Introduction

Cholecalciferol, also known as vitamin D₃, 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 D₃ (i.e. calcidiol), and then in the kidneys to 1,25-dihydroxyvitamin D₃ (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 D₃² by means of various analytical methods, including liquid chromatography, radioimmunoassay, and electrochemiluminescence immunoassay.³

A more sensitive analytical procedure, i.e. ultrahigh performance liquid chromatography coupled with tandem mass spectrometry, has allowed the active form of vitamin D₃ (1,25-dihydroxyvitamin D₃) 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 bioequivalence⁶ 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 D₃ and of the parent compound.
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 requires the conclusions concerning bioequivalence be drawn only from $C_{\text{max}}$ and from $\text{AUC}_{0-t}$ 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 D₃

In the past, our CRO has investigated serum concentrations of 25-hydroxyvitamin D₃, after repeated oral administration of cholecalciferol 800 IU/day for four days, and 1600 IU/day for 30 days (Marzo A., 2004, unpublished data). In these trials, serum concentrations of 25-hydroxyvitamin D₃ 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 D₃ in serum over a period of 112 days after administration. Results showed a base-line concentration of $27.1\pm7.7$ ng/mL (mean$\pm$SD), a $C_{\text{max}}$ of $42.0\pm9.1$ ng/mL (mean$\pm$SD), $t_{\text{max}}$ of seven days and a period to restore base-line concentration of 84 days or more.2

Subtracting base-line values from $C_{\text{max}}$ 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 D₃ 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*392=16 subjects; with the base-line subtraction, the pool size should be increased to 0.34*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. 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.

From the data of Xie et al. 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 D₃, the evaluation of pool size could produce similar values.

**Conclusions**

Comparison of the two approaches, bioassay 25-hydroxyvitamin D₃ 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 D₃ 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 D₃ 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 cholecalciferol, 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 to bioassay and to restrict the bioequivalence conclusion to the parent compound only favors the bioassay of cholecalciferol.

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 D₃: one in fasting status and the other after food, in both cases with 50,000 IU and subtracting baseline.

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.

**References**

6. European Medicines Agency (EMA). Note for guidance


