COLLEGE TER BEOORDELING VAN GENEESMIDDELEN

# Public Assessment Report

# Scientific discussion

# Calvigor 25.000 IU and 50.000 IU, soft capsules and orodispersible films (cholecalciferol)

# (NL/H/5172/001-004/DC)

# Date: 18 May 2022

**This module reflects the scientific discussion for the approval of Calvigor. The procedure was finalised at 4 January 2022. For information on changes after this date please refer to the 'steps taken after finalisation' at the end of this PAR.**

# List of abbreviations

| | |
|---|---|
| $1,25(OH)_2D$ | 1,25-dihydroxyvitamin D |
| $1,25(OH)_2D_3$ | Vitamin $D_3$ |
| 1α-OHase | 25-hydroxyvitamin D-1α-hydroxylase |
| 24-OHase | 25-hydroxyvitamin D-24-hydroxylase |
| ASMF | Active Substance Master File |
| BMD | Bone mineral density |
| BW | Bodyweight |
| cAMP | Cyclic adenosine monophosphate |
| Ca/P | Calcium/phosphorus |
| CEP | Certificate of Suitability to the monographs of the European Pharmacopoeia |
| CHMP | Committee for Medicinal Products for Human Use |
| $C_{max}$ | Peak plasma concentration |
| CMD(h) | Coordination group for Mutual recognition and Decentralised procedure for human medicinal products |
| CMS | Concerned Member State |
| DBP | Vitamin D binding protein |
| EDMF | European Drug Master File |
| EDQM | European Directorate for the Quality of Medicines |
| EEA | European Economic Area |
| ERA | Environmental Risk Assessment |
| GH | Growth hormone |
| GI | Gastrointestinal |
| HDL | High-density lipoprotein |
| ICH | International Conference of Harmonisation |
| IM | Intramuscular |
| MAH | Marketing Authorisation Holder |
| MED | Minimal erythema dose |
| Ph.Eur. | European Pharmacopoeia |
| PK | Pharmacokinetic |
| PL | Package Leaflet |
| PNMT | Phenylethanolamine-N-methyl transferase |
| PTH | Parathyroid hormone |
| RH | Relative Humidity |
| RMP | Risk Management Plan |
| SmPC | Summary of Product Characteristics |
| RANKL | Receptor activator of NFκB ligand |
| TSE | Transmissible Spongiform Encephalopathy |
| VDD | Vitamin D deficient |
| VDR | Vitamin D receptor |
| VDRE | Vitamin D response elements |
| VDR/RXR | Vitamin D receptor/retinoic X receptor |

# I. INTRODUCTION

Based on the review of the quality, safety and efficacy data, the Member States have granted a marketing authorisation for Calvigor 25.000 IU and 50.000 IU, soft capsules and orodispersible films, from IBSA Farmaceutici Italia S.r.L.

The product is indicated for the initial treatment of clinically relevant vitamin D deficiency in adults.

A comprehensive description of the indications and posology is given in the SmPC.

This decentralised procedure concerns a well-established use application of the medicinal product Calvigor. The active substance of Calvigor, Vitamin D3 (cholecalciferol) is in "well-established" medicinal use within the EU for more than 10 years, with recognized efficacy and an acceptable level of safety. The composition of the proposed medicinal product is similar to the composition of other products that have been widely used world-wide for the same indications, and it is expected to have the same pharmacokinetic/pharmacodynamic characteristics with Vitamin D3 products described in the submitted literature and no significant differences in efficacy or safety.

The concerned member state (CMS) involved in this procedure was Italy.

The marketing authorisation has been granted pursuant to Article 10a of Directive 2001/83/EC, a so called bibliographic application based on the well-established medicinal use of Vitamin D3 (cholecalciferol). This type of application does not require submission of the results of pre-clinical tests or clinical trials if it can be demonstrated that the active substance of the medicinal product has been in well-established medicinal use within the community for at least ten years, with recognised efficacy and an acceptable level of safety.

# II. QUALITY ASPECTS

## II.1 Introduction

Calvigor 25.000 IU and 50.000 IU, soft capsules are oval-shaped gelatine capsules, with a yellowish shell containing a yellow oil.

Calvigor 25.000 IU orodispersible film
Rectangular, flexible, opaque light orange film.

Calvigor 50.000 IU orodispersible film
Square, flexible, opaque light orange film.

Calvigor contains as active substance either 25.000 IU or 50.000 IU of cholecalciferol, equivalent to 0.625 mg or 1.25 mg vitamin D3 respectively.

The capsules are packed in blisters and boxes.

The orodispersible films are packed in sachets.

The excipients are:

Calvigor 25.000 IU and 50.000 IU, soft capsules
*Capsule fill* – refined olive oil.

*Capsule shell* – glycerol (422), gelatine, sorbitol – liquid (non crystalising), medium chain triglycerides (MCT oil) and soybean lecithin.

Calvigor 25.000 IU and 50.000 IU, orodispersible films
Refined olive oil, purified water, maltodextrin, hydroxypropylbetadex, copovidone, mannitol (E421), glycerin (E422), polysorbate 80 (E433), glycerol monolinoleate, titanium dixoxide (E171), sucralose (E955), orange flavour, ascorbic acid (E300), DL-alfa tocopherol (E307) and sunset yellow (E110).

The two capsule and orodispersible film strengths are dose proportional.

## II.2 Drug Substance

The active substance is cholecalciferol, an established active substance described in the European Pharmacopoeia (Ph.Eur.). The active substance is a crystalline powder and it is practically insoluble in water, freely soluble in ethanol (96 per cent), and soluble in trimethylpentane and in fatty oils. Polymorphism is not relevant as the active substance is present in solution within the final finished product.

The CEP procedure is used for the active substance. Under the official Certification Procedures of the EDQM of the Council of Europe, manufacturers or suppliers of substances for pharmaceutical use can apply for a certificate of suitability concerning the control of the chemical purity and microbiological quality of their substance according to the corresponding specific monograph, or the evaluation of reduction of Transmissible Spongiform Encephalopathy (TSE) risk, according to the general monograph, or both. This procedure is meant to ensure that the quality of substances is guaranteed and that these substances comply with the Ph.Eur.

Manufacturing process
A CEP has been submitted; therefore no details on the manufacturing process have been included.

Quality control of drug substance

The active substance specification is considered adequate to control the quality and meets the requirements of the monograph in the Ph.Eur. Batch analytical data demonstrating compliance with this specification have been provided for three batches.

Stability of drug substance
Stability data on the active substance have been provided for eight batches stored at 25°C/60% RH (accelerated, six months), and six batches are stored at 5°C (long-term, 60 months) in accordance with applicable European guidelines demonstrating the stability of the active substance for 60 months. None of the tested parameters shows any changes at both storage conditions. Based on the data submitted, a retest period could be granted of 60 months when stored below 8°C (refrigerator).

## II.3 Medicinal Product

**Capsules**
Pharmaceutical development
The product is an established pharmaceutical form and its development is adequately described in accordance with the relevant European guidelines. The choice of excipients is justified and their functions explained.

Manufacturing process
The manufacturing process is described in three stages: shell phase preparation, fill phase preparation and encapsulation. The manufacturing process is considered a non-standard manufacturing process considering the low content unit dose preparations and has been validated according to relevant European/ICH guidelines. Process validation data on the product have been presented for three full scaled batches in accordance with the relevant European guidelines.

Control of excipients
The excipients comply with Ph.Eur. requirements except for lecithin which complies with the USP requirements. These specifications are acceptable.

Quality control of drug product
The finished product specifications are adequate to control the relevant parameters for the dosage form. The specification includes tests for appearance, average fill weight, average capsule weight, uniformity of dosage units, disintegration, peroxide value, acid value, cholecalciferol identification, cholecalciferol assay, impurities and microbial control. Limits in the specification have been justified and are considered appropriate for adequate quality control of the product. Satisfactory validation data for the analytical methods have been provided. Batch analytical data from three full scaled batches per strength from the proposed production sites have been provided, demonstrating compliance with the specification. An adequate risk evaluation on presence of nitrosamine impurities is provided.

Stability of drug product

Stability data on the product have been provided for one pilot scaled batch (25,000 IU), two pilot scaled batches (50,000 IU) and three production scaled batches per strength. Pilot batches have been stored at 30°C/65% RH (six months) and 40°C/75% RH (six months). Production scaled batches have been stored at 25°C/60% RH (12 months) and 30°C 65% RH (12 months) in accordance with applicable European guidelines demonstrating the stability of the product for 24 months (extrapolated based on the available 12 months long-term data). Photostability studies were performed in accordance with ICH recommendations and showed that the product is not stable when exposed to light directly and not stable when exposed to light when stored in the blister. None of the tested parameters show any trends in the results. On basis of the data submitted, a shelf life was granted of 24 months when stored at temperature below 30°C in the original package in order to protect from light.

Specific measures concerning the prevention of the transmission of animal spongiform encephalopathies

Scientific data and/or certificates of suitability issued by the EDQM for gelatine has been provided and compliance with the Note for Guidance on Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via medicinal products has been satisfactorily demonstrated.

**Orodispersible films**

Pharmaceutical development

The product is an established pharmaceutical form and its development is adequately described in accordance with the relevant European guidelines. The choice of excipients is justified and their functions explained. Selection of a flavour was tested. Optimization of the film flexibility by introducing co-povidone. Improvement of dissolution and introduction of surfactant and antioxidants were investigated. Further optimization of the formulation by adjusting the ratio cholecalciferol and cyclodextrin.

Manufacturing process

The manufacturing process is described in four main steps: mixing of active substance and excipients, drying and formation, cutting, final film formation and packaging. The manufacturing process has been validated according to relevant European/ICH guidelines. Considering that cholecalciferol (Vit D3) orodispersible films are unit dose preparations containing the active substance in low content, makes this a specialized dosage form. Process validation data on the product have been presented for three full scaled batches in accordance with the relevant European guidelines.

Control of excipients

The excipients, except orange flavour and colourant E110 sunset yellow comply with Ph.Eur. requirements. In-house specifications for orange flavour and colourant sunset yellow are provided. The specifications for the excipients are in general adequate,

Quality control of drug product

The finished product specifications are adequate to control the relevant parameters for the dosage form. The specification includes tests for appearance, size (surface area), average

weight, identification of cholecalciferol, uniformity of dosage units, cholecalciferol assay, impurities, vitamin C and E identification and assay, loss on drying, dissolution test, sealing of the sachet and microbial control. Limits in the specification have been justified and are considered appropriate for adequate quality control of the product. Satisfactory validation data for the analytical methods have been provided. Batch analytical data from three full scaled batches per strength from the proposed production sites have been provided, demonstrating compliance with the specification. An adequate risk evaluation on presence of nitrosamine impurities is provided.

Stability of drug product

Stability data on the product have been provided for three production scaled batches per strength stored at 25°C/60% RH (12-18 months) and 30°C/65% RH (6-12 months) and 40°C/75% RH (six months). Production scaled batches have been stored at 25°C/60% RH (12 months), 30°C/65% RH (12 months) and 40°C 75% RH (six months) in accordance with applicable European guidelines demonstrating the stability of the product for 24 months (extrapolated based on the available 12 months long-term data). Photostability studies were performed in accordance with ICH recommendations and showed that the product is not stable when exposed to light. On basis of the data submitted, a shelf life was granted of 24 months when stored at temperature below 30°C in the original package in order to protect from light.

Specific measures concerning the prevention of the transmission of animal spongiform encephalopathies

Scientific data and/or certificates of suitability issued by the EDQM for gelatine has been provided and compliance with the Note for Guidance on Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via medicinal products has been satisfactorily demonstrated.

### II.4 Discussion on chemical, pharmaceutical and biological aspects

Based on the submitted dossier, the member states consider that Calvigor has a proven chemical-pharmaceutical quality. Sufficient controls have been laid down for the active substance and finished product.

No post-approval commitments were made.

# III. NON-CLINICAL ASPECTS

## III.1 Pharmacology

### III.1.1 Primary pharmacodynamics

Photobiogenesis of vitamin D

Vitamin $D_3$ is a derivative of 7-dehydrocholesterol (provitamin $D_3$), the immediate precursor of cholesterol. Exposure of human skin to solar UVB radiation (wavelengths: 290–315 nm) leads to the conversion of 7-dehydrocholesterol to previtamin $D_3$ in the skin (*Wacker et al. 2013*). Previtamin $D_3$ is then rapidly converted to vitamin $D_3$ (cholecalciferol) by temperature- and membrane-dependent processes. Previtamin $D_3$ is biologically inactive, but it undergoes a spontaneous temperature-dependent molecular rearrangement of its conjugated triene system (three double bonds) to form the thermally stable 9,10-secosterol, vitamin $D_3$ or cholecalciferol. At body temperature it takes approximately 24 hours for previtamin $D_3$ to be converted completely into vitamin $D_3$. Wide changes in skin surface temperature do not affect the rate of this conversion because the process occurs in the actively growing layers of the epidermis, where the temperature is relatively stable. Also the changes in the core body temperature have little effect on this reaction. Once vitamin $D_3$ is synthesized, it is translocated from the epidermis into the circulation by the vitamin D binding protein (DBP). Thus, vitamin $D_3$ is made in the skin from the provitamin for many hours after a single sun exposure. 25(OH)D is a summation of both vitamin D intake and vitamin D that is produced from sun exposure (*Holick 2009*). However, excessive exposure to sunlight degrades previtamin $D_3$ and vitamin $D_3$ into inactive photoproducts (*Wacker et al. 2013*). The amount of vitamin D production in the skin depends on the incident angle of the sun and thus on latitude, season and time of the day. It is highest when the sun is in the zenith and a flattening of the incident angle leads to a reduced vitamin D production (*Wacker et al. 2013*). Whole body exposure to sunlight with one minimal erythema dose (MED), i.e., the minimal dose leading to pink coloration of the skin 24 hours after exposure, leads to vitamin D levels comparable to oral intake of 10.000 I.U. to up to 25.000 I.U. vitamin $D_2$. However, sun exposure during most of the winter at latitudes above and below ~33 degrees North and South, respectively, does not lead to any production of vitamin $D_3$ in the skin. Other factors influencing the cutaneous vitamin D production adversely are an increase in skin pigmentation, aging, especially age > 65 years and the topical application of a sunscreen.

The nuclear vitamin D receptor

The biological actions of vitamin $D_3$ ($1,25(OH)_2D_3$) are mediated by the vitamin D receptor (VDR) (*Christakos et al. 2016*). VDR belongs to the steroid receptor family which includes receptors for retinoic acid, thyroid hormone, sex hormones, and adrenal steroids. The general and severe end organ resistance to vitamin $D_3$ observed in patients with mutations in the gene encoding VDR suggests that most of the cellular actions of $1,25(OH)_2D_3$ are mediated through interactions of the hormone with its nuclear receptor (*Whitfield et al. 2003*).

The VDR is composed of two principal domains. The $1,25(OH)_2D_3$-receptor complex interacts with the retinoic acid X receptor to form a heterodimeric complex that binds to specific DNA sequences, termed the vitamin D response elements (VDREs) (*Nguyen et al. 2004*). This interaction alters the transcription of genes: as an example, the calcium-binding protein is synthesized in the small intestine, thus increasing the GI absorption of calcium, while in bone osteocalcin, osteopontin and alkaline phosphatase are produced.

While most of the classical effects of $1,25(OH)_2D_3$ involve this genomic pathway, several other effects may be observed very rapidly, within seconds to minutes. These effects are not blocked

by inhibitors of transcription or translation, suggesting a direct action of the vitamin $D_3$ at the membrane level. $1,25(OH)_2D_3$ may also have some extranuclear effects in the target tissues, such as increased transport of calcium from extracellular to intracellular space, mobilization of intracellular calcium pools and enhancement of phosphatidylinositol metabolism. Attempts have been made to identify the biochemical mechanisms underlying the non-classical rapid effects of $1,25(OH)_2D_3$, but the data are still controversial. A first set of data supports the hypothesis that VDR itself mediates also the $1,25(OH)_2D_3$ rapid effects. Among those findings are the observations that functional VDR expression and rapid response to $1,25(OH)_2D_3$ in enterocytes have a simultaneous onset during rat post-natal development, and that the generation of transgenic mice lacking the first zinc finger of the VDR blocks rapid and long-term responses of osteoblasts to $1,25(OH)_2D_3$. However, other data suggest that the presence of a functional nuclear VDR is not compulsory. For instance, persistent rapid effects of $1,25(OH)_2D_3$ on protein kinase C activation and calcium increase in osteoblasts of transgenic mice expressing a non-functional VDR, have been observed (*Nguyen et al. 2004*).

The rapid and long-term effects of $1,25(OH)_2D_3$ in cultured skin fibroblasts from three patients with severe vitamin D-resistant rickets and one age-matched control, have been compared (*Nguyen et al. 2004*). Patients bear homozygous missense VDR mutations that abolished either VDR binding to DNA (patient 1, mutation K45E) or its stable ligand binding (patients 2 and 3, mutation W286R). In patient 1 cells, $1,25(OH)_2D_3$ (1 pm-10 nm) had no effect on either intracellular calcium or 24-hydroxylase (enzyme activity and mRNA expression). In contrast, cells bearing the W286R mutation had calcium responses to $1,25(OH)_2D_3$ and 24-hydroxylase responses to low (1 ppm - 100 ppm) $1,25(OH)_2D_3$ concentrations (activity, CYP24, and ferredoxin mRNAs) similar to those of controls. The blocker of calcium channels, verapamil, impeded both rapid (calcium) and long term (24-hydroxylase activity, CYP24, and ferredoxin mRNAs) responses in patient and control fibroblasts. In conclusion, the comparison of rapid and long-term responses to $1,25(OH)_2D_3$ in three fibroblast models expressing normal, W286R-mutated, or K45E-mutated VDR provides evidence that also the rapid effects of $1,25(OH)_2D_3$ on intracellular calcium require the presence of classical VDR.

Metabolites of vitamin D

Once vitamin $D_3$ has entered the circulation, either coming from gastrointestinal (GI) absorption or by synthesis in the skin, it is transported to the liver by means of a specific $\alpha_1$ globulin (DBP). Vitamin D in the circulation is bound to the vitamin D binding protein which transports it to the liver where vitamin D is converted by the vitamin D-25-hydroxylase to $25(OH)D_3$. $25(OH)D_3$ is one of the major circulating metabolites with a half-life of approximately 21 days. The hepatic 25-hydroxylation of vitamin $D_3$ is regulated by a product feedback mechanism: the regulation, however, is not tight and, therefore, an increase in the dietary intake or endogenous synthesis of vitamin $D_3$ is reflected by increased plasma concentrations of $25(OH)D_3$. $25(OH)D_3$ is not biologically active *in-vivo*, although it may be active *in-vitro* at high concentrations. It is biologically inactive and must be converted in the kidneys by the 25-hydroxyvitamin D-1α-hydroxylase (1α-OHase) to its biologically active form 1,25-dihydroxyvitamin D [$1,25(OH)_2D$]. Thus, 1α-OHase catalyses this rate-limiting hormonally regulated step in the bioactivation of vitamin D. The expression of this enzyme in human kidneys has been investigated (*Takeyama et al. 1997*). The mRNA and protein for 1α-OHase

are expressed mostly in the proximal tubules, but they are also found in distal tubules and in collecting ducts. The other key sites of 1α-OHase expression along the nephron were the medullary collecting ducts and the papillary endothelium. Western blots and enzyme-activity studies using primary cell culture confirmed the presence of 1α-OHase in human cortical and medullary renal tissue.

Serum phosphorus, calcium, fibroblast growth factors (FGF-23) and other factors can either increase or decrease the renal production of $1,25(OH)_2D$. 1,25(OH)2D feedback regulates its own synthesis and decreases the synthesis and secretion of parathyroid hormone (PTH) in the parathyroid glands. Then, the renal production of $1,25(OH)_2D$ enhances the effects of PTH in increasing the plasma calcium concentration, and lowering the plasma concentrations (and presumably also the renal intracellular concentrations) of phosphate. Moreover, $1,25(OH)_2D$ increases the expression of the 25-hydroxyvitamin D-24-hydroxylase (24-OHase) to catabolise $1,25(OH)_2D$ to the water soluble biologically inactive calcitroic acid which is excreted in the bile (*Wacker et al. 2013*). $1,25(OH)_2D$ enhances intestinal calcium absorption in the small intestine by stimulating the expression of the epithelial calcium channel and the calbindin 9K (calcium binding protein). $1,25(OH)_2D$ is recognized by its receptor in osteoblasts causing an increase in the expression of receptor activator of NFκB ligand (RANKL). Its receptor RANK on the preosteoclast binds RANKL which induces the preosteoclast to become a mature osteoclast. The mature osteoclast removes calcium and phosphorus from the bone to maintain blood calcium and phosphorus levels. Hence, adequate calcium and phosphorus levels promote the mineralization of the skeleton (*Wacker et al. 2013*).

In addition to the renal conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$, there is also a peripheral metabolism of vitamin $D_3$, which is still – at least partly – unexplored. An extra-renal production of $1,25(OH)_2D_3$ has been in fact demonstrated (*Takeyama et al. 1997*). A 1α-OHase activity has been found in tissues such as normal skin (stratum basalis) and sarcoid lymph nodes. Immunohistochemistry has shown that 1α-OHase is expressed in decidual cells, and also in trophoblasts and syncytiotrophoblasts, thus suggesting a role of 1α-OHase in placentation and feto-placental calcium homeostasis. Novel sites for 1α-OHase expression include the parathyroid, pancreas, adrenal medulla, colon and cerebellum, with negative tissues including the heart, liver and adrenal cortex (*Takeyama et al. 1997*).

Moreover, it has been discovered that some cells, such as chondrocytes, skin keratinocytes and fibroblasts, and intestinal and melanoma cells, not only have the property to hydroxylate exogenous $25(OH)D_3$ at the C-1α position, thus producing $1,25(OH)_2D_3$, but they can also hydroxylate vitamin $D_3$ and one of its metabolites at the C-25 position (*Lehmann et al. 1998*). This relatively new pathway of the epidermal vitamin $D_3$ metabolism could be of great importance for further studies on the regulation of growth, differentiation and apoptosis of keratinocytes, including immunological processes.

Vitamin D and bone metabolism

The effects of vitamin $D_3$, as well as of its deficiency, on the bone metabolism are well known. Vitamin D increases the serum inorganic calcium and phosphate concentrations by increasing the absorption of calcium and phosphorus from intestine, and reducing the loss of calcium in

the urines. The bone mass density (BMD) might be therefore reduced as a consequence of a reduced bioavailability of biologically active vitamin $D_3$ (*Holick 2006*).

In the bone, the $1,25(OH)_2D_3$ elicits physiological responses at both the genomic and nongenomic levels. Osteoblasts, which are secretory cells, produce a variety of bone matrix proteins and actively participate in the mineralization process under the influence of $1,25(OH)_2D_3$. From a biochemical perspective, the effects of vitamin $D_3$ on osteoblasts are very important. In fact, the rate of bone formation and resorption is largely determined by the numbers of bone-forming (osteoblasts) and bone-resorbing (osteoclasts) cells present in the basic multicellular unit responsible for the regeneration of the adult skeleton. Similarly to other regenerating tissues, the number of bone cells is controlled by changes not only in the production of mature cells but also in their survival. Recent evidence indicates that apoptosis (programmed cell death) represents the most common fate of osteoblasts during physiologic bone remodelling, and agents that influence the rate of bone formation and bone mass control osteoblast apoptosis *in-vitro*. It has been reported (*Morales et al. 2004*) that $1,25(OH)_2D_3$ and growth hormone (GH) in combination significantly increased the number of cultured cells UMR 106, a rat clonal cell line with osteoblast-like phenotypic properties. The effect was associated with decreased apoptosis and altered cell cycle distribution. While inhibition of apoptosis was mainly the effect of $1,25(OH)_2D_3$, the increased proliferation was the main effect of GH. These findings are confirmed by the observation that the $1,25(OH)_2D_3$ increases the apoptosis of mouse chondrocytes, where VDR deficiency decreased apoptosis. To investigate the mechanism behind the antiapoptotic effects exerted by $1,25(OH)_2D_3$ and GH, the activities of caspase-3, -8 and –9 were measured in the same series of experiments. The results showed that caspase-3, a down-stream effector caspase, is inhibited in cells treated with $1,25(OH)_2D_3$ and GH. The inhibition of caspase-3 was seen after 48 hours of treatment with $1,25(OH)_2D_3$ and/or GH. In addition, both the receptor-mediated caspase-8 and the mitochondria-dependent caspase-9 were inhibited after 48 hours with the same hormonal treatment. These data support the hypothesis that $1,25(OH)_2D_3$, in combination with GH, increases the amount of osteoblast-like cells. This is mediated by an antiapoptotic effect exerted mainly by $1,25(OH)_2D_3$, as well as a proliferative effect of GH.

Another study (*Zanello et al. 2004*) has investigated the responses of osteoblasts to $1,25(OH)_2D_3$ and the role played by a functional classic VDR in eliciting these responses. The $1,25(OH)_2D_3$ was reported to modulate the osteoblast gene expression of bone matrix proteins and to modify the electrical state of the plasma membrane through rapid nongenomic mechanisms still not fully understood. To elucidate whether the VDR is required for the $1,25(OH)_2D_3$-promoted electrical responses, the $1,25(OH)_2D_3$ modulation of ion channel activities was studies in calvarial osteoblasts isolated from VDR knockout and wild type mice. Authors concluded that, at least in calvarial osteoblasts, the $1,25(OH)_2D_3$ modulates the ion channel activities only in cells with a functional VDR and that this effect is coupled to exocytosis. This is a demonstration of the requirement of a functional classic VDR for the rapid hormonal modulation of electric currents linked to secretory activities in a target cell.

The effects of vitamin $D_3$ on osteoblasts support its use in the treatment of defects of mineralization and in the prevention of osteoporosis. In fact, it has been suggested that the

incidence of bone fractures caused by osteoporosis could be inversely related to the peak BMD achieved during development (*Hirano et al. 2002*). To verify this hypothesis, tests were performed in female Sprague-Dawley rats (*Hirano et al. 2002*) to investigate whether the BMD could be increased during post-natal development, and which drugs would be effective in suppressing the rate of decrease in BMD by continuous administration from childhood. At the age of 3 months, 168 rats were divided into five groups and fed with regular diet added with: A) vitamin $K_2$ (50 mg/kg/day); B) vitamin D (0.2 mg/kg/day three times a week); C) calcium (1.8%); D) vitamin $K_2$ (50 mg/kg/day) + calcium (1.8%) + vitamin D (0.2 mg/kg/day three times a week). In the control group, 40 rats had free access to a regular diet. Dual-energy X-ray absorptiometry was used to measure the BMD of the femoral epiphysis and microcomputed tomography to analyze its fine structure. The average BMD increased rapidly with age and reached a peak at the age of 8 months.

In the group treated with vitamin $D_3$, the mean (± SD) BMD at the ages of 6, 8, 12 and 16 months was 0.3196 ± 0.026, 0.3200 ± 0.031, 0.3270 ± 0.024, and 0.302 ± 0.022 g/cm², respectively, and thus the BMD for this group peaked at the age of 12 months. The rate of decrease in BMD from the peak to the age of 16 months was 8.9%. There were significant differences in BMD between the age of 3 months and the ages of 6.8 and 12 months. Among the other groups, the peak BMD was the highest in group C (calcium 1.8%) and the rate of decrease the smallest in group D (vitamin $K_2$ 50 mg/kg/day + calcium 1.8% + vitamin D 0.2 mg/kg/day three times a week). The results of this study suggest that the peak BMD of humans can be raised by consuming sufficient amounts of vitamin $D_3$ and calcium continuously from childhood, and that this diet may suppress the rate of decrease in BMD, thus ultimately preventing bone fractures caused by osteoporosis.

### III.1.2 Secondary pharmacodynamics

Over the course of the last decades, it has become increasingly clear that the effects of $1,25(OH)_2D_3$ are not limited to the maintenance of calcium and phosphate homeostasis (*Christakos et al. 2016*). While the effects of vitamin $D_3$ on the calcium/phosphate metabolism and bone mineralization are well known, its physiologic role in other tissues and organs, such as brain, heart, pancreas, mononuclear cells, activated lymphocytes, and skin, remains unknown and still not fully elucidated.

Although the vitamin exhibits potent antiproliferative and prodifferentiation effects, there is currently little evidence that vitamin $D_3$ deficiency results in major disorders in these organs and cellular systems.

## III.2 Pharmacokinetics

### III.2.1 Pharmacokinetics

Pharmacokinetic (PK) data in animal support the data observed in humans. The PK of $1,25(OH)_2D_3$ has been investigated by Muindi and coworkers (*Muindi et al. 2004*) with refer to the antitumor activity of the vitamin $D_3$ in some experimental animal models. The objective of

the study was in fact to determine the plasma calcitriol PK in normal mice at doses of calcitriol that are active in suppressing tumor growth. The plasma $1,25(OH)_2D_3$ concentrations were examined in normal C3H/HeJ mice after a single intraperitoneal injection dose of 0.125 or 0.5 µg $1,25(OH)_2D_3$/mouse. The blood samples were collected from groups of 5-9 mice at each time point up to 24 hours after $1,25(OH)_2D_3$ administration. The plasma $1,25(OH)_2D_3$ concentrations were measured by radioimmunoassay method, and their diurnal variations were determined by collecting blood samples in the morning (9:00-11:00 a.m.) and in the evening (4:00-9:00 p.m.). Median baseline plasma $1,25(OH)_2D_3$ concentration measured in the morning and in the evening averaged 0.082 ng/ml and 0.067 ng/ml, respectively ($p<0.01$). After 0.125 and 0.5 µg dosing, the peak plasma $1,25(OH)_2D_3$ concentrations ($C_{max}$) were 12.0 ng/ml and 41.6 ng/ml, respectively. The corresponding areas under the curve ($AUC_{0-24h}$) were 47.0 and 128.0 ng*h/ml. No dose-related changes in time to $C_{max}$ and apparent total plasma clearance were observed.

### III.2.2 Distribution

After absorption, vitamin $D_3$ (cholecalciferol) enters the blood via chylomicrons of lymph, where it is associated mainly with a specific α-globulin (DBP). Vitamin $D_3$ is then accumulated in the liver within a few hours, where it is metabolized to its 25-hydroxylated derivative $25(OH)D_3$. The 25-hydroxylated derivatives of cholecalciferol also circulate associated with the same α-globulin, and they are stored in fat and muscle for prolonged periods. The $25(OH)D_3$ may be distributed into milk after large doses of cholecalciferol (*AHFS 2007*).

### III.2.3 Metabolism

In the liver, cholecalciferol is converted in the mitochondria to its 25-hydroxy derivative ($25(OH)D_3$) by the enzyme vitamin $D_3$ 25-hydroxylase, that is regulated by the plasma concentrations of vitamin $D_3$ and its metabolites.. $25(OH)D_3$ is one of the major circulating metabolites with a half-life of approximately 21 days. In the kidneys, the $25(OH)D_3$ is further hydroxylated at the 1 position by the enzyme vitamin $D_3$ 1-hydroxylase to their active form, $1,25(OH)_2D_3$ (calcitriol) (*AHFS 2007*).

### III.2.4 Excretion

The circulating half-life of the 25-hydroxy metabolite is about ten days to three weeks and that of the $1,25(OH)_2D_3$ is about four to six hours. Activity of the vitamin $D_3$ 1-hydroxylase enzyme requires molecular oxygen, magnesium ion, and malate and is regulated principally by PTH in response to serum concentrations of calcium and phosphate, and perhaps by circulating concentrations of $1,25(OH)_2D_3$. Other hormones (i.e., cortisol, estrogens, prolactin, and growth hormone) also may influence the metabolism of cholecalciferol. The metabolites of vitamin $D_3$ are excreted principally in bile and faeces. Although some vitamin $D_3$ that is excreted in bile is reabsorbed in the small intestine, enterohepatic circulation does not appear to be important mechanism for conservation of the vitamin. Following oral or intravenous (IV) administration of a single dose of radiolabeled calcitriol, 19-41% of radioactivity is recovered in urine within six to ten days (*AHFS 2007*).

## III.3 Toxicology

### III.3.1 Single dose toxicity

The acute toxicity of cholecalciferol has been evaluated in rabbit as part of an assessment of alternative toxins to sodium monofluoroacetate and pindone for large-scale rabbit control in the South Island of New Zealand. After a dose-ranging study spanning the known $LD_{50}$ values for other mammals (up to 400 mg/kg), an acute toxicity study established an oral $LD_{50}$ of 9 mg/kg and a $LD_{95}$ of 18 mg/kg for the rabbit, which proved very susceptible to cholecalciferol (*Eason 1993*). The 1α-hydroxylated derivative of cholecalciferol (alfacalcidol; $1(OH)D_3$) has been reported to have the following oral $LD_{50}$ in mouse, rat and dog, respectively (the data are reported as range between males and females): 476 - 440 µg/kg; 340 - 720 µg/kg; > 500 µg/kg. The $LD_{50}$ in mouse and dog after intravenous administration of alfacalcidol are 71 - 56 µg/kg, and > 200 µg/kg, respectively (*Makita et al. 1976*).

### III.3.2 Repeat dose toxicity

Information on the repeated dose toxicity of vitamin $D_3$ analogues are available for its 1α-hydroxylated derivative ($1(OH)D_3$), which was orally administered to Wistar rats at doses ranging between 0.5 and 50 µg/kg/die for 4 consecutive weeks. Control rats (a group of 80 animals, 15 males and 15 females of which underwent a recovery period) were treated with saline. The animals showed a slackening of the body weight gain when treated with 12.5, 25 and 50 µg/kg/die of $1(OH)D_3$. At these doses, an increased concentration of calcium in urines was also observed, as well as a reduced concentration of phosphates and proteinuria, but only at the dose of 12.5 µg/kg/d. At the doses of 12.5 and 50 µg/kg/d, leukocytosis and increased plasma concentrations of calcium were observed. The histological examination revealed scattered lesions of several organs, that disappeared within 50 days after the end of treatment (recovery). The investigators concluded that the most characteristic findings of $1(OH)D_3$ were necrotic lesions of the intima of coronary arteries, as well as of GI tract and striatal muscle fibres. The dose of 2.5 µg/kg/die for 30 consecutive days was considered the no-observed-adverse-effect level (*Makita et al. 1976*).

In other experiments (*Mortensen et al. 1993*), Sprague-Dawley rats were fed either a standard diet with 0.9/0.7% calcium/phosphorus (Ca/P) or a semisynthetic low-calcium diet with 0.5/0.4% Ca/P and treated orally for 28 days with $1(OH)D_3$ or $1,25(OH)_2D_3$, at the dose levels of 0.2 and 2.0 µg/kg/day. The high dose caused a severe hypercalcaemia with retarded growth, nephrosis, and structural bone changes in rats fed the standard diet. The same dose caused only slight hypercalcaemia without growth retardation or bone changes, and it only minimally affected the kidneys in rats fed the low-calcium diet. Hypercalcaemia with less pronounced pathological changes was found in the standard diet low-dose rats, whereas no hypercalcemia or pathological changes were found in the low-calcium diet low-dose group. The rats fed the low-calcium diet tolerated $1(OH)D_3$ at dose levels up to 10 times higher than rats on the standard diet. The use of diets low in calcium and low in phosphorus will thus allow the administration of higher dosages of vitamin D compounds without causing

hypercalcaemia. This may permit a better evaluation of the pharmacologic and toxic effects not directly associated with the calcium-regulating properties of vitamin D metabolites and analogues.

Tischler and coworkers (*Tischler et al. 1999*) treated Charles River Crl:CD BR rats with 0 (control), 5.000, 10.000 and 20.000 I.U./kg/day of vitamin $D_3$ (corresponding to 20 mg/kg, 40 mg/kg and 80 mg/kg, respectively) by oral intubation. Rats were killed after 4, 8, 12, or 26 weeks of treatment, following a final week of labeling with bromodeoxyuridine (BrdU) using a mini-pump. In fact, the study was not aimed at investigating the toxicity of cholecalciferol, but to prospectively testing the relationship between mitogenicity and focal proliferative lesions in the adrenal medulla by using the vitamin $D_3$ model, and to determining the early events in the pathogenesis of these lesions. Nevertheless, the results of the study can be useful in the global evaluation of the safety of use of cholecalciferol. Adrenal sections were double-stained for BrdU and phenylethanolamine-N-methyl transferase (PNMT) to discriminate epinephrine from norepinephrine cells or for vesicular acetylcholine transporter (VAchT) to identify cholinergic nerve endings. Cholecalciferol caused a 4-5-fold increase in BrdU labeling at week 4, diminishing to a 2-fold increase by week 26. An initial preponderance of labeled epinephrine cells gave way to a preponderance of labeled norepinephrine cells. By week 26, 17/19 (89%) animals receiving the 2 highest doses of vitamin $D_3$ had focal adrenal medullary proliferative lesions, in contrast to an absence of lesions in control rats. The lesions encompassed a spectrum including BrdU-labeled "hot spots" not readily visible on hematoxyllin/eosin sections, hyperplastic nodules, and pheochromocytomas. Lesions were usually multicentric, bilateral, and peripheral in location, and almost all were PNMT-negative. The lesions were not cholinergically innervated, suggesting autonomous proliferation. Hot spots, hyperplastic nodules, and pheochromocytomas appear to represent a continuum rather than separate entities. Their development might involve selective responses of chromaffin cell subsets to mitogenic signals, influenced by both innervation and corticomedullary interactions. A number of non-genotoxic compounds that induce pheochromocytomas in rats are known to affect calcium homeostasis.

The long-term effects of a $24,25(OH)_2D_3$ feeding were examined in Wistar rats (14-week-old, male, 20 rats/group) receiving a powder diet containing 0 or 5 ppm $24,25(OH)_2D_3$ for 57 weeks (*Ikezaki et al. 1999*). Final body weights and total food consumption were comparable between the groups. Urinary calcium levels were significantly increased by the administration of $24,25(OH)_2D_3$ at weeks 3, 22 and 56, although the levels of serum calcium did not differ between the groups at the termination of week 57. In the $24,25(OH)_2D_3$ group, weights of the adrenals and femurs were significantly increased. Histopathologically, this was found due to thickening of cortical bone in the femurs, and medullary hyperplasia and pheochromocytoma of the adrenals. Immunohistochemically, proliferating cell nuclear antigen-labeling indices for intact adrenal medulla, medullary hyperplasia and pheochromocytoma in the $24,25(OH)_2D_3$ group were respectively 1.82 ± 1.21, 5.88 ± 4.13 and 16, all higher than that for the adrenal medulla in the control group (0.87 ± 0.67). These results indicate that $24,25(OH)_2D_3$ at a dose with which serum calcium is not chronically increased, causes thickening of the cortex of the femur, and development of adrenal proliferative lesions, suggesting that rats may be too sensitive for results to be relevant to human risk assessment.

Finally, two studies in pigs (*Haschek et al. 1978*) and calves (*Mullen et al. 1979*) are available from literature. Two groups of weanling pigs (*Haschek et al. 1978*), injected with $^{45}Ca$, were fed diets containing optimal calcium and phosphorus, and vitamin $D_3$ at 1.320 I.U./kg (5.28 mg/kg) feed in the control group, and 825.000 I.U./kg (3.300 mg/kg) feed in the test group. The groups were further subdivided with 2 pigs in each subgroup, with survival times of 1, 2, 3, 4, 7, and 14 days. Pigs fed the high level of vitamin $D_3$ lost weight, and anorexia, weakness, rough hair coat and labored breathing were observed. Hypercalcemia began at 12 hours and progressed rapidly after 2 days. Radioisotope studies interpreted in the light of histopathologic findings indicated that bone was the primary source of increased plasma calcium. Calcium was released at a rapid rate into blood from prelabeled bone that was undergoing necrosis; it was also removed from blood and deposited into bone at a slower rate due to decreased apposition. Histopathologic examination of bones from test pigs showed regressive changes in the osteocytes, chondrocytes and osteoblasts, which began within 1 day of treatment and resulted in evidence osteopenia within 7 days. Arrested osteocytic osteolysis led to the appearance of cementing lines and to chondroid core retention. Further regressive changes in the osteocytes resulted in osteocytic death and osteonecrosis with subsequent osteoclasia and osteopenia. Retardation and arrest of cartilage maturation as well as osteoblastic deficiency contributed to the osteopenia. The osteopenia was further evidenced by decreased specific gravity and ash content per unit volume of humerus. The initial negative effect on the osteocytes, chondrocytes and osteoblasts is attributed to a direct toxic effect of excessive dietary vitamin $D_3$ since hypoparathyroidism and hypercalcitoninism, which occur secondarily to hypercalcemia, could not account for the rapid appearance of this effect, nor are they known to induce osteocytic death. The release of bone calcium and the resulting hypercalcemia in vitamin $D_3$ suggested that toxicosis is due to a direct toxic effect of the vitamin, or its metabolites, on the osteocyte resulting in osteonecrosis, rather than to increased resorption. Degeneration, with subsequent inflammation, but without calcification, was observed in the kidneys and in the lungs. Epithelial cells, basement membranes, and smooth muscle were affected. This demonstrated that degeneration is the primary soft tissue lesion in vitamin $D_3$ toxicosis, and that the subsequent calcification is therefore dystrophic. Degenerative changes occurred in the parathyroid glands within 1 day of treatment resulting in necrosis, inflammation and atrophy within 4 days. Relative fibrosis was seen as the parenchyma receded. The parathyroid gland changes were considered a direct effect of vitamin $D_3$ toxicity since they occurred with only mild hypercalcemia and since necrosis of parathyroid cells has not been demonstrated with hypercalcemia either in vivo or in vitro.

In the study in calves (*Mullen et al. 1979*), groups of 2 calves were treated with 15 µg/kg of $1(OH)D_3$ by intramuscular (IM) injection on 4 occasions at 7 day intervals. Anorexia and reduced water consumption persisted for 48 hours after each treatment. No clinical signs of iridocyclitis or any other lesions of the eyes were present at any time either macroscopically or microscopically. After the first treatment serum aspartate transaminase and glutamate dehydrogenase activities increased, serum alkaline phosphatase activity decreased, serum concentrations of calcium and phosphate increased, and magnesium concentrations decreased. The reduced serum magnesium concentrations and increased calcium and phosphate concentrations were maintained for the duration of the experiment, but there was

no evidence of a cumulative effect of successive treatments. Blood urea concentrations increased after the 3rd treatment. The gross pathology at post mortem examination was similar to that reported after vitamin $D_3$ supplementation.

### III.3.3 Reproductive and developmental toxicity

The potential of $1,25(OH)_2D_3$ for teratogenesis and other detrimental effects on the reproductive performance was investigated in rat and rabbits orally treated with 0.02, 0.08 and 0.3 µg/kg/day in Neobee oil (*McClain et al. 1980*). In the teratogenesis studies, female rats were treated since Day 7 to Day 15 of pregnancy, and killed on Day 21, while the female rabbits were treated since Day 7 to Day 18 and killed on Day 29. In the studies of fertility and reproduction, male rats were treated for 60 days before and during mating, while the females were treated for 14 days before mating, as well as for all the duration of pregnancy and lactation. A group of female animals was killed on Day 13, while the others were left to deliver. Finally, in the studies of fetal and post-natal development, female rats were mated with untreated males and treated since Day 15 of pregnancy to Day 21 of lactation.

No maternal mortality was observed except for one dam that was sacrificed moribund at the medium dose of 0.08 µg/kg/day. This rat had large calculi in the bladder and the distal end of each ureter. There did not appear to be any adverse effect on fertility at the dosages used. The pregnancy rate in the high dose (83%) was slightly lower than controls (100%); however, this difference was not statistically significant. In the groups of rats sacrificed on Day 13 of gestation, no significant differences were noted in the average viable litter size, the average number of implants and corpora lutea or the resorption rates between treated and control groups.

In rats allowed to deliver in both reproduction studies no substantial differences were noted in the average litter size, the percentage of pregnant dams that cast a litter (Gestation Index), the percentage of pups that survived to lactation Day 4 (Viability Index), or the percentage of pups that survived beyond lactation Day 4 to lactation Day 21 (Lactation Index). The average pup weights at birth and throughout lactation were similar in all dose groups and no external, visceral or skeletal abnormalities were noted except for one pup in the mid-dose groups which exhibited a rudimental tail.

The dams showed a dose-dependent hypercalcemia and hypophosphatemia. A slight but statistically significant increase was noted in the serum urea nitrogen in one study. Pups at weaning showed hypercalcemia and a slight trend toward increased bone ash. This latter effect paralleled the degree of hypercalcemia in the pups which was greater in the perinatal/postnatal study.

The pretreatment parameters of the percentage pregnant, and the numbers of corpora lutea and implants were within normal limits. No differences were noted in the average litter size, the fetal body weight, or the resorption rate between treated and control groups. No external abnormalities were noted in fetuses nor were there any substantial differences noted in the incidence of visceral or skeletal abnormalities. The visceral abnormalities noted included

enlarged atria and dilatation of the renal pelves observed in a few fetuses from all groups and a small indentation of the palate in one control fetus.

Three rabbits in the high-dose group of 0.3 µg/kg/day, one rabbit in the low-dose group and one rabbit in the control group died. Two of the three high-dose rabbits that died, had histopathological evidence of hypervitaminosis D (focal renal tubular calcification). In addition, focal calcification of the lungs and stomach was seen during the treatment period in rabbits at the high dose as compared to a weight gain in controls. A reduction in the average litter size and an increase in the resorption rate that were considered to be biologically significant were observed in the high-dose group; however, these differences were not statistically significant. The percentage of viable pups that survived a 24-hour incubation period (Viability Index) was significantly decreased in the high-dose group as compared to controls.

The overall incidence of external, visceral, and skeletal abnormalities was comparable in all groups; however, one litter from each of the mid- and high-dose groups had fetuses with multiple abnormalities. Open eyelids, microphthalmia, cleft palate, shortened long bones, curvature of the paws, pes caves, shortened ribs, and sternabral defects were seen in nine fetuses of the affected litter in the mid dose group.

One litter in the high-dose group contained six fetuses with abnormalities that included open eyelids, shortened long bones, and shortened ribs. Another in the high-dose group was found to have severe renal and uterine abnormalities (agenesis of the left kidney with an enlarged and abnormal right kidney and agenesis of the left uterine horn) which may have secondarily affected the sole fetus in the right uterine horn that showed multiple craniofacial malformations. No statistically significant differences were noted between the treated groups and the control group in either the numbers of litters or the number of fetuses displaying abnormalities. Due to the low incidence of litters involved, the lack of a clear dose response and statistical significance, it was uncertain whether or not the one litter observed in each of the mid- and high-dose groups with multiple abnormalities were related to compound administration; however, this possibility could not be excluded.

Overall, the results of these experiments showed that calcitriol, at dosages of up to 0.3 µg/kg/day (30x the usual dose in humans) did not adversely affect reproduction in the rat. Calcitriol in the rabbit produced maternal and some fetotoxic effects at 0.3 µg/kg/day but not at 0.02 or 0.08 µg/kg/day (8x the usual human dose).

Horii and co-workers (*Horii et al. 1992*) investigated in the female rats treated with $1,25(OH)_2D_3$: a) the effect on the estrous cycle (no treatment for 2 weeks, treatment for 3 weeks and recovery for 2 weeks); b) the effect on the maintenance of pregnancy (treatment for 2 weeks before mating and during the gestation period). In both groups, the levels of calcium, calcitonin, PTH and progesterone in serum were measured, and histopathological examination of the thyroid, parathyroid, ovary and uterus was carried out. The following results were observed: 1) disturbance of the estrous cycle, 2) hypofunctional changes in the corpus luteum in the ovary, and the epithelium, endometrium and uterine gland in the uterus

with a decrease in the serum progesterone level and 3) hypercalcemia with a decrease in calcitonin or PTH levels in serum with morphological changes including atrophy and cyst formation in the parathyroid. However, the above changes were reversible, and recovery was observed after administration of the compound was discontinued. These results indicate that the hypercalcemia caused by $1,25(OH)_2D_3$ disrupts endocrinological homeostasis that in turn temporarily disrupts the female reproductive system. Furthermore, it was suggested that $1,25(OH)_2D_3$ itself directly influences on endocrinological organs (hypothalamus, pituitary, parathyroid and thyroid) and reproductive organs (ovary and uterus).

The potential toxicity of vitamin $D_3$ and its hydroxylated derivatives, i.e. alfacalcidol $1(OH)D_3$ and calcitriol $1,25(OH_2)D_3$) was studied by administration of these compounds at three different doses to weanling C57BL/6J mice over a 4-week period (*Crocker et al. 1985*). Vitamin D was administered at 10, 50 and 250 I.U./kg, $1(OH)D_3$ and $1,25(OH)_2D_3$ were administered at the doses of 10, 50 and 250 ng/kg. Drug effects on calcium were monitored by serum calcium and urine calcium/creatinine ratio determinations. Tests of renal function included serum creatinine, 24-hour urine volume, urinary protein, and glucose excretion, and histological evaluation of renal tissue. At 2 weeks, there was no significant change in serum creatinine, irrespective of treatment. Treatment with vitamin D itself did not change serum calcium at any of the three doses. Serum calcium was significantly elevated in the groups receiving 50 and 250 ng of $1(OH)D_3$ compared to controls. Increased serum calcium was also observed in the animals receiving the two higher doses of $1,25(OH)_2D_3$, but the changes were not statistically significant. The 50 ng dose of $1(OH)D_3$ and $1,25(OH)_2D_3$ produced similar increases in calcium/creatinine ratios, but the 250 ng dose of $1(OH)D_3$ determined a more substantial calciuric response. The increase in the urinary calcium/creatinine ratio for $1,25(OH)_2D_3$ was less than for $1(OH)D_3$ and was only significant for the group receiving the 50 ng dose.

By 4 weeks, serum calcium was significantly increased in all groups that received either $1(OH)D_3$ or high-dose $1,25(OH)_2D_3$. There was a consistent trend toward higher serum calcium in all animals receiving $1(OH)D_3$ when compared to equivalent doses of $1,25(OH)_2D_3$, but only at 250 ng doses was the serum calcium significantly different from all other animals. Apart from the high-dose group, urine volume was the same in all animals. No change in the urinary calcium excretion was noted in the vitamin D-treated animals. On the other hand, a significant calciuria was noted in the animals receiving either 50 or 250 $1(OH)D_3$. The investigators concluded that $1(OH)D_3$ is more toxic than calcitriol in the mouse and suggest that the degree of toxicity is correlated to the degree of hypercalcemia and to the vitamin D metabolite used.

In other tests (*Kistler 1980*), the oral administration for 5 days of an excess of $1,25(OH)_2D_3$ at doses of 1, 5, and 25 µg/kg to rats, beginning at the age of 2 or 10 days, produced dose-dependent reductions in weight development and additional calcification near the skeleton. Alizarin red S stained skeleton revealed calcific deposits near the bones of the head, near the neural arches, between the ribs, along the bones both of the fore limbs and, to a lesser extent, of the hind limbs. Histologically, the deposits appeared to be localized primarily in the sub-epithelial connective tissues. Starting treatment with $1,25(OH)_2D_3$ (25 µg/kg for 5 days) at the age of 20 days produced additional calcification in 1 out of 8 rats at only one location (lower jaw). Additional calcification as described above could no longer be induced by $1,25(OH)_2D_3$ in

30-day-old rats using doses up to 25 µg/kg and 10 daily treatments. The investigators concluded that the sensitivity of young rats to $1,25(OH)_2D_3$-induced additional calcification, which differs in localization from that observed in adult rats, decreases with the maturation of the animals.

The effects on the serum calcium and phosphorus and on kidney calcium were determined in lactating rats and their suckling pups after the mothers had received high doses of vitamin $D_3$ or $1,25(OH)_2D_3$ (*Dostal et al. 1983*). High dietary vitamin $D_3$ intake (1200 µg/g diet) or daily oral doses of vitamin $D_3$ (1 µg/g bodyweight or BW) to vitamin-replete (+D) lactating rats for 8 or 12 days caused significant increases in serum calcium in the mothers (1-2 mg/dl) and in their suckling pups (1.5 mg/dl). Daily oral doses of $1,25(OH)_2D_3$ (2 ng/g BW) to +D lactating rats caused a similar increase in serum calcium in the mothers, but did not affect the serum calcium of the pups. The administration of a high dose of $1,25(OH)_2D_3$ to vitamin D-deficient lactating rats or high doses of vitamin $D_3$ to +D rats caused no change in milk calcium, magnesium or phosphate. Milk from +D rats given high doses of $^3$H-vitamin $D_3$ (1 µg/g BW) contained mostly $^3$H-vitamin $D_3$ (85%) and a small amount of $^3$H-hydroxyvitamin $D_3$ (6%). The results indicate that high doses of vitamin $D_3$, but not $1,25(OH)_2D_3$, given to +D lactating rats can cause hypercalcemia in the suckling pups. The hypercalcemic effect on the pups observed after vitamin $D_3$ treatment of the mother was probably a result of transport of toxic amounts of primarily vitamin $D_3$ into the milk and is not due to altered mineral composition of the milk.

Recently, a study (*Fu et al. 2017*) has investigated whether vitamin D deficiency has an impact on testicular development and spermatogenesis in mice. In the control group and vitamin D deficient (VDD) diet group, dams and their pups were fed with standard-chow diet and VDD diet, respectively. Interestingly, testicular weight and sperm quality were reduced, testicular germ cell proliferation was suppressed, and the percentage of mature seminiferous tubules was decreased in VDD diet-fed mice. Moreover, testicular testosterone synthesis enzymes were down-regulated in VDD diet-fed mice. Correspondingly, serum and testicular T levels were reduced in VDD diet-fed mice. Importantly, fertility index was reduced and live fetuses are decreased when both males and females were fed with VDD diet. These results provided evidence that vitamin D deficiency impairs testicular development and spermatogenesis. Despite the evidence from these tests, whether vitamin D deficiency impairs male reproduction in humans remains a matter of debate.

## III.4 Ecotoxicity/environmental risk assessment (ERA)

Since the product Calvigor contains a (natural) vitamin, vitamin D3, and, moreover, is intended for generic substitution, this will not lead to an increased exposure to the environment. An environmental risk assessment is therefore not deemed necessary.

## III.5 Discussion on the non-clinical aspects

The submission is intended for well-established use. As such, the MAH has not provided additional non-clinical studies and further studies are not required. An overview based on literature review is, thus, appropriate. The effects of vitamin D are well known, and the

literature on pharmacology, pharmacokinetics and toxicology has been adequately reviewed in the MAH's non-clinical overview.

# IV. CLINICAL ASPECTS

## IV.1 Pharmacokinetics

### IV.1.1 Absorption

Orally administered cholecalciferol is readily absorbed from the gastrointestinal tract with a systemic bioavailability of about 80%. The bile is required for absorption of liposoluble vitamins, which may be reduced in patients with hepatic, biliary or gastrointestinal disease, associated with malabsorption syndromes. Since cholecalciferol is fat soluble, it is incorporated into chylomicrons and absorbed via the lymphatic system. Approximately 80% of ingested vitamin D is absorbed through this mechanism, mainly in the small intestine.

Normal combined plasma concentrations of 25-hydroxycholecalciferol (calcidiol) and 25-hydroxyergocalciferol, which are the major circulating metabolites of cholecalciferol and ergocalciferol, have been reported to range from 8-80 ng/ml, depending on the assay used, and vary with exposure to ultraviolet light. Circulating concentrations of the 25-hydroxy metabolites generally increase with increasing intake of ergocalciferol or cholecalciferol. However vitamin D absorption is not affected by vitamin D status.

### IV.1.2 Distribution

After absorption, cholecalciferol enters the blood via chylomicrons of lymph and then associates mainly with a specific α-globulin (vitamin D-binding protein; DBP), synthesized by the liver. The hydroxylated metabolites of cholecalciferol also circulate associated with the same α-globulin. A direct study of the binding capacity of the purified DBP for added 25(OH)D3 showed that the isolated DBP had a high affinity for 25(OH)D3, with an apparent maximum binding capacity of one molecule of 25(OH)D3 per molecule of protein. It has also been recently demonstrated that the circulating levels of DBP are not affected by vitamin D supplementation and are not associated with the free fraction of 25-OHD. Since DBP has a single sterol binding site, only approximately 5% of the total human plasma is occupied with vitamin D compounds. Therefore, under normal physiological conditions, nearly all circulating vitamin D compounds are protein bound, which has a great influence on vitamin D pharmacokinetics. In pregnancy the free 1,25-(OH)D3 index remains normal up to 35 weeks of gestation, but during the last weeks of gestation, this increases in both maternal and cord serum. Once vitamin D enters systemic circulation from lymph via the thoracic duct or from skin, it accumulates in the liver within a few hours. The vitamin disappears from plasma with a half-life of elimination (t1/2) of 20–30 hours but is stored in fat depots for prolonged periods. The lipophilic nature of vitamin D explains its adipose tissue distribution and its slow turnover in the body (half-life of approximately 2 months).

As for other hormones, free and bioavailable (not protein-bound) 25(OH)D is more strongly correlated with BMD than total 25(OH)D. The vitamin D excreted in bile may be reabsorbed in the small intestine. This entero-hepatic circulation does not appear to be an important mechanism for conservation of the vitamin. Only small amounts of cholecalciferol appear in breast milk.

### IV.1.3 Elimination

Excretion

The circulating half-life of the 25(OH)D3 metabolite is of approximately two to three weeks, and that of the 1,25(OH)2D3 is about four to six hours. The two metabolites of cholecalciferol are excreted principally in bile and faeces, with a small percentage found in the urine. Although some vitamin D that is excreted in bile is reabsorbed in the small intestine, enterohepatic circulation does not appear to be an important mechanism for conservation of vitamin.

Metabolism

Whether derived from diet or endogenously synthesized, vitamin D requires modification to become biologically active. The small amount of circulating cholecalciferol is converted by the liver cells to its 25-hydroxy derivative, 25(OH)D3 (calcifediol), by the vitamin D 25-hydroxylase. In the kidneys, the 25(OH)D3 is further hydroxylated at the 1 position by the enzyme vitamin D 1α-hydroxylase to its active form, 1,25(OH)2D3 (calcitriol). The primary active metabolite of the vitamin is calcitriol, the product of two successive hydroxylations of vitamin D.

In the liver, cholecalciferol is converted in the mitochondria to its 25-hydroxy derivatives by the enzyme vitamin D 25-hydroxylase. Vitamin D 25-hydroxylase activity is regulated in the liver by concentrations of vitamin D and its metabolites. Therefore, increases in the systemic circulation of the 25-hydroxy metabolites following exposure to sunlight or ingestion of vitamin D are relatively modest compared with cumulative production or intake of the vitamin. Serum concentrations of non-hydroxylated vitamin D are short-lived as a result of storage in body fat or metabolism in the liver.

After production in the liver, 25(OH)D enters the circulation and is carried by the vitamin D–binding globulin. Final activation to calcitriol occurs primarily in the kidney but also takes place in other sites, including keratinocytes and macrophages. The enzyme system responsible for 1α-hydroxylation of 25(OH)D (CYP1α, 25-hydroxyvitamin D3-1α-hydroxylase, 1α-hydroxylase) is associated with mitochondria in proximal tubules. Vitamin D 1α-hydroxylase is subject to tight regulatory controls that result in changes in calcitriol formation appropriate for optimal calcium homeostasis. Dietary deficiency of vitamin D, calcium, or phosphate enhances enzyme activity. 1α-Hydroxylase is potently stimulated by PTH and probably also by prolactin and oestrogen. Conversely, high calcium, phosphate, and vitamin D intakes suppress 1α-hydroxylase activity. Regulation is both acute and chronic, the latter owing to changes in protein synthesis. PTH increases calcitriol production rapidly via a cyclic adenosine

monophosphate (cAMP)-dependent pathway. Hypocalcaemia can activate the hydroxylase directly in addition to affecting it indirectly by eliciting PTH secretion.

Hypophosphatemia greatly increases 1α-hydroxylase activity. Calcitriol controls 1α-hydroxylase activity by a negative-feedback mechanism that involves a direct action on the kidney, as well as inhibition of PTH secretion. The plasma t1/2 of calcitriol is estimated at 3-5 days in humans. Calcitriol and 25-OHD are hydroxylated to 1,24,25(OH2)D and 24,25(OH)2D, respectively, by another renal enzyme, 24-hydroxylase, whose expression is induced by calcitriol and suppressed by factors that stimulate the 25-OHD-1α-hydroxylase. Both 24-hydroxylated compounds are less active than calcitriol and presumably represent metabolites destined for excretion.

### IV.1.4 Bridging data

The Vitamin D3 25.000 I.U. and 50.000 I.U. orodispersible films and soft capsules proposed by the MAH are both oral oil-based formulations. Since the highly lipophilic nature of Vitamin D3 does not allow to dissolve it in the water-based mixture of the excipients of the orodispersible film, Vitamin D3 was added in the mixture as oil solution in order to promote its complete and homogeneous dispersion in the mass. The refined olive oil, used to solubilize Vitamin D3 during the mixture preparation, is the same excipient comprised in oily solutions already available on the market, like DIBASE oral solution. As such, even though the proposed orodispersible film is a solid dosage form, it can be retained as comparable to oily liquid dosage forms already authorized as drugs, from a pharmaceutical viewpoint.

More importantly, cholecalciferol (Vitamin D3) is fat soluble and is readily absorbed from the gastrointestinal tract following oral administration, since it is incorporated into chylomicrons and absorbed via the lymphatic system. Approximately 80% of ingested vitamin D is absorbed through this mechanism, mainly in the small intestine (AHFS 2010), and none of the excipients used in the manufacturing of cholecalciferol-containing products is known to affect neither the gastrointestinal transit, nor the absorption of vitamin D3, or the in vivo solubility (e.g. co-solvents) or the in vivo stability of the active substance. Human in vivo data indicate that the absorption of vitamin D is not influenced by the type of formulation chosen. Like other liposoluble vitamins, intestinal absorption of vitamin D is thought to be enhanced if taken concurrently with foods containing lipids. However, a more recent study (Cavalier et al. 2016) showed how significant differences between fasting vs. fed conditions following administration of vitamin D3 from an oily solution should not be expected.

A systematic literature search was conducted in order to examine the impact of different matrix delivery systems on the bioavailability of vitamin D3 (cholecalciferol), with a focus on clinical studies that directly compared the cholecalciferol bioavailability from different vehicles, including cholecalciferol fortified food as well as food supplements. Randomised studies (Natri et al.2006, Holvik et al. 2007, Biancuzzo et al. 2010, Coelho et al. 2010, Frankling et al. 2020) and a recent systematic review (Grossmann et al. 2010) evaluating the effects of vehicle substances on vitamin D bioavailability did not show critical differences among different nutritional and pharmaceutical preparation with regard of the absorption of vitamin

D. Apparently, vitamin D in an oil vehicle were reported to produce a greater 25(OH)D response than vitamin D in a powder or an ethanol vehicle in healthy subjects.

Since Vitamin D insufficiency is a widespread public health problem, functional food fortified with this vitamin during processing, such as milk, cheese, bread, cereals and orange juice has received increasing attention in recent years and is recommended by the World Health Organisation (Allen et al. 2006). Fortified food is already implemented in countries such as Canada, US and the UK, where cholecalciferol is added to margarine, fluid milk and breakfast cereals (Calvo et al. 2004). It has been shown that there is a good bioavailability of cholecalciferol from fortified foods. Several randomized clinical trials attested how circulating levels of 25(OH)D increased in a dose-dependent manner with increased intake of cholecalciferol–fortified foods. For instance, Biancuzzo et al. (2010) conducted a randomized placebo-controlled double-blind study in healthy adults who received 1000 IU cholecalciferol in orange juice or in capsule containing lactose, magnesium stearate, and silicon dioxide for 11 weeks: no significant difference in serum 25(OH)D3 was observed between subjects who consumed cholecalciferol–fortified orange juice and cholecalciferol capsules. Natri e al. (2006) examined the cholecalciferol bioavailability from low-fiber wheat bread and high-fiber rye bread compared with cholecalciferol supplement (tablets) and concluded that the added cholecalciferol was stable and bioavailable since it increased serum 25(OH)D concentration as effectively as the cholecalciferol supplement.

Recently, the consumption of oral cholecalciferol supplements has been increasing and various delivery systems (e.g. lipids/oil-based, cellulose and lactose vehicles) are available on the market. According to the results of the published literature review, substantial differences in the bioavailability of cholecalciferol supplements across different categories of vehicles were not found. Holvik et al. (2007) conducted a randomized study in healthy young adults in which they compare bioavailability of 10 µg cholecalciferol from water-miscible tablets (Vitaplex ABCD, Cederroth AS, Revetal, Norway) and fish oil capsules administered over a 4 weeks' period of daily supplementation. The authors concluded that fish oil capsules and multivitamin tablets containing the same dose of cholecalciferol, produced a similar mean increase in serum 25(OH)D concentration.

A recent post-hoc re-analysis of data from an observational study conducted on 206 immunodeficient patients with serum 25-(OH)D concentrations <75 nmol/L compared the effectiveness of tablets (Divisun 800 IU,1600 IU/day) versus oil-drops (Detremi,1500 IU/day) in raising 25-(OH)D in plasma for twelve months. They found that cholecalciferol administered as oil drops was equally effective as powdered tablets in raising serum 25(OH)D concentrations (Frankling et al. 2020). Similar results were obtained by Coelho et al. (2010) that conducted a randomized, open clinical trial in which the bioavailability of cholecalciferol in capsules (in lactose excipient) was compared to oily drops, both containing cholecalciferol 66,000 UI. The study conducted in nuns living in a closed community with very low sun exposure showed that increase in serum 25(OH)D concentrations were very similar with the two formulations.

Overall, based on the literature review it can be concluded that cholecalciferol is highly bioavailable and that the impact of the matrix in which it is supplied can be regarded as negligible.

### IV.1.5 Bioequivalence study

The MAH conducted a bioequivalence study in which the pharmacokinetic profile of the test product Cholecalciferol 25000 IU orodispersible film (IBSA Farmaceutici Italia Srl, Italy) is compared with the pharmacokinetic profile of the reference product DIBASE, oral solution (Abiogen Pharma S.p.A., Italy).

The choice of the reference product in the bioequivalence study has been justified by comparison of dissolution results. The formula and preparation of the bioequivalence batch is identical to the formula proposed for marketing.

Bioequivalence studies

*Design*

A single dose, randomised, parallel group, open-label bioavailability study was carried out under fasted and fed conditions in 48 healthy subjects, aged 44-57 years. Each subject received a single dose (25.000 IU) of one of the two cholecalciferol formulations (test product under fasted or fed conditions, the reference product under fed conditions). The parallel design was chosen due to the long half-life of the investigated analyte 25(OH)D3, i.e. 21-30 days. Subjects were divided in three groups: Test$(T)_{fed}$, Test$(T)_{fast}$ Reference$(R)_{fed}$. The test product was orally administered with 20 ml water after a ten hour fasting period ($T_{fast}$) or after a light meal ($T_{fed}$). The reference product was fully drunk by subjects after a light meal ($R_{fed}$).

Blood samples were collected pre dose and 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72, 144, 312, 480 and 648 hours after administration of the products.

The design of the study is acceptable.

*Analytical/statistical methods*

The analytical method has been adequately validated and is considered acceptable for analysis of the plasma samples. The methods used in this study for the pharmacokinetic calculations and statistical evaluation are considered acceptable.

*Results*

All 48 subjects were eligible for pharmacokinetic analysis.

**Table 1. Pharmacokinetic parameters (non-transformed values; arithmetic mean ± SD, $t_{max}$ (median, range)) of baseline corrected 25(OH)D3 under fasted and fed conditions.**

| Treatment N=16 per group | $AUC_{0-t}$ (ng.h/ml) | $C_{max}$ (ng/ml) | $t_{max}$ (h) |
|---|---|---|---|
| $T_{fed}$ | 2364.80 ± 1336.97 | 6.68 ± 2.03 | 144 (36–312) |
| $T_{fast}$ | 2244.38 ± 1144.26 | 7.23 ± 1.48 | 42 (2-480) |
| $R_{fed}$ | 2150.52 ± 1622.76 | 6.61 ± 2.62 | 48 (12–312) |
| | **$AUC_{0-t}$ (PE% – 90% CI)** | **$C_{max}$ (PE% – 90% CI)** | |
| $T_{fed}$ vs $R_{fed}$ | 124.60 (84.84 – 183.00) | 104.95 (83.91 – 131.25) | -- |
| $T_{fed}$ vs $T_{fast}$ | 106.34 (76.78 – 147.28) | 90.11 (77.21 – 105.17) | -- |

**$AUC_{0-t}$** area under the plasma concentration-time curve from time zero to t hours
**$C_{max}$** maximum plasma concentration
**$t_{max}$** time for maximum concentration
**CI** confidence interval
**PE** point estimate

**ln-transformed values*

## IV.2 Pharmacodynamics

### IV.2.1 Primary pharmacodynamics

Vitamin D exists in two forms: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). Vitamin D2 is found in plants, being the product of UVB (290 to 315 mm) irradiation of ergosterol; it can be consumed as a supplement or fortified foods. Vitamin D3, a product of UVB irradiation of 7-dehydrocholesterol, is synthesized in the human epidermis or consumed in the form of oily fish, fortified foods, or a supplement (*Holick 2006*).

Vitamin D is converted in the liver to 25(OH)D, which is the major circulating metabolite of vitamin D. In the kidney, 25(OH)D is then converted by 1α-hydroxylase to its active form, 1,25-dihydroxyvitamin D [1,25(OH)2D], which plays a vital role in maintaining bone and muscle health by regulating calcium metabolism. The activity of the vitamin D 1α-hydroxylase enzyme requires molecular oxygen, magnesium ion and malic acid, and it is primarily regulated by the parathyroid hormone (PTH) in response to low serum concentrations of calcium and phosphate (*Holick 2006; Holick et al. 2008*).

The effects of vitamin D are mediated by an intracellular VDR. Circulatory 1,25(OH2)D crosses the cell membrane and cytoplasm and reaches the nucleus, where it binds to the VDR. The

VDR-bound 1,25(OH2)D in turn binds to the retinoic acid x-receptor and serves as a nuclear transcription factor, altering gene function and inducing protein synthesis.

The 1,25(OH2)D-VDR complex triggers an increase in GI absorption of both calcium and phosphorus. In addition, vitamin D is involved in bone formation, resorption, and mineralization and in maintaining neuromuscular function. Circulating 1,25(OH)2D reduces serum PTH levels directly by decreasing parathyroid gland activity and indirectly by increasing serum calcium. The 1,25(OH)2D also regulates bone metabolism in part by interacting with the VDR in osteoblasts to release biochemical signals, leading to formation of mature osteoclasts. These reabsorb bone tissue with secondary release of calcium and phosphate into the circulation. In addition, either directly or indirectly, 1,25(OH)2D regulates over 200 genes, including those involved in renin production in the kidney, insulin production in the pancreas, release of cytokines from lymphocytes, production of cathelicidin in macrophages, and growth and proliferation of both vascular smooth muscle cells and cardiomyocytes (*Lee et al. 2008*).

The functional outcomes of effects related to the interaction between vitamin D status and calcium intake have been extensively investigated. These effects fall into 3 broad categories: 1) synergistic effects of vitamin D status and calcium intake on calcium absorption; 2) effects of calcium intake on vitamin D status; and 3) largely observational data suggesting an association between calcium and vitamin D status and non-skeletal outcomes, such as cancer (*Heaney 2008*).

### IV.2.2 Secondary pharmacodynamics

From a molecular mechanism of action perspective, recent research has indicated that the genomic mechanism of 1,25(OH)2D3 action involves the direct binding of the 1,25(OH)2D3 activated vitamin D receptor/retinoic X receptor (VDR/RXR) heterodimeric complex to specific DNA sequences. Numerous VDR co-regulatory proteins have been identified, and genome-wide studies have shown that the actions of 1,25(OH)2D3 involves the regulation of gene activity at a range of locations many kilobases from the transcription start site. The structure of the liganded VDR/RXR complex has been recently characterized using cryoelectron microscopy, X-ray scattering, and hydrogen deuterium exchange. These recent technological advances will result in a more complete understanding of VDR coactivator interactions, thus facilitating cell and gene specific clinical applications. Although the identification of mechanisms mediating VDR-regulated transcription has been one focus of recent research in the field, other topics of fundamental importance include the identification and functional significance of proteins involved in the metabolism of vitamin D. CYP2R1 has been identified as the most important 25-hydroxylase, and a critical role for CYP24A1 in humans was noted in studies showing that inactivating mutations in CYP24A1 are a probable cause of idiopathic infantile hypercalcemia. In addition, studies using knockout and transgenic mice have provided new insight on the physiological role of vitamin D in classical target tissues as well as evidence of extra-skeletal effects of 1,25(OH)2D3 including inhibition of cancer progression, effects on the cardiovascular system, and immunomodulatory effects in certain autoimmune diseases. Some of the mechanistic findings in mouse models have also been observed in

humans. The identification of similar pathways in humans could lead to the development of new therapies to prevent and treat disease *(Christakos et al. 2016*).

### IV.2.3 Pharmacodynamic interactions with other medicinal products or substances

The MAH has submitted information regarding the interaction of Calvigor with several medicinal products and/or substances; antacids *(Drugdex 2007*), cardiac glycosides (*AHFS 2010*), cholestyramine/colestipol (*AHFS 2010; Drugdex, 2007*), orlistat (*AHFS 2010*), thiazide diuretics (*AHFS 2010*) and phenobarbital/phenytoin (*AHFS 2010; Drugdex 2007*). This is considered acceptable.

## IV.3 Clinical efficacy

### IV.3.1 Dose-response studies and main clinical studies

Use of 25,000 & 50,000IU vitamin D

In the following study (*Wijinen et al. 2015*), 30 nursing home patients with 25(OH)D levels <50 nmol/l were randomized to receive vitamin D3 in divided doses of 50,000IU twice a week, followed by a monthly maintenance dose of either 50,000 or 25,000IU(loading dose group) or 800IU daily (daily dose group) for 26 weeks, showed that levels of 25(OH)D >75 nmol/L at week 5 were reached in 79 % of the 14 loading dose patients, but in none of the 13 daily dose patients ($p<0.001$). At week 26, 25(OH)D levels >75 nmol/L were reached in 83 % of the 12 loading dose patients and in 30 % of the 10 daily dose patients ($p<0.05$). In this study in nursing home patients with severe 25(OH)D deficiency, an individualized calculated cholecalciferol loading dose was likely to be superior to a daily dose of cholecalciferol 800IU in terms of the ability to rapidly normalize vitamin D levels.

Initial treatment and prevention of vitamin D deficiency

A study aimed at investigation vitamin D status and supplementation in ambulatory patients (*Leidig-Bruckner et al. 2011*) indicates that supplementation with higher (2,000-3,000IU/day) than recommended vitamin D dosages is required to achieve a relevant increase of 25(OH)D and normalisation of PTH. Patients with the commonly recommended vitamin D supplementation of about 500-1,000IU/day often did not achieve a relevant increase of 25(OH)D levels thus indicating that higher dosages of vitamin supplementation (i.e. 2,000-3,000IU/day, that can be reproduced with weekly doses of 20,000-25,000IU) are required in vitamin D-deficient patients.

Another recent study (*Diamond et al. 2013*) has compared the effects of 5,000IU/day and 2,000IU/day of vitamin D in 30 patients with serum 25(OH)D ≤ 50 nmol/L. Treatment of vitamin D deficiency for 3 months with oral cholecalciferol 5,000IU daily was more effective than 2,000IU daily in achieving optimal serum 25(OH)D concentrations.

Similar findings were reported in a double-blind, randomized clinical trial that enrolled adults 65 years of age and older (*Lagari et al. 2012*), who were to receive either 400 or 2,000IU vitamin D3 daily for 6 months. The study showed that, regardless of baseline 25(OH)D level, a

6-month vitamin D3 supplementation with 400 IU daily resulted in low 25(OH)D in most individuals, while 2,000IU daily maintained 25(OH)D levels within an acceptable range in most people on this regimen.

A recent specific study (*Schleck et al. 2015*) has been carried out in 150 healthy adults with low serum 25(OH)D who received an oral loading dose of 50,000 (group 1), 100,000 (group 2) or 200,000 (group 3) IU of vitamin D3 at Week 0, followed respectively by 25,000, 50,000 or 100,000IU at Week 4 and Week 8. This study demonstrated a linear dose-response relationship with an increase in 25(OH)D levels proportional to the dose administered. Authors concluded that a loading dose of 200,000IU vitamin D3 followed by a monthly dose of 100,000IU is the best dosing schedule tested in this study to quickly and safely correct the vitamin D status.

These findings were confirmed in another study (*Mazahery et al. 2015*) that has evaluated the effect of monthly 50,000IU or 100,000IU vitamin D supplements on vitamin D status in premenopausal women. This study has shown that vitamin D deficiency/insufficiency is highly prevalent also in middle-age premenopausal women and has provided confirmatory evidence that monthly 100,000 IU vitamin D for 6 months is more effective than 50,000 IU in achieving the target of serum 25(OH)D ≥ 30 ng/ml.

The efficacy of a high unit dose of 50,000IU vitamin D was evaluated in a retrospective study (*De Jong et al. 2013*) in 381 consecutive patients with hip fractures. The replacement of 50,000IU oral cholecalciferol daily for 3 days with 50,000IU oral cholecalciferol daily for 7 days increased vitamin D plasma levels rapidly and consistently.

The adequacy of a monthly regimen of 50,000IU vitamin D has been confirmed in another recent study (*Dalle Carbonare et al. 2018*) conducted in a cohort of 44 adults with low levels of 25(OH) vitamin D3 at baseline, who were randomised to receive 7 drops (1750 IU/day) vs. 50,000IU/month for 6 months (22 patients in each group). The two regimens were found to be equivalent in terms of serum calcium and 25(OH)D concentration at 6 months.

A placebo-controlled study (*Kearns et al. 2015*) showed that a dose of 250,000IU of vitamin D3 given once in November to prevent the wintertime decline, resulted in a robust increase in serum 25(OH)D after 5 days, but it was unable to sustain this increase after 90 days. The authors concluded that a larger or more frequent dosing regimen may be needed for long-term vitamin D sufficiency.

Another recent trial (*Cipriani et al. 2013*) has evaluated the long-term effects of a single dose of 600,000IU of vitamin D2 and D3 administered by oral or IM route in 24 subjects with hypovitaminosis D. In this study, an oral dose of 600,000IU of vitamin D2 or D3 was initially more effective in increasing serum 25(OH)D than the equivalent IM dose and was rapidly metabolized.

A randomized, cross-over study (*Bruyere et al. 2015*) has been conducted in 100 volunteers aged ≥ 50 years to compare a once-monthly administration of 25,000IU vitamin D3 to a daily

administration of a fixed-dose combination of 1000 mg calcium carbonate + 800IU In this study, a once-monthly administration of 25,000IU vitamin D3 was preferred over a once-daily administration of a fixed-dose combination of 800 IU vitamin D3 and calcium, with a better compliance but without any significant difference in the increase in vitamin D levels.

The adequacy of vitamin $D_3$ 50,000IU given monthly has been confirmed in a study (*De Niet et al. 2018*) conducted in 60 subjects with vitamin D deficiency who were randomised to receive this dose regimen or 2000IU vitamin $D_3$ daily, to reach the same cumulative dose of vitamin $D_3$ in each treatment group (150,000IU). In this study, a monthly administration of 50,000IU vitamin $D_3$ was associated with a rapid normalization of 25(OH)$D_3$ in deficient subjects. A daily administration of the same cumulative dose was similarly effective but took two weeks longer to reach the desirable level of 20 ng/ml.

Another recent study (*Takacs et al. 2017*) has evaluated the effects of daily 1,000 IU, weekly 7,000 IU and monthly 30,000 IU vitamin D3 given for 3 months in 64 adult subjects with vitamin D deficiency (25(OH)D <20 ng/ml). In this study the daily, weekly and monthly administrations of daily equivalent of 1,000 IU of vitamin D3 provides equal efficacy profiles.

Another placebo-controlled study (*Saleh et al. 2017*) that investigated the effect of a high dose of a single 100,000IU dose of vitamin D3 on circulating concentrations of 25(OH)D3 and its metabolites. In this study, the administration of a single high dose of 100,000IU vitamin D3 led to a significant increase in concentrations of 25(OH)D3 and its metabolites. However, the high inter-individual variation in the 25(OH)D3 response to supplementation suggested that any given dose of vitamin D is unlikely to achieve optimal vitamin D status in all treated individuals.

A systematic literature review (*Kearns et al. 2014*) was aimed at investigating the efficacy of a single large bolus dose to treat vitamin D deficiency. The results have shown that large, single doses of vitamin D consistently increased serum 25(OH)D concentrations in several vitamin D-sufficient and D-deficient populations. Vitamin D3 doses ≥ 300,000IU provided optimal changes in serum/plasma 25(OH)D and PTH concentrations.

## IV.4 Clinical safety

Hypervitaminosis

Most vitamin D supplements and fortified foods do not pose a risk of toxicity unless taken inappropriately. Physiologic mechanisms limit the formation and metabolism of vitamin D; therefore, the potential for vitamin D toxicity due solely to sun exposure is highly unlikely. Consequently, unless intentionally ingested in very large doses, vitamin D toxicity is rare. Nonetheless, at 25(OH)D serum concentrations greater than 150 ng/ml, vitamin D toxicity has been reported and is most commonly manifested as hypercalcemia and hypophosphatemia.

Initial symptoms related to hypercalcemia include diarrhoea, constipation (primarily in children/adolescents), nausea, vomiting, anorexia, polyuria, polydipsia, nocturia, weakness/fatigue, headache, and mental changes. More chronic manifestations include

proteinuria and renal impairment; soft tissue calcification in kidneys (with nephrolithiasis and/or nephrocalcinosis), heart, vessels and skin; hypertension and possibly arrhythmias; worsening of GI symptoms; pancreatitis, and psychotic symptomatology. Renal failure and death may occur after prolonged use of high *doses (Drugdex, 2007*).

Nonspecific signs and symptoms of hypercalcemia and hypercalciuria, such as nausea, vomiting, anorexia, weight loss, cardiac arrhythmias, polyuria, and kidney stones may occur if the patient develops significant electrolyte abnormalities (*Haines et al 2012*).

Hypervitaminosis D may occur with relatively small doses in some individuals. The risk of toxicity is particularly evident in infants and children, where hypercalcemia may occur in hypersensitive infants with doses as low as 400 IU daily. Mental retardation, elfin facies, linear-growth retardation, and renal failure have been described after chronic administration (several weeks or months) of 20,000 to 60,000IU daily in adults, and of 2,000 to 4,000IU daily in children (*Drugdex, 2007*). However, in one study, 75 children with rickets were safely treated with vitamin D 1.200,000IU (600,000IU IM at enrolment and at 12 weeks), alone or in combination with calcium (1,000 mg daily) (*Thacher et al, 1999*).

Kidney toxicity

Decreased renal function without hypercalcemia has been reported in patients with hypoparathyroidism after long-term vitamin D analogue therapy. Before therapy with vitamin D analogues is initiated, serum phosphate concentrations should be evaluated to avoid ectopic calcification: the serum calcium/phosphorus ratio should not exceed 70 prior to initiate treatment with cholecalciferol. Because administration of vitamin D analogues may increase phosphate absorption, patients with renal failure may require adjustment in the dosage of aluminium-containing antacids used to decrease phosphate absorption (*AHFS 2010*).

Lipid abnormalities

Dyslipidemic effects of cholecalciferol, characterized by decreases in high-density lipoprotein (HDL) cholesterol and increases in low-density lipoprotein cholesterol, have been observed when the vitamin was given alone in postmenopausal women. Beneficial lipid changes of hormone replacement therapy have been blunted by addition of cholecalciferol. In one study, an increase in the HDL/total cholesterol ratio of 45% was seen after one year of estrogen/norethindrone replacement therapy. The increase was only 25% when this therapy was combined with cholecalciferol, which was similar to the increase observed with placebo (*Drugdex 2007*).

It has been suggested that excess vitamin D may be linked to heart disease, with a possible contribution to the pathogenesis of hypertriglyceridemia and atherogenic dyslipidaemia through inflammation (*Guasch et al. 2012*), but there is little evidence for the association of vitamin D administration and dyslipidemia.

Safety levels of administered Vitamin D (overdose)
Vitamin D toxicity is mostly described in the literature due to irrational or accidental use of vitamin D of more than 1000 µg (40,000IU)/day and serum 25(OH)D concentration above 250 nmol/L.

Recent trials that have investigated the effects of high unit doses of vitamin D given as single bolus or intermittent doses have provided reassurance on the safety of the newly proposed Vitamin D3 25,000IU and 50,000IU orodispersible films and soft capsules formulations. The tolerable upper intake level, i.e. the highest recommended daily intake established by the IOM for vitamin D in adults, is 4,000IU/day (IOM 2010).

In children, the tolerable upper intake level increases progressively from 1,000IU/day in infants (aged 0–6 months) to 4,000IU/day in children aged 9 years or older. A daily intake below this limit is unlikely to pose a risk of harm, but intakes above this limit may increase the risk of hypercalcemia (or clinical hypercalciuria in the absence of hypercalcemia) (*Glade 2012, Cranney et al 2008, Hatchcock et al 2007, Haines et al 2012, Schleck et al 2015, Mazahery 2015, Kearns et al 2015, McNally et al 2015, Kearns et al 2014*).

The neutral effect of long-term high-dose vitamin D on blood pressure was demonstrated in a study (*Scragg et al. 2014*).

Although high-dose vitamin D supplementation appears to be safe, numerous reports of accidental or ill-informed consumption of very large doses of vitamin D have been published (*Hathcock et al. 2007*). Many of the patients in these reports developed hypercalcemia with significant symptoms. Doses consumed in these reports were typically greater than 50,000IU/day, and the patients often had underlying comorbidities that predisposed them to develop vitamin D toxicity and hypercalcemia.

## IV.5 Risk Management Plan

The MAH has submitted a risk management plan, in accordance with the requirements of Directive 2001/83/EC as amended, describing the pharmacovigilance activities and interventions designed to identify, characterise, prevent or minimise risks relating to Calvigor.

**Table 2. Summary table of safety concerns as approved in RMP**

| | |
|---|---|
| Important identified risks | - None |
| Important potential risks | - None |
| Missing information | - None |

The member states agreed that routine pharmacovigilance activities and routine risk minimisation measures are sufficient for the risks and areas of missing information.

### IV.6 Discussion on the clinical aspects

This national procedure concerns a well-established use application for Calvigor. For this authorisation, reference is made to literature. One bioequivalence study was submitted. Risk management is adequately addressed. Altogether it is considered that efficacy of cholecalciferol in the initial treatment of clinically relevant vitamin D deficiency in adults has been established as the majority of studies in subjects showed statistically significant and clinically relevant results. Finally, it is considered that the safety issues that are identified are adequately addressed in the SmPC.

## V. USER CONSULTATION

The package leaflet (PL) has been evaluated via a user consultation study in accordance with the requirements of Articles 59(3) and 61(1) of Directive 2001/83/EC. The language used for the purpose of user testing the PL was English. The test consisted of a pilot test with two participants, followed by two rounds with ten participants each. The questions covered the following areas sufficiently: traceability, comprehensibility and applicability. The results show that the PL meets the criteria for readability as set out in the Guideline on the readability of the label and package leaflet of medicinal products for human use.

## VI. OVERALL CONCLUSION, BENEFIT/RISK ASSESSMENT AND RECOMMENDATION

Calvigor 25.000 IU and 50.000 IU, soft capsules and orodispersible films have a proven chemical-pharmaceutical quality. Calvigor has an adequate efficacy and safety profile and is considered widely established.

Bioequivalence has been shown to be in compliance with the requirements of European guidance documents.

The Board followed the advice of the assessors.

There was no discussion in the CMD(h). Agreement between member states was reached during a written procedure. The concerned member states, on the basis of the data submitted, considered that essential similarity has been demonstrated for Calvigor with the reference product, and have therefore granted a marketing authorisation. The decentralized procedure was finalised with a positive outcome on 4 January 2022.

## STEPS TAKEN AFTER THE FINALISATION OF THE INITIAL PROCEDURE - SUMMARY

| Procedure number* | Scope | Product Information affected | Date of end of procedure | Approval/ non approval | Summary/ Justification for refuse |
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# LITERATURE REFERENCES

AHFS Drug Information. Vitamin D Analogs General Statement. American Society of Health-System Pharmacists, Bethesda, 2010.

Allen L, Benoist B, Dary O, Hurrell R. Guidelines on food fortification with micronutrients. WHO. ISBN 9241594012

Biancuzzo RM, Young A, Bibuld D, Cai MH, Winter MR, Klein EK, Ameri A, Reitz R, Salameh W, Chen TC, Holick MF. Fortification of orange juice with vitamin D(2) or vitamin D(3) is as effective as an oral supplement in maintaining vitamin D status in adults. Am J Clin Nutr. 2010 Jun;91(6):1621-6. doi: 10.3945/ajcn.2009.27972. Epub 2010 Apr 28. PMID: 20427729; PMCID: PMC2869510.

Bruyère O, Deroisy R, Dardenne N, Cavalier E, Coffiner M, Da Silva S, De Niet S, Reginster JY. A phase IV, two-armed, randomized, cross-over study comparing compliance with once-a-month administration of vitamin D3 to compliance with daily administration of a fixed-dose combination of vitamin D3 and calcium during two 6-month periods. Osteoporos Int 2015;26(12):2863-68.

Calvo MS, Whiting SJ, Barton CN. Vitamin D fortification in the United States and Canada: current status and data needs. Am J Clin Nutr. 2004 Dec;80(6 Suppl):1710S-6S. doi: 10.1093/ajcn/80.6.1710S. PMID: 15585792.

Cavalier E, Jandrain B, Coffiner M, Da Silva S, De Niet S, Vanderbist F, Souberbielle JC. A Randomised, Cross-Over Study to Estimate the Influence of Food on the 25-Hydroxyvitamin $D_3$ Serum Level after Vitamin $D_3$ Supplementation. Nutrients 2016;8(5).

Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: Metabolism, Molecular Mechanism of Action, and Pleiotropic Effects. Physiol Rev 2016;96(1):365-408.

Cipriani C, Romagnoli E, Pepe J, Russo S, Carlucci L, Piemonte S, Nieddu L, McMahon DJ, Singh R, Minisola S. Long-term bioavailability after a single oral or intramuscular administration of 600,000 IU of ergocalciferol or cholecalciferol: implications for treatment and prophylaxis. J Clin Endocrinol Metab 2013;98(7):2709-15.

Coelho IM, Andrade LD, Saldanha L, Diniz ET, Griz L, Bandeira F. Bioavailability of vitamin D3 in non-oily capsules: the role of formulated compounds and implications for intermittent replacement. Arq Bras Endocrinol Metabol. 2010 Mar;54(2):239-43. doi: 10.1590/s0004-27302010000200022. PMID: 20485915.

Cranney A, Weiler HA, O'Donnell S, Puil L. Summary of evidence-based review on vitamin D efficacy and safety in relation to bone health. Am J Clin Nutr 2008;88:S5139.

Crocker JFS, Muhtadie SF, Hamilton DC, Cole DEC. The comparative toxicity of vitamin D metabolites in the weanling mouse. Toxicol Applied Pharmacol 1985;80:119-26.

Dalle Carbonare L, Valenti MT, Del Forno F, Piacentini G, Pietrobelli A. Vitamin D Daily versus Monthly Administration: Bone Turnover and Adipose Tissue Influences. Nutrients 2018;10(12).

de Jong A, Woods K, Van Gestel L, Suresh M, Porteous M. Vitamin D insufficiency in osteoporotic hip fracture patients: rapid substitution therapy with high dose oral cholecalciferol (vitamin D3). Acta Orthop Belg 2013;79(5):578-86.

De Niet S, Coffiner M, Da Silva S, Jandrain B, Souberbielle JC, Cavalier E. A Randomized Study to Compare a Monthly to a Daily Administration of Vitamin $D_3$ Supplementation. Nutrients 2018;10(6).

Diamond T, Wong YK, Golombick T. Effect of oral cholecalciferol 2,000 versus 5,000 IU on serum vitamin D, PTH, bone and muscle strength in patients with vitamin D deficiency. Osteoporos Int 2013;24(3):1101-5.

Dostal LA, Boass A, Toverud SU. Effects of high doses of vitamin D3 and 1,25-dihydroxyvitamin D3 in lactating rats on milk composition and calcium homeostasis of the suckling pups. Endocrinology 1983; 112:1631-8.

Drugdex Drug Evaluation. Vitamin D. 2007.
Glade MJ. A 21st century evaluation of the safety of oral vitamin D. Nutrition 2012;28(4):344-56.

Eason CT. The acute toxicity of cholecalciferol to the European rabbit, Oryctolagus cuniculus. Wildl Res 1993;20:173-6.

Fu L, Chen YH, Xu S, Ji YL, Zhang C, Wang H, Yu DX, Xu DX. Vitamin D deficiency impairs testicular development and spermatogenesis in mice. Reprod Toxicol 2017;73:241-9.

Helde Frankling M, Norlin AC, Hansen S, Wahren Borgström E, Bergman P, Björkhem-Bergman L. Are Vitamin $D_3$ Tablets and Oil Drops Equally Effective in Raising S-25-Hydroxyvitamin D Concentrations? A Post-Hoc Analysis of an Observational Study on Immunodeficient Patients. Nutrients. 2020 Apr 26;12(5):1230. doi: 10.3390/nu12051230. PMID: 32357579; PMCID: PMC7282031.

Grossmann RE, Tangpricha V. Evaluation of vehicle substances on vitamin D bioavailability: a systematic review. Mol Nutr Food Res 2010;54(8):1055-61.

Guasch A, Bullo M, Rabassa A. Bonada A, Del Castillo D, Sabench F, Salas-Salvadó J. Plasma vitamin D and parathormone are associated with obesity and atherogenic dyslipidemia: a cross-sectional study. Cardiovasc Diabetol 2012;11:149.

Haines ST, Park SK. Vitamin D supplementation: what's known, what to do, and what's needed. Pharmacotherapy 2012;32(4):354-82.

Haschek WM, Krook L, Kallfelz FA, Pond WG. Vitamin D toxicity. Initial site and mode of action. Cornell Vet 1978;68:324-64.

Hathcock JN, Shao A, Vieth R, Heaney R. Risk assessment for vitamin D. Am J Clin Nutr 2007;85 (1): 6-18.

Heaney RP. Vitamin D and calcium interactions: functional outcomes. Am J Clin Nutr 2008;88(2):541S-4S.

Hirano J, Ishii Y. Effects of vitamin K2, vitamin D, and calcium on the bone metabolism of rats in the growth phase. J Orthop Sci 2002;7:364-9.

Holick MF. High prevalence of vitamin D inadequacy and implications for health. Mayo Clin Proc 2006;81:353-73.

Holick MF, Chen TC. Vitamin D deficiency: a worldwide problem with health consequences. Am J Clin Nutr 2008;87(Suppl):1080-6.

Holick MF. Vitamin D status: measurement, interpretation and clinical application. Ann Epidemiol 2009;19(2):73-8.

Holvik K, Madar AA, Meyer HE, Lofthus CM, Stene LC. A randomised comparison of increase in serum 25-hydroxyvitamin D concentration after 4 weeks of daily oral intake of 10 µg cholecalciferol from multivitamin tablets or fish oil capsules in healthy young adults. Br J Nutr 2007;98(3):620-5.

Horii I, Takizawa S, Fujii T. Effect of 1,25-dihydroxyvitamin D3 on the female reproductive system in rats. J Toxicol Sci 1992;17:91-105

Ikezaki S, Nishikawa A, Furukawa F, Tanakamaru Z, Nakamura H, Mori H, Hirose M. Influences of long-term administration of 24R, 25-dihydroxyvitamin D3, a vitamin D3 derivative, in rats. J Toxicol Sci 1999;24:133-9.

IOM, US Institute of Medicines, “Dietary Reference Intakes for Calcium and Vitamin D”, 2010.

Kearns MD, Alvarez JA, Tangpricha V. Large, single-dose, oral vitamin d supplementation in adult populations: a systematic review. Endocr Pract 2014;20(4):341-51.

Kearns MD, Binongo JN, Watson D, Alvarez JA, Lodin D, Ziegler TR, Tangpricha V. The effect of a single, large bolus of vitamin D in healthy adults over the winter and following year: a randomized, double-blind, placebo-controlled trial. Eur J Clin Nutr 2015;69(2):193-7.

Kistler A. Effects of excess 1,25-dihydroxycholecalciferol in young rats. Arch Toxicol 1980;43:155-61.

Lagari VS, Gómez-Marín O, Levis S. Differences in vitamin D3 dosing regimens in a geriatric community-dwelling population. Endocr Pract 2012;18(6):847-54.

Lee JH, O'Keefe JH, Bell D, Hensrud DD, Holick MF. Vitamin D deficiency: an important, common and easily treatable cardiovascular risk factor? J Am Coll Cardiol 2008;52:1949-56.

Lehmann B, Pietzsch J, Kampf A, Meurer M. Human keratinocyte line HaCaT metabolizes 1α-hydroxyvitamin D3 and vitamin D3 to 1α,25-dihydroxyvitamin D3 (calcitriol). J Dermatol Sci 1998;18:118-27.

Leidig-Bruckner G, Roth HJ, Bruckner T, Lorenz A, Raue F, Frank-Raue K. Are commonly recommended dosages for vitamin D supplementation too low? Vitamin D status and effects of supplementation on serum 25-hydroxyvitamin D levels--an observational study during clinical practice conditions. Osteoporos Int 2011;22(1):231-40.

Makita T, Uotani Y, Izawa Y, Kawashima H, Hashimoto Y. Toxicologic studies of the hormonal form of vitamin D3: acute and subacute toxicity of 1α-hydroxycholecalciferol. Toxicol Appl Pharmacol 1976;36:323-9.

Mazahery H, Stonehouse W, von Hurst PR. The effect of monthly 50,000 IU or 100,000 IU vitamin D supplements on vitamin D status in premenopausal Middle Eastern women living in Auckland. Eur J Clin Nutr 2015;69(3):367-72.

McClain RM, Langhoff L, Hoar RM. Reproduction studies with 1α,25-dihydroxyvitamin D3 (calcitriol) in rats and rabbits. Toxicol Appl Pharmacol 1980;52:89-98.

McNally JD, Iliriani K, Pojsupap S, Sampson M, O'Hearn K, McIntyre L, Fergusson D, Menon K.. Rapid normalization of vitamin D levels: a meta-analysis. Pediatrics 2015;135(1):e152-66.

Morales O, Samuelsson MK, Lindgren U, Haldosen LA. Effects of 1α,25-dihydroxyvitamin D3 and growth hormone on apoptosis and proliferation in UMR 106 osteoblast-like cells. Endocrinology 2004;145:87-94.

Mortensen JT, Brinck P, Binderup L. Toxicity of vitamin D analogues in rats fed diets with standard or low calcium contents. Pharmacol Toxicol 1993;72:124-7.

Muindi JR, Modzelewski RA, Peng Y, Trump DL, Johnson CS. Pharmacokinetics of 1α,25-dihydroxyvitamin D3 in normal mice after systemic exposure to effective and safe antitumor doses. Oncology 2004;66:62-6.

Mullen PA, Bedford PGC, Ingram PL. An investigation of the toxicity of 1α-hydroxy-cholecalciferol to calves. Res Vet Sci 1980;27:275-9.

Natri AM, Salo P, Vikstedt T, Palssa A, Huttunen M, Kärkkäinen MU, Salovaara H, Piironen V, Jakobsen J, Lamberg-Allardt CJ. Bread fortified with cholecalciferol increases the serum 25-hydroxyvitamin D concentration in women as effectively as a cholecalciferol supplement. J Nutr. 2006 Jan;136(1):123-7. doi: 10.1093/jn/136.1.123. PMID: 16365070.

Nguyen TM, Lieberherr M, Fritsch J, Guillozo H, Alvarez ML, Fitouri Z, Jehan F, Garabedian M. The rapid effects of 1,25-dihydroxyvitamin D3 require the vitamin D receptor and influence 24-hydroxylase activity: studies in human skin fibroblasts bearing vitamin D receptor mutations. J Biol Chem 2004;279:7591-7.

Saleh L, Tang J, Gawinecka J, Boesch L, Fraser WD, von Eckardstein A, Nowak A. Impact of a single oral dose of 100,000 IU vitamin D3 on profiles of serum 25(OH)D3 and its metabolites 24,25(OH)2D3, 3-epi-25(OH)D3, and 1,25(OH)2D3 in adults with vitamin D insufficiency. Clin Chem Lab Med 2017;55(12):1912-21.

Schleck ML, Souberbielle JC, Jandrain B, Da Silva S, De Niet S, Vanderbist F, Scheen A, Cavalier E. A Randomized, Double-Blind, Parallel Study to Evaluate the Dose-Response of Three Different Vitamin D Treatment Schemes on the 25-Hydroxyvitamin D Serum Concentration in Patients with Vitamin D Deficiency. Nutrients 2015;7(7):5413-22.

Scragg R, Slow S, Stewart AW, Jennings LC, Chambers ST, Priest PC, Florkowski CM, Camargo CA Jr, Murdoch DR. Long-term high-dose vitamin D3 supplementation and blood pressure in healthy adults: a randomized controlled trial. Hypertension 2014;64(4):725-30.

Takács I, Tóth BE, Szekeres L, Szabó B, Bakos B, Lakatos P. Randomized clinical trial to comparing efficacy of daily, weekly and monthly administration of vitamin D3. Endocrine 2017;55(1):60-5.

Takeyama K, Kitanaka S, Sato T, Kobori M, Yanagisawa J, Kato S. 25-Hydroxyvitamin D3 1α-hydroxylase and vitamin D synthesis. Science 1997;277:1827-30.

Thacher TD, Fischer PR, Pettifor JM, Lawson JO, Isichei CO, Reading JC, Chan GM. A comparison of calcium, vitamin D, or both for nutritional rickets in Nigerian children. N Engl J Med 1999;341:563-68.

Tischler AS, Powers JF, Pignatello M, Tsokas P, Downing JC, McClain RM. Vitamin D3-induced proliferative lesions in the rat adrenal medulla. Toxicol Sci 1999;51:9-18.

Wacker M, Holick MF. Vitamin D - effects on skeletal and extraskeletal health and the need for supplementation. Nutrients 2013;5(1):111-48.

Whitfield GK, Dang HT, Schluter SF, Bernstein RM, Bunag T, Manzon LA, Hsieh G, Dominguez CE, Youson JH, Haussler MR, Marchalonis JJ. Cloning of a functional vitamin D receptor from the lamprey (Petromyzon marinus), an ancient vertebrate lacking a calcified skeleton and teeth. Endcrinology 2003;144:2704-16.

Zanello LP, Norman AW. Rapid modulation of osteoblast ion channel responses by 1α,25(OH)2-vitamin D3 requires the presence of a functional vitamin D nuclear receptor. Proc Natl Acad Sci USA 2004;101:1589-94.