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Effect of Freezing-thawing and Storage Time on Some Specific Human Enzymes

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Authors' contributions

This work was carried out in collaboration between all authors. Author CNE designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors PN and FCE managed the analyses of the study. Authors JNE and OGC managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The present research was designed and conducted to study the effect of freezing-thawing and storage time on some specific human enzymes. The enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) were analysed immediately after sample collection, after undergoing freeze thaw at -4°C , -20°C and -70°C at day 0 and after 7 days of storage at -4°C , -20°C and -70°C . A total of 50 healthy males and 50 healthy females were used for the study and sample collection was by pooled serum. Our results show that there was no statistically significant difference ($p>0.05$) between AST, ALT, ALP and CPK levels obtained after freeze-thaw at -4°C , -20°C and -70°C at day 0 when compared with the control for both males and females. Also, no statistically significant difference ($p>0.05$) was seen in the levels of AST, ALT, ALP, CPK analysed

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after 7 day storage at -4°C , -20°C and -70°C in both males and females when compared with the control. However, there was a significant difference ($p<0.05$) in the levels of LDH obtained after freeze-thaw at -4°C both at day 0 and after 7 days of storage. In conclusion, the results showed that the specific enzymes studied were most stable when stored at -70°C for 7 days assuming sample analysis is not carried out shortly after sample collection.

Keywords: Human serum; freeze; enzymes; thaw; storage.

1. INTRODUCTION

One of the greatest challenges faced in veterinary and human medical laboratory practice is the method and period of storage of samples as to ensure the stability of serum biochemical analytes. In most laboratories, samples are either stored in the refrigerator door at about $4-8^{\circ}\text{C}$ or in the deep freezer at about -20°C for an unlimited period. Hence, the temperature at which biochemical samples are stored has a great influence on the results obtained from a biochemistry laboratory [1].

Most clinical chemistry laboratories in tertiary institutions receive a lot of samples on a daily basis that may not be processed on the same day due to the following reasons: equipment breakdown, lack of reagents, power failure, acquisition of samples after work hours or over the weekends, need to save cost or also the need to store samples for future analysis or to confirm previous results [1]. The above reasons and the recurrent need to carry out research analysis in batches necessitate that samples are subjected to different periods of storage before analysis [2]. Such long-time storage before use exposes the samples to repeated freeze-thaw cycles which may adversely affect the results of clinical chemistry analysis [3,4].

Enzymes are protein molecules that are involved in the catalysis of biochemical reactions without themselves being altered or destroyed at the end of the reaction. They increase the rate of metabolism in the body. Disease diagnosis in human and veterinary medicine is pivoted on the measurement of serum enzymes levels which serve as markers for cellular damage [3]. Creatine phosphokinase (CPK), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), lactate dehydrogenase (LDH), ornithine carbamoyl transferase (OCT) and 5^1 - Nucleotidase (5^1NT) are routinely used as a tool for the diagnosis of myocardial and liver diseases [5,6]. Fluctuations of these enzyme levels are mainly the result of the leakage of

such enzymes from the cytosol into the blood stream.

Many studies abound which examined the effect of freezing-thawing and storage time on some biochemical analytes using methods that are best described as outdated [7,8] and most of these studies were carried out with animal blood samples [3,9-11]. However, there is paucity of information regarding the stability of the routinely used clinical chemistry analytes including the effect of storage for a prolonged time at a temperature as low as -70°C as well as the effect of repeated freeze-thaw cycles. This research therefore was carried out to assess the effect of freezing-thawing at different temperatures of -4°C , -20°C and -70°C as well as storage for 7 days on some specific human enzymes (AST, ALT, ALP, CPK, LDH).

2. MATERIALS AND METHODS

2.1 Study Design

A total of 100 subjects comprising 50 males and 50 females who presented at the outpatient department of Imo State University Medical Centre between March 2018 and May 2018 for pre-admission screening were randomly selected for this study. The samples collected from each subject was only for laboratory investigations. Informed written consent was obtained from all the subjects and Imo State University ethical committee approved the study. All procedures were conducted in accordance with the guidelines as stipulated in the Helsinki declaration on human experimentation.

2.2 Sample Collection and Analysis

Fasting venous blood (total of 10 mL) was collected in the morning using the standard blood collection technique. Venepuncture of the median cubital vein in the cubital fossa was done with a 10mL syringe and needle (21g x 1 ½ inches – 0.8mm x 40mm) manufactured by Anhui Kenning Industrial (Group) Co. Ltd. Anhui

province china. The blood specimen was transferred into 10 mL plain plastic specimen containers. Serum was harvested after the samples were allowed to stand at room temperature for about 30 to 45 minutes and following clot formation and centrifugation at 6000 revolutions per minute for 5 minutes. The serum sample of each patient was divided into 7 aliquots. The first aliquot was immediately assayed for the following enzymes AST, ALT, ALP, LDH and CPK within an hour of serum separation to serve as control. The samples were analysed 8 hours after sample collection and freeze-thawing at -4°C – 20°C and -70°C and served as basal fresh values (day 0). The remaining aliquots were stored at -4°C , -20°C and -70°C for 7 days before analysis. Prior to analysis of the frozen samples, the samples were left to stand at room temperature to thaw and repeatedly inverted to allow for proper mixing. All enzyme assays were performed using Ecoline-Merck diagnostic Kits (Merck specialties pvt. Ltd, Mumbai) on an auto blood analyzer (micro lab 200) at the laboratory of Imo State University Medical Centre Owerri.

2.3 Statistical Analysis

Data obtained from this study was analysed using statistical package for social science (IBM-SPSS), version 21.0 for windows. A test of significance was done using students t-test, and analysis of variance (ANOVA) was done at 0.05

level of significance. Results were expressed as mean \pm standard deviation and presented in tables.

3. RESULTS

Table 1 shows the values of serum enzyme levels (AST, ALP, LDH, and CPK) obtained from fresh female blood samples analysed shortly after collection (control) and then on the same day of collection (day 0) after freezing and thawing at -4°C , -20°C and -70°C while Table 2 shows values of serum enzymes obtained from the analysis of female blood samples after undergoing storage for 7 days at different temperatures of -4°C , -20°C and -70°C . There was no statistically significance difference ($p>0.05$) between the serum enzyme levels obtained shortly after sample collection and the serum enzyme levels measured after undergoing freezing at -4°C , -20°C and -70°C and thawing 8 hours after sample collection. However, the level of LDH obtained after the analysis of fresh sample at -4°C was significantly lower than the control ($p<0.05$).

Also, the values obtained after 7 days storage at -4°C , -20°C and -70°C were not significantly different ($p>0.05$) from the control. The level of LDH obtained after 7 days storage at -4°C was, however, significantly ($p<0.05$) lower when compared with the control.

Table 1. Effect of freezing-thawing on enzymes of fresh female blood samples

Enzymes	Control	-4°C ,	-20°C	-70°C
AST (IU/L)	10.81 \pm 0.10	7.22 \pm 0.02	8.15 \pm 0.03	9.42 \pm 0.03
ALT (IU/L)	8.12 \pm 0.50	5.19 \pm 0.02	5.24 \pm 0.02	7.81 \pm 0.05
ALP (IU/L)	65.26 \pm 0.30	53.40 \pm 0.02	54.51 \pm 0.02	62.09 \pm 0.04
LDH (IU/L)	195.21 \pm 0.20	165.61 \pm 0.06*	184.29 \pm 0.01	190.16 \pm 0.03
CPK (IU/L)	52.54 \pm 0.40	43.27 \pm 0.01	39.28 \pm 0.03	50.44 \pm 0.04

Values are mean \pm SD; n = 50

Values marked with asterisk are statistically different from control ($p<0.05$).

Table 2. Effect of 7 day storage at different temperatures on enzymes of female blood samples

Enzymes	Control	-4°C ,	-20°C	-70°C
AST (IU/L)	10.81 \pm 0.10	6.10 \pm 0.20	7.66 \pm 0.30	9.20 \pm 0.20
ALT (IU/L)	8.12 \pm 0.50	5.41 \pm 0.24	6.81 \pm 0.10	7.32 \pm 0.10
ALP (IU/L)	65.26 \pm 0.30	49.60 \pm 0.28	50.25 \pm 0.30	60.48 \pm 0.40
LDH (IU/L)	195.21 \pm 0.20	164.56 \pm 0.81*	180.36 \pm 0.62	190.20 \pm 0.56
CPK (IU/L)	52.54 \pm 0.40	40.15 \pm 0.36	42.17 \pm 0.32	49.16 \pm 0.40

Values are mean \pm SD; n = 50

Values marked with asterisk are statistically different from control ($p<0.05$).

Table 3. Effect of Freezing-thawing on enzymes of fresh male blood samples

Enzymes	Control	- 4°C,	- 20°C	- 70°C
AST (IU/L)	9.26 ± 0.15	6.38 ± 0.11	7.82 ± 0.03	9.15 ± 0.03
ALT (IU/L)	7.62 ± 0.13	4.41 ± 0.21	5.54 ± 0.02	6.62 ± 0.05
ALP (IU/L)	61.46 ± 0.32	52.38 ± 0.38	54.27 ± 0.42	58.49 ± 0.61
LDH (IU/L)	190.22 ± 0.54	171.62 ± 0.60*	175.36 ± 1.15	185.28 ± 0.30
CPK (IU/L)	51.38 ± 0.42	44.21 ± 0.25	48.64 ± 0.43	50.46 ± 0.62

Values are mean ± SD; n = 50

Values marked with asterisk are statistically different from control (p<0.05)

Table 4. Effect of 7 day storage at different temperatures on enzymes of male blood samples

Enzymes	Control	- 4°C,	- 20°C	- 70°C
AST (IU/L)	9.26 ± 0.15	6.18 ± 0.30	7.46 ± 0.30	9.05 ± 0.22
ALT (IU/L)	7.62 ± 0.13	5.32 ± 0.20	6.74 ± 0.13	7.11 ± 0.10
ALP (IU/L)	61.46 ± 0.32	49.21 ± 0.30	50.72 ± 0.40	59.83 ± 0.44
LDH (IU/L)	190.22 ± 0.54	164.12 ± 0.85*	179.20 ± 0.75	191.14 ± 0.62
CPK (IU/L)	51.38 ± 0.42	40.81 ± 0.32	42.39 ± 0.30	48.80 ± 0.34.

Values are mean ± SD; n = 50

Values marked with asterisk are statistically different from control (p<0.05)

Tables 3 and 4 show the values of serum enzyme levels obtained from the analysis of freshly collected male blood sample shortly after collection (control) and 8 hours after samples collection after freezing –thawing at - 4°C, - 20°C and - 70°C and the values of serum enzymes level obtained from analysis of male blood samples after a 7 day storage at - 4°C, - 20°C and - 70°C respectively. The pattern of variation of results obtained from the analysis of male blood sample at day 0 and day 7 and at various temperatures are similar to those of females.

4. DISCUSSION

The role of sample storage in the practice of medical laboratory science and human clinical pathology cannot be over emphasized [2,12-16]. Most of these studies were on animals with limited study on human subjects [17-21]. Sample handling before harvesting of serum, storage temperature, method of analysis may explain the difference in the results of the studies cited.

In our study, we evaluated the effect of freezing-thawing and storage time on some specific human enzymes. We observed a decrease in the concentration of AST, ALT, ALP, LDH and CPK in both males and females after analysis of both fresh samples (8 hours after sample collection) and samples stored at different temperatures for 7 days. However, these changes were statistically and clinically insignificant except for LDH which showed a statistically significant

decrease. Our findings are consistent with those of [17,21] where it was stated that serum analytes were more stable at -70°C than - 20°C save for lactate dehydrogenase and serum amylase which showed statistically significant changes.

AST levels were found to be stable for the period of 7 days when stored at - 4°C, - 20°C and - 70°C. Highest stability was observed when AST was stored at - 70°C and this is in accord with the work of [17]. Our findings on serum AST level agree with those of [1,3,22]. Also, AST activity was seen to be stable after [23] subjected human plasma sample to 10-15 freeze-thaw cycles after storage at - 80°C. Our study, however, is not in accord with that of [1] where significant statistical and clinical differences were reported between serum amylase levels in fresh samples and samples stored at - 20°C for 7,15 and 30 days. Changes in enzyme stability may be attributable to the existence of different isoforms of the enzyme. The activities of ALT and ALP were observed to be stable when stored at - 4°C, - 20°C and - 70°C for 7 days. Our findings agree with those of [1,17]. These variations in the activities of these enzymes may be due to the existence of different isozymes which differ in their density at different temperatures [3].

The storage of human serum for 7 days at - 20°C and - 70°C and thawing did not affect the activity of LDH. This is supported by the findings of Sydney et al. [24] where storage of human plasma for 4-6 weeks at - 90°C did not alter LDH

activity. Also, our observation when LDH was stored at -4°C is in accord with the study of [25,26], who described significant instability of LDH activity when stored at -8°C to 10°C . We did not find any evidence to believe that freeze-thaw of fresh samples of human serum or after storage for 7 days has any harmful effect on the stability of CPK. Our results are consistent with those of Clark et al. [12,24]. Our findings however, differ from those of the [27,28] who described substantial losses of enzyme activity of CPK isozymes during storage at -80°C . Existence of isoforms of CPK which differ in their density at different temperatures may be responsible for this variation [3].

5. CONCLUSION

This present research shows that with the exception of lactate dehydrogenase, most liver and muscle serum enzymes are stable when stored at -4°C , -20°C or -70°C . Sample analysis should be done as soon as possible after collection; otherwise they (samples) are most stable when stored at -70°C for 7 days. In the absence of -70°C freezer, storage at -20°C for 7 days should be an alternative.

ETHICAL ISSUE AND CONSENT

The samples collected from each subject was only for laboratory investigations. Informed written consent was obtained from all the subjects and Imo State University ethical committee approved the study. All procedures were conducted in accordance with the guidelines as stipulated in the Helsinki declaration on human experimentation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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