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DETERMINATION OF VITAMIN D METABOLITES AND THEIR EPIMERS IN OBESE EMIRATI POPULATION

Bashar Hiatham Alzohily

United Arab Emirates University

College of Science

Department of Chemistry

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Bashar Hiatham Alzohily

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Chemistry

Under the Supervision of Dr. Iltaf Shah

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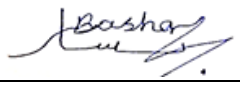
Declaration of Original Work

I, Bashar Hiatham Alzohily, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Determination of Vitamin D Metabolites and their Epimers in Obese Emirati Population*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Iltaf Shah, in the College of Science at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

Student's Signature:  Date: 09/01/2021

Advisory Committee

1) Advisor: Dr. Iltaf Shah

Title: Associate Professor

Department of Chemistry

College of Science

2) Co-advisor: Dr. Asma Al Menhali

Title: Assistant Professor

Department of Biology

College of Science

3) Co-advisor: Dr. Mohammed Al-Meetani

Title: Professor

Department of Chemistry

College of Science

Approval of the Master Thesis

This Master Thesis is approved by the following Examining Committee Members:

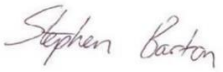
1) Advisor (Committee Chair): Dr. Iltaf Shah
   Title: Associate Professor
   Department of Chemistry
   College of Science

   Signature  Date 09/01/2021

2) Member: Dr. Nayla Munawar
   Title: Assistant Professor
   Department of Chemistry
   College of Science

   Signature  Date 09/01/2021

3) Member (External Examiner): Dr. Stephen J Barton
   Title: Associate Professor
   Department of Pharmacy and Chemistry
   Institution: Kingston University London, UK

   Signature  Date 09/01/2021

This Master Thesis is accepted by:

Dean of the College of Science: Professor Maamar Benkraouda

Signature Maamar Benkraouda Date January 18, 2021

Dean of the College of Graduate Studies: Professor Ali Al-Marzouqi

Signature Ali Hassan Date Januray 18, 2021

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Abstract

Vitamin D deficiency is a global issue, and despite all-year-round sunshine, it is a big problem in the UAE. Obesity or overweight refers to an accumulation of excessive fat that may harm health; so, it is considered a worldwide health concern. The accurate measurement of vitamin D active forms is a problematic issue. The aim of this study was to develop and validate a UHPLC-MS/MS method for the determination of vitamin D metabolites and their epimers and then apply the new method for the determination of vitamin D levels in the obese Emirati population. The vitamin D epimers and other metabolites were determined by UHPLC-MS/MS system, model 8060. The methods include the collection of blood samples from 729 individuals, divided into 277 baselines (vitamin D deficient volunteers), 277 follow-ups (supplemented volunteers), and 175 healthy volunteers, extraction of vitamin D and epimers from plasma samples, method development, and validation. The vitamin D metabolites and their epimers have been separated by UHPLC-MS/MS instrument. The method validation criteria were found in an acceptable range. Epimers of vitamin D metabolites were found to cause an overestimation of 25OHD and give false-positive results in the obese Emirati population. The results showed that baseline, follow-up, and healthy volunteers are deficient in vitamin D's major metabolites. UHPLC-MS/MS has superior selectivity. This new vitamin D blood test has been successfully applied to estimate the vitamin D and epimer levels in the obese Emirati population, and the results showed that the current vitamin D estimation methods are overestimating the actual results. This new method will have far-reaching applications in any future clinical studies.

Keywords: Vitamin D, vitamin D metabolites, epimers of vitamin D, UHPLC-MS/MS, vitamin D blood test, obese Emirati population.

Title and Abstract (in Arabic)

تحديد مستقلبات فيتامين د ومصاوغاتهم الصنوية في السكان الإماراتيون البدناء

الملخص

يعد نقص فيتامين د مشكلة عالمية وعلى الرغم من سطوع الشمس طوال العام، إلا أنه يمثل مشكلة كبيرة في الإمارات العربية المتحدة. تشير السمنة أو زيادة الوزن إلى تراكم الدهون الزائدة التي قد تضر بالصحة؛ لذلك، يعتبر مصدر قلق صحي عالمي. يعتبرالقياس الدقيق لأشكال فيتامين د النشطة قضية إشكالية. الهدف من هذه الدراسة هو تطوير والتحقق من صحة طريقة (UHPLC-MS/MS) لتحديد مستقلبات فيتامين د ومصاوغاتهم الصنوية ثم تطبيق الطريقة الجديدة لتحديد مستويات فيتامين د في السكان الإماراتيين البدناء. تم قياس كمية المصاوغات الصنوية لفيتامين د والمستقلبات الأخرى بواسطة نظام (UHPLC-MS/MS)، نموذج 8060. تشمل الطرق جمع عينات الدم من 729 فردًا (منهم 277 فرداً من المتطوعون اللذين يعانون من نقص فيتامين د، و277 فرداً من المتطوعون المكملون بالمكملات الغذائية، و 175 فردًا من المتطوعون الأصحاء)، واستخراج فيتامين (د) و المصاوغات الصنوية من عينات البلازما، وتطوير هذه الطريقة والتحقق من صحتها. تم فصل مستقلبات فيتامين د ومصاوغاتهم الصنوية بواسطة تقنية (UHPLC-MS/MS). وكانت معايير التحقق من صحة الطريقة في نطاق مقبول. وُجِد أن المصاوغات الصنوية لمستقلبات فيتامين د تسبب تقديرًا مفرطًا لـ25OHD وتعطي نتائج إيجابية خاطئة في السكان الإماراتيون البدناء. أوضحت النتائج أن المتطوعون الذين يعانون من نقص فيتامين د، والمتطوعون المكملون بالمكملات الغذائية، والمتطوعون الأصحاء يعانون من نقص في المستقلبات الرئيسية لفيتامين د. يتميز (UHPLC-MS/MS) بانتقائية فائقة. تم تطبيق اختبار الدم الجديد لفيتامين (د) بنجاح لتقدير مستويات فيتامين (د) و المصاوغ الصنوي في السكان الإماراتيون البدناء، وأظهرت النتائج أن طرق فيتامين د الحالية تبالغ في تقدير النتائج الفعلية. سيكون لهذه الطريقة الجديدة تطبيقات بعيدة المدى في أي دراسات سريرية مستقبلية.

مفاهيم البحث الرئيسية: فيتامين د، مستقلبات فيتامين د، المصاوغات الصنوية لفيتامين د، UHPLC-MS/MS، فحص فيتامين د في الدم، السكان الإماراتيون البدناء.

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Dedication

To my beloved parents, family and friends

Table of Contents

List of Tables

List of Figures

List of Abbreviations

1α,25(OH)$_2$D2	1-α-25 dihydroxyvitamin-D3
1α,25(OH)$_2$D3	1-α-25 dihydroxyvitamin-D3
25OHD2	25-hydroxyvitamin-D2
25OHD3	25-hydroxyvitamin-D3
3-epi-25OHD2	3-epi-25-hydroxyvitamin-D2
3-epi-25OHD3	3-epi-25-hydroxyvitamin-D3
7αC4	7-α-hydroxy-4-cholesten-3-one
BGLAP	Bone Gamma-Carboxy Glutamic Acid-Containing Protein
BMI	Body Mass Index
Caco-2	Colon Adenocarcinoma Cells
CYP24A1	Cytochrome P450 Family 24 Subfamily A Member 1
CYP27B1	Cytochrome p450 27B1
CYP2R1	Cytochrome P450 2R1
DBP	Vitamin D-Binding Protein
DEQAS	Vitamin D External Quality Assessment Scheme
DNA	Deoxyribonucleic Acid
EIA	Enzyme Immunoassay
ESI	Electrospray Ionization
FDA	U.S. Food and Drug Administration
HepG2	Human Hepatoblastoma Cells
HPLC	High Performance Liquid Chromatography

IDS	ImmunoDiagnostic Systems
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LLC-PK1	Porcine Kidney Cells
LOD	Limit of Detection
LOQ	Limit of Quantitation
MG-63	Human Osteosarcoma
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
PTH	Parathyroid Hormones
QCH	Quality Control High
QCL	Quality Control Low
QCM	Quality Control Medium
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
RXR	Retinoid X Receptor
SD	Standard Deviation
SNPs	Single-Nucleotide Polymorphisms
SP-B	Surfactant Protein-B
SRM	Standard Reference Material
UHPLC–MS/MS	Ultra-High-Performance Liquid Chromatography–Tandem Mass Spectrometry
UPSFC–MS/MS	Ultra-Performance Supercritical Fluid Chromatography–Tandem Mass Spectrometry
UV-B	Ultraviolet-B

VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element

Chapter 1: Introduction

1.1 Overview

Obesity or overweight refers to an accumulation of excessive fat that may harm health; so, it is considered a worldwide health concern [1]. Obesity is associated with developing different diseases, including hypertension, cardiovascular disease, diabetes mellitus, and cancer; therefore, it will increase the mortality rate and morbidity. Also, obesity could lead to social discrimination and less physical activity [2, 3]. Genetics and eating behavior are factors leading to obese development [4, 5]. Various studies confirmed a relationship between vitamin D and obesity; however, the causal relationship has not yet been elucidated [6]. The possible mechanism for the inverse relationship between Body Mass Index (BMI) and vitamin D serum levels is the volumetric dilution of vitamin D. Although the vitamin D quantity in lean and obese people are similar, the vitamin D concentration in obese people is lower due to the dilution of vitamin D in an immense volume [7]. However, if low 25-hydroxyvitamin-D (25OHD) levels in obese subjects are caused by volumetric dilution, this means that 25OHD serum levels could be increased upon weight loss. Nevertheless, studies about weight loss illustrate inconsistent results. For instance, Mason et al. [8] reported an insignificant increase of 25OHD serum levels in a large number of obese and overweight postmenopausal volunteers who undergo a diet and exercise plan for a weight loss. Namely, there was an increase in 25OHD of 2.1, 2.7, 3.3, and 7.7 ng/ml for participants who loss in their weight <5%, 5-9.9%, 10-14.9% and >15%, respectively [8]. One study found that vitamin D supplementation increases the 25OHD serum concentration in lean subjects more than in obese subjects [9].

According to Drincic et al. [10], the adjustment of vitamin D supplementation has to be conducted in obese subjects, which could eliminate the difference in increasing the 25OHD serum level upon supplementation between lean and obese subjects . Another study reported an excess amount of body fat could deactivate an essential hormonal pathway for skeletal health. For instance, the pathways for the synthesis of active metabolites of vitamin D could be inhibited by a leptin hormone derived from adipocytes [11].

Vitamin D is a prohormone; it is fat-soluble and derived from cholesterol. Vitamin D is obtained from two sources: the first source is sunlight or artificial ultraviolet-B (UV-B) exposure and the second source is from food like fish, eggs, milk, and cereals, or vitamin D supplements [12, 13]. Exposure to UVB is a major source of vitamin D, and limited exposure to UVB radiation can negatively impact vitamin D synthesis in the skin [14]. It is also known that people with darker skin require more prolonged sun exposure compared to light-skinned people. For example, the dark skin has more amounts of melanin pigment compared to the light skin, which causes in reducing the ability of skin to produce vitamin D from sunlight [15]. In some countries in the world, despite the year-round sunlight, there is a high prevalence of vitamin D deficiency [14, 16, 17]. Vitamin D deficiency is correlated with the development of many diseases, including rickets, osteomalacia, arthritis, diabetes, dementia, Parkinson's, Alzheimer's, and cardiovascular diseases [18]. Furthermore, vitamin D deficiency is also associated with aging, obesity, skeletal muscle weaknesses, and metabolic syndrome diseases. The role of vitamin D epimers in disease onset and progression is not clearly understood, but it is known that epimers have a potent role as compared to their corresponding non-epimeric forms [12, 14, 19-22].

There are two primary metabolites of vitamin D, vitamin D3 and vitamin D2, which are collectively known as vitamin D [18, 23]. Vitamin D3 is formed from its precursor 7-dehydrocholesterol in the skin by ultraviolet B light (medium wavelength, 290-315 nm). In the first step, 7-dehydrocholesterol is converted to pre-vitamin D3 which is followed by conversion of pre-vitamin D3 to vitamin D3, as shown in Figure 1 [24-26]. The second step is governed by the conversion of vitamin D3 to 25OHD3 in the liver via the 25-hydroxylase (CYP2R1) enzyme, as shown in Figure 1 [27]. In the liver, the 3-epimerase enzyme also converts 25OHD3 to 3-epi-25OHD3. 25OHD3 and 3-epi-25OHD3 are converted into 1α,25$(OH)_2$D3 in the kidneys via the action of the enzyme 1α-hydroxylase (CYP27B1), as shown in Figure 1 [28, 29]; 3-epimerase enzymes also convert 25OHD3 into 3-epi-1α,25$(OH)_2$D3. Moreover, 3-epi-25OHD3 also becomes converted into 3-epi-1α,25$(OH)_2$D3 in the kidneys. Likewise, 1α,25$(OH)_2$D3 is converted into 3-epi-1α,25$(OH)_2$D3 via the action of the epimerase enzyme. Sometimes, however, 25OHD3 and 1α,25$(OH)_2$D3 undergo further oxidation using enzyme epimerases and are converted into epimeric forms like 3-epi-25OHD3, 3-epi-1α,25$(OH)_2$D3, and 3-epi-24R,25$(OH)_2$D3.

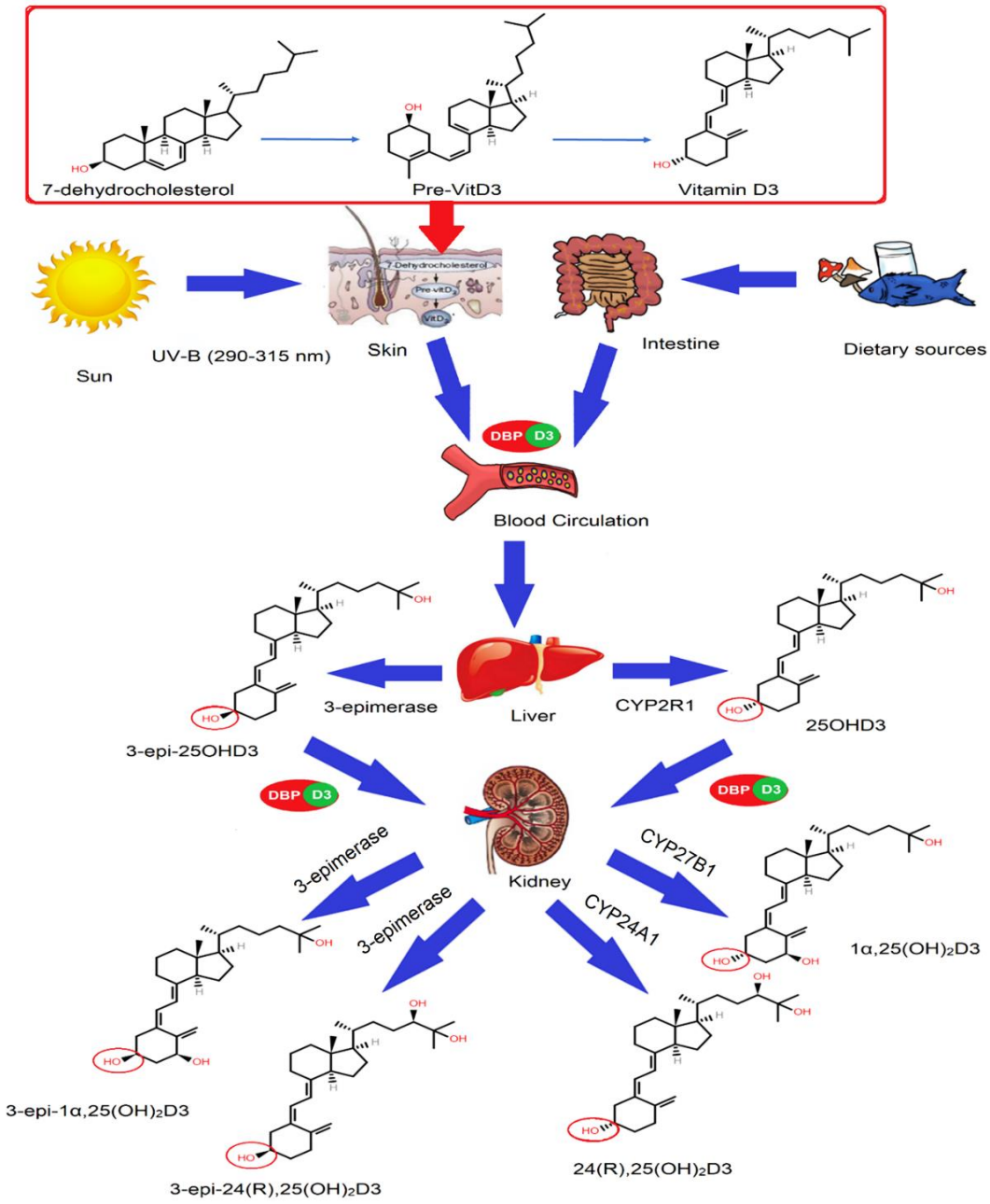


Figure 1: Sources and metabolism of vitamin D. Sunshine activate a chemical reaction in the plasma membrane of dermal fibroblasts and epidermal keratinocytes in the skin, producing an unstable form, 7-dehydrocholesterol, which forms pre-vitamin D3 and, upon thermal isomerization, produces a stable vitamin D3 form. Dietary sources are another source of vitamin D3 and D2. Hydroxylation of vitamin D3 occurs in the liver using enzyme CYP2R1, mainly forming 25OHD3. In the kidney, 25OHD3 undergoes further hydroxylation at the C-1α or C-24 positions. The CYP27B1 enzyme is responsible for C-1α hydroxylation, while CYP24A1 is responsible for C-24 hydroxylation. CYP24A1 can inactivate 25OHD3 to produce 24R,25$(OH)_2$D3, while 3-epimerase enzyme could inactivate the major metabolites (25OHD3, 24R,25$(OH)_2$D3, and 1α,25$(OH)_2$D3) in the epimerization process by changing the orientation of only one group [30].

Recently it was found that C3-epimers of vitamin D may have an important role to play in the body. The exact source of C3-epimers is not known but it was found that oral supplementation of vitamin D can cause increased production of epimers in mice but not in humans, the 25OHD3 levels and its epimer were determined in this study by using LC-MS/MS [31]. Moreover, it is known that all major vitamin D metabolites can be epimerized at the C3 position, with higher amounts in infants [32]. Furthermore, mothers and newborns are known to have high levels of C3-epimers [33]. Recent genetic models also show that the genetic determinants and potential factors of C3-epimers differ from those of non-C3-epimers [34]. It is also of importance that the C3-epimers can cause an overestimation of vitamin D status in routine laboratory tests [18]. However, very few labs in the world take into account that measurements can be misleading due to overlapping C3-epimers and that these co-eluting C3-epimers can be separated from vitamin D metabolites using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) techniques [35]. Studies have shown that routine vitamin D blood tests for healthy adults are not significantly affected by epimeric interference using LC-MS/MS assays [36]. However, in the present study, the baseline (vitamin D deficient volunteers) and follow-up samples (supplemented volunteers) were added and compared with healthy samples. The vitamin D supplements could increase the epimer levels more than the major form of vitamin D; therefore, the epimers will significantly affect routine vitamin D blood tests. Moreover, the routine vitamin D blood tests are not affected by epimers when LC-MS/MS techniques are employed in vitamin D blood tests due to the separation of vitamin D epimers from major forms by these techniques.

In neonatal human keratinocytes, Reddy et al. [37] has concluded that 3-epi-1α,25$(OH)_2$D3 is metabolized through the C-24 oxidation pathway to produce three

polar compounds: 3-epi-1α,24,25(OH)$_3$D3, 3-epi-24-oxo-1α,25(OH)$_2$D3, and 3-epi-24-oxo-1α,23(S),25(OH)$_3$D3, while C-23 oxidation of 3-epi-1α,25(OH)$_2$D3 produces 3-epi-1α,23(S),25(OH)$_3$D3, as shown in Figure 2 [24, 37]. The C-24 oxidation pathway is catalyzed by the 1α,25(OH)$_2$D3-24-hydroxylase (CYP24) enzyme. The biological activity of 3-epi-1α,25(OH)$_2$D3 and its metabolites have not yet been identified [37].

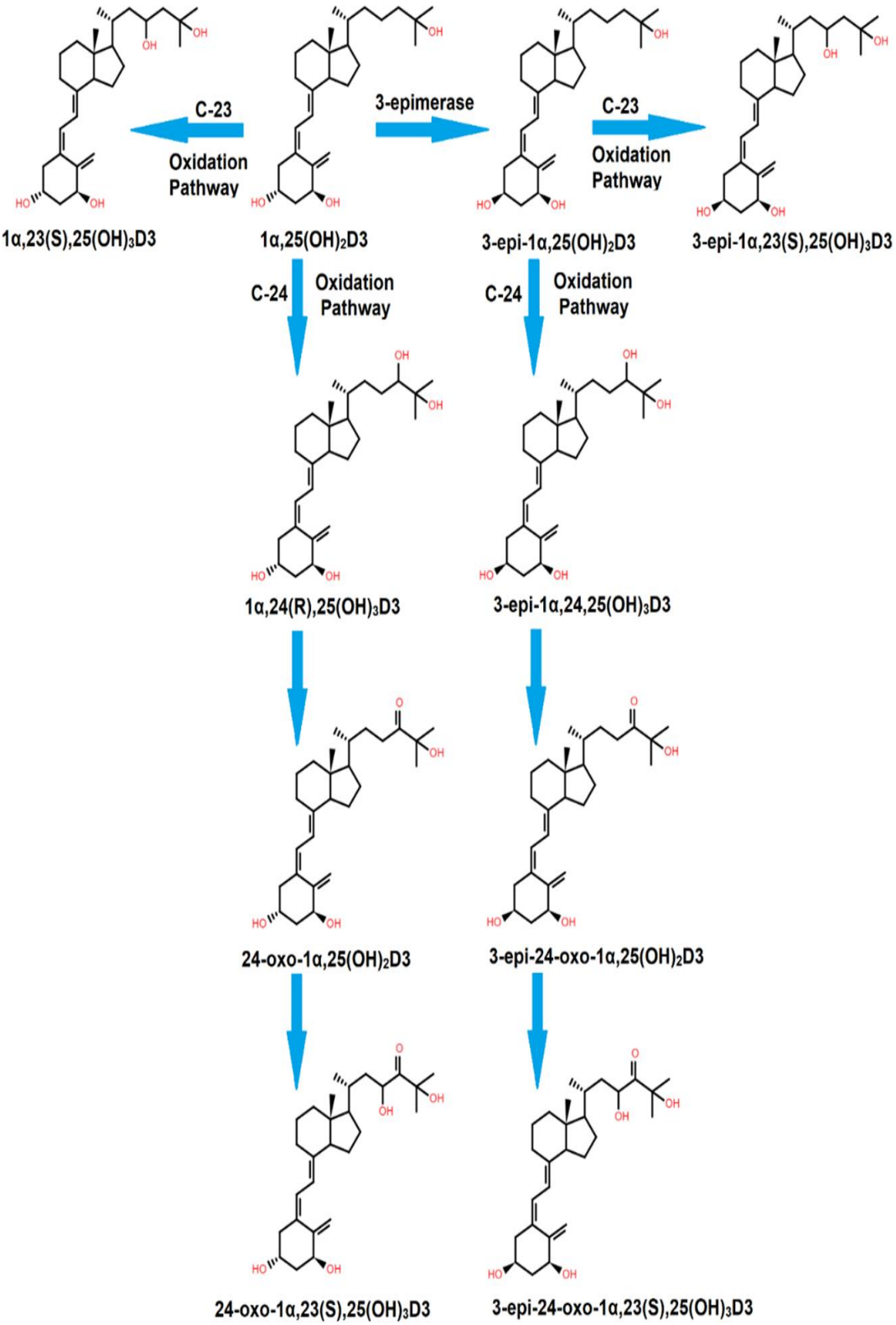


Figure 2: The C-23 and C-24 oxidation pathways for 1α,25(OH)$_2$D3 and its epimer [30].

In the epimerization pathway, for example, the intermediate metabolites of major vitamin D precursors are converted to epimers, which undergo the same hydroxylation and oxidation events by the same enzymes when compared to the standard metabolic pathway, which also subsequently leads to the production of epimers like 3-epi-25OHD3, 3-epi-1α,25$(OH)_2$D3, and 3-epi-24(R),25$(OH)_2$D3 [24, 38-42].

To write this chapter, some electronic databases were searched, including NCBI PubMed, Google Scholar, SciFinder, and ScienceDirect, for English language articles. This was done using keywords such as epimers of vitamin D, C3-epimers, metabolism of vitamin D epimers, epimerization pathway for standard metabolism of vitamin D, the function of the epimer of 1α,25$(OH)_2$D, detection and quantitative analysis of vitamin D epimers, and the effects of epimers on routine analysis of total circulating vitamin D (25OHD) and its determination in serum. Due to an increased interest in epimers of vitamin D and their possible relationship with health and disease, it was decided to carry out this subjective literature survey.

1.2 C3-Epimerization of Vitamin D

The epimers of 25OHD3 and 1α,25$(OH)_2$D3 were also found in rats when given pharmacological doses of 25OHD3 and 1α,25$(OH)_2$D3 supplements over a period of time [38, 42]. The epimerase enzyme (25OHD3-3-epimerase) is known to be responsible for the epimerization of 25OHD3 at the C3 location in the endoplasmic reticulum of liver, bone, and skin cells. However, the gene responsible for encoding the epimerization enzyme has not yet been identified [28]. NADPH is also utilized as a cofactor for the action of 25OHD3-3-epimerase in the microsomes of osteoblastic UMR-106 cells (these are transplantable rat osteogenic sarcoma cell lines, and these

cell lines are responsive to PTH, prostaglandins, and bone-resorbing steroids like vitamin D). Furthermore, epimerase enzymes can carry out the epimerization process of 1α,25$(OH)_2$D3, and 24(R),25$(OH)_2$D3 but not at the same rate as 25OHD3. Moreover, the observation shows that this process is irreversible [28, 43].

It is known that the epimerization process for 25OHD3, 24(R),25$(OH)_2$D3, and 1α,25$(OH)_2$D3 occurs in some specific culture cells, including quiescent human colon adenocarcinoma cells (Caco-2 colon carcinoma cells), human hepatoblastoma cells (HepG2 cells), human osteosarcoma (MG-63) [44], bovine parathyroid cells [41], and porcine kidney cells (LLC-PK1) [44]. Vitamin D supplementation has recently become common, and more studies are recommended to determine the origin of epimers, either produced by exogenous sources or when metabolized endogenously; additionally, it will be beneficial to understand the nature and properties of the enzymes or tissues responsible for the epimerization process to obtain an in-depth understanding of epimers [24]. A recent study reported that 25OHD3, 1α,25$(OH)_2$D3, and 24,25$(OH)_2$D3 can be respectively epimerized into 3-epi-25OHD3, 3-epi-1α,25$(OH)_2$D3, and 3-epi-24,25$(OH)_2$D3 in many different cell cultures types, such as LLC-PK1, Caco-2, UMR-106, Hep-G2, and MG-63 cells, but at different proportions, which were determined and quantified using ^{1}H NMR spectroscopy and LC-MS techniques. For example, the C3 epimerization of 25OHD3 and 1α,25$(OH)_2$D3 mostly occurs in equivalent amounts in UMR-106, LLC-PK1, and Caco-2. However, compared to other epimers, Hep-G2 and MG-63 cells mostly produce 3-epi-25OHD3, while 3-epi-24,25$(OH)_2$D3 is the least produced of the epimers [38].

1.3 Role of Epimers in Calcium, Phosphorus and PTH Homeostasis Compared to Parent Metabolites

As mentioned earlier, 1α,25$(OH)_2$D3 is considered to be the most effective vitamin form; its principal function is to increase the amount of calcium and phosphate to normal levels in plasma, and it is also required to optimize bone health. Moreover, 1α,25$(OH)_2$D3 suppresses parathyroid hormones (PTH) by binding to VDR, thereby inhibiting gene expression and cell proliferation and causing the levels of calcium to increase [13, 24]. It is known that 3-epi-1α,25$(OH)_2$D3 has fewer calcemic effects than non-epimeric forms of vitamin D [45] but, compared to other epimeric forms of vitamin D, it is the most efficient [38]. It was also recently discovered that 3-epi-25OHD3 and its calcemic effects are higher than those of 3-epi-24,25$(OH)_2$D3 [38]. 3-epi-25OHD3 and 3-epi-1α,25$(OH)_2$D3 have less affinity toward DBP and even lower affinity for VDR compared to primary metabolites 25OHD3 and 1α,25$(OH)_2$D3, which will lead to a reduction in the ability of epimers to induce calcium transport, as well as a much-reduced gene expression in the human colonic carcinoma cell line, Caco-2 [46, 47]. For example, the bone gamma-carboxy glutamic acid-containing protein (BGLAP, osteocalcin) and the CYP24 gene are activated by 3-epi-1α,25$(OH)_2$D3 but at a much lower rate than 1α,25$(OH)_2$D3 [24, 38, 48]. Furthermore, 3-epi-1α,25$(OH)_2$D3 has less control on the antiproliferation and differentiation of cells compared to 1α,25$(OH)_2$D3, such as HL-60 promyelocytic leukemia cells, keratinocytes, and rat UMR-106 osteosarcoma cells [24, 28]. Moreover, parathyroid hormone secretions can be suppressed by 3-epi-1α,25$(OH)_2$D3 in bovine parathyroid cells with almost the same potency as its parent metabolite [41]. Moreover, some biological studies conducted on pulmonary alveolar type II cells show that 3-epi-1α,25$(OH)_2$D3 can boost the synthesis of surfactant phospholipids and activate gene

expression to yield an increase in the synthesis of surfactant protein-B (SP-B) [24, 45]. Also, 3-epi-1α,25(OH)$_2$D3 has greater metabolic stability than 1α,25(OH)$_2$D3 [24, 49]. For example, the rate of metabolism (side-chain oxidation) by CYP24A1 in human keratinocytes for 3-epi-1α,25(OH)$_2$D3 is less than that of its parent metabolite [28, 50, 51]. The biological activity of 3-epi-25OHD in humans has not yet been identified [52]. The biological activity of the C3-epimer has been demonstrated in most in vitro models; however, the physiological functions of the C3-epimer remain unclear despite having been studied using in vivo models [24, 46, 53].

The C3-epimer has been shown to possess some calcemic and non-calcemic regulatory effects compared to its non-epimeric form. Compared to the respective 25OHD3 and 1α,25(OH)$_2$D3 forms, the C3-epimers (3-epi-25OHD3 and 3-epi-1α,25(OH)$_2$D3) bind to DBP at about 36-46% and VDR at 2-3%. However, 3-epi-1α,25(OH)$_2$D3 induces BLGP (osteocalcin) VDR-binding downstream at only ~15%, as well as CYP24 gene expression, compared to 1α,25(OH)$_2$D3. Similarly, 3-epi-1α,25(OH)$_2$D3 has been shown to possess more differentiation or antiproliferative activities (approximately 30% and 10%) than the non-epimeric compound. It is also known that the parathyroid hormone is suppressed by 3-epi-1α,25(OH)$_2$D3 and that epimers are responsible for inducing phospholipid synthesis in pulmonary alveolar type II cells in comparable amounts to the non-epimeric form. Moreover, 3-epi-1α,25(OH)$_2$D3 has been proposed to have higher metabolic stability than 1α,25(OH)$_2$D3, despite having non-equivalent VDR binding [24]. Due to its weak interactions with VDR, the vitamin D epimer will remain in a free form, which might affect bodily function and give false-positive results using normal measurement methods.

1.4 The Potency of Epimerization in Microsomal Fractions

Bone cells (UMR-106) were used to collect subcellular fractions (homogenate, nuclei, mitochondria, and microsome fractions), and when 1α,25$(OH)_2$D3 was incubated with these fractions, the potency of epimerization was observed to be the highest in microsomal fractions compared to others. Moreover, when comparing the epimerization activity of liver cells (HUH-7, HepG2), colon cells (Caco2), and bone cells (MG-63, UMR-106), it was found that the highest proportions of epimerization were found in the microsomal fractions for UMR-106 cells [43]. Recently, it was found that 3-epi-24,25$(OH)_2$D3 could be formed from the epimerization of 24,25$(OH)_2$D3, and this reaction is catalyzed by 3α-HSD and β-HSD (studied in the presence of testosterone in Pseudomonas bacteria), where the nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADPH) act as coenzymes in the reaction [54]. It was also found that this reaction could be modified for non-epimeric forms, like 24,25$(OH)_2$D3, which could also be catalyzed by the same enzymes (3α-HSD and β-HSD) [43]. Furthermore, it was noted that the cytosol is the region where β-HSD and α-HSD could be most efficient [55-57], and it was reported that 3α-HSD and β-HSD enzymes could also be responsible for epimerization in microsomal fractions but in very small proportions [43].

1.5 The Role of DBP, VDR, and Genetics in Epimerization

The differences in the molecular structures of 25OHD3 and its epimer lie in the configuration of only one functional group at a specific carbon (C-3), whereby the hydroxyl groups in 3-epi-25OHD3 and 3-epi-1α,25$(OH)_2$D3 have different orientations in space compared to 25OHD3 and 1α,25$(OH)_2$D3, respectively (C-3α vs. C-3β) [28, 35]. The vitamin D-binding protein (DBP) and vitamin D receptor (VDR)

are two proteins that are central to the metabolism and mechanism of action related to the circulation of the vitamin D metabolite, 1α,25$(OH)_2$D3 [28, 58]. The vitamin D-binding protein (DBP) transports vitamin D metabolites through blood vessels toward different tissues, and these metabolites rarely circulate in free form. The liver is one of the organs that are responsible for producing DBP. Liver, intestinal, or renal diseases will lead to a decrease in DBP, which will cause a reduction in vitamin D metabolites and produced epimers. However, this does not mean that people with low DBP are deficient in vitamin D as the free form of vitamin D could be within the normal range [58]. When active 1α,25$(OH)_2$D3 reaches a target cell, it is released from the DBP. After that, it is attached to vitamin D receptors on the cells, and the target cells will uptake this free active metabolite 1α,25$(OH)_2$D3 inside the cell; the metabolite will then either be rapidly metabolized by the 1α,25$(OH)_2$D-24-hydroxylase (CYP24A1) enzyme through the C-24/23 oxidation pathway leading to the formation of other metabolites, or it will be re-attached to VDR [24, 28, 58]. The VDR will then go through conformational changes within the nucleus that will allow other transcriptional factors to combine with 1α,25$(OH)_2$D3, which will ultimately influence gene transcription. To initiate gene expression, the active vitamin D–VDR complex must interact with retinoid X receptor (RXR), which recognizes the selective or promoter sites of DNA and transcription begins; however, transcriptional activity and VDR binding affinity were found to be weaker for 3-epi-1α,25$(OH)_2$D3 than for 1α,25$(OH)_2$D3. Therefore, it was concluded that 3-epi-1α,25$(OH)_2$D3 performs gene regulation in the same manner as its non-epimeric analogue, but less efficiently. It was also concluded that epimers will have limited binding capabilities to DBP and VDR which will, in turn, influence gene transcription. This difference in orientation (C-3α

vs. C-3β) likely makes this affinity and potency differ between 1α,25(OH)$_2$D3 and its epimer, as shown in Figure 3 [38, 58, 59].

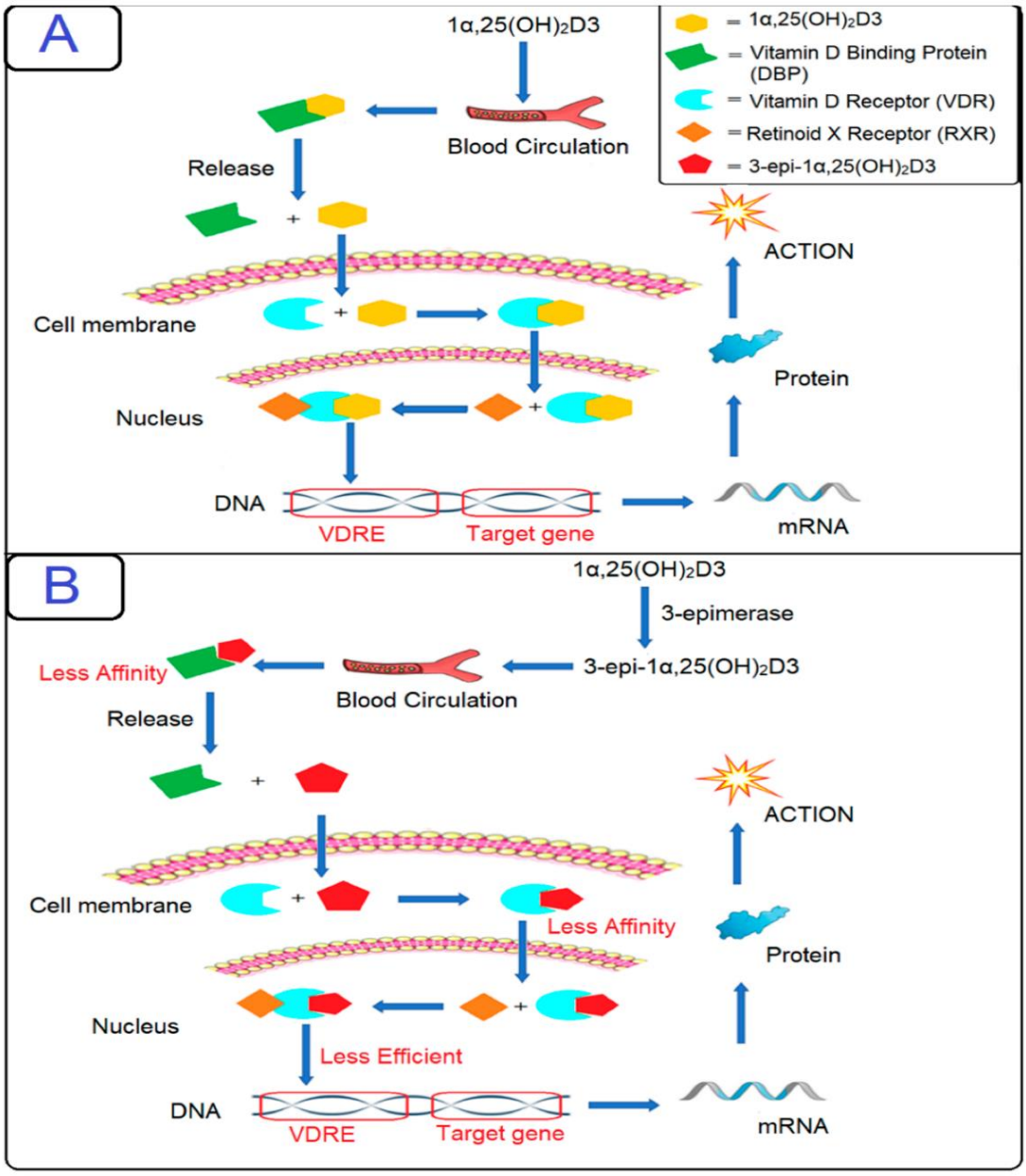


Figure 3: Comparison between 1α,25(OH)$_2$D3 and 3-epi-1α,25(OH)$_2$D3 in terms of gene regulation. (A) When 1α,25(OH)$_2$D3 is released from DBP, it will cross the cell membrane and enter the target cell to bind with VDR. The 1α,25(OH)$_2$D3–VDR complex undergoes translocation to the nucleus and performs conformational changes in order to link with other transcriptional factors and heterodimerize with RXR. After that, the 1α,25(OH)$_2$D3–VDR–RXR complex binds to the vitamin D response element (VDRE); then, the transcription of RNA begins for the specific genes that are then expressed as different proteins responsible for vitamin D homeostasis. (B) We assume that 3-epi-1α,25(OH)$_2$D3 will carry out the same function but at a slower rate. The 3-epi-1α,25(OH)$_2$D3 complex could likely perform the same gene regulation as its non-epimeric forms but at a lower rate [30].

For example, VDR binds with an active vitamin D metabolite to induce genomic action which inhibits growth in renal osteodystrophy, breast, or prostate primary tumor cells, lymphomas, psoriasis, or in autoimmune diseases and osteoporosis [58, 60]. However, the role of epimers in these contexts remains unclear. A recent study hypothesized that genetic polymorphisms in the vitamin D-related gene pathway cause variation in C3-epimer levels. In this study, candidate single-nucleotide polymorphisms (SNPs) with regard to C3-epimer levels were investigated. Interestingly, it was noted that participants carrying a minor T allele exhibited a tendency to increase their levels of C3-epimer, while those carrying a minor G allele tended to produce a decreased level of both non-C3-epimers and C3-epimers [34].

1.6 Vitamin D Epimer Levels in Humans

The source of vitamin D epimers, whether from endogenous metabolism, diet, or supplementation, remains unclear [61]. Previously, it was found that, if endogenously synthesized, the immaturity of renal and hepatic tissues could be responsible for epimerization of the major metabolites of vitamin D. Subsequently, a new study has found that there is no difference in the epimer levels between healthy adults and adults with compromised liver function. For example, these studies want to find if the epimerization process could take place either in the liver or kidney. They discovered no difference in epimer levels between healthy adults who have intact liver and adults who have compromised liver; therefore, the epimerization could take place in the kidney or even in the skin before the vitamin D being further hydroxylated [32, 46]. Previously, it was found that supplements of vitamin D3 are not a possible source of epimerization and, therefore, do not constitute a potential cause [62]. However, a recent study has shown that epimer levels are proportionally higher with oral

supplementation of vitamin D compared to the ultraviolet irradiation of mice skin. This was not the case in humans; for instance, the epimer levels could be increased either by vitamin D supplements or by the ultraviolet irradiation of human skin or both; however, the mechanism was not identified yet [31]. A recent study where oral supplementation was compared with sunshine exposure in an animal model vs. human model found that the total levels of epimers (3-epi-25OHD3) were higher with oral supplementation than with sunlight exposure in an animal model while in human model, oral supplementation and sunlight exposure were increased the levels of epimers. Further experiments showed that sun exposure causes a decline in epimer levels in the animal model; however, the mechanism for this process was not identified. Nonetheless, it was noted that several variables might be responsible for the breakdown or production of epimers. It was found that CYP2R1 gene expression in mice livers is greater with vitamin D supplements compared to sunshine irradiated mice [31].

It is not known whether the epimerization process happens before or after hydroxylation. The epimerization process takes place in various other tissue types, including kidney, colon, liver, and bone cells, and the degree of epimerization is different between these tissues [31, 38]. Epimerization could also occur in the skin and lead to the epimerization of pre-vitamin D3, and therefore, the expression of 25-hydroxylase (CYP2R1) will be less efficient, thus leading to a decrease in 3-epi-25OHD3 production [31]. One study found a considerable increase in the expression of the CYP24A1 gene in the renal cells of mice exposed to sunlight (UV-irradiated) compared to those with vitamin D supplements [63]. When the activity of CYP24A1 enzyme increases, the catabolism of 25OHD3, 1α,25$(OH)_2$D3, and the corresponding epimeric forms increases. Thus, these epimers could also be decreased by an increase

in the activity of the CYP24A1 enzyme [31]. Recently, it was found that vitamins D3 and D2 can also be hydroxylated by the CYP11A1 enzyme, which leads to the production of a few new vitamin D metabolites, 20, 22$(OH)_2$D3 or D2 and 20OHD3 or D2. Thus, it was found that CYP27A1, CYP27B1, and CYP24A1 can also carry out further hydroxylation [64, 65]. The activity of the CYP11A1 enzyme has been observed in epidermal keratinocytes, where 20, 22$(OH)_2$D3 and 22OHD3 were formed in higher proportions [66]. If true, CYP11A1 may be activated by UVB exposure, and the availability of the vitamin D3 substrate for classical hydroxylation to form 25OHD3 and its epimer could be lower in the absence of sunlight [31].

Vitamin D metabolism is affected by different characteristics of humans and mice. The first characteristic is the structure of the skin. For instance, the melanocytes in human skin are present at the basal layer of the epidermis, while in mice, melanocytes are present in the hair follicle and the dermis. Mice also have a higher pelage density compared to humans; furthermore, the permeability and fragility of the stratum corneum are higher for rodents than for humans [67-69]. Moreover, humans are diurnal animals, while mice are nocturnal animals, which means that mice depend mostly on vitamin D supplements for their vitamin D stores, while humans rely on UVB exposure to synthesize vitamin D [31]. Singh et al. [32] also determined the C3-epimer by using LC-MS/MS, though not in all ages—only among those less than one year old .

1.7 C-3 Epimer Levels in Newborn and Adults

The epimer for children (<1 year) accounted for 8.7–61.1% of overall 25OHD. It was also hypothesized that the formation of epimers might depend on the maturity of the liver because the number of epimers is reduced in the blood of the infants

compared to adults [32]. Moreover, van den Ouweland et al. [70] established that the percentage of the C3-epimer of 25OHD was up to 60% higher in infants compared to only 22% in adults . Moreover, Studies noticed that adult pregnant women have higher concentrations of epimers (80% detectable epimers) compared to blood donors (43% detectable epimers). In this study, 3-epi-25OHD3 was found in all pregnant women and their cord blood, comprising 6.0% and 7.8% of 25OHD3, respectively. When the epimer was not accounted for in vitamin D estimations, 38% of women and 80% of newborns were classified as having an insufficient concentration (<75 nmol/L). However, with the epimer included in the estimation of insufficiency, 33% of women and 73% of neonates were found to have sufficient levels of vitamin D. It is assumed that a high use of dietary supplements in women could have contributed to the formation of high levels of epimers in both maternal and cord blood [33, 71, 72].

Vitamin D deficiency is generally regarded as 30 nmol/L or lower, while sufficient vitamin D levels for healthy people range between 40 and 80 nmol/L [36]. The range of absolute epimer concentrations for the infant population is 0–230 nmol/L, with an average of 18.2 nmol/L, while the range for epimer concentration in the adult population is around 0–22.5 nmol/L, with a mean value of 4.3 nmol/L. It is concluded that as the infants grow older, their epimers levels start decreasing. These levels remain approximately constant during their entire adult life. The percentage of C3-epimers of the total 25OHD3 ranged between 0% and 61.1% for infants and 0% and 47% for adults, with an average of 21.4% for infants and 5.9% for adults, respectively [24, 73, 74]. According to Chailurkit et al. [75], the concentration of vitamin D epimer was greater in males than in females. According to Keevil et al. [74], insufficient levels of vitamin D are estimated as below 50 nmol/L, while the epimer concentration could be equal to 2.5 nmol/L. Therefore, clinical interpretation is not hugely affected in adults

or children [24, 74]. Nonetheless, when the epimers were quantified, a percentage of infants and adults were misclassified as sufficient, with values determined at 9% and 3%, respectively [76]. Keevil et al. [74], concluded that the epimer for 25OHD3 performs a significant role in clinical applications; however, it could have a minimal effect on routine LC–MS/MS measurements. It was also shown that high levels of epimers can be found in pregnant women and their newborns, and it was suggested that the role of epimers in characterizing vitamin D status in pregnancy and infancy is imperative [33]. Others suggest that clinicians should determine the C3-epimer during routine analysis, especially among pediatric and infant populations [24]. The production of vitamin D epimer in humans is variable [63]. Moreover, levels of 3-epi-25OHD3 production could be related to gender, age, and living areas (rural areas vs. urban areas) [74].

1.8 Techniques for Measurement of Vitamin D Metabolites and Epimers

The epimers of vitamin D have been investigated in many studies [18, 35, 62, 73, 74, 76-80]. Immunoassay techniques have difficulty separating the D2 form of vitamin D from D3 forms, and these techniques can only give the total 25OHD value in a measurement. For example, these techniques are based on the binding between an antigen (analyte) and its antibody. The antibodies for 25OHD3, 25OHD2, and their epimers are the same [35, 81-84]. The differentiation between 25OHD2 and 25OHD3 in most immunoassay techniques is not possible, as these techniques consider both as one entity [85]. Current LC–MS/MS techniques can separate D2 forms from D3 forms, but they have difficulty in separating the interference caused by the epimeric and isobaric metabolites. In some vitamin D LC–MS/MS with electrospray ionization techniques, the ionization potency of 3-epi-25OHD3 is higher than that of 25OHD3,

which will lead to an overestimation of 3-epi-25OHD3 if it is not separated from its non-epimeric components [28]. These techniques lack specificity, which affects the estimation of major vitamin D metabolites and will lead to false-positive results [86, 87]. Therefore, it is essential to separate epimers from the primary active forms of vitamin D that can overlap and compromise the accuracy of the method [18]. In the past 10 years, innovations in analytical techniques have had the beneficial ability to separate vitamin D forms from co-eluting epimers, and there are many published methods, which can separate the epimers of vitamin D, as shown in Table 1 [18, 35, 61]. The separation of epimers from vitamin D metabolites is considered a challenge as these epimers have the same mass-to-charge ratios as non-epimeric metabolites. Further, mass differentiation is not possible using most standard LC–MS techniques as these epimers require chromatographic separation [24, 88].

Table 1: Methods used for separation of vitamin D epimer in the past 10 years.

Year	MS/MS instrument-ionization mode	Mobile Phase -column	Run Time (min)	Reference
2010	API 4000/APCI	Isocratic mobile phase (34% H_2O + 66% methanol) – Zorbax SB CN column	40	[89]
2011	API 4000/APCI	methanol/H_2O/formic acid -Zorbax cyanopropyl HPLC column.	14	[90]
2012	API-3000/ESI	acetonitrile +0.3% formic acid/ammonium acetate (2 mM) + 0.1% formic acid – Ultrason ES-OVM chiral column +Zorbax SB-C18 column	20	[91]
2013	API 4000 QTRAP/APCI	H_2O/Methanol - Phenomenex Kinetex PFP column	7.0	[62]
2014	Waters Xevo TQ-S/ESI	H_2O/Methanol – BEH - Phenyl UPLC column	< 5	[92]
2015	API 5500 QTRAP/ESI	0.1% formic acid in water/0.1% formic acid in methanol – waters pentafluorophenyl (PFP) column	10	[93]
2016	Agilent 6410/ESI	ammonium formate in H2O/ammonium formate in MeOH –Pursuit PFP +Poroshell 120 EC-C18 column	28	[94]
2017	API 4000/ESI	0.1% formic acid in water, methanol - Raptor FluoroPhenyl column	7	[95]
2018	Agilent 6460/ESI	3 mM ammonium formate in H_2O/ 3 mM-ammonium formate in methanol – PFP column	35	[96]
2019	Shimadzu 8050/ESI	0.1% formic acid in water/0.1% formic acid in acetonitrile – Kinetex F5 column	8	[80]

It is known that LC–MS/MS is the most recommended method for analysis of vitamin D, especially in young infants, due to its ability to detect and quantify epimeric and non-epimeric metabolites separately in plasma [28, 97]. One study illustrated that ultra-performance supercritical fluid chromatography-tandem mass spectrometry (UPSFC–MS/MS) could be used as an alternative technique to Ultra-High-performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC–MS/MS)

for the quantification of vitamin D metabolites in clinical applications. Furthermore, this technique has advantages compared to HPLC, which utilizes CO_2 as supercritical fluid; therefore, UPSFC –MS/MS is more cost-effective than HPLC [79].

According to the literature, there are four columns widely used and tested according to their resolution, selectivity, efficiency, and analysis time in order to separate 25OHD3 from its epimer; these columns are COSMOSIL cholester (stationary phase: cholester), Kinetex F5 (stationary phase: pentafluorophenyl), Kinetex biphenyl (stationary phase: biphenyl), and COSMOSIL pentabromophenyl (stationary phase: pentabromophenyl) [80]. Cholester columns do not separate epimers of 25OHD3, while F5 and Biphenyl columns can separate the epimers. However, the analysis time for biphenyl is longer than that of the F5 column. Also, the width of the peak by biphenyl is larger. Pentabromophenyl, on the other hand, can separate the epimers. Nonetheless, the analysis time for pentabromophenyl is longer than that for the F5 column. Also, the width of the peak in pentabromophenyl is larger. COSMOSIL pentabromophenyl could be improved if the particle size becomes smaller, as in F5, subsequently reducing the time and improving the resolution and peak shape. Lastly, the F5 column is the best column for separating 25OHD3 and its epimer [80]. According to Singh et al. [32], a longer 5-dinitrobenzoyl-(R)-phenylglycine column (Chirex-PGLY and DNB 250 mm × 4.6 mm) gave good separation of the epimers of 25OHD. Unfortunately, the C3-epimer was not determined in all ages but only for those less than one year old. Schleicher et al. [78] used ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) together with a pentafluorophenyl (PFP) column to determine the epimers.

1.9 Quality Assurance in Vitamin D Metabolites and C-3 Epimers Determination

Recently, the International Vitamin D External Quality Assessment Scheme (DEQAS) survey noted that liquid chromatography-tandem mass spectrometry (LC–MS/MS), immunoassays, DBP-based assays, and HPLC are common assays that can differentiate between 25OHD3 and its epimers. Around 14 methods were selected and compared in this article. These methods are EIA (enzyme immunoassay), Abbott Architect, chromatographic ligand binding assay, automated IDS (ImmunoDiagnostic Systems), DiaSorin RIA (radioimmunoassay), DiaSorin Liason Total immunoassay, DIA source immunoassay, HPLC, LC–MS/MS, IDS RIA, IDS-iSYS, IDS EIA, Roche Total 25OHD, and Siemens ADVIA Centaur. Some of those methods can recognize 3-epi-25OHD3 among 25OHD3, including LC–MS/MS, HPLC, Roche Total 25OHD, DIA source, and chromatographic ligand binding assay [24]. Gallo et al. [97] determined that IDS-EIA and DiaSorin-RIA could be challenging to use for the measurement of 25OHD3 because other vitamin D metabolites could interfere with 3-epi-25OHD3 and 24,25$(OH)_2$D3, which could lead to inaccurate measurements of 25OHD3. A recent study highlighted that the enzyme-linked immunosorbent assay (or a commercial chemiluminescence vitamin D assay) and the LC–MS/MS method are not equally able to evaluate vitamin D status in humans. This is because the majority of commercial assay kits cannot distinguish between 25OHD and its epimer, which leads to an overestimation of vitamin D levels as compared to LC–MS/MS, due to the interfering epimers [18].

In order to improve the accuracy and separation of 25OHD measurements in plasma, a Standard Reference Material (SRM) was developed by the National Institute of Standards and Technology (NIST) in cooperation with the National Institutes of

Health's Office of Dietary Supplements (NIH-ODS). SRM 972 contains four different concentration levels of vitamin D serum samples, and each level has different ratios of vitamin D metabolites (25OHD3, 25OHD2, 3-epi-25OHD3). The standard measurement for the SRM was carried out under three methods using isotope-dilution (ID) mass spectrometry at the Centers for Disease Control and Prevention (CDC) and the NIST [52]. The columns that were assessed in these measurements were C18 and cyano columns, and it was observed that cyano is a better column than C18 for resolving 25OHD3 and its epimers [52]. The Vitamin D External Quality Assessment Scheme (DEQAS) also uses NIST standards to ensure the analytical reliability of 25OHD, 1,25$(OH)_2$D, and 3-epi-25OHD3 assays [98]. These studies highlight the fact that the epimers of vitamin D should be separated from the vitamin's non-epimeric components to properly determine circulating vitamin D levels in humans. This is especially important if one has to analyze samples from pregnant women, newborns under 1-year-old, and people with liver and kidney disorders. LC–MS/MS techniques should be implemented in all the labs around the world, especially in Middle Eastern countries where these techniques are non-existent. It is also very important that the current methods in laboratories be standardized across the world to account for the interference caused by epimers and isobars. This will help to accurately address the epidemic caused by vitamin D deficiency.

1.10 Objectives of this Study

This study will give qualitative and quantitative information of vitamin D metabolites in blood samples. Moreover, it will give an idea for clinical laboratories about the importance of separation and differentiation between vitamin D metabolites and their epimers. These epimers can overlap and interfere with 25OHD leading to

overestimation and false-positive results during analysis in Immunoassay and commercial chemiluminescence vitamin D assay method. The main aims of this study are as follow: -

1. To develop, validate and improve the UHPLC-MS/MS method that could accurately detect and quantitate different metabolites of vitamin D and separate the epimers and isobars.
2. To apply the developed and validated UHPLC-MS/MS method for accurate measurement of blood vitamin D levels in obese Emirati population.
3. To determine if supplementation could increase either the level of major vitamin D metabolites or their epimers or both in follow-up serum samples.

Chapter 2: Materials and Methods

2.1 Materials

Materials, standards and other chemical solvents were purchased from various companies. Vitamin-D3, 25-hydroxyvitamin-D3 (25OHD3), 3-epi-25-hydroxyvitamin-D3 (3-epi-25OHD3), 25-hydroxyvitamin-D2 (25OHD2), 3-epi-25-hydroxyvitamin-D2 (3-epi-25OHD2), 1-α-25 dihydroxyvitamin-D3 (1α,25$(OH)_2$D3), 1-α-25-dihydroxyvitamin-D2 (1α,25$(OH)_2$D2), 25-hydroxyvitamin D3 (6, 19, 19-d3) and Albumin from human serum were purchased from Green Oasis Medical Equipment Installation and maintenance (Gomet) (Al-Ain, UAE), Vitamin-D2 and 7-α-hydroxy-4-cholesten-3-one (7αC4) were obtained from LABCO LLC (Dubai, UAE), hexane, LC-MS-grade water and LC-MS-grade methanol, ethyl acetate, deionized water, formic acid, ammonium formate and phosphate buffered saline were obtained from Emirates Scientific and Technical Supplies L.L.C. (ESTS) (Dubai, UAE). Ascentis Express F5 column (dimensions: 150 mm × 2.1 mm × 2.7 μm) was received from Gomet (Al-Ain, UAE).

2.2 Methods

2.2.1 Preparation of Standards Solution

The stock solutions of each vitamin D metabolite and internal standard (25-hydroxyvitamin D3 (6, 19, 19-d3)) were prepared in methanol with a concentration of 1 mg/ml. All the stock solutions were kept and stored in amber vials at -80℃. Furthermore, all the standards solutions of vitamin D metabolites should be kept in the dark due to the fact that vitamin D metabolites are very sensitive to light; therefore, they could be degraded. The working standards solution of vitamin D metabolites and

internal standard were prepared by serial dilution of stock solutions in methanol to desired concentrations.

2.2.2 Collection of Blood Samples

The blood samples of people were collected from 729 individuals, consisting of 277 baselines, which are vitamin D deficient volunteers, 277 follow-ups who are supplemented volunteers, and 175 healthy volunteers. These individuals (baselines and follow-ups) were 73 males (age range 18-82) and 204 females (age range 18-65), while healthy volunteers were 8 males (age range 18-29) and 167 females (age range 18-65). The serum of these blood samples was extracted after 15 minutes of centrifugation at 2200-2500 rpm. These serum samples were stored and kept at -80°C in labeled plastic amber vials until analysis. The College of Medicine in the United Arab Emirates University approved this recent Vitamin D study sample with the ethics approval number (AAHEC-3-17-055).

2.2.3 Plasma Samples Extraction Method

The frozen human serum samples were taken from the refrigerator and thawed at room temperature for around 20 minutes. After that, the serum samples were vortexed, and 500 µl of each sample was transferred into a new test tube to begin a liquid-liquid extraction. Afterward, 20 µl of working internal standard with a concentration of 1 µg/ml was added to all calibrants, quality controls, and samples except for the blank. The calibrants and quality controls of vitamin D metabolites mixture were prepared in albumin from human serum (artificial serum), which contained phosphate-buffered saline. All vitamin D metabolites and internal standard in all calibrants, quality controls, and samples were extracted by adding 1.0 ml of

hexane: ethyl acetate (9:1); then, this mixture was vortexed for a few seconds. After that, this mixture was centrifuged at 4000 rpm for 20 minutes at standard conditions. The mixture's centrifugation leads to separate two layers: organic layer or supernatant layer and aqueous layer; it is known that vitamin D is fat-soluble; therefore, it will be extracted toward the organic layer. Pasteur pipettes were utilized to separate or transfer the supernatant layer into a new amber glass vial. Then, further extraction was performed once more to the remaining lower layer. All the extract layers from the extraction processes were pooled together into a new amber vial. Afterward, the extracts were dried at room temperature in a sample concentrator, which perform a flow of air toward the extracts. Lastly, 100 µl of methanol: water (75:25, v/v) mixture LC-MS/MS grade was added to re-suspend the residue and then transferred to LC-MS/MS amber vial for the analysis by the instrument, as shown in Figure 4.

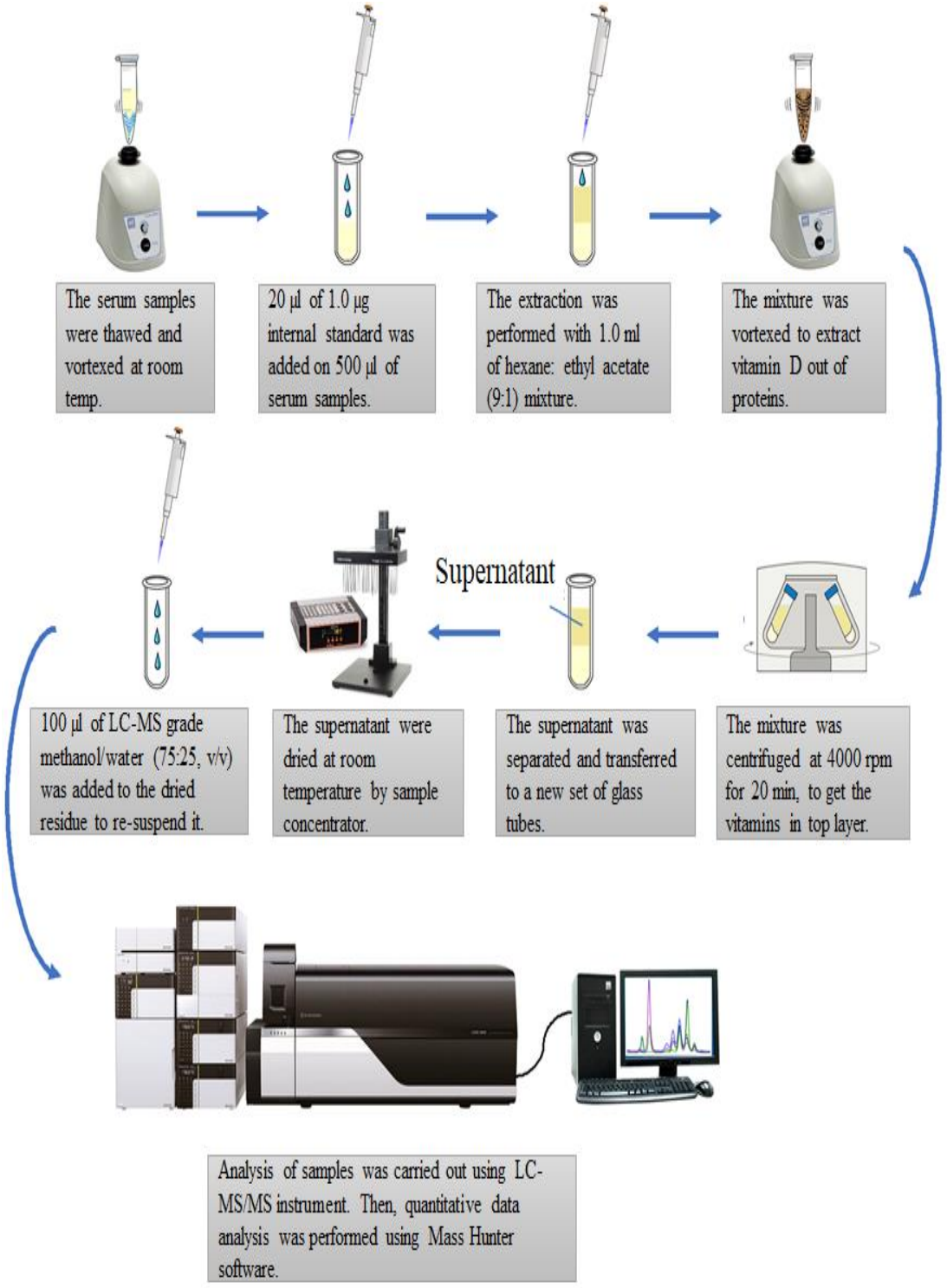


Figure 4: Vitamin D extraction diagram. The diagram illustrates the Liquid-Liquid Extraction method used to extract vitamin D metabolites and their epimers from human serum samples.

2.2.4 Ultra-High-Pressure Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS) System

The UHPLC-MS/MS system is composed of a Nexera Ultra High-Pressure Liquid Chromatography (UHPLC) combined with Triple Quadrupole Mass Spectrometer (Shimadzu, model 8060, Japan). The UHPLC (Nexera X2) system is an advanced technology that provides an ultra-high-speed LC analysis. It consists of a pump, auto-sampler, column oven, and degasser controlled by UHPLC-MS/MS system. The UHPLC system has a higher-pressure tolerance, which enables using a column with a small diameter and small particle size. Moreover, the auto-sampler part has a high injection speed, and the system can use multi-solvents at the same time. The Tandem Mass Spectrometer system consists of a triple quadrupole with a newly developed UF-Qarray ion guide that increases the UHPLC-MS/MS sensitivity by enhancing the ion signal's intensity and reducing noise. UHPLC-MS/MS-8060 is a highly sensitive instrument from Shimadzu company that provides a new level of speed and responsiveness.

The separation of vitamin D metabolites and their epimers was achieved on a reversed-phase analytical column Ascentis Express F5 column with a particle size 2.7 µm, an inner diameter of 2.1 mm, and a length of 150 mm. The analytical column was connected with a guard column for increasing the life of the analytical column and physical filtration. The injection volume for analysis into the UHPLC-MS/MS system was 5 µl. After each sample injection, the sample contamination was minimized by a wash program to rinse the needle with a methanol/water (75:25, v/v) mixture. The column's temperature was kept at 30℃, and the flow rate of the mobile phase was set to 0.5 ml/min. Two mobile phases were used during analysis by the instrument: Mobile

Phase A consisted of 5 mM ammonium formate and 0.1% formic acid in LC-MS grade water, while Mobile Phase B consisted of 5 mM ammonium formate and 0.1% formic acid in LC-MS grade methanol. The optimum chromatography of vitamin D metabolites and their epimers was achieved by using a binary mobile phase gradient pump which was set as follow: 75% of B and 25% of A were kept from 0:0 to 11:0 min, followed by increasing in B to 100% while decreasing in A to 0.0% at 15:00 min that maintained for one minute. After that, mobile phase B was decreased to 75% while mobile phase A was increased to 25% at 16:10 min and maintained until 20:00 min, which is the end of the mobile phase gradient elution profile, as shown in Figure 5.

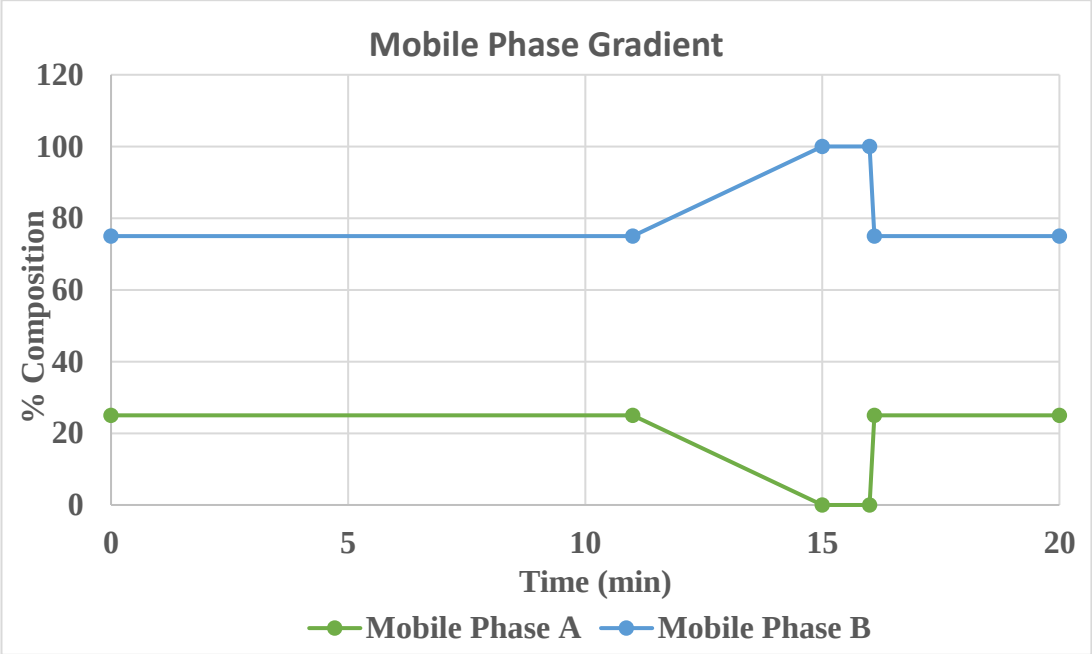


Figure 5: Mobile phase gradient elution profile. A separation of vitamin D metabolites and their epimers employs two mobile phases or solvents: Mobile Phase A contained 0.1% formic acid or 5mM ammonium formate, and Mobile Phase B contained 0.1% formic acid or 5mM ammonium formate. These two mobile phases differ significantly in polarity. Moreover, the composition of solvents changes during the time analysis leads to the optimum chromatography for vitamin D metabolites and their epimers.

A Tandem Mass Spectrometer system was managed using the positive electrospray ionization (ESI) mode for all vitamin D metabolites and their epimers.

Shimadzu's Lab Solutions software was used to control the mass spectrometry parameters. The software was also utilized to control UHPLC parameters (flow rate, mobile phase gradient, injection volume, column's temperature, and pump) and handle or analyze the data, as shown in Table 2.

Table 2: UHPLC-MS/MS parameters and conditions. The table illustrates the factors and conditions used to achieve an optimum chromatography for vitamin D metabolites and their epimers.

UHPLC Parameters	**UHPLC Conditions**
Stationary Phase	Ascentis Express F5 column (dimensions: 150 mm × 2.1 mm × 2.7 µm)
Mobile Phase A	Water for LC-MS + 5mM ammonium formate + 0.1% formic acid
Mobile Phase B	Methanol for LC-MS + 5mM ammonium formate + 0.1% formic acid
Injection Volume (µl)	5.00
Flow Rate (ml/min)	0.50
Column's Temperature (℃)	30.00
Pump mode	Binary Gradient
MS Parameters	**MS Conditions**
Ion Source	Electrospray Ionization (ESI)
ESI Mode	Positive
Nebulizing gas flow (L/min)	2.00
Drying gas flow (L/min)	8.00
Heating gas flow (L/min)	8.00
Interface temp. (℃)	300.00
Heating block temp. (℃)	400.00

2.2.5 Method Development and Validation

U.S. Food and Drug Administration (FDA) suggested that the bioanalytical method should be developed and validated by optimizing the conditions and

procedures for the extraction and detection of the analyte and its metabolites. Moreover, the bioanalytical parameters that are calibration curve, quality control samples, specificity, sensitivity, accuracy, precision, recovery, linearity and stability have to be involved in the method development and validation to ensure the method's suitability for the analysis of study samples [99, 100]. Three quality controls (QCs), which are Quality Control High (QCH), Quality Control Medium (QCM), and Quality Control Low (QCL), were used to determine linearity, stability, intra and inter-day accuracy, intra and inter-day precision. The concentration for each QC level of some vitamin D metabolites (vitamin D3, Vitamin D2, 25OHD and its epimer and 7αC4) was 80 ng/ml, 40 ng/ml and 20 ng/ml for QCH, QCM, and QCL, respectively; while each QC level for 1α,25$(OH)_2$D was 0.8 ng/ml, 0.4 ng/ml and 0.2 ng/ml for QCH, QCM, and QCL, respectively. Each QC level was prepared six times, along with the calibration curve for each validation run. All calibrants and quality control samples were prepared in a biological matrix (serum) that was a mixture of albumin from human serum and phosphate-buffered saline at a concentration of 60 g/L.

The extraction efficiency was evaluated by determining the recovery's percentage. The recovery was implemented by preparing two sets of quality controls where each set consisted of three levels of quality control (QCH, QCM, QCL) and each level was prepared six times. In the first set, the quality control samples for QCH, QCM, and QCL were spiked with methanol, then the internal standard was added to all QC samples; however, in this set, the extraction method that is shown in Figure 4 was not performed; so, this set was called unextracted QC samples. In the second set, the quality control samples for QCH, QCM, and QCL were spiked with blank serum, then the internal standard was added to all QC samples; after that, the extraction method was performed; therefore, this set was called extracted QC samples. The area

under the normal peak in the chromatogram for the extracted QC samples was compared with the unextracted QC samples; then, the recovery's percentage was calculated. The intactness of analyte (stability) was performed by preparing four sets of quality controls in one day where each set consisted of three levels of quality control (QCH, QCM, QCL) and each level was prepared six times. In all four sets, the quality control samples for QCH, QCM, and QCL were spiked with blank serum; then, these sets were stored at -20℃ except the first set where the internal standard was added to all QC samples; then, the extraction method was performed. After 24 hr, the rest three sets were taken out from the refrigerator and thawed at room temperature. In the second set, the internal standard was added to all QC samples; then, the extraction method was performed while the rest two sets were returned to the refrigerator and stored at -20℃. After 24 hr, the rest two sets were taken out from the refrigerator and thawed at room temperature. In the third set, the internal standard was added to all QC samples; then, the extraction method was performed while the rest one set was returned to the refrigerator and stored at -20℃. After 24 hr, the rest one set was taken out from the refrigerator and thawed at room temperature. In the fourth set, the internal standard was added to all QC samples; then, the extraction method was performed. Afterward, the concentration results of QCs were compared between three freeze/thaw cycles at 0 hr, 24 hr, 48 hr, and 72 hr. The specificity experiment was operated by analyzing six blank serum samples; then, each sample's chromatogram was compared with other samples to detect interferences (e.g., matrix components and impurities).

Chapter 3: Results and Discussion

3.1 Chromatogram of Major Vitamin D Metabolites and Epimers with their Respective Retention Times

The Ascentis Express F5 column under the optimum conditions, which were controlled by the UHPLC part, provides a good separation of major vitamin D metabolites and their epimers and isobars with a normal peak shape, as shown in Figure 7. It can be noticed that the method can separate the major vitamin D metabolites from their epimers and isobar, which are 3-epi-25OHD (epimer) and 7αC4 (isobar). Although the major vitamin D metabolites and their epimers have similar structure and mass, there is a difference in retention time between them, as shown in Table 3. When the tandem mass spectrometry (MS/MS) is used by itself to analyze 25OHD and its epimer and isobar, the mass of 25OHD overlaps with its epimer and isobar, which leads to false-positive results. For instance, under or overestimation of actual 25OHD concentration occurred due to co-elution and sharing the exact mass with its epimer and isobar. If the epimers of 25OHD and its isobar have the same precursor to product ion transition or Multiple Reaction Monitoring (MRM), it will be difficult for tandem mass spectrometry by itself to distinguish between co-eluting metabolites for accurate estimation of 25OHD, as shown in Figure 6. Therefore, the UHPLC part with MS/MS part provides a powerful combination for bioanalytical analysis.

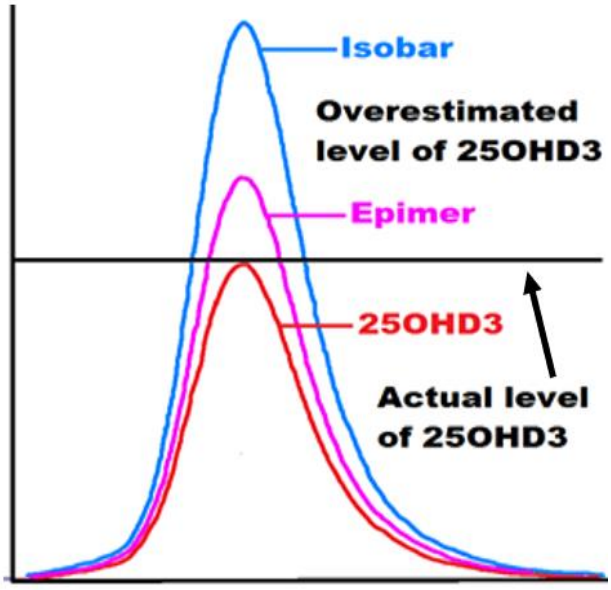


Figure 6: Overestimation of 25OHD3 with its epimer and isobar by mass spectrometry analysis.

According to Figure 7, the universally measured vitamin D metabolites (25OHD3 and 25OHD2) have been separated from their epimers. In this case, the UHPLC-MS/MS instrument has solved the limitations of two techniques, which are Immunoassay and mass spectrometry. These limitations have been resolved by chromatographic separation of 25OHD2 and 25OHD3 and their epimers based on their polarities using the reverse phase column, Ascentis Express F5 column (dimensions: 150 mm × 2.1 mm × 2.7 µm). Furthermore, although 25OHD2 and 3-epi-25OHD3 have almost the same retention time, their ionization behaviors are different.

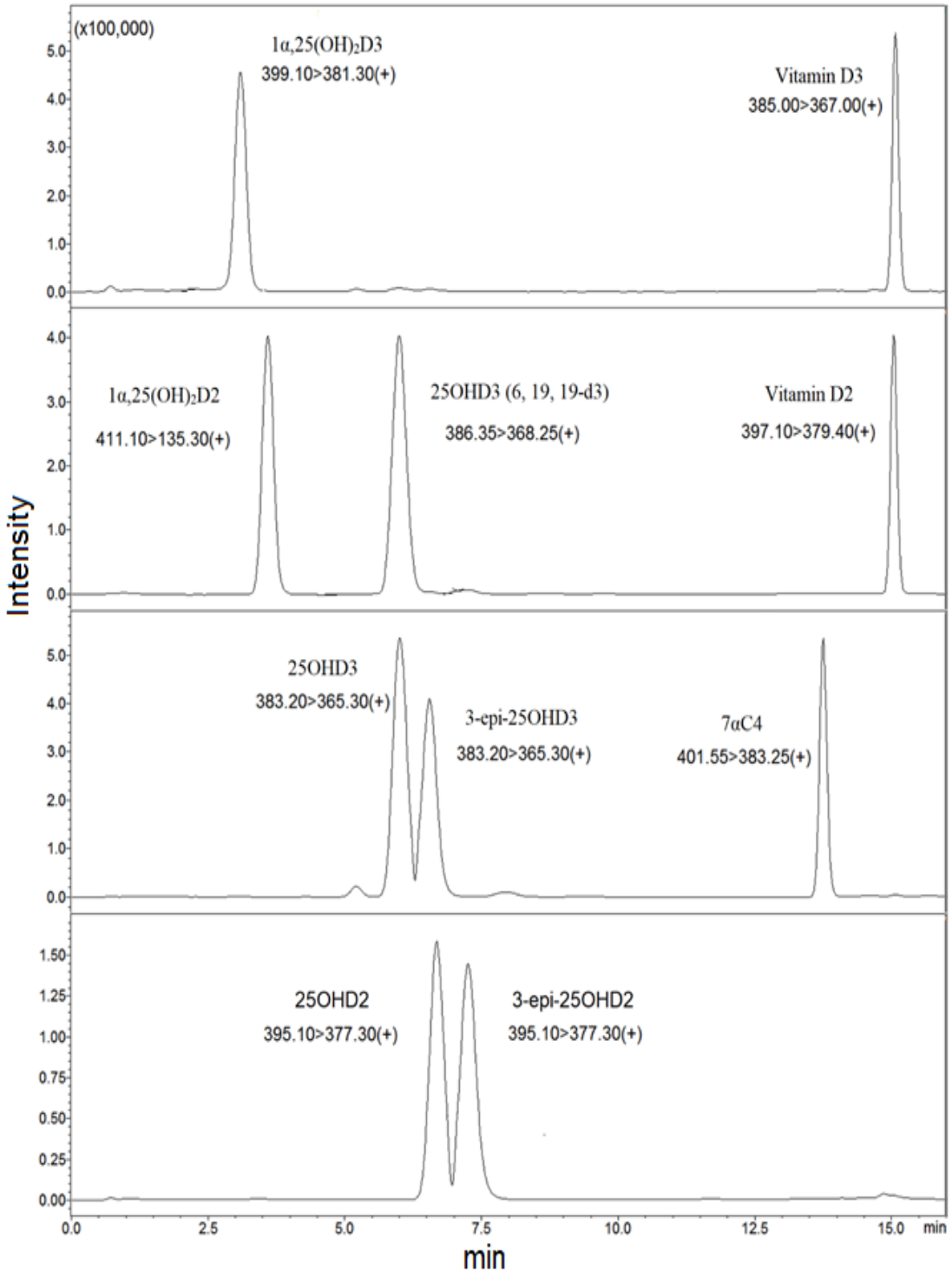


Figure 7: A representative chromatogram of vitamin D metabolites with epimers and isobar. The vitamin D metabolites with epimers and isobar have been separated by UHPLC-MS/MS instrument.

According to Table 3, although D3 and D2 have almost similar retention time and polarities, their masses are different, leading to separate them based on mass to

charge (m/z) ratios. 25OHD and its epimer have the same mass to charge (m/z) ratios; however, their polarities are slightly different that can be separated in the UHPLC part using a reverse-phase column. Although 25OHD3, 3-epi-25OHD3, and 7αC4 have the same masses, their retention times differ. Figure 7 and Table 3 illustrate that the UHPLC-MS/MS separates the vitamin D metabolites and their epimers based on mass to charge (m/z) ratios and polarities.

Table 3: The names, structures, and masses of vitamin D metabolites and epimers with their respective retention times. Although some metabolites have similar structure and mass, the retention time of each metabolite is different.

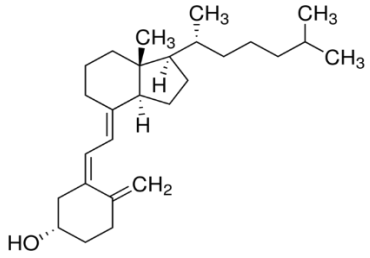
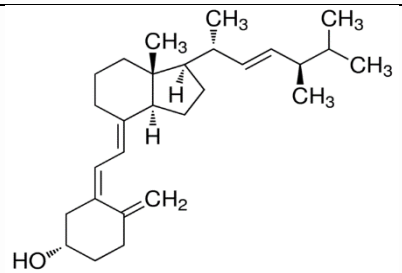
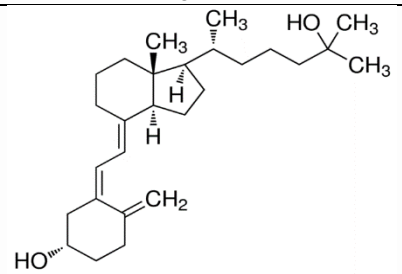
Name	**Vitamer Structure**	**Mass (g/mol)**	**Retention Time (min)**
Cholecalciferol (Vitamin D3)	 $C_{27}H_{44}O$	384.64	15.104
Ergocalciferol (Vitamin D2)	 $C_{28}H_{44}O$	396.65	15.072
25-Hydroxyvitamin D3 (25OHD3)	 $C_{27}H_{44}O_2$	400.64 382.62 ($-H_2O$)	6.008

Table 3: The names, structures, and masses of vitamin D metabolites and epimers with their respective retention times. Although some metabolites have similar structure and mass, the retention time of each metabolite is different (cont'd).

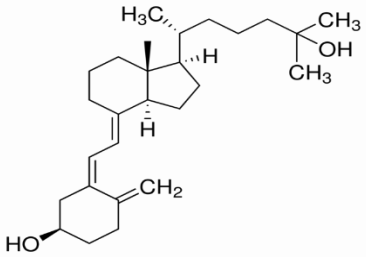
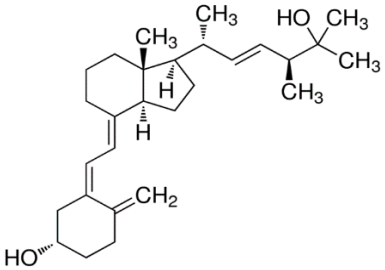
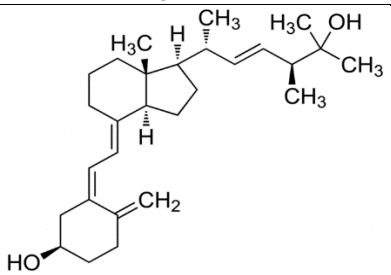
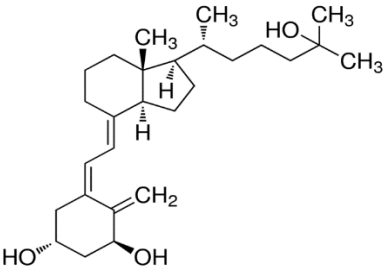
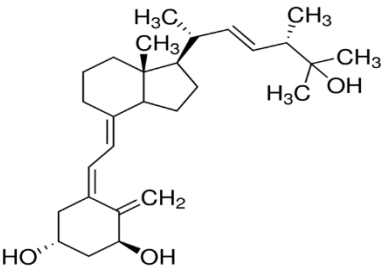
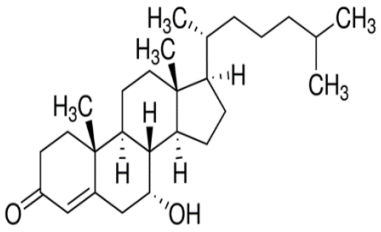
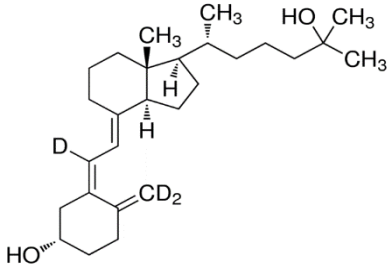
Name	Vitamer Structure	Mass (g/mol)	Retention Time (min)
3-epi-25-Hydroxyvitamin D3 (3-epi-25OHD3)	 $C_{27}H_{44}O_2$	400.64 382.62 ($-H_2O$)	6.552
25-Hydroxyvitamin D2 (25OHD2)	 $C_{28}H_{44}O_2$	412.65 394.63 ($-H_2O$)	6.682
3-epi-25-Hydroxyvitamin D2 (3-epi-25OHD2)	 $C_{28}H_{44}O_2$	412.65 394.63 ($-H_2O$)	7.257
1α,25-Dihydroxyvitamin D3 ($1\alpha,25(OH)_2D3$)	 $C_{27}H_{44}O_3$	416.64 398.62 ($-H_2O$)	3.108
1α,25-Dihydroxyvitamin D2 ($1\alpha,25(OH)_2D2$)	 $C_{28}H_{44}O_3$	428.65 410.63 ($-H_2O$)	3.452

Table 3: The names, structures, and masses of vitamin D metabolites and epimers with their respective retention times. Although some metabolites have similar structure and mass, the retention time of each metabolite is different (cont'd).

Name	Vitamer Structure	Mass (g/mol)	Retention Time (min)
7α-Hydroxy-4-cholesten-3-one (7αC4)	 $C_{27}H_{44}O_2$	400.64	13.763
25-Hydroxyvitamin D_3 (6,19,19-d_3)	 $C_{27}D_3H_{41}O_2$	403.66 385.64 (-H_2O)	5.983

3.2 Determination of Method Validation Parameters

The method validation parameters, which are intra and inter-day precision, intra and inter-day accuracy, recovery, linearity, specificity, and stability, were calculated by analyzing six QC samples at three different concentration levels (QCL, QCM, and QCH). The QCs values were used to calculate the percent of intra and inter-day precision using the following equation:

$$\%CV = \frac{(Standard\ deviation)}{(mean)}\ x\ 100$$

The percent of intra and inter-day accuracy was calculated using the following equation:

$$\%Accuracy = \frac{(mean\ value)}{(nominal\ value)}\ x\ 100$$

The extracted and unextracted QCs values were used to calculate the percent of absolute recovery using the following equation:

$$\%\text{Recovery} = \frac{(mean\ extracted\ QC\ values)}{(mean\ unextracted\ QC\ values)}\ x\ 100$$

The instrument's sensitivity toward vitamin D metabolites was measured using the Lower Limit of Detection (LOD). The LOD was determined by comparing the peak's intensity for the lowest concentration of the analyte to the chromatogram's noise level. The lowest concentration of an analyte should give response higher three times than the background or noise level. The linear range of some vitamin D metabolites was 0.5-100 ng/ml while for 1α,25$(OH)_2$D was 0.015 – 1 ng/ml with a very good regression value (linearity), as shown in Table 4.

The method validation parameters were found in an acceptable range for each QC level. For example, the percentage of the coefficient of variations and accuracy indicates that the method used to analyze vitamin D metabolites and their epimers has good precision and accuracy for intra and inter-day. Moreover, the method illustrates a good recovery percentage, which explains that the extraction method is efficient in extracting vitamin D metabolites and epimers from serum samples, as shown in Table 4. Various studies were reported different LOD and LOQ values. For instance, Müller et al. [101] reported the LOQ for 25OHD3 and its epimer of 0.1 and 1.0 ng/ml, respectively. Moreover, according to Ronda et al. [102] , the LOD for 25OHD3 and its epimer was 0.68 and 0.76 ng/ml, while LOQ was 1.36 and 1.52 ng/ml, respectively, by using LC-MS/MS 6490. Also, the LOD and LOQ were reported for 25OHD3 and its epimer by using LC-MS/MS 6410; the LOD was 0.8 ng/ml while LOQ was 1.4 ng/ml for both metabolites. Another study reported the LOD and LOQ values; for instance, the LOD for 25OHD3 and its epimer was 0.075 and 0.09 ng/ml while LOQ

was 0.25 and 0.3, respectively [94]. Table 4 shows the values of LOD and LOQ for nine metabolites of vitamin D by UHPLC-MS/MS 8060, which explains that the method is extremely sensitive. Moreover, it illustrates the percent of intra and inter-day precision and accuracy results among six quality control samples for each quality control level. Furthermore, the values of percentage recovery were calculated by comparing areas under the normal curve in the chromatogram for the extracted and unextracted quality control samples. A Low Limit of Detection (LOD) was determined from the lowest concentration of vitamin D metabolite that can be detected by the instrument.

Table 4: The values of method validation parameters for each quality control level.

N	Vitamin D metabolites	Conc. (ng/ml)	Intra-day		Inter-day		% Recovery	LOD (ng/ml)	LOQ (ng/ml)	Linearity	Linear Range (ng/ml)
			Precision (%CV)	% Accuracy	Precision (%CV)	% Accuracy					
1	Vitamin D3	QCH 80	1.31	98.9	2.89	90.2	77	0.05	0.5	0.998	0.5 - 100
		QCM 40	2.86	99.4	2.33	89.4	75				
		QCL 20	4.13	87.1	5.32	89.8	98				
2	Vitamin D2	QCH 80	9.4	89.4	9.6	89.3	88	0.05	0.5	0.997	0.5 - 100
		QCM 40	11.3	97.5	13.3	94.8	89.3				
		QCL 20	6.3	99.2	11.3	93.2	105				
3	25OHD3	QCH 80	3.5	99.1	2.9	99.2	86	0.025	0.5	0.998	0.5 - 100
		QCM 40	2.7	97.3	3.7	99.8	89				
		QCL 20	6.1	99.9	7.4	98.1	111				
4	3-epi-25OHD3	QCH 80	3.3	89.1	8.9	99.9	80	0.05	0.5	0.998	0.5 - 100
		QCM 40	3.1	87.3	10.9	87.6	96				
		QCL 20	3.5	89.2	4.3	98.5	86				
5	25OHD2	QCH 80	3.3	88.7	3.8	89.3	82	0.025	0.5	0.998	0.5 - 100
		QCM 40	2.7	99.9	3.9	98.9	99				
		QCL 20	2.8	99.8	3.4	101.2	115				
6	3-epi-25OHD2	QCH 80	3.2	89.9	2.2	88.3	86.5	0.05	0.5	0.998	0.5 - 100
		QCM 40	4.9	84.6	2.7	87.1	97				
		QCL 20	7.1	85.8	3.5	88.5	89				
7	1α,25$(OH)_2$D3	QCH 0.8	3.6	99.7	2.6	88.9	88	0.01	0.015	0.997	0.015 - 1
		QCM 0.4	4.2	86.4	4.4	87.3	82				
		QCL 0.2	4.4	89.2	5.6	88.5	97				
8	1α,25$(OH)_2$D2	QCH 0.8	3.9	99.9	4.3	87.8	86	0.01	0.015	0.993	0.015 - 1
		QCM 0.4	4.4	99.4	6.3	98.9	87				
		QCL 0.2	3.2	98.9	5.2	89.8	89.1				
9	7αC4	QCH 80	4.1	79.9	3.1	88.1	92.45	0.1	0.5	0.999	0.5 - 100
		QCM 40	2.3	88.6	3.1	85.3	97				
		QCL 20	3.2	98.3	5.4	86.2	92				

The analysis of six blank serum samples determined the specificity of the method. For instance, six blank serums were prepared without vitamin D metabolites; then, the blank serums were analyzed by UHPLC-MS/MS instrument. After that, the chromatogram for each blank serum was compared and overlaid with other blank's chromatograms in order to reveal any co-eluting or interfering peaks, which could have the same retention time as the vitamin D metabolites. It was found that there are no interfering or co-eluting peaks generated from any interferences in the serum. There was only a noise level without any strange peaks, which could interfere with the vitamin D peak and lead to overestimating the result, as shown in Figure 8.

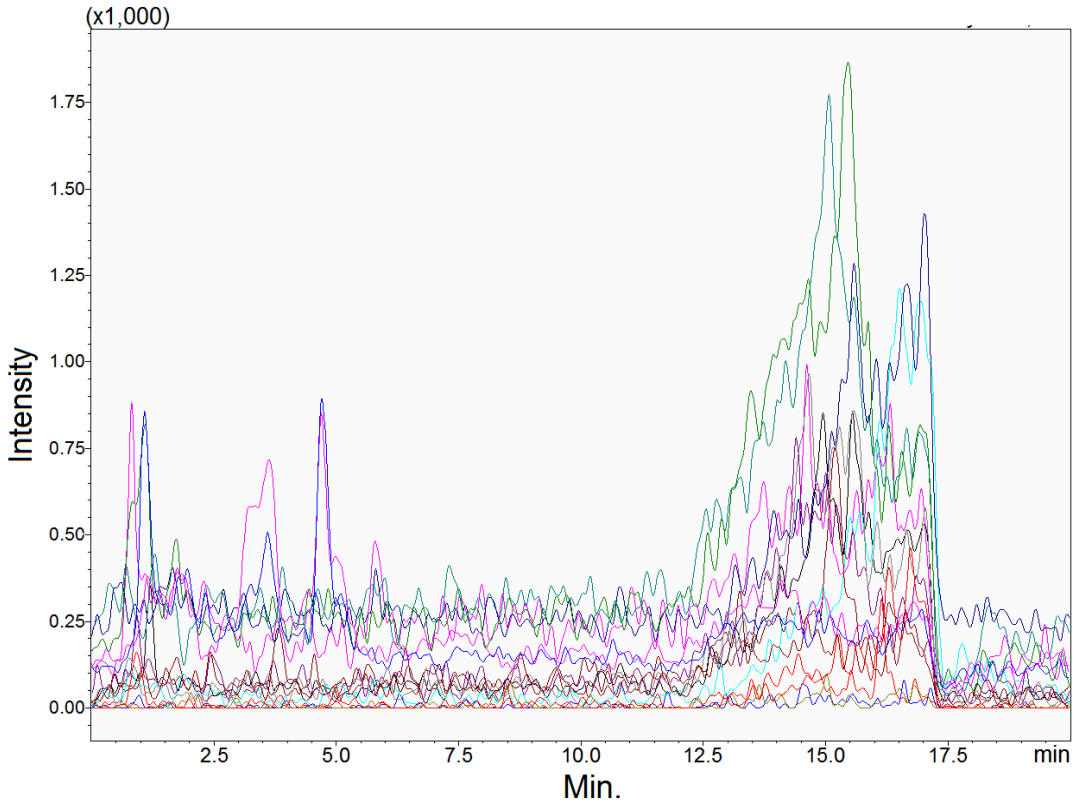


Figure 8: Overlaid chromatograms of six blank serum samples. The specificity experiment results illustrate that the interfering or co-eluting peaks did not exist at the respective retention times of vitamin D metabolites.

The stability of the vitamin D metabolites and their epimers was assessed by analyzing six QC samples of each concentration level (QCH, QCM, and QCL). The

QC samples were analyzed over 24 hr, 48 hr, and 72 hr (three sequential freeze/thaw cycles); then, the results were compared between each other and with time zero. Figure 9 shows the percentage change in the intactness of vitamin D metabolites and their epimers over three freezes/thaw cycles. The vitamin D3 exhibited more than 30% change in 24 hr, while for 48 hr and 72 hr, the percentage change was around 20. The stability of vitamin D2 was almost the same as vitamin D3. The 25OHD3 illustrated a higher percentage change in 24 hr compared to 48 hr and 72 hr, while its epimer was around 20% of all three freezes/thaw cycles. The highest percent of change was observed by 3-epi-25OHD2 in 48 hr and 72 hr compared to all vitamin D metabolites. The rest of the metabolites (25OHD2, 1α,25$(OH)_2$D3, 1α,25$(OH)_2$D2, and 7αC4) have fluctuated around 20%. Ultimately, the results of stability or intactness of vitamin D metabolites and their epimers revealed that these metabolites are not very stable with consecutive cycles of freezing and thawing.

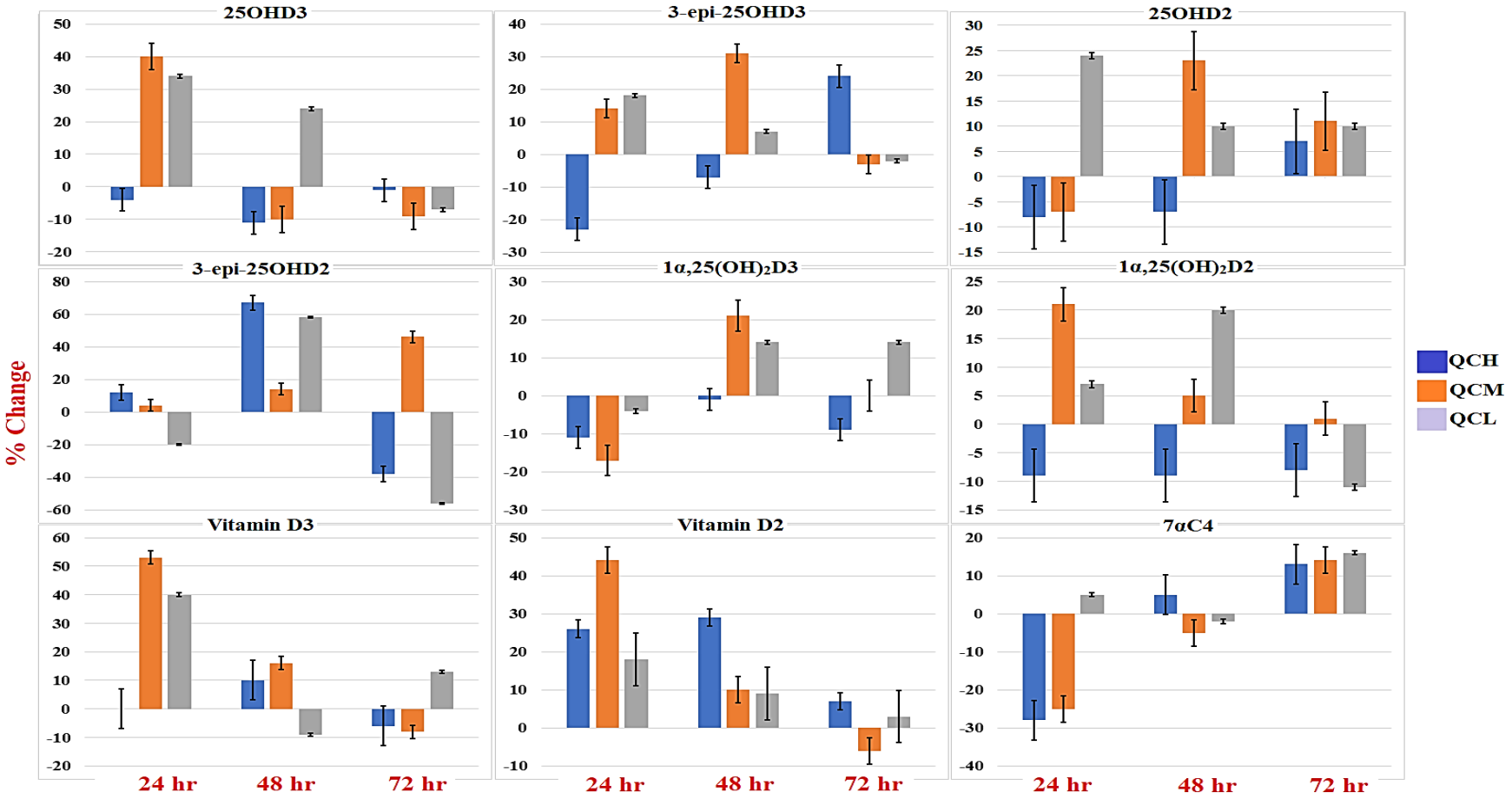


Figure 9: Vitamin D metabolites stability over three consecutive freeze/thaw cycles (24 hr, 48 hr, 72 hr). The Quality Control High (QCH), Quality Control Medium (QCM), and Quality Control Low (QCL) are represented by blue, orange, and grey bars at 24 hr, 48 hr, and 72 hr intervals, respectively. The values of six quality control samples for each level (QCH, QCM, and QCL) were used to estimate the stability of each vitamin D metabolite over three consecutive freeze/thaw cycles. The range of % change in vitamin D metabolites stability was between 0 and 60.

3.3 Identification of Precursor and Product Ions (Multiple Reaction Monitoring, MRM) for Vitamin D Metabolites and their Epimers

The precursor to product ion transition and collision energy of the epimers were the same as 25OHD3 and 25OHD2; therefore, the tandem mass spectrometry by itself cannot differentiate between 25OHD and its epimer, which lead to cause overestimation of 25OHD and give false-positive results. The remaining metabolites of vitamin D revealed different MRM and collision energies, as shown in Table 5. An accurate and highly sensitive UHPLC-MS/MS-8060 instrument facilitates the determination of vitamin D metabolites and epimers accurately during the analysis time. Mass spectrometry analysis was operated on the MS/MS-8060 (Triple Quadrupole Mass Spectrometer) system in positive electrospray ionization (ESI^+) mode for all vitamin D metabolites and epimers due to that the instrument was highly sensitive when the vitamin D metabolites are positively charged. The precursor ions of 25OHD and its epimer, 1α,25$(OH)_2$D3, 1α,25$(OH)_2$D2, and 25OHD3 (6, 19, 19-d3) metabolites were most sensitive when these metabolites lose water molecule with simultaneous production of $[M+H-H_2O]^+$. Moreover, the MRM mode gives the most sensitive product ions. The 25OHD3, 3-epi-25OHD3 (epimer), 7αC4 (isobar) have the same masses without losing water molecules. These factors lead to under- and over-estimation of 25OHD and give false-positive results; therefore, the separation of these coeluting interfering components is crucial for accurate measurement of vitamin D. Table 5 illustrates the selected MRM transitions along with their collision energies.

Lastly, the developed and validated method for the analysis of vitamin D metabolites and epimers revealed that UHPLC-MS/MS technique was accurate, reproducible, specific, quick, reliable, and robust. Furthermore, the method can distinguish between major metabolites of vitamin D and their epimers and isobars;

therefore, the method will be more reliable for the quantitative and qualitative analysis of vitamin D in human serum samples.

Table 5: The names of the analytes, masses, precursor and product ions along with their collision energies. Some vitamin D metabolites have the same MRM and collision energies.

No.	Analytes	Mass (g/mol)	Precursor (m/z)	Product (m/z)	Collision energy (eV)
1	Vitamin D3	384.64	385 385 385	367 259 91	-13 -16 -55
2	Vitamin D2	396.65	397.1 397.1	379.4 69	-17 -29
3	25OHD3	400.64 382.62 (-H_2O)	383.2 383.2	365.3 107.1	-15 -30
4	3-epi-25OHD3	400.64 382.62 (-H_2O)	383.2 383.2	365.3 107.1	-15 -30
5	25OHD2	412.65 394.63 (-H_2O)	395.1 395.1	377.3 81.1	-17 -38
6	3-epi-25OHD2	412.65 394.63 (-H_2O)	395.1 395.1	377.3 81.1	-17 -38
7	1α,25$(OH)_2$D3	416.64 398.62 (-H_2O)	399.1	381.3	-14
8	1α,25$(OH)_2$D2	428.65 410.63 (-H_2O)	411.1 411.1	135.3 133.1	-13 -12
9	7αC4	400.64	401.55 401.55 401.55	383.25 97.1 177.2	-16 -29 -23
10	25OHD3(6,19,19-d3)	403.66 385.64 (-H_2O)	386.35 386.35 386.35	368.25 257.2 95.2	-15 -183 -35

3.4 Analysis of Human Serum Samples (Baseline, Follow-up and Healthy)

In many clinical laboratories, the 25OHD is considered the best metabolite of vitamin D to determine the vitamin D status in human blood samples. Measurement of total 25OHD is defined as vitamin D status; total 25OHD refers to 25OHD3 and

25OHD2 with their epimers. The measurement of 1α,25$(OH)_2$D metabolite is more complicated than 25OHD for several reasons: the short half-life of 1α,25$(OH)_2$D, which is between 4 – 15 hr compared to its parent 25OHD, which is between 21 – 30 days, instability of 1α,25$(OH)_2$D and presence at a low concentration within the circulation (picomolar concentration) [103, 104]. Different organizations proposed the reference values of vitamin D; therefore, the normal level of vitamin D in the blood may differ slightly between these organizations. For instance, the optimum level of 25OHD, 20 ng/ml, was proposed by the institute of medicine, while the vitamin D council considered the optimum level between 40 and 80 ng/ml [36, 105]. One of the guidelines mentioned the reference value of total 25OHD and 1α,25$(OH)_2$D in the blood. For example, the optimum levels of total 25OHD are between 25 and 80 ng/ml while the optimum range of 1α,25$(OH)_2$D in males and females is 18 – 64 pg/ml and 18 – 78 pg/ml, respectively [106]. Table 6 illustrates the normal range of 25OHD levels in serum that was proposed by different studies. The vitamin D results of male and female patients are illustrated separately in Figures 10 and 11.

Table 6: The normal range of vitamin D in serum proposed in various studies.

Study	**Deficient (ng/ml)**	**Insufficient (ng/ml)**	**normal (ng/ml)**	**Excess (ng/ml)**
[107]	<10	10 - 20	20 - 30	>200
[13]	<12	12 - 20	20 - 50	>50
[108]	<20	21 - 29	30 - 100	>100
[109]	<10	10 - 30	30 - 80	>100
[110]	<20	21 - 29	30 - 60	>150
[111]	<10	10 - 30	30 - 100	>100

Different guidelines have been published by various studies about serum vitamin D levels for good health; therefore, it seems that there is no consensus about an adequate level of vitamin D [13, 107-111], as shown in Table 6.

The concentration of vitamin D metabolites is deficient in the baseline patients. When vitamin D deficient volunteers (baseline samples) take vitamin D supplements, the levels of vitamin D metabolites have been increased (follow-up samples), as shown in Figures 10 and 11. Although the levels of vitamin D metabolites in follow-up samples have increased, most of them were still lower than the normal range proposed by the studies, Table 6. 3-epi-25OHD2 concentration was not detected. The vitamin D3 concentration in the follow-up samples of both males and females is high; therefore, either 25OHD3 or its epimer should be increased significantly when vitamin D3 is converted to 25OHD3 by CYP2R1 enzyme in the liver (Chapter 1). On the other hand, this study shows that 25OHD3 is increased slightly in the follow-up samples because vitamin D3 could probably be converted to other forms. For example, a few new vitamin D metabolites such as 20, 22$(OH)_2$D3, and 22OHD3 could be produced in epidermal keratinocytes upon hydroxylation of vitamin D3 by CYP11A1 enzyme [64-66]. Moreover, UVB light may activate the CYP11A1 enzyme; thus, it will lead to a decrease in the vitamin D3 substrate availability for classical hydroxylation to form 25OHD3 and its epimer [31].

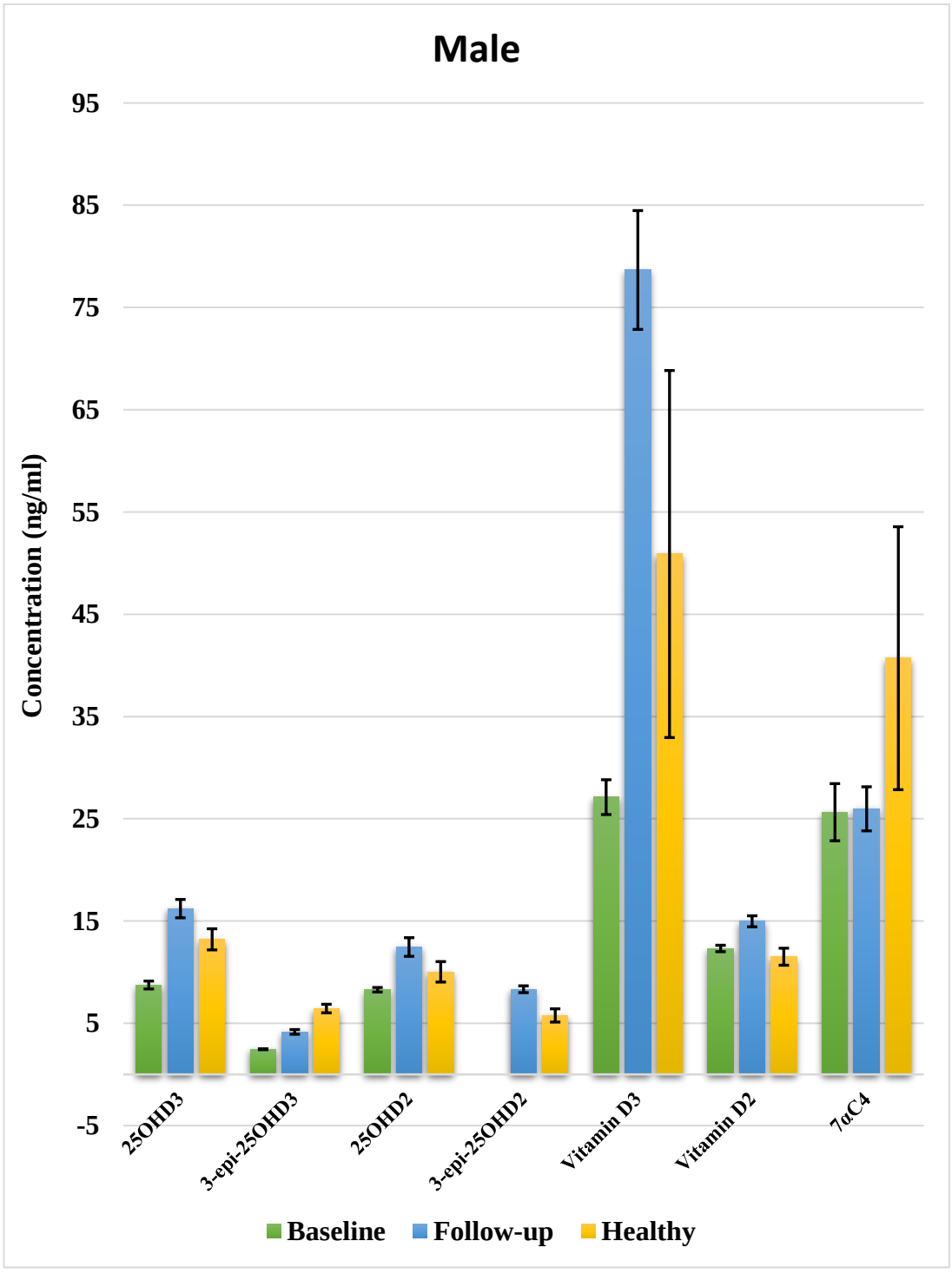


Figure 10: A comparison between vitamin D metabolites concentration in male blood samples. 3-epi-25OHD2 concentration was not detected in baseline samples while $1\alpha,25(OH)_2D$ was not detected in the baseline, follow-up, and healthy samples. The standard errors of the mean were represented by the error bars in the graph.

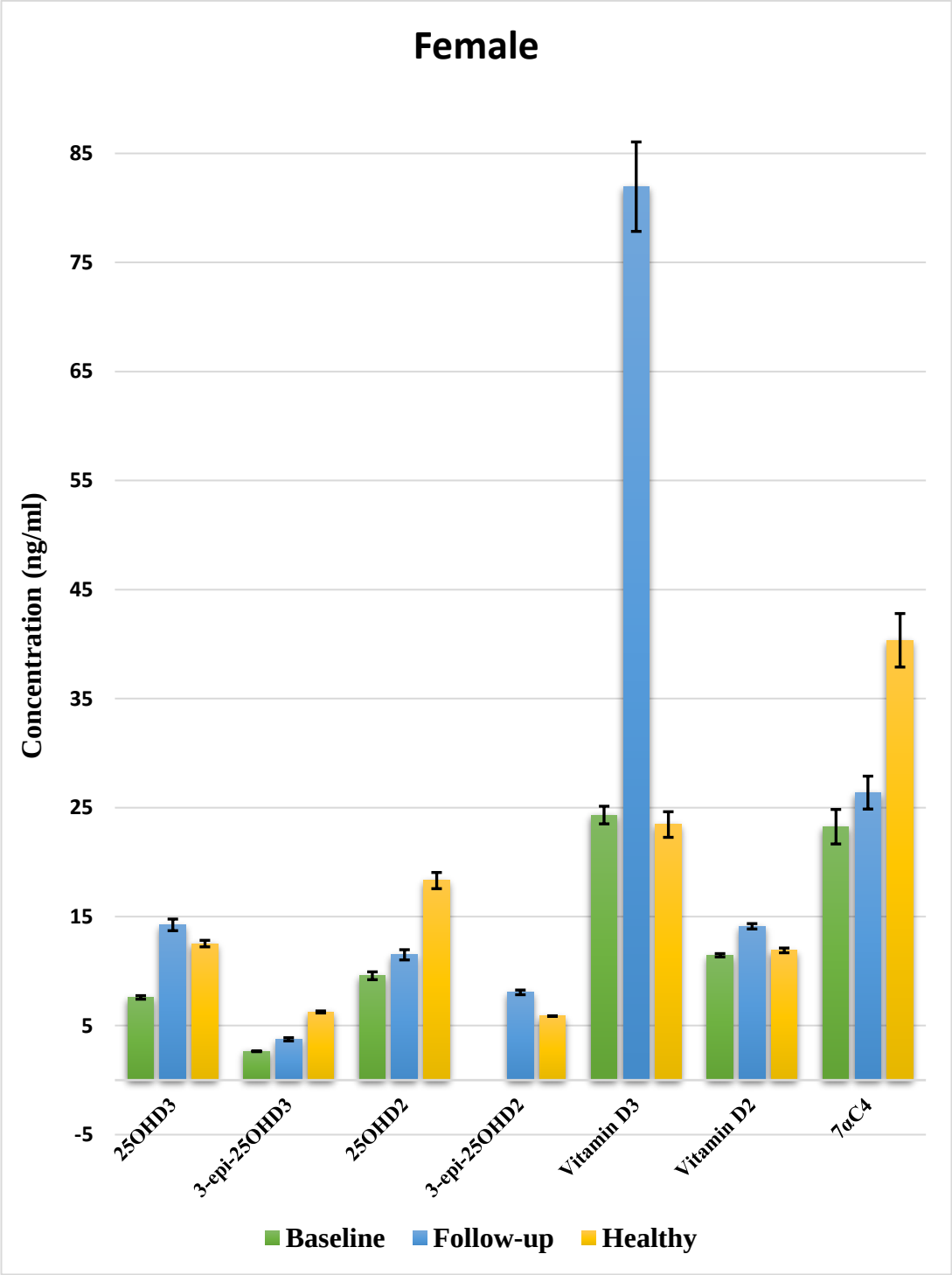


Figure 11: A comparison between vitamin D metabolites concentration in female blood samples. 3-epi-25OHD2 concentration was not detected in baseline samples while 1α,25(OH)$_2$D was not detected in the baseline, follow-up and healthy samples. The standard errors of the mean were represented by the error bars in the graph.

The UHPLC-MS/MS method can separate and distinguish the epimers and isobars of vitamin D, which caused an interference during the analysis of the major

forms of vitamin D in human blood samples; therefore, this method leads to giving an actual result due to its ability to measure and distinguish the major metabolites of vitamin D (25OHD3 and 25OHD2) accurately without combining the epimers with these major metabolites. Table 7 illustrates the results of vitamin D metabolites in baseline, follow-up, and healthy samples of mixed gender volunteers (male + female).

When the vitamin D deficient volunteers (baseline) were supplemented, the levels of all vitamin D metabolites and epimers were increased; however, the increase was not sufficient to achieve the normal range proposed by the studies (Table 6), as shown in Table 7. Although the vitamin D3 was increased significantly in follow-up samples, the levels of other vitamin D3 metabolites and epimers were not increased sufficiently, which may explain that vitamin D3 was being converted not only to major form (25OHD3) but also to other forms such as 20, 22$(OH)_2$D3, and 22OHD3 [64-66]. Probably, this can be resolved by increasing the dietary vitamin D intake to achieve the normal range proposed by the studies (Table 6).

According to Table 7, if the components of total 25OHD (25OHD3 + 3-epi-25OHD3 + 25OHD2 + 3-epi-25OHD2) are combined together in the follow-up and healthy samples, the total 25OHD will be in the sufficient range according to the studies (Table 6). Moreover, the total 25OHD for follow-up and healthy samples is in the normal range (25 – 80 ng/ml) according to Mosby's Diagnostic and Laboratory Test Reference [106]. The immunoassay technique will consider 25OHD3, 3-epi-25OHD3, 25OHD2, and 3-epi-25OHD2 as one entity; so, it will combine the components of total 25OHD and overestimate the results.

Table 7: A comparison between the average concentrations of vitamin D metabolites and epimers for baseline, follow-up and healthy mixed-gender volunteers**.** The last column shows the average concentrations in ng/ml and the standard deviation (SD). The symbol N represents the total number of analyzed samples of baseline, follow-up, and healthy volunteers. 3-epi-25OHD2, 1α,25$(OH)_2$D3 and 1α,25$(OH)_2$D2 were not detected.

No.	Analytes	Sample Type	N	Mean ± SD
1	Vitamin D3	Base line	277	25.03 ± 12.40
		Follow-up	277	81.09 ± 56.31
		Healthy	175	25.07 ± 21.96
2	Vitamin D2	Base line	277	11.68 ± 2.33
		Follow-up	277	14.34 ± 3.84
		Healthy	175	11.88 ± 2.79
3	25OHD3	Base line	277	7.89 ± 2.65
		Follow-up	277	14.76 ± 7.65
		Healthy	175	12.56 ± 3.84
4	3-epi-25OHD3	Base line	277	2.60 ± 0.48
		Follow-up	277	3.85 ± 2.10
		Healthy	175	6.26 ± 1.19
5	25OHD2	Base line	277	9.25 ± 4.52
		Follow-up	277	11.73 ± 6.94
		Healthy	175	18.18 ± 9.58
6	3-epi-25OHD2	Base line	277	n.d.
		Follow-up	277	8.13 ± 2.94
		Healthy	175	5.88 ± 0.22
7	1α,25$(OH)_2$D3	Base line	277	n.d.
		Follow-up	277	n.d.
		Healthy	175	n.d.
8	1α,25$(OH)_2$D2	Base line	277	n.d.
		Follow-up	277	n.d.
		Healthy	175	n.d.
9	7αC4	Base line	277	23.88 ± 22.94
		Follow-up	277	26.27 ± 20.75
		Healthy	175	40.36 ± 31.84

Figure 12 illustrates that the epimers and isobars of 25OHD3 account for a significant proportion, which will lead to an overestimation of actual 25OHD3 concentrations due to co-elution and sharing the exact mass with its epimers (3-epi-25OHD3) and isobars (7αC4). According to the guidelines proposed by some studies (Table 6), when the epimers and isobars are excluded from the universally measured 25OHD, the baseline, follow-up, and healthy volunteers are considered deficient

25OHD3 and 25OHD2; as shown in Figure 13. In contrast, follow-up and healthy volunteers are considered insufficient according to the guidelines of other studies (Table 6). The UHPLC-MS/MS method excludes and differentiates the epimers and isobars that cause an interference leading to overestimate an actual concentration of major vitamin D metabolites (25OHD3 and 25OHD2) and gave false-positive results; as shown in Figure 12. In the immunoassay technique, 25OHD3 and 25OHD2 with their epimers are considered one entity; according to Figure 12, when 25OHD3 and 25OHD2 with their epimers are combined, the baseline samples are in an insufficient range while follow-up samples are in a normal range. Healthy volunteers are in the normal range of vitamin D when the epimers are accounted. Interestingly, the 25OHD3 and 25OHD2 concentrations, which were around 12.56 ng/ml and 18.18 ng/ml, respectively, in the healthy volunteers were even less than the adequate value proposed by different studies (Table 6); as shown in Figure 13, probably, the vitamin D is diluted in an immense volume of obese volunteers. In the mass spectrometry technique, 25OHD3, 3-epi-25OHD3, and 7αC4 have the same masses; therefore, they are recognized as one entity. In Figure 12, if 25OHD3, 3-epi-25OHD3, and 7αC4 are gathered, the baseline, follow-up, and healthy samples will be in the sufficient range; therefore, the results will be overestimated. This study displays that UHPLC-MS/MS technique erases and excludes the epimers and isobar interferences; accordingly, it can separate epimers and isobar from major vitamin D metabolites based on their mass to charge ratios (m/z) and polarities, leading to give actual results.

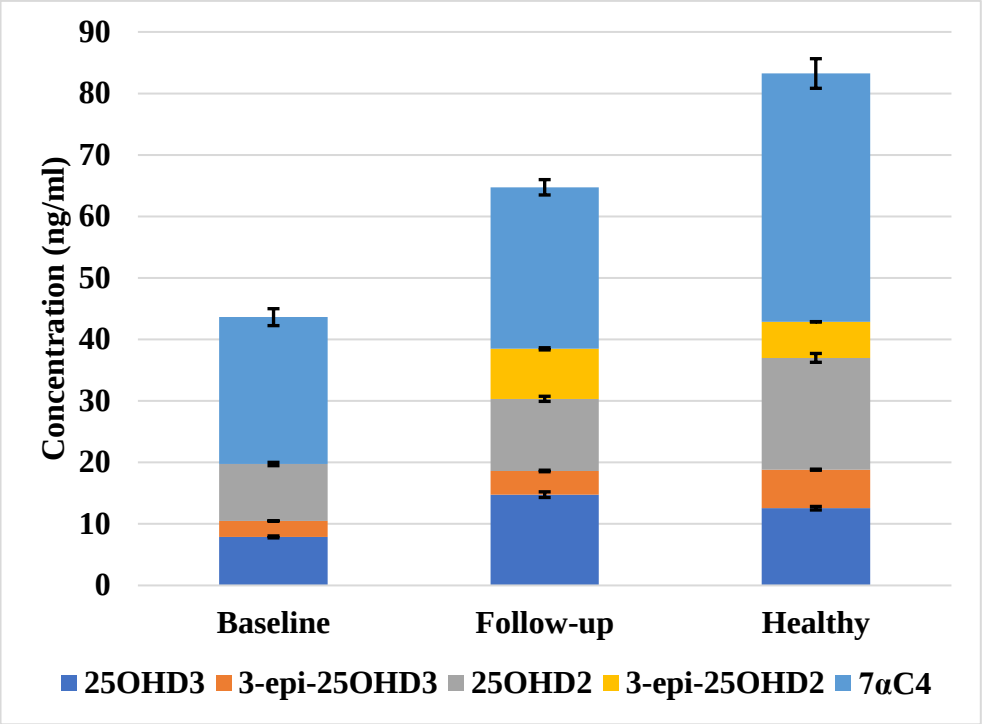


Figure 12: The bar chart illustrates a comparison of major vitamin D metabolites and their epimers and isobars at the baseline, follow-up, and healthy mixed-gender samples. The stacked columns show the combination between major forms of vitamin D (25OHD3 + 25OHD2) along with the co-eluting epimers (3-epi-25OHD3 + 3-epi-25OHD3) and the isobar (7αC4). The standard errors of the mean were represented by the error bars in the graph.

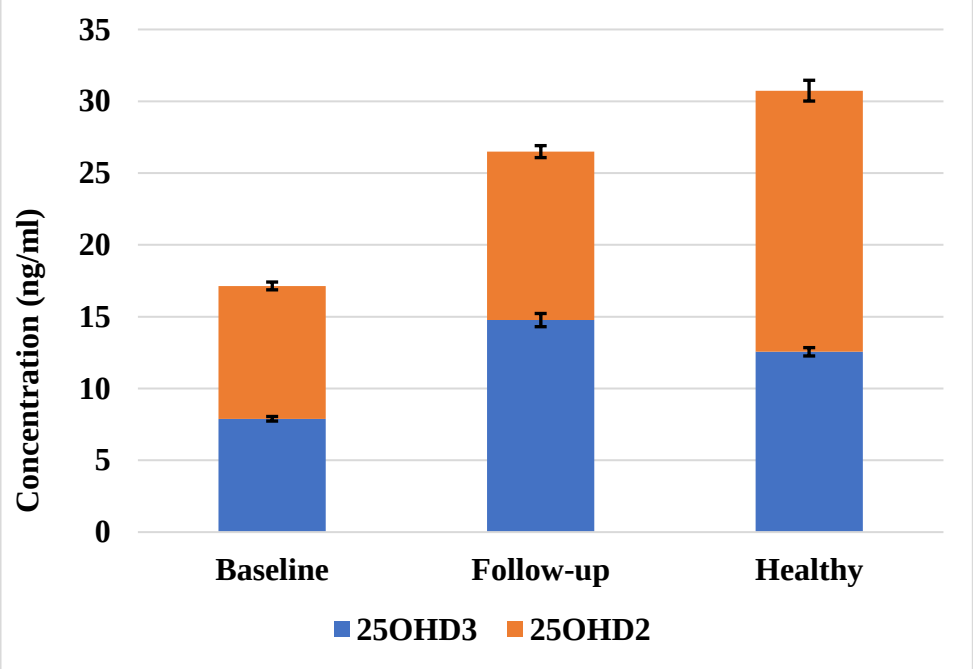


Figure 13: The bar chart illustrates a comparison of major vitamin D metabolites (25OHD3 + 25OHD2) at the baseline, follow-up, and healthy mixed-gender samples. The stacked columns show the combination of these major forms. The standard errors of the mean were represented by the error bars in the graph.

Chapter 4: Conclusion

The Immunoassay technique is one of the first techniques used in measuring vitamin D. It is widely used in clinical laboratories; however, it has many limitations. One of the main challenges that face this technique is the low specificity to assess vitamin D metabolites. For example, this technique measures total 25OHD and $1\alpha,25(OH)_2D$ concentrations because it measures D2 and D3 as one entity and cannot distinguish between them [85]; therefore, this technique is not in agreement with the US Food and Drug Administration (FDA), which stipulates that 25OHD3 and 25OHD2 should be detected in 25OHD assays [112].

High-Performance Liquid Chromatography (HPLC) was considered until lately as the standard technique for quantifying vitamin D [83, 113]. HPLC was preferred in terms of accuracy and precision more than Immunoassay. It was able to solve the problem of separating 25OHD2 and 25OHD3 levels. Another advantage for HPLC and LCMS is that they can extract 25OHD and remove the binding protein by either liquid-liquid extraction or solid-phase extraction. The HPLC method has limitations as well; for example, it requires large sample volumes and has less sensitivity for low levels of vitamin D metabolites compared to LC-MS/MS [114].

The best available technique to quantify and distinguish between 25OHD3 and 25OHD2, along with their epimers, is the LC-MS/MS. It can eliminate most technical problems of HPLC and immunoassays methods. What gives LC-MS/MS preference is the high specificity and sensitivity of it. LC-MS/MS has superior selectivity compared to the other methods due to its ability to differentiate between different metabolites via chromatographic separation and mass transition differences. The compounds that have differences in polarities and mass to charge (m/z) ratios can be separated by LC-

MS/MS method; moreover, this method has very Low Limits of Quantitation (LOQ), which gives a higher sensitivity. LC-MS/MS is the main technique for quantification of vitamin D metabolites in clinics due to its accuracy and precision [115].

Epimers are compounds that have different arrangements around a carbon atom with the same chemical formula. The epimerization pathway of vitamin D occurs at C-3 during the standard metabolic pathway [24]. The separation of epimers and isobars from effective forms of vitamin D metabolites is crucial because epimers peaks can interfere with the standard peaks for major metabolites, which affect the measurement of the actual concentrations of vitamin D. 3-epi-25OHD3 is the most common epimer. Two main compounds can affect the analysis of the universally measured 25OHD and interfere with it as isobars. These compounds are 1α-hydroxyvitamin D3(1αOHD3, exogenous pharmaceutical compound) and 7-α-hydroxy-4-cholesten-3-one (7αC4, endogenous bile acid precursor) [35]. In this study, when the total 25OHD concentration is measured in the blood, the epimers and isobars of 25OHD contribute to a significant proportion of total 25OHD; subsequently, they will lead to an overestimation of actual levels of 25OHD; as shown in Figure 12.

Most of the existing methods of vitamin D analysis can lead to an overestimation of vitamin D levels due to interfering epimers, especially among infant groups and pregnant women. The determination of vitamin D metabolites and vitamin D epimers is not very reliable due to cross-reactivity issues. Studies have shown that LC–MS/MS is the gold standard method to quantify and separate epimers, bypassing the issues caused by their interference. All major vitamin D metabolites can be epimerized at the C3 position. It is known that very few labs in the world account for misleading measures due to overlapping C3-epimers. It is also known that routine vitamin D blood tests for healthy adults will not be significantly affected by epimeric

interference using LC–MS/MS assays. Recent genetic models also show that the genetic determinants and potential factors of C3-epimers differ from those of non-C3-epimers.

Moreover, mice have higher epimer concentrations than humans, and the epimerization process for mice with orally ingested vitamin D3 is higher than that for mice irradiated by UVB light. It was established that oral supplementation of vitamin D could cause increased production of epimers in mice but not in humans. Under clinical laboratory conditions, time and throughput are essential concerns. For example, separating isomers by chromatographic analysis using HPLC, LC–MS, and LC–MS/MS is time-consuming and utilizing chiral or CN columns could lead to compromised throughput. However, PFP columns could be more suitable. The biological origin of these epimers has not yet been clearly identified, and more clinical research is required [24, 31, 63, 73, 76-78, 116, 117]. The C3-epimers of vitamin D may well have an important role to play in clinical research, and more research is warranted. Finally, the method used in our study could be useful to study vitamin D metabolites and their epimers in hair samples; also, it could be useful to study the relationship between vitamin D and other diseases such as Covid-19.

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List of Publications

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