Fluorescence Sensor for the Quantification of Unbound Bilirubin Concentrations

Bob Barrett: This is the podcast from *'Clinical Chemistry'*. I am Bob Barrett. An access of bilirubin in the blood or hyperbilirubinemia is the cause of jaundice or yellowing of the skin in infants. When assessing hyperbilirubinemia in these infants with jaundice, total serum bilirubin is measured. However, it's only the unbound bilirubin that crosses the blood-brain barrier and can be neurotoxic. Therefore, measuring this free form of bilirubin is crucial to properly treating these infants.

A method exists to measure plasma concentrations of free-form bilirubin, but this measurement is relatively complex and the assay is not routinely used.

In the May 2012 issue of '*Clinical Chemistry*' Dr. Alan Kleinfeld, a member of the Torrey Pines Institute for Molecular Studies and President of FFA Sciences in San Diego and his colleagues described a new fluorescence assay for quantifying free-form bilirubin in plasma. Dr. Kleinfeld is our guest in this podcast.

Doctor, it's clear that unbound but not albumin bound bilirubin gets into the brain. However, total and unbound bilirubin are related. So why is it important to measure unbound bilirubin rather than total bilirubin for assessing the risk of neurotoxicity in babies?

Dr. Alan Kleinfeld: Well, there are several critical issues that make unbound bilirubin a more accurate gauge of neurotoxicity. First, the amount of bilirubin that gets into the brain from the blood is proportional to the blood concentration of unbound bilirubin, but not the total bilirubin or albumin bound bilirubin concentration.

So because of the albumin's high affinity for bilirubin, the bilirubin equilibrium leads to unbound bilirubin concentrations that are more than 10,000 times smaller than the total bilirubin concentration. So a measurement of total bilirubin doesn't provide the unbound bilirubin concentration, unless you also know the albumin concentration and the degree to which albumin binding sites for bilirubin are available.

The ability of bilirubin to bind albumin is strongly dependent on the presence of drugs and other metabolites that combine to albumin and can now alter the affinity of bilirubin binding sites. This kind of competition can result in complete decoupling of the unbound and total bilirubin concentrations.

For example, in the presence of increasing concentrations of free fatty acids, the unbound bilirubin levels can increase to well above what are considered toxic levels with no change

in the total bilirubin concentration. Therefore, the potential toxicity of bilirubin can best be determined by direct measurement of unbound bilirubin.

Bob Barrett: What are the clinical studies that support the superiority of unbound versus total bilirubin in assessing neonatal neurotoxicity?

Dr. Alan Kleinfeld: Almost all clinical studies that have compared the utility of unbound to total bilirubin have found that unbound is superior. Perhaps, the most informative studies are those in which early bilirubin toxicity was detected by abnormalities observed by auditory brainstem measurements in newborns. These studies found that unbound, but not total bilirubin accurately predicted the presence of such abnormalities, which occur in regions of the brain known to be sensitive to bilirubin toxicity.

This suggests that unbound bilirubin would detect babies at risk for neurotoxicity that would otherwise be missed by measurements of total bilirubin.

Recent studies have also addressed the opposite diagnostic errors that are false positives. In these new studies, newborns presented with total bilirubin levels so high that the condition was a medical emergency and required a transfusion to exchange out high bilirubin blood.

However, these babies also received an auditory brainstem test before the exchange transfusion was administered. And based upon these measurements, about 80% of the transfusions dictated by total bilirubin levels may have been unnecessary.

In contrast, if the treatment decisions have been based on unbound bilirubin measurements, fewer transfusions would have been performed and less than 30% of those would have been unnecessary.

So taken together, the clinical studies indicate that unbound bilirubin measurements to assess the risk of toxicity should have reduced both false negative and false positive rates relative to total bilirubin measurements.

Bob Barrett: Well doctor, from what you have described it would be expected that unbound would be superior to total for identifying babies at risk for bilirubin neurotoxicity. Why then hasn't the FDA cleared Arrows device using peroxidase-mediated oxidation of bilirubin to determine unbound bilirubin as the standard of care?

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Dr. Alan Kleinfeld: Well, there are several issues that complicate accurate determinations of the concentration of unbound bilirubin with the Arrows device.

First, the peroxidase assay determines the rate of bilirubin oxidation, rather than measuring the unbound bilirubin concentration directly. As a result, determination of the unbound bilirubin concentration with the Arrows device requires substantial patient blood volume. This, together with blood specimens that are needed for other tests, may especially for preemies be large enough as to require a blood transfusion to prevent anemia.

In addition, the peroxidase assay is sensitive to interference from hemoglobin as well as conjugated bilirubin and bilirubin photoisomers. And lastly, plasma samples in the Arrows assay are diluted 40-fold. This degree of dilution can lead to measurements reporting erroneously low unbound bilirubin concentrations, especially when drugs and metabolites are present that compete with bilirubin for binding to albumin.

In contrast, the unbound bilirubin sensor measures unbound bilirubin concentration directly in a single measurement that in the '*Clinical Chemistry*' publication use plasma samples diluted 25-fold.

Furthermore, the sensor reveals little or no sensitivity to hemoglobin, and may not be sensitive to conjugated bilirubin and its photoisomers. Moreover, in preliminary studies the assay has been configured for measurements with small volumes of undiluted plasma.

Bob Barrett: Well doctor, this certainly sounds important. Can you give us some idea how this new approach to determining the unbound bilirubin concentration was discovered?

Dr. Alan Kleinfeld: Well, the bilirubin sensor is composed of a fluorescently labeled fatty acid binding protein and its development represents a natural progression from our work on unbound free fatty sensors.

In the course of generating fatty acid specific probes, we produced thousands of mutants that did not bind fatty acids. Because the importance of unbound bilirubin had been established by previous investigators, we searched among these fatty acid non-responders for probes that responded to bilirubin.

We obtained several hits, carried out further mutations to improve specificity for un-conjugated bilirubin and eventually obtained the sensor described in the *'Clinical Chemistry'* article. This sensor is largely independent of the

concentration of total bilirubin as well as other potential interference, including free fatty acids.

Bob Barrett: Doctor, you have said that fatty acids may compete with bilirubin for binding to albumin and that unbound fatty acid concentrations may exceed those of bilirubin. Now this suggests that increasing unbound fatty acid levels might increase unbound bilirubin levels. Under what conditions would these factors be important in the diagnosis and treatment of neonates?

Dr. Alan Kleinfeld: Well, that's a very important issue. As it turns out especially in premature babies, nutrition is administered intravenously and the major source of calories is provided by Intralipid, which is an emulsion of oil. As this emulsion flows through the baby's circulation, enzymes in the blood produce free fatty acids from the triglycerides in the oil. And depending upon a variety of factors that include the gestational age and weight of the baby, free fatty acids may substantially increase the unbound bilirubin concentration by decreasing bilirubin binding to albumin.

Under these conditions the unbound bilirubin can increase to concentrations that are likely neurotoxic, while the total bilirubin concentration remains unchanged at low levels.

Moreover, in addition to the neurotoxicity of unbound bilirubin, at high levels the unbound free fatty acids may also be toxic. In preliminary studies, we found that unbound fatty acid concentrations may increase more than a thousand-fold above normal in Intralipid neonates and at least in in-vitro studies, these level of unbound free fatty acids may be cardiotoxic and may disrupt immune function.

Bob Barrett: Well, finally doctor, how would you expect the unbound bilirubin sensor to change clinical practice?

Dr. Alan Kleinfeld: Given the biochemical fundamentals and the supporting clinical studies, the risk of bilirubin toxicity should eventually be assessed by unbound, rather than by total bilirubin measurements. If the studies hold up, adoption of unbound bilirubin should lead to improved outcomes because neurotoxicity currently missed would be treated and healthcare costs would be lowered, because unnecessary therapies would be reduced.

Most likely the test would be used first for premature newborns, where rates of jaundice are higher than in term babies. Moreover, in ongoing work we've developed versions of the sensor that function in undiluted plasma. These sensors have been tested successfully in a disposable cartridge with 20 µL of plasma and might be able to function with similar or lower volumes of whole blood.

This should greatly simplify measuring unbound bilirubin in the NICU.

Bob Barrett: Dr. Alan Kleinfeld is a member of the Torrey Pines Institute for Molecular Studies and President of FFA Sciences in San Diego. He has been our guest in this podcast from *'Clinical Chemistry'*.

I'm Bob Barrett, thanks for listening.

Total Duration: 11 Minutes