Simultaneous Determination Of BUN-Creatinine as Kidney Function Biomarkers in Blood using a Microfluidic Paper-based Analytical Devices

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Abstract. In this paper, we describe a Microfluidic Paper-based Analytical Devices (μPADs) for the simultaneous quantification of two important biomarkers of kidney function in blood. This paper provides a simple, disposable, portable, and inexpensive colorimetric method for the quantification of Blood Urea Nitrogen (BUN) and serum creatinine (sCRE). BUN detection is based on the Berthelot reaction, in which urea is converted to ammonium by the use of urease, ammonium ion which then reacts with a mixture of salicylate, sodium nitroprusside, and hypochlorite to yield a blue-green chromophore. sCRE detection is based on the Jaffe reaction, in which sCRE under alkaline conditions react with picrate ions forming an orange complex. The intensity of the color formed is proportional to the BUN & sCRE concentration in the sample. Various experimental parameters were optimized to achieve the best performance of the μPADs. There were no significant differences between the results obtained using the μPADs and a comparative method.

Keyword. BUN, creatinine, kidney, microfluidic paper-based analytical devices.

1. Introduction
Chronic Kidney Disease (CKD) can originate from acute kidney injury (AKI) cases or is purely a CKD case. The paradigm of AKI cases has changed a lot, around 15-20% of cases of AKI will develop into advanced CKD in 24 months [1]. CKD is a public health problem throughout the world. The main results of chronic kidney disease, whatever the cause, develops kidney failure, complications of decreased kidney function, and cardiovascular disease. A lot of research shows this can be prevented or delayed by early detection and treatment. Unfortunately, CKD is underdiagnosed and treated early, in part because there is no agreement on the definition and classification of stages in the development of CKD and the availability of simple detection devices [2].

Nitrogen compounds that accumulate in the blood (BUN) and serum creatinine (sCRE) are routinely used as biomarkers for the initial stages of AKI [3]. These two biomarkers are also useful in the diagnosis of CKD where the blood BUN-sCRE ratio increases [4].

Metabolism gives the end result in the form of urea and creatinine. Dietary proteins and alteration of tissue proteins produce primary urea metabolites, whereas muscle creatin catabolism produces creatinine. Both are small molecules (60 and 113 Dalton each) which are distributed throughout the body fluid [5].

UV Vis Spectroscopy [6], High Performance Liquid Chromatography (HPLC) [7], Surface Enhanced Raman Spectroscopy (SERS) [8], Impedimetric Biosensor [9], Potentiometric Biosensors [10], Chronoamperometry [11], Slide Commercial Colorimetric Test on Smartphone Platforms [12],...
Microfluidic Systems [13], Graphene Quantum Dots (GQDs) - Enzymatic Determination based on Fluorescent Sensing [14], and Amperometric Biosensors [15] commonly used in BUN determination. UV Vis Spectroscopy [6], High Performance Liquid Chromatography [16], Potentiometric Biosensors [17], Surface Enhanced Raman Spectroscopy (SERS) [18], Liquid Chromatography-Mass Spectrophotometry (LCMS) [19], Use Microfluidic Chips [20], Chronoamperometry [11], Highly Sensitive TITP-CZE [21], Amperometric Biosensor [22], Non-Enzymatic Electrochemical Sensors [23], Sequential Injection in Valve Mixing (SI-VM) [24], Micro Paper-based 3D Analytical Devices (3D-mPADs) [25] commonly used in determining sCRE. These methods mostly require expensive tools with special skills, not disposable, not portable. Simple methods, a disposable and portable device are needed in areas with limited tools and limited skills. Those needs can be provided by microfluidic Paper-based Analytical Devices (μPADs).

μPAD is a single analytic system with all the components needed for analytical detection (immobilized reagents, sample transportation, and detection systems) on paper. Filter paper or paper chromatography with the main component of cellulose needed as an immobilization medium [26].

A simple, disposable, portable, and inexpensive colorimetric method for quantification of Blood Urea Nitrogen (BUN) and serum creatinine (sCRE) is designed simultaneously based on the Berthelot reaction and the Jaffe reaction. In the Berthelot reaction, urea is converted to ammonium using urease, ammonium ion which then reacts with a mixture of salicylate, sodium nitroprusside, and hypochlorite to produce blue-green chromophore. In the Jaffe reaction, sCRE in alkaline conditions reacts with picric ions to form an orange complex. The color intensity formed is proportional to the concentration of BUN & sCRE in the sample, measured using CorelDRAW software. The proposed analytic device can be used to evaluate kidney function and help prevent kidney failure.

2. Experimental

2.1. Chemicals and apparatus

Creatinine was purchased from Sigma-Aldrich, US. Creatinine colorimetric kit reagent was purchased from Glory Diagnostics, Spain. Urea was purchased from Merck, Germany. Urea colorimetric kit reagent was purchased from Biolabo, France. Whatman No.1 filter paper was purchased from GE Healthcare, UK. ColorQube 8570 Solid Ink Color Printer was purchased from Xerox, US. Micropipette was purchased from Thermo Fisher Scientific, US.

2.2. Design and fabrication of μPADs

The μPAD pattern was designed using CorelDraw, consist of two detection zones, BUN and sCRE simultaneously. Each detection zone had a diameter of 12 mm, with μPADs dimensions of 30 x 16.5 mm. Wax was applied to the surface of Whatman No.1 filter paper with ColorQube 8570 Solid Ink Color Printer, heated on a hot plate at 150°C for 2 minutes. The wax that melts and penetrates to the filter paper becomes a hydrophobic barrier (Figure 1(a)). The backside of the μPADs was coated with tape.

2.3. Colorimetric detection of BUN-sCRE

Simultaneous colorimetric detection procedure is schematically illustrated in Figure 1(b). The colorimetric kit reagents were used for BUN and sCRE quantification. First, 1 μL R1 (urease 15 KUI/L, salicylate 31 mmol/L, and nitroprusside 1.67 mmol/L) was dropped into the BUN detection zone, followed by 1 μL R2 (sodium hypochlorite 7 mmol/L and sodium hydroxide 62 mmol/L), forming a colorless side. On the other hand, 1 μL R1 (25 mmol/L picric acid) was dropped into the sCRE detection zone, followed by 1μL R2 (300 mmol/L phosphate buffer, pH 12.7 and SDS 2.0 g/L), forming the yellow side. 1 μL standard solutions or blood serum were dropped into each detection zone and filled the detection zone with capillary action, incubated for 45 minutes. The color of the reaction changes from colorless to blue-green for the determination of BUN and yellow to orange for the determination of sCRE. Finally, the μPADs were captured using a scanner (HP Deskjet 2135 Ink Advantage).
2.4. Image processing for quantification

CorelDRAW-64 bit software was used to measure color intensity. The intensity of red, green, or blue is proportional to the concentration of BUN and sCRE.

2.5. Analysis of BUN-sCRE in serum samples

To analyze BUN-sCRE in serum samples, human serum samples were collected from Saiful Anwar General Hospital. Whole blood was collected in vacutainer tubes. After the collection, whole blood was centrifuged at 3000 rpm for 30 minutes. The resulting supernatant is designated serum. After centrifugation, it is important to immediately transfer the liquid component (serum) into a clean polypropylene tube using a Pasteur pipette. If the serum is not analyzed immediately, the serum should be apportioned into 0.5 mL aliquots, and stored at -20°C or lower.

![Figure 1](image)

**Figure 1.** (a) The design of the µPADs. (b) Simultaneous colorimetric detection procedure for BUN-sCRE. First, R1 (urease 15 KUI/L, salicylate 31 mmol/L, and nitroprusside 1.67 mmol/L) was dropped into the BUN detection zone, followed by R2 (sodium hypochlorite 7 mmol/L and sodium hydroxide 62 mmol/L), forming a colorless side. On the other hand, R1 (25 mmol/L picric acid) was dropped into the sCRE detection zone, followed by R2 (300 mmol/L phosphate buffer, pH 12.7 and SDS 2.0 g/L), forming the yellow side. Standard solutions or blood serum were dropped into each detection zone and filled the detection zone with capillary action, incubated for 45 minutes. Finally, the color of the reaction changes from colorless to blue-green for the determination of BUN and yellow to orange for the determination of sCRE.

3. Results and Discussion

3.1. Optimization of the penetration temperature and penetration time

The penetration temperature and penetration time of wax were investigated. Paper with the wax pattern was heated on the hot plate at 120°C, 130°C, 140°C, 150°C, 160°C for 30 seconds, 60 seconds, 90 seconds, and 120 seconds. The penetration time of 30 seconds, 60 seconds, and 90 seconds doesn't
produce perfect penetration. As shown in Figure 2, for temperature 120°C to 140°C, the inner diameter on the front side is always smaller than the inner diameter on the back side, which means that the penetration of the wax only takes place horizontally, not vertically, except on 150°C. The penetration temperature of 150°C and the penetration time of 120 s provided perfect wax penetration, where the inner diameter on the front side is equal to the inner diameter on the back side.

![Figure 2](image)

**Figure 2.** Optimization of the penetration temperature (t=120 seconds) (n=3).

### 3.2. Optimization of the order of addition of the reagents

The order of addition of the reagents and samples was investigated. As shown in Figure 3, for both, BUN and sCRE determination, the best spot color intensity was achieved when R1 was added first, followed by R2, and then blood serum sample.

![Figure 3](image)

**Figure 3.** Optimization of the order of addition of the reagents (a) BUN and (b) CRE (n=3).

### 3.3. Optimization of reaction time

The reaction time was investigated. The reaction time is immediately recorded after the color change and the solution in the detection zone is completely dry. The μPADs was captured using a scanner (HP Deskjet 2135 Ink Advantage) at 45 minutes, 60 minutes, 75 minutes, and 90 minutes. As shown in Figure 4, color intensity increases proportionally to 75 minutes. But the optimal reaction time is determined at 45 minutes for efficiency and the reason for the instability of the formation of the blue-green complex in the BUN colorimetric reaction. The color formed from the BUN colorimetric reaction gradually disappears.

![Figure 4](image)
3.4. Linear range

The calibration curve was based on all optimizations (Figure 5). The linear range for determination of BUN is 10-100 mg/dL ($R^2 = 0.9905$). The linear range for determination of sCRE is 10-100 mg/dL ($R^2 = 0.9928$).

![Figure 5. Calibration curve for (a) BUN determination and (b) sCRE determination (n=3).](image)

3.5. Color charts

![Figure 6. Color charts of (a) BUN and (b) sCRE.](image)
Color charts were made to facilitate the use of μPADs without other tools. The results obtained can be compared directly with the available color charts as shown in Figure 6. The normal range of BUN concentration in blood serum is 5 to 20 mg/dL. The normal range of sCRE concentration in blood serum is 0.8 to 1.5 mg/dL for male and 0.5 to 1.1 mg/dL for female.

3.6. Selectivity

![Figure 7](image-url) Selectivity of (a) BUN determination and (b) sCRE determination (n=3).

The selectivity of BUN-sCRE colorimetric determination was investigated, the specific color intensity between BUN reagents and standard BUN is shown in Figure 7(a) and the specific color intensity between CRE reagents and CRE standards is shown in Figure 7(b). Reagents used are specific to the analytes that determined.

3.7. Determination of BUN-sCRE in blood serum

The BUN-sCRE μPADs were used for simultaneous determination of BUN-sCRE in human serum samples (Table 1).

| Table 1. Concentration of BUN-sCRE in serum samples using the proposed method and the standard method RSSA* (n=3). |
|---------------------------------------------------------------|
| Blood Serum | BUN Concentration/ mg/dL | sCRE Concentration/ mg/dL |
| | Proposed Method | Standard Method (RSSA* Lab Report) | Proposed Method | Standard Method (RSSA* Lab Report) |
| Sample 1 | 100 | 105.60 | 10 | 13.05 |
| Sample 2 | 60 | 63.80 | 10 | 15.56 |

*RSSA (Saiful Anwar General Hospital)

4. Conclusions

A simple, disposable, portable, and inexpensive colorimetric method on μPADs for the simultaneous quantification of Blood Urea Nitrogen (BUN) and serum Creatinine (sCRE) were successfully developed.

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