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Comparing bilirubin assay technologies with implications for improving the quality of patient care

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Most clinical laboratories today measure total and direct bilirubin and calculate indirect bilirubin. However, at Primary Children's Medical Center and our affiliated healthcare network, Intermountain Health Care, in Salt Lake City, Utah we are using a different method which measures both conjugated and unconjugated bilirubin. We believe this approach provides important advantages to patient care.

The technology used to measure bilirubin in most clinical laboratories today is based on the diazo method first described by Jendrassik and Gróf in 1938. The estimation of conjugated and unconjugated fractions is based on the relative insolubility of unconjugated bilirubin in aqueous solution. It does not react with the diazo reagent unless an "accelerator" (caffeine and sodium benzoate in the Jendrassik-Gróf method) is included to increase its solubility. Conjugated bilirubin, which is much more soluble, reacts "directly" with the diazo reagent without an accelerator. Without an accelerator, only conjugated bilirubin reacts and the result is "direct bilirubin". With an accelerator, all the bilirubin reacts and the result is "total bilirubin". Unconjugated bilirubin is estimated "indirectly" as the difference between total and direct.

These statements are correct only to a first approximation, because unconjugated bilirubin does react, although very slowly, even without an accelerator. Reaction conditions for direct bilirubin assays are chosen to minimize the contribution of unconjugated bilirubin, but under these conditions the reaction with conjugated bilirubin does not go to completion. Because of this trade-off, direct bilirubin assays report a small amount of direct bilirubin in specimens with lots of unconjugated bilirubin and essentially no conjugated (such as in a newborn), and most direct bilirubin assays underestimate conjugated bilirubin. Many efforts have been made to adjust the reaction conditions (time, temperature, pH, reagent concentration, etc.) so that all the conjugated bilirubin reacts but none of the unconjugated bilirubin does. This ideal has not been reached. As a result, the separation of bilirubin into direct and indirect fractions based on solubility is always an approximation.

A century ago, we did not know that direct bilirubin was conjugated and indirect bilirubin was not. The distinction was purely empirical, and it was useful because it correlated with disease. Today we know considerably more about bilirubin and its various forms. High Performance Liquid Chromatography (HPLC) has been used to separate bilirubin into four peaks:

* Alpha, which is unconjugated bilirubin;

* Beta, which is bilirubin singly conjugated, usually to glucuronic acid;

* Gamma, which is doubly conjugated bilirubin;

* Delta, which is bilirubin covalently bound to albumin.

HPLC is impractical for routine bilirubin determinations. However, tests available on the Ortho-Clinical Diagnostics VITROS 950 and 250 Clinical Chemistry Systems measure three distinct bilirubin fractions which match the molecular species identified by HPLC. The VITROS test method for bilirubin consists of two separate test slides. The VITROS TBIL slide measures total bilirubin by a diazo method. The VITROS BuBc slide simultaneously measures unconjugated (Bu) and conjugated (Bc) bilirubin by use of a mordant (a substance used in the textile and photographic industries to bind dyes to solid supports such as fabric or film). In the presence of the mordant, the visible spectra of conjugated and unconjugated bilirubin are different, allowing measurement of both species from a single slide. With the VITROS bilirubin test methods, unconjugated bilirubin (Bu) corresponds to the HPLC alpha peak; conjugated bilirubin (Bc) corresponds to the sum of the beta and gamma peaks; total bilirubin (TBIL) includes all HPLC fractions, and the difference between TBIL and the sum of Bu+Bc corresponds to the delta peak by HPLC (Figure 1). This is the technology we use in our laboratory.

<table>
<tr><td>HPLC Peak</td><td>Alpha</td><td>Beta</td><td>Gamma</td><td>Delta</td></tr>
<tr><td rowspan="2">Bilirubin Species</td><td>Unconjugated</td><td>Singly Conjugated</td><td>Doubly Conjugated</td><td>Conjugated to albumin</td></tr>
<tr><td colspan="4">Total Bilirubin (TBIL)</td></tr>
<tr><td rowspan="2">VITROS Methods</td><td>Unconjugated (Bu)</td><td colspan="2">Conjugated (Bc)</td><td>Delta (Total-(Bu+Bc))</td></tr>
<tr><td colspan="4">Total Bilirubin</td></tr>
<tr><td>Traditional Methods</td><td>Indirect (Total – Direct)</td><td>Gray Zone*</td><td colspan="2"></td></tr>
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Clinical effectiveness of the assay

There are two differences between VITROS and traditional bilirubin determinations which may aid in patient diagnosis and monitoring. First, bilirubin fractions measured by VITROS test methods correspond closely with the molecular species, as separated by HPLC. Second, delta bilirubin (covalently bound to albumin) is not included in conjugated bilirubin measured by the VITROS BuBc slide, as it is in total (VITROS TBIL) and direct bilirubin tests measured by diazo methods.

In obstructive jaundice, conjugated bilirubin is not excreted into the bile and consequently re-enters the bloodstream. Once in circulation, it slowly binds covalently to albumin forming delta bilirubin. This non-enzymatic process is analogous to the formation of glycohemoglobin from glucose and hemoglobin. Delta bilirubin has the same half-life as albumin, 2-3 weeks. After successful therapy for a patient with obstructive jaundice, conjugated bilirubin is expected to drop sharply and immediately. However, because of its long half-life, delta bilirubin remains elevated for weeks after the obstruction has cleared. Because they include delta bilirubin, total and direct

bilirubin results also remain elevated, falsely suggesting the patient needs further treatment. On the other hand, with the VITROS BuBc slide, conjugated bilirubin (Bc) drops quickly, correctly indicating the patient is recovering.

A test that measures the distinct forms of bilirubin is also valuable in the early detection of rare but serious diseases of newborns, especially biliary atresia. Generally, the sooner these diseases are identified, the more effectively they can be treated. In biliary atresia, the infant's bile ducts are progressively destroyed. Often surgery can partially restore bile flow, but a liver transplant is usually the only definitive cure. Traditionally, biliary atresia is suspected when physiologic jaundice of the newborn fails to clear after 2-4 weeks or if stools become gray or white (acholic). If only total bilirubin is used to monitor physiologic jaundice, a small but significant increase in conjugated bilirubin that could give an early tip-off to biliary atresia remains undetected. However, if jaundiced infants are followed with the VITROS BuBc test, a single test which provides two results on the VITROS Systems, the elevation in conjugated bilirubin is immediately apparent. For this reason, our hospital's gastroenterologists prefer the VITROS BuBc test method, rather than total or "neonatal" bilirubin, as the primary bilirubin test for neonates.

Other problems with traditional bilirubin assays include bilirubin's susceptibility to photodegradation, and interference due to hemolysis. Light degrades bilirubin, especially unconjugated bilirubin, in vivo during phototherapy and, even more rapidly, in vitro after a specimen has been collected unless it is protected from light. Although much remains to be learned about this complex issue, in our hands, it appears that the VITROS BuBc test method is less susceptible to photodegradation of the specimen, and to hemolysis interference, than traditional total bilirubin assays, and therefore gives a better estimation of the bilirubin level in patients receiving phototherapy and in hemolyzed or photodegraded specimens.

Interpreting BuBc Assays

At Intermountain Health Care, we encourage physicians to use the VITROS BuBc test method for routine bilirubin testing. Our efforts to educate them about the interpretation of this test include the following points:

* Unconjugated bilirubin. Interpret as you would indirect bilirubin. Our upper limits of normal are 3.4 mg/dL (58 (mol/L) at birth, 15 mg/dL (257 (mol/L) at 2-3 days, 7 mg/dL (120 (mol/L) at 7 days, and 1.1 mg/dL (19 (mol/L) at 1 month and over. Elevated levels indicate increased production of bilirubin, a failure to conjugate bilirubin, or both.

* Conjugated bilirubin. Interpret as you would direct bilirubin, recognizing that direct is less specific than Bc and includes delta. Our reference ranges are less than 0.6 mg/dL (10 (mol/L) for the first week and less than 0.3 mg/dL (5 (mol/L) thereafter. Elevated levels indicate cholestasis at the level of the hepatocyte or the intra- or extrahepatic biliary tree. Levels rise soon after bile flow is interrupted and fall back to normal soon after flow is reestablished. Elevated levels in neonates are always pathologic and warrant immediate investigation.

* Situation when the sum of Bu plus Bc exceeds total bilirubin. This apparently aberrant result sometimes generates inquiries from clinicians. We explain that this anomaly is due to differences in the analytical specificity of these two quite different bilirubin methods, and that hemolysis and/or

photodegradation of the specimen may contribute to the differences. In such cases, we believe the conjugated (Bc) and unconjugated (Bu) results from the BuBc slide reflect the patient's status more accurately than the VITROS TBIL (total bilirubin) slide result.

In many cases, the clinical accuracy and effectiveness of different assay technologies are similar enough that laboratories are free to select a technology based on non-clinical criteria. But in our opinion, the analytical specificity of the VITROS BuBc test method is a compelling reason for its use by any lab, but especially labs that perform pediatric testing.

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