Measuring Free Bilirubin: The Clinical Perspective

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In 1959, Gerald Odell first proposed that albumin and tissue compete for binding bilirubin and that this competition is mediated by a very low concentration of free bilirubin (Bf) \(^1\) (bilirubin not bound to plasma proteins). He argued that displacement of albumin-bound bilirubin by binding competitors could explain the reported high incidence of kernicterus in premature infants receiving sulfonamide prophylaxis \(^1\). This sentinel publication led to a flurry of proposed binding tests, most of which were nonspecific competitive-binding assays, until 1974, when Jacobsen and Wennberg presented a method for measuring Bf in infant plasma that was based on the observation that only unbound bilirubin serves as a substrate for enzymatic oxidation by horseradish peroxidase \(^2\).

Several studies from Japan, where the peroxidase assay had been adapted to a semiautomated instrument, identified a clear relationship of Bf with acute neurotoxicity in premature infants \(^3\) and with acute auditory impairment in term infants \(^4\). Enthusiasm was tempered in the US by a perceived difficulty in performing the assay and by confusion regarding the poor correlation between Bf and outcome in premature babies who died with kernicterus at very low total bilirubin (Bt) concentrations \(^5\). The reported high incidence of death of infants with kernicterus was isolated to specific care centers: Very high Bf/Bt ratios in affected infants indicated very poor binding, and the phenomenon of lethal kernicterus at low Bt concentrations largely disappeared after cessation of benzyl alcohol use as a preservative in multiple-dose vials or solutions for parenteral therapy \(^6\). Kernicterus is still occasionally observed at low Bt concentrations in babies with sepsis or renal failure, conditions associated with impaired albumin binding of bilirubin and other ligands.

Interest in measuring Bf declined during the 1970s after the development of passive immunization to prevent Rh hemolytic disease \(^7\) and the introduction of phototherapy as an effective treatment for hyperbilirubinemia \(^8\). With the aggressive use of phototherapy, frank kernicterus became a rare event in affluent nations. Concern about jaundice was placed on the back burner until a perceived resurgence of kernicterus in term/near-term infants led the American Academy of Pediatrics (AAP) to issue new guidelines in 2004 to improve early recognition of babies at risk for severe hyperbilirubinemia \(^9\). Intervention guidelines based on the Bt concentration (adjusted downward with a number of coexisting conditions such as sepsis, hemolysis, low albumin, low gestational age) were little changed, and the AAP panel acknowledged that the uncertainty in risk assessment required treating a large number of babies to prevent a single adverse outcome.

Evidence to support the rather specific recommendations was surprisingly sparse, being limited to <120 patients with a variety of acute and/or persistent adverse auditory or neurologic outcomes reported in scattered case studies and reports \(^10\).

There are good theoretical reasons and an increasing body of clinical evidence to suggest that risk assessment based on Bf or a combination of Bt, albumin, and Bf will improve the identification of babies in need of treatment \(^11\). In this issue of Clinical Chemistry, Huber and colleagues describe a new approach to Bf measurement based on competitive binding of bilirubin to a high-affinity probe coupled to a sensitive fluorophore \(^12\). In contrast to the peroxidase assay, which requires measuring a reduction in the Bt concentration and extrapolating that to the initial Bf-dependent oxidation rate, the high-affinity modified free fatty acid receptor can be used at a very low concentration so that the equilibrium Bf concentration is minimally perturbed. The developers have carefully documented the potential pitfalls in Bf measurement and have used model sera in validating the method by comparing it with the peroxidase method. The simplicity of the method and the potential for point-of-care instrumentation are very attractive and worthy of clinical trials that compare proposed markers for intervention.

The clinical application of predictive markers is complicated by vagaries in the behaviors of albumin binding and bilirubin chemistry, notably the effects of plasma dilution and photoisomerization on Bf assays and the interpretation of results. Plasma dilution

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\(^5\) Nonstandard abbreviations: Bf, free bilirubin; Bt, total bilirubin; AAP, American Academy of Pediatrics.
creases the binding affinity of albumin for several substrates, including bilirubin, thereby leading to different Bf values for the same sample, depending on the assay conditions (13). More importantly, dilution diminishes the displacing effect of binding competitors (e.g., sulfonamides), leading to an underestimation of risk (2, 5). Both the peroxidase method as usually performed and the proposed method suffer from this potential limitation. A modified peroxidase method that uses minimally diluted samples mitigates this problem (14) but, as with the fluorescent probe, it has not been evaluated in jaundiced newborns considered to be at risk for kernicterus.

A more evasive issue is the effect of photoisomers on Bf measurement and clinical application of the results. Under intensive phototherapy, photoisomers, predominantly (4Z,15E)-bilirubin-IXα, may constitute >20% of Bt. There is evidence that photoisomers have a lower affinity for both the fluorescent probe and peroxidase than native (4Z,15Z)-bilirubin-IXα, but they may also have a lower binding affinity for albumin. The consequence of these competing forces with the peroxidase method is controversial (15) and is evaluated only partially with the fluorescent-probe system. Even more unsettled are the questions of whether photoisomers bind competitively with native bilirubin, whether they cross the blood–brain barrier efficiently, and whether they are toxic or revert to the toxic ZZ conformation when bound to tissue.

Resolving the interaction of these factors experimentally would be formidable and maybe impossible, but perhaps unnecessary. The bottom line is whether a marker predicts outcome in severely jaundiced newborns and whether the discrimination of babies at risk under various neurotoxicity risk scenarios is substantially improved by using the measured Bf concentration or some combination of the Bf concentration with the Bt concentration or the bilirubin/albumin ratio. The design and execution of well-crafted observational studies are challenging, and long-term outcomes can be confounded by interventions and comorbid events.

The toxic threshold for Bt is lower in premature infants, in part because of the lower serum albumin concentrations, and there is some evidence that the toxic threshold of Bf is lower as well (3). Unfortunately, studies that relate the Bf concentration to outcome in premature infants are complicated both by the widespread prophylactic use of phototherapy and by the high incidence of unrelated neurodevelopmental difficulties that attend the extrauterine existence of the premature neonate of very low birth weight. Evaluating paradigms for intervention in term/near-term infants in the US or Europe is no less daunting, because aggressive phototherapy has led to a very low incidence of disease and the scattered distribution of affected infants precludes adherence to a comprehensive protocol.

Hearing loss, acute bilirubin encephalopathy, and residual kernicterus remain major problems in many developing countries where severe hyperbilirubinemia due to delayed treatment is common. Cairo University Children’s Hospital in Egypt, for example, admits about 250 babies per year with Bt concentrations >25 mg/dL (428 μmol/L), a value identified as critical by the AAP, and has already provided important information regarding risk factors for acute bilirubin encephalopathy (16). Such venues with a high prevalence of disease and a mature research capacity may be able to provide the quality evidence needed to evaluate the clinical application of binding assays and to define more-appropriate paradigms for clinical decisions. Lacking such studies, we will continue to depend on “state of the art” expert opinion rather than evidence-based guidelines. Some say that’s good enough.

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References