

Review on Different Methods of Serum Bilirubin Estimation

Abstract

It is quite essential to estimate serum bilirubin in neonates with jaundice. The early prediction of jaundice is usually by observing the yellowish colour of the skin. Various methods of estimation of bilirubin have been developed and used for assessing the risk of jaundice.

Jaundice is the common neonatal problem, that with the timely diagnosis, effective treatment and proper counselling to the mother can decrease the neonatal morbidity. Proper neonatal examination, breastfeeding advice to the mother, identifying the high risk groups, proper history elicitation, quick diagnosis and early intervention can reduce the incidence of jaundice and also prevent long term complications. The main goal is to prevent the risk of severe hyperbilirubinemia and for prevention of kernicterus. Hence there is a need for a sensitive, rapid and a reliable method for the assessment of serum bilirubin. These methods include both invasive and non-invasive analysis. Invasive methods involve estimation of serum bilirubin by obtaining a plasma or serum sample and non-invasive include the assessment of bilirubin using various instruments without the requirement of blood sample from the neonate.

As the invasive method of bilirubin estimation needs more time for analysis to yield a result, many non invasive methods are being studied to find an effective tool which can give fast and accurate results, thus making it an effective screening tool. This review article throws light on the different methods of estimating bilirubin levels in a neonate.

KEYWORDS: Serum bilirubin, Hyperbilirubinemia, Neonatal Jaundice, Bilirubin Encephalopathy, Transcutaneous bilirubin

Introduction

Jaundice is one of the common causes for neonatal ICU admission[1]. Jaundice is mainly a normal condition that happens due to raised levels of bilirubin in the blood, which can cause serious damage to the brain resulting in a poor neurological sequelae and also death. Hence it is essential to diagnose early and intervene accordingly.

Jaundice is mainly of two types in neonates, physiological when it appears in the first week of life between Day 2 and Day 7 and other is Pathological jaundice which appears within the first 24 hours of life or after the one week of life. Jaundice or hyperbilirubinemia can be either due to raised unconjugated bilirubin or raised conjugated bilirubin.

Unconjugated bilirubin can cause hazardous effects if it gets accumulated in cell organelles. Our body has natural mechanisms to protect against bilirubin toxicity, these mechanisms include binding of serum bilirubin to serum albumin, and rapid bilirubin uptake, conjugation of the unconjugated bilirubin, and finally hepatic clearance of bilirubin. All these innate protective mechanisms help in preventing toxic effects of hyperbilirubinemia, however, these effects occur mostly in neonates with a high percentage of unconjugated bilirubin and babies with genetic disorders that affect bilirubin conjugation.

High bilirubin levels usually more than 20 mg/dL in a newborn may result in clinical picture suggestive of brain damage, these clinical features vary from subtle neurologic deficiencies to severe encephalopathy or permanent bilirubin-induced neurologic damage (BIND) also known as kernicterus eventually leading to death [2]. The concentration of serum bilirubin where neurotoxicity features clinically manifest is mainly based on numerous factors like protein-binding property of bilirubin, transporters of bilirubin in the central nervous system, and enzymes in the brain that oxidize bilirubin [3].

In Neonates unconjugated hyperbilirubinemia is more common. Incidence being, 60% in term neonates and 85% in preterm neonates[4].

In Neonates, hyperbilirubinemia is first noticed through, dermal icterus. It usually is noticed in the face and descends downwards to the rest of the body with palms and soles being the last to be affected[5,6]

Timely assessment of jaundice is essential for early diagnosis and treatment. Multiple methods for bilirubin estimation have evolved over the last few decades[7].

Serum bilirubin more than 2 to 2.5mg/dl usually manifests as yellowish discoloration of skin and sclera whereas it is termed latent jaundice when serum bilirubin ranges from 1 to 2 mg/dl [7].

Causes of Hyperbilirubinemia

Hyperbilirubinemia in neonates can be of two types: conjugated and unconjugated type of hyperbilirubinemia. The following two flow charts give the etiology of the same.

Fig 1. Causes of conjugated hyperbilirubinemia

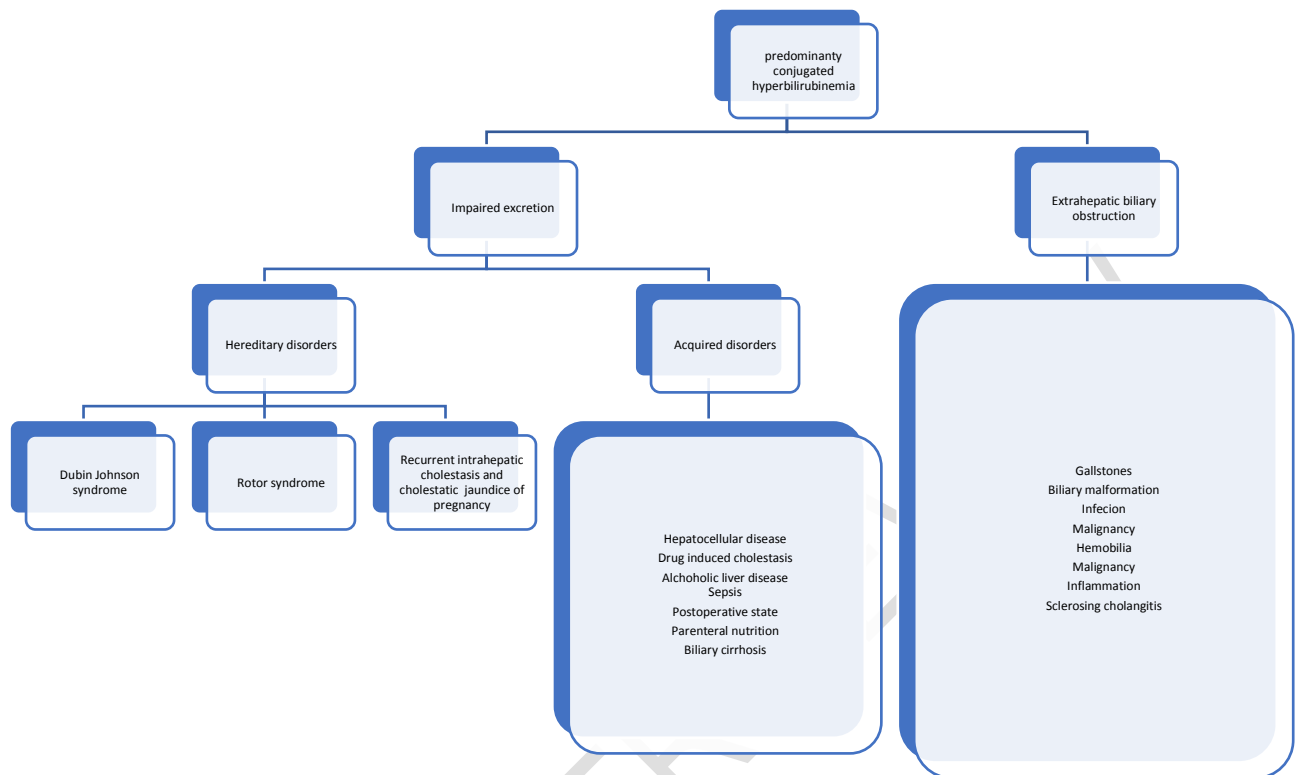
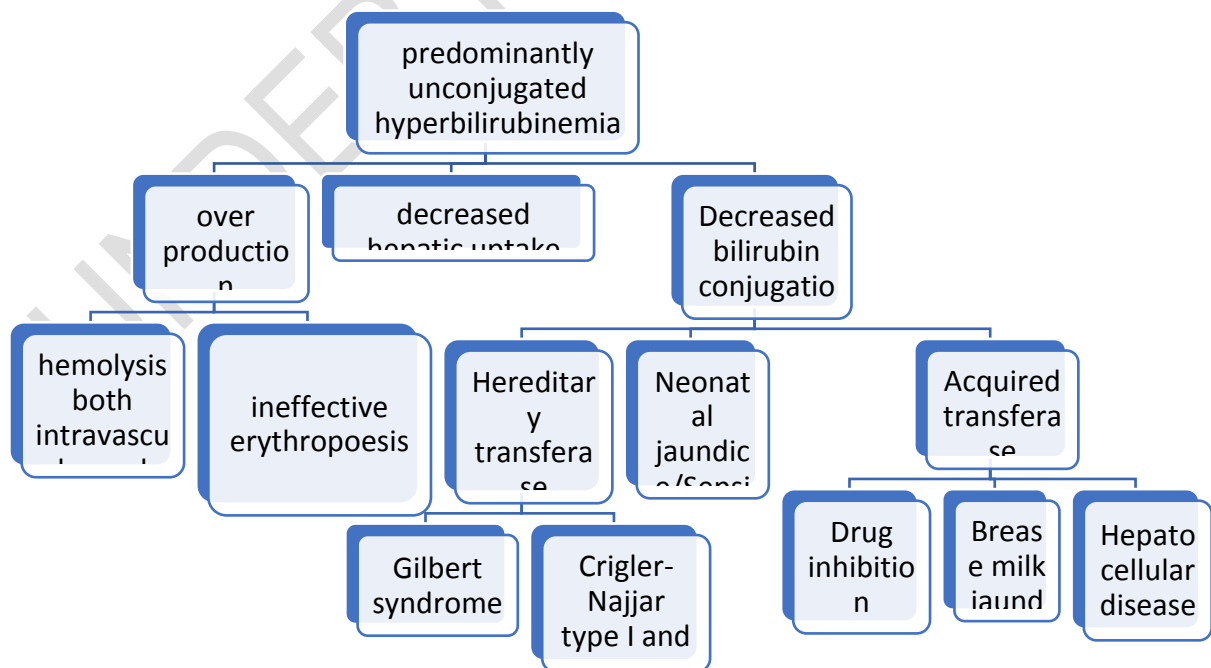


Fig 2. Causes of unconjugated hyperbilirubinemia



The unconjugated bilirubin is not bound to albumin hence it can easily cross the blood brain barrier and affect the central nervous system. Therefore it is essential to diagnose and treat unconjugated hyperbilirubinemia, especially in neonates as it can cause irreversible damage to the brain and increase morbidity.

History of development of Bilirubin estimation methods

1883 marked its importance when Ehrlich used a diazotized sulfanilic acid as a reagent which is also known as Ehrlich's reagent to treat bilirubin present in urine in order to produce a red blue pigment, hence introducing diazo reaction. This diazo reaction forms the foundation of bilirubin evaluation.

Van Den Bergh and Muller were the pioneers who found that when normal serum bilirubin is treated with a Ehrlich reagent it forms a reaction. They observed that when the bile pigment is treated with diazo reagent in the presence of alcohol, it has a different effect than that in the absence of alcohol. When alcohol is added, bilirubin reacts with the diazo reagent and this bilirubin is called indirect or unconjugated bilirubin.

Whereas when the bilirubin reacts in the absence of a diazo reagent it is direct or conjugated bilirubin. This differential estimation helps us to classify jaundice and follow the particular line of management. [8]

Spectrophotometric method

White et al. proposed another alternative for estimating bilirubin levels using spectrophotometry. They used a diluted serum and measured the optical densities at 455 and 575 nanometre in a phosphate buffer. Then they used the difference to obtain a value that is proportional to the amount of bilirubin in the blood. This value is also independent of the haemoglobin content. The only shortcoming of this method is source error due to non bilirubin yellow pigments. [9]. But the blood of a newborn contains very small amounts of carotenoids along with bilirubin unlike that of adults, hence this method becomes a rapid and convenient method of assessing hyperbilirubinemia.

Mertz and West modified this method by estimating unconjugated bilirubin alone. This is done by precipitating conjugated bilirubin with eighty percent acetone. [10]

Thus bilirubin obtained by spectrophotometric and diazo methods show good agreement with that of infants plasma bilirubin levels especially in the first seven days of life, with an exception of infants with inspissated bile syndrome due to accumulation of conjugated bilirubin in the blood. [11]

Diazo Method

Different ways of serum bilirubin estimation has been developed over the decades. Most of these methods are based on Van Den Bergh's diazo reaction method.

1. Van Den Bergh, Malloy and Evelyn reaction : Serum direct bilirubin is treated with Ehrlich's diazo reagent in an aqueous solution to form pink to reddish blue compound (azobilirubin). A study has been performed using diazo reagent. Through this calorimetric estimation, a reading of 450nm of optical density has been observed [8]. Thus giving an appropriate value of serum bilirubin.
2. In the year 1938, the Diazo method has been modified by Jendrassik and Grof using Caffeine benzoate. This has been used as accelerator to release bilirubin from a protein complex thus allowing it to react with diazotized sulfanilic acid. In order to convert red acid bilirubin to a compound that is green coloured, tartrate buffer is used. This has shown a reading of 607nm using Ilford filter in a colorimeter [11]. Hemoglobin and carotene have minimal absorbance at this wavelength.
3. Powell in the year 1944 used diazo reagent along with sodium benzoate urea solution in the blood and the color densities were interpreted using Ilford filter at a wavelength of 625 nanometre. Plasma bilirubin up to 5mg/dl is usually assessed by this method[11].

Diazo method is not an accurate method for bilirubin estimation especially for detecting low serum bilirubin levels. Direct bilirubin overestimates at lower bilirubin levels and underestimates at higher bilirubin levels. So even if there is a slight increase in unconjugated bilirubin it is not detected, this can be a major shortcoming especially in bilirubin estimation of conditions like Gilbert's syndrome. So, this method was further modified in which alkaline methanolysis of serum bilirubin was done following which bilirubin methyl esters are extracted. Then these extracted esters are separated further by chromatography and spectrophotometric determination at a wavelength of 430 nm. 20mg/dl is the maximum linearity limit of the diazo method.

Major problem that arises in using the diazo method is that the diazo method uses artificial standards for matching the colour which are proven to be unsatisfactory and another problem is the extraneous coloured substances that can give erroneous values.

Peroxidase Method

Horseradish peroxidase is used to catalyse oxidation of unconjugated bilirubin and conjugated bilirubin to form colourless products at simple dilutions of 1:40 [12]. Bilirubin that is bound to albumin does not undergo oxidation [13] and the rate of oxidation is slower as compared to the rate of dissociation of bound complexes [14,15]. Thus the present method can only be applicable on where there is a high percentage of predominantly unconjugated bilirubin.

Peroxidase-Diazo Method

Usually a 25ml of serum sample is added to a mixture of horseradish peroxidase and peroxide. Time is given for the reaction to occur before doing the diazo test. The sulfanilic acid present denatures the horseradish peroxidase, thereby halting the oxidation of the bilirubin. Bilirubin that undergoes oxidation forms byproducts that are negative for diazo reaction whereas the non-oxidised bilirubin forms diazo derivatives [16].

High performance liquid chromatography

In the year 1916, Van Den Bergh and Muller observed that the serum samples of patients with obstructive jaundice reacted directly and that of the samples from haemolytic anaemia patients reacted indirectly in the presence of an accelerator. The pigment which reacted directly was called as conjugated bilirubin, also called as direct bilirubin. The indirectly reacting pigment was identified as unconjugated or indirect bilirubin. Direct measurements over estimates conjugated bilirubin at low concentration and vice versa at high concentration. To nullify these limitations alkaline methanolysis was done. Alkaline methanol treatment is done to form mono and dimethyl esters from their respective glucuronide conjugates. However, the Unconjugated bilirubin cannot undergo this reaction. It is extracted in to methyl ester derivatives along with chloroform. These pigments can be separated and quantified by high performance liquid chromatography and it is detected spectrophotometrically in the affluent. Direct measurement of individual pigment is obtained by using an internal standard and calibration of the method with reference bilirubin [17].

Newer methods of Bilirubin Assay

Newer methods for assessment of serum bilirubin, both direct and total bilirubin works based on the principle of measuring the reduction of absorbance at 450nm, produced by bilirubin oxidase. The degree of bilirubin oxidation is easily measured because both total bilirubin and direct bilirubin are oxidised at two different PH of 7.2 and 3.7 respectively. Reducing substances, anticoagulants and haemoglobin

showed no apparent interference. Values measured using the new method were in good correlation with the values obtained by Malloy-Evelyn's method [18].

Non-Invasive Methods

All the spectrophotometric methods require blood samples of the neonates for bilirubin assessment. This causes pain and trauma to the neonate. Also there will be lab to lab variability in the bilirubin analysers. Hence many researches and studies have developed and demonstrated non-invasive methods of bilirubin assessment through measurement of yellowness of the skin of a jaundiced neonate especially use of Transcutaneous bilirubinometer.

Principle of Transcutaneous Bilirubinometer :

Studies showed a high correlation of transcutaneous bilirubin with that of total serum bilirubin. In order to measure the Transcutaneous bilirubin, a device has been used. This works by measuring specific wavelength of the light that has been reflected after permeating light into the skin of the neonate.

Different transcutaneous bilirubinometers measure different wavelengths. The meter analyses the optical spectrum, the wavelength which belongs to, which are further converted to electrical signals by a photocell and they are analysed by a microprocessor to generate a bilirubin value.

Spectral reflectance in a neonate is mainly from melanin, dermal maturity, haemoglobin and bilirubin. In the early days, transcutaneous bilirubinometer was utilised only for a few wavelengths, there was also no assessment of dermal maturity and melanin content. So populations of different ages and races required separate analysis, hence there was a requirement of multiple conversion tables for various population.

Then a new product was introduced known as Bilicheck TM which performs a spectral analysis up to hundred different wavelengths. It does so by quantifying the absorbance of known spectral wavelengths.

These available transcutaneous bilirubinometers were divided into two categories

- 1.) Multi wavelength spectral reflectance meters (Bilicheck TM):

This device is measured to generate five readings and it should be calibrated prior to use. It is applied used on the forehead and the measurements are in micromole per litre (19). Correct pressure should be applied in order to get an apt reading.

Advantages of Bilicheck TM:

- It is found to give accurate readings for newborn of all races and ages, hence there is a no necessity of separate conversion tables for different population
- It is optimised for measuring bilirubin present in venous blood.
- Results are displayed in the respective standard units.
- Proper skin/tip contact is ensured by the small 0.5cm platform provided by the optical tip.

Disadvantage of bilicheck:

- Changing the tip for each measurement, also adds to the cost of operation.



Fig 3. bilicheck

- 2.) Two wavelength spectral reflectance meters – here the two wavelengths are 460nm and 540 nm [10].



Fig 4. wavelength spectral reflectance meters

The Konica Minolta JM-103 or Dräger JM-103 requires calibration daily. Long and short optical paths of JM-103 should read within between +1.0 to -1.0 of reference value. The device is used by positioning on the forehead of the infant skin and measurement is taken. The tip is then cleaned with alcohol wipes before the next infant. Accuracy of both the sternum and forehead has been validated. [20].

JM-105 is also a type of jaundice meter. It is nothing but an upgraded version of JM-103 and it includes bigger touch screen, greater storage and transmission functionality. [21]



Fig 5. jaundice meter

Procedure for measuring transcutaneous Bilirubin

The basic principle of all bilirubinometers remains the same with a different but detailed operating procedure. Every bilirubinometer will have an optic head, this is placed in such a way that there is full contact of the entire area of the optic head with that of the baby's skin. Gentle pressure should be exerted on the skin by the bilirubinometer to read the levels of the bilirubin.

Bilicheck TM differs from the working procedure of other meters. It involves the measurement of bilirubin at five different sites of the neonate before the result gets displayed.[22]

Site of measurement

The most commonly used sites of measurement of transcutaneous bilirubin are the forehead and upper sternum . It could be used against any bony prominence if assessment of different sites is required. However, it is noted that the correlation of the bilirubin values with that of the serum is poor in other areas as compared to that of the forehead and the upper sternum. Hyperemia at the site of measurement can affect the results. Measurement of bilirubin against bruises, birthmarks and subcutaneous hematoma are avoided as it can lead to an erroneous interpretation.[22]

Clinical use of transcutaneous Bilirubinometer

It is difficult to sample all the icteric babies in the postnatal ward to assess hyperbilirubinemia. For one baby in order to assess hyperbilirubinemia on different postnatal days of life it may require multiple samples, subjecting the baby to pain and trauma multiple times. It requires laboratory setup and also the reports of serum bilirubin values takes time. So it is essential to have a non-invasive assessment tool for hyperbilirubinemia in a postnatal ward. It acts as a screening tool for all neonates. It reduces the burden of trauma and also allows faster analysis of hyperbilirubinemia. Many studies and researches have found good correlation between total serum bilirubin and transcutaneous bilirubin, hence rendering it an effective tool.[22]

Conclusion

Among all the methods transcutaneous bilirubinometer method of bilirubin assessment has been increasingly used for neonates due to it's non invasive nature,

reduction of patient and parental distress, ability to deliver the result fast and also reliable result that is very essential for preliminary diagnosis of hyperbilirubinemia.

It can be effectively used for screening purposes in a postnatal ward. Neonates, especially pre-term babies are more prone for hyperbilirubinemia and since their blood brain barrier is immature it can easily develop kernicterus. It is essential to intervene before it can cause permanent damage to the brain. This is done mainly by phototherapy. Phototherapy causes structural and configurational isomerization of bilirubin into simpler products which can be easily excreted from the body. There are other pharmacotherapy also such as giving intravenous immunoglobulin for jaundice due to isoimmune haemolytic anemia, albumin infusion and phenobarbitone. Exchange transfusion is also one of the treatment modalities for hyperbilirubinemia. We follow Bhutani's chart for phototherapy and exchange transfusion in term neonates whereas we use NICE guidelines in preterm neonates.

Initially most babies underwent exchange transfusion, but after increased use of transcutaneous bilirubinometer, the exchange transfusion rate has reduced and so did the phototherapy.

Hence transcutaneous bilirubinometer is an effective assessment tool of hyperbilirubinemia.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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