بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Effects of Storage Temperature and Duration on Glucose Levels in Serum and Plasma

تأثير درجة الحرارة ومدّة الحفظ على مستوى سكر الجلكوز فى مصل وبلازما الدم

A dissertation submitted in partial fulfillment for the requirement of M.Sc degree in Medical Laboratory Sciences (Clinical Chemistry)

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الآيـــــة

بسم الله الرحمن الرحيم

قال تعالى:

(وَلَقَد آتَينَا دَاوُودَ وَسُلَيمانَ عِلماً وَقَالَا الحَمدُ لِلَّهِ الَّذِي فَضَّلَنَا عَلَى كَثِيرٍ مِن عِبَادِهِ المُؤمِنِينَ)

سورة النمل (15)

Dedication

I dedicate this dissertation work to my family and many friends. Special feelings of gratitude to my loving mother, whose words of encouragement were always there to support me while crossing this road.

I also dedicate this work to my brothers and sister. Finally, I dedicate this work to my friends who put their efforts and helped in completing this research.

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Grateful thanks to Allah at first, who gave me the health, strength and patience to conduct this study.

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Abstract

Background: Glucose is the main energy source for humans. The nervous system - including the brain- completely depends on glucose for energy. Glucose is considered to be one of the most important parameters in routine analysis and monitoring for diseases such as diabetes mellitus.

Objectives: This study was done to evaluate the effects of storage temperature and duration on the levels of the blood glucose in serum and plasma.

Materials and methods: The study was conducted in Khartoum state from the period of March to August 2018. Including 100 healthy volunteers ages range between 18 and 28 years. The blood samples were dispensed into plain containers for serum preparation and containers with suitable quantity of fluoride oxalate anticoagulant for plasma preparation.

The serum and plasma were used for estimation of glucose using chemical method by automated spectrophotometry and data were analyzed using (SPSS) in computer program.

Results: There was a significant decrease in blood glucose levels in serum and in plasma stored at room temperature and at $4^{o}C$.

Conclusion: From this study it appears that the stability of serum glucose stored at room temperature is lost within 2 hours and within 24 hours when stored at $4^{o}C$.

The plasma was found to be stable at room temperature for not more than 6 hours, and 48 hours when stored at $4^{o}C$.

مستخلص الأطروحة

الخلفية: يعتبر سكر الجلكوز مصدر الطاقة الرئيسي للإنسان ، الجهاز العصبي ومن ضمنه الداغ يعتمد كلياً على الجلكوز كمصدر للطاقة ، ويعتبر الجلكوز أحد أهم الوحدات في التحليلات الروتينية ومتابعة الأمراض كالسكري.

الأهداف: هدفت هذه الدراسة لتقييم تأثير درجة الحرارة ومدة الحفظ على مستوى سكر الجلكوز في مصل وبلازما الدم.

المواد والأساليب: تم اجراء هذه الدراسة في الفترة مابين مارس الى أغسطس 2018 في ولاية الخرطوم. وشملت مائة متطوع صحيح تراوحت أعمارهم بين 18 و 28 سنة. تم وضع عينات الدم في حاوية فارغة لاستخلاص المصل وحاوية أخرى تحتوي على كمية مناسبة من اوكسلايد الفلوريد لاستخلاص البلازما.

تم استخدام المصل والبلازما في قياس الجلكوز باستخدام الطريقة الكيميائية عبر جهاز قياس الضوء الطيفي الآلي ، وتم تحليل البيانات باستخدام برنامج الحزمة الاحصائية للعلوم الاجتماعية على الحاسوب.

النتائج: كان هناك نقصان دال احصائياً للجلكوز في المصل والبلازما المحفوظتان في درجة حرارة الغرفة وفي الثلاجة.

الخلاصة: من هذه الدراسة تظهر أن استقرارية الجلكوز في المصل المخزن في درجة حرارة الغرفة هي ساعتين أو أربع وعشرين ساعة ان خُزن في الثلاجة.

أما الجلكوز في البلازما فوُجد مستقراً في حرارة الغرفة لفترة لا تتجاوز الست ساعات ، وثمانٍ وأربعين ساعة إن خُزن في الثلاجة.

Table of contents

List of tables

List of figures

Chapter One

Introduction, Rationale and Objectives

1.1 Introduction:

Glucose is the main energy source for humans. The nervous system, including the brain, completely depends on glucose from the surrounding extracellular fluid for energy. Nervous tissues do not have the ability for carbohydrates storage; therefore, it is important to maintain a steady supply of glucose to these tissues. When the concentration falls below a certain level, the nervous tissue loses the primary energy source and are incapable of maintaining normal function (Bishop *et al*., 2010).

Specimen collection and handling can result in inaccurate test results, which can lead to the mismanagement of a patient. Some of the most common errors include clerical errors, hemolyzed samples, and incorrect use of anticoagulants. Hemolysis from a venipuncture can lead to erroneous results for an array of analytes (Kitchens, 2006).

The temperature and time at which a sample is stored can have a great influence on prescription drug concentration, analytes values, and enzyme stability in the blood sample. For instance, Bennetto *et al*. (2004) proved that variable temperatures and lengths of storage time can alter the stability of neviraprine, a highly prescribed HIV treatment.

Analytes values as creatinine, phosphorus, potassium, aspartate aminotransferase, and alanine aminotransferase in blood samples can also be affected by variables such as temperature (Rehak and Chiang, 1988).

Blood samples kept in serum or plasma at 25ºC have significant changes in nine of the blood profile tests included in the comprehensive metabolic panel. Glucose, potassium, creatinine, total protein, calcium, albumin,

alanine aminotransferase, and CO_2 are all affected by storage duration factor (Boyanton and Blick, 2002).

The effects of blood storage time on the accuracy of comprehensive metabolic panel results were investigated by Jessica L. Kitchens (2006), and concluded a significant change in parameters results including glucose.

1.2 Rationale:

Blood glucose estimation has always been an easy and a productive mean for the diagnosis and short term prediction of diabetes complications and hypoglycemia.

In America alone 1.5 million individuals are diagnosed with diabetes each year (ADA, 2017). In a developing country like ours, laboratory personnel are constantly faced with the problems of inconsistent power supply such that specimens for analysis are left on the laboratory bench at ambient temperature for several hours or even days before analysis can be done.

This study aims to investigate the changes in glucose levels in plasma and serum samples stored at $4^{o}C$ and $25^{o}C$.

In Sudan there is no published study concerned with the impact of storage temperature or duration on blood glucose in serum and plasma.

1.3 Objective:

1.3.1General Objective:

To study the effects of storage temperature and duration on glucose levels in serum and plasma.

1.3.2 Specific Objectives:

1 -To estimate glucose concentration from serum and plasma samples stored at room temperature after 0, 2, 6 and 12 hours after collection.

2- To estimate glucose concentration from serum and plasma samples stored at $4^{o}C$ after 24, 48, 72 hours after collection.

3-To detect the presence of statistically significant difference between baseline values and later estimation points made at room temperature and at $4^{o}C$.

Chapter Two

Literature review

2.1 Carbohydrates:

Carbohydrates are aldehyde or ketone derivatives of polyhydric alcohols or any substance derived from them. They are widely distributed both in plants and in animal tissues. In plants, they are produced by photosynthesis. Carbohydrates constitute about 60% of our diet. They are important for: Energy production, Formation of structural elements in animal and plant cells, and formation of glycolipids (carbohydrates combined with lipids) and glycoproteins (carbohydrates combined with protein); both enter in the structure of cell membrane and form the ground substances between tissues (Said Oraby, 2013).

2.1.1 Classification of carbohydrates:

All carbohydrates are compounds of carbon, hydrogen and oxygen. They can be classified in many different ways. One common way is according to the size of the molecule into:

A. Monosaccharides: These are the simplest carbohydrate molecules. The most commonly occurring monosaccharides in food are glucose, fructose and galactose.
B. Disaccharides: These sugars are formed when two monosaccharide molecules join together with the removal of one molecule of water. They have the general formula $C_{12}H_{22}0_{11}$. Examples of disaccharides are sucrose (glucose and fructose), lactose (glucose and galactose) and maltose (2 molecules of glucose).
C. Oligosaccharides: Oligosaccharides are carbohydrate molecules that have longer carbon chain lengths than sugars (mono and

disaccharides) but are shorter than polysaccharides. They generally have carbon chains of between 3 and 10 carbon molecules. Generally, humans do not have enzymes that digest oligosaccharides so that they pass through the digestive tract and may be metabolised by gut bacteria.

D. Polysaccharides: Polysaccharides are made up of many monosaccharide molecules (usually glucose), joined together. They have the general formula $(C_6H_{10}0_5)_n$ where `n´ is a large number. Examples of polysaccharides are starch, glycogen (the form in which glucose is stored in the body), and cellulose, beta glucan and pectin (components classed as dietary fiber) (BNF, 2012).

2.1.2 Digestion of carbohydrates:

The goal of carbohydrate digestion is to break down all disaccharides and complex carbohydrates into monosaccharides for absorption, although not all are completely absorbed in the small intestine (e.g., fiber).

Digestion begins in the mouth with salivary amylase released during the process of chewing. There is a positive feedback loop resulting in increased oral amylase secretion in people consuming diets high in carbohydrates. The amylase is synthesized in the serous cells of the salivary glands. Amylase breaks starches into maltose and polysaccharides. Amylase is sensitive to pH and thus is inhibited in the acidic environment of the stomach. Only 5% of starch is broken down by salivary amylase due to limited exposure. Salivary amylase has increased importance in two groups; infants with decreased pancreatic amylase production in the first 9 months and children with pancreatic insufficiency from cystic fibrosis or other etiologies. Minimal carbohydrate digestion occurs in the stomach due to the inactivation of

amylase in the acidic environment. Pancreatic amylase is released from acinar cells into the small intestine in concert with other enzymes under the stimulus of secretin and Cholecystokinin (CCK) and continues the process of carbohydrate digestion. Amylase targets the α-1,4 bonds of complex carbohydrates and is unable to break terminal bonds or α-1,6 bonds. Starch is digested in the small intestine to simple components derived from branched amylopectin (maltose, maltotriose and α-limit dextrins). Oligosaccharides and disaccharides are digested by specific enzymes in the microvillus membrane (brush border).

Brush border enzymes are synthesized in the endoplasmic reticulum and glycosylated in the Golgi apparatus of the enterocyte. They are then trafficked to the apical membrane where they are anchored at the surface by a transmembrane segment. The anchored enzymes are active following cleavage of a small residue at the extracellular N-terminal end. Disaccharidases are protected from proteolysis by glycosylation and are found in higher concentration in villus enterocytes of the proximal small bowel. These enzymes include maltase (digests maltose to glucose and glucose), sucrase (digests sucrose to fructose and glucose), trehalase (digests trehalose to glucose and glucose), lactase (digests lactose to galactose and glucose) and isomaltase (de-branching enzyme digests α1,6 bonds of limit dextrin to produce glucose). Glucose does not require any additional digestion (Christine Hurtado, 2016).

2.1.3 Absorption of carbohydrates:

Once carbohydrates are digested, the products must be absorbed and transported to the portal circulation. Digestion and absorption are typically coupled, with the enzymes closely located to the appropriate transporters. Glucose absorption occurs in the small intestine via the SGLT-1 transporter

(sodium glucose co-transporter). Fructose absorption is completed via the GLUT5 transporter by facilitated diffusion. Glucose and galactose are actively transported from the small intestine lumen by the sodium glucose transporter SGLT-1 located in the brush border of the small intestine. The transporter is more prevalent in the duodenum and jejunum. Glucose transport is driven by a sodium gradient across the apical cell membrane generated by the Na^+/K^+-ATPase pump located in the basolateral membrane of the enterocyte. The Na^+/K^+-ATPase pump creates a low intracellular sodium concentration by transporting 3 Na^+ ions out of the cell and 2 K^+ ions into the cell. The SGLT-1 transporter utilizes the sodium gradient. Two Na^+ ions bind to the outer face of the SGLT-1 transporter which results in a conformational change permitting subsequent glucose binding. The two Na^+ ions and the glucose molecule are then transferred to the cytoplasmic side of the membrane following another conformational change that involves rotation of the receptor. The glucose is released first followed by the sodium ions. The sodium is transported from high to low concentration (with concentration gradient) and at the same time allows the carrier to transport glucose against its concentration gradient. The Na^+ ion is subsequently expelled by Na^+/K^+-ATPase pump to maintain the gradient. The SGLT-1 transporter undergoes another conformational change resulting in the binding sites again being exposed at the apical surface. This action can occur one thousand times per second. Much of the glucose transported into the cell passes out of the cell at basolateral surface by facilitated diffusion via GLUT-2. Sodium ions and accompanying anions and water follow the glucose, maintaining iso-osmolarity. A small portion of the glucose is utilized by the cell. Facilitated diffusion is the mechanism for fructose transport. Facilitated diffusion utilizes a carrier protein to achieve transport

at rates greater than simple diffusion and does not rely on concentration gradients. GLUT-5 is present on the apical membrane of the brush border throughout the small intestine with increased density in the proximal small intestine. Little fructose is metabolized in the cell. Both GLUT-2 and GLUT-5 are present at the basolateral membrane to transport fructose to the portal circulation (Christine Hurtado, 2016).

2.1.4 Fate of absorbed carbohydrates:

Monosaccharides (glucose, galactose and fructose) resulting from carbohydrate digestion are absorbed and undergo the following:

A. **Uptake by liver:**

After absorption the liver takes up sugars, where galactose and fructose arc converted into glucose.

B. **Glucose utilization by tissues:**

Glucose undergoes one of three fates:

A-Oxidation mainly through major pathways for the production of energy.

B- Stored in the form of glycogen and fat.

C- Converted to substances of biological importance such as ribose, lactose, fructose (in semen), or glucuronic acid (Said Oraby, 2013).

2.2 Glucose:

Glucose is a major energy substrate. It is the only utilizable source of energy for some tissues such as erythrocytes and, in the short term, the central nervous system. Many tissues are capable of oxidizing glucose completely to carbon dioxide; others metabolize it only as far as lactate, which can be converted back into glucose, principally in the liver and also in the kidneys, by gluconeogenesis. Even in tissues capable of completely oxidizing glucose, lactate is produced if insufficient oxygen is available. The body's sources of glucose are dietary carbohydrate and endogenous production by

glycogenolysis and gluconeogenesis. Glycogen is stored in the liver and skeletal muscle, but only the former contributes to blood glucose (Marshall *et al*., 2012).

2.2.1 Regulation of blood glucose:

The concentration of blood glucose is regulated by multiple pathways with multiple hormones (Said Oraby, 2013).

During fasting state, a progressive decline in blood glucose concentration is stopped by glycogenolysis in the liver. A small amount of glucose also may be derived from synthesis within the kidneys. These organs possess the enzymes which are necessary to produce glucose by both gluconeogenesis and glycogenolysis.

The concentration of glucose in the blood is normally maintained within a narrow interval by hormones that modulate the movement of glucose within the body. These include insulin, which decreases blood glucose, and the counter regulatory hormones (glucagon, epinephrine, cortisol, and growth hormone), (Carl *et al*., 2008).

2.2.2 Disorders of glucose metabolism:

2.2.2.1 Hypoglycemia:

Clinical hypoglycemia is a plasma or serum glucose concentration low enough to cause symptoms and/or signs, including impairment of brain function (Philip Cryer *et al*., 2009).

Symptoms of hypoglycemia are categorized as neuroglycopenic (the result of brain glucose deprivation) and neurogenic or autonomic (largely the result of the perception of physiological changes caused by the sympathoadrenal discharge triggered by hypoglycemia). Some neurogenic symptoms, such as palpitations, tremor, and arousal/anxiety, are adrenergic whereas others,

such as sweating, hunger, and paresthesias, are cholinergic (Towler *et al*., 1993).

Neuroglycopenic symptoms range from behavioral changes, fatigue, and confusion to seizure and loss of consciousness, *i.e.* functional brain failure (Cryer, 2007).

Seemingly complete recovery after the glucose level is raised is the rule, although on rare occasions neurological recovery is delayed. Profound, prolonged hypoglycemia can cause brain death (Cryer, 2007).

In healthy individuals, symptoms of hypoglycemia develop at a mean plasma glucose concentration of approximately 55 mg/dl (3.0 mmol/liter) (Cryer, 2001).

because of the provision of alternative circulating fuels to the brain (specifically ketones), lower plasma glucose concentrations occur in healthy individuals, particularly in women and children, without symptoms or signs during extended fasting (Cryer, 2001).

A hypoglycemic etiology may be apparent (*e.g.* in a patient with insulin-treated diabetes) or a diagnostic challenge (*e.g.* in a seemingly well individual with an insulinoma). On the other hand, in a person who does not have diabetes mellitus an unequivocally normal plasma glucose concentration [*e.g.* more than 70 mg/dl (3.9 mmol/liter)] during a symptomatic episode indicates that those symptoms are not the result of hypoglycemia (Philip Cryer *et al*., 2009).

2.2.2.2 Hyperglycemia:

Hyperglycemia is defined as an excess of glucose in the bloodstream, often associated with diabetes mellitus (OED, 2018).

In healthy individuals, during a hyperglycemia state, insulin is secreted by the β-cells of the pancreatic islets of Langerhans. Insulin enhances

membrane permeability to cells in the liver, muscle, and adipose tissue. It also alters the glucose metabolic pathways. Hyperglycemia, or increased plasma glucose levels, is caused by an imbalance of hormones (Bishop *et al*., 2010).

Diabetes mellitus can be defined as a group of metabolic disorders characterized by a chronic hyperglycemic condition resulting from defects in insulin secretion, insulin action or both. Permanent neonatal diabetes is caused by glucokinase deficiency, and is an inborn error of the glucose-insulin signaling pathway (Njolstad *et al*., 2003).

Signs and symptoms include polydipsia (excessive thirst), polyphagia (increased food intake), polyuria (excessive urine production), rapid weight loss, hyperventilation, mental confusion, and possible loss of consciousness (due to increased glucose to brain). Complications include microvascular problems such as nephropathy, neuropathy, and retinopathy. Increased heart disease is also found in patients with diabetes (Bishop *et al*., 2010).

2.2.2.2.1 Type 1 diabetes mellitus:

Type 1 diabetes mellitus is a chronic autoimmune disease associated with selective destruction of insulin-producing pancreatic β-cells. The onset of clinical disease represents the end stage of β-cell destruction leading to type 1 diabetes mellitus (Ozougwu *et al*., 2013).

The autoimmune destruction of pancreatic β-cells, leads to a deficiency of insulin secretion which results in the metabolic derangements associated with type 1 diabetes. In addition to the loss of insulin secretion, the function of pancreatic α-cells is also abnormal and there is excessive secretion of glucagons. Normally, hyperglycemia leads to reduced glucagons secretion, however, in patients with IDDM, glucagons secretion is not suppressed by hyperglycemia (Raju and Raju, 2010).

2.2.2.2.2 Type 2 diabetes mellitus:

Type 2 diabetes is the predominant form of diabetes and accounts for at least 90% of all cases of diabetes mellitus (Gonzalez *et al*., 2009). It's a heterogeneous disorder caused by a combination of genetic factors related to impaired insulin secretion, insulin resistance and environmental factors such as obesity, over eating, lack of exercise, and stress as well as aging (Kaku, 2010).

2.2.2.2.3 Gestational diabetes:

Gestational Diabetes Mellitus (GDM) is carbohydrate intolerance of variable severity with onset or first recognition during pregnancy (Metiger and Cuustan, 1998).

Women with GDM are at a significantly increased risk of subsequent diabetes, predominantly type 2. The cumulative incidence of type 2 diabetes after GDM varies among populations, ranging from 40% to 70% (Carl *et al*., 2008).

2.2.2.2.4 Other types of diabetes:

This subclass includes patients in whom hyperglycemia is due to a specific underlying disorder, such as (1) genetic defects of β-cell function; (2) genetic defects in insulin action; (3) disease of the exocrine pancreas; (4) endocrinopathies (e.g., Cushing disease, acromegaly, and glucagonoma); (5) the administration of hormones or drugs known to induce β-cell dysfunction or impair insulin action (e.g., glucocorticoids); (6) infections; (7) uncommon forms of immune-mediated diabetes; or (8) other genetic conditions (e.g., Down syndrome). This was formerly termed secondary diabetes (Carl *et al*., 2008).

2.2.3 Methods for estimation of glucose:

2.2.3.1 Hexokinase Methods:

Hexokinase (HK) methods are based on a coupled enzyme assay that uses HK and glucose-6-phosphate dehydrogenase(G-6-PD).

Glucose is first phospholylated by ATP in the presence of HK and Mg^{2+}. The glucose-hephosphate formed isoxidized by G-6-PD to 6-phosphogluconate in the presence of nicotinamide-adenine dinucleotide phosphate (NADP"). The amount of reduced NADP (NADPH) produced is directly proportional to the amount of glucose in the sample and is measured by the increase in absorbance at 340nm.

Sources of interference include hemolyzed samples, drugs, bilirubin, and lipemia (Carl *et al.*, 2008).

2.2.3.2 Glucose Oxidase Methods:

Glucose oxidase catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide. Addition of the enzyme peroxidase and a chromogenic oxygen acceptor, such as o-dianisidine, results in the formation of a colored compound that is measured. Glucose oxidase is highly specific for β-D-glucose. Because 36% and 64% of glucose in solution are in the *a*- and β-forms, respectively, complete reaction requires mutarotation of the *a*- to β-form. Some commercial preparations of glucose oxidase contain an enzyme, mutarotase, that accelerates this reaction. Otherwise, extended incubation time allows spontaneous conversion.

The second step, involving peroxidase, is much less specific than the glucose oxidase reaction. Various substances, such as uric acid, ascorbic acid, bilirubin, hemoglobin, tetracycline, and glutathione, inhibit the reaction (presumably by competing with the chromogen for H202), producing lower values.

2.2.3.3 Glucose Dehydrogenase Method:

The enzyme glucose dehydrogenase catalyzes the oxidation of glucose to gluconolactone. Mutarotase is added to shorten the time necessary to reach equilibrium. The amount of NADH generated is proportional to the glucose concentration. The reaction appears to be highly specific for glucose, shows no interference from common anticoagulants and substances normally found in serum, and provides results in close agreement with hexokinase procedures.

2.2.3.4 Implanted Sensors technique:

Several implanted biosensors have been developed. The most widely studied method is an electrochemical sensor that uses glucose oxidase. This sensor is implanted either intravenously or subcutaneously. Alternatives to enzymes are being developed, including artificial glucose "receptors". Implantation of a needle type of sensor into the subcutaneous tissue induces inflammatory responses that alter the sensitivity of the device. Microdialysis with hollow fibers or ultrafiltration with biologically inert material has been used to minimize this problem.

2.2.3.5 Minimally invasive glucose monitoring:

The devices are based on the observation that the concentration of glucose in the interstitial fluid correlates with blood glucose concentration. For example, the principle of the Gluco Watch is the application of a low-level electric current on the skin. This induces movement by electro-osmosis of glucose across the skin where it is measured by a glucose oxidase detector. Glucose concentrations in transdennal fluid and plasma are highly correlated.

2.2.3.6 Noninvasive Glucose Monitoring:

Near-infrared spectroscopic devices measure either the absorption or reflection of light from subcutaneous tissue, but many substances interfere. A computer, individually calibrated, screens out interfering information to obtain the glucose result. Similar limitations have prevented successful use of light scattering. Photoacoustic spectroscopy, which uses pulsed infrared light, is a newer technique that shows some promise. (Carl *et al.*, 2008).

2.3 Effects of storage duration and temperature on glucose concentration:

The results of blood glucose determinations can be strongly affected by the method of storage and handling of the blood samples between the time of collection and the time of analysis (Young and Bermes, 1999). Improperly handled or stored samples could generate results that may mislead the clinician into wrong judgment/diagnosis (Clark *et al*., 2003).

Since prolonged contact of plasma or serum with cells is a common cause of spurious test results, plasma and serum should ideally be separated from cells as quickly as possible to prevent on going metabolism of cellular constituents as well as active and passive movement of analytes between the plasma or serum and cellular compartments (Ono *et al*., 1981; Murphy *et al*., 2000). Standard guidelines for blood sample handling and processing states that serum or plasma should be physically separated from contact with cells as soon as possible unless conclusive evidence shows that longer contact times do not contribute to result inaccuracy. A maximum time of two hours from the time of sample collection to the time of separation of serum and plasma from cells was also recommended (NCCLS Infobase, 1995; Young and Bermes, 1999).

Plasma glucose concentrations must be measured with a reliable laboratory method, not with self-monitoring of blood glucose. Although a distinctly low, reliably measured plasma glucose concentration obtained in the absence of recognized symptoms or signs should not be ignored, that finding raises the possibility of "pseudohypoglycemia"— an artifact of continued glucose metabolism by the formed elements of the blood after the sample is drawn. That may occur when the blood sample is collected in a tube that does not contain an inhibitor of glycolysis and separation of the plasma (or serum) from the formed elements is delayed, particularly in the setting of erythrocytosis, leukocytosis, or thrombocytosis (Cryer, 2008).

2.4 Previous studies:

Rapid blood separation was found to be superior to florid for preventing in vitro reductions in measured blood glucose concentration (Shi *et al*., 2009). And the serum samples if analyzed immediately were better in accuracy than plasma samples with antiglycolytic agent which are analyzed with loiter (Turchiano *et al*., 2013; Waring *et al*., 2007).

Attention to proper blood handling, processing, and storage procedures, and avoidance of hemolysis are important in blood clinical analyses and in the proper interpretation of experimental results (Morris *et al.*, 2002). Different storage times were also found to be contributing to a significant reduction in neonatal blood glucose levels along with the length of storage duration (Xiao-Li Zhu *et al.*, 2017)

The effectiveness of sodium florid as a glycolysis inhibitor on blood glucose measurement was questioned. The analysis of paired blood samples with and without antiglycolytic agent was needed to conclude a significant reduction in the delayed plasma glucose concentration (Lee *et al.*, 2009).

Chapter Three

Materials and methods

3.1 Materials:

3.1.1 Study design:

This was an analytical case study.

3.1.2 Study area:

The current study was conducted in Khartoum state from the period of March to August 2018.

3.1.3 Study population:

Apparently healthy individuals.

3.1.4 Sampling:

3.1.4.1 Sample size:

The study was conducted on 100 apparently healthy subjects.

3.1.4.2 Sampling technique:

Samples were collected using simple random technique.

3.1.5 Inclusion criteria:

Apparently healthy individuals from Khartoum state, ages range between 18 and 28 years from both genders were enrolled in this study.

3.1.6 Exclusion criteria:

Individuals suffering from jaundice, gout or and under medications such as ascorbic acid, salicylate, and beta lactam antibiotics were excluded from this study.

3.1.7 Ethical consideration:

Verbal informed consent was obtained from each enrolled subject. Ethical approval was obtained from research committee of the university.

3.1.8 Data collection

A structured questionnaire was designed to provide personal and medical information about the study subjects.

3.1.9 Blood sampling and processing:

6 ml of venous blood was collected from each enrolled subject. The blood samples were dispensed into each of the following collection tube:

a-half (3 ml) was added into plain containers, allowed to stand at room temperature for 15 minutes to clot, after which it was centrifuged at 3000 rpm for 5 minutes, and the serum from it was separated into another plain container

b-The other half was added into a container with suitable quantity of florid oxalate anticoagulant and centrifuged immediately after collection at 3000 rpm for 5 minutes, and the plasma from it was dispensed into a plain container.

3.2 Methodology:

The plasma and serum samples from each patient were analyzed for glucose concentrations immediately after separation to obtain the baseline values.

The plasma and serum samples were then divided into two equal parts: one part was kept in a refrigerator at 4ºC while the other was kept in the laboratory bench at room temperature (25-30ºC). Further analysis on the samples kept at room temperature was carried out after 2, 6, and 12 hours. Samples stored at 4ºC were analyzed after 24, 48, and 72 hours after separation.

3.2.1 Estimation of blood glucose:

Serum and plasma glucose were determined by an automated glucose oxidase method using a full automated instrument (mindray BS-200). The kits supplied by (SPINREACT, Spain).

Principle of the method:

Glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid. The formed hydrogen peroxide (H_2O_2), is detected by a chromogenic oxygen acceptor, phenol-aminophenzone in the presence of peroxidase (POD). The intensity of the color formed is proportional to the glucose concentration in the sample.

Reagent and procedure refer to appendix III page

3.2.2 Quality control Method:

Controls level I and level II were used with each patch to ensure the validity of the results.

3.2.3 Statistical analysis method:

The analysis of results was done using SPSS, and the Microsoft Excel computer program. Paired T-test was used for comparison between glucose levels at different temperatures and times.

Chapter Four

Results

This study was conducted on 100 healthy subjects of the same age group (18-28 years old) from both genders to investigate the effects of storage duration on glucose levels in serum and plasma stored at room temperature (25-30^{o}C) and at refrigerator temperature (4^{o}C).

The results were compared according to the objectives of the study as illustrated below:

Table (4-2) Shows subsequent significant decrease (P = 0.000) in glucose concentration in serum samples stored at 25-30^{o}C compare with that immediately measured (Zero time) which indicate instability of glucose in serum after two hours storage at room temperature.

Table (4-3) Shows subsequent significant decrease (P = 0.000) in glucose concentration in serum samples stored at 4^{o}C compare with that immediately measured (Zero time) which indicate instability of glucose in serum after 24 hours storage at 4^{o}C

Table (4-4) Shows subsequent significant decrease (P = 0.000) in glucose concentration in plasma samples stored at 25-30^{o}C after 6 hours compare with that immediately measured (Zero time) which indicate instability of glucose in plasma after 6 hours storage at room temperature.

Table (4-5) Shows subsequent significant decrease (P = 0.000) in glucose concentration in plasma samples stored at 4^{o}C compare with that immediately measured (Zero time) after 48 hours which indicate instability of glucose in serum after 48 hours storage at 4^{o}C.

Table (4-1) descriptive data.

Temperature	Duration of storage	Mean concentration (mg/dl)	
		plasma	serum
Baseline value	0 hour	99.13 ± 14.07	95.83 ± 13.60
Room temperature ($25^{o}C$)	2 hours	99.06 ± 14.09	94.55 ± 13.67
	6 hours	98.65 ± 14.03	91.87 ± 13.86
	12 hours	97.62 ± 14.08	87.7 ± 14.15
Refrigerator temperature ($4^{o}C$)	24 hours	99.03 ± 14.10	94.34 ± 13.53
	48 hours	98.32 ± 14.08	92.07 ± 13.55
	72 hours	96.87 ± 14.15	89.6 ± 13.58

Table (4-2) The percentage change in glucose concentration in serum samples stored at 25-30^{o}C compared with that immediately measured (Zero time)

Duration of storage (hours)	Percentage (%) change in serum glucose concentration stored at room temperature	P value
2	- 1.37 ± 0.88	0.000**
6	- 4.23 ± 1.88	0.000**
12	- 8.67 ± 3.22	0.000**

Analyzed by paired T-test.

**: significance at less than 0.01level.

Table (4–3) The percentage change in glucose concentration in serum samples stored at 4^{o}C compared with that immediately measured (Zero time)

Duration of storage (hours)	Percentage change in serum glucose concentration stored at 4^{o}C (%)	P value
24	- 1.57 ± 0.85	0.000**
48	- 3.98 ± 1.38	0.000**
72	- 6.59 ± 2.07	0.000**

Analyzed by paired T-test.

**: Significance at less than 0.01 level

Table (4-4) The percentage change in glucose concentration in plasma samples stored at 25-30^{o}C compared with that immediately measured (Zero time)

Duration of storage (hours)	Percentage (%)change in plasma glucose concentration stored at room temperature.	P value
2	- 0.08 ± 0.56	0.210^{ns}
6	- 0.49 ± 0.70	0.000**
12	- 1.55 ± 0.97	0.000**

Analyzed by paired T-test.

**: significance at less than 0.01 level, ns: no significant difference.

Table (4-5) The percentage change in glucose concentration in plasma samples stored at $4^{o}C$ compare with that immediately measured (Zero time)

Duration of storage (hours)	Percentage (%) change in plasma glucose concentration stored at $4^{o}C$	P value
24	- 0.11 ± 0.70	0.141^{ns}
48	- 0.83 ± 0.80	0.000^{**}
72	- 2.33 ± 1.20	0.000^{**}

Analyzed by paired T-test.

**: Significance at less than 0.01 level, ns: no significant difference.

Chapter Five

Discussion, conclusions and recommendations

5.1 Discussion:

Glucose is considered to be one of the most important parameter in routine analysis and monitoring for disorders such as diabetes mellitus. This study was primarily designed to estimate glucose concentrations in healthy Sudanese individuals in an attempt to spot the effects of storage duration and storage temperature on serum and plasma glucose levels.

This study showed a significant reduction in serum glucose at room temperature (25-30^{o}C) within the first two hours of storage (*p.* value 0.000) and this result agrees with (Nwosu and Nwani, 2008) and (Oddoze *et al*., 2012) indicating instability of glucose concentration in serum after two hours of storage at room temperature, and disagrees with (Marjani, 2006) who found a significant reduction only after 24 hours of storage.

The results also show a significant reduction in serum glucose stored at 4^{o}C after twenty-four hours (*p.* value 0.000) and this finding came in agree with (Kitchen, 2006) and (Oddoze *et al*., 2012) indicating instability of glucose concentration in serum after twenty-four hours storage at 4^{o}C. and disagrees with (Marjani,2006) and (Nwosu and Nwani, 2008) who found that reduction wasn't significant until 48 hours of storage.

For plasma, the study proved that plasma stored at 25-30^{o}C loses its stability within the first six hours (*p.* value 0.000) and this result agrees with (Nwosu and Nwani, 2008). Unlike its stability at 4^{o}C which was lost within the second day of storage (*p.* value 0.00) and this finding came in disagree with (Nwosu and Nwani, 2008) who found it to be stable until the third day.

5.2 Conclusion:

The stability of serum glucose stored at room temperature was found to be sensitive to delay within 2 hours, while the sensitivity for serum glucose stored at $4^{o}C$ within 24 hours of storage.

The plasma was found to be stable at room temperature for no more than 6 hours, and 48 hours when stored at $4^{o}C$.

5.3 Recommendations:

- Serum and plasma samples should be processed timely and the analysis done immediately for glucose measurement.
- more studies can be applied to compare the effects of different anticoagulant on glucose levels.
- Follow-on studies would benefit from the inclusion of pathological subjects.
- More analytes such as potassium, lactate dehydrogenase, and phosphorus could be studied.
- More studies can be applied to evaluate clot contact time effect.

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Appendices (I)

بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Effects of Storage Temperature and Duration on Glucose Levels in Serum and Plasma

Questionnaire

No ( )

A. General information:

Name: ……. Age: ……..years

Gender: Male ☐ Female ☐

B. Clinical information:

Do you have any of the following conditions?

Gout No ☐ Yes ☐

Liver disease No ☐ Yes ☐

Are you under medications? No ☐ Yes ☐

C. Laboratory investigation:

A. Results obtained at room temperature ($25^{o}C – 30^{o}C$):

	Serum	Plasma
After 0 hour		
After 2 hours		
After 6 hours		
After 12 hours		

B. Results obtained at $4^{o}C$:

	Serum	Plasma
After 24 hours		
After 48 hours		
After 72 hours		

Date:…… Signature:……

Appendices (II)

Mindray BS-200

Appendices (III)

SPINREACT

CE

GLUCOSE -LQ

Glucose

GOD-POD. Liquid

Quantitative determination of glucose
IVD

Store at 2-8ºC

PRINCIPLE OF THE METHOD
Glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid. The formed hydrogen peroxide (H_2O_2), is detected by a chromogenic oxygen acceptor, phenol, 4 – aminophenazone (4-AP) in the presence of peroxidase (POD):

$$\beta\text{-D-Glucose} + O_2 + H_2O \xrightarrow{GOD} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + \text{4-AP} \xrightarrow{POD} \text{Quinone} + H_2O$$

The intensity of the color formed is proportional to the glucose concentration in the sample[1,2].

CLINICAL SIGNIFICANCE
Glucose is a major source of energy for most cells of the body; insulin facilitates glucose entry into the cells.
Diabetes is a disease manifested by hyperglycemia; patients with diabetes demonstrate an inability to produce insulin[1,2,6].
Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

REAGENTS

R	TRIS pH 7.4	92 mmol/L
	Phenol	0,3 mmol/L
	Glucose oxidase (GOD)	15000 U/L
	Peroxidase (POD)	1000 U/L
	4 – Aminophenazone (4-AP)	2,6 mmol/L

PREPARATION
The reagent is ready to use.

STORAGE AND STABILITY
All the components of the kit are stable until the expiration date on the label when stored tightly closed at 2-8ºC, protected from light and contaminations prevented during their use.
Do not use reagents over the expiration date.

SIGNS OF REAGENT DETERIORATION:
- Presence of particles and turbidity.
- Blank absorbance (A) at 505 nm ≥ 0,32

ADDITIONAL EQUIPMENT
- SPIN640 / SPIN640Plus Autoanalyzer.
- General laboratory equipment.

SAMPLES
Serum or plasma, free of hemolysis[1].
Serum should be removed from the clot as quickly as possible.
Stability of the sample: Glucose in serum or plasma is stable at 2-8º for 3 days.

QUALITY CONTROL
Control sera and calibrators are recommended to monitor the performance of assay procedures: SPINTROL H Calibrator, SPINTROL H Normal and Pathologic (Ref. 1002011, 1002120 and 1002210).
If control values are found outside the defined range, check the instrument, reagents and technique for problems.
Each laboratory should establish its own Quality Control scheme and corrective actions if controls do not meet the acceptable tolerances.

BARCODED REAGENTS LOAD MUST BE PRECEDED OF A SPINREACT "DATABASE" COPY INTO THE ANALYZER SOFTWARE. IT IS AVAILABLE UNDER REQUEST TO SPINREACT.

SPIN640 APPLICATION

TEST INFORMATION		REAGENT VOLUME			
N0	**	Vol. R1	300		
Test	GLU	Vol. R2			
Full Name	Glucose	Vol. R3			
Standard nº	1	Vol. R4			
SAMPLE VOLUME		**RESULT SETUP**			
Vol. Sample Stand.	3	Decimal	1	Slope	1
Vol. Sample Increas.		Unit	mg/dL	Inter.	0
Vol. Sample Dec					
REACTION PARAMETERS					
Reac. Type	End Point	Direction	Increase		
Pri. Wave.	505	Reagent Blank	10-11		
Sec. Wave.		React. Time	79-80		

SPIN640Plus APPLICATION

EDIT PARAMETERS					
Test	GLU	No.	**		
Full name	GLUCOSE	Print name	GLU		
Reac. Type	End Point	Direction	Increase		
Pri. Wave.	505	Sec. Wave			
Unit	mg/dL	Decimal		0.1	
Reagent Blank	10 - 11	React. Time	51 - 52		
Vol. Sample	3 ul	R1	300 ul		
Increased		R2			
Decreased		R3			
Sample blank		R4			

The Calibration is stable until 36 days. After this period the Calibration must be performed again in order to obtain good results.

REFERENCE VALUES[1]
Serum or plasma:
60 – 110 mg/dL ≙ 3,33 – 6,10 mmol/L
These values are for orientation purpose; each laboratory should establish its own reference range.

PERFORMANCE CHARACTERISTICS
Measuring range: From *detection limit* 0,3709 mg/dL to *linearity limit* 500 mg/dL.
If the concentration is greater than linearity limit dilute 1/2 the sample with ClNa 9 g/L and multiply the result by 2.
Precision:

	Intra-assay (n=20)		Inter-assay (n=20)	
Mean (mg/dL)	98,5	264,6	92,5	250
SD	0,5754	1,2733	2,76	6,44
CV (%)	0,59	0,48	2,98	2,57

Sensitivity: 1 mg/dL = 0,0039 (A).
Accuracy: Results obtained using SPINREACT reagents (y) did not show systematic differences when compared with other commercial reagent (x).
The results obtained using 50 samples were the following:
Correlation coefficient (r)²: 0,99492.
Regression equation: y=1,104x - 1,249.
The results of the performance characteristics depend on the analyzer used.

NOTES
1. Calibration with the aqueous standard may cause a systematic error in automatic procedures. In these cases, it is recommended to use a serum Calibrator.
2. Use clean disposable pipette tips for its dispensation.

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PACKAGING

Ref: MD41011	Cont.	R: 6 x 40 mL

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