Reagentless Estimation of Urea and Creatinine Concentrations Using Near-Infrared Spectroscopy for Spot Urine Test of Urea-to-Creatinine Ratio

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Abstract In this work, we developed a measurement system that uses LEDs to estimate multiple components such as urea and creatinine in spot urine samples using near-infrared spectroscopy, considering future transition to LED light sources. In this study, we chose LEDs with 10 standard wavelengths (1400–2300 nm, in 100 nm increments). A multiple regression analysis using all combinations of 10 wavelengths was performed. We prepared glucose-added urine samples (GAU, urine samples from 10 healthy adults, each mixed with glucose). Wavelength selection was performed by comparing the minimum standard error of prediction (SEP, calculated from actual concentration and predicted concentration) for each wavelength combination. We obtained high accuracy for estimating urinary urea and creatinine levels (SEP: 42.4 mg/dl and 7.34 mg/dl, respectively) using four wavelengths for urea including two wavelengths showing negative absorbance, and five wavelengths for creatinine. Furthermore, an extremely high correlation coefficient ($\gamma > 0.99$) was obtained for both components. We calculated urea concentration, creatinine concentration, and urea-to-creatinine ratio using this optical, reagentless method. The low SEP and high $\gamma$ show that our method is suitable for practical determination of urea-to-creatinine ratio. Thus, this method of analyzing urine samples using NIR spectroscopy can be used to assess protein intake in CKD patients.

Keywords: reagentless, near-infrared spectroscopy, urine components, urea-to-creatinine ratio, spot urine.


1. Introduction

Japan is currently facing two major challenges: aging society and declining birthrate. The proportion of aged people (65 years and above) was 26.7% in 2015 and will increase to 33.4% in 2035 according to population projections [1]. In addition, the percentage of national health expenditure on Japanese people affected by lifestyle-related diseases is increasing. The Ministry of Health, Labor, and Welfare in Japan promotes primary prevention of lifestyle-related diseases including cancers and diabetes [2]. In preventing the progression of chronic kidney disease (CKD), diet therapy is crucial not only for patients in Japan but also all over the world. Hence, several reports relating to progressive preventive measures by limiting protein intake in CKD patients have been published [3–6]. Fouque and Laville [3] investigated the effectiveness of reducing protein intake in patients with CKD and reported that reduced protein intake decreases the occurrence of renal death by 32% compared to higher or unrestricted protein intake. Kovesdy and Kalantar-Zadeh [4] reported that low protein intake may mitigate uremic symptoms and allow conservative management of advanced CKD without the need to initiate dialysis. On the other hand, the effect of a two- to three-year intervention of dietary protein restriction on the progression of nondiabetic kidney disease remains inconclusive, as reported by Levey et al. [5]. However, their findings are consistent with the hypothesis that continued adherence to low-protein diet is associated with a beneficial effect. Hansen et al. [6] reported that moderate dietary protein restriction improves prognosis in type 1 diabetic patients with progressive diabetic nephropathy in addition to the beneficial effect of antihypertensive treatment. For measuring the excretion of urine components, 24-hour urine
collection is the general method adopted. However, urin-
ary urea-to-creatinine ratio in spot urine is effective for
assessing dietary protein intake [7], and the sodium-
to-creatinine ratio measured using multiple spot
urine samples allows direct estimation of 24-h sodium excretion [8]. Kanno et al. [9] reported the feasibility of
using urea nitrogen concentration determined from spot
urine for estimating daily protein intake. In this regard,
spot urine measurement can be equally useful compared
to 24-hour urine collection. Many methods are adopted
to measure urine components in spot urine samples, in-
cluding enzyme electrode method and urine dipstick test.
However, the methods have some disadvantages because they measure only a single component, use a qualitative
complicated procedure, and are unsanitary. To solve the
above-mentioned problems, many studies related to the
measurement of urine components using near-infrared
(NIR) spectroscopy have been conducted [10–14]. Pez-
zaniti et al. [10] investigated the feasibility of NIR spec-
troscopy for quantitating the concentrations of urea, crea-
tinine, glucose, ketone, and protein in urine. However,
it is difficult to create a high precision calibration model. We examined the possibility of using LEDs for the measurement of urine components. Mea-
surements using LEDs have been reported. Giovenzana
et al. [16] designed and tested an LED device that used only four wavelengths in the VIS–NIR region for rapid estimation of ripening parameters of white grapes. Schnable et al. [17] reported a portable instrument with LED arrays that used six wavelengths for VIS–NIR ab-
sorption and turbidity analyses.

The aim of this study were to examine the feasibility of reagentless estimation of urea and creatinine in spot
urine samples using an optical measurement method and
to investigate the feasibility of measuring urea-to-creati-
nine ratio without 24-h urine collection. Our final goal is
to develop a practical LED system for monitoring multi-
ple components in spot urine samples, which allows pa-
tients to assess and manage their body conditions them-
selves. Urinary urea and creatinine levels are markers of
kidney function. Urea is measured to estimate protein intake in CKD patients and to determine nitrogen bal-
ance, and these two parameters are indicators of protein
change and/or adequacy of feeding in a controlled set-
ting [18]. Creatinine is measured to estimate total excre-
tion of other urine components. Moreover, the creati-
nine-height index (CHI) is calculated from 24-h urine
creatinine, and is thought to provide an estimate of lean
body mass [19].

2. Materials

2.1 Preparation of single urea and creatinine solu-
tions

In this study, we aimed to verify the peak or sensitive
wavelengths of urea and creatinine by measuring the
spectra of the respective single solutions. Therefore, we
prepared single urea and creatinine solutions as de-
scribed below. Table 1 lists the structural formulas of
urea and creatinine. We weighed the appropriate amounts
of urea powder (JIS Special Grade, Wako Pure Chemical
Industries, Ltd., Japan) and creatinine powder (Wako
Special Grade, Wako Pure Chemical Industries, Ltd., Ja-
pan). The mixture of pure water and each single powder
was agitated for at least 20 min to prepare the solution.
For each powder, solutions of five different concentrations (urea: 1000–5000 mg/dl in 1000 mg/dl increments, creatinine: 500–2500 mg/dl in 500 mg/dl) were prepared. Both solutions thus prepared are hereinafter referred to as “sample A.” Normal levels of urea and creatinine in urine are 200–400 mM (1200–2400 mg/dl) and 6–20 mM (68–226 mg/dl) [20].

2.2 Urine samples
The Medical Ethics Committee of Kanazawa University approved the study and informed consent was obtained from the participants. We prepared urine samples with the addition of glucose (GAU: glucose-added urine) for use as simulated spot urine samples because patients with lifestyle-related diseases are likely to have diabetes. The normal level of glucose in urine is 0 mg/dl [20]. Thus, glucose was added to urine samples to higher concentrations than the normal level as described below.

Urine obtained from 10 healthy adults (10 men, 21–23 years, 61.67 ± 5.63 kg, see Table 2) were used. Each urine sample was mixed with glucose powder (JIS Special Grade, Wako Pure Chemical Industries, Ltd., Japan) to obtain 11 serial concentrations (0–500 mg/dl, in 50 mg/dl increments). Thus, a total of 110 samples were obtained. The actual concentrations of urea and creatinine were measured by a clinical laboratory method (error range: ±10%) using a BioMajesty system (JCA-BM8060, JEOL Ltd, Japan). Urea concentration was measured by a urease and leucine dehydrogenase method [21] and creatinine by an enzymatic method [22]. The GAU samples are hereinafter referred to as “sample B.”

2.3 Optical system
We measured the spectral energy of each sample using an FT-IR spectrometer (Spectrum One, PerkinElmer, Inc., USA) for the entire wavelength range (750–2500 nm, wavelength resolution: 1.667 nm) at room temperature of approximately 25°C. To reduce the signal-to-noise ratio, we used the averaged value of the spectral energy of 10 measurements. Each measurement takes approximately 3 s and all measurements take 30 s. The steps to measure the absorbance are as follows. (1) NIR light (I0) is irradiated onto a flow-through cell (optical path length: 0.5 mm) filled with sample or pure water. (2) Transmitted light of pure water (Ir: reference) and samples (Im: measured) is measured. (3) Differential absorbance (ΔAbs) is calculated using equation (1).

\[ \Delta \text{Abs} = \log_{10} \frac{I_0}{I_m} - \log_{10} \frac{I_0}{I_r} = \log_{10} \frac{I_r}{I_m} \]  

Multiple regression analysis is performed using the calculated ΔAbs and the actual concentration.

3. Methods
The spectrometric method is well suited to determine the quantities of multiple components in a liquid mixture [23]. Because the Lambert–Beer law applies for the VIS region, if the path length is constant, absorbance is directly proportional to the concentration of the component in the sample. However, in the NIR region, it is difficult to obtain the absorbance accurately for a sample at a specific concentration because of overlapping of the absorption wavelength ranges of multiple components. Therefore, we used ΔAbs because it indicates the characteristic of each solute in a solution. We calculated ΔAbs using equation (1) in each optical measurement. The used the ΔAbs at 1060 nm for correction of measured ΔAbs. The ΔAbs of 1060 nm was set at zero because ΔAbs at this wavelength has a small concentration dependence of each component. A commercially available LED (OSA Opto Light GmbH, Germany) was used to emit light at this wavelength. To identify the wavelengths for analysis, we chose 10 commercially available LED wavelengths (1400–2300 nm, in 100 nm increments; LED Microsensor NT LLC, Russia) and performed a multiple regression analysis using all combinations of the 10 wavelengths (total: 1023 sets). We used the R programming language (version 3.3.1) [24] and calculated the predicted concentration by leave-one-out cross-validation. The predicted concentration in each GAU sample was calculated based on the calibration equation obtained using the actual concentrations measured by the clinical laboratory method. Prediction accuracies be-
between the actual concentrations and the predicted values were assessed using the standard error of prediction (SEP) and the correlation coefficient (γ).

4. Results

Figure 1 shows the $\Delta Abs$ spectra of single urea and creatinine solutions obtained using sample A at various concentrations. It should be noted that negative values of $\Delta Abs$ are seen in specific wavelength regions (encircled parts). In spectral measurement of solutions using pure water as the solvent, $\Delta Abs$ increases with increasing concentration of the solute and negative absorbance is also confirmed.

Figure 2 shows the $\Delta Abs$ spectra obtained using sample B. Comparing Fig. 1 and Fig. 2, some common sensitive wavelength ranges are observed. The actual concentrations of urea obtained by the clinical laboratory method were 640.2–2797.9 mg/dl. The actual concentrations of creatinine were 40.1–298.8 mg/dl.

Figure 3 compares the minimum SEP of each wavelength combination using sample B. Table 3 shows the combination of wavelengths for each “number of applied wavelength” shown in Fig. 3.

Figure 4 shows the correlation plots of urea and creatinine using sample B, showing the distribution of actual concentrations versus predicted concentrations using a combination of four wavelengths for urea and a combination of five wavelengths for creatinine (to be discussed below). The dotted lines in the plots show the measurement accuracy of the clinical laboratory method. It should be noted that extremely high correlation coefficients (γ > 0.99) can be attained in estimating concentrations of urea and creatinine using only four and five wavelengths, respectively.

Figure 5 shows the correlation plot between the actual and predicted urea-to-creatinine ratios obtained using sample B. The dotted lines in the figure show the calculated error range (maximum possible error: urea/creatinine-1 = 1.1/0.9 × 100–100 = 22.2%; minimum
possible error: urea/creatinine-1 = 0.9/1.1 \times 100–100 = \sim 19.2\% \) obtained from the measurement accuracy of the clinical laboratory method.

5. Discussion

First, we describe the negative absorbance shown in Fig. 1. The creatinine concentrations of sample A were prepared at approximately 10 times the normal level. The reason was to facilitate predictability and to improve visibility of peaks in the spectra. When water is used as solvent and VIS as light source, according to the Lambert–Beer law, there is no absorption by water and it is impossible that \( \Delta Abs \) has a negative value. On the other hand, in the NIR region, there is a possibility that the molar absorptivity of solute (\( \epsilon_s \)) is larger than that of solvent, and hence negative absorbance with increasing solute concentration is possible. Solute concentration is normally estimated from the positive peak of absorbance. However, even in the wavelength ranges showing negative \( \Delta Abs \) (negative absorbance regions), useful information about the concentration of solute could be obtained.

Hence, we did not exclude these regions, but incorporated them for estimating the concentration of urine components, based on a report by Amerov et al. [25] that negative absorbance occurs by replacement of water molecules and solute molecules.

Second, the optimal wavelength combinations for urea and creatinine measurements were decided based on Fig. 3. In Fig. 3a, the SEP was 42.4 mg/dl for the combination of 1400, 1800, 2000, 2200 nm. This SEP is lower than those (43.2–384.8 mg/dl) obtained using other four-wavelength combinations. In Fig. 3b, the SEP was 7.34 mg/dl for the combination of 1600, 1800, 2000, 2100, 2200 nm. This SEP is lower than those (8.23–22.2 mg/dl) obtained for other five-wavelength combinations. This means that, using only seven wavelength (1400, 1600, 1700, 1800, 2000, 2100 and 2200 nm), we can estimate the concentration of urea and creatinine with high accuracy. The number of the wavelength could

Table 3 | Wavelength combinations for the “numbers of applied wavelengths” for urea (a) and creatinine (b) shown in Fig. 3.

<table>
<thead>
<tr>
<th>Number of applied wavelength</th>
<th>Wavelengths combination for analysis</th>
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<tbody>
<tr>
<td>10</td>
<td>1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300</td>
</tr>
<tr>
<td>9</td>
<td>1400, 1500, 1600, 1700, 1800, 2000, 2100, 2200, 2300</td>
</tr>
<tr>
<td>8</td>
<td>1400, 1500, 1600, 1700, 1800, 2000, 2100, 2200, 2300</td>
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</tr>
<tr>
<td>2</td>
<td>1800, 2200</td>
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<tr>
<td>1</td>
<td>2200</td>
</tr>
</tbody>
</table>

(a) Urea

(b) Creatinine

Fig. 3 Comparison of minimum SEP of each wavelength combination for urea (a) and creatinine (b) obtained using sample B. The appropriate wavelength combination for analysis is selected based on this graph.
be reduced to six, however, slight deterioration in accuracy were seen. And therefore, we chose the above seven wavelengths considering higher accuracy.

Third, Figure 4a shows extremely high accuracy of urea prediction using the NIR spectroscopic method when the clinical laboratory method was used to obtain the actual concentrations. Table 4 shows the functional groups corresponding to the selected wavelengths for urea and creatinine [26]. Williams and Norris [26] summarized the known functional groups for the corresponding wavelengths. Because each selected wavelength in Table 4 corresponds to a functional group, our wave-
length selection reflects the molecular structure. However, negative absorbance was observed for the wavelengths of 1400 nm and 1800 nm, and these wavelengths were not used for measurement in previous studies [10–14]. Several papers reported the reason of negative absorbance region. Amerov et al. [25] reported that negative absorbance occurs due to replacement of the water molecule by a molecule of the solute. We carried out an analysis using nine wavelengths except 1400 nm to confirm the usefulness of 1400 nm for urea concentration estimation. The minimum SEP obtained using four wavelengths and not 1400 nm (44.4 mg/dl) indicated approximately 4.5% deterioration compared with the minimum SEP (42.4 mg/dl) obtained using a combination of the four selected wavelengths. In the analysis of excluding 1800 nm, the minimum SEP obtained using four wavelengths excluding 1800 nm (48.9 mg/dl) indicated approximately 15.3% deterioration compared with the minimum SEP (42.4 mg/dl) obtained using the selected four-wavelength combination. These results indicate that 1400 nm and 1800 nm are both useful wavelengths for urea concentration estimation.

Fourth, glucose is expected to reduce the accuracy of estimating urea and creatinine concentrations. However, even when glucose was added to the solution, we achieved high prediction accuracy for each component. Figure 6 shows the correlation plots between actual glucose concentration and predicted urea or creatinine concentration. There is no correlation between actual glucose concentration and predicted urea or creatinine concentration. Thus, glucose concentration does not affect the prediction accuracy of urea and creatinine. Figure 4 confirms that the correlation coefficients of urea and creatinine are greater than 0.9. Especially, in the case of urea, all plots are within the error range, thereby demonstrating the measurement accuracy of the clinical laboratory method. In the case of creatinine, 88 plots out of 110 plots are within the error range. The results suggest that highly accurate prediction of urea and creatinine concentrations is obtained. Moreover, the predicted urea-to-creatinine ratio correlates with the actual value. These results indicate the feasibility of assessing dietary protein intake by the NIR spectroscopic method. In general, 24-h urine is used for analysis, because it is important to evaluate the secretion of urine components per day in CKD patients. However, using NIR spectroscopy, it is possible to assess the protein intake of CKD patients by analyzing spot urine samples.

Fifth, this study proposes a reagentless measurement method of multiple urine components using an optical method. Reagent denaturation adversely affects measurement accuracy, and a measurement system using reagents is difficult to use at patients’ home. Our proposed reagentless measurement system can be used at home. Moreover, because we can estimate the concentrations of urine components faster, this method may be used for pooled urine for point-of-care-testing.

Sixth, because the experiment was performed with only spot urine samples, the results obtained cannot be compared with other studies performed using 24-h urine samples. However, Fujita et al. [27–28] studied urine volume estimation using a noncontact method in the toilet. When this method for urine volume estimation is established, approximate daily urine volume can be estimated from one urine volume, and we may obtain the equivalent data of 24-h urine from spot urine samples. On the other hand, even if urine volume cannot be obtained, studies of measurements using spot urine samples are increasing [7–9]. Middendorf et al. [29] reported that urea nitrogen/creatinine excretion ratio
determined using a spot urine specimen obtained 5 h after the patient’s last meal of the day can be used to accurately calculate the urinary urea excretion for the previous 24-h period. This indicates that spot urine measurement is comparable to 24-h urine measurement. In the future, it can be expected that spot urine measurements would be a more effective method to estimate the total excretion of urine components. Moreover, using this method, other urine components can be quantitatively measured. If this is achieved, this method will be useful in clinical practice for predicting, for example, the albumin-to-creatinine ratio for incident diabetes in adults at high risk of developing type 2 diabetes [30].

6. Conclusion

In this study, we measured urinary urea, creatinine, and urea-to-creatinine ratio using an optical method in the NIR region. Using this method, we developed a calibration model for high-accuracy estimation of concentrations of components in urine samples obtained from human subjects. The wavelengths selected for the calibration model correspond to the functional groups of urea and creatinine. Results of the analysis indicated that absorbances at 1400 nm and 1800 nm provide concentration information. Moreover, we confirmed the feasibility of optical measurements of urinary urea, creatinine, and urea-to-creatinine ratio in CKD patients. Thus, this method of analyzing urine samples using NIR spectroscopy can be used to assess protein intake in CKD patients.

Acknowledgement

This work was partly supported by JSPS KAKENHI Grant Numbers 26282119, 20300195, and 15H02798. The authors would like to thank donors of urine samples, and the graduates of the Division of Mechanical Science and Engineering, Kanazawa University for their technical assistance.

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