DESIGN OF A SKIN-TONE INCLUSIVE TECHNIQUE FOR THE NON-INVASIVE TRANSCUTANEOUS MEASUREMENT OF BILIRUBIN

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On my honor as a University student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

Design of a Skin-Tone Inclusive Technique for the Non-Invasive, Transcutaneous Measurement of Bilirubin

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Abstract

Neonatal jaundice is a common condition caused by a build-up of bilirubin in the blood; approximately 50% of term and 80% of preterm infants develop jaundice in their first week of life. Relevantly, up to 10% of term and 25% of preterm neonates require phototherapy, which involves the use of blue light as a phototherapeutic treatment to reduce the serum concentration of bilirubin in the blood (TSB) by photoisomerizing the bilirubin in the skin (TcB). Currently, bilirubin levels in neonates of darker skin tones are frequently overestimated by non-invasive, transcutaneous bilirubinometers; this makes the predictive utility of TcB screening lower in this racial population. Overestimation of TcB increases the likelihood that darker-skinned neonates undergo unnecessarily long phototherapeutic treatment protocols that can deplete essential nutrients, disrupt their thermochemical environment, and separate them from the mother. The overestimation is due to the high degree of overlap between the absorption spectra of bilirubin and melanin, which makes it difficult to determine whether variations in the reflectance spectra collected by bilirubinometers are attributable to bilirubin specifically. We address this issue by using blue light to stimulate a photoisomerization reaction that converts bilirubin into lumirubin—a colorless substance. By tracking the rate of decay of bilirubin using changes in absorbance over time, the resultant decay curve makes it possible to determine the original concentration of bilirubin in the skin irrespective of melanin concentration. Our project establishes a proof of concept for the viability of this method. In our research we present valuable background information, collect the UV-Vis spectra of relevant skin chromophores, create and validate a computational model of the rate of change of bilirubin, and construct a simple physical model—all of which provide support for the continued development and refinement of this method.

Keywords: bilirubin, melanin, absorbance, photoisomerization, phototherapy, bilirubinometer

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Introduction

Neonatal jaundice is a common condition caused by a build-up of bilirubin—a byproduct of hemoglobin destruction—in the blood. The build-up of bilirubin concentration (hyperbilirubinemia) is what contributes to many of the common signs regularly associated with jaundiced patients, including notable yellowing of the eyes and skin in patients with lighter skin tones. Approximately 50% of term and 80% of preterm infants develop jaundice in their first week of life[1]. Careful and accurate monitoring of bilirubin concentrations is of critical importance, as untreated or unnoticed hyperbilirubinemia can lead to encephalopathy, hearing loss, and kernicterus—a permanently disabling neurological condition characterized by choreoathetoid cerebral palsy, upward gaze paresis, enamel dysplasia of deciduous teeth, sensorineural hearing loss, and dyssynchrony spectrum disorder[2].

Phototherapy

In order to prevent the sequelae associated with hyperbilirubinemia, up to 10% of term and 25% of preterm neonates require phototherapy for treatment, which involves the use of blue light as a phototherapeutic treatment to reduce the serum concentration of bilirubin in the blood (TSB) by photoisomerizing and reducing the cutaneous concentration of bilirubin in the skin (TcB)[3]. The blue light employed in phototherapy stimulates the photoisomerization of bilirubin into a second conformation of bilirubin termed lumirubin. While bilirubin is typically stored in fatty deposits in the tissue, lumirubin is much more soluble in the aqueous solutions present in the circulatory system. This makes it easier to break down and pass through the body without needing to be processed by the liver, which is not fully developed in neonates and is the primary contributor to hyperbilirunemia[4].

Existing Measurement Methods

Two methods are currently employed for monitoring and diagnosing hyperbilirubinemia. The current gold standard method involves measuring TSB directly by obtaining venous or heel stick blood samples; while accurate, this procedure is painful, invasive, and poses notable health risks such as an increased risk of hospital-acquired infections—particularly in neonates[5]. Other risks of heel pricks include the accidental puncturing of the heel bone or joint disease, which can cause damage to cartilage and bone. To add, in order to continuously monitor TSB and track increases and decreases in its concentration, healthcare workers must often repeatedly prick newborns at the same site, which complicates the healing process in a patient category that is already prone to infection due to their underdeveloped immune systems[6].

Alternatively, to mitigate these health risks, transcutaneous bilirubin screening measures TcB non-invasively by using a handheld bilirubinometer. Transcutaneous bilirubinometry directs specific wavelengths of light into the skin and measures the reflectance spectrum of the light that returns to the device; the reflectance spectrum varies based on the wavelengths of light that are absorbed by components in the skin such as bilirubin, hemoglobin, and melanin[7]. The proportion of light absorbed by the skin is used as an indicator of the concentration of bilirubin present. These transcutaneous measurements work to provide a quantitative risk assessment for infants prone to severe hyperbilirubinemia or bilirubin encephalopathy and allow for timely clinical decisions in areas with limited access to laboratory screenings[8].

Inherent Inadequacy of Bilirubinometers

Currently, bilirubin levels in neonates of darker skin tones (with higher cutaneous melanin concentrations) are frequently overestimated, making the predictive utility of TcB screening lower in this racial population[9]. This can be explained by the high degree of overlap between the absorption spectra of bilirubin and melanin as seen in *Figure 1*; the relative absorption due to melanin is noticeably high near the peak absorbance of bilirubin—which is near the ideal wavelength at which bilirubin concentration would be most easily detected or monitored. Even considering other locations along the spectra, the relative absorption due to melanin is higher

at all locations. As a result, higher cutaneous melanin concentrations can effectively mask fluctuations in bilirubin absorption and make it more difficult to accurately determine bilirubin concentrations in the skin[10,11]. The overlap typically leads to the overestimation of TcB, since the increase in absorption is generally interpreted as a higher bilirubin concentration by most bilirubinometers[7].

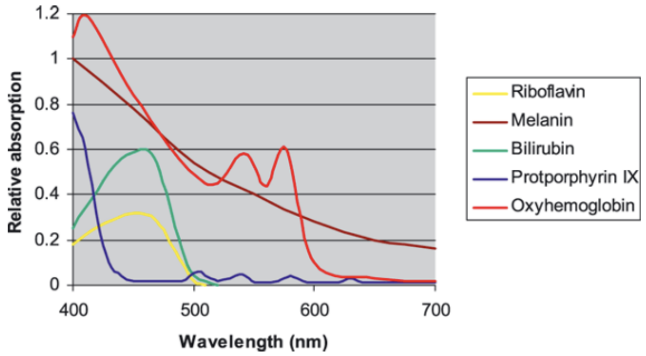

Figure 1: Relative absorption spectra of common cutaneous chromophores at wavelengths between 400 and 700 nm (Mahmoud et al., 2008).

The overestimation of bilirubin measurements poses several risks, including an increased propensity to be prescribed phototherapeutic treatment to treat hyperbilirubinemia. While historically this treatment has been considered generally safe, recent studies have indicated that various side effects may have gone unaccounted for. These include notable short-term effects such as imbalances in the neonatal thermal environment, water loss, electrolyte disturbance, induction of modified liver function and development of cholestasis known as "bronze baby syndrome," and disordering of the newborn's circadian rhythm, and effects associated with the reduction of early-stage maternal-infant interactions[12]. To add, some studies have suggested that long-term side effects and impacts may be at play, such as melanocytic nevi and skin cancer, allergic diseases, and retinal damage[12].

Considering the aforementioned overestimation of bilirubin concentration in neonates of darker skin tone, their increased likelihood of being prescribed phototherapeutic treatment indicates the unintentional implementation of racial bias into bilirubinometers. The racial bias ingrained into the current method used to attain efficient transcutaneous bilirubin measurements disproportionately affects these individuals, limits their access to equivalent quality care, and contributes to macroscale problems with race-based health disparities in the United States.

Overview of Solution

The goal of this research is to provide a proof of concept for a non-invasive, skin-tone-inclusive method for measuring TcB. The overlap in absorbance between melanin and bilirubin makes it difficult to determine whether modulations in the reflectance spectra collected by bilirubinometers can be attributed to bilirubin specifically. Our data suggest that a combination of photobleaching bilirubin—as is done in phototherapy—and traditional reflectance-based measurements can be used to remedy the negative influence of melanin on TcB measurements.

This novel procedure involves continuously photoisomerizing the bilirubin at a small test site on the skin, likely on the pinna of the ear, similar to a bilirubinometer currently on the market—the Bilicare device[13]. The photoisomerization of bilirubin in response to phototherapeutic light causes it to convert to lumirubin. While phototherapy is typically employed in an effort to help neonates easily excrete excess concentrations of bilirubin, the conversion of bilirubin to lumirubin is also recognized for its role in "photobleaching" newborns [14]. Lumirubin is not only more soluble in aqueous solutions, but also absorbs significantly less light. The method described capitalizes on this change in absorbance; at specified time intervals throughout the photobleaching process, the transmittance of 460 nm light will be recorded through the pinna of the ear. As the bilirubin in the skin decreases at the measurement site, the transmittance plot is expected to reach a horizontal asymptote as the bilirubin is fully photoisomerized to lumirubin. Computational analysis of the decay curve and final horizontal asymptote should make it possible to determine the initial cutaneous concentration of bilirubin—TcB. The concentration of

bilirubin in the skin of the pinna correlates significantly with TSB; TcB values determined using our proposed method should therefore make it possible to accurately approximate serum concentrations of bilirubin and help physicians make clinical decisions regarding the requirement of phototherapeutic treatment[13].

We here describe a series of physical and computational experiments that were performed to corroborate a proof of concept for this method. The most important of these included the development of a computational framework to theoretically describe and predict the rate of conversion of bilirubin to lumirubin and the collection of spectrophotometric data for relevant skin chromophores—especially bilirubin, lumirubin, and melanin. The latter task involved determining the absorption spectrum of lumirubin by first fully photoisomerizing bilirubin, and comparing the collected spectra of all chromophores to published spectra available in the literature when available. Lastly, a simple physical model was built in order to validate/adjust the aforementioned computational model, confirm the relationship between absorption and actual bilirubin concentration, and perform other tests to assess the potential clinical viability of the technique.

The differentiating factor between our proposed method and existing devices is that our technical solution accounts for the absorbance of light due to melanin and other skin chromophores so that race and complexion are not determinants of healthcare quality.

Results

The first approach was to verify the absorbance spectra of the three main chromophores of interest (melanin, bilirubin, oxy-hemoglobin) that we would review in the literature. By creating solutions of the three chromophores we were able to read their absorbance spectra using the ThermoFisher NanoDrop oneC. The figure below showed the comparison between our collected results and the literature values. The melanin data match very well (Figure 2). The bilirubin data varies but at the important range of wavelengths (445-480 nm) the data does match (Figure 3). The hemoglobin was excluded because the extinction coefficient of hemoglobin and the concentration in the skin are so small that any physiological concentrations of melanin and bilirubin would wash out any effect in the absorbance spectrum of all the chromophores combined.

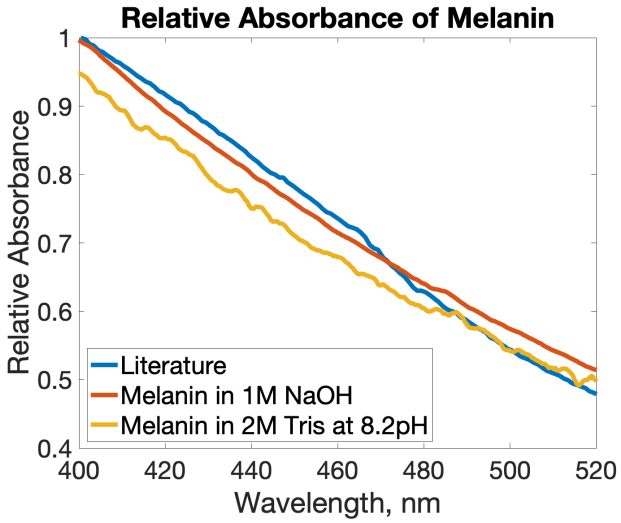

Figure 2. Absorption spectra of melanin in NaOH and Tris buffer solutions.

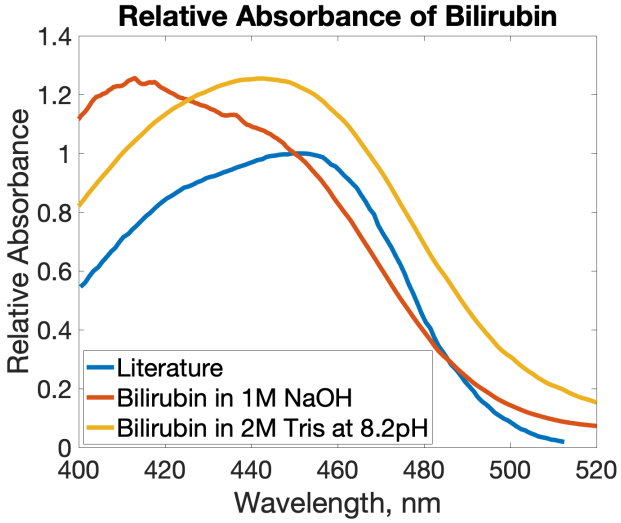

Figure 3. Absorption spectra of bilirubin in NaOH and Tris buffer solutions.

We selected a 253 mW, 470nm LED array from Thorlabs to use as our light source. We found that 470 nm light would maximize the amount of light that converts bilirubin to lumirubin. This LED array is 38mm in

diameter which was big enough to light an entire cuvette without needing a lens.

We needed to create solutions of bilirubin and melanin that were closer to physiological concentrations, so the introduction of a tris-buffer was added to lower the pH to 8.2. Then, to verify how stable bilirubin was in an 8.2 pH solution 2 different storage conditions were tested simultaneously (Light and No light). The figure below shows the decay of the bilirubin's absorbance over time. With this information, we concluded that the buffered bilirubin solution does not rapidly degrade in a buffered solution (Figure 4).

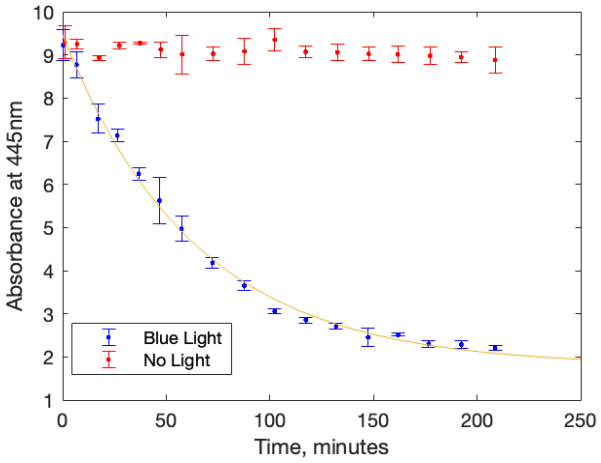

Figure 4. Sample of bilirubin in 2M Tris buffer at 8.2pH being exposed to the blue light array (blue light) and in the dark (no light). Bilirubin does not rapidly degrade over time.

To create a way to predict the bilirubin concentration in a solution, buffered bilirubin solutions of varying concentrations were synthesized (0.011, 0.05, 0.1 mg/ml). These concentrations cover the extreme variation in the actual physiological concentration of bilirubin in the skin. Then these solutions were placed into a 3-D printed chamber that placed the ThorLab's light array aligned with a cuvette of 4ml of each solution.

Each of the solution's absorbance spectra decreased at the 460 nm wavelength. We selected 460nm since our computational model indicated that this wavelength had the greatest change in absorbance in melanin and bilirubin solutions. The computational model was based on findings from Nii et al. which indicated that the conversion from bilirubin to lumirubin could be modeled as a simple, first-order differential equation (see Methods and Material for an in-depth description). This change in absorbance at 460 nm is then plotted over time. Noticing that the curves generated show an exponential decay, we fitted exponential decay curves to the data using Matlab and noted the alpha, or initial degree of absorbance—which we expected to relate closely with concentration. The alpha value represents the amount of change in absorbance due to photoconversion bilirubin if you complete phototherapy for an infinite amount of time. We also directly took the delta in absorbance between the starting and ending concentrations, which we called delta the method. We were able to generate a linear regression model for both the alpha and delta values. Both models had a high R-squared value (>.99).

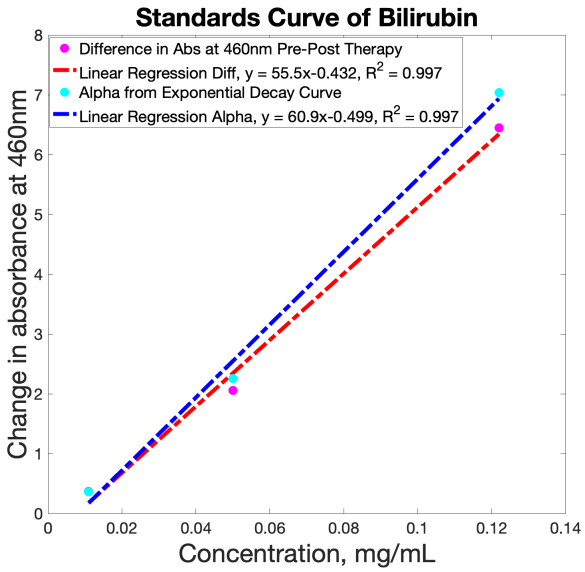

Figure 5. Linear standard curves that relate initial bilirubin concentration to change in absorbance at 460 nm and the initial absorbance, alpha, from a fitted exponential decay curve. These will be used to estimate bilirubin concentration from the phototherapy procedure.

The next step in validating the novel procedure was adding varying mixtures of both melanin and bilirubin to verify the regression could accurately predict the bilirubin concentration independent of the melanin concentration. To accomplish this validation we created

4 mixtures of high/low (0.05mg/ml and 0.2mg/ml) melanin and high/low (0.05mg/ml and 0.1mg/ml) bilirubin. These concentrations were decided since they represent the range of concentrations that are expected physiologically. Ideally, more combinations should be tested with higher concentrations of melanin but this research was focused on proof of concept and struggled to dissolve melanin at concentrations greater than 0.5mg/ml (see Challenges & Limitations section). After the four solutions were photobleached, exponential decay curves were fitted (see Figures 4 & 5).

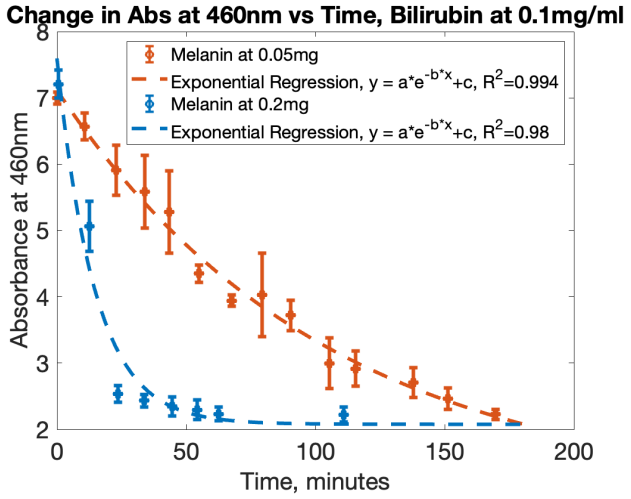

Figure 5. Absorbance at 460 nm during blue light exposure. Bilirubin concentration is 0.1mg/mL

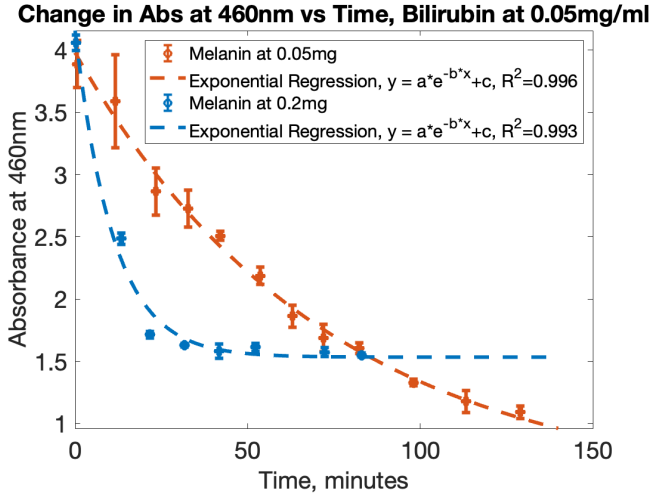

Figure 6. Absorbance at 460 nm during blue light exposure. Bilirubin concentration is 0.05mg/mL

Both the high melanin solutions photobleach much more rapidly than we have seen in both when generating the standards curve and in the low melanin groups. We are currently unsure of the cause of this; our first guess would be due to the tris buffer breaking down and the pH of the solution being much more basic, which may catalyze the degradation process.

The alpha and delta values were used to estimate the initial concentration by the standard curves (see Table 1). Overall, the percent error was 9.44%+/-10.3%. However, this was mostly due to the low melanin/low bilirubin solution, which photo bleached much quicker than expected and seems to be an outlier in this group. Excluding that solution the percent error goes down to 4.5% +/-2.9%. The fitted alpha value was expected to perform better than directly measuring the delta because it should be more representative of the total absorbance due to bilirubin. In the small sample size, it is difficult to confirm this hypothesis but the alpha value is twice as accurate in the high concentration of melanin. Additionally, there does not appear to be a difference in accuracy in the high melanin concentration solution, which gives us hope that localized phototherapy could be used as a bilirubin measurement technique.

		Melanin Concentration			
		0.05 mg/ml		0.2 mg/ml	
Bilirubin Concentration	0.05 mg/ml	Delta	0.0582 (16.3%)	Delta	0.053 (5.9%)
		Alpha	0.0661 (32.1%)	Alpha	0.0513 (2.5%)
	0.1 mg/ml	Delta	0.0937 (6.3%)	Delta	0.0976 (2.4%)
		Alpha	0.1086 (8.6%)	Alpha	0.0988 (1.2%)

Table 1. Estimated concentration of bilirubin using both methods. The delta method takes the difference in absorbance at 460 nm before and after phototherapy. The alpha method fits an exponential decay curve to the data and the initial amount coefficient, alpha, is used. The percent error accompanies the estimated concentration.

Methods & Materials

Optimal Light Wavelength for Photobleaching

Below, Equations 1 and 2 were derived to find the expected extinction of bilirubin for a given wavelength (λ). The percent absorbance for bilirubin is based on summing the total relative absorbances at a given

wavelength, as in Figure 1. Then, the absorbance of bilirubin at the given wavelength is divided by the

$$1.\ E_{Br}(\lambda) = A_{\%Br}(\lambda)\phi_{Br}(\lambda)N_p$$

$$2.\ A_{\%Br}(\lambda) = \frac{A_{Br}(\lambda)}{\Sigma_i^M A_i(\lambda)}$$

Equations 1&2: Two derived equations for calculation the expected extinction rate of bilirubin ($E_{Br\%}(\lambda)$).

summation yielding the percent absorbance of the bilirubin at a specific wavelength. This percentage is multiplied by the quantum yield of the conversion of bilirubin to lumirubin for a given wavelength as seen in Equation 2. The quantum yield is the ratio between the number of isomerization events for a specific conversion and the total number of possible isomerization events possible (i.e. based on the number of photons). Finally, these two values are multiplied by the number of photons. The product is the total number of bilirubin molecules converted into lumirubin for a specific wavelength of light. In order to find the optimal wavelength for maximal bilirubin extinction, we used quantum yield data from Agati et al and absorbance data from Mahmoud et al. Since the number of protons is independent of wavelength, the term is unnecessary for the optimization of the equation. By plotting the product of the percent absorbance of bilirubin and the quantum yield of bilirubin with respect to wavelength and finding the maximum value gave approximately 470 nm as the optimal wavelength as seen in *Supplemental Figure 1*.

Bilirubin and Melanin Stock and Test Solution Preparation

Using stock solution protocols found on Sigma Aldrich, we used a 1M NaOH solution to dissolve both bilirubin and melanin. An ultrasonic bath was used to dissolve bilirubin into the basic solution. A rotating mixer in a cold room was used to dissolve melanin into solution. However, small aggregates of melanin remained even after 50x dilution. Therefore, the concentration of the melanin stock solution was estimated using an extinction coefficient spectrum from the Oregon Medical Laser Center in Portland[15]. The 10mm absorbance at 300 nm was used to estimate the concentration of the melanin stock solution. The test solutions were created by dissolving the stock solution in a 2M Tris buffer at 8.2pH.

Confirmation of Absorbance Spectra of Melanin and Bilirubin

Solutions of bilirubin at 1 mg/ml and melanin at 0.5ml/ml were created using the above procedure. Then these solutions were diluted by 5 times into the Tris buffer. After obtaining absorbance spectrums of all 4 solutions using 5uL from a ThermoScientific NanoDrop OneC spectrophotometer, the absorbance data was exported into Matlab. To generate the absorbance spectra plots, the data from the NaOH solutions was scaled to a relative absorbance of 1 at 450 nm for bilirubin and 400 nm for melanin. The absorbance data for the Tris solutions was multiplied by 5 after being scaled. This adjustment was done to understand the shape of the spectra, not the absolute magnitude. These were compared to spectra found in Mahmoud et al[10].

Phototherapy Procedure

Fill a spectroscopy cuvette with 4mL of a test solution and place it within the 3D printed holder, which places the cuvette directly against the LED array. The LED array is a 4.0 mW/cm^2 array centered at 470 nm purchased from Thorlabs. Once the test solution is inserted into the holder, 5uL is removed and tested on ThermoScientific NanoDrop OneC. Full absorbance spectrum data, from 200 nm to 800 nm, is generated 3 times for each time point. Between measurements, the device was covered to be shielded from ambient light.The test is continued until the absorbance spectrum stops changing between time points. Then the data is imported into Matlab. The change in absorbance is calculated by subtracting the last reading from the initial reading. The decay curves were created by determining the average times and absorbance values at 460 nm. The error bars represent the standard deviation of the 3 absorbance values. Exponential decay curves are fitted using Matlab's built-in fitting function. The function is “a*exp(-b*x)+c”, with the starting values a=4, b=0.05, and c=1. Bilirubin is estimated using a linear standard

curve which was created after photobleaching 3 solutions of bilirubin, 0.011mg/ml, 0.05mg/ml, and 0.122mg/ml. Linear regressions were performed on both the fitted exponential decay alpha value and the difference in 10mm absorption at 460 nm before and after phototherapy.

Computational Model

The computational model is a time-iterative model that estimates the concentrations of the differing chromophores during phototherapy: hemoglobin, melanin, and bilirubin's photoisomers[10]. The computational model uses a reaction rate found in Nii et al[16]., which is derived from a simple reaction equation: d[LR]/t='k' [EZ-BR]. Then using the relative absorbance spectrums of each chromophore, composite absorbance spectrums were created using the relative concentration to weigh each chromophore. The model estimates the skin absorption spectra at time intervals and creates concentration vs time curves (see Supplemental Figure 2). It also generates absorbance decay curves at specific wavelengths which can be compared to our experimental data.

Discussion

Significance & Innovation

Up until now, a lack of consideration for the influence of melanin on transcutaneous bilirubin measurements led to the perpetuation of inequality in healthcare by selectively marginalizing patients with darker skin tones. Existing bilirubinometers have a tendency to overestimate bilirubin concentration, resulting in a variety of consequences—most notably the prescription of excessive phototherapeutic treatments, which is associated with several (aforementioned) consequences. The only present means of avoiding this involves repeated heel stick blood samples—which also carry notable negative consequences, such as increasing the risk of hospital-acquired infections. In either case, hyperbilirubinemic neonates of darker complexions (especially Black patients) are currently left severely disadvantaged.

The significance of finding a solution to this problem is clear, and the results are very promising for future endeavors to remedy the negative relationship between skin color and neonatal healthcare. Overall, the results suggest that bilirubin concentrations can be accurately estimated by detecting changes in absorbance in response to the photoisomerization of bilirubin at a small test site. In our physical model, this remained true even after the addition of influential skin chromophores such as melanin that contribute significantly to the existing technical issues associated with bilirubinometers.

The novel combination of phototherapy and traditional spectrometry described offers several notable value propositions, such as:

- Mitigating the impact of race and skin tone on TcB measurements.
- Reducing the likelihood that neonates are prescribed phototherapeutic treatment unnecessarily as a result of overestimated TcB.
- Introduces a fundamentally new diagnostic method that employs common treatment-oriented technologies in a non-traditional way. This introduces a new methodology for solving future diagnostic issues attributable to variable patient demographics.
- Generating new knowledge regarding the rate of photoisomerization and decay of bilirubin in the skin that may be applied to solve other problems—such as difficulties measuring TcB non-invasively after phototherapy due to the dissonance between TcB and TSB concentrations. Currently, TcB is no longer representative of TSB after phototherapy due to the photobleaching of the skin.
    - Much of the information related to bilirubin, its conversion to lumirubin, and its processing out of the body is treated as a "black box" in engineering.

Challenges and Limitations

One of the most notable challenges faced while generating the data presented involved dissolving skin chromophores such as bilirubin and melanin under

physiological conditions—let alone physiological pH. This is largely attributable to the fact that most of the skin chromophores considered (especially bilirubin and melanin) are not naturally found in aqueous solutions, but rather in subcutaneous fat stores. Working with melanin was particularly difficult, and small specks or aggregates were extremely difficult to dissolve in melanin stock solutions regardless of the pH or concentration—even in strong bases. Even in cases where the skin chromophores were fully dissolved, the applicability of the results may be limited by the fact that the testing occurred under conditions that were distinct from physiological conditions.

The influence of the buffer spectra on the chromophore stock solutions also presented notable hurdles when collecting absorbance data. This is largely because the buffers considered—including Tris—often had relatively strong absorption peaks within the UV-Visible light range.

Another area of consideration is the relatively small sample size. This is in part attributable to the time scale associated with the full photoisomerization of bilirubin to lumirubin. While it was expected to be on the order of minutes, the multiple hours required for each test limited the number of samples the team was able to run within the time allotted for the project. The regular variance in absorption data collected using the ThermoFisher NanoDrop oneC may also be important to consider. It proved difficult to pipette the same volume of the solution onto the reader for every experiment, mainly due to the scale and sample size. Furthermore, bubbles in the liquid sample may have impacted data as well.

Future Work

The apparent exponential decay observed as bilirubin was photoisomerized into lumirubin is of particular relevance. The estimated bilirubin concentrations showcased in the results section were calculated either using the total change in absorbance by bilirubin before and after phototherapy, or by using values based on the full exponential curves fitted to the decay data. Contrary to the relatively short time frame expected, the photoisomerization of bilirubin regularly took several hours to fully convert to lumirubin and reach a horizontal asymptote in the decay curve. However, in the future, it would be ideal to be able to measure TcB within a much shorter time frame. An ideal method would use a smaller number of data points collected during the first few minutes of blue light therapy in order to predict the entire exponential decay curve in real time and estimate TcB on a shorter timescale. Alternatively, a brighter light could be used to accelerate the rate of photoconversion. However, more research needs to be done regarding the potential negative impacts associated with brighter light; a safety limit for the timescale employed in this technique has not yet been established.

Improvements could be made to the physical model employed, such as using a photodiode that measures the transmittance of light at a particular wavelength rather than using the Nanodrop oneC to reassess the entire spectrum at specific time intervals. This is beneficial because it would not only help generate more data points for future decay curves, but also be more representative of the eventual (ideal) handheld prototype device this technique is being designed for. Having more data points would likely make it easier to accurately predict complete decay curves on a shorter timescale. It is also worth considering a wider range of bilirubin and melanin concentrations in future tests to confirm that the proposed method remains viable for “edge” cases—at particularly high or low concentrations. Future tests should also consider the influence of the other known skin chromophores present in the skin—especially hemoglobin. Monitoring changes in the pH of the test solution may also be useful to ensure that the cuvette conditions remain as representative of physiological conditions as possible.

It may also be worth establishing that the decay rate of bilirubin observed is best represented by an exponential decay curve. Other curves were not strongly considered (such as polynomial decay curves) and may yield values that more accurately measure TcB.

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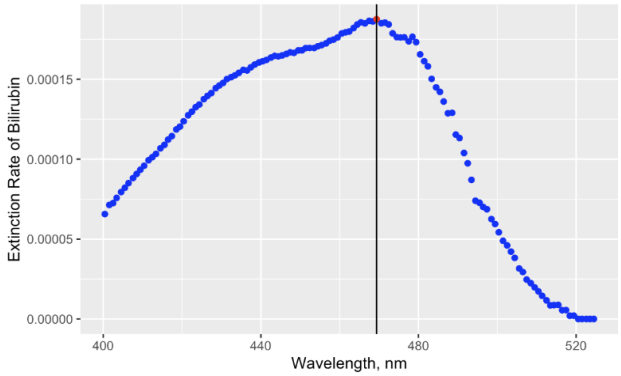
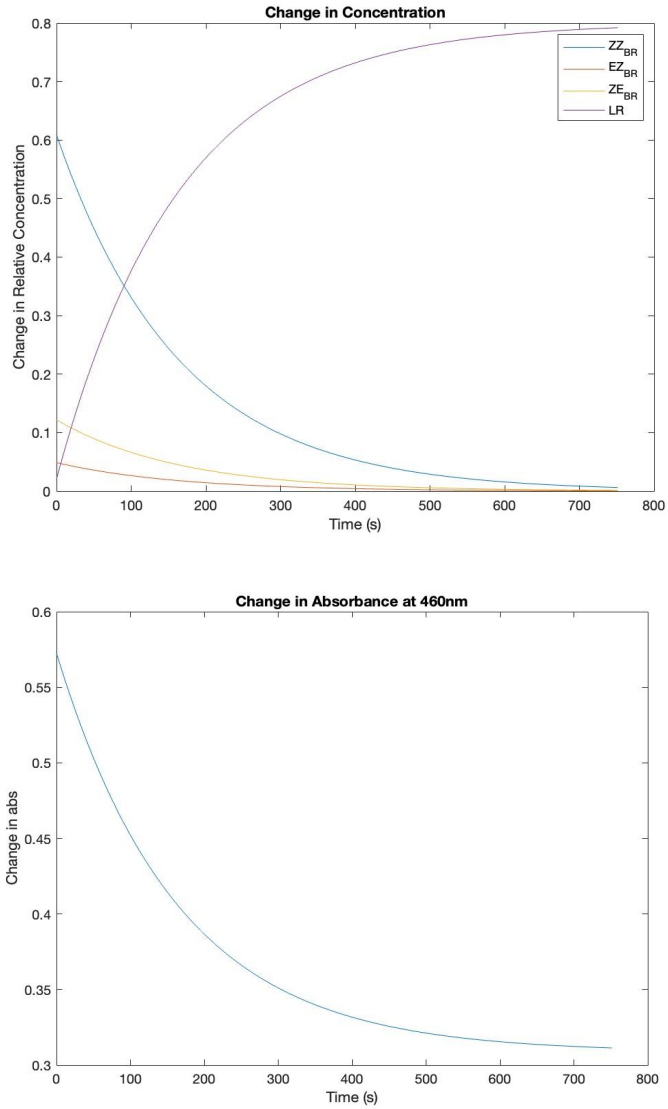
Supplemental Figures



Supplemental Figure 1. Calculated extinction rate of bilirubin plotted against wavelength. Relevant data sourced from *Quantum yield and skin filtering effects on the formation rate of bilirubin*[17].



Supplemental Figure 2. Both figures were created using the computational model of the phototherapy reaction in Matlab. (Top) Plot of the relative concentrations of ZZ_BR, ZE_BR, EZ_BR, and LR throughout a simulated phototherapy duration. (Bottom) Change in absorbance at 460 nm throughout the duration of a simulated phototherapy duration. 460nm was selected because it has the greatest change in absorbance and thus will be the easiest to detect in a physical model.

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