We describe a new kinetic assay for determining urea in serum or urine with use of urease (EC 3.5.1.5) and leucine dehydrogenase (EC 1.4.1.9). The latter enzyme is suitable for the kinetic assay of NH$_4^+$ because its $K_m$ value for NH$_4^+$ at pH 8.75 is large ($\sim$500 mmol/L). Interference from endogenous NH$_4^+$ in serum or urine is obviated by subtraction of the asayed endogenous NH$_4^+$ value in a sample blank. For serum, within-assay CVs (n = 10) were 0.39–0.58%; day-to-day CVs (n = 10) were 1.56–2.30%. In urine, within-assay CVs (n = 10) were 0.86–1.15%. Analytical recovery of urea (0.893–71.4 mmol/L) added to patients’ sera (urea 6.14 mmol/L) was 99.2–105.2%. The calibration curve for serum was linear through zero for urea concentrations up to 142.9 mmol/L and for urine up to 714.3 mmol/L. No influences of added ammonium ion, bilirubin, hemoglobin, ascorbic acid, or Intralipid were observed. The regression equations for this method ($y$) and conventional methods ($x$ = Determiner-LUN for serum assays, Serotec UUR-R for urine) were: $y = 1.016x - 0.12$ mmol/L ($r = 0.999$, $S_{yx} = 0.34$ mmol/L, $n = 100$) for sera, and $y = 1.070x - 12.6$ mmol/L ($r = 0.998$, $S_{yx} = 7.41$ mmol/L, $n = 100$) for urine.

Urea nitrogen (UN) in serum or urine has been measured by many methods [1, 2] that are based on enzymatic reaction with urease (EC 3.5.1.5) and glutamate dehydrogenase (EC 1.4.1.4). Endogenous NH$_4^+$ in serum and urine is a potential interference, because NH$_4^+$ produced from urea by urease is determined in the reaction system. The NH$_4^+$ in serum and urine has been eliminated with the glutamate dehydrogenase and 2-oxoglutaric acid before addition of urease. But in some urine that has a large amount of endogenous NH$_4^+$, the elimination of the NH$_4^+$ may not be complete, even in samples diluted 10–20-fold.

Other approaches to avoiding interference from NH$_4^+$ have been described. To eliminate much of the NH$_4^+$ in urine, a recycling system (NADH$\rightarrow$NAD$^+$) with isocitrate dehydrogenase (EC 1.1.1.42) has been used [3]. Alternatively, ADP (produced from ATP by urease [4]) can be determined instead of NH$_4^+$. However, the cost of urea determination in these methods is very high; moreover, the former method was linear only to urea concentrations of $\sim$10 g/L, and the latter method was carried out at 376 nm, a wavelength unavailable on many automated analyzers.

We investigated a new enzymatic kinetic assay of serum or urine UN by using leucine dehydrogenase (LED; EC 1.4.1.9) and urease [5, 6]. Interference from endogenous NH$_4^+$ in serum and urine is avoided by subtracting the endogenous NH$_4^+$ value assayed in a sample blank.

### Assay Principle

Endogenous NH$_4^+$ in serum or urine is allowed to react with 2-ketoisohexanoic acid, NADH, and LED. The reaction rate at which NADH is oxidized to NAD$^+$ depends on the amount of the endogenous NH$_4^+$ measured (I).

\[ \text{I} \quad \text{Sample} + \text{R1} \]

\[ \text{NH}_4^+ + 2\text{-ketoisohexanoic acid} \]

\[ \xrightarrow{\text{LED}} \text{NADH} \rightarrow \text{NAD}^+ \]

Next, urease is added to the reaction system, and the oxidation rate of NADH to NAD$^+$ by both the NH$_4^+$ produced from urea and the endogenous NH$_4^+$ is mea-
UN in the sample is calculated from the differences of oxidation rate between I and II.

(II) Sample + R1 + R2

\[
\text{Urea} + 2\text{H}_2\text{O} + 2\text{H}^+ \xrightarrow{\text{Urease}} \text{NH}_4^+ + \text{CO}_2
\]

\[
\text{NH}_4^+ + 2\text{-ketoisohexanoic acid} \rightarrow \text{t-leucine} + 2\text{H}_2\text{O}
\]

\[
\text{LED} \rightarrow \text{NAD}^+ + \text{NADH}
\]

**Materials and Methods**

**Apparatus.** This proposed method and the conventional (comparison) methods were performed with the Hitachi Model 7150 automated analyzer.

**Reagents.** Urease (111 kU/g; \(K_m(\text{NH}_4^+) = 500 \text{ mmol/L}\) [5]) were purchased from Toyobo. Analytical-grade ammonium chloride, l-ascorbic acid, urea, and NADH were from Wako Pure Chemical Industries. 2-Ketoisohexanoic acid sodium salt was from Nacalai Tesque (Kyoto, Japan); \(N,N\)-bis(2-hydroxyethyl)glycine (Bicine) from Dojindo Labs (Kumamoto, Japan); bilirubin from Sigma Chemical Co., and Intralipid 10% from KabiVitrum AB.

Reagent 1 (R1) for the new method contained 2-ketoisohexanoic acid 3.0 mmol/L, \(\beta\)-NADH 0.3 mmol/L, and LED 1.5 kU/L in 100 mmol/L Bicine buffer (pH 8.75). Reagent 2 (R2) contained urease, 70 kU/L, in R1.

Calibrators were physiological saline, with and without 17.86 mmol/L UN.

**Assay procedure.** UN in serum or urine was measured with the Rate-Analysis system B mode of the Hitachi 7150 automated analyzer, as shown Fig. 1. Serum (15 \(\mu\)L) or urine (3 \(\mu\)L) and R1 (300 \(\mu\)L) were mixed, and the reaction rate was measured at measurement points 6–24, from 1 min to 5 min after the mixing (test I). Next, R2 (100 \(\mu\)L) was added and the reaction rate was measured at points 30–50 (from 6 to 10 min) at 340 nm (test II). The lag time, to allow the urease reaction to proceed sufficiently, was set at 1 min (measurement points 25–30). A linear calibration curve based on the absorbance of the 0 (physiological saline) and 17.86 mmol/L UN calibrators was used to estimate the UN concentrations of the samples.

**Comparison method.** The enzymatic method eliminated endogenous \(\text{NH}_4^+\) in serum. The Determiner-LUN reagent kit from Kyowa Medex (Tokyo, Japan) was used for serum UN determination; another enzymatic method eliminated endogenous \(\text{NH}_4^+\) in urine, the Serotec (Sapporo, Japan) UUR-R reagent kit for urine UN determination.

**Samples.** L-Consera N (Nissui Pharmaceutical Co., Tokyo, Japan) was used as control serum. Patients’ serum and urine samples were from patients in Nagoya University hospital.

**Results**

**Optimization studies**

Optimization studies of this proposed method were carried out with the calibrator (UN 17.86 mmol/L), a patient’s serum (UN 18.29 mmol/L), and L-consera N control serum (UN 5.82 mmol/L).

**Effects of pH.** The effect of pH on the serum UN determination was examined in 100 mmol/L Bicine buffer at various pH values (8.00, 8.25, 8.50, 8.75, 9.00, and 9.25). With increasing pH values, the reaction rate (A/min) for assay of the calibrator increased, but the UN values of the patient’s serum and L-consera N remained virtually unchanged (Fig. 2a). We chose to use Bicine buffer, pH 8.75.

**Effects of 2-ketoisohexanoic acid.** We examined the effects of concentrations of 2-ketoisohexanoic acid on the serum UN determination. With increasing concentrations of 2-ketoisohexanoic acid (0.3, 0.5, 1.0, 3.0, 5.0, and 10.0 mmol/L), the reaction rate (A/min) of the calibrator increased, reaching almost the maximum at 5.0–10.0 mmol/L 2-ketoisohexanoic acid, whereas the UN values of the patient’s serum and L-consera N were almost unchanged (Fig. 2b). We chose 10 mmol/L as the 2-ketoisohexanoic acid con-

**Fig. 1. Assay procedure.**
concentration in R1 and R2 to obtain the maximum reaction rate.

Effects of LED activity. With increasing LED activity (0.1, 0.2, 0.5, 1.0, 2.0, and 4.0 kU/L), the rate of reaction ($A_{min}$/min) of the calibrator increased proportionally, whereas UN values of the patient’s serum and L-consera N did not indicate much change (Fig. 2c). We added 1.5 kU/L LED to R1 and R2.

Effect of urease activity. With increasing urease activity (5.0, 10.0, 20.0, 50.0, 70.0, and 100.0 kU/L), the reaction rate of the calibrator ($A_{min}$/min) increased, with the maximum occurring at 70–100 kU/L, whereas the UN values of the patient’s serum and L-consera N did not show any marked change at urease of 50 kU/L or greater, as shown in Fig. 2d. We thus added 70 kU/L urease to R2.

Time course. Typical time courses in this proposed kinetic assay of serum UN are shown for the calibrator, the patient’s serum, and L-consera N in Fig. 3. The first reaction is the kinetic assay of the endogenous NH$_4^+$.
ASSAY EVALUATION

**Precision.** The serum UN assay samples were calibrator, L-consera N, and a patient’s serum, which contained 17.91, 5.83, and 18.17 mmol/L UN, respectively. For the urine UN assay, we used a calibrator and two patients’ urines, with respective UN contents of 17.99, 93.68, and 575.49 mmol/L. Within-assay CVs were measured with n = 10, and day-to-day CVs were determined from assays performed on 10 days (n = 10). As shown in Table 1, serum UN within-assay CVs ranged from 0.39% to 0.58%; day-to-day CVs were 1.56–2.30%; in urine UN determinations, within-assay CVs (n = 10) were 0.86–1.15%.

**Detection limit.** We examined the detection limit of this UN determination by assaying physiological saline (NaCl 9 g/L, the 0 UN calibrator) 10 times. The result (mean ± SD) was 0.012 ± 0.018 mmol/L. The detection limit, mean for physiological saline + 3.0 SD, was 0.066 mmol/L.

**Table 1. Precision of the proposed UN assay.**

<table>
<thead>
<tr>
<th></th>
<th>Calibrator</th>
<th>L-Consera N</th>
<th>Patient’s serum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within-assay serum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean, mmol/L</td>
<td>17.91</td>
<td>5.83</td>
<td>18.17</td>
</tr>
<tr>
<td>SD, mmol/L</td>
<td>0.095</td>
<td>0.034</td>
<td>0.071</td>
</tr>
<tr>
<td>CV, %</td>
<td>0.53</td>
<td>0.58</td>
<td>0.39</td>
</tr>
<tr>
<td><strong>Day-to-day serum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean, mmol/L</td>
<td>18.04</td>
<td>6.03</td>
<td>18.55</td>
</tr>
<tr>
<td>SD, mmol/L</td>
<td>0.282</td>
<td>0.139</td>
<td>0.406</td>
</tr>
<tr>
<td>CV, %</td>
<td>1.56</td>
<td>2.30</td>
<td>2.19</td>
</tr>
<tr>
<td><strong>Within-assay urine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean, mmol/L</td>
<td>17.99</td>
<td>93.68</td>
<td>575.49</td>
</tr>
<tr>
<td>SD, mmol/L</td>
<td>0.206</td>
<td>1.044</td>
<td>4.929</td>
</tr>
<tr>
<td>CV, %</td>
<td>1.15</td>
<td>1.11</td>
<td>0.86</td>
</tr>
</tbody>
</table>

*n = 10 each.*

**Analytical recovery.** For UN additions of 0.89, 1.79, 2.68, 3.57, 7.14, 10.71, 17.86, 26.79, 35.71, 53.57, and 71.43 mmol/L to patients’ serum (UN 6.14 mmol/L), from 99.2% to 105.2% was recovered (mean 102.2%).

**Linearity of calibration curve.** We examined the linearity of the calibration curve in duplicate with the UN calibrators. The calibration curve was straight up to at least 142.9 mmol/L for serum UN determinations and up to 714.3 mmol/L in urine UN determinations (Fig. 4).

**Interferences.** Various substances were examined for their potential effects on this UN determination. One volume of examined substance was mixed with nine volumes of patient’s serum (UN 16.29 mmol/L), L-consera N (UN 5.82 mmol/L), or a patient’s urine (UN 324.1 mmol/L).

Ammonium chloride was added to the patient’s serum and L-consera N at concentrations of 0.056–0.56 mmol/L, and to the patient’s urine at 55.6–166.7 mmol/L. Assay of UN was virtually unaffected (Fig. 5). Bilirubin, hemoglobin, ascorbic acid, and Intralipid were added to the patient’s serum and L-consera N in the same way and assayed. We found no interference with serum UN determination from bilirubin up to 0.342 mmol/L, hemoglobin 0.059 mmol/L, ascorbic acid 1.14 mmol/L, and 50-fold-diluted (0.2%) Intralipid.

**Correlation.** Possible correlation between this proposed method (y) and the comparison methods (x) was examined (Fig. 6). The correlation between values obtained with our method and the Determiner LUN kit for 100 patients’ sera was: $y = 1.016x - 0.12$ mmol/L ($r = 0.999, \text{Sy}_x = 0.34$ mmol/L); comparison with the Serotec UUR-R kit for 100 patients’ urines gave: $y = 1.070x - 12.6$ mmol/L ($r = 0.998, \text{Sy}_x = 7.41$ mmol/L).

Discussion

After detailed optimization studies, we investigated the new enzymatic kinetic assay of serum or urine UN determination by LED and urease with the Hitachi 7150.

![Fig. 4. Calibration curve for serum UN (a) and urine UN (b) determinations.](image-url)
automated analyzer, which automatically calculated the difference between the endogenous NH₄⁺ reaction (test I) and the reaction of both the endogenous NH₄⁺ and the NH₄⁺ produced from urea by urease (test II). Characteristic features of this method are:

1) Endogenous NH₄⁺ in serum or urine does not affect the UN determination, so that urine, even when containing a high concentration of endogenous NH₄⁺, need not be diluted before assaying.

2) This method yields linear results for large concentrations of UN—up to 142.9 mmol/L in serum and up to 714.3 mmol/L in urine—because the large $K_m$ value of LED for NH₄⁺, ~500 mmol/L, is suitable for the kinetic assay of NH₄⁺.

3) No influences of added ammonium ion, bilirubin, hemoglobin, ascorbic acid, or Intralipid were observed.

4) Good precision, reasonable analytical recovery without interference, and good correlation with conventional methods were observed.

The procedure takes 10 min and should be applicable to other discrete automated analyzers. The reagents could be prepared as liquid reagents. We therefore conclude this proposed method may be useful in routine clinical diagnosis.

**References**


