Ann. For. Res. 65(1): 630-652, 2022

ANNALS OF FOREST RESEARCH
https://www.e-afr.org/

AN EXPLORATION AND EVOLUTION OF BILIRUBIN PHYSIOLOGY AND ANALYSIS

Kabita Kumari[1*], Sharvan Kumar Pahuja[2], Sanjeev Kumar[3]

[1,2]Department of Instrumentation and Control Engineering, Dr B R Ambedkar National Institute of Technology, Jalandhar, Punjab-144011, India

[3]Biomedical Instrumentation Unit, CSIR-Central Scientific Instruments Organisation (CSIR-CSIO), Chandigarh, India

*Corresponding author : kkabita208@gmail.com

Abstract

Bilirubin is a yellow tetrapyrrole molecule found in the gastrointestinal systems, and it is produced when haemoglobin (Hb) is degraded. For treating various liver disorders like jaundice, serum bilirubin in the body is a testing marker. Jaundice develops when the serum bilirubin level is more significant than 2.0 to 2.5 mg/dl. Examining different forms of bilirubin, i.e. conjugated (direct) bilirubin, unconjugated (indirect) bilirubin, and total bilirubin, helps the physician identify the cause and metabolic disorder of jaundice. Inconsistent bilirubin production and removal results in lasting neurologic consequences (kernicterus). In this paper, we have presented a brief introduction to jaundice, the physiological mechanism of bilirubin, its types and causes, clinical approaches toward patients having jaundice, i.e. the conventional method being practiced in clinical laboratories, and various non-invasive systems in the point-of-care settings along with their advantages and disadvantages. Information on bilirubin production and elimination with tracking of bilirubin levels may help to guide the proper clinical management of jaundice. The primary focus is on the progression of established methodologies and techniques to newer ones capable of measuring bilirubin in biological materials.

Keywords- Bilirubin, Biliverdin, Jaundice, Kernicterus, Neonates, Transcutaneous bilirubinometer.

1. Introduction

Jaundice is a frequent physical sign that appears in the first week of human life. It is a common symptom in hepatitis infections A, B, C, and D. As per the fact sheet of the world health organization (WHO), updated in 2020, about 325 million people are struggling with this liver disease and is responsible for more than 1.3 million deaths due to severe or persistent consequences of the liver. Also, from data **of the year 2019, an approx. 2.4 million deaths were reported in the first month of life, and India recorded the highest number of newborn deaths, with 522 deaths per 1,000 lives [1].** In the case of neonates, an approx. 60% of the term (37- 42 weeks of gestation) and 80% of preterm (less than 37 weeks of gestation) infants developed jaundice in the very first age of the life, and some breastfed infants, i.e. roughly 10% of one month old continue to exhibit jaundice. According to the global burden of disease in the early neonatal period, jaundice accounts for 1309.3 deaths per 1,00,000 and globally ranked seventh on the cause of mortality [2].

While in the case of adults generally, jaundice is rare but it can cause severe problems when it is present.

The name jaundice is acquired from the French word 'Jaune,' which corresponds to 'yellow colour' [3]. The condition in which the skin, the mucous membrane, and the sclera of the human body become yellow is called jaundice. This yellowish hue develops in the body when the amount of bilirubin in the blood increases, known as hyperbilirubinemia. Clinically when the bilirubin level reaches 34.2 mol/L or 2 mg/dL, the physical appearance of jaundice occurs. The usual quantity of bilirubin in the blood of a fit person is between (0.3–1.9) mg $dL^{-1,}$ and it consists of conjugated bilirubin (0.1– 0.4 mg dL^{-1}), unconjugated bilirubin (0.2– 0.7 mg dL^{-1}) and a minimal quantity of unconjugated bilirubin that is not bounded to protein (free bilirubin) [4]. Deficiency of iron (anaemia) and coronary artery disease (CAD), among other conditions, are associated with low serum bilirubin levels, which may be used to diagnose diabetic retinopathy [5]. In comparison to adults, the bilirubin level in children was 10-fold higher, and excess bilirubin was related to liver infections as neonate's liver has a limited metabolic capacity and cannot metabolize significant levels. Because bilirubin is harmful to the early newborn brain, serum bilirubin levels might be evaluated continually using standard bilirubin levels, newborn days after delivery, and weight after birth. Additionally, abnormal hyperbilirubinemia results in bilirubin sublimation in the basal ganglia, culminating in kernicterus, i.e. bilirubin induced neurologic dysfunction (BIND) [6]. Kernicterus is characterized by mental impairment, athetotic cerebral palsy, nerve deafness, and upward gaze paralysis. According to a report, at least 4,81,000 term/near-term neonates are affected by hyperbilirubinaemia each year, with 1,14,000 dying and an additional 75,400 surviving with kernicterus [7]. In low- and middle-income nations, jaundice is a primary cause of infant death, mental damage, and other health problems.

2. Bilirubin

Bilirubin is the primary product of heme breakdown that comes from the catabolism of haemoglobin in the reticuloendothelial system [8]. The liver secretes it in the vertebrates, giving characteristic colour (yellowish) to the waste products (feces). Bilirubin is chemically composed of four pyrroles or pyrrole-like rings (tetrapyrrole) with molar mass 584.673 $gmol^{-1}$ and $C_{33}H_{36}N_4O_6$ as a chemical formula (Fig 1).

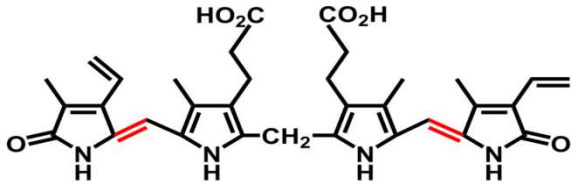

Fig 1. Chemical structure of bilirubin

In the formation process of bilirubin, about 80% of bilirubin is formed from the breakdown of haemoglobin. The remaining 20% is composed of heme-containing proteins such as myoglobin, cytochromes, enzyme, peroxidase, and tryptophan pyrrolase. Bilirubin synthesis is associated with a two-step sequential catalyst degeneration event in reticuloendothelial cells. The liver is composed of hepatic and kupffer cells, which absorbs heme and act on it via heme oxygenase

(present in kupffer cells). This action liberates a supplemental form of iron by accelerating the oxidation of the alpha carbon bond (Cα); as a result of this reaction, equimolar carbon monoxide is formed and is discharged by the lungs. Hence a green pigment 'biliverdin' is formed. Biliverdin is a green tetrapyrrolic bile pigment that makes the colour of bile green.

Further, before releasing nicotinamide adenine dinucleotide phosphate (NADPH), cytosolic enzyme biliverdin reductase acts on biliverdin, leading to the formation of bilirubin, an orange-yellow pigment, as shown in Fig 2. Bilirubin's final structure is severely compressed in the presence of hydrogen bonds and is thus unsolved in diluted aqua solutions at neutral pH. Its insoluble nature is due to its bond to albumin.

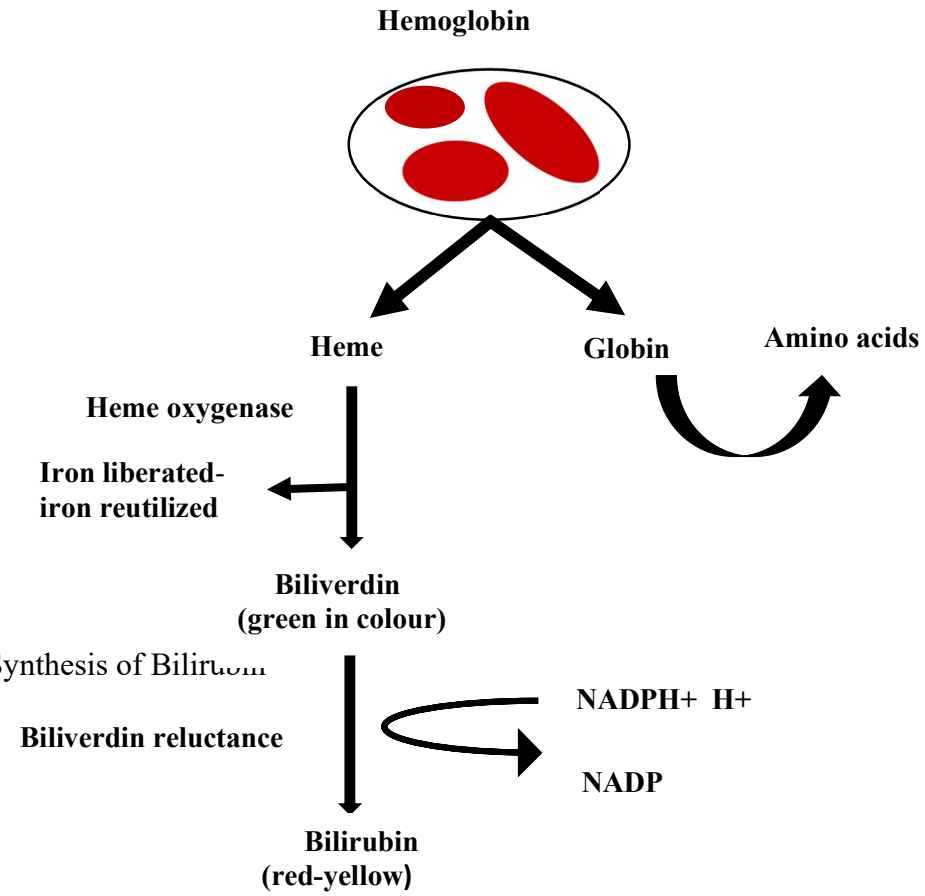

Fig 2. Synthesis of Bilirubin

2.1 Metabolism of Bilirubin

A person's daily bilirubin production is generally around (0.2-0.3) grams [9]. There are various steps involved in the metabolism of bilirubin which includes: i) production of bilirubin, ii) uptake by the hepatocytes (albumin binding), iii) hepatic transport mechanism, iv) detoxication into bile and v) degradation into the digestive tract. The process of bilirubin production has been discussed in Section 2.

(i) Uptake by the hepatocytes

When a water-insoluble (unconjugated) form of bilirubin is produced in the blood plasma, it is absorbed by albumin, acts as a carrier all over the body. Albumin's attachment to bilirubin is extensive, and in optimal conditions, unconjugated bilirubin is not detected in the plasma as free or unbound (non-albumin bound) bilirubin. Albumin prevents the escaping of bilirubin from the blood vessel and its excretion in tissues. The albumin-bilirubin compound can then reach the sinusoidal surface through hepatic circulation. This approach permits the bilirubin pigment to enter the liver without binding to albumin. It assures that the concentration of unconjugated bilirubin linked to albumin in the venous circulation can be consistently measured. The binding of albumin to bilirubin is reversible.

(ii) Mechanism of hepatic (liver) transport

Hepatocytes are a kind of parenchymal cells present in the liver, which plays a vital role in the metabolism process, purification and protein synthesis. The largest gland in the human body is the liver, with approximately (70-85%) of its volume covered by parenchymal cells [10]. Hepatocytes have both front and back faces, which secretes special proteins into the circulation. These proteins bind with bilirubin, called bilirubin-bind-up-protein (ligandin); this prevents the diffusion of bilirubin to microcirculation back to many tissues. Passive diffusion and receptor-mediated endocytosis (cells absorb protein, hormones metabolites etc.) are two distinct methods for transporting bilirubin from the liver sinusoids to hepatocytes. The majority of unconjugated bilirubin enters hepatocytes via the periportal area. Some fraction of conjugated and unconjugated bilirubin within the hepatocytes is transported back into the sinusoidal space and taken up downstream to sinusoidal flow. This takeup is done by organic anion transporting polypeptide family (OATP), i.e., 1A and 1 B. Some conjugated bilirubin absconded in reuptake into hepatocytes gets discharged in the urine. The binding of bilirubin to cytoplasmic protein, i.e. glutathione S-transferases, diminishes the efflux of internalized bilirubin.

(iii) Conjugation

Bilirubin conjugates within hepatocytes that can convert glucose into glucuronic acid (highly polar compound), and this conjugation is caused by the special enzyme uridine-diphosphoglucuronic glucuronosyltransferase (UDPGT). So, glucuronidation is a rate-limiting process in the bilirubin metabolism and eradication. UDPGT converts bilirubin into a water-soluble form (conjugated bilirubin) and facilitates its production over the biliary canalicular system and biliary excretion. There are numerous UDPGT isoforms; however, a functionally significant glucuronidation isoform is UDPGT1A1. The enzyme attaches two glucuronoside molecules to the propanoic acid side chains of bilirubin. The resulting products are bilirubin monoglucuronide (BMG) and bilirubin diglucuronide (BDG), expelled by the hepatocyte. The essential proportion of these conjugates in bile is 85-% BDG and 15% BMG, which is soluble and called conjugated bilirubin [11]. Acquired unconjugated hyperbilirubinemias, Gilbert's syndrome, and the rarer Crigler-Najjar type I and II syndromes are caused by mutations in UDPGT [12]. The conversion of bilirubin to a water-soluble state necessitates the dissolution of hydrogen bonds, which is essential for its elimination via the

liver and kidney. This is accomplished by conjugating the propanoic acid side chains with glucuronic acid [13].

(iv) Detoxication into bile

Four known transporters sited in the canalicular membrane efflux carries the excretion process of conjugated bilirubin. However, the multidrug resistance-associated protein 2 (MRP2), an ATP (Adenosine Triphosphate Dependent Proteinase) dependent transport protein, is predominant in conjugated bilirubin canalicular secretion. By MRP3, a small fraction of conjugated bilirubin is transported into a tiny endothelium-lined space, i.e. sinusoids and portal circulation, which may be reabsorbed into hepatocytes via organic anion transport protein 1B1 and 1B3 (OATP1B1 and OATP1B3). As a result, unconjugated and conjugated bilirubin may escape the cytoplasm of hepatocytes and be transported throughout the body. On the other hand, only conjugated bilirubin has the ability to enter the bile. The rate at which it is absorbed is controlled by the hepatic toxins excretory capacity of conjugated bilirubin. The conjugation process changes the properties of bilirubin by making it water-solvable. And increases the molecule's size and inhibits bilirubin's reabsorption because of its hydrophilic nature and higher molecular weight via the intestinal mucosa. Thus, conjugation aids in eliminating harmful toxins from the body and reduces bilirubin's affinity for albumin.

(v) Deterioration in the gastrointestinal tract

During bilirubin circulation, a tiny amount of bilirubin is selectively reabsorbed across the small intestine epithelium's lipid barrier and experiences enterohepatic circulation. While conjugated bilirubin is not absorbed from the colon, there is no additional metabolic activity, and a minimal amount of deconjugation occurs. When conjugated bilirubin passes through the gastrointestinal tract (GIT) (a distal portion of the small intestine), it quickly dwindles and gets deconjugated by gut microbiota to a series of molecules known as *urobilinogen*. A significant part of it is more distrained to the last part of the "gut", where it is converted into *stercobilinogen,* and eventually, it is passed into the faecal matter. Also, a significant portion of urobilinogen is reabsorbed to be filtered into urine or undergo an intrahepatic cycle. These chemicals are colourless but turn bright yellow when oxidised to produce urobilin, giving faeces its peculiar colouration.

A reduction usually follows the excretion of bilirubin from the body in its content in the blood. This procedure leads to bilirubin recapitulation back into the circulation, manifested by 'jaundice.'. Most of the conjugated bilirubin in serum will be bound to serum, and a few per cent remain free to be filtered through the kidneys. However, some will form complexes that cannot be filtered through the kidneys. This condition has been seen the patients with severe prolonged cholestasis. Daily urinary and faecal excretion is about 4mg to 280mg, respectively [13].

When the amount of bilirubin in the blood is less metabolized, free (unbound) plasma bilirubin concentration increases as in infants due to an immature glucuronidation potential. This permits

bilirubin to efficiently cross blood-brain-barrier (BBB) by diffusion, that accumulates in the brain, causing neurotoxic (poisonous to nerve tissue) consequences including kernicterus (yellow-strained nucleus) [14]. Within this framework, bilirubin's glucuronidation in tissues plays a vital role in controlling its passive transportation along BBB, since only unbounded or unconjugated bilirubin can reach the brain [15]. In singlelayer grown cell lines, massive amounts of free bilirubin disrupt the permeability and secretory activities of human brain microvascular endothelial cells, causing BIND (Bilirubin Induced Neurological Dysfunction) [16].

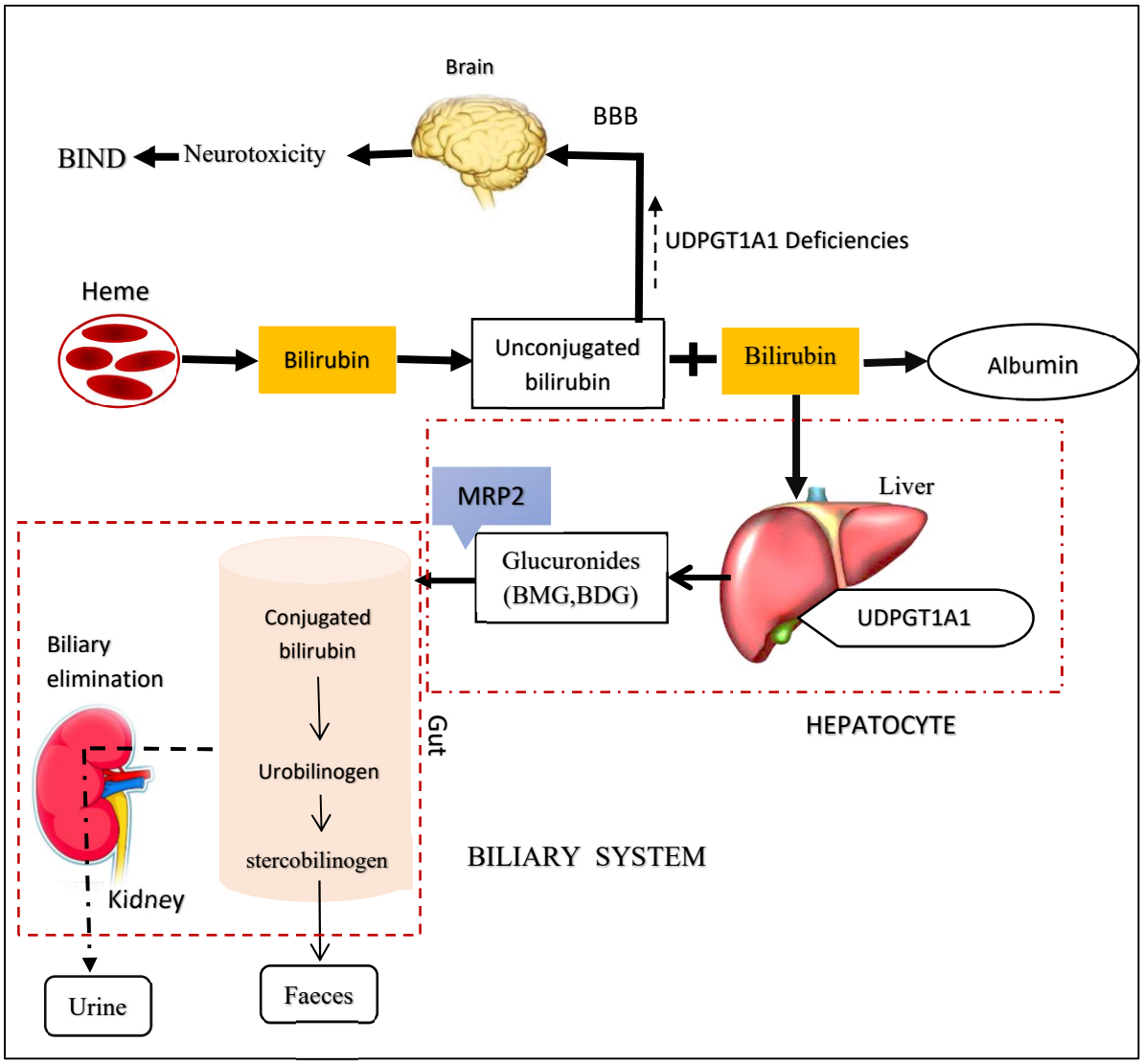

Fig.3: Schematic representation of bilirubin metabolic pathway

2.2 Different Types of Jaundice

(i) <u>Pre-hepatic</u>

An **extravagant disintegration of red blood cells, which bury the liver's ability to conjugate bilirubin** causes pre-hepatic jaundice. However, with normal hepatic function, the liver can

quickly eliminate excess bilirubin; thus, this jaundice is usually mild and moderate. Because the amount of bile pigments entering the gut is more than usual, the amount of urobilinogen reabsorbed from the gut is likewise higher, raising its levels in the urine. A slight increase in serum conjugated bilirubin is also common. Other than enhanced haemolysis, the only causes of pre-hepatic jaundice are specific genetic disorders that induce congenital hyperbilirubinaemia.

(ii) Hepatic

Either poor absorption or conjugation generally causes hepatic jaundice. Flavaspidic acid ($C_{24}H_{30}O_8$), used to treat tapeworm infestation, may cause unconjugated hyperbilirubinemia as it competes with bilirubin for binding to ligandin. Bilirubin transport from the blood to the bile is less efficient when the liver cells are damaged. Hepatocellular jaundice is the medical term for this illness. This condition can be caused by various factors such as viral hepatitis, primary liver cancer, and damage caused by drugs or poisons. It can also be caused by intrahepatic obstruction. Unconjugated hyperbilirubinemia occurs in neonates due to undeveloped hepatic enzyme glucuronosyltransferase and insufficiency of intestinal bacteria that limit bilirubin conversion to urobilinogen. [17].

(iii) Post Hepatic

Hepatic excretory function abnormalities run in families -

a) *Dubin-Johnson syndrome (DJS)* - Dubin et al. and Sprinz et al. early described DJS in 1954, reporting numerous cases with chronic low-grade jaundice (primarily conjugated hyperbilirubinemia) and black livers in healthy young adults [18], [19]. It is likely to be mediated by a hereditary impairment in conjugated bilirubin canalicular transport caused by a mutation in the MRP2 gene that leads to faulty MRP2 transporter production. Contrary to acquired hepatobiliary disease and Rotor syndrome, a small amount of diconjugated bilirubin than monoconjugated bilirubin is found in the serum. This inverted fraction is thought to be distinctive and indicative of homozygous (having identical alleles from both parents.) patients.

b) *Rotor syndrome (RS)*- RS was primarily considered one of the variants of DJS, which was first described in 1948 by Rotor et al. [20]. There are more monoconjugates in RS Serum conjugated bilirubin than diglucuronide conjugates. Several functional tests indicated that patients with RS have a deficit in hepatic uptake and storage rather than biliary excretion. RS is an autosomal recessive condition [21].

Table1: Different potential causes of pre-hepatic, post-hepatic and hepatic jaundice.

Type of Jaundice	Mechanism involved	Causes
Pre-hepatic	Excessive heme liberation	• shortage in lifespan of RBCs • hemolytic anemia

		• G6PD deficiency • spherocytosis • malaria
Hepatic	• Improper liver functioning • Obstruction of small bile ducts • Abnormal secretion	• reduction in hepatic bilirubin uptake • hepatic tumors • liver cirrhosis • viral hepatitis • congenital enzymatic defects • alcoholic hepatitis • gall stones • leptospirosis • autoimmune liver disease • liver cancer
Post-hepatic	Mechanical obstruction of large bile ducts or digestive tracts	• Biliary atresia • Gall stones • Infection in biliary tree • Pancreatic cancer • Pancreatitis • Cholestasis of pregnancy • drugs

(iv) <u>Defects in hepatic excretory function</u>

a) *Drug induced and postoperative*: When the bile ducts left the liver an obstruction occurs that leads to extrahepatic cholestatic jaundice. Because of the absence of bilirubin in the gut, not any part of it is reabsorbed into the bloodstream and eliminated as urobilinogen by the kidneys. This indicates that the urine has little or no pigment. However, as bile pigment levels in the blood arises, it is expelled in the urine, which results in a dark-colored urine after reaching to renal threshold [22].

b)*Hepatitis and Cirrhosis*: Hepatitis is an inflammation of the liver that can be caused by a variety of reasons, including well-known viruses like hepatitis B. Cirrhosis is also a condition in which functioning scar tissue replaces good liver tissue. The liver is exceptional in its ability to heal after injury, although this process is disrupted by recurrent injury or persistent infections, such as chronic hepatitis [23]. The liver eventually loses its ability to function correctly, and scarring develops. Cirrhosis refers to long-running liver scarring, mainly caused by chronic hepatitis. Hepatitis can be reversible (curable); however, cirrhosis is not. Thus, bilirubin's primary goal in serum fractionation is to separate hepatic and biliary obstructive disease characterized primarily by unconjugated hyperbilirubinemia.

3. Role of bilirubin in neonates

The common neonatal jaundice are physiologic and pathologic. And if it presents within the first 24 hours after birth, it is considered pathologic jaundice. An approximately(6-8)mg per kg per day bilirubin is being produced by newborns. The bilirubin production rate in neonates is more than twice that of adult bilirubin production, owing to relative polycythemia and higher red blood cell turnover in neonates[22]. Bilirubin production typically declines to the adult level within 10 to 14 days after birth [24]. The newborn's bilirubin load arises first, after increased bilirubin production, since red blood cells have a higher density. Secondly, by slower bilirubin clearance due to deficiency in the uridine diphosphatase glucuronosyltransferase (UGT) enzymes [25]. This enzyme has roughly 1% of the adult liver's activity in newborns. If the amount is excessively high at delivery, an infant's blood may be examined numerous times in the first few days of life to evaluate liver function. Jaundice in a newborn can be severe and life-threatening if left untreated.

4. Evaluation methods Of Jaundice

The most prevalent reason for jaundice is a systemic disorder and its associated metabolic abnormalities. In most cases, the patient's serum total bilirubin is fractionated into two or three components: Direct or conjugated bilirubin is bilirubin that has been linked to glucuronic acid, a glucose-derived acid, by the liver. When Bilirubin is not attached to glucuronic acid is called indirect, or unconjugated, bilirubin. A combination of both direct and indirect bilirubin together in the blood is called total bilirubin. There are various methods that are used for the analysis of bilirubin. In clinical laboratories, where the most pressing problems of human disease are addressed, the analytical processes have always been valued for their speed and easy procedures[26]. Therefore, comparing the yellow colour of serum to that of a yellow standard was widely used to measure serum bilirubin for many years. So, clinically there are two existing methods for measuring bilirubin, i.e. total serum bilirubin (TSB) and transcutaneous bilirubin meter (TcB). TSB is an invasive (skin penetration) method, and it is measured preferably by blood tests. In contrast, TcB is a non-invasive method used for initial screenings since it can be easily operated [27]. Total bilirubin is found by summing direct and indirect bilirubin. The indirect amount is calculated by taking the difference between the direct and total values to distinguish between different types of jaundice.

Total and direct bilirubin estimation is essential for diagnosing diseases associated with hyperbilirubinemia, which clinically appears as icterus. Bilirubin is a well-known marker commonly included in biochemical testing for individuals suffering from liver impairment or other conditions. Many factors can cause hyperbilirubinemia: An increase in the destruction of red blood cells, i.e. hemolysis, neonatal jaundice, inefficient erythropoiesis (production of red blood cells), and hepatic illness are all linked to total bilirubin (viral or drug-induced hepatitis, cirrhosis) [28]. Because instruments for testing bilirubin levels in serum and treating neonates who have jaundice are widely available in high-resource countries, jaundice morbidity and mortality are extremely rare. In low-resource countries, however, jaundice remains a significant cause of newborn illness

and mortality [29]. Hence, a bilirubin measuring device is low cost and can be readily available for low resource countries. Because of the numerous disorders linked to substantial bilirubin elevations, there is a need to develop new innovative diagnostic approaches to aid in the detection of the diseases caused by it. Several existing conventional techniques for estimating conjugated, unconjugated and total bilirubin have been thoroughly examined reviewed in paper [30].

4.1 Spectrophotometry method

Spectrophotometry is used for measuring the amount of a solute in a solution when a certain amount of light rays passes through it, causing the solute to absorb some significant amount of light. Each substance ingests or diffuses light in a specific wavelength range according to the fundamental rule. The measurement of bilirubin in newborn serum via spectrophotometry is a quick and straightforward procedure requiring only a small number of samples for research. In this process, bilirubin is combined with sulfanilic acid to produce red azodipyrrole. Various attempts were made to reduce the intervention in the process brought on by chemicals in serum and urine. A procedure for analyzing data was devised in the presence of the biocatalytic system hemoglobin/glucose oxidase/glucose in one such effort. When combined with H2O2, both myoglobin and haemoglobin exhibit pseudocatalytic activity. This process was initiated by using poly(vinyl alcohol) as a biocompatible material. In comparing the spectrophotometric method with diazo, it is found that total bile pigment level in plasma is higher for adults paper [17]. The approach is based on bilirubin absorption at 454 nm; however, haemoglobin equally absorbs bilirubin at both 454 and 528 nm emission wavelengths. The influence of hemolysis was demonstrated by subtracting the absorbance from higher to lower wavelengths, yielding a value that could be attributed primarily to bilirubin [31]. However, the occurrence of additional sorts of bilirubin and chromophores (chemical groups) in older children and adults limits this method for its usage in neonates aged below 2–3 weeks. One of the significant factors that can cause erroneous results in spectrophotometric tests is the presence of non-yellow pigments [32]. So, a four channel 'SIEMA' i.e. simultaneous injection efficient mixing flow analysis system, was designed to allow the simultaneous analysis of both urobilinogen and bilirubin from a urine sample. A syringe pump, connectors, holding coils, mixing coils, and spectrometer make up the designed system. Two of the four channels of this SIEMA system were used to measure urobilirubin, while the other two were utilized to determine bilirubin. Using this spectrophotometric approach has been deemed the optimum solution for measuring bilirubin and urobilinogen in urine samples [33]. The same developed system can be used to trace the amount of bilirubin and creatinine in the urine sample. Creatinine is produced when creatinine phosphate is broken down during the metabolism process of muscle.

It is continuously created inside the body and eliminated in little amounts through the urine. The kidneys are responsible for removing creatinine from the body completely. The creatinine removal, which indicates the glomerular filtration rate (GFR), is clinically significant for renal function can

be calculated using creatinine levels in the blood and urine [34]. The colour reactions for bilirubin and creatinine detection were found by the diazo and Jaffé reactions [35].

A novel method for total bilirubin based on the absorption of numerous wavelengths were developed, and accordingly, an algorithmic rule for calculation was assessed. Using this method, specimens comprising varying quantities of unconjugated bilirubin (UB), conjugated bilirubin (CB), and delta (protein-bound) bilirubin (BD), accuracy was tested. Thus, to overcome the limitations observed in earlier spectroscopic methods providing reliable output. One of the primary purposes of the research was to assess the accuracy and precision of the created approach using a radiometer and blood gas analysis/co-oximetry (ABL 735) to measure total bilirubin concentration in plasma [36]. Atef M. Attia et al.[37]developed a multi-component spectrophotometric approach to measure the accurate blood concentrations of total bilirubin (TB), oxyhemoglobin (HbO2), and methemalbumin (Mha) in patients with haemolytic jaundice. Experimentally, a preparation method for the serum sample was developed and compared with the diazo assay. Theoretically, formulas for calculating the principal components (TB, HbO2, and Mha) in human sera have been derived. The author suggests this method provides highly sensitive and accurate because it could detect TB, HbO2, and Mha concentrations as low as 6.86, 0.162, and 2.252 µmol/l [38].

Benefits: Specific or Precise, consistent and fast technique compared to process used in schedule.

Drawbacks: Low sensitivity and specificity; results get affected by substances reacting with colours.

Table2: Multicomponent spectrophotometric method expressing amounts of total bilirubin (TB), oxyhemoglobin (HbO_2) and methemalbumin (Mha) in patients with haemolytic jaundice

Parameters	Neonates (sample size,n=4)	Adults (sample size,n= 12)	P value
HbO_2 (µ mol/l)	**57.965± 48.805 (25.436 –130.365)**	**2.069±1.024 (0.162–3.206)**	**<0.001**
TB (µ mol/l)	**174.582 ± 95.855 (83.003 – 284.093)**	**10.020±3.648 (6.86–19.873)**	**<0.00005**
Mha (µ mol/l)	**51.493± 28.929 (13.951–83.759)**	**5.983±1.833 (2.252–8.026)**	**<0.00005**

The two-tailed probability of differences between the means of two groups is indicated by p-value, and values are expressed as ± S.D. (standard deviation).

4.2 Diazo method

The diazo method (Jendrassik Grof) is one of the most extensively used methods for measuring different forms of bilirubin that was initially developed in 1883 by Ehrlich. He discovered a red-

blue coloured pigment when he treated bilirubin in urine with diazo reagent [39]. Van den Bergh's introduction of the diazo reaction for serum bilirubin in 1918 led to broad acceptance of the method for measuring the pigment in serum. In this reaction, two isomeric pigments are formed with absorbance maxima of 530 nm, resulting in direct bilirubin. The unconjugated bilirubin needs an accelerator to solubilize by adding a small organic molecule to the unconjugated bilirubin. It can then displace it from albumin. This method provides a result within one minute of reading; however, it is pH sensitive, so its output got affected. There have been multiple modifications over time in the diazo method. Malloy and Evelyn (1937) introduce a fifty percent methanol accelerator that combines with total bilirubin in the serum to produce a pink to the reddish purple coloured substance (azodipyrrole). This was used to determine bilirubin at 540nm absorption. In the diazo reaction for determining total bilirubin level, a mixture of caffeine and benzoate in acetate is utilized as an accelerator. The National Committee for Clinical Laboratory Standards in the United States recommended this approach for total bilirubin estimation [40].

An automated diazo approach involves incubating serum/plasma with a diazo reagent at pH 1.7–2.0 to produce a diazonium salt. The azobilirubin isomers are formed when the resultant product combines with bilirubin. The conjugated bilirubin is the most common type transformed by diazotized sulfanilic acid (approximately five percent of unconjugated bilirubin may react well). The intensity of azobilirubin's red colour intensity is measured around 600 nm and is proportional to the conjugated bilirubin concentration. Following accelerants (e.g., caffeine, sodium benzoate, or methanol), diazo reagents interact with unconjugated bilirubin., allowing the total bilirubin concentration to be determined. While the diazo methods are low-cost and easy to automate, they have some drawbacks, such as lipemia, intervention with low amounts of hemolysis [41].

Benefits: Acceptable inter-laboratory transferability, reproducible and reliable.

Drawbacks: pH dependency compromises the precision, delay in producing results, blood sample requirement is high.

4.3 Vanadate Oxidase method

The vanadate oxidase method can be accomplished by using vanadic acid as an oxidizing agent. This technique is based on bilirubin oxidation to biliverdin, oxidized to produce uncoloured products. Total bilirubin (Bt) is oxidized by vanadate in the presence of detergent to create biliverdin and, is further converted to colourless compounds using the vanadate oxidase method. Removal of detergent leads to the determination of direct bilirubin (Bd) and decreases the optical density of Bt or Bd. Bt and Bd values are compared in blood samples from clinically healthy dogs, monkeys, and rats. As assessed by the Diazo and vanadate oxidase techniques, these are spiked with panels of varying concentrations of commercially available bilirubin standards. Interference from coexisting serum substances is minimal when using the vanadate oxidase method. The vanadate method can be used as an alternative to the Diazo method for samples containing interfering chemicals because it is simpler and faster [42]. Although the vanadate oxidase approach has been used in medicines for humans for a long time, it has only been used in animals on a few occasions [43]. An electronic instrument has been designed to use a vanadic acid-

based photometric method. This is used to measure the bilirubin through the skin. The vanadate oxidase method involves mixing specimen with reagent at about pH 3, converting DBIL to biliverdin, lowering the yellow absorbance (main wavelength 450 nm, sub-wavelength 546 nm) [41]. The vanadate oxidase methods for measuring direct bilirubin experience minimal hemolysis interference [44].

Benefits: Lower interferences by substances like hemolysis, good correlation with the diazo method, more comprehensive analytical measurement range.

Drawbacks: Open analyzer channels are required for running, high cost, required calibration before testing with unconjugated bilirubin in human serum.

4.4 High-Performance Liquid Chromatography (HPLC) method

Chromatographic approaches appear suitable for examining the distribution of distinct bilirubin species in serum and their clinical importance. The four bilirubin fractions (unconjugated, mono and diglucuronide, delta-bilirubin) can be quickly separated and quantified using HPLC procedures. Bilirubin fractions in serum comprise a δ bilirubin (δB) or bilirubin-albumin bond that is covalently linked to serum albumin, bilirubin monoglucuronide (BMG) form, bilirubin diglucuronide (BDG) form, and unconjugated bilirubin (UCB) form, according to various high-performance liquid chromatography (HPLC) analysis. In paper [45], HPLC methods have been used for separating and quantifying four different fractions of bilirubin, i.e. δB, BDG, BMG and UCB in untreated human serum. Here a multimode column has been used that can isolate four fractions of serum bilirubin by isocratic elution. Specifically, this method was an accurate quantitative HPLC approach that could be used to compare the accuracy and features of different bilirubin measurement routines. The total bilirubin concentration is consistent if measured by the HPLC method compared to the values obtained using the method by Jendrassik-Grof [46]. A reverse-phase HPLC was employed to quantify at least three bilirubin species in bile and four species in human serum. The approach provides semi-quantitative data on the concentrations of the four species in serum, demonstrating that they differ significantly amongst diseased serum. The column's separation efficiency decreased as the number of analyses increased. This method is commonly used to analyze serum globulins without using sodium sulfate. As increasing serum protein became adsorbed to the silica gel particles in the column [47]. An HPLC based ultra-sensitive method has been developed for biliverdin and free bilirubin findings in human serum. Here consistent reverse phase HPLC combined with thermal lens spectrometric detection(TLS) system is excited by krypton laser at 470nm. It facilitates the separation of IX-α biliverdin and IX-α bilirubin compounds within eleven minutes; step gradient elution is built by changing mobile phase composition to improve sensitivity. This system shows the concentration ratio of unbound biliverdin with free bilirubin , and it provides crucial information that helps in preventing some pathological conditions in humans.

Benefits: High resolution and reproducibility, compassionate and precise, enable the identification of the type of bilirubin.

Drawbacks: Requirement of costly chemicals, proficiency in management.

4.5 Fluorometric method

When a beam of light hits a particular material, it emits visible light or radiation, called fluorescence. And substances showing this phenomenon is known as fluorescent substances. Fluorescence is spontaneous, starts immediately after light absorption, and stops when the incident light is cut off. In a fluorometric enzymatic study, chemical derivatization of bilirubin oxidase (BOx) with a fluorescein derivative (FS) produces an artificial enzyme (BOx–FS), with excitation at 487nm and emission at 520 nm, respectively. In this reaction, the changes within the fluorescence of the Box-FS enzyme depends on the type and concentration of bilirubin. A mathematical model has been developed to optimize the changes in fluorescence parameters. The concentration range in which the model can be applied is up to 12mg/l with a precision of about four percent can be built up to measure three different types of bilirubin (free, conjugated and albumin-bound bilirubin). This approach eliminates the problem of free enzyme fluorescence in the spectrum area (which is highly susceptible to interferences) while allowing for a more extensive linear response range. This work aims to develop a method that combines the various aspects of the kinetic profile method to determine direct bilirubin with a multivariate alignment method. It was performed by inspecting different kinetic behaviour of direct and total bilirubin. Because the fluorescence was monitored using a traditional luminometer, only kinetic discrimination was possible [48]. A non-enzymatic fluorescence quenching method was used for determining free bilirubin. The method was performed using human serum albumin (HAS) with a minimum detection limit of (248±12)nm gold nanoclusters stabilised by human serum albumin were used in blood samples., each size approx. 1.0nm (HSA-AuNCs) as a fluorescent probe. A robust binding constant of 0.55×10^6 L/mole was achieved between HSA-AuNCs and bilirubin. The fluorescence response from λ_{530}nm to λ_{745}nm of HAS-AuNCs, which is excited at λ_{380}nm, was monitored. It doesn't get affected over a wide pH range up to (6-9) and a temperature range of (25-50)°C and thus, offers to analyze the sample under considerable medial biological conditions. The colorimetric method has also been used for finding free bilirubin since the free bilirubin is oxidized with a limit of detection of (200±19)nM to a colourless compound. The peroxidative action of HSA-AuNCs assists the assay's progress that can be visually monitored [49]. A novel fluorescent post-synthetic metal-organic frameworks (UIO-66-PSM) sensor was designed. Post-synthetic modification (PSM) is a valuable method for introducing functional groups into MOFs to be used as fluorescence sensors. Following three factors are considered while designing the sensor: (a) UIO-66-NH2 possesses unsaturated metal sites (Zr) that can interact with free bilirubin carboxyl groups. (b) 2,3,4-trihydroxybenzaldehyde (THBA)was used in MOF because it contains a lot of hydroxyl groups, which help with water solubility and provide free bilirubin recognition sites. (c) As UIO-66-PSM has so many π electrons, it tends to create a π -π contact with the aromatic ring of free bilirubin. The interactions between the UIO-66-PSM and free bilirubin may affect this sample's fluorescence activity, resulting in efficient fluorescence resonance energy transfer (FRET). The MOF (metal-organic framework) based fluorescent probe unveils low detection limit, wide linear with a fast response time and high selectivity towards free bilirubin

without being affected by the interferences of other biomolecules and metal ions. This system offers invigilation of free bilirubin in human serum samples and has been clinically used to diagnose jaundice [50].

Benefits: Relatively sensitive and therefore can recognize less quantity of compounds.
Drawbacks: Collision instead of fluorescence may cause loss of energy**, very sensitive concerning the changes in the environment, their structure get changes and can be modified by changing solvents.**

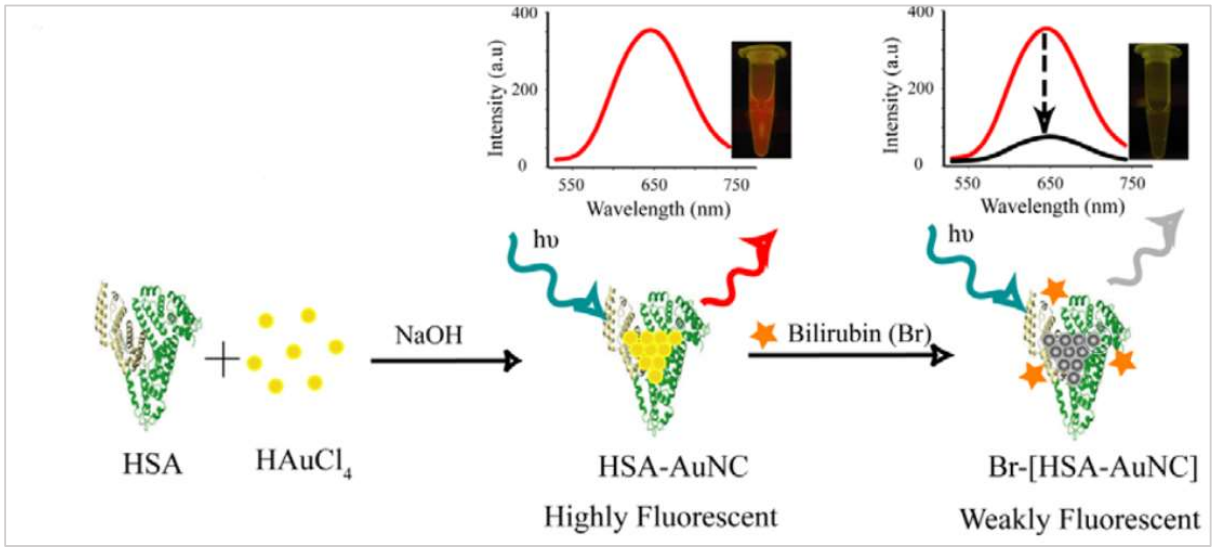

Fig.4: HSA-AuNCs as fluorometric probe for free bilirubin estimation [49].

5. Non-invasive determination of Bilirubin

In India, various factors such as physiological and infectious conditions, blood group incompatibility, breast milk and G-6PD deficiency can cause neonatal jaundice [51]. Higher bilirubin levels can cause severe neurotoxicity in newborns, such as kernicterus, as well as damage to the white matter of the adult brain. Kernicterus is a prominent severe state of jaundice. It describes the yellow nuclei and settling of unconjugated bilirubin in the brain's basal ganglia. According to a 2012 report by the United Nations Children's Fund, twenty-one children die every minute in most undeveloped and developing nations, mainly from preventable causes such as newborn jaundice. As a result, these factors present an ominous scenario for jaundice care, necessitating a comprehensive evaluation of bilirubin levels. The American Academy of Pediatrics has issued guidelines on early diagnosing and monitoring hyperbilirubinemia. [52].

The measurement of total serum bilirubin (TSB) is frequently required in treating jaundiced newborns and is currently considered the standard gold method. Blood tests are the most accurate way to quantify total serum bilirubin (TSB), as they require drawing of blood, but these tests are invasive. Invasive blood sampling causes blood loss and infection at the sample site, agonizing and distressing for neonates. Besides, the traditional method is costly, complex, time-devastating, and dilatory, preventing prompt diagnosis in underdeveloped countries [53]. These issues have prompted researchers to look forward to a non-invasive, repeatable method for estimating jaundice that is progressively replaced by transcutaneous bilirubinometers. Transcutaneous bilirubinometers are often used for initial screenings since they are non-invasive, simple, and have fast detection capability. Also, repeated measurements can be taken on the same subject. The operating principle of TcB is based on measuring the excitation of light with a specific wavelength directed into the skin of the subject, i.e. yellowness of the skin is measured by analyzing the spectrum of light reflected by the subject's skin and subcutaneous tissues. In different transcutaneous bilirubinometers, the number of wavelengths used varies. TcB readings have previously been reported to coordinate well with TSB levels, with correlation values ranging from 0.77 to 0.97 [54]. Some lucratively available TcB meters include Philips Bilicheck, BiliMed, Drager JM105, and Bilistick. Yamanouchi et al. were the first to introduce transcutaneous bilirubinometry into medical practice in 1980 [55]. This device generated a number index that was further correlated with TSB. However, the performance of TcB meters gets variated by race, gestational age, birth weight, skin colour also with the preterm and term deliveries. As TcB reflects mainly the extravascular space and may get affected by thickness and pigmentation of the skin.

SpectRx Inc developed the Bilicheck, and for accurate TcB measurement, BiliChek uses multiple wavelength technology, i.e. between 400 nm and 760 nm, to account for variances in skin pigmentation and haemoglobin. It is also accompanied by a disposable tip that must be attached to the probe before the TcB measurement. Then comes the second generation TcB device, i.e. BiliMed (Med and Led,Gilly, Belgium), works by examining four separate wavelength windows. Unlike Bilicheck it doesn't require any disposable material. Gwendoline et.al evaluated the accuracy of TcB measures taken with the Draeger JM 103 device in assessing jaundice in black Zimbabwean babies against the conventional diazo technique. The study was performed on a newborn's sternum and forehead between the age ranged from 28 to 42 weeks. The correlation coefficient between serum and TcB at sternum was 0.77 and at forehead was 0.7. Hence, validating JM103 and can be used as a screening tool for measuring jaundice in this population [56]. In a comparative study [57], bilirubin concentration was determined employing transcutaneous devices with Bilicheck, BiliMed and JM-103, in random order. It has been found that both Minolta JM-103, Bilicheck System (Philips) were equally reliable screening tools for hyperbilirubinemia in multiracial neonatal populations but not with BiliMed.

A spectrum-based non-invasive device has been developed to accurately measure bilirubin in newborns. Here, the measurement site is the subject's nail illuminated by an optical fiber guide , then the amount of light reflected was collected and sent to a spectrometer. The correlation coefficient between the developed TcB device and TSB was 0.95. Hence, the device is suitable for detecting bilirubin in a wide range of physiological circumstances can be accurately and precisely predicted [52]. Polley et al. developed an optical device for the online monitoring of jaundice in human subjects. The device measures the optical spectrum of the conjunctiva and derived pigment concentration by using diffused reflection measurement. An optical-fibre-based setup is created to measure the absorption spectrum from (400-800)nm and monitor the level of bilirubin. The results reveal a strong correlation between relative absorption at 460 and 600 nm and the bilirubin content measured in the serum [58]. A new generation of colorimetric-based methods has been used to detect the concentration of bilirubin in a person. The method involves taking images using a smartphone camera with a built-in light source. Also, a colorimetry-based wearable sensor is designed for the real-time detection of jaundice, having oxygen saturation related to total haemoglobin of the blood (SpO_2) and heart rate sensing functionalities. This device may aid in the early diagnosis of newborn apnea and other life-threatening disorders. A list of TcB devices investigated along with their critical performances (LOD)and bilirubin determination under different principles are included in table 3.

Table 3: TcB devices investigated with sample volume, operating principle and LOD

TcB Device	**Principle of Measurement**	**Sample Volume**	**Level of Detection (LOD)**	**References, (Year)**
JM-102 (Minolta/Hill-Rom Air-Shields)	Reflectance based on two wavelengths analysis	101	(19.9±2.8) mg/dl	[59], (2002)
Bilitest (BB 77)	Reflectance bichromatic photometry	241	>13 mg/dl	[60], (2008)
Bilicheck (SpectRx Inc.)	Intensity of light at multiple wave-lengths	223	>12.2 mg/dl	[61], (2011)
Twin Beam	Dual wavelength photometry	110	(.0113- 4.062) mg/dl	[62], (2015)
JM-103	Optical density of the skin at two wavelengths	5075	>15 mg/dl	[63], (2017)

Bili Capture	Optical imaging/ capturing of conjunctival image using camera	100	(8.6 -18.1) mg/dl	[64], (2019)
JM-105	Two wavelengths and dual optical path system	217	>7.91 mg/dl	[65], (2020)
Ajo-Neo	Spectrometry based technique	1968	>15 mg/dl	[66], (2020)

6. Conclusion

The condition of jaundice is a source of concern, and especially for newborns, it's a common cause of mortality and morbidity. Therefore, clinicians should take precautionary for the proper diagnosis and treatment of the disease. This study describes the physiology of jaundice, i.e. metabolism of bilirubin and its synthesis and types and causes. It also presents the different conventional with their operating principle, advantages, and disadvantages used to evaluate jaundice. Among the different methods, spectrophotometry, diazo, vanadate oxidase, high-performance liquid chromatography, and fluorometric are the accustomed methods already in practice. However, the spectrophotometric technique is mainly employed despite the fact that these approaches have some drawbacks, such as the necessity for pre-treatment, distillation, and large sample quantities. To overcome these limitations, the demand for transcutaneous methods has become widespread due to their non-penetrating, rapid, convenient, and reliable nature. Based on the principle, mass production of different diagnostic devices has been manufactured by many companies, which are helpful at birthing centres and in outpatient settings. However, research on this subject is ongoing to develop innovative ways of bilirubin determination as available devices vary depending on the environment. The introduction of simple, fast, and cost-effective devices for effective measurement and treatment is essential and has yet to develop appropriately in low and middle-income countries. Several attempts have been made to employ advanced techniques to meet such requirements. Also, incorporating the devices with the Internet of Things (IoT) such as smartphones and Bluetooth could enhance the device applications in remote and resource limiting areas.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Funding

None

References

[1] "World Health Organization." https://www.who.int/.
[2] B. O. Olusanya, S. Teeple, and N. J. Kassebaum, "The contribution of neonatal jaundice to global child mortality: Findings from the GBD 2016 Study," Pediatrics, vol. 141, no. 2, pp. 2016–2020, 2018, doi: 10.1542/peds.2017-1471.
[3] C. Article, "Jaundice in the Adult Patient," 2004.
[4] B. Batra, S. Lata, J. S. Rana, and C. Shekhar, "Biosensors and Bioelectronics Construction of an amperometric bilirubin biosensor based on covalent immobilization of bilirubin oxidase onto zirconia coated silica nanoparticles / chitosan hybrid film," Biosens. Bioelectron., vol. 44, pp. 64–69, 2013, doi: 10.1016/j.bios.2012.12.034.
[5] J. O. Chung, S. Park, D. J. Chung, and M. Y. Chung, "Relationship between anemia, serum bilirubin concentrations, and diabetic retinopathy in individuals with type 2 diabetes," pp. 1–5.
[6] B. A. M. Van Der Geest, I. M. Theeuwen, I. K. M. Reiss, E. A. P. Steegers, and J. V Been, "Assessing knowledge and skills of maternity care professionals regarding neonatal hyperbilirubinaemia : a nationwide survey," vol. 0, pp. 1–13, 2021.
[7] R. Mori et al., "newborn : incidence and impairment estimates for 2010 at regional and global levels," vol. 74, no. December, 2013, doi: 10.1038/pr.2013.208.
[8] A. Kalakonda, B. A. Jenkins, and S. John, "Physiology , Bilirubin," pp. 10–13, 2021.
[9] V. Narwal et al., "Sensing and Bio-Sensing Research Bilirubin detection by different methods with special emphasis on biosensing : A review," Sens. Bio-Sensing Res., vol. 33, no. June, p. 100436, 2021, doi: 10.1016/j.sbsr.2021.100436.
[10] Z. Zhou, M. Xu, and B. Gao, "Hepatocytes : a key cell type for innate immunity," no. September 2015, pp. 301–315, 2016, doi: 10.1038/cmi.2015.97.
[11] S. Louis and J. Lopez, "Carl A . Burtis , Edward R . Ashwood and David E . Bruns (eds): Tietz Textbook of Clinical Chemistry and Molecular Diagnosis," vol. 28, no. 1, pp. 104–105, 2013, doi: 10.1007/s12291-012-0287-7.
[12] J. M. Kirk, "Review Article Neonatal jaundice : a critical review of the role and practice of bilirubin analysis," no. Figure 1.
[13] N. Yazigi and W. F. Balistreri, "N. Yazigi and W.F. Balistreri," pp. 2–3.
[14] S. Gazzin, N. Strazielle, and C. Tiribelli, "Transport and metabolism at blood – brain interfaces and in neural cells : relevance to bilirubin-induced encephalopathy," vol. 3, no. May, pp. 1–13, 2012, doi: 10.3389/fphar.2012.00089.
[15] A. F. Mcdonagh, "Seminars in Fetal & Neonatal Medicine Controversies in bilirubin biochemistry and their clinical relevance," Semin. Fetal Neonatal Med., vol. 15, no. 3, pp. 141–147, 2010, doi: 10.1016/j.siny.2009.10.005.
[16] M. Ouzzine, S. Gulberti, N. Ramalanjaona, and J. Magdalou, "The UDP-glucuronosyltransferases of the blood-brain barrier : their role in drug metabolism and detoxication," vol. 8, no. October, pp. 1–12, 2014, doi: 10.3389/fncel.2014.00349.

[17] D. P. V. Puppalwar, "Review on 'Evolution of Methods of Bilirubin Estimation,'" IOSR J. Dent. Med. Sci., vol. 1, no. 3, pp. 17–28, 2012, doi: 10.9790/0853-0131728.
[18] R. S. Nelson and F. Knox, "PERSISTENT NONHEMOLYTIC HYPERBILIRUBI-NEMIA ASSOCIATED WITH LIPOCHROME- LIKE PIGMENT IN LIVER CELLS : REPORT OF FOUR CASES *," pp. 952–962, 1954.
[19] M. D. and F. B. J. I N Dubin, "Chronic Idiopathic Jaundice with unidentified pigment in Liver Cells." .
[20] F. A. Rotor AB ML, "Familial nonhemolytic jaundice with direct van den Bergh reaction," Acta Med Phil, vol. 5, pp. 37–49, 1948.
[21] N. Memon, B. I. Weinberger, T. Hegyi, L. M. Aleksunes, N. Brunswick, and N. H. Park, "HHS Public Access," vol. 79, no. 3, pp. 378–386, 2016, doi: 10.1038/pr.2015.247.Inherited.
[22] S. L. H. M.D., Eugene Braunwald; M.D., Anthony S. Fauci; M.D., Kurt J. Isselbacher; M.D., Joseph B. Martin; M.D., Jean D. Wilson; Kasper, Dennis L.; M.D., Harrison's Principles of Internal Medicine. Companion Handbook, 1999.
[23] D. Schuppan, N. H. Afdhal, and B. Israel, "Seminar Liver cirrhosis," vol. 371, 2008.
[24] L. M. Gartner and M. Herschel, "JAUNDICE AND BREASTFEEDING," Pediatr. Clin. North Am., vol. 48, no. 2, pp. 389–400, 2001, doi: 10.1016/S0031-3955(08)70032-4.
[25] B. M. Jaundice, "Breastfeeding and Breast Milk Jaundice," no. I, pp. 213–217.
[26] R. M. Clemons, "ISSUES IN NEWBORN CARE," vol. 27, no. 1, pp. 251–267, 2000.
[27] I. Morioka, O. Id, K. Fujioka, O. Id, and O. Articles, "Evaluation of BiliCare," pp. 0–2, doi: 10.1111/ijlh.12426.
[28] N. Textbook, Nelson Textbook of. .
[29] P. A. Keahey, M. L. Simeral, K. J. Schroder, M. M. Bond, and P. J. Mtenthaonnga, "Point-of-care device to diagnose and monitor neonatal jaundice in low-resource settings," pp. 10965–10971, 2017, doi: 10.1073/pnas.1714020114.
[30] L. Ngashangva, V. Bachu, and P. Goswami, "Journal of Pharmaceutical and Biomedical Analysis Development of new methods for determination of bilirubin," J. Pharm. Biomed. Anal., vol. 162, pp. 272–285, 2019, doi: 10.1016/j.jpba.2018.09.034.
[31] A. D. Method, S. C. Kazmierczak, F. Robertson, P. G. Catrou, K. P. Briley, and L. Bill, "Bilirubin in Newborns : Comparison with HPLC and an," pp. 1096–1097.
[32] Malloy HT and Evelyn KA, "The determination of bilirubin with the photometric colorimeter," J. Biol. Chem, no. 119, pp. 481–490, 1937.
[33] J. Vichapong, R. Burakham, N. Teshima, S. Srijaranai, T. Sakai, and M. Chemistry, "Analytical Methods," no. 9, pp. 4–9, 2013.
[34] A. S. Levey, "Assessing Kidney Function — Measured and Estimated Glomerular Filtration Rate," pp. 2473–2483, 2006.
[35] K. Ponhong, N. Teshima, K. Grudpan, and J. Vichapong, "Talanta Successive determination of urinary bilirubin and creatinine employing simultaneous injection effective mixing fl ow

analysis," Talanta, vol. 133, pp. 71–76, 2015, doi: 10.1016/j.talanta.2014.05.065.

[36] O. F. Laterza, C. H. Smith, T. R. Wilhite, and M. Landt, "Accurate direct spectrophotometric bilirubin measurement combined with blood gas analysis," vol. 323, pp. 115–120, 2002.

[37] A. M. M. Attia et al., "A multi-component spectrophotometric method for simultaneous determination of total bilirubin, oxyhemoglobin, and methemalbumin in human sera," J. Appl. Biol. Biotechnol., vol. 8, no. 1, pp. 38–46, 2020, doi: 10.7324/JABB.2020.80107.

[38] A. M. M. Attia et al., "A multi-component spectrophotometric method for simultaneous determination of total bilirubin , oxyhemoglobin , and methemalbumin in human sera," vol. 8, no. 01, pp. 38–46, 2020, doi: 10.7324/JABB.2020.80107.

[39] "Verhalten des H a r n s z u S u l f o d i a z o b e n z o l . ~ a c h P. E h r l i c h **)," p. 514.

[40] B. T. Doumas et al., "CandidateReferenceMethodfor Determination of Total Bilirubin in Serum : Developmentand Validation," vol. 31, no. 11, pp. 1779–1789, 1985.

[41] N. Dhungana, C. Morris, and M. D. Krasowski, "Operational impact of using a vanadate oxidase method for direct bilirubin measurements at an academic medical center clinical laboratory," Pract. Lab. Med., vol. 8, no. May, pp. 77–85, 2017, doi: 10.1016/j.plabm.2017.05.004.

[42] M. Ameri, H. Schnaars, J. Sibley, and D. Honor, "Comparison of the vanadate oxidase method with the diazo method for serum bilirubin determination in dog , monkey , and rat," vol. 123, pp. 120–123, 2011, doi: 10.1177/104063871102300121.

[43] F. Beppu, Y. Niwano, T. Tsukui, and M. Hosokawa, "Single and repeated oral dose toxicity study of fucoxanthin (FX), a marine carotenoid , in mice," 2009.

[44] M. S. Devgun and C. Richardson, "Direct bilirubin in clinical practice — Interpretation and haemolysis interference guidance reassessed," Clin. Biochem., pp. 10–12, 2016, doi: 10.1016/j.clinbiochem.2016.09.005.

[45] S. Osawa, S. Sugo, T. Yoshida, T. Yamaoka, and F. Nomura, "An assay for separating and quantifying four bilirubin fractions in untreated human serum using isocratic high-performance liquid chromatography," vol. 366, pp. 146–155, 2006, doi: 10.1016/j.cca.2005.09.031.

[46] S. Osawa, S. Sugo, T. Yoshida, T. Yamaoka, and F. Nomura, "An assay for separating and quantifying four bilirubin fractions in untreated human serum using isocratic high-performance liquid chromatography," Clin. Chim. Acta, vol. 366, no. 1–2, pp. 146–155, 2006, doi: 10.1016/j.cca.2005.09.031.

[47] E. Easper and T. Ambrose, "Separation of bilirubin species' in serum and bile by high-performance reversed-phase liquid chromatography," 1981.

[48] Y. Andreu, M. Ostra, C. Ubide, J. Galba, S. De Marcos, and J. R. Castillo, "Study of a fluorometric-enzymatic method for bilirubin based on chemically modified bilirubin-oxidase and multivariate calibration," vol. 57, pp. 343–353, 2002.

[49] M. Santhosh, S. R. Chinnadayyala, A. Kakoti, and P. Goswami, "Author ' s Accepted

Manuscript," Biosens. Bioelectron., 2014, doi: 10.1016/j.bios.2014.04.003.

[50] Y. Du, X. Li, and Q. Jia, "Highly Sensitive and Selective Sensing of Free Bilirubin Using Metal-Organic Frameworks-Based Energy Transfer Process," 2017, doi: 10.1021/acsami.7b09091.

[51] S. Rathore, C. K. Vk, and R. Sharashchandra, "Journal of Ayurveda and Integrative Medicine A critical review on neonatal hyperbilirubinemia-an Ayurvedic perspective," J. Ayurveda Integr. Med., vol. 11, no. 2, pp. 190–196, 2020, doi: 10.1016/j.jaim.2018.08.006.

[52] A. Halder et al., "A Novel Whole Spectrum-Based Non-Invasive Screening Device for Neonatal Hyperbilirubinemia," IEEE J. Biomed. Heal. Informatics, vol. 23, no. 6, pp. 2347–2353, 2019, doi: 10.1109/JBHI.2019.2892946.

[53] N. Bosschaart et al., "Limitations and opportunities of transcutaneous bilirubin measurements," Pediatrics, vol. 129, no. 4, pp. 689–694, 2012, doi: 10.1542/peds.2011-2586.

[54] J. A. Taylor, A. E. Burgos, V. Flaherman, and E. K. Chung, "Discrepancies Between Transcutaneous and Serum Bilirubin Measurements," vol. 135, no. 2, 2015, doi: 10.1542/peds.2014-1919.

[55] Veeranki S. R, Varshney M. (2022). Intelligent Techniques and Comparative Performance Analysis of Liver Disease Prediction, International Journal of Mechanical Engineering, 7(1), 489-503. https://kalaharijournals.com/ijme-vol7-issue-jan2022part2.php,

[56]Dr. Ritika Malik, Dr. Aarushi Kataria and Dr. Naveen Nandal, Analysis of Digital Wallets for Sustainability: A Comparative Analysis between Retailers and Customers, International Journal of Management, 11(7), 2020, pp. 358-370.

[57]Hersh F. Mahmood, Hooshang Dabbagh, Azad A. Mohammed, Comparative study on using chemical and natural admixtures (grape and mulberry extracts) for concrete, Case Studies in Construction Materials, Volume 15, 2021.

[58]Masood Abu-Bakr, Hersh F. Mahmood, Azad A. Mohammed, Investigation of metakaolin and steel fiber addition on some mechanical and durability properties of roller compacted concrete, Case Studies in Construction Materials, Volume 16,2022, e01136.

[59]I. Yamanouchi and Y. Yamauchi, "Transcutaneous Bilirubinometry: Preliminary Studies of Noninvasive Transcutaneous Bilirubin Meter in the Okayama National Hospital," vol. Li, no. 2, 2020.

[60] G. Lilly, T. Chimhini, S. Chimhuya, and V. Chikwasha, "Evaluation of transcutaneous bilirubinometer (DRAEGER JM 103) use in Zimbabwean newborn babies," pp. 1–7, 2018, doi: 10.1186/s40748-017-0070-0.

[61] F. Raimondi et al., "Measuring transcutaneous bilirubin : a comparative analysis of three

devices on a multiracial population," 2012.

[62] N. Polley and B. R. Choudhury, "Development and optimization of a noncontact optical device for online monitoring of jaundice in human subjects," doi: 10.1117/1.JBO.20.6.067001.

[63] B. Check, M. J. Meter, and N. Population, "Original Article Improved Transcutaneous Bilirubinometry :," pp. 12–14, 2002, doi: 10.1038/sj/jp/7210592.

[64] G. Bertini, S. Pratesi, E. Cosenza, and C. Dani, "Transcutaneous Bilirubin Measurement : Evaluation of Bilitest TM," pp. 101–105, 2008, doi: 10.1159/000107351.

[65] A. Cecilia and M. Draque, "Transcutaneous Bilirubin in Exclusively Breastfed Healthy Term Newborns Up to 12 Days of Life," vol. 128, no. 3, 2015, doi: 10.1542/peds.2010-3878.

[66] K. Grohmanna et al., "Bilirubin measurement for neonates: Comparison of 9 frequently used methods," Pediatrics, vol. 117, no. 4, pp. 1174–1183, 2006, doi: 10.1542/peds.2005-0590.

[63] B. O. Olusanya, C. A. Mabogunje, D. O. Imosemi, and A. Emokpae, "Transcutaneous bilirubin nomograms in African neonates," pp. 1–12, 2017, doi: 10.1371/journal.pone.0172058.

[64] M. R. Rizvi, F. M. Alaskar, R. S. Albaradie, N. F. Rizvi, and K. Al-Abdulwahab, "A novel non-invasive technique of measuring bilirubin levels using bilicapture," Oman Med. J., vol. 34, no. 1, pp. 26–33, 2019, doi: 10.5001/OMJ.2019.05.

[65] U. C. A. C. M. F. T.-L. E. G. M. L. González-durán, "Accuracy of transcutaneous bilirubin on covered skin in preterm and term newborns receiving phototherapy using a JM-105 bilirubinometer," J. Perinatol., pp. 226–231, 2020, doi: 10.1038/s41372-019-0557-9.

[66] A. Halder et al., "Large scale validation of a new non-invasive and non-contact bilirubinometer in neonates with risk factors," Sci. Rep., vol. 10, no. 1, pp. 1–14, 2020, doi: 10.1038/s41598-020-67981-9.