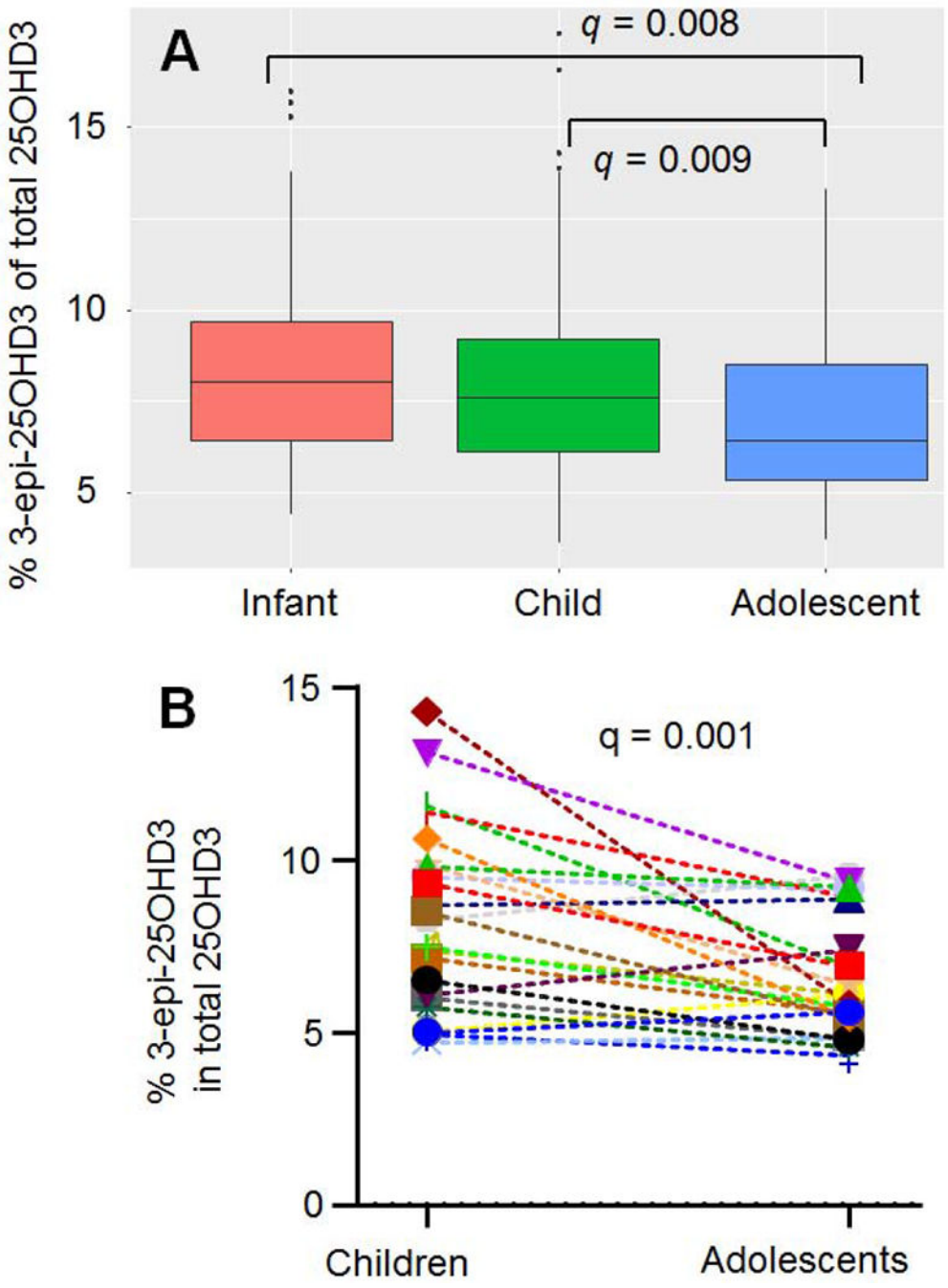


**Figure 3). The percentage of 3-epi-25OHD3 of total 25OHD3 (%3-epi) decreases significantly after the onset of puberty.**

**A)** Box plot comparing vitamin D levels in healthy infants (n =41), children (n =255), and adolescents (n =61). **B)** Time series plot from 23 subjects with blood drawn in both the child and adolescent age groups reveals %3-epi decreases in 17/23 (74%) of subjects ($q$ =0.001).

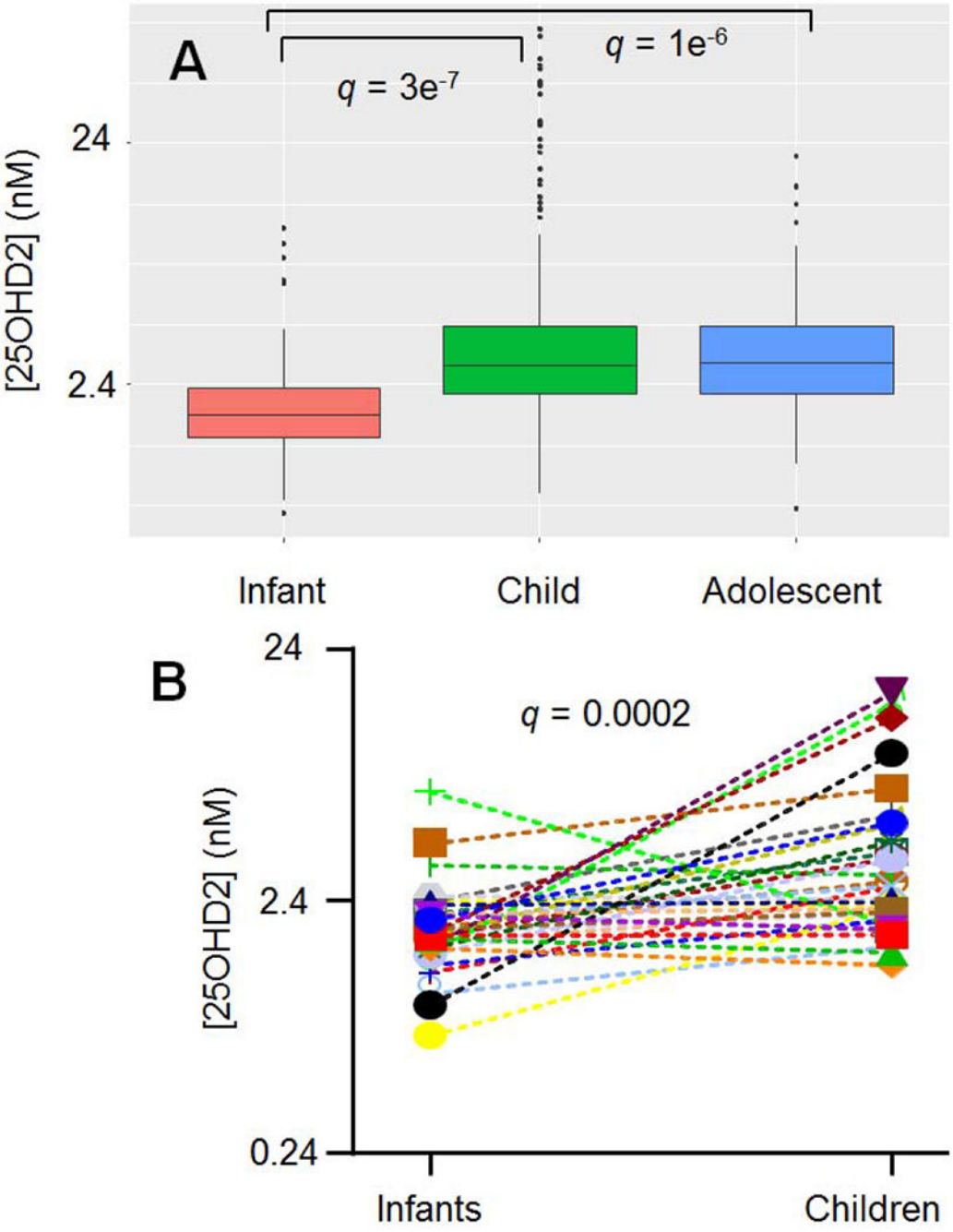


**Figure 4). The concentration of 25OHD2 increases significantly after infancy, remaining elevated throughout adolescence.**

**A)** Box plot comparing 25OHD2 concentrations (nM) in healthy infants (n =41), children (n =255), and adolescents (n =61). **B)** Time series plot from 32 subjects with blood drawn in both the infant and child age groups reveals 25OHD2 increases in 24/32 (75%) of subjects ($q$ =0.0002).

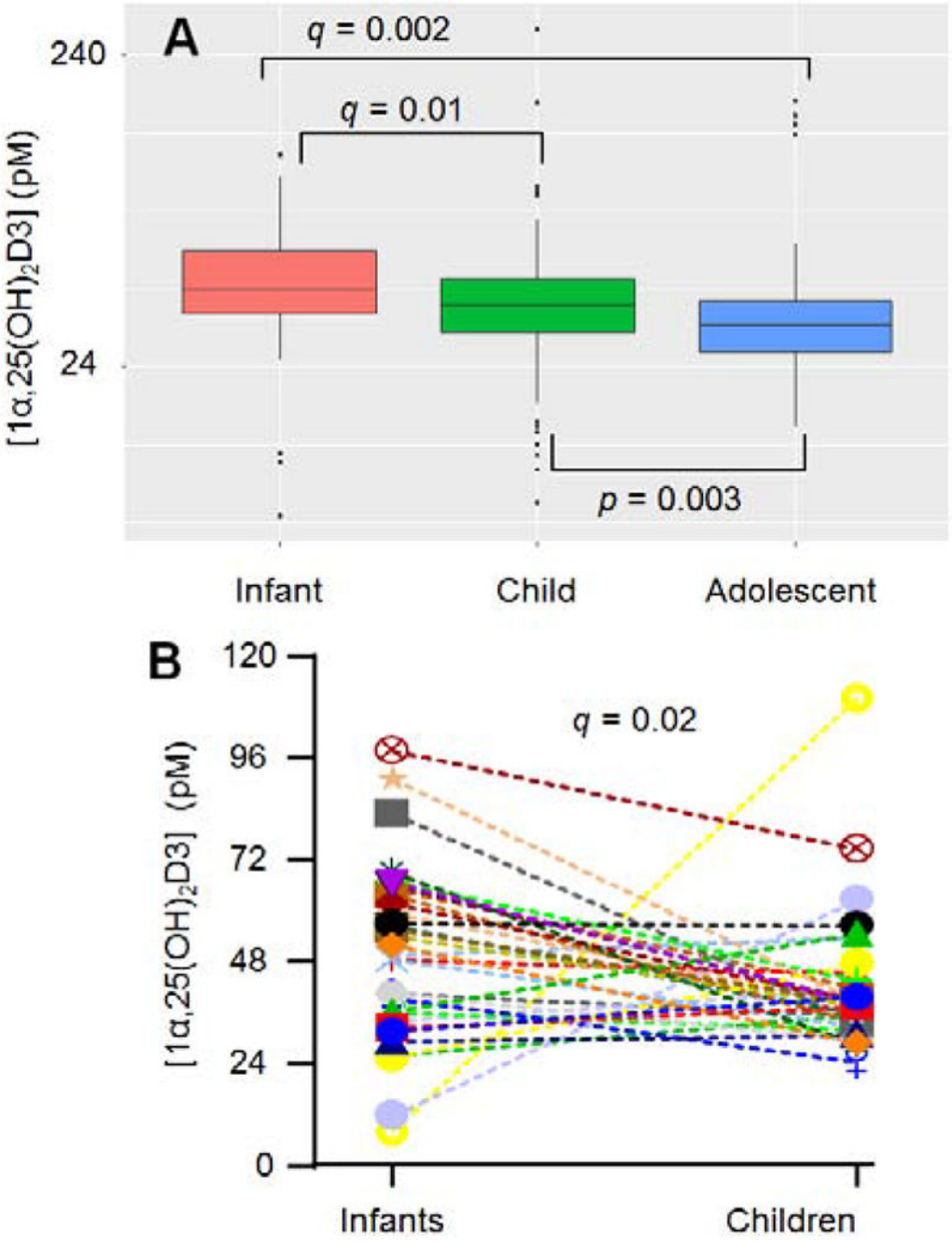


**Figure 5. Circulating concentration of 1α,25(OH)2D3 decreased significantly after infancy and continued to decrease in subsequent age-groups.**

**A)** Boxplot comparing 1α,25$(OH)_2$D3 concentration in healthy infants (n =41), children (n =255), and adolescents (n =61). **B)** 22/32 (69%) of subjects had a lower concentration of 1α,25$(OH)_2$D3 as children compared to their infant sample(s) ($q$ = 0.02). The change in 1α,25$(OH)_2$D3 concentration from 23 subjects observed as children and adolescents was not significant.

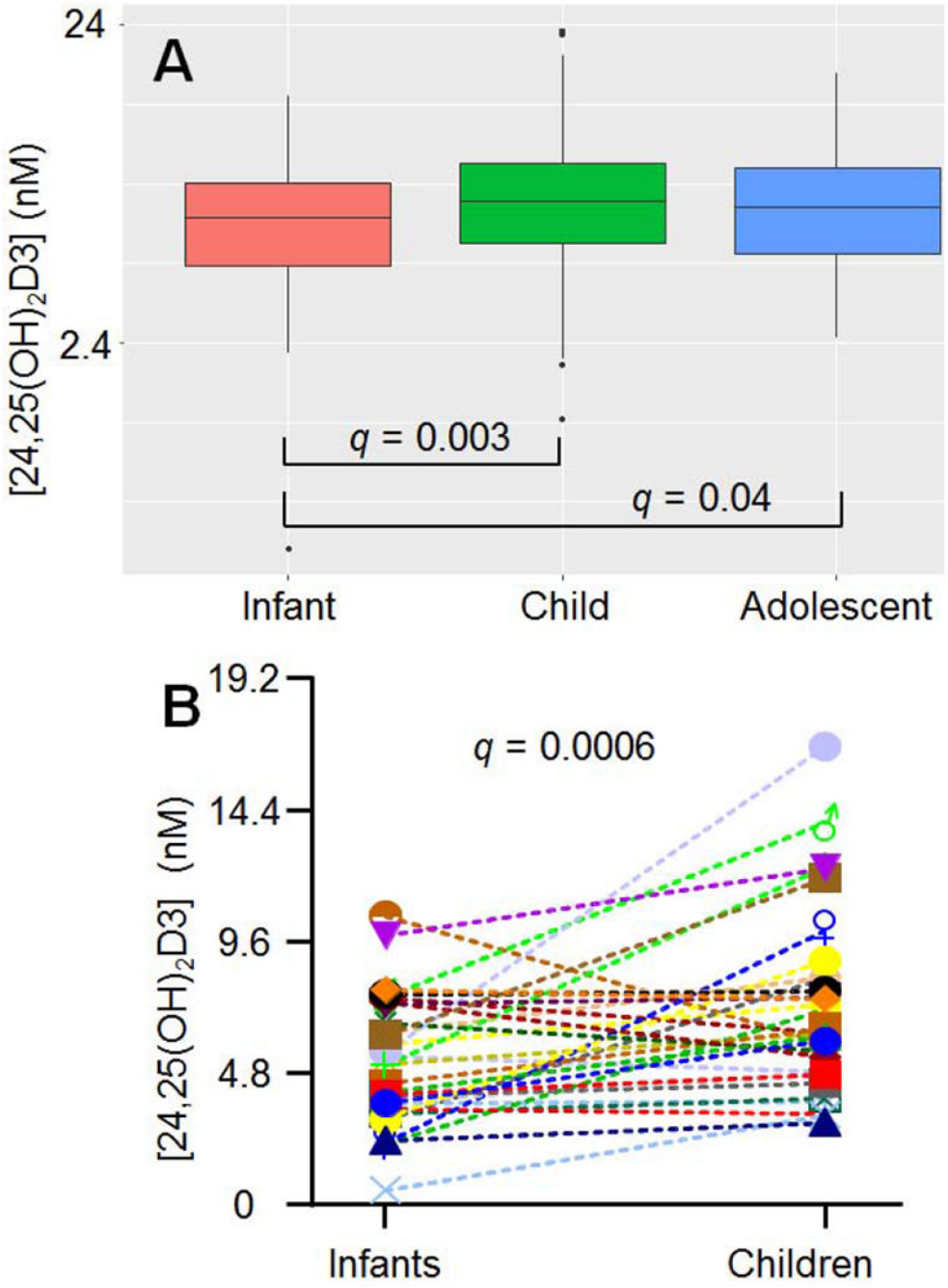


**Figure 6. The concentration of 24R,25(OH)$_2$D3 increases significantly after infancy, remaining increased through adolescence.**

**A)** Boxplot comparing 24R,25(OH)$_2$D3 concentrations (nM) in healthy infants (n =41), children (n =255), and adolescents (n =61). **B)** Time series plot from 32 subjects with blood drawn in both the infant and child age groups reveals 24R,25(OH)$_2$D3 concentration increases in 24/32 (75%) of subjects ($q$ =0.0006).

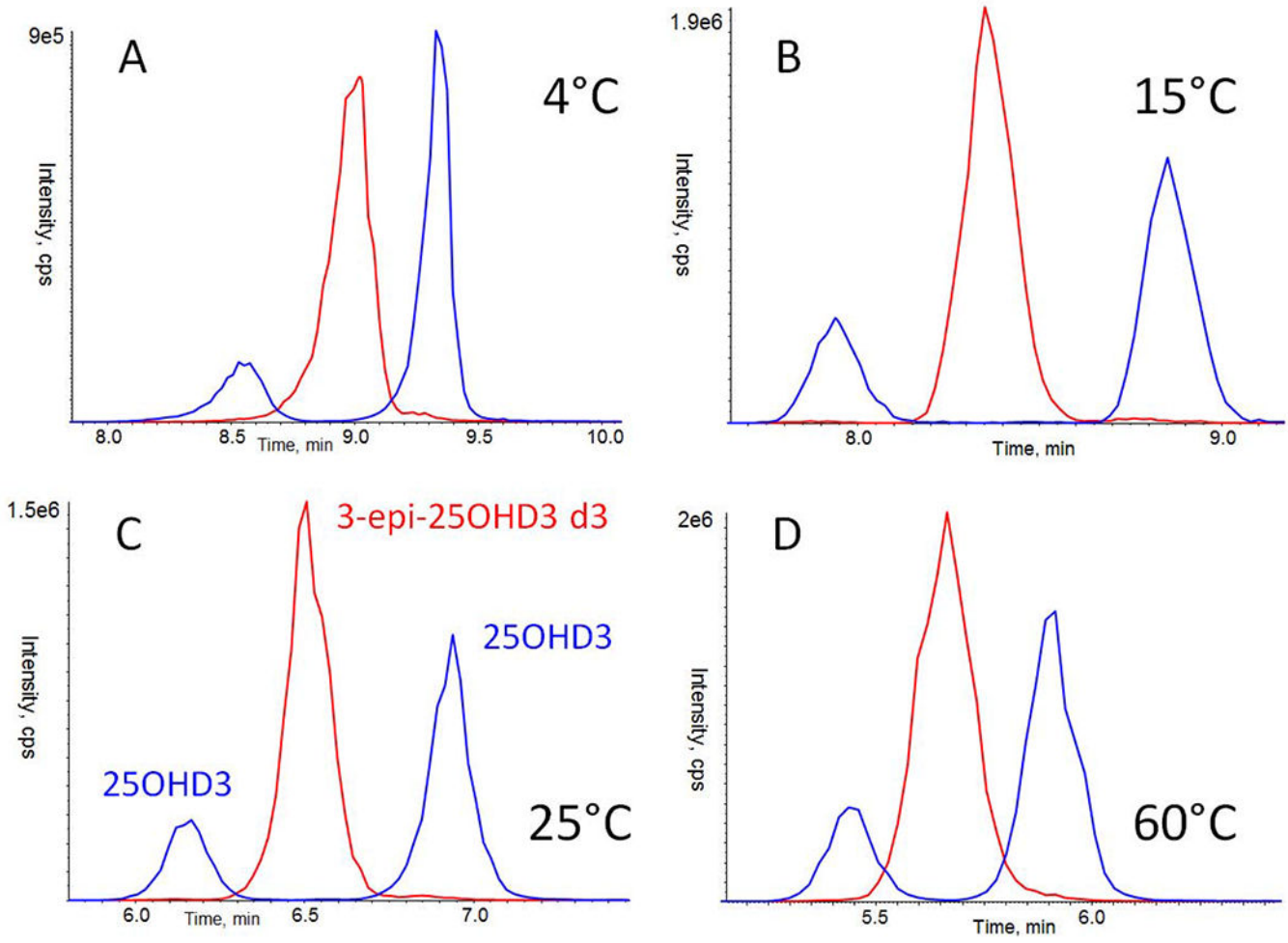


**Figure 7). Separation of 3-epi-25OHD3 from 25OHD3 is affected by column temperature. Extracted ion chromatograms of 25OHD3 (blue) and deuterium labeled 3-epi-25OHD3 d3 (red). Deuterated 3-epi-25OHD3 was used to differentiate the C3-epimer from native 25OHD3.**

**A)** Cooling the column to 4°C caused peak fronting and poor resolution. **B)** The column maintained at 15°C proved to yield optimal separation of 3-epi-25OHD3 from 25OHD3. **C)** Column temp of 25 °C yielded fairly good separation of epimers, but resolution was decreased compared to 15°C. **D)** Increasing column temperature to 60°C further decreased chromatographic resolution.

**Table 1)**

**Linear range determined by a 24-point, serial diluted, calibration curve.**

Linear range of concentrations from ten standards in calibration curve, values reported in nanomolar (nM), 5μL injected onto column. Coefficients of determination ($R^2$ value) were calculated automatically by Sciex software Multiquant 3.0.2.

| Metabolite | Linear Conc. Range (nM) | $R^2$ Value |
|---|---|---|
| **1α,25(OH)$_2$ D3** | 0.007-30 | 0.992 |
| **3-epi-25(OH) D3** | 0.240-250 | 0.992 |
| **24R,25(OH)$_2$ D3** | 0.060-960 | 0.998 |
| **25(OH) D3** | 0.120-970 | 0.990 |
| **23R,25(OH)$_2$ D3** | 0.120-960 | 0.994 |
| **D3** | 0.008-32.5 | 0.995 |
| **3-epi-25(OH) D2** | 0.120-240 | 0.991 |
| **1α,25(OH)$_2$ D2** | 0.060-15 | 0.993 |
| **25(OH) D2** | 0.240-960 | 0.990 |
| **D2** | 0.031-31.5 | 0.995 |

**Table 2)**

**A blank matrix (4% BSA in PBS) was spiked with known concentrations of vitamin D metabolites, at four concentrations (replicates; n=5).**

Samples were then analyzed and quantified on three consecutive days to test the method's intra- and inter-day precision and accuracy. Interday and intraday precision and accuracy were <15% at all concentrations. Vitamin D concentrations near previously published values are highlighted in green.

| Vitamin D Metabolites | Spiked Conc. (nM) | Detected Concentration [Mean (SD), nM] | Intraday Accuracy (%) | Intraday Precision (%) | Interday Accuracy (%) | Interday Precision (%) |
|---|---|---|---|---|---|---|
| **3-epi-25OH Vit D3** | 0.2496 | 0.277 (0.030) | **10.8** | **8.8** | **10.8** | **10.7** |
| | 2.496 | 2.485 (0.152) | **1.1** | **6.5** | **0.5** | **6.1** |
| | 24.96 | 24.69 (1.98) | **2.9** | **7.1** | **1.1** | **8.0** |
| | 249.6 | 230.89 (11.46) | **7.5** | **5.1** | **7.5** | **5.0** |
| **1α,25(OH)$_2$ Vit D3** | 0.012 | 0.0124 (0.002) | **11.3** | **9.8** | **10.5** | **10.3** |
| | 0.12 | 0.119 (0.014) | **3.1** | **12.0** | **1.2** | **12.1** |
| | 1.2 | 1.198 (0.094) | **0.4** | **8.0** | **0.1** | **7.8** |
| | 12 | 11.44 (0.70) | **4.7** | **5.3** | **4.7** | **6.1** |
| **24R,25(OH)$_2$ Vit D3** | 0.048 | 0.051 (0.003) | **7.0** | **5.8** | **7.0** | **6.7** |
| | 0.48 | 0.497 (0.028) | **5.3** | **3.4** | **3.5** | **5.6** |
| | 4.8 | 4.75 (0.19) | **1.0** | **4.2** | **1.0** | **3.9** |
| | 48 | 42.83 (1.08) | **10.8** | **2.6** | **10.8** | **2.5** |
| **25OH Vit D3** | 0.250 | 0.285 (0.038) | **14.6** | **11.5** | **14.1** | **13.2** |
| | 25.0 | 28.186 (1.531) | **12.4** | **4.9** | **12.4** | **5.4** |
| | 250.0 | 274.77 (8.57) | **10.1** | **3.2** | **10.1** | **3.1** |
| | 2500.0 | 2588.84 (117.7) | **3.7** | **4.0** | **3.7** | **4.5** |
| **25OH Vit D2** | 0.3635 | 0.374 (0.020) | **2.8** | **5.2** | **2.8** | **5.5** |
| | 3.635 | 3.693 (0.148) | **1.9** | **3.8** | **1.6** | **4.0** |
| | 36.35 | 36.76 (1.14) | **1.2** | **3.1** | **1.1** | **3.1** |
| | 363.5 | 322.91 (11.24) | **11.2** | **3.2** | **11.2** | **3.5** |
| **Vit D3** | 0.039 | 0.040 (0.005) | **4.7** | **12.5** | **3.3** | **13.0** |
| | 0.390 | 0.386 (0.034) | **2.6** | **8.9** | **1.0** | **8.7** |
| | 3.90 | 3.867 (0.455) | **1.8** | **12.4** | **0.9** | **11.8** |
| | 39.0 | 39.35 (4.87) | **5.5** | **11.4** | **0.9** | **12.4** |
| **Vit D2** | 0.038 | 0.039 (0.004) | **2.8** | **11.1** | **2.8** | **10.9** |
| | 0.378 | 0.376 (0.021) | **2.5** | **5.0** | **0.5** | **5.5** |
| | 3.78 | 3.95 (0.435) | **4.4** | **10.9** | **4.3** | **11.0** |
| | 37.82 | 39.13 (4.74) | **3.4** | **11.8** | **3.4** | **11.4** |
| **23R,25(OH)$_2$ Vit D2** | 0.24 | 0.248 (0.031) | **7.5** | **10.6** | **3.5** | **12.4** |
| | 2.4 | 2.548 (0.273) | **7.6** | **5.0** | **4.3** | **8.7** |
| | 24 | 25.65 (2.31) | **6.3** | **6.6** | **6.3** | **8.8** |
| | 240 | 237.43 (17.91) | **5.4** | **4.0** | **1.1** | **7.5** |

| Vitamin D Metabolites | Spiked Conc. (nM) | Detected Concentration [Mean (SD), nM] | Intraday Accuracy (%) | Intraday Precision (%) | Interday Accuracy (%) | Interday Precision (%) |
|---|---|---|---|---|---|---|
| **1α,25(OH)$_2$ Vit D2** | 0.023 | 0.023 (0.002) | **3.7** | **5.7** | **1.1** | **7.6** |
| | 0.233 | 0.240 (0.029) | **5.7** | **11.5** | **2.9** | **12.0** |
| | 2.33 | 2.432 (0.298) | **6.4** | **8.6** | **3.7** | **11.6** |
| | 23.33 | 23.60 (2.128) | **5.6** | **5.9** | **1.2** | **9.0** |

**Table 3)**

**Extraction recoveries of standards spiked into a blank matrix of 4% bovine serum albumin in phosphate buffered saline and extracted by the same sample preparation method described.**

Recoveries were calculated by comparing samples with standards added prior to extraction, to samples where standards were added after extraction. Five concentrations were tested and six replicates were used per concentration. Concentrations near previously published endogenous blood plasma values are highlighted in green.

| Metabolite | Conc. (nM) | Extraction Recovery (%) | Metabolite | Conc. (nM) | Extraction Recovery (%) |
|---|---|---|---|---|---|
| **1α,25(OH)$_2$ Vit D3** | 0.054 | 72.5 | **3-epi 25OH Vit D2** | 0.24 | 50.5 |
| | 0.27 | 80.9 | | 1.21 | 58.0 |
| | 0.54 | 83.9 | | 2.42 | 55.8 |
| | 5.40 | 79.1 | | 24.2 | 61.0 |
| | 54.0 | 76.1 | | 242.3 | 59.7 |
| **1α,25(OH)$_2$ Vit D2** | 0.052 | 72.1 | **25OH Vit D3** | 0.50 | 57.2 |
| | 0.26 | 67.8 | | 2.50 | 62.1 |
| | 0.52 | 72.1 | | 4.99 | 59.0 |
| | 5.25 | 84.0 | | 49.9 | 65.0 |
| | 52.5 | 73.0 | | 499.2 | 64.0 |
| **24R,25(OH)2 Vit D3** | 0.12 | 72.5 | **25OH Vit D2** | 0.24 | 48.8 |
| | 0.60 | 80.0 | | 1.21 | 59.7 |
| | 1.20 | 77.3 | | 2.42 | 54.9 |
| | 12.0 | 72.0 | | 24.2 | 62.0 |
| | 120.0 | 79.6 | | 242.3 | 60.5 |
| **23R,25(OH)2 Vit D3** | 0.120 | 78.0 | **Vit D3** | 0.26 | 2.7 |
| | 0.60 | 80.6 | | 1.30 | 3.2 |
| | 1.20 | 75.9 | | 2.60 | 1.9 |
| | 12.0 | 73.4 | | 26.0 | 3.7 |
| | 120.0 | 78.5 | | 260.0 | 3.3 |
| **3-epi 25OH Vit D3 d3** | 0.25 | 58.8 | **Vit D2** | 0.25 | 3.0 |
| | 1.25 | 67.0 | | 1.26 | 3.7 |
| | 2.50 | 59.5 | | 2.52 | 2.7 |
| | 25.0 | 64.9 | | 25.2 | 5.1 |
| | 249.6 | 66.1 | | 252.1 | 4.7 |

**Table 4)**

**The assay's accuracy and precision over the course of 21-day analysis. BioIVT control sample analyzed after every ten patient samples.**

Ten NIST SRM972a samples were assayed to monitor accuracy. Concentrations from each pair of blinded samples (n=22) were plotted and linear fit was applied. The resulting slope and Pearson's correlation values were reported above.

| External QC Samples | | | 22 Blind Sample Pairs | |
|---|---|---|---|---|
| **Metabolite** | **BioIVT %RSD** | **NIST Accuracy** | **Pearson's Correlation (R)** | **Slope** |
| **1α,25(OH)$_2$D3** | 15.8 | N/A | 0.82 | 0.85 |
| **24R,25(OH)$_2$D3** | 10.3 | 14.9 | 0.95 | 1.02 |
| **25OHD3** | 12.9 | 8.4 | 0.86 | 1.01 |
| **3-epi-25OHD3** | 10.0 | 12.8 | 0.94 | 1.09 |
| **25OHD2** | 15.2 | 102.5 | 0.97 | 0.91 |
| **D3** | N/A | N/A | 0.75 | 1.33 |