Methods for the Measurement of Vitamin D Metabolites and Studies on Their Relationships in Health and Disease

A thesis submitted to the University of East Anglia in accordance with the requirements for the degree of

Doctor of Philosophy

By

Jonathan Chung Yan Tang

Department of Medicine, Norwich Medical School, Faculty of Medicine and Health Sciences, University of East Anglia

2019

Abstract

The prevalence of vitamin D deficiency in the general population has become a major public health problem. Vitamin D deficiency might have significant consequences not only to bone health but possible to other autoimmune, infectious, cancer and cardiovascular diseases. The explosion of interests in vitamin D had sparked a massive increase in the number of laboratory requests for the measurement of serum 25 hydroxyvitamin D (25(OH)D). This in turn highlighted problems of the methodologies available for measuring vitamin D metabolites.

The aim of the study was to develop and fully validate quantitative assays for measuring serum vitamin D metabolites by Liquid Chromatography Tandem Mass Spectrometry (LC/MS-MS). The methods were used to perform analysis on samples collected for vitamin D research studies to establish relationships between the metabolites and determine reference intervals.

Using solid phase extraction to remove phospholipids in sample matrix and derivatisation to enhance sensitivity, assays were successfully developed for 25(OH)D3/D2, C3-epi-25(OH)D3/D2, and for the dihydroxyvitamin D 24,25$(OH)_2$D3/D2 and 1,25$(OH)_2$D3/D2. The performance characteristic of the assays satisfied industry standards for method validation. Results showed a high prevalence of C3-epi-25(OH)D3 (87.7%) in paediatric samples that resulted in misclassification of total 25(OH)D status in 10.4% of cases. Serum 25(OH)D showed a significant correlation with 24,25$(OH)_2$D (r^2=0.754, p<0.001), but not with 1,25$(OH)_2$D (r^2=0.1034). The reference intervals (2.5-97.5 percentile) for 25(OH)D:24,25$(OH)_2$D ratio was established between 7-23. Loess fitting showed an increase in the 25(OH)D:24,25$(OH)_2$D ratio at 25(OH)D <50 nmol/L; evidence of reduced catabolic activity during low vitamin D status. In contrast, when high dose vitamin D3 was supplemented, serum 24,25$(OH)_2$D was found to be grossly elevated to counteract against excessive vitamin D and prevent toxicity. Using the 1,25$(OH)_2$D:24,25$(OH)_2$D/25(OH)D ratio model, this thesis was the first to demonstrate a relationship between the three metabolites, and the association with PTH concentration.

This thesis has provided new insights to the vitamin D metabolism that will further our understanding and appreciation of its role in health and pathophysiological conditions. The methods developed have provided an analytical platform for many large scale studies in musculoskeletal research and other areas of science; the publications and citations are a testament to the impact of this research.

Table of Contents

Figures

Tables

Declaration

The method development studies were conceived and designed following discussions with my supervisor, Professor William Fraser. I conducted all method development, sample and data analyses.

The vitamin D supplementation studies described in Chapter 8 was jointly led by Prof. William Fraser with Dr Lanja Saleh (University Hospital of Zurich, Switzerland), Prof. Graeme Close (Liverpool John Moores University), and Prof. Helen MacDonald (University of Aberdeen). The Army study described in Chapter 7 and 9 was conducted jointly with Prof. Julie Greeves OBE (Directorate of Manning, UK Ministry of Defence), Dr Sarah Jackson (Army Medical Directorate), and Prof. Neil Walsh (Bangor University). I was a named contributor and project co-investigator in the studies. Sample collection was undertaken by the technical support teams from the respective institutions.

Ethics Statement

The collection, processing and storage of all data, human blood and tissue samples were in accordance with the Data Protection Act 2018 and the Human Tissue Act 2004. I am registered with the Health and Care Professions Council and is entitled to practise as a Biomedical Scientist. I have completed Good Laboratory Practice and Good Clinical Practice training with certification.

Samples used for method development were collected and processed in accordance with generic ethical approval for assay development. All samples were anonymised at the point of access.

The trial studies described in Chapter 7-9 were conducted in accordance with the declaration of Helsinki and Good Clinical Practice guidelines. Ethical approvals were obtained before the start of the studies, and informed consent was obtained from all participants prior to enrolment.

Publications

First authored publications

1. **Tang, J.C.Y.**, Jackson, S., Walsh, N.P., Greeves, J., Fraser, W.D. & Bioanalytical Facility, t. The dynamic relationships between the active and catabolic vitamin D metabolites, their ratios, and associations with PTH. Sci Rep 9(1), 6974 (2019).
2. **Tang JCY**, Nicholls H, Piec I, Washbourne CJ, Dutton JJ, Jackson S, Greeves J, Fraser WD. Reference intervals for serum 24,25-dihydroxyvitamin D and the ratio with 25-hydroxyvitamin D established using a newly developed LC-MS/MS method. *J Nutr Biochem.* 2017;46:21-29.
3. Owens DJ, **Tang JC**, Bradley WJ, Sparks AS, Fraser WD, Morton JP, Close GL. Efficacy of High-Dose Vitamin D Supplements for Elite Athletes. *Med Sci Sports Exerc.* 2017;49(2):349-356.

Co-authored publications

1. O'Leary, T.J., Izard, R.M., Walsh, N.P., **Tang, J.C.Y.**, Fraser, W.D. & Greeves, J.P. Skeletal macro- and microstructure adaptations in men undergoing arduous military training. Bone 125, 54-60 (2019).
2. Fraser, W.D., **Tang, J.C.Y.**, Dutton, J.J. & Schoenmakers, I. Vitamin D Measurement, the Debates Continue, New Analytes Have Emerged, Developments Have Variable Outcomes. Calcif Tissue Int (2019).
3. Macdonald, H.M., Reid, I.R., Gamble, G.D., Fraser, W.D., **Tang, J.C.** & Wood, A.D. 25-Hydroxyvitamin D Threshold for the Effects of Vitamin D Supplements on Bone Density: Secondary Analysis of a Randomized Controlled Trial. J Bone Miner Res 33(8), 1464-1469 (2018).
4. Parekh, D., Dancer, R.C.A., Scott, A., D'Souza, V.K., Howells, P.A., Mahida, R.Y., **Tang, J.C.Y.**, Cooper, M.S., Fraser, W.D., Tan, L., Gao, F., Martineau, A.R., Tucker, O., Perkins, G.D. & Thickett, D.R. Vitamin D to Prevent Lung Injury Following Esophagectomy-A Randomized, Placebo-Controlled Trial. Crit Care Med 46(12), e1128-e1135 (2018).
5. Carswell, A.T., Oliver, S.J., Wentz, L.M., Kashi, D.S., Roberts, R., **Tang, J.C.Y.**, Izard, R.M., Jackson, S., Allan, D., Rhodes, L.E., Fraser, W.D., Greeves, J.P. & Walsh, N.P. Influence of Vitamin D Supplementation by Sunlight or Oral D3 on Exercise Performance. Med Sci Sports Exerc 50(12), 2555-2564 (2018).

6. Saleh, L., **Tang, J.**, Gawinecka, J., Boesch, L., Fraser, W.D., von Eckardstein, A. & Nowak, A. Impact of a single oral dose of 100,000 IU vitamin D3 on profiles of serum 25(OH)D3 and its metabolites 24,25(OH)2D3, 3-epi-25(OH)D3, and 1,25(OH)2D3 in adults with vitamin D insufficiency. Clin Chem Lab Med 55(12), 1912-1921 (2017).
7. Macdonald, H.M., Gryka, A., **Tang, J.C.Y.**, Aucott, L.S., Fraser, W.D. & Wood, A.D. Longevity of daily oral vitamin D3 supplementation: differences in 25OHD and 24,25(OH)2D observed 2 years after cessation of a 1-year randomised controlled trial (VICtORy RECALL). Osteoporos Int 28(12), 3361-3372 (2017).
8. Hanusch, B.C., Tuck, S.P., McNally, R.J.Q., Wu, J.J., Prediger, M., Walker, J., **Tang, J.**, Piec, I., Fraser, W.D., Datta, H.K. & Francis, R.M. Does regional loss of bone density explain low trauma distal forearm fractures in men (the Mr F study)? Osteoporos Int 28(10), 2877-2886 (2017).
9. Earl, K.E., Sakellariou, G.K., Sinclair, M., Fenech, M., Croden, F., Owens, D.J., **Tang, J.**, Miller, A., Lawton, C., Dye, L., Close, G.L., Fraser, W.D., McArdle, A. & Beadsworth, M.B.J. Vitamin D status in chronic fatigue syndrome/myalgic encephalomyelitis: a cohort study from the North-West of England. BMJ Open 7(11), e015296 (2017).
10. Cleal, J.K., Hargreaves, M.R., Poore, K.R., **Tang, J.C.Y.**, Fraser, W.D., Hanson, M.A. & Green, L.R. Reduced fetal vitamin D status by maternal undernutrition during discrete gestational windows in sheep. J Dev Orig Health Dis 8(3), 370-381 (2017).
11. Ball, N., **Tang, J.**, Fraser, W.D. & Dutton, J. Development of a quantitative LC-MS/MS analysis for measurement of circulating 25-hydroxyvitamin D using Volumetric Absorptive Microsampling (VAMS). Mass Matters (82), 18-21 (2017).
12. He CS, Fraser WD, **Tang J**, Brown K, Renwick S, Rudland-Thomas J, Teah J, Tanqueray E, Gleeson M. The effect of 14 weeks of vitamin D3 supplementation on antimicrobial peptides and proteins in athletes. *Journal of sports sciences.* 2016;34(1):67-74.
13. Owens, D.J., Sharples, A.P., Polydorou, I., Alwan, N., Donovan, T., **Tang, J.**, Fraser, W.D., Cooper, R.G., Morton, J.P., Stewart, C. & Close, G.L. A systems-based investigation into vitamin D and skeletal muscle repair, regeneration, and hypertrophy. Am J Physiol Endocrinol Metab 309(12), E1019-1031 (2015).
14. Owens, D.J., Webber, D., Impey, S.G., **Tang, J.**, Donovan, T.F., Fraser, W.D., Morton, J.P. & Close, G.L. Vitamin D supplementation does not improve human skeletal muscle contractile properties in insufficient young males. European journal of applied physiology 114(6), 1309-1320 (2014).
15. MacDonald, H.M., Wood, A.D., **Tang, J.C.** & Fraser, W.D. Comparison of vitamin D(2) and vitamin D(3) supplementation in increasing serum 25-hydroxyvitamin D status: a systematic review and meta-analysis. Am J Clin Nutr 96(5), 1152-1153; author reply 1153-1154 (2012).

Poster abstract publications

1. Saleh, L., **Tang, J.**, Gawinecka, J., Boesch, L., Fraser, W.D., von Eckardstein, A. & Nowak, A. Impact of a single oral dose of 100,000 IU vitamin D3 on profiles of serum 25(OH)D3 and its metabolites 24,25(OH)2D3, 3-epi-25(OH)D3, and 1,25(OH)2D3 in adults with vitamin D insufficiency. Clinica Chimica Acta 2019; 493 (Suppl 1): S1-S776. Presented at EuroMedLab. Barcelona, Spain Poster #W416
2. Schoenmakers, I., Piec, I., Baban, S., Barebring, L., Green, D., Washbourne, C., **Tang, J.C.Y.**, Fraser, W.D. & Augustin, H. Gestational hypercalcemia: Prevalence and biochemical profile. J Bone Miner Res 2019; (Suppl 1): 185-186. Presented at The American Society of Bone and Mineral Research. Orlando, FL, USA Poster #SUN-13
3. **Tang, J.C.Y.**, Jackson, S., Izard, R.M., Oliver, S.J., Piec, I., Washbourne, C.J., Walsh, N.P., Greeves, J. & Fraser, W.D. Vitamin D insufficiency and elevated vitamin D metabolite ratios (VMR) are associated with increased risk of injuries: Results from the British army lower limb injury prevention (ALLIP) study. J Bone Miner Res 2018; 33 (Suppl 1): 168. Presented at The American Society of Bone and Mineral Research. Montreal, QC, Canada Poster #SAT-0757
4. **Tang, J.**, Nicholls, H., Ball, N., Dutton, J., Piec, I., Rudge, J., Washbourne, C. & Fraser, W.D. Assessment of vitamin D status using MitraTM volumetric absorptive microsampling (VAMS) device. J Bone Miner Res 2017; 32 (Suppl 1) Presented at American Society of Bone and Mineral Research. Denver, CO, USA Poster #SA0253
5. Kashi D.S, Wentz L.M, Oliver S.J, Carswell A.T, Aw Yong X.H, Potter C, Ward M.D, Edwards J.P, **Tang J**, Jackson S, Izard R.M, Fraser W.D, Greeves J.P, and Walsh N.P. Vitamin D sufficiency improves hepatitis B vaccine response in male Army recruits. Presented at the International Sport and Exercise Nutrition conference, 2017.
6. Greeves, J.P., O'Leary, T.J., **Tang, J.**, Jackson, S., Fraser, W.D., Walsh, N.P. & Izard, R.M. Associations between vitamin D status and bone density and microarchitecture at the distal tibia in British Army infantry recruits. The American College of Sports Medicine 2017; 49 (5): S289 1432. Presented at The American College of Sports Medicine. Denver, CO, USA
7. Carswell A.T Wentz L.M, Oliver S.J, Kashi D.S, Potter C, Ward M.D, Aw Yong X.H, **Tang J**, Jackson S, Izard R.M, Fraser W.D, Greeves J.P, and Walsh N.P. Vitamin D status is an important predictor of aerobic performance in male and female Army recruits. Paper presented at: International Sport and Exercise Nutrition conference 2017.
8. **Tang, J.**, Nicholls, H., Piec, I., Dutton, J., Washbourne, C., Jackson, S., Greeves, J. & Fraser, W.D. Establishing reference intervals of serum 24,25-dihydroxyvitamin D and 25-hydroxyvitamin D-to-24,25-dihydroxyvitamin D ratio by LC-MS/MS. J Bone Miner Res

2016; 31 (Suppl 1) Presented at American Society of Bone and Mineral Research. Atlanta, GA, USA Poster #SA0188

9. **Tang, J.**, Nicholls, H., Dutton, J., Piec, I., Washbourne, C., Saleh, L., Nowak, A., Close, G.L., Macdonald, H.M. & W.D., F. Changes in serum 25-hydroxyvitamin D, 24,25-dihydroxyvitamin D and 1,25-dihydroxyvitamin D in response to three vitamin D3 supplementation regimens. Endocrine Abstracts 44 P72 2016; 44 Presented at Society for Endocrinology BES 2016. Brighton, UK Poster #P72
10. Carswell, A.T., Wentz, L.M., Oliver, S.J., Kashi, D.S., Potter, C., Ward, M.D., Aw Yong, X.H., **Tang, J.**, Jackson, S., Izard, R.M., Fraser, W.D., Greeves, J.P. & Walsh, N.P. Vitamin D status is an important predictor of aerobic performance in male and female Army recruits. International Sports and Exercise Nutrition Conference 2016; 27 (Suppl 1) Presented at International Sport and Exercise Nutrition conference. Newcastle upon Tyne, UK Poster #S17
11. **Tang, J.**, Nicholls, H., Budnik-Zawilska, M., Dutton, J., Piec, I., Washbourne, C. & Fraser, W.D. Examining phospholipids interference in LC-ESI-MS/MS measurements of 25-hydroxyvitamin D in long term storage samples. J Bone Mineral Research 2015; 30 (Suppl 1): SA0244. Presented at The American Society of Bone and Mineral Research. Seattle, USA Poster #SA0244
12. **Tang, J.**, Nicholls, H., Budnik-Zawilska, M., Brookes, P., Dutton, J., Piec, I., Washbourne, C. & Fraser, W.D. Profiling C3-Epi-25-Hydroxyvitamin D3 concentrations in paediatric populations as determined by LC-MS/MS. J Bone Mineral Research SU0246 2015; 30 (Suppl 1) Presented at The American Society of Bone and Mineral Research. Seattle, USA Poster #SU0246
13. **Tang, J.**, Nicholls, H., Dutton, J., Piec, I., Washbourne, C., Saleh, L., Nowak, A., Close, G., Macdonald, H.M., Jackson, S., Greeves, J. & Fraser, W.D. Simultaneous LC-MS/MS measurement of 24,25-hydroxyvitamin D and 25-hydroxyvitamin D provide a new perspective on the assessment of vitamin D status. J Bone Mineral Research 2015; 30 (Suppl 1) Presented at The American Society of Bone and Mineral Research. Seattle, USA Poster #LB-MO0026
14. Washbourne, C., Green, D., Fisher, E., Piec, I., **Tang, J.** & Fraser, W.D. Quantification of total 25-hydroxyvitamin D: A comparison between the Elecsys (R) Vitamin D Total assay and LC-MS/MS. J Bone Miner Res 2014; 29 (Suppl 1): S313. Presented at The American Society of Bone and Mineral Research. Houston, Texas, USA Poster #SU0351
15. **Tang, J.**, Washbourne, C., Piec, I. & Fraser, W.D. Assessment of C3-Epi-25-Hydroxyvitamin D concentration in adult serum: LC-MS/MS determination using [$^{2}H_{3}$] 3-epi-25OHD3 internal standard and NIST traceable commercial 3-epi-25OHD calibrators.2014; 52 (11):

eA205–eA379. Presented at The Clinical Chemistry and Laboratory Medicine (CCLM) Focus. Liverpool, UK Poster #W11

Grant awards

1. **Association of vitamin D and iron status with injury risk, health and physical performance in British Army recruits,** Fraser, W. & Tang, J. 1/09/13 → 31/10/16, £350,000, Army Recruiting and Training Division, UK Ministry of Defence
2. **Development of Mitra-microsampling (VAMS) for biochemical analysis** Fraser, W. & Tang, J. 1/12/16 → 30/11/17 £20,104, Neoteryx LLC
3. **Vitamin D and CardiOvascular Risk (VICtORy)** Fraser, W. & Tang, J. 1/12/16 → 30/04/17 £6,988, University of Aberdeen
4. **Examining the effects of both carbohydrate and calorie restriction on muscle adaptations to a twice per day exercise protocol** Fraser, W. & Tang, J. 1/12/16 → 31/01/17 £4,950, Liverpool John Moores University
5. **Impact of a single oral dose of 100,000 IU vitamin D3 on profiles of serum 25(OH)D3 and its metabolites 24,25(OH)2D3, 3-epi-25(OH)D3, and 1,25(OH)2D3 in adults with vitamin D insufficiency** Fraser, W. & Tang, J. 1/09/16 → 31/08/17 £420, University Hospital of Zurich
6. **Development of quantitative LC-MS/MS analysis for whole blood 25-hydroxyvitamin D using Volumetric Absorptive Microsampling (VAMS).** Tang, J. 1/07/16 → 30/09/16 £1,500, British Mass Spectrometry Society

Oral presentations

1. Vitamin D insufficiency and elevated vitamin D metabolite ratios (VMR) are associated with increased risk of injuries: Results from the British army lower limb injury prevention (ALLIP) study. Endocrine Abstracts 2018; 59 Presented at Society for Endocrinology BES. Glasgow, UK Oral presentation #OC1.2

2. Profiles of 25 hydroxyvitamin D and its metabolite 24,25 dihydroxyvitamin D and 1,25 dihydroxyvitamin D in vitamin D3 supplementations studies. Bone Abstracts 2016; 5 Presented at The 43rd Annual European Calcified Tissue Society Congress. Rome, Italy Oral presentation #OP19

3. From benchside to bedside: How development of samples preparation techniques advanced vitamin D research. 2016; Presented at 1st British Mass Spectrometry Society Clinical and forensic special interest group meeting. Runcorn, UK

4. Evaluation of the MitraTM micro-sampling device against dried blood spot cards for measurement of 25-hydroxyvitamin D3 by LC-MS/MS. 2016; Presented at Microsampling Workshop. London, UK

5. Fast chromatographic separation of 3-epi-25-hydroxyvitamin D for more accurate measurement of 25-hydroxyvitamin D by LC-MS/MS. 2013; Presented at the British Mass Spectrometry Society Annual Meeting. Eastbourne, UK

Acknowledgements

This thesis is dedicated to my wife Emma, daughters Kimberley and Megan for their love, endless support and encouragement.

My sincere gratitude to my supervisors who guided me through the process to completion.

Professor William D Fraser

Professor Garry John

Special thank you to Mr John Dutton, who has inspired and mentored me, and for years of conversations helping me overcome challenges in developing liquid chromatography tandem mass spectrometry methods.

and for the tremendous support I have received from

The team at the Bioanalytical Facility, University of East Anglia

Abbreviations

1,25(OH)$_2$ D	1α,25-dihydroxyvitamin D3
1,25(OH)$_2$ D3	1α,25-dihydroxyvitamin D3
1,25(OH)$_2$ D2	1α,25-dihydroxyvitamin D2
1,25(OH)$_2$D3-^{2}H$_3$	1α,25-dihydroxyvitamin D3-[6,19,19,2H$_3$]
25(OH)D	25-hydroxyvitamin D
25(OH)D3	25-hydroxyvitamin D3
25(OH)D2	25-hydroxyvitamin D2
25(OH)D3-^{2}H$_6$	25-hydroxyvitamin D3-[26,26,26,27,27,27,-^{2}H$_6$]
25(OH)D2-^{13}C$_3$	25-hydroxyvitamin D2-[^{13}C$_3$]
24R,25(OH)$_2$D3	24R,25-dihydroxyvitamin D3
24S,25(OH)$_2$D2	24S,25-dihydroxyvitamin D3
24R,25(OH)$_2$D3-^{2}H$_6$	24R,25-dihydroxyvitamin D3-[^{2}H$_6$]
23,25(OH)$_2$D3	23,25-dihydroxyvitamin D3
25,26(OH)$_2$D3	25,26-dihydroxyvitamin D3
3-epi-25(OH)D3-^{2}H$_3$	3-Epi-25-Hydroxyvitamin –D3-[6,19,19-^{2}H$_3$]
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionisation
C3-epi-25(OH)D3	C3-Epimer 25-hydryoxyvitamin D3
C3-epi-25(OH)D2	C3-Epimer 25-hydryoxyvitamin D2
CI	Confidence interval
CID	Collision induced dissociation
CLIA	Chemiluminescent immunoassay
CV	Coefficient of variation
CV$_{between}$	Between-run coefficient of variation
CV$_{total}$	Total coefficient of variation
CV$_{within}$	Within-run coefficient of variation

CYP24A1	Cytochrome P450, family 24, subfamily A, polypeptide 1
d6	hexadeuterated
DEQAS	Vitamin D External Quality Assessment Scheme
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylenediamine tetraacetic acid
ESI	Electrospray ionisation
HPLC	High performance liquid chromatography
IQC	Internal quality control
IS	Internal standard
ISO	International Organization for Standardization
IU	International unit
ID	Isotope dilution
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLE	Liquid liquid extraction
LoQ	Limit of quantification
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
m/z	mass-to-charge ratio
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NIST	National Institute of Standards and Technology
RF	Radio frequency
RIA	Radioimmunoassay
Rpm	Revolutions per minute

RT	Room temperature
Standard deviation	SD
SI-units	"Système International d'Unités" (International System of Units)
S/N	Signal-to-noise ratio
SRM	Standard reference material
SLE	Supported liquid extraction
UPLC	Ultra high performance liquid chromatography
UV	Ultraviolet
°C	Degrees centigrade
+ve	Positive
-ve	Negative

Chapter 1 Introduction

1.1 The Physiology of vitamin D

The vitamin D family is categorised as either cholecalciferols (vitamin D3) or ergocaliferols (vitamin D2). Vitamin D is mainly synthesised in the skin by exposure to sunlight, where UVB exposure converts 7-dehydrocholesterol to vitamin D3 (Fig. 1.0). Once it enters the circulation, vitamin D_3 is metabolised to 25-hydroxyvitamin D_3 (25(OH)D3) in the liver by 25-hydroxylase and then further metabolised in the kidneys to the most biologically active form 1α,25-dihydroxyvitamin D3 ($1,25(OH)_2D3$) by the enzyme 1-hydroxylase. $1,25(OH)_2D3$ increases intestinal absorption of calcium and phosphate, regulates synthesis and secretion of parathyroid hormone (PTH), and promotes bone mineralisation. An alternative pathway produces 24R,25-dihydroxyvitamin D3 ($24,25(OH)_2D3$) by 24-hydroxylase. 80-90% of circulating total 25(OH)D is derived from skin exposure to UVB and 10-20% ingested in the diet. 25(OH)D3 in the diet is mainly from animal sources (oily fish, fish oils, dairy products, meat) and 25(OH)D2 is mainly obtained from irradiated plants (green leafy vegetables, mushrooms, fungal contamination of foodstuffs). Total 25(OH)D is the combination of all circulating forms of 25(OH)D3 and 25(OH)D2.

1.2 Clinical significance of vitamin D

The clinical effects of vitamin D have been a hot topic among the scientific community, with regular reports appearing in the news. Low vitamin D status is prevalent in the UK, particularly in young and elderly populations and in ethnic minorities[1]. Severe vitamin D deficiency leads to rickets in children, and osteomalacia in adults. Vitamin D deficiency can cause a reduction in serum ionised calcium resulting in secondary hyperparathyroidism which can promote an increase in bone turnover to release calcium and phosphate from bone and results in decreased mineralisation of newly formed osteoid. The long-term effect of this mineralisation defect exacerbates the reduction of bone mass, contributing to increased fracture incidence.

Beyond bone diseases, epidemiological studies have shown that deficient levels of total 25(OH)D are associated with higher risk of colon[2], prostate[3,4] and breast cancer[5]. Cardiovascular disease[6], autoimmune diseases[7], diabetic nephropathy[8] and schizophrenia are also associated with vitamin D deficiency. The wide association is due to the high abundance of vitamin D receptors[9] in tissues, allowing $1,25(OH)_2D$ to directly or indirectly regulate cell growth, induce apoptosis and control metabolism.

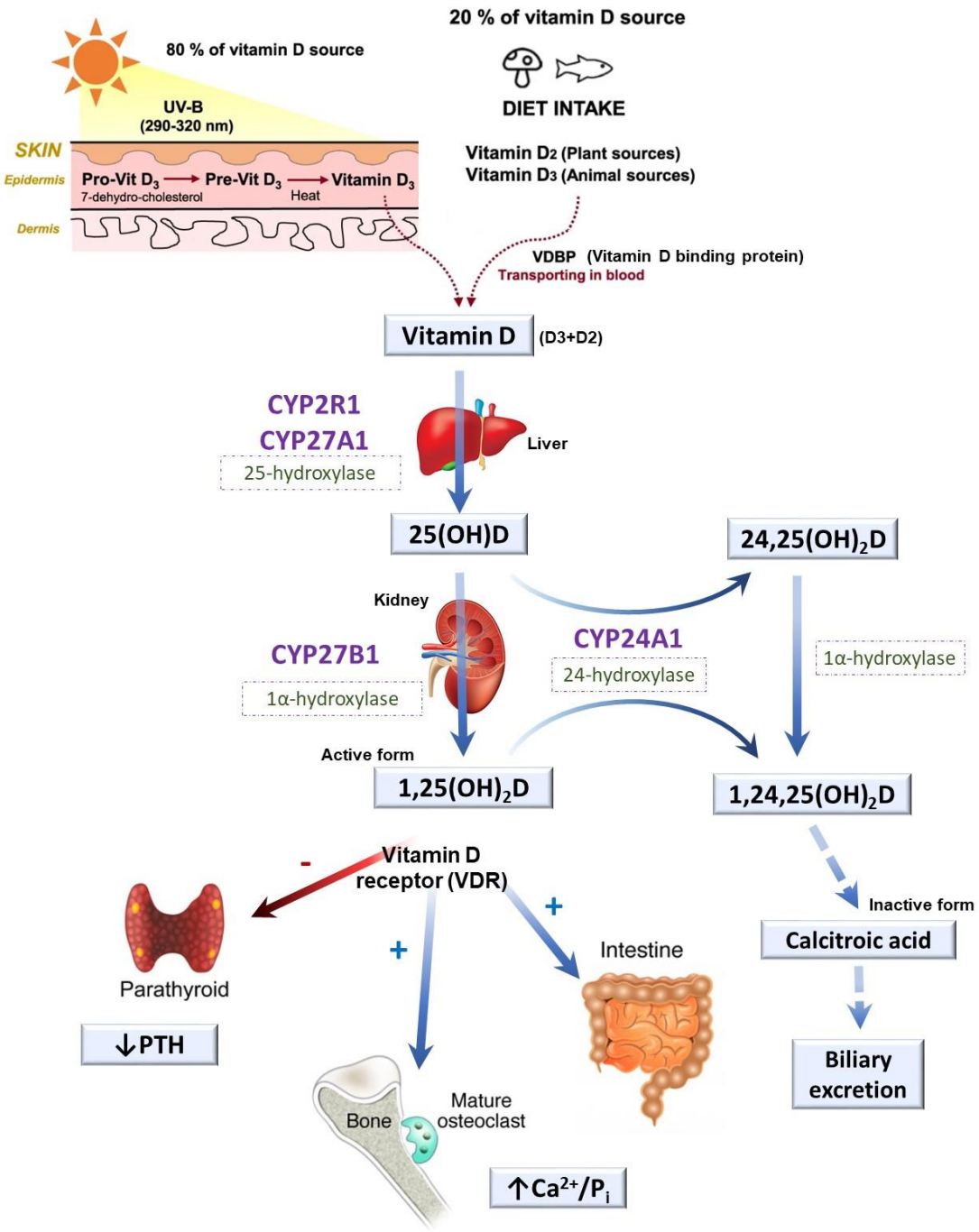


Figure 1.0 Vitamin D metabolic pathway.

$1,25(OH)_2D$ is thought to play an important role in pathophysiological conditions, even more so than that of 25(OH)D. Whereas it is well established that measurement of circulating total 25(OH)D is the best indicator of vitamin D status, measurement of the biologically active hormone $1,25(OH)_2D$ can be a valuable diagnostic and therapeutic tool.

1.3 Vitamin D metabolites of interest

1.3.1 25-hydroxyvitamin D

Measurement of 25(OH)D is accepted as a reliable clinical indicator of vitamin D status in humans. The steady circulating serum level and long half-life of approximately 2 to 3 weeks make 25(OH)D an ideal diagnostic indicator of vitamin D deficiency and monitoring target in supplementation therapy. The Royal Osteoporosis Society (ROS)[10] in agreement with the Institute of Medicine (IOM)[11] defined insufficient vitamin D status as a serum 25(OH)D <50 nmol/L but advocated that 25(OH)D concentration should exceed 75 nmol/L, in order to maximise the effect on calcium, bone and muscle metabolism. Serum 25(OH)D <30 nmol/L is highlighted by IOM as deficient, whereas ROS adopted <25 nmol/L as the severely deficient threshold. In the UK, the Scientific Advisory Committee on Nutrition (SACN) that advises the government on diet and health published in its report *Vitamin D and Health 2016*[12] the population recommended reference nutrient intake (RNI) of 10 micrograms (400 IU) of vitamin D per day, for everyone in the general population aged 4 years and older throughout the year regardless of sunlight exposure. The RNI and safe intakes were developed to ensure that the majority of the UK population has sufficient 25(OH)D to protect musculoskeletal health.

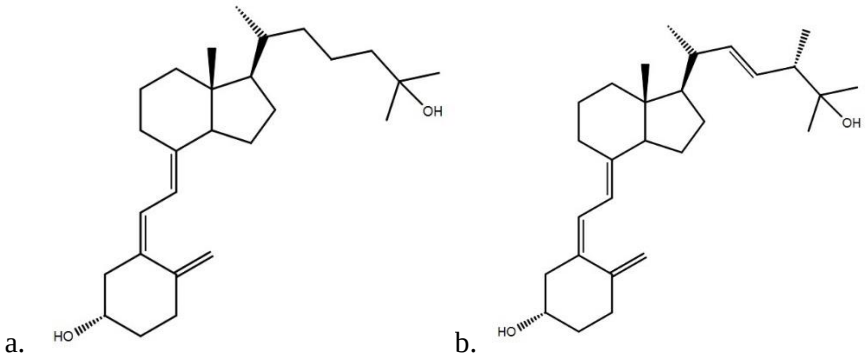


Figure 2.1 Structure of **(a)** 25-hydryoxyvitamin D3 and **(b)** 25-hydryoxyvitamin D2

Two forms of 25(OH)D exist (Figure 2.1) being 25(OH)D3, which is mainly derived from skin exposure to UVB and to a minor extent from diet and supplements, and 25(OH)D2, which is mainly derived from supplements and to a small extent from dietary origin. When measuring 25(OH)D, it is important the assay methodology has co-specificity for 25(OH)D3 and 25(OH)D2, as both metabolites are metabolically active, more so for 25(OH)D3. The serum concentration of 25(OH)D3 is higher than 25(OH)D2 due to the cutaneous synthesis by UVB radiation and broader sources of vitamin D3 available in foods and supplements derived from animal origin. 25(OH)D2

is less prevalent though can be present in individuals taking vitamin D2 supplements derived from plant products.

Biochemical assessment of vitamin D status is frequently performed on patients with bone diseases and musculoskeletal symptoms such as osteomalacia and widespread chronic pain. For asymptomatic individuals, daily 400 IU vitamin D3 supplement is recommended by the Public Health England (PHE) for specific population groups that are at risk of vitamin D deficiency; in particular to older people aged >65 years, pregnant and breastfeeding women, infants and young children aged <5 years, and people who lack sunlight exposure (e.g. housebound)[13]. For patients presenting with severe vitamin D deficiency, a loading regimen up to a total of approximately 300,000 IU can be prescribed either as weekly or daily split doses, followed by a maintenance regimen of 800-2000 IU per day (up to a maximum of 4000 IU daily) starting one month after loading regimen[10]. Although such dosing regimen is unlikely to result in toxicity, clinicians are advised that certain groups of patients with increased vitamin D sensitivity may be at risk of developing toxicity or adverse side effects; in conditions due to genetic abnormalities in vitamin D metabolism, co-morbidities such as CKD, granuloma-forming diseases or hyperparathyroidism. Assessment of vitamin D status by measurements of serum 25(OH)D, adjusted calcium and PTH (for hyperparathyroidism patients) are recommended to monitor treatment and compliance with medication.

Vitamin D toxicity is rare; severe cases can manifest through chronic hypercalcaemia. Less severe cases can be identified by hypercalciuria, if left untreated could increase the risk of forming renal stones. There are reports of increased incidents of falls and fractures in the older population associated with the use of high doses of vitamin D administered annually or monthly[14,15]. Current evidence suggests the correlation of risks vs benefit effects of vitamin D exhibit a U-shaped curve, rather than a J-shaped curve. Although higher doses of vitamin D supplement were more effective in reaching 25(OH)D concentration of 50 nmol/L, high supplementation levels can be detrimental to the patients. Vitamin D replacement regime must be carefully optimised under medical supervision, based on the characteristics of the patient, in combination with measurements of 25(OH)D and other downstream metabolites of vitamin D.

1.3.2 C3-Epimer 25-hydroxyvitamin D

25(OH)D3 and 25(OH)D2 can be metabolised through an alternate C3 epimerisation pathway, into C3-epi-25(OH)D3 and C3-epi-25(OH)D2 (Fig. 1.2), and further into their respective C3-epi-1,25$(OH)_2$D forms[16]. Reports had shown that C3-epi-1,25$(OH)_2$D3 is nearly as potent as 1,25$(OH)_2$D3 in suppressing PTH secretion, but has reduced calcaemic properties[17]. The biological relevance of C3-epi-25(OH)D remains to be elucidated. Studies have found detection

of the epimer in 23% of infant sera less than 1 year old accounting for up to 61% of total 25(OH)D concentration when determined using a LC-MS/MS method[18].

The 3-epi-25(OH)D3 metabolite is identical in chemical structure to 25(OH)D3 except for molecular asymmetry at the OH position on C3. As both forms have the same mass precursor to product ion transitions, insufficient separation of the C3-epimer could lead to overestimation of 25(OH)D3[19]. Several studies have been published using cyano-propyl (SB-CN)[20], chiral[21] and pentafluorophenyl (PFP)[22] columns to resolve C3-epi-25(OH)D chromatographically. However, long run times from 6.4 to 40 minutes making such assays difficult to implement in routine clinical laboratories.

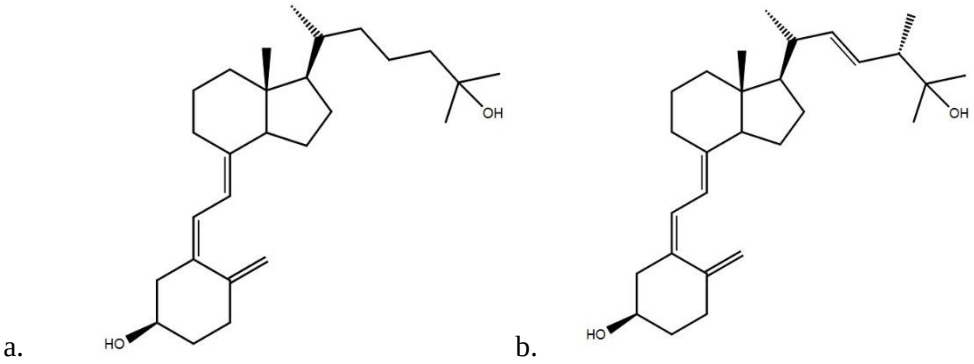


Figure 3.2 Structure of **(a)** C3-Epimer 25-hydryoxyvitamin D3 and **(b)** C3-Epimer 25-hydryoxyvitamin D2

Epidemiologic studies on general population had shown serum concentration of C3-epi-25(OH)D is closely related with 25(OH)D that is independent of vitamin D binding protein and genetic influences[23,24]. There is considerable variability in serum C3-epi-25(OH)D concentration between individuals and substantial difference in the production of the metabolite in response to vitamin D supplementation. Case reports had shown that patients who took high doses of over-the-counter cholecalciferol and ergocalciferol were found with substantial serum levels of C3-epi-25(OH)D3 and C3-epi25(OH)D2[25-27]. Such cases highlight the need to evaluate the use of non-prescribed forms of vitamin D supplements.

1.3.3 1α,25-dihydroxyvitamin D

$1,25(OH)_2D$ (calcitriol) (Fig. 1.3) is the physiologically active form of vitamin D. It is a product of the enzymatic hydroxylation of 25(OH)D by 1α-hydroxylase that takes place primarily in the kidneys. The physiological regulation of $1,25(OH)_2D$ is more tightly regulated than 25(OH)D, when serum 25(OH)D stores decline, $1,25(OH)_2D$ is maintained by increasing PTH stimulation

of 1α-hydroxylation. Measurement of serum 1,25$(OH)_2$D should be considered upon suspicion of deficiency or excess of 1,25$(OH)_2$D leading to hypo- or hypercalcaemia, hypoparathyroidism and hypomagnesemia. Decreased concentrations are found in patients with renal insufficiency, vitamin D dependent rickets (1α-hydroxylase deficiency), X-linked hypophosphataemia and hypoparathyroidism. Increased 1,25$(OH)_2$D concentrations are found in vitamin D-deficient rickets (end-organ resistance), granulomatous disorders and hereditary hypophosphataemic rickets. It is important to stress that serum concentration of 1,25$(OH)_2$D does not reflect the nutritional vitamin D status of an individual.

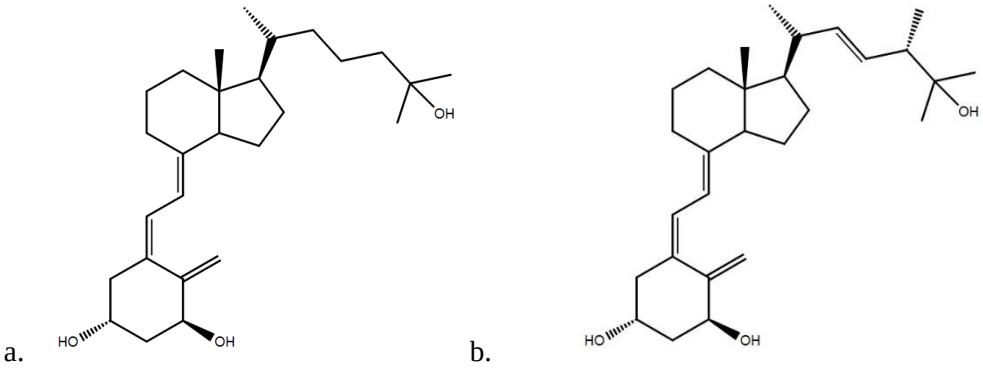


Figure 1.3 Structure of **(a)** 1α,25-dihydroxyvitamin D3 and **(b)** 1α,25-dihydroxyvitamin D2

1,25$(OH)_2$D measurements can be used in clinical settings or disease-focused patient studies. The use in healthy population-based or vitamin D supplementation studies is rare; serum concentration does not correlate with 25(OH)D, except in patients with chronic kidney disease (CKD)[28], where a greater association is observed between 1,25$(OH)_2$D and 25(OH)D dependent upon the severity of the renal impairment. Despite being the most biologically active form of vitamin D metabolite, 1,25$(OH)_2$D is not used for the assessment of vitamin D status. Serum concentration is tightly regulated by the hydroxylation enzymes expressed by the actions of CYP27B1 and CYP24A1, and influenced by PTH and FGF23. Mutations of the CYP27B1 and CYP24A1 genes can result in excessive production of serum 1,25$(OH)_2$D and develop into a persistent state of hypercalcaemia – a condition known as Idiopathic Infantile Hypercalcaemia (IIH). Clinical manifestations of IIH depend largely on the age of diagnosis. Infants present with weight loss, failure to thrive, vomiting, dehydration, lethargy and hypotonia. Adults with total or partial CYP24A1 mutations most frequently present with nephrolithiasis and/or nephrocalcinosis. Distinguishable biochemical profile of IIH includes hypercalcaemia, low PTH and elevated 1,25$(OH)_2$D. Serum 25(OH)D concentration can remain normal as the condition is affecting inadequate disposal of 1,25$(OH)_2$D and not excessive substrate. Low serum concentration of

24,25$(OH)_2$D and elevated 25(OH)D:24,25$(OH)_2$D vitamin D metabolite ratio (VMR) have proved to be useful in identifying patients with CYP24A1 mutations. Further details to be presented in 1.3.4.

Before the introduction of fully automated platforms, measurement of serum 1,25$(OH)_2$D in hospital laboratories have mostly relied on the use of manual immunoassays with a radioisotopic (I-125) or enzyme label. Two commercially available methods have recently been launched by Diasorin and IDS, both developed to be run on their own proprietary automated systems. The DiaSorin LIAISON® XL chemiluminescent immunoassay utilises the ligand-binding domain (LBD) of the vitamin D receptor for the capture of 1,25$(OH)_2$D molecule, followed by a murine monoclonal antibody detection system that recognises the conformation change produced by the LBD-1,25$(OH)_2$D complex[29]. The method exploits the reaction conditions where binding differential of VDR highly favours 1,25$(OH)_2$D over other forms of hydroxylated vitamin D, thus negates the need for immunoextraction. The Immunodiagnostic Systems (IDS) iSYS 1,25 VitDXp method involves an onboard immunopurification that has been modified from the IDS mini-immunocapsule extraction approach. The two-stage assay utilises an anti-1,25$(OH)_2$D antibody-coated magnetic particles to extract and enrich 1,25$(OH)_2$D in the sample, followed by competitive binding against a 1,25$(OH)_2$D-labelled conjugate with a sheep anti-1,25$(OH)_2$D antibody for detection and amplification[30].

Due to the low concentration of 1,25$(OH)_2$D in circulation (reference intervals 48-150 pmol/L) and its poor ionisation properties, LC-MS/MS methodology for 1,25$(OH)_2$D require high-end mass spectrometry instruments. To achieve adequate assay sensitivity, extensive sample preparation to enrich sample load by either a manual immunoaffinity step[31,32] or online two-dimensional chromatography[33,34]. Alternatively, assay sensitivity can be boosted by increasing the ionisation efficiency of 1,25$(OH)_2$D using a derivatisation agent. Several described in the literature are based on a substituted form of 1,2,4-triazoline-3,5-dione (TAD) at the 4-position (4-X-TAD). Most commonly used derivatisation agents are PTAD (4-phenyl-1,2,4-triazoline-3,5-dione), DMEQ-TAD (4-[2-(3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxo-2-quinoxalinyl)ethyl]-TAD)[35] and Amplifex™ Diene[36]. These Cookson-type dienophilic agents react to the *s-cis-diene* moiety of vitamin D molecule via the Diels-Alder reaction to form a larger complex with increasing ionisation potential depending on the number of proton-affinitive functional groups available in the resultant reaction. The improvement in sensitivity and specificity enable differential analysis of vitamin D metabolites, expanding the applicability in research and diagnostic investigations.

1.3.4 24,25-dihydroxyvitamin D

24,25$(OH)_2$D is formed via C24-hydroxylation of 25(OH)D by cytochrome P450 24-hydroxylase enzyme CYP24A1 (cytochrome P450, family 24, subfamily A, polypeptide 1). CYP24A1 plays an important role in vitamin D catabolism, however, the biological function of 24,25$(OH)_2$D remains unclear besides being the apparent inactive catabolite of 25(OH)D. A recent publication suggested the measurement of serum 24,25$(OH)_2$D in conjunction with 25(OH)D shows promise as a novel marker of 25(OH)D catabolism and predictor of serum 25(OH)D response to vitamin D supplementation[37]. 24,25$(OH)_2$D is quantitatively most abundant circulating vitamin D metabolite besides 25(OH)D, with human serum concentrations between 0.7 and 24 nmol/L[38]. Studies have shown serum 24,25$(OH)_2$D correlated positively with serum 25(OH)D levels[37]. It is important to note the difference between the R- and the S- form, 24S,25$(OH)_2$D is the epimer form of 24R,25$(OH)_2$D (Fig. 1.4).

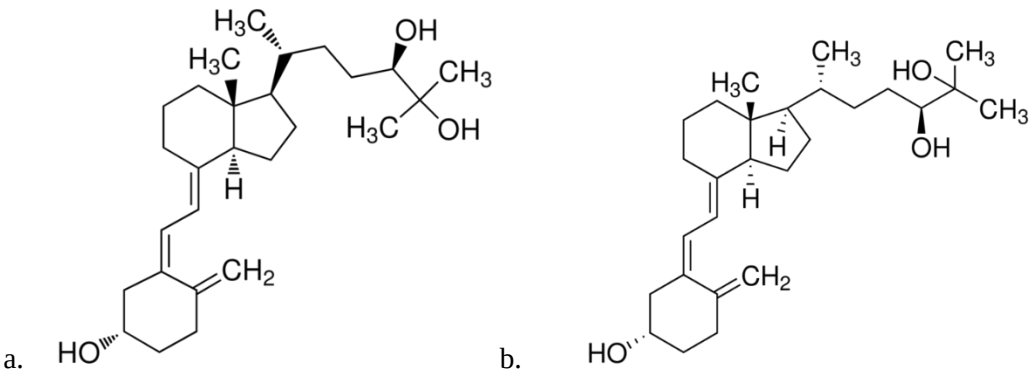


Figure 1.4 Structure of **(a)** 24R,25-dihydroxyvitamin D3 and **(b)** 24S,25-dihydroxyvitamin D3.

Measurement of serum 24,25$(OH)_2$D was first reported in the late 1970s using competitive protein binding assays[39,40] in animal and *in vitro* models to study the healing properties of 24,25$(OH)_2$D on bone[41]. When HPLC methods with UV detection became the method of choice for 25(OH)D, the improvement in assay sensitivity had remained insufficient to detect serum 24,25$(OH)_2$D, which circulates at concentrations between 1-13 nmol/L[42-44], 10-fold lower than 25(OH)D. Shimizu et al. first reported a chemical derivatisation method for 24,25$(OH)_2$D using a Cookson-type dienophilic agent which reacts to the *s-cis-diene* moiety of vitamin D via the Diels-Alder reaction; forming a conjugated diene derivative for detection by HPLC with a fluorometric detector[45]. However, despite the improvement in assay sensitivity, studies in the 1980-90s have found limited biological activity of 24,25$(OH)_2$D in humans[46-48], and it was considered to be an inactive catabolic product of the vitamin D pathway[49]. The revival of interest in 24,25$(OH)_2$D measurement was brought about after Genome-wide studies have pinpointed *CYP24A1* as one of the major genetic determinants of variability in 25(OH)D[50]. Publications have shown *CYP24A1*

defects in children with IIH[51]; in adults with nephrolithiasis and nephrocalcinosis[52]; and in patients with chronic kidney disease[53], are associated with low serum 24,25$(OH)_2$D concentration, and the genetic defect can be highlighted by an elevated 25(OH)D:24,25$(OH)_2$D VMR. 25(OH)D:24,25$(OH)_2$D VMR can indicate the vitamin D catabolic status of an individual and highlight 24-hydroxylase deficiency due to genetic abnormalities of *CYP24A1*. In a healthy population, 25(OH)D:24,25$(OH)_2$D VMR is between 7-23, and increases when vitamin D status becomes insufficient[54]. A ratio of >80 is associated with patients with heterozygous or biallelic mutations of *CYP24A1*[43,55]. Moderate elevation of 25(OH)D:24,25$(OH)_2$D VMR due to partial inactivity of CYP24A1 is associated with renal impairment[53,56] and bone disorders[57]. 25(OH)D:24,25$(OH)_2$D VMR has an advantage for being less susceptible to seasonal fluctuation[58]; allowing interpretation using fixed reference intervals irrespective of the time of the year.

1.4 Current Issues relating to the measurement of vitamin D metabolites

25(OH)D

The dramatic explosion of interests in vitamin D sparked a massive increase in the number of laboratory requests for the measurement in serum. This, in turn, has highlighted problems of the methodologies available for measuring vitamin D metabolites. The ability to measure the two major forms of vitamin D (Cholecalciferol (D3) and ergocalciferol (D2) with precision and accuracy are essential to correctly identify the vitamin D status of an individual and to monitor the effectiveness of vitamin D treatment. Proficiency testing bodies such as the International Vitamin D External Quality Control Scheme (DEQAS)[59] have been monitoring the accuracy of 25(OH)D and 1,25$(OH)_2$D assays. Their recent report suggests measurement of 25(OH)D by a liquid chromatography tandem mass spectrometry (LC-MS/MS) method using NIST-aligned calibration standards exhibits the least inter-laboratory imprecision in comparison with immunoassays[60]. LC-MS/MS methods are capable of distinguishing the D3 and D2 form of vitamin D by their mass-to-charge (m/z) ratio, allowing the source of vitamin D to be identified. Immunoassays have variable cross-reactivity with 25(OH)D2 and D3, some methods may significantly underestimate an individual's true vitamin D status. In the literature, comparison studies between commercial immunoassays with LC-MS/MS methods have shown negative biases in the results generated by immunoassay in samples containing 25(OH)D2, reflecting the varying degree of antibody cross-reactivity with the D2 metabolite[61-63]. Discrepancies in the interpretation of vitamin D status from different immunoassays were also reported[64]; with some methods identified significantly more hypovitaminosis cases than others. Disparity can also occur in specific population groups; Dowling et al. had reported over-estimation of 25(OH)D measurements in the Roche assay due to the presence of elevated 24,25$(OH)_2$D in patient samples[65]. Hara et al. had reported falsely elevated 25(OH)D in serum samples from in infants and postpartum women produced by the Diasorin radioimmunoassay (RIA)[66]. Several studies have reported low serum concentration of 25(OH)D in pregnant women are associated with increased risk of pre-eclampsia, gestational diabetes and other complications of pregnancy[67]. The negative association could be exaggerated by the method of 25(OH)D quantitation. Vitamin D binding protein (VDBP) is known to be elevated during pregnancy; incomplete dissociation of 25(OH)D from VDBP by immunoassays can result in under-recovery of 25(OH)D and thus underestimate the actual value. Heijboer et al. had reported an inverse relationship between VDBP concentrations and deviations of 25(OH)D values from the LC-MS/MS assay[68]. Elevated VDBP can also be observed in patients presenting with inflammatory disease such as rheumatoid arthritis and acromegaly[69]. The use of oestrogen-containing hormonal contraceptive can increase the serum concentration of VDBP similar to pregnancy. Chemical solvents used in the sample

pretreatment step in LC-MS/MS methods ensure complete dissociation of 25(OH)D from its binding proteins, hence LC-MS/MS technique offers higher specificity than immunoassays, thus the capability to recognise different forms of vitamin D simultaneously. It is therefore considered to be the reference method for measuring 25(OH)D.

C3-epi-25(OH)D

In the DEQA 2012 review[70] published the results of an experimental study conducted to assess the ability of the methods to distinguish C3-epi-25(OH)D3 from 25(OH)D3. Two serum samples were sent out to the scheme participants, both originated from the same base pool, with one sample spiked with exogenous C3-epi-25(OH)D3 (51 nmol/L). The study report (Fig 1.5) showed that all commercial immunoassays, except for the Roche competitive protein binding (CPB) based assay, did not cross-react with the epimer and produced similar results on the base and spiked samples. For chromatography-based methods (HPLC and LC-MS/MS), due to the identical molecular weight and structure, routine methods that employ the use of a C18 analytical column are unable to chromatographically resolve the epimer forms from 25(OH)D. Instead, the epimer peaks co-elute with 25(OH)D3 and 25(OH)D2. The report showed major positive bias in the sample spiked with C3-epi-25(OH)D3, overestimating the concentration of 25(OH)D3.

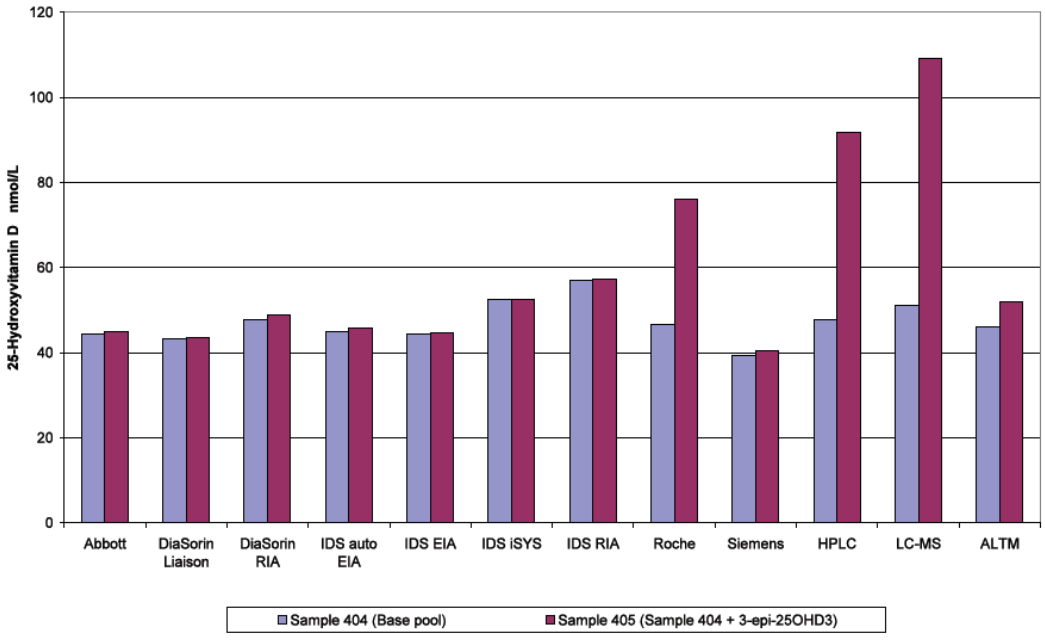


Figure 1.5 DEQAS returns of the base pool (sample 404) and the base pool spiked with C3-epi-25(OH)D3 (sample 405) illustrating positive bias observed in HPLC, LCMS and Roche CPB method groups. (Graph was adapted from[70])

Despite the alarming concerns over the isobaric interference that may cause overestimation of total 25(OH)D by HPLC, LC-MS/MS and Roche CPB methods, it is not clear the impact this interference may have on the assessment vitamin D status in patients. There was little evidence on the clinical significance and the prevalence of endogenous C3-epi-25(OH)D3, which had been shown to behave differently to the exogenous form of the same compound. Van den Ouweland et al. had reported that the Roche CPB method displayed minimal recognition in samples containing natural endogenous C3-epi-25(OH)D3; whereas the cross-reactivity to exogenous C3-epi-25(OH)D3 added to human serum was approximately 51%[71]. There are several evaluation studies in the literature that reported variable differences in the significant of C3-epi-25(OH)D using a plethora of 'in-house' chromatography-based UV or MS/MS methods, but the lack of standardised calibration material and appropriate isotopic-labelled internal standard have undermined the accuracy of the results produced.

$1,25(OH)_2D$

Long term assay performance between all the immunoassay and LC-MS/MS methods are closely monitored by DEQAS. The lack of cross-assay reference standards has yet to be addressed. Since the introduction of fully automated platforms by DiaSorin and IDS, manual radioisotopic immunoassays have been on a gradual decline. The shift has reduced inter-laboratory variability, but raised concern over the dominance of the two methods, particularly with the DiaSorin method placing a heavy influence on the DEQAS scheme and potentially masking method-specific bias.

One study compared the IDS iSYS, Diasorin LIAISON assays with a LC-MS/MS method found a marked difference in the assay performance. The DiaSorin assay performed better in terms of accuracy, sensitivity and imprecision compared to the IDS iSYS assay. Total imprecision was 5.2% or less for the DiaSorin assay but reached 20.1% for the IDS iSYS assay. $1,25(OH)_2D$ concentrations measured with the DiaSorin assay showed a strong correlation with $1,25(OH)_2D$ levels measured by LC-MS/MS and a good agreement with method-specific means on DEQAS samples. By contrast, the IDS iSYS method overestimated $1,25(OH)_2D$ concentrations in human serum, particularly at higher concentrations[72].

Reference ranges have been established using immunoassays in healthy adults and patient groups[73,74], but wide intervals reported cast concerns over assay specificity, given circulating concentration is tightly regulated by the body. Higgins et al.[75] reported high disparity between the DiaSorin and LC-MS/MS methods in pooled neonates and infants samples with elevated $1,25(OH)_2D$; a positive bias of up to 26.5% was observed in immunoassay results, 3-epi-$1,25(OH)_2D$ was ruled out as a potential source of bias.

LC-MS/MS methodologies for 1,25$(OH)_2$D has remained confined to few laboratories with high-end instruments. Using a SCIEX 6500+ instrument, Ivison et al. have developed an LC-MS/MS method that employs the use of immunoaffinity extraction followed by derivatisation with Amplifex™ reagent[76]. The method performed with imprecision CV of <15.6%. Analysis of DEQAS samples showed a negative bias compared with the all lab trimmed mean of –13.8% and the specific method group (average –7.75%). A negative bias was observed across the concentration range found in 78 patient samples in comparison to a commercial RIA (mean –47.8%).

24,25$(OH)_2$D

Efforts to standardise and improve inter-laboratory comparability of 24,25$(OH)_2$D assays are in working progress. NIST SRM972a and 2973 in frozen human serum are available with certified values for 24,25$(OH)_2D_3$ between 3.39-7.51 nmol/L[77]. DEQAS has introduced a pilot scheme since April 2015, but due to the small number of participants, albeit using LC-MS/MS methods, high inter-laboratory variability was observed, with CVs ranging between 19-29%[78]. Wise et al. reported lower variability in five laboratories using isotopically labelled 24,25$(OH)_2D_3$ as internal standard[79]. The availability of a reference-traceable, matrix-matched, multi-point calibration standards would improve method accuracy and commutability of results between laboratories.

1.5 Conclusion

Over the past decade there have been major advances in the development of analytical techniques for measuring vitamin D metabolites. The breakthrough in LC-MS/MS technology; with its superior sensitivity and specificity, has created new grounds for improving assay methods. New metabolites have been identified, and some known metabolites that were previously thought to have little biological actions have remerged with new evidence. Much more work remains to be done to better our understanding and appreciation of vitamin D metabolism, its role in calcium/bone homeostasis, and clinical significance in health and pathological conditions.

Chapter 2 Aims and objectives

The aim of this PhD thesis was to develop and validate quantitative assays for serum vitamin D metabolites by LC/MS-MS, and utilise the newly developed methods to perform analysis on study samples to investigate the relationships between the metabolites of interests. This research has focused on the major circulating vitamin D metabolites; 25(OH)D, C3-epi-25(OH)D, $24,25(OH)_2D$, $1,25(OH)_2D$, and their respective D3/D2 forms. The performance characteristic of the assays must satisfy industry standards for method validation. The assays will be accurate, have excellent reproducibility (low inter and intra assay), have sufficiently low quantification/detection limits, show correlation with existing methodologies and perform well in an external proficiency testing scheme. The project poses many challenges; due to the complex nature of the sample matrix, small sample volume, potential interference from other endogenous and exogenous components within the sample matrix such as phospholipids must be addressed. Development of effective sample extraction strategy is a key component of this research. The scope of development includes the use of dried blood spots and novel microsampling techniques.

Once the assays are established, the objectives that followed were to determine the serum concentration and reference intervals of the vitamin D metabolites in adults and children from both healthy and patient populations. Using samples collected from three vitamin D3 supplementation studies with contrasting dosage regimen; the changes in metabolite concentration in response to the dose and frequency of administration were investigated.

This thesis provides evidence on the intricate relationships between active and catabolic forms of vitamin D metabolites, giving insights into the mechanism and consequences of vitamin D status, and potential interactions in various pathophysiological conditions.

Chapter 3 Materials and Methods

3.1 Materials

3.1.1 Reagents

LCMS grade water, acetonitrile and methanol, and analytical grade formic acid, n-heptane and propan-1-ol were obtained from Fisher Scientific (Loughborough, UK). Zinc sulphate was purchased from Sigma-Adrich (Dorset, UK). Isolute® SLE+ 96-well Supported Liquid Extraction plates were supplied by Biotage (Uppsala, Sweden).

3.1.2 Standard materials and calibration solutions

Stock solutions were prepared by spiking certified reference 25(OH)D3, 25(OH)D3, C3-epi-(OH)D3, 1,25$(OH)_2$D3 1,25$(OH)_2$D2, 24R,25$(OH)_2$D3 and 24,25$(OH)_2$D2 (IsoSciences, King of Prussia, PA, USA) in LCMS grade methanol and were stored at -20˚C. NIST SRM 972a traceable human lyophilised multilevel serum calibrators for 25(OH)D3/D2 and C3-epi-(OH)D3/D2 were purchased from Chromsystems (München, Germany). 1,25$(OH)_2$D3/D2 lyophilised calibrators and LC-MS/MS ImmuTube® (Immundiagnostik AG, Bensheim, Germany) were used in the immunoaffinity extractions.

3.1.3 Preparation of calibration standards

Methanolic stock calibration standard solutions were prepared by dissolving 1 mg of the powder compound or 1 mL of the aqueous standards in 1 mL of LCMS grade methanol. Working standards were made from diluting the stock solutions in two consecutive 1 in 100 dilutions by methanol followed by vitamin D depleted serum to achieve the top serum calibration standard. The spiked serum standards were aliquoted into 0.5 mL portions and stored at -20ºC until the day of analysis. Each batch of aliquoted standards was assigned with a lot number and date of preparation recorded. Lyophilised commercial serum calibrators (Chromsystems) were made up by reconstituting in 1 mL of LCMS grade water and allowed to stand for 15 minutes before use. Reconstituted standards were aliquoted into 100 µL microcentrifuge tubes and frozen at -20˚C for use at a later date.

3.1.4 Internal quality control

Lyophilised Internal quality control (IQC) materials for 25(OH)D3/D2 were purchased from Chromsystems (München, Germany) and UTAK (Grifols, Valencia, CA, USA). C3-epi-

25OHD3/D2 bi-level controls were supplied by Chromsystems. Calf serum pool containing endogenous 25(OH)D3/D2 was obtained from Lorne Laboratories (Danehill, Berkshire, UK). Vitamin D depleted serum purchased from BBI Solutions (Cardiff, UK) was used as the base pool for spiking in-house vitamin D metabolites standards and controls.

3.1.5 Preparation of internal quality control

All commercially available quality control materials were prepared in accordance with manufacturer instructions. Chromsystems bi-level serum controls and UTAK serum controls were prepared by reconstituting with 1 mL and 5 mL of LCMS grade water, respectively. Before a new batch of IQC material was introduced the new materials were analysed over 20 separate runs for each analyte to obtain the mean, SD and %CV values. Aliquots were stored at -20˚C before use.

3.1.6 Isotopic labelled internal standards

Isotopic labelled 25-hydroxy vitamin D3-[26,26,26,27,27,27,-$^{2}H_{6}$], 25-hydroxy vitamin D2-[$^{13}C_{3}$], 3-Epi-25-Hydroxyvitamin-D3-[6,19,19-$^{2}H_{3}$], 1α,25-dihydroxyvitamin D3-[6,19,19,2H_{3}] and 24R,25-dihydroxyvitamin D3-[$^{2}H_{6}$] (IsoSciences, King of Prussia, PA, USA) were used as internal standards.

3.1.7 Human sera

Human serum samples containing endogenous vitamin D metabolites, used during method validation for the determination of assay recovery, limit of quantification, method comparison and assessment of concentrations in adults were obtained from the Department of Laboratory Medicine at the Norfolk and Norwich University Hospital NHS Trust. The samples were treated in agreement with the local ethical guidelines for assay development[80]. All samples were stored at -20˚C before use.

3.1.8 Material and laboratory instrumentation

1.5mL microcentrifuge tubes (Starlab, Milton Keynes, UK) and 96-well propylene microtitre and 2 mL deep well plates (Corning Life Sciences, Amsterdam, Netherlands) were used in sample pre-treatment steps. Sample homogenisation was performed using a roller (Bibby Scientific, Stone, UK), microtitre plate shaker (Heidolph, Schwabach, Germany) and multi-tube vortexer (Luckham, USA). The mobile phase was degassed using an ultrasonic bath (Fisher Scientific, Loughborough, UK). Stock standard solutions were weighed using analytical balance (Sartorius,

Goettingen, Germany), calibrated using test weights traceable to UKAS metrology certification, allowing the lowest measurements at 0.1 mg. Centrifugation was performed using Heraeus Megafuge 40R refrigerated centrifuge (Thermo Scientific, Finland). The temperature of the fridges, freezers, room temperature cabinets and laboratory preparation area were continuously monitored and recorded by wireless temperature probes (t-Scan, London, UK).

3.2 Instrumentation and conditions

3.2.1 Instrumentation

The instrument setup for LC-MS/MS analysis was performed using a Rheos Allegro UPLC pump (Flux Instruments, Switzerland) connected to a Micromass Quattro Ultima Pt mass spectrometer (Waters Corp., Manchester, UK) (Fig. 3.1). The UPLC system was equipped with a binary solvent manager operated via Janeiro® II software. A Waters® 2777 Sample manager (Waters Corp., Milford, MA, USA) equipped with 3-drawer cooler stack regulated at 10˚C was used for sample injection.

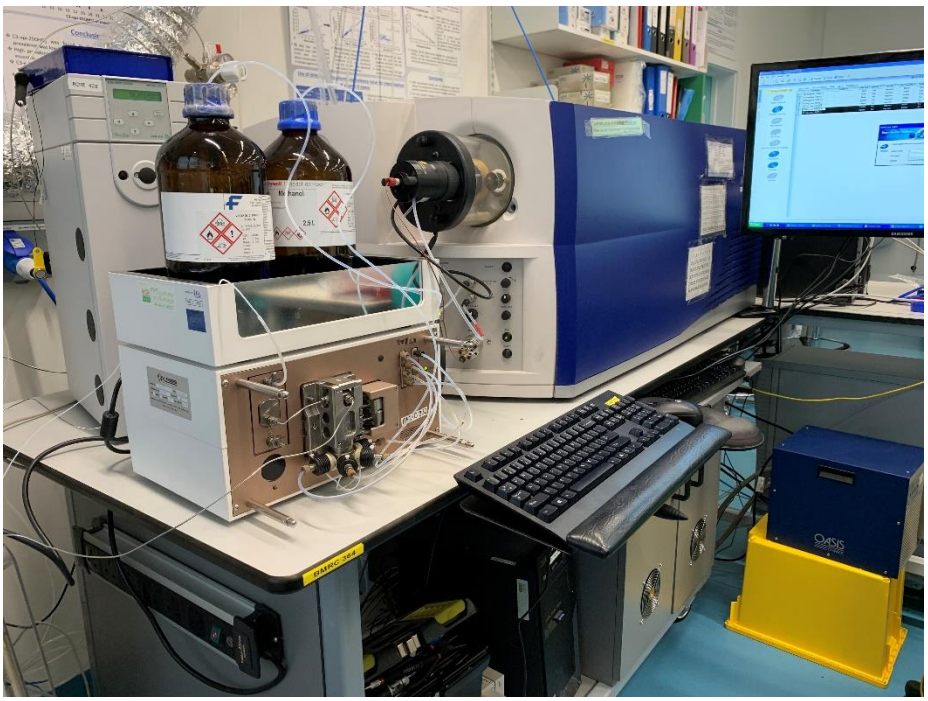


Figure 3.1 Micromass Quattro Ultima Pt tandem mass spectrometer (picture right) with Rheos Allegro UPLC pump (left).

3.2.2 Liquid chromatography conditions

Serum extracts were injected into the liquid chromatography system via a sample manager into the UPLC system. Chromatographic separation of 25(OH)D3/D2 was achieved using the Sunfire® C18 50 × 2.1mm, 3.5 µm column (Waters Corp., Milford, MA, USA) reversed-phase column heated at 55˚C and protected by a 2µm, 6.35mm × 24mm guard cartridge. A gradient elution profile was set up with a column flow rate at 0.4 mL/min. At the start of the gradient, the mobile phase consisted of 12%:88% (A) LCMS grade Water in 0.1% formic acid and (B) LCMS grade methanol in 0.1% formic acid, gradually increased to 99% (B) then returned back to starting gradient in 4.5 minutes. Chromatographic separation of C3-epi-25(OH)D3/D2 required the use of a solid core phenylfluoropropyl (PFP) 100 × 2.1mm, 2.6 µm column (Restek, PA, USA). The gradient elution profile was optimised at (A) at 25% and (B) 75%. Solvent divert was employed to divert the ion suppression region of the separation to waste in order to minimise contamination to the source of the mass spectrometer.

3.2.3 Mass spectrometric conditions

Mass spectrometric analysis of vitamin D metabolites and their respective internal standards was performed using triple quadrupole tandem mass spectrometer with an integrated Electrospray ionisation (ESI) source operated in positive mode. The capillary voltage was kept at 3.0 kV and RF lens 1 and 2 were set at 0.1. Source temperature was maintained at 85˚C. Nitrogen was used as both nebuliser gas and as desolvation gas, the flow rate was set at 30 L/hr and 850 L/hr, respectively. Desolvation temperature was kept at 250˚C. Sample cone voltage and collision energies were optimised for each compound (Table 3.1). Argon was used as the collision gas. Sample analysis was performed in multiple reaction monitoring (MRM) mode with a dwell time of 0.2 seconds. The transitions of each of the compounds were identified by direct infusion of methanolic standards into the ion source via a T-connector to determine their respective precursor to product quantifier and qualifier ion transitions (Table 3.1).

Compound	voltage (kV)		m/z	
	Cone	Collision	Precursor	Product
25(OH)D3 / C3-Epi-25-25(OH)D3	35	12	401	383 (Quan) 365 (Qual)
25-(OH)D3-[26,26,26,27,27,27,-$^{2}H_6$]	35	22	407	107
C3-Epi-25(OH)D3-[6,19,19-$^{2}H_3$]	35	22	415	107
25(OH)D2 / C3-Epi-25(OH)D2	35	8	413	270 (Quan) 395 (Qual)
25(OH)D2-[$^{13}C_3$]	35	22	416	107
1,25$(OH)_2$D3	35	10	399	381 (Quan) 135 (Qual)
1,25$(OH)_2$D2	35	8	411	392 (Quan) 135 (Qual)
1,25$(OH)_2$D3-[6,19,19,2H_3]	35	8	402	384
24R,25$(OH)_2$D3	35	10	417	398 (Quan) 121 (Qual)
24R,25$(OH)_2$ D3-[$^{2}H_6$]	35	8	423	121

Table 3.1 The precursor to product quantifier and qualifier ion transitions of vitamin D metabolites and their respective isotopic labelled internal standards.

3.2.4 Software

MassLynx v4.1 and QuanLynx software (Waters Corp., Milford, MA, USA) were used for system control, data acquisition, baseline integration and peak quantification.

3.2.5 Statistical Data analysis

Results were recorded in Microsoft Excel spreadsheet. Statistical analysis for determination of inter and intraassay coefficient of variation (%CV), linear regression graphs, box and whisker plots and Bland-Altman plots were generated using SPSS v.23 (IBM Corporation, NY, USA) and GraphPad Prism 8 (GraphPad, San Diego, CA, USA). All data were visually examined and checked for transcriptional, pre/post analytical errors before statistical analysis.

3.3 Analytical procedures

3.3.1 Sample pre-treatment procedures

Before the start of each experiment, all reagents were mixed thoroughly by gentle inversion before use. For solvent-based reagents, all refrigerated reagents were left on the bench to reach room temperature (18-25˚C). Frozen serum/plasma samples were thawed, mixed, and centrifuged at 4000 ×g for 5 minutes before use.

3.3.2 Isotopic dilution protein precipitation

100 µL of calibration/quality control/sample was placed in 1.5 mL microcentrifuge tubes, followed by 100 µL of 0.1 M zinc sulphate in water. 200 µL of cold LCMS grade acetonitrile containing [$^{2}H_{6}$]-25(OH)D3 and [$^{13}C_{2}$]-25(OH)D2 at concentrations of 450 nmol/L was added to each tube. All tubes were immediately capped and mixed vigorously on a vortex mixer for one minute to ensure homogeneity, and then incubated in 4˚C for 45 minutes. After incubation, all tubes were mixed and centrifuged at 10,000 ×g for 5 minutes. 200 µL of the supernatant was transferred to a microtitre plate which was then heat sealed with aluminium foil before injection into the LC system.

3.3.3 Phree™ Phospholipid depletion plate

100 µL of calibration/quality control/sample was placed directly onto the bed of the plate, followed by 600 µL of internal standard mixture in 60% (v/v) acetonitrile and water. The plate was placed on a waste collection plate and mixed vigorously on a plate vortexer for 10 minutes. Nitrogen gas was applied at 6 bar of pressure on a positive pressure manifold to remove the eluent to waste. The plate was then transferred onto a fresh 2 mL 96-deep well collection plate with 100 µL of 0.1 M zinc sulphate dispensed into each well. 300 µL of acetonitrile was added onto the bed of the phospholipid removal plate; nitrogen pressure was applied to push the eluent into the collection plate containing the zinc sulphate. The plate was heat-sealed with aluminium foil after a brief vortex and then placed onto the sample manager for analysis.

3.3.4 Supported liquid extraction (SLE)

100 µL of calibration/quality control/sample was placed in 1.5 mL microcentrifuge tubes, followed by 200 µL of internal standard mixture in 50% (v/v) propan-1-ol and water. The mixture was mixed before loading onto the SLE plate, the plate was placed on top of a 2 mL 96-deep well collection plate and left to stand for 5 minutes to allow the mixture to soak into extraction bed.

The plate was washed twice with 750 µL of n-heptane and the eluent collected. A positive pressure manifold delivering nitrogen gas at 6 bar of pressure was applied to the bed of the plate after each wash step to remove residual eluent. The collected eluents were dried down under a constant stream of nitrogen gas on a sample concentrator heated at 37 ˚C. The dried plate was reconstituted in 70% (v/v) methanol in water and then vortexed and heat sealed with aluminium foil before LC-MS/MS analysis.

3.4 Method Validation

Method validation criteria is based on the 2018 U.S. Food and Drug Administration (FDA)[81] and 2012 European Medicines Agency (EMA)[82] guidelines on bioanalytical method validation

3.4.1 Ion suppression

An Ion suppression experiment was performed by post-column infusion of a methanolic vitamin D mixture containing approximately 1 µg/mL of each metabolite via a T-junction at a flow rate of 20 µL/min, during which samples of extracted phosphate buffered saline (PBS) and human serum were injected via the liquid chromatography system at 0.4 mL/min.

3.4.2 Imprecision

Intra and inter-assay imprecision of the assay were assessed by running QC materials a minimum of 6 times within a single run and separately over a defined period. Variation was expressed in terms of standard deviation (SD) and coefficient of variation percentage (%CV). The acceptance criteria defined that %CV should not exceed 10% and 15% at the lower limit of quantification.

3.4.3 Linearity

The linearity of the method was evaluated by analysing stock standards made up from reference calibration solutions. 25(OH)D3/D2 and C3-epi-25(OH)D3 concentrations ranging from 0 to 500 nmol/L were prepared and analysed. The calibration curve was constructed by plotting the ratio of analyte peak area to the internal standard peak area against the concentration of their respective standards. Calibration curves were accepted as linear if the weighted linear regression produced a correlation coefficient (r^2) value of >0.950000.

3.4.4 Extraction efficiency

Serum samples containing low, medium and high endogenous concentrations of vitamin D metabolites were aliquoted and spiked with two different concentrations of 25(OH)D3/D2 and C3-epi-25(OH)D3 at 50 nmol/L and 150 nmol/L. Assay recovery was determined by calculating the percentage of the measured value against the sum of endogenous value plus spiking concentration.

3.4.5 Lower limit of quantification and detection

Lower limit of quantification (LLoQ) was determined by the lowest concentration quantifiable with a precision CV of 15% over a minimum of 3 replicates with a minimum analyte peak area 10x the response compared to a blank (signal:noise ratio of 10:1). The lower limit of detection (LLoD) was determined by the lowest concentration that produced a peak signal-to-noise ratio of 10:1.

3.5 Assay acceptance criteria

3.5.1 LC-MS/MS measurement procedures and assay acceptance criteria

Calibration standards and IQC materials were analysed with each batch of samples. The concentration of the QCs ranged from the lowest level (within 20% of the LLoQ) to the high end of the analytical limit (within 20% of the top calibration standard). The QCs provided the basis of accepting or rejecting the batch. The assay was accepted when QC results fall within ±2SD from the target value. An assay is rejected when a QC failed to fulfil one of the following Westgard[83] rejection criteria.

- 1_{3s} **Rejection rule:** detection of random error. If one data point exceeds ±3SD the run is considered out of control.

- 2_{2s} **Rejection rule:** detection of systematic error. If two data points for the same level in a single run exceed ±2SD, the run is considered out of control.

- R_{4s} **Rejection rule:** detection of random error. If two data points in the same run are separated by 4SD the run is considered out of control.

- 10X **Rejection rule:** detection of systematic error. If 10 data points in or across runs were on the same side of the mean the run is considered out of control. This violation often indicates the deterioration of assay reagents and should trigger recalibration or equipment maintenance.

Chapter 4 The prevalence of C3-epimer 25-hydroxyvitamin D in adult and paediatric samples

4.1 Background

C-3 epimer of 25 hydroxyvitamin D3 (C3-epi-25(OH)D3) is produced in the liver by the epimerisation pathway of 25-hydroxy vitamin D3. It differs from 25(OH)D3 in the configuration of the hydroxyl group at the third carbon (C-3) position (Fig. 4.1). Reddy et al. first described that vitamin D3 can alternatively be metabolised through a C-3 epimerisation pathway that parallels the standard metabolic pathway in neonatal human keratinocytes[84]. Both *in vitro* and *in vivo* studies have shown that intermediate metabolites of vitamin D3 can be epimerised and that these epimers can be further metabolised by the same enzyme responsible for hydroxylation and oxidation events as in the standard pathway, producing C3-epi-25(OH)D3 and subsequently C3-epi-1α,25$(OH)_2$D3 and C3-epi-24(R),25$(OH)_2$D3[16,84-86].

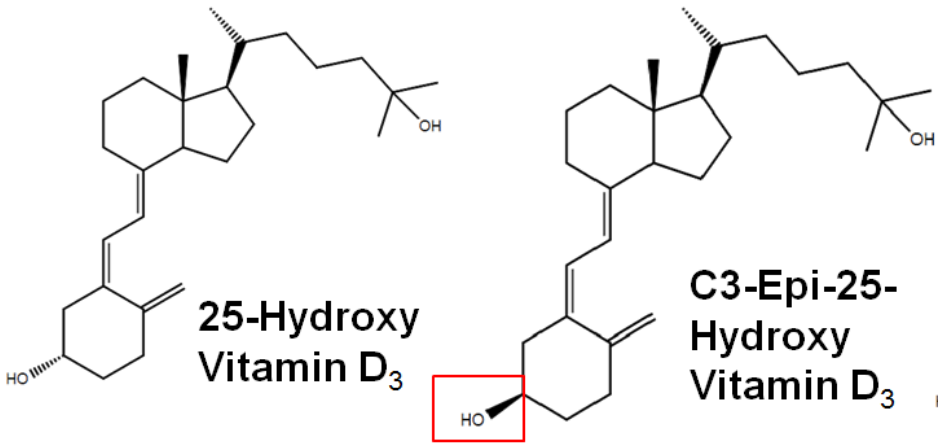


Figure 4.1 Structural configuration of C3-epi-25(OH)D3 differs from 25(OH)D3 in the hydroxy group at the third carbon (C-3) position.

The biological relevance of C3-epi-25(OH)D remains to be elucidated. It has been documented that C3-epi-25(OH)D3 is present in children and adults[18,87,88]. Although higher concentrations have been observed in neonates, C3-epi-25(OH)D3 has been reported to be present in adults and children under 1 year of age at 3-5% of the respective 25(OH)D3 concentrations[87].

Despite the lack of evidence on the clinical significance of C3-epi-25(OH)D, concerns have been raised that isobaric interference may result in over-estimation of total 25(OH)D when measured by liquid chromatography tandem mass spectrometry (LC-MS/MS). As both forms share the same m/z precursor to product ion transitions, standard triple quadrupole instruments are unable to distinguish the epimeric form by the m/z. The alternative is to perform chromatographic separation before MS detection. C3-epi-25(OH)D3 co-elutes with 25(OH)D3 on all C18 reverse phase analytical columns commonly used in routine clinical laboratories. If not chromatographically separated, C3-epi-25(OH)D3 present in serum can contribute significantly to the total 25(OH)D3 concentration. In the literature, studies on the prevalence of C3-epi-25(OH)D3 in human infant and adult sera have used relative percentages[22,89], others used absolute concentrations C3-epi-25(OH)D3 but often the analytical methods are poorly characterised. To achieve baseline separation of C3-epi-25(OH)D3 from 25(OH)D3, the use of chiral columns[18,21], cyano-propyl (SB-CN)[20,87,90] and pentafluorophenyl (PFP)[71,88,91,92] columns have been used. Accurate quantitation of C3-epi-25(OH)D3 also requires reference-traceable calibration standards to be analysed on each run, ideally with the use of deuterated C3-epi-25(OH)D as internal standard. At the time of writing this report, a commercial source of NIST-aligned standards materials for C3-epi-25(OH)D3/D2 and isotopic labelled C3-epi-25(OH)D3-[6,19,19-$^{2}H_{3}$] standards (Fig 4.2) have recently become available.

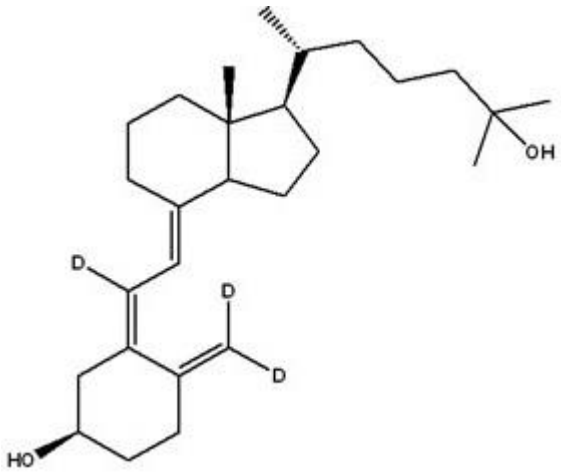


Figure 4.2 Molecular structure of C3-epi-25(OH)D3-[6,19,19-$^{2}H_{3}$]. MW = 415.63, molecular formula = C28H44O2. D indicates the location of the deuterium (^{2}H) label.

The aim of the study was to assess the occurrence of C3-epi-25(OH)D3 in adult and paediatric serum samples. The first objective was to develop and validate a LC-MS/MS method to resolve and quantify C3-epi-25(OH)D3 from 25(OH)D3 using the newly available NIST SRM972a

traceable C3-epi-25(OH)D3 commercial standards to calibrate the assay. The second objective was to analyse adult and paediatric serum samples to establish the prevalence of C3-epi-25(OH)D3. The results were compared against non-epimer resolving method to assess the effect of C3-epi-25(OH)D3 on the interpretation of vitamin D status in the chosen populations.

4.2 Materials and methods

4.2.1 Serum sample collection

Serum from 839 adults (age mean, range: 37 yrs, 25-57) and 179 paediatric samples (age mean, range: 8 yrs, 0-14) were selected at random from end of healthcare requests at the Department of Laboratory Medicine, Norwich and Norfolk University Hospital. Blood samples were collected in serum vacutainer tubes (Becton Dickinson, Oxford, UK) and centrifuged for 10 minutes at 3,000x g to obtain the serum. The serum layer was aliquoted into a polystyrene tube and stored at -20°C until analysis. All samples were anonymised at point of access and processed in accordance with generic ethical approval for assay development[80].

4.2.2 Materials, calibration standards and controls

NIST SRM972a traceable C3-epi-25(OH)D commercial standards and quality control materials (Chromsystems, München, Germany). LCMS grade water, acetonitrile, methanol and formic acid were obtained from Fisher Scientific (Loughborough, UK). Zinc sulphate was purchased from Sigma-Adrich (Dorset, UK). Certified C3-epi-25(OH)D3 ethanolic standard and isotopic labelled [$^{2}H_{3}$]-C3-epi-25(OH)D3 (IsoSciences, King of Prussia, PA, USA).

4.2.3 Sample preparation and LC-MS/MS procedures

Serum was extracted by isotopic dilution protein precipitation procedure as described in Chapter 3.3.2 Isotopic dilution protein precipitation. LC conditions for non-epimer resolving method using C18 column as described in Chapter 3.2.2. For epimer method, [$^{2}H_{3}$]-C3-epi-25(OH)D3 (500 nmol/L) was added to the acetonitrile mixture that contains [$^{2}H_{6}$]-25(OH)D3 and [$^{13}C_{2}$]-25(OH)D2. To resolve C3-epi-25(OH)D3/D2 from 25(OH)D3/D2, the extracted samples were chromatographed using a pentafluorophenyl (PFP) 2.6µm 100 x 2.1mm I.D. solid core particle column (Restek, PA, USA). A new gradient elution program was developed (Table 4.1); the mobile phase consisted of 25:75 (A) water and (B) methanol in 0.1% formic acid for the first 9 minutes of run time, then increased to 100% B to cleanse and remove residue from the column. The flow rate was maintained at a constant rate of 0.4 mL/min. The total run time was 13 minutes.

Mass spectrometer conditions were performed in positive electrospray ionisation with MS/MS in multiple reaction monitoring mode as described in 3.2.3 Mass spectrometric conditions. The precursor to product ion transitions of 401>383 was used for detection and quantification.

Steps	Time (minute)	Water with 0.1% formic acid (A) %	Methanol with 0.1% formic acid (B)%	Flow rate (mL/min)
1	Initial	25	75	0.4
2	9.0	25	75	0.4
3	9.1	0	100	0.4
4	11.0	0	100	0.4
5	11.1	30	70	0.4
6	13.0	30	70	0.4

Table 4.1 Gradient elution program for the C3-epi-25(OH)D3 assay

4.2.4 Method validation and quality control procedures

Assay validation criteria is described in Chapter 3.4 and 3.5. Four pools of human sera were collected to form the base pool for spiking experiments and to test for extraction efficiency.

4.2.5 Statistical data analysis

Data exploration, frequency distribution histograms, and statistical analyses were performed using Statistical Package for Social Science (SPSS) version 22.0.0.2 (IBM, NY, USA) and GraphPad Prism 8 (GraphPad, San Diego, CA, USA). Method comparisons were assessed using Passing-Bablok regression. A correlation is deemed satisfactory if the regression produced an r^2 ≥0.9.

4.3 Results

Full mass spectra from direct infusion of pure C3-epi-25(OH)D3 were recorded and compared against the spectra separately obtained from 25(OH)D3 (fig. 4.3). The two compounds produced identical precursor m/z 401 and water loss product ions m/z at 383 and 365. This confirms the MS is unable to distinguish between the two compounds.

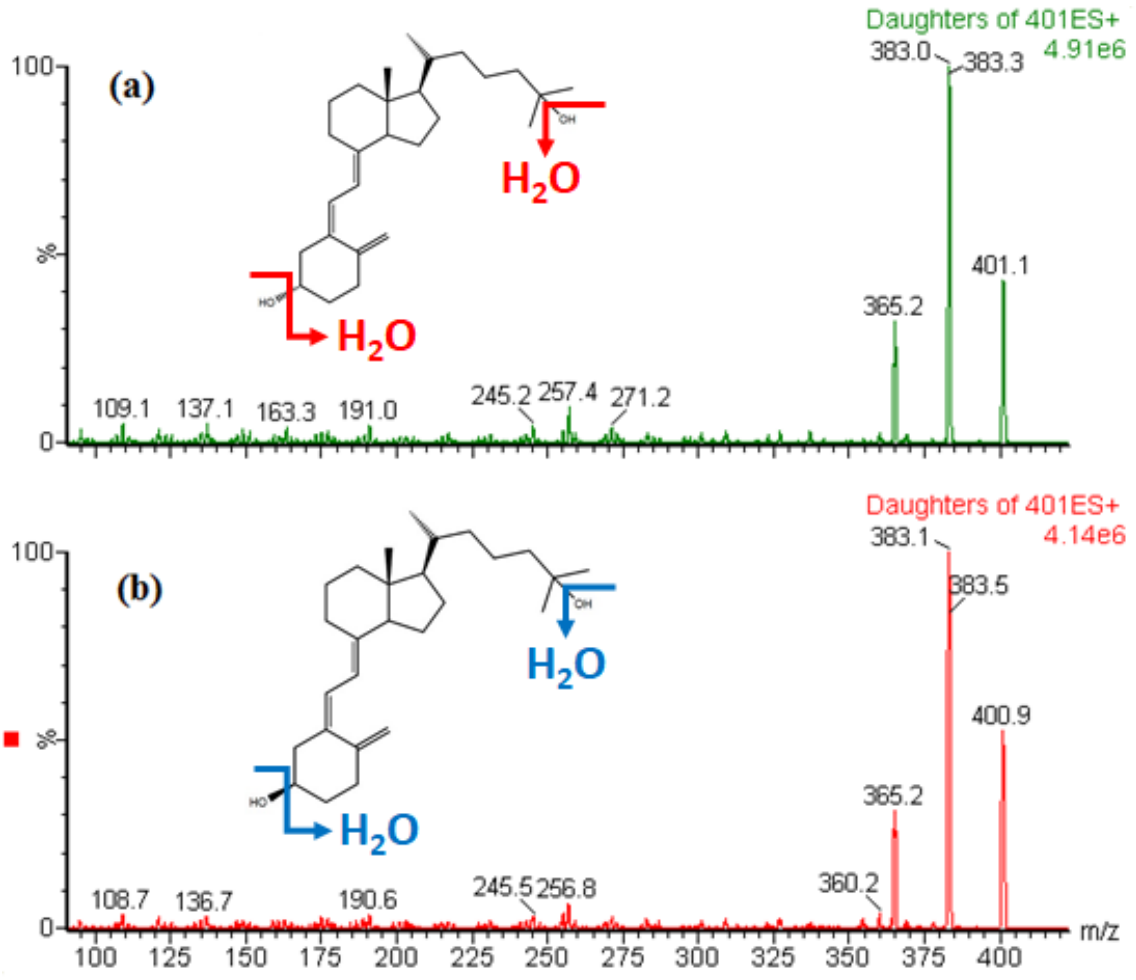


Figure 4.3 Mass spectrums of (a) 25(OH)D3 and (b) C3-epi-25(OH)D3 with identical precursor ion m/z of 401 and product ion m/z of 383 (x1 H_2O loss) and 365 (x2 H_2O loss).

Chromatographic separation of 25(OH)D from C3-epi-25(OH)D was necessary to quantify these compounds. A serum sample spiked with internal standards was extracted and analysed using the new gradient elution programme on the pentafluorophenyl (PFP) column. The chromatographic peaks obtained were authenticated against the pure aqueous standards. The PFP analytical column produced clean, well-defined C3-epi-25(OH)D3 and C3-epi-25(OH)D2 peaks that were fully resolved from 25(OH)D3 and 25(OH)D2 at the baseline (Fig. 4.4).

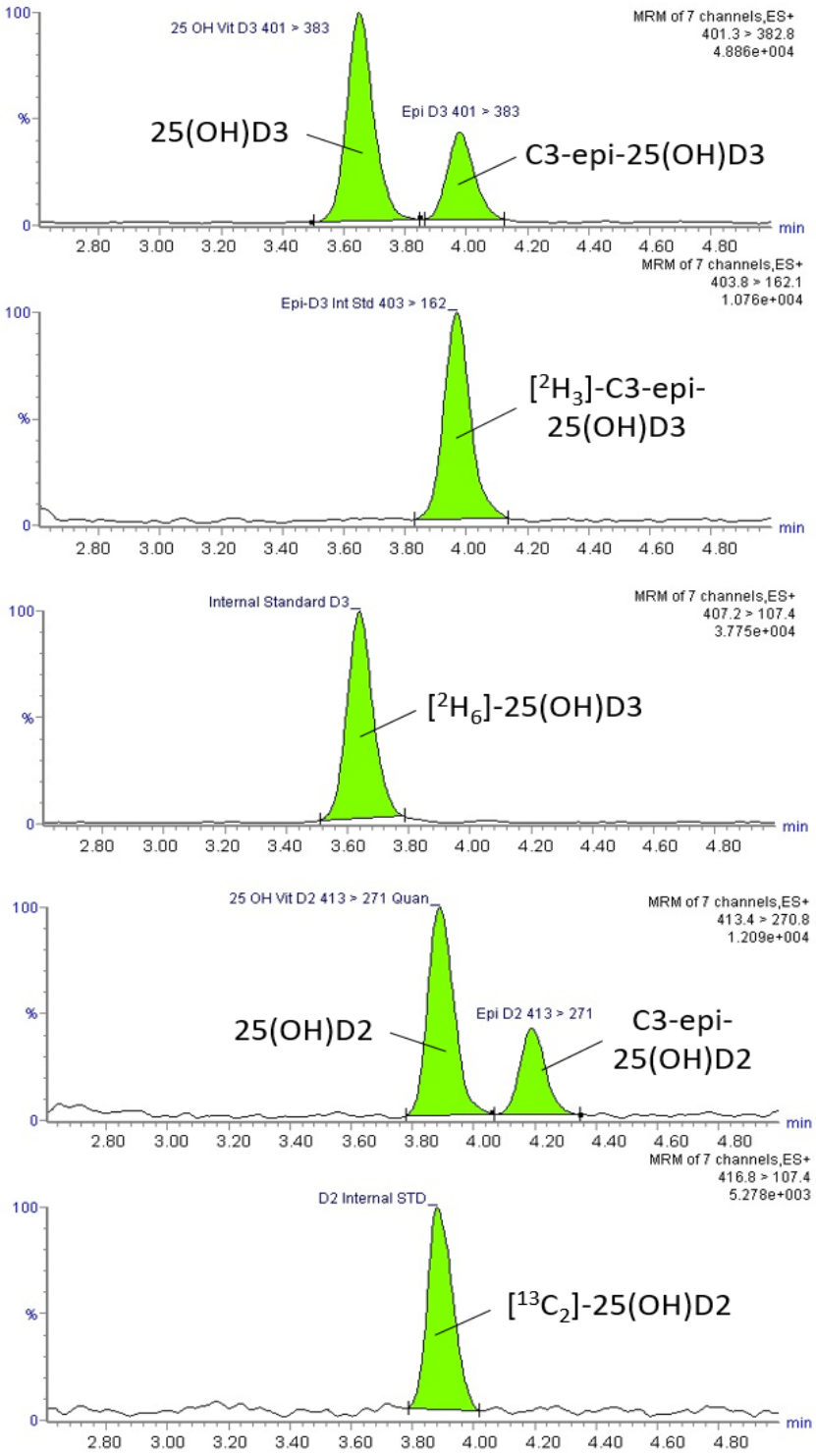


Figure 4.4 Chromatogram depicts the separation of a sample containing C3-epi-25(OH)D3/D2, 25(OH)D3/D2 and the respective isotopic internal standards recorded simultaneously in a single injection.

7.3.1 Method validation

Ion suppression study

An ion suppression study was performed by post column infusion of a pure methanolic C3-epi-25(OH)D3 solution via a T-junction at a flow rate of 20 µL/min, during which samples of an extracted phosphate buffered saline (PBS) blank and a human serum were injected into the source via the LC system at 0.4 mL/min. Results showed a region of baseline suppression occurred during the first 3.2 minutes. Analyte peaks were eluted from 3.6 min onwards. Solvent divert was subsequently employed to set the period of ion suppression to waste. A 100% methanol column wash step was introduced at the end of gradient cycle to remove residual waste.

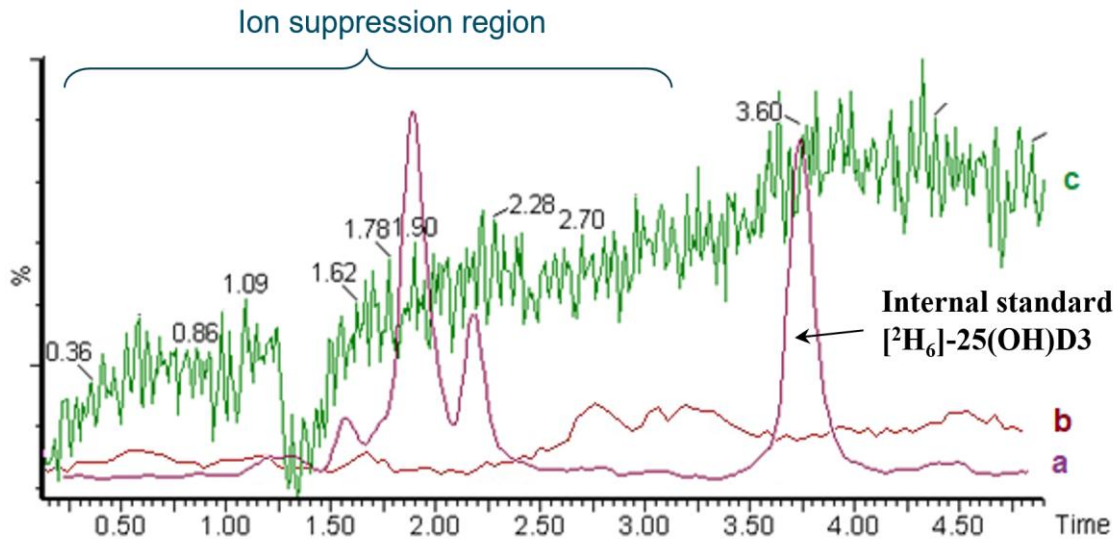


Figure 4.5 Ion suppression study. Reduction in baseline signal was observed during co-injections of extracted serum sample (**a**), phosphate buffered saline blank (**b**), with post-column infusion of 25(OH)D3 (**c**). Ion suppression occurred during 0-3.2 min, prior to the elution of [$^2H_6$]-25(OH)D3 peak at 3.6 min.

Assay imprecision

The imprecision of the LC-MS/MS assay is summarised in Table 4.3 and 4.4. Intra and inter-assay imprecision were assessed by running QC materials 10 times within a single run and separately over a two month period. The intra-assay run imprecision (%CV) ranged from 5.6 to 9.9%. The inter-assay imprecision (%CV) ranged from 6.8 to 12%.

	Level 1 (nmol/L)				**Level 2 (**nmol/L)			
	C3-epi-25(OH)D3	C3-epi-25(OH)D2	25(OH)D3	25(OH)D2	C3-epi-25(OH)D3	C3-epi-25(OH)D2	25(OH)D3	25(OH)D2
Mean	42.4	45.3	32.5	43.9	64.8	61.9	101.8	88.1
SD	3.0	2.5	3.0	3.1	6.4	4.9	5.7	5.7
%CV	7.1	5.6	9.3	7.0	9.9	7.8	5.6	6.5

Table 4.2 Intra-assay imprecision of the LC-MS/MS method (n=10).

	Level 1 (nmol/L)				**Level 2 (**nmol/L)			
	C3-epi-25(OH)D3	C3-epi-25(OH)D2	25(OH)D3	25(OH)D2	C3-epi-25(OH)D3	C3-epi-25(OH)D2	25(OH)D3	25(OH)D2
Mean	43.6	35.4	34.5	37.0	62.5	60.8	107.6	94.6
SD	3.0	3.0	3.2	4.4	5.9	6.6	10.2	11.3
%CV	6.8	8.4	8.3	12.0	9.4	10.8	9.5	11.9

Table 4.3 Inter-assay imprecision of the LC-MS/MS method (n=10).

Linearity

Standard curves of C3-epi-25(OH)D3, C3-epi-25(OH)D2, 25(OH)D3 and 25(OH)D2 were generated by plotting the analyte peak area over the internal standard peak area ratio against the concentration of standards ranging from 0-200 nmol/L (Fig. 4.6). Linear regression analysis consistently gave r^2 value of >0.998.

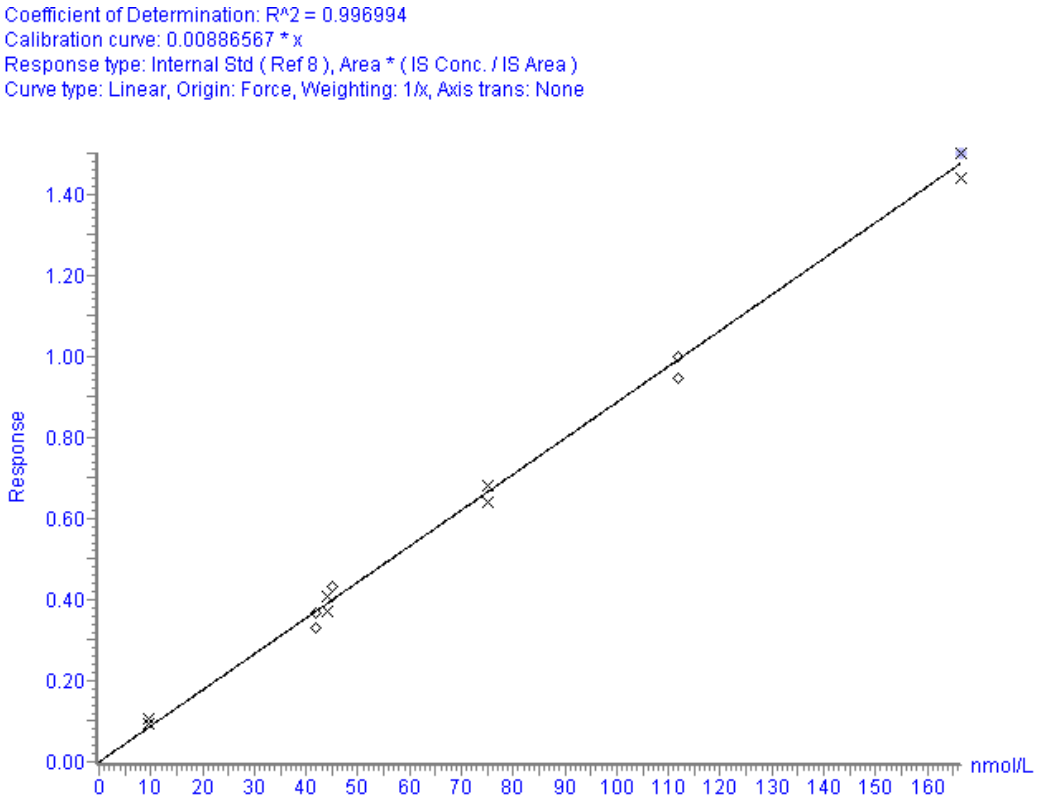


Figure 4.6 Typical standard curves of C3-epi-25(OH)D3. (×) represents standards points and (◊) represent quality controls.

Extraction efficiency

Extraction recovery was evaluated by determining the concentration of C3-epi-25(OH)D3 recovered from the spiked prior to extraction. Mean recovery was 103.3% (range 97.0-107%) (Table 5). Results indicated that the extraction procedure was able to efficiently extract C3-epi-25(OH)D3 from matrix.

	Endogenous C3-epi-25(OH)D3 present (nmol/L)	Amount spiked (nmol/L)	Measured value (nmol/L)	% Recovery
Sample 1	15	50	67.0	103.1
Sample 2	33.2	50	81.0	97.4
Sample 3	19.2	100	112.0	94.0
Sample 4	34.8	100	126.0	93.5

Table 4.4 Spiked recovery of C3-epi-25(OH)D3.

The lower limit of quantification (LLoQ)

Aliquots of vitamin D depleted serum were spiked with C3-epi-25(OH)D3 at concentrations ranged between 0.1 to 10 nmol/L. Each standard was analysed five times to determine the reproducibility of the measured value. The lowest C3-epi-25(OH)D3 concentration that produced a peak signal-to-noise ratio of 10:1 with CV of 15% was 2.1 nmol/L. The upper limit of quantification (ULoQ) was defined by the highest standard of the calibration curve.

Interference study

To investigate possible interference from other vitamin D metabolites the LC-MS/MS method was subjected to cross-reactivity testing. An aliquot of vitamin D depleted serum was spiked with 100 nmol/L of 25(OH)D3, 25(OH)D2, 1α,25 $(OH)_2$D3, 1α,25 $(OH)_2$ D2 and 24(R),25 $(OH)_2$ D was analysed for C3-epi-25(OH)D3. No peak signal was detected at the m/z values corresponding with the precursor to product transitions for C3-epi-25(OH)D3 (401 > 383, 401 > 365) and C3-epi-25(OH)D2 (413 > 270, 413 > 395). 25(OH)D3/D2 peaks were detected on the epimer transitions but not present at the retention time window of C3-epi-25(OH)D3 and C3-epi-25(OH)D2.

4.3.2 Prevalence and concentration of C3-epi-25(OH)D in adult serum samples and the relative proportion to 25(OH)D

Serum from 839 clinical samples for routine 25(OH)D measurement were analysed for C3-epi-25(OH)D. Results showed 25(OH)D3 was present in all 839 sera [mean (±SD) 45.9 (25.5), ranged from 1.9 to 165 nmol/L and normally distributed (Fig.4.7). C3-epi-25(OH)D3 was present in 733 (87.4%) of samples. The median (±SD) C3-epi-25(OH)D3 concentration was 1.9 (2.8) nmol/L (Fig. 4.8). The distribution of C3-epi-25(OH)D3 concentration was left-skewed; 98% of samples were observed with values <10 nmol/L. No C3-epi-25(OH)D2 was detected in any samples. The highest C3-epi-25(OH)D3 value observed was 45.6 nmol/L in a patient supplemented with vitamin D in whom no 25(OH)D2 was detected and the 25(OH)D3 was 141 nmol/L.

The percentage proportion of C3-epi-25(OH)D3 to 25(OH)D3 was calculated in all the samples. The mean percentage of C3-epi-25(OH)D3/25(OH)D3 was 4.8%, range up to 37.8%. A concentration-dependent relationship (r^2=0.3988, p<0.001) was observed between the two compounds. In general, higher concentrations of C3-epi-25(OH)D3 can be found in samples containing high concentration of 25(OH)D3, but varied greatly between individuals. (Fig. 4.9).

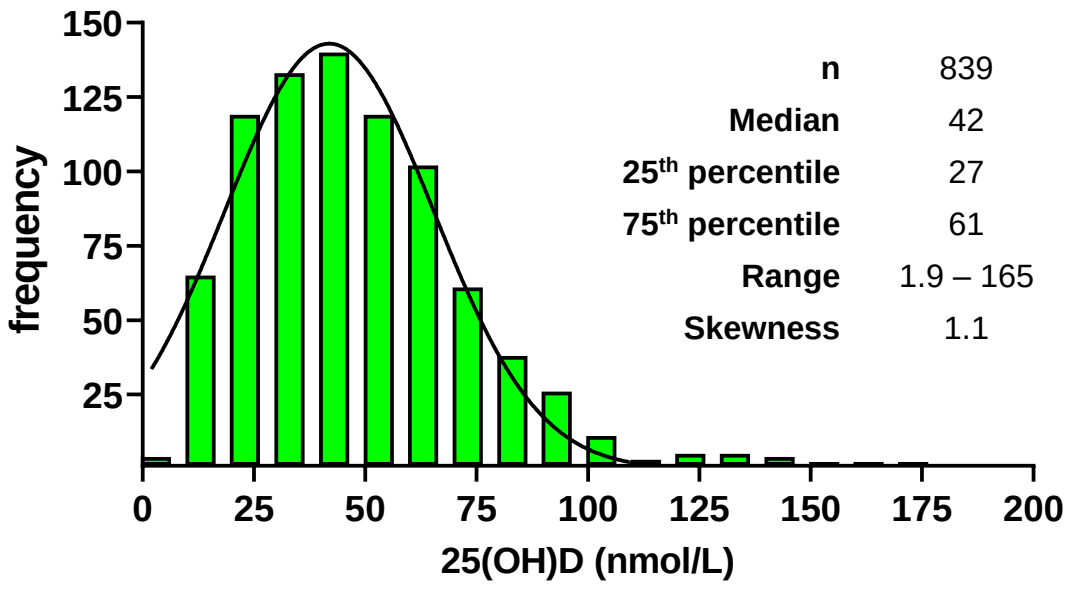


Figure 4.7 Distribution of 25(OH)D3 concentration in the adult cohort.

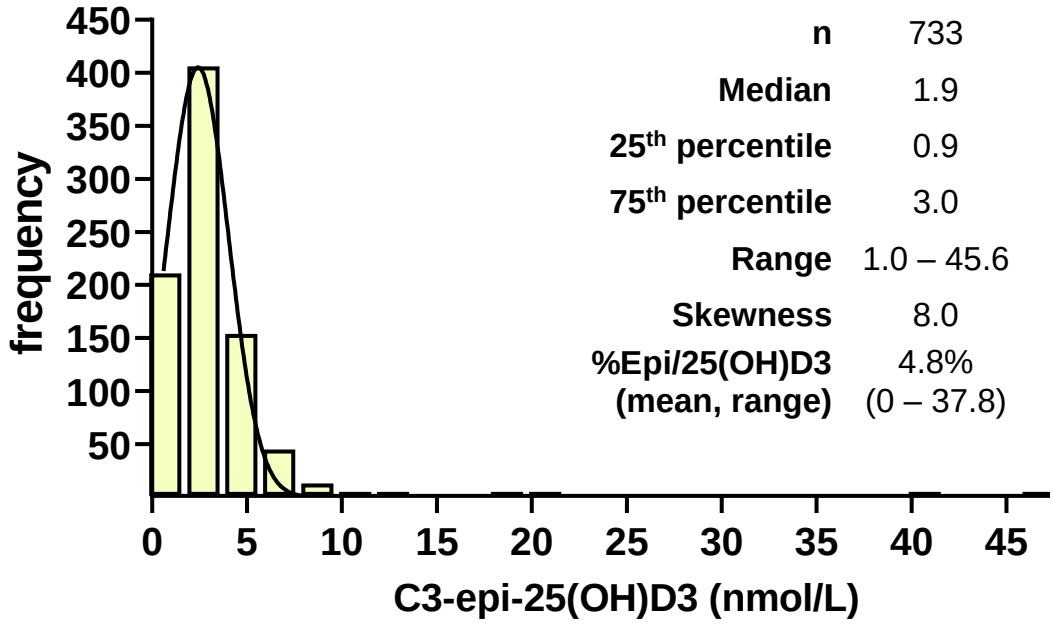


Figure 4.8 Distribution of C3-epi-25(OH)D3 concentration in the adult cohort.

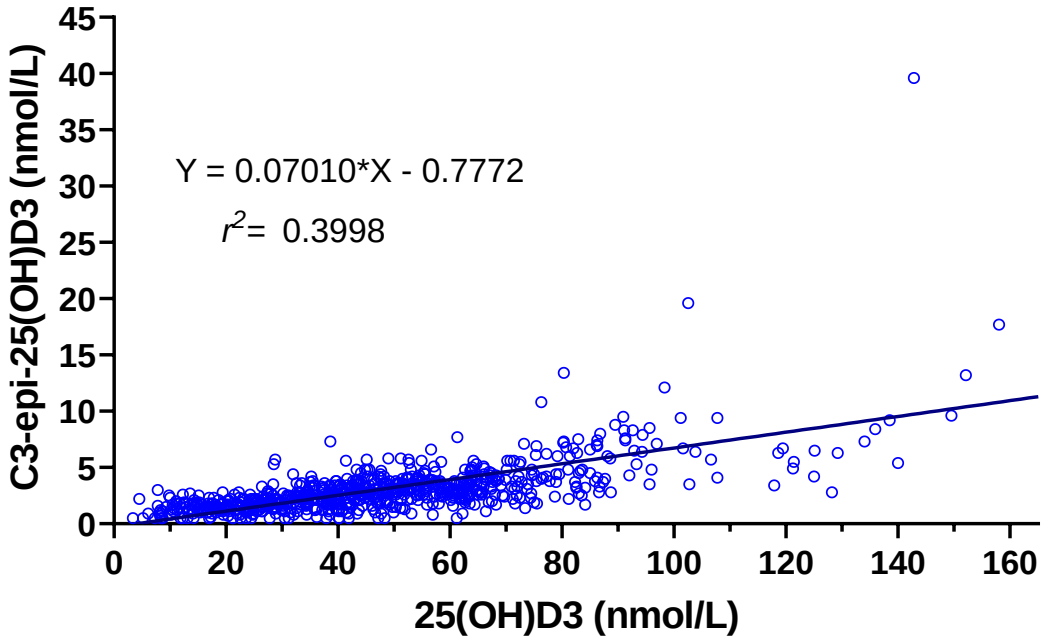


Figure 4.9 Relationship between serum concentrations of C3-epi-25(OH)D3 and 25(OH)D3 in the adult cohort. Linear regression showed goodness of fit r^2 of 0.3998, $p<0.001$.

4.3.3 Prevalence and concentration of C3-epi-25(OH)D in paediatric serum

Analysis of 179 paediatric samples (age mean, range: 8 yrs, 0-14) showed C3-epi-25(OH)D3 was present in 157 out of 179 (87.7%) of samples. The 25(OH)D3 concentration was normally distributed, with a mean (range) of 74.5 (7.9-314.4) nmol/L (Fig. 4.10). The C3-epi-25(OH)D3 distribution was left-skewed similar to the adults, with a higher median concentration (range) of 7.7 (1.0-50.8) nmol/L than adults. 47.2% of samples had C3-epi-25(OH)D3 >10 nmol/L. No C3-epi-25(OH)D2 was detected in samples.

The mean percentage of C3-epi-25(OH)D3/25(OH)D3 was 12%, range up to 54.4%. A concentration-dependent relationship (r^2=0.4497, p<0.001) was observed between the two metabolites. A substantial inter-dividual variability existed across the concentration range (Fig. 4.12) .

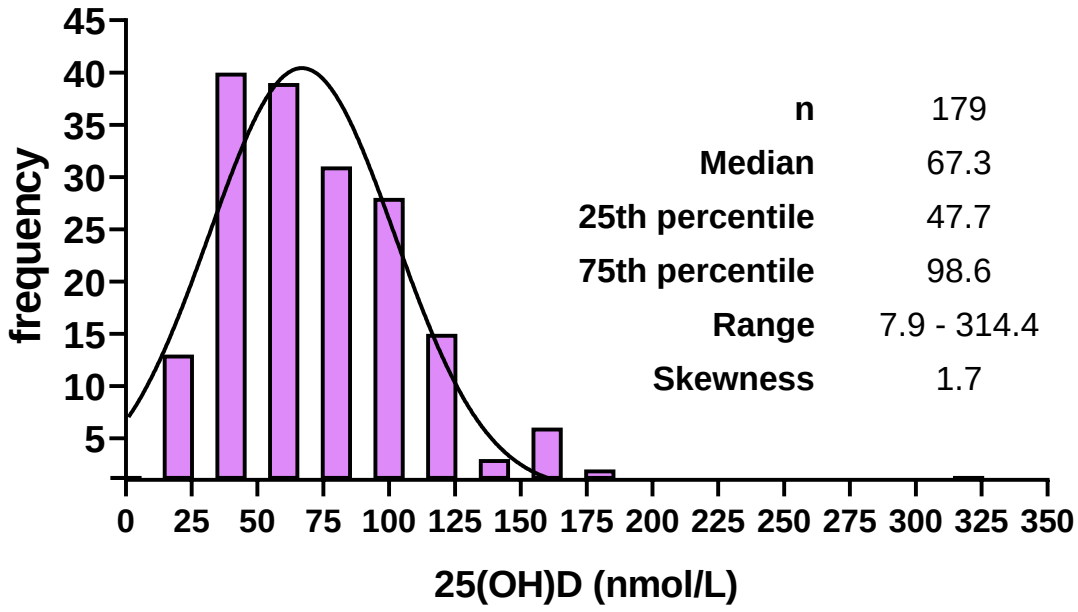


Figure 4.10 Distribution of 25(OH)D3 concentration in the paediatric cohort.

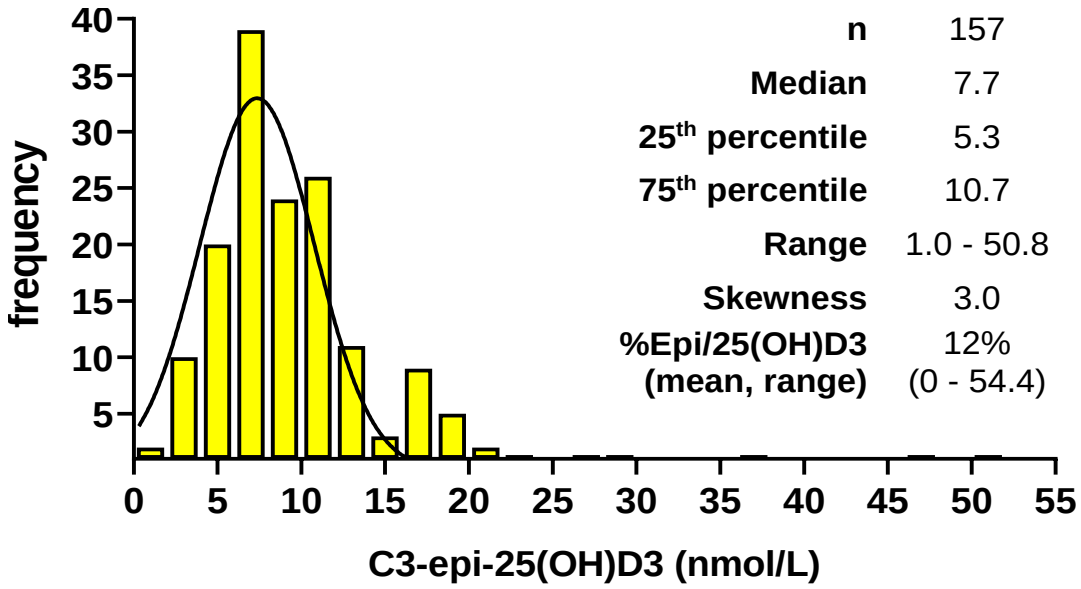


Figure 4.11 Distribution of C3-epi-25(OH)D3 concentration in the paediatric cohort.

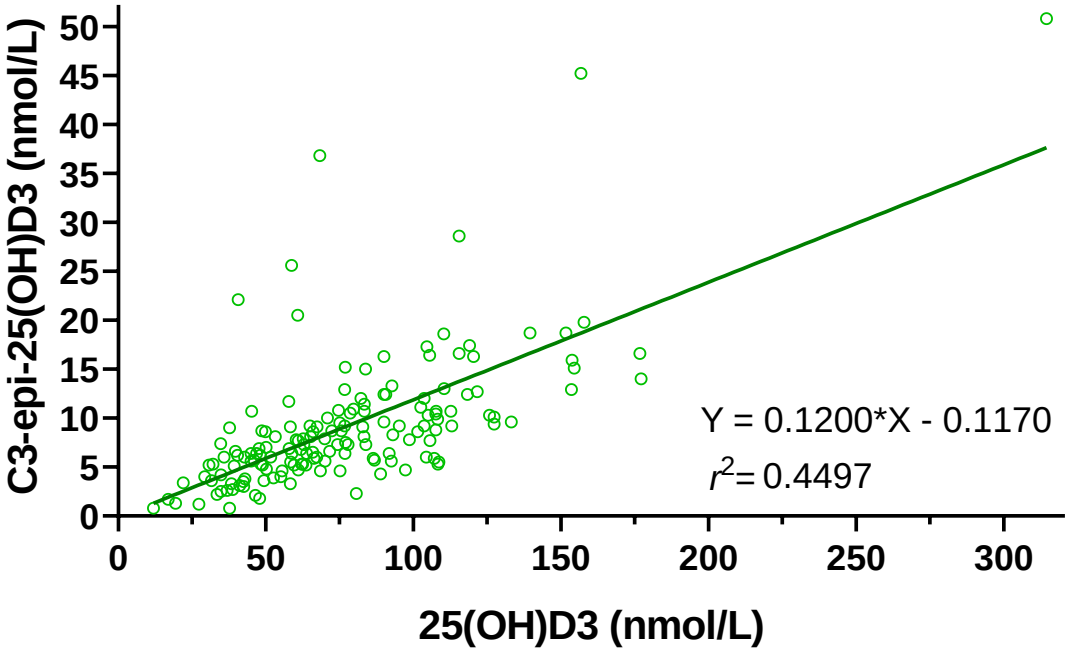


Figure 4.12 Relationship between serum concentrations of C3-epi-25(OH)D3 and 25(OH)D3 in the paediatric cohort. Linear regression showed a goodness of fit r^2 of 0.4497, $p<0.001$.

4.3.4 Changes to the interpretation of vitamin D status with resolving C3-epi-25(OH)D

All adult and paediatric samples were retested by LC-MS/MS using a C18 column, which does not resolve the epimer forms of 25(OH)D. The 25(OH)D values obtained by the non-epimer resolving method and the epimer resolving method (excluded C3-epi-25(OH)D) were grouped according to the Institute of Medicine (IOM) vitamin D thresholds[93], which defined the vitamin D status of an individual with serum 25(OH)D <30 nmol/L as deficient; between 30-50 nmol/L as insufficient; and >50 nmol/L as sufficient. Based on these definitions, in the adult cohort of 733 out of 837 samples with detectable C3-epi-25(OH)D, resolving the epimer had reduced the percentage of the cohort identified with vitamin D sufficient status by 4.4%, and increased those interpreted as deficient and insufficient by 1.8% and 2.6% respectively. In the paediatric cohort of 157 out of 179 samples (undetectable C3-epi-25(OH)D were excluded), resolving the epimer had decreased the percentage of the cohort classified as vitamin D sufficient status by 10.4%, and increased those categorised as deficient and insufficient by 10% and 0.4% respectively (Table 4.4).

Vitamin D status definitions	**Adults**		**Children**	
	Non-epimer resolved (no. of cases, % in cohort)	**Epimer resolved (n, Δ%)**	**Non-epimer resolved (no. of cases, % in cohort)**	**Epimer resolved (n, Δ%)**
<30 nmol/L, Deficiency	181 (24.7%)	194 (↑1.8%)	6 (3.8%)	23 (↑10.0%)
30-50 nmol/L, Insufficiency	245 (33.4%)	265 (↑2.6%)	36 (22.9%)	39 (↑0.4%)
>50 nmol/L, sufficiency	307 (41.9%)	275 (↓4.4%)	115 (73.2%)	105 (↓10.4%)

Table 4.4 Interpretation of vitamin D status in the adult (n=733) and paediatric (n=157) samples according to the U.S. Institute of Medicine guidelines. The number of cases classified as vitamin D deficient, insufficient and sufficient based on their respective serum 25(OH)D3 concentration. Resolving C3-epi-25(OH)D from 25(OH)D resulted in a reduction of the number of cases identified as vitamin D sufficient and increased the number of insufficient and deficient cases.

4.4 Discussion and conclusion

This chapter describes the development and validation of a LC-MS/MS method that utilised gradient chromatography by PFP bonded phase column to separate and quantify C3-epi-25(OH)D3/D2 and 25(OH)D3/D2 in human serum. Positive electrospray ionisation (ESI) coupled with tandem quadrupole MS/MS mass analyser provided sensitive detection of low concentration of C3-epimer present in serum, whereas the PFP column provided the selectivity to distinguish the structural difference of the epimeric form of 25(OH)D.

The fluorinated stationary phase in the PFP column interacts with the compounds based on multiple retention mechanisms, including hydrophobic interactions, π-π, dipole-dipole, H-bonding and shape selectivity, hence able to distinguish the epimeric form of 25(OH)D which proved problematic on C18 columns. The non-porous, solid core particles were capable of rapid column equilibration, thus enabling fast recovery of column back pressure. As a result, the total injection-to-injection run time of 13 minutes was achieved.

The assay satisfied the acceptance criteria for precision, linearity, extraction recovery and lower limit of quantification, as described by guidelines commonly adopted by the industry as standards of performance. Using SRM 972 traceable calibration standards ensures all significant components that may give rise to analytical measurement uncertainty are minimised; thus the components of uncertainty that do not contribute significantly to the bias of the test result can be neglected. The use of [$^2H_3$]-C3-epi-25(OH)D3 as internal standard further enhanced assay recovery and accuracy of the results produced.

Although the concentration of serum C3-epi-25(OH)D3 in both adult and paediatric samples were found to be low, it was present in the majority (87.4% and 87.7%) of the samples tested. The biological activity of C3-epi-25(OH)D3 remains unclear; Brown *et al.* had found C3-epi-25(OH)D3 can be converted to C3-epi-1,25$(OH)_2$D3 with similar potency as 1,25$(OH)_2$D3 in suppressing PTH section, but with lower calcaemic properties[17,94]. Many studies have reported a higher concentration of C3-epi-25(OH)D3 in infants and adolescent populations than in adults[89,95-98]. Findings from the paediatric samples collected in this study from patients in the Norfolk region echoed those reported in the publications; the median C3-epi-25(OH)D concentration was found to be 4-fold higher in the paediatric cohort than the adults, at a rate that is 2x higher than the difference observed in 25(OH)D. The relationship between C3-epi-25(OH)D and 25(OH)D exhibited in a concentration-dependent manner, in which greater concentration of C3-epi-25(OH)D3 were often found in higher concentrations of 25(OH)D3. However, the relationship between the two metabolites do not correlate in a linear fashion, and high inter-individual variability existed. A common observation made from the clinical information obtained on the adult samples with a concentration of C3-epi-25(OH)D3 >25 nmol/L revealed all the patients

were receiving vitamin D supplementation, though the exact dosage and frequency of intake were unclear. Upon review of publications found case reports of substantial serum levels of C3-epi-25(OH)D3 and C3-epi25(OH)D2 were found in patients taking high doses of over-the-counter cholecalciferol[26] and ergocalciferol[27]. A probable explanation is that some patients may have higher epimerase activity than found in most individuals, thus produce considerably more epimeric forms of 25(OH)D from vitamin D supplementation. The high inter-individual variability observed are likely to be influenced by the regulating genes and the level of enzyme expression/activity.

The clinical utility of C3-epi-25(OH)D3 remains to be clarified. However, given C3-epi-25(OH)D3 possesses a degree of biological activity, it would be beneficial to the interpreting clinicians to know the serum concentration of C3-epi-25(OH)D3 in their patients; either as a standalone measurement or inclusion into the 25(OH)D result as "total". The concerning issue is over the standardisation of 25(OH)D3 measurements across all clinical laboratories. Immunoassays are unable to determine the epimer forms of 25(OH)D, whereas chromatography methods (HPLC and LC-MS/MS) are able to distinguish or combine C3-epi-25(OH)D with 25(OH)D. The difference in the method's capability to detect C3-epi-25(OH)D will affect method harmonisation and therefore must be addressed as part of the ongoing effort to standardise measurements across all assay types.

The final objective of this study was to determine how resolving the epimer might change the interpretation of vitamin D status. In the adult samples, a small percentage of patients (4.4%) classified as vitamin D sufficient were re-classed as insufficient (2.6%) and deficient (1.8%) when C3-epi-25(OH)D3 is separated. A higher percentage was observed in the paediatric samples; 10.4% of patients were recategorised into a class below. More concerning is the increase in the number of cases identified as vitamin D deficient. The finding showed C3-epi-25(OH)D3 had resulted in an overestimation of patient's vitamin D status, particularly in the infant and paediatric population where the changes were considerably greater. It would be advisable for laboratories that provide routine vitamin D testing service to the paediatric population to quantify C3-epi-25(OH)D3 using the method as described in this study.

A major strength of the study is the large sample size used in the adult and paediatric cohorts. It allows observations to be made with confidence and subtle changes to be identified. Also, the use of LC-MS/MS, which has emerged as the 'gold-standard' methodology for 25(OH)D measurement, together with the use of reference-traceable standard materials and isotopic-labelled internal standards ensures the commutability of the result findings from this study to the broader scientific community. The limitations are that the two groups of samples were chosen

based on the age of the patients as the only inclusion criteria; influencing factors such as ethnicity, pregnancy, pathology and use of mediation/supplementation were not controlled.

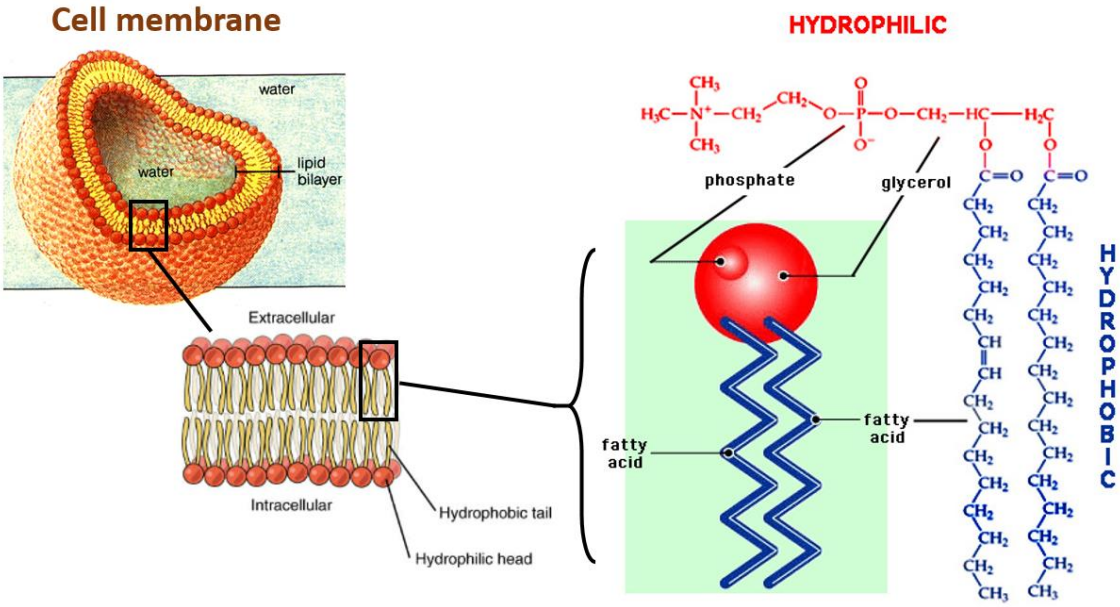
In conclusion, this chapter described a method for the sensitive detection and quantification of C3-epi-25(OH)D3 in human sera. The epimer form of 25(OH)D3 was found to be present in the majority of patient samples tested, and higher concentration was observed in the paediatric samples than in the adults. A concentration-dependent relationship exists between C3-epi-25(OH)D3 and 25(OH)D3, though with substantial variability between individuals. The study has highlighted the potential for non-epimer resolving methods to overestimate the vitamin D status of an individual, particularly in the paediatric population. Current clinical evidence does not provide sufficient support for routine C3-epi-25(OH)D3 measurement as part of the vitamin D status assessment. The described LC-MS/MS method would facilitate future studies into the physiological importance of the epimeric forms of 25(OH)D.

Chapter 5 Improved LC-MS/MS method for measurement of serum 25(OH)D using phospholipids depletion and derivatisation techniques

5.1 Background

In recent years, the field of Clinical mass spectrometry has been making fast progress, Liquid chromatography tandem mass spectrometry (LC-MS/MS) has now become the preferred method for routine hospital and pharmaceutical industry for analysis of biological fluids. Analysis of endogenous biological compounds poses many challenges; the complex nature of sample matrix, low analyte concentration, potential interference from other similar endogenous and exogenous components within the sample matrix, all of which require a high level of theoretical knowledge and practical skills and application in liquid chromatography and mass spectrometry techniques to tackle these issues. Instrument manufacturers offer powerful and ultra-sensitive LC-MS/MS equipment but at high prices that are often out of reach for most hospital and academic research laboratories. Alternative strategies to gain analytical sensitivity can be through reducing interference from sample matrix (i.e. background noise) and enhancing compound signal strength, resulting in a net increase in signal-to-noise ratio.

Phospholipids are highly abundant in biological membranes; their strong presence in the biological matrix can be a significant source of analytical errors[99]. Phospholipids are a major component of all cell membranes forming the lipid bilayers (Fig.5.1). It is composed of ester or amide derivatives of glycerol (phosphoglycerides) or sphingosine (sphingomyelins) with fatty acids and phosphoric acid. The structure constitutes a hydrophilic phosphate head and one or two hydrophobic fatty acid ester tails. The choline-esterified form called phosphatidylcholine, commonly referred to as lecithin was one of the first phospholipid identified in biological tissue, and considered the dominant form of phospholipids in plasma that cause significant matrix ionisation effects during LC-MS/MS analysis[100]. Lysophospholipid is referred to any phospholipids with one fatty acid chain; whereas sphingomyelin is composed of an amide-linked fatty acid, and a phosphatidylcholine (Fig. 5.2).



Sources: http://biology4alevel.blogspot.ro/2014/08/10-lipids.html
https://biologydictionary.net/semipermeable-membrane/

Figure 5.1 Schematic illustration of a phospholipid.

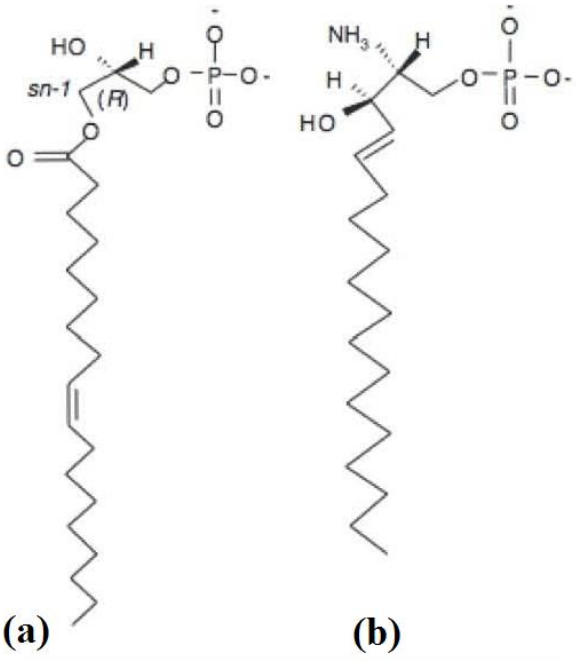


Figure 5.2a-b Structure of **(a)** Lysophosphtidic acid and **(b)** Sphinogsine-1-phosphate.

Endogenous phospholipids in biological fluids present a major problem in LC-MS/MS analysis. Due to their strong retention characteristics in reverse phase chromatography, phospholipids tend not to elute as discrete peaks and are often very difficult to separate from analytes of interest. This co-elution often leads to areas of suppression or enhancement in the chromatogram, that in turn can cause analytical errors. The Guidance for Industry on Bioanalytical Method Validation published by the U.S. Food and Drug Adminstration[81] states that "Matrix effects on ion suppression or enhancement or on extraction efficiency in LC-MS and LC-MS/MS based procedures should be addressed." However, the specific methods to evaluate matrix effects are left to the discretion of the investigator. In this regard, many researchers have described methods to study and control matrix effects[100-105]. Numerous approaches have been presented in the literature mostly involving the use of solid phase extraction (SPE), such sample clean-up techniques provide effective removal of phospholipids and other interfering compounds, but the procedures are often time-consuming and involve the use of expensive extraction columns and solvents. Recently, new sample phospholipids depletion tools in a 96-well plate format have become commercially available, offering fast and effective matrix clean up at a fraction of the cost of SPE. The first objective of this study was to investigate ways to detect and monitor phospholipids in serum samples and to develop sample clean-up procedures using plate-based phospholipid depletion tools for LC-MS/MS analysis of 25(OH)D3.

The second objective was to study the use of chemical derivatisation technique to enhance the ionisation strength of 25(OH)D3. The sensitivity or detectability of a compound depends upon the number of ionisable groups available in the analyte. The hydrophobic nature of 25(OH)D with two hydroxyl groups (Chapter 1 fig. 1.0) meant ion generation in the harsh LC-MS/MS environment is often suboptimal and result in loss of water (Chapter 4 figure 4.3). Shimizu et al. first reported a chemical derivatisation method for 25(OH)D using a Cookson-type dienophilic agent. It reacts to the s-cis-diene moiety of vitamin D via the Diels-Alder reaction; forming a conjugated diene derivative for detection by HPLC with a fluorometric detector[45]. Derivatisation enhances the MS detection by increasing the molecular weight of the analyte, moving it away from the low molecular weight compounds, and increases the number of reactive functional groups available for ionisation and fragmentation.

This chapter presents the development of methods for eliminating phospholipids and sample derivatisation for measurement of 25(OH)D3. The newly developed method was evaluated for the improvement shown in the quality of the results produced.

5.2 Materials and methods

5.2.1 Serum samples

All fresh and stored serum samples used for method development, comparison and storage studies were obtained from clinical research studies with ethical approvals. All samples were anonymised at the point of access and processed in accordance with generic ethical approval for assay development[80]. Method comparison was conducted using serum samples previously analysed for 25(OH)D3 and 25(OH)D2 by isotopic dilution protein precipitation LC-MS/MS method (described in Chapter 3.32) and then retested by the new methods. Aliquots of serum sample were stored at -20°C unless obtained fresh. Before each batch of analysis, samples were defrosted at room temperature, mixed and centrifuged at 2000 x g for 10 minutes.

5.2.2 Materials and preparation of calibration standards, controls and samples.

Preparation procedures are detailed in Chapter 3. In brief, isotopic dilution protein precipitation method was performed using zinc sulphate and acetonitrile (Chapter 3.3.2). Depletion of phospholipids was performed using Phree™ (Phenomenex, Torrance, USA) in 96-well format (Chapter 3.3.3) and Isolute® SLE+ Supported Liquid Extraction plate (Biotage, Uppsala, Sweden) with bed mass of 400 µL (Chapter 3.3.4). 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) and methylamine were used for derivatisation and adduct formation (Sigma-Aldrich, Dorset, UK). Preparation of 25(OH)D3 calibration standards, quality control materials, and isotopic-labelled internal standards are as described in Chapter 3.1.2-5.

5.2.3 Detecting phospholipids in samples

Phospholipids transitions were acquired in positive electrospray ions (ESI+) multiple reaction monitoring (MRM) mode using common product ion trimethylammonium-ethyl phosphate (m/z 184). The 18 most abundant phospholipid precursor ions (Table 5.1) were identified from the literature and chosen in this study.

Sample clean-up procedures developed using Phree phospholipid depletion plate and SLE are described in Chapter 3.3.3 and 3.3.4. Sample extraction was carried out on a PRESSURE +96® positive pressure manifold (Biotage, Uppsala, Sweden) with nitrogen gas supplied from a nitrogen generator (Peak, Scotland, UK) at a flow rate of 30 L/min. Sample evaporation was carried out on a microplate evaporator (Evaporex, Apricot designs, Ca, USA) distributing heated nitrogen gas at 50˚C via a stainless steel needle assembly into the deep well plate.

m/z transitions			
Lysophospholipids	Phospholipids		
494.4 > 184	184 > 184	758.5 > 184	786.6 > 184
496.4 > 184	701.7 > 184	786.6 > 184	806.6 > 184
520.4 > 184	703.7 > 184	760.5 > 184	808.7 > 184
522.4 > 184	732.8 > 184	784.6 > 184	810.9 > 184
524.4 > 184	756.5 > 184		

Table 5.1 Phospholipids m/z transitions.

5.2.4 Sample derivatisation with PTAD

PTAD was prepared by placing 1g of 4-phenyl-1,2,4-triazoline-3,5-dione into 500 mL of acetonitrile. Derivatisation took place by adding 50 μL of PTAD in acetonitrile solution into dried down samples. The plate was vortexed and allowed to incubate for 30 minutes at room temperature in the dark. 50 μL of LC-MS grade water was added and mixed to stop the reaction.

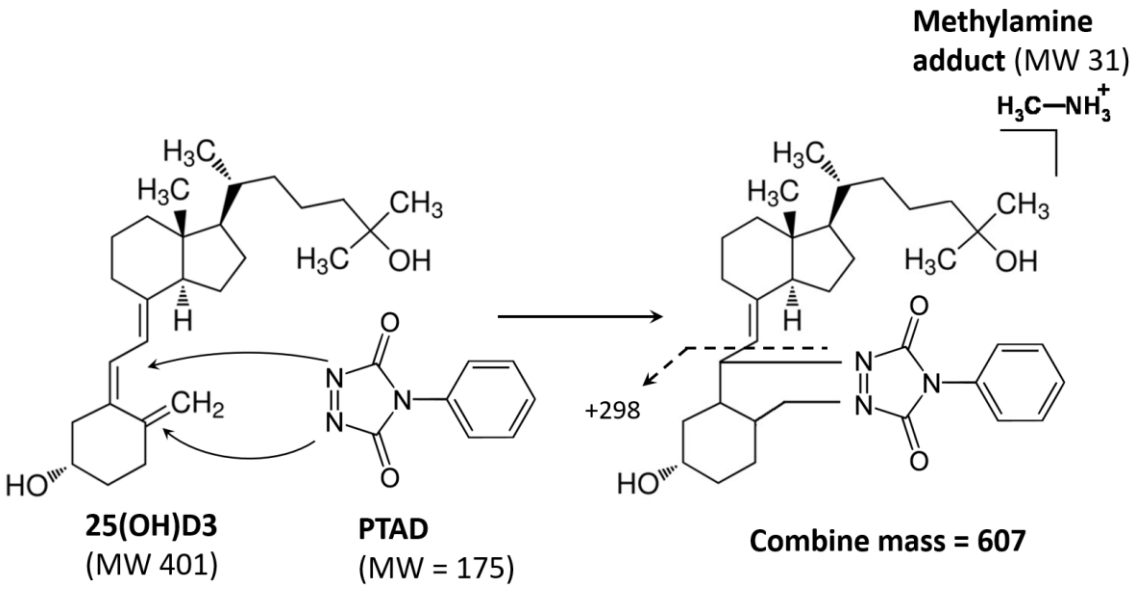


Figure 5.3 The dienophilic reaction of 25(OH)D3 with PTAD and formation of methylamine adduct.

5.2.5 Liquid chromatography Tandem mass spectrometry

Analytical equipment and instrumentation conditions for non-derivatised methods were unchanged from the details described in Chapter 3.2. For PTAD derivatised method, liquid chromatography was performed on reverse phase chromatography using a UHPLC binary pump (Flux Instruments, Switzerland) which delivered the mobile phases at a flow rate of 0.4 mL/min through a core-shell C18 50 × 2.1mm, 2.6 µm column (Restek, PA, USA) that was heated to +55°C. Chromatographic separation was performed using a solvent gradient which began at 50:50 (v:v) of water containing 0.2 mM methylamine in 0.1% formic acid (A) and methanol containing 0.2 mM methylamine in 0.1% formic acid (B). Mobile phase B was steadily increased to 99% over 2 minutes 10 seconds then returned to the original conditions after three minutes. Under these conditions, the 25(OH)D3 peak was eluted at 1.75 minutes. Micromass® Quattro Ultima™ Pt tandem mass spectrometer (Waters Corp., Milford, MA, USA) operating in multiple reaction monitoring was used to analyse the samples with precursor to product transitions, 607>298 for 25(OH)D3 and 613>298 for 25(OH)D3-[$^{2}H_{6}$] (Fig. 5.3) (Table 5.2). MassLynx version 4.1 and QuanLynx were used for data acquisition and peak integration.

Parameters	**Setting**
Ion source	Electrospray positive
Capillary voltage	3.0 kV
Cone energy	35 kV
Collision energy	20kV
Nebuliser gas flow rate	30 L/hr
Desolvation gas flow rate	850 L/hr
Source temperature	85°C
Desolvation gas temperature	250°C

Table 5.2 Mass spectrometer settings.

5.2.6 Statistical analysis

Data exploration, bar charts and box-whisker plots were performed using GraphPad Prism 8 (GraphPad, San Diego, CA, USA). Passing-Bablok regression generated linear equations were used to derive the PEVs from raw DBS and VAMS values. Method comparisons were assessed using Passing-Bablok regression and residuals in Bland-Altman plots. A correlation is deemed satisfactory if the regression produced an $r^2 \geq 0.9$.

5.3 Results

5.3.1 Phospholipids in human sera

An aliquot of human sera was extracted by isotopic dilution protein precipitation procedure, phospholipids and lysophospholipids were eluted using LC gradient conditions and MS/MS parameters optimised for 25(OH)D3/D2. Solvent divert valve was disabled to allow acquisition of the entire LC separation and all 18 phospholipid transitions to be recorded. Results showed phospholipids were present throughout the trace (Fig. 5.4), gradient elution from 88% methanol:water increased to 100% on a reverse phase C18 analytical column failed to resolve phospholipids into a distinct peak; no clear phospholipid-free region can be identified. The majority of lysophopholipids were eluted in the first 2 minutes of the gradient, followed by phospholipids. The late retention of phospholipids is due to the hydrophobicity of the two fatty acid chains present.

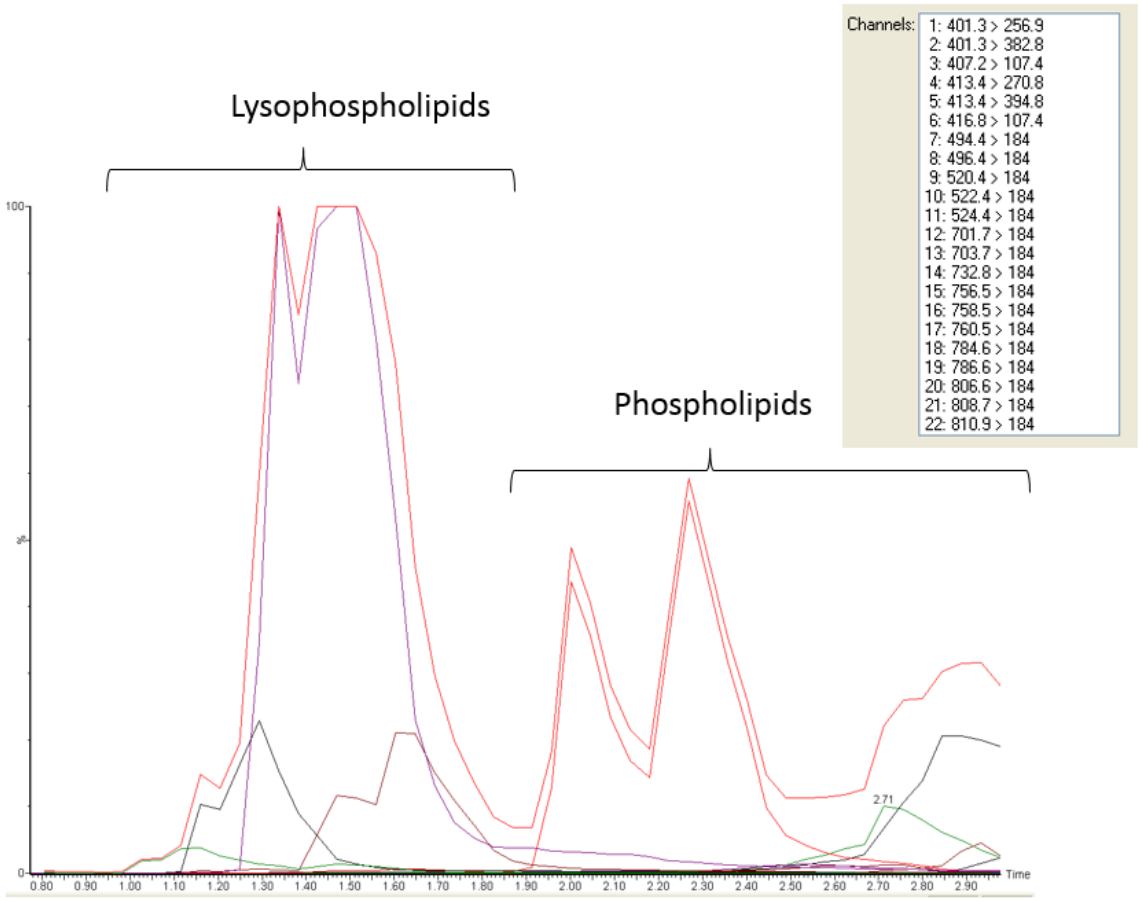


Figure 5.4 Elution profile of phospholipids and Lysophospholipids in LC-MS/MS analysis of a protein-precipitated human serum.

To monitor 18 phospholipids ion transitions in every MRM experiment is not practical. To acquire such a large number of transitions would also decrease instrument sensitivity. For that reason, the most abundant phospholipid transition m/z 522 > 184 that co-eluted with 25(OH)D3 and 25(OH)D2 was chosen to be the monitoring transition in all future sample analysis. This approach allowed phospholipids to be monitored by just one transition in an MRM experiment without affecting sensitivity. An example of a human serum sample extracted by isotopic dilution protein precipitation and then analysed by LC-MS/MS is shown (Fig.5.5).

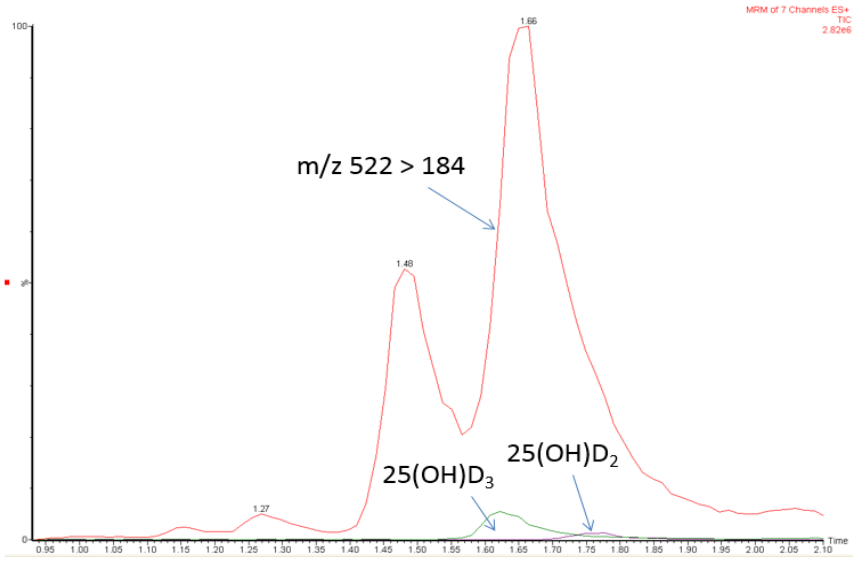


Figure 5.5 Chromatographic trace showing elution of lysophospholipids transition m/z 522.4 > 184 during the separation of 25(OH)D3 and 25(OH)D2.

5.3.2 Phospholipids in long term storage samples

Fresh serum samples and samples stored for up to 72 months at -20˚C (n = 140) were analysed retrospectively using isotopic dilution protein precipitation method with lysophospholipids monitored using *m/z* transition 522 > 184 (Fig 5.6). The month 72 samples were re-tested using SLE+ extraction plates.

Results showed an upward trend in phospholipids peak area count with the increasing length of sample storage. A near tenfold increase was observed in serum samples stored for 6, 12 and 18 months compared with fresh samples; whereas an average 90-fold increase was observed in 24 samples and ten thousand-fold increase in phospholipids were observed in 48 and 72 months samples. Re-analysis of the month 72 samples using SLE+ extraction plates showed a reduction of the phospholipids peak area count similar to those observed in fresh samples.

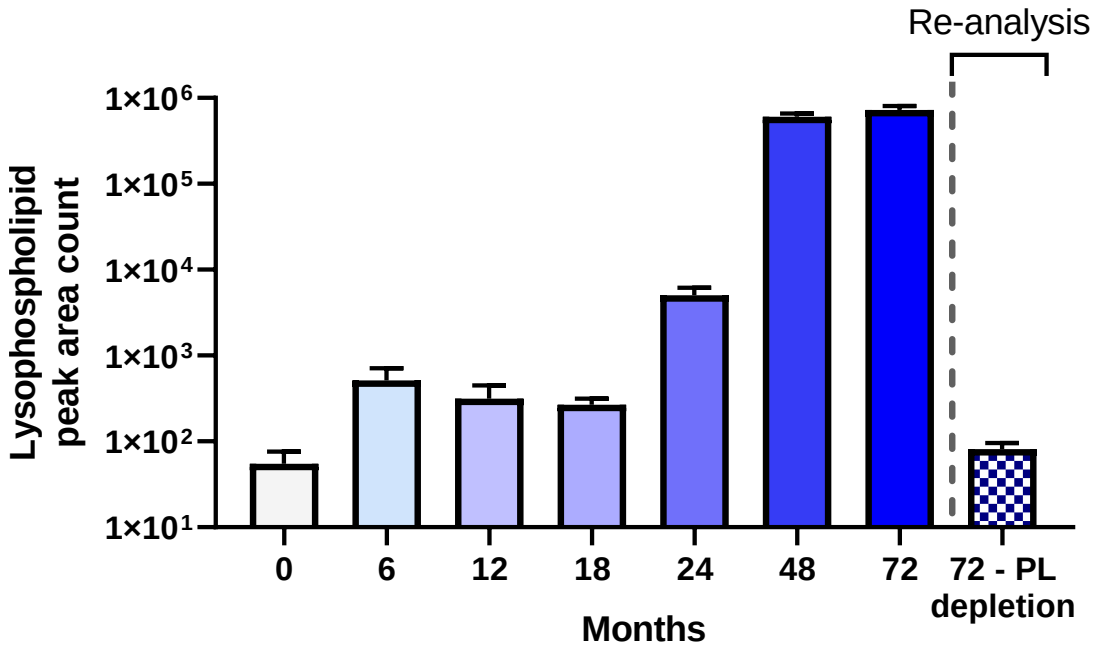


Figure 5.6 Bar chart showing the median and 95% CI of lysophospholipids present in serum samples stored up to 72 months. Month 0 to 72 samples were analysed using isotopic dilution protein precipitation method with lysophospholipids transitions monitored at 522>184. Month 72 samples were re-tested using phospholipid depletion method (SLE), a reduction of lysophospholipids was observed.

5.3.3 Phospholipids depletion method and the effects on analyte recovery

The first phospholipids depletion procedure was developed using Phree extraction plates incorporating the isotopic dilution protein precipitation method. During initial experiments, in-plate precipitation was trialled where human sera, zinc sulphate and acetonitrile internal standard mixture were placed directly into the 96-wells (Fig 5.7a). The mixture in the plate was vortexed vigorously for 10 minutes before eluted off by positive pressure using nitrogen gas. The procedure was simple to perform and produced clean extracts. LC-MS/MS analysis showed complete elimination of Lysophospholipids. However, the procedure failed to achieve adequate recovery for 25(OH)D3; peak shapes were poor and baseline noise was high. Repeat experiment found the viscosity of serum to be a key factor affecting sample flow through. Therefore, a sample dilution step with 60% (v/v) of acetonitrile/internal standard mixture in water solution was introduced to increase the solubility of the loading mixture. A neat acetonitrile wash step was also added to improve analyte recovery before injection into LC-MS/MS (Fig 5.7b).

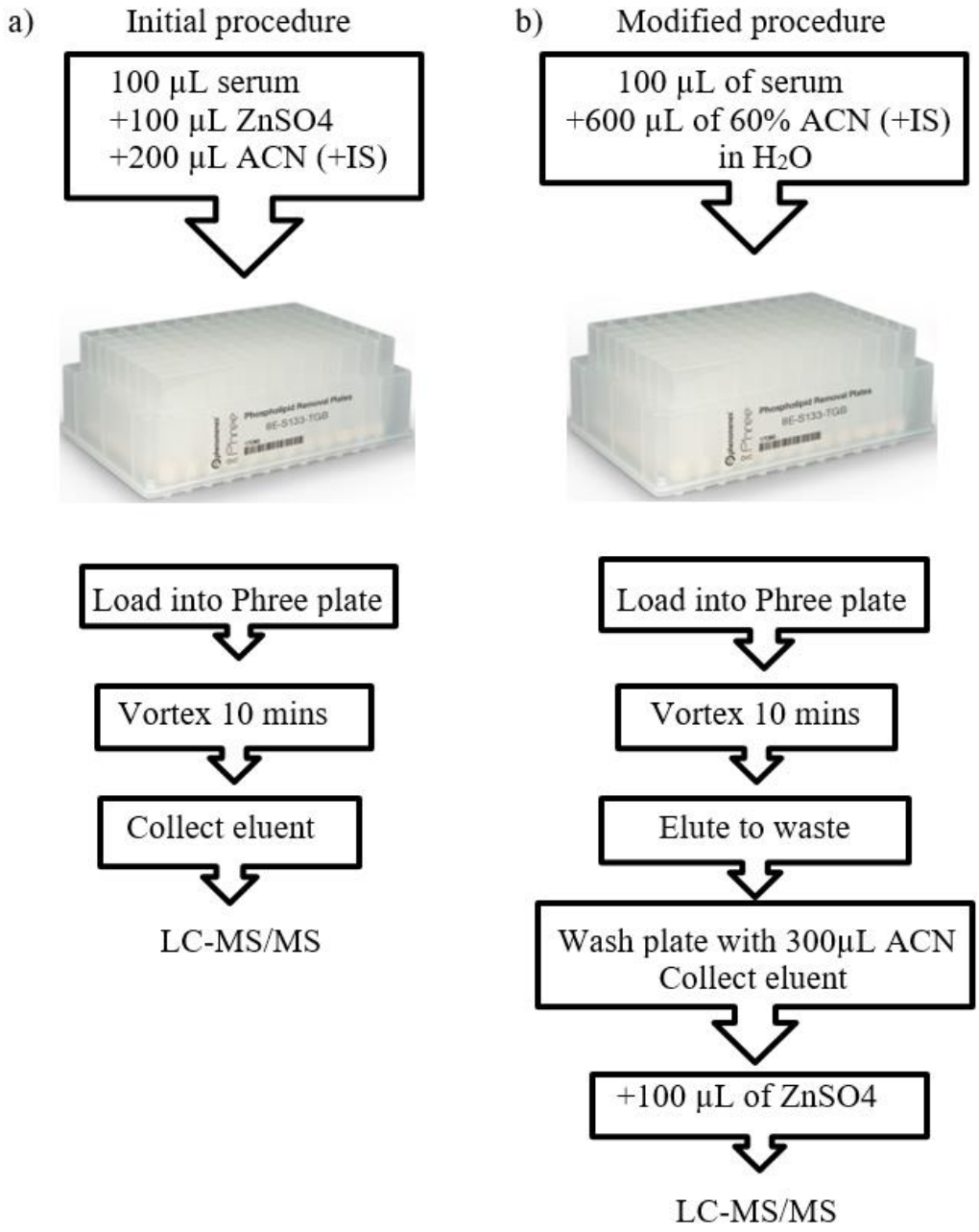


Figure 5.7a-b Schematic presentation of the initial and modified procedures using the Phree phospholipid depletion plate.

Figure 5.8 and 5.9 demonstrate the impact of phospholipid depletion and improvement observed in the analyte peak signal. Using the Phree plate, lysophospholipid was eliminated and a tenfold increase in 25(OH)D3 peak area count compared with isotopic dilution protein precipitation method was observed. A reduction in background noise was found in the Phree-extracted samples. In the long-term, removing phospholipids from samples will reduce contamination on the LC

column and MS source, allowing for longer intervals between instrument maintenance and reduce the risk of system downtime.

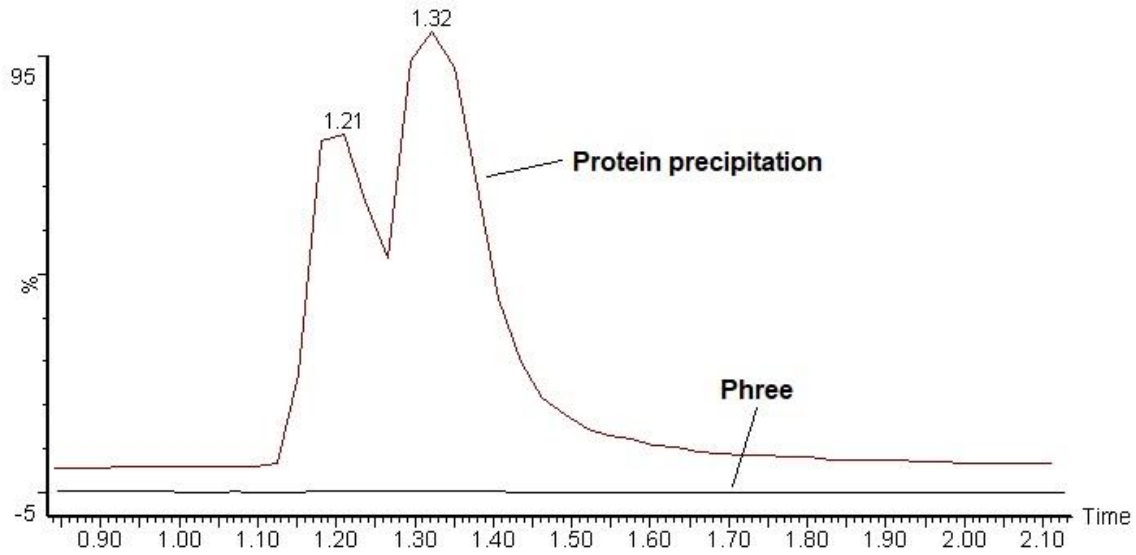


Figure 5.8 Lysophospholipid trace monitored at m/z 522>184 was observed from a sample prepared by isotopic dilution protein precipitation method. It is eliminated by the Phree method.

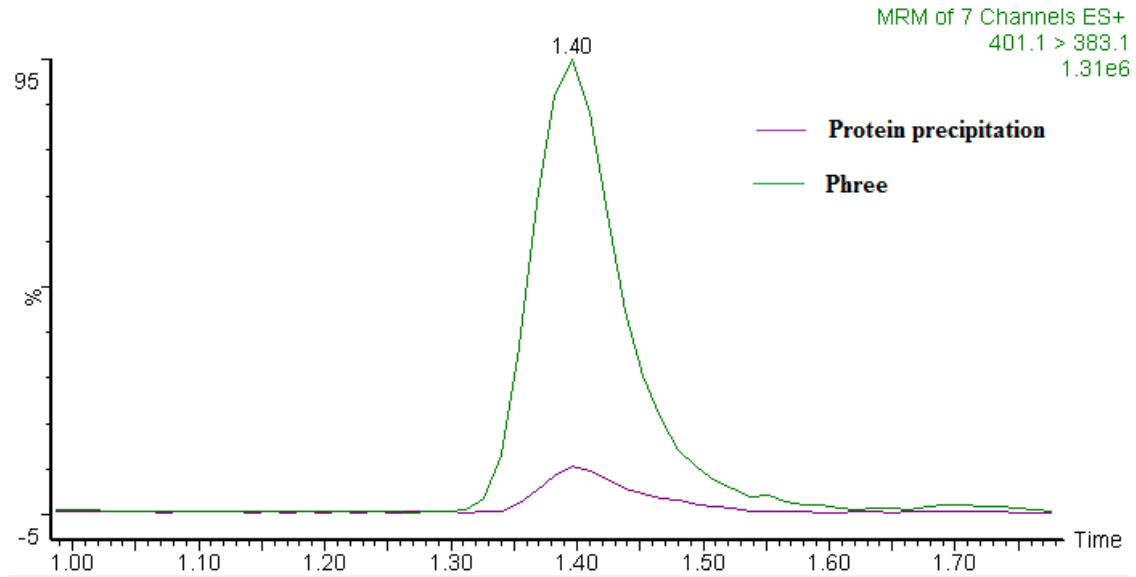


Figure 5.9 An overlap of two 25(OH)D3 peaks produced by Phree and protein precipitation procedure.

5.3.4 Measurement of 25(OH)D3 using SLE and comparison with isotopic dilution protein precipitation method.

A second phospholipids depletion procedure was developed using SLE+ extraction plates. SLE was able to eliminate phospholipids and improve analyte peak signal as shown using the Phree plate. It was easier to perform as SLE and do not require a sample wash step and in-plate precipitation. SLE was therefore chosen to be the method for validation. Standard curves for 25(OH)D3 were analysed using Chromsystems calibration standards. Over the 13 test batch performed, the calibration curves were linear with regression r^2 value of >0.998. Intra-assay imprecision on repeat measurement of IQC (n = 6) showed %CV of <6% across the concentration range. Inter-assay imprecision determined by analysis of 13 runs of the tri-level IQC showed %CV (mean concentration, ±SD) for 25(OH)D_3 were 7.3% (45 nmol/L, ±3.3) and 9.0% (100.3 nmol/L, ±9.0), respectively. The method participated in the DEQAS scheme between 2014-2015, results produced by this method were within ±15% of target value (Fig. 5.10) and met the performance target set by the DEQAS advisory panel for proficiency certification.

Method comparison was carried out using 135 fresh serum samples previously analysed for 25(OH)D3 by isotopic dilution protein precipitation method. The comparison between the SLE and isotopic dilution protein precipitation methods showed good correlation with the line of best fit generated a slope of 1.0151 and r^2 value of 0.9785 (Fig. 11a) Bland-Altman plot demonstrated a small negative bias of -0.1% produced by the SLE method (Fig. 11b).

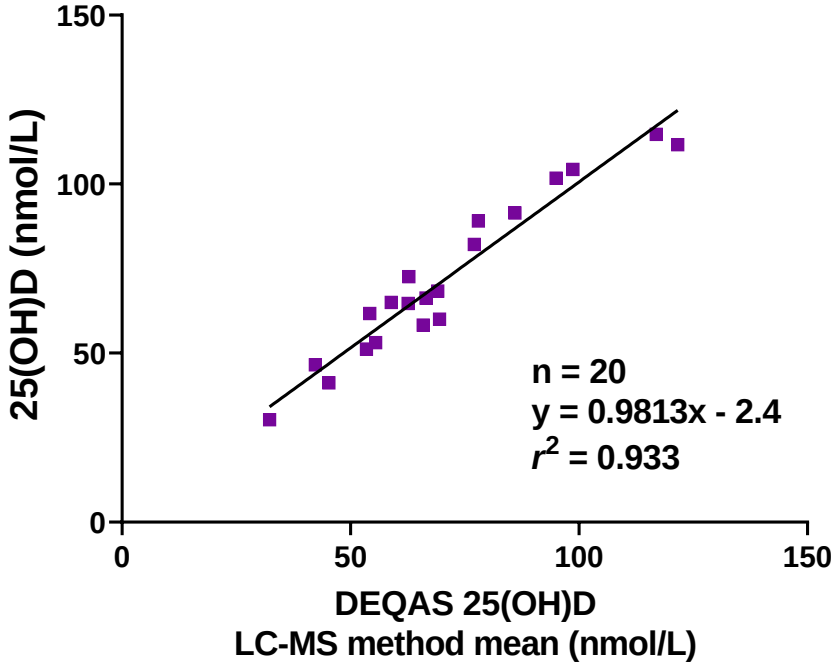


Figure 5.10 Comparison of DEQAS returns with the LC-MS method group mean.

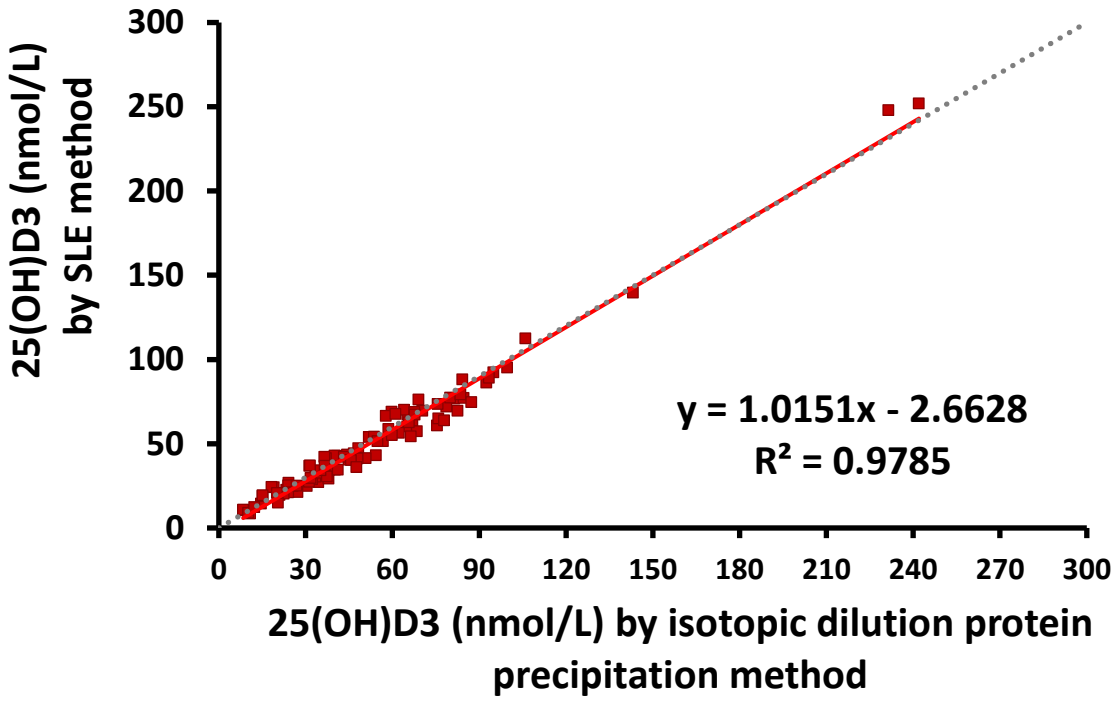


Figure 5.11a Comparison of 25(OH)D3 concentrations on fresh samples (n =135) measured using SLE and isotopic dilution protein precipitation methods. Linear regression is represented by the solid red line, the dashed grey line represents the line of identity (y = x).

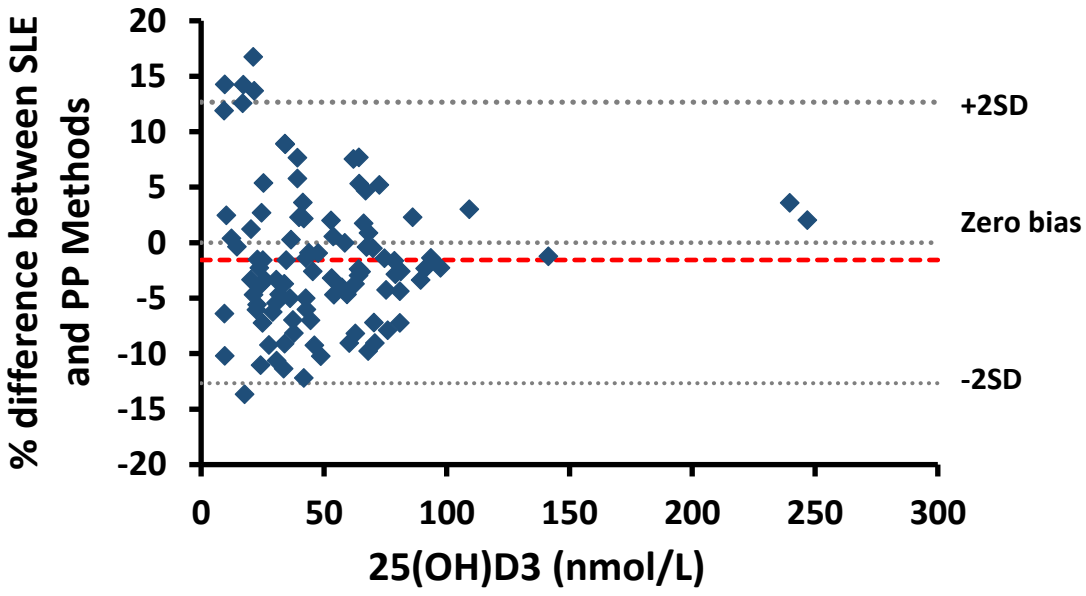


Figure 5.11b Bland-Altman plot showing the percentage bias between the two methods. The dashed red line represents the average bias of -0.1%. The dashed lines represent zero bias and ±2SD limits of agreement.

A parallel comparison was performed on 158 serum samples stored at -20°C for 5 years (Fig 5.12). Samples were analysed by SLE and isotopic dilution protein precipitation methods, results were compared against the original values obtained when the sample was fresh. Protein precipitation method showed 32% positive bias against the original values ($p < 0.001$) (Fig 5.13a).; whereas SLE method did not show a significant difference (Fig 5.13b). It was concluded that the bias was caused by the high level of phospholipids present after prolonged storage.

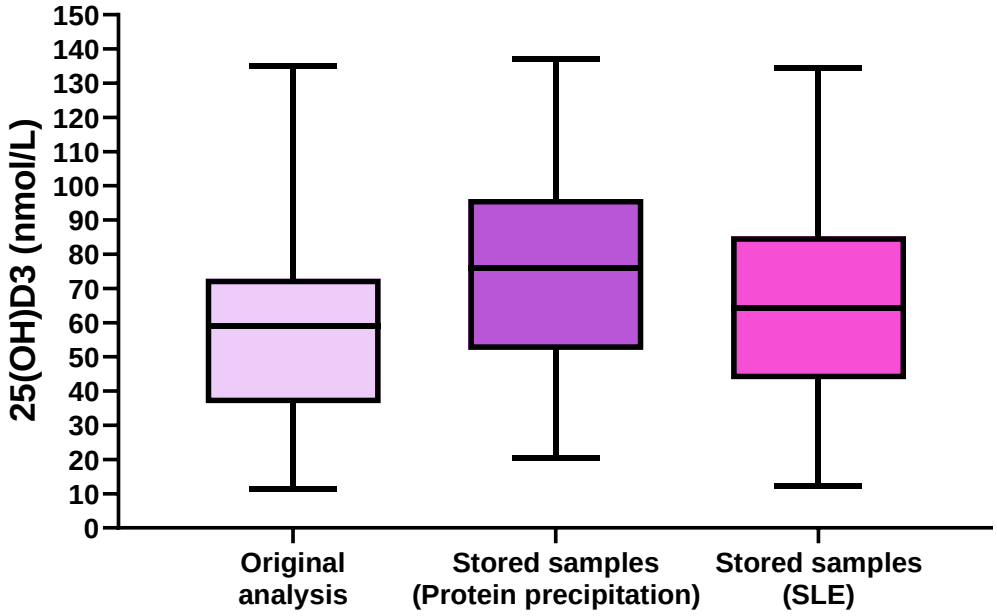


Figure 5.12 Box-whisker plot comparing the difference in the distributions of 25(OH)D3 values originally obtained fresh and re-analysed by phospholipids depletion (SLE) and non-phospholipids depletion (protein precipitation) methods after five years of storage. Box represent median and interquartile; whiskers represent the minimum and maximum range.

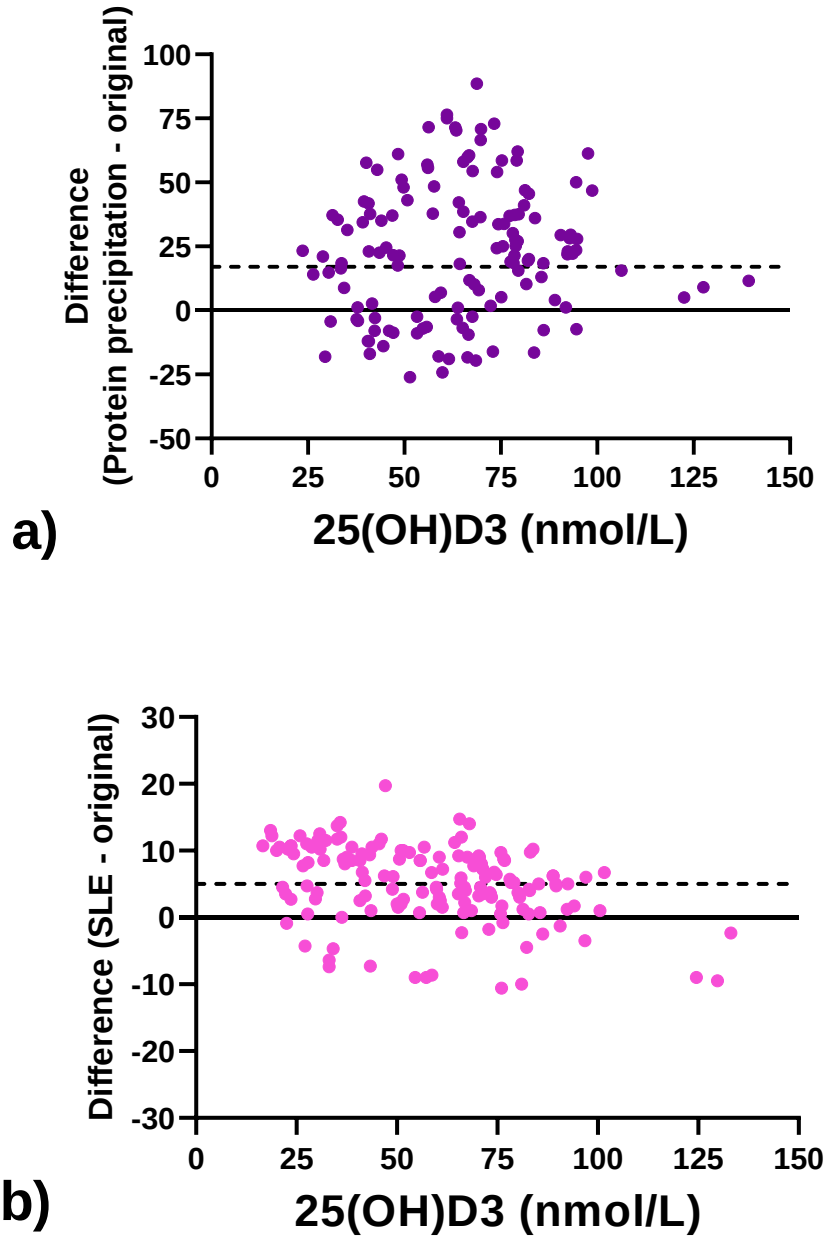


Figure 5.13a-b Bland-Altman plots showing the difference in 25(OH)D3 concentrations between **(a)** protein precipitation, **(b)** SLE methods with the values obtained in the original analysis. The dashed line represents the average bias.

5.3.5 Combining SLE with PTAD derivatisation

The SLE method (Fig. 5.14a) was further modified to incorporate a PTAD derivatisation step (Fig. 5.14b). LC-MS/MS analysis of a serum sample showed the SLE with PTAD derivatisation produced a 25(OH)D3 peak area in the $x10^6$ region; increase in an order of magnitude compared with SLE without derivatisation. The preparation and extraction procedure were programmed into the Extrahera™ automation system (Biotage, Uppsala, Sweden) to enable high-throughput processing. Method validation results are described in Chapter 6 and 7.

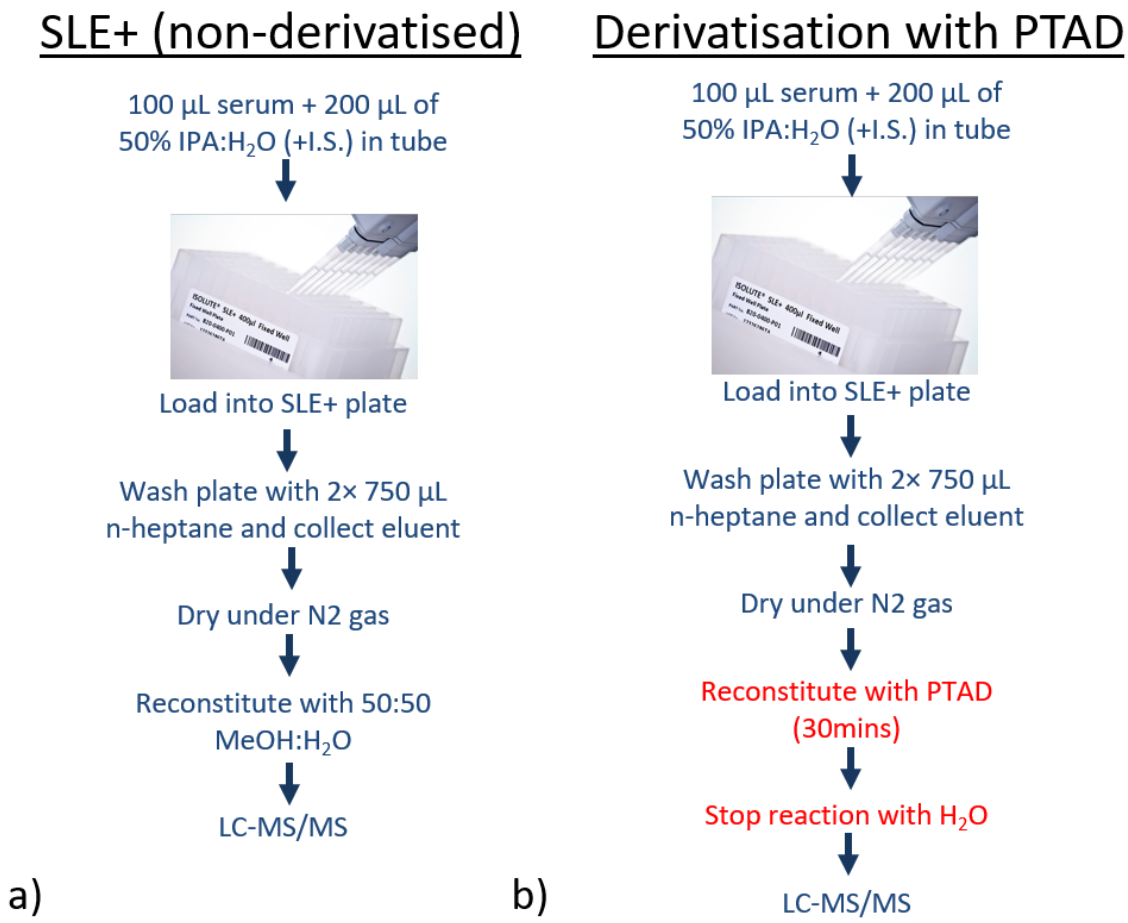


Figure 5.14a-b Schematics of the sample preparation procedure for **(a)** the first SLE method developed and **(b)** modified with PTAD derivatisation steps added.

5.4 Discussion and conclusion

Phospholipids are a major source of analytical interference in LC-MS/MS methods. Isotopic dilution protein precipitation may be a quick and easy sample preparation technique to perform, but it is not effective in removing phospholipids from human serum matrix. The results have shown that the high abundance of phospholipids accumulated with prolonged storage can have a profound effect on 25(OH)D measurements. In this study, two sample phospholipids clean-up procedures for the application of 25(OH)D3 quantification were successfully developed. Both SLE and Phree methods were found to be robust for routine use and can be automated with the use of a semi-automated plate processor to increase analytical precision and throughput. The SLE method had few steps than Phree and therefore chosen as the base method of further modification.

Due to the lipophilic nature of 25(OH)D, it is not possible to separate lipids in a reversed phase chromatography system. Reduction of phospholipid content was necessary in order to reach the low circulating concentration of vitamin D metabolites. SLE is a new sample preparation tool that is similar to traditional liquid-liquid extraction (LLE). Each well is packed with a modified form of diatomaceous earth. Serum samples were pre-treated with 1:2 sample/aqueous mixture of propan-1-ol and water 50% (v/v). Propan-1-ol disrupts and releases 25(OH)D from binding protein; whereas water increases the solubility of the serum, allowing the diatomaceous earth layer to retain the aqueous portion, leaving serum behind in a simple partition. A number of organic solvents were investigated in the elution step including acetonitrile, methyl *tert*-butyl ether and dichloromethane. n-heptane was found to give cleaner extract and higher recovery and was therefore selected as an optimum solvent for elution. A combination of single, double and triple washing steps was also investigated; double washing with 750µL of n-heptane was found to be sufficient to elute 25(OH)D from the plate. Analysis of the third eluent collection confirmed the absence of residual analyte.

Derivatisation with PTAD greatly enhanced the sensitivity of the assay. Vitamin D metabolites have low ionisation efficiency and readily dissociate into water loss product ions at low collision energy. The advantage of the PTAD derivatisation is that it adds proton-affinitive oxygen and nitrogen molecules (Fig. 5.3) to the structure of vitamin D, increasing the ionisation potential. Derivatisation shifts the compound to a higher mass range, where background interference from low molecular weight species is relatively low. The combination of SLE with PTAD derivatisation had substantially improved assay sensitivity. It is possible to detect 25(OH)D in samples with sub-10 nmol/L concentrations that were previously undetectable with isotopic dilution protein precipitation method, suggesting better analyte recovery. Also notable was the increase in the presence of 25(OH)D2 in samples. 25(OH)D2 is thought to have quicker clearance than 25(OH)D3[106,107] and lower tissue bioavailability[108]; hence serum concentrations of the plant-

derived vitamin D is much lower than 25(OH)D3. Serum concentration 25(OH)D2 in non-supplemented individuals are often close to the lower limit of the analytical range that it is undetectable in the majority of clinical specimens. Improvement in assay detection and quantification of 25(OH)D3 and 25(OH)2 achieved by using the newly developed phospholipid depletion methods will provide better quality results for the assessment of vitamin D status. Individuals with total 25(OH)D <30 nmol/L and <50 nmol/L as defined by the Institute of Medicine (IOM)[11] as the thresholds for vitamin D deficiency and insufficiency may be reinterpreted as such the consequence may impact on the clinical management of patient treatment.

In conclusion, this chapter describes a series of method developments on sample preparation to enhance LC-MS/MS detection of 25(OH)D3. Sample clean-up using supported liquid extraction (SLE) is highly effective for the removal of proteins and phospholipids. Derivation with a Cookson-type dienophilic agent increased the ionisation efficiency and greatly enhanced the peak signal.

Chapter 6 Development and validation of an LC-MS/MS method for 25-hydroxyvitamin D3 in dried blood samples using a volumetric microsampling device

6.1 Background

The high prevalence and the increasing awareness of vitamin D deficiency as a public health concern have led to an increase in demand for measurement of 25-hydroxyvitamin D (25(OH)D)[109]. Many laboratories offer services directly to the general public for the assessment of vitamin D status[110,111]. The use of finger prick dried blood spot (DBS) microsampling techniques for general wellness assessment and in clinical diagnostics has gained popularity as an alternative to venous sampling.

DBS microsampling techniques are commonly used in epidemiological studies and clinical/pre-clinical pharmacokinetic studies[112]. In hospital laboratories, DBS analysis is routinely performed for screening programmes such as neonatal inborn errors of metabolism[113]. DBS sampling is a convenient and less invasive blood sampling method than venepuncture; whilst the low sample volume requirement (typically 10-50 μL) is ideally suited for use in paediatric practice and elderly population[114]. A phlebotomist or nurse would not be required to perform venepuncture for patients who are able to self-sample; reducing time and the inconvenience of attending hospital appointments. The reduced storage and shipping requirements allow patients to send their samples via the post, which could streamline the process of transporting samples to the laboratory and improve efficiency. Also, dried blood samples carry a lower risk of infection from pathogens than a wet sample as some viruses are deactivated on the filter paper due to disruption of the viral envelope[115].

There are limitations that have prevented the widespread use of DBS sampling techniques; difficulty in controlling the amount of blood applied onto the filter paper and the level of haematocrit (Hct) present can affect the spot size. The viscosity of the blood sample can influence the dispersion on the filter paper; low Hct samples can disperse more easily on the filter paper than those with high Hct, creating a larger spot size[116]. Variability in analyte concentrations between central and peripheral punches has been reported[117], which could reduce the accuracy of measurements when sub-punches are taken[118].

This chapter describes a liquid-chromatography mass spectrometry (LC-MS/MS) method for measurement of 25-hydroxycholecalciferol (25(OH)D3) in dried blood samples. Measurements of 25(OH)D3 from blood samples collected by Mitra® volumetric absorptive microsampler (VAMS™) (Neoteryx, Torrance, CA, USA) were compared against a conventional paper-based method against plasma concentrations. LC-MS/MS is considered the gold standard technique for the measurement of vitamin D metabolites due to its high sensitivity and specificity[119], hence the method of choice in this study.

6.2 Materials and methods

6.2.1 Serum sample collection

Whole blood samples collected in K3EDTA vacutainer tubes (Becton Dickinson, Oxford, UK) from 167 patient samples were selected at random from routine full blood count requests at the Department of Laboratory Medicine, Norwich and Norfolk University Hospital. Blood samples were stored at room temperature. All samples were anonymised at point of access and processed in accordance with generic ethical approval for assay development[80]. The plasma samples were obtained by centrifugation for 10 minutes at 3,000x g. Plasma was aliquoted into a polystyrene tube and stored at -20°C until analysis.

6.2.2 Materials, calibration standards and controls

LCMS grade water, formic acid, methanol, isopropanol (IPA) and analytical grade n-heptane were purchased from Fisher Scientific (Loughborough, UK). 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) and methylamine (Sigma-Aldrich, Dorset, UK) were used for derivatisation and adduct formation. Whatman® 903 Protein saver cards and Harris UniCore 3.0mm puncher were purchased from GE Healthcare (Cardiff, UK). Mitra® VAMS™ were provided by Neoteryx (Torrance, CA, USA). ISOLUTE SLE+ plates were purchased from Biotage (Uppsala, Sweden). Certified 25(OH)D3 and 25(OH)D3-[$^{2}H_{6}$] standards were purchased from IsoSciences (PA, USA).

Preparation of whole blood 25(OH)D3 calibration standards and quality controls

Matrix-matched calibration standards were prepared from certified standards spiked into vitamin D-free whole blood. Vitamin D-free whole blood was prepared in-house from washing packed cells using 0.9% saline solution; a 50 mL pool of EDTA whole blood was centrifuged at 1500 x

g for 15 minutes, the plasma layer was removed and replaced with an equal volume of 0.9% saline solution. The packed cells were washed with the saline on a carousel rotator for 20 minutes and then centrifuged again under the same conditions. This procedure was repeated three times. After the final wash, the saline layer removed was replaced with vitamin D depleted serum (BBI solutions, Cardiff, UK) and made up to the volume equivalent to the original discarded plasma. Certified 25(OH)D3 standards were spiked into vitamin D-free whole blood to concentrations of 125, 100, 75, 50, 25 and 17 nmol/L. All the standards including a sample of the blank (vitamin D-free whole blood) were analysed in singleton in each run.

Blood spot and VAMS microsampling procedure

Whole blood samples were thoroughly mixed before microsampling to ensure homogeneity. For VAMS sampling, the 10 µL fixed volume (product number 10006) was used. The device was placed on the surface of blood until the absorbent tip became red indicating full saturation (Figure 6.1a). For DBS filter paper, 10 µL of whole blood was pipetted into the centre of the spotting circle. Samples collected by both devices were left to dry for 18 hours at room temperature before being stored at -20^{o}C and analysed within 24 hours. Prior to analysis, DBS samples were obtained from the DBS spot by one of two extraction techniques; 1) cutting a disc around the edge of the blood spot to obtain the whole blood spot (wDBS) (Figure 6.1b), and 2) by making two 3 mm sub-punches (spDBS) (Figure 6.1c). The spots were then placed into separate glass tubes. The absorbent tip from the VAMS was detached and placed into a glass tube.

Three patient sample pools containing low (10.6 nmol/L), medium (57.7 nmol/L) and high (82.0 nmol/L) concentrations of 25(OH)D3 were prepared. Each QC sample was analysed over ten separate runs to obtain a mean target value and ±2SD limits. Westgard QC rules[83] were applied as acceptance criteria for each batch of analysis.

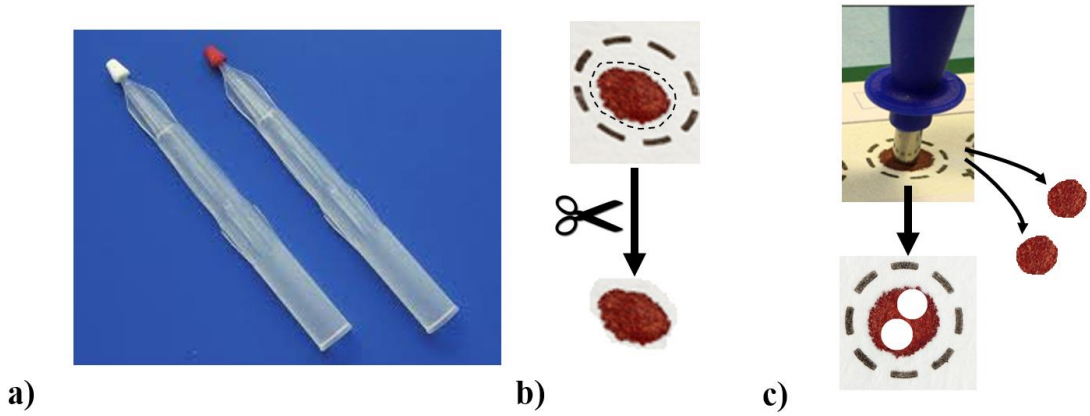


Figure 6.1a-c **(a)** a 10 µL Mitra VAMS before and after saturation with blood (**b)** cutting a whole blood spot **(c)** two 3 mm DBS sub-punch discs.

6.2.3 Sample preparation procedure for LC-MS/MS

Sample preparation procedure was modified from the derivatisation method described in Chapter 5.3.5. To each glass tube containing a DBS or VAMS sample, 20µL of internal standard solution consisting of 50 nmol/L of 25(OH)D3-[$^{2}H_{6}$] in IPA: water 50:50 (v:v) was added, followed by 150µL of H_2O. The tubes were sonicated in a water bath (Fisher Scientific, Loughborough, UK) at +60^{o}C for 60 minutes. The aqueous extracts were transferred into a new set of glass tubes, leaving behind the extracted spots/VAMS tips in the primary tubes. 150 µL of IPA was added to the primary tubes, vortexed by an orbital shaker at 300 rpm for 30 mins at room temperature, then transferred to the corresponding secondary tubes containing the aqueous extracts. The combined IPA/aqueous extracts (300µL), together with the spots/VAMS tips were loaded onto an ISOLUTE SLE+ plate. The plated samples were eluted twice with 750µL of n-heptane using the Extrahera automation system (Biotage, Uppsala, Sweden). Positive pressure was applied after each wash. The eluent was collected into a 96 deep well plate and then dried for 30 minutes under nitrogen heated at +45˚C.

The dried extracts were reconstituted using 50 µL of 1.1 mmol/L of PTAD in acetonitrile and incubated for 30 minutes while protected from light. The reaction was stopped by the addition of 50 µL of H_2O. After a brief vortex, the plate was heat sealed using a 20 µm pierceable foil. 20 µL of the extract was injected into the LC-MS/MS.

6.2.4 Liquid chromatography Tandem mass spectrometry

Liquid chromatography was performed as described in Chapter 5.2.5. In brief, chromatographic separation was performed using a core-shell C18 50 × 2.1mm, 2.6 µm column (Restek, PA, USA) that was heated to +55°C. Mobile phase (A) was water and methanol (B), both contained 0.2 mM methylamine in 0.1% formic acid containing. Gradient started at a flow rate of 0.4 mL/min with 50% Mobile phase B increased to 100% over 2 minutes 10 seconds then returned to the original conditions after three minutes. The 25(OH)D3 peak was eluted at 1.75 minutes. Micromass Quattro Ultima Pt tandem mass spectrometer (Waters Corp., Milford, MA, USA) was used to analyse the samples with precursor to product transitions, 607>298 for 25(OH)D3 and 613>298 for 25(OH)D3-[$^{2}H_{6}$]. MassLynx version 4.1 and QuanLynx were used for data acquisition and peak integration.

6.2.5 Method validation

For method validation, the guidelines on linearity, accuracy, precision, recovery and cross-validation procedures from the 2013 guidance produced by the U.S Food and Drug Administration (FDA)[81] and the 2012 guideline from the European Medicines Agency (EMA)[82] were followed. In addition, the effects from Hct in the blood matrix on 25(OH)D3 measurements and sample storage stability were investigated.

6.2.6 Linearity, accuracy, precision and recovery

Calibration standards prepared in wDBS, spDBS and VAMS, ranging from 0-125 nmol/L were included at the start of each run. Calibration curves were constructed by the ratio of analyte peak area to internal standard peak area on the y-axis against the weighted concentration (1/x) of the standards on the x-axis. The acceptable limit of linearity was defined as r^2 >0.98. Accuracy was assessed by the deviation of the assayed value of a calibration standard against its nominal value. Acceptance criteria are ≤15% of the nominal values, and ≤20% at the lower limit of quantification (LLoQ)[82].

Recovery was determined by analysing extracted samples spiked with fixed concentrations of 25(OH)D3 and repeated three times on separate runs. The percentage recovery was calculated using the expected concentration for each sample. The average recovery was determined from the mean of the three runs.

6.2.7 Lower limit of quantification and detection

To evaluate the intra-assay precision, ten replicates of the four samples covering concentrations between 7.6-70.9 nmol/L of 25(OH)D3 were analysed within a single run. The precision was expressed in percentage of coefficient of variation (%CV) for each sample. To assess the inter-assay precision, three QC pools were analysed across eleven runs over a 50-day period. The acceptance criteria were an intra-assay and an inter-assay CV of <15%. LLoQ was determined by analysis of a series of eleven standards, with concentrations ranging from 1.2 to 95.7 nmol/L of 25(OH)D3. Each standard was tested six times within a run. The LLoQ was defined as the lowest concentration with a CV of ≤20%, with the peak signal-to-noise ratio >5. The lower limit of detection (LLoD) was determined by the lowest concentration that produced a peak area of at least five times the area produced by the blank.

6.2.8 Effects of haematocrit on 25(OH)D3 measurements

The effects of Hct on plasma 25(OH)D3 concentrations were determined by two volume displacement studies. Firstly, three patient whole blood EDTA samples containing 25(OH)D3 (Hct) concentrations of 7.0 nmol/L (0.40 L/L), 27.6 nmol/L (0.40 L/L) and 43.2 nmol/L (0.51 L/L) were centrifuged for 5 minutes at 4000 rpm to separate the plasma. 10% of the plasma volume was removed from the top layer, and the residual sample was re-homogenised by gentle rotation for 10 minutes. Duplicate wDBS, spDBS and VAMS samples were taken from the homogenised blood. The process was repeated, taking 10% of plasma volume until the plasma layer was removed. DBS and VAMS samples were dried overnight at room temperature and extracted as described in 6.2.3. Secondly, washed plasma-free packed cells were added to patient blood samples in stepwise increments until full saturation. At each step after homogenisation, DBS and VAMS samples were collected for analysis.

6.2.9 Method comparison

EDTA blood samples from 97 patients with Hct level ranging between 0.32-0.55 L/L were sampled by wDBS, spDBS and VAMS. 25(OH)D3 concentrations from each microsampling technique were compared against the plasma concentrations. The validated plasma 25(OH)D3 assay described in Chapter 5.3.4 was calibrated using commercial 25(OH)D3 standards (Chromsystems, Müchen, Germany) traceable to reference source NIST SRM972a[77]. The inter-assay CV was ≤9%, the analytical range was 0.5-200 nmol/L, with an LLoQ of 0.1 nmol/L. The mean assay recovery was 96 ±2%. The assay showed <8% accuracy bias against NIST reference method on the Vitamin D external quality assessment (DEQAS) scheme.

6.2.10 Establishing the DBS-to-plasma equivalency values

To determine the DBS-to-plasma equivalent values (PEV) for wDBS (PEV_{wDBS}), spDBS (PEV_{spDBS}) and VAMS (PEV_{VAMS}), raw DBS and VAMS 25(OH)D3 concentrations were plotted against their respective plasma concentrations using a simple linear regression model. The regression equations from the microsampling techniques were used to transform raw DBS and VAMS 25(OH)D3 concentrations from a new second set of patient samples (n=70) into PEVs. Passing-Bablok regression, Lin's concordance correlation (CCC) and Bland-Altman difference plots were constructed for PEV_{wDBS}, PEV_{spDBS} and PEV_{VAMS} against the plasma 25(OH)D3 concentrations to assess the strength of the correlation.

6.2.11 Storage stability

To test the stability of 25(OH)D3 in storage, multiple DBS and VAMS samples from three pools of blood at 25(OH)D3 concentrations of 8.5, 27.9 and 72.1 nmol/L were prepared. Samples were dried for 18 hours and stored at -20°C and analysed at intervals up to 209 days. Changes in 25(OH)D3 concentration were expressed as the average percentage difference from day 0 to the day analysed.

6.2.12 Statistical analysis

Data exploration, scatter plots, and statistical analyses were performed using Statistical Package for Social Science (SPSS) version 22.0.0.2 (IBM, NY, USA). Passing-Bablok regression generated linear equations were used to derive the PEVs from raw DBS and VAMS values. Method comparisons were assessed using Passing-Bablok regression, CCC analysis and Bland-Altman plots. A correlation is deemed satisfactory if the regression produced an $r^2 \geq 0.9$.

6.3 Results

6.3.1 Assay validation

Calibration curves for wDBS, spDBS, VAMS and the plasma assay were linear from 0-125 nmol/L (r^2 >0.98). Precision profiles of both intra- and inter-assays CVs were <10% except for wDBS and spDBS at the lowest 25(OH)D3 concentrations (Table 6.1 and 6.2). The average intra- and inter-assay CVs were higher in spDBS than wDBS and VAMS across the concentrations tested. VAMS showed the least variability in comparison with wDBS and spDBS. LLoQ for wDBS, spDBS and VAMS were 1.6, 2.6 and 1.5 nmol/L, respectively. LLoD for wDBS, spDBS and VAMS were 0.8, 1.4 and 0.5 nmol/L, respectively. VAMs showed the best recovery results (Table 6.3). The lowest analyte recovery was observed in wDBS (78.9%) and the highest was found in spDBS (120%).

Sample	Mean 25(OH)D3 nmol/L (%CV)		
	wDBS	**SpDBS**	**VAMS**
1	7.5(16.1)	8.5 (13.6)	7.0 (7.6)
2	23.4 (8.8)	23.2 (9.0)	20.5 (6.7)
3	43.6 (9.2)	45.0 (12.0)	40.3 (6.4)
4	64.2 (6.5)	66.5 (11.0)	59.5 (7.7)

Table 6.1 Intra-assay precision. Ten replicates of four samples were analysed within a single run. The precision is expressed in percentage of coefficient of variation (%CV) for each sample.

	wDBS	SpDBS	VAMS
	Mean 25(OH)D3 nmol/L (%CV)		
Low QC	9.7 (12.9)	12.6 (14.8)	10.3 (9.5)
Medium QC	59.0 (6.9)	60.8 (13.5)	64.2 (7.1)
High QC	81.1 (7.2)	95.7 (10.8)	91.4 (5.7)
average %CV	9.0	13.0	7.5

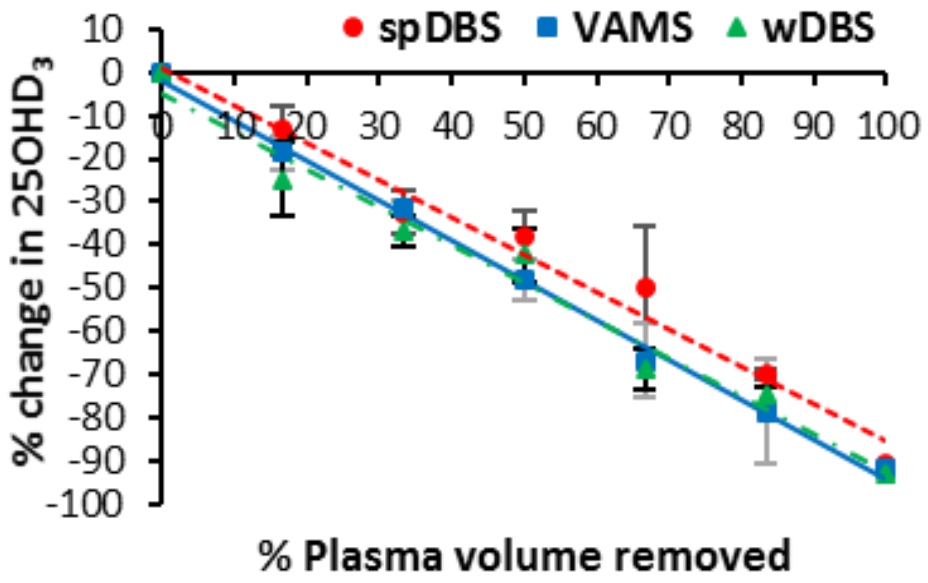
Table 6.2 Inter-assay precision. Three QC pools containing 25(OH)D3 analysed in eleven runs over a 50-day period

Expected 25(OH)D3 nmol/L	Mean recovery (%)		
	wDBS	SpDBS	VAMS
8.5	94.5	110.2	98.4
21.0	90.0	108.6	104.0
37.5	93.3	97.1	101.7
62.5	89.2	96.7	100.7
87.5	90.3	104.6	95.4
106.3	91.8	102.5	99.6
Overall average (range, %CV)	91.5 (78.9-102.4, 6.0)	103.8 (88.3-120, 9.2)	101.0 (93.4-112.4, 5.9)

Table 6.3 Assay recovery of the microsampling techniques. Fixed quantities of 25(OH)D3 were spiked into whole blood before microsampling, recovery is determined by the amount of 25(OH)D3 recoverable from the spiked amount. Each spiked sample was assayed three times over separate runs.

6.3.2 Volume displacement effects by haematocrit

25(OH)D3 concentrations showed a linear correlation with the decrease in plasma volume in wDBS, spDBS and VAMS (Fig. 6.2a). It demonstrates that 25(OH)D3 in blood is present primarily in the extracellular fluid (ECF) compartment, the intracellular space contained <1.7%. When plasma-free packed cells were added to a whole blood sample until saturation, a reduction in 25(OH)D3 concentrations were found in all three microsampling techniques that were proportional to the increase in Hct in blood (Fig 6.2b). The results demonstrated the displacement effect of plasma volume by Hct; whilst the plasma volume remained unchanged, the increasing level of Hct prevented the uptake of 25(OH)D3 into the microsampling devices. The finding indicated the concentration of 25(OH)D3 in the whole blood sample is dependent upon the level of Hct present, and that measurements using microsampling devices must be corrected for the level of Hct.



a)

Figure 6.2a The effect of plasma volume on whole blood 25(OH)D3 concentration. **(a)** Stepwise removal of plasma volume from the packed cells proportionally reduced 25(OH)D3 concentrations in wDBS, spDBS and VAMS samples. Results are represented as mean ± SEM.

b)

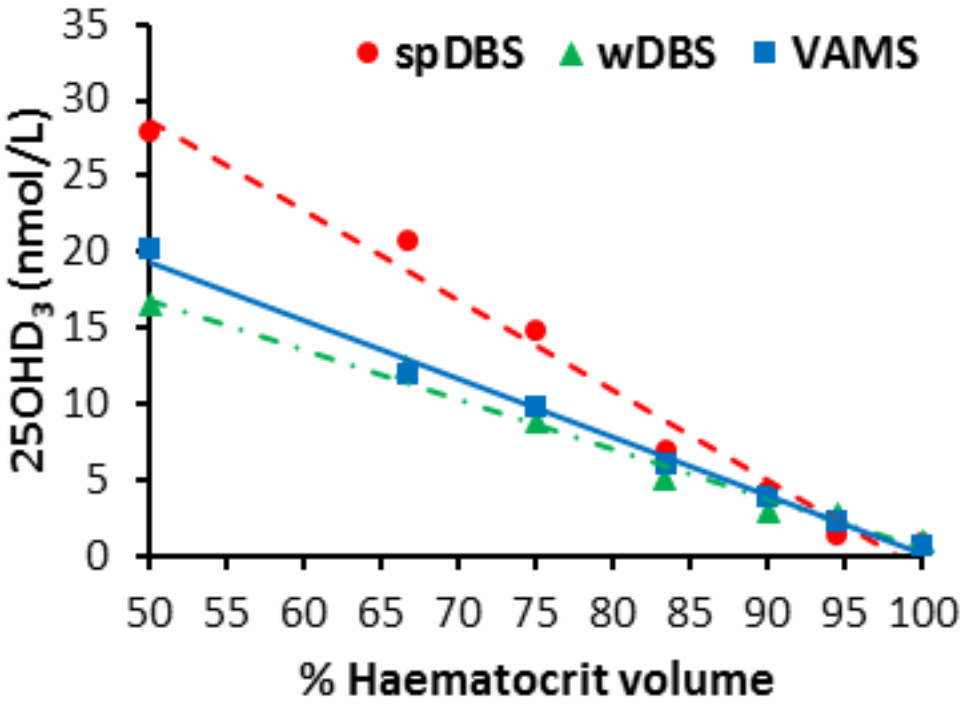
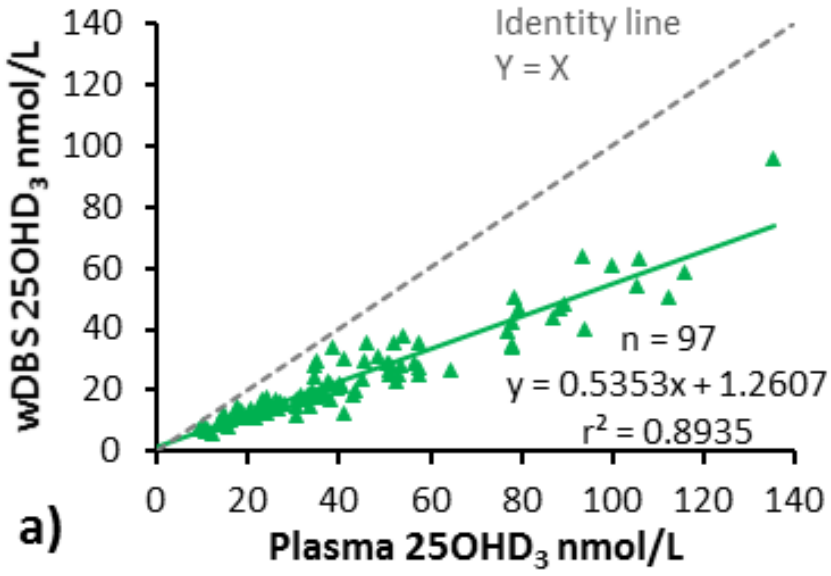
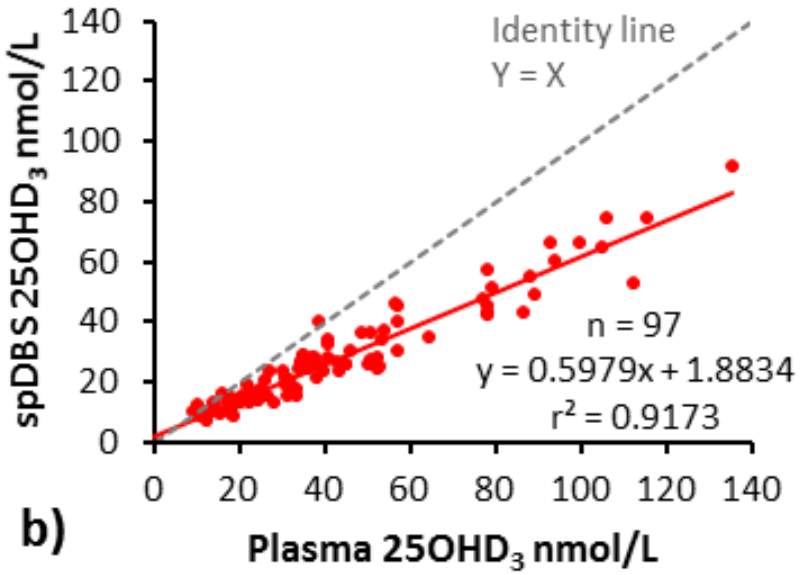


Figure 6.2b The displacement effect of plasma volume by Hct. Plasma-free packed cells were added to a whole blood sample in incremental steps until full saturation. Reduction in 25(OH)D3 concentrations were found to be proportional to the increase in Hct in blood.

6.3.3 Comparison between dried blood and plasma 25(OH)D3 measurements

EDTA samples (n = 97) with Hct values ranging from 0.32-0.55 L/L had 25(OH)D3 concentrations measured in DBS/VAMS and plasma. wDBS, spDBS and VAMS were positively correlated with plasma concentration with $r^2 = 0.893$; $r^2 = 0.917$ and $r^2 = 0.870$, respectively (Fig. 6.3a-c). A negative bias was found in values produced using microsampling devices. On average, the 25(OH)D3 concentrations in wDBS, spDBS and VAMS were 41%, 33% and 44% lower respectively than in plasma.





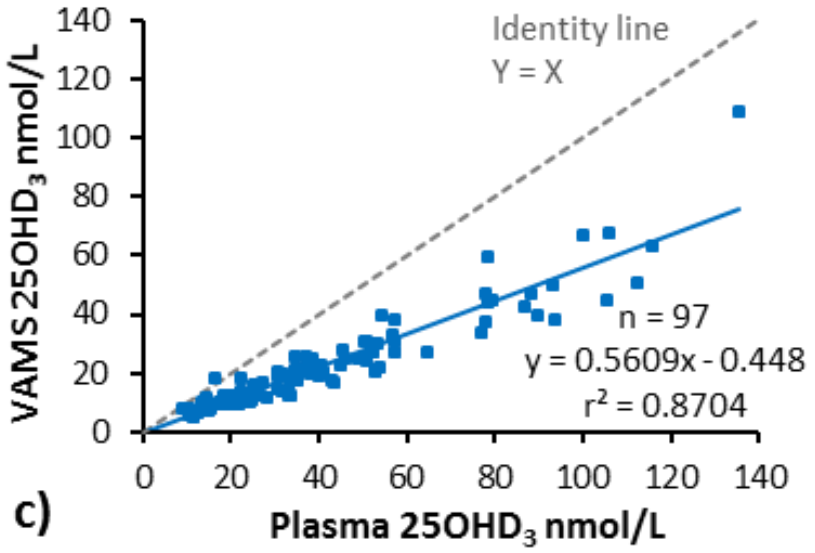
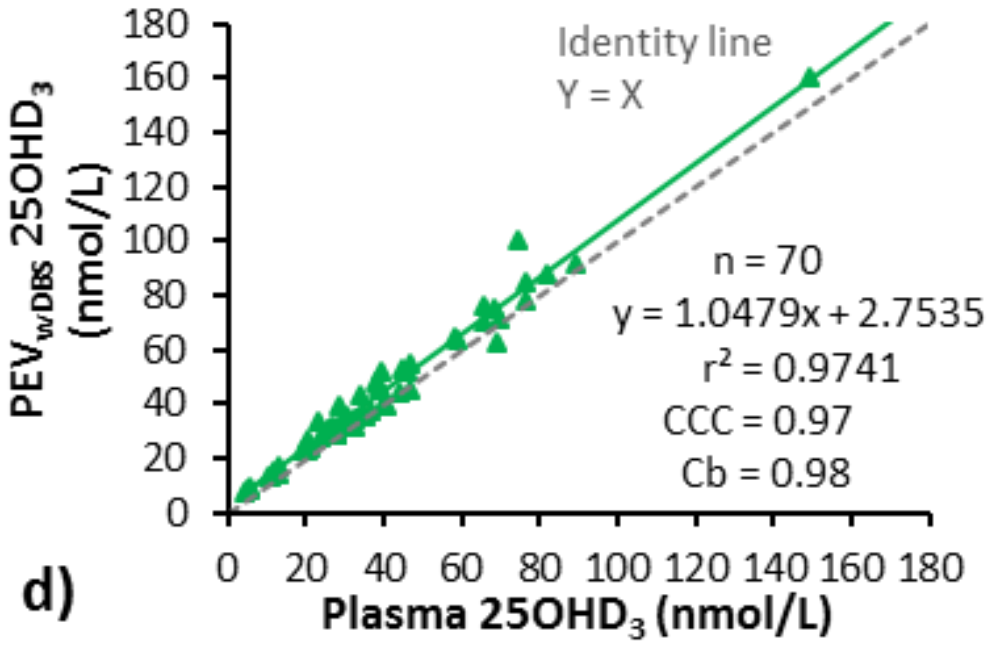


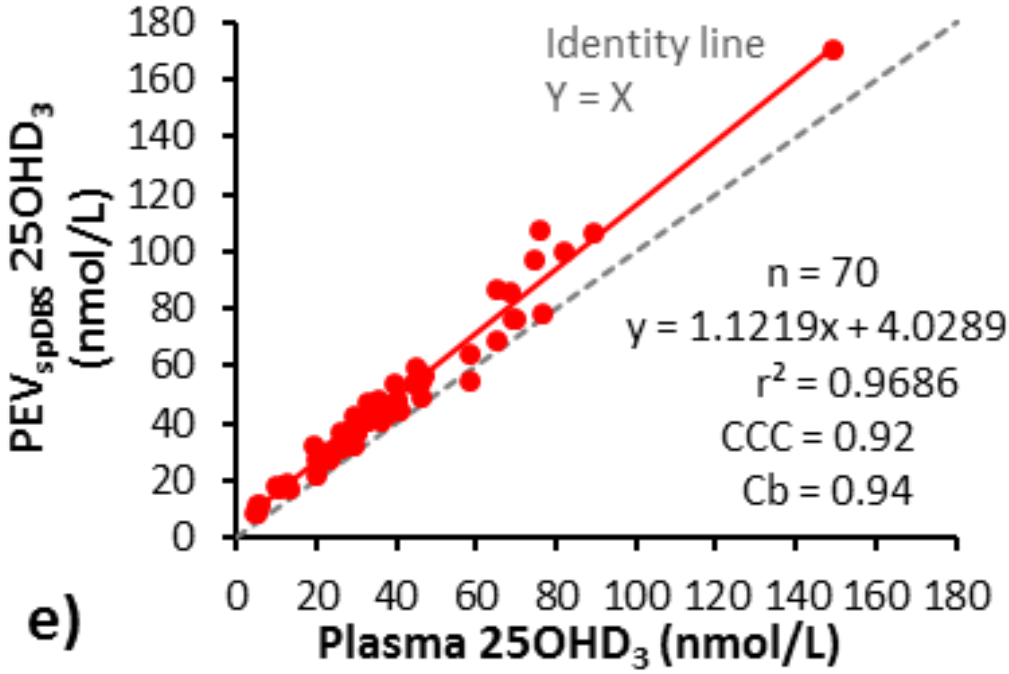
Figure 6.3a-c Comparison of 25(OH)D3 concentrations from 97 patient samples (Hct range 0.32-0.55 L/L) collected in **(a)** wDBS, **(b)** spDBS, and **(c)** VAMS with plasma. 25(OH)D3 concentrations produced from microsampling devices were negatively biased against plasma concentration. On average, the bias between the microsampling devices (solid colour lines) against the line of identity (grey dashed line) was -39.3%.

6.3.4 Dried blood-to-plasma equivalency values and their interpretability of vitamin D status

Linear regression analyses obtained the following dried blood-to-plasma equivalency equations: $PEV_{wDBS} = (wDBS + 1.26)/0.54$; $PEV_{spDBS} = (spDBS + 1.88)/0.6$; and $PEV_{VAMS} = (VAMS - 0.45)/0.56$. Using these equations, the raw 25(OH)D3 concentrations of wDBS, spDBS and VAMS obtained from a new set of 70 samples had their respective PEVs calculated. Passing-Bablok regression, correctional bias factor (Cb) and CCC analyses performed on PEV_{wDBS}, PEV_{spDBS} and PEV_{VAMS} against the plasma concentrations (Fig. 6.3d-f) showed good agreements with minimal deviation from the line of identity (y = x). However, the Bland-Altman plots showed overestimation of 25(OH)D3 concentrations by PEVs compared with plasma 25(OH)D3 concentrations(Fig. 6.3g-i). The average bias for PEV_{wDBS} and PEV_{VAMS} were +12.3% and +9.2%, respectively; PEV_{spDBS} showed the highest bias at +24.3%. The assay bias was negatively associated with the increase in Hct levels (Fig. 6.4a-c). For PEV_{VAMS} and PEV_{wDBS} zero assay bias was observed at Hct level of 0.48 L/L and 0.51 L/L respectively. In PEV_{spDBS}, a constant positive bias was observed across the entire Hct range.


Identity line
Y = X
n = 70
y = 1.0479x + 2.7535
r² = 0.9741
CCC = 0.97
Cb = 0.98
PEV wDBS 25OHD3 (nmol/L)
Plasma 25OHD3 (nmol/L)
0
20
40
60
80
100
120
140
160
180

d)


Identity line
Y = X
n = 70
y = 1.1219x + 4.0289
r² = 0.9686
CCC = 0.92
Cb = 0.94
PEV sPDBS 25OHD3 (nmol/L)
Plasma 25OHD3 (nmol/L)
0
20
40
60
80
100
120
140
160
180

e)

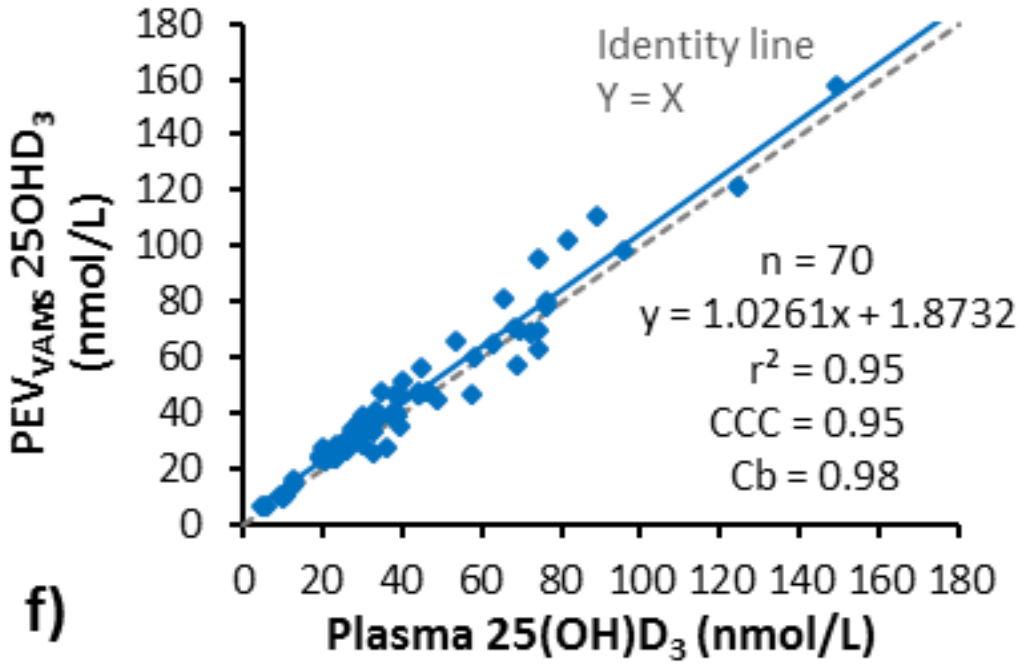
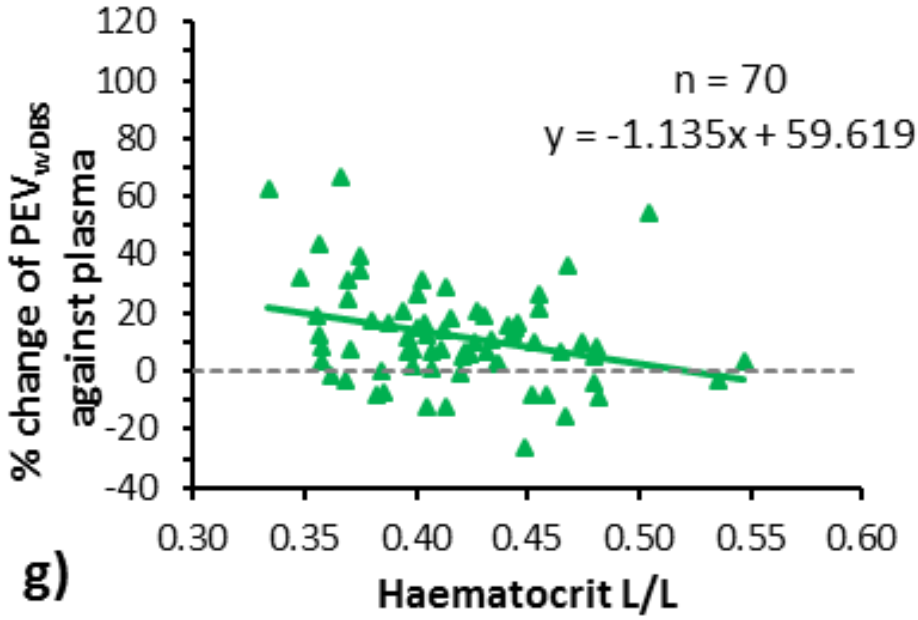


Figure 6.3d-f Passing-Bablok regression and Lin's concordance correlation analyses performed on **(d)** PEV_{wDBS}, **(e)** PEV_{spDBS} and **(f)** PEV_{VAMS} against plasma concentrations (n=70). CI: confidence interval; CCC: concordance correlation coefficient; r: Pearson correlation coefficient; Cb: correctional bias.



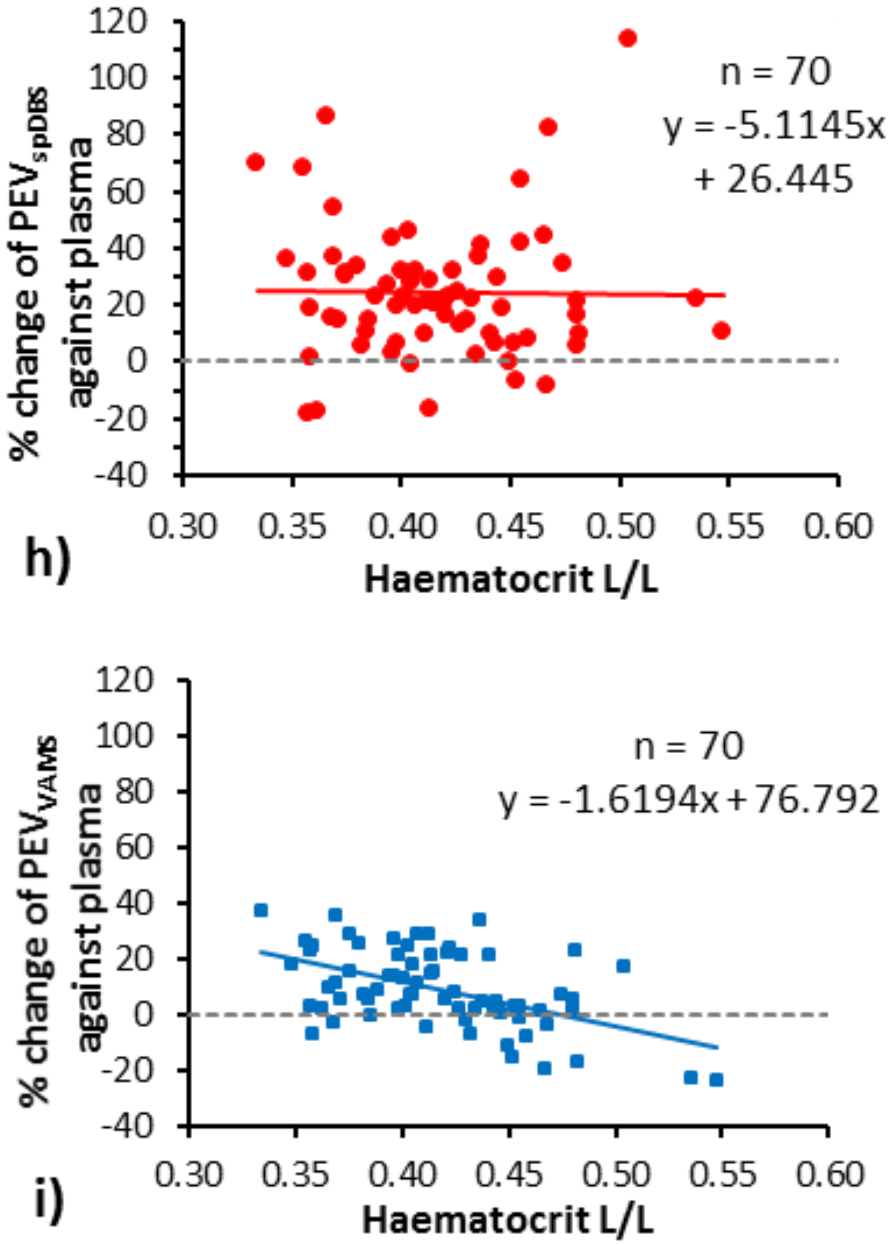
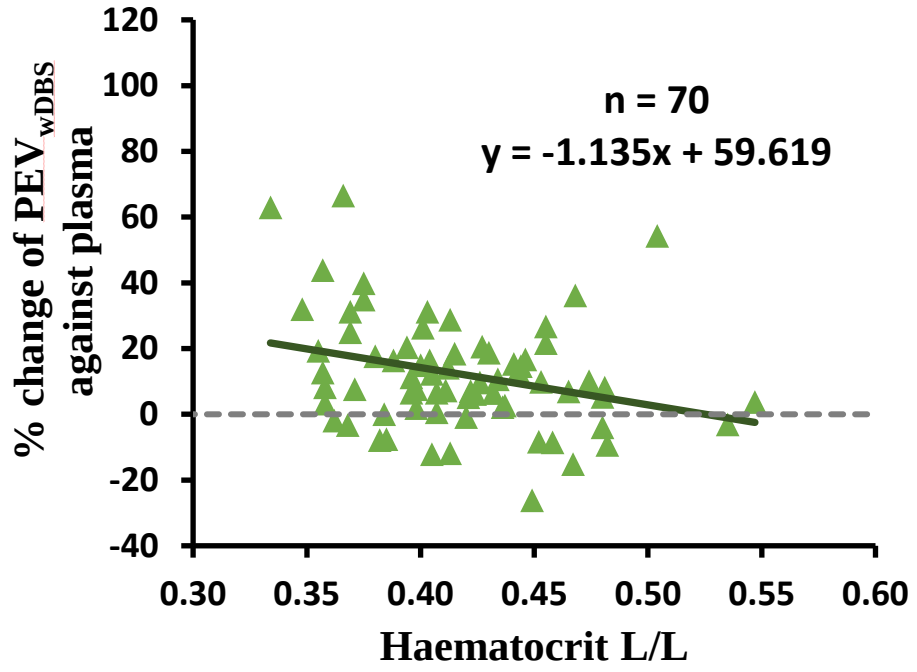
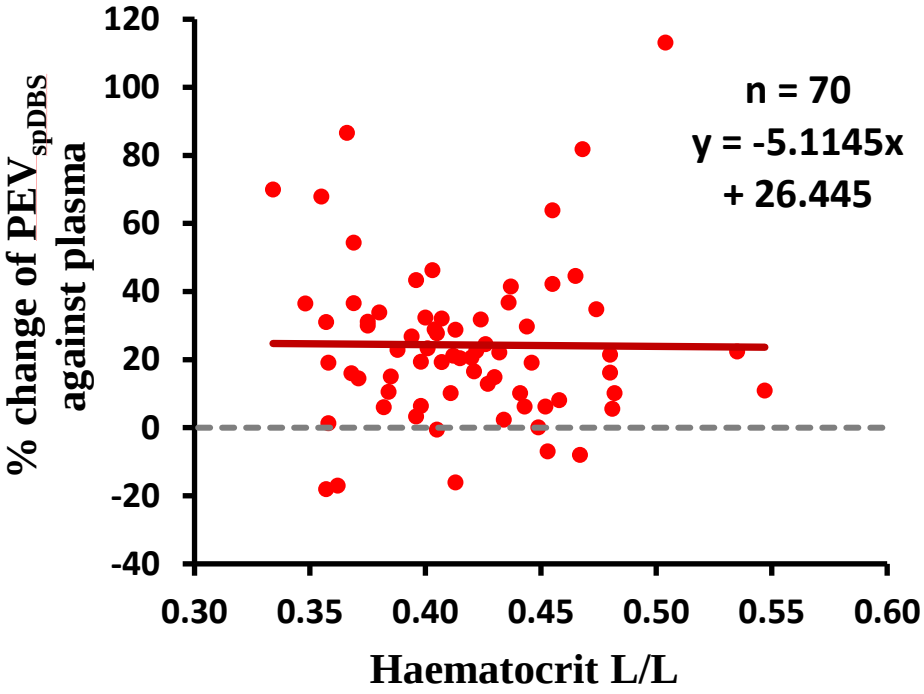


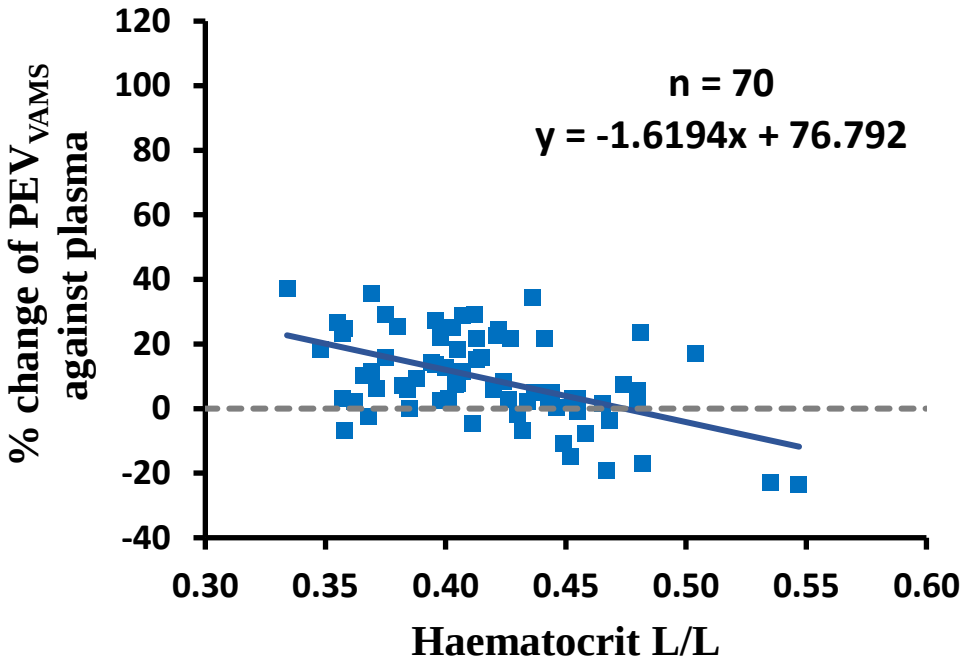
Figure 6.3g-i Bland-Altman plots showing the percentage difference between **(g)** PEV_{wDBS}, **(h)** PEV_{spDBS} and **(i)** PEV_{VAMS} with plasma 25(OH)D3 concentrations of each microsampling methods.


n = 70
y = -1.135x + 59.619
% change of PEV wDBS against plasma
Haematocrit L/L
120
100
80
60
40
20
0
-20
-40
0.30
0.35
0.40
0.45
0.50
0.55
0.60

a)


n = 70
y = -5.1145x
+ 26.445
% change of PEV spDBS against plasma
Haematocrit L/L
120
100
80
60
40
20
0
-20
-40
0.30
0.35
0.40
0.45
0.50
0.55
0.60

b)



c)

Figure 6.4a-c The percentage difference between a) PEV_{wDBS}, b) PEV_{spDBS} and c) PEV_{VAMS} with plasma concentration against the respective haematocrit level in each sample.

The Institute of Medicine (IOM) guidelines[93] defined the vitamin D status of an individual with serum 25(OH)D (sum of 25(OH)D3 + 25(OH)D2) <30 nmol/L as deficient; between 30-50 nmol/L as insufficient; and >50 nmol/L as sufficient. Based on these definitions, in this cohort of 70 samples with none detectable 25(OH)D2, measurements of plasma 25(OH)D3 concentrations identified 27 (38.6%) cases of vitamin D deficiency, 24 (34.3%) cases of insufficiency, and 19 (27.1%) individuals with sufficient vitamin D status (Table 6.4). PEVs showed an overall underestimation of individuals with deficient vitamin D status, and an overestimation of individuals with insufficient and sufficient vitamin D status. Between the three microsampling techniques, PEV_{VAMS} showed the least difference in the interpretation of vitamin D status.

Vitamin D status definitions	**Plasma (no. of cases, % in cohort)**	**PEV_{wDBS} (n, Δ%)**	**PEV_{spDBS} (n, Δ%)**	**PEV_{VAMS} (n, Δ%)**
<30 nmol/L, Deficiency	27 (38.6%)	20 (↓10%)	15 (↓17.1%)	24 (↓4.3%)
30-50 nmol/L, Insufficiency	24 (34.3%)	29 (↑7.1%)	31 (↑10%)	26 (↑2.9%)
>50 nmol/L, sufficiency	19 (27.1%)	21 (↑2.9%)	24 (↑7.1%)	20 (↑1.4%)

Table 6.4 Interpretation of vitamin D status in the cohort (n=70) according to the U.S. Institute of Medicine guidelines. The number of cases classified as vitamin D deficient, insufficient and sufficient by the respective plasma 25(OH)D3 concentration and the corresponding PEV produced from each sampling method.

6.3.5 Storage stability over 209 days

25(OH)D3 in both wDBS and VAMS was stable up to 209 days when stored at -20°C. No significant differences were observed between day 0 and each time point (Fig. 6.5).

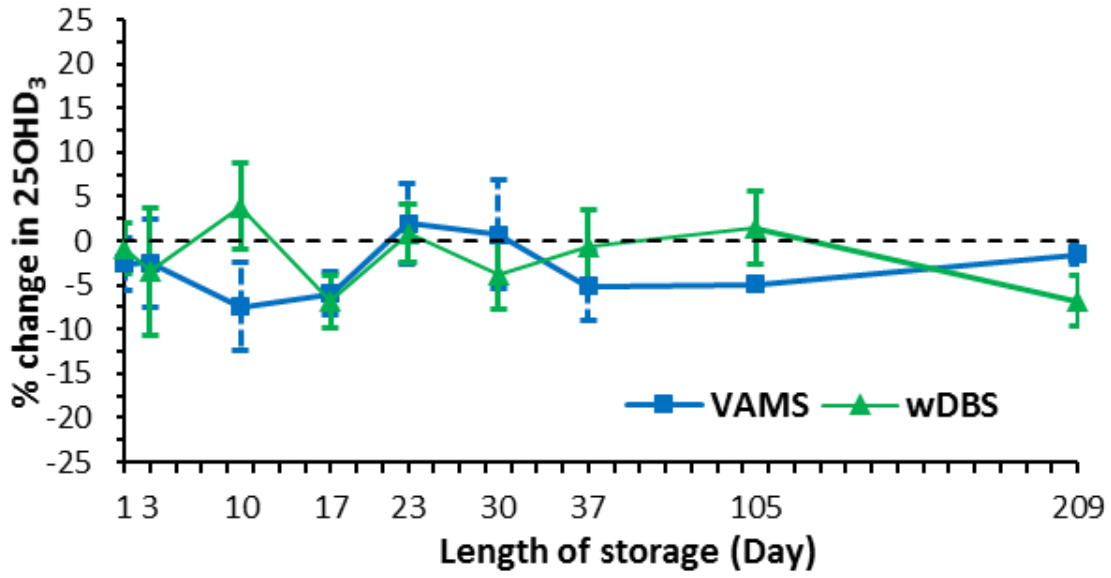


Figure 6.5 A 209-day stability plot. Each time point represents the mean (range) percentage change of 25(OH)D3 concentration from day one in samples collected by wDBS and VAMS stored at -20°C.

6.4 Discussion and conclusion

The prime objective of the study was to develop an LC-MS/MS method to determine 25(OH)D3 concentrations in DBS from filter paper and VAMS. A robust sample extraction procedure was developed with derivatisation to enhance the sensitivity of the LC-MS/MS method and improve the precision of 25(OH)D3 measurements. The assay was validated against published acceptance criteria for clinical and research use. The method demonstrated excellent linearity, recovery and intra/inter-assay precision. The quality of measurements can be affected by pre-analytical factors associated with the use of dried blood samples. Kvaskoff *et al.*[120] showed that due to the non-uniform nature of blood diffusion on the filter paper, 25(OH)D3 concentrations from a sub punch taken from the centre of the blood spot could vary to a sub punch of the same sample taken from around the edge of the spot. To address this issue, this study evaluated two common approaches for the extraction of blood spots; sub punches (spDBS) and removal of the entire blood spot from a fixed volume of sample (wDBS). wDBS showed better performance than spDBS; with lower intra- and inter-assay CVs, lower variability in recovery, and the respective PEV_{wDBS} showed a stronger correlation with plasma concentration.

In comparison with wDBS and spDBS, VAMS was superior in both reproducibility and recovery of 25(OH)D3; the fixed volumetric device also produced better assay precision. spDBS and wDBS showed higher intra/inter-assay CVs than VAMS and greater variability in assay recovery, despite the fact that DBS samples were prepared from a fixed 10 μL of blood pipetted directly onto the filter paper in a controlled laboratory environment. In practice, a person carrying out self-sampling would not be able to control the blood volume on the filter paper; the variability in sample volume and spot sizes would likely have a greater negative impact on assay performance and compromise the quality of results.

The effects of blood sample Hct on 25(OH)D3 measurements was tested, by adding packed red cells and removing plasma in incremental steps. The results showed that 25(OH)D3 concentrations decreased proportionally as Hct increased; and also when plasma volume is reduced. The findings are consistent with those of Kvaskoff *et al*.[120] who, using isotopic I-125 labelled 25(OH)D3, showed that 98% of 25(OH)D3 was present in the serum fraction, and concluded that because the majority of 25(OH)D3 is bound to vitamin D binding protein, 25(OH)D3 is virtually absent from intra-cellular and membrane components of the erythrocytes. However, despite assurance from the fact that Hct does not alter the physiological concentration of 25(OH)D3 in circulation, the displacement effect of Hct can prevent the uptake of 25(OH)D3 into the microsampling devices, resulting in biased correlations between raw DBS and VAMS values with plasma concentrations as shown in Figure 6.3a-c. To adjust for the bias, regression models were established to transform values into a clinically-relevant scale. The accuracy of the

DBS/VAMS-to-plasma equivalent PEVs were blindly tested on a new set of samples; the results showed a strong agreement with the plasma values suggesting that the proposed equation models can be used to obtain reliable and interpretable measurements of 25(OH)D3.

The use of mathematical conversions to adjust for Hct in LC/MS-MS assays for DBS have been reported[120-123], but the use of VAMS was not studied. The formulae also require measurement of individual Hct, unlike the proposed formula which can be applied without the need for Hct measurement. Using the exact Hct value may improve the diagnostic accuracy of the results, but venous blood is required. The results demonstrated the suitability of using PEV in the interpretation of vitamin D status and found PEV_{VAMS} to be the most accurate method in comparison to PEV_{spDBS} and PEV_{wDBS}. Therefore, VAMS is recommended as the microsampling method of choice for the determination of 25(OH)D3 and the assessment of vitamin D status.

A limitation of the study was that this study did not determine the rate of absorption of Hct into VAMS and assumed equal uptake of intra and extracellular contents. Given 25(OH)D3 exists primarily in the ECF compartment of blood, and the uptake of sample by the microsampling device is dependent upon the amount of Hct present. Denniff and Spooner[123] demonstrated that there is no preferential absorption of plasma over blood cells when using VAMS. Similarly, a multi-laboratory study[118] showed no notable change of blood volume absorbed by VAMS across the Hct range. This supporting evidence suggests that VAMS can provide an accurate representation of the sample constituents.

This study was limited to the use of venous blood samples, in order to obtain Hct measurements. Further study will be required to cross-validate agreement between capillary and venous samples, and to investigate the potential effects which may result from bacterial or tissue fluid contamination at the site of puncture. Jensen *et al.*[124] demonstrated using LC-MS/MS, a strong agreement of 25(OH)D3 concentrations between capillary and venous blood (r^2 = 0.9963), with a small average positive bias of 2.0 nmol/L in capillary blood.

A major strength of the study was the large sample size. However, the samples were selected at random; hence a limited Hct range between 0.32-0.55 L/L was obtained. Low or high Hct levels may result from blood transfusion, the use of medications and pathological conditions[125-128].

In conclusion, this study provides validation of microsampling methodologies for measurement of 25(OH)D3 and interpretation of vitamin D status. VAMS demonstrated benefits over the conventional paper-based method; the consistency in sampling volume, ease of use without the need for sub punches, and preservation of sample constituency are all advantages of VAMS. The results provided evidence that samples collected from VAMS produced better assay performance for the assessment of vitamin D status. The use of an empirically-derived model to transform DBS

values into clinically-relevant equivalency improves the interpretability and may broaden the applicability of DBS sampling particularly in vulnerable populations.

Chapter 7 Reference intervals for serum 24,25-dihydroxyvitamin D and the ratio with 25-hydroxyvitamin D established using LC-MS/MS profile analysis

7.1 Background

The vitamin D endocrine system plays a major role in human biological functions. The vitamin D pathway produces a large number of metabolites including 25-hydroxyvitamin D (25(OH)D), and 1α,25-dihydroxyvitamin D (1,25(OH)D). Each vitamin D metabolites exists in two major forms: cholecalciferol (D3) is derived from sunlight (UVB) exposure on the skin and meat products, and ergocalciferol (D2) is derived from dietary plant sources. The synthesis of the metabolites are controlled by 25-hydroxylase in the liver and 1-hydroxylase in the kidney. Severe deficiency of serum 25(OH)D results in decreased production of $1,25(OH)_2D$ which is strongly associated with bone and neuromuscular diseases[9]. Epidemiological studies have shown an inverse relationship between serum 25(OH)D concentrations with a broad spectrum of disease states, including cardiovascular, cancer, diabetes, immunological and psychiatric disorders[4,5,7,8,129-131]. However, evidence from vitamin D intervention trials supporting the beneficial effects of vitamin D supplementation on disease outcome is largely inconclusive[132-135]. The contradictory evidence indicates that the catabolism of 25(OH)D is a multistep process. Recent attention has centred on an alternative pathway of 25(OH)D metabolism driven by 24-hydroxylase producing 24,25-dihydroxyvitamin D ($24,25(OH)_2D$). Studies have found limited biological activity of $24,25(OH)_2D$ in humans, and similarly the metabolite 1,24,25-trihydroxyvitamin D ($1,24,25(OH)_3D$) which was considered to be an inactive excretory product of the vitamin D pathway[136]. The revival of interest in $24,25(OH)_2D$ is partly as a result of altered vitamin D catabolism discovered in patients with chronic kidney disease (CKD)[137], where impaired renal production of $1,25(OH)_2D$ was found to be associated with decreased CYP24A1 (cytochrome P450, family 24, subfamily A, polypeptide 1) activity. The *CYP24A1* gene is expressed in most tissues in the body[138,139]; it encodes the 24-hydroxylase enzyme that catalyses the conversion of 25(OH)D and $1,25(OH)_2D$ into 24-hydroxylated products[136]. The transcription of the *CYP24A1* gene is stimulated by the phosphate-regulating hormone fibroblast growth factor-23 (FGF23) and suppressed by PTH[137,140]. As $24,25(OH)_2D$ is the main product of 25(OH)D

catabolism by CYP24A1, the measurement of serum 24,25$(OH)_2$D concentration can be useful in identifying patients with loss-of-function mutations in *CYP24A1*. The relative ratio of serum 25(OH)D to 24,25$(OH)_2$D concentration may serve as an indicator of vitamin D catabolic status.

Recent advances in methods for measurement of vitamin D metabolites, in particular with assays for measurement 25(OH)D, have led to the development of a range of semi- and fully automated assays capable of measuring large numbers of samples. Competitive protein binding assays and enzyme immunoassays using chemiluminescent detection-based systems in fully automated platforms appeal to hospital laboratories due to their ease of use, speed and efficiency[141]. However, immunoassays have variable cross-reactivity with other vitamin D metabolites and are unable to distinguish the two major forms of vitamin D (D3 and D2). This results in some immunoassays significantly underestimated an individual's true vitamin D status[61]. Liquid chromatography tandem mass spectrometry (LC-MS/MS) methods quantify vitamin D metabolites based on the compound-specific precursor to product ion mass-to-charge (m/z) transitions. They can distinguish each hydroxylation metabolite and their respective D3 and D2 forms. Proficiency testing bodies such as the International Vitamin D External Quality Control Scheme (DEQAS) have reported that measurement of 25(OH)D by LC-MS/MS methods using traceable calibration standards from the National Institute of Standards and Technology (NIST) exhibit the lowest inter-laboratory imprecision in comparison with immunoassays[60]. LC-MS/MS is now considered to be the reference method for measurement of 25(OH)D.

The aim of the study was to develop a robust and sensitive assay to measure 25(OH)D and 24,25$(OH)_2$D simultaneously in human serum samples. The objective was to apply the assay to measure vitamin D metabolites in a large cohort of healthy individuals and patients to examine the clinical correlation of 24,25$(OH)_2$D with 25(OH)D. The goal was to establish from a cohort of healthy individuals: 1) reference intervals for serum 24,25$(OH)_2$D and 25(OH)D:24,25$(OH)_2$D ratio; 2) to determine the diagnostic accuracy of 24,25$(OH)_2$D measurement as an indicator for vitamin D status; and 3) to identify a 24,25$(OH)_2$D cut-off threshold when vitamin D status is sufficient; i.e. serum 25$(OH)_2$D concentration ≥50 nmol/L, as defined by the U.S Institute of Medicine (IOM) and the UK Royal Osteoporosis Society (ROS)[10,11]. Recent publications have reported reference intervals of 25(OH)D:24,25$(OH)_2$D ratio on smaller cohorts[42,142,143], or post-menopausal women supplemented with vitamin D[55]. This chapter describes the reference intervals from a large cohort of unsupplemented individuals using a statistical approach supported by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) working group[144].

7.2 Materials and methods

7.2.1 Serum sample collection

The characteristics of the subjects included in the study are shown in Table 7.1. Serum samples used in the healthy cohort (n=1996) were obtained from young British Army recruits during initial training (ClinicalTrials.gov Identifier NCT02416895). The study was approved by the UK Ministry of Defence research ethics committee (MODREC). Written informed consent was obtained from all study participants. The participants were from Caucasian population and each required to complete details in a health questionnaire, including the use of medications and supplements. Individuals taking calcium and vitamin D supplements were excluded from the study. Samples were collected between June 2014 and March 2016.

Serum samples from patients (n=294) were collected from residual samples following their course of routine care in accordance with generic ethical approval[80]. The patient samples collected were routine requests for 25(OH)D measurement received by the Department of Laboratory Medicine at the Norfolk and Norwich University Hospital between February 2015 to March 2016. From the clinical information available, the cohort contained patients from Caucasian population presented with fatigue/wellness-check (42.6%), osteoporosis (23.9%), primary hyperparathyroidism (19.9%), liver disease (5.7%), chronic renal failure (6.2%) and unknown (1.7%). Use of vitamin D supplements or drugs known to affect mineral metabolism were reported in 32% of the patients. All samples were anonymised to the researchers at the point of access. Blood samples were collected into serum gel separator tube (BD Vacutainer) and centrifuged immediately. After a 10 minute centrifugation at 3,000 x g, serum layer was aliquoted into a separate polystyrene tube and stored at -20°C until analysis.

		Healthy subjects	Patients	ρ-value
n		1996	294	0.001
Gender	**Male**	1492	177	0.001
	Female	504	117	0.001
Age, years (range)		23 (18-32)	52 (2-95)	0.001
25(OH)D3, nmol/L		63.7±29.4	50.4±50.7	0.001
25(OH)D2, nmol/L		ND	5.4±9.5	
24,25(OH)$_2$D3, nmol/L		5.7±3.4	4.1±5.2	0.001
24,25(OH)$_2$D2, nmol/L		ND	2.4±1.1	
25(OH)D:24,25(OH)$_2$D		13±4.3	17±7.4	0.001

*Data are shown in mean±SD, ND: none detected

Table 7.1 Characteristics of the subjects included in the study.

7.2.2 Materials, calibration standards and controls

SRM972a traceable 25(OH)D3 and 25(OH)D2 serum based calibrators (Chromsystems, München, Germany) and internal quality controls (IQC) (UTAK Laboratories, CA, USA) were analysed in each run. Certified pure standards for 24,25$(OH)_2$D3, 24,25$(OH)_2$D2 were used for the preparation of spiked standards and deuterated standards 25(OH)D3-[$^2H_6$] and 24,25$(OH)_2$D3-[$^2H_3$] (IsoSciences, King of Prussia, PA, USA) were used as internal standards. Deionised water, methanol, acetonitrile and formic acid were LCMS grade, n-heptane and isopropanol were analytical grade (Fisher Scientific, Loughborough, UK). 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) and methylamine (Sigma-Aldrich, Dorset, UK) were used for derivatisation and adduct formation. Vitamin D metabolites in frozen human serum (Standard Reference Material 972a) was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA).

7.2.3 Sample preparation procedure for LC-MS/MS

Sample preparation and extraction procedures were based on the PTAD derivatisation method described in Chapter 5.3.5. Samples were processed using the Extrahera™ automation system (Biotage, Uppsala, Sweden), under positive pressure supplied from a nitrogen generator (Peak, Scotland, UK) at a flow rate of 30 L/min. In a 96 position 2mL deep well plate, 100 µL of

calibration standards, IQC materials or serum samples were diluted with 200 µL of pre-treatment solution consisting of deuterated internal standards in isopropanol:water 50:50 (v/v). After mixing, the samples were loaded onto ISOLUTE® supported liquid extraction (SLE+) 400 µL plate (Biotage). Elution was carried out by adding two cycles of 750 µL of n-heptane, both cycle of eluents were collected into corresponding deep well plate. Positive pressure was applied at each stage to remove residual solvent. Samples were then dried under a gentle stream of nitrogen gas heated to 45°C. Derivatisation took place by adding 50 µL of 1.1 mmol/L PTAD in acetonitrile, into all wells. The plate was vortexed and allowed to incubate for 30 minutes at room temperature in the dark. 50 µL of water was then added and mixed to stop the reaction. 20 µL of the derivatised extracts was injected into the LC-MS/MS. Using this sample preparation procedure, a batch of 96 samples was processed in one hour.

7.2.4 Liquid chromatography

Extracted samples were injected into LC-MS/MS by Waters® 2777 Sample manager (Waters Corp., Milford, MA, USA) equipped with 3-drawer cooler stack regulated at 10˚C. Chromatographic separation was achieved using a core-shell C18 50 × 2.1mm, 2.6 µm, reversed-phase (Restek, Bellefonte, PA, USA) column heated at 55˚C. An in-line 2µm, 6.35mm × 24mm guard filter was used to protect the column. A gradient elution profile was set up using a binary UPLC pump (Flux Instruments, Switzerland) to deliver mobile phase at a flow rate of 0.4 mL/min. At the start of the gradient, the mobile phase consisted of 50:50 (v/v) of (A) water containing 0.2 mM methylamine in 0.1% formic acid and (B) methanol containing 0.2 mM methylamine in 0.1% formic acid. The gradient was gradually increased to 99% of methanol mobile phase (B) then returned to the starting gradient at 4 minutes. Solvent divert was employed to divert ion suppression regions of the separation to waste in order to minimise contamination to the source of the mass spectrometer.

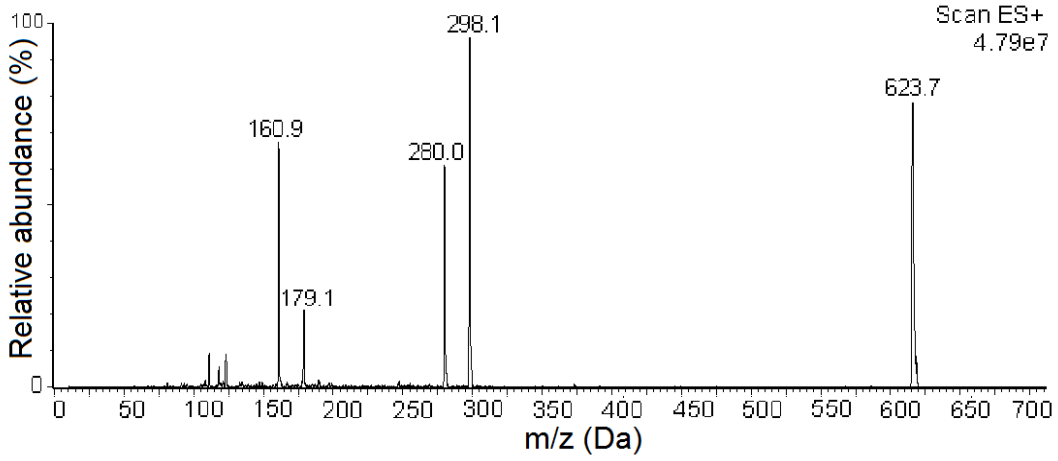
7.2.5 Tandem mass spectrophotometry analysis

LC-MS/MS analysis of vitamin D metabolites was performed using Micromass Quattro Ultima Pt electrospray ionisation (ESI) tandem mass spectrometer (Waters Corp., Milford, MA, USA). MassLynx version 4.1 and QuanLynx software (Waters Corp., Milford, MA, USA) were used for system control, data acquisition, baseline integration and peak quantification. Optimisation of MS/MS parameters was accomplished by direct infusion of derivatized standards (Table 7.2). The capillary voltage was set at 3.0 kV and RF lenses 1 and 2 were set at 0.1. Source temperature was maintained at 90˚C. Nitrogen was used as both nebuliser gas at a flow rate of 30 L/hr and as

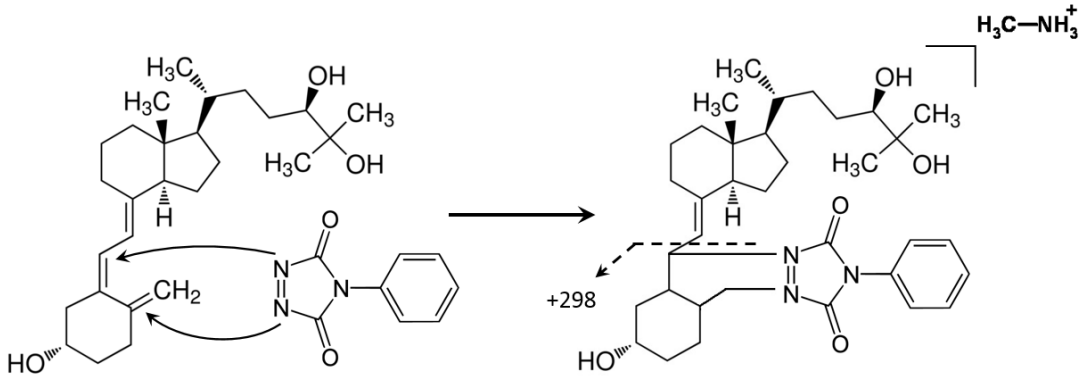
desolvation gas at a flow rate of 850 L/hr at 120˚C. Sample cone voltage and collision energy for all vitamin D metabolites were 35kV and 25kV respectively. Argon gas was applied to the collision cell during the Collision Induced Dissociation (CID) process. The precursor to product ion transitions for each compound was ascertained based on the molecular weight of the methylamine adduct of PTAD derived products (Fig. 7.1a and b).

Parameter	**Setting**
Ion source	Electrospray positive
Capillary voltage	3.0 kV
Cone energy	35 kV
Collision energy	20kV
Nebuliser gas flow rate	30 L/hr
Desolvation gas flow rate	850 L/hr
Source temperature	85°C
Desolvation gas temperature	250°C
m/z transitions	
25(OH)D3	607>298
25(OH)D3	619>298
24,25$(OH)_2$D3	623>298
24,25$(OH)_2$D2	635>298
25(OH)D3-[$^2H_6$]	613>298
24R,25$(OH)_2$D3-[$^2H_3$]	626>316

Table 7.2 Mass spectrometer parameter settings and MRM precursor to product ion transitions for 25(OH)D3/D2 and 24,25$(OH)_2$D3/D2.



a)



b)

Figure 7.1a-b **(a)** Collision-induced dissociation spectra of PTAD-derivatized 24,25$(OH)_2$D3 (M+CH_3NH_3+ at m/z 623). The precursor to product ion transition m/z 623>298 was utilised for MRM. **(b)** The dienophilic reaction of 24,25$(OH)_2$D3 with PTAD and formation of methylamine adduct.

7.2.6 Method validation

Method validation guidance was partially followed from the 2013 U.S Food and Drug Administration (FDA)[145] and the 2012 European Medicines Agency (EMA)[146]. Human sera based calibration standards for 25(OH)D3 and 25(OH)D2 (Chromsystems) were used to ensure traceability to SRM972. Where commercial calibration material was not available for 24,25$(OH)_2$D3 and 24,25$(OH)_2$D2, certified pure standards were spiked gravimetrically into vitamin D depleted human serum, representing the same biological matrix as samples. IQC materials were incorporated into each batch, the concentrations of IQCs were spread across the analytical range; from the base level at three times the concentration of lower limit of quantification (LLoQ) to top level at the upper assay limit[145]. IQC provided the basis of accepting

or rejecting the batch, the assay acceptance criteria require over half of the IQC samples analysed in each batch produce results within 15% from their respective target value, and 20% at LLoQ[146].

7.2.7 Linearity

Serum standards with known concentrations ranged from 0-180 nmol/L for 25(OH)D3 and 25(OH)D2, and 0-19.3 nmol/L for 24,25$(OH)_2$D3 and 24,25$(OH)_2$D2 were analysed at the start and end of every batch. A standard curve was generated by plotting the ratio of analyte peak area to internal standard peak on the y-axis against the weighted (1/*x*) concentration of their respective standards on the x-axis. The goodness-of-fit of a standard curve is justified by linear regression that produced a correlation coefficient (r^2) value of >0.980.

7.2.8 Accuracy, precision and recovery

The accuracy of an analytical method can be influenced by sample matrix[102,103], endogenous components such as phospholipids in biological fluids are a major source of ion suppression in LC-MS/MS analysis[147]. Spike and recovery experiments were performed at low, medium and high concentrations across the analytical range to test the effects of biological matrix and sample types (serum and EDTA plasma) on the efficiency of sample extraction. A multiple reaction monitoring (MRM) transition using the 184 Da product ion of phospholipids was included in the acquisition method to monitor the level of phospholipids present in the sample matrix. Imprecision of the assay was assessed by ten consecutive measurements of IQC materials within a single run (intra-assay) and repeatedly (n = 53) over a three month period (inter-assay). Precision is expressed as coefficient of variation (CV), acceptance criteria defined the intra-assay CV limit of <10% and cumulative inter-assay CV limit <15%. Accuracy was assessed using NIST SRM 972a materials; method bias was measured against the certified values for 25(OH)D3, 25(OH)D2 and 24,25$(OH)_2$D3.

7.2.9 Lower limit of quantification and detection

A precision profile was carried out to determine the lower limit of quantification (LLoQ) and detection (LLoD) of the assay. Samples containing 25(OH)D3, 25(OH)D2, 24,25$(OH)_2$D3 and 24,25$(OH)_2$D2 at concentrations of 0.05, 0.12, 0.19, 0.41, 0.81 and 1.24 nmol/L were each analysed six times, the CVs of each sample were plotted against their respective concentration. The analyte peak must produce a signal-to-noise (s/n) ratio of 10:1, the LLoQ was defined as the concentration at which the CV ≤22%.

7.2.10 Method comparison

The 25(OH)D3 values generated by the new multi-analyte, PTAD derivatised method was compared against the single analyte non-derivatised LC-MS/MS method previously described in Chapter 5.3.4. Serum samples (n=630) that were previously analysed for 25(OH)D3 were retested by the new method. Samples from the vitamin D external quality assurance scheme (DEQAS)[148] were analysed, and results compared against the LC/MS method mean. 24,25$(OH)_2$D3 measurements from samples distributed between April 2015 to January 2019 (n = 75) were submitted to the proficiency testing scheme for variability assessment. NIST-certified SRM 972a standards were tested for accuracy. Passing-Bablock regression was used to assess the comparability of results between methods; a correlation is deemed satisfactory if the linear regression produced a correlation coefficient (r^2) value of >0.90.

7.2.11 Statistical data analysis

Scatterplots, residual plots, box-whisker graphs and Loess curve were constructed and analysed by Statistical Package for the Social Science (SPSS) version 22.0.0.1 (IBM, New York, USA). Passing-Bablock regression analysis and one-way ANOVA were used to establish the inter-quartile range and compare variables. Loess curve fitting was employed to explore nonlinear relationships between variables and identify inflection points. Statistical significance was defined as p <0.05. Frequency distribution histograms of the data were visually examined and checked for transcriptional, pre/post analytical errors before exclusion for statistical analysis. Outliers were identified as outside the 2.5% and 97.5% of the population and removed to establish the Trimmed Mean.

7.2.12 Partitioning for gender

The mean and standard deviation of the data were divided according to gender. Harris and Boyd's standard deviate test[149] was used to determine the need for gender partition.

Deviate test: $Z = \bar{x}2 - \bar{x}1/[(\sigma 1^2/n1) + (\sigma 2^2/n2)]^{1/2}$

$$Z(critical) = 3[(n1 + n2/240)]^{1/2}$$

($\bar{x}$: mean, σ: standard deviation, n: sample size, 1: male, 2: female)

Partitioning is required if the statistical $Z \geq Z(critical)$. In this study, the $Z(critical)$ value is 8.65.

7.2.13 Establishing the reference intervals and cut-off value for clinically significant change

The approach followed for the determination of reference intervals was supported by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) working group[144]. Statistical analysis was performed using SPSS Statistical package, based on non-parametric method, which does not assume Gaussian distribution. Assessment of the lower and upper reference limits were estimated at 2.5th and 97.5th percentiles of the reference distribution. Determination of the cut-off value for clinically significant change was estimated using the Jacobson and Truax method of analysis[150,151]. By categorising the total 24,25$(OH)_2$D concentrations obtained from the cohort of healthy subjects into two subgroups of serum total 25(OH)D concentration 1) less than 50 nmol/L (vitamin D deficient/insufficient) or 2) greater than 50 nmol/L (vitamin D sufficient), the decision cut-off value was calculated as follows:

$$decision\ threshold = \frac{(\bar{x}1 \times \sigma 2) + (\bar{x}2 \times \sigma 1)}{\sigma 2 + \sigma 1}$$

($\bar{x}$: mean, σ: standard deviation, 1: vitamin D deficient/insufficient , 2: vitamin D sufficient group)

The diagnostic sensitivity and specificity of the cut-off was assessed by using the Receiver Operating Characteristics (ROC) curve. In this method, the total 24,25$(OH)_2$D concentrations obtained from the healthy subjects were grouped according to total 25(OH)D concentration of 1: greater (true negatives), or 0: less than 50 nmol/L (true positives). The ROC curve was generated by plotting sensitivity (true positive rate) against 1 – specificity (false-positive rate). The accuracy of the test is measured by the area under the ROC curve (AUC), an AUC value of >0.9 is classified as a highly accurate test.

7.3 Results

Chromatographic peaks for 24,25(OH)$_2$D3, 24,25(OH)$_2$D2, 25(OH)D3 and 25(OH)D2 were eluted at 1.39, 1.42, 1.68 and 1.73 minutes respectively (Fig. 7.2). Total injection-to-injection run time was achieved in 4 minutes. An ion suppression test was conducted by post-column infusion of an aqueous mixture of vitamin D metabolites with simultaneous injection of an extracted pooled serum. Baseline signals were found to be depressed during 0 - 0.8 minutes of the chromatographic run and elevated between 2.8 – 3.2 minutes. A switch valve was employed to divert parts of the sample where ion suppression and ion enhancement occurred to waste.

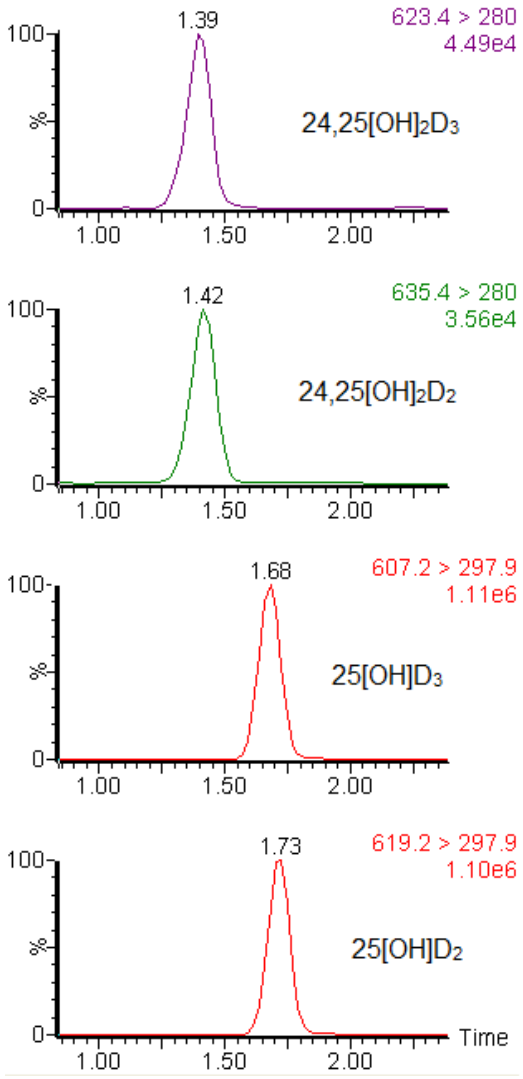


Figure 7.2 Chromatogram from an extracted serum sample containing 64.7 nmol/L of 25(OH)D3, 46.1 nmol/L of 25(OH)D2, 6.4 nmol/L of 24,25(OH)$_2$D3 and 4.5 nmol/L of 24,25(OH)$_2$D2.

7.3.1 Method validation, recovery efficiency and removal of phospholipids

Assay validation is summarised in Table 7.3a-b. The assay achieved adequate linearity (Fig. 7.4a-d), sensitivity, precision, accuracy, and demonstrated reproducible recovery to satisfy method validation criteria. Injections of aqueous blank after running samples of spiked standards containing 1 μmol/L of 24,25$(OH)_2$D3, 24,25$(OH)_2$D2, 25(OH)D3 and 25(OH)D2 affirmed the absence of carry-over of analytes. Injection of a single analyte standard confirmed no cross interference in the other analyte transitions. Levels of phospholipids in the sample matrix were monitored by the acquisition of m/z transitions 522>184 that co-eluted with the vitamin D metabolites (re. Chapter fig. 5.5). Lysophospholipid peak was completely eliminated in samples extracted using the supported liquid extraction method.

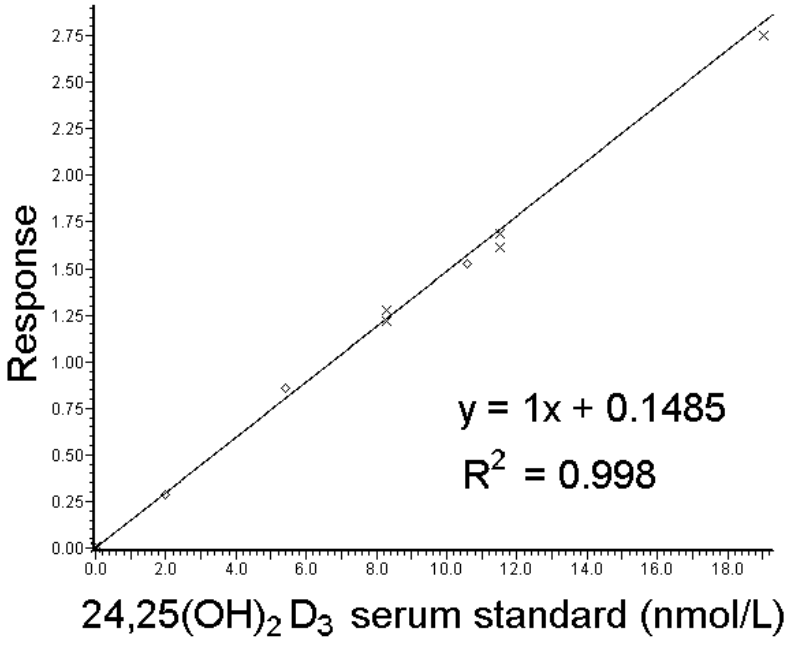
	24,25$(OH)_2$D3	**24,25$(OH)_2$D2**	**25(OH)D3**	**25(OH)D2**
Linearity, nmol/L	0-25	0-25	0-250	0-250
Typical r^2	0.985	0.983	0.997	0.995
Intra assay imprecision mean (±SD) in nmol/L, %CV	1.9 (±0.19) 10.3% 5.8 (±0.44) 7.6%	2.1 (±0.16) 7.6% 6.5 (±0.5) 7.7%	32.5 (±3.0) 9.3% 88.1 (±5.7) 6.5% 180.4 (±16.5) 9.1%	24.8 (±1.6) 6.6% 64.9 (±3.2) 4.9% 140.6 (±4.7) 3.3%
Inter assay imprecision mean (±SD) in nmol/L, %CV	2.0 (±0.21) 10.5% 6.1 (±0.51) 8.3% 14.8 (±1.16) 7.9%	1.8 (±0.20) 11% 5.7 (±0.61) 10.7% 19.4 (±1.95) 10.0%	27.1 (±2.9) 10.9% 66.2 (±6.0) 9.1% 169.7 (±15.2) 8.9%	25.0 (±2.0) 8.0% 72.3 (±5.7) 7.9% 148.9 (±9.4) 6.3%
LLoQ, nmol/L	0.1	0.8	0.1	0.1
Bias against NIST 972a values, mean (range)	-1.2% (-7.5 to +4.4%)	-	-0.4% (-7.5 to +2.6%)	+3.3% (+1.5 to +5%)

Table 7.3a LC-MS/MS assay characteristics.

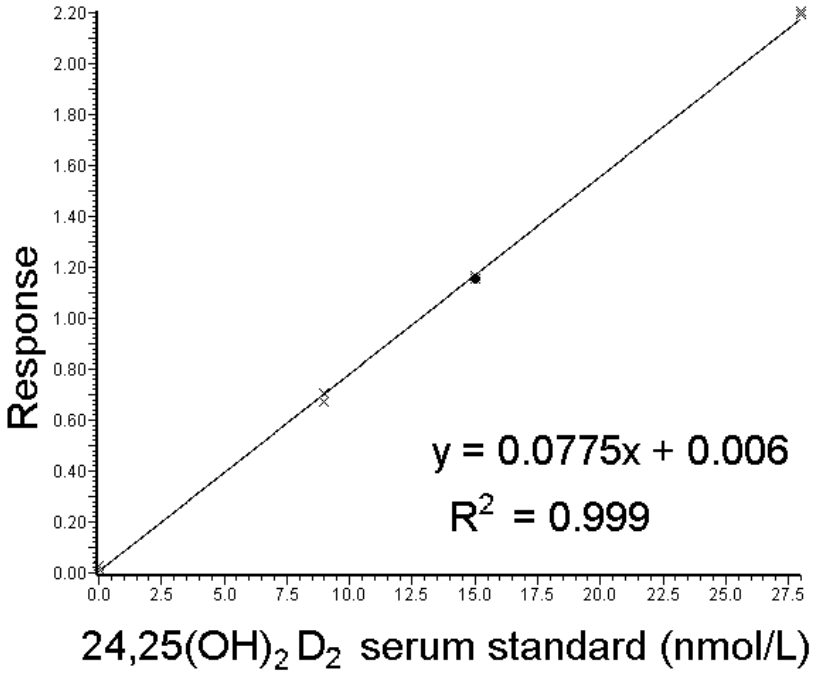
Sample matrix + concentration spiked in nmol/L	Mean % recovery (±SD)			
	24,25(OH)$_2$D3	**24,25(OH)$_2$D2**	**25(OH)D3**	**25(OH)D2**
Depleted serum + 1.2	97% (±4)	108% (±3)	96% (±2)	102% (±4)
Depleted serum + 12.2	94% (±4)	97% (±2)	101% (±4)	107% (±2)
Depleted serum + 250	-	-	103% (±1)	95% (±4)
Pooled EDTA plasma + 1.2	96% (±5)	107% (±3)	99% (±2)	94% (±3)
Pooled EDTA plasma + 12.2	103% (±3)	99% (±2)	91% (±2)	104% (±2)
Pooled EDTA plasma + 250	-	-	98% (±2)	101% (±3)

Concentrations of pooled EDTA plasma: 14.3 nmol/L 25(OH)D3, 10.2 nmol/L 25(OH)D2, 1.3 nmol/L 24,25(OH)$_2$D3, 1.2 nmol/L 24,25(OH)$_2$D2.

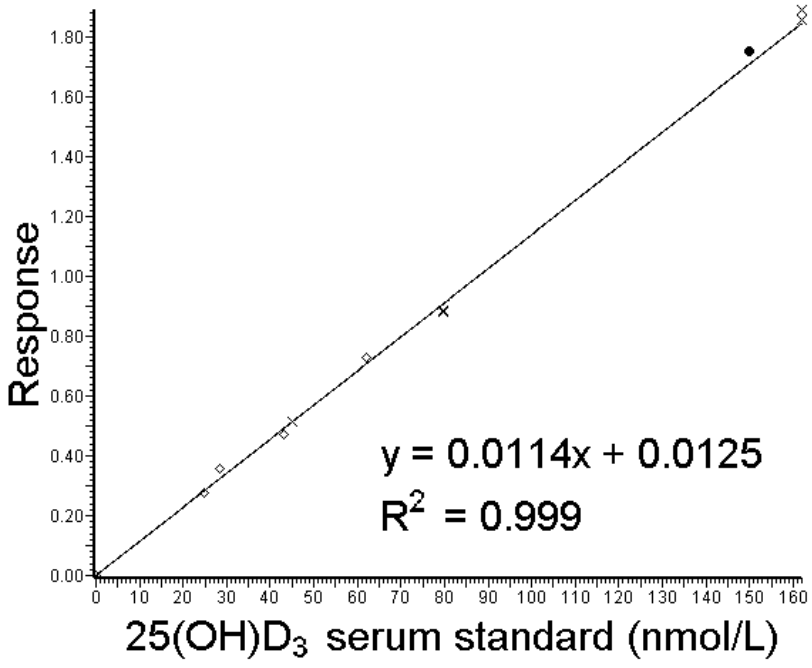
Table 7.3b Recovery efficiency was determined by spiking known quantities of each compound into vitamin D depleted serum and into a base pool of EDTA plasma containing endogenous vitamin D metabolites. Each spiked sample was aliquoted and analysed over ten separate runs.



a)


Response
y = 0.0775x + 0.006
R2 = 0.999
24,25(OH)2 D2 serum standard (nmol/L)

b)


Response
y = 0.0114x + 0.0125
R2 = 0.999
25(OH)D3 serum standard (nmol/L)

c)

d)

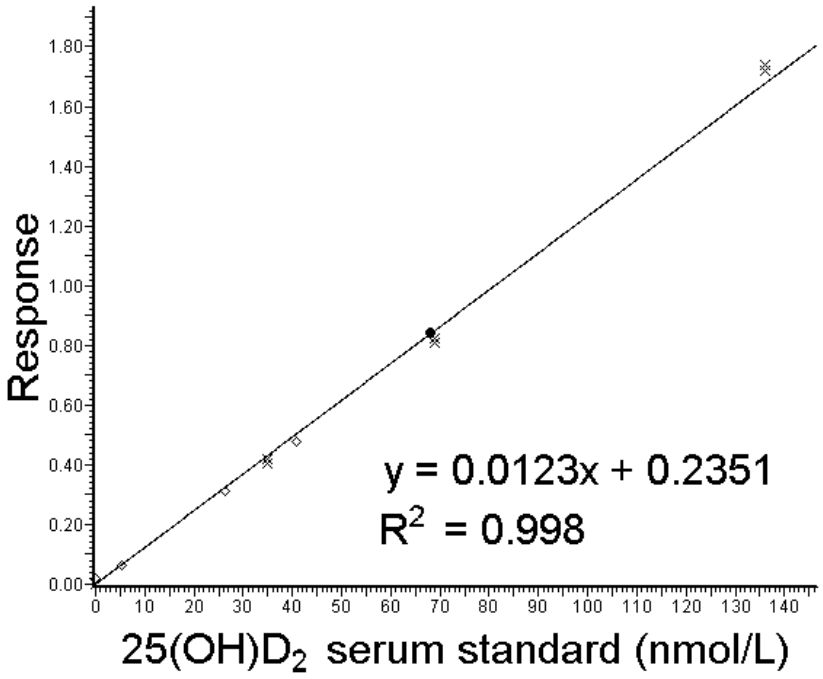
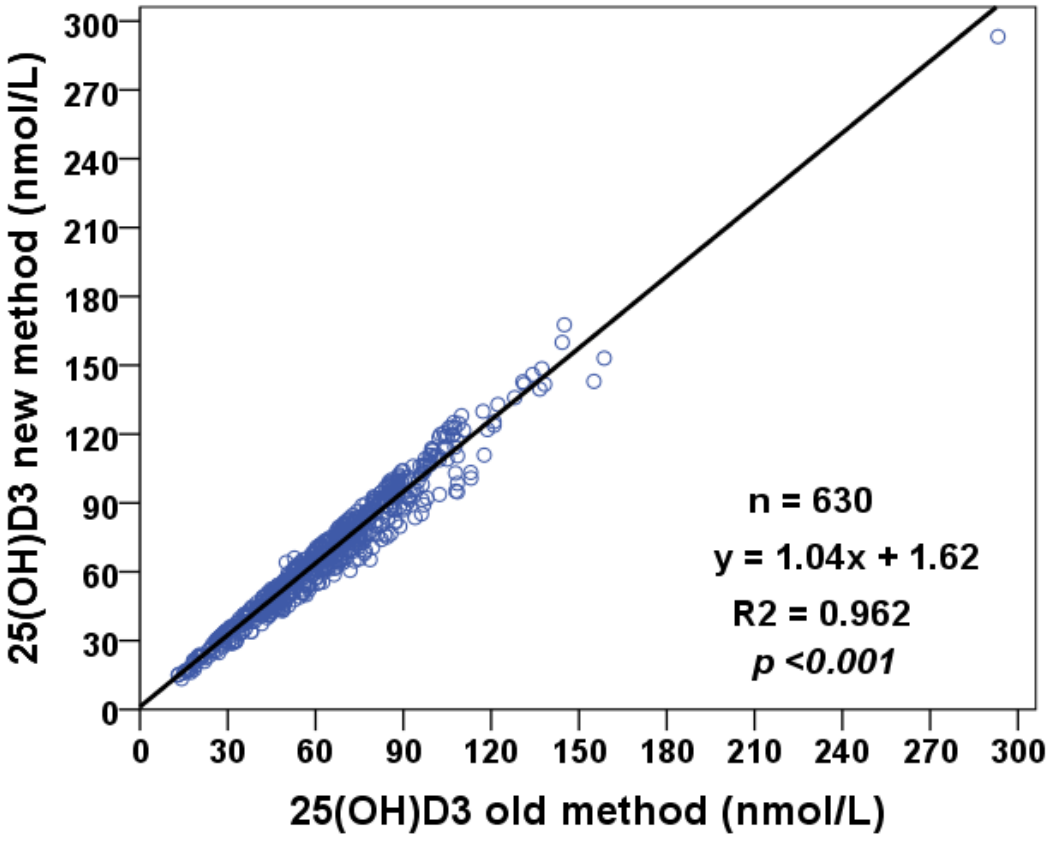


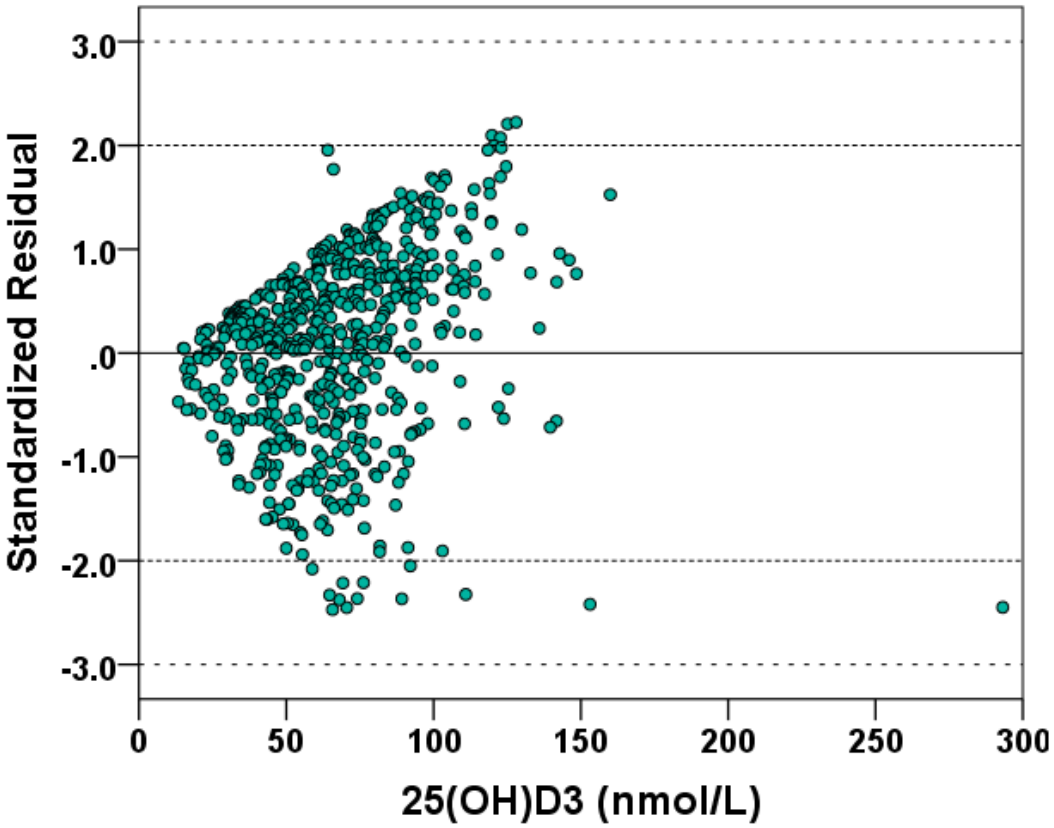
Figure 7.3a-d Typical standard curves constructed by plotting the response of each standard on the y-axis against their respective concentrations (nmol/L) on the x-axis. The response of each compound was determined by the ratio of the peak area of the standards to the peak area of the deuterated internal standards. Regression analysis showed a typical correlation coefficient r2 > 0.99.

7.3.2 Method comparisons

To assess the comparability of the new method, 630 serum samples previously analysed for 25(OH)D3 by the non-derivatised LC-MS/MS method were retested using the new method. Passing-Bablock regression analysis (Fig. 7.5a) showed a strong correlation with the previous method; the line of best fit generated a slope of 1.04 (r^2 = 0.962, p<0.001). Residual plot (Fig. 7.5b) showed no systematic bias. Evaluation of variability in measurements of 24,25$(OH)_2$D3 was performed by comparing the results produced from DEQAS samples against mean concentrations submitted by other participants. Passing-Bablock Regression (Fig. 7.6a) and residual plots (Fig. 7.6b) illustrated the correlation and difference in values measured by the new method with other registrants of DEQAS. A significant linear association (r^2 = 0.926, p<0.001) was found and, the residual plot showed no systematic bias or concentration-dependent variance between the methods.

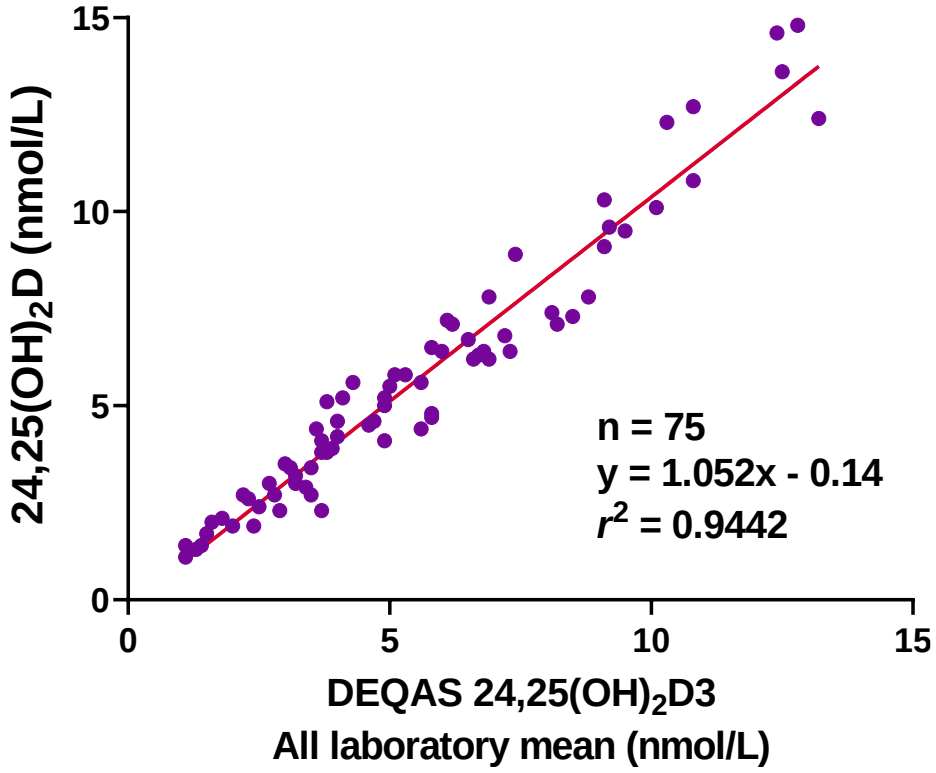


a)

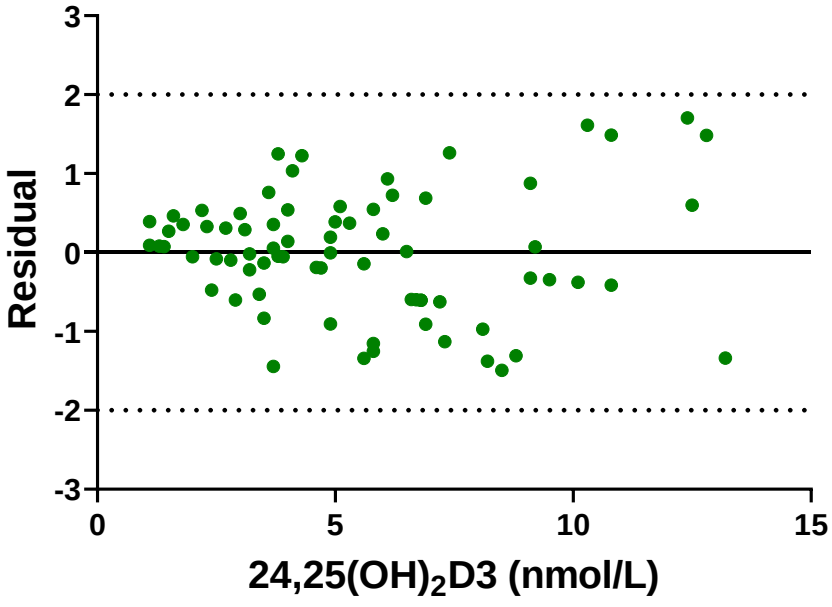


b)

Figure 7.5a-b **(a)** Comparison of 25(OH)D3 of the new method (n=630) showing a strong correlation (r^2 =0.962) with the previous method. **(b)** Standardised residual plot showing the variability of 25(OH)D3 measurements between the new and previous method. The dashed lines represent ±SD limits of agreement.



a)



b)

Figure 7.6a-b **(a)** Comparison of 24,25(OH)$_2$D3 values submitted to DEQAS between April 2015 to Jan 2019 (n=75) with other DEQAS participants. The red line in the graph represents the fitted regression line. **(b)** Standardised residual plot showing the variability of 24,25(OH)$_2$D3 measurements. The dashed lines represent 95% limits of agreement. All values were within ±2SD.

7.3.3 Partitioning for gender

The healthy male and healthy female groups were subjected to the Harris and Boyd deviate test. The Z values determined for total 25(OH)D, total 24,25$(OH)_2$D, and 25OHD:24,25$(OH)_2$D ratio were 1.61, 4.0 and 5.86, respectively. Each calculated Z value is $\leq Z(critical)$ value of 8.65. Therefore, partitioning for gender is not required in establishing the reference intervals.

7.3.4 Reference intervals in a healthy population

Samples from healthy individuals with plasma intact parathyroid hormone (PTH) or adjusted serum calcium concentration (ACa) of greater or less than the healthy reference range (PTH: 1.6-6.9 pmol/L, ACa 2.2-2.6 mmol/L) were excluded. Based on the exclusion criteria, 1996 samples (1492 male, 504 female, age 18-32yrs, mean BMD T-score 1.2±0.1) were included in the statistical study. The upper 97.5th and lower 2.5th reference limits for total (sum of D3 + D2) 25(OH)D, 24,25$(OH)_2$D, and 25OHD:24,25$(OH)_2$D ratio were established according to the non-parametric method of analysis (Table 7.4). No outlier was found. The ROC curve (Fig. 7.7) produced an AUC value of 0.943, which classified 24,25$(OH)_2$D as an excellent test to discriminate individuals with vitamin D insufficiency from adequate status.

Parameters	**Total cohort (n = 1996)**	**Reference interval (2.5-97.5 percentile)**	**Diagnostic cut-off for vitamin D replete**
24,25$(OH)_2$D, nmol/L	0.4 - 51.1	1.1 - 13.5	>4.2*
25(OH)D, nmol/L	6.9 – 293.7	18.9 – 126.4	
25(OH)D: 24,25$(OH)_2$D ratio	2 - 59	7 - 23	

*At sensitivity = 88.8%, specificity 86.3%

Table 7.4 Summary of reference intervals and cut-off values for adequate vitamin D status.

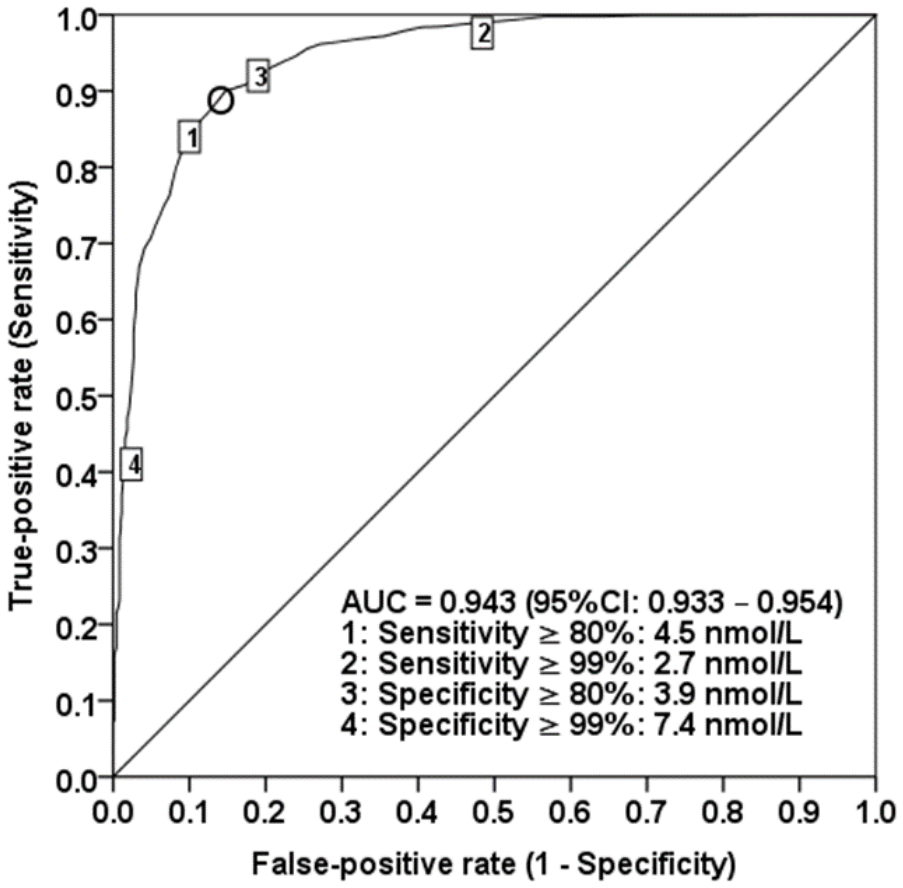


Figure 7.7 Diagnostic performance of 24,25$(OH)_2$D in the assessment of vitamin D status. Receiver Operating Characteristic (ROC) curve depicts diagnostic performance sensitivity and specificity levels. (O) represents the decision threshold for vitamin D replete at 24,25$(OH)_2$D concentration of 4.2 nmol/L (sensitivity = 88.8%, specificity = 86.3%). The diagonal line is the line of no discrimination.

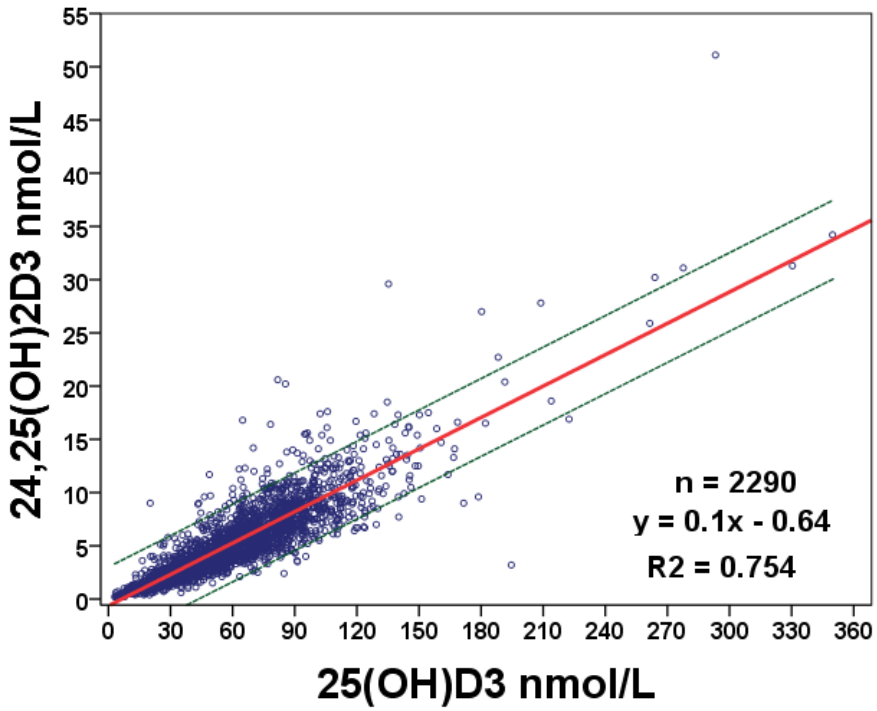
7.3.5 24,25$(OH)_2$D cut-off value for clinically significant change in vitamin D status.

Determination of the cut-off value for clinically significant change using the Jacobson and Truax[150,151] method approach has estimated total 24,25$(OH)_2$D of >4.2 nmol/L to be the threshold value for vitamin D replete status, i.e. serum total 25(OH)D concentration ≥50 nmol/L. The decision cut-off has a true positive rate (sensitivity) of 88.8%, and false positive rate (specificity) of 86.3%, as indicated by the ROC curve (Fig. 7.7).

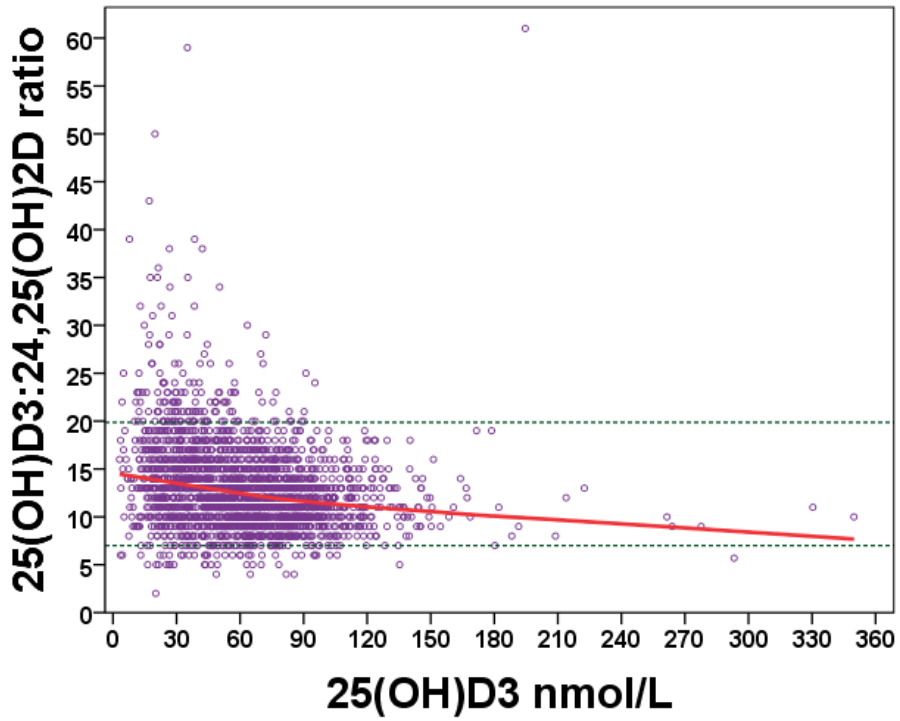
7.3.6 Relationship between serum 25(OH)D, 24,25$(OH)_2$D and 25(OH)D:24,25$(OH)_2$D ratio among the healthy and patient population

In total, 2290 samples collected from healthy volunteers (n = 1996) and patients (n = 294) were analysed for serum vitamin D metabolites and included in the statistical analysis. 25(OH)D3 was

quantified in all samples with a concentration range (mean±SD) between 3.1 – 349.8 nmol/L (62±33.2 nmol/L), from which 24,25$(OH)_2$D3 were quantified in 99.8% of samples. 24,25$(OH)_2$D3 concentrations ranged between 0.2 – 51.1 nmol/L (5.5±3.7 nmol/L). 25(OH)D2 was present in 12.9% of samples, with a range between 0.1-74.5 nmol/L (5.4 ±9.5 nmol/L), of which 1.1% contained 24,25$(OH)_2$D2 concentrations between 1.3 – 5.0 nmol/L (2.4 ±1.1 nmol/L). Statistical analyses were performed on total (sum of D3 + D2) values, the distributions were untrimmed, and no outlier was removed. A concentration-dependent relationship was observed between serum 24,25$(OH)_2$D and 25(OH)D (Fig. 7.8a), linear regression analysis indicated [24,25$(OH)_2$D] = 0.1 x [25(OH)D] -0.64; r^2 value of 0.754. In contrast, the ratio of 25(OH)D:24,25$(OH)_2$D (Fig. 7.8b) showed an indirect relationship with 25(OH)D; Loess fitting showed a downward trend in the ratio of 25(OH)D:24,25$(OH)_2$D as serum 25(OH)D concentrations increased. Analysis of variance between the subgroups showed 25(OH)D and 24,25$(OH)_2$D concentrations in patients were significantly lower than healthy individuals (p<0.001), whereas the relative ratio of 25(OH)D:24,25$(OH)_2$D was significantly higher in patients (p<0.001). The Box-whisker plots of total 25(OH)D:24,25$(OH)_2$D ratio against serum total 25(OH)D grouped into eight concentration categories with an equal number of cases (Fig. 7.9) revealed a highly significant increase (p<0.001) in ratio occurred at 25(OH)D concentration below 50 nmol/L.



a)



b)

Figure 7.8a-b The relationship of serum total 25(OH)D with **(a)** serum total 24,25(OH)2D, (b) total 25(OH)D:24,25$(OH)_2$D ratio. Sample from a cohort of mixed patients and healthy individuals (n = 2290). The solid red line in **(a)** represents the linear regression line, with 95% confidence intervals (dashed lines). Loess fitted curve in **(b)** depicted in the solid red line (99% point fit), dashed lines represent the lower and upper reference intervals (7-23).

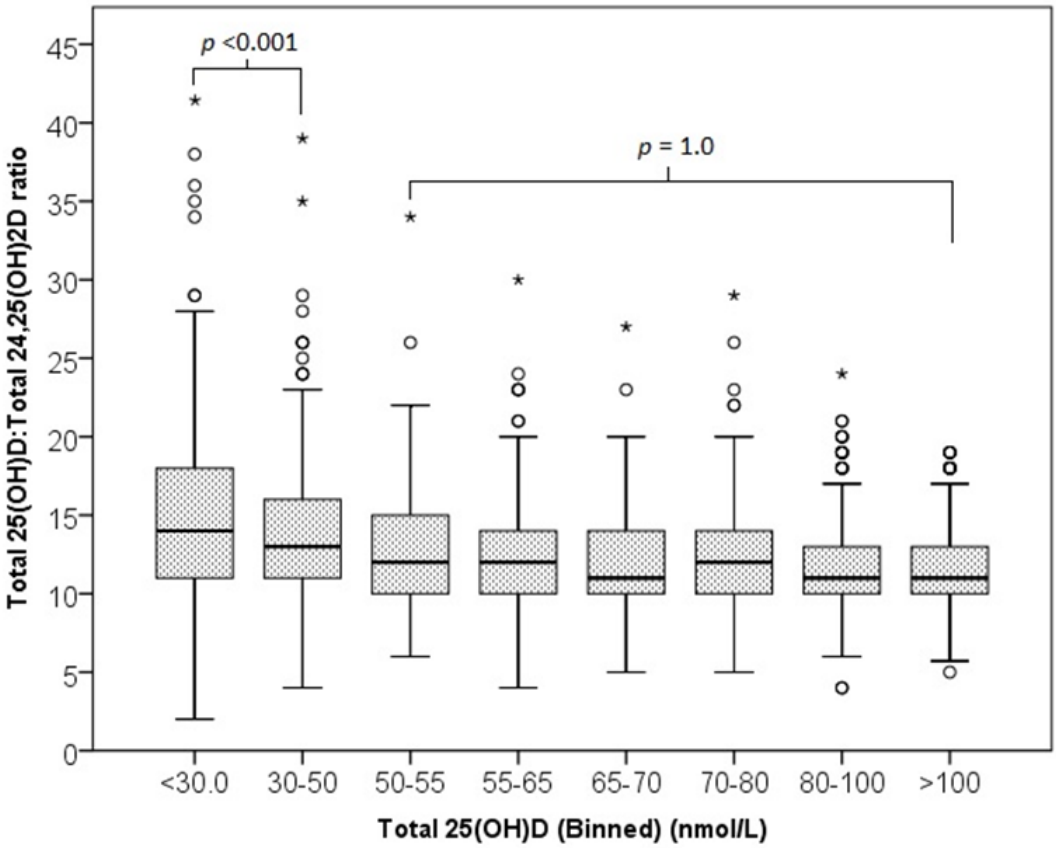


Figure 7.9 25(OH)D:24,25(OH)$_2$D ratio profile of 1996 healthy subjects against 25(OH)D bins. Each bin contains an equal number of subjects to illustrate the significantly elevated ratio found in those with serum 25(OH)D <50 nmol/L. Box and whiskers represent the median, interquartile range and 95% population intervals.

7.3.7 CYP24A1 mutation on patients with elevated 25(OH)D:24,25(OH)$_2$D ratio.

Using the established reference intervals, a sample from the patient group was identified with an elevated 25(OH)D:24,25(OH)$_2$D ratio of 32; clinical records revealed the patient presented with hypercalcaemia and elevated serum 1,25(OH)D of 293 pmol/L (reference range 43-144 pmol/L). Retrospective genetic analysis confirmed biallelic pathogenic mutations in *CYP24A1* (c.823T>C, p.W275R and c.1315C>T p.R439C).

7.4 Discussion and conclusion

This chapter describes a robust LC-MS/MS assay for simultaneous measurement of 24,25$(OH)_2$D3, 24,25$(OH)_2$D2, 25(OH)D3 and 25(OH)D2. Sample clean-up using supported liquid extraction (SLE) and PTAD derivatisation first described in Chapter 5 enabled detection of 24,25$(OH)_2$D3 and 24,25$(OH)_2$D2. Assay sensitivity is paramount in order to achieve the detection limit required to measure 24,25$(OH)_2$D; particularly in CYP24A1 deficient patients with low total 25(OH)D. The new method has demonstrated good recovery, intra/inter-assay precision, linearity and accuracy against international reference standards. Certified standards spiked into vitamin D depleted human sera were used to maintain matrix composition between calibration standards and samples. Evaluation of the DEQAS 24,25$(OH)_2$D3 returns from 2015-2019 distributions were compared against the mean values from all participants. The new assay showed good agreement with other methods; all returned values were within ±2SD of the mean concentration from other laboratories. The author must stress that the small number of participants currently in the DEQAS 24,25$(OH)_2$D3 scheme and the disparity in results between laboratories undermine the reliability of the statistics. NIST standard reference materials used in this study provided a mean to assess method accuracy; however the certified values for 24,25(OH)D3 were limited between 3.39-6.38 nmol/L[77]. The availability of reference materials covering a wider concentration range would improve method accuracy and commutability of results between laboratories.

The strong association between serum concentrations of 25(OH)D and 24,25$(OH)_2$D demonstrated in this study echo the findings from the previous reports[37,42,53,55,143]. The concentrations of 24,25$(OH)_2$D exhibited a positive, concentration-dependent relationship, which was on average 10% of the concentrations of 25(OH)D. A significant increase in 25(OH)D:24,25$(OH)_2$D ratio at serum 25(OH)D concentration below 50 nmol/L was observed. The decrease in relative production of 24,25$(OH)_2$D may result from downregulation of CYP24A1 in response to the decline in circulating 25(OH)D3, in order to facilitate catabolism to 1,25(OH)D. The reverse is observed in individuals with high concentrations of serum 25(OH)D; where production favours 24,25(OH)D at the expense of 1,25$(OH)_2$D which may act to prevent toxicity.

The usefulness of 24,25$(OH)_2$D measurement as a surrogate marker of vitamin D status was investigated. It showed high diagnostic accuracy. The 24,25$(OH)_2$D cut-off value of 4.2 nmol/L can predict an individual's vitamin D status with good diagnostic sensitivity and specificity; a value below the cut-off indicating insufficiency and the likelihood of metabolism favouring 1,25$(OH)_2$D; whereas a value above the cut-off indicates sufficient vitamin D status with 25(OH)D catabolism likely to favour 24,25$(OH)_2$D production.

The clinical utility of dual measurements to obtain absolute values as well as a ratio of 25(OH)D to 24,25$(OH)_2$D is valuable not only in the assessment of vitamin D catabolic status but also to provide an assessment of CYP24A1 activity. Based on a cohort of healthy young adult subjects, the normal reference interval for 25(OH)D:24,25$(OH)_2$D ratio was defined between 7 to 23. In comparison with current publications, the upper limit is lower than those observed by others[42,142,143], which may be reflective of the subject population, selection criteria, and analytical method employed in this study. The reference interval provides an indication of an individual's vitamin D catabolism with a ratio value below 7 indicating elevated activity of 24-hydroxylase. Such individuals may be at risk of developing resistance to vitamin D treatment. A ratio above 23 indicates enzyme under activity and possible genetic abnormalities of *CYP24A1*. One patient in the cohort was detected with an elevated ratio (32) and hypercalcaemia who on genetic testing turned out to have a biallelic mutation of *CYP24A1*. Molin et al.[43] reported patients with heterozygous or biallelic mutations of *CYP24A1* are associated with 25(OH)D:24,25$(OH)_2$D ratios >80; whereas patients with a single mutant allele or no *CYP24A1* mutation have ratio values <25. Moderate elevation of 25(OH)D:24,25$(OH)_2$D ratio may be a result of substrate depletion from underlying diseases caused by renal impairment[53], bone disorders[57] or pathological conditions associated with an increased concentration of FGF23[56], all of which affect calcium and phosphate homeostasis resulting in an imbalance state of vitamin D catabolism. Detection of an elevated 25(OH)D:24,25$(OH)_2$D ratio, especially in patients with nephrocalcinosis or renal stones, may indicate a requirement to restrict or avoid vitamin D supplementation in order to prevent hypercalcaemia and further stone formation.

In conclusion, a robust LC-MS/MS method for the simultaneous measurement of 25(OH)D and its metabolite 24,25$(OH)_2$D have been successfully developed. The reference intervals of 24,25$(OH)_2$D and 25(OH)D:24,25$(OH)_2$D ratio was determined from serum analysis of a large cohort of healthy young adults. The 25(OH)D:24,25$(OH)_2$D ratio exhibited a remarkable consistency across the entire range of 25(OH)D concentrations. A significant increase in 25(OH)D:24,25$(OH)_2$D ratio in individuals with insufficient 25(OH)D was observed. The established reference intervals for 25(OH)D:24,25$(OH)_2$D ratio can help clinician in the investigation of patients presenting with diseases and conditions resulted from loss of CYP24A1 activity and help optimise supplementation therapy based on individual differences in serum vitamin D metabolite concentration ratio in order to achieve the best response.

Chapter 8 Profiles of 25 hydroxyvitamin D and its metabolites 24,25-dihydroxyvitamin D and 1,25-dihydroxyvitamin D in vitamin D3 supplementation studies

8.1 Introduction

Vitamin D deficiency continues to be the subject of public health concerns. The lack of sunlight (UVB) exposure for the synthesis of vitamin D and the limited amount of vitamin D intake from diet lie behind the concerns about vitamin D deficiency in the UK. In 2016, the Scientific Advisory Committee of Nutrition (SACN) recommended that all adults in the UK have 400 IU daily vitamin D[12], leading to the Public Health England recommendation that the general population should consider taking vitamin D supplements in the winter. This recommendation was based on structured literature reviews and metanalysis of population observational studies establishing beneficial associations between vitamin D status and many health outcomes; and intervention with vitamin D supplementation. Whilst the evidence on vitamin D deficiency-associated negative impacts on health outcomes are overwhelming, vitamin D intervention and dose-ranging studies have been less convincing, therefore questioning the role of vitamin D in health[152]. In clinical settings, it is common practice to administer high doses of vitamin D to patients presented with osteomalacia and vitamin D deficiency to ameliorate symptoms[153]. In professional sports, it is commonplace to supplement athletes with vitamin D supplementation, often without basal concentrations being assessed. Perhaps even more concerning is the use of single, ultra-high dose vitamin D3 capsules up to 50,000 IU (1250 μg), being used on a weekly or monthly basis, to raise serum 25(OH)D level and achieve the perceived beneficial effects in a short amount of time. Such practices could lead to vitamin D toxicity and increase the risks of developing hypercalcaemia, hypercalciuria and renal stone formation[154].

Using vitamin D3 supplement to achieve a target 25(OH)D concentration is often the accepted approach in intervention studies. Studies have shown individuals with lower baseline 25(OH)D concentration (≤30 nmol/L) given vitamin D3 supplement benefitted from greater outcomes[155,156]. On the contrary, Smith et al. have shown that higher doses of up to 4800 IU/day increase the incidence of falls in elderly women[157]. The confounding effects of vitamin D supplement prompt for a better understanding of the metabolism of 25(OH)D, particularly in the active and catabolic

forms of vitamin D metabolites. 1,25$(OH)_2$D is the active, hormonal form and has the highest biological activity among the circulating vitamin D metabolites. Insufficient clinical studies focused on the catabolic form 24,25$(OH)_2$D, which circulates at 1/10 of the concentration of 25(OH)D[54], and is the first step of the degradation pathway (Fig. 8.1). 24,25$(OH)_2$D is converted from 25(OH)D by enzyme 24-hydroxylase, the production of which is stimulated by the expression of CYP24A1 (cytochrome P450, family 24, subfamily A, polypeptide 1) found in most tissues in the body. The same enzyme also converts 1,25$(OH)_2$D into 1,24,25-trihydroxyvitamin D (1,24,25$(OH)_3$D); another key exit point in the vitamin D excretion pathway. Studies suggest the production of 1,25$(OH)_2$D from 25(OH)D is 24,25$(OH)_2$D dependent[37,55,136]. Genetic mutations of the CYP24A1 gene resulting in the reduced or total loss of 24-hydroxylase function are associated with hypercalcaemic conditions[158-160]. Measurement of serum 24,25$(OH)_2$D can indicate CYP24A1 activity, and the vitamin D catabolic status of an individual.

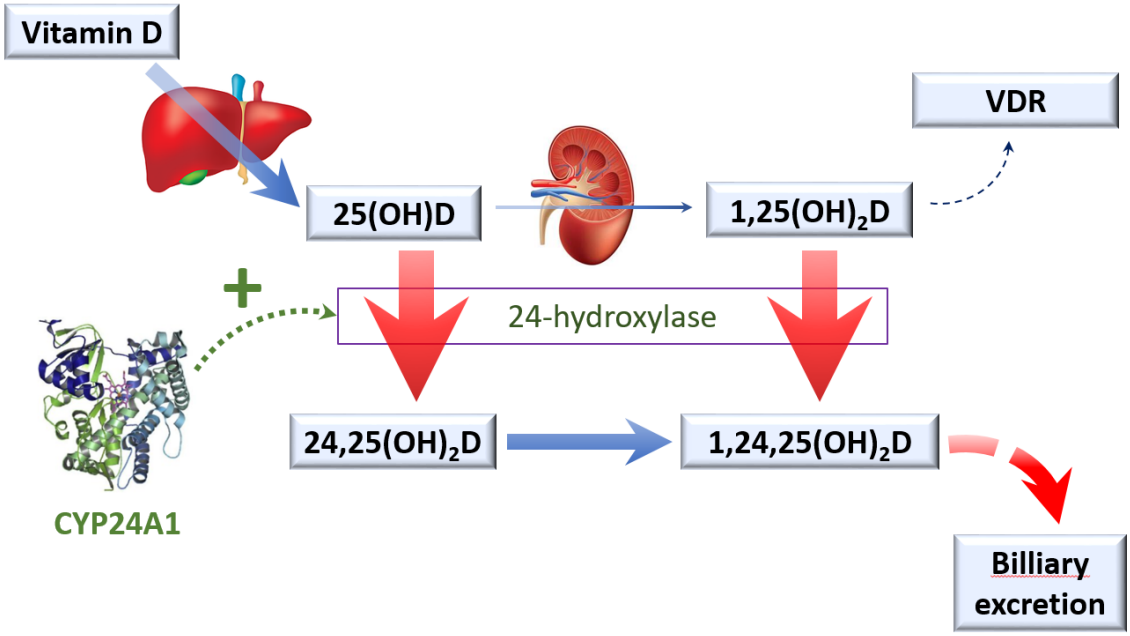


Figure 8.1 CYP24A1-induced conversion of 25(OH)D and 1,25$(OH)_2$D by enzyme 24-hydroxylase, indicated by red arrows.

This chapter presents the profile of vitamin D metabolite measurements from three vitamin D3 supplementation studies; with contrasting dosing regimen where participants were supplemented with either single high bolus, high weekly or a low daily dose of vitamin D3. The current study is aimed to characterise the serum responses of the major vitamin D metabolites; 25(OH)D, 1,25$(OH)_2$D3, 24,25$(OH)_2$D, and the 25(OH)D:24,25$(OH)_2$D vitamin D metabolite ratio (VMR)

to oral vitamin D supplementation. The previous chapter (ch. 7.3.6) described in non-supplemented individuals, serum concentration of $24,25(OH)_2D$ is strongly dependent upon 25(OH)D. When the vitamin D status become insufficient, serum $24,25(OH)_2D$ concentration is reduced significantly. The reverse is hypothesised that supplementation would increase serum concentrations of the three vitamin D metabolites, but a greater difference in $24,25(OH)_2D$ response to high than low doses.

8.2 Materials and methods

The following three vitamin D supplementation studies carried out between 2013-2017 were led by Dr Lanja Saleh, University Hospital of Zurich, Switzerland (study 1[161]), Prof. Graeme Close, Liverpool John Moores University (study 2[162]), and Prof. Helen MacDonald, University of Aberdeen (study 3[163]). I was a major contributor and project co-investigator alongside Prof. William Fraser in all three studies. I performed samples analysis using the LC-MS/MS method described in Chapter 7 for measurements of 25(OH)D and $24,25(OH)_2D$[54]. Measurement of total $1,25(OH)_2D$ were performed using an enzyme immunoassay kit (IDS, Boldon, UK) according to the manufacturer instructions.

8.2.1 Study 1 – A prospective, randomised, double-blinded, placebo-controlled study of single 100,000 IU bolus dose in healthy adults with vitamin D deficiency/insufficiency

Study design

The clinical study was conducted at the University Hospital of Zurich, Switzerland (latitude 47°22′ N) (ClinicalTrials.gov Registry number NCT02022475) in accordance with the declaration of Helsinki and Good Clinical Practice guidelines; the study protocol was approved by the Zurich Cantonal Ethical Committee and Swissmedic. Informed consent was obtained from all participants before enrolment.

Participants (n = 107) [age 20–50 years, body mass index (BMI) 18–25 kg/m2], who were identified at a screening visit with low vitamin D status (serum 25(OH)D3 <50 nmol/L, in accordance with the recommendation of the Institute of Medicine[11]), were randomised to receive either a single 100,000 IU oral dose of vitamin D3 (n = 52) or placebo (n = 55). Blood samples were obtained immediately before treatment and at four weeks after supplementation. All blood samples were collected into serum gel separator tube (BD Vacutainer) and centrifuged

immediately at 3,000 x g, serum layer was aliquoted into a separate polystyrene tube and stored at -80°C until analysis. All samples were anonymised to the researchers at the point of access.

8.2.2 Study 2 – Prospective, randomised, 35,000 vs 70,000 IU/week over 12 weeks in elite athletes

Study design

Elite male professional team sport athletes (n = 42) volunteered to participate in the study (mean ±SD; age 26±3 yr, height 1.86±0.6 m, weight 101.5±11 kg, fat mass 11.4%±3%). Participants underwent a medical screening and provided full informed consent before inclusion into the study. Volunteers who self-reported to be taking vitamin D supplements, using sunbeds, or injured at the time of the study were excluded from the study. Ethical approval was granted by the ethics committee of Liverpool John Moores University (Ethics code 12/SPS/047). The study was conducted at latitude 52°N during the winter months to limit sunlight exposure.

Participants were randomly allocated to either 35,000 or 70,000 IU per week supplement of vitamin D3 (Maxi Nutrition, UK). The supplemental doses chosen represent widely reported supplement strategies in elite sporting settings. Weekly supplements were taken orally as a bolus in capsule form, ingestion of capsules was monitored by the club staff to track compliance. Supplementation continued for 12 weeks at which point supplementation was ceased to monitor the response of vitamin D metabolites to withdrawal. Sunlight exposure was minimal during the trial because of the latitude at which the participants were based. Blood samples were collected before supplementation, at 6, 12, and 18 weeks (withdrawal). Blood was collected in serum gel separator tube (BD Vacutainer) and then separated by centrifugation at 3,000 x g for 15 min at 4°C. Serum extracted was stored at -80°C until analysis.

8.2.3 Study 3 - One year prospective 400 and 1000 IU/day in postmenopausal women

Study design

The VICtORy (Vitamin D and cardiovascular risk) randomised controlled trial (RCT) was conducted at the University of Aberdeen, UK; ethical permission was obtained from Grampian ResearchEthics Committee (08/S0802/73), and the follow-up RECALL study was provided by North of Scotland Research Ethics Committee (12/NS/0013). All participants gave informed consent.

Participants (n = 287) were healthy, non-smoking postmenopausal women (mean±SD; age 64.7±2.1 yr) residing in the northeast of Scotland (latitude 57°N). The women were randomised to receive either a daily dose of 400 IU, 1000 IU of vitamin D3 or placebo for 12 months. Blood samples were collected at 0, 2, 4, 6, 8, 10, 12 and 13 mth (withdrawal). The participants were invited for the RECALL visit at 2 and 3 years after the original study had ended. Those who took part (n = 159, mean±SD; age 67.6±2.3 yr) confirmed that they had not taken vitamin D supplements since the supplementation study concluded and did not take extended holidays abroad >1 month before the RECALL visits. Results of the VICtORy RCT showed no difference in markers of cardiovascular risk, inflammation, glucose tolerance (homeostatic model assessment) and blood pressure after one year between the two treatment groups[164].

8.2.3 Statistical analysis

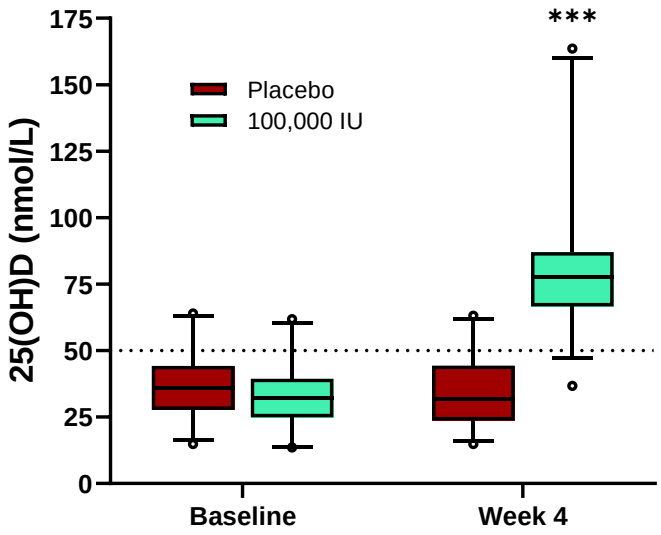
All statistical analyses and Box-whisker plots were constructed and analysed by GraphPad Prism 7 (GraphPad, San Diego, CA, USA). Frequency distribution histograms of the data were visually examined and checked for transcriptional, pre/post analytical errors before exclusion for statistical analysis. Outliers were identified as outside the 2.5% and 97.5% of the population and removed from statistical analysis. Non-parametric two-tailed t-test was used to determine the difference between groups. Statistical significance was defined as $p < 0.05$.

8.3 Results

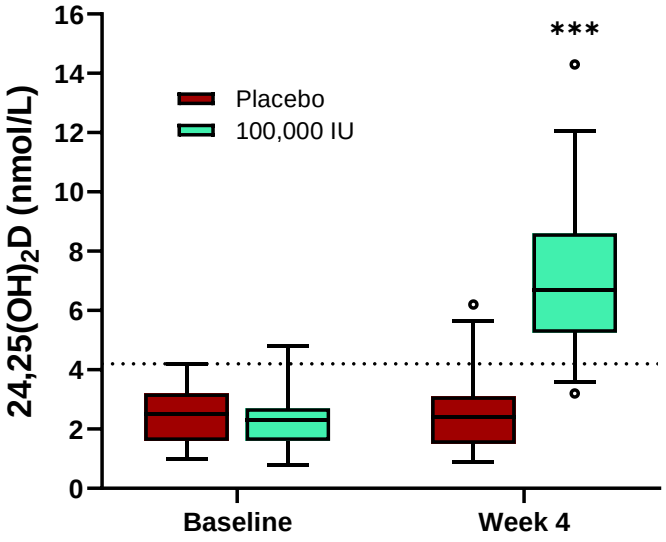
Data analyses on 25(OH)D and 24,25$(OH)_2$D were performed on the respective total (sum of D3 + D2) values. Immunoassay for 1,25$(OH)_2$D cross-reacts 100% with 1,25$(OH)_2$D3 but under recovers 1,25$(OH)_2$D2 at 39%. As none of the study samples was found to have significant 25(OH)D2 and 24,25$(OH)_2$D2 concentrations; effects from assay under-recovery was deemed negligible.

8.3.1 Study 1

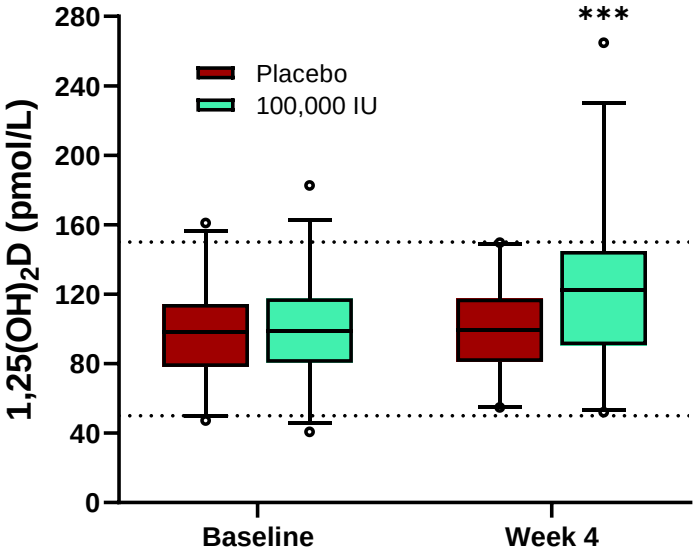
Vitamin D metabolites concentrations at baseline and 4 weeks after treatment with placebo (n = 52) or a single oral dose of 100,000 IU vitamin D3 (n = 55) are presented in Figure 8.2. Increase in serum concentrations of vitamin D metabolites at week 4 in the supplement group was highly significant ($p<0.001$). The majority of participants in both groups began with vitamin D deficient/insufficient status (25(OH)D <50 nmol/L). Those received a single dose of 100,000IU had achieved 25(OH)D >50 nmol/L after 4 weeks; whereas no significant changes were observed in the placebo group (Fig 8.2a). The response to high dose vitamin D3 in the supplement group resulted in an average 2.4-fold increase in 25(OH)D (mean±SD; 33.4 ±11.7 to 80.2 ±20.8 nmol/L) and 24,25$(OH)_2$D concentrations (mean±SD; 2.4 ±1.0 to 6.9 ±2.0 nmol/L)(Fig. 8.2b), and a 1.3-fold increase in 1,25$(OH)_2$D (mean±SD; 99.1 ±27.4 to 125.1 ±42.2 pmol/L) (Fig. 8.2c) compared to baseline. Of those participants received the high dose treatment, 21% had elevated 1,25$(OH)_2$D above the upper reference interval of 150 pmol/L. Although 25(OH)D and 24,25$(OH)_2$D showed a similar average increase, the 25(OH)D:24,25$(OH)_2$D VMR in the supplement group showed a significant decrease (mean±SD; 15 ±3 to 12 ±3, $p<0.001$) (Fig. 8.2d), suggesting the increase in serum 24,25$(OH)_2$D is relatively higher than 25(OH)D.


175
150
125
100
75
50
25
0
25(OH)D (nmol/L)
Placebo
100,000 IU
***
Baseline
Week 4

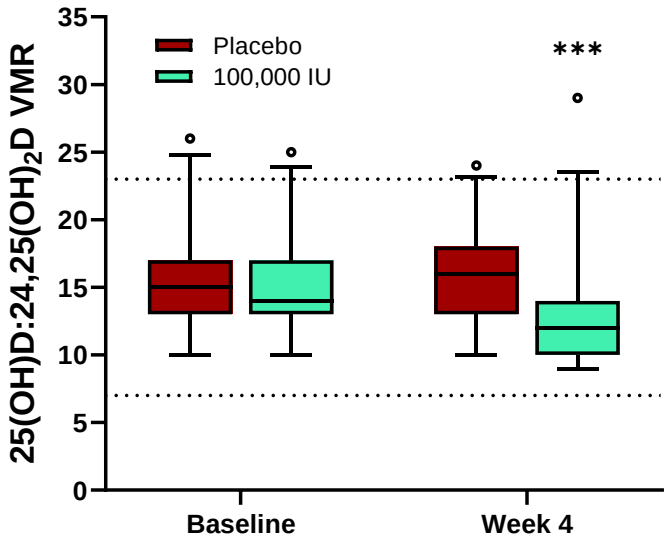
a)


16
14
12
10
8
6
4
2
0
24,25(OH)2D (nmol/L)
Placebo
100,000 IU
***
Baseline
Week 4

b)


280
240
200
160
120
80
40
0
1,25(OH)2D (pmol/L)
Placebo
100,000 IU
***
Baseline
Week 4

c)



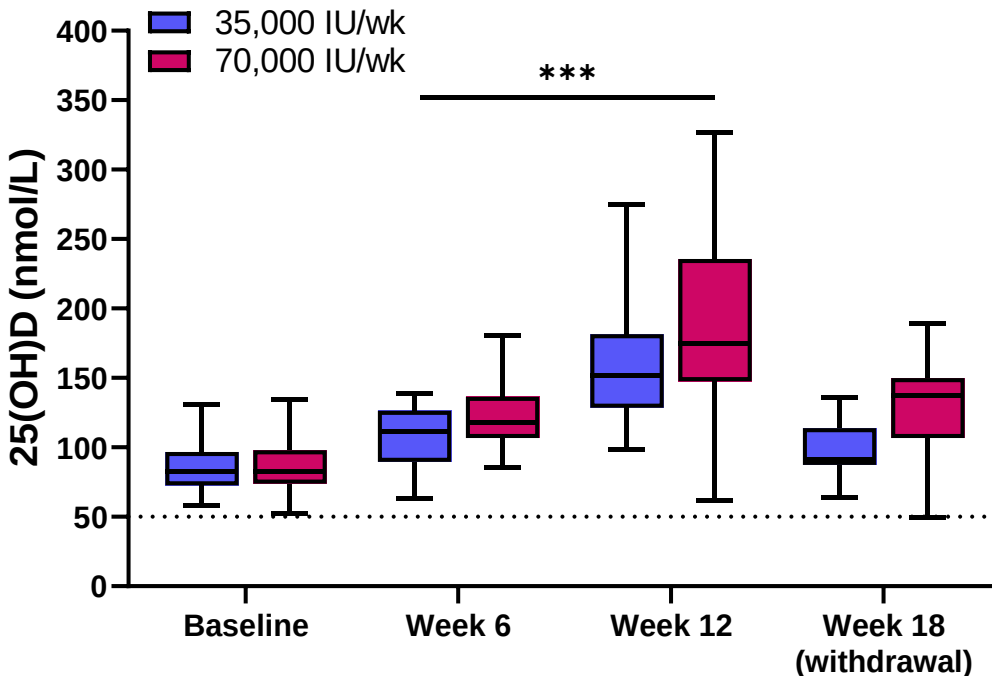
d)

Figure 8.2 Serum concentrations of a) 25(OH)D, b) 24,25$(OH)_2$D, c) 1,25$(OH)_2$D, and d) 25(OH)D:24,25$(OH)_2$D VMR, at baseline and 4 weeks after a single 100,000 IU oral dose of vitamin D3 or placebo. Box and whiskers represent the median, interquartile range and 95% population intervals. Dotted lines represent a) b) the equivalent vitamin D insufficiency thresholds, c) d) the lower and upper reference intervals. ***denotes significance at the $p<0.001$ level compared with baseline.

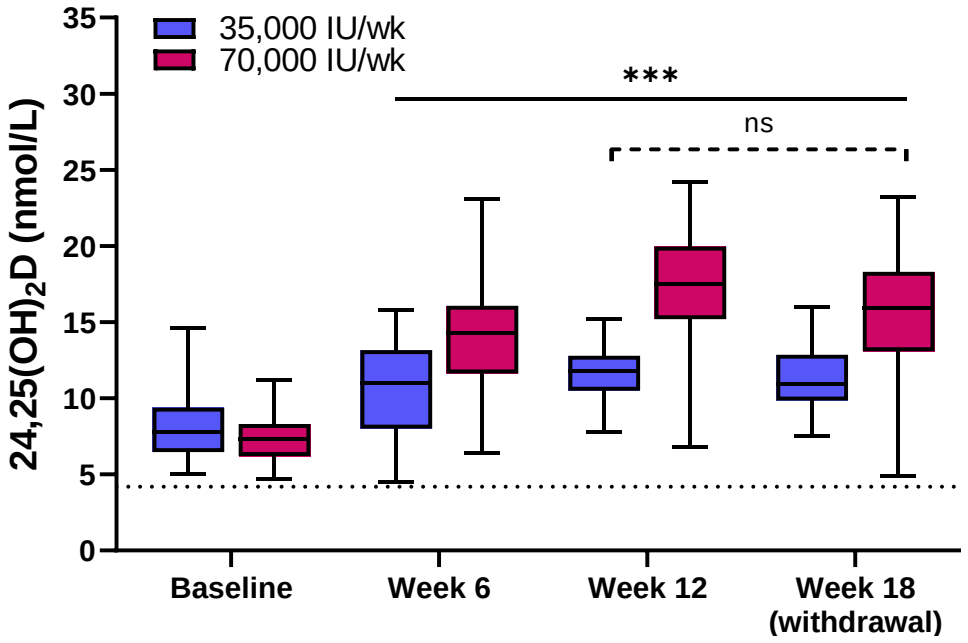
8.3.2 Study 2

Vitamin D metabolite concentrations during the 18-week study are presented in Figure 8.3. Participants in both treatment groups (35,000 IU/wk, n = 22) or (70,000 IU/wk, n = 19) began with sufficient vitamin D status (25(OH)D mean±SD, 86.0 ±19.2) at baseline (pre-treatment). Serum concentrations of 25(OH)D and 24,25$(OH)_2$D increased progressively during the 12-week treatment phase by an average 1.3-fold at week 6 and 2.1-fold at week 12 compared to baseline ($p <0.001$) (Fig. 8.3 a-b). At week 18, six weeks after the last treatment, 25(OH)D concentrations decreased to the levels observed in week 6. In contrast, 24,25$(OH)_2$D concentrations in both treatment groups did not show significant reduction after the withdrawal. 1,25$(OH)_2$D (Fig. 8.3c) showed significant increase in concentration that peaked at week 6 for the 35,000 IU/wk group (mean±SD; baseline 88.7 ±44.2 to peak 141.3 ±57.8 pmol/L) and at week 12 for the 70,000 IU/wk group (mean±SD; baseline 104.8 ±35.9 to peak 141.6 ±51.6 pmol/L). By week 18, 1,25$(OH)_2$D concentrations in both groups returned to baseline. Of those participants received the 35,000 IU/wk treatment, 33.3% had elevated 1,25$(OH)_2$D above the upper reference interval; 47.1% in the 70,000 IU/wk treatment group.

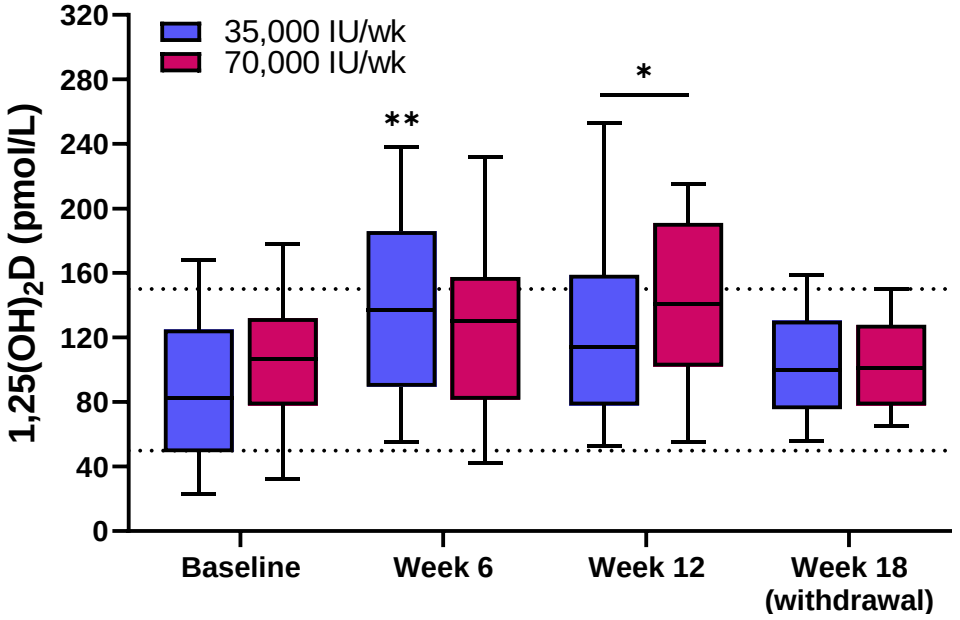
At baseline, the 25(OH)D:24,25$(OH)_2$D VMR (Fig. 8.3d) in both groups began in the lower quartile of the reference interval (between 7-23), suggesting a pre-existed activation of 24,25$(OH)_2$D production. At week 6, the ratio remained unchanged in the 35,000 IU/wk group whereas a significant reduction in the 70,000 IU/wk treatment group was observed; evident of a further increase in 24,25$(OH)_2$D production. At week 12, the 25(OH)D:24,25$(OH)_2$D VMR in both group showed a contradictory increase, suggesting the production of 24,25$(OH)_2$D had reached the limit of saturation. Once the supplement was withdrawn, the rapidly declining 25(OH)D but continuously elevated 24,25$(OH)_2$D resulted in a suppressed 25(OH)D:24,25$(OH)_2$D VMR that was significantly lower at week 18 than baseline in both treatment groups.



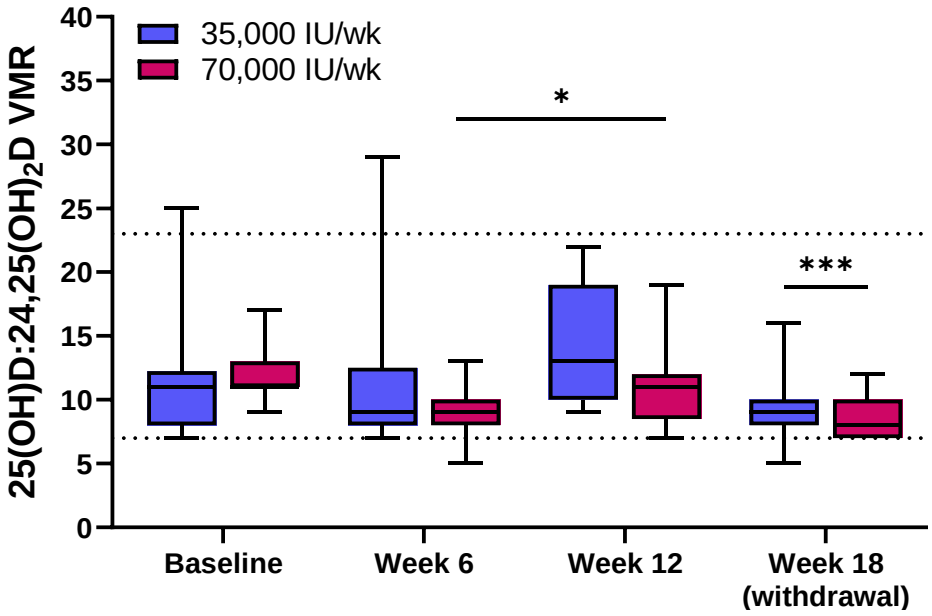
a)


35,000 IU/wk
70,000 IU/wk
***
ns
24,25(OH)2D (nmol/L)
35
30
25
20
15
10
5
0
Baseline
Week 6
Week 12
Week 18
(withdrawal)

b)


35,000 IU/wk
70,000 IU/wk
**
*
1,25(OH)2D (pmol/L)
320
280
240
200
160
120
80
40
0
Baseline
Week 6
Week 12
Week 18
(withdrawal)

c)



d)

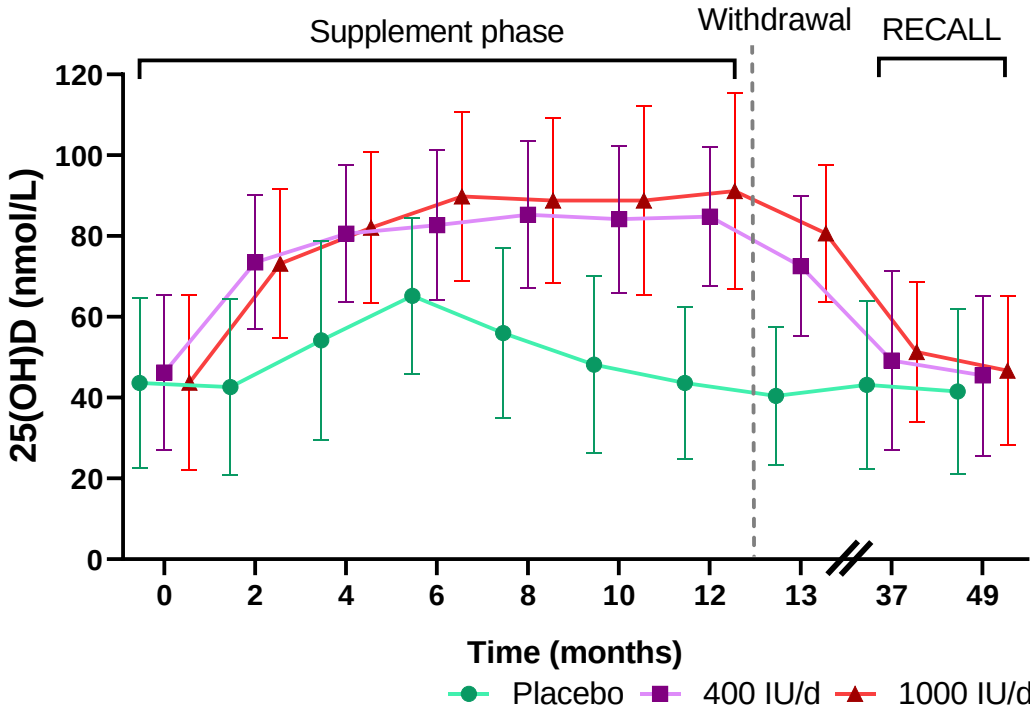
Figure 8.3 Serum concentrations of a) 25(OH)D, b) $24,25(OH)_2D$, c) $1,25(OH)_2D$, and d) 25(OH)D:$24,25(OH)_2D$ VMR with treatment of either 35,000 or 70,000 IU/week from baseline to 12 weeks. Supplementation was stopped after week 12 in both groups. Box and whiskers represent the median, interquartile range and 95% population intervals. Dotted lines in 8.3(a-b) represent the equivalent vitamin D insufficiency thresholds, in 8.3(c-d) represent the lower and upper reference intervals. *denotes significance digits for p-value at 0.05(*), 0.002(**), <0.001 (***) compared with baseline. No significant changes in $24,25(OH)_2D$ were observed in both groups between week 12 (before final dose) and 18 (6 weeks post dose), as depicted by the dashed bracket in 8.3b.

8.3.1 Study 3

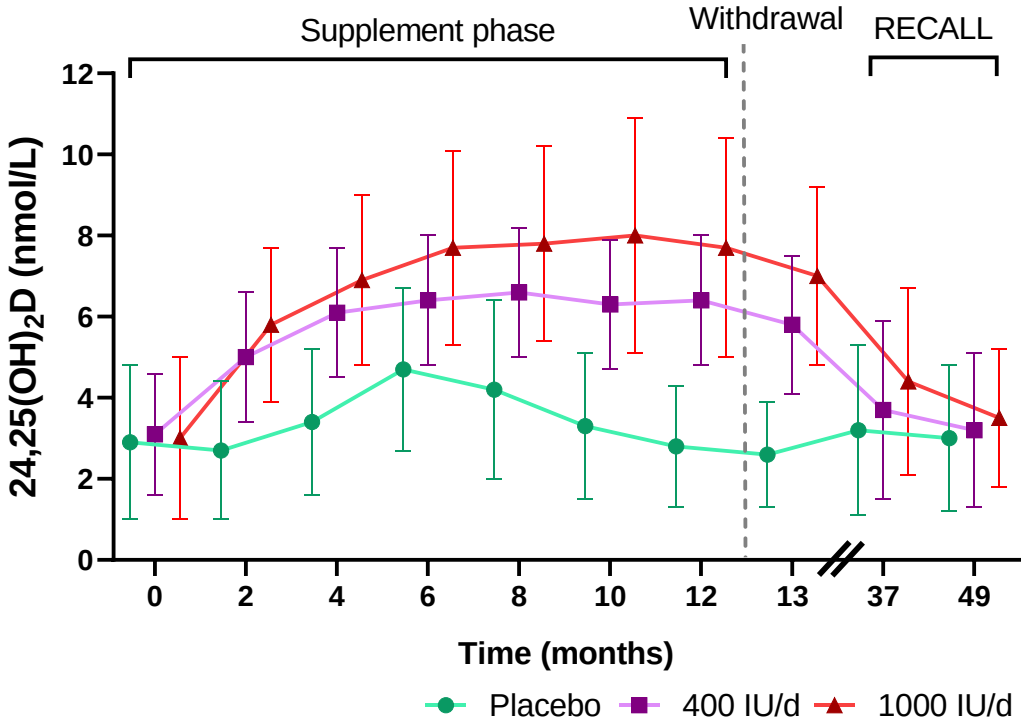
In the VICtORy RCT, 265 of the 287 women (placebo n = 90, 400 IU/day n = 85, 1000 IU/day n = 90) completed the 12-months supplemental phase, followed by a one-month withdrawal of treatment. Of the 265 women, 159 from each group (placebo n = 52, 400 IU n = 49, 1000 IU n = 58) attended the RECALL visits at 37 and 49 months. The vitamin D metabolites concentrations throughout the VICtORy and RECALL visits are presented in Figure 8.4. At the start of the study (January), all the women had insufficient vitamin D status (25(OH)D mean±SD; 43.6 ±21.1). Serum concentrations of 25(OH)D and $24,25(OH)_2D$ increased steadily in the first

six months of treatment, to reach peak 25(OH)D (mean±SD; placebo 65.2 ±19.4, 400 IU 82.7 ±18.5, 1000 IU 89.8 ±20.9); and 24,25$(OH)_2$D (mean±SD; placebo 4.7 ±2.0, 400 IU 6.1 ±1.6, 1000 IU 7.7 ±2.2) (Fig. 8.4a-b). The increase in daylight hours from January to June contributed to the rise in 25(OH)D in all groups, with a greater increase observed in those received the active treatment than placebo. Serum concentrations of 25(OH)D and 24,25$(OH)_2$D in the 400 IU and 1000 IU groups had remained in a consistent state throughout month 8-12; whereas the placebo group saw a progressive decline back to baseline level. In the RECALL visits, 2 and 3 years post withdrawal of treatment, 25(OH)D and 24,25$(OH)_2$D in all groups had returned to the baseline levels.

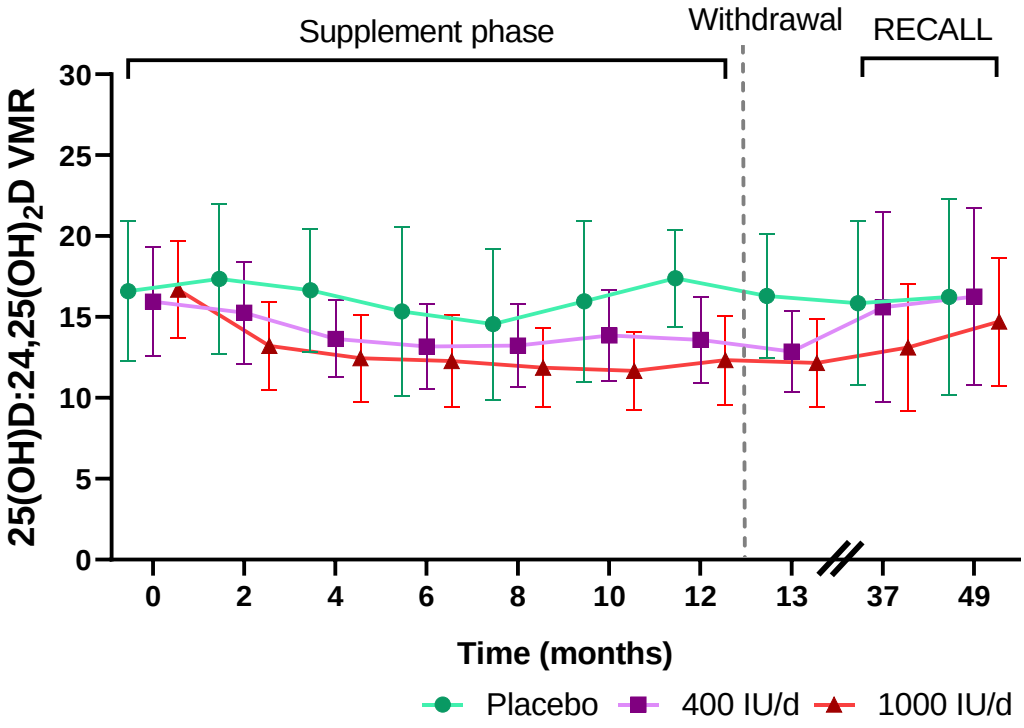
Comparing between the 400 IU and 1000 IU groups, while there was no significant difference in the increase in 25(OH)D during the supplemental phase, there was a distinct difference in 24,25$(OH)_2$D concentrations. The 25(OH)D:24,25$(OH)_2$D VMR plot (Fig. 8.4c) showed greater depression in the 1000 IU than 400 IU group; the data demonstrated that 400 IU/day dosing regime achieved similar serum 25(OH)D concentration as in the 1000 IU/day, but without stimulating 24,25$(OH)_2$D production to the same extent. Serum 1,25$(OH)_2$D concentrations across the time points were consistent; no significant changes were detected (Fig. 8.4d).



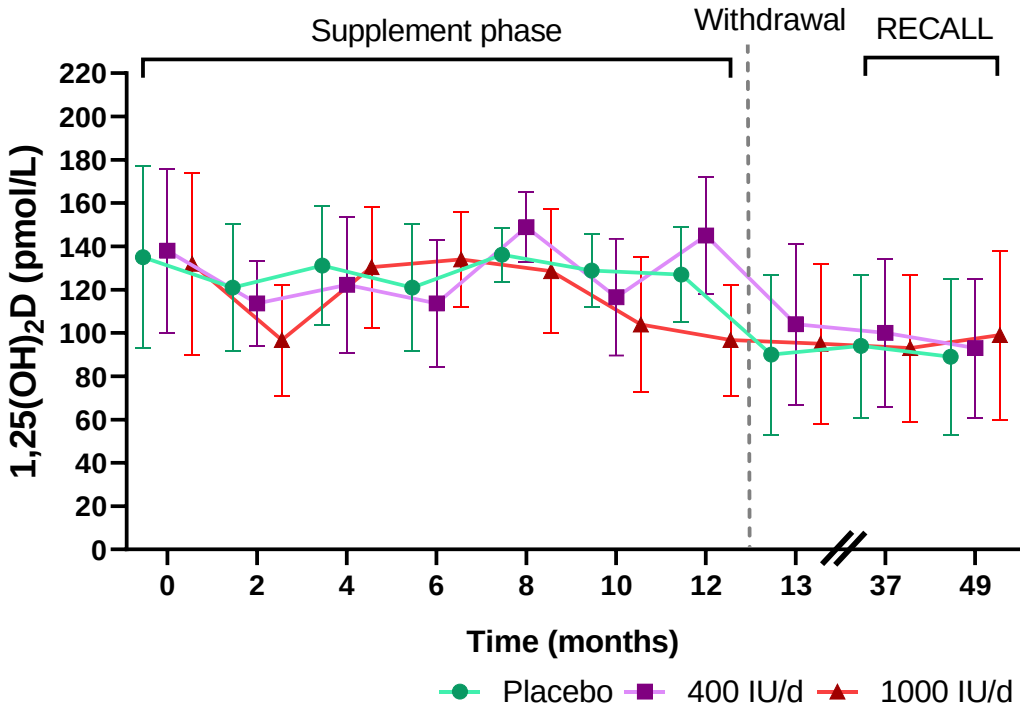
a)


Supplement phase
Withdrawal
RECALL
24,25(OH)2D (nmol/L)
0
2
4
6
8
10
12
0
2
4
6
8
10
12
13
37
49
Time (months)
Placebo
400 IU/d
1000 IU/d

b)


Supplement phase
Withdrawal
RECALL
25(OH)D:24,25(OH)2D VMR
0
5
10
15
20
25
30
0
2
4
6
8
10
12
13
37
49
Time (months)
Placebo
400 IU/d
1000 IU/d

c)



d)

Figure 8.4 Serum concentrations of a) 25(OH)D, b) 24,25$(OH)_2$D, and c) 25(OH)D:24,25$(OH)_2$D VMR, and d) 1,25$(OH)_2$D at 0, 2, 4, 6, 8, 10, 12 mths(treatment phase), 13 mth(withdrawal), 37 and 49 mths(RECALL). Participants received treatment of either placebo, 400 or 1000 IU/day of vitamin D3 from baseline (January) for 12 months. Treatment terminated after month 12. Points on graphs represent mean serum concentration, and error bars represent 95% confidence intervals.

Study	Dosing regimen	Mean percentage change from baseline to peak concentration			
		25(OH)D	24,25$(OH)_2$D	1,25$(OH)_2$D	25(OH)D: 24,25$(OH)_2$D VMR
1	Single 100,000 IU	+140 %	+191.9 %	+26.2%	-19.3 %
2	35,000 IU/week	+88.3 %	+41.4 %	+59.3%	-18.6 %
	70,000 IU/week	+118 %	+134.6 %	+35.1%	-29.4 %
3	400 IU/day	+84.6 %	+6.6 %	+5.1 %	-10 %
	1000 IU/day	+108.5 %	+8.0 %	+1.5 %	-19.4 %

Table 8.1 Summary of the percentage change in vitamin D metabolites and ratio from baseline to peak concentration in the studies.

8.4 Discussion and conclusion

Dose and timing are two important factors to consider for vitamin D supplementation. Evidence in the literature to support effective vitamin D replacement therapy is inconsistent and continue to be a subject of debate. The three vitamin D supplementation studies described in this chapter demonstrate the effects of dosing modality on the three major circulating vitamin D metabolites. It also demonstrated the use of 25(OH)D:24,25$(OH)_2$D VMR to obtain additional information on the efficacy of treatments that is not obvious when using absolute concentrations.

Daily administration of low dose vitamin D3 is a physiologically gentle approach to correct vitamin D deficiency. Participants in study 3 who received the active low dose treatments achieved vitamin D repletion without over stimulation of serum 24,25$(OH)_2$D and 1,25$(OH)_2$D (Table. 8.1). However, both daily 400 IU and 1000 IU regimens took six months to reach maximum concentration at steady state. In contrast, a single high dose of 100,000 IU of vitamin D3 in study 1 achieved similar 25(OH)D concentrations as in study 3 after four weeks. Single bolus administration has the advantage of better patient compliance with the treatment, but findings from study 1 showed high dose of vitamin D3 supplementation resulted in grossly elevated serum 24,25$(OH)_2$D (+192% from baseline) and 1,25$(OH)_2$D (+26.2% from baseline). The finding is evident that 24,25$(OH)_2$D excretory pathway is 'switched on' by CYP24A1 to divert excess 25(OH)D to waste in order to prevent overproduction of 1,25$(OH)_2$D. Such mechanism can be observed in study 2; where weekly high dose treatment on vitamin D replete

subjects had provoked a surge in 24,25$(OH)_2$D and 1,25$(OH)_2$D concentrations. In comparison with the single dose treatment study 1, the continuous weekly treatment regimen in study 2 showed a greater increase in 1,25$(OH)_2$D (up to +59.3% from baseline). The notable lack of proportional response in 24,25$(OH)_2$D by week 12 (Fig. 8.3b) suggests the excretory pathway had reached the breaking point of saturation, thus unable to stop the further increase in 1,25$(OH)_2$D observed. More concerning is the profound elevation of 24,25$(OH)_2$D had remained high six weeks after the withdrawal of treatment; where 25(OH)D and 1,25$(OH)_2$D concentrations had receded to baseline levels, but 24,25$(OH)_2$D showed no significant decrease beyond the expectant half-life of 7.5 days[165]. Such phenomenon suggests the counteracting measures for vitamin D toxicity had remained stimulated to protect against lateral effects from the aftermath of vitamin D overexposure. This mechanism appears to prevent toxicity was less evident in low and regular dosage of vitamin D in study 3.

There was no adverse event of vitamin D toxicity reported in all three studies, likely because the participants were recruited from healthy populations. Ultra-high doses of vitamin D administered annually or monthly had shown to be associated with increased incidents of falls and fractures in the older population[14,15]. Although higher doses of vitamin D supplement were more effective in reaching 25(OH)D concentration of 50 nmol/L, the greater risk of falls and fractures suggests the correlation of risks vs benefit effects of vitamin D exhibit a U-shaped curve, rather than a J-shaped curve. Based on the findings described in this chapter, and observations from literature, the best approach would be a loading dose followed by a daily maintenance dose, chosen based on the characteristics of the patient, in combination with baseline profile of vitamin D metabolite/ratio measurements. Such an approach represents an effort to define a tailored treatment to vitamin D replacement therapy. Vitamin D therapy should be optimised base on individual differences in serum vitamin D metabolite concentrations to give the best response. Blanket high-dose approaches to supplementation should be avoided. Future research should aim to establish the appropriate dose and frequency of administration to achieve a positive increase in both 25(OH)D and 1,25$(OH)_2$D while limiting the increase in 24,25$(OH)_2$D concentrations.

In conclusion, increasing vitamin D supplementation results in an increase in metabolites, but relative difference in the production of 24,25$(OH)_2$D and 1,25$(OH)_2$D was observed with the modality of treatment. The results indicate that, in high supplementation of vitamin D, the metabolism favours the production of 24,25$(OH)_2$D rather than 1,25$(OH)_2$D to prevent toxicity. Such mechanism to prevent toxicity was less evident in low and regular dosage of vitamin D. Caution is advised when using extremely high supplementation levels can be detrimental to the patients. The use of vitamin D metabolite ratio can provide insight that may begin to explain the lack of optimal response to increasing supplementation with vitamin D.

Chapter 9 The dynamic relationships between the active and catabolic vitamin D metabolites, their ratios, and associations with PTH

9.1 Background

The vitamin D pathway is a dynamic system, and its functional role in bone health and other diseases is the subject of intense research. Associations of vitamin D deficiency with a broad spectrum of disease states have drawn attention from the scientific community and increasing awareness of the general population. Despite vitamin D deficiency being a global public health concern, the approach through improving vitamin D status by supplementation, dietary intake and increased sunlight exposure, has resulted in mixed outcomes[152,166,167]. The contradictory evidence has prompted studies on the metabolites of vitamin D. The most abundant metabolite in circulation is 25-hydroxyvitamin D (25(OH)D), which exists in two major forms: 25-hydroxycholecalciferol (25(OH)D3) and 25-hydroxyergocalciferol (25(OH)D2). Measurement of serum total 25(OH)D (D3+D2) is the barometer of vitamin D status; concentrations ≤30 nmol/L and between 30-50 nmol/L are defined as deficient and insufficient, respectively by the U.S Institute of Medicine (IOM)[11]. 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) is synthesised by the hydroxylation of 25(OH)D through the actions of 1α-hydroxylase produced in the renal tubules. $1,25(OH)_2D$ is the most biologically active form of vitamin D and circulates in pmol/L concentration; it controls intestinal absorption of calcium and phosphate, stimulates osteoclast activity, and helps regulate the release of parathyroid hormone (PTH). Although $1,25(OH)_2D$ is derived from 25(OH)D, there is no direct correlation in serum concentrations between the two vitamin D metabolites except in patients with chronic kidney disease (CKD)[28], where a greater association is observed between $1,25(OH)_2D$ and 25(OH)D, dependent upon the severity of the renal impairment. The lack of a direct relationship, despite their close proximity in the metabolic pathway, is due to the tight regulation of the hydroxylation enzymes expressed by the actions of CYP27B1 and CYP24A1. CYP24A1 produces 24-hydroxylase that converts 25(OH)D into 24,25-dihydroxyvitamin D ($24,25(OH)_2D$). The transcription of the *CYP24A1* gene is stimulated by the phosphate-regulating hormone fibroblast growth factor-23 (FGF23), and when PTH is suppressed. The combination increases serum $24,25(OH)_2D$. In Chapter 7 (ch. 7.3.6) first described a concentration-dependent relationship between serum 25(OH)D and $24,25(OH)_2D$[54]. In Chapter 7.3.7 reported a patient, presenting with idiopathic infantile hypercalcaemia (IIH), who

was diagnosed with biallelic *CYP24A1* mutations resulting in the inability to produce $24,25(OH)_2D$ from 25(OH)D, and had an elevated serum $1,25(OH)_2D$ and a persistent state of hypercalcaemia. The use of 25(OH)D:$24,25(OH)_2D$ vitamin D metabolite ratio (VMR) can be a valuable tool in identifying such pathological conditions resulting from impaired CYP24A1 function[43,55]. The use of VMR in the population can provide an assessment of the vitamin D catabolic status; thus allowing a targeted approach to vitamin D supplementation[168].

This chapter describes a novel approach to the interpretation of serum 25(OH)D and $1,25(OH)_2D$ concentrations that incorporate $24,25(OH)_2D$ values. Using data from a large cohort of healthy young adults as the reference population to provide evidence on the intricate relationships between active and catabolic forms of vitamin D metabolites, and the influence on PTH.

9.2 Materials and methods

The following study carried out between 2013-2018 was led by Prof. Julie Greeves OBE (Directorate of Manning (UK Ministry of Defence), Dr Sarah Jackson (Army Medical Directorate), Prof. William Fraser (University of East Anglia), and Prof Neil Walsh (Bangor University). I was a named contributor and project co-investigator in the study, and I performed sample and data analysis using methods as described below.

9.2.1 Study design

The study received ethics approval from the UK Ministry of Defence Research Ethics Committee and was conducted in accordance with the Declaration of Helsinki (2013). The characteristics of the subjects included in the study are shown in Table 9.1. In total, 2252 new British Army recruits at the start of phase one training volunteered for the study. Written informed consent was obtained from all study participants, and each required to complete a detailed health questionnaire, including medical history and the use of supplements. All recruits undertook physical and cognitive testing, and a detailed medical examination before joining the army. The British Army entry requirements restrict individuals with chronic medical conditions; therefore, this study population represents a medically screened, disease-free, and physically fit population. In the analysis, individuals who reported the use of calcium and vitamin D supplements (including multivitamins and cod liver oil) were excluded from statistical analysis. Also excluded were participants who reported injury and illness before recruitment; conditions such as being underweight, eating disorders, or those with a history of bone fracture. 940 participants were included in the final statistical analyses. The majority of participants were from a Caucasian

population (92.9%), with a minority from a diverse ethnicity (Asian 1.6%, Black 1.7%, Chinese 0.1%, mixed 3%, others 0.7%).

	Male	**Female**
n	652	288
Mean age, years (range)	21.7 (18-32)	22.1 (18-32)
Height, m	1.77 (6.4)	1.66 (5.9)
body mass, kg	75.9 (9.8)	64.7 (7.5)
Body mass index (BMI)	24.1 (2.6)	23.4 (3.3)
Total body BMD (g/cm^2)	1.24 (0.10)	1.16 (0.09)

*Data shown in mean ±SD otherwise stated

Table 9.1 Baseline characteristics of the subjects included in the study.

9.2.2 Sample collection

Blood samples were collected during June 2014 to March 2016. Venous blood samples were obtained from the participants at the start of 14-week long basic military training. Sample collections were scheduled on a monthly basis to balance the seasonal variations. Each intake comprised, on average (range), 86 (43-120) participants. Blood samples were collected into serum gel separator tube and EDTA plasma container (BD Vacutainer). Samples were centrifuged immediately after collection at 3,000x g for 10 minutes. Plasma/serum layers were aliquoted into a separate polystyrene tube and stored at -20°C until analysis. All samples were anonymised to the researchers at the point of access.

9.2.3 Ethical approval

The study was approved by the UK Ministry of Defence research ethics committee (MODREC 165/Gen/10 and 692/MoDREC/15). ClinicalTrials.gov Identifier NCT02416895.

9.2.4 LC-MS/MS measurements of serum 25(OH)D and 24,25(OH)$_2$D

Liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed as described[54,161,162,169]. The method quantified 25(OH)D3, 25(OH)D2, 24,25(OH)$_2$D3 and 24,25(OH)$_2$D2 simultaneously from a single injection. 25(OH)D3 and 25(OH)D2 were calibrated

using commercial standards (Chromsystems, München, Germany) traceable to standard reference material SRM972a from the National Institute of Science and Technology (NIST)[77,79], and showed linearity between 0-200 nmol/L. The inter/intra-assay coefficient of variation (CV) was ≤9%, the lower limit of quantification (LLoQ) of 0.1 nmol/L. The assay showed <8% accuracy bias against NIST reference method on the Vitamin D external quality assessment (DEQAS) scheme. 24,25$(OH)_2$D3 and 24,25$(OH)_2$D2 were calibrated using in-house spiked standards traceable to NIST SRM972a. The assay is linear between 0-25 nmol/L; inter/intra-assay CV was ≤11%, LLoQ of 0.1 nmol/L for 24,25$(OH)_2$D3 and 0.8 nmol/L for 24,25$(OH)_2$D2.

9.2.5 Measurements of serum 1,25$(OH)_2$D

The DiaSorin LIAISON® XL 1,25$(OH)_2$D chemiluminescent immunoassay (Stillwater, MN, USA) method was used. The sandwich assay utilises a recombinant fusion protein for the capture of 1,25$(OH)_2$D molecule and a murine monoclonal antibody detection system. The assay measures total 1,25$(OH)_2$D between 12-480 pmol/L, the inter/intra-assay CV was ≤9.2%. The mean assay recovery was 94±2%. On the Vitamin D external quality assessment (DEQAS) scheme, the assay showed ≤8.5% bias against method-specific mean and ≤9.1% bias against all method mean.

9.2.6 Biochemical analysis

Intact PTH and albumin-adjusted calcium (ACa) were analysed on the COBAS® (Roche Diagnostics, Mannheim, Germany) platform. PTH in EDTA plasma was measured using electrochemiluminescence immunoassay (ECLIA), the inter-assay CV was ≤3.8% across the analytical range of 1.2-5000 pg/mL. Total calcium and albumin were measured based on spectrophotometric methods. The inter-assay CV for Ca was ≤1.6%, albumin was ≤1.1%. ACa value is calculated using the equation $ACa = (-0.8 \times [Albumin] - 4) + [Total\ Ca]$.

9.2.7 Statistical analysis

Descriptive statistics, scatterplots, ROC and LOWESS curves were constructed and analysed by Statistical Package for the Social Science (SPSS) version 22.0.0.1 (IBM, New York, USA) and GraphPad Prism 8 (GraphPad, San Diego, CA, USA). Univariate and multivariable linear regression analyses and one-way ANOVA were used to estimate associations. LOWESS curve fitting was used to explore nonlinear relationships between variables. Kruskal-Wallis independent analysis and Spearman's rho were used to establish associations in non-parametric variables. Statistical significance was defined as $p < 0.05$. Frequency distribution histograms of the data

were visually examined and checked for transcriptional and pre/post analytical errors before exclusion for statistical analysis. Confidence interval (CI) was established at 95% of the population. Circannual rhythm analysis was performed by population-mean cosinor analysis, based on cosinor-fitting equation $y = \text{MESOR} + \text{Amplitude} \times \cos(\text{Frequency}(x) + \text{acrophase})$. Midline estimate statistic of rhythm (MESOR), defined as the rhythm-adjusted mean value. Acrophase is the difference (time) between MESOR and peak value in the cosine curve.

9.3 Results

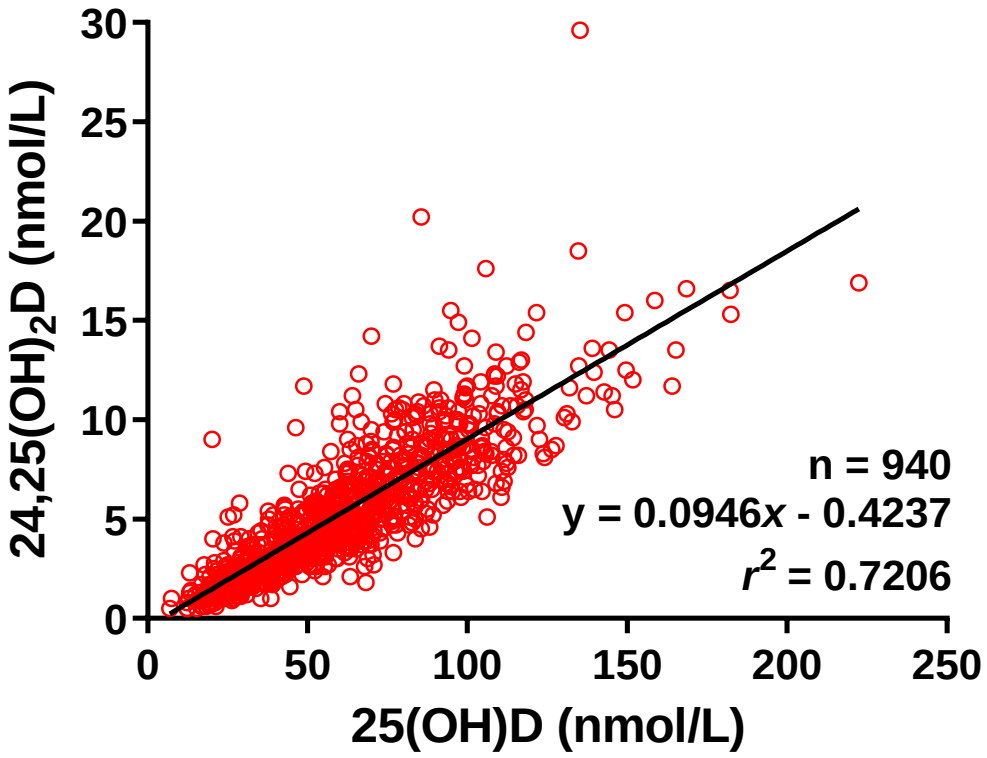
Results from 940 participants were included in the data analysis. Statistical analyses on 25(OH)D and 24,25$(OH)_2$D were performed on the respective total (sum of D3 + D2) values, the distributions were untrimmed, and no outlier was removed. Summary of the distribution of biochemical profile is shown in Table 9.2. 25(OH)D2 was found in 57.8% of the subjects, mean (range) of 4.2 nmol/L (0.6-29.1). 24,25$(OH)_2$D2 was found in 0.4% of the subjects, mean (range) 1.5 nmol/L (1.2-1.8).

Profile	mean	SD	Min	2.5th Percentile	25th Percentile	Median	75th Percentile	97.5th Percentile	Max
25(OH)D, nmol/L	62.4	29.8	6.9	18.1	39.8	59.2	81.0	130.9	222.5
24,25$(OH)_2$D, nmol/L	5.4	3.3	0.5	1.0	2.9	4.9	7.4	13.0	29.6
1,25$(OH)_2$D, pmol/L	138.8	39.6	32.3	71.9	111.0	135	161.0	229.7	380.0
25(OH)D:24,25$(OH)_2$D VMR	13	4	2	7	10	12	15	25	39
1,25$(OH)_2$D:24,25$(OH)_2$D VMR	38	33	5	9	18	28	45	132	300
Intact PTH, pmol/L	3.7	1.2	1.0	1.9	2.9	3.5	4.3	6.8	11.4
ACa, mmol/L	2.38	0.07	2.00	2.20	2.32	2.40	2.41	2.50	2.60

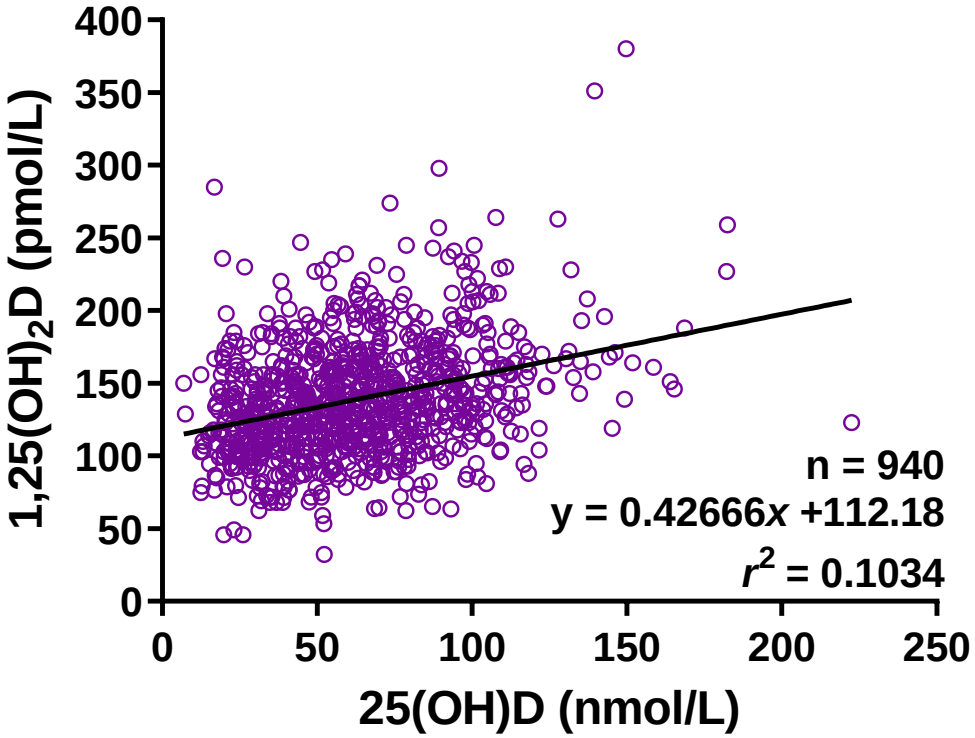
Table 9.2 Distribution of biochemical measurements performed in the study.

9.3.1 24,25(OH)$_2$D and 25(OH)D

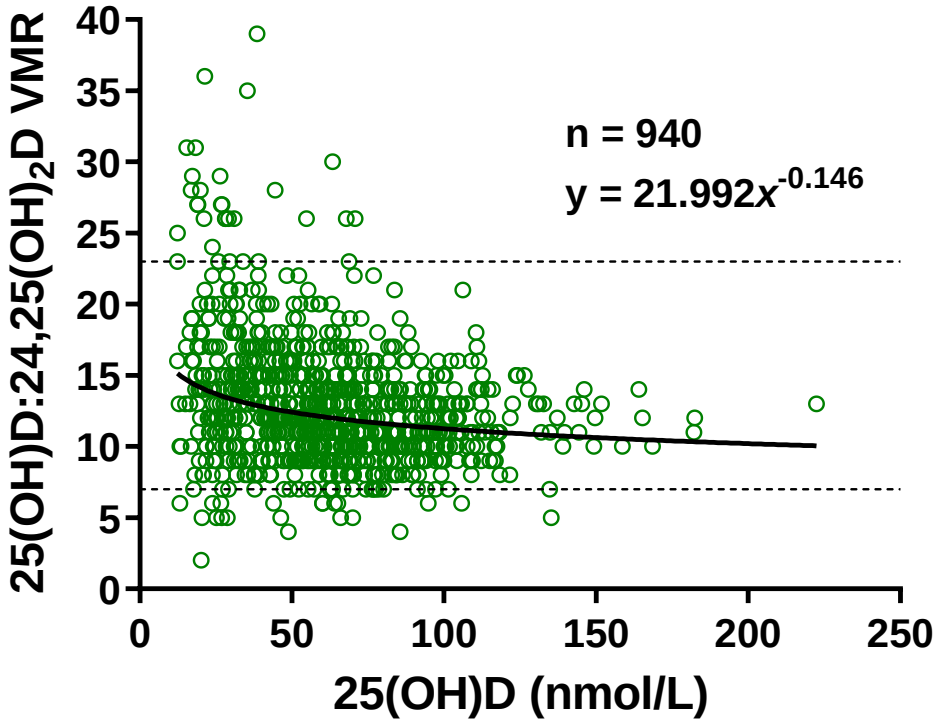
The mean concentration of 24,25(OH)$_2$D was on average 9.5-fold lower than 25(OH)D. Linear regression analysis (Fig. 9.1a) showed a directly proportional relationship between 24,25(OH)$_2$D and 25(OH)D concentrations: $[24{,}25(\mathrm{OH})_2\mathrm{D}] = 0.0946 \times [25(\mathrm{OH})\mathrm{D}] - 0.42$; $r^2 = 0.7206$. Using this equation, the derived serum 24,25(OH)$_2$D concentration of ≥4.3 nmol/L is equivalent to the IOM vitamin D replete status (i.e. 25(OH)D of 50 nmol/L), and 24,25(OH)$_2$D concentration of ≤2.4 nmol/L is equivalent to deficiency status (i.e. 25(OH)D of ≤30 nmol/L).



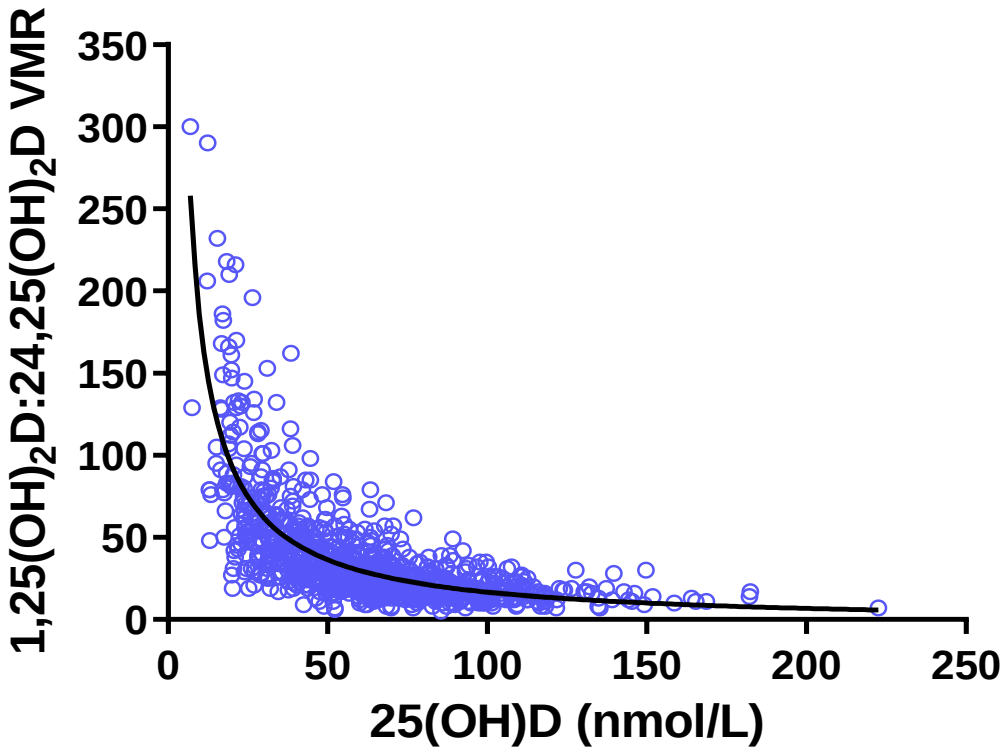
a)


1,25(OH)2D (pmol/L)
400
350
300
250
200
150
100
50
0
0
50
100
150
200
250
25(OH)D (nmol/L)
n = 940
y = 0.42666x +112.18
r2 = 0.1034

b)


25(OH)D:24,25(OH)2D VMR
40
35
30
25
20
15
10
5
0
0
50
100
150
200
250
25(OH)D (nmol/L)
n = 940
y = 21.992x-0.146

c)



d)

Figure 9.1a-d Non-parametric correlations of **(a)** 24,25$(OH)_2$D **(b)** 1,25$(OH)_2$D **(c)** 25(OH)D:24,25$(OH)_2$D VMR and **(d)** 1,25$(OH)_2$D:24,25$(OH)_2$D VMR, against their respective 25(OH)D concentration. Solid lines in **(a)** and **(b)** represent linear regression line. LOWESS fitted curve in **(c)** and **(d)** (99% point fit). The mean 24,25$(OH)_2$D and 1,25$(OH)_2$D concentrations, 25(OH)D:24,25$(OH)_2$D VMR and 1,25$(OH)_2$D:24,25$(OH)_2$D VMR represent 8.7%, 222.4%, 20.8% and 60.9% of their respective 25(OH)D concentration. Assay lower limit of quantification (LLoQ): 25(OH)D and 24,25$(OH)_2$D=0.1 nmol/L, 1,25$(OH)_2$D=12 pmol/L.

9.3.2 1,25$(OH)_2$D and 25(OH)D

Despite a direct enzymatic conversion of 25(OH)D to 1,25$(OH)_2$D, there was no significant correlation in serum concentrations between these two vitamin D metabolites (Fig. 9.1b). This finding is consistent with published studies; 1,25$(OH)_2$D can directly inhibit the expression of 1α-hydroxylase, and indirectly inhibit by suppressing PTH and stimulating FGF23 production[170,171]. This negative feedback system provides an essential safeguard mechanism against hypercalcaemia. Hence 1,25$(OH)_2$D concentration is unaffected by the circulatory concentration of 25(OH)D.

9.3.3 25(OH)D:24,25$(OH)_2$D VMR and vitamin D status

The 25(OH)D:24,25$(OH)_2$D VMR showed an indirect relationship with 25(OH)D (Fig. 9.1c); LOWESS fitting showed a steady increase in 25(OH)D:24,25$(OH)_2$D VMR with the decline in 25(OH)D concentration. One-way ANOVA showed a significant increase in 25(OH)D:24,25$(OH)_2$D VMR ($p>0.001$) at 25(OH)D below 50 nmol/L (Fig. 9.2). The greatest increase was observed when 25(OH)D concentration decreased below ≤30 nmol/L. The decrease in relative production of serum 24,25$(OH)_2$D in response to the decline in 25(OH)D suggests down-regulation of CYP24A1.

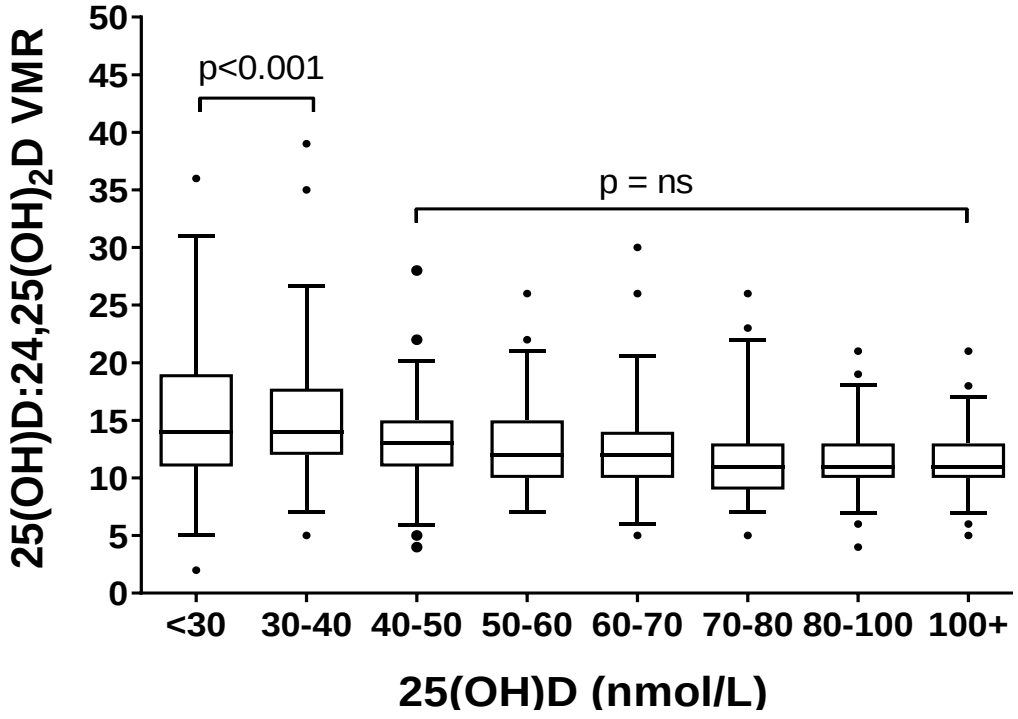


Figure 9.2 Distribution of 25(OH)D:24,25$(OH)_2$D VMR by 25(OH)D intervals. Each interval contains an equal number of subjects to illustrate the significantly elevated ratio in those with serum 25(OH)D ≤50 nmol/L. Box and whiskers represent the median, interquartile range and 95% population intervals.

9.3.4 1,25$(OH)_2$D:24,25$(OH)_2$D VMR and vitamin D status

Vitamin D status, as indicated by 25(OH)D concentrations, revealed an exponential negative correlation (r^2Exp=0.582) with 1,25$(OH)_2$D:24,25$(OH)_2$D VMR (Fig. 9.1d). *Post hoc* analysis

identified a significant increase in $1,25(OH)_2D:24,25(OH)_2D$ VMR at 25(OH)D ≤60 nmol/L (Fig. 9.3). Using the Jacobson and Truax[150,151] method to determine the cut-off value for clinically significant change[54], $1,25(OH)_2D:24,25(OH)_2D$ VMR of ≥35 was estimated to be the predictive threshold value for vitamin D insufficiency, and ≥51 to be the predictive threshold for vitamin D deficiency. The threshold values were determined from subject samples collected in the winter months (January to April) due to the seasonal variation of 25(OH)D. Receiver Operating Characteristic (ROC) curves generated from data collected between January to April produced area under the curve (AUC) values of 0.88 and 0.86, indicating the VMR cut-offs are excellent at discriminating individuals with vitamin D insufficiency and deficiency. The $1,25(OH)_2D:24,25(OH)_2D$ VMR at 35 and 51 achieved true positive rate (sensitivity) at 80% and 78%, respectively, and false positive rate (specificity) of 82% and 74%, respectively. (Fig. 9.4a and b).

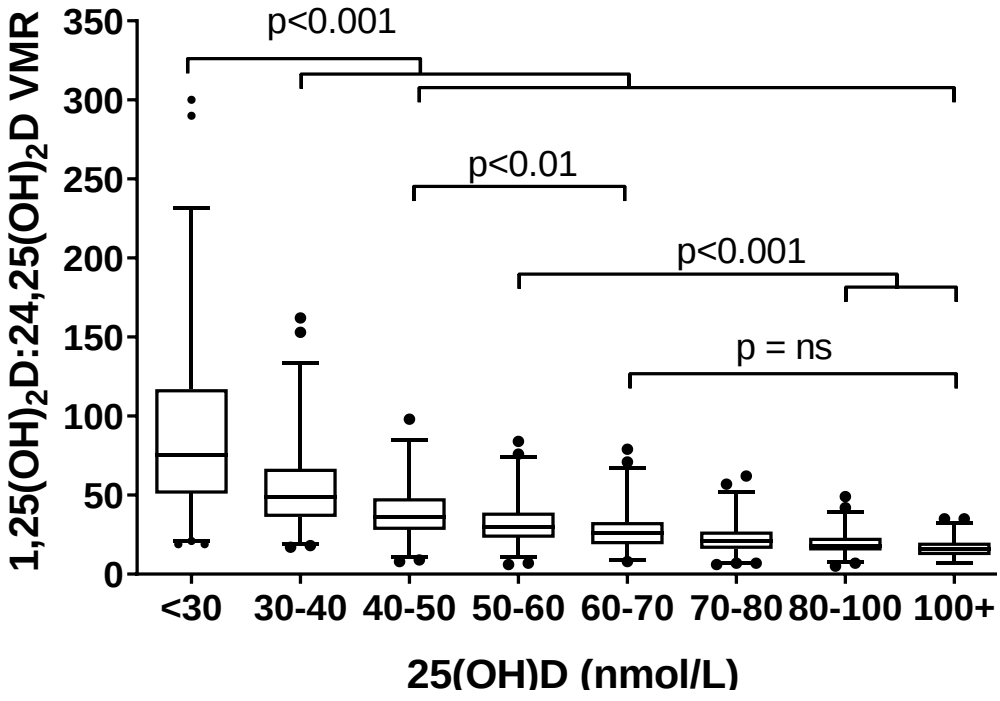
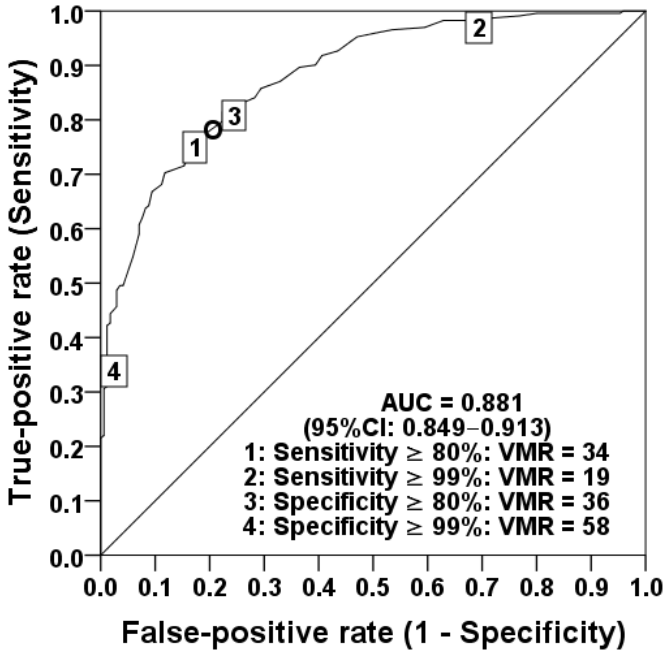
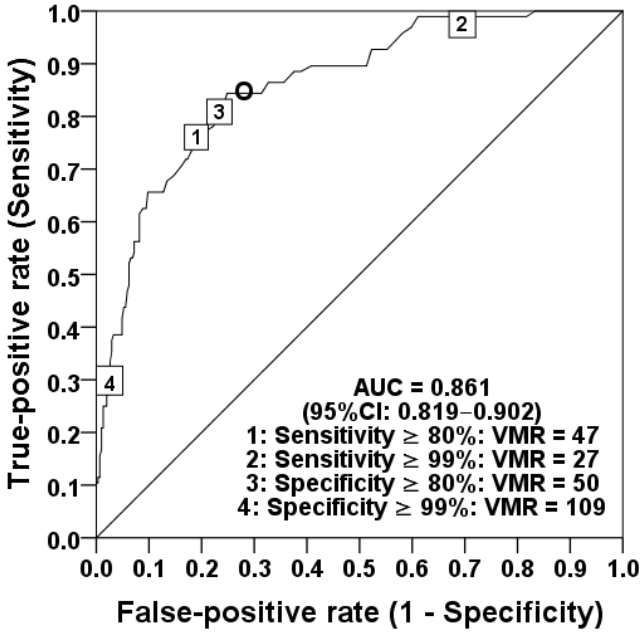


Figure 9.3 Distribution of $1,25(OH)_2D:24,25(OH)_2D$ VMR by 25(OH)D intervals. It demonstrates the exponential increase in $1,25(OH)_2D:24,25(OH)_2D$ VMR with the decrease in serum 25(OH)D. Box and whiskers represent the median, interquartile range and 95% population intervals. Each interval contains an equal number of subjects.



a)



b)

Figure 9.4a-b Diagnostic performance of $1,25(OH)_2D$:$24,25(OH)_2D$ VMR in the assessment of vitamin D status during winter months (Jan-April) (n = 402). Receiver Operating Characteristic (ROC) curve depicts diagnostic sensitivity and specificity levels. (**O**) represents decision threshold for **(a)** vitamin D replete (i.e. 25(OH)D ≥50 nmol/L), $1,25(OH)_2D$:$24,25(OH)_2D$ VMR threshold value of 35 (sensitivity = 80%, specificity = 78%), **(b)** vitamin D insufficiency (i.e. 25(OH)D ≥30 nmol/L), $1,25(OH)_2D$:$24,25(OH)_2D$ VMR threshold value of 51 (sensitivity = 82%, specificity = 74%). The diagonal lines represent the line of no discrimination.

9.3.5 $1,25(OH)_2D$:$24,25(OH)_2D$ VMR and PTH

Circulating PTH is influenced by 25(OH)D and $1,25(OH)_2D$, and *vice versa*. Based on this assumption, a hypothesis was made that PTH concentration changes with $1,25(OH)_2D$:$24,25(OH)_2D$ VMR and 25(OH)D. To test the hypothesis, median PTH concentrations were established from grid analysis based on groupings of $1,25(OH)_2D$:$24,25(OH)_2D$ VMR and 25(OH)D in ascending order (Table 9.3). A decrease in PTH concentration was observed from the high $1,25(OH)_2D$:$24,25(OH)_2D$ VMR (100+) and low 25(OH)D (<30 nmol/L) group, to the low $1,25(OH)_2D$:$24,25(OH)_2D$ VMR (<30) and high 25(OH)D (100+ nmol/L) group. Using Kruskal-Wallis independent non-parametric analysis to test the distribution of PTH across all groups, a highly significant ($p>0.001$) change in PTH concentration was found across the $1,25(OH)_2D$:$24,25(OH)_2D$ VMR and 25(OH)D categories, hence the null hypothesis was rejected. Using Spearman's rank correlation coefficient (2-tailed) to assess monotonic functions between variables, significant positive correlations were evident between VMRs and PTH ($1,25(OH)_2D$:$24,25(OH)_2D$ VMR rho = 0.249, $p>0.001$ and 25(OH)D:$24,25(OH)_2D$ VMR rho = 0.134, $p>0.001$); whereas vitamin D metabolites showed significant negative correlations with PTH (25(OH)D rho = -0.287, $p>0.001$, $24,25(OH)_2D$ rho = -0.282, $p>0.001$ and $1,25(OH)_2D$ rho = -0.87, $p>0.001$). The statistical significance remained unchanged after adjustment for BMD and BMI as covariates.

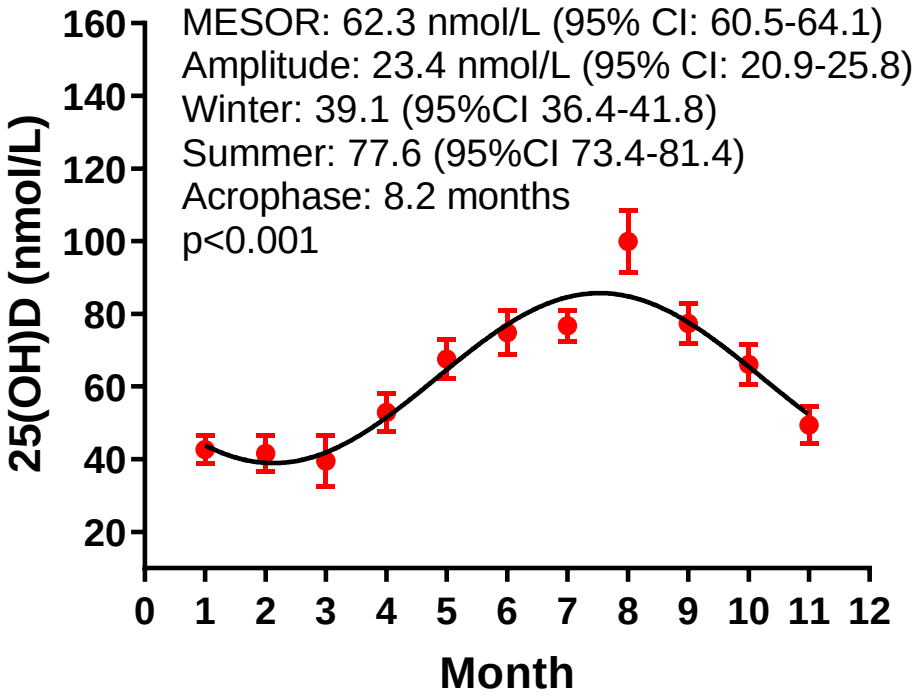
Median PTH, pmol/L		**25(OH)D, nmol/L**			
		<30	**30-50**	**51-100**	**100+**
$1,25(OH)_2D$:$24,25-(OH)_2D$ VMR	**100+**	5.7 (0.2)*	5.4 (0.5)*	-	-
	51-100	4.3 (0.2)*	3.8 (0.2)	3.4 (0.3)	-
	30-50	4.0 (0.2)*	3.7 (0.1)	3.5 (0.1)	2.7 (0.2)*
	<30	3.8 (0.3)	3.9 (0.1)	3.3 (0.1)*	3.2 (0.1)*

*denotes significance at the $p<0.05$ level.

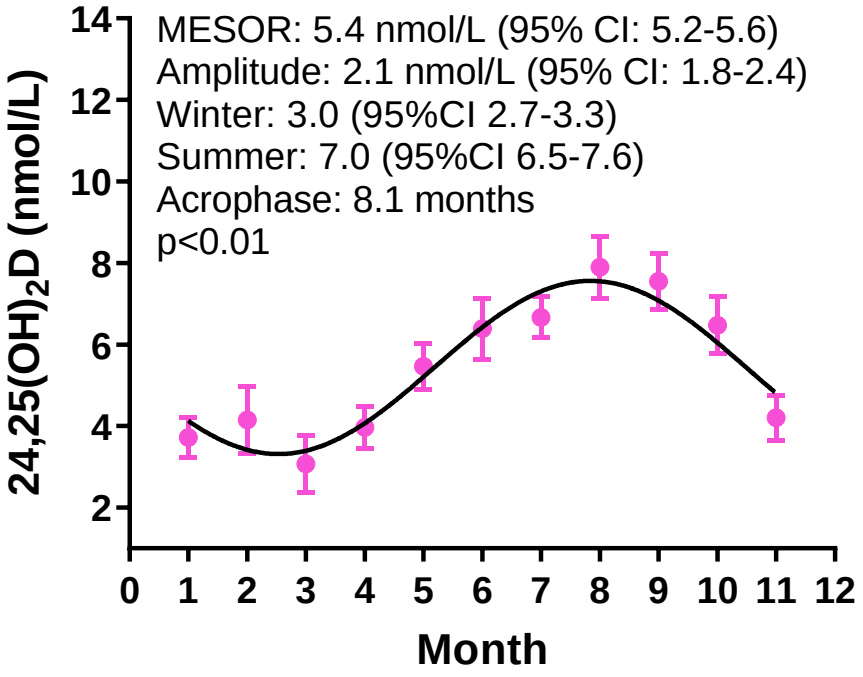
Table 9.3 Median (SEM) PTH concentrations in categories of increasing $1,25(OH)_2D$:$24,25(OH)_2D$ VMR and 25(OH)D. One-way ANOVA showed PTH concentrations decreased significantly ($p>0.001$) from high $1,25(OH)_2D$:$24,25(OH)_2D$ VMR/low 25(OH)D to low $1,25(OH)_2D$:$24,25(OH)_2D$ VMR/high 25(OH)D.

9.3.5 Circannual variations in vitamin D metabolites and VMRs

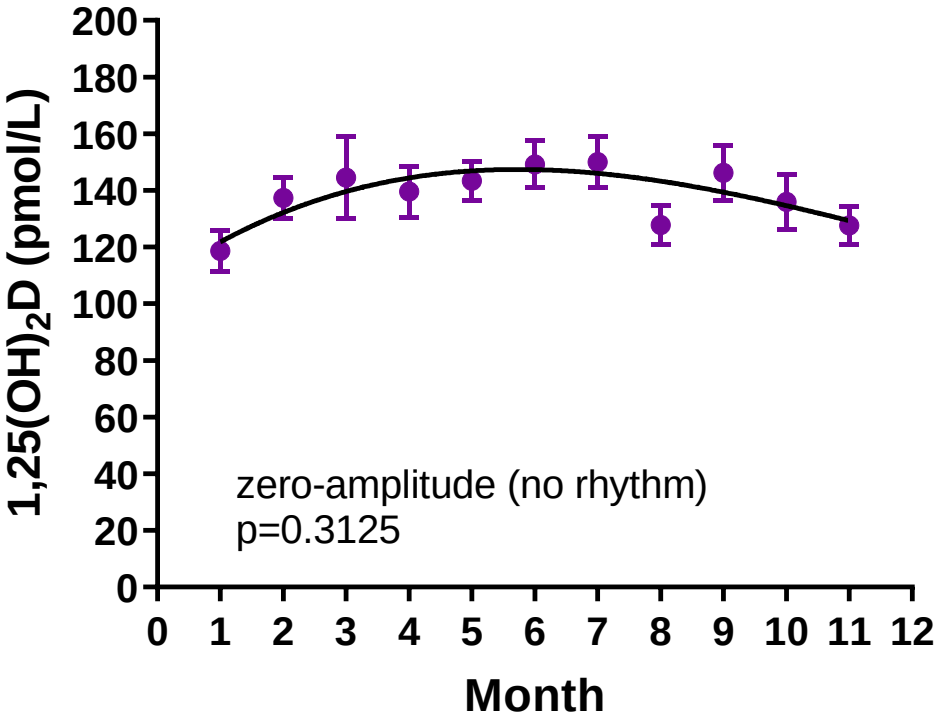
Cosinor-fit curves (Fig. 9.5a-f) show significant circannual rhythm for 25(OH)D ($p<0.001$), $24,25(OH)_2D$ ($p<0.01$), 25(OH)D:$24,25(OH)_2D$ VMR ($p<0.001$), $1,25(OH)_2D$:$24,25(OH)_2D$ VMR ($p<0.001$) and PTH ($p<0.05$). No significant rhythm was observed for $1,25(OH)_2D$ ($p=3.125$). The rhythm observed for 25(OH)D is consistent with previous reports[172,173]. $24,25(OH)_2D$ showed a similar peak (July-Aug) and nadir (Jan-Mar) pattern as for 25(OH)D. 25(OH)D:$24,25(OH)_2D$ VMR and $1,25(OH)_2D$:$24,25(OH)_2D$ VMR exhibited patterns in the opposite direction, with peak (Mar-April) and nadir (Aug-Sept) suggesting that the production of $24,25(OH)_2D$ is relatively higher during summer/early autumn months. Acrophase, defined as the lag time between rhythm-adjusted mean and peak cycle value, was on average (SD) of 8.1(0.3) months for all vitamin D metabolites except for $1,25(OH)_2D$. A low amplitude, circasemiannual PTH secretory rhythm was observed, with an acrophase of 3.5 months.



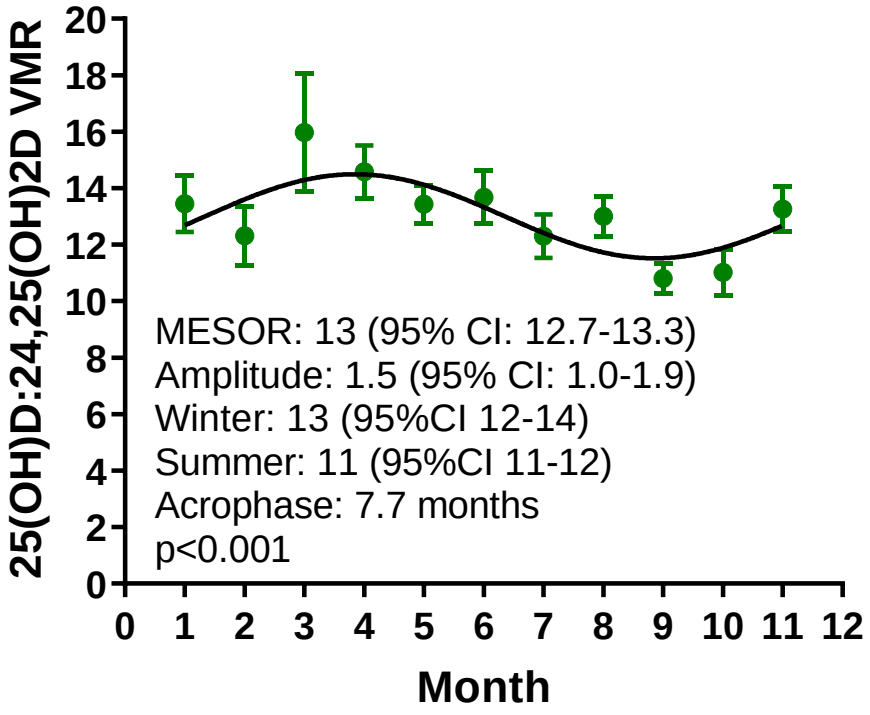
a)


MESOR: 5.4 nmol/L (95% CI: 5.2-5.6)
Amplitude: 2.1 nmol/L (95% CI: 1.8-2.4)
Winter: 3.0 (95%CI 2.7-3.3)
Summer: 7.0 (95%CI 6.5-7.6)
Acrophase: 8.1 months
p<0.01
24,25(OH)2D (nmol/L)
Month

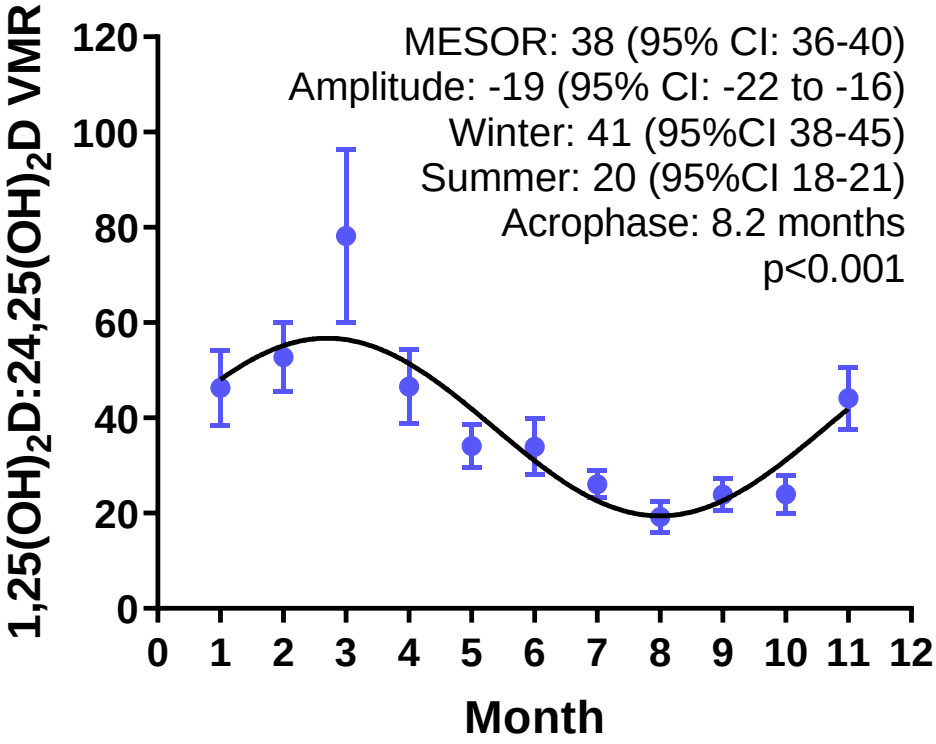
b)


zero-amplitude (no rhythm)
p=0.3125
1,25(OH)2D (pmol/L)
Month

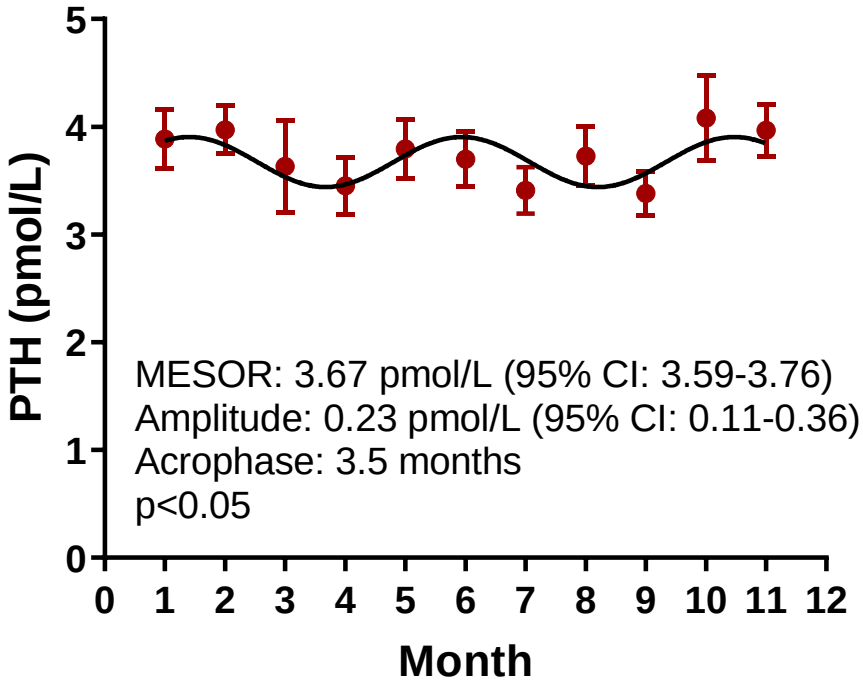
c)


25(OH)D:24,25(OH)2D VMR
20
18
16
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Month
MESOR: 13 (95% CI: 12.7-13.3)
Amplitude: 1.5 (95% CI: 1.0-1.9)
Winter: 13 (95%CI 12-14)
Summer: 11 (95%CI 11-12)
Acrophase: 7.7 months
p<0.001

d)


1,25(OH)2D:24,25(OH)2D VMR
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Month
MESOR: 38 (95% CI: 36-40)
Amplitude: -19 (95% CI: -22 to -16)
Winter: 41 (95%CI 38-45)
Summer: 20 (95%CI 18-21)
Acrophase: 8.2 months
p<0.001

e)



f)

Figure 9.5a-f Cosinor-fit circannual rhythm for **a)** 25(OH)D, **b)** 24,25$(OH)_2$D, **c)** 1,25$(OH)_2$D, **d)** 25(OH)D:24,25$(OH)_2$D, **e)** 1,25$(OH)_2$D:24,25$(OH)_2$D, **f)** PTH. Error bars represent 95% CI.

8.4 Discussion and conclusion

This study is the first to demonstrate a relationship between serum concentrations of 25(OH)D and 1,25$(OH)_2$D when expressed as a relative ratio with serum 24,25$(OH)_2$D. The findings provide evidence that the conversion of 25(OH)D to 1,25$(OH)_2$D is associated with the catabolism of 25(OH)D to 24,25$(OH)_2$D, which can be assessed by the measurement of serum 24,25$(OH)_2$D and its derived VMR.

The inverse exponential correlation between 1,25$(OH)_2$D:24,25$(OH)_2$D VMR and 25(OH)D provides insight into the dynamics of vitamin D metabolites in healthy, young adults; when vitamin D status is sufficient, serum concentrations of 1,25$(OH)_2$D and 24,25$(OH)_2$D are maintained in relative proportion and showed no significant change beyond the sufficient threshold. In contrast, when vitamin D status is insufficient, a progressive and highly significant increase in 1,25$(OH)_2$D:24,25$(OH)_2$D VMR is evidence that the production of serum 1,25$(OH)_2$D is favoured over 24,25$(OH)_2$D as the availability of vitamin D precursors in circulation diminishes. The data imply two possible regulatory functions of the 24,25$(OH)_2$D pathway; in hypervitaminosis, the pathway is 'switched on' to allow excess 25(OH)D to be converted to 24,25$(OH)_2$D. The 24-hydroxylase pathway results in the formation of calcitroic acid for excretion. In hypovitaminosis, the 24,25$(OH)_2$D pathway is partially inactivated to conserve 25(OH)D and to maintain an adequate supply of substrate for conversion to 1,25$(OH)_2$D. Although the biological activity of 24,25$(OH)_2$D is yet to be fully elucidated, its role in vitamin D catabolism appears certain. Low serum concentrations of 24,25$(OH)_2$D and elevated 25(OH)D:24,25$(OH)_2$D VMR is useful in identifying patients with loss-of-function *CYP24A1* mutations[42,43,55]. Chapter 7 described a case of biallelic *CYP24A1* mutation in a patient presenting with hypercalcaemia, elevated serum 1,25$(OH)_2$D concentration (293 pmol/L, reference range 43-144 pmol/L), and elevated 25(OH)D:24,25$(OH)_2$D VMR of 32. On diagnosis, the patient's 1,25$(OH)_2$D:24,25$(OH)_2$D VMR was 212 (1.6 times the upper 97.5th percentile of 132), which was attributed to supplementation with vitamin D. One month after treatment for hypercalcaemia and cessation of vitamin D supplement, serum 1,25$(OH)_2$D was within the reference range, the 1,25$(OH)_2$D:24,25$(OH)_2$D VMR decreased to 130 (below the 97th percentile), and the 25(OH)D:24,25$(OH)_2$D VMR remained elevated at 35.

A major finding of this study was the link between vitamin D metabolites and VMRs with the distribution of PTH. To the best of the author's knowledge, this is the first such report in a human population study. It is widely accepted that the PTH concentration is associated with 25(OH)D, but not with the active 1,25$(OH)_2$D. This is due to the tight regulatory mechanisms, and the regulatory processes that take place via the vitamin D receptor (VDR) to activate intracellular transport of calcium and stimulate PTH secretion. Using the 1,25$(OH)_2$D:24,25$(OH)_2$D VMR and

25(OH)D model (Fig. 9.1d), it shows that individuals with low 25(OH)D (≤50 nmol/L), normal $1,25(OH)_2D$ but high $1,25(OH)_2D$:$24,25(OH)_2D$ VMR (≥101) have significantly higher PTH concentration than those at the opposite end of the spectrum (Fig. 9.6). An interpretation of the finding supports a biological role of $24,25(OH)_2D$ other than as a catabolic metabolite of vitamin D. Relative high production of $24,25(OH)_2D$ may reduce the bioactivity of 25(OH)D and $1,25(OH)_2D$, particularly extra-renal production of $1,25(OH)_2D$, to down-regulate the secretion of PTH whilst maintaining $1,25(OH)_2D$ concentrations within the strict boundaries required for appropriate calcium homeostasis. Relatively low $24,25(OH)_2D$ could enhance the anabolic effects of vitamin D metabolism, by stimulating PTH production. The biological action of $24,25(OH)_2D$ on the inhibition of PTH secretion was first reported in animal and *in vitro* models in the late seventies[174,175]. More recently there is increasing evidence supporting physiological functions of $24,25(OH)_2D$ on bone and cartilage[49,176,177] in promoting fracture healing, and protection against cartilage damage. The existence of a $24,25(OH)_2D$-specific nuclear or membrane receptor has been reported[178], but its function has yet to be elucidated. Given that CYP24A1, the enzyme responsible for the production of $24,25(OH)_2D$, is present in most tissues with VDR, understanding the mechanisms controlling the production of $24,25(OH)_2D$ relative to other vitamin D metabolites may have significance beyond vitamin D catabolism, potentially shaping vitamin D supplementation strategies.

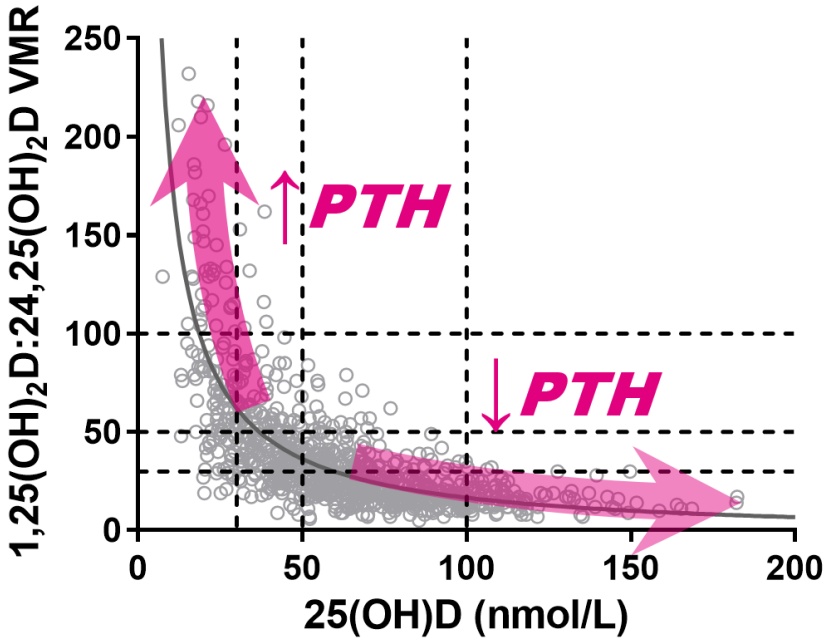


Figure 9.6 Figure depicts the distribution of PTH concentration in the $1,25(OH)_2D$:$24,25(OH)_2D$ VMR/25(OH)D model.

Mapping the circannual rhythms of vitamin D metabolites and VMRs is an important component of this study. In the literature, reports on longitudinal studies (Macdonald et al.)[172,173,179], describing the changes in serum 25(OH)D and 24,25$(OH)_2$D throughout a year in vitamin D supplemented or non-supplemented subjects. In the VICtORy (Vitamin D and CardiOvascularRisk)[172] and VICtORy RECALL[173] randomised controlled studies performed using a group of postmenopausal women residing in the northeast of UK, the placebo group showed a two-fold increase in serum 25(OH)D in peak summer months (July-August), compared to the nadir in late winter months (January-March). The younger cohort of healthy individuals in this study showed similar trends; 24,25$(OH)_2$D had a propensity to fluctuate with 25(OH)D throughout the year, with changes between summer and winter months, as indicated by a lower 25(OH)D:24,25$(OH)_2$D VMR during January to March than during July to September. Serum 1,25$(OH)_2$D displayed no rhythm and was within the reference range throughout the year. The circannual variation of 1,25$(OH)_2$D:24,25$(OH)_2$D VMR was dependent on 24,25$(OH)_2$D, with a peak-to-nadir difference of 19; such sharp demarcation between seasons would inevitably create uncertainty when using 1,25$(OH)_2$D:24,25$(OH)_2$D VMR in diagnostic decision-making. In contrast, 25(OH)D:24,25$(OH)_2$D VMR is less susceptible to seasonal fluctuation, allowing the use of the VMR with fixed reference intervals irrespective of the time of the year.

A major strength of the data is the chosen cohort; with participants attending blood sampling visits at strictly controlled time intervals and that the vitamin D metabolites were measured using gold-standard methodologies. The participants are well-defined, largely from a similar social-economic background, and exposed to the same level of fitness training, diet, and frequency of outdoor activities. The relative homogeneity of the subjects, in combination with the inclusion criteria, formed the basis of a reference population of which important changes in analytes can be identified. The limitations are that the findings are observational and based on baseline sampling at the start of training. Also, the cohort represents young adults of Caucasian extraction (92.9%), and cannot be extrapolated to the wider population of mixed ethnicity. The predictive threshold values were established based on the equivalent vitamin D status as described by IOM, and not based on data generated by the study as it was not randomly controlled. Measurement of vitamin D binding protein (VDBP) and free 25(OH)D were not performed due to the ethnic homogeneity of the population, factors that may influence VDBP levels (e.g. oral contraceptive use in female recruits) were not excluded.

In conclusion, the present analysis characterises the absolute and relative concentrations of the active and catabolic form of vitamin D metabolites in a well-defined young, healthy and physically fit population. The use of VMRs provides insight into the metabolic pathway and the variations exhibited throughout the year. The proposed a three-dimensional model incorporating 1,25$(OH)_2$D, 24,25$(OH)_2$D and 25(OH)D measurements demonstrated a strong correlation

between the metabolites and PTH. Such modelling could help establish vitamin D-adjusted PTH reference intervals, and ultimately contribute to the goal of a “Treat to target” approach to vitamin D supplementation.

Chapter 10 Immunoaffinity extraction and DAPTAD derivatisation for LC-MS/MS quantification of serum 1,25-dihydroxyvitamin D.

10.1 Background

1,25-dihydroxyvitamin D ($1,25(OH)_2D$), the most biologically active form of vitamin D, mediates its pleiotropic effects through the ubiquitous vitamin D receptor (VDR) that binds to vitamin D response elements in target genes to regulate their transcription. $1,25(OH)_2D$ has potent antiproliferative and cell differentiation-inducing activities in addition to its classical role in the absorption of calcium and phosphate from the intestine, and the retention of calcium from renal excretion and bone mineralisation. Deficiency of $1,25(OH)_2D$ occurs in chronic kidney disease–mineral and bone disorder (CKD-MBD), vitamin D dependent rickets (1α-hydroxylase deficiency), X-linked hypophosphataemia and hypoparathyroidism. Excessive production of $1,25(OH)_2D$ occurs in vitamin D-deficient rickets (end-organ resistance), granulomatosis disease (e.g. sarcoidosis), primary hyperthyroidism, ectopic production (malignancy), and genetic defects resulting in the inactivation of *CYP24A1* gene in children and adults. Serum $1,25(OH)_2D$ can be measured to monitor therapy with vitamin D analogues (calcitriol), 1α-hydroxyvitamin D (alpha calcidol) and calcimimetics used to treat patients with CKD-MBD and metabolic bone disease[180].

The low concentration of serum $1,25(OH)_2D$ (pmol/L), its lipophilic nature in combination with the co-existence of other forms of dihydroxyvitamin D metabolite in circulation pose a difficult challenge for separation and quantification. Immunoassays (radioisotopic or enzyme-labelled) are commercially available, but concerns over cross-reactivity with other dihydroxyvitamin D metabolites have limited their application. Many LC-MS/MS assays have been described in the literature (presented in chapter 1.3.3) that combines an immunoaffinity approach using $1,25(OH)_2D$ antibodies to capture the analyte, followed by derivatisation to enhance the ionisation efficiency of the molecule for MS detection. However, such a method still requires a high-end sensitive LC-MS/MS instrument that is capable of performing the separation and detection. In January 2019, the Bioanalytical Facility at UEA purchased a Waters® Xevo TQ-XS tandem mass spectrometer with the Acquity I-class UPLC LC system (Fig. 10.1). It is one of the top-end, class-leading LC-MS/MS system currently available in the market. The aims of the study were to use this system to develop and validate a robust method to measure $1,25(OH)_2D3$ and $1,25(OH)_2D2$

in serum and plasma, and perform sample comparison with the existing immunoassay method that is currently in routine service.

Figure 10.1 Waters Xevo TQ-XS tandem mass spectrometer (picture right) with Acquity I-class UPLC system with integrated sample organiser (left).

10.2 Materials and methods

10.2.1 Serum sample collection

Serum patient samples (n=69) were collected from residual samples following their course of routine care in accordance with generic ethical approval[80]. The samples were selected at random from routine requests for 1,25$(OH)_2$D measurement received by the Department of Laboratory Medicine at the Norfolk and Norwich University Hospital. All samples were anonymised at the point of access. Blood samples were collected into serum gel separator tube (BD Vacutainer) and centrifuged immediately. After a 10 minute centrifugation at 3,000 x g, serum layer was aliquoted into a separate polystyrene tube and stored at -20°C until analysis.

10.2.2 Reagents, standards and controls

Certified pure ethanolic standards for $1,25(OH)_2D3$, $1,25(OH)_2D2$ (IsoSciences, King of Prussia, PA, USA) were spiked into vitamin D depleted serum (BBI Solutions, Cardiff, UK) to create a series of calibration standards with concentrations ranged between 30-900 pmol/L. Three pools of human sera containing $1,25(OH)_2D3$ and $1,25(OH)_2D2$ at 35, 75 and 300 pmol/L were analysed with each batch of samples as controls. A carbon-13 labelled $1,25(OH)_2D3$-25,26,27-$^{13}C_3$ (Cerilliant, Round Rock, TX, USA) was used as internal standard. Chromasolv™ LC-MS grade deionised water and methanol (Honeywell Riedel-de Haen™, Seelze, Germany) were used in the mobile phases. Formic acid, ethanol were LCMS grade, ethyl acetate and isopropanol were analytical grade (Fisher Scientific, Loughborough, UK). ImmuTube® $1,25(OH)_2D$ LC-MS/MS kit (cat# LM1100, ImmunoDiagnostik, Bensheim, Germany) was used for sample extraction. Stock derivatisation reagent was synthesised by combining 40 mg of 4-[4-(Dimethylamino)phenyl]-1,2,4-triazolidine-3,5-dione (DAPTAD) (Santa Cruz Biotechnology, Dallas, TX, USA) with 60 mg of Iodobenzene diacetate (Sigma-Aldrich, Dorset, UK) in 40 mL of ethyl acetate, the mixture was placed on a magnetic mixer at room temperature for 3 hours until the colourless solution turned red (Fig. 10.2).

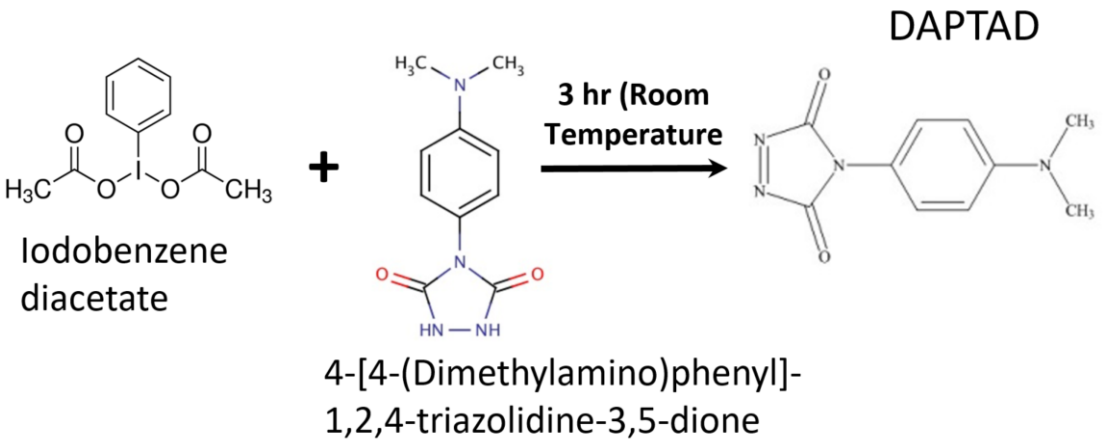


Figure 10.2 Synthesis of DAPTAD.

10.2.3 Sample preparation procedure for LC-MS/MS

An outline of the sample extraction procedure is shown in figure 10.3. The immunoextraction step was performed according to the kit manufacturer's instruction, 300 µL of samples was used instead of the recommended 500 µL. Test samples, calibration standards and quality control materials were placed into the respective spin column containing 100 µL of $1,25(OH)_2D$ antibody slurry, followed by 200 µL of $1,25(OH)_2D3$-25,26,27-$^{13}C_3$ in 50:50 isopropanol/water internal

standard mixture (100 pmol/L) added into each column. The isopropanol releases the protein-bound form of $1,25(OH)_2D$. The columns were capped and placed onto a vertical carousel rotator for a 1-hour incubation at room temperature while rotated end-over-end at 15 rpm. After the incubation, the columns underwent three repeat cycles of wash through with 500 µL of the kit wash solution performed in a centrifuge set at 550 xg for 2 minutes. The outlet of the columns was removed to allow the waste to drain into a discard tube while the antibody-bound $1,25(OH)_2D$ remained inside the column. After the wash cycles, 250 µL of the kit elution reagent was added into each column and centrifuged at 550 xg for 2 minutes. The eluents were collected into a fresh set of glass tubes and then dried under nitrogen gas in a sample concentrator heated at 60°C.

The working DAPTAD solution was made up fresh on the day by diluting the stock 1 in 5 with ethyl acetate, 100 µL was used to reconstitute the dried sample tubes. After a 1-hour incubation, the dienophilic reaction was terminated by adding 50 µL of ethanol (Fig. 10.4) into each tube. The tubes were returned to the sample concentrator for a second dry down under nitrogen gas heated at 60°C. The dried tubes were reconstituted with 50 µL of methanol followed by 50 µL of water. After a brief vortex, 30 µL of the derivatised extract were injected into the LC-MS/MS.

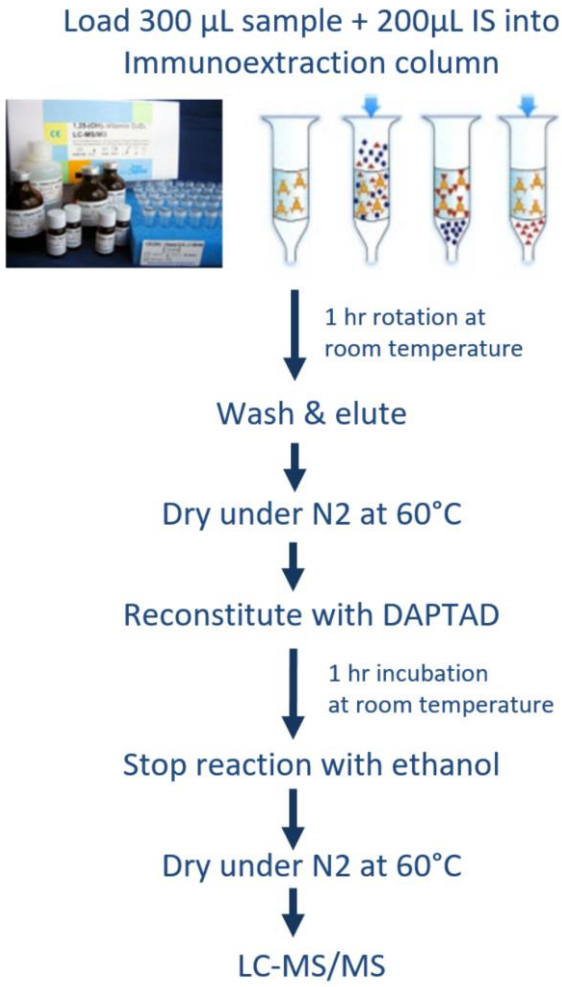


Figure 10.3 Schematic representation of the immunoextraction and sample derivatisation procedure.

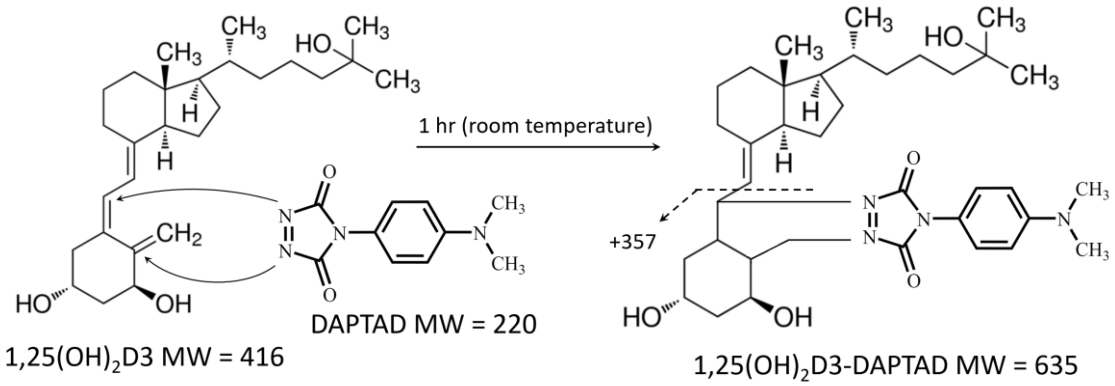


Figure 10.4 The dienophilic reaction of $1,25(OH)_2D3$ with DAPTAD and formation of the derivatised product.

10.2.4 Liquid chromatography

Extracted samples were injected into LC-MS/MS by Waters® Acquity I-class UPLC system (Waters Corp., Milford, MA, USA) equipped with a 10-drawer cooler stack regulated at 15˚C. Chromatographic separation was achieved using a CORTECS™ core-shell C18 50 × 2.1mm, 2.7 µm, reversed-phase (Waters Corp., Milford, MA, USA) column heated at 50˚C. Waters VanGuard pre-column C18 1.7µm. 5 x 2.1mm was used to protect the column. A gradient elution profile was set up to deliver the mobile phase at a flow rate of 0.4 mL/min (Fig. 10.5). At the start of the gradient, the mobile phase consisted of 50:50 (v/v) of (A) water containing in 0.1% formic acid and (B) methanol in 0.1% formic acid. The gradient was gradually increased to 100% of the mobile phase (B) then returned to the starting gradient at 4 minutes. Solvent divert was employed to the divert ion suppression region of the separation to waste to minimise contamination to the source of the mass spectrometer.

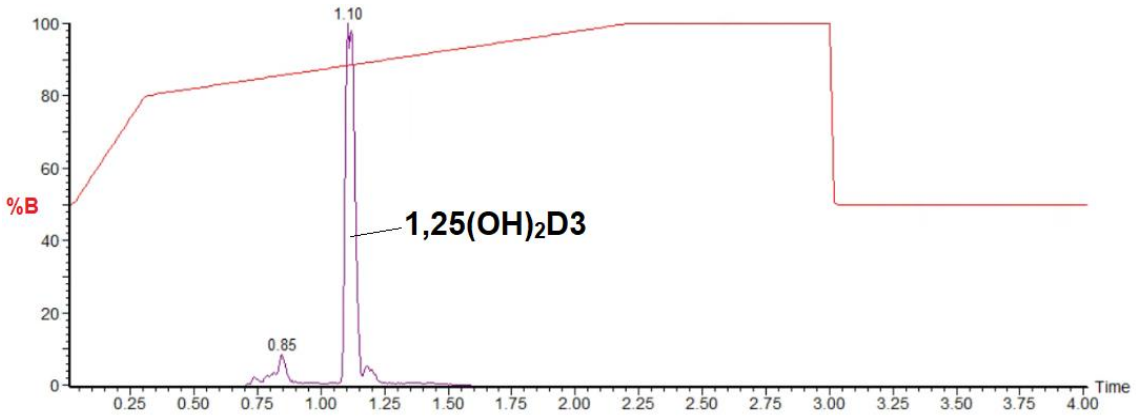


Figure 10.5 The percentage composition of mobile phase B (methanol in 0.1% formic acid) during a 4-minute gradient run. 1,25$(OH)_2$D3 peak eluted at 1.10 minutes.

10.2.5 Tandem mass spectrophotometry analysis

LC-MS/MS analysis of vitamin D metabolites was performed using Waters Xevo TQ-XS tandem mass spectrometer (Waters Corp., Milford, MA, USA). MassLynx version 4.2 and QuanLynx software (Waters Corp., Milford, MA, USA) were used for system control, data acquisition, baseline integration and peak quantification. Optimisation of MS/MS parameters was accomplished by direct infusion of derivatised standards (Table 10.1). Argon gas was applied to the collision cell during the Collision Induced Dissociation (CID) process. The precursor to product ion transitions for each compound was ascertained based on the molecular weight of the DAPTAD derived products (Fig. 10.6).

Parameter	Setting
Ion source	Electrospray positive
Capillary voltage	1.7 kV
Cone energy	30 V
Collision energy	27 eV
Nebuliser gas flow rate	7.0 Bar
Cone gas flow rate	150 L/hr
Desolvation gas flow rate	600 L/hr
Collison gas flow rate	0.18 mL/min
Source temperature	85°C
Desolvation gas temperature	200°C
m/z transitions (Precusor>Product)	
$1,25(OH)_2D3$	635>357
$1,25(OH)_2D2$	647>357
$1,25(OH)_2D3$-[$^{13}C_3$]	638>357

Table 10.1 Mass spectrometer settings and MRM precursor to product ion transitions for $1,25(OH)_2D3/D2$.

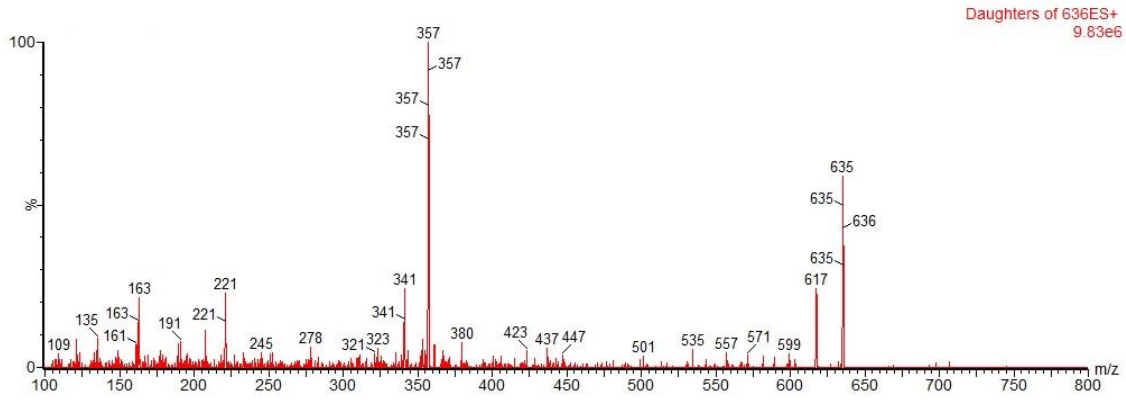


Figure 10.6 Mass spectrum showing the precursor ion mass of DAPTAD-derivatised $1,25(OH)_2D3$ (m/z 635) and fragmentation product ion (m/z 357).

10.2.6 Method validation

Method validation was validated for linearity, lower limits of quantification/detection (LLoQ/D), accuracy, precision and recovery following the 2013 U.S Food and Drug Administration (FDA)[145] and the 2012 European Medicines Agency (EMA)[146] guidance. NIST traceable standards for $1,25(OH)_2D3$, $1,25(OH)_2D2$ are not available, certified pure standards for $1,25(OH)_2D3$ and $1,25(OH)_2D2$ were spiked gravimetrically into vitamin D depleted human serum to create a six-point calibration. Three controls were analysed with each batch of sample analysis. The controls provide the basis of accepting or rejecting the batch, the assay acceptance criteria require over half of the IQC samples analysed in each batch produce results within 15% from their respective target value, and 20% at LLoQ[146].

10.2.7 Linearity

Serum standards with known concentrations ranged from 0-900 pmol/L for $1,25(OH)_2D3$ and $1,25(OH)_2D2$ were analysed at the start and end of every batch. A standard curve was generated by plotting the ratio of analyte peak area to internal standard peak on the y-axis against the weighted ($1/x$) concentration of their respective standards on the x-axis. The goodness-of-fit of a standard curve is justified by linear regression that produced a correlation coefficient (r^2) value of >0.980.

10.2.8 Accuracy, precision and recovery

Due to the lack of NIST reference standards, the accuracy of an analytical method was assessed by spike and recovery performed at low, medium and high concentrations across the analytical range. Method bias was assessed using DEQAS samples and compared against the mean values generated by other participating laboratories. A multiple reaction monitoring (MRM) transition using the 184 Da product ion was included in the acquisition method to monitor the level of phospholipids present in the sample matrix. Imprecision of the assay was assessed by six consecutive measurements of the control materials within a single run (intra-assay) and repeatedly over one month (inter-assay). Imprecision was expressed as the coefficient of variation (CV), acceptance criteria defined the intra-assay CV limit of <10% and cumulative inter-assay CV limit <15%.

10.2.9 Lower limit of quantification and detection

A precision profile was carried out to determine the lower limit of quantification (LLoQ) and detection (LLoD) of the assay. Samples containing $1,25(OH)_2D3$ and $1,25(OH)_2D2$ at

concentrations of 10, 15, 20, 25 and 30 pmol/L were each analysed six times, the CVs of each sample were plotted against their respective concentration. The analyte peak must produce a signal-to-noise (s/n) ratio of 10:1, the LLoQ was defined as the concentration at which the CV ≤15%.

10.2.10 Method comparison

The 1,25$(OH)_2$D3 values generated by the newly developed method was compared against the DiaSorin LIAISON® XL 1,25$(OH)_2$D chemiluminescent immunoassay (Stillwater, MN, USA). Serum samples (n=69) that were previously analysed by the immunoassay were retested by the new method. Samples from the DEQAS external quality assurance scheme (n=21) were analysed, and results compared against the LC/MS method mean. Passing-Bablock regression was used to assess the comparability of results between methods; a correlation is deemed satisfactory if the linear regression produced a correlation coefficient (r^2) value of >0.90.

10.2.11 Statistical data analysis

Passing-Bablock regression plots and bias plots were constructed and analysed by GraphPad Prism 8.2 (GraphPad, San Diego, CA, USA) analysis. Statistical significance was defined as p <0.05. All numerical data were visually examined and checked for transcriptional, pre/post analytical errors before statistical analysis.

10.3 Results

Chromatographic peaks for 1,25$(OH)_2$D3 and 1,25$(OH)_2$D2 were eluted at 1.12 and 1.14, minutes, respectively (Fig. 10.7). No phospholipids peak was detected during this period.

An ion suppression test was conducted by post-column infusion of an aqueous mixture of 1,25$(OH)_2$D3 with simultaneous injection of an extracted pooled serum or phosphate buffered saline blank (Fig 10.8). Moderate reduction of baseline signals was observed during the first minute of the chromatographic run before the 1,25$(OH)_2$D3 peak. The period of ion suppression was diverted to waste to prevent contamination of the instrument source. Phospholipids in the sample matrix monitored by the acquisition of m/z transitions 184>184 did show significant presence during the peak elution period.

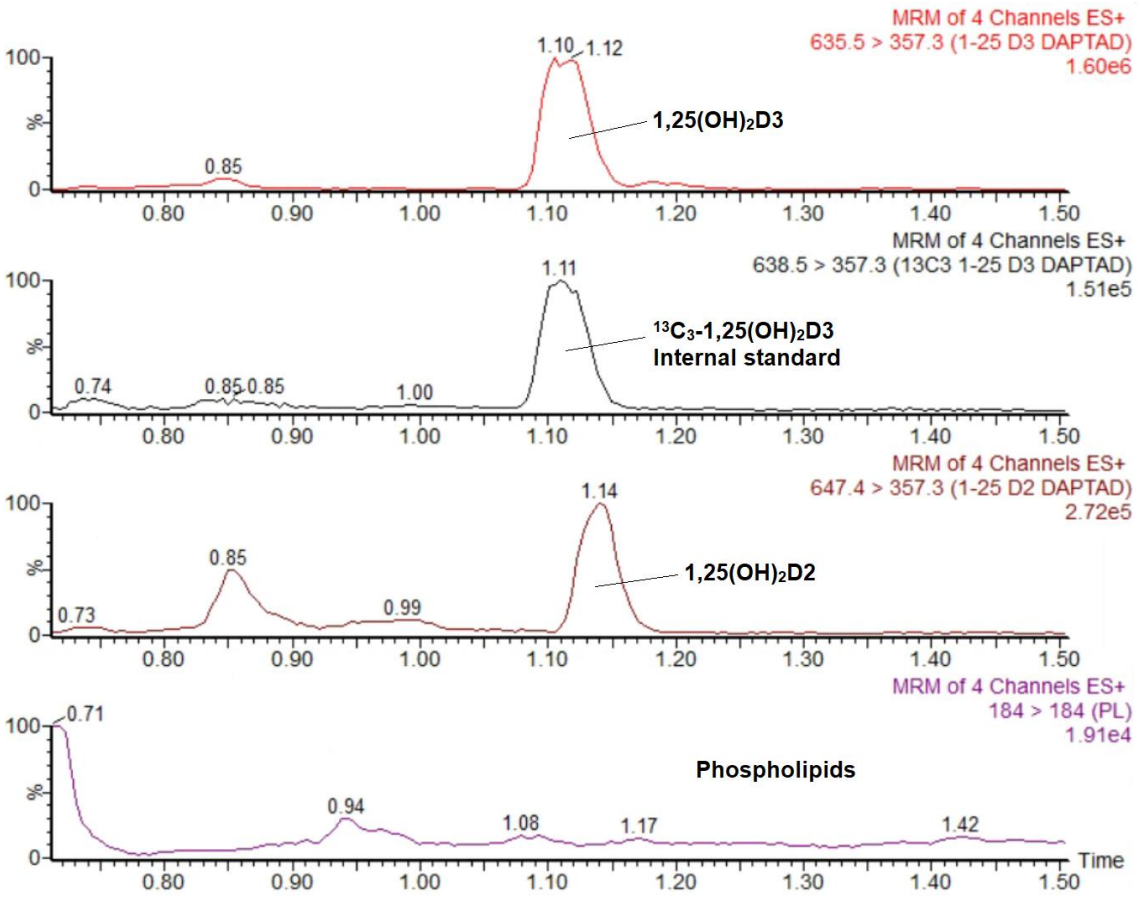


Figure 10.7 Chromatogram from a patient serum sample containing 154.5 pmol/L of 1,25$(OH)_2$D3 and 27.1 pmol/L of 1,25$(OH)_2$D2.

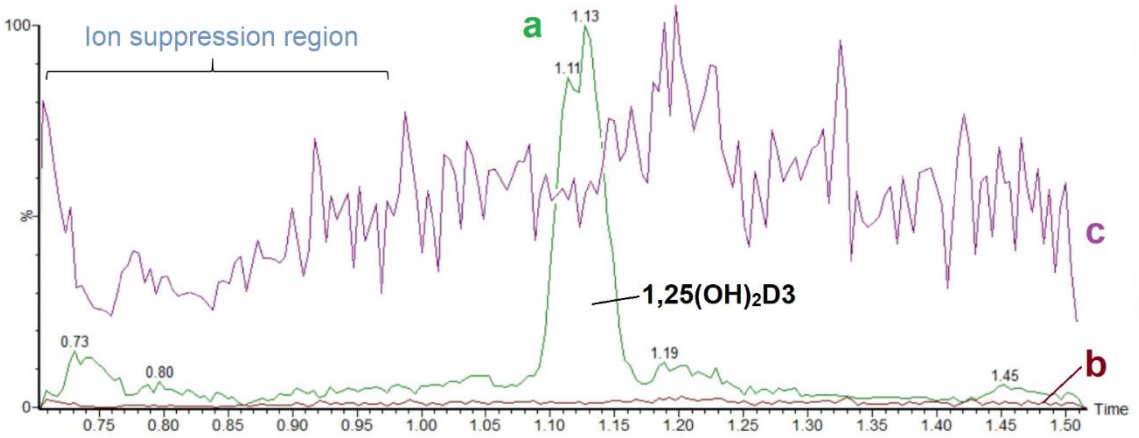


Figure 10.8 Ion suppression test. Chromatographic trace recorded during co-injection of (**a**) extracted serum sample, or (**b**) phosphate buffered saline blank with post-column infusion of 1 nmol/L of pure 1,25$(OH)_2$D3 (**c**). Moderate ion suppression occurred within the first minute of the run, prior to the elution of 1,25$(OH)_2$D3 peak at 1.13 min.

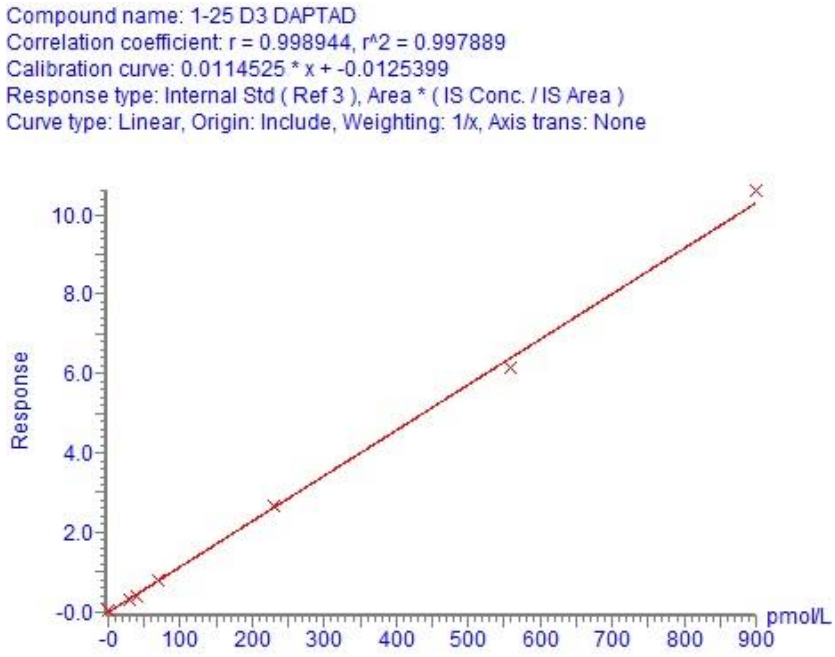
10.3.1 Assay performance

Method validation results are summarised in Table 10.2. The assay achieved adequate linearity (Fig. 10.9a-b), sensitivity, precision, and demonstrated reproducible recovery to satisfy method validation criteria. Carry-over testing confirmed no peaks were present during the analysis of a blank sample following an injection of a sample spiked with high concentrations (1 μmol/L) of 1,25$(OH)_2$D3 and 1,25$(OH)_2$D2. No interfering peaks were found between the analyte transitions. The LLoQ of the method was established by the analysis of a series of samples that contained a decreasing concentration of analytes. Results showed the lowest 1,25$(OH)_2$D3 and 1,25$(OH)_2$D2 concentrations determined with a CV of 15% were 20 pmol/L (Fig. 10.10a-b).

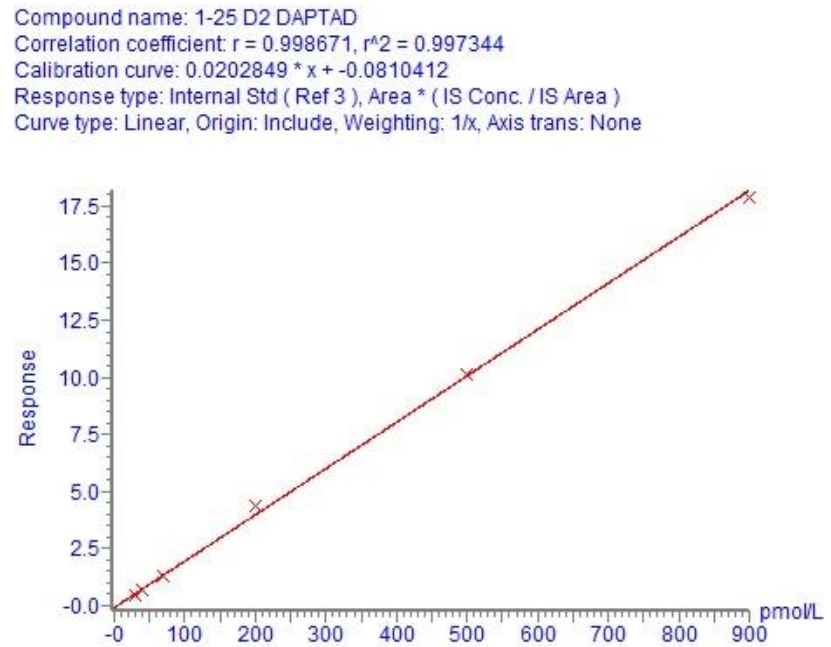
		$1,25(OH)_2D3$	$1,25(OH)_2D2$
Linearity, pmol/L (typical r^2)		0-900 (0.977)	0-900 (0.985)
LLoQ (LLoD), pmol/L		20 (16)	20 (12)
Imprecision conc. (±SD), %CV	**Intra (n=6)**	37.1 (±3.5) 9.4%	39.7 (±3.6) 9.0%
		75.6 (±5.6) 7.4%	81.5 (±4.9) 6.0%
		305.5 (±6.2) 2.0%	434.7 (±11) 2.5%
	Inter (n=6)	40.3 (±3.6) 9.0%	42.4 (±4.2) 9.8%
		75.3 (±5.6) 7.4%	89.6 (±4.9) 5.5%
		302.8 (±10.3) 3.4%	280.2 (±8.5) 3.0%
Recovery spiked conc. pmol/L (mean recovery% ±SD)	**Spiked into vitamin D depleted serum**	+50 (109%±2.1)	+50 (115%±6.5)
		+100 (93%±6.5)	+100 (91%±4.6)
		+300 (92%±8.2)	+300 (94%±1.2)
	Spiked into pooled EDTA plasma*	+50 (103%±2.5)	+50 (97%±3.5)
		+100 (108%±5.6)	+100 (105%±5.6)
		+300 (93%±6.2)	+300 (95%±6.2)

*Pooled EDTA plasma contained 164.3 pmol/L of $1,25(OH)_2D3$ and 19.2 nmol/L of $1,25(OH)_2D2$

Table 10.2 Performance characteristics of the DAPTAD $1,25(OH)_2D$ LC-MS/MS method. Recovery was determined by spiking known amount of $1,25(OH)_2D3$ and $1,25(OH)_2D2$ into vitamin D depleted serum and a pool of EDTA plasma containing endogenous $1,25(OH)_2D$. Each spiked sample was analysed three times in separate runs.

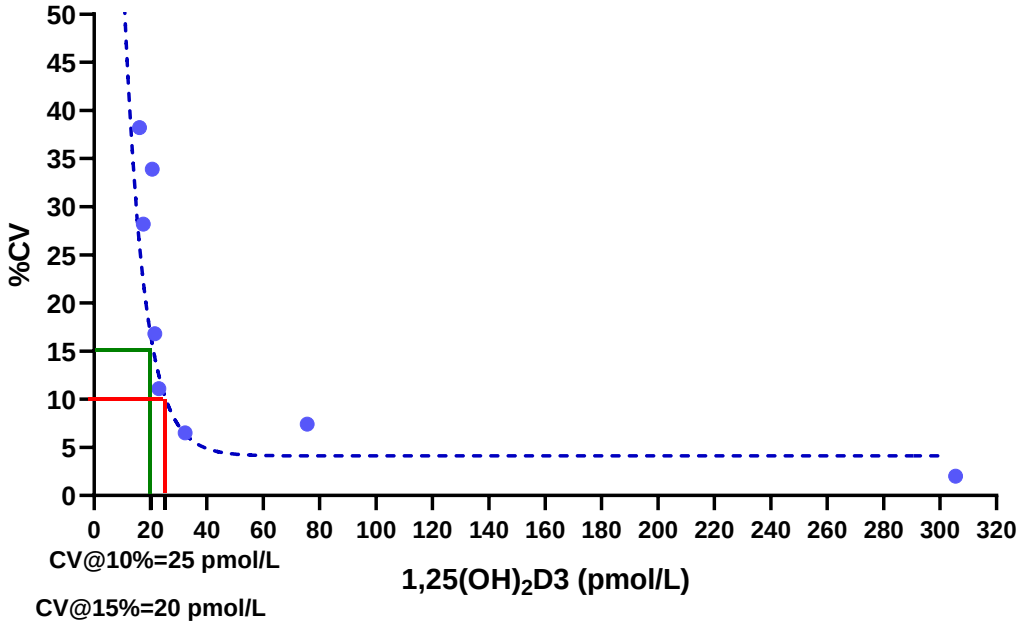


a)

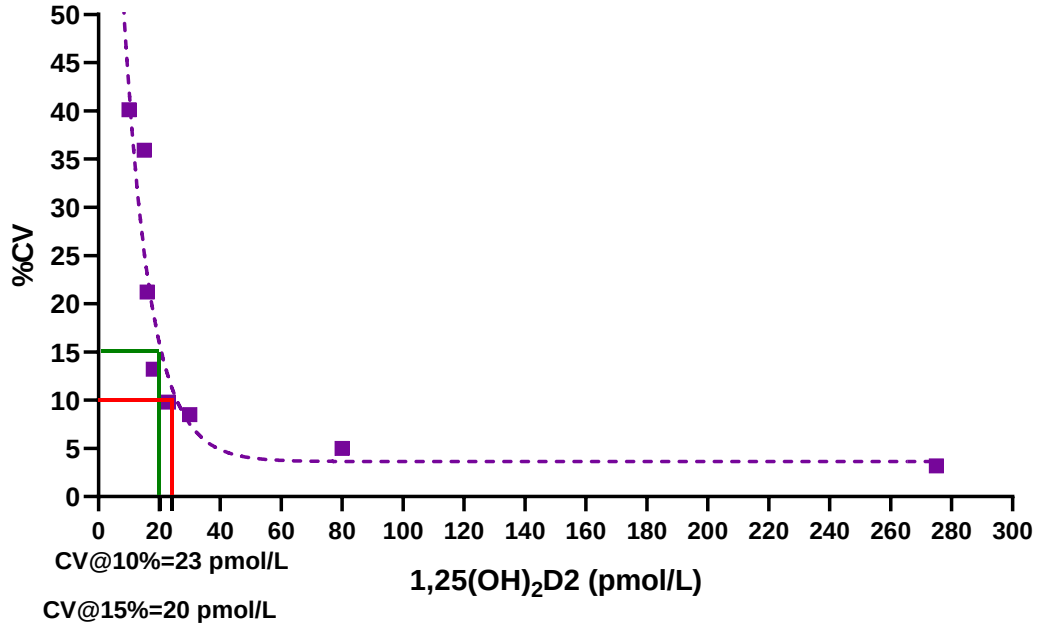


b)

Figure 10.9a-b Typical standard curves for **a)** 1,25$(OH)_2$D3 and **b)** 1,25$(OH)_2$D2 constructed by plotting the response of each standard on the y-axis against their respective concentrations (nmol/L) on the x-axis. The response of each compound was determined by the ratio of the peak area of the standards to the peak area of the deuterated internal standards. Regression analysis showed a typical correlation coefficient r^2 >0.99.



a)

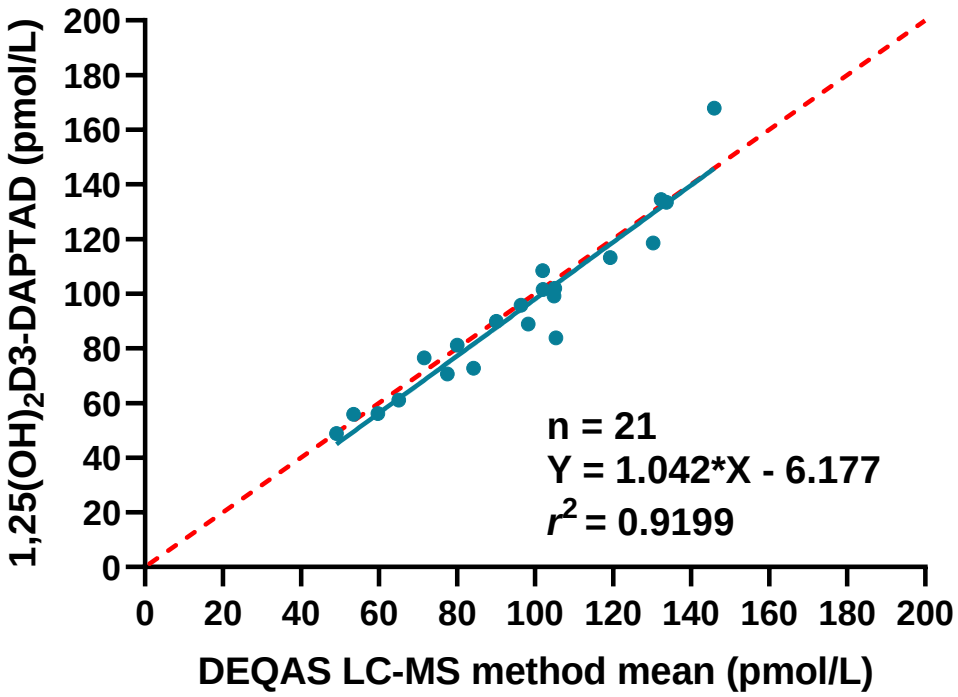


b)

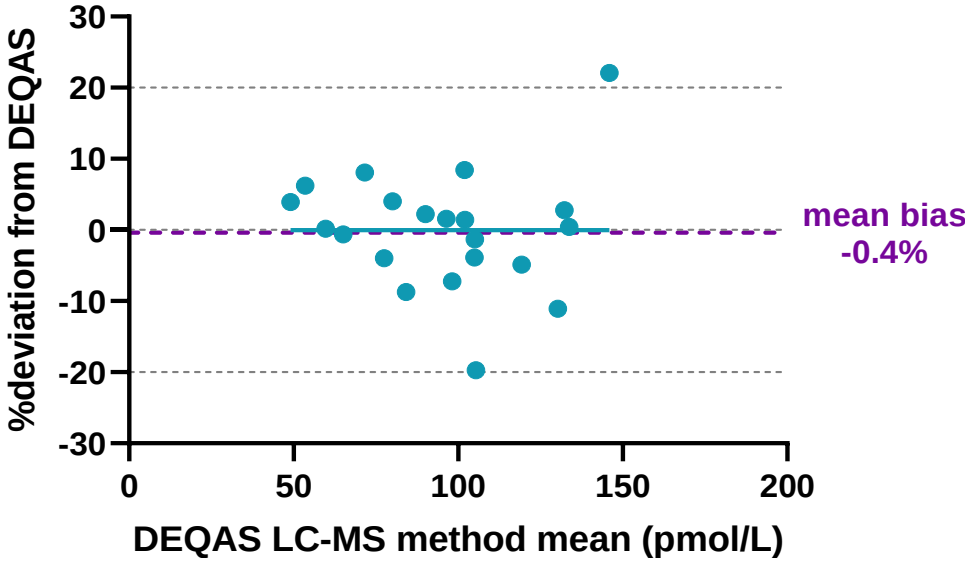
Figure 10.10a-b Method imprecision profile for **a)** 1,25$(OH)_2$D3 and **b)** 1,25$(OH)_2$D2 . The reproducibility across the analytical range of the assay is expressed by plotting the coefficient of variation (CV) against their respective mean concentration (pmol/L) on the x-axis. Each concentration was analysed three times over separate runs. The concentration of which CV=10% and 15% are indicated by red and green lines. The LLoQ was determined at the CV of 15%.

10.3.2 Method comparisons

To assess the comparability of the new method, 18 DEQAS samples and 52 serum samples previously analysed by the DiaSorin LIAISON XL $1,25(OH)_2D$ chemiluminescent immunoassay was tested by the new method. Passing-Bablock regression analysis between the $1,25(OH)_2D3$ values produced by the DAPTAD LC-MS/MS method with the DEQAS LC-MS method group mean (Fig. 10.11a) showed a highly significant correlation $y = 1.042x - 6.177, r^2 = 0.9199$, p<0.001. Bland-Altman residual plot (Fig. 10.11b) showed a small mean (±SD) bias of -0.4% (±8.4). None of the values deviated outside the ±30% from the mean and therefore met the DEQAS performance proficiency criteria.



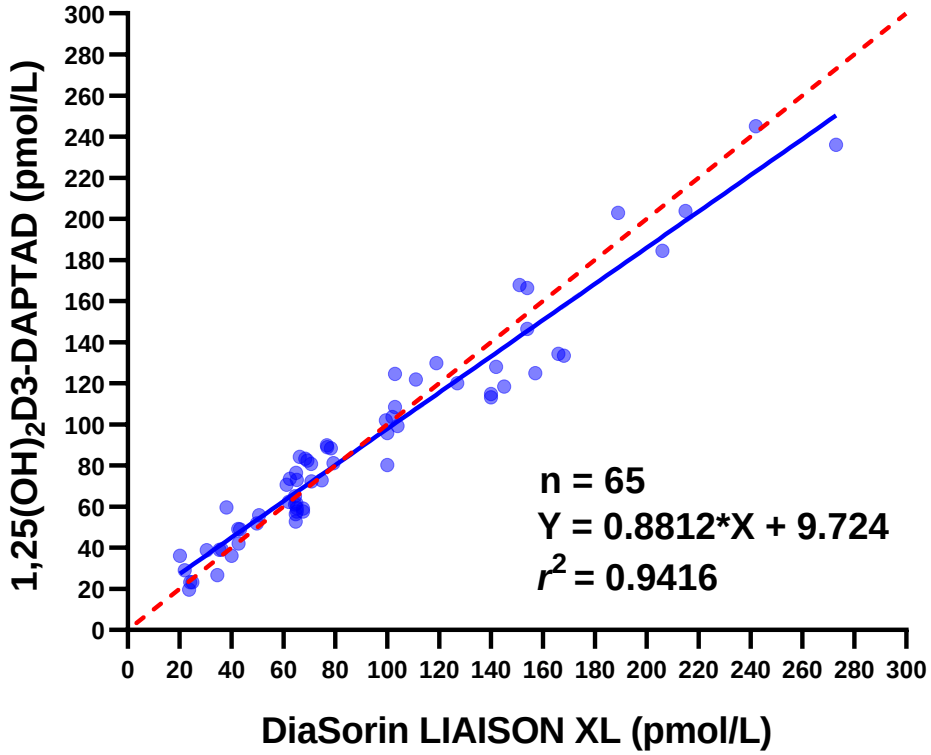
a)



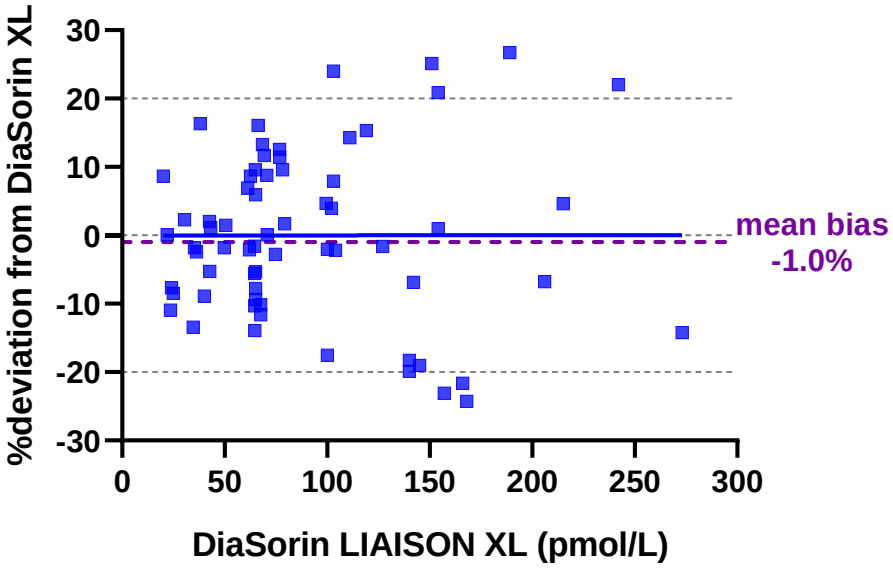
b)

Figure 10.11a-b Comparison of 1,25(OH)$_2$D3 between the new method and DEQAS LC-MS method group mean (n=21). **(a)** Passing-Bablock regression analysis showed a highly significant correlation (r^2=0.9199, p<0.001). **(b)** Bland-Altman residual plot showing the percentage deviation of the measurements with DEQAS LC-MS group mean values. The trend line is represented in blue; purple dashed lines represent the mean bias. The dotted lines represent ±20% limits of agreement. The red dashed line in **(a)** represents the line of identity.

Comparison of with the DiaSorin immunoassay also showed a highly significant correlation $y = 0.8812x - 9.724, r^2 = 0.9416, p < 0.001$ (Fig 10.12a). Four of the 69 samples tested by the immunoassay below the detection limit (<12 pmol/L) were also below the limit for the LC-MS/MS assay (<16 pmol/L). Bland-Altman residual plot showed a small mean (±SD) bias of -1% (±12.3) (Fig. 10.12b). In contrast with the DEQAS comparison, greater variability was observed between the LC-MS/MS and immunoassay results; the line of best fit in figure 10.12a showed a greater negative bias towards values >150 pmol/L produced by the LC-MS/MS. The evidence suggests the variance is method specific.



a)



b)

Figure 10.12a-b **(a)** Comparison of results generated by the LC-MS/MS against Diasorin immunoassay (n=69). Four samples with 1,25(OH)$_2$D concentration below the detection limits assayed both methods were not shown in the plot. The red line in the graph represents the fitted regression; red dashed line is the line identity. **(b)** Bland-Altman plot showing the percentage deviation of the LC-MS/MS from immunoassay. The dotted lines represent ±20% limits of agreement. The purple dashed line represents the mean bias.

10.4 Discussion and conclusion

This final chapter fulfils the most challenging objective set out in this PhD study, that is to develop an LC-MS/MS assay for the measurement of serum $1,25(OH)_2D3$, and $1,25(OH)_2D2$. The poor ionisation properties of $1,25(OH)_2D$ and its low circulating concentration in serum require extensive sample preparation to enrich and extract the compounds from the matrix, followed by derivatisation to transform $1,25(OH)_2D$ into a robust complex for ionisation. There were compromises; the immunoextraction step is a long (1 hour) process that required a relatively large volume (300 µL) of serum/plasma sample, which could be a concern for paediatric patients. The procedure involved lengthy manual handling steps that cannot be easily adapted by automated platforms. Also, the immunoaffinity spin columns were costly at £20 per test. On the contrary, the DAPTAD was synthesised from relatively inexpensive raw materials; the derivatisation reagent was <£1 per test. A comparable alternative with Amplifex™ Diene reagent (SCIEX) is commercially available at £10 per test. During the initial development period, attempts were made to use the SLE sample extraction and PTAD derivatisation as described in chapter 5, but the procedure failed to achieve sufficient sensitivity to detect samples with $1,25(OH)_2D3$ concentration less than 50 pmol/L, which is necessary for analysis of samples from patients with chronic kidney disease and vitamin D-dependent rickets.

The new Waters Acquity Xevo TQ-XS tandem mass spectrometer system had shown to be superior to the Micromass Quattro Ultima Pt system described in the previous chapters. The low internal dwell volume in the Acquity LC/autosampler system helped reduce band dispersion and peak distortion. Noticeable improvement in the base peak width was observed; peaks were eluted within $1/10^{th}$ of a minute using the Acquity system (Fig. 10.7), whereas the Microssmass system operated in the same conditions eluted peaks in ½ of a minute (chapter 7 fig. 7.2). Higher peak resolution concentrates the sample in the column, allowing for a sharper, more focused delivery to the source of the mass spectrometer, thus enhancing the sensitivity. Derviatisation is a key step; the Xevo TQ-XS instrument, similar to other high-end instruments of the latest generation, features an off-axis ion source and transfer optics (StepWave™) that actively removes interfering compounds in order to increase signal strength. Such design creates a harsh environment that is suboptimal for $1,25(OH)_2D$. Without derivatisation, water loss product ions are formed from the fragmentation of the two hydroxyl groups in the 1α and 25 positions. Water loss products are less specific and can be prone to interference by other compounds with similar chemical structure. Derviatisation also increased the m/z to a higher mass range where it is less prone to interference by other small molecules.

The method was compared against DEQAS and the DiaSorin immunoassay platform. Although the sample size for the DEQAS comparison was small (n=21), the target values were based on

the mean values generated by LC-MS/MS methods, providing a more method-specific comparison. The $1,25(OH)_2D3$ results produced by the new method showed a strong correlation with the DEQAS LC-MS/MS consensus, with 19 out of 21 results (90.5%) showed less than 10% deviation. The comparison with the DiaSorin immunoassay using routine patient samples provided a different perspective on a larger sample size (n=69). The new method correlated with the DiaSorin immunoassay with a negative bias that was more significant in sample concentration >150 pmol/L. More considerable variability was observed between the methods, with 42 out of 69 (60.9%) samples showed less than 10% deviation with the immunoassay. The disparity is likely due to the cross-reactivity of the antibodies used in the immunoassay and the difference in the serum matrix components (e.g. vitamin D binding protein) associated with the patient's pathophysiological conditions. The samples were collected anonymously; therefore, it was not possible to ascertain whether the reasons for the disparity was related to the diagnosis. There are reports of assay bias published in the literature; Higgins et al.[75] reported high disparity between the DiaSorin and LC-MS/MS methods in pooled neonates and infants samples with elevated $1,25(OH)_2D$, a positive bias of up to 26.5% was observed in immunoassay results, 3-epi-$1,25(OH)_2D$ was ruled out as a potential source of bias. Ivison et al. reported a negative bias (mean -47.8%, range -160 to 15%, n=78) of their LC-MS/MS method with the IDS radioimmunoassay[76]. Spanaus and Von Eckardstein evaluated the Diasorin immunoassay with a LC-MS/MS method using 93 patient samples[72], and their results showed a mean bias of 2.3% (95%CI: -29.2 to 33.7%), which was not dissimilar to results produced by the new method.

The $1,25(OH)_2D2$ method was validated alongside $1,25(OH)_2D3$, but few patient samples with endogenous $1,25(OH)_2D2$ were identified; therefore, it was not possible to carry out a comparison study. Future study would be to collect samples with $1,25(OH)_2D2$ present from routine requests and send the samples to another LC-MS/MS laboratory for a blind comparison. Currently, there is no routine service for $1,25(OH)_2D2$ available in the UK.

In conclusion, this final chapter describes the successful development and validation of an LC-MS/MS method for the quantification $1,25(OH)_2D3$ and $1,25(OH)_2D2$ in serum and plasma. The immunoaffinity column extraction combined with DAPTAD sample derivatisation procedure was robust and reproducible to provide a low to medium throughput routine analytical service. The new method performed well against the Diasorin immunoassay and other LC-MS method users, concerns over the disparity of results observed in some patient samples will require further investigations to determine the source of bias.

Chapter 11 Conclusion and summary of research impact

This PhD thesis described a series of successful method developments using LC-MS/MS techniques to provide accurate and precise measurements for the major circulating vitamin D metabolites, and the applications in research and clinical context. All the methods had satisfied the performance validation criterion. The dried blood 25(OH)D method using microsampling device described in Chapter 6 extends the application further; allowing self-sampling at home or at remote locations where access to phlebotomy service is limited, whilst the low sample volume requirement is ideally suited for use in paediatric practice and elderly population. This thesis is the first to provide supporting evidence that a relationship exists between 25(OH)D with the active ($1,25(OH)_2D$) and the catabolic ($24,25(OH)_2D$) forms of vitamin D (Chapter 9). The finding had led to the successful publication in *Scientific Reports* in early 2019[181]. The assay for simultaneous measurement of $24,25(OH)_2D$ and 25(OH)D (Chapter 7) published in the *Journal of Nutritional Biochemistry* in 2017[54] have been providing routine service for NHS patients, and have supported many collaboration research studies, resulting in important findings and publications that had significant impacts in several areas of science.

Underpinning research

The introduction of a high-throughput extraction technology combined with the excellent sensitivity of the LC-MS/MS has allowed large numbers of samples to be analysed for several clinical trial studies. The $24,25(OH)_2D$ and 25(OH)D assay, using a biochemical technique to remove the built-up of phospholipids in sample matrix (Chapter 5), allowed accurate measurement of vitamin D metabolites in long term storage samples. Chapter 8 described three vitamin D3 supplementation studies with different dosing modality; the VICtORY study (400 IU and 1000 IU/day) was collected over a 2-year period, retrospective sample analysis could not have been possible without adequate phospholipids depletion. Results from the study had formed the basis for a compare and contrast study with the other two high dose vitamin D supplementation studies; where a single 100,000 IU bolus and weekly (35,000 and 70,000 IU/wk) had shown gross elevation of serum $24,25(OH)_2D$ as a result of excessive intake of vitamin D3. The publications that followed[161,162,173] provided a stark warning to the scientific community, clinicians and sports nutritionist that administration of high dose vitamin D supplement may not be beneficial to the

individual, but the practice can be detrimental to a person's health. The importance of the finding was such that I was selected to give an oral presentation at the 2016 European Calcified Tissue Society Congress (Rome, Italy)[169], and poster presentations at the American Society of Bone and Mineral Research conference (Atlanta, USA)[182] and the British Endocrine Society meeting (Brighton, UK)[183]. In the follow-up publication on the secondary analysis of the VICtORY study, the daily low dose of vitamin D3 treatment showed significant improvement in spine and hip BMD in postmenopausal women with baseline 25(OH)D ≤30 nmol/L, whereas no treatment effect was observed in women with 25(OH)D ≥30 nmol/L[179]. It was recommended that future vitamin D studies on bone health should focus on individuals with 25(OH)D in this concentration range. The finding from this research and the recommendation that 25(OH)D should be measured by tandem mass spectrometry method to distinguish 25(OH)D3 and D2 were endorsed by the Royal National Osteoporosis '*Vitamin D and Bone Health. A Practical Clinical Guideline For Patient Management*'[10](Specific references to UEA work with Prof. William Fraser are on pages 2, 7).

The studies performed at UEA to date that involved the use of the vitamin D metabolite assays developed in this thesis had contributed to important research in other areas of medicine; significant association of vitamin D status with, or effect on, disease, are extensive and include respiratory disease[184,185], pregnancy[186], chronic fatigue syndrome/myalgic encephalomyelitis[187], renal transplant recipients[188], cannabis use[189], fetal development (animal model)[190]. Research undertaken at UEA in 2013 had established that vitamin D2 supplementation is not effective as vitamin D3 in changing clinical outcomes[172]. The finding had a significant effect on public health, resulting in new approaches to vitamin D supplementation and interpretation of vitamin D deficiency. This research has been cited extensively and incorporated into several meta-analyses of the role of vitamin D in disease, and in attempts to define the optimal therapeutic thresholds for treatment and prevention of disease.

Impact on patient care and the NHS economy

Before the development of LC-MS/MS methodology, 25(OH)D was measured by a variety of immunoassays with poor standardisation that could result in over or underestimation of results resulting in erroneous diagnosis and incorrect treatment. All immunoassays have poor antibody cross-reactivity with 25(OH)D2 resulting in underestimation of 25(OH)D2 and, depending on 25(OH)D3 assay standardisation, total 25(OH)D status. The superior specificity and selectivity of mass spectrometry detection allowed measurement of vitamin D metabolites with precision, accuracy and sensitivity. Co-determination of 24,25(OH)D and 25(OH)D with their respective D3 and D2 forms from a single sample analysis enabled the assessment of the individual's vitamin D storage and catabolic status. The use of 25(OH)D:24,25(OH)D vitamin D metabolite ratio can

identify patients with potential *CYP24A1* mutations and hence the avoidance of unnecessary and expensive patient investigation for malignancy as a cause of hypercalcaemia.

In recent years, hospital laboratories have experienced a marked, year-over-year increase in requests for 25(OH)D measurement[191,192]. The demand on workload while under significant financial constraints, plus deskilling of the laboratory workforce and increasing regulatory requirements has seen many hospital laboratories returning to immunoassays. LC-MS/MS methodology must be rapid and cost-effective. Since 2015, the 24,25$(OH)_2$D and 25(OH)D assay has been providing a routine service for patients from the Norfolk and Norwich University NHS hospitals, with typical turnaround <3 days. The assay is also offered as part of the Supraregional Assay and Advisory service (SAAS) for other NHS and private hospitals in the UK. The cost per sample for an LC-MS/MS 25(OH)D analysis is £10.50, which is significantly below the NICE estimated national average of £17[193]. The Bioanalytical Facility at UEA currently processes around 10,000 patient samples per annum, representing a yearly cost saving of £65,000 for the NHS.

The assay has consistently met the performance target set by the Vitamin D External Quality Assessment Scheme (DEQAS) and achieved proficiency certificates since the method was implemented.

Impact on the MOD Army training programme and in sports science

Based on the underpinning research outlined in the previous section, the vitamin D metabolite assays described in this thesis has supported collaborative programmes of work with the Army Health and Physical Performance Research (APRC). The British Army recruits up to four thousand new soldiers every year, the high physical demands in the initial military training combined with periods of energy deficit and sleep deprivation increase the risk of injury and illness to recruits resulting in lost training days. Up to 20.4% of Army recruits suffer from musculoskeletal injuries and up to 10% suffer skeletal stress fracture[194]. Previous pilot studies had shown a high prevalence of vitamin D insufficiency in the British Army[195]. The Army Lower Limb Injury prevention programme (ALLIPP) was established in 2013 by the UK Ministry of Defence (MoD) to study the association of vitamin D status with injury risk, health and physical performance in British Army Recruits. A subset of baseline data collected in this large scale (n=2292) study was described in Chapter 9. The follow-up data analysis showed 38% of the participants identified as vitamin D insufficiency (25(OH)D <50 nmol/L) were associated with increased risk (odds ratio) of training-related stress fracture SFx(1.03), medial tibial stress syndrome (MTSS) (1.26), upper limb trauma (1.02), respiratory infections (1.13); and upper limb

overuse injuries (3.18) and prolonged the length of recovery (3.49) (data not included as part of this PhD thesis). I was selected to give an oral presentation at the 2018 British Endocrine Society meeting (Brighton, UK)[196]. The MOD has now recognised that a high percentage of recruits have significant vitamin D deficiency, and that female recruits, in particular, have a higher predisposition to injury. This has prompted a review of the nutritional recommendations for all recruits in training and an alteration in the training programme for servicewomen in ground close combat (GCC) roles[197].

In sports science research, LC-MS/MS technology is not available to the majority of researchers in this field. The methods described in this thesis have facilitated many research studies in sports science; contributing to the discovery of the beneficial effects of vitamin D supplement on physical performance in elite professional athletes. Positive associations with skeletal muscle regeneration[198] but not on muscle function[199], improved resistance to infections[111], enhanced exercise endurance (1.5-mile run time was approximately 0.5 seconds faster for every 1 nmol/L increase in 25(OH)D concentration[200]) have been published.

Conclusion

This thesis presented the development of methods to measure the major circulating vitamin D metabolite 25(OH)D, $1,25(OH)_2D$ and $24,25(OH)_2D$. Using state of the art LC-MS/MS technology and robust sample preparation techniques to enhance sensitivity, accuracy and precision, results from the studies have demonstrated the role that each metabolite plays in maintaining homeostasis. This study is the first to describe a relationship between the three vitamin D metabolites; using serum concentrations and their respective ratio to give a mechanistic view of the physiological response during states of deficiency and toxicity. The established reference intervals and diagnostic thresholds can be utilised clinically for the assessment of vitamin D metabolic status, and in the investigation for the cause of hypercalcaemia. The newly developed methods have provided the analytical platform for many large scale studies, results of which have, and will continue to help further our understanding and appreciation of the vitamin D metabolism as part of the hormonal network; expanding our knowledge base in the field of musculoskeletal research and other areas of science. The publications and citations are a testament to the far-reaching impact from the product of this programme of research.

References

1. Lanham-New, S.A., Buttriss, J.L., Miles, L.M., Ashwell, M., Berry, J.L., Boucher, B.J., Cashman, K.D., Cooper, C., Darling, A.L., Francis, R.M., Fraser, W.D., de Groot, C.P., Hypponen, E., Kiely, M., Lamberg-Allardt, C., Macdonald, H.M., Martineau, A.R., Masud, T., Mavroeidi, A., Nowson, C., Prentice, A., Stone, E.M., Reddy, S., Vieth, R. & Williams, C.M. Proceedings of the Rank Forum on Vitamin D. *Br J Nutr* **105**(1), 144-156 (2011).
2. Jenab, M., Bueno-de-Mesquita, H.B., Ferrari, P., van Duijnhoven, F.J., Norat, T., Pischon, T., Jansen, E.H., Slimani, N., Byrnes, G., Rinaldi, S., Tjonneland, A., Olsen, A., Overvad, K., Boutron-Ruault, M.C., Clavel-Chapelon, F., Morois, S., Kaaks, R., Linseisen, J., Boeing, H., Bergmann, M.M., Trichopoulou, A., Misirli, G., Trichopoulos, D., Berrino, F., Vineis, P., Panico, S., Palli, D., Tumino, R., Ros, M.M., van Gils, C.H., Peeters, P.H., Brustad, M., Lund, E., Tormo, M.J., Ardanaz, E., Rodriguez, L., Sanchez, M.J., Dorronsoro, M., Gonzalez, C.A., Hallmans, G., Palmqvist, R., Roddam, A., Key, T.J., Khaw, K.T., Autier, P., Hainaut, P. & Riboli, E. Association between pre-diagnostic circulating vitamin D concentration and risk of colorectal cancer in European populations:a nested case-control study. *BMJ* **340**, b5500.
3. Gilbert, R., Metcalfe, C., Fraser, W.D., Donovan, J., Hamdy, F., Neal, D.E., Lane, J.A. & Martin, R.M. Associations of circulating 25-hydroxyvitamin D with prostate cancer diagnosis, stage and grade. *Int J Cancer* **131**(5), 1187-1196 (2012).
4. Gilbert, R., Martin, R.M., Beynon, R., Harris, R., Savovic, J., Zuccolo, L., Bekkering, G.E., Fraser, W.D., Sterne, J.A. & Metcalfe, C. Associations of circulating and dietary vitamin D with prostate cancer risk: a systematic review and dose-response meta-analysis. *Cancer Causes Control* **22**(3), 319-340 (2011).
5. Mayor, S. Lack of vitamin D raises risk of heart disease and worsens prognosis for some cancers. *BMJ* **336**(7635), 62 (2008).
6. Lavie, C.J., Lee, J.H. & Milani, R.V. Vitamin D and cardiovascular disease will it live up to its hype? *J Am Coll Cardiol* **58**(15), 1547-1556 (2011).
7. Hewison, M. An update on vitamin D and human immunity. *Clin Endocrinol (Oxf)* **76**(3), 315-325 (2012).
8. Thomas, M.C. & Cooper, M.E. Into the light? Diabetic nephropathy and vitamin D. *Lancet* **376**(9752), 1521-1522 (2010).
9. Holick, M.F. Vitamin D deficiency. *N Engl J Med* **357**(3), 266-281 (2007).
10. Francis, R.M., Aspray, T.J., Fraser, W.D., Macdonald, H.M., Patel, S., Mavroeidi, A., Schoenmakers, I. & Stone, M. Vitamin D and bone health: a practical clinical guideline for patient management. Royal Osteoporosis Society 2018 Dec 2018 [cited 24 June 2019]; 2:[Available from: https://theros.org.uk/media/100231/nos_vitamin_d_and_bone_-health_in_adults_web.pdf.
11. Ross, A.C., Manson, J.E., Abrams, S.A., Aloia, J.F., Brannon, P.M., Clinton, S.K., Durazo-Arvizu, R.A., Gallagher, J.C., Gallo, R.L., Jones, G., Kovacs, C.S., Mayne, S.T., Rosen, C.J. & Shapses, S.A. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J Clin Endocrinol Metab* **96**(1), 53-58 (2011).
12. SACN. Vitamin D and Health report. Scientific Advisory Committee on Nutrition. (Public Health England, London: TSO, 2016).
13. Vitamin D - advice on supplements for at risk groups. Department of Health and Social Care 2016 [cited 27th June 2019]; Available from: https://www.gov.uk/government/news/phe-publishes-new-advice-on-vitamin-d.
14. Sanders, K.M., Stuart, A.L., Williamson, E.J., Simpson, J.A., Kotowicz, M.A., Young, D. & Nicholson, G.C. Annual high-dose oral vitamin D and falls and fractures in older women: a randomized controlled trial. *JAMA* **303**(18), 1815-1822 (2010).

15. Bischoff-Ferrari, H.A., Dawson-Hughes, B., Orav, E.J., Staehelin, H.B., Meyer, O.W., Theiler, R., Dick, W., Willett, W.C. & Egli, A. Monthly High-Dose Vitamin D Treatment for the Prevention of Functional Decline: A Randomized Clinical Trial. *JAMA Intern Med* **176**(2), 175-183 (2016).
16. Kamao, M., Tatematsu, S., Hatakeyama, S., Sakaki, T., Sawada, N., Inouye, K., Ozono, K., Kubodera, N., Reddy, G.S. & Okano, T. C-3 epimerization of vitamin D3 metabolites and further metabolism of C-3 epimers: 25-hydroxyvitamin D3 is metabolized to 3-epi-25-hydroxyvitamin D3 and subsequently metabolized through C-1alpha or C-24 hydroxylation. *J Biol Chem* **279**(16), 15897-15907 (2004).
17. Brown, A.J., Ritter, C.S., Weiskopf, A.S., Vouros, P., Sasso, G.J., Uskokovic, M.R., Wang, G. & Reddy, G.S. Isolation and identification of 1alpha-hydroxy-3-epi-vitamin D3, a potent suppressor of parathyroid hormone secretion. *J Cell Biochem* **96**(3), 569-578 (2005).
18. Singh, R.J., Taylor, R.L., Reddy, G.S. & Grebe, S.K. C-3 epimers can account for a significant proportion of total circulating 25-hydroxyvitamin D in infants, complicating accurate measurement and interpretation of vitamin D status. *J Clin Endocrinol Metab* **91**(8), 3055-3061 (2006).
19. Flynn, N., Lam, F. & Dawnay, A. Enhanced 3-epi-25-hydroxyvitamin D3 signal leads to overestimation of its concentration and amplifies interference in 25-hydroxyvitamin D LC-MS/MS assays. *Ann Clin Biochem* **51**(Pt 3), 352-359 (2014).
20. Tai, S.S., Bedner, M. & Phinney, K.W. Development of a candidate reference measurement procedure for the determination of 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 in human serum using isotope-dilution liquid chromatography-tandem mass spectrometry. *Anal Chem* **82**(5), 1942-1948 (2010).
21. Shah, I., James, R., Barker, J., Petroczi, A. & Naughton, D.P. Misleading measures in Vitamin D analysis: a novel LC-MS/MS assay to account for epimers and isobars. *Nutr J* **10**, 46 (2011).
22. van den Ouweland, J.M., Beijers, A.M. & van Daal, H. Fast separation of 25-hydroxyvitamin D3 from 3-epi-25-hydroxyvitamin D3 in human serum by liquid chromatography-tandem mass spectrometry: variable prevalence of 3-epi-25-hydroxyvitamin D3 in infants, children, and adults. *Clin Chem* **57**(11), 1618-1619 (2011).
23. Kubiak, J.M., Grimnes, G., Cashman, K.D., Kamycheva, E., Dowling, K., Skrabakova, Z. & Jorde, R. C3-epimerization of 25-hydroxyvitamin D increases with increasing serum 25-hydroxyvitamin D levels and shows a high degree of tracking over time. *Clin Biochem* **54**, 61-67 (2018).
24. Engelman, C.D., Bo, R., Zuelsdorff, M., Steltenpohl, H., Kirby, T. & Nieto, F.J. Epidemiologic study of the C-3 epimer of 25-hydroxyvitamin D(3) in a population-based sample. *Clinical nutrition* **33**(3), 421-425 (2014).
25. Granado-Lorencio, F., Blanco-Navarro, I., Perez-Sacristan, B., Donoso-Navarro, E. & Silvestre-Mardomingo, R. Serum levels of 3-epi-25-OH-D3 during hypervitaminosis D in clinical practice. *J Clin Endocrinol Metab* **97**(12), E2266-2270 (2012).
26. Granado-Lorencio, F., Rubio, E., Blanco-Navarro, I., Perez-Sacristan, B., Rodriguez-Pena, R. & Garcia Lopez, F.J. Hypercalcemia, hypervitaminosis A and 3-epi-25-OH-D3 levels after consumption of an "over the counter" vitamin D remedy. a case report. *Food Chem Toxicol* **50**(6), 2106-2108 (2012).
27. Wiebe, D. & Binkley, N. Case report: Three patients with substantial serum levels of 3-epi-25(OH)D including one with 3-epi-25(OH)D2 while on high-dose ergocalciferol. *J Clin Endocrinol Metab* **99**(4), 1117-1121 (2014).
28. Levin, A., Bakris, G.L., Molitch, M., Smulders, M., Tian, J., Williams, L.A. & Andress, D.L. Prevalence of abnormal serum vitamin D, PTH, calcium, and phosphorus in patients with chronic kidney disease: results of the study to evaluate early kidney disease. *Kidney Int* **71**(1), 31-38 (2007).
29. Valcour, A., Zierold, C., Podgorski, A.L., Olson, G.T., Wall, J.V., DeLuca, H.F. & Bonelli, F. A novel, fully-automated, chemiluminescent assay for the detection of 1,25-

dihydroxyvitamin D in biological samples. *J Steroid Biochem Mol Biol* **164**, 120-126 (2016).
30. Tran, J., Bautista, D., Seres, Z., Cornaut, L., Gundlach, T., Rousseau, A. & Griesser, H. 1,25-Dihydroxy vitamin D assay with on-board sample purification on the IDS-iSYS automated system. Bone Abstracts 2014; 3: PP81. Presented at European Calcified Tissue Society Congress. Czech Republic, Prague Poster
31. Yuan, C., Kosewick, J., He, X., Kozak, M. & Wang, S. Sensitive measurement of serum 1alpha,25-dihydroxyvitamin D by liquid chromatography/tandem mass spectrometry after removing interference with immunoaffinity extraction. *Rapid Commun Mass Spectrom* **25**(9), 1241-1249 (2011).
32. Zittermann, A., Ernst, J.B., Becker, T., Dreier, J., Knabbe, C., Gummert, J.F. & Kuhn, J. Measurement of Circulating 1,25-Dihydroxyvitamin D: Comparison of an Automated Method with a Liquid Chromatography Tandem Mass Spectrometry Method. *Int J Anal Chem* **2016**, 8501435 (2016).
33. Abu Kassim, N.S., Shaw, P.N. & Hewavitharana, A.K. Simultaneous determination of 12 vitamin D compounds in human serum using online sample preparation and liquid chromatography-tandem mass spectrometry. *Journal of chromatography. A* **1533**, 57-65 (2018).
34. Casetta, B., Jans, I., Billen, J., Vanderschueren, D. & Bouillon, R. Development of a method for the quantification of 1alpha,25(OH)2-vitamin D3 in serum by liquid chromatography tandem mass spectrometry without derivatization. *Eur J Mass Spectrom (Chichester)* **16**(1), 81-89 (2010).
35. Kaufmann, M., Morse, N., Molloy, B.J., Cooper, D.P., Schlingmann, K.P., Molin, A., Kottler, M.L., Gallagher, J.C., Armas, L. & Jones, G. Improved Screening Test for Idiopathic Infantile Hypercalcemia Confirms Residual Levels of Serum 24,25-(OH)2 D3 in Affected Patients. *J Bone Miner Res* **32**(7), 1589-1596 (2017).
36. Muller, M.J., Stokes, C.S., Lammert, F. & Volmer, D.A. Chemotyping the distribution of vitamin D metabolites in human serum. *Sci Rep* **6**, 21080 (2016).
37. Wagner, D., Hanwell, H.E., Schnabl, K., Yazdanpanah, M., Kimball, S., Fu, L., Sidhom, G., Rousseau, D., Cole, D.E. & Vieth, R. The ratio of serum 24,25-dihydroxyvitamin D(3) to 25-hydroxyvitamin D(3) is predictive of 25-hydroxyvitamin D(3) response to vitamin D(3) supplementation. *J Steroid Biochem Mol Biol* **126**(3-5), 72-77 (2011).
38. Bosworth, C.R., Levin, G., Robinson-Cohen, C., Hoofnagle, A.N., Ruzinski, J., Young, B., Schwartz, S.M., Himmelfarb, J., Kestenbaum, B. & de Boer, I.H. The serum 24,25-dihydroxyvitamin D concentration, a marker of vitamin D catabolism, is reduced in chronic kidney disease. *Kidney Int* **82**(6), 693-700.
39. Haddad, J.G., Jr., Min, C., Mendelsohn, M., Slatopolsky, E. & Hahn, T.J. Competitive protein-binding radioassay of 24,25-dihydroxyvitamin D in sera from normal and anephric subjects. *Arch Biochem Biophys* **182**(2), 390-395 (1977).
40. Horst, R.L., Shepard, R.M., Jorgensen, N.A. & DeLuca, H.F. The determination of 24,25-dihydroxyvitamin D and 25,26-dihydroxyvitamin D in plasma from normal and nephrectomized man. *J Lab Clin Med* **93**(2), 277-285 (1979).
41. Ornoy, A., Goodwin, D., Noff, D. & Edelstein, S. 24, 25-dihydroxyvitamin D is a metabolite of vitamin D essential for bone formation. *Nature* **276**(5687), 517-519 (1978).
42. Ketha, H., Kumar, R. & Singh, R.J. LC-MS/MS for Identifying Patients with CYP24A1 Mutations. *Clin Chem* **62**(1), 236-242 (2016).
43. Molin, A., Baudoin, R., Kaufmann, M., Souberbielle, J.C., Ryckewaert, A., Vantyghem, M.C., Eckart, P., Bacchetta, J., Deschenes, G., Kesler-Roussey, G., Coudray, N., Richard, N., Wraich, M., Bonafiglia, Q., Tiulpakov, A., Jones, G. & Kottler, M.L. CYP24A1 Mutations in a Cohort of Hypercalcemic Patients: Evidence for a Recessive Trait. *J Clin Endocrinol Metab* **100**(10), E1343-1352 (2015).
44. de Boer, I.H., Sachs, M.C., Chonchol, M., Himmelfarb, J., Hoofnagle, A.N., Ix, J.H., Kremsdorf, R.A., Lin, Y.S., Mehrotra, R., Robinson-Cohen, C., Siscovick, D.S., Steffes, M.W., Thummel, K.E., Tracy, R.P., Wang, Z. & Kestenbaum, B. Estimated GFR and

circulating 24,25-dihydroxyvitamin D3 concentration: a participant-level analysis of 5 cohort studies and clinical trials. *Am J Kidney Dis* **64**(2), 187-197 (2014).
45. Shimizu, M., Gao, Y., Aso, T., Nakatsu, K. & Yamada, S. Fluorometric assay of 25-hydroxyvitamin D3 and 24R,25-dihydroxyvitamin D3 in plasma. *Anal Biochem* **204**(2), 258-264 (1992).
46. Sebert, J.L., Fournier, A., Lambrey, G., Moriniere, P., Decourcelle, P.H., Makdassi, R. & De Fremont, J.F. [Does 24,25 dihydroxycholecalciferol have a physiological and pathophysiological role?]. *Nephrologie* **3**(3), 133-141 (1982).
47. Castro-Errecaborde, N., de la Piedra, C., Rapado, A., Alvarez-Arroyo, M.V., Torres, R. & Traba, M.L. Correlation between serum osteocalcin and 24,25-dihydroxyvitamin D levels in Paget's disease of bone. *J Clin Endocrinol Metab* **72**(2), 462-466 (1991).
48. Schwartz, Z., Soskolne, W.A., Atkin, I., Goldstein, M. & Ornoy, A. A direct effect of 24,25-(OH)2D3 and 1,25-(OH)2D3 on the modeling of fetal mice long bones in vitro. *J Bone Miner Res* **4**(2), 157-163 (1989).
49. St-Arnaud, R. & Glorieux, F.H. 24,25-Dihydroxyvitamin D--active metabolite or inactive catabolite? *Endocrinology* **139**(8), 3371-3374 (1998).
50. Wang, T.J., Zhang, F., Richards, J.B., Kestenbaum, B., van Meurs, J.B., Berry, D., Kiel, D.P., Streeten, E.A., Ohlsson, C., Koller, D.L., Peltonen, L., Cooper, J.D., O'Reilly, P.F., Houston, D.K., Glazer, N.L., Vandenput, L., Peacock, M., Shi, J., Rivadeneira, F., McCarthy, M.I., Anneli, P., de Boer, I.H., Mangino, M., Kato, B., Smyth, D.J., Booth, S.L., Jacques, P.F., Burke, G.L., Goodarzi, M., Cheung, C.L., Wolf, M., Rice, K., Goltzman, D., Hidiroglou, N., Ladouceur, M., Wareham, N.J., Hocking, L.J., Hart, D., Arden, N.K., Cooper, C., Malik, S., Fraser, W.D., Hartikainen, A.L., Zhai, G., Macdonald, H.M., Forouhi, N.G., Loos, R.J., Reid, D.M., Hakim, A., Dennison, E., Liu, Y., Power, C., Stevens, H.E., Jaana, L., Vasan, R.S., Soranzo, N., Bojunga, J., Psaty, B.M., Lorentzon, M., Foroud, T., Harris, T.B., Hofman, A., Jansson, J.O., Cauley, J.A., Uitterlinden, A.G., Gibson, Q., Järvelin, M.R., Karasik, D., Siscovick, D.S., Econs, M.J., Kritchevsky, S.B., Florez, J.C., Todd, J.A., Dupuis, J., Hyppönen, E. & Spector, T.D. Common genetic determinants of vitamin D insufficiency: a genome-wide association study. *Lancet* **376**(9736), 180-188 (2010).
51. Streeten, E.A., Zarbalian, K. & Damcott, C.M. CYP24A1 mutations in idiopathic infantile hypercalcemia. *N Engl J Med* **365**(18), 1741-1742; author reply 1742-1743 (2011).
52. Jacobs, T.P., Kaufman, M., Jones, G., Kumar, R., Schlingmann, K.P., Shapses, S. & Bilezikian, J.P. A lifetime of hypercalcemia and hypercalciuria, finally explained. *J Clin Endocrinol Metab* **99**(3), 708-712 (2014).
53. Bosworth, C.R., Levin, G., Robinson-Cohen, C., Hoofnagle, A.N., Ruzinski, J., Young, B., Schwartz, S.M., Himmelfarb, J., Kestenbaum, B. & de Boer, I.H. The serum 24,25-dihydroxyvitamin D concentration, a marker of vitamin D catabolism, is reduced in chronic kidney disease. *Kidney Int* **82**(6), 693-700 (2012).
54. Tang, J.C.Y., Nicholls, H., Piec, I., Washbourne, C.J., Dutton, J.J., Jackson, S., Greeves, J. & Fraser, W.D. Reference intervals for serum 24,25-dihydroxyvitamin D and the ratio with 25-hydroxyvitamin D established using a newly developed LC-MS/MS method. *J Nutr Biochem* **46**, 21-29 (2017).
55. Kaufmann, M., Gallagher, J.C., Peacock, M., Schlingmann, K.P., Konrad, M., DeLuca, H.F., Sigueiro, R., Lopez, B., Mourino, A., Maestro, M., St-Arnaud, R., Finkelstein, J.S., Cooper, D.P. & Jones, G. Clinical utility of simultaneous quantitation of 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D by LC-MS/MS involving derivatization with DMEQ-TAD. *J Clin Endocrinol Metab* **99**(7), 2567-2574 (2014).
56. Stubbs, J.R., Zhang, S., Friedman, P.A. & Nolin, T.D. Decreased conversion of 25-hydroxyvitamin D3 to 24,25-dihydroxyvitamin D3 following cholecalciferol therapy in patients with CKD. *Clinical journal of the American Society of Nephrology : CJASN* **9**(11), 1965-1973 (2014).

57. Edouard, T., Husseini, A., Glorieux, F.H. & Rauch, F. Serum 24,25-dihydroxyvitamin D concentrations in osteogenesis imperfecta: relationship to bone parameters. *J Clin Endocrinol Metab* **97**(4), 1243-1249 (2012).
58. Tang, J.C.Y., Jackson, S., Walsh, N.P., Greeves, J., Fraser, W.D. & Bioanalytical Facility, t. The dynamic relationships between the active and catabolic vitamin D metabolites, their ratios, and associations with PTH. *Scientific Reports* **9**(1), 6974 (2019).
59. Carter, G.D., Berry, J., Durazo-Arvizu, R., Gunter, E., Jones, G., Jones, J., Makin, H.L.J., Pattni, P., Sempos, C.T., Twomey, P., Williams, E.L. & Wise, S.A. Hydroxyvitamin D assays: An historical perspective from DEQAS. *J Steroid Biochem Mol Biol* **177**, 30-35 (2018).
60. Carter, G.D. 25-Hydroxyvitamin D assays: the quest for accuracy. *Clin Chem* **55**(7), 1300-1302 (2009).
61. Glendenning, P., Taranto, M., Noble, J.M., Musk, A.A., Hammond, C., Goldswain, P.R., Fraser, W.D. & Vasikaran, S.D. Current assays overestimate 25-hydroxyvitamin D3 and underestimate 25-hydroxyvitamin D2 compared with HPLC: need for assay-specific decision limits and metabolite-specific assays. *Ann Clin Biochem* **43**(Pt 1), 23-30 (2006).
62. Freeman, J., Wilson, K., Spears, R., Shalhoub, V. & Sibley, P. Performance evaluation of four 25-hydroxyvitamin D assays to measure 25-hydroxyvitamin D2. *Clin Biochem* **48**(16-17), 1097-1104 (2015).
63. Wyness, S.P. & Straseski, J.A. Performance characteristics of six automated 25-hydroxyvitamin D assays: Mind your 3s and 2s. *Clin Biochem* **48**(16-17), 1089-1096 (2015).
64. Karvaly, G., Meszaros, K., Kovacs, K., Patocs, A., Sipak, Z. & Vasarhelyi, B. Looking beyond linear regression and Bland-Altman plots: a comparison of the clinical performance of 25-hydroxyvitamin D tests. *Clin Chem Lab Med* **55**(3), 385-393 (2017).
65. Dowling, K.G., Hull, G., Sundvall, J., Lamberg-Allardt, C. & Cashman, K.D. Improved accuracy of an tandem liquid chromatography-mass spectrometry method measuring 24R,25-dihydroxyvitamin D3 and 25-hydroxyvitamin D metabolites in serum using unspiked controls and its application to determining cross-reactivity of a chemiluminescent microparticle immunoassay. *Journal of chromatography. A* **1497**, 102-109 (2017).
66. Hara, K., Ikeda, K., Koyama, Y., Wada, Y. & Hasegawa, T. Comparison of serum 25-hydroxyvitamin D levels between radioimmunoassay and liquid chromatography-tandem mass spectrometry in infants and postpartum women. *J Pediatr Endocrinol Metab* **31**(10), 1105-1111 (2018).
67. Christesen, H.T., Elvander, C., Lamont, R.F. & Jorgensen, J.S. The impact of vitamin D in pregnancy on extraskeletal health in children: a systematic review. *Acta Obstet Gynecol Scand* **91**(12), 1368-1380 (2012).
68. Heijboer, A.C., Blankenstein, M.A., Kema, I.P. & Buijs, M.M. Accuracy of 6 routine 25-hydroxyvitamin D assays: influence of vitamin D binding protein concentration. *Clin Chem* **58**(3), 543-548 (2012).
69. Bouillon, R., Schuit, F., Antonio, L. & Rastinejad, F. Vitamin D Binding Protein: A Historic Overview. *Front Endocrinol (Lausanne)* **10**, 910 (2019).
70. Review 2012. *Vitamin D External Quality Assessment Scheme (DEQAS)* **London, UK**(2012).
71. van den Ouweland, J.M., Beijers, A.M., van Daal, H., Elisen, M.G., Steen, G. & Wielders, J.P. Evaluation of 3-epi-25-hydroxyvitamin D3 cross-reactivity in the Roche Elecsys Vitamin D Total protein binding assay. *Clin Chem Lab Med* **52**(3), 373-380 (2014).
72. Spanaus, K. & von Eckardstein, A. Evaluation of two fully automated immunoassay based tests for the measurement of 1alpha,25-dihydroxyvitamin D in human serum and comparison with LC-MS/MS. *Clin Chem Lab Med* **55**(9), 1305-1314 (2017).
73. Souberbielle, J.C., Cavalier, E., Delanaye, P., Massart, C., Brailly-Tabard, S., Cormier, C., Borderie, D., Benachi, A. & Chanson, P. Serum calcitriol concentrations measured with a new direct automated assay in a large population of adult healthy subjects and in various clinical situations. *Clin Chim Acta* **451**(Pt B), 149-153 (2015).

74. Carpenter, T.O., Herreros, F., Zhang, J.H., Ellis, B.K., Simpson, C., Torrealba-Fox, E., Kim, G.J., Savoye, M., Held, N.A. & Cole, D.E. Demographic, dietary, and biochemical determinants of vitamin D status in inner-city children. *Am J Clin Nutr* **95**(1), 137-146 (2012).
75. Higgins, V., Truong, D., White-Al Habeeb, N.M.A., Fung, A.W.S., Hoffman, B. & Adeli, K. Pediatric reference intervals for 1,25-dihydroxyvitamin D using the DiaSorin LIAISON XL assay in the healthy CALIPER cohort. *Clin Chem Lab Med* **56**(6), 964-972 (2018).
76. Ivison, F.M., Hinchliffe, E., Howarth, N., Pickersgill, M. & Tetlow, L. Development of a mass spectrometry method for 1,25-dihydroxy vitamin D3 using immunoextraction sample preparation. *Ann Clin Biochem*, 4563219847225 (2019).
77. Phinney, K.W., Tai, S.S., Bedner, M., Camara, J.E., Chia, R.R.C., Sander, L.C., Sharpless, K.E., Wise, S.A., Yen, J.H., Schleicher, R.L., Chaudhary-Webb, M., Maw, K.L., Rahmani, Y., Betz, J.M., Merkel, J., Sempos, C.T., Coates, P.M., Durazo-Arvizu, R.A., Sarafin, K. & Brooks, S.P.J. Development of an Improved Standard Reference Material for Vitamin D Metabolites in Human Serum. *Analytical chemistry* **89**(9), 4907-4913 (2017).
78. Carter, G.D., Ahmed, F., Berry, J., Cavalier, E., Durazo-Arvizu, R., Gunter, E., Jones, G., Jones, J., Phinney, K., Sempos, C.T., Twomey, P.J., Williams, E.L. & Wise, S.A. External Quality Assessment of 24,25-dihydroxyvitamin D3 (24,25(OH)2D3) assays. *J Steroid Biochem Mol Biol* **187**, 130-133 (2019).
79. Wise, S.A., Tai, S.S., Nelson, M.A., Burdette, C.Q., Camara, J.E., Hoofnagle, A.N., Laha, T.J., Carter, G.D., Jones, J., Williams, E.L., Barclay, Z.J., Jones, G., Kaufmann, M., Binkley, N., Kapoor, A., Ziegler, T., Cashman, K.D., Dowling, K.G. & Sempos, C.T. Interlaboratory Comparison for the Determination of 24,25-Dihydroxyvitamin D(3) in Human Serum Using Liquid Chromatography with Tandem Mass Spectrometry. *J AOAC Int* **100**(5), 1308-1317 (2017).
80. Governance arrangements for research ethics committees: a harmonised edition. *Department of Health UK* (2011).
81. Bioanalytical Method Validation Guidance for Industry. *U.S. Department of Health and Human Services Food and Drug Administration* **Final version**, CVM GFI #145 (2018).
82. Guideline on bioanalytical method validation. *European Medicines Agency. Committee for Medicinal Products for Human Use (CHMP)* **London, UK**(2012).
83. Westgard, J.O., Barry, P.L., Hunt, M.R. & Groth, T. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin Chem* **27**(3), 493-501 (1981).
84. Reddy, G.S., Muralidharan, K.R., Okamura, W.H., Tserng, K.Y. & McLane, J.A. Metabolism of 1alpha,25-dihydroxyvitamin D(3) and its C-3 epimer 1alpha,25-dihydroxy-3-epi-vitamin D(3) in neonatal human keratinocytes. *Steroids* **66**(3-5), 441-450 (2001).
85. Rehan, V.K., Torday, J.S., Peleg, S., Gennaro, L., Vouros, P., Padbury, J., Rao, D.S. & Reddy, G.S. 1Alpha,25-dihydroxy-3-epi-vitamin D3, a natural metabolite of 1alpha,25-dihydroxy vitamin D3: production and biological activity studies in pulmonary alveolar type II cells. *Mol Genet Metab* **76**(1), 46-56 (2002).
86. Kamao, M., Tatematsu, S., Reddy, G.S., Hatakeyama, S., Sugiura, M., Ohashi, N., Kubodera, N. & Okano, T. Isolation, identification and biological activity of 24R,25-dihydroxy-3-epi-vitamin D3: a novel metabolite of 24R,25-dihydroxyvitamin D3 produced in rat osteosarcoma cells (UMR 106). *Journal of nutritional science and vitaminology* **47**(2), 108-115 (2001).
87. Lensmeyer, G., Poquette, M., Wiebe, D. & Binkley, N. The C-3 epimer of 25-hydroxyvitamin D(3) is present in adult serum. *J Clin Endocrinol Metab* **97**(1), 163-168 (2012).
88. Strathmann, F.G., Sadilkova, K., Laha, T.J., LeSourd, S.E., Bornhorst, J.A., Hoofnagle, A.N. & Jack, R. 3-epi-25 hydroxyvitamin D concentrations are not correlated with age in a cohort of infants and adults. *Clin Chim Acta* **413**(1-2), 203-206 (2012).

89. Stepman, H.C., Vanderroost, A., Stockl, D. & Thienpont, L.M. Full-scan mass spectral evidence for 3-epi-25-hydroxyvitamin D(3) in serum of infants and adults. *Clin Chem Lab Med* **49**(2), 253-256 (2011).
90. Keevil, B. Does the presence of 3-epi-25OHD3 affect the routine measurement of vitamin D using liquid chromatography tandem mass spectrometry? *Clin Chem Lab Med* **50**(1), 181-183 (2011).
91. Schleicher, R.L., Encisco, S.E., Chaudhary-Webb, M., Paliakov, E., McCoy, L.F. & Pfeiffer, C.M. Isotope dilution ultra performance liquid chromatography-tandem mass spectrometry method for simultaneous measurement of 25-hydroxyvitamin D2, 25-hydroxyvitamin D3 and 3-epi-25-hydroxyvitamin D3 in human serum. *Clin Chim Acta* **412**(17-18), 1594-1599 (2011).
92. Baecher, S., Leinenbach, A., Wright, J.A., Pongratz, S., Kobold, U. & Thiele, R. Simultaneous quantification of four vitamin D metabolites in human serum using high performance liquid chromatography tandem mass spectrometry for vitamin D profiling. *Clin Biochem* **45**(16-17), 1491-1496 (2012).
93. Ross, A.C., Manson, J.E., Abrams, S.A., Aloia, J.F., Brannon, P.M., Clinton, S.K., Durazo-Arvizu, R.A., Gallagher, J.C., Gallo, R.L., Jones, G., Kovacs, C.S., Mayne, S.T., Rosen, C.J. & Shapses, S.A. The 2011 Dietary Reference Intakes for Calcium and Vitamin D: what dietetics practitioners need to know. *J Am Diet Assoc* **111**(4), 524-527 (2011).
94. Brown, A.J., Ritter, C., Slatopolsky, E., Muralidharan, K.R., Okamura, W.H. & Reddy, G.S. 1Alpha,25-dihydroxy-3-epi-vitamin D3, a natural metabolite of 1alpha,25-dihydroxyvitamin D3, is a potent suppressor of parathyroid hormone secretion. *J Cell Biochem* **73**(1), 106-113 (1999).
95. Yazdanpanah, M., Bailey, D., Walsh, W., Wan, B. & Adeli, K. Analytical measurement of serum 25-OH-vitamin D(3), 25-OH-vitamin D(2) and their C3-epimers by LC-MS/MS in infant and pediatric specimens. *Clin Biochem* **46**(13-14), 1264-1271 (2013).
96. Tapan, S., Sertoglu, E. & Uyanik, M. Importance of C-3 epimer of 25-hydroxyvitamin D in dried blood spots of neonatal population. *Int J Cancer* **137**(3), 750 (2015).
97. Cho, S.E., Kim, S., Kim, Y.D., Lee, H., Seo, D.H., Song, J., Um, T.H., Cho, C.R., Kim, N.H. & Hwang, J.H. Measurement of serum 3-epi-25-hydroxyvitamin D3, 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 in infant, paediatric and adolescent populations of Korea using ultra-performance liquid chromatography-tandem mass spectrometry. *Ann Clin Biochem* **54**(5), 530-538 (2017).
98. Ooms, N., van Daal, H., Beijers, A.M., Gerrits, G.P., Semmekrot, B.A. & van den Ouweland, J.M. Time-course analysis of 3-epi-25-hydroxyvitamin D3 shows markedly elevated levels in early life, particularly from vitamin D supplementation in preterm infants. *Pediatr Res* **79**(4), 647-653 (2016).
99. Annesley, T.M. Ion suppression in mass spectrometry. *Clin Chem* **49**(7), 1041-1044 (2003).
100. Little, J.L., Wempe, M.F. & Buchanan, C.M. Liquid chromatography-mass spectrometry/mass spectrometry method development for drug metabolism studies: Examining lipid matrix ionization effects in plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* **833**(2), 219-230 (2006).
101. King, R., Bonfiglio, R., Fernandez-Metzler, C., Miller-Stein, C. & Olah, T. Mechanistic investigation of ionization suppression in electrospray ionization. *Journal of the American Society for Mass Spectrometry* **11**(11), 942-950 (2000).
102. Taylor, P.J. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry. *Clin Biochem* **38**(4), 328-334 (2005).
103. Chambers, E., Wagrowski-Diehl, D.M., Lu, Z. & Mazzeo, J.R. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *J Chromatogr B Analyt Technol Biomed Life Sci* **852**(1-2), 22-34 (2007).
104. Ismaiel, O.A., Halquist, M.S., Elmamly, M.Y., Shalaby, A. & Karnes, H.T. Monitoring phospholipids for assessment of matrix effects in a liquid chromatography-tandem mass

spectrometry method for hydrocodone and pseudoephedrine in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* **859**(1), 84-93 (2007).
105. Ismaiel, O.A., Halquist, M.S., Elmamly, M.Y., Shalaby, A. & Thomas Karnes, H. Monitoring phospholipids for assessment of ion enhancement and ion suppression in ESI and APCI LC/MS/MS for chlorpheniramine in human plasma and the importance of multiple source matrix effect evaluations. *J Chromatogr B Analyt Technol Biomed Life Sci* **875**(2), 333-343 (2008).
106. Armas, L.A., Hollis, B.W. & Heaney, R.P. Vitamin D2 is much less effective than vitamin D3 in humans. *J Clin Endocrinol Metab* **89**(11), 5387-5391 (2004).
107. Romagnoli, E., Mascia, M.L., Cipriani, C., Fassino, V., Mazzei, F., D'Erasmo, E., Carnevale, V., Scillitani, A. & Minisola, S. Short and long-term variations in serum calciotropic hormones after a single very large dose of ergocalciferol (vitamin D2) or cholecalciferol (vitamin D3) in the elderly. *J Clin Endocrinol Metab* **93**(8), 3015-3020 (2008).
108. Heaney, R.P., Recker, R.R., Grote, J., Horst, R.L. & Armas, L.A. Vitamin D(3) is more potent than vitamin D(2) in humans. *J Clin Endocrinol Metab* **96**(3), E447-452 (2011).
109. Mula-Abed, W.A. 25- hydroxyvitamin d: explosion in clinical interest and laboratory requests. *Oman Med J* **24**(4), 239-241 (2009).
110. Robinson-Cohen, C., Zelnick, L.R., Hoofnagle, A.N., Lutsey, P.L., Burke, G., Michos, E.D., Shea, S.J.C., Tracy, R., Siscovick, D.S., Psaty, B., Kestenbaum, B. & de Boer, I.H. Associations of Vitamin D-Binding Globulin and Bioavailable Vitamin D Concentrations With Coronary Heart Disease Events: The Multi-Ethnic Study of Atherosclerosis (MESA). *J Clin Endocrinol Metab* **102**(8), 3075-3084 (2017).
111. He, C.S., Fraser, W.D., Tang, J., Brown, K., Renwick, S., Rudland-Thomas, J., Teah, J., Tanqueray, E. & Gleeson, M. The effect of 14 weeks of vitamin D3 supplementation on antimicrobial peptides and proteins in athletes. *Journal of sports sciences* **34**(1), 67-74 (2016).
112. Solomon, S.S., Solomon, S., Rodriguez, I.I., McGarvey, S.T., Ganesh, A.K., Thyagarajan, S.P., Mahajan, A.P. & Mayer, K.H. Dried blood spots (DBS): a valuable tool for HIV surveillance in developing/tropical countries. *Int J Std Aids* **13**(1), 25-28 (2002).
113. Health Professional Handbook: A guide to newborn blood spot screening for healthcare professionals. *UK Newborn Screening Programme Centre, Department of Health* (Review December 2015)(2012).
114. Lehmann, S., Delaby, C., Vialaret, J., Ducos, J. & Hirtz, C. Current and future use of "dried blood spot" analyses in clinical chemistry. *Clinical Chemistry and Laboratory Medicine* **51**(10), 1897-1909 (2013).
115. Deglon, J., Thomas, A., Mangin, P. & Staub, C. Direct analysis of dried blood spots coupled with mass spectrometry: concepts and biomedical applications. *Anal Bioanal Chem* **402**(8), 2485-2498 (2012).
116. Wilhelm, A.J., den Burger, J.C. & Swart, E.L. Therapeutic drug monitoring by dried blood spot: progress to date and future directions. *Clin Pharmacokinet* **53**(11), 961-973 (2014).
117. O'Mara, M., Hudson-Curtis, B., Olson, K., Yueh, Y., Dunn, J. & Spooner, N. The effect of hematocrit and punch location on assay bias during quantitative bioanalysis of dried blood spot samples. *Bioanalysis* **3**(20), 2335-2347 (2011).
118. Spooner, N., Denniff, P., Michielsen, L., De Vries, R., Ji, Q.C., Arnold, M.E., Woods, K., Woolf, E.J., Xu, Y., Boutet, V., Zane, P., Kushon, S. & Rudge, J.B. A device for dried blood microsampling in quantitative bioanalysis: overcoming the issues associated with blood hematocrit. *Bioanalysis* **7**(6), 653-659 (2015).
119. Vogeser, M. Quantification of circulating 25-hydroxyvitamin D by liquid chromatography-tandem mass spectrometry. *J Steroid Biochem Mol Biol* **121**(3-5), 565-573 (2010).

120. Kvaskoff, D., Ko, P., Simila, H.A. & Eyles, D.W. Distribution of 25-hydroxyvitamin D3 in dried blood spots and implications for its quantitation by tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* **901**, 47-52 (2012).
121. Eyles, D., Anderson, C., Ko, P., Jones, A., Thomas, A., Burne, T., Mortensen, P.B., Norgaard-Pedersen, B., Hougaard, D.M. & McGrath, J. A sensitive LC/MS/MS assay of 250H vitamin D-3 and 250H vitamin D-2 in dried blood spots. *Clinica Chimica Acta* **403**(1-2), 145-151 (2009).
122. Spooner, N., Denniff, P., Michielsen, L., De Vries, R., Ji, Q.C., Arnold, M.E., Woods, K., Woolf, E.J., Xu, Y., Boutet, V., Zane, P., Kushon, S. & Rudge, J.B. A device for dried blood microsampling in quantitative bioanalysis: overcoming the issues associated blood hematocrit. *Bioanalysis* **7**(6), 653-659 (2015).
123. Denniff, P. & Spooner, N. Volumetric Absorptive Microsampling: A Dried Sample Collection Technique for Quantitative Bioanalysis. *Analytical chemistry* **86**(16), 8489-8495 (2014).
124. Jensen, M.E., Ducharme, F.M., Theoret, Y., Belanger, A.S. & Delvin, E. Assessing vitamin D nutritional status: Is capillary blood adequate? *Clin Chim Acta* **457**, 59-62 (2016).
125. Lahsaee, S.M., Ghaffaripour, S. & Hejr, H. The Effect of Routine Maintenance Intravenous Therapy on Hemoglobin Concentration and Hematocrit during Anesthesia in Adults. *Bull Emerg Trauma* **1**(3), 102-107 (2013).
126. Zhong, Y., Lin, S.L. & Schooling, C.M. The effect of hematocrit and hemoglobin on the risk of ischemic heart disease: A Mendelian randomization study. *Prev Med* **91**, 351-355 (2016).
127. Walton, B.L., Lehmann, M., Skorczewski, T., Holle, L.A., Beckman, J.D., Cribb, J.A., Mooberry, M.J., Wufsus, A.R., Cooley, B.C., Homeister, J.W., Pawlinski, R., Falvo, M.R., Key, N.S., Fogelson, A.L., Neeves, K.B. & Wolberg, A.S. Elevated hematocrit enhances platelet accumulation following vascular injury. *Blood* **129**(18), 2537-2546 (2017).
128. Delgado-Corcoran, C., Wolpert, K.H., Lucas, K., Bodily, S., Presson, A.P. & Bratton, S.L. Hematocrit Levels, Blood Testing, and Blood Transfusion in Infants After Heart Surgery. *Pediatr Crit Care Med* **17**(11), 1055-1063 (2016).
129. Jenab, M., Bueno-de-Mesquita, H.B., Ferrari, P., van Duijnhoven, F.J., Norat, T., Pischon, T., Jansen, E.H., Slimani, N., Byrnes, G., Rinaldi, S., Tjonneland, A., Olsen, A., Overvad, K., Boutron-Ruault, M.C., Clavel-Chapelon, F., Morois, S., Kaaks, R., Linseisen, J., Boeing, H., Bergmann, M.M., Trichopoulou, A., Misirli, G., Trichopoulos, D., Berrino, F., Vineis, P., Panico, S., Palli, D., Tumino, R., Ros, M.M., van Gils, C.H., Peeters, P.H., Brustad, M., Lund, E., Tormo, M.J., Ardanaz, E., Rodriguez, L., Sanchez, M.J., Dorronsoro, M., Gonzalez, C.A., Hallmans, G., Palmqvist, R., Roddam, A., Key, T.J., Khaw, K.T., Autier, P., Hainaut, P. & Riboli, E. Association between pre-diagnostic circulating vitamin D concentration and risk of colorectal cancer in European populations:a nested case-control study. *BMJ* **340**, b5500 (2010).
130. McGrath, J., Saari, K., Hakko, H., Jokelainen, J., Jones, P., Jarvelin, M.R., Chant, D. & Isohanni, M. Vitamin D supplementation during the first year of life and risk of schizophrenia: a Finnish birth cohort study. *Schizophr Res* **67**(2-3), 237-245 (2004).
131. Robbins, J., Petrone, A.B., Gaziano, J.M. & Djousse, L. Dietary vitamin D and risk of heart failure in the Physicians' Health Study. *Clinical nutrition* **35**(3), 650-653 (2016).
132. Wactawski-Wende, J., Kotchen, J.M., Anderson, G.L., Assaf, A.R., Brunner, R.L., O'Sullivan, M.J., Margolis, K.L., Ockene, J.K., Phillips, L., Pottern, L., Prentice, R.L., Robbins, J., Rohan, T.E., Sarto, G.E., Sharma, S., Stefanick, M.L., Van Horn, L., Wallace, R.B., Whitlock, E., Bassford, T., Beresford, S.A., Black, H.R., Bonds, D.E., Brzyski, R.G., Caan, B., Chlebowski, R.T., Cochrane, B., Garland, C., Gass, M., Hays, J., Heiss, G., Hendrix, S.L., Howard, B.V., Hsia, J., Hubbell, F.A., Jackson, R.D., Johnson, K.C., Judd, H., Kooperberg, C.L., Kuller, L.H., LaCroix, A.Z., Lane, D.S., Langer, R.D., Lasser, N.L., Lewis, C.E., Limacher, M.C., Manson, J.E. & Women's

Health Initiative, I. Calcium plus vitamin D supplementation and the risk of colorectal cancer. *N Engl J Med* **354**(7), 684-696 (2006).
133. Jackson, R.D., LaCroix, A.Z., Gass, M., Wallace, R.B., Robbins, J., Lewis, C.E., Bassford, T., Beresford, S.A., Black, H.R., Blanchette, P., Bonds, D.E., Brunner, R.L., Brzyski, R.G., Caan, B., Cauley, J.A., Chlebowski, R.T., Cummings, S.R., Granek, I., Hays, J., Heiss, G., Hendrix, S.L., Howard, B.V., Hsia, J., Hubbell, F.A., Johnson, K.C., Judd, H., Kotchen, J.M., Kuller, L.H., Langer, R.D., Lasser, N.L., Limacher, M.C., Ludlam, S., Manson, J.E., Margolis, K.L., McGowan, J., Ockene, J.K., O'Sullivan, M.J., Phillips, L., Prentice, R.L., Sarto, G.E., Stefanick, M.L., Van Horn, L., Wactawski-Wende, J., Whitlock, E., Anderson, G.L., Assaf, A.R., Barad, D. & Women's Health Initiative, I. Calcium plus vitamin D supplementation and the risk of fractures. *N Engl J Med* **354**(7), 669-683 (2006).
134. Cauley, J.A., Chlebowski, R.T., Wactawski-Wende, J., Robbins, J.A., Rodabough, R.J., Chen, Z., Johnson, K.C., O'Sullivan, M.J., Jackson, R.D. & Manson, J.E. Calcium plus vitamin D supplementation and health outcomes five years after active intervention ended: the Women's Health Initiative. *J Womens Health (Larchmt)* **22**(11), 915-929 (2013).
135. Racovan, M., Walitt, B., Collins, C.E., Pettinger, M., Parks, C.G., Shikany, J.M., Wactawski-Wende, J., Manson, J.E., Moreland, L., Wright, N., Jackson, R. & Howard, B.V. Calcium and vitamin D supplementation and incident rheumatoid arthritis: the Women's Health Initiative Calcium plus Vitamin D trial. *Rheumatol Int* **32**(12), 3823-3830 (2012).
136. Jones, G., Prosser, D.E. & Kaufmann, M. 25-Hydroxyvitamin D-24-hydroxylase (CYP24A1): its important role in the degradation of vitamin D. *Arch Biochem Biophys* **523**(1), 9-18 (2012).
137. Petkovich, M. & Jones, G. CYP24A1 and kidney disease. *Curr Opin Nephrol Hypertens* **20**(4), 337-344 (2011).
138. Schuster, I. Cytochromes P450 are essential players in the vitamin D signaling system. *Biochim Biophys Acta* **1814**(1), 186-199 (2011).
139. Prosser, D.E. & Jones, G. Enzymes involved in the activation and inactivation of vitamin D. *Trends Biochem Sci* **29**(12), 664-673 (2004).
140. Shimada, T., Hasegawa, H., Yamazaki, Y., Muto, T., Hino, R., Takeuchi, Y., Fujita, T., Nakahara, K., Fukumoto, S. & Yamashita, T. FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. *J Bone Miner Res* **19**(3), 429-435 (2004).
141. Fraser, W.D. & Milan, A.M. Vitamin D assays: past and present debates, difficulties, and developments. *Calcif Tissue Int* **92**(2), 118-127 (2013).
142. Cashman, K.D., Hayes, A., Galvin, K., Merkel, J., Jones, G., Kaufmann, M., Hoofnagle, A.N., Carter, G.D., Durazo-Arvizu, R.A. & Sempos, C.T. Significance of serum 24,25-dihydroxyvitamin D in the assessment of vitamin D status: a double-edged sword? *Clin Chem* **61**(4), 636-645 (2015).
143. Berg, A.H., Powe, C.E., Evans, M.K., Wenger, J., Ortiz, G., Zonderman, A.B., Suntharalingam, P., Lucchesi, K., Powe, N.R., Karumanchi, S.A. & Thadhani, R.I. 24,25-Dihydroxyvitamin d3 and vitamin D status of community-dwelling black and white Americans. *Clin Chem* **61**(6), 877-884 (2015).
144. van den Ouweland, J.M., Beijers, A.M., Demacker, P.N. & van Daal, H. Measurement of 25-OH-vitamin D in human serum using liquid chromatography tandem-mass spectrometry with comparison to radioimmunoassay and automated immunoassay. *J Chromatogr B Analyt Technol Biomed Life Sci* **878**(15-16), 1163-1168 (2010).
145. Kadavil, J. Guidance for industry bioanalytical method validation. *U.S. Department of Health and Human Services Food and Drug Administration* **draft guidance Version 1**(2013).
146. Antoni, F.A. Interactions between intracellular free Ca2+ and cyclic AMP in neuroendocrine cells. *Cell Calcium* **51**(3-4), 260-266 (2012).
147. Silvester, S. & Smith, L. Profiling phospholipid elution in reversed-phase LC-MS/MS bioanalytical methods in order to avoid matrix effects. *Bioanalysis* **4**(8), 879-895 (2012).

148. Carter, G.D., Carter, R., Jones, J. & Berry, J. How accurate are assays for 25-hydroxyvitamin D? Data from the international vitamin D external quality assessment scheme. *Clin Chem* **50**(11), 2195-2197 (2004).
149. Harris, E.K. & Boyd, J.C. On dividing reference data into subgroups to produce separate reference ranges. *Clin Chem* **36**(2), 265-270 (1990).
150. Jacobson, N.S., Roberts, L.J., Berns, S.B. & McGlinchey, J.B. Methods for defining and determining the clinical significance of treatment effects: description, application, and alternatives. *J Consult Clin Psychol* **67**(3), 300-307 (1999).
151. Jacobson, N.S. & Truax, P. Clinical significance: a statistical approach to defining meaningful change in psychotherapy research. *J Consult Clin Psychol* **59**(1), 12-19 (1991).
152. Bolland, M.J., Grey, A. & Avenell, A. Effects of vitamin D supplementation on musculoskeletal health: a systematic review, meta-analysis, and trial sequential analysis. *The lancet. Diabetes & endocrinology* **6**(11), 847-858 (2018).
153. Vitamin D deficiency in adults - treatment and prevention. Management of vitamin D deficiency or insufficiency in adults. National Institute for Health and Care Excellence (NICE) [Online] 2018 September 2018 [cited 29th April 2019]; Available from: https://cks.nice.org.uk/vitamin-d-deficiency-in-adults-treatment-and-prevention#!scenario.
154. Tebben, P.J., Singh, R.J. & Kumar, R. Vitamin D-Mediated Hypercalcemia: Mechanisms, Diagnosis, and Treatment. *Endocr Rev* **37**(5), 521-547 (2016).
155. Macdonald, H.M., Reid, I.R., Gamble, G.D., Fraser, W.D., Tang, J.C. & Wood, A.D. 25-Hydroxyvitamin D Threshold for the Effects of Vitamin D Supplements on Bone Density: Secondary Analysis of a Randomized Controlled Trial. *J Bone Miner Res* (2018).
156. Macdonald, H.M., Wood, A.D., Aucott, L.S., Black, A.J., Fraser, W.D., Mavroeidi, A., Reid, D.M., Secombes, K.R., Simpson, W.G. & Thies, F. Hip bone loss is attenuated with 1000 IU but not 400 IU daily vitamin D3: a 1-year double-blind RCT in postmenopausal women. *J Bone Miner Res* **28**(10), 2202-2213 (2013).
157. Smith, L.M., Gallagher, J.C. & Suiter, C. Medium doses of daily vitamin D decrease falls and higher doses of daily vitamin D3 increase falls: A randomized clinical trial. *J Steroid Biochem Mol Biol* **173**, 317-322 (2017).
158. Carpenter, T.O. Cyp24a1 Loss of Function: Clinical Phenotype of Monoallelic and Biallelic Mutations. *J Steroid Biochem Mol Biol* (2017).
159. Schlingmann, K.P., Kaufmann, M., Weber, S., Irwin, A., Goos, C., John, U., Misselwitz, J., Klaus, G., Kuwertz-Broking, E., Fehrenbach, H., Wingen, A.M., Guran, T., Hoenderop, J.G., Bindels, R.J., Prosser, D.E., Jones, G. & Konrad, M. Mutations in CYP24A1 and idiopathic infantile hypercalcemia. *N Engl J Med* **365**(5), 410-421 (2011).
160. Fencl, F., Blahova, K., Schlingmann, K.P., Konrad, M. & Seeman, T. Severe hypercalcemic crisis in an infant with idiopathic infantile hypercalcemia caused by mutation in CYP24A1 gene. *Eur J Pediatr* **172**(1), 45-49 (2013).
161. Saleh, L., Tang, J., Gawinecka, J., Boesch, L., Fraser, W.D., von Eckardstein, A. & Nowak, A. Impact of a single oral dose of 100,000 IU vitamin D3 on profiles of serum 25(OH)D3 and its metabolites 24,25(OH)2D3, 3-epi-25(OH)D3, and 1,25(OH)2D3 in adults with vitamin D insufficiency. *Clin Chem Lab Med* **55**(12), 1912-1921 (2017).
162. Owens, D.J., Tang, J.C., Bradley, W.J., Sparks, A.S., Fraser, W.D., Morton, J.P. & Close, G.L. Efficacy of High-Dose Vitamin D Supplements for Elite Athletes. *Med Sci Sports Exerc* **49**(2), 349-356 (2017).
163. Macdonald, H.M., Gryka, A., Tang, J.C.Y., Aucott, L.S., Fraser, W.D. & Wood, A.D. Longevity of daily oral vitamin D3 supplementation: differences in 25OHD and 24,25(OH)2D observed 2 years after cessation of a 1-year randomised controlled trial (VICtORy RECALL). *Osteoporos Int* (2017).
164. Wood, A.D., Secombes, K.R., Thies, F., Aucott, L., Black, A.J., Mavroeidi, A., Simpson, W.G., Fraser, W.D., Reid, D.M. & Macdonald, H.M. Vitamin D3 supplementation has no effect on conventional cardiovascular risk factors: a parallel-group, double-blind, placebo-controlled RCT. *J Clin Endocrinol Metab* **97**(10), 3557-3568 (2012).

165. Leeuwenkamp, O.R., van der Wiel, H.E., Lips, P., van der Vijgh, W.J., Barto, R., Greuter, H. & Netelenbos, J.C. Human pharmacokinetics of orally administered (24 R)-hydroxycalcidiol. *Eur J Clin Chem Clin Biochem* **31**(7), 419-426 (1993).
166. Martineau, A.R., Jolliffe, D.A., Hooper, R.L., Greenberg, L., Aloia, J.F., Bergman, P., Dubnov-Raz, G., Esposito, S., Ganmaa, D., Ginde, A.A., Goodall, E.C., Grant, C.C., Griffiths, C.J., Janssens, W., Laaksi, I., Manaseki-Holland, S., Mauger, D., Murdoch, D.R., Neale, R., Rees, J.R., Simpson, S., Jr., Stelmach, I., Kumar, G.T., Urashima, M. & Camargo, C.A., Jr. Vitamin D supplementation to prevent acute respiratory tract infections: systematic review and meta-analysis of individual participant data. *BMJ* **356**, i6583 (2017).
167. Khaw, K.T., Stewart, A.W., Waayer, D., Lawes, C.M.M., Toop, L., Camargo, C.A., Jr. & Scragg, R. Effect of monthly high-dose vitamin D supplementation on falls and non-vertebral fractures: secondary and post-hoc outcomes from the randomised, double-blind, placebo-controlled ViDA trial. *The lancet. Diabetes & endocrinology* **5**(6), 438-447 (2017).
168. Binkley, N., Lappe, J., Singh, R.J., Khosla, S., Krueger, D., Drezner, M.K. & Blank, R.D. Can vitamin D metabolite measurements facilitate a "treat-to-target" paradigm to guide vitamin D supplementation? *Osteoporos Int* **26**(5), 1655-1660 (2015).
169. Tang, J., Nicholls, H., Dutton, J., Piec, I., Washbourne, C., Saleh, L., Nowak, A., Close, G.L., Macdonald, H.M., Jackson, S., Greeves, J. & Fraser, W.D. Profiles of 25 hydroxyvitamin D and its metabolite 24,25 dihydroxyvitamin D and 1,25 dihydroxyvitamin D in vitamin D3 supplementations studies. Bone Abstracts 2016; 5 Presented at The 43rd Annual European Calcified Tissue Society Congress. Rome, Italy Oral presentation #OP19
170. Armbrecht, H.J., Boltz, M.A. & Hodam, T.L. PTH increases renal 25(OH)D3-1alpha -hydroxylase (CYP1alpha) mRNA but not renal 1,25(OH)2D3 production in adult rats. *Am J Physiol Renal Physiol* **284**(5), F1032-1036 (2003).
171. Collins, M.T., Lindsay, J.R., Jain, A., Kelly, M.H., Cutler, C.M., Weinstein, L.S., Liu, J., Fedarko, N.S. & Winer, K.K. Fibroblast growth factor-23 is regulated by 1alpha,25-dihydroxyvitamin D. *J Bone Miner Res* **20**(11), 1944-1950 (2005).
172. MacDonald, H.M., Wood, A.D., Tang, J.C. & Fraser, W.D. Comparison of vitamin D(2) and vitamin D(3) supplementation in increasing serum 25-hydroxyvitamin D status: a systematic review and meta-analysis. *Am J Clin Nutr* **96**(5), 1152-1153; author reply 1153-1154 (2012).
173. Macdonald, H.M., Gryka, A., Tang, J.C.Y., Aucott, L.S., Fraser, W.D. & Wood, A.D. Longevity of daily oral vitamin D3 supplementation: differences in 25OHD and 24,25(OH)2D observed 2 years after cessation of a 1-year randomised controlled trial (VICtORy RECALL). *Osteoporos Int* **28**(12), 3361-3372 (2017).
174. Chertow, B.S., Baker, G.R., Henry, H.L. & Norman, A.W. Effects of vitamin D metabolites on bovine parathyroid hormone release in vitro. *Am J Physiol* **238**(4), E384-388 (1980).
175. Henry, H.L., Taylor, A.N. & Norman, A.W. Response of chick parathyroid glands to the vitamin D metabolites, 1,25-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol. *J Nutr* **107**(10), 1918-1926 (1977).
176. Boyan, B.D., Hyzy, S.L., Pan, Q., Scott, K.M., Coutts, R.D., Healey, R. & Schwartz, Z. 24R,25-Dihydroxyvitamin D3 Protects against Articular Cartilage Damage following Anterior Cruciate Ligament Transection in Male Rats. *PloS one* **11**(8), e0161782 (2016).
177. Gal-Moscovici, A., Gal, M. & Popovtzer, M.M. Treatment of osteoporotic ovariectomized rats with 24,25(OH)2D3. *Eur J Clin Invest* **35**(6), 375-379 (2005).
178. St-Arnaud, R. CYP24A1-deficient mice as a tool to uncover a biological activity for vitamin D metabolites hydroxylated at position 24. *J Steroid Biochem Mol Biol* **121**(1-2), 254-256 (2010).
179. Macdonald, H.M., Reid, I.R., Gamble, G.D., Fraser, W.D., Tang, J.C. & Wood, A.D. 25-Hydroxyvitamin D Threshold for the Effects of Vitamin D Supplements on Bone Density:

Secondary Analysis of a Randomized Controlled Trial. *J Bone Miner Res* **33**(8), 1464-1469 (2018).
180. Kidney Disease: Improving Global Outcomes, C.K.D.M.B.D.U.W.G. KDIGO 2017 Clinical Practice Guideline Update for the Diagnosis, Evaluation, Prevention, and Treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). *Kidney Int Suppl (2011)* **7**(1), 1-59 (2017).
181. Tang, J.C.Y., Jackson, S., Walsh, N.P., Greeves, J., Fraser, W.D. & Bioanalytical Facility, t. The dynamic relationships between the active and catabolic vitamin D metabolites, their ratios, and associations with PTH. *Sci Rep* **9**(1), 6974 (2019).
182. Tang, J., Nicholls, H., Piec, I., Dutton, J., Washbourne, C., Jackson, S., Greeves, J. & Fraser, W.D. Establishing reference intervals of serum 24,25-dihydroxyvitamin D and 25-hydroxyvitamin D-to-24,25-dihydroxyvitamin D ratio by LC-MS/MS. J Bone Miner Res 2016; 31 (Suppl 1) Presented at American Society of Bone and Mineral Research. Atlanta, GA, USA Poster #SA0188
183. Tang, J., Nicholls, H., Dutton, J., Piec, I., Washbourne, C., Saleh, L., Nowak, A., Close, G.L., Macdonald, H.M. & W.D., F. Changes in serum 25-hydroxyvitamin D, 24,25-dihydroxyvitamin D and 1,25-dihydroxyvitamin D in response to three vitamin D3 supplementation regimens. *Endocrine Abstracts* 44 P72 2016; 44 Presented at Society for Endocrinology BES 2016. Brighton, UK Poster #P72
184. Parekh, D., Dancer, R.C.A., Scott, A., D'Souza, V.K., Howells, P.A., Mahida, R.Y., Tang, J.C.Y., Cooper, M.S., Fraser, W.D., Tan, L., Gao, F., Martineau, A.R., Tucker, O., Perkins, G.D. & Thickett, D.R. Vitamin D to Prevent Lung Injury Following Esophagectomy-A Randomized, Placebo-Controlled Trial. *Crit Care Med* **46**(12), e1128-e1135 (2018).
185. Dancer, R.C., Parekh, D., Lax, S., D'Souza, V., Zheng, S., Bassford, C.R., Park, D., Bartis, D.G., Mahida, R., Turner, A.M., Sapey, E., Wei, W., Naidu, B., Stewart, P.M., Fraser, W.D., Christopher, K.B., Cooper, M.S., Gao, F., Sansom, D.M., Martineau, A.R., Perkins, G.D. & Thickett, D.R. Vitamin D deficiency contributes directly to the acute respiratory distress syndrome (ARDS). *Thorax* **70**(7), 617-624 (2015).
186. Lawlor, D.A., Wills, A.K., Fraser, A., Sayers, A., Fraser, W.D. & Tobias, J.H. Association of maternal vitamin D status during pregnancy with bone-mineral content in offspring: a prospective cohort study. *Lancet* **381**(9884), 2176-2183 (2013).
187. Earl, K.E., Sakellariou, G.K., Sinclair, M., Fenech, M., Croden, F., Owens, D.J., Tang, J., Miller, A., Lawton, C., Dye, L., Close, G.L., Fraser, W.D., McArdle, A. & Beadsworth, M.B.J. Vitamin D status in chronic fatigue syndrome/myalgic encephalomyelitis: a cohort study from the North-West of England. *BMJ Open* **7**(11), e015296 (2017).
188. Lynch Cronin, I., Byrne, F., Doyle, R., Fraser, W.D., Chipchase, A. & Eustace, J.A. The Effect of Short-Term Vitamin D Supplementation on Calcium Status in Vitamin D Insufficient Renal Transplant Recipients at Risk of Hypercalcemia. *J Ren Nutr* **29**(3), 181-187 (2019).
189. Sophocleous, A., Robertson, R., Ferreira, N.B., McKenzie, J., Fraser, W.D. & Ralston, S.H. Heavy Cannabis Use Is Associated With Low Bone Mineral Density and an Increased Risk of Fractures. *Am J Med* **130**(2), 214-221 (2017).
190. Cleal, J.K., Hargreaves, M.R., Poore, K.R., Tang, J.C.Y., Fraser, W.D., Hanson, M.A. & Green, L.R. Reduced fetal vitamin D status by maternal undernutrition during discrete gestational windows in sheep. *J Dev Orig Health Dis* **8**(3), 370-381 (2017).
191. The rise and rise of vitamin D. Clinical laboratory International. 2014 [cited 10 Sept 2019; Available from: https://www.clinlabint.com/detail/clinical-laboratory/the-rise-and-rise-of-vitamin-d/.
192. Bilinski, K. & Boyages, S. The rise and rise of vitamin D testing. *BMJ* **345**, e4743 (2012).
193. Costing statement: Vitamin D: increasing supplement use among at-risk groups (PH56). National Institute for Health and Care Excellence (NICE). 2014 [cited 10/9/19; Available from: https://www.nice.org.uk/guidance/ph56/resources/costing-statement-69288013.

194. Sharma, J., Greeves, J.P., Byers, M., Bennett, A.N. & Spears, I.R. Musculoskeletal injuries in British Army recruits: a prospective study of diagnosis-specific incidence and rehabilitation times. *BMC Musculoskelet Disord* **16**, 106 (2015).
195. Greeves, J., Wickes, C. & Fraser, W.D. Vitamin D Status and Bone Health in Female Army Recruits. J Bone Miner Res 2005; 20 (Suppl 1): 154. Presented at The American Society of Bone and Mineral Research. Nashville, Tennessee, USA Poster #SA307
196. Tang, J.C.Y., Jackson, S., Izard, R.M., Oliver, S.J., Piec, I., Washbourne, C.J., Walsh, N.P., Greeves, J. & Fraser, W.D. Vitamin D insufficiency and elevated vitamin D metabolite ratios (VMR) are associated with increased risk of injuries: Results from the British army lower limb injury prevention (ALLIP) study. Endocrine Abstracts 2018; 59 Presented at Society for Endocrinology BES. Glasgow, UK Oral presentation #OC1.2
197. Women in ground close combat findings paper. Ministry of Defence. 2016 [cited 11/9/19; Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/536423/20160615-WGCC-COSIfindings-Public_FINAL.pdf.
198. Owens, D.J., Sharples, A.P., Polydorou, I., Alwan, N., Donovan, T., Tang, J., Fraser, W.D., Cooper, R.G., Morton, J.P., Stewart, C. & Close, G.L. A systems-based investigation into vitamin D and skeletal muscle repair, regeneration, and hypertrophy. *Am J Physiol Endocrinol Metab* **309**(12), E1019-1031 (2015).
199. Owens, D.J., Webber, D., Impey, S.G., Tang, J., Donovan, T.F., Fraser, W.D., Morton, J.P. & Close, G.L. Vitamin D supplementation does not improve human skeletal muscle contractile properties in insufficient young males. *European journal of applied physiology* **114**(6), 1309-1320 (2014).
200. Carswell, A.T., Oliver, S.J., Wentz, L.M., Kashi, D.S., Roberts, R., Tang, J.C.Y., Izard, R.M., Jackson, S., Allan, D., Rhodes, L.E., Fraser, W.D., Greeves, J.P. & Walsh, N.P. Influence of Vitamin D Supplementation by Sunlight or Oral D3 on Exercise Performance. *Med Sci Sports Exerc* **50**(12), 2555-2564 (2018).