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Effects of Sample Dilution, Peroxidase Concentration, and Chloride Ion on the Measurement of Unbound Bilirubin in Premature Newborns

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Abstract

Objectives—To assess the effects of sample dilution, peroxidase concentration, and chloride ion (Cl⁻) on plasma unbound bilirubin (B_f) measurements made using a commercial peroxidase methodology (UB Analyzer) in a study population of ill, premature newborns.

Design and Methods—B_f was measured with a UB Analyzer in 74 samples at the standard 42-fold sample dilution and compared with B_f measured at a 2-fold sample dilution using a FloPro Analyzer. B_f was measured at two peroxidase concentrations to determine whether the peroxidase steady state B_f (B_{fss}) measurements were significantly less than the equilibrium B_f (B_{feq}), in which case it was necessary to calculate B_{feq} from the two B_{fss} measurements. B_f was also measured before and after adding 100 mmol/L Cl⁻ to the UB Analyzer assay buffer.

Results—B_{feq} at the 42-fold dilution was nearly 10-fold less than but correlated significantly with B_{feq} at the 2-fold dilution (mean 8.2±5.2 nmol/L versus 73.5 ±70 nmol/L, respectively, p<0.0001;

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correlation $r=0.6$). The two UB Analyzer B_{fss} measurements were significantly less than B_{feq} in 42 of 74 (57%) samples, and Cl^- increased B_{feq} in 66 of 74 (89%) samples by a mean of $82\pm 67\%$.

Conclusions— B_{fss} measured by the UB Analyzer at the standard 42-fold sample dilution using assay buffer without Cl^- and a single peroxidase concentration is significantly less than the B_{feq} in undiluted plasma. Accurate B_f measurements can be made only in minimally diluted serum or plasma.

Keywords

newborn jaundice; hyperbilirubinemia; unbound bilirubin; peroxidase test; bilirubin/albumin binding; free bilirubin

Introduction

Newborn jaundice (unconjugated hyperbilirubinemia) is a common, transient, and usually benign neonatal condition that on rare occasions is associated with acute neurological injury (bilirubin encephalopathy) resulting in chronic neurological impairment (kernicterus) or death [1]. Subtle neurological damage manifesting later in life may also occur [2]. Although kernicterus is usually preventable, its continued occurrence underscores the shortcomings of current treatment guidelines and warrants continued efforts to improve the laboratory identification of those jaundiced newborns most likely to develop bilirubin toxicity [3].

The plasma or serum total bilirubin concentration (TBC) is the conventional laboratory criterion used for evaluating and treating jaundiced newborns [4-7]. Evidence in both animals [8] and humans [9-11], however, suggests that the minute fraction of TBC not bound to albumin, referred to as the unbound or “free” bilirubin concentration (B_f), correlates more strongly with bilirubin toxicity than the TBC. It has been proposed that these results justify further investigations into the clinical use of B_f measurements [12].

We are participating in a prospective phototherapy study involving sick, jaundiced premature newborns and sponsored by the National Institute of Child Health and Human Development Neonatal Research Network (NRN) that for the reasons stated above includes measuring both TBC and B_f . We selected the UB Analyzer for our B_f measurements as it is the only commercially available instrument with reagents for measuring B_f in addition to TBC that is approved by the Federal Drug Administration. The UB Analyzer uses the peroxidase method [13,14] and measures B_f at a 42-fold sample dilution, a single peroxidase concentration, and without chloride ion (Cl^-) in the assay buffer [14]. Several studies using artificial serum and/or pooled plasma samples indicate that B_f may be affected by sample dilution [15-18], Cl^- concentration [18-21], and when measured by the peroxidase test, the peroxidase concentration used [17,22,23].

For these reasons we felt it important to document and characterize the potential confounding effects of sample dilution, peroxidase concentration, and Cl^- on the UB Analyzer B_f measurements in our study population. Our findings in a subset of study plasma samples are presented in this report.

Methods

TBC and B_f levels were measured in plasma samples from extremely low birth weight newborns (birth weight ≤ 1000 g) enrolled for the NRN study from 8 study centers between September 2002 and April 2005. The study was approved by each local Institutional Review Board and parental informed consent was obtained. On day of life 5 ± 2 days blood (0.5 mL) was collected in lithium-heparin gel tubes (CapiJect, Terumo Medical Corporation, Somerset, NJ). The samples were immediately centrifuged for at least 1 min at $13,000\times g$ to allow the gel to separate

the plasma from the red blood cells and avoid possible contamination of the plasma with haemoglobin during freezing and thawing of the samples. They were then stored at -20°C until shipped on dry ice to Stanford University, Stanford, CA where they were stored at -20°C until analyzed. Due to sample volume and time constraints we arbitrarily elected to study the effects of Cl^{-} in the first 100 of the 700 samples eventually analyzed. All the data reported in this study are specific to these 100 NRN samples.

Since the NRN study did not include testing to determine the possible impact of sample handling on B_f , NRN TBC and B_f results were compared with TBC and B_f measured immediately after phlebotomy and centrifugation in a similar cohort of newborns at California Pacific Medical Center, San Francisco, CA (CPMC). TBC and B_f were measured routinely at CPMC between 1998 and 2002. Approval for extracting TBC, B_f , and demographic data without patient identifiers from CPMC medical records was approved by the CPMC Institutional Review Board.

TBC and B_f were measured by the peroxidase method at a 42-fold sample dilution (NRN and CPMC samples) using a UB Analyzer (Arrows Co., Ltd., Osaka, Japan) [14] and at a 2-fold sample dilution (NRN samples only) using a FloPro Analyzer (Global FIA, Fox Island, WA) [22]. Limited sample volumes and instrument configuration issues made it impractical to use the UB Analyzer to measure B_f at both sample dilutions. The UB Analyzer and FloPro Analyzer differ primarily in reagent handling and light path lengths, and comparison TBC values are not statistically different between the instruments [22]. Since both instruments obtain B_f from integrated rate equations that require measuring the change in TBC (change in absorbance at 460 nm corrected for haemoglobin) over time [14,22], it seems unlikely that any differences in B_f with sample dilution would be solely due to intrinsic differences in the instruments. In addition, a decrease in B_f with sample dilution was originally documented using a single instrument [16], and the phenomenon, which is not unique to B_f [24], has been confirmed by ultrafiltration studies [18].

Freeze-dried horseradish peroxidase/glucose oxidase enzyme provided for the UB Analyzer by the manufacturer was dissolved in 3.0 mL of the diluent provided and used for both instruments. The manufacturer does not provide the peroxidase concentration or activity so we measured the product of the rate constant for the peroxidase catalyzed oxidation of bilirubin by peroxide and (unknown) peroxidase concentration (k_p) at 37°C and a 24,562-fold dilution of the stock peroxidase as described previously [13,17,22]. The k_p was $1.02 \pm 0.12 \text{ min}^{-1}$ ($n=16$) or $25,053 \pm 2,940 \text{ min}^{-1}$ for the undiluted stock peroxidase, which is 0.025 min^{-1} per g/L of peroxidase assuming the commercial peroxidase concentration is 1 g/L as described originally for the peroxidase method [13]. Defining a unit of peroxidase activity as oxidizing 1 mol of bilirubin per min at 37°C (peroxidase activity is usually given as purpurogallin units), the bilirubin oxidation velocity of 3 mol/L per min we observed at the 24,562-fold stock peroxidase dilution indicates the commercial stock peroxidase contains 73,686 peroxidase units/L.

Effect of Sample Dilution on B_f

The effect of sample dilution on the measured B_f was studied by comparing the B_f measurements at the 42-fold dilution (UB Analyzer) with those at the 2-fold dilution (FloPro Analyzer).

Effect of Peroxidase Concentration on B_f

The equilibrium B_f ($\text{B}_{f\text{eq}}$) falls to a steady state B_f ($\text{B}_{f\text{ss}}$) during the peroxidase test as shown in the equilibrium and steady state schematics below where K is the bilirubin/albumin equilibrium association constant. In the steady state, when the bilirubin oxidation rate (rate 2

below) is not insignificant compared with the rate at which bilirubin dissociates from albumin (rate 1 below), B_{fss} is significantly less than B_{feq} [13,17,22,23].

$$\text{Equilibrium : albumin-bound bilirubin} \xrightleftharpoons{K} B_{feq} + \text{free albumin}$$

$$\text{Steady State : albumin-bound bilirubin} \xrightarrow{\text{rate 1}} B_{fss} \xleftarrow{\text{rate 2}} B_{feq} \rightarrow \text{oxidized bilirubin}$$

This potential error is readily detected by measuring B_f at more than one peroxidase concentration [17,22,23]. If rate 2 is insignificant compared with rate 1, B_{fss} will not change significantly with peroxidase concentration and, since the precision of the B_{fss} measurement does not vary with peroxidase concentration [22], each B_{fss} measurement is considered a replicate and B_{feq} is taken as the mean of the two B_{fss} measurements. Otherwise B_{fss} at a peroxidase concentration lower than the original will be significantly greater than the original or, conversely, B_{fss} at a peroxidase concentration higher than the original will be significantly less than the original. In these circumstances, B_{feq} must be calculated from the individual B_{fss} measurements [17,23] as shown below.

For the UB Analyzer, B_{fss} was measured at two peroxidase concentrations by first measuring it according to the manufacturer's instructions ($B_{f-100\%}$) and then repeating the measurement at half the manufacturer's recommended peroxidase concentration ($B_{f-50\%}$). TBC for both B_f measurements was calculated by the instrument from the light absorbance at 460 nm (A_{460}) corrected for haemoglobin interference following addition of 25 L of plasma to 1000 L of the 0.1 M sodium/potassium phosphate assay buffer provided and displayed in mg/dL (1 mg/dL = 17.1 mol/L) [14]. Then 25 L of stock peroxidase (1.85 peroxidase units) for the $B_{f-100\%}$ determination or 25 L of the stock peroxidase diluted 1+1 with 0.1 M sodium phosphate buffer, pH 7.4 (0.93 peroxidase units) for the $B_{f-50\%}$ determination are added and the respective B_f calculated from the decrease in A_{460} corrected for haemoglobin interference over time [14] and displayed in g/dL (1 g/dL = 17.1 nmol/L). The displayed $B_{f-50\%}$ is doubled since the instrument calculates B_f assuming undiluted stock peroxidase was used. When $B_{f-50\%}$ was < 1.05 · $B_{f-100\%}$ (the error of the method is 5%), B_{feq} was taken as the mean of the two measurements. Otherwise B_{feq} was calculated using the following equation [23]:

$$B_{feq} = \frac{0.5 \cdot B_{f-50\%}}{1 - \left(\frac{0.5 \cdot B_{f-50\%}}{B_{f-100\%}} \right)}$$

A similar strategy was used when measuring B_f at the 2-fold sample dilution using the FloPro Analyzer. For these measurements, 8 L of plasma was combined with 8 L of 0.2 mol/L sodium/potassium phosphate buffer, pH 7.4, containing 2000 mg/L (11.5 mmol/L) of glucose, 300 mol/L of hydrogen peroxide, 100 mmol/L of Cl^- (5% as the potassium salt and 95% as the sodium salt), and 0, 0.02, or 0.04 peroxidase units to give a final reaction volume of 16 L [22]. Cl^- was included in the buffer because plasma Cl^- ranges from about 90 to 110 mmol/L, making it impossible to measure B_f at a 2-fold plasma dilution without significant amounts of Cl^- present. The 100 mmol/L buffer Cl^- concentration was selected as the Cl^- effect on B_f appears saturated at Cl^- concentrations above 75 mmol/L [25], and diluting the plasma 2-fold with buffer containing 100 mmol/L of Cl^- maintains assay Cl^- levels near those in the undiluted plasma and well above 75 mmol/L. The TBC was calculated from the A_{460} corrected for haemoglobin interference [22] in the run with no peroxidase units in the buffer, and the $B_{f-100\%}$ and $B_{f-50\%}$ were calculated from the change in A_{460} over time corrected for haemoglobin interference with 0.04 and 0.02 peroxidase units in the buffer, respectively. B_{feq} was calculated from $B_{f-100\%}$ and $B_{f-50\%}$ as described above for the UB Analyzer, except

that a 10% rather than 5% error was assumed for the FloPro Analyzer measurements [22]. The entire analysis at the 2-fold sample dilution utilized 24 L of plasma.

Effect of Cl^- on B_f

The effect of Cl^- on B_f was determined with the UB Analyzer by measuring B_f before and after the addition of 100 mmol/L of Cl^- to the assay buffer supplied by the manufacturer. Without Cl^- in the assay buffer, at the 42-fold sample dilution used by the UB Analyzer the Cl^- would be about 2-3 mmol/L during the B_f measurement.

Effect of Sample Handling on B_f

The effect of freezing, thawing, and storage on B_f was assessed by comparing the mean binding constant (K) for the NRN samples with that of the CPMC samples. K (L/mol) was calculated for samples in which TBC, B_f , and albumin were measured using:

$$K = \frac{TBC - B_f}{B_f(\text{albumin} - TBC + B_f)}$$

For calculating K , TBC (mg/dL), B_f (g/dL), and albumin (g/dL) were converted to mol/L by dividing by 0.0585, 58.5, and 0.006612, respectively.

Albumin was measured on the NRN study samples by the manual bromcresol purple method [26]. Albumin was measured by the clinical laboratory at CPMC using an automated bromcresol green method.

Data are presented as mean $\pm$ SD, (minimum to maximum). ANOVA and t-tests were used to compare continuous data, and standard regression methods were used to compare linearly-related data.

Results

Twenty six samples of the first 100 NRN samples were excluded because of excessive haemolysis due to improper centrifugation or insufficient volume, leaving 74 samples for study. These 74 samples had minimal haemolysis as indicated by the absorbance at 578 nm [14], and the results from these samples were compared with those of 76 samples from 62 newborns obtained from the CPMC cohort at mean of 5 days of age. In one NRN patient, albumin was not measured due to insufficient sample volume. Patients from CPMC and the NRN study had mean birth weights of 799 ± 148 g (406-1000) and 775 ± 141 g (501-1000), respectively. The mean gestational ages were 26 ± 2 wks (23-31) and 26 ± 2 wks (23-33), respectively.

TBC (124.7 ± 37.6 mol/L, 58.1-129.3) as measured by the UB Analyzer was not significantly different from that measured using the FloPro Analyzer (124.7 ± 37.6 mol/L, 56.4-166.7). However, the mean TBC (111.1 ± 42.7 mol/L, 47.8-259.8) for the CPMC samples (UB Analyzer) was statistically lower (ANOVA) than the TBC from the NRN samples. The mean albumin concentration in the NRN samples was significantly greater (t-test) than that in the CPMC samples (469 ± 151 mol/L, 77-847 versus 424 ± 76 mol/L, 77-711, respectively, 1 mol/L = 0.066 g/L). However, the TBC/albumin molar ratios were not significantly different between the groups, with a mean of 0.29 ± 0.15 , (0.10-1.26) for the NRN samples and 0.26 ± 0.10 , (0.11-0.59) for the CPMC samples.

Effect of Sample Dilution Concentration on B_f

Sample dilution had a dramatic effect on the B_f . As seen in Table 1 the mean $B_{f_{eq}}$ at the 2-fold sample dilution used by the FloPro Analyzer was about an order of magnitude greater than that measured at the 42-fold dilution used by the UB Analyzer. Interestingly, however, there is a

statistically significant linear correlation between the B_{feq} values at the two dilutions (Figure 1). Figure 2 shows B_{f} as a function of TBC for the UB Analyzer, and the scatter indicates the considerable variability in bilirubin-albumin binding (i.e. K) in the population, offering one possible explanation for the poor correlation between bilirubin toxicity and the TBC alone [12]. The FloPro Analyzer data (not shown) showed similar variability between TBC and B_{f} with $r=0.25$.

Effect of Peroxidase Concentration on B_{f} measurement

For the UB Analyzer, $B_{\text{f-50\%}}$ exceeded $B_{\text{f-100\%}}$ by more than 5% (see Methods) in 42/74 (57%) of the NRN samples and 37/76 (49%) of the CPMC samples. The $B_{\text{f-100\%}}$, $B_{\text{f-50\%}}$, and B_{feq} measurements for the NRN samples are summarized in Table 1. The $B_{\text{f-50\%}}$ averaged $20 \pm 14\%$ (6-67) higher than $B_{\text{f-100\%}}$ in the 42 NRN samples and $17 \pm 9\%$ (6-50) higher in the CPMC samples. As shown in Figure 3, the difference between B_{feq} and $B_{\text{f-100\%}}$ for the NRN samples increases significantly as $B_{\text{f-100\%}}$ increases, as indicated by the significantly positive linear regression equation.

For the FloPro Analyzer, $B_{\text{f-50\%}}$ was greater than 110% of $B_{\text{f-100\%}}$ in 43/74 (58%) of the samples (Table 1), but the difference between B_{feq} and $B_{\text{f-100\%}}$ did not increase significantly as $B_{\text{f-100\%}}$ increased (Figure 4).

Effect of Cl^- on B_{f} (UB Analyzer)

The $B_{\text{f-feq}}$ determined with Cl^- added to the UB Analyzer reaction buffer was 13 ± 7.5 nmol/L (1.9-39) and significantly greater than the mean of 8.2 ± 5.2 nmol/L (1.0-26) without the addition of Cl^- ($p < 0.0001$, mean difference 0.0051 mol/L). The discrepancy between B_{feq} and $B_{\text{f-100\%}}$ also significantly increased as $B_{\text{f-100\%}}$ increased with Cl^- present (data not shown). While the B_{feq} with and without Cl^- present were significantly different, they were highly correlated (Figure 5).

Effect of Sample Processing on B_{f}

The K determined for the NRN samples on the UB Analyzer (spun, frozen, shipped, and thawed before analysis) was 70 ± 67 L/mol (11-506) and not significantly different from that of 69 ± 43 L/mol (12-217) determined on the samples from CPMC that were analyzed immediately following phlebotomy and centrifugation. The variances were statistically different due to the one NRN sample with $K = 506$ L/mol. If that sample is eliminated, the variances are not statistically different with the NRN mean K 63 ± 42 L/mol (11-210). The mean B_{feq} for the CPMC samples was 7.9 ± 7.0 nmol/L (0.9-42) as compared with 8.2 ± 5.6 nmol/L (0.7-26) for the NRN samples ($p=0.56$, NS).

Results and Current Exchange Transfusion Criteria

In populations of newborns similar to those in this study, a $\text{TBC} \geq 171$ mol/L (10 mg/dL), a $\text{TBC/albumin molar ratio} \geq .45$ (≥ 4.0 mg/g), or a $B_{\text{f-100\%}}$ (UB Analyzer) ≥ 17 nmol/L (≥ 1.0 g/dL) have all been recommended as criteria for exchange transfusion [4,9,11]. In the 74 NRN samples analyzed with the UB Analyzer, a $\text{TBC} \geq 10$ mg/dL occurred in 9 samples, a $\text{TBC/albumin ratio} \geq 4$ mg/g in 4 samples, and a $B_{\text{f-100\%}} \geq 1.0$ in 1 sample (the TBC in this sample was 11.2 mg/dL and the TBC/albumin ratio was 3.9 mg/g). Table 2 shows the other exchange transfusion variables in the 9 samples in which the TBC was ≥ 10 mg/dL. There was considerable variation in binding in these samples as indicated by the high coefficient of variation for K (Table 2, Figure 2).

Discussion

While it has been suspected for many years that measurement of bilirubin/albumin binding would be a useful adjunct in the management of newborn jaundice, robust methods for doing so remain elusive. The peroxidase method seems ideally suited for this task, but as seen in this study, peroxidase measurements of B_f are highly dependent on reaction conditions. We confirmed in plasma from ill premature newborns the earlier observations made in artificial serum and pooled plasma that B_f is altered by sample dilution, Cl^- , and the concentration (activity) of peroxidase used.

The variation in B produced by dilution and Cl^- appears due to intrinsic changes in bilirubin/albumin binding (i.e. K), but the variation in B_{fss} due to excess peroxidase activity is attributable to the method itself and can be corrected. Since this methodological error for the UB Analyzer worsens as $B_{f-100\%}$ increases, B_f should always be measured at two or more peroxidase concentrations when using this instrument.

Increase in K for bilirubin/albumin binding with sample dilution has been demonstrated by other binding methods [15,18], and this phenomenon has also been reported with other ligands thought to bind at the bilirubin site [24]. K is the ratio of the rate at which B_f and free albumin combine to form the bilirubin-albumin complex to the rate at which bilirubin dissociates from its complex with albumin (rate 1 in steady state scheme in Methods) [23]. We calculated rate 1 in samples where both $B_{f-100\%}$ and $B_{f-50\%}$ significantly underestimated B_{feq} [23] and found it to be much faster at the 42-fold dilution (mean 5.5/min at the 42-fold dilution versus 1.4/min at the 2-fold dilution). Since K is about 10-fold higher at the 42-fold dilution, this implies that dilution must increase the rate at which B_f and free albumin combine to form the bilirubin-albumin complex about 50-fold.

The lower B_{feq} at the 42-fold sample dilution is due in part to dilution of plasma Cl^- . Sample dilution may also attenuate the effects of drugs or endogenous compounds that weakly interfere with bilirubin/albumin binding [17,22,27]. Since many drugs used in neonatal practice have this potential [28], this may cause an even weaker correlation between B_f measurements by the UB Analyzer and the actual B_{feq} in the undiluted plasma [27].

The levels of B_f we found in minimally diluted plasma often exceeded the 4 g/dL bilirubin solubility limit proposed as an upper limit of B_f when measured by the UB Analyzer [1]. Nonetheless, B_f levels well above 4 g/dL would be expected at the K values of about $5-9 \times 10^6$ L/mol as determined by the peroxidase test and other methods [15,18] when physiologic levels of albumin are used. Calculations of central nervous system B_f in Gunn rat pups suggest that it ranges between 70 and 700 nmol/L (about 4 to 40 g/dL) [29]. How albumin concentration or other *in vivo* plasma and tissue factors may affect bilirubin solubility, which is extremely limited in non-albumin containing aqueous solutions at pH 7.4 [30], remains to be determined.

B_f measurements made after freezing, storing, and thawing samples did not seem significantly different from those made in a cohort of freshly obtained samples as assessed by comparing K and B_f between the two groups. It was also encouraging to find that B_f measurements in samples from similar patient populations analyzed with the same methodology and instrumentation at different institutions over different time periods were quite similar. We did find that several NRN samples had excessive haemolysis due to inadequate centrifugation prior to freezing, but when spun properly, the gel in the sample collection tubes prevented any haemoglobin produced by freezing and thawing the red blood cells from contaminating the plasma. It would be useful to investigate further how best to prepare and store samples when immediate B_f analysis cannot be performed.

In summary, sample dilution significantly decreases B_f by intrinsically altering bilirubin/albumin binding, diluting Cl^- , and possibly attenuating the effects of any weakly binding drugs or exogenous compounds present that interfere with bilirubin/albumin binding [16,22,27]. Even though our B_f measurements in plasma diluted 42-fold correlated with B_f at the 2-fold dilution ($r=0.6$), accurate measurement of plasma bilirubin/albumin binding requires minimal sample dilution.

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ABBREVIATIONS

A_{460} , light absorbance at 460 nm; B_f , free (unbound) bilirubin concentration; B_{feq} , equilibrium free bilirubin concentration; B_{fss} , steady state free bilirubin concentration; $B_{f-50\%}$, free bilirubin determined at 50% of the $B_{f-100\%}$ peroxidase concentration; $B_{f-100\%}$, free bilirubin measured at the higher of two (100%) peroxidase concentrations; Cl^- , chloride ion; CPMC, California Pacific Medical Center; K , bilirubin/albumin equilibrium association binding constant; k_p , product of the rate constant for bilirubin oxidation and peroxidase concentration; NRN, Neonatal Research Network; TBC, total bilirubin concentration.

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Figure 1.

B_{feq} measured by the FloPro Analyzer (2-fold sample dilution) versus B_{feq} measured by the UB Analyzer (42-fold sample dilution). The correlation is significant and the regression equation, regression coefficient, and level of significance are shown.

Figure 2.

B_{feq} measured by the UB Analyzer as a function of the TBC. The regression equation for B_{feq} versus TBC is shown and the variability in bilirubin-albumin binding in the population is reflected in the correlation coefficient of 0.41.

Figure 3.

The difference between $B_{f-100\%}$ and B_{feq} as a function of $B_{f-100\%}$ as measured with the UB Analyzer. The regression line, which increases significantly with increasing $B_{f-100\%}$, is shown along with the regression equation and correlation coefficient.

Figure 4.

The difference between $B_{f-100\%}$ and B_{feq} as a function of $B_{f-100\%}$ as measured with the Global FloPro Analyzer. The regression line does not increase significantly with increasing B_{f-100} .

Figure 5.

UB Analyzer measurements of B_{feq} with and without Cl^- in the reaction buffer. B_{feq} with Cl^- is plotted against B_{feq} without Cl^- . The regression equation, correlation coefficient, and level of significance are shown.

Table 1

B_{f-100%}, B_{f-50%}, and B_{feq} (nmol/L, mean±SD) measured in 74 NRN samples at 42- and 2-fold sample dilutions. The B_{feq} is obtained from the B_{f-100%} and B_{f-50%} as described in the Methods. The range is the minimum to maximum value.

Sample Dilution	METHOD			
	UB Analyzer 42-fold	B _{feq} **	0.04 (B _{f-100%}) 51±46 10-381	FloPro analyzer 2-fold
Peroxidase Units	1.85 (B _{f-100%})	B _{feq} **	0.04 (B _{f-100%})	B _{feq} **
B _{res}	6.3±3.6	8.2±5.3	51±46	74±70
Range	0.7-20.1	0.7-26.0	10-381	5.5-457

* B_{f-50%} > B_{f-100%} (p< 0.05, t-test)

** B_{feq} > B_{f-100%} and B_{f-50%} (p<0.001, ANOVA)

Binding variables (UB Analyzer) for 9 of 74 NRN samples with TBC ≥ 10 mg/dL (171 mol/L). Current exchange transfusion criteria recommended for this group of babies include TBC ≥ 10 mg/dL [4], $B_{f-100\%} \geq 1.0$ g/dL (17 nmol/L) [9,11], and TBC/albumin ratio ≥ 4 mg/g (0.45 TBC/albumin molar ratio) [4].

Table 2

TBC, mg/dL	B_{eq} , g/dL	$B_{f-100\%}$, g/dL	$B_{f-50\%}$, g/dL	Albumin, g/L	TBC/albumin, mg/g	K, L/ μ mol
14.0	0.18	0.18	0.18	45	3.1	113
13.0	1.52	0.62	0.88	28	4.6	24
12.9	1.14	0.57	0.76	56	2.3	15
12.3	0.71	0.49	0.58	54	2.3	23
11.2	1.17	1.18	1.16	29	3.9	25
10.5	1.10	0.63	0.80	42	2.5	17
10.3	0.55	0.46	0.50	44	2.3	30
10.2	0.53	0.52	0.54	23	4.4	58
10.0	0.77	0.52	0.62	23	4.3	40
Mean $\pm$ SD	11.6 $\pm$ 1.5	0.85 $\pm$ 0.41	0.57 $\pm$ 0.26	38 $\pm$ 13	3.3 $\pm$ 1.0	38 $\pm$ 31