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FEASIBILITY STUDY — VITAMIN D LOADING DETERMINATION BY FTIR-ATR

E. Simana[a], *B.Sc., Electrical Engineering, eran.simana@gmail.com*
R. Simian[a], *B.Sc., Electrical Engineering, ronensimian@gmail.com*
S. Portnoy[b], *PhD, Biomedical Engineering, portnoys@tauex.tau.ac.il*
A. Jaffe[c], *M.D, PhD, Endocrinologist, jaffe@hy.health.gov.il*
B. Z. Dekel[a], *PhD, Electrical Engineering, benziond@ruppin.ac.il*
[a]*Ruppin Academic Center, Emek-Hefer, 40250, Israel*
[b]*Occupation Therapy Department, Tel-Aviv University, Tel-Aviv, Israel*
[c]*Endocrinology and Diabetes Unit, Hillel Yaffe Medical Center, Hadera, Israel*

Purpose: *The aim of the present study was to develop a simple and accurate way to measure vitamin D levels. Vitamin D nowadays is measured by a variety of methods which their common drawbacks are expensive equipment and the need for high trained technical staff. In this research we measured vitamin D levels by means of Fourier transform infra red method in conjugation with the evanescent wave spectroscopy technique, in order to develop a simpler vitamin D measurement method.* ***Methods:*** *Blood samples were collected from patients with vitamin D deficiency at five intervals before and up to 16 days after they took a dose of 200,000 IU vitamin D_3. Samples were measured by the conventional bio-chemical method and by the evanescent wave spectroscopy means.* ***Results:*** *Correlation was found between the vitamin D levels measured by the traditional method and by the evanescent wave spectroscopy technique. The absorption lines occurred prominently in the IR spectral regions of the Amide I (≈1650 cm^{-1}),Amide II (≈1530 cm^{-1}) and the (≈3400 cm^{-1}) absorption band which is attributed to the hydroxyl group indicated by the O-H stretch. In addition, the examination of the blood samples using the evanescent wave spectroscopy with clustering techniques facilitated the discrimination between vitamin D deficiency and normal vitamin D levels.* ***Practical relevance:*** *This study demonstrates the potential of using the Fourier transform infra red method in conjugation with the evanescent wave spectroscopy techniquecoupled with multivariate analysis as a non-expensive, rapid and accurate alternative to the routine methodologies.*

Keywords *— FTIR-ATR Spectroscopy, Mid Infrared, Vitamin D.*

Introduction

It has been well-known for decades that vitamin D is activated in the skin by ultraviolet B radiation and that its main activities are calcium balanceby affectingcalciumabsorption, secretion, bone metabolism and control of PTH. Although, how much vitamin D is produced in the body under given ultraviolet B exposure conditions is still discussed [1]. In the last decade, it has been suggested that vitamin D plays an important role in various diseases, e.g. osteoporosis, rickets, diabetes mellitus, cardiovascular disease, multiple sclerosis, different forms of cancer and a number of mental disorders, including depression [2].

Vitamin D exists in two main physiological forms [3], vitamin D_2 and vitamin D_3, which are hydroxylated by liver enzymes into 25-hydroxyvitamin D_2 ($25(OH)D_2$) and 25-hydroxyvitamin D_3 ($25(OH)D_3$), respectively. Their level in plasma is considered the best indicator of physiological vitamin D status.

Current health systems make daily use of several analytical methods which are usually applied to measure 25(OH)D. Among which are the radioimmunoassay method,the LIASONE which is an automated antibody and microparticle-based chemiluminescent immunoassay method, the high performance liquid chromatography and recently the liquid chromatography tandem mass spectrometry method which is accepted as the reference method of choice for 25(OH)D determination. The main drawbacks to radioimmunoassay are the expense and hazards of preparing and handling the radioactive antigen, and the requirement of specially trained personnel and labs. The chromatography techniques also require expensive equipment and a great deal of technical experience. The above drawbacks increase in third world countries.

Hence, there is great need for a fast, low-cost, safe, simple and accurate method for measuring vitamin D or at least one that will indicate vitamin D status as normal, low level or severely deficient.

In recent decades, fourier transform infrared (FTIR) spectroscopy with or without attenuated total reflection (ATR) accessory, is a field that has undergone significant development for its utilization as a simple, reproducible, accurate diagnosis tool [4] in which nondestructive and minimum sample preparation is required. In addition, these techniques provide molecular-level information allowing investigation of functional groups, bonding types, and molecular conformations. Spectral bands in vibrational spectra are specific and provide direct information about the biochemical composition [5].

Fourier transform infrared has also been applied in a variety of medical fields, e.g. hematology [6], and diseases of various organs, e.g. laryngeal

cancer [7], colorectal cancer [8], lung cancer [9], liver cancer [10], and many others. Furthermore, FTIR spectral analysis has been used to characterize proteins [11], nucleic acids [12] and a variety of diseases by locating their spectral fingerprint [13] in the infrared spectrum. FTIR technique has also been applied in the detection of low level quantities (in the range of 50 ng/ml), of the hormone progesterone at levels of 17 to 76 ng/ml by recognizing the functional groups of ketone, methyl and methylketone at 1724, 1375 and 1354 cm^{-1} wavenumbers respectively [14]. Furthermore, spatially resolved FTIR spectroscopy of single oral mucosa cells has also been investigated [15]. FTIR technique has been used to measure low level concentrations of materials in air, like ethene which was obtained with a limit of detection of 1.1 ppb (in the range of 1 ng/ml) in air samples in the Austrian Alps [16].

Vitamin D has characteristic absorption spectra in the mid-IR range 3–30 μm, due to the excitation of fundamental rotational and vibrational transitions in this spectral range [17]. Therefore, simple and reliable optical sensing methods, such as mid-IR evanescent wave spectroscopy, can be used for their detection. This method is of particular interest due to its intrinsic molecular specificity, robustness and the possibility to deal with liquids such as blood which have a very high absorption in the mid-IR. ATR spectroscopy has long been used as a method for chemical and biological analysis [18]. The method is based on the principle of optical absorption of an evanescent wave outside a waveguide. When light is totally internally reflected in a waveguide there is an evanescent wave that decays exponentially outside the waveguide over a distance of a few wavelengths. If a liquid like plasma blood is placed on the waveguide, the evanescent wave may be partially or totally absorbed and the transmission through the waveguide is reduced. This will occur at specific wavelengths that correspond to the absorption peaks of the sample. Therefore, by measuring the transmission of the waveguide, at different wavelengths, one is actually measuring the absorption spectrum of the sample.

The main purpose of this study was to employ FTIR-ATR spectroscopy to determine vitamin D values several times before and during 16 days after a loading of 200,000 IU vitamin D_3 was taken. This intra-subject study design was chosen since it eliminates unique physiological features specific to each subject and enhances the vitamin D measurement reliability.

Materials and Methods

In this study, 11 healthy subjects (mean and SD age (36±13) years old) with vitamin D deficiency (mean and SD (17.5±3.4) ng/ml) participated. Each subject read and signed an informed consent form pretrial, approved by the hospital Helsinki committee. For each patient a venous blood sample was taken five times (Helsinki no. 009-13-HYMC and NIH no. NIC01770262). Plasma was obtained by centrifugation of whole blood and stored at –20 °C before further analysis. The first blood sample was taken before the patient swallowed a dose of 200,000 IU of vitamin D_3. Then blood samples were taken at 4 hours, 24 hours, 48 hours and 16 days after the loading. Each plasma blood sample 10 ml was divided equally into two samples. 5 ml of the sample was transferred to the biochemical laboratory in order to measure the vitamin D level by the LIAISON 25 OH vitamin D total Assay (DiaSorin Inc., USA). The second 5 ml sample was transferred to the electro-optical laboratory for Mid-IR examination. For analysis, the frozen plasma samples were thawed at room temperature for 60 min. Each specimen was transferred to the IR-spectrometer and a spectrum was obtained.

An Oriel MIR 8025 FTIR spectrometer with PIKE HATR attachment containing a Zinc Selenide (ZnSe) crystal was used to acquire IR-spectra (8 cm^{-1} spectral resolution within the 5000–1000 cm^{-1} region, co-added for 100 scans). Prior to use and between each specimen, the ATR crystal was washed with ethanol and dried with tissue paper. In addition, background and saline absorption spectra were also taken prior to the examination of each new specimen. Spectra were analyzed for baseline correction, smoothing and converted into absorbance by eFTIR software (Essential FTIR™).

Raw spectra were processed employing principal components analysis (PCA) principal coordinates analysis (PCoA) and linear discriminant analysis (LDA) using the PAST software package (University of Oslo, Norway). Grouping of spectra into clusters and the extent to which these clusters correspond to classes of the sample was calculated. PCA and PCoA are based on the assumption that variation implies information: it replaces the original wavenumbers between 1000 to 5000 cm^{-1} with just a very few significant or principal components. Each plasma blood spectrum obtained, is replaced by a "score"; thus in a scores plot, each set of measurements (spectrum) appears as a single point in n-dimensional space. PCA and PCoA allow one to distinguish between vitamin D levels.

Principal components analysis was also used for data reduction and processing of the output using LDA. In LDA, new variables are found such that the ratio of the between-cluster variance to the within-cluster variance is maximized, so that the clusters display maximum separation. LDA also allows the choice of predetermined classes to be taken into account during the derivation of clusters.

Results and Discussion

Vitamin D levels

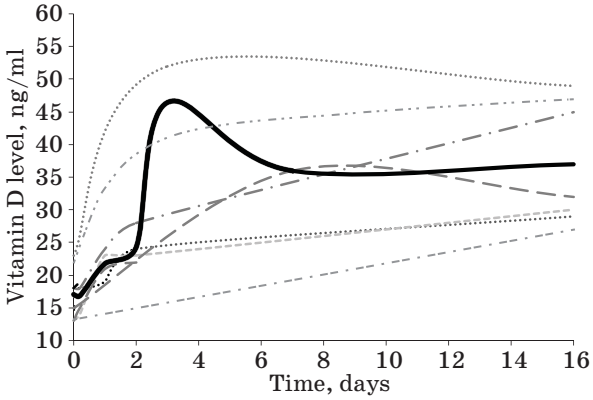
Total vitamin D during the 16 days after the loading, was determined by the LIAISON 25, using chemiluminescent immunoassay technology. During the first days following the loading, total vitamin D levels were rising (Fig. 1); however, from the end of week 1, values were monotonically decreasing, as has been shown in previous studies [19].

Infrared spectrums of blood plasma samples

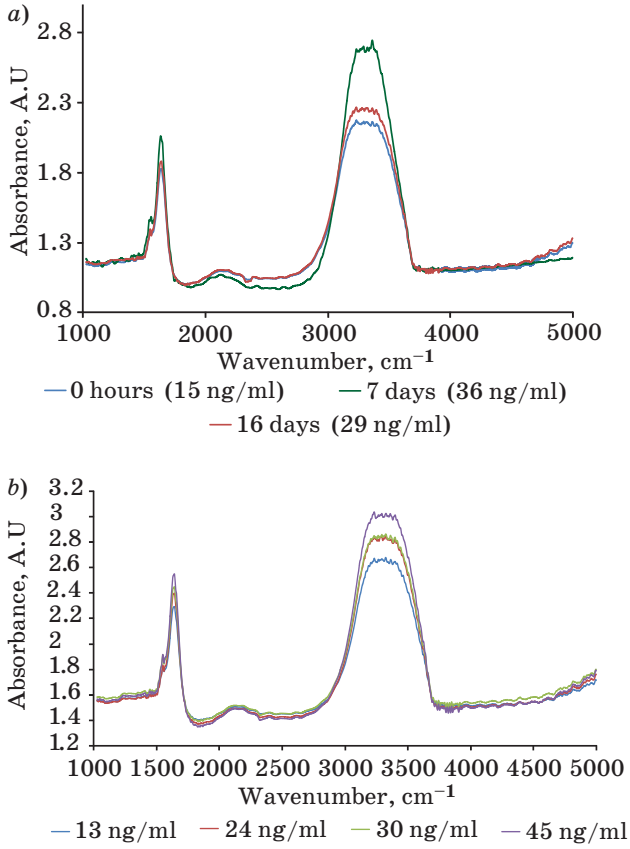
Infrared spectra were taken for all plasma blood samples of the subjects. Fig. 2, *a* shows the spectra of a representative subject (male, age 50 years old, vitamin D levels at the time of recruitment 15 ng/ml) of a set of blood plasma samples after saline subtraction. This set is for spectra taken before the vitamin D_3 loading, 7 days and 16 days post loading.

The vitamin D levels as measured by the LIAISON 25 system were 15 ng/ml at 0 hours, 32 ng/ml at 7 days and 36 ng/ml after 16 days, respectively. It can been seen that spectral differences between plasma samples concern mainly the wavenumber range of the Amide I and II at 1550 and 1640 cm^{-1} and the (3300 to 3400 cm^{-1}) absorption band which is attributed to the hydroxyl group indicated by the O-H stretch. This strong absorption band of the OH group compared to the other relative strength of the absorbance of the vitamin D spectrum is most likely attributable to an alcohol group in the molecule [20]. The spectral line at 3300 cm^{-1} has similarly been attributed to vitamin D in the literature [21]. The set in Fig. 2, *b* is for spectra taken during the 16 days after loading from a variety of subjects with vitamin D range from 13 to 45 ng/ml.

This demonstrates the same behavior as was shown in Fig. 2, *a*: as vitamin D levels increase, absorption lines in the ranges 1550, 1640 and 3300 cm^{-1} increase respectively.



■ *Fig. 1.* Vitamin D levels vs. time after oral vitamin D_3 loading. The solid line represents the average of all patients, while the dashed and dotted lines represent individual patient samples

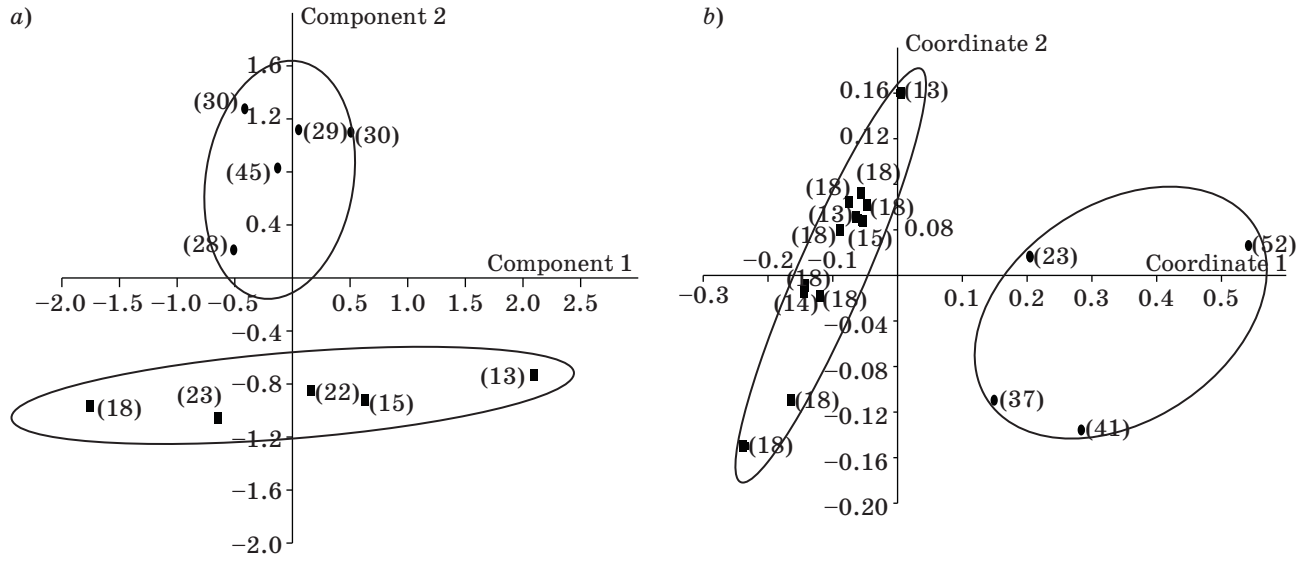


■ *Fig. 2.* Infrared spectrum of blood plasma with various values of vitamin D levels of a single subject (*a*) and for four different subjects (*b*)

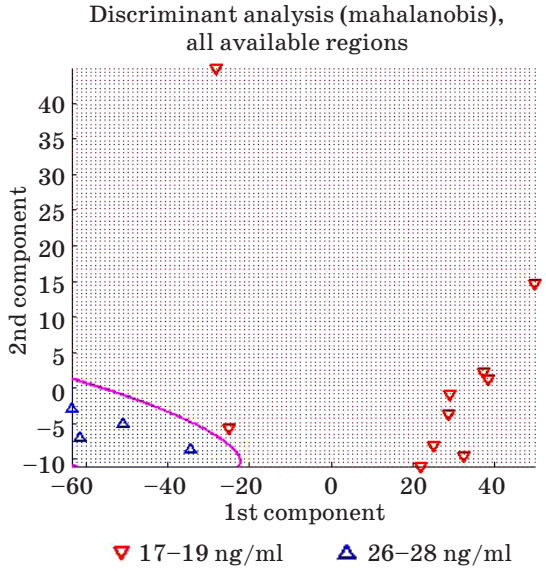
PCA/PCoA-LDA analysis of blood plasma samples

Although visual differences in spectral peak intensities and/or positions were not observed markedly between spectra for different vitamin D levels, small differences were noted throughout the 1600 and the 3300 cm^{-1} range, as seen in Fig. 2. These spectral differences were exploited for tissue classification with the multivariate statistical techniques of PCA/LDA. In Fig 3, *a* and *b* a good separation was observed between two classes of vitamin D levels, as the "low" level (vitamin D deficiency) is in the range of 20 ng/ml and the "high" level (normal vitamin D values) is in the range of 30 ng/ml.

We analyzed spectra in the region of 1000 to 5000 cm^{-1} using LDA for the differences between two vitamin D levels (the low level (vitamin D deficiency) was less than 20 ng/ml and the high level (normal vitamin D levels) was above 26 ng/ml). As shown in Fig. 4, PCA-LDA provides clearer clustering and separation between these two vitamin D levels.



■ *Fig. 3.* PCA (*a*) and PCoA (*b*) scores plot, classed by vitamin D levels shown in parentheses. Class I: levels below 23 ng/ml and Class II: levels between 28 to 45 ng/ml



■ *Fig. 4.* LDA scores plots, classed by "low" — vitamin D deficiency levels and "high" — normal vitamin D levels

Conclusions

This preliminary study shows that measuring the mid-IR spectrum of plasma blood using FTIR/ATR spectroscopy technique creates a simple, direct and rapid quantitative analysis method for vitamin D levels.

Our results show that vitamin D levels can be assigned specifically to spectral bands of the Amide I and II and the O-H stretch at 3400 cm^{-1}; clustering analysis with PCA/PCoA-LDA facilitated the identification of and the discrimination between various vitamin D_3 25OH levels.

Although, our sampling data is small, we can conclude that the Mid-IR spectral method for the determination of vitamin D_3 25OH is a feasible method that has the advantage of being simple, non-expensive, unhazardous, and accurate, so that it can be an alternative to the methods currently in use. Further studies are needed to verify these findings.

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