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Effects of Storage Temperature and Time on Clinical Biochemical Parameters from Rat Serum

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Serum is often frozen and banked for analysis at a later date. This study assessed the stability of 17 analytes in rat serum during refrigeration at 4 °C and extended storage at –20 °C (frost-free and nonfrost-free freezers) and –70 °C. Samples were analyzed by using an automated dry-slide chemistry analyzer at time 0 and then stored as aliquots for analysis at time points including day 7, 30, 90, and 360. After 7 d of refrigeration, only creatine kinase activity had varied by more than 10% of the starting value. Freezing at –70 °C was clearly superior to –20 °C where changes were observed in CO_2 as early as day 30 and alanine aminotransferase as early as day 90. Samples stored in frost-free and nonfrost-free –20 °C freezers did not differ significantly through day 90. Factors such as storage time and temperature should be considered when designing any retrospective study.

Experimental design frequently necessitates the use of frozen samples for retrospective studies. The planning for multiple experimental time points often results in samples that will be analyzed together at a later date and thus subjected to different periods of storage before analysis. Acquisition of samples after hours or on weekends often results in a few days of refrigeration before analysis. In addition, many investigators may not have access to –70 °C storage or may use frost-free –20 °C freezers without being aware that doing so may affect their experimental results.

Studies of human blood samples, including stability analyses for refrigeration and the effects of freeze–thawing,[1-4,6,8,11] have resulted in guidelines for storage. Surprisingly, data from such reports in veterinary medicine are rather scarce, with only a few reports on the effects of storage on canine and avian samples.[5,9,12,13] Notably, studies in the veterinary literature indicate that stability differs among species, including humans. The present study examined the stability of 17 biochemical analytes in rat serum after refrigeration and freezing at 2 temperatures and at multiple time points.

Materials and Methods

Animals and housing. All animals were maintained in accordance with the temperature and humidity recommendations of the *Guide for the Care and Use of Laboratory Animals* at the facilities of the University of Miami, which are AAALAC-accredited.[7] All experimental procedures were approved by the university's Animal care and use committee. As part of the university's rodent health program, sentinel rats are maintained on dirty bedding and screened quarterly for the following agents: Sendai virus, rat coronavirus (sialodacryoadenitis) virus, pneumonia virus of mice, Kilham rat (H1) virus, *Mycoplasma pulmonis*, rat parvovirus, rat minute virus, and parasitic infections. Once a year, the panel is extended to include the following agents: Thieler murine encephalomyelitis virus, *Encephalitozoon cuniculi*, mouse adenovirus, reovirus, hantavirus, and cilia-associated respiratory *bacillus*. All results from the sentinel rats were negative during the course of this investigation.

For this study, Sprague–Dawley rats were obtained from a commercial vendor (Harlan, Indianapolis, IN) and were kept conventionally housed in groups of 2 animals per cage using nonautoclaved bedding (Aspen, Harlan Teklad, Madison WI) and microisolation tops. Rats were given ad libitum access to rodent chow (Lab Diet 5001, PMI International, Richmond, IN) and municipal water by bottle.

Sample collection, storage, and analysis. Rats were euthanized by CO_2 overdose, and blood was collected by cardiac puncture and placed in clot tubes (Terumo Capiject, VWR, West Chester, PA). The blood was allowed to clot for 30 min and the serum was separated by centrifugation. All samples were free of hemolysis and lipemia.

Each study sample was a unique sample pooled from 2 individual animals. A total of 16 rats were bled on a single day for the first study to create 8 serum samples which were analyzed at all time points for the refrigeration, –20 °C, and –70 °C comparison. A total of 20 rats were bled on a single day for the second study to create 10 serum samples that were analyzed in the study comparing –20 °C freezers (frost-free versus nonfrost-free). The serum was analyzed for the day 0 time point and then aliquoted in freezer appropriate O-ring enclosure freezer tubes for storage at either 4 °C (range, 2 °C to 4 °C), –20 °C nonfrost-free (range, –18 °C to –22 °C), –20 °C frost-free (range, –16 °C to –25 °C) or –70 °C (range, –72 °C to –76 °C). The –20 °C freezers were a frost-free freezer that goes through a preprogrammed warm-up cycle to prevent the buildup of frost and a –20 °C freezer that does not have this option and maintains a stable temperature. Samples were analyzed on days 7, 30, 90, and 360. The freezers and refrigerator were monitored for temperature through the use of thermometers and high temperature alarms. All equipment is on automatic emergency power back up.

All chemistry analyses were conducted by using a dry-slide chemistry analyzer (Vitros 250, Ortho, Rochester, NY). Quality assurance controls representing high and low values were run on the analyzer throughout the study. The following analyses were performed: glucose, BUN, sodium, potassium, chloride,

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total CO_2, amylase, lipase, calcium, phosphorus, total protein, albumin, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, creatine kinase, and alkaline phosphatase.

Statistical analysis. The mean and standard error of samples was calculated, and percentage differences from the starting value were reported. Differences greater than 10% were not only statistically significant (by using paired *t* test methodology) and represented changes greater than 2 standard deviation of the starting values but also represented the minimal difference that was considered to have clinical significance in the interpretation of the biochemical analysis. All analyses were conducted using GraphPad Prism 4 software (La Jolla, CA).

Results

After samples had been refrigerated for 7 d, several analytes showed slight changes: CO_2 decreased by 9.4%, creatine kinase activity by 11.1%, and lactate dehydrogenase by 8.0% ($P < 0.05$) (Table 1). Significant changes were not detected in samples frozen at –20 °C (frost-free) and –70 °C through day 7. However, creatine kinase activity dropped by 59% and alanine aminotransferase activity decreased by 54% by day 360 in the frost-free freezer ($P < 0.05$). Other changes ($P < 0.05$) occurred in CO_2, lipase, calcium, and alkaline phosphatase. In the samples stored at –70 °C, none of the chemistry markers had changed by more than 10% by day 360.

In a second study, samples were stored in frost-free and nonfrost-free freezers at –20 °C and analyzed on days 30 and 90 (Table 2). Samples in both freezers showed decreases of more than 10% in CO_2, and levels were even lower on day 90 ($P < 0.05$). Overall, analytes did not differ between the two –20 °C freezers through day 90.

Discussion

Storage of samples has been recognized as an important factor in human clinical pathology.[1-4,6,8,10] Differences among the cited studies may be due to differences in sample handling before serum separation, storage temperatures, and test methodologies. In the veterinary literature, similar studies have been limited to canine and avian blood samples, which revealed interspecies differences in storage stability.[5,9,12,13] Multispecies data from our laboratory (not shown) support this premise.

In the current study, refrigeration of rat serum for 7 d resulted in greater than a 10% decrease in creatine kinase activity. The activity of this enzyme in canine serum showed similar declines after storage at room temperature for 3 d.[12] In addition, concentrations of CO_2 in rat serum decreased by 9.4% over 7 d of refrigeration. This observation is consistent with findings from studies using human serum samples, for which loss of CO_2 to the ambient atmosphere has been suggested as a cause for the differing concentrations.[8,10]

Freezing rat serum samples at –70 °C resulted in only modest changes in the analyte levels, with changes of less than 10% even after 360 d of storage. This finding is consistent with the human literature, which contains reports of analyte stability for 5 y.[2] In addition, for many enzymes, storage in liquid nitrogen is superior to storage at –20 °C.[4] Canine serum and plasma reportedly were more stable at –70 °C vs. -20ºC over 240 d of storage, although both lactate dehydrogenase and amylase activity showed clinically significant changes (>10%).[13]

Prolonged freezing of samples of rat serum in a frost-free –20 °C freezer led to many changes in analyte levels. These changes may be related to the overall higher temperature (relative to –70 °C). In the rat samples, alanine aminotransferase showed early significant decreases after 90 d. By day 360, greater than 50% decreases in alanine aminotransferase and creatine kinase were apparent, as were other less dramatic changes in lipase and alkaline phosphatase, for example. In the canine storage studies, alanine aminotransferase, alkaline phosphatase, and creatine kinase decreased, whereas amylase increased.[13] Changes in enzyme activity are hypothesized to occur due to instability of enzyme isoforms.[6] In addition, CO_2 and calcium levels both showed changes of more than 10%. The CO_2 changes appear consistent with those in the refrigerated samples. The observed increase in calcium after 360 d has not been reported previously.

To address differences between nonfrost-free and frost-free –20 °C freezers, we evaluated rat serum stored for 30 or 90 d. Notably, no significant differences were found between the freezers through the day 90 time point, with CO_2 reaching a 18.3% decrease at day 90 in the nonfrost-free freezer compared with 21.5% in the frost-free freezer. Because most of the marked changes in analyte levels occurred between days 90 and 360, short-term (30 d) storage at –20 °C may be acceptable.

Throughout the multiple time points we assessed, the standard errors associated with the results were consistent among analytes. That is, no single sample showed marked instability. Because we obtained the samples from normal healthy rats, this result was expected. We presume that samples from clinically ill rats would behave similarly, but differences in enzyme isoforms and perhaps higher or lower starting values may be associated with particular sample instabilities. Further studies should be conducted to address this issue.

The present results show that, with the exception of creatine kinase activity, common biochemical analytes in rat serum are stable under refrigeration for 7 d. Whenever possible, prolonged sample storage should occur at –70 °C. If a –70 °C freezer is not available, –20 °C storage for as long as 90 d is acceptable for common analytes except CO_2 and alanine aminotransferase. Retrospective studies and those requiring storage and batch analysis should accommodate these storage restrictions.

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Table 1. Effects of prolonged refrigeration and freezing on biochemical analytes in rat serum

Analyte	Mean ± SE, day 0	Mean (n = 9 or 10) percentage change from day 0 value		
		Refrigeration, day 7	Frost-free –20°C freezer, day 360	Freezing –70°C, day 360
Glucose, mg/dL	153.3 ± 7.0	1.6	(5.8)	1.3
Blood urea nitrogen, mg/dL	21.25 ± 0.67	0.2	6.5	(0.3)
Sodium, mmol/L	145.8 ± 0.5	(1.0)	1.3	1.1
Potassium, mmol/L	5.61 ± 0.07	(0.7)	(1.2)	1.2
Chloride, mmol/L	97.8 ± 0.5	(0.2)	3.3	2.4
Carbon dioxide, mmol/L	32.5 ± 0.07	(9.4)[a]	(31.1)[a]	4.3
Amylase, U/L	1243.0 ± 37.6	1.4	0.9	(0.6)
Lipase, U/L	91.5 ± 2.7	(0.9)	(19.1)[a]	4.4
Calcium, mg/dL	11.03 ± 0.08	0.8	17.0[a]	3.0
Phosphorus, mg/dL	8.64 ± 0.15	(0.5)	6.8	1.4
Total protein, g/dL	6.35 ± 0.04	(4.1)	(2.4)	0.5
Albumin, g/dL	3.70 ± 0.04	(2.7)	6.8	(1.9)
Aspartate transaminase, U/L	111.9 ± 3.5	(3.8)	(7.1)	8.8
Alanine transaminase, U/L	92.6 ± 1.6	0.4	(54.0)[a]	(3.6)
Lactate dehydrogenase, U/L	887.0 ± 54.2	(8.0)[a]	2.8	3.3
Creatine kinase, U/L	178.1 ± 19.2	(11.1)[a]	(59.3)[a]	(2.7)
Alkaline phosphatase, U/L	208.9 ± 13.2	(2.5)	(22.9)[a]	5.7

Values in parentheses indicate decreases from day 0 value.
[a]Values are significantly ($P < 0.05$) different from day 0 value.

Table 2. Effects of storage in frost-free and nonfrost-free –20°C freezers on biochemical analytes in rat serum

Analyte	Mean ± SE, day 0	Mean (n = 10) percentage change from day 0 values			
		Frost-free freezer		Nonfrost-free freezer	
		day 30	day 90	day 30	day 90
Glucose, mg/dL	153.3 ± 7.0	0.6	0.7	2.5	0.3
Blood urea nitrogen, mg/dL	21.25 ± 0.67	0.6	3.6	4.8	(1.2)
Sodium, mmol/L	145.8 ± 0.5	(1.9)	(1.7)	(0.1)	(2.4)
Potassium, mmol/L	5.61 ± 0.07	1.1	0.4	3.1	(0.1)
Chloride, mmol/L	97.8 ± 0.5	2.6	(1.8)	5.1	(3.0)
Carbon dioxide, mmol/L	32.5 ± 0.07	(12.1)[a]	(21.5)[a]	(10.7)[a]	(18.3)[a]
Amylase, U/L	1243.0 ± 37.6	(1.0)	(2.6)	(2.5)	(4.7)
Lipase, U/L	91.5 ± 2.7	0.4	(0.7)	2.8	(0.3)
Calcium, mg/dL	11.03 ± 0.08	1.2	(1.7)	2.8	(0.8)
Phosphorus, mg/dL	8.64 ± 0.15	(1.6)	(3.3)	0.6	(0.6)
Total protein, g/dL	6.35 ± 0.04	2.0	4.9	3.4	4.5
Albumin, g/dL	3.70 ± 0.04	1.7	3.6	5.5	1.1
Aspartate aminotransaminase, U/L	111.9 ± 3.5	(1.8)	(2.8)	1.1	(4.0)
Alanine aminotransaminase, U/L	92.6 ± 1.6	(4.8)	(8.8)	(2.0)	(10.3)
Lactate dehydrogenase, U/L	887.0 ± 54.2	0.3	1.8	2.5	0.6
Creatine kinase, U/L	178.1 ± 19.2	(1.3)	(5.7)	(1.0)	(2.7)
Alkaline phosphatase, U/L	208.9 ± 13.2	(1.3)	(5.6)	(1.9)	(3.6)

Values in parentheses indicate decreases from day 0 value.
[a]Values are significantly ($P < 0.05$) different from day 0 value.

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