REVIEW ARTICLE

Translation Investigation

Bilirubin binding in jaundiced newborns: from bench to bedside?

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Background: Bilirubin-induced neurologic dysfunction (BIND) is a spectrum of preventable neurological sequelae in jaundiced newborns. Current total plasma bilirubin (B_T) concentration thresholds for phototherapy and/or exchange transfusion poorly predict BIND. Methods: The unbound (free) bilirubin (B_f) measured at these B_T thresholds provides additional information about the risk for BIND. B_f can be readily adapted to clinical use by determining B_f population parameters at current B_T thresholds. These parameters can be established using a plasma bilirubin binding panel (BBP) consisting of B_T, B_f, and two empiric constants, the maximum B_T (B_{Tmax}) and the corresponding equilibrium association bilirubin constant (K). Results: B_{Tmax} and K provide the variables needed to accurately estimate B_f at $B_T < B_{Tmax}$ to obtain B_f at threshold B_T in patient samples. Once B_f population parameters are known, the BBP in a newborn can be used to identify poor bilirubin binding (higher B_f at the threshold B_T compared with the population) and increased risk of BIND. Conclusion: The BBP can also be used in jaundice screening to better identify the actual B_T at which intervention would be prudent. The BBP is used with current B_T thresholds to better identify the risk of BIND and whether and when to intervene.

Clinicians have known for decades that newborns with hyperbilirubinemia and excessive bilirubin production or poor plasma bilirubin binding have an increased risk of bilirubin-induced neurologic dysfunction (BIND, kernicterus).[1–5] Both conditions increase brain bilirubin exposure, excessive production by increasing the bilirubin load,[4, 6–8] and poor plasma bilirubin binding by increasing the plasma levels of unbound (free) bilirubin (B_f), which is the bilirubin species moving freely between the vascular and extravascular spaces.[1–3, 9–14]

The elevated B_f resulting from poor bilirubin binding forces a greater percentage of the accumulating bilirubin load (bilirubin produced – bilirubin excreted) into the extravascular space, exposing the brain to more bilirubin.[2, 5, 9, 10, 12, 14] The vascular bilirubin level and plasma total bilirubin concentration (B_T) will be correspondingly lower.[1, 3, 9, 10] If Andersen et al.[1] had measured B_f in their premature newborns given daily sulfisoxazole or tetracycline, they would have found it to be about twice as high in those receiving sulfisoxazole versus those receiving tetracycline at any B_T.[9] A greater load of bilirubin was therefore needed in the sulfisoxazole cohort to reach a given B_T e.g., >12 mg/dL,[9] and the increased extravascular bilirubin lead to BIND occurring at B_T well below that at which BIND would ordinarily occur in this population.[9, 15] Clearly, both poor bilirubin binding and the size of the bilirubin load were important factors in determining whether newborns receiving sulfisoxazole actually developed BIND.[3, 9, 14, 16]

The axiom (not hypothesis) emanating from these elderly data is: both B_T and B_f contribute important information about the risk of BIND in hyperbilirubinemic newborns. B_T reflects the size of the bilirubin load, and B_f provides information about the percentage of the bilirubin load to which the brain is exposed at that B_T. Bilirubin binding varies considerably in newborn and adult populations, making it impossible to gauge the B_f from the magnitude of B_T.[9, 10, 16–18]

Currently, the hour-specific B_T is used to assess the size of the bilirubin load,[19] and if coupled with B_f measurements, clinicians would have even more information about the brain bilirubin exposure and the risk of BIND.[16, 20] Recent studies suggest the risk of BIND in term newborns with $B_T \geq 30$ mg/dL is about 10%.[21–23] Since there is considerable uncertainty as to when an exchange transfusion should be initiated in these newborns,[21–25] measuring bilirubin binding would be more helpful in identifying those needing the procedure.

Remarkably, despite the accumulating studies documenting the importance of B_f in assessing the risk of BIND,[26–34] bilirubin binding has not found its way to the bedside except in Japan,[35] where B_T and B_f are used routinely and successfully for many years.[30–33] Elsewhere, B_T alone guides treatment with phototherapy and/or exchange transfusion using B_T thresholds derived from ever-evolving clinical experience and expert consensus.[36–38] Unfortunately, this approach has resulted in excessive treatment, erratic clinician compliance, and considerable confusion without eliminating BIND.[39–53] Adding bilirubin binding to the clinical armamentarium is unlikely to resolve all these issues, but it will certainly offer some improvements for the current situation.

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Table 1. Bilirubin binding variables[54, 55, 64]

Variable, units	Parameter	Comments, relationships	Test
B_T, mg/dL	Total plasma bilirubin (≅bound bilirubin)	$B_T - B_f \approx B_T$	CL HRP
B_f, µg/dL	Unbound (free) bilirubin		HRP FP
A_T, g/dL	Plasma albumin	1 g of albumin = 8.8 mg bilirubin (mole/mole)	CL
BBC, mg/dL	B_T at which ALL plasma bilirubin binding sites are occupied	Determined by titrating sample with bilirubin[69]	No Test
HMF 'BBC', mg/dL	Concentration of binding sites where bound bilirubin fluoresces		HMF
B_{Tmax}, mg/dL	Empiric mass action binding constant	Used with K to calculate B_f at $B_T < B_{Tmax}$	HRP
K, dL/µg	Equilibrium association binding constant for B_{Tmax}	$K = \frac{B_T}{B_f(B_{Tmax} - B_T)}$	Calculated

CL clinical laboratory, *HRP* peroxidase test, *HMF* hematofluorometry jematofluorometry, *FP* fluorescent probe
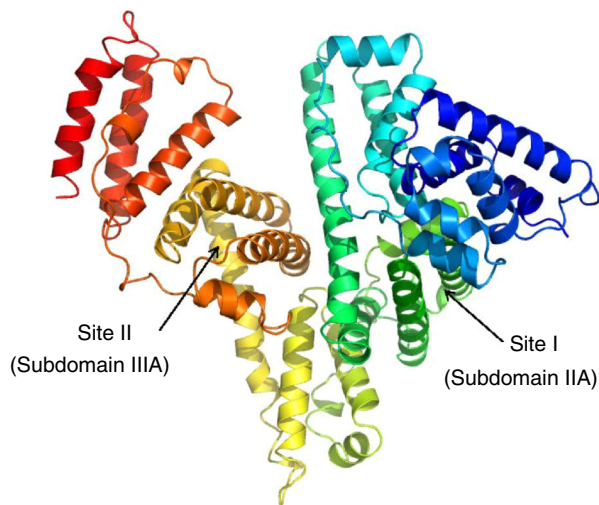

Fig. 1 Schematic of the albumin molecule showing two bilirubin binding sites. Bilirubin bound to site I fluoresces and its equilibrium association constant (K_{hi}) is about 10-fold more tightly than site II[69]
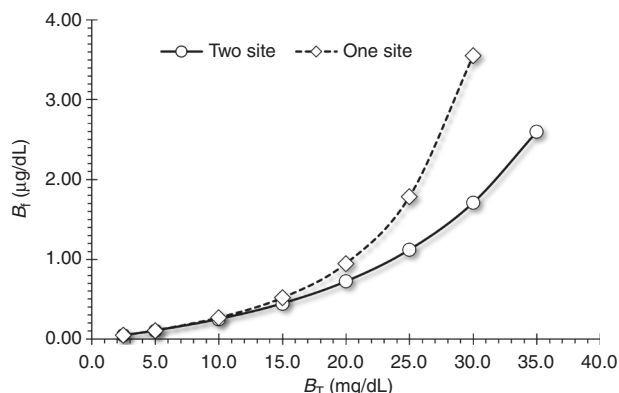

Fig. 2 The change in B_f as B_T increases at an albumin concentration of 3.5 g/dL and assuming a single site with $K_{hi} = 1.71$ dL/µg (100 L/µmol) or two binding sites with $K_{hi} = 1.71$ dL/µg and $K_{lo} = 0.171$ dL/µg (see Fig. 1). The individual points were calculated using standard binding equations.[62, 63] The lower affinity binding site significantly impacts the change in B_f over the clinically relevant range of B_T

The clinical use of bilirubin binding has been effectively blocked because clinicians have been asking the wrong questions. B_T was the original and only clinical laboratory (CL) test available for quantifying newborn jaundice, and it was several years before clinically suitable 'bilirubin binding tests' were developed.[5, 54–56] Clinicians then asked for proof that the 'binding tests' were superior to B_T as predictors of BIND, which was not forthcoming.[57–59] The better question would have been 'How well does a jaundiced newborn who has reached a threshold B_T bind bilirubin?' That question provides a natural pathway for bringing bilirubin binding to the bedside that is not dependent on problematic prospective studies comparing how well 'binding tests' and B_T predict BIND.

In this Review, we argue that the key to bringing bilirubin binding safely and expeditiously to the bedside is to determine the population parameters for B_f at current B_T thresholds. The B_f population data will serve as the reference metric[60] for quantifying how well a jaundiced newborn binds bilirubin as an additional factor in determining their risk of BIND, irrespective of whether the incidence of BIND is known. B_f population parameters at threshold B_T levels can be obtained by using a bilirubin binding panel (BBP) to measure bilirubin binding in about 400 patients from the population of interest.[20, 61] In our paradigm, both B_T and B_f are included in the BBP.

Two additional variables are needed in the BBP, the maximum B_T (B_{Tmax}) and its corresponding equilibrium association constant (K). B_{Tmax} is an empirical mass action upper limit of B_T in a sample that is used with K to calculate B_f at any $B_T < B_{Tmax}$ (Equation 1). B_{Tmax} and K are necessary to calculate B_f at the threshold B_T level since the B_T of a sample will rarely be the threshold B_T. It is important to note that B_{Tmax} is not the plasma bilirubin binding capacity (BBC), i.e., the B_T level at which ALL the plasma bilirubin binding sites are occupied. There are no tests for determining BBC although the term continues to be mistakenly applied to 'bilirubin binding test' endpoints.[17, 55] The BBP variables and other binding measurements are summarized in Table. 1.

The mass action chemical and mathematical relationships hematofluometry, not jematofluometry, could not get system to eliminate J and add H between the BBP components (B_T, B_f, B_{Tmax}, and K) are shown below.[62, 63] B_T is the bound bilirubin concentration, since B_f is orders of magnitude less than B_T (µg/dL versus mg/dL) and bound bilirubin = $B_T - B_f \cong B_T$. The unoccupied binding sites in the derivation of Equation 1 are $B_{Tmax} - B_T$.[63]

$$(B_{Tmax} - B_T) + B_f \overset{K}{\underset{}{\leftrightarrows}} B_T$$
$$K = \frac{B_T}{B_f(B_{Tmax} - B_T)}$$
(1)

A practical issue is how the BBP variables are obtained. B_T has long been measured by the clinical laboratory (CL), and clinically suitable methods for measuring B_f are available.[35, 54, 64] K is calculated from B_T, B_f, and B_{Tmax} using Equation 1. B_{Tmax}, however, is a 'new' variable introduced herein to avoid the long-standing misconception that albumin has only one bilirubin binding site that is clinically relevant.[17, 55, 64–68] The advantage of using the 'single-site' model is that the simplest mass action equations apply (e.g., Equation 1), and measured variables such as the concentrations of albumin or albumin-bound bilirubin that fluoresces[55, 68]

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Table 2. Summary of bilirubin binding measurements before and after sample bilirubin enrichment in 110 samples from 72 newborns 24 to 34 weeks' GA (median 30 weeks) with birthweights 0.483–2.490 kg (median 1.305 kg). The B/A_T molar ratios varied from 0.10 to 0.76 and 0.31 to 1.45 before and after sample bilirubin enrichment, respectively

	B_T[a] (mg/dL)	CL A_T (g/dL)	HMF[b] 'BBC' (mg/dL)	HRP B_f (µg/dL)	Enriched (HRP)	
					B_T (mg/dL)	B_f (µg/dL)
Mean	7.4	2.4	24.5	0.46	14.3	1.79
SD	2.6	0.3	5.8	0.28	4.0	1.55
Range	1.7−15.7	1.5−3.2	11.7−42.5	0.06−1.66	6.3−23.2	0.42−10.6
Median	7.4	2.4	24.1	0.38	13.7	1.28
25th	5.8	2.2	20.7	0.28	11.2	0.93
75th	9.0	2.7	27.5	0.59	17.3	1.95

[a]Average of CL B_T and HRP B_T before bilirubin enrichment.
[b]The instrument readout is 'BBC' referring to the maximum concentration of bound bilirubin that fluoresces, not the plasma BBC

Table 3. B_f calculated at the enriched B_T for the 110 samples in Table 2 using Equation 1 and B_{Tmax} estimated using either the CL A_T (8.8 × A_T in g/dL) or the HMF 'BBC' and their corresponding K's determined using Equation 1

	CL B_{Tmax} mg/dL	CL K dL/µg	CL B_f µg/dL	[a]HMF 'BBC' mg/dL	HMF KdL/µg	HMF B_f µg/dL
n	97	97	97	100	100	100
Mean	22.0	1.47	2.54[b]	24.9	1.25	1.97[c]
SD	2.4	0.60	5.63	5.7	0.54	3.29
Range	16.7–28.2	0.38–3.02	0.19–3.77	11.7–42.5	0.33–3.12	0.19–25.8

[a]BBC = B_{Tmax} = concentration of binding sites where bound bilirubin fluoresces
[b]Paired t-test $p = 0.035$ with corresponding 97 measured B_f (mean = 1.45 ± 0.98 µg/dL)
[c]Paired t-test $p = 0.13$ with corresponding 100 measured B_f (mean = 1.58 ± 1.13 µg/dL)

Table 4. Hyperbilirubinemia treatment guidelines for newborns <35 weeks' GA[38]

Gestational age (weeks)	B_T (mg/dL)	
	Phototherapy	Exchange transfusion
<28$0/7$	5–6	11–14
28–29$6/7$	6–8	12–14
30–31$6/7$	8–10	13–16
32–33$6/7$	10–12	15–18
34–34$6/7$	12–14	17–19

The inaccuracy of the 'single-site' model in predicting B_f at non-sample B_T is illustrated using bilirubin binding data from 72 newborns < 35 weeks' GA.[70] Table 2 contains a summary of the data before and after sample bilirubin enrichment. The concentration of binding sites where the bound bilirubin fluoresces (HMF 'BBC') was measured using hematofluorometry (HMF).[55] Table 3 shows the B_f predicted using the CL albumin (A_T) or the HMF 'BBC' as B_{Tmax} in Equation 1, with the corresponding K's calculated using the sample B_f before bilirubin enrichment. As expected from Fig. 2, both predicted higher average B_f at the enriched B_T compared with the measured B_f, and B_f could not be calculated in several samples because the enriched B_T exceeded the B_{Tmax} determined using the CL A_T or the HMF test.

An empirical B_{Tmax} that does not require assumptions about the actual bilirubin binding sites and will provide accurate estimates of B_f at non-sample $B_T < B_{Tmax}$ can be readily obtained from the B_T and B_f measured before and after sample bilirubin enrichment (B_{T1}, B_{f1} and B_{T2}, B_{f2}). B_{Tmax} and K in Equation 1 are both considered unknowns, and Equation 1 is solved for B_{Tmax} using Equation 2 (70). Enriching samples with bilirubin to B_T above the relevant B_T thresholds and re-measuring B_T and B_f to obtain B_{Tmax} and K for accurate estimates of B_f at threshold $B_T < B_{Tmax}$ will not require substantially more sample volume or time for testing.[17, 54, 71]

$$B_{Tmax} = \frac{B_{T1}B_{T2}(B_{f2}-B_{f1})}{B_{T1}B_{f2} - B_{T2}-B_{f1}} \qquad (2)$$

Once B_f population parameters are available, the BBP can be used to help determine how best to treat a newborn that has reached or exceeded a B_T threshold and also as a screening tool[72] to individualize the threshold B_T as warranted. Table 4 shows current B_T threshold guidelines for phototherapy and exchange transfusion in newborns <35 weeks' GA. Consider a clinician (armed with bilirubin binding) confronting a <28-week GA newborn that has reached the phototherapy B_T threshold of 5 mg/dL. Assume the median B_f for the population at $B_T = 5$ mg/dL is 0.30 µg/dL with the 25th percentile 0.15 µg/dL and the 75th percentile 0.45 µg/dL. If the newborn's B_f is >0.30 µg/dL phototherapy is indicated and if it is >0.45 µg/dL there is considerable cause for concern as the binding is 'poor' relative to the population. If the median B_f for the population at the threshold exchange transfusion is 1.0 µg/dL,[34] B_{Tmax} and K from the newborn's BBP and the B_f of 1.0 µg/dL can be used in Equation 1 to calculate the actual B_T at which the B_f would reach 1.0 µg/dL. This approach provides considerably more information about the risk of BIND even though the actual risk is unknown and individualizes care.

It is important to note that B_f varies with the local environment, temperature, sample dilution, GA, illness, and the presence of endogenous or exogenous ligands competing for bilirubin

can be designated as the 'BBC' and substituted for B_{Tmax} in Equation 1(17,55,64-68). Unfortunately, Equation 1 will not provide accurate estimates of B_f at non-sample B_T using the 'BBC' and corresponding K provided by these other methods.

Albumin has at least two bilirubin binding sites as shown in Fig. 1.[69] The ~10-fold difference in their affinity for bilirubin as quantified by their respective association constants, $K_{hi} > 10·K_{lo}$[62, 63, 69] is used to justify the assumption that only the high affinity site where bound bilirubin also fluoresces is clinically relevant.[18, 54, 67] Fig. 2 shows that both sites will significantly impact the change in B_f as B_T increases, invalidating the assumption that only the high affinity site is clinically relevant.

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binding sites.[9, 10, 14, 17, 73–80] Therefore, comparisons of outcome and B_f across institutions will require measuring B_f under standard conditions. B_{Tmax} and K, although empirical with respect to specific bilirubin binding sites, are critical binding components required for reliable determination of B_f at relevant threshold B_T. The use of the BBP will individualize care and as patient data accumulates may help better clarify the relationships between neonatal bilirubin exposure and BIND. In the meantime, the BBP can provide additional guidance for the clinicians caring for jaundiced newborns.

SUMMARY

BIND is a spectrum of neurological disorders affecting jaundiced newborns and unpredictably related to the magnitude of B_T. The BBP (B_T, B_f, B_{Tmax}, and K) can provide important additional information about the risk of developing BIND and the need for treatment at threshold B_T, irrespective of whether the actual incidence of BIND at or above that B_T is known. The BBP components can be obtained using minimal modification of well described, clinically suitable methods, and when configured as described in this Review, provide accurate estimations of B_f at $B_T < B_{Tmax}$. The BBP can be used to establish B_f population parameters at current threshold B_T that are the metric for quantifying how well a newborn binds bilirubin, which provides important additional information the risk of BIND and need for intervention The BBP is useful as a screening tool as well as for determining the urgency of treatment in newborns reaching or exceeding threshold B_T. This approach to the clinical of use of bilirubin binding augments current clinical practice within the context of established B_T thresholds and helps individualize care.

ACKNOWLEDGEMENTS

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

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