# Effect of storage time, temperature and repeated freezing and thawing on serum total bilirubin

Shankary, S.*1 Arasaratnam, V.2 and Kandeepan, K.2

*1Unit of Allied Health Sciences, Faculty of Medicine, University of Jaffna;*
*2Department of Biochemistry, Faculty of Medicine, University of Jaffna.*
**Shankary.selvarajah@yahoo.com*

**Abstract - Inadvertent delay and reanalysis may be required in bilirubin measurement due to heavy workload, transport and inadequate working staff. Hence samples have to be stored and the bilirubin concentration may need to be determined in serum samples which have to be repeatedly frozen and thawed. The objective of this study was to evaluate the effect of storage time, temperature and repeated freeze-thaw cycles on serum total bilirubin level in pooled normal serum. Bilirubin was measured by diazo-sulphanilic acid method. Following baseline measurement, serum was divided and stored in dark at 25°C and 4°C for 1, 2, 4, 6 and 8 hours respectively. Aliquots and a tube to evaluate multiple freeze-thaw cycles were stored without light exposure at -20°C for 1, 2, 3, 4, and 5 days. The total bilirubin level in serum samples stored at 25°C, 4°C and -20°C at different time intervals, did not show significant difference in mean bilirubin concentration when compared with the baseline value ($p>0.05$). However, the bilirubin concentration in serum sample subjected to 2nd, 3rd, 4th and 5th freeze-thaw cycles showed significant ($p<0.05$) reduction in bilirubin level when compared with the baseline value. Serum bilirubin level has shown a strong negative correlation with time at higher temperatures than at lower temperatures [25°C (r=-0.943), at 4 °C (r=-0.493) and at -20°C (r=-0.355)]. Serum samples subjected to multiple freeze-thaw cycles showed decrease in bilirubin concentration with increasing number of freeze-thaw cycles (r= -986). The results indicated that serum can be stored for total bilirubin estimation without light exposure at 4°C and 25°C for at least 8 hours and at -20°C for at least 5 days. However, repeated freeze- thaw cycles significantly decreased the total bilirubin concentration after 2nd freeze-thaw cycle.**

**Keywords: Bilirubin, repeated freezing and thawing, temperature**

## I. INTRODUCTION

Accurate measurement of bilirubin is clinically important to reveal the functions of hepatic and biliary systems, haemolytic diseases, transfusion reactions and inherited bilirubin metabolic disorders. The pre-analytical phase including handling of sample and storage-related effects is the most critical part in all biochemical analysis. In practice, delays might occur due to workload and inadequate working staff. Samples collected in peripheral areas are sometimes assessed in central laboratories with delay and exposed to various temperatures. Samples are stored to reanalyze and to confirm the previous results. Bilirubin decreases gradually as the temperature and time increases [4]. Bilirubin can be destroyed by light, oxygen & heat and repeated freeze - thaw cycles. This study has examined the stability of serum bilirubin at room temperature, 25, 4 & -20°C and with repeated freeze-thaw cycles.

## II. Materials and Methods

*Serum specimen*

This is a laboratory based experimental study. Non-fasting blood samples (10mL) were collected from three healthy volunteers after obtaining the informed written consent. Serum samples were pooled and baseline bilirubin concentration was measured.

Aliquots of serum were stored for 1, 2, 4, 6 and 8 hours at room temperature (25°C) and refrigeration (4°C). Aliquots and a tube to evaluate multiple freeze-thaw cycles were stored without light exposure at -20°C for 1, 2, 3, 4, and 5 days. All the tubes were covered with black paper.

*Analytical method*

Diazo sulphanilic acid method was used for the estimation of serum total bilirubin [5].

*Ethical Clearance*

Ethical clearance was obtained from the Ethics Review Committee of the Faculty of Medicine, University of Jaffna.

*Statistical analysis*

Data were analysed in Statistical Package of Social Science (SPSS) Version 21. Statistically significant differences compared with baseline (0 hour) were determined by one-way ANOVA and Tukey's honestly significant difference (HSD) post hoc test. Spearman Rank Correlation was used to correlate the time with bilirubin concentration at room temperature and refrigeration. Pearson correlation was used to correlate the time and number of repeated freeze-thaw cycles with bilirubin concentration stored in freezer. Results were considered significant when p value was $< 0.05$.

## III. Results and Discussion

Baseline bilirubin concentration of pooled serum was measured. The time of the measurement taken was considered as 0 hour. The mean bilirubin concentration was 0.526 (±0.023) mg/dL (normobilirubinemic).

Total serum bilirubin concentration in the serum samples stored at room temperature (25°C), and refrigeration (4°C) showed no significant difference from that of the baseline

serum bilirubin level ($p>0.05$) up to 8 hours, while those stored at -20°C showed no significant difference up to 5 days. However, when the serum sample was repeatedly frozen and thawed the serum total bilirubin concentration was significantly ($p<0.005$) decreased after 2nd, 3rd, 4th and 5th cycles.

The results indicated that there is significantly ($p<0.01$) strong negative correlation ($r = -0.943$) between storage time and bilirubin concentration at 25oC, while at 4oC ($r = -0.493$) and -20oC ($r = -0.355$) moderately negative correlation was found between bilirubin concentration and storage time and it was statistically not significant ($p>0.05$). Figures 1-4 illustrating the results.

The current study confirms the previous findings [3,6], that bilirubin in serum sample without light exposure is relatively stable at room and refrigeration temperature up to 8 hours. However, this study showed a significant negative trend in bilirubin concentration with time at 25°C, while some studies showing unchanged concentration. It may be due to the compensation of loss by the turbidity formed by the bacterial multiplication at the room temperature which leads to increase absorbance. In this study serum blank was used to avoid the interference by turbidity. The loss of bilirubin can be reduced by refrigeration at 4°C and -20°C. In the presence of either light, elevated temperature or oxygen the conversion of bilirubin to biliverdin is accelerated.

In the present study, bilirubin concentration in the serum was stable after the first cycle of thawing (24 h) when compared with values obtained from fresh samples. However statistically significant ($p<0.005$) differences were found after 2nd 3rd 4th and 5th freeze-thaw cycles. A strong negative significant ($p<0.001$) correlation ($r= -0.986$) was found between the number of repeated freeze-thaw cycles and bilirubin concentration.

In the present study, time dependent statistically insignificant decreases within five freeze-thaw cycles were observed. In contrast to a previous study [1], which showed a fluctuated pattern in loss of bilirubin, our study showed continuous decrease of estimated bilirubin concentration through five freeze-thaw cycles. It may be due to the appearance of turbidity after thawing, which was caused by the formation of cryoprecipitate of various serum components, primarily lipids may interfere with analytical methods [2]. However, in the current study serum blank was performed to avoid the interference by turbidity and lipemic samples.

Freeze-thaw cycles contribute to degradation and creation of insoluble precipitates. During the freezing of serum, the water will freeze first and become less dense, resulting in accumulation of other serum components like proteins and salt at the bottom of the container. This can cause precipitation and possible denaturation of proteins especially if the freezing process is slow.

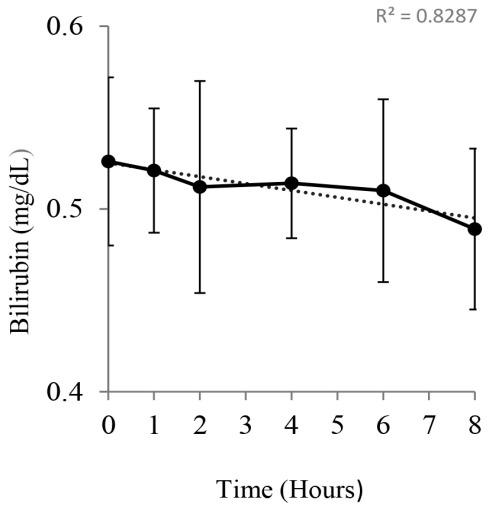


Figure 1: Changes in mean total bilirubin concentration in serum stored at 25°C (Error bars: +/- 2SD) (Trend is indicated by dot lines)

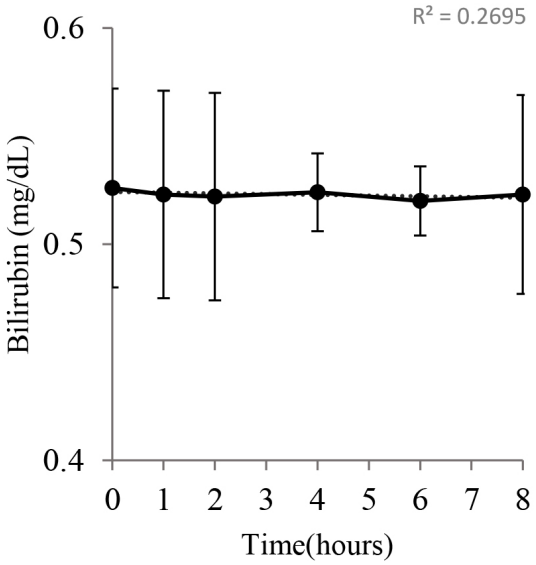


Figure 2: Changes in mean total bilirubin level in serum stored at 4(±1)°C (Error bars: +2SD) (Trend is indicated by dot lines)

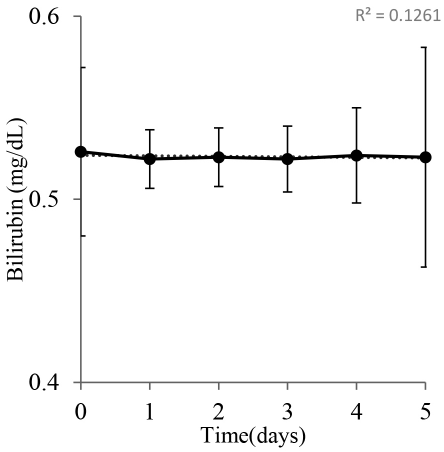


Figure 3: Changes in mean total bilirubin level in serum stored at -20°C (Error bars: +/- 2SD) (Trend is indicated by dot lines)

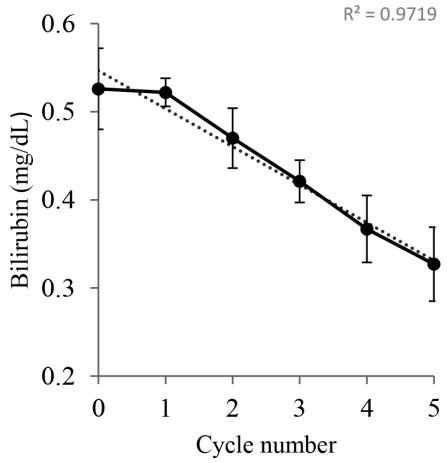


Figure 4: Changes in mean total bilirubin level in serum with multiple freeze thaw cycles (Error bars: +/- 2SD) (Trend is indicated by dot lines).

**IV. Conclusion**

The serum can be stored before the estimation of bilirubin without light exposure even at 25°C and at 4°C for at least to 8 hours and at -20°C for at least 5 days in aliquots, if there is any delay in analysis. Serum should not be exposed to more than one freeze-thaw cycle and has to be frozen as aliquots.

Recommendation Further studies can be carried out on more than 8 hours of storage at room temperature to find the maximum possible time to store the serum sample.

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