JOURNAL FOR INNOVATIVE DEVELOPMENT IN PHARMACEUTICAL AND TECHNICAL SCIENCE

Volume-2,Issue-11 (Nov-2019)
ISSN (O) :- 2581-6934

QUANTIFICATION OF HEMOGLOBIN IN HUMAN BLOOD TO DISTINGUISH PERSONS FROM ONE ANOTHER

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ABSTRACT-Blood is one of the principal sources of evidence in crime scene investigations. The level of hemoglobin in human blood is known to hardly fluctuate over time in a healthy adult patient. Using a method like the sodium lauryl sulfate (SLS) method for hemoglobin level determination is beneficial because SLS converts any other derivates of hemoglobin into methemoglobin. The amount of hemoglobin would be determined using the linear correlation between the absorbance of methemoglobin and its amount in blood samples. Those curves differ significantly from sample to sample. Therefore, a differentiation can be observed between the blood samples of different originators. Ultraviolet-Visible spectrophotometry and an image processing software were utilized to quantify the amount of hemoglobin in different human whole blood samples. The data obtained was used to plot different graphs, each one representing an individual. A distinction between a fresh blood sample and an aged blood sample is determined using enzyme-linked immunosorbent assay (ELISA) by tracking the change in absorbance of horseradish peroxidase (HRP) present in blood serum. Based on the results obtained, a clear difference in change of absorbance between a fresh and one-week old blood serum is observed. Future development and further analysis may lead to the conception of a small device using the method of quantification of hemoglobin proposed in this project, with which forensic investigators would be able to use directly on-site and get an idea of how many people where on a crime scene.

Keywords: hemoglobin, human blood, Ultraviolet Visible spectrophotometry, image processing softwar

Introduction

Hemoglobin in human blood

The level of hemoglobin in human blood is known to barely fluctuate over time in a healthy adult patient. However, it differs from individual to individual (Gedye 382). Moreover, several methods to determine the level of hemoglobin in blood are known and widely used. Nowadays, those methods are mostly used for medical and clinical research, to detect the presence of anemia for example (Lamhaut 548). One of the most-used methods for measuring hemoglobin levels is based on photometric detection of a specific form of hemoglobin, cyanmethemoglobin. HemoCue can be found as an alternative to this technique and developed a photometric method based on the determination of another form of hemoglobin, azidemetahemoglobin (Rosenblit 108). However, using a method like the sodium lauryl sulfate (SLS) method for hemoglobin determination is beneficial because it will convert hemoglobin into methemoglobin in the order of oxyhemoglobin, hemochrome, and methemoglobin with its oxidative activity which does not create toxic wastes such as KCN unlike the cyanide hemoglobin method, which is part of the well- known methods for hemoglobin determination (Oshiro 83). The conversion will useful when the amount of hemoglobin is determinate by UV-Vis spectrophotometry.Different applications of digital camera in analytical chemistry have been developed in more than a decade, especially smartphone cameras. Indeed, for example, smartphones are used for spectrometer for colorimetric biosensing application (Wang 3233), proton concentration measurements conducted on pH paper (Chang 549) and colorimetric paper sensor array for the detection and discrimination of explosives using a smartphone (Salles 2047).

The spectral sensitivity of cameras as a function of wavelength can be divided in three peaks: one peak in the blue region, one peak in green and one peak in the blue region of the visible absorption spectrum (Jiang 2013).

Enzyme-linked immunosorbent assay

For more than a century, enzyme-linked immunosorbent assay (ELISA) has been used to identify blood samples. According to Cattaneo, this assay has been used to detect human albumin, IgG, beta thromboglobulin in bloodstains and also the stability of protein in dried blood (140). Nowadays, horseradish peroxidase is commonly used in ELISA due to its production of colored products, whose absorbances can be analyzed using UV-Vis spectroscopy (Beyzavi 145).

Purpose/Goal

In this project, it was hypothesized that the amount of hemoglobin from different blood samples would be determined using the SLS method and differ so significantly from sample to sample that a differentiation can be made between the blood samples of different originators. It was believed that UV-Vis spectrophotometry, as well as image processing analyses, would demonstrate the potential applicability of SLS method combined with a small device to get confirmation as quickly as possible that bloodstains left on crime scenes belong to one or more individuals. The motivation for using a smartphone camera is to determine if it could be used as a field-instrument to replace a UV-Vis spectrophotometer, which is a bulky instrument. Indeed, a smartphone is a very portable device, which can take pictures with great resolution and can run image processing software to possibly get results instantly. It was also hypothesized that a distinction between a fresh blood sample and an aged one would be determined using enzyme-linked immunosorbent assay by tracking the change in absorbance of HRP present in blood serums (Cattaneo 139).

2.1 Materials and methods

Before using the SLS method, human hemoglobin calibration curve was plotted. Human hemoglobin lyophilized powder was purchased from Sigma-Aldrich. Four standard solutions from 0.1 mg/mL to 0.5 mg/mL of hemoglobin in phosphate buffer (pH = 7.41) were prepared. Normal K2- EDTA whole blood from ten different donors were purchased from ProMedDx. The samples were stored in a freezer.

Table 1. Blood donors' sex and age

Sample number	Sex	Age (years old)
1	Male	53
2	Male	52
3	Male	55
4	Male	31
5	Male	55
6	Male	64
7	Male	28
8	Male	28
9	Male	59
10	Male	22

To determine the amount of hemoglobin in human whole blood sample, the following steps were taken: a 2.08 mM Sodium Lauryl Sulfate- Phosphate buffer (pH = 7.41) working solution was prepared, which was then added to Eppendorftubes in which different volumes of human blood, 1.00 µL, 1.09 µL, 1.21 µL, 1.35 µL, 1.52 µL, 1.75 µL, 2.06 µL, 2.51 µL, and 3.20µL, were added to a final solution of 1.6 mL.

Table 2. Dilution factor of each standard solution

Volume of blood in tube (µL)	Dilution Factor
1.00	1600
1.09	1463
1.21	1325
1.35	1188
1.52	1050

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1.75	913
2.06	775
2.51	638
3.20	500

The solutions were incubated at room temperature for 15 minutes before adding each solution to the wells of the plates. As a control for image processing, a 3.50 mg/mL of Supernatural Kitchen red food dye solution in deionized water was prepared.Using clear 96-well plates, absorption curves of hemoglobin and absorbance values for the different volumes of blood were recorded by spectrophotometer SpectraMax Plus 384 Absorbance Plate Reader and pictures of each plate were taken from an iPhone 8 Plus camera and analyzed using the image processing software Fiji ImageJ.

Results and discussion

Plotting known concentrations of human hemoglobin standard solutions against their absorbances provide to this project its shape. Figure 1 shows that the strongest absorption peak of absorption of the human hemoglobin solutions peaks at a wavelength of 405 nm. Because there is the most signal there, the following graph only track absorbance at 405 nm (figure 2). Therefore, the absorbance values of the four standard solutions of human hemoglobin were read at 405 nm. Figure 2 demonstrates that the plot of a calibration curve depending on the amount of hemoglobin is linear, revealing that the relationship between the amount of hemoglobin and the absorbance is directly proportional. The coefficient of determination R2 is equal to 0.997. The value is quite close to 1, which demonstrates how well the regression model fits the data obtained. Therefore, the use of UV-Vis spectrophotometry is justified. UV-Vis spectrophotometry was applied to real human blood samples whose hemoglobin amounts are unknown, however the trendline expected for their curves is linear. Figure 3 shows that the strongest absorption peak of hemoglobin in human blood samples is at a wavelength of 415 nm. Because there is the most signal there, the following graphs only track absorbance at 415 nm (figures 4 to 6). This value is close to 1, which shows that the linear regression fits the data obtained. In figure 5, the trendline of the absorbance versus the amount of hemoglobin in human blood sample 2 has a coefficient of determination R2 equal to 0.9755. This value is close to 1, which shows that the linear regression fits the data obtained, however it is not equal or greater than 0.99, which can be explained by the data point for 1.21 µL of blood in final solution being a bit further away from the trendline. In figure 6, the trendline of the absorbance versus the amount of hemoglobin in human blood sample 3 has a coefficient of determination R2 equal to 0.9861. This value is close to 1, which shows that the linear regression fits the data obtained. In figure 7, the trendline of the absorbance versus the amount of hemoglobin in human blood sample 4 has a coefficient of determination R2 equal to 0.948. This value is close to 1, which shows that the linear regression fits the data obtained, however it is not equal or greater than 0.99, which can be explained by the data points for 2.06 and 2.51 µL of blood in final solution being a bit further away from the trendline. In figure 7, the trendline of the absorbance versus the amount of hemoglobin in human blood sample 5 has a coefficient of determination R2 equal to 0.9861. This value is close to 1, which shows that the linear regression fits the data obtained.Pictures taken from a smartphone camera (figures 3 to 8) were analyzed using an image processing software, whose ability to measure mean gray area was used to calculate the ratio between control, the red dye, and the blood samples in SLS-Phosphate buffer. Those challenges should be addressed in the future. Having a consistency in the parameters and settings of this process, such as the parameters of the camera and the lighting of the surroundings when taking pictures of the wells, is key to improve this method. The use of a smartphone camera was to determine if it could be used as a field-instrument to replace UV-Vis instrument, that is why smartphone pictures results are compared to the standard curves measured by the UV-vis.

CONCLUSION

This method shows promising results. Further studies should be conducted to develop the method and support the idea of using hemoglobin level determination on crime scenes to help differentiate blood samples from different individuals.

In order to do so, blood samples from more donors should be analyzed. Moreover, more replicates for each standard should

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be prepared to lower errors. In this project, it was shown that fresh and aged blood samples can be differentiate. To take a step further, to be able to obtain more realistic results, an analysis and comparison between the amount of hemoglobin in fresh blood and aged and/or dried blood samples should be done. Being able to know how old the blood sample analyzed for hemoglobin quantification is will help with the comparison between bloodstains left on crime scene at different times.

A small device that forensic investigators would be able to use directly on-site and would give them an idea of how many people where on a crime scene would shorten the time spent during investigations, getting samples sent and analyzed to a laboratory. Future research could lead to the adoption of those devices by every forensic investigator in the country and serve as an indispensable tool to solve a crime.

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APPENDIX A

Figure 1. Absorption spectra of human hemoglobin standard solutions

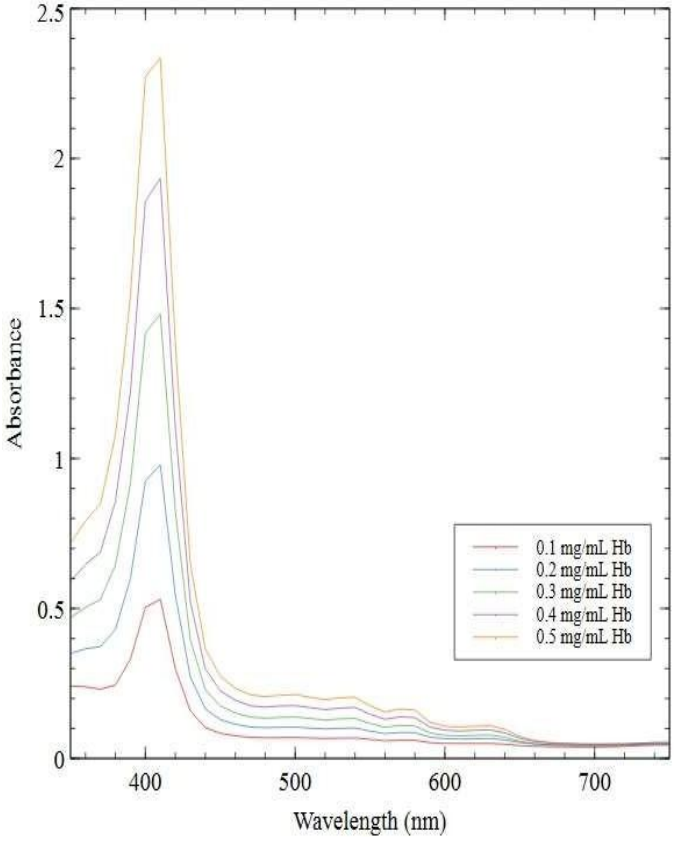
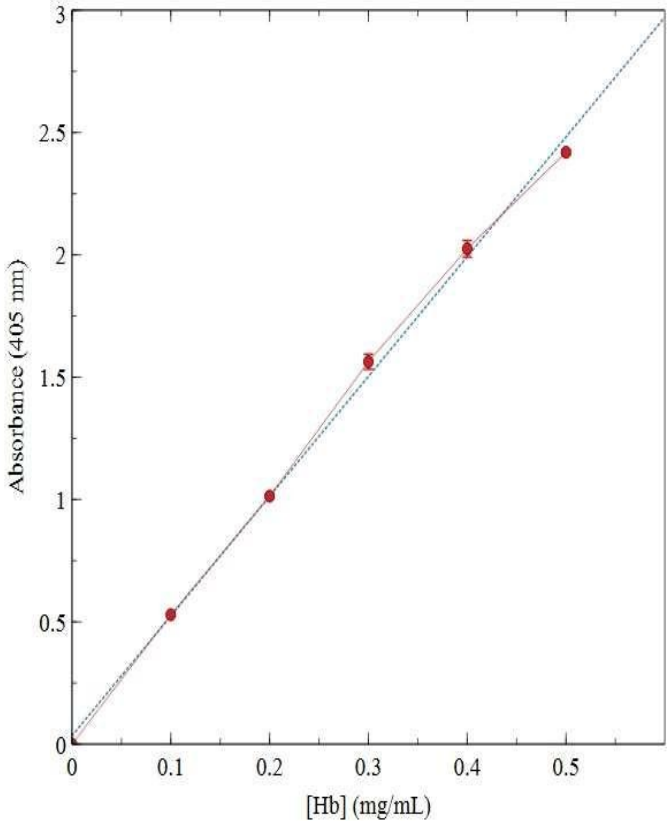


Figure 2. Concentration of human hemoglobin v. Absorbance (at 405 nm)



$y = 4.8951x + 0.0345$

$R^2 = 0.997$

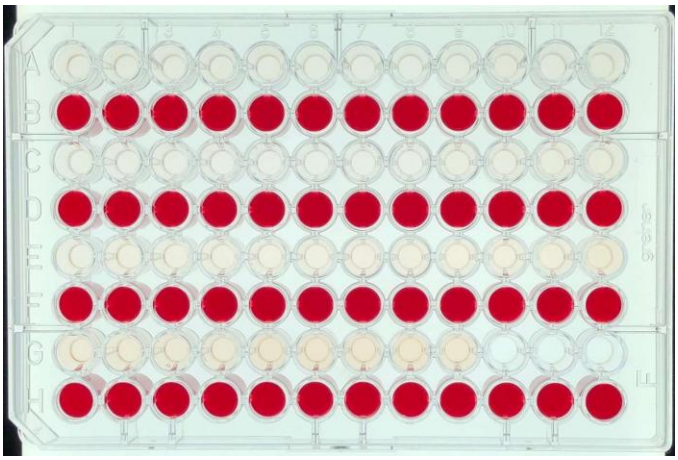


Figure 3. Plate of blood sample 1

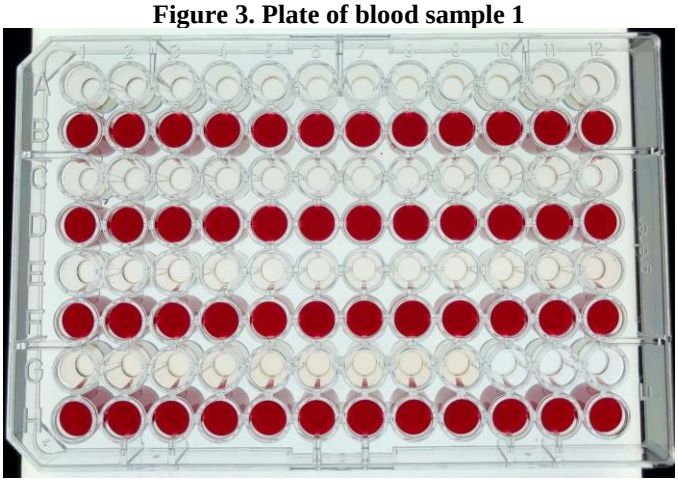
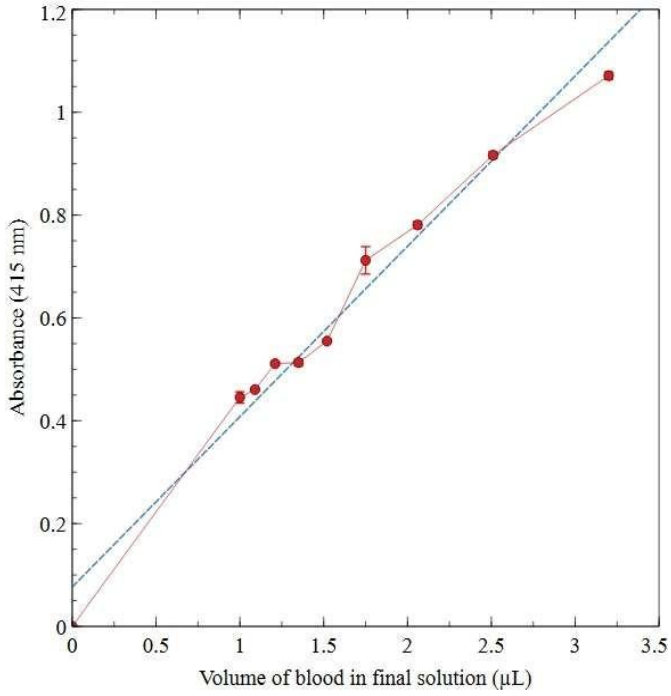


Figure 4. Plate of blood sample 2

Figure 5. Absorbance v. amount of hemoglobin in human blood sample 9 at 415 nm



$y = 0.3314x + 0.0765$

$R^2 = 0.9777$

Figure 6. Intensity ratio v. amount of hemoglobin in human blood sample 10

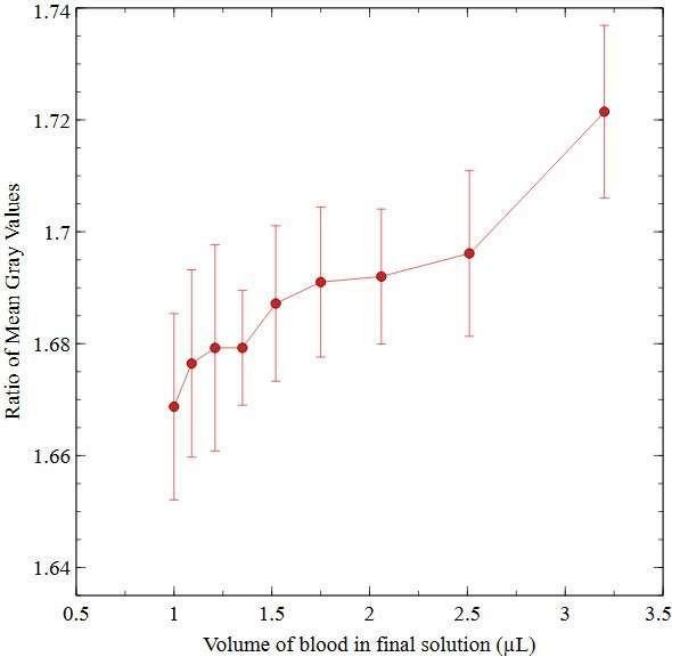
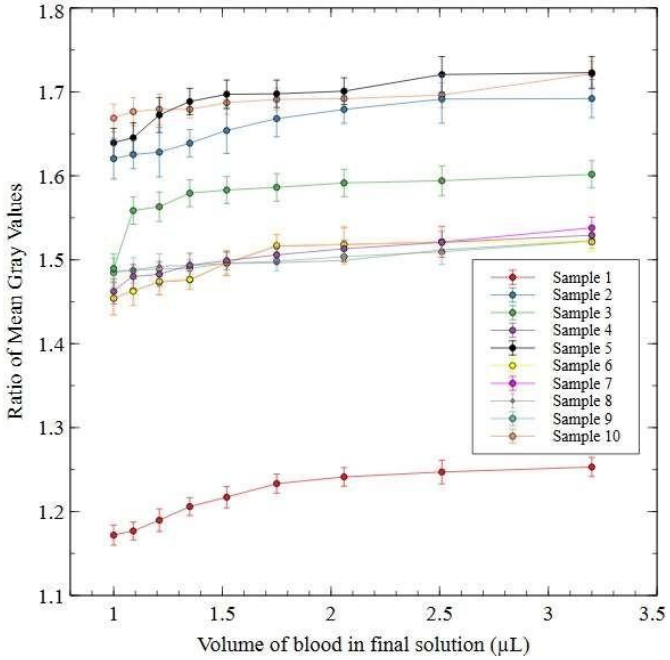


Figure 7. Intensity ratio v. amount of hemoglobin in human blood samples



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