Quantitative Proteinuria Screening UV Method to Check Kidney Health Status

By

Yadeno Yazachew

A Thesis Submitted to

The School of Biomedical Engineering

Presented in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Biomedical Engineering

(Bioinstrumentation)

JIMMA UNIVERSITY, SCHOOL OF GRADUATE STUDIES OF JIMMA, ETHIOPIA

Jan 2019

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Advisor: Olu Emanuel Femi (PhD.)
Co-Advisor: Amare Gessesse (PhD.)

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DECLARATION

I hereby declare that this thesis entitled **Quantitative Proteinuria Screening UV Method to Check Kidney Health Status** submitted to Jimma Institute of Technology School of Biomedical Engineering in partial fulfillment of the requirements for the Degree of Master of Science in Biomedical Engineering, Bioinstrumentation specialization area is entirely my work except paraphrased or quoted work whose sources are appropriately cited.

Author: Yadeno Yazachew Sign_____ Date_____

On behalf of the School of Biomedical Engineering at the Jimma Institute of Technology, we the mentors of this research **Quantitative Proteinuria Screening UV Method to Check Kidney Health** and me, the evaluator, confirm that this research has approved as MSc thesis for the Student.

Mentors

Name: Dr. Olu Emanuel Femi Sign_____ Date_____

Name: Dr. Amare Gessesse Sign_____ Date_____

External Evaluator

Name _____ Signature_____ Date_____

Internal Evaluator

Name _____ Signature_____ Date_____

Chair of Bioinstrumentation Signature_____ Date_____

DEDICATION

This study is dedicated to in loving memory of my lovely little sister Hawi Yazachew tragically taken from me 1 year ago, 16th Oct. 2017 aged 20 by a car accident.

"Though no more you here; you are Always near Still Missed, Still Loved, Still Very Dear and Remembered Everyday Deep in My Heart."

ABSTRACT

Quantitative Proteinuria Screening UV Method to Check Kidney Health Status

Yadeno Yazachew

Jimma University, 2018

At end-stage renal failure need of dialysis or a kidney transplant is a must to treat the renal failure; which cannot be a better solution for developing countries like Ethiopia. However, if chronic kidney disease is early detected it is possible to prevent, control disease progression and minimize complication of the disease. Chronic kidney disease can be screened using a measurement of protein level in urine. Urine tests are noninvasive and enough to get general information about kidney status. The existing urinalysis methods for kidney function tests are a semi-quantitative method (dipstick), and immunological based assay methods. Dipsticks are not sensitive for microalbuminuria (albumin in the urine between 30 and 300 mg/day), measures qualitatively and not precise whereas Immunological based assays are very expensive and complex. The main Objective of this research is to develop a quantitative proteinuria screening UV method to check kidney health status based on protein denaturing properties. Different laboratory experiments have been conducted to determine the best denaturing agent and check the performance of the proposed method. 23% Diluted Sulfuric acid reagent was used as best denaturing reagent to detect protein in the urine. Thirty-three standard solutions prepared to resemble patient urine sample with different protein concentration. The method proposed in this study has a linear equation ($y = 0.00695x + 0.18932$, $r = 0.9958$), moderate sensitivity, 94.93 % accuracy and can be used for the quantitative determination of low and high levels of Protein in human urine for screening and diagnosing kidneys condition.

Keywords: Chronic Kidney Disease, Denaturation, Microalbuminuria, Proteinuria

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Nobody has been more important to me in the pursuit of this study than my fiancé, Sisay Midaso and my family whose love and guidance are with me in whatever I pursue.

TABLE OF CONTENTS

DECLARATION i

DEDICATION ii

ABSTRACT iii

ACKNOWLEDGMENT iv

LIST OF FIGURES viii

LIST OF TABLES ix

LIST OF ABBREVIATIONS x

CHAPTER 1 1

INTRODUCTION 1

1.2 Background of the Study 1

1.2 Statement of the Problem 2

1.3 Objectives 3

1.3.1 General Objective 3

1.3.2 Specific Objective 3

1.4 Significance of the Study 3

1.5 Organization of the Thesis 3

CHAPTER 2 5

CHRONIC KIDNEY DISEASE AND PROTEIN 5

2.1 Kidneys 5

2.2 Chronic Kidney Disease 5

2.3 Kidney Disease Symptoms 6

2.4 Kidney Failure 6

2.5 Diabetic and Kidney Disease 7

2.6 High Blood Pressure & Kidney Disease 7

2.7 Human Urine 8
2.8 Albuminuria and Proteinuria for Screening Kidney Health 8
2.9 Proteins and Denaturing Agents 10
2.10 Existing Albuminuria/Proteinuria Measuring Methods and their Gaps 12
2.11 Chronic Kidney Disease (CKD) Management 15
CHAPTER 3 17
METHOD AND MATERIALS 17
3.1 Experimental Design (Method) 17
3.2 Materials 17
3.2 The Criteria's for Satisfactory Reagent Analysis 18
3.3 Standard Solution Preparation 18
3.3.1 Stock Solution 19
3.3.2 Working Solution 19
3.4 Beer-Lambert Law 20
3.4.1 Determining the Wavelength of Maximum Absorption 23
3.4.2 Calibration Curve 24
3.5 Typical Validation Characteristics 25
3.5.1 Linear Regression 25
3.5.2 Residual Plot 25
CHAPTER 4 27
RESULTS AND DISCUSSION 27
4.2 Determining Best Denaturing Reagent 27
4.2.1 Denaturing Albumin using Sulfuric Acid 27
4.3 Determining Wavelength for Maximum Absorption 30
4.3.1 Results of Best Three Selected λmax 32

4.4 Comparing the Three Wavelength Results 39
4.5 Performance Analysis 40
CHAPTER 5 43
SUMMARY AND CONCLUSION 43
5.1 Summary and Conclusion 43
5.2 Future Work 44
REFERENCE 46
APPENDIX 50

LIST OF FIGURES

Figure 1: Types of bonding interaction in protein 11
Figure 2: Urine Dipstick 13
Figure 3: Sulfosalicylic turbidity result 13
Figure 4:Steps of the proposed method 17
Figure 5: The prepared stock solution 19
Figure 6: The twenty-one Prepared working solutions (standard solutions) 20
Figure 7: UV- Vis Spectrophotometer principle 21
Figure 8:Multiskan GO UV-Vis Spectrophotometer 22
Figure 9:Working principle of Multiscan GO UV spectrophotometer 23
Figure 10: The Twenty one prepared albuminuria concentrations (working solutions) 24
Figure 11: Human serum albumin structure alpha helix using SPDB Viewer 28
Figure 12: 3D picture of different bonding interaction and amino acids contained in HSA 28
Figure 13: Comparing the results obtained from 88% and 23% concentrated sulfuric acid 29
Figure 14: Data analysis using origin software (at 318nm) 34
Figure 15: Data analysis using origin software (at 318nm) 36
Figure 16: Data analysis using origin software (at 318nm) 38
Figure 17: Error bars plotted to estimate error of each reading 40
Figure 18: Transfer function of the proposed method 44

LIST OF TABLES

Table 1: Normal urine contents..... 8
Table 2:Thresholds for defining Proteinuria and Albuminuria..... 9
Table 3: Degree of Turbidity 14
Table 4: Highest absorbance's achieved at different albuminuria concentration 30
Table 5: Absorbance value record of 21 samples at 318 nm 32
Table 6: Absorbance Value record of 21 samples at 322 nm 35
Table 7: Absorbance value record of 21 samples at 336 nm 37
Table 8: Comparing the 3 wavelength outputs 39
Table 9: Testing the design with unknown concentration 41
Table 10: Specifications of the design 42
Table 11: Comparing the developed Method with the existing methods 44

LIST OF ABBREVIATIONS

BCG: Bromo Cresol Green

BCP: Bromo Cresol Purple

BSA: Bovine Serum Albumin

CKD: Chronic Kidney Disease

ELISA: Enzyme Linked Immuno Sorbent Assay

ESRD: End Stage Renal Disease

eGFR: Estimated Glomeruli Filtration Rate

LMIC: Low Middle Income Countries

LOD: Limit of Detection

NKDEP: National Kidney Disease Education Program

QL: Quantitation Limit

UAE: Urinary Albumin Excretion

UPE: Urinary Protein Excretion

UV-VIS: Ultra Violet Visible

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Any person at any age can get kidney disease. But, some people are more likely to progress into chronic kidney disease (CKD) than others, especially diabetic and hypertensive patients. Chronic kidney disease is damage of the kidney and is manifested by abnormal albumin excretion or a gradual decrease in kidney function over time. Mortality due to CKD is on the rise showing a 32% increase in the ten years period from 2005 and 2015 thus causing 1.2 million deaths worldwide. This shows that chronic kidney disease is an increasingly global public health problem having an estimated prevalence of 8% -16% [1]. Most of the time kidney diseases do not show symptoms. When kidney's performance is less than 15%, kidneys no longer work well enough to keep a person alive, and need treatment which often involves either dialysis or a kidney transplant. But, these treatments are prohibitively expensive and unaffordable by most families. Thus people in resource-poor countries like in Ethiopia families with a kidney patient are subjected to bear huge costs and that creates enormous social and financial problems in these countries [2]. Thus, if someone in a family has a kidney failure the individual and the families could easily despair [3]. However, the good news is that it is possible to control CKD if it is properly diagnosed at an early stage of development (stage 1 and 2) and reduce its chance of changing into CKD [4].

Urinalysis is a test used to screen the kidney using proteinuria concept [5]. Proteinuria is an important biomarker to diagnose kidney disease [6]. Existing methods used to detect protein in urine are dipsticks and immunological based assays. Dipsticks detect albuminuria/proteinuria qualitatively [7]. However, the definitive diagnosis of microalbuminuria/ microproteinuria requires quantitation of albumin/protein excretion by immunological based assays [8]. The most available screening tool for proteinuria in healthcare facilities is dipstick, but it is qualitative and not sensitive for microalbuminuria range [9, 10]. Lim and colleagues 2014 [8] have checked urine dipstick diagnostic accuracy for proteinuria and proofed that urine dipstick has low sensitivity.

The goal of this study is to design a method that detects protein in the urine to check the kidney health status. The method proposed is aimed to design a simple UV detection system to quantify protein found in urine with minimized incubation time, reagent cost and also detect microalbuminuria range.

1.2 Statement of the Problem

Chronic kidney disease is an increasingly becoming global public health problem. At end-stage renal failure (ESRD) patients need treatment either through dialysis or a kidney transplant. For people living in developing countries like Ethiopia these are not easy and affordable solutions. But the good thing is, that it is possible to stop or delay progression to CKD if a person knows the kidney status before the commencement of kidney failure. A kidney can be screen through urine analysis. Urinalysis tests are based on the use of Dipsticks and Immunologically-Based Assays [5]. Immunologically-Based Assays are advanced medical equipment with high specificity and accuracy for albuminuria concentration. However, those methods need sample preprocessing that takes a long time through a tedious procedure and involve the use of expensive medical equipment. In developing countries, these tests are not affordable to be used by hospitals due to a reagent, and device expensiveness. The Most commonly used urinalysis method for CKD screening is dipstick. On the other hand, dipsticks are qualitative, user dependent and not sensitive for microalbuminuria range (albuminuria concentration between 30 and 300 mg/L), being sensitive only when protein excretion exceeds 300–500 mg/L (macroalbuminuria).

Considering the current situation in most health care facilities in Ethiopia where hospitals are overcrowded with patients at all times, health centers (clinics) not having the services (due to device cost) and people do not have the habit of regular health checkup, control, detection, and prevention of kidney failure is a huge challenge. Therefore developing cost-effective and better method that could minimize hospital workload, improve kidney urinalysis test service in health centers, increase sensitivity to detect microalbuminuria range which dipsticks normally fail to detect and spread of the improved urinalysis service across the country healthcare facilities will encourage people to make regular health checkup which could have substantial national benefits.

1.3 Objectives

1.3.1 General objective

The general objective of this study is to design a quantitative proteinuria screening UV method to check kidney health status.

1.3.2 Specific objective

- To determine the best protein denaturation procedure and prepare reagent (denaturing agent).
- To design a method that gives appropriate output relying on the denaturalized protein.
- To detect protein from urine based on a method designed.
- To evaluate the result Sensitivity and Accuracy.

1.4 Significance of the Study

This research mainly focuses on developing noninvasive quantitative urine test method using the proteinuria concept. The significance of this study is to increase CKD screening device availability (with fair cost) and sound quality in all health care centers. This will improve quality healthcare service, and it will motivate healthcare professionals to work with individuals to prepare a treatment plan in order to minimize the risk of getting kidney failure and prevent the kidney disease from advancing. This can happen when kidney test service is widely available with minimized cost and fast laboratory service. Good self-management plays a significant role to stop or delay kidney failure which will reduce the economic burden associated with renal failure. Therefore the proposed method is not only to diagnose kidney progress but also to create awareness about good self-management which will influence people to have a trend of regular health checkup.

1.5 Organization of the Thesis

This thesis is segregated into six chapters, in sequential order, to help the reader to grasp and understand the work quickly. From Chapter 1, the reader will get the overall background, the problem, the purpose and significance of the research work. On Chapter 2, physiology,

pathology, the biomarker for chronic kidney disease, literature and other related concepts that are essential to the project will be discussed. Chapter 3 will explain the method and material used to conduct this study and also the parameters used to evaluate the reagent and the system to detect protein in the urine. The next chapter, Chapter 4, is all about result and discussion, and all the research achievements will be found with adequate explanation. The last chapter, Chapter 5, summarizes all the chapters before it, discusses the strong sides of the project, points out the limitations and lists future steps. Additional information's, some mathematical formulas, and tables (data's) are included in the Appendix part at the end of the document.

CHAPTER 2

CHRONIC KIDNEY DISEASE AND PROTEIN

2.1 Kidneys

The kidneys play a vital role in sustaining general health and wellbeing. They differentiate non-recyclable waste from recyclable waste, 24 hours a day, cleaning the blood. Twenty up to thirty-three (20-33%) percent of all blood leaving the heart passes into the kidneys to be filtered before flowing to the rest of the body's tissues. A human being has two kidneys, each weighing around 150 grams. Kidneys have about 1.5 million filters called nephrons [11]. Nephrons filters waste from the blood in the form of urine. Due to this analysis of the urine is used to check the kidneys condition. Some of the main action of kidneys are waste excretion, water level balancing, blood pressure regulation, red blood cell regulation, and acid regulation [12].

2.2 Chronic Kidney Disease

Chronic kidney disease (CKD) is a condition that reduces kidney function over a period. Kidneys can get damaged by a physical injury or due to diseases like diabetes or high blood pressure. Once kidneys are damaged, they cannot filter blood as they should. Kidney disease has five stages. Treating kidney disease at early stages help keep from kidney failure [13]. CKD is common among adults where a study conducted in the United States estimated that 30 million people or 15% of US adults to have CKD. Of that, 48 % are severely reduced kidney function but are not on dialysis and thus are not aware of having CKD. In general, most people (96%) people with kidney damage or mildly reduced kidney function are not aware of that they have CKD [14]. Mortality due to CKD has been shown to be on the rise. In ten years between 2005 and 2015, there were 1.2 million deaths worldwide. CKD is increasingly becoming a serious global public health issue, with an estimated overall prevalence of 8% -16%. This corresponds to nearly 500 million affected individuals, of whom 78% (387.5 million) reside in low-income to middle-income countries [1]. Kidney disease does not happen overnight. It happens slowly, and in stages, as its early stages most people do not exhibit any symptoms and may not aware that anything is wrong [15]. Kidney disease can often be slowed down or completely stopped if it is detected early. However, if not treated early enough kidney disease can gets resulting in

accumulation of high-level wastes, and make the person feel sick and ultimately result in kidney failure[16]. When this happens, the kidneys no longer function well enough to keep the person alive unless the patient gets such treatment as dialysis or kidney transplant.

The Causes of Chronic Kidney Disease

People are at high risk of getting CKD if they have diabetes, high blood pressure, a family member with kidney failure, or 60 years old [17]. Of these, the two common causes of kidney disease that increase the chance for kidney disease are diabetes and high blood pressure. Therefore, to avoid damage to the kidney, a person at high risk of kidney disease must get tested for it [18].

2.3 Kidney Disease Symptoms

Most people know they have kidney; after the kidney is severely damaged that patients detect symptoms such as a feeling of tiredness or short of breaths, trouble thinking clearly, losing appetite, have trouble to sleep, having dry and itchy skin, or muscle cramping at night. Therefore, to avoid complications related to kidney failure, it is essential to test 'apparently normal' individuals for any sign for kidney malfunction, especially for high-risk individuals (diabetics and hypertensive) [19]. However, not everyone who is at high-risk experience kidney failure. To reduce the chance of kidney failure, people, especially those that are considered at high risk could reduce the chances for kidney failure doing the following.

It is possible to reduce the chances of getting kidney disease.

- Do regular health checkup.
- Control blood sugar if a person has diabetes.
- Control blood pressure if a person has high blood pressure.
- Exercise regularly.
- Not indulging in activities like smoking and drinking alcohol.

2.4 Kidney Failure

Depending on how severe it is, the effects of kidney disease can be minor and may not show any symptom, and if it is not controlled well in time, it could result in serious health problems. Thus

untreated kidney disease could turn into the end-stage renal disease (ESRD), which is a total and permanent failure of the kidney. When the kidneys fail, the body retains fluid resulting in a buildup of harmful wastes. Especially, inability to remove potassium from the bloodstream could lead to abnormal heart rhythms and sudden death. Once there is kidney failure it cannot be cured. The only options that patient has been to have dialysis or carry out a kidney transplant.

2.5 Diabetic and Kidney Disease

Kidneys filter the blood and keep some things which the body need and remove wastes that the body must get rid of. Kidneys filter blood with tiny blood vessels known glomeruli. In diabetic patients, high blood sugar level could, over time, clog and damaging these tiny blood vessels, and prevent normal blood flow. In the absence of enough blood, a kidney became injured, and albumin (a type of protein) pass the filters and ends up in the urine where it should not be. When this happens, it is called diabetic kidney disease (or diabetic nephropathy). Once the kidneys are hurt like this, they cannot be fixed. Thus if diabetic kidney disease is not treated early, it can lead to kidney failure. The good news is that diabetic kidney disease does not happen fast. Sometimes it takes many years. This means there is a time to treat and protect the kidneys [20]. According to study Assah and Mabanya 2017 [21] it is estimated that in Africa, on average, about 14.2 million adults in the age range of 20-79 have diabetes. Until 2040 this number is expected to grow more than double increasing the likelihood of kidney failure resulting in a higher burden CKD patients in the future. Ethiopia is among the top four countries with the highest adult diabetic population in Sub-Saharan Africa [22].

2.6 High Blood Pressure & Kidney Disease

High blood pressure (hypertension) is another factor that contributes to kidney failure. It is a force of blood pushing blood vessels as blood moves in the body. Factors that increase the force are high blood volume and, narrow, stiff or clogged blood vessels. Hypertension can reduce the kidneys' ability to function correctly. This is because of the force of blood stretches blood vessels and this stretching scars and weakens tiny blood vessels (glomeruli) in the kidneys. A study carried out in in the United States showed that high blood pressure is the second leading cause of kidney failure [23].

2.7 Human Urine

Urination is a process which liquid byproduct of the body (urine) secreted by the kidneys and excreted through the urethra. Urine mainly contains water (95%), but it also includes other metabolic waste components like urea (60.06 g/mol, molecular weight), creatinine (113.12 g/mol molecular weight) and others. If kidneys are injured the ability of the nephron to reabsorb or filter different components of blood plasma will be altered and excreted in urine. Table 1, shows normal metabolic wastes in order of decreasing concentration (normal chemical composition of urine):

Table 1: Normal urine contents

Urine component name	**Concentration g/L**	**Molecular weight g/mol**
Urea	9.3	60.06
Chloride	1.87	35.453
Sodium	1.17	23
Specific gravity	1.010 -1.030	-
Potassium	0.750	39
Creatinine	0.670	113.12

2.8 Albuminuria and proteinuria for Screening Kidney Health

If urine contains high levels of protein, it is indicative of kidney problems [24]. Proteinuria refers to the existence of any protein in urine whereas albuminuria refers to more concentration of a protein called albumin in urine. Since Albumin is large, a healthy kidney does not filter out albumin as a waste. As Julian and colleagues 2009 [25] discussed that presence of protein in urine shows the progress of kidneys not functioning appropriately due to renal diseases or other medical conditions . Having traces of albumin in urine is called microalbuminuria (concentration range between 30–300 mg/L), and When there are high levels of albumin in the urine, one is said to suffer from macroalbuminuria (concentration above 300 mg/L) [26]. Under normal conditions, albumin concentration in urine should be between 0-30 mg/L or protein concentration in urine must lie between 0-150 mg/L. If urinalysis results show high levels of albumin or protein, other diagnostic tests must be conducted to analyze the kidney function and measures should be taken

to bring back albumin/protein within the normal range [27]. Cassia and colleagues 2016 [28] in detail explained that proteinuria and albuminuria are powerful predictor of renal risk in patients with type 2 diabetes and hypertension. Table 2 shows typical thresholds for defining proteinuria and albuminuria [24].

Table 2: Thresholds for defining Proteinuria and Albuminuria

Stage	**GFR(glomerular filtration rate) ml/min**	**Classification of abnormal urinary albumin excretion**		
		UAE (urinary albumin excretion) mg/day(24 hr.)	**UPE (urinary protein excretion) mg/day(24 hr.)**	**Spot Urine Albumin (mg/L) Gender: both Male and Female**
1. Hyperfiltration	Normal (>90)	< 30	< 150	<30
2. Microalbuminuria	Kidney damage with mild decrease in GFR (60-89)	30-300	150-500	30-300
3. Macroalbuminuria (Over Proteinuria)	Moderate decrease in GFR (30-59)	>300	>500	>300
4.Progressive Nephropathy	Sever reduction in GFR (15-29)	Increasing	Increasing	Increasing
5. ESRD (End Stage Renal Disease)	Kidney failure (<15 ml/min)	Massive	Massive	Massive

*Adapted from the American National Kidney Foundation's KDOQI guidelines for evaluation and stratification of chronic kidney disease (*www.kidney.org*).*

Proteins which have less than 20 kDa molecular weight (kiloDalton in which 1 Dalton= 1 atomic mass unit) pass the filtration barrier with no difficulties. Compounds of 60–70 kDa mainly kept in the capillary lumen because of their substantial molecular weight and albumin have 69 kDa of molecular mass. That is why albumin is used as an indicator of kidney health. About 20 percent of customarily excreted protein is a low-molecular-weight type such as immunoglobulins (molecular weight about 20,000 Daltons), 40 percent is high-molecular-weight albumin (about

69,000 Daltons), and 40 percent is made up of Tamm-Horsfall mucoproteins secreted by the distal tubule [25].

Proteinuria is composed of albumin and globulin from the plasma. Because albumin is the dominant type of protein that appear in the urine the term albuminuria may be used exchangeable with proteinuria [29]. Proteinuria may include albumin, globulins, immunoglobulin light chains, and low molecular-weight proteins. Microalbuminuria refers to the measurement of small quantities of albumin in the urine. In other words, microalbuminuria alludes to a small urine albumin concentration, typically <300 mg/L, which is undetectable by semi-quantitative dipstick tests.

2.9 Proteins and denaturing agents

Proteins are polymers that contain amino acids linked by peptide bonds. The amino acids contain carboxylic acid and amine group both linked through a central carbon atom and R (a side group) that largely determines the chemical properties of the amino acid (chemical formula H2N-CHR-COOH). Twenty essential amino acids found in proteins and the R groups represent acidic, alkaline, polar, or non-polar properties to the various amino acids [30]. Amino acids have different chemical properties that are used to measure proteins using densitometric or spectrophotometric methods [31].

Types of Plasma Proteins:

The three significant fractions of plasma proteins are known as albumin, globulin, and fibrinogen. On a more exceptional resolution by electrophoresis, these fractions separated as follows:

- Albumin – 55.2%
- α1-Globulin – 5.3% (α1-Antitrypsin, TBG, Transcortin)
- α2-Globulin – 8.6% (Haptoglobulin, ceruloplasmin, α2- macroglobulin)
- β-Globulin – 13.4% (β1-transferrin, β-lipoprotein)
- ¥-Globulin – 11.0% (Antibodies)
- Fibrinogen – 6.5%

Albumin is the most abundant class of plasma proteins. Albumin is synthesized in the liver and consists of a single polypeptide chain of 584 amino acids having a molecular weight of 69,000 and soluble in water. Albumin facilitates the vital function of regulating the fluid volume and the oncotic pressure of the blood. The presence of different amino acids (residues) makes the molecule highly charged with a positive, negative charge and also to have hydrophilic and hydrophobic properties. Amino acids found in albumin and their chemical properties are listed in Appendix I. Albumin dominantly found in urine; therefore, Bovine Serum Albumin (BSA) was used as protein to conduct this study.

As discussed above proteins are polymers of amino acids. The R-groups of the amino acid may be negatively charged, positively charged, nonpolar or polar. The sequence of amino acids is the primary structures. Folded state of the protein defined by the secondary and tertiary structures of proteins. Proteins in their native conformations held by a combination of forces: ionic interactions, disulfide bridges, hydrogen bonds, and hydrophobic interactions. The native conformation is the state in which the protein is most active and functional. Disrupting these forces cause a conformational change of protein either temporarily or permanently and it is called denaturation. Disrupting the secondary and tertiary structures changes the solubility of the protein in solution. Denaturing agents like acids, bases, heat, pH changes, heavy metal salts, and alcohols cause denaturation. In denaturation process bonding interaction that held secondary structure and tertiary structure are disrupted, but primary structures remain the same (Figure 1). The most common consideration in the denaturation process is the precipitation of the protein.

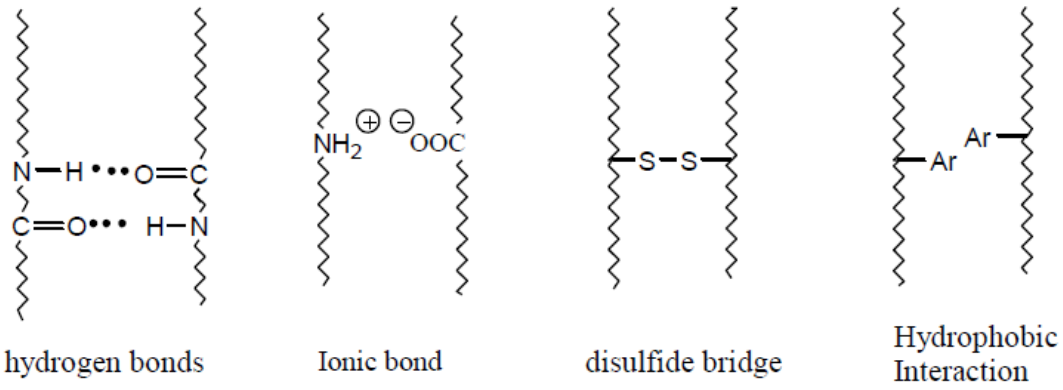


Figure 1: Types of bonding interaction in protein

Heat disrupts hydrogen bonds and non-polar hydrophobic interactions by increasing the kinetic energy and causing the molecules to vibrate so rapidly and violently.

Bases and acids disrupt salt bridges. When base or acid added to protein double replacement reaction takes place where positive and negative ions from the new base or acid change partners with the positive and negative ions in the salt. Albumin proteins can be denatured using strong acid. Hydrogen bonding and hydrophobic bonding interactions support the tertiary structure of albumin proteins. Those intermolecular forces will be disrupted by the addition of strong acids and lost tertiary structure which denaturation occur (proteins precipitate, forming a white solid).

Heavy metal salts: like the acids and bases, heavy metal salts can denature proteins. Heavy metal salts contain metals with high atomic weights like Pb+2, Ag+1, Hg+2, Cd+2, etc. Heavy metals disrupt disulfide bonds due to their high affinity and attraction for sulfur which leads to denaturation of proteins. The reaction between a heavy metal salt and protein result an insoluble metal protein salt (precipitation).

2.10 Existing albuminuria/proteinuria measuring methods and their gaps

Two semiquantitative and one quantifiable method are commonly used to detect albumin: the dipstick method, sulfosalicylic acid precipitation, and homocue albumin analyzer.

There is no absolute photometric protein concentration assay [32]. All methods have advantages and disadvantages when sensitivity, specificity, the accuracy, the measurable range of concentration, the time required for the measurement and the presence of materials interfering with the measurement, are considered [33]. Mc Taggart et al. 2014 [34] in their review concluded that quantitative point of care testing (POCT) are more accurate than semiquantitative POCT in ruling out albuminuria. Semiquantifiable and immunological based assays are generally employed methods to detect protein in the urine.

Semi quantifiable methods

Two semi-quantitative methods are dipsticks and sulfosalicylic acid test. A dipstick is a test strip used to detect the presence of albumin in the urine (Figure 2). If protein exists in urine, it shows color change. Dipsticks work based on the acid-binding dyes bromocresol green (BCG) and bromocresol purple (BCP) these dyes react with acidic residues on all proteins. The test needs

careful observation to interpret the result obtained (user dependent). Dipsticks designed to measure directly without any pretreatment of samples. The procedure involves a 5-minute incubation. However, conventional dipsticks detect albumin in concentrations > 300 mg/L predominantly and, thus, do not detect microalbuminuria concentration which commonly found in patients with excreting 30 – 300 mg/L. Cassia et al 2016 [28] mentioned an alkaline or concentrated urine sample may cause a false-positive reading (pH dependent) on dipsticks. Result interpretation for dipstick has shown in the Appendix II.

Figure 2: Urine Dipstick

The sulfosalicylic acid test requires the addition of 2.5 ml of the supernatant to 7.5 ml of 3% sulfosalicylic acid. The method detect proteinuria qualitatively and result is interpreted from the turbidity obtained from the reaction shown in Figure 3.

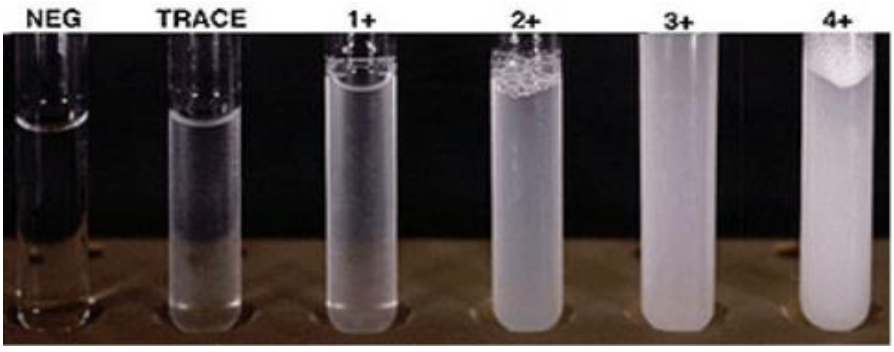


Figure 3: Sulfosalicylic turbidity result

It is an old method but still used in some clinical laboratories [35]. The degree of turbidity is quantified as follows in Table 3.

Table 3: Degree of Turbidity

Protein (mg/dl)	**Degree of Turbidity**
0	Clear
1-10	Opalescent
15-30	Can read print through tube
40-100	Can read only black lines
150-400	No visible black line
>500	Flocculent

Immunologically-Based Assays:

There are three laboratory methods immunonephelometry, immunoturbidimetry, and radioimmunoassay have been used for the confirmation and measurement of microalbuminuria [34].

Immunonephelometry: Albumin in the urine sample attach with an antibody to human albumin and form an antigen-antibody reaction. Light scatter from this reaction is analyzed optometrically to provide microalbuminuria concentration information.

Immunoturbidimetry: human albumin and Albumin in a urine sample, compete for a monoclonal antibody that aggregates the latex particles consequently, the amount of aggregation inversely proportional to the amount of albumin in a urine sample. The aggregation amount is measured optometrically and converted mathematically to a microalbuminuria concentration.

Radioimmunoassay: Albumin in the urine sample displaces isotopically-labeled human albumin that has an antibody bound to it. The amount of albumin in the sample is in inverse proportion to the amount of labeled albumin that remains bound to the antibody. Radioactivity measurement will be used to separate the "free" and "bound" labeled albumin. Radioactive counts are compared with a calibration or standard curve to provide microalbuminuria concentration.

HemoCue urine albumin analyzer is a point of care testing (POCT) instrument that works based on immunochemical antigen-antibody reaction to measure albuminuria concentration

quantitatively. Then turbidity is measured by the HemoCue albumin 201 photometer. The linear regression analysis of the method show good correlation in the range of 5-150 mg/L for the HemoCue system ($y = 0.8557x + 0.2497y$, $r = 0.97$). Still, now it is the only POCT system for urine albumin on the market that delivers quantitative results. In the study Lloyd et al. 2011 [36] mentioned The HemoCue is easy to use and display results in two minutes, but expensive. Since albuminuria concentration measurement range for this POCT is between 5 to 150 mg/L dilution with normal saline should be made to measure above albuminuria concentration 150 mg/L. Lloyd et al. concluded that the system is somewhat time-consuming and may be difficult for an inexperienced health professional in the health center, not only this HemoCue reagent has to be refrigerated and visually turbid samples should only be measured after centrifugation (need preprocessing) which may create difficulties in clinic settings. Generally, several methods have been proposed in different kinds of literature for the determination of urinary albumin. Among these, the best methods are based on immunoassays, due to their high sensitivity and selectivity. The most sophisticated immunological method with tedious washing steps known so far is the enzyme-linked immunosorbent assay which requires an incubation time of approximately 210 minutes. The radioimmunoassay "gold-standard" method but not widely used because of its health hazards. Currently, the widely used immunological based assay in clinical laboratories is the immunoturbidimetric method. Nonetheless, automation of immunoassays has significant advantages in measuring proteinuria, these machines are expensive, costing between $50,000 and $200,000, consume large amounts of expensive antibody reagent and are not portable devices[37].

2.11 Chronic Kidney Disease (CKD) Management

Berns et al. 2014 [19] listed diabetic nephropathy and hypertensive nephrosclerosis as the most common causes of ESRD in developing countries and presented reasons why it is increasing so fast. As Beladi-Mousavi et al. ESRD is elevating possibly because of lack of awareness among patient and late referral of patients with CKD to the nephrologists. Everyone with risk factors of CKD such as diabetes mellitus, high blood pressure, metabolic syndrome, family history of CKD and proteinuria are in danger of kidney failure. Berns et al. suggest that routine CKD screening is very useful to slow down disease progression. Many researchers explained kidney function could be assessed through measurement of albumin levels in the urine. J Feehally and colleagues

recommend early detection of chronic kidney disease is very important and explained the role of estimated glomerular filtration rate as part of comprehensive management. Djukanović 2010 [38] mention screening for CKD is the only effective approach prevent and control kidney failure. The ultimate goal of CKD management is to prevent disease progression, minimize complications, and promote quality of life [39]. Microalbuminuria is an early sign of progressive renal disease in individuals with diabetes, hypertensive and cardiovascular disease. Health care professionals should give more attention to the early detection and treatment of individuals with microalbuminuria for the cost-effective benefit to prevent CKD, kidney failure [40].

Healthy person excretes lower than 150 mg protein or 30 mg albumin per day. Overt proteinuria or macroalbuminuria is the amount that is readily detectable by routine screening methods, generally greater than 500 mg protein or 300 mg albumin. To detect CKD at early stage researchers, have turned their attention to the urinary albumin excretion (UAE) and suggest microalbuminuria (excretion of 30–299 mg albumin/day) is very useful than macroproteinuria [41]. George and colleagues 2017 [42] concluded that CKD screening should be a policy priority in low-income to middle-income countries to achieve cost-effective prevention.

CHAPTER 3

METHOD AND MATERIALS

3.1 Experimental Design (Method)

To do this research, the following approach was used. The method has four main steps; first best denaturing reagent has been selected based on amino acids chemical properties of albumin, mainly nonpolar amino acids. Then reagent sensitivity and concentration tested. After selecting and moderating the reagent, maximum wavelength (λ max) determined using standard solutions based on Beer-Lambert law (theory). Finally, a calibration curve has been prepared using selected λ max. Not least but last the design capability was tested using unknown standard solutions. Figure 4 shows the main experiment steps that have been taken to conduct the study.

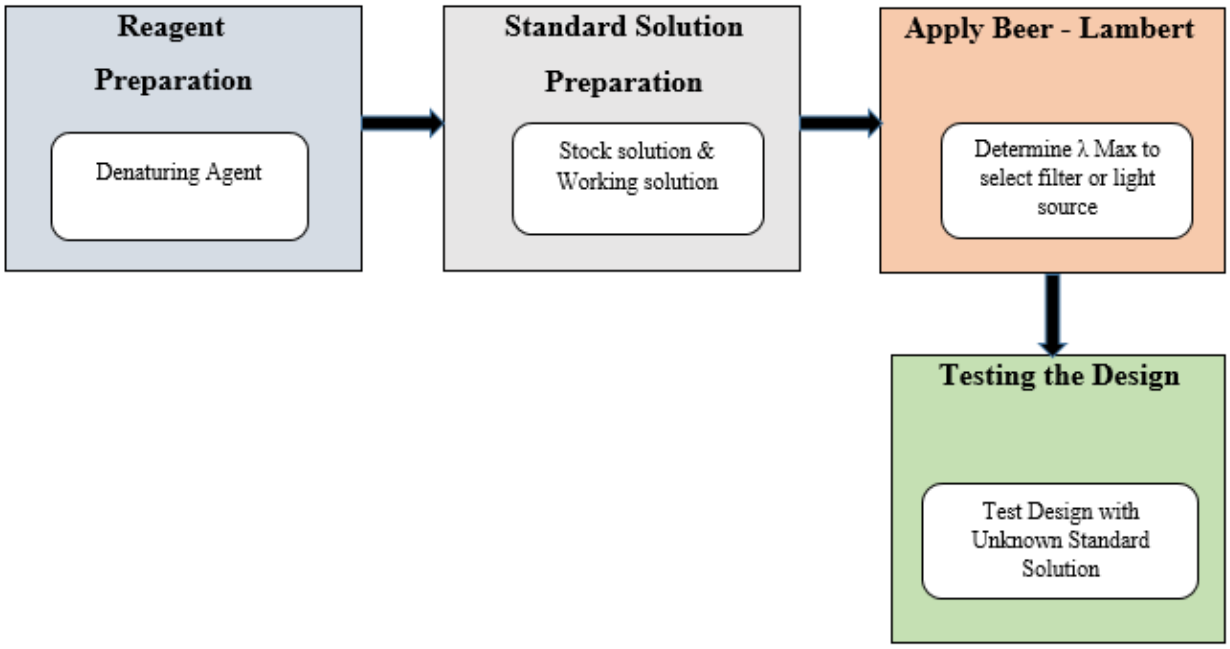


Figure 4: Steps of the proposed method

3.2 Materials

Materials used to perform all the experiments to do this research are listed as follows:

Proteins: Bovine Serum Albumin (BSA) Bovostar.

Solvent: Human urine, distilled water

Reagents: Heavy metal salts like silver nitrate and mercury chloride, sulfuric acid, acetic acid, hydrochloric acid, sodium hydroxide.

Software: Origin data analysis and graphing software and SPDBV software.

The brand, company, model and other information's about the materials used to conduct this study is attached in Appendix III.

3.2 The Criteria's for Satisfactory Reagent Analysis

To get satisfactory results, the process of reagent analysis needs careful operations. Since the color or turbidity development between albumin in urine and selected reagent involves the diverse type of reactions, the selected reagent was tested before applying the method for a particular application. The criteria's considered to select reagent for this study are mentioned as follows.

1. Specificity of the turbidity reactions

To make sure reagent is specific for only albumin (protein); another complex-forming compound of urine components (especially urea) with different concentration was introduced alone with a reagent to observe the reaction output weather if there are precipitation and any color change.

2. Proportionality between turbidity and concentration

Light intensity should increase linearly with the concentration of the albumin. The system should follow Beer's law when UV spectrophotometer is used.

3. Stability of the turbidity and clarity of the solutions

To take accurate readings the turbidity created should be stable and for precise measurement to be made the period over which maximum absorbance remains constant should be long enough.

4. Reproducibility and sensitivity

The reagent preparation procedure must give reproducible results under specific experimental conditions.

3.3 Standard Solution Preparation

A standard solution is a solution containing an accurately known concentration of an element or a substance, a known weight of solute is dissolved to make a specific volume. It is prepared using a standard substance. To conduct this study known concentration of albuminuria solution was prepared by following standard solution protocol. Standard solution categorized into two: stock solution and working solution.

3.3.1 Stock Solution

An obvious concentrated solution that will be diluted to a lower concentration for laboratory use is called stock solution. Preparing Stock solution in order to conserve materials, save preparation time, reduce storage space, and improve the accuracy in preparing lower concentration solutions. To experiment 100 ml of a stock solution was prepared. 100ml was enough to do a test repeatedly five times.

Procedure: 1000 mg Bovine Serum Albumin which is in the form of powder was measured using weight balancer then the weighed BSA was added in a beaker and pour normal urine in a beaker until the solution reaches to the 100ml line. The solution was mixed and dissolved using magnetic stirrer. After the protein is dissolved completely, the stock solution is ready to be used; Figure 5 shows the prepared stock solution to do the test.

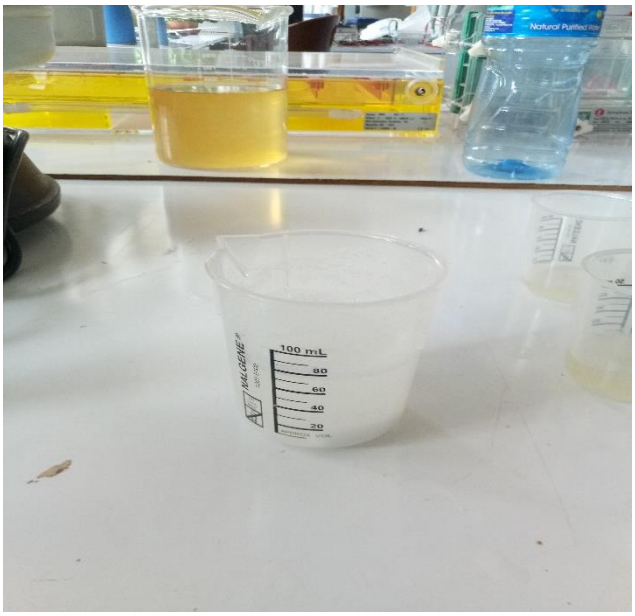


Figure 5: The prepared stock solution

3.3.2 Working Solution

Working Solution is a solution made for actual use in the lab, made from diluting stock solutions. Therefore 21 Working solutions prepared from stock solution. The 21 Working solutions were used as a replica of standard urinary albumin concentration (<30mg/L as representing normal people, 30-299 mg/L as representing microalbuminuria stage, >300 mg/L as representing Macroalbuminuria stage. Therefore different concentration samples were selected based on

standard thresholds with 300 mg/L interval. To make preparation comfortable mg/L was converted to mg/dl.

Samples were taken starting from 0 mg/L to 6000mg/L (0 mg/dl to 600 mg/dl). The interval taken between 21 working solutions was 300 mg/L or 30 mg/dl (0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 450, 480, 510, 540, 570, 600 mg/dl). Figure 6 shows the prepared 21 working solutions. A table that shows how albumin concentration in urine is moderated found in Appendix IV.

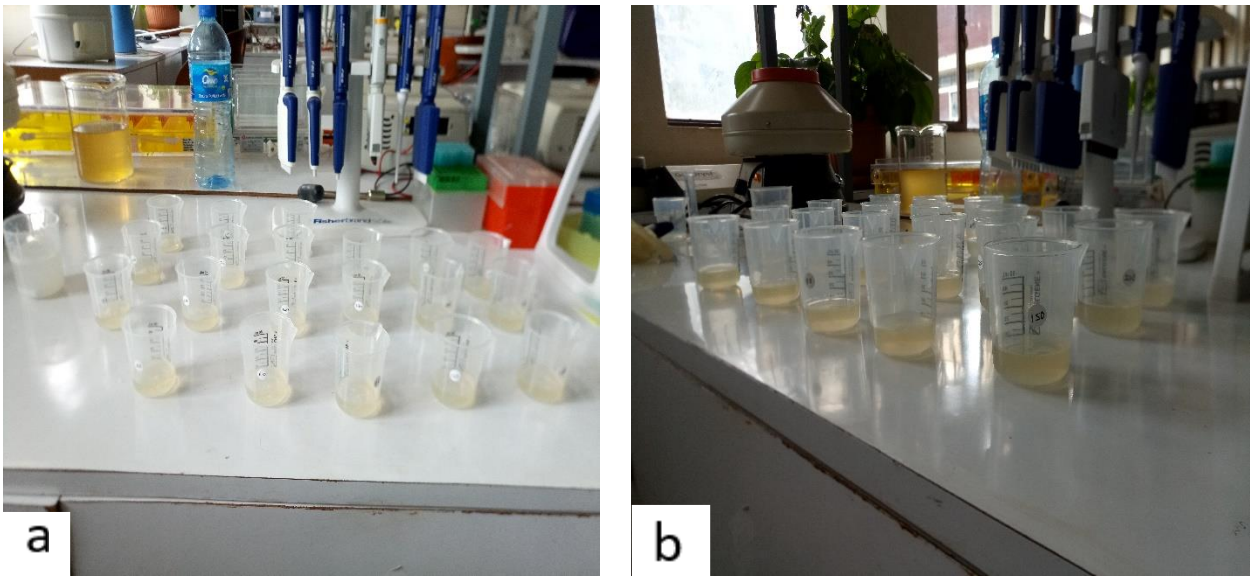


Figure 6: The twenty-one Prepared working solutions (standard solutions) as replica of patient urine sample with albuminuria concentration range b/n 0 -6000 mg/dl.

3.4 Beer-Lambert Law

The basic concept of turbidity measurement depends upon the following principal:

- Measure the ratio of the intensity of light scattered at right angles by the suspended matter to the intensity of the incident light.
- Measure the ratio of the light transmitted through the solution in a straight line to the intensity of the incident light.
- Measure the depth at which an object disappears from view beneath the surface of the solution.

From the above concepts of turbidity measurement techniques; the second technique of measuring solution was used; which is measuring the ratio of the light transmitted through the

solution in a straight line to the intensity of the incident light. This technique uses spectrophotometric analysis. The basic law that rules the quantitative spectrophotometric analysis is the Beer-Lambert law. Beer's law states that the intensity of light of monochromatic radiation decreases exponentially as the amount of absorbing molecules increases. Lambert's law states that the intensity of light of monochromatic radiation decreases as it passes through a medium of uniform thickness. A combination of these two laws gives the Beer-Lambert law.

Theory: When electromagnetic radiation is passed through a sample, certain characteristic wavelengths are absorbed by the sample. As a result, the intensity of the transmitted light is decreased (Figure 7). The measurement of the decrease in intensity of radiation is the basis of Ultraviolet-Visible Spectrophotometer (UV-Vis Spectroscopy). Thus the UV-Vis spectroscopy compares the intensity of the transmitted light with that of incident lights.

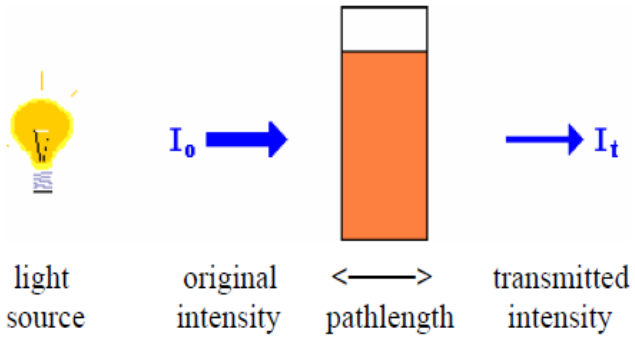


Figure 7: UV- Vis Spectrophotometer principle

According to the Beer-Lambert's law; when a beam of monochromatic light of intensity Io passes through a medium that contains an absorbing substance, the intensity of transmitted radiation I depend on the length of the absorbing medium and the concentration of the solution. Mathematically it can be represented as:

$A = \log (I_o / I) = \varepsilon cl$ …….. (1)

Where, Io = Intensity of incident light

I = Intensity of transmitted light

A = Absorbance (OD)

l = Length of the absorbing medium

c = Concentration of the solution

ε = Molar absorption coefficient or molar extinction coefficient

The molar absorption coefficient or molar extinction coefficient is the absorbance of a solution having the unit concentration (c = 1M) placed in a cell of unit thickness (l = 1 cm). Absorbance is also called optical density (OD). Thus, for a particular wavelength λ, the absorbance of a solution is directly proportional to the concentration of a solution.

Measuring device: Ultraviolet-Visible Spectrophotometer (UV-Vis Spectroscopy). The Multiskan GO (Figure 8) is a high-quality monochromator based UV/VIS spectrophotometer used in different research applications including nucleic acid, protein and ELISA assays. It is used in spectral scanning, and kinetic measurements to measure absorbance in the 200 – 1000 nm wavelength range.

Figure 8: Multiskan GO UV-Vis Spectrophotometer

The principle of operation of measuring device

A monochromatic (filter) will select the exact wavelength of the spectrum of the light source. The light source in this device is a Xenon flash lamp. An optical fiber guide light to the cuvette optics. The light is measured simultaneously by the reference detector and the measurement detector placed after the sample. In cuvette measurement always a separate zero measurement is done manually before measuring the sample (zeroing). The available wavelength range is 200 – 1000 nm. Figure 9 shows the operating principle of the Multiskan GO.

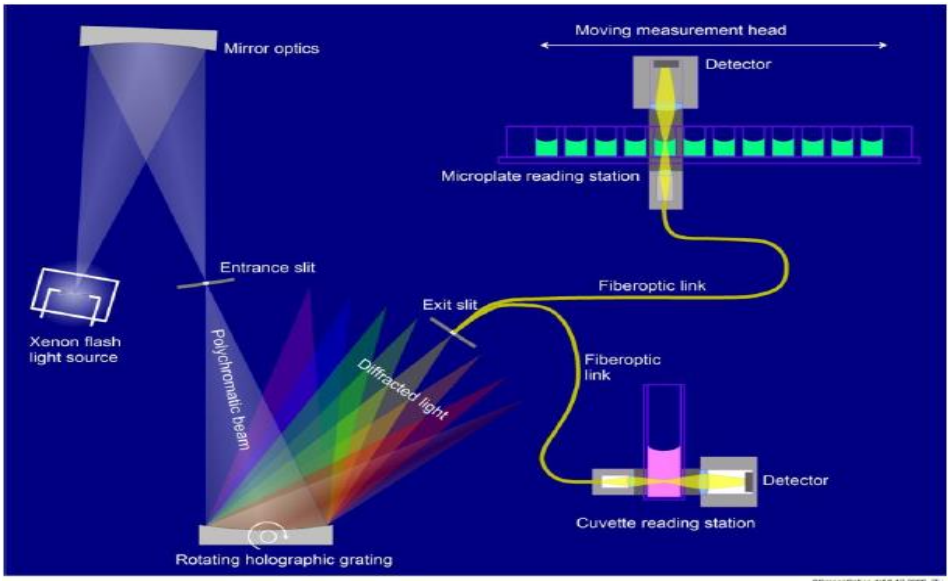


Figure 9: Working principle of Multiscan GO UV spectrophotometer

3.4.1 Determining the wavelength of maximum absorption

For this study determining λ max (wavelength of maximum absorption) using a UV-Vis Spectroscopy was the first step to do calibration curve. If there is high absorbance of light by a solution, it means the transmitted light is low. The light wavelength at which absorbance is more is the wavelength at which the solution is most sensitive to concentration changes, and it is called maximum wavelength or λ max. The albuminuria solutions of different strength were supplied to UV-Vis Spectroscopy absorbance and measured at different wavelengths (starting from 200 nm to 1000 nm). The minimum transmittance at a particular wavelength will correspond to maximum absorbance which will give λ max. [Important: a blank solution was always adjusted to 100% before each wavelength measurement (zeroing). This is because the extinction coefficient also changes with the blank solution wavelength. The blank solution used to conduct this study was 2.5 ml pure urine mixed with 0.5 ml reagent placed in the cuvette which will give a volume of 3 ml].

Twenty-one Albuminuria solutions (working solution) of different strength were prepared. Among 21 samples ten more concentrated samples were selected and supplied to UV-Vis Spectroscopy to measure absorbance at different wavelengths. These are 330mg/dl, 360mg/dl, 390 mg/dl, 410mg/dl, 450mg/dl, 480mg/dl, 510mg/dl, 540mg/dl, 570mg/dl and 600mg/dl (Figure 10). The reason was to identify which wavelength achieve maximum absorption. Lambda max refers to the wavelength along the absorption spectrum where a substance has its

strongest photon absorption. A blank solution was always adjusted to 100% before each wavelength measurement.

Figure 10: The Twenty one prepared albuminuria concentrations (working solutions)

Procedure: steps followed to get λmax

Step 1: The UV-Vis Spectroscopy were calibrated

Step 2: Transmittance of the blank solution was always adjusted to 100% before each wavelength measurement. All measured sample absorbance was measured in comparison to the baseline level. Zeroing performed directly zero the instrument with the Blank sample. The blank sample used for this study is 0.5 ml reagent and 2.5 ml pure urine mixed in a cuvette.

Step 3: The device set to multiple measurements. Each sample was measured with starting wavelength 200 and end wavelength 1000nm.

Step 4: 2.5 ml albuminuria representing 330mg/dl mixed with 0.5ml reagent were measured for 200 up to 1000 nm. 5 peak maximum absorbance were recorded. This test ran for the other nine albuminuria concentration samples (330,360,390,420,450,480,510,540,570,600 mg/dl).

3.4.2 Calibration curve

A plot between absorbance (A) and concentration (c) is expected to be a straight line plot, passing through the origin, shows that Beer-Lambert's law is obeyed. This plot, known as a calibration curve, can also be employed in finding the concentration (or strength) of a given solution [43].

3.5 Typical validation characteristics

3.5.1 Linear Regression

Linear regression was typically performed using a computer program called origin data analysis and graphing software. A linear regression analysis, an R^2 value is the square of the correlation coefficient (r). R^2 provides information about how far away the y values are from the predicted line. A perfect line has an R^2 value of 1, and most calibration curves R^2 values are above 0.95. Generally, in practice, a value of R^2 greater than 0.990 is considered satisfactory.

3.5.2 Residual Plot

A residual is a difference between an obtained y value, and the y value calculated using the equation of the fitted line. The residuals give information on how well the line fits the data. The smallest sum of the squared residuals of line, best represents the linear relationship between the x (concentration) and y (absorbance) variables. Interpretation of the standard outputs (the typical validation characteristics) from a regression analysis used to know sensitivity, accuracy, limit of linearity, standard deviation, limit of detection, limit of quantitation [44].

1. Sensitivity

In linear calibration curve measure of sensitivity is the slope of the graph. The slope is capable of telling the change in signal as concentration changes. A larger slope indicates a more sensitivity.

2. Accuracy

To achieve a reasonable accuracy R^2 must be very close to 1.0 for quantitative results in analytical chemistry. Accuracy is how close measured value is to expected (true) value.

Accuracy = Measured value – True value

The minimum acceptable value of R^2 depends on the accuracy required. As a rough rule of thumb, if an accuracy of about 0.5% is needed, R^2 should be 0.9998; if a 99% accuracy or 1% error is well enough, an R^2 should be 0.997; and if a 95 % accuracy or 5% error is acceptable, an R^2 of 0.97 will do.

3. Limit of Linearity

The linear range is the range of concentrations that the instrument gives a linear response. Outside this range, the response is not acceptable, and it is called limit of linearity.

4. Limit of Detection

The smallest amount of concentration that can be determined from the noise is called Limit of detection. The detection limit is the lowest concentration of a solution of an element that can be detected with 95% certainty. The limit of detection (minimum detectable concentration) can be calculated using calibration curve information and expressed as

LOD=3*S.D/m

Where S.D. is the standard deviation of the noise

6. Limit of Quantitation

Minimum level at which the analyte can be quantified with acceptable accuracy and precision. Quantitation limit (QL) may be expressed as: QL= 10S.D/m

CHAPTER 4

RESULTS AND DISCUSSION

4.2 Determining Best Denaturing Reagent

Different reagents were used as proteins denaturants and cause precipitation. These include sodium hydroxide, sulfuric acid, hydrochloric acid, and acetic acid. Additionally metallic salts like mercury chloride and silver nitrate were also used. After selecting the best denaturing agent, the precipitation process was optimized in terms of reagent concentration and the potential danger it may cause was also considered. Finally, reagent specificity to albumin, the proportionality between turbidity and concentration, reproducibility and stability of the turbidity were evaluated in relation to time. Albumin solutions representing different albuminuria concentration mixed with each reagent. Among the reagents that caused a protein to precipitation, sulfuric acid was selected for further study due to the exothermic reaction and fast precipitation formed. Metallic salts Hydrochloride acid, acetic acid, and sodium hydroxide, although caused precipitation, but not as effective as sulfuric acid for the detection of urine protein concentrations of 200 mg/L.

4.2.1 Denaturing albumin using sulfuric acid

Sulfuric acid composed of the elements sulfur, oxygen, and hydrogen, with molecular formula H_2SO_4. It is a colorless, odorless, and viscous liquid that is soluble in water, in a reaction that is highly exothermic. Denaturing albumin in urine using sulfuric acid has two outputs. One is precipitation formed, and the other one is it gives an exothermic reaction. Urine is composed of 95% water and 5% of other urine constituents. The reaction of water (H_2O) and sulfuric acid (H_2SO_4) is very exothermic. A hydrogen bond is a weak bond that forms between a hydrogen atom and an oxygen atom in the amino acids Figure 12. The albumin becomes denatured when its normal shape gets deformed because some of the hydrogen bonds are broken. When a protein is exposed to an acid or if heat is applied the weak hydrogen bonds could break. Therefore the heat generated from exothermic reaction between water and sulfuric acid could improve protein precipitation. As albumin deform or unravel parts of the structure (hydrophobic part of the albumin) that were hidden away get exposed and form bonds with each other, so they coagulate

(stick together) and become insoluble in water (precipitate). Figure 11 shows human serum albumin structure.

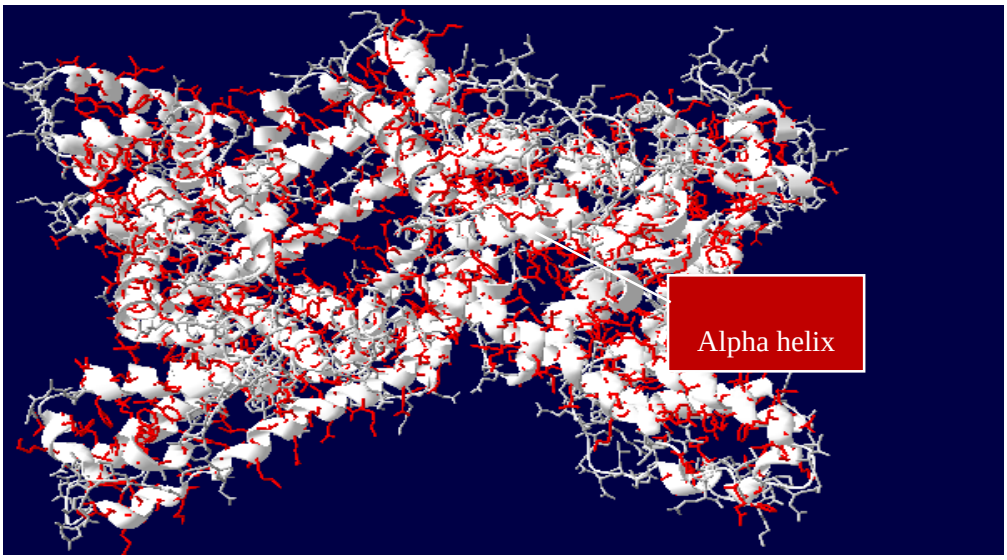


Figure 11: Human serum albumin structure alpha helix using SPDB Viewer

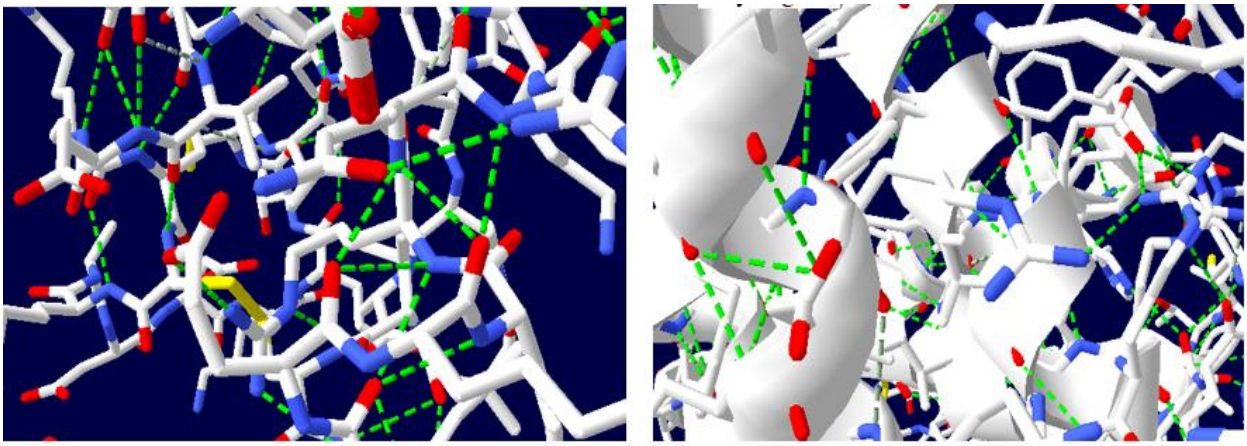


Figure 12: 3D picture of different bonding interaction and amino acids contained in human serum albumin

Nitrogen
Oxygen
Carbon
Hydrogen

4.2.1.2 Moderating concentration of the selected reagent

Concentrated sulfuric acid is hazardous to skin and other materials. In addition it is very strong acid and very reactive it has high probability to make reaction with 5% urine waste components

specially urea. To minimize the reagent's effect and get a better specificity for albumin, number of tests were performed. This helps to achieve a best-denaturing result, less error and risk minimized. So, different concentrations of sulfuric acid was prepared and applied in albumin containing urine. Thus twenty different sulfuric acid concentrations ranging from 3% to 98% with an interval of 5 were used (3, 8, 13, 18, 23, 28, 33, 38, 43, 48, 53, 58, 63, 68, 73, 78, 83, 88, 93 , 98 %). The selection was random because the intention of moderating the reagent was to minimize the risks associated using the reagent and increase its specificity (as long as it denature large molecule diluting sulfuric acid reagent will decrease its danger).

Among the twenty reagent concentrations 23% concentrated sulfuric acid was selected than the others that shows acid specificity for urine components is minimized, and acid specificity for albumin (protein) is increased. As Figure 13 shows for reagent concentration above 48% sulfuric acid the color change came from the reaction between urine and its components especially light scarlet color is in the presence of urea compound in urine. The reaction caused by 23% sulfuric acid reagent was stable for more than 1 hour showed better stability (no color change and precipitation was stable), with reaction temperature 34 °C and has capability of denaturing 200mg/L albuminuria sample which shows it has good sensitivity of detecting micro albuminuria range or lower albuminuria concentration . Figure 13 shows the results obtained from the laboratory experiment for 88 % and 23 % compared with pure urine while the reagent is moderated.

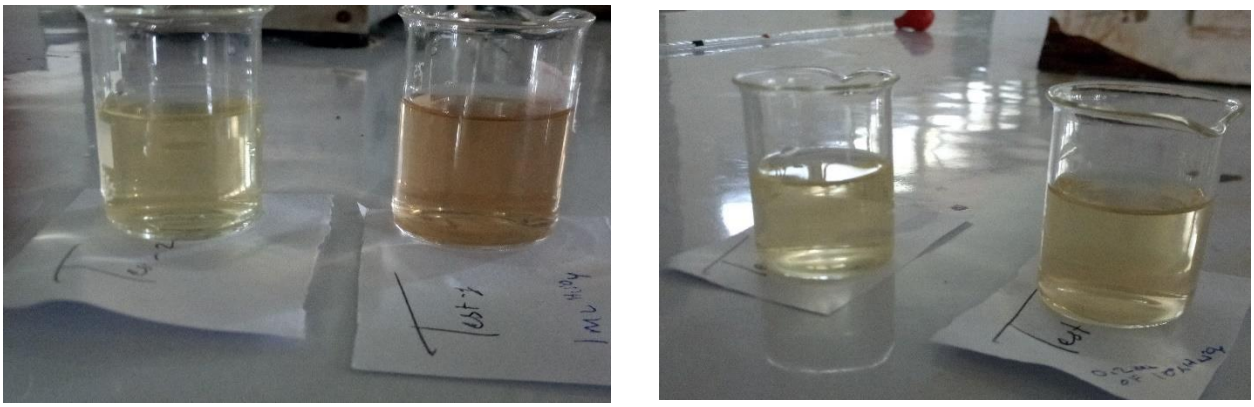


Figure 13: Comparing the results obtained from 88% and 23% concentrated sulfuric acid while moderating reagent a) comparing urine without reagent and urine reacted with 88% concentrated sulfuric acid b) comparing urine without reagent and urine reacted with 23% concentrated sulfuric acid.

Sample volume and Temperature

To measure sample using UV/VIS Spectrophotometer cuvette were used and sample volume was 3ml (0.5 ml reagent with 2.5 ml urine sample) the temperature was 34 degree celsius.

4.3 Determining Wavelength for Maximum Absorption

Twenty-one working solutions were prepared from BSA stock solution as a replica of different albumin concentration of human urine. Urine used to prepare standard solutions were tested in the universal advanced medical laboratory and found to be normal urine. Thirteen type of tests were performed using Coomber urine test strip to screen urine condition. The 13 tests were Protein, Blood, Glucose, Ketone, Bilirubin, Urobilinogen, Leucocyte, Nitrite, pH, Specific gravity, WBC, RBC, Cast, and Crystal). For this study, all the urine samples were taken from one person. For every test performed, 24 hour collected urine was used. In the collection process, there were some instructions followed on how to collect the urine and prevent any contamination. For each day urine collected; intake fluid was 1.5 liter of water. The result of 13 urine test is attached in Appendix V.

To determine λmax of the solution 10 highly concentrated solutions selected and supplied to UV/VIS Spectrophotometer and maximum absorbance along with the wavelength recorded as it is shown in Table 4.

Table 4: Highest absorbance's achieved at different albuminuria concentration

Albuminuria concentration mg/dl	330	360	390	420	450	480	510	540	570	600
λmax (nm)	315	320	322	336	348	360	318	378	396	398
O.D (Absorbance)	2.576	2.565	2.982	2.976	2.663	2.808	2.821	2.763	2.783	2.708

As Table 4 shows all of the measured higher concentrations of the albuminuria solution samples give similar absorption spectra the reason the wavelength is increasing is because of the amount of delocalization of electron in molecule increases. The maximum absorbance is moving to

longer wavelengths as delocalization increases. This leads maximum absorbance to move to shorter frequencies and less energy as the amount of delocalization increases. The albuminuria solution absorbs throughout the ultra-violet region but particularly it strongly absorbs in between about 315 and 340 nm with a peak about 336 nm. As Table 4 shows the highest absorbance was achieved by 390 mg/dl concentration at 322nm, the second absorbance reading was 420 mg/dl at 336 nm with 2.936 absorbance. Lastly, 510mg/dl with the absorbance of 2.8210 at 318nm selected. After the best three λmax were selected, the absorbance or OD of different albuminuria concentration solutions at those λ maxes was measured by starting with the lowest concentration of the solution and move to the highest. For each solution, the absorbance at the wavelength of strongest absorption using the same cuvette for each one were measured. Then a graph plotted of the absorbance against concentration which is a calibration curve.

4.3.1 Results of best three selected λmax

4.3.1.1 λ max =318 nm

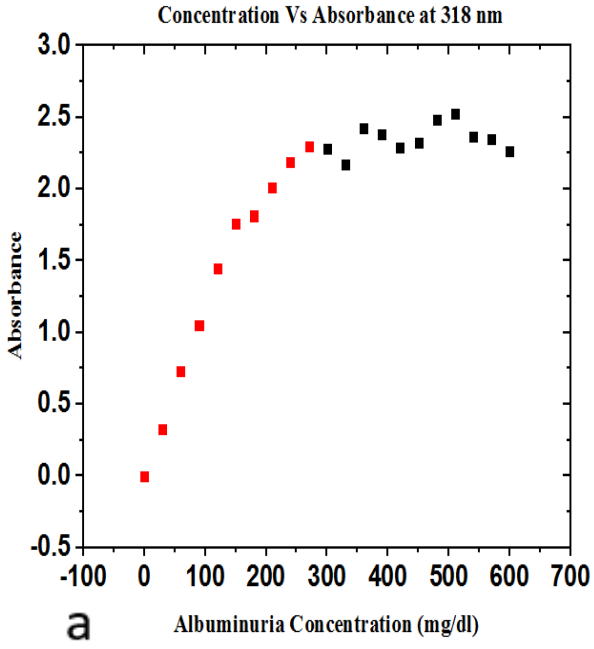
Single wavelength measurement was selected and set at maximum wavelength value, 318 nm. Table 5 shows the absorbance value recorded for 21 different concentration samples.

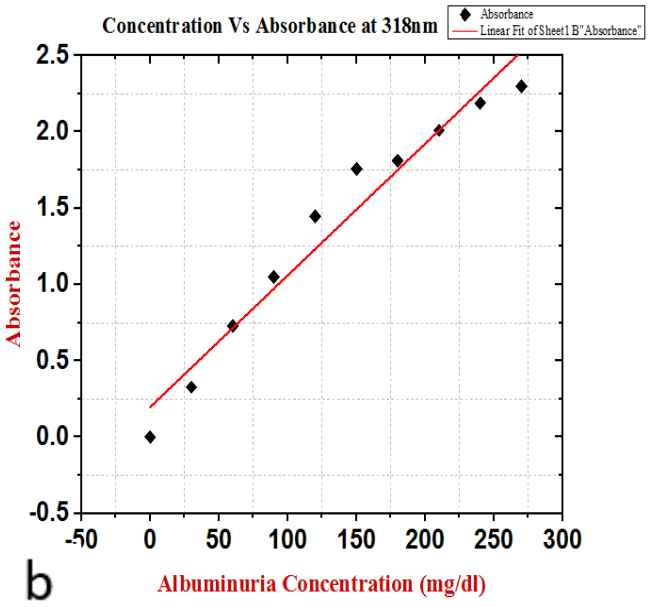
Table 5: Absorbance value record of 21 samples at 318 nm

Sample	Albuminuria concentration (mg/dl)	Optical Density (O.D) Absorbance
1	0	0
2	30	0.3277
3	60	0.728
4	90	1.0488
5	120	1.4475
6	150	1.7568
7	180	1.8124
8	210	2.0092
9	240	2.1881
10	270	2.2982
11	300	2.2765
12	330	2.1717
13	360	2.4219
14	390	2.3803
15	420	2.2904
16	450	2.319
17	480	2.4847
18	510	2.821
19	540	2.3644
20	570	2.3504
21	600	2.2604

As Figure 14 shows absorbance data taken from UV- Vis spectroscopy was plotted opposed to each other on a diagram to check if a correlation exists between albuminuria concentration and absorbance reading. According to the scatter plot of instrument response data versus concentration; the linear relationship between instrument response and analyte concentration was achieved up to 270 mg/dl (Figure 14a). Therefore data are taken above 270 mg/dl were removed because the calibration curve selected should verify that absorbance of different concentration is linearly related to concentration. The linear regression analysis was applied to establish the equation (i.e., $y = mx + b$, where m is the slope of the line and b is its intercept with the y-axis) that best describes the linear relationship between absorbance and albuminuria concentration (Figure 14b). Linear regression gives the equation or values of m and b which best describe the relationship between the data sets. As Figure 14c illustrates the slope of the line is 0.00863 and y-intercept is 0.19678. Finally, the relationship was described by the equation of the line $y=0.00863x+0.19678$. For this study their values were obtained using origin data and graphical analysis software.

As it is discussed in chapter 3, a residual is a difference between an obtained y value, and the y value calculated using the equation of the fitted line. In figure 14d it can be shown that the residuals indicate how well the line fits the data. The smallest sum of the squared residuals of line, best represents the linear relationship between the x (concentration) and y (absorbance) variables.





Equation	y = a + b*x		
Weight	No Weighting		
Residual Sum of Squares	0.24028		
Pearson's r	0.97895		
Adj. R-Square	0.95314		
		Value	Standard Error
Absorbance	Intercept	0.19678	0.10186
	Slope	0.00863	6.3601E-4

c

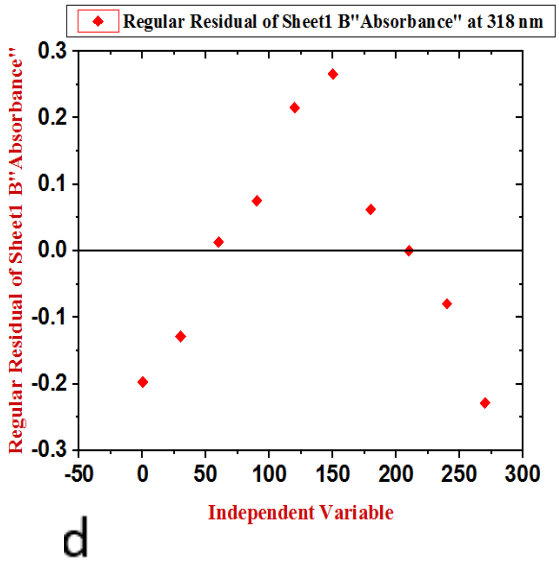


Figure 14: Data analysis using origin software (at 318nm): a) shows Scatter plot of instrument response versus concentration: the horizontal axis represent the Albuminuria concentration and the vertical axis represent the absorbance. b) Linear regression applied on selected data appearance. c) Typical output from a regression analysis using origin lab software. d) Shows plot of the residuals.

4.3.1.2 λmax = 322 nm

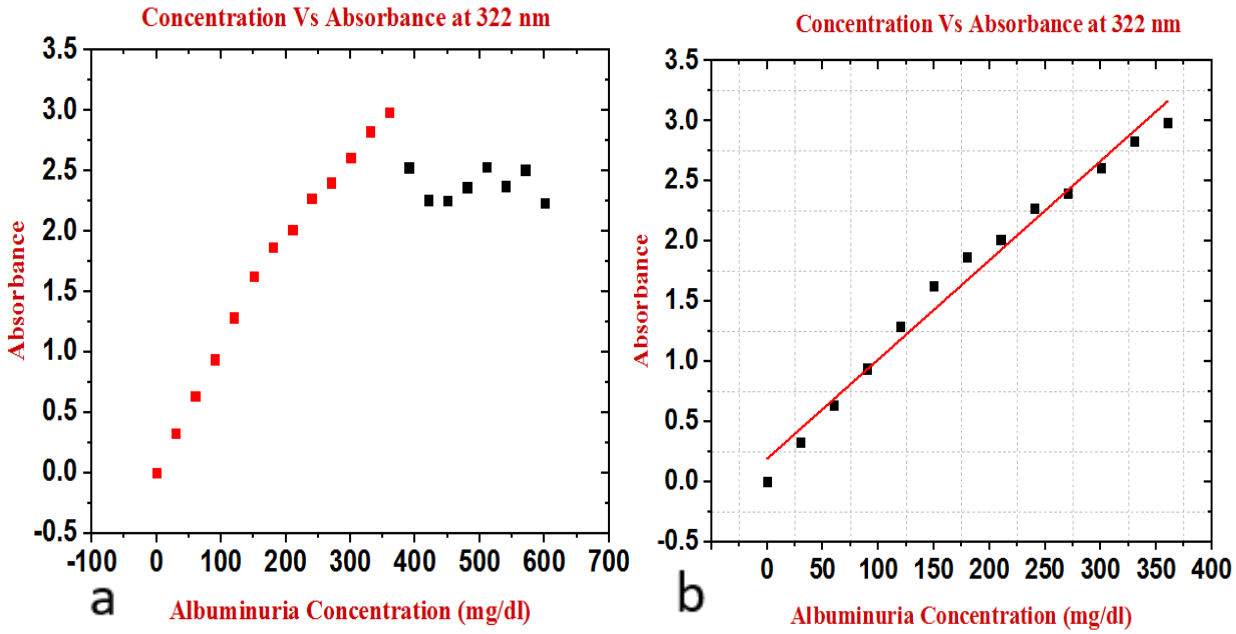
Single wavelength measurement was selected and set at maximum wavelength value, 322 nm. Table 6 shows the Absorbance value recorded for 21 different concentration samples.

Table 6: Absorbance Value record of 21 samples at 322 nm

Sample	Albuminuria concentration (mg/dl)	Optical Density (O.D) Absorbance
1	0	0
2	30	0.3269
3	60	0.6328
4	90	0.939
5	120	1.2868
6	150	1.625
7	180	1.8711
8	210	2.0135
9	240	2.2743
10	270	2.4016
11	300	2.6093
12	330	2.8266
13	360	2.901
14	390	2.5268
15	420	2.2568
16	450	2.2511
17	480	2.3634
18	510	2.5294
19	540	2.3751
20	570	2.5091
21	600	2.2357

The procedure that was used to measure 21 albuminuria concentrations at λmax 322 nm is the same as the procedure used to measure λmax 318 nm. Scatter plot of instrument response data is

shown in Figure 15. A linear relationship between instrument response and analyte concentration was achieved up to 360 mg/dl it is marked with red color in Figure 15a. The equation established by the applied linear regression analysis were y = 0.00825x + 0.19098, (Figure 15b). A residual was calculated using the equation of the fitted line. Figure 15d shows a plot of the residuals.



Equation	y = a + b*x		
Weight	No Weighting		
Residual Sum of Squares	0.20153		
Pearson's r	0.99108		
Adj. R-Square	0.98063		
		Value	Standard Error
Absorbance	Intercept	0.19098	0.07095
	Slope	0.00825	3.34439E-4

c

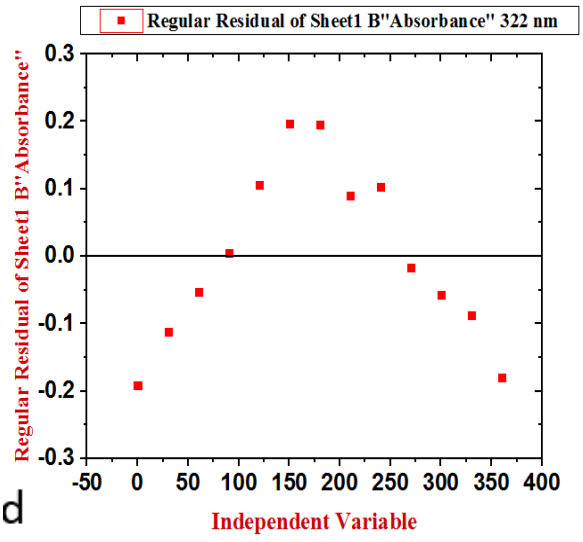


Figure 15: Data analysis using origin software (at 318nm): a) shows Scatter plot of instrument response versus concentration: the horizontal axis represent the Albuminuria concentration and the vertical axis represent the absorbance. b) Linear regression applied on selected data appearance. c) Typical output from a regression analysis using origin lab software. d) Shows plot of the residuals.

4.3.1.3 λmax = 336 nm

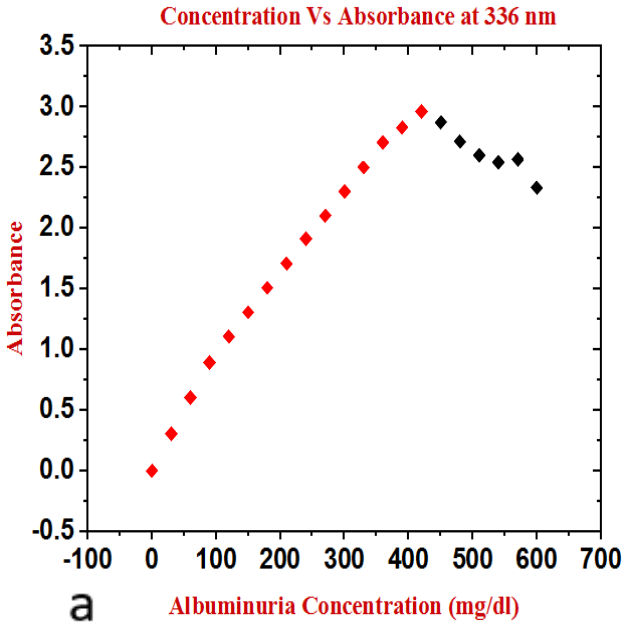
Set the device on a single wavelength and enter the maximum wavelength value, 336 nm and record value for different concentration representing samples (Table 7).

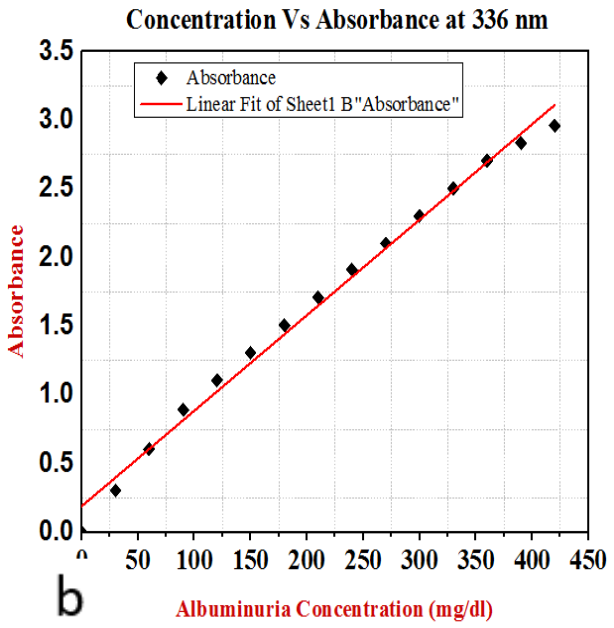
Table 7: Absorbance value record of 21 samples at 336 nm

Sample	**Albuminuria concentration (mg/dl)**	**Optical Density (O.D) Absorbance**
1	0	0
2	30	0.3041
3	60	0.605
4	90	0.8925
5	120	1.1054
6	150	1.3066
7	180	1.5078
8	210	1.7067
9	240	1.911
10	270	2.1023
11	300	2.3024
12	330	2.5009
13	360	2.705
14	390	2.8311
15	420	2.9603
16	450	2.8722
17	480	2.7127
18	510	2.6016
19	540	2.541
20	570	2.567
21	600	2.333

The procedure that was used to measure 21 albuminuria concentrations at λmax 336 nm is the same as the procedure used to measure λmax 318 nm and λmax 322 nm. Scatter plot of instrument response data is shown in Figure 16. The linear relationship between instrument

response and analyte concentration was achieved up to 420 mg/dl it is marked with red color in Figure 16a. The equation established by the applied linear regression analysis was y = 0.00695x + 0.18932, which describe the relationship between the data sets (Figure 16b). A residual is calculated using the equation of the fitted line. Figure 16d shows the plot of the residuals.





Equation	y = a + b*x		
Weight	No Weighting		
Residual Sum of Squares	0.10303		
Pearson's r	0.9958		
Adj. R-Square	0.99097		
		Value	Standard Error
Absorbance	Intercept	0.18932	0.04377
	Slope	0.00695	1.77345E-4

c

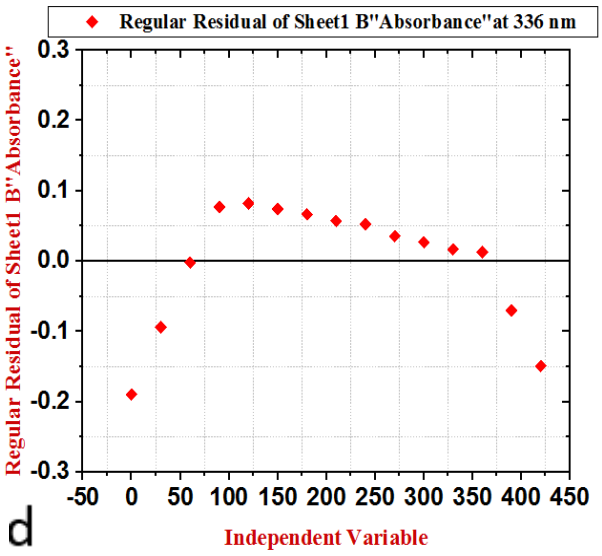


Figure 16: Data analysis using origin software (at 318nm): a) shows Scatter plot of instrument response versus concentration: the horizontal axis represent the Albuminuria concentration and the vertical axis represent the absorbance. b) Linear regression applied on selected data appearance. c) Typical output from a regression analysis using origin lab software. d) Shows plot of the residuals.

4.4 Comparing the three wavelength results

After reagent was selected λmax was determined to achieve the primary objective of this study. The result of the three best wavelengths (λmax) was obtained, and their performance was evaluated as it is shown in Table 8. This helps to decide what type of filter and light source will be used to design the system.

Table 8: Comparing the 3 wavelength outputs

Filter (wavelength)	**318 nm**	**322 nm**	**336 nm**
Residual sum of square	0.24028	0.20153	0.10303
r	0.97895	0.99108	0.9958
R^2	0.95314	0.98063	0.99097
Intercept	0.19678	0.19098	0.18932
Slope (sensitivity)	0.00863	0.00825	0.00695
Intercept standard error	0.10186	0.07095	0.04377
Slope standard error	6.3601E-4	3.34439E-4	1.77345E-4
Detected range of albuminuria concentration (limit of linearity)	(0-270) mg/dl	(0-360) mg/dl	(0-420) mg/dl

As Table 8 illustrates the residual sum of a square of the three wavelengths is good but since the line that gives the smallest sum of the squared residuals (R), best represents the linear relationship between the x (concentration) and y (absorbance). Therefore wavelength λmax=336 nm best represent the linear relationship between concentration and absorbance. The correlation coefficient (r) ranges from –1, a perfect negative relationship, through zero (no relationship), to +1, a perfect positive relationship and from the table above, greater r value is achieved at 336 nm with an r value of 0.9958. Wavelength 336 nm has better R^2 value 0.99097. The larger slope indicates a more sensitive measurement, but in this case, the sensitivity of wavelength 336nm is lower than the other two. In sensitivity range, the achieved sensitivity at 336 nm is categorized under moderate sensitivity. The limit of linearity or albuminuria detection range at 336 nm is wide. Concentration starting from 0 mg/dl to 420 mg/dl is linear but for concentration above 420 mg/dl or 4200 mg/L system won't give response. Both intercept and slope standard errors of 336

nm are minimal compared to that of 322 nm and 318 nm. Therefore based on the above typical outputs obtained from Table 8 wavelength 336 nm was chosen as maximum absorbance wavelength to design the system to achieve general objective of this study. Absorbance measurements were taken for each concentration five times, and mean error was calculated so that error bars can be plotted of the standard deviation of those measurements to estimate of the error of each reading. Figure 17 shows the error bars of the absorbance reading when test is repeatedly done.

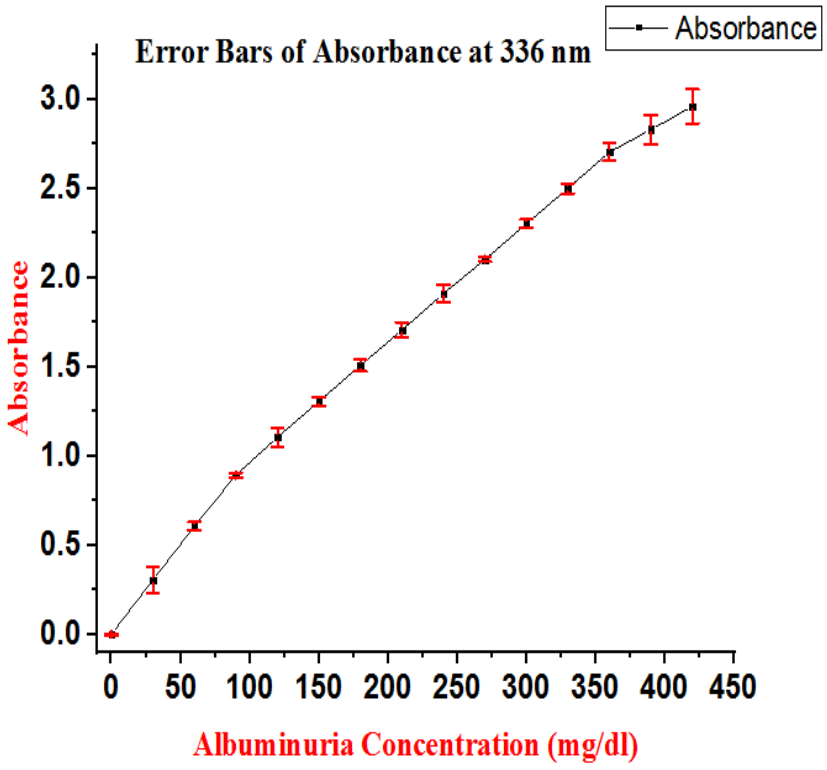


Figure 17: Error bars plotted to estimate error of each reading

4.5 Performance Analysis

The standard working solution of known concentration was prepared; the table that shows how those concentrations were moderated attached in Appendix VI. Table 9 shows the difference between the measured (expected) absorbance of selected albuminuria concentration using Multiskan GO UV/VIS spectrophotometer and absorbance calculated from the equation obtained to check the accuracy of the system. The absorbance reading using Multiskan GO UV/VIS spectrophotometer which is listed in Table 9 is the average result obtained form 5 time

repeatedly measured selected known albuminuria concentrations. Therefore based on the data's obtained accuracy of the proposed method is 94.93 %. Linear regression equation is

Y= 0.00695x + 0.18932.

Table 9: Testing the design with unknown concentration

Sample	λmax (nm)	Albuminuria Concentration (mg/dl)	Optical Density (O.D) Absorbance	Absorbance Value obtained from developed equation	Error
1	336	20	0.2327	0.32838	0.09568
2	336	80	0.7283	0.74555	0.01725
3	336	190	1.4691	1.51035	0.04125
4	336	130	1.0310	1.09318	0.06218
5	336	220	1.6648	1.71893	0.05413
6	336	310	2.3241	2.34468	0.02058
7	336	370	2.7286	2.76185	0.03325
8	336	412	2.9625	3.05387	0.09137
9	336	45	0.4874	0.5022	0.0148
10	336	100	0.8082	0.8846	0.0764

As it mentioned in chapter 3 evaluating the results of the regression analysis using origin data analysis and graphing software to carry out the linear regression will result in many different statistical parameters and possibly a table or plot of the residuals. The meaning and interpretation of each of the common outputs (the typical validation characteristics) from a regression analysis used to know sensitivity, accuracy, limit of linearity, standard deviation, limit of detection, limit of quantitation. Table 10 shows the output of parameters used to evaluate 336 nm wavelength using common outputs from regression analysis (specification of the proposed method). The formula and definition of the parameters explained in detail in chapter 3.

Table 10: Specifications of the design

	Parameter	$\boldsymbol{\lambda max = 336}$ **nm**
1	Linear regression	Y= 0.00695x+0.18932
2	Standard deviation	S.D = 0.04377
3	Sensitivity	0.00695 (moderate sensitivity)
4	Accuracy	5.0689 % error or (94.93 % Accurate)
5	Limit of linearity	0 mg/L – 4200 mg/L
6	Limit of detection	18.89 mg/dl or 188.9 mg/L
7	Limit of quantitation	62.98 mg/dl or 629.8 mg/L

The results gained from table 10 illustrates that the system function is linear which is Y=0.00695x+0.18932. Low standard deviation and moderate sensitivity. The system can detect both microalbuminuria and macroalbuminuria. The smallest concentration of a solution of an element that can be detected with 95% certainty (limit of detection) is 18.89mg/dl or 188.9 mg/L and the smallest amount at which analyte can be measured with acceptable accuracy and precision (limit of quantitation) is 62.98 mg/dl or 629.8 mg/L. The accuracy of the designed system is 94.93%. This shows using 23% concentrated sulfuric acid it is possible to quantitatively measure proteinuria to tell the kidneys condition.

CHAPTER 5

SUMMARY AND CONCLUSION

5.1 Summary and Conclusion

Different approaches have been proposed in the literature to detect protein in the urine. In this study Sulfuric acid was selected as best denaturing reagent due to the precipitated protein (turbidity) and exothermic reaction observed. The reagent used for this study was able to detect microalbuminuria range as well as macroalbuminuria range in comparison to other well established method as shown in Table 11. The linear regression analysis was applied to establish the equation (i.e., $y = mx + b$, where m is the slope of the line and b is its intercept with the y-axis) that best describes the linear relationship between absorbance and albuminuria concentration. Based on the reagent output UV based detection method was designed and tested with unknown concentration using Multiskan GO UV/VIS spectrophotometer.

The proposed urine albumin detection method does not need any urine preprocessing procedure (centrifugation) as compared to HemoCue albumin analyzer. The sample volume is 3 ml, and incubation time is 30 min. Turbidity is proportional to the concentration of albumin in urine. Turbidity achieved has good sensitivity for 336 nm wavelength (measuring range 188.9 - 4200 mg/L). The linear regression analysis demonstrated a good correlation in the range of 188.9 - 4200 mg/L for the proposed system ($y = 0.00695 + 0.18932$, $r = 0.9958$). As Table 11 summarizes the developed method has better performance than dipstick and better advantage than the Homocue albumin analyzer even though there are some draw backs that should be improved in future.

In conclusion, a simple, quantitative, and effective detection method in microalbuminuria range was developed for screening kidney health status. This method measures urine protein quantitatively, which allows for clinical decision-making and follow-up of patients. Both spot and timed urine specimens can be used with this system.

Table 11: Comparing the developed Method with the existing methods

Type of Method	Availability	Spot urine albumin mg/L detection range	Transfer function	Draw Backs of the method
Dipstick	Found in all clinics	>300 mg/L	-	User dependent , pH dependent, unable to detect if protein is in between 30 and 300 mg/L,
Homocue Albumin Analyzer	Costy and not easily available (antigen-antibody reaction)	5-150 mg/L	Y=mx+b (linear)	Need sample preprocess , reagent has to be refrigerated, time consuming, to measure above 150 mg/L dilution factor is used, need experience
The developed UV Method	Reagent is sulfuric acid (available)	188.9 – 4200 mg/L	Y=mx+b (linear)	Nature of the reagent, moderate sensitivity,

5.2 Future Work

This research can be a primary input to construct the hardware part of the protein detecting device since the basic necessary components to construct the device are identified. Using the equation or transfer function obtained from the graphical analysis (figure 18) it is possible to code the system.

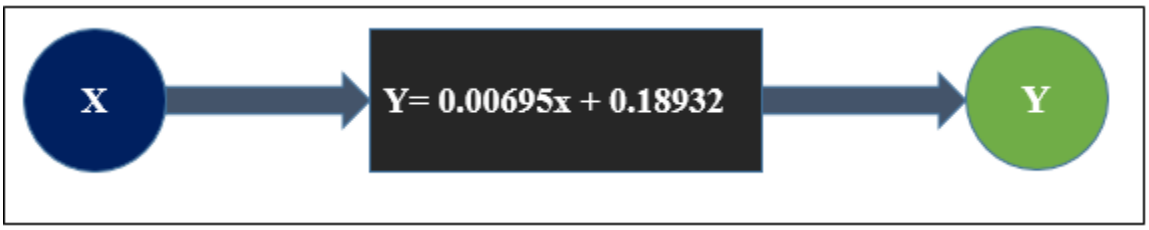


Figure 18: Transfer function of the proposed method

This study can be used as a reference to design variety simple UV/VIS Spectrophotometer to diagnose different type of diseases depending on reagent behavior. If the system is implemented the cost per test compared to immunological based assay method will be very low since reagent used for this study is available in Ethiopia (Awash Melkasa Sulfuric Acid and Aluminum Sulphate PLC) and cost of the device will be very fair if it is designed to only diagnose kidney health. In future depending on the obtained result the device will be design and sensitivity will be improved.

Limitations and challenges of the study

Due to the unavailability of quantitative proteinuria/albuminuria measuring device it was difficult to perform clinical trial. For this study 23% concentrated sulfuric acid was used as reagent but still it has to be modified in future for the sake of safety and minimizing risk.

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APPENDIX

I) Amino acids found in albumin and their chemical properties

Rank in Abundance	Type of amino acid	Properties	
63	Alanine	Non polar aliphatic aminoacids	189 non polar aliphatic amino acids found in albumin
61	Leucine		
39	Valine		
12	Glycine		
8	Isoleucine		
6	Methionine		
35	Cysteine	Poalr amino acids	150 polar,uncharged amino acids found in albumin
30	Threonine		
25	Proline		
23	Glutamine		
22	Serine		
15	Asparagine		
60	Glutamate	Negatively charged amino acids	99 negatively charged amino acids found in albumin
39	Aspartate		
58	Lysine	Positively charged amino acids	97 positively charged amino acids found in albumin
23	Arginine		
16	Histidine		
30	Phenylalanine		49 non polar aromatic amino acids found in albumin
18	Tyrosine		
1	Tyrpthophan		
	Total = 584 amino acids in albumin		

I) Dipstick test result interpreting table

Dipstick designation	24 hour urine Protein concentration/Day(**mg/24hr**)	24 hour urine collection Albumin concentration /Day (**mg/24hr**)	Spot urine albumin sample Concentration/Liter (**mg/L**)	Concentration/Deciliter (**mg/dl**)	Stage
Trace	-	-	-	5-20	Microalbuminuria (Microproteinuria)
1+	<500	<300	<300	<30	
2+	500 – 1000	300-1000	1000	100	Macroalbuminuria (Macroproteinuria)
3+	1000-2000	1000-2000	3000	300	
4+	>2000	>2000	10000	>1000	

III) Laboratory equipment's used to do lab experiments

Laboratory equipment's used: Weight balancer, magnet stirrer, 100 ml graduated beakers, 25 ml graduated beakers, test tubes, 27 Cuvettes, 5ml, and 2ml pipette, Micropipette, Spatula,

Laboratory equipments	Brand	Country	Model
Electronic Weighing Balance	METTLER TOLEDO	Switzerland	ME203E
Water Distiller	NUVE	Turkey	ND 8
Graduated beaker	NALGENE	USA	
micropipette	Fisher	USA	
Cuvette	ELKay	USA	
Magnet stirrer	JANWAY 1000		

Appendix IV

A table below shows how albumin concentration in urine is moderated or samples prepared. Procedure: Each working solution volume was 12.5 ml. This is because preparing 12.5 ml working solution was enough to run a test repeatedly 5 times since only 2.5 ml urine was needed to perform 1 test. From table 4.1 for example to prepare sample 4 which is 300 mg/L albuminuria concentration (marked sample in table 4.1) first 300 mg/L was converted to 30 mg/dl using standard unit conversion but the concentration is the same. 1 dl = 100 ml, to get 12.5 ml volume of 30mg/dl albuminuria concentration, both numerator and denominator divided by factor 8 which will give 3.75mg/12.5ml albuminuria. Bear in mind still concentration didn't change. Next step was determining 3.75mg albumin from stock solution. So if 1000 mg albumin found in 100 ml volume what about 3.75mg. Computing for 3.75 mg will give 0.375ml albumin from stock solution. Mix 0.375ml from stock solution and add 12.125 ml urine from urine jar to get 0.375/12.5ml which can represent 300mg/L or 30mg/dl albuminuria solution. The rest concentrations prepared following this protocol. Column 4 and 5 in the table shows the concentration of albumin taken from stock solution and urine solution respectively.

Working solution sample based on Standard Albumin Urine excretion (UAE) Spot		Albumin concentration in spot urine(mg/dl)	Albumin concentration in spot urine (mg / 12.5 ml)	Stock solution Taken in ml to prepare mg/12.5ml albumin urine concentration	Urine solution added to prepare mg/12.5ml albumin urine concentration
sample	mg/L				
1	0	0	0	0	12.5
2	100	10	1.25	0.125	12.375
3	200	20	2.5	0.25	12.25
4	300	30	3.75	0.375	12.125
5	600	60	7.5	0.75	11.75
6	900	90	11.25	1.125	11.375
7	1200	120	15	1.5	11
8	1500	150	18.75	1.875	10.625
9	1800	180	22.5	2.25	10.25
10	2100	210	26.25	2.625	9.875
11	2400	240	30	3	9.5
12	2700	270	33.75	3.375	9.125
13	3000	300	37.5	3.75	8.75
14	3300	330	41.25	4.125	8.375
15	3600	360	45	4.5	8
16	3900	390	48.75	4.875	7.625
17	4200	420	52.5	5.25	7.25
18	4500	450	56.25	5.625	6.875
19	4800	480	60	6	6.5
20	5100	510	63.75	6.375	6.125
21	5400	540	67.5	6.75	5.75
22	5700	570	71.25	7.125	5.375
23	6000	600	75	7.5	5

Albuminuria concentration in mg/dl	Albuminuria concentration (mg/12.5 ml)	Albuminuria concentration (mg/3ml)	Albuminuria concentration in molarity (mole/L)
10	1.25	0.3	$4.35E^{-6}$
20	2.5	0.6	$8.69E^{-6}$
30	3.75	0.9	$1.31E^{-5}$
60	7.5	1.8	$2.61E^{-5}$
90	11.25	2.7	$3.91E^{-5}$
120	15	3.6	$5.22E^{-5}$
150	18.75	4.5	$6.52E^{-5}$
180	22.5	5.4	$7.83E^{-5}$
210	26.25	6.3	$9.13E^{-5}$
240	30	7.2	$1.04E^{-4}$
270	33.75	8.1	$1.17E^{-4}$
300	37.5	9	$1.3E^{-4}$
330	41.25	9.9	$1.43E^{-4}$
360	45	10.8	$1.56E^{-4}$
390	48.75	11.7	$1.69E^{-4}$
420	52.5	12.6	$1.83E^{-4}$
450	56.25	13.5	$1.96E^{-4}$

Appendix V

Table below shows a laboratory report from the universal advanced medical laboratory (result certificate taken from the laboratory).

Test type (name)	Result
Protein	Negative
Blood	Negative
Glucose	Negative
Ketone	Negative
Bilirubin	Negative
Urobolinogen	Negative
Leucocyte	Negative
Nitrite	Negative
pH	6.5
Specific gravity	1.010
WBC	3-5 HPF
RBC	0-2/HPF
Cast	Not seen
Crystal	Not seen

Appendix VI.

Table below shows how known standard solution with different concentration prepared.

working solution based on standard albumin urine excretion (uae)	Albumin concentration in spot urine(mg/dl)	Albumin concentration in spot urine (mg / 12.5 ml)	Stock solution Taken to prepare ml/12.5ml albumin urine concentration	Urine solution added to prepare mg/12.5ml albumin urine concentration
1	20	2.5	0.25	12.25
2	80	10	1	11.5
3	130	16.25	1.625	10.875
4	190	23.75	2.375	10.125
5	220	27.5	2.75	9.75
6	310	38.75	3.875	8.625
7	370	46.25	4.625	7.875
8	412	51.5	5.15	7.35