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 D₂) and cholecalciferol (vitamin D₃). 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 synthesis¹⁻⁵.

Vitamin D is largely biologically inert and is metabolized to its dihydroxy active forms 1α, 25(OH)₂D₃ and 24,25(OH)₂D₃. Both of these metabolites are obtained in vivo by hydroxylation at carbon 25 of the vitamin D₃ molecule in the liver⁴.

CHEMICAL CHARACTERISTICS

Ergocalciferol (C₂₇H₄₄O)
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 (C₂₇H₄₆O)
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 ergocalciferol¹.

METHODS
Several methods have been developed for the analysis of vitamin D and metabolites using spectrophotometric, electrochemical and chromatographic methods⁶⁻¹⁰. 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 D₂ and
D₃ makes the method very sensitive. This technique is simple and rapid, however, it require a pure sample, free of UV-absorbing impurities, for accurate determination¹. The method has been used for the determination of high potency vitamin D after chromatographic separation¹¹.

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 mcg¹². The various colorimetric methods for the determination ergocalciferol and cholecalciferol have been reviewed¹³,¹⁴. These methods are based on the development of color with sulphuric acid, antimony trichloride trifluroacetic 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 ng¹. HPLC methods have been developed for the determination of the hydroxyl metabolites of ergocalciferol and cholecalciferol and biological fluids¹⁵-¹⁸. Ergocalciferol, and cholecalciferol in their 25-hydroxy metabolites have been determined by UV detection in plasma with a detection limit of 500 ng/L¹⁹. HPLC has been considered as the best method of separation of vitamin D components and their metabolites²⁰. According to the British Pharmacopeia method²¹, 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 mixtures²²-²⁵. The urinary metabolites of cholecalciferol in man have been identified by Higashi et al.²⁶ using HPLC-MS. A clinical assay of 25(OH)₂D₃ metabolite in biological fluids have been developed using HPLC-MS technique²⁷.

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.
REFERENCES


