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Elucidation of stability profiles of common chemistry analytes in serum stored at six graded temperatures

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Abstract

Background: Many reports address the stability of biochemical analytes in serum. However, studies covering a wide range of storage temperatures are unavailable. Using equipment enabling precise temperature control, we investigated the effect of six different storage temperatures on serum analytes.

Methods: Serum specimens from seven healthy volunteers were obtained and divided into multiple aliquots for storage at -30 , -20 , -10 , 0 , 4 , and 25 °C. On days 1, 3, 7, 14, 28 and 56, the aliquots stored at each temperature were relocated to a deep freezer maintained at -80 °C. On day 60, all aliquots were measured collectively for 13 major chemistry analytes.

Results: (1) At 25 °C, alanine aminotransferase (ALT), creatine kinase (CK), aspartate aminotransferase (AST) and total bilirubin (TBil) were very unstable especially on day 7 and later. (2) At ≤ 4 °C, alkaline phosphatase (ALP), γ -glutamyltransferase (GGT), amylase (AMY), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), triglyceride (TG), TBil and complement component-4 (C4) were generally stable and were very stable at 25 °C until day 14. (3) Between -20 and 4 °C, especially at -10 °C, test results of ALT, AST and lactate dehydrogenase (LDH) showed prominent decreases, but their stability was greatly improved at -30 °C. (4) In contrast, the value of complement component-3 (C3) increased at ≥ -20 °C. (5) At -30 °C, test results of all analytes were generally very stable except for ALT and CK, which showed noticeable reductions in activity after 14 days.

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Conclusions: This is the first study to assess the stability of serum analytes at six graded temperatures simultaneously. Each analyte has a unique stability pattern for a range of temperatures.

Keywords: bilirubin; complement C3; complement C4; deep freeze; enzymes; HDL-cholesterol; LDL-cholesterol; stability; triglyceride.

Introduction

There have been many studies on the effects of specimen storage at various temperatures on laboratory test results. Short-term stability of human serum specimens stored in a refrigerator (at 4 °C) and/or at room temperature (RT) have been compared for common biochemical analytes [1–4]. Most reports showed variable degrees of instability of the test results for lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and complement component-3 (C3) at 4 °C and RT, but the results for creatine kinase (CK) and alkaline phosphatase (ALP) have been mixed, especially at RT.

Longer-term stability of human specimens has been examined for storage at -20 °C [5–9], -25 °C [10], and -30 °C [11] in comparison to 4 °C and/or RT. Most reports showed a gradual decrease of LDH, ALT, CK, and an increase of the C3 test results in the frozen states over a period of 28–90 days. Some inconsistencies in the results among these reports appear to be attributable to differences in the storage period and target temperature set by each investigator.

Despite efforts to keep biobanked material at a stable temperature, accidental or inadvertent fluctuations of storage temperature, especially at or near the freezing range (0 to -30 °C), may occur. As previous studies have only covered a single temperature in the range, we aimed to determine the stability of serum analytes from freshly drawn blood samples at six graded temperatures simultaneously: -30 °C and -20 °C (freezing temperatures), -10 °C (semi-freezing temperature), 0 °C and 4 °C (near-freezing temperature), and 25 °C (RT). The target tests we chose were 13 major clinical chemistry analytes whose

molecular stability during storage for future re-testing is generally viewed with some concern. The time periods of serum storage examined ranged from 0 to 56 days.

Materials and methods

Preparation of serum specimens and conditions for storage

We obtained approximately 150 mL of blood from each of seven healthy volunteers aged 20 to 65 years old. The blood was drawn four times in increments of 40, 40, 40 and 30 mL through a butterfly needle into a 50 mL disposable syringe, and each portion was poured into a conical centrifuge tube (Corning Inc., USA). After centrifugation at $1200 \times g$ for 10 min, pooled serum from each individual was divided into 38 aliquots. Each aliquot was assigned a specified storage temperature (one from -30 , -20 , -10 , 0 , 4 and 25 °C) and storage duration (one from 1, 3, 7, 14, 28 and 56 days). Therefore, a total of 6×6 aliquots were allocated to cover all conditions. Two more aliquots (for duplicate measurement) were allocated for storage at -80 °C for a fixed duration of 60 days for determination of base value (see below for the concept of collective measurement). The total number of aliquots was 266 (seven individuals $\times$ 38). For storage containers, we used 1.2 mL CryoTubes with an outer cap (Nunc: Thermo Fisher Scientific, USA).

Temperature-controlled storage equipment

For storage at -80 °C, we used a VT-208 deep freezer (Nihon Freezer Co., Japan). For storage at -30 , -10 , and 0 °C, we used a portable incubator box capable of precise temperature control (Cryo Porter™ CS-80CP; Scinics Corp., Tokyo, Japan). This box can maintain a specified preset temperature over a range of -80 to 50 °C with a precision of ± 0.2 °C when it is housed in a space at RT. For storage at 25 °C, we used an incubator box (MIR-154-PJ; Panasonic Healthcare Co., Tokyo, Japan) also capable of precise temperature control. All of these temperature-controlled storage devices possess a visual display with beep functions for current temperature and alarm status to allow for external monitoring. For storage at -20 °C and 4 °C, we used a high-grade freezer/refrigerator (SJ-23TM; Sharp Corp., Japan), for which we confirmed the stability of the temperature within a precision of ± 0.5 °C (SD) as specified by the manufacturer, by external monitoring using a PC-3300 digital thermometer (Sato Keiryoki Mfg. Co., Tokyo, Japan).

Target analytes and measurements

After the assigned duration of storage (1, 3, 7, 14, 28 or 56 days) at a given temperature was completed, each aliquot was relocated to the deep freezer for storage at -80 °C until the time of collective measurement on day 60. Therefore, the length of storage at -80 °C was 60 days – (days of storage at a given temperature). For example, if an aliquot was assigned for storage at 4 °C for 14 days, then it was stored for another $60 - 14 = 46$ days at -80 °C. This scheme of collective measurement was essential to eliminate any influence of between-day assay variations in the analysis of storage stability.

On day 60, all specimens were thawed in batches by placing a rack of sample containers into a 37 °C water bath for 10 min for collective measurements of the following 13 biochemistry analytes using a Beckman Coulter AU480 analyzer: ALT, aspartate aminotransferase (AST), ALP, LDH, γ -glutamyltransferase (GGT), amylase (AMY), CK, triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), total bilirubin (TBil), C3 and complement component-4 (C4). The assay methods and analytical precision for all 13 analytes are listed in Table 1. Internal quality control was performed by daily measurement of three levels of quality control (QC) sera provided for the AU480 analyzer. The within- and between-day coefficients of variation (CVs) listed in the table were based on the results for QC specimens near the normal levels.

Data analyses

We assumed that all of the analytes were stable when stored in the deep freezer (at -80 °C) (see the Discussion for the validity of this assumption). Therefore, test results of the specimens stored continuously at -80 °C until day 60 (the time of collective measurements) were regarded as the values at day 0. The time-dependent alterations in values of the aliquots stored at the other temperatures are expressed as percent change (% Δ) from the values at day 0. For testing of the differences in values on each day from those on day 0, Wilcoxon signed rank test was performed with Bonferroni correction for the multiplicity of testings [12] applied per analyte per storage temperature: i.e., each P value was multiplied by 6 (the number of storage durations). For evaluation of the practical significance of % Δ , we referred to the CV_i or within-individual CV posted on Westgard's web site (<https://www.westgard.com/biodatabase1.htm>). We regard $0.5 \times CV_i$, which corresponds to the so-called allowable limit of error, as a guide value for interpreting the magnitude of the observed % Δ .

The analyses and graphical presentation of the results were created by use of StatFlex Ver 6.0 (Artech, Co., Osaka, Japan).

Ethical considerations

This study was approved by the Ethical Committee of Yamaguchi University Graduate School of Medical Sciences for Faculty of Health Sciences in June 2015 (No. 199-2015). Informed consent for the provision of blood samples was obtained from each volunteer.

Results

Serial changes in the test results over time for the 2-month period at each of the six storage temperatures are shown in Figure 1 for six selected analytes: ALT, LDH, CK, HDL-C, LDL-C and C3. The averages of % Δ in the measured values among the seven individuals for days 14, 28, and 56 are shown at the top of each panel. The major findings were as follows:

1. ALT showed the most prominent time-dependent decrease in test results at 25 °C, then at -10 °C, followed by -20 °C and -30 °C. It is notable that ALT activity was rather stable at 4 °C until day 14.

Table 1: List of assay methods and imprecision near the mid-normal ranges.

Abbrev	Analytes	Method	Reagent	Traceability	Mean of QC specimen	Within-day CV	Between-day CV
AST	Aspartate aminotransferase	JSCC method (no addition of pyridoxal phosphate)	Beckman Coulter	JCCLS CRM-001b	43.4 U/L	2.39	0.89
ALT	Alanine aminotransferase	JSCC method (no addition of pyridoxal phosphate)	Beckman Coulter	JCCLS CRM-001b	44.3 U/L	1.11	0.61
LDH	Lactate dehydrogenase	JSCC method (lactate to pyruvate, OD amplified)	Beckman Coulter	JCCLS CRM-001b	253 U/L	1.39	4.99
ALP	Alkaline phosphatase	JSCC method (kinetic rate method (using AMP buffer))	Beckman Coulter	JCCLS CRM-001b	155.9 U/L	0.77	3.02
GGT	Gamma-glutamyl transferase	JSCC method (GluCANA)	Beckman Coulter	JCCLS CRM-001b	59.2 U/L	1.57	3.04
CK	Creatine kinase	JSCC method	Beckman Coulter	JCCLS CRM-001b	92.3 U/L	0.88	2.23
AMY	Amylase	JSCC method (substrate: ethylidene-G7-PNP)	Beckman Coulter	JCCLS CRM-001b	156.2 U/L	1.01	0.00
TG	Triglyceride	Timed endpoint enzyme colorimetry (glycerol-removed)	Beckman Coulter	JCCRM 223,224	1.61 mmol/L	0.64	0.70
HDL-C	HDL-cholesterol	Timed endpoint direct method	Beckman Coulter	JCCRM 223,224	1.40 mmol/L	1.00	1.21
LDL-C	LDL-cholesterol	Timed endpoint direct method	Beckman Coulter	JCCRM 224	1.56 mmol/L	1.08	2.55
TBil	Total bilirubin	Enzymatic method	Beckman Coulter	NIST SRM916	23.4 μ mol/L	0.93	0.44
C3	Complement Component 3	Turbidimetric method	Beckman Coulter	CRM470	0.92 g/L	2.56	1.98
C4	Complement Component 4	Turbidimetric method	Beckman Coulter	CRM470	0.17 g/L	1.72	1.40

CV, coefficient of variability; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; JSCC, Japan Society of Clinical Chemistry; OD, optical density. Between-day CVs are net values subtracted of within-day component of variations.

- LDH activity showed a prominent reduction at -10 , 0 , and 4 $^{\circ}\text{C}$ in that order of magnitude. In contrast, the activity was relatively stable at -30 $^{\circ}\text{C}$ and at 25 $^{\circ}\text{C}$ until day 14.
- Interestingly, CK activity was more stable at -10 , 0 , and 4 $^{\circ}\text{C}$ than at -30 or -20 $^{\circ}\text{C}$, in sharp contrast to that of ALT or LDH. However, its activity rapidly decreased when stored at 25 $^{\circ}\text{C}$ for more than 7 days.
- Test results of TG were notably stable if it was stored at 0 $^{\circ}\text{C}$ or below for the entire 2-month period. A tendency for the values to decrease slightly was noted at 4 and 25 $^{\circ}\text{C}$ after 7 days.
- Test results of HDL-C were very stable during storage at all of the low-temperature zones between -30 $^{\circ}\text{C}$ and 4 $^{\circ}\text{C}$. However, at 25 $^{\circ}\text{C}$, HDL-C notably showed a biphasic pattern of a slight increase by 9% on day 3 with subsequent slight decrease after storage for longer than 14 days.
- In sharp contrast to the other analytes, C3 showed a storage-dependent increase in values at all temperatures and especially above -10 $^{\circ}\text{C}$. The increase in C3 values occurred more rapidly at 25 $^{\circ}\text{C}$ even on day 3.

The serial changes over time for all of the other analytes are shown in Supplementary Figure 1.

Figure 2 provides a summary of the serial changes in values over time at each storage temperature, which are shown in Supplementary Figure 1. The average $\%\Delta$ in value on each day from that at day 0 is represented by a colored bar in each cell. The level of statistical significance for each change is expressed by three different grades: $p < 0.05$ by bold font, $p < 0.01$ by light gray background, and $p < 0.001$ by gray background in each cell.

Overall, we obtained the following findings:

- At -30 $^{\circ}\text{C}$, all 13 analytes were generally very stable although ALT and CK showed minor reductions in their activities after storage of longer than 14 days.
- At 25 $^{\circ}\text{C}$, ALT, CK, AST, and TBil were very unstable especially after day 7.
- The values of ALP, GGT, AMY, HDL-C, LDL-C, TG, and TBil were generally very stable when these analytes were stored at 4 $^{\circ}\text{C}$ or below. They were also stable at 25 $^{\circ}\text{C}$ for 7–14 days except for TBil, which showed a tendency to rapidly decrease in value even by day 3.

4. ALT, AST, and LDH showed a prominent decrease in test results at -20 to 4 °C and especially at -10 °C.
5. C3 showed a time-dependent gradual increase in values at temperatures above -20 °C. C4 also showed a similar pattern of increase although to a much lesser degree.

Discussion

Experimental design

There have been a host of reports on the storage stability of bioactive substances in serum. Even when limiting the reports to those dealing just with the biochemical analytes evaluated in the present study, there are still a number of them [1, 2, 4, 5, 11, 13–15]. However, after carefully assessing their results, we noted large between-day fluctuations of values or non-monotonous/inconsistent time courses of the changes [16, 17]. Such a tendency was noted especially

in those reports published in the 1970s or earlier. Therefore, in this discussion, we limited our consideration to those studies that were relevant to our experimental conditions and were conducted with well-controlled measurements after the 1980s.

Almost all of the previous reports examined storage stability under two or three conditions, i.e. -20 °C and 4 °C and/or RT. Because we sometimes experience accidental or inadvertent fluctuations of storage temperature, we thought it necessary to analyze a wider range of temperatures and determine the degree of latitude allowable in the temperature settings. The present study is the first report, to the best of our knowledge, to investigate six different temperatures in parallel for storage stability of 13 major chemistry analytes whose molecular stability during storage for future re-testing is generally viewed with some concern. For storage at -30 , -10 and 0 °C, we used a portable incubator box that is capable of precise temperature control to an accuracy of ± 0.2 °C. For the other temperatures, we used a reliable freezer, refrigerators, and an incubator with an accuracy of ± 0.5 °C as

Figure 1: Change profiles of analytes over 2 months at six storage temperatures.

For six selected analytes, the time courses of test results for the seven sera are shown for each of the six storage temperatures. The data point for day 0, which corresponds to the value of the specimen continuously stored at -80 °C, is not shown. For days 14, 28 and 56, the average rates of change (%) from day 0 are posted at the top of each graph.

Figure 1: (continued)

confirmed by digital thermometer. Therefore, we believe our results are reproducible despite the wide temperature range covered. We would emphasize that the evaluation of the specific storage temperatures of -10 and 0 °C with the use of reliable equipment for common biochemistry analytes was not done in the past.

Before discussing the implications of our study findings, we first need to assess the validity of our basic assumption that serum specimens are stable for all analytes if stored in a deep freezer (at -80 °C), and therefore, test results of the specimens maintained at -80 °C can be regarded as basal values (values at day 0).

Ikeda et al. [1] analyzed the stability of 28 major biochemistry analytes in serum during 7 days of storage at -80 °C, -20 °C, 4 °C, and RT. All 13 of our analytes except C3 and C4 were included in their study, and all were shown to be stable at -80 °C for 7 days. Zander et al. [2] also analyzed the stability of 32 biomarkers in serum and plasma at 4 °C, -20 °C, -80 °C and -130 °C for a duration of 90 days. Four of our analytes: ALT, LDH, CK, and C3, were included in their study and were shown to be stable at or below -80 °C for 90 days by their criteria of significant fluctuation set as a change ratio of >3 times the between-day analytical CV and $p < 0.01$.

More recently, Haslach et al. [18] examined the storage stability of 26 biochemical analytes in serum at -75 °C for 14–21 days and the effect of up to 30 repetitions of artificial fluctuations of temperature between -65 to -75 °C. They observed no effect of temperature fluctuation at this narrow range but did show statistically significant changes in some of the analytes. All of our analytes except for LDL-C, C3, and C4 were included in their study. Only AMY was reported to show statistically significant decreases of 0.5% and 1.1% at days 14 and 21, respectively, which we regard as practically insignificant compared with the allowable limit of error of 7.2% for AMY. No consistent changes were reported for our other analytes at -75 °C during storage for 21 days. Based on these assessments, we think it rational to assume the virtual stability of all of our analytes stored in the deep freezer at -80 °C over the 60-day period or less.

Comparison with findings of previous reports

The lowering of serum LDH activity during storage at 4 °C and -20 °C is well known [1, 2, 19]. These changes were mainly attributed to the instability of isozymes LDH2–5,

Temp	Days	ALT	AST	ALP	LDH	GGT	AMY	CK	TG	HDL-C	LDL-C	T-Bil	C3	C4
−30 °C	3	−0.7	−4.7	1.2	−1.3	−1.0	−0.2	−2.4	0.5	−0.3	−0.2	1.6	−0.8	−0.5
	7	−4.5	1.9	−0.4	−1.8	−1.3	0.3	−3.7	0.6	−0.1	−0.5	2.0	1.1	−1.2
	14	−9.3	−3.9	0.5	−2.7	0.4	0.4	−6.1	0.0	1.1	−0.2	2.4	1.4	−0.1
	28	−8.2	−3.6	0.1	−4.1	−1.2	0.1	−7.1	0.5	0.1	0.2	0.0	2.6	−0.1
	56	−21.6	−3.4	−0.2	−8.6	−0.6	0.2	−9.4	0.2	−0.3	0.0	0.4	3.9	−0.8
−20 °C	3	−12.6	−3.4	1.2	−6.4	−0.5	−0.4	−1.2	1.1	−0.4	0.5	2.5	0.7	0.4
	7	−21.5	−7.2	0.3	−5.3	−2.4	0.1	−2.4	0.4	0.3	0.7	1.9	1.6	0.0
	14	−25.9	−4.8	−0.4	−8.3	0.7	0.0	−3.8	−0.2	0.2	1.3	2.3	4.6	1.0
	28	−39.1	−6.8	−2.3	−8.1	−1.0	0.0	−6.1	0.7	−0.6	1.6	2.5	9.3	1.5
	56	−43.4	−16.5	−3.2	−8.0	−0.4	0.3	−9.6	0.5	−1.2	1.5	−0.2	12.6	2.4
−10 °C	3	−22.2	−3.2	0.2	−26.4	−1.4	−0.6	0.1	1.0	−0.1	0.0	2.5	1.9	4.5
	7	−35.8	−5.7	0.0	−41.3	−3.0	−0.1	−1.5	0.3	−0.9	−0.9	2.2	8.7	3.8
	14	−51.7	−9.4	−1.4	−59.4	−1.5	−0.4	−1.2	0.8	−0.8	−1.6	2.5	14.9	4.6
	28	−46.6	−13.3	−2.4	−59.5	0.1	−0.1	−2.2	−0.1	−1.0	−1.9	0.8	14.0	3.4
	56	−49.5	−24.3	−6.3	−53.2	0.3	0.5	−2.1	−0.1	−1.9	−3.6	−2.7	16.4	4.2
0 °C	3	0.1	−3.5	0.1	−14.4	−1.3	−0.9	−0.5	0.2	−2.0	0.2	2.6	−1.8	1.8
	7	−9.3	−2.9	−0.7	−22.1	−1.6	−0.8	−0.3	−0.1	−1.2	0.6	1.9	2.4	3.9
	14	−10.4	−4.7	−0.6	−29.7	0.0	−1.4	−0.6	−0.4	−1.2	1.1	1.3	10.3	5.7
	28	−18.2	−15.8	−0.8	−37.6	0.7	−0.5	−2.2	−1.8	−3.0	1.2	−2.0	16.6	5.0
	56	−31.4	−25.0	−1.1	−48.4	−1.7	−0.4	−3.6	−3.4	−4.7	−0.8	−6.6	17.7	4.1
4 °C	3	−2.8	−4.2	0.3	−9.2	−0.6	−0.9	−1.8	0.8	−1.7	0.2	2.3	0.2	1.1
	7	−2.3	−7.4	−0.5	−15.6	0.4	−1.2	0.4	−0.8	−0.6	1.5	1.8	5.2	2.2
	14	−3.3	−8.5	−0.7	−21.6	1.9	−0.2	−0.4	−2.3	−1.1	2.0	0.8	12.6	4.7
	28	−10.3	−15.3	−0.1	−29.7	−0.2	−0.2	−3.0	−3.6	−2.3	2.2	−1.4	16.4	5.8
	56	−30.0	−25.9	−1.1	−41.9	1.3	−0.4	−4.2	−4.1	−5.4	1.6	−9.9	17.8	6.0
25 °C	3	−9.0	−8.1	2.5	−2.0	−0.6	−0.6	−1.4	−4.1	9.0	0.5	−27.0	10.2	5.1
	7	−14.7	−18.5	2.1	−2.5	0.3	−1.3	−6.2	−4.5	4.9	−2.4	−42.2	15.5	8.4
	14	−51.0	−33.6	3.9	−2.4	1.1	−1.7	−25.1	−6.5	−2.1	−10.1	−82.2	16.0	10.9
	28	−91.0	−65.0	0.4	−6.6	−4.1	−1.2	−63.7	−6.9	−8.5	−17.9	−86.4	15.6	9.0
	56	−94.3	−94.9	−10.3	−24.1	−8.8	−1.6	−88.6	−9.7	−13.4	−18.7	−87.4	16.1	10.0
ALE		11.48	6.54	6.72	4.26	11.06	7.40	11.51	9.57	5.61	5.46	8.95	4.11	8.64

Figure 2: Effects of specimen storage period and temperature on laboratory test results. Values and lengths of color bars listed for day 3 to day 56 are averages of change ratios (%) compared to the base value (test result of the specimen stored at −80 °C for 60 days). Statistical significance of the change from the base value: $p < 0.05$ = bold font; $p < 0.01$ = light gray background; $p < 0.001$ = gray background. The allowable limit of error (ALE) that was calculated as $0.5 \times CV_i$ (within-individual CV) for each analyte is shown in the bottom row. The values of change ratios exceeding the ALE are marked in blue font.

especially LDH4–5, whereas LDH1 is relatively stable at these temperatures [19].

We found LDH activity to be fairly stable at 25 °C and −30 °C at least for the first 14 days. Our finding for the former temperature is consistent with past reports [1, 19]. In contrast, LDH activity was very unstable at temperatures between −20 °C and 4 °C and was least stable at −10 °C followed by 0 °C, 4 °C and −20 °C. This finding at 0 °C is new. In summary, for long-term storage of serum for LDH (≥ 1 month), it would be best to store serum specimens in a deep freezer. Storage at temperatures between −10 °C to 4 °C, even for a short time, should be avoided, whereas storage at 25 °C seems to cause no problems up to 14 days.

Serum ALT activity after storage for a few days or more is well known to be unstable both at RT (22–25 °C) [1, 4] and −20 °C [1, 2, 4] but is relatively stable at 4 °C [4, 11, 15]. However, the relative magnitude of instability across

various temperature conditions has not been clarified. Our results clearly showed that storage temperatures between −20 °C and −10 °C were the least recommended for ALT even for a period of 3 days. Progressive instability after 7–14 days was also noted for temperatures at −30 °C, 0 °C and 25 °C. In contrast, ALT remained relatively stable at 4 °C for up to 14 days. Meanwhile, we confirmed the stability of ALT activities at −80 °C as we did for the other 12 analytes (data not shown). In summary, serum specimens for ALT testing can be kept at 4 °C for up to 14 days and are best stored in a deep freezer for any longer period. Storage at −20 to −10 °C does not appear to be appropriate even for a few days.

Our findings for the pattern of instability for AST were similar to those of ALT but were much less prominent except at 25 °C. In addition to the reports of the stability of AST activity at 4 °C [1], one report found a 51.3% reduction

in plasma within 3 days [20]. However, our results at 4 °C showed much smaller reductions of only 4% at day 3 and 8.5% at day 14. It is notable that this reduction at 4 °C is relatively stronger than that of ALT. In conclusion, serum for AST needs to be stored at -30 °C or below. No other storage temperature appears to be appropriate even though the magnitude of the changes is relatively small compared with that of ALT.

For CK, there are multiple reports of either a slight [2] or prominent [3] lowering of its activity at -20 °C. At 25 or 20 °C, there are multiple reports of decreased activity [1, 3], and at 4 °C, the reports are mixed, showing either no change [21, 22] or a decrease [1, 3]. The decreased activity at -20 and 4 °C was attributed to all three isozymes of CK [3]. In contrast, Friedman et al. reported no changes in the isozyme profile at -20 and 4 °C after 3 months of storage [22]. From our study, we noted only a slight decrease in activity at -20 °C: 3.8% on day 14 and 6.1% on day 28. A similar decrease was also noted at -30 °C. In contrast, CK activity was very stable at storage temperatures between -10 and 4 °C compared to storage at -30 or -20 °C. It is difficult to explain this paradoxical reversal of the freezing effect, unlike that in the other enzymes, but we assume that restricted mobility of CK molecules under a deeper freezing condition is harmful to its structure and thus causes the decrease in activity. However, at 25 °C, CK activities decreased progressively after day 7. In conclusion, specimens for testing of CK can be stored at -10, 0, or 4 °C for up to 14 days, but storage at -30, -20, and 25 °C for more than 7 days should be avoided.

For ALP, all of the recent reports showed the stability of ALP activity at RT (25 or 20 °C), 4 °C [1, 2, 15] and -20 °C [1]. The present study essentially confirmed the findings for these temperatures and also showed its stability at -30 °C, -10 °C and 0 °C. Therefore, serum for retesting ALP can be stored over the full range of temperatures at least for 1 month.

The high stability of GGT and AMY even after long storage at RT and at 4 and -20 °C is well known [1, 5, 11, 14]. We confirmed their stability over the full range of temperatures for at least 1 month, as noted above for ALP.

We noted a prominent decrease in TBil at 25 °C within a few days, but there were no noticeable changes in serum TBil at the other temperatures except for a minor decrease observed when specimens were left at 0 °C or 4 °C for more than 28 days. In interpreting these results, it is important to note that we took special care to avoid the well-known degradation of the molecule by ultraviolet rays during storage. Therefore, the findings we obtained appeared to be primarily attributable to temperature-dependent

molecular change. In our literature search, we noted one report of a similar decrease in TBil at 25 °C even within a few days [1]. However, at 4 °C, there is a report of a reduction within hours [20] unlike our finding of the stability of TBil until day 28.

For C3, its peculiar pattern of a gradual increase with storage has been well documented at 18–22 °C and at 4 and -20 °C [2, 23]. This has been attributed to its degradation into C3b and C3c during storage [23]. We showed a similar increase even at temperatures between -10 and 0 °C. The same tendency was also observed for C4, but the trend was much less pronounced. In the literature, serum C4 was reported to both increase [24] or decrease [23] at 4 °C. We assume that the discrepancy is attributed to differences in antibody specificities against the degradation products.

There have been numerous reports on the stability of the lipids TG, HDL-C and LDL-C because of the strong clinical demand for laboratory testing of these analytes. Most studies examined stability at -20 or 4 °C, and their results are mixed [9, 25–28]. Pini et al. [29] showed increases of HDL-C and LDL-C by 4% and 7%, respectively, after 7 days of storage both at -20 and 4 °C, whereas Tiedink and Katan [30] reported a decrease of both LDL-C and HDL-C by <4.1% after 11 weeks of storage at -20 °C. Furthermore, Bausserman et al. [31] found no changes at -20 °C for up to 1 month or at 30 days. We found all three lipids to be very stable at any temperature below 4 °C for 4 weeks. At 25 °C, however, test results for TG decreased by 4% on day 3 and by 7% on day 28, and those for HDL-C showed a biphasic pattern with an increase of 9% on day 3 but a decrease of 8.5% on day 28. Regarding the increase in HDL-C, Pini et al. reported that if the specimens are left at RT, LCAT (lecithin:cholesterol acyltransferase) in serum acts on HDL to transiently increase the cholesterol content [29]. We conclude that all three lipids are stable for at least 2 months if stored at or below -10 °C. However, storage of serum at RT even for a few days does not appear to be appropriate because of the pseudo-increase in HDL-C.

Limitations

One limitation of this study is that we examined the storage stability of major biochemical analytes from only seven healthy volunteers. Although we observed the overall consistency of the time-serial change patterns among the individuals, each checked for statistical significance, we need to be cautious in generalizing our results because we may have missed a peculiar profile of changes in some

individuals, the elucidation of which requires the analysis of a larger number of individuals. Furthermore, we only covered test results of healthy individuals and therefore cannot deny the possibility that test results of the analytes at higher than normal levels may show a different profile of changes.

The other limitation is that the evaluation of the effects of storage at a temperature of 25 °C for a period of 7 days or longer has no practical meaning due to the feasibility of microbial contamination of the specimens. However, we did not note any visible changes in the appearance of the specimens during the study period because all specimen containers were tightly sealed.

Conclusions

This is the first comprehensive study to elucidate storage stability profiles of serum specimens of common biochemistry analytes over six graded temperatures: −30, −20, −10, 0, 4 and 25 °C. Serum specimens freshly sampled from seven healthy volunteers were divided into multiple aliquots and stored in chambers accurately controlled at all six temperatures. Then, after 1, 3, 7, 14, 28 and 56 days, aliquots designated for a given storage time and temperature were relocated one by one to a deep freezer (at −80 °C). To eliminate any influence of between-day assay variations, all measurements were then collectively performed on day 60.

Serum ALT activity was reduced by storage at all temperature ranges, especially at −20, −10, and 25 °C. AST activity showed a similar but much less prominent pattern of reduction. LDH activity was unstable at −10, 0, and 4 °C in that order of magnitude. CK activity was peculiar in that it was rather stable at temperatures between −10 and 4 °C but decreased when stored at −20 or 25 °C for 2 weeks or more. Other enzymes, GGT, AMY and ALP, were very stable over the entire temperature range. TG, HDL-C and LDL-C were very stable when stored below 4 °C, but some fluctuations were noted at 25 °C. TBil was very stable if stored at 4 °C or below, but a prominent decrease in test results was noted at 25 °C. In contrast, C3 and C4 showed storage-dependent increases in their values for any temperature above −20 °C.

We could elucidate a clearer picture of storage-dependent changes in the test results across a wide range of storage temperatures for 13 major laboratory tests. These results should be useful for clinical laboratories in determining optimal conditions for storing serum specimens of each analyte for future retesting.

Author contributions: YS and KI designed the research. YS conducted the experiment and measured all of the specimens under the guidance of KI. YS and KI performed data analyses. KI and YS wrote the manuscript. All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

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