

The effect of storage time and freeze-thaw cycles on the stability of serum samples

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Abstract

Introduction: Optimal storage of serum specimens in central laboratories for a long period for multicenter reference interval studies, or epidemiologic studies remains to be determined. We aimed to examine the analytical stability of chemistry analytes following numerous freeze-thaw and long term storage.

Materials and methods: Serum samples were obtained from 15 patients. Following baseline measurement, sera of each subject were aliquoted and stored at -20 °C for two experiments. A group of sera were kept frozen for up to 1, 2 and 3 months and then analyzed for stability. The other experiment consisted of one to ten times of freeze and thaw cycles. Total of 17 chemistry analytes were assayed at each time point. The results were compared with those obtained from the initial analysis of fresh samples. Median or mean changes from baseline (T_0) concentrations were evaluated both statistically and clinically according to the desirable bias.

Results: Of the analytes studied, aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), gamma-glutamyl transferase (GGT), direct bilirubin, glucose, creatinine, cholesterol, triglycerides, high density lipoprotein (HDL) were stable in all conditions. Blood urea nitrogen (BUN), uric acid, total protein, albumin, total bilirubin, calcium, lactate dehydrogenase (LD) were changed significantly ($P < 0.005$).

Conclusions: As a result, common clinical chemistry analytes, with considering the variability of unstable analytes, showed adequate stability after 3 months of storage in sera at -20 °C, or up to ten times of freeze-thaw cycle. All the same, such analysis can only be performed for exceptional cases, and this should be taken into account while planning studies.

Key words: preanalytical phase; serum; stability; storage; temperature

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Introduction

Optimal storage of serum specimens in biobanks for a long time for multicenter reference interval studies, or epidemiologic studies remains to be determined.

Specimens shipped from multicenter laboratories are usually stored in a central laboratory for further analysis. Parameters that may vary across collections include; i) delays in shipping frozen samples; ii) power cut or voltage fluctuations of the freezers; and iii) using frost-free freezer that goes through numerous defrost cycles, which may result in loss of some analytes.

Pre-analytical phase is the most critical part of the total analytical process which has impacts on patient reports in clinical chemistry testing (1). It is possible in practice to reanalyze the samples stored to confirm the previous results or to perform additional analysis, however the stability of the analytes must be assured before giving results, or before establishing new investigations. Furthermore, analyzing unsuitable samples often cause high cost expenditures and enlargement of total testing process.

Manufacturers allow the usage of sera stored frozen and thawed only for once, and advise to dis-

perse sera into the single usage aliquots before freezing. However, the number and volume of the aliquots must be taken into account regarding the handling and freezer space costs while planning the studies.

There are various studies examined how storage conditions affected the stability of various serum components (2-5). However, there is less information on the stability of commonly used chemistry analytes in human sera.

The present study examined the stability of 17 routine chemistry analytes in sera after storing at a designated temperature and freeze-thawing at multiple time points.

Materials and methods

Subjects

This study included fifteen out-patients attending the clinics of our hospital. The specimens collected from each patient were for the laboratory testing ordered by the physician. No additional blood was taken from the subjects. All were informed for the study and signed the informed consent. The procedures were in accordance with the guidelines of the Helsinki Declaration on human experimentation.

Study design

Fasting venous blood (totally 25.5 cc blood) was collected in the morning into three 8.5 mL plastic evacuated tubes (BD Vacutainer Systems, Becton-Dickinson, Plymouth, UK). The sample tubes were left in upright position for 30 min at room temperature for complete clot formation. All were then centrifuged at $1800 \times g$ for 10 minutes (according to the instruction of the tube manufacturer). Serum samples were checked visually for hemolysis and lipemia for possible interferences (6,7). Sera of each subject were pooled into a plain tube, and were aliquoted into 1.5-mL Eppendorf (Eppendorf, Milano, Italy) tubes. Composing of 13 aliquots for each patient (three for storage, 10 for freeze-thaw), remaining sera were used for the patients' requested tests. Following baseline measurement (T_0), all were kept frozen until analysis for two experiments below:

- Ten samples of each subject were frozen at -20°C . After 24 hours, all frozen sera in the Eppendorf tubes of each patient were thawed at room temperature for approximately 1 h until completely thawed, and then mixed properly with automatic pipettes before analysis (freeze-thaw 1).
- Samples were immediately re-frozen at -20°C for the next study day. This cycle was repeated for ten consecutive days ($T_{1d}, T_{2d}, T_{3d}, T_{4d}, T_{5d}, T_{6d}, T_{7d}, T_{8d}, T_{9d}, T_{10d}$) to yield freeze-thaw processing.
- A group of sera were stored frozen at -20°C for up to 1, 2 and 3 months, and then analyzed for stability in singleton at three time intervals (T_{1m}, T_{2m}, T_{3m}).

Samples stored at -20°C were kept in a freezer equipped with a temperature recorder (Figure 1).

Methods

Assays were performed on the Abbott Aeroset analyzer (Abbott, Wiesbaden, Germany) with Abbott reagents at the Department of Clinical Biochemistry Laboratory of Ataturk Training and Research

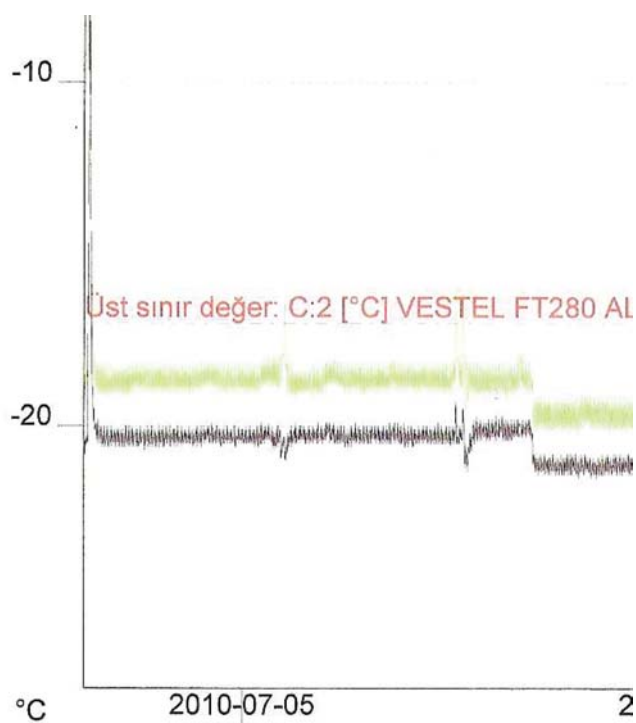


FIGURE 1. The temperature graph of the freezer during the freeze-thaw period.

Hospital in Izmir, Turkey. The following biochemical constituents were assayed:

- metabolites: BUN (blood urea nitrogen), calcium, creatinine, direct bilirubin, glucose, total bilirubin, uric acid;
- proteins: total protein, albumin;
- lipids: triglycerides, cholesterol, HDL (high density lipoprotein);
- enzymes: ALT (alanine aminotransferase), AST (aspartate aminotransferase), CK (creatin kinase), GGT (gamma-glutamyl transferase), LD (lactate dehydrogenase).

Intra-assay and inter-assay CV's were performed with pooled patient sera different from the study subjects (Table 1). Inter-assay variation was assessed from 20 determinations (with 2 aliquots each day) on ten consecutive study days, and intra-assay variation was calculated from eight se-

quential determinations obtained from the first day of the study period.

Quality control was performed each day before studying aliquots with two levels of control materials (Bio-Rad Laboratories, Milano, Italy).

Statistical analysis

Stabilities of analytes after freeze thaw cycles and after storage were assessed by percentage change from T₀ for paired groups (T₀-T_{1d}, T₀-T_{2d}, etc. and T₀-T_{1m}, T₀-T_{2m}, etc). Bias was calculated by the formula:

$$[(C_x - C_1) / C_1] \times 100\%$$

C₁: the mean or median of the T₀ sample;

C_x: the mean or median of the experimented sample.

TABLE 1. Methods, intra and inter-assay coefficient of variations.

Analyte	Method	Intra-assay CV (%)	Inter-assay CV (%)
Albumin (g/L)	Bromcresol green	2.0	1.4
ALT (U/L)	UV without P5P	0.7	2.8
AST (U/L)	UV without P5P	0.5	1.5
Bilirubin, direct (µmol/L)	Diazotization	1.7	3.1
Bilirubin, total (µmol/L)	Diazonium ion	0.5	0.9
BUN (mmol/L)	Urease, UV	1.6	3.5
Calcium (mmol/L)	Arsenazo III	0.4	1.0
Cholesterol (mmol/L)	CHOD-PAP	0.6	0.9
Creatinine (µmol/L)	Alkaline picrate	0.7	0.4
CK (U/L)	UV-NAC activated	0.3	1.0
Glucose (mmol/L)	Hexokinase	0.7	0.8
GGT (U/L)	Gamma-glutamyl-carboxyl nitroanilide	0.5	0.8
HDL (mmol/L)	Direct, non-immunologic	5.1	3.6
LD (U/L)	Lactate-pyruvate, UV	0.8	1.6
Protein, total (g/L)	Biuret	1.3	1.1
Triglyceride (mmol/L)	GPO-PAP	1.0	1.4
Uric acid (µmol/L)	Uricase, PAP	0.8	0.6

Inter-assay variation was assessed from totally 20 determinations (with 2 determinations each day) on 10 consecutive study days, and intra-assay variation was assessed from 8 determinations, measured on a single assay.

ALT - alanine aminotransferase; AST - aspartate aminotransferase; BUN - blood urea nitrogen; CK - creatine kinase; GGT - gamma-glutamyl transferase; HDL - high density lipoprotein; LD - lactate dehydrogenase.

Median or mean changes from T₀ concentrations were evaluated both statistically and clinically according to the desirable bias taken from the Westgard QC (8), which was first published at 1999 and updated at 2012 by Ricos et al.

The distribution of the variables was determined using Shapiro-Wilk normality test.

In normally distributed groups, results were presented as mean ± standard deviation. The significant differences from T₀ value were determined by Paired samples t-test. For non-Gaussian groups, median variations from T₀ was determined by non-parametric Friedman test and Wilcoxon signed rank test using licensed statistical package for Windows, Version 15.0, SPSS Inc. (Chicago, IL, USA). P value < 0.005 (0.05/11 = 0.0045) for freeze-thaw cycles and P < 0.013 (0.05/4 = 0.0125) for storage period were considered statistically significant according to Bonferroni correction for multiple comparisons.

Results

The results and variations for two experiments are presented in table 2 and figures 2 and 3.

Freeze-thaw cycles

After samples had been frozen and thawed up to ten times, there were no statistically significant differences for AST, ALT, GGT, cholesterol, glucose, creatinine, direct bilirubin and HDL levels.

Statistically significant changes were not clinically significant for triglyceride and CK. Sera should not go through more than one freeze-thaw cycle for total bilirubin (P < 0.005). Among the analytes studied, total protein and uric acid were found as the least stable tests (Table 2). Albumin concentration was not altered statistically as long as seven freeze-thaw cycles and increased afterwards. BUN and calcium were stable up to three freeze-thaw and LD was found as stable for five cycles evaluating together the significance clinically and statistically. Statistically significant changes were marked in bold in table 2.

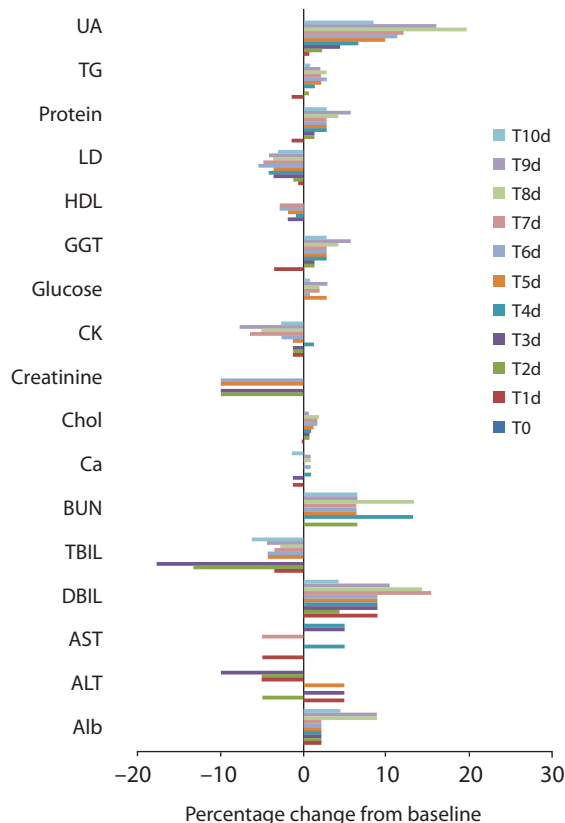


FIGURE 2. Percentage change in concentrations during 10 days of freeze-thaw period.

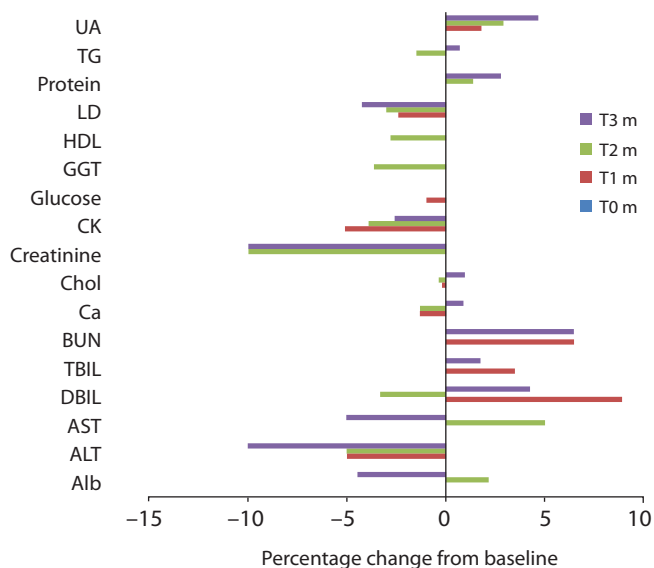


FIGURE 3. Percentage change in concentrations during storage for 1-3 months at -20 °C.

Alb - albumin; ALT - alanine aminotransferase; AST - aspartate aminotransferase; BUN - blood urea nitrogen; Ca - calcium; CK - creatine kinase; Chol - cholesterol; D BIL - direct bilirubin; GGT - gamma-glutamyl transferase; HDL - high density lipoprotein; LD - lactate dehydrogenase; T BIL - total bilirubin; TG - triglyceride; UA - uric acid; T0 - baseline, T1d-10d - 1-10 corresponding days of freeze-thaw cycle, T1m-3m - corresponding months of storage.

TABLE 2. Percentage of mean ± standard deviation and median (interquartil) changes from T₀ values (% change from T₀; baseline value) at each freeze-thaw cycle and storage period compared to maximum desirable bias.

Analyte	DB(± %)	T0	T1d	T2d	T3d	T4d	T4d	T6d	T7d	T8d	T9d	T10d	T1m	T2m	T3m
Albumin (g/L)		45 (38-47)	45 (37-48)	46 (38-48)	46 (38-48)	46 (38-47)	46 (39-48)	46 (38-48)	46 (38-49)	49 (41-50)	49 (40-51)	47 (39-49)	45 (37-47)	46 (37-46)	43 (36-46)
%	1.3		0	2.2	2.2	2.2	2.2	2.2	2.2	8.9	8.9	4.4	0	2.2	-4.4
P		0.132	0.480	0.102	0.102	1.000	0.206	0.414	0.102	0.001	0.001	0.006	0.029	0.206	0.001
ALT (U/L)		20 (13-104)	21 (12-106)	19 (12-109)	21 (12-107)	20 (11-107)	21 (13-103)	20 (12-103)	19 (12-100)	19 (13-97)	18 (11-97)	20 (12-105)	19 (10-103)	19 (12-100)	18 (10-101)
%	11.4		5	-5	5	0	5	0	-5	-5	-10	0	-5	-5	-10
P		0.527	0.166	0.782	0.782	0.782	0.593	0.763	0.017	0.061	0.057	0.206	0.001	0.002	0.001
AST (U/L)		20 (16-132)	19 (17-135)	20 (15-141)	20 (16-138)	21 (17-139)	20 (16-141)	20 (17-140)	19 (17-141)	20 (15-141)	21 (17-141)	21 (16-134)	20 (14-139)	21 (15-133)	19 (13-130)
%	5.4		-5	0	0	5	0	0	-5	0	5	5	0	5	-5
P		0.285	0.480	0.480	1.0	0.527	0.480	0.031	0.317	0.317	0.018	1.0	0.431	1.0	0.001
Bilirubin, total (µmol/L)		11.3 ± 2.74	10.9 ± 2.75	9.8 ± 2.65	9.3 ± 2.91	11.3 ± 2.75	10.8 ± 2.62	10.8 ± 2.57	10.9 ± 3.08	11.0 ± 3.01	10.8 ± 3.03	10.6 ± 2.74	11.7 ± 2.43	11.3 ± 2.51	11.5 ± 2.43
%	11.4		-3.5	-13.3	-17.7	0	-4.4	-4.4	-3.5	-2.7	-4.4	-6.2	3.5	0	1.8
P		<0.001	<0.001	<0.001	<0.001	<0.001	0.039	0.010	0.118	0.155	0.041	0.001	0.028	0.609	0.904
Bilirubin, direct (µmol/L)		3.93 (3.3-5.6)	4.28 ± 0.67	4.10 ± 0.87	4.28 ± 0.84	4.28 ± 0.99	4.25 ± 0.99	4.28 (3.76-6.33)	4.53 ± 0.80	4.50 ± 0.74	4.34 ± 0.68	4.10 (3.25-5.13)	4.28 ± 0.67	3.80 (3.42-5.30)	4.10 ± 0.63
%	14.2		8.9	4.3	8.9	8.9	8.9	8.9	15.3	14.3	10.4	4.3	8.9	-3.3	4.3
P		0.379	0.754	0.754	0.887	0.774	0.888	0.140	0.165	0.182	0.668	0.777	0.598	0.084	0.629
BUN (mmol/L)		5.36 (3.57-11.07)	5.36 (3.93-10.71)	5.71 (3.57-10.71)	5.36 (3.93-10.71)	6.07 (3.93-11.78)	5.71 (4.28-11.42)	5.71 (4.28-11.42)	5.71 (4.28-11.78)	5.71 (4.28-11.78)	6.07 (4.28-11.78)	5.71 (3.57-11.42)	5.71 (3.93-11.78)	5.71 (3.57-11.42)	5.71 (3.57-11.07)
%	5.5		0	6.5	0	13.3	6.5	6.5	6.5	13.3	6.5	6.5	6.5	0	6.5
P		0.705	1.00	1.00	0.317	0.002	0.008	0.003	0.003	0.001	0.002	0.015	0.002	0.096	0.429
Calcium (mmol/L)		2.33 (1.95-2.38)	2.30 (1.93-2.38)	2.33 (1.95-2.40)	2.30 (1.93-2.38)	2.35 (2.00-2.45)	2.33 (2.00-2.40)	2.35 (2.03-2.42)	2.33 (2.00-2.38)	2.35 (2.03-2.45)	2.35 (2.00-2.40)	2.30 (1.95-2.40)	2.30 (1.93-2.38)	2.30 (1.95-2.38)	2.35 (1.93-2.40)
%	0.8		-1.3	0	-1.3	0.9	0	0.9	0	0.9	0.9	-1.3	-1.3	0.9	
P		0.008	0.763	0.763	0.006	0.001	0.366	0.007	0.096	0.001	0.006	0.739	0.705	0.025	0.148
Cholesterol (mmol/L)		4.88 ± 0.58	4.87 ± 0.59	4.92 ± 0.53	4.92 ± 0.54	4.93 ± 0.53	4.94 ± 0.53	4.96 ± 0.53	4.96 ± 0.54	4.97 ± 0.53	4.91 ± 0.53	4.88 ± 0.56	4.87 ± 0.60	4.86 ± 0.59	4.93 ± 0.54
%	4.0		-0.2	0.8	0.8	1.0	1.2	1.6	1.6	1.8	0.6	0	-0.2	-0.4	1.0
P		0.085	0.317	0.317	0.313	0.188	0.141	0.052	0.035	0.034	0.489	1.000	0.536	0.280	0.019

Analyte	DB (± %)	T0	T1d	T2d	T3d	T4d	T4d	T4d	T6d	T7d	T8d	T9d	T10d	T1m	T2m	T3m
Creatinine (µmol/L)	88.4 (70.7-247.5)	88.4 (70.7-247.5)	79.6 (61.9-256.4)	79.6 (61.9-256.4)	79.6 (61.9-256.4)	79.6 (70.7-247.5)	88.4 (70.7-256.4)	88.4 (70.7-256.4)	88.4 (70.7-256.4)	88.4 (70.7-256.4)	88.4 (70.7-256.4)	88.4 (70.7-256.4)	88.4 (70.7-247.5)	88.4 (61.9-256.4)	79.6 (70.7-229.8)	79.6 (70.7-247.5)
%	4.0	0	-10	-10	-10	-10	-10	-10	-10	0	0	0	0	0	-10	-10
P	1.0	1.0	0.655	1.00	1.00	0.655	0.564	0.564	0.317	0.180	0.020	0.102	0.414	0.317	0.317	0.564
CK (U/L)	78 (59-796)	77 (58-790)	77 (55-793)	77 (53-791)	77 (53-791)	79 (54-790)	77 (52-789)	77 (52-789)	76 (53-792)	73 (52-791)	74 (52-790)	72 (50-789)	76 (52-793)	74 (56-800)	75 (58-790)	76 (59-797)
%	11.5	-1.3	-1.3	-1.3	-1.3	1.3	-1.3	-1.3	-2.6	-6.4	-5.1	-7.7	-2.6	-5.1	-3.9	-2.6
P	0.063	0.285	0.285	0.046	0.046	0.109	0.009	0.009	0.003	0.001	0.001	0.001	0.001	0.015	0.001	0.004
Glucose (mmol/L)	5.94 (5.38-11.88)	5.94 (5.27-11.93)	5.99 (5.05-11.54)	5.99 (5.05-11.60)	5.99 (5.05-11.60)	5.94 (5.33-11.60)	6.11 (5.38-11.49)	6.11 (5.33-11.77)	5.99 (5.33-11.54)	6.05 (5.33-11.77)	6.05 (5.50-11.71)	6.11 (5.44-11.71)	5.99 (5.38-11.77)	5.88 (5.33-11.93)	5.94 (5.27-11.99)	5.94 (5.50-11.66)
%	2.2	0	0.8	0.8	0.8	0	2.9	1.9	0.8	1.9	1.9	2.9	0.8	-1.0	0	0
P	0.058	0.285	0.285	0.564	0.564	0.782	0.088	0.088	0.405	0.166	0.071	0.053	0.083	0.009	0.143	0.176
GGT (U/L)	28 (16-92)	27 (16-95)	29 (16-97)	28 (16-98)	28 (16-98)	29 (16-97)	29 (16-98)	29 (16-98)	28 (16-99)	28 (16-98)	28 (16-99)	28 (15-96)	28 (15-98)	28 (16-95)	27 (15-93)	28 (12-90)
%	10.8	-3.6	3.6	0	0	3.6	3.6	3.6	0	0	0	0	0	0	-3.6	0
P	0.739	0.564	0.564	1.000	1.000	0.206	0.132	0.132	0.132	0.058	0.083	0.206	0.564	0.957	0.005	0.004
HDL (mmol/L)	1.08 ± 0.19	1.08 ± 0.19	1.08 ± 0.19	1.06 ± 0.19	1.06 ± 0.19	1.07 ± 0.18	1.06 ± 0.16	1.06 ± 0.16	1.05 ± 0.17	1.05 ± 0.16	1.08 ± 0.18	1.08 ± 0.18	1.08 ± 0.18	1.08 ± 0.19	1.05 ± 0.18	1.08 ± 0.18
%	5.2	0	0	-1.9	-1.9	-0.9	-1.9	-1.9	-2.8	-2.8	0	0	0	0	-2.8	0
P	0.728	0.933	0.933	0.174	0.174	0.517	0.380	0.380	0.067	0.054	0.797	0.896	0.719	0.742	0.004	0.658
LD (U/L)	167 (141-286)	166 (140-289)	165 (139-285)	161 (137-282)	161 (137-282)	160 (132-280)	161 (134-279)	161 (134-279)	158 (134-280)	159 (133-281)	161 (132-280)	160 (133-279)	162 (135-282)	163 (138-286)	162 (139-281)	160 (134-282)
%	4.3	-0.6	-1.2	-3.6	-3.6	-4.2	-3.6	-3.6	-5.4	-4.8	-3.6	-4.2	-3.0	-2.4	-3.0	-4.2
P	0.285	0.008	0.008	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.060	0.001	0.003
Protein, total (g/L)	72 (61-76)	71 (60-76)	73 (60-76)	73 (61-77)	73 (61-77)	74 (61-77)	74 (62-78)	74 (62-78)	74 (62-77)	74 (63-78)	75 (62-79)	76 (64-79)	74 (62-77)	72 (60-76)	73 (62-77)	70 (60-74)
%	1.2	-1.4	1.4	1.4	1.4	2.8	2.8	2.8	2.8	2.8	4.2	5.6	2.8	0	1.4	2.8
P	0.004	0.004	0.157	0.005	0.005	0.056	0.001	0.001	0.001	<0.001	0.001	0.009	0.025	0.053	0.033	0.001
Triglyceride (mmol/L)	1.45 ± 0.27	1.43 ± 0.26	1.46 ± 0.28	1.45 ± 0.29	1.45 ± 0.29	1.47 ± 0.28	1.48 ± 0.29	1.48 ± 0.29	1.49 ± 0.29	1.48 ± 0.28	1.49 ± 0.28	1.48 ± 0.28	1.46 ± 0.28	1.45 ± 0.27	1.43 ± 0.27	1.46 ± 0.27
%	10.7	-1.4	0.7	0	0	1.4	2.1	2.1	2.8	2.1	2.8	2.1	0.7	0	-1.4	0.7
P	0.041	0.366	0.366	0.782	0.782	0.014	0.011	0.011	0.008	0.007	0.004	0.002	0.052	0.376	0.026	0.228
Uric acid (µmol/L)	275 ± 41	277 ± 40	281 ± 42	287 ± 41	287 ± 41	293 ± 40	302 ± 39	302 ± 39	306 ± 40	308 ± 39	329 ± 39	319 ± 43	298 ± 43	280 ± 41	283 ± 42	288 ± 44
%	4.9	0.7	2.2	4.4	4.4	6.6	9.8	9.8	11.3	12.0	19.6	16.0	8.4	1.8	2.9	4.7
P	0.207	0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.001	<0.001

All concentrations and activities are written as mean ± SD and median (interquartile ranges). Shapiro Wilks test was used for normality, and for Gaussian distributed values, Paired samples t test, for non-Gaussian distributed values, Friedman and Wilcoxon signed rank tests were used to compare the repeated measures with a post-hoc Bonferroni correction. P < 0.005 values for freeze thaw cycles, P < 0.013 for storage period, and % variations exceeded the maximum desirable bias (5) are written in bold font. DB - desirable bias; T0 - baseline, T1d-T10d - 1-10 corresponding days of freeze-thaw cycle, T1m-3m - 1-3 months of storage period; ALT - alanine aminotransferase; AST - aspartate aminotransferase; BUN - blood urea nitrogen; CK - creatine kinase; GGT - gamma-glutamyl transferase; HDL - high density lipoprotein; LD - lactate dehydrogenase.

Storage at -20 °C

Except from albumin, total protein and BUN levels, there were no significant variations for sera stored until 3 months. Statistical and clinical significances were evaluated for all analytes.

Albumin and total protein concentration changed significantly ($P < 0.013$) after 3 months of storage. BUN concentration showed variability during storage (Table 2).

Discussion

This study examined the storage and repeated freeze thaw effects on chemistry analytes pre-analytically. According to our findings, 14 analytes after storage and 10 analytes following freeze-thaw cycles showed clinically equivalent results.

In a comprehensive study by Jackson *et al.* (9) clinically negligible biases were obtained in most of the routine clinical chemistry parameters following repeatedly freeze-thaw cycles between 0 to 24 hour. Cray *et al.* (10), experimented the storage stability of rat sera at -20 °C for glucose, BUN, calcium, total protein, albumin, AST, ALT, LD and CK when stored in nonfrost freezer up to 90 days. In agreement with Jackson and Cray (9,10), we found glucose concentrations as stable through the whole experiment. However in a study (11), glucose concentrations were found as increased from 11.8 to 14.0% in human serum refrozen and thawed even once.

Protein stability is problematic. Fast freezing with slow thawing resulted in severe damage in proteins, accordingly, slow freezing and fast thawing process is advised to prevent protein denaturation in aqueous solutions (12). In human fluids, long time storage in freezer is advised instead of thawing samples repeatedly (13). The variations for albumin and total protein were found as outside the desirable bias (8) during storage and freezing thawing cycles in this study, however the bias could not be explained by denaturation, as the concentrations were measured as increased.

In accordance with the current study, Paltiel and Comstock *et al.* (14,15) reported the stability of serum cholesterol and triglyceride concentrations

during storage in freezer and following freeze thaw cycles. The present study confirms the findings of Beekhof *et al.* (16), who found a good stability of HDL up to 6 months of storage at -20 °C.

Paltiel *et al.* (14), subjected the human plasma samples to several freeze thaw cycles after storage at -80 °C and AST activity was determined as stable within the 10-15 freeze-thaw cycles. Throughout the multiple time points we assessed, all of the enzyme levels measured were biased insignificantly, except from LD activity.

Our results indicate an appreciable increase in BUN values over time, however BUN instability has been observed in a recent study as a large percentage decrease (15.6% average) when samples were stored at -20 °C (17).

The estimated percentage change in uric acid concentrations per freeze thaw cycle showed an increasing trend over time as this analyte was demonstrated as unstable after 48 hours in human serum stored at -4 °C in a recent study (18).

In the present study, time dependent decreases during storage and within the 10 times of freezing cycle were statistically insignificant for creatinine assay.

Total bilirubin concentration was found as stable up to three months of storage at -20 °C, unlike to a study in which authors demonstrated decreases both at -20 °C and at -80 °C (19) after storage even for two weeks. Although total bilirubin concentrations significantly decreased due to freeze-thaw cycles in the present study, practically serum is not stored for this analyte assay for future use in laboratories, considering its photo-sensitivity. In the CALIPER study (17), similar to our study, significant negative slope was observed for total bilirubin in samples stored at -20 °C with a low baseline concentration for a few months, and that relative large bias was attributed to the low baseline analyte concentrations.

The limitation of this study is that the results are based on small patient group size. Secondly, all time measurements were done once because of economical reasons. The pH of the samples was not measured to evaluate the possible bacterial

growth in the specimen which may cause erroneously elevated results for urea. Determinations were done with the defined analyzer with its original reagents. Therefore, these results might not be universally reproducible with other testing systems.

As a result, common clinical chemistry analytes, with considering the variability of unstable analytes, showed adequate stability after 3 months of storage in sera at -20 °C, or up to ten times of freeze-thaw cycle. All the same, such analysis can only be performed for exceptional cases, and results must be interpreted with great attention.

Potential conflict of interest

None declared.

References

1. Simundic AM, Lippi G. Preanalytical phase – a continuous challenge for laboratory professionals. *Biochem Med* 2012;22:145-9.
2. Männistö T, Surcel HM, Bloigu A, Ruokonen A, Hartikainen AL, Järvelin MR, et al. The effect of freezing, thawing, and short- and long-term storage on serum thyrotropin, thyroid hormones, and thyroid autoantibodies: implications for analyzing samples stored in serum banks. *Clin Chem* 2007;53:1986-7. <http://dx.doi.org/10.1373/clinchem.2007.091371>.
3. Gao YC, Yuan ZB, Yang YD, Lu HK. Effect of freeze-thaw cycles on serum measurements of AFP, CEA, CA125 and CA19-9. *Scand J Clin Lab Invest* 2007;67:741-7. <http://dx.doi.org/10.1080/00365510701297480>.
4. Hsing AW, Comstock GW, Polk F. Effect of repeated freezing and thawing on vitamins and hormones in serum. *Clin Chem* 1989;35:2145.
5. Zivkovic AM, Wiest MM, Nguyen UT, Davis R, Watkins SM, German JB. Effects of sample handling and storage on quantitative lipid analysis in human serum. *Metabolomics* 2009;5:507-16. <http://dx.doi.org/10.1007/s11306-009-0174-2>.
6. Koseoglu M, Hur A, Atay A, Cuhadar S. Effects of hemolysis interference on routine biochemistry parameters. *Biochem Med* 2011;21:79-85.
7. Calmarza P, Cordero J. Lipemia interferences in routine clinical biochemical tests. *Biochem Med* 2011;21:160-6.
8. Westgard QC. Desirable specifications for total error, imprecision, and bias, derived from intra- and inter-individual biological variation. Available at: <http://www.westgard.com/biodatabase1.htm#11>. Accessed January 2012.
9. Jackson C, Best N, Elliott P. UK Biobank pilot study: stability of haematological and clinical chemistry analytes. *Int J Epidemiol* 2008;37:116-22. <http://dx.doi.org/10.1093/ije/dym280>.
10. Cray C, Rodriguez M, Zaias J, Altman NH. Effects of storage temperature and time on clinical biochemical parameters from rat serum. *J Am Assoc Lab Anim Sci* 2009;48:202-4.
11. Flood A, Pfeiffer R, Mai V, Remaley A, Lanza E, Schatzkin A. The effects of freeze-thaw cycles on serum measurement of insulin and glucose in epidemiologic studies. *Annals of Epidemiol* 2002;12:528. [http://dx.doi.org/10.1016/S1047-2797\(02\)00391-5](http://dx.doi.org/10.1016/S1047-2797(02)00391-5).
12. Cao E, Chen Y, Cui Z, Foster PR. Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions. *Biotechnol and Bioeng* 2003;82:685-90. <http://dx.doi.org/10.1002/bit.10612>.
13. Mitchell B, Yasui Y, Li CI, Fitzpatrick AL, Lampe PD. Impact of freeze-thaw cycles and storage time on plasma samples used in mass spectrometry based biomarker discovery projects. *Cancer Informatics* 2005;1:98-104.
14. Paltiel L, Rønningen KS, Meltzer HM, Baker SV, Hoppin JA. Evaluation of freeze thaw cycles on stored plasma in the Biobank of the Norwegian Mother and child cohort study. *Cell Preserv Technol* 2008;6:223-30. <http://dx.doi.org/10.1089/cpt.2008.0012>.
15. Comstock GW, Burke AE, Norkus EP, Gordon GB, Hoffman SC, Helzlsouer KJ. Effects of repeated freeze-thaw cycles on concentrations of cholesterol, micronutrients, and hormones in human plasma and serum. *Clin Chem* 2001;47:139-42.
16. Beekhof PK, Gorshunskaya M, Jansen EH. Long term stability of paraoxonase-1 and high-density lipoprotein in human serum. *Lipids Health Dis* 2012;11:53. <http://dx.doi.org/10.1186/1476-511X-11-53>.
17. Brinc D, Chan MK, Venner AA, Pasic MD, Colatonia D, Kyriakopoulou L, Adeli K. Long-term stability of biochemical markers in pediatric serum specimens stored at -80 °C: A CALLPER Substudy. *Clin Biochem* 2012;45:816-26. <http://dx.doi.org/10.1016/j.clinbiochem.2012.03.029>.
18. Cuhadar S, Atay A, Koseoglu M, Dirican A, Hur A. Stability studies of common biochemical analytes in serum separator tubes with or without gel barrier subjected to various storage conditions. *Biochem Med* 2012;22:202-14.
19. Amin SB, Ahlfors C. Effect of storage and freezing on unbound bilirubin measurement. *Clin Chim Acta* 2008;396:56-7. <http://dx.doi.org/10.1016/j.cca.2008.06.023>.