

EFFECT OF FREEZING AND STORAGE TIME OF BLOOD SERUM OF RAT IN THE DETERMINATION OF BIOCHEMICAL PARAMETERS

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RESUMO

O presente estudo apresenta relevância significativa para o contexto laboratorial de pesquisa, em que diversas amostras são armazenadas para análises bioquímicas posteriores. O objetivo foi avaliar a estabilidade de testes bioquímicos em soro de ratos Wistar mantidos em diferentes temperaturas por até 360 dias. A pesquisa foi realizada na Faculdade de Medicina da UNESP, Unidade de Pesquisa Experimental (UNIPLEX), em Botucatu – SP. Foram utilizados soros de 30 ratos machos Wistar, com aproximadamente quatro meses de idade. As análises foram feitas imediatamente após a coleta (tempo zero) e após 30, 180 e 360 dias de armazenamento a -20°C e -80°C. Avaliaram-se colesterol, albumina, ureia, proteína total, triglicerídeos e glicose. Observou-se que tempo e temperatura influenciam alguns parâmetros, exceto glicose, que se manteve estável por 360 dias. Os resultados indicam que temperaturas muito baixas nem sempre garantem melhor preservação das amostras.

Palavras-chave: Estabilidade bioquímica; Temperatura; Modelo experimental; Confiabilidade analítica.

ABSTRACT

This study holds significant relevance for the experimental research setting, where numerous samples are stored for subsequent biochemical analyses. The aim was to evaluate the stability of biochemical tests in serum from Wistar rats maintained at different temperatures for up to 360 days. The study was conducted at the School of Medicine, São Paulo State University (UNESP), Experimental Research Unit (UNIPLEX), Botucatu – SP, Brazil. Serum samples were obtained from 30 male Wistar rats, approximately four months old. Analyses were performed immediately after collection (time zero) and after 30, 180, and 360 days of storage at -20°C and -80°C. The analytes assessed were cholesterol, albumin, urea, total protein, triglycerides, and glucose. The results showed that time and temperature influence some biochemical parameters, except for glucose, which remained stable for 360 days. The findings indicate that extremely low temperatures do not necessarily ensure better preservation of samples.

Keywords: Biochemical stability; Temperature; Experimental model; Analytical reliability.

1. INTRODUCTION

Biochemical tests conducted using serum and blood plasma are important tools for aiding in the diagnosis of diseases affecting domestic animals (Clinical Biochemistry of Domestic Animals, 1997). The biochemical profile serves as an indicator of protein, energy, and mineral

metabolism, as well as potential alterations in the function of the liver, kidneys, pancreas, bones, muscles, heart, central nervous system, and gastrointestinal tract (González, 2003; Mundim et al., 2004). Clinical and preclinical studies use biochemical analyses as a research tool; therefore, performing accurate and reliable analyses is highly

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relevant. Several factors can lead to false results, such as hemolysis, temperature, collection method, and, often, the type of storage. In scientific studies, samples are frequently stored until analysis, making their preservation a determining factor for obtaining valid results.

Storage time may range from days to months or even years, and temperature can also fluctuate during this period, as immediate analysis after collection is often impractical, especially in production animals. In veterinary medicine, little has been published so far regarding the stability of whole blood and many biochemical markers (Davy; Jackson; Walker, 1984; De Oliveira et al., 2011; Devanapalli; Bermingham; Mahajan, 2002; Ehsani et al., 2008). In a study conducted by Fernandes et al. (2001) using 928 canine serum and plasma samples, it was found that blood serum is more stable than plasma under all tested storage conditions (Fernandes; Teixeira; Santos, 2001).

According to Russell and Roussel (2007), analyte stability varies, but most remain stable at refrigeration temperature (4°C) for 24 to 36 hours (Russell; Roussel, 2007). If a longer period is required before processing, serum can be frozen at -20°C, since repeated freeze-thaw cycles should be avoided as they affect the stability of various substances, especially enzymes. Currently, several storage options are available, ranging from 4°C to -80°C; however, not all laboratories or research centers have access to these systems. Lower temperatures, such as -80°C freezers, are more expensive and less accessible, whereas -20°C

freezers are more affordable in terms of both acquisition and maintenance.

Therefore, this study aimed to evaluate the influence of storage time at -80°C and -20°C on rat serum samples for the determination of cholesterol, albumin, urea, total protein, triglycerides, and glucose.

2. MATERIALS AND METHODS

2.1 BIOLOGICAL MATERIALS

1. Blood samples of 30 healthy rat, 4 months old, males.

2.2 MATERIALS

1. Sterile containers without anticoagulants.
2. Sodium thiopental

2.3 PROCEDURE

A. Blood samples

- I. **Collect samples from the heart in sterile containers without anticoagulant.**
 - a. Thirty male Wistar rats were used and subjected to exsanguination by cardiac puncture. The animals were housed in the UNIPLEX vivarium for 15 weeks until they reached an approximate weight of 400 grams. They were kept under controlled conditions, including a temperature of $22 \pm 2^\circ\text{C}$, humidity of $70 \pm 10\%$, air exchange, and a 12-hour light/dark cycle. They had free access to filtered water and standard laboratory-grade Purina chow.
 - b. The animals were anesthetized to a deep anesthetic plane using sodium thiopental at a dose of 300 mg/kg administered intraperitoneally

(Pharmaceuticals Used in Laboratory Animals – Anesthetics and Analgesics – Fiocruz Manual). The depth of anesthesia was monitored and recorded based on the presence or absence of specific reflexes (RN33, (Brasil. Conselho Nacional de Controle de Experimentação Animal (CONCEA), 2016)).

c. Once the animals no longer exhibited interdigital and tail pinch reflexes, cardiac puncture was performed, and all blood was aspirated.

d. At the conclusion of the procedure, death was confirmed, and the carcasses were discarded in a white opaque container and stored in the carcass disposal freezer until incineration. Please note that this description refers to standard procedures in biomedical research, which are subject to strict ethical and legal regulations to ensure the welfare of the animals used in experiments.

I. Whole blood

a. The whole blood was centrifuged at 2,500 rpm for 10 minutes to obtain the serum.

b. The serum from each animal was divided into four aliquots, placed in Eppendorf tubes, and properly labeled.

c. The aliquot labeled as “time zero” was processed immediately after centrifugation. Samples were placed in the analyzer for determination of the selected biochemical parameters.

d. The remaining aliquots were stored in freezers at -80°C and -20°C , both equipped with a

frost-free defrosting system, until processing after 30, 180, and 360 days of storage.

B. Biochemical parameters analyzed

1. Cholesterol – enzymatic colorimetric method (BioClin; Quibasa Química Básica Ltda., Belo Horizonte, MG, Brazil)
2. Albumin – colorimetric method (BioClin; Quibasa Química Básica Ltda., Belo Horizonte, MG, Brazil)
3. Urea – fixed-time kinetic method (BioClin; Quibasa Química Básica Ltda., Belo Horizonte, MG, Brazil)
4. Total protein – colorimetric method (BioClin; Quibasa Química Básica Ltda., Belo Horizonte, MG, Brazil)
5. Triglycerides – enzymatic colorimetric method (BioClin; Quibasa Química Básica Ltda., Belo Horizonte, MG, Brazil)
6. Glucose – enzymatic colorimetric method (BioClin; Quibasa Química Básica Ltda., Belo Horizonte, MG, Brazil)

C. Equipment

Laboratory analyses were performed using an automatic biochemical analyzer (BS-200, Mindray, Shenzhen, China).

D. Software and Datasets

BS-200 operational software, version 02.00.00.

2.4 DATA ANALYSIS

To assess the performance of biochemical parameters at the two storage temperatures over one year, linear mixed-effects models (LMMs) were built to evaluate the effects of temperature, time, and their interaction on each parameter. Extreme values were removed, and data were log-transformed to achieve normality. Individual rats were included as a random factor to account for repeated measures, while temperature, time, and their interactions were treated as fixed effects. Comparisons between storage times were made relative to the baseline (time zero) for each biochemical parameter.

The LMMs were fitted using the *lme4* package (Bates et al., 2015). The significance of each explanatory variable was tested with the *Anova* function in the *car* package (Fox; Weisberg, 2011). Post hoc comparisons based on the LMMs were performed using the *emmeans* package to detect pairwise differences relative to time zero ($\alpha = 0.05$; (Lenth, 2023)).

Marginal and conditional R^2 values were compared among models for each parameter, with and without interaction terms, and results from the selected models were reported. All statistical

analyses were performed in R (R Core Team, 2023).

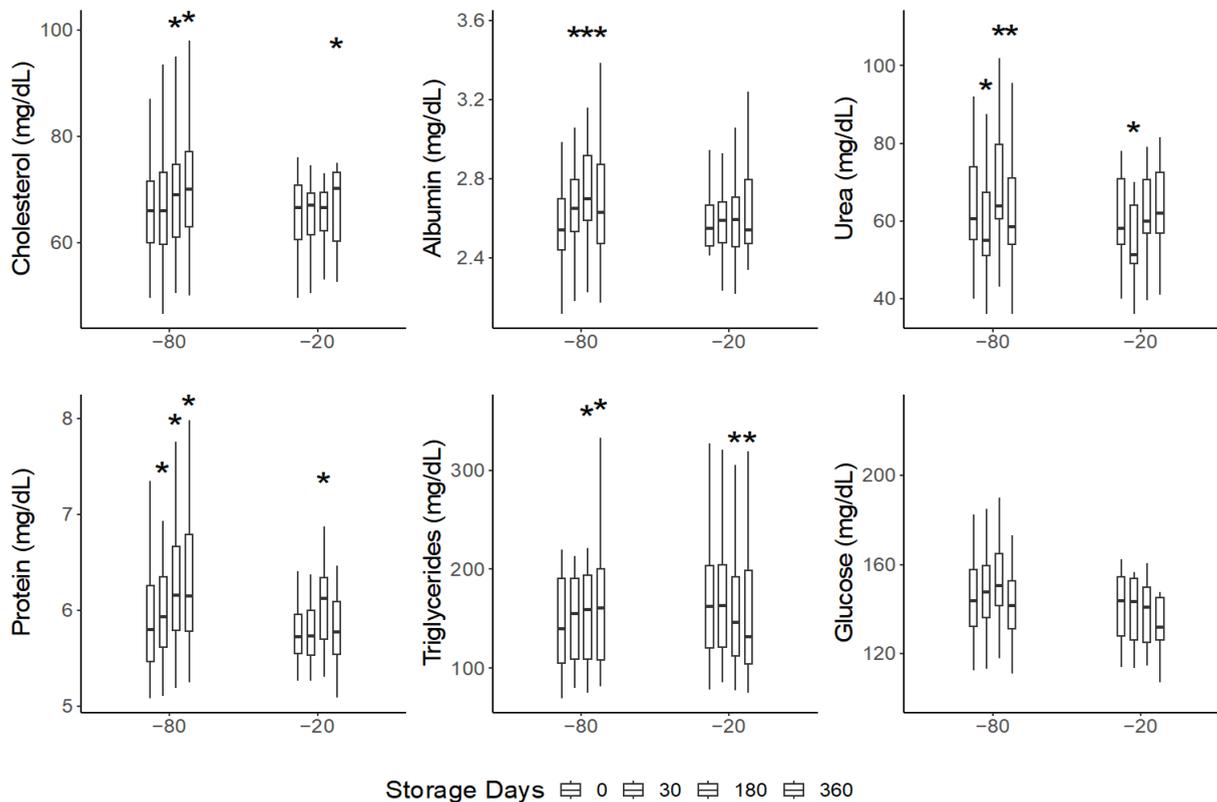
3. RESULTS AND DISCUSSION

Figure 1 shows the comparison of all biochemical parameters analyzed over the storage period. No significant differences were observed between treatments (-20°C vs. -80°C ; all $P > 0.05$). Changes were mainly related to storage time. Urea levels decreased at 30 days for both temperatures, and triglycerides decreased after 180 days. The other significant changes were associated with increased values over time.

At -80°C , albumin, urea, and total protein did not remain stable over time, showing significant differences compared with baseline (time zero). Serum cholesterol and triglycerides remained stable up to 180 days of storage, while glucose exhibited stability at all evaluated time points.

At -20°C , urea levels were not stable at any storage time. Total protein and triglycerides remained stable up to 180 days, while cholesterol was stable at 0, 30, and 180 days. Glucose also remained stable throughout all storage periods.

Figure 1. Comparison of all the biochemical parameters analyzed: cholesterol, albumin, urea, protein, triglycerides, and glucose, stored at two temperatures (-80 and -20°C), and assessed along the time (Storage days, colors of the bars). Different measures in each temperature are shown with *.



Most of the serum samples analyzed in our laboratory are frozen prior to analysis. However, according to the manufacturer's recommendations, the biochemical kits are validated for use with fresh samples. Therefore, this study aimed to verify the reliability of using frozen serum samples.

At -80°C , storage beyond 30 days altered the results of cholesterol and triglycerides, while albumin, urea, and total protein were not stable at any time. Glucose, however, showed no differences across all storage times, indicating high stability. At -20°C , urea should be measured in fresh

samples, as freezing led to significant changes. Triglycerides and total protein were stable up to 180 days of storage. For total protein, a significant increase was observed at 180 days compared with baseline (T0). A similar finding was reported by Oliveira (2016) in equine serum, where an increase in total protein was observed after 30 days of storage at -20°C , followed by a decrease at 60 and 90 days (Oliveira et al., 2016). Despite these fluctuations, the mean values remained within the reference range, corroborating the present findings for rat serum. Albumin and glucose remained stable at all evaluated times and temperatures. Although

studies assessing the stability of these analytes in rat serum are scarce, similar results were reported by Kuchmak et al. (1982) for human serum samples stored at -20°C for five years, which showed no alterations (Kuchmak; Taylor; Olansky, 1982).

4. FINAL CONSIDERATIONS

This study provides valuable information on the biochemical stability of rat serum samples stored under different temperature conditions over one year. Overall, glucose showed excellent stability at both -20°C and -80°C , while triglycerides and total protein remained reliable for up to 180 days, particularly at -20°C . In contrast, urea and albumin exhibited significant time-dependent alterations, indicating that these analytes should preferably be measured in fresh samples.

These findings reinforce the importance of validating storage conditions for biochemical analyses, especially in experimental models where sample reuse and long-term storage are common. The results may support laboratory protocols aiming to minimize pre-analytical variability and ensure analytical reliability.

Future studies should extend these observations by including additional biomarkers, evaluating shorter thawing cycles, and testing serum stability across other animal models and storage conditions.

5. ACKNOWLEDGMENTS

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6. COMPETING INTERESTS

The authors declare there is no conflict of interest.

7. ETHICAL CONSIDERATIONS

The project was approved by the Animal Experimentation Ethics Committee of the São Paulo State University (UNESP), Botucatu Medical School, under protocol number 1262.

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