Letter to the Editor

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Long-term stability of 25-hydroxyvitamin D: importance of the analytical method and of the patient matrix

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Twenty-five hydroxy vitamin D (25(OH)D) is known to be “solid as a rock” [1]. Lissner et al. [2] have showed that vitamin D metabolites in blood stored at 24 °C for up to 72 h remained intact. Other studies on the stability of 25(OH)D in plasma or serum that has undergone many freeze-thaw cycles have reported the same stability [3, 4]. Bruce Hollis has mentioned that he used the same pooled human 25(OH)D internal controls stored at –20 °C for 10 years with no detectable degradation of either compound (without showing any data) [5]. If other authors have presented stability results on long-term studies at –20 °C, but with a single method [6, 7], long-term storage studies at –80 °C including different methods and patients suffering from different diseases are however scarce. In this study, we evaluated the stability of serum 25(OH)D after 5 years of storage at –80 °C using three immuno-assays and a liquid chromatography coupled with 2 mass spectrometers in tandem (LCMS/MS) method in five different type of patients.

To perform this stability study, we used leftover serum samples obtained in 20 patients suffering from chronic kidney diseases (CKD), seven healthy subjects, 20 hemodialyzed patients, 14 third trimester pregnant women and 20 osteoporotic women. These samples had been measured in February 2016 in a single batch with Liaison XL (DiaSorin, Saluggia, Italy), Cobas e411 (Roche, Mannheim, Germany) and Lumipulse 1200 (Fujirebio, Tokyo, Japan) instruments and with the LCMS/MS from the CHU de Liège

certified by the Vitamin D Standardization-Certification Program (VDSCP) of the Centers for Disease Control and Prevention (CDC) [8]. These samples had been kept less than 1 month at –20 °C. Directly after measurement, the samples were put in a –80 °C storage device until February 2021 where they were all measured in a single batch after thawing and ultracentrifugation. The CHU de Liège laboratory is accredited against the ISO 15189 and participates to the DEQAS survey for the DiaSorin and Fujirebio assays and to the DEQAS and CAP surveys for the VDSCP traceable LCMS/MS.

The 25(OH)D concentration of the samples ranged from 1.4 to 71.5 ng/mL with the LCMS/MS in 2016 (median: 33.4 ng/mL, p25 and p75: 26.6–41.5 ng/mL, respectively). No sample contained quantifiable concentrations of 25(OH)D2. After 5 years of storage at –80 °C, we observed a median increase of 4.7% (95% confidence interval: 3.5; 6.3%) with the LCMS/MS, 8.5% (95% CI: 5.9; 10.2%) with the Fujirebio Lumipulse and 8.9% (95% CI: 6.2%; 10.7%) with the Roche Cobas. On the contrary, we observed a decrease of –6.6% (95% CI: –8.6%; –4.6%) with the DiaSorin Liaison XL (Figure 1).

Figure 2 presents the percentages of variation according to the patients’ health status. The results obtained with the LCMS/MS method after storage did not seem to be affected by the health status of the patient: the changes observed in CKD patients and healthy subjects were not different from 0% whereas an increase of approximately 5% was observed for the other subgroups. For the Lumipulse, an important increase of 20 and 17% was observed in CKD patients and pregnant women, respectively, whereas a significant lower increase of 5% was observed in the other subgroups. With the Cobas, results observed in pregnant women also showed an important increase of about 20% whereas a rise of about 5% was observed in the other categories of patients (excepted in healthy subjects where the change was not different from 0). Finally, the Liaison XL results were particularly affected by CKD and hemodialyzed patients with a decrease higher than 10%, but stability was not influenced by pregnancy or in healthy individuals (Figure 2).

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Figure 1: Mean percentage of 25(OH)D variation after five years of storage at -80°C for five years according to the analytical method.

To the best of our knowledge, we present here for the first time the results of a long-term (5 years) stability study of serum samples kept at -80°C according to the health status of the subjects. Of particular interest, we also used three different immunoassays and a VDSCP traceable LCMS/MS to measure 25(OH)D in this study. The first valuable observation was that two immunoassays (Fujirebio and Cobas) as well as the LCMS/MS method presented a slight but statistically significant increase whereas the Liaison XL presented a significant decrease over time. Many reasons can explain why the concentration of a sample can vary over storage time. The first one could be analytical variation, notably linked to changes in reagent versions over time. The LCMS/MS we used in 2016 was the commercially

Figure 2: Percentage of variations of 25(OH)D after five years of storage at -80°C according to the method and health status of the subjects.

available Chromsystems method which was certified by the CDC VDSCP since 2015. In 2021, we used a in-house developed LCMS/MS method [8] which was also certified since 2019 by the CDC VDSCP. Using a highly controlled VDSCP traceable LCMS/MS minimizes (without totally eliminating) the analytical variation over time. In this study, we also used the version II of the Roche assay which had recently been launched and was still available in 2021. For the two other immunoassays, we are not aware of any significant changes in the kit formulation. Other reasons potentially explaining a difference over time is the concentration of the sample due to loss of water and, of course, degradation of the analyte. The LCMS/MS and two immunoassays confirm that a slight concentration has occurred in the samples whereas a third immunoassay behaved differently. We have no clear explanation for this phenomenon, but this could be linked to the variation of the sample matrix. Indeed, the second interesting observation of this study is that we presented the variation over time according to the health status of the subjects. If the LCMS/MS was poorly affected, as expected, this was not really the case of the immunoassays. For the Lumipulse, CKD patients (but not hemodialyzed patients) and pregnant women clearly behaved differently. Variations in the Cobas were particularly perceptible in pregnant women whereas 25(OH)D measured in CKD and hemodialyzed patients was particularly decreased over time with the Liaison XL. Matrix effects are known to affect immunoassays for 25(OH)D measurement, especially in third trimester pregnant women and in patients suffering from chronic kidney diseases [9–13]. These results show that vitamin D binding protein (DBP) or uremic media could have changed over storage time and have affected some immunoassays at various degrees. In healthy subjects, however, the results remained in the $\pm 5\%$ of the original value with all the methods.

In conclusion, our results show that the variation of 25(OH)D concentrations after 5 years of storage at -80°C is small, but statistically significant. It could rather be due to sample concentration than to sample degradation even if the probable variation of the sample matrix over time could affect the immunoassays differently. The LCMS/MS presents similar results over time in all the subjects' categories whereas this is not the case with the immunoassays.

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Competing interests: EC is consultant for DiaSorin and Fujirebio (methods used in this study).

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Ethical approval: The local Institutional Review Board deemed the study exempt from review.

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