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TOTAL BILIRUBIN REAGENT

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For the quantitative determination of total bilirubin in serum.

INTRODUCTION
Bilirubin is a metabolite of the heme portion of 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 reticuloendothelial system (RES). Bilirubin is then released into the bloodstream where it binds tightly to albumin and is transported to the liver. Upon uptake by the liver, bilirubin is conjugated with glucuronic acid to form bilirubin mono and diglucuronide which are water soluble metabolites. The metabolites are then excreted in the bile.[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) obstruction of the biliary tract e.g.. gallstones.[1]

Most chemical methods for the determination of total bilirubin is based on the reaction between diazotized sulfanilic acid and bilirubin to produce azobilirubin which absorbs maximally at 560 nm. Such tests are often run in the presence and absence of an organic solvent e.g., methanol to distinguish free bilirubin from conjugated bilirubin on a differential solubility basis.[2]

PRINCIPLE
Bilirubin reacts with diazotized sulfanilic acid to produce azobilirubin which has an absorbance maximum at 560 nm in the dimethyl sulfoxide (DMSO) solvent. The intensity of the color produced is directly proportional to the amount of total bilirubin concentration present in the sample.

REAGENTS
1. Total Bilirubin Reagent: Sulfanilic Acid 32mM. Hydrochloric Acid 165mM. DMSO 7 M.
2. Bilirubin Nitrite Reagent: Sodium Nitrite 60mM.
3. Bilirubin Calibrator: N- 1 -Naphthylethylenediamine dihydrochloride salt (5 mg/dl).

PRECAUTIONS
1. For In Vitro Diagnostic Use.
2. Specimens should be considered infectious and handled appropriately.
3. Do not pipet reagents by mouth. Avoid contact reagent with eyes, skin and clothing. Do not ingest. Wash hands after use.

REAGENT PREPARATION
Total bilirubin working reagent. Add 0.05 ml (50 µl) of sodium nitrite reagent per 1.0 ml of total bilirubin reagent and mix reagent. Example: 0.5ml sodium nitrite/l0ml total bilirubin reagent, 1ml sodium nitrite/20ml total bilirubin reagent, etc.

REAGENT STORAGE
1. Store reagent and calibrator at refrigerator temperature (2 - 8°C).
2. Combined working reagent can be stored for up to eight (8) hours when kept in an amber bottle at room temperature.
3. Do not freeze reagents.
4. Avoid exposure to direct sunlight.

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

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

INTERFERENCES
1. Young et al. give an exhaustive list of drugs and other substances known to affect the circulating level of bilirubin.[4]
2. In this assay, as in 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. Total Bilirubin reagent.
2. Sodium Nitrite reagent.
3. Bilirubin Calibrator.

MATERIALS REQUIRED BUT NOT PROVIDED
1. Cuvettes
2. Pipettes
3. Timers
4. Appropriate automated chemistry analyzer or spectrophotometer capable of measuring at 560 nm.

TOTAL BILIRUBIN PROCEDURE (AUTOMATED)
Refer to appropriate instrument application instructions.

TOTAL BILIRUBIN PROCEDURE (MANUAL)
1. Label test tubes, "Blank, Standard, Control, Patient". Each tube requires a **blank** tube.
2. Dispense 1.0ml of total bilirubin reagent to all **blank** tubes.
3. Prepare a working reagent. See "Reagent Preparation."
4. Dispense l.0ml of the working reagent into the labeled test tubes.
5. Add 0.1ml (100 µl) of each standard, control and sample to its respective tube. Mix well.
6. Allow all tubes to stand for five (5) minutes at room temperature.
7. Set the wavelength of the instrument at 560nm. Zero with reagent blank.(Wavelength range:500-550).
8. Read and record absorbance of all tubes.

- USE MULTI PURPOSE CALIBRATOR TO REPLACE STANDARD.

PROCEDURE NOTE
1. The final color produced is stable for 60 minutes.

2. For pediatric samples with bilirubins over 3.0 mg/dl, use 0.05ml (50 µl) of sample and then multiply the result by two (2).
3. If the spectrophotometer being used requires a final volume greater than l.0ml for accurate readings, 3ml of reagent and 0.2ml (200 µl) of sample could be used.

CALCULATIONS

Abs. = absorbance

$$\frac{\text{Abs. of unknown - Abs. of blank}}{\text{Abs. of calibrator - Abs. of calibrator blank}} \times \text{Conc of calibrator} = \text{Total bilirubin (mg/dl)}$$

Example:

unknown	= 0.565
Absorbance of unknown blank	= 0.024
Absorbance of calibrator	= 0.480
Absorbance of calibrator blank	= 0.004
Concentration of calibrator	= 5.0 mg/dl

Then

$$\frac{0.575 - 0.024}{0.480 - 0.004} \times 5 = \frac{0.551}{0.476} \times 5 = 5.7 \text{ mg/dl}$$

PROCEDURE LIMITATIONS

1. Serum 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

It is recommended that commercially available control material with known total bilirubin values be included in each set of assays.

EXPECTED VALUES[5]

Infant (after one month) and adults 0.2-1.0 mg/dl.

PERFORMANCE

1. Linearity: 20 mg/dl
2. Sensitivity: Based on an instrument resolution of A = 0.001, this procedure has a sensitivity of 0.01 mg/dl.
3. Comparison: Studies between the present method and similar method yielded a correlation of $R^2 = 0.99$ and a regression equation of $y = 1.13x - 0.09$.

Day to Day precision: Two commercial control sera were assayed for a period of 30 days and the following day to day precision was obtained.

	Level I	Level II
Mean (mg/dl) N = 22	0.68	6.41
S.D.	0.05	0.63
C.V.	7.3%	9.7%

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

	Level I	Level II
Mean (mg/dl)	0.67	6.28
S.D.	0.02	0.15
C.V.	2.9%	2.3%

REFERENCES

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2. Walters, M. Gerand, H., *Microchem J.* 15; 231 (1970). Michaelson, M.: *Sand., J., J. Clin. Lab. Invest.* (Suppl. *49)* 13. 1 (1961).
4. Young, D.S., et. al.: *Clin. Chem.* 21, 10 (1975). Gambino, S.R., et. al.: Bilirubin Assay (Revised), Commission on Continuing Education. *Am. Soc. of Clin. Path.,* Chicago (1968).

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