DEVELOPMENT OF A TECHNIQUE TO DETERMINE BLOOD HEMOLYSIS DUE TO LONGER STORAGE AT VARIOUS VISIBLE LIGHT WAVELENGTHS

by

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LIST OF ABBREVIATIONS

ADC	Analog-to-digital converter
ALP	Alkaline phosphatase
ALT	Alanine transaminase
A-mode	Amplitude mode
ANOVA	Analysis of variance
Ar	Argon
AST	Aspartate transaminase
B-mode	Brightness mode
CBC	Complete blood count
Cd	Cadmium
CK	Creatine kinase
CPDA	Citrate phosphate dextrose anticoagulant
DC	Direct current
EDTA	Ethylenediamine tetraacetic acid dipotassium salt
FHP	Free hemoglobin plasma
GGT	γ-glutamyl transferase
GRAN	Granulocytes
HCT	Hematocrit
He	Helium
Hg	Mercury
HGB	Hemoglobin
In	Indium

IR	Infrared
Kr	Krypton
LCD	Liquid crystal display
LDH	Lactate dehydrogenise
LYM	Lymphocytes
MCH	Mean cell hemoglobin
MCHC	Mean cell hemoglobin concentration
MCV	Mean cell volume
M-mode	Motion mode
MPV	Mean platelet volume
Na	Natrium
PCT	Platelet crit
PCV	Packet cell volume
PDW	Platelet distribution width
PLT	Platelet
RBC	Red blood cell
RDW	Red blood cell distribution width
Rh	Rhesus
SD	Standard deviation
UV	Ultraviolet
WBC	White blood cell
Zn	Zinc

LIST OF SI UNITS

UNIT SYMBOL	UNIT NAME	QUANTITY
μL	microliter	Volume
μm	micrometer	Length
$\mu W/cm^2$	micro watt per centimeter square	Irradiance
^0C	degree Celsius	Temperature
cm	centimeter	Length
fL	femtoliters	Volume
g	gram	Mass
g/dL	gram per decilitre	Mass per volume concentration
k/μL	thousand per microliter	Count per volume
kHz	kilohertz	Frequency
m/s	meter per second	velocity
MHz	megahertz	Frequency
mm	millimeter	Length
nm	nanometer	Length
pg	picograms	Mass
V	volt	Voltage

PEMBANGUNAN TEKNIK BAGI MENENTUKAN HEMOLISIS DARAH DISEBABKAN PENYIMPANAN LAMA PADA PELBAGAI JARAK GELOMBANG CAHAYA NAMPAK

ABSTRAK

Kaedah piawai makmal klinikal untuk menentukan hemolisis darah adalah dengan menggunakan emparan dan kemudian pemeriksaan visual dilakukan ke atas hasil pecahan plasma sama ada ia telah tercemar atau tidak. Pada masa ini, masih tidak kedapatan kaedah yang praktikal untuk menentukan hemolysis secara terus ke atas darah. Tujuan penyelidikan ini adalah untuk membangunkan teknik bagi menentukan hemolisis pada keseluruhan darah disebabkan penyimpanan yang lama pada jarak gelombang cahaya nampak dan juga mengkaji faktor-faktor yang menjejaskan pola-pola penyerapan dan pemancaran cahaya yang disebabkan oleh proses hemolisis. Pelbagai jenis kumpulan darah ABO telah diambil daripada 100 pasien dari Pusat Sejahtera USM. Lapan sumber cahaya dengan jarak gelombang dalam julat 300 nm hinnga 700 nm digunakan untuk dipancarkan ke atas sampel darah. Bacaan keamatan cahaya yang melalui darah dikesan dan bilangan sel-sel darah juga diperolehi menggunakan mesin analisa hematologi untuk 25 hari berturut-turut. Penemuan menunjukkan kepekatan plasma hemoglobin bebas dan terbitan sel darah merah penting dalam penyerapan dan pemancaran gelombang cahaya nampak, oleh itu, mampu menunjukkan tahap hemolisis dalam sampel darah. Penyelidikan ini berguna untuk menentukan sama ada darah telah mengalami hemolisis dan dapat menunjukkan tahap hemolisis apabila diuji pada keseluruhan darah. Tambahan, teknik ini boleh digunakan di mana emparan tidak boleh didapati.

DEVELOPMENT OF A TECHNIQUE TO DETERMINE BLOOD HEMOLYSIS DUE TO LONGER STORAGE AT VARIOUS VASIBLE LIGHT WAVELENGTHS

ABSTRACT

Standard clinical laboratory method for determining blood hemolysis is to spin the sample in centrifuge and then, visually examine the plasma fraction being contaminated or not. Currently, there is no practical method for determining whether a sample is hemolyzed which operates on whole-blood. The purpose of this research is to develop a technique to determine blood hemolysis due to longer storage at various visible light wavelengths and also to study the factors that affect the patterns of light absorption and transmission caused due to the hemolysis. Various types of ABO blood group samples were taken from 100 patients in the USM Health Centre. Eight different light sources with wavelengths in the range of 300 nm to 700 nm were used to irradiate the. Intensity readings were detected through the blood and the count of the blood cells were also acquired for 25 consecutive days using the automated hematology analyzer. The finding shows that concentration of free hemoglobin plasma and red blood cells derivatives play an important role in absorbance and transmittance of visible light wavelengths, thus, indicated the level of hemolysis in the whole-blood sample. This research is useful in determining whether the blood sample is hemolyzed and also can indicate the level of the hemolysis when tested on the whole-blood. Furthermore, this technique can be used where a centrifuge is not available.

CHAPTER 1

INTRODUCTION

1.1 Background

1.1.1 Composition of blood

Blood is a complex and unique fluid of variable composition through the heart, arteries, capillaries, and veins, known as the vascular system of the body. It is a tissue in which cellular constituents are suspended in liquid medium performing specialized functions. As with other suspensions, the components of blood can be separated by filtration, however, the most common method of separating blood is to centrifuge (spin) it. Three layers are visible in centrifuged blood as shown in Figure 1.1.

Blood is composed of two main parts, the straw-coloured liquid portion called plasma and the other part is formed elements composed of blood cells. Plasma makes up 55% of the blood. The main plasma protein groups are albumins, globulins, and fibrinogens. The primary gases in plasma are oxygen, carbon dioxide, and nitrogen (John C. Da Costa, 1905). The blood cells are suspended in the plasma, making up approximately 45% of total blood volume and include erythrocytes or red blood cells (RBCs), leukocytes or white blood cells (WBCs), and thrombocytes or platelets (PLTs). Figure 1.2 shows details of blood constituents. Blood cells consist of 99% red blood cells, with white blood cells and platelets making up the remainder. The average pH value of blood is 7.40. Blood is slightly denser and approximately 3-4 times more viscous than water. Blood volume is variable, but tends to be about 8% of body weight. Factors such as body size, amount of adipose tissue, and electrolyte concentrations all affect volume. The average adult has about 5 liters of blood.

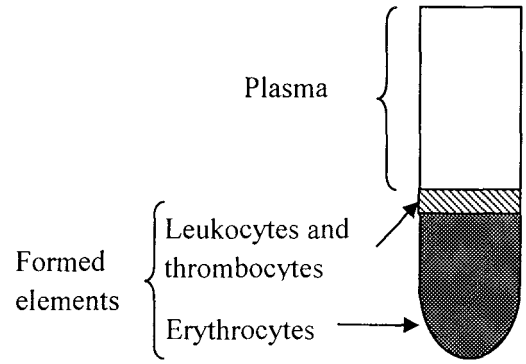


Figure 1.1 Composition of blood (National Cancer Institute, 2008).

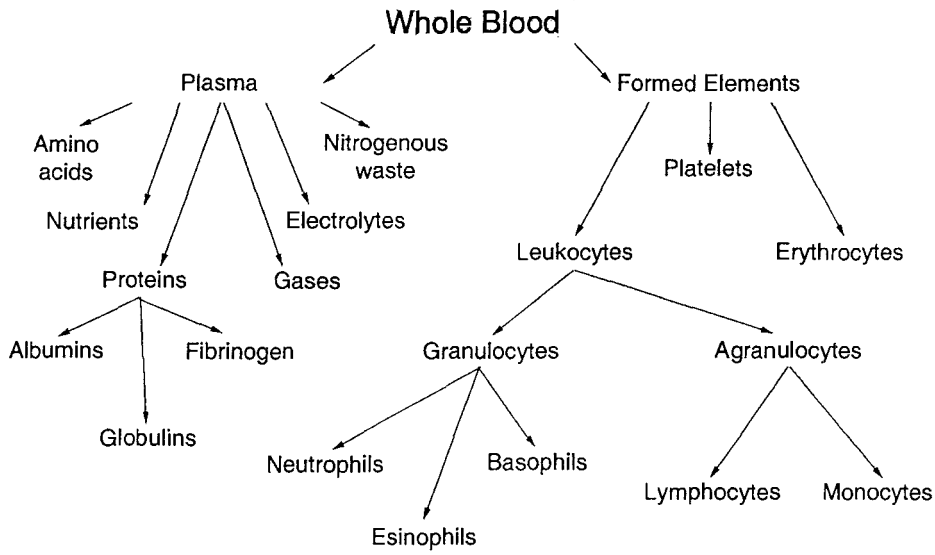


Figure 1.2 Constituents of blood (virtual cancer centre, 2002).

a. Erythrocytes (RBCs)

Erythrocytes are filled with hemoglobin (Hb), a biomolecules that can bind to oxygen. They take up oxygen in the lungs or gills and release it while squeezing through the body's capillaries. The blood's red colour is due to the colour of hemoglobin. In humans, RBCs develop in bone marrow; take the form of flexible biconcave disks, non-nucleated, and the ability to synthesize protein. RBCs have an average life span only about 120 days (Tortora & Derrickson, 2006).

A typical human RBC disk has a diameter of 6-8 μm and a thickness of 2 μm, much smaller than most other human cells. A normal RBC has a volume 90 fL. About a third of that volume is hemoglobin, a total of 270 million hemoglobin molecules, which each carrying four heme groups whose iron atoms temporarily link to oxygen molecules in the lungs or gills and release them throughout the body (Don & Timothy, 2004).

Adult humans have roughly $2\text{-}3x10^{13}$ RBCs at any given time; women have about 4 to 5 million erythrocytes per microliter or blood and men about 5 to 6 million; people living at high altitudes with low oxygen tension will have more. In humans, hemoglobin in the RBCs is responsible for the transports most of the oxygen and part of the carbon dioxide in the blood (Tortora & Derrickson, 2006).

b. Leukocytes (WBCs)

Leukocytes are commonly known as white blood cells (WBCs) because of their lack of colour in unstained preparations. WBCs are cells of the immune system defending the body against both infection disease and foreign materials. They are nucleated cells that have an average diameter of 8 to 12 μm. The number of WBCs in the blood is often an indicator of disease. There are normally between $4x10^9$ and $11x10^9$ white blood cells in a liter of blood, making up approximately 1% of blood in a healthy adult (Alberts et al., 2008). They can be divided into granulocytes and agranulocytes. The former have cytoplasms that contain organelles that appear as coloured granules through light microscopy. Granulocytes consist of neutrophils, eosinophils and basophils. In contrast, agranulocytes do not contain granules. They

consist of lymphocytes and monocytes (Sherwood, 2004). They are differentiated by the specific nuclear and cytoplasmic staining properties.

c. Thrombocytes (PLTs)

Thrombocytes or platelets (PLTs) are small fragments of bone marrow cells and are therefore not really classified as cells themselves. Like RBCs, platelets have no nucleus. Unstained platelets appear as small hyaline structure with diameter of approximately 2 μm. If the number of platelets is too low, excessive bleeding can occur, however, if the number of platelets is too high, blood clots can form (thrombosis), which block blood vessels, and may cause a stroke and/or a heart attack. An abnormality or disease of the platelets is called a thrombocytopathy (Maton et al. 1993). The physiological range for platelets is 150-400x10^9 per liter. The lifespan of circulating platelets is 7-10 days (Sunitha & Muniratham, 2008).

d. Plasma

Blood plasma is a clear, yellowish fluid that accounts for about 55% of the total volume of the blood. The chemical nature of plasma is very complex. It consists of 90-92% water, 7% proteins (albumin, globulin, and fibrinogen), carbohydrate (glucose), lipids (fats, lecithin, and cholesterol), dissolved gases (oxygen, carbon dioxide, and nitrogen), non-protein nitrogenous substances, and less than 1% of inorganic salts. Blood plasma is a fluid portion of the blood before clotting occurs, prepared simply by spinning a tube of fresh blood centrifuge until the blood cells fall to the bottom of the tube. Plasma which fibrinogen has been removed is called serum.

Blood and separated serum are the most common specimens taken to investigate the etiology of communicable diseases. Blood is also separated into serum for the detection of genetic material, specific antibodies, antigens, or toxin (Caribbean Epidemiology Centre, 2006). For processing of most specimens for diagnosis of viral pathogens, generally, serum is preferable to unseparated blood.

1.1.2 Functions of blood cells and plasma

Each component of blood is uniquely capable of performing one or more functions. Together, these components provide the maintenance of a relative biological constancy or integrity and are known as homeostasis. Once the blood cells reach full maturity, they enter the bloodstream and begin fulfilling their functions. Tables 1.1 and 1.2 (Tortora & Derrickson, 2006) summarize the functions of formed elements in blood and blood plasma respectively.

Table 1.1 Summary of formed elements in blood and their functions.

Formed elements	**Functions**
Erythrocytes (RBCs)	Hemoglobin within RBCs transports most of the oxygen and part of the carbon dioxide in the blood.
Leukocytes (WBCs)	Combat pathogens and other foreign substances that enter the body.
Granular leukocytes	
Neutrophils	Phagocytosis. Destruction of bacteria with lysozyme, defensins and strong oxidants, such as superoxide anion, hydrogen peroxide and hypochlorite.
Eosinophils	Combat the effects of histamine in allergic reactions, phagocytise antigen – antibody complexes and destroy certain parasitic worms.
Basophils	Liberate heparin, histamine and serotonin in allergic reactions that intensify the overall inflammatory response.

Table 1.1 Continued

Formed elements	Functions
Agranular leukocytes	
Lymphocytes (T cells, B cells and natural killer cells)	Mediate immune responses, including antigen – antibody reactions. B cells develop into plasma cells, which secrete antibodies. T cells attack invading viruses, cancer cells and transplanted tissue cells. Natural killer cells attack a wide variety of infectious microbes and certain spontaneously arising tumor cells
monocytes	Phagocytosis (after transforming into fixed or wandering macrophages)
Thrombocytes (PLTs)	Form platelet plug in homeostasis; release chemicals that promote vascular spasm and blood clotting

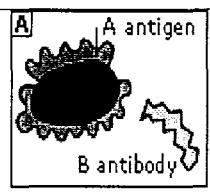
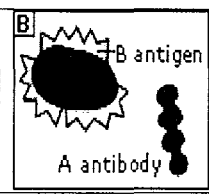
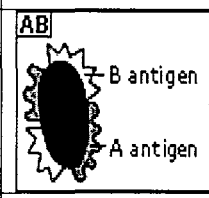
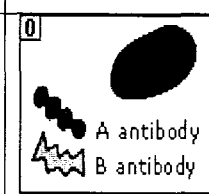
Table 1.2 Substances in blood plasma and their functions.

Constituent	Functions
Water (91.5%)	Acts as solvent and suspending medium for components of blood; absorb, transport and released heat.
Plasma Protein (7.0%)	Exert colloid osmotic pressure, which helps maintain water balance between blood and tissue and regulates blood volume.
Albumins	Smallest and most numerous blood plasma proteins; functions as transport protein for several steroid hormones and for fatty acids.
Globulins	Antibodies (immunoglobulins) help attack viruses and bacteria. Alpha and beta globulins transport iron, lipids and fat-soluble vitamins.
Fibrinogen	Plays essential role in blood clotting
Other solutes (1.5%)	
Electrolytes	Helps maintain osmotic pressure and play essential roles in the function of cells
Nutrients	Products of digestion pass into blood for distribution to all body cells
Gases, regulatory substances and waste products	

1.1.3 Blood types and rhesus (Rh) factor

The differences in human blood are due to the presence or absence of certain protein molecules called antigens and antibodies. The antigens are located on the surface of the RBCs and the antibodies are in the blood plasma. Individuals have different types and combinations of these molecules. The blood groups belong to depends on inheritance from parents. According to the ABO blood typing system there are four different kinds of blood types: A, B, AB, or O (Table 1.3) (Marieb & Hoehn, 1998).

Table 1.3 Blood types determined by the ABO blood group antigens present on RBCs (National Cancer Institute, 2008).

	Group A	**Group B**	**Group AB**	**Group O**
RBC type				
Antibodies present	Anti-B	Anti-A	None	Anti-A and Anti-B
Antigens present	A antigens	B antigens	A and B antigens	None

Many people also have a so called Rh factor on the red blood cells surface. This is also an antigen and those who have it are called Rh+ and Rh- for those without. A person with Rh- blood does not have Rh antibodies naturally in the blood plasma, however, Rh antibodies can be develop in the blood plasma if he or she receives blood from a person with Rh+ blood, whose Rh antigens can trigger the production of Rh antibodies. A person with Rh+ blood can receive blood from a person with Rh- blood without any problems.

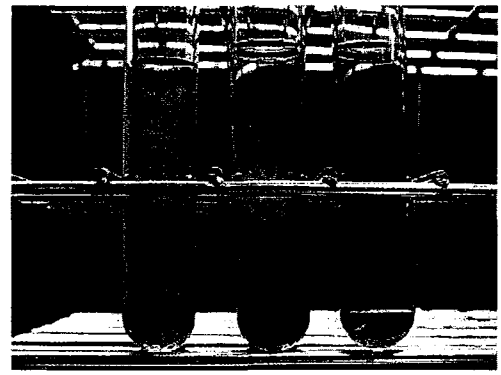


Figure 1.3 Red blood cells with (right) and without (left and middle) hemolysis.

In vitro hemolysis is often graded as mild, moderate or gross. A slight hemolysis has little effect on most test values. The gross hemolysis causes a slight dilutional effect on analytes present at a lower concentration in the red cells compared to plasma. However, a marked elevation may be observed for analytes present at a higher concentration in red cells than in plasma. Table 1.4 displays the effect of hemolysis on some chemistry test result (Calgary Laboratory Services, 2005).

Table 1.4 Effect of hemolysis on some chemistry test result.

Degree of change in analyte	**Test result increased by hemolysis**	**Test result decreased by hemolysis**
Slight change	Phosphate, Total Protein, Albumin, Magnesium, Calcium, Alkaline Phosphatase (ALP)	Haptoglobin, Bilirubin
Noticeable change	ALT, CK, iron	Thyroxine (T4)
Significant change	Potassium (K+), Lactate Dehydrogenise (LDH), AST, free hemoglobin in plasma	Troponin T

In vivo, red cells are carried and protected by the plasma, which helps to regulate the body temperature, controlled pH, adequate glucose supply and removed of metabolic waste. In the protected environment, the life span of the red cells is about 120 days. *In vitro*, series of changes occur that alters the physiological properties. To ensure that blood retains its *in vivo* environment involves anticoagulants, preservative, storage temperature, shipping and transport conditions. Lowering of the storage temperature and adding the preservatives do help in reducing the changes but changes do occur and known as storage lesion (Christopher et al., 2001).

Clinical laboratories must improve the preanalytical phase, a phase highly susceptible to mistakes (Jones, 1997). In some reports, hemolyzed specimens, the most common reason for rejection, account for nearly 60% of rejected specimens, fivefold more than the second most common cause (Plebani & Carraro, 1997). It is suggested that appropriate analytical methods be used to quantify the levels of hemoglobin in the blood samples. This will avoid unnecessary rejection of blood samples that are within the acceptable level that is recognized by regulatory agencies for the licensure of additive solutions for the long-term storage of RBCs.

Since the colour of the blood sample changes due to the hemolysis, visible light sources with photometer will be used to study the level of blood hemolysis as a reversible and non destructive testing method to the blood. Besides that, this research will focused on the prolong storage hemolysis using the light transmittance and absorbance technique in the whole blood sample. So, the different between intensity of light transmission will reveal.

1.2 Problems statement

The most common causes of hemolyzed specimens are improper specimen collection, storage or transport. Specific examples include forceful aspiration during venipuncture, prolonged tourniquet time, vigorous shaking or mixing, centrifugation of a serum specimen before completion of clotting, and prolonged storage or delayed transport at room temperature.

The standard clinical chemistry laboratory method for determining if a blood sample has significant hemolysis is to spin the sample in a centrifuge to separate the plasma fraction from the red blood cells, and then visually examine the plasma fraction. Hemolysis results in the plasma fraction being contaminated with hemoglobin, which gives an obvious red colour to the otherwise yellow plasma. In a typical clinical chemical laboratory, virtually all blood tests performed are based on plasma measurements (Davis, 1995). Accordingly, centrifuging the blood sample is a satisfactory procedure to determine hemolysis in the clinical laboratory. On the other hand, centrifugation process itself generates heat that can cause unnecessary damage to the cells which means it is a destructive and non-reversible process to the sample. In general, operational costs for a centrifuge are relatively low, but the machines themselves are expensive to purchase and maintain.

Currently, there is no practical method for determining whether a blood sample is hemolyzed when tested with any analytical system which operates on whole-blood as a non-destructive process, and is used at the patient's bedside, where a centrifuge is not available. So, this research will encompass a method of estimating the concentration of hemoglobin in a whole-blood sample, and correspondingly, the number of hemolyzed red blood cells per unit volume or concentration of hemolyzed

red blood cells in a whole-blood sample by using the light absorbance and transmittance technique.

1.3 Research objectives

This study aims to have three objectives. Firstly, is to develop a technique to determine blood hemolysis due to longer storage at various visible light wavelengths. Secondly, is to study the effect of light absorption on blood hemolysis with time and lastly to study the effect of light transmission on blood hemolysis with time.

1.4 Scope of research

The blood samples to be used in this research will be fresh human blood samples from the patients who are either students or staff in the Universiti Sains Malaysia. Chronic or problematic blood patients will be excluded from this research.

Visible light of wavelengths between 300 nm and 700 nm will be employed to irradiate the blood samples. A-mode ultrasound will be utilized to measure the ultrasound velocity in the hemolysed blood samples. Hematology analyzer will be used to measure the complete blood count for 25 days consecutively.

1.5 Outline of thesis

This thesis is divided into several chapters. The chapter on introduction includes general background of research, problems of research, research objectives, scope of research and the outline of each chapter in this thesis.

Chapter 2 is mainly concern about some theory of this research. It includes the theory of visible light and its optical properties, spectral absorption by human blood and also about ultrasonic absorption and velocity in biological tissue. This chapter also includes literature review on previous and current research.

The following chapter mentions the equipments employed in this experiment. The features and functions of each equipment are included in this chapter.

Chapter 4 highlights the procedure of each experiment, beginning with the selections of the materials and samples, preparations of the samples and finally the details of the experimental setup and calibration. Chapter 5 will focus on the results and discussion and also the finding of this research. The conclusion appears in Chapter 6 with suggestions on further work.

CHAPTER 2

THEORY AND LITERATURE REVIEW

2.1 Visible light and its optical properties

The visible spectrum is the portion of the electromagnetic spectrum that is visible to or can be detected by the human eye. A typical human eye will respond to wavelength from about 380 to 750 nm (Cecie, 2003). A light-adapted eye generally has its maximum sensitivity at around 555 nm, in the green region of the optical spectrum.

The wavelength which related to frequency and energy of the light determined the perceived colour in Table 2.1. Some sources vary these ranges pretty drastically, and the boundaries of them are somewhat approximate as they blend into each other. The edges of the visible light spectrum blend into the ultraviolet and infrared levels of radiation (Mark, 2004).

Table 2.1 Range of wavelengths for visible spectrum.

Colour	**Wavelength**
Violet	380 – 450 nm
Indigo	420 – 450 nm
Blue	450 – 495 nm
Green	495 – 570 nm
Yellow	570 – 590 nm
Orange	590 – 620 nm
Red	620 – 750 nm

Visible light waves consist of a continuous range of wavelengths or frequencies. When a light wave with a single frequency strikes an object, a number of things could happen. The light wave could be absorbed by the object, in which case its energy is converted to heat. The light wave could also be reflected or transmitted by the object.

It is more usual that visible light of many frequencies or even all frequencies are incident towards the surface of objects. When this occurs, objects have a tendency to selectively absorb, reflect or transmit light of certain frequencies. That is, one object might reflect green light while absorbing all other frequencies of visible light. Another object might selectively transmit blue light while absorbing all other frequencies of visible light. The manner in which visible light interacts with an object is dependent upon the frequency of the light and the nature of the atoms of the object. The absorbance of a sample is proportional to the thickness of the sample and the concentration of the absorbing species in the sample.

Reflection and transmission of light waves occur because the frequencies of the light waves do not match the natural frequencies of vibration of the objects. When light waves of these frequencies strike an object, the electrons in the atoms of the object begin vibrating. But instead of vibrating in resonance at large amplitude, the electrons vibrate for brief periods of time with small amplitudes of vibration; then the energy is reemitted as a light wave. If the object is transparent, then the vibrations of the electrons are passed on to neighbouring atoms through the bulk of the material and reemitted on the opposite side of the object. Such frequencies of light waves are said to be transmitted. If the object is opaque, then the vibrations of the electrons are not passed from atom to

atom through the bulk of the material. Rather the electrons of atoms on the material's surface vibrate for short periods of time and then reemit the energy as a reflected light wave (Henderson, 1996).

A relationship between the absorption of light in a purely absorbing medium and the thickness of the medium was first determined by Bouguer (1729). Some years later Lambert (1760) derived mathematical expression for the relationship, known as the Lambert-Bouguer Law or Beer-Lambert Law which describes how successive layer dl of the medium absorbs the same fraction dI / I of the incident intensity I for a constant μ_a, the latter known as the absorption coefficient with units of mm^{-1}.

Thus,

$$\frac{dI}{I} = \mu_a dl \tag{2.1}$$

For incident intensity I_0, therefore, the transmitted intensity I through a distance or thickness l will be

$$I = I_0 e^{-\mu a l} \tag{2.2}$$

From equation (2.2), the transmittance T and absorbance A can be calculated by;

$$T = I/I_0 \tag{2.3}$$

$$A = \text{Log}\,(1/T) \tag{2.4}$$

The percentage of transmission is

$$\%\ T = I/I_0 \times 100 \tag{2.5}$$

2.2 Spectral absorption in biological tissues; blood

Most colours in biological tissues arise from natural pigments. A pigment is a molecule that absorbs visible light of a certain colour. Heme, the molecule in blood that makes it red, absorbs blue and green light. Only red light passes through or gets reflected. The pigments can be assumed as selective filters that allow only certain wavelengths of light to reach our eyes (Thompson, Stewart & Rodriguez, 2008).

Human blood is always red. The tone is bright red when it is oxygenated (or oxygen rich) and a darker red when it is lacking oxygen or oxygen poor. Although blood has a high absorption in a very broad region (300-900 nm) it has a strong and narrow absorption band with a maximum wavelength around 415 nm as shown in Figure 2.1. Absorption is weakest above 600 nm (Hortola, 1992). This results in the reflection of visible light and the appearance of red wavelength of the visible light. Absorption by blood is mainly determined by the concentration and oxygen saturation (SO_2) level of hemoglobin since the absorption of water is low.

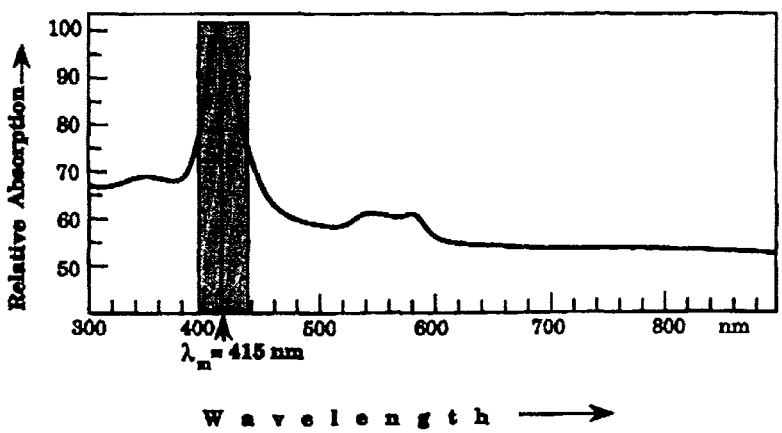


Figure 2.1 Relative absorption of visible range in blood (Hortola, 1992).

When the electromagnetic wave of optical ray encounters biological tissue, there will be multiple effects of reflectance, absorption, and scattering due to inhomogeneities of the sample. In this study, even though, each tissue has its own optical absorption spectra characteristic, one can approximate the optical properties with that of water, due to the fact that water is the major composition of human body which accounts > 70%. Both water and saline solution transmit well in the visible range and the absorption is high in the UV and IR. Tissue shows similar strong absorption in UV and the IR region. However, in blood there are strong absorption in the visible range due to chromospheres such as hemoglobin and bilirubin. The penetration of these three regions of electromagnetic wave is shown in Figure 2.2 where the epidermis is about 50-150 μm in thickness, the dermis (1000-4000 μm), with collagen and elastic fibres produced by fibroblasts, blood and lymph vessels, hair follicles, sweat and sebaceous glands, smooth muscles, and nerves. The last part is subdermal tissues which consist of a fat layer.

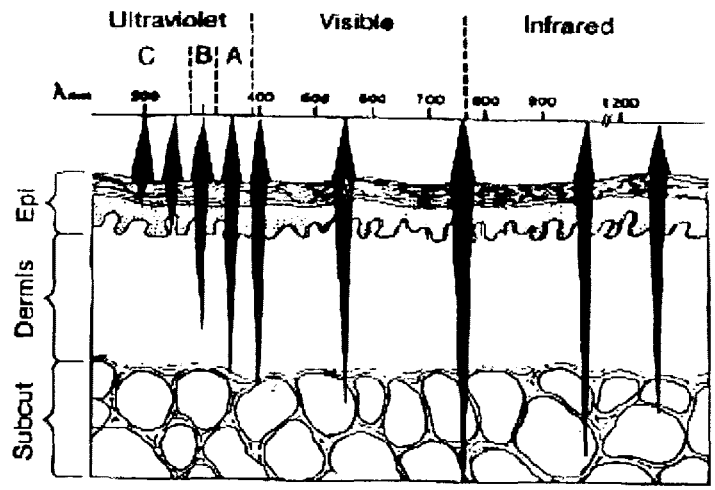


Figure 2.2 Penetration of UV, visible and IR range in the skin (Cheong, Prahl & Welch, 1990).

Due to the difference in isotropic and intracellular contents, different types of tissue do show markedly different optical and thermal properties. It thus required in depth investigation for the clinical application of optical methods. Some of these properties may depend on the water content.

The red blood cell concentration influences the absorption as well as the transmission behaviour of blood (Kim & Keller, 2003). The Beer-Lambert Law states that the concentration of a substance in solution is directly proportional to the absorbance, A, of the solution, given by

Absorbance, A = (constant) x (concentration) x (cell length)

The law is only true for monochromatic light, which is light of a single wavelength or narrow band of wavelength, and provided that the physical or chemical state of the substance does not change with concentration. When monochromatic radiation passes through a homogenous solution in a cell, the intensity of the emitted radiation (I) depends upon the thickness (*l*) and the concentration (*C*) of the solution (Figure 2.3).

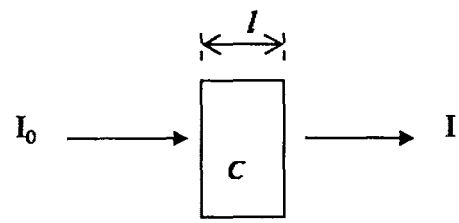


Figure 2.3 Incident intensity, Io passes through the solution with concentration C at thickness *l*. I is the transmitted intensity.

Mathematically, absorbance is related to percentage transmittance T by the expression:

$$A = \log_{10}(I_0/I) = \log_{10}(100/T) = kCl \qquad (2.6)$$

where l is the length of the radiation path through the sample, C is the concentration of absorbance molecules in the path, and k is the extinction coefficient; a constant dependent only the nature of the molecule and the wavelength of the radiation (Figure 2.4). Applying equation (2.6), the relation between absorbance, A and transmittance, T is shown as;

0% T = ∞ A

0.1% T = 3.0 A

1.0% T = 2.0 A

10% T = 1.0 A

100% T = 0 A

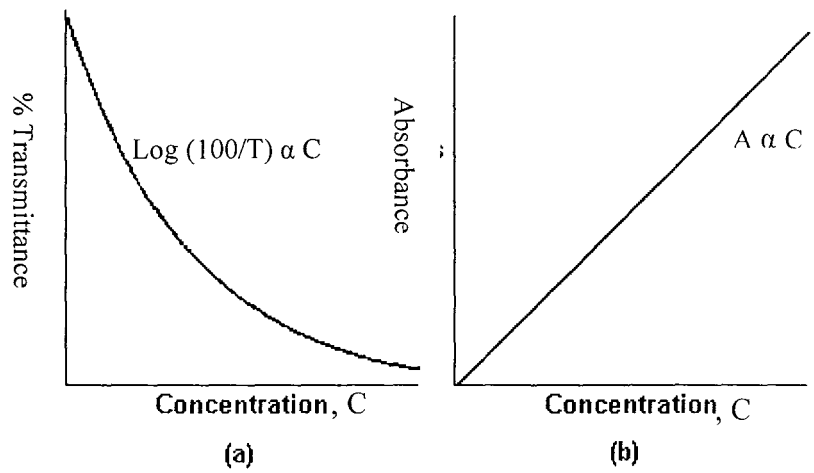


Figure 2.4 (a) Percentage of light transmittance T versus concentration C.
(b) Absorbance A of light versus concentration C.

2.3 Ultrasonic absorption and velocity in biological tissue

Amplitude-mode ultrasound is used as an additional technique to study the hemolysis level in whole-blood sample. Ultrasound is the second most utilized diagnostic imaging modality in medicine, second only to conventional X-ray and is critically important diagnostic tool of any medical facility. Ultrasound is a form of nonionizing radiation and is considered safe to the best present knowledge, less expensive, produces images in real time and also portable. Besides that, ultrasound also has several drawbacks. Chief among them are; organs containing gases and bony structure cannot be adequately imaged without introducing specialized procedure and it also depends on operator skill.

Ultrasound is a wave characterized by such parameter as pressure, medium (particle) velocity, medium displacement, density and temperature. It differs from a sound wave in that its frequency is higher than 20 kHz. The audible range of human ear is from 20 Hz to 20 kHz. Ultrasound is a wave, thus, requires a medium in which to travel and thus cannot propagate in a vacuum. As an acoustic wave propagates through an inhomogeneous medium such as biological tissues, part of its energy will be lost due to absorption and scattering and part of its energy will be lost due to specular reflection at the boundary. The ultrasonic images are formed from the specularly reflected echoes due to planar interfaces. However, according to Anson et al. (1989) it does not necessarily preclude significant scattering effects because the dimension of blood cells is much less than a wavelength. Moreover, investigations by Shung and Thiemen (1993) have shown that scattering contributes little to attenuation at most, a few percentage

points in most soft tissues. Therefore, it is safe to say that absorption is the dominant mechanism for ultrasonic attenuation in biological tissues.

There are several types of ultrasound imaging modes which is amplitude-mode (A-mode), brightness-mode (B-mode), motion-mode (M-mode), two dimension-real time (2D-real time) and Doppler modes. In this study, an A-mode ultrasound is used to measure the ultrasonic velocity in whole-blood sample. When the ultrasound beam encounters an anatomical boundary, the received sound impulse is processed to appear as a vertical reflection of a point. On the display, it will looks like spikes of different height or the amplitude as shown in Figure 2.5. The intensity of the returning impulses determined the height of the vertical reflection and the time it took for the impulse to make the round trip would determine the space between the verticals. The distance between these spikes can be measured accurately by dividing the speed of sound in tissue (1540 m/s) by half the sound travel time.

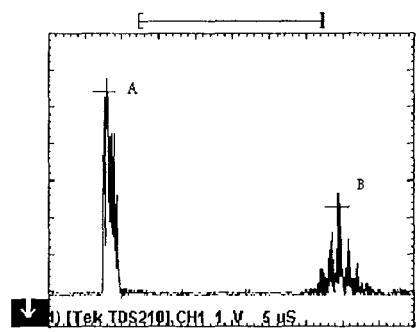


Figure 2.5 A-mode ultrasound display with the two spikes A and B (Anaesthesia UK, 2004).

The acoustic velocity in a medium is a sensitive function of the temperature but its dependence on frequency is minimal over the frequency range 1 to 15 MHz. Ultrasound velocity in biological media is also dependent both on the protein concentration of biological liquids composing the biological tissue and on the external

pressure; factors that are influencing tissue density. However, the influence of these factors is depending on temperature. It is reported by Carstensen and Schwan (1953) that the absorption of ultrasound in blood is caused primarily by protein content of blood and also due to the vary presence of intact cells in suspension.

The ultrasonic study of liquid mixtures has been gaining importance in assessing the nature of molecular interaction. Many researchers have used ultrasonic techniques for studying the conformational fluctuations of protein molecules. Barnes et al. (1988) studied the absorption of low-frequency ultrasound in aqueous solutions of hemoglobin, myoglobin and bovine serum albumin, at 200 – 1200 kHz and pH 2 – 11, to study the absorption of ultrasound with a constant temperature of 20 ^{0}C. Besides that, Arumugam et al. (1998) also discovered that the transformation from monomer to the polymeric form of collagen also results in increase of the attenuation and velocity with concentration. Thus, the interaction between the collagen molecules may be responsible for the observed increase in ultrasonic velocity, viscosity and relaxation time.

The literature reveals a continuing interest in the measurement of the ultrasonic properties of biological systems. Hence, it might be of interest to extend the ultrasonic study to free hemoglobin plasma in hemolysis blood.

2.4 Literature review

The invention of blood hemolysis analyzer relates generally to device and methods that are used for measuring the concentration of hemoglobin in bodily fluids such as blood, plasma, serum and urine (Tarasev, 2007). In addition, the invention provides

methods and devices for evaluating erythrocyte membrane deformation or fragility, erythrocyte hemolysis and hematocrit levels in a fluid sample. This method allows direct measurement of plasma hemoglobin concentration by scanning spectroscopic methods using visible and near infrared portions of the electromagnetic spectrum. The analyzer can also be used to evaluate the relative amount of rupture erythrocytes thus providing an indirect measure of erythrocyte membrane fragility, based on cell-free Hb concentration in plasma. Each of the Hb derivatives has a characteristic absorption. Oxyhemoglobin has the maximum absorbance 415 nm, deoxyhemoglobin at 430 nm, carboxyhemoglobin at 419 nm, and methemoglobin at 405 nm. These absorbance peak wavelengths can be used to identify the hemoglobin derivatives in a sample and the hemolyzed blood can be investigated. However not all changes in membrane fragility result in erythrocyte rupture and increased cell-free Hb. The limit in this invention is that centrifugation to remove the erythrocytes is required before analysis can take place, and thus, requires an access to laboratory equipment and do not provide the speed necessary for rapid analysis.

Davis (1995) estimated the concentration of Hb in a whole blood sample, and correspondingly the number of hemolyzed red blood cells per unit volume or concentration of hemolyzed red blood cells in a whole blood sample, by comparing the colour hue of the separated fraction with a number of different colour hues on a chart. The chart displays a number of characteristic colour hues corresponding to the colours associated with range of predetermined Hb concentration in plasma. The detection step may alternatively be performed with the aid of a reflectance meter, the meter providing a reading that is a function of the concentration of extracellular hemoglobin present in the