# Clinical Analysis of Vitamin D and Metabolites

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## ABSTRACT

Vitamin D (mainly ergocalciferol and cholecalciferol) and their metabolites exist in a complex environment in biological systems. The identification and determination of the individual vitamin Ds in these systems require specific methods. The purified components of vitamin D can be determined by UV spectrometry and colorimetry. However the best method for the separation and determination of ergocalciferol and cholecalciferol and their metabolites is high-performance liquid chromatography. This technique in combination with mass spectrometry (LC-MS) can be used for the simultaneous separation and identification of vitamin D components and metabolites. The technique is also useful for the identification of vitamin D in steroidal mixture.

## INTRODUCTION

Vitamin D is a group of fat-soluble compounds containing ergocalciferol (vitamin $D_2$) and cholecalciferol (vitamin $D_3$). The D vitamins possess antirachitic activity and are involved in the maintenance of calcium and phosphorous homeostasis. Calcium and phosphorous are required for a wide variety of biological processes including muscle contraction, nerve pulse transmission, blood clotting, and membrane structure. Vitamin D is also important for insulin secretion, hair growth, muscle function, immune response and melanin synthesis[1-5].

Vitamin D is largely biologically inert and is metabolized to its dihydroxy active forms 1α, 25$(OH)_2$ $D_3$ and 24,25$(OH)_2$ $D_3$. Both of these metabolites are obtained in vivo by hydroxylation at carbon 25 of the vitamin $D_3$ molecule in the liver[1].

## CHEMICAL CHARACTERICTICS1

### *Ergocalciferol ($C_{28}$ $H_{44}O$)*

The molecule is unsaturated containing three double bonds. In alcoholic and hexane solutions, it exhibits an absorption maximum at 264-265 nm with a molar absorptivity of 18,300 $M^{-1}$ $cm^{-1}$. It is insoluble in water and soluble in benzene, chloroform, ethanol and acetone. The molecule is unstable in light and is oxidized on exposure to air.

### *Cholecalciferol ($C_{27}$ $H_{44}O$)*

It possesses four double bonds and exhibits and absorption maximum at 265 nm in ethanol or hexane with a value of molar absorptivity as 19,400 $M^{-1}cm^{-1}$. The solubility and stability characteristics are the same as that of ergocalciferol[1].

## METHODS

Several methods have been developed for the analysis of vitamin D and metabolites using spectrophotometric, electrochemical and chromatographic methods[6-10]. However, these methods lack the sensitivity and selectivity of biological assays. The important methods of the analysis of vitamin D and metabolites are presented in the following sections:

### Spectrometric Methods:

### a. Ultraviolet Spectrometry

The conjugated triene system possessed by vitamin D secosteroids imparts high absorption around 264 nm which can be used for the determination of the vitamin. The high molar absorptivity of the vitamin $D_2$ and

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$D_3$ makes the method very sensitive. This technique is simple and rapid, however, it require a pure sample, free of UV-absorbing impurities, for accurate determination[1]. The method has been used for the determination of high potency vitamin D after chromatographic separation[11].

**b. Colorimetry**

Colorimetric methods have extensively been used for the determination of vitamin D. One of the sensitive methods involves isomerisation of vitamin D to isotachysterol and development of color with antimony trichloride. The detection limit of vitamin D with the method is 1-1000 mcg[12]. The various colorimetric methods for the determination ergocalciferol and cholecalciferol have been reviewed[13,14]. These methods are based on the development of color with sulphuric acid, antimony trichloride trifloroacetic acid stannous chloride, iodine-ethylene-dichloride and other reagents.

**c. High Performance Liquid Chromatography (HPLC)**

HPLC is nowadays a most widely used technique for the determination of vitamin D, its metabolites and related compounds. This technique has the advantage of separation of vitamin D components and their metabolites followed by determination. The sensitivity of detection of vitamin D is ~5 ng[1]. HPLC methods have been developed for the determination of the hydroxyl metabolites of ergocalciferol and cholecalciferol and biological fluids[15-18]. Ergocalciferol, and cholecalciferol in their 25-hydroxy metabolites have been determined by UV detection in plasma with a detection limit of 500 ng/L[19]. HPLC has been considered as the best method of separation of vitamin D components and their metabolites[20]. According to the British Pharmacopeia method[21], ergocalciferol and cholecalciferol are determined by liquid chromatography.

High performance liquid chromatography has been used in combination with mass spectrometry (HPLC-MS) for the simultaneous separation and identification of vitamin D components and other steroidal mixtures[23-25]. The urinary metabolites of cholecalciferol in man have been identified by Higashi et al.[26] using HPLC-MS. A clinical assay of $25(OH)_2D_3$ metabolite in biological fluids have been developed using HPLC-MS technique[27].

## DISCUSSION

This review presents a literature survey on the physicochemical characteristics and the methods of analysis of vitamin D components, ergocalciferol and cholecalciferol and their various metabolites. These compounds exist as a complex mixture and the determination of a particular component can not be carried out by the UV spectrophotometric method as a result of interference from other components. In some cases colorimetric methods are suitable for selective determination of individual vitamin D components. The HPLC method could be considered to be the best method for separation, identification and determination of various vitamin D components, their metabolites and related compounds using UV detector. The application of HPLC method has facilitated accurate determination of ng quantities of vitamin D in biological fluids. A combination of liquid chromatography-mass spectrometry (LC-MS) has the advantage of separation as well as identification of individual vitamin D components in a mixture. The technique may be useful in the detection of vitamin D metabolites in blood circulation.

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