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Preanalytical variables affect for bilirubin measurement in case of neonatal hyperbilirubinemia at tertiary care hospital

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INTRODUCTION:

Hyperbilirubinemia is the result of an imbalance between the production and excretion of bilirubin by the liver. Various types of disorders, both acquired and congenital, can cause neonatal hyperbilirubinemia. Bilirubin in high concentration is toxic to the brain because bilirubin can penetrate the blood-brain barrier and might cause irreversible neurological damage. [1] Typically, bilirubin concentration increases within 96-120 hours after birth, peaks on day 5 to 7 and then decreases. The physiological jaundice is caused by increased foetal haemoglobin catabolism that accompanies an immature liver as well as intensified enterohepatic circulation. [2] Monitoring of serum bilirubin concentration is essential during early neonatal life. Approximately 60% of term babies and 85% of preterm babies develop clinically apparent jaundice. [3]

Accurate measurement of bilirubin in blood or serum is very important for both diagnostic purposes and therapeutic monitoring of small babies with hyperbilirubinemia. Although bilirubin is one of the most commonly performed laboratory measurements in new-borns, its measurement remains remarkably inaccurate. Common problems faced in neonatal Bilirubin estimation are low sample volume, hemolysis and exposure of sample to light due to delay in transport or analysis.[4,5,6]

Bilirubin photo isomers are rapidly produced from (ZZ)-bilirubin when it is exposed to light at a particular wavelength. [7] Bilirubin photoisomers include (EZ)-bilirubin, (ZE)-bilirubin, and (EE)-bilirubin, which are configurational isomers, and (EZ)-cyclobilirubin and (EE)- cyclobilirubin, which are structural isomers. In physiologic human serum, (ZE)-bilirubin as a photoisomer is present in the largest amount next to (ZZ)- bilirubin, and (ZE)-bilirubin/(ZZ)- bilirubin ratio is about 0.1 in serum of neonates with unconjugated hyperbilirubinemia. [8]

Different methods of bilirubin estimation shows result differently with various fractions like α,β,y and 8 bilirubin. Bilirubin photo isomers, which are unconjugated bilirubin, have been reported to act as substrates in this DB assay method. Especially, (4Z, 15E)-bilirubin ((ZE)- bilirubin) is produced rapidly by exposing to light, and increase in (ZE)-bilirubin was correlated to increase in DB in vitro. Therefore, caution is necessary in the interpretation of the DB values obtained by this method in serum samples from unconjugated hyperbilirubinemia neonates, because the serum samples have already contained a large amount of bilirubin photo isomers and (ZZ)-bilirubin. [9, 10]

Pre-analytical errors are known in bilirubin estimation and there more studies needed to demonstrate effect of preanalytical error in neonates. In this study, we evaluated that the effect of delay in analysis and storage conditions on Serum bilirubin levels in neonates. However, the stability of bilirubin in serum or plasma exposed to light was only partly tested [11-16].

Material Method:
Cross sectional study carried out in SSG Hospital, Baroda, Gujarat between November 2020 to January 2021.Study is approved by Institutional Scientific and Ethics Committee.Twenty- two serum samples of neonates with Total bilirubin level more than 3 mg/dl were included in the study. These samples have at least extra one ml of serum after analysis. Hemolysed sample, QNS (Quantity Not Sufficient) sample were excluded. Each serum sample was transferred into two transparent aliquots. One set was left on working desk in well-illuminated air-conditioned laboratory room and other set was stored in refrigerator. After 24 hours, serum total bilirubin (TB) and direct bilirubin (DB) estimated by a Chemistry Analyzer (ERBA EXCEL 600, Germany) using a timed endpoint diazo method. Total bilirubin was measured using the same method in the presence of caffeine, benzoate, and acetate as accelerators to form azobilirubin. Indirect bilirubin (IB) calculated in all the samples of both the sets.

The samples divided into two groups. Group I included initial readings of TB, DB & IB; Group II (A) - readings after 24 hrs from the samples left on working desk; and Group II (B) - readings after 24 hrs from the samples stored in refrigerator.

Statistical Test:
The differences between the baselines and all subsequent measurements were analyzed with analysis of variance (ANOVA) with a post hoc Dunnett's test and $p < 0.05$ was considered statistically significant. The allowable total error for bilirubin was <0.4 mg/dl or 20% according to Clinical Laboratory Improvement Act criteria.

Result:
After 24 hours, in the samples kept in well-illuminated air-conditioned room (Group II (A)), the value of TB decreased significantly (p value=0.004) whereas DB increased significantly (p value=0.0006) and no significant difference in IB was found (p=0.05). (Table/Fig 1)

In samples stored in refrigerator which is Group II (B), there was no significant variations in results observed, p value of TB, DB and IB were p=0.857, p=1.0 and IB p=0.99, respectively.

Direct bilirubin significantly higher in Group II (A), Total bilirubin was significantly lower in Group II (A) compared Group I and no difference in indirect bilirubin value after exposed to illuminated air-conditioned Laboratory room. In samples stored in refrigerator, no significant variations in results were observed.

Discussion:
The stability of bilirubin in blood samples is one of the factors that affect the accuracy of the test. It is well known that bilirubin is photosensitive substance, undergoing both photoisomerization and photooxidation and the latter is much slower than the former [17]. In order to prevent these reactions, clinical laboratories generally protect specimens for bilirubin tests from light exposure. It was recommended that bilirubin measurement should be performed immediately on drawing the specimen, or after keeping in a refrigerator for up to 2 h before examination [18].

In present study, we evaluated that the effect of delay in analysis and storage conditions on serum bilirubin levels in neonates and we found out that DB is increased when serum of neonates with unconjugated hyperbilirubinemia is exposed to room light for 2 hours.

Okada et al. also found that after exposure to room light, TB decreased significantly ($P<0.01$) from 16.3±3.56 (mean±SD) to 13.6±4.18 mg/dL and DB increased significantly ($P<0.01$) from 0.61±0.4 to 2.36±1.15 mg/dL. [19].
In O'Hagan's study, a serum sample containing 9.0 mg/dl was left exposed to direct sunlight for 1 h (from 2:45 P.M. to 3:45 P.M.) on a bright spring day in Australia. It was found that the bilirubin concentration dropped to 4.8 mg/dl [18].O'Hagan et al. did put a serum sample on a shaded bench for 24 h and they found that the bilirubin level dropped

from 0.8 mg/dl to 0.25 mg/dl. However, they tested only one sample and the concentration of bilirubin in that sample was within normal reference interval.

Alina G. et al. demonstrates that in plasma samples with elevated total bilirubin concentrations, bilirubin is stable for at least 24 h without light protection at room temperature in the laboratory. The discrepancy could be due to different sample containers used in Alina G. et al. and O'Hagan's studies. In Alina G. et al study, author used labeled plastic Vacutainer tubes with plasma separator gel. [20]. In the case of Alina G. et al study, the specimens were left in the capped original plastic vacutainer tubes with plasma separator gel and printed identification label covering more than 2/3 area of the tube and placed in a tube rack on the laboratory bench with exposure to normal laboratory lighting. This reflects the usual laboratory practice, as in the case that the testing process is delayed and the specimens are not aliquoted, but left in their original tube on the laboratory bench. so the different bilirubin stability between Alina G. et al study and Rehak's may be due to different specimen tubes, the process of sample handling, and partial protection from light exposure provided by the label and the cap.

A more recent study, published by Rehak et al., recommends that exposure of specimens to light should be limited to less than 2 h, as the losses of bilirubin are far greater that 20% [16]. In this study, however, the selected serum specimens were individually aliquoted into plastic tubes and exposed to fluorescent lighting at ambient temperature.

S.kawamoto et.al. also found that total bilirubin and direct bilirubin concentrations were 10.73 mg/dL with significant a decrease to 10.60 mg/dL and 0.69 mg/dL with a significant increase to 0.78 mg/dL.[21].
The allowable total error for total bilirubin is 0.4 mg/dl or 20% according to CLIA criteria.
One cause of this decrease in TB may be that this assay method is based on the change in absorbance: The absorbance near 450nm decreased when photoisomers was produced.[22].

It is consider that increase in DB is caused by the disappearance of bilirubin photoisomers which causes the decrease in absorption at 455 nm. The most abundant photoisomer in clinical serum samples, (ZE)-bilirubin-IXa, changes to (ZZ)-bilirubin-IXa under natural environmental condition, during phototherapy or acidic conditions. [23]. Another cause may be bronze baby syndrome, which is considered to result from the polymerization of cyclobilirubin due to its accumulation in the body. [24] Because the samples exposed to environmental light appeared somewhat browned, and this appearance resembled that of serum in bronze baby syndrome, which is an adverse effect of phototherapy in neonates. Previous studies have shown that direct bilirubin undergoes isomerization and hydrolysis of glucuronic acid esters of bilirubin, which could artifactually inflate the proportion of unconjugated bilirubin present [25].

Indeed, we found that direct bilirubin was more sensitive to light exposure at room temperature than total bilirubin with differences in an average recovery of 8% at 24 h and 13% at 48 h. On the other hand, prolonged exposure to light causes photoisomerization, increasing direct- reacting bilirubin in bilirubin [26]. Also, 8-bilirubin, which is covalently linked to albumin, can directly react with diazo reagent. In addition, use of wetting agents or incorrect pH buffers may increase the amount of unconjugated bilirubin measured as direct bilirubin [27].

Conclusion:
The Bilirubin concentration changes on exposure to light, TB decreases whereas DB increases. The follow up, surveillance and intervention in jaundiced newborn are based on TB values; spurious underestimation might lead to withholding of necessary therapy or over estimation of DB may lead to fallacious diagnosis of Physiological jaundice as Pathological jaundice. The possible alterations in bilirubin results should be taken into account in interpretation of neonatal hyperbilirubinemia. More stringent and uniform recommendation are required for Bilirubin estimation with emphasis on control of preanalytical conditions.

Recommendation:
Utmost care is required in transport and storage of samples for Bilirubin estimation. They must be transported without delay in cold transport boxes and should be kept in dark environment while awaiting analysis.

Limitation:
Present study establishes that change in concentration of bilirubin on exposure to light which is a preventable pre-analytical error in estimation. The limitations of the study were that sample size was small and analysis has not been done at serial intervals. Further study can be done to quantify change in the level of photoisomers in the serum by HPLC method.

References

1. Milinkovi N, Ignjatovi S,Singh N. Uncertainty of measurement in laboratory medicine. J Med Biochem 2018; 37: 279-88.

2. Ullah S, Rahman K, Hedayati M. Hyperbilirubinemia in Neonates: Types, Causes, Clinical Examinations, Preventive Measures and Treatments: A Narrative Review Article. Iran J Public Health 2016; 45(5): 558-68.
3. Berkwitt A, Osborn R, Grossman M. The utility of inpatient rebound bilirubin levels in infants readmitted after birth hospitalization for hyperbilirubinemia. Hosp Pediatr 2015; 5(2): 74-8.
4. Cvitkovic L, Mesic R. Various preanalytical variables and their effects on the quality of laboratory results Diabetologia Croatica [Internet]. 1999 [cited 2010 Jan 3];282 Available from http://www.idb.hr/diabetologia/99no2-2.html. [Google Scholar]
5. Murphy JM, Browne RW, Hill L, Bolelli GF, Abagnato C, Berrino F, et al. Effects of transportation and delay in processing on the stability of nutritional and metabolic biomarkers. Nutr Cancer, 2000;37(2):155-160. [PubMed] [Google Scholar]
6. Dale JC, Hamrickk HJ. Neonatal bilirubin testing practices: Reports from 312 laboratories enrolled in the College of American Pathologists Excel Proficiency Testing Program. Arch Pathol Lab Med. 2000;124(10):1425-1428. [PubMed] [Google Scholar]
7. Itoh S, Onishi S, Isobe K, et al. Wavelength dependence of the geometric and structural photoisomerization of bilirubin bound to human serum albumin. Biol Neonate 1987; 51: 10- 17
8. Itoh S, Sumitani T, Sugihara S, et al. Effects of bilirubin photoisomers on direct bilirubin concentrations estimated by bilirubin oxidase method. J Jpn Pediatr Soc 1993; 97: 687-691.
9. Onishi S, Isobe K, Itoh S, et al. Metabolism of bilirubin and its photoisomers in newborn infants during phototherapy. J Biochem 1986; 100: 789-795.
10. Spandana, M. and Vagha, J. (2022) "Review on Different Methods of Serum Bilirubin Estimation", Journal of Pharmaceutical Research International, 34(3B), pp. 34-41. doi: 10.9734/jpri/2022/v34i3B35390
11. O'hagan JE, Hamilton T, Le Breton EG, Shaw AE. Human serum bilirubin; an immediate method of determination and its application to the establishment of normal values. Clin Chem 1957:3(5):609-23.
12. Ihara H, Aoki Y, Aoki T, Yoshida M. Light has a great effect on direct bilirubin measured by the bilirubin oxidase method than by the diazo method. Clin Chem 1990;36(6):895-7.
13. Ihara H, Nakamura H, Aoki Y, Aoki T, Yoshida M. In vitro effects of light on serum bilirubin subfractions measured by high-performance liquid chromatography: comparison with four routine methods. Clin Chem 1992;38:2124-9.
14. Landt M, Hortin GL, Smith CH. Monoject amber samplette free bilirubin photolysis. Clin Chem 1989;35(5):889-90.
15. Wu TW, Dappen GM, Spayd RW, Sundberg MW, Powers DM. The Kodak Ektachem clinical chemistry slide for measurement of bilirubin in newborns: principle and performance. Clin Chem 1982;26(12):2366-72.
16. Rehak NN, Cecco SA, Hortin GL. Photolysis of bilirubin in serum specimens exposed to room lighting. Clin Chim Acta 2008;387(1-2):181-3.
17. McDonagh AF. Photolysis and photoisomerization of bilirubin in serum specimens exposed to room lighting. Clin Chim Acta 2008;393(2):130.
18. O'hagan JE, Hamilton T, Le Breton EG, Shaw AE. Human serum bilirubin; an immediate method of determination and its application to the establishment of normal values. Clin Chem 1957;3(5):609-23.
19. Okada H., Kou K, Itoh S. Effects of bilirubin photoisomers on the measurement of direct bilirubin by the bilirubin oxidase method. Annals of Clinical Biochemistry 2018; 55(2): 276- 280.
20. Sofronescu AG, Loebs T, Zhu Y. Effects of temperature and light on the stability of bilirubin in plasma samples. Clin Chim Acta. 2012 Feb 18:413(3-4):463-6. doi: 10.1016/j.cca.2011.10.036. Epub 2011 Nov 13. PMID: 22108388.
21. S. Kawamoto, K Koyano, M Ozaki. Effects of bilirubin configurational photoisomers on the measurement of direct bilirubin by the vanadate oxidation method. Annals of Clinical Biochemistry 2021; 58(4).
22. Itoh S, Isobe K, Onishi S. Accurate and sensitive high-performance liquid chromatographic method for geometrical and structural photoisomers of bilirubin IXa using the relative molar absorptivity values.J Chromatogr A 1999; 848: 169-177.
23. Itoh S, Kusaka T, Imai T, et al. Effects of bilirubin and its photoisomers on direct bilirubin measurement using bilirubin oxidase. Ann Clin Biochem 2000; 37: 452-456.
24. Onishi S, Itoh S, Isobe K, et al. Mechanism of development of bronze baby syndrome in neonates treated with phototherapy. Pediatrics 1982; 69:273-276
25. McDonagh AF. Photolysis and photoisomerization of bilirubin in serum specimens exposed to room lighting. Clin Chim Acta 2008;393(2):130.
26. Ihara H, Shino Y, Hashizume N, et al. Effect of light on total and direct bilirubin by an enzymatic bilirubin oxidase assay. J Anal Biol Sci 1997;20:349-54.
27. Doumas BT, Wu TW. The measurement of bilirubin fractions in serum. Crit Rev Clin Lab Sci 1991;28:415-45.

Table:

Table 1: Bilirubin level in comparison in Group I and Group II A

		Group – I	Group – II A
Total bilirubin	**Mean ± SD**	12.08 ± 5.35	11.30 ± 4.95
	Range	3.3 – 23.2	3.22 – 20.0
	P Value	------	0.004
Direct	**Mean ± SD**	1.41 ± 0.50	1.66 ± 0.53
	Range	0.6 – 2.7	1.0 – 2.9
	P Value	-----	0.0006
Indirect	**Mean ± SD**	10.67 ± 5.36	9.64 ± 4.86
	Range	1.9 – 21.3	1.6 – 18.2
	P Value	-----	0.05

Table2: Bilirubin level in comparison in Group I and Group II B

		Group – I	Group – II B
Total bilirubin	**Mean ± SD**	12.08 ± 5.35	12.08 ± 5.38
	Range	3.3 – 23.2	3.42 – 23.3
	P Value	------	0.857
Direct	**Mean ± SD**	1.41 ± 0.50	1.41± 0.52
	Range	0.6 – 2.7	1.0 – 2.8
	P Value	-----	1.0
Indirect	**Mean ± SD**	10.67 ± 5.36	10.67 ± 5.4
	Range	1.9 – 21.3	1.3 – 21.4
	P Value	-----	0.99

Figure:

Figure 1: Change in Concentration of TOTAL BILIRUBIN

In Samples kept on working desk

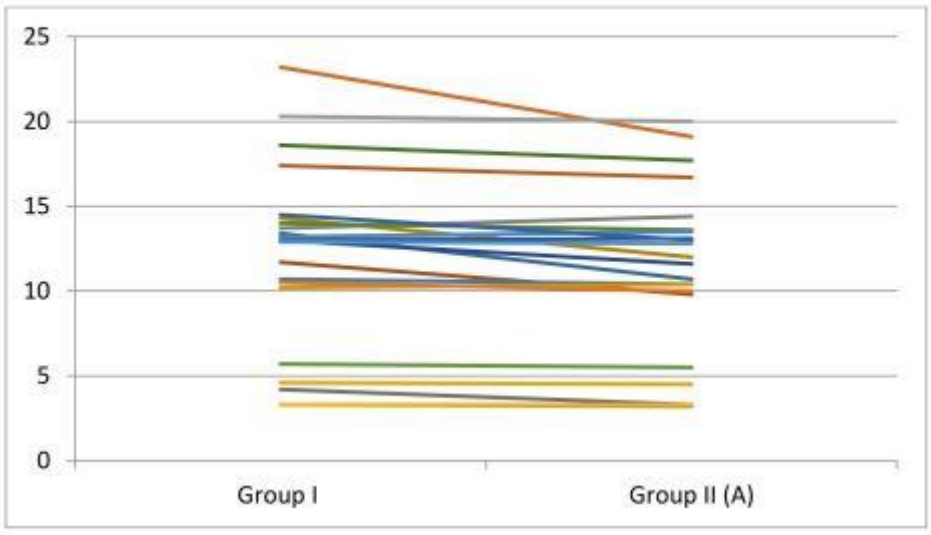


Figure 2: Change in Concentration of TOTAL BILIRUBIN

In Samples kept in Refrigerator

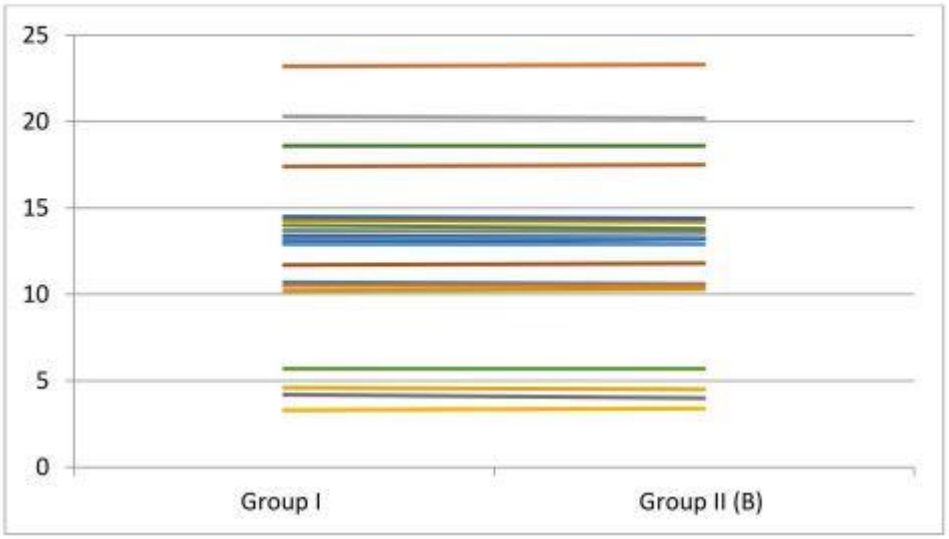


Figure 3 : Change in Concentration of DIRECT BILIRUBIN

In Samples kept on working desk

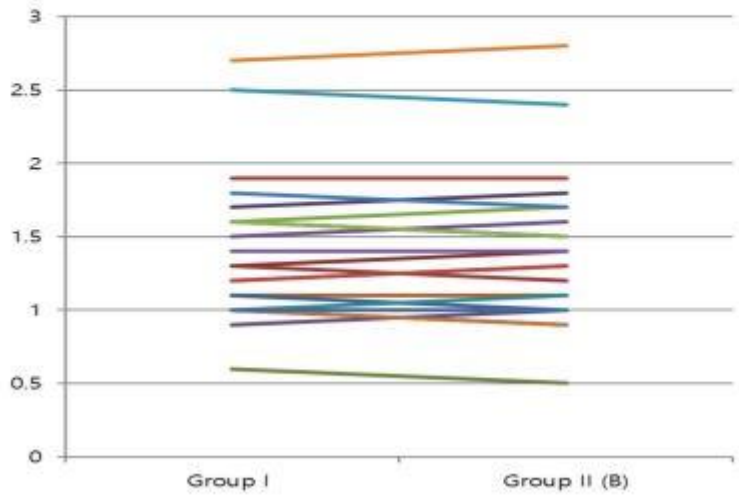


Figure 4 : In Samples kept in Refrigerator

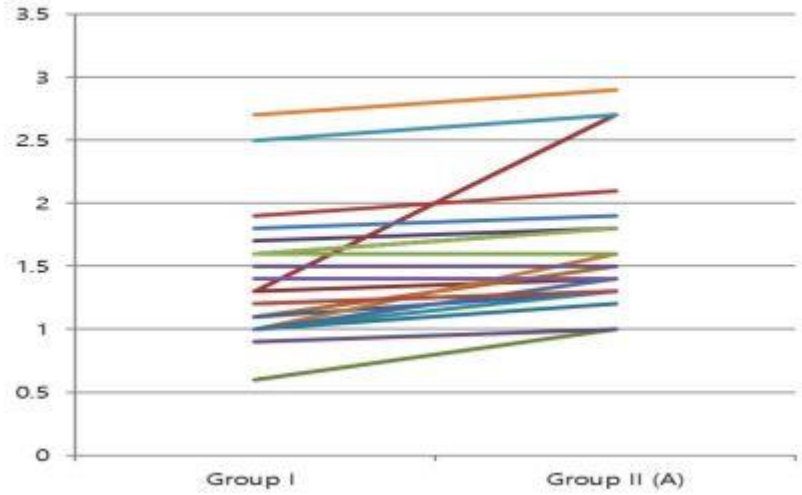


Figure 5: Change in Concentration of INDIRECT BILIRUBIN

In Samples kept on working desk

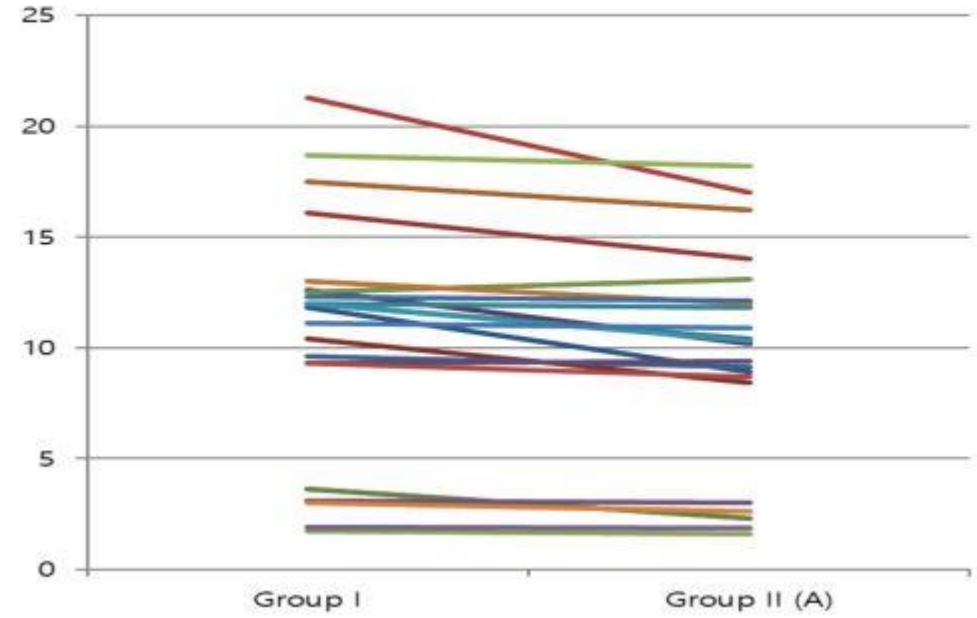
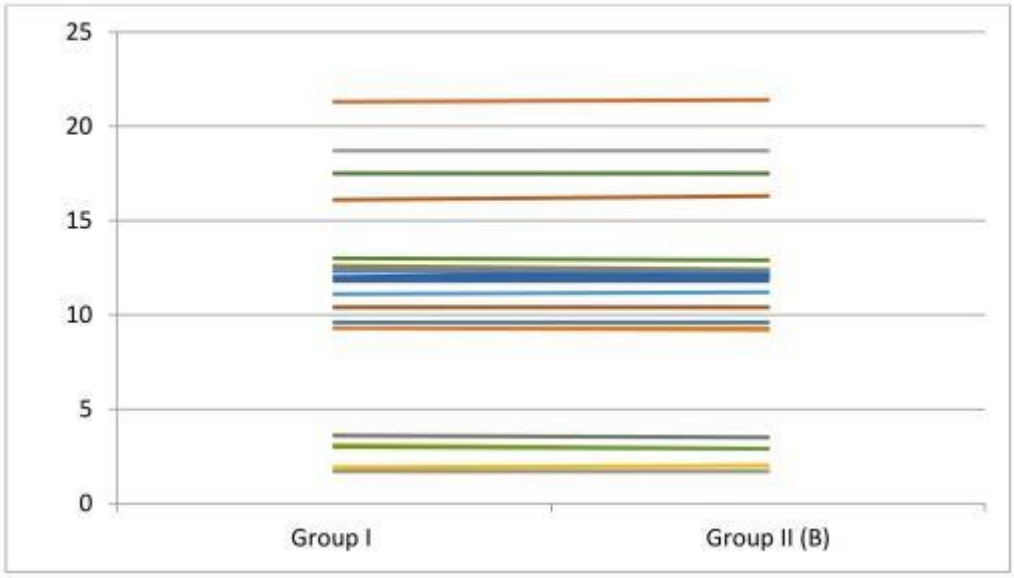


Figure 6: Change in Concentration of INDIRECT BILIRUBIN

In Samples kept in Refrigerator



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Are there any relevant conflicts of interest?

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