DEVELOPMENT OF PREPARATION METHODS FOR GLYCATED BIOMOLECULES

WANUTCHAYA DUANGINTA

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Doctor of Philosophy in Biomedical Sciences - (Type 2.1)
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Thesis entitled "Development of Preparation Methods for Glycated Biomolecules"
By Wanutchaya Duanginta

has been approved by the Graduate School as partial fulfillment of the requirements for the Doctor of Philosophy in Biomedical Sciences - (Type 2.1) of Naresuan University

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ABSTRACT

Blood materials are required for quality assurance and control of hemoglobin A_{1C} (HbA_{1C}) measurements. This study presents an optimal *in vitro* glycation condition for preparing blood materials for HbA_{1C} with the desired high HbA_{1C} content and commutable are needed for performance evaluations to ensure the quality of HbA_{1C} results. Whole blood in CPDA-1 from blood bank was washed with 0.85% normal saline, erythrocytes were incubated with various concentrations of D-glucose in phosphate buffer saline at 37 °C for up to 120 hours for *in vitro* glycation. Processed blood materials from *in vitro* glycation were tested for homogeneity and stability following ISO Guide 35. Twenty-five clinical blood samples and nine blood materials were commutability tested using six methods in reference and clinical laboratories, as well as two point-of-care devices. Unweighted means based on commutability were used to characterize HbA_{1C} in blood materials. Incubating erythrocytes with 400 mM D-glucose for 15 hours at 37 °C resulted in a significant (p<0.001) increase (p<0.001) in HbA_{1C} in blood materials, with a remaining Hct ranging from 38% to 42%. Hemoglobin A_{1C} in blood materials was stable at 3.8±0.8 °C for 70 days and stable at 8.1 to 23.5 °C during transportation. Six out of nine blood samples had commutable HbA1C measurement results between enzymatic and turbidimetric immunoassays, whereas three out of nine were non-commutable. The commutability of HbA_{1C} in blood materials showed a variance and dependence on the

preparation methods, level of HbA_{1C}, and measurement principles. An optimal condition for *in vitro* glycation by incubation of erythrocytes with 400 mM D-glucose for 15 hours at 37 °C was able to generate HbA_{1C} material with intact erythrocytes that is sufficiently stable and commutable between enzymatic and turbidimetric immunoassay. Therefore, this condition is suitable for the preparation of blood material for HbA_{1C} immunoassays.

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Wanutchaya Duanginta

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Introduction

Background and Rationale

The prevalence of diabetes mellitus (DM) has increased worldwide, and also in Thailand. The International Diabetes Federation (IDF) reported in 2019 that 436 million adults worldwide had diabetes, with this result projected to continue to 700 million by 2045 (Saeedi, Petersohn et al. 2019). The increasing prevalence of diabetes was attributed to a poor lifestyle, an increase in obesity, an increase in the elderly, and an improvement in diabetes screening. Daily blood glucose (BG) monitoring is beneficial for glycemic control and preventing complications in diabetic patients through dietary intervention and changes in behavior. The dawn back of BG testing is a variation during the day and cannot be used to predict long-term glycemic control. Diabetes is a growing global health concern. In 2000, diabetes affected an estimated 171 million people worldwide; by 2011 this had increased to more than 366 million and numbers are expected to exceed 552 million by 2030. The highest prevalence of diabetes overall is anticipated to occur in the Middle East and North Africa due to rapid economic development, and changes in lifestyle patterns in the region (Alotaibi A, 2017). Diabetes is a metabolic disease with multiple etiologies that is characterized by hyperglycemia caused by defects in insulin secretion, insulin action, or both and is associated with disruptions in carbohydrate, fat, and protein metabolism. One of the most important features of diabetes is that nearly half of all diabetics have household heredity factors.

The inability of the pancreas to produce enough insulin, as well as the body's inefficient use of insulin, are both pathologic causes of diabetes (Han W, 2018). The three common types of diabetes are Type 1 Diabetes Mellitus (T1DM), Type 2 Diabetes Mellitus (T2DM), and Gestational Diabetes Mellitus (GDM). Type 2 diabetes is the most common type of diabetes, afflicting 90-95% of diabetics. Since insulin resistance (IR) in peripheral tissues has caused hyperglycemia and hyperinsulinemia to coexist for a long time, hyperglycemia is the most prominent clinical syndrome that characterizes T2DM. Patients require insulin treatment only when cells are out-of-function and cause a deficiency of endogenous insulin (Chang SC, 2016). Type 2 DM more than doubles the risk of an ischemic stroke. Furthermore, ischemic stroke is more severe in T2DM patients and is associated with poor functional outcomes and a high risk of death. High blood glucose levels provide enough energy for cancer cells, stimulate their proliferation, and hasten the progression of cancer development. Hyperglycemia is frequently observed for many years before clinical diabetes is diagnosed. It can take many years to progress from early metabolic abnormalities that precede diabetes, such

as impaired fasting glucose (IFG) and impaired glucose tolerance, to diabetes (Siddiqui Z, 2018). Hyperglycemia is the most common cause of protein glycation. It occurs both exogenously and endogenously, causing damage to the biological system and contributing to the progression of diabetes-related complications. Non-enzymatic glycation involves the Amadori reaction via Schiff base rearrangement, which results in the formation of advanced glycation end products (AGEs). Secondary oxidative stress is produced when AGEs interact with receptor for advanced glycation end products (RAGEs). To contribute to the development of diabetic complications, AGEs form intracellular glycation (glycated hemoglobin) and extracellular cross-linking (glycated albumin) not only with proteins but also with some other endogenous key molecules such as lipids and nucleic acids. According to recent research, AGEs interact with plasma proteins such as albumin, fibrinogen, globulins, and collagen to form various types of AGEs. Biomarkers for glycemic control include hemoglobin A_{1C}, glycated albumin (GA), and fructosamine. Glycation is a general spontaneous process in proteins which has significant impact on their physical and functional properties. The formation of AGEs is dependent on both sugar and protein concentrations. Increases in temperature, pH, and exposure time of sugars to the proteins also play a significant role in the rate of AGE formation.

Glycated hemoglobin (Hb) or hemoglobin A_{1C} (HbA_{1C}) is a hemoglobin compound produced from the reaction between glucose and amino groups on the beta chain of hemoglobin inside red blood cells. HbA_{1C} is a reliable marker for monitoring long-term diabetes for approximately 3 months (Little RR 2009). Hemoglobin is a type of protein in red blood cells and contains a heme inside. Hemoglobin A_{1C} is formed when hemoglobin (Hb) reacts with glucose in the blood, is stable more than blood glucose, and is linked to microvascular complications. The American Diabetes Association (Association, 2022), recommend $HbA_{1C} < 6.5\%$ for good glycemic control to prevent complications in DM patients and $HbA_{1C} \geq 6.5$ with clinical sign and symptom for diagnosis of DM.

Although, measurement of HbA_{1C} has more advantages than measuring blood glucose. However, there are limitations in DM patients with blood disorders such as thalassemia and hemoglobinemia so, fructosamine or glycated albumin (GA) monitoring is another option for DM care. The limited reliability of HbA_{1C} levels in chronic kidney disease (CKD), use of fructosamine and glycated albumin as alternative proposed markers of glycemic control for monitoring diabetes patients with CKD. Finally, GA has been shown to be more accurate than HbA_{1C} in diabetic patients undergoing hemodialysis, is more reliable in neonatal diabetes monitoring and in pregnancy and has also a substantial relationship to certain types of diabetes complications such as nephropathy and coronary artery disease. Glycated albumin values are spread over a wider scale with respect to those of HbA_{1C} (Montagnana M, 2013). Fructosamine, a glycated serum protein measure, represents the average glycemia over the previous 2-3 weeks. Fructosamine was found to be strongly

correlated with other glycemic control measures such as HbA_{1C}, fasting glucose, the oral glucose tolerance test, and albumin and total protein concentrations (Innes KE, 2011).

In most cases, the technologies used in HbA_{1C} laboratory instruments have recently enabled the immediate availability of rapid and accurate HbA_{1C} measurements such as HPLC, immunoassay, and enzymatic reactions, but each has limitations due to interference with genetic variants, such as HbS and HbC (Chaudhury S, 2017, Gupta S, 2017, Rohlfing C, 2016, and Murat C, 2019). Measurement procedures were developed by diagnostic reagent manufacturers, they attempt to design measurement values accurately for the intended patient samples. The reagents or materials processed should be commutable between different analyzers according to CLSI EP14-A3 (Karl DV, 2014), while those that non-commutable. The sample matrix can have an impact on the quality of patient care by providing inaccurate imprecision of accuracy. Whole blood samples from a single donor from a single healthy and diabetic patient were used in a European study to evaluate the performance of the HbA_{1C} test using the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) model ($EurA_{1C}$, 2018). Fresh blood was preferable to a lyophilized matrix, but there were limitations in bulk production from single fresh blood samples and in obtaining HbA_{1C} at high concentrations by following human ethics. Pooled blood material obtained from multiple DM patients to obtain high HbA_{1C} levels were limited due to low stability to hemolysis and interference from hemoglobinopathies (Wongsri P, 2017). Lyophilized HbA_{1C} materials are stable at freezing temperatures but may not be suitable for some meters designed to measure whole blood samples.

Previous methods had prepared HbA_{1C} from many studies, such as incubating human erythrocytes with 50 mmol/L of glucose and 10 mmol/L of malondialdehyde (MDA) at 37^{o}C for 24 hours to increase hemoglobin glycation (Selvaraj N, 2006). It was discovered that incubating 10 mg/mL of Haemoglobin with 0.1 M D-glucose for three days, glycates Hb and produces HbA_{1C} (Chen X, 2017). *In vitro* glycation incubation of hemoglobin with urea at 37^{o}C for 5 days to increase HbA_{1C} levels. An enhancer such as ascorbic acid had did not affect erythrocyte hemolysis or glycated Hb ((Sadowska-Bartosz I 2015). Several previous studies reported the optimal conditions for *in vitro* glycation to prepare blood materials for HbA_{1C} from the fresh human blood sample at 37°C, but with differences in precursor, incubation time, preservative, and kept the final products at -20°C or in lyophilized form (Smith RJ, 1982, Sadowska-Bartosz I, 2015, Selvaraj N, 2006, Siddiqui Z, 2018). The limitations of prepared HbA_{1C} materials from previous studies in terms of mass production were not yet clear, shelf life, the solution to stop glycation reaction, the effect of red cell hemolysis at -20^{o}C or lyophilized form, and characterization of HbA_{1C} based on commutability.

Wongsri P, 2017, had prepared HbA_{1C} from DM patients for use as a proficiency testing (PT) material. The limitations the high rate of hemolysis from using DM red blood cells during transportation, and storage, high risk of infectious diseases, and

human ethics from using a remaining specimen from the laboratory. Hemoglobin A_{1C} levels from diabetic patients are still difficult to prepare, resulting in less production, long periods of glycation, sterilized conditions in preparation, variation in glucose concentration in study methodology, and lack of storage mixture in -20^{o}C to stop the reaction. The majority of the limitations methods for glycation products require additional research. Processed blood materials (GLY materials) derived from *in vitro* glycation procedures could be used to mass-produce HbA_{1C} materials. As a result, glycation methods may be a viable option for producing HbA_{1C} and fructosamine materials on a large scale with specific values and stability comparable to commercial products.

Research Aims

1. To determine the influence parameters and conditions that effect the quality of human red blood cells in CPDA-1 from the blood bank.
2. To investigate the optimal conditions for *in vitro* glycation among glucose, and hemoglobin (intracellular glycation).
3. To investigate the optimal conditions for *in vitro* glycation among glucose, and albumin (extracellular glycation).
4. To determine homogeneity and stability of HbA_{1C} and fructosamine in glycation products by following ISO 17034-Reference material producer and Guide 3-Guidance for characterization and assessment of homogeneity and stability.
5. To characterized HbA_{1C} and fructosamine in prepared materials according to ISO 17034-Reference material producer and Guide 35-Guidance for characterization and assessment of homogeneity and stability.
6. To determine HbA_{1C} by advance technique by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS)

Scope of Research

This study aims to develop preparation methods of materials by using *in vitro* glycation between glucose and hemoglobin in erythrocytes and glycation between glucose and albumin in plasma. This study focuses on select high-quality human blood in CPDA-1 from a blood bank for *in vitro* glycation. The optimal conditions for *in vitro* glycation techniques will be used particularly concentration of glucose with specific conditions for glycations that are shorter incubation time but produce higher values products. Obtainable materials are homogeneity and stability of HbA_{1C} and fructosamine according to the requirements ISO 17034-Reference material producer and ISO Guide 35-Guidance for assessment of homogeneity, stability, and characterization. Preparation methods from this study will provide more facility to produce material with novel techniques, low cost, blood materials ready for use, and

these material like a human sample. To provide an analysis method using advanced techniques with MALDI-TOF MS conditions for HbA_{1C} assays are being developed.

Preliminary Agreement

1. The American Diabetes Association (ADA)

ADA annually updates the Standards of Medical Care in Diabetes to provide clinicians, patients, researchers, payers, and other interested parties with evidence-based recommendations for the diagnosis and management of patients with diabetes. There are several ways to diagnose diabetes. The HbA_{1C} test measures your average blood glucose for the past 2 - 3 months. The advantages of being diagnosed this way are that you don't have to fast or drink anything. Diabetes is diagnosed at an $HbA_{1C} \geq$ 6.5%, fasting blood glucose ≥126 mg/dL, and Oral Glucose Tolerance Test (also called the OGTT) ≥ 200 mg/dL (Association, 2022). Hyperglycemia condition is the prime cause of protein glycation. It occurs exogenously and endogenously both that may harm the biological system and contribute to the progression of diabetes-associated complications. Non-enzymatic glycation involves the Amadori reaction via Schiff base rearrangement that finally forms AGEs.

2. Glycation

Glycation is a spontaneous non-enzymatic reaction of free reducing sugars with free amino groups of proteins, DNA, and lipids that forms Amadori products. Hyperglycemia has an important role in the pathogenesis of diabetic complications by increasing protein glycation and the gradual build-up of AGEs in body tissues. These AGE form on intra- and extracellular proteins, lipids, nucleic acids, and possess complex structures (Ahmed N, 2005). Advance glycation end products contribute to a variety of microvascular and macrovascular complications through the formation of cross-links between molecules in the basement membrane of the extracellular matrix and by engaging the receptor for advanced glycation end products (Goldin A, 2006). Glycated hemoglobin or HbA_{1C} is a compound between glucose and amino groups on the β-chain of hemoglobin. Hemoglobin A_{1C} is a marker for monitoring DM during about 3 months (Gilbert TR, 2021).

3. External Quality Assurance (EQA) and Internal Quality Control (IQC)

External Quality Assurance and Internal Quality Control, encompass the extent and complexity of examination procedures used by the laboratory. Both IQC and EQA are distinct processes that contribute to the overall quality of laboratory test procedures. The laboratory management shall monitor the results of external quality assessment and participate in the implementation of corrective actions when control criteria are not fulfilled. Interlaboratory comparison programs shall be in substantial agreement with ISO 17043-Proficiency testing (ISO 17043).

4. International Standards Organization (ISO)

International Standards Organization has been developed for point-of-care- testing (POCT) generally, and for specific material items. The international standard for POCT was ISO 22870, Point-of-care testing-Requirements for quality and competence. This standard, produced by the International Organization for Standardization, gives requirements applicable to POCT and is intended to be used in conjunction with Medical Laboratories-Requirements for quality and competence. The requirements of ISO 22870 apply when POCT is carried out in a hospital, a clinic, or a health care organization providing ambulatory care. Moreover, ISO 17034:2017 specifies general requirements for the competence and consistent operation of reference material producers. To cover the production of all reference materials, including certified reference materials as well as ISO Guide 35:2017-guidance for characterization and assessment of homogeneity and stability (ISO Guide 35:2017).

5. Control materials

The International Federation of Clinical Chemistry (IFCC) defines a control solution or control material as a "specimen or solution which is analyzed for quality control purposes, not for calibration". We use the term control material or control product to refer to an available control solution, usually commercially, in liquid, frozen, or lyophilized form, packaged in small bottles suitable for use daily. Control materials are widely available today for most laboratory tests.

6. Reference material (RM)

Reference material is sufficiently homogeneous and stable concerning one or more specified properties, which has been established to be fit for its intended use in a measurement process (ISO Guide 30:2015).

7. Certified reference material (CRM)

Certified reference material is characterized by a metrologically valid procedure for one or more specified properties, accompanied by a reference material certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability (ISO Guide 35:2017).

8. Whole blood materials

Whole blood refers to a mixture of blood cells and lymph or plasma. Blood is an essential liquid organ, a mixture of cellular elements, colloids and crystalloids. As different blood components have different relative densities, sediment rates and sizes they can be separated when centrifugal force is applied. The whole blood which is a mixture of cells, colloids and crystalloids can be separated into red cells, white cells, and platelets (~45% of volume) suspended in plasma amounts of ~55% of volume (Basu D, 2014). Whole blood was used for glycation techniques in this study.

9. Commutability

The commutability is intended to be developers of commercial diagnostic tests as well as laboratory-developed tests, manufacturers of meaning interval sample sets and

QC samples, and PT or EQA providers. The commutability may also be useful to all clinical laboratory professionals wishing to investigate a processed sample's commutability. Analyze both patient and processed samples at the same time, using three or more replicates of each sample in the batch, with the replicates in order. A property of given reference material, demonstrated by the closeness of agreement between the relation among the measurement results for a stated quality in this material, obtained according to two measurement procedures, and the relation obtained among the measurement results for other specified materials (Karl DV, 2014). The commutability of an RM relates to the ability of the RM, characterized by one measurement procedure (usually a reference procedure) as a calibrator or quality control (QC) material for a second measurement or testing procedure applied to routine test materials. This is particularly important because different measurement procedures can respond very differently to different types of test materials. Commutability assessment is not required for all RMs but is required for some important classes of RM (ISO Guide 35:2017).

10. Characterization

Characterization refers to the determination of the property values of the relevant properties of an RM, as part of the production process. The characterization of an RM is described in Clause 9. For CRMs, certified values are accompanied by a statement of measurement uncertainty (ISO Guide 35:2017).

Research Hypothesis

In vitro glycation is a non-enzymatic reaction that produces between glucose and an amino group on the beta chain to stimulate HbA_{1C} levels inside red blood cells. These theories are used to design the optimal conditions for *in vitro* glycation using erythrocytes, which include glucose concentration, temperature, incubation time, and additive solution to stop the reaction. Processed blood materials from *in vitro* glycation may have sufficient homogeneity and stability on a large scale, and it is anticipated that these GLY materials are most likely human blood samples and commutable with various principal analyzer.

Chapter 2

Literature Review

1. Diabetes mellitus (DM)

Diabetes mellitus (DM) was a common cause of death for many adults worldwide. Diabetes mellitus is a global endemic with rapidly rising rates in both developing and developed countries. Cho NH, 2018 estimate global diabetes prevalence, diabetes deaths, and public health expenditures due to diabetes present a significant social impact. There is an increasing need for governments to implement policies that ensure appropriate access to treatment for all diabetics. Diabetes mellitus is a carbohydrate metabolism disease that belongs to the metabolic disorder's family, characterized by hyperglycemia caused by underutilized glucose over a long period of time. Many patients did not have their risk factors and complications monitored at the recommended intervals while fasting blood glucose, HbA_{1c}, triglyceride, and cholesterol levels were measured. Diabetes can be classified into the following general categories (Association, 2022).

1. Type 1 diabetes; due to autoimmune beta-cell destruction, usually leading to absolute insulin deficiency.

2.Type 2 diabetes; due to a progressive loss of beta-cell insulin secretion frequently on the background of insulin resistance.

3. Gestational diabetes mellitus (GDM); diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation

4. Specific types of diabetes due to other causes, e.g., monogenic diabetes syndromes (such as neonatal diabetes and maturity-onset diabetes of the young), diseases of the exocrine pancreas (such as cystic fibrosis and pancreatitis), and drug- or chemical-induced diabetes (such as with glucocorticoid use, in the treatment of HIV/AIDS, or after organ transplantation).

Type 2 diabetes affects millions of people worldwide but type 1 diabetes affects a significant minority (5%). The classification of diabetes as Type 1 or Type 2 is largely determined by two factors, how old a person is when they develop diabetes, as well as the presence of antibodies that attack insulin-producing beta cells. Type 1 diabetes is the most common type, but it does not always start in childhood, and it is recognized by the presence of antibodies that block insulin production. It necessitates ongoing monitoring and treatment.

2. American Diabetes Association

According to the American Diabetes Association, diabetes is typically diagnosed based on three major criteria, which include:1) fasting plasma glucose (FPG $\geq$ 126 mg/dL), 2) 2-h plasma glucose value ($\geq$ 200 mg/dL), and 3) after a 75-g oral

glucose tolerance test (OGTT) and hemoglobin A_{1C} ($HbA_{1C} \geq 6.5\%$) (Duda SA 2018). Because HbA_{1C} is an indicator of long-term glycemic control, it has recently been recommended by the American Diabetes Associations as a possible substitute for fasting blood glucose for diabetes diagnosis (Association, 2022).

However, the HbA_{1C} level is affected by an abnormal erythrocyte life span, which may occur in iron deficiency anemia. Glycated albumin (GA) would be used in patients that has anemia or hemoglobinopathies and whose clinically measured HbA_{1C} level is inaccurate. Glycated albumin is a ketoamine formed by non-enzymatic glycation of serum albumin. It is expressed as a percentage of serum GA to total serum albumin. For several reasons, glycated albumin is a more useful marker than HbA_{1C}. According to Yong-ho, GA has been found to reflect fasting blood glucose levels more accurately than HbA_{1C}. Furthermore, GA levels, but not HbA_{1C} levels, correlated with β-cell insulin secretion, reflecting fluctuations in glucose concentration. The extracellular-intracellular glucose dynamics have no effect on GA. An albumin has a half-life of about 15 days thus, glycated albumin is a better indicator of short-term glycemic control than HbA_{1C}, and iron metabolism has no effect on glycated albumin. Because gestational diabetes mellitus (GDM) frequently causes iron deficiency anemia, HbA_{1C} may be insufficient for assessing glycemic control in these women. As a result, glycated albumin could be a useful marker for glycemic control during pregnancy. Glycated albumin levels can be measured using affinity chromatography, ion exchange chromatography, and high-performance liquid chromatography (HPLC) (Furusyo N, 2013). When the glucose level in the blood is higher than normal, glucose molecules become attached to the N-terminals of hemoglobin in red blood cells, a process known as glycation, which results in the formation of HbA_{1C}.

3. Whole Blood and Red Blood Cells

3.1 Whole Blood

Whole blood is composed of red blood cells (RBCs) as shown in **Figure 1**, white blood cells, and platelets (45 % of the volume) suspended in plasma (55% of the volume). Red blood cells, or erythrocytes, transport oxygen from the lungs to your body's tissues and return carbon dioxide to the lungs to be exhaled. Platelets, also known as thrombocytes, are blood cell fragments that interact with clotting proteins to stop or prevent bleeding. Plasma is a fluid that is 92% water, 7% vital proteins like albumin, gamma globulin, anti-hemophilic factor, and other clotting factors, and 1% mineral salts, sugars, fats, hormones, and vitamins. Red blood cells play an important role in your health by transporting oxygen throughout your body. Red blood cells are round with a flattish, indented center, similar to doughnuts without a hole.

3.2 Red blood cell

The red blood cell is approximately 8 μm in diameter when circulating through large vessels. The red blood cell membrane is essential for maintaining cellular functions in the body's only non-nucleated cell. The RBCs membrane achieves its key

structural properties by being deformable but stable, as well as having a unique biconcave shape with a high surface area to volume ratio.

Hemoglobin (Hb) is the protein found inside RBCs that transports oxygen as shown in Figure 2. Hb is an iron-containing complex protein found in red blood cells. Red blood cells also remove carbon dioxide from your body and transport it to your lungs for exhalation. The bone marrow is where red blood cells are created. They usually live for about 120 days before dying. Hemolysis is the breakdown or disruption of the integrity of the RBCs membrane, resulting in hemoglobin release (Sowemimo SO, 2002).

The red blood cell membrane can be thought of as a lipid membrane attached to an underlying skeleton. Protein profiles for approximately 52% of the membrane mass, lipid profiles for 40%, and carbohydrates profile for 8% (Sant RP, 2014). The erythrocyte membrane. A model of the major proteins of the erythrocyte membrane is shown: α and β spectrin, ankyrin, band 3 (the anion exchanger), 4.1 (protein 4.1) and 4.2 (protein 4.2), actin, and glycophorin as shown in Figure 3 (Gallagher P, 2015). The primary sugar found in blood and the body's primary source of energy, also known as blood sugar. When blood sugar levels rise, such as after a meal, the pancreas releases insulin. Insulin enters the bloodstream and ensures that the sugar found in our food and beverages is transported from our blood to our cells, where it is converted into energy for the body.

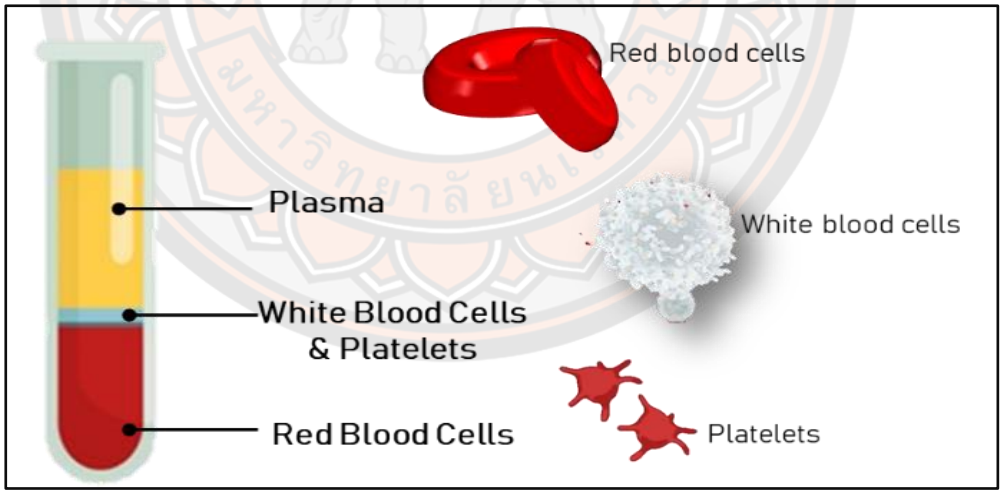


Figure 1. The component of whole blood

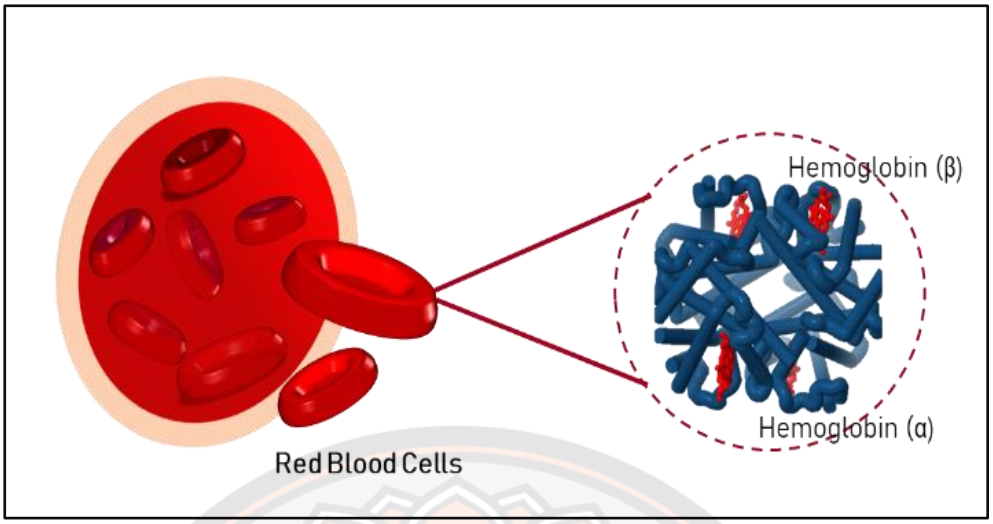


Figure 2. Hemoglobin complex protein in RBCs

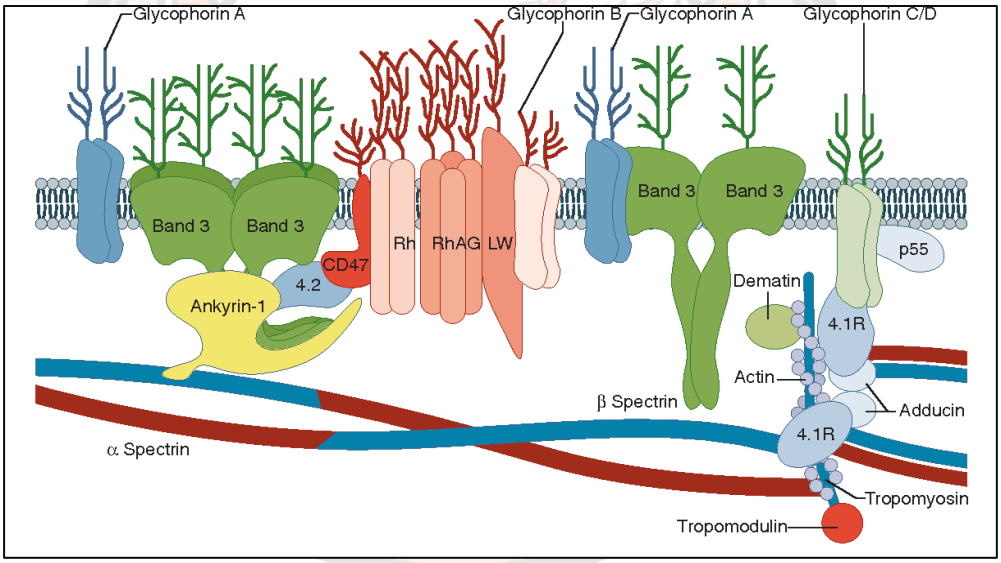


Figure 3. Structure of erythrocyte membrane

3.3 Glucose transporter

Glucose is a primary energy source for most cells as well as a key substrate in many biochemical reactions. The majority of mammalian cells import glucose via a facilitative diffusion process mediated by a family of membrane transport proteins. A large family of structurally related transport proteins known as glucose transporters regulates the entry of glucose molecules into cells. Glucose transporters, which is found on the membrane of small intestinal cells, is capable of absorbing glucose from the intestinal content. There are two types of glucose transporters: sodium-glucose linked transporters (SGLTs) and facilitated diffusion glucose transporters (GLUTs) as shown in **Figure 4** (Navale AM, 2016).

3.3.1 Sodium-glucose linked transporter-1 (SGLT1)

The Sodium-glucose linked transporter-1 (SGLT1) has 14 transmembrane helices, with both the COOH and NH_2 terminals facing the extracellular space. Sodium-glucose linked transporters symport (transport glucose in the same direction as sodium ions). To transport glucose against its concentration gradient, SGLTs must rely on the sodium concentration gradient generated by the sodium-potassium ATPase as a source of chemical potential. The SGLTs are present on the luminal surfaces of cells lining the small intestine they absorb glucose from dietary sources. There are Types and characteristics of sodium-glucose transporters show in **Table 1**.

Table 1. Types and characteristics of sodium-glucose transporters (SGLT)

SGLT Type	Location	Function	Nature
SGLT1	Apical membranes of small intestine cells	Absorption of glucose from intestine content	High affinity
SGLT2	Proximal convoluted tubule of nephron	Reabsorption of plasma glucose	Low affinity, High capacity
SGLT3	Intestine, testes, uterus, lung, pancreas	Controlling glucose levels in gut and brain	Low affinity, High capacity
SGLT4	Intestine, kidney, liver, brain, lung	Absorption and reabsorption of mannose	Nature not known
SGLT5	Kidney cortex	Transport of glucose and galactose	Low affinity
SGLT6	Brain, kidney, intestine	Preferred substrate is D-chiro-inositol	High affinity for myo-inositol

SGLT; Sodium-glucose link transported (sodium-dependent glucose transporter)

3.3.2 Facilitative glucose transporters (GLUTs)

Facilitative glucose transporters are proteins that have 12 membrane-spanning regions with intracellular amino and carboxyl terminals. The amino acid sequences of GLUT proteins have been found to share 28-65 % identity with GLUT-1. The GLUTs use a facilitated diffusion mechanism to transport glucose across the plasma membrane. The classical of GLUTs transporters were show in **Table 2**.

Table 2. Classification of facilitative glucose transporters (GLUTs)

Class	Type	Location / function
I	1	Associated with the bidirectional transport of glucose controlled by hormones, such as thyroid hormone
	2	On the hepatocyte membrane regulates the entry and exit of glucose
	3	Mainly present in the brain. It has high affinity to transfer glucose into cells
	4	An insulin-responsive glucose transporter that is found in the heart, skeletal muscle, adipose tissue, and brain
II	5, 7, 9, 11,	Located on cells of the small intestine, testes, and kidney
III	6	Mainly expressed in brain and spleen cells and in peripheral leukocytes
	8	A high-affinity transporter, while fructose and galactose inhibit this transport
	12	Expression on the surface of cardiomyocytes is not insulin dependent,

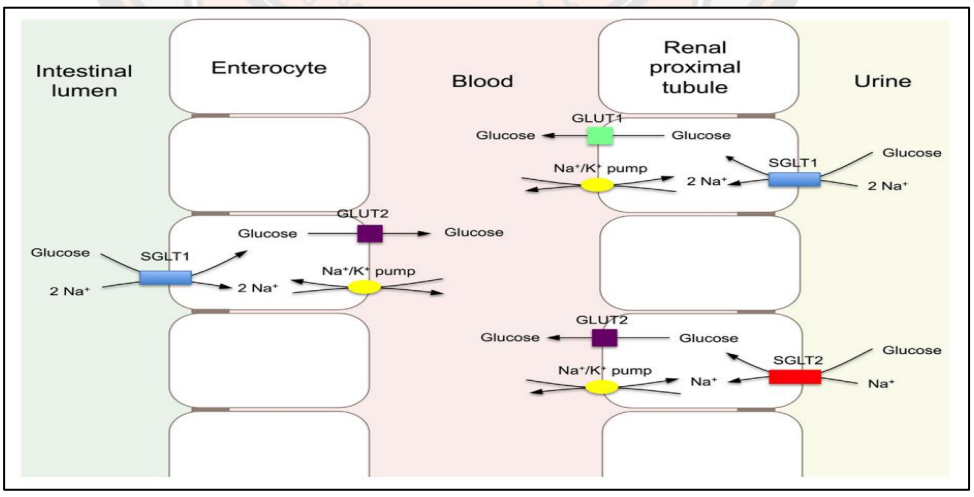


Figure 4. Active sodium glucose transporters in the intestine and in proximal renal tubules **(**SGLT2 = sodium-glucose cotransporter 2; GLUT2 = glucose transporter 2, (Baud G, 2016)

4. Glycation

Several chronic diseases, such as chronic kidney disease (CKD) and diabetes mellitus, are characterized by increased non-enzymatic activity. The most visible reaction is glycation, which is triggered by the initial binding of glucose and other sugars to protein amino groups, followed by Amadori rearrangement and its subsequent reactions (for example, oxidation, cleavages, molecular rearrangements, and formation of crosslinks). Glycation first generates stable Amadori products such as glycated hemoglobin (HbA_{1C}) and serum fructosamine, followed by a group of complex compounds known as "advanced glycation end-products" (AGEs; for example, carboxymethyllysine and pentosidine). Glycation and glycoxidation (oxidation in the formation of AGEs) rates are higher in diabetics due to hyperglycemia, as well as in patients with carbamylation-derived product; CKD (Nicolas C, 2018). Glycation-derived products are being studied as HbA_{1C} biomarkers in routine glycemic control evaluations (Mosca A, 2013).

4.1 Glycation reaction

The major non-enzymatic post-translational modification mechanisms (Figure 5.) that alter and damage the structure and function of biological macromolecules, particularly proteins, are oxidation due to oxidative stress and glycation due to carbonyl stress. Protein glycation is not the same as glycosylation, which is an enzyme-catalyzed reaction with site-specific carbohydrate addition (Zhang Q, 2009). The glycation products are a diverse group of reactive glycotoxins with aggressive and oxidative properties. The following are the characteristics of protein glycation products (Taghavi F, 2017).

1. Schiff base product: Schiff base is an unstable covalent linkage product of sugars and free amino groups in lysine and arginine residues. This conformational potential contributes to the formation of intra- and intermolecular cross-linking in proteins.

2. Amadori product: The result of Schiff base rearrangement, as early glycation products play a dominant role in diabetes complications. These compounds differ structurally from AGE products. The main Amadori adduct in extracellular proteins that stimulate fructosyl lysine receptors is fructosyl lysine (Salazar R,1995).

3. AGEs: Amadori products are produced as AGE by a complex reaction that includes condensation, dehydration, rearrangement, oxidation, and potential fragmentation. The effect of AGEs on tissue destruction occurs via two main pathways: a) cross-linking pathways and b) activation of several inflammatory signaling pathways. The accumulation of AGE products is a direct cause of long-term DM complications. However, AGEs may also be formed by non-oxidative processes after direct binding of carbonyl compounds produced from sugar derived metabolites, such as glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Protein glycation, the non-enzymatic modification of proteins by reducing sugars, is a major

cause of hyperglycemia-related toxicity in diabetes. Glycation is an uncontrollable physicochemical process that disrupts haemostatic balance in redox systems (Kaur J, 2018).

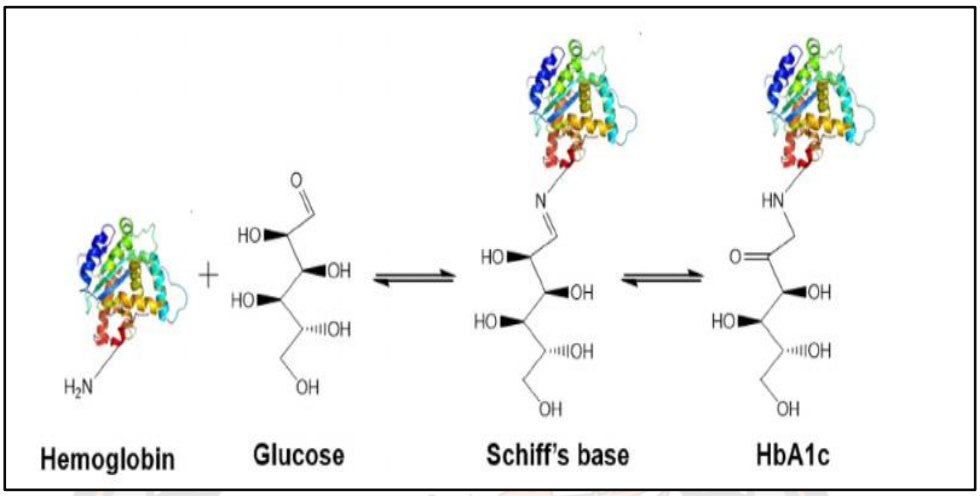


Figure 5. The process of HbA1C by glycation of Hemoglobin

4.2 Analytic Systems for HbA_{1C}

Hemoglobin A_{1C} (HbA_{1C}) is formed by non-enzymatic glycation of the hemoglobin beta chain's N-terminal valine, and the rate of the glycation reaction is proportional to the concentration of glucose (Strickland SW, 2017). Hemoglobin A_{1C} which is hemoglobin (Hb) that has been glycated by glucose in the blood, was used as a biomarker for average blood glucose levels over a period of 2-3 months. HbA_{1C} levels in all patients should be kept below 6.5% (48 mmo/mol) to avoid the development of long-term microvascular complications. Blood glucose levels have no effect on HbA_{1C} levels on their own. The person is affected by hemolytic anemias, hemoglobinopathies, acute and chronic blood loss, pregnancy, uremia, erythrocyte life span variation, and iron deficiency anemia (Urrechaga E, 2018). A model for HbA_{1C} formation was used to predict the relationship between average glucose (AG) and $\%HbA_{1C}$ under conditions of altered red blood cell lifetime (RCL) (Molinaro R, 2017). Kameyama M 2018 suggested a linear relationship between average glucose and HbA_{1C}. The measurement of glycosylated hemoglobin is the gold standard procedure for assessing long-term glycemic control in people with diabetes mellitus. Since the 1970s, many analytic methods have been developed that consider taking advantage of differences in electrical charge between HbA_{1C} and hemoglobin A_0, as well as structural differences between glycated and non-glycated forms of hemoglobin. There are methods based on difference in charge, with isoelectric point of HbA_{1C} and A_0 differs slightly. This implies that they have a different electrical charge under certain analytic conditions, and that they can be separated on that basis, and methods based on structural difference. The difference in molecular structure becoming widely available such as enzymatic

Assays, affinity Chromatography, and immunochemical assays. The **Table 3**. Show principles are used for the measurement of HbA_{1C}.

Table 3. Analytic Systems for HbA_{1C}

Difference in charge	
Chromatography based HPLC assay	Assay uses an HPLC instrument and ion exchange or affinity column to separate HbA_{1C} molecules from another hemoglobin molecules (Jeppsson JO, 1986).
Capillary electrophoresis	Separation of HbA_{1C} and A_0 occurs because one positively charged amino group in the HbA_{1C} molecule by attachment of glucose moiety. Secondly, Hb analysis as anions in alkaline conditions with selectivity to HbA_{1C} induced by a cis-diol interaction (Koval D, 2011).
Ion-exchange chromatography	In cation-exchange HPLC, a type of ion-exchange-HPLC, separation of hemoglobin molecules is based on charge, and hemoglobin molecules are positively charged. Red blood cells are lysed and passed through a negatively charged resin packed in a column. Positively charged hemoglobin molecules interact with the negatively charged resin, so the negatively charged molecules move at a faster rate (Priya S, 2020).
Difference in structure	
Boronate affinity	The basic interaction for boronate chromatography is esterification between boronate ligands and cis-diols configuration formed by stable glucose attachments to Hb. As the glycated and non-glycated products elute through a column, the hemoglobin interacts with boronic acid giving

	2 peaks: glycated (GHb) and non-glycated hemoglobin (Priya S, 2020).
Immunoassay	The antibody binds to the N-terminal glycated tetrapeptide or hexapeptide group. The design of the assay varies, from immunoturbidimetry to latex-enhanced competitive immunoturbidimetry and enzymatic detection (Weykamp C, 2009).
Enzyme based enzymatic assay	Direct Enzymatic HbA_{1C} Assay™ glycated valines serves as substrates for a specific recombinant fructosyl valine oxidase (FVO) enzyme. This is measured using a horseradish peroxidase (POD) catalyzed reaction and a suitable chromogen.
Liquid chromatography-isotope dilution tandem mass spectrometry (LC-IDMS/MS)	IFCC devised a reference measurement method for the determination of HbA_{1C} based on enzymatic cleavage of the hemoglobin molecule to obtain the β-*N*-terminal hexapeptides, followed by LC-IDMS/MS measurement of HbA_0 and HbA_{1c} using two "signature" hexapeptides for calibration (Liu H, 2015).

Conversion factors for IFCC compared to each of the designated comparison methods (DCMs) including the NGSP are shown below in **Table 4**. In 2007, the IFCC recommended that IFCC HbA_{1C} be expressed as mmol HbA_{1C}/mol Hb. With these new units, the master equation changes to NGSP = [0.09148 * IFCC] +2.15.

Table 4. Conversion factors for IFCC unit

DCM	From IFCC to DCM	From DCM to IFC
NGSP (USA)	NGSP = (0.09148*IFCC) + 2.152	IFCC = (10.93*NGSP) - 23.50
JDS/JSCC (Japan)	JDS = (0.09274*IFCC) +1.724	IFCC = (10.78*JDS) - 18.59
Mono-S (Sweden)	Mono-S = (0.09890*IFCC) + 0.884	IFCC = (10.11*Mono-S) - 8.94

DCM = Designated comparison methods, JDS = The Japan Diabetes Society

IFCC= The International Federation of Clinical Chemistry and Laboratory Medicine

NGSP= The National Glycohemoglobin Standardization Program

The relationships between NGSP and IFCC are listed in **Table 5** as well as with eAG (mmol/L and mg/dL) based on linear regression analysis was: eAG(mg/dl) = (28.7*HbA_{1C}) - 46.7, r^2=0.84

Table 5. The relationships between NGSP and IFCC HbA_{1C} as well as with eAG.

IFCC HbA_{1C} (mmol/mol)	NGSP HbA_{1C} (%)	eAG (mg/dL)	eAG (mmol/l)
31	5	97	5.4
42	6	126	7.0
53	7	154	8.6
64	8	183	10.2
75	9	212	11.8
86	10	240	13.4
97	11	269	14.9
108	12	298	16.5

(http://www.ngsp.org/docs/ifccstd.pdf

4.3 International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)

There are different assays available to measure the proportion of HbA_{1C} in blood. The International Federation of Clinical Chemistry (IFCC) established a Working Group (IFCC WG-HbA_{1C}) in 1995 to achieve international standardization of HbA_{1C} measurement. The major accomplishments are as follows: a) A reference measurement procedure based on purified primary calibrators has been established; b) a global network of reference laboratories has been established; and c) work on implementing traceability to the IFCC reference system has begun. The current issue is HbA_{1C} traceability to the IFCC reference system as shown in **Figure 6**.

4.4 IFCC reference method

Liu H, 2015 using enzymatic proteolysis was applied to sample preparation, followed by LC-IDMS/MS measurement of hemoglobin A_0 (HbA_0) and HbA_{1C}, using two "signature" hexapeptides for calibration. The developed LC–IDMS/MS method was used in participation in an IFCC ring trial for reference laboratories for HbA_{1C}. Figure 7. Schematic of LC-IDMS/MS procedure for HbA_{1C} Measurement. Part (a) represents the determination of the concentrations of VEc and GEc calibration standard solutions, and part (b) represents the measurement of HbA_{1C} in hemolysate sample (Liu H, 2015).

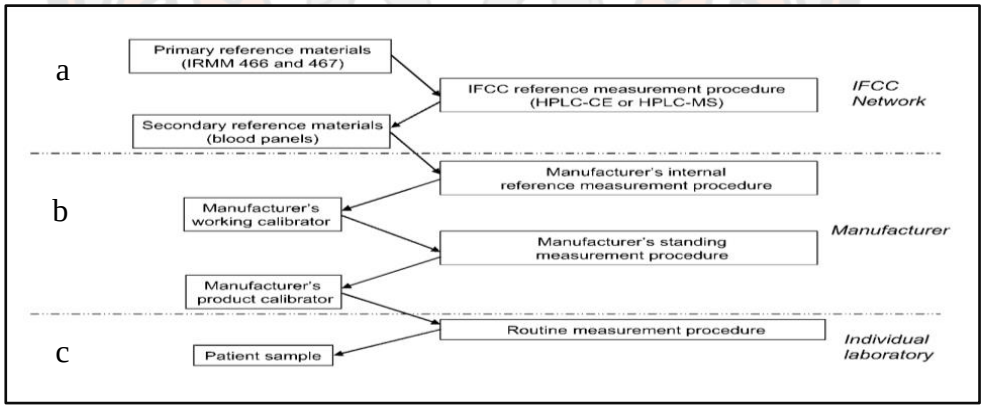


Figure 6. IFCC reference measurement system and traceability chain for HbA_{1C}

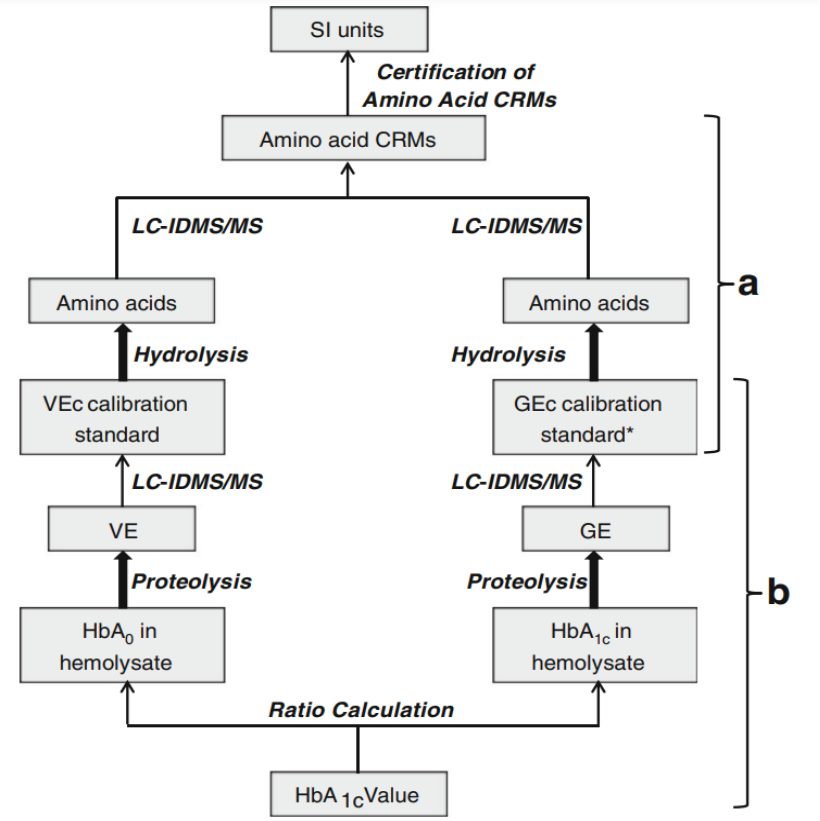


Figure 7. Schematic representation of LC-IDMS/MS procedure for HbA_{1C} Measurement

4.5 Interference from Hb variants

Hemoglobin A_{1C} levels are influenced by many factors of genetic, hematological, and disease-related factors, including sickle cell disease and thalassemia, as well as conditions that shorten the lifespan of the erythrocyte (e.g., hemolytic anemia), and high levels have been linked to iron deficiency. The limitations of HbA_{1C} as a glycemic control measure imply that there are niches for other biomarkers that could be used when HbA_{1C} cannot because they do not suffer from the same limitation. Fructosamine (total glycated serum protein) and glycated serum albumin (GA) are the focus. The interferences for measuring HbA_{1C} is shown in **Table 6**.

Table 6. Comparison of principle for HbA_{1c} measurement

Principle	Advantages	Limitation
Ion-exchange chromatography	No interference from Hb variants	-Variable interference from hemoglobinopathies -False positive may come from HbE
Boronate affinity chromatography	No interference from HbS, HbC, HbE, HF	Variable interference from Hemoglobinopathies conjugated with total glycated hemoglobin
Capillary electrophoresis	No interference from HbS, HbC, HbE	Variable interference from HbF
Immunoassay	No interference from HbD, HbE	Variable interference from HbF, if HbF>30%

(http://www.ngsp.org/interf.asp)

Hemoglobin types, hemoglobin F (HbF; fetal hemoglobin), hemoglobin C (HbC), and hemoglobin S (HbS)

4.6 Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry (MS) (MALDI-8020, Mass Spectrometer)

New approaches to testing HbA_{1C}, such as Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry (MS) was show in Figure 8, could be used instead of traditional chromatographic techniques. A few reports characterize hemoglobin as a new, quick, accurate, and simple to characterize protein and peptide from biological sources using MALDI-TOF MS. Furthermore, MALDI-TOF MS is not yet employed in routine laboratory HbA1C quantification. MALDI-TOF is typically used for research, quality control and analysis of proteins & peptides, as well as other biological and organic samples.

Key features of the instrument

1. Linear mode (positive-ion) MALDI-TOF
2. 200 Hz solid-state laser, 355 nm
3. Load-lock chamber for fast sample introduction
4. UV laser-based source cleaning (patented)
5. Small footprint/benchtop design
6. Quiet operation (55 db)

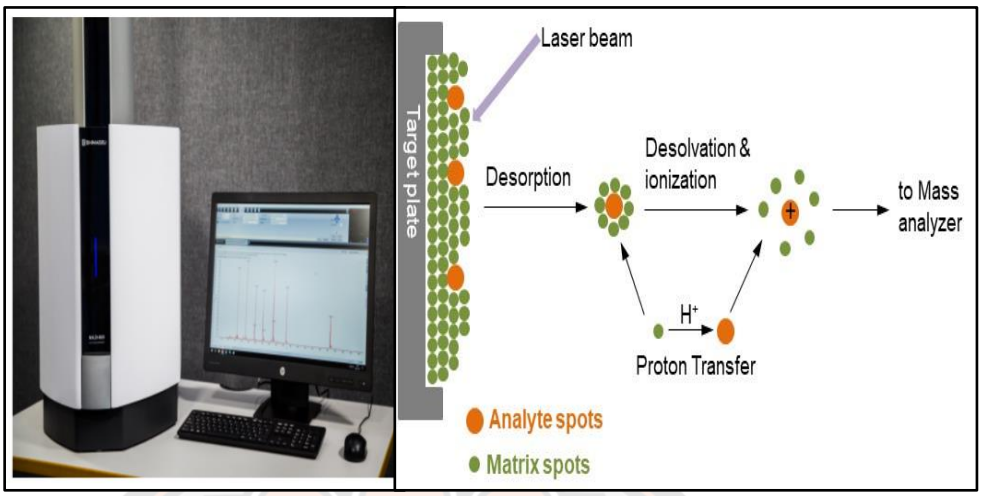


Figure 8. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)

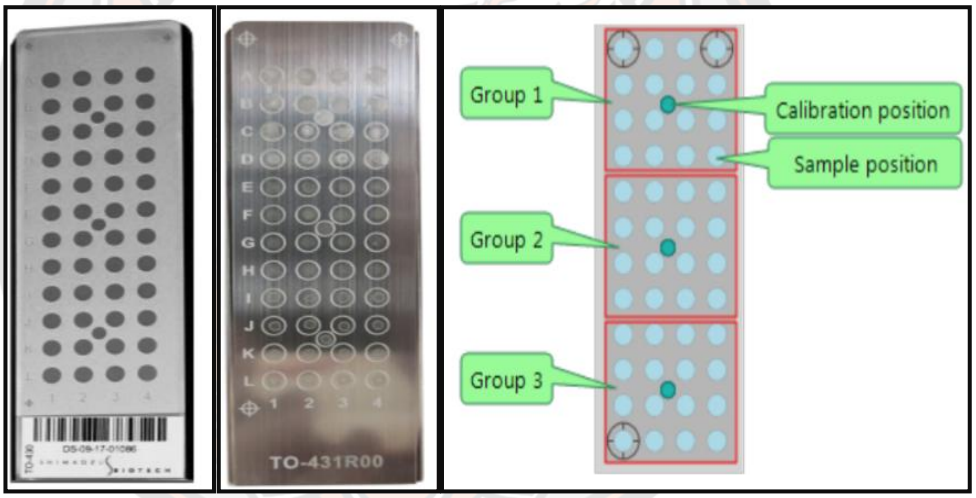


Figure 9. Disposable targets (Left) and SR48 steel targets (Middle)

MALDI-TOF MS was used in this study to precisely quantify the molecular mass of β-globin and α-globin to compute HbA_{1C} in NGSP or IFCC units and compare with laboratory methods (Pais RJ, 2019, Xu A, 2021, and Anping X, 2020). MALDI-TOF is typically used for research, quality control and analysis of proteins & peptides, as well as other biological and organic samples (SHIMADZU). Samples are introduced into the instrument using target plates which can be 'spotted' with a range of different samples up to the maximum the plate allows. There are two types of plate as show in **Figure 9**.

5. Glycated albumin and Fructosamine

Albumin contains 585 residues amino acid, including 59 lysine residues that may be glycated. Lys-525 is the primary glycation site in mature albumin, accounting for approximately 33% of total albumin glycosylation. Albumin also contains 24 arginine that can be glycated. As a result, the term "glycated albumin" (GA) encompasses a wide range of molecular species resulting from various albumin glycation patterns, influencing the accuracy of some analytical methods for GA determination. The half-life of albumin is approximately 2-3 weeks, which is sufficient to allow the formation of advanced, stable glycation end products. Notably, albumin glycation can impair its ability to bind a variety of compounds, including fatty acids, hormones, and drugs (M 2019). Due to the limited reliability of HbA_{1C} levels in CKD, fructosamine and glycated albumin have been proposed as alternative proposed markers of glycemic control for monitoring diabetes patients with CKD. Finally, GA has been shown to be more accurate than HbA_{1C} in diabetic patients receiving hemodialysis, to be more reliable in neonatal diabetes monitoring and in pregnancy, and to have a significant relationship with certain types of diabetes complications such as nephropathy and coronary artery disease (CAD). In comparison to HbA_{1C}, GA values are distributed over a broader range (Montagnana M, 2013).

Fructosamine, a glycated serum protein measure, represents the average glycemia over the previous 2-3 weeks. Fructosamine was found to be strongly correlated with other glycemic control measures such as HbA_{1C}, fasting glucose, and the oral glucose tolerance test (Innes KE 2011). Fructosamine concentrations have a strong relationship with albumin and total protein concentrations. Except in patients with liver cirrhosis, hyperthyroidism, and nephritic syndrome, where albumin had prolonged half-lives. Glycated albumin (GA) represents the short-term glycemic index of two to three weeks and showed lower values in subjects with elevated BMI, body mass, or visceral adipose tissue, as well as infants (Raghav A, 2018). The various methods for measurement of GA and fructosamine reported so far are laborious, and unsuitable for routine clinical use. Glycated albumin determinations were following.

High-performance liquid chromatographic: The separating albumin from other serum proteins is difficult, serum glycated protein has been measured rather than glycated albumin. The HPLC method described here for measuring glycated albumin is quick and precise, requires a small sample, and is automatable. The interaction of the cis-diol groups of the non-glycated protein's glycosyl moieties with boronic acid is responsible for the separation of glycated and non-glycated protein (Shima K, 1988).

Fluorescence spectroscopy: has been used to distinguish between glycated and un-glycated albumin, as well as to characterize the effect of antioxidants on glycation-induced protein changes.

Colorimetric assay using nitroblue tetrazolium (NBT): this assay is based on a kinetic color reaction associated with the ability of ketoamines to reduce NBT to the corresponding formazan in alkaline medium (Rodríguez-Segade S, 2017).

Raman spectroscopy: can accurately discriminate glycated albumin from the un-glycated variant, even at low μM concentrations. The proposed method provides with completely reagent-free, translating this promising technology is to assess its predictive diagnostic value in multi-component mixtures, and combination with recent studies of Raman-based characterization of protein glycosylation (Dingari NC, 2012).

Liquid chromatography: combined with tandem mass spectrometry (LC-MS/MS); using trypsin digestion of proteins in plasma produced a large number of proteotypic peptides from albumin, including KQTALVELVK amino group, which was detected both glycated and non-glycated protein (Brede C, 2016).

Enzymatic method: proteinase hydrolyzes glycated albumin to glycated amino acids, and ketoamine oxidase oxidizes the glycated amino acids to produce hydrogen peroxide, which is quantified. The percentage of glycated albumin in total albumin is used to calculate glycated albumin (Kouzuma T, 2002).

Electrochemical Immunoassay: developed a new nanozyme-based electrochemical immunoassay method with urchin-like Pt nanozymes (uPtNZs) and applied them to colorimetric and electrochemical assays for sensitive determination of GA in total human serum albumin (tHSA) using 3,3′,5,5'-tetramethylbenzidine (TMB) and thionine as substrates, respectively (Choi H, 2020).

6. International Organization for Standardization of reference material producers

6.1 ISO 17034; General requirements for the competence of reference material producers (ISO 17034, 2016)

The International Organization for Standardization (ISO) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. ISO has published ISO 17034:2016 on the general requirements for the competence of reference material producers (RMPs). The production of RMs is a key activity for the improvement and maintenance of a worldwide coherent measurement system. RMs with different characteristics are used in measurements, such as calibration, quality control, proficiency testing and method validation, as well as for the assignment of values to other materials. Certified reference materials (CRMs) are also used to confirm or establish metrological traceability to conventional scales. However, statistical methods for the assessment of the homogeneity and stability of reference materials for qualitative properties are not covered.

6.2 ISO Guide 35:2017; Guidance for characterization and assessment of homogeneity and stability (ISO Guide 35, 2017)

International Organization for Standardization (ISO). Reference materials-Guidance for characterization and assessment of homogeneity and stability. ISO/Guide 35:2017 explains concepts and provides approaches to the following aspects of the production of reference materials (**Figure 10**).

- assessment of homogeneity
- assessment of stability and the management of the risks associated with possible stability issues related to the properties of interest
- characterization and value assignment of properties of a reference material
- evaluation of uncertainty for certified values
- establishment of the metrological traceability of certified property values

The methodology for the assessment of the homogeneity and stability of reference materials are following (Charun Y, 2018).

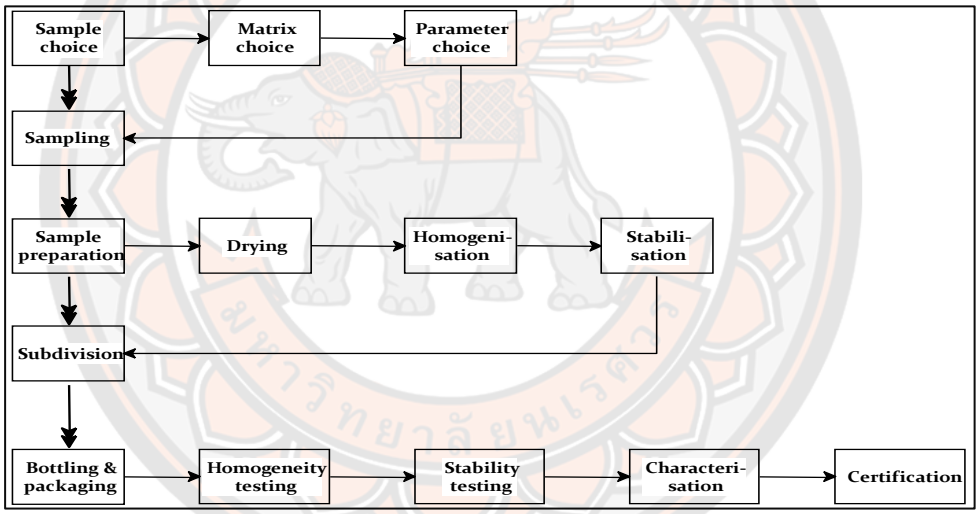


Figure 10. Production and certification process

6.2.1. Assessment of homogeneity

The RMP shall carry out an assessment of the homogeneity of any candidate RM in its final packaged form to ensure its fitness for purpose. Assessment of homogeneity can include the use of prior evidence (including prior experimental evidence), the conduct of an experimental homogeneity study on the candidate RM or both. In most cases, provided an experimental study is necessary. Guidance on the need for an experimental number of homogeneity study is provided in ISO Guide 35.

In most cases, experimental homogeneity tests require measurements of a representative number of randomly chosen units. The units can be chosen for example by random selection, stratified random selection or systematic selection from a random start point. For certified values, homogeneity shall be quantified as an uncertainty value

contribution to the certified value or shall be shown to a negligible contribution to the uncertainty of the certified values.

Homogeneity testing is of the highest importance for the certification of reference materials, as it should demonstrate the validity of the certified values and their uncertainties in the analysis of individual units or portions thereof. The primary aim of such homogeneity testing is to verify, on a reasonable number of units, that all units of the certified reference material (CRM). However, for both between-unit and within-unit homogeneity testing, it is observed that tests are usually only carried out with the intention of verifying the hypothesis of no significant between-samples variation (Pauwels J, 2001).

1. Between-units homogeneity testing

When appropriate, the assessment of the homogeneity of the candidate CRM may be performed on randomly chosen units, but in general it is preferred to carry out between-units homogeneity testing on samples which are taken from production either in a systematic or in a random.

2. Within-unit homogeneity testing

CRMs are not prepared in portions for single use, and (small) test portions of a single unit are used for analysis (J 1988). To check whether sub-samples from the same unit are equally "identical" in terms of their certified properties.

The homogeneity uncertainty was calculated based on ISO Guide 35, the ANOVA of the raw peak areas of the main component was performed and mean squares within each group (MS within) and among the groups (MS among) were calculated. Standard deviations between bottles (U_{bb} (1)) were then calculated by use of equation (1), where n represents the number of measurements per bottles:

$$U_{bb} = \frac{\sqrt{\text{MS among} - \text{MS within}}}{n} \quad (1)$$

In the case of insufficient repeatability of the measurement method, the influence of analytical variation on the standard deviation between units (U_{bb} (2)) was calculated and used as the estimate of inhomogeneity. The U_{bb} (2) was calculated by using equation (2)

$$U_{bb}\,(2) = \sqrt{\frac{\text{MS within}}{\text{n}}} \times \sqrt[4]{\frac{2}{V\ MS\ within}} \quad (2)$$

6.2.2. Stability monitoring

As with assessing homogeneity, one of the key features of any RM is the stability of the properties of interest, a further refinement of the material currently contained in ISO Guide 35 production. Stability testing of candidate RMs is performed over a period of time so that the behavior of the properties of the material is known. The stability of some physical property RMs can improve over time. Approaches to stability testing can be widely different. The simplest approach is to take two

measurements at different points in time and to draw conclusions about the stability over the elapsed time.

Most reference materials are stored for extended periods at the RM producer's premises or by distributors. Since stability assessment cannot usually anticipate all changes that may occur, it is usually necessary, as a part of managing the risks associated with possible instability, to monitoring the property values of materials held for extended periods. An alternative method for stability testing of candidate certified reference materials (CRMs) utilizing "isochronous" measurements based on a storage design (storing samples at different temperatures for different time periods), at the end of the study, is compared to "classical" designs of stability studies using reference time or reference temperature. Therefore, in most cases, a short-term stability study of the possible behavior of the material during transport as well as a long-term stability study of the probable behavior of the material under controlled storage conditions (Figure 11 (Charun Y, 2018).

1) Classical stability study (Long-term) Long-term stability studies of the actual CRM upon storage are generally an integrated part of the certification project, and typically cover a storage period of two years.

2) Isochronous stability study (Short-term) Experimental study of reference material stability in which units exposed to different storage conditions and times are measured in a short period of time.

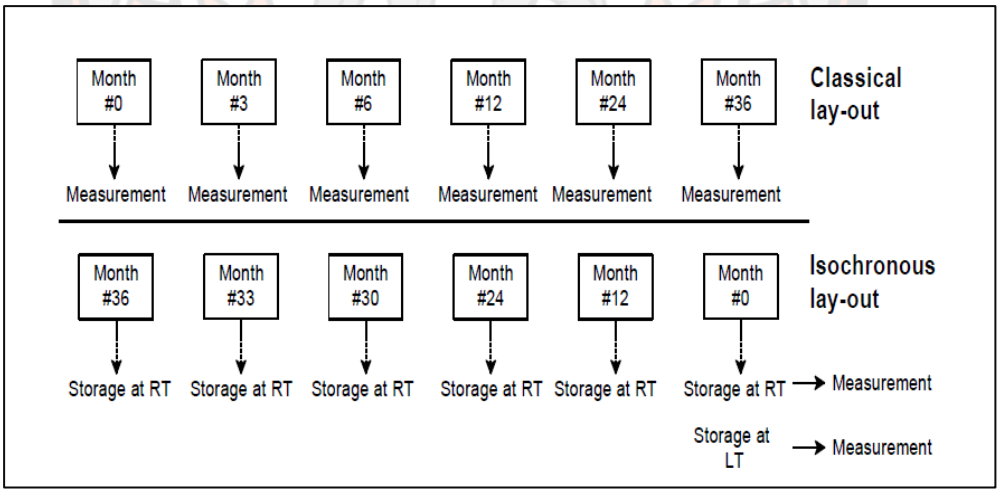


Figure 11. Long-term stability study

7. Commutability

The manufactures of diagnostic reagents were developed measurement procedures, they attempt to design them so that they will report measurand values accurately for the intended patient samples. These measurement procedures may not be designed to produce accurate results when nonpatients samples such as external quality

assessment samples, proficiency testing samples, or QC samples are measured. Because such nonpatient sample matrixes typically undergo some processing and spiking of additional components, and therefore are altered in some manner, measurand results may not reflect the accuracy that would be observed for patient samples. Processed samples that recover like patient samples are called commutable, while those that do not are called non-commutable (Karl DV, 2014).

Clinical and Laboratory Standard Institute (CLSI), guideline will be helpful in exploring differences in the test material results between measurement procedures, especially when such materials serve as a bias fir determining measurement procedure performance. The data evaluation used an ordinary linear regression or OLR (**Figure 12**) for results of the measurement procedures, whereas this uses the Deming regression model (**Figure 13**). Commutability is determined by comparison of the measured result for a proceed sample to the "scatter" of results for a representative set of patient samples measured using two measurement procedures. The mean value for the processed samples is compared with the scatter of patient's sample means about the regression line through patient's sample results.

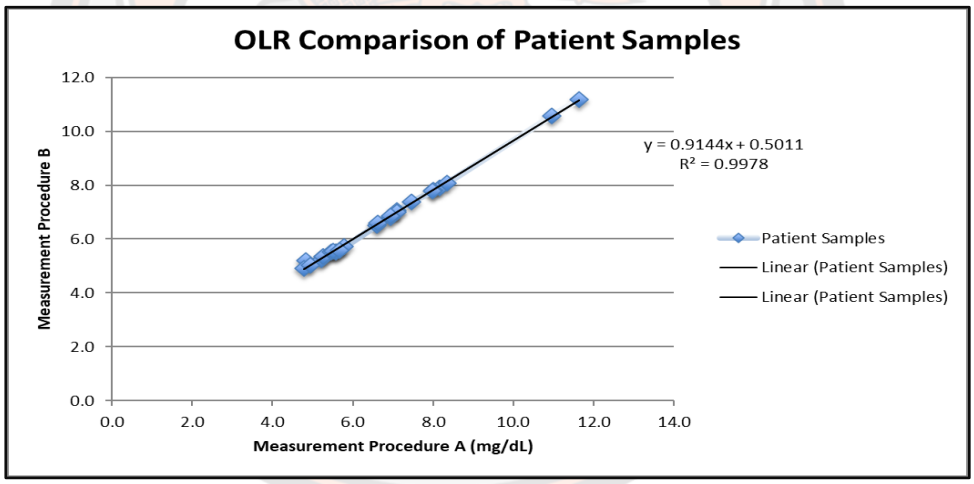


Figure 12. Ordinary Linear Regression or OLR

Plot means of replicates of the patient samples with measurement procedure B results on the y-axis and measurement procedure A results on the x-axis.

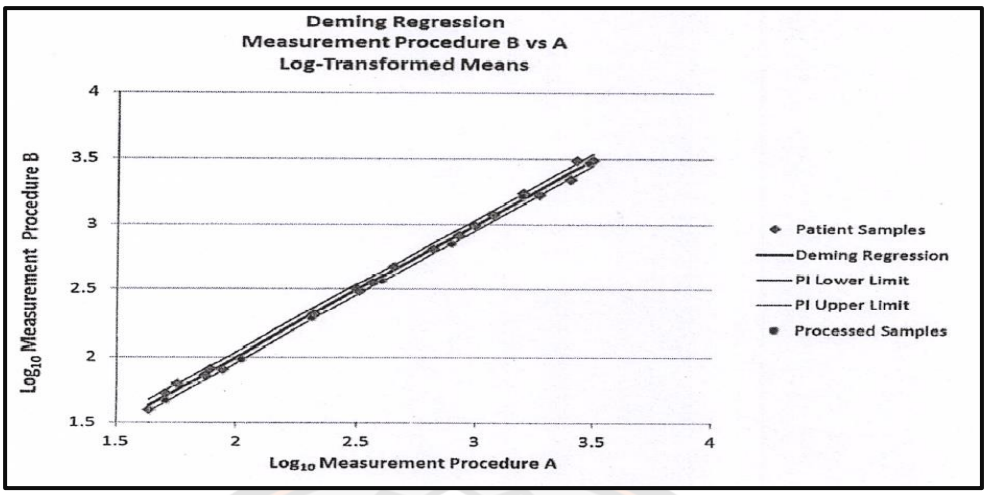


Figure 13. Deming regression analysis

Comparison of processed samples to the patient's sample. Deming regression line between procedure A and B to constructs 95% prediction interval (PI) for the regression relationship between results for patients measured by these two procedures represents the limits within which a future result will fall with a probability of 95%.

8. Characterization (Guide 35:2017)

Characterization is a method to determine uncertainty then used for assigned value the chemical concentration in materials. Characterization can be achieved by using one or several methods in one or several labs. ISO 17034 lists several basic approaches to characterization.

8.1 Characterization using a single reference measurement procedure (as defined in ISO/IEC Guide 99) in a single laboratory

8.1.1 Characterization of a non-operationally defined measurand using two or more methods of demonstrable accuracy in one or more competent laboratory.

8.1.2 Characterization of an operationally defined measurand using a network of competent labs. A basic model for batch characterization (3).

$$X_{CRM} = ychar + \delta homo + \delta lts \quad (3)$$

X_{CRM}= property value

y_{char}=property value obtained from the characterization of the batch

δ_{hom}= an error due to heterogeneity

δ_{lts}= an error for stability effect under storage condition

$$U_{CRM} = \sqrt{uchar + uhomo + ults} \quad (4)$$

8.1.3 Value transfer from a RM to a closely matched candidate RM performed using a single measurement procedure performed by one laboratory

8.1.4 Characterization based on mass or volume of ingredients used in the preparation of the RM.

9. Previous studies for *in vitro* glycation research

Previous research had discovered that the red blood cells of diabetic patients, particularly those in the poor control group with high HbA_{1C} levels, were easily deformed due to the loss of their deformity properties. It is frequently found in red blood cells during transport. There was still a long way to go before developing blood sample preparations with constant HbA_{1C}. In the industry, it is possible to produce large quantities. It is applicable to all analyzers. The material's quality is comparable to that of foreign-sourced blood (Wongsri P, 2017).

This study focuses on the conditions of glycated hemoglobin in whole blood samples (**Table 7**) and conditions of glycate human serum albumin (**Table 8**). Then discuss the biochemistry of glycated proteins, the methods of measuring glycated HbA_{1C}, it a clinical application, and it a relationship to the complications of diabetes mellitus.

Table 7. Conditions for intracellular glycation between glucose and hemoglobin.

Reference	Conditions
Smith RJ, 1982	Erythrocyte were culture for 8 days in 90% Earle's balanced salt solution, 10% bovine serum, phenol red (glucose indicator), pH 7.4, and 5% CO_2. The result show HbA_{1C} concentration was found to increase linearly with time during 8 days of incubation. The rate of formation of HbA_{1C} increased linearly as glucose concentration was increased. The non-enzymatic glycosylation of hemoglobin is influenced by factors other than glucose, including oxygen tension and 2,3-diphosphoglycerate levels.
Selvaraj N ,2006	Human erythrocytes in phosphate buffered saline (pH 7.4) were incubated with 5 or 50 mmol/L glucose. Erythrocytes were incubated with either lipoic acid or taurine and then exposed to glucose concentration of either 5 or 50 mmol/L. To clarify if lipid peroxides per sec enhances the glycated hemoglobin level, an *in vitro* study was performed by incubating erythrocyte suspension containing either 5 or 50 mmol/L glucose with or without malondialdehyde. The level of glycated hemoglobin in erythrocyte incubated in the presence of malondialdehyde was increased significantly when compared to erythrocyte incubated with baseline.
Sadowska-Bartosz I, 2015	Peripheral blood from a healthy donor was collected in sodium citrate as an anticoagulant. The blood was centrifuged with 2000xg for 10 min at 4ºC. The pellet was washed three times with phosphate buffered and were suspended to final hematocrit of 10% in PBS containing 5-, 50- or 100-mM glucose in the absence and in the presence of ascorbic acid at 37ºC for 24 and 48 hours. Ascorbic acid did not affect erythrocyte hemolysis, glycation of hemoglobin and erythrocyte membranes.

Siddiqui Z 2018	Hemoglobin was modified with varying concentration of D-ribose (1, 2, 5 and 10 mM) at 100 mM phosphate buffer saline (PBS), pH 7.4 under sterile conditions. The reaction mixture was incubated at 37°C for varying time periods 1-21 days. The native Hb was served as control. The reaction mixture was stored at -20°C for further use. The stability of glycated Hb was disturbed due to glycation by D-ribose. Due to structural and conformational changes, functional property of glycated Hb could also be compromised.
Fluckiger R, 2018	Human erythrocyte was incubated at 20°C for 10 days without or with 0.5 M glucose or for 7 days without or with 0.5 M ribose in phosphate buffer. At 0.5 M of the glucose although not physiologic was used as accelerate the nonenzymatic glycation process which proceeds at a slow pace *in vivo*. Incubations were carried out in NaCl, pH 7.4 with gentle rocking. To prevent glycoxidation from occurring, the incubation mixtures were blanketed with nitrogen and the tubes kept sealed. Human erythrocyte was incubated with glucose for a long period time and storage in liquid nitrogen.
Silva AMN, 2018	Human holo-serotransferrin was incubated with 0, 10-, 20-, 100- and 500-mM D-glucose at 37°C in pH 7.4 PBS buffer and 0.02% sodium azide was added. Two hundred microliters aliquots were collected after 14-days incubation. Glycation occurs away from the iron-binding sites and, despite the observed iron release.

Table 8. Conditions for extracellular glycation between glucose and protein

Reference	Conditions
Behroozi J, 2014	Human hemoglobin from freshly drawn blood was dialysis, protein concentrations and bovine serum albumin (BSA) was used as standard. Hemoglobin (10 mg/ml) was incubated with 40 mM of glucose in a 50 mM phosphate buffer with pH 7.4. Bee venom was used as an anti-glycating agent. Incubation was carried out at 37°C for 5 weeks in a shaker incubator. Then, sampling was conducted to store at -70°C until processing. Bee venom has a significant anti-glycation effect, which can restrain glycation-induced alteration in the secondary structure and function of hemoglobin.
Sadowska-Bartosz I, 2015	Bovine Serum Albumin was incubated with 0.5 M, fructose or ribose in phosphate buffer, pH 7.4, in the absence or in the presence of 0.05, 0.1, 0.2, 0.5 and 1 mM at 37^{o}C for 6 days. The BSA was also incubated with various concentrations of ascorbic acid under the same conditions. The samples were incubated in Eppendorf tubes; azide was used as a preservative. Ascorbic acid enhanced glycoxidation of BSA by glucose and induced BSA glycation in the absence of sugars.
Thomas CJ, 2017	To glycate osteocalcin *in vitro*, synthetic intact human osteocalcin was incubated either with HBS alone or HBS with 1.43 M ribose or glucose at 60^{o}C for 2 or 4 h. After incubation, osteocalcin was buffer exchanged to 50 mM ammonium bicarbonate using 5,000 MWCO spin columns (3x washes with 13,000 rpm centrifugation) to remove excess sugar and HBS. The levels of glycated osteocalcin fragments released from bone during bone resorption.

Chandhury S, 2017	Gramma B-crystallin was incubated in phosphate buffer, pH 7.4 at 37^{o}C for 28 days in the presence of D-glucose in the dark under sterile conditions. A solution of sodium azide was added before incubation to prevent bacterial growth. B-crystallin without D-glucose was incubated under similar conditions and treated as a control for this study. One milliliters aliquot from each set was withdrawn on 7, 14, 21 and 28 days of incubation. The incubation period was extended up to 60 days and check the possibility of protein oligomerization/aggregate formation. Human B-crystallin was determined by *in vitro* glycation study in a solution of high glucose content for up to 28 days.
Villa M, 2017	Fifty mg/mL BSA in 0.2 M PBS pH 7.8, containing antibiotics and protease inhibitors. The solution was glycated in vitro, under sterile conditions at 37^{o}C, with increasing D-glucose concentrations (0, 5, 25, 125, 250, 500 mM) for 4 weeks or with 500 mM D-glucose for increasing time of exposure (2, 4 or 8 weeks). BSA glycated in vitro with increasing D-glucose concentrations for a fixed time or with a fixed D-glucose concentration. *In vitro* gBSA with 500 mM D-glucose for 8 weeks proved to be the richest in fluorescent AGEs detection and was chosen for the in vitro experiments.
Fatima S, 2017	Six hundred micromoles of HSA were incubated with glucose (1.8 M) at 37^{o}C for 30 days. A sodium azide solution of was added to avoid bacterial contamination. A modified commercial fructosamine assay kit was used to determine the level of glycation. The level of glycation was checked after every 5 days on the basis of the difference in molecular weight by 12% SDS-PAGE. The extent of glycation of HSA, as measured by fructosamine assay kit. Most of the increase in glycation occurred for 20 days and measured by SDS-PAGE.

Summary of *in vitro* glycation methods divided to 2 glycation methods: intracellular glycation (HbA_{1C}) and extracellular glycation (GA). Intracellular glycation method, blood from healthy donor was centrifuged with Normal saline (0.85%) and supernatant were removed. Erythrocytes were modified with varying concentration of glucose of D-glucose in phosphate buffer saline (PBS), pH 7.4 under sterile conditions. A solution of sodium azide was added to prevent antibacterial. The reaction mixture was incubated at 37^{o}C for 120 hours. The product from *in vitro* glycation as a processed blood materials or GLY materials were keep in additive solution in an optimal temperature to keep stability of HbA_{1C}. Finally, GLY materials were evaluated using a variety of primary analyzers for commutability studies and material characterization of processed blood materials assigned values. For extracellular glycation method, human serum albumin was incubated with D-glucose at 37^{o}C for increasing time to exposure 48 hours. Antibacterial was added to avoid bacterial contamination. Glycation product was determined by using turbidimetric immunoassay. The sample were test following ISO Guide 35 for stability and homogeneity test and characterization to assigned value of concentration of HbA_{1C} or fructosamine in processed materials before applied in proficiency testing (PT) program.

To create HbA_{1C} on an industrial scale, the optimal conditions for *in vitro* glycation were applied, and the PT provider sent these products to participants in the PT program. The findings of the participants were analyzed for z-sore to assess the laboratory's performance.

Chapter 3

Materials and Methods

A summary of the research was prepared, defined the value and applied as a reference material in laboratory to prove the results of diagnosis. *In vitro* glycation was initiated using blood from a blood bank under sterile conditions. The optimal conditions for *in vitro* glycation to generate processed blood materials for HbA_{1C} tests with various incubation time, glucose concentration, explored while keeping intact erythrocytes and completely preventing glycation by chemical preservation at low storage temperature. Based on commutable data, processed blood materials were evaluated for homogeneity, stability, and characterization of HbA_{1C}. The processed blood materials were produced following ISO 17034; General requirements for the competence of reference material producers (ISO 17034, 2016) and data were analyzed following the requirement of ISO Guide 35:2017; Guidance for characterization and assessment of homogeneity and stability (ISO Guide 35, 2017). This study was approved for human research ethics (IRB No. 0139/62) from Naresuan University. The overview of this research divided into 7 studies there are:

Study 1: To determine influence parameters and conditions effect to quality of human red blood cell in CPDA-1 from blood bank.

1.1 Determination quality of human red blood cells and plasma

1.2 To study the characteristics of whole blood before using *in vitro* glycation

Study 2: To investigate the optimal conditions for *in vitro* glycation among glucose, and hemoglobin (intracellular glycation).

2.1 Substrate for *in vitro* glycation

2.2 Temperature for *in vitro* glycation

2.3 Incubation times

2.4 Glucose concentrations

2.5 Inhibited solution and storage conditions for HbA_{1C} from *in vitro* glycation

2.6 Hemoglobin typing of processed blood materials

Study 3: To investigate the optimal conditions for *in vitro* between glucose and albumin (extracellular glycation).

3.1 Glucose concentrations

3.2 Incubation times

Study 4: **Homogeneity and stability test of HbA_{1C} and fructosamine in *in vitro* glycation product by following ISO 17034-Reference material producer and Guide 35-Guidance for characterization and assessment of homogeneity and stability.**

4.1 Homogeneity test of HbA_{1C} in processed blood materials

4.2 Homogeneity test of fructosamine in processed plasma

4.3 Stability of HbA_{1C} in processed blood materials

4.4 Stability of fructosamine in processed plasma

Study 5: **Commutability study of processed blood materials to investigate differences in test material results between measurement procedures parallel with clinical blood samples**

5.1 Hemoglobin A_{1C} measurement in clinical blood sample and in processed blood materials

5.2 Comparisons of HbA_{1C}

5.3 Commutability evaluation

Study 6: **Characterized HbA_{1C} in blood materials according to Guide 35-Guidance for characterization and assessment of homogeneity and stability.**

6.1 Hemoglobin A_{1C} in processed blood materials were measured by six measurements

6.2 Characterization of HbA_{1C}

Study 7: **To determine HbA_{1C} by advance technique by Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS)**

7.1 MALDI-TOF MS conditions and blood sample preparation

7.2 Prospective HbA_{1C} measurement in native blood samples with optimal conditions MALDI-TOF MS

Study 1: To determine influence parameters and conditions effect to quality of human red blood cell in CPDA-1 from blood bank.

1.1 Determination quality of human red blood cells and plasma

Determination of the parameters and conditions that influence the quality of human red blood cells obtained from a blood bank. The parameters used to analyze the quality of red blood cells including of color, size and form of red blood cells, lifetime, hematocrit, and non-hemolysis. This research will focus on preparing a high-quality red blood cell prior to the glycate experiment. The overview of experiment design was show in Figure 14.

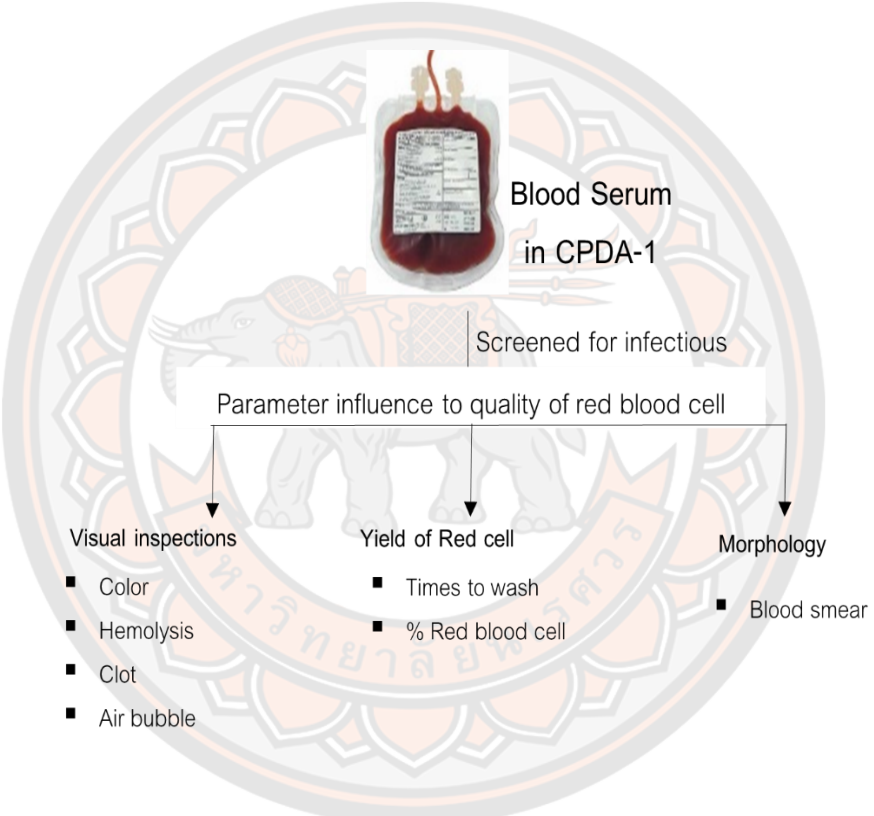


Figure 14. The influent parameters were used to determine the quality of human red blood cell from blood bank

Methods

Three hundred milliliters of whole blood (WB) in citrate phosphate dextrose adenine (CPDA-1) donated from a blood bank has expired was maintained at 2-8^{o}C until the experiments. All whole blood samples are tested for infectious markers before being used in this study. Then, was tested for %HbA_{1C}, blood smear, and %hematocrit. Whole blood sample was centrifuged to separate the plasma from the red blood cells, and the supernatant will be discarded. The red blood cells were saved for a future study (*in vitro* glycation).

Study subject

Inclusion criteria

The WB from National Blood Bank Centre has negative results infectious disease marker, Hepatitis B, Hepatitis C, HIV and Syphilis. Hemolysis is not found by visualize inspection.

Exclusion criteria

The WB from National Blood Bank Centre has positive results infectious disease marker, Hepatitis B, Hepatitis C, HIV and Syphilis. Hemolysis 3^{+}, in this cause can not to preparation for *in vitro* glycation.

Blood samples in CPDA-1 bags are stored at 2-8°C for determining the parameters that influence to red cell quality after the expiration date. The inclusion and exclusion criteria were shown in **Table 9.**

Table 9. Inclusion and exclusion criteria to select quality of red blood cell

Criteria	Inclusion	Exclusion
Color	Red color of blood and light-yellow liquid of plasma	green color
Hemolysis serum	≤ 3+	≥ 3+
Clot	absent	present
Air bubble	absent	present
Time for washing RBC	≤ 3	≥ 3
Yield of RBC	40-50%	≤40 %
Morphology	normocytic/size and normal color	abnormal RBC Size

Data analysis

The WB inclusion criteria expired less than 90 days after the blood specimen was collected in a CPDA-1 bag and used as a blood sample for screening parameter influence. The color will be red and clear, hemolysis will be less than 3+ to prevent red cell damage, clots and air bubbles will not appear, whole blood can be washed less than 3 times to save cost, red blood cell yield will be 40-50%, and morphology will be

normal. After passing these parameters, the complete blood as well as a blood sample were used in study 2 (*in vitro* glycated hemoglobin).

Table 10. Data analysis for acceptable quality of red blood cells

Study	Criteria	Non-Expired (Control) (n=50)	Expired < 1 month (n=50)	Expired < 2 months (n=50)	Expired < 3 months (n=50)
Observe	Color				
	Hemolysis				
	serum				
	Clot				
	Air bubble				
	Yield of RBCs				
Washing time	Time				
Yield	Yield of RBCs				
Morphology	Blood smear				
Results	“Pass” or “Not pass”				

1.2 To study the characteristics of whole blood before using *in vitro* glycation

To investigate the characteristics of RBCs before, during, and after washing with 0.85% NS. Red blood cells (RBCs) should be high quality with a high yield, an expired date less than 2 months, washing time less than 3 times to save cost, red cells that were intact and WB present without hemolysis.

Method

One milliliter of whole blood was placed in 15 mL centrifuge tubes and washed suspended with 0.85 % of NS then centrifuge at 3,500 rpm for 5 mins. Remove the upper solution and repeat the process until supernatant was clear. The method was shown in **Figure 15**.

Study subject

Inclusion criteria

The whole blood sample was passed requirement of study 1.1 and expired less than 1, 2, 3 months, respectively (expiration date classified by date from drawback).

Exclusion criteria

The whole blood sample was not passed requirement of study 1.1 and expired more than 3 months.

Data analysis

The number of spins until clear was used as the criterion for consideration. The number of cells remaining after washing until clear, as well as the yielding of RBCs remaining after washing until clear, were criteria for blood selection.

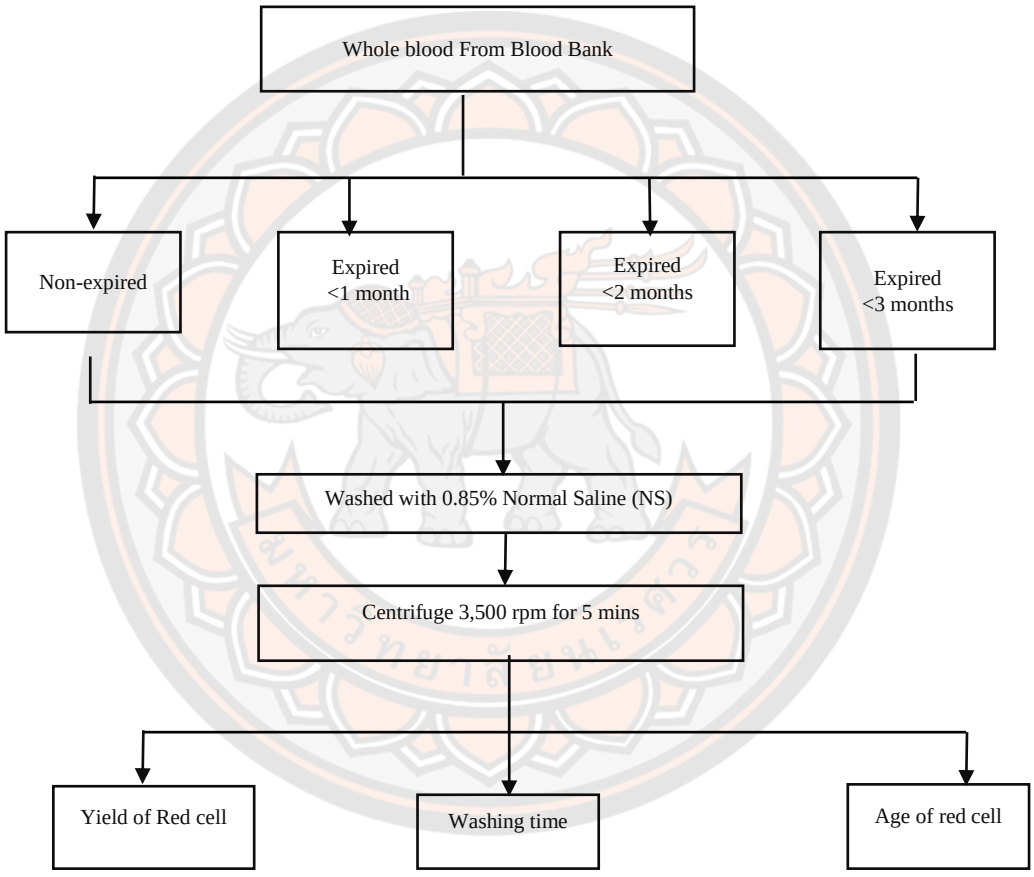


Figure 15. Diagram characteristics of whole blood before using in vitro glycation

Study 2: To investigate the optimal conditions for *in vitro* glycation among glucose, and hemoglobin (intracellular glycation)

Determination of the optimal conditions for *in vitro* glycation with high quality of red blood cells passing criteria study 1 for glycation between hemoglobin and glucose. The whole blood was centrifugation and supernatant were removal. Erythrocytes washed three time with 0.85% NS and then suspended in various concentration of glucose in PBS buffer pH 7.4. A solution of sodium azide was added for antibacterial, and 38-40% of hematocrit was adjusted in final volume. The mixture reaction was incubated at 37ºC for a periods time. The products were estimated by boronate affinity chromatography. This experiment focusses on optimal conditions for in vitro glycation were divided to 6 studies.

2.1 Substrate for in vitro glycation
2.2 Temperature for in vitro glycation
2.3 Incubation times
2.4 Glucose concentrations
2.5 Inhibited solution and storage conditions for HbA_{1C} from *in vitro* glycation
2.6 Hemoglobin typing of processed blood materials

2.1 Substrate for *in vitro* glycation

Method

Three hundred milliliters donation blood from blood bank and passing criteria from study 1.1 and 1.2. The red blood cell maintains a high quality of properties, size, and shape of RBCs. One milliliter of whole blood sample was added in 15 mL of centrifuge tube then, separated into 2 phases of plasma and red blood cell pellet phase by centrifugation 3,500 rpm for 5 minutes. RBCs were collected and suspended in 0.85% normal saline (NS) until supernatant was clear. Red blood cells and whole blood were used as a substrate for *in vitro* glycation and incubated with 100 mM final concentration of D-glucose at 37ºC for 5 days under sterile conditions. Sodium azide was added for antibacterial. The HbA_{1C} products were measured for 5 duplicates for each day by boronate affinity chromatography. The percentage of Hct was measured by centrifugation for hemolysis observation. The method was prepared following **Table 11**.

Table 11. The conditions for the selection of substrates for *in vitro* glycation

Chemicals	Whole blood		Red blood cell	
	Control	100 mM D-Glucose	Control	100 mM D-Glucose
Whole blood (mL)	100	100	-	-
Red blood cells (mL)	-	-	100	100
200 mM of D-Glucose (mL)	-	100	-	100
pH 7.4 PBS (mL)	100	-	100	-
Temperature (°C)	37	37	37	37
Incubation time (days)	5	5	5	5

2.2 Temperature for *in vitro* glycation

The ideal incubation temperature at 37°C was reported. Since blood from the Blood Bank was kept at 2-8°C, donated blood kept at 37°C or 2-8°C may be effects on %HbA_{1C} and cell hemolysis.

Method

The conditions for temperature investigation were show in **Table 12**. Three hundred milliliters of whole blood (WB) were suspending and centrifugation at 3,500 rpm for 5 minutes, and RBCs were collected (RBCs preparation describe in study 1.2). In this study, WB or RBCs was incubated with and without D-Glucose at 37°C or 2-8°C for 5 days (phosphate as a control). Hemoglobin A_{1C} and Hct levels were measured five times using boronate affinity chromatography and centrifugation, respectively.

Table 12. Temperature effects on *in vitro* glycation are investigated in this condition.

Chemicals	Whole blood		Red blood cell	
	Control	100 mM D-Glucose	Control	100 mM D-Glucose
Whole blood (mL)	100	100	-	-
Red blood cells (mL)		-	100	100
200 mM of D-Glucose	-	100	-	100
pH 7.4 PBS (mL)	100		100	-
Incubated at 37°C or 2-8°C				

2.3 Incubation times

Previous research has found that more than 15-20 days for incubation period of glycation effects to cause RBC rupture. It also has an impact on the analysis as well. Hct should be at least 15%, according to some principles. Long-term glycation has an impact on production costs, production time, and cell integrity. The purpose of this study was to determine the effect of incubation times during incubation WB or RBCs with D-glucose.

Method

The blood is centrifuged (3,500g, 5 mins), plasma and leukocyte buffy coat are separated, and pellet is washed three times with Normal saline (0.85% NSS). The experiment was designed with four conditions with WB or RBCs were incubated with PBS or D-Glucose. Erythrocytes were incubated with 400 mM D-glucose at 37 ^{o}C for 5 days. Sodium azide (0.02%) was added as a preservative to all processed blood materials. HbA_{1C} level was determined by turbidimetric immunoassay method, and Hct level was determined by centrifuge method at baseline and 120 hours after the start of the study. The experiment was designed in Table 13. Percentage of HbA_{1C} was measured for 5 times by boronate affinity chromatography and Hct was measured by centrifugation at 0, 1, 2, 3, 4, and 5 days.

Table 13. The conditions of incubation times for *in vitro* glycation of WB and RBC

Chemicals	**WB**		**RBC**	
	Control	**400 mM D-Glucose**	**Control**	**400 mM D-Glucose**
Whole blood (mL)	100	100	-	-
Red blood cells (mL)		-	100	100
800 mM of D-Glucose	-	100	-	100
pH 7.4 PBS (mL)	100		100	-
Incubation time (days)	5	5	5	5
Temperature (^{o}C)	37	37	37	37

2.4 Glucose concentrations

The preliminary *in vitro* glycation study discovered RBCs incubated with D-Glucose for 15 hours at 37°C were optimized. The concentration of glucose is the factor influencing glycation. In this study, glucose was used at concentrations of 0 (PBS), 50, 100, 125, 150, 200, 300, 400, and 500 mM with PBS serving as a control.

Method

Whole blood was centrifuged at 3,500g for 5 min, and RBCs pellet was washed approximately three times with 0.85% NS. Erythrocytes were incubated with 0 (PBS), 50, 100, 150, 200, 250, 300, 400 and 500 mM of D-glucose in pH 7.4 PBS, and 0.02% sodium azide was added as a preservative in reaction solutions. For each hour, HbA_{1C} in processed blood materials from *in vitro* glycation (GLY materials) was determined by boronate affinity method, and Hct was determined by centrifugation from the baseline (day 0). The data from optimal glucose concentrations for *in vitro* glycation determination were evaluated by paired-t-test compared with vehicle control for each hour ($p < 0.001$).

2.5 Inhibited solution and storage conditions for HbA_{1C} from *in vitro* glycation

When the temperature, glucose concentration, incubation times, and appropriate substrate were all optimal, the glycation reaction occurred. In vitro glycation processing until RBCs ruptured or hemolysis occurred. The inhibited solution was important to maintain the HbA_{1C} and Hct approximately 38-42%. The processed blood from *in vitro* glycation at -20°C to maintain stability of HbA_{1C} in previous report. This research design for the laboratory scale, and then it scaled up for stability testing.

Method

Red blood cells were incubated with 400 mM of D-glucose then Processed blood materials were suspended in inhibited solution-1, inhibited solution-2, or non-inhibited solution. Processed blood materials (GLY materials) were aliquoted into 0.5 mL in vials and stored at three different conditions (**Figure 16**).

Non-inhibited solution: processed blood materials (GLY materials) were aliquoted 0.5 mL into vial and kept at 2-8°C (at -20°C as a control).

Inhibited solution-1: processed blood materials (GLY materials) were centrifuge to remove excess of glucose solution, and RBCs was suspended in inhibited solution-1. The GLY materials were aliquoted 0.5 mL into vials and kept at 2-8°C (at -20°C as a control).

Inhibited solution-2: processed blood materials (GLY materials) were centrifuge to remove excess of glucose solution, and RBCs was suspended in inhibited solution-1. The GLY materials were aliquoted 0.5 mL into vials and kept at 2-8°C (at -20°C as a control).

Ten vials of GLY materials were randomized for homogeneity assay (ISO Gude35:2017). The methodology for homogeneity test is describe in **APPENDIX A, B and C**. Five vials were random for stability assay (ISO Guide 35:2017). The methodology for stability test is describe in **APPENDIX D, E and F**. Hemoglobin A_{1C} and Hct in the GLY materials each condition was monitored stability continuously for 38 days (n=5) by statistics following ISO Guide 35 ($T_{calculation} < T_{critical}$).

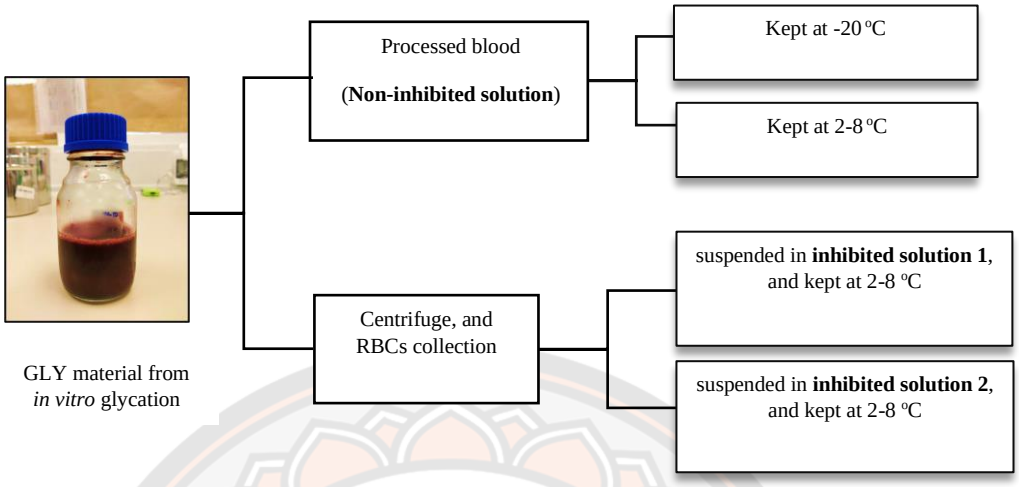


Figure 16. Conditions of inhibited solution for stop in vitro glycation

2.6 Hemoglobin typing of processed blood materials

Capillary electrophoresis was used to check abnormal Hb variants in processed blood materials (NDM-1) was prepared from erythrocytes suspended in 0.85 (Wongsri P, 2017), and GLY material was prepared from *in vitro* glycation.

Method

Processed blood material (NDM-1) was prepared from whole blood by suspending in 0.85% NS, GLY-1 was prepared from *in vitro* glycation was randomized and screened for Hb typing to check for abnormal Hb variants expected to affect the analysis or erythrocytes when stored at low glucose concentration in a refrigerator. Hemoglobin typing determined by capillary electrophoresis methods with reference laboratory that certified by ISO 15189:2012.

Study 3: To investigate the optimal conditions for *in vitro* between albumin and glucose (extracellular glycation)

3.1 Glucose concentrations

Plasma from blood Bank was incubated with D-Glucose at 0 (control), 50, 100, 150, 200, 250, and 300 mM. Sodium azide was added for anti-bacterial. Plasma solutions were incubated at 37^{o}C for 48 hours, and then fructosamine, total protein, and albumin were measured. Fructosamine was measured by turbidimetric immunoassay. The increasing of fructosamine, total protein, and albumin were significant with $p < 0.05$ by paired t-test when compared with control (0 mM of D-glucose). The conditions for fructosamine investigation were show in **Table 14**.

3.2 Incubation times

Plasma from blood Bank was incubated with D-Glucose at 0 (control), 100, 200, and 300 mM at 37^{o}C for 48 hours, and fructosamine was measured by turbidimetric immunoassay. The significant difference evaluated with $p < 0.05$ by paired t-test when compared with control (0 mM of D-glucose).

Table 14. Conditions for studying fructosamine

Chemical	Control	Glucose concentrations (mM)					
		50	100	150	200	250	300
Plasma (mL)	-	4.0	8.0	12.0	16.0	20.0	24.0
500 mM of D-Glucose	39.2	35.2	31.2	27.2	23.2	19.2	15.2
0.02% Sodium azide	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Incubation time (hr.)	48						

Table 15. Conditions for incubation time of fructosamine

Chemical	Glucose concentrations (mM)			
	Control	100 mM	200 mM	300 mM
Plasma (mL)	98.0	78.0	58.0	38.0
500 mM of D-Glucose	0.0	20.0	40.0	60.0
0.02% SA	2.0	2.0	2.0	2.0
Incubation time (hr.)	48			

Study 4: Homogeneity and stability test of HbA_{1C} and fructosamine in glycation product by following ISO 17034-Reference material producer and Guide 35-Guidance for characterization and assessment of homogeneity and stability.

Homogeneity and stability test was followed ISO 17034-reference material producer and Guide 35-Guidance for characterization and assessment of homogeneity and stability (**Figure 17**). The number of units required for homogeneity and stability testing is determined at random from processed blood production. The ISO Guide 35:2017 guideline having determined homogeneity of RM with the between-unit and within-unit standard deviation, the RM producer should confirm that the variation within and between units is sufficiently small for the intended use of the material. Such confirmation may include, for example by use of an $F_{calculation} < F_{critical}$, that the between-unit term is not statistically significant at the 95% level of confidence.

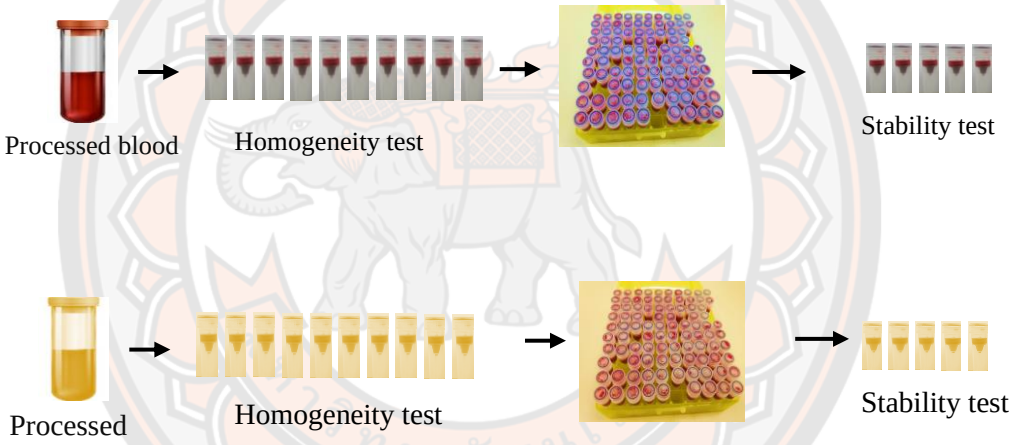


Figure 17. Determination homogeneity and stability test of HbA_{1C} in processed blood materials and processed plasma (Fructosamine)

4.1 Homogeneity test of HbA_{1C}

Homogeneity test can refer either to variation of a property value between separate units of the material, or to variation within each unit. It is always necessary to assess the between-unit variation.

Method

Three processed blood materials were prepared following;

1. A processed blood material processed plasma (GLY-1) was produced by incubating erythrocytes with 400 mM of D-Glucose at 37°C for 15 hours. The GLY-1 materials were centrifuged to remove the excess of glucose solution from supernatant, and the erythrocytes were suspended in inhibited solution-1 and kept at 2-8°C.

2. A processed blood material from fresh blood sample from a single DM patient (FDM-1) was collected in a K_3EDTA tube, and 0.5 mL was aliquoted into a vial and stored at 2-8^{o}C.
3. A processed blood material (NDM-1) was prepared from a pooled non-DM blood sample was suspended in 0.85%NS (P 2017) and kept at 2-8^{o}C.

The GLY materials were aliquoted and refrigerated until the experiments. Three processed blood materials (GLY-1, FDM-1, and NDM-1) from three different procedure were evaluated for homogeneity of HbA_{1C}. Hemoglobin A_{1C} levels and Hct in three blood samples were measured and analyzed 5 replicates in accordance with ISO Guide 35 (ISO Guide 35:2017) by turbidimetric immunoassay and centrifugation method, respectively.

4.2 Homogeneity of fructosamine

Processed plasma (PPM) was aliquoted and kept in a refrigerated until the experiments. Three processed plasmas (PPM-1, PPM-2, and PPM-3) were evaluated for homogeneity. Fructosamine in three processed plasmas were measured and analyzed in accordance with ISO Guide 35 (ISO Guide 35:2017) by turbidimetric immunoassay.

Data analysis of homogeneity test

1. The selection of a minimum of 10 units is to be used for measurements. The number of items should be sufficient to give a reasonable estimate of the between-unit variance. However, examining only 10 units might not provide sufficient information therefore increases the number of units examined as the total number of units produced, N_{prod} increases recommendations are between $\sqrt[3]{\text{Nprod}}$ and $\sqrt[3x3]{\text{Nprod}}$

$$N_{\min} = \max\left(\sqrt[10,3]{Nprod}\right)$$

2. Random stratified sampling from each segment is most appropriate when there is no known ordering, or for small batches. Processed plasma sample were random by using stratified random (n=10) and HbA_{1C} measurement in duplicate.

3. The Cochran's critical value (**Table 16)** at 99% (ISO Guide 35:2017) was removed as an outlier from the technical explanation error and calculated for both within-sample and between-sample variation.

4. Calculate F_{test} and $F_{critical}$ ($C_{critical}$ at 95%) by using ANOVA single factor. If, $F_{test} < F_{critical}$, the products will be homogeneity.

Table 16. The Cochran's critical value

	95% critical values ($\alpha = 0.05$)			99% critical values ($\alpha = 0.01$)		
	n			n		
k	2	3	4	2	3	4
3	0.9669	0.8709	0.7978	0.9933	0.9423	0.8832
4	0.9064	0.7679	0.6839	0.9675	0.8643	0.7816
5	0.8411	0.6838	0.5981	0.9277	0.7885	0.6958
6	0.7806	0.6161	0.5321	0.8826	0.7218	0.6259
7	0.7269	0.5612	0.48	0.8373	0.6644	0.5687
8	0.6797	0.5157	0.4377	0.7941	0.6152	0.5211
9	0.6383	0.4775	0.4028	0.7538	0.5727	0.4812
10	0.6018	0.445	0.3734	0.7169	0.5358	0.4471
11	0.5696	0.4169	0.3482	0.683	0.5036	0.4207
12	0.5408	0.3924	0.3265	0.652	0.4751	0.3922
13	0.515	0.3709	0.3075	0.6236	0.4498	0.3698
14	0.4917	0.3517	0.2907	0.5976	0.4272	0.3499
15	0.4706	0.3346	0.2758	0.5737	0.4069	0.3321
16	0.4514	0.3192	0.2625	0.5516	0.3885	0.3162
17	0.4339	0.3053	0.2505	0.5313	0.3718	0.3018
18	0.4178	0.2927	0.2396	0.5124	0.3566	0.2887
19	0.4029	0.2811	0.2296	0.4949	0.3426	0.2767
20	0.3892	0.2705	0.2206	0.4786	0.3297	0.2657
21	0.3764	0.2607	0.2122	0.4634	0.3178	0.2557
22	0.3645	0.2516	0.2046	0.4492	0.3068	0.2462
23	0.3535	0.2432	0.1974	0.4358	0.2966	0.2376
24	0.3431	0.2354	0.1908	0.4233	0.2871	0.2296
25	0.3334	0.2281	0.1847	0.4115	0.2782	0.2222

The null hypothesis of homogeneity of variance is rejected if $c > C_{\text{critical}}$.

(Quality Assurance in the Analytical Chemistry Laboratory - Brynn H.)

4.3 Stability of HbA_{1C} in processed blood materials

Two types of (in)stability are particularly relevant in the production of reference materials: 1) the short-term stability or stability under transportation, and 2) long-term stability of the material during the period of validity under storage conditions.

Methods

Short-term stability or transportation stability; thirty vials of GLY-1 were randomly select and packed into a container with ice packs and kept at ambient indoor temperatures for 3 trials. The temperatures inside a container were monitored by calibrated data loggers for 5 days per a trial. The GLY-1 were measured for HbA_{1C} and Hct for each day. The stability of HbA_{1C} in GLY-1 were compared by statistics following ISO Guide 35.

4.3.1 Alternative storage for short periods stability; was measured for HbA_{1C} by randomly selection of 5 samples to assessment of stability was also

investigated the stability of HbA_{1C}. Material from optimal *in vitro* glycation (GLY-1, GLY-2, GLY-3), fresh blood from a single DM patient was collected in a K_3EDTA tube (FDM-1), and pooled non-DM blood from a donor (NDM-1). One-way ANOVA was calculated for $T_{calculation}$ and $T_{critical}$ were reported.

4.3.2 Long-term stability of processed blood materials (GLY-1, GLY-2, GLY-3, NDM-1), and commercial QC (CQC-1, and CQC-2) were aliquot and kept at 2-8°C. The classical approach for stability study was performed under reproducibility conditions. The stability study of HbA_{1C} in processed blood material was performed for 0, 7, 14, 21, 35, 49, 70 and 98 days using 5 tubes of each sample in duplicate analysis HbA_{1C} and Hct.

4.4 Stability of fructosamine in processed plasma

Long-term stability of fructosamine was measured for 0, 7, 14, 21, and 35 days using 5 tubes of processed plasma in duplicate analysis.

Data analysis

Statistical analysis (**Figure 18**) was carried out by one-way ANOVA. Mean of sample are calculated, and plot regression line confidence interval. Hemoglobin A_{1C} was stable when $T_{calculation}$ was less than $T_{critical}$ for the short-term stability test (ISO Guide 35:2017), and slope was significantly different from zero for long-term stability test ($p < 0.05$).

$$t_{Cal} = \frac{|Slope|}{|SD\ slope|}$$

Regression by using LINEST			
Slope (b1	0.20436	0.15531	Slope (b0)
Standard error of the	1.252511	11.3083	Standard error of the slope (sb0)
r^2	0.002044	0.87616	SYX
F	0.026621	13	df
SS regression	0.020436	9.97956	SS residual
tcal	0.16316		
tcritical	2.160369		
Summary :	Stability		

Figure 18. Formulation for stability test (ISO Guide 35)

Study 5: Commutability study of processed blood materials to investigate differences in test material results between measurement procedures parallel with clinical blood samples

Twenty-five clinical blood samples and six processed blood materials were examined for HbA_{1C} with six measurement procedures in triplicate. The characteristic of reference laboratories and measurement methods were shown in **Table 17**. Different type of measurements was used to examine HbA_{1C};

- TI-RL1: Turbidimetric immunoassays from reference laboratories certified by NGSP
- EN-RL1: Enzymatic immunoassay from reference laboratories certified by NGSP
- BA.HPLC-CL1: Boronate affinity Hight-Performance-Liquid-Chromatography
- TI-CL2: Turbidimetric immunoassays in routine clinical laboratories
- BA-POCT1: Boronate affinity point of care (POC) devices in routine clinical laboratories
- FI-POCT2: Fluorescent immunoassays point of care (POC) devices in routine clinical laboratories

Table 17. Specification of measurement analysers for HbA_{1c} testing

Measurement methods	Laboratories	Brand/model	Range
Turbidimetric immunoassay	TI-RL1*	Cobas c513	2.0-16.0%
Enzymatic immunoassay	EN-RL1*	Abbott	2.0-16.0%
Boronate affinity-High performance liquid chromatography	BA.HPLC-CL1	Premier Hb9210	3.8-18.5%
Turbidimetric immunoassay	TI-CL2	Cobas c111	4.2-20.1%
Boronate affinity	BA-POCT1	Clover A1C	4.0-14.0 %
Fluorescent immunoassay	FI-POCT2	Finecare	4.0-14.0 %

*Certified for Hemoglobin A_{1c} testing by NGSP level-1

5.1 Hemoglobin A_{1C} measurement in clinical blood sample and in processed blood materials

Twenty-five clinical blood samples from volunteers (there were age of 20- >60 years old, <6.4%=12 volunteers, 6.5-9.0%= 11 volunteers, and >9.1%= 2 volunteers),

including healthy and DM patients, were collected in a K_3EDTA tube and stored in the refrigerator.

Processed blood materials: NDM-1 was prepared from whole blood by suspending in 0.85% NS (Wongsri P, 2017), three materials from *in vitro* glycation; GLY-1, GLY-2, GLY-3 were prepared from *in vitro* glycation, and two commercial QC; CQC-1, CQC-2 were purchased commercially. All six blood materials were measured randomly and in triplicate (CLSI EP14-A3:2014) by six methods.

5.2 Comparisons of HbA_{1C}

Hemoglobin A_{1C} levels in clinical blood samples, processed blood materials, and commercial QC were evaluated for mean, standard deviation (SD), median, and lower and upper 95% confidence intervals (CI). Analysis of Variance (ANOVA); Turkey statistic was used to compare HbA_{1C} mean values of six different measurement procedures. A statistically significant $p < 0.05$ was used.

5.3 Commutability evaluation

All six blood materials were measured in triplicate by immunoassay and enzymatic immunoassay that certified by NGSP level 1. Results were used to computed according to CLSI EP14-A3 with ordinary regression linear (ORL). Hemoglobin A_{1C} in clinical blood samples were plot the means of triplicate of patient's samples with measurement procedure B results on the y-axis and measurement procedure A results on the x-axis can be used to perform an initial evaluation. To calculating Deming regress parameters and plot the 95% prediction interval (95% PI) based upon the patient samples. Then, plot the average of each measurement procedures' result (X_{pc}, Y_{pc}) on the same graph for each of processed samples. When X_{pc}, Y_{pc} results fall inside the PI limits, then that unique processed blood sample is considered to commutable; otherwise, it is non-commutable. The data visualization by distribution of mean is describe in **APPENDIX G and H**.

Study 6: Characterized HbA_{1C} in prepared materials according to Guide 35-Guidance for characterization and assessment of homogeneity and stability.

The commutability of a reference materials (RM) relates to the ability of the RM, characterized by one measurement procedure (usually a reference procedure) to act as a calibrator or quality control (QC) material for a second measurement or testing procedure applied to routine test materials. The characterization was evaluated based on commutability study. Uncertainty (u) was evaluation according to ISO Guide 35 (ISO Guide 35:2017).

6.1 Hemoglobin A_{1C} in processed blood materials were measured by six measurements

Blood samples

Processed blood materials: 1) NDM-1 was obtained from previous conditions (Wongsri P, 2017), 2) three blood materials from *in vitro* glycation; GLY-1, GLY-2 and GLY-3 (Duanginta W, 2023), 3) three unprocessed blood random from clinical blood sample; NBS-1, NBS-2, and NBS-3, and 4) two commercial QC: CQC-1, and CQC-2 were aliquot five hundred microliters in each vial and kept at 2-8^{o}C until experiments.

Method

All blood samples (NDM-1, GLY-1, GLY-2, GLY-3, NBS-1, NBS-2, NBS-3, CQC-1, and CQC-2) were transport to reference and clinical laboratories under 2-8^{o}C. Blood samples were measured for HbA_{1C} with six measurement methods (TI-RL1, EN-RL1, BA.HPLC-CL1, TI-CL2, BA-POCT1, and FI-POCT2) in triplicate.

Data analysis

The finding was analyte for commutability following CLSI EP14-A3:2014.

6.2 Characterization of HbA_{1C}

Data analysis

The percentage of HbA_{1C} in blood samples measured by each method were calculated for the mean and SD (mean±SD). The unweighted mean and uncertainty (unweighted mean±U) were calculated following ISO Guide 35 based on commutability. The analysis of variance for uncertainty evaluation is describe in **APPENDIX I**.

Study 7: To determine HbA_{1C} by advance technique by Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS)

7.1 MALDI-TOF MS conditions and blood sample preparation

7.1.1 Development of MALDI-TOF mass spectrometry conditions

Mass spectra were applied acquired with a MALDI mass spectrometer set in a linear, positive mode, mass range 5,000-20,000, manual positioning spots, 50 shot accumulate, laser replication rate with 200 Hz, 1500 pulsed extract/focus mass, 6,000 blank mass, no subtract baseline, 100 laser energy 15 K voltage, 2 KHz laser frequency, 1500 pulsed extract/focus mass, Gaussian smoothing method, 20 smoothing filter widths, 200 filter width, and 200 peak widths.

7.1.2 Calibrations

Six level of Lyphochek hemoglobin A_{1C} linearity set was purchased from Bio-Rad (Irvine, CA, USA). This blood based HbA_{1C} reference standards vary systematically in their $\%HbA_{1C}$. Refill each vial with 0.5 mL of deionized water. Replace the stopper and leave the control to stand for 5 to 10 minutes. To ensure homogeneity, gently swirl the vial several times before sampling, and kept in a refrigerator until experiment.

7.1.3 Measurement sample and preparation

Six level of standard Lyphochek hemoglobin A_{1C} linearity set, three level of Liquicheck diabetes control was purchased from Bio-Rad (Irvine, CA, USA). This blood based HbA_{1C} was diluted with in DI water. One hundred clinical blood sample were collected in K_3EDTA tubes in 3.5 ml. The MALDI-TOF MS specimens were chosen to span the range of percent HbA_{1C} found in the blood of normal, treated, and unmanaged diabetic patients. Whole blood samples spun down, and erythrocytes were diluted in DI water. Prior to MALDI-TOF MS the samples were analyzed by a clinically validated by boronate affinity-High Performance Liquid Chromatography method (Boronate-HPLC). All blood samples mixed with 1:1 with 10 mg/mL sinapinic acid (30% CH_3CN, 0.1% TFA) until homogenize. Twenty microliters of this matrix mixture were spotted in 4×analytical replication onto a disposable stainless steel MALDI target (4 × 12 sample array). Matrix plates were dried in an incubator and run in 5 replications for glycated α-hemoglobin (G-αH) and glycated β-hemoglobin (G-βH).

7.1.4 Data analysis

The area of glycated α-hemoglobin (G-αH) and glycated β-hemoglobin (G-βH) were converse to $\%HbA_{1C}$ with linear equation of standard Lyphocheck 6 levels.

7.2 Prospective HbA_{1C} measurement in clinical blood samples with optimal conditions MALDI-TOF MS

Accuracy

Quality control and three clinical blood samples randomly from volunteers were used to evaluate accuracy %G-βHb from MALDI-TOF MS intra-plates along the %CVs calculated for intra-sample technical replicates (n = 5). Results were expressed in both NGSP (%) and IFCC units (mmol/mol).

7.2.1 Standard curve for glycated-βHb

Five spot-averaged spectra were calibrated using the standard masses αH, G-αHb, βHb, and G-βHb subunits was show m/z at 15127, 15289, 15868, and 16030, respectively (Stephen JH, 2016). respectively. Estimates of the quantity of G-αHb and G-βHb by generated are presented as a ratio of the percent of each chain using the following formula: glycated-αHb = 100 * glycated-αHb/(αHb + glycated-αHb) and glycated-βHb =100 *glycated-βHb/(βHb + glycated-βHb), respectively.

7.2.2 Accuracy and imprecision of MALDI-TOF MS

Accuracy was evaluated by analyzing external quality control samples and comparison against the boronate-HPLC. Biases between HbA_{1C} results obtained with MALDI-TOF-MS and target values were calculated.

Three samples with low (<6.4%), medium (6.4-9.0%), and high HbA_{1C} levels (>9.0%) were used to evaluate precision of the method. For intra-assay imprecision, samples were run in 20 technical replications on a target plate. The three native blood samples with different HbA_{1C} levels were tested 20 runs within plate. Results were expressed in both NGSP (%) and IFCC units (mmol/mol).

7.2.3 Clinical sample comparisons

One hundred clinical sample results were computed for paired t-test to compare means between %G-βH from MALDI-TOF MS and $\%HbA_{1C}$ from boronate-HPLC at $p < 0.05$, construct a regression model that assumes a linear relationship between results of two methods, and show mean difference between two methods were evaluate

Chapter 4

Results

Study 1: To determine influence parameters and conditions effect to quality of human red blood cell in CPDA-1 from blood bank.

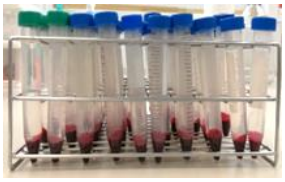
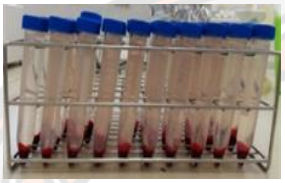
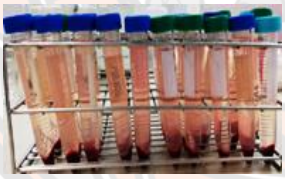
1.1 Determination quality of human red blood cells and plasma

The whole blood (WB) used to create whole blood material was donor blood that was required in study 1. The obtaining WB from a blood bank in order to prepare materials using blood samples that had non-expired as a control and had expired less than 1, <2, and <3 months were studied. To assessing cell quality, the color of the blood sample should be bright red, not dark, black, or green, clear of clots, no air bubbles, and there are no cell rupture or hemolysis because hemolysis would deform the cells. As a result, the glycation process can be affects.

1.2 To study the characteristics of whole blood before using *in vitro* glycation

Whole blood that passed the test in study 1.1 were suspended in 0.85% NS until the upper phase was clear and RBCs were collected. The results of suspending RBCs in 0.85% NS were show in Table 18. The results show age of RBCs in non-expired WB was 28.6±2.3 days, while expired WB <1, <2, and <3 months was 45.8±9.3, 66.8±7.0, and 106.6±6.7 days, respectively. Non-expired WB show washing times 1.4±0.5 days, while expired WB show washing times 3.0±0.5, 4.0±0.4, and 7.0±0.5 times, respectively. The yielding RBCs of non-expired WB was 1.0±0.1, while expired WB with 1, 2, and 3 months was yielded 0.7±0.1, 0.5 ±0.1, and 0.3±0.1 mL, respectively. Whole blood samples expired less than two months have a clear color, get high percentage yield of RBCs, and washing less three times. When a WB is expired more than three months displays black color of cells with hemolysis, and washed for 7 times because cells were damaged prior to the reaction, this reaction may be effect on *in vitro* glycation. So, this study concludes that, WB expired less than 2 months was used for future experiment.

Table 18. The washing times and yield RBCs remaining after centrifugation of non-expired and expired whole blood sample (n=20).

Expired (days)	Color of RBCs	Characteristics of RBCs (Mean ± SD)		
		Age (days)	Washing time (times)	Yield (mL)
Non expired (Control) (n=5)		28.6 ± 2.3	1.4 ± 0.5	1.0 ± 0.1
Expired < 1 month (n=5)		45.8 ± 9.3	3.0 ± 0.5	0.7 ± 0.1
Expired < 2 months (n=5)		66.8 ± 7.0	4.0 ± 0.4	0.5 ± 0.1
Expired < 3 months (n=5)		106.6 ± 6.7	7.0 ± 0.5	0.3 ± 0.1

Expired as of the expiration date (not including of 35 days after drawback from volunteers)

Study 2: To investigate the optimal conditions for *in vitro* glycation among glucose, and hemoglobin (intracellular glycation)

Whole blood (WB) was centrifuged, and pellet was washed three times with Normal saline (0.85% NS). Then, RBCs and WB were used as a substrate. Moreover, incubation times, various concentration of D-glucose, and inhibited solution were investigated. A solution of sodium azide (0.02%) was added before incubation at 37 ^{o}C, pH 7.4. The optimal conditions shown many advantages more than publications such as high yield production, short time for glycated, low cost and non-hemolysis can be capable with many principles. There are divided to 5 factors in these studies include substrate for initiation, incubation times, concentrations of D-glucose, inhibited solution to stop reaction, and storage condition of processed blood materials for HbA_{1C} testing.

2.1 Substrate for *in vitro* glycation

Red blood cells were washed with 0.85% NS and whole blood in CPDA-1 bag were used as a substrate. Whole blood or RBCs was incubated with 100 mM final concentration of D-glucose at 37^{o}C for 5 days. Hemoglobin A_{1C} was estimate for each day by boronate affinity chromatography.

At 100 mM final concentration of D-glucose of whole blood materials and RBCs were significant difference of HbA_{1C} after incubation for 5 days (Table 19). Whole blood incubated with 100 mM of D-glucose at 0, 1, 2, 3, 4 and 5 day was 4.2±0.07, 6.7±0.07*, 8.7±0.14*,12.2±0.21*, 13.7±0.21* and 14*%, respectively. Red blood cells incubated with 100 mM of D-glucose show HbA_{1C} was 4.2±0.07, 7.2±0.07*, 8.4±0.07*, 12.3±0.14*, 13.9±0.07*and 14*%, respectively. Hemoglobin A_{1C} in processed blood materials from *in vitro* glycation at 37^{o}C for 5 days is show in **Figure 19**.

Table 19. Hemoglobin A1C in processed blood material after in vitro glycation was measured by boronate affinity chromatography.

Days	Mean ± SD (n=5) Whole blood materials		Mean ± SD (n=5) Red blood cells	
	Control	100 mM of D-Glucose	Control	100 mM of D-Glucose
0	4.2±0.07	4.2±0.07	4.2±0.07	4.2±0.07
1	4.2±0.00	6.7±0.07*	4.1±0.00	7.2±0.07*
2	4.3±0.07	8.7±0.14*	4.1±0.00	8.4±0.07*
3	4.4±0.07	12.2±0.21*	4.1±0.07	12.3±0.14*
4	4.4±0.00	13.7±0.21*	4.1±0.07	13.9±0.07*
5	4.5±0.00	14.0±0.00*	4.2±0.00	14.0±00*

*Significant difference at *p*-value < 0.05 when compared with base line (day 0) by paired t-test

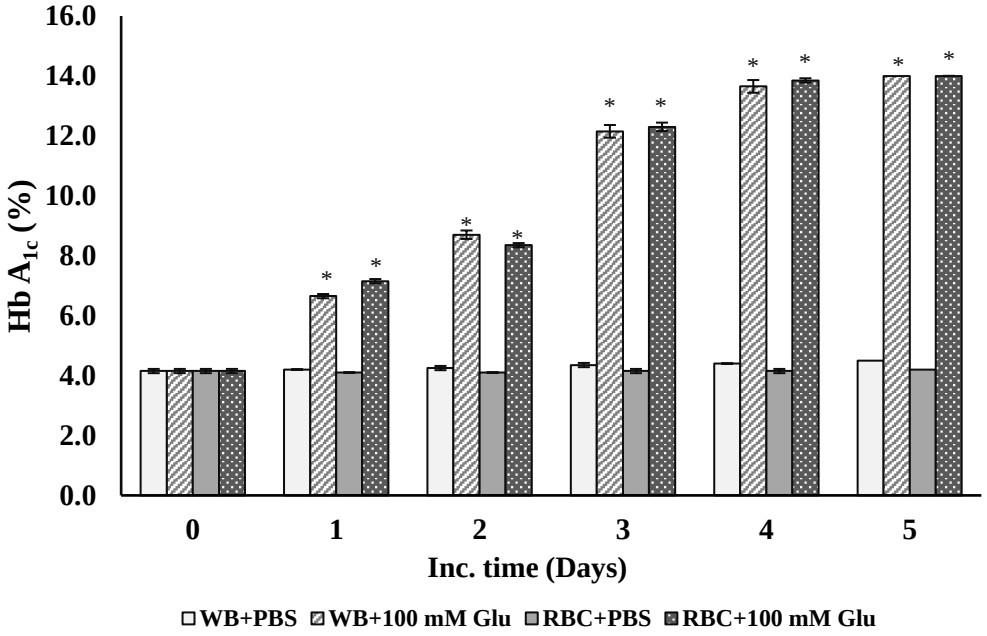


Figure 19. Hemoglobin A1c in processed blood materials after 5 days

Table 20 present hematocrit after incubate WB and 100 mM of D-glucose at 0, 1, 2, 3, and 4 day was 61±1.41, 51±0.71*, 31±1.41* and 15±0.71*%, respectively. Hematocrit was 52±0.71, 37±1.41*, and 13±0.71*%, respectively. At day 4 and day 5,

red cells was hemolysis 100%. Hematocrit in processed blood materials from *in vitro* glycation at 37ºC for 5 days is show in **Figure 20**.

Table 20. Hematocrit in processed blood material after *in vitro* glycation was measured by centrifugation.

Days	Mean ± SD (n=5) Whole blood materials		Mean ± SD (n=5) Red blood cells	
	Control	100 mM Glu	Control	100 mM Glu
0	61±1.41	61±1.41	52±0.71	52±0.71
1	47±1.41	51±0.71*	50±0.71	37±1.41*
2	31±0.71*	31±1.41*	47±0.71	13±0.71*
3	11±1.41*	15±0.71*	45±141*	0
4	0	0	45±0.71*	0
5	0	0	40±0.00*	0

*Significant difference at p value < 0.05 when compared with base line (day 0) by paired t-test

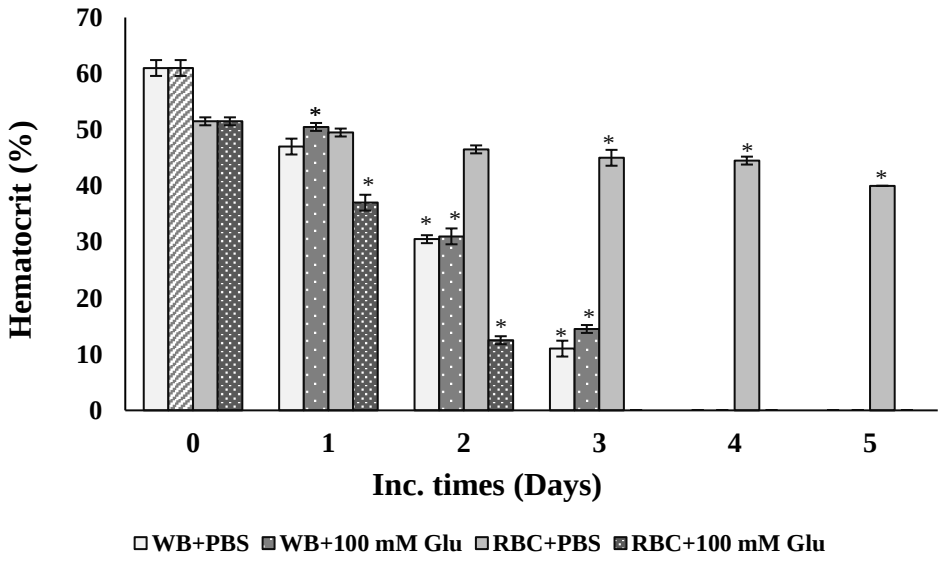


Figure 20. Hematocrit in processed blood materials after 5 days incubation

Five day of glycation study revealed that hemolysis was increased in all four conditions. The Hct was reduced significantly at $p < 0.05$. As a result, the incubation period of the WB sample influences red blood cell hemolysis. The incubation time of the samples must be investigated in the following step. There was an increase in HbA_{1C} in the sample in order for RBCs to remain intact without hemolysis. To reduce production costs and production time any more than possible. As a result, using either

whole blood or red blood cell as a substrate to produce HbA_{1C} results that differed from controls.

2.2 Temperature for *in vitro* glycation

Incubation temperature at 37ºC or 2-8°C has an effect to $\%HbA_{1C}$ and cell hemolysis when WB or RBCs was incubated with and without D-glucose at 37°C or 2-8°C. The results were show in **Table 21, Table 22, Figure 21, Figure 22, Figure 23** and **Figure 24**.

Table 21. The effect of temperature on the $\%HbA_{1C}$ from *in vitro* glycation of WB or RBCs with D-Glucose.

Temperature (ºC)	Days	Mean ± SD Whole blood materials		Mean ± SD Red blood cells	
		Control	100 mMGlu	Control	100 mMGlu
37	0	4.2±0.07	4.2±0.07	4.2±0.07	4.2±0.07
	1	4.1±0.00	6.6±0.21*	4.1±0.00	7.4±0.28*
	2	4.2±0.00	8.7±0.21*	4.1±0.00	8.7±0.35*
	3	4.2±0.21	11.9±0.28*	4.2±0.07	12.6±0.28*
	4	4.3±0.14	13.3±0.57*	4.2±0.00	13.8±0.21*
	5	4.4±0.14	14.0±0.00*	4.2±0.00	14.0±0.00*
2-8	0	4.1±0.14	4.3±0.07	4.2±0.00	4.2±0.07
	1	4.2±0.07	4.4±0.00	4.1±0.14	4.1±0.00
	2	4.2±0.07	4.4±0.00	4.3±0.07	4.1±0.14
	3	4.3±0.07	4.4±0.07	4.1±0.07	4.2±0.00
	4	4.3±0.07	4.5±0.14	4.2±0.07	4.4±0.07
	5	4.4±0.07	4.5±0.14	4.1±0.07	4.5±0.07

*Significant difference at p value < 0.05 when compared with base line (day 0) by paired t-test

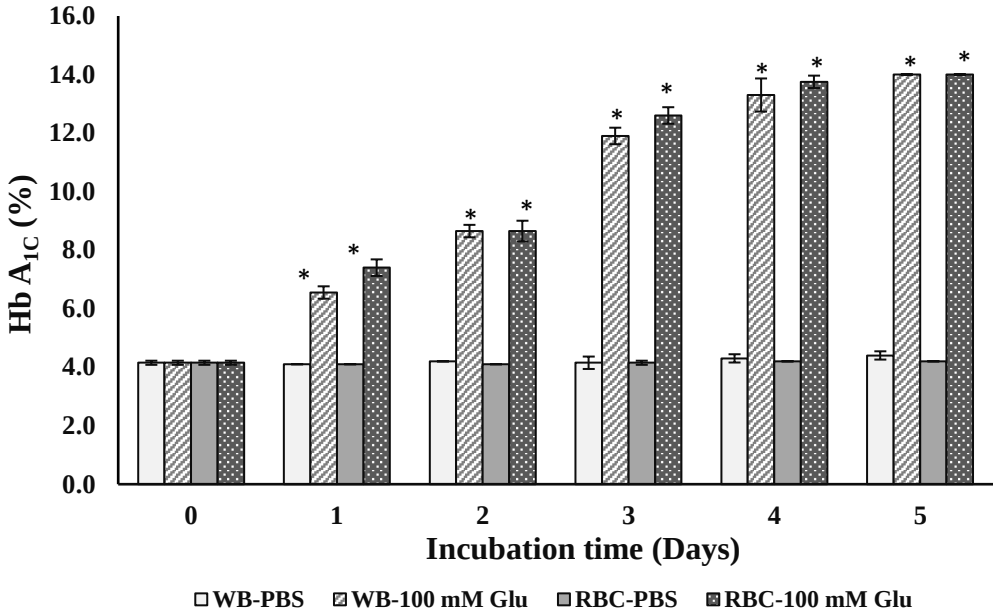


Figure 21. Hemoglobin A_{1C} after incubation WB or RBCs with and without D-Glucose at 37^{O}C for 5 days

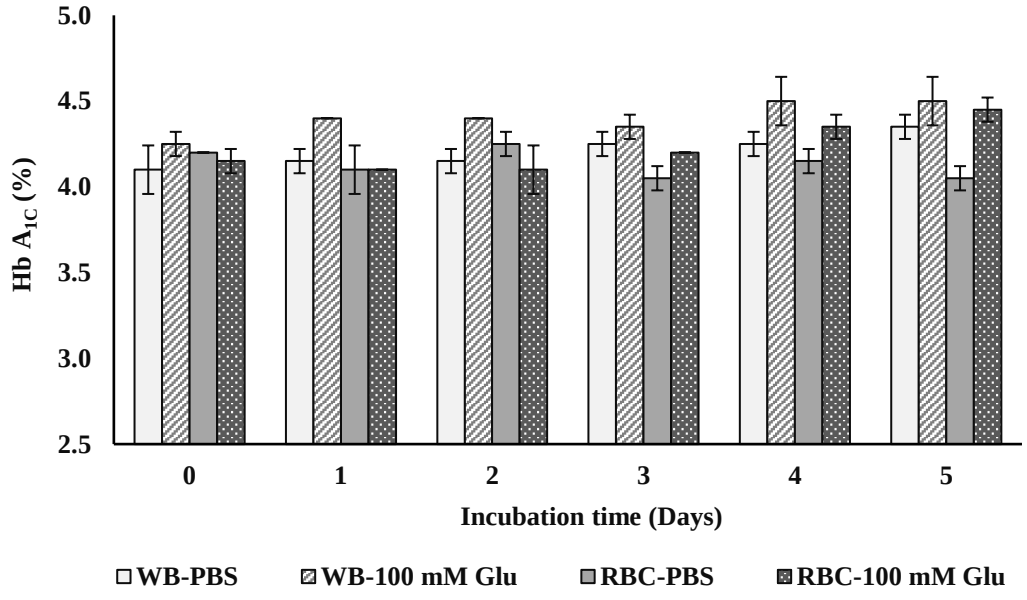


Figure 22. Hemoglobin A_{1C} after incubate WB or RBCs with and without D-Glucose at 2-8 ^{O}C for 5 days

Table 22. The effect of temperature on the %Hct from *in vitro* glycation of WB or RBCs with D-Glucose.

Temperature (°C)	Days	Mean ± SD (n=5) Whole blood materials		Mean ± SD (n=5) Red blood cells	
		Control	100 mM Glu	Control	100 mM Glu
37	0	61±071	61±0.71	53±0.71	52±0.00
	1	48±0.71*	45±0.41*	41±0.71*	31±1.41*
	2	27±0.71*	21±1.41*	24±2.12*	18±0.71*
	3	0	0	0	0
	4	0	0	0	0
	5	0	0	0	0
2-8	0	61±0.71	61±1.41	53±0.71	53±0.71
	1	57±0.71	57±1.41	51±0.71	51±0.71
	2	52±0.00*	56±0.71	50±0.71	51±1.41
	3	48±0.71*	55±0.71	46±1.41*	47±1.41
	4	47±0.71*	53±0.00*	41±1.41*	46±0.71*
	5	45±0.71*	50±0.00*	39±2.12*	40±0.71*

*Significant difference at p value < 0.05 when compared with base line (day 0) by paired t-test

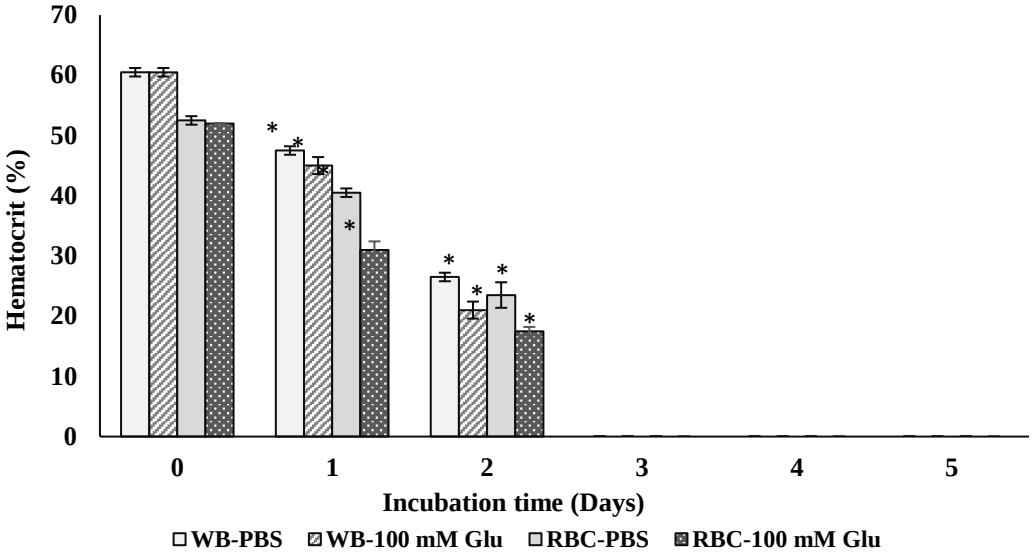


Figure 23. Hematocrit after incubate WB or RBCs with and without D-Glucose at 37 ^{O}C for 5 days

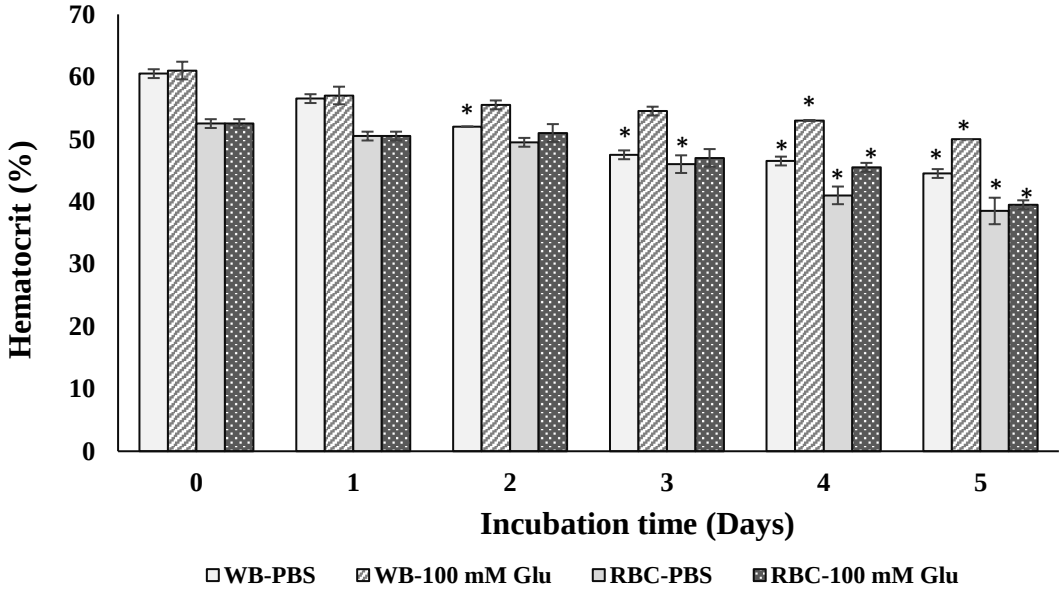


Figure 24. Hematocrit after incubate WB or RBCs with and without D-Glucose at 2-8 ^{O}C for 5 days

Hemoglobin A_{1C} increased significantly after 5 days of incubation at 37 ^{o}C with WB and 100 mM D-glucose was 4.2±0.07, 6.6±0.21*, 8.7±0.21*, 11.9±0.28*, 13.3±0.57* and 14.0±0.00*%, and decreasing of Hct was 61±0.71, 45±0.41* and 21±1.41*%, respectively. Hemoglobin A_{1C} increased significantly after 5 days of incubation at 37 ^{o}C with RBCs and 100 mM D-glucose was 4.2±0.07, 7.4±0.28*, 8.7±0.35*, 12.6±0.28*, 13.8±0.21*, and 14.0±0.00*% and show decreasing of Hct was

52±0.71, 31±1.41* and 18±0.71*%, respectively. Hematocrit cannot be measured on days 4 and 5 because the cells were hemolyzed completely.

At 2-8 ^{o}C in a refrigerator of incubation between WB and 100 mM of D-glucose for 5 days was increasing significant difference of HbA_{1C} 4.3±0.07, 4.4±0.00, 4.4±0.00, 4.4±0.07, 4.5±0.14 and 4.5±0.14 %, respectively. Hematocrit was decreasing significant difference was 61±0.71, 57±1.41, 56±0.71, 55±0.71, 53±0.00* and 50±0.00*%, respectively. When incubated RBCs and 100 mM of D-glucose for 5 days was increasing significant difference of was found HbA_{1C} 4.2±0.07, 4.1±0.00, 4.1±0.14, 4.2±0.00, 4.4±0.07 and 4.5±0.07%, and Hct was decreasing significant difference 53±0.71, 51±0.71, 51±1.41, 47±01.41, 46±0.71* and 40±0.71*%, respectively.

Hemoglobin A_{1C} was higher than in the WB group after 24 hours incubation. After 5 days of incubation, Hct was reduced, and hemolysis occurred. Incubation at 2-8°C, the HbA_{1C} level did not increase, and no glycation occurred. Hematocrit was not statistically significant at 0.05. Glycation was suitable when RBCs were incubated with 100 mM glucose at 37°C. The issue using WB in glycation with 1) the default Hct cannot be adjusted in the same condition at the start of the reaction and 2) WB pack in CPDA-1 bag that may influence glycation reaction.

2.3 Incubation times

Whole blood from the Blood Bank was centrifuged and RBCs were collected after screening quality of RBCs. Red blood cells were incubated with 400 mM D-Glucose for 5 days at 37oC at 0, 1, 2, 3, 4, and 5 days, HbA1C and Hct levels were measured for 5 times measurement. The data was shown in Table 23.

Hemoglobin A1C (Table 23) was produced from incubated between WB and D-Glucose at 0, 1, 2, 3, 4, and 5 days was 4.1±0.14, 6.9±0.21*, 8.5±0.42*, 11.8±0.28*, 13.7±0.35* and 14.0*%, respectively. Hemoglobin A1C was increasing significant difference at p-value < 0.05 at the third day of incubation. Incubation between RBCs and D-Glucose increasing of HbA1C 0, 1, 2, 3, 4, and 5 days was 4.1±0.14, 7.1±0.14*, 8.2±0.28*, 12.2±0.28*, 13.5±0.64*, and 14.0*%, respectively. Hemoglobin A1C was increasing significant difference (p-value < 0.05) at first day of incubation. Incubation between PBS and WB or incubated with RBCs there was no significant difference (p-value > 0.05).

Hematocrit (Table 24) was measured after 5 days of incubation between WB and 100 mM of D-Glucose at 0, 1, 2, and 3 days was 53±0.70, 44±2.83, 31±0.71* and 14±0.00*%, Hct was decreasing significantly (p-value < 0.05). Incubation between RBCs and D-Glucose, Hct was decreasing significantly (p-value < 0.05) was 53±0.71, 35±0.71* and 12±0.71*%, respectively. At day 4 and 5 were hemolysis 100%, Hct was not appear.

Table 23. The percentage of HbA_{1c} after incubation for 5 days

Days	mean ± SD (n=5) Whole blood materials		mean ± SD (n=5) Red blood cells	
	Control	100 mM Glucose	Control	100 mM Glucose
0	4.1±0.14	4.1±0.14	4.1±0.14	4.1±0.14
1	4.2±0.00	6.9±0.21*	4.4±0.07	7.1±0.14*
2	4.3±0.00	8.5±0.42*	4.4±0.00	8.2±0.28*
3	4.3±0.07	11.8±0.28*	4.4±0.14	12.2±0.28*
4	4.3±0.00	13.7±0.35*	4.6±0.00	13.5±0.64*
5	4.5±0.07	14.0±0.00*	4.6±0.07	14.0±0.00*

*Significant difference at p-value < 0.05 when compared with base line (day 0) by paired t-test

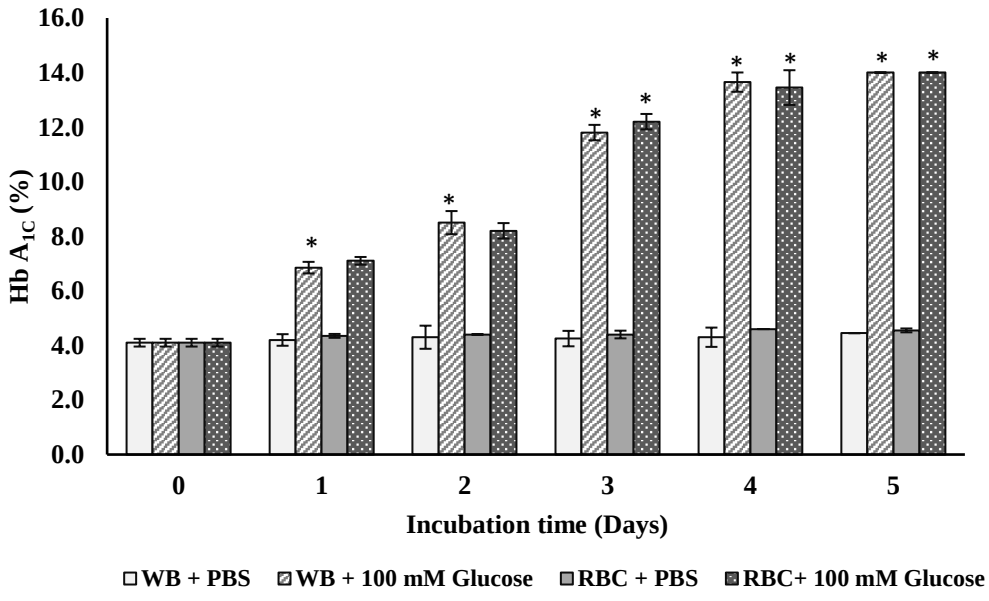


Figure 25. Hemoglobin A_{1C} after incubate WB or RBCs with and without D-Glucose at 37 ^{O}C for 5 days

Table 24. The percentage of Hct after incubation for 5 days

Days	Mean ± SD (n=5) Whole blood materials		Mean ± SD (n=5) Red blood cells	
	Control	100 mM Glucose	Control	100 mM Glucose
0	53±0.71	53±0.71	53±0.71	53±0.71
1	44±0.71	44±2.83	48±0.71	35±0.71*
2	31±0.71*	31±0.71*	47±0.71	12±0.71*
3	11±1.41*	14±0.0*	45±0.71*	0
4	0	0	43±0.71*	0
5	0	0	36±1.41*	0

*Significant difference at *p*-value < 0.05 when compared with base line (day 0) by paired t-test

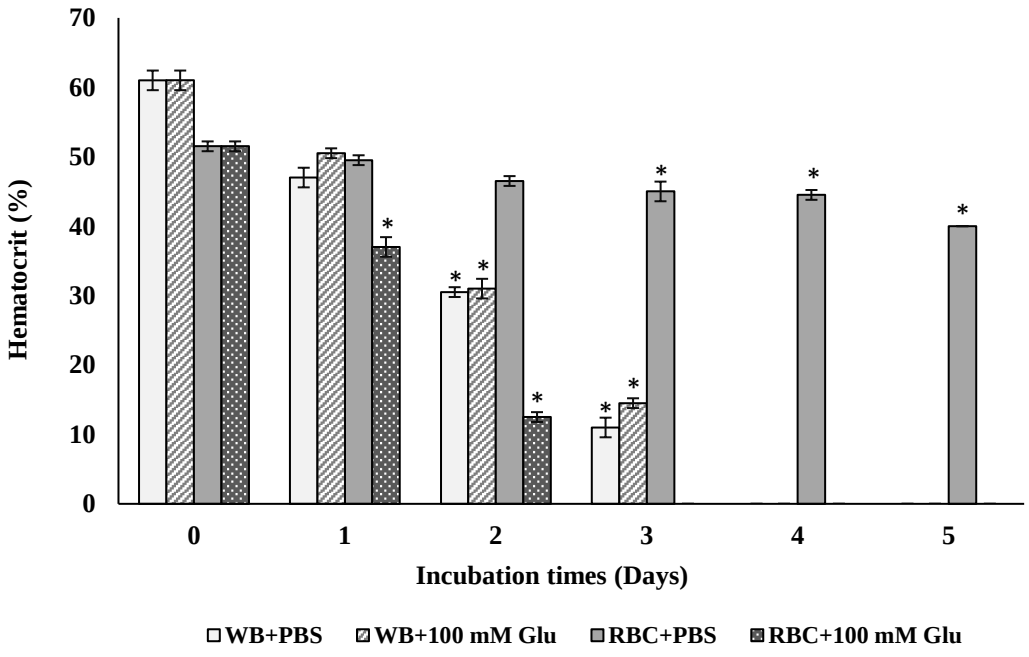


Figure 26. Hematocrit after incubate WB or RBCs wit and without D-Glucose at 37 ^{O}C for 5 days

Red blood cells incubated with 100 mM of D-Glucose increased significant difference at first day at *p*-value < 0.05, whereas WB increased significant difference at the third day of incubation times. A day of incubation, RBCs were increasing significant difference HbA_{1C} from baseline and Hct levels are close to native blood samples (38-42%). The glycation process should be rapid, save on harvesting, and red cells should behave similarly to native or clinical samples, with no hemolysis. As a result, using RBCs as a precursor in the glycation process could be a viable option. Then, RBCs were incubated with 400 mM D-Glucose at 37^{o}C for 120 hours to skip the incubation time.

Erythrocytes were suspended in 0.85% NS and incubated for 120 hours at 37 °C with 400 mM of D-glucose. The percentage of HbA_{1C} increased significantly and the Hct value decreased significantly (P<0.001) when the incubation time was extended. After incubating erythrocytes with D-glucose for 120 hours at 37 ^{o}C, maximum HbA_{1C} and 38-42% Hct were achieved at incubation time of 15 hours (**Table 25**). **Figure 27** demonstrates the changes in HbA_{1C} and Hct.

Table 25. The percentage of HbA_{1c} and Hct after incubation red blood cells with 400 mM of D-glucose for 120 hours

Inc. Times (hr.)	HbA_{1C} (%)					Mean± SD	Hematocrit (%)					Mean± SD
	#1	#2	#3	#4	#5		#1	#2	#3	#4	#5	
0	4.5	4.5	4.5	4.5	4.5	4.5± 0.59	53	53	54	53	54	53±1.49
6	4.8	4.7	4.7	4.8	4.8	4.8±0.70	50	50	51	51	50	50±3.58
9	7.2	6.9	6.9	6.5	7.4	7.0±0.25*	48	48	47	46	48	47±4.20*
15	7.5	7.6	7.4	7.5	7.8	7.6±0.49*	40	40	41	41	40	40±6.11*
24	8.0	7.9	8.0	7.9	7.9	7.9±0.74*	30	32	32	30	32	31±4.12*
48	8.5	8.7	8.2	8.3	8.7	8.5±0.90*	12	11	11	12	11	11±2.31*
72	11.8	11.5	12.0	11.3	11.6	11.6±0.33*	0	0	0	0	0	0±0.00*
120	14.0	14.0	14.0	13.8	13.8	13.9±0.14*	0	0	0	0	0	0±0.00*

* Significant difference at *p-value* at 0.001 when compared with a base line (0 hour) by paired t-test.
The standard deviation was calculated using three batches of experiments from different WB donors.

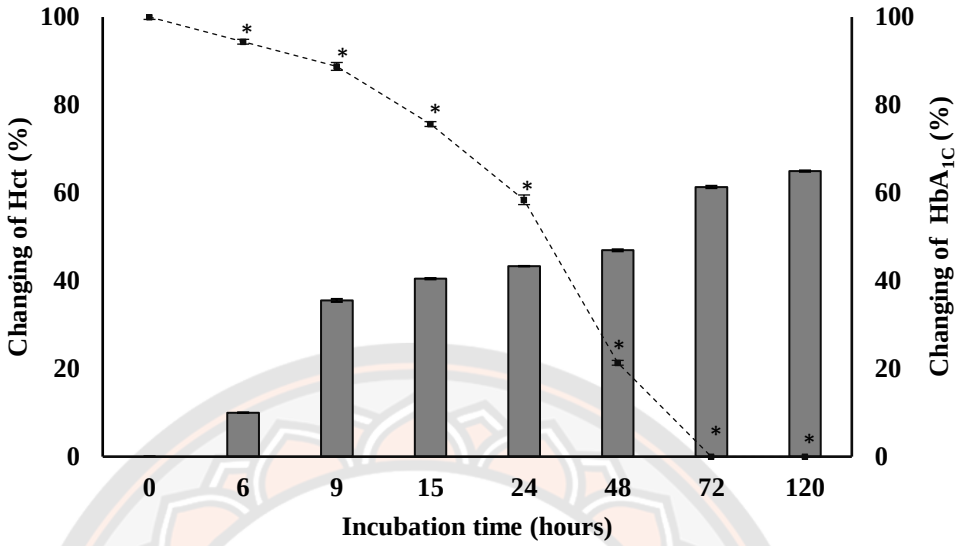


Figure 27. The increasing of %HbA1C and decreasing of %Hct during incubation for 120 hours

The optimum condition for *in vitro* glycation were using RBCs as a precursor and incubate for 15 hours, which resulted in an increase in HbA_{1c} and a decrease in Hct. Red cells were intact after 15 hours of incubation, same as clinical samples (38-42%). The effect of D-glucose concentration on *in vitro* glycation was investigated in study 2.4.

2.4 Glucose concentration

Glycation is influenced by the concentration of glucose. Glucose was used at concentrations of 0, 50, 100, 125, 150, 200, 300, 400 and 500 mM in PBS (PBS as a control). The results were shown in **Table 26**.

Table 26. The effects of glucose concentration for glycated hemoglobin

Batch	Time (hr.)	%HbA_{1C} (Mean ± SD)								
		PBS	50 mM	100 mM	150 mM	200 mM	250 mM	300 mM	400 mM	500 mM
1	0	6.1±0.38	6.2±0.16	6.1±0.38	6.2±0.09	6.2±0.09	6.4±0.24	6.2±0.16	6.1±0.11	6.4±0.08
	15	6.1±0.26	6.2±0.10	8.0±0.10*	8.6±0.10*	9.7±0.10*	9.6±0.10*	11.3±0.10*	11.4±0.10*	10.4±0.18
2	0	5.7±0.26	5.6±0.32	5.8±0.00	5.7±0.18	5.7±0.18	5.6±0.22	5.7±0.18	5.7±0.18	5.7±0.00
	15	5.7±0.31	5.8±0.32	7.5±0.00*	8.0±0.23*	8.3±0.36*	8.5±0.33*	9.9±0.58*	10.3±0.60*	9.5±0.19*
3	0	5.5±0.24	5.6±0.19	5.6±0.41	5.7±0.09	5.8±0.18	5.6±0.23	5.6±0.21	5.7±0.08	5.5±0.08
	15	5.7±0.29	5.6±0.25	7.7±0.21*	8.0±0.00*	8.5±0.10*	8.8±0.10*	9.3±0.20*	10.1±0.10*	9.6±0.16*

*Significant difference of %HbA_{1C} at *p-value* < 0.001 when compared various of D-glucose with PBS by paired t-test.
Red blood cells for *in vitro* glycation batch-1, batch-2, and batch-3 were from different blood donor from blood bank.

Erythrocytes were suspended in 0.85% NSS and incubated RBCs with various of D-Glucose concentration 0 (PBS), 50, 100, 150, 200, 250, 300, 400, and 500 mM at 37 ^{o}C for 15 hours. Hemoglobin A_{1C} in processed blood materials after RBCs incubated with 0(PBS), 50, 100, 150, 200, 250, 300, 400, and 500 mM of D-glucose for batch-1 was 6.1±0.26, 6.2±0.10, 8.0±0.10*, 8.6±0.10*, 9.7±0.10*, 9.6±0.10*,11.3±0.10*, 11.4±0.10, and 10.4±0.18*%, respectively. Hemoglobin A_{1C} of batch-2 was 5.7±0.31, 5.8±0.32, 7.5±0.00*, 8.0±0.23*, 8.3±0.36*, 8.5±0.33*, 9.9±0.58*, 10.3±0.60, and 9.5±0.19*%, respectively. For batch-3 show HbA_{1C} was 5.7±0.29, 5.6±0.25, 7.7±0.21*, 8.0±0.00*, 8.5±0.10*, 8.8±0.10*, 9.3±0.20*, 10.1±0.10, and 9.6±0.16*%, respectively. Hemoglobin A_{1C} was significantly (p<0.001) higher than PBS (control group) and higher than base line (at 0 hour). The *in vitro* glycation techniques resulted increase more than 50% HbA_{1C} value from baseline when incubated with 400 mM D-glucose for 15 hours.

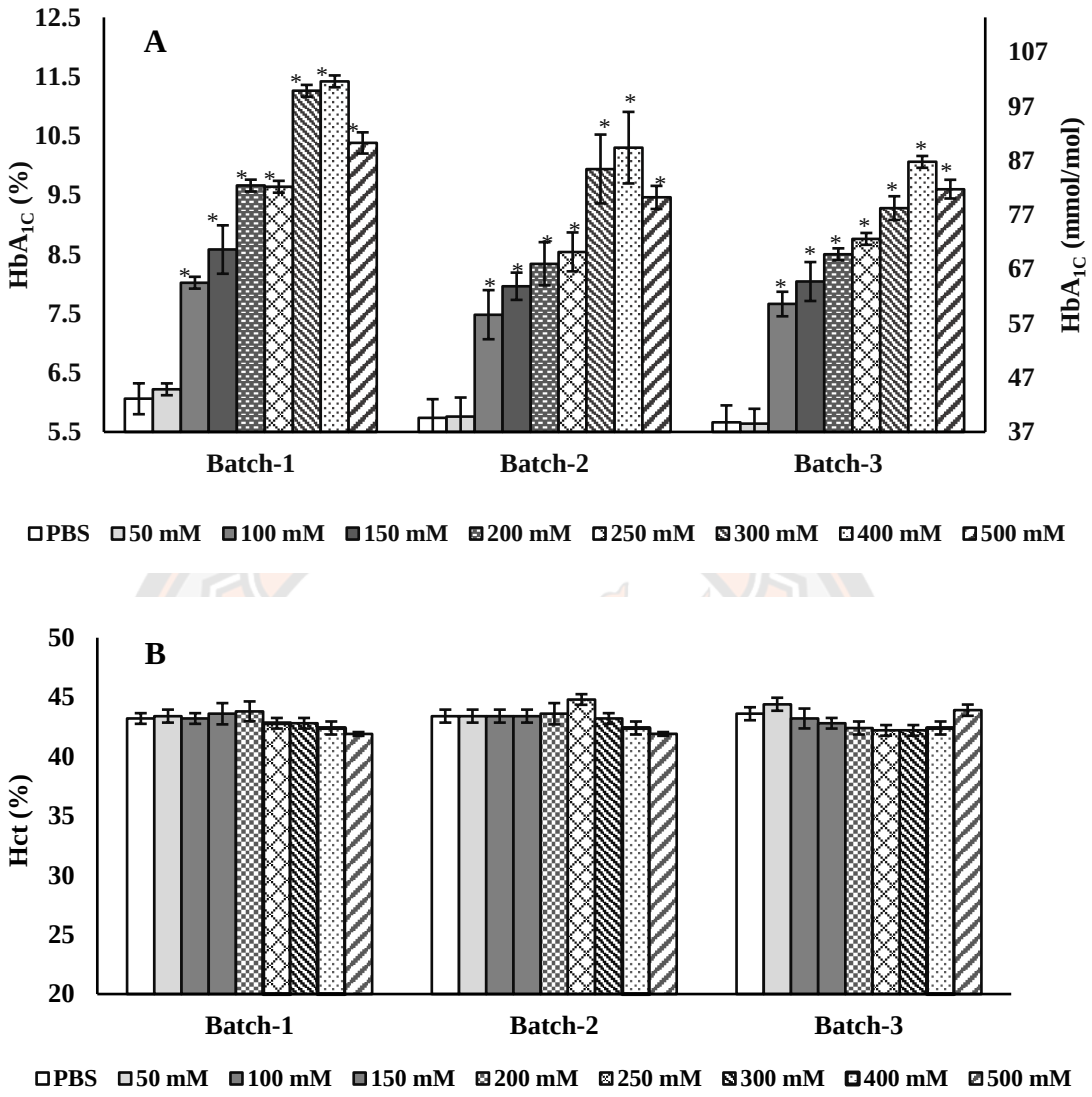


Figure 28. Glucose concentration was influence to level of HbA_{1C} (A) and Hct (B) after 15 hours incubation times

In vitro glycation is accelerated at optimal temperature, glucose concentration, suitable incubation time, and suitable substrate are present. As the reaction progressed and red blood cells ruptured, the inactivation process became key to maintaining hemoglobin A_{1C} levels and Hct stability throughout the measurement period. The study 2.5 was determining the inhibited solution to stop *in vitro* glycation reaction by using inhibited solution with low glucose concentrations.

2.5 Inhibited solution and storage conditions for HbA_{1C} from *in vitro* glycation

Whole blood donor in CPDA-1 bag was suspending in 0.85% NS until upper phase was clear. Erythrocytes was incubated with 400 mM of D-glucose at 37 ^{o}C for 15 hours and then this processed blood material was removed excess of D-glucose solution, aliquoted 0.5 mL into 100 vials, and stored at three different conditions 1) non-inhibited at 2-8^{o}C 2) suspended in inhibited solution-1, and 3) suspended in inhibited solution-2. Three processed blood material were measured for HbA_{1C} and Hct for 10 days. Stability was determined randomly with 5 vials for each day when $T_{critical}$ was less than $T_{calculate}$ using the LINEST statistic following ISO Guide 35.

Figure 29 represents HbA_{1C} production on a laboratory scale. Hemoglobin A_{1C} was elevated after incubating RBCs with 400 mM D-glucose for 15 hours at 37^{o}C during the reaction. The processed blood materials from *in vitro* glycation were aliquoted and stored at -20^{o}C, and it showed significant differences in HbA_{1C} and Hct with LINEST Guide 35 ($T_{calculation} > T_{critical}$). Some primary analyzers may be influenced by red blood cell hemolysis at -20^{o}C. The excess of D-glucose was removed from GLY materials and kept RBCs in inhibited solution -1, HbA_{1C} remained stable over the period of the experiment (10 days). On the other hand, processed blood materials in inhibited solution-2, HbA_{1C} was significantly different at day 6, and Hct was stable for the entire 10-day experiment. As a result, the elevated HbA_{1C} level following glycation should be stable for a period. The processed blood materials should be stored in the proper conditions with optimal glucose concentration in inhibited solution and kept at optimal temperature to maintain Hct like a human blood. Therefore, the optimal conditions for HbA_{1C} production by *in vitro* glycation were maintained by keeping processed blood materials in inhibited solution-1 at 2-8^{o}C.

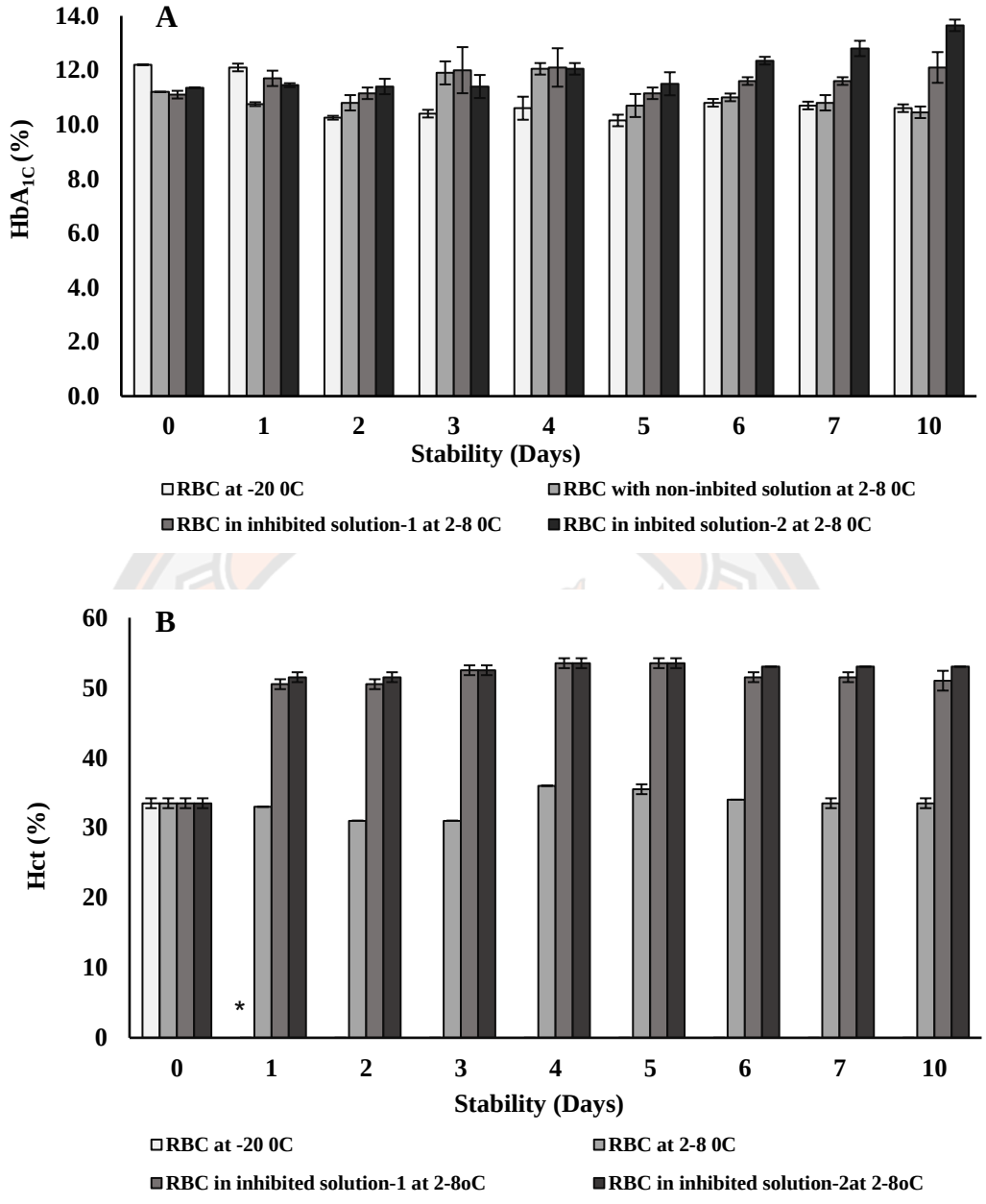


Figure 29. HbA_{1C} (A) and Hct (B) in processed blood materials kept in different inhibited solution on laboratory scale

In an industrial scale, processed blood materials were aliquot 0.5 mL in 100 vials and kept in different conditions at 2-8^{o}C including in inhibited solution-1, inhibited solution-2, and non-inhibited solution. Hemoglobin A_{1C} and Hct were measured for each day for 38 days with boronate affinity and centrifugation, respectively. The level of HbA_{1C} (A) and Hct (B) is show in **Figure 30**. The parenthesis represents IFCC unit in mmol/mol of HbA_{1C}.

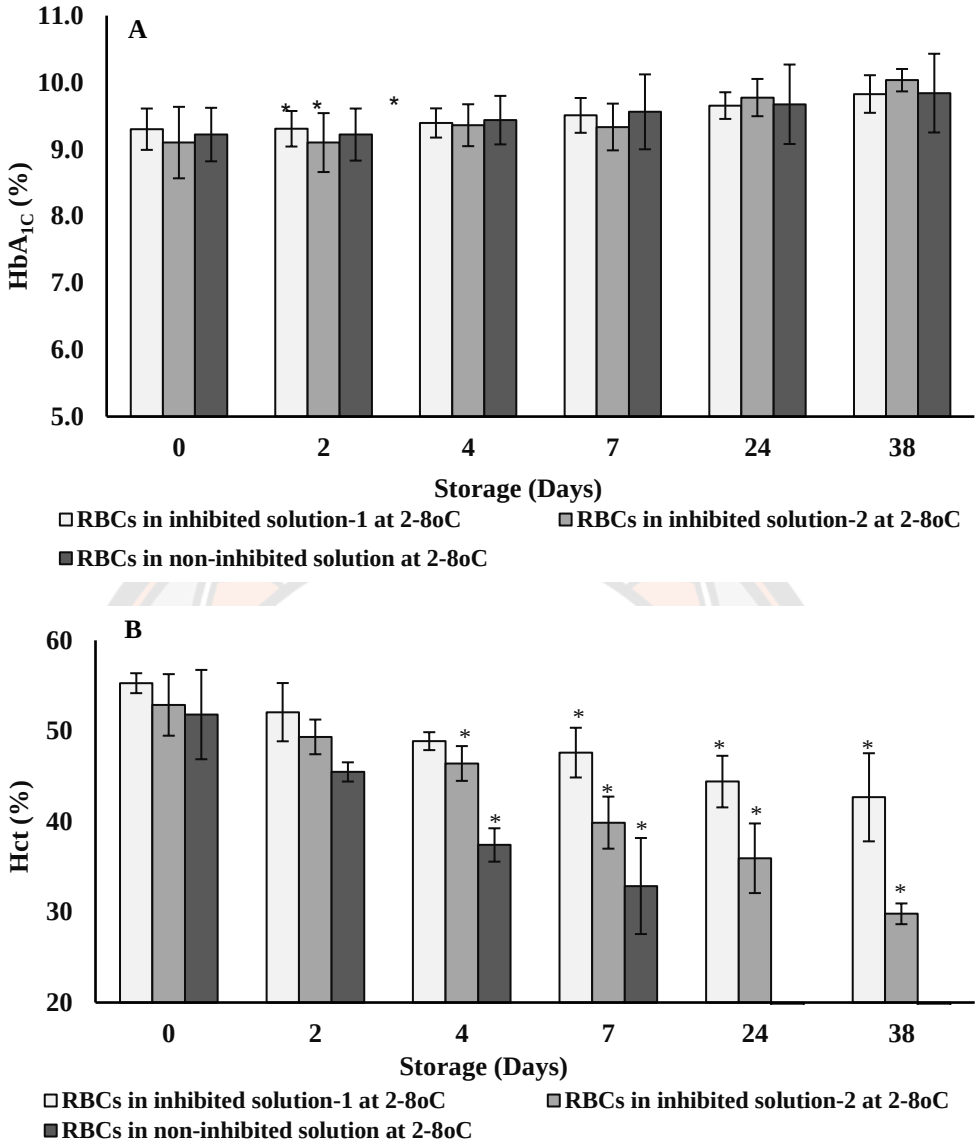


Figure 30. Hemoglobin A_{1C} (A) and Hct (B) in processed blood materials kept in different inhibited solution with different blood donors in scale up.

Hemoglobin A_{1C} in processed blood material kept in inhibited solution-1 at 0, 2, 4, 7, 24, and 38 day was 9.3±0.3(78), 9.3±0.3(78), 9.4±0.2(79)*, 9.5±0.3(80)*, 9.7±0.2(82)* and 9.8±0.3(84)*, respectively. Hematocrit was decreasing significantly was 55±1.1, 52±3.2, 49±1.0*, 48±2.7*, 44±2.8 and 43±4.9*, respectively. Hemoglobin A_{1C} in processed blood material kept in inhibited solution-2 at 0, 2, 4, 7, 24, and 38 day was 9.1±0.5(76), 9.1±0.3(76), 9.4±0.3(79)*, 9.3±0.3(79)*, 9.8±0.3(83)* and 10.0±0.2(86)*, respectively. Hematocrit was decreasing significantly was 53±3.4, 49±3.2*, 46±1.9*, 40±2.9*, 36±3.8* and 30±1.1*%, respectively. Hemoglobin A_{1C} in processed blood material kept in non-inhibited solution at 0, 2, 4, 7, 24, and 38 day was 9.2±0.4(77),9.2±0.4(77), 9.4±0.4(80)*, 9.6±0.6(81)*, 9.7±0.6(82)*, and 9.8±0.6(84)*,

respectively. Hematocrit was decreasing significantly was 52±4.9, 45±1.1*, 37±1.8* and 33±5.3*, respectively. At day 24 and 38, RBCs was hemolysis 100% lead to Hct was zero. Significant difference was determined by using Guide 35 ($T_{calculation} < T_{critical}$).

Processed blood materials kept in inhibited solution-1 remained HbA_{1C}, and Hct was approximately 38-42% as a human blood it was stable more than those kept in inhibited solution-2 or non-inhibited solution, and Hct was significantly decreased with Guide 35 statistics.

2.6 Hemoglobin typing of processed blood materials

Processed blood material (NDM-1) was prepared from pool of non-DM blood donor, then suspended in 0.85% NS, and erythrocytes were suspended in inhibited solution-1 with low glucose concentration and kept vials at 2-8^{o}C until experiments. Processed blood materials GLY-1 was prepared from *in vitro* glycation were sent to reference laboratory to determine Hb variants in processed blood materials. Capillary electrophoresis was used to check abnormal Hb variants in processed blood material. The NDM-1 show HbA 97.10% and A_2A 2.90% and GLY-1 show HbA 97.0% and A_2A 3.0% by capillary electrophoresis methods with reference laboratory that certified by ISO 15189:2012. The identified A_2A as the most common variant type in HbA_{1C} material during glycation procedures (**Figure 31**), these HbA_{1C} material had no effect on A_2A typing within erythrocytes and were unaffected by A_{1C} measurements.

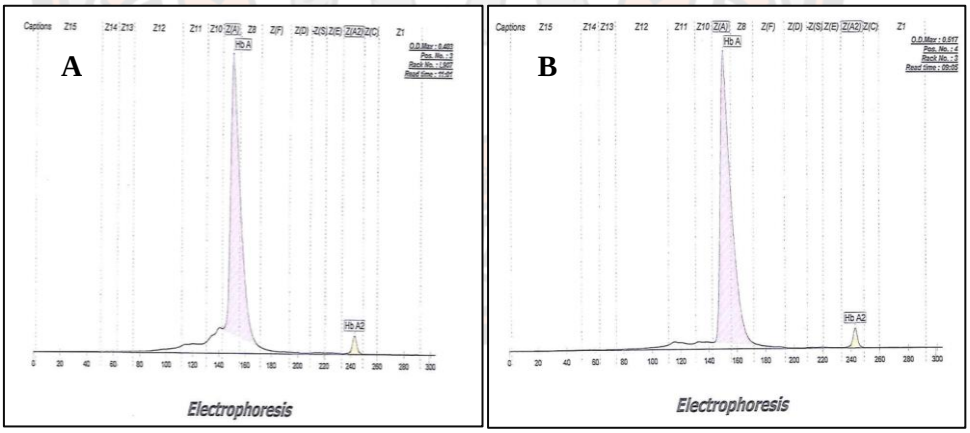


Figure 31. Hemoglobin typing of NDM-1 material (A) and GLY-1 material (B)

Study 3: To investigate the optimal conditions for *in vitro* between albumin and glucose (extracellular glycation)

3.1 Glucose concentrations

Plasma incubated with D-Glucose concentration at 0 (control), 50, 100, 150, 200, 250, and 300 mM at 37 ^{o}C for 48 hours. The level of Fructosamine, and albumin were shown in **Table 27**.

Table 27. Fructosamine and albumin levels after 48 hours of *in vitro* glycation with various glucose concentrations

Glucose concentration	**mean±SD**	
(mM)	Fructosamine (umol/L)	Albumin (g/dL)
Control	316±0.71	3.3±0.00
50	520±0.00*	2.9±0.00
100	625±2.83*	2.5±0.00
150	668±0.71*	2.3±0.00
200	823±0.00*	1.9±0.00
250	693±2.12*	1.4±0.00
300	675±0.00*	1.2±0.00

*Significant difference at $p<0.05$ by paired t-Test when compared with base line (control).

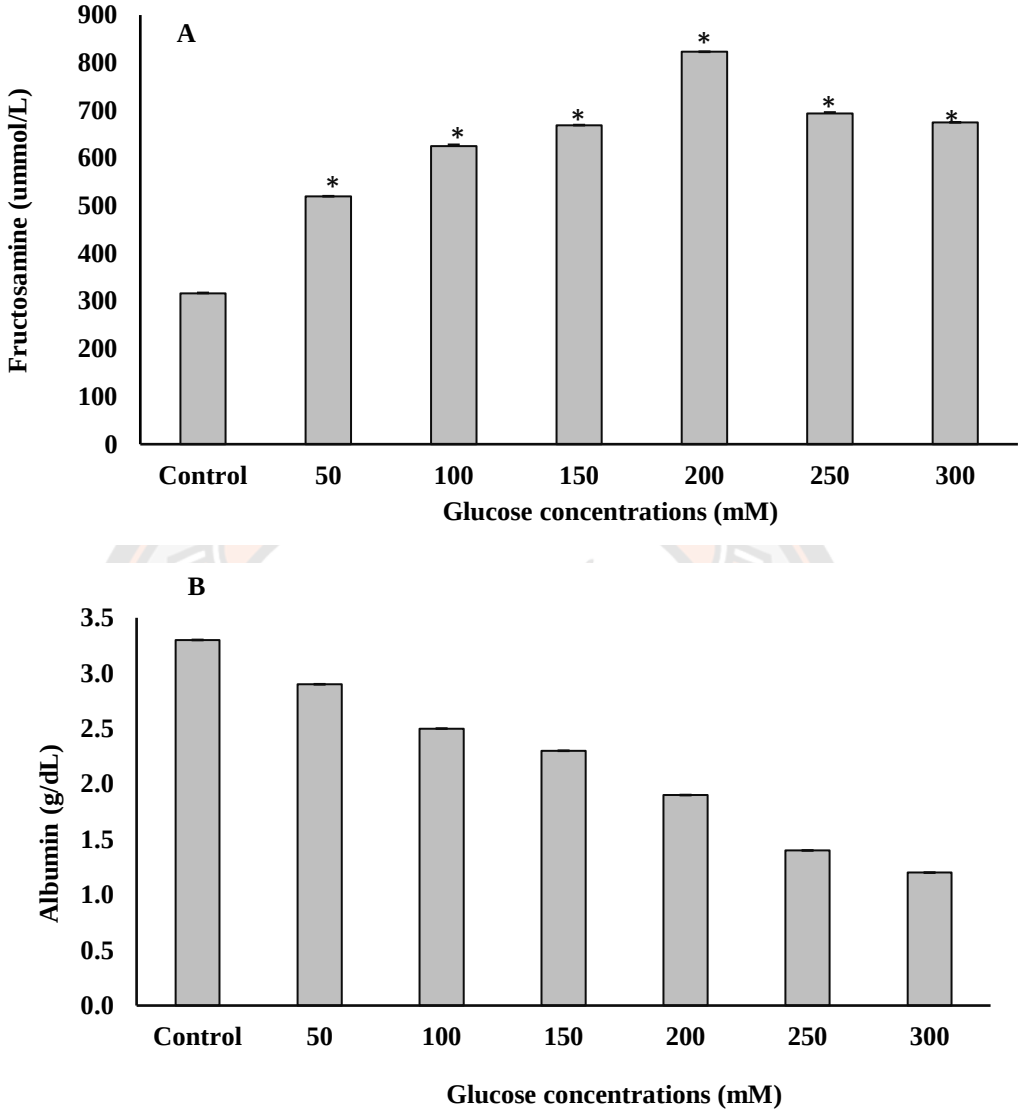


Figure 32. Fructosamine and albumin levels after 48 hours of in vitro glycation with various glucose concentrations

Fructosamine from 48 hours incubation times at 0, 50, 100, 150, 200, 250, and 300 mM was 316±0.71, 520±0.00*, 623±2.83*, 669±0.71*, 823±0.00*, 693±2.12*, and 675±0.00* umol/L, respectively. Albumin was 3.3±0.00, 2.9±0.00, 2.5±0.00, 2.3±0.00, 1.9±0.00, 1.4±0.00, and 1.2±0.00 g/dL, respectively. The paired t-Test revealed that plasma incubated with D-glucose at 50, 100, 150, 200, 250, and 300 mM for 48 hours increased significantly different fructosamine ($p < 0.05$) and decreasing not significant difference ($p > 0.05$) of albumin. At 200 mM D-glucose, there was highest and significant difference of fructosamine from the control.

3.2 Incubation times

Plasma incubated with 0 (control), 100, 200, and 300 mM of D-Glucose concentration at 37^{o}C for 48 hours. The level of fructosamine, total protein, and albumin was measured by immunoassay method. The results were shown in **Table 28**.

Table 28. The level of fructosamine, total protein, and albumin after 48 hours of *in vitro* glycation between plasma and various glucose concentrations

Glucose concentration (mM)	Mean ± SD		
	Fructosamine (umol/L)	Total protein (mg/dL)	Albumin (g/dL)
Control	379±0.00	5.0±0.00	2.7±0.42
100	611±1.41*	5.5±0.07	2.8±0.00
200	717±0.71*	4.0±0.00	2.0±0.00
300	674±2.83*	3.0±0.07*	1.4±0.00*

*Significant at $p < 0.05$ by paired t-Test when compared with base line (control)

Fructosamine from 48 hours of incubation times with D-glucose 0, 100, 200, and 300 mM was 379±0.00, 611±1.41*, 717±0.71*, and 674±2.83* umol/L, respectively, total protein was 5.5±0.00, 5.5±0.07, 4.0±0.00, and 3.0±0.07*g/dL, respectively, and albumin was 2.7±0.42, 2.8±0.00, 2.0±0.00, and 1.4±0.00* g/dL, respectively. The paired t-Test revealed that plasma incubated with D-glucose at 50, 100, 150, 200, 250, and 300 mM for 48 hours increased significantly different fructosamine (p<0.05) and decreasing of total protein and decreasing of albumin. At 200 mM D-glucose, there was a significant difference in fructosamine from the control. The optimal conditions for fructosamine production from *in vitro* glycation was used plasma incubated with D-glucose at 200 mM at 37 ^{o}C for 48 hours.

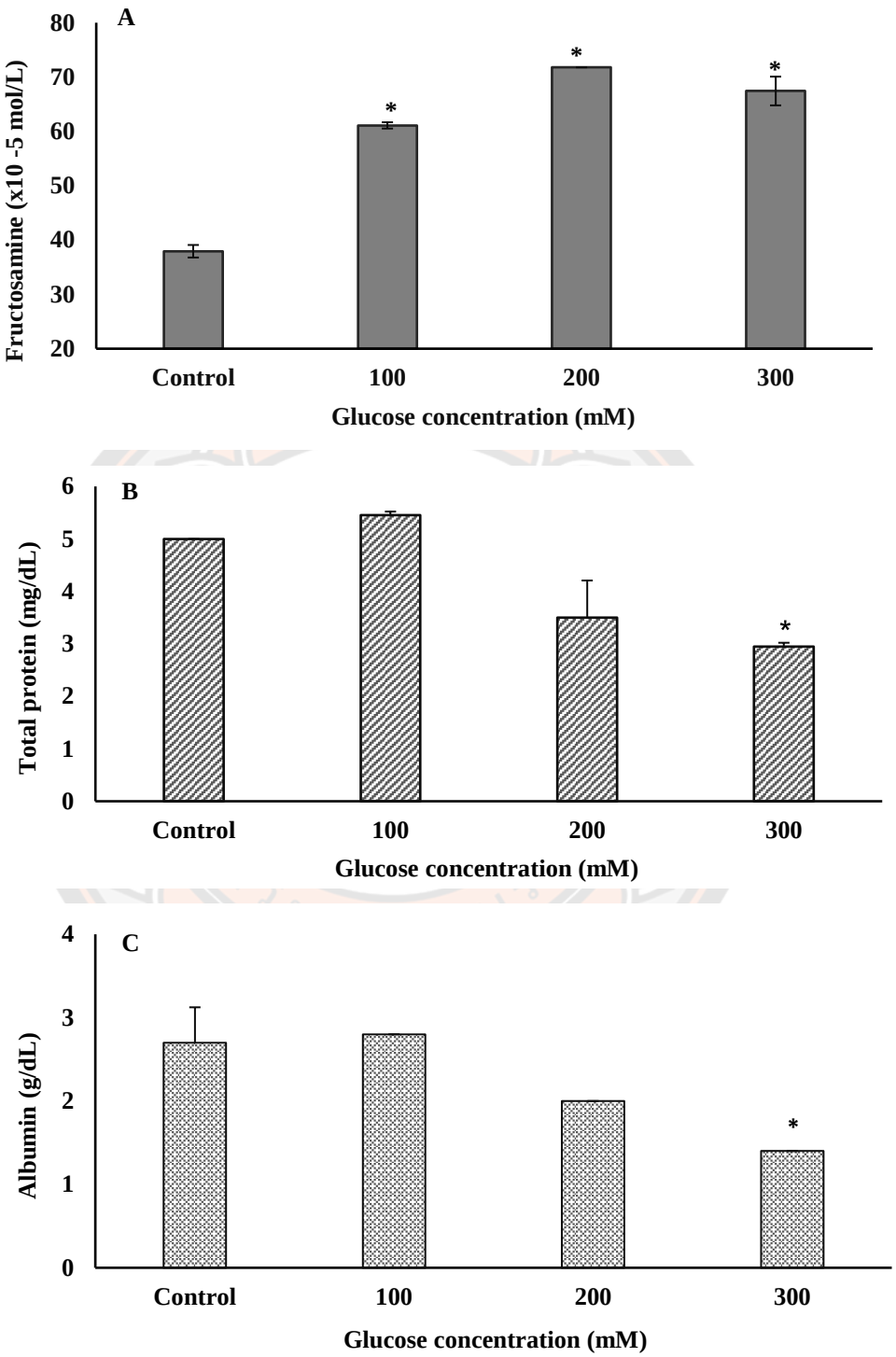


Figure 33. Fructosamine, total protein, and albumin after incubate plasma with 0 (control), 100, 200, and 300 mM for 48 hours

Study 4: Homogeneity and stability test of HbA_{1C} and fructosamine in *in vitro* glycation product by following ISO 17034-Reference material producer and Guide 35-Guidance for characterization and assessment of homogeneity and stability.

Processed blood material (GLY-1, GLY-2, GLY-3) from *in vitro* glycation, a fresh blood sample: (FDM-1) from single DM patient was collected in an K_3EDTA tube, and pooled DM blood sample (NDM-1) were prepared by following the procedure in a previous study (Wongsri P, 2017), while 0.5 mL was aliquoted and kept in a refrigerator until the experiments. Hemoglobin A_{1C} in processed blood material was homogeneously measured and analyzed in accordance with ISO Guide 35 (ISO GUIDE 35:2017).

4.1 Homogeneity test of HbA_{1C}

Processed blood materials (GLY-1, GLY-2, GLY-3) were prepared from *in vitro* glycation, FDM-1 was prepared from single DM donor, and NDM-01 was prepared from single non-DM blood donor were aliquot in vials and kept at 2-8ºC. Sampling processed blood materials by random 10 vials for homogeneity test. Outlier was checked by R/R total more than Critical at 95% CI was show in **Table 29**. The result was not showing any outliers from these studies. **Table 30** shows the results of the $F_{calculation}$ and $F_{critical}$ using one-way ANOVA single factor. The results show that $F_{calculation}$ (0.604) and $F_{critical}$ (3.020) and standard uncertainty was 0.03%, indicating that this material was homogeneous because $F_{calculation}$ was less than $F_{critical}$. All samples including of processed plasma were computed in the same step. The homogeneity of processed blood material was shown in **Table 31**.

Table 29. The Cochran's critical value for evaluated outliers is set at 95%

No.	Rep#1	Rep#2	Rang^2 (R)	R/R total	C>Critical
1	7.9	7.9	0.0000	0.00000	pass
2	8.0	8.0	0.0000	0.00000	pass
3	7.9	8.0	0.0050	0.00063	pass
4	8.0	7.9	0.0050	0.00063	pass
5	7.9	8.0	0.0050	0.00063	pass
6	8.0	8.0	0.0000	0.00000	pass
7	8.0	7.8	0.0200	0.00251	pass
8	8.0	8.0	0.0000	0.00000	pass
9	7.9	8.0	0.0050	0.00063	pass
10	8.0	7.9	0.0050	0.00063	pass
Grand mean			7.955		
Critical at 95%CI			0.6018		

Table 30. Data of GLY-2 was analyzed by One-way ANOVA single factor

Groups	Count	Sum	Average	Variance
Row 1	2	15.8	7.9	0
Row 2	2	16.0	8.0	0
Row 3	2	15.9	7.95	0.005
Row 4	2	15.9	7.95	0.005
Row 5	2	15.9	7.95	0.005
Row 6	2	16.0	8.0	0
Row 7	2	15.8	7.9	0.02
Row 8	2	16.0	8.0	0
Row 9	2	15.9	7.95	0.005
Row 10	2	15.9	7.95	0.005

ANOVA

Source of Variation	SS	df	MS	F
Between Groups	0.0245	9	0.002722	0.604938
Within Groups	0.045	10	0.0045	
Total	0.0695	19		

Processed blood materials: NDM-1, FDM-1, GLY-1, GLY-2, and GLY-3 were homogeneity after being aliquoted in small vials and kept in refrigerated. The homogeneity of CQC-1, and CQC-2 were not shown. The results of homogeneity ($F_{calculation} < F_{critical}$) evaluated from ANOVA were summarized in **Table 31**. Processed blood materials: NDM-1, FDM-1, GLY-1, GLY-2, and GLY-3 were homogeneity and stable with $T_{calculation} < T_{critical}$ for 35, 21, 70, 68, and 64 days, respectively. The commercials QC (CQC-1 and CQC-2) in lyophilized form were stable more than 3 years at 2-8^{o}C with unopened or stable for 7 days with opened. GLY-1, GLY-2, GLY-3 were prepared from *in vitro* glycation, FDM-1 was prepared from single DM donor, and NDM-1 was prepared from single non-DM blood donor, CQC-1 and CQC-2 were purchased from commercials.

4.2 Homogeneity test of fructosamine

Table 32. Homogeneity and stability test of fructosamine in processed plasma blood materials

Processed plasma materials	Homogeneity test		Stability test		Long-term stability (Day)
	$F_{calculation}$	$F_{critical}$	$T_{calculation}$	$T_{critical}$	
PPM-1	0.304	4.414	1.785	2.306	14
PPM-2	0.435	4.414	2.282	2.306	14
PPM-3	0.131	4.414	1.894	2.306	14

PPM were processed plasma from *in vitro* glycation between plasma and glucose at optimal conditions

4.3 Stability of HbA_{1C} in processed blood materials

4.3.1 Short-term stability or stability under transportation

To simulate temperature in an ice pack box, as well as transport HbA_{1C} material (GLY-1) to participants over a five-day period. After day 3 of transportation, HbA_{1C} levels increased significantly ($T_{calculation} < T_{critical}$), data is shown in Figure 34. Temperatures were measured five days using data loggers embedded in the pack box. The trial-1 temperatures were 7.2, 16.8, 23.4, 24.4, and 28.1^{o}C, respectively. The trial-2 temperatures were 9.0, 17.8, 23.5, 24.5, and 25.2^{o}C, respectively. The trial-3 temperatures were 8.0, 17.2, 23.5, 24.2, and 25.2^{o}C, respectively. The HbA_{1C} level increased when the temperature (Figure 35) goes up to 23.5^{o}C on the third day because of red cell hemolysis in processed blood materials. Processed blood materials should be carried to participants over a three-day period.

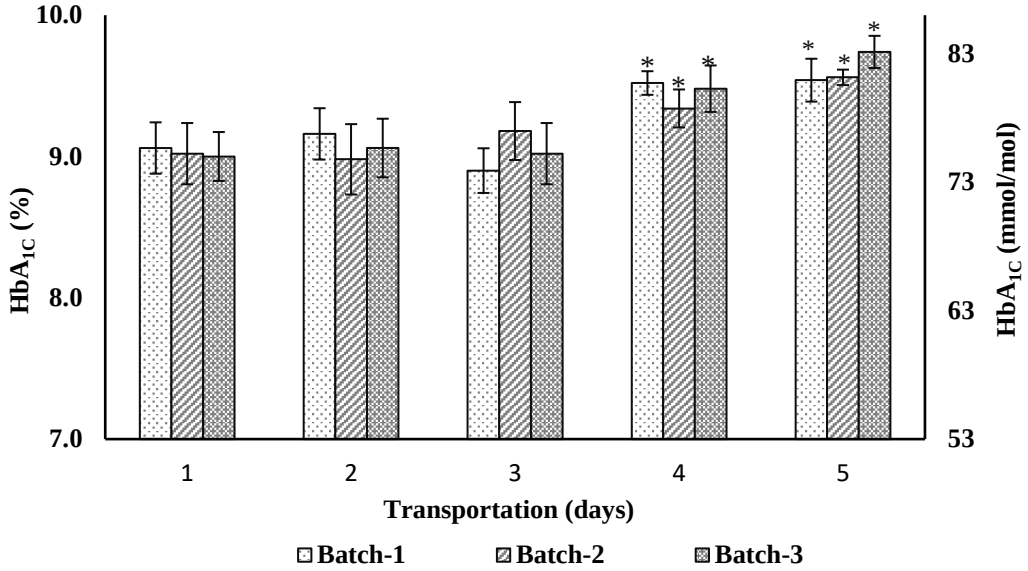


Figure 34. The increase in HbA_{1C} during a 5-day transport with three different batches. Error bars represent standard deviation

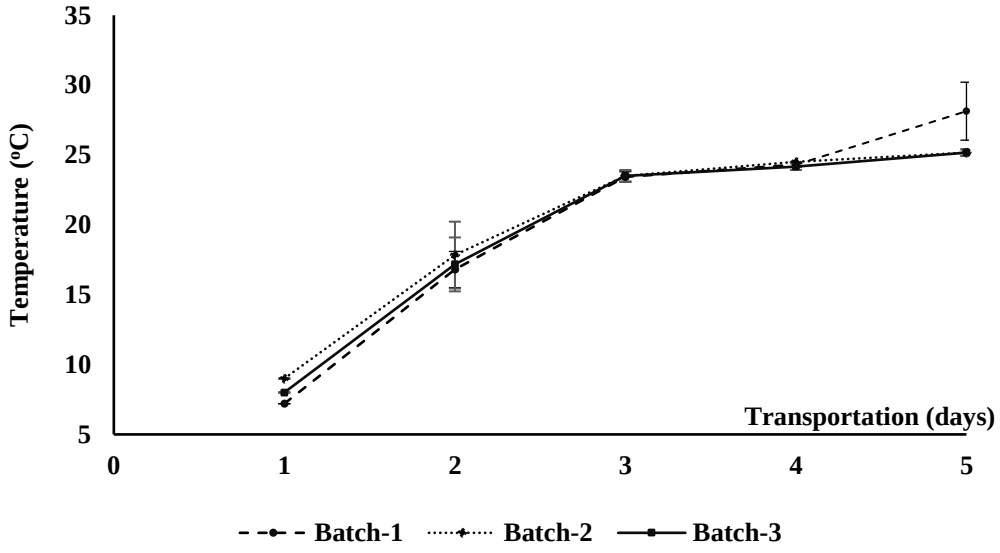


Figure 35. Temperature in container during transportation processed blood materials in 5 days

4.3.2 Long-term stability

Randomly selecting 5 samples at 0, 1, 14, 28, 32 days and measured for HbA_{1C}. The results were evaluated by a regression analysis was also used to assess stability for evidence of a linear trend. The result is show in **Figure 36**. The slope is most likely not significantly different from zero ($p < 0.05$). There is no distinction between the first and final observations. This GLY materials demonstrated stability over a 70-day period.

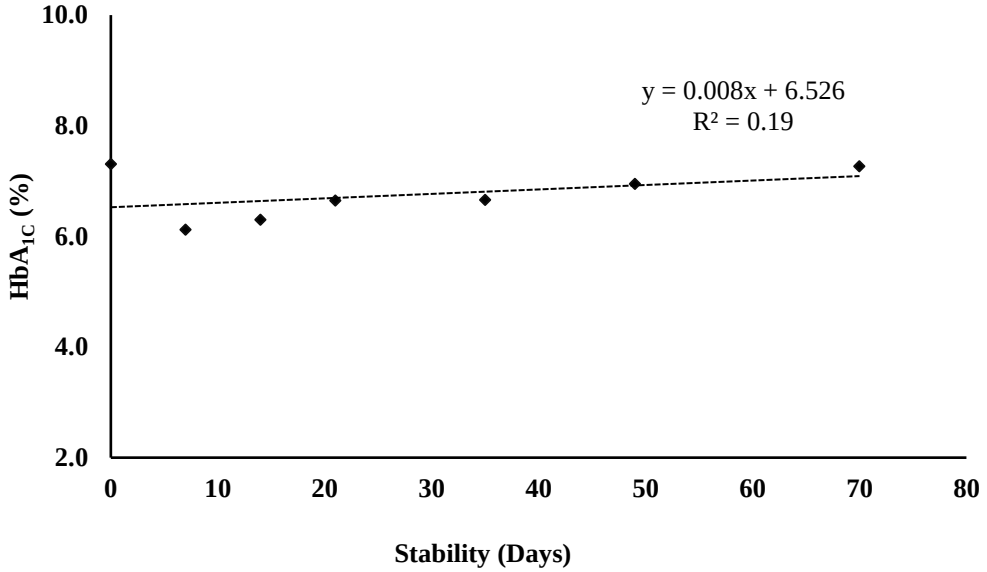


Figure 36. Linear regression analysis of GLY-1 materials

Processed blood materials: GLY-1, GLY-2, GLY-3, FDM-1, and NDM-1 were HbA_{1C} measured until instability. **Figure 37** illustrates the self-life stability of processed blood materials from *in vitro* glycations were found to be more stable than other materials. As a result, this condition is suitable for the preparation of HbA_{1C} reference material. The processed blood, GLY-1, GLY-2, GLY-3, FDM-1, and NDM-1 were demonstrated self-life stability for 70, 68, 64, 21, and 35 days, respectively.

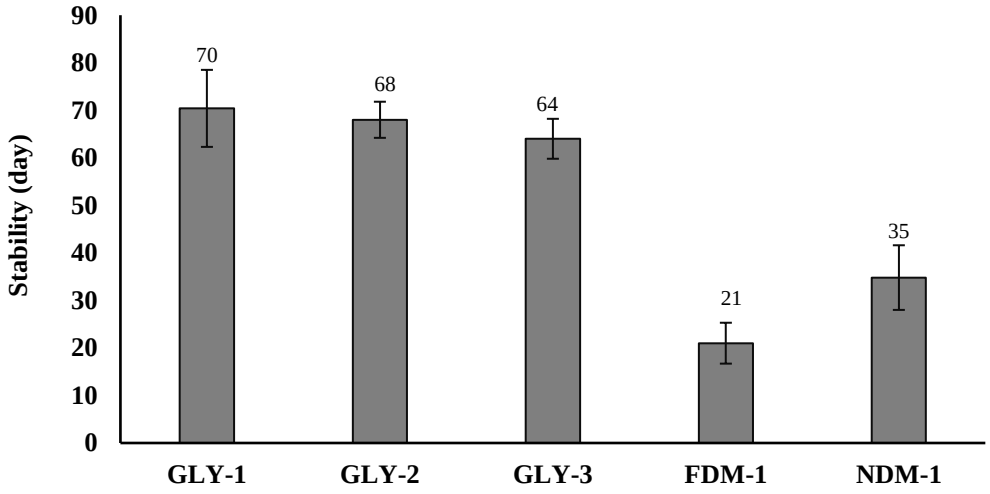


Figure 37. Long-term stability of HbA_{1C} in blood materials. The error bar represents the standard deviation of the mean of the day of stability

4.4. Stability of fructosamine in processed plasma

Long-term stability of fructosamine in processed plasma was demonstrated stable over 14 days by statistical evaluation using ISO Guide 35 to predict fructosamine shelf life (**Figure 38)**.

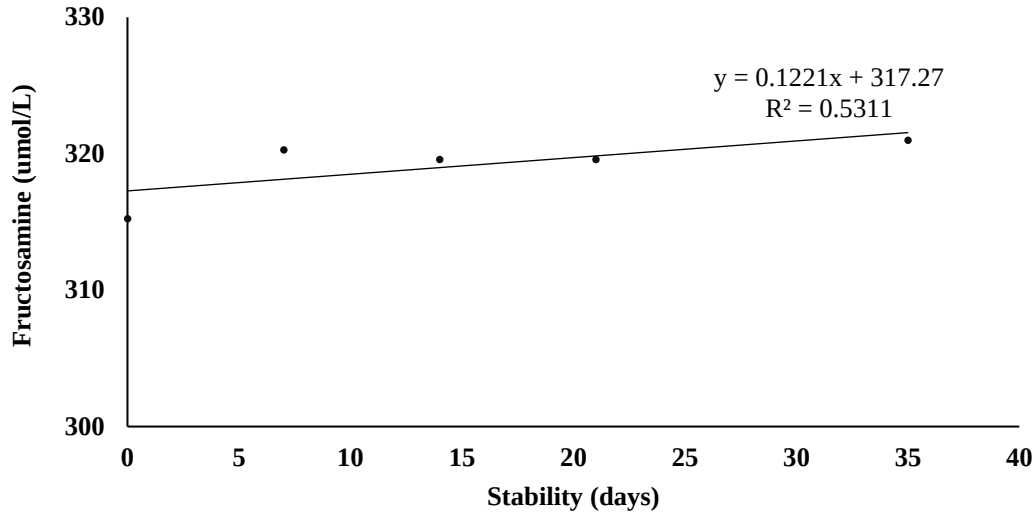


Figure 38. Long-term stability test of fructosamine in processed plasma

Study 5: Commutability study of processed blood materials to investigate differences in test material results between measurement procedures parallel with native samples

5.1 Hemoglobin A_{1C} measurement in clinical blood sample and in processed blood materials

Analyze the clinical blood samples and processed blood materials using six measurement analyzers. The study of commutability analyzed 25 patient samples (HbA_{1C} ranged from 4.5-12.6% in the NGSP unit, 26-114 mmol/mol in the IFCC unit) using six HbA_{1C} measurement methods to create a 95% prediction interval (PI). The processed blood materials were evaluated by using various clinical analyzers in accordance with standard CLSI EP14-A3 evaluation model. Six methods were measure HbA_{1C} in 25 clinical blood samples and 6 processed blood materials, average of $\%HbA_{1C}$ are summarized in **Table 33.**

5.2 Comparisons of HbA_{1C}

Hemoglobin A_{1C} levels in twenty five native blood samples were show mean (**Table 34**) from TI-RL1, EN-RL1, BA.HPLC.-CL1, TI-CL2, FI.POCT-CL2, and BA.POCT-CL2 was show %HbA1C in NGSP and IFCC in parenthesis 6.6(49), 6.7(50), 6.7(50), 6.7(50), 7.0(53), and 6.5(48)%, respectively. Six processed blood materials: NDM-01, GLY-01, GLY-02, GLY-03, CQC-01, and CQC-02 was 7.2(55), 7.1(54), 8.2(66), 7.1(54), 8.8(73) and 7.2(55) HbA_{1C}%, respectively. Two commercial QC blood materials was 7.3(56), 6.8(51), 6.9(52), 7.2(55), 7.3(56) and 7.3 (56) HbA_{1C}%, respectively. All sample were not difference significant at $p > 0.05$ from six different measurement procedures.

Table 33. Average of %HbA_{1C} were measured by six measurement methods

Sample	Average ± SD of HbA_{1C} (%)					
	TI-RL1*	EN-RL1*	BA.HPLC-CL1	TI-CL2	BA-POCT1	FI-POCT2
AHSN-009	4.9±0.03	4.8±0.02	4.8±0.00	4.5±0.12	5.0±0.06	4.9±0.00
AHSN-014	5.0±0.05	5.0±0.00	5.0±0.00	4.9±0.35	5.3±0.12	4.9±0.06
AHSN-003	5.2±0.01	4.8±0.05	4.8±0.00	5.0±0.00	5.3±0.35	4.8±0.06
AHSN-011	5.2±0.03	5.2±0.02	5.2±0.00	5.0±0.12	5.6±0.00	5.0±0.06
AHSN-010	5.3±0.01	5.3±0.02	5.3±0.00	4.9±0.00	5.5±0.00	5.1±0.06
AHSN-016	5.4±0.01	5.3±0.03	5.3±0.06	5.3±0.23	5.6±0.17	5.2±0.00
AHSN-006	5.5±0.02	5.6±0.00	5.6±0.00	5.5±0.00	6.3±0.06	5.6±0.00
AHSN-007	5.5±0.02	5.5±0.01	5.6±0.06	5.5±0.06	5.7±0.06	5.4±0.06
AHSN-002	5.5±0.06	5.6±0.01	5.5±0.00	5.6±0.00	5.6±0.23	5.6±0.12
AHSN-005	5.5±0.01	5.5±0.00	5.6±0.00	5.6±0.00	6.0±0.23	5.4±0.00
AHSN-027	5.6±0.01	5.7±0.07	5.5±0.06	5.6±0.06	5.8±0.17	5.5±0.23
AHSN-004	5.7±0.02	5.8±001	5.6±0.00	5.7±0.06	6.0±0.17	5.7±0.06
AHSN-030	6.5±0.02	6.6±0.04	6.7±0.12	6.6±0.06	6.6±0.12	6.2±0.12
AHSN-026	6.6±0.01	6.6±0.01	6.7±0.06	6.6±0.06	7.0±0.17	5.9±0.17
AHSN-019	6.8±0.02	6.9±0.01	6.9±0.06	6.8±0.23	6.8±0.12	6.8±0.17
AHSN-028	6.9±0.05	6.9±0.00	6.9±0.00	7.2±0.06	7.9±023	7.0±0.17
AHSN-023	7.1±0.02	7.1±0.04	7.1±0.00	7.0±0.12	7.1±0.06	6.4±0.12
AHSN-022	7.0±0.03	7.1±0.02	7.2±0.00	7.2±0.17	7.4±0.06	6.6±0.17
AHSN-029	7.4±0.00	7.5±0.00	7.3±0.06	7.3±0.00	7.8±0.06	7.2±0.17
AHSN-020	7.8±0.03	8.0±0.01	7.9±0.00	7.8±0.06	8.3±0.06	8.2±0.12
AHSN-024	7.8±0.04	8.0±0.08	7.8±0.06	7.8±0.06	8.1±0.06	8.5±0.06
AHSN-018	7.9±0.02	8.2±0.01	8.1±0.00	7.9±0.06	8.1±0.17	6.6±0.17
AHSN-025	8.1±0.02	8.3±0.02	8.1±0.00	8.7±0.06	7.9±0.17	7.9±0.00
AHSN-021	10.6±0.05	11.0±0.00	10.9±0.00	10.9±0.06	12.0±0.12	10.1±0.06
AHSN-031	11.2±0.0.5	11.6±0.05	11.2±0.12	11.6±0.12	11.1±0.12	12.6±0.06
NDM-1	5.6±0.03	5.5±0.01	5.4±0.00	5.6±0.06	5.5±0.06	5.1±0.12
GLY-1	8.7±0.04	8.8±0.01	10.9±0.06	8.7±0.06	11.5±0.00	9.2±0.12
GLY-2	6.6±0.01	6.6±0.03	7.4±0.00	6.3±0.06	7.6±0.06	7.1±0.17
GLY-3	7.9±0.02	7.7±0.02	8.9±0.06	7.8±0.06	10.7±0.12	7.3±0.06
CQC-1	5.1±0.00	4.6±0.02	4.7±0.00	5.2±0.00	5.5±0.17	5.6±0.06
CQC-2	9.4±0.03	9.0±0.01	9.1±0.00	9.3±0.06	9.0±0.00	9.0±0.06

*Reference laboratories certified by NGSP Level-1; turbidimetric immunoassays methods (TI-RL1) and enzymatic immunoassay methods (EN-RL1). A clinical laboratory-1 certified by LA; boronate affinity-HPLC (BA.HPLC-CL1). Clinical laboratory-2 certified by ISO 15189; turbidimetric immunoassay (TI-CL2), POCT devices in routine clinical laboratories 2; boronate affinity (BA-POCT1), and fluorescent immunoassay (FI-POCT2).

Table 34. Hemoglobin A_{1C} in clinical blood samples and processed blood materials were examine by six different methods

Methods	Mean%HbA1C (mmol/mol)					
	TI-RL1*	EN-RL1*	BA.HPLC-CL1	TI-CL2	BA-POCT1	FI-POCT2
Clinical blood samples (<6.4%, n=36)						
Mean	54 (35)	5.3 (35)	5.3 (35)	5.3 (35)	5.6 (38)	5.3 (35)
SD	0.2	0.3	0.3	0.4	0.4	0.3
Minimum	4.9 (30)	4.8 (29)	4.8 (29)	4.5 (26)	5.0 (32)	4.8 (29)
Maximum	5.7 (39)	5.8 (40)	5.6 (38)	5.7 (39)	6.3 (45)	5.7 (38)
Median	5.4 (36)	5.4 (35)	5.4 (35)	5.4 (35)	5.6 (38)	5.3 (34)
Lower 95%CI	4.9 (30)	4.7 (27)	4.7 (28)	4.5 (26)	4.9 (31)	4.6 (27)
Upper 95%CI	5.9 (41)	6.0 (42)	5.9 (41)	6.0 (43)	6.3 (46)	5.9 (41)
Clinical blood samples (6.5 - 7.4%, n=21)						
Mean	6.9 (52)	7.0 (53)	7.0 (53)	6.9 (52)	7.2 (56)	6.6 (49)
SD	0.3	0.3	0.5	0.3	0.5	0.5
Minimum	6.5 (47)	6.6 (49)	6.7 (49)	6.6 (48)	6.6 (49)	5.9 (41)
Maximum	7.4 (57)	7.5 (58)	7.3 (57)	7.3 (56)	7.9 (62)	7.2 (56)
Median	6.9 (52)	6.9 (52)	6.9 (52)	7.0 (53)	7.1 (54)	6.6 (49)
Lower 95%CI	6.3 (45)	6.4 (46)	6.5 (47)	6.3 (46)	6.3 (45)	5.7 (39)
Upper 95%CI	7.5 (58)	7.6 (59)	7.5 (58)	7.6 (59)	8.2 (66)	7.5 (59)
Clinical blood samples (> 7.5%, n=21)						
Mean	8.9 (74)	9.2 (77)	9.0 (75)	9.1 (76)	9.2 (77)	9.0 (75)
SD	1.6	1.7	1.6	1.7	1.8	2.1
Minimum	7.8 (61)	8.0 (64)	7.8 (62)	7.8 (61)	7.9 (63)	6.6 (49)
Maximum	11.2 (99)	11.6 (104)	11.2 (99)	11.6 (104)	12.0 (107)	12.6 (115)
Median	8.0 (64)	8.3 (67)	8.1 (65)	8.3 (67)	8.2 (66)	8.4 (68)
Lower 95%CI	5.8 (40)	5.9 (41)	5.8 (40)	5.7 (38)	5.7 (38)	4.8 (29)
Upper 95%CI	12.0 (108)	12.5 (113)	12.2 (110)	12.5 (114)	12.8 (121)	13.2 (121)
Processed blood materials (n=21)						
Mean	7.2 (55)	7.1 (54)	8.2 (66)	7.1 (54)	8.8 (73)	7.2 (55)
SD	1.2	1.2	2.0	0.0	2.4	1.5
Minimum	5.6 (38)	5.5 (37)	5.4 (36)	5.4 (36)	5.5 (37)	5.1 (32)
Maximum	8.7 (72)	8.8 (73)	10.9 (96)	8.7 (72)	11.5 102)	9.2 (77)
Median	7.0 (53)	7.1 (54)	8.2 (66)	7.0 (53)	9.2 (77)	7.2 (55)

Lower 95%CI	4.8 (2)	4.7 (28)	4.1 (21)	7.1 (54)	4.1 (21)	4.3 (23)
Upper 95%CI	9.6 (81)	9.6 (81)	12.2 (110)	7.1 (54)	13.6 (125)	10.1 (87)
Commercial blood materials (n=6)						
Mean	7.3 (56)	6.8 (51)	6.9 (52)	7.2 (55)	7.3 (56)	7.3 (56)
SD	2.1	2.2	2.2	2.0	1.8	1.7
Minimum	5.1 (32)	4.6 (27)	4.7 (28)	5.2 (33)	5.5 (37)	5.6 (38)
Maximum	9.4 (79)	9.0 (75)	9.1 (76)	9.3 (78)	9.0 (75)	9.0 (75)
Median	7.3 (56)	6.8 (51)	6.9 (52)	7.2 (55)	7.3 (56)	7.3 (56)
Lower 95%CI	3.0 (9)	2.4 (12)	2.5 (12)	3.2 (11)	3.8 (18)	4.0 (20)
Upper 95%CI	11.6 (103)	11.2 (99)	11.3 (100)	11.3(100)	10.8 (95)	10.6 (92)

*Method certified for hemoglobin A_{1C} testing by NGSP (National Glycohemoglobin Standardization Program) Level-1. TI-RL1: Turbidimetric Immunoassay); EN-RL1: Enzymatic Immunoassay; BA.HPLC-CL1: Boronate Affinity HPLC, TI-CL2: Turbidimetric Immunoassay Clinical Laboratory, BA.POCT-CL2: POC device with boronate affinity; FI.POCT-CL2: POC device with fluorescent immunoassay.
n: amount of sample as triplicate measurements. IFCC unit was given in parentheses (mmol/L)

5.3 Commutability evaluation

The data were evaluated for Ordinary Regression Linear (ORL) comparison of clinical blood samples (**Figure 39**). The examine the distribution of means of HbA_{1C} in clinical blood samples obtained using measurement turbidimetric immunoassay method (TI-RL1) and enzymatic immunoassay method (EN-RL1). Hemoglobin A_{1C} was tends increase with increasing measurand content. The processed blood materials and commercials QC materials were evaluated with measurement TI-RL1 and EN-RL1 provide the same results. Calculating Deming regress parameters and plot the 95% prediction interval (95% PI) based upon the clinical blood samples. The Deming regression plot between TI-RL1 and EN-RL1 is show in **Figure 40.** The solid line represents upper and lower prediction intervals (PI) at 95%. Twenty five native blood samples, four processed blood materials, and two commercial QC were found to be similar to the mean from 6 methods: TI-RL1, EN-RL1, BA.HPLC-CL1, TI-CL2, BA-POCT1, and FI-POCT2. Ordinary Regression Linear (ORL) comparison of clinical blood samples with measurement procedure TI-RL1 and EN-RL1. The ORL slope was 0.99 show a high correlation between two methods. Three processed blood materials fell within upper and lower 95% PI were considered commutable. Deming regression plot to predict the commutability other materials is summarized in **Table 35.**

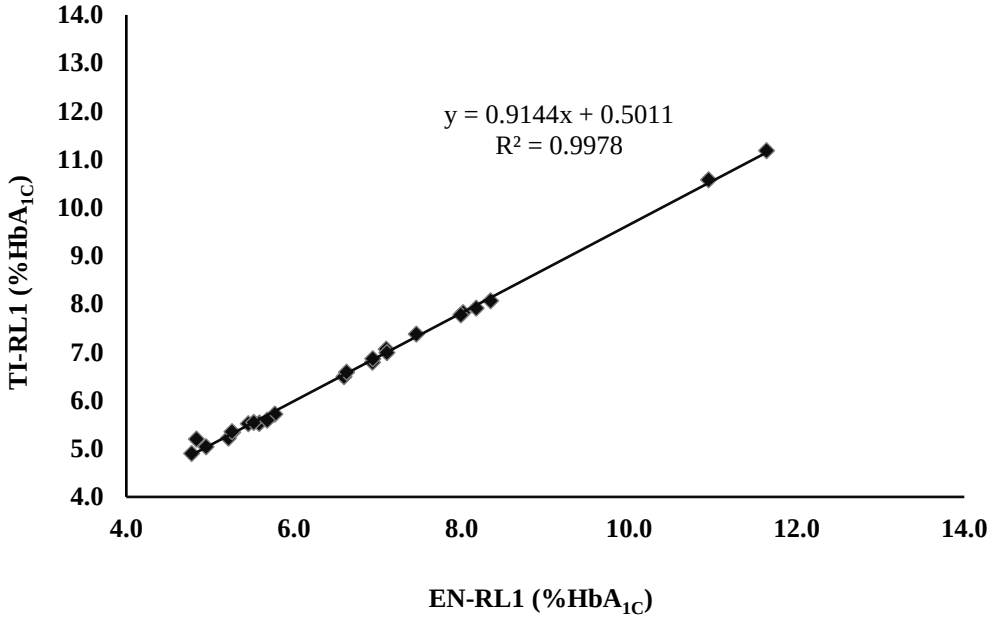


Figure 39. Ordinary Regression Linear (ORL) comparison of clinical blood samples

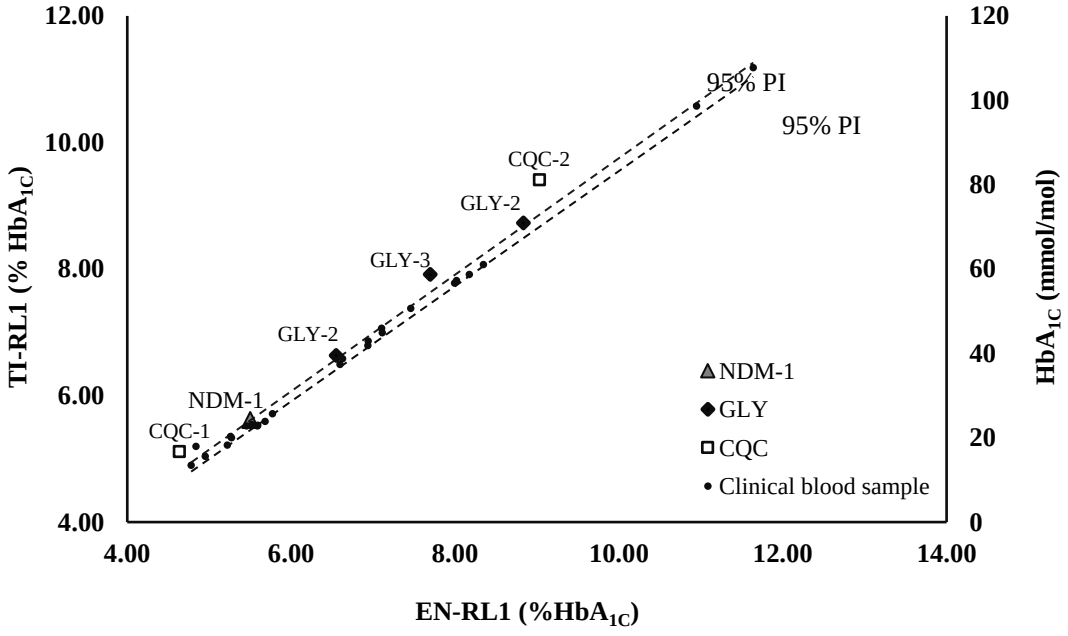


Figure 40. Deming regression of commutability study between TI-RL1 and EN-RL1

Table 35. Commutable of HbA_{1c} in processed blood materials, and mean difference from reference analyzers

Blood samples	Methods A	Methods B					
		TI-RL1*	EN-RL1*	BA.HPLC-CL1	TI-CL2	BA-POCT1	FI-POCT2
NDM-1	TI-RL1	-	C (2%)	C (4%)	C (0%)	C (2%)	C (9%)
	EN-RL1	C (2%)	-	C (2%)	C (4%)	C (0%)	C (7%)
GLY-1	TI-RL1	-	C (0%)	NC (12%)	C (5%)	NC (15%)	NC (8%)
	EN-RL1	C (0%)	-	NC (6%)	C (5%)	NC (15%)	NC (8%)
GLY-2	TI-RL1	-	NC (3%)	NC (13%)	C (1%)	NC (35%)	NC (8%)
	EN-RL1	NC (3%)	-	NC (12%)	C (1%)	NC (39%)	C (5%)
GLY-3	TI-RL1	-	C (1%)	NC (25%)	C (0%)	NC (32%)	C (6%)
	EN-RL1	C (1%)	-	NC (24%)	C (1%)	NC (30%)	C (5%)
CQC-1	TI-RL1	-	NC (11%)	NC (8%)	C (2%)	C (8%)	NC (10%)
	EN-RL1	NC (11%)	-	C (2%)	NC (13%)	NC (20%)	NC (22%)
CQC2	TI-RL1	-	NC (4%)	NC (3%)	C (1%)	NC (4%)	NC (4%)
	EN-RL1	NC (4%)	-	C (1%)	C (3%)	C (0%)	C (2%)

C: commutable, and NC; non-commutable

Percentages of mean difference between method A and method B was show in parenthesis. Reference laboratories certified by NGSP Level-1 ; turbidimetric immunoassays methods (TI-RL1) and enzymatic immunoassay methods (EN-RL1). A clinical laboratory-1 certified by LA; boronate affinity-HPLC (BA.HPLC-CL1). Clinical laboratory-2 certified by ISO 15189; turbidimetric immunoassay (TI-CL2), POCT devices in routine clinical laboratories 2; boronate affinity (BA-POCT1), and fluorescent immunoassay (FI-POCT2). NC; materials with non-commutable. A blood material: NDM-1 was prepared from pool non-DM blood, three processed blood materials: GLY-1, GLY-2, GLY-3 was prepared from *in vitro* glycation, two commercial QC materials. CQC-1, and CQC-2.

Four processed blood materials, two commercial blood QC materials, and twenty-five clinical blood samples were used to investigate differences in measurement procedures. The results obtained were evaluated based on CLSI EP14-A3. Three of four blood materials, NDM-01 (5.6%, 37 mmol/mol), GLY-1 (6.6%, 48 mmol/mol), and

GLY-3 (8.7%, 70 mmol/mol) fall in 95% prediction interval (PI) and were found to be commutable between turbidimetric immunoassay (TI-RL1) and enzymatic immunoassay (EN-RL1). Processed blood materials, GLY-1 and GLY-3 were prepared from *in vitro* glycation as intact erythrocytes at 2-8^{o}C, like human blood, and were commutable between two different measurement methods. On the other hand, GLY-12 (7.9%, 61 mmol/mol) and two commercial materials, CQC-1 (5.1%, 31 mmol/mol) and CQC-2 (9.4%, 77 mmol/mol) were demonstrated fall out of the 95% PI, indicating that were non-commutable. As a result of these findings, characterization based on a commutable was developed and used the target value for performance evaluation.

Study 6: Characterized HbA_{1C} in prepared materials according to Guide 35-Guidance for characterization and assessment of homogeneity and stability.

The processed blood samples were sufficient homogeneity and stability test as required by ISO 13528:2015. All samples were measured by six methods in clinical laboratories, then comparisons, and characterization to assigned value of HbA_{1C}.

Characterization of HbA_{1C}

The samples were "commutable" when locate within the 95% prediction interval (PI) range was assessed for uncertainty by characterization (unweighted mean). Target values of hemoglobin A_{1C} in processed blood materials from means, SD and characterization from unweighted means with uncertainty (**Table 36**).

Table 36. Target values of hemoglobin A_{1C} in processed blood materials from means, SD and characterization from unweighted means with uncertainty

Blood material	Mean ± SD (%HbA_{1C})						Unweighted mean ± U (%HbA_{1C}) Based on commutability			
	TI-RL1*	EN-RL1*	BA.HPLC-CL1	TI-CL2	BA-POCT1 (n=3)	FI-POCT2 (n=3)	TI-RL1, EN-RL1, FI-POCT2	TI-RL1, FI-POCT2	EN-RL1, FI-POCT2	TI-RL1, EN-RL1, BA-POCT1, FI-POCT2
NDM-1	5.6±0.0	5.5±0.0	5.4±0.0	5.6±0.1	5.5±0.1	5.1±0.1	5.4±0.3	5.3±0.5	5.3±0.4	5.4±0.1
GLY-1	6.6±0.0	6.6±0.0	7.4±0.0	6.3±0.1	7.6±0.1	7.1±0.2	6.8±0.4	6.9±0.5	6.8±0.6	7.0±0.2
GLY-2	7.9±0.0	7.7±0.0	8.9±0.1	7.8±0.1	10.7±0.1	7.3±0.1	7.7±0.5	7.6±0.7	7.5±0.5	8.4±0.3
GLY-3	8.7±0.0	8.8±0.0	10.9±0.1	8.7±0.1	11.5±0.0	9.2±0.1	8.9±0.3	9.0±0.5	9.0±0.4	9.6±0.2
CQC-1	5.1±0.0	4.6±0.0	4.7±0.0	5.2±0.0	5.5±0.2	5.6±0.1	5.1±0.2	5.4±0.2	5.1±0.3	5.2±0.2
CQC-2	9.4±0.0	9.0±0.0	9.1±0.0	9.3±0.1	9.0±0.0	9.0±0.1	9.1±0.1	9.2±0.1	9.0±0.0	9.1±0.1
NBS-1	5.2±0.0	5.2±0.0	5.2±0.0	5.0±0.1	5.6±0.0	5.0±0.1	5.2±0.0	5.1±0.0	5.1±0.0	5.3±0.1
NBS-2	7.9±0.0	8.2±0.0	8.1±0.0	7.9±0.1	8.1±0.2	6.6±0.2	7.6±0.3	7.3±0.3	7.4±0.4	7.7±0.2
NBS-3	11.2±0.0	11.6±0.1	11.2±0.1	11.6±0.1	11.1±0.1	12.6±0.1	11.8±0.2	11.9±0.3	12.1±0.2	11.6±0.2

U; Expanded uncertainty of characterization by following ISO Guide-35. Turbidimetric immunoassay (TI-CL2), POCT devices in routine clinical laboratories 2; boronate affinity (BA-POCT1;), and fluorescent immunoassay (FI-POCT2). Processed blood material: NDM-1 was prepared from pool non-DM blood, and GLY-1, GLY-2, GLY-3 was prepared from in vitro glycation. CQC-1, CQC-2 were available from commercial.

Study 7: To determine HbA_{1C} by advance technique by MALDI-TOF MS

7.1 MALDI-TOF MS conditions and blood sample preparation

Quality control and clinical blood sample were used to evaluate accuracy $\%HbA_{1C}$ from MALDI-TOF intra-plates along the % CVs calculated for intra-native blood sample 20 replicates (n = 3, <6, 6.1-9.0, and >9.1% HbA_{1C}). Results were expressed in both NGSP (%) and IFCC units (mmol/mol). The precision was show in **Table 37**.

Table 37. Precisions of MALDI-TOF MS with optimal acquired of MALDI-TOF MS

Sample	Mean ± SD	%CV
QC sample		
Level-1	3.4±1.2	36.3
Level-2	8.2±2.4	29.5
Level-3	17.4±5.1	29.4
Clinical sample (%HbA_{1C} range)		
<6.0	5.8±1.2	20.6
6.1 – 9.0	7.8±1.2	15.7
>9.1	10.8±1.9	17.3

7.2 Prospective HbA_{1C} measurement in native blood samples with optimal conditions

Six replicate analyses for 20 clinical samples were measured by MALDI-TOF MS and boronate HPLC. The results of were plot between %glycated β-Hb from MALDI-TOF MS and $\%HbA_{1C}$ from boronate HPLC (**Figure 41**).

Comparison of MALDI-TOF MS

Comparison of HbA_{1C} ranging from 4.0-7.0, 7.1-9.0, >9.1 and 4.0- > 9.1 was show mean difference between MALDI-TOF MS and boronate HPLC was -0.8 (0.659), -0.3(0.818), -0.2(0.03), and -0.6% (P<0.001), respectively (**Table 38**). The p value was given in parenthesis. For each range of $\%HbA_{1C}$ in clinical blood sample were not significant difference ($p > 0.05$) but for 4.0- >9.1% was shown significant difference at $p < 0.05$. The **Figure 42.** present correlation between $\%HbA_{1C}$ measured by boronate-HPLC and $\%HbA_{1C}$ was measured by MALDI-TOF MS was R^2 =0.72.

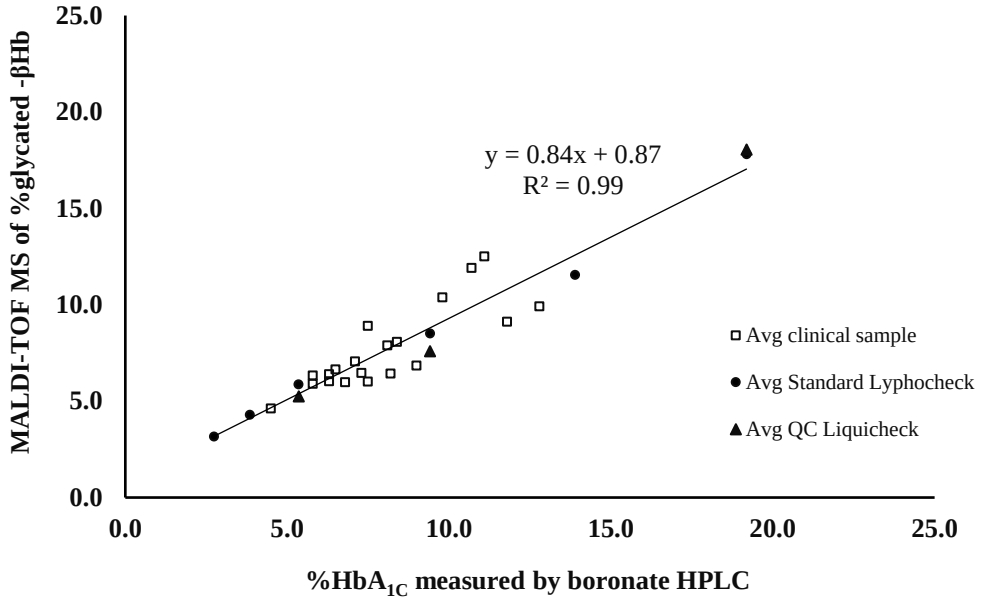


Figure 41. Plot shows % glycated-βHb the MALDI-TOF MS measurements from 6 replicate analysis for 20 clinical blood samples

Table 38. Comparison between %G-βH from MALDI-TOF MS and %HbA_{1C} from boronate HPLC method

%HbA_{1C} range	**MALDI-TOF MS %G-βH**	**MALDI-TOF MS % HbA_{1C}**	**Boronate HPLC %HbA_{1C}**	**Mean paired difference (%)**	**r *(p-value)***
4.0 - 7.0 (n= 54)	6.4	6.6	5.9	-0.8	0.003 (0.659)
7.1 - 9.0 (n=15)	7.9	8.3	8.1	-0.3	0.004 (0.818)
≥ 9.1 (n=12)	10.1	11.0	10.7	-0.2	0.030 (0.562)
4.0 - >9.1 (n= 81)	7.2	7.6	7.0	-0.6	0.440 (< 0.001)

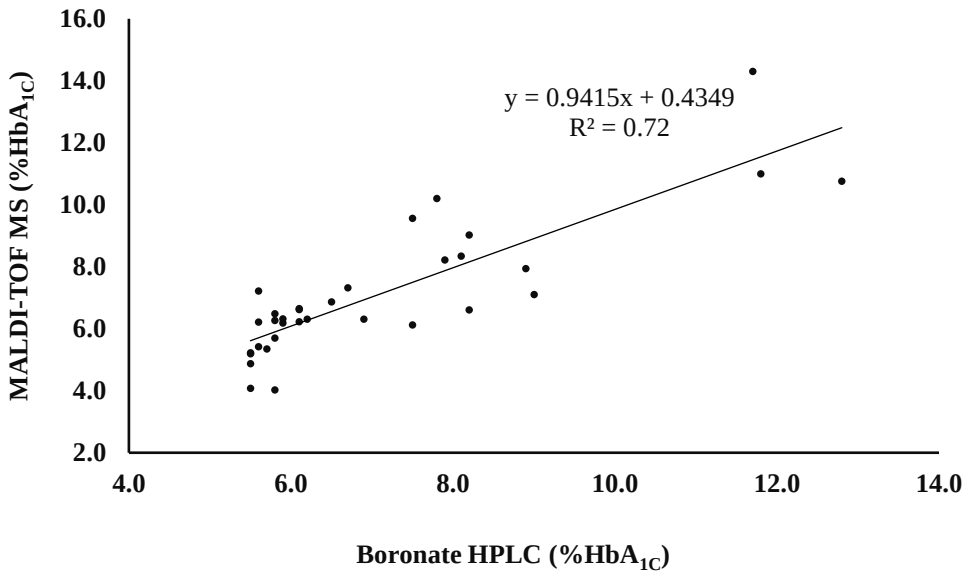


Figure 42. Correlation between %HbA$_{1C}$ measured by HPLC and %HbA$_{1C}$ was measured by MALDI-TOF MS

Chapter 5

Discussion

Glycation is the non-enzymatic covalent bonding of protein, lipid, or other molecules with reducing sugars such as glucose or fructose. The non-enzymatic reaction's end product is known as the advance glycation end product (AGE). The glucose level was used to assess, control, or monitor diabetic patients, as well as to determine hemoglobin A_{1C} (HbA_{1C}). The binding of sugars to the hemoglobin chain inside red blood cells to catalyze the glycation reaction is known as glycation. A high glycation efficiency should be selected when selecting a good quality of RBCs. As a result, the substrate is necessary. The sugar binds to Hb in red blood cells under normal conditions, glycation can occur.

Determination quality of human red blood cells and plasma with assessing cell quality, the color of the blood sample should be bright red, not dark, black, or green, clear of clots, no air bubbles, and there are no cell rupture or hemolysis less than 3+because hemolysis would deform the cells. As a result, the glycation process can be affects. To study the properties of whole blood with non-expired, and expired blood donor less than 1, <2, and <3 months get high percentage yield of RBCs and washing for three times. Whole blood that had been expired more than three months was washed three times, resulting in a percentage yield loss. In this study used whole blood from a blood donor expired within two months.

The optimal conditions for *in vitro* glycation among erythrocytes and 400 mM of D-glucose was incubated for 15 hours to increase HbA_{1C} significant different ($P<0.05$). Then glycation reaction was inhibited by resuspending washed erythrocytes in low glucose concentration solutions-1 and adjusting Hct level by approximately 38-42%. Hematocrit had decreased significantly after 9 hours of incubation, whereas Hct in processed blood material remained at 38-42% after 15 hours of incubation. At 38-42%Hct similar human blood component. The processed blood materials at low glucose concentration (pH of 7.4) and kept processed blood material at 2-8°C. Glycations between glucose and hemoglobin can be inhibited by a combination of inhibition solution and low temperature. After inhibition of *in vitro* glycation, HbA_{1C} level was stable in processed blood materials for 70 days using trend analysis according to ISO Guide 35. The processed blood materials were stable longer than materials were prepared from pooled patient blood DM (35 days), and from a fresh DM blood donor (21 days). However, the shelf life of HbA_{1C} in commercially available products (Bio-Rad laboratories, 2014 and Randox Laboratories Ltd., 2021) higher than 12 months with unopened, and stable for 7 days with opened in a refrigerator.

In vitro glycation for the preparation of processed blood materials with intact erythrocytes for HbA_{1C} assays has been successfully optimized. The use of quality red

cells, 400 mM D-glucose in PBS buffer pH 7.4, incubate at 37°C, 15-hour incubation time, inhibition of *in vitro* glycation with an inhibited solution at low glucose concentration, and storage processed blood materials in a refrigerator. Processed blood material was homogeneous, stable, and commutable with clinical samples. Under optimal conditions, in vitro glycation increased the amount of HbA_{1C} in processed blood material by at least 70%.

There are several point-of-care (POC) devices for HbA_{1C} testing (Hirst JA 2017, Whitley HP 2015, Bruegel M 2018), and a POC device has been shown to be sufficiently accurate and precise for the diagnosis of DM. The matrix of commercial products in freeze-dried form, which requires a purification process. Lyophilized blood may not be suitable for use with some POC devices that require a minimum Hct in the test samples (Lenters-Westra E, 2017). Processed blood materials from *in vitro* glycation with optimal conditions obtain intact erythrocytes like-human blood samples with an Hct value of 38-42%. In Germany, Belgium, the Netherlands (Kaiser P, 2016), Norway (Sølvik UØ, 2013) and other countries, whole blood materials are used in proficiency testing programs. Only 250 mL of blood can be donated from volunteers, which limitations in $EurA_{1C}$ Trials ($EurA_{1C}$, 2018). Under optimal conditions, our *in vitro* glycation technique could produce processed blood material for HbA_{1C} testing in bulk production more than 350 mL per batch, which is sufficient for use as test material distributed to at least 600 participants in an external quality assurance (EQA) or proficiency testing program (PT).

Processed blood materials (NDM-1) were made from a units of blood donated by a blood donor (Wongsri P, 2017), and GLY-1, GLY-2, and GLY-3 were prepared from in vitro glycation for HbA_{1C} assays (Duanginta W, 2023). The whole blood was suspended with 0.85% Normal saline (NS) until supernatant was clear. Erythrocytes were collected and incubated with 400 mM of D-glucose under optimal conditions. All processed blood materials were homogeneity and stability according to Guide 35 and ISO 13528. Fresh blood is the ideal blood materials, but its stability limits use because properties change over time depending on shipping conditions ($EurA_{1C}$, 2018). The short-term stability study confirmed the percentage of HbA_{1C} in processed blood materials was stable for three days during shipment at temperatures ranging from 8.1 to 23.5°C.

The commutability (Liu H, 2019) of HbA_{1C} in processed blood material should be investigated before the material was used in different clinical analyzers. The use of non-commutable materials for HbA_{1C} testing in interlaboratory studies did not result in comparable performance between analyzers and agreement of HbA_{1C} value with standard methods (Delatour V, 2020). Processed blood materials from *in vitro* glycation were commutable between enzymatic and turbidimetric immunoassay. However, one of the four processed blood materials dropped out of the 95% PI range with an absolute 4+ hemolysis might from a preparation error, while the other three processed blood material had less than 1+ hemolysis. The obtained results were analyzed using the

Deming regression plot according to CLSI EP14-A3. The two-sided of 95% prediction interval (PI) was construct from mean of clinical blood samples. Percentages of HbA_{1C} in processed blood materials and commercial materials shows similar behavior as in clinical blood samples are called commutable, while those that do not are called non-commutable. The commutability of HbA_{1C} materials was used to calculate the certified value or characterization.

To be used for HbA_{1C} test quality control, the concentration of HbA_{1C} in processed blood materials should be close to true values and stable sufficiently. Diagnosis of diabetes mellitus (DM) should be done with clinical laboratory equipment and a method traceable to standard methods provided by the National Glycohemoglobin Standardization Program (NGSP) or the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) based on metrological traceability concepts (John WG, 2007). The uncertainty (u) in commutable GLY materials materials characterized by reference laboratories was 0.5%, which is less than three times the desired bias (Westgard QC, 2021). Limitations of this study were;1) 1) An optimal in vitro glycation condition could not provide HbA_{1C} greater than 9% without hemolysis, and 2) percentage increase in HbA_{1C} from baseline was measured using a boronate affinity POC device under optimal conditions for in vitro glycation, which was higher than an immunoassay.

Hemoglobin A_{1C} (HbA_{1C}) can be used as a diagnostic test for diabetes if stringent quality assurance tests are in place, assays are standardized to criteria closely associated to international reference values. A European study that approached at the performance of hemoglobin A_{1C} (HbA_{1C}) assays from 24 different manufacturers in 17 different countries with fresh whole blood and lyophilized hemolysate samples derived from the same pool ($EurA_{1C}$, 2018). Noklus laboratory were prepared IQC and EQA on a HbA_{1C} POC instrument with control material from person with and without diabetes (patient-like material). Many Point-of-care testing (POCT) are widely used instruments with a variety of measured principles and advanced technology. As a result, the evaluation of commutable materials was observed. In these studies, was to investigate commutability, assign a target value by characterization, and evaluate laboratory performance in GLY materials.

ISO Guide 35:2017 defined materials of natural origin or with complex matrices, such as foodstuffs, soils, and alloys, are typically heterogeneous in composition. Reference materials (RM) prepared from such heterogeneous materials should therefore be subjected to an experimental homogeneity study (ISO Guide 35:2017, page 10). The first task in an RM production project is the acquisition of a sufficient amount of starting material(s) with the desired properties. The production of materials with particular properties is considered briefly in

- the number of units needed for the homogeneity study
- the number of units needed for the stability study

- the number of units required for monitoring stability over the expected lifetime of the material

Various measurements were used to examine HbA_{1C} in blood from volunteers and HbA_{1C} in processed blood materials to investigate bias of HbA_{1C}. The results revealed that the percent HbA_{1C} in native blood samples from human blood specimens was similar ($p>0.05$, Tamhane). Commutability was a characteristic of a reference material that correlates to the closeness of agreement between results for a reference material and results for clinical samples was evaluated by CLSI EP14-A3 (2014). The GLY-01, GLY-03, and NDM-01 were show a good commutable and GLY-03 was poor commutable between various different analyzers. Erythrocytes were hemolyzed during glycation method in processed blood materials from in vitro glycation. It had an impact on commutability with various analyzers. The commutability of processed EQA materials was highly heterogeneous and commonly insufficient to assess the accuracy of HbA_{1C} assays (Delatour V, 2020). The pool of blood donors should be used for processed blood materials production when up to industrial scale. The results conclude that processed blood materials from in vitro glycation are sufficient for commutability studies with clinical analyzers. The production should be producing the appropriate amount of $\%HbA_{1C}$. It may affect the measurement. This study suggested that the commutability of processed blood materials should be investigated by various clinical analyzers. However, commutability of processed blood materials that were prepared from *in vitro* glycation was not reported before. Processed blood materials production is important during manufacturing development to anticipate and possible assessment of commutability.

However, in recent years questions of methodological improvements and standardizations by the National Glycohemoglobin Standardization Program (NGSP) and the International Federation of Clinical Chemists (IFCC) to assess HbA1C traceability. However, in recent years, the National Glycohemoglobin Standardization Program (NGSP) and the International Federation of Clinical Chemists (IFCC) have raised concerns about the methodological improvements and standardizations used to assess HbA_{1C} traceability. Material characterization is the process of defining the chemical properties of one or more components of a substance or device material. Characterization can be achieved by using a single reference measurement procedure and using a network of competent laboratories. In this study, characterization with competent laboratories were successful for the HbA_{1C} test. The data were collected and unweighted mean calculation.

The limitations in this study were: 1) processed blood materials were not delivered to the IFCC for HbA_{1C} testing, so it must rely on reference methods and characterization based on a commutable method to assign value. Therefore, the problems were solved by finding a method to assign values by using characterization and using competent laboratories and finding for a new technology to investigate HbA_{1C} in processed blood materials. New methods, such as matrix-assisted laser

desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS), could be used as an alternative to existing chromatographic techniques for measuring HbA_{1C}. A few reports characterize hemoglobin as a new protein using mass spectrometry and matrix-assisted laser desorption/ionization (MALDI) and 2) control is difficult due to transportation limitations at temperatures ranging from 2 to 8 degrees Celsius. This research presents a protocol for an assay based on MALDI-TOF MS for the measurement and relative quantitation of glycated-βHb directly from whole blood.

Conclusions

Quality control materials for HbA_{1C} at a high level are difficult to prepare from patient blood due to limitations in mass production as well as the instability of glycated hemoglobin. This study assessed the performances of processed blood materials from *in vitro* glycation for HbA_{1C} testing as well as determined conditions for *in vitro* glycation for fructosamine. The *in vitro* glycation in this study was divided to two methods, intracellular *in vitro* glycation for HbA_{1C} production, and extracellular *in vitro* glycation for fructosamine production. A proper blood sample from a blood bank which A2A on a hemoglobin typing and $HbA_{1C} \leq 5.0\%$ was used for glycation.

To examine the criteria and factors that affect the quality of human RBCs in CPDA-1 from the blood bank. Human blood was expired less than two months was determined high quality by visual inspection, which included bright red color, less than 3^+ hemolysis, no air bubble and clot blood, washing time less than three times, and high percent yield. The high quality of cells was used as a substrate for *in vitro* glycation. HbA_{1C} (processed blood materials) were incubated at 37^{o}C for 15 hours among RBCs and 400 mM of D-glucose after being washed with 0.85% NS, suspended in inhibited solution-1 with low glucose concentrations, and final product was stored at 2-8^{o}C. The investigation the optimal conditions for extracellular glycation (fructosamine) between glucose and plasma protein incubated with 200 mM of D-glucose at 37^{o}C for 48 hours. Fructosamine increased significantly by 1.6 times from the baseline line. The homogeneity and stability of HbA_{1C} and fructosamine from *in vitro* glycation products were tested by following Guide 35-Guidance for characterization and assessment of homogeneity and stability. Processed blood and processed plasma materials were stable for 70 days and 14 days in a refrigerator, respectively.

The commutability was designed for commercial diagnostic test makers as well as laboratory developed tests useful to all clinical laboratory professionals wishing to investigate a processed sample's commutability (CLSI EP14-A3:2014). The commutability of HbA_{1C} in processed blood materials with difference from six various analyzers were non-commutable and commutability between enzymatic and turbidimetric immunoassays regarding the reference laboratory (RL) evaluations. Material characterization is the method of evaluating the chemical properties of one or more components of a substance. In this investigation, characterization with competent laboratories was effective since HbA_{1C} tests were performed and an unweighted mean

computation was performed. HbA_{1C} content in blood material should be closer to the theoretical values using a method traceable to standard methods of the National Glycohemoglobin Standardization Program (NGSP) or the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) based on metrological traceability concepts if HbA_{1C} materials are used for quality control. To determine HbA_{1C} by advance technique by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) was applied. The initial MALDI-TOF-MS settings were found to be suitable for producing high quality peaks in method development studies. HbA_{1C} measurement using MALDI-TOF-MS could be a low-cost alternative to diabetes screening. MALDI-TOF-MS produces HbA_{1C} results comparable to boronate affinity chromatography laboratory methods based on our current proof-of-concept method. More research is needed to improve pre-analytic processing to reduce imprecision, as well as additional comparison/calibration using higher order NGSP certified material.

This condition was appropriate for preparing HbA_{1C} blood material rather than fresh blood or pool DM blood donor blood materials because there were limitations in bulk production, low stability, and obtaining HbA_{1C} at high concentrations in accordance with human ethics. Processed blood materials were created and used for quality control, verification, validation, and proficiency testing of HbA_{1C}.

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APPENDIXS

A Homogeneity studies

Need for an experimental homogeneity study; materials of natural origin or with complex matrices, such as foodstuffs, soils, ores, and alloys, are typically heterogeneous in composition. Although the magnitude of between-un it differences can sometimes be small or even negligible after homogenization, in other cases, between-unit differences can remain larger than the uncertainty arising from characterization. RMs prepared from such heterogeneous materials should therefore be subjected to an experimental homogeneity study (ISO Guide 35:2017).

B Minimum number of units for a homogeneity study

Homogeneity test: examining only 10 units might not provide sufficient information about the onset of trends near the ends of a lengthy processing run. Current best practice therefore increases the number of units examined as the total number of units produced, Nprod increases. Typical recommendations are between $\sqrt[3]{\text{Nprod}}$ and $3x\sqrt[3]{\text{Nprod}}$. Taken together with the degrees of freedom requirement above, this leads to a recommended minimum number of units Nmin for a homogeneity study of materials characterized for a quantitative property given by the equation. where max(..,..) indicates the maximum of the terms within the parenthesis. (ISO Guide 35:2017, 7.4.1).

$N_{min}=\max\left(\sqrt[3]{\text{Nprod}}\right)$

C Check for sufficient homogeneity

Having determined the between-unit and (where appropriate) within-unit standard deviation, the RM producer should confirm that the variation within and between units is sufficiently small for the intended use of the material. Such confirmation may include, for example:

a) comparison of the between-unit and within-unit standard deviation to the uncertainty associated with characterization to confirm that the standard deviation(s) are small compared with the characterization uncertainty (for example, Sub< uchar/3);

b) calculation of the combined uncertainty CRM of the certified value, with allowance for heterogeneity, and confirmation that the uncertainty of the certified value is acceptable for the intended use;

c) for reference materials where the combined uncertainty CRM will not be calculated, checking that the between-unit standard deviation SR is small compared with the typical interlaboratory reproducibility standard deviation SR in the field of use. Ideally Sbb should be less than SR/3;

d) confirmation, for example by use of an F test, that the between-unit term is not statistically significant at the 95% level of confidence.

A method of confirming that the between-unit term is not significantly greater than a predetermined upper limit is given in ISO 13528. (ISO Guide 35:2017, 7.10)

D Classical stability studies-Intermediate conditions of measurement

In the classical stability study, individual samples prepared at the same time (i.e.as a batch), under identical conditions, are measured as time elapses (e.g. one sample immediately, one after three months, the next one after s ix months, etc.). This design, in which the measurements are carried out under intermediate conditions of measurement (sometimes called within-laboratory reproducibility conditions), can lead to a relatively high uncertainty when instability of the measurement system contributes significantly to the dispersion of the measurement results. (ISO Guide 35:2017, 8.3.2.1)

E Transportation or other short-term stability studies

Transportation or other short-term stability studies is a property of the material referring to stability under expected transport conditions. For this, the behavior of the material and its property values are studied under (as a minimum) the intended conditions for packaging and transport. (ISO Guide 35:2017, 8.3.4.1)

F Long-term stability studies

Long-term stability studies are conducted to assess stability under storage conditions specified for the lifetime of the product. Real-time long-term studies typically last 12 months or more; accelerated studies are typically shorter but include more extreme conditions. The period of validity of the certificate is also ensured by stability monitoring after release (ISO GUIDE 35:2017, 8.3.4.2).

The regression parameters can be computed using the procedures by suitable software. The calculations provide estimates b_o and b_1 respectively, of the true intercept and slope $\beta 0$ and $\beta 1$, together with the corresponding standard errors s_{bo} and s_{b1}, which can be used in subsequent statistical tests and in uncertainty evaluation (ISO GUIDE 35:2017, 8.5.2.4).

G Measurement procedure (commutability)

Using the other measurement procedure, analyze (as a single analytical run or batch) the sample 20 or more patient samples, with the same processed samples randomly interspersed among patient samples. Analyze the patient samples and processed samples at the same time using each of measurement procedures. Analyze three or more replicates of each sample with the replicates in sequence in the batch (CLSI EP14-A3:2014, 2.3.1).

H Data visualization by distribution of Means

Perform Deming linear regression analysis using the patients sample means (or transformed means, as appropriate). Graph the means of measurement procedure B as the y-value and the means of measurement procedure A as the X-value. The patient sample means obtained from both procedures used in the Deming regression analysis will be calculated from either transformed or untransformed replicated based on the

pattern of the scatter of results noted above. The processed samples will be treated the same as the patient samples and plotted on the same graph using different symbols (CLSI EP14-A3:2014, 2.4.2).

I Evaluation without the laboratories' uncertainties

Use of analysis of variance for uncertainty evaluation; Analysis of variance (ANOVA) may be used as a tool to process the data. The use of ANOVA can be particularly helpful when assessing uncertainty components such as the between-bottle homogeneity or the between-laboratory standard deviation. Otherwise, the mean of means may be computed for these strategies instead.

Evaluation without the laboratories' uncertainties; Where Formula was used to calculate the certified value, where the data set means follow an approximately normal distribution and no weighting is applied, the standard deviation of the mean of the p data set means yi can be applied as uchar (ISO Guide 35:2017, A.2.5.1). where s(y) denotes the standard deviation of the p data set mean values.

$$u_{char} = \frac{s(y)}{\sqrt{p}} = \frac{1}{\sqrt{p}} \cdot \frac{\sqrt{\sum(y|_i - y_{char})^2}}{P-1}$$

If the results do not follow a normal distribution and cannot be transformed into normally distributed data, a metrologically and statistically sound approach that is consistent with the observed distribution should be applied.