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Newborn Jaundice Technologies: Unbound Bilirubin and Bilirubin Binding Capacity In Neonates

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Abstract

Neonatal jaundice (hyperbilirubinemia), extremely common in neonates, can be associated with neurotoxicity. A safe level of bilirubin has not been defined in either premature or term infants. Emerging evidence suggest that the level of unbound (or “free”) bilirubin has a better sensitivity and specificity than total serum bilirubin for bilirubin-induced neurotoxicity. Although recent studies suggest the usefulness of free bilirubin measurements in managing high-risk neonates including premature infants, there currently exists no widely available method to assay the serum free bilirubin concentration. To keep pace with the growing demand, in addition to reevaluation of old methods, several promising new methods are being developed for sensitive, accurate, and rapid measurement of free bilirubin and bilirubin binding capacity. These innovative methods need to be validated before adopting for clinical use. We provide an overview of some promising methods for free bilirubin and binding capacity measurements with the goal to enhance research in this area of active interest and apparent need.

Keywords

Free bilirubin; total binding capacity; reserve binding capacity; binding affinity

I. Bilirubin Induced Neurotoxicity

Unconjugated hyperbilirubinemia, or jaundice, is one of the most common problems for neonates, including premature infants. Jaundice may lead to neurotoxicity including but not limited to sensori-neural deafness, auditory neuropathy, athetoid cerebral palsy, supranuclear gaze palsy, neonatal seizures, and apnea. (1–3) However, despite extensive research, the total serum bilirubin (TSB) level, the traditional parameter to evaluate and manage jaundice, has not been useful as a sensitive and specific predictor of neurological outcomes. (1, 4–9) This is not surprising because several biochemical and physiological factors are involved in the pathogenesis of bilirubin-induced neurotoxicity.(10)

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Bilirubin, a product of hemoglobin degradation, exists in the blood mostly bound to albumin. Bilirubin-albumin binding is a function of the concentrations of bilirubin and albumin, and the binding affinity for bilirubin (strength of bilirubin binding to albumin). The fraction of unbound bilirubin (also referred to as nonalbumin-bound or free bilirubin, B_f) increases significantly as the TSB approaches the binding capacity of albumin (1 gram of competent adult albumin binds 8 mg of bilirubin) or when the apparent albumin affinity decreases. The binding affinity may be decreased in the presence of sepsis, acidosis, hypoxia, free fatty acids and various albumin binding drugs. (1) According to current theory, B_f is capable of crossing the intact blood brain barrier and causing subsequent neuronal damage.(11) According to “free bilirubin thinking”, the B_f concentration determines the distribution of bilirubin between the tissues and vascular space and therefore the risk of bilirubin-induced neurotoxicity.(12) There exists overwhelming clinical evidence to support this B_f theory. (1, 2, 13–19) The results of many human neonatal studies have suggested that the neurological outcome of hyperbilirubinemia correlate better with serum or plasma B_f than the TSB. (1, 2, 13–19) Although recent studies suggest usefulness of the B_f level in managing high-risk neonates including premature infants, there currently exists no widely available sensitive and accurate method for measurement of B_f . Because of its potential importance for improved routine clinical care, there has been renewed interest in developing methods for accurate and rapid measurement of B_f in serum or blood.

II. Properties of Bilirubin and Bilirubin Binding to Albumin

Most of the both the older and new methods for determination of B_f in serum, plasma, or blood are based on well known properties of bilirubin as described below.

Unconjugated bilirubin, a linear tetrapyrrole, is amphipathic, i.e., it is hydrophobic but has ionizable groups. Its bichromophoric tetrapyrrole structure, two dipyrroles separated by a saturated linkage, leads to its yellow color. The predominant native form of unconjugated bilirubin in its usual form (4Z,15Z-IX α isomer) has its two rigid dipyrrole units internally hydrogen bonded to each other such that no polar (or ionizable) groups are exposed making it quite insoluble in water.(20, 21) Because of low solubility in water, unconjugated bilirubin can form dimers and higher aggregates. (21) Solubility increases with increasing pH due to the ionization of the acidic groups on the molecule. (20, 21) It is easy to appreciate that hydrophobic and amphipathic native form of unconjugated bilirubin can readily cross biomembranes. The native bilirubin or unbound unconjugated bilirubin (4Z15Z-IX α isomer) is neurotoxic.(22, 23) It is not known if other isomers of bilirubin are neurotoxic.(24)

Unconjugated B_f is essentially non-fluorescent in most solvents. Its fluorescence in water, in the range of 500–550nm (green light), has a quantum yield of less than 10^{-4} . (25, 26) This is due to the dominance of rapid nonradiative de-excitation processes including isomeric rearrangements and conformational transitions in the excited state that lead to rapid decay (25, 27). In low temperatures these isomerizations are greatly slowed, leading to an increase in the fluorescence quantum yield. The fluorescence of unconjugated B_f increases by binding to its native binding site on albumin.(28–30). The albumin binding apparently slows down the competing isomerization pathways leading to fluorescence yields of greater than 10^{-3} , at least 100 times that of B_f .(25, 31)

There are at least two types of binding sites for bilirubin and its photoisomers on albumin. (26) The strongest and key site for native bilirubin has a very high binding constant (affinity), $\sim 1.4 \times 10^7$ l/mol at 37° C, and can be considered a specific site.(32, 33) Other, secondary sites have very much smaller binding constants. The specific binding site is protected by the protein, and neither modest pH nor salt concentration changes affect the

fluorescence yield. (26) It should be noted that the affinity of albumin for bilirubin is reduced with increasing temperature. (31, 34, 35)

There is abundant experimental evidence that because of both the large quantity of albumin and its very large affinity for bilirubin, the level of B_f in serum is controlled essentially by the albumin binding. (12, 33) There appears to be a simple picture that bilirubin binding to other sites on albumin or other serum sites such as red blood cells is relatively unimportant to the B_f level. That is, consideration has, to a very good approximation, only to be given to the following equilibrium:

Where 'R' represents the concentration of albumin with empty high-affinity sites or the reserve albumin binding capacity. While 'B' represents the concentration of albumin with bilirubin bound to high-affinity site. The total albumin binding capacity, 'C', is the total amount of albumin capable of strongly binding bilirubin ($R + B$). Using these symbols the mass action equation can be written as:

$$K = B/(B_f \times R), \text{ where } K \text{ is the binding constant for the high affinity site.}$$

$$\text{The } B_f \text{ is then } = (B/R)(1/K) \quad (\text{equation 1})$$

This equation predicts a linear relationship between B_f and B/R which is, in fact, observed. (33) Until the albumin binding is significantly saturated, the TSB and B should be about the same. As B becomes comparable and greater than R , there is significant bilirubin in the red cells. (36) For $B = R$, the red cell content of bilirubin can be 2 mg/dl. Alternatively using a similar integrated equation, specifically while using instrumentations such as UB analyzer or Zone fluidics that directly measures B_f , the apparent binding constant or affinity (K) can be calculated as:

$$\frac{TSB - B_f}{B_f ([\text{Albumin}] - TSB + B_f)} = K \quad (\text{equation 2})$$

Finally it needs be pointed out that because the photoisomers of bilirubin, which can be present at levels approaching 20% of the TSB during phototherapy, will lead to deviations in all the methods for measuring B_f and binding capacity because of differences in physical properties, chemical reactivities and binding affinities. (37, 38) While more investigations need to be done to draw well documented conclusions, it appears at this time that such deviations should not significantly reduce the clinical usefulness of many of the methods described below.

It is important to keep in mind that albumin-bilirubin binding is reversible and relatively fast. That is, there is a dynamic equilibrium with bilirubin going onto albumin and coming off continuously. (39) The lifetime of the albumin-bilirubin complex before dissociation is of the order of a second at normal serum pH and temperature. (39) As bilirubin leaves the blood compartment more bilirubin is dissociating from the albumin bound pool.

III A. Measurement of Total Binding Capacity, Reserve Binding Capacity and Calculated Free Bilirubin

Several methods have been developed to measure total and reserve bilirubin binding capacity and indirectly estimate B_f using the equation 1 (described before).(40–52) The Table 1 summarizes some of the binding assays that have been reported (48, 53) ; however, because of potential usefulness we provide in-depth description of hematofluorometry.(33, 54, 55)

The simplest fluorometric method that makes use of bilirubin's natural fluorescence is being redeveloped for a point- of- care (POC) system that uses less than 100 μ l of whole blood and precludes the need for separation of serum or dilution of the specimen. Furthermore no quantitative additions of reagents or exact quantities of specimen need be used making the method quite easy to use. The method and prototype instruments were evaluated in a number of clinical sites in its previous laboratory bound embodiment. (33, 54, 55)

The method makes use of the observation that essentially only the bilirubin bound (B) to albumin at the high affinity site produces a fluorescence signal that can be measured. The 'B' concentration is close to TSB since the B_f concentration is much smaller until albumin binding is saturated. The reserve binding capacity (R) can be measured by adding excess bilirubin to the blood, with the increase in fluorescence being due to the newly bound bilirubin. This method measures bilirubin binding capacity and presumably takes into account the competent albumin concentration and the presence of competitors for bilirubin binding sites in the blood. The hematofluorimetry results for binding capacity and a calculated value of B_f were shown to be in excellent agreement with other more complex methods including the peroxidase (1:42 fold dilution), the 2-(4'-hydroxybenzene)azobenzoic acid (HBABA) and the Sephadex methods (33, 54, 55) and may provide essential information to assess risk of bilirubin-induced neurotoxicity in high-risk neonates. In the original embodiment of the hematofluorometer a third drop of blood was used to obtain the Total Blood Bilirubin (TBB) through the addition of a small amount of the surfactant dodecyltrimethylamine oxide. This surfactant extracts bilirubin from all blood sites, including the red cells, and renders all the bilirubin fluorescent, albeit with a much lower yield than albumin bound bilirubin. In this way the bilirubin in secondary or weaker binding sites including the red cells can be assayed. A significant amount of this weakly or loosely bound (normally non-fluorescent) bilirubin indicates that the albumin binding is being significantly saturated. A linear correlation of the difference between the TBB and B (TBB-B), which represents this more loosely bound bilirubin, and the B_f was found in clinical samples with the loosely bound bilirubin being up to 8 mg/dl when the B_f is about 20 nM.

Based on this principle and using modern optoelectronic technology, a hemotofluorometer and cuvettes with automatic reagent addition are being redeveloped for POC evaluation of bilirubin binding capacity and calculated B_f . The hemoglobin content or hematocrit of the specimen must also be measured to reference the results to the serum volume. This methodology, if successfully developed as a POC system, promises to be faster, less expensive, and extremely useful for POC evaluation of at-risk infants, especially in the developing world settings. As for use with apparently healthy neonates its value may be as a standard pre-discharge evaluation of binding capacity so as to identify neonates who should be followed up early should they become jaundiced at home.

III B. Direct Methods for Free Bilirubin Determination

i) Enzyme Based Methods

i.a) Bilirubin oxidase method—Andreu *et al.* (56) developed a technique using a bilirubin oxidase/fluorescein conjugate to differentially assay B_f , albumin bound bilirubin and conjugated bilirubin in serum. The fluorescence of the modified enzyme, which has excitation and emission maxima at 487 and 520nm, exhibits changes with time depending on the “type” and concentration of bilirubin. This required individual calibrations for “type” of bilirubin. While this method is useful in obtaining a simultaneous estimate of the three “types” of bilirubin, it is not sufficiently accurate or sensitive to be used for B_f assays in clinical samples.

i.b) Modified Peroxidase Method Using UB Analyzer—Currently the most commonly used technique, presently considered the “gold standard”, for serum B_f measurement is the so-called “peroxidase method”, introduced by Jacobsen and Wennberg in 1974. (57) An appropriate concentration of the peroxidase enzyme is used to obtain values for both TSB and B_f levels from the kinetics. The concentration of B_f is determined from the rate of horse radish peroxidase-catalyzed oxidation of B_f (bound bilirubin does not react) by a peroxide, hydrogen peroxide or ethyl hydrogen peroxide.(57) The bilirubin oxidation rate is derived from the time course of the diminution of the bilirubin color. The method directly assays B_f and is not confounded by bilirubin bound to secondary or low-affinity binding sites as it may with methods described in Table 1.

A standardized dedicated spectrophotometer instrument for B_f measurement using the peroxidase method, the UB Analyzer (UB-A1, Arrows Co. LTD, Osaka, Japan) has been approved for use by the FDA.(58) A 42-fold dilution must be made to the serum sample (25 μ l), which can alter intrinsic bilirubin binding properties and mask the presence of binding competitors to albumin. To decrease underestimation of B_f secondary to sample dilution, a second set of B_f measurement is made using half the initial concentration of peroxidase (modified peroxidase method) and both automated readouts of steady state B_f are used to derive the final equilibrium B_f using an algorithm developed for the purpose.(59) The modified peroxidase method has been recently validated.(35) These developments over the years have led to an expanding utility of peroxidase method for clinical research.(2, 13–16, 18, 37, 60–63) Several studies have demonstrated that the B_f measured using the peroxidase method is more sensitive and specific predictor of neurotoxicity than TSB and/or bilirubin:albumin molar ratio. (2, 13–18, 60)

i.c) Peroxidase Method Using Zone Fluidics—The Zone Fluidics instrumentation (Global FIA mini-FloPro) also executes a horseradish peroxidase/glucose oxidase (HRP/GO) assay to measure the serum B_f . (64) However, the instrumentation uses a minimally diluted sample compared to the UB analyzer. The mini-FloPro analyzer performs a repeatable, automated measurement of B_f in a controlled system. Signals measured by the spectrophotometer within the analyzer are fed directly into the Zone Fluidics software, which then calculates the equilibrium B_f concentration based on measurements at three different HRP/GO concentrations.(64)

The instrument creates “zone stacks” for analyzing B_f concentration by aspirating first an air bubble, followed by 8 μ L of sample, 4 μ L of R3 (phosphate buffer with glucose and hydrogen peroxide), 4 μ L of R2+R1 (HRP/GO enzyme and phosphate buffer, respectively), and lastly another air bubble. These reaction stacks are mixed and then transported to the spectrometer for analysis. The small sample dilution (2-fold dilution) and automation of sampling, mixing, and measurement intrinsic to Zone Fluidics are all factors that potentially contribute to a more precise measurement of B_f .(59) However, compared to UB analyzer,

the peroxidase method using FloPro analyzer is cumbersome, very time consuming and often difficult because the enzyme dilution to be used depends on the expected B_f concentration of the sample. To date, no jaundice associated neurological outcome studies have been reported using this instrumentation.

The peroxidase method (UB analyzer and Zone fluidics) has some technical and practical limitations that have hindered its routine use and more or less confines it to an expert clinical laboratory. There are issues with standardization of the enzyme activity and the stability of the prepared enzyme reagent. Moreover, as with other binding assays (Table 1), there is a possibility of interference with B_f measurement by direct or conjugated bilirubin and photoisomers. (37, 65)

ii) Fluorometric Method

A fluorometric assay in developmental phase utilizes fluorescently labeled fatty acid binding protein mutants (BL22) as probes that have a high specificity and sensitivity for B_f . Acrylodan is the fluorophore that gives the probe its fluorescence. Bilirubin quenches acrylodan fluorescence by resonance energy transfer, the absorbance of bilirubin overlaps with the emission of acrylodan. The probe has an excitation wavelength of 375 nm, and the emission wavelength of interest is 550 nm. This carefully chosen protein probe specifically binds bilirubin and appears to be insensitive to free fatty acids and other metabolites normally found in serum.

A handheld fluorometer, FFAu Meter, with dedicated settings, has been developed for this assay by FFA Sciences (San Diego, CA). Each measurement requires 8 μ L of serum to which a quantitative addition of buffer and the probe is added manually. The fluorescence signal over time is collected by the instrument and automatically transferred into a spreadsheet for calculation of the B_f . A single measurement of the B_f takes between 1 and 2 minutes. Although this method is not automated, the fast turnaround rate and low sample volume requirement make it an attractive method for use with premature infants.

iii) Metal-Enhanced Fluorescence

Metal-Enhanced Fluorescence (also called Surface Enhanced Fluorescence) is a phenomenon by which metallic particles can interact with fluorophores to increase greatly (up to 10^6) the yield of fluorescence from a normally poorly fluorescent substance. (66). Normally, when an organic chromophore is on or close to the surface of any metal its fluorescence is precluded because of rapid transfer of the excitation energy in the chromophore to the metal. However, for some metals, for example silver, when the chromophore is in just the right distance range of the surface and oriented in a particular way there can be an immense enhancement of the fluorescence rate and a concomitant increase in the fluorescence yield because deactivation processes, such as photochemistry, cannot compete. (67)

This technology was recently applied to the development of a specific assay for B_f in neonatal serum in the presence of a high background of serum albumin bound bilirubin (> 99 %) using nanoparticles of silver. Based on preliminary findings, the silver particle based assay provides for a 10^3 fold increase in B_f sensitivity as compared to a non-silvered assay. The metal-enhanced fluorescence B_f assay was constructed as shown (Fig 1). Silver nanostructures are immobilized on either glass or plastic supports. A thin polymer layer is then coated and immobilized on both the silvered and non-silvered portions of the glass / plastic supports. The polymer film contains an optimized amount of human serum albumin to bind any freely diffusing B_f . The polymer is chosen such that bilirubin, can readily diffuse into the polymer film and bind with the human serum albumin, but once bound cannot

diffuse out from the film due to the cross-linking density of the polymer. The larger molecules such as bilirubin-bound to albumin cannot diffuse into the polymer film. In essence, the polymer film acts as a membrane through which only B_f diffuses and found not to be fouled by surface bound human serum albumin. The weak fluorescence of B_f is increased upon complexation with human serum albumin. Due to the close proximity of silver, it is enhanced even further. This technique under developmental phase is being further evaluated to account for the effect of temperature, pH of the sample, sample size, albumin concentration, timing, and other related factors on this bilirubin binding assay.

Conclusions

Unbound, unconjugated serum bilirubin has been an elusive target for facile quantitative assays. Its concentrations in newborn infants are much lower than that of total serum bilirubin. Yet, it is the level of unbound bilirubin that controls the rate at which bilirubin can escape the blood compartment and presents danger to the infant because of its neurotoxic effects. Although the modified peroxidase method has been most commonly used for clinical studies, and has been shown to correlate with outcomes specific to bilirubin induced neurotoxicity, it is somewhat cumbersome and is, no doubt, confined to the specialized clinical laboratory. The newer techniques, including improved hematofluorometry, briefly described here appear promising and may not only provide rapid, sensitive measurement of free bilirubin concentration, but also a practical approach for routine assessment in clinical laboratories, out-patient clinics and at point of care throughout the world. One or more of these promising techniques under development may in future become an alternative basis for the clinical assessment and management of hyperbilirubinemia in neonates.

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Abbreviations

TSB	total serum bilirubin
B_f	free bilirubin
POC	point- of- care
HBABA	2-(4'-hydroxybenzene) azobenzoic acid
TBB	total blood bilirubin
SI	saturation index
RBC	reserve binding capacity
TBC	total binding capacity

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Fig 1.
Metal-Enhanced Fluorescence

Table 1

Summary of Commonly Used Indirect Methods for Bilirubin-Binding Assays

Method	Mechanism	Measurement	Potential limitations
Sephadex column elution method (53)	Bilirubin loosely bound to albumin is absorbed to Sephadex columns while tightly bound bilirubin remain attached to albumin	RBC and TBC	Direct bilirubin binds readily to columns. Does not differentiate between loosely bound and B _f . The estimation does not correlate well with other methods. (53) Requires complex series of steps.
Fluorescence quenching method (46, 47)	Fluorescence quenching of albumin by bilirubin. Measures both albumin bound and non-albumin (red cell bound) bilirubin	TBC and Binding affinity	Cumbersome, complex calculation for binding parameter. Required dilution even greater than with peroxidase method which may alter binding properties and mask binding competitors.
Salicylate Saturation Index (36, 48, 51)	Changes in optical density at 460 nm caused by addition of salicylate (Saturation Index, SI) which displaces bilirubin from binding sites	Binding affinity. SI > 8% is abnormal	Measured in diluted sera. Variable results because of inherent excess risk in laboratory error. The maximum achievable SI is 14%.
Difference Spectroscopy (40, 50)	The assay quantitates the shift in the absorbance spectra of bilirubin at 482 nm when sample containing albumin is added to a solution containing excess bilirubin	RBC	Requires severe sample dilution. The assay has not been compared with other methods
2-(4'-hydroxybenzene)azo benzoic acid (HBABA Dye) binding method (44, 54)	Measures empty bilirubin binding sites on bilirubin-bound albumin compared to bilirubin-free albumin by the shift in the absorbance spectrum of the dye. The method assumes that the dye does not displace bilirubin bound to albumin.	RBC compared to standard	Non specific binding does not distinguish competition by other anions for albumin sites not specific for bilirubin. Does not account for decrease in binding affinity by competitors. Paradoxical effect of pH on bilirubin binding.
Fluorescent Dye Method using Direct yellow 7 (DY7) dye (42)	Dye binds to specific bilirubin binding sites on albumin and fluoresces when bound to albumin	TBC and RBC	The pH of the buffer used in the assay should be the same as the pH of the blood sample. Photooxidation of bilirubin with dye exposure may overestimate TBC and RBC.
Hematofluorometer (31, 33, 54, 55)	Bilirubin bound to high-affinity site (Bound) on albumin fluoresces at 520 nm. Further increase in fluorescence on addition of bilirubin measures RBC at high affinity site (Reserve). Finally, addition of a surfactant extracts bilirubin from all binding sites and measures total blood bilirubin	TBC and RBC, The SI (Bound/RBC) at high affinity binding site of X 10 is an indirect estimate of B _f (r =0.73)	May be inaccurate in the presence of photoisomers (with phototherapy), conjugated bilirubin, and competitors for high affinity albumin binding sites.

RBC indicates reserve binding capacity; TBC indicates total binding capacity,