REVIEW ARTICLE

CLINICAL SIGNIFICANCE AND METHODS OF ANALYSIS OF VITAMIN A

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ABSTRACT

Vitamin A is a group of unsaturated nutritional organic compounds, that includes retinol, retinal, retinoic acid, and several provitamin A carotenoids, among which beta-carotene is the most important. Most compounds within the vitamin A family are soluble in fat and are essential for numerous processes within the body. Vitamin A (retinol) and derivatives play an essential role in metabolic functioning of the retina, the growth of bone, reproduction, and the immune response. Dietary vitamin A is derived from a variety of carotenoids found in plants. It is enriched in the liver, egg yolk and the fat component of dairy products. It is required for normal vision, reproduction, embryonic development, cell and tissue differentiation and immune function in animals and humans. Vitamin A deficiency (VAD) is also known to be involved with different specific disease conditions such as xerophthalmia, polynephritis, keratomalacia and ulceration leading to irreversible blindness along with increased susceptibility to infections and abnormalities in reproduction. Vitamin A is sensitive to light and air and its ester forms are more susceptible to oxidation. The important analytical techniques used for the analysis of the vitamin A and its derivatives are high-performance liquid chromatography (HPLC), spectrophotometry and voltammetry. These methods have been found to be accurate and precise and have been applied successfully to the analysis of drug formulations.

Keywords: Vitamin A, vitamin A deficiency, clinical significance, stability, analytical methods.

1. INTRODUCTION

Vitamin A (retinol, axerophthol) was discovered in the early 1900s by McCollum and colleagues at the University of Wisconsin and independently by Osborne and Mendel at Yale University. They extracted a minor ether-soluble fraction from fish oils, milk or meat and called it "fat-soluble". Vitamin A refers to compounds with the biological activity of retinol. These include the provitamin A carotenoids that are present in diet by green, yellow and orange vegetables and some fruits. The preformed vitamin A, retinyl esters and retinol present in the food of animal origin and in the organ meat such as liver, eggs and dairy products are also rich in vitamin A. Vitamin A deficiency has shown to cause several specific diseases including xerophthalmia, squamous metoplasia of epithelial and mucosal tissues and increased susceptibility to infections[1].

Vitamin A is an essential micronutrient for all vertebrates. The vitamin is required for normal vision, reproduction, embryonic development, cell and tissue differentiation and immune function. Dietary vitamin A is ingested in two forms, i.e., preformed vitamin A (retinyl esters and retinol) and provitamin A carotenoids (α-carotene, β-carotene and β-cryptoxanthene). The amount of vitamin A obtained from these forms varies among animal species and humans[2].

Excessive dietary intake of vitamin A produces symptoms of acute and chronic toxicity, including teratogenicity in developing features. Normally, toxicity results from indiscriminate use of pharmaceutical supplements and not from the consumption of normal diets[3].

2. CLINICAL SIGNIFICANCE

There is an emerging awareness about Vitamin A deficiency (VAD) in the recent years. WHO showed concern for successful eradication of severe consequences of VAD globally. In addition to the

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well-known effects on humans to serve as a chromophore in sight, vitamin A status plays a significant role in essential biological processes. It is a vital component of food and its absence causes a high risk of disease and death. To combat VAD, breast feeding must be encouraged in babies as breast milk is a natural source of vitamin A. Vitamin A deficiency is characterized by changes in the tissues of the eye that ultimately result in irreversible blindness. Clinical symptoms are collectively referred to as xerophthalmia[4], and include the following:

1. Night blindness—the inability to see in dim light.
2. Conjunctival and corneal xerosis.
3. Keratomalacia—ulceration and scarring of the cornea that leads to loss of vision.

Other symptoms include skin lesions, loss of appetite, epithelial keratinization, lack of growth, and increased susceptibility to infections[4-6]. Human status assessment methods include dietary assessment, assessment of content of liver, plasma, and breast milk, and functional assessment by dark adaptation and conjunctival impression cytology[6]. Vitamin A toxicity (hypervitaminosis A) can occur because of high intake from either food high in vitamin A or high-potency supplements. Toxicity to retinoids has been classified as acute, chronic, and teratogenic[4,7,8]. Common teratogenic defects include physical malformations, heart, kidney, and thymic disorders, and central nervous system disorders[7]. Vitamin A plays a significant role in the following diseases and processes:

2.1. Allopecia

Vitamin A has many roles in human biology with respect to hair. Endogenous retinoids are involved in the pathogenesis of alopecia areata (AA) which is an autoimmune disease that attacks anagen hair follicles. Vitamin A regulates both the hair cycle and immune response to alter the progression of AA[9]. Hypovitaminosis A from inadequate vitamin A intake causes hair loss and the dietary vitamin A may have a role in maintaining alopecias[10].

2.2. Skin Disease

Vitamin A, and more specifically, retinoic acid, appears to maintain normal skin health by switching on genes and differentiating keratinocytes (immature skin cells) into mature epidermal cells[11]. Exact mechanisms behind pharmacological retinoid therapeutical agents in the treatment of dermatological diseases are being researched. For the purpose treatment, the most prescribed retinoid drug is 1,3-cis retinoic acid (isotretinoin). It reduces the size and secretion of the sebaceous glands. Although it is known that 40 mg of isotretinoin will break down to an equivalent of 10 mg of acyl-CoA: retinol acyltransferase (ARTA) the mechanism of action of the drug (original brand name Accutane) remains unknown and is a matter of some controversy. Isotretinoin reduces bacterial numbers in both the ducts and skin surface. This is thought to be a result of the reduction in sebum, a nutrient source for the bacteria. Isotretinoin reduces inflammation via inhibition of chemotactic responses of monocytes and neutrophils[12]. Isotretinoin has also been found to initiate remodeling of the sebaceous glands; triggering changes in gene expression that selectively induce apoptosis[13].

Psoriasis is significantly associated with increased serum levels of vitamin A and α-carotene and reduced sugar intake. Therefore it is necessary to monitor nutritional status in psoriasis patients to evaluate the effect of nutrition on psoriasis progression and the modifying role of treatments[14].

2.3. Reproduction and Development

Vitamin A is very important for the eyes of developing embryo. Generally it is believed that all-trans retinoic acid (RA) is the form of vitamin A that supports both male and female reproduction as well as embryonic development. According to recent studies vitamin A participates in a signaling mechanism to initiate meiosis in the female gonad during embryogenesis, and in the male gonad postnatally. It is also useful for the maintenance of

the male genital tract and spermatogenesis. Both nutritional and genetic approaches are being used to elucidate the vitamin A-dependent pathways upon which these processes depend[15].

2.4. Effect on Eyes

The eye is an immunologically privileged and profoundly immunosuppressive environment. It is concluded that RA in the eye plays a dual role: in vision and in immune privilege. Nevertheless, primed effector T cells are relatively insensitive to aqueous humor, helping to explain their ability to induce uveitis despite an inhibitory ocular microenvironment[16].

2.5. Polynephritis

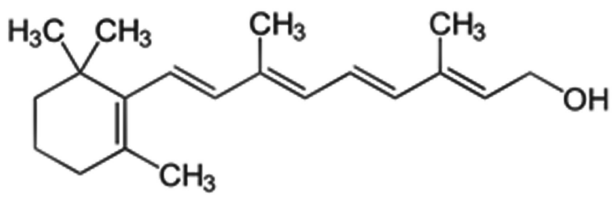
Vitamin A was useful in decreasing the amount of the injury and scarring following the polynephritis. Vitamin A can also be used in conjunction with other treatments in the management of acute pyelonephritis in children[17]. Studies suggest that therapy using retenoids is a novel approach to the treatment of the patients with lupus nephritis[18].

3. STRUCTURE AND PHYSICOCHEMICAL PROPERTIES OF VITAMIN A

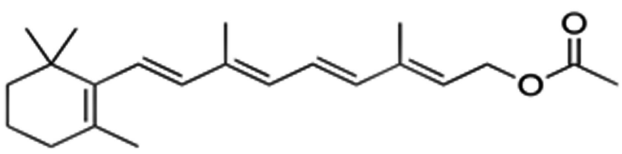
Some of the physicochemical properties of vitamin A are as follows[19]:

Structural formula

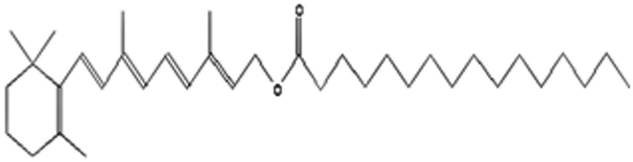
Alcohol: R=OH



Acetate: $R=COCH_3$



Palmitate: $R=CO(CH_2)_{14}CH_3$



Empirical formula

Alcohol: $C_{20}H_{30}O$
Acetate: $C_{22}H_{32}O_2$
Palmitate: $C_{36}H_{60}O_2$

Molecular weight

Alcohol: 286.4
Acetate: 328.5
Palmitate: 524.9

Description

Alcohol: Yellow to orange crystalline solid
Acetate: Bright yellow crystalline powder
Palmitate: Yellow oil, or crystalline mass

Solubility

Alcohol: Insoluble in water or glycerol, soluble in alcohol, readily soluble in ether, chloroform, acetone and fats and oils.

Melting point

Alcohol: 62-64 °C
Acetate: 57-58 °C
Palmitate: 28-29 °C

Absorption maxima

(Absolute ethanol)

Alcohol: 324-325 nm (A1%, 1cm 1835)
Acetate: 326 nm (A1%, 1cm 1550)
Palmitate: 325-328 nm (A1%, 1cm 975)

4. STABILITY OF VITAMIN A

Vitamin A is sensitive to light and air and is very susceptible to oxidation in pharmaceutical dosage forms. Vitamin A esters are more stable towards oxidation than the alcohol. Irradiation of vitamin

A leads to the rearrangement of double bonds to form inactive isomeric compounds, which are responsible for much of the yellow color of degrading vitamin A[3,20].

Vitamin A is unstable on exposure to light[21]. The photodegradation of the vitamin is wavelength-dependent in the ultraviolet region[22,23]. The use of antioxidants is effective in increasing the stability of vitamin A formulations. Refrigeration and exclusion of oxygen retards the degradation of the vitamin[24]. It should be stored in air tight and light resistant containers[25-26].

5. METHODS OF ANALYSIS OF VITAMIN A

The various techniques used for the determination of Vitamin A include spectrometry (ultraviolet and visible), spectrofluorimetry, high-performance liquid chromatography (HPLC) and electrochemical method. The earlier methods used for the determination of vitamin A have been reviewed[27]. The details of the analytical methods of vitamin A, based on various techniques, are given as follows:

5.1. Chromatographic Methods

Since mid-1970s in pharmaceuticals, the method of choice for determining vitamin A and the provitamin A carotenoids in food has been suggested as HPLC. In the 1995 edition of Official Methods of Analysis of AOAC (Association of Official Agricultural Chemists) International, HPLC methods have been introduced for the first time for the determination of vitamin A[27] and milk-based infant formula[28].

Of the vitamin A commonly found in foods, the esterified form of both trans-retinol and smaller amounts of 1,3-cis retinol are usually present in significant quantities. For the analysis of vitamin A-fortified foods, HPLC can be applied to determine either the total retinol content or the added retinyl ester (acetate or palmitate), depending on the extraction technique employed. The vitamin A activity of plant foods is usually based on the HPLC determination of the three most ubiquitous provitamins, namely α-carotene and β-carotene, and β-cryptoxanthene. It is necessary to separate the provitamins from other carotenoids and to quantify them individually. An obvious prerequisite to accurate quantitation is the conclusive identification of the provitamins. To assess the effects of processing on the nutritional value of a plant food with respect to vitamin A activity, the various isomeric forms of provitamin A carotenoids present in both the fresh and processed states must be accurately measured. In such investigations it must be demonstrated that the analytical procedure does not itself cause trans–cis isomerization of carotenoids[29].

The HPLC methodology for carotenoids analysis depends on their known distribution in plant tissues, which can be classified into three main groups:

(1) those in which the vitamin A value is due almost exclusively to β-carotene (e.g., green leafy vegetables, peas, broccoli, sweet potatoes, tomatoes, water-melon, mango);

(2) those in which primarily α- and β-carotene account for the vitamin A value (e.g., carrots, some varieties of squash); and

(3) those in which β-cryptoxanthin and β-carotene are the major contributors (e.g., cashew, apple, peach, persimmon, loquat)[29].

A novel, simple and fast reversed-phase HPLC/UV method has been developed. This method has been optimized for various chromatographic conditions, and validated according to international guidelines for simultaneous determination of all-trans-retinol and α-tocopherol in human serum using retinyl acetate as internal standard. Different particulate reversed-phase chromatographic columns have been evaluated in order to select the best column in terms of sensitivity, selectivity, resolution and short run time of both the analytes. It has been concluded that

3 micron columns are better to be used in clinical set up as well as in laboratories for the separation of these analytes in a shorter time as compared with 5 micron columns. The method has been validated and applied for the analysis of all-trans-retinol and α-tocopherol in the serum of human volunteers[30].

A HPLC method has been used to determine two biochemical indicators, i.e. serum retinol and serum retinol-binding protein (RBP), currently recommended for determining whether vitamin A deficiency (VAD) is a public health problem. After consideration of the data sets and the original rationale for previously proposed cut-offs, a cut-off for serum retinol concentration has been proposed at <0.70 μmol/L (20 μg/dL) in 15% of the sampled population. This cut-off should be applied to a representative group of preschool age children. For serum RBP, a cut-off cannot be reliably specified, because available data are too few and too variable. However, because serum RBP concentration correlates well with serum retinol concentration, it can be used to determine whether VAD is a public health problem in those populations for which the relationship between serum concentrations of retinol and RBP has been established. More efforts to establish a reliable cut-off for RBP is warranted, because analysis, in particular radial immuno-diffusion (RID), is relatively simple and inexpensive. Although HPLC and RID analyses of vitamin A are being done in a laboratory, methods are being developed for assessing serum retinol and RBP under more remote conditions[31].

A robust HPLC/UV method has been used to quantify retinyl esters (RE), retinol (ROL) and retinal (RAL) applicable to diverse biological samples, with lower limits of detection of 0.7 pmol, 0.2 pmol, and 0.2 pmol, respectively, and linear ranges >3 orders of magnitude. These assays function well with small, complex biological samples (10–20 mg tissue). Coefficients of variation ranged from: intra-day, 5.9–10.0%; inter-day, 5.9–11.0%. The results of the assays provided sensitive and rigorous quantification of endogenous RE, ROL, and RAL to elucidate retinoid homeostasis in disease states, such as Alzheimer's disease, type 2 diabetes, obesity, and cancer[32].

A novel ultra-performance liquid chromatographic (UPLC) method used for the determination of retinol and α-tocopherol in human serum has been compared with the conventional HPLC with particulate and monolithic C (18) columns. In UPLC a sub-two-micron particle-hybrid C (18) stationary phase was used for separation, in contrast with a five-micron-particle packed column and a monolithic column with a highly porous structure. Methanol has been used as mobile phase for isocratic elution of the compounds in the three methods. Detection is performed for retinol at 325 nm and for α-tocopherol at 290 nm. Analysis time, sensitivity, mobile-phase consumption, validation data, and cost have critically compared for these different chromatographic systems. Although cost and mobile-phase consumption seem to make UPLC the method of choice, use of the monolithic column resulted in almost the same separation and performance with a slightly shorter analysis time. These methods are alternatives and, in routine laboratory practice, more economical means of analysis of large numbers of biological samples than use of a traditional particulate column[33]. A HPLC method has also been used for simultaneous determination of retinoids and tocopherols in human serum for monitoring anticancer therapy[34].

A reversed-phase high-performance liquid chromatographic (RP-HPLC) method has been described for the simultaneous determination of retinol, α-tocopherol and retinyl palmitate in plasma. Plasma containing an internal standard (tocol) is deproteinized with ethanol, then extracted with n-hexane. The organic layer is removed and evaporated under a nitrogen stream, and chromatographed on a reversed-phase RP-18 column using a

water/acetonitrile-ethyl acetate/2-propanol gradient solvent system over 15 min at 305 nm. The recovery exceeded 93%. The detection limit was 0.1 µg/ml for retinol, 1.3 µg/ml for α-tocopherol and 0.95 µg/ml for retinyl palmitate, respectively. The reproducibility, precision and accuracy were less than 8% for all analytes. The small sample requirement, the simplicity of extraction, the short run-time and the good reproducibility make this procedure particularly useful for monitoring retinol and α-tocopherol supplementation in premature newborns[35].

5.2. Spectrometric Methods

Formerly, few foods were routinely analyzed for vitamin A, but recent emphasis on nutrient requirements, nutrient labeling, and use of dietary convenient foods has created need for determining vitamin A in a variety of foods. There are many vitamin A methods, some suitable for certain products only. For regulatory purposes, the FDA specifies the AOAC method where it is applicable. However, some food analysts and organizations continue with their own vitamin A methods. If possible, a single, widely applicable general method of analysis should be used for vitamin A in foods. Vitamin A may be determined by spectrophotometric, colorimetric, and fluorometric procedures. Sometimes chromatography is required as an important part of the method. If vitamin A content is high enough and extracts sufficiently free of interfering substances, spectrophotometric or flurometric methods are satisfactory[36].

Simple and sensitive spectrometric methods in the visible range for the assay of retinol have been developed. One method is based on the reaction of retinol with iodine to give a molecular charge-transfer complex. The retinol acts as n-electron donor and iodine as σ-electron acceptor. The second method depends on the formation of a highly colored stable radical anion between retinol and 7,7,8,8-tetracyanoquinodimethane (TCNQ) as π-electron acceptor. Beer's law is obeyed over the retinol concentration range of 2.5–26 µg/ml. The average recovery and average standard deviation of the method is 99.99 ±1.13% with retinol-iodine and 100.001 ± 1.31% with retinol-TCNQ. A kinetic study has been performed by heating retinol at 50 °C for different periods of time. The results obtained by plotting log c against time indicate that thermal decomposition of retinol is of first-order. The results obtained are in good agreement with those obtained by the official method. The developed procedures have been found to be simple, accurate and precise and can be used for the determination of retinol in the presence of its degradation products. The proposed procedures have been applied successfully to the analysis of drug formulations[37].

An enzymatic determination of vitamin A in pharmaceutical formulations with spectrometric detection has been reported. In this method soluble and immobilized alcohol dehydrogenase has been used, that was isolated from rabbit liver. The reaction is based on the oxidation of retinol and simultaneous reduction of NAD^+ to NADH followed by spectrometric detection at 340 nm. The calibration graph is linear over the range of 2.0–10 µM with correlation coefficient of 0.9992 (n = 5) for soluble and immobilized alcohol dehydrogenase, respectively, with relative standard deviations (n = 3) in the range of 0.5–1.2%. The limit of detection is lower than 1.0 µM. This proposed method is applied to determine vitamin A in pharmaceuticals, and the results obtained are in reasonable agreement with the labeled amount. The results have been compared using a reference spectrometric method, and no significant difference has been found between the results of the two methods[38].

The spectrometric analysis of retinol derivatives and estimation of their analytical usefulness for simultaneous determination in synthetic mixtures and pharmaceuticals have been studied. The analysis of

the zero-order and the second-order derivative spectra of retinol acetate, α-tocopherol acetate and coenzyme Q_{10} have also been reported. The aim of this study is to develop a simple and rapid method for the determination of retinol acetate, tocopherol acetate and coenzyme Q_{10} in pharmaceuticals without involving any preparation operations like separation or masking. The values of second derivative amplitude at 212 nm for α-tocopherol, 351 nm for retinol and 222 nm for coenzyme Q_{10} have been used for the construction of calibration graphs. Beer's law is obeyed in the concentration range 0.5–20, 0.5–7.5 and 0.5–30 μg ml^{-1} for retinol acetate, tocopherol acetate and coenzyme Q_{10}, respectively. The elaborated procedures have successfully been applied to the simultaneous determination of studied compounds in their binary synthetic mixtures and in commercial preparations with high reliability and repeatability. Spectral properties of retinol acetate allow the determination of its contents in ternary mixture which includes Vitamin E and coenzyme Q_{10}[39].

5.3. Electrochemical Methods

A voltammetric method has been developed for the determination of vitamin A in pharmaceutical preparations. In this method, the voltammetric oxidation of vitamin A at a carbon paste electrode has been used to determine the vitamin A content of some pharmaceutical preparations. Chromatography is not required as a clean-up procedure, therefore time is gained and a potential source of errors is eliminated. In the mixture of preparations containing α-tocopherol in large amounts, interfering in the most widely used methods, is rendered electrochemically inactive in the range of potentials that is of interest. This is achieved by acetylating all of the tocopherols that are present in the sample in alcohol form. The oxidation waves of vitamin A are linearly proportional to the concentration range studied. This method is used for the alcohol and acetate derivatives of vitamin A[40].

6. CONCLUSION

The present review provides information on certain aspects of vitamin A and its derivatives, including the clinical significance, physicochemical properties, stability and analytical methods used for their determination in clinical samples and pharmaceutical products. Vitamin A is one of the most useful component of the food required for body functions and that it should be included in daily diet according to the recommended daily amounts (RDA) for all age groups to achieve a better life style. It protects the body from many life threatening diseases by strengthening the immune system efficiency and the organism's ability to respond to pathogens, antigens, and mitogens, effectively. Due to its requirement from infants to pregnant women for normal body functioning and high immune response, its use would surely be helpful in reducing subclinical deficiency symptoms. Vitamin A stability is also an important factor since it is very sensitive to light and air and undergoes degradation. The use of antioxidants, refrigeration and exclusion of oxygen is effective in increasing the stability of vitamin A formulations. The UV-visible absorption characteristics of vitamin A may directly be utilized for assay purpose or for detection in HPLC assays. For pharmaceuticals, the method of choice for determining vitamin A and the provitamin A carotenoids in food is HPLC which allows the separation of these vitamins in food material, supplements and pharmaceutical preparations followed by its determination.

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