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Monitoring Bilirubin Binding Parameters in a Cohort of Japanese Neonates

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Abstract

The status of bilirubin binding to albumin (Alb) is central to personalized management of unconjugated newborn hyperbilirubinemia, especially those at risk of bilirubin-induced neurologic dysfunction. Our objectives were to: (1) compare assays of bilirubin binding using hematofluorometry (Hmf) and the glucose oxidase-peroxidase (GOD-POD) methods; and (2) elucidate the relationships between bilirubin binding parameters and gestational age (GA). 169 blood samples were obtained from 98 Japanese newborns with GAs ranging from 22-40 wks. Apparent serum unbound bilirubin (UB) concentrations were determined by GOD-POD. Ratios of bound bilirubin (BB) to reserve Alb binding capacity for bilirubin (RABC) were determined by Hmf. Bilirubin binding capacity (BBC) by Hmf was compared to the calculated BBC (or 8.8 × Alb) using Alb levels as measured by the clinical laboratory. UB was compared with the ratio of total serum/plasma bilirubin (TB) to ((8.8 × Alb)-TB). Linear regression analyses were performed for all comparisons. BBC was lower than that expected when assuming one-to-one binding of bilirubin to Alb and the divergence of (8.8 x Alb) and BBC increased at earlier GAs. A strong correlation between UB and BB/RABC was found (r=0.83; P<0.001) and was independent of GA and unaltered after infants were subcategorized by phototherapy and drug use. UB and TB/((8.8 × Alb)-TB) also strongly correlated (r=0.84), but was significantly GA- dependent. The strong correlation between apparent UB and BB/RABC demonstrates a practical equivalence of the two methods, with UB and BB/RABC well correlated over the entire GA range. While the TB/Alb ratio may provide a reliable assessment of risk of bilirubin neurotoxicity, it appears increasingly unreliable as GA decreases.

Introduction

Besides its transport and osmotic pressure stabilizing functions, albumin (Alb) can bind unconjugated bilirubin to sequester the toxin within the vascular space and to buffer the level of unbound bilirubin (UB) that can cross membranes and enter cells. Consequently, a low Alb level that is reflective of a low bilirubin binding capacity (BBC), increases an infant's risk of developing bilirubin-induced neurologic dysfunction (BIND) by reducing an infant's ability to "tolerate" a given bilirubin load [1-6]. A reduced Alb level (or hypoalbuminemia) is characteristic of neonates at early gestational ages (GAs) [7-9], and considered a major cause for the higher risk of BIND in infants at early GAs at any given total serum/plasma bilirubin (TB) level [10,11]. But neither the Alb level alone nor the bilirubin: Alb molar ratio (BAMR) appears to be reliable predictors of an infant's risk for developing BIND [10-13]. Previous studies using various direct assays have shown that the binding of bilirubin in newborns is lower than that expected from one-to-one binding of bilirubin to Alb as reported for adults and that this difference appears to increase as GA decreases [14-16].

Hematofluorometry (Hmf), based upon bilirubin fluorescence, can measure BBC directly in whole blood [15,17-19]. Furthermore, Hmf specifically determines the capacity of the single strong Alb site to bind bilirubin [15,17-21]. Hmf can also assay the bilirubin bound to Alb (BB) in blood. The difference between BBC and BB (or BBC -BB) is the reserve Alb binding capacity (RABC). Within the limits of the single-site binding model, the ratio of BB/RABC is proportional to the UB level with the proportionality constant being 1/Ka, where Ka is the affinity constant of the strong site [17,22,23].

The so-called "peroxidase" (POD) method developed by Jacobsen and Wennberg [24] for the measurement of UB in sera or plasma has been in limited use for decades [3,17,22]. Automated colorimetric devices (Arrows Co, Osaka, Japan) for this assay and for TB have been used to determine the clinical utility of this assay for UB. The general conclusion of the various investigators is that UB appears to be a better predictor of BIND than TB alone, especially in the small and early GA infants [2,3,25-27]. Currently, the limited use of the "POD" assay is due to the general unavailability of these systems for routine use outside of Japan where they are widely used. It was, therefore, of interest to compare BB/RABC by

Hmf using a prototype device (Bili- 4, Aviv Biomedical, Inc, Lakewood, NJ, USA) and UB by POD using colorimetry (Arrows-2 Analyzer). Although such a comparison was made years ago using the original Aviv Hmf system and the original, non-automated POD assay [23], here we present data from a neonatal population spanning a full range of GAs, using the same blood samples collected for measurements of Alb, BBC, BB, and UB.

Methods

Sample collection

169 blood samples from 98 newborns (born from May 2015 to June 2016) were collected in serum separator tubes after phlebotomy for routine laboratory tests and stored at room temperature in the dark until use within 3 hrs. Parental informed consent was obtained prior to blood collections. After sampling for a complete blood count including hemoglobin (Hb), Hmf assays by the Bili-4 Hematofluorometer were performed using whole blood. Then, serum was separated and 20 µl was immediately measured for TB and UB using a UB Analyzer-2. All assays were performed using blood from the same draw and under subdued light.

The study protocol was approved by the ethical committee in Kobe University Graduate School of Medicine (No. 1772) and was in accordance with the current revision of the Helsinki Declaration.

Hematofluorometric (Hmf) assay

The Hmf assay has been described in detail previously [18,23,28]. In brief, 25 µl of whole blood was transferred to a small microfuge vial containing 20-µg bilirubin disodium salt, which was stored at -20°C and warmed to room temperature prior to use. After approximately 30 sec to allow for complete bilirubin to Alb binding, the vial was vortexed. A 15-µl aliquot was then placed on a slide, which was inserted into the hematofluorometer. Another 15-µl aliquot of whole blood (without the bilirubin reagent) was placed directly on another slide inserted into the instrument. The two slides were placed in the instrument within 15 sec of each other to avoid differential settling of red blood cells. BB, BBC, RABC, and BB/RABC values are displayed almost immediately. The Bili-4 Hematofluorometer was calibrated according to each manufacturer's instructions and as follows: normal adult blood was spiked with known amounts of bilirubin and additionally checked for TB using a Reichert Unistat colorimetric instrument (Buffalo, NY) by the clinical laboratory.

GOD-POD method [29-31]

This method is based upon the premise that UB is rapidly oxidized to colorless compounds by POD in the presence of hydrogen peroxide derived from glucose by mediation of glucose oxidase-peroxidase (GOD) and TB concentrations are then determined by direct spectrophotometry at 460 nm for Alb-bound bilirubin. Under these experimental conditions where bilirubin oxidation follows first-order kinetics, the rate constant is determined by measuring the oxidation velocity of bilirubin in the absence of Alb. The initial velocity is estimated from the time required for a 20% decrease in the initial TB concentration. Apparent UB is then calculated from the initial velocity of bilirubin loss and the ratio of the POD concentration to that in the standard assay solution containing Alb-free bilirubin [30,31]. In brief, the procedure is as follows: a cuvette containing 1 ml of phosphate buffer and a metal stirrer is warmed and then placed into the optical unit of the analyzer. 20 µl of serum is then added. Within 30 sec, the TB value is displayed. 25 µl of enzyme solution (GOD and POD, 3.2 units/sec, respectively) is then added. Within 2 min, the apparent UB value is displayed. The UB Analyzer-2 was calibrated according to each manufacturer's instructions using manufacturer-supplied standard calibrators.

Other assays

Hb levels were measured by the sodium lauryl sulfate-hemoglobin method using a XN 9000 instrument (Sysmex, Inc., Kobe, Japan) [32]. Conjugated (direct) serum bilirubin concentrations were measured by the bilirubin oxidase method using IatroLQ D-bil kits (Unitika Co., Okazaki, Japan) [33]. Serum Alb concentrations were measured using the modified bromocresol purple method (Kainos Laboratories, Inc., Tokyo, Japan) [34].

Results

Clinical demographics and sample characteristics

Clinical demographics and characteristics for the 98 enrolled newborns and the 169 blood samples are shown in Table 1. Newborns with various birth weights (BWs) and GAs were included. Of the 169 blood samples, 20 (12%) samples were from newborns treated with phototherapy and 78 (46%) were from newborns treated with drugs. Eighty (47%) samples were from newborns not treated with phototherapy and any drugs. No samples with high conjugated (direct) bilirubin levels were included. Also, there were no samples from newborns treated with lipids or with hemolysis.

Precision

To assess precision, a sub-cohort of 14 samples with different concentrations of bilirubin was randomly chosen for precision analyses (7 for Hmf and 7 for GOD-POD) (see Supplementary Table 1). Mean CVs for BB, BBC, RABC, BB/RABC, TB, and apparent UB were 5.2 ± 1.8%, 7.3 ± 3.0%, 10.9 ± 4.5%, 10.6 ± 4.0%, 2.7 ± 1.2%, and 5.5 ± 1.7%, respectively.

BBC, Alb, and GA: For one-to-one binding, 8.8 mg of bilirubin can be bound to 1 gm of Alb, and therefore (8.8 × Alb) has been used clinically as an estimate of BBC. Values for (8.8 × Alb) and BBC obtained for the entire cohort were plotted as a function of GAs in completed weeks (cGA) and shown in Figure 1. The level of (8.8 × Alb) and the slope of the regression line (0.38 mg/dl/wk, r = 0.56) are in close agreement with other reports [7,14,16,28]. When the postpartum age (mean of 5

Table 1: Demographics and clinical characteristics of enrolled newborns (N= 98) and blood samples (N = 169)

		Number (%)
Birth weight (g)	< 1000	8 (8)
	1000 to 2499	39 (40)
	> 2500	51 (52)
Gestational age (wks)	22 to 27	9 (9)
	28 to 36	37 (38)
	37 to 41	52 (53)
Male		48 (49)
Small for gestational age		18 (19)
Delivery mode	Vaginal delivery	47 (48)
	Cesarean-section	51 (52)
Nationality	Japanese	98 (100)
Hemolysis		0 (0)
		Median (range) or number (%)
Postnatal age when sampling (days)		5 (0-147)
Corrected gestational age when sampling (wks)		36 (22-43)
Laboratory Data	TB by GOD-POD method (mg/dl)	10.9 (0.8-20.9)
	UB by GOD-POD method (μg/dl)	0.49 (0.01-1.42)
	BB by Hmf	9.2 (2.1-18.1)
	BBC by Hmf	25.5 (11.0-38.5)
	Conjugated (direct) bilirubin (mg/dl)	0.3 (0.1-0.5)
	Alb (g/dl)	3.3 (1.9-4.2)
	Hb (g/dl)	15.0 (7.8-21.0)
Clinical Data	Infants treated with/without phototherapy	20 (12) / 149 (88)
	Infants treated with* / without drugs	78 (46) / 91 (54)
	Infants treated without phototherapy and drugs	80 (47)
	Infants given the following drugs*	
	Ferric pyrophosphate	31 (18)
	Calcium gluconate	31 (18)
	Dopamine	17 (10)
	Ampicillin	17 (10)
	Dobutamine	16 (9)
	Amino acid preparation	15 (9)
	Amikacin	8 (5)
	Caffeine	6 (4)
	Activated vitamin D	5 (3)
	Fluconazole	4 (2)
	Levothyroxine	4 (2)
	Furosemide	3 (2)
	Phenobarbital	3 (2)
	Spironolactone	2 (1)
	Indomethacin	1 (1)
	Fentanyl	1 (1)
	Midazolam	1 (1)
	Cefmetazole	1 (1)
	Lipid	0 (0)

days) was added to the cGA, the slope of the linear regression (data not shown) was nearly identical (0.37 mg/dl/wk). Because most management guidelines for neonatal hyperbilirubinemia are designed specifically for infants greater than 35 wks GA [10], we compared the linear regression lines for infants with cGAs below and above 35 wks (data not shown) and found the slopes to be 0.61 and 0.07 mg/dl/wk, respectively. The linear regression line and correlation coefficient (r = 0.71) for BBC *versus* cGA (Figure 1) was also consistent with previous studies [7,14,16,28]. The plot of BBC *versus* cGA + postpartum age (data not shown) exhibited a similar linear regression (slope = 0.72 *vs* 0.74 mg/dl/wk when corrected for postpartum age). Similar to that observed for the (8.8 × Alb) *versus* cGA relationship, the slopes of the regression lines for the BBC levels (data not shown) were different for cGAs above and below 35 wks with slopes of 0.11 and 0.93 mg/dl/wk, respectively. All in

all, on average, BBC was found to be much lower than (8.8 × Alb) at early cGAs and increases with cGA and comparable with (8.8 × Alb) at term.

By plotting the ratio of the mean BBC to the mean (8.8 × Alb) as a function of cGA, the divergence of BBC from (8.8 x Alb) with decreasing GA is more clearly shown (Figure 2). The linear regression (r=0.93, slope=0.015 mg/dl/wk) indicates that BBC is only 70% of that expected from (8.8 x Alb) at 21 wks GA but increases to almost 100% at term.

Because the various data comparisons with cGA showed little differences from those with cGA + postpartum age, which averaged 5 days, only analyses based on cGA are reported below.

Comparison of UB (by GOD-POD) with BB/RABC (Hmf): The single strong site model for bilirubin-Alb binding predicts a linear relationship between UB and BB/RABC with a zero intercept (see Eq. 2 in Appendix). Figure 4 shows that UB measured by the GOD-POD method and BB/RABC by Hmf correlated well (r = 0.83; P < 0.001) for the entire cohort. Comparable correlations (data not shown) were also observed for four sample subsets: samples from infants treated with (r=0.91) or without phototherapy (r=0.83); and infants treated without phototherapy and with (r=0.81) or without drugs (r=0.83).

The slope of the regression line for the plot of UB (in nM) *vs* BB/RABC for all samples was 13.7 nM (Figure 3). The y-intercept was very close to zero (–0.01) and indicates no systematic bias in both method and that both methods show negligible signals in the absence of bilirubin. The slopes of the regression lines for the four above-mentioned sample subsets were also comparable (13.7 ± 0.3). When the ratio of (BB/RABC)/UB (both in nM) *versus* cGA was plotted (data not shown), the slope was –0.0004/wk, demonstrating that GA did not affect these assays. This was confirmed when we found nearly identical regression lines of the UB and BB/RABC plots when stratified by cGAs above and below 35 wks: for 22 to 27 wks, slope = 11.6 nM) (Figure 4A) and for 38 to 43 wks, slope = 12.2 nM (data not shown).

Comparison of UB with TB/((8.8 × Alb) – TB): If we assume that (8.8 × Alb) is equal to the BBC and apply the single strong site binding model, then UB as a function of TB/((8.8 x Alb) – TB) should also be linear. We found that the correlation coefficient (r=0.84, Figure 5) was similar to that between UB and BB/RABC (Figure 4A), and with a y-intercept of 2 nM. However, in the single site model, a y-intercept of zero is expected. Unlike the plot of (BB/RABC)/UB *versus* GA, [TB/((8.8 × Alb) – TB)]/UB (both in nM) *versus* cGA (data not shown) yielded a regression line with a significant slope of 0.04/wk (r = 0.55). This significant dependence on GA was confirmed from our plots of UB *versus* TB/((8.8 × Alb) – TB) when stratified by cGAs: for 22 to 27 wks, r=0.92, slope=21.2 nM (Figure 4B) and for 38 to 43 wks, r=0.92, slope=11.4 nM, data not shown). While the high cGA regression line was similar to those of both late and early cGA

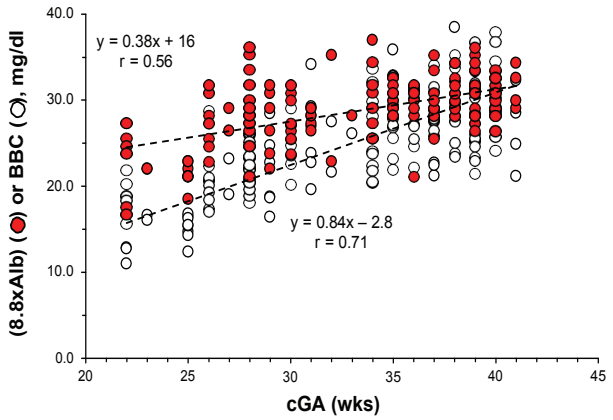

Figure 1: Plot of corrected GA vs BBC (mg/dl) by hematofluorometry (○) and assumed Alb capacity (mg/dl) (●) calculated by multiplying the Alb level (g/dl) by 8.8 and their linear regressions (r = 0.71 and 0.56, respectively).

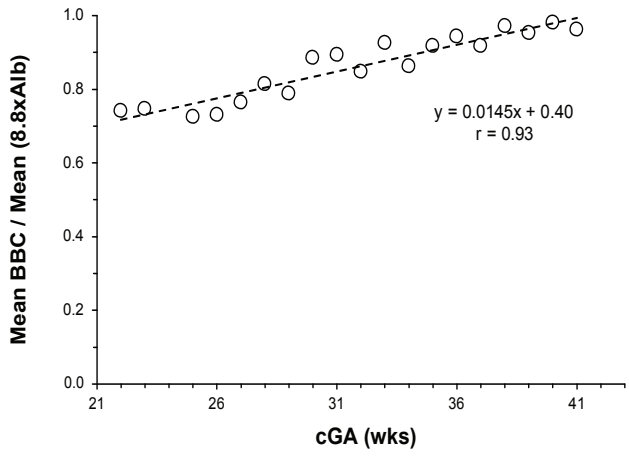

Figure 2: The ratio of the mean BBC to the mean (8.8xAlb) at each completed week of gestation (cGA, slope = 0.015 mg/dl/wk; r = 0.93).

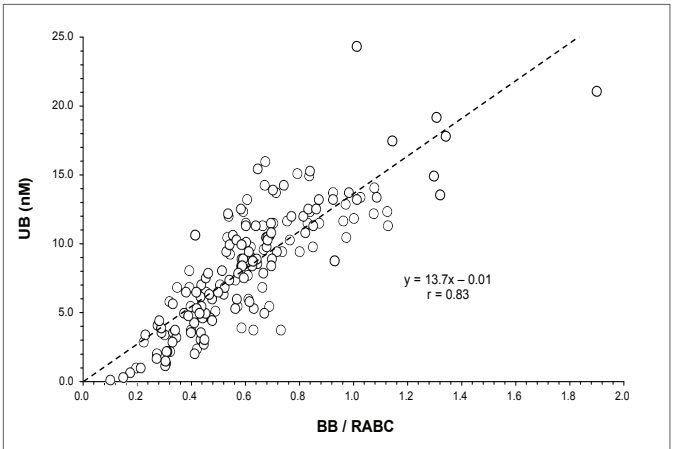

Figure 3: Linear correlation between the apparent UB (UB Analyzer-2) and BB/RABC (Bili-4 Hematofluorometer) for all samples (r = 0.83, slope = 13.7 nM).

for UB *versus* BB/RABC, the early cGA regression line has a slope that was nearly two times larger. This difference could be due to either a reduced binding affinity at early GAs or to (8.8 × Alb) being an overestimate of BBC at early GAs, but a good estimate of BBC at later GAs. A quantitative test of the latter can be done by using the measured average ratios of BBC to (8.8 × Alb) at each GA week as shown in Figure 2. Application of this "correction" yields the results shown in Figure 4C, where the slope of the early cGA regression line (11.4 nM) was now very nearly equal to the slope of the "corrected" late GA range plot (11.8 nM) (data not shown) and of the corresponding plots of UB *versus* BB/RABC. All the correlations between UB and BB/RABC and "corrected" TB/(8.8 × Alb) - TB) have high r-values, which indicate that UB and BB/RABC and "corrected" TB/((8.8 × Alb) - TB) correlate well for both the early and late GA ranges.

Discussion

The premise that BBC does not always correlate with Alb levels (and therefore (8.8 × Alb)) in neonates was based on three different methods for measuring BBC [15,16]. In 2014 [28], BBC and Alb assayed using the bromocresol green method showed that BBC was almost always lower than (8.8 × Alb) and that the BBC/(8.8 × Alb) ratio increased with GA. The correlation of BBC with GA showed an r-value of 0.34 with a slope of 0.11 mg/g/wk, and BBC/(8.8 × Alb) ratios near 0.70 at 26 wks and near 0.90 at 40 wks. In contrast to these past studies, we used the more specific bromocresol purple method [34] to measure Alb, our neonatal population was genetically much more homogeneous, and BBC values were means of at least two determinations. It is, perhaps, for these reasons that the regression line of BBC/(8.8 × Alb) *versus* GA, while having a similar slope (0.13 mg/g/wk) had a significantly better correlation of 0.62 ($P < 0.002$), and comparable BBC/(8.8 × Alb) ratios of 0.72 at 22 wks and 0.98 at 41 wks.

There have been concerns that Hmf reports only the bilirubin that is bound to Alb (i.e., BB) at its primary binding site [19-21]. However, it has been shown that BBC values in neonatal sera using Hmf are nearly identical to those obtained by Sephadex column staining or to UB using GOD-POD methods [18].

Other binding sites of Alb have been hypothesized that may affect BBC/(8.8 × Alb) ratios, and therefore not accounted for by Hmf. For example, deficient bilirubin binding in the fetus would allow the transfer of bilirubin across the placental barrier. Is a portion of what is assayed as Alb, a different (fetal) form that does not bind bilirubin well or at all, and diminishes with maturation? Or is there an extremely strong competitor for primary binding, or an allosteric effector that distorts the primary site, that is present at early GAs and decreases with GA. While there are negative findings for a non-binding fetal form of Alb [35], there is evidence for a strong competitor or allosteric effector [36].

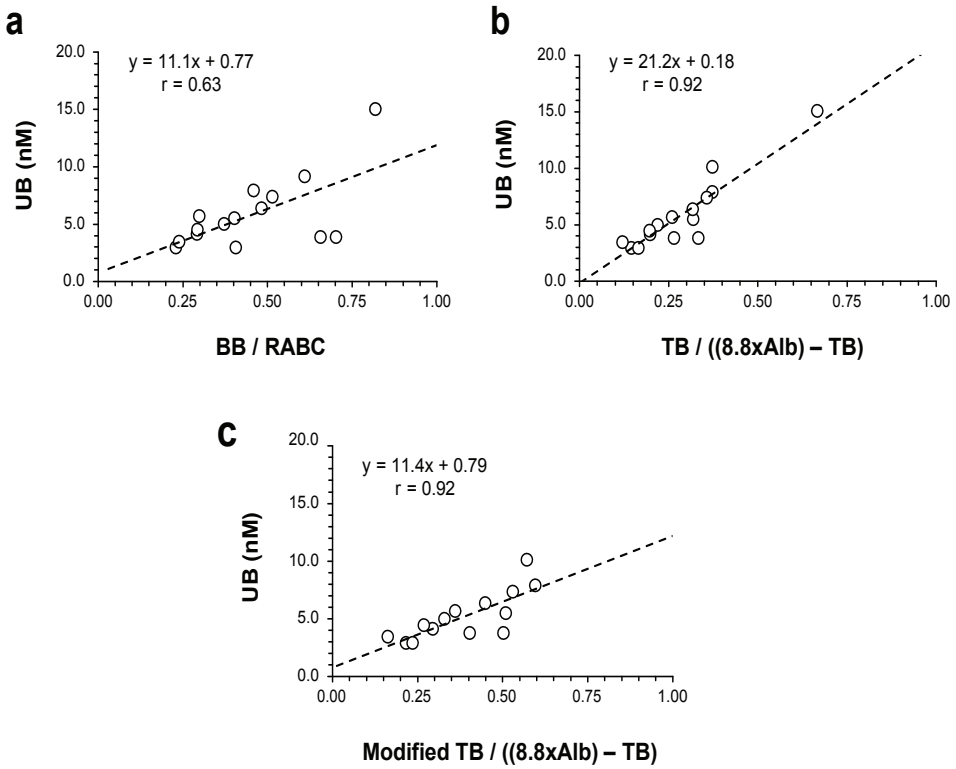

Figure 4: Linear correlation between: A. UB and BB/RABC for infants (slope = 11.6 nM); B. UB and TB/((8.8xAlb) – TB) (slope = 21.0 nM); and C. UB and modified TB/((8.8xAlb) – TB) (slope = 11.4 nM) for infants at cGA between 22 to 27 wks.

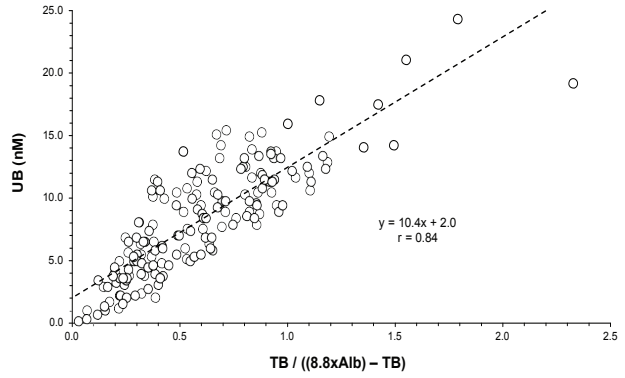

Figure 5: Linear correlation between the apparent UB (UB Analyzer-2) and TB/((8.8xAlb) – TB) (r = 0.40).

Because the UB level is more than 100-fold less than TB, direct methods to measure UB has been elusive. However, several indirect methods have been developed [17,22,23]. The automated POD method that has been used in many studies [2,14,16,22,25-27] and Hmf. An acceptable correlation of results for the two methods has been reported decades ago [23].

The precision analysis showed the CVs of UB and BB/RABC assays to be 5.5% and 10.6%, respectively, with an r-value of 0.83 and comparable (r=0.87) to a previous study [23].

It is documented that the binding affinity of Alb for bilirubin varies with the Alb concentration, especially at low concentrations [37]. The UB Analyzer-2 method measures serum diluted 52-fold so that Alb levels are also 52 times lower than physiological levels and in the range of large variation of binding affinity [37]. Hmf uses undiluted blood samples and, over the range of Alb found in neonatal sera, there is insignificant change in affinity. The variation in the affinity constant with the Alb in diluted sera used in the UB Analyzer-2 method may be one cause of difference between the methods.

BBC measurements can be confounded if the added bilirubin displaces a significant competitor for primary binding. Such known competitors include free fatty acids and some drugs. None of the infants in the study received lipids or known drug competitors. Samples with abnormal levels of unconjugated bilirubin were excluded from the present study, precluding the inaccuracies of both methods for samples with significant levels of direct bilirubin.

The single strong site model for bilirubin-Alb binding is supported by much biochemical and biophysical evidence [18-20,22]. There are certainly secondary sites on Alb for binding bilirubin, but these have affinities in the order of 100 times smaller. Thus, the relationship of UB to the ratio (BB)/(strong binding capacity) can be well estimated by the relationships shown in the appendix, which explains how the values of Ka obtained from the linear correlations of UB with BB/RABC can be compared (see below) with those obtained from correlations of UB with TB/((8.8 × Alb) – TB).

This study demonstrated that there is a correlation between apparent UB (nM) and BB/RABC. However, the slope of 13.7 nM was different from the slope of 10.5 nM, which was previously observed in the comparison of the early prototype Hmf system [23] and apparent UB determined using the original POD method [23,24], but the difference of 18% was not unreasonable. In those previous studies, apparent UB was measured by the original non- automated POD, and not by the automated GOD-POD method, which uses H2O2 and GOD [31]. Furthermore, the serum dilution factor (52x), substrates, and POD concentrations and protocols were also different from the original POD method [23,24]. To better define the slope (that is, the Ka value that relates UB and BB/RABC), a larger dataset and careful co-calibration of the two methods are required. Presently, it appears that a provisional common scale for UB (nM) and BB/RABC is approximated by multiplying BB/RABC by about 13.

When we calculated the linear correlation of UB with TB/((8.8 × Alb) – TB) in our cohort, we found that it was similar to the correlation between UB and BB/RABC. However, the regression line had a significant intercept (2 nM), and thus indicated a bias of UB over TB/(8.8 × Alb – TB). This bias might be due to the divergence between BBC and (8.8 × Alb) at earlier GAs. When the data for TB/((8.8 × Alb) – TB) was stratified by GA, it was observed that the slopes of the regressions of UB *vs* TB/((8.8 × Alb) – TB) were distinctly different for the earlier (22 to 27 wks, slope=21 nM) and latest (38 to 43 wks, slope=11.2 nM) GA subgroups. That is, assuming that (8.8 × Alb) is a valid proxy for RABC, the mean binding affinity of the early GA group would have to be half that of the late GA group. This result is significantly different from that observed for the early and late GA groups for UB *versus* BB/RABC for which the slopes were comparable (11.7 ± 0.5 nM). The observed divergence of BBC and (8.8 × Alb) suggests that a "correction" of the (8.8 × Alb) values could resolve this difference. When the "correction" was made for each GA week using the values of the regression line of figure 2, the slopes of the resulting regressions of UB *vs* (8.8 × Alb - TB) for the early and late GA subgroups were found to be in good agreement (insignificant intercepts, slopes of 11.4 and 11.8, respectively).

Conclusion

It is believed that effective bilirubin binding can vary from infant to infant even in the absence of binding site competitors. Although the affinity of the strong binding site is comparable from infant to infant, the fraction of Alb that is able to bind bilirubin varies from infant to infant and that fraction becomes, on average, significantly larger as GA decreases. Our direct observation of an increasing divergence of BBC from (8.8 × Alb) at early GAs coupled with the solid evidence that Hmf specifically assays the capacity of the strong site, leads us to favor the latter view.

This observation is consistent with what is seen in clinical practice, where there is difficulty in assessing risk for developing BIND in infants at early GAs. Using the Alb level alone as a

proxy for BBC, has been used as a rough estimation, but with limitations [10-13]. Apparently, early GA infants suffer two deficiencies that can reduce BBC: (1) they generally possess low Alb levels; and (2) a significant portion of their Alb may not be able to strongly bind bilirubin.

For term and near-term infants where (8.8 × Alb) and BBC appear to be comparable, the use of the TB/(8.8 × Alb) ratio can be used to assess risk. At early GAs where the divergence of (8.8 × Alb) and BBC can be very large, the TB/(8.8 × Alb) ratio is not an accurate measure of risk. The recent consensus [11] action levels for infants <35 wks GA are consistent with our findings and offer a significant improvement in risk evaluation for early GA infants. However, in the age of precision medicine, the variation in individual BBC levels observed at any GA strongly suggests the utility of individual assays of UB and/or BB/RABC.

Future clinical studies are needed to establish guidelines for the management of neonatal hyperbilirubinemia based upon an infant's bilirubin binding status and not on their TB level alone.

Conflict of Interest

All authors state that they have no conflicts of interest.

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Appendix

The relationship of UB to the ratio (BB)/(strong binding capacity)

$$UB = (1/K_a)(BB/\text{strong binding capacity}), \quad \text{(Eq 1)}$$

where K_a is the strong site affinity constant.

If UB and BB/RABC, as assayed in this study, are accurate, then:

$$UB = (1/K_a)(BB/RABC) \quad \text{(Eq 2)}$$

and if 8.8xAlb is an accurate proxy for BBC, then:

$$UB = (1/K_a)(BB/RABC) = (1K_a)BB/(8.8 \times Alb - BB), \quad \text{(Eq 3)}$$

or, since on the average BB ~ TB, then:

$$UB = (1/K_a)[TB/(8.8 \times Alb - TB)]. \quad \text{(Eq 4)}$$

Thus, values of K_a can be obtained from the linear correlations of UB with BB/RABC and compared with those obtained from correlations of UB with TB/(8.8 × Alb - TB).