Chapter 4
Optical Spectroscopy for Clinical Diagnostics of Hyperbilirubinemia

4.1. Introduction

Recent world health organization (WHO) fact sheets (updated in June 2014) on global statistics of hepatitis (A, B, C & E) show that out of more than 400 million detected cases of potentially life-threatening liver infection, more than 1.3 million people die every year due to acute or chronic consequences of advanced liver damage. The global statistics of child mortality due to liver malfunction are also very alarming. It is stated in United Nations Children’s Fund report (2012) that twenty one children die per minute, mostly from preventive causes including neonatal jaundice, in most underdeveloped/developing countries. Jaundice is a yellowish pigmentation of the skin and conjunctiva caused by high blood bilirubin levels [1, 2] and is an indicator of liver disease such as hepatitis or liver cancer [3]. An early diagnosis of the neonatal and maternal (particularly due to hepatitis E) jaundice is a proven means of prevention and cure.

The current gold standard to measure the total serum bilirubin (TSB) is determined from a blood sample obtained in an invasive way. Although the method is approved for monitoring jaundice [4, 5], it has several drawbacks. Invasive blood sampling is painful and stressful for the neonates, resulting in blood loss and an increased risk of osteomyelitis [5-7] and infection at the site of sampling. In addition, a factor which is of particular concern is that in the developing world, the conventional method is expensive, laborious, time consuming and dilatory which prevents the possibility of immediate diagnosis [5]. In the case of neonates, the possible alternative for invasive blood sampling is a transcutaneous bilirubinometer (BiliChek and JM-103 are the commercial version of the device) that provides instantaneous cutaneous bilirubin concentration (TcB). The method is based on optical spectroscopy that relates the amount of light absorption by bilirubin (yellow skin) to the concentration of bilirubin in the skin. Since the discovery of the method in 1980 [8], several more devices have been developed in order to improve the accuracy of the device. However, even after 30 years
of development [5], no subcutaneous bilirubinometer can replace blood sampling for the following reasons. The first is the variation of accuracy in different skin color. Most importantly, the bilirubin measured by transcutaneous bilirubinometry (TcB) is a completely different physiological parameter from TSB in blood because TcB consists for over 99% of the concentration of extravascular bilirubin. Due to largely unpredictable processes that regulate the supply and clearance of bilirubin in the extravascular space, one-to-one comparison of the TcB with TSB is impossible. Therefore, an uncertainty in the replacement of blood sampling by TcB still exists. To date there are few other techniques described in the literature for non invasive assessment of bilirubin level in adults i.e., assessment of the jaundice by image acquisition of both the eyes of the patients [9-11]. The system is not capable of making quantitative estimation of bilirubin and is not portable either. It is important to note that in adults, the elevated level of bilirubin and its oxidative products cause various serious diseases including Gilbert syndrome (> 6 mg/dL), Crigler-Najjar type I disease (> 30 mg/dL) [12] and bilirubin-induced neurologic dysfunction (BIND) [13]. Severe neurotoxicity in case of neonates (Kernicterus) and damage in white matter of adult brain are also the consequences of higher bilirubin levels [14]. In case of Hepatitis E infection in pregnant women, associated hyperbilirubinemia itself is found to increase the risk of preterm delivery [15].

In order to surmount the above mentioned limitations of a noninvasive bilirubin monitoring device, the following two strategies are viable alternatives: (1) a medical approach, requiring extensive risk analysis for the predictive value of TcB for mortality/morbidity. (2) A technological approach, where measurement volume of the device is essentially confined to intra vascular space, enabling a one-to-one comparison of TcB and TSB. Our present work basically adopts the latter strategy where the spectroscopic signal essentially comes from the vascular bed of bulbar conjunctiva [16]. As the sclera, duly covered by transparent conjunctiva is white in all human subjects across variety of races, the accuracy of the proposed device is independent of skin color. The light power in the visible region (400-700 nm) which is required (~20 microwatts) for such investigation is much lower than that used in commercially available ophthalmoscope (~100 microwatts) for regular eye check-up, given the sensitivity of the state of the art spectrograph used in the proposed device. Thus the features of the setup which make the device distinct from the existing non-invasive devices for jaundice detection are as follows: (1) directly monitors amount of bilirubin in blood (intensity
of the absorption peak at 460 nm) with extremely high precision without any interference from other pathological condition. (2) Non-contact device does not need any mechanical attachment to the subject, which is very important for the friendly use of the device in neonates/young infants and also virus infected maternal subjects. (3) Signal from conjunctiva, which is white in all human subjects independent of skin color offers uniform sensitivity across different communities in a country. (4) Very limited or almost no training would be required for the healthcare provider. Moreover, the ease of operation with precision in the detection strategy offers future development of the device for low-cost diagnosis of jaundice with minimal manual intervention.

4.2. Result and Discussion

4.2.1. Development and Optimization of a Non-contact Optical Device for Online Monitoring of Jaundice in Human Subjects [17]: The diffused reflectance spectroscopy based absorbance setup (Patented, Patent No. 467/KOL/2009) for monitoring the spectral response of the conjunctiva is represented in Figure 4.1a. A white light source (Model No. LS-450) and a spectrograph (Model No. STS-VIS) with wavelength resolution of 0.47 nm (both are from Ocean Optics, Florida) were used in our study. Lab-grade optical fibers from Ocean Optics were used for the transmission and collection of light to and from the sample (Conjunctiva) respectively. The light from the source is transmitted through the 6 surrounding fibers (Figure 4.1a, excitation fiber) and is incident on the conjunctiva while the single fiber, in the middle of the probe (Figure 4.1a, detection fiber) collects the diffused light and sends it back to the spectrograph. The corresponding spectral response as generated in the spectrograph is then transferred to a laptop computer through USB interface where it is processed in our developed software. The wavelength calibration of our setup has been established with a He-Ne Laser (632.8 nm), fluorescent lamp and emission/absorption of a number of dyes including aqueous bilirubin solution as shown in Figure 4.1b [18, 19]. The comparative spectral response of a normal volunteer and a jaundice patient is represented in the Figure 4.1b. A distinct difference in their spectral appearance is visible; the contribution of yellow pigment deposited in the conjunctiva of the jaundice patients is higher compared to the normal volunteer.
A total of 90 patients arrived at the pathology section for Liver Function Test (LFT) in the Calcutta Medical Research Institute (CMRI) hospital, Kolkata were recruited in our study. Data were collected in two stages: first, for calibration of the device; second, for measuring the precision of the software driver device in contrast to the standard biochemical method. Soon after the blood sample collection, the volunteers were taken for the bilirubin assessment.

Figure 4.1. (a) Schematic representation of our working device. The light from the source is transmitted through the six excitation fibers of the excitation arm and incident on the subject (conjunctiva). The diffused light is collected by the detection fiber and transmitted through detection arm to the spectrograph. The spectral response corresponding to the conjunctiva is processed and generated in the laptop computer. (b) The comparative spectral response of conjunctiva of a normal volunteer and jaundice patient has been represented. An absorption spectrum of aqueous bilirubin solution is also included as reference.
using our setup with a 5 min time window. Due to the noninvasive and non contact nature of the test there is no need of disinfecting the measuring probe. Approval of the local medical ethical committee (Ref: IEC/07/2014/APRV/23) and informed consent from the patient’s legally authorized representative were obtained. Blood samples were taken only for clinical reasons and were obtained by professional technicians from CMRI hospital. A wide variety of age group of the recruited patients with mean age of 45 years (SD 14 years) with different skin tones were the subjects of the present study.

For the calibration purpose, 60 patients were incorporated in this set of study. We studied statistically significant number of patients (n=30) for the assessment of the calibrated device. After placing the probe close to the conjunctiva (~2 cm apart) of the eye, the device acquires data and displays bilirubin value. The information is stored and a comprehensive medical report is generated for further study. In order to establish the potential of the device in terms of reproducibility, 20 patients from the total 30 patients in this stage were repetitively examined by our device by two independent examiners.

**Figure 4.2.** Flow chart of the software designed in LABVIEW platform for non-contact online monitoring of bilirubin level in humans (see text for details).
The optomechanical components have been connected to a laptop computer using a USB interface. The spectrometer (STS-VIS), which is the active detector in this set-up, has been programmed on LabVIEW platform and can be modified for user defined data acquisition. The online display of the acquired data has been used to analyze the data quality and assess the medical condition of the patients. Finally, the bilirubin level of the patient is displayed with a suggested medical attention on the monitor of DAQ laptop computer. The software for automatic data acquisition has been designed in LabVIEW platform. Figure 4.2 shows the sequential program flow or the algorithm of the developed software. The instrument is first re-initialized to its power on status programmatically to remove any previous custom settings. The software then sets the proper integration time for data acquisition to build up the right S/N ratio of the acquired data. This can either be set manually or automatically as decided by the software using an iterative algorithm. For a particular distance between the probe and the reference surface the software adjusts the integration time using the mentioned iterative algorithm until the peak count reaches the maximum allowed value (here 14000). The information is acquired through the raw socket of the USB port and the size of the array is determined, thus the wavelength array is calculated on the basis of instrument specifications. The “dark spectrum” and “reference spectrum” which can either be pre-acquired or can be determined in-situ are then loaded for spectrum processing. The software now acquires data, produces the processed spectrum, generates an online graph and displays the appropriate bilirubin value. The bilirubin value is calculated using the calibration equation. The data safety level of the patient is determined by the differential absorption values of wavelength 460 nm and 600 nm. The online display also suggests the condition of the patient being within or above the safety limits. The information is stored and a comprehensive medical report is generated for offline use for medical practitioners and patients. Complete care has been taken for the software to be simple on the front panel for the ease of operation even with non scientific personnel having no or minimal medical or instrumentation knowledge.

The stored data (stage I, n = 60) were then processed to find the correlation between the TSB level of the volunteer with the spectral information obtained from the conjunctiva of the eye. It has already been reported that the spectral contribution near 460 nm wavelength is due to bilirubin, the yellow pigment [19-21]. Different characteristic wavelengths over the
conjunctival spectrum were selected for assessment but it was found that the differential absorbance of 460 nm (a) to 600 nm (a) and ratiometric values of 470 nm (a) to 576 nm (a) were found to be more consistent with the TSB level. The differential absorbance of 460 nm to 600 nm (a-a) was chosen as the index value (x) to calibrate the setup with the TSB level. The dependency of the of index value (x) with TSB level is represented in the Figure 4.3a. The correlation coefficient (r) is found to be 0.84; P<0.0001, which shows a significant relationship between the two methods (TSB and x). Further calibration was done in order to

![Figure 4.3 Calibration](image)

**Figure 4.3. Calibration:** (a) The dependency of the total serum bilirubin (TSB) value from blood test with the index value from our instrument (n=60) has been represented graphically. The correlation between them is found to follow a second order polynomial equation with R² value of 0.89. (b) Correlation between the TSB value from blood test and from our instrument (correlation factor (r) = 0.96) with the 95% confidence limits and the 95% prediction interval have been represented.
achieve a nearly perfect relationship. The $x_i$ value is found to follow second order polynomial equation $y_i = 74.67x_i^2 - 7.686x_i + 0.748$ (calibration equation), where $y_i$ is the individual TSB level. We used this calibration equation to calculate bilirubin level from the spectral information ($x_i$) obtained by our device. This modification greatly improved the correlation to almost perfect (correlation coefficient, $r=0.96$; $P<0.0001$). Corresponding linear regression curve is represented in the Figure 4.3b with Pearson correlation coefficient, $r=0.96$; $P<0.0001$ and $F=627.1$; slope 0.932; y intercept 0.118.

In order to find the statistical significance of the non contact optical device for online assessment of the bilirubin level correlation and regression analyses were used [22-24]. We have also used the Bland-Altman method for assessing agreement between the conventional biochemical technique and our non contact optical device [25]. Two crucial factors decide whether a new method can be used interchangeably with an already established method: the amount of agreement between the methods and its clinical evaluation. We compared our proposed non-invasive bilirubin detection method to an established biochemical method using the approach described by Bland and Altman [25, 26] in order to assess the statistical agreement. 30 patients (stage II, n=30) of all age groups were included in our study. Linear regression analysis and Bland-Altman plots are shown in Figure 4.4. Data obtained from the linear regression analysis (Figure 4.4a) show that the two methods show strong correlation as the Pearson correlation coefficient, $r=0.99$; $P<0.0001$ and $F=1588$; slope 1.026; y intercept 0.018.

For adequate comparison of the two methods the difference in measurement of the two methods are plotted against their average (Figure 4.4b). The mean difference between the two methods is depicted as a horizontal line and is rated as bias. The other two horizontal lines represent limits of agreement which explains that 95% of the differences were assumed to lie within these limits. The results exhibit reasonable agreement between our proposed method and the conventional pathological method of bilirubin detection. The difference in two methods (conventional-proposed) has mean value of -0.06 mg/dL and SD value of 0.182. The limits of agreement are from -0.42 to 0.30 mg/dL. Hence, it can be inferred that for 95% of individuals, a measurement by our method would be between 0.42 units less and 0.30 units greater than a measurement by the conventional method. This small difference has no serious clinical significance in the diagnosis of jaundice. The mean value of the differences indicates a
small bias of approximately -0.06 mg/dL. The 95% confidence interval (CI) for the bias represented in Figure 4.4b is -0.12 to 0.00. As the CI includes 0.00, the bias is statistically insignificant [27]. The negative bias along with CI indicates that the predominant tendency of our instrument is to overestimate the bilirubin levels, so dangerous clinical errors are unlikely to occur. In addition, the coefficient of variation (CV) between our method and conventional biochemical method found to be 1.81% which is comparable to the CV range of 0.35-1.96%
for laboratory chemical analyzers in repeatability studies [28]. This clearly states the bias to be non-significant in clinical diagnosis.

In order to establish the potential of the device in terms of reproducibility, 20 patients were repetitively examined by our device. We found excellent precision between the bilirubin

![Graph showing reproducibility](image)

**Figure 4.5. Reproducibility:** (a) The linear regression plot of the total serum bilirubin (TSB) level measured successively by two different observers (b) Bland-Altman analysis: Reproducibility in measuring the TSB level by the device (see text).
levels detected from the same subject by two independent observers. Mean and SD were almost the same in both observations and the intra-class correlation values were highly significant ($r=0.98; P<0.0001$). Linear regression analysis also illustrates the accuracy of the two measurements ($F=557.8; \text{slope } 1.04; \text{y intercept } -0.06$) (Figure 4.5a). Furthermore, the Bland-Altman plot of the two successive measurements by two different observers is represented in Figure 4.5b (mean 0.01 mg/dL and SD 0.18). The bias should be zero for an ideal instrument [25]. However, in our case the bias is 0.01 mg/dL and CV in between repetitive measurements is 0.79%, which have insignificant contributions in clinical diagnosis.

4.3. Conclusion

In conclusion, we have demonstrated that the conjunctiva could be a targeted organ to diagnose jaundice independent of race, age and sex by using a simple diffused reflection measurement technique. Based on the aforementioned principle, we have also developed a non-invasive, easy, expeditious, reliable and practical device for routine measurement of bilirubin level. Although serum bilirubin measurements are still required for precise diagnosis, the proposed device has the potential to reduce frequent blood sampling. The setup would be particularly useful for the initial screening of the patients for the blood test and routine examination of the prognosis of some therapeutic strategies including phototherapy in neonates. It has to be noted that evaluation of the instrument with a much larger data set with a wide range of serum bilirubin concentration, various degrees of medical severity, and a variety of age groups including neonates are our immediate future plan. We have also realized that there is an enormous scope for the development of the setup including the use of two-color LED (460 nm and 600 nm) instead of spectrograph in a very low-cost version. Different calibration equation for different age group of subjects would also increase the sensitivity in measurement. In the future, our study is expected to find relevance in quick, non contact diagnosis of jaundice in rural areas as well as in urban clinics.
References


