Estimation of various fractions of bilirubin in cases of neonatal jaundice

Padmanabhan P[1,*], Hotkar KN[2], Nagarkar VD[3], Jangle SN[4]

[1]Associate Professor, [2,3]Assistant Professor, [4]Professor & HOD, Dept. of Biochemistry, Rural Medical College, Pravara Institute of Medical Sciences, Maharashtra

***Corresponding Author:**
Email: preetipadmanabhan@gmail.com

Abstract

Background & Objectives: Neonatal jaundice causes yellow discoloration of the skin and sclera of the infants, caused by the accumulation of bilirubin in the skin and the mucous membrane.

Before discharge the newborn should be examined, monitored and risk due to high bilirubin which maybe genetic be identified to prevent brain damage.

Neonatal bilirubin was measured using micro slide technology, whereas Diazo method by Wet chemistry was utilized on the Erba analyzer.

Methods: Neonatal bilirubin (N-Bil) was measured using micro slide technology on Vitros 250 (Ortho Clinical Diagnostics USA) Dry Chemistry analyzer. Wet technology was measured using wet technology based on diazo principle by a kit performed on Erba analyzer.

Results: Direct Bilirubin showed significantly higher values than conjugated bilirubin (Bc) values and shows poor correlation. Cross reaction may occur between Direct bilirubin and Bc which is clinically misleading.

Conclusion: Neonatal bilirubin (N-Bil) is better parameter than total bilirubin (T Bil) in assessing neonatal jaundice in newborns. The slide provides bichromatic accurate readings of unconjugated bilirubin (Bu) at 460nm and conjugated bilirubin (Bc) 420 nm without photometric interference from hemoglobin and albumin. Diazo method is the most widely used method for bilirubin estimation till date. The Kodak Ektachem (Eastman Kodak company Rochester NY 14650) dry –slide method directly estimates unconjugated(Bu) and conjugated bilirubin (Bc) by a rapid automated method developed in the 1980's.Bc concentrations are a marker of variety of infections, metabolic or liver conditions.

Keywords: Neonatal bilirubin, Unconjugated bilirubin, Conjugated bilirubin, Total bilirubin, Direct bilirubin, Jaundice

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Introduction

Jaundice is a common pathology among infants caused by the combination of increased haem catabolism and physiological immaturity of the liver in conjugating bilirubin and its excretion[(1)]. Jaundice is the most common cause for morbidity in the first week of life, occurring in 60% of term and 80% of pre-term new born. The disease is also the common reason for readmission after discharge from birth hospitalization[(2)].

Neonatal jaundice results in the yellow discoloration of the skin and sclera of the infants, caused by the accumulation of bilirubin in the skin and the mucous membrane[(3)]. Although it is often a natural and temporary condition, some infants develop severe hyperbilirubinemia, in which unconjugated bilirubin in the serum may cross the blood- brain barrier and cause bilirubin encephalopathy (acute bilirubin intoxication) or kernicterus (chronic bilirubin intoxication). Before discharge, the new born should be examined, monitored and the risk due to high bilirubin which maybe genetic also therefore should be identified to prevent brain damage(Ichiro et al;2010)[(4)].

Causes such as inadequate breast milk intake, imbalance between bilirubin production and conjugation as well as mutation of the gene encoding bilirubin conjugation have been associated with hyperbilirubinemia[(5,6)]. An association between bilirubin levels as subsequent risk of hyperbilirubinemia has been reported[(7)]. The hour – specific evaluation of total serum bilirubin and risk of early discharge assessment by using predictive nomogram of hour based record of bilirubin has been proved beneficial in predicting severe hyperbilirubinemia[(8)]. Cases of prolonged neonatal jaundice could be dangerous or even result in worst consequences and cause major health problem in neonates. Close monitoring of bilirubin is essential during early neonatal life (Muhammad et al; 2011)[(9)].

Kuenzle et al in 1966[(10)] have reported four species of bilirubin in serum in order of elution from the chromatographic column as unconjugated bilirubin (α), monoconjugated bilirubin (β), diconjugated bilirubin (γ) and albumin bound species (δ).

Bilirubin species are also classified according to reactivity in "diazo method" as "indirect reacting" or unconjugated bilirubin (Bu).The δ –fraction reacts directly in the diazo method[(11)].

Diazo method is the most widely used method for bilirubin estimation till date. The Kodak Ektachem (Eastman Kodak Company, Rochester NY 14650) dry-slide method directly measure unconjugated (Bu) and conjugated bilirubin (Bc) by a rapid automated method developed in the 1980's[12,13,14].

The slide provides bichromatic accurate readings of unconjugated bilirubin (Bu) at 460 nm and conjugated bilirubin (Bc) at 420 nm without photometric interference from haemoglobin and albumin. Hence Bu slide quantitatively separates unconjugated and conjugated bilirubin, not including delta bilirubin, with minimal interference from haemoglobin[18]. Today, this forms the basis of dry chemistry developed on Vitros Clinical Chemistry analyzer for bilirubin estimation as "BuBc slide" by Ortho Clinical Diagnostics[15].

Increased catabolism of fetal haemoglobin and deficiency of conjugating enzyme glucuronidase results in decreased capacity of the liver to conjugate bilirubin and hence results in elevated levels of unconjugated bilirubin. Hence, the major bilirubin fraction in the neonatal specimen is Bu. In healthy infants conjugated bilirubin result is expected to be close to 0mg/dl. Delta bilirubin is negligible in neonates who are less than 14-21 days old, however its presence is associated with elevated conjugated bilirubin (Bc). In neonates, no clinical utility for delta bilirubin has been reported. Only Ortho Clinical Diagnostics has methods on its Vitros analyzers that provide total, unconjugated and conjugated bilirubin results. The total bilirubin (T-Bil) slide on the Vitros instruments measures all fractions of bilirubin by a diazo method, while BuBc slide measures unconjugated (α –bilirubin or Bu) and a sum of monoglucuronide (β- bilirubin and γ- bilirubin respectively or Bc) by direct spectrophotometry. The Vitros neonatal method (N-Bil or BuBc slide) is a sum of Bu and Bc; it does not include δ- bilirubin[16].

Material and Methods

Prior to the study, permission was obtained from the Institutional Ethical Committee. The study was approved and permitted by Institutional Ethical Committee by Registration No: PIMS/RMC/2015/103. The type of study is descriptive –cross-sectional type.

40 newborns admitted as patients suffering from neonatal jaundice at Pravara Rural Hospital, Loni, Maharashtra were selected for the study. Blood was drawn using vacutainers (Becton–Dickinson USA) containing a clot activator and the serum obtained were used for the estimation of TBil and Direct bilirubin.

Serum was separated from blood samples of newborns aged 1-5 days not receiving phototherapy received in Biochemistry Division of Central Clinical Laboratory. Dry Chemistry of Ortho Clinical Diagnostics analyzed on Vitros 250 fully Automated Analyzer was utilized for analyzing bilirubin fractions in serum. Neonatal Bilirubin (NBil) was measured using micro slide technology on Vitros 250 (Ortho Clinical Diagnostics, USA) Dry chemistry analyzer. Total bilirubin (TBil) and Direct bilirubin (DBil) was measured using Diazo method by Wet technology using Kit manufactured by Trans asia Biomedicals on ERBA-CHEM -7 analyzer[17,18,19].

Results

The results of the present study (Table 1) indicate that total bilirubin measured by Wet chemistry is slightly overestimated value than the total bilirubin estimated by Dry Chemistry. This is probably due to the overestimation of total bilirubin by Dry Chemistry whereas considerable accuracy and precision was maintained by Dry Chemistry.

Reporting the results for Bu, Bc and neonatal bilirubin (NBIL) provides the pediatrician a complete picture. Total Bilirubin (TBIL) maybe monitored but proves to be beneficial after 30 days. Presence of delta bilirubin is associated with an elevated Bc result. In neonates therefore no clinical utility for delta bilirubin has been reported.

Delta bilirubin (δ) is negligible in neonates less than 14 -21 days old. According to Table 1 the Direct Bilirubin values are higher than the conjugated bilirubin by Dry Chemistry which indicates the presence of delta bilirubin in the neonatal samples. Wet Chemistry measures direct bilirubin which is equivalent to conjugated bilirubin since the delta bilirubin is not considered separately by Wet Chemistry.

Reference interval for the various fractions of bilirubin in neonates in mg/dl as measured on Vitros analyzer systems are reported as:

Unconjugated bilirubin – 0.5 -10.5 mg/dl

Conjugated bilirubin - 0.0-0.6 mg/dl

Neonatal bilirubin- 1.0-10.5 mg/dl

Neonatal bilirubin reported by dry chemistry is the sum of unconjugated and conjugated bilirubin. The primary advantage of separate reporting of neonatal bilirubin values is possible by Vitros System of Ortho Clinical Diagnostics and hence the values of neonatal bilirubin would be beneficial to the clinician.

Dry Chemistry utilized in the present study shows very accurate and precise readings for unconjugated bilirubin, since the slide used for the estimation measures solely the unconjugated bilirubin. There is major influence of unconjugated bilirubin on the total bilirubin value which explains the reason for overestimation by Wet Chemistry.

Table 1: **Serum Bilirubin in Neonatal Jaundice by Dry Chemistry and Wet Chemistry**

Dry Chemistry(Mean±SD of 40 patients)						**Wet Chemistry (Mean±SD of 40 patients)**		
Total Bilirubin mg/dl	**Direct Bilirubin mg/dl**	**Unconjugated Bilirubin Bu mg/dl**	**Conjugated BilirubinBc mg/dl**	**Neonatal Bilirubin= Bu + Bc (mg/dl)**	**Delta Bilirubin T BIL – (Bu+Bc)**	**Total Bilirubin mg/dl**	**Unconjugated Bilirubin mg/dl**	**Direct Bilirubin mg/dl**
11.8±3.38	0.5±0.44	11.4±3.31	0.2±0.17	11.6±3.32	0.2±0.35	12.0±3.45	10.6±4.01	1.4±1.60

Serum Bilirubin Levels in Neonates

Laboratory evaluation of serum bilirubin is an essential tool in assessing neonatal jaundice in neonates to follow photo therapy. Detection of even a marginal increase in serum bilirubin levels in neonates is critical in the management for following photo therapy. Determination of serum bilirubin by HPLC method is considered to be the best method for assessing neonatal jaundice. The so called ''gold standard'' methods are laborious, complex and expensive and not in practical use. Till date, the assessment of serum bilirubin in neonates is based solely upon the estimation of total bilirubin (TBil) by Diazo method with accelerator and direct bilirubin (DBil) Diazo method without accelerator. Adjustments of the dosage of use of light units for following phototherapy are based upon the value of TBil. TBil and DBil determinations are affected by interferences of hemolysis and lipemia in serum samples. Diazo methods compare poorly across platforms and are variably sensitive to substantial amount of unconjugated bilirubin (Bu) in neonatal blood[20].

Neonatal bilirubin (NBil) estimation by dual spectrometric method (Micro-slide technology) using mordant for the binding of bilirubin fractions (unconjugated bilirubin Bu and conjugated bilirubin Bc) has been proposed as a sensitive parameter for assessing neonatal jaundice in neonates. Mordant separates the reflectance maxima of conjugated bilirubin at 420 nm and unconjugated bilirubin at 460nm. Bu is not affected by classical errors of DBil assays and Bc by delta bilirubin (DelBil). Spreading layer of micro-slide (technology of Vitros slide) is protecting estimation from hemolysis and lipaemia effects.

In the present study, we aim to compare the efficacy of NBil and TBil in the assessment of neonatal jaundice in neonates and comparison between Bc and DBil with Wet technology.

Statistical analysis

Statistical analysis was performed using Med Calc software. The correlation between the NBil and TBil was calculated by Pearson's correlation coefficient. The agreement between NBil and TBil, Bc and DBil were assessed using Bland – Altman plots.

Results

I. Comparison of Neonatal Bilirubin (NBil) with Total bilirubin in neonates carried out by Dry chemistry.

The present study (Table 2) shows, TBil level has been found to be higher in the studied group when compared with the NBil level. The mean level of NBil was 11.53±3.31 mg/dL while TBil level was 12.18±3.47 mg/dl. There was an excellent correlation between NBil and TBil with $r = 0.97$, $p<0.001$ (Fig. 1). The Bland and Altman analysis (Fig. 2) showed an agreement between the two methods within the studied range. The mean difference between two methods was - 4.2 mg/dl (Fig. 2)

Table 2: Characteristics of Neonate samples

S. No	Parameter	N	Mean±SD (mg/dl)	95% Cl (mg/dl)
1	NBil	40	11.53±3.31	10.47 – 12.59
2	Bc	40	0.18±0.19	0.118 – 0.242
3	Bu	40	11.35±3.30	10.30 – 12.41
4	T.Bil	40	12.18±3.47	10.69 – 12.82
5	In-Dir.Bil	40	10.61±3.86	9.37 – 11.84
6	Dir.Bil	40	1.42±1.59	0.91 – 1.93

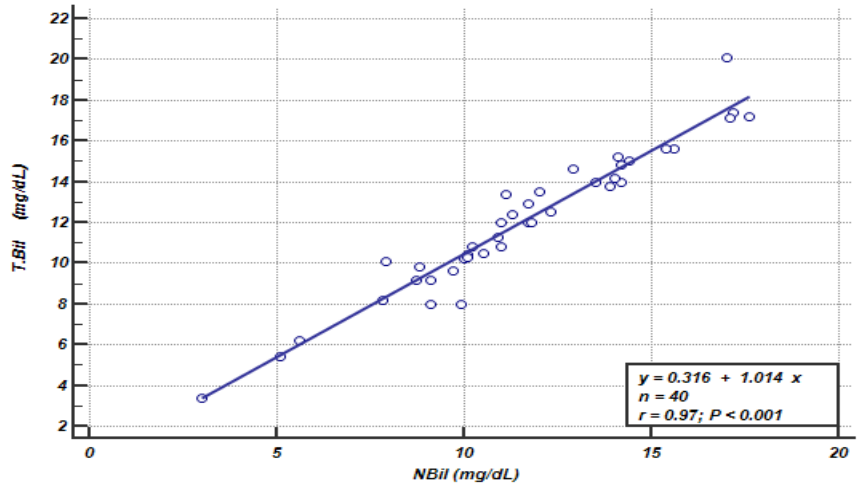

Fig. 1: Correlation between NBil and TBil

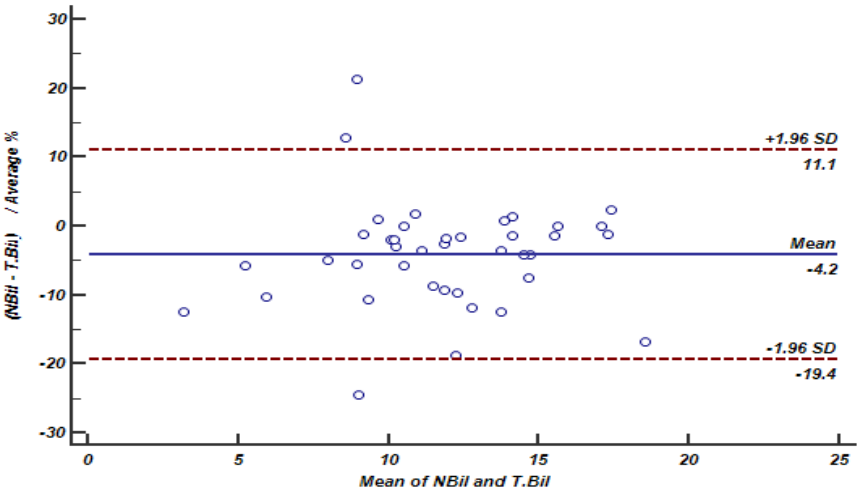

Fig. 2: Bland and Altman plot showed the mean difference between NBil and TBil

II. Comparison of conjugated bilirubin (Bc) with DBil (Direct Bilirubin) in neonates: The study has demonstrated that the DBil levels in neonates were higher when compared with Bc levels (Table 2) and there was poor correlation between conjugated bilirubin (Bc) and Direct Bilirubin (Fig. 3).

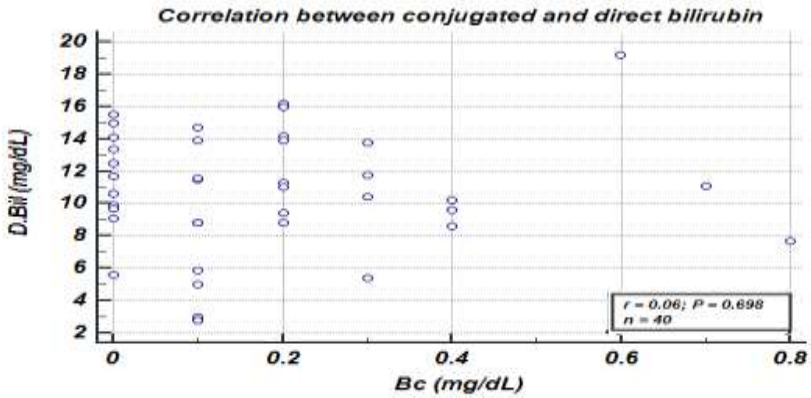

Fig. 3: Correlation between Bc and DBil

In this study, we have compared the performance of NBil and TBil, Bc and DBil, for the assessment of neonatal jaundice in neonates. There was an excellent correlation between NBil and TBil. However the Bland and Altman analysis showed that the mean difference between the NBil and TBil was - 4.2 mg/dl. This is a clear indication that dependence of TBil could mislead to monitoring bilirubin levels in newborns on phototherapy as well as missing to detect Bc for following phototherapy. Similar results have been reported by Doumas et. al.[21] Total Bilirubin levels showed positive bias from 0.1 mg/dl upto 1mg/dL or negative bias as compared to J-G reference method (unpredictable behavior) on neonate samples[21]. The rise in the TBil could be due to haemolysis which affects the TBil measurement. Another factor to be is considered Hb interference with the diazo methods by chemical as well as photometric method.

In our present study, DBil showed significantly higher values than Bc values and poor correlation between these two parameters was noticed in our studied group. The results clearly demonstrated that DBil may be affected by cross reaction with Bu which is clinically misleading. Studies by Stanley et. al in 2011 and Lo et.al in 2007 reported that DBil assays measure all Bc (monoglucucoronide and di glucucoronide and delta) as well as some Bu[22,23].

Discussion

Neonatal jaundice is commonly associated with high morbidity and mortality[24]. Under normal conditions, the level of bilirubin rises at birth and signs of jaundice become visible, usually reaching the peak value between 2nd & 4th day. However the level starts to decrease between 6th and 8th day of life[25]. This variant of jaundice is called physiological and results in defective transport of bilirubin into hepatocytes[26,27]. Low activity of bilirubin conjugating enzyme[28], excessive load of bilirubin to the liver than its ability to conjugate due to its immaturity at birth which is a resultant of haemolysis from blood type compatibilities[29].

According to the study by Muhammad et al in 2011[30] jaundice is common among infants and therefore hyperbilirubinemia should be diagnosed and appropriately treated in order to reduce risk of morbidity and mortality as well as risk of kernicterus and cerebral palsy in the infants which survive.

However, if symptoms of jaundice persist after third day of life it is suggestive of severe form of hyperbilirubinemia. Continuation in the rise of bilirubin levels leads to acute jaundice, kernicterus and cerebral palsy[25].

Recent medical reports and studies indicate (indicate) re-occurrence of kernicterus from a state of near morbidity in case of neonatal jaundice which is of major concern to pediatricians[31].

It is concluded from both laboratory investigations and epidemiological studies, that unconjugated bilirubin is toxic to the central nervous system. ”Kernicterus” is characterized by convulsions, hypotonia, high-pitched cry and fever in newborns affected with jaundice. The clinical symptoms include choreoathetosis, asymmetric spasticity, paresis of upward gaze and neurogenic hearing loss[32,33,34].

The clinical condition in which the concentration of unconjugated bilirubin exceeds the bilirubin binding capacity of albumin, resultant of severe haemolysis, displacement of albumin bound to bilirubin by sulfisoxazole or free fatty acid leads to hypoxemia, hypercarbia and hyperosmolar conditions in the newborn. In newborn the auditory pathway is prone to damage from moderate to severe hyperbilirubinemia resulting in sensineural hearing loss.[35,36,37,38,39,40]

Vitros neonatal bilirubin is estimated as the sum of Bu and Bc, this value is clinically equivalent to total bilirubin except when delta bilirubin is present. As the name suggests, neonatal bilirubin is intended for use in neonates. Although delta bilirubin is not common in neonates its existence is not impossible. Therefore caution should be exercised while interpreting neonatal bilirubin as total bilirubin[41].

In vivo exposure to light may alter bilirubin’s chemical and spectral properties due to formation of photobilirubin in neonates receiving intensive phototherapy thereby exhibiting elevated Bc resultant of in-vivo formation of photobilirubin. The advantage of Vitros method utilizing Dry Chemistry is that conjugated bilirubin (Bc) measurement is less susceptible to the photodegradation of the sample and gives accurate value of conjugated bilirubin in neonates.

When phototherapy is monitored by BuBc slide, it is a necessity to report unconjugated bilirubin, conjugated bilirubin and neonatal bilirubin. To report neonatal bilirubin just as the sum of Bu and Bc is to ignore or overlook useful information. Elevated conjugated bilirubin is always pathologic, and it is an important necessity to notice it as quickly as possible. Many cases of conjugated hyperbilirubinemia can be treated more effectively and at the earliest, this is applicable to biliary atresia, an uncommon but particularly serious cause of hyperbilirubinemia in neonates[42].

High δ bilirubin (> 50% of total bilirubin) in newborn was reported to be associated with intra and extra- hepatic cholestasis, biliary cirrhosis, biliary atresia and hepatitis[41].

Extrahepatic biliary atresia describes a condition in which the bile ducts are damaged and blocks the bile which contains conjugated bilirubin to drain into the intestinal tract, as a result conjugated bilirubin is elevated. This bile drainage problem can be surgically repaired using the Kasai procedure so that the bile drainage is sent directly to the bowel[23].

The Vitros method of Ortho Clinical Diagnostic is unique. The BuBc slide utilizes a mordant which produces a spectral difference between conjugated and unconjugated bilirubin. During phototherapy treatment structural photoisomers formed from bilirubin not detected from traditional total and direct bilirubin methods are detected by the Vitros BuBc slide.

Hence laboratories must strive to be efficient as well as proficient in measuring the bilirubin in the neonates in order to provide proper interventions by clinicians and execute proper diagnosis and appropriate treatment to the newborns on the basis of accurate determinations of the various fractions of bilirubin[43].

Conclusion

To conclude, the results have clearly shown that neonatal bilirubin (NBil) is better parameter than total bilirubin (TBil) in assessing neonatal Jaundice in newborns. Currently, almost all hospitals in India rely upon the results of total bilirubin (TBil), in neonates following photo therapy. The inclusion of neonatal bilirubin (NBil) parameter would certainly help avoiding mislead to monitoring bilirubin levels in newborns on phototherapy and neonates may have more serious diseases that require prompt intervention, because increased conjugated bilirubin (Bc) concentrations are a marker of variety on infectious, metabolic, and /or liver conditions.

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