



Clin Biochem Rev. 2016 Feb; 37(1): 27–47.

PMCID: PMC4810759

Fat-Soluble Vitamins: Clinical Indications and Current Challenges for Chromatographic Measurement

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Abstract

Fat-soluble vitamins, including vitamins A, D and E, are required for a wide variety of physiological functions. Over the past two decades, deficiencies of these vitamins have been associated with increased risk of cancer, type II diabetes mellitus and a number of immune system disorders. In addition, there is increasing evidence of interactions between these vitamins, especially between vitamins A and D. As a result of this enhanced clinical association with disease, translational clinical research and laboratory requests for vitamin measurements have significantly increased. These laboratory requests include measurement of 25-OHD (vitamin D), retinol (vitamin A) and α -tocopherol (vitamin E); the most accepted blood indicators for the assessment of body fat-soluble vitamin (FSV) status. There are significant obstacles to precise FSV measurement in blood. These obstacles include their physical and chemical properties, incomplete standardisation of measurement and limitations in the techniques that are currently used for quantification. The aim of this review is to briefly outline the metabolism and interactions of FSV as a prelude to identifying the current challenges for the quantification of blood vitamins A, D and E.

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Introduction

Vitamins are small organic compounds, generally obtained from the diet, that are essential in very small amounts for diverse biological functions. The thirteen known vitamins are divided into two classes based on their relative solubility in water and fat. The fat-soluble vitamins (FSV) A, D, E and K, are absorbed in the intestine in the presence of fat. Classical deficiencies of these vitamins can manifest clinically as night blindness (vitamin A), osteomalacia (vitamin D), increased oxidative cell stress (vitamin E) and haemorrhage (vitamin K). Recent studies have identified additional potential actions for FSV, particularly vitamins A and D.^{1,2} Deficiencies of these two FSV have also been indirectly linked to cancer, type II diabetes mellitus and a number of immune system disorders.^{2,3} With the increased awareness of the potential

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role that these vitamins play, laboratory requests have significantly increased in the last decade.⁴ The aim of this review is to briefly outline the metabolism and interactions of FSV as a prelude to highlighting the current challenges for the quantification of blood vitamins A, D and E. Hence, the abbreviation 'FSV' is used to indicate vitamins A, D and E throughout this review.

Go to: 

Vitamin A

The term 'vitamin A' is used to refer to retinol and related compounds that exhibit the biological activity of retinol. The main forms of vitamin A are retinol, retinoic acid and retinal, while the main liver storage form is retinyl palmitate.⁵ (Figure 1). The re-surfing interest in vitamin A relates to retinoid acid's actions as an endocrine and paracrine hormone. Retinoic acid is thought to be essential for embryonic stem cell differentiation and development, and in maintaining healthy structure and function of epithelial cells.⁶ In addition, retinoic acid may have a role in vitamin A metabolism in the liver.⁷ Whilst vitamin A's best known role relates to sight, it is also speculated that vitamin A has a role in the regulation of the macronutrient metabolism of carbohydrates, lipids and proteins.⁸ This vitamin is also thought to inhibit the growth of tumour cells *in vitro*⁹ and may play a role in controlling cell division and differentiation, as well as cell apoptosis.¹⁰ Exemplifying this, Manna et al. (2000) linked the carboxylic form of vitamin A, all-trans-retinoic acid, with human lung cancer cell apoptosis.¹¹

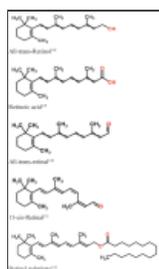


Figure 1.

Chemical structure of vitamin A and its derivatives.

Metabolism

The major source of this vitamin is dietary, either as preformed vitamin A (mainly as retinyl esters) from animal sources, or as provitamin A compounds (carotenoids, especially β -carotene) from pigmented vegetables and fruits.^{12,13} Normally 70–90% of vitamin A is absorbed by the gut in the presence of intestinal juice and bile salts.¹⁴

In the intestinal lumen, retinyl esters are hydrolysed to retinol and free fatty acids by the brush-border retinyl ester hydrolase prior to being taken up by enterocytes (small intestinal absorptive cells).¹⁵ On the other hand, β -carotene is passively diffused into the enterocytes and its absorption efficiency is associated with the quantity of dietary fat intake.¹⁶ In the enterocytes the majority of β -carotene is symmetrically cleaved to retinal by 15,15'-monooxygenase, then converted to retinol by retinal reductase.¹⁷ Most of the retinol in enterocytes, obtained from retinoid and carotenoid, is esterified into retinyl esters by lecithin:retinol acyltransferase or acyl-CoA:retinol acyltransferase.⁸ Later, retinyl esters are incorporated with chylomicrons and secreted into the lymphatic system,¹⁸ (Figure 2). Also, small quantities of dietary retinoids are converted to retinoic acid, which are absorbed directly into the blood circulation.^{12,19}

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Figure 2.

General scheme for Vitamin A metabolism.

Approximately 70% of dietary retinoids are taken up by the liver, especially parenchymal cells, and stored as retinyl palmitate; the predominant retinyl ester.¹⁴ In parenchymal cells, retinyl esters are re-hydrolysed into retinol by a number of enzymes, including retinyl ester hydrolase. The retinol released from the liver can be mediated to a variety of cells, where it is metabolised to various forms of vitamin A (such as retinal and retinoic acid) for a variety of physiological functions²⁰ (Figure 2).

The hydrophobic nature of vitamin A means that it requires carriers for transportation. The extracellular transportation of vitamin A mainly occurs through binding with retinol-binding protein (RBP) and thyroxine binding-protein transthyretin (TTR). The retinol-RBP-TTR complex is not only essential for vitamin A solubility, but is also essential for vitamin A protection against oxidation and esterification.^{21,22} Intracellular unesterified retinol is transported by binding with cellular RBP type I (CRBP-I) and cellular RBP type II (CRBP-II). Other intracellular proteins include: cellular retinoic acid-binding proteins (CRABP-I and CRABP-II), which are involved in the transport of retinoic acid and cellular retinal-binding protein (CRALBP) for retinal transportation.²²

Pathophysiology

The classic role of vitamin A in dim-light vision is well understood. Circulating retinol reaches the retinal pigments in the epithelial cells of the eye, where it is esterified to retinyl esters. By hydrolysis and isomerisation processes, retinyl esters are converted to 11-cis-retinol, then oxidised to 11-cis-retinal which binds with the protein opsin in the rods (i.e. the sensitive light cells that allow for dim-light vision) to form a complex called rhodopsin. When rhodopsin is exposed to a photon of light, 11-cis-retinal is isomerised to all-trans-retinal. All-trans-retinal is then disassociated from the complex, and photochemical events are triggered; consequently, the brain deduces that a visual event has occurred.⁶

Vitamin A also has a clear role in immune function. Vitamin A deficiency causes dryness and keratinisation in epithelial cells of the skin, the respiratory, gastrointestinal and urogenital tracts all of which are initial preventative systems against infection. Furthermore, this deficiency disrupts neutrophil development, increases inflammatory cytokine release by macrophages, and decreases the number of natural killer cells and their lytic activity. These disruptions and changes lead to a decrease in the body's ability to eliminate infectious agents.² As a result, communities that suffer from vitamin A deficiency may also have a high prevalence of infection.²³

The World Health Organization considers the high prevalence of vitamin A deficiency to be a serious public health problem in lower socioeconomic communities.²⁴ About fifty per cent of preschool-aged children and pregnant mothers are at risk of vitamin A deficiency worldwide.²⁴ In the twenty year period between 1995 and 2005 vitamin A deficiency (serum retinol <0.7 µmol/L) was estimated to affect 190 million pre-school children and 19.1 million pregnant women globally.²⁴ This manifests clinically as night blindness, xerophthalmia and recurrent infections.⁶ In these communities, the

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underlying cause of vitamin A deficiency is chronic poor nutritional intake.

On the other hand, high storage levels of vitamin A in the body may cause hypervitaminosis A and vitamin A toxicity, which is rare but serious. The toxicity of vitamin A is caused from excessive vitamin A intake, especially from vitamin supplementation. The clinical manifestations of vitamin A toxicity include nausea, vomiting, headache, dizziness and blurred vision.¹² However, as vitamin A is fat-soluble, slow chronic ingestion is not necessarily reflected in the serum vitamin A level.^{12,25}

Biochemical Markers

Blood (serum or plasma) retinol is routinely used as a biochemical indicator for vitamin A status; particularly to distinguish deficient from replete levels in individuals. The level of retinol in the blood is homeostatically regulated, and declines only when liver vitamin A storage is severely depleted. It is thus a valuable indicator of the depletion of liver vitamin A storage. In addition to retinol, other analytical tests, such as tests for β -carotene, accompanied with clinical symptoms, may be used for the diagnosis of individual vitamin A deficiency.²³ Serum retinol is also a reliable indicator to estimate the status of vitamin A in population based studies.^{23,26}

Go to:

Vitamin D

Vitamin D is a prohormone that has been associated, through direct and indirect links, with a number of pathologies.²⁷ The most abundant form of vitamin D in blood is 25-hydroxyvitamin D. The active form of vitamin D is 1,25-dihydroxyvitamin D3 (1,25-(OH)₂D3), (Figure 3). Historically, vitamin D deficiency is associated with rickets in children and osteomalacia in adults.²⁸ Low levels of vitamin D are linked with bone fractures²⁹ and other clinical manifestations.³⁰ During the last two decades, a large number of studies have focussed on the biological roles of vitamin D. The optimum blood level of vitamin D is associated with the overall health of bone, skin and the immune response.¹ Vitamin D status has been correlated with risk status for cardiovascular disease and stroke.³¹ In addition, low levels of vitamin D were observed in patients with respiratory infections and HIV, which may relate to the role of vitamin D in immunity.³² Low levels of vitamin D and its metabolites have also been indirectly linked to the development of breast cancer. The active form of vitamin D, (1,25-(OH)₂D3), and the vitamin D receptors (VDRs) are postulated to have a regulatory effect on normal and breast cancer cell growth and differentiation.^{33,34} In addition, 1,25-(OH)₂D is thought to have a role in TNF- α expression, which induces apoptosis of cancer cells.^{35,36}

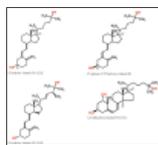


Figure 3.

Chemical structure of some vitamin D metabolites.

Vitamin D deficiency is a public health problem in many countries.³⁷ While it was previously thought that this deficiency was common only in countries that lacked a sunny climate for most of the year, progressive research findings have revealed that this deficiency is worldwide, with a higher prevalence in some ethnicities and in some geographical locations. For example, although

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Saudis enjoy a sunny climate most of the year, one study found that more than 87 % of 834 healthy adult Saudi men (aged between 20–74 years) had vitamin D deficiency, especially older and obese men.³⁸ A study in the USA, with 12,862 adult participants (over 20 years old), demonstrated a prevalence rate of vitamin D deficiency of more than 41%; especially among African Americans and Hispanic Americans, with rates of 82.1% and 62.9%, respectively.³⁹ In Australia, according to the AusDiab study, with samples collected in 1999 and 2000 from 11,247 Australian adults (over 25 years old), vitamin D deficiency was estimated at 31%, while vitamin D insufficiency was estimated at 73%.⁴⁰ Data such as provided in these examples has informed government policy and lead to health initiatives to monitor and decrease the prevalence of vitamin D deficiency in specific population groups.

Metabolism

There are two main forms of vitamin D: ergocalciferol (also called vitamin D2) and cholecalciferol (also known as vitamin D3). Vitamin D2 is provided by plants, which are subjected to ultraviolet irradiation, or by dietary supplementation. While a small quantity of vitamin D3 is obtained from food derived from animals, the greatest natural source of this vitamin is endogenous synthesis during sunlight exposure.⁴¹ This endogenous synthesis is influenced by many factors such as skin thickness and colour, the period of sunlight exposure and the season.^{3,41–3}

The C9–C10 of pro-vitamin D3 (7-dehydrocholesterol) is broken down to form pre-vitamin D3 in the malpighian layer of the skin during exposure to ultraviolet radiation (UVR) between 280 nm and 315 nm wavelengths to synthesise endogenous vitamin D3. Unlike dietary supplements, continuous exposure to UVR does not lead to vitamin D toxicity, because the excess amount of pre-vitamin D3 converts to lumisterol and tachysterol.⁴⁴ This process is reversed when pre-vitamin D3 levels fall.^{45,46} After that, previtamin D3 is spontaneously isomerised to vitamin D3. Once synthesised, vitamin D circulates through the bloodstream by binding with vitamin D binding protein (DBP) to reach the liver^{45,46} (Figure 4).



Figure 4.

General scheme for Vitamin D metabolism.

In the liver, vitamin D is metabolised to 25-hydroxyvitamin D (25-OHD), also known as calcidiol, by a number of hepatic cytochrome P450 enzymes, especially CYP27A and CYP2R1.⁴⁵ After being formed, 25-OHD mediates through the blood stream to the kidneys for further hydroxylation. In the kidneys, CYP27B1 (25-hydroxyvitamin D-1 α -hydroxylase) converts calcidiol to the biological active metabolite 1,25-dihydroxyvitamin D (1,25-(OH)₂D), also known as calcitriol.⁴⁷ Cells such as bone, activated macrophages and epidermal keratinocytes^{48,49} can also locally produce 1,25-(OH)₂D. The 24,25-dihydroxyvitamin (24,25-(OH)₂D) can be formed in the kidneys through the activity of CYP24A1 (25-OHD-24-hydroxylase)^{46,50} (Figure 4).

The 1,25-(OH)₂D binds with the vitamin D receptor (VDR), which is expressed by many cell types.⁵¹ Through this binding, vitamin D's actions include the stimulation of intestinal calcium absorption, cell differentiation and insulin secretion. This interaction between 1,25-(OH)₂D and the VDR is essential for calcium absorption in the intestinal cells and for osteoblastogenesis.⁵² In this process 1,25-(OH)₂D induces the maturation of

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preosteoclasts into osteoclasts, which have a role in maintaining calcium and phosphorus levels in the blood by stimulating bone resorption.⁴⁷ Furthermore, by binding to VDR in some cells, 1,25-(OH)₂D regulates the gene expression of upstream protein synthesis, such as osteocalcin and 24-hydroxylase expression, and downstream production effects such as inflammatory markers (e.g., IL-2 and IL-12).⁵¹ In addition, although 1,25-(OH)₂D is mainly synthesised in the kidneys through stimulation by parathyroid hormone (PTH), it also causes the parathyroid gland to decrease its hormonal production and secretion of PTH.⁴⁴ High levels of 1,25-(OH)₂D in the blood can also trigger a negative feedback process to decrease its production. This is through an increase in the synthesis of CYP24A1, which converts 1,25-(OH)₂D to an inactive form of calcitric acid. Then calcitric acid, which is water soluble, is eliminated into the bile.⁴⁷ Many factors, such as serum phosphorus, calcium and fibroblast growth factor 23, have negative or positive effects on 1,25-(OH)₂D synthesis in the kidneys.⁴⁷

Biochemical Markers

25-OHD is considered to be the best biomarker to assess vitamin D status in blood. This metabolite has several advantages as a biochemical indicator, including: (1) it reflects both dietary and endogenous vitamin D; (2) it is an inactive metabolite, and is not tightly regulated; (3) the 25-OHD concentration is relatively high compared with other metabolites - for example, its concentration is 1,000 times more highly concentrated than 1,25-(OH)₂D; and (4) the half-life of 25-OHD is relatively long (about three weeks). Compared to 25-OHD₂ levels in the blood, 25-OHD₃ is usually the more abundant metabolite.^{42,53}

Go to: 

Vitamin E

Vitamin E is a fat-soluble antioxidant metabolite and an essential dietary factor. There are two naturally occurring groups of vitamin E: tocopherols and tocotrienols. Each have four isomers (α , β , γ and δ) based on the position and number of the methyl groups on the chromanol ring. In most cases, the main source of vitamin E is from diets that are rich in γ -tocopherol; however, α -tocopherol is the dominant form in the bloodstream and linked with many biological activities in humans and animals (Figure 5).

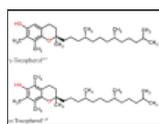


Figure 5.

Chemical structure of γ -tocopherol and α -tocopherol.

Although the health importance of vitamin E is mostly related to its antioxidant properties, it has recently also been correlated with non-antioxidant activities.⁵⁴⁻⁷ Vitamin E is important for the normal morphology of erythrocytes and is thought to be involved in slowing the aging process, since it is essential for the elimination of reactive oxygen species (ROS), which are involved in cell destruction.⁵⁸ Furthermore, this vitamin inhibits platelet aggregations, and therefore it may play a protective role against the atherosclerotic process and cardiovascular disease.⁵⁹⁻⁶¹ It has also been suggested that vitamin E has a protective role against arthritis, cataracts, neurological disease and immunological disorders.^{58,62}

Vitamin E has been associated with cancer prevention, because of its

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involvement in a variety of biological activities, including anti-oxidation, anti-proliferation and anti-inflammation. This is thought to be through vitamin E's role in protecting membrane polyunsaturated fatty acids and plasma lipoproteins from free radical attack⁶³ and γ -tocopherol's role in NO₂ detoxification.⁶⁴ This vitamin has also been linked with suppression of the synthesis of TNF, IL-1, IL-6 and IL-8.⁵⁷ In addition, in human breast cancer cell lines vitamin E has been shown to have apoptotic and growth inhibitory effects on the cancer cells.^{65,66}

Vitamin E deficiency is most commonly associated with genetic or malabsorption disorders e.g. cystic fibrosis, chronic hepatitis and gastrointestinal disorders.⁵⁴ This is because most food sources contain vitamin E. Despite this, epidemiologically, vitamin E deficiency is actually more common in developing countries than in industrial countries. This is due to inadequate vitamin intake and the high prevalence of infectious diseases that relate to oxidative stress processes, such as malaria and AIDS.⁶² As an example, vitamin E deficiency was recently estimated at 55.5% in Thai adults aged over 60 years.⁶⁷ In other small-scale studies, the prevalence for vitamin E deficiency was estimated at 15.6% in Jordanian children and 64% in urban Greeks aged over 65 years.^{68,69}

Metabolism

The small intestine is the site of vitamin E absorption in humans.⁷⁰ Absorption is enhanced in the presence of dietary fat and the food matrix.⁷¹ Intestinal absorption of vitamin E requires mixing the vitamin with micelles under the effects of biliary and pancreatic secretions to enhance vitamin E solubility. Consequently, enterocytes passively absorb micelles, which integrate with chylomicrons, and secrete into the lymph system.^{59,64,70} Chylomicrons are enriched with cholesterol and different types of vitamin E, such as α -tocopherol and γ -tocopherol.⁷² In the circulatory system, chylomicrons are hydrolysed by lipoprotein lipase to mediate vitamin E transport to target tissues such as brain and muscle. As a result of chylomicron hydrolysis, chylomicron remnants, which still contain vitamin E, are also formed.⁵⁹

Vitamin E is metabolised in the liver and only α -tocopherol is re-secreted into the blood circulation; facilitated by the hepatic α -tocopherol transfer protein (α -TTP). This protein maintains the concentration of α -tocopherol in the blood; therefore, α -TTP gene defects are associated with vitamin E deficiency.^{64,70} Blood α -tocopherol is transferred by lipoproteins such as very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) to target tissues⁷³ (Figure 6).

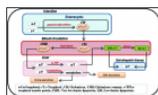


Figure 6.

General Scheme for Vitamin E metabolism.

Biochemical Markers

The blood level of α -tocopherol is the common marker used to indicate vitamin E status. It is speculated that α -tocopherol varies according to gender, age and the blood level of lipids, especially cholesterol. Therefore, the ratio of α -tocopherol to total cholesterol has also been suggested as a biomarker.⁷⁴ Alpha-tocopherol concentration in the plasma does not reflect vitamin E intake, since α -TTP selectively re-secretes α -tocopherol to the blood.⁶⁴

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Practically speaking, blood α -tocopherol is an acceptable indicator of vitamin E status.^{62,75}

Go to: 

Fat-Soluble Vitamin Interaction

The interaction of vitamin A and vitamin D functions has been observed in animals and humans.^{76,77} Studies in rats demonstrated that a high intake of vitamin A attenuated the toxicity of hypervitaminosis D.⁷⁶ In another study conducted in humans and based on a nested case-control model, the blood level of 25-OHD3 was inversely associated with colorectal cancer among individuals who had lower retinol intake.⁷⁸ Vitamin D deficiency (<50 nmol/L) and a high level of retinol (>2.8 μ mol/L) have also been associated with a high risk of osteoporotic fractures.⁷⁹

There is evidence that supplementation of one FSV has an impact on other FSV levels in blood. Vitamin D3 supplementation (800 IU/D for 6 months) alone or with calcium (2 g/d for 6 months) increased 25-OHD3 levels by 48% and decreased α -tocopherol by 14%. Serum 25-OHD2 levels decreased by 48% with vitamin D3 supplementation but this was indicated as being statistically insignificant. Vitamin D3 supplementation, however, had no significant effects on retinol levels among 85 study subjects.⁸⁰

FSVs are absorbed in the small intestine through different, but inter-related, mechanisms.⁸¹ Based on experiments made in an *in vitro* cell line culture (Caco-2 TC7, a cell line derived from colon carcinoma but resembling the enterocytes that line the small intestine), Goncalves and colleagues found that vitamin E significantly improved the absorbance of vitamin A but also significantly decreased the absorbance of vitamin D. In contrast, both vitamins A and D were shown to have negative effects on the absorbance of vitamin E. Furthermore, it was reported that vitamin A reduced both vitamin D and E uptake significantly.⁸¹ The group hypothesized that when there is concomitant consumption of vitamin A and E, the antioxidant properties of vitamin E helped to prevent vitamin A oxidation and therefore serve to enhance vitamin A absorption; this is at the expense of vitamin E absorption.⁸¹

Whilst vitamins A and E have been routinely measured together, it is actually the interaction of vitamins A and D at the molecular level that is currently generating research interest related to their regulatory roles in gene expression. 1,25-(OH)₂-D₃ forms a complex with the vitamin D receptor (VDR) which then can form a heterodimer with the retinoid X receptor (RXR), this then triggers gene expression. Retinoic acid, and endocrine receptors such as thyroid hormone receptors, can also form a heterodimer with RXR which in turn regulates gene expression. Given the common link of heterodimer formation involving RXR and high doses of vitamin A, it is postulated to attenuate heterodimer formation of VDR and RXR. This hypothesis is supported by an *in vitro* study, where the heteromeric interaction of VDR and RXR was influenced by the presence of 1,25-(OH)₂-D₃ and inhibited by high concentrations of 9-cis retinoic acid.⁸²

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Quantification of Soluble Vitamins in Blood

Clinical laboratory requests for FSV measurement, especially for vitamin D (25-OHD), have risen dramatically during the last decade. For example, in Australia, the number of laboratory requests for vitamin D measurement increased from 23,000 in 2000 to 2.2 million in 2010.⁸³ Immunoassay (for vitamin D) and high performance liquid chromatography (HPLC) (for vitamins A and E) are the most common analytical principles used for blood FSV measurement.

Laboratory medicine best practice guideline: vitamins a, e and the carotenoids in blood. [Clin Biochem Rev. 2014]

Overview of the *in vitro* stability of commonly measured vitamins and carotenoids in whole blood [Ann Clin Biochem. 2015]

Effect of temperature and light on the stability of fat-soluble vitamins in whole blood over several days [Int J Epidemiol. 2004]

Effects of delayed sample processing and freezing on serum concentrations of selected nutritional indicators [Clin Chem. 2008]

Effect of temperature and light on the stability of fat-soluble vitamins in whole blood over several days [Int J Epidemiol. 2004]

Review Traceability in laboratory medicine. [Clin Chem. 2009]

Review Vitamin E deficiency in developing countries. [Food Nutr Bull. 2011]

Bias and random error in retinol measurements of laboratories in countries with populations with mild to severe deficiency [Clin Chem. 2002]

Is calibration the cause of variation in liquid chromatography tandem mass spectrometry testosterone? [Ann Clin Biochem. 2013]

Review Standardization in laboratory medicine: new challenges. [Clin Chim Acta. 2005]

Implementation of standardization in clinical practice: not always an easy task. [Clin Chem Lab Med. 2012]

Review Standardization in laboratory medicine: new challenges. [Clin Chim Acta. 2005]

Traceability as a unique tool to improve standardization in laboratory medicine. [Clin Biochem. 2009]

25-hydroxyvitamin D: a difficult analyte. [Clin Chem. 2012]

Preparation and value assignment of standard reference material 968e fat-soluble vitamins, α [Anal Bioanal Chem. 2012]

The Joint Committee for Traceability in Laboratory Medicine (JCTLM): a global approach to promote traceability [Clin Biochem Rev. 2007]

For vitamin D analysis, the automated immunoassay systems predominate as they offer advantages of higher throughput, faster turnaround time and easier operating and troubleshooting abilities, compared to chromatography based applications. However, unsatisfactory bias and imprecision have been reported in various automated immunoassay platforms.⁵³ In addition, most immunoassays are unable to distinguish between 25-OHD2, epi-25OHD3 and 25-OHD3.⁸³ This problem may be related to the specificity and sensitivity of antibodies targeting these small molecules with very similar structures. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques can quantify each of these vitamins, including the identification of the epimer of 25-OHD3.

By contrast to vitamin D, requests for the measurement of vitamins A and E is less frequent and the routine analysis by HPLC is also more technically challenging. Variation of results obtained for vitamins A and E between laboratories remains a confounder for the broad application of recommended levels for health.²⁵ As part of the process of harmonisation, evidence based recommendations have recently been developed for the analysis of these vitamins.⁷⁵ These guidelines highlight the need for the implementation of a higher order reference method, in line with what is already available for vitamin D, and LC-MS/MS has been proposed as an appropriate method principle.

LC-MS/MS was introduced to clinical biochemistry screening and diagnostic laboratories as an emerging technique in the late 1990s. It is currently considered a more robust technique compared to immunoassay.^{83,84} The LC-MS/MS system is based on coupling liquid chromatography (LC), an eluting power used for physical analyte separation, with mass spectrometry (MS), which is a high resolution metabolite-selective detector. More recently, large- and medium-sized clinical laboratories have used LC-MS/MS systems for drug monitoring, newborn screening, endocrinology and metabolism applications (e.g. vitamin D).⁸⁵

The most common mass spectrometer in the clinical biochemistry diagnostic laboratory uses multiple mass quadrupole spectrometers.⁸⁶ With these advances in technology, LC-MS/MS can now be used to create highly specific and sensitive methods to measure the majority of FSV found in serum/plasma samples. Despite the purported advantages of the LC-MS/MS, several variables affect LC separation, such as the mobile phase (composition, flow rate, gradient and isocratic timing) and the column (size, type, temperature and pressure). The MS analysis is also influenced by the parameters selected, such as the voltage applied in the quadrupoles and the collision cell. As a result, LC-MS/MS system parameters need to be optimised to provide the most sensitive transition for each analyte.⁸⁷ During method development and optimisation, method validation and method performance criteria must be considered.^{75,88-90} Whilst many quantification methods for FSV using chromatographic techniques have been published, few have included detailed method validation and performance criteria (Table 1).^{75,88}

Table 1.
Specification and performance of published methods.

Comparison of three commercial calibrators for alpha-tocopherol using liquid chromatography-ta [Clin Biochem. 2013]

Reference materials and commutability. [Clin Biochem Rev. 2007]

Matrix effects in clinical analysis: commutability of control materials between the Ektachem, Beckm [Clin Chim Acta. 1981]

Measurement of 25-hydroxyvitamin D3 (25OHD3) and 25-hydroxyvitamin D2 (25OHD2) in human [Clin Chim Acta. 2008]

Why commutability matters. [Clin Chem. 2006]

Exogenous versus endogenous recovery of 25-hydroxyvitamins D2 and D3 [J Steroid Biochem Mol Biol. 2010]

Commutability of calibration and control materials for serum lipase. [Clin Chem. 2001]

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The accurate and precise quantification of FSV is still a significant challenge for clinical laboratories. This is largely due to the nature of FSV molecules and their metabolites, the availability of acceptable reference materials, reference measurement procedures (reference methods) and reference laboratories. Consequently, there is significant variation in the results obtained between clinical laboratories.^{83,84,91} As a result, it has been difficult to reach agreement on the optimal recommended level of these vitamins for health. The definitions of vitamin insufficiency, deficiency and severe deficiency therefore remain unclear. There has also been debate as to whether all current analytical techniques are fit for their intended purpose of diagnosing and monitoring the pathologies associated with FSV deficiency. In this section, we outline these challenges and, where practical, suggest what action is required to move forward.

Challenge 1: Physiology

The blood concentration of FSV range from pmol/L levels for 1,25-(OH)₂D₃ and vitamin K, to nmol/L for 25-OHD₃ and μmol/L for retinol and α-tocopherol.^{92,93} This range of concentrations adds to the challenge of simultaneous vitamin measurement by chromatographic techniques. Also for the vitamins at the lowest concentration levels, insufficient analytical sensitivity by some techniques remains a challenge. Also the ability of methods to differentiate between vitamins with similar structure and molecular weights is essential for specificity. Consequently, the specificity and sensitivity of techniques used in detection of these metabolites are critical for accurate and precise quantification.

Challenge 2: Binding Proteins

Another problem related to the analytical technique is that the majority of these vitamin metabolites are small (less than 500 Da) hydrophobic compounds that are mediated in the blood by binding with relatively large proteins (for instance, VDBP is around 50 kDa). The dissociation of a vitamin from its binding protein is therefore essential as part of vitamin measurement. This step in the analysis may significantly contribute to the technique's sensitivity and specificity.^{83,94}

Challenge 3: Stability

The stability of FSV in blood, especially vitamin A (retinol) and vitamin E (α-tocopherol), represents a gap in our knowledge. Although several factors, such as sample storage and transportation, are known to have an impact on vitamin stability, precisely how they do so remains inconclusive.⁷⁵ Currently, FSV in blood samples are treated as labile analytes, especially in the cases of retinol and α-tocopherol. As a result, a specific protocol for sample collection, transport and storage is used to control several crucial factors, such as light exposure, temperature, storage conditions and time.^{25,95,96}

The data on FSV stability is limited, and some studies' results contradict each other. For instance, one study indicated that changes in whole blood retinol and α-tocopherol at room temperature (RT) for 72h were -9.8% and -1.0%, respectively.⁹⁷ Another study reported that changes in whole blood retinol and α-tocopherol at RT after 1 week were 1.8% and 4.8%, respectively.⁹⁶ Because of the limited availability of FSV stability data, every clinical laboratory has its own procedure for dealing with blood samples, from patient sample collection to sample analysis. To address this challenge, evidence based stability data is required to support the appropriate handling both pre-analytically and during analytical processing of FSV.

Challenge 4: Standardisation

Harmonisation, and where practical standardisation with demonstrated traceability, is important for FSV in order to provide the same result and interpretation, irrespective of the laboratory in which the measurement is performed. Standardisation thus would help significantly in considering analytical results regardless of time, location or the measurement system used to obtain the result.⁹⁸ In practice, there are variations in the results obtained, even when using the same measurement technique for the same patient sample.^{62,91} This not only potentially affects daily patient care but also affects population-based vitamin deficiency assessment and the determination of common reference intervals or decision limits. In addition, this may cause misinterpretation or contradiction of results performed in different geographical places and/or at different times. This has recently been exemplified with LC-MS/MS measurement of serum testosterone, where different methods were found to be a source of inter-laboratory variation.⁹⁹ This poor comparison is in part due to the limitation in successful standardisation.¹⁰⁰

The standardisation of analyte measurement relies on five main pillars: (1) reference materials, (2) reference measurement procedures (reference method), (3) reference laboratories, (4) reference intervals or recommended levels and decision limits, and (5) external quality assurance programs.¹⁰¹ The reference measurement procedure is the procedure used to assign certified values of a reference material as a primary calibrator (pure analyte) or as a secondary calibrator (analyte in human clinical samples). This reference material can be used to assign values to commercial calibrators. Routine laboratory medicine subsequently uses validated commercial calibrators to measure analytes in human samples. Ideally, analytical results obtained from routine laboratory tests are therefore traceable to these references.^{100,102}

Standardisation initiatives for the measurement of FSV were first successfully introduced in the late 1980s. The National Institute of Standards and Technology (NIST) produced the standard reference material SRM 968 in 1989, and in 2009 added SRM 972 to support the standardisation of FSV measurement.^{94,103} Further important efforts for the standardisation of clinical analyte measurement came through establishing The Joint Committee for Traceability in Laboratory Medicine (JCTLM) in 2002. The JCTLM was formed as a result of collaboration between the International Committee for Weights and Measures (CIPM), the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC) and the International Laboratory Accreditation Cooperation (ILAC). The JCTLM plays a significant role in promoting the standardisation of analyte measurement through identifying appropriate reference materials, measurement procedures and laboratories.¹⁰⁴ In regard to reference materials for vitamin D, the original NIST SRM 972 material is no longer available and hence is no longer listed in the JCTLM database.¹⁰⁵ This material has been replaced by the new version SRM 972a; which is not listed yet on the JCTLM database. The NIST SRM 968e is recognised as reference materials for vitamins A and E by the JCTLM.¹⁰⁶ In the context of primary reference materials, the challenge is now to provide commutable reference materials for all routinely measured FSV.

Challenge 5: Reference Measurement Procedures

Although standardisation efforts for the measurement of FSV have been made, there are still gaps in the process for establishing and maintaining traceability. Recently, the isotope dilution liquid chromatography-mass spectrometry method has been recognised by the JCTLM as a reference measurement procedure for 25-OHD₂ and 25-OHD₃, although not for the epimer of 25-OHD₃.¹⁰⁷ The challenge now is to develop and list on the JCTLM database reference measurement procedures for vitamins A, E and epi-25OHD₃ in

blood.¹⁰⁷

Challenge 6: Commercial Calibrator Information

Individual commercial calibrators for the chromatographic analysis of FSV are currently available from a limited number of manufacturers. Many clinical laboratories use these commercial calibrators. The package inserts for these commercial calibrators state that they are traceable to available NIST reference materials for FSV. Theoretically, the availability of these commercial calibrators traceable to primary reference materials should minimise variation in patient results between laboratories. However, to assign a value to a commercial calibrator also requires an appropriate analytical method and an understanding of the uncertainty of measurement. Currently a number of manufacturers do not routinely provide their customers with details of the method performance used in the traceability process and also how the calibrator matrices are prepared. Our previous study investigated three commercial calibrators for serum/plasma α -tocopherol. Results showed discrepancies between the observed concentration versus manufacturer expected concentration; although they were all traceable to a NIST reference material.¹¹⁶ The challenge here is for the analytical community to “encourage” the FSV commercial calibrator manufacturers to provide this information routinely as part of the purchase process.

Challenge 7: Commutability of Reference Material

Both method performance and matrix treatment can affect the patient results obtained using these calibrators.^{108–114} Questions have therefore been raised regarding the trueness and traceability of commercial calibrators. Different matrices in calibrators could alter assay imprecision and bias.¹¹⁵ Demonstrated commutability of both the reference materials and commercial calibrators is essential for method harmonisation.¹¹⁰ For example, two levels of the NIST SRM-972 for vitamin D analytes were non-commutable for all routine analytical methodologies because these levels contained non-human serum and exogenous analytes.¹¹⁷ The importance of commutability has been demonstrated through comparisons between patient results obtained using commutable calibrators, which were contradicted by results generated using non-commutable calibrators demonstrates this.¹⁰⁹ The challenge is to demonstrate commutability of all reference materials, including that of commercial calibrators and external quality assurance material for FSV.

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Conclusions

Traditionally we have measured individual fat soluble vitamins to look for isolated deficiencies in relation to disease. Now there is increasing evidence of interactions between these vitamins, especially between vitamins A and D and it would be useful to measure these FSV simultaneously within the one analytical method. Translational clinical research and diagnostic laboratory requests for the measurement of these vitamins has significantly increased, but whilst there are still gaps in the traceability chain, comparison of results between laboratories and over time is limited. Therefore, it is necessary for all parties (including clinical institutes for measurement standardisation, and manufacturers of *in vitro* diagnostic medical devices) to work cooperatively in order to address the current challenges related to standardisation, with clear traceability and demonstrated commutability.

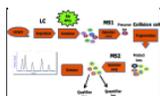


Figure 7.

Schematic of a sample progressing through a liquid chromatography-tandem mass spectrometer.

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Acknowledgments

This manuscript was developed as part of the work conducted by Dr Ali Albahrani during his PhD candidature. Accordingly, we wish to thank the General Administration of Medical Services -Ministry of Interior (Saudi Arabia) for offering an academic scholarship to Dr Albahrani to complete his PhD study. We also acknowledge Dr Peter Roche and Dr Victor Rotarou for their support, help and valuable guidance during Dr Albahrani's PhD candidature. We would like to give special praise and recognition for RMIT Clinical Biochemistry -Agilent Technologies Mass Spectrometry Collaboration Laboratory to support this work.

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Footnotes

Competing Interests

None declared. There was no specific grant for this work. This work was part of a PhD project which was supported in kind by Agilent Technologies as part of the RMIT Clinical Biochemistry - Agilent Mass Spectrometry Collaboration.

Contributors Statement

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this manuscript. This manuscript was generated as part of the work conducted by Dr Ali Albahrani during his PhD candidature investigating "A Novel Simultaneous Quantification Method for Fat-Soluble Vitamins using Liquid Chromatography - Tandem Mass Spectrometry for Clinical Applications". Dr Albahrani wrote the first draft of this manuscript, which largely forms the basis of Chapter One of his thesis. Dr Greaves provided scientific input into this work and reviewed the draft of this manuscript as primary supervisor for Dr Albahrani's PhD candidature. Both authors contributed, reviewed, edited and approved the final manuscript.

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