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DIRECT AND TOTAL BILIRUBIN

INTENDED USE
For the quantitative determination of direct and total bilirubin in serum.

INTRODUCTION
Bilirubin is a metabolite of the heme proteins , mainly hemoglobin . Normally it is excreted into the intestine and bile from the liver. The site of the catabolism of hemoglobin is the reticuleondothelial system (RES). Bilirubin is then released into the bloodstream where it binds tightly to albumin to the liver. Upon uptake by the liver , bilirubin is conjugate with glucuronic acid to form bilirubin mono and diglucuronidewhich are water-soluble metabolites. The metabolites will react with aqueus diazo reagent and are commonly referred to as 'direct bilirubin". [1]
Elevation of total serum bilirubin may occur due to (1) excessive hemolysis or destruction of the red blood cells e.g. hemolytic disease of the newborn, (2) liver diseases e.g. hepatitis and cirrhosis (3) obstration of the biliary tract e.g. gallstones .[1] There is information in the literature indicating elevated levels of direct bilirubin in patients with liver or biliary tract disease, even though, total bilirubin levels are normal. [2] Therefore , the greatest diagnostic value of direct bilirubin assays stem from their ability to indicate occult liver disease.
Most chemical methods for the determination of total bilirubin are based on the reaction between diazotized sulfanilic acid and bilirubin to produce azobilirubin, which absorbs maximally at 560 nm. Such test are often run in the presence and absence of an organic solvent e.g., methanol to distinguish free bilirubin from conjugate bilirubin on a differential solubility basis. [3]

PRINCIPLE
Bilirubin reacts with diazotized sulfanilic acid produce azobilirubin, which has an absorbance maximum at 56nm in the aqueous solution . The intensity of the color produced is directly proportional to the amount of direct bilirubin concentration present in the sample. The subsequent addiotn of methods accelerates the reaction of unconjugate bilirubin in the serum , and a value of total bilirubin is is obtained after five (5) minutes. The total bilirubin value represents the sum of bilirubin glucoronide (direct) and the unconjugated (indirect) bilirubin The color produced measured at 560 nm is proportional to the amount of the total bilirubin concentration present in the sample.

REAGENT
1. Bilirubin Reagent: Sulfanilic Acid 32 nM, Hydrochloric Acid 165 mM.
2. Bilirubin Nitrite Reagent : Sodium Nitrite 60mM.
3. Bilirubin Calibrator : N-1- naphtylenediamine dihydrochloride salt (5mg/dl).
4. Methanol Reagent : Absolute methanol, reagent grade.

WARNING AND PRECAUTIONS
1. For in vitro use
2. Specimens should be considered infec tious and handle appropriately.
3. Do not pipette reagents by mount. Avoid contact reagent with eyes, skin and clothing . Do not ingest. Wash hand after use.

REAGENT STORAGE
1. All reagents are stored at room temperature (15 - 30°C).
2. Do not freeze reagents.
3. Avoid exposure to direct sunlight .

REAGENT DETERIORATION
The reagent should be discarded if:
1. Sodium Nitrite reagent has a yellow discoloration.
2. Reagent fails to achive assigned assay values of fresh control sera.

SPECIMEN COLLECTION AND STORAGE
1.Hemolysis interference with the test , i.,e hemolyzed samples should be avoided since they mat give falsely low values .[4]
2. All specimens for this assay must be carefully protected from light
3. Bilirubin in serum is stable for 4-7-7 days when stroed in the dark at 2-8ºC.[1]

ITERFERINCE SUBSTANCES
1.Young et al ., give an exhaustive list of drugs and other substances known to affect the circulating level of bilirubin .
2. In this assay , as an all laboratory procedures, materials, which come in contact with specimens should be clean and free of contamination by heavy metals, detergents, and other chemicals.

MATERIALS PROVIDED
1.Bilrubin reagent
2. Bilirubin Nitrite reagent
3. Bilirubin Calibrator
4. Methanol reagent

MATERIAL REQIURED BUT NOT PROVIDED
1.Cuvettes
2. Pipettes
3. Timer
4. Appropriate automated chemistry analyzer or spectrophotometer capable of measuring at 560 nm

MANUAL ENDPOINT PROCEDURE
NOTE: Due to the critical time of the direct Bilirubin Reaction, process each patient separately . Serum blank must be prepared for each patient and control.
1.Label test tube, " Blank, Standard, Control, Pateint " Each tube requires a blank tube.
2. Dispense 2.8 ml of Bilirubin Reagent to all tubes.
3. Add 50µl (0.05 ml) of bilirubin Nitrite Reagent to all tubes, mix and let stand for one (1) minute. (DO NOT add Bilirubin Nitrite to the blanks).
4. Transfer 200 µl (0.2 ml) of serum to its respective tube, gently mix and set timer for one (1) minutes (Use distilled water for blank tube).
5. Set the wavelength of the photometer at 560 nm and zero with blank tube (Wavelength range :500-550nm).
6. After exactly one (1) minute, record absorbance and use this to calculate Direct Biirubin .
7. Add 3.0 ml of Methanol Reagent to all tubes , mix by inversion and let stand five (5) minutes
8. After five (5) minutes, read and recor absorbance of all tubes.
9. Use this to calculate Total Bilirubin.

* TC MULTI-PURPOSE CALIBRATOR MAY BE USED TO REPLACE STANDARD.

PATIENT BLANK

1.Label test tubes"Blank, Standard , Control, Patient " , ect.
2.Dispense 2.8 ml of Bilirubin Reagent into All tubes.
3.Add 50 µl(0.05ml) of distilled water to all tubes.
4. Follow above procedure (steps 4-8).

STABILITY OF ENDPOINT REACTION

Direct bilirubin color formation is stable for thrity (30) minutes whereas the total bilirubin color formation is stable for sixty (60) minutes.

CALCULATIONS

Abs.=absorbance

$$\frac{\text{Abs. of unknown-Abs.of blank}}{\text{Abs. of calibrator –Abs. of calibrator blank}} \times \text{Concentration of calibrator (mg/dl)} = \text{Bilirubin (mg/dl)}$$

Example:

Absorbance of unknown	=0.132
Absorbance of unknown	=0.120
Absorbance of calibrator	=0.450
Absorbance of calibrator blank	=0.000
Concentration of calibrator	=5.0mg/dl

Then

$$\frac{0.132-0.120}{0.450-0.000} \times 5 = \frac{0.012 \times 5}{0.450} = 0.13 \text{mg/dl}$$

LIMITATIONS

1.Sera with values above 20 mg/dl must be diluted 1:1 with isotonic saline . reassayed and the final answer multiplied by two(2).
2. Serum hemoglobin levels of up to 1.0 g/dl do not interfere with results .

QUALITY CONTROL

Normal and abnormal control sera of known concentrations direct and total bilirubin should be analyzed routinely with each group of unknown specimens.

EXPECTED VALUES[5]

Direct up to 0.5 mg/dl
Total up to 1.0 mg/dl
It is recommended that esch laboratory establish is ownrange of expected values, since differences exist between instruments laboratories and local population.

PERFORMANCE CHARACTERISTICS

1. Linearity: 20 mg/dl.
2.Sensitivity: Based on a instrument resolution of 0.001 absorbance , the present procedure has sensitivity of 0.010 mg/dl.
3.Comparison : A comparison study between the present method with an available commercial product using the same identical method on twenty (20) fresh serum samples and two commercial coefficient of 0.97 and regression equation of:y=0.98x+0.001.
4. Preciion :

Run-to-run : Two commercial control sera were assayed for a period of 21 days and the following day-to-day precision was obtained.

	Level 1	Level 2
Mean (g.dl) N=16	0.20	7.2
S.D.	0.02	0.36
C.V.	10.0%	5.8%

Within Run: Two Commercial control sera were assayed 20 times and the following within run precision was obtained.

	Level 1	Level 2
Mean (mg/dl)N=20	0.24	7.6
S.D.	0.02	0.36
C.V.	10%	5.8%

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