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A LAB-ON-A-PAPER FOR LOW-COST AND DISPOSABLE DETECTION OF URIC ACID AND BLOOD IN URINE SAMPLE SIMULTANEOUSLY

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Abstract:-

This work presents a low-cost and disposable paper based microfluidic system for detection of uric acid and blood in urine simultaneously. Detection is achieved by using a colorimetric or visual indicator. Immobilized specific reagents designed for the parameter under consideration act as capture molecules on the surface of the detection zone of a microfluidic system made of paper (cellulose). Fe-(III)tris-(1,10)-phenantroline and Tetramethylbenzidinecumene hydroperoxide and have been immobilized using absorption method for blood and uric acid detection respectively. For blood detection, the detection zone will be change from pale blue to dark blue, due to presence of hemoglobin, which in turn, resulted in oxidized reaction of tetramethylbenzidine by cumene hydroperoxide. While for uric acid detection zone, the color change from yellow to orange/red due to complexation of Fe(III)tris-(1,10)-phenantroline with uric acid in urine sample. The capability of lab on paper for detection of blood and uric acid in urine samples has been demonstrate successfully.

Keywords:- *Lab-on-a-paper, Visual detection, Blood, Uric acid, Urine, Disposable sensor.*

1. INTRODUCTION

Hematuria is a symptom that is characterized by the presence of blood or red blood cells (erythrocytes) in urine [1]. Given the fairly common case, the necessary detection is need in early stage [2]. In one of the study mentioned that 22.5% of men aged 28 to 57 years positive with hematuria [3]. Moreover, in another study mentioned that hematuria occurred in over 10% of the total population [4].Macroscopic or gross hematuria can be seen by naked eye, whereas microscopic hematuria can only be detected by chemical tests, and confirmed by microscopic examination of urine sediment [5]. Diagnosis hematuria by microscopic examination of urine sediment requires a long time and requires skilled personnel [6]. Therefore, there is need to developed diagnostic methods that are rapid and practical. One of the commont method is benzidine. This method has been used widely for detecting blood in biological fluids [7]. However, benzidine reagents are carcinogenic and toxic, so it is necessary to use other safer reagents, such as tetrametilbenzidin [8].

Uric acid is the end product of purine metabolism. Uric acid has no physiological function of so-called waste product [9] both in the blood and urine [10]. However, if the amount is excessive, it can cause gout, [11] which is characterized by pain and the incidence of microscopics hematuria [12].

Various laboratory analysis techniques have been developed to detect uric acid. The method used for the detection of uric acid is based on uricase [13]. However, the method is still relatively expensive and complex. To solve these problems, the use of complex reagent, i.e. iron (III) -tris (1,10-phenantroline) that can be used to detect uric acid [14]. This method, using tetrametilbenzidine to detect blood and uric acid in urine using a solution of complex iron (III) -tris (1,10-phenantroline) still need long procedure and wet analysis as well as the volume of biological samples requires quite a lot. Therefore, there need an alternative form of testing of biological fluids that use less sample volume and low-cost [15].

One alternative technology that can be used is lab on paper based chemical sensor. This chemical sensor is a device contains a reagent that can react with a specific analyte resulting physico-chemical changes in proportional to the concentration of the analyte [16]. Here, lab on paper is a paper-based chemical that contains immobilised reagents capable of detecting analyte using nude eye. This lab on paper is able to detect the levels of uric acid and blood in urine samples simultaneously. Urine is used as a sample, since it easy to be taken, and also urine composition changes during the occurrence of disease or dysfunction before blood composition changes significantly [17].

2. METHOD

2.1. Materials and reagents

Materials used in this study include rubber based ink (CV. Cipta color Jaya), Whatman filter paper (Cat No. 1001 150), and chemical used such as uric acid, hemoglobin, 3, 3 ', 5' 5 tetramethylbenzidine (TMB), cumene hydroperoxide, iron (III) chloride pentahydrate ($FeCl_3.6H_2O$), Tris (1.10 Phenantroline), H_2SO_4 and Na_2CO_3 were purcahased from sigmaaldrich (UK). All solution were used double distilled water where required.

2.2. Preparation of reagents and solutions

For the detection of blood, it prepared using citrate buffer at pH 4-7, while for the detection of uric acid was prepared in citrate buffer at pH 5-8. Standard hemoglobin solution of 0.15 ppm - 7.5 ppm is made by dissolving a certain amount of hemoglobin standard to 10 ml of distilled water to obtain the desired concentration. While the standard of uric acid concentration of 1000 ppm was made by dissolving 100 mg of uric acid with 100 mg. Na_2CO_3, then dissolved in citrate buffer at pH 6 to 100 mL and then diluted where required. Real samples are prepared by placing urine, while still fresh in a clean container that included the patient's name and the time of holding the urine samples.

2.3. Fabrication of lab on paper

The rabber based ink were used to make pattern of lab on paper as shown in Fig. 1. This can be fabricated by placing filter paper under the screen pattern, the ink is poured on top of the screen and then leveled with a pressed strongly. The filter paper that has been patterned with the ink is dried. The lab on chip then cut cleanly as shon in Fig. 1.

The TMB reagent (20000 ppm) prepared by dissolving 80 mg TMB in 4 ml of acetic acid glacial. Cumene hydroperoxide 3% was prepared by mixing 1 ml of cumene hydroperoxide 30% in 9 ml of distilled water, afterword, they both mixed and placed in reagent zone in the lab on paper for the detection of blood. While for uric acid, a complex of 1000 ppm iron (III) - tris (1,10 Phenantroline) were prepared by mixing a solution of 1000 ppm iron (III) and 1000 ppm tris (1,10 Phenantrolin) by volume ratio of 1: 3. Complex of Iron (III) - Tris (1.10 Phenantroline) in 1000 ppm then diluted to a desired concentration of 100 to 900 ppm.

Then, this mixture of solution was placed in the reagent zone as given in Fig. 1.

Fig. 1. Lab on paper with Fe(III)-tris(1,10) phenatroline (left) for uric acid detection and tetramethylbenzidine-cumene hydroperoxide for blood detection.

2.4 Optimization parameters

Optimum condition of parametrs affects color changes as sensor response in the lab on paper, that occur after the reagents react with the analyte. In order to achieve optimum conditions, the optimization of lab on paper need to be performed includeing optimization of sample volume, volume and reagent concentration, as well as pH of the buffer.

2.5. Application

The lab on paper were in urine sample of patients to examined the levels of uric acid and blood in the urine sample, along with their assay in the Clinical Laboratory "ELISA" of dr. Soebandi general hospital, Jember. The results of the analysis of blood and uric acid levels in the urine sample are compared with standard method used in clinical laboratory.

3. RESULTS AND DISCUSSION

3.1. Optimization parameters

Sample volume

Here, we try to optimise volume of sample between 10 to 80 µL, that can be accomodate by lab on paper. The optimised volume was tested with red ink to show the volume of the sample as depicted in Fig. 2. The result show that the optimum sample volume was 80 µL. Therefore, this volume was used for further measurements.

Figure 2. Optimization of sample volume in lab on chip between 10-80 µL.

Reagen volume

Optimization of reagent volume was performed by studying number of volume required to accupy the sensing zone either for uric acid and blood, as depicted in Fig. 3. Based the result optained, the optimum reagent volume was found to be 0,5 µL and 1 µL for uric acid reagent and blood respectively. Therefore, these value were used for further immobilization of reagent in the sensing zone.

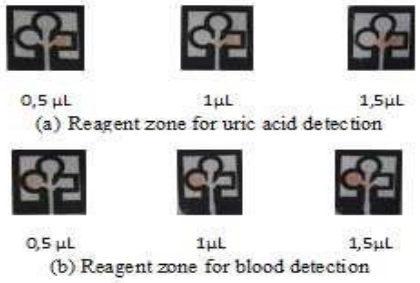

Figure 3. Optimazation of reagent volume at 0.5-1.5 µL.

Reagent concentration

The optimization of reagent for blood detection usin TMB was performed by immobilised the TMB concentration on the reagent zone in the range concentration between 100-20000 ppm of TMB in the presence of Cumene hydroperoxide (3 %). The result is given in Table 1, whre the each concentration of TMB tested was reacted with hemoglobin (7,5 ppm). Based on Table 1, it shown that TMB at cocentration of 20000 ppm gave best response toward hemoglobin, since it give the highest color change from pale blue to dark blue, compared to other concentration tested. Therefore, the TMB concentration at 20000 ppm was used for further lab on paper fabrication for blood detection.

Tabel 1. Effect of TMB concentration toward hemoglobin detection.

[TMB] (+*Cumene hydroperoxide* 3 %)	Before reaction with hemoglobin (7,5 ppm)	After reaction with hemoglobin (7,5 ppm)	*Δ mean Blue*
100 ppm			2,6
500 ppm			2,8
1000 ppm			3,6
2500 ppm			4,0
5000 ppm			4,7
10000 ppm			5,5
20000 ppm			9,1

The optimization of reagent concentration for uric acid was performed by immobilized the complex reagent of Fe (III) – tris (1,10-phenantrolin) at the concentration range of 100 – 1000 ppm in the reagent zone for uric acid detection, afterward it reacted with uric acid at1000 ppm, the result has been given in Table 2. Based on Table 2, it was found that the optimum reagent for Fe(III) – Tris (1,10-Phenantrolin) at the concentration of 1000 ppm, since it gave the highest color change from pale yellow to red orange compared to others. Therefore, the Fe (III) – Tris (1, 10-Phenantrolin) concentration at 1000 ppm was used for further lab on paper fabrication for uric acid detection.

Tabel 2. Effect of Fe (III) – Tris (1,10-Phenantrolin) concentration towards uric acid.

Fe (III) – Tris (1,10-Phenantroline)	Before reaction with uric acid (1000 ppm)	After reaction with uric acid (1000 ppm)	*Δ mean Red*
100 ppm			15,8
200 ppm			18,2
300 ppm			24,4
400 ppm			25,3
500 ppm			25,6
700 ppm			27,0
800 ppm			27,8
900 ppm			32,2
1000 ppm			36,8

PH Buffer

The effect of pH buffer toward blood detection was performed by reacting the TMB (20000 ppm) at various pH toward hemoglobine in the pH range 4-7, as shown in Fig 4. Based on this result, the optimum pH buffer for this reaction was found at pH 5. This is due to the fact that this pH gave the highest color change from pale blue to dark blue, compare to other pH tested in this case. Therefore, this pH value was used for further lab on paper fabrication for detection of blood.

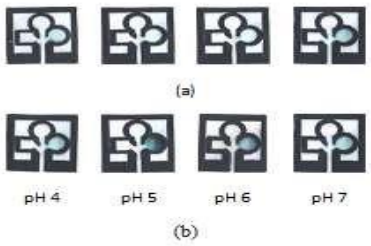

Figure 4. Effect of pH Buffer toward blood detection, before (a) and after (b) reaction of TMB (20000 ppm) toward hemoglobine (7,5 ppm).

While the effect of pH buffer toward uric acid detection was performed by reacting Fe(III) – Tris (1,10-Phenantrolin) at 1000 ppm with uric acid (1000 ppm) in various pH ranging from 5 to 8, as given in Fig. 5. Based on Fig. 5, it shows that the optimum for this reaction was found at pH 6. This is due to the fact that this pH value gave the highest color change form pale yellow to red orange, compare to other pH buffer tested in this case. Therefore, this pH was used for further lab on paper fabrication for detection of uric acid.

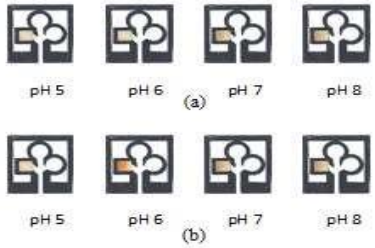

Figure 5. Effect of pH Buffer toward uric acid detection, before (a) and after (b) reaction of Fe (III) – Tris (1, 10-Phenantrolin) (1000 ppm) toward uric acid (1000 ppm).

3.2. Analytical characteristics

Response time

The response time of lab on paper towards each analyte was performed by observing the time taken to fully color change for each analyte (hemoglobine and uric acid). For blood detection, 7,5 ppm hemoglobin was tested toward reagen for blood detection (TMB and cumene hydroperoxide) by dipping the lab on paper toward this sample solution. Based triplicate measurements, it can be stated that 2 min is required to fully color change from pale blue to dark blue. While for uric acid, 1000 ppm uric acid was tested toward sensing zone of immoblised Fe(III)–tris(1,10-Phenantrolin) in triplicate measurement, by dipping the lab on paper in this sample. It can be stated that the time required to fully color change from pale yellow to red orange was 5 min. Therefore, these response times were used for further measurements, when measuring blood and uric acid simultaneouly.

Linear range

The linear range of lab on paper towards blood and uric acid detection can be given in Figures 6a and b for blood and uric acid respectively. The linear range for blood can foud in the range concentration of 0, 15 - 7, 5 ppm has been given in Fig. 6a, with the linearity eaquation $\Delta mean\ blue = 0{,}855\ [hemoglobin] + 3{,}71$ with correlation coeficient (r) of 0,992. While for uric acid, the linear range was found in the concentration range of 100 - 1000 ppm, with the linear equation is $\Delta\ mean\ red = 0{,}046\ x - 9{,}017$, wiith correlation coeficient (r) of 0,996. It show that the r value show that the sensor response depend on the hemoglobin concetration [18]. Based Figure 6 (a and b), it can be also found that the limit of detection for blood detection in term of hemoglobine at the concentration 0,15 ppm, while for uric acid the limit of detection was found at 100 ppm. These figure of LOD shows that the lab on paper could be apply for clinical sample, since the LOD within the clinical value of blood and uric acid in real urine sample.

Recovery and reproducibility

Before the lab on paper was tested to the real sample, it recovery value toward blood and uric acid was tested simultaneouly using simulated sample. The measurement of the recovery value (%) was tested in triplicate as given in Table 3. It can stated that the recovery (%) was 94, 53 % for hemoglobine(Hb) and 98,27 % for uric acid respectively. Since the recovery value within accepted range of the recovery value [19], thus the lab on paper can be stated has good accuracy in detection of hemoglobine and uric acid simultaneouly.

The reproducibiliy of the lab on paper was tested toward concentration range of hemoglobine of 0,30 – 7,50 ppm, with triplicate measurement, and gave good reproducibility at 0.802%. While for uric acid, also give good reproducibility at 1.017% at triplicate measurement. Thus, it can be stated that the lab on paper has good precicion toward hemoglobin and uric acid detection simultaneously.

Table 3. Recovery value (%) of lab on paper towards hemoglobine(Hb) and uric acid simultaneously in simulated sample.

[Analyte] (ppm)		Sensor Response	sensor response value		Found (ppm)		% recovery (%)	
Hb	Uric acid		Δ *mean Blue*	Δ *mean Red*	Hb	Uric acid	Hb	Uric acid
0,30	200		4,0	-	0,33	-	110,00	-
			3,4	-	0,35	-	116,67	-
			3,5	-	0,24	-	80,00	-
0,45	300		4,1	-	0,44	-	97,78	-
			4,1	-	0,44	-	97,78	-
			4,2	-	0,55	-	122,22	-
0,60	400		4,2	8,0	0,55	369,93	91,67	92,48
			4,2	8,8	0,55	387,33	91,67	96,83
			4,3	9,2	0,67	396,02	111,67	99,01
0,75	500		4,3	13,3	0,67	485,15	89,33	97,03
			4,4	13,7	0,78	493,85	104,00	98,77
			4,5	12,4	0,89	465,59	118,67	93,12
1,50	600		4,9	19,6	1,34	609,07	89,33	101,51
			5,0	19,1	1,46	611,24	97,30	101,87
			5,1	18,3	1,57	593,85	104,67	98,98
3,00	700		6,2	23,6	2,81	709,07	93,67	101,30
			6,0	19,9	2,59	628,63	86,30	89,80
			6,4	20,2	3,04	635,15	101,33	90,74
4,50	800		7,6	27,1	4,39	785,15	97,56	98,14
			7,5	28,0	4,28	804,72	95,11	100,59
			7,0	28,6	3,72	817,76	82,61	102,22
6,00	900		8,5	32,5	5,41	902,54	90,17	100,28
			8,9	35,4	5,86	965,59	97,67	107,29
			8,6	32,1	6,09	893,85	101,50	99,32
7,50	1000		-	36,8	-	996,02	-	99,60
			-	36,2	-	982,98	-	98,29
			-	35,4	-	965,59	-	96,56
Average							94,53	98,27

Intereference

Since, the lab on paper would be tested for blood and uric acid in urine sample. Therefore, interference study was carried out in particulary with other interference that contain in urine, such as urea and salt (NaCl). After with add the intereference at the ratio 1: 100 toward interferences, it can be stated that urea and NaCl did not interference with hemoglobine (0.3 ppm) detection in this case. While for uric acid, these interferences also did not give interference in the ration 1: 100, when 400 ppm uric acid detected. Thus, it can be stated that the lab on chip can be used for detection of blood and uric acid in urine sample, since urea and NaCl did not give significant interference at the ratio 1:100.

Life time

The life time of the lab on paper that can be used was studied by placing the lab on paper in plastik sealed container along with silica. Then, it stored in room temperature (± 28ºC) and chiller condition (± 4ºC). The lab on paper in room temperature was stabil up to three days, while in the chiller condition can be stabil up to 3 weeks. Therefore, it can be suggested that for longger stability of lab on paper should be stored at chiller condition.

3.3. Applications

Tabel 4 shows the result of lab on paper in determination of the concentration of blood and uric acid simultaneously in urine sample of patient. The result also compared with standard method used by clinical laboratory "ELISA" of Dr. Soebandi general Hospital, Jember.

Tabel 4. Result of uric acid determination of the patients tested.

No	Patient Number	Clinical Laboratory	Lab on paper	Note
1	962	Erythrocytes (50/µL) Uric acid (3.8 mg/dL)		Hemoglobin 1,5 ppm Uric acid 4.0 mg/dL
2	036	Erythrocytes (250/µL) Uric acid (8,2 mg/dL)		Hemoglobin 6,0 ppm Uric acid 8.0 mg/dl
3	008	Erythrocytes (25/µL) Uric acid (2.7 mg/dL)		Hemoglobin 0,75 ppm Uric acid 3.0 mg/dl
4	999	Erythrocytes (50/µL) Uric acid (12,3 mg/dL)		Hemoglobin 1,5 ppm Uric acid 12.2 mg/dL
5	010	Erythrocytes (50/µL) Uric acid (3.2 mg/dL)		Hemoglobin 1,5 ppm Uric acid 3.0 mg/dL
6	254	Erythrocytes (250/µL) Uric acid (3.4 mg/dL)		Hemoglobin 6,0 ppm Uric acid 3.2 mg/dL
7	257	Erythrocytes (0/µL) Uric acid (7,8 mg/dL)		Hemoglobin 0 ppm Uric acid 8.0 mg/dL
8	264	Erythrocytes (250/µL) Uric acid (4.2 mg/dL)		Hemoglobin 6,0 ppm Uric acid 4.0 mg/dL
9	288	Erythrocytes (0/µL) Uric acid (5.0 mg/dL)		Hemoglobin 0 ppm Uric acid 5.0 mg/dL
10	301	Erythrocytes (25/µL) Uric acid (5.2 mg/dL)		Hemoglobin 0,75 ppm Uric acid 5.0 mg/dL
11	250	Erythrocytes (250/µL) Uric acid (3.7 mg/dL)		Hemoglobin 6,0 ppm Uric acid 4.0 mg/dL
12	987	Erythrocytes (10/µL) Uric acid (2,3 mg/dL)		Hemoglobin 0,30 ppm Uric acid 2.2 mg/dL

Based on the Table 4, it can be stated particularly for uric acid determination that the result between lab on paper and standard method by clinical laboratory are in good agreement for all urine sample tested. However, for blood detection in urine, since the result unit is different, where the clinical laboratory used erythrocyte as number blood cells per µL, while in lab on paper, we used hemoglobin as blood concentration in ppm (µg/mL), somehow it difficult to compare the results. However, when no erythrocyte found in urine sampel like in sample no. 7 and 9, the lab on paper also show the same result, since no color change on lab on paper.

CONCLUSIONS

This paper presents a low-cost and disposable paper based microfluidic analysis system for detection of uric acid and blood in urine simultaneously. Detection is achieved by using a colorimetric indicator. Fe-(III)tris-(1,10)-phenantroline and Tetramethylbenzidine-cumene hydroperoxide have been immobilized using absorption method for blood and uric acid detection respectively. For blood detection, the detection zone has be change from pale blue to dark blue, due to presence of hemoglobin. While for uric acid detection zone, the color change from yellow to orange/red, due to the presence of uric acid in the sample. In optimised condition the lab on paper has recovery (%) of 94, 53 % for blood detection and 98, 27 % for uric acid with high reproducibility (RSD) < 2 %, in the linear range of deteksi 0, 30-7,5 ppm for blood and 400-1000 ppm for uric acid, where measurement can be performed simultaneouly.

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