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# Effect of Repeated Freezing and Thawing on 18 Clinical Chemistry Analytes in Rat Serum

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**In a preclinical research laboratory, using serum samples that have been frozen and thawed repeatedly is sometimes unavoidable when needing to confirm previous results or perform additional analysis. Here we determined the effects of multiple cycles of refrigeration or freezing and thawing of rat serum at 3 temperature conditions for different storage times on clinical chemistry analytes. Serum samples obtained from adult Wistar rats were stored at 2 to 8 °C and −10 to −20 °C for as long as 72 h and at −70 °C for as long as 30 d. At different time points (24, 48, and 72 h for samples stored at 2 to 8 °C or −10 to −20 °C and 1, 7, and 30 d for samples stored at −70 °C), the samples were brought to room temperature, analyzed, and then stored again at the designated temperature. The results obtained after each storage cycle were compared with those obtained from the initial analysis of fresh samples. Of the 18 serum analytes evaluated, 14 were stable without significant changes, even after 3 freeze–thaw cycles at the tested temperature ranges. Results from this study will help researchers working with rat serum to interpret the biochemical data obtained from serum samples that have been frozen and thawed repeatedly.**

Repeated cooling or freezing and thawing has long been assumed to affect the biochemical analytes of serum samples. However sometimes the use of such samples is unavoidable, as when needing to confirm the previous results or analyze additional analytes or at the time of instrument failure. The stability of biochemical analytes in human,[1,7] canine,[10] and rat serum[3] that has been stored and thawed only once for analysis has been addressed. In addition, the effect of repeated freezing and thawing on selected analytes in the serum and plasma of humans,[2,4] baboons,[5] and canines[9] has been reported previously. However similar information is unavailable for rats (*Rattus norvegicus*), a common laboratory animal used in various in vivo pharmacology and toxicity studies of drugs and pesticides.

Typically serum is used to assess the effects of drugs on clinical chemistry analytes in these studies. Often, researchers need to reanalyze a few or all of the serum analytes to confirm conclusions or previous results. In addition, in the case of instrument failure, serum samples may need to be stored for future analysis. Under such circumstances, serum samples may need to be frozen and thawed repeatedly. Generally, serum samples for clinical chemistry analysis are refrigerated (2 to 8 °C) or frozen (−10 to −20 °C) for short-term durations and stored at −70 °C for long periods. In view of these practical situations, we investigated the effect of 3 freeze–thaw cycles on 18 serum clinical chemistry analytes that are evaluated routinely during nonclinical studies of drugs or chemicals in rodents.[11]

## Materials and Methods

**Housing and animal husbandry.** Apparently healthy 20- to 25-wk-old male Wistar rats were obtained inhouse from the Animal Research Facility of Zydus Research Centre (Ahmedabad, India), where they were bred and reared under controlled environmental conditions. The health of the rat colony was monitored regularly. Rats were housed in individually ventilated polysulfone cages with adequate floor space and controlled environmental conditions (temperature, 22 to 25 °C; humidity, 55% to 60%) according to AAALAC specifications. This facility is accredited by AAALAC. Rats were given a commercial pellet feed (Amrit Feeds Pvt Sangli, Maharashtra, India) of standard nutritional composition and purified drinking water ad libitum.

**Study design.** Blood (2 mL per rat) was collected through the retroorbital plexus from 30 rats under light ether anesthesia. Blood was allowed to clot for 45 to 60 min and centrifuged at 3000 × *g* for 10 min to separate the serum. Three pooled samples of serum from 10 rats each were prepared. Each pooled sample was divided into 10 aliquots. All of the aliquots were analyzed immediately for 18 serum clinical chemistry analytes on the same day. Ten samples each were stored at different temperatures: refrigeration (2 to 8 °C), freezing (−10 to −20 °C), and deep-freezing (−70 °C). The samples stored at refrigeration temperature and −10 to −20 °C were analyzed after bringing them to room temperature at 24, 48, and 72 h of storage, whereas the samples stored at −70 °C were thawed and analyzed after 24 h and 7 and 30 d. After each analysis, all samples were returned to their respective storage conditions. Here we use term 'cycle' to define the freezing or cooling of samples and bringing those samples to room temperature. The temperatures of the laboratory (22 ± 5 °C), refrigerators, freezer, and deep-freezer were monitored and recorded daily to ensure that all temperatures were within prescribed limits.

**Clinical chemistry analysis.** Clinical chemistry analysis was done by using a Daytona autoanalyzer (Randox Laboratories, Crumlin, UK) as described previously.[6] Analytes were evaluated according to standard methods as per manufacturer's (Randox Laboratories, Crumlin, UK) instructions as detailed in our previous publication.[6] Serum levels of electrolytes such as sodium, potassium, and chloride were analyzed by using an

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**Table 1.** Effect of 3 cooling cycles on clinical chemistry analytes of rat serum at 2 to 8 °C (refrigeration temperature)

| | | Cooling cycle 1 (24 h) | | Cooling cycle 2 (48 h) | | Cooling cycle 3 (72 h) | |
|---|---|---|---|---|---|---|---|
| Analyte (unit) | Fresh sample | Mean ± 1 SD | % difference | Mean ± 1 SD | % difference | Mean ± 1 SD | % difference |
| Glucose (mg/dL) | 152.0 ± 1.3 | 154.8 ± 0.7 | 1.8 | 158.8 ± 0.9 | 4.5 | 159.6 ± 1.3 | 5.0 |
| Triglyceride (mg/dL) | 139.8 ± 2.3 | 143.6 ± 2.2 | 2.7 | 151.9 ± 1.5 | 8.7 | 153.8 ± 2.5 | 10.0 |
| Total cholesterol (mg/dL) | 46.4 ± 0.7 | 47.2 ± 1.3 | 1.7 | 46.8 ± 0.7 | 0.9 | 46.4 ± 0.6 | 0.1 |
| HDL cholesterol (mg/dL) | 20.1 ± 0.5 | 20.2 ± 0.7 | 0.4 | 20.4 ± 0.3 | 1.4 | 19.7 ± 0.7 | −2.2 |
| AST (U/L) | 127.7 ± 1.2 | 128.2 ± 1.7 | 0.5 | 130.5 ± 1.4 | 2.2 | 130.1 ± 0.9 | 1.9 |
| ALT (U/L) | 23.7 ± 0.6 | 24.3 ± 1.2 | 2.2 | 25.2 ± 0.9 | 6.2 | 24.4 ± 0.5 | 2.7 |
| ALP (U/L) | 119.3 ± 1.1 | 119.5 ± 0.5 | 0.1 | 119.6 ± 0.8 | 0.2 | 117.7 ± 0.9 | −1.3 |
| Total bilirubin (mg/dL) | 0.16 ± 0.03 | 0.17 ± 0.01 | 3.1 | 0.16 ± 0.01 | 1.0 | 0.16 ± 0.01 | −1.0 |
| Total protein (g/dL) | 5.8 ± 0.1 | 5.8 ± 0.1 | −1.4 | 5.9 ± 0.1 | 1.1 | 5.9 ± 0.1 | 0.3 |
| Albumin (g/dL) | 3.7 ± 0.1 | 3.8 ± 0.1 | 1.8 | 3.8 ± 0.0 | 3.2 | 3.8 ± 0.0 | 2.3 |
| Urea (mg/dL) | 48.1 ± 1.4 | 47.3 ± 0.9 | −1.6 | 48.1 ± 0.8 | 0.2 | 49.2 ± 0.9 | 2.4 |
| Uric acid (mg/dL) | 1.8 ± 0.1 | 1.9 ± 0.0 | 3.6 | 2.0 ± 0.1 | 6.4 | 2.0 ± 0.1 | 6.4 |
| Creatine kinase (U/L) | 1335.2 ± 16.5 | 1213.4 ± 53.6 | −9.1 | 1223.1 ± 24.6 | −8.4 | 1200.2 ± 12.9 | −10.1 |
| Calcium (mg/dL) | 10.3 ± 0.2 | 10.2 ± 0.1 | −1.1 | 10.6 ± 0.2 | 3.2 | 10.6 ± 0.1 | 3.1 |
| Phosphorus (mg/dL) | 6.5 ± 0.1 | 6.6 ± 0.1 | 2.8 | 6.2 ± 0.1 | −3.9 | 6.2 ± 0.1 | −3.4 |
| Sodium (mmol/L) | 138.9 ± 0.5 | 139.3 ± 0.4 | 0.3 | 139.2 ± 0.4 | 0.3 | 139.4 ± 0.3 | 0.4 |
| Potassium (mmol/L) | 3.9 ± 0.0 | 3.9 ± 0.0 | 0.4 | 3.9 ± 0.0 | 0.5 | 3.9 ± 0.0 | 0.3 |
| Chloride (mmol/L) | 102.2 ± 0.3 | 101.8 ± 0.4 | −0.4 | 102.0 ± 0.2 | −0.2 | 102.6 ± 0.5[a] | 0.4 |

Data are given as mean ± 1 SD ($n$ = 10), with the percentage difference relative to the value after immediate analysis
[a]$P < 0.05$ as compared with value after immediate analysis of fresh sample.

**Table 2.** Effect of 3 freeze–thaw cycles on clinical chemistry analytes of rat serum at −10 to −20 °C (freezing temperature)

| | | Freeze–thaw cycle 1 (24 h) | | Freeze–thaw cycle 2 (48 h) | | Freeze–thaw cycle 3 (72 h) | |
|---|---|---|---|---|---|---|---|
| Analyte (unit) | Fresh sample | Mean ± 1 SD | % difference | Mean ± 1 SD | % difference | Mean ± 1 SD | % difference |
| Glucose (mg/dL) | 151.3 ± 1.2 | 154.4 ± 1.4 | 2.0 | 157.6 ± 1.4[b] | 4.2 | 155.9 ± 1.6 | 3.0 |
| Triglyceride (mg/dL) | 188.5 ± 1.2 | 192.0 ± 1.0 | 1.8 | 201.8 ± 4.0 | 7.0 | 202.4 ± 3.2 | 7.4 |
| Total cholesterol (mg/dL) | 48.2 ± 0.3 | 49.0 ± 0.5 | 1.6 | 49.4 ± 0.6 | 2.4 | 48.6 ± 0.7 | 0.9 |
| HDL-C (mg/dL) | 20.8 ± 0.7 | 21.5 ± 0.5 | 3.8 | 21.4 ± 0.6 | 3.2 | 21.5 ± 0.6 | 3.6 |
| AST (U/L) | 113.8 ± 1.5 | 114.2 ± 1.1 | 0.3 | 116.0 ± 1.1 | 1.9 | 115.3 ± 1.2 | 1.3 |
| ALT (U/L) | 28.6 ± 0.4 | 28.9 ± 0.7 | 0.9 | 28.8 ± 0.3 | 0.5 | 27.8 ± 0.5 | −2.9 |
| ALP (U/L) | 145.3 ± 1.2 | 145.1 ± 1.6 | −0.1 | 144.0 ± 1.6 | −0.9 | 141.5 ± 1.6 | −2.6 |
| Total bilirubin (mg/dL) | 0.19 ± 0.02 | 0.19 ± 0.01 | −0.9 | 0.18 ± 0.03 | −6.9 | 0.20 ± 0.01 | 2.6 |
| Total protein (g/dL) | 6.2 ± 0.1 | 6.2 ± 0.1 | −1.1 | 6.4 ± 0.1 | 2.4 | 6.2 ± 0.1 | 0.0 |
| Albumin (g/dL) | 3.90 ± 0.0 | 3.92 ± 0.0 | 0.4 | 3.98 ± 0.0[b] | 2.1 | 3.97 ± 0.1[a] | 1.7 |
| Urea (mg/dL) | 43.7 ± 0.4 | 43.5 ± 0.7 | −0.5 | 42.4 ± 2.6 | −3.1 | 44.5 ± 0.7 | 1.8 |
| Uric acid (mg/dL) | 1.5 ± 0.1 | 1.5 ± 0.1 | 0.0 | 1.6 ± 0.1 | 5.6 | 1.6 ± 0.1 | 3.3 |
| Creatine kinase (U/L) | 1206.4 ± 14.4 | 1129.0 ± 45.8 | −6.4 | 1185.0 ± 19.0 | −1.8 | 1156.5 ± 19.4 | −4.1 |
| Calcium (mg/dL) | 10.3 ± 0.1 | 9.9 ± 1.4 | −4.2 | 10.9 ± 0.2 | 5.3 | 10.8 ± 0.1 | 4.4 |
| Phosphorus (mg/dL) | 6.2 ± 0.1 | 6.2 ± 0.0 | 0.8 | 5.8 ± 0.1 | −6.8 | 5.8 ± 0.1 | −6.5 |
| Sodium (mmol/L) | 139.5 ± 0.3 | 139.5 ± 0.2 | 0.0 | 139.4 ± 0.4 | −0.1 | 139.5 ± 0.3 | 0.0 |
| Potassium (mmol/L) | 3.9 ± 0.0 | 3.9 ± 0.0 | 0.2 | 3.9 ± 0.0 | 0.1 | 3.9 ± 0.0 | 0.0 |
| Chloride (mmol/L) | 102.7 ± 0.2 | 102.6 ± 0.1 | 0.0 | 102.7 ± 0.5 | 0.0 | 103.9 ± 0.5[b] | 1.2 |

Data are given as mean ± 1 SD ($n$ = 10), with the percentage difference relative to the value after immediate analysis.
[a]$P < 0.05$ as compared with value after immediate analysis of fresh sample.
[b]$P < 0.01$ as compared with value after immediate analysis of fresh sample.

Instalyte analyzer (ERBA, Mumbai, India) and the ion-selective electrode method.

All necessary precautions were taken to avoid any preanalytical, analytical, and postanalytical errors. Appropriate quality control (as per ISO15189: 2007) procedures were followed to ensure the integrity of the data. Validated instruments were used to analyze the samples in this study. All of the samples were analyzed in an ISO15189:2007-certified clinical pathology laboratory (Zydus Research Center).

**Table 3.** Effect of 3 freeze–thaw cycles on clinical chemistry analytes of rat serum at –70 °C (deep-freezing temperature)

| | | Freeze–thaw cycle 1 (24 h) | | Freeze–thaw cycle 2 (7 d) | | Freeze–thaw cycle 3 (30 d) | |
|---|---|---|---|---|---|---|---|
| Analyte (unit) | Fresh sample | Mean ± 1 SD | % difference | Mean ± 1 SD | % difference | Mean ± 1 SD | % difference |
| Glucose (mg/dL) | 156.7 ± 2.0 | 160.1 ± 2.6 | 2.2 | 158.6 ± 1.5 | 1.3 | 144.2 ± 3.4 | –7.9 |
| Triglyceride (mg/dL) | 155.0 ± 2.8 | 158.9 ± 1.2 | 2.5 | 161.0 ± 1.0[a] | 3.9 | 152.7 ± 3.3 | –1.5 |
| Total cholesterol (mg/dL) | 50.5 ± 0.7 | 50.5 ± 1.1 | 0.0 | 50.9 ± 0.9 | 0.9 | 53.4 ± 2.9 | 5.8 |
| HDL cholesterol (mg/dL) | 22.3 ± 0.5 | 22.7 ± 0.5 | 2.0 | 22.1 ± 0.6 | –0.7 | 23.1 ± 0.7 | 3.8 |
| AST (U/L) | 114.5 ± 1.4 | 115.0 ± 1.3 | 0.5 | 115.7 ± 1.3 | 1.1 | 117.1 ± 2.3 | 2.3 |
| ALT (U/L) | 26.5 ± 0.6 | 26.7 ± 0.6 | 0.8 | 26.7 ± 0.8 | 0.7 | 26.3 ± 0.8 | –0.8 |
| ALP (U/L) | 125.9 ± 0.9 | 125.5 ± 1.0 | –0.4 | 123.7 ± 1.1 | –1.7 | 125.9 ± 2.5 | 0.0 |
| Total bilirubin (mg/dL) | 0.17 ± 0.01 | 0.17 ± 0.01 | –0.4 | 0.16 ± 0.01 | –6.1 | 0.19 ± 0.02 | 6.3 |
| Total protein (g/dL) | 6.5 ± 0.1 | 6.4 ± 0.1 | –1.8 | 6.4 ± 0.1 | –1.3 | 6.5 ± 0.1 | 0.0 |
| Albumin (g/dL) | 4.0 ± 0.1 | 4.0 ± 0.1 | 0.0 | 4.1 ± 0.1[b] | 2.5 | 3.9 ± 0.1 | –2.5 |
| Urea (mg/dL) | 42.5 ± 0.8 | 42.3 ± 0.4 | –0.5 | 40.9 ± 0.3 | –3.8 | 38.3 ± 0.9 | –9.9 |
| Uric Acid (mg/dL) | 1.9 ± 0.0 | 1.9 ± 0.0 | 0.9 | 2.0 ± 0.0 | 5.3 | 2.0 ± 0.0 | 6.1 |
| Creatine kinase (U/L) | 1149.0 ± 12.4 | 1125.4 ± 18.9 | –2.0 | 1114.4 ± 17.7 | –3.0 | 1151.2 ± 21.8 | 0.2 |
| Calcium (mg/dL) | 10.7 ± 0.1 | 10.6 ± 0.1 | –0.9 | 10.9 ± 0.1 | 2.2 | 10.8 ± 0.1 | 1.1 |
| Phosphorus (mg/dL) | 5.9 ± 0.0 | 5.9 ± 0.1 | –0.6 | 5.7 ± 0.1 | –3.7 | 5.5 ± 0.1 | –7.1 |
| Sodium (mmol/L) | 139.5 ± 0.2 | 139.5 ± 0.2 | 0.0 | 140.1 ± 0.3 | 0.4 | 140.3 ± 2.0 | 0.6 |
| Potassium (mmol/L) | 3.9 ± 0.0 | 3.9 ± 0.0 | 0.1 | 3.9 ± 0.0 | 0.7 | 3.9 ± 0.0 | 1.1 |
| Chloride (mmol/L) | 102.3 ± 0.2 | 102.4 ± 0.2 | 0.1 | 102.6 ± 0.3 | 0.2 | 103.8 ± 1.4 | 1.4 |

Data are given as mean ± 1 SD ($n$ = 10) with the percentage difference relative to the value after immediate analysis.
[a]$P < 0.05$ as compared with value after immediate analysis of fresh sample.
[b]$P < 0.01$ as compared with value after immediate analysis of fresh sample.

**Table 4.** Intraassay, interassay, and interday precision

| Analyte (unit) | Intraassay | Interassay | Interday |
|---|---|---|---|
| Glucose (mg/dL) | 2.6 | 2.6 | 3.2 |
| Triglyceride (mg/dL) | 2.7 | 2.9 | 3.6 |
| Total cholesterol (mg/dL) | 2.4 | 2.4 | 3.2 |
| HDL cholesterol (mg/dL) | 1.7 | 1.7 | 3.6 |
| AST (U/L) | 2.9 | 2.8 | 2.7 |
| ALT (U/L) | 3.4 | 3.3 | 3.1 |
| ALP (U/L) | 1.8 | 2.0 | 3.1 |
| Total bilirubin (mg/dL) | 19.5 | 20.3 | 4.9 |
| Total protein (g/dL) | 1.0 | 1.3 | 2.7 |
| Albumin (g/dL) | 1.7 | 1.8 | 2.3 |
| Urea (mg/dL) | 8.1 | 8.2 | 2.9 |
| Uric acid (mg/dL) | 7.5 | 10.9 | 1.5 |
| Creatine kinase (U/L) | 1.3 | 2.0 | 4.1 |
| Calcium (mg/dL) | 1.8 | 1.8 | 3.2 |
| Phosphorus (mg/dL) | 0.6 | 0.94 | 2.6 |
| Sodium (mmol/L) | 0.3 | 0.28 | 1.1 |
| Potassium (mmol/L) | 0.4 | 0.43 | 1.3 |
| Chloride (mmol/L) | 0.2 | 0.24 | 1.2 |

Data are given as coefficients of variation.

Intraassay precision (as a coefficient of variation) was estimated by analyzing a rat serum sample 10 times in single assay run, whereas interassay precision was estimated by analyzing 10 samples in 2 assay runs. Interday precision was calculated from 6 mo of data of internal quality control samples (Randox Laboratories). Coefficients of variation were calculated by using the formula CV = SD × 100 / mean value.

**Statistical analysis.** Paired $t$ test was applied to compare the statistical significance of differences between data obtained after each of 3 cooling or freezing cycles and values obtained from fresh samples on day 0 by using Prism software (version 4, GraphPad Software, San Diego, CA). Data were analyzed at the 5% and 1% levels of significance. The means, SD, and percentages of difference between the means from fresh and stored samples were calculated by using Excel (Microsoft, Redmond, WA).

## Results

**Refrigeration (2 to 8 °C).** After 3 cycles of refrigeration of serum and subsequently bringing it to room temperature, changes in serum chloride (0.4%) were statistically significant at the 5% level. After the first cycle, changes in all the analytes were less than 10%; the maximal change occurred in creatine kinase (−9.1%). After the second cycle, the maximal change was found in triglycerides (8.7%), whereas after third cycle, creatine kinase (10.1%) showed the greatest change compared with fresh samples (Table 1).

**Freezing (−10 to −20 °C).** After the first cycle, creatine kinase showed the greatest change (−6.4%) relative to the value from fresh samples, whereas triglycerides had the largest variation after the second (7%) and third (7.4%) freeze–thaw cycles. Differences at the 1% level of significance occurred in chloride (1.2%) after third cycle and in glucose (3%) and albumin (2.1%) after the second cycle; albumin also showed a difference (1.7%) at the 5% level of significance after the third cycle (Table 2).

**Deep-freezing (−70 °C).** Differences at 5% level of significance were present in triglyceride (3.9%) and albumin (2.5%) after the second freeze–thaw cycle. Maximal changes occurred in triglyceride (2.5%) after the first cycle, total bilirubin (−6.1%) after the second cycle, and glucose (−7.9%) after the third cycle (Table 3).

Precision data (intraassay, interassay, and interday coefficients of variation) are provided in Table 4.

## Discussion

In the present study, all the analytes tested in rat serum were stable after the first cycle (24 h) at all 3 temperatures (refrigeration, freezing, and deep-freezing), compared with values obtained from fresh samples. The stability of analytes in rat serum after a single thaw following as long as 7 d of storage has been reported previously.[3] After the second freeze–thaw cycle, glucose was increased after storage at −10 to −20 °C, albumin was increased after storage at −10 to −20 °C and −70 °C, and triglyceride was increased after storage at −70 °C. After the third cycle, chloride (2 to 8 °C and −10 to −20 °C) and albumin (−10 to −20 °C) showed significant changes compared with values obtained from fresh samples. These data indicate that changes in these analytes are not consistent across all cycles and storage temperatures tested. The transient increases in glucose, triglyceride, and albumin only after the second cycle cannot be attributed to the effect of repeated freezing and thawing, because similar changes did not occur after the third freeze–thaw cycle. Similar incidental changes that have been considered clinically insignificant are previously reported for glucose, urea, sodium, potassium, chloride, calcium, phosphorus, and ALT in canine plasma samples.[9] Similar insignificant changes have occurred in cholesterol and HDL cholesterol after repeated freeze–thawing of baboon serum[5] and in cholesterol, micronutrients, and hormones after repeated thawing of human plasma and serum samples.[2]

Whatever the reason, the statistically significant changes obtained indicate that chloride is unstable after 3 freeze–thaw cycles unless the serum sample is stored at deep-freezing temperatures and albumin is unstable after 2 or 3 freeze–thaw cycles when stored at −10 to −20 °C. The 14 analytes other than glucose, chloride, albumin, and triglyceride were stable after storage at all temperatures tested and 3 freeze–thaw cycles. The stability of triglyceride, sodium, cholesterol, and AST in human plasma after 10 freeze–thaw cycles has been reported earlier.[8]

In conclusion, most of the clinical chemistry analytes in rat serum are stable after storage at a wide range of temperatures (2 °C to −70 °C) and after as many as 3 freeze–thaw cycles. However, inconsistent yet significant changes in some of the analytes merit careful interpretation during the analysis of clinical chemistry data obtained from repeatedly frozen serum samples of rats. In addition, our results do not rule out possible effects of multiple thawing on serum analytes from diseased or abnormal rats. This relative stability of analytes in rat serum does not negate the preferred use of fresh samples.

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