# Open questions on bioequivalence: the case of cholecalciferol

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## ABSTRACT

Cholecalciferol or vitamin $D_3$ is an endogenous substance with the typical problems of bioavailability/bioequivalence of this class of substances. A previous trial has shown that, if administered orally at a dose of 800-10,000 IU and bioassayed as its 25-hydroxy metabolite, a slow absorption without an evident peak shape can be expected. However, if administered orally at a dose of 100,000-300,000 IU, this drug shows a well-defined peak shape of its metabolite 25-hydroxyvitamin $D_3$ (which is exceptionally long-lasting) with time to peak ($t_{max}$) at seven days and a kinetic profile requiring 84 days or more to restore pre-dose base-line levels. A recent paper has described a new bioassay of cholecalciferol in serum with a serum concentration-time curve after oral administration of 70 µg (2800 IU). An evident peak shape with $C_{max}$ of approximately 4 ng/mL was achieved at 12-24 h after administration. Baseline was restored after 96 h. However, these pharmacokinetic data were obtained in only one subject. This approach for bioassay of the parent drug seems to be more suitable for bioequivalence trials of cholecalciferol, even if more data concerning application are needed to adequately prepare a bioequivalence protocol. Therefore, the case of cholecalciferol appears to be extremely complex and remains one of the unanswered questions concerning bioequivalence that has not been taken into consideration in operating guidelines.

## Introduction

Cholecalciferol, also known as vitamin $D_3$, is marketed as an oily solution for oral or intramuscular (i.m.) administration, at strengths of 10,000 IU/mL, 100,000 IU/mL and 300,000 IU/mL (Figure 1).

When absorbed in the systemic circulation, cholecalciferol is first metabolized in the liver to 25-hydroxyvitamin $D_3$ (*i.e.* calcidiol), and then in the kidneys to 1,25-dihydroxyvitamin $D_3$ (*i.e.* calcitriol), which is the active form of cholecalciferol.[1]

The bioavailability of cholecalciferol is usually evaluated by bioassaying its hepatic metabolite, 25-hydroxyvitamin $D_3$[2] by means of various analytical methods, including liquid chromatography, radioimmunoassay, and electrochemiluminescence immunoassay.[3]

A more sensitive analytical procedure, *i.e.* ultra-high performance liquid chromatography coupled with tandem mass spectrometry, has allowed the active form of vitamin $D_3$ (1,25-dihydroxyvitamin $D_3$) to be evaluated; for the moment, this is only present in serum at a very low concentration.[4]

Recently, Xie *et al.*[5] have published a report of a bioassay of parent cholecalciferol after a chemical derivatization by tandem mass spectrometry. This approach had not previously been considered because cholecalciferol is inactive and studies focused on evaluating its metabolite. The last European Medicines Agency (EMA) guideline on bioequivalence[6] clearly states that bioequivalence (BE) requirements must be restricted to the parent compound, excluding further metabolites, either biologically active or inactive.

Our contract research organization (CRO) was asked to plan bioequivalence trials for approval of two oral generic formulations of cholecalciferol (10,000 and 300,000 IU/mL) both in oily solutions, according to the abridged new drug application (ANDA) procedure. This paper discusses the problems encountered in planning these two BE projects. Both approaches are considered: bioassay of 25-hydroxyvitamin $D_3$ and of the parent compound.

## Requirements of guidelines on bioequivalence

Section 5.1.2 of the 2001 EMA guidelines[6] and Appendix II of the 2010 EMA up-dated guidelines[7]

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discuss the BE waiver for oral solutions, with the restriction to aqueous solutions at the time of administration. Being an oily solution, cholecalciferol is excluded from this waiver.

Therefore, according to EMA guidelines, the application of the ANDA procedure to the two oral generic formulations of cholecalciferol 10,000 and 300,000 IU/mL would require a demonstration of BE, *i.e.* a comparison of test *vs* reference. In addition, considering that cholecalciferol is an endogenous substance, operating guidelines would require that the demonstration of BE should be applied to plasma concentrations obtained after a base-line subtraction.[7]

The latest EMA guideline on bioequivalence[7] requires the conclusions concerning bioequivalence be drawn only from $C_{max}$ and from $AUC_{0-t}$ of the parent compound, thus excluding data of either active or inactive metabolites.

In addition, as cholecalciferol is marketed at doses of 10,000, 100,000 and 300,000 IU/mL, any investigation into bioequivalence must take this into consideration, even if no definitive data on linear/non-linear kinetics are available.

## Expectations of bioassaying 5-hydroxyvitamin $D_3$

In the past, our CRO has investigated serum concentrations of 25-hydroxyvitamin $D_3$, after repeated oral administration of cholecalciferol 800 IU/day for four days,[8] and 1600 IU/day for 30 days (Marzo A., 2004, unpublished data). In these trials, serum concentrations of 25-hydroxyvitamin $D_3$ proved to be constant or to fluctuate around average base-line values, without showing any peak shape.

Ilahi *et al*. investigated the pharmacokinetics of 100,000 IU cholecalciferol, administered as a single oral dose to 30 healthy subjects and bioassayed 25-hydroxyvitamin $D_3$ in serum over a period of 112 days after administration.[2] Results showed a base-line concentration of 27.1±7.7 ng/mL (mean±SD), a $C_{max}$ of 42.0±9.1 ng/mL (mean±SD), $t_{max}$ of seven days and a period to restore base-line concentration of 84 days or more.[2]

Subtracting base-line values from $C_{max}$ gives a net increase of 42.0-27.1=14.9 ng/mL. At an oral dose of 10,000 IU, the net increase expected assuming linear pharmacokinetics should be 1/10 above 14.9, namely 1.5 ng/mL, *i.e.* hardly distinguishable from baseline. With lower doses, such as 800 or 1600 IU, the net increase is expected to be nil, as in fact previously observed by our group.[8]

A test *vs* reference comparison of bioequivalence of cholecalciferol at low oral doses of 1000-10,000 IU would produce serum concentrations of 25-hydroxyvitamin $D_3$ after administration that are indistinguishable from pre-dose baseline; thus, bioequivalence is not demonstrable either with or without base-line subtraction.

As with higher doses, at an oral dose of 100,000 IU the bioequivalence trial would involve the following issues.

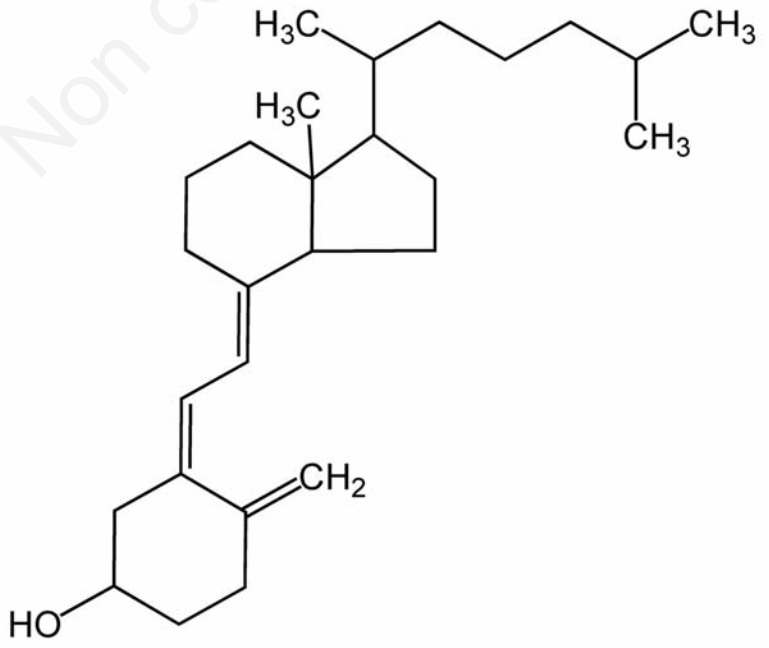


**Figure 1. Chemical structure of cholecalciferol.**

- With crossover design: i) period of blood sampling should last approximately four months, which is the period needed to explore the post-dose serum concentration-time behavior. A second study period could continue for a *washout* of 4-8 months, so that the whole crossover study should last 12-16 months; ii) the pool size hypothesized with an intrasubject coefficient of variation ($CV\%_{intra}$) of approximately 20%, namely without base-line subtraction, should be $0.20^2*392=16$ subjects; with the base-line subtraction, the pool size should be increased to $0.34^2*392=46$.[9]
- With the two-parallel group design: i) a pool size of approximately 40 subjects, without base-line subtraction, and approximately 100 subjects or more with base-line subtraction; ii) blood samples should be taken over at least a 3-month period.

## Expectations of bioassaying parent compound

Among the literature reporting studies on the parent compound, recently Xie *et al*.[5] describe the analytical conditions to bioassay cholecalciferol in serum using a deuterated internal standard. The authors explain this new approach to bioassay blood samples and report serum concentration of cholecalciferol of 2800 IU in one subject after a single dose. Baseline was approximately 1 ng/mL and the peak of around 4 ng/mL appeared 12-24 h after dosing.[5]

From the data of Xie *et al*.[5] we are not able to calculate the pool size of a bioequivalence trial as this requires the intrasubject coefficient of variation (CV%). If CV were similar to that found by other authors assaying 25-hydroxyvitamin $D_3$, the evaluation of pool size could produce similar values.

## Conclusions

Comparison of the two approaches, bioassay 25-hydroxyvitamin $D_3$ or the parent compound, leads to the following considerations.

At the low-dose range, *i.e.* 1000-10,000 IU, the bioassay of 25-hydroxy vitamin $D_3$ will only produce fluctuations of base-line concentrations, whereas the evaluation of the parent compound should produce a clear shape of serum concentration-time profile.

At higher strengths, *i.e.* 100,000/300,000 IU, the bioassay of 25-hydroxyvitamin $D_3$ will produce an extremely long-lasting serum concentration-time profile that is not useful for bioequivalence trials.

However, the absence of data concerning application does not allow us to consider the possible advantages of the bioassay of parent compound over the metabolite approach.

The long duration of the metabolite production (7 days after dosing) could lead us to hypothesize, also for cholecolciferol, a long-lasting decrease in serum concentrations that would parallel the long-lasting behavior of the metabolite, mainly at doses of 100,000 IU or over.

The need to subtract baseline is common to both approaches, which leads to an increase of approximately 3 times the pool size of volunteers in comparison with the procedure without base-line subtraction.

The specific request of the last EMA guideline on bioequivalence[5] to bioassay and to restrict the bioequivalence conclusion to the parent compound only favors the bioassay of cholecolciferol.

However, this approach, even if more appropriate, in this specific case has the disadvantage of poor literature application data, which would require a previous pilot trial to produce useful information to correctly plan the subsequent pivotal trial.

Given this, the US Food and Drug Administration (FDA) suggested two trials to study the bioequivalence of ergocalciferol, a structurally related vitamin $D_2$: one in fasting status and the other after food, in both cases with 50,000 IU and subtracting baseline.[10]

Considering the various doses involved, and the evidence that with increasing the dose, levels also increase and their profile is delayed without the evidence of linear/non-linear kinetic profile, the dose at which to demonstrate bioequivalence must be decided, selecting one that would cover all the dose range reported by the summary of product characteristics.

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