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 synthesis.

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 liver.

CHEMICAL CHARACTERISTICS

Ergocalciferol (C₂₈H₄₄O)
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 ergocalciferol.

METHODS
Several methods have been developed for the analysis of vitamin D and metabolites using spectrophotometric, electrochemical and chromatographic methods. 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 determination. The method has been used for the determination of high potency vitamin D after chromatographic separation.

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. The various colorimetric methods for the determination ergocalciferol and cholecalciferol have been reviewed. 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. HPLC methods have been developed for the determination of the hydroxyl metabolites of ergocalciferol and cholecalciferol and biological fluids. Ergocalciferol, and cholecalciferol in their 25-hydroxy metabolites have been determined by UV detection in plasma with a detection limit of 500 ng/L. HPLC has been considered as the best method of separation of vitamin D components and their metabolites. According to the British Pharmacopeia method, 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. The urinary metabolites of cholecalciferol in man have been identified by Higashi et al. using HPLC-MS. A clinical assay of 25(OH)₂D₃ metabolite in biological fluids have been developed using HPLC-MS technique.

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


