ORIGINAL RESEARCH ARTICLE

# Understanding the Difference in Various Fractions of Serum Bilirubin while Estimated by Wet Chemistry and Dry Chemistry Method and its Importance in Neonatal Jaundice

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*Received on:* 20 February 2022; *Accepted on:* 01 September 2022; *Published on:* 03 January 2023

## Abstract

**Aim:** The aim of this study is to find out the percentage of variation of bilirubin values in neonates by two different techniques: Dry chemistry versus wet chemistry method, and also to establish that unconjugated bilirubin ≠ indirect bilirubin and conjugated bilirubin ≠ direct bilirubin.

**Materials and methods:** This comparative study was conducted over a period of 6 months from October 2019 to March 2020. Ethical approval was taken from Institutional Ethical Committee. Informed and written consent were taken from the parents of the enrolled neonates. A total of 195 blood samples were randomly collected from neonates (<14 days) admitted with neonatal hyperbilirubinemia in Department of Pediatrics for estimation of serum bilirubin fractions by two techniques, i.e., traditional wet chemistry and dry chemistry. The statistical analysis of data was performed by using software package SPSS version 16 and Microsoft Excel 2019.

**Results:** In our study, mean total bilirubin measured by dry chemistry method Total bilirubin ($TBil_d$), neonatal bilirubin (BuBc/NBil) estimated by dry chemistry method, and total bilirubin estimated by wet chemistry method ($TBil_w$) were 12.42 mg/dL, 12.21 mg/dL, and 11.72 mg/dL, respectively.

**Conclusion:** Total bilirubin estimated by dry and wet chemistry methods differ due to difference in the principle of methods by which they are estimated in laboratories. Neonatal bilirubin (BuBc/NBil) may be preferred over total bilirubin estimated by wet chemistry as dry chemistry is estimating unconjugated and conjugated fractions of bilirubin, while in wet chemistry, direct bilirubin is estimated and indirect bilirubin is calculated. Hence, the term unconjugated bilirubin cannot be interchanged with indirect bilirubin and conjugated bilirubin as direct bilirubin.

**Clinical significance:** Measurement of serum bilirubin among neonates with hyperbilirubinemia is an essential part for diagnosis and monitoring of neonatal jaundice. Both dry and wet chemistry methods correlated well, any method can be used for measurement of bilirubin, but switching the method while monitoring serum bilirubin levels during treatment must be discouraged.

**Keywords:** Conjugated bilirubin, Dry chemistry, Hyperbilirubinemia, Serum bilirubin, Unconjugated bilirubin, Wet chemistry.

*Indian Journal of Medical Biochemistry* (2022): 10.5005/jp-journals-10054-0200

## Introduction

Bilirubin, an endogenous compound formed by the catabolism of heme in heme oxygenase system of reticuloendothelial cells which on the basis of high-pressure liquid chromatography (HPLC) is divided into four fractions: unconjugated (α), mono-glucuronide conjugated (β), di-glucuronide conjugated (γ), and delta (δ) bilirubin. Unconjugated bilirubin (Bu or α) is the fraction of bilirubin that is not conjugated with glucuronic acid but noncovalently bound to albumin.[1] Conjugated bilirubin (Bc or β + γ) is the fraction having one or two glucuronic acids, and UDP-glucuronic acid serves as an active donor of glucuronic acid. While delta bilirubin results due to spontaneous reaction between mono- and di-glucuronides with albumin (bilirubin covalently bound to albumin). Delta bilirubin is slowly cleared off from circulation and is not seen in neonates (<14 days).

Neonatal hyperbilirubinemia is defined as the elevated serum bilirubin levels in newborns due to accumulation of unconjugated bilirubin.[2] Broadly, neonatal jaundice is divided into three types: inherited, unconjugated, and conjugated neonatal hyperbilirubinemia. Among these, unconjugated hyperbilirubinemia is the commonest while conjugated hyperbilirubinemia is typically associated with diseases, e.g., extrahepatic biliary obstruction. For diagnosing neonatal hyperbilirubinemia, two methods are available: visual estimation gives a rough guide for level of bilirubin in newborns as it is prone to error, especially in newborns with hyperpigmented skin,[3] while measurement of serum bilirubin provides the accurate value for hyperbilirubinemia, which guides clinician's diagnosis of hyperbilirubinemia in newborn.[4]

According to the American Academy of Pediatrics,[5] total serum bilirubin should be measured on every newborn suspected of neonatal jaundice within 24 hours of birth, or at a minimum, before

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**How to cite this article:** Singh KK, Singh K, Kumar M. Understanding the Difference in Various Fractions of Serum Bilirubin while Estimated by Wet Chemistry and Dry Chemistry Method and its Importance in Neonatal Jaundice. Indian J Med Biochem 2022;26(1):9–14.

**Source of support:** This work was funded under Intramural Seed (Student) Fellowship (2019) by King George's Medical University

**Conflict of interest:** None

the newborn is discharged from the hospital. The decision to retest newborn is a clinical judgment determined by the zone in which the total bilirubin falls, the age of newborn, other risk factors, and the expected course of hyperbilirubinemia. Hence, estimation of serum bilirubin plays an important role in neonatal jaundice. Analytical methods available for estimation of bilirubin are[6] – spectrophotometric method, commonly used Diazo method's, peroxidase method, and gold standard HPLC method.[7] Other methods for bilirubin estimation are dry chemistry method (BuBc/slides) also known as neonatal bilirubin (NBil) slides based on direct spectrophotometry and noninvasive method – transcutaneous bilirubinometer. Though, the estimation of serum bilirubin by commonly used methods provides an accurate level of bilirubin, improvement in currently used laboratory methods is still needed. The aim of this study is to find out the percentage of variation of bilirubin values in neonates by two different techniques: Dry chemistry versus wet chemistry method and also to establish that unconjugated bilirubin ≠ indirect bilirubin and conjugated bilirubin ≠ direct bilirubin.

## Materials and Methods

This comparative study was conducted over a period of 6 months from October 2019 to March 2020 in the Department of Biochemistry, King George's Medical University, Lucknow, in collaboration with Department of Pediatrics, King George's Medical University, Lucknow. Ethical approval was taken from Institutional Ethical Committee (Ref. code: 98th ECE IIB IMR-F/P5). Informed and written consent were taken from the parents of the enrolled neonates. A total of 195 blood samples were randomly collected from neonates (<14 days) admitted with neonatal hyperbilirubinemia in Department of Pediatrics for estimation of serum bilirubin fractions by two techniques, i.e., traditional wet chemistry and dry chemistry. One ml blood sample was collected in plain vacutainer under aseptic conditions. After half an hour, the sample was centrifuged and serum separated for estimation of bilirubin. Out of 195 samples, 158 were estimated by both the techniques, and 37 samples were quantity not sufficient (QNS).

### Measurement of Serum Bilirubin

Serum bilirubin was measured in each sample by two different methods:

*Method 1*: Wet chemistry method based on colorimetric diazo method[8] TBiland direct bilirubin (DBil) were estimated in each blood sample on Selectra (Make: ELITECH, Model: Pro-M) autoanalyzer. Indirect bilirubin was calculated by the formula TBil – DBil.

*Method 2:* Dry chemistry method[9]
Unconjugated bilirubin (Bu), conjugated bilirubin (Bc), and total bilirubin (TBil) were measured in each blood sample on Vitros350 analyzer. Bu and Bc measure fractions by direct spectrometry, whereas total bilirubin is measured by a diazo method.

### Statistical Analysis

The statistical analysis of data was performed by using software package SPSS version 16 and Microsoft Excel 2019. Paired *t*-test was used to compare mean of total bilirubin, unconjugated bilirubin (Bu), and conjugated bilirubin (Bc) estimated by dry chemistry technique with total bilirubin, indirect bilirubin, and direct bilirubin estimated by traditional wet chemistry method. To study the relationship between total bilirubin estimated by wet and dry chemistry method, indirect bilirubin and Bu, direct bilirubin and Bc, and Pearson's correlation coefficient was applied. *p*-values <0.05 was considered statistically significant. To assess the difference between Bu and indirect bilirubin, Bc and direct bilirubin, neonatal bilirubin (Bu and Bc) and total bilirubin by wet chemistry, neonatal bilirubin (Bu and Bc) and total bilirubin by dry chemistry, the Bland–Altman method was used, using average difference ± 1.96 SD as the 95% limits of agreement.

## Results

In this study, 158 samples collected from neonates with neonatal hyperbilirubinemia were analyzed for estimation of serum bilirubin fractions by two different techniques. Mean serum bilirubin level of bilirubin fractions estimated by wet chemistry (Bu, Bc, and $TBil_d$) and dry chemistry ($TBil_w$, direct Bil, and indirect Bil) method is shown in Table 1. Comparison of serum bilirubin fractions estimated by two methods is detailed in Table 2a. Correlation between bilirubin fractions is shown in Figures 1A to D. Further, 158 samples were divided into two groups based on the reference range used for conjugated bilirubin, i.e., 0.0–0.6 mg/dL. One group consisted of conjugated bilirubin level below 0.6 mg/dL and other with level more than 0.6 mg/dL, descriptive and comparative analysis of which is detailed in Tables 1 and 2b, respectively. The Bland–Altman plot to assess the difference in bilirubin fractions by two methods (wet and dry chemistry) is shown in Figures 2A to D.

## Discussion

Neonatal jaundice in healthy as well as preterm infants is encountered commonly by the Pediatrician and for its management, monitoring of serum bilirubin is an essential part. In 2004, American Academy of Pediatrics revised the guidelines for the management of hyperbilirubinemia and proposed the universal total serum bilirubin measurement for risk assessment. As the visual assessment is not reliable, estimation of accurate serum bilirubin plays a critical role in the treatment of neonatal jaundice and timely intervention. For the measurement of serum bilirubin, laboratory methods available are Spectrophotometric, Diazo, Peroxidase, Peroxidase Diazo, and HPLC. With the development in the field of technology, newer methods, for example, dry chemistry and noninvasive (transcutaneous bilirubinometer), are now also available. Estimation of serum bilirubin fractions shows marked variations in between laboratories as well as in between methods used in various laboratories. Hence, it is suggested while interpreting serum bilirubin results, clinicians should carefully check the method used for its estimation in laboratory and also not to switch the method during the follow-up period.

In our study, mean total bilirubin measured by dry chemistry method ($TBil_d$), neonatal bilirubin (BuBc/NBil) estimated by dry chemistry method, and total bilirubin estimated by wet chemistry method ($TBil_w$) were 12.42 mg/dL, 12.21 mg/dL, and 11.72 mg/dL, respectively (Table 1). $TBil_w$ levels were found to be lower as compared to $TBil_d$ and NBil which were statistically significant as shown in Table 2a. In contrast to our study, Berska et al. observed that mean NBil values were lower when compared to TBil estimated with dry and wet chemistry though the difference was not statistically significant.[10] In 2018, Kumar et al. observed no difference in mean level of NBil and TBil estimated by wet chemistry.[11] Similarly, Padmanabhan et al. found that NBil measured using microslide of dry chemistry showed lower values when compared to total bilirubin estimated by wet chemistry diazo method.[12] In 2004, Lo et al. reported higher NBil values by

**Table 1:** Descriptive analysis of serum bilirubin fractions estimated by wet and dry chemistry

| | *Mean* | *Standard deviation* | *Median* | *Minimum* | *Maximum* | *N* |
|---|---|---|---|---|---|---|
| $TBil_d$ | 12.42 | 4.07 | 12.35 | 1.70 | 28.10 | 158 |
| Bu | 11.59 | 3.97 | 11.75 | 0.80 | 23.60 | 158 |
| Bc | 0.62 | 2.09 | 0.00 | 0.00 | 16.20 | 158 |
| NBil (BuBc) | 12.21 | 4.41 | 12.1 | 0.8 | 33.4 | 158 |
| $TBil_w$ | 11.72 | 3.99 | 11.57 | 0.99 | 32.75 | 158 |
| Indirect bil | 10.68 | 3.55 | 10.86 | 0.79 | 24.89 | 158 |
| Direct bil | 1.05 | 1.63 | 0.69 | 0.20 | 13.47 | 158 |
| Conjugated bilirubin reference range 0.0–0.6 mg/dL | | | | | | |
| $TBil_d$ | 15.49 | 3.59 | 12.2 | 1.7 | 22 | 132 |
| Bu | 11.32 | 3.44 | 11.65 | 0.8 | 21.3 | 132 |
| Bc | 0.11 | 0.18 | 0.00 | 0.00 | 0.6 | 132 |
| NBil (BuBc) | 11.24 | 3.18 | 11.51 | 0.99 | 19.29 | 132 |
| $TBil_w$ | 10.55 | 3.12 | 10.83 | 0.79 | 18.61 | 132 |
| Indirect Bil | 0.70 | 0.18 | 0.67 | 0.2 | 1.41 | 132 |
| Direct Bil | 15.49 | 3.59 | 12.2 | 1.7 | 22 | 132 |
| Conjugated bilirubin reference range >0.6 mg/dL | | | | | | |
| $TBil_d$ | 15.08 | 5.24 | 15.1 | 6.5 | 28.1 | 26 |
| Bu | 12.98 | 5.87 | 14.15 | 2.5 | 23.6 | 26 |
| Bc | 3.22 | 4.33 | 1.45 | 0.7 | 16.2 | 26 |
| NBil (BuBc) | 14.14 | 6.31 | 13.55 | 4.3 | 32.75 | 26 |
| $TBil_w$ | 11.35 | 5.26 | 11.68 | 0.87 | 24.89 | 26 |
| Indirect Bil | 2.88 | 3.58 | 1 .21 | 0.65 | 13.47 | 26 |
| Direct Bil | 15.08 | 5.24 | 15.1 | 6.5 | 28.1 | 26 |

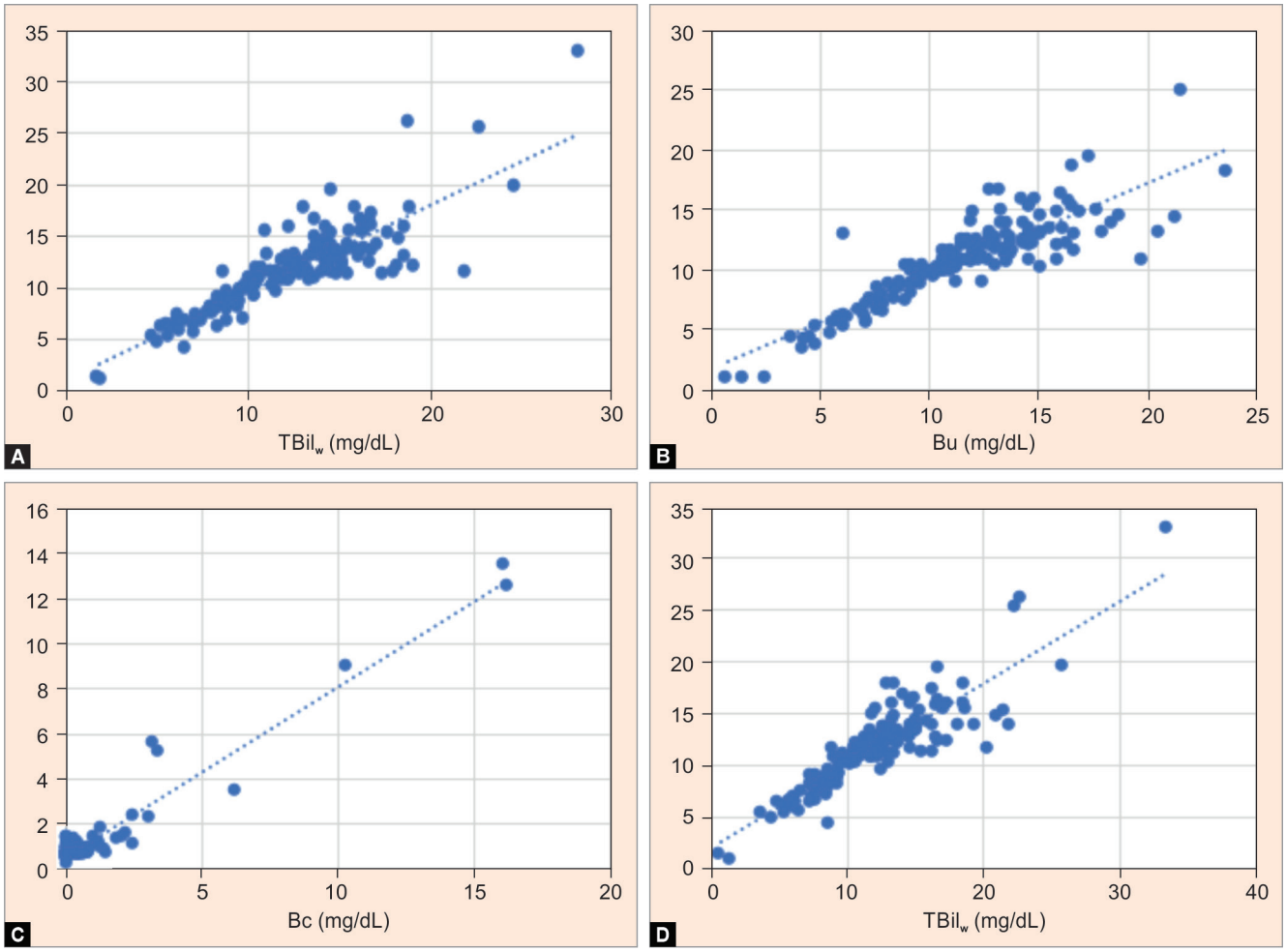
**Table 2a:** Comparison of serum bilirubin fractions estimated by wet and dry chemistry

| | | *Paired difference* | | | | |
|---|---|---|---|---|---|---|
| *Pair* | | *Mean* | *SD* | *Std. error mean* | *t* | *p-value* |
| 1 | $TBil_d$–$TBil_w$ | 0.70 | 2.18 | 0.17 | 4.04 | **<0.001** |
| 2 | Bu–indirect Bil | 0.91 | 1.95 | 0.16 | 5.89 | **<0.001** |
| 3 | Bc–direct Bil | –0.42 | 0.66 | 0.05 | 8.03 | **<0.001** |
| 4 | NBil–$TBil_w$ | 0.49 | 2.09 | 0.35 | 2.97 | **0.0035** |
| 5 | NBil–$TBil_d$ | –0.21 | 1.52 | 0.12 | 1.72 | 0.0871 |

Bold values and *p*-value <0.05 is considered as statistically significant, <0.01 as highly significant and <0.001 as very highly significant

using BuBc slide of dry chemistry than the TBil values estimated by using TBil slide of same technology.[13] However, in our study, mean difference between NBil and $TBil_d$ using same technology was only –0.20, which was not statistically significant (Table 2a). Though the NBil and $TBil_d$ are estimated by dry chemistry method, the principle of NBil is based on direct spectrophotometry while $TBil_d$ is on diazo method. Also, $TBil_d$ of dry chemistry and $TBil_w$ of wet chemistry are measured by diazo method, but in dry chemistry, concentration of total bilirubin is measured at two wavelengths by reflectance spectrophotometry, and the relationship between absorbance and concentration is not linear, while diazo method of wet chemistry uses transmission spectrophotometry and follow Lambert–Beer law which shows linear relationship between absorbance and concentration. Thus, the principle of NBil, $TBil_d$, and $TBil_w$ differ from each other which may result in variation of total bilirubin concentration when estimated by dry and wet chemistry methods. As in neonates <14 days, delta bilirubin is negligible, NBil slide may be opted over $TBil_d$ slide for estimation of bilirubin fractions (Bu and Bc).

Serum bilirubin fractions, when estimated by dry and wet chemistry methods, were highly correlated, as shown in Figures 1A to D. In Bland–Altman plot analysis, the difference between NBil and $TBil_d$ was –0.207 mg/dL with limits of agreement ranging from –3.17 to 2.762 mg/dL. But the plot showed greater disagreement between NBil and $TBil_w$ results varied from –3.6 mg/dL to 4.58 mg/dL with a mean difference of 0.493 mg/dL (Fig. 2D). Berska et al. also observed difference of 0.4903 mg/dL between NBil and $TBil_w$ with limit of agreement ranging from –1.67 mg/dL to 2.48 mg/dL, however, their study difference between NBil and $TBil_d$ was 0.716 mg/dL. Contrary to our findings, Kumar et al. found difference of only 0.004 mg/dL between NBil and TBil, estimated by wet chemistry. Padmanabhan et al. reported the difference between NBil and $TBil_w$ in percentage as –4.2%, and limit of agreement ranged from –19.4 to 11.1%, while in our study, mean difference was 3.15%. We also compared $TBil_w$ and $TBil_d$ by Bland–Altman plot which showed a difference of 0.7009 mg/dL, and limit of agreement ranged from –3.574 to 4.976 mg/dL between the two methods (Fig. 2A).



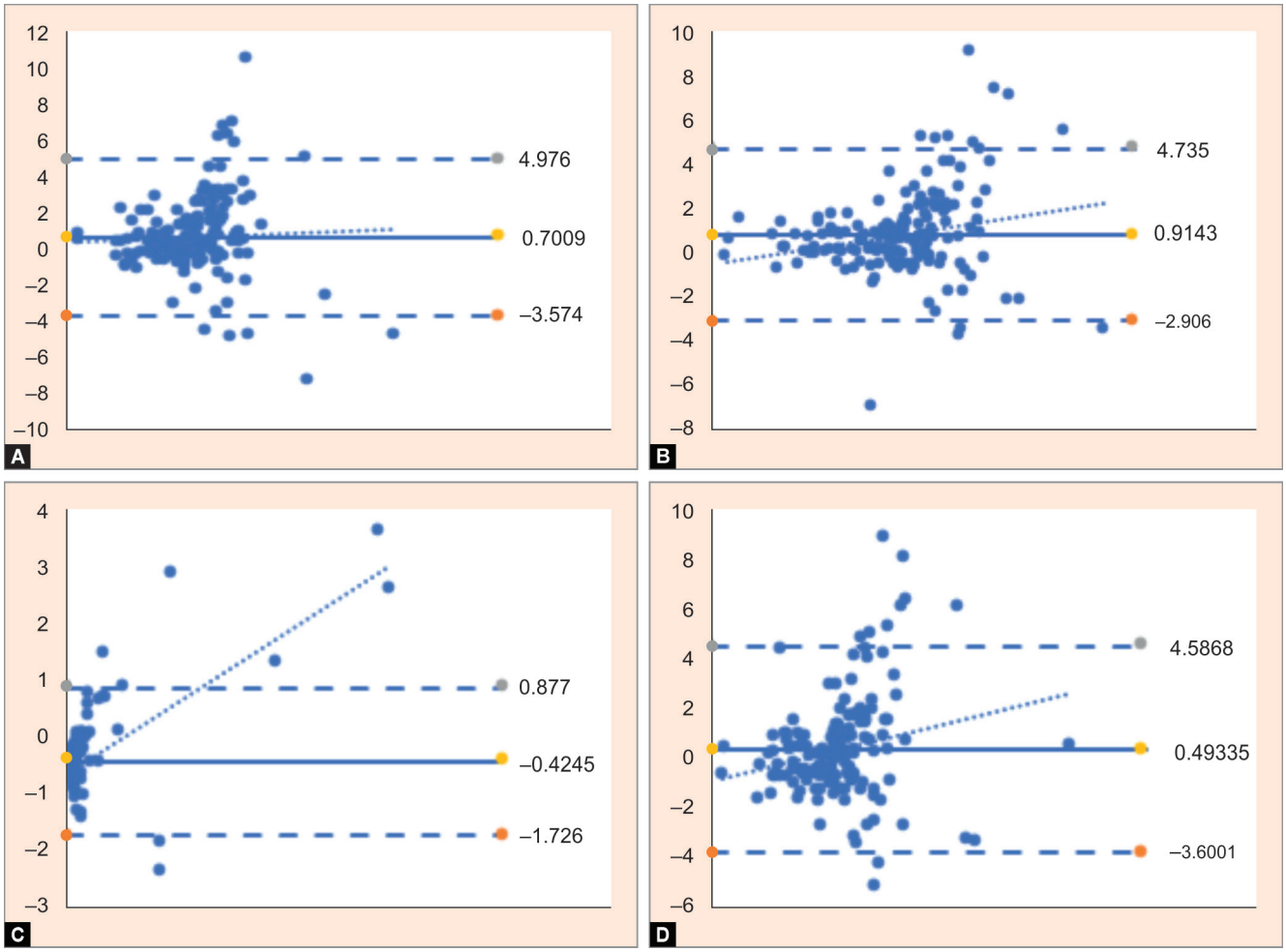

**Figs 1A to D:** Correlation between serum bilirubin fractions. (A) $TBil_d$ and $TBil_w$; (B) Bu and indirect bilirubin; (C) Bc and direct bilirubin; (D) NBil and $TBil_w$

**Table 2b:** Comparison of serum bilirubin fractions estimated by wet and dry chemistry (categorized on the basis of conjugated bilirubin reference range)

| | | *Paired difference* | | | | | |
|---|---|---|---|---|---|---|---|
| *Pair* | | *Mean* | *SD* | *Std. error mean* | *t* | *Df* | *p-value* |
| Conjugated bilirubin 0.0–0.6 mg/dL | | | | | | | |
| 1 | $TBil_d$–$TBil_w$ | 0.65 | 2.08 | 0.18 | 3.60 | 131 | **0.0004** |
| 2 | Bu–indirect Bil | 0.77 | 1.67 | 0.15 | 5.31 | 131 | **<0.0001** |
| 3 | Bc–direct Bil | –0.59 | 0.22 | 0.02 | 30.43 | 131 | **<0.0001** |
| Conjugated bilirubin >0.6 mg/dL | | | | | | | |
| 1 | $TBil_d$–$TBil_w$ | 0.94 | 2.65 | 0.52 | 1.81 | 25 | 0.0820 |
| 2 | Bu–indirect Bil | 1.63 | 2.93 | 0.57 | 2.84 | 25 | **0.0088** |
| 3 | Bc–direct Bil | 0.43 | 1.26 | 0.25 | 1.73 | 25 | 0.0946 |

Bold values and *p*-value <0.05 is considered as statistically significant, <0.01 as highly significant and <0.001 as very highly significant

Mean level of Bu was 11.59 mg/dL, while mean indirect Bil level measured by wet chemistry was 10.68 mg/dL, significantly lower as shown in Table 2a (pair 2). Mean Bc level was 0.62 mg/dL, and direct Bil level was significantly higher ($t = 8.033$; $p < 0.0001$) with mean difference of –0.4246 mg/dL (Table 2a, pair 3). Similarly, Padmanabhan et al. observed lower indirect bilirubin and higher direct bilirubin levels as compared to Bu and Bc of dry chemistry method.[12] In our study, the Bland–Altman plot showed difference of 0.914 mg/dL between Bu and indirect Bil of wet chemistry, and limit of agreement ranged from –2.906 to 4.735 mg/dL (Fig. 2B). The plot between Bc and direct bilirubin showed difference of –0.4245 mg/dL with lower and upper limits of agreement as –1.726 and 0.877 mg/dL, respectively (Fig. 2C). In the traditional wet chemistry method, a small percentage of indirect bilirubin reacts with diazotized sulfanilic acid even in absence of accelerator and results in higher level of direct bilirubin and




**Figs 2A to D:** Bland–Altman plot analysis. (A) $TBil_d$ and $TBil_w$; (B) Bu and indirect bilirubin; (C) Bc and direct bilirubin; (D) NBil and $TBil_w$

lower indirect bilirubin level, as observed in our study, which shows that two terms are not interchangeable as also reported in a retrospective study.[14]

Further, we formed two groups based on Bc reference range, group I having all those samples with Bc results between 0 and 0.6 mg/dL, and group II those samples in which Bc results were >0.6 mg/dL. Based on this criterion, mean and SD of serum bilirubin fractions estimated by wet and dry chemistry method is shown in Table 1. In group I comparison of $TBil_d$ vs $TBil_w$, Bu vs indirect Bil, and Bc vs direct Bil showed statistically significant differences between wet and dry chemistry (Table 2b), however, on analyzing for correlation study, both the method correlated significantly. In group II, no significant difference was found between $TBil_d$–$TBil_w$ and Bc–direct Bil, but statistically significant difference was observed between Bu–Indirect Bil of dry and wet chemistry methods (Table 2b), but serum bilirubin fractions showed a good correlation between two methods.

## Conclusion

Measurement of serum bilirubin among neonates with hyperbilirubinemia is an essential part for diagnosis and monitoring of neonatal jaundice. Total bilirubin estimated by dry and wet chemistry methods differs due to differences in the principle of methods by which they are estimated in laboratories. Neonatal bilirubin (BuBc/NBil) may be preferred over total bilirubin estimated by wet chemistry as dry chemistry is estimating unconjugated and conjugated fractions of bilirubin, while in wet chemistry, direct bilirubin is estimated and indirect bilirubin is calculated. Hence, the term unconjugated bilirubin cannot be interchanged with indirect bilirubin and conjugated bilirubin as direct bilirubin. The term direct and indirect were named due to the property of bilirubin fraction, which react in van den berg reaction. However, as both dry and wet chemistry methods correlated well, any method can be used for measurement of bilirubin, but switching the method while monitoring serum bilirubin levels during treatment must be discouraged.

## Acknowledgment

Authors would like to acknowledge the support of our technical staff while conducting this study. We are most thankful to the King George's Medical University, Lucknow, Uttar Pradesh, for encouraging the undergraduate students to participate in research work and providing intramural grant for this research.

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