BILIRUBIN
DCA Method

BACKGROUND

Bilirubin is formed in the reticuloendothelial system during the degradation of aged erythrocytes. The heme portion from hemoglobin and from other-containing proteins is removed, metabolized to bilirubin, and transported as a complex with serum albumin to the liver. This process accounts for about 80% of bilirubin formed daily. Other sources of bilirubin include the breakdown of myoglobin and cytochromes and the catabolism of immature red cells in the bone marrow. In the liver, bilirubin is conjugated with glucuronic acid for solubilization to form conjugated or direct bilirubin for subsequent transport through the bile duct into the digestive tract where it is metabolized by bacteria to a group of products collectively known as stercobilinogen. Total bilirubin is the sum of the conjugated and unconjugated fractions. Pre-hepatic diseases or conditions such as hemolytic disease or liver diseases resulting in impaired entry, transport or conjugation within the liver cause elevation of unconjugated (indirect) bilirubin. Monitoring of bilirubin in newborns, particularly if premature, has special importance since the hepatic handling of bilirubin is immature leading to elevated unconjugated bilirubin. If not bound to albumin, unconjugated bilirubin is able to cross the blood brain barrier more easily, increasing the risk of cerebral damage. Total bilirubin is elevated in conditions causing obstruction of the bile duct, hepatitis, cirrhosis, in hemolytic disorders and several inherited enzyme deficiencies. There is information indicating elevated levels of direct bilirubin in patients with liver or biliary tract disease, even though, total bilirubin levels are normal. Therefore, the greatest diagnostic value of direct bilirubin assays stem from their ability to indicate occult liver disease.

ASSAY PRINCIPLE

Bilirubin present in the sample reacts with diazotized Dichloroaniline to form a coloured azocomplex whose intensity at 546 nm (540-560 nm) is directly proportional to the analyte concentration.

EXPECTED VALUES

<table>
<thead>
<tr>
<th>Direct Bilirubin</th>
<th>Adults and infants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Up to 0.2 mg/dl</td>
</tr>
<tr>
<td></td>
<td>Up to 3.4 μmol/l</td>
</tr>
</tbody>
</table>

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference range. For diagnostic purposes, the bilirubin results should always be assessed in conjunction with the patient’s medical history, clinical examination, and other findings.

REAGENTS

- Reagent Preparation & Stability
  - All reagents are stable up to the expiry date given on label when stored at room temperature.

- Serum.
  - For direct bilirubin plasma specimen may be used, but the only accepted anticoagulants are heparin and oxalate.

- Specimen Preparation & Stability
  - The specimen of choice is serum. Specimens should be assayed promptly after collection, since direct bilirubin is reportedly unstable. If testing is delayed, specimens should be protected from exposure to light. Bilirubin remains stable in serum samples for 2 days at room temperature, 4 days at 4°C, or 3 months at ~20°C, if care is taken to prevent exposure to light.

PROCEDURE

Wavelength: 546 nm
Cuvette: 1 cm light path
Temperature: 20-25°C
Zero adjustment: against specimen blank
Specimen: Serum

Method 1

<table>
<thead>
<tr>
<th>Specimen blank</th>
<th>Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>R₁ 1000 μL</td>
<td>1000 μL</td>
</tr>
<tr>
<td>R₂ 100 μL</td>
<td>One drop</td>
</tr>
<tr>
<td>Specimen</td>
<td>100 μL</td>
</tr>
</tbody>
</table>

Mix well, let stand 1 minutes at 37°C (Maximum 2 minutes)

Method 2: Icetric or pediatric specimen

<table>
<thead>
<tr>
<th>Specimen blank</th>
<th>Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>R₁ 1000 μL</td>
<td>1000 μL</td>
</tr>
<tr>
<td>R₂ 20 μL</td>
<td>One drop</td>
</tr>
<tr>
<td>Specimen</td>
<td>20 μL</td>
</tr>
</tbody>
</table>

Mix well, let stand 1 minutes at 37°C (Maximum 2 minutes)

Measure the absorbance of specimen (Aspecimen) against specimen blank.

INTERFERING SUBSTANCES

- Anticoagulants:
  - The only accepted anticoagulants are heparin and oxalate for direct bilirubin.

- Drugs:
  - Young in 1990 has published a comprehensive list of drugs and substances, which may interfere with this assay.

- Hemolysis:
  - Avoid hemolyzed specimens. Even slight hemolysis interferes with the test. Haemoglobin interference is dependent on both analyte and hemoglobin concentration.

- Lipemia:
  - Avoid lipemic specimens. Even slight lipemia interferes with the test.

CALCULATION

Calculate the bilirubin concentration by using the following formulae:

For method 1

Direct Bilirubin Concentration= Specimen absorbance X 15.2 =mg/dl

For method 1

Direct Bilirubin Concentration= Specimen absorbance X 71.2 =mg/dl

Unit conversion

mg/dl x 17.1= μmol/dl

QUALITY CONTROL

It is recommended that controls (normal and abnormal) be included in:
- Each set of assays, or
- At least once a shift, or
- When a new bottle of reagent is used, or
- After preventive maintenance is performed or a clinical component is replaced.

Commercially available control material with established bilirubin values may be routinely used for quality control.

Failure to obtain the proper range of values in the assay of control material may indicate:
- Reagent deterioration,
- Instrument malfunction, or
- Procedure errors.

The following corrective actions are recommended in such situations:
- Repeat the same controls.
- If repeated control results are outside the limits, prepare fresh control serum and repeat the test.
- If results on fresh control material still remain outside the limits, then repeat the test with fresh reagent.
- If results are still out of control, contact Vitro Technical Services.
**WARNING & PRECAUTION**

- Vitro bilirubin reagent is for in vitro diagnostic use only. Normal precautions exercised in handling laboratory reagents should be followed.
- The reagent and sample volumes may be altered proportionally to accommodate different spectrophotometer requirements.
- Valid results depend on an accurately calibrated instrument, timing, and temperature control.
- All specimens must be protected from light. Serum bilirubin will decrease 50% in one hour if kept at room temperature and at direct sunlight.
- Don’t use the reagent if it is turbid.

**PERFORMANCE CHARACTERISTICS**

**Imprecision**
Reproducibility was determined using in an internal protocol. The following results were obtained.

<table>
<thead>
<tr>
<th></th>
<th>Direct Bilirubin</th>
<th>Direct Bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Within Run</strong></td>
<td><strong>Between Day</strong></td>
</tr>
<tr>
<td>Number of samples</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Mean (mg/dl)</td>
<td>0.78</td>
<td>0.8</td>
</tr>
<tr>
<td>SD (mg/dl)</td>
<td>0.015</td>
<td>0.01</td>
</tr>
<tr>
<td>CV (%)</td>
<td>1.31</td>
<td>1.63</td>
</tr>
</tbody>
</table>

**Sensitivity**
The sensitivity is defined as the change of analytical response (ΔA/min) per unit change in analyte concentration at a pathlength of 1 cm.
When run as recommended the sensitivity of this assay is 0.01 mg/dl (0.17 mol/l).

**LINEARITY**
When run as recommended, the assay is linear up to 20 mg/dl (0.342 mmol/l) for method 1 up to 100 mg/dl (1.71 mmol/l) for method 2. If result exceeds 20 mg/dl (0.342 mmol/l) perform use method number 2.

**BIBLIOGRAPHY**