Method Development and Validation of Vitamin D_2 and Vitamin D_3 Using Mass Spectrometry

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Abstract

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Vitamin D has long been known to maintain bone health by regulating calcium and phosphorous homeostasis. In recent years, scientists have discovered additional physiological roles for vitamin D. The complex interaction between the active vitamin D hormone and its metabolic precursors continues to be a rich area of research. Fundamental to this research is the availability of accurate and precise assays. Few published assays for vitamins D_2 and D_3 have contained sufficient details on method validation or performance characteristics. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay developed for this thesis has undergone a rigorous validation and proven to yield a sensitive and specific method that exceeds the capabilities of all previously published methods. Developing and validating a novel assay is often complicated by the lack of established acceptability standards. This thesis explores this challenge, specifically for establishing meaningful interpretations and qualification standards of the lower limit of the measuring interval. Altogether, future research focused on vitamins D_2, D_3 and the Vitamin D pathway can benefit from this robust LC-MS/MS assay and the associated quality parameters outlined in this thesis.

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Chapter 1:

Introduction

Publications focusing on the association of vitamin D deficiency and various disease pathways have increased significantly over the last few years. The concentrations that define sufficiency (or deficiency) for the active hormone, its precursors, and its metabolites are still debated. For example, 25-hydroxyvitamin D (25(OHD)) is the precursor to the active hormone, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), and is often used as the measure of vitamin D status because it has the longest half-life. The concentration defining sufficiency for 25(OH)D varies between medical institutions and reference laboratories, ranging from >20 ng/mL[1] to >30 ng/mL[2]. 25(OH)D and $1,25(OH)_2D$ are commonly tested in the clinical laboratory and numerous testing methods for these compounds have been documented, some of which are immunoassays, high performance liquid chromatography (HPLC), and liquid chromatography-tandem mass spectrometry (LC-MS/MS)[3]. In contrast, there are limited publications focused on quantifying the metabolites upstream of 25(OH)D, specifically vitamin D_2 and D_3. None of the documented vitamin D_2/D_3 assays utilize mass spectrometry. The objective of this thesis was to develop and validate an LC-MS/MS assay that quantifies vitamin D_2 and D_3.

Vitamin D is complex and consists of several important precursors in addition to the active hormone (see Figure 1.1 and Table 1.1). Vitamin D_3, also called cholecalciferol (hereafter referred to as vitamin D_3), is synthesized in humans from 7-dehydrocholesterol (a cholesterol precursor) in the skin or consumed through the diet. 7-dehydrocholesterol absorbs UVB radiation, specifically between 280 and 320 nm, and is transformed to previtamin D_3. Previtamin D_3 undergoes thermal isomerization to vitamin D_3.[4] If the skin is exposed to excessive sunlight, previtamin D_3 can undergo transformation to inactive

metabolites, some of which are lumisterol$_3$ and tachysterol$_3$ (discussed in chapter 2).[5] Vitamin D_2, also called ergocalciferol (hereafter referred to as vitamin D_2), is produced in plants and consumed through the diet. Vitamin D_2 is produced in plants from the phototransformation of ergosterol after sunlight exposure. The conversion of ergosterol to vitamin D_2 is analogous to the conversion of 7-dehydrocholesterol to previtamin D_3 in human skin.

Vitamin D_2 and D_3 are transported to the liver by vitamin D-binding protein where 25-hydroxylase catalyzes the reaction of vitamin D_2 and vitamin D_3 to 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 (collectively referred to as 25(OH)D), respectively. 25-hydroxylase is a microsomal cytochrome P450 (CYP) enzyme of which there are several isoforms. Each of these isoforms can hydroxylate vitamin D_2 and vitamin D_3.[6] 25(OH)D has a longer half-life and higher concentration compared to vitamin D_2, vitamin D_3, and active hormones. The active vitamin D hormones are 1,25-dihydroxyvitamin D_2 and 1,25-dihydroxyvitamin D_3 (collectively referred to as 1,25$(OH)_2$D) and are produced from an additional hydroxylation by 1α-hydroxylase, mainly in the kidneys.[7] The proximal convoluted tubules in the kidneys are the main source of this enzyme; however, other tissues and cell types, like keratinocytes, have the ability to produce 1,25$(OH)_2$D.[4] 25(OH)D and 1,25$(OH)_2$D are both catabolized by 24-hydroxylase (CYP24A1) to inactive metabolites, such as 24,25-dihydroxyvitamin D_2 and 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2$D], and 1,24,25-trihydroxyvitamin D_2 and 1,24,25-trihydroxyvitamin D_3 [1,24,25$(OH)_3$D].[8]

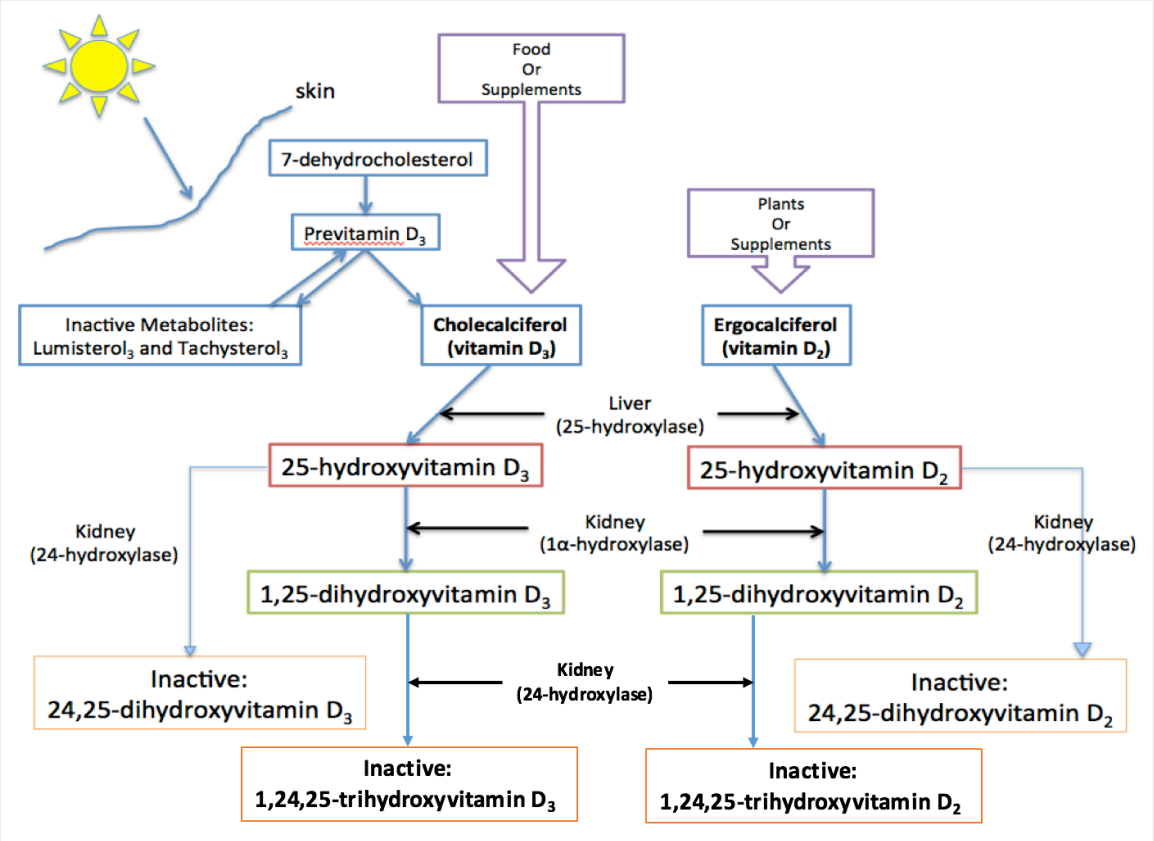

Figure 1.1. Vitamin D Pathway. Vitamin D_3 is produced after sunlight exposure and vitamin D_2 is consumed through the diet. They are hydroxylated in the liver to become 25(OH)D. The active hormone ($1,25(OH)_2D$) is produced from a second hydroxylation in the kidney. 25(OH)D and $1,25(OH)_2D$ are further hydroxylated to inactive metabolites $24,25(OH)_2D$ and $1,24,25(OH)_3D$.

Metabolite	Abbr./ Common Name	MS Assay?	Indication	Reference Range
Vitamin D_2	D_2/ Ergocalciferol	No	Supplementation compliance, diet, and sunlight exposure	N/A
Vitamin D_3	D_3/ Cholecalciferol	No		
25-hydroxyvitamin D_2	25(OH)D/ Calcidiol*	Yes	Vitamin D status	20-50 ng/mL †
25-hydroxyvitamin D_3	25(OH)D/ Calcidiol*	Yes		
1,25-dihydroxyvitamin D_2	$1,25(OH)_2D$/ Calcitriol**	Yes	(active hormone) chronic kidney disease	17-72 pg/mL †
1,25-dihydroxyvitamin D_3	$1,25(OH)_2D$/ Calcitriol**	Yes		

Table 1.1. Vitamin D metabolites with their abbreviations and common names. The table includes: whether or not a mass spectrometry assay existed prior to the publication of this thesis, how the metabolites are used, and reference ranges (if established). This table is not an exhaustive list of all vitamin D metabolites.
*Collectively referred to as 25(OH)D
**Collectively referred to as $1,25(OH)_2D$
† Reference Range from UW Medicine[1]

The classical function of $1,25(OH)_2D$ is to maintain calcium and phosphorous homeostasis through several mechanisms. $1,25(OH)_2D$ stimulates osteoclasts to resorb bone, enhances absorption of calcium and phosphate from the small intestine, and decreases calcium excretion in the kidneys. $1,25(OH)_2D$ also stimulates the endocrine system by binding to the vitamin D receptor (VDR). In addition to bone, intestine, and kidney, VDRs are found in many tissues such as the pancreas, parathyroid, skin, and mammary epithelium.[7] The widespread presence of VDRs suggests that $1,25(OH)_2D$ also has noncalcaemic or non-classical functions. The non-classical functions are not well understood and further research is needed to understand this complex pathway.

In a study by Bouillon, et al.,[6] VDR-deficient and vitamin D deficient mice showed an increased sensitivity to autoimmune diseases, an increased susceptibility to oncogene- and chemocarcinogenic-induced cancers, and an increased risk of

cardiovascular disease. Knockout mice experiments led Bouillon, et al. to estimate that nearly 3% of human genes may be regulated directly or indirectly by the actions of $1,25(OH)_2D$ on the endocrine system. While this is likely an overestimation, it does implicate $1,25(OH)_2D$ in a variety of cellular functions including growth regulation, DNA repair, differentiation, apoptosis, membrane transport, metabolism, cell adhesion, and oxidative stress.[6]

Despite promising animal studies, many cross-sectional studies have yielded variable results on vitamin D intake and disease pathology in humans, particularly those with oncology applications. Bouillon, et al., suggest that the variable results are a function of the variability in diet and vitamin D fortification of food. A limitation in many of these studies is the lack of sensitive and specific assays. The ability of studies to generate meaningful conclusions is contingent upon quality assays.

Historically, vitamin D_2 and D_3 were measured by competitive protein binding assays (CBPA), high-performance liquid chromatography (HPLC), and radioimmunoassay (RIA). [9-12] These assays are complicated with lengthy sample preparations and they lack specificity and sensitivity. Antibody-based techniques, such as CBPA, were unable to distinguish between vitamin D_2 and D_3, often times under- or over-estimating total vitamin D.[13] Immunoassays are practical for their high throughput ability and user-friendly platforms; however, interference from inactive vitamin D metabolites and vitamin D epimers (3-epi-25(OH)D) reduce accuracy. Additionally, RIA introduces problems related to handling of radioactivity and the radioactive labels have limited shelf-life. Though these methods may be good for screening, more reliable methods are needed for diagnostic purposes.

More recently, mass spectrometry has gained popularity in the clinical laboratory for small molecule analysis. Mass spectrometry is a highly specific method considered to be the "gold standard" for quantitation of many small molecules. Furthermore, liquid chromatography-tandem mass spectrometry (LC-MS/MS) increases specificity, sensitivity and reproducibility for vitamin D assays.[14]

Mass spectrometry separates molecules based on the mass to charge (m/z) ratio. The first mass spectrometer was developed in the early 20th century by Sir J.J. Thomson.[15] Thomson successfully separated ions based on their different parabolic trajectories in an electromagnetic field. Many individuals continued to make revolutionary discoveries, and mass spectrometry continued to evolve into the more complex instruments that are now used in many clinical laboratories. Most notably, John B. Fenn earned the 2002 Nobel Prize in Chemistry for the electrospray ionization of biomolecules, a technique used in the development of the vitamin D_2 and D_3 assay. Mass spectrometers can be broken down into four major components: the sample inlet, ionization source, mass analyzer, and ion detector (Figure 1.2). Numerous techniques and methods exist for each component; however, emphasis will be placed on the methods used during the development of the vitamin D_2 and D_3 assay in this thesis.

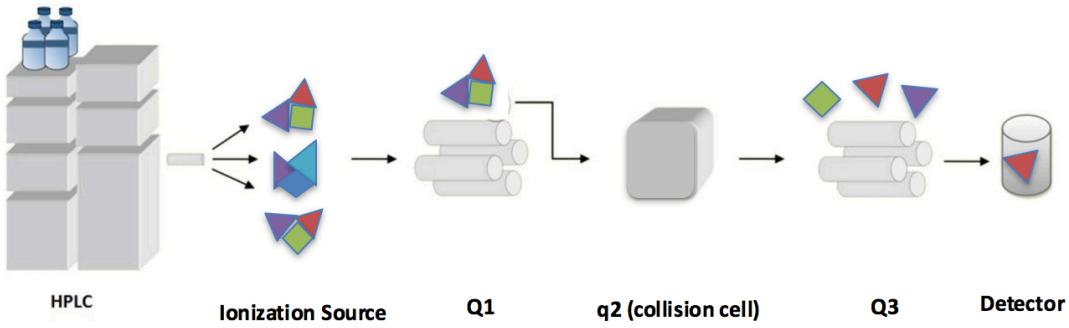

Figure 1.2. Liquid chromatography tandem mass spectrometry. The sample is separated by HPLC and ionized using electrospray ionization (ESI). Parent ions are selected based on the *m/z* ratio in the first quadrupole (Q1). The parent ions enter the second quadrupole, which functions as the collision cell (q2). Inert gas collides with the parent ion creating fragments or daughter ions. All fragments enter the third quadrupole and are separated by their *m/z* ratios. Ions passing through Q3 will make it to the detector.

Serum, plasma, or whole blood based LC-MS/MS methods typically include sample preparation to remove interfering matrix components, such as phospholipids. The vitamin D_2 and D_3 assay utilizes liquid-liquid extraction before samples are introduced to the column. Columns packed with silica particles are used to introduce a sample into the ionization source, in addition to separating sample components. The silica beads (with a non-polar coating) in the column comprise the stationary phase of chromatographic separation. Separation also relies on selecting the proper mobile phase. In the case of liquid chromatography (LC), a high-pressure liquid mobile phase forces the sample through the column. The stationary and mobile phase properties can be altered to exploit the chemical properties of the analyte of interest. The mobile phase flow rate and composition defines the amount of time the analyte is retained on the solid phase. The vitamin D_2 and D_3 assay utilizes a reverse-phase LC column and a gradient elution mobile phase. The reverse-phase LC column is non-polar, which tightly binds vitamin D_2

and D_3. The gradient mobile phase allows the user to control when vitamin D_2 and D_3 will separate from the other sample components. The gradient elution begins with a polar mobile phase and a non-polar mobile phase is gradually introduced. The non-polar properties in the mobile phase decrease the affinity of vitamin D_2 and D_3 from the stationary phase and the infusion pump delivers the sample to the ionization source, serving as the sample inlet.

Ionization is critical for analysis of molecules using mass spectrometry. Ionized particles can be selectively separated by the mass analyzer based on their mass-to-charge ratio (m/z). Many ionization techniques exist, some of which are: electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), matrix-assisted laser desorption ionization (MALDI), electron ionization (EI), and chemical ionization (CI). EI (used in gas chromatography mass spectrometry) and ESI are the most common ionization sources used for biomedical mass spectrometry. ESI is utilized for the vitamin D_2 and D_3 assay and will therefore be the ionization source detailed in this chapter. ESI uses a combination of voltage, heat, and vacuum to produce successively smaller droplets from liquid eluting off the column.[16] The voltage creates a spray of very small droplets. Excessive solvent is removed, concentrating the analytes and thereby increasing the charges accumulating on the droplet surface. The concentration of positive charges will cause a natural repulsion between charges, resulting in a Coulombic explosion. Ions that were once on the droplet surface are then liberated into the gas phase as the repulsion of the charges exceeds the forces of the surface tension, allowing the ions to enter into the mass spectrometer for analysis.

Once the sample components are ionized, the charged particles enter the mass analyzer. The ions are separated based on their mass-to-charge (m/z) ratio. Various methods of separation exist, some of which are: time of flight (TOF), quadrupole, quadrupole ion trap, and Fourier transform ion cyclotron resonance. Tandem mass analysis is the ability of an analyzer to isolate the parent ion, fragment the ion, and then detect the daughter or fragmented ion. The use of a tandem mass analyzer increases the specificity of the method and is utilized in the vitamin D_2 and D_3 assay. Quadrupoles have four parallel rods connected to a radio frequency (RF) generator and a DC potential.[15] These charges alternate at a set frequency such that balanced attraction and repulsion of the ion of interest maintains a stable flight path between the pairs of rods. The amount of positive or negative charge and the frequency at which the charges alternate is optimized for the m/z ratio of the ion. By using a tandem quadrupole instrument, the first quadrupole (Q1) is set to the parent ion (vitamin D_2 and vitamin D_3). The second quadrupole (q2) functions as a collision cell containing inert argon gas. The gas collides with parent ions, creating the daughter ions. During the method development phase, the collision energy of the argon gas is optimized for the daughter ions. The fragmented ions created in the second quadrupole pass on to the third quadrupole (Q3), which is set to the specific daughter ion m/z. The ions that pass through the third quadrupole will reach the detector. The specific parent and daughter ion pair is referred to as a transition.

Once the ions are separated in the mass analyzer, they are detected by creating a current signal. The most commonly used detector is the electron multiplier. An electron multiplier has a series of dynodes at increasing potentials that produce a cascade of

electrons. A dynode is an electrode in a vacuum tube, serving as an electron multiplier through secondary emission.[15] This allows for signal amplification, meaning a single incident ion will generate a very large signal. Other detectors that can be used are: Faraday cup, photomultiplier conversion dynode (PMT), array detector, and charge detector. The vitamin D_2 and D_3 assay uses a PMT detector which is similar to the electron multiplier in concept, but instead of a series of dynodes, a phosphorous screen is used. When ions impact the phosphorous screen, photons are released and detected by a photomultiplier. The PMT is sealed in a vacuum, resulting in a long detector lifetime. As with any method, there are disadvantage and advantages for each detector, but a detector that is robust, sensitive, and has a long lifetime is ideal in the clinical laboratory.

Although LC-MS/MS improves sensitivity and specificity compared with immunoassays and competitive protein binding assays, several limitations remain. Jeffrey Lai, et al.,[17] cites significant interlaboratory variations in LC-MS/MS measurements. Major factors for this variation were found to be interference from ion suppression and inactive isomers, and poor assay standardization. These limitations are addressed in the next several chapters, and the method development and validation of this assay is outlined in detail. This assay was developed and validated using the CLSI document C62-A *Liquid Chromatograph-Mass Spectrometry Methods*. Prior to its publication in October 2014, no other CLSI document existed which specifically addressed LC-MS methods. C62-A provides guidelines for method development, pre-validation, and validation of LC-MS assays. By using CLSI document C62-A throughout the entire process, we have ensured this method is reliable and highly reproducible. All experiments were thoughtfully designed and data were rigorously analyzed for acceptability. The purpose

of developing this assay was to add to the clinical picture especially in studies involving vitamin D supplementation and specific outcomes.

Defining vitamin D sufficiency and deficiency depends on the availability of robust assays. The relationship between the parent vitamin D (vitamin D_2 and D_3) and 25(OH)D is influenced by genetic variations in the cytochrome P450 enzymes, vitamin D-binding protein concentration, and disease states that alter the pathway. In populations where the vitamin D pathway is altered, measuring vitamin D_2 and D_3, may more accurately reflect the vitamin D status and may even help identify individuals with an abnormal vitamin D biometabolism. For example, if acquired disorders such as hyperparathyroidism lead to a reduced level of 25(OH)D, analyzing only 25(OH)D concentration may lead a clinician to incorrectly prescribe a higher dose of vitamin D supplementation. Measuring vitamin D_2 and D_3 provides the clinician with a more accurate indicator of vitamin D status. Furthermore, in certain populations where vitamin D metabolism is abnormal (i.e. chronic kidney disease patients) monitoring 25(OH)D or $1,25(OH)_2D$ to track response to vitamin D supplementation may not be useful. Instead, measuring vitamin D_2 and D_3 may more accurately reflect vitamin D supplementation compliance and response to treatment.[18]

Chapter 2:

Quantification of Vitamin D_2 and Vitamin D_3 in Human Serum to Evaluate Exposure

Abstract

Background: Vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) are the precursors to 25-hydroxyvitamin D [25(OH)D]. 25(OH)D currently serves as the marker for vitamin D status, but concentrations of 25(OH)D have more inter individual variability compared to vitamin D_2 and D_3. The concentration of 25(OH)D does not appear to increase drastically until a particular vitamin D_2/D_3 threshold is achieved. A superior assay is need to investigate the complex interaction between the precursors (vitamin D_2 and D_3) and 25(OH)D. The liquid chromatography-tandem mass spectrometry assay developed in this chapter can address these limitations.

Methods: Sample preparation consisted of alkalinization, liquid-liquid extraction using 90:10 n-heptane: ethyl acetate, and derivatization with 4-phenyl-1,2,4-triazoline-3,5-dione. Analytes were resolved using reversed-phase Pentafluorophenyl Propyl (PFPP) column and quantified using positive ion electrospray ionization–tandem mass spectrometry. Hexadeuterated vitamin D_2 and D_3 in methanol were used as internal standards. Method validation followed the guidelines outlined in CLSI document C62-A.

Results: Vitamin D_2 within-run imprecision (CV) was 6.6% and between-run imprecision (CV) was 6.9% from 0.13 to 84.79 ng/mL. Vitamin D_3 within-run imprecision (CV) was 9.0% and between-run imprecision (CV) was 9.3% from 0.12 to 88.81 ng/mL. The lower limit of measuring interval (LLMI) was 0.140 ng/mL for vitamin D_2 and 0.156 ng/mL for vitamin D_3. The analytical measurement interval (AMI) of the assay was linear over the range 0.07 to 130 ng/mL with a Pearson correlation (r^2) of 0.99 for D_2 and D_3. The average recovery was 88% for vitamin D_2 and 96% for vitamin D_3.

Conclusions: A fully validated sensitive, specific, and user friendly LC-MS/MS method was developed.

1. Introduction

Vitamin D is a key component of phosphorous and calcium homeostasis and adequate concentrations of vitamin D are necessary to prevent a variety of maladies, like childhood rickets or osteomalacia. Vitamin D deficiency has also been linked to cardiovascular disease, obesity, diabetes, autoimmunity, cancer, and all-cause mortality.[19] Vitamin D_3 is made from 7-dehydrocholesterol in the skin after exposure to UVB radiation in humans, or it is consumed through the diet. Vitamin D_2 is made in plants from the phototransformation of ergosterol after exposure to sunlight and is absorbed in the human GI tract during digestion. Both vitamin D_2 and D_3 are transported to the liver, mostly by vitamin D-binding protein, and are hydroxylated to 25-hydroxyvitamin D_2 ($25(OH)D_2$) and 25-hydroxyvitamin D_3 ($25(OH)D_3$). $25(OH)D_2$ and $25(OH)D_3$ are transported to the kidneys where they are enzymatically converted to the active hormones 1,25-dihydroxyvitamin D_2 and 1,25-dihyxroxyvitamin D_3 through an additional hydroxylation.[4] Currently, circulating levels of $25(OH)D_2$ and $25(OH)D_3$ (will henceforth be referred to collectively as 25(OH)D) are used to determine a patient's vitamin D status. There are several methods used by clinical laboratories to measure a patient's 25(OH)D levels with LC-MS/MS being the most sensitive and specific. Preliminary data shows vitamin D_3 seems to be a more precise and accurate measure of an individual's response to vitamin D_3 supplementation when compared to 25(OH)D concentration (unpublished data, collaboration between Hoofnagle and Kratz laboratories). In this study, 8 vitamin D deficient people received 4,000 IU/day of oral vitamin D_3 for 3

months and 7 vitamin D deficient people received 2,000 IU/day. Plasma vitamin D_3 and 25(OH)D concentrations were measured at baseline and at 3 months. Each dosing strategy resulted in an increase in both vitamin D_3 and 25(OH)D, but the vitamin D_3 increased in a dose response pattern and concentrations were less variable compared to 25(OH)D (see Figure 2.1). Many studies have demonstrated that 25(OH)D concentration is influenced by the concentration of vitamin D-binding protein, affinity of the vitamin D-binding protein to vitamin D, and by differences in gene expression of the vitamin D metabolizing P450 enzymes.[20-23] Due to this variability, vitamin D_3 may be more predictive of nutritional adequacy compared to 25(OH)D concentration.

Prior to the publication of CLSI document C62-A *Liquid Chromatography-Mass Spectrometry Methods*, the development of LC-MS/MS assays lacked standardization. C62-A provides a road map to developing LC-MS assays, emphasizing preverification, which is a quick way to test a newly developed method before allocating time and resources to a full verification. Preverification steps include basic performance parameters such as specificity and selectivity, matrix effects, carryover, and precision. The assay is then rigorously tested with the verification process. The verification includes determining the lower limit of the measuring interval (LLMI), linearity, imprecision, assay interference, and trueness. Using C62-A as a guide throughout vitamin D_2 and vitamin D_3 method development will facilitate reproducibility between laboratories.[16]

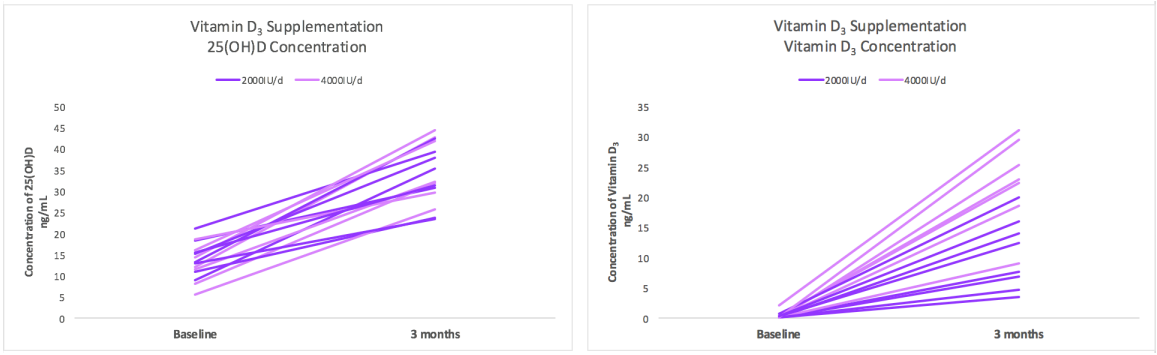

Figure 2.1 Eight vitamin D deficient people received 4,000 IU/day of oral vitamin D_3 daily for 3 months and 7 vitamin D deficient people received 2,000 IU/day daily for 3 months. The left graph is 25(OH)D measured at baseline and at 3 months for each dosing group. The right graph is plasma vitamin D_3 concentration measured at baseline and at 3 months. The vitamin D_3 concentrations at 3 months were statistically different between dosing groups (p=0.003).

2. Materials and methods

2.1 Chemicals and reagents

Chemicals and reagents were purchased from Fisher Scientific, unless otherwise noted. Stock internal standards [cholecalciferol-d6 (D_3-d6) and ergocalciferol-d6 (D_2-d6), Medical Isotopes] were prepared at 50 ng/mL in methanol and were diluted in methanol to make the working internal standard solutions at 5 ng/mL. The derivatizing reagent, 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD; Sigma- Aldrich), was prepared at 0.5 g/L in acetonitrile.

2.2 Sample Preparation

All samples were prepared in a 96-deep well plate (Greiner Bio-One, NC). Serum, calibrators, or control materials (200 µL) was added to 200 µL of 1 M sodium hydroxide. Samples were covered with a PTFE/silicone cover (Agilient) and vortexed for 15 s and then incubated for 15 min at room temperature (RT) before the addition of 200 µL of internal standard (5 ng/mL). Following, the plate was covered and vortexed for 15 s. Vitamin D_2 and vitamin D_3 were extracted with 1 mL 90:10 *n*-heptane: ethyl acetate.

Samples were covered and vortexed at maximum speed (speed 10) for 5 min and then centrifuged at 2000 rpm (493 *g*) for 4 min in a plate rotor (Beckman Coulter, CA). The plate was fitted with a liquid transfer gasket [24] and another 96-deep well plate. The bottom aqueous layer was frozen by placing samples in a dry-ice acetone bath, creating a vacuum sealing the two plates together. After 50 min, the organic layer was transferred to the second 96-well plate by inverting and tapping the two plates (see Figure 2.2). The organic layer was dried using nitrogen in a TurboVap (Biotage, NC, plate temperature 35°C, gas flow 30) for 40 min. The residue was derivatized with 100 µL PTAD (0.5 g/L).[25] The 96-deep well plate was covered, vortexed for 15 s, and incubated at RT for 15 min. HPLC-grade water (100 µL) was added to quench the PTAD.

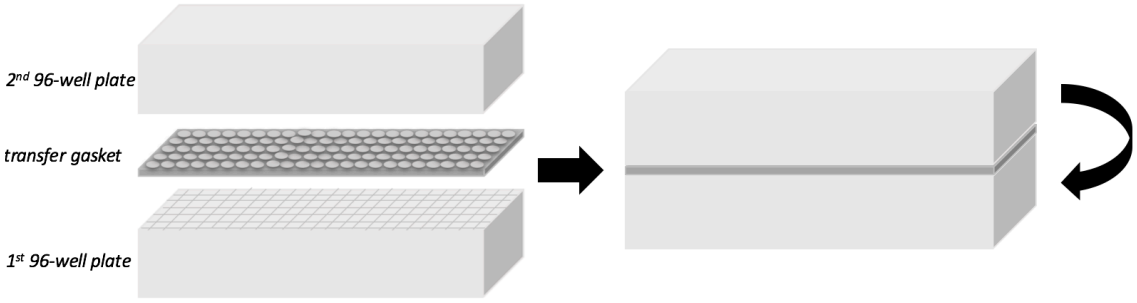

Figure 2.2. This figure represents the 96-deep well plate with prepared samples (bottom), fitted with a liquid transfer gasket and a second 96-deep well plate (top). Once the bottom aqueous layer is frozen, the plates are inverted. By tapping the plate, the organic layer is transferred to the second 96-deep well plate.

2.3 Sample Extraction

To improve vitamin D_2 and D_3 extraction, solvent mixtures (n=24) were created by mixing various concentrations of the following: *n*-heptane, ethyl acetate, toluene, and methyl tert-butyl ether. The resulting solvent mixtures had polarity indexes ranging from 0 to 4.4. Extraction efficiency and matrix effect both impact the recovery of vitamin D_2 and D_3. The ability for each solvent mixture to extract vitamin D_2 and D_3 was evaluated

using a serum pool (matrix) and spiking in 20 ng/mL vitamin D_2 and D_3 (in methanol) either before or after extraction. Spiking in vitamin D_2 and D_3 before extraction evaluates extraction efficiency, while spiking in vitamin D_2 and D_3 after extraction assesses matrix effect. An equivalent amount of methanol was spiked into the serum pool before analysis as a control ("unspiked").

To evaluate the solvents, two peak area ratio calculations were used. The first equation (1) determined the extraction efficiency *(prespiked-unspiked)/(postspiked-unspiked)*. The second equation (2) evaluated the matrix effect *[(postspiked-unspiked)-unextracted]/unextracted*. "Prespiked" examines the efficiency and reproducibility of the analyte extraction, "unspiked" accounts for the endogenous analyte in the pooled serum, "postspiked" examines the impact of matrix effect on the recovery of the analyte, and "unextracted" represents a matrix-free sample that is not affected by the extraction process.

Each solvent mixture was used in an unspiked, prespiked, and postspiked sample. To create the unextracted sample, we added the 20 ng/mL spike solution to our internal standard. The unextracted samples represent the best possible recovery for both vitamin D_2 and D_3.

2.4 Chromatography and mass spectrometry

The derivatized extract (40 μL) was injected onto a reversed-phase liquid chromatographic column (2.1 mm x 50 mm, 1.9 μm, 140 Å PFPP UPLC column; Restek). Using a linear gradient technique, the mobile phase progressed from 50% methanol, 0.1% formic acid (VWR), 0.5 mM methylamine (Sigma Aldrich) in water to 70% methanol, 0.1% formic acid, 0.5 mM methylamine in water over 3.5 min at a flow

rate of 0.4 mL/min. The column was cleaned with 95% methanol, 0.1% formic acid, 0.5 mM methylamine for 1 min at 1.0 mL/min and then re-equilibrated at the starting conditions for 1 min at 1.0 mL/min. Total instrument analysis time was 6 min per injection. Analytes were quantified using multiple reaction monitoring on a Waters Xevo triple quadrupole tandem mass spectrometer (transitions: vitamin D_3, 591.4>298.09, 298.1, and 298.11; vitamin D_2, 603.4>298.09, 298.1, and 298.11; vitamin D_3-d6, 597.4> 298.09, 298.1, and 298.11; vitamin D_2-d6, 609.4>298.09, 298.1, and 298.11). Quantification was performed using MassLynx 4.0 software (Waters) using integrated peak area ratios of vitamin D_2/D_2-d6 or vitamin D_3/ D_3- d6. Three transitions (298.09, 298.10, and 298.11 differing by 0.01 m/z centered around the nominal mass in Q3) were monitored for all analytes and internal standards to reduce the Poisson noise and improve assay precision (Table 2.1). Instrument-specific cone and collision parameters were derived experimentally (Table 2.2).

Transition Ion Monitoring		
	1 Transition CV%	**3 Transitions (sum) CV%**
Vitamin D_2	13.7%	5.9%
Vitamin D_3	13.6%	9.5%

Table 2.1 During method development we began monitoring three product ion transitions (298.09, 298.1, 298.11) for vitamins D_3, D_2, D_3-d6, and D_2-d6. By monitoring three ion transitions vs. one ion transition (298.1) we minimize Poisson noise and improve the CV (%) of the assay. Ten samples near the estimated 110% LLMI for vitamin D_2 and D_3 were used to generate the data. LLMI is a term used in C62-A to describe the lowest measurand concentration at which all defined performance characteristics are met.

Mass Spectrometer Parameters	
Capillary	3.50 kV
Desolvation Temperature	500°C
Desolvation Gas	1000 L/h
Cone Gas	30 L/h
Collision Gas Flow	0.15 mL/min

Table 2.2 Mass spectrometer parameters were determined empirically during method development on a Waters Xevo triple quadrupole tandem mass spectrometer. Parameters were optimized for the maximum performance of the assay.

2.5 Calibration and quality control

Calibrators contained vitamin D_2 (Medical Isotopes) and vitamin D_3 (Sigma Aldrich) at 0.096, 0.48, 0.96, 28.8, and 96 ng/mL. The two lowest concentration calibrators were made using fetal bovine serum (FBS; Gibco, Mexico), while the remaining calibrators were made using MSG4000 (Golden West Biologicals). Aliquots were stored at -20°C prior to use. Calibration curves were fit to a linear model with 1/x weighting.

2.6 Method validation

Evaluation of method performance included specificity and selectivity, ion suppression, carryover, lower limit of the measuring interval (LLMI), linearity, imprecision, interference, and trueness according to CLSI guideline C62-A, and are detailed below.

2.6.1 Specificity and Selectivity

To verify specificity and selectivity acceptability, FBS was used as the double-blank matrix due to nearly undetectable endogenous vitamin D_2 and D_3 concentrations. The lowest calibrator (standard F; 0.096 ng/mL) had the closest concentration to the expected LLMI. Specificity and selectivity were acceptable if background peaks in the double blank were absent or <20% of the peak area for the analyte at the LLMI or ≤ 5% of the internal standard at the expected retention time.

2.6.2 Matrix effects

Matrix effects and ion suppression were investigated in two ways. The first was an admixture study using 16 patient samples. Eight samples were designated "A" and eight samples were designated "B" (i.e. 1A, 1B, 2A, 2B, etc.). They were mixed together in the following concentrations: A (100%), A:B (80%:20%), A:B (50%:50%), A:B (20%:80%), and B (100%). The expected concentrations were compared to the observed concentrations. Matrix effects were considered insignificant if the slope of the observed vs. expected concentrations was 0.95-1.05, demonstrating assay parallelism and minimal ion suppression. The second experiment was a T-infusion experiment.[26, 27] Derivatized analyte was directly infused into the mass spectrometer to establish a stable total ion count (TIC). Two different extracted serum pools each were injected while simultaneously infusing derivatized analyte. The TIC was inspected visually to evaluate for ion suppression at analyte retention times.

2.6.3 Carryover

Carryover was assessed by injecting 10 replicates of a low-concentration sample (0.11 ng/mL vitamin D_2 and 0.13 ng/mL vitamin D_3) and calculating the mean. In the same run, we alternately injected five replicates of a single high-concentration sample (182.1 ng/mL vitamin D_2 and 179.4 ng/mL vitamin D_3) followed by the low-concentration sample (0.11 ng/mL vitamin D_2 and 0.13 ng/mL vitamin D_3) and calculated the mean of the low concentration samples. Means of the first and second set of low-concentration samples were compared by Student's *t*-test. A bias of <10% was used to define acceptable carryover (i.e., <0.006% carryover).

2.6.4 Analytical sensitivity

The LLMI was determined from 40 replicates of five samples close to our hypothesized limit of detection, over five runs. The concentrations tested were 0.05, 0.10, 0.125, 0.15, and 0.25 ng/mL. The CV was calculated for each concentration and plotted on the y-axis with expected concentration on the x-axis. The LLMI was considered acceptable at a total allowable imprecision (CV_{total}) of 20%.

2.6.5 Analytical measuring interval

The analytical measuring interval (AMI) is defined by the concentration range that sustains linearity when quantified by the assay. In the same run, 11 samples were tested in quadruplicate. The sample with the lowest concentration (0.07 ng/mL) was created by spiking fetal bovine serum (FBS) with unlabeled vitamin D_2 and D_3. The sample with the highest concentration (130 ng/mL) was created by spiking MSG4000 with unlabeled vitamin D_2 and D_3. The low and high concentration samples were mixed in various amounts to create a series of nine evenly-spaced concentrations (13.06, 26.06, 39.05, 52.04, 65.04, 78.03, 91.02, 104.01, and 117.01 ng/mL for vitamin D_2 and D_3). The AMI was defined as (1) the concentrations at which the recovery was 80-120% and all other performance characteristics were met, (2) the concentrations at which Pearson correlation coefficient (r^2) of observed vs. expected concentrations was ≥ 0.99, and (3) the concentrations across which least squares regression using a power function (i.e., $y=Ax^n$, where y is the observed concentration and x is the expected concentration) resulted in an exponent between 0.95 and 1.05.

2.6.6 Precision

Within-run imprecision, between-day imprecision, and CV_{total} were determined using five

replicates of samples at four different concentrations (0.13, 0.98, 45.25, and 84.79 ng/mL for vitamin D_2; 0.12, 0.99, 48.84, and 88.81 ng/mL for vitamin D_3) spanning the AMI tested on each of five days. A CV_{total} of 20% was considered acceptable near the LLMI, and a CV_{total} of 15% was considered acceptable for all other concentrations.

2.6.7 Interferences

Assay interference from conjugated or unconjugated bilirubin, hemolysis (20 mg/dL), triglycerides (1000 mg/dL), hemoglobin (500 mg/dL), and protein (12 g/dL) was assessed using patient sera spiked using the ASSURANCE Interference Test Kit (Sun Diagnostics). Creatinine interference was assessed by mixing three patient samples with high creatinine concentrations (9.0, 10.3, and 13.4 mg/dL) with a low creatinine concentration patient pool. The high creatinine samples had low endogenous vitamin D_2 (below the LLMI) and vitamin D_3 (0.69-1.39 ng/mL). The low creatinine samples were spiked with unlabeled vitamin D_2 (17.65-18.33 ng/mL) and vitamin D_3 (33.09-33.94 ng/mL). A bias of < 15% between adultered and unadultered specimens was considered acceptable. To test for analytical interference with the isobaric inactive metabolites lumisterol$_2$, lumisterol$_3$, tachysterol$_2$, and tachysterol$_3$ we derivatized 20 ng/mL of each metabolite in triplicate to ensure that each could be derivatized and ionized (data not shown). We then spiked the 28.8 ng/mL calibrator with 5% total volume of a high- or low-concentration stock solution (each analyte at 600 ng/mL or 20 ng/mL in MSG4000, respectively) to achieve a final concentration of 30 ng/mL and 1 ng/mL, for all four potentially interfering metabolites. A recovery of 80-120% for vitamin D_2 and D_3 in the spiked calibrator was considered acceptable.

2.6.8 Trueness

Trueness was established using a spike and recovery experiment with a patient pool and three spiking solutions at: 1, 5, and 30 ng/mL. Stock solution concentrations of vitamin D_2 (10.4 μg/mL) and vitamin D_3 (12.0 μg/mL) were confirmed by uv-spectrophotometry (Beckman Coulter, CA) at 265 nm with extinction coefficients of 18,900 for vitamin D_2 and 18,300 vitamin D_3.[28] These stock solutions and spiking solutions were made separately from the calibrators and calibrator stock solutions. A recovery of 80-120% was considered acceptable.

2.6.9 Robustness testing

To evaluate the robustness of the method, our validation experiments spanned three columns with different lot numbers. The retention time, peak height, peak width, and resolution of the system suitability samples were used to additionally evaluate column performance. The method was considered robust if the retention time between columns did not vary more than ±2.5%.[29]

2.6.10 Stability and tube type appropriateness

Stability of endogenous analytes was determined for multiple tube types from three different individuals: red top (standard serum tube), gold top (serum with gel separator), purple top (EDTA anticoagulant, no gel separator), and green top (lithium heparin anticoagulant, no gel separator). Fresh serum from the red top tubes were used as the comparator for tube-type acceptability and analyte stability. Tube-types were evaluated to ensure the anticoagulant and/or gel separators did not cause interference. Stability of vitamin D_2 and D_3 was evaluated at 48 h over the following temperatures: room temperature, 2-8°C, -20°C, and -80°C or at 90 d (-20°C). Freeze-thaw stability was

evaluated over three freeze-thaw cycles. Bias <15% comparing fresh serum in the red top tubes to all other tube types at each temperature was considered acceptable.

Stability of the extracts was determined after evaporation (stored in the plate at -20°C) and after reconstitution (post-preparative; stored in the plate on the autosampler overnight). The post-preparative samples were prepared in duplicate on day 1. One replicate was analyzed on day 1 and day 2, testing analyte stability when exposed to air (pierced). The other replicate was only analyzed on day 2 and remained sealed (unpierced). Bias <15% comparing day 1 to either pierced or unpierced was considered acceptable.

3. Results

3. 1 Sample Extraction

The most effective extraction mixture was 90:10 *n*-heptane: ethyl acetate. This extraction mixture maximized extraction efficiency while minimizing ion suppression. The final extraction solvent mixture had an extraction efficiency of 65% for vitamin D_2 and 62% vitamin D_3 and matrix effect of 4% for vitamin D_2 and 3% for vitamin D_3 (Figure 2.3).

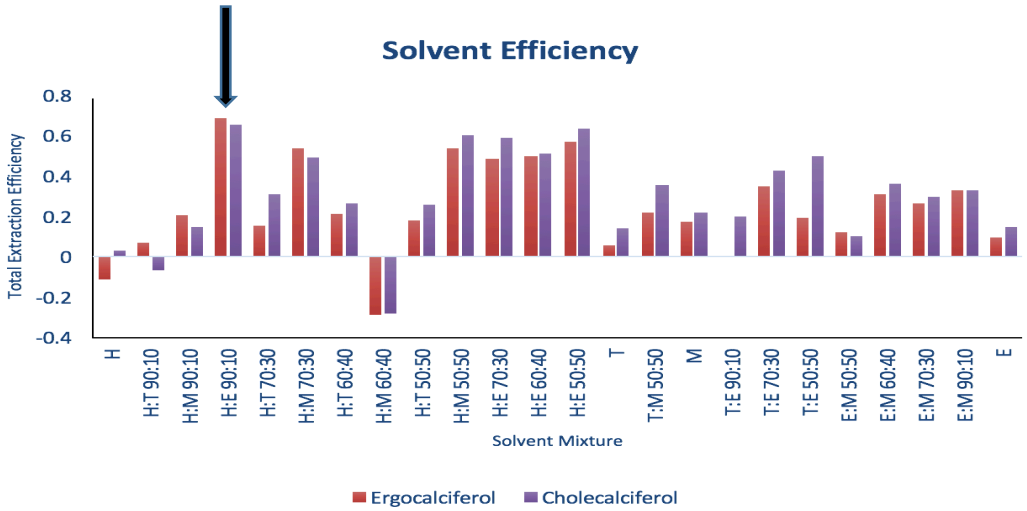

Figure 2.3. Solvent efficiency was highest with 90:10 *n*-heptane: ethyl acetate. Several solvent mixtures (n=24) were evaluated for the extraction efficiency of vitamin D_2 and vitamin D_3. Extraction efficiency was calculated using the equation *(prespiked-unspiked)/(postspiked-unspiked)*. Matrix effects (bias) were calculated using the equation *[(postspiked-unspiked)-unextracted]/unextracted*. The direction of the bias (positive vs. negative) dictated if it was added or subtracted from extraction efficiency. The arrow denotes the selected extraction solvent.

3.2 Assay Performance

To assess specificity and selectivity, we assayed FBS as a double blank matrix. A double blank matrix should have undetectable vitamin D_2 and D_3 and does not contain internal standard. Essentially, this is a sample that does not produce a signal in the transitions we are monitoring. Background peak areas in FBS were 1.02% of standard F (0.096 ng/mL) for vitamin D_2 and 7.28% of standard F for vitamin D_3. Background peaks in the internal standard (IS) areas were 0.03% for both vitamin D_2 and D_3 (Table 2.3) indicating that the assay had acceptable specificity and selectivity.

Specificity and Selectivity				
	Vitamin D_2		Vitamin D_3	
	Area	*IS Area*	*Area*	*IS Area*
Mean FBS	3.0	17.7	19.7	14.0
Standard F	293	55407	270	44587
% of FBS/Standard F	1.02%	0.03%	7.28%	0.03%

Table 2.3. Specificity and selectivity of the method were determined by measuring noise in a double blank. Fetal bovine serum (FBS) and the low standard (standard F; 0.096 ng/mL) was selected to represent a sample near the LLMI. Specificity was calculated by taking the signal generated from the FBS sample and divided it by the signal generated from standard F for either vitamin D_2 or D_3. This "% of FBS/standard F" should be relatively low in the transitions monitored, indicating specificity and selectivity of the assay.

Ion suppression was tested by LC-MS/MS analysis of an extracted serum pool and extracted FBS along with a post-column infusion of derivatized vitamin D_2 and D_3. No significant ion suppression was observed at the retention time of the analytes (Figure 2.4). Minimal matrix ion suppression was compensated for by use of a coeluting internal standard when analyte/internal standard ratios were used for quantitation. The second experiment evaluating matrix effects was the admixing study of 8 pairs of patient samples, creating 40 total samples. To assess linearity, all pairs were plotted on one graph and evaluated the slope and correlation coefficient (r^2) for vitamin D_2 and vitamin D_3. The slopes for the observed concentrations (y-axis) vs. the expected concentrations (x-axis) were 1.02 for vitamin D_2 and 0.99 for vitamin D_3. The r^2 values were 0.99 for both analytes (see Figures 2.5 and 2.6). Average recovery from the admixing study was 88% for vitamin D_2 and 96% for vitamin D_3. These two experiments indicate minimal ion suppression observed for this method.

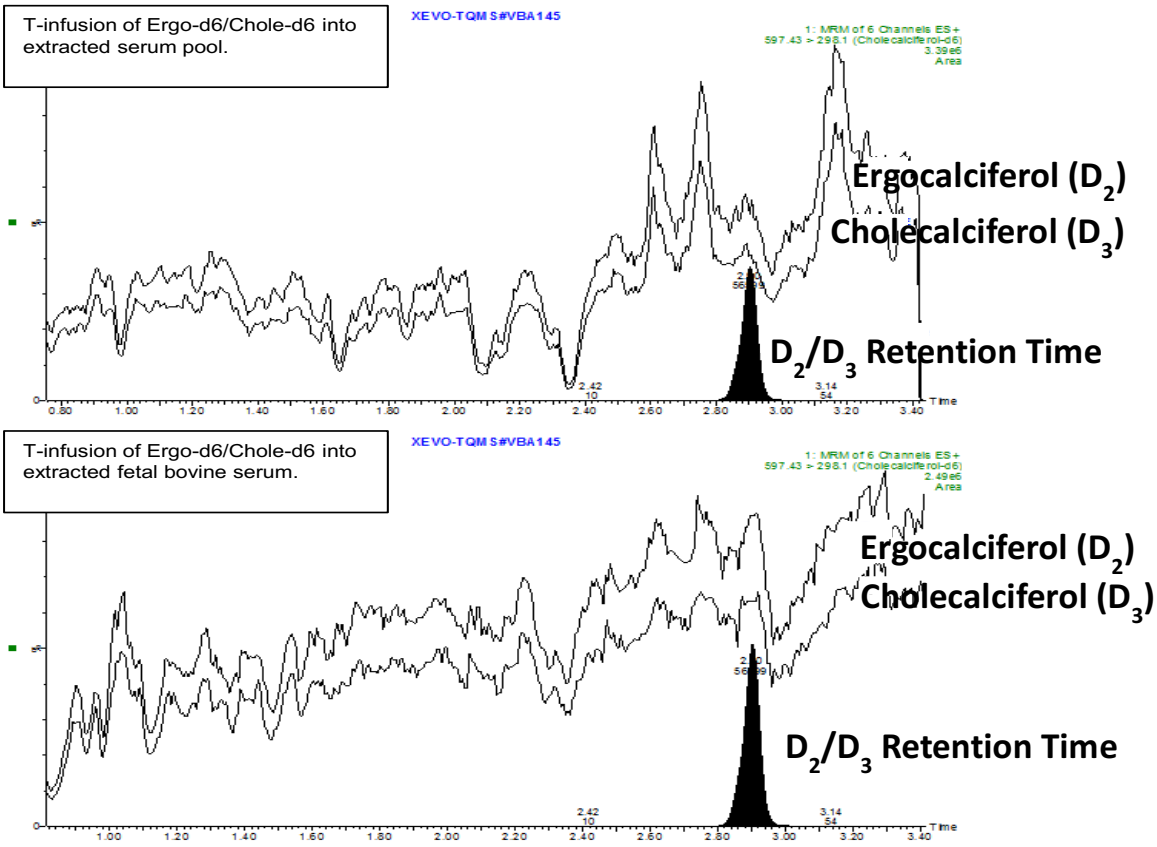

Figure 2.4 Derivatized vitamin D_2 and D_3 were directly infused into the mass spectrometer to establish a stable total ion count (TIC). The top figure illustrates an extracted serum pool and the bottom figure shows an extracted fetal bovine serum (FBS) sample. Each serum pool was injected concurrently while infusing derivatized vitamin D_2 and D_3, and the TIC was observed for any decrease in signal to indicate ion suppression. For reference, a representative chromatogram of vitamin D_3/D_2 is overlaid in solid black.

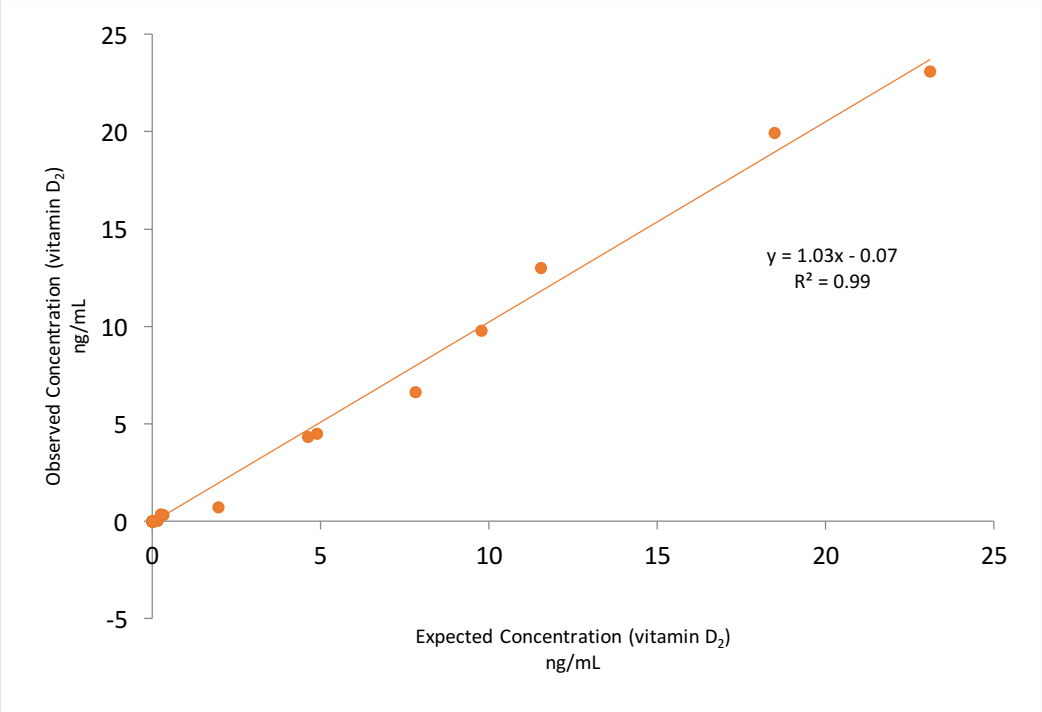

Figure 2.5 Vitamin D_2 admixing study. Patient samples (n=16) were mixed in specific ratios. Eight samples were designated "A" and eight samples were designated "B" (i.e. 1A, 1B, 2A, 2B, etc.). They were mixed together in the following concentrations: A (100%), A:B (80%:20%), A:B (50%:50%), A:B (20%:80%), and B (100%). The vitamin D_2 concentration in many samples was below the LLMI, accounting for the limited data points in this figure. The average recovery for vitamin D_2 was 88% (n=40).

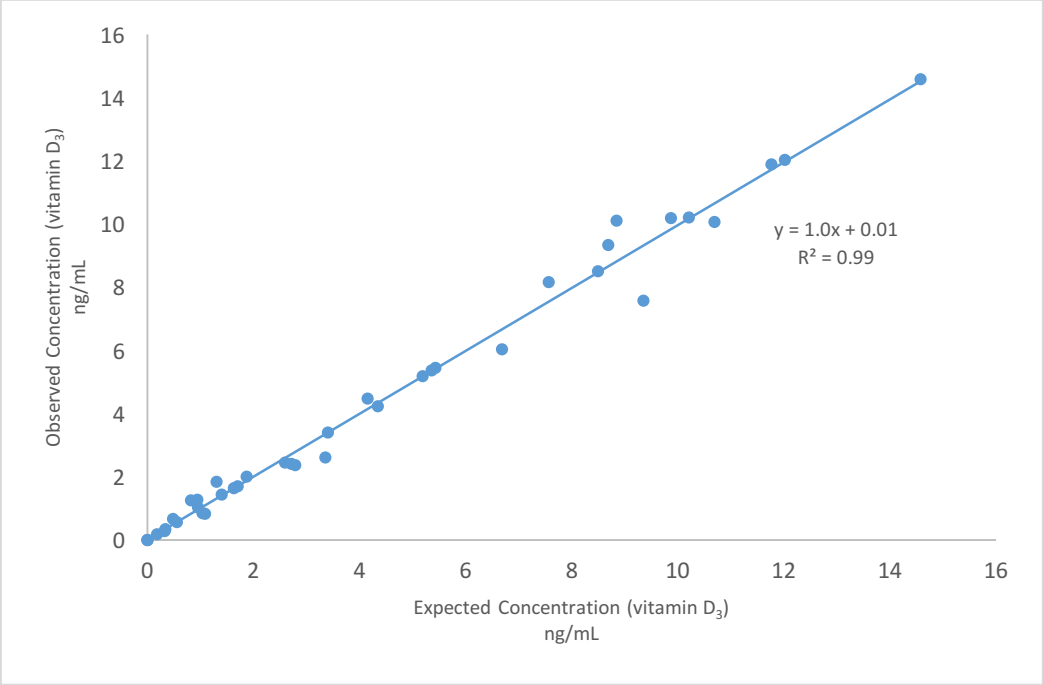

Figure 2.6 Vitamin D_3 admixing study. Patient samples (n=16) were mixed in specific ratios. Eight samples were designated "A" and eight samples were designated "B" (i.e. 1A, 1B, 2A, 2B, etc.). They were mixed together in the following concentrations: A (100%), A:B (80%:20%), A:B (50%:50%), A:B (20%:80%), and B (100%). The average recovery for vitamin D_3 was 96% (n=40).

Carryover was 0.044% for vitamin D_2 and 0.039% for vitamin D_3 ($p<0.01$ for both). Any sample that is between the LLMI (0.140 ng/mL for vitamin D_2 and 0.156 ng/mL for vitamin D_3) and 1.1 ng/mL and immediately follows a sample >25 ng/mL will have a positive bias of >10%. The clinical relevance of vitamin D_2 and D_3 concentrations at the LLMI are currently unknown. These samples should be repeated following a blank to eliminate bias due to carryover.

The LLMI was 0.140 ng/mL for vitamin D_2 and 0.156 ng/mL for vitamin D_3 (Figures 2.7 and 2.8). Total imprecision was evaluated using a CV_{total} of 20%, which is a generally acceptable imprecision for the LLMI of most laboratory assays. CV_{total} is expressed as a percentage and Jones defines CV_{total} as $(CVa^2 + CVi^2)^{1/2}$. [30] CVa is the analytical coefficient of variation and CVi is the within-person biological variation. No biological

variation studies exist for vitamins D_2 and D_3 so we modified the CV_{total} calculation to be *[[(mean within run CV)^2 + (mean between run CV)^2]$^{1/2}$]*.

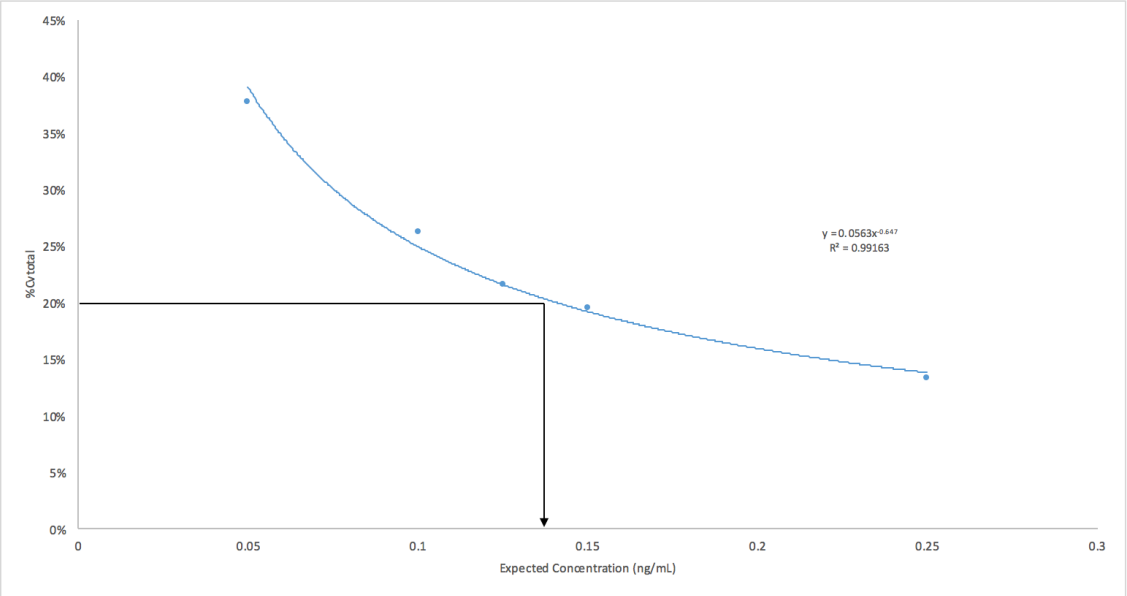

Figure 2.7 The LLMI for vitamin D_2 was derived by testing five concentrations near the expected LLMI in replicates of eight for five days. The CVtotal (%) (y-axis) vs. expected sample concentration (x-axis) was plotted and a trend line was added. The CVtotal (%) acceptability for this assay is 20%. LLMI for vitamin D_2 is 0.140 ng/mL.

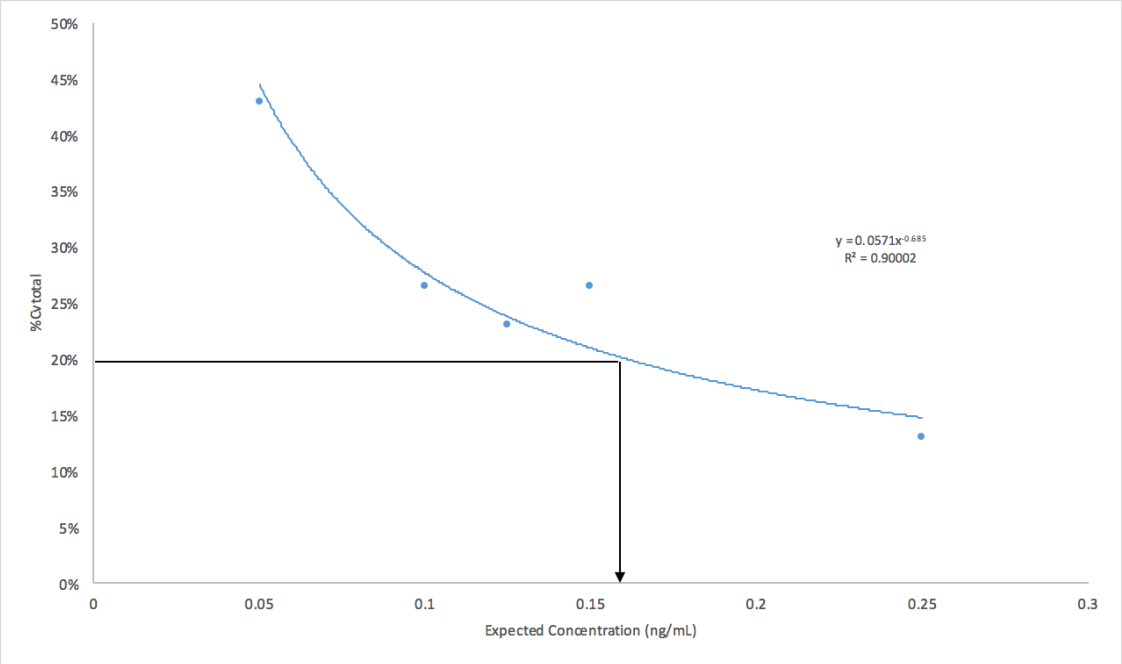

Figure 2.8 The LLMI for vitamin D_3 was derived by testing five concentrations near the expected LLMI in replicates of eight for five days. The CVtotal (%) (y-axis) vs. expected sample concentration (x-axis) was plotted and a trend line was added. The CVtotal (%) acceptability for this assay is 20%. LLMI for vitamin D_3 is 0.156 ng/mL.

The AMI of the assay was confirmed between 0.140-130 ng/mL for vitamin D_2 and 0.156-130 ng/mL for vitamin D_3. The recovery for each concentration ranged from 98-115%. The data was fitted with a power curve and the exponent was 0.97 and 0.96 for vitamin D_2 and D_3, respectively. The correlation coefficient (r^2) of expected vs. observed concentrations was 0.99 for both vitamin D_2 and D_3. The y-intercepts for each analyte were not statistically different from zero (p=0.56 for vitamin D_2 and 0.07 for vitamin D3; Figures 2.9 and 2.10).

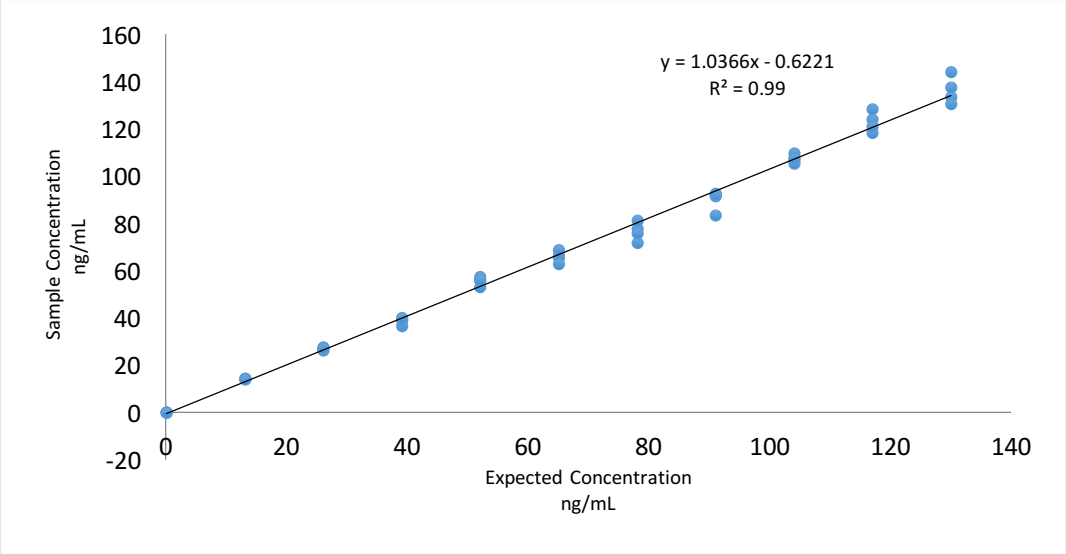

Figure 2.9 Vitamin D_2 is linear from 0.140-130 ng/mL. In the same run, 11 samples were tested in quadruplicate. The sample with the lowest concentration (0.07 ng/mL) was created by spiking fetal bovine serum (FBS) with unlabeled vitamin D_2 and D_3. The sample with the highest concentration (130 ng/mL) was created by spiking MSG4000 with unlabeled vitamin D_2 and D_3. The low and high concentration samples were mixed in various amounts to create nine evenly-spaced concentrations. Observed concentration (y-axis) vs. expected concentration (x-axis) is plotted and fitted with a linear regression line. The y-intercept is not statistically different from zero (p=0.56).

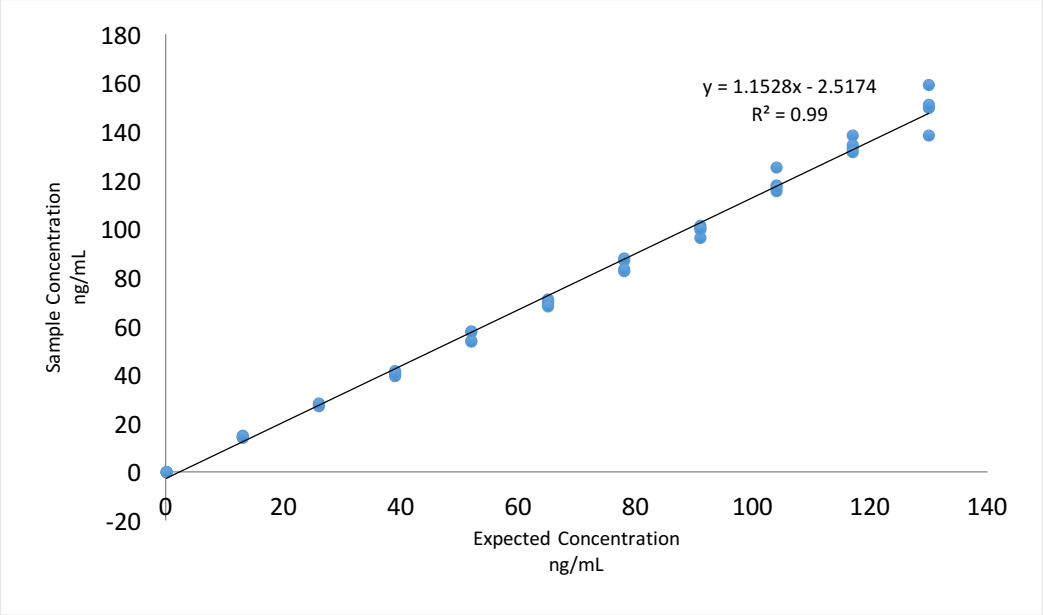

Figure 2.10 Vitamin D_3 is linear from 0.156-130 ng/mL. In the same run, 11 samples were tested in quadruplicate. The sample with the lowest concentration (0.07 ng/mL) was created by spiking fetal bovine serum (FBS) with unlabeled vitamin D_2 and D_3. The sample with the highest concentration (130 ng/mL) was created by spiking MSG4000 with unlabeled vitamin D_2 and D_3. The low and high concentration samples were mixed in various amounts to create nine evenly-spaced concentrations. Observed concentration (y-axis) vs. expected concentration (x-axis) is plotted and fitted with a linear regression line. The y-intercept is not statistically different from zero (p=0.07).

Assay imprecision is shown in Table 2.4. The observed concentrations at which precision was evaluated ranged from 0.13 to 84.79 ng/mL for vitamin D_2 and 0.12 to 88.81 ng/mL for vitamin D_3. Within-run imprecision CVs were 2.3% to 14% for vitamin D_2 and 3.5% to 22% for vitamin D_3. Between-run imprecision CVs were 2.4% to 14.6% for vitamin D_2 and 4.4% to 19.5% for vitamin D_3. While the CV_{total} for vitamin D_3 exceeds the 20% acceptability, the level 1 concentration is lower than the LLMI of 0.156 ng/mL.

Within-Run and Between-Run Total Imprecision					
Sample	**Within-Run Mean (ng/mL)**	**Within-Run CV(%)**	**Between-Run Mean (ng/mL)**	**Between-Run CV(%)**	**CV total(%)**
Ergocalciferol (D_2)					
Level 1	0.12	14.0 (0.02)	0.12	14.6 (0.02)	20.2 (0.02)
Level 2	0.96	6.1 (0.06)	1.02	6.4 (0.06)	8.9 (0.06)
Level 3	44.57	2.3 (1.02)	45.67	2.4 (1.09)	3.3 (1.06)
Level 4	82.48	4.1 (3.46)	86.74	4.3 (3.67)	5.9 (3.57)
Cholecalciferol (D_3)					
Level 1	0.10	22.0 (0.03)	0.12	19.5 (0.02)	29.2 (0.02)
Level 2	0.94	6.8 (0.07)	0.97	9.2 (0.09)	11.4 (0.08)
Level 3	47.97	3.5 (1.68)	48.22	4.5 (2.21)	5.7 (1.94)
Level 4	88.05	3.5 (3.14)	90.39	4.4 (3.86)	5.6 (3.50)

Table 2.4 Four concentrations of samples were made using fetal bovine serum (FBS) or MSG4000 and spiked with unlabeled vitamin D_2 and D_3. Each level was tested in replicates of five each day, for five days. Total CV (%) is $[[(\text{mean within run CV})^2 + (\text{mean between run CV})^2]^{1/2}]*100$. Each CV% is followed by the standard deviation in parentheses.

Possible interference by total protein, triglycerides, hemolysis, unconjugated bilirubin, conjugated bilirubin, and creatinine was evaluated. None of these substances interfere at the concentrations tested (Table 2.5).

Bias (%) of interfering analyte			
Interference	**Concentration tested**	**Ergocalciferol (D_2)**	**Cholecalciferol (D_3)**
Protein	12 g/dL	-2%	-1%
Triglyceride	1000 mg/dL	5%	-4%
Hemolysate	500 mg/dL	-3%	-1%
Unconj. Bili	20 mg/dL	-1%	-3%
Conj. Bili	20 mg/dL	7%	-2%
Creatinine	13.42 mg/dL*	-1%†	-1%†

Table 2.5 Assay interference (except creatinine) was assessed using patient serum spiked using ASSURANCE™ Interference Test Kit. Creatinine interference was assessed using a mixing study involving three patient samples with high creatinine concentrations and a patient pool low in creatinine. The high creatinine samples were low in vitamin D_2 and D_3 concentration and the low creatinine serum pool had high vitamin D_2 and D_3 concentration. The expected concentrations of vitamin D_2 and D_3 were calculated and compared against the observed vitamin D_2 and D_3 concentrations. Percent bias was calculated using: *[(observed-expected)/expected]*100*.
*Creatinine interference assessed over many concentrations, the highest tested was 13.42 mg/dL.
† Bias at all concentrations were averaged.

Possible isobaric interference from lumisterol and tachysterol was analyzed and found not to interfere at the concentrations listed in Table 2.6 The average recovery was 94% for vitamin D_2 and 99% for vitamin D_3.

Recovery (%) of Standard B			
		Ergocalciferol (D_2)	**Cholecalciferol (D_3)**
1 ng/mL spike	Lumisterol	94%	97%
	Tachysterol	93%	100%
30 ng/mL spike	Lumisterol	93%	98%
	Tachysterol	97%	99%

Table 2.6 Isobaric interference by lumisterol$_2$/lumisterol$_3$ (lumisterol) and tachysterol$_2$/tachysterol$_3$ (tachysterol) was evaluated using two spike solutions at 20 and 600 ng/mL. Spike solutions were prepared with MSG4000 and 1 µg/mL stock solutions of lumisterol and tachysterol, separately. A 10 µL volume of each spike solution was added to an aliquot of standard B pre-extraction. The final spike concentrations tested were 1 and 30 ng/mL for both lumisterol and tachysterol. The recovery of standard B was calculated by dividing the concentration of vitamin D_2 and D_3 from the spiked sample by the concentration of vitamin D_2 and D_3 from standard B without the spike.

In assessing trueness, one evaluates accuracy or mean systematic error. Using a recovery calculation allows us to determine if the observed concentration is near the expected concentration. Certified reference materials are not available for vitamin D_2 and D_3 so trueness was instead evaluated using a spike and recovery experiment. For all three spiking concentrations tested, the recovery was 89% to 90% for vitamin D_2 and 108% to 114% for vitamin D_3 (see Table 2.7). The stock solutions used to create the spiking solutions were confirmed spectrophotometrically and were separate than the stock solutions used to create the calibrators.

Recovery (%)		
	Vitamin D_2	**Vitamin D_3**
1ng/mL spike	89%	114%
5ng/mL spike	90%	113%
30ng/mL spike	89%	108%

Table 2.7 Trueness of the vitamin D_2 and D_3 assay was evaluated using three spike solutions at 20, 100, and 600 ng/mL. Spike solutions were prepared with MSG4000 and unlabeled vitamin D_2 and D_3. Aliquots of a patient pool (n=15) were analyzed to establish native vitamin D_2 and D_3 concentrations. MSG4000 was also analyzed in replicates of five to determine initial vitamin D_2 and D_3 concentrations. A 20 µL volume of each spike solution was added to an aliquot of the serum pool pre-extraction, in replicates of five. The final spike concentrations tested were 1, 5, and 30 ng/mL. Percent recovery was calculated by: *[(pre-extraction spike-native)/expected concentration]*100*.

Freeze-thaw stability results are listed in Table 2.8. QC samples frozen at -20°C for 24hrs were used as the comparison for bias calculations. There was no evidence of degradation of vitamin D_2 and D_3 after 3 freeze-thaw cycles.

Freeze-Thaw Stability				
	2nd Freeze-Thaw Cycle (-20°C)		3rd Freeze-Thaw Cycle (-20°C)	
	D_2	D_3	D_2	D_3
QC Low	-9%	2%	3%	-6%
QC High	-7%	-5%	10%	-11%

Table 2.8 Freeze-thaw stability study was completed using low and high QC samples tested in triplicate over three days. Samples frozen at -20°C for 24hrs were used as the comparison for bias calculations. Low QC samples were made with fetal bovine serum (FBS) and high QC samples were made with MSG4000. Unlabeled vitamin D_2 and D_3 were spiked into each low and high serum pool.

The influence of tube type on measured vitamin D_2 and D_3 is listed in Table 2.9. All three tube types evaluated (gold top, purple top, and green top) had a bias of 2%, -3%, and -3%, respectively, compared to serum collected in red top tubes (no anticoagulant; n=3). The data from the temperature stability study conducted with these tubes can be found in Table 2.10. Vitamin D_2 concentrations were well below the LLMI in all individuals and the data was not included. It is not unexpected that vitamin D_2 concentrations in individuals is below the LLMI. The tube types evaluated were all acceptable for use with this assay and the analyte stability at the various storage conditions was satisfactory.

Tube Type	Bias (%)
Serum Separator Tube (gel)	2%
EDTA (no gel)	-3%
Lithium Heparin (no gel)	-3%

Table 2.9 Serum was collected from three individuals. Serum collected in red top (no anticoagulant or gel) tubes was used as the comparator. Gold top (serum separator with gel), purple top (EDTA without gel), and green top (lithium heparin without gel) tubes were evaluated. Gel separators allow cells and serum/plasma to be separated after centrifugation. Concentrations of vitamin D_2 were below LLMI and not included in this data. Percent bias of vitamin D_3 was determined by: *[("tube type"-red)/red]*100*.

Temperature Stability Study of Vitamin D_3				
Tube Type	**RT**	**2-8°C**	**Frozen (-20°C)**	**Frozen (-80°C)**
No anticoagulant (no gel)	3%	0%	-8%	-10%
Serum Separator Tube (gel)	1%	-2%	-12%	-11%
EDTA (no gel)	-1%	-2%	-12%	-8%
Lithium Heparin (no gel)	3%	1%	-7%	-6%

Table 2.10 Serum was collected from three individuals into red top (no anticoagulant or gel), gold top (serum separator with gel), purple top (EDTA without gel), and green top (lithium heparin without gel) tubes. The freshly collected samples were evaluated after remaining at various temperatures for 48 hours. Gel separators allow cells and serum/plasma to be separated after centrifugation. Measurements at t_0 from each tube was used as the comparison for bias calculations. Concentrations of vitamin D_2 were below LLMI and not included in this data. Percent bias for vitamin D_3 was determined by: *[("temperature"-fresh)/fresh]*100.*

Results from evaluating plate stability are listed Table 2.11. The average bias for vitamin D_2 and D_3 for each set of samples was less than 15%. Samples remain stable through freezing and after 24hrs in the analyzer.

Post-Preparative Stability		
	Vitamin D_2	**Vitamin D_3**
Post-Preparative Plate (pierced)	13%	10%
Post-Preparative Plate (unpierced)	-7%	9%
Frozen Plate	-10.6%	5.3%

Table 2.11 Patient samples (n=24) were prepared in duplicate and on two separate 96-deep well plates. All samples were spike with unlabeled vitamin D_2 and D_3. One plate contained two sets of patient samples and only the first set of samples were analyzed on day 1 and again on day 2. These samples are represented as "post-preparative plate (pierced)." The second set of samples were analyzed only on day 2 and the bias was calculated using results from day 1. These samples are represented as "post-preparative plate (unpierced)." The frozen plate was prepared day 1, but frozen after evaporation. On day 8, the frozen plate was evaporated again and the same procedure was followed through analysis. The percent bias for each was calculated using concentrations*:[(post-preparative - fresh)/fresh]*100*. The average bias for vitamin D_2 and D_3 was calculated for each set of samples.

4. DISCUSSION

This novel vitamin D_2 and D_3 LC-MS/MS assay was fully validated and rigorously tested according to CLSI C62-A. While many LC-MS/MS are subject to interference from phospholipids, we have proven minimal matrix interference with our post-column infusion experiment and admixing study. We also optimized our extraction method using innovative equations to evaluate the extraction efficiency of 24 distinctive solvent mixtures. Challenges faced during the development of a novel assay include, but are not limited to, a lack of certified reference materials and individual biological variation studies to aid in the evaluation of trueness and total imprecision acceptability. Despite these challenges, we used alternate methodologies to prove trueness, and scholarly articles to define an acceptable CV_{total} for this assay. Ruggedness of our assay was evaluated using columns with different lots, and we demonstrated acceptable suitability and stability conditions through multiple experiments.

Vitamin D assays are certainly not novel; however, there are limited publications for vitamin D_2 and D_3 determinations. In 1971, one of the original assays for vitamin D_3 was developed to determine its primary storage site. This method used thin-layer chromatography and tissue samples from rats.[31] Since this time, methods for vitamin D_3 include radioimmunoassay, HPLC coupled with UV quantitation, and HPLC with a competitive protein binding assay.[9, 11, 12, 32] The majority of previously published methods use samples other than serum or plasma, such as tissue and milk, and involve complicated procedures and impractically long analysis times. Standardized validation practices and well-defined performance acceptability is lacking from all publications we examined. This newly-developed vitamin D_2 and D_3 assay is the first that uses LC-MS/MS with

internal standard for both analytes for serum and plasma specimens. We have demonstrated an LLMI down to 0.140 for vitamin D_2 and 0.156 ng/mL for vitamin D_3 and proven validation through exhaustive testing according CLSI C62-A.

Vitamin D testing and research has increased exponentially in the last five to ten years, yet the relationship between vitamin D_2/D_3 and 25(OH)D has not been fully explored primarily because a robust and precise method to quantify vitamin D_2/D_3 was unavailable. There are limited studies evaluating vitamin D supplementation dose and subsequent vitamin D_2/D_3 and 25(OH)D concentrations. Humans make vitamin D_3 after sun exposure, but the concern regarding sun exposure and skin cancer has led to the recommendation of limited sun exposure. In place of sun exposure, vitamin D supplements largely supply our total dietary vitamin D and yet there are limited studies defining effective vitamin D doses. Our preliminary findings, conducted in collaboration with Dr. Mario Kratz, bring about more questions regarding the vitamin D pathway. The difference between baseline and final concentrations each for vitamin D_3 and 25(OH)D was much more pronounced when measuring vitamin D_3, and not as much when measuring 25(OH)D. 25(OH)D concentration is subject to variation due to differing concentrations and binding affinity of vitamin D binding protein, and enzymatic activity of the cytochrome P450 enzymes involved in the vitamin D pathway.[20] The decreased variability in vitamin D_3 concentration in individuals receiving supplementation may indicate a need to monitor this analyte as a more precise measurement of an individual's response to supplementation. Heaney, et al., examines the concentration of 25(OH)D as a function of vitamin D_3 concentration and observes a standard enzyme kinetic relationship.[33]The threshold concentration we must achieve with vitamin D_2 and D_3 to

make a sufficient quantity of 25(OH)D has yet to be determined, but the development of this LC-MS/MS assay, as outlined here, can facilitate defining this concentration. Furthermore, this assay can be used to identify individuals with an abnormal vitamin D metabolism and may provide additional insight into pathology.

Before the publication of C62-A, no other comprehensive CLSI guidance existed that focused specifically on LC-MS/MS assays. C62-A provides a straightforward template for preverification and verification standards. In some cases, acceptability criteria are not clearly defined and applicable references must be consulted. We determined acceptable bias to be less than or equal to 15% based on clinical relevancy and past practice, and a CV of <20% near the LLMI or <15% at all other concentrations. If CLSI documents did not contain explicit acceptability criteria, we used generally accepted best practices. The lack of specific evaluation criteria allowed us the flexibility to define what we considered to be acceptable and clinically relevant. The fully validated method is a specific and selective LC-MS/MS method for measuring serum levels of vitamin D_2 and D_3 at nanomolar concentrations. In addition to providing a reproducible method, this assay has a short analysis time compared with previously published methods. With the development of this assay, research on the significance of vitamin D_2 and D_3 serum concentrations in conjunction with disease states and supplementation compliance can be pursued.

Chapter 3:

Lower Limit of the Measuring Interval

Laboratory tests should be developed and validated with two main purposes: how relevant is the assay and what do the results indicate? The purpose of developing a vitamin D_2 and D_3 assay was discussed in previous chapters, but the significance of the results has not yet been examined. In order to define a relevant result, reference values are used as a comparator. CLSI document C28-A3 defines reference values as values associated with good health or with some other pathological condition.[34] Currently, no published data exists which defines a reference interval for vitamin D_2 or D_3. While validating this assay, a reference interval was not defined. Vitamin D_3 is a naturally occurring molecule that can also be taken exogenously. In this case, a reference interval may erroneously be used as a substitute for intake or sun-exposure recommendations. In lieu of a reference interval to guide the development of a measuring interval that includes clinically relevant concentrations, a wide measuring interval only restricted by instrument performance and chosen parameters was validated.

The measuring interval of an assay is defined as a range of concentrations that can be measured under specific conditions.[16] Each measuring interval has a lower limit and upper limit. These values are simply the lowest and highest concentrations measured at which all defined performance characteristics are met. This chapter will specifically focus on the lower limit of the measuring interval (LLMI), or the lowest concentration that can be reliably quantified. The performance characteristics of the assay must be defined for a result to be trustworthy.

The Centers for Medicare and Medicaid Services (CMS) issued a federal regulation "Standards for Certification: Laboratory Requirements" as a way to enforce The Clinical Laboratory Improvement Amendments (CLIA) law passed by Congress.

CLIA regulations establish quality standards for laboratory testing performed on human specimens, including body fluids and tissues, for the purpose of diagnosis, prevention, or treatment of disease, or assessment of health.[35] CLIA '88 outlines allowable error criteria for about 75 analytes, but did not include vitamin D_2 or D_3. Additionally, CLSI document C62-A does not explicitly define what is considered acceptable, allowing the user some flexibility. Several different acceptability criteria were explored: a total coefficient of variation (CV_{total}) of 20%, a CV_{total} of 36%, and a total allowable error (TE) of 36%. Ultimately, a CV_{total} of 20% was used to determine the LLMI. The latter two acceptability criterion will be explored later in the chapter.

Before establishing acceptability criteria, all sources of error or variation which may affect results must be considered. Laboratory analyses for individuals are subject to several sources of variation, including biological, preanalytical, analytical, and postanalytical variation.[36] Biological variation consists of within-person and between-person variation. Preanalytical variation refers to the sample collection and processing. The analytical variation is the bias and imprecision of the assay. Postanalytical variation involves reporting and interpretation of results. Assuming preanalytical and postanalytical errors remain constant for all analytes, the biological and analytical sources of variation can then be considered.

From 1999 to 2002, National Health and Nutrition Examination Survey (NHANES) conducted an analysis of 34 laboratory analytes to estimate within-person coefficients of variation (CV_w) and between-person coefficients of variation (CV_B).[36] More exhaustive databases also exist that analyze hundreds of analytes.[37] Knowing CV_w is clinically useful when determining an allowable CV_{total}, because CV_{total} is defined as

$[(CVa)^2 + (CVw)^2]^{1/2}$, where CV_a is the analytical coefficient of variation (bias and imprecision) and CV_w is the within-person coefficient of variation. NHANES found a wide range of CV_w for all analytes studied. At the extreme ends, the CV_w for sodium was 1% while the CV_w for triglycerides was 27.8%. Armed with information on the CV_w, realistic goals for bias and imprecision can be established. For example, if the CV_{total} is arbitrarily set to some value, this translates to different standards for CV_a depending on the analyte CV_w.

Biological variation studies do not exist for vitamin D_2 or D_3. Instead of using the above calculation for CV_{total}, it was defined as $[(\text{mean within run CV})^2 + (\text{mean between run CV})^2]^{1/2}$. Using a CV_{total} of 20% allows the LLMI to be defined in terms of precision. Precision is a measure of agreement between repeated measurements. More specifically, precision is a measure of random error. Accuracy is the closeness between the observed concentration and the actual concentration, often thought of as mean systematic error.[38] Systematic error is further divided into constant and proportional error. The constant error component is estimated by the mean bias observed in the test method, and the proportional error is calculated using recovery, together representing the accuracy of the method. Typically, the LLMI would be determined using a measure of precision and accuracy, but true accuracy is usually determined by comparing results to a reference method or using certified reference material. This concept of true bias will be explored in more detail later. In most assays, as the measured concentration decreases, the precision also decreases (CV_{total} increases). Consistently distinguishing signal from noise at extremely low concentrations becomes harder to reproduce. Finding a perfect intersection where the CV_{total} is

acceptable and the measured concentration is medically relevant can be challenging.

CLSI document EP17 *Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures* was used as a guide for the experimental design.[39] The hypothesized LLMI was 0.1 ng/mL and five sample concentrations around 0.1 ng/mL were created: 0.05, 0.1, 0.125, 0.15, and 0.25 ng/mL. All samples were created using fetal bovine serum (FBS) and unlabeled vitamin D_2 and D_3. FBS has virtually undetectable concentrations of vitamin D_2 and D_3, whereas pooled human serum generally has a vitamin D_3 concentration around 5 ng/mL. The vitamin D pathway in ruminants is comparable to humans, making our use of FBS acceptable as a proxy matrix.[10] Vitamin D_3 is not detectable in fetal calf serum because vitamin D_3 is only made in the skin after sunlight exposure. Adult bovine serum, like pooled human serum, contains impractically high endogenous concentrations of vitamin D_2 and vitamin D_3. Each concentration of the LLMI samples was tested in eight replicates per day for five days. Additionally, this experiment was conducted over two different column lots. The different column lots add another variable to the experiment and enhance the robustness of the results. The data were examined for outliers or unusual results using the residual plots of observed concentrations (y-axis) vs. expected concentrations (x-axis). At the end of five days the mean, standard deviation (SD), and error were calculated (Table 3.1).

LLMI evaluation using 5 concentrations of samples						
SAMPLE (ng/mL)	**MEAN (ng/mL)**	**SD (ng/mL)**	**BIAS (ng/mL)**	**TE (ng/mL)**	**TE(%)**	**CV_{total}(%)**
Ergocalciferol (D_2)						
0.05	0.055	0.012	0.005	0.028	56.75	37.78
0.1	0.110	0.019	0.010	0.047	47.29	26.24
0.125	0.131	0.019	0.006	0.045	35.88	21.57
0.15	0.160	0.022	0.010	0.054	36.03	19.48
0.25	0.265	0.023	0.015	0.062	24.78	13.36
Cholecalciferol (D_3)						
0.05	0.055	0.012	0.005	0.029	58.92	42.96
0.1	0.115	0.017	0.015	0.049	48.54	26.46
0.125	0.133	0.020	0.008	0.048	38.66	23.07
0.15	0.171	0.033	0.021	0.086	57.24	26.41
0.25	0.278	0.022	0.028	0.073	29.03	13.07

Table 3.1 Each sample was made using fetal bovine serum (FBS) spiked with unlabeled vitamin D_2 and D_3. The spiking solution was created using stock solution with known concentrations (measured using a Beckman spectrophotometer set to 265nm and extinction coefficients of 18,900 and 18,300 for vitamin D_2 and D_3, respectively). Each sample was tested in replicates of eight per day for five days. The LLMI was determined by plotting CV_{total} (%) (y-axis) vs. sample concentration (x-axis). Bias is the expected concentration-observed concentration. TE is $|Bias| + 2SD$. TE (%) is *(TE/expected concentration)*100*. CV_{total}(%) is $[[(\text{mean within run CV})^2 + (\text{mean between run CV})^2]^{1/2}]*100$. The acceptable CV_{total} (%) for this assay is 20%.

To determine the LLMI for both vitamin D_2 and D_3, CV_{total} (y-axis) was plotted against the expected sample concentration (x-axis). (See Figure 3.1 and 3.2.) The data was fit with a power function and the sample concentration for vitamin D_2 and D_3 was calculated where the CV_{total} was 20%. The LLMI is 0.140 ng/mL for vitamin D_2 and 0.156 ng/mL for vitamin D_3.

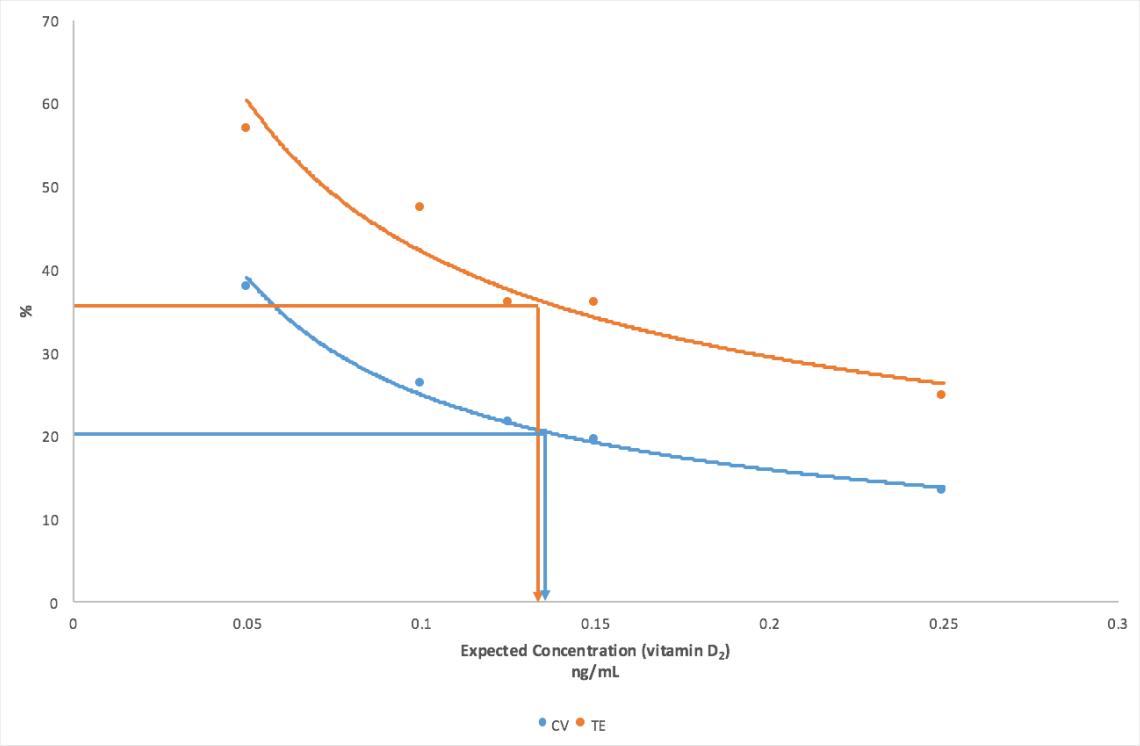

Figure 3.1 The LLMI for vitamin D_2 was derived by testing five concentrations near the expected LLMI in replicates of eight for five days. The CV_{total} and TE as % (y-axis) vs. expected sample concentration (x-axis) was plotted and trend lines were added. When the TE (%) acceptability is set at 36%, LLMI for vitamin D_2 is 0.135 ng/mL. When the CV_{total} (%) acceptability for this assay is 20%, the LLMI for vitamin D_2 is 0.140 ng/mL.

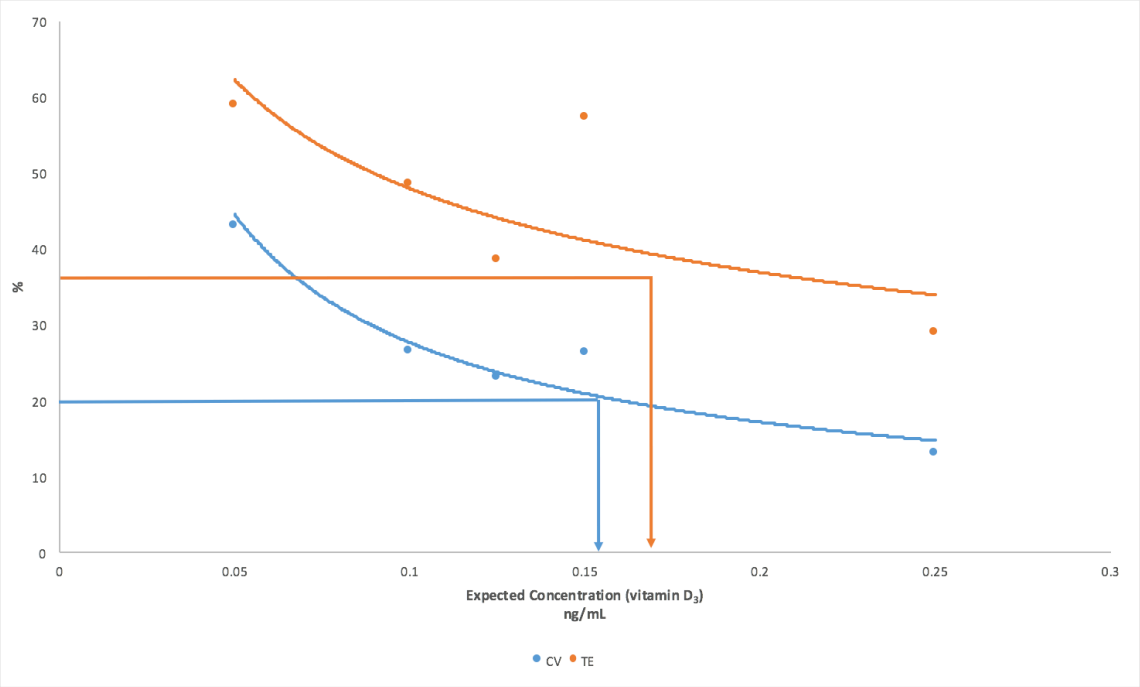

Figure 3.2 The LLMI for vitamin D_3 was derived by testing five concentrations near the expected LLMI in replicates of eight for five days. The CV_{total} and TE as % (y-axis) vs. expected sample concentration (x-axis) was plotted and trend lines were added. When the TE (%) acceptability is set at 36%, LLMI for vitamin D_3 is 0.165 ng/mL. When the CV_{total} (%) acceptability for this assay is 20%, the LLMI for vitamin D_3 is 0.156 ng/mL.

As mentioned in the beginning of the chapter, other acceptability criteria considered were a CV_{total} goal of 36% and a TE of 36%. Referring back to the main purpose for developing an assay-what do the results indicate? For many analytes, medical decision limits exist at various concentrations within the AMI. For example, when monitoring a therapeutic drug, a low or high concentration communicates very specific information to the physician. Based on the laboratory results, the physician may choose to change medications or dosages. For every medical decision limit, acceptability criteria are established. Medical decision limits are not currently established for vitamin D_2 or D_3, which led to the hypothesis that a doubling in concentration at the LLMI is clinically relevant. To differentiate 0.10 ng/mL from 0.20 ng/mL, for example, this is a 100% difference. Using the equation from a paper by Jones the performance goals at the LLMI

are established, *CD (critical difference)* = $2.77*CV_{total}$.[30] The critical difference is the smallest difference between sequential laboratory measurements associated with an actual change in concentration. Specifically, results for serial draws in a patient will not be considered different unless this critical difference is achieved. CV_{total}, as Jones defines it, is $CV_{total} = (CVa^2 + CVi^2)^{1/2}$. CV_a is the analytical coefficient of variation, and CV_i is the within-person biological variation. As discussed previously, within-run and between-run coefficients of variation were used in lieu of CV_a and CV_i. When the critical difference is set to 100%, CV_{total} becomes 36%. Using the power curves generated in Figure 3.1 and 3.2, the concentration where CV_{total} is 36% is calculated. The LLMI is 0.057 ng/mL for vitamin D_2 and 0.072 ng/mL for vitamin D_3. At this concentration for each analyte, other performance characteristics for the assay could not be met, specifically trueness and linearity. With a total imprecision of 36%, a doubling of concentration is detected; however, the precision requirements for establishing trueness and linearity are not achieved. According to the definition of LLMI, all performance characteristics must be met, which is violated when CV_{total} is 36%.

Achieving a precise and accurate result depends on the magnitude of error in the assay. To obtain a low enough concentration for relevant purposes, the total analytical error must be minimized. The total analytical error refers to all assay errors from all sources arising during the data collection process.[40] The main types of error contributing to the total analytical error are systematic error and random error. Simply stated, the systematic error is a measure of accuracy. Accuracy is the agreement between the observed value and the expected or true value. The random error is a measure of precision, which is the closeness of agreement in repeated sampling. Combining both

random error and systematic error into a total analytical error is more clinically relevant. Westgard notes that providers interpreting laboratory results do not think in terms of random or systematic errors separately.[41] Instead, calculating the total analytical error provides more relevant diagnostic information, allowing the provider to make the best decision.

The final acceptability criteria considered was a total allowable error (TE) of 36%. Again, using the equation *CD (critical difference)* $= 2.77 * CV_{total}$, TE replaces CV_{total}. According to Westgard, TE is defined *as* $|Bias| + 2SD$.[38] The absolute value of bias is an estimate of accuracy. Bias is the difference between the expected concentration and the observed concentration. Precision is estimated by "2SD" where SD is the standard deviation or measure of spread of the values. When the standard deviation is multiplied by 2, this approximates the 95% confidence interval in which 95% of the results are expected to fall by chance alone. The TE is calculated using the bias and standard deviation data in Table 3.1. Figure 3.1 and 3.2 show TE (%) on the y-axis vs. expected concentration on the x-axis. By using the acceptability criteria of 36% for TE, the LLMI is 0.135 ng/mL for vitamin D_2 and 0.165 ng/mL for vitamin D_3. Evaluating data using precision and accuracy is preferable; however, the bias calculated from this experiment is not a true representation of the assay's accuracy. The material used to create each sample was made using vitamin D_2 and D_3 stock solutions with concentrations determined spectrophotometrically. The same stock solutions were used to make the calibrators. Additionally, this experiment was conducted on one instrument, with one operator, and one set of calibrators. In fact, recalibration of the assay may eliminate the calculated bias altogether. If the focus was on the life of the assay and it is

assumed that the trueness study estimates potential bias over time, then using a TE of 36% as the acceptability criteria to determine the LLMI is appropriate.

Without existing studies evaluating vitamin D_2 and D_3 concentrations associated with either health or pathology, it may seem tedious to examine the small differences in LLMI concentrations calculated for each criterion. Often times laboratory scientists may be inclined to standardize performance considerations for all analytes as an uncomplicated approach to assuring quality results. In other words, for a simplified assurance of quality results, it may be tempting to create a single performance standard for all testing platforms (i.e. LLMI for all assays is determined using a CV_{total} of 10%). Standardization ensures the production of reliable and comparable results across laboratories and ensures the reproducibility of results.[42] Quality assurance includes all aspects of performance, including preanalytical, analytical, and postanalytical processes. While each process is relevant and deserves fastidious attention, preanalytical and postanalytical processes will have comparable standards for each testing platform in the laboratory. For instance, preanalytical variability due to phlebotomy techniques are assumed to be relatively similar for all tests, while freeze-thaw stability will need to be evaluated for each analyte. The analytical process requires intentional thought and decisions should be supported by the peer reviewed scientific literature. In lieu of guidelines and regulations outlining specific performance parameters, one must carefully determine what the results communicate about the patient and then decide how to make these results the most reliable.

Novel assays without preexisting reference methods or certified reference materials must measure performance using less common approaches. One must first

determine the purpose of the assay and then strive to deliver meaningful results. Using the equation *CD (critical difference) = 2.77*CV_{total}*, the CD can be calculated when CV_{total} is 20%. At the LLMI, the smallest difference between sequential laboratory results associated with a true change is 55.4%. This means that 0.140 ng/mL and 0.218 ng/mL for vitamin D_2 and 0.156 ng/mL and 0.242 ng/mL for vitamin D_3 can be differentiated. Until the search for the perfect vitamin D concentration is exhausted and vitamin D supplementation is optimized, tedious interpretation of the limitations of this assay cannot be overlooked.

Chapter 4:

Conclusion

History of Vitamin D testing

Rickets was first described in the 15th century as a deficiency in bone mineralization resulting in deformities. By the end of the 19th century, the incidence of rickets in England began to increase, most likely owing to the limited sunlight exposure and increased pollution during the industrial revolution.[7] Then, in 1913 after experiments by Sir Edward Mellanby, scientists hypothesized a micronutrient was capable of curing and preventing rickets. In these experiments performed on dogs, Sir Edward Mellanby discovered that cod liver oil could reverse rickets. At this time, vitamin A had been discovered and its ability to prevent xerophthalmia was well known. Mellanby observed that cod liver oil not only reversed rickets, but also cured xerophthalmia. This discovery led him to incorrectly report that vitamin A was responsible for the observed antirachitic effects. In 1922, McCollum, et al., were not convinced that vitamin A was the source of the observed calcium-depositing capabilities. McCollum, et al., theorized that this calcium-depositing capability was the result of some new and undetermined fat-soluble molecule. To test this theory, they destroyed vitamin A in cod liver oil by oxidation and noted that while its antirachitic activity remained, xerophthalmia developed.[43] This experiment was the first indication that some new and undetermined vitamin was responsible for restoring calcium homeostasis.

The discovery that vitamin D was not exclusively a vitamin was made by Hess and Unger in 1921. They demonstrated this finding by exposing rachitic children to sunlight, thereby reversing rickets within four months.[7] Vitamin D_2 was identified in irradiation experiments on plant material in 1930. Askew, et al., irradiated and distilled ergosterol, fed the resulting crystals to rats, and noted that the crystals exhibited

antirachitic activity.[44] In 1937, Windaus and Bock identified another form of vitamin D that was produced in the skin, which they called vitamin D_3.[7] It was not until 1978 that Esvelt, et al., identified vitamin D_3 using mass spectrometry.[45] Since this time, many laboratory methods, such as HPLC, RIA, and thin-layer chromatography, have been developed to isolate and quantify vitamins D_2 and D_3.

This thesis outlined the development and validation of an LC-MS/MS method for detecting vitamin D_2 and vitamin D_3. The criteria for data acceptability were clearly stated and all aspects of the assay were rigorously tested. The performance of this assay is consistent because of the thorough validation process in accordance with CLSI C62-A, an absent quality in previously published methods for vitamins D_2 and D_3. Without a standardized validation process, the reproducibility of the assay is not guaranteed, translating into limited applicability. Currently, this assay is used for research purposes, but the procedure can be adapted for the clinical laboratory. A highly sensitive and specific assay to detect vitamin D_2 and D_3 may elucidate vitamin D physiology.

After many years of vitamin D research, the medical community has yet to uncover everything there is to know about vitamin D or why vitamin D deficiency continues to plague millions of people. Ganji, et al., compared NHANES data collected from 1988-1994 to 2001-2006, and concluded that vitamin D deficiency prevalence has increased.[46] More than one-third of the U.S. population has serum 25(OH)D concentrations of <20 ng/mL. Despite adjusting for assay changes and drifts overtime, Ganji, et al., could not account for the lower serum 25(OH)D concentrations in the U.S. population. Many theories have been presented, but it is most likely a result of the increased prevalence of overweight and obese individuals. It has been reported in many

studies that vitamin D is sequestered in adipose tissue, but the mechanism is still unclear.[47] Despite the clear statistical evidence that vitamin D deficiency continues to afflict many individuals, the recommended daily allowance (RDA) of vitamin D supplementation is not sufficient enough to correct this issue.[48] Current RDA is based on limited, and sometimes misinformed, historical data. The inaccurate association between idiopathic infantile hypercalcemia and hypervitaminosis D began in post-WWII Britain. In 1966, excessive vitamin D intake was viewed by the medical community as the cause of supravalvular aortic stenosis (SAS) syndrome. The assumption that excessive vitamin D intake could result in such devastating outcomes led to a conservative RDA of vitamin D. The Institute of Medicine (IOM) issued RDA guidelines for vitamin D in 1997 of 200IU to 2000IU per day, and only recently amended the RDA to 400IU to 4000IU per day. Studies focused on vitamin D supplementation and adverse outcomes have shown that 400IU per day is not effective in producing an adequate 25(OH)D concentration.[48] Clearly, more studies are needed to explore safe and effective supplementation strategies needed for all individuals. This LC-MS/MS assay can be deployed for use in future studies to monitor compliance and metabolism of individuals enrolled in these studies.

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