Drew University

College of Liberal Arts

Investigating and Improving the Accuracy of Bilirubin Measurement in Neonates

A Thesis in Biochemistry and Molecular Biology

by

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ABSTRACT

Icterus or newborn jaundice can progress to severe hyperbilirubinemia in 8-10% of healthy newborn infants and may result in *kernicterus* or irreversible brain damage. Although recent studies suggest the usefulness of bilirubin measurements in managing high-risk neonates including premature infants, to date, no validated clinical chemistry methods for the accurate and precise determination of neonatal bilirubin are available. Our study aimed to establish a methodology for clinical use through a combination of analytic techniques. A state-of-the-art system of size exclusion chromatography (SEC) and reversed-phase high-performance liquid chromatography (RP-HPLC) with visible (Vis) and ultraviolet (UV) detection was utilized. Acquisition wavelengths were configured to measure at λ=460 nm, consistent with the yellow-orange pigment of bilirubin, and λ=280 nm, consistent with the absorption of serum proteins, most notably albumin, bilirubin's carrier protein. Patient samples were diluted with an aqueous mobile phase, spun, and filtered using a 0.2 µm membrane. This method enabled the determination of total bilirubin (TBIL) concentrations through SEC, along with a baseline separation of all three species of bilirubin (Bu, mono, and di Bc) and the respective degradation product (lumirubin) in a 30-minute gradient elution run excelling in ultra-high sensitivity through RP-HPLC. Since bilirubin binds to the carrier protein albumin, TBIL concentrations were determined from a single chromatographic peak in a record time of 11.54 minutes using SEC. Results of several chromatographic separations of patient samples indicated a wide variation in the relative proportions of the four bilirubin fractions observed. A correlation of the sum of the areas of bilirubin peaks produced by RP-HPLC was found with the total bilirubin value obtained by a standard reference procedure using 97% Alfa Aesar. Both techniques afford high precision and accuracy with an SEC linearity test yielding an R^2 value of 1 over a targeted concentration range

(0 - 32 mg/dL) and an RP-HPLC relative standard deviation (RSD) of 0.35% over a concentration range of clinical interest (0 - 100 mg/dL). Thus, this analytical methodology could identify the components of bilirubin in its various forms exceeding the precision and accuracy of recent clinical techniques cost-effectively. Because the effects of bilirubin toxicity are often devastating and irreversible, quickly determining TBIL concentrations in newborns is essential for the appropriate diagnosis and management of hyperbilirubinemia. Thus, laboratory testing can play a pivotal role in treating affected neonates.

LIST OF ABBREVIATIONS AND ACRONYMS

AAP: American Academy of Pediatrics

Bu: Unconjugated bilirubin

Bc: Conjugated bilirubin

BVR: Biliverdin reductase

CAP: College of American Pathologists

LC-MS: Liquid chromatography-mass spectroscopy

LR: Lumirubin

OCD: Ortho Clinical Diagnostics

SEC: Size exclusion chromatography

TBIL: Total bilirubin

UDPGT: Uridine-diphosphoglucuronic glucuronosyltransferase

RP-HPLC: Reversed-phase high-performance liquid chromatography

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1. INTRODUCTION

Hyperbilirubinemia, the result of a buildup of bilirubin in the blood, is a common occurrence in neonates, particularly term and late preterm infants (Bhutani et al. 2013). Neonatal jaundice typically occurs as a part of normal newborn physiology 3 – 5 days after birth and is characterized by yellow-pigmented skin and increased bilirubin blood concentrations (Lo 2010). Normally, this final product of heme degradation binds to serum albumin and is transported to the liver where it is conjugated with glucuronic acid and gets secreted in bile (Barrett et al. 2013). However, within the first few days of life, glucuronic activity is minimal resulting in an increase in bilirubin concentrations in most newborns (Lo 2010; Young et al. 2013; Watchko et al. 2017). Although most jaundice is benign, critical hyperbilirubinemia has the potential to cause long-term neurological impairment or *kernicterus*, a form of brain damage due to the deposition of bilirubin in the basal ganglia and brainstem nuclei (Bhutani et al. 2013). Infants who survive run the risk of developing movement disorders, gaze abnormalities, and auditory abnormalities. *Kernicterus* is a preventable cause of mental retardation, and its prevention is significantly easier and cost-effective if diagnosed promptly and properly managed. Thus, bilirubin measurement must efficiently identify those infants at risk. Bilirubin levels in newborns are unlike other laboratory measurements. During the first week of life, an increase in bilirubin production and a decrease in bilirubin elimination cause total bilirubin (TBIL) levels to rise rapidly (Bhutani et al. 2015). Thus, jaundice, which may be preventable, is the leading cause of hospital readmission during that period (Young et al. 2013). Pediatricians and scientists have been working on a prediction method for neonatal hyperbilirubinemia for decades (Young et al. 2013). While several new quantification

methodologies have been developed, they have yet to perfect the accuracy and precision of bilirubin measurement (Lo 2010).

1.1 Structure, Origin, and Role of Bilirubin

Bilirubin has been studied since the eighteenth century because the assessment of serum bilirubin concentration can aid in the diagnosis and prognosis of patients, regardless of age, with liver or bile duct disorders (Sanchez et al. 2016). A catabolic product of heme metabolism, roughly 85% of bilirubin originates from the breakdown of hemoglobin from senescent red blood cells and prematurely destroyed erythroid cells in the bone marrow (Hinds et al. 2018). The remainder originates from the turnover of heme-containing proteins found in the liver and muscles: myoglobin, hemoglobin, pyrrolase, cytochrome, and others (Ngashangva et al. 2019). In mammals, bilirubin is the principal product of the degradation of the heme moiety of hemoglobin and other hemoproteins (Kalakonda et al. 2021). The metabolite is synthesized by a two-stage sequential catalytic degradation reaction that occurs in the cells of the reticuloendothelial system, notably the spleen (Kalakonda et al. 2021). Reticuloendothelial cells are macrophages responsible for the maintenance of the blood, through the destruction of old or abnormal cells. In the adult human, for instance, erythrocytes have a lifetime of approximately three months and their destruction produces about 300 milligrams (mg) of bilirubin daily (Lathe et al. 1972).

To begin the metabolism of bilirubin, the porphyrin ring of heme is opened by selective enzymatic oxidation of the α-bridge carbon by a microsomal enzyme designated heme oxygenase (Barrett et al. 2013). This leads to the formation of a green tetrapyrolic pigment known as biliverdin (Figure 1). The oxidized α-bridge carbon is liberated as carbon monoxide (Barrett et al. 2013).

Biliverdin is rapidly converted by a second enzyme, biliverdin reductase (BVR), to the orange-yellow pigment bilirubin, the chromophore responsible for the coloration in various forms of jaundice. Neonates have an increased turnover of erythrocytes causing them to produce more than twice the adult daily amount of bilirubin (Watchko et al. 2017). Given their deficiency in their ability to conjugate and clear bilirubin, there is an increased risk of kernicterus (Bhutani et al. 2013).

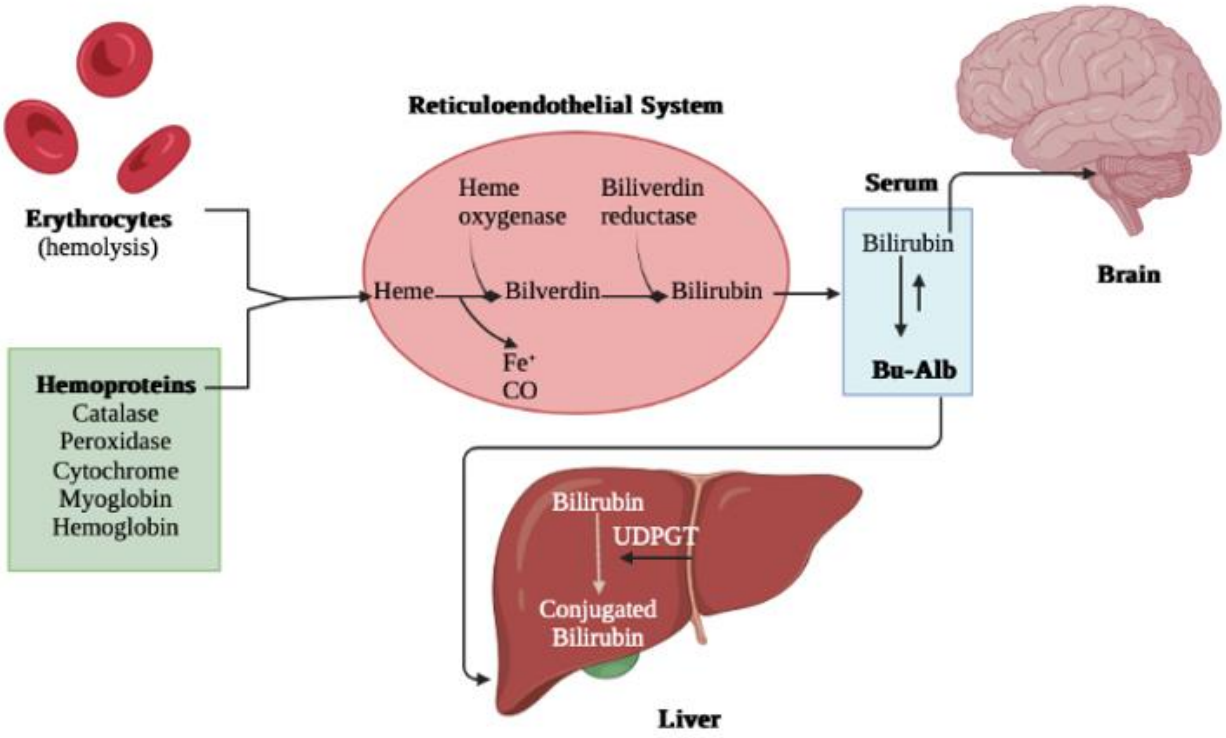
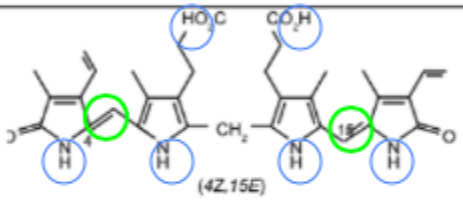
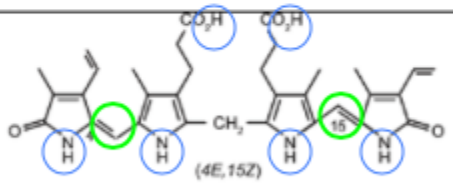


Figure 1: Metabolism of heme to form bilirubin. Bilirubin is formed as a breakdown product of heme. In phagocytic cells of the reticuloendothelial system, heme oxygenase opens the tetrapyrrole ring to produce the intermediate biliverdin. Biliverdin is then reduced to bilirubin by biliverdin reductase. In the liver, bilirubin becomes conjugated through the enzymatic activity of uridine-diphosphoglucuronic glucuronosyltransferase preventing bilirubin from penetrating the brain. Bilirubin is then encapsulated by the carrier protein albumin. Figure created using BioRender.com.

Unconjugated bilirubin is insoluble in water. At physiological pH, the highly polar carboxyl groups of the propionic acid are hydrogen-bonded internally to the nitrogen atoms of the pyrrole rings (Lo 2010). Four stereoisomers of unconjugated bilirubin coexist (McDonagh et al. 2010). The intramolecular hydrogen bonds stabilize the Z-Z and E-E stereoisomers and prevent their interaction with water (Table 1). The other structural isomers, E-Z-bilirubin, and Z-E-bilirubin, formed by the rupture of certain hydrogen bonds, occur from rotation around the double bonds between the pyrrole rings of bilirubin (McDonagh et al. 2010).

Table 1: Structural and geometric isomers of unconjugated bilirubin

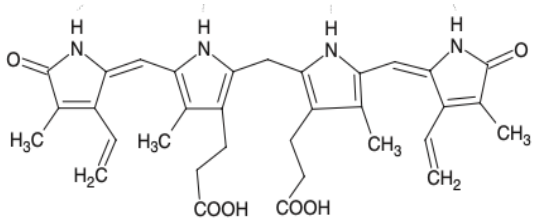
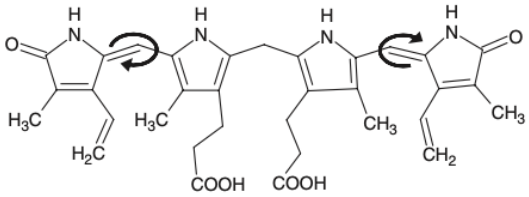
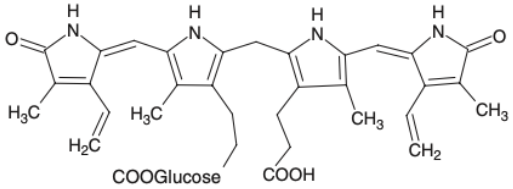
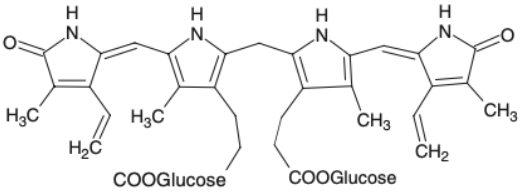
Bilirubin Structural Isomer	Geometric Isomer	Structure
III-α	EE	
XIII-α	ZE	
IX- α	EZ	
IX- α	ZZ	

Blue circles represent intramolecular hydrogen bonding between H-N and H-O. Green circles represent the variations in the double bonds across the various Bu stereoisomers. Suppose two higher-priority substituents occur on the same side of the double bond, in that case, the configuration of the bond is Z. If the substituents arise on opposite sides of the double bond, the configuration is E. Chemical structures created using ChemDraw.

All the stereoisomers of unconjugated bilirubin are transported from the reticuloendothelial system to the surface of the liver where bilirubin uridine diphosphate glucuronyl transferase (UDPGT) catalyzes the covalent bonding of glucuronic acid (McDonagh et al. 2010). It is this enzyme that is inactive at birth and requires a few days to be induced (McDonagh et al. 2010;

Dosch et al. 2019). The process of glucuronidation is one of the many crucial detoxification mechanisms of the human body. The enzyme esterifies one or two glucuronide moieties to bilirubin producing both mono- and di- glucuronide bilirubin at a ratio of 1:4 (Dosch et al. 2019). The process of conjugation alters the physicochemical properties of bilirubin and renders the molecule water-soluble. The remaining species of bilirubin are unconjugated bilirubin (Bu) and lumirubin (LR), isomers of Bu that undergoes a conformational change when exposed to light (Table 2) (Lo 2010; McDonagh et al. 2010; Dosch et al. 2019).

Table 2: Four naturally occurring species of bilirubin comprise total bilirubin

Bilirubin Specie	Abbreviation	Structure
Unconjugated	Bu	
Lumirubin	LR	
Mono-glucuronide conjugated	Mono Bc	
Di-glucuronide conjugated	Di Bc	

Bu and LR are considered unconjugated bilirubin whereas both Bc forms are considered conjugated bilirubin. The arrows on the LR structure represent the product of the conformational change that occurs when Bu is exposed to a photon source. Structures created using ChemDraw.

Once conjugated, bilirubin is secreted into bile, which is released from the gallbladder (McDonagh et al. 2010). However, at birth, nearly all the bilirubin in a newborn's blood is in the unconjugated form (McDonagh et al. 2010). Within 24 - 48 hours, however, mono-conjugated bilirubin appears in the bile followed by the deconjugated form (Lo 2010; McDonagh et al. 2010).

As mentioned previously, the lack of a fully functional conjugation system in newborns ultimately leads to increased bilirubin concentrations, commonly observed as physiologic jaundice (Lo 2010; Watchko et al. 2017).

When bilirubin, in all occurring forms, is released into the plasma, it is taken up by albumin which serves as its transporter throughout the body (Lo 2010). The binding affinity for albumin to bilirubin is extremely high (9.5×10^7 M^{-1}), and under ideal conditions, no free (non-albumin bound) unconjugated bilirubin is seen in the plasma (Gray and Stroupe 1978). The binding of albumin limits the escape of bilirubin from the vascular space and prevents its precipitation and deposition in tissues, most notably, the brain. Conjugation is mandatory to render bilirubin aqueous soluble and facilitate its secretion across membranes and excretion into bile (Kalakonda et al. 2021).

1.2 The Albumin-Bilirubin Complex

One of the important functions of serum albumin is to protect the individual, particularly the newborn, from the toxic effects of bilirubin (Gray et al. 1978). The albumin-bilirubin complex increases the amount of bilirubin carried per volume of plasma and minimizes the diffusion of bilirubin into extrahepatic tissues, thereby preventing bilirubin toxicity. *In vitro* studies indicate that bilirubin is less toxic when bound to albumin than when free in solution (Cowger et al. 1974). Once bilirubin is released into the plasma, it is taken up by albumin which serves as its transporter throughout the body. Ultimately, the binding of albumin to bilirubin limits the escape of bilirubin from the vascular system, minimizes glomerular filtration, and prevents its precipitation and deposition in tissues. When the albumin-bilirubin complex reaches the liver, the highly permeable

hepatic circulation allows the complex to reach the surface of the hepatocyte, disassociate from the albumin, and enter the liver (Kalakonda et al. 2021). This inefficiency often provides the opportunity to measure the concentration of unconjugated bilirubin bound to albumin in venous circulation as the binding of albumin to bilirubin is reversible.

1.3 Importance of Accurate and Precise Bilirubin Measurements in Neonates

About 60% of term neonates and 80% of preterm neonates are diagnosed or develop jaundice during their first week of life (Rennie et al. 2010). An increased turnover of erythrocytes leads to the production of more than twice the amount of bilirubin produced daily by an adult (Duke Pediatrics 2018). Increased enterohepatic circulation and a transient deficiency in the ability to conjugate and clear bilirubin are additional causes of hyperbilirubinemia and neonatal jaundice. The causes of neonatal hyperbilirubinemia can be categorized into ones that result in increased bilirubin production, and ones that decrease the clearance of bilirubin (UMHS Neonatal Hyperbilirubinemia Guideline 2020). For instance, hemolytic disease results in an increased turnover of erythrocytes and thus, an increase in bilirubin metabolism while prematurity at birth leads to an underdeveloped liver incapable of clearing excess bilirubin (Table 3).

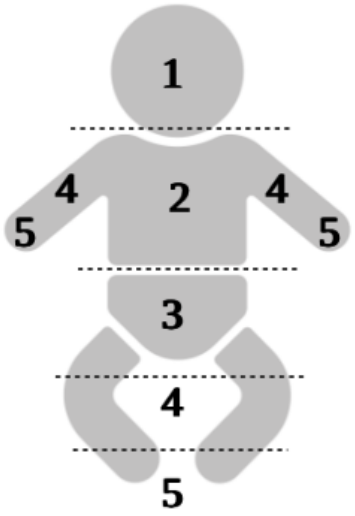
Table 3: Causes of neonatal hyperbilirubinemia

Common Causes of Hyperbilirubinemia	Rare Causes of Hyperbilirubinemia
Increased Bilirubin Production Hemolytic disease (Rh/ABO incompatibility) Polycythemia Sepsis *Decreased Clearance of Bilirubin* Prematurity Inborn errors of metabolism (e.g., Gilbert Syndrome)	Poor response to phototherapy Recurrent admissions for hyperbilirubinemia Direct hyperbilirubinemia Prolonged jaundice (>14d for a term; >21d for preterm infants) Family history of non-immune hemolytic diseases

Adapted from 2020 UMHS Neonatal Hyperbilirubinemia Guideline

In clinical chemistry terms, serum bilirubin is classified as either indirect or direct (Martelanc et al. 2013). Indirect serum bilirubin is unconjugated and thus it is poorly soluble in physiological solutions while direct serum bilirubin is conjugated and soluble (Temme et al. 2001). Supra-physiological amounts of indirect serum bilirubin are neurotoxic in neonatal development, while, at lower levels, bilirubin is regarded as a potent endogenous antioxidant. The buildup of unconjugated hyperbilirubinemia due to a developing liver and jaundice is frequently an issue for neonates. If untreated, jaundice may lead to neurotoxicity including but not limited to sensorineural deafness, auditory neuropathy, athetoid cerebral palsy, and neonatal seizures (Amin et al. 2005). *Kernicterus* refers to the neurological consequences of the deposition of unconjugated bilirubin in brain tissue (Amin et al. 2005). Subsequent damage and scarring of the basal ganglia and brainstem nuclei may occur.

The schema for grading the extent of jaundice based on pigmentation (Figure 2) is based on visual observations of skin and body and is used to estimate bilirubin concentrations. Currently, visual estimation of bilirubin levels that assess the degree of jaundice is one of the methods used to clinically quantify hyperbilirubinemia; however, it is considered prone to error, especially in heavily pigmented skin (Duke Pediatrics 2018). Despite extensive research, total bilirubin (TBIL), the traditional parameter to evaluate and manage jaundice, has not been useful as a sensitive and specific predictor of hyperbilirubinemia of neurological outcomes since it does not provide insight into the cause of bilirubin buildup (Scheidt et al. 1991). Improvement in the currently used laboratory methods is urgently needed (Lo et al. 2004).



Grade	Extent of Jaundice	[Bilirubin] mg/dL
0	None	0
1	Face and neck only	4-6
2	Chest and back	6-8
3	Abdomen below umbilicus to knees	8-12
4	Arms and legs below knees	12-14
5	Hands and feet	>15

**The estimation of the degree of jaundice can lead to errors especially in darkly pigmented infants.*

Figure 2: Neonatal jaundice grading schema. The extent of jaundice is based on visual observations of the neonate's body. Adapted from Duke Department of Pediatrics (2018).

Bilirubin levels in newborns are unlike other laboratory measurements (Stevenson et al. 2001). The continuously shifting balance between bilirubin production and clearance produces hourly fluctuations in total bilirubin (TBIL) levels that are unique to the neonatal period. By day 8, the 50th percentile for TBIL levels in healthy newborns is approximately 8 - 9 mg/dL and the

95th percentile is approximately 15 - 17.5 mg/dL (Stevenson et al. 2001). While these ranges are generally harmless, bilirubin can penetrate the blood-brain barrier should levels exceed 25-30 mg/dL which results in chronic bilirubin encephalopathy or *kernicterus* (Shapiro et al. 2012).

Hospital stays for newborns are becoming shorter. Monitoring jaundice and measuring TBIL levels when infants remained in the hospital for three or more days after birth was easier (Maisels et al. 2009). Today, infants delivered vaginally are often discharged well before 48 hours, and in some cases, before 24 hours (Maisels et al. 2009). Bilirubin levels tend to peak on the fourth or fifth day after birth illustrating the importance of interpreting TBIL levels according to the infant's age in days. The identification and management of jaundiced infants have thus transformed from an inpatient to an outpatient problem, warranting supplemental follow-ups. Generally, it is recommended that healthcare practitioners retest bilirubin levels two to three days following discharge from the hospital (Watchko et al. 2017). Follow-up testing of infants discharged with higher-than-normal bilirubin levels routinely occurs and the decision to retest newborns is a clinical judgment determined by the zone in which the total serum bilirubin falls (Tables 4 and 5), the age of the infant, other known risk factors, and the expected course of hyperbilirubinemia (Maisels et al. 2009; Lo 2010; Watchko et al. 2017). Risk factors fall into one of three categories: major, minor, or decreased.

Table 4: Risk factors for hyperbilirubinemia in neonates

Major Risk Bu>25 mg/dL	Predischarge Bu levels in critical zone	Jaundice observed within 24 hours of birth	Blood group or Rh factor incompatibility and hemolytic diseases	GA of 35-36 weeks	Phototherapy treatment of a sibling	Bruising	Exclusive breast feeding	East Asian Parentage
Minor Risk 20<Bu<24 mg/dL	Predischarge bilirubin levels in high intermediate risk zone	Jaundice observed before discharge		GA 37-38 weeks	Previous sibling with jaundice		Maternal age >25 years of age	Male Gender
Decreased Risk Bu<20 mg/dL	Bilirubin levels in low-risk zone			GA >41 weeks	Discharge from hospital after 72 hours		Exclusive bottle feeding	Black Ethnicity

Risk factors are classified from most to least critical
**All levels are assessed using Total Serum Bilirubin (TsB) measurements*
**GA: Gestational Age*
**Hemolytic disease: blood disorder that occurs when the blood of a mother and a baby are incompatible*

Adapted from Lo 2010. See supplemental materials for a detailed explanation of these risk factors.

Table 5: Total bilirubin concentrations that constitute hyperbilirubinemia according to age and birth weight

Age (Hours)	Birth Weight 2000-2500g	Birth Weight >2500g
0-24	>5 mg/dL	>5 mg/dL
25-48	>8 mg/dL	>12 mg/dL
49-72	>12 mg/dL	>15 mg/dL
73-96	>14 mg/dL	>17 mg/dL
97-120	>14 mg/dL	>17 mg/dL

1.4 Development of Current Methods for Measuring Bilirubin

Diazo Methods

In 1916, Van den Bergh and Muller first developed the diazo reaction to determine total bilirubin concentrations in serum (Figure 3). They discovered that diazotized sulfuric acid added to serum from hepatitis patients yielded a red color immediately but produced little to no color when added to serum from jaundiced neonates. When alcohol, an accelerator or promoter of the diazo coupling reaction, was added to the jaundiced neonate serum, however, color developed more quickly (Lo 2010). The researchers coined the adult sera "direct" for direct-reacting and that in the neonatal sera "indirect" because it required an accelerator. Since then, researchers have developed many variations of the diazo method with the Doumas modification of the Jendrassik-Grof method extensively studied (Bhutani et al. 1999; Lo 2010). Ultimately, caffeine was implemented to remove Bu from the albumin complex (Bhutani et al. 1999; Lo 2010). Due to azobilirubin, the product formed from the reaction of diazonium and bilirubin, with an absorbance of 600 nm, through spectroscopy, various bilirubin concentrations could be determined (Bhutani et al. 1999; Lo 2010). The concentrations of the total, direct, and indirect bilirubin must be calculated based on the reactions to give concentrations of total, unconjugated, and conjugated bilirubin. Issues with the diazo method arise as not only is a reaction required for the measurement of bilirubin, but calculations must also be made to account for the different species of bilirubin reacting or not reacting. These factors introduce inconsistencies and ultimately reduce accuracy and precision.

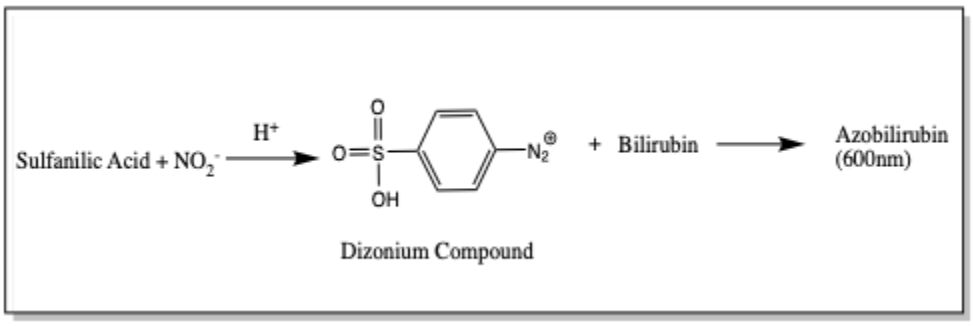


Figure 3: Scheme of Diazo method reaction. Sulfanilic acid reacts with NO_2^- under acidic conditions and forms the diazonium compound. This compound can react with bilirubin to form azobilirubin which absorbs at 600 nm. This reaction scheme was created using ChemDraw.

Oxidation Methods

Bilirubin may be oxidized by a chemical compound. The concomitant decrease in absorbance at 450 - 460 nanometer (nm) is proportional to the concentration of bilirubin. With bilirubin oxidase, total bilirubin is measured near pH 8 and direct bilirubin near pH 4 (Doumas et al. 1987; Kosaka et al. 1987). At pH 10, bilirubin oxidase selectively oxidizes the two glucuronide groups and approximately 5% of unconjugated bilirubin (Doumas et al. 1987; Kosaka et al. 1987). To quantify serum bilirubin concentrations an analyzer is used and calibrated using vanadate. While this method is said to be more reliable than the Diazo method, inaccuracies of ±2 mg/dL were reported across clinical laboratories and were attributed to errors in calibrating the analyzer (Doumas et al. 1999).

Direct Spectrophotometry

Direct spectrophotometry is based on measuring the absorbance of bilirubin near 460 nm and the absorbance of hemoglobin between 454 and 540 nm (Wennberg et al. 1973). The concentration of bilirubin is calculated by solving a system of two simultaneous equations with two unknowns. Direct spectrophotometry is affected by the protein matrix or the materials that create an affinity for binding such as agarose. Ultimately, calibrators made in matrices other than

human serum are not suitable because the absorption maximum of unconjugated bilirubin varies which may increase or decrease the absorptivity. Therefore, a given control or calibrator preparation may appear to yield more than one "true" value for bilirubin leading to confusion surrounding the prognosis of neonatal jaundice and the appropriate course of treatment.

Transcutaneous Bilirubinometer

The first bilirubin meter (icterometer) for a noninvasive approach to estimating bilirubin in blood was a reflectance photometer (Yamanouchi et al. 1980). This approach utilizes reflectance densitometry, a light source that focuses defined light on a sample (National Academy of Clinical Biochemistry 2007). For the most part, bilirubin results show a fair correlation up to 10 mg/dL but are highly inconsistent at higher levels and warrant additional measurements and calculations (National Academy of Clinical Biochemistry 2007). The reliability of transcutaneous bilirubin meters was determined in neonates aged 2 to 31 days in the pediatric emergency department (Grabenhenrich et al. 2014; Ercan & Ozgun 2018). Measurements were considered unreliable as this method underestimated TBIL by 2.4 mg/dL (SD=2.1 mg/dL, R^2=0.8467) and had a specificity of ~62.1% (Grabenhenrich et al. 2014; Ercan & Ozgun 2018). Furthermore, the effects of infants' inherent skin pigmentation on transcutaneous bilirubin measurements with the photometer have not been well defined, and further studies are needed to determine its potential role in screening and managing infants with hyperbilirubinemia ((National Academy of Clinical Biochemistry 2007). More extensive studies and measurement methods need to be completed to accurately determine bilirubin levels as the overestimation of bilirubin could potentially lead to unnecessary therapy, and the underestimation of bilirubin could lead to the withholding of necessary therapy and the development of kernicterus (Subcommittee on Hyperbilirubinemia 2004).

1.5 Treatment of Neonatal Hyperbilirubinemia

The most common treatment for acute hyperbilirubinemia is phototherapy (Subcommittee on Hyperbilirubinemia 2004). Based on bilirubin concentration and limited evidence, the American Academy of Pediatrics (AAP) provides an age-specific practice guideline with recommendations for intervention. Many different phototherapy units are available, as well as several different lamps emitting different wavelengths of light at various intensities (Subcommittee on Hyperbilirubinemia 2004). Phototherapy entails exposing the neonate to blue light with a wavelength of 450 nm in a specially designed bed or blanket (Subcommittee on Hyperbilirubinemia 2004; Lo 2010). When bilirubin absorbs the blue light, a photochemical reaction occurs and disrupts the internal hydrogen bonding of unconjugated bilirubin and results in several different water-soluble isomers of bilirubin, allowing them to be cleared from circulation in the bile (Subcommittee on Hyperbilirubinemia 2004; Lo 2010). Ultimately, this wavelength disrupts the hydrogen bonds of bilirubin and isomerizes the ZZ isomer of Bu to the EE isomer of lumirubin making it water-soluble. Fortunately, this procedure is relatively simple and safe. Not only does it decrease bilirubin levels, but also prevents the newborn from undergoing an exchange blood transfusion. In rare cases, however, bilirubin levels are high enough to warrant this invasive treatment with a relatively high risk of unwanted effects such as fever, hemolysis, and blood incompatibility. Treatment options are assessed by physicians using the recommendations for intervention by the AAP. These guidelines have specific cutoffs for various treatments (Subcommittee on Hyperbilirubinemia 2004). Intensive phototherapy and exchange transfusion are initiated after carefully studying the infants' age, TBIL levels, and neurotoxicity risk factors such as asphyxia or acidosis (Figure 4). These infants can fall into one of three categories: low, risk, or high risk according to assessment.

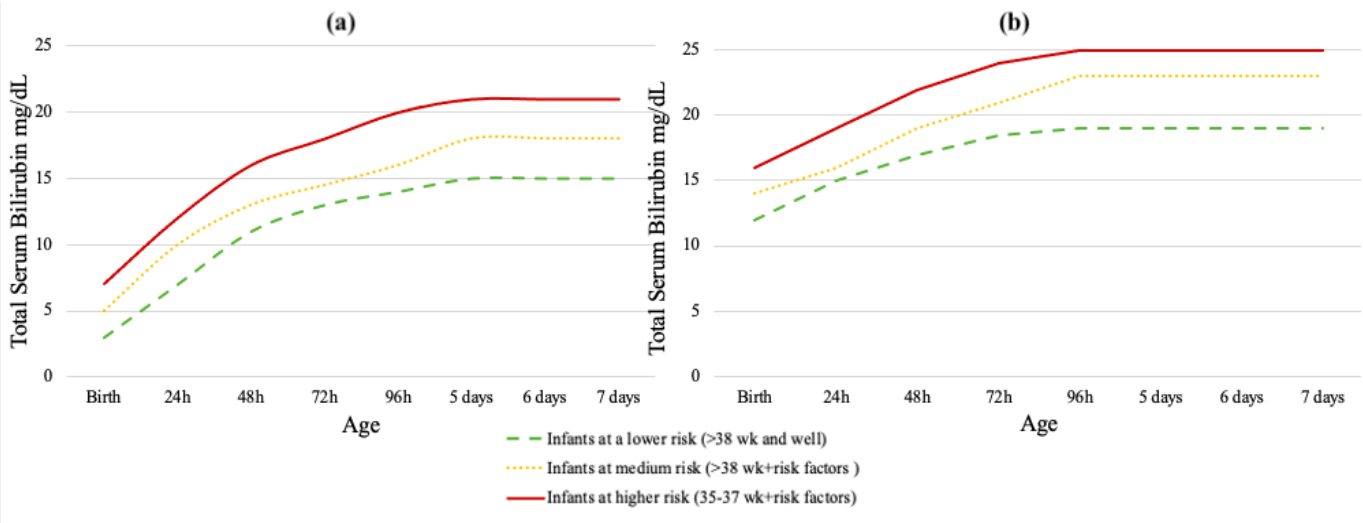


Figure 4: Treatment guidelines for neonatal jaundice. Intensive phototherapy (a) and exchange transfusion (b) thresholds based on neurotoxicity risk factors (isoimmune hemolytic disease, G6PD deficiency, asphyxia, significant lethargy, temperature instability, sepsis, acidosis, albumin <3.0 g/dL). Red corresponds to infants at higher risk, yellow corresponds to infants at medium risk, and green corresponds to infants at a lower risk. Adapted from UMHS Neonatal Hyperbilirubinemia Guideline (2020).

1.6 Discrepancies in Methodologies Utilized for the Analytical Measurement of Bilirubin

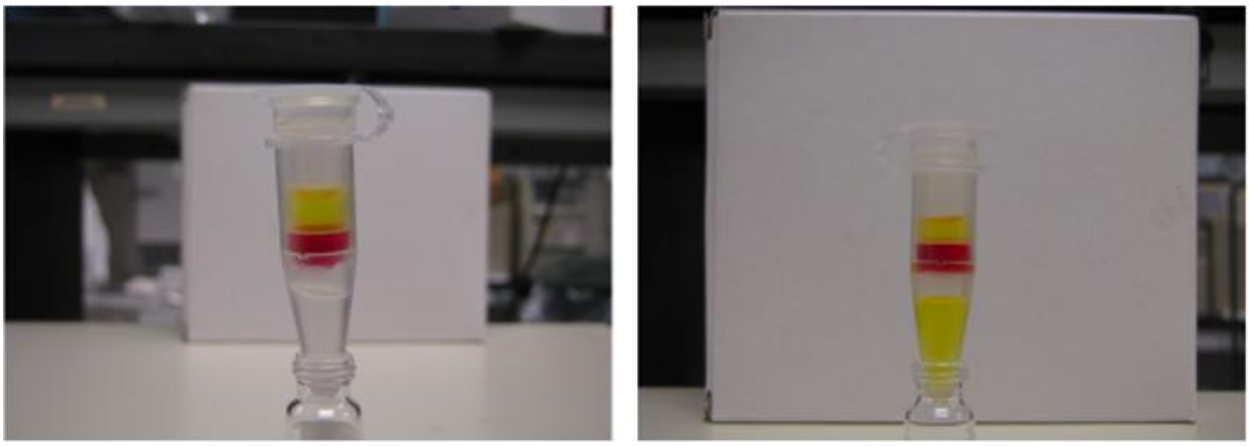
Annually, the College of American Pathologists (CAP) issues a neonatal bilirubin (NBIL) survey to compare the performance of laboratories that perform these assays. Unknown specimens are analyzed by participating hospitals and reference laboratories, and the results are reported to CAP (Lo 2004). Each laboratory is then issued a report of its performance as well as a summary of the results of all participating laboratories. The reported results varied between ±3.5% to 5.1% (Lo 2004). In recent years, the annual CAP survey has shown the VITROS® BuBc assay from Ortho Clinical Diagnostics (OCD) to have a positive bias versus other methods. This assay is utilized to provide measurements of both conjugated and unconjugated bilirubin fractions. Inconsistencies have been noted in several publications, including Clinical Chemistry (Routh 1976). The demonstrated positive bias indicates a potential drift in the VITROS® BuBc assay

accuracy. Ultimately, the assay produced results closer to that of the reference method when the bilirubin concentration was low (8.65 mg/dL), but deviated from the reference method when the bilirubin concentration was high (~23 mg/dL) (Lo & Doumas 2011). With the severe risk of some of the complications that hyperbilirubinemia causes, their findings were extremely significant. For the most part, bilirubin concentrations that exceed 15 mg/dL often present an imminent danger to the neonate. Accurate measurements of bilirubin are critical in preventing irreversible damage. Given the presented differences, treatment courses could have varied drastically on high-risk neonates. For instance, a 2 mg/dL increase in bilirubin could mean the difference between phototherapy and a full blood transfusion, based on the nomograms published by AAP (2010). Achieving accuracy of the BuBc assay is critical to patient health. For this reason, several aspects of the BuBc assay were investigated, including the reference method used for calibration.

The reference method previously used to support the BuBc slide was a combination of the total bilirubin (TBIL) method and an HPLC method developed by Lauff and colleagues in 1981 to quantify TBIL. The strategy was to use the TBIL reference to obtain a total bilirubin concentration, and then determine the relative percentages of each bilirubin species from HPLC. The HPLC method reported relative values for monoglucuronide (monoBc), diglucuronide (diBc), and unconjugated bilirubin (Bu) species (Lauff et al. 1981). The relative amounts of each species were then multiplied by the TBIL concentration to determine the absolute amounts of each fraction. However, the Lauff HPLC is now obsolete as the HPLC column is no longer available. Additionally, insufficient characterization of the data led to incorrect assignments of peaks in the chromatograms, and the buffers used introduced unnecessary exposure to dehydrating solvents including 2-methoxy ethanol and 2-propanol (Shugrue & Connolly 2014).

Other studies found even greater discrepancies when looking into the measurement of total bilirubin in human adult serum samples. While measurements seemed to agree throughout different laboratories when using synthetic standard reference materials, serum samples were observed to have discrepancies reaching 30% (Apperloo et al. 2005). The differences were attributed to the high presence of conjugated bilirubin which was not being measured properly and not accounted for in the synthetic material. These discoveries highlight some of the overlooked flaws in current measurements of bilirubin.

To better investigate the accumulating discrepancies appearing in the literature, Ortho Clinical Diagnostics (OCD) attempted to explore new ways to measure bilirubin. All Bu in adult and neonate human blood is tightly bound to the carrier protein albumin, but it was unknown whether that was true for the other bilirubin components (Mosallam et al. 2019). HPLC work suggested Bc and LR were also tightly bound to albumin. Bilirubin samples from patient serum were centrifuged with a 30,000 amu filter which prevented unconjugated bilirubin (534 atomic mass units (amu)) from going through since it was albumin-bound (67,000 amu) (Figure 5a). The bilirubin appeared yellow in the solution and did not pass through the filter until acetonitrile was added to precipitate albumin, centrifuged, and stopped halfway through the spinning hence the yellow color observed above the filter (Figure 5b).



(a) (b)

Figure 5: Adult bilirubin samples before (a) and after (b) precipitation of albumin with acetonitrile. Samples were obtained from Ortho Clinical Diagnostics, Raritan, NJ, and the experiment was reproduced at Drew University (data not shown).

1.7 A Novel Approach to Bilirubin Quantification

Historically, the separation of bilirubin into conjugated and unconjugated fractions provided an important tool for the diagnosis of jaundice (Duke Pediatrics 2018). Progress in chromatography has further allowed the identification of four species of bilirubin. Unlike in adults, there is little information on the significance of these species of bilirubin in jaundiced neonates (Duke Pediatrics 2018). Despite improvements during the past decades in both the precision and accuracy of clinical chemistry analyses concerning bilirubin measurements in neonates, there have been repeated discrepancies and inconsistencies across clinical laboratories (Lo 2010). The impact of bilirubin toxicity can be devastating and accurately determining bilirubin levels is pivotal for the treatment and management of neonates. In the last few years, several HPLC methods have been reported for the separation of bilirubin in biological fluids, yet these methods have not resolved sensitivity, selectivity, and proper sample preparation workflow (Baranano et al. 2002). To

overcome this limitation in the analysis of bilirubin species in patient and synthetic samples, we seek to establish that a combination of precise execution techniques including Size Exclusion Chromatography (SEC), and complementary analysis using Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) can provide accurate and reproducible analytical measurements of bilirubin and its species in patient serum samples.

New techniques can be used to quantify TBIL and bilirubin species. SEC can be utilized to determine total bilirubin (TBIL) concentrations from one chromatographic peak. This technique offers an accurate and precise quantification of TBIL and can be utilized as an initial test hours after birth. Given the sensitivity of SEC, the practical implications of this methodology become clear; several of the medical complications can be avoided, hospitals and clinics can minimize costs, and the mother and neonate can return home post-delivery timely. Additionally, RP-HPLC instrumentation can be utilized to separate peaks and identify various bilirubin conformations if SEC measurements indicate hyperbilirubinemia. The quantification of individual bilirubin species can provide insight into the causes of neonatal jaundice. For instance, healthcare practitioners can narrow the causes down to enzyme deficiency (e.g., uridine diphosphate glucuronosyltransferase (UDPGT)), abnormalities of the liver (e.g., prematurity or blockage), or hypoalbuminemia (decreased production of albumin).

1. METHODS

2.1 Size Exclusion Chromatography

Chemicals and Standards

HPLC gradient grade water, 99% pure 0.05 M monobasic potassium phosphate (KH_2PO_4), 99% pure 0.05 M dibasic potassium phosphate (K_2HPO_4), and 0.025% sodium azide (NaN_3) were purchased from Fisher Scientific (Waltham, MA). 30% monomeric bovine serum albumin (BSA) and patient serum bilirubin samples were obtained from Ortho Clinical Diagnostics Manufacturing (OCD). All patient samples and standards were stored in amber glassware at -80 ℃ until use.

Mobile Phase Preparation

An aqueous mobile phase with a buffer system, preservative, and albumin was utilized. 1 L was prepared before stirring and a 0.45 μm vacuum filtration using 8.17 g of 0.05 M K_2HPO_4, 6.80 g of 0.05 M KH_2PO_4, 0.025% NaN_3, 1 mL of 30% BSA/L, and approximately 1000 mL of HPLC grade water. Both K_2HPO_4 and KH_2PO_4 ensure that the system remains neutral (pH=7) while NaN_3 prohibits contamination and microbial growth. HPLC-grade water enhances chromatographic performance, decreases background signals, and minimizes negative or unknown peaks during elution (Chang et al. 2011). 1 mL of bovine serum albumin from Ortho Clinical was included to correct chromatographic inconsistencies and bilirubin carryover.

SEC Standard Solution Preparation

A protein standard mix for SEC offering full coverage of the molecular weight range from 15 kDa to 600 kDa was utilized as it quantifies it for the molecular mass determination of most proteins, notably bilirubin, and albumin. The protein mix from Fisher Scientific came in 2 mL tubes containing lyophilized protein powder and all necessary buffer ions: thyroglobulin, ϒ globulin (bovine), ovalbumin, myoglobin, and vitamin B_{12}. 5 mg of the calibration standard was placed into a glass vial and 1 mL of HPLC water was added before agitation and injection. This rapid procedure made column calibrations and system suitability tests possible.

Preparation of Albumin Standard

The relative retention time of the human albumin peak was measured using synthetic 30% Bovine Serum Albumin (BSA). A 1:100 diluted sample was prepared in a 0.2 μm Pall Nanosep MF centrifugal spin filter using 100 μL of 30% BSA was added to 900 μL of water, and spun at 12,000 rotations per minute (RPM) for 10 minutes at 4 °C. The sample was employed as a location marker in the chromatogram.

Patient Sample Selection, Preparation, and Sequence

Serum samples were collected hours after delivery and have been preserved at -80 ℃ for the past decade. All the patient samples were obtained from Ortho Clinical Diagnostics (OCD). Anonymous patient and synthetic bilirubin samples were randomly selected to test with a diversity of Bu, Bc, and TBIL concentrations.

To prepare patient samples a 1:10 dilution was done by adding 100 µL of sample to 900 µL of the mobile phase. Additional albumin was added to the patient samples to prevent the dissociation of the polar constituents and ensure that a single peak was observed chromatographically. This peak has clinical significance and plays a pivotal role in identifying total bilirubin concentrations in patients. All the tested samples were placed into amber glassware to protect the specimens from the light, which could facilitate metabolism and falsely lower bilirubin levels. On average, the rate of bilirubin degradation over 24 hours for samples stored in the light ranges from 0.0001 to 0.0048 astronomical units per hour (AU/h) (mean=0.0020 AU/h) while the rate is significantly lower ($p<0.01$) in the dark ranging from 0 to 0.0024 AU/h (mean=0.0005 AU/h) (Foroughi et al. 2010).

The sample queue consisted of duplicate injections of the mobile phase followed by five replicate injections of the SEC Protein Standard Mix. All the patient samples were injected in duplicate for consistency, repeatability, and precision with the SEC's measurement.

SEC Analysis

SEC analysis was performed to measure samples of total bilirubin bound to albumin and samples of pure albumin using a commercial Agilent 1100 Series System (Agilent Technologies, Waldron, Germany) with deuterium and tungsten lamps (Figure 6). ChemStation (for LC 3D system Rev. B01. 03 204, Agilent Technologies 2001-2005) was used for the evaluation of the collected data.

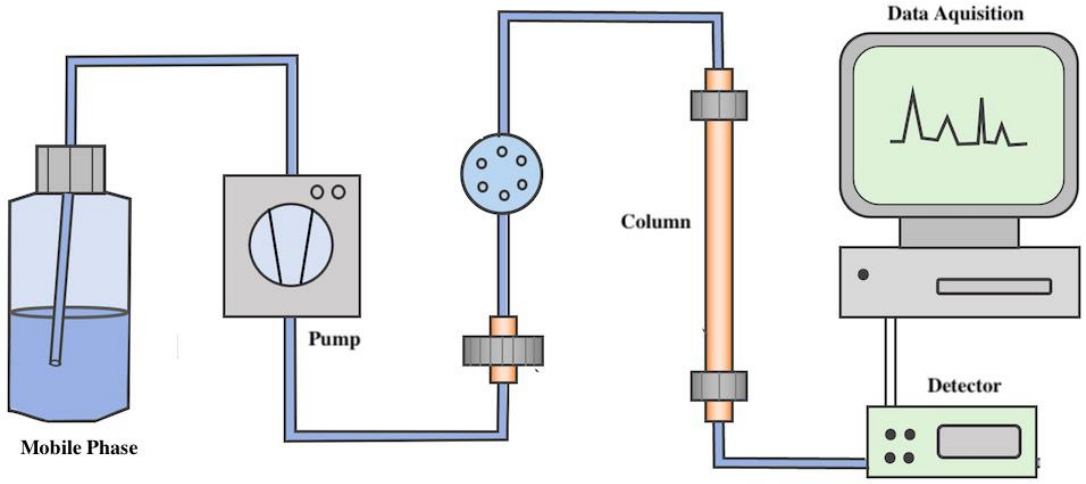


Figure 6: Schematic representation of SEC system. The underlying principle of SEC is that particles of different sizes elute through the stationary phase (silica beads in the column) at different rates with the aid of a mobile phase. This results in the detection and separation of a solution of particles. The data acquisition system then produces peaks that correspond to each particle. Figure created using BioRender.com.

The following parameters were configured before each sample run using the Tosoh Bioscience TSKgel G3000SWxL column from Sigma Aldrich and were determined from previous studies conducted by Lauff and colleagues (1981). The SEC column was 0.78 cm x 30 cm and was packed with 5 μm silica beads. The system maintained a constant temperature of 30 °C. The solvent gradient was pumped through the column at a fixed rate of 0.8 mL/min for a complete 30-minute run. The pure albumin and bilirubin bound to albumin components present in the 20 μL sample injections were detected at wavelengths of 280 and 460 nm. The 280 nm wavelength is consistent with the serum proteins like albumin while the 460 nm wavelength is consistent with the yellow-orange pigment of bilirubin. The injection volume resulted in chromatogram peaks twice the size of those obtained when smaller injections were employed; thus, generating better system precision. Additionally, the four major analogs have a comparable extinction coefficient according to the Beer-Lambert Law (Chang et al. 2011).

Bilirubin Identification and Quantification

The sample peak representing the albumin-bilirubin complex was identified based on its retention time (t_R) value obtained after SEC analysis and was compared with the t_R value obtained after the analysis of the albumin retention marker. Accordingly, the external standard calibration curve was generated from the SEC Protein Standard Mix by plotting the retention times for each protein compound (thyroglobulin, ovalbumin, and myoglobin) versus the natural log of the molecular weight for each of the previous proteins. This permitted the determination of albumin's expected elution time.

2.2 Reversed-Phase High-Performance Liquid Chromatography

Chemicals and Standards

HPLC gradient grade water, 99.9% pure acetonitrile (CH_3CN), 99.7% extra dry dimethyl sulfoxide (DMSO), 98+% pure formic acid (CH_2O_2), and (Alfa Aesar) synthetic bilirubin (97% purity) were purchased from Fisher Scientific (Waltham, MA). 30% monomeric bovine serum albumin (BSA) and patient serum bilirubin samples were obtained from Ortho Clinical Diagnostics Manufacturing (OCD). All patient samples and standards were stored in amber glassware at -80 ℃ until use.

Preparation of the Two Mobile Phase System

RP- HPLC utilized a two-mobile phase system: an aqueous solvent (A) followed by a non-polar acetonitrile (B). To prepare mobile phase A, 100 mL of acetonitrile (CH_3CN), 20 mL of dimethyl sulfoxide (DMSO), 2 mL of formic acid (CH_2O_2), and 890 mL of HPLC grade water were combined. DMSO serves a critical role as it is one of the few solvents capable of completely solubilizing bilirubin in solution while formic acid acts as a buffer and maintains a solution pH of 2.5 (data not shown). The acidic nature of the solution enhances the shape of the chromatogram peaks and further resolves the broad albumin peak from other bilirubin-related peaks. Acetonitrile and water are solvents of choice since they maintain low viscosity and have low UV cutoffs. This creates minimal interference with the intended peaks and ensures maximum resolution of the chromatography (Burdick and Jackson 2004). To prepare mobile phase B, 950 mL of acetonitrile, 20 mL of DMSO, 2 mL of formic acid, and 50 mL of HPLC-grade water were combined. Both mobile phases were made when needed and were stored at room temperature. Before usage, each mobile phase was stirred to guarantee homogeneity and properly filter and degas the solution.

Preparation of the Bilirubin Standard Solution

Two standards were prepared from the Alfa Aesar synthetic standard (97.0% purity) using approximately 100.0 mg of the standard; the material was dissolved in 100 mL of DMSO using a 100mL amber volumetric flask. Both standards were prepared in duplicate. The standard was used to test the quality and traceability of the reference material that is used to validate and calibrate the HPLC instrument. A standard curve (0 - 100 mg/dL) was prepared to assess linearity and to calculate the limit of detection. Repeatability was assessed in each chromatographic queue along with two preparations of the standard (5 repeats of standard A and 2 repeats of a second preparation

standard B). When the two standards are compared, confidence can be achieved concerning the proper weighing of the samples.

To prepare the RP-HPLC synthetic bilirubin standard, approximately 75% of a 100-mL amber glass volumetric flask was filled to the top with DMSO. 100.0 mg ± 0.5 mg of synthetic bilirubin was placed into the flask and the solution was stirred with a stir bar for 30 minutes to ensure dissolution. The solution was placed in the dark to prevent bilirubin degradation until HPLC testing was conducted. Photodegradation was observed after testing a control sample that was exposed to light (data not shown).

The standard solution was further diluted 1:10 in DMSO to remain consistent with the preparation of the sample. Dilution is critical for limiting the contamination of the HPLC column and conserving valuable patient samples while maintaining sufficient sensitivity. The stock solution was preserved in amber glassware for three months at 25 ℃ while sample solutions were preserved in amber vials for up to one week.

Preparation of the Albumin Retention Marker

To measure the relative retention of the human albumin peak, an additional synthetic Bovine Serum Albumin (BSA) from Ortho Clinical Diagnostics (OCD) was utilized. This secondary standard was adopted since all patient samples were complexed with human serum albumin. HPLC samples were prepared by diluting 30% liquid monomeric BSA 1:100 with HPLC grade water yielding a 0.3% albumin sample. Bovine albumin (583 amino acids) and human albumin (585 amino acids) are extremely alike to each other and have comparable RP-HPLC chromatographic properties (Ketrat et al. 2020). According to the Basic Alignment Search Tool

(BLAST), BSA and HSA have 76% amino acid sequence homology which indicates common ancestry and similar structure/function.

Patient Sample Selection, Preparation, and Sequence

From one hundred samples obtained from Ortho Clinical Diagnostics (OCD) with a diversity of Bu, Bc, and LR concentrations, sixteen patient serum vials were selected to dilute and test for bilirubin levels. Hours after delivery, serum samples were collected, and have been preserved at -80 ℃ for the past decade. All the patient samples were diluted 1:10 with mobile phase A.

The sample queue consisted of duplicate injections of a mobile phase blank followed by five replicate injections of the standard bilirubin solution (10 mg/dL). All the patient samples were injected in duplicates to verify the HPLC's measurement.

HPLC Analysis

HPLC analyses were conducted using the commercial *Agilent 1100* Series System (Agilent Technologies, Waldbronn, Germany) consisting of a quaternary pump (G1311A QUAT PUMP), degasser (G1322A), thermostatic autosampler (G1313A ALS) with a 200-µL injection loop and a diode-array detector (G1315A DAD) (Figure 7). ChemStation (for LC 3D system Rev. B01. 03 204, Agilent Technologies 2001-2005) was used for the evaluation of the collected data. In each run, 10 µL of each sample was injected. The flow rate was set to 0.8 mL/min, the column temperature was adjusted to 30 ℃, and the acquisition wavelengths were set to measure both 280 and 460 nm. 280 nm because it is consistent with the absorption of serum proteins like albumin and 460 nm is consistent with the yellow-orange pigment of bilirubin. (Chang et al. 2011). The

four major bilirubin analogs have a comparable extinction coefficient according to the Beer-Lambert Law (Chang et al. 2011). Separations were performed on a stainless-steel reverse phase Waters XBridge BEH300 C18 column (150 mm x 4.6 mm I.D.) with a particle size of 3.5 µm and C18 bonded silica beads.

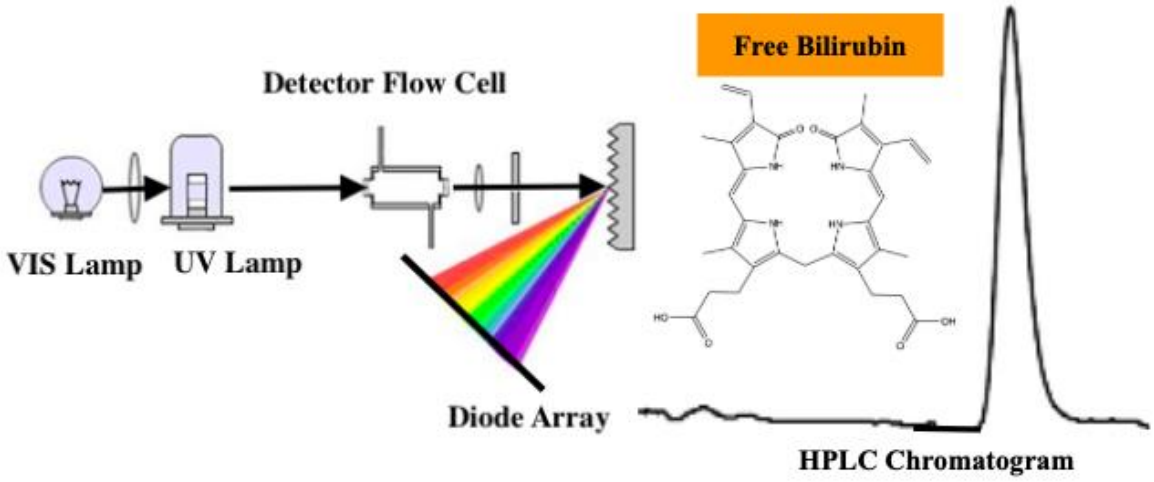


Figure 7: Schematic representation of HPLC system. RP-HPLC relies upon the reversible adsorption of solute molecules with changing levels of hydrophobicity. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase. As molecules are pumped through the column, peaks are generated. Both visible (VIS) and Ultraviolet (UV) light are utilized in the determination of bilirubin species as the acquisition wavelength was configured to 280 and 460 nm. Figure was created using BioRender.com.

Bilirubin Identification and Quantification

The sample peak representing bilirubin was identified based on the retention time (t_R) value obtained after HPLC-DAD (diode array detector) analysis and was compared with the t_R value obtained after the analysis of the bilirubin standard solution. Accordingly, the external standard calibration curve was generated using six data points covering a 0 - 100 mg/dL range. The

calibration curve for the HPLC-DAD system was obtained by plotting the average of the integrated areas versus the concentration (mg/dL) of the sample.

2. RESULTS AND DISCUSSION

3.1 Size Exclusion Chromatography

Size Exclusion Chromatography Rational

Given the premise that all bilirubin species are bound to the carrier protein albumin, all the masses of the bilirubin species can be treated as though they are a mass approximately equal to that of albumin as the mass of bilirubin species alone (534 – 936 amu) are negligible compared to the mass of albumin (67,000 amu) (Table 6) (Jacobsen & Brodersen 1983; Iskander et al. 2014; Perlman et al. 2018). Thus, all bilirubin species can be detected at ~67,000 amu through SEC. This technique separates large compounds and proteins according to their respective molecular weights with a low enough separation to group all bilirubin-albumin complexes into one chromatographic peak.

Table 6: Nominal atomic weights of bilirubin-albumin complexes

Species of Bilirubin	Atomic Weight of species (amu)	Atomic Weight of species when albumin-bound (amu)
Unconjugated Bilirubin (Bu)	534	67,021
Lumirubin (LR)	534	67,021
Monoglucuronide Conjugated Bilirubin (mono Bc)	760	67,197
Diglucuronide Conjugated Bilirubin (di Bc)	936	67,373

Data were obtained from SciFinder-CAS.org.

Chromatography system

Our earlier SEC studies utilized a mobile phase system consisting of 0.05 M monobasic potassium phosphate (KH_2PO_4), 99% pure 0.05 M dibasic potassium phosphate (K_2HPO_4), and 0.025% sodium azide (NaN_3). However, reproducibility did not meet expectations due to the absence of bilirubin in the mobile phase. This led to the dissociation of the albumin-bilirubin

complex and bilirubin carryover occurred between injections within the column indicating dissociation of the albumin-bilirubin complex, particularly at 460 nm, but not at 280 nm (Figure 8). Since there was no carryover seen between injections at 280 nm, it was concluded that the bilirubin interacted with the column and remained bound to the silica beads due to its hydrophobic nature, but that the albumin elutes as expected. Furthermore, it was observed that the bilirubin would not elute from the column until a subsequent sample containing BSA was injected. The BSA served as a carrier protein that encapsulated the bilirubin species and rendered them polar. This adjustment facilitated the elution of all bilirubin species complexed to albumin in a single chromatographic peak. Injections of mobile phase and DMSO showed no bilirubin, but when albumin or Bu-albumin sample was injected, the bilirubin would elute separately from the column dissociating from the albumin-bilirubin complex. To counteract this issue, a few different mobile phase conditions were tested, including varying concentrations of BSA in the mobile phase and aided in the maintenance of the desired stable albumin-bilirubin complex.

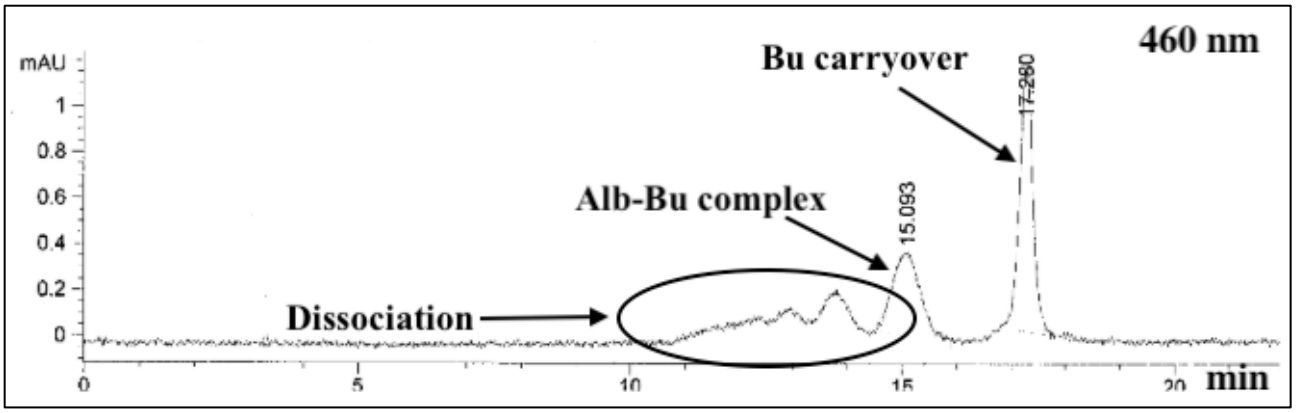


Figure 8: SEC chromatogram of a random bilirubin sample. Dissociation of the albumin-bilirubin complex is observed. Bilirubin carryover is seen at 17.280 min signifying the need for mobile phase adjustments.

In SEC analysis, adding albumin stabilizes the albumin-bilirubin complex and is especially important for Bc and Bu bilirubin fractions (Jacobsen & Brodersen 1983). According to Le

Chatelier's Principle "if a constraint (such as a change in pressure, temperature, or concentration of a reactant) is applied to a system in equilibrium, the equilibrium will shift to counteract the effect of the constraint". An "n-fold" addition of albumin drives the system's equilibrium to the right, preventing the dissociation of the complex and any bilirubin carryover (Figure 9).

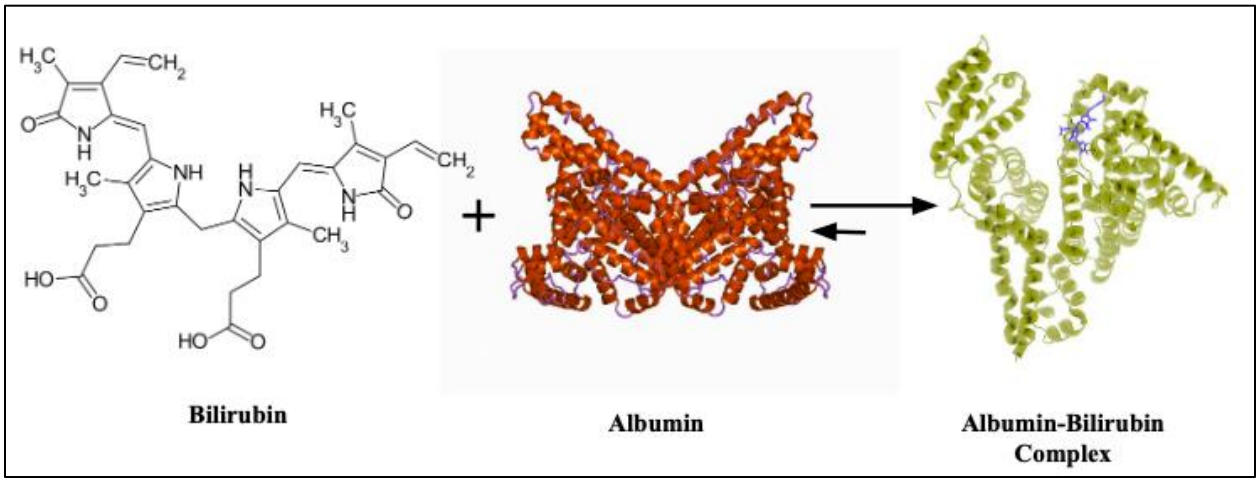


Figure 9: Albumin-bilirubin complex formation. "N-fold" added albumin drives the equilibrium to the right and permanently prevents the dissociation of the albumin-bilirubin complex which is critical in SEC analysis. Protein images of albumin (file 1AO6) and the albumin-bilirubin complex (file 2VUE) are available from RCSB Protein Data Bank.

Bovine Serum Albumin (BSA) was employed due to its analogous functions and similar physicochemical properties to Human Serum Albumin (HSA) (Jacobsen & Brodersen 1983). To determine the appropriate amount of BSA to be added to the mobile phase, a precision study was performed under different mobile phase conditions. A total of six bilirubin fractions were tested including both synthetic (NB01) and patient samples (Patients N3 N2 N10, and JC NC). These fractions were labeled such that the anonymity of the patients was respected after collection and distribution. Varying concentrations of 30% monomeric liquid BSA were introduced into 1 L of the mobile phase (0-10,000 µL/L). Percent RSD values were calculated for each sample (Figure 10). The results demonstrated that both 1000 µL/L and 2000 µL/L provided optimal %RSD values

(<3% respectively). Ultimately, these concentrations were sufficient to completely encapsulate the bilirubin and form a stable complex.

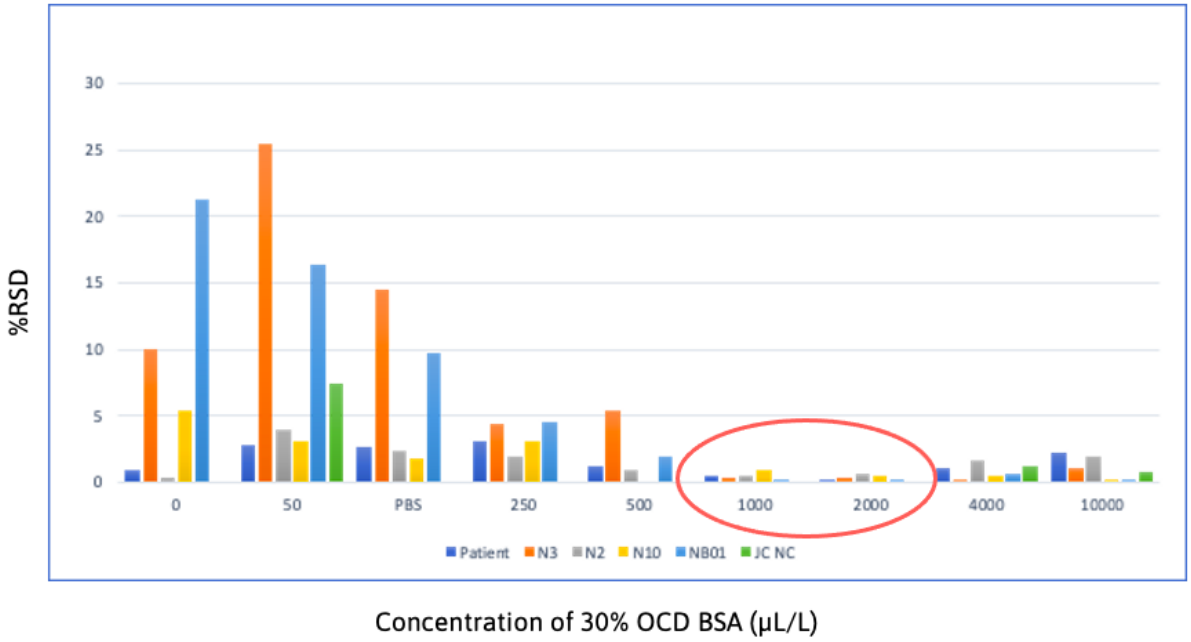


Figure 10: SEC precision under different mobile phase conditions. Both 1000 µL/L and 2000 µL/L provided optimal %RSD values (<3% respectively). PBS refers to phosphate buffered saline (albumin free). Figure was generated in Microsoft® Excel.

A recovery test was performed by comparing the total bilirubin concentrations tested using the mobile phase with varying concentrations of liquid BSA (Figure 11). The aim was twofold: to establish which concentration of BSA would limit the dissociation of the albumin-bilirubin complex and to further confirm the previous precision results (Figure 10). As seen by the sum peak for 0, 0.05, 0.25, 0.5, 1 and 2 mL/L. 1 mL of BSA/L in the mobile phase offered maximum recovery when compared to the other concentrations tested. Thus, the recipe for the mobile phase was adjusted accordingly.

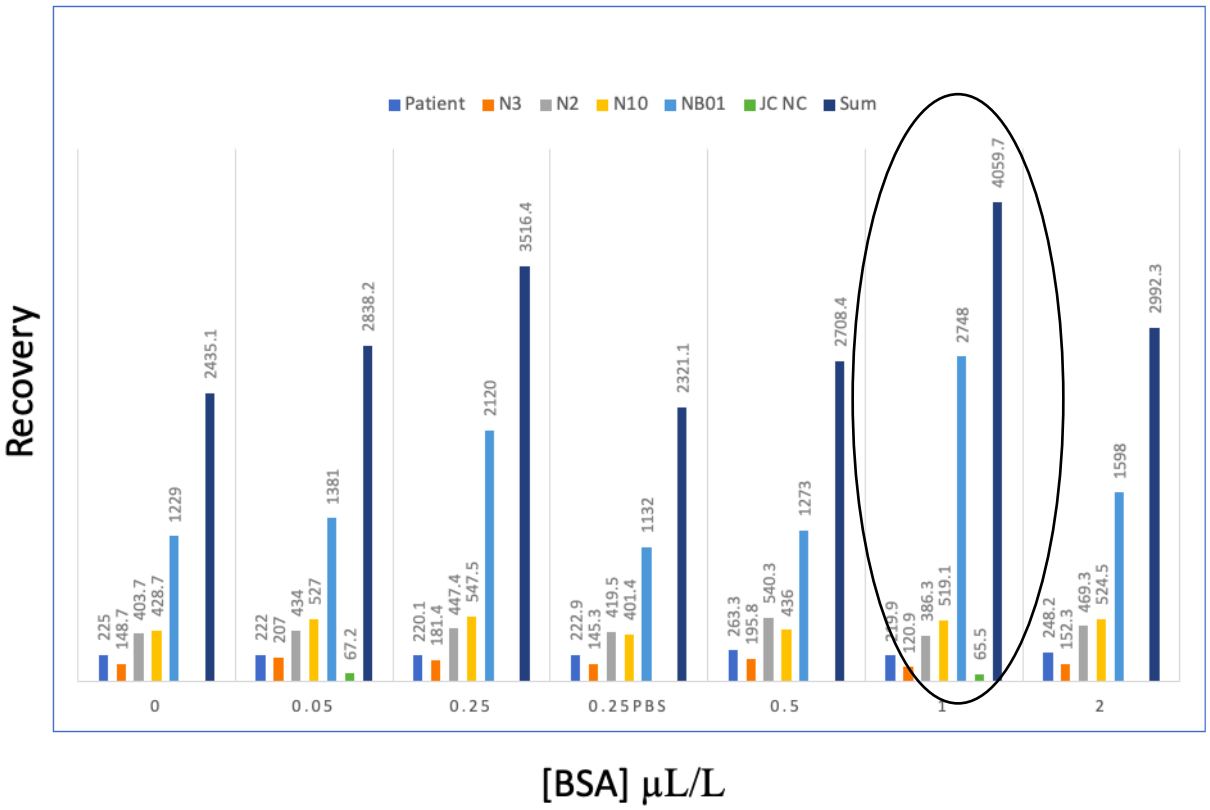


Figure 11: Recovery of patient samples with varying concentrations of liquid BSA in the SEC mobile phase. 1 mL/L provided an optimal recovery. PBS refers to phosphate buffered saline (albumin free). Figure was generated in Microsoft® Excel.

A bilirubin sample was run using the updated mobile phase recipe containing 1 mL of 30% liquid BSA. On the chromatogram (Figure 12), a single peak is visible at ~11 minutes, the same elution time as albumin signifying the resolution of the carry-over issue as no other peak is visible at 460 nm. Not only does the importance of BSA presence within the mobile phase become apparent, but this also demonstrates the significance of the albumin-bilirubin complex. The single chromatographic peak observed signifies that bilirubin is entirely encapsulated by the carrier protein albumin and that the updated mobile phase contains sufficient albumin to prevent the complex from dissociating while testing samples. It also confirms the results of the recovery test and that 1 mL/L of BSA is optimal.

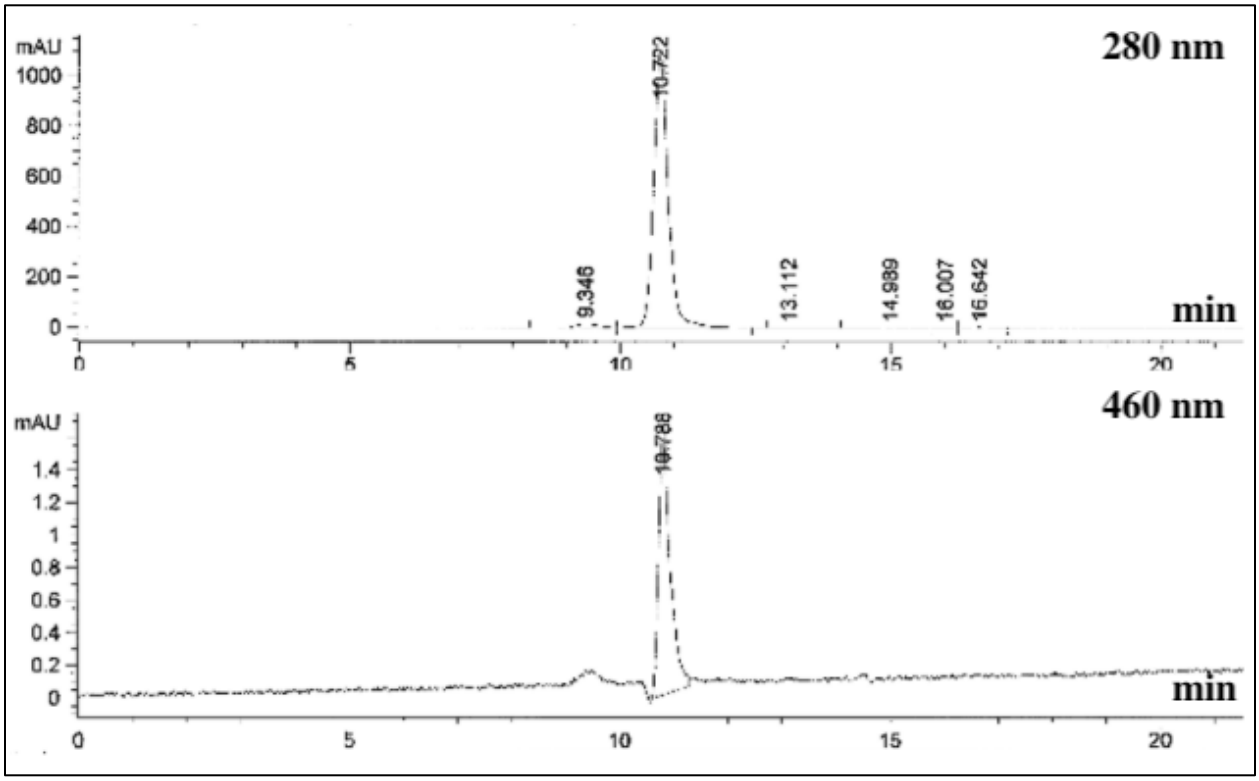


Figure 12: SEC chromatogram for a bilirubin sample using the mobile phase containing 1 mL/L of liquid BSA. The additional peaks observed at 280 nm correspond to other serum proteins such as tryptophan.

As seen previously and in our experiments, in SEC analysis, adding albumin stabilizes the albumin-bilirubin complex and is especially important for Bc and Bu bilirubin fractions (Jacobsen & Brodersen 1983). Keeping this in mind and taking into consideration Le Chatelier's Principle, a concentration of liquid BSA was added to all patient sample vials as a means of prohibiting the dissociation of the albumin-bilirubin complex. Trial experiments were conducted by introducing two varying concentrations of liquid BSA: 4.4 g/L and 44 g/L, respectively. In previous experiments, the Bc-albumin complex began to dissociate due to the polarity and additional glucuronide groups (Jacobsen & Brodersen 1983). This conclusion was made based on the increased size of the Bc bilirubin species which requires additional BSA to be completely encapsulated. A comparison of the trial run of the neonatal patient sample 9 demonstrated that 44 g/L of liquid BSA presented the least amount of dissociation as seen by the absence of the peak of

14.805 min (Figures 13 & 14). Thus, 44 g/L of liquid BSA was incorporated into each patient vial prepared. Ultimately, 44 g/L of albumin ensured that all the components (Bc, Bu, and LR) remained complex in solution.

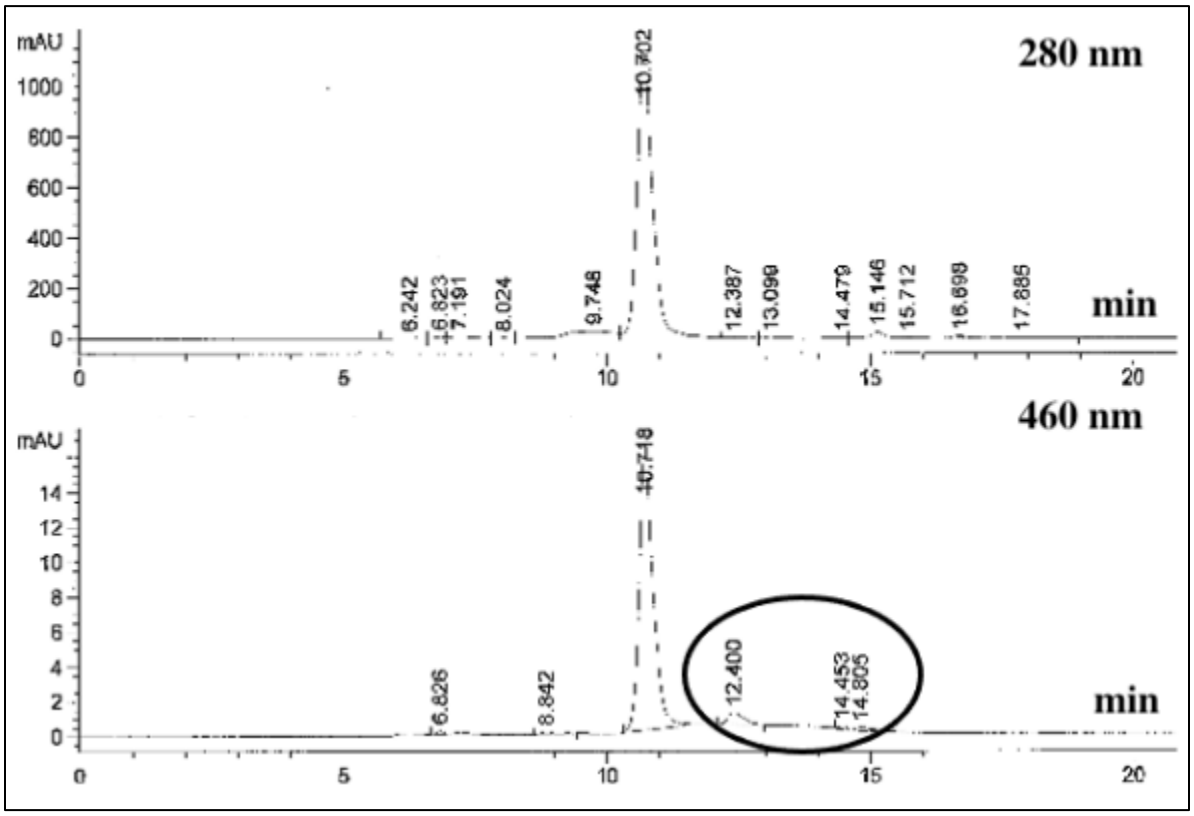


Figure 13: SEC chromatogram demonstrating the albumin-bilirubin complex (4.4 g/L BSA). Alb-Bu peak elutes at 10.718 minutes**.**

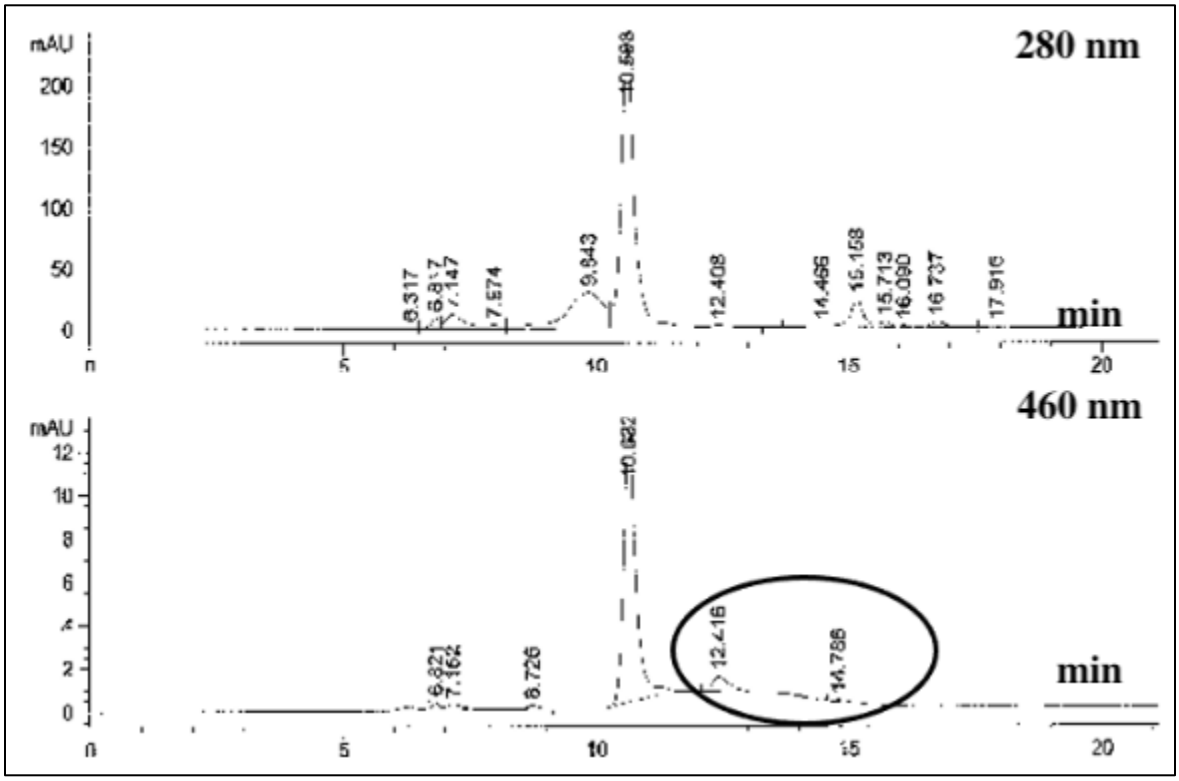
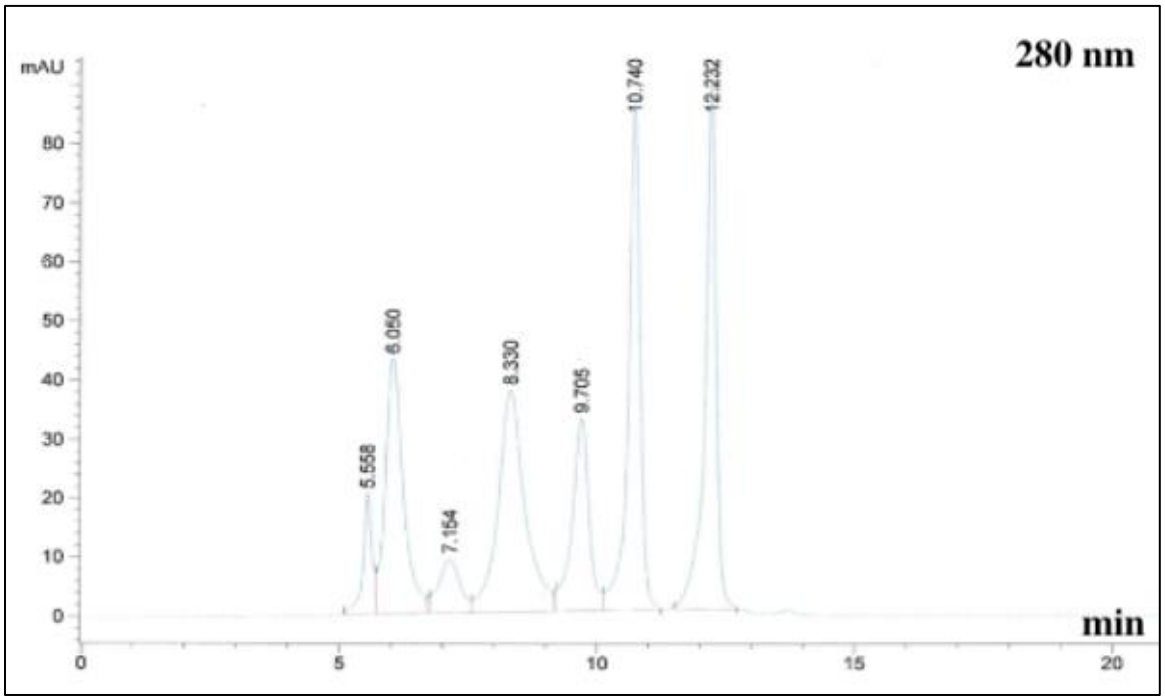


Figure 14: SEC chromatogram demonstrating the albumin-bilirubin complex (44 g/L). Alb-Bu peak elutes at 10.718 minutes**.**

Column performance

With the proposed SEC configuration and methodology, column lifetime was dramatically improved. No significant degradation to the column resolution was observed and retention times were remarkably stable throughout the multiple sequence injections (Figure 15). This was determined by the sharpness of the peaks produced and by comparing the elution times to the reference values provided by the manufacturer of the Tosh Bioscience TSKgel G3000SWxL column from Sigma Aldrich.

(a)



(b)

Compound	Retention Time (min)	Log Molecular Weight
Thyroglobulin	6.05	5.83
Gamma Globulin (bovine)	8.30	5.20
Ovalbumin	9.40	4.64
Myoglobin	10.70	4.23
Vitamin B12	12.20	3.13

Figure 15: SEC column performance. Size Exclusion Test Mix standard chromatogram demonstrating the quality and performance of the column (a) and compound retention times (min) and the logarithm of their corresponding molecular weights.

To experimentally deduce the elution time of albumin, an SEC calibration curve was generated by plotting the logarithm of molecular weight of each compound present in the SEC test mix standard versus their corresponding retention time. This experiment was conducted after the mobile phase recipe was updated to ensure accurate results. A line of best fit was fitted to the data obtained and using the equation generated y= -0.378 x + 9.17 and the molecular weight of albumin (67,000 amu), the data demonstrated that the protein is expected to elute at approximately 11.54 minutes (Figure 16).

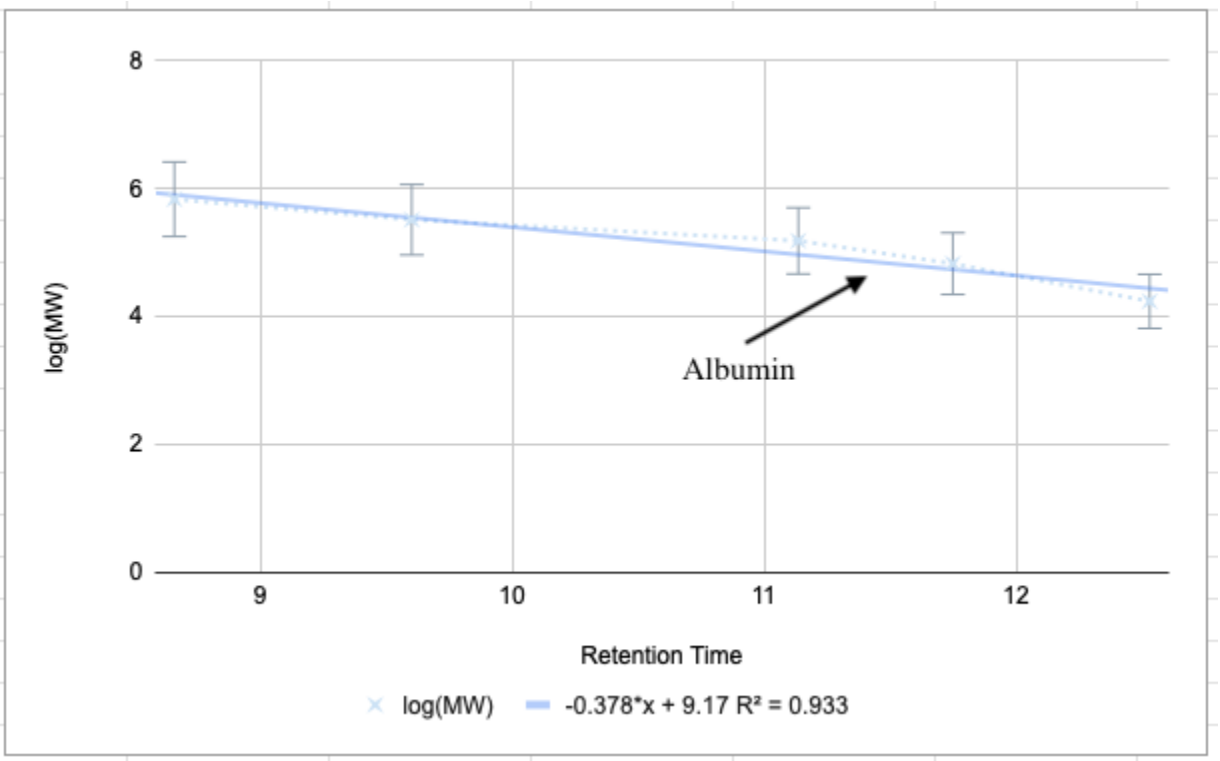


Figure 16: Calibration curve for SEC test mix standard. In each case, 10 µL of the diluted standard was injected. The line was fitted using a linear line of best fit (Microsoft® Excel) with R^2=0.933.

Significance of the Albumin Injection

Given the premise that all bilirubin species are bound to the carrier protein albumin, all the masses of the bilirubin species can be treated as though they are a mass approximately equal to that of albumin, considering that the large mass of albumin (67,000 amu), and the masses of the bilirubin species (534-936 amu) are relatively negligible. 20 µL of 30% liquid BSA was injected to confirm the albumin elution time. The sample was run at both 280 and 460 nm wavelengths, respectively. At 280 nm, albumin eluted as a single peak at approximately 11.5 minutes supporting the prediction generated by the calibration curve (Figure 17). No activity was observed at 460 nm as expected due to the absence of bilirubin and most proteins, notably albumin, absorb in the 280 nm wavelength.

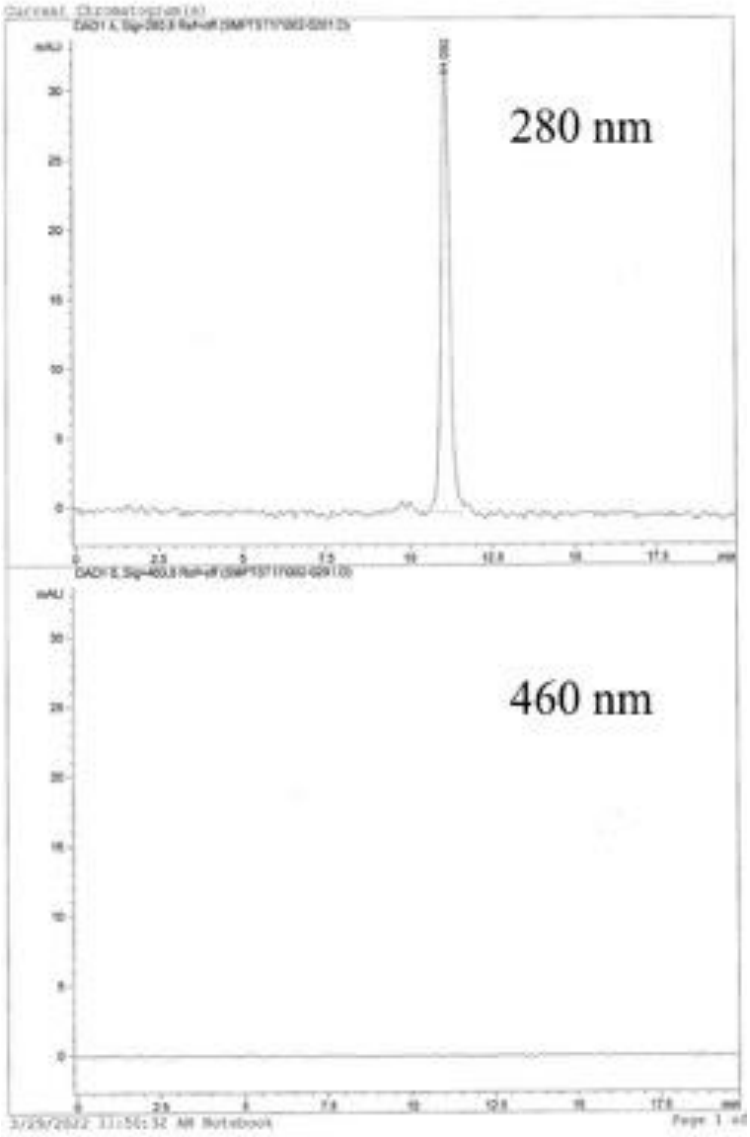


Figure 17: Chromatogram of a pure albumin standard. Albumin (MW=66,500 amu) elutes as a single peak at approximately 11.5 min. No activity is detected at 460 nm.

Determining Linearity from Samples

To determine the accuracy and precision of the SEC of patient and surrogate standards, linearity testing was carried out on Bu-BSA samples over a range of concentrations ranging from 2 to 32 mg/dL. The range of concentrations chosen represents target concentrations commonly seen in clinical settings. The average peak area of five consecutive injections was plotted against

the target concentrations before the data were fitted with a line of best fit (Figure 18). The R^2 value was 1.0 demonstrating exceptional performance across a wide range of concentrations that exceeds current laboratory techniques several folds (Doumas et al. 1987; Granbenhenrich et al. 2014; Ecran & Ozgun 2018).

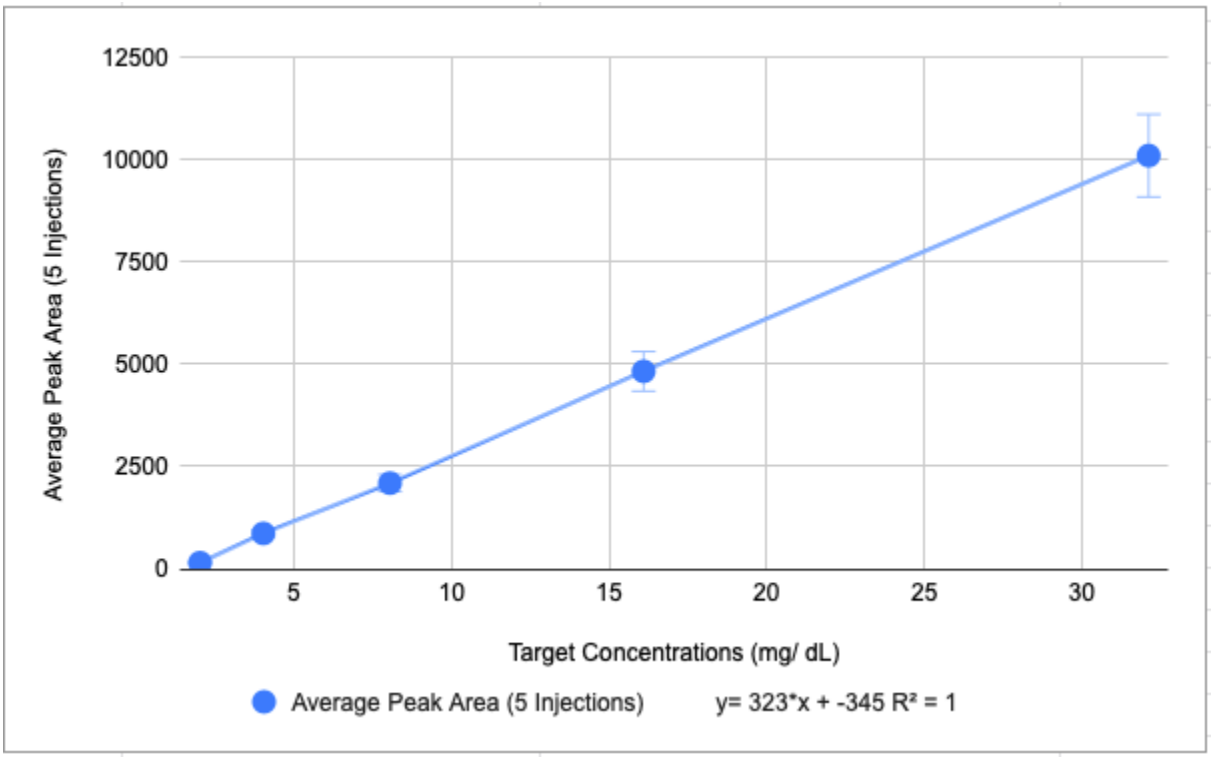
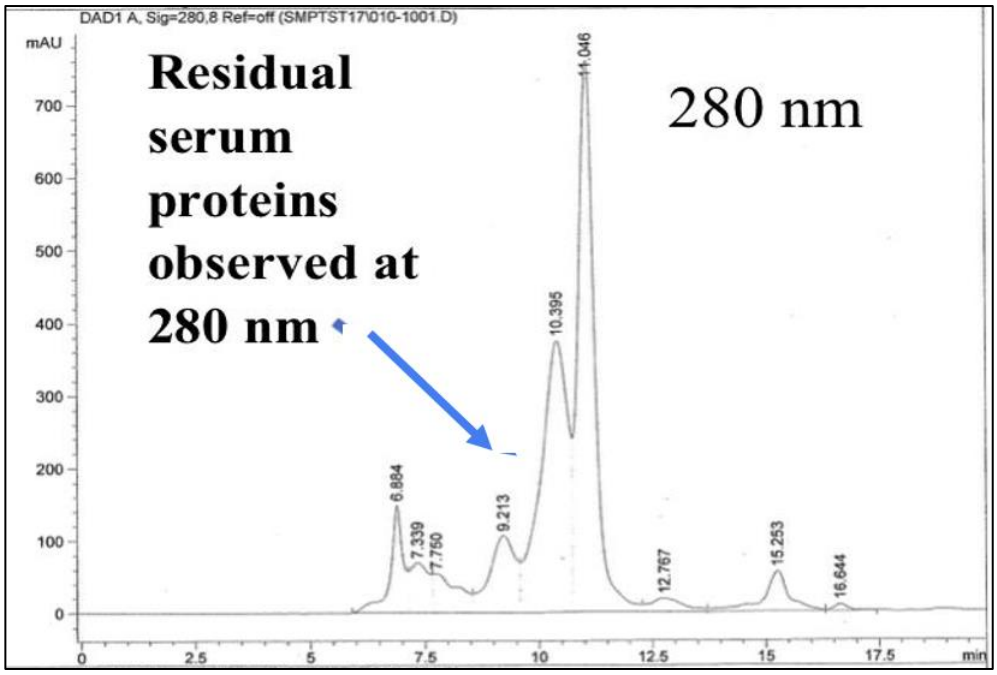


Figure 18: Accuracy and linearity testing of Bu-BSA samples. Samples were prepared for accuracy testing over a target concentration range of 2-32 mg/dL. The line was fitted using a linear line of best fit (Microsoft ® Excel) with R^2=1 demonstrating linearity in this range.

Identification of Total Bilirubin Peak in Patient Samples

A neonatal patient sample was run to determine the concentration of TBIL at both 280 and 460 nm, respectively. At 280 nm, residual serum proteins were observed. Albumin appeared as a single, and tall peak that eludes at ~11 minutes for 280 nm (Figure 19). The remaining peaks correspond to serum proteins such as tryptophan, hemoglobin, and myoglobin (Kalakonda et al.

2021). At 460 nm, only total bilirubin conjugated to albumin was observed and eluted at the same time as albumin. This further confirms the presence of an albumin-bilirubin complex, and the potential for SEC to measure TBIL concentrations. The concentration of the patient sample was calculated by utilizing a reference standard and drawing a comparison using a suitable standard and average peak area produced by the albumin-bilirubin complex. The TBIL concentration of neonatal sample 7 was estimated to be [TBIL]=2.5 mg/dL. This value was expected given the area of the peak produced by the albumin-bilirubin complex at 460 nm.



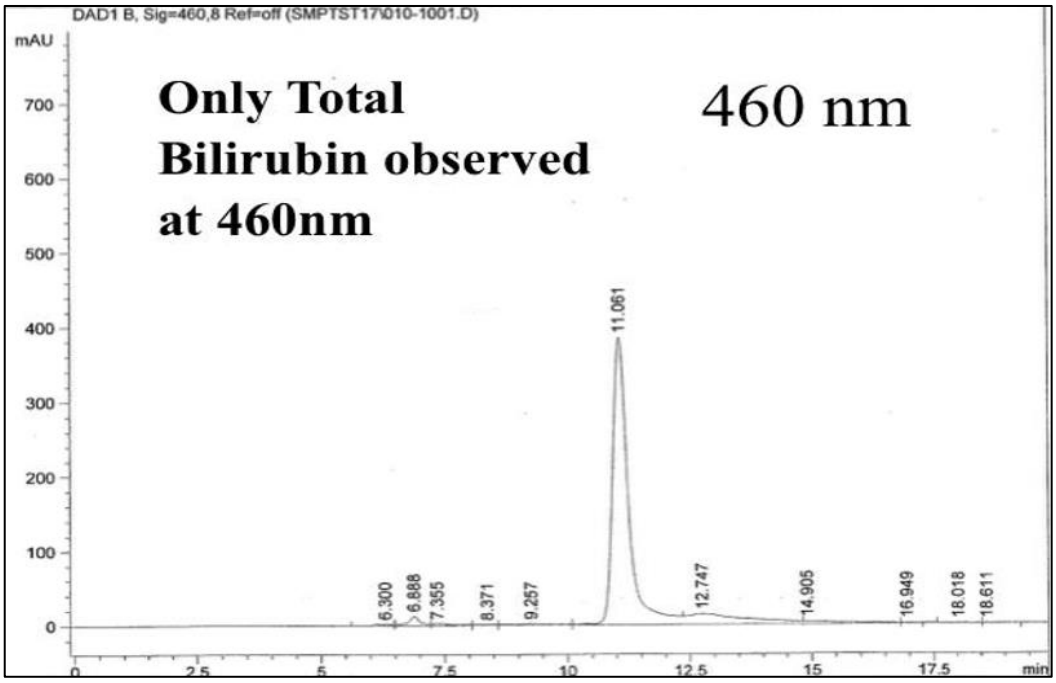


Figure 19: Chromatogram of the neonatal patient sample 7

3.2 Reversed-Phase High-Performance Liquid Chromatography

RP-HPLC Chromatography System

A binary RP-HPLC mobile phase system consisting of an aqueous solution (A) (100 mL CH_3CN, 20 mL DMSO, 2 mL CH_2O_2, and 890 mL of HPLC grade H_2O) and a more organic solution (B) (950 mL CH_3CN, 20 mL DMSO, 2 mL CH_2O_2, and 50 mL HPLC grade H_2O) was utilized for sample data acquisition. The transition between both hydrophilic (90% aqueous buffer/10% CH_3CN) to the more hydrophobic phase (95% CH_3CN/5% aqueous buffer) enabled the gradient to successfully be eluted from the RP column. In other words, as the concentration of mobile phase B increases, the more hydrophobic molecules eluded off the column at specific and consistent retention times (Figures 20, 23, 24, and 26). This binary mobile phase system was designed according to Broderson (1979) to maximize the function of the HPLC instrumentation

and differs greatly from the original parameters outlined in 1981 by Lauff and colleagues (Table 7).

In the mobile phase system, acetonitrile is less viscous (0.38 centipoise (cp)) than isopropanol (2.4 cp); thus, it significantly reduces the pressure and preserves the functionality of the system. Additionally, the UV cutoff for acetonitrile (190 nm) is lower than isopropanol (205 nm) generating minimal interference with the intended peaks (Figure 19). This composition ensured maximal resolution of the chromatography and produced discernable sharp peaks allowing for the precise differentiation of bilirubin species. The present chromatography system has enabled the elution and separation of both albumin and unconjugated bilirubin species (Bu and LR) due to the presence of DMSO, one of the very few solvents capable of entirely solubilizing bilirubin in solution (Table 8) (Brodersen 1979).

Table 7: Comparison of previous versus current mobile phase parameters utilized in RP-HPLC instrumentation

Parameter	1981	Current
Solvent	Isopropanol/Phosphoric Acid	Acetonitrile
Mobile Phase A	5% Isopropanol	10% Acetonitrile
Mobile Phase B	95% Isopropanol	95% Acetonitrile

Note: 1981 parameters were established by Lauff and colleagues' paper.

Table 8: Approximate solubility of bilirubin in various solvents at 25 ℃

Solvent	Solubility /µM	Solvent	Solubility/µM
n-Hexane	0	Methanol	0
Cyclohexane	0	Ethanol	0
n-Heptane	0	1-Propanol	0
Liquid paraffin	0	2-Propanol	0
N, *N*-Dimethylformamide (DMF)	800	1-Butanol	0
Benzene	22	1-Pentanol	0
Toluene	50	Cyclohexanol	0
Xylene	45	Olive Oil	1.3
Water pH 7.0	≈0.001	Ether	1
Pyrrole	150	2-Methoxyethanol	15
Pyridine	580	Lard	2
Dimethyl Sulfoxide (DMSO)	**>10,000**	Acetone	10
Dichloromethane	1,800	Butanone	22
Chloroform	2,500	2-Pentanone	25
Carbon tetrachloride	40	4-Methyl-2-pentanone	15
Formamide	2,300	2,6 Dimethyl-4-heptanone	8
N, *N*-Dimethylformamide (DMF)	800	Ethyl Acetate	9

Adapted from Broderson (1979)

Additionally, the mobile phase was adjusted to include formic acid which was necessary for a reproducible chromatographic separation of bilirubin species and consistent sample recovery (Broderson 1979). Before the addition of formic acid to the mobile phase, there was a substantial loss of resolution as seen by the poor quality of the peaks observed at 460 nm (Figure 20). Peak broadening and tailing became progressively more severe, and the absolute area of the peaks decreased. To disentangle the bilirubin species from the albumin-bilirubin complex, 2 mL CH_2O_2 was introduced into the system in place of ammonia acetate (NH_4Ac) which lowered the pH from 5.5 to 2.5. The acidic nature of the solution enhanced the shape of the chromatogram peaks and further ensured quality resolution (Figure 23).

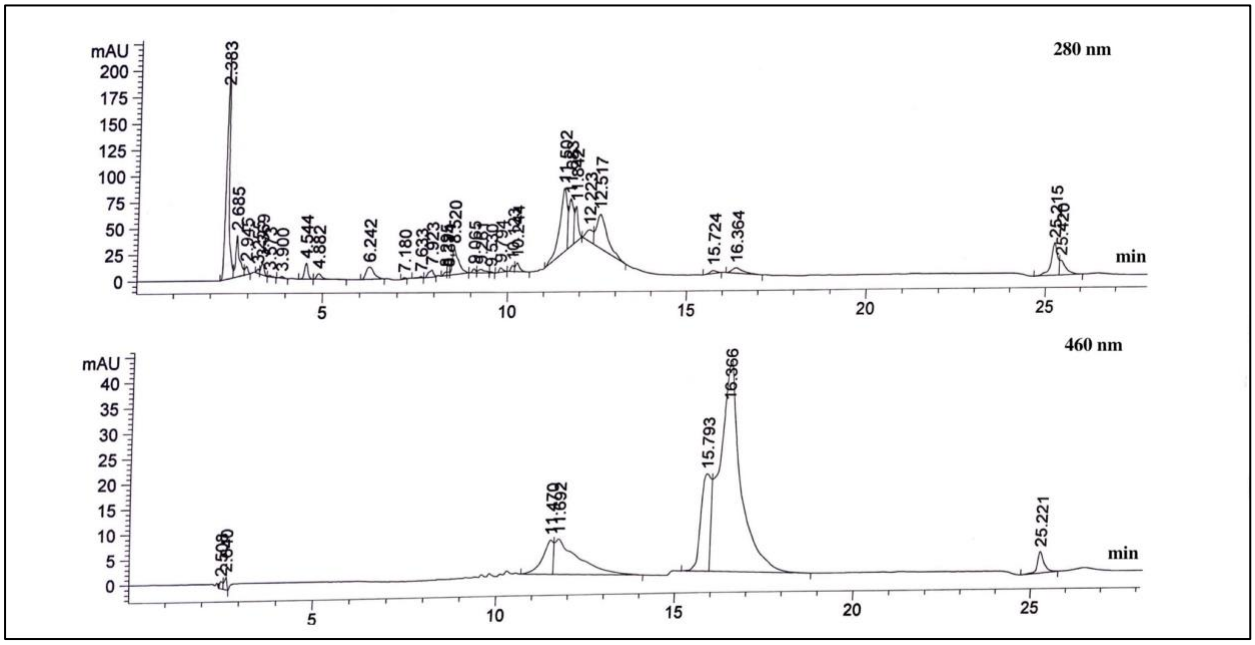


Figure 20: Neonate 9 patient chromatogram before the addition of CH_2O_2

Column Performance

No significant degradation to the column resolution was observed and retention times were remarkably stable throughout the multiple sequence injections (Figure 21). With the proposed RP-HPLC configuration and methodology, column lifetime was dramatically improved from lasting a few experimental runs (1 – 3) to multiple (>12).

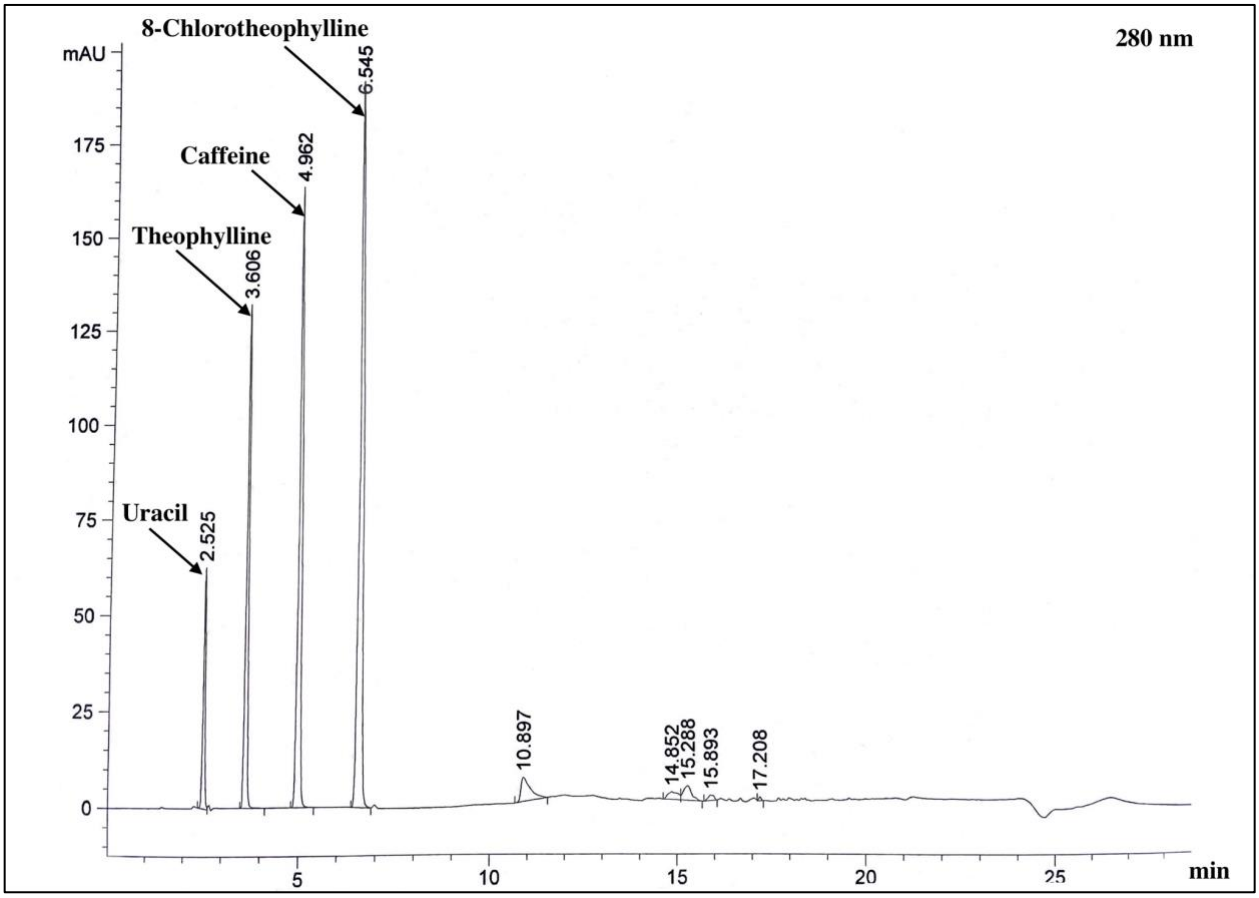


Figure 21: PharmAssist reference caffeine standard chromatogram

Standard Calibration Curve for Bilirubin

In our experiments, the diluted standards were automatically injected by the RP-HPLC autoinjector in overnight runs and yielded a linear calibration curve (Figure 22). All standards were

prepared and diluted for injection at the same time and remained in the automatic injector in incandescent light before injection. This procedure produced a linear calibration of area versus concentration with R^2<0.98 over a clinical range of interest (0-100 mg/dL). Peak heights were not generally used for quantification because of band broadening at higher concentrations. Instead, average peak areas were used as they provided a more accurate means for calculating bilirubin concentrations. The addition of formic acid at an acidic pH along with BSA minimized oxidation and increased the stability of standard solutions of bilirubin when diluted with DMSO for injection into the chromatography column.

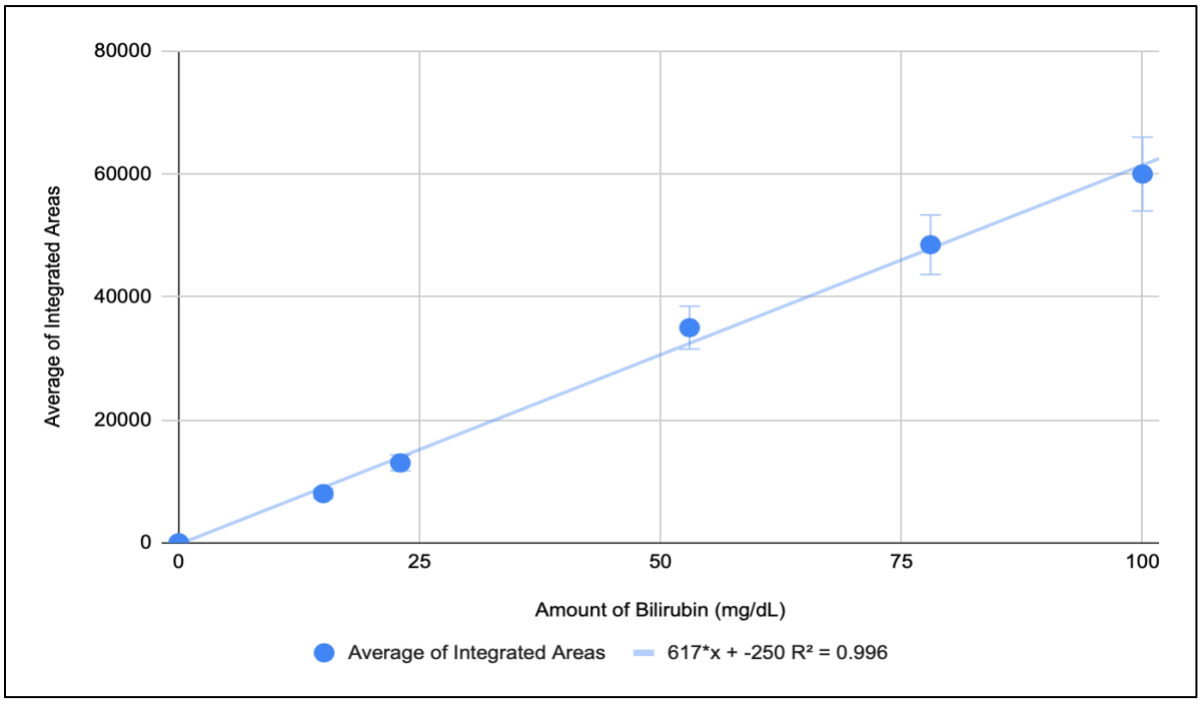


Figure 22: Bilirubin calibration curve. Concentrations are for standard solutions diluted 1:10 with DMSO. In each case, 10 µL of the diluted standard was injected. Figure made in Microsoft® Excel R^2=0.996.

Patient Sample Selection and Analysis

To determine the composition and presence of bilirubin species, sixteen patient samples and five synthetic standards were selected at random. The peak areas were obtained by chromatographic analysis with the quantification of bilirubin peaks solely. The patient total bilirubin concentrations [TBIL] were determined based on the anointed reference standard, 97% Alfa Aesar. According to the chromatographic peaks produced at 460 nm (Figure 23), the composition of the patient sample demonstrates the presence of Bu and LR; there was more Bu than LR which was expected. Similar results were obtained for the remaining three patient samples (data not shown). All the neonate samples received from Ortho Clinical Diagnostics were collected after birth. After birth, neonates require 24 – 48 hours to conjugate bilirubin (McDonagh et al. 2010). Thus, the free Bu peak is per the composition of a typical serum sample. The LR peak is due to light exposure and is expected with the handling and preparation of samples for testing. On the chromatogram with the synthetic bilirubin standard (Figure 24), a single chromatographic peak at 23.106 minutes is observed at 460 nm as expected since the sample contains Bu. The sample tested, NB-01, is a synthetic bilirubin standard. The synthetic nature of the sample can be confirmed by the presence of two small satellite peaks on either side of a tall sharp peak and a lack of LR peaks.

For two of the samples tested, the small absorbance on the 460 nm trace at the retention for albumin was mostly due to the slight inherent yellow color of the bovine serum albumin and not to unconjugated bilirubin (McDonagh et al. 2010). This observation confirms the disentanglement of the bilirubin species from the carrier protein albumin. This can be seen by a small peak at approximately 10.794 minutes, the same elution time as albumin at 280 nm (Figure 23). This peak

was minimally enhanced when compared with an injection of the albumin standard and was proportional to the amount added. Thus, in this chromatography system, the interaction of the column with bilirubin and albumin was strong enough to break up the complex known to exist between unconjugated bilirubin and albumin in an aqueous solution. Ultimately, the combination of silica beads in the column and the binary mobile phase system containing DMSO was sufficient to disrupt the albumin-bilirubin complex and render bilirubin and its species soluble in solution.

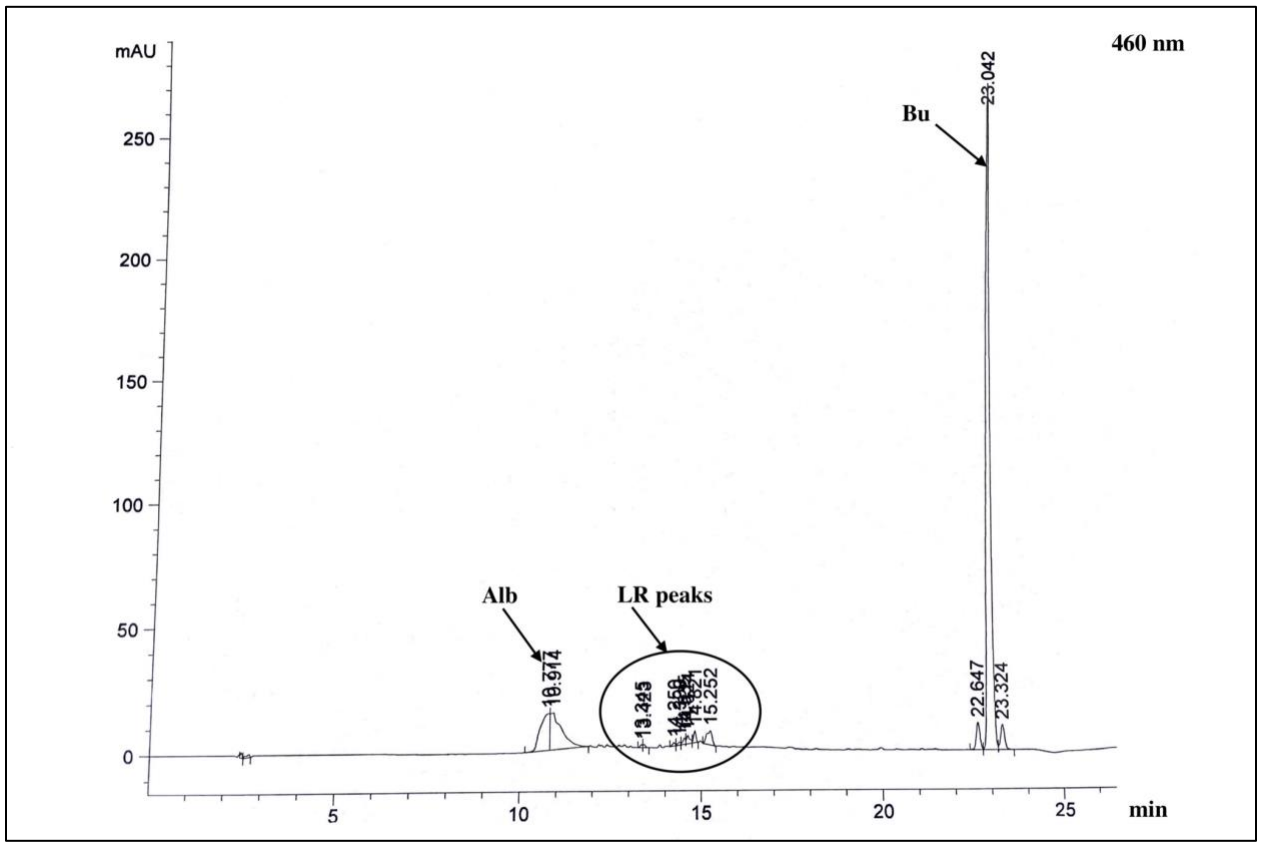


Figure 23: Neonate 9 patient chromatogram. HPLC separation demonstrates the presence of unconjugated bilirubin (Bu) and lumirubin (Lu) at 460 nm.

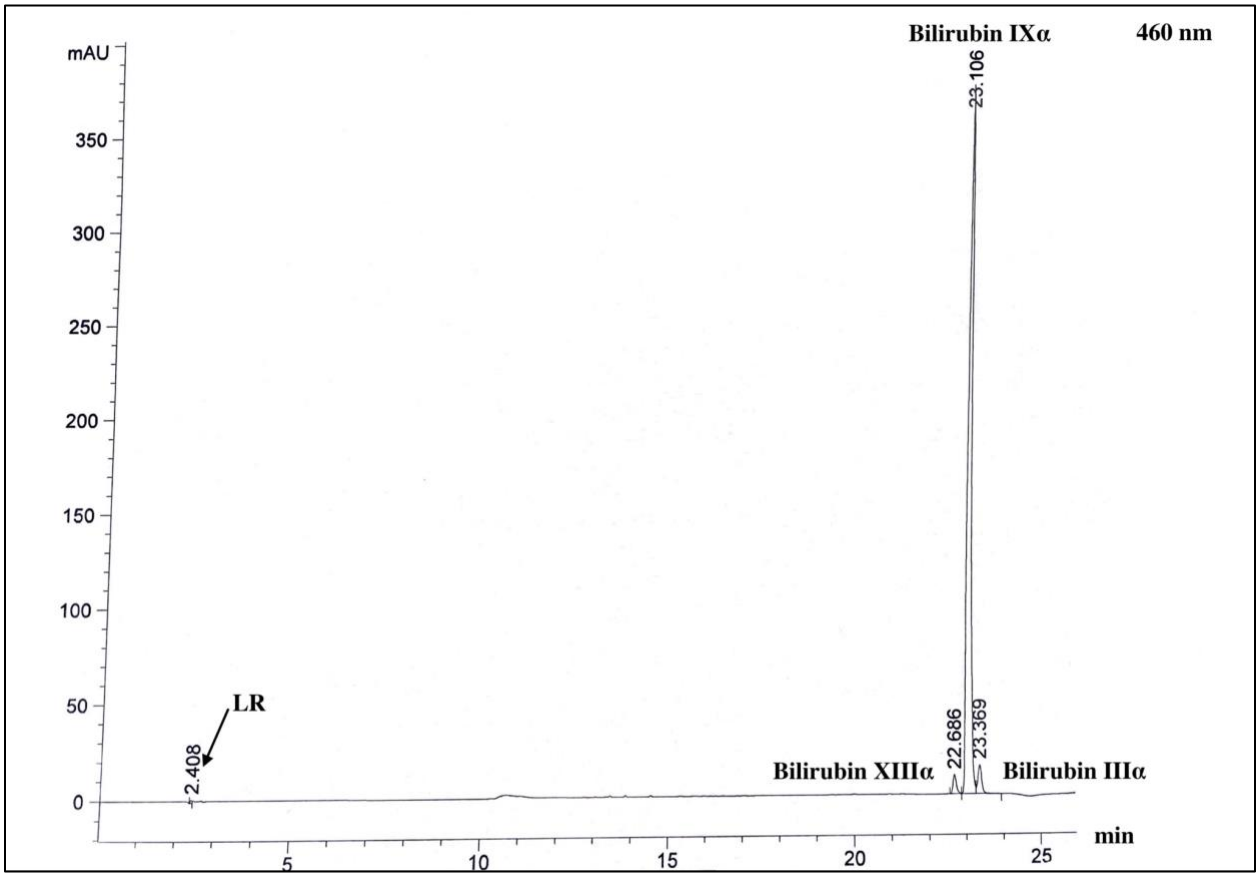


Figure 24: Chromatogram of a synthetic bilirubin sample NB-01 2012. HPLC separation of bilirubin (Bu or Bilirubin IXα) is observed, and satellite peaks (Bilirubin XIIIα and Bilirubin IIIα) confirm the synthetic nature of the sample.

Precision and Accuracy of RP-HPLC Testing

To ensure the accuracy of RP-HPLC testing, various criteria were instituted, and statistical values were calculated. Through the configuration of the RP-HPLC and the methodology used, data integrity was established through a blank baseline produced by the instrument to begin and end each sequence (Figure 25). High resolution was demonstrated with the PharmAssist caffeine standard test solution. Reproducibility was determined/verified through multiple injections (n = 5)

of 10.41 mg/dL of bilirubin standard B (Figure 26). The criterion for RSD is <2.0% and the actual RSD was 0.356% (Avg=97.157, SD=0.342).

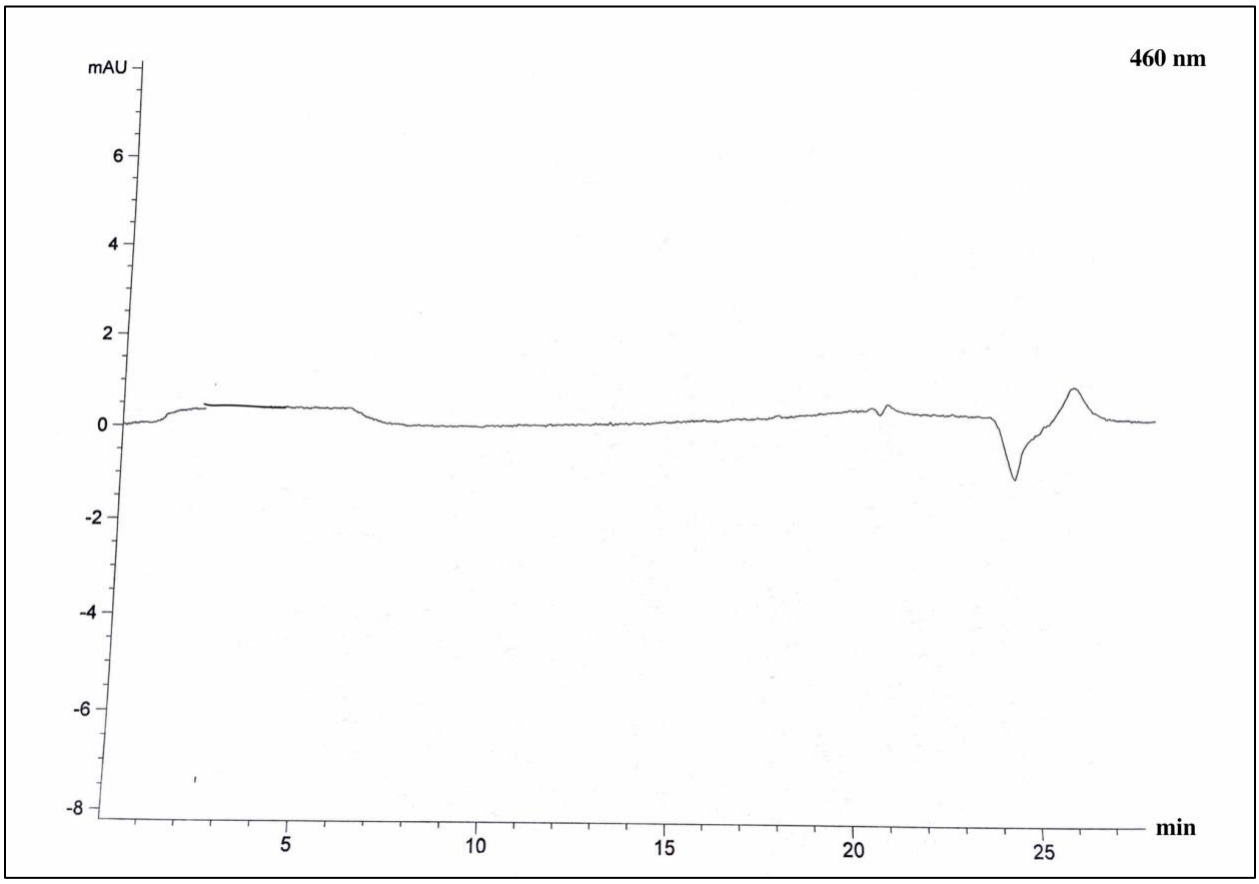


Figure 25: Reference blank chromatogram. Mobile phase A was tested and supports a zero-intercept baseline.

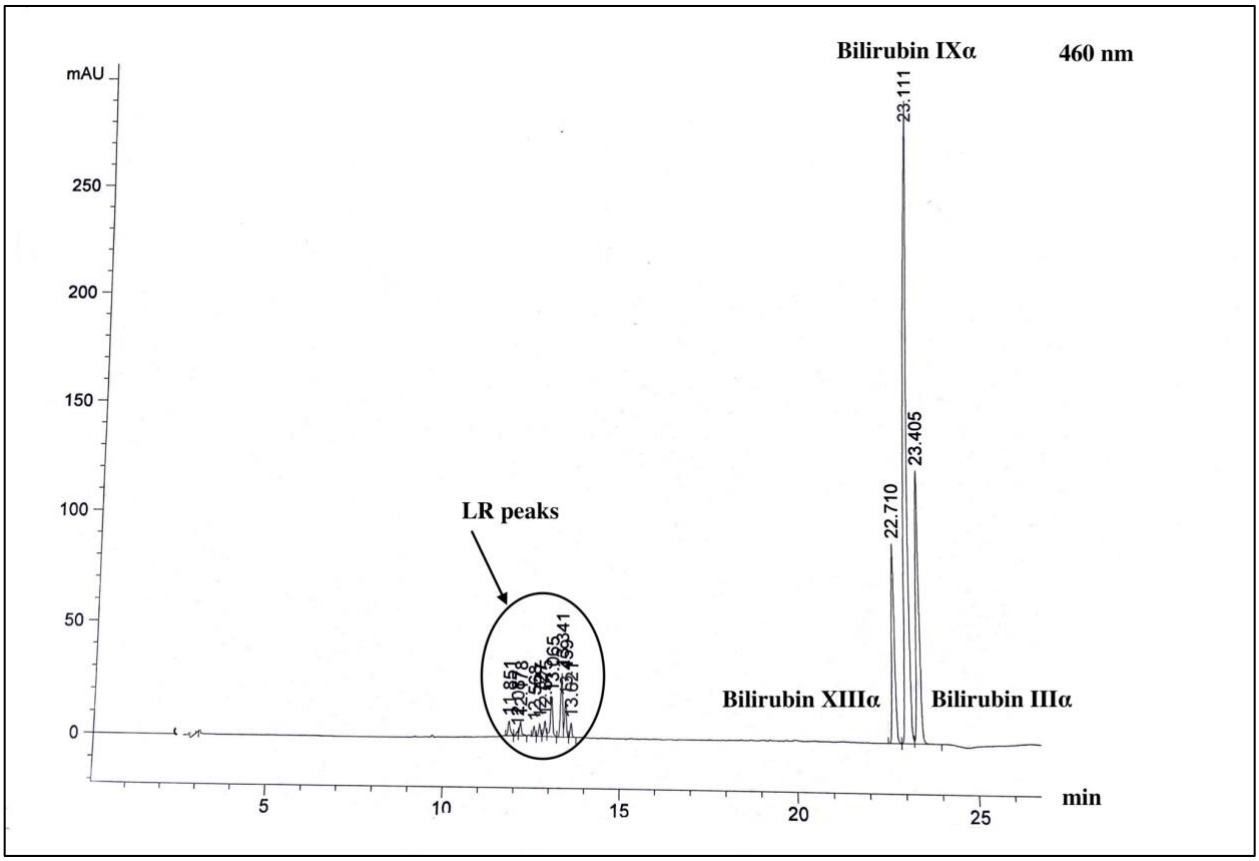


Figure 26: Bilirubin secondary standard B chromatogram. Example of one of five injections utilized to test accuracy, precession, and reproducibility at 460 nm. Unconjugated bilirubin (Bu) with satellite peaks and Lumirubin (LR) are observed.

3.3 Comparison of SEC and RP-HPLC Results to Current Clinical Methodologies

In 1960, Mather stated that "*Bilirubin determinations are perhaps the most notoriously unreliable of any clinical chemistry"*. Twenty-two years later, Watkinson and colleagues came to the same conclusion (1982). Studies conducted by Vreman, and colleagues revealed that the accuracy of measuring serum bilirubin can be difficult to achieve even in university hospitals (1996). For one specimen, the reported concentrations ranged from 110 to 210 mg/L once all the laboratories' results were included (Vreman et al. 1996); this is an issue because treatment for neonatal jaundice is dependent on TBIL concentrations. To enhance commutability and facilitate

bias and accuracy comparisons, specimen NB-01was manufactured and distributed by CAP using human serum and spiked with unconjugated bilirubin to stimulate a genuine hyperbilirubinemia neonatal sample (Subcommittee on Hyperbilirubinemia 2004). All clinical manufacturers and laboratories are encouraged to use CAP reference standards to yearly assess their performance and to investigate differences observed in comparison with other methods. Among the various method peer groups for these manufacturers, the range of peer group means for bilirubin on 2004 sample NB-01 was from a low value of 20.33 mg/dL to a high value of 25.81 mg/dL (Subcommittee on Hyperbilirubinemia 2004). The range of mean biases from the reference method target value was -12.3% to +11.3%, in contrast with the recommended allowable error limits of ±10% (Doumas et al. 1985; Subcommittee on Hyperbilirubinemia 2004).

To illustrate the accuracy and precision of the proposed SEC and RP-HPLC methodologies, the results from the proficiency testing data from the CAP survey were compared to values obtained in this pilot study for the 2012 synthetic bilirubin sample NB-12 (Table 9). Our proposed methodologies were compared to both the Diazo and direct spectrophotometry methods. All the data demonstrated a wide variation across laboratories and methods concerning TBIL values. Values ranged from 2.3 mg/dL to 8.7 mg/dL with an approximate fourfold difference highlighted. The values from both the SEC and RP-HPLC, 15.68 mg/dL respectively, agreed with each other confirming the reliability of our methods in quantifying bilirubin.

Table 9: Comparison of CAP NB-12 2012 TBIL Values Across Laboratories Using Various Analytical Techniques

Method	TBIL (mg/dL)
CAP	14.62
All laboratories	8.7
DuPont aca (DS)	2.4
DuPont Dimension (DZ)	2.3
Beckman Synchron (DZ)	2.6
BMD/Hitachi (DZ)	3.3
Ektachem (DS)	3.8
Ektachem (DZ)	4.0
Dade Paramax (DS)	4.4
LS/JC (RP-HPLC)	15.68 (TBIL=Bu+Bc=10.4+5.28)

Adapted from the College of American Pathologists Neonatal Bilirubin survey 2012. DS is direct spectrophotometry and DZ is the Diazo method. LS/JC refers to the SEC and RP-HPLC methodologies developed by Laila Serraj and Dr. Jonathan Crowther.

The differences observed across these methodologies can be explained. The Diazo method calls for a particular reaction to measure bilirubin, and numerous calculations must be performed to take into consideration the different species of bilirubin that may or may not respond, and the interference from the tiniest amounts of hemolysis is a main drawback of direct spectrophotometry (Lo et al. 2004). Additionally, transcutaneous techniques, which are most often employed in clinical practice, typically maintain accuracy up to 10 mg/dL but are variable after that. SEC and RP-HPLC maintain reliability up to 32 mg/dL and 100 mg/dL, respectively. Moreover, the BuBc assay's accuracy has decreased, with reported variations of 0.1 - 2.16 mg/dL and variations of 24

– 30% seen among samples because of inconsistent calibration (Ercan et al. 2018). These variations can influence the treatment of the neonate and their prognosis. These approaches are unreliable when the likelihood of irreparable newborn problems occurring is above this threshold.

3. CONCLUSIONS AND FUTURE DIRECTIONS

Prolonged icterus is a common problem in neonatology. Up to 60% of full-term neonates and as many as 80% of premature neonates develop jaundice during their first week of life (Rennie et al. 2010). For the most part, phototherapy is initiated when TBIL levels are between 10 and 15 mg/dL while blood transfusions are necessary when TBIL levels surpass 15 mg/dL (Rennie et al. 2010). However, treatment guidelines may differ when considering additional factors such as weight, maternal-fetal ABO group incompatibility, or ethnicity (Duke Pediatrics 2018).

The accurate determination and differentiation of the different bilirubin species are useful for diagnostic purposes; however, most bilirubin measurements are hampered by inconsistencies which place neonates at risk for developing *kernicterus* (Martelanc et al. 2013). To address this issue, our study sought to demonstrate that size exclusion chromatography and complementary analysis using reversed-phase high-performance liquid chromatography could accurately and precisely quantify total bilirubin and individual bilirubin species in patient and synthetic samples. Results from this pilot study have demonstrated that a robust and reliable candidate for a semi-automatized setup for measuring the various total and individual bilirubin fractions in a specialized laboratory handling samples from clinics with expertise in biliary disease has been established. The methodology was optimized to provide accurate, precise, and reproducible bilirubin measurements across a clinical range of interest.

Size Exclusion Chromatography (SEC) enabled the determination of total bilirubin concentrations by observing a single chromatographic peak at 460 nm in a thirty-minute run. The single peak represented the coupled albumin-bilirubin complex. This was confirmed by comparing the elution time of the complex to that of albumin. Both chromatograms illustrated similar elution times of approximately 11.54 minutes throughout all the tested samples. Reliable results were verified through the utilization of a protein test mix to ensure the highest standards of system performance and equilibration. The retention times of the compounds contained in the mix were compared to the values provided by the manufacturers and were found to be identical (Table 10). Additionally, two replicate injections were performed for each patient sample and five replicate injections for each standard tested to verify that reproducibility and accuracy were consistent throughout the experiment. The linearity testing illustrated accuracy across a wide range of target concentrations (0 - 32 mg/dL). This range is of clinical importance as most neonatal jaundice concentrations are contained within it (Duke Pediatrics 2018). Statistical testing was performed, and a line of best fit was plotted. The coefficient of determination (R^2) obtained was equal to 1 signifying that the SEC measurements were indeed precise the maximum R^2 value possible is 1.

Table 10: Comparison of SEC test mix compound retention times from Sigma Aldrich to those obtained experimentally

Compound	Expected Retention Time (min)	Actual Retention Time (min)
Thyroglobulin	6.05	6.05
Gamma Globulin (bovine)	8.29	8.30
Ovalbumin	9.40	9.40
Myoglobin	10.68	10.70
Vitamin B12	12.20	12.20

Complementary analysis using Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) enabled the separation of two of the four bilirubin species from albumin: unconjugated and lumirubin. Before the sequence run, mobile phase blanks were injected, all patient samples were diluted 1:10, and a resolution standard (PharmAssist) was run to demonstrate system equilibration and monitor the quality of the reverse-phase column. The PharmAssist chromatogram produced sharp and well-defined peaks. The compounds contained in the solution also eluted at the times set forth by the manufacturer. These results signified that the column was intact and that the RP-HPLC system was operating at peak standards. Additionally,

replicate injections of each tested sample enabled reproducible results. To further confirm this matter, a statistical analysis was performed to determine the relative standard deviation (RSD) of the test results and an RSD<1% was achieved. Generally, the lower the RSD, the smaller the spread of the results obtained and the higher their precision. The bilirubin calibration curve illustrated accuracy across a wide range of target concentrations (0 - 100 mg/dL). This range is of clinical importance and exceeds the range provided by current diagnostics (Grabenhenrich et al. 2014; (Ercan & Ozgun 2018). Additionally, this methodology allowed for the determination of the composition and presence of bilirubin species, notably, unconjugated and lumirubin species. Not only can this method provide insight into the causes of hyperbilirubinemia, but it can also shed light on the effects of phototherapy or exposure to a photon source making it a robust candidate for clinical chemistry analyses.

Both SEC and complementary analysis using RP-HPLC yielded accurate, precise, and reproducible results in comparison to current clinical treatments (Table 9). For instance, the neonatal jaundice grading schema is arbitrary, subjective, and prone to error, especially in heavily pigmented skin (Duke Pediatrics 2018). Moreover, the Diazo method requires a specific reaction for the measurement of bilirubin, and a variety of calculations must be made to account for the different species of bilirubin reacting or not reacting. One of the major limitations of this method is the interference from the slightest levels of hemolysis (Lo et al. 2004). These factors introduce inconsistencies and significantly reduce accuracy and precision. Besides, transcutaneous methods, the most used in clinical practice, tend to show a fair correlation up to 10 mg/dL, but are highly inconsistent above these levels warranting additional measurements and calculations. Higher level measurements are pivotal since *kernicterus* develops when TBIL levels surpass 15 mg/dL (Duke Pediatrics 2018). Studies have demonstrated that this method has a low sensitivity (62.1%) and R^2

of 0.8467 (Grabenhenrich et al. 2014; Ercan & Ozgun 2018). Furthermore, the BuBc assay has reduced accuracy with reported differences in concentration of 0.1 - 2.16 mg/dL (Ecran et al. 2018). These inconsistencies were observed 24-30% of the time across samples due to calibration inconsistencies (Ercan et al. 2018). With the likelihood of irreversible neonatal complications occurring above the 15 mg/dL threshold, it becomes clear that these methods are unreliable. The results of our study demonstrate that the accuracy and precision of bilirubin quantification are maintained up to 32 mg/dL using SEC and up to 100 mg/dL using RP-HPLC. Given these encouraging results and that data integrity was secured through amber glassware, duplicate injections, and quality synthetic standards, this methodology has the potential to be readily applied to other clinical laboratories. Additionally, the sensitivity of the SEC and RP-HPLC methodologies and the ranges of measurement (0-32 mg/dL and 0-100 mg/dL) respectively, can ensure that neonates obtain the appropriate treatment and have a promising prognosis.

The practical implications of these technologies are straightforward. Both SEC and RP-HPLC can easily be implemented across clinical laboratories and hospital settings. These methodologies can be used to accurately and timely assess TBIL and individual bilirubin species concentrations. For instance, SEC can be utilized as an initial diagnostic for determining the extent of hyperbilirubinemia. TBIL concentrations can inform health care practitioner's decisions to implement phototherapy or a blood transfusion according to treatment guidelines. Accurate measurements are critical as they can influence the prognosis of the neonate and mitigate medical complications like *kernicterus* from developing. RP-HPLC can be used as a later diagnostic for narrowing down the potential causes of hyperbilirubinemia and can serve as a deciding factor for treatment next steps (e.g., surgery for a bile duct blockage) (Duke Pediatrics 2018). Choosing a diagnostic because it is cost efficient, widespread, or rapid is not enough. It is pivotal to consider

the well-being and health of the neonate. Healthcare providers, scientists, and laboratory technicians should be mindful and cautious of the ramifications that may result from faulty measurements provided by erroneous methods and point-of-care devices. Both methodologies, SEC and RP-HPLC, would require basic training of personnel and minimal maintenance. Ultimately, deciding which diagnostic to implement, therefore, requires balancing the costs and benefits associated with each and becomes evident that the methodologies proposed make strong candidates.

Future work on bilirubin standardization could be applied. Given the small sample size employed in this pilot study, it would be advantageous to expand the size of patient samples tested using SEC and RP-HPLC. More recently collected samples could be studied since those employed in this pilot study were collected and stored for over a decade, and we are unaware if this may have influenced the presented results. Also, given the sensitivity and capacity of RP-HPLC to quantify the various species of bilirubin, it would be interesting to expand this methodology to testing complex and adult bilirubin serum samples that contain measurable levels of mono and di Bc species. The assessment of serum bilirubin concentration can aid in the diagnosis and prognosis of patients with liver or bile duct disorders (Sanchez et al. 2016). There is a potential for RP-HPLC to detect abnormalities that may be related to the liver and bile, such as cancer, cirrhosis, or blockage of the bile ducts. Besides, additional work could focus on shortening the length of both SEC and RP-HPLC runs. Ideally, runs could be adjusted to less than ten minutes (Martelanc et al. 2013). This is critical, especially, in neonates presenting TBIL concentrations above 15 mg/dL as they are at increased risk of developing *kernicterus* and starting immediate treatment is pivotal in minimizing medical complications (Duke Pediatrics 2018).

Moreover, future work could apply the Standard Reference Material (SRM) from National Standard Testing to mass spectroscopy (MS) to expand on the work conducted by Jasprova and colleagues (2020). The molecular weights of the four bilirubin species are known: 584 amu for unconjugated bilirubin and lumirubin, 936 amu for di-conjugated bilirubin, and 760 amu for mono-conjugated bilirubin. LC-MS can be utilized to further confirm the identity of the bilirubin species peaks observed on the RP-HPLC chromatograms. Not only will this further afford exact identification and quantification of bilirubin species exceeding current clinical assays and instrumentation, but this method has the potential to monitor neonates receiving phototherapy treatment while improving our understanding of both the kinetics and biology of bilirubin phototherapy products. Ultimately, LC-MS would enable the separation of bilirubin species, notably lumirubin, by their physical and chemical properties, then allow for the identification of the components within each peak based on their mass spectrum (Jasprova et al. 2020). This is critical since it would enable one to determine the number of photoproducts present in a serum sample. Although phototherapy is widely used and considered relatively safe for neonatal infants, some risks can be associated with the treatment such as increased mortality rates in those neonates treated with aggressive phototherapy and the late-onset of solid tumors in infants, a phenomenon related to DNA damage caused by the blue-green light utilized (Jasprova et al. 2020). Thus, LC-MS work could shed light on this phenomenon and aid in mitigating these ramifications.

Additionally, research has demonstrated that albumin serves as a carrier protein for numerous endogenous and exogenous compounds including steroids, fatty acids, and thyroid hormones in the blood (Merlot et al. 2014). Since SEC enables the detection of TBIL in a single chromatographic peak due to the albumin-bilirubin complex, it may be possible to apply this methodology to the measurement of steroid hormones like estrogen or testosterone as well as

thyroid hormones like triiodothyronine (T3) or thyroxine (T4). Like bilirubin, these hormones are insoluble in physiological solution and are dependent on the albumin to serve as a carrier protein. Ultimately, the same principles of the SEC methodology would be applicable to these hormones. This could prove beneficial in diagnosing a myriad of hormonal disorders (Sitteri et al. 1982).

In summary, by the application of the presented SEC and RP-HPLC methods, determination of TBIL and individual bilirubin species is no longer allusive as stated recently in the literature (Martelanc et al. 2013). This opens new frontiers in clinical and preclinical investigations of bilirubin. It must be pointed out that the lack of appropriate bilirubin analysis has allowed for an increase in *kernicterus* and hyperbilirubinemia associated complications. It should be taken in consideration that the SEC and RP-HPLC methods developed in this work could be easily applied in clinical medicine and can accurately and precisely quantify bilirubin with ultra-high sensitivity and specificity.

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SUPPLEMENTAL MATERIALS

Risk Factors for Neonatal Hyperbilirubinemia

Premature delivery: (Boskabadi et al. 2020)

- Neonatal hyperbilirubinemia caused by premature delivery is a maternal factor affecting the incidence rate of neonatal jaundice and accounts for 30% of children.
- It is related to the maturity of uridine diphosphate glucuronosyltransferase. Gestational age is positively correlated with the activity of the enzyme.
- Premature delivery may lead to delayed first feeding, insufficient breast milk, infant nutritional disorders, reduced feeding frequency, and other problems, which will lead to increased concentrations of bilirubin in the newborn's blood.

Maternal-fetal ABO group incompatibility, Bruising, and G6PD deficiency: (Thielemans et al. 2018)

- ABO blood group incompatibility, G6PD enzyme deficiency, premature delivery, and Rh blood group incompatibility are the most common risk factors for early neonatal jaundice.
- Bruising causes a rapid breakdown of red blood cells thus, increasing circulating levels of bilirubin.

Exclusive breastfeeding: (Scrafford et al. 2013)

- Breast milk jaundice is related to the presence of glucuronosyltransferase inhibitors in the mother's colostrum and the lack of bilirubin reuptake inhibitors in the infant's small intestine.
- A partial reason for increased unconjugated bilirubin in breast milk jaundice is that breast milk contains more reactive glucuronic acid, which increases intestinal and liver circulation.
- The increased release of fatty acids from triglycerides, catalyzed by lipoprotein lipase, may interfere with hepatic uptake and conjugation of bilirubin.

East Asian Parentage: (Huang et al. 2004)

- Variations in the UGT1A1 gene which is located at chromosome 2q37 are a frequent risk factor. The frequency of the A(TA)7TAA allele in the promoter area of the UGT1A1 gene is lower for Taiwanese (14.3%), Chinese (16.2%), Malaysians (18.8%), and Japanese (10.0-16.8%) in comparison with whites (35.7-41.5%).
- Variations in the expression of this gene result in decreased enzyme activity and ultimately, a delayed elimination of bilirubin and occurrence of hyperbilirubinemia.

Black Ethnicity: (Golden 2017)

- Infants of black ethnicity display multiple variants in the *UGT1A1* and *SLCO1B1* genes that result in decreased bilirubin metabolism.
- G6PD deficiency occurs in roughly 12% of African American males.

Male Gender: (Clarke 1995)

- Dysfunction of the placenta can be a factor associated with male fetus pregnancies. This theory is enforced by the fact that XY blastocysts and embryos grow at an accelerated rate when compared to XX chromosome bearers which alter metabolic processes and the clearance of excess bilirubin.

Putting it all Together: The AAP Guidelines Recommendations to help standardize care.

1. Promote and support successful breastfeeding.
2. Establish nursery protocols for the identification and evaluation of hyperbilirubinemia.
3. Measure the total serum bilirubin or transcutaneous bilirubin level of infants jaundiced in the first 24 hours.
4. Interpret all bilirubin levels according to the infant's age in hours.
5. Recognize that infants at less than 38 weeks' gestation, particularly those who are breastfed, are at higher risk of developing hyperbilirubinemia and require closer surveillance and monitoring.
6. Perform a systematic assessment of all infants before discharge for the risk of severe hyperbilirubinemia.
7. Provide parents with written and verbal information about newborn jaundice.
8. Provide appropriate follow-up based on the time of discharge and risk assessment.
9. Treat newborns, when indicated, with phototherapy or exchange transfusion.

SEC Analytical Test Worksheet

Date: **02/20/23**	**Notebook #, Page LS 32**
Brief Experiment Description: To prepare a final sample queue for SEC testing for accuracy and reproducibility across patient and synthetic standards.	
Mobile Phase A: 0.05M Monobasic Potassium Phosphate pH7, 0.05M Dibasic Potassium Phosphate, 0.05 NaN3	**Date Prepared** **02/20/23**
HPLC Column and Serial # TSKgel G3000SWXL (014GA045876)	**Flow Rate 0.8 ml/min**
Sequence Name: **RP1 2021-06-08 \RP1.S**	**Method Name: SEC_1122.M**
System Suitability: **PharmAssist Standards**	**Blank: Mobile Phase**

Sample Queue

Vial #	Injection Volume	# of Inj	Sample
1	20ul	2	Mobile Phase Blank
2		2	SEC Std.
3		5	Std. C
4		2	Std. B
5		2	Albumin+B12
6		2	1
7		2	2
8		2	3
9		2	4
10		2	5
11		5	Std C
12		2	6
13		2	7
14		2	8
15		2	9
16		2	10
17		2	Std. C
18		1	Mobile Phase Blank
19		1	Inj. Flush

HPLC Analytical Test Worksheet

Date: **02/20/23**			**Notebook #, Page LS 32**
Brief Experiment Description: To prepare a final sample queue for HPLC testing for accuracy and reproducibility across patient and synthetic standards.			
Mobile Phase A: 90(Water):10 (CH3CN)0.02 formic acid **B: 5(water):95(CH3CN) - 0.02 formic acid** **Both Bottles had 10mL/L DMSO**			**Date Prepared** **02/20/23**
HPLC Column and Serial # XBridge Peptide BEH C18 (01383227816129)			**Flow Rate 0.8 ml/min**
Sequence Name: **RP1 2021-06-08 \RP1.S**			**Method Name: RPMethod.M**
System Suitability: **PharmAssist Standards/ 10 mg/dL Std. C**			**Blank: DMSO/Mobile Phase**
Sample Queue			
Vial #	**Injection Volume**	**# of Inj**	**Sample**
1	**20ul**	**2**	**MP A**
2		**2**	**DMSO Blank**
3		**2**	**PharmAssist Std**
4		**5**	**Std C**
5		**2**	**Std B**
6		**2**	**NB-01**
7		**2**	**NAZ-1**
8		**2**	**JC-1**
9		**2**	**JC-2**
10		**2**	**1**
11		**2**	**7**
12		**1**	**Std C**
13		**2**	**9**
14		**2**	**16**
15		**2**	**23**
16		**2**	**28**
17		**2**	**55**
18		**1**	**Std C**
19		**2**	**77**
20		**2**	**53**
21		**2**	**67**
22		**2**	**78**
23		**2**	**90**
24		**2**	**82**
25		**2**	**Albumin**
26		**2**	**MP A**