ANALYSIS OF THE ABSORPTION EFFICIENCY OF DIFFERENT FORMS OF VITAMIN D3

Mārtiņš Borudušķis
University of Latvia, Faculty of Biology

Anna Ramata – Stunda
University of Latvia, Faculty of Biology

Guna Bīlande
University of Latvia, Faculty of Medicine, Jūrmala Hospital, Ltd, Aiwa clinic, Ltd

Dr. med. Aleksejs Derovs
Physician-internist, gastroenterologist Riga Stradiņš University, associate professor
Riga East Clinical University Hospital, Out-Patient Department
Latvian Maritime Medicine Centre

Vitamin D has been shown to play an essential role in the proper functioning of the human body, currently making it the only vitamin with proven necessity of supplementation, and in certain regions physician associations support its regular use in accordance with the recommended usage of the specific formula, while monitoring the blood levels of vitamin D.[1,2] The debate as to the best form of vitamin D supplementation, including forms that are absorbed in the oral cavity, has been ongoing for several years, since various formulae of vitamin D have become commercially available.
This is especially important for patients with malabsorption syndrome, regardless of its genesis. Several studies in various countries have been conducted in order to examine the efficiency of various forms, and some of these studies are summarised in this article.

Despite the malabsorption of vitamin D in patients with gastrointestinal and liver diseases, vitamin levels in the body are often neglected in these patients. Although vitamin D is often prescribed for them, sufficient absorption of this medicine has not been documented.[3]

It is known that vitamin D is fat-soluble, and its relative bioavailability when used in solid form (capsules) may cause adverse conditions, as the process of its secretion is a factor that limits absorption speed, keeping in mind that bioavailability is determined not only by pharmacologically active molecules, but also the formulae and excipients used.

It is estimated that the use of an oral spray results in the rapid and complete absorption of tiny microdroplets of vitamin D3 through the mucous membrane of the cheek into the multiple capillaries and veins located near the surface of the tissue.

Taking the aforementioned into consideration, the objective of the study by MC Satia et al. was to compare the absorption of vitamin D3 in the form of an ingestible soft gelatine capsule (1,000 IUs per capsule) and a buccal spray (500 IUs per dosage shot) in healthy subjects and patients with intestinal malabsorption syndrome. The study of this working group included 40 subjects (including 12 control cases). The study consisted of two phases:

First, patients were randomised into groups, receiving one form of vitamin D3 for 30 days (total daily dose of 1,000 IUs), followed by a 30 day elimination period and in the second phase the subject groups were rotated, i.e., the ones, who had been given capsules were rotated to the buccal spray group and vice versa, continuing vitamin D3 treatment for another 30 days.

As a result of the study, the authors concluded that the buccal spray form was able to significantly improve the average serum concentration of vitamin D as compared to the soft gel capsule both in healthy subjects (1.9 times) and in patients with intestinal malabsorption syndrome (2.6 times). [4]

Despite being commercially available, little is known about the efficiency of peroral vitamin D, which is mostly absorbed in the oral cavity, under the tongue and in the mucous membranes of the palate, rather than the gastrointestinal tract. [5] The latest research has demonstrated that ingestible vitamin D3 can ensure an accelerated path of absorption in comparison to capsules, and can prove effective in people suffering from digestive tract malabsorption.[4] Due to the lipophilic nature of vitamin D, ingestible sprays containing this micronutrient usually contain a TAG carrier, as well as solubilising excipients, such as α-Tocopherol and oleic acid, which promote the passive absorption of the micro-emulgated solution into systemic circulation.[6] This is achieved through the distribution of capillary beds in the peroral submucosa.[7]

Therefore, a group of authors from Ireland performed a randomised study, which included 22 healthy subjects who received 3,000 IUs (75 µg) of vitamin D3 (in the form of capsules or an oral spray). Similarly to the previous study, the authors divided each subject into one of the groups that received a certain form of vitamin D3, followed by a 10 week elimination period, after which the form of vitamin D3 received by each group was changed to the opposite and the subjects continued to receive it for another 4 weeks. The authors concluded that vitamin D3 in the form of an oral spray was an equally efficient alternative to the capsule form, and suitable for healthy adults. [8]

Another study worth mentioning was conducted by colleagues from the USA, Salvia-Trujillo et al., who evaluated the influence of in vitro and in vivo lipid droplet size on the peroral bioavailability of an encapsulated emulsion of vitamin D2. Various sections of the GIT were modelled and analysed: the oral cavity, the stomach, the small intestine. Interestingly, the in vitro study showed that smaller

lipid droplets are digested quicker thus invoking the faster formation of mixed micelles, which are able to dissolve the vitamin. This effect could be explained by the observed increase of the bioavailability of vitamin D2 as the droplet size decreased. On the contrary, the in vivo study demonstrated higher absorption of vitamin D2 for emulsions with the largest droplet size. [9]

The aforementioned results demonstrate that more research is needed, involving a higher number of subjects in order to evaluate several factors that may have an impact on vitamin D3 absorption.

A study CONDUCTED in Latvia

Lastly, it is crucial to also reflect the results of a study conducted in Latvia this year. The objective of the *in vitro* study was to investigate the permeability and safety of various vitamin D3 sprays available on the market in the mucous membranes of the mouth within various time intervals and to demonstrate the buccal-sublingual absorption of the active substance cholecalciferol (vitamin D3). The study was conducted on the basis of the Laboratory of Bioanalytical Methods and Biodosimetry of the Faculty of Biology of the University of Latvia and InCell, Ltd, and with the support of "pharm&med" Ltd.

Tissue cultures and culture media

The study was performed by using EpiOral tissue cultures (MatTek, Cat. No. ORL-212 ver. 3.0, Lot No. 31634 and Cat. No. ORL-200 ver. 3.0, Lot No. 31634 to obtain a total of 36 tissue samples). The tissue cultures were cultured in the growth media recommended by the manufacturer: ORL-200 Assay Media (MatTek, Cat. No. ORL-200-ASY, Lot No. 112919TVKD) by using 6-well sterile tissue culture plates (MatTek, Cat. No. MW-15-003-0027). All operations related to the tissue culture and assayable sample application were performed in a laminar flow cabinet. Tissues were cultured in a Binder incubator at 37 °C, 5% CO_2.

Assayable samples and controls

The following samples were used in the study (see table below)

Additionally, fluorescein diacetate – FDA (Sigma, 7378) was used as a control compound, and the negative control was tissue for which the specific sample volume was replaced with a phosphate buffer – PBS (pH 7.4). All samples were taken in equal doses.

Application of assayable samples on EpiOral tissue

Upon receipt, in accordance with the manufacturer's recommendations, the tissue was separated from the agar and placed into a pre-prepared medium (1 ml volume) which was transferred to a 6-well plate. The tissue was incubated overnight (12-16 h) at 37 °C, 5% CO_2. After a night of incubation a fresh 1 ml medium was replaced. The apical surface of the tissue was rinsed twice with 400 mcl of PBS (MatTek, TC-PBS, phosphate-buffered saline, pH 7.4).

After these manipulations, the tissue samples are ready for testing. Prior to applying the samples on the tissue, each product type was sprayed into 1.5 ml test-tubes. A microdropper was used to transfer 40 µL of each sample in order to cover the entire apical surface of the tissue. Uncoated tissue was used as the negative control for the study, adding 40 mcl of phosphate buffered saline, the comparative control being tissue treated with fluorescein diacetate (dissolved in dimethyl sulfoxide (DMSO)). After two hours the tissue inserts were photographed using a Canon EFS digital camera with an 18-55 mm lens, and microscopic assessment was also performed using a Leica DMI4000B microscope; images were obtained using the camera integrated into the microscope and program Axio Vision 4.8. After 30 minutes and after two hours, the tissue medium was collected in aliquots of 0.5 ml for further quantitative analysis of the absorption of vitamin D3. The experiment was repeated three times – each assayable sample, as well as the controls were applied on three tissue samples.

Assessment of tissue viability

Tissue viability was assessed in accordance with the protocol recommended by the supplier of the tissue, using an MTT (3-4.5-Dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide) reduction test. The test was performed with an MTT reactant concentrate (MatTek, Cat. No. MTT-100-CON, Lot No. 102219TVKA), solvent (MatTek MTT-100-DIL) and MTT extraction liquid (MatTek, MTT-100-EXT), and 24-well plates (MatTek, MW-15-003-0028). The MTT working solution was prepared in a laminar flow cabinet by transferring the MTT concentrate to the solvent. 300 µL of MTT working solution were transferred to each well of the 24-well plate and the plate was placed at 37 °C. After 6 h of incubation with samples that developed coating and control samples, the tissue material was rinsed three times with 400 µL of PBS (MatTek, TC-PBS) to remove the coating. The tissues rinsed in the laminar flow cabinet were transferred to a pre-prepared 24-well plate with MTT reactant and incubated for 3 hours at 37 °C, 5% CO_2. The solution assumed a violet colour in proportion to the amount of living cells in the tissue. Shortly before the end of the incubation period, the MTT extraction liquid was transferred to another 24-well

1st table | **Tested samples**

Code	Content of assayable samples: key
LYL EFFUSIO® formula placebo	Placebo — water-based without active substance
LYL EFFUSIO® formula	Water-based, cholecalciferol integrated into nanoparticles
LYL COMPOSITUM® formula placebo	Placebo — water-based without active substance
LYL COMPOSITUM® formula	Water-based, cholecalciferol
LYL BASIS® formula placebo	Placebo — oil-based without active substance
LYL BASIS® formula	Oil-based, cholecalciferol
LYL EXCELSIS® formula placebo	Placebo — water-based without active substance
LYL EXCELSIS® formula	Water-based cholecalciferol integrated into liposomes
LYL MICRO® formula placebo	Placebo — water-based without active substance
LYL MICRO® formula	Water-based, cholecalciferol integrated into microemulsion

plate, placing 2 ml of the liquid in each well. The tissue was removed from the MTT solution, dried with filter paper and transferred to the extraction liquid. The tissue was incubated in the extraction liquid overnight — 12 to 16 hours at room temperature, ensuring that the plate was protected from light. After incubation, the tissue was removed from the extraction liquid and disposed of, the extraction liquid was mixed thoroughly and transferred to a 96-well plate with 200 µL per well in 3 technical reiterations (three measurements performed for each individual tissue sample). The optical density was registered at a wavelength of 570 nm using a TECAN 200 Pro microplate reader. Background correction was performed by subtracting absorption at a wavelength of 650 nm.

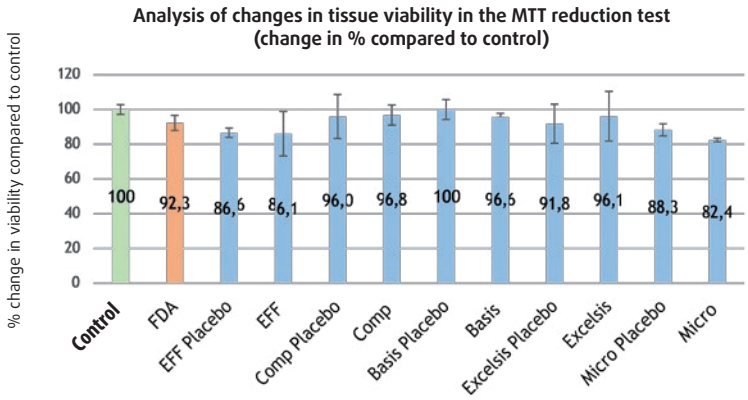
Assessment of the diffusion of cholecalciferol (vitamin D3) with the use of membranes

The diffusion through membranes (0.45mm Cat. No. PR04114, Lot No. R8EA76013 from Millipore) was assessed by using an automatic 6-cell vertical transdermal diffusion system. Manufacturer: Hanson Research Corporation, model: 6 cell "Vision Microette diffusion test system". The receptor fluid used in the vertical diffusion cells was a 70% ethanol/water compound with a total volume of 10 ml, sample transfer volume of 100 ml, membrane surface in contact with sample — 0.64 cm2. The system was set to collect samples of 0.5 ml once every 30 minutes for 3 hours and 16 hours at a permanent temperature regime of 37 °C and mixing at 100 rpm. Samples were automatically collected into chromatography vials for immediate quantification of cholecalciferol (vitamin D3).

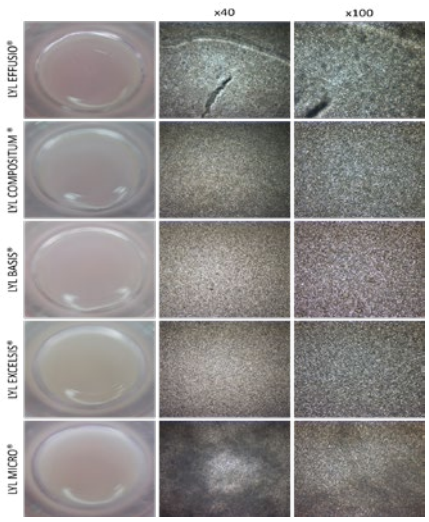
Results of the chromatographic analysis

Cholecalciferol (vitamin D3) was quantified by using an ultra-high performance liquid chromatography (UHPLC) device Agilent 1290 Infinity series with a diode-array detector (DAD) connected to a high-resolution mass spectrometer Agilent 6230 TOF LC/MS. The chromatographic analysis was performed by using a Kinetex C18, 3.00×100 mm, 2.6 µm (Phenomenex) column. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). Flow rate was 0.3 mL min-1 and the following gradient program was used: 0 min, 50% B; 3.0 min, 50% B; 5.0 min, 98% B; 10 min, 98% B; 11 min, 50% B; 15 min, 50% B. Column thermostat temperature was 40 °C and sample injection volume – 5 µL. The analyte was detected with DAD at 265 nm and the mass spectrometer. Mass spectrometry data: electrospray in positive ion mode (ESI+), drying gas temperature 325 °C and flow rate 12 mL/min, fragmentation voltage 130 V, spray pressure 40 psi, scanning range m/z 100 – 1000. Internal mass calibration solution – 121,050873 m/z and 922,009798 m/z (G1969-85001 ES-TOF Reference Mass Solution Kit, Agilent Technologies) was used during all tests. The obtained chromatograms were processed using MassHunter Qualitative Analyses B.07.00 data processing software.

1st picture — **Changes in EpiOral tissue viability 2 hours after the application of test samples, changes are reported as relative change (%) in relation to the negative control. Negative control – uncoated tissue, FDA – fluorescein diacetate**



2nd picture — **Image. EpiOral microscopic analysis 2 h after the application of assayable samples, at 40x and 100x magnification**



Sample preparation and analysis

In order to perform quantification, six standard solutions of vitamin D3 were prepared with the following concentrations: 0.1; 0.5; 1.0; 5.0; 10.0; 25.0 µg mL-1. Initially, a primary standard solution with a concentration of 1000 µg mL-1 was prepared using an analytically pure cholecalciferol reference substance (Sigma-Aldrich) and 96% ethanol (Sigma-Aldrich).

Results

Coverage effect on tissue viability

The results show that differences between samples are minor and indicate that the assayable samples have no significant impact on tissue viability. The results obtained are illustrated in the figure below. None of the samples exhibit a cytotoxic effect, overall changes in viability of the assayed samples are minor, allowing one to make the assumption that the assayable material is not cytotoxic. Minor fluctuations in the results can be caused by the density of the specific composition and slightly different mechanical properties, which may have slightly affected gas exchange in the tissue and the viability thereof.

Microscopic and macroscopic assessment of the EpiOral tissue cultures

After incubation with forming samples containing vitamin D3, the tissue cultures

2nd table | **The concentration or absolute volume of cholecalciferol (vitamin D3) in control samples is given in the image below**

Control samples	UV, area ms	D3 γ, µg/mL
LYL EFFUSIO®	207.59	4.34
LYL COMPOSITIUM®	97.56	2.04
LYL BASIS®	162.85	3.41
LYL EXCELSIS®	2.94	0.06
LYL MICRO®	94.22	1.97

were analysed with a microscope and the camera integrated into the microscope was used to document the structure of the tissue samples. In addition, the apical surface of the tissue was also digitally photographed to assess and document macroscopically identifiable changes in tissue structure. The microscope images, as well as the macroscopic assessment of the apical surface of the tissue demonstrate that the assayable samples have no effect on tissue structure. The microscopic analysis showed cobble-like cell morphology as is typical of EpiOral tissue. These observations confirm the data already obtained from the tissue viability test, that the assayed coating forming component samples do not have a negative effect on tissue viability, furthermore indicating that the samples also have no effect on tissue structure and integrity.

Quantitative assessment of cholecalciferol (vitamin D3) diffusion through membranes

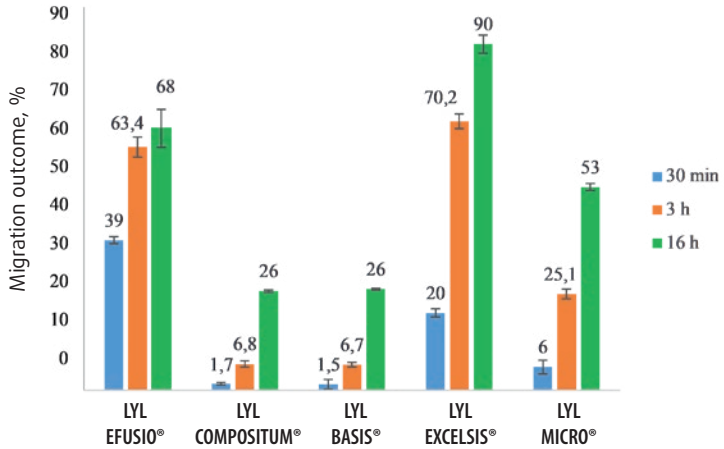
An automated vertical diffusion cell system was selected for the relative comparison of cholecalciferol (vitamin D3) diffusion efficiency among various samples by using cellulose membranes as a barrier as described in the method. Timing points selected: at 30 minutes and slightly extended – at 2 hours, as well as at 16 hours. The result of migration was calculated by determining the concentration of vitamin D3 in control samples and assayable samples. The concentration determined in the control samples was assumed to be 100% – maximum concentration, which can diffuse through membranes. Statistical processing of the results among the technical reiterations was performed to calculate the standard deviation. According to the results, the best migration of cholecalciferol was observed in the samples with LYL EFFUSIO® and LYL MICRO®, although the highest numerical results specifically in regard to the migration percentage ratio were seen in the sample with LYL EXCELSIS®, however, this indicator is not to be considered objective, since the absolute volume of cholecalciferol that had passed through the membrane was miniscule, which creates a higher percentage result at lower detection in assayable samples. Although the concentration of cholecalciferol in the assayable samples differs significantly, the absolute volume of cholecalciferol that could have migrated through the membrane, and the comparison of the LYL EFFUSIO® with the LYL EXCELSIS® sample demonstrates that the difference observed is 72 times in favour of the former, as well as more than 32 times in favour of LYL MICRO®, which results in incomparable values.

This study led to the following conclusions

- The assayed compositions containing vitamin D3 do not have a significant negative effect on the viability of epithelial tissue in the mucous membranes of the mouth, which demonstrates the safety of the formulations.
- In order to characterise the migration efficiency through cellulose membranes of vitamin D3 encapsulated in liposomes (LYL EXCELSIS® formula), the testing system should be optimised, including the use of solutions modelling the human saliva environment.
- The best results of vitamin D3 migration through cellulose membranes at all tested points in time were observed for LYL EFFUSIO® and LYL MICRO® formulae.

Taking into consideration the fact that practically all in vivo studies are performed abroad on different populations, the absorption results obtained in an in vivo study in Latvia assessing various forms of oral absorption of vitamin D3 will be published in the next issue. This study illustrates the efficiency of forms of vitamin D3 specifically in the population of Latvia and reveals which form of vitamin D3 can effectively increase vitamin D blood levels within only 30 days.

Fig. 3. | **Percentages of cholecalciferol (vitamin D3) migration in the samples**



References

1. Holick M & Chen T (2008) Vitamin D deficiency: a worldwide problem with health consequences. Am J Clin Nutr 87 1080S-1086S.
2. Mithal A, Wahl DA, Bonjour J, et al. (2009) Global vitamin D status and determinants of hypovitaminosis D. Osteoporos Int 20, 1807-1820.
3. Lo CW, Paris PW, Clemens TL, Nolan J, Holick MF. Vitamin D absorption in healthy subjects and in patients with intestinal malabsorption syndromes. Am J Clin Nutr. 1985 Oct; 42(4):644-9.
4. MC Satia, AG Mukim, KD Tibrewala, MS Bhavsar. A randomized two way cross over study for comparison of absorption of vitamin D3 buccal spray and soft gelatin capsule formulation in healthy subjects and in patients with intestinal malabsorption. Nutr J. 2015; 14: 114.
5. Narang N & Sharma J (2011) Sublingual mucosa as a route for systemic drug delivery. Int J Pharm Pharm Sci 3, 18–22.
6. Strickley RG (2004) Solubilizing excipients in oral and injectable formulations. Pharm Res 21, 201–230.
7. Kalepu S, Manthina M & Padavala V (2013) Oral lipid-based drug delivery systems – an overview. Acta Pharm Sin B 3, 361–372.
8. Joshua J. Todd, Emeir M. McSorley, L. Kirsty Pourshahidi et al. Vitamin D3 supplementation in healthy adults: a comparison between capsule and oral spray solution as a method of delivery in a wintertime, randomised, open-label, cross-over study. British Journal of Nutrition (2016), 116, 1402–1408.
9. L. Salvia-Trujillo, B. Fumiaki, Y. Park and D. J. McClements. The influence of lipid droplet size on the oral bioavailability of vitamin D2 encapsulated in emulsions: an in vitro and in vivo study: 12:39. Food Funct. 2017 Feb 22;8(2):767-777.