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Liquid Chromatography Microfluidics for Detection and Quantification of Urine Albumin Using Linear Regression Method

Patricia B. Cruz, Catrina Jean G. Valenzuela, Analyn N. Yumang

***Abstract*—**Nearly a hundred per million of the Filipino population is diagnosed with Chronic Kidney Disease (CKD). The early stage of CKD has no symptoms and can only be discovered once the patient undergoes urinalysis. Over the years, different methods were discovered and used for the quantification of the urinary albumin such as the immunochemical assays where most of these methods require large machinery that has a high cost in maintenance and resources, and a dipstick test which is yet to be proven and is still debated as a reliable method in detecting early stages of microalbuminuria. This research study involves the use of the liquid chromatography concept in microfluidic instruments with biosensor as a means of separation and detection respectively, and linear regression to quantify human urinary albumin. The researchers' main objective was to create a miniature system that quantifies and detect patients' urinary albumin while reducing the amount of volume used per five test samples. For this study, 30 urine samples of unknown albumin concentrations were tested using VITROS Analyzer and the microfluidic system for comparison. Based on the data shared by both methods, the actual vs. predicted regression were able to create a positive linear relationship with an R^2 of 0.9995 and a linear equation of $y = 1.09x + 0.07$, indicating that the predicted values and actual values are approximately equal. Furthermore, the microfluidic instrument uses 75% less in total volume – sample and reagents combined, compared to the VITROS Analyzer per five test samples.

Keywords—Chronic kidney disease, microfluidics, linear regression, VITROS analyzer, urinary albumin.

I. Introduction

CKD is the increasing loss of kidney functionality that gradually progresses over time to End-Stage Renal Disease (ESRD) [1]. The kidney is responsible for filtering excess fluids and wastes in the blood, which are removed through urination. The number of people in the world being diagnosed with CKD is only increasing each year exponentially [2]. CKD has five known stages, and each stage is identified depending on the gradual loss of kidney functions. There are a lot of undiagnosed early-stage CKD cases (first and second stage) because, during these stages, kidney functions are silently being damaged before symptoms show [3]. Early stages of CKD are diagnosed by performing various tests on the patient's blood pressure, serum creatinine, and detection of albumin in the urine. Performing tests on the urine to detect albumin is considered a non-invasive medical procedure. Non-invasive methods are more considered by both medical professionals and patients compared to invasive medical procedures since non-invasive procedures do not require cutting or injecting the body [4]. Albumin is a protein that should only be present in the human blood and should be absent in human urine [5]. Presence of small amounts of albumin in urine is known as microalbuminuria [6]. Traditionally, the urine dipstick method is used to detect albuminuria wherein a chemically treated strip is dipped in the patient's urine for one to two minutes until a color reaction is observed. Then it is compared to the accepted standard color range that corresponds to a urinary albumin concentration [7]. However, the accuracy of the dipstick is not yet proven and completely accepted when detecting albuminuria since the positive predictive values are low and is still being debated [8].

Existing studies that focused on various chemical and biological approaches in quantifying urinary albumin include: high performance liquid chromatography (HPLC) [9], and immunochemical methods such as immunoturbidimetry (IT) [10], on-chip immunoassay [11], immunonephelometric (IN), radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA) [12]. According to [13], HPLC is considered the most reliable and effective since it quantifies the total intact albumin by protecting the immunoreactivity of albumin and detecting the immune-unreactive albumin that cannot be achieved in immunochemical methods. However, according to [14], methods such as mass spectrophotometry, IT, and HPLC have yielded the same results in their study with the use of the same standard curve. The difference between these methods arises because of their differences in the standard curve used in the quantification. Moreover, with the advancement of technology, detecting and quantifying urinary albumin can be achieved using electrophoresis [15], mass spectrometry [16], microfluidics [17], and so on. There are related studies that combine traditional assays and new biosensors, [18] creating a better method and a more significant impact in determining the albumin present in the urine with lesser sample size and volume of eluents. In this study, the concept of liquid chromatography is used for the system.

Existing methods for quantifying urinary albumin such as, which is composed of a mobile phase reservoir, pumping system, sample injector, column and a detector [19], often comes in large scale machinery or requires large volumes of chemicals. Using large scale machinery also means the

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necessary resources such as chemicals and biological samples for the quantification of urinary albumin are greater in terms of volume [20]. Microfluidics can perform various chemical or biological procedures using lesser volume of chemicals and biological samples through manipulation and control of fluids in the micrometer channel [21].

The core purpose of this study is to detect and quantify the urinary albumin concentration by integrating the concept of the HPLC method, microfluidics, and biosensing technology. The researchers specifically aim: (1) to develop a syringe pump, UV spectrophotometer biosensor, and microfluidic instruments for the mobile phase and stationary phase in order to reduce the chemicals used, (2) to develop a software application that will display and compute for urinary the albumin concentration, and (3) to compare the amount of chemicals used per five sample testing for both the reverse-phase HPLC microfluidics system (RPHPLC-MS) and the laboratory method.

With this study, the determination of urinary albumin concentration can be a significant help in the medical field by introducing new methods for the determination of a developing CKD in places where medical machinery is not present. This study can also aid medical missions conducted by the government by giving new methods for quantifying urine albumin and raising awareness in the rising death rates due to CKD. Although there were existing studies conducted purposely for the determination of the albumin and or protein in the urine, this paper can be used to further the study in determining and diagnosing microalbuminuria.

This study is limited with the detection of albumin found in human urine sample. Sample tests will only be conducted using urine samples with microalbuminuria. Also, this study covers the quantification of the urine albumin concentration using the concept of an HPLC method incorporated in microfluidics and biosensing technology for prognosis of microalbuminuria with a limitation of 3.4 mg/dL and 114 mg/dL as its lowest and highest range respectively. Further quantification above and below the said range can be detected with warning but cannot be quantified. Lastly, the microfluidic instrument is compared with the VITROS Analyzer, a technology used in the public hospital Philippine General Hospital (PGH) for detection of microalbuminuria.

II. Methodology

A. Conceptual Framework

The conceptual framework of the system, as illustrated in Fig. 1, starts with the insertion of the mobile phase eluents (water, acetonitrile, 0.1 M sodium hydrogen phosphate, and trifluoroacetic acid) and urine sample to the microfluidic instrument via syringe pump and pipette, respectively. The eluents with the urine sample will be processed using the same concept as the reverse-phase HPLC in the microfluidic instrument. The fractions accumulated will be placed in the UV spectrophotometer for the detection of urinary albumin absorbance. A standard curve using a linear regression method will be used for the quantification of urinary albumin to generate the absorbance vs. concentration graph and compute for the corresponding concentration of urinary albumin.

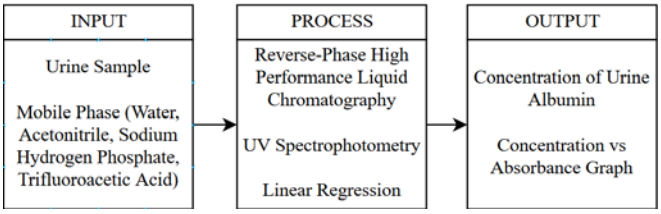


Fig. 1 RPHPLC Microfluidics System Conceptual Framework

B. Linear Regression

The relationship between absorbance x and the concentration y was determined using linear regression wherein values are predicted for a dependent variable y for every known independent variable x. Equation (1) expresses the format of the standard curve equation, where b is the computed y-intercept, m is the slope, and y is the absorbance calculated using the Beer-Lambert law. Furthermore, linear regression was applied to verify the value of R^2. The correlation coefficient R^2 identifies the linear relationship between a set of data [22]. A value of R^2 approximately equal to 1 state that the relationship is positively linear [23], and the sets of the value of concentration x, and absorbance y, are precisely the best fit.

$$y = mx + b \tag{1}$$

In the actual vs. predicted linear regression, The R^2 will determine whether the predicted value and the actual value has a perfect relationship ($R^2 = 1$), to no relationship ($R^2 = 0$). The closest the R^2 to the value of 1, the stronger the relationship is. Conversely, the further the R^2 to the value of 1, the weaker the relationship. R^2 can be obtained by (2). The slope and the y-intercept will also indicate the similarity of the predicted values and the actual values.

$$R^2 = \frac{\sum(\hat{y}-\bar{y})^2}{\sum(y-\bar{y})^2} \tag{2}$$

C. Hardware Development

Fig. 2 illustrates the hardware block diagram of the system. There are three main components, namely the: syringe pump, the UV spectrophotometer, and the microfluidics of the mobile phase and stationary phase. The syringe pump holds the four mobile phases which will pass through the mobile phase and stationary phase microfluidics instrument and to the sample cuvette of the UV spectrophotometer. The Arduino Nano that controls the syringe pump is composed of an ATmega328P microcontroller. Although, it has a lower power consumption, the performance of the 8-bit microcontroller is still reasonably high [24]. The pipette with the urine sample will supply the said sample of the patient into the microfluidic instrument. The water, acetonitrile, 0.1M sodium hydrogen phosphate, trifluoroacetic acid and urine sample will pass through the stationary phase for separation of the analytes. The absorbance of the resulting fractions was detected by the UV

spectrophotometry. The result from the UV sensor was transferred to the computer using the Arduino Uno for computation and processing of the absorbance of urine albumin concentration.

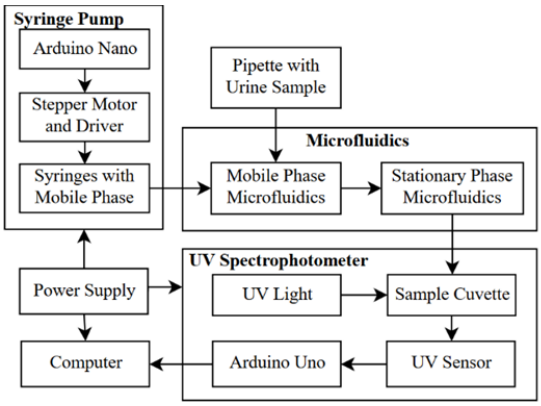


Fig. 2 Hardware Block Diagram

D. Liquid Chromatography Microfluidics

The liquid chromatography microfluidics of the system is divided into two parts, namely the mobile phase and stationary phase. The mobile phase microfluidics has an inner diameter of 800 µm while the stationary phase microfluidics has an inner diameter of 900 µm. The flow rate of the system was set to 30 µL/min, while the injected sample volume was 5 µL, and the total run time for each sample is approximately 16 minutes and 30 seconds.

E. Hardware Setup

Fig. 3 RPHPLC Microfluidics System Hardware Setup

In Fig. 3, the RPHPLC-MS hardware setup is illustrated. The mobile phases are placed on the syringe pump. Tubes from the syringe pump are inserted to the four inlets of the RPHPLC-MS 3D-printed mobile phase and the outlet of the mobile phase is connected to the metal stationary phase using another tube. The inlet for the urine is at the center of the mobile phase microfluidics. The syringe pump is set to run approximately 16 minutes and 30 seconds. The fraction is collected through a quartz cuvette and will be placed in the 254 nm spectrophotometer for quantification.

F. Software Development

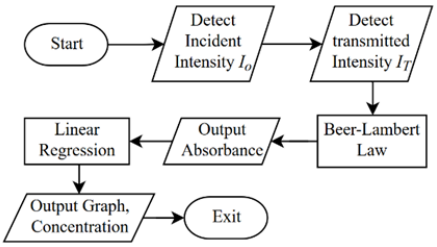


Fig. 4 Quantification of Urine Albumin Flowchart

As described in Fig. 4, the initial process is to detect the incident intensity I_o of distilled water followed by the detection of transmitted intensity I_T of the urine sample. After acquiring the standard curve, the absorbance and concentration of the urine albumin can be obtained using Beer-Lambert law (4) and linear regression, respectively. From there, the detected absorbance, and computed urine albumin concentration were plotted.

$$T = \frac{I_T}{I_o} \tag{3}$$

$$A = -\log_{10} T = -\log_{10} \frac{I_T}{I_o} \tag{4}$$

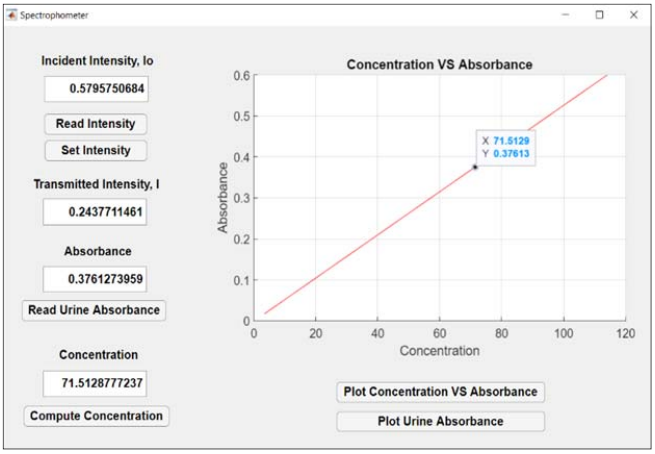
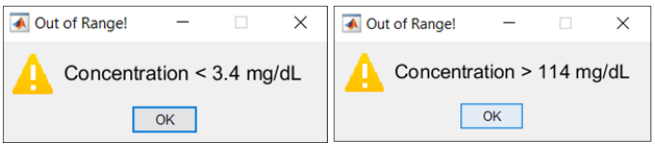


Fig. 5 Graphical User Interface



(a) (b)

Fig. 6 Out of Range Warning (a) Less than 3.4 mg/dL (b) Greater than 114 mg/dL

The program and Graphical User Interface of the application were developed using MATLAB R2019a as shown in Fig. 5. MATLAB software has numerous modules and functions capable of multiple variables, and multidimensional array computation [25]. Moreover, the program is set to have a maximum and minimum concentration that it can quantify,

depending on the range of the standard curve. The program will display warnings when samples with urinary albumin concentration below and above the range are detected.

G.Handling of Test Samples

The medical technician collects samples and observes proper handling of the urine. The VITROS Analyzer was then used for the quantification of the urine albumin for the laboratory method with run time up to 10 minutes. A 3 mL of centrifuged sample will be provided to the researchers for testing of the urine sample using the RPHPLC-MS. Fig. 7 shows some of the urine samples of patients diagnosed with microalbuminuria.

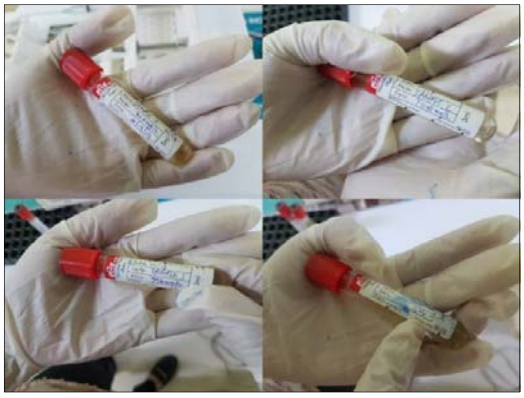


Fig. 7 Urine Test Samples with Labels

The recommended storage time of these urines can only be up to 24 hours in room temperature, to 7 days when properly refrigerated and handled to have the best result in detecting and quantifying urinary albumin.

H.Data Gathering

The data gathering for the urinary albumin of the patient was run through VITROS Analyzer and the RPHPLC Microfluidics System. VITROS Analyzer uses IT as its method in quantifying urinary albumin. Additionally, the process for both methods is supervised by a medical technician and a resident doctor. As illustrated in Fig. 8, the standard curve equation acquired using linear regression is as follows: $y = -0.0005 + 0.0052x$ with an R^2 of 0.9683. The data points used for the standard curve ranged from 3.4 mg/dL to 114 mg/dL.

The researchers gathered urine samples from the Department of Laboratories in PGH. Since the standard curve for the study has already been gathered, urine samples were tested accordingly with the help of medical technicians and a resident doctor. The following procedures are done to test the system for detection and quantification of urine albumin:

1. Fill the four syringes of the syringe pump with their respective mobile phases,
2. Attach the syringe tubing to the mobile phase microfluidics instrument,
3. Collect 5 µL volume of the centrifuged urine sample using the pipette,
4. Activate syringe pump and the spectrophotometer,
5. Dispense the urine sample to the urine inlet of the mobile phase microfluidic instrument,
6. After the stationary phase, collect protein fractions using the sample quartz cuvette,
7. Place the sample cuvette in spectrophotometer, and
8. Using MATLAB Application, gather and record the resulting absorbance and concentration of sample urine albumin.

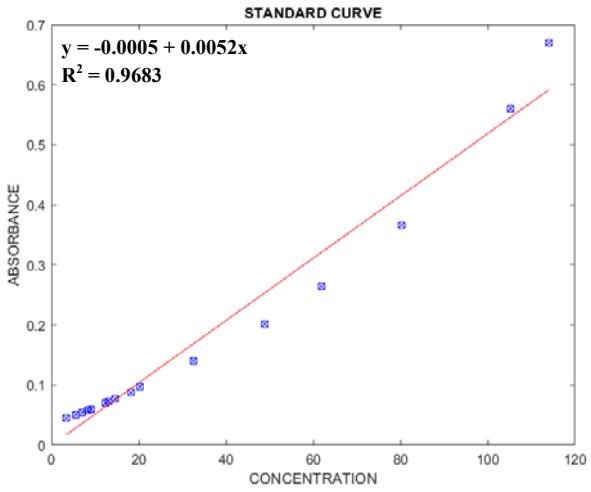


Fig. 8 Standard Curve of Urinary Albumin

III. Results and Discussion

The comparison of the result of VITROS Analyzer (actual value) and the RPHPLC-MS (predicted value) are shown in Table I. The comparison of the two methods was performed using linear regression by determining the linear relationship between the actual and predicted values. The actual and predicted values are expected to be approximately equal.

As illustrated in Fig. 9, the Actual Concentration and Predicted Concentration shows a positive linear relationship with an R^2 of 0.9995. The linear equation $y = 1.0972x + 0.0669$ obtained from the linear regression indicates that the actual and the predicted values are almost equivalent since the value of slope m is approximately equal to 1 and the y-intercept b is almost equivalent to 0.

Table II shows the concentration of urine albumin that went out of range. The VITROS Analyzer has a < 0.6 and > 19 mg/dL min-max range while the RPHPLC-MS has < 3.4 and > 114 mg/dL for its min-max range. In the RPHPLC Microfluidics system, it has an error of "Out of Range" (OOR) when a concentration was < 3.4 or > 114 mg/dL. On the other hand, for the VIRTOS Analyzer, sample 1, sample 3, and sample 4 were diluted and tested again by medical technicians with the ratio of 1:6. Thus, giving a > 19 mg/dL per fraction concentration, resulting to a concentration that is > 114 mg/dL.

The comparison of the VITROS Analyzer and the RPHPLC-MS for volume used per five sample tests is shown in Table III. The ratio of volume used per five samples was identified to be 1:3.9842. This ratio means that the RPHPLC-MS uses approximately 75% less than the VITROS Analyzer.

RPHPLC-MS method requires less volume of sample and reagents enabling more tests to be done with the excess urine not just for the quantification of microalbuminuria but also for the other tests that uses urine as the biological sample.

TABLE I
COMPARISON OF VITROS ANALYZER AND RPHPLC-MS FOR QUANTIFICATION OF URINE ALBUMIN

	Urine Albumin Concentration (VITROS Analyzer) mg/dL	Urine Albumin Concentration (RPHPLC-MS) mg/dL
1	13.17	14.6362
2	7.12	7.8911
3	3.4	3.6576
4	14.56	16.5674
5	5.63	6.1704
6	9.15	9.9722
7	18.31	19.9490
8	12.4	13.4716
9	8.4	9.1264
10	3.97	4.3271
11	77.6	87.4451
12	104.6	113.1829
13	22.64	24.8644
14	19.42	21.4611
15	6.93	7.8041
16	12.0	13.1932
17	5.72	6.2603
18	20.67	22.8433
19	19.0	21.0408
20	7.35	8.0644
21	11.2	12.2832
22	13.4	14.5389
23	7.56	8.2633
24	9.24	10.1503

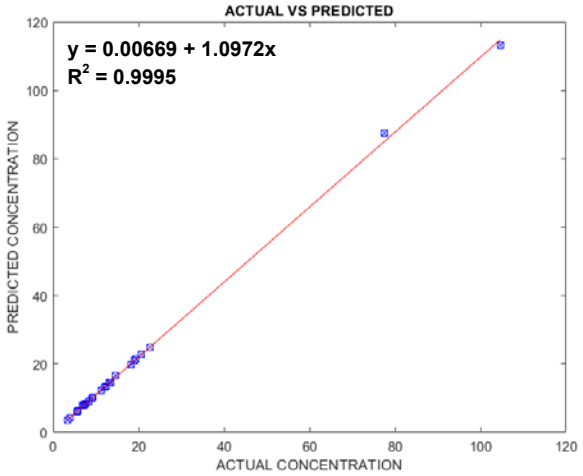


Fig. 9 Actual Concentration VS Experimental Concentration

IV. CONCLUSION

The detection and quantification of urinary albumin were made possible using the concept of RPHPLC in a microfluidic system, a biosensor, and a software program for the display of the results. The urinary albumin concentration for both the VITROS Analyzer and the RPHPLC Microfluidics system has obtained a significant linear regression relationship with an R^2 of 0.9995, approximately equal to 1. It can be concluded that the RPHPLC microfluidic system is comparable with the VITROS Analyzer when detecting and quantifying urinary albumin based on the actual vs. predicted regression where y-intercept is 0.0669 and slope of the linear equation is 1.0972, thus making the predicted values almost equal to the actual values, y = x. Moreover, the RPHPLC microfluidic system was able to lessen the volume per five sample testings since it only uses a total of 2.525 mL, sample and reagents combined, as compared to the approximately 10.060 mL VITROS Analyzer is using. Therefore, the volume required by the RPHPLC Microfluidics system is 75% less compared to the VITROS Analyzer.

TABLE II
COMPARISON OF VITROS ANALYZER AND RPHPLC-MS FOR QUANTIFICATION OF URINE ALBUMIN THAT WENT OUT OF RANGE (OOR)

No. of Sample	Quantification of Urine Albumin (VITROS Analyzer) mg/dL	Quantification of Urine Albumin (RPHPLC-MS) mg/dL
1	> 114 (1:6)	OOR > 114
2	< 0.6	OOR < 3.4
3	> 114 (1:6)	OOR > 114
4	> 114 (1:6)	OOR > 114
5	2.62	OOR < 3.4
6	0.89	OOR < 3.4

TABLE III
VOLUME USED IN TEST PER FIVE SAMPLES OF UNKNOWN CONCENTRATION OF URINE ALBUMIN

Method	Volume Used for Unknown Concentration of Urine Albumin (mL)
RPHPLC-MS	2.525
VITROS Analyzer	10.060

V. FUTURE WORKS

The study is intended to help aid and redefine current methods in quantifying and detecting urine albumin. For the improvement of the system, the microfluidics instruments should be more ergonomic in design. It is recommended to use a strong type of connector to reduce the spillage of chemicals. Moreover, since the spectrophotometer is limited to only 254 nm, it is suggested to make use of a light source and detector that has a wider range of wavelengths from ultraviolet light to infrared light to extend quantification and detection of different proteins and analytes. Furthermore, the creation of a standard curve is recommended to have a wider range to cover all possible value of urine albumin, whether it is microalbuminuria or macro albuminuria. Lastly, to reduce the amount of errors in the proper handling of the fraction of a urine sample, it is also suggested that the design of the system will be in one flow wherein fractions of samples will directly pass through the biosensor.

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