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Impact of ultra-low temperature long-term storage on the preanalytical variability of twenty-one common biochemical analytes

https://doi.org/10.1515/cclm-2022-0063
Received January 22, 2022; accepted April 11, 2022;
published online April 26, 2022

Abstract

Objectives: Retrospective studies frequently assume analytes long-term stability at ultra-low temperatures. However, these storage conditions, common among biobanks and research, may increase the preanalytical variability, adding a potential uncertainty to the measurements. This study is aimed to evaluate long-term storage stability of different analytes at <−70 °C and to assess its impact on the reference change value formula.

Methods: Twenty-one analytes commonly measured in clinical laboratories were quantified in 60 serum samples. Samples were immediately aliquoted and frozen at <−70 °C, and reanalyzed after 11 ± 3.9 years of storage. A change in concentration after storage was considered relevant if the percent deviation from the baseline measurement was significant and higher than the analytical performance specifications.

Results: Preanalytical variability (CV_P) due to storage, determined by the percentage deviation, showed a noticeable dispersion. Changes were relevant for alanine aminotransferase, creatinine, glucose, magnesium, potassium, sodium, total bilirubin and urate. No significant differences were found in aspartate aminotransferase, calcium, carcinoembryonic antigen, cholesterol, C-reactive protein, direct bilirubin, free thryroxine, gamma-glutamyltransferase, lactate dehydrogenase, prostate-specific antigen, triglycerides, thyrotropin, and urea. As nonnegligible, CV_P must remain included in reference change value formula, which was modified to consider whether one or two samples were frozen.

Conclusions: After long-term storage at ultra-low temperatures, there was a significant variation in some analytes that should be considered. We propose that reference change value formula should include the CV_P when analyzing samples stored in these conditions.

Keywords: biobank; long-term storage; magnitude stability; preanalytical variability; reference change value; ultra-low temperatures.

Introduction

The use of archived and frozen stored samples for analytical assays is a very common practice in clinical and research laboratories. Many retrospective clinical studies measure analytes in thawed samples that have been previously stored frozen during very different times [1, 2]. These samples are usually stored for short periods. However, in some cases, samples derive from long-term storage in controlled conditions such as in biobanks [3–5]. Both handling and storage conditions determine sample quality after thawing [6, 7]. In fact, some international standards, such as ISO 15189 for clinical Laboratories [8] and ISO 20387 for biobanking institutions [9], establish specific requirements about handling and long-term preservation of the samples to minimize preanalytical variability, although it cannot be completely eliminated. Consequently, it is necessary to guarantee that the analyte to be measured is stable throughout the whole storage period to provide reliable results [7].

It is known that several factors affect stability of the different analytes in stored samples [7]. Among them, temperature and time may be the most relevant ones [3, 6]. Several studies have addressed the stability of analytes in specimens stored at −20 °C [10, 11] and during shorts periods of time (less than one year) [10]. For some analytes not stable at these temperatures, some authors recommend the storage at ultra-low temperatures, such as <−70 °C [12].

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However, there is little evidence about long-term stability at <−70 °C [1, 13, 14], like in biobanks or research collections [1, 15].

The uncertainty of the analyte quantification is determined by the sum of biological, preanalytical, and analytical variabilities [16, 17]. In some experiments such as those addressed for biological variation calculation [18], preanalytical conditions are standardized. Consequently, the preanalytical component could be considered constant and its variability could be interpreted as negligible. However, when using stored frozen samples the preservation conditions could introduce a new and not constant preanalytical variability and a potential added uncertainty to the measurement [13].

The reference change value (RCV) enables the assessment, at a predetermined probability, of the change between two successive results on the same patient, not explained by biological and analytical variability [19]. Its mathematical formula includes the coefficient of analytical variation (CV_A) and the within-subject biological variation (CV_I) [20]. For the estimation of RCVs, it is usually assumed that measurements are done with the same method and preanalytical component is thus considered negligible. However, this could not be a valid assumption if one of the samples suffers a different process, such as being kept frozen for a certain period of time and then thawed for analysis.

The first aim of this study was to examine the long-term freezing stability of different common biochemical analytes at <−70 °C. We also aimed to assess if the most widely used RCV formula should take into account this preanalytical variability.

Materials and methods

Samples and storage conditions

Samples were collected from 2005 to 2015 in Vacutainer® serum tubes with separator gel and immediately delivered to the laboratory. Samples were allowed to clot for 30 min at room temperature, centrifuged at 2000 *g* for 10 min. Serum was aliquoted into Eppendorf® tubes, frozen at <−70 °C in a freezer (MDF-U53V, Sanyo) during more than 5 years (11 ± 3.9 years, Table 1) with external monitoring temperature control. A total of 60 stored serum specimens from different patients were selected based on previous analytical data to cover analytical measurement range. Samples from donors with ages <18 years were excluded. Also, to avoid interference, lipemic or hemolyzed samples were excluded from the study.

At the time of reanalysis, samples were thawed at room temperature and centrifuged at 18 °C and 2000 *g* for 10 min (Eppendorf Centrifuge 5810R). All the analysis were immediately performed by singlicate.

Sample collection and storage was approved by the local Ethics Committee (project 14/2004 and 111/2010 and collection ISCIII C.0003132). Samples were anonymized, so clinical factors, ethnicity, sex, or age have not been considered in this study.

Biochemical measurements

To minimize analytical bias and to avoid the possible lack of interchangeability, only analytes that could be measured by the same methodology and the same provider than in the initial quantifications were selected. All measurements were performed in a Cobas 8000 analyzer (Roche Diagnostics, Switzerland) according to the manufacturer's instructions, except for baseline measurements of creatinine, potassium, sodium, urate, and urea, which were performed in a Modular P analyzer, also from Roche Diagnostics. Quality control procedures were applied and verified on a daily basis before analytical process using two distinct control materials (PreciControl, Roche Diagnostics).

Stability of the analytical performance for each analyte was evaluated through the evolution of the coefficient of analytical variation (CV_A). This CV_A was calculated as the mean of the coefficients of variation of the quality control results within a year period corresponding to the paired analysis dates (Table 1).

A detailed list of analytes and measurement methods are presented in Table 1. The same reagents and consumables were used and there was no drift of internal quality control over the course of the study period.

Statistical analysis

Shapiro–Wilk normality tests were performed for each magnitude, for both initial and after long-term-storage results. Normally distributed variables were compared with paired two tail t tests. For data non-normally distributed, non-parametric Wilcoxon tests were used.

The percent deviation (PD%) defined the deviation due to the loss of stability during storage [21]. The PD% for each analyte was calculated using the following equation [22]:

$$\mathrm{PD\%} = 100 \times \frac{C_T - C_0}{C_0}$$

Being the concentration of the analytes obtained in the initial measurement in fresh samples C_0 at time 0, and C_T the concentration measured at time T after long-term storage at <−70 °C. Hence, positive values of PD% indicate an increase of the concentration of a magnitude and negative values imply decreasing results. PD% values were compared to 0, which implies no change at all [22].

We considered the analytical performance specification (APS) for imprecision based on biological variation at the desirable level, as a criteria value for interpreting the effect of the observed PD%. This APS was calculated as $0.5 \times CV_I$ [23], where CV_I was obtained from the European Federation of Clinical Chemistry and Laboratory Medicine Biological Variation Database [24].

We considered an analytical relevant effect in concentration after long-term storage if: (1) PD% was significant and (2) median PD% absolute value was higher than APS.

Table 1: Magnitudes and characteristics of measurement methods.

Analyte	n	Years stored	Method	Analytical range	Mean CV_A, %
ALT, µkat/L	15	6	Enzyme-kinetic assay acc. to IFCC without pyridoxal phosphate activation	0.08, 11.7	1.9
AST, µkat/L	15	6	Enzyme-kinetic assay acc. to IFCC without pyridoxal phosphate activation	0.08, 11.7	3.3
Calcium, mmol/L	15	6	Colorimetric assay with NM-BAPTA and EDTA complexes	0.2, 5	1.4
CEA, µg/L	15	6	Electrochemiluminescence immunoassay	0.3, 1,000	3.3
Cholesterol, mmol/L	15	6	Enzymatic colorimetric test, Trinder endpoint reaction	0.1, 20.7	1.8
Creatinine, µmol/L	15	>10	Kinetic colorimetric assay, Jaffé method	15, 2,200	2.5
CRP, nmol/L	15	6	Particle-enhanced immunoturbidimetric assay	5.7, 3,332	2.4
Direct bilirubin, µmol/L	15	6	Colorimetric diazo method	1.2, 236	1.9
Free T4, pmol/L	19	6	Electrochemiluminescence immunoassay	0.5, 100	3.2
GGT, µkat/L	15	6	Enzymatic colorimetric assay acc. to IFCC	0.05, 20	1.7
Glucose, mmol/L	15	6	Enzymatic colorimetric with hexokinase	0.85, 45	2.2
LDH, µkat/L	15	6	Enzyme-kinetic assay acc. to IFCC	0.17, 16.7	1.7
Magnesium, mmol/L	15	6	Colorimetric endpoint method with diazonium salt	0.1, 2	2.7
Potassium, mmol/L	30	>10	Indirect potentiometry	1.5, 10.0	0.9
PSA, µg/L	18	8	Electrochemiluminescence immunoassay	0.006, 100	3.1
Sodium, mmol/L	30	>10	Indirect potentiometry	80, 180	1.1
Total bilirubin, µmol/L	15	6	Colorimetric diazo method	2.5, 650	3.4
Triglycerides, mmol/L	15	6	Enzymatic colorimetric test, Trinder endpoint reaction	0.1, 10	1.9
TSH, mUI/L	15	6	Electrochemiluminescence immunoassay	0.005, 100	3.1
Urate, µmol/L	30	>10	Enzymatic colorimetric test with uricase	11.9, 1,487	1.6
Urea, mmol/L	30	>10	Kinetic test with urease and glutamate dehydrogenase	0.5, 40	2.3

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CEA, carcinoembryonic antigen; CRP, C-reactive protein; Free T4, free thyroxine; GGT, gamma-glutamyl transferase; LDH, lactate dehydrogenase; n, number of samples; PSA, prostate-specific antigen; TSH, thyroid stimulating hormone; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine; NM-BAPTA, 5-nitro-5'-methyl-(1,2-bis(*o*-aminophenoxy) ethan-*N*,*N*,*N*′,*N*′-tetraacetic acid; alternative name for NM-BAPTA, 5-nitro-5'-methyl-BAPTA; EDTA, ethylenediaminetetraacetic acid.

The RCV were calculated using the following equation described by Harris and Yasaka [19]:

$$RCV = \sqrt{2} \times Z \times \sqrt{\left(CV_I^2 + CV_A^2\right)}$$

where Z-score is the two-sided statistic parameter with a value of 1.96 for a 95% confidence interval.

All the calculations and graphs were performed using GraphPad Prism 5 Software. $p<0.05$ was considered statistically significant.

Results

Biomarkers stability after long term storage

The stability of the performance of the analytical systems remained constant throughout the years, without significant changes in imprecision (CV_A) for all studied analytes (data not shown). The medians and ranges of the initial concentrations and after the storage period of all the studied analytes are shown in Table 2.

No significant differences in concentrations were found after long-term storage in aspartate aminotransferase, calcium, carcinoembryonic antigen (CEA), C-reactive protein, free thyroxine, gamma-glutamyltransferase, glucose, lactate dehydrogenase, prostate-specific antigen (PSA), total bilirubin and thyrotropin (thyroid stimulating hormone, TSH). However, a statistically significant decrease ($p<0.05$) between the initial concentration and after long-term storage was observed in alanine aminotransferase (ALT), cholesterol, creatinine, direct bilirubin, magnesium, urea, and urate. By contrast, potassium, sodium, and triglycerides levels were significantly ($p<0.05$) higher after storage compared to initial concentrations.

In relation to this, we analyzed the change in the measurand concentrations after storage as PD% (Table 2). We observed that there was a noticeable dispersion in the PD %, as shown in Figure 1. PD% was significant for the same analytes in which we observed statistical different concentration except for urea, glucose total bilirubin, and TSH.

To evaluate the relevance of these changes in concentration due to storage, we further assessed the median PD% for each analyte in relation to the corresponding APS [23]. The absolute value of median PD% was higher than the APS for ALT, creatinine, glucose, magnesium, potassium, sodium, total bilirubin and urate (Table 2).

In those analytes that presented relevant changes due to long-term storage, the effect of freezing for a very short time (7 days) was also analyzed (Supplementary material). As expected, there were no statistical differences between the concentration of analytes measured at time 0 and after 7 days of storage at <−70 °C. In addition, changes due to

Table 2: Variation of the concentrations of 21 biochemical analytes after prolonged storage at <−70 °C and relevance of changes.

Analyte	CV_I, %	Median initial concentration (range)	Median storage concentration (range)	p-Value	Median PD% (Q1, Q3)	p-Value	$\lvert PD\% \rvert > 0.5 \times CV_I$	Relevant effect of storage
ALT, µkat/L	19.4	0.43 (0.23, 3.58)	0.25 (0.12, 2.40)	0.004	−36.8 (−49.1, −24.4)	<0.001	Yes	Yes
AST, µkat/L	12.3	0.45 (0.27, 2.02)	0.38 (0.23, 2.02)	0.076	−5.0 (−11.0, 1.1)	0.101	No	No
Calcium, mmol/L	2.1	2.29 (2.12, 2.54)	2.29 (2.12, 2.39)	0.484	0.0 (−3.2, 1.1)	0.514	No	No
CEA, ng/mL	12.7	45.6 (2.9, 420.6)	42 (2.1, 394.5)	0.326	−5.6 (−11.8, 0.8)	0.268	No	No
Cholesterol, mmol/L	5.95	5.6 (3.0, 7.4)	5.4 (3.0, 7.2)	0.003	−1.7 (−9.6, −0.8)	0.002	No	No
Creatinine, µmol/L	5.97	97.3 (35.4, 822.3)	88.4 (53.1, 786.9)	<0.001	−9.1 (−14.2, −6.6)	<0.001	Yes	Yes
CRP, nmol/L	42.4	211 (27, 1,470)	202 (20, 1,430)	0.119	−3.8 (−11.8, 0.8)	0.068	No	No
Direct bilirubin, µmol/L	29.8	5.8 (1.9, 22.1)	3.4 (1.5, 18.1)	0.013	−17.8 (−28.6, −12.5)	<0.001	No	No
Free T4, pmol/L	5.7	23.1 (11.2, 33.2)	23.3 (11.7, 34.7)	0.326	−0.9 (−5.4, 3.9)	0.258	No	No
GGT, µkat/L	13.4	1.9 (0.4, 10.5)	1.7 (0.3, 10.5)	0.440	0 (−9.5, 2.6)	0.102	No	No
Glucose, mmol/L	5.6	5.8 (4.1, 9.5)	6.5 (4.2, 10.1)	0.079	6.4 (4.4, 9.9)	0.007	Yes	Yes
LDH, µkat/L	11	3.3 (2.4, 19.5)	3.3 (2.5, 15.2)	0.852	0.6 (−4.6, 5.6)	0.787	No	No
Magnesium, mmol/L	3.6	0.86 (0.62, 0.99)	0.82 (0.59, 0.99)	0.036	−2.9 (−4.8, 0.0)	0.011	Yes	Yes
Potassium, mmol/L	4.6	4.3 (3.6, 5.4)	4.4 (3.9, 5.4)	0.004	5.5 (0.9, 7.9)	0.003	Yes	Yes
PSA, µg/L	18.1	38.5 (0.2, 65.7)	38.2 (0.2, 61.3)	0.061	−1.2 (−3.9, 0.2)	0.162	No	No
Sodium, mmol/L	0.6	140.5 (131.0, 146.0)	141.9 (132.0, 145.3)	<0.001	1.4 (0.3, 2.3)	<0.001	Yes	Yes
Total bilirubin, µmol/L	21.8	11.5 (3.1, 28.4)	9.2 (2.6, 23.3)	0.076	−12.9 (−21.7, −7.5)	0.007	Yes	Yes
Triglycerides, mmol/L	19.9	1.05 (0.79, 2.18)	1.23 (0.84, 2.34)	<0.001	7.5 (5.7, 17.2)	<0.001	No	No
TSH, µUI/mL	29.3	2.60 (0.04, 19.8)	2.1 (0.02, 20.1)	0.337	−8.2 (−21.0, 3.4)	0.014	No	No
Urate, µmol/L	8.6	288 (149, 547)	274 (137, 512)	<0.001	−5.5 (−7.1, −3.8)	<0.001	Yes	Yes
Urea, mmol/L	12.1	7.5 (3.3, 22.8)	7.2 (3.7, 21.4)	0.036	−2.2 (−5.5, 2.7)	0.200	No	No

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CEA, carcinoembryonic antigen; CRP, C-reactive protein; CV_I, within-subject biological variation; Free T4, free thyroxine; GGT, gamma-glutamyl transferase; LDH, lactate dehydrogenase; PD%, percent deviation; PSA, prostate-specific antigen; TSH, thyroid stimulating hormone.

7-days storage (7-days PD%) neither exceed the APS nor the CV_A for any of the analytes measured except for potassium and ALT, which were lower than 1.5 CV_A.

Storage effect in the estimation of the reference change value

Finally, we aimed to investigate if this variability had an impact in RCV assessment.

Due to the characteristics of our model, all other preanalytical variability is negligible except for the variability due to long-term storage (V_P). Consequently, as this affects the total variability (V_T), it should remain included with the analytical (V_A) and the intraindividual (V_I) variability:

$$V_T = V_P + V_A + V_I$$

and, expressed in terms of CV:

$$CV_T^2 = CV_P^2 + CV_A^2 + CV_I^2$$

where CV_A is the coefficient of analytical variation, CV_I is the within-subject biological variation, and CV_P refers to the variability due to the long-term storage, which can be assimilated to the median PD%.

If we consider preanalytical variability as a non-negligible component, CV_P should remain included in the formula (RCV_P) [20]:

$$RCV_P = \sqrt{2} \times Z \times \sqrt{CV_P^2 + CV_A^2 + CV_I^2}$$

if both samples compared were kept frozen.

In addition, the formula RCV_P should be adjusted if one sample was not subjected to storage, as follows:

$$RCV_P = \sqrt{2} \times Z \times \sqrt{CV_P^2/2 + CV_A^2 + CV_I^2}$$

The variability in the change of concentration due to the long-term storage at <−70 °C was determined by PD%, so PD% is equal to CV_P. Using this parameter, then we calculated the corresponding the RCV_P for the analytes,

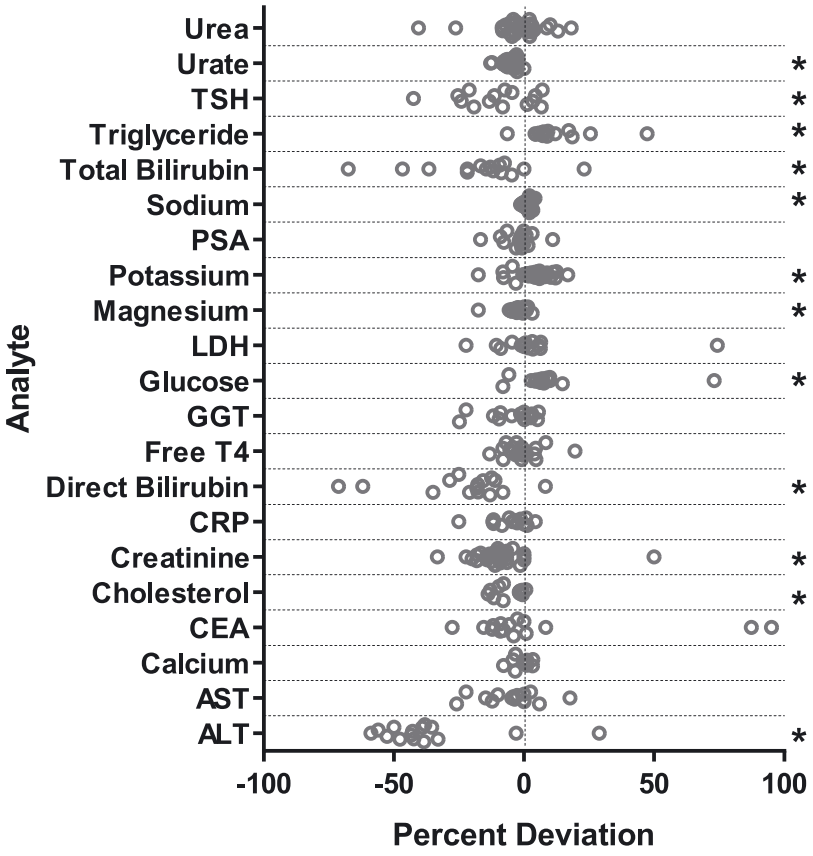

Figure 1: Percent of deviation in the concentration of the analytes after long-term storage at <−70 °C.
*p-Value<0.05 in relation to the initial value.

either if only one or the two samples were kept frozen (Table 3). For example, if one sample is obtained from a biobank and the other is recent, the difference in CEA concentration to be considered significant should be higher than 38.0% (RCV_P), no higher than 36.4% (RCV). As expected, those analytes with significant PD% showed much higher RCV_P than RCV due to the instability in these conditions.

Discussion

This study evaluated the effects of long-term storage at very low temperature on several analytes commonly used in clinical routine. To the best of our knowledge, this is the first study evaluating the impact of ultra-low temperature and long-term preservation as it happens in biobanks, on different biochemical analytes. We used the percent deviation, PD%, to assess the variation [21, 22] and the APS to estimate the relevance of the alteration [23]. Accordingly, we have shown that long-term storage at <−70 °C can cause a relevant effect on the concentrations of ALT, creatinine, glucose, magnesium, potassium, sodium, total bilirubin and urate, as the deviations were significant and higher than the APS.

Previous studies have also provided insights into the effects of various preanalytical conditions on biomarker stability, including centrifugation, protease inhibitors, temperature, and time [6, 7]. Our results agree with some previous studies and expand these previous findings to a storage time up to 15 years [12, 23, 25]. Given that our results were obtained with unchanged methodology, and similar analytical performance, we suggest that the changes in analytes concentrations over time could be the effect of the progressive alteration of the samples despite low-temperature storage. It is interesting to observe, that the concentrations can decrease, but also increase, as described by other authors for sodium and potassium [3, 26]. Alteration in analyte concentrations after long-term storage could be explained by molecular aggregation, precipitation or degradation, ice damage, oxidation, dehydration, reaction with other molecules, or even interaction with the walls of the cryotubes [7, 27]. Biochemical degradation of proteins can alter their activity and/or antibody reactivity. Although these processes can be negligible during short periods, they can be relevant after many years of storage [28].

Table 3: Reference change value including storage variability.

Analyte	RCV, %	RCV_P, %	
		One sample kept frozen	Two samples kept frozen
ALT	54.0	90.1	115.4
AST	35.3	36.6	37.9
Calcium	7.0	7.0	7.0
CEA	36.4	38.0	39.5
Cholesterol	17.2	17.5	17.9
Creatinine	17.9	25.3	30.9
CRP	117.7	118.0	118.2
Direct bilirubin	82.8	89.8	96.4
Free T4	18.1	18.2	18.3
GGT	37.4	37.4	37.4
Glucose	16.7	20.9	24.3
LDH	30.9	30.9	30.9
Magnesium	12.5	13.7	14.8
Potassium	13.0	16.9	20.1
PSA	50.9	51.1	51.0
Sodium	3.5	4.4	5.1
Total bilirubin	61.2	66.2	70.8
Triglycerides	55.4	57.3	59.2
TSH	81.7	83.2	84.8
Urate	24.2	26.5	28.6
Urea	34.1	34.4	34.7

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CEA, carcinoembryonic antigen; CRP, C-reactive protein; Free T4, free thyroxine; GGT, gamma-glutamyl transferase; LDH, lactate dehydrogenase; PSA, prostate-specific antigen; RCV, reference change value, RCV_P, reference change value with preanalytical variability; TSH, thyroid stimulating hormone.

In our model preanalytical variables, except storage, and biological variation are cero as aliquots derived from the same sample. However, we observed an important variation in analyte concentrations due to storage that should be considered when interpreting the results. Our results agree with others showing that PSA was stable after storage at <–70 °C for 7 years, but there was a systematic and random error that increased with the concentration [13]. In addition, in the CALIPER study, direct bilirubin was determined stable at <–70 °C in a period of 13 months and an increased CV was observed in samples with low analyte concentration [1]. We have gone a step further and applied this observed variation to estimation of a significant change.

Furthermore, as PD% exceeded the CV_A the variation found in the analytes concentrations would not be due to the analytical imprecision but to the effect of the long-term storage at <–70 °C. This variation due to the storage conditions (CV_P) could change depending on the temperature and time of storage. In addition, no variation was observed when storage was reduced to 7 days at <–70 °C, so we can rule out that the alterations van be explained by the process of freezing and thawing. Other CV_P should be calculated for storage at different temperatures, such as –20 °C. In addition, it is possible that the same magnitude in other type of matrix can also show different CV_P.

The formula of the RCV has been commonly used to compare concentrations of a magnitude in the same samples after a period of time, such as for method comparison or new interference studies, or for retrospective analysis of consecutive results [29]. This formula is usually simplified to not include the variability due to preanalytical conditions. However, in numerous situations, frozen samples are thawed and used for analytical comparison with previous results. Furthermore, modifications in analyte concentrations due to long-term storage could be especially important in omics studies. As we have shown before, there is an added variability when measuring the concentration of an analyte after long-term storage. Therefore, we consider that when applied to results obtained from samples archived in these conditions, the RCV should include this variable as this preanalytical variation cannot be minimized or neglected. For this reason, we propose this formula for samples that have been stored at very low temperature for a long term period [20]. We used the classical formula of Harris and Yasaka to estimate the RCV [19], but similar considerations can be applied when using other approaches for the calculation [30, 31].

A limitation of this work is that samples were not analyzed in a single run to avoid analytical bias and that the analyses were not performed with replicates [21]. However, the analytical imprecision remained stable throughout the years as observed from the internal quality control data. Nevertheless, we cannot fully rule out some CV_A underestimation. Although the imprecision could be higher for low concentrations, the concentrations of the quality control material corresponded to the range of concentrations of the analyzed samples.

In conclusion, our study demonstrates that long-term storage at <–70 °C can result in a significant change in the levels of several common biochemical analytes. In addition, storage causes a variation in the concentration of the analytes that should be considered when using the RCV if at least one of the samples has been stored frozen for a long time. Similarly, this preanalytical variability should be studied for analytes' concentrations obtained from samples stored at other temperatures and other conditions.

Acknowledgments: We thank Sonia Irisarri, Ruben del Campo, and Dra. María Romero for their technical assistance.
Research funding: This study was partially supported by Roche Diagnostics, Barcelona, Spain.
Author contribution: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Competing interests: Authors state no conflict of interest.
Informed consent: Not applicable.
Ethical approval: Sample collection and storage was approved by the local Ethics Committee (project 14/2004 and 111/2010 and collection ISCIII C.0003132).

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Supplementary Material: The online version of this article offers supplementary material (https://doi.org/10.1515/cclm-2022-0063).