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Development of a Pipette-tip-based Colorimetric Biosensor for On-site Diagnosis of Blood Urea Nitrogen

Konoka Nakamura,[1] Hayate Kitazume,[1] Shoji Yamamoto,[2] Hizuru Nakajima,[2] Yukiko Moriiwa,[1] Atsushi Shoji,[1] Akio Yanagida,[1] and Kazuhiro Morioka[1*]

[1]Graduate School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
[2]Department of Applied Chemistry, Graduate School of Urban Environmental Sciences, Tokyo Metropolitan University, 1-1 Minami-Osawa, Hachioji, Tokyo 192-0397, Japan

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A urea quantification method integrated with a pipette-tip-based biosensor and a smartphone was developed for the on-site diagnosis of blood urea nitrogen. When a sample solution containing phenol red is drawn into a pipette tip with urease immobilized on its inner wall, the color of the solution changes owing to an enzymatic reaction. The urea concentration can be quantified by photographing the pipette tip with a smartphone and quantifying the color intensity of the solution. In actual urea measurements, the detection limit (3σ) was estimated to be 1.3 mg/dL, indicating that the method has sufficient performance to quantify urea in serum. Furthermore, the method was found to reduce sample consumption and analysis time to 1/10 and 1/3, respectively, compared with conventional methods using microwell plates. This method requires only a micropipette and a smartphone to carry out measurements and does not require bench-top precision equipment or special training. Therefore, the method is expected to develop into a practical analytical platform for biomarker diagnostics that can be used in the field.

1. Introduction

Blood urea nitrogen (BUN) is a well-established biomarker for acute kidney injury[(1)] and chronic kidney disease.[(2)] BUN is typically measured in blood tests to assess renal function[(3,4)] and the normal BUN concentration range in healthy individuals has been defined as 5–20 mg/dL.[(4,5)] BUN can be calculated from the quantitative values of urea, the main product of nitrogen metabolism, which is excreted by the human kidney and liver. In the presence of urease, the conversion of urea to ammonia and bicarbonate ions is facilitated. Given that BUN accounts for approximately half (28/60, 0.446) of blood urea,[(6,7)] the normal range of urea level in the blood is estimated to be around 11 to 43 mg/dL, based on the normal BUN concentration range in healthy individuals. In clinical settings, serum urea is typically quantified using a 96-well microplate and a microplate reader. These measurements are conducted in well-equipped

*Corresponding author: e-mail: kmorioka@toyaku.ac.jp
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hospital laboratories or external testing centers by skilled technicians. Consequently, it frequently takes a significant amount of time from sample collection to obtaining test results. If BUN can be readily measured at the point of specimen collection, such as at the bedside or in a community clinic, results can be obtained immediately, allowing for the monitoring of the patient's health status. An easy-to-use, cost-effective, and rapid urea measurement platform for on-site diagnosis that does not necessitate the use of large, expensive, and specialized equipment or trained expertise is desired.

Pipette-tip biosensors[8–10] integrate the functions of pipetting and biosensing, offering the advantages of low cost and disposable use, the easy handling of low-volume liquids, reduced sampling errors,[11] and the parallelization of analysis using a multipipette.[12] These advantages suggest that pipette-tip biosensors can be developed as on-site analytical tools in a wide range of fields, including the medical, environmental, and food industries. Previously, we developed an enzyme-linked immunosorbent assay method that combines a capture antibody immobilized on a pipette tip with a homemade fluorescence detection system. This method has been used to quantify IgA, a known biomarker in saliva.[13] In this study, we demonstrated that antibodies can easily adsorb on the surfaces of commercially available pipette tips. By applying this method to the immobilization of enzymes, it was postulated that pipette-tip biosensors can be utilized for the measurement of urea. Furthermore, the combination of a pipette-tip biosensor and a colorimetric readout technique using a smartphone[14–18] can provide a simple urea measurement platform that can be performed on-site.

In this study, a simple and rapid urea determination method was developed by using a pipette tip biosensor in combination with a smartphone. When a mixed solution of sample and pH indicator is sucked into a urease-immobilized pipette tip, ammonia and bicarbonate ions are produced as a result of the hydrolysis of urea contained in the sample by the urease in an enzymatic reaction. The color of the solution changes owing to an increase in the pH of the solution, and the measurement is carried out by analyzing the color intensity of the image of the solution taken with a smartphone. Measurements of serum samples spiked with urea were carried out and the potential for this method to be used for the analysis of real samples was demonstrated.

2. Materials and Methods

2.1 Reagents and materials

All reagents utilized in this study were of an analytical reagent grade unless otherwise stated. Purified water was used in experiments, obtained from a PURELAB flex3 (Veolia Jenets K.K., Tokyo, Japan). Hydrochloric acid, sodium dihydrogen phosphate, disodium hydrogen phosphate, and sodium chloride were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). All other reagents were obtained from Fujifilm Wako Pure Chemical Corp. (Osaka, Japan), except for human serum, which was sourced from Sigma-Aldrich (MO, USA).

2.2 Preparation of pipette-tip biosensor

A 187.5 U/mL urease solution was prepared using 5 mM phosphate buffered saline (PBS) (pH 7.4) containing 0.14 M NaCl. A 5 µL volume of this solution was aspirated with a micropipette (Research plus V, Eppendorf Japan, Tokyo, Japan) into a transparent pipette tip (epT.I.P.S. 0.5–10 µL, standard, Eppendorf Japan, Tokyo, Japan). Incubation was carried out at room temperature for 1 h to allow the urease to physically adsorb on the inner wall of the pipette. Subsequently, the solution in the pipette tip was then drained, followed by the suction and draining of 7.5 µL of PBS (pH 7.4), repeated three times to remove any non-adsorbed urease. Finally, 10 µL of PBS (pH 7.4) was then sucked into the tip and the enzyme-immobilized surface was kept wet until immediately before use.

2.3 Measurement of urea

A schematic illustration of the urea measurement procedure is shown in Fig. 1. Five microliters of a 1:1 by volume mixture of sample and 0.04% (w/v) phenol red (PR) reagent was sucked into the enzyme-immobilized pipette tip and incubated at room temperature for 10 min. Subsequently, images of the pipette tips with the solution were then captured using a smartphone (iPhone 15 Pro, Apple Inc., CA, U.S.A.). The signal intensity of the area near the tip of the pipette tip was then calculated using the free color analysis software ImageJ. The color of the solution containing PR changes from red to pink at pH 7.4 to 8.2. The color image was divided into red, green, and blue (RGB) images using the ImageJ function "color split", and the signal intensity was calculated by comparing the intensity of blue to that of green (Blue/Green, hereafter referred to as B/G).[19] The change in B/G value is known to be related to the change in the absorbance of PR at its maximum absorption wavelengths (430 nm and 550 nm), and the increase in B value and the decrease in G value with increasing solution pH are attributed to PR absorbing green light more strongly.[20]

2.4 Conventional urea assay with a 96-well plate

Conventional assays were performed with a commercially available colorimetric assay kit (DetectX Urea Nitrogen Colorimetric Detection Kit, Arbor Assays, MI, U.S.A.) using 96-well

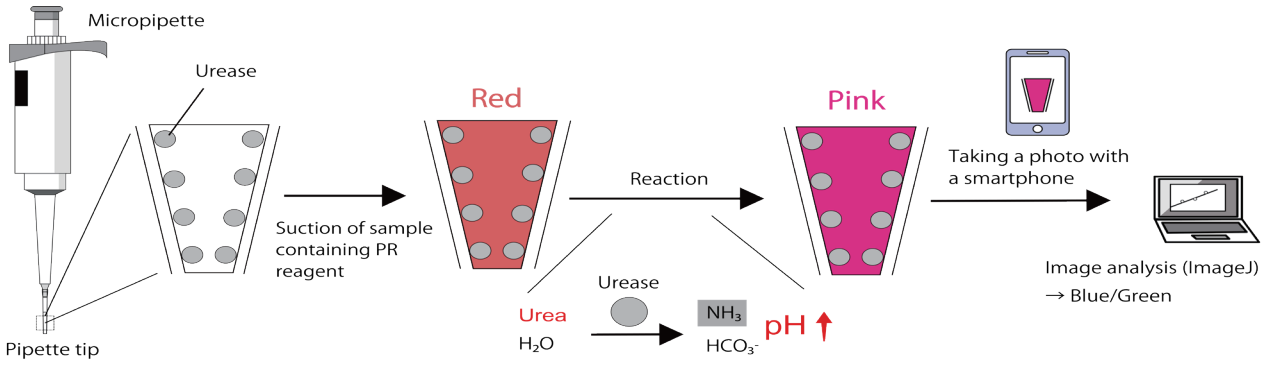


Fig. 1. (Color online) Schematic diagram of urea measurement.

plates. The assay was performed according to the instructions in the kit manual,[21] and the urea solutions prepared in PBS (pH 7.4) were used as standard solutions. Initially, 50 μL of the sample solution was added to each microwell. Subsequently, 75 μL of each of the supplied Color Reagent A and Color Reagent B was added to each microwell, and microwells were then covered with a plate seal and incubated at room temperature for 30 min. Finally, the absorbance at 450 nm was quantified using a microplate reader (SH-9000Lab, Hitachi High-Tech Corp., Tokyo, Japan).

2.5 Measurement of urea in human serum

The quantification of urea in human serum was conducted using an internal standard, following the methodology described in a commercially available urea assay kit.[22] Frozen human serum was allowed to thaw completely at room temperature prior to use. A 250 μL volume of serum was subjected to centrifugation at 12000 rpm for 5 min using a spin column (Amicon Ultra-0.5 mL, 100K, Merck Milipore Ltd.). The resulting filtrate was then diluted fivefold with PBS (pH 7.4). The sample and standard solutions were prepared by mixing the diluted solution with PBS or 50 mg/dL urea solution at a volume ratio of 4:1, respectively. The mixture of the sample solution and PR reagent at a volume ratio of 1:1 was aspirated into a urease-immobilized pipette tip and an untreated pipette tip, and the resulting values were recorded as I_{sample} and I_{blank}, respectively. Subsequently, a mixture of the standard solution and PR reagent at a volume ratio of 1:1 was measured using a urease-immobilized pipette tip to obtain $I_{standard}$. The sample urea concentration was calculated using the following equation:

$$[Urea]=\frac{(I_{sample})-I_{blank}}{(I_{standard}-I_{sample})}\times\frac{[standard]}{4}\times n\ , \tag{1}$$

where I_{sample}, I_{blank}, and $I_{standard}$ are the B/G values obtained by measuring the sample, sample blank, and standard, respectively. n is the sample dilution factor. The concentration of the internal standard is designated as [*standard*]. The volume of the internal standard is four times lower than that of the sample, and thus the internal standard concentration should be divided by four.

3. Results and Discussion

3.1 Optimization of PR concentration

Colorimetric assays using pH indicators such as PR[23] and bromothymol blue (BTB)[24] have been reported as methods for the determination of urea. Since the type and concentration of the pH indicator are strongly related to the quantitative concentration range and detection sensitivity, it is important to find the appropriate conditions. First, to select the type of pH indicator, the measurement of 0–25 mg/dL urea solution was carried out using PR or BTB as the indicator, and the color change of the solution was observed. The results are shown in Fig. 2(a). In the measured

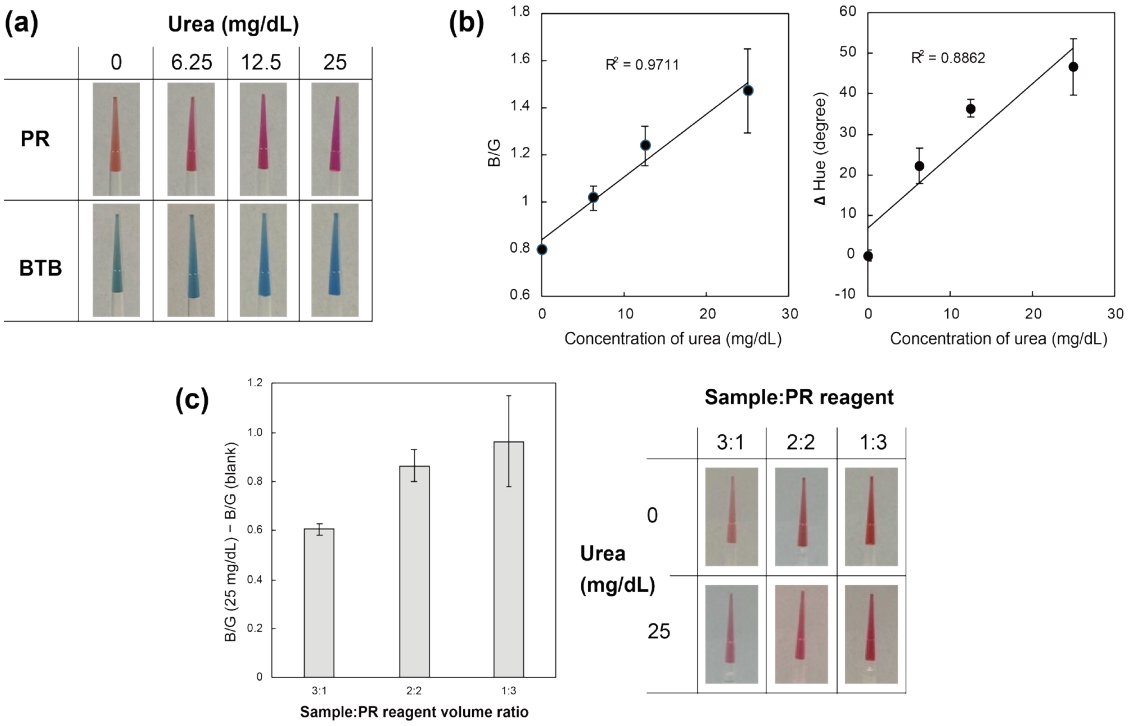


Fig. 2. (Color online) Selection of pH indicator conditions. The final concentration of both PR and BTB was 0.02% (w/v), and the reaction time was 10 min. (a) Digital images obtained by measuring 0–25 mg/dL urea solution using PR or BTB. (b) Calibration curves obtained by plotting B/G values (left) or Hue values (right) against urea concentration. (c) Intensity versus volume ratio of sample and PR reagent. The vertical axis represents the measured value of the 25 mg/dL urea solution minus that of the blank solution. The reaction time was 10 min. Error bars indicate the standard deviation for three trials. The photographs on the right are the digital images obtained in the measurement.

concentration range, the color change from red to pink could be observed by the naked eye when PR was used. On the other hand, when BTB was used, the color of the solution did not change markedly from blue at concentrations above 6.25 mg/dL. The pH of serum was around 7.4 and the hydrolysis reaction of urea in the presence of urease increased pH. Therefore, the use of BTB with a pH discoloration range of 6.0–7.6 results in a shorter dynamic range. From this viewpoint, PR with a wider pH discoloration range (6.8–8.2) was used as the pH indicator.

Next, the color analysis method was investigated, the *B*/*G* and Hue values were calculated from the images obtained from the measurement of 0–25 mg/dL urea solution, and a calibration curve for urea was constructed [Fig. 2(b)]. The *B*/*G* value showed good linear response to urea concentration (R^2 = 0.9711), while the Hue value increased gradually with urea concentration (R^2 = 0.8862). In the images, the color of the solution in the pipette tip changed from red to pink to a darker pink with increasing urea concentration. Hue values can be obtained by converting from RGB values and can represent differences in the "type" of color, but do not fully reflect the "shade" of the color (generally, the shade of a color is expressed by "saturation" and "value"). On

the other hand, *B*/*G* values correlate with the degree of absorption of green light by the solution, suggesting that the color shade is also represented in the values. On the basis of the results obtained, it was decided to use the *B*/*G* value as a signal in this study.

To determine the optimal PR concentration, 5 μL of a mixture of 0.04% (w/v) PR reagent and sample (blank solution or 25 mg/dL urea solution) at varying volume ratios was introduced into a pipette tip with urease immobilized on the inner wall, and the color intensity of the solution was quantified. The relationship between the volume ratio of the PR reagent to the sample and the intensity is shown in Fig. 2(c). Upon the visual observation of the solution within the pipette tip, it was observed that the solution remained red when a blank solution was utilized as a sample. Conversely, when 25 mg/dL urea solution was employed as a sample, the color of the solution changed from red to pink. This change was attributed to an increase in the pH of the solution, resulting from the production of ammonia by the hydrolysis reaction of urease. When the PR reagent ratio was high, the color change with solution pH change was greater, but so was the standard deviation. When the color of the solution was darker, the difference in signal intensity between the center of the pipette and near the side of the pipette was greater, suggesting that slight differences in the position of analysis caused the variation in signal intensity. Conversely, at a lower PR reagent ratio, the difference in intensity between the blank and urea solutions was minimal owing to the pale color of the solution. Although the relative standard deviation (*RSD*) values were smaller than those of the other conditions, this condition consumed more samples. In addition, assuming application to the determination of urea in serum, there were concerns about the effect of interference on the measurement, since serum at low dilution factors contains many foreign substances. On the other hand, a volume ratio of 1:1 (= 2:2) between the PR reagent and the sample [i.e., a final PR concentration of 0.02 % (w/v)] resulted in large color changes and a small standard deviation. Therefore, from this experiment, we concluded that the appropriate final PR concentration is 0.02% (w/v).

3.2 Optimization of urease concentration

The amount of immobilized urease is an important parameter affecting the sensitivity of the assay. In this experiment, urease was immobilized on the wall of the pipette tip by physisorption, a method similar to that used to immobilize antibodies in a previous report.[13] Urease-immobilized pipette tips prepared with urease solutions of different concentrations (0–1500 U/mL) were used to measure the 25 mg/dL urea solution. The intensity increased with urease concentration, reaching a maximum at a concentration of 187.5 U/mL (Fig. 3). The intensity decreased slightly at concentrations above 187.5 U/mL. When the amount of urease immobilized on the inner wall of the pipette tip increases, the pH in the vicinity of the urease increases locally owing to the formation of large amounts of ammonia. It is assumed that the reduced urease activity results in a decrease in the amount of ammonia produced, which in turn results in a decrease in signal intensity. On the basis of these results, 187.5 U/mL was selected as the appropriate urease concentration for high sensitivity and low reagent consumption.

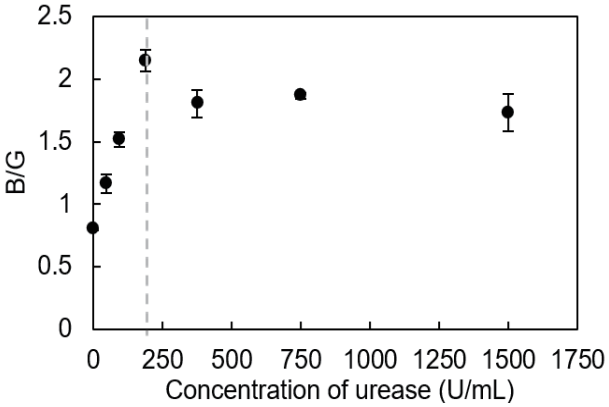


Fig. 3. Relationship between urease concentration and intensity. The sample used was 25 mg/dL urea solution. The reaction time was 10 min. Error bars indicate the standard deviation for three trials.

3.3 Reaction time

In the context of on-site diagnostics, where immediate results are required, short reaction times are of paramount importance. To investigate the effect of incubation time on the intensity reaction, mixtures of indicator and samples (12.5 or 25 mg/dL urea solution) were aspirated into a urease-immobilized pipette tip, and the intensity was measured after various incubation times (0–20 min). The results demonstrated that the *B*/*G* values increased with incubation time, with the values remaining almost constant after 10 min (Fig. 4). The *B*/*G* value of 12.5 mg/dL is almost constant after 10 min, indicating that all the urea in the solution is consumed in the enzymatic reaction. In consideration of the optimal balance between sensitivity and reaction time, the selected incubation time for this study was 10 min.

3.4 Concentration dependence

To evaluate the concentration dependence, urea solutions with concentrations ranging from 0 to 100 mg/dL were measured. The obtained calibration curve for urea is shown in Fig. 5(a). The color intensity exhibited a positive correlation with the urea concentration [Fig. 5(b)], reaching a plateau at concentrations above 25 mg/dL. This nearly constant value can be attributed to the formation of significant quantities of ammonia in solution, which elevated the pH of the solution and exceeded the upper limit of the PR discoloration range (pH 8.2). The calibration curve was drawn in the concentration range of 0 to 25 mg/dL, demonstrating good linearity with a correlation coefficient of R^2 = 0.9997. The limit of detection (*LOD*) of urea, defined as three standard deviations (3σ), and the *RSD* (n = 3) values were estimated to be 1.3 mg/dL and 1.1 to 11%, respectively. The normal concentration range for BUN is known to be 5–20 mg/dL,[4,5] which can be calculated as 11–43 mg/dL as serum urea concentration from the formula.[6,7] As the *LOD* values obtained by the present method are below this urea concentration range, the method has potential applicability for the diagnosis of BUN. The repeatability (intra-day and inter-day variations) of the present method was evaluated using 6.25, 12.5, and 25 mg/dL urea

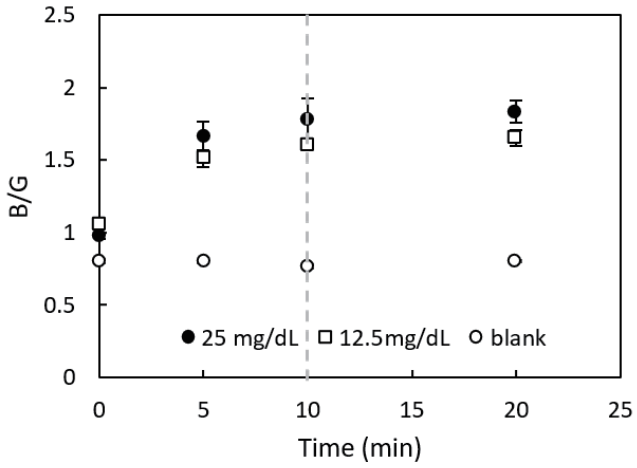
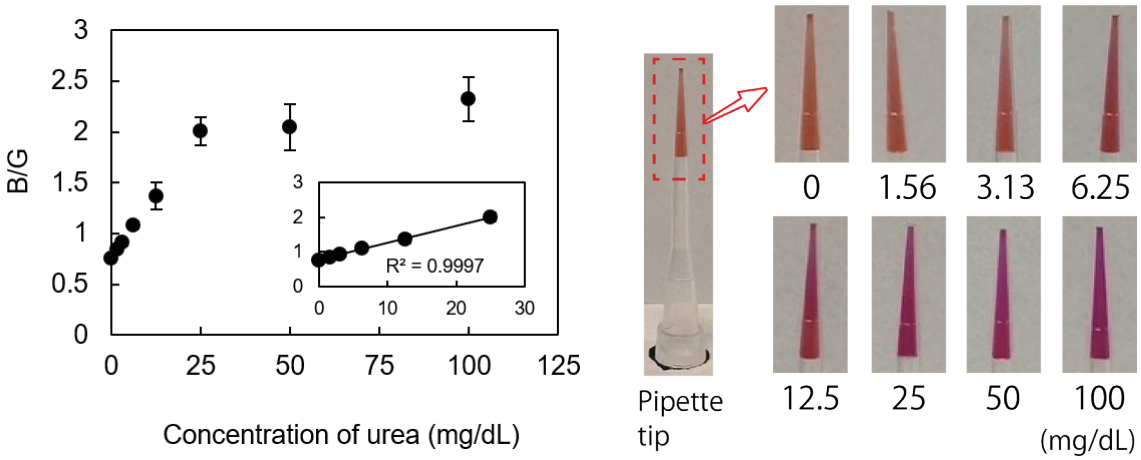


Fig. 4. Change in intensity with reaction time. The samples used were blank and 12.5 and 25 mg/dL urea solutions. Error bars indicate the standard deviation as in hte other figure captions for three trials.



(a) (b)

Fig. 5. (Color online) (a) Calibration curve for urea derived from the intensity. The reaction time is 10 min. Error bars indicate the standard deviation for three attempts. (b) Photographs of pipette tip after the reaction.

solutions, and the intra-day (n = 3) and inter-day (n = 3) variations were estimated to be 3.2–9.8 and 11–18%, respectively.

3.5 Comparison with conventional urea assay

Conventional urea assays was performed with a 96-well plate, and the obtained results were compared with those obtained by the present method. The calibration curve for urea obtained by the conventional assay is shown in Fig. 6. The calibration curve demonstrated satisfactory linearity with a correlation coefficient of R^2 = 0.987 within the concentration range of 0 to 12.5 mg/dL. The estimated *LOD* (3σ) and *RSD* (n = 4) values were 0.46 mg/dL and 1.4–13%, respectively. A comparison table between the method developed in this study and other methods is presented in Table 1. The microplate method and microplasma optical emission

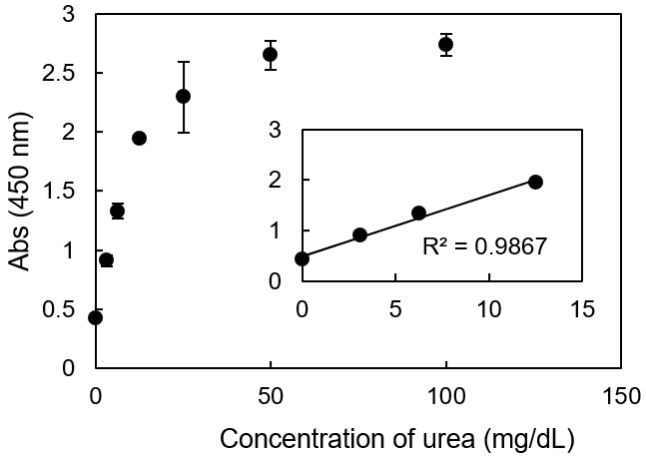


Fig. 6. Calibration curve for urea derived from conventional urea assay. Error bars indicate the standard deviation for three trials.

Table 1
Comparison of this method with other methods.

Method	Reaction field	*LOD* of urea	*RSD* (%)	Range	Sample volume (μL)	Reaction time (min)	Detection tool	Reference number
Present method	Pipette tip	1.3 mg/dL (3σ)	1.1–11 (n = 3)	1.3–25 mg/dL	2.5	10	Smartphone	-
Conventional method (96-well plate)	Microwell	0.46 mg/dL (3σ)	1.4–13 (n = 3)	0.46–12.5 mg/dL	50	30	Plate reader	-
AZOSTIX	Strip	20 mg/dL	Unknown	20–130 mg/dL	A drop of blood	1	Color chart (Semi-quantitative)	[24]
Microplasma optical emission spectrometry	Vial	0.03 mM	~3.3 (n = 11)	0.1–10 mM	20	5	Handheld instrument	[25]
Paper-based analytical device	Paper	Unknown	Unknown	10–100 mg/dL	1	45	Scanner	[26]

spectrometry[25] have advantages of high detection sensitivity and broad dynamic range. However, these methods require the consumption of higher amounts of samples, the utilization of sophisticated analytical equipment, and highly trained personnel for accurate measurement. On the other hand, the AZOSTIX strip[24] or paper-based analytical devices[26] can be operated without sophisticated analytical equipment and require a small sample volume. However, these portable sensors cannot measure urea concentration below 10 mg/dL, making it difficult to evaluate low BUN levels as an indicator of liver damage. The present method can be easily performed using a micropipette and a smartphone and has sufficient detection sensitivity, dynamic range, rapidity, and simplicity for urea testing. The present method with these performance characteristics has the potential to be applied to the on-site diagnosis of BUN.

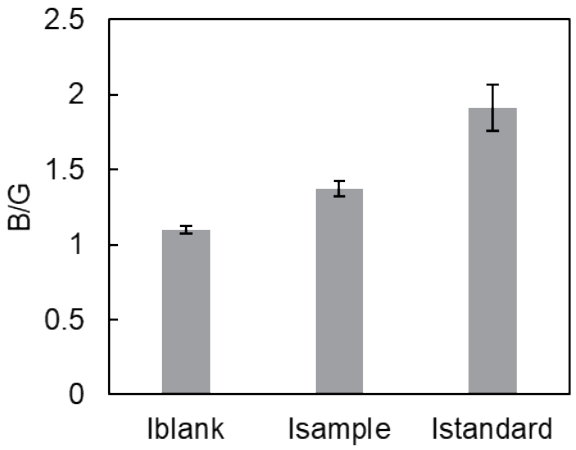


Fig. 7. Each value obtained in measurements using serum samples. Error bars indicate the standard deviation for three trials.

3.6 Quantification of urea in human serum

The measurement of urea by this method is based on changes in the pH of the solution containing the sample. Therefore, the pH of the serum may affect the measurement. To address this, internal standards were used for the determination of urea. When 50 mg/dL urea solution was used as an internal standard solution, the quantitative value of urea was estimated to be 33.6 ± 10.1 mg/dL (n = 3) from the parameters in Fig. 7. These results were in general agreement with those obtained in the conventional assay (30.8 ± 0.9 mg/dL, n = 3). The results indicate that this method can be used for the determination of urea in blood. The concentration range of urea that can be measured by this method is 1.3–25 mg/dL, with serum samples diluted fivefold. Serum samples with higher urea concentrations can be measured by diluting them further.

4. Conclusions

In this study, a urea measurement method using a urease-immobilized pipette tip biosensor and a smartphone was developed. This method is straightforward and does not necessitate the use of sophisticated or costly instrumentation. Additionally, this method is advantageous for the on-site diagnosis of urea nitrogen in blood samples, as it requires reduced sample consumption and a short analysis time in comparison with conventional methods. As the developed measurement method is based on capturing images with a smartphone, the present method is adaptable to various analytical techniques combining enzymes and chromogenic substrates and is expected to be applied to the on-site measurement of various biomarkers.

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